Se-Kwon Kim *Editor*

Handbook of Anticancer Drugs from Marine Origin

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Preface—Anticancer Drugs from Marine Origin

Over the years, the invention of new compounds are isolated by using advanced technology has expanded significantly. There are number of compounds developed from marine environment for the treatment of various diseases. Increasing evidence suggested that anticancer drug discovery leads from the marine environment. This book combines the knowledge about the compounds isolated from marine environment and their product development. This handbook is divided into five parts.

Chapter 1 provides general introduction and sponges, seaweed, microbes, tunicates and other miscellaneous compounds derived from marine organisms.

Part I—Sponges (Chaps. 2–6), described the sponge derived drugs represent one of the most promising sources of research for finding new anticancer drugs. These chapters discusses about the anticancer and angiogenesis inhibitors isolated from marine sponges and mechanism of action and preclinical and clinical studies.

Part II—About the marine algae derived compounds on cancer targets (Chaps. 6–11)—explained the compounds isolated from algae species, amelioration and anti tumor effect of a tertiary sulfonium compound, dimethylsulfoniopropionate, carotenoids, polysaccharides etc and the possible mechanisms of action are described. Also the health benefits of seaweeds biological roles and potential benefits for female cancers to be discussed in this part.

Part III—Provides (Chaps. 12–17) the details about marine microbial derived compounds for cancer therapeutics. The antitumor compounds isolated from marine microbes such as fungi, bacteria and actinobacteria are discussed.

Part IV—Discusses (Chap. 18) with marine tunicate derived compounds for cancer therapeutics.

Part V—The final part of the book covers others marine organisms derived compounds and mechanism of actions. In this part sources of the marine compounds, pyridoacridine alkaloids, triterpene glycosides, meroterpenoids for cancer targets such as microtubules, apoptosis, angiogenesis and also discovery and computer-aided drug design studies of the anticancer marine triterpene sipholanes as novel P-gp and Brk modulators to be discussed in these chapters.

This book provides details about compounds isolation, chemistry, and application in detail. Hence, anticancer drugs from marine origin are important for academic research, pharmaceutical, nutraceutical and biomedical industries. I would

like to acknowledge Springer publisher, for their encouragement and suggestions to get this wonderful compilation related marine drugs for cancer treatment. I would also like to extend my sincere gratitude to all the contributors for providing help, support, and advice to accomplish this task.

Busan, South Korea Prof. Se-Kwon Kim

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Chapter 1
Introduction to Anticancer Drugs from Marine Origin

Se-Kwon Kim and Senthilkumar Kalimuthu

Abstract The chemical and biological diversity of the marine environment is extraordinary resource for the discovery of new anticancer drugs. Recent technological and methodological advances in elucidation of structure, synthesis, and biological assay have resulted in the isolation and clinical evaluation of various novel anticancer agents from marine pipeline. To understanding the marine derived anticancer compounds are useful in pharmaceutical industry and clinical applications. The marine sponges, algae, microbes, tunicates and other species from the marine pipeline are the important sources for biological active compounds. The past decade has seen a dramatic increase in the number of preclinical anticancer lead compounds from diverse marine life enter human clinical trials.

Keywords Anticancer · Algae · Sponges · Marine · Bioactive compounds

1.1 Introduction

Cancer is a dreadful human disease, increasing with changing life style, nutrition, and global warming. A report released by the World Health Organization (WHO) showed that an estimated 12.7 million people were diagnosed with cancer globally and about 7.6 million people died of it in 2008. As estimated in this report, more than 21 million new cancer cases and 13 million deaths are expected by 2030. Although cancer accounts for around 13 % of all deaths in the world, more than 30 % of cancer deaths can be prevented by modifying or avoiding key risk factors [1]. However, almost all of the chemotherapy drugs currently in the market cause serious side effects. Natural products and their derivatives represent more than 50 % of all the drugs in clinical use of the world. Higher plants contribute not less than 25 % of the total. Almost 60 % of drugs approved for cancer treatment are of natural origin.

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Although marine compounds are underrepresented in current pharmacopoeia, it is anticipated that the marine environment will become an invaluable source of novel compounds in the future [2].

Marine nutraceuticals can be derived from a vast array of sources, including marine plants, microorganisms, and sponges. Marine nutraceutical products currently promoted to various countries include fish oil, chitin, chitosan, marine enzymes and chondroitin from shark cartilage, sea cucumbers and mussels. Polysaccharides derived from alga, including alginate, carrageenan and agar are widely used as thickeners and stabilizers in a variety of food ingredients. In addition, Omega PUFA (Polyunsaturated fatty acid) is an important ingredient to the nutraceutical industry [3]. It has been proven that Omega-PUFA, especially eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA) play a significant role in number of aspects of human health [4].

More than 70% of our planet's surface is covered by oceans. An exciting "marine pipeline" of new anticancer clinical and preclinical agents has emerged from intense efforts over the past decade to more effectively explore the rich chemical diversity offered by marine life. The chemical adaptations generally take the form of so-called "secondary metabolites," and involve such well known chemical classes as terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids, and a multitude of mixed biogenesis metabolites. In addition, and unique to the marine environment, is the relatively common utilization of covalently bound halogen atoms in secondary metabolites, mainly chlorine and bromine, presumably due to their ready availability in seawater [5, 6]. Marine compounds that act as hallmarks of cancer presented namely self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replication, sustained angiogenesis and tissue invasion and metastasis [7–11].

1.2 Sponges

Marine sponges for the past decades have been considered as a very fertile field for the discovery of bioactive natural chemical substances with respect to the diversity of their primary and secondary chemical components and metabolites. Marine sponges (Porifera) are the oldest metazoan group, having an outstanding importance as a living fossil [12]. There are approximately 8000 described species of sponges and perhaps twice as many un-described species [13, 14]. Sponges inhabit every type of marine environment, from polar seas to temperate and tropical waters and also thrive and prosper at all depths. They show an amazing variety of shapes, sizes and colours. Giant barrel sponges can reach up to 70 in. in height, while another tiny encrusting sponge may only be half of an inch long. Sponges are sessile organisms. However, due to their cellular plasticity, many sponges reorganize their bodies continuously and move during this process very slowly [14]. Marine sponges through evolutionary and ecological long term changes often contain diverse microbial communities (bacteria, archaea, microalgae, fungi) which comprise as much as 40% of the sponge volume and can contribute significantly to host metabolism

(e.g., via photosynthesis or nitrogen fixation). The ecological and evolutionary importance of sponge-microbe associations can be mirrored by their enormous biotechnological potential producing a great range of bioactive metabolites [15, 16]. Scientist has discovered more than 5000 species and also there are more than 8000 marine sponges on Earth.

Marine sponges have been ranked at the top with respect to the discovery of bioactive compounds with potential pharmaceutical applications. The diversity in chemical structures of sponge-derived metabolites is related to an equally diverse pattern of activities. The chemical diversity of sponge natural products is remarkable, including unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives (which are frequently halogenated) [17]. In the field of natural products chemistry and research suggest that sponges have the potential to provide future drugs against some important diseases, such as viral diseases, malaria, inflammations, immunosuppressive diseases and various malignant neoplasms [5, 18–20]. In the last few years there are several other candidates from marine natural compounds in the pipeline for evaluation in Phase I–III clinical trials for the treatment of various cancers [21, 22]. From the previous studies, marine natural compounds from sponge species were undergoing preclinical and clinical trials (I, II, III) for anticancer activity. Among the compounds were discodermolide, hemiasterlins A & B, modified halichondrin B, KRN-70000, Alipkinidine (alkaloid), fascaphysins (alkaloid), isohomohalichondrin B, Halichondrin B, Laulimalide/Fijianolide, 5-methoxyamphimedine (alkaloid) and Variolin (alkaloid) [16]. In recent years, new marine-derived antiangiogenic agents have been widely investigated. At least 43 marine-derived natural products and their derivatives have been reported to display antiangiogenic activities, mediated by distinct or unknown molecular mechanisms [16, 23].

The first successful sponge-derived pharmaceutical drugs were the nucleosides spongothymidine and spongouridine which were isolated from *Tectitethya crypta* [24]. A derivative of these nucleosides, Ara-C (also known as 1-beta-D-Arabinofuranosylcytosine or cytarabine) is documented as the first marine derived anticancer agent that is recently used for the treatment of leukemia [25, 26]. An overview (2011) retrieved scientific papers identifying 39 compounds from marine sponges with apoptosis-inducing anticancer properties [27]. Renieramycins are members of the tetrahydroiso-quinoline family that were isolated from marine sponges belonging to genera *Reniera* induces apoptosis through p53-dependent pathway and may inhibit progression and metastasis of lung cancer cells [28]. Monanchocidin is a novel polycyclic guanidine alkaloid isolated from the marine sponge *Monanchora pulchra* that promote cell death in human monocytic leukemia (THP-1), human cervical cancer (HeLa) and mouse epidermal (JB6 Cl41) cells [29]. Smenospongine, a sesquiterpene aminoquinone, from the sponge *Smenospongia* sp. have antiproliferetive and antiangiogenic activities [30]. The macrocyclic lactone polyether spongistatin 1 was isolated from the marine sponge *Spongia* sp. [31], inhibit mitosis, microtubule assembly and inducing cytotoxic cell death in numerous cancer cell lines [32]. Recently, scientists purified a lectin from the marine sponge *Cinachyrella apion* (CaL) have hemolytic, cytotoxic and antiproliferative proper-

ties and cell death in tumor cells [33]. Heteronemin, a marine sesterterpene isolated from the sponge *Hyrtios* sp., inhibits chronic myelogenous leukemia cells by regulating cell cycle, apoptosis, mitogen-activated protein kinases (MAPKs) pathway and the nuclear factor kappaB (NF-kappaB) signaling cascade [34]. Still there are number of anticancer compounds is isolated and screened form marine sponges.

1.3 Algae

Algae are relatively undifferentiated organisms which, unlike plants, have no true roots, leaves, flowers or seeds. They are found in marine, freshwater and terrestrial habitats. Their size varies from tiny microscopic unicellular forms of 3–10 μm (microns) to large macroscopic multicellular forms up to 70 m long and growing at up to 50 cm per day. Most of the algae are photosynthetic organisms that have chlorophyll. Marine macroalgae are important ecologically and commercially to many regions of the world, especially in Asian countries such as China, Japan and Korea [35]. Phytoplankton, seaweeds and symbiotic dinoflagellates (unicellular, biflagellate organisms) in corals and sea anemones are marine algae. Seaweeds are classified as green algae (Chlorophyta), brown algae (Phaeophyta), red algae (Rhodophyta) and some filamentous blue-green algae (Cyanobacteria). Most of the seaweeds are red (6000 species) and the rest known are brown (2000 species) or green (1200 species). Seaweeds are used in many maritime countries as a source of food, for industrial applications and as a fertilizer. Industrial utilization is at present largely confined to extraction for phycocolloids, industrial gums classified as agars, carrageenans and alginates. Carrageenans, extracted from red seaweeds such as Chondrus, Gymnogongrus, and Eucheuma among others, are used to provide particular gel qualities. Alginates are derivatives of alginic acid extracted from large brown algae such as Laminaria. They are used in printers' inks, paints, cosmetics, insecticides, and pharmaceutical preparations.

Seaweeds have been one of the richest and most promising sources of bioactive primary and secondary metabolites [36]. The algae synthesize a variety of compounds such as carotenoids, terpenoids, xanthophylls, chlorophyll, vitamins, saturated and polyunsaturated fatty acids, amino acids, acetogenins, antioxidants such as polyphenols, alkaloids, halogenated compounds and polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol and fucoidan [36, 37]. These compounds probably have diverse simultaneous functions for the seaweeds and can act as various functions including anticancer effects. The seaweeds are the rich source of carotenoids, the most notable being β-carotene, α-carotene, fucoxanthin, astaxanthin, canthaxanthin, zeaxanthin and lutein has been reported as effective antioxidants. Seaweed carotenoids are powerful antioxidants associated with the prevention of cardiovascular, neurodegenerative diseases and also cancer. The carotenoids have been extensively studied and the consumption of the dietary carotenoids has been correlated with cancer prevention [38, 39]. Also, amelioration effect of green sea algae derived compound

dimethylsulfonioacetate (DMSP) has shown that on stress and aging closely related to cancer, solid and free cell cancer, and neural degeneration caused by brain cancer with model animals.

1.4 Microbes

Microbes, like this single-celled marine phytoplankton, make up a staggering 90%of the ocean's total biomass. Marine microbes are tiny organisms that live in marine environments and can only be seen under a microscope. They include cellular life forms such as bacteria, fungi and plankton along with the viruses that freeload on the cellular life forms. There are more than a billion micro-organisms living in each litre of seawater, and it is now known that microbes dominate the abundance, diversity and metabolic activity of the ocean. Marine microbes are having huge biochemical diversity and rich source of novel drugs. Marine microbial compounds are an important source for drug development [40]. Marine bacteria are one of the important sources for many bioactive compounds, antibiotics and pharmaceuticals. They are usually found in the marine sediments and also found to be associated with the marine organisms [41]. Despite a limited number of marine microbial antitumor agents currently on the market or in clinical trials, there are strong evidences that some promising marine natural compounds in clinical trials as well as some approved marine-derived anticancer agents produced by invertebrates, in fact metabolic products of their associated microorganisms, or derived from a diet of prokaryotic microorganisms [42, 43].

Meroterpenoids are a class of secondary metabolites in which the terpenoid moieties are linked to molecules from different biosynthetic pathways. Meroterpenoids containing quinones are also widespread in marine microorganisms, with prenylated naphtoquinones and reduced hydroquinone analogues are reported from marine microorganisms especially fungi and actinomycetes [44]. Meroterpenoids especially those with anticancer activity, produced by all types of marine-derived microorganisms. Marine fungi are also reported as a potential source for bioactive compounds. Polyketide synthases are a class of enzymes that are involved in the biosynthesis of secondary metabolites. The microbe's derived compounds are potential use for anticancer research.

Actinomycetes are one of the most efficient groups of secondary metabolite producers, they exhibit a wide range of biological activities and also anticancer effects. Several species have been isolated and screened from the soil in the past decades. Among its various genera, *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora* and *Actinoplanes* are the major producers of commercially important biomolecules [45]. Actinomycetes are virtually unlimited sources of new compounds with many therapeutic applications and hold a prominent position due to their diversity and proven ability to produce novel bioactive compounds [46]. In the search for bioactive compounds from actinomycetes collected from the deep-sea water in Toyama Bay, two new glycosylated polyketides were isolated from the

culture extract of *Micromonospora* sp., the arisostatin A and arisostatin B, respectively [47, 48]. Arisostatins are the new members of tetrocarcin-type cytotoxic compounds. Arisostatin A showed a potent cytotoxic effect on human cancer cells and activates caspase 3, a key effector protease responsible for apoptosis induction [49].

Marine fungi have proven to be untapped resources for the rich and promising source of novel antibacterial, antiplasmodial, anti-inflammatory antiviral and anticancer agents. Most of the fungi grow in unique and extreme environments therefore they have the ability to generate unique and unusual secondary metabolites [50]. Toluquinol is derived from marine fungus interferes with one of the hallmarks of cancer described by Hanahan and Weinberg by impairing the unlimited replicative potential, characteristic of tumor cells. Toluquinol represses the proliferation of the promyelocytic leukemia HL60 cell line, fibrosarcoma HT1080 cell line and colon adenocarcinoma HT29 cell line. The IC50 values, which represent the concentrations of toluquinol yielding a 50 % of cell growth, were lower than 10 μM in the three cell lines and also inhibits angiogenesis of cancer [51]. Diketopiperazines (DKPs) of marine resources, especially those isolated from marine-derived fungi, have been paid increasing attention for their diversity in chemical structure and bioactivity. Halimide ((-)-phenylahistin) is a fungal prenylated DKP isolated from *Aspergillus ustus* NSC-F038 and arrested the cancer cell cycle of P388 in the G2/M phase [52].

1.5 Tunicates

Tunicates are also known as *urochordates,* belong to the subphylum Tunicata or Urochordata. Tunicates have been shown as a primitive model organism to study immunodefense since the innate immune system has been hypothesized as an important functional component that may partially explain the lack of metastatic tumors in invertebrates [53]. Marine-derived compounds have reached clinical trials as antitumor from tunicates such as didemnin B, Aplidine, and ecteinascidin 743. Didemnin B (DB), a cyclic depsipeptide from the compound tunicate *Tridemnum solidum*, was the first marine-derived compound to enter Phases I and II clinical trials. The Phase II studies, sponsored by the U. S. National Cancer Institute, indicated complete or partial remissions with non-Hodgkins lymphoma, but cardiotoxicity caused didemnin B to be dropped from further study. The closely related dehydrodidemnin B (DDB, Aplidine) was isolated in 1988 from a second colonial tunicate, *Aplidium albicans*, and spectroscopic studies assigned a structural formula in which a pyruvyl group in DDB replaced the lactyl group in DB and syntheses of DDB have been achieved. Aplidine is more active than DB and lacks DB's cardiotoxicity. The second family of tunicate-derived antitumor agents are the ecteinascidins (ETs), from the mangrove tunicate *Ecteinascidia turbinata*. The antitumor extracts of *E. turbinata* were first described in 1969, but the small amount of ETs in *E. turbinate* prevented their isolation for over a decade. Phase II clinical trials with ET 743 are underway [54].

1.6 Miscellaneous

In recent years, marine natural product bioprospecting has yielded a considerable number of drug candidates. Research into the ecology of marine natural products has shown that many of these compounds have anticancer function [43]. Apart from sponge, algae, tunicate, microbes other marine organisms include sea cucumber, sear hare, mollusks and Bryozoans derived marine natural products also has a anticancer function include microtubule-interfering agents, DNA-interactive agents, phosphatase inhibitors *etc*. Alkaloids pyridoacridines isolated from various marine sources have been reported to possess significant cytotoxicity against cultured cells, and the family as a whole seems to be of great interest as a source of new lead structures for the development of future generation of therapeutic agents [55]. Sea cucumbers are one of the marine animals which are important as human food source, and sea cucumber extracts have been used for over-the-counter dietary health supplements [56, 57]. Triterpene glycosides from sea cucumbers demonstrate that wide spectrum of biological effects, such as antifungal, antitumor, hemolytic, cytostatic, pro-apoptotic and immunomodulatory activities. Frondoside A and Cucumariosides showed cancer preventive effects on both *in vitro* and *in vivo* models [58–60]. The dolastatins were originally reported from the Indian Ocean sea hare, *Dolabella auricularia*. Subsequently, a number of dolastatins and related molecules were isolated from filamentous marine cyanobacteria, which are the natural diet of the sea hares [61]. The dolastatins is the most active molecule in inhibiting cancer cell growth [62].

Meroterpenes are a class of natural products that exhibit a remarkable chemical diversity. This rich chemistry is a consequence of their mixed biosynthesis, as they are composed of an aromatic moiety/carbohydrate residue and also a terpenoid portion that can range from one to nine isoprene units. Prenylated quinone/hydroquinone derivatives are amongst the most numerous and widespread in marine environment [63, 64]. Meroterpenes are not exclusive to marine organisms, being found also in many terrestrial species. This type of compounds has various biological functions including anticancer effects. In the marine environment, the main sources of meroterpenes are brown algae, microorganisms, soft corals and marine invertebrates, such as sponges or ascidians [64]. Number of bacteria and cyanobacteria associated with the marine sponges have been found to be the sources of antibiotics and other bioactive compounds and it has been reported that the wider biosynthetic capabilities of sponges are associated with their symbiotic microorganisms [65]. IB-96212, a 26-membered macrolide that contains a spiroketal lactone structure, is produced by the actinomycete, *Micromonospora* sp. L-25-ES25-008, isolated from a sponge, collected from the Indian Ocean near the coast of Mozambique [66] and showed cytotoxic activity against mouse leukemia P-388 and human lung nonsmall cell A-549, colon adenocarcinoma HT-29 and melanoma MEL-28 cell lines [67]. Cembrane-type diterpenoids are a large and structurally varied group of natural products isolated from both terrestrial and marine organisms [68]. In the marine environment, coelenterates of the orders Alcyonacea and Gorgonacea are recognized as the most prominent source of cembranoids, which usually exhibit cyclic

ether, lactone, or furane moieties around the cembrane framework [69–71]. The diterpenoids of the cembrane family have been shown to biomedical perspective, cytotoxicity is the most remarkable property of this class of diterpenoids [72].

1.7 Research Scope

Marine environments play a vital role in exploring and studying various marine resources and isolation, characterization and applications of biological active compounds from marine field. The sea covers over 70 % of the earth's surface and large proportion of the sea offers untapped sources of potential drugs with promising activities due to a large diversity of marine habitats and environmental conditions (nutrient availability, sunlight presence, and salinity levels). In the area of marine research, a recent census of marine life that involved the participation of 2700 scientists from over 80 nations assessed the diversity, distribution and abundance of marine life resulted in the discovery of over 6000 potentially novel species (Census of marine life. http://www.comlorg/about-census).

The anticancer research progress in throughout the world including Republic of Korea, Japan, India, China, Singapore, Malaysia, Australia and USA, as well as others countries also in importance as a research priority for finding new anticancer compounds from marine sources. However, advances in drug discovery are expected to encourage applications from the marine field. A major task of marine is to develop an efficient process for the discoveries of novel molecules from the marine environment. The huge level of marine biodiversity of marine organism makes them a prime target for the productions of enzymes and bioactive molecules for the treatment of various diseases including cancer. Biochemical studies of marine organisms are an important task for the discovery of new drug molecules and biological tools and management of biodiversity. These research efforts, it is clear that the marine environment represents an important source of unknown natural compounds whose medicinal potential must be evaluated. Almost 50 % of the antitumor agents approved in the last 50 years of the twentieth century were either compounds derived from natural sources or (semi-) synthetic analogs of these products. Natural compounds remain a high output source of promising chemotherapeutic or chemopreventive agents in current cancer research. In addition to PharmaMar, other pharmaceutical companies including Bedford, Enzon, Eisai Inc., Novartis, Aventis, Eli Lilly, Abbott In"azyme, Pfizer and Taiho Pharmaceuticals Co., have therapeutic compounds of marine origin under development.

1.8 Organization of Handbook

This handbook combines the knowledge about the compounds isolated from sponge, algae, microbes, tunicatesetc and also methods, product development, industrial and biomedical applications. This handbook is divided into five parts. The first part of

the book comprises the introduction, sponges, microbes, algae, tunicates and other miscellaneous compounds derived from other marine organisms. The second part deals with sponge derived drug discovery represent one of the most promising sources of leads in the research of new cancer drugs. These chapters provide an overview of the angiogenesis inhibitors isolated from marine sponges based on the available information regarding their primary targets or mechanism of action and antitumour effect of triterpenoids, cyclic peptides and cyclodepsipeptides also discussed. Moreover, marine sponge derived compound eribulin with respect to its clinical pharmacology, pharmacokinetics, pharmacodynamics, mechanism of action, metabolism, preclinical studies and clinical trials. The third part of the book introduces about the marine algae derived compounds on cancer targets. In this amelioration and anti tumor effect of a tertiary sulfonium compound, dimethylsulfoniopropionate, from green sea algae and the various biological functions including anticancer effects of the seaweed carotenoids such as fucoxanthin etc. and the possible mechanisms of action are described. Fucoidan, a sulfated polysaccharides isolated from brown algae, anticancer and antimetastatic action are described. The health benefits of marine algae have been intensively investigated for human. The seaweeds biological roles and potential benefits for female cancers to be discussed in this part.

The fourth part of the book provides the details about marine microbial derived compounds for cancer therapeutics. In this chapter provide evidence on the antitumor compounds isolated from marine microbes such as fungai, bacteria and actinobacteria. The fifth part of the book dealt with marine tunicate derived compounds for cancer therapeutics. Finally the sixth part of the book covers others marine organisms derived compounds for cancer to be discussed. In this part deals the structures and sources of the isolated marine pyridoacridine alkaloids, as well as the mechanisms underlying the cytotoxicity of certain naturally occurring marine pyridoacridines. Anticancer effects of triterpene glycosides, Frondoside A and Cucumarioside A2–2 isolated from sea cucumbers. Discovery and computer-aided drug design studies of the anticancer marine triterpene sipholanes as novel P-gp and Brk modulators. Molecular targets of anticancer agents from filamentous marine cyanobacteria. Cytotoxic terpene-purines and terpene-quinones from the sea cytotoxic triterpene glycosides from sea cucumbers. Meroterpenes from marine invertebrates chemistry and application in cancer. Marine sponge derived actinomycetes and their anticancer compounds. Advances of microtubule-targeting small molecular anticancer agents from marine origin. Targeting cellular proapoptotic agents from marine sources. Cytotoxic cembrane diterpenoids. Pederin, psymberin and the structurally related mycalamides biological activities, P-gp inhibitory activity from marine sponges, tunicates and algae.

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Chapter 2
Triterpenoids as Anticancer Drugs from Marine Sponges

Yong-Xin Li and Se-Kwon Kim

Abstract Natural products provide an important source of new therapeutic drugs and biochemical tools. In the last decades researchers of natural products chemistry focused their research in a wide variety of bioactive compounds from marine species. Marine sponges have been considered as a very fertile field for the discovery of bioactive natural chemical substances with respect to the diversity of their primary and secondary chemical components and metabolites. Triterpenoids are the most abundant secondary metabolite present in marine sponges. A large number of triterpenoids are known to exhibit cytotoxicity against a variety of tumor cells as well as anticancer efficacy in preclinical animal models. Therefore, triterpenoids from marine sponges leads to be used in the pharmaceutical industry as new chemical classes of anticancer agents.

Keywords Triterpenoids · Anticancer agents · Marine natural products · Marine sponges

2.1 Introduction

Natural products have served as important chemical prototypes for the discovery of new molecules, and continue to be the most promising source of drug leads, especially in the anticancer field [1]. In the last decades researchers of natural products chemistry focused their research in a wide variety of bioactive compounds from marine species. Marine sponges for the past decades have been considered as a

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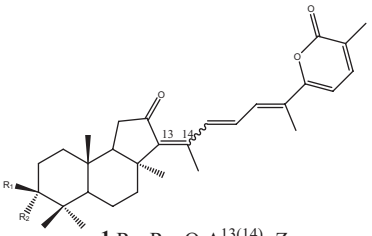
very fertile field for the discovery of bioactive natural chemical substances with respect to the diversity of their primary and secondary chemical components and metabolites [2]. Marine sponges have a bright potential in anticancer drug discovery as they represent a major source of new antitumor and anticancer drugs [3]. Triterpenoids are structurally diverse organic compounds, characterized by a basic backbone modified in multiple ways, allowing the formation of more than 20,000 naturally occurring triterpenoid varieties. Several triterpenoids, including ursolic and oleanolic acid, betulinic acid, celastrol, pristimerin, lupeol, and avicins possess antitumor and anti-inflammatory properties [4]. Triterpenoids are terpenoid derivatives of natural products containing about thirty carbon atoms, and their structures are considered to be derived from acyclic precursor squalene [5, 6]. Triterpenoids are the most abundant secondary metabolite present in marine sources, such as marine sponges [7, 8]. During a last few years, great number of biologically active triterpenoids is found to have cytotoxicity against a variety of tumor cells [9, 10]. More than 20,000 triterpenoids has been isolated and identified from natrue, which belongs to chemical groups such as, squalene, lanostane, dammarane, lupane, oleanane, ursane, hopane [11, 12]. This chapter summarizes the anti-cancer triterpenoids isolated from marine sponge, that includes isomalabaricane-type triterpenoids (stellettins, stelliferins, and geoditins), and their potential anti-cancer activity. Therefore, this chapter brings insights to marine triterpenoids as potent candidates to be developed as pharmaceuticals against tumor progression.

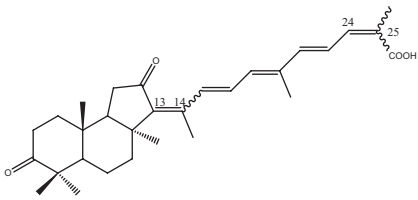
2.2 Triterpenoids from marine Sponge

Isomalabaricane-type triterpenoids are a rare group of triterpenoids with unique skeleton, often found in marine sponges.Isomalabaricane-type triterpenes were first reported from a Fijian collection of the sponge *Jaspis stellifera* and the Somalian marine sponge *Stelletta* sp. Since then, they have been isolated from several genera of marine sponges belonging to the order Astrophorida including members of the genera *Rhabdastrella*, *Stelletta*, *Jaspis*, and *Geodia* [13] The cytotoxic isomalabaricane-type triterpenoids stellettins A-K (**1–13**) have been reported from the marine sponge species of the genus *Jaspis* [14], *Stelleta* [15–17], and *Rhabdastrella* [18]. Stellettin A (**1**) and B (**2**), were isolated from the sponge *Stelletta tmuis* collected from Hainan Island, China in 1994. Stellettin A was significantly toxic to P388 leukemia cells, exhibiting an ED_{50} value of 0.001 μg/ml [19]. Furthermore, Liu et al. have demonstrated that stellettin A and stellettin B induce cytotoxicity in HL-60 cells treated for 24 h at 3 μM concentration [20]. The cytotoxic isomalabaricane triterpenoids stellettins A-G (**1–7**) have been examined at the National Cancer Institute (Australia) against 60 cell lines. Stelletin C (**3**) and D (**4**) were the most potent derivative with a mean panel GI_{50} of 0.09 μM. The stelletin E (**5**) and F (**6**) pair was approximately 10-times less potent (mean GI_{50} of 0.98 μM) [13, 15].

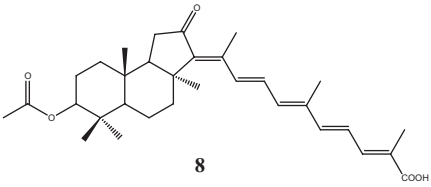
The isomalabaricane triterpenes, Stellettin A-D (**1–4**), stellettin H (**8**) and stellettin I (**9**) with and rhabdastrellic acid-A (**14**), have been isolated from the marine sponge *Rhabdastrella globostellata*, collected from the Philippines. These compounds have shown selective cytotoxicity towards $p21^{WAF1/Cip1}$-deficient human colon tumor(HCT-116) cells [21].



1 R_1, R_2=O $\Delta^{13(14)}$=Z
2 R_1, R_2=O $\Delta^{13(14)}$=E
3 R_1, R_2=$OCOCH_3$, $\Delta^{13(14)}$=Z
4 R_1, R_2=$OCOCH_3$, $\Delta^{13(14)}$=E



5 Δ13(14)=Z, Δ24(25)=E
6 Δ13(14)=E, Δ24(25)=Z
7 Δ13(14)=Z, Δ24(25)=Z
14Δ13(14)=E, Δ24(25)=E



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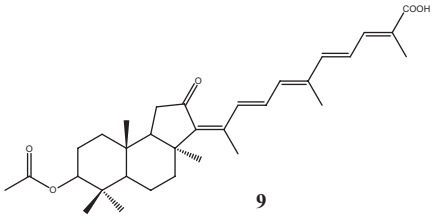
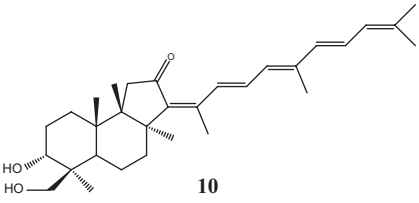


9

The cytotoxic isomalabaricane triterpenoids Stelletin J (**10**) and K (**11**) from *Rhabdastrella globostellata* has shown activity in an assay measuring stabilization of the binding of DNA with DNA polymerase *β*. However, stelletin J (**10**) and K (**11**) displayed varying levels of activity toward the A2780 ovarian cancer cell line, revealing structure-based effects on both the level of cytotoxicity and DNA-polymerase *β* binding [22].



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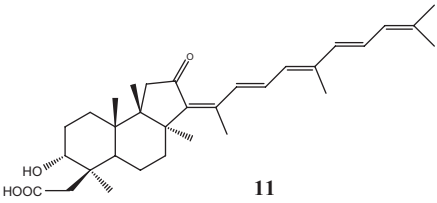
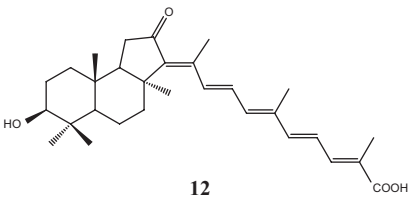


11

Stelletin L (**12**) and M (**13**) were isolated from the marine sponge *Stelletta tenuis* collected in the South China Sea and both compounds exhibited significant cytotoxic activity against stomach cancer cells (AGS) *in vitro* [17].



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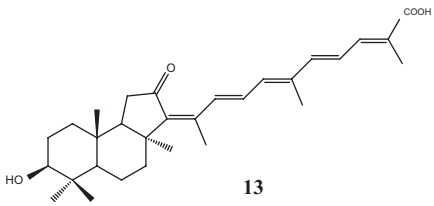
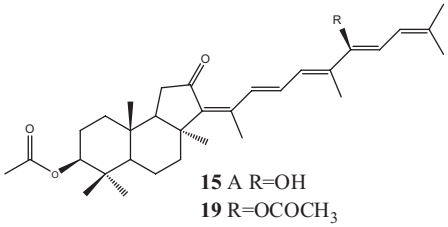
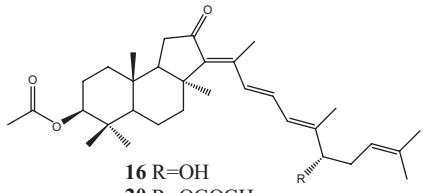


13

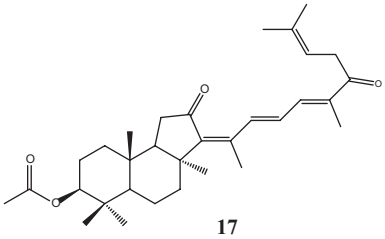
Stelliferins A–F (**15–20**), antineoplastic isomalabaricane triterpenes were isolated from the Okinawan marine sponge *Jaspis stellifera* [23]. The isomalabaricane triterpenes, stelliferin G (**21**), 29-hydroxystelliferin A (**22**), 29-hydroxystelliferin E (**23**) together with the known triterpene 3-epi-29-hydroxystelliferin E (**24**), 13E-29-hydroxystelliferin E (**25**), 29-hydroxystelliferin B (**26**), 13E-stelliferin G(**27**), and 13E-3-epi-29-hydroxy-stelliferin E (**28**), were isolated from the organic extract of the sponge *Jaspis sp*. collected in the South Pacific ocean. All compounds were tested against melanoma (MALME-3M) and leukemia (MOLT-4) cells. The mixtures of 29-hydroxystelliferin B (**26**) and 13E-stelliferin G (**27**) have shown highest growth-inhibitory [(IC_{50}) 0.11, 0.23 μg/mL, respectively)] activities against MALME-3M [24].



15 A R=OH
19 R=$OCOCH_3$



16 R=OH
20 R=$OCOCH_3$



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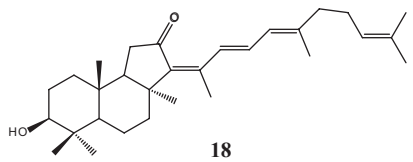

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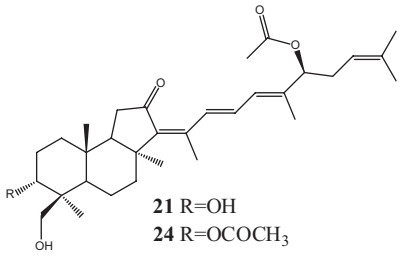
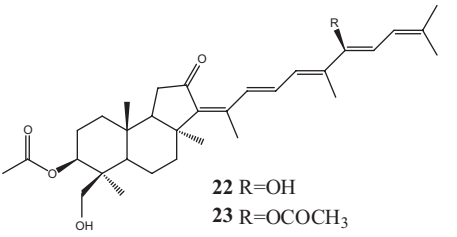
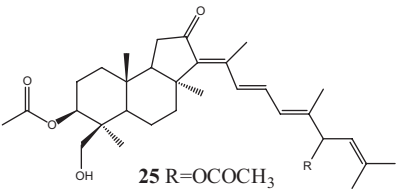


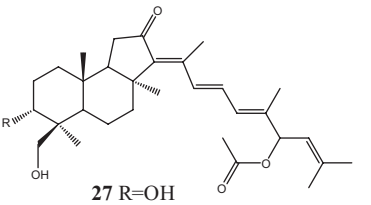
21 R=OH
24 R=$OCOCH_3$



22 R=OH
23 R=$OCOCH_3$

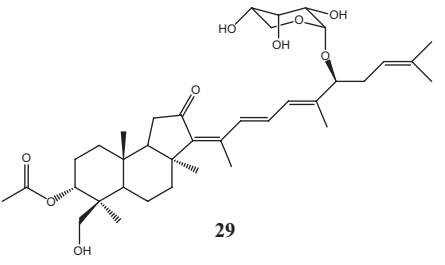


25 R=$OCOCH_3$
26 R=OH



27 R=OH
28 R=$OCOCH_3$

Moreover, stelliferin riboside (**29**) and 3-epi-29-acetoxystelliferin E (**30**) isomalabaricane triterpenoids were isolated from an extract of the sponge *Rhabdastrella globostellata* which was active in an assay measuring stabilization of the binding of DNA with DNA polymerase β. Two compounds have shown to induce 29 and 23 % binding, respectively [22].



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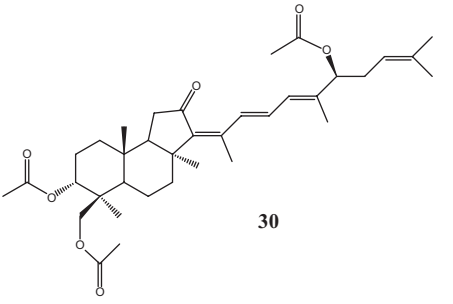
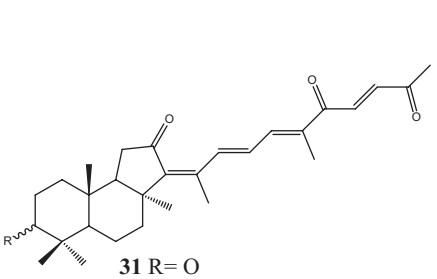


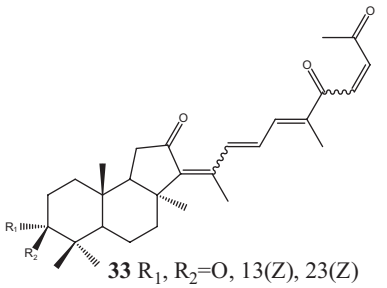
30

Four isomalabaricane triterpenes, geoditin A (**31**), geoditin B (**32**), isogeoditin A (**33**), and isogeoditin B (**34**) were isolated from marine sponge *Rhabdastrella aff. distincta*. All compounds were tested against a small panel of human tumor cell lines [18]. Geoditin A (**31**) and geoditin B (**32**) have also been isolated from marine

sponge *Geodia japonica*. Geoditin A was the most cytotoxic to HL60 cells [IC 50 Z3 mg/ml (<6.6 mM)], and geoditin B exhibited relatively weak cytotoxicity [25].

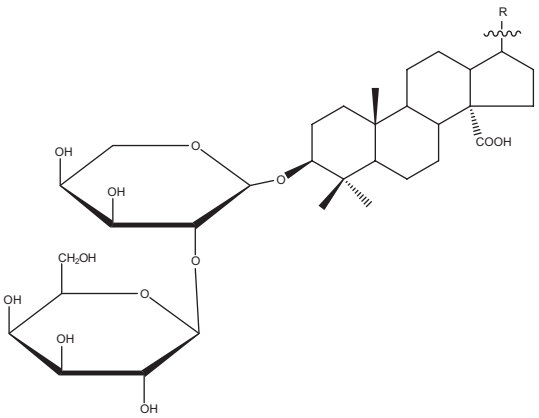


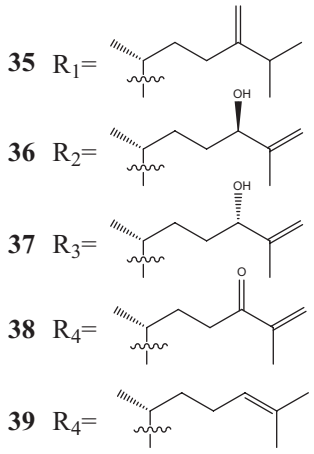
31 R= O
32 R= β -OAc



33 R_1, R_2=O, 13(Z), 23(Z)
34 R_1=H, R_2=OAc, 13(Z), 23(Z)

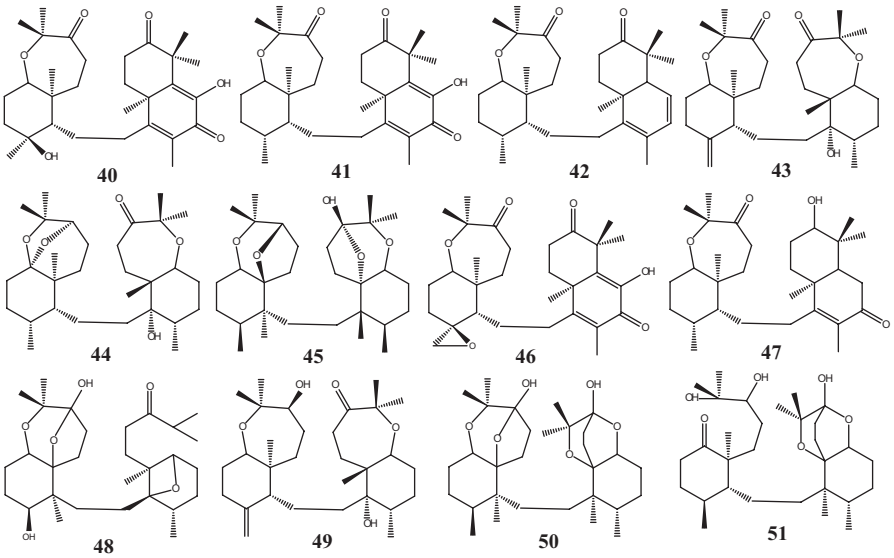
Five cytotoxic triterpene glycosides, erylosides F1-F4 (**35–38**), and erylosides F (**39**) were isolated from the sponge *Erylus formosus* collected from the Mexican Gulf (Puerto Morelos, Mexico). Four compounds induced the early apoptosis of Ehrlich carcinoma cells, where erylosides F3 have shown the highest activity at a concentration of 100 μg/mL [26].

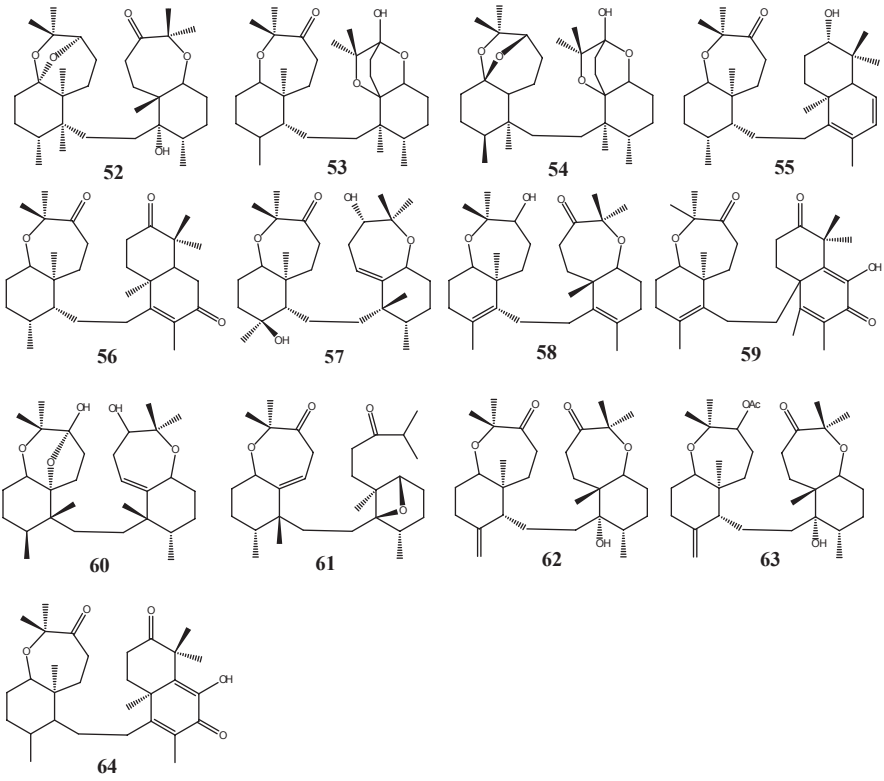




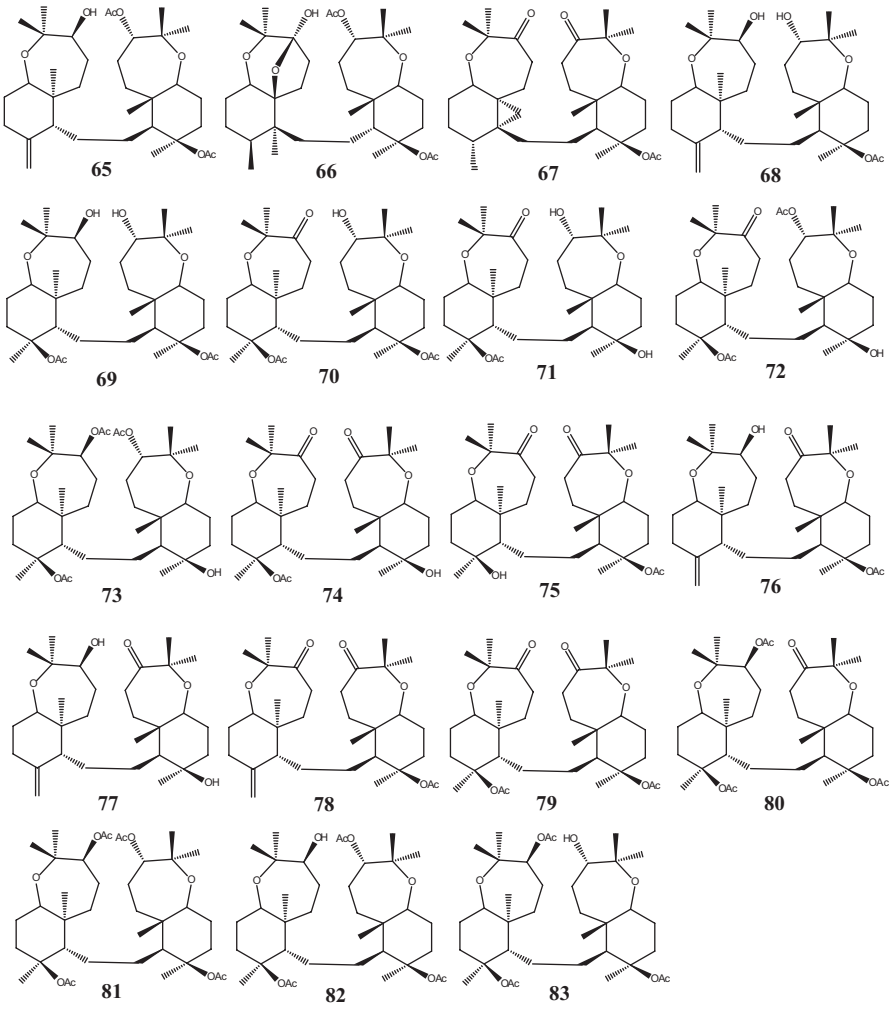
35 R_1=
36 R_2=
37 R_3=
38 R_4=
39 R_4=

The special group of triterpenoids named sodwanones, sodwanones A-I (**40–48**) and sodwanones K-W (**49–61**), have been isolated from the Indo-Pacific sponge *Axinella wltneri* [27]. Sodwanones G (**46**), H (**47**), and I (**48**) have been found to have cytotoxic activity. The compounds have shown cytotoxicityactivity against cell cultures of P-388 murine leukemia, A-549 human lung carcinoma, HT-29 human colon carcinoma, and MEL-28 human melanoma. Sodwanones G (**46**), H (**47**), I (**48**) showed high specificity towards human lung carcinoma cell line A-549, where the specificity of sodwanone G was prominent (**46**) [28]. The cytotoxic triterpenes, sodwanones K(**49**), L (**50**), and M (**51**) were found to be cytotoxic to P-388 murine leukemiacells [29]. The biological activity of sodwanone S (**57**) was evaluated against 13 human tumor cell lines [30]. Sodwanone V (**60**) inhibited both hypoxia-induced and iron chelator (1, 10-phenanthroline)-induced HIF-1 activation in T47D breast tumor cells (IC_{50} 15 μM), and sodwanone V (**60**) was the only sodwanone that inhibited HIF-1 activation in PC-3 prostate tumor cells (IC_{50}15 μM). Sodwanone A (**40**) and sodwanone T (**58**) inhibited hypoxia-induced HIF-1 activation in T47D cells (IC_{50} values 20–25 μM), and sodwanone V (**60**) showed cytotoxicity to MDA-MB-231 breast tumor cells (IC_{50} 23 μM). Sodwanone derived compounds, 3-epi-sodwanone K (**62**), 3-epi-sodwanone K 3-acetate (**63**), 10,11-dihydrosodwanone B (**64**) have been isolated from *Axinella sp*., and **62** and **64** also inhibited hypoxia-induced HIF-1 activation in T47D cells (IC_{50} values 20–25 μM) and **63** was cytotoxic to T47D cells (IC_{50} 22 μM) [31].

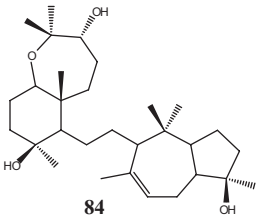




Raspacionin triterpinoids (**65–83**), raspacionin (**65**), raspacionins A (**66**), raspacionins B (**67**), 21-Deacetyl-raspacionin (**68**), 10-Acetoxy-21-deacetyl-28-hydroraspacionin (**69**), 10-Acetoxy-21-deacetyl-4-oxo-28-hydroraspacionin (**70**), 10-Acetoxy-15,21-dideacetyl-4-oxo-28-hydroraspacionin (**71**), 10-Acetoxy-15-deacetyl-4-oxo-28-hydroraspacionin (**72**), 10- Acetoxy-4-acetyl-15-deacetyl-28-hydroraspacionin (**73**), 10-Acetoxy-15-deacetyl-4–21- dioxo-28-hydroraspacionin (**74**), 10-hydroxy-4,21-dioxo-28-hydroraspacionin (**75**), 21-oxo-raspacionin (**76**), 15-deacetyl-21-dioxo-raspacionin (**77**), 4,21-dioxo-raspacionin (**78**), 10-acetoxy-4, 21-dioxo-28-hydroraspacionin (**79**), 10-acetoxy-4-acetyl-21-oxo-28-hydroraspacionin (**80**), 10-acetoxy-4-acetyl-28-hydroraspacionin (**81**), 10-acetoxy-28-hydroraspacionin (**82**), 10-acetoxy-21-deacetyl-4-acetyl-28-hydroraspacionin (**83**), have been isolated from red sponge, *Raspaciona aculeuta Johnston* (family *Raspailiidae*), and from the Mediterranean sponge *Raspaciona aculeata*. All the compounds have showed cytotoxicity against MCF-7 tumor cell line with IC_{50} values between 4 and 8 μM [32–34].



The Red Sea sponge Siphonochalina *siphonella* is a rich source of sipholane triterpenoids including sipholenols (A, C-L) (**84**, **85–94**), sipholenones (A, E) (**95**, **96**), and siphonellinols (C, D, E) (**97**, **98**, **99**). Sipholenol A (**84**) and sipholenone A (Sipholenol B) are the major sipholane triterpenoids [35]. Sipholenol A was found to have increased the sensitivity of resistant KB-C2 cells [36]. Sipholenol A (**84**), sipholenol I (**91**), sipholenol L (**94**), sipholenone A (**95**), sipholenone E (**96**), siphonellinol C (**97**), and siphonellinol D (**98**) have found to show potent reversal of multidrug resistance in cancer cells that over expressed P-glycoprotein. These compounds enhanced the cytotoxicity of several P-glycoprotein substrate anticancer drugs, and significantly reversed the multidrug resistance phenotype in P-glycoprotein-overexpressing multidrug resistant cancer cells KB-C2 and KB-V1 in a dose-dependent manner [37, 38].



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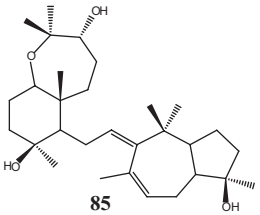

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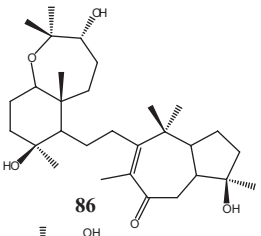

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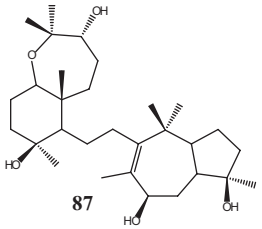

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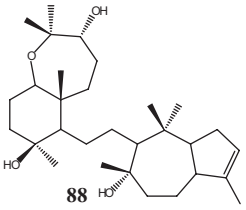

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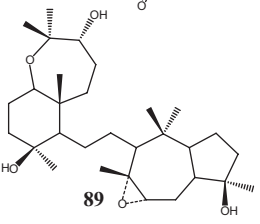

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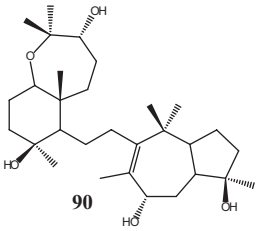

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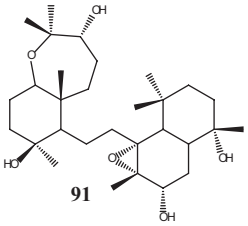

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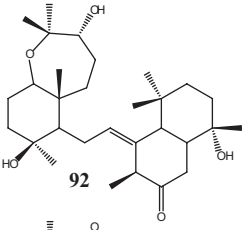

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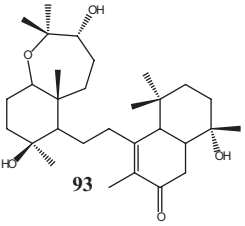

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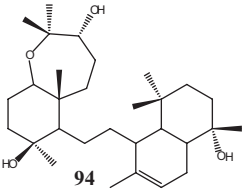

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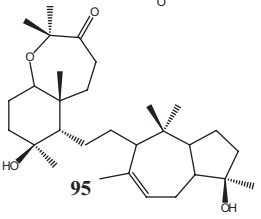

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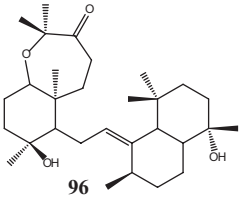

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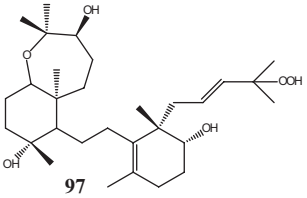

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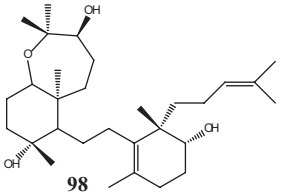

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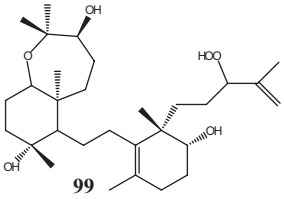

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2.3 Summary

Marine sponges is the most dominant group responsible for discovering a large number of natural compounds, that have been used as template to develop therapeutic drugs. Cytotoxic drugs have an effect of preventing the rapid growth and division of cancer cells. During a last few years, great numbers of biologically active triterpenoids are found to have cytotoxicity against a variety of tumor cells. This chapter summarizes the anti-cancer triterpenoids isolated from marine sponge that includes isomalabaricane, sodwanones, raspacionin, sipholane triterpenoids and their potential anti-cancer activity. Therefore, marine sponges are considered a rich source of chemical diversity and health benefits for developing drug candidates that can be supported to increase the healthy life span of humans.

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Chapter 3
Marine Sponge Derived Antiangiogenic Compounds

Ana R. Quesada, Beatriz Martínez-Poveda, Salvador Rodríguez-Nieto and Miguel Ángel Medina

Abstract Angiogenesis plays an essential role in tumor growth, invasion, and metastasis. Inhibition of angiogenesis has become a major challenge in the development of new anticancer agents, with a countless number of antiangiogenic strategies being tested in preclinical and clinical trials. Nowadays the clinical development of antiangiogenic therapies seems to be unstoppable, not only for cancer, but also for an increasing number of non-neoplasic angiogenesis-related diseases. Although most of the natural compounds previously described as inhibitors of angiogenesis have been isolated from plants and terrestrial microorganisms, increasing attention is being paid to the development of marine-derived antiangiogenic agents. Marine organisms produce interesting and singular pharmacological lead compounds, derived from the large diversity of marine habitats and environmental conditions. Among the many different types of marine organisms used as a source for drug discovery, sponges represent one of the most promising sources of leads in the research of new cancer drugs. There are different strategies for angiogenesis intervention, based on the modulation of any of the different steps of the angiogenic process. In this chapter, we will provide an overview of the angiogenesis inhibitors isolated from marine sponges based on the available information regarding their primary targets or mechanism of action.

Keywords Marine sponge · Angiogenesis inhibitors · Cancer · Aeroplysinin-1

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3.1 Introduction: Angiogenesis in Health and Disease

The term angiogenesis designates the process of formation of new blood vessels from other pre-existing ones. The new vessels are formed by a monolayer of endothelial cells, covering the inside surface of all the vessels, irrespective of their caliber. In mature capillaries, endothelial cells are wrapped by a sheet of extracellular matrix or basement membrane, and are tightly linked to pericytes and smooth muscle cells, which confer stability to the vessel.

Blood vessels form the largest network in our body, being responsible for the supply of nutrients to the whole organism. Angiogenesis is highly activated in embryo and the growing individuals. Nevertheless, in the adult, most blood vessels remain quiescent, the proliferation rate of endothelial cells being very low compared to many other cell types in the body. So, for example, just a 0.01 % of all the endothelial cells of a normal adult vessel are dividing at any given moment, to be compared with the 14 % of epithelial cells within the cell cycle of division [1]. Although angiogenesis is usually blocked, it has to take place in some physiological processes to fulfill the requirements of nutrition and excretion of growing tissues, both along the ontogenetic development and in the adult. Physiological exceptions in which angiogenesis occur under tight regulation are found in wound healing, bone repair and blood flow restoration after an insult or injury. In females, angiogenesis is also present during the reproductive cycle (*corpus luteum* formation, endometrial vascularization) and during pregnancy (placental development) [2].

Being so crucial for physiological functions, angiogenesis must be carefully controlled in order to maintain health. This control is achieved by a tight balance between positive and negative regulatory molecules. When angiogenesis-stimulating growth factors are produced in excess of angiogenic inhibitors, the balance is tipped in favour of blood vessel growth, connecting the so called “angiogenic switch”. In contrast, when inhibitors surpass stimulators, angiogenesis is stopped.

A persistent and deregulated angiogenesis is related to diseases such as cancer, age-related macular degeneration, proliferative retinopathies, psoriasis and rheumatoid arthritis. Moreover, many other inflammatory, allergic, infectious, traumatic, metabolic or hormonal disorders, are characterized by excessive vessel growth that could be related to an upregulated angiogenesis [3, 4]. For this reason angiogenesis research has attracted broad attention in the field of pharmacological, with more than 500 million people worldwide predicted to benefit from pro- or anti-angiogenesis treatments [4]. Today angiogenesis is considered as an organizing principle in drug discovery, allowing the development of therapeutics for one disease to help the development of therapeutics for others [5]. Supporting this idea, drugs that were initially developed for the treatment of cancer patients, have now received the approval for the treatment of age-related macular degeneration, and are being tested in the treatment of a growing number of non-neoplastic diseases.

Antiangiogenesis was proposed as a cancer therapy over 30 years ago by Judah Folkman [6]. Therapeutic strategies based on this new approach, focused to the activated endothelial cells, would be applicable to a wide variety of tumors and because of low mutagenic potential of endothelial cells, tumors do not develop resistance to the effects of many of these inhibitors [7, 8]. Nowadays angiogenesis is widely recognized as a hallmark of cancer [9, 10]. The angiogenic switch can occur at any stage of tumor progression, depending on both the type of tumor and its microenvironment. Many tumors start growing in an avascular phase until they reach a steady state level of proliferating cells. At this point, the angiogenic switch is connected to ensure exponential tumor growth [11]. Moreover, the newly formed vessels allow a pathway for tumor cells to evade the primary tumor and colonize secondary sites. At a given moment, micrometastasis could activate angiogenesis to grow. An extensive number of inhibitors of angiogenesis have been identified. Although the first results arising from the clinical setting made doubt about the real potential of this strategy for the treatment of cancer, now the role of angiogenesis in tumor progression and metastasis is widely accepted, with a continuously increasing number of antiangiogenic drugs and treatment being approved for their clinical use. The accumulating clinical evidences of antiangiogenic therapies in extending survival in patients with advanced cancers and supplying new strategies for the treatment of blindness and other angiogenesis-dependent pathologies has propelled the interest in the clinical development of angiogenesis inhibitors, making of antiangiogenesis one of the more active fields in Pharmacology.

3.2 Tumor Angiogenesis is a Complex Process

Angiogenesis is tightly regulated by a number of molecules that inhibit or activate any of the angiogenesis steps. When resting endothelial cells are activated by an angiogenic signal, they are stimulated to release proteases that will allow them to degrade the extracellular matrix, migrate, proliferate, avoid apoptosis that could be triggered by the loss of survival signals and, finally, differentiate to form new vessels. The quiescent or activated state of the endothelial cell will be a consequence of the activating or inhibiting signals received in a given moment, what will determine the endothelial expression pattern and behaviour. The angiogenic transformation of the endothelium is a complex process involving the activation of very diverse intracellular signaling pathways [12]. Those pathways are related and interconnected, being redundant in some cases. Figure 3.1 illustrates this complexity by showing the interaction of the signaling pathways of some relevant modulators of angiogenesis. A comprehensive understanding of the molecular mechanism of angiogenesis will result in the design of more effective therapeutic strategies.

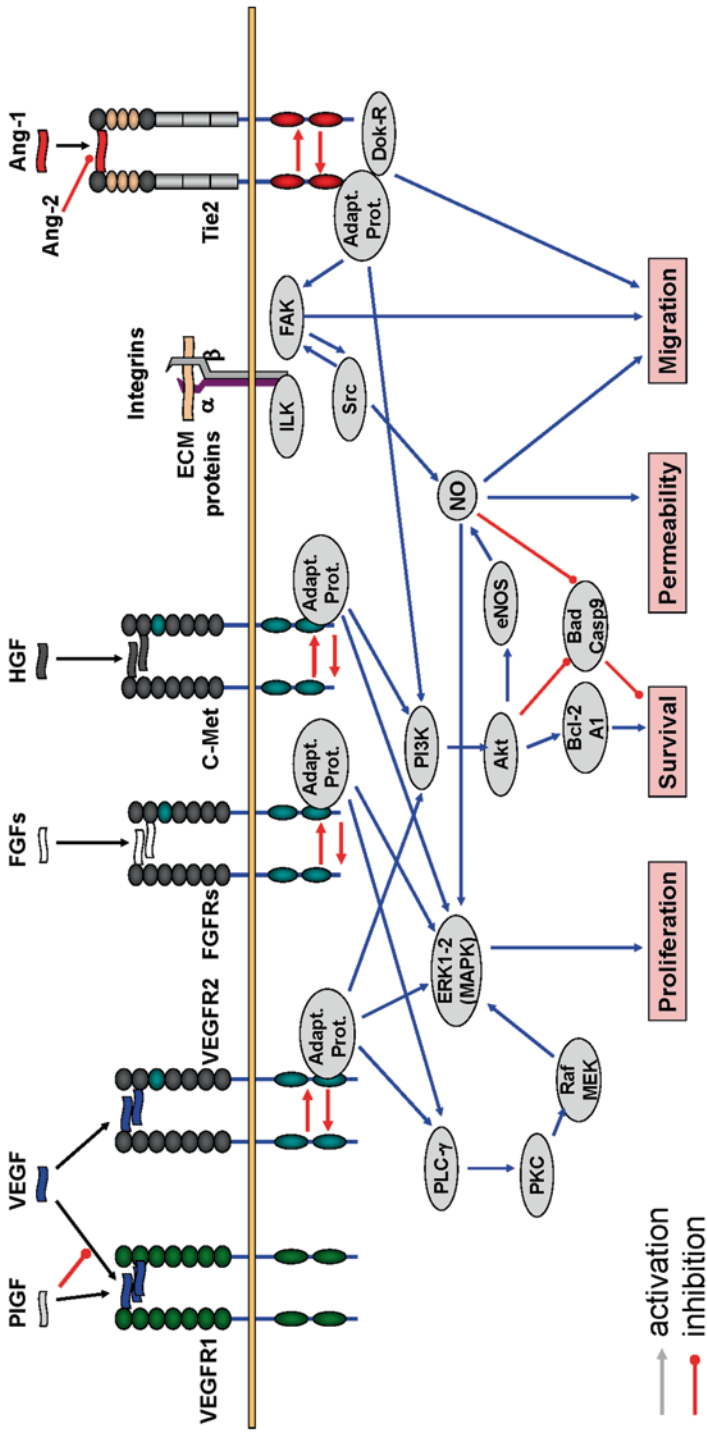

Fig. 3.1 Main pathways for signal transduction in angiogenesis. Ligand binding induces dimerization and autophosphorylation of tyrosine kinase receptors (VEGFR2, FGFRs, c-Met, Tie-2). Receptor activation brings on the recruitment of adaptor proteins, starting a cascade of signals resulting in proliferation, improved survival, loss of intercellular adhesions and migration of endothelial cells. Integrin receptors also contribute to the generation of these intracellular signaling pathways, depending on their binding to proteins of the extracellular matrix. (Adapted from [31])

3.2.1 Tumor Hypoxia may Connect the Angiogenic Switch

Initial tumor stages show a continuous tumor growth without being accompanied by supportive angiogenesis. This growth leads to hypoxic regions inside the tumor. The Hypoxia Inducible Factors (HIFs) mediate transcriptional responses and can promote tumor progression by altering cellular metabolism [13, 14]. Hypoxia has been shown to be an important stimulus for angiogenesis, promoting tumor and stromal cell secretion of potent proangiogenic growth factors via the activation of HIFs. Upregulation of the HIF system is observed in many common cancers and occurs by a multiplicity of genetic and environmental mechanisms. In addition to activation by hypoxia, HIF-induced angiogenesis is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways, as well as after the inactivation of tumor-supressor genes, so that it can be considered a consequence of the oncogenic malignization process [15].

HIFs are heterodimeric transcription factors composed of alpha and beta subunits, which belong to the basic helix-loop-helix family of transcription factors. The beta subunit is constitutively expressed, while the alpha subunit is tightly oxygen-regulated. There are three types of HIFs, HIF-1, HIF-2, and HIF-3, each of them encoded by different genes. Regulation of HIF activity is mediated primarily through the stability of the alpha subunit: under conditions of abundant oxygen, HIF-α proteins are translated but rapidly degraded. On the contrary, in the absence of oxygen, HIF-α proteins stabilize, accumulate, and migrate to the nucleus, where they associate with beta subunits, forming the HIF-1 and HIF-2 heterodimers. By binding to specific HRE (hypoxia response elements) in their promoters, these heterodimers may induce the expression of at least 150 genes encoding proteins that regulate cell metabolism, survival, motility, basement membrane integrity, angiogenesis, hematopoiesis, and other functions. Some relevant activators of angiogenesis, including the vascular endothelial growth factor (VEGF) are included among these genes [16]. In such a situation, angiogenic factors acts on surrounding vessels in order to promote angiogenesis towards the hypoxic focus and alleviate oxygen insufficiency.

Efforts are currently under way to develop targeted cancer therapeutics to hypoxia-activated pathways, and in particular to the search for HIF-1 inhibitors as new antitumoral drugs [16–18].

3.2.2 VEGF-A Plays a Central Role in the Control of Angiogenesis

Among the growth factors that are involved in the angiogenesis process, VEGF seems to play the most relevant role. The family of VEGFs in mammals is composed of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and the placental growth factor (PlGF). Among the different isoforms of VEGF, VEGF-A is the one that mainly

controls tumor angiogenesis [19, 20]. Tumor cells are the major source of this angiogenic factor within the tumor microenvironment. VEGF-A overexpression has been associated with tumor progression and poor prognosis in several tumor systems, including colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, breast cancer, prostate cancer, lung cancer and melanoma [21–23].

VEGF-A plays a central role in the control of angiogenesis. It promotes proliferation, migration, differentiation and survival of the endothelial cells, as well as the activation of the mechanisms of extracellular matrix degradation and vascular permeability. The expression of the VEGF-A gene is regulated by multiple factors, including the cellular hypoxia, and a number of extracellular signals such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF)-1 and several cytokines [24].

All the VEGF isoforms share common tyrosine kinase receptors (VEGFR1 or Flt-1, VEGFR2 or KDR/Flk-1, VEGFR3 or Flt-4). Binding of VEGF-A to the receptor VEGFR2 is probably the main extracellular signal triggering an angiogenic response in endothelial cells. This binding leads to the receptor dimerization and autophosphorylation of the intracytoplasmic domains in specific tyrosine residues located in the carboxy-terminal region, what activates a tyrosine kinase cascade involving various intracellular proteins, particularly phosphatidyl-inositol-3′ kinase [25]. The main VEGFR2-induced proliferative pathway is mediated by the extracellular signal regulated kinase (ERK)-MAPK cascade. VEGFR2 also activates the Akt/PKB pathway, involved in both, the control of cellular survival by inhibiting pro-apoptotic pathways such as B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue (BAD) and Caspase 9, and activation of endothelial nitric oxide synthase (eNOS), implicated in the increase in vascular permeability and cellular migration [12, 26].

VEGFR1 is a positive regulator of monocyte and macrophage migration, and has been described as a positive and negative regulator of VEGFR2 signaling capacity. Finally, VEGF signaling is also modulated through neuropilins, which act as VEGF co-receptors, and by heparan sulfate and integrins [27, 28].

The clinical relevance of VEGF-A for tumor growth can explain why most of the efforts in the development of antiangiogenic drugs have been aimed at neutralizing the activation of endothelial cells by this angiogenic factor, either by blocking VEGF-A or by inhibiting the activation of VEGFR2 [29]. The most successful approach to block VEGF relies on the production of humanized neutralizing antibodies (Genentech/Roche's bevacizumab®), which neutralize the biologically active forms of VEGF-A [30]. Since the first approval in 2004, bevacizumab indications have increased notoriously. Drugs able to inhibit VEGFR2 activation represent a different approach for therapeutic inhibition of angiogenesis. This is the case of an increasing number of low molecular weight tyrosine kinase inhibitors, including sunitinib, sorafenib, pazopanib and axitinib, being approved for the treatment of cancer patients [24, 31].

3.2.3 VEGF-A and Endothelial Cells are not the only Target in Antiangiogenesis

In spite of the above mentioned success of anti VEGF therapies, a critical analysis of the clinical data have shown up several limitations of these therapeutic strategies: the efficacy of a given antiangiogenic drug relies on the type of tumors, no survival benefit is obtained in anti-VEGF monotherapy trials, and only moderate benefits are observed in combination with chemotherapy [32]. The limited clinical success met by anti-angiogenic monotherapies is explained by the high complexity of angiogenesis regulation. Pro and anti-angiogenic factors form a complex network made by multiple, complementary, overlapping and independent signaling pathways. The "angiogenic switch" is controlled by the signals received by endothelial cells from their environment, signals whose transduction pathways will form not only cascades leading to gene transcription but also a network of cross-talks which will determine the final behavior of the cell [12] (Fig. 3.1).

A variety of angiogenesis inducers have been described, some of them specifically acting on endothelial cell, such as the VEGF family and the angiopoietins, whereas others, such as TNF-α and TGF-β may indirectly affect angiogenesis by the release of direct-acting factors from macrophages, endothelial or tumor cells. The angiopoietin family includes four ligands (angiopoietin-1, angiopoietin-2 and angiopoietin-3/4) and two corresponding tyrosine kinase receptors (Tie1 and Tie2). The opposing effects of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) binding with their specific receptor Tie2 play a crucial role in the regulation of the angiogenic remodelling and vessel stabilization that take place after VEGF action [33]. Tie2 signaling promotes vessel assembly and maturation by facilitating recruitment and high association with mural cells and mediating survival signals for endothelial cells. An agonist-antagonist interplay of Ang-1 and Ang-2 allows the Tie-2 signaling pathway to be regulated with a high degree of spatial and temporal precision. Ang-1, secreted by endothelial support cells, activates of Tie2 by tyrosine phosphorylation whereas Ang-2 inhibits the Ang-1 mediated Tie2 activation [34, 35]. In this way, Ang-1 maintains the endothelium in a quiescent state until localised expression of Ang-2 primes the vascular endothelium to exogenous cytokine, such as VEGF, allowing vascular remodelling [36]. In the absence of the mitogenic signal from VEGF, however, endothelial cells are more likely to undergo apoptosis, leading to vessel regression [37]. Although clinical evidences support a relevant role of the angiopoietin-Tie system in the connection of the angiogenesis switch in tumors, the high complexity of the process may difficult the design and development of clear therapeutic strategies to target this pathway [38].

Some other factors, such as the members of the FGF family, activate a broad range of target cells besides endothelial cells. FGF, a superfamily with 19 ligands and four receptors regulates a plethora of developmental processes, including brain patterning, branching morphogenesis and limb development. The prototype member of this group, FGF-2, one of the first angiogenic peptides to be characterized, is expressed at low levels in almost all organs and tissues examined, and found in

many cultured cell types, including fibroblasts, endothelial, smooth muscle, and glial cells [39]. FGF signaling, mediated by FGFRs, follows a classic receptor tyrosine kinase signaling pathway and plays a key role in the maintenance of vascular integrity [40]. A deregulation at various points of its cascade results in malignancy. Members of FGF family have been proposed to participate in the appearance of phenotypic resistance to anti-VEGFR2 therapy, involving vascular regrowth in a VEGF-independent second wave of angiogenesis [41].

The hepatocyte growth factor (HGF) is a protein secreted by fibroblasts and perivascular cells, which is involved in a number of developmental processes, and shows angiogenic activity *in vivo* and *in vitro* [42, 43]. The HGF receptor is encoded by the proto-oncogen c-Met, a tyrosine-kinase receptor mainly expressed by epithelia and also present in the endothelium, where it is induced by hypoxia. HGF binding to c-Met induces dimerization, autophosphorylation, activation of tyrosine kinase catalytic activity and docking of substrates such as Gab-1, Grb2, Shc and c-Cbl [44]. The proliferative response induced in endothelial cells by HGF involves activation of the ERK-MAPK pathway and requires of eNOS function (the endothelial isoform of the nitric oxide synthase) [45]. On the other hand, the antiapoptotic effect of the HGF/c-Met axis is mediated by PI3K/Akt activation [46]. c-Met is frequently expressed in cancer, its activation resulting in increased proliferation, invasion and survival of cancer cells. Thus c-Met is an interesting target for inhibition of both, tumor growth and tumor angiogenesis [47].

In order to establish a functional vascular network, during the sprouting process endothelial cells must undergo a functional specialization. The specialized endothelial tip cells follow guidance signals and migrate extensively into avascular tissues while trailing stalk cells must stay connected to the patent blood vessel [48]. Such organization is under control of the Dll4(Delta-like 4)/Notch signaling pathway, which sets a hierarchy in receptiveness of cells to VEGF-A. Notch receptor are large transmembrane proteins that regulate cell fate in multiple lineage when are activated by their ligands expressed on adjacent cells [49]. During sprouting angiogenesis, tip cell formation is the default response to VEGF (vascular endothelial growth factor), whereas the stalk cell phenotype is acquired through Dll4/Notch-mediated lateral inhibition. Notch signaling plays an important role for in determining how an endothelial cell responds to VEGF regulating multiple components of the VEGFR system. In addition, VEGF signals regulate the expression of both, Notch and Dll4. The interplay between VEGF and Dll4/Notch signaling, which has revealed to be critical for the angiogenic process, opens exciting possibilities for combined therapies simultaneously targeting both pathways [50–52].

Even when most studies on angiogenesis and tumor growth and on anti-angiogenesis as a therapeutic approach have paid attention to endothelial cells, based on the observation that many tumor cell lines directly stimulate the endothelium, other cell types are involved in angiogenesis modulation. Stroma cells, including fibroblastic, immune and inflammatory cells infiltrating the tumor, including monocytes/macrophages, mast cells, neutrophils, and T lymphocytes, secrete angiogenesis activators, angiogenesis inhibitors and chemokines that significantly contribute to the modulation of angiogenesis [53–55]. The interaction between pericytes and

endothelial cells is important for the maturation, remodelling and maintenance of the vascular system via the secretion of growth factors or modulation of the extracellular matrix [56, 57]. Pericyte recruitment, in response to growth factors such as platelet-derived growth factor (PDGF) is part of the development of normal and mature functional capillaries [58]. Tumor vessel pericytes have multiple abnormalities, including a bizarre morphology, lower density, disorganized cytoplasmic processes that extend away from the blood vessel wall into tumor tissue, and a looser connection with endothelial cells [59]. VEGF-targeting therapies are mostly active on immature vessels lacking pericyte coverage, and pericyte mediated survival signals confer resistance to VEGF antagonists, what advises the use of multitargeted approaches to reach an effective inhibition of tumor angiogenesis [60]. The convenience of targeting both, endothelial cells and pericytes, by combined inhibition of VEGF and PDGF signaling may help to explain the clinical success of multikinase inhibitors [61–63].

A final thought should be devoted to the multiple roles played by the extracellular matrix (ECM) in the angiogenesis process. Behind a classical structural role hides a powerful conductor to shape the branching pattern of vessels. ECM, by interacting with angiogenic factors, such as VEGF-A, modulates their availability, their gradient organization and signaling properties. Also, by engaging a specific array of integrins, ECM can indirectly signal through the Notch pathway by controlling Dll4 ligand expression. ECM mechanically influences cellular tension and cytoskeleton organization, required for sprouting, and provides structural and functional support. Moreover, ECM is a source of anti-angiogenic peptides which tune the angiogenic response in tissues by liberating endogenous inhibitors of angiogenesis, many of which are fragments generated by partial proteolysis of naturally occurring ECM proteins [64–66].

In conclusion, the high complexity of tumor angiogenesis, regulated by multiple, complementary, overlapping and independent pathways of multiple factors secreted by tumor cells and their surrounding host stromal cells, advises the use of effective combination therapies that block multiple angiogenic pathways simultaneously to slow down or reverse the progression of malignant cancer [63]. Alternative angiogenic pathways, including those of PlGF, PDGF, Ang/Tie, FGF, HGF and Notch, among others, would therefore provide new pharmacological target for the development of angiogenesis inhibitors that could prolong the duration of anti-VEGF treatments and extend their clinical benefits.

3.3 Marine Sponges as a Source of Antiangiogenic Compounds

The vast majority of the natural compounds that have been previously described as inhibitors of angiogenesis have been isolated from plants and terrestrial microorganisms, mainly due to their higher availability and because their therapeutic effects had been previously known in folk traditional medicines [67]. However,

increasing attention is being paid to the development of marine-derived antiangiogenic agents, probably fuelled by the increase in the number of marine-derived anticancer drugs which are being successfully used for cancer therapy, including trabectedin (PharmaMar's Yondelis®), which represents the first anticancer drug isolated from a marine source [68–71]. Nowadays is widely acknowledged that marine organisms produce interesting and singular pharmacological lead compounds, derived from the large diversity of marine habitats and environmental conditions. Many marine organisms have adapted to survive in extreme environments by developing chemical means of defence. Marine organisms have evolved to produce toxic metabolites or to obtain them from marine microorganisms, being an unexplored and prolific source of molecular diversity, and yielding an increasing number of products of the highest interest for drug discovery [72]. Since these compounds are normally released into the water and rapidly diluted they need to be highly potent to have any effect [73]. Over the last few years, samples from the marine environment have been screened for a variety of compounds with different biological activities. Among the many different types of marine organisms used as a source for drug discovery, sponges represent one of the most promising sources of leads in the research of new cancer drugs [74]. The actual source of the bioactive secondary metabolites remains unsolved, since the microorganisms harboured by the sponges on their surfaces, in their canal systems, and in their intercellular spaces, may contribute up to 40% of their total cellular content and are probably the major source of the compounds isolated from marine sponges [75].In this regard, some angiogenesis inhibitors isolated from marine sponges have been described by us and others [76–81]. There are different strategies for angiogenesis intervention, based on the modulation of any of the different steps of the angiogenic process. Although in many cases the molecular mechanisms of the antiangiogenic activity of the marine sponge-derived compounds is not fully known, for the purposes of this chapter we have tried to classify them according to the step of angiogenesis they are acting on (Fig. 3.2).

3.3.1 Compounds that Inhibit the Activation of the Angiogenic Switch

Several compounds have been described to act on the mechanisms employed by hypoxic tumor cells to promote angiogenesis through the HIF-1-dependent induction of VEGF. Mycothiazole (Fig. 3.3), a metabolite isolated from a *Petrosaspongia mycofijiensis* sponge, inhibited hypoxic HIF-1 signaling in tumor cells in the nanomolar range, what correlated with the suppression of hypoxia-stimulated VEGF secretion by tumor and angiogenesis in vitro. Nevertheless, the high neurotoxicity of this compound, that selectively suppresses the mitochondrial respiration at complex I (NADH-ubiquinone oxidoreductase) prevents its use as an anticancer drug [82]. In the same way, the potential therapeutical application of the lipophilic 2,5-disubstituted pyrroles HIF inhibitors, from the marine sponge *Mycale* sp., is limited by

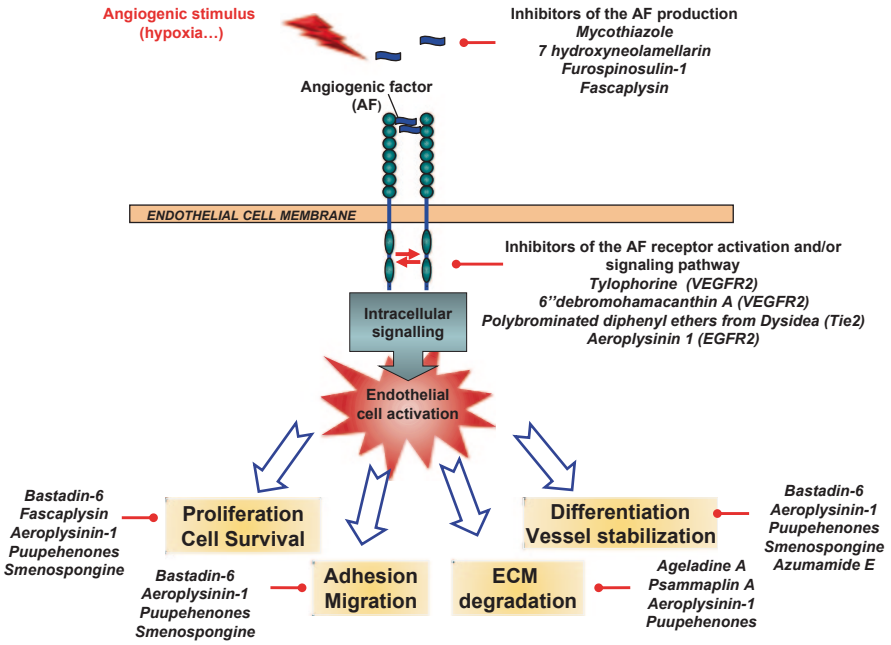

Fig. 3.2 Mechanism of action of some sponge derived-antiangiogenic compounds

the high toxicity expected from their inhibitory activity of mitochondrial respiration [83].

7-hydroxyneolamellarin A, isolated from the marine sponge *Dendrilla nigra* was found to inhibit hypoxia-induced HIF-1 activation at micromolar concentrations and the hypoxic induction of VEGF in T47D human breast tumor cell [84]. Further investigation on the mechanism of action and toxicities of this compound are required to establish its potential as a molecular-targeted antitumor agent.

Furospinosulin-1 (Fig. 3.3), a furanosesterterpene from the Indonesian marine sponge *Dactylospongia elegans*, exhibited hypoxia-selective growth inhibition and antitumor activity. Nevertheless very high concentrations of this compound were required to slightly inhibit the HIF-1α accumulation and VEGF production by hypoxic cancer cells. These results suggest that the hypoxia-selective growth inhibition of cancer cells by furospinosulin-1 is not derived from inhibition of HIF-1α function. Mechanistic analysis of furospinosulin-1 revealed that the observed hypoxia-selective growth inhibition may be a result of inhibition of IGF-2 expression [85].

Fascaplysin (Fig. 3.3), a red pigment originally isolated from Fijian marine sponge *Fascaplysinopsis sp*. exhibited broad range of bioactivities including the specific inhibition of cyclin-dependent kinase-4 CDK4/D1 [86]. This carboline class alkaloid caused a decrease in the number of capillary plexus formation according to concentrations in CAM, and suppressed VEGF expression in this model. Moreover, it inhibited the VEGF expression and secretion by human hepatocarcinoma cells

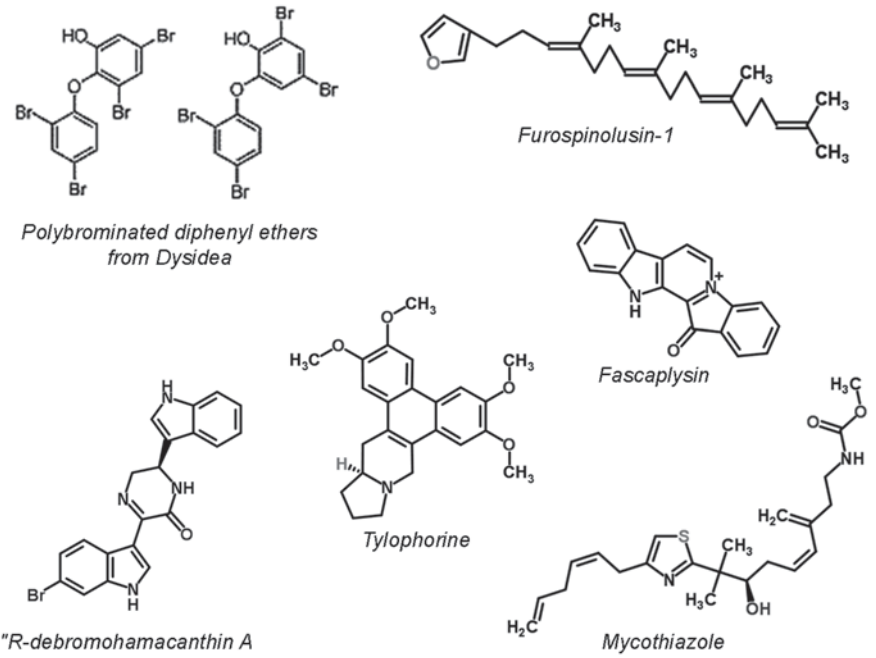

Fig. 3.3 Some sponge derived-compounds that inhibit the activation of the angiogenic switch

BeL-7402, suggesting that a blockade of VEGF expression could be involved in the antiangiogenic activity of this compound [87]. The down-regulation of some cell adhesion molecules and the decreased expression of endothelial cell markers-CD31 in a sarcoma mice model reinforce the conclusion that fascaplysin can inhibit angiogenesis in tumor tissue [88].

Tylophorine (Fig. 3.3), a phenanthraindolizidine alkaloid isolated from *Tylophora indica* has been shown to inhibit VEGF-stimulated endothelial cell proliferation, migration and tube formation in vitro, as well as neovascularization in a sponge implant angiogenesis assay in vivo and further attenuated tumor associated angiogenesis. Tylophorine inhibited VEGFR2 binding with VEGF, attenuated VEGFR2 tyrosine kinase activity, and further downregulated VEGFR2-mediated signaling pathway. Molecular docking simulation indicated that tylophorine could interact with the ATP-binding sites of VEGFR2 kinase domain. All these data indicate that tylophorine exerts potent anti-angiogenesis activities via specifically targeting VEGFR2 and its signaling pathway, suggesting that it could be a promising candidate for development of anti-angiogenesis agents [89].

6″-debromohamacanthin A (Fig. 3.3), an active component of the hamacanthins found in a few marine sponges, including *Spongosorites sp*., significantly inhibited the VEGF-induced cell proliferation, migration and tube formation in endothelial cells. Antiangiogenic activity of this compound was confirmed in the microvessel sprouting of mouse aortic rings ex vivo model. 6″-debromohamacanthin A suppressed

the VEGF-induced expression of MAPKs (p38, ERK and SAPK/JNK) and the PI3K/AKT/mTOR signaling pathway, suggesting that it could inhibit angiogenesis by targeting the VEGFR2 signaling pathways in endothelial cells [90].

As mentioned before, the success of VEGF targeting strategies depends on the pericyte coverage of capillaries, maintained through PDGFR signaling. PDGFR inhibitors act simultaneously on the regulation of interstitial fluid pressure, a common feature of solid tumors and is thought to impede transcapillary transport of chemotherapy, as well as in the function of pericytes, making endothelial cells more susceptible to anti-VEGF drugs [91, 92]. Recently, a number of hamacanthin-derived pyrazin-2(1H)-ones have shown interesting properties as lead for the further development of highly potent and selective PDGFRβ-inhibitors. Modeling studies showed the core moiety of hamacanthins to bind in the ATP binding pocket of the receptor tyrosine kinase, and suggested a straightforward strategy towards potent PDGFRβ binders [93].

As previously stated, Ang-2 regulates tumor angiogenesis in cooperation with VEGF as well as Ang1 through the Tie2-dependent pathways. Two polybrominated diphenyl ethers isolated from the crude extract prepared from a sponge of the *Dysidea* genus were reasonably potent inhibitors of Tie2 kinase exhibiting IC50 values at the micromolar range [94]. No data about the putative antiangiogenic activity of these compounds have been reported. New evidence suggesting multiple roles for Ang-2 in angiogenesis in physiologic processes and invasive phenotypes of cancer cells during progression of human cancers should be taken into account in the clinical development of Ang-2 inhibitors [95]

Other kinase inhibitors from marine sponges that could potentially be used to inhibit angiogenesis include some EGFR inhibitors (reviewed in [96]) and c-Met inhibitors, such as simplextone C, isolated from the South China Sea sponge *Plakortis simplex* [97]. Nevertheless in most cases, there are not available data regarding their putative antiangiogenic activity.

Aeroplysinin-1, a brominated metabolite extracted from the marine sponge-*Aplysina aerophoba*, is a potent and selective inhibitor of angiogenesis ([76]; Figs. 3.5 and 3.6). Previous reports suggested that this compound inhibited the tyrosine kinase activity of EGF receptor in vitro and in vivo [98, 99], although this effect has been questioned by others [100]. Nevertheless, data from our laboratory indicate that the mechanism of action of aeroplysinin-1 does not rely on a direct inhibition of the autophosphorylation of the angiogenic factors receptors, but on a selective effect on activated endothelial cells [78].

3.3.2 Compounds that Inhibit the Extracellular Matrix Remodelling

To initiate the formation of new capillaries, endothelial cells of existing blood vessels must loosen inter-endothelial cell contacts, to relieve periendothelial cell support and break down the surrounding ECM. Breakdown of the ECM is mediated by

several proteinase families, including plasminogen activators (such as urokinase plasminogen activator (uPA) and its inhibitor, PAI-1), matrix metalloproteinases (MMPs and tissue inhibitors of metalloproteinases (TIMPs)), chymases, heparanases, tryptases, cathepsins, and kallikreins (and their inhibitors kallistatin) [101, 102]. The uPAs and tPAs are serine proteases that convert plasminogen into plasmin. Plasmin has broad substrate specificity and degrades several ECM components, including fibrin, fibronectin, laminin, and the protein core of proteoglycans. In addition, plasmin can also degrade the ECM indirectly through activation of pro-matrix metalloproteinases (pro-MMPs) [103]. The interaction of uPA with its receptor (uPAR) concentrates the enzyme activity to the focal attachment sites on the cell surface and stimulates signal transduction through the uPAR, leading to induction of cell migration and invasion [104].

MMPs are a family of zinc-containing proteases that degrade ECM proteins and are critical in vascular remodeling, cellular migration, and sprout formation. MMP activity is upregulated in endothelial cells during inflammation, wound healing and tumor growth, what shows their important role in both physiologic and pathologic angiogenesis [102]. MMPs may be either secreted as inactive pro-peptides which are often activated by a cascade of cell surface protease activity, or membrane bound (MT-MMP). In theory, MMPs are one of the most promising targets to fight cancer and other diseases, due to the dual benefits of inhibiting tumor angiogenesis and metastasis, as these processes both require significant ECM degradation. But clinical results show that things are not so simple [105]. In addition to their role in extracellular matrix turnover and cancer cell migration, MMPs regulate signaling pathways that control cell growth, inflammation, or angiogenesis and may even work in a non-proteolytic manner [106]. These pleiotropic activities may explain why proteinases and their receptors and inhibitors often have activities that are context- and concentration-dependent and may have contributed to the disappointing results obtained in clinical trials with inhibitors of proteases [31].

The activity of both PAs and MMPs is controlled at different levels: (i) by proteolytic activation, since they are secreted as proenzymes; (ii) by their respective endogenous inhibitors, PAIs and TIMPs, and (iii) their expression is up-regulated by angiogenic growth factors and and cytokines. Examples of marine sponge derived compounds acting on any of those levels can be mentioned.

Ageladine A (Fig. 3.4), from the marine sponge *Agelas nakamurai Hoshino* inhibited in vitro the migration of endothelial cells, as well as the vascular organization model on type-I collagen gel using mouse vascular progenitor cells. Ageladine A has shown inhibition at micromolar levels against various matrix metalloproteinases, including MMPs- 1, 2, -8, -9, -12, and -13. The inhibition mechanism of this compound is presumed to be different from those of other MMP-2 inhibitors, since ageladine A was not capable to chelate Zn^{2+} [107]. As a novel structural and biological lead in this field, has spurred the interest for total synthesis [108, 109]. Recently, some ageladine A analogs showing more potent MMP-12 inhibitory activity than the natural product have been synthetized [110–112].

The enzyme aminopeptidase N (APN, also known as CD13) is a Zn^{2+} dependent membrane-bound ectopeptidase that degrades preferentially proteins and peptides

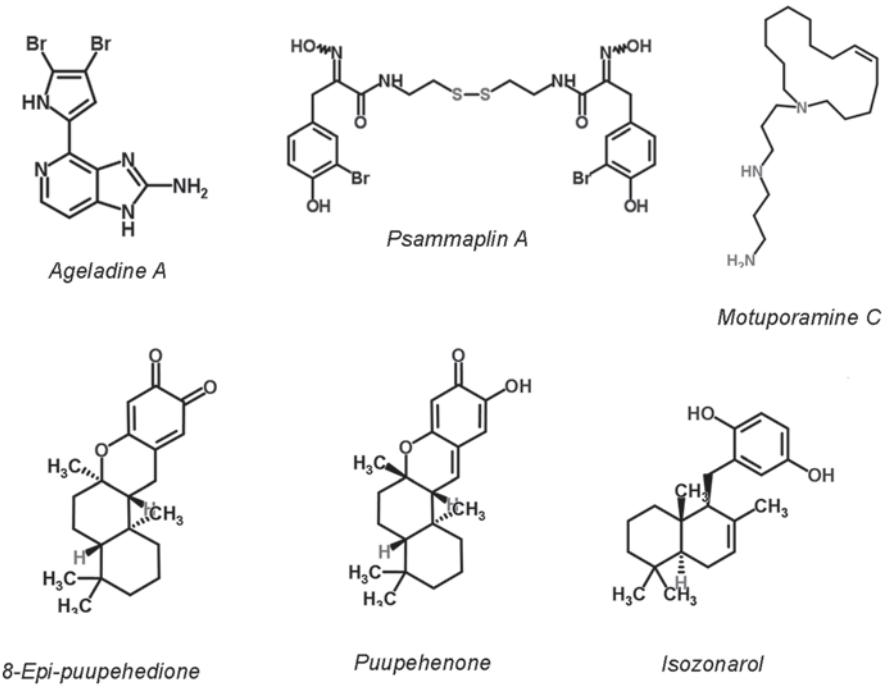

Fig. 3.4 Some sponge derived-compounds that inhibit the extracellular matrix remodeling

with a N-terminal neutral amino acid. Recent experimental data indicate that APN is an important regulator of endothelial morphogenesis during angiogenesis and suggest that this protease could be considered a novel target in the search for new antiangiogenic drugs [113, 114]. Psammaplin A (Fig. 3.4) is a brominated tyrosine derived natural product that moderately inhibited chitinases and displayed antifungal activity [115]. The wound-activated conversion of psammaplin A sulfate to psammaplin A has been proposed as an example for activated chemical defense, i.e., the rapid conversion of precursor molecules to defensive compounds following tissue damage in marine sponges [116]. Psammaplin A is a non-competitive inhibitor of APN, inhibiting the proliferation of several cancer and endothelial cells and suppressing the invasion and tube formation of endothelial cells stimulated by FGF2. The inhibitory effect of psammaplin A on APN activity may be crucial for its anti-angiogenic activity [117]. The interest of psammaplin as a novel anticancer drug, acting on multiple molecular targets, does not retrict to its antiangiogenic activity. In addition to the previously reported activity as a topoisomerase II inhibitor, this compound has been described as a new histone deacetylase and DNA methyltransferase inhibitor, epigenetic modifiers in the silencing of tumor supressor genes [118]. Because of their multi(epi)target features and their action in ex vivo samples, psammaplin A and its derivatives are attractive molecules for the modulation of epigenetic disorders [119, 120]. Recent pharmacokinetics and tissue distribution

studies indicate a preferential distribution of psammaplin A to lung, suggesting the potential of this compound as a lung cancer treatment agent [121].

The motuporamines are a family of relatively simple macrocyclic alkaloids containing a spermidine-like substructure, isolated from the sponge *Xestospongia exigua* [122]. The antiangiogenic motuporamine C (Fig. 3.4) neither inhibited angiogenesis through toxic or antiproliferative effects, nor induced apoptosis in endothelial cells. Experimental data suggest that the drug acts to inhibit cell-mediated degradation of extracellular matrix and/or inhibit cell motility [123]. Motuporamine C inhibited both tumor cell invasion and VEGF-induced endothelial cell angiogenesis at doses that were not cytotoxic to cancer cell lines and human umbilical vein endothelial cells (HUVECs), what might be relevant with respect to the possible therapeutic usefulness as an antiangiogenic or antimetastatic drug.

Alternative mechanisms of inhibition of the ECM degradation by endothelial cells consist of the use of drugs that either inhibit the expression, secretion or activation of proteases, or increase those of their inhibitors. Among them, we could mention some marine derived angiogenesis inhibitors identified in our laboratory: aeroplysinin-1 and puupehenone related compounds.

The production of aeroplysinin-1 from an enzymatic cleavage of the brominated oxazoline alkaloids reservoir activates in the sponges a chemical defense mechanism from preys and from the invasion of bacterial pathogens after wound [124, 125]. Aeroplysinin-1 exerts a pleiotropic effect on activated endothelial cells, acting on several steps of angiogenesis (Fig. 3.6). Recently, the conformational properties of aeroplysinin-1 in aqueous solution have been determined by means of a combined experimental and theoretical Raman optical activity and vibrational circular dichroism study, allowing the visualization of the subtle interplay among the flexible groups of the molecule, in particular the two polar hydroxyls [126]. Aeroplysinin-1 inhibited the proteolytic capability of endothelial cells by displacing their proteolytic balance towards antiproteolysis. MMP-2, constitutively secreted by endothelial cells, is probably the main MMP involved in angiogenesis, contributing to trigger tumor angiogenesis in vitro e and in vivo [127]. Gelatin zymography studies showed that the concentration of MMP-2 in the medium conditioned by aeroplysinin-1 treated endothelial cells was clearly lower than that of untreated cell. Furthermore, the treatment with aeroplysinin-1 also affected the balance of PA/PAI levels, other system that has been involved in extracellular matrix remodeling occurring in angiogenesis [128]. Aeroplysinin-1 induced an important decrease in the expression of PA protein and a parallel increase in the expression of PAI protein in both conditioned media and cell extracts. Taking the results obtained with zymographies altogether, they clearly show that aeroplysinin-1 induces a shift in the proteolytic balance towards antiproteolysis [76]. Since invasion is dependent on extracellular matrix remodelling capabilities, this inhibitory effect strongly suggests that the two key extracellular membrane remodeling enzymes expressed by endothelial cells, namely, MMP-2 and uPA could be a key target of the pharmacological action of aeroplysinin.

Results of the inhibition of MMP-2 expression after incubation with aeroplysinin-1 were consistently reproduced in HUVEC and other three different human

endothelial cells tested (RF-24, HMEC and EVLC-2), irrespective of their origins [129]. Moreover, expression studies suggest that aeroplysinin-1 decreases the expression levels of MMP-1 in endothelial cells. MMP-1, also known as interstitial collagenase, is the only enzyme able to initiate the breakdown of the interstitial collagens (types I, II and III), and it is involved in the pathogenesis of inflammatory diseases and is upregulated by inflammation [130–132].

Puupehenone, isolated from the deep water marine sponge *Stronglyophora hartmani*, and related compounds (Fig. 3.4) have been reported to display a wide range of important biological functions including antiviral, antifungal, antimalarial and antitumor activities. Some members of this family exhibited antiangiogenic properties in vitro and in vivo, inhibiting the capability of endothelial cells to form capillary-like tubes and to remodel and invade extracellular matrix. The antiangiogenic activity of 8-epipuupehedione, isozonarol and 8-epi-9,11-dehydropuupehediol could be related to a decrease in the expression of uPA protein in endothelial cells [77]. Moreover, the capability of 8-epipuupedione to inhibit the extracellular matrix remodeling potential does not restrict to endothelial cells. Our results showed that this compound inhibited MMP-2 secretion by HL-60 promyelocytic leukaemia cells, whereas it was not a direct inhibitor of the MMP enzymatic activity. 8-epipuupehedione strongly inhibited the urokinase production and induced the expression of the specific endogenous inhibitor of urokinase by HL-60 cells, reinforcing the hypothesis that this compound is able to shift the cell proteolytic balance towards antiproteolysis and suggesting its potential for the treatment of promyelocytic leukaemia [133].

3.3.3 Compounds that Inhibit One or Several Specific Functions of Activated Endothelial Cells

In vitro assays provide a valuable tool for assessing effects of anti-angiogenic agents and have been used in the selection of new putative antiangiogenic drugs (reviewed in [134, 135]). Cell culture techniques can be used to identify those endothelial cell functions affected by the antiangiogenic compound. In vitro models of angiogenesis have focused to date predominantly on migration, proliferation and tubule formation by endothelial cells in response to exogenous inhibitory or stimulatory agents. The available information regarding the mechanism of the antiangiogenic activity of a given compound has been restricted by the methodological capabilities of any given research group with focus on one or several steps of the angiogenesis process.

Following matrix degradation, endothelial cells move, via a process called chemotaxis, along a gradient of angiogenesis-inducing factor(s) such as VEGF. Cell motility is of particular interest in the design of anti-cancer therapeutics, as cell migration is required for both tumor invasion and tumor angiogenesis (reviewed in [136]). Assays that allow measurement of endothelial cell motility in response to added factors include the modified Boyden chamber assays (simplified by the use of transwells) and the scratch or “wound healing” assay [137]. Some sponge derived

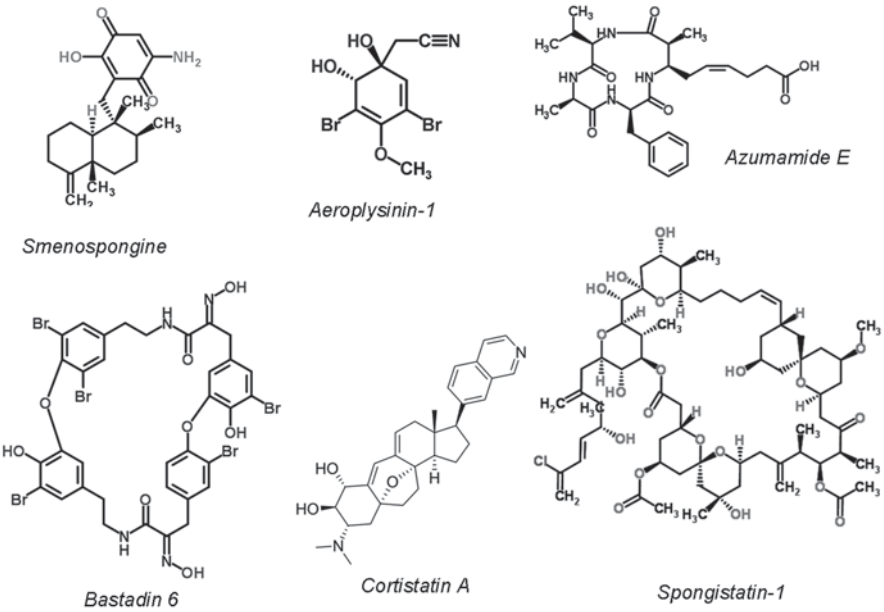

Fig. 3.5 Some sponge derived-compounds that inhibit one or several specific functions of activated endothelial cells

compounds that have been reported to inhibit endothelial cells migration include the above mentioned aeroplysinin-1, pupehenone and derivatives, and smenospongine, a sesquiterpene aminoquinone isolated from the Indonesian sponge *Dactylospongia elegans*, which also showed multifaceted antitumor activities on leukemia cells ([138]; Figs. 3.4 and 3.5).

Angiogenesis requires the assembly of endothelial cells into vessel tubes. The tube formation stage of angiogenesis can be modelled in vitro by plating endothelial cells with extracellular matrix components. The most widely used assay for the identification of endothelial cell morphogenesis involves plating endothelial cells on Matrigel, an extracellular matrix isolated from Engelbreth–Holm–Swarm mouse sarcoma cells. This assay is easy to handle and can be adapted for large-scale screening. For this reason it has been one of the favourite tools for the identification of new antiangiogenic drug [139]. This is the case of compounds such as aeroplysinin-1 and puupehenones, which were selected by their capability to inhibit endothelial cell morphogenesis at non toxic concentrations, and further characterized for their in vitro and in vivo antiangiogenic activity. Another example could be that of azumamides A–E, a group of histone deacetylase inhibitors isolated from the marine sponge *Mycale izuensis* [140]. Histone deacetylases play a key role in regulating gene expression by deacetylating histones and their inhibitors have generated strong interest as antitumor agents, because of their effect on apoptosis, cell cycle arrest and inhibition of angiogenesis [141]. Azumamide E (Fig. 3.5) exhibited

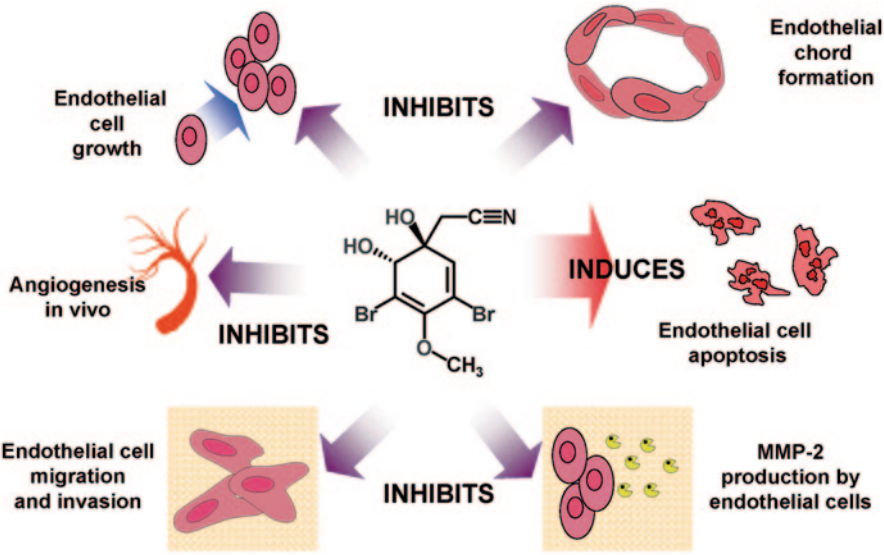

Fig. 3.6 Aeroplysinin-1 inhibits angiogenesis in vitro and in vivo

the highest antiangiogenic activity in an in vitro vascular organization model, what correlated with the histone deacetylase inhibitory activity [142].

Endothelial cell proliferation, combined with increased survival, supplies the cells that make up a new vessel. The antiproliferative effect of the antiangiogenic drugs can be measured by direct cell counts, quantification of DNA synthesis, or assessment of metabolic activity when the culture medium contains normal levels of serum and/or growth factors. Several of the best characterized anti-angiogenic compounds were initially detected and selected for their capability to interfere with endothelial cell growth. This is the case of the selective inhibitor of endothelial cell proliferation TNP-470, a synthetic analog of fumagillin with enhanced anti-angiogenic properties [143]. However, the desirable endothelial cell specificity of this effect is not a common feature [144]. A number of compounds isolated from marine sponges have demonstrated to inhibit selectively the endothelial cell growth, either by an inhibition of cell proliferation, or by induction of endothelial cell apoptosis.

Cortistatin are a group of steroidal alkaloids isolated from the marine sponge *Corticium simplex* on the basis of bioassay guided separation. Cortistatins A–D exhibited selective inhibition of endothelial cells (HUVEC) proliferation [145]. The most potent member of the family, cortistatin A (Fig. 3.5) demonstrated a remarkable selectivity index of more than 3000-fold in comparison with normal human dermal fibroblast (NHDF) and several tumor cells (KB3-1, K562 and Neuro2A). Cortistatin A also inhibited the VEGF-induced migration of HUVECs and the FGF-induced tube formation on Matrigel [146]. Although the activation of ERK1/2 MAP kinase and p38 MAP kinase pathway by angiogenic factors are the main responsible of the increase in proliferation and migration in activated endothelial cells,

cortistatin A showed no effect on VEGF-induced phosphorylation of ERK1/2 and p38 in HUVEC. Nevertheless, the phosphorylation of an unidentified 110 kDa protein in HUVECs was inhibited by the treatment with cortistatin A and has been postulated to be involved in the mechanism of action of this compound.

In addition to cortistatin A, ten related natural products have been isolated from the same sponge, one of which, cortistatin J, also exhibited good antiangiogenic activity in vitro [147, 148]. To date, the exact cellular target of the cortistatins has not been determined, nor in vivo studies have been reported. Although several total syntheses of cortistatin A have been published, very small quantities of the synthetic compound have been prepared [149]. The structural complexity of this compound has aimed to the design and synthesis of cortistatin analogues that have equal or greater biological activity, but are structurally less complex and more readily available for further biological studies than the natural product [150].

Globostellatic acid X methyl esters and isomarabarican-type triterpenes, isolated from *Rhabdastrella globostellata*, exhibited selective anti-proliferative activity as well as bFGF-induced tubular formation and VEGF-induced migration in HUVECs [151]. It induced apoptosis in endothelial cells, and the antiproliferative activity did not correlated to an inhibition of the VEGF-induced phosphorylation of ERK1/2 in HUVECs. Some structurally simplified model compounds of globostellatic acid X methyl ester have been synthesized, although their antiproliferative activity was lower than the original compound [152].

Spongistatin 1 (Fig. 3.5), the most cytotoxic member of the spongistatin family, is a macrocyclic lactone polyether isolated from a *Spongia* species [153]. Showing an average IC50 value of 0.12 nM against the National Cancer Institute's panel of 60 human cancer cell lines, exhibited low cytotoxicity against quiescent human fibroblasts. Consistent with a microtubule-targeting mechanism of action, spongistatin 1 caused mitotic arrest in cancer cells [154]. Spongistatin 1 induces apoptotic cell death through both caspase-dependent and -independent mechanisms, and positive results from an orthotopic in vivo model of human pancreatic cancer suggest its potential as a promising experimental drug [155, 156]. Spongistatin 1 showed strong antiangiogenic effects in vitro and in vivo. As in cancer cells, probably the most relevant effect of this compound on endothelial cells is the inhibition of cell proliferation, which is exerted at non-toxic concentrations. The antiangiogenic effect of spongistatin 1 seems to be due to nonmitotic effects and have been proposed to hint toward an as yet unknown mechanism of tubulin antagonism during angiogenesis: the inhibition of protein kinase Cα (PKCα) translocation [157].

Antiangiogenic therapy can inhibit further endothelial proliferation in a tumor bed or induce endothelial cell apoptosis, depending on the potency of the antiangiogenic therapy versus the total angiogenic output which it must overcome [158]. It has been suggested that endothelial cell apoptosis, induced by a variety of mechanisms, might be responsible for the angiogenesis-inhibitory activity of a number of endogenous and exogenous angiogenesis inhibitors including thrombospondin-1, pigment epithelium-derived factor, adiponectin, neovastat, and IB05204, among many others [159–162]. During apoptosis, extrinsic or intrinsic signals activate caspases, which in turn induce DNA fragmentation, DNA budding, and

the chromatin condensation characteristic of programmed cell death (reviewed in [163]). The induction of endothelial apoptosis can also be a relevant mechanism of the antiangiogenic activity of some sponge isolated compounds.

Fascaplysin (Fig. 3.3) inhibited human umbilical vein endothelial cell proliferation [87] and activated apoptosis in those cells. Fascaplysin arrested cell cycle at G1 and induced apoptosis in HUVEC cells in a dose- and time-dependent manner, in addition to blocking VEGF in the anti-angiogenesis process. The apoptogenic activity of fascaplysin was exerted at lower concentrations than those required to inhibit proliferation, indicating that the induction of apoptosis through the mitochondrial pathway could be a relevant mechanism of its antiangiogenic activity. The induction of active caspase-3, and decreases of procaspase-8, Bid, and the ratio of Bax/Bcl-2 further supported this conclusion [164].

Bastadin 6 (Fig. 3.5), a macrocyclic tetramer of a brominated tyrosine derivative, was isolated from the marine sponge, *Ianthella basta*. Bastadin 6 exhibited selective inhibition of endothelial cells (HUVEC) proliferation in comparison with normal fibroblast (3Y1) or several tumor cells (KB3-1, K562 and Neuro2A). Bastadin 6 also inhibited the VEGF- or FGF-induced tubular formation and VEGF-induced migration. The in vivo antiangiogenic activity of this compound was demonstrated by the VEGF- or FGF-induced in vivo neovascularization in a corneal micropocket model. Bastadin showed no effect on the VEGF-induced auto-phosphorylation of VEGFR1 and VEGFR2. The anti-angiogenic effect of bastadin 6 could be related to selective induction of apoptosis [165]. Structure-activity relationship studies revealed that both the oxime moiety and bromine atoms are needed for the anti-proliferative activity of bastadin 6 on HUVEC [166]. Recently, a synthetically derived 5,5′-dibromohemibastadin-1 about ten times less toxic than the natural cyclic bastadins, has been shown to display also in vitro growth inhibitory activity in cancer cells and anti-angiogenic properties [167].

Induction of endothelial apoptosis seems also to be relevant to the antiangiogenic activity of aeroplysinin-1. In vivo studies, carried out with a modified chorioallantoic membrane assay developed in our laboratory, suggested that the apoptogenic properties of aeroplysinin-1 are largely directed against the endothelial cells [168]. Experimental data revealed that incubation with this compound induces several changes in endothelial cells which are indicative of apoptosis, including chromatin condensation and nuclear fragmentation, an increase in the percentage of cells with sub-diploid DNA content, and an increase in the annexin V+ subpopulations. Interestingly, the same doses of aeroplysinin-1 did not cause any observable effect in the human HCT-116 and HT-1080 tumor cell lines, which confirms a selective induction of apoptosis in endothelial cells [78]. Treatment of endothelial cells with aeroplysinin-1 induces activation of caspases-2, -3, -8 and -9, the cleavage of apoptotic substrates such as poly (ADP-ribose) polymerase and lamin-A in a caspase-dependent mechanism, and the cytochrome c release from mitochondria indicating a key role of the mitochondria in the apoptogenic activity of this compound. Aeroplysinin-1 prevented phosphorylation of the BH3-only pro-apoptotic protein Bad in HUVE cells in a dose-dependent manner, but not in HCT-116 cells, reinforcing the previously shown data regarding the endothelial selectivity of aeroplysinin-1

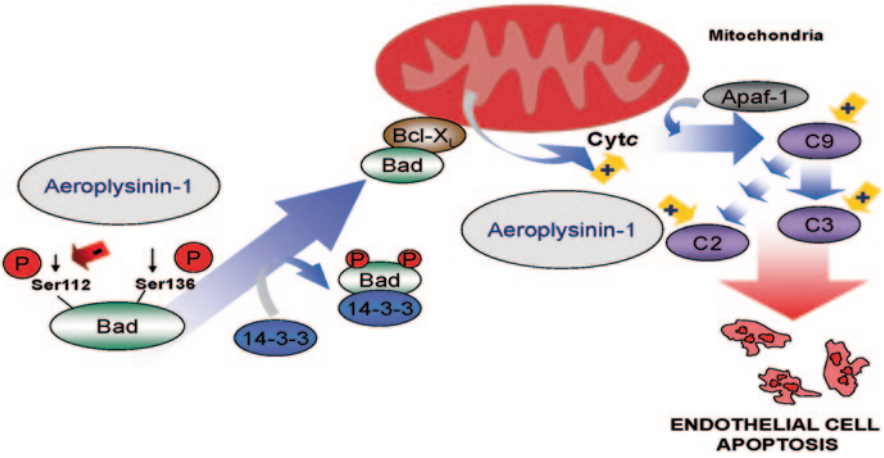

Fig. 3.7 Aeroplysinin-1 induces apoptosis in endothelial cells by activating the mitochondrial pathway. (Adapted from [78])

apoptogenic activity. Taken together, those results led us to suggest a mechanism by which aeroplysinin-1, through inhibition of Bad phosphorylation, could orchestrate the decision to undergo endothelial apoptosis by mitochondria permeabilization, cytochrome c release and further activation of the caspases proteolytic cascade (Fig. 3.7).

3.4 Concluding Remarks

Targeting angiogenesis is an exciting field of biomedicine, with relevant clinical implications. The role of angiogenesis in tumor progression and metastasis is widely accepted, with a continuously increasing number of antiangiogenic drugs and treatment being approved for their clinical use. Yet clinical results have shown up several limitations of the antiangiogenic monotherapies, explained by the high complexity of the tumor angiogenesis mechanisms. Angiogenesis regulation is exerted by multiple factors secreted by tumor cells and their surrounding host stromal cells, modulated by extracellular matrix and controlled by multiple complementary, overlapping and independent pathways. Growing evidence advises the use of multitargeted approaches to reach an effective inhibition of tumor angiogenesis. In the last years, the increasing exploration of marine organism as a source of drug candidates have yielded a list of new natural products able to inhibit angiogenesis in vitro and in vivo. The number of marine-derived inhibitors of angiogenesis will increase under the light of the the cellular and molecular basis of angiogenesis. A deeper knowledge of the primary targets or mechanism of action of those compounds will help to develop rational approaches for their use alone or in combination treatments for cancer and other diseases characterized by abnormal vasculature.

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Chapter 4
Marine Sponge Derived Eribulin in Preclinical and Clinical Studies for Cancer

Umang Swami, Umang Shah and Sanjay Goel

Abstract Eribulin mesylate is a completely synthetic, structurally simplified, macrocyclic ketone analogue of Halichondrin B. Halichondrin B is complex, natural, macrocyclic polyether derived from marine sponges. Eribulin has been approved by United States Food and Drug Administration in 2010 as a third line therapy for metastatic breast cancer patients, who have previously been treated with an anthracycline and a taxane. It has a microtubule dynamics inhibitory action. Preclinical studies have demonstrated a broad spectrum of anti-tumor activity in various cancer cell lines and synergistic action with multiple anticancer agents. It has also undergone clinical trials in non-small cell lung cancer, pancreatic, prostate, bladder, head and neck cancers, sarcomas and ovarian and other gynecological tumors. Various combination trials are currently ongoing. The predominant side effects are neutropenia and fatigue, which are manageable. This article reviews the available information on eribulin with respect to its clinical pharmacology, pharmacokinetics, pharmacodynamics, mechanism of action, metabolism, preclinical studies and clinical trials.

Keywords Breast cancer · EMBRACE · Eribulin · E7389 · Halichondrin B · Halaven™ · Microtubule inhibitor · NSC 707389

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4.1 Introduction

Halichondrin B (NSC 609395) is a natural large polyether macrolide which was originally isolated from a rare marine Japanese sponge, Halichondria okadai Kadota by Hirata and Uemura in 1985. That time 600 kg of Halichondria okadai Kadota yielded 12.5 mg of Halichondrin B after complex steps of isolation and purification. It generated a great interest due to its exquisite anticancer activity against murine cancer cells both *in vitro* and *in vivo* [1, 2]. Subsequently it was isolated from more commonly available sponges belonging to the Axinella, Phakellia and Lissodendoryx families [3–5]. Due to its potent anticancer activity, halichondrin B [2, 6] was one of the first agents to be tested using the novel United States (US) National Cancer Institute's (NCI) 60-cell line screen and was compared with other known antimitotic and anticancer agents [7, 8]. It was found that though halichondrin B had similar antiproliferative patterns, its tubulin-based mechanism of action was unique among all other known classes of antitubulin agents. It completely inhibited β tubulin β^s intra chain cross link formation (and enhanced β^* formation), enhanced bis-5, 5′-[8-(N-phenyl)-amino-naphthalene-1-sulfonic acid] binding to tubulin, noncompetitively inhibited vinblastine binding to tubulin, and had no effect on iodoacetamide alkylation of tubulin sulfhydryl groups; yet it did not stabilize or inhibit colchicine binding to tubulin [8–10]. However, even after the confirmation of its potent anticancer activity [7, 11] further studies hit a road block due to lack of availability of sufficient quantities of halichondrin B and its slow and costly procurement from marine sponges. Progress again started in 1998 when Dr. Yoshito Kishi at Harvard developed a completely synthetic halichondrin B. It was also discovered that its cytotoxicity was a function of the macrocyclic lactone C1-C38 moiety [8, 12]. Thereafter Eisai Research Institute licensed the technology and accomplished the synthesis of the resulting drug, eribulin (Halaven®, E7389, NSC 707389) [13, 14].

Eribulin mesylate is a structurally simplified, completely synthetic, non-taxane, macrocyclic ketone analogue of halichondrin B [15]. Although eribulin is characterized as an antitubulin drug, its tubulin interactions appear to be quiet distinct from other drugs. It inhibits microtubule dynamics via a novel mechanism of action [16–18], which is thought to involve binding to a unique binding site on tubulin [18], resulting in the suppression of microtubule polymerization, without affecting depolymerization along with sequestration of tubulin into nonfunctional aggregates [16]. By inhibiting mitotic spindle formation, eribulin causes irreversible mitotic block, which ultimately leads to cell cycle arrest in the G2-M phase and induces apoptosis [16–19]. Due to the novel mechanism of action of eribulin, which is distinct from other known classes of tubulin-targeted agents, and its encouraging preclinical profile, it was hypothesized that eribulin may have efficacy in patients with malignancies that were resistant to other tubulin-targeted agents. Eribulin was presented to NCI, Drug Development Group in 1998 and entered phase I clinical trials in 2002. On November 15, 2010, U. S. Food and Drug Administration approved eribulin for treatment of patients with metastatic breast cancer (MBC) who have previously received an anthracycline and a taxane in either the adjuvant or

metastatic setting, and at least two chemotherapeutic regimens for the treatment of metastatic disease [20]. Phase II trials with eribulin have been conducted in breast, non-small cell lung cancer, salivary gland, pancreatic, prostate, head and neck cancer, bladder/urothelium and kidney dysfunction and ovarian and related gynecological malignancies. It has also undergone phase III clinical trials in MBC, soft-tissue sarcoma and non-small cell lung cancer [21].

The purpose of this article is to review the available information on eribulin with respect to preclinical studies, clinical pharmacology, mechanism of action, pharmacokinetic and pharmacodynamic properties, results of various phase I, II, III trials, clinical efficacy for breast cancer, adverse-effect profile and dosage and administration. Data from studies using eribulin in combination with other chemotherapeutic agents are also reported.

4.2 Pharmacology

Eribulin mesylate (also known as eribulin mesilate, INN codename E7389, and before that, ER-086526 and B1939, US NCI designation NSC-707389)is a white crystalline hygroscopic powder with a molecular weight of 826.0 (729.9 for free base) and molecular formula of $C_{40}H_{59}NO_{11}\cdot CH_4O_3S$ [14]. The structures of halichondrin B and eribulin are given in Figs. 4.1 and 4.2.

Chemical structure names [14]:

- (2R,3R,3aS,7R,8aS,9S,10aR,11S,12R,13aR,13bS,15S,18S,21S,24S,26R,28R,29aS)-2- [(2S)-3-amino-2-hydroxypropyl]-3-methoxy-26-methyl-20, 27-dimethylidenehexacosahydro- 11,15:18,21:24,28-triepoxy-7,9-ethano-12,15-methano-9H, 15H-furo[3,2- i]furo[2′,3′:5,6]pyrano[4,3-b][1,4]dioxacyclopentacosin-5(4H)-one methanesulfonate (salt)

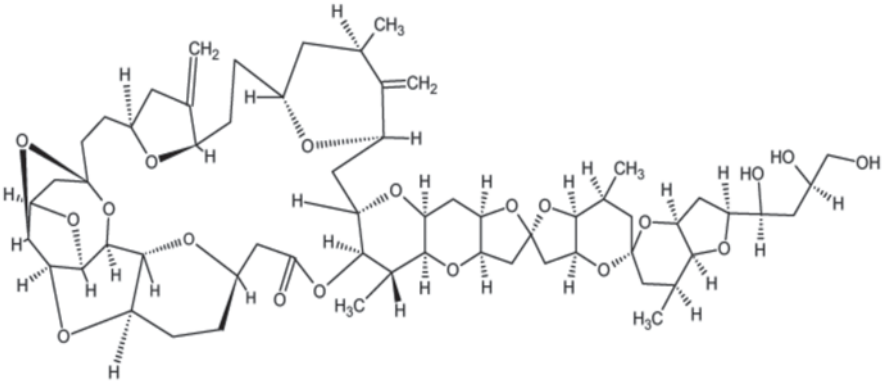

Fig. 4.1 Chemical structure of halichondrin B

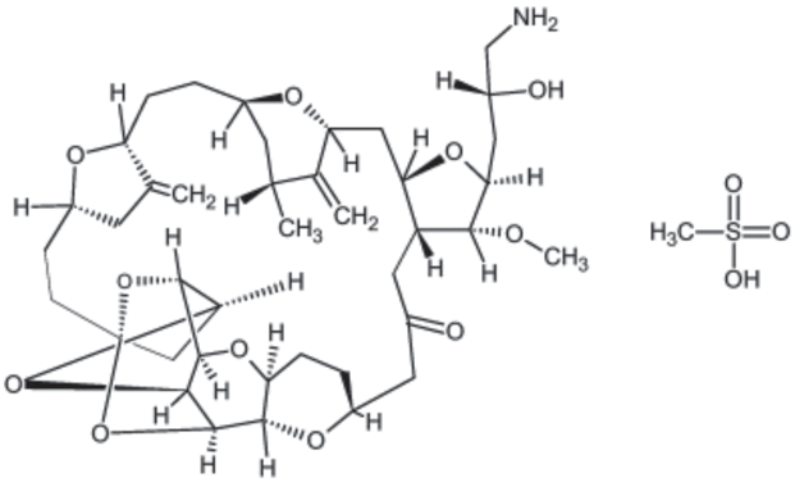

Fig. 4.2 Chemical structure of eribulin mesylate

- 11,15:18,21:24,28-Triepoxy-7,9-ethano-12,15-methano-9H,15H-furo[3,2-i]furo[2′,3′:5,6]pyrano[4,3-b][1,4]dioxacyclopentacosin-5(4H)-one, 2-[(2S)-3-amino-2-hydroxypropyl]hexacosahydro-3-methoxy-26-methyl-20,27-bis(methylene)-, 2R,3R,3aS,7R,8aS,9S,10aR,11S,12R,13aR,13bS,15S,18S,21S,24S,26R,28R,29aS)-, methanesulfonate (salt)

The structure of eribulin is of a simplified macrocyclic ketone in which the C1 lactone ester of halichondrin B is replaced by ketone, C31 methyl is replaced by methoxy, the tricyclic C29-38 system is replaced by a single five membered ring and the entire C39-C54 polyether side-chain is removed. Eribulin is freely soluble in water, methanol, ethanol, 1-octanol, benzyl alcohol, dimethylsulfoxide, N-methylpyrrolidone, ethylacetate and dichloromethane, soluble in acetone, sparingly soluble in acetonitrile, practically insoluble in tert-butylmethyl ether, n-heptane and n-pentane. In Britton-Robinson buffer, E7389 is freely soluble at pH 3–7, soluble at pH 9 and slightly soluble at pH 11. Eribulin is stored at 25°C/77°F (not to be frozen). Eribulin, which is prepared in an aqueous solution, has a short infusion time, can be administered with or without dilution, does not require steroid or antihistamine premedications and protection from light, is not necessary. These are some of the distinct advantages of eribulin as compared to currently approved microtubule-targeting agents [14, 20].

Halaven® is marketed as a clear colorless injection concentrate with each vial containing 0.44 mg/mL of eribulin which equates to 0.5 mg/mL eribulin mesylate solved in a mixture of ethanol (4%) and water for injection (95.8%) [22]. While in US, the content of eribulin mesylate is declared; in Europe labeling refers to the content of the active substance eribulin. This had lead to dosing errors. The recommended dose according to the EU product information for eribulin treatment is 1.23 mg/m^2 eribulin equivalent to 1.4 mg/m^2 eribulin mesylate. Eribulin should be

administered i.v. within 2 to 5 min in concentrations varying from 0.018 to 0.18 mg/mL (0.020–0.205 mg/mL eribulin mesylate) after dilution with 0.9% sodium chloride solution [22]. The product summary states that the undiluted injection solution is stable for 4 h, or 24 h when withdrawn into a plastic syringe and stored at room temperature (up to 25 °C) or under refrigeration [23]. Diluted Halaven® solutions should not be stored longer than 24 h under refrigeration, "unless dilution has taken place under controlled and validated aseptic conditions [22]. In one study eribulin mesylate injection (0.5 mg/mL) was physico-chemically stable over a period of 28 days after first puncture of the vial [22]. After dilution with 0.9% sodium chloride vehicle solution, ready-to-administer eribulin mesylate injection solutions (0.205 mg/mL in polypropylene syringe) and infusion solutions (0.02 mg/mL in prefilled polypropylene/polyethylene bags) were physico-chemically stable for a period of at least four weeks either refrigerated or stored at room temperature. However, for microbiological reasons storage under refrigeration is recommended [22].

4.3 Preclinical Studies

4.3.1 In vitro *Studies*

In the *in vitro* studies, eribulin inhibited cell growth at sub to low nmol/L IC_{50} values (0.09–9.5 nmol/L) in a wide variety of established human cancer cell lines, including MDA-MB-231, -435, -468 and HCC1806 breast cancers, HT-29, COLO 205 and DLD-1 colon cancers, H23, H441, H520 and H522-T1 non-small cell lung carcinomas (NSCLC), NCI-H82 small cell cancer, DU 145 and LNCaP prostate cancers, U937 histiocytic lymphoma, FaDu pharyngeal squamous cell carcinoma (head and neck cancer), A2780/1A9 ovarian cancer, MES-SA uterine sarcoma, HL-60 promyelocytic leukemia, and LOX melanoma [8, 14, 24].

Eribulin was found to inhibit MDA-MB-435 cell growth at sub-nM levels (IC_{50} = 0.09 nM) with greater potency than paclitaxel (IC_{50} = 2.5 nM) or vinblastine (IC_{50} = 0.59 nM) [8]. Similar low, nanomolar or subnanomolar activity was seen for eribulin against colon cancer COLO 205 and DLD-1, melanoma LOX, prostate cancer DU 145 and LNCaP, leukemia HL-60 and lymphoma U-937 cells, with mean IC_{50} value 1.8 nM (ranging from 0.09 to 9.5 nM) and was found to be roughly 2–4 fold more potent than vinblastine and paclitaxel (two tubulin based antimitotic agents run as internal standards in each experiment) and similar to its natural precursor halichondrin B. Though growth of several human cancer cell lines were inhibited at sub-nM concentrations, eribulin didn't show cytotoxic effects with concentrations up to 1 μM against dormant IMR-90 human fibroblasts. This indicates that growth inhibition by low- or sub-nM levels of eribulin is specific for proliferating cells and not secondary to nonspecific cytotoxicity [8]. Experiments on the NSCLC cell lines Calu-1 (p53-null) and A549 (p53 wild type) showed p53-independent anticancer activity of eribulin in the 0.5 pM range which is clinically achievable [25]. Though eribulin showed virtually similar activity as

natural halichondrin B in NCI 60 cell line screen [8, 24], it was found to be more potent in its interactions with tubulin (inhibition of assembly, nucleotide exchange on β-tubulin, radiolabeled vinblastine binding and radiolabeled dolastatin 10 binding) [18] and *in vivo* studies as well as less toxic as seen in granulocyte-macrophage colony forming units [26].

In *in vitro* studies on SK-BR-3 cell lines eribulin demonstrated synergistic activity when combined with many conventional drugs like gemcitabine, cisplatin, epirubicin, trastuzumab, docetaxel and vinorelbine. It showed additive effects when combined with carboplatin and antagonistic effects when combined with 5′-DUFR [27]. Interestingly eribulin in combination with carboplatin demonstrated antagonism in some NSCLC cell lines while additive response in others [24]. Studies with paclitaxel resistant ovarian cancer sublines A2780/1A9, 1A9PTX10 and 1A9PTX22 revealed that eribulin retained full *in vitro* potency in cells harboring beta-tubulin mutations that leads to substantial resistance to taxanes [28]. Eribulin has been tested against the Pediatric Preclinical Testing Program (PPTP) *in vitro* cell line panel at concentrations from 0.1 nM to 1.0 μM and demonstrated cytotoxic activity, with a median relative IC50 value of 0.27 nM, (range < 0.1–14.8 nM) [29].

The effect of eribulin on cell growth and PI3K pathway activity with or without mTOR inhibitor, RAD001 has been studied in triple-negative and HER2-expressing breast cancer using MDA468, BT549 and SKBR3 breast cancer cell lines. Inhibition of pAkt expression was seen in both MDA468 and SKBR3 cells on treatment with eribulin in varying concentrations. Standard dilutions of eribulin in combination with log dilutions of RAD001 resulted in marked synergistic growth inhibition (combination indices <<1) in both MDA468 and BT549 cells. A dose related suppression of pAkt along with complete inhibition of pS6K1 was shown on Western blot analysis of MDA468 cells treated with combination of erubulin and RAD001, while RAD001 alone increased pAkt. The study showed dose related inhibition of Akt activation and cell growth inhibition in triple negative breast cancer and HER2 cell lines treated with eribulin alone or combined with RAD001. The study also showed a significant synergistic growth inhibition with combination treatment and reversal of the pAkt feedback response with mTOR inactivation [30].

In vesicle motility assays in isolated squid axoplasm eribulin, vincristine, paclitaxel and ixabepilone inhibited anterograde (conventional kinesin-dependent) fast axonal transport, with the potency being vincristine = ixabepilone > paclitaxel = eribulin [31]. However, in contrast to vincristine and ixabepilone, eribulin and paclitaxel did not inhibit retrograde (cytoplasmic dynein-dependent) fast axonal transport. Similarly, significant inhibitory effects was exerted by both vincristine and ixabepilone in an *in vitro* microtubule gliding assay consisting of recombinant kinesin (kinesin-1) and microtubules composed of purified bovine brain tubulin, whereas paclitaxel and eribulin had insignificant effects. These results suggest that inhibition of microtubule-based fast axonal transport may be a significant contributor to neurotoxicity induced by antitubulin agents, and different classes of drugs may affect fast axonal transport through different mechanisms [31].

4.3.2 In vivo *Studies*

In *in vivo* studies of breast, lung, ovary, colon, melanoma, pancreatic, and fibrosarcoma human tumor xenograft models in mice, eribulin demonstrated tumor regressions, remissions and an increased lifespan at dose levels below the maximum tolerated dose (MTD) [8, 14]. It showed significant and superior *in vivo* anticancer efficacy in MDA-MB-435, COLO 205 and LOX cell lines (in NIH:OVCAR-3 model, significant only) at much lower doses (0.05–1 mg/kg i.v. or i.p.) as compared to paclitaxel, run at empirically determined MTD levels. In the MDA-MB-435 model, treatment with 0.25–1.0 mg/kg eribulin resulted in actual regression of measurable tumors by day 14, >95% inhibition at day 42 with no evidence of cytotoxicity. All doses of eribulin were found to be either equally efficacious (0.25, 0.5 mg/kg) or superior (1 mg/kg) to paclitaxel (MTD = 25 mg/kg) in the breast cancer model. Similarly in LOX melanoma model eribulin (0.05 mg/kg) inhibited tumor growth by 78% on day 17 and higher doses resulted in complete tumor suppression. Eribulin (0.5 mg/kg) treated mice showed a significantly delayed tumor regrowth rate as compared to paclitaxel (12.5 mg/kg), with 30% becoming tumor free by day 17 and remained so for additional 7 months. Eribulin was found to be more potent in NIH: OVCAR-3, COLO 205, LOX and MDA-MB-435 models by 20, 40, 50 and 100 folds, respectively as compared to paclitaxel running at MTD levels based on complete tumor suppression [8]. More importantly, eribulin showed significantly wider *in vivo* therapeutic windows (5-fold in LOX and 4-fold in MDA-MB-435 models), in comparison to paclitaxel (<2.0 in the LOX model and 1.7 in the MDA-MB-435 model). This wide therapeutic window leads to the possibility of augmenting dosage, 4–5 times above fully tumor-suppressive dose which can subsequently lead to more complete eradication of residual tumor cells and can contribute to substantial *in vivo* efficacy. Though the reasons for this unusually wide therapeutic window of eribulin are not currently known, it might also explain its superiority over paclitaxel due to latter's small therapeutic window [8, 24].

Dose scheduling studies with eribulin on MDA-MB-435 showed that maximal efficacy and minimal toxicity is achieved with moderate intermittent dosing such as Q2D × 3 [× 3 weeks], Q4D × 3 and Q7D × 3 [32]. Further studies with Q4D × 3 schedule in HT-1080 (fibrosarcoma) mice xenograft model showed long-lasting tumor regression at all doses (1.3–4.0 mg/kg). Treatment with eribulin resulted in 10 of 10 (100%) and 9 of 10 (90%) mice being rendered tumor free by day 38 and day 42 at dose 1.7 mg/kg (maximal tolerated dose) and 1.3 mg/kg, respectively. In PANC-1 (pancreatic cancer) xenograft models also doses from 0.4 to 4 mg/kg produced long lasting remissions with Q4D × 3 schedule [32]. In MDA-MB-435 (breast cancer) xenograft model Q4Dx3 treatment at 0.375–1.5 mg/kg/dose led to complete tumor regression in 14 of 15 animals with 24–41 day remission. Similarly, in NCI-H522 (lung cancer) xenograft model Q4D × 3 treatment at 0.375–1.5 mg/kg/dose led to complete tumor regression in 14 of 15 animals with at least 37-day remission [26].

Synergistic *in vivo* activity of eribulin was tested with gemcitabine, doxorubicin, docetaxel, and carboplatin [24, 32]. Eribulin 0.1–0.4 mg/kg and q4d × 3 schedule, which has a limited tumor inhibitory effect when combined with gemcitabine 120–270 mg/kg and q3d × 4 schedule, which has only tumor stasis effect induced significant regression in H522 NSCLC xenografts [32]. However eribulin combination with doxorubicin was not synergistic in the MDA-MB-435 xenograft model [24, 32].

In vivo activity of eribulin against HT-1080 fibrosarcoma, U251 glioblastoma, SR-475 head and neck cancer, SK-LMS-1 leiomyosarcoma, NCI-H322M and NCI-H522 non-small cell lung cancer (NSCLC), PANC-1 pancreatic cancer, and NCI-H82 small cell lung cancer (SCLC) xenografts was examined in single-schedule studies at dose levels of 0.19–4.0 mg/kg using q2d × 3(× 3), q4d × 3, q4d × 4, and q7d × 2 schedules [33]. The MTD values (or maximal at or below MTD values) ranged from 0.8–1.7 mg/kg. At these dosing levels *in vivo* antitumor response included tumor growth inhibition, stasis, and regression. Several studies showing regression also yielded long-term tumor-free survivors. Eribulin effectiveness showed model-to-model variability which appeared to be unrelated to dose or administration schedule. The study also evaluated administration schedule dependence by directly comparing q1d × 5, q2d × 3(× 3), q4d × 3, and q7d × 3 schedules in the MDA-MB-435 breast cancer xenograft model, using conditions of equivalent total dosing over the course of the experiment. The results suggested the following order of effectiveness and tolerability: q2d × 3(× 3) > q4d × 3 ≈ q7d × 3 **>>** q1d × 5. Therefore, a moderately intermittent dosing showed optimal preclinical effectiveness, which seems consistent with the approved intermittent clinical schedule for eribulin (days 1 and 8 of a 21-day cycle). In summary the results indicate that eribulin has a broad spectrum preclinical antitumor activity and maximum effectiveness and optimal tolerability can be obtained using moderately intermittent dosing schedules [33].

Eribulin has also been tested against the PPTP *in vivo* xenograft panels at a dose of 1 mg/kg (solid tumors) or 1.5 mg/kg (ALL models) using a q4dx3 schedule repeated at day 21 and was well tolerated. All 43 xenograft models were considered evaluable for efficacy. In total, 18 of 35 (51 %) solid tumor xenografts showed objective responses. Complete responses (CR) or maintained CR were observed in panels of Wilms tumor, Ewing sarcoma, rhabdomyosarcoma, glioblastoma, osteosarcoma and all eight ALL xenografts. As compared to control eribulin induced significant differences in event-free survival distribution in 29 of 35 (83 %) of the solid tumors and in 8 of 8 (100 %) of ALL xenografts. Eribulin activity pattern in solid tumor panels was found to be equal or superior to that observed previously with vincristine [29].

Eribulin has shown to induce less neuropathy in female BALB/c mice than paclitaxel or ixabepilone at equivalent MTD-based doses [34]. It showed no statistically significant adverse effect on caudal and digital nerve conduction velocity and amplitude in caudal and digital nerves in mice at any dosage including MTD. Whereas, in comparison, both paclitaxel and ixabepilone treated mice at MTD demonstrated statistically significant reduction in caudal and digital nerve conduction velocity and amplitude. Also the pathological changes in mice at equivalent half MTD or

higher doses in dorsal and sciatic root ganglia were milder and less frequent with eribulin as compared to ixabepilone and paclitaxel [34].

The same group later studied the induction of additional polyneuropathy by paclitaxel as compared to eribulin when administered to female BALB/c mice with preexisting paclitaxel-induced polyneuropathy [35]. In this study, paclitaxel at 0.75 × MTD (22.5 mg/kg) on a Q2D × 3 regimen was initially given for 2 weeks. After 2 weeks a second chemotherapy of 0.5 MTD eribulin (0.875 mg/kg) or paclitaxel (15 mg/kg) on a similar regimen was administered. Initial paclitaxel treatment resulted in a significant decrease in caudal nerve conduction velocity (averaging 19.5 ± 1 and 22.2 ± 1.3 %, $p < 0.001$) and amplitude (averaging 53.2 ± 2.6 and 72.4 ± 2.1 %, $p < 0.001$) versus vehicle when measured 24 h or 2 weeks after dosing cessation, respectively. In the second phase additional 0.5 MTD paclitaxel further reduced caudal nerve conduction velocity and amplitude (by 11 ± 2.1 and 59.2 ± 5 %, $p < 0.01$, respectively) relative to immediately before initiation of the second regimen. On the other hand, 0.5 MTD eribulin caused no further decrease in caudal nerve conduction velocity. This suggests that eribulin as compared to additional paclitaxel treatment, to mice with preexisting paclitaxel-induced polyneuropathy has limited additional damaging effects at 6 weeks. This may suggest a reduced tendency to exacerbate preexisting paclitaxel-induced polyneuropathy with eribulin in a clinical setting [35].

4.3.3 Pharmacodynamics and Mechanism of Action

Halichondrin B, the parent compound of eribulin is a large polyether macrolide, which binds tubulin at a site close to the vinca site altering depolymerization [7], with no effect on colchicine binding [9]. It binds only with β-tubulin, with no involvement of the α-subunit in the binding interaction [36]. It has shown to inhibit the formation of an intra-chain cross-link between two sulfhydryl groups in β-tubulin, does not affects alkylation of tubulin sulfhydryl groups by iodoacetamide (unlike vinblastine) and enhances the exposure of hydrophobic areas on the tubulin molecule. This unique mechanism of cytotoxicity of the halichondrins is secondary to a specific conformational effect as a function of the macrocyclic lactone C1-C38 moiety [8–10] which suppresses microtubule growth without an effect on microtubule shortening. This is in contrast to conventional anti-tubulin agents like taxanes, epothilones, and vinca alkaloids which inhibit both growth and shortening of microtubules. Though Halichondrin B is supposed to sequester tubulin into nonfunctional aggregates (as seen with eribulin) [16], a recent study found no Halichondrin B-induced tubulin aggregation [36].

Eribulin is a strong competitive inhibitor of halichondrin B and non competitive inhibitor of vinblastin and dolastatin 10 binding to tubulin [36]. The antimitotic mechanism of eribulin is different from other microtubule-targeted agents such as vinblastine, vincristine and paclitaxel as revealed in various experiments on human breast cancer MCF7 and human osteosarcoma U-2 OS cells [16, 37, 38]. Eribulin

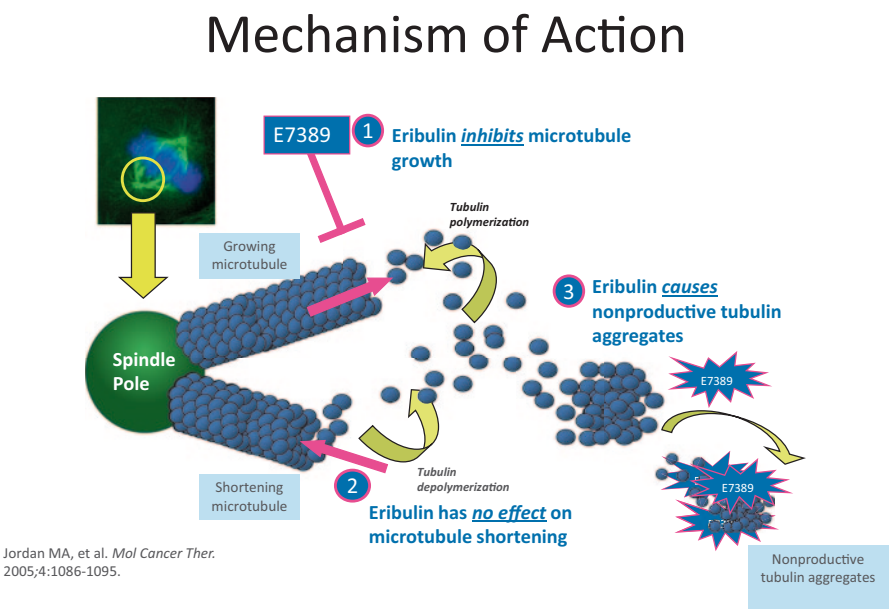

Fig. 4.3 Mechanism of action of eribulin mesylate. (From: Swami U, Chaudhary I, Ghalib MH, Goel S (2012) Eribulin—a review of preclinical and clinical studies. Crit Rev Oncol Hematol Feb 81(2):163–184)

exerts its antiproliferative action via a novel tubulin targeted mechanism, which predominantly involves inhibiting microtubule dynamics and tubulin polymerization into microtubules rather than shortening (unlike other antitubulin agents) (Fig. 4.3) [8, 16, 37, 38]. It acts via an end poisoning mechanism. At its lowest effective concentration, eribulin may suppress mitosis by either directly binding as unliganded eribulin to microtubule ends or by competing with unliganded soluble tubulin for addition to growing microtubule ends by inducing tubulin aggregates. This leads to formation of abnormal mitotic spindles which cannot pass the metaphase/anaphase checkpoint. It results in microtubule growth inhibition and tubulin sequestration into non-functional aggregates [16]. It inhibits cancer cell growth via induction of irreversible complete mitotic block at G_2-M (prometaphase blockage), disruption of mitotic spindles formation and initiation of apoptosis following prolonged mitotic blockage [8, 19].

Eribulin is believed to inhibit tubulin polymer formation by binding to either the interdimer interface or the β-tubulin subunit alone. It does not bind to both α- and β-tubulin [39, 40]. Eribulin potently inhibits microtubule dynamics, with only a single bound eribulinmolecule per two microtubules inducing a 50% reduction in growth rate [41]. Eribulin at 100 nM (concentration that inhibits microtubule plus end growth by 50% or the concentration approximately 10 times higher than that minimally induces complete G2/M blocks) suppresses dynamic instability at microtubule plus ends by binding with high affinity but does not suppress dynamic

instability at microtubule minus ends [14, 39]. Also significant correlation has been demonstrated between βIII tubulin expression levels in cancer cell lines and sensitivity to eribulin in studies which suggests that tumors expressing higher levels of βIII tubulin isotype may be more responsive to eribulin [42]. This may help as βIII tubulin gene is found to confer resistance to [43] and is inducible by [44] various antitubulin agents like vinorelbine and paclitaxel. Also its over expression has been correlated either with low response rates in patients treated with regimens containing taxanes or vinorelbine or with reduced survival in patients with non -small cell lung cancer, breast, ovarian, and gastric cancers, and in cancers of unknown primary site [45].

4.4 Pharmacokinetics and Metabolism

4.4.1 Pharmacokinetics

The first-in-man phase I trial was conducted by California Cancer Consortium, with eribulin administered as a 1–2 min weekly bolus every 3 weeks out of 4. The pharmacokinetics results demonstrated a tri-phasic elimination with prolonged terminal half-life of 36–48 h. At the MTD, plasma levels of eribulin were above concentrations required for *in vitro* cytotoxicity for >1 week. After 48 h a mean of 10 ± 1 % of the dose was recovered in the urine. Fluorescent immunohistochemistry analyses of serial tumor biopsies of patients treated at MTD demonstrated that eribulin disrupts microtubule structure in tumors *in vivo* [46, 47]. Another study was conducted at our site with a 1 h i.v. infusion of eribulin mesylate on days 1, 8 and 15 of 28 day cycle. As demonstrated in Fig. 4.4, eribulin pharmacokinetics was linear and dose-proportional over the dosing range of 0.25–1.4 mg/m^2. Eribulin exhibited consistent pharmacokinetic parameter estimates between the first and third i.v. doses administered on days 1 and 15 at each dose level. The plasma concentration-time profile demonstrated a rapid distribution phase with a mean distribution half-life of ≈0.43 h followed by a slower elimination phase with a half-life of 38.7 h. Again urinary excretion of eribulin was found to be minimal with 5 to 6 % of the administered dose eliminated in urine over a 72-h period after a single dose [10]. Eribulin mesylate administered as a 1 h i.v. infusion every 21 days also showed linear kinetics with terminal half-life of 2 days, rapid extensive volume of distribution, slow-to-moderate clearance and slow elimination, with non- renal clearance (≈7 % of the drug excreted unchanged in the urine) [48]. In a separate study, eribulin (5 min i.v. bolus on day 1 and 8 every 21 days) exhibited triphasic pharmacokinetics over the dosing range of 0.7–2.0 mg/m^2 with no difference observed between day 1 and day 8 pharmacokinetic profiles. Eribulin showed a long terminal half-life (36.4–59.9 h), a low systemic clearance (1.32–2.37 L/h/m^2), a high volume of distribution (105.6–143.0 L/m^2) and minima urinary excretion (5.01–12.88 % over 72-h) [49].

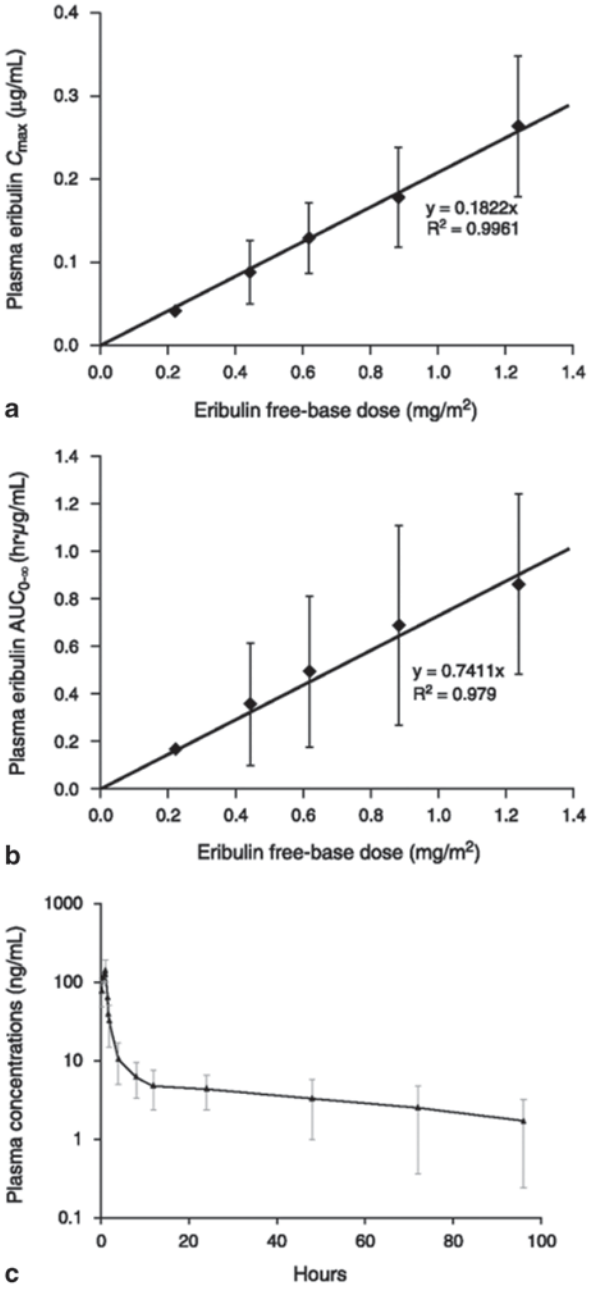

Fig. 4.4 **a** Plasma Cmax versus dose following a 1-h infusion of eribulin on day 1. **b** plasma area under the concentration-time curve (AUC0–8) versus dose following a 1-h infusion of eribulin on day 1. **c** plasma concentration versus time profile for the 1.0 mg/m^2 treatment group ($n = 9$). (From: Goel S, Mita AC, Mita M, et al (2009) A phase I study of eribulin mesylate (E7389), a mechanistically novelinhibitor of microtubule dynamics, in patients with advanced solid malignancies. Clin Cancer Res 15(12):4207–4212)

Table 4.1 Eribulin pharmacokinetics comparison in normal, mild and moderate hepatic impairment [50]

	Normal hepatic function ($N=6$)	Mild hepatic impairment (Child- Pugh A) ($N=7$)	Moderate hepatic impairment (Child-Pugh B) ($N=5$)
Dose on Day 1 (mg/m^2)	1.4	1.1	0.7
Clearance (L/h)	4.57	2.75	2.06
Elimination half life (h)	36.1	41.1	65.9
Mean dose normalized Cmax (ng/ml/mg)	72	83.9	100
Mean dose normalized $AUC_{(0-\infty)}$ (ng.hr/mL/mg)	229	420	646

In a phase I study done on patients with liver impairment, eribulin was generally safe and well tolerated. Hepatic impairment decreased clearance and prolonged elimination half-life. The mean dose-normalized C_{max} of eribulin was similar in the Child-Pugh A group [1.15-fold; 90% Confidence Interval (CI) 0.81–1.63] and the Child-Pugh B group (1.29-fold; 90% CI 0.89–1.89) compared to normal hepatic function. The mean dose-normalized $AUC_{(0-\infty)}$ increased 1.75-fold (mild) (90% CI: 1.15–2.66) in the Child-Pugh A group and 2.48-fold (90%CI: 1.57–3.92) in the Child-Pugh B group, when compared to the normal hepatic function group. In summary, eribulin exposure increased with worsening hepatic function. A comparative data is presented in Table 4.1 [50]. Due to this a lower starting dose is recommended in patients with mild (Child-Pugh A) and moderate (Child-Pugh B) hepatic impairment [23].

To study the effect of CYP3A4 inhibitors on the plasma pharmacokinetics of eribulin, a randomized, open-label, two treatments, two sequences, crossover phase I study with ketoconazole was performed in patients with advanced solid tumors [51]. Group one received 1.4 mg/m^2 eribulin mesylate (day 1), followed by 0.7 mg/m^2 eribulin mesylate plus 200 mg ketoconazole (day 15), and 200 mg ketoconazole alone (on day 16) of a 28-day cycle. Group 2 received 0.7 mg/m^2 eribulin mesylate plus 200 mg ketoconazole (day 1) and 200 mg ketoconazole (day 2), followed by 1.4 mg/m^2 eribulin mesylate (day 15) of a 28-day cycle. A half dose of eribulin with ketoconazole was given due to safety concerns. Pharmacokinetic sampling was performed up to 144 h following administration of eribulin. In total, 12 patients were enrolled and 10 patients (four in group one, six in group two) were evaluable for pharmacokinetic sampling. Co-administration of ketoconazole had no effect on single dose exposure to eribulin (ratio of geometric least square means: $AUC(0-\infty)=0.95$, 90% CI: 0.80–1.12 and $C_{max}=0.97$, 90% CI: 0.83–1.12). Ketoconazole showed no effect on clearance and elimination half-life of eribulin [51].

An open-label, two-center, non-randomized phase I study was conducted to determine the effect of rifampicin, a CYP3A4 inducer, on the plasma pharmacokinetics of eribulin mesylate in patients with advanced solid tumors for whom no standard treatment was available [52]. Eribulin mesylate (i.v. 1.4 mg/m^2) was administered on days 1 and 15 and oral rifampicin 600 mg on days 9–20 of a 28 day cycle. Pharmacokinetic sampling was performed up to 144 h following administration.

Subsequently, patients were allowed to continue eribulin on days 1 and 8 of a 21 day cycle. Overall 14 patients were enrolled and 11 patients were evaluable for pharmacokinetic analysis. Co-administration of rifampicin had no effect on single dose exposure to eribulin (geometric least square means ratio: AUC(0-∞) = 1.10, 90% CI 0.91–1.34, C_{max} = 0.97, 90% CI 0.81–1.17) [52]. Therefore pharmacokinetic analyses show that CYP3A4 inhibitors and inducers have no effect on eribulin exposure [51, 52]. These results show that drug-drug interactions are not expected between eribulin and other CYP3A4 inhibitors or inducers and eribulin mesylate can be safely co-administered with them. The pharmacokinetic analyses of combination trial of eribulin with carboplatin suggested absence of any interaction [53].

A phase II study evaluated eribulin mesylate (1.4 mg/m^2 i.v. bolus on days 1 and 8 of 21 day cycle) pharmacokinetics in heavily pretreated, locally advanced or MBC patients. Eribulin pharmacokinetics was best described by a three-compartment model, with elimination from the central compartment. As seen in above other studies distribution was rapid with slow elimination. In a typical patient with adequate organ functions [AST < ULN and creatinine clearance = 101 mL/min (Cockroft-Gault)], clearance was 2.98 L/h and central volume of distribution was 3.72 L (V1). Volumes of the two peripheral compartments were 3.60 and 126 L and inter-compartmental clearances were 2.7 and 5.6 L/h, respectively. The inter-patient clearance variability was 57% which ranged 26–98% for other parameters with a 21% residual error (proportional). In patients with elevated AST, clearance on an average was 38% lower and positively correlated with the renal function. Appreciable interpatient pharmacokinetic variability was observed in the study, a minor fraction of which was explained by measures of liver and renal function [54].

4.4.2 Metabolism

A phase I, open-label, nonrandomized, single-center mass balance study was conducted on 6 patients with advanced solid tumors using a flat 2 mg (approximately 80–90 μCi) dose of [^{14}C] eribulin acetate administered as a 2–5 min bolus injection on day 1. Blood, urine, and fecal samples were collected from day 1–8 or until sample radioactivity was ≤1% of the administered dose. The study didn't find any major metabolite of eribulin in plasma. The elimination half-life of eribulin (45.6 h) was comparable to total radioactivity (42.3 h). Eribulin is primarily eliminated unchanged in feces, whereas urine constitutes a minor elimination route [55].

The IC_{50} of eribulin on P-gp mediated digoxin transport activity is estimated to be greater than 10 μmol/L, indicating that eribulin is a weak P-gp inhibitor. In the presence of ≥1 μmol/L eribulin the transport of digoxin is reduced [14]. Eribulin is a substrate for P-gp drug efflux pump. Thus it shows reduced *in vitro* activity against multidrug resistant cells which overexpress P-gp drug efflux pump [56]. Although eribulin is a P-gp drug efflux pump substrate it retains full *in vitro* activity against taxane-resistant PTX10 and PTX22 human ovarian cancer cells. which are resistant due to β-tubulin mutations, suggesting that eribulin may show clinical effectiveness in taxanes refractory tumors harboring β-tubulin mutations [28, 57].

In animal studies also unchanged drug was found to be the major component in plasma, bile, urine and feces following i.v. dosing in intact and bile duct cannulated rats and dogs. Eribulin is not strongly bound to mouse, rat, dog or human plasma protein with a species difference. This suggests that variability in albumin or α1-acid glycoprotein will not significantly affect eribulin pharmacokinetics [14, 24].

The major enzyme responsible for the human hepatic metabolism of eribulin *in vitro* appears to be CYP3A4. Predominantly four isomeric monohydroxylates are formed via CYP3A4 mediated reactions [14]. No significant inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 was detected with eribulin at concentrations up to 5 μM [14]. Eribulin does not induce CYP1A and CYP3A expression or activities [58]. In a manner similar to terfenadine, eribulin acts as a CYP3A4 substrate competitor and not as a mechanism-based inhibitor [58]. Although eribulin competitively and reversibly inhibits nifedipine dehydration, testosterone 6-beta-hydroxylation and R-warfarin 10-hydroxylation activities of recombinant CYP3A4 (apparent Ki 3–17 μM), it does not induce or inhibit hepatic CYP3A4 activity nor does it inhibits CYP3A4-mediated metabolism of various therapeutic agents, including carbamazepine, diazepam, Tamoxifen, paclitaxel, midazolam, or terfenadine at clinically relevant concentrations [24, 58]. Therefore, it would not be expected to inhibit the metabolism of concurrently administered drugs that are metabolized by CYP3A4 or CYP1A, suggesting a minimal risk of drug–drug interactions [14, 58].

4.5 Clinical Trials: Safety and Efficacy

4.5.1 Phase I Studies

The first phase I trial conducted at the California Cancer Consortium on patients with refractory or advanced solid tumors [46], used a rapid titration design with real-time pharmacokinetic analysis to guide dose escalation [59]. Starting dose was 0.125 mg/m^2/week. Forty patients (38 evaluable) entered the study. Median age was 61 years. The rapid escalation phase, which enrolled single patient cohorts with intra- and inter-patient dose doubling till toxicity was observed, ended with a grade 3 elevation of alkaline phosphatase at a dose of 0.5 mg/m^2/week. The second phase consisted of a standard 3 + 3 dose escalation schedule, which ended at 2.0 mg/m^2/week with two dose limiting toxicities (DLT's); one grade 3 febrile neutropenia and one grade 4 neutropenia. Other serious non-hematological toxicities included hypoglycemia, hypophosphatemia, and fatigue. The MTD was 1.4 mg/m^2/wk. Two partial responses (PR, NSCLC; bladder) and three marginal responses (NSCLC, breast, and thyroid) were observed. Twelve patients had stable disease (SD, median of 4 months; range 2–14 months) as best response [46].

In a second phase I clinical trial conducted jointly by CTRC and our institution [10], patients received a 1-h i.v. infusion of eribulin mesylate on days 1, 8 and 15 of a 28-day cycle. Thirty-two patients were treated following the NCI accelerated

titration scheme design 4B [59]. The median numbers of cycles administered were two (range, 1–10). At highest dose level of 1.4 mg/m^2 in the study, DLT of grade 4 neutropenia was seen in two patients and one of these patients also experienced grade 3 fatigue. Three other patients at the dose level of 1.4 mg/m^2 experienced grade 3 neutropenia (not DLT) leading to the omission of the planned week 3 dose in cycle 1. These events showed that eribulin cannot be administered on the planned schedule at 1.4 mg/m^2; therefore, the MTD in this study was regarded as 1.0 mg/m^2. The most common eribulin-related adverse events were fatigue (all grades 53%, 13% grade 3, no grade 4), nausea (41% all grade 1/2), and anorexia (all grades 38%, 3% grade 3, no grade 4). Eribulin related grade 3/4 toxicities included neutropenia (19%), fatigue (13%), anorexia (3%), anemia (3%) and vomiting (3%). Eribulin was found to be fairly safe, was associated with a low incidence of neuropathy (25%, all grade 1/2) or cumulative toxicities (as seen in 12 patients treated for $\geq$3 cycles) and with an absence of hypersensitivity reaction. An unconfirmed PR (lasting 79 days) in a patient with cervical cancer and SD (range 39–234 days) in 10 patients was achieved [10].

Another phase I trial enrolled 21 patients with advanced solid tumors and used a 1 h infusion on day 1 of 21-day cycle [48]. Doses ranged from 0.25 to 4 mg/m^2 and dose escalation involved two-stage design based on design 3B [59]. Febrile neutropenia as DLT was seen in all three patients at 4 mg/m^2, two of the three patients at 2.8 mg/m^2 (on one dose reduction) and one of the seven patients treated at 2.0 mg/m^2 dose levels. Therefore MTD with this schedule was established at 2.0 mg/m^2. The most frequently reported drug related adverse events (all grades) were neutropenia (38%), fatigue (33%, all grade 1 or 2), alopecia (33%, all grade 1 or 2), febrile neutropenia (29%), anemia (24%, all grade 1) and nausea (19%, all grade 1). Seven patients experienced treatment related serious adverse events which included grade 3 hyponatremia (one patient), grade 4 febrile neutropenia (five patients) and grade 4 febrile neutropenia, grade 2 pyrexia, and grade 3 infection (one patient). Eribulin associated neutropenia exhibited a nadir of 7–15 days after the first treatment with recovery to normal by the end of the 21-day cycle. A noteworthy hematological toxicity in the study was grade 1 thrombocytopenia (10%). Only one grade 1 peripheral neuropathy occurred at 4-mg/m^2. An unconfirmed PR in NSCLC patient at 4 mg/m^2 (received four cycles) was observed. Twelve patients experienced stable disease as best response with a median duration of 86 days (range 47–386 days) [48].

A fourth single-center, non-randomized, open-label, dose-escalation phase I study involved the use of a 5-min eribulin i.v. bolus on day 1 and 8 of 21 days cycle for the first time in 15 Japanese patients. Three, three, six and three patients were treated at 0.7, 1.0, 1.4 and 2.0 mg/m^2 drug cohorts, respectively. A median of two cycles (range 1–15) were administered with seven patients receiving more than four cycles. Five of 15 patients experienced neutropenia or febrile neutropenia as DLTs. The most commonly observed Grade 3/4 toxicities were neutropenia (67%), lymphocytopenia (20%), febrile neutropenia (33%), and fatigue (13%) which can be easily managed. Of the 14 evaluable patients PR in three patients (two with NSCLC and one with head & neck cancer) at 1.4 mg/m^2 and SD (more than 12 weeks) in four patients were observed. The recommended phase II dose was 1.4 mg/m^2

Table 4.2 Summary of eribulin as single agent dose finding phase I trials

Trial	Total patients	Schedule	Maximum tolerated dose (mg/m^2)	Dose limiting toxicity (DLT)
Synold et al. [46]	40	Weekly bolus 3 weeks out 4	1.4	Febrile neutropenia and neutropenia
Goel et al. [10]	32	1-hr weekly infusion 3 weeks out of 4	1.0	Neutropenia
Tan et al. [48]	21	1-hr infusion on day 1 of 21-day cycle	2.0	Febrile neutropenia
Minami et al. [49]	15	Weekly bolus 2 weeks out of 3	1.4	Febrile neutropenia and neutropenia
Synold et al. [60]	21	Weekly on day 1 and 8 every 21 days in renal dysfunction	Moderate-1.4 Severe-1.4	Moderate- No Severe-Grade 3 weakness + fatigue

administered on days 1 and 8 every 3 weeks and MTD was 2 mg/m^2 [49]. A summary of the above phase I trials in solid tumors in presented in Table 4.2.

In a phase I trial on patients with liver dysfunction the most common treatment-emergent adverse events were alopecia (12/18), fatigue (7/18) and neutropenia (6/18). In total six patients experienced treatment related Grade ≥ 3 toxicities. There were no cycle one dose reductions, treatment-related serious adverse events or discontinuations during the study phase of the study (cycle one only). Half (9/18) patients had stable disease as best tumor response [50]. In a first-in-human phase I study of eribulin in patients with renal dysfunction and advanced urothelial cancer (UC), eribulin mesylate was given in tiers of 0.7, 1.0 and 1.4 mg/m^2 intravenously on days 1 and 8, every 3 weeks with dose escalation in 3 + 3 design. Eligibility criteria included patients with ≤ 2 prior regimens. Overall 21 patients were treated and a median number of six cycles (range 0–16) were administered. The MTD in moderate renal dysfunction ($\geq$40–59 mL/min, Cockroft-Gault) arm was 1.4 mg/m^2 with no DLTs. In severe renal dysfunction (20–40mL/m, not needing dialysis) arm 1 out of 6 patients treated at 1.4 mg/m^2 had a DLT of grade 3 weakness and fatigue. Of the 20 evaluable patients, three achieved PR and nine had SD. Of these nine patients, two patients had unconfirmed PR on 6 week scans but progressive disease at 12 weeks. Median progression-free survival (PFS) was 4.1 months (range 2.8–8.8 months) and median survival was 9.7 months (range 7.1–19.7 months) at a median follow-up of 11 months. Important toxicities included grade 3/4 neutropenia (five patients), febrile neutropenia (one patient), grade 1 sensory neuropathy seven patients) and grade 1 transaminitis (eight patients). Tolerance of full doses of eribulin and encouraging activity may fulfill an unmet need across a spectrum of cancers in patients with renal dysfunction [60]. A lower starting dose is recommended for patients with moderate renal impairment (creatinine clearance 30–50 ml/min) [23].

A phase I combination study of eribulin and gemcitabine was conducted in patients with advanced solid tumors based on synergistic action in H522 NSCLC xenografts. Twenty-one patients were treated and median number of administered cycles were two (range 1–8). One PR in ovarian cancer was obtained. Eight patients

had SD with four showing minor response (two NSCLC, one endometrial and one head and neck cancer). The DLTs were grade 3 diarrhea (one patient) and grade 3 dizziness/fatigue (one patient). Other drug related grade 3/4 treatment related toxicities were neutropenia (six patients), leukopenia (three patients), anemia (two patients) and thrombocytopenia (two patients). Recommended phase II dose is eribulin 1.0 g/m^2 and gemcitabine 1000 mg/m^2 every 3 weeks [61].

A phase I study of eribulin in combination with cisplatin was conducted patients with advanced solid tumors. Eribulin was administered on days 1, 8 and 15 every 28 days with cisplatin administered on day 1 every 28 days which was later changed to eribulin on days 1 and 8 and cisplatin day 1 every 21 days due to inability to administer treatment on day 15 (due to neutropenia). Eligibility criteria included patients with normal organ function and <2 prior chemotherapy regimens. Of the 36 patients treated till now, the median age was 61 years and a median number of cycles was three (1–8) administered. Three patients experienced DLT's on the 28-day cycle which included grade 4 febrile neutropenia, grade 3 anorexia/fatigue/hypokalemia; and grade 3 stomatitis/fatigue. Three patients had DLTs on 21-day schedule which included grade 3 hypokalemia/hyponatremia, grade 4 mucositis and grade 3 hypokalemia. The MTD of the combination was determined to be eribulin 1.2 mg/m^2 and cisplatin 75 mg/m^2 (one patient out of six with a DLT). Two patients had an unconfirmed PR (pancreatic, breast) and two had a confirmed PR (esophagus, transitional bladder) [62].

Another open label, phase I combination trial of eribulin was conducted with carboplatin in patients with advanced refractory solid tumors. Carboplatin was given as 30-min i.v. infusion and eribulin as a 2–5-min i.v. bolus, separated by 1 h. In stage one, eribulin mesylate (0.7, 0.9, 1.1, and 1.4 mg/m^2) was dose escalated with carboplatin at a fixed AUC of 5 in a 3+3 design in two schedules, differing by the order of administration. In stage two, eribulin mesylate (1.1 and 1.4 mg/m^2) was dose escalated with carboplatin at a fixed AUC of 6 using the preferred schedule from stage one. Fifty two patients with a median age of 60 years (range 38–80 years) were treated. In stage one, a DLT of diarrhea was experienced in one out of five patients at 1.4 mg/m^2 in the carboplatin-first schedule and in 0/3 patients in the eribulin-first schedule, defining the MTD as 1.4 mg/m^2 (same as the monotherapy dose) with eribulin given first. In stage two, eribulin was given first and DLTs occurred in 1/6 patients (febrile neutropenia) at 1.1 mg/m^2 and in 2/3 patients (febrile neutropenia, neutropenia) at 1.4 mg/m^2, defining the MTD as 1.1 mg/m^2. Most frequent adverse events were neutropenia (52%; 40% grade 3/4), thrombocytopenia (29%; 13% grade 3/4), fatigue (58%; 4% grade 3/4), and nausea (40%; no grade 3/4). One complete response (CR) in tonsillar cancer and two PRs in prostate cancer were observed by RECIST [53]. Eribulin has shown antitumor activity in patients with advanced NSCLC and the combination of eribulin and carboplatin demonstrated additive activity in lung cancer cell lines [27, 63, 64]. This led to the extension arm to investigate the efficacy and safety of the combination in chemo-naïve patients with advanced NSCLC (stage IIIB or IV) with measurable disease. Eribulin mesylate (1.1 mg/m^2 i.v.) was administered on days 1 and 8 every 21 days, along with carboplatin (AUC 6 i.v.) on day 1 as per recommended phase II dose. Twelve patients

(11 male, one female) with a median age of 66.5 (range 42–74) years were enrolled. Among the 11 patients evaluable for efficacy, objective response rate (ORR) was 27.3 % (all PRs), disease control rate (DCR) was 63.6 %, median overall survival (OS) was 12.1 months (range 1.6–12.1, 5 patients still alive at the time of study presentation); progression-free survival (PFS) was 4.2 months (0.03^{+}–5.8^{+}, upper value censored as 1 patient still responding at final visit) and duration of response (DOR) was 2.9 (2.8–3.1^{+}) months. The most common grade 3/4 adverse effects included thrombocytopenia, neutropenia, febrile neutropenia and anemia in six, five, four and three patients, respectively. The combination of eribulin and carboplatin warrants further studies with consideration to specific histological subgroups [65].

A 3 + 3 phase Ib study was conducted to assess the combination of eribulin and cyclophosphamide in patients with taxane-resistant MBC for potential application in adjuvant setting. Eribulin was administered in 2 escalating doses on day 1 and 8, with cyclophosphamide 600 mg/m^2 on day 1 every 21 days. Eligibility criteria included peripheral neuropathy ≤ grade 1. Six patients with MBC were enrolled. Median age was 50 (47–63) and all patients had prior taxane exposure. Median number of prior treatment for MBC was 5 (1–8). Five patients were hormone receptor positive, 2 were HER2 + and one patient was triple-negative. No dose limiting toxicities were observed and the recommended phase II dose was eribulin 1.4 mg/m^2 on day 1 and day 8 with cyclophosphamide day1 600 mg/m^2. The only Grade 3/4 non-DLT observed at this dose was neutropenia, requiring G-CSF support in cycle one in two of three pts. All grade toxicities included neutropenia (50 %), thrombocytopenia, fatigue, nausea, peripheral neuropathy, rash, mucositis, alopecia (33 % each), and elevated liver enzymes (17 %). Patients received a median of 5.5 cycles (range 3–13), with three patients still on treatment at the time of presentation of the study. Two partial responses (33 %) and 4 SD (67 %) were seen. Two patients stopped study treatment for quality of life and continued eribulin alone. Of the only two patients who met threshold of > 5 circulating tumor cells/7.5 ml at baseline; a mean decrease of 90.5 % at the start of cycle two was seen. Due to the acceptable toxicity and promising activity in MBC of the regimen, a phase II study in MBC is underway [66].

A phase Ib, open-label dose-escalation study was conducted to determine the MTD of eribulin in combination with capecitabine. Eribulin mesylate (2–5 min i.v.) by Schedule 1 (1.2, 1.6 or 2.0 mg/m^2 on Day 1) or Schedule 2 (0.7, 1.1 or 1.4 mg/m^2 on Days 1 and 8), in combination with twice-daily oral capecitabine (1000 mg/m^2 oral BID, days 1–14 every 21 days) was administered to patients with advanced solid refractory tumors. Thirty four patients with median age 62 years and 15 patients with median age 61 years were recruited in Schedules 1 and 2, respectively. No unexpected toxicities were seen with the combination. The MTD for eribulin mesylate was 1.6 for Schedule 1 and 1.4 mg/m^2 for Schedule 2, in combination with capecitabine 1000 mg/m^2 twice-daily. Eribulin pharmacokinetics was dose proportional and independent of schedule or capecitabine co-administration. Similarly combination with eribulin had no effect on the disposition of capecitabine and its metabolites. Schedule 2 MTD (1.4 mg/m^2 days 1 and 8) due to high dose intensity was selected for evaluation in a Phase II breast cancer study [67].

To assess the effect of eribulin on cardiac repolarization, an open-label, single-arm, phase I study was conducted. Patients with advanced solid tumors received eribulin mesylate (1.4 mg/m^2) on days 1 and 8 of a 21-day cycle. The primary objective was to assess the impact of eribulin on patients' ECGs with a focus on cardiac repolarization, as measured by the change in QT/QTc interval, and through a pharmacokinetic/pharmacodynamic analysis to investigate any potential correlation between QTc and eribulin plasma concentration. Twenty-six patients were enrolled. QTcNi (QTcNi = QT + bi*(1000-RR), where RR is 60/(HR) and bi subject-specific slope) was more effective than QTcF (QTc interval derived using Fridericia's formula, QTcF = QT/(RR)$^{0.33}$) in correcting for heart-rate dependency of the QT interval. On Day 1, mean ΔQTcNi were ~0 at all time-points. On day 8 an apparent time-dependent increase in ΔQTc was observed. The changes from baseline were larger and more variable, without clear relation to plasma levels of eribulin. On day 8 predose ΔQTcNi was 5 ms, post-infusion mean values ranged from 2 to 9 ms (largest mean ΔQTcNi at 6 h). Results from QTcF were with higher values on day 8 with pre-dose ΔQTcF of 9 ms and all post-dose ΔQTcF varying from the pre-dose by ± 6 ms (range 3–11 ms) [68]. No new or unexpected adverse events were reported. Eribulin demonstrated an acceptable safety profile with a minor QTc prolongation which is not expected to be of clinical concern in the targeted patient population [68]. But due to QT prolongation on day 8 it is advised to avoid eribulin in congenital long QT syndrome [23].

In the phase I study of eribulin mesylate with ketoconazole described above, all 12 patients were evaluable for safety. The most frequently reported treatment related adverse events were fatigue and nausea, each reported in 8/12 (66.7%) patients. Ten patients were evaluable for response of which 7 achieved stable disease as best overall response [51]. In the phase I study of eribulin mesylate with rifampicin described above, all 14 patients were evaluable for toxicity. The most common treatment-related grade ≥3 adverse events were neutropenia (29% Grade 3 and 14% Grade 4), leucopenia (21% Grade 3 and 7% Grade 4) and fatigue (21% Grade 3). One patient with breast cancer had PR and 4 patients had SD as best response [52].

Due to activity as monotherapy in urothelial carcinoma [69], eribulin has been evaluated in combination with gemcitabine and cisplatin as first-line therapy for locally advanced or metastatic bladder cancer in a phase Ib/II study. In the phase Ib part of this open-label, multicenter study, three ascending doses of eribulin (i.v. on days 1 and 8 every 21 days) were administered to determine the MTD with standard doses of gemcitabine (1000 mg/m^2, days 1 and 8 every 21 days) and cisplatin (70 mg/m2, day 1). Nine patients with median age 59 years entered phase Ib. Eribulin was administered at 0.7 mg/m^2 (n = 3) or 1.0 mg/m^2 (n = 6), with standard dose of gemcitabine and cisplatin. One DLT of grade 4 thrombocytopenia was observed at 1.0 mg/m^2. The 1.4 mg/m^2 dose was not explored due to an investigator consensus on high probability of developing severe hematologic toxicity DLTs. Thus, the MTD was neither achieved nor defined. The recommended phase II dose of eribulin in combination with gemcitabine and cisplatin was 1.0 mg/m^2. The most common adverse events at this dose were nausea (83%), neutropenia (83%), fatigue (83%), thrombocytopenia (83%), anemia (83%), and anorexia (50%). Overall response for

both cohorts was 89 % with two CRs (one confirmed, one unconfirmed) and six PRs (four confirmed, two unconfirmed) including one CR (unconfirmed) and four partial responses (PR) (all confirmed) at 1.0 mg/m^2. Thus combination is feasible with encouraging activity. The phase II part is currently ongoing in which patients are to be randomized in 1:1 to eribulin with gemcitabine and cisplatin, or gemcitabine and cisplatin alone [70].

4.5.2 Phase II Trials

4.5.2.1 Breast Cancer

Further development of eribulin in phase II trials initially continued with the bolus 1.4 mg/m^2/week dose on days 1, 8, and 15 of a 28-day cycle schedule as recommended by the first phase I trial [46]. However due to neutropenia in many patients on day 15 leading to dose interruption or omission, treatment schedule was modified to days 1 and 8 of a 21-day cycle which appeared to be more favorable [71, 72].

The first phase II trial of eribulin in breast cancer was a large, open-label, single-arm; Simon's two-stage, multicenter study with 23 sites in USA. It enrolled patients with MBC who had received prior therapy with at least an anthracycline and a taxane. The primary objective was to assess the ORR. Secondary objectives were to evaluate DOR, PFS, OS, and safety. Patients received eribulin mesylate (1.4 mg/m^2, 2-to 5-min i.v. infusion) on days 1, 8, and 15 ofa 28-day cycle. But as discussed above, due to recurrent neutropenia on day 15, treatment schedule was modified with eribulin on days 1 and 8 of a 21-day cycle. Patients received a median number of 4 prior chemotherapies (range 1–11 regimens). Overall 103 patients with a median age of 55 years (range 32–84) were treated. In the per-protocol population(n=87), observed ORR (all PRs) was 11.5 %and CBR was 17.2 % on independent review. The median DOR was 171 days (5.6 months; range, 44-363 days), PFS was 79 days(2.6 months; range 1–453 days), and OS was 275 days (9.0 months; range 15- 826 days). The six-month PFS and OS were 25.9 % and 67.8 %, respectively. The most common grade 3/4 drug-related toxicities were neutropenia (64 %), leukopenia (18 %), fatigue (5 %), peripheral neuropathy (5 %) and febrile neutropenia (4 %). The 21 day schedule had a more acceptable toxicity profile than the 28-day schedule [71].

The second, Eisai sponsored phase II trial was also a non-randomized, open label, single arm, multicenter (n=78) trial that assessed the efficacy and safety of eribulin (1.4 mg/m^2 administered as a 2–5 min i.v. bolus on days 1 and 8 of a 21-day cycle) in locally advanced or MBC patients previously treated with an anthracycline, a taxane and capecitabine. Eligibility criteria included two–five prior chemotherapeutic regimens including an anthracycline, a taxane, and capecitabine with at least one administered for advanced/metastatic disease. The primary objective was assessment of ORR, by independent review using RECIST criteria. The secondary objectives were assessment of DOR, PFS, OS and safety. Of the 299 patients enrolled, 291 received eribulin (safety population) but 269 patients met key inclusion

criteria (eligible population) and evaluated for efficacy. The eligible population had a median age of 56 years (range 26–80) with a median of 4 prior chemotherapy regimens. In total 71 % were ER + and/or PgR +, 11 % were HER2/neu + and 20 % were triple (ER, PgR and HER2) negative. A median number of 4 (range 1–27) cycles of eribulin were administered. The investigator determined ORR was 14.1 % (1 CR, rest all PRs) with a CBR of 19.7 %. Independent review determined ORR was 9.3 % (all PRs) and CBR was 17.1 %. Stable disease was seen in 46.5 % patients. The median DOR was 3.5 months (106 days; range 42–258 days), median PFS was 2.6 months (78; 1–398 days) and median OS was 10.4 months (315; 19–604 days). The six-month PFS and OS rates were 12.4 % and 72.3 %, respectively. Subgroup analyses revealed a better CBR (21.4 %) with ER + and/or PgR + subgroup as compared to the triple negative (ER/PgR/HER2 negative) subgroup (3.7 %). The most frequently reported treatment-related grade 3/4 treatment related adverse events were neutropenia (54 %; febrile neutropenia: 5.5 %), leukopenia (14.1 %), and asthenia/fatigue (10 %; no grade 4). Grade 3 peripheral neuropathy occurred in 6.9 % of patients with no grade 4. No death was "probably" related and one death with unknown cause was considered "possibly" related to eribulin [72].

Both studies showed a lower incidence of neuropathy ~ 32 % (26 % grade 1/2, 5–7 % grade 3 and no grade 4) with eribulin. Thus eribulin was associated with a tolerable level of neuropathy, and pre-existing neuropathy usually did not worsen with eribulin treatment [71, 72]. However, it should be noted that both trials excluded patients with more than grade 2 neuropathy which calls for caution in patients with prior taxane exposure and residual neuropathy.

Another single-arm, multicentre, open-label phase II study evaluated the efficacy and safety of eribulin in Japanese patients with locally advanced or MBC previously treated with an anthracycline and a taxane. Patients with ≤ 3 prior chemotherapies in metastatic setting, including an anthracycline and a taxane; relapse during or within a year after neoadjuvant or post-operative adjuvant chemotherapy immediately before the study, or progression during or within 6 months of last chemotherapy in the metastatic setting were included in the study. The primary efficacy endpoint was ORR by independent review using RECIST criteria. Eighty four patients were enrolled, 81 patients received eribulin and 80 patients constituted the eligible population. The eligible population had a median age of 54 years (range 31–72 years) and had received a median of three prior chemotherapy regimens (range 1–5). Sixty five percent were ER and/or PgR positive, 11.3 % HER2/neu-positive and 27.5 % were triple negative. Eribulin was given as 1.4 mg/m^2 i.v. bolus on days 1 and 8 of a 21-day cycle. The median duration of treatment was 85 days (range 1–435) with a median of 5 cycles (range 1–20) were received per patient. Dose reductions, omissions and delays were seen in 27 (33.3 %) 54 (66.7 %) and 29 (35.8 %) patients, respectively. No dose interruptions were required. The ORR was 21.3 % (95 % CI 12.9–31.8; all PRs), SD occurred in 30 patients (37.5 %) and the CBR (CR + PR + SD ≥ 6 months) was 27.5 % (95 % CI 18.1–38.6). Median DOR was 3.9 months (95 % CI 2.8–4.9), PFS was 3.7 months (95 % CI 2.0–4.4) and OS was 11.1 months (95 % CI 7.9–15.8). Neutropenia (95.1 %), leukopenia (74.1 %) and febrile neutropenia (13.6 %) comprised the most frequent treatment-related grade

Table 4.3 Phase II trials of eribulin in metastatic breast cancer patients

	Vahdat et al. [71]	Cortes et al. [72]	Aogi et al. [73]
Protocol population	87	269	80
PR (%)[1]	11.5	9.3	21.3
ORR % (95 % CI)	11.5 (5.7–20.1)	9.3 (6.1–13.4)	21.3 (12.9–31.8)
CBR %	17.2	17.1	27.5
Median DOR (months)	5.6	4.1	3.9
Median PFS (months)	2.6	2.6	3.7
Median OS (months)	9	10.4	11.1
Most common grade 3/4 toxicities (%)	Neutropenia, leukopenia, fatigue	Neutropenia, leukopenia, fatigue	Neutropenia, leukopenia, febrile neutropenia
Grade 3 neuropathy (%)[a]	5	6.9	3.7
Grade 3/4 neutropenia (%)	64	54	95.1
Febrile neutropenia (%)	4	5.5	13.6

CBR Clinical benefit rate (defined as CR + PR + SD ≥ 6 months), *CI* Confidence Interval
PFS Progression free survival, *OS* Overall survival, *PR* Partial response
DOR Duration of response1- No Complete response (CR) in all 3 trials
RR Overall objective response rate = [CR + PR/number of eligible patients]
[a] No grade 4 peripheral neuropathy in any trial

3/4 adverse events. The incidence of Grade 3 peripheral neuropathy was 3.7 % (no grade 4) [73]. A comparison of above three trials is presented in Table 4.3.

A multicenter, phase II, single-arm study has been conducted to evaluate the combination of eribulin and capecitabine for adjuvant treatment in post-menopausal estrogen receptor–positive (ER+) early-stage breast cancer. In the study stage I-II, HER2 negative, ER+, female breast cancer patients received eribulin mesylate (1.4 mg/m^2 i.v.) on day 1 and 8 and capecitabine (900 mg/m^2, oral BID) on days 1–14 of 21 day cycle, for four cycles. The study was to be considered feasible if 80 % of patients were able to achieve the target relative dose intensity (RDI) of at least 85 % of the regimen and lower 95 % confidence boundary (LCI) was above 70 %. Of the 67 patients enrolled, 88 % patients completed four cycles of treatment. The median age was 62 years (range 28–80) and 64 patients were evaluable for feasibility. The study met its primary endpoint with a feasibility rate of 81 % (95 % LCI: 71 %) and average RDI of 91 %. Capecitabine dose adjustments (RDI-88 %) were comparatively higher than that of eribulin (RDI-93 %). Capecitabine had higher dose reductions (36 % vs. 21 %), missed doses (85 % vs. 8 %), discontinuations due to adverse effects (16 % vs. 10 %) as compared to eribulin. Most common Grade 3/4 adverse effects with dose reductions and drug discontinuation were hand foot syndrome (12 % and 8 %), neutropenia (8 % and 3 %), neuropathy (8 % and 2 %) and gastrointestinal disorders (6 % and 3 %) patients, respectively. Overall 14 (21 %) patients had an SAE with 12 (18 %) requiring hospitalization. The adjuvant combination treatment of eribulin with capecitabine can be given safely with a majority of patients achieving full dosing regimen. An alternative schedule of capecitabine with 7 week on and off with this regimen is currently being explored [74].

A phase II, multicenter, randomized, open label study was recently conducted is USA to compare the incidence and severity of neuropathy associated with eribulin and ixabepilone in MBC patients [75]. The primary objective was to assess the incidence of neuropathy in patients treated with eribulin or ixabepilone. Eligibility criteria included MBC, prior taxane therapy, at least one chemotherapy for advanced disease and pre-existing neuropathy < grade 2. The study was designed to detect a difference in neuropathy rate of 35% for eribulin versus 63% for ixabepilone (OR 0.316, 80% power, 0.05 two-sided significance level). Eribulin mesylate (1.4 mg/m^2, i.v. over 2–5 min) on days 1 and 8 of each 21-day cycle, and ixabepilone (starting dose 32 or 40 mg/m^2 as per approved labeling or at 32 or 20 mg/m^2 in patients with mild or moderate hepatic impairment, respectively as per approved labeling as a 3-hr i.v. infusion) on day 1 of each 21-day cycle was administered. The intent-to-treat population comprised 104 patients randomized (1:1) to eribulin mesylate (1.4 mg/m^2, 2–5 min intravenous on days 1 and 8) or ixabepilone (40 mg/m^2, 3 h intravenous on day 1) on a 21-day cycle. Overall 101 patients in the safety population received a median of 5.0 eribulin and 3.5 ixabepilone cycles. Fewer patients on eribulin as compared to ixabepilone experienced neuropathy (any grade) (33.3% vs. 48.0%), and peripheral neuropathy (31.4% vs. 44.0%). However after controlling for baseline pre-existing neuropathy (Grade 0 or 1) and number of prior chemotherapies (≤ 3, >3) as binary variables, no significant difference in overall incidence of neuropathy and peripheral neuropathy between both treatments was found ($p = 0.1284$ and $p = 0.1632$, respectively). Importantly compared with ixabepilone, fewer patients on eribulin discontinued treatment due to neuropathy (3.9 vs. 18.0%) or adverse events in general (11.8 vs. 32.0%). Fewer patients on eribulin experienced Grade 3 neuropathy as compared to ixabepilone (9.8% vs. 22.0%). No Grade 4 neuropathy was reported in either arm. The study found that eribulin tends to have a later time to onset (35.9 weeks vs. 11.6 weeks) as well as a later time to resolution (48 vs. 10 weeks) of neuropathy. This was believed to be due to greater number of treatment cycles and longer duration of treatment with eribulin as compared to ixabepilone. Other adverse events were comparable in the study. In intent to treat population the ORR for eribulin as compared to ixabepilone was 15.4% versus 5.8%, CBR was 26.9% versus 19.2%, disease control rate was 67.3% versus 55.8% and median PFS was 104 days versus 95 days, respectively. In conclusion, after controlling for pre-existing neuropathy and number of prior chemotherapies, the differences in the incidence of neuropathy with eribulin and ixabepilone were not statistically significant. With eribulin neuropathy onset was found to occur later and resolve later. Compared to ixabepilone, fewer patients receiving eribulin discontinued treatment due to neuropathy (3.9% vs. 18.0%) or due to adverse events in general (11.8% vs. 32.0%). However caution should be taken with above result interpretations due to small sample size and lack of power in the study to detect the observed magnitude of differences between the two treatment arms [75].

Eribulin has also been investigated in combination with ramucirumab (IMC-1121B), a fully human IgG1 monoclonal antibody directed against the vascular endothelial growth factor receptor 2 in a multicenter phase II study. Eligibility criteria included females with locally recurrent or MBC, received 2–4 prior cytotoxic chemotherapy regimens in the locally recurrent or metastatic setting including

anthracycline and taxane, HER-2-directed treatment or not a candidate for HER-2-directed treatment in HER-2 positive patients and normal LVEF. Patients were randomized in 1:1 to receive eribulin (1.4 mg/m^2 days 1 and 8 of 21 day cycle) or ramucirumab with eribulin (eribulin 1.4 mg/m2 on days 1 and 8; ramucirumab 10 mg/kg day 1; of 21 day cycle, RAM + E). Patients were stratified by triple-negative breast cancer and prior antiangiogenic therapy status. The planned accrual is 134 patients. On interim safety analysis of 13 evaluable patients (RAM + E = 8, eribulin five) grade 3 febrile neutropenia (one patient), grade 3 odynophagia (one patient), and grade 3 neutropenia (four patients) were seen with RAM + E whereas grade 4 neutropenia (one patient) was seen in eribulin arm. The trial is currently continuing unmodified [76].

4.5.2.2 Non-small Cell Lung Cancer

Based on activity in xenograft models, an open-label, single-arm, phase II study of eribulin was conducted in patients with advanced NSCLC that have progressed after platinum-based doublet chemotherapy. Eribulin mesylate (1.4 mg/m^{2}2–5 min i.v. infusion) was administered on days 1, 8, and 15 of a 28-day cycle which was later changed to 21 day schedule (eribulin on days 1 and 8 of a 21-day cycle) due to dose delays and omissions on day 15 secondary to myelosuppression like in phase II breast trials [71]. Patients were enrolled in two strata based on prior taxane exposure. A total of 103 patients (83 with prior taxane therapy and 20 taxane naïve) were treated of which 77 were on 28 day schedule and 26 on 21 day schedule. The median age was 65 years (range 40–83 years). The primary efficacy endpoint was ORR evaluated using RECIST by independent radiologic review. A median number of 3 cycles (range 1–15) were administered. The most common grade 3/4 drug-related toxicities were adverse events were neutropenia (49 %), fatigue (11 % grade 3, no grade 4) and leucopenia (6 %). The 21-day schedule was well-tolerated. The ORR (all PRs) was 9.7 % (95 % CI: 4.8–17.1), with 10.8 % PR (95 % CI: 5.1–19.6) in taxane pre-treated, and 5 % PR (95 % CI: 0.1–24.9) in taxane naïve patients. Overall disease control rate (PR + SD) was 55.3 % (95 % CI: 45.2–65.1) and median PFS was 3.4 months (95 % CI: 2.4–3.6). Median DOR was 5.8 months (range, 1.6–9.6 + months), and median OS was 9.4 months. The 6 month and one-year survival rate were 61 and 46 %, respectively [77].

Another phase II trial of eribulin in prior taxane-treated NSCLC patients was conducted under the sponsorship of National Cancer Institute under a contract with the California Cancer Consortium [78]. Eligibility criteria included histologically or cytologically confirmed stage IIIB or IV NSCLC with measurable disease, recurrent or had progressed after treatment, previous treatment with platinum-based therapy and a taxane and no more than two prior cytotoxic chemotherapy regimens given for either metastatic disease or as adjuvant therapy. Eribulin was administered as 1.4 mg/m^2 i.v. over 1–2 min on day 1 and 8 of a 21 day schedule. Patients were classified in two strata based on taxane-sensitivity. The primary endpoint was ORR and secondary endpoints included PFS and OS. In the taxane sensitive stratum (progression > 90 days after taxane), 45 patients were enrolled; PR in three patients

(7%) and SD in 11 patients (24%) for at least 3 months was achieved. Median number of cycles was four (range 1–23), median PFS was 2.9 months (95% CI 2.5–4.8) and OS was 12.6 (95% CI 9.9–17.5). In the taxane resistant stratum (progression during or <90 days after taxane) 21 patients were enrolled, SD in 4 (29%) patients for at least 3 months but no response was achieved. Median number of cycles was 2 (range 1–8), SD was seen in 6 patients (29%), median PFS was 1.2 months (95% CI 1.1–2.9) and median OS was 8.9 months (95% CI 5–15.4). Grade 3 or 4 drug related toxicities seen were predominantly hematological, 55% patients had Grade 3 or four neutropenia though only one patient had Grade 3 febrile neutropenia. No Grade 4 neuropathy was seen and two patients had grade three neuropathy. The ORR in the study was 5% with median duration of response of 7.8 months Thus eribulin showed encouraging activity in taxane sensitivity cohort [78].

4.5.2.3 Prostate Cancer

In the first multicenter, open-label, two-stage design, single-arm, phase II study in metastatic castration resistant prostate cancer (CRPC) patients, eribulin was administered as 1.4 mg/m^2 i.v. bolus (2–5 min infusion) on days 1 and 8 of a 21-day cycle. Patients were evaluated in two separate strata based on prior taxane exposure. Patient were eligible if they had documented PSA progression (defined as two consecutive rises in PSA of minimum 3 PSA taken ≥1 week apart with the last value being ≥5 ng/ml) despite maintenance of castrate-level testosterone with orchiectomy or luteinizing hormone-releasing hormone analog and appropriate antiandrogen withdrawal. Primary efficacy endpoint was prostate-specific antigen (PSA) response rate, based on Bubley criteria. Secondary endpoints were duration of PSA response, ORR, PFS, OS and safety. In total 108 patients with median age of 71 years (range 47–91) were evaluable for safety. Results are summarized in Table 4.4. Treatment

Table 4.4 Comparison of metastatic castration-resistant prostate cancer patients stratified by prior taxane therapy treated with eribulin [63]

	Taxane-naïve	Taxane-pretreated
Safety/efficacy evaluable population	58/58	50/47
Median age (range) years	72.5 (53–91)	68.0 (47–89)
Median eribulin cycles (range)	4.0 (1–47)	3.0 (1–16)
PSA response (≥50% decline) % (95% CI)	22.4% (12.5–35.3)	8.5% (2.4–20.4)
No. of Patients with measurable disease (%)	33 (56.9)	29 (61.7)
No. of patients with PR (%)	5 (15.2)	0
No. of patients with SD (≥12 weeks) (%)	25 (75.8)	20 (69.0)
ORR (95% CI)	15.2 (5.1–31.9)	0
Median OS in months (range)	20.8 (2.2^+–32.4^+)	15.0 (1.0^+–32.4^+)
Median PFS in months (range)	2.1 (0.03^+–32.2^+)	1.9 (0.03^+–9.9)

PFS Progression free survival, *OS* Overall survival, *PR* Partial response
ORR Overall objective response rate=[CR+PR/number of eligible patients], *CI* Confidence Interval
$^+$ censored observation

related grade 3/4 toxicities in taxane- naïve and taxane-pretreated safety population were neutropenia (22.4 and 40.0%, respectively), leucopenia (8.6 and 16%, respectively), fatigue (6.9 and 8.0%, respectively; no grade 4), and peripheral neuropathy (0.0 and 6.0%, respectively; no grade 4). The results demonstrated some eribulin activity in metastatic CRPC patients with taxane naïve disease [63].

In a second multicenter ECOG trial, 119 progressive metastatic CRPC patients were treated with eribulin on the same dose and schedule as earlier trial. Overall 116 patients were eligible for the primary response evaluation. Median age of patients was 70 years and median number of treatment cycles was 4 (range 1–20+). This non-comparative study stratified patients to either a chemonaïve, prior-taxane only, or two prior cytotoxic chemotherapy arms. The trial was powered to detect a 50% PSA reduction using Consensus Criteria in at least 40 vs. 20% (90% power, one-sided $\alpha = 0.10$) for the chemonaïve stratum and 25 vs. 10% (power 87%, one-sided $\alpha = 0.10$) for the taxane and two prior cytotoxic chemotherapy strata. In the chemonaïve arm (41 patients) 24% had ≥50% PSA response, 15% had SD for more than 9 weeks and 8% (of 26 patients with measurable disease) showed response. Median duration of PSA response was 7.1 months and median OS (in months) was not reached. In prior taxane arm (51 patients) 10% had ≥50% PSA response, 20% had SD for more than 9 weeks and 3% (of 38 patients with measurable disease) showed response. Median duration of PSA response was 3.6 months and median OS was 11.4 months. In the "two prior cytotoxic chemotherapy regimens" arm (51 patients) 4% had ≥50% PSA response, 29% had SD for more than 9 weeks and 8% (of 13 patients with measurable disease) showed response. Median duration of PSA response can't be evaluated and median OS was 13.7 months. Among eligible patients who received treatment, 65 had dose modifications (both planned and unplanned). Treatment related grade 3/4 adverse events (frequency ≥10%) in taxane naïve, prior taxane and two prior chemotherapies, respectively were neutropenia (52, 50, 68%), leukopenia (33, 44, 52), fatigue (17, 8, 12%) and sensory neuropathy (14, 16, 4%), respectively. In the chemonaïve strata PSA response rate was 24%, much below the anticipated 40% rate. Thus although eribulin demonstrated some activity in metastatic CRPC with taxane-naïve disease, the ORR was not sufficient to warrant further studies in CRPC [64].

4.5.2.4 Ovarian Cancer

Based on the activity of eribulin in NIH:OVCAR-3 human epithelial ovarian cancer xenograft model, a multi-centric phase II study in recurrent epithelial ovarian, fallopian tube or peritoneal cancer was conducted. Eribulin mesylate was administered as 1.4 mg/m^2 infusion over 15 min on days 1 and 8 of every 21 days cycle. The eligibility criteria for patients included epithelial ovarian, fallopian tube or peritoneal cancer with measurable disease and ≤2 prior cytotoxic regimens. Patients were stratified into two separate cohorts namely platinum resistant (progression-free interval from last platinum-based therapy <6 months) and platinum sensitive (progression-free interval from last platinum-based therapy ≥6 months). The primary

Table 4.5 Comparison of platinum-resistant and platinum-sensitive ovarian cancer patients treated with eribulin [79]

	Platinum resistant	Platinum sensitive
Patients enrolled/evaluable	37/36	37/37
Median age years (range)	61 (38–80)	60 (45–77)
Median no. of cycles delivered (range)	2 (1–10)	6 (1–51)
Partial response (%)	2/36 (5.5%)	7/37 (19%)
Stable disease (%)	16/36 (44%)	21/37 (57%)
Median PFS (months) (95% CI)	1.8 (1.4–2.8)	4.1(2.8–5.8)
Median OS (months) (95% CI)	18 (11–25)	26 (21–38)

PFS Progression free survival, *OS* Overall survival, *CI* Confidence Interval

objective was to determine the ORR. The results are presented in Table 4.5. No complete response was seen in either cohort. The main treatment related Grade 3/4 toxicities were neutropenia and leucopenia in platinum resistant (42 and 33%) and sensitive (54 and 30%) cohorts, respectively [79].

The effect of weekly administration of bevacizumab (2 mg/kg) with eribulin (1 mg/m^2) and oxaliplatin (30 mg/m^2) in recurrent or refractory serous ovarian carcinoma patients pretreated with paclitaxel and carboplatin is being evaluated. Thirteen patients have been treated of which one patient is still under treatment of two cycles. All patients were pretreated with paclitaxel and carboplatin, nine patients (75%) were pretreated with platinum containing regimen within 6 months and ten patients (83%) received three or more regimens of chemotherapy. Of the 12 evaluable patients, two patients (17%) had CR, one patient (8%) had a PR and three patients (25%) had a SD based on RECIST criteria. The ORR and CBR were 25 and 50%, respectively. Median PFS was 3 months (range: 1–5 months). Grade 3/4 hematological adverse effects were observed in two patients (17%), grade 3 edema and hypo albuminemia were observed in one patient (8%), respectively. The combination showed significant activity with mild toxicity in the target population and warrants further study [80].

4.5.2.5 Sarcoma

This phase II study assessed the efficacy and safety of eribulin mesylate in four independent strata of patients with soft tissue sarcoma namely leiomyosarcoma, adipocytic, synovial and other defined soft tissue sarcomas. Patients with intermediate or high grade, histologically proven, advanced or metastatic soft-tissue sarcomas with ≤ 2 previous chemotherapies (up to two single agents or one combination) for advanced disease and documented progression within last 6 months were eligible. Eribulin, 1.4 mg/m^{2}[22] (i.v. infusion) was given over 2–5 min on days 1 and 8 every 3 weeks. The primary end point was the progression-free survival at 12 weeks. Secondary endpoints included OS, PFS, time to progression, response to therapy, time to onset and duration of response, and incidence and severity of adverse events. A Simon two-stage design was applied (P1: 40%; P0: 20%; $\alpha = \beta = 0.1$) per stratum. A

Table 4.6 Comparison of eribulin efficacy in sarcoma subtypes [81]

	Leiomyosarcoma	Adipocytic sarcoma	Synovial sarcoma	Other soft tissue sarcoma
Number of patients	40	37	19	32
Median age years (range)	60.1 (27.9–81.4)	59.2 (32.7–75.2)	42.3 (208–73.9)	55.9 (18.0–83.3)
Evaluable patients	38	32	19	26
No. of patients progression free at 12 weeks (%)	12 (31.6)	15 (46.9)	4 (21.1)	5 (19.2)
Median PFS in months (95% CI)	2.9 (2·4–4·6)	2.6 (1·7–6·2)	2.6 (2.3–4.3)	2.1 (1.4–2.9)
6-month OS (%) (95% CI)	86.8 (71.2–94.3)	74.6 (55.5–84.6)	71.1 (43.7–86.8)	52.9 (31.2–70.7)

PFS-Progression Free Survival OS-Overall Survival CI-Confidence Interval

median of four cycles per patient (range of 1–43+) cycles were given. A tabulated result of different strata is presented in Table 4.6. Important grade 3/4 treatment-related toxicities included leucopenia, neutropenia, anemia and fatigue in 35, 52, 7 and 7% of patients respectively. The results showed that eribulin has a tolerable profile in pretreated patients with soft tissue sarcomas and showed responses ranging from CR (one adipocytic), PR to SD in various subgroups. Further studies are warranted in leiomyosarcoma and adipocytic sarcoma subgroups, as the progression free survival at 12 weeks reached predefined statistical targets [81]. Further analysis to identify potential biomarkers revealed that overall expression of 62 transcripts including *ALS2CR11* (uncorrected $p < 0.001$) and 26 miRNAs ($p < 0.05$) differed significantly between non-responders and responders [82]. A randomized phase 3 trial in patients with leiomyosarcoma and adipocytic sarcoma has been initiated [81].

4.5.2.6 Pancreatic Cancer

Based on eribulin activity in pancreatic cancer models, an open label, multi-centric, single-arm, phase II study in pancreatic cancer as second line therapy was conducted. Eligibility criteria included locally advanced or metastatic pancreatic adenocarcinoma with measurable disease and evidence of disease progression after gemcitabine therapy. The study used a Simon 2-stage design and the primary endpoint was ORR (CR + PR). Eribulin mesylate (1.4 mg/m^2 i.v.) was administered on days 1 and 8 every 3 weeks. Overall 15 patients were enrolled, 14 received treatment and 12 were evaluable for response. The median age of accrued patients was 62 years and they received a median number of two cycles (range 1–16). No response was observed and therefore study was closed at the end of stage 1. Stable disease as best response was seen in 42% (5/12) patients. Of these 5 patients, 3 had SD for 12 cycles or greater (range 12–16 cycles). Median time to progression was 1.4 months, and median OS was 6.1 months. Drug related grade 3/4 toxicities

included neutropenia, leucopenia and fatigue in 29, 21 and 14 %, patients respectively. Though no response was seen, due to tolerable profile and long term disease control further studies of eribulin in pancreatic cancer may be warranted [83].

4.5.2.7 Urothelial Tract Cancer (UC) and Renal Insufficiency (UCD)

Eribulin has minimal renal excretion (< 10 % excreted in urine) and antitubulin agents have activity in UC. Therefore after encouraging activity of eribulin in UC in phase I renal dysfunction [60], subsequent phase II part of this multi-centric trial was conducted. As per protocol, initially, only patients without renal insufficiency were to be enrolled until the phase II dose (MTD) was proved safe. Accrual of patients with renal insufficiency would then begin at this dose. In the first part of phase II trial, patients with normal creatinine or calculated creatinine clearance ≥ 60 mL/min and any histologically or cytologically confirmed urothelial cancer without prior cytotoxic therapy for advanced disease (though allowed neo and adjuvant therapies) were accrued. Eribulin mesylate (1.4 mg/m^2 i.v.) was administered on day 1 and 8 every 21 days. The primary objective was ORR evaluation as defined by RECIST in a 2-stage design (requiring ≥ 2 responses in 21 patients to proceed to a total of 41 pts). The secondary objectives were PFS, OS and safety assessment. In total 40 patients with median age of 67 years entered the trial. Accrued patients included transitional (35), adenocarcinoma (3), squamous (1) and small cell (1) cancer. Overall 72.5 % patients had prior neo/adjuvant chemotherapy. The percentage of patients in Bajorin risk groups 0, 1 and 2 were 30, 57 and 13 %, respectively. Based on 37 evaluable patients, ORR was found to be 38 % (one CR and 14 PR). Of the 15 responses 13 were in transitional cell cancer patients (37 % response rate). A response rate of 34 % was observed in patients with prior neo/adjuvant chemotherapies. At median follow-up of 19.8 months, median PFS was 3.9 months and median OS was 9.4 months. There was a significant correlation between PFS and Bajorin risk group ($p = 0.028$ for trend). Important drug related grade 3/4 toxicities included neutropenia, hyponatremia, hyperglycemia, leg fatigue with aching and sensory neuropathy in 20, four, three, one and one patient, respectively. The results showed eribulin as a single agent has significant activity in UC to warrant further investigations. Final results of the study are awaited. A per last reports accrual was ongoing for patients with creatinine clearance < 40 mL/min in whom the 1.4 mg/m^2 dose was tolerable [69].

4.5.2.8 Squamous Cell Carcinoma of the Head and Neck

A multi-centric phase II trial to evaluate response of eribulin mesylate in patients with metastatic or recurrent squamous cell carcinoma of the head and neck (SCCHN) was conducted with NCI collaboration. The primary objective was assessment of response rate (confirmed CR and PR). Secondary objectives were PFS, OS and safety/toxicity assessment. Eligibility criteria included patients with no

prior chemotherapy for metastatic or recurrent SCCHN. Induction or adjuvant therapy was limited to one. A total of 40 eligible patients with median age of 61.2 years and Zubrod Performance Status of 0 (48 %) and 1 (53 %) were enrolled. Thirty-three patients (83 %) had metastatic disease. Primary tumor sites included oropharynx (15 patients), lip/oral cavity (12 patients), larynx (six pateints), hypopharynx (four patients), other/unknown (two patients) and nasopharynx (one patient). Patients received eribulin mesylate at 1.4 mg/m^2 on Days 1 and 8 of an every 21-day cycle. Common grade 3/4 toxicities included lymphopenia, leucocytopenia, neutropenia, hyponatremia, fatigue, diarrhea and dyspnea in 15, 13, 10, 5, 5, 5 and 5 % of population, respectively. One treatment-related death due to pulmonary hemorrhage was observed at treatment initiation. Among 40 assessable patients, two confirmed and one unconfirmed PRs were observed and 19 patients had SD. An estimated confirmed response rate of 5 % (95 % CI: 1–17 %) was observed. The estimated median PFS was 3 months (95 % CI: 1–3 months) and estimated median OS was 7 months (95 % CI: 5–10 months). Thus although eribulin was well tolerated in metastatic or recurrent SCCHN, it did not show any significant efficacy in terms of median PFS [84].

4.5.3 Phase III Studies

The encouraging results of eribulin in MBC patients led to its investigation in two randomized, controlled phase III trials in patients with locally advanced/recurrent or MBC previously treated with an anthracycline and a taxane.

The first of these trials, "EMBRACE" (Eisai Metastatic Breast Cancer Study Assessing Physician's Choice Versus E7389; NCT00388726; Study 305), was an open-label, global, multicenter (137), randomized, controlled, parallel-group phase III study in heavily pretreated MBC patients to compare OS with eribulin to real-life choices. Inclusion criteria included women (≥18 years) with hystologically or cytologically confirmed, locally recurrent or MBC previously treated with 2–5 prior chemotherapies (≥2 for advanced disease), including an anthracycline and a taxane. Patients were randomized in 2:1 to eribulin (1.4 mg/m^2 i.v., 2–5 min on days 1 and 8 of a 21-day cycle) or treatment of physician's choice (TPC; defined as any single-agent chemotherapy or hormonal or biological treatment approved for the treatment of cancer and to be administered according to local practice; radiotherapy; or symptomatic treatment alone). Patients as well as investigators were not masked to treatment allocation. The primary endpoint was to compare OS. Secondary endpoints were ORR, PFS, DOR by independent masked review. Tumor assessments were performed every 8 weeks (±1 week), or sooner if there was a suspicion of disease progression. In all, 762 (508 eribulin, 254 TPC) patients with median age of 55 years (range 27–85 years) were randomized. Of these 16 % were HER2-positive and 19 % were triple-negative. Median number of prior chemotherapy regimens was four (range 1–7) and 73 % of them had received prior capecitabine.

Table 4.7 Summary of results of EMBRACE trial as assessed by independent review [85]

	Eribulin	TPC
No. of patients randomized/ treated/measurable disease	508/503/468	254/247/214
Age (range)	55·0 (28–85)	56·0 (27–81)
Number of HER2 (combined FISH and IHC tests) positive patients (%)	83 (16%)	40 (16%)
Triple (ER, PgR, HER2) nega tive patients (%)	93 (18%)	51 (20%)
Patients with prior Capecitabine (%)	370 (73%)	189 (74%)
Median duration of treatment, months (range)	3·9 (0·7–16·3)	2·1 (0·03–21·2) for chemotherapy (n=238), and 1·0 month (0·8–6·2) for hormone therapy (n=9).
Median PFS, months (95% CI)	3.7 (3.3–3.9)	2·2 (2·1–3·4) HR 0·87 (95% CI: 0·71–1·05), p=0·137
Complete response (%)	3 (1%)	0
Partial response	54 (12%)	10 (5%)
Stable disease	208 (44%)	96 (45%)
ORR (%, 95% CI)	57 (12%; 9·4–15·5)	10 (5%; 2·3–8·4) p=0.002
CBR (%, 95% CI)	106 (23%; 18·9–26·7)	36 (17%; 12·1–22·5)
Median DOR (months) (95% CI)	4·2 (3·8–5·0)	6·7 (6·7–7·0) (p=0·159)
Median OS (months) (95% CI)	13·1 (11·8–14·3)	10·6 (9·3–12·5) HR 0·81 (95% CI: 0·66–0·99; p=0·041)

CBR-Clinical benefit rate (defined as CR+PR+SD>6 months), *CI* Confidence Interval
PFS-Progression free survival, *OS* Overall survival
DOR-Duration of response
ORR-Overall objective response rate=[CR+PR/number of eligible patients]

Table 4.7 presents summarized results from EMBRACE study. Important adverse events with eribulin were asthenia/fatigue (all grades 54, 8% grade 3, 1% grade 4), neutropenia (all grades 52, 21% grade 3, 24% grade 4) and peripheral neuropathy (all grades 35, 8% grade 3 and <1% grade 4). With TPC important adverse events were asthenia/fatigue (all grades 40, 10% grade 3, no grade 4), neutropenia (all grades 30, 14% grade 3, 7% grade 4) and nausea (all grades 28, 2% grade 3, no grade 4). Overall 25% patients on eribulin and 26% of patients on TPC experienced serious adverse events and adverse events leading to treatment discontinuation occurred in 13% of eribulin patients and 15% of TPC patients. The most common adverse event leading to eribulin discontinuation was peripheral neuropathy, occurring in 24 (5%) of 503 patients. The study met its primary endpoint with a significant improvement of OS by a median of 2.5 months in patients treated with eribulin as compared to real world choices. Moreover, eribulin related toxicities were easily manageable, and are acceptable for a single chemotherapy agent to be used in this late-line setting. Though ORR, a secondary end point was statistically significant, PFS another secondary endpoint, did not reach statistical significance in independent review. However on investigator review the difference between treatment groups was significant with similar median PFS (eribulin 3.6 months vs.

TPC 2.2 months, HR 0·76, 95% CI 0·64–0·90; $p=0·002$). This was because fewer patients were censored in investigator review as compared to independent review (127 vs. 241). The response rate to eribulin, though significant (12% with eribulin vs. 5% with TPC; $p=0.002$), was on the lower side [85]. The pre-defined subgroup analyses showed that as compared to TPC, eribulin maintained an OS benefit irrespective of hormone receptor status, extent of disease and prior therapy [86]. Also though the study had a limited number of old patients, survival outcomes with eribulin were independent of age and toxicity did not appear to be greater in older patients [87].

An analysis on EMBRACE data was done to determine the effects of dose modification due to adverse events on duration of therapy. Overall, 462 patients had a treatment-related adverse event and 204 (44.2%) had dose modification (61.8% had dose delay only, 17.6% had reduction only, and 20.6% both). Interestingly most patients were Caucasian (92.4%). Patients with dose modification had a higher number of chemotherapy cycles (mean 7.36 (±4.56) vs. 5.71 (±3.68), $p<0.001$), remained on treatment for a longer duration (median 143 days vs. 105 days, $p<0.001$) and had a better median PFS (130 vs. 92 days based on independent review). However, after adjusting for the length of treatment exposure using time-dependent and landmark approaches no statistically significant correlation was found between PFS and dose modification. Thus, delaying or reducing eribulin dosage in patients who experience adverse effects may allow patients to remain on therapy for a longer duration. However further studies are warranted to confirm the impact on overall efficacy and safety [88].

Another open-label, randomized, multicenter, controlled, parallel-group phase III study (NCT00337103; Study 301) has been conducted to assess eribulin as a second-line therapy for metastatic disease. Patients were randomized 1:1 to eribulin mesylate (1.4 mg/m^2 i.v., days 1 and 8 of a 21 day cycle) or capecitabine (2.5 g/m^2/day orally BID, days 1–14 of a 21 day cycle). Eligibility criteria included women (≥18 years) with histologically or cytologically confirmed locally advanced or MBC with ≤3 previous chemotherapeutic regimens (but ≤2 regimens for locally advanced or MBC) including an anthracycline and a taxanes with documented progression. Eligible patients were receiving study drug as 1st, 2nd, or 3rd line therapy for advanced disease [89]. Tumor assessments are carried out every 2 cycles (each of 3 weeks' duration) and every third cycle subsequently starting with cycle 15 [14]. The primary co-endpoints were OS and PFS and pre-specified statistical significance at final analysis for eribulin versus capecitabine were $p\leq0.0372$ for OS and $p<0.01$ for PFS. Secondary endpoints included ORR, quality of life measured using the EORTC questionnaire, DOR, 1-, 2- and 3-year survival, and safety. The study was stratified for HER 2 status and geographic region. In total 1102 patients with a median age of 54.0 years (range 24–80) were randomized to eribulin and capecitabine. Overall 27.2% patients received study treatment as their 1st line, 57.4% as 2nd line and 14.7% as 3rd line chemotherapy in the setting of metastatic disease. Results are summarized in Table 4.8. The most commonly encountered adverse effects (>20% all grades) with eribulin and capecitabine were neutropenia (54.2 vs. 15.9%), hand-foot syndrome (0.2 vs. 45.1%) alopecia (34.6 vs.

Table 4.8 Summary of results of Phase III study of eribulin mesylate versus capecitabine [89]

	Eribulin	Capecitabine
Number of randomized patients	554	548
HER2- patients	375	380
Median no. of cycles	6	5
Median OS (months)	15.9	14.5 (HR 0.879; 95% CI 0.770–1.003; $p=0.056$)
Median PFS on independent review (months)	4.1	4.2 (HR 1.079; 95% CI 0.932–1.250; $p=0.305$)
ORR (independent review)	11.0% (95% CI 8.5–13.9)	11.5% (95% CI 8.9–14.5; $p=0.849$)
OS for HER2(–) pts (months)	15.9	13.5 (HR 0.838; 95% CI 0.715–0.983; $p=0.030$)

PFS Progression free survival, *OS* Overall survival, *CI* Confidence Interval
ORR Overall objective response rate = [CR + PR/number of eligible patients]

4.0%), leukopenia (31.4 vs. 10.4%), diarrhea (14.3 vs. 28.8%), and nausea (22.2 vs. 24.4%), respectively. Though eribulin demonstrated a slightly improved OS, it didn't meet the pre-defined criteria for statistical significance and it didn't show a benefit in PFS. The study didn't show superiority of eribulin over capecitabine [89]. Global Health Status/Quality of life scores and cognitive functioning improved significantly more with eribulin whereas emotional functioning improved significantly more with capecitabine. Gastrointestinal side effects like nausea, vomiting and diarrhea were less severe in eribulin arm whereas systemic side effects and upset due to hair loss were more with capecitabine [90]. Subgroup analyses suggest that patients with triple negative, ER negative, HER2 negative disease, only non-visceral disease, with >2 organs involved, who had progressed >6 months after last chemotherapy, or who had received an anthracycline and/or a taxane in the metastatic setting appeared to benefit more from treatment with eribulin compared with capecitabine [91]. However, further studies are warranted to address these hypotheses.

4.6 Conclusions

Eribulin mesylate is a novel drug which has shown a significant activity and efficacy in heavily pretreated refractory breast cancer patients in phase III trials and has also progressed in phase II trials in several cancer subtypes. On November 15, 2010, eribulin was approved as a third line therapy for patients with MBC, previously treated with an anthracycline and a taxane by the U.S. Food and Drug Administration [20]. Eribulin received European Commission approval on 17 March 2011 based on the results of the Phase III EMBRACE study and is now available in more than 50 countries worldwide [92]. The recommended dose of eribulin mesylate in normal, mild hepatic (Child Pugh A), moderate hepatic (Child Pugh B) and moderate renal impairment (creatinine clearance 30–50 mL/min) is 1.4, 1.1, 0.7 and 1.1 mg/m^2, re-

spectively, as a 2–5 min i.v. bolus on days 1 and 8 of 21-day cycle [20]. A liposomal formulation of eribulin is currently undergoing clinical development. Apart from the trials mentioned above eribulin is also being tested either as a single agent or in combination with multiple agents like carboplatin, sorafenib, cetuximab, POL6326, PLX3397, cyclophosphamide, trastuzumab, pemetrexed, lapatinib, and erlotinib in various phase I/II trials for various malignancies like advanced or recurrent salivary gland cancer, cervical cancer, lung cancer, head and neck cancer, colon cancer, bladder cancer, cancer of urothelium and kidney dysfunction and breast cancer (as neoadjuvant therapy, adjuvant therapy, and as a first and second line agent) [21].

The main treatment related toxicities are fatigue and neutropenia which are manageable and the incidence of peripheral sensory neuropathy is comparatively low. The differences in the incidence of neuropathy with eribulin and ixabepilone were not statistically significant. Onset of neuropathy tended to occur later with eribulin and resolve later according to phase II study results [75]. Also the incidence of grade 3/4 neutropenia with eribulin was comparatively less than with ixabepilone, vinorelbine and docetaxel as observed from cumulative data of phase III trials. Eribulin showed a statistically significant OS [85] which was not seen with a recently approved drug, ixabepilone.

In an effort to produce second generation structurally modified analogues with increased oral bioavailability, brain penetration and efficacy against multidrug resistant tumors as well as attenuated P-gp mediated drug efflux via minimizing the P-gp susceptibility, new drug analogs with attenuated basicity of the nitrogen substituent have been developed. The results suggested that introduction of amines with low basicity in the C32 side chain, or otherwise increasing lipophilicity in this portion of eribulin, leads to the identification of analogs with greatly reduced susceptibility to P-gp mediated drug efflux. These compounds showed moderate oral bioavailability, high potency and oral *in vivo* activity. These novel compounds may thus provide candidates for the treatment of a wider variety of human cancers [93]. A series of eribulin analogues was also evolved in silico through iterative atom-based enumeration employing a genetic algorithm-derived survival function to minimize predicted P-gp-mediated drug efflux. These new computer-inspired derivatives were found to exhibit high cell growth inhibitory activity and to be among the least sensitive to P-glycoprotein-mediated drug efflux in the eribulin series, thereby validating this approach to in silico molecular design [94]. Sensitivity analysis of liposomal formulation of eribulin mesylate (E7389-LF) showed that adjusting liposomal formulation would lead to decreased C(max) and much longer half-life of ultracentrifuged mice plasma (UCM) E7389, which might result in better efficacy and lower toxicity with better therapeutic profile[95].

Although eribulin was approved in U.S. and Europe, it was rejected by National Institute of Health and Clinical Excellence (NICE) in the United Kingdom based on its cost effectiveness. Following the EMBRACE trial, the estimated cost per cycle of eribulin was £1738 compared to £1335, £1599, £1429, and £740 for the costs per cycle for treatment of physician's choice (TPC), vinorelbine, gemcitabine and capecitabine respectively with incremental costs for eribulin of £5586, £5177, £4041, and £12,779 compared with TPC, gemcitabine, vinorelbine and

capecitabine respectively. This resulted in incremental cost-effectiveness ratios for eribulin of £46,050 per quality-adjusted-life year (QALY) gained vs. TPC, £35,602 vs. vinorelbine, £27,183 vs. gemcitabine and £47,631 vs. capecitabine. The NICE review committee concluded that eribulin could not be considered a cost-effective use ofNational Health Service (NHS) resources even if all of the criteria for being a life-extending, end-of-life treatment were met [96]. However, eribulin represents a promising new treatment option as single-agent chemotherapy in patients' with solid tumors and in particular, the chemotherapy pretreated breast cancer patients. Results of other phase I/II/III trials and novel combinations [21] are awaited.

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Chapter 5
Antitumour Effect of Cyclodepsipeptides from Marine Sponges

Rosa Lemmens-Gruber

Abstract Marine natural compounds with cyclodepsipeptidic structure are of great interest in drug discovery. The most intensively studied sponge-derived cyclodepsipeptide jasplakinolide (jaspamide) and its analogues are generally accepted as actin-polymerising and actin-stabilising drugs. Jasplakinolide is a potentially useful pharmacological tool for the study of actin organisation and dynamics in living cells. Also neamphamides and geodiamolides were tested to possess potent cytotoxic activities. These compounds represent an ideal starting point for scaffold mining and it is believed that additional screening of natural and unnatural jasplakinolide compounds and other cyclic depsipeptides will provide a route for significant future innovation.

Keywords Cyclodepsipeptides · Marine sponges · Jaspakinolide analogues · Jaspamide · Neamphamides · Geodiamolides · Cytotoxicity

5.1 Introduction

Studies on marine natural products have led to the discovery of a variety of compounds with known or novel pharmacological and toxicological activities. These new organic molecules and their structurally modified analogues may be used as medicines, or as biochemical, physiological or pharmacological tools in biomedical research.

A major challenge in the drug development of marine natural products is the difficulty in supplying sufficient material for pre-clinical and clinical evaluation due to natural limitations of the source organism. Marine products are frequently endowed with complex organic structures that complicate their total syntheses in large scale to meet the demands of a drug development programme.

The first marine natural product that was tested in clinical trials as an anticancer agent was didemnin B. It was the most potent analogue of the cyclic depsipeptides isolated from the Caribbean tunicate *Tridemnum solidum*. However, in the

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end it failed to demonstrate effective antitumor activity, but showed cardiac and neuromuscular toxicities [1, 2]. A structurally related compound, dehydrodidemnin B, also named aplidine or plitidepsin, was originally isolated from the Mediterranean tunicate *Aplidium albicans* and has been shown to be more potent and less toxic than didemnin B [3, 4]. Plitidepsin is currently in multiple phase II and III trials for the treatment of various cancers [5–9]. Kahalalide F was isolated from the mollusks *Elysia rufescens* and *Spisula polynyma* and from the green alga *Bryopsis* sp. [10]. In phase I and II clinical trials, kahalalide F provided a benefit to patients with advanced androgen-refractory prostate cancer and other advanced tumours [11–14]. Thus, marine natural compounds with cyclodepsipeptidic structure are of great interest in drug discovery.

5.2 Jasplakinolide (Jaspamide)

Jasplakinolide, isolated from the marine sponge *Jaspis splendens* and also known as jaspamide, is a cyclic depsipeptide with a 15-carbon macrocyclic ring containing three amino acid residues, L-alanine, *N*-methyl-2-bromotryptophan, and β-tyrosine (Fig. 5.1 and Table 5.1). These residues in the backbone and the aromatic electrons of the β-tyrosine residue are important during binding of jasplakinolide to positively charged species. Such binding could cause conformational changes in both jasplakinolide and target proteins, and affect their properties [15].

5.2.1 Structure of Jasplakinolde Analogues

Two new jasplakinolide derivatives Q and R (Fig. 5.1 and Table 5.1), together with the parent compound jasplakinolide, have been isolated from the marine sponge *Jaspis splendens* collected in Kalimantan, Indonesia [16]. Additional jasplakinolide

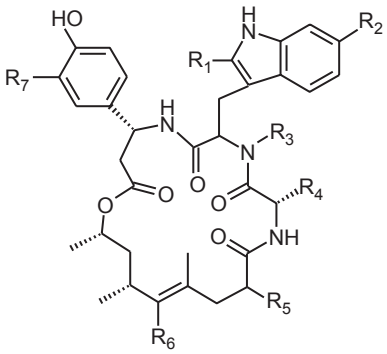
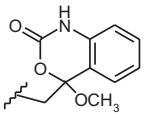
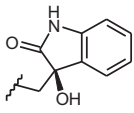
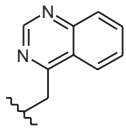


Fig. 5.1 Structure of jasplakinolides

Table 5.1 Residues referring to Fig. 5.1 (asterisks for R_1 mark residues shown in Table 5.2)

Jasplakinolide and analogues	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Jasplakinolide	Br	H	CH_3	CH_3	CH_3	H	H
B	Br	H	CH_3	CH_3	CH_3	O	H
C	Br	H	CH_3	CH_3	CH_3	OH	H
D	Br	H	CH_3	CH_2 CH_3	CH_3	H	H
E	Br	H	CH_3	CH_2OH	CH_3	H	H
F	Br	H	CH_3	CH_3	H	H	H
J	Br	H	CH_3	CH_3	H	H	H
M	Br	H	H	CH_3	CH_3	H	H
P	*	H	CH_3	CH_3	CH_3	H	H
Q	H	H	CH_3	CH_3	CH_3	H	H
R	Br	Br	CH_3	CH_3	CH_3	H	H
S	*	H	CH_3	CH_3	CH_3	H	H
T	*	H	CH_3	CH_3	CH_3	H	H
U	*	H	CH_3	CH_3	CH_3	O	H
V	Br	H	CH_3	CH_3	CH_3	H	OH

Table 5.2 Structure of residues R_1 in jasplakinolides P, U, S and T

R_1 of jasplakinolide analogues		
Jasplakinolide P and U	**Jasplakinolide S**	**Jasplakinolide T**
		

analogues were isolated from two taxonomically distinct marine sponges including two *Auletta* spp. and one *Jaspis splendens*. This led to the identification of jasplakinolide and eleven jasplakinolide analogues including seven new analogues, 21-epi-jasplakinolides P and S, jasplakinolides S, T, U, C_a and C_b (Fig. 5.1, Tables 5.1 and 5.2). Five of the new compounds are oxidized tryptophan derivatives of jasplakinolide, including a unique quinazoline derivative jasplakinolide T (Fig. 5.1, Tables 5.1 and 5.2) [17].

Recently new jasplakinolide analogues were referred to the National Cancer Institute's Biological Evaluation Committee. In total, 12 congeners including six known jasplakinolides and new open chain derivatives as well as structures with modified β-tyrosine residues were identified (jasplakinolide V, Tables 5.1 and 5.2) and evaluated for biological activity. For a number of congeners cytotoxicity in the nM range and potent microfilament disruption activity was observed.

Structure–activity requirements and actin-binding models based on jasplakinolide and chondramide C were evaluated. The importance of the macrolide geometry for this binding model was established, however, some new insights were provided; e.g. two acyclic analogues exhibited similar potency to jasplakinolode in colon, CNS and lung cancer cell lines [18].

5.2.2 Mode of Action of Jasplakinolide

Jasplakinolide is a potent inducer of actin polymerisation *in vitro* [19]. Actin is a ubiquitous eukaryotic cytoskeletal protein, critical for many aspects of cell activity. In addition to maintaining cell morphology, it is required for cell motility, cell division, and intracellular transport [20]. The dynamics of the globular actin to fibrous actin transition may be critical to many cellular functions including cell division. Competitive binding studies suggest that jasplakinolide binds to fibrous actin. It was suggested that jasplakinolide might exert its cytotoxic effect *in vivo* by inducing actin polymerisation and/or inhibiting depolymerisation of actin filaments [19]. Jasplakinolide disrupts the actin cytoskeleton of normal and malignant mammalian cells with no significant effects on phagocytic activity [21]. The effect of jasplakinolide on the *in vitro* proliferation and differentiation (the expression of the differentiation marker CD16 and CD14 B was up-regulated) of leukaemic cell lines and blast cells was comparable to that of cytosine arabinoside [22].

Jasplakinolide is generally accepted as an actin-polymerising and actin-stabilising drug. It stabilises actin filaments *in vitro,* but *in vivo* it can disrupt actin filaments and induce polymerisation of monomeric actin into amorphous masses. The effect of jasplakinolide on the actin cytoskeleton is reversible even at high concentrations [23].

Jasplakinolide is a potentially useful pharmacological tool for the study of actin organisation and dynamics in living cells, since it induces actin polymerisation *in vitro* and, unlike phalloidin, is membrane permeable. Jasplakinolide continues to be employed in studies investigating the role of the actin cytoskeleton in various cell processes. Participation of the actin cytoskeleton in the transduction of proliferative signals has been established. To address the possibility that actin also participates in the transduction of apoptotic signals, the response of the murine interleukin 2 (IL-2) dependent T cell line CTLL-20 to treatment with jasplakinolide upon IL-2 deprivation was studied. Jasplakinolide was not toxic to CTLL-20 cells, nor was apoptosis induced in the presence of exogenous recombinant human IL-2. However, actin stabilisation at IL-2 deprivation enhanced apoptosis. This effect of jasplakinolide correlated with its ability to stabilise polymerised actin. This enhancement occurred upstream of the induction of caspase-3-like activity and could be inhibited by the overexpression of the anti-apoptotic protein Bcl-x_L. These data suggest that the actin cytoskeleton plays an active role in modulating lymphocyte apoptosis induced by cytokine deprivation [24]. In addition, in the HL-60 human promyelocytic leukaemia cell line jasplakinolide-induced loss of viability by programmed cell death was accompanied by neutral endopeptidase/CD10 expression on the surface of the apoptotic cells. HL-60 cells normally do not express detectable amounts of

neutral endopeptidase/CD10 on their surface or in the cytoplasm. This implies an association between apoptosis induction and CD10/neutral endopeptidase expression in myeloid cell lines. Thus, jasplakinolide induces cell death via apoptosis through caspase-dependent and -independent pathways [25].

In addition, it has been demonstrated that various transformed cell lines, such as human leukaemia Jurkat T cells, murine T lymphoma EL-4 cells, murine myeloma SP-2/0 cells, murine macrophage-like J774.1 cells and murine fibroblast L cells, are more sensitive to jasplakinolide-induced apoptosis than normal, non-transformed cells such as murine thymocytes and spleen T cells [26].

Results from the clonogenic assay for jasplakinolide showed little toxicity to HCT-116 cells at 2 and 24 h dosing schedules. However, the desired therapeutic effect was achieved at 0.004 μM with continuous exposure for 7 days. This outcome indicates that jasplakinolide would require a chronic dosing schedule for maximum therapeutic effectiveness *in vivo*. The maximum tolerated dosage of jasplakinolide has been determined as 0.625 mg/kg. Subsequent pharmacokinetic studies are in progress. The authors plan further therapeutic evaluation of jasplakinolides B, E, and V. These compounds represent an ideal starting point for scaffold mining and it is believed that additional screening of natural and unnatural jasplakinolide compounds will provide a route for significant future innovation [18].

5.2.3 Toxicological Studies with Jasplakinolide

In contrast to microtubules, which have been targeted successfully with anti-tumour drugs such as taxol-like compounds and the vinca alkaloids, very few actin-targeting drugs have been characterised. To date, no actin targeting drugs have been used in clinical trials due to their severe cytotoxicity. One reason for this cytotoxicity is that drugs such as cytochalasins and latrunculins disrupt actin microfilaments in both non-tumour and tumour cells [27, 28]. In mouse tumour models and acute toxicity studies in rats and dogs, jasplakinolide had a narrow margin of safety. It was assumed that the observed toxicity was due to cardiotoxicity. Thus, jasplakinolide was tested in a patch clamp assay to determine its effect on cardiac ion channels. At a concentration of 10 μM jasplakinolide inhibited Kv1.5 activity almost completely. In addition, jasplakinolide also inhibited other channels such as Cav1.2, Cav3.2, and HCN2. However, the hERG channel was only minimally affected. Contractility in spontaneously contracting human cardiomyocytes was decreased by jasplakinolide. The cardiotoxic effects of jasplakinolide were compared with that of the known cardiotoxicant mitoxantrone, and confirmed by multiparameter fluorescence imaging analysis [29].

5.2.4 Cytotoxic Effects of Jaslakinolide Analogues

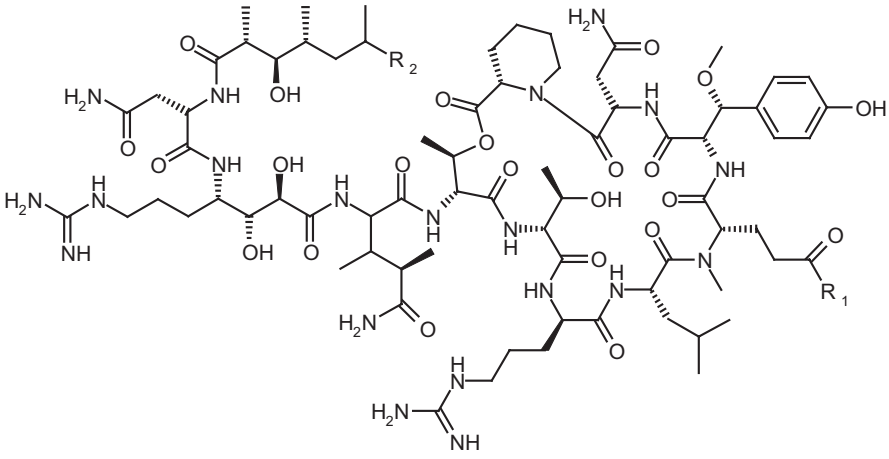
Two new jasplakinolide derivatives Q and R, together with the parent compound jaspamide have been isolated from the marine sponge *Jaspis splendens* collected in

Kalimantan (Indonesia). The new derivatives inhibited the growth of mouse lymphoma cell line (L5178Y) in vitro with IC_{50} values of < 0.1 µg/ml [16].

Jasplakinolides B, E, P and S as well as 21-epijasplakinolides P and S were evaluated in the NCI 60 cell line screen, and all compounds were tested in a microfilament disruption assay. Jasplakinolide B exhibited potent cytotoxicity. A 50% reduction in proliferation of human colorectal adenocarcinoma (HCT-116) cells was observed at concentrations less than 1 nM. However, jasplakinolide B did not exhibit microfilament-disrupting activity at 80 nM [17].

5.3 Neamphamides

In the search for potential antitumour compounds from marine organisms, a single fraction derived from a pre-fractionated extract of the Australian sponge *N. huxleyi* was identified as having potent activities against lung (A549), cervical (HeLa), and prostate (LNCaP and PC3) cancer cell lines [30]. The authors reported the isolation and structure elucidation of three new cyclic depsipeptides, neamphamides B, C, and D (Fig. 5.2), along with their cytotoxic activity. The cytotoxicity of neamphamides B−D was evaluated on a panel of human cancer and non-cancer cell lines. The results demonstrated that these cyclic depsipeptides possessed potent cytotoxic activities, with IC_{50} values ranging from 88 to 370 nM. To study the cytotoxic effects of these compounds in more detail, a real-time assessment of cell function or



Neamphamide A: R_1=NH_2, R_2=H
Neamphamide B: R_1=NH_2, R_2=CH_3
Neamphamide C: R_1=OH, R_2=CH_3
Neamphamide D: R_1=NH_2, R_2=C_2H_5

Fig. 5.2 Structure of neamphamides

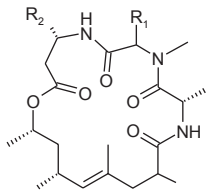
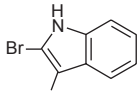
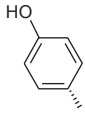
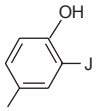
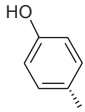
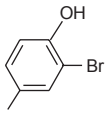
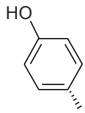
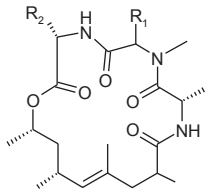
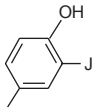
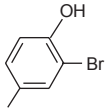
viability using impedance readout was performed. All three compounds were tested on A549, PC3, and NFF cells at 100, 200, and 300 nM. The real-time cell analysis failed to identify any mechanism of cytotoxicity. Thus, confocal imaging was then used to identify the causes. The image showed that the effect was due to increased cell numbers. Taken together with the real-time cell analysis there was a prolonged increase in cell numbers for 72 h. Thus, this class of compounds should be treated cautiously as cytotoxic compounds, as they may cause cell proliferation at sub-cytotoxic doses.

5.4 Geodiamolides

The cyclic depsipeptides geodiamolides A, B, H and I were isolated and characterized from the Caribbean marine sponge *Geodia* sp. [31, 32]. Geodiamolides A and B presented antifungal activity [31], while geodiamolide H was active against cancer cell lineages (lung, HOP 92; central nervous system, SF-268; ovarian, OV car-4; kidney, A498 and UO-31; breast, MDA-MB-231/ATCC and HS 578T), although geodiamolide I was considered inactive in the same screening [32]. Compounds were also isolated from the marine sponge *G. corticostylifera*, collected on the Brazilian coast, for studies on the anti-proliferative effects of these peptides in sea urchin eggs (*Lytechinus variegatus*), and T47D and MCF7 human breast cancer cells lineages. Using fluorescence techniques and confocal microscopy the effects of the geodiamolides A, B, H and I on cancer cell and normal cell lineages (human fibroblasts and rat liver cells) cytoskeleton and nucleus were studied. In the breast cancer cells experiments, the values of EC_{50} for the geodiamolides A, B, H and I were obtained in nM range. Geodiamolides A and H were more effective against T47D cells, while geodiamolides B and A had a stronger effect on MCF7 cells. In T47D cells geodiamolides A, B, H and I act upon F-actin, disorganizing the filaments and gathering them in the cytoplasm, in a dose-dependent manner, and in a way similar to other depsipeptides such as jaspamide and dolastatins. At the concentration of 100 ng/ml (135–170 nM), nuclei were displaced from central position in the cytoplasm and their shape changed when compared to control cells, while microtubule organization remained unchanged. Disorganization of microfilaments of T47D cells induced by the geodiamolides A and H was perceived within 2 h of the treatment, and progressed along the incubation time. In normal cells lines, only geodiamolide A induced a slight disorganization of the human fibroblasts microfilaments at 100 ng/ml concentration, while geodiamolide H did not cause cytoskeleton alterations. The same concentration of both peptides had no effect on rat liver epithelial cells (BRL-3A) F-actin, thus indicating the biomedical potential of these compounds [33].

Further experiments with geodiamolide H and breast cell cultures (normal and tumoral) growth in a 3D environment showed that the peptide induced striking phenotypic modifications in the Hs578T cell line, a poorly differentiated and aggressive cell line, and disruption of the actin cytoskeleton. The peptide seemed to revert

Table 5.3 Structural similarities between jasplakinolide and geodiamolides [33]

Basic structure	R_1	R_2	cyclodepsipeptide
			Jasplakinolide
			Geodiamolide H
			Geodiamolide I
		-CH_3	Geodiamolide A
		-CH_3	Geodiamolide B

Hs578T malignant phenotype and impaired the migration and invasive behaviour of this cell line [34]. Geodiamolides were not cytotoxic for normal cell lines and did not affect the normal distribution pattern of the microfilaments. Microfilaments may be involved on gap junction communication maintenance at the cell membrane. Both geodiamolides A and H (200 nM concentration for 2 h) increased the length of gap junction plaques in HTC-Cx43-GFP cells [35].

Connexins are membrane proteins that form gap junction channels between adjacent cells. Connexin 43, the most widely expressed member of the connexin family, has a rapid turnover rate, and its degradation involves both the lysosomal and ubiquitin–proteasome pathway. The goal of this work was to study the effects of geodiamolides, natural peptides from marine sponge that normally are involved with microfilament disruption, on connexin assembly or degradation in the plasma membrane. Among the four peptides tested, only 200 nM geodiamolide H statistically enhanced the length of gap junction plaques (38.4%) without changing the actin filaments organization. At 400 nM geodiamolide drastic microfilament disruption was observed. Under these conditions, the cells became rounded, and gap junction plaques were absent. Geodiamolide A also showed activity in the gap junction plaque size; however, its effect was less pronounced (25.4%). Further experiments in presence of the fungal antibiotic brefeldin A were performed in order to uncouple events leading to gap junction assembly from those related to gap junction removal. Gap junction plaques were drastically reduced after treatment with geodiamole H and brefeldin A, indicating that geodiamolide H affects mainly the delivery pathway of connexion 43 protein [35].

5.5 Sponge-derived Cyclodepsipeptides with Cytotoxic Activity

There is a number of cyclodepsipeptides with strong *in vitro* cytotoxic effects, however, with lacking *in vivo* data (Table 5.4).

Table 5.4 Sponge-derived cyclodepsipeptides with *in vitro* cytotoxic effects

Cyclodepsipeptide	Sponge	Cell line	IC_{50}	Reference
Arenastatin A analogues	*Dysidea arenaria*	KB 3-1	0.18 ng/ml	[36]
		In vivo mouse model	1 mg/kg	
Halipeptins D	*Leiosella cf. Arenifibrosa*	HCT-116	7 nM	[37]
Scleritodermin A	*Scleritoderma nodosum*	HCT116	1.9 μM	[38]
		HCT116/VM46	5.6 μM	
		A2780	0.94 μM	
		SKBR3	0.67 μM	
Theopapuamides	*Theonella swinhoei*	CEM-TART	0.5 μM	[39]
		HCT-116	0.9 μM	
Spongidepsin	*Spongia* sp.	J774.A1	0.56 μM	[40]
		WEHI-164	0.42 μM	
		HEK-293	0.66 μM	
Seragamides A–E	*Suberites japonicus*	NBT-T2	0.064–0.58 μM	[41]
		(BRC-1370)		

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Chapter 6
Cytotoxic Cyclic Peptides from the Marine Sponges

Toshiyuki Wakimoto, Karen Co Tan, Hiroki Tajima and Ikuro Abe

Abstract To date, a significant number of cyclic peptides have been isolated from the marine sponges. Their structures often contain non-proteinogenic amino acids, some of which are derived from the biosynthetic pathway mixed with polyketides synthase. Halogenation, *N*-formylation, and racemaization to D-isomers were also frequently observed. Here we review the structural features of cytotoxic cyclic peptides from marine sponges. The cyclic peptides and depsipeptides were classified into different cyclization ways. The recent progress on the studies of their mode of action and biosynthesis was also included.

Keywords Cyclic peptide · Marine sponge · Nonribosomal peptide · Ribosomal peptide · Polyketide

6.1 Introduction

The marine sponges are prolific sources of cyclic peptides and depsipeptides (including ester bonds as part of their backbone) with unusual amino acids. The unique biological activity of these macrocycles originates from their structural complexity and ability to form rigid conformations in solution, which are therefore sought after as promising lead compounds for drug discovery. In particular, the reduction in conformational freedom brought about by macrocyclization often results in specific biological activities. Indeed, the fact that linearization abolishes the activity, has been known in some cyclic peptides. Although many reviews on natural compounds from marine environments have been published, we will focus here only on selected sponge-derived cytotoxic cyclic peptides and analogues either discovered or syn-

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thesized. Moreover, recent progress in biosynthetic studies on peptide secondary metabolites will also be described. It has become apparent that cyclic peptides were classified into ribosomal peptides and nonribosomal peptides. Nonribosomal peptide synthetases (NRPSs) are large multimodular biocatalysts, which are sometimes mixed with multimodular polyketide synthases (PKS) [1]. The hybrid pathway composed of NRPS and PKS often involves the biosynthesis of non-proteinogenic amino acids, leading to non-ribosomal peptide natural products with new and unusual structural motif. Most recently, the some modification processes of ribosomal peptides were also found to be comparable to those of nonribosomal peptides [2]. Taking into account the biosynthetic pathway, the cyclic peptides and depsipeptides are classified into two different cyclization ways, head-to-tail and head-to-side-chain, respectively. In addition, other unique cyclic peptides such as side-chain-to-tail heterodetic cyclopeptides, imidazole-bridged cyclopeptides, and dilactones are also included.

6.2 Cyclopeptides

6.2.1 Head-to-tail Cyclopeptides

This section aims to roughly classify the structurally diverse family of N-to-C-macrocylic peptides that have been characterized from marine sponges. Generally, natural product peptides can be subclassified on the basis of their biosynthetic origin: non-ribosomal (NRPS) and ribosomal peptides. Nonribosomally-produced peptides usually possess non-proteinogenic amino acids and the final product may be modified by tailoring enzymes via epimerization, hydroxylation, methylation, heterocyclization, and/or oxidative cross linking, among others, during or after the nonribosomal peptide assembly [3]. In this section, the following putative macrocyclic NRPS metabolites will be discussed: azumamides, barangamides, ceratospongamides, mutremdamides, and perthamides. On the other hand, ribosomally-synthesized cyclic peptides or cyanobactins consist exclusively of proteinogenic amino acids, and are produced by cyanobacteria, including *Prochloron* sp. and *Anabaena* sp. Many of them feature heterocyclized amino acids, such as thiazoline, methyloxazoline, and oxazoline, or their oxidized counterparts. However, homodetic cyanobactins or those which do not possess heterocyclized amino acids also exist, in which case, at least one proline residue is always present. Occasionally, post-translational modification may also involve prenylation of serine, threonine or tyrosine residues [4]. Under this category, the following putative cyanobactins from marine sponges shall be tackled: axinastatin, euryjanicins, hymenamides, malaysiatin, myriastramides, phakellistatins, and waiakeamide.

Azumamides are cyclic tetrapeptides isolated from the marine sponge *Mycale izuensis*. They were noted to possess an unusual β-amino acid, 3-amino-2-methyl-5-nonedioic acid (or 9-amide), and were found to potently inhibit histone deacetylase (HDAC), an enzyme involved in the regulation of DNA transcription.

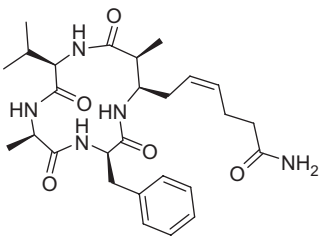
Consequently, HDAC is thought to be a potential target for anticancer agents, as demonstrated by the antiangiogenic effects of azumamide A *in vitro* [5].

Barangamides were first discovered from the marine sponge *Theonella swinhoei*, along with both new and known depsipeptides, theonellapeptolides. These cyclic undecapeptides have the same amino acid sequence as the cyclic portion of theonellapeptolides II, differing only in that the latter undergoes macrolactonization through the hydroxyl group of threonine, while the former forms the macrocycle through a peptide bond. Both peptide series bear several D-amino acids, N-methylated amino acids, as well as β-amino acids. Despite this, only the theonellapeptolides displayed cytotoxicity against L1210 tumor cells, while the barangamides were inactive [6, 7].

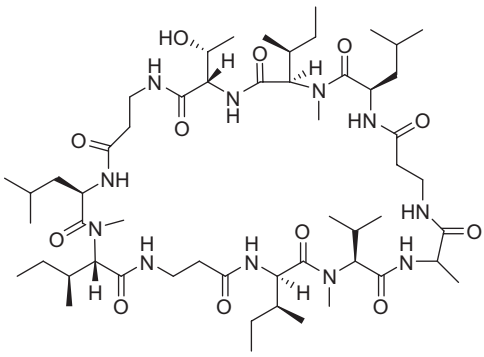
Ceratospongamides are cyclic heptapeptides having thiazole and methyloxazoline heterocycles. The *cis,cis*- and *trans,trans*-isomers were concomitantly obtained from the marine red alga *Ceratodictyon spongiosum* containing the symbiotic sponge *Sigmadocia symbiotica*. This was an unprecedented case of isolating two stable non-interconverting proline amide rotamers from nature. Although the real producer of these metabolites is still unknown, their biosynthesis is thought to proceed through a non-ribosomal pathway. This is well-suggested by the results of the synthetic studies conducted by Deng and Taunton, where if macrocyclization was carried out before threonine heterocyclization, only the *cis, cis*-isomer was synthesized. The chronology of this synthetic scheme mirrors that of the ribosomally-produced cyanobactins, where macrocylization precedes heterocyclization, and as a result, only one geometric isomer could be expected. In contrast, when heterocyclization was conducted before macrocylization, it became possible to obtain both geometric isomers, in a ratio that closely resembled their occurrence in nature. In terms of biological activity, only the *trans, trans*-isomer potently inhibited the secreted phospholipase A2 enzyme, suggesting its anti-inflammatory activity [8, 9].

Mutremdamide A (perthamide C) is a cyclic depsipeptide first characterized from deep-water specimens of *T. swinhoei*. Furthermore, it possesses a sulphated and carbamoylated asparginine, an unusual 3-amino-2-hydroxy-6-methylheptanoic acid (Ahmha), and a rare *o*-tyrosine residue. It is structurally similar to perthamide B isolated from the same species, in which the aforementioned residues have been replaced with β-hydroxyaspargine, 3-amino-2-hydroxy-6-methyloctanoic acid, and 3-bromotyrosine, respectively. Perthamide B has been reported to be cytotoxic, while perthramides C and D exhibited potent anti-inflammatory activity *in vivo* [10–12] (Fig. 6.1).

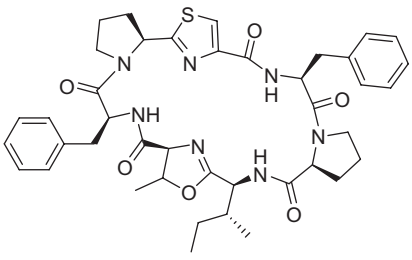
Axinastatins are N-to-C macrocyclic all-L-peptides that were isolated from the marine sponge *Axinella* sp. Axinastatins 1–4 are heptapeptides with two proline residues, while axinastatin 5 is an octapeptide having three proline residues. This group of peptides have been shown to be cytotoxic against murine and human cancer cell lines suggesting that the presence of high proportions of proline residues may be an important structural requirement for bioactivity. Moreover, conformational studies of axinastatins 1–4 indicated that the two prolines in common in positions two and six most strongly influenced the heptapeptide conformation, regardless of the solvent [13–18]. Two years after the discovery of axinastatin 1, another cyclic



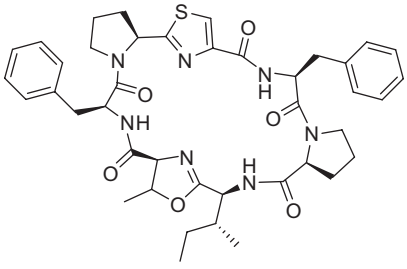
Azumamide A



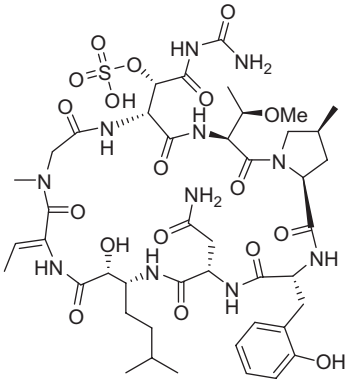
Barangamide A



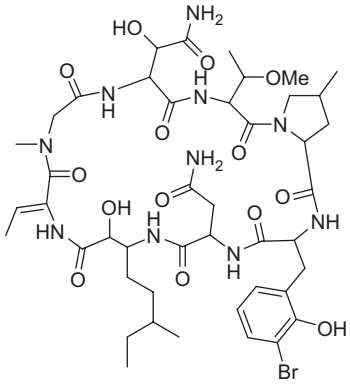
cis,cis-ceratospongamide



trans,trans-ceratospongamide



Mutremdamide A



Perthamide B

Fig. 6.1 Structures of head-to-tail cyclopeptides

heptapeptide, malaysiatin was reported from the marine sponge *Pseudoaxinyssa spongue*, whose concentrated extracts showed both cytotoxic and antimicrobial activities. The proposed structure was claimed to possess a unique valine homotripeptide and a prolylproline fragment: cyclo-(-Asn-Pro-Pro-Phe-Val-Val-Val) [19]. In comparison with axinastatin 1, cyclo-(-Asn-Pro-Phe-Val-Val-Pro-Val), the only difference was their amino acid sequence. However, through chemical synthesis of both peptides and comparison with the natural products, only the structure of axinastatin 1 was confirmed to be accurate, while malaysiatin's structure was found to be identical with that of axinastatin 1 [20].

Dominicin is a cyclic octapeptide that was first characterized from the Caribbean marine sponge *Eurypon laughlini*. It was isolated again from another Caribbean marine sponge *Prosuberites laughlini*, along with the cycloheptapeptide euryjanicins. Dominicin was demonstrated to inhibit a leukemia and a renal cancer cell line, while euryjanicin A inhibited a non-small cell lung and renal cancer cell line. Euryjanicins B-D were only weakly cytotoxic against the 60-tumor cell line panel of the National Cancer Institute. Such loss in activity of proline-rich peptides was explained to may have been caused by conformational changes during the isolation process or otherwise their ability to complex with highly toxic natural products, which can only be detected by biological methods [21–25].

Hymenamides A–F are heptapeptides, while hymenamides G, H, J, and K are octapeptides isolated from the Okinawan marine sponge *Hymeniacidon* sp. Among this series of cyclic proline-rich peptides, hymenamides B and J demonstrated cytotoxicity against L1210 murine leukemia cells and KB human epidermoid carcinoma cells, while hymenamide H exhibited cytotoxicity only against the L1210 cells. On the other hand, hymenamides A, C, D, E, G and K did not show cytotoxicity against these cells. Furthermore, hymenamides A, B, C and E were shown to have antifungal activity against *Cryptococcus neoformans*, while hymenamides A and B were active against *Candida albicans* [26–29].

Myriastramides are cyclic octapeptides and were the first peptidic metabolites characterized from the Philippine marine sponge *Myriastra clavosa*. Myriastramides A and B are structurally similar, both possessing an isoprenyl ether group on tyrosine. However, whereas the former is directly prenylated, the latter bears an exomethylene and a chlorine atom. In contrast, the aromatic residue in myriastramide C is tryptophan, rather than tyrosine. Although the crude extracts of the sponge displayed selective toxicity towards NCI's 60-cell line human antitumor screen, myriastramide A failed to show any cytotoxicity, while the other two derivatives yielded insufficient amounts for further cytotoxicity screening [30].

Phakellistatins are proline-rich homodetic type macrocyclic peptides. The first one of this series, phakellistatin 1, is a heptapeptide obtained from two marine sponges, *Phakellia costata* and *Stylotella aurantium*. Phakellistatins 2–6, 13–14, and isophakellistatin 3 consist of 7 amino acids. Phakellistatin 2 from *P. carteri* has the same amino acid composition as phakellistatin 1, but the sequence is different. On the other hand, phakellistatin 3 and its isomer isophakellistatin 3 only differ on the hydroxyl group orientation at the photo-tryptophan indole ring juncture. These compounds discovered from *P. carteri* represent the first example of natural

product peptides with photo-oxidized tryptophan. Interestingly, between these two isomers, only phakellistatin 3 inhibited cancer cell growth. Furthermore, in contrast with other phakellistatins, which are generally all-L cyclopeptides, phakellistatin 4, characterized from *P. costata*, bears a threonine in D-form, confirmed by chiral GC analysis of its *N*-pentafluoropropionyl isopropyl ester-derivatized hydrolysate. Next, phakellistatin 5 and 14, isolated from *P. costata* and *Phakelli* sp., contain methionine and methionine sulfoxide units, respectively. In addition, phakellistatin 14 possesses a unique β-methoxyaspartic acid residue. Moreover, decapaptides, phakellistatins 7–9 and phakellistatin 12, have also been characterized from *P. costata* and *Phakellia* sp., respectively. They represent the first cyclic decapeptides with cell growth inhibitory activity. Lastly, octapeptides, phakellistatins 10–11 have also been characterized from *Phakellia* sp. All phakellistatins exhibited cancer cell growth inhibitory activities [31–41].

A cyclic hexapeptide, waiakeamide, was described from the sponge *Ircinia dendroides*. Its structure consists of a thiazolylphenylalanine and two methionine sulfoxides. Although it did not exhibit any biological activity, a few years later, it was re-isolated from another sponge *Haliclona nigra*, together with two other cytotoxic analogues, haligramides A and B. Haligramide A possesses two methionines in place of the methionine sulfoxides in waiakeamide, while haligramide B has one methionine and one methionine sulfoxide. The structures of these three peptides were eventually confirmed by their oxidation to a common bis-sulfone derivative. The haligramides demonstrated cytotoxicity against various tumor cell lines [42, 43] (Fig. 6.2).

6.2.2 Head-to-side-chain Cyclopeptides

This section focuses on some of the head-to-side chain cyclised peptides that have been described from marine sponges of the genus *Theonella*, *Discodermia*, *Microscleroderma*, *Psammocinia*, and *Ircinia*. The first two genera both belong to the family Theonillidae (Order Lithistida), the third one belongs to the family Scleritodermidae (Order Lithistida), while the latter two belong to family Irciniidae (Order Dictyoceratida). *Theonella* species have been an abundant source of bioactive peptides, including cyclotheonamides (A-E, E2, E3), orbiculamide, oriamides, keramamides, motuporin, and microsclerodermins. Meanwhile, under the *Discodermia* species, we shall be covering the calyxamides and discobahamins. Lastly, we shall also be discussing about the cyclocinamides and cycotheonamides E4-E5 from *Psammocinia* and *Ircinia*, respectively.

Cyclotheonamides A–E, E2 and E3 are potent serine protease inhibitors. These cyclic pentapeptides bearing a β-linked diaminopropionic acid (Dpr) have been reported from either *Theonella* sp. or *T. swinhoei*. The characteristic and rare amino acids, vinylogous tyrosine (V-Tyr) and α-ketohomoarginine (K-Arg), in their structures have been demonstrated by X-ray crystallography to be important for the binding of cyclotheonamide A with human α-thrombin, a serine protease crucial

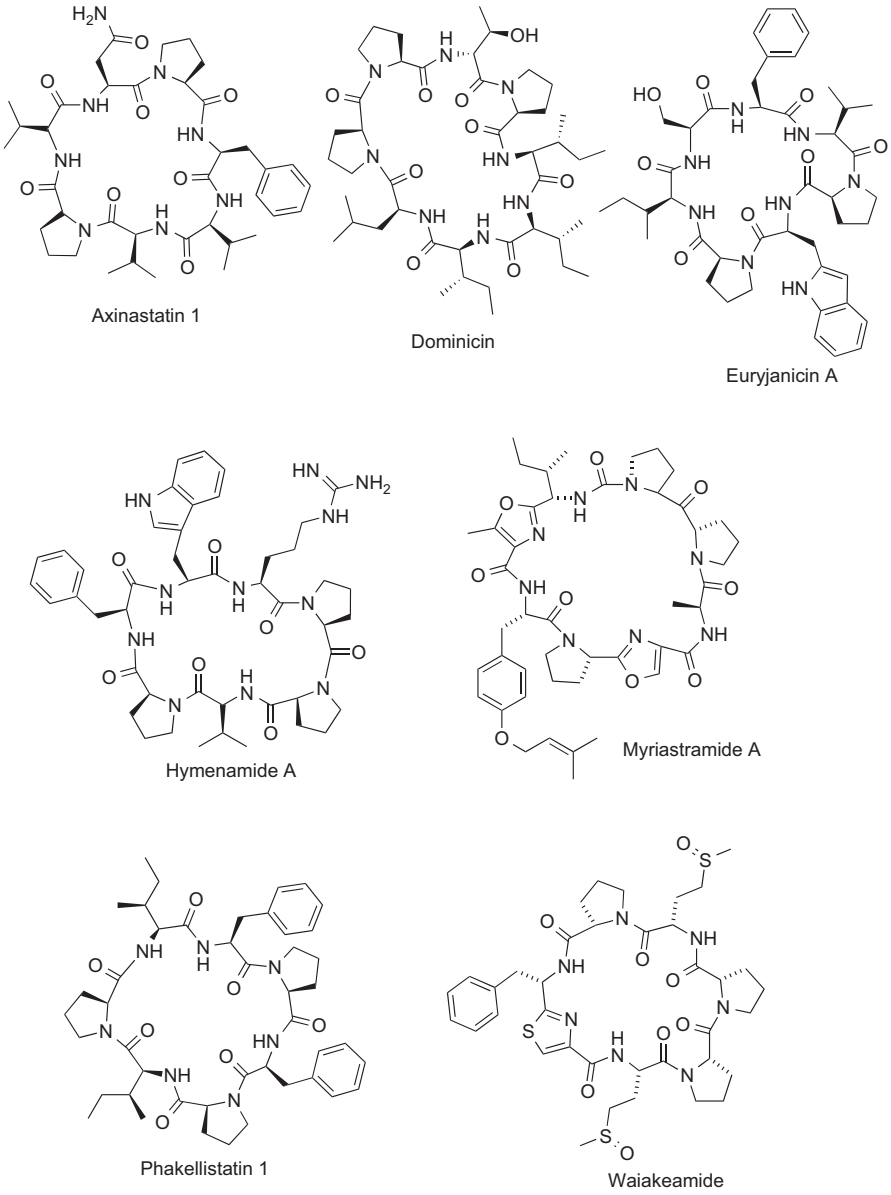


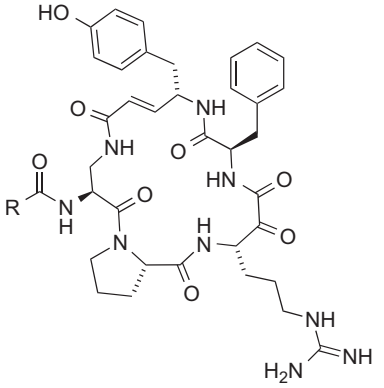
Fig. 6.2 Structures of head-to-tail cyclopeptides

for the regulation of thrombosis and hemostasis. Their ability to inhibit other serine proteases has also been reported, showing that cyclotheonamide A has enhanced specificity towards trypsin, while cyclotheonamides E-E3 prefer thrombin. Such difference in specificity was explained by the X-ray crystallography results and

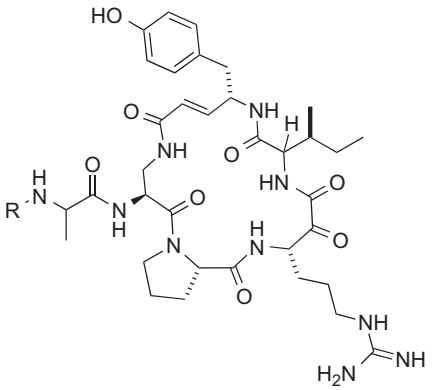
the structural differences among the analogues. The *N*-formyl group in cyclotheonamide A is replaced by an *N*-acetyl group in cyclotheonamide B. Cyclotheonamide C is a dehydrogenated analogue of cyclotheonamide A, where V-Tyr possesses an additional unsaturation. Next, the D-phenylalanine residue in cyclotheonamide A is replaced by a leucine residue in cyclotheonamide D. On the other hand, in cyclotheonamides E, E2, and E3, this D-phenylalanine residue is substituted with D-isoleucine, while the *N*-formyl group is replaced by an *N*-phenylacetylalanyl, an *N*-benzoylalanyl, and an *N*-isovalerylalanyl group, respectively. The phenylalanine-isoleucine substitution explains the loss of aromatic interaction of cyclotheonamides E-E3 with Tyr 39 and Tyr 41 in trypsin, while the presence of bulky acylated alanyl residue increases the hydrophobic interaction with Ile 174 in thrombin. Because of the potential of these compounds as antithrombotic agents, the total synthesis of cyclotheonamide A has also been described. Interestingly, four years after the discovery of cyclotheonamides E2 and E3, two other analogues, cyclotheonamides E4 and E5 were isolated from a different family of marine sponge, *Ircinia* sp. Both cyclotheonamides E4 and E5 bear an *N*-3-methylpentanoyl moiety in place of the *N*-isovaleryl group in cyclotheonamide E3. However, only cyclotheonamide E4 contains the characteristic V-Tyr residue, whereas cyclotheonamide E5 possesses an additional hydroxyl substitution ortho to V-Tyr's hydroxyl group. These two cyclotheonamides were reported to potently inhibit tryptase, which is a protease released from mast cells during allergic reactions. Consequently, these natural products are eyed for their potential use for the treatment of allergic diseases, including asthma [44–51].

Orbiculamide A is a cytotoxic cyclic peptide from *Theonella* sp. Its structure consists of three unique amino acid residues, 2-bromo-5-hydroxytryptophan (BhTrp), theonalanine, and theoleucine. Theoalanine contains an oxazole ring, which derives from the carbonyl of an alanine residue, while theoleucine is an α-keto-β-amino acid, which is reminiscent of the α-ketoamide, K-Arg, in cyclotheonamides. Aside from these, the cyclic portion of this peptide also consists of proline and δ-linked ornithine, while the side chain consists of a (*S*)-3-methylpentanoyl moiety [52]. Oriamide is another cytotoxic cyclic peptide from *Theonella* sp. Such as in the case of other cyclic peptides from *Theonella*, it was found to contain an α-ketoamide, 3-amino-2-keto-4-methyl hexanoic acid (AKMH). Furthermore, the presence of an unprecedented amino acid, 4-propenoyl-2-tyrosylthiazole (PTT), was established. Along with AKMH and PTT, the cyclic part of oriamide also includes cysteic acid, a β-linked Dpr, and norvaline. In addition, the side chain consists of one residue each of alanine and glycine, and a 2,5-dihydroxybenzoyl-protected *N*-terminus. However, this paper did not present their data regarding cytotoxicity [53] (Fig. 6.3).

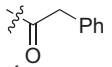
Keramamides are a series of tryptophan-containing head-to-chain cyclic peptides from the Okinawan marine sponge *Theonella* sp. We have classified them into the (1) oxazole-containing keramamides B–E and M–N, (2) thiazole-containing keramamides F–H, and J–K, and for the sake of completeness, we have also arbitrarily included (3) ureido-containing keramamides A and L. For the first subgroup, keramamides B–E are closely related to orbiculamide A, where the latter's (*S*)-3-methylpentanoyl side chain is changed to a (2*S*,3*S*)-2-hydroxy-3-methylpentanoyl moiety



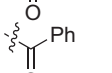
Cyclotheonamide A R = H
Cyclotheonamide B R = Me



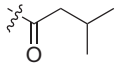
Cyclotheonamide E R =

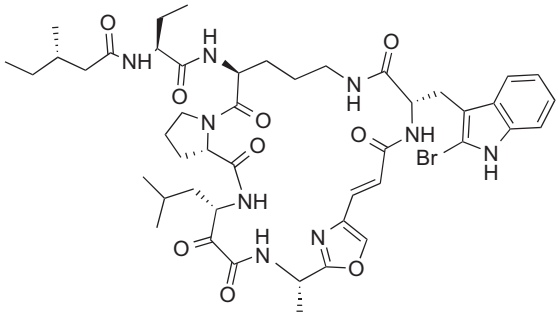


Cyclotheonamide E2 R =

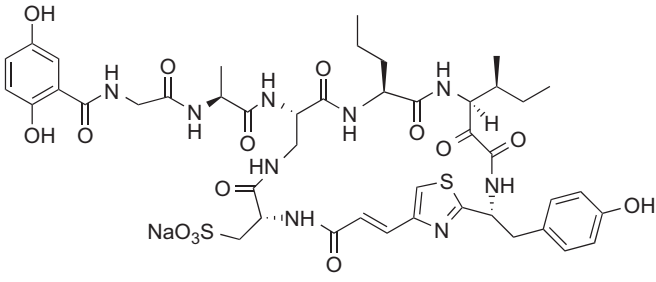


Cyclotheonamide E3 R =





Orbiculamide A



Oriamide

Fig. 6.3 Structures of head-to-side-chain cyclopeptides

(Hmp). On the other hand, keramamides M and N are rare sulphate ester congeners of the Hmp residue of keramamides D and E, respectively. For the next subgroup, all of them possess unusual amino acids, including isoserine (Ise), β-linked Dpr, AKMH, and (*O*-methylseryl)thiazole. In addition, keramamides F and G possess an α, β-dehydrotryptophan. The gross structures of these two keramamide congeners were judged to be the same, but with slight differences in the ^{13}C chemical shifts in the AKMH residue. Alkaline peroxidation, followed by acid hydrolysis of these two peptides converted AKMH to isoleucine in the D-form for keramamide F and in the L-form for keramamide G. Then, keramamides H and K contain rare modified tryptophan residues, BhTrp and (1-Me)Trp, respectively. Finally, for the third subgroup, keramamides A and L possess unusual features, such as the presence of a ureido bond and a 6-chloro-5-hydroxy-*N*-methyltryptophan (MeCtrp). In terms of bioactivities, keramamide A was reported to inhibit sarcoplamic reticulum Ca^{2+}-ATPase; keramamides B–D inhibited the superoxide generation response of human neutrophils; and keramamides E–F, K–N demonstrated cytotoxicity *in vitro* against KB human epidermoid carcinoma cells and L1210 murine leukemia cells, while keramamides G, H, and J displayed weak activity against these cell lines [54–59].

Motuporin (nodularin V) is a cytotoxic cyclic pentapeptide isolated from a Papua New Guinea marine sponge *T. swinhoei*. Its structure closely resembles that of an equipotent phosophatase inhibitor, nodularin (nodularin R), which was isolated from fresh-water blue-green cyanobacterium *Nodularia spumigena*. Both contain the β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda), which is also present in the structurally-related heptapeptide cyanobacterial toxin, microcystin-LR, from the cyanobacterium *Microcystis aeurigonsa*. The only difference between the two nodularins is the substitution of a polar arginine residue in nodularin R with a nonpolar valine residue in motuporin. These data suggest that the arginine residue is not significant for biological activity. Further details about the structural basis of how protein phosphatases are inhibited by motuporin and dihydromicrocystin were revealed by the crystal structure of these toxins complexed with human protein phosphatase-1c (γ-isoform). Indeed, phosphatase inhibitors are valuable tools for understanding these enzymes, which play important roles in regulating intracellular signalling pathways. Furthermore, as for motuporin, it is an especially attractive target for total synthesis because of its relative scarcity, high potency, and unique structure. Consequently, there have been a couple of reports for the total synthesis of motuporin. On the other hand, as for nodularin R and microcystin, their complete gene clusters, encoding integrated nonribosomal peptide-polyketide megasynthases, have been sequenced and characterized from their respective cyanobacterial producers [60–69] (Fig. 6.4).

Microsclerodermins are antifungal cyclic peptides from Lithistid sponges, *Microscleroderma* sp. and *Theonella* sp. Microsclerodemins are a family of hexapeptides, having only three amino acids, glycine, *N*-methylglycine and (3*R*)-4-amino-3-hydroxybutyric acid (GABOB), in common for all members. The variable units include (1) tryptophan, which is formoylated in microsclerodermins A, B, and E, chlorinated in microsclerodermins C-D, and α,β-desaturated in microsclerodermins G and I; (2) the unusual 3-amino-pyrrolidone-4-acetic acid, which is either hydrox-

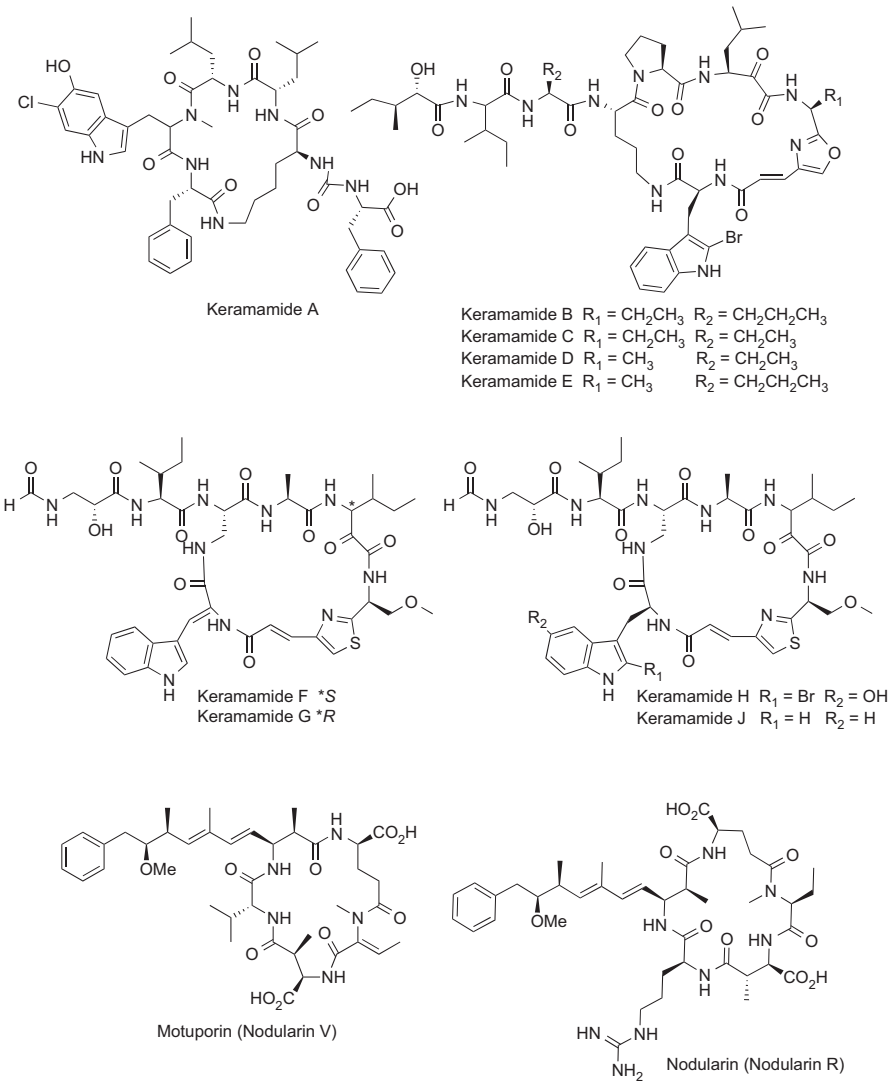


Fig. 6.4 Structures of head-to-side-chain cyclopeptides

ylated or dehydrated; and (3) different ω-aromatic 3-amino-2,4,5-trihydroxyacids. With respect to bioactivity, aside from antifungal activity, microsclerodermins F-I also displayed antitumor activity against HCT-116 cell line. Finally, their production has been attributed to a filamentous bacterium of the family Beggiatoaceae, which occurs as mats in deep-water environments [70–72].

Calyxamides are cytotoxic cyclic peptides isolated from the marine sponge *Discodermia calyx*. Their structures are very similar to the thiazole-containing keramamides, in which the components of the cyclic portion are entirely the same,

except for the tryptophan moiety, which is 5-hydroxytryptophan for calyxamides. In addition, the *N*-terminal amino acid in the side chain is glutamine, instead of Ise, but is also *N*-formylated. Another similar point is that calyxamides A and B possess the same planar structure, but are isomeric at the 3-position of the AKMH residue, just as in the case of keramamides F and G. The microbial producer of the calyxamides is suspected to be *Candidatus* Endotheonella sp., whose presence in *D. calyx* was confirmed by 16s rDNA sequence analysis of the sponge DNA metagenome. This endosymbiont has also been identified from *T. swinhoei*, which may rationalize the similarity of the secondary metabolites from these two sponges [73].

Discobahamins are structurally similar to calyxamides and oxazole-containing keramamides, wherein the BhTrp unit in the latter is changed for a 5-hydroxytryptophan. The Hmp-protected *N*-terminus is common among the discobahamins and keramamides B-E and M-N, while the characteristic (*O*-methylseryl)thiazole, AMKH, and δ-linked ornithine are common among these two series of compounds, as well as the calyxamides. Lastly, the discobahamins are reported to possess weak antifungal activity against *Candida albicans* [74].

Cyclocinamides A and B are minor head-to-side chain cyclohexapeptides, which were isolated at different times and by separate groups from two distinct marine sponges *Psammocinia* and *Corticium*, respectively. Their unique β2αβ2α tetrapeptide cyclic core includes a β-linked Dpr, aspargine, isoserine, and 5-bromotryptophan, while the side chain consists of a glycine unit protected by a distinct proline-derived *N*-methylchloropyrrole for cyclocinamide A or *N*-methyldichloropyrrole for cyclocinamide B. During the first isolation of cyclocinamide A in 1997 by Crews' group, the determination of its absolute structure could not be completed by chiral TLC analysis of its hydrolysate, where only two of the four chiral centers' stereochemistries were identified as 7*S*, 14*S* for 5-bromotryptophan and aspargine, respectively. From the two remaining chiral centers, there were still four possibilities, (4*S*, 11*S*), (4*S*, 11*R*), (4*R*, 11*R*), and (4*R*, 11*S*). In efforts to elucidate cyclocinamide A's complete structure, Grieco and Reilly successfully synthesized nominal (4*R*, 11*R*)-cyclocinamide A, while Postema and Liu were able to synthesize the nominal (4*R*, 11*S*)-diastereomer. Both synthetic studies concluded that the natural product was different from the synthetic materials. Years later, in 2008, recollection of the cyclocinamide-containing sponge lead to the isolation of a little more of the compound, whose acid hydrolysate was derivatized by Marfey's method. This resulted to the supposed complete assignment of the absolute structure of cyclocinamide A as all-*S*. However, in 2012, Konopelski et al., who prepared the two remaining unsynthesized nominal cyclocinamide A's, suggested that the natural product did not match the spectra of either the 4*S*, 11*S*- or 4*S*, 11*R*-diastereomer. Moreover, cyclocinamide B characterized by Ireland et al. (2007) [79], was assigned a 4*S*, 7*R*, 11*S*, 14*R* stereochemistry by Marfey's and modified Marfey's analyses. Because of these conflicting results, the complete structures of the cyclocinamides remain elusive. As for biological activity, only cyclocinamide A demonstrated potent cytotoxicity and selective cytotoxicity against solid tumors, while cyclocinamide B exhibited no cytotoxicity against HCT-116 cells [75–80] (Fig. 6.5).

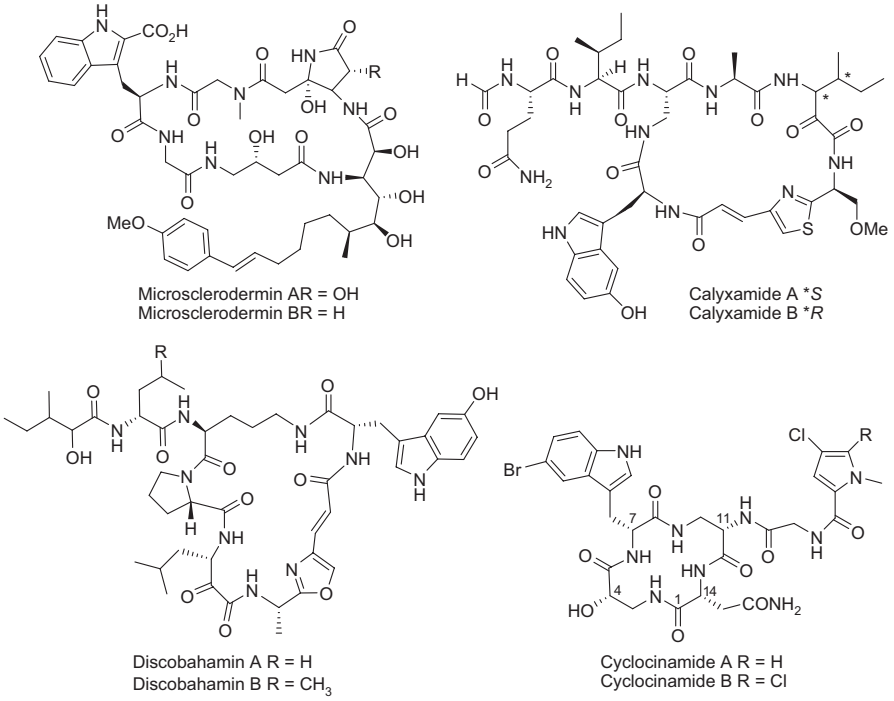


Fig. 6.5 Structures of head-to-side-chain cyclopeptides

6.3 Cyclodepsipeptides

6.3.1 Head-to-tail Cyclodepsipeptides

Jasplakinolide, also known as jaspamide, is a cytotoxic cyclodepsipeptide from the marine sponge, *Jaspis splendens*. Its characterization was first reported in 1986 by two separate groups, Crews et al. [81], and the Ireland–Faulkner–Clardy consortium [82]. Since then, it has also been isolated from taxonomically distinct sponges, *Auletta* [83, 84] and *Hemiastrella minor* [84, 85]. The structure of this polyketide-peptide (PKS-NRPS) metabolite consists of a 19-membered macrocylic lactone, containing an L-Ala-D-N-Me-2-BrTrp-L-β-Tyr tripeptide fragment, linked to an ω-hydroxyacid. Its complete structure was elucidated by NMR spectral [81] and X-ray crystallographic analyses [81, 82], and later confirmed by total synthesis [86].

This unique cytotoxin possesses several bioactivities, such as antifungal, insecticidal, and anthelmintic properties [81, 82]. Most notably, its antiproliferative activity is attributed to its ability to alter actin assembly, inducing cell death. Actin is a cytoskeletal protein ubiquitously found in eukaryotic cells. Its dynamic interconversion between its two forms, filamentous (F-actin) and globular (G-actin) states, is suggested to be crucial for regulating various cellular functions including

cell division [84, 87–88]. Previous reports have provided evidence that jasplakinolide, binds competitively *in vitro* with phalloidin to F-actin. Phalloidin, on the other hand, is a bicyclic heptapeptide derived from the poisonous mushroom *Amanita phalloides*. Although bearing little structural resemblance, the Ala-Trp fragment present in both phalloidin and jasplakinolide are implicated in their actin filament-stabilizing ability, and are representative examples of this class of actin-targeting natural products [87, 89]. Jasplakinolide, in contrast to other actin-targeting substances, has exhibited selective induced apoptosis towards transformed cell lines than normal, nontransformed cells. In addition, its cytotoxicity against HL-60 (human promyelocytic leukemia) cells, Jurkat T (immortalized human T lymphocytes) cells, EL-4 (murine lymphoma) cells, SP-2/0 (mouse hybridoma: B lymphocyte) cells, and J774.1 (mouse ascites reticulum) cells has been demonstrated [84, 90]. It has also exhibited cytotoxicity against breast and prostatic cancers [87]. As such, it was considered to be a potential antineoplastic agent until it was withdrawn from preclinical evaluation due to severe toxicity [91, 92]. Consequently, investigations on both natural and synthetic analogues of jasplakinolide still continue in hope of finding a pharmacologically useful drug.

The jasplakinolide family so far consists of nearly 20 analogues and has been classified into two groups based on the hybridization of C-31, i.e. sp^3-hybridized in Group 1 and sp^2-hybridized in Group 2 [83, 93, 94]. In addition, related actin-targerting cyclodepsipeptides have been characterized from other marine sponges, such as geodiamolides from *Geodia* [95] and *Cymbastela* and seragamides from *Suberites* [96]. Interestingly, a structurally similar family of 18-membered cyclodepsipeptides, the chondramides, have been isolated from terrestrial myxobacterium [94, 97]. Furthermore, similarly bioactive congeners have been synthesized [92], persistently providing insights to structure-activity relationships, substrate binding mechanisms, and drug optimization studies. Based on these, a number of generalizations about the structure-bioactivity patterns of jasplakinolide congeners have been summarized by Crews et al., citing the importance of the *S*-configuration at C-9 to maintain optimal activity and the β-tyrosine unit for protein binding, among others [94]. These valuable pieces of information contribute to overcoming the challenges of drug development and to the further optimization of modified jasplakinolide analogues that hold promise for cancer chemotherapy (Fig. 6.6).

6.3.2 Head-to-side-chain Cyclodepsipeptides

The depsipeptide is defined as the peptides containing ester bonds in place of at least one of the amide bonds. Usually, the ester bond is involved in the macrocycle linkage between the carboxyl group in C-terminal amino acid and an amino acid bearing hydroxyl group such as serine and threonine. When serine or threonine is located at an internal position of the peptide sequence, the macrocyclic ring can be closed in a head-to-side-chain fashion. The first head-to-side chain cyclodepsipeptides from marine sponge was discodermin A originally isolated from the

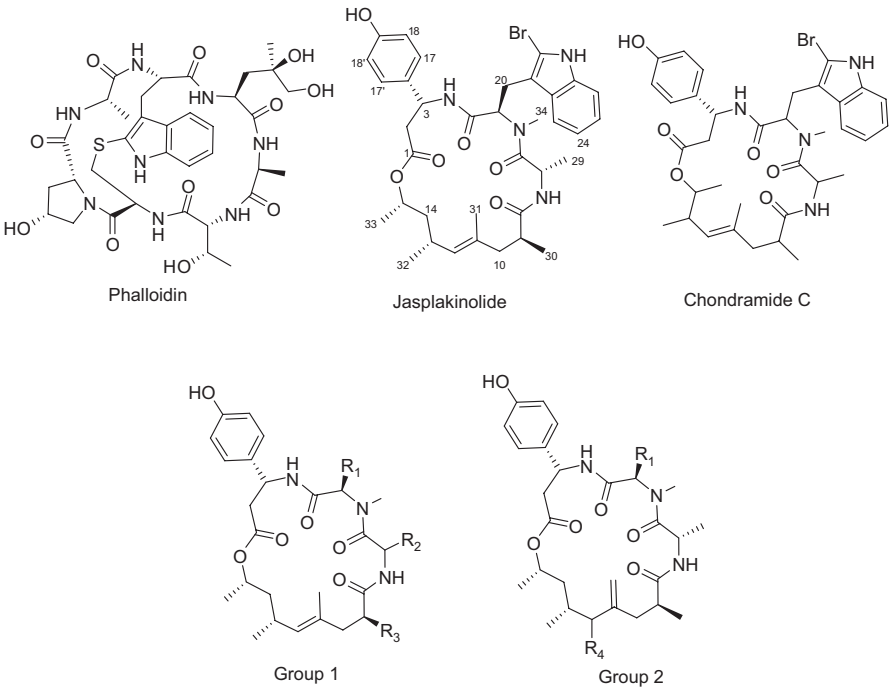


Fig. 6.6 Structures of head-to-tail cyclodepsipeptides

marine sponge *Discodermia kiiensis* in 1984 [98, 99]. Since then, the related derivatives with high structural similarity had been isolated from taxonomically remote sponges. Discodermins [99–102] and polydiscamide A [103] were from the genus *Discodermia*; polydiscamides B–D [104] from the genus *Ircinia*; halicylindramides [105, 106] from the genus *Halichondria*; microspinosamide [107] from the genus *Sidonops*; corticiamide [79] from the genus *Corticium*. Their structures can be classified into two groups; discodermins, halicylindramides and corticiamide are tetradecapeptides with 19-membered macrolactone, and polydiscamides and microspinosamide are tridecapeptides with 16-membered macrolactone. The presence of formyl group on N-terminus, L-The residue on the branching point and adjacent D-cysteic acid residue are identical in both tetradecapeptides and tridecapeptides. Both L- and D-forms, as well as *N*-Me amino acids such as Sar are found in the structures. Discodermins and halicylindramides show cytotoxicity against P388 cells with IC_{50} in the sub-micromolar range as well as antimicrobial activity. The hydrophobic *N*-terminal sequence composed of six successive amino acids such as L-*t*-Leu and L-β-MeIle resemble the *N*-terminal portion of 48-mers polytheoneamides, which exhibit potent cytotoxicity by forming unimolecular ion channel in cell membranes [108, 109]. As alluded to this, Karaki and co-workers demonstrated that discodermin A enhanced the permeability of the plasma membrane [110]. On the other hand, the linear analogs, secohalicylindramide B and halicylindramide E were

no longer cytotoxic, which suggested that the macrolactone ring is also essential for the cytotoxicity [105]. More recently, tridecapeptides, polydiscamides B-D were disclosed to be potent agonists against human sensory neuron-specific G protein coupled receptor [104]. Microspinosamide has cytoprotective activity against HIV-1 *in vitro* [107] (Fig. 6.7).

Callipeltins A–C were originally isolated from the New Caledonian Lithistida sponge *Callipelta* sp. by Minale and co-workers [111]. Noteworthy is the acylation of the *N*-terminal unit with unique polyketide-derived hydroxyacid moieties and the presence of previously unknown amino acid residues such as 3,4-dimethylglutamine. Callipeltin A was first reported to exhibit anti-HIV and anti-fungal activities [111]. Later, it was reported that callipeltins A and B exhibit broad-spectrum cytotoxicity against the tumor cell lines [112]. However, cytotoxicity of the acyclic congener, callipeltin C was significantly diminished [112], thus suggesting that the macrolactone ring is important for the cytotoxicity as is the case for halicylindramides. Recently it was found that callipeltin A is a selective and powerful inhibitor of the Na/Ca exchanger and a positive inotropic agent in guinea pig left atria [113]. In addition to callipeltins A-C, more congeners callipeltins D–M were isolated from a different sponge, *Latrunculia* sp. [114, 115]. All these new derivatives were truncated and linear in structure except for callipeltin L, which contains a unique 8-membered ring formed between carboxyl group at C-terminal and β-hydroxyl group on tyrosine residue. The unusual structural features of these peptide metabolites and the interesting biological activities have attracted considerable interest among the synthetic chemistry community, culminating in the total synthesis of callipeltin B, D and E [116–118]. The Papua New Guinea marine sponge *Neamphius huxleyi* contained closely related depsipeptide, neamphamide A [119]. Another congener, neamphamide B, was isolated from a Japanese marine sponge *Neamphius* sp., [120] and neamphamides B–D from Australia collection of the marine sponge *Neamphius huxleyi* [121]. Whereas the macrocyclic region of neamphamide A is composed of a 25-membered ring, both callipeltin A and neamphamides B-D share the same ring size of 22-memebers. All neamphamides bear a unique L-homoproline reside in place of L-*N*-MeAla residue at the C-terminal of callipeltin A and inhibited the growth of human cell lines with IC_{50} of sub-nanomolar range. The homoproline residue is also found in similar depsipeptides, papuamides and mirabamide. Papuamides A-D are cytotoxic, antiviral cyclic depsipeptides isolated from the marine sponge *Theonella swinhoei* and *mirabilis* [122]. New derivatives papuamides E and F were also isolated from the genus *Melophlus* [123]. Mirabamides, isolated from the Micronesian sponge *Siliquariaspongia mirabilis* represent the a recent example of this class. The distinguishable feature of all these depsipeptides is β-methoxytyrosine (β-MeOTyr) present in callipeltin A–C, neamphamide, papuamide and mirabamides. This unique substructure evoked the formation of a quinone methide intermediate by elimination of methanol from β-MeOTyr, which might be the principal source of the cytotoxicity. However, synthetic desmethoxylcallipeltin B lacking β-methoxy group at the tyrosine residue also showed cytotoxicity comparable to that of callipeltin B, leading to the conclusion that the quinone methide intermediate is unlikely to be essential for the cytotoxicity [124]. This is rather

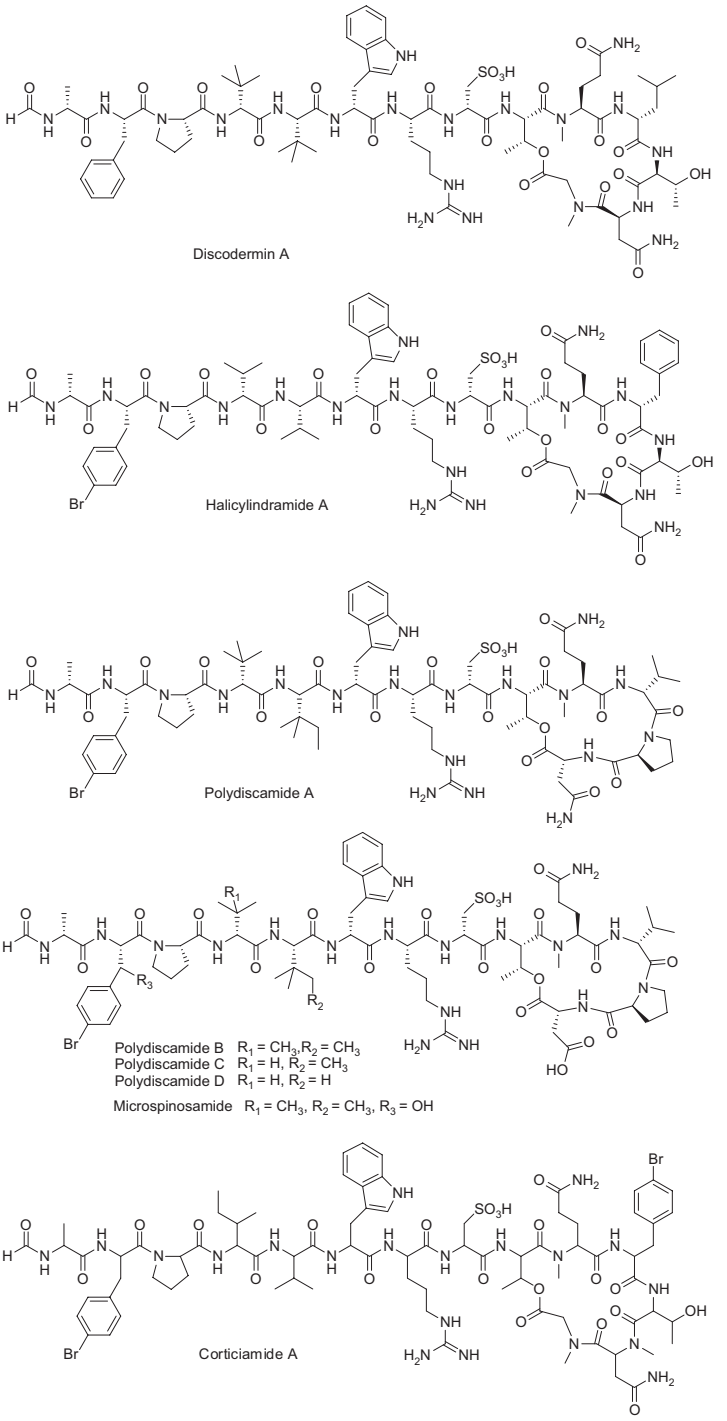


Fig. 6.7 Structures of head-to-side-chain cyclodepsipeptides

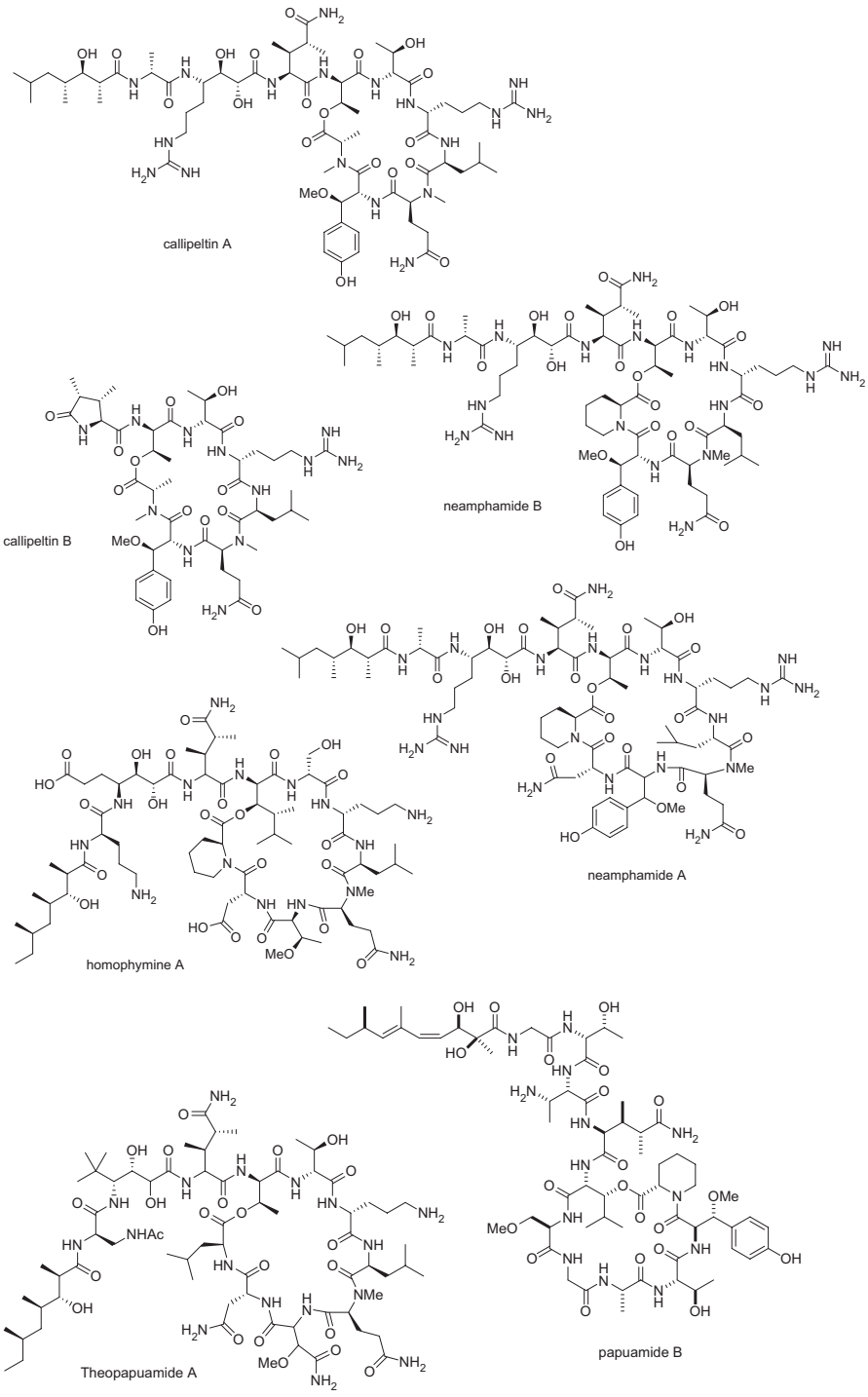


Fig. 6.8 Structures of head-to-side-chain cyclodepsipeptides

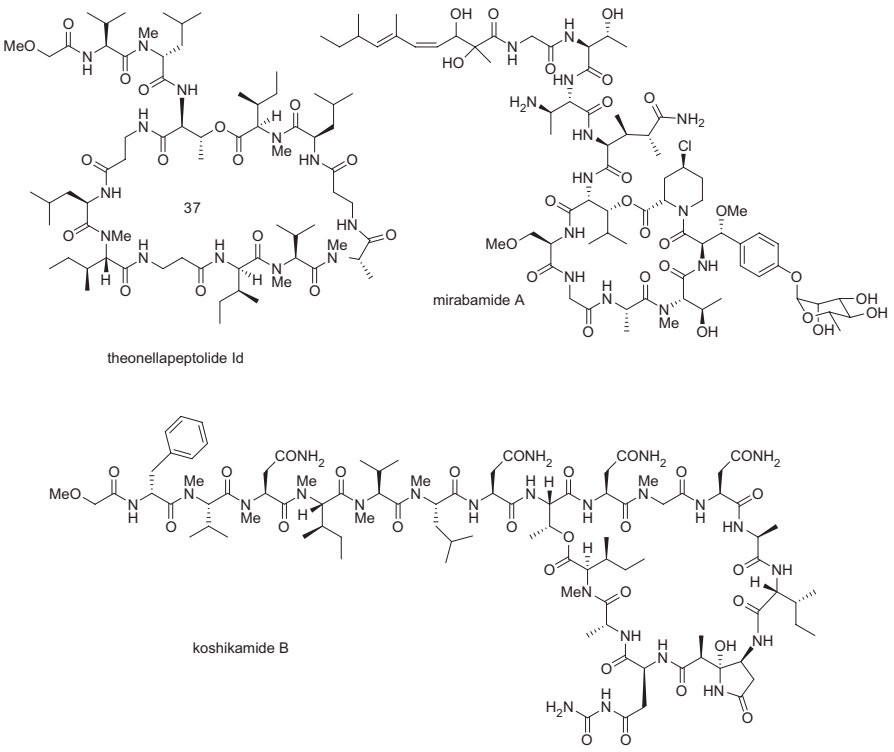


Fig. 6.8 (continued)

reasonable because the related depsipeptides, both homophymine and theopapuamide lacking β-MeOTyr, retain considerable cytotoxicity.

Other classes of head-to-side chain cyclodepsipeptides are theonellapeptolides and koshikamides, both of which were orginally isolated from the genus *Theonella*. Theonellapeptolides are tridecapeptide containing a 37-membered lactone ring. They constitute the members of a growing class of depsipeptides from sponges. Of note, apart from sponge-derived head-to-side-chain cyclodepsipeptides, there are several other important cytotoxic peptides including kahalalides from mollusk and didemnins from tunicates (Fig. 6.8).

6.4 Others

6.4.1 Side-chain-to-tail Cyclopeptides

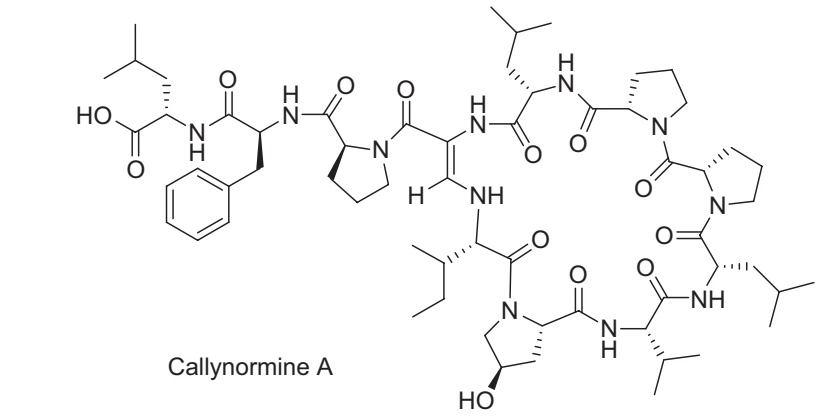
The side chain-to-tail type cyclopeptides are rarely-observed in natural sources. In 2004, callynormine A was reported as a new class of peptides by Kashman and co-workers [125]. Callynormine A was isolated from a Kenyan marine sponge,

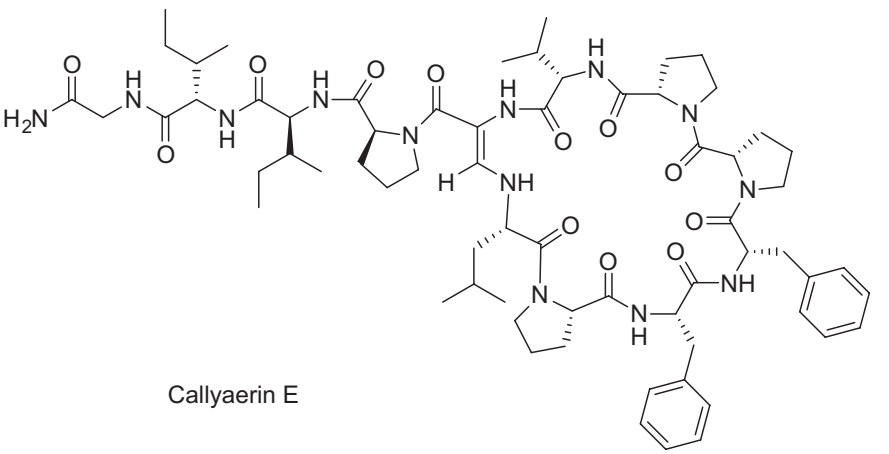
Callyspongia abnormis. The undecapeptide structure was composed of three Leu, three Pro, Phe, Val, Ile and two non-proteinogenic amino acids including γ-hydroxyproline and latent formylglycine. All amino acids were determined to be L-forms based on Murphy's analysis. The most unprecedented feature of the structure is the α-amido-β-aminoacrylamide functionality for the linkage of macrocylization, which was confirmed by X-ray structure. It was envisioned that the formylglycine embedded in the nascent linear peptide chain is a suitable acceptor of the amino group on the *N*-terminal residue, Ile. The condensation between aldehyde and *N*-terminal amino group generates the conjugated acrylamide functionality through Schiff base formation. Thus, callynormine A is a novel class of side-chain-to-tail cyclopeptide in which the endiamino moiety served as a heterodetic linkage. Although no biological activity of callynormine A was reported, same class of peptides was reported to be cytotoxic, soon later. In 2008, Proksch and co-workers reported callyaerin G as an analogue of callynormine A, from the Indonesian sponge *Callyspongeia aerizusa* [126]. The succeeding report by same group included the isolation and structure elucidation of callyaerin A-F and H. All callyaerins contain several proline residues, of which one proline was always positioned at the side chain and adjacent to macrocyclic ring. The basic structural unit of the callyaerins comprises a cyclic peptide with a linear peptide side chain, both of variable size, linked through an α-amido-β-aminoacrylamide functionality. This functional group in a peptide has to date only been described from the sponge of the genus *Callyspongia* [127]. Among eight derivatives, callyaerin E exhibited the most potent cytotoxicity against the L5178Y cell line with ED_{50} values of 0.39 μM. Considering lesser activity of the remaining congeners, increasing the number of proline residues in the cyclic moiety seemed to enhance the cytotoxicity, while replacement of a proline with a hydroxyproline would reduce the cytotoxicity (Fig. 6.9).

6.4.2 Imidazole-bridged Peptides

Aciculitins are cytotoxic and antifungal cyclopeptides isolated from the lithistid sponge *Aciculites orientalis* by Faulkner et al. in 1996 [128]. The structural feature of these peptides is a bicyclic peptide moiety containing an unusual histidinotyrosine bridge, to which C13-C15 2,3-dihydroxy-4,6-dienoic acids having D-lyxose at 3-position are attached. At the same time, aciculitamides, artifacts generated during the isolation process, were also found in the same sponge. In aciculitamides, the imidazole ring is oxygenated at 2′-position and methanol is added to the same ring at 4′-position. Interestingly, while aciculitins are cytotoxic to HCT-116 cell line (IC_{50}: 0.5 μg/mL), aciculitamides didn't show any cytotoxicity. This fact suggests the importance of the imidazole ring for bioactivity.

Theonellamides are cytotoxic and antifungal cyclopeptides from marine sponge *Theonella* sp., which was reported by Fusetani et al. in 1989 and in 1995 [129, 130]. Similarly, two related compounds have been isolated from the sponge *Theonella swinhoei*; theonegramide [131] and theopalauamide [132]. Their structures





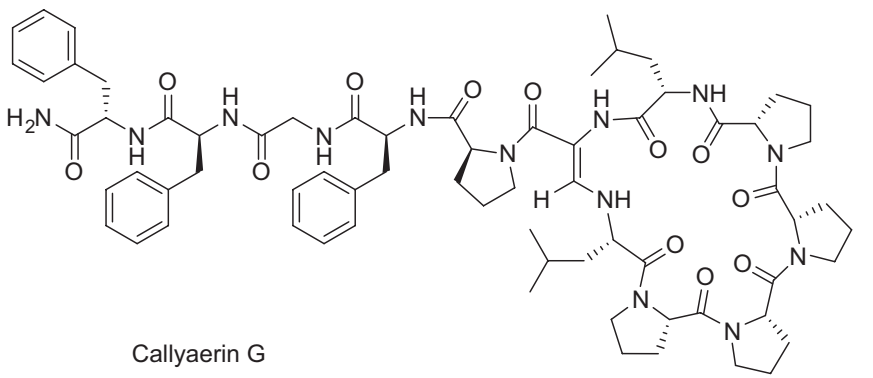
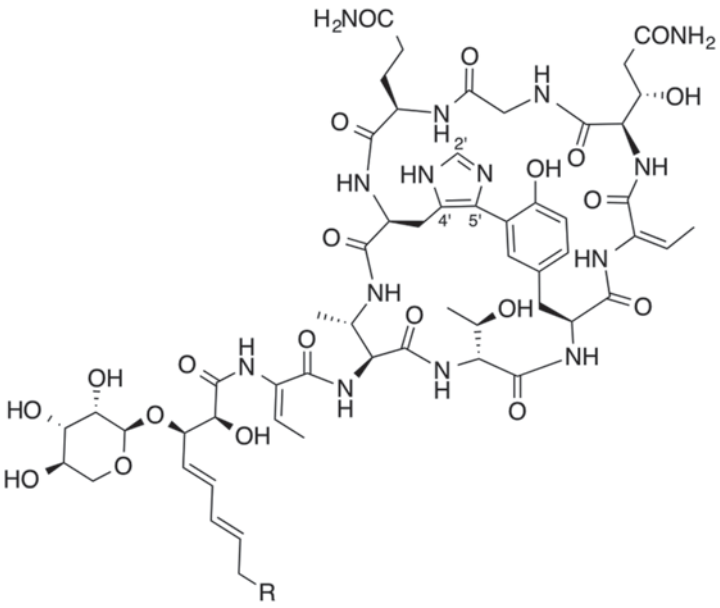


Fig. 6.9 Structures of side-chain-to-tail heterodetic cyclopeptides

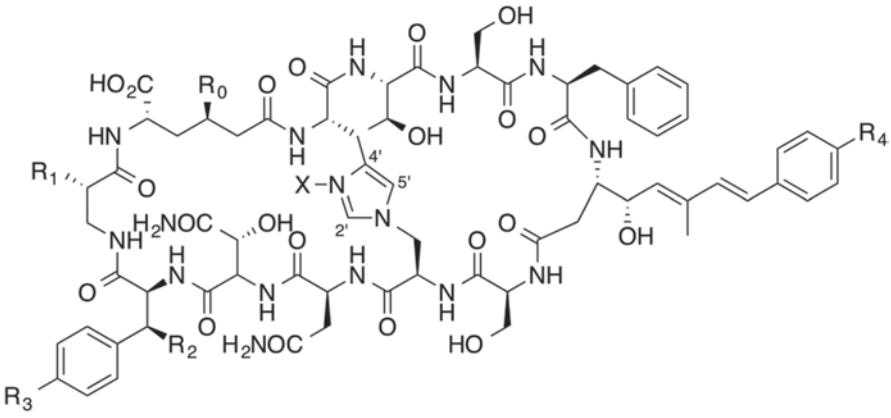
are characterized by a histidinoalanine residue that bridges the macrocyclic peptide ring. Notably, while the carbon atom at 5′-position in the imidazole ring is connected to a tyrosine residue in aciculitins, the nitrogen at 1′-position is connected in theonellamide analogues. Four of the eight analogues including theonegramide and theopalauamide are glycosylated at the π-nitrogen of the imidazole ring. Theonellamides A–F showed moderate cytotoxicity against P388 murine leukemia cells with IC_{50} values of 5.0, 1.7, 2.5, 1.7, 0.9, and 2.7 μg/mL, respectively [129, 130]. This suggests that the glycosyl groups show little effect on cytotoxicity and that the characteristic bicyclic peptide framework is responsible for biological activity. Recently, it has been revealed that theonellamides recognize 3β-hydroxysterol-containing membranes, induce glucan overproduction, and damage cellular membrane [133]. Further study turned out that they directly recognize the 3β-OH moiety and facilitate their binding to bilayer membranes [134] (Fig. 6.10).

6.4.3 Dilactones

Arenastatin A is a potent cytotoxic cyclodepsipeptide isolated from the Okinawan marine sponge *Dysidea arenaria* by Kitagawa et al. in 1994 [135]. The structure of this compound is characterized by a 16-membered macrocyclic dilactone, in which both of the ester linkages are formed by an L-leucic acid. This macrocycle exhibits extremely potent cytotoxicity (IC_{50}:5 pg/mL) against KB cells [135]. Synthetic approach of this compound has elucidated the cytotoxicity to be ascribable to inhibition of microtubule assembly through binding to rhizoxin/maytansine site [136, 137]. On the other hand, it possesses little *in vivo* anti-tumor activity through intravenous administration due to its lability in mouse serum arising from the cleavage of a 15,20-ester bond [138]. In addition, structure-activity relationship studies have revealed that each epimer of 7,8-epoxide, 6-methyl, and OMe-tyrosine lost cytotoxicity at concentration below 0.1 μg/mL [139]. In contrast, a 15-epimer synthesized from D-Leu showed moderate cytotoxicity (IC_{50}: 20 ng/mL). Therefore, several analogues derived from changing the L-leucic acid moiety or the 15,20-ester bond were synthesized, including a 15,20-triamide analogue (IC_{50}: 6 ng/mL, [138], a carba analogue (IC_{50}: 70 ng/mL, [140]), a deoxo analogue (IC_{50}: 40 ng/mL, [140]), and a 15-*t*-butylanalogue (IC_{50}: 10 ng/mL, [141]). All of these analogues turned out to be more stable than arenastatin A, while the 15,20-triamide analogue was almost insoluble in polar solvents. Thereafter, some soluble triamide analogues have been synthesized with polar substituents on the phenyl ring [142]. Among them, two analogues with diethylamine and piperazine moieties on the phenyl group have shown the strongest cytotoxicity (IC_{50}: 0.18 and 1.5 ng/mL, respectively) with good solubility and stability. The *in vivo* anti-tumor activity assay of the diethylamine analogue through intraperitoneal administration in subcutaneously implanted murine sarcoma S180 cells has revealed that it inhibited tumor growth at a dose of 1 mg/kg without acute toxicity, the efficacy of which was comparable to that of doxorubicin (positive control).



Aciculitin A R = C_5H_{11}
Aciculitin B R = C_6H_{13}
Aciculitin C R = C_7H_{15}



	R_0	R_1	R_2	R_3	R_4	X
Theonellamide A	OH	OH	Me	Br	H	β-D-Gal
B	OH	OH	Me	Br	H	
C	OH	H	H	H	Br	
D	OH	H	H	Br	Br	β-L-Ara
E	OH	H	H	Br	Br	β-D-Gal
F	OH	H	H	Br	Br	
Theonegramide	H	OH	Me	Br	H	β-D-Ara
Theopalauamide	H	OH	Me	Br	H	β-D-Gal

Fig. 6.10 Structures of imidazole-bridged cyclopeptides

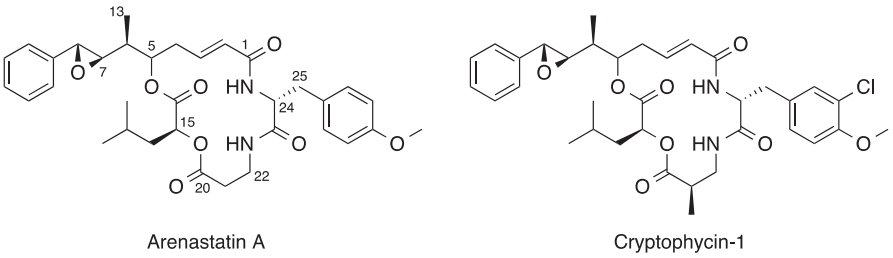


Fig. 6.11 Structures of dilactones

Arenastatin A is also known as cryptophycin-24, which belongs to the cryptophycin natural product family consisting of more than 25 analogues [143–145]. Cryptophycin-1 has firstly been reported as an antifungal depsipeptide from a cultured cyanobacterium of *Nostoc* sp. by Merck Sharp & Dohme Research Laboratories, which has shown antifungal activity against the genus *Cryptococcus* [146]. Cryptophycin-1 shows not only *in vitro* cytotoxicity but also *in vivo* anti-tumor activity because a C21-methyl group prevents hydrolysis of the 15,20-ester linkage. Recently, Sherman and co-workers identified the biosynthetic gene cluster of cryptophycin from the cyanobacterium *Nostoc* sp. ATCC 53789 [147]. The gene cluster encoded mixed NRPS and PKS biosynthetic enzymes, in which the thioesterase (TE) was isolated and its function was evaluated with a series of linear intermediate substrates. The TE substrate flexibility as well as its ability to catalyze hydrolysis or macrocyclization between C-2 ester and C-16 hydroxyl groups demonstrated an efficient chemoenzymatic synthesis of cryptophycins and arenastatins (Fig. 6.11).

6.5 Summary and Future Prospects

In the past three decades, numerous cyclic peptides have been discovered from marine sponges as described above, which attracted growing interest due to unique chemical structures as well as due to pronounced biological activities. In addition, some of them exhibit potential value as primary structures for the development of anti-cancer agents. The frequent occurrence of bioactive peptides, especially in sessile marine invertebrates such as sponges, is usually interpreted as chemical defense that protects these organisms against biotic stress factors such as predation, infection by pathogens, or overgrowth by fouling organisms [148]. It has long been deduced that the real producer of these peptides could be symbiont bacteria rather than sponge itself [149, 150]. The recent progress in biosynthetic research makes it possible to investigate the biosynthesis gene cluster of sponge-derived peptides or polyketides, revealing that they are most likely of bacterial origin [151–154]. Even though the symbiont bacteria would usually be difficult to be cultivated in standard laboratory conditions, the biosynthetic gene clusters might be useful for heterologous expression in a suitable cultivable host. If engineering technology of

biosynthetic enzymes such as non-ribosomal peptide synthetase becomes generally available, a vast variety of unique peptides would be generated according to the scaffolds of sponge-derived bioactive peptides. This was exemplified to some extent, by the pioneering works on some ribosomal peptides [2] and non-ribosomal peptides [147]. Furthermore, the solid phase peptide synthesis has culminated in advancement highly efficient synthesis of complex peptides composed of non-proteinogenic amino acids [155, 156]. In future, the combination of chemical synthetic and biosynthetic technologies has been anticipated to accelerate the development of anticancer drugs derived from the defensive peptides evolving from sponge-microbe association.

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Chapter 7
Fucoidan, A Sulfated Polysaccharides from Brown Algae as Therapeutic Target for Cancer

Senthilkumar Kalimuthu and Se-Kwon Kim

Abstract Cancer is most common in worldwide and badly threaten to human's life. Unfortunately drugs, which are used for cancer therapy, are toxic and affect not only cancer cells but also normal cells and tissues. At last decade marine brown algae attract much attention because they represent a rich and easily regenerated source of polysaccharides with various structures and biological activities. Fucoidan sulfated polysaccharides (FCSPs) extracted from seaweeds, mainly brown macro-algae, are known to possess essential bioactive properties, notably growth inhibitory effects on tumor cells. Cellular damage induces growth arrest and tumor suppression by inducing apoptosis, the mechanism of cell death depends on the magnitude of DNA damage following exposure to anticancer agents. Apoptosis is mainly regulated by cell growth signaling molecules. Fucoidan was shown to induce cytotoxicity of various cancer cells, induces apoptosis, and inhibits invasion, metastasis and angiogenesis of cancer cells. Hence, this chapter deals the potential role of fucoidan on cancer therapeutics.

Keywords Fucoidan · Cancer · Apoptosis · Metastasis · Algae

7.1 Introduction

Cancer is a leading cause of death in worldwide. It's a diverse group of diseases characterized by uncontrolled growth and spread surrounding tissues and metastasize to other tissues and organs. Cancer results from a mutation in the chromosomal

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DNA of a normal cell, which can be triggered by both external factors (tobacco, alcohol, chemicals, infectious agents and radiation) and internal factors (hormones, immune conditions, inherited mutations, and mutations occurring in metabolism) promotes cancer progression. Taking into account the rising trend of the incidence of cancers of various organs, effective therapies are urgently needed to control human malignancies. Last decades various drugs arise from marine environment.

The marine environment contains a various number of plants, animals, and microorganisms, which have a wide diversity of natural products[1]. Among these natural sources, marine algae has great frontier for pharmaceutical and medical research. In the field of cancer research have exposed promising compounds, isolated from natural sources, with proven anticancer activity. Seaweeds have great potential as a supplement in functional food or for the extraction of compounds. Seaweeds are known for their richness in polysaccharides, minerals and certain vitamins, but they also contain bioactive substances including polysaccharides, proteins, lipids, polyphenols etc [2, 3] and also used for the development of new pharmaceutical agents [4]. The Phaeophyceae or brown algae, is a large group of mostly marine multicellular algae, including of many seaweeds play an important role in marine environments, both as food and for the habitats they form. Brown seaweed is constitute a part of the conventional diet in several Asian countries, especially in Japan and Japanese cohort study support that the intake of seaweeds has been associated with lower mortality from chronic diseases including cancer [5].

7.2 Polysaccharides from Algae

Marine algae contain huge amounts of polysaccharides, remarkably cell wall structural, also mycopolysaccharides and storage polysaccharides [2, 6]. The cell wall and storage polysaccharides are species specific. Polysaccharides are polymers of simple sugars (monosaccharides) linked together by glycosidic bonds, and they have numerous commercial applications in products such as thickeners, food, feed, stabilizers, emulsifiers, beverages etc. [7, 8]. The total polysaccharide concentrations in the seaweed species of interest range from 4 to 76% of dry weight [3]. Brown algae substantially differ from algae of other divisions and terrestrial plants in the structure of carbohydrates. Seaweeds have low lipid, high carbohydrate and more dietary fibers. The cell wall polysaccharides mainly consist of cellulose and hemicelluloses, neutral polysaccharides, and are thought to physically support the thallus in water. Green algae contain sulphuric acid polysaccharides, sulphated galactans and xylans, brown algae contains alginic acid, fucoidan (sulphated fucose), laminarin (β-1,3 glucan) and sargassan and red algae contains agars, carrageenans, xylans, floridean starch (amylopectin-like glucan), water-soluble sulphated galactan, as well as porphyran as mucopolysaccharides located in the intercellular spaces [2, 6]. The components of galactose, glucose, mannose, fructose, xylose, fucose and arabinose were found in the total sugars in the hydrolysates. The glucose content was 65, 30 and 20% of the total sugars in an autumn sample of 50 individual plants

of Saccharina, Fucus (serratus and spiralis) and Ascophyllum, respectively [9]. Several other polysaccharides are present in and utilized from seaweed, e.g. furcellaran, funoran, ascophyllan and sargassan. Brown algae mainly contain *L*-fucose and sulfate ester groups containing polysaccharides called fucoidan.

7.2.1 Structure and Composition of Fucoidan

During the last decade, numerous bioactive polysaccharides with interesting functional properties has discovered from marine algae [10]. Brown algae are highest sources of structurally diverse polysaccharides with various biological activities. Fucoidan is a sulfated polysaccharide (MW: average 20,000) found in the brown seaweed and also various species of brown algae such as mozuku, kombu, limu moui, bladderwrack, wakame, and hijiki (variant forms of fucoidan have also been found in animal species, including the sea cucumber). The monosaccharide composition and structure of algal fucoidans appear to depend on algal species, the methods of isolation and purification of polysaccharides harvest from brown seaweeds [11]. Structurally, fucoidan is a heparin-like molecule with a substantial percentage of L-fucose, sulfated ester groups, as well as small proportions of D-xylose, D-galactose, D-mannose, and glucuronic acid [12]. The polysaccharide was named as "fucoidin" was first isolated from marine brown algae by Kylin in 1913 [13]. Now it is named as "fucoidan" according to IUPAC rules, but also called as fucan, fucosan or sulfated fucan [14]. The structures and compositions of fucoidan vary among different species of brown seaweed and fucoidan are complex in their chemical composition. Low molecular weight fucoidan (LMWF) has more biological actions than native fucoidan. The pharmacological effects of fucoidans vary with their molecular weight, which is generally classified as low (< 10 kDa), medium (10–10,000 kDa), or high > 10,000 kDa [15]. Thus low molecular weight fucoidans, mainly contained fucose residues and a large amount of sulfate groups, possessed higher anti-tumor activity than high-molecular weight heterofucans with low degree of sulfation [16, 17].

Fucose-containing sulfated polysaccharides (FCSPs) designate a group of diverse polysaccharides that can be extracted from brown seaweeds of the class Phaeophyceae. The saccharides are naturally assembled from monosaccharides having different types of linkages, at varying positions in the carbohydrate ring, forming straight chain or branched polymers. Further modifications such as alkylation, phosphorylation, and sulfation provide an additional structural complexity. The high degree of variability among biologically relevant sulfated carbohydrates is attributed to a number of factors. There is inconsistency among sulfated monosaccharides (i.e., glucose (Glc), galactose (Gal), the corresponding N-acetyl (NAc) amines GlcNAc, GalNAc, and mannose among others), the total number of sulfate moieties, and the hydroxyl(s) to which the sulfate group(s) are linked (i.e., 2-O, 3-O, 4-O, and 6-O sulfates); furthermore, the varied structure of the underlying oligosaccharide moiety. The most studied FCSPs, originally called fucoidin, fucoidan

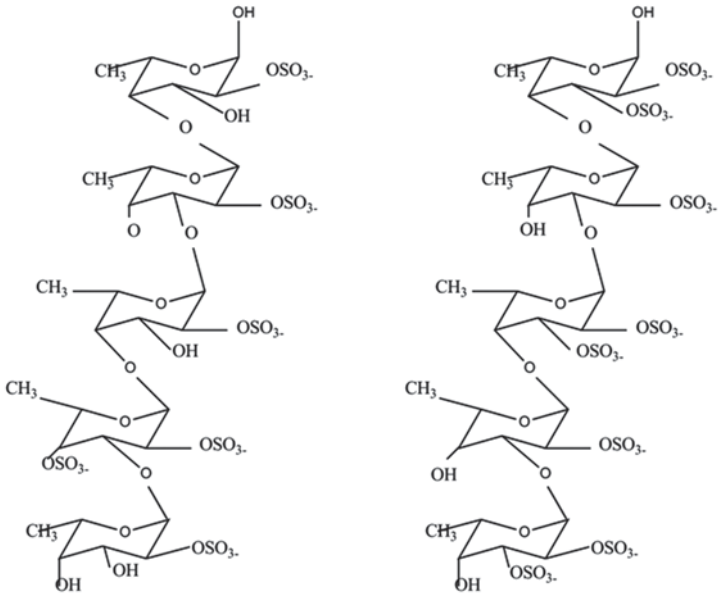

Fig. 7.1 Structure of fucoidan

or just fucans, have a backbone built of (1→3)-linked α-L-fucopyranosyl residues or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues [18, 19]. These fucopyranosyl residues may be substituted with short fucoside side chains or sulfate groups at C-2 or C-4, and may also carry other minor substitutions, e.g., acetate, xylose, mannose, glucuronic acid, galactose, or glucose [20–22]. FCSPs also include sulfated galactofucans with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units. In addition to sulfate these backbone residues may be substituted with fucosides, single fucose substitutions, and/or glucuronic acid, xylose or glucose substitutions [21]. The compositional and structural features of FCSPs differ significantly among seaweed species and that these features are markedly influenced by the conditions used to extract them [20, 23]. Fucoidan extracted from brown seaweed algae *Fucus vesiculosus* [24], *Ascophyllum nodosum* [25], *Sargassum kjellmanianum* [26], *Sargassum thunbergii* [27], *Cladosiphon okamuranus Tokida* [28], in which the percentage of L-fucose ranged from 12.6 to 36.0%, and the percentage of sulfate content from 8 to 25%. The isolated and determined structural characteristics of the fucoidans from brown algae *Saccharina cichorioides*, *Fucus evanescens*, and *Undaria pinnatifida* [29–31] and *S. cichorioides* consisted of (1→3)-α-L-fucose residues, fucoidan from *F. evanescens* contained alternating (1→3)- and (1→4)-α-L-fucose, while fucoidan from *U. pinnatifida* was built up of (1→3)- and (1→4)-linked α-L-fucose and β-D-galactose residues [32, 33]. The structure of fucoidan is depicted in Fig. 7.1 (Fig. 7.2).

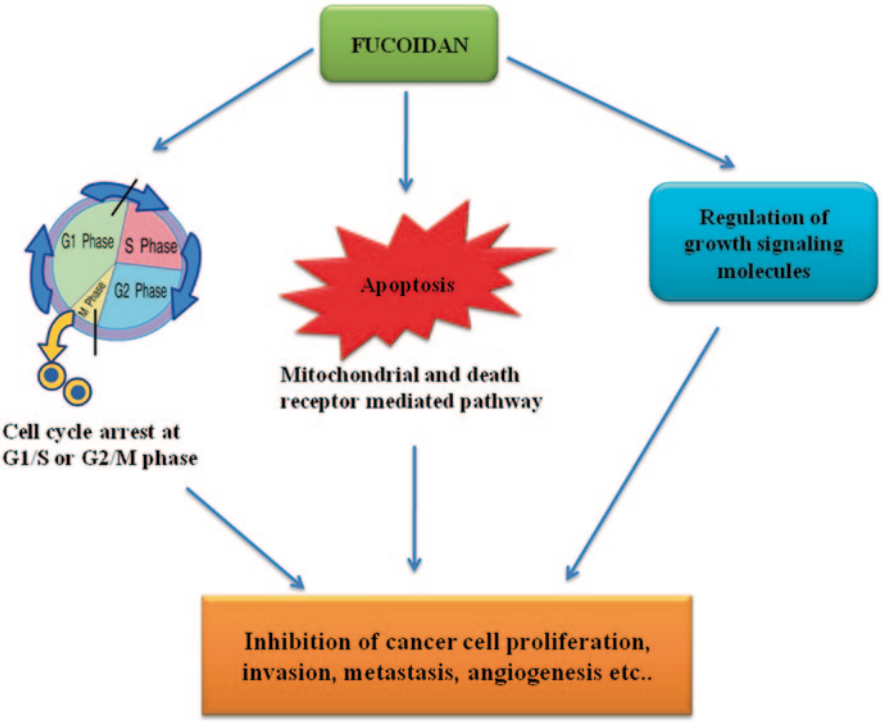

Fig. 7.2 Overview of fucoidan regulation

7.3 Biological Aspects of Fucoidan

Various researches done and many have to do with fucoidan to evaluate the medicinal and cosmeceutical potentials [34, 35]. The bioactivity of fucoidan depends on nature and extent for sulphation, which must be taken care during extraction. Because of harshness of extraction method, the sulphation pattern may be destroyed and the bioactivity may be lost [36]. Fucoidan or FCSPs from seaweed have potential biological activity including antitumor and immunomodulatory [37–39], antivirus [40], antithrombotic and anticoagulant [41], anti-inflammatory [42], and antioxidant effects [43], as well as their effects against various renal [44], hepatic [45] and uropathic disorders [46]. The fucoidan was isolated from brown seaweed such as *Undaria* and *Laminaria* showed that anticoagulant, antiviral and anticancer properties etc [47, 48]. Sulphated polysaccharides from the marine algae *Porphyra haitanesis* [49], *Ulva pertusa* [50], *F. Vesiculosus* [51], *Laminaria japonica* [52] and *Ecklonia kurome* [53] has demonstrated to possess as antioxidant activity. From the *L. japonica*, extracted fucoidan has commercially important algae species in China. Three sulphated polysaccharide fractions was successfully isolated through anion exchange column chromatography and their antioxidant activities investigated

employing various established *in vitro* systems, including superoxide and hydroxyl radical scavenging activity, chelating ability and reducing power [54]. Fucoidan stimulates the immune system in several ways, and the biological effects are related to their ability to modify cell surface properties [55]. Oral intake of the fucoidans present in dietary brown seaweed might take the protective effects through direct inhibition of viral replication and stimulation of the immune system (innate and adaptive) functions [56]. The mechanism of antiviral activities of fucoidan is to inhibit viral sorption so as to inhibit viral-induced syncytium formation. Sulphate is necessary for the antiviral activity, and of (1 → 3)-linked fucopyranosyl units appears to be very important for the anti-herpetic activity of fucoidan [57].

The venous antithrombotic activity of LMW fucans (LMWF) has compared with a low-molecular-weight heparin in the Wessler rabbit model and exhibited a better ratio antithrombotic effect/hemorrhagic risk [1, 58, 59]. This antithrombotic activity may, in part, be explained by the decrease of tissue factor expression in the media of denuded arteries and the significant increase of plasma TFPI (tissue factor pathway inhibitor) released from endothelial cell [60, 61]. Vasculogenesis is the process of blood vessel formation occurring by the production of endothelial cells. Fucoidan induces endothelial progenitor cell (EPCs) proliferation, migration and differentiation into capillary-like structures on Matrigel [62]. Low molecular weight fucoidan (LMWF) is act through stromal cell-derived factor (SDF-1), which when stimulated EPCs in hind limb ischemia [63, 64]. LMWF can also promote tissue rebuilding parameters such as signaling by heparin-binding growth factors (FGF-2, VEGF) and collagen processing in fibroblasts, smooth muscle cells or endothelial cells in culture. LMWF can bind fibrillar collagens provide protection and signal promotion of heparin binding growth factors to improve biocompatibility of purified cancellous bone substitute. Indeed, it was demonstrated that LMWF mimics and restores the properties of bone non collagenous matrix (proteoglycans, glycoproteins) that were eliminated by drastic purification process during design of the biomaterial, to regulate soluble factors for bioavailability [65]. LMWF has sulfate content higher than 20% was found to exert profound anticoagulant activity as well as antiproliferative effects on fibroblast cell line (CCL39) in a dose-dependent fashion [66].

A high molecular weight is required to achieve anticoagulant activity as fucoidan needs a long sugar chain in order to be able to bind the thrombin (coagulation protein in the blood stream). Heparin is a biomolecule containing highly sulfated glucosaminoglycan that is widely used as an injectable anticoagulant. It has reported that the anticoagulant mechanisms of fucoidan are related to both antithrombin and heparin cofactor II-mediated activity [67]. The structural and anionic characteristics of fucoidan are similar to those of heparin. Heparin stimulates production of hepatocyte growth factor (HGF), which have key role in tissue regeneration. Fucoidan and fucoidan-derived oligosaccharides have similar ability to stimulate production of hepatocyte growth factor (HGF). This induction of HGF by heparin or fucoidan and their oligosaccharide derivates occurs primarily at the level of translation. Thus, fucoidan may be useful to protect tissues and organs from various injuries and diseases, via mechanisms involving HGF [68].

Fucoidan from *L. japonica* reduced serum total and LDL-cholesterol and triglycerides and raised HDL-cholesterol in a hyperlipidemic rat model [69]. Fucoidan treatment led to less severe symptoms in the early stages of *Staphylococcus aureus* triggered arthritis in mice, but delayed phagocyte recruitment and decreased clearance of the bacterium [70]. Also, injection of fucoidan into sensitized mice with before hapten challenge reduced the contact hypersensitivity reactions [71]. Furthermore, recruitment of leukocytes into cerebrospinal fluid in a meningitis model is reduced by fucoidan [72], as is IL-1 production in a similar model [73]. Also, fucoidan has studied in bone tissue engineering. Recent studies evidenced that hydroxyapatite-fucoidan (HApF) nanocomposite may be promising biomaterial and could be used for bone tissue engineering studies [74].

7.4 Fucoidan on Cancer

7.4.1 Anticancer Effect of Fucoidan

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in Worldwide [75]. Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. The National Cancer Institute estimates that "approximately 11.4 million Americans with a history of cancer were alive in January 2006." In 2012, about 577,190 Americans are expected to die of cancer, more than 1500 people a day. Cancer is the second most common cause of death in the US, exceeded only by heart disease, accounting for nearly 1 of every 4 deaths (Source: Cancer Facts and Figs. 2012 of the American Cancer Society). Accordingly, research must continue to progress to improve existing therapies and to develop novel cures. For many years, research has essentially focused on plants and terrestrial microorganisms, mainly because of these specimens are easily available and folk traditions have described beneficial effects from their use. Several different therapeutic strategies such as chemotherapy, radiation therapy, surgery or combinations have been used to treat different types of cancer. Unfortunately, several of these treatments provide only minimal benefits; moreover, complications and long term side effects of these treatments [76, 77]. Chemotherapy is one of the mostly used therapeutic modalities for the treatment of cancer, but it does not achieve a satisfactory therapeutic result. Recently, a great deal of interest has been developed in the nutraceutical compounds from the pharmaceutical industries to isolate natural anti-tumor compounds from marine resources. The therapeutic potential of natural bioactive compounds such as polysaccharides, especially fucoidan is now well documented, and combined activity of this compound useful for the development of a new generation of therapeutic measures against cancer from brown seaweeds [12].

Many studies evidenced that fucoidan have anti-tumour effects, but its mode of action is not fully understood. Fucoidan inhibits proliferation and induce apoptosis of human lymphoma HS-Sultan cells [24]. Brown seaweeds fucoidan from

Table 7.1 Anticancer effects of fucoidans from marine brown algae

Species	Effects	Reference
Fucus vesiculosus	Lymphoma, Leukaemia, Lung (HS-Sultan cells, HL-60, THP-1 cells, A549 cells)	[24, 102, 111]
Saccharina japonica and *Undaria pinnatifida*	Bladder and Melanoma (T-47D and SK-MEL-28)	[29]
Cladosiphon novae-caledoniae	Breast (MCF-7 and MDA-MB-231), Cervical carcinoma (HeLa), Fibrosarcoma (HT1080)	[82, 84]
Cladosiphon okamuranus	Liver (Huh7 and HepG2 cell line)	[86]

Eclonia cava, *Sargassum hornery*, and *Costaria costata* showed that inhibit human melanoma and colon cancer cells [78]. Human malignant melanoma cancer cell (SK-MEL-28 and SK-MEL-5) growth was inhibited by native fucoidan isolated from *Fucus evanescens* [30]. Fucoidans derived from *L.* saccharin*a*, *L. digitata, F. serratus, F. distichus* and *F. vesiculosus* strongly blocked MDA-MB-231 breast carcinoma cell adhesion and implications in tumor metastasis[79]. Alekseyenko et al. reported that the antitumor and antimetastatic activities of fucoidan from *Fucus evanescens* in C57Bl/6 mice with transplanted Lewis lung adenocarcinoma. Fucoidan after single and repeated administration in a dose of 10 mg/kg produced moderate antitumor and antimetastatic effects [37]. The animals were fed with a diet containing 1 % fucoidan from Mekabu for 10 days and subcutaneously (*s.c.*) inoculated with A20 leukemia cells. Thereafter, the mice were fed with the diet containing fucoidan for 40 days. Mekabu fucoidan inhibited tumors by 65.4 % [38]. Native and oversulfated FCSPs derived from *Cladosiphon okamuranus* (Chordariales) was analyzed using 1H NMR spectroscopy and sulfation produced 4-mono-O-sulfo-L-fucopyranose the oversulfated FCSPs contained 2,4-di-, 2-mono-, and 4-mono-O-sulfo-L-fucopyranose that sulfate content and the positioning of sulfate groups, e.g., 2,4-di- vs. 4-mono, might be important for the anti-proliferative activity of fucoidan in a human leukemia cell line (U937) [80]. Sulfated polysaccharides from brown seaweeds *S. japonica* and *U. pinnatifida* possessed high antitumor activity and inhibit proliferation and colony formation of breast cancer and melanoma cell lines [29]. Fucoidan inhibits the proliferation of prostate cancer (PC-3) cells [81].

LMWF mediated the broad-spectrum growth inhibition of human carcinoma cells, including HeLa cervix adenocarcinoma, HT1080 fibrosarcoma, K562 leukaemia, U937 lymphoma, A549 lung adenocarcinoma and HL-60 [82] and also inhibits the angiogenesis of HT 1080 fibrosarcoma cells and induces apoptosis of MCF-7 breast cancer cells [82, 83]. LMWF induces apoptosis through mitochondrial mediated pathways in MDA-MB-231 breast cancer cells and also evidenced that the interrelated roles of Ca^{2+} homeostasis, mitochondrial dysfunction and caspase activation [84]. Some of the anticancer brown algae are listed in Table 7.1.

7.4.2 Effects of Fucoidan on Cell Cycle and Apoptosis

The cell cycle is the mechanism by which cells divide. It is a high energy demanding process that requires an encompassed and ordered series of events to guarantee

the correct duplication and segregation of the genome. This process involves four sequential phases that go from quiescence (G0 phase) to proliferation (G1, S, G2, and M phases) and back to quiescence [85]. Increasing knowledge on the cell cycle deregulations in cancers has promoted the introduction of marine bioactive compounds, which can either modulate signaling pathways leading to cell cycle regulation or directly alter cell cycle regulatory molecules, in cancer therapy. Fucoidan at 1.0 mg/ml concentration increased the G0/G1-phase population in hepatocarcinoma cell line (Huh7) accompanying by a decrease in the S phase, suggesting that fucoidan may cause the cell cycle arrest at the G0/G1 phase [86]. Fucoidan suppressed cell proliferation and arrest cell cycle in HCC cell lines (HAK-1A, KYN-2, KYN-3) and increased number of cells in the G2/M phase at 72 h after the addition of the fucoidan (22.5 μg/ml) [87]. Also identified that fucoidan induced the accumulation of cells in G1/S phase of the cell cycle of HUT-102 cells (T-cell lymphoma) [28] and non-small-cell human bronchopulmonary carcinoma (NSCLC-N6) cells [25]. The growth-inhibitory function of fucoidan on human T-cell leukemia virus type 1 (HTLV-1) infected T cells by the induction of cell cycle arrest in the G1 phase was accompanied by downregulation of cyclin D2 and also downregulation of *c-myc* and pRb phosphorylation in HTLV-1-infected T-cell line [28].

Apoptosis is critically important for the survival of multicellular organisms [88]. The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Impairment of this native defense mechanism promotes aberrant cellular proliferation and the accumulation of genetic defects, ultimately resulting in tumorigenesis [89]. Apoptosis is mediated through death receptor pathway (extrinsic) and mitochondrial pathway (intrinsic) are the two major well-studied apoptotic processes [90, 91]. In the extrinsic pathway, stimulation of death receptors, such as Fas and tumor necrosis factor receptor-1, leads to clustering and formation of a death-inducing signaling complex, which include the adaptor protein Fas-associated death domain (FADD) and initiator caspases, such as caspase-8. Activated caspase-8 directly activates downstream effector caspases, such as caspase-3 and -7 [92]. Also, caspase-8 can cleave Bid (Bcl-2 interacting protein) into tBid (truncated Bid), which interacts with proapoptotic protein Bax, lead to the accumulation of Bax in mitochondria promotes release of cytochrome c into the cytosol [93–95]. In the intrinsic pathway, death receptors transmit death signals to the mitochondria, resulting in the release of several mitochondrial intermembrane space proteins, such as cytochrome c, which associate with Apaf-1 and procaspase-9 to form the apoptosome. Activated caspase-9 can cleave and activate effector caspases, such as caspase-3 and -7 [96]. Studies showed that fucoidan, extracted from *Cladosiphon okamuranus*, strongly antiproliferative and apoptotic effects on MCF-7 cells in a dose-dependent manner but not affect proliferation of normal cells of human mammalian epithelial (HMEC) cells. The characteristics of apoptotic cell death are induction of chromatin condensation, fragmentation of nuclei and DNA, and cleavage of specific proteins. Fucoidan induced accumulation of sub-G1 population, chromatin condensation, and internucleosomal fragmentation of DNA [97], these are representative features of apoptosis, was shown in MCF-7 cells. Effector caspases, such as caspase-3 or -7, activate DNase, resulting in fragmentation of DNA in response

to various apoptotic stimuli. MCF-7 cells show a defect in caspase-3 but express caspase-7, which is an executioner caspase capable of cleaving PARP (Poly (ADP-ribose) polymerase) [98]. Activation of caspase-7 and PARP cleavage are hallmarks of apoptosis in MCF-7 cells [99]. Cleavage of PARP and activation of caspase-7 were induced after treatment with fucoidan in MCF-7 cells and that caspase-7 inhibitor z-DEVD-fmk canceled fucoidan-induced apoptosis [24, 80]. Fucoidan (Fucoidan extract) increased mitochondrial depolarization by up-regulates the expression of pro- apoptotic proteins Bax and Bad, and down-regulates the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl in MCF-7 cells [82].

Caspase-8 plays a crucial role in apoptosis triggered by the interaction of ligand with death receptors (DR), such as Fas, tumor necrosis factor receptor (TNFR), and TNF-related apoptosis inducing ligand receptor (TRAILR). Bid is directly cleaved by caspase-8, whereupon the C-terminal BH3 domain containing a fragment of Bid called as tBid, could triggers cytochrome c release and leading to caspase-9 activation. Fucoidan activate Bid into tBid in MCF-7 cells. Therefore, it is also suggested that caspase-8 activates the tBid-related apoptotic pathway, leading to caspase-9 activation as well as direct activation of capase-7 in fucoidan-treated cells. The activated caspase-9 then proteolytically cleaves and activates executioner caspase-3, -6, and -7 [100]. Activation of caspase-9 appears to be a branched pathway of apoptotic cell death, originating from caspase-8 during fucoidan-induced apoptosis. Fucoidan treatment induced the activation of extrinsic pathway-related proteins, DR5 and caspase-8, as well as the activation of the intrinsic pathway through the decrease of Bcl-2, the increase of Bax, and the activation of caspase-9, which were followed by the activation of caspase-3 and the cleavage of poly(ADP-ribose)-polymerase (PARP) in prostate cancer cells [81]

The FCSPs derived from *F. vesiculosus* enhances mitochondrial membrane permeability of human colon cancer cells *in vitro*, and to induce cytochrome c and Smac/Diablo release from the mitochondria [101]. Jin et al. [102] reported that the intracellular levels of apoptotic proteins modulated in fucoidan-treated HL-60 cells. The active forms of caspases-8, caspase-9, caspase-3 and PARP increased in response to fucoidan in a dose-dependent manner. The anti-tumor effects of fucoidan accompanied by the activation of the caspase pathway and the down-regulation of the ERK pathway [24, 28]. LMWF induces a caspase independent, mitochondrial-mediated apoptotic pathway in ER-positive MCF-7 cells [82]. Zhang et al. [84] reported that LMWF induces a sustained collapse of mitochondrial membrane potency, release of cytochrome c, and down regulation of antiapoptotic proteins Bcl-2, Mcl-1, Bcl-xl, and activation of caspase-9, caspase-7, caspase-3 in MDA-MB-231 cells. Another mechanism of apoptosis is changes in Ca^{2+} signaling can affect cell proliferation and differentiation. LMWF evoked a rapid increase in the intracellular Ca^{2+} level in MDA-MB-231 cells, and this increase was significantly inhibited by the Ca^{2+} chelator, BAPTA-AM [1,2-Bis(2-aminophenoxy)ethane-N, N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)] suggested that the disruption of Ca^{2+} homeostasis in the apoptotic process [84]. Calpain is a protein belonging to the family of calcium-dependent, non-lysosomal cysteine proteases present in the cytosol as the inactive proenzyme, procalpain, which translocates to the cell membrane

in the presence of Ca^{2+}. The substrate for calpain is α-spectrin (or fodrin), which cleaved and play a role in regulating membranestructure, cell shape and linking the cytoskeleton to the plasma membrane or intracellular vehicles [103]. The detection of calpain induced α-spectrin proteolytic fragments has great importance between calpain activation and the earliest stage of apoptosis. The E-64d calpain inhibitor reduced the production of the 150 kDa fragment, these findings suggests that both calpain and caspases participated in LMWF induced apoptosis [84].

Ale et al. [39] proposed a mechanism for the inhibition of proliferation and induce apoptosis of melanoma cells by FCSPs that activation of macrophages via membrane receptors, which leads to the production of cytokines that enhance natural killer (NK) cell activation. Activated NK cells release Granzyme B and perforin through granule exocytosis into the space between NK cells and melanoma cells to initiate caspase cascades in melanoma cells [39]. The antitumor activity of fucoidan from *Fucus vesiculosus* was markedly inhibited the growth of HCT-15 cells (human colon carcinoma cells) by several apoptotic events such as DNA fragmentation, chromatin condensation and increase in the population of sub-G1 cells and mediates apoptosis signaling through mitochondrial pathway [104].

7.4.3 Effects of Fucoidan on Cell Growth Signaling Molecules

Various compounds exert chemopreventive and chemotherapeutic effects through the inhibition of phosphorylation of membrane receptors, including receptor tyrosine kinases (RTKs), EGFR and platelet-derived growth factor receptor (PDGFR). They are involved in the transduction of mitogenic signals across the plasma membrane and the regulation of cell growth and proliferation. Mitogen activated protein kinase (MAPK) pathways are involved in cellular proliferation, differentiation, and apoptosis[105, 106]. Fucoidan treatment increased the phospho-ERK1/2 level, decreased the phospho-p38 level and decreased the phosphor-form of PI3K/Akt [81]. ERK1/2 pathway is involved in the invasive or migratory behavior of a number of malignancies [107–109]. PI3K overexpression is highly correlated with the development, invasion, and metastasis of non small-cell lung cancer [110]. Fucoidan inhibits the phosphorylation of PI3K-Akt in time and concentration-dependent manners. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth and proliferation, cell motility, cell survival, protein synthesis, and transcription. Fucoidan effectively down regulates the expression of MMP-2 through the inhibitions of PI3K-Akt-mTOR as well as ERK1/2 signaling pathways in A549 human lung cancer cells. Additionally, 4E-BP1 and p70S6K, two immediate downstream targets of mTOR and indicators of mTOR activity, were also significantly suppressed [111]. NF-κB and AP-1 are transcription factors that regulate the expressions of numerous genes associated with many important biological and pathological processes, including cancer and it has reported that inhibition of NF-κB and AP-1 results in the suppression of tumor initiation, promotion and metastasis [112, 113]. Fucoidan inhibits the phosphorylation of Iκ-Bα and increased

the total p65 in the cytosolic fraction and its decreased in nuclear fraction in lung cancer cells A549 [111].

Fucoidan suppressed the phosphorylation of ERK and JNK under the control of EGF. Interestingly, c-fos and c-jun transcriptional activities were inhibited by fucoidan, leading to inhibition of activator protein-1 (AP-1) activity and cell transformation induced by EGF [114]. AP-1 is a transcription factor involved in cellular proliferation and transformation [115]. AP-1 complexes are formed by dimmers of Jun proto-oncogenes or heterodimers of the Jun family members with the Fos proto-oncogene family members. AP-1 binds to a specific target DNA site in the promoters of several cellular genes and mediates immediate early gene expression involved in a diverse set of transcriptional regulation processes [116]. Fucoidan suppress the AP-1 activation through inhibition of JunD expression in an HTLV-1-infected T-cell line, thereby inhibits HTLV-1-infected T-cell proliferation [28]. An up-regulation of the Wnt/β-catenin signaling pathway has a pivotal role in the development and progression of prostate cancer [117]. Fucoidan decreased the β-catenin, a key molecule of Wnt/β-catenin signaling pathway. Fucoidan also induced the activation of GSK-3β, a regulator of β-catenin, which was followed by the decrease of c-myc and cyclin D1, β-catenin target genes. To confirm the role of the Wnt/β-catenin signaling pathway, GSK-3β, an important upstream regulator of β-catenin, was inhibited using the GSK-3β inhibitor LiCl. Pretreatment with LiCl, fucoidan restored β-catenin levels to untreated control levels. These results suggested that fucoidan might regulate β-catenin through GSK-3β activation, and that the fucoidan effect on the apoptosis induction could be associated with the down-regulation of the Wnt/β-catenin signaling pathway [81].

7.4.4 Fucoidan on Metastasis and Angiogenesis

Metastasis is the spread of a disease from one organ or tissue to another non-adjacent organ or tissue. It is regulated by numerous signaling pathways in both the cancer cells and microenvironment. Metastasis is a leading cause (up to 90%) of cancer-related deaths. The primary tumor, invade surrounding tissues and intravasate into blood and/ or lymphatic systems and extravasate from the vasculature and subsequently settle and colonize at the target organs. Matrix metalloproteinases (MMPs) play a key role in tumor metastasis. The degradation of extracellular matrix (ECM) is crucial for cellular invasion, indicating the inevitable involvement of matrix degrading proteinases for the process. Fucoidan suppressed MMP-2 activity and protein expression with increasing concentrations of fucoidan in A549 (lung) cancer cells [111]. Fucoidan inhibit metastasis via MMP-2 suppression as well as decreased the expression and secretion of a vascular endothelial growth factor (VEGF) [83]. The anti-metastatic activity of fucoidan was also proven in the animal model of experimental transplanted Lewis lung carcinoma (LLC) cells [37]. Fucoidan was shown to regulates the function of several cell surface proteins involved in migration and cell adhesion, including integrins, VEGF 1 and 2, P-selectin and neuropilin-1 [118–120].

Angiogenesis is the new blood capillaries formation in physiological and pathological processes [121, 122]. Tumor growth is requires for angiogenesis to supply nutrients and oxygen. Anti-angiogenic therapy has become an effective strategy for inhibiting tumor growth. Inhibition of angiogenesis can leads to target for cancer therapy [123]. Oversulfated fucoidan enhances anti tumor and anti angiogenic effects on cancer [124]. Fucoidan and oversulfated fucoidan significantly suppressed the mitogeneic and chemotactic actions of VEGF. Vascular endothelial growth factor (VEGF) is an interesting inducer of angiogenesis and lymphangiogenesis, because it is a highly specific mitogen for endothelial cells. SDF-1 (stromal cell-derived factor-1) is a small cytokine belonging to the chemokine family that designated as Chemokine (C-X-C motif) ligand 12 (CXCL12). The receptor for this chemokine is CXCR4. Targeting the CXCL12/CXCR4 pathway is a logic strategy in cancer therapy [125, 126]. Fucoidan inhibited cell growth more prominently in Huh7 human hepato cancer cells by suppressing chemotaxin CXCL12 and CXCR4 [86]. Both native and oversulfated FCSPs have anti-angiogenic actions *in vivo* and *in vitro* anti-proliferative effects against B16 melanoma cells, Sarcoma-180 and Lewis lung carcinoma cells. The interaction of oversulfated FCSPs with VEGF165 occurred with high affinity and resulted in the formation of highly stable complexes, thereby interfering with the binding of VEGF165 to vascular endothelial growth factor receptor-2 (VEGFR-2). The study showed that both native and oversulfated FCSPs were able to suppress neovascularization in mice implanted Sarcoma-180 cells; and that both FCSPs types inhibited tumor growth through the prevention of tumor-induced angiogenesis, but the data indicated that sulfation tended to give more potent effects [124]. Fucoidan inhibited both human glioblastoma (T98G) and THP1 (acute monocytic leukemia) cell-induced angiogenesis [127].

Using a human umbilical vein endothelial cell (HUVEC)-based cell culture model, the anti-angiogenic activity of fucoidan extracted from the brown seaweed *Undaria pinnatifida* showed significant inhibition of cell proliferation, cell migration, tube formation and vascular network formation. Also *Ex vivo* angiogenesis assay demonstrated that 100 μg/ml of fucoidan caused significant reduction in microvessel outgrowth. Western blot and RT-PCR analyses indicated that at 400 μg/ml, fucoidan significantly reduced the expression of the angiogenesis factor VEGF-A in the suppression of angiogenesis activity [128]. Recent studies also showed that fucoidan, significantly reduces tumor volume and the number of metastatic lung nodules in the 4T1 xenograft model. The mechanisms involved in the reduction of tumor growth in 4T1-bearing mice, demonstrated that fucoidan suppresses *in vitro* cell proliferation, colony formation, expression of epithelial to mesenchymal transition (EMT) biomarkers and blocks cell migration and cell invasion [129]. The molecular network of transforming growth factor β (TGF-β) receptors (TGFRs) plays an important role in the regulation of the EMT in cancer cells. Using 4T1 and MDA-MB-231 cells, fucoidan effectively reverses TGF-R induced EMT morphological changes, upregulates epithelial markers, downregulates mesenchymal markers and decreases the expression of transcriptional repressors Snail, Slug and Twist. Fucoidan decreases TGF-RI and TGF-RII proteins and affects downstream signaling molecules, including Smad2/3 phosphorylation and Smad4 expression.

The study was shown to identify a novel mechanism for fucoidan antitumor activity, namely regulation of the EMT via modulation of TGFR/Smad-dependent signaling, which leads to an inhibition of breast cancer cell growth *in vitro* and *in vivo*. The findings indicated that fucoidan is a potential therapeutic agent for breast cancer and acts via an ubiquitin-dependent degradation pathway that affects the TGFR/Smad/Snail, Slug, Twist and EMT axes [129].

7.5 Conclusions

Brown seaweeds contains fucoidans are complex and heterogeneous, and have various structures, but not been very clear until now. Fucoidan has various biological activities which include antitumor, immunomodulatory, antiviral, antithrombotic, anticoagulant, antithrombotic, antioxidant and antilipidemic activity. Fucoidan inhibits cancer cell proliferations by inducing cell cycle arrest, inducing apoptosis, inhibiting cell migration, invasion and regulating signaling molecules. Deeply studying the structure of fucoidans and exploring the relationship activity and structure are useful for developing and utilizing the brown algae resource. Further *in vivo* study and clinical investigations are necessary for the application of fucoidan as a novel therapeutic agent and alternative remedy for anticancer therapy.

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Chapter 8
Seaweeds-Derived Bioactive Materials for the Prevention and Treatment of Female's Cancer

Ratih Pangestuti and Se-Kwon Kim

Abstract Seaweeds are a famous delicacy in Eastern hemisphere. In addition to its food value, structurally diverse biologically active molecules with great pharmaceutical and biomedical potential have been reported to be present in the seaweeds. The epidemiologic studies have correlates the seaweeds consumption and health benefit effects on female subject. Furthermore, seaweeds and its derived components have extensively been investigated for their anti-cancer anti-viral activities. As seaweeds have the potential to both benefit health and improve food acceptability, seaweeds and its metabolites offer exciting potential as ingredients in the development of functional foods, nutraceuticals and pharmaceuticals. This contribution highlights seaweeds health benefits effects for cancer in female subject.

Keywords Seaweeds · Female · Health · Cancer

8.1 Introduction

The wide diversity of marine organisms is being recognized as rich sources of functional materials [1]. Among marine organisms, seaweeds are still identified as under-exploited plant resources; although they have long been used as food sources and traditional remedies. In Eastern hemisphere, seaweeds have always been of particular interest as marine food sources. Seaweeds accounted for more than 10 % of *Japanese* diet with average consumption reached an average 1.4 kg/person/year [2]. In Korea, new mothers are served with *miyeok-guk* (seaweeds soup) after deliver their babies up to 37 days [3]. Korean believes that *miyeok-guk* provide complete nutrition to help the new mother to recover and regain their energy.

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Fig. 8.1 Seaweeds delicacies. Several kinds of popular seaweeds delicacies in East Asia

Seaweeds have been demonstrated as rich sources of structurally diverse biologically active molecules with great pharmaceutical and biomedical potential therefore it represents one of the most nutritious plant foods [4]. The tradition and everyday habits of seaweeds consumption has made possible a large numbers of epidemiological studies showing the health benefits linked to seaweeds consumption. Considering combination with international diet-related chronic disease incidences, a significant environmental factors including dietary difference between populations varying in seaweeds consumption have revealed. For example, several epidemiologic studies show that seaweeds consumption correlates with low breast cancer rates in Japan and China compared to North America and Europe [5, 6]. It has also been reported that neurodegenerative cases in East Asian countries were lower than Europe ($p < 0.0004$) [7].

General utilization of seaweeds in food products has grown steadily since the early 1980s. In recent years, consumers in developed countries are turning to more natural and nutritional foods, particularly seaweeds (Fig. 8.1). Seaweeds have recently been approved in France for human consumption, thus opening for the food and fisheries industries. During 2003, it was estimated that about one million tons of seaweeds were harvested in thirty-five countries mainly as food sources. Several seaweeds species are consumed by human directly after only minor pre-processing such as drying. *Porphyra* sp. which is commercially known as nori or lavers are the most widely consumed among edible red seaweeds worldwide. Among green seaweeds,

sea lettuces are the most common, ubiquitous, and environmentally important genera. Sea lettuces comprise the genus *Ulva*, a group of edible green seaweeds which is widely distributed along the coasts of the world's oceans and often found in the mid and upper tidal zones. Sea lettuces or sometimes termed as green laver are found in tidal and near tidal seawater worldwide, generally anchored to rocks or other algae. They are easily identified by its paper thin, semi translucent, and vibrant green color. Most sea lettuces are gathered wild as it grows prolifically wherever there are sufficient nutrients, but some is farmed. Many species of *sea lettuces* are reported to be tolerant of organic and metal pollution; hence, if we consumed, we need to make sure they are collected far from any potential sources of pollution.

In recent years, nutritional value, and potential health benefits of marine algae have been intensively investigated and reviewed. In spite of extensive studies and reviews on nutritional value, and potential health benefits of marine algae for human, there is little available literature focusing on potential benefits role of seaweeds in female subject. Hence, this contribution focuses on biological roles of seaweeds and presents an overview of their potential benefits for female health.

8.2 Seaweeds-Derived Bioactive Materials for the Treatment and Prevention of Female's Cancer

8.2.1 Anti-breast Cancer Activities

Breast cancer is the second most common frequent cancer in the world, and is by far the most leading cause of cancer-related death among female worldwide [8, 9]. Globally, more than 1 million patients are diagnosed annually, representing around 10% of all newly diagnosed cancer cases [10]. Geographical variation incidence and mortality rates of breast cancer suggest that the known risk factors for breast cancer may vary and that environmental factors are of greater importance than genetic factors [11]. For example, the mortality rate for premenopausal breast cancer is almost four times greater in the Western world, compared with Eastern and one importance difference in the diet between these two populations is higher consumption of fish and seaweeds in the Eastern populations [7].

Traditional Eastern breast cancer medicine has long used seaweeds as a cancer treatment to "soften" tumors and "reduce" nodulation. Teas et al. demonstrated that diet containing 5% *Laminaria angustata* significantly delays the tumors occurrence in female Sprague-Dawley rats induced with the carcinogen 7,12-dimethylbenz(a) anthracene (DMBA) [12]. Furthermore, Yamamoto et al. reported that only 2% of *Porphyra tenera*, *L. religiosa or L. japonica* var ochotensis is necessary to obtain the same protective effect [13]. In accordance, wakame *(Undaria pinnatifida)* and mekabu (sporophyll of wakame) have been showed to reduce the incidence, multiplicity and size of breast tumors in female Sprague-Dawley rats induced with DMBA [14, 15]. Considering that wakame and mekabu is particularly rich in

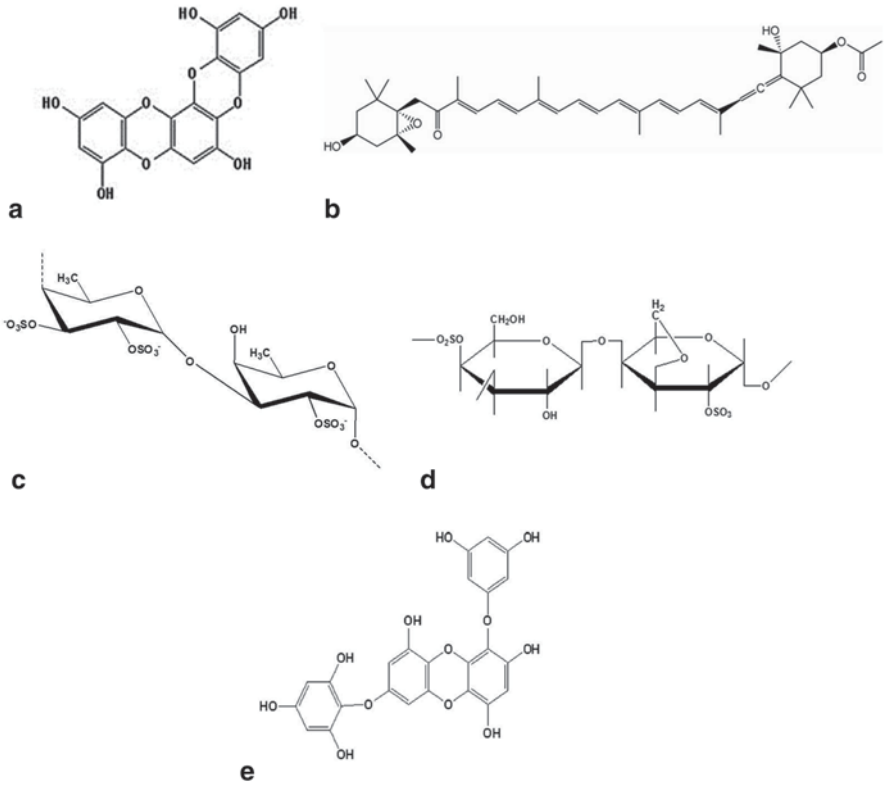

Fig. 8.2 Chemical structures of seaweeds derived-bioactive molecules. Dioxinodehydroeckol (a); fucoxanthin (b); fucoidan (c); ι–carrageenan (d); 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin (e)

iodine, Funahashi et al. suggested that the cancer inhibition was brought about by the iodine. A number of studies have reported the importance of iodine in the treatments for mammary gland dysfunctions. Iodine deficiency have been demonstrated to alters the structure and function of mammary gland in virgin rats, and iodine components (I_2) is effective in diminishing ductal hyperplasia and perilobular fibrosis secondary to this iodine deficiency [16]. Similarly, I_2 treatment of patients with benign breast disease is accompanied by a significant bilateral reduction in breast size, which further leads to a remission of disease symptoms. The importance of I_2 in the treatments for mammary gland dysfunctions has been corroborated in human and animal models [17].

Dioxinodehydroeckol (Fig. 8.2a), a phloroglucinol derivative from *Ecklonia cava,* has a potential inhibitory effect against growth of human breast cancer cells MCF-7 via induction of apoptosis [18]. One μg/ml mekabu strongly induced apoptosis in three human breast cancer cell line (MCF-7, T-47D, and MDA-MB-231), the induction of apoptosis even greater than 5-fluorouracil, a chemotherapeutic agent frequently used in human breast cancer clinics. More recently, fucoxanthin

(Fig. 8.2b) the brown pigment which colors kelp and other brown seaweeds induced strong cytotoxicity against breast cancer MCF-7 cells with IC_{50} = 11.5 μg/ml [19]. In addition, sulfated polysaccharides derived from brown algae, fucoidan (Fig. 8.2c) induced apoptosis in breast cancer 4T1 cells through down-regulation of VEGF. Furthermore, intraperitoneal injection of fucoidan in mice breast cancer models reduced the tumor volume and weight significantly [20]. The enhanced antitumor efficacy was associated with decreased angiogenesis and increased induction of apoptosis. Apoptosis is a key process in cancer development and progression which can be characterized through distinct set of morphological and biochemical progresses. Inactivation of apoptosis has been considered to be one of six fundamental hallmarks of cancer; therefore, apoptosis is a major target of cancer therapy development up to present [21]. Hence, development of molecules derived from marine algae which promote apoptosis in breast cancer cells by targeting both the intrinsic and extrinsic apoptotic pathways may lead to the development of effective breast cancer therapies.

8.2.2 Anti-Estrogen Dependent Cancers

Estrogen-dependent cancers are among the leading causes of morbidity and mortality in American female [22]. Increased incidence of these cancers is predicted in the future, and the need for primary prevention is clear. Epidemiological studies demonstrated that estrogen dependent breast cancers (ER+) are among the highest in Western, industrialized countries, while rates are much lower in China and Japan [23, 24]. Due to some research study, low estrogen-dependent cancers rates have been attributed to the soy-rich and seaweeds diets inherent among Asian populations [25]. As an example, dietary intake of *Alaria esculenta* (L) and soy protein has been reported to modify estrogen and phytoestrogen metabolism in healthy postmenopausal female [25]. In another female pilot study, Skibola et al. demonstrated that intake of *Fucus vesiculosus* (bladderwrack) significantly increased the total number of days of the menstrual cycle, reduced circulating 17*β*-estradiol levels, and elevated serum progesterone levels in premenopausal female with abnormal menstrual cycling histories [26]. Moreover, *F. vesiculosus* have been demonstrated to modulate endocrine hormones in female sprague-dawley rats and human luteinized granulose cells [27]. Hence, it may assumed that intake of seaweeds may contribute to the lower estrogen circulating level which may correlate to the lower incidence of hormone-dependent cancers in female.

Cervical cancer is the second most common cancer in female worldwide and more female die annually because of cervical cancer rather than from AIDS [28, 29]. It is the principal cancer of female in most developing countries, where 80% cases occur [29]. Recent reports demonstrated that several marine algae species: *Palmaria palmate* (dulse), *Laminaria setchellii, Macrocystis integrifolia, Nereocystis leutkeana, Udotea flabellum and Udotea conglutinate* extracts were able to inhibit cervical cancers cell proliferations *in vitro* [6, 30, 31]. Fundamentally, cancer is the

increase in tumor cell number, and thus tumor burden, which ultimately accounts for the adverse effects on the host. The goal of most current cancer therapy is to reduce the number of tumor cells and to prevent their further accumulation. Hence, antiproliferative activity of marine algae in cervical cancer cells demonstrated potential of marine algae as therapeutic agent for cervical cancer treatment.

Additionally, formation of cancer cells in human body can be directly induced by free radicals and natural anticancer drugs as chemopreventive agents have gained a positive popularity in treatment of cancer. Therefore, seaweed derived-radical scavenging molecules such as phlorotannins, carotenoids, sulfated polysaccharides and carmamol derivatives can be used indirectly to reduce cancer formation in the female body. Antioxidant activity of seaweeds and its bioactive molecules have been determined by various methods such as ferric thiocyanate (FTC), thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2′-azinobis-3-ethylbenzothizoline-6-sulfonate (ABTS) radical scavenging effect, singlet oxygen quenching activity, lipid peroxide inhibition, superoxide radical and hydroxyl radical scavenging assays [1].

Seaweeds and seaweed derived-metabolites have shown promising anticancer activities and hence, seaweeds have a great potential to improve female health by being a part of anticancer functional foods and nutraceuticals. However, future studies focusing on the synergistic benefits of consuming different seaweeds, intake timing, recommended amounts of seaweeds consumption, and preparation methods for seaweeds is needed to maximize the desired protective effect in cancer managements.

8.2.3 Anti-human Papilloma Virus as a Prevention of Cervical Cancer

Human papilloma virus (HPV) is a sexually transmitted virus from the papillomavirus family. HPV infections of the cervix and vagina have emerged as the most common sexually transmitted diseases among young sexually active populations. In the US, an estimated 75% of the reproductive-age populations have been infected with genital strains of HPV [32]. HPV Infection by certain HPV types (especially 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 58, 59 and 68 types) in female genital has been associated with cervical cancer and cervical dysplasia [33, 34]. For this reasons, the diagnosis of symptomatic genital warts caused by the HPV and HPV prevention strategies have skyrocketed during the last three decades.

The first generation of HPV vaccine is currently available on the market to prevent HPV infection [35]. However, high cost of vaccine has been a cause for concern and will be too expensive for use in the developing world. Moreover, there is considerable uncertainty about the most effective strategy for vaccination and the likely impact of vaccination on HPV and cancer incidence [34]. Therefore, the search for potential anti-HPV candidates containing higher inhibitory activity and fewer prices has rise great interest in pharmaceutical industries. In this regard, natural bioactive compounds and their derivatives are potential source for the develop-

ment of functional foods as new generation anti-HPV therapeutics which is more effective, less side effects and less expensive.

Seaweeds contain significant quantities of complex structural sulfated polysaccharides which have been demonstrated as potent inhibitors of wide variety of viruses, including [36–38]. Carrageenan, a sulfated linear polysaccharides of d-galactose and 3,6-anhydro-d-galactose extracted from the *Rhodophyceae*, has been used in food products for centuries. Recently, carrageenan has been shown to bear anti-HPV activity *in vitro* [36]. Buck et al. noted that carrageenan particularly ι–carrageenan (Fig. 8.2d) inhibit HPV three orders magnitude more potent than heparin, a highly effective model for HPV inhibitor [29]. Carrageenan acts primarily by preventing the binding of HPV virions to cells and block HPV infection through a second, post attachment heparin sulfate-independent effect. Those mechanism is consistent by the fact that carrageenan resembles heparin sulfate, which is known as HPV-cell attachment factor. Furthermore, some of milk-based products block HPV infectivity *in vitro*, even when diluted million-fold [39]. In another study, carrageenan has been reported to inhibit genital transmission of HPV in female mouse model of cervicovaginal [40, 41]. Carrageenan was able to generate antigen-specific immune responses and antitumor effects in female (C57BL/6) mice vaccinated with HPV-16 E7 peptide vaccine [42].

Carrageenan can be an alternative source of novel therapeutic candidate for HPV by being a part of food additives. There are numerous advantages of carrageenan over other classes of anti-HPV agents, such as relatively low production costs, broad spectrum of anti-HPV properties, low cytotoxicity, safety, wide acceptability and novel modes of action, suggest carrageenan are promising candidates in the near future.

8.2.4 Anti-Obesity as Cancer Prevention Strategies

Obesity is a major risk factor for developing cancer, roughly equivalent of tobacco use, and both are potentially reversible. Around one-fifth of cancer deaths in the United States are associated with obesity. Further, obese cancer patients do worse in surgery, with radiation or chemotherapy. While the links among obesity, diabetes and heart disease are well-known, there is considerably less public awareness that obesity is also directly tied to several cancers. These include some of the toughest to treat: postmenopausal breast; cervical; ovarian; colon and rectal; endometrial, esophageal; thyroid; gallbladder; kidney; and pancreatic cancer. Studies also show higher recurrence and mortality rates for overweight and obese patients and survivors.

Obesity may occur in any gender; however, it is more likely to occur in female [43, 44]. Females of industrialized and developing countries are experiencing increases in the prevalence of obesity, diabetes, and high cholesterol [45, 46]. A detrimental effect of obesity on female reproductive system has also been demonstrated consistently. Furthermore, it is reported that media and socio-cultural continues to pressures young female to be thin which promotes body dissatisfaction, eating disturbance, depression and negative effect in young female [47]. Therefore,

Table 8.1 Soluble, insoluble and total fiber (% dry weight) in some edible seaweeds

Seaweeds	Soluble Fibre	Insoluble Fibre	References
Hijiki fusiformis	16.3	32.9	[49]
Himanthalia elongate	25.7	7.0	[49]
Eisenia bicyclis	59.7	14.9	[49]
Undaria pinnatifida	17.31	16.26	[50]
Lamina riadigitata	9.15	26.98	[50]
Fucus vesiculosus	9.80	40.29	[50]
Durvillaea antarctica	27.7	43.7	[51]
Porphyra teneri	14.56	19.22	[50]
Chondrus crispus	22.25	12.04	[50]
Ulva lactuca	21.3	16.8	[2]
Enteromorpha sp	17.2	16.2	[2]

female may pay a higher health price for *obesity*. Despite these increases and detrimental effect, prevention opportunities exist to improve female's health during their reproductive years and beyond and to improve the health of future generations. Many categories of natural and synthetic compounds which demonstrated as anti-obesity drugs have been used by female to reduce their weight. However, synthetic anti-obesity agents are believed to have certain side effects such as unacceptable tachycardia, hypertension, improve lipid blood levels, improve glucose metabolism and disturbance of female reproductive system [48]. Hence, more scientific efforts have been dedicated to study medicinal foods that can act as anti-obesity agents.

In the last four decades, researchers have found that soluble dietary fibers are negatively associated with obesity. Seaweeds is particularly rich two different types of fiber, soluble and insoluble (Table 8.1). As an example, *Eisenia bicyclis* contains more than 50% soluble fiber of its dry weight; the other brown algae species, *Fucus vesiculosus* contained more than 40% insoluble fiber per dry weight [49, 50]. In human body, soluble and insoluble fiber acts in a very different way. Consumption of marine algae soluble fiber such as carrageenan, agar, and alginate are primarily associated with hypocholesterolemic and hypoglycemic effects [52]. Alginates consumptions have been shown to modulate appetite and energy intake in human models of acute feeding. Upon reaction with gastric acid (acid-soluble calcium source), alginates undergo ionic gelation to form an alginate gel that can slow gastric emptying, stimulate gastric stretch receptors and reduce intestinal nutrient uptake and influence the glycaemic response [53]. In accordance, ingesting calcium-gelled, alginate-pectin twice per day has been reported to reduce spontaneous food intake in overweight and obese female [54]. Hence, the use of alginate to address the features important in the development of overweight and obesity is therefore shown to be clinically effective. Furthermore, insoluble fiber such as cellulose, xylans, mannans are associated with excretion of bile acids, increase fecal bulk and decrease intestinal transit time [2, 55]. Previous studies have also suggested that seaweed polysaccharides may be used at fat substitute in a range of food applications in order to produce an end-product with reduced total fat content, while still allowing for a product with improved moisture retention and consistency. The use of seaweed

polysaccharides has been shown to facilitate the production of low fat versions of meat-based, starch-based, fat-based and fruit/vegetable-based products [56].

Maeda et al. reported that dietary intake of fucoxanthin significantly attenuates the weight gain of white adipose tissue (WAT) and expressed Uncoupling Protein 1 (UCP1) in diabetic/obese KKA^y female mice [57, 58]. The potential involvement of fucoxanthin in attenuate the weight gain of WAT may correlate to the presence of unusual double allenic bonds at C-7′ position [59]. WAT is the predominant type of adipose tissue and commonly calles "fat" in mammals [60]. Besides its role in energy storage, WAT is now recognized as an endocrine and active secretory organ through its production of biologically active mediators termed, adipokines [61]. Excess production of adipokines includes proinflammatory factors and chemokines, has been linked with obesity and plays an important role in the development of obesity related disease. Therefore, fucoxanthin activity to attenuate the weight gain of WAT in female mice demonstrated potential of fucoxanthin for the prevention and treatment of obesity and diabetes particularly in female subject. Dioxinodehydroeckol and 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin (Fig. 8.2e), two phloroglucinol derivatives isolated from *E. cava* have significantly inhibits adipocyte differentiation in 3T3-L1 cells suggesting its potential use as a functional ingredient in obesity managements [62].

Seaweeds may serve as a potential candidate of functional foodstuffs for obesity management. Hence, negative effect in female subject particularly to prevent cancer can be minimized by the application of seaweeds and seaweeds-derived bioactive materials in functional foods, nutraceuticals and pharmaceuticals. Additionally, seaweeds may develop as a new approach for the treatment of obesity in addition to currently available anti-obesity agents. Therefore, seaweeds would be a potent natural source for the development of foods and pharmaceuticals for the cancer prevention in female subject.

8.3 Conclusions

The wide range of biological activities associated with bioactive materials derived from seaweeds such as phlorotannins, alginates, sulfated polysaccharides and carotenoids have potential to expand its nutritional and health beneficial value of seaweeds in food industries. Seaweed and seaweed-derived bioactive materials enrichment not only benefit the nutritional value of a food product, but also benefit the product in terms of improving the shelf-life and in some cases actually improving the sensorial properties. Furthermore, the wide diversity of seaweeds and numerous undiscovered unique metabolites present in seaweeds are interesting sources to increase numbers of novel functional foods which beneficial for cancer in female subjects. Accordingly, possibilities of designing new medicinal foods or nutraceuticals and pharmaceuticals for cancer prevention derived from seaweeds are promising. As such, nutrition or health researchers should collaborate early on with food technologists/food industry in order to design and develop suitably appealing products with these ingredients.

Although side effects of seaweed and seaweed-derived functional ingredients have been limited in chronic animal and short-term clinical studies, sustained clinical trials are needed to establish the safety of seaweeds at anticancer doses.

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Chapter 9
Antitumor and Antimetastatic Effects of Marine Algal Polyphenols

Fatih Karadeniz and Se-Kwon Kim

Abstract Recently, bioactive substances from marine organisms have gained notable attention from various fields throughout the globe. Among marine organisms, algae have been studied widely for the isolation and characterization of biologically active components and polyphenols are one of the most abundant among them. Vast majority of algal polyphenols are consisted of phlorotannins which are derived mainly from brown algae and shown to possess numerous bioactivities such as antioxidant, anti-inflammatory, antidiabetic, antihypertensive, anti-allergic and so on. Moreover, marine polyphenols are reported to act against tumor growth and show anti-cancer properties. Results indicate that these substances demonstrate varying mechanisms of action and significant activities towards cancer and tumor-related complications. Herein, some recent findings towards the anticancer and antimetastatic characteristics of marine algal polyphenols are reviewed. Their efficiency, source and molecular mechanisms are presented.

Keywords Algal polyphenols · Anti-cancer · Antitumor · Antimetastatic · Marine algae · Phlorotannins

9.1 Background

Among all life-threatening diseases of modern world, cancer has stirred enormous difficulties for the fields of medicine and immunology. Discovery and development of novel and efficient compounds from natural sources have been the key aspect

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of concern for researches, especially in the pharmaceutical field. Nature contains a broad variety of organisms including microbial inhabitants and lithosphere, atmosphere and hydrosphere altogether present an excellent source of distinctive chemical compounds with great potential to be used as therapeutic agents. Present trends credit drugs derived from natural sources to have notable impact on the antitumor agent discovery approach [1]. Natural products are considered to possess this giant potential due to their bioavailability, specific and strong binding to drug targets, ability to bind proteins with minimal entropy loss. In addition, it has been reported that compounds of natural origin are known to be adoptive to diverse conformations in aqueous and lipophilic environments [2].

The utilization of organisms present in folk medicine is being widely investigated worldwide. Among all traditional medicinal organisms, plant materials occupy a large part of natural products which are being recognized for their renowned bioactivities such as anticancer, antiviral, antioxidant and antibacterial. Starting from ancient times, folk medicines use marina algae intensely, and in this regard several species, especially brown algaehave emerged as abundant source of nutrition, hence consumed as a kind of seasoned vegetable in various coastal areas worldwide [3–5]. Moreover, it has been treated as a source of natural marine product due to its biological activity in a broad range. Marine algae already reported to contain various phlorotannin derivates, which have been regarded as potential pharmacological polyphenols [6–7]. Phlorotannins are oligomeric form of phloroglucinol and have been revealed to have antioxidant, antibacterial, anti-inflammation, anti-allergy, anti-matrix metalloproteinase (MMP), apoptosis-induction, and so on [8–10].

9.2 Phlorotannins

Phlorotannins are natural compounds which are formed by the polymerization of 1,3,5-trihydroxybenzene (phloroglucinol) monomer units and are known to be biosynthesized through acetate–malonate pathway. Highly hydrophilic phlorotannins are found to be between 126 Da and 650 kDa molecular weight [11]. Phlorotannins of different molecular weights are mainly accumulated in marine brown algae which contains a wide range of phloroglucinol-based polyphenols. There four main types of phlorotannins based on linkage of monomers, namely fuhalols (phlorotannins with an ether linkage), fucols (with a phenyl linkage), fucophloroethols (with an ether and phenyl linkage) and eckols (with a dibenzodioxin linkage). Reports indicate several isolated and elucidated phlorotannins from marine sources such as phloroglucinol, eckol, fucodiphloroethol G, phlorofucofuroeckol A, 7-phloroeckol, dieckol, and 6,6′-bieckol [12]. In addition, triphloroethol A, 8,8’-bieckol, and 8,4’”-dieckol have been isolated. Marine brown alga *Ecklonia cava* is extensively studied and promoted to be a rich and dependable source of phenolic compounds in comparison to other brown algae [13]. Stressful conditions of marine environments and herbivore danger are considered to be battled by phlorotannins in case of brown algae. Owing to the health beneficial various biological activities of marine brown

algae, phlorotannins are reported to be important compounds for future development and discovery of therapeutic agents.

The bioavailability of plant polyphenols have already been studied and discussed in vivo [14–16]. According to these reports it could be said that around 70% out of consumed polyphenolics amount has been shown potent bioavailability. However, these reports mostly direct the issues through mouse models systems which present a need for further researches that investigates phlorotannin bioavailability in human subjects.

9.3 Anti-cancer Effect of Marine Algal Polyphenols

Harada and Kamei [17] showed that phlorotannins exhibit anticarcinogenic effects. Study presented that brown alga *Laminaria japonica* fractionated phlorotannin extract (PE) has shown considerable anti-proliferative activity in the hepatocellular carcinoma cells (BEL-7402) and also on leukemic cell lines (P388) with the IC_{50} values of 120 and >200 μg/ml, respectively. Microscopic observations have revealed that the morphologic features of tumor cells treated with PE and 5-fluorouracil (a commercial chemotherapy drug) are markedly different from the normal control group suggesting the anti-proliferative effect of PE [18]. Moreover, dioxinodehydroeckol which was isolated from *E. cava* has shown to possess a notable anti-proliferative effect on human breast cancer cells (MCF-7). Dioxinodehydroeckol inhibited the proliferation of MCF-7 cells with rates of approximately 25, 40, 53, 56 and 64% at concentrations of 1, 5, 10, 50 and 100 μM, respectively, compared to the control group. Study credited the potential anti-proliferative activity of dioxinodehydroeckol to its ability to induce of apoptosis through nuclear factor kappa-light-chain-enhanced activated B cells (NF-κB) family and NF-κB dependent pathway [19]. In another research, *E. cava* has been subjected to enzymatic extraction along crude polyphenolic and polysaccharide fractions. All aforementioned fractions of *E. cava* have been evidently shown to possess antiproliferative and antiradical activities. Especially the CphF at an IC_{50} of 5.1 μg/ml has inhibited cell proliferation in murine colon cancer cell line (CT-26) significantly. A nuclear cell staining assay suggested that this anti-proliferative effect of CphF is associated with apoptotic cell demise in CT-26 [20]. The direct correlation between the anti-proliferative effect of the algae and their polyphenolic content is evidently documented. In this context, the anti-proliferative effects of red alga, *Palmaria palmate* and three kelp Laminaria setchellii, Macrocystis integrifolia, Nereocystis leutkeana extracts has been studied on human cervical adenocarcinoma cell line (HeLa cells). HeLa cell proliferation was inhibited between 0 and 78% by *P. palmate*; 0 and 55% by *L. setchellii* and 0 and 69% by *M. integrifolia* and *N. leutkeana* at 0.5–5 μg/ml algal extract concentration range. This investigation suggests the effectiveness of polyphenolic compounds in controlling tumor growth and brings front a fact that marine algae could serve beneficial for anticancer properties [21]. In addition, in vivo tests also presented valuable data regarding antitumor

effects of polyphenols. Dietary inclusion of brown algal polyphenols in pre-tumor bearing mouse feeding at the rates of 0.1 and 0.5% has notably reduced tumor proliferation by 45 and 56% and tumor mass by 54 and 65%, respectively, for each application rate. Moreover, the topical application of polyphenols at 3 and 6 mg has significantly decreased tumor proliferation by 60 and 46% and tumor mass by 66 and 57%, respectively. In case of action mechanism, it is believed that brown algal polyphenols inhibit the cyclooxygenase-2 activity and cell proliferation hence preventing the tumor progression [22].

9.4 Marine Algal Polyphenols as MMP Inhibitors

Matrix metalloproteinase enzymes (MMPs) play a significant role in the digestion of extra cellular matrix components, hence directly associated with chronic inflammation, wrinkle formation, arthritis, osteoporosis, periodontal diseases, tumor invasion, and metastasis in pathological conditions. During current decade, a detailed presentation of MMP inhibitory effects of phlorotannins derived from *E. cava* has been documented for the first time [23]. In this report, a novel gelatin digestion assay was able to visualize complete inhibition of bacterial collagenase-1 activity with introduction of 20 μg/ml of *E. cava* extract during preliminary screening assays. In addition, a sensitive fluorometric assay has been carried out and it showed that phlorotannin content of E. cava can specifically inhibit both MMP-2 and MMP-9 activities ($p < 0.001$) at 10 μg/ml. Also, artificially induced activities of MMP-2 and MMP-9 in human dermal fibroblast and HT 1080 cells have been successfully suppressed by E. cava extract in a comparable manner to that of positive control doxycycline. More interestingly, EC extract did not exert any cytotoxic effect even at 100 μg/ml, anticipating, its potential use as a safe MMP inhibitor. Therefore, it can easily be suggested that phlorotannins would be a potent natural source for the development of therapeutic agents against MMP and cancer.

In another research, 3-(3, 4-dihydroxy-phenyl)-acrylic acid phenethyl ester (caffeic acid phenethyl ester, CAPE) has also been isolated and characterized as biologically active compound with health benefits from methanol extracts prepared from roots of *Rhodiola sacra* and quadrifida [24, 25]. In this regard, Lee et al. evidently proposed that these active compounds can down regulate artificially enhanced MMP-9 activities, indicating a notable antitumor effect [26].

Comparison of 29 seaweed extracts in regard to their inhibitory efficiencies on transcriptional activities of MMP-1 expression has been performed by Joe et al. [27]. Research has concluded that the eckol and dieckol from Ecklonia species have showed strong inhibition of both NF-κB and AP-1 reporter activity, which were well related with their abilities to inhibit MMP-1 expression. In addition, MMP-1 expression was dramatically attenuated by treatment with the eckol or dieckol.

It has been also known that matrix metalloproteinases (MMPs) are crucial components in photoaging of the skin, especially due to high and long exposure to ultraviolet A. Reports indicate that enhanced activity of MMPs and increased

photoaging appear to be stimulated by UV-irradiation-associated generation of reactive oxygen species (ROS). Ryu et al. demonstrates that the alga Corallina pilulifera methanol extract which has been shown a high phenolic content, reduced the expression of UV-induced MMP-2 and -9 of human dermal fibroblasts in a dose dependent fashion, and has also exhibited strong antioxidant activity by scavenging free radicals [28].

In a murine asthma model, it has been documented that MMP-9 expression was significantly reduced by the administration of *E. cava* extracts. And it has been presented that *E. cava* extracts were able to notable suppress the cytokine signaling-3 (SOCS-3) expression and reduce the increased eosinophil peroxidase (EPO) activities [29].

Also aforementioned phlorotannins, namely eckol, dieckol, 6,6′-bieckol and 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxy-phenoxy)-2,4,9-trihydroxydibenzo-1,4,-dioxin were also extracted from brown alga, *E. cava*, and in regard it has been reported that these compounds were able to inhibit the expression of MMP-1, -3 and -13 induced by proinflammatory cytokines [30].

In short, polyphenols, especially from marine sources, have excellent MMP inhibitory activities; however potent cytotoxicity of polyphenols come front as a major drawback. Therefore, the pharmaceutical applications of these MMP inhibitors are usually limited.

In this regard, future researches should turn their attention to reduce their toxicity levels by altering the chemical structure in a way to preserves bioactivity while converting the compound to be biologically safe. Following these improvements, MMP inhibitor polyphenols will gain a huge potential to be used in clinical applications.

9.5 Conclusions

In conclusion, due to the abundant presence and distinctive chemical distribution of polyphenols among marine sources, especially algae, future focus should be directed to biological and pharmacological of novel polyphenols from marine sources with higher efficiency against cancer and less cytotoxicity to non-cancer cells. It is recommended to screen phlorotannins from other marine macro algae and evaluate their anticancer activities as a comparative study. With the latest advancements in the fields of molecular biology and biochemistry, a sophisticated approach to study the interactions of polyphenols with human cellular systems could prove beneficial in understanding and altering parameters like bioavailability and cytotoxicity of these compounds. Also, detailed studies on molecular interactions of phenolic compounds involved in the management of various human diseases would pave the way for development of novel therapeutic agents with superior efficiency. On the other hand, broadening the ways and studies that are developed to screen more biologically efficient polyphenols will definitely provide promising drug candidates for pharmaceutical purposes.

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Chapter 10
Seaweed Carotenoids for Cancer Therapeutics

Meganathan Boominathan and Ayyavu Mahesh

Abstract Carotenoids are naturally occurring fat soluble organic pigments that are found in phototrophic organisms. In the marine environment, carotenoids are wide spread in the seaweeds. Seaweeds are the important sources of bioactive compounds which have numerous beneficial effects such as antioxidant, antimicrobial, anti-inflammation, and anti-cancer activities. The most notable and predominant seaweed carotenoids, such as β-carotene, astaxanthin and fucoxanthin have remarkable biological functions and applications in human health. Cancer chemoprevention is the most crucial strategy in cancer control. Dietary intake of carotenoids has proven to reduce the risk of variety of cancers. Epidemiological investigations have evidenced that there is a clear link between diet and cancer risk. β-carotene, main dietary source of vitamin A have enhanced antioxidant activity and the β-carotene supplementation has shown suppressive effects on carcinogenesis. Astaxanthin, a ketocarotenoid is a potent antioxidant and also has various positive attributes including anti-inflammatory and anticancer activity. Fucoxanthin is a predominant carotenoid in brown algae known to possess antioxidant, anti-inflammatory, anticancer, antidiabetic, and antiangiogenic activities. These seaweed carotenoids are important pharmaceutical compounds, which might be a promising anticancer marine drug. In this chapter, the various biological functions including anticancer effects of the seaweed carotenoids and the possible mechanisms of action are described.

Keywords Carotenoids · Seaweed · Fucoxanthin · Cancer · β-carotene

10.1 Introduction

Cancer, a major threat among the human population has shown rapid increase with changing environment and life style. Cancer cells adapt to the toxic environment and keeps on divide to forming mass of cells without any dominance, and these factor

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makes difficult to cure cancer. Cancer prevention has gained attention as there were no effective treatments available to reduce mortality rates among common carcinomas [1]. Chemotherapy kills fast growing cancer cells and the naturally derived compounds like polyphenols, carotenoids and flavonoids are preferred as a cancer drug [2]. WHO stated that, 80% of the drugs used in health care are derived from natural resources [3]. Marine floras occupying 90% of the ocean biomass, serves as a reservoir for marine drugs and these are remain nature's best source of chemicals [4]. In Ancient, days the marine floras have been used as traditional medicines in India, China, Japan, and some European countries. Marine algae have been used as ayurvedic medicines in China and Egypt [5, 6]. Dietary carotenoids have proven to show the beneficial effects on human health. A large number of new bioactive compounds derived from seaweeds have clinical significance possessing anticancer, anti-inflammatory, antibacterial activity and other medicinal properties [7–9]. First report of marine algae used as anticancer medicine (algasol T331) has been reported in 1960s in Italy. However, the systematic investigation began in the mid -1970s. Ever since algal based drugs [10] and marine pharmaceuticals have gained importance [11].

Carotenoids are natural pigments, which belonging to chemical group of tetraterpenoids with linear C_{40} molecular backbone. Carotenoids have numerous conjugated double bonds and cyclic end groups and hence exist in a variety of stereoisomer's with different chemical and physical properties. Geometric forms are commonly found, also this kind of isomers are inter-convertible in solution. These isomers make difference in their melting points, stability and solubility [12, 13]. Carotenoids are categorized as four group; (a) vitamin A precursors that does not produce pigments, β-carotene; (b) pigments which contain partial vitamin A activity, cryptoxanthin; (c) non-vitamin A precursors that pigment poorly, violaxanthin and neoxanthin; (d) non-vitamin A precursors that produce pigments, lutein, zeaxanthin and canthaxanthin.

The seaweeds are the rich source of carotenoids, the most notable being β-carotene, α-carotene, fucoxanthin, astaxanthin, canthaxanthin, zeaxanthin and lutein (Fig. 10.1), which have all been reported to be effective antioxidants. Seaweed carotenoids are powerful antioxidants which have been associated with the prevention of cardiovascular, neurodegenerative diseases and cancer. As antioxidants, carotenoids can protect the body from oxidative stress, a physiological state that is putatively involved in the development of cancer. The carotenoids have been extensively studied and the consumption of the dietary carotenoids has been correlated with cancer prevention [14, 15]. Several studies have been reported that the anticancer activity of β-carotene, α-carotene, canthaxanthin, astaxanthin and lutein [16–19]. The antioxidant, anti-inflammation and anticancer activity of fucoxanthin has also been reported recently [8, 9]. Chemoprevention is the most crucial strategy for controlling cancer development, molecular mechanism based cancer prevention using carotenoids is an attractive approach. This review describes the anticancer properties of seaweed carotenoids indicating the molecular targets and their mechanism and action on various cancers.

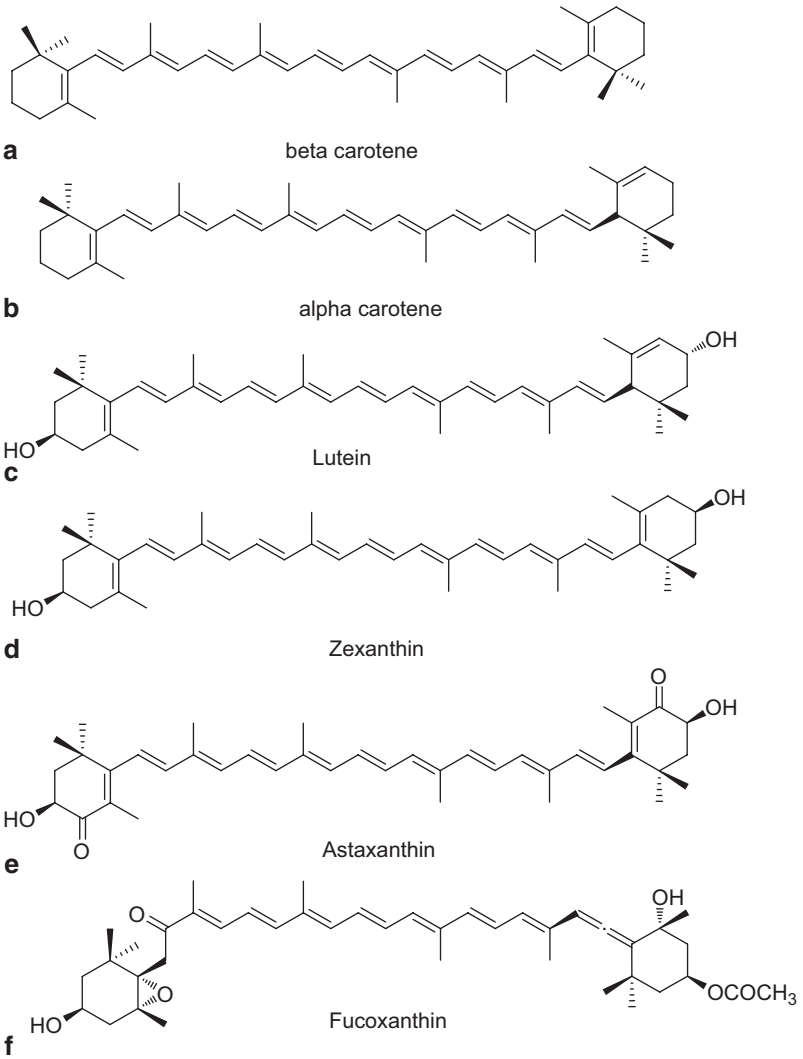

Fig. 10.1 Chemical Structure of few seaweed carotenoids

10.2 Distribution of Seaweed Carotenoids

Seaweeds are photoautotrophic, multicellular marine algae, classified as Chlorophyta (green algae), Rhodophyta (red algae), and Phaeophyta (brown algae) based on their pigments and chemical composition [20]. Number of carotenoids have been isolated and characterised in seaweeds. Most of them have functions in photosynthesis and intermediates of carotenogenesis. The seaweed carotenoid

profiles are served as a chemotaxonomic marker for taxonomic classification of various seaweeds [13]. Seaweeds has considerable source of bioactive compounds as they have numerous biological activities such as antioxidant, antimicrobial, anti-inflammation and anti-cancer [21, 22].

Allene (C=C=C=) a unique structure found in carotenoids such as fucoxanthin in brown algae and 9′-*cis* neoxanthin in green algae. Acetylene (C≡C) also a unique structure found only in seaweeds; diadinoxanthin and diatoxanthin in *Heterokontophyta*. Acetylated carotenoids (–O–CO–CH_3) include fucoxanthin and dinoxanthin are mainly found in *Heterokontophyta*. These carotenoids are particularly found in certain algal divisions and classes [23–25].

Rhodophyta (red algae) are divided into two groups based on their carotenoids composition; the unicellular Rhodophyta containing higher amount of β-carotene and zeaxanthin, and the macrophytic Rhodophyta containing lesser amount α-carotene and lutein, β-carotene, violaxanthin and fucoxanthin. Phaeophyceae (brown algae) has β-carotene, zeaxanthin, violaxanthin and fucoxanthin. Chlorophyceae (green algae) contain β-carotene, violaxanthin, 9′-cis neoxanthin and lutein. Loroxanthin and siphonaxanthin, derivatives of lutein are class specific carotenoids found in chlorophyceae [23, 26].

10.3 Molecular Targets for Cancer Prevention

Cancer can be defined as the uncontrollable proliferation of cells having the ability to migrate and forming the cell mass throughout the body [27]. Cancer cells are aberrant and they use aggressive strategies for entering the blood stream and also various parts of the body. The primary target of cancer cells are cell adhesion molecules such as E-cadherins, which helps in keeping the cells where they belong. Tissue invasion and metastasis are the major part in cancer development, which makes them lethal. Matrix metalloproteinase's (MMPs) and vascular endothelial growth factor (VEGF) plays essential role in angiogenesis and metastasis. They help the cancer cells to spread throughout the body [27–30].

Oncogenes and tumor suppressor genes plays a crucial role in cancer development. Loss of functions of tumour suppressor or oncogenes activation are due to abnormal epigenetic modification influenced by HDACs (histone deaetylases), HATs (histone acetyl transferases), and DNA methyl transferases. These enzymes can serve as a target for cancer therapeutics. Activation of oncogenes can be deprived by interfering through binding to the proteins active site and with signalling cascades of oncogenic proteins. CDK, Chk inhibitors, topoisomerase and antimitotic agents are assistive for controlling abnormal proliferation of cells [30–32]. Apoptosis, programmed cell death can serve as useful function in reverting cellular immortality. Nevertheless advanced tumours are resistant to apoptotic cell death [33, 34].

Anticancer agents target various stages of cancer development. Tissue oxidative stress and Cellular damage can be prevented by antioxidants. Antioxidant plays a vital role in chemoprevention and reduces the chance of tumor formation. A potent

anticancer drug should have antioxidant, anti-apoptotic, anti-angiogensis, anti-inflammatory, antimitotic activity and anti-metastasis properties for suppressing cancer cell progression and prevention.

10.4 Anticancer Effects of Seaweed Carotenoids

The failure of conventional chemotherapy to attain a reduction in the mortality rates of common cancers, which indicates a critical need for new approaches to control cancer development [1, 35]. Cancer chemoprevention grown vigorously as it focuses on the drug discovery from seaweeds that helps in preventing tumour development [36]. It has been estimated that one-third of cancer preventing agents are dietary supplements and they have been tested for their potent chemo preventive activity in cancer induced animal models [7, 37, 38]. A large number of naturally occurring antioxidant compounds have shown chemo preventive activity [17], decreases mutagenesis and thereby carcinogenesis by decreasing the oxidative damage of the DNA [39]. Dietary carotenoids play a major role in reducing oxidative stress and it has been directly correlated with the reduced risk of cancer development. Major carotenoids with potent antioxidant activity have been evaluated for their chemo preventive effects of cancer, which includes β-carotene, lutein, zeaxanthin, fucoxanthin and astaxanthin [8, 9, 16–19].

10.4.1 Anticancer Activity of β-carotene

β-Carotene is a major source of vitamin A, acts as provitamin A dietary supplements. It has various biochemical functions as hormones, antioxidants, and mediators of cell signalling and regulators of cell and also plays a role in tissue growth and differentiation. The antioxidant properties of the algal carotenoids have also been shown to play a role in preventing oxidative stress [40]. β-carotene is an antioxidant rich compound, prevents the carcinogenesis by preventing DNA damage induced by free radicals [41]. Furthermore, experimental studies strongly suggest that β-carotene could prevent the onset of cancers, especially lung cancer [42]. The β-carotene implementation studies carried out in Chinese population that suffered from a diet deficient in vitamins and mineral salts, led to reduced incidence of total mortality from gastrointestinal cancer [43].

Lung cancer is one of the leading causes of death. Many research studies have been conducted worldwide to access the potential of β-carotene and its association with lung cancers. Majority of these studies examined the effect of carotenoids against lung cancer and smokers. A study reported that smokers with high β-carotene level had a higher risk of lung cancer and other smoking related cancers, whereas non-smokers with high β-carotene intake had a lower risk [41, 44–46].

β-carotene is inversely associated with colorectal cancer risk. Several research studies revealed that dietary intake of β-carotene rich food may or may not assistant in developing colon cancer and their association with cancer development is unknown and also smokers who consume high β-carotene have an increased risk of colon cancer [47, 48].

β-carotene has also play a protective role in the prevention of oral cancer and associated cancers, whereas some etiological reports suggest that smoking and alcohol consumption may enhance the risk of oral cancer [48]. Smoking and alcohol intake is associated with decreased serum carotenoids level and continuous intake of fruits and vegetables with high β-carotene have cancer inhibitory activities in mouth and throat cancer and also in oral leukoplakia with reduced mortality rate. Continuous intake of β-carotene supplements significantly reduced the risk of occurrence of stomach and oesophageal cancer and also lowered the mortality rate up to 16% [48]. Various research studies evidenced the functional role of β-carotene and vitamin A and reduced risk of breast cancers. Consumption of carotenoids rich foods have been directly associated with the lower risk of development of premenopausal breast cancer [49, 50].

10.4.2 Anticancer Activity of Lutein and Zeaxanthin

Lutein and zeaxanthin are the principle constituents of the macular pigments of the retina. The macula lutea "yellow spot" in the retina is serving as an essential component for visual activity. Lutein is a dihydroxy-form derivative of α-carotene, while zeaxanthin is the dihydroxy-form of β-carotene. Lutein and zeaxanthin are found in seaweeds including rhodophyta, chlorophyceae and phaeophyceae [29]. Lutein and zeaxanthin selectively accumulate in the macula of the human retina and thought to have antioxidant function [51, 52] and filters blue light [53] which helps in the protection of eyes from oxidative stresses, which may lead to age-related macular degeneration, leading cause of blindness and cataracts [54].

Lutein and zeaxanthin are vital nutrient supplements, which play a crucial role in prevention of stroke and lung cancer. A study conducted in U.S reported that patients consuming high amount of carotenoids including β-carotene, lutein and zeaxanthin shown significant reduction in lung cancer [55]. In another study carried out among 20 South Pacific populations, an inverse association between lutein and lung cancer has been found. Fijians who have consuming an average of 25 mg of lutein daily were shown lower risk of lung cancer than other populations in South Pacific countries [56].

Carotenoids are the key players of scavenging free radicals and their anti-mutagenic activity prevent tumour development and reduce cancer risk [57]. The antioxidant potential of lutein, zeaxanthin and β-carotene are quench peroxy radicals and prevent oxidative damage [58, 59]. Another study using multilamellar liposomes depicted that consumption of carotenoids in combination (lutein or lycopene) rather than singly, evoke stronger antioxidant activity [60]. The anti-carcinogenic

activity of lutein has been evidenced by its ability to interact with mutagens such as aflatoxin B1 (AFB1) [61] and 1-nitropyrene [62] and by stimulating certain genes involved in T-cell transformations [63].

Evolution of epidemiologic studies has revealed the protective effect of lutein in specific cancers, yet no link has been observed between plasma lutein and zeaxanthin concentrations in gastric cancer [64]. Intake of dietary lutein and colon cancer showed inverse association among men and women, whereas significant reduction in risk was demonstrated in younger age group [47].

In case control study, reduced risk of breast cancer has been invariably associated with increasing serum concentration of lutein and zeaxanthin [65]. Increased concentration of lutein and zeaxanthin in breast adipose tissue is significantly associated with the decreased risk of cancer [66]. Anti-mutagenic properties of carotenoids from green algae *Chlorococcum humicola* have been evaluated and it has shown significant reduction ($P < 0.005$) against BAP induced mutagen and zeaxanthin showed inhibition up to 30–60% *in vivo* [67]. The lutein has also shown 50–60% reduction of histidine revertants and comparatively zeaxantion to a lesser extent.

10.4.3 Anticancer Activity of Fucoxanthin

Fucoxanthin was first isolated from the marine brown seaweeds Fucus, Dictyota, and Laminaria by Willstätter and Page [68]. Englert [69] has been determined the complete structure, that including chirality of fucoxanthin and its derivatives (Fig. 10.2). Fucoxanthin, allelic carotenoids is a naturally occurring brown and orange-coloured pigment belongs to the class of non-provitamin A carotenoids, contributes more than 10% of the total production of carotenoids in marine environment [70]. It belongs to the class of 40-carbon organic molecules which can be categorized into two groups: xanthophylls (molecular structure containing oxygen group) and carotenes (without oxygen group). Fucoxanthin has a unique molecular structure with characteristic allenic bond and conjugated carbonyl group in their polyene backbone similar to neoxanthin, dinoxanthin and peridinin [71]. This allenic bond mostly found in carotenoids and is responsible for their higher antioxidant potential [72].

Fucoxanthin has several known biological activities such as anticarcinogenic property, which includes apoptosis induction on cancer cells, anti-inflammatory and antioxidant activity [73–75]. Figure 10.3 described the anticancer activity of the fucoxanthin and its derivative on various cancers and their mechanism action. The antioxidant properties are known to be the primary origin for their beneficiary effects on health [76]. The extracts of brown alga such as *Hizikiafusiformis, Cladosiphon okamuranus, Undaria pinnatifida,* and *Sargassum fulvellum*, rich source of fucoxanthin facilitated DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity [77].

In an *in vitro* study, fucoxanthin and its metabolite fucoxanthinol exhibited higher antioxidant activity and comparatively halocynthiaxanthin showed lower antioxidant activity. The hydroxyl radical scavenging activity of fucoxanthin was higher than that of fucoxanthinol and halocynthiaxanthin, but singlet oxygen quenching

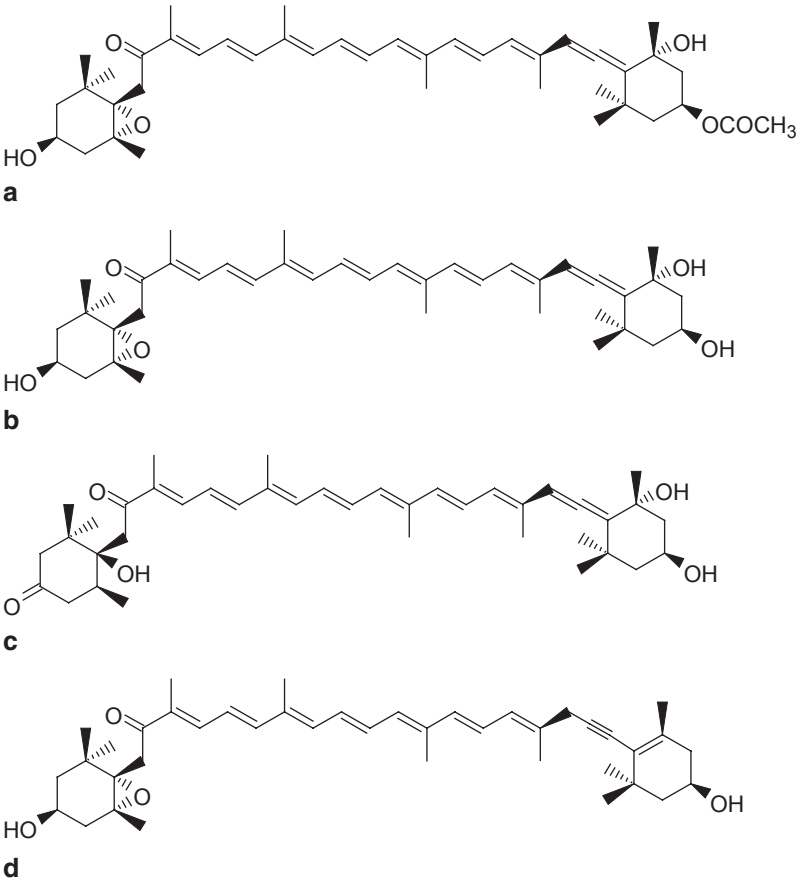

Fig. 10.2 Chemical structure of Fucoxanthin and derivatives. **a** Fucoxanthin. **b** Fucoxanthinol. **c** Amaroauciaxanthin A. **d** Halocynthiaxanthin

ability was comparatively lower than β-carotene [72]. Even though halocynthiaxanthin showed very low scavenging activity, it showed relatively greater suppressive effect on superoxide anion and nitric oxide generation from differentiated human promyelocytic HL-60 cells and mouse macrophage RAW 264.7 cells [78].

It has been reported that, under anoxic conditions fucoxanthin effectively reacted with DPPH than other carotenoids such as β-carotene, β-cryptoxanthin, zeaxanthin, licopene and lutein. Nevertheless, only a part of fucoxanthin consumed DPPH under aerobic conditions [79]. A comparative study on the effects of fucoxanthin and β-carotene on oxidative stress indicators and in their role in suppressing lipid peroxidation in retinol deficiency rats showed increased catalase and glutathione transferase activity and decreased Na+K+-ATPase activity, thereby protecting membranes against lipid peroxidation caused by retinol deficiency [80, 81]. Fucoxanthin exercised its antioxidant potential through its pro-oxidant activities, which reportedly enhanced the expression of HO-1 and NQO1 in murine hepatic BNL CL.2 cells by

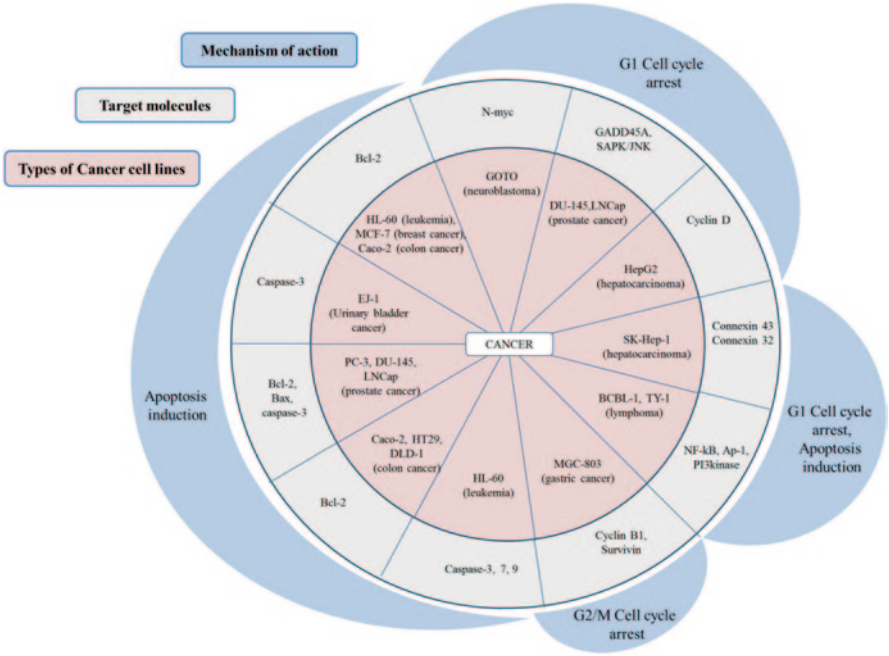

Fig. 10.3 Anti-cancer effects of fucoxanthin and fucoxanthinol on various types of cancer cells and their mechanism of action

activating the Nrf2/ARE system [82]. Many nutrigenomics studies demonstrated the exceptional role of fucoxanthin in cell metabolism and this feature enhances their role in health effects [83].

10.4.3.1 Apoptosis Induction

Apoptosis plays a crucial role in the suppression of tumour cells. Fucoxanthin is an efficient drug to inhibit progression of cancer cells by inducing apoptosis. A substantial antiproliferative activity of fucoxanthin has been studied on human promyelocytic leukemia HL-60 cell line, which found to induce apoptosis on HL-60 cells [84]. It has induces the apoptosis through caspase-9 and caspase-3 activation in HL-60 cell line [85]. Although many carotenoids including astaxanthin, siphonaxanthin, neoxanthin, and violaxanthin have shown cytotoxic effects on HL-60 cells, the inhibitory effect of fucoxanthin was remarkable compared with other carotenoids [86]. Halocynthiaxanthin and fucoxanthinol, metabolites of fucoxanthin have shown significant antiproliferative and apoptosis-inducing effects on HL-60 cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells [87]. Fucoxanthin have shown reduced the viability of Caco-2, HT-29, and DLD-1 cells (human colon cancer cell lines) by inducing apoptosis in a dose dependent manner and it has been

accomplished due to the suppression of Bcl-2 protein [88]. In another study, fucoxanthin has been shown to induce apoptosis by caspase-3 activity and suppressing the Bax and Bcl-2 proteins level in prostate cancer cell line [89]. Fucoxanthin has been demonstrated the antiproliferative activity on EJ-1 cells (human urinary bladder cancer) by inducing apoptosis, characterized by increased percentage of hypodiploid cell and caspase-3 activity [90].

10.4.3.2 Antiproliferative Effect

The metabolites of fucoxanthin (fucoxanthinol and amarouciaxanthin A), has significantly reduced the viability of PC-3 human prostate cancer cells. The cytotoxic effect on PC-3 cells rendered by fucoxanthin and fucoxanthinol may be due to the presence of 5, 6-epoxide structure [91]. Adult T-cell leukemia is a malignancy of mature CD4+T cells caused by human T-cell leukemia type 1 virus. Ishikawa et al. [92] has been demonstrated the strong antiproliferative effect of fucoxanthin and its derivative fucoxanthinol on human T-cell leukemia virus type 1-infected T-cell lines and adult T-cell leukemia cells.

10.4.3.3 Cell Cycle Progression

The role of fucoxanthin, which inhibited human neuroblastoma GOTO cells growth by suppressing N-*myc* gene expression and arresting the cell cycle G0/G1 phase has been reported by Okuzumi et al. [93]. Fucoxanthin has also inhibited the growth of DU145 cells, by inducing cell cycle arrest at G1 phase and GADD45A expression. The increased expression of GADD45A(protooncogene) might be the cause of G1 cell cycle arrest by fucoxanthin [94]. Das et al. [95] has been demonstrated the growth inhibitory effect of fucoxanthin against hepatocellular carcinoma (HepG2 cells), which reported that inhibition was mainly because of arresting the cell cycle G0/G1 phase and also suggested that suppression of cyclin D/cdk4 activity may be related to anticancer activity.

Fucoxanthin significantly inhibited the rapid multiplication of human hepatoma SK-Hep-1 cells by effectively increasing the expression of connexion 43 and connexion 32, enhanced gap junctional intercellular communication, thereby increased the intracellular calcium level which effectively lead to G0/G1 cell cycle arrest [96]. Recent studies of fucoxanthin on human adenocarcinoma MGC-803 cells reported that, fucoxanthin induced G2/M phase cell cycle arrest and also induced apoptosis by down-regulating the Cyclin B1 expression through JAK/STAT pathway, and thereby inhibited MGC-803 cell proliferation [73]. Fucoxanthin and fucoxanthinol induced G1 phase cell cycle arrest and also inhibited the activation of NF-*k*B and activator protein-1, phosphatidyl inositol and activator protein-1, phosphatidyl inositol 3-kinase and have been shown to reduce the viability of cells in primary effusion lymphoma BCBL-1 and TY-1 [97].

10.4.4 Anticancer Effect of Astaxanthin

Astaxanthin plays an essential role in photoprotection and prevention of oxidative stress. *Haematococcus pluvialis*, the green alga produces the highest amount of astaxanthin accumulation, attaining up to 4% of dry weight. It produces astaxanthin in response to environmental stress such, as high light intensity, salt stress, and nitrogen limitation [98]. Astaxanthin have been used as feed for farm-raised salmon and chickens as it supplements salmon and egg yolks their yellow or orange pigment. These supplements started out as a pigmentation additive, and now turned out to as a powerful antioxidant.

The ketocarotenoid astaxanthin (3, 3-dihydroxy-4, 4-diketo-carotene) derived from a central phytoene backbone of 40 carbon atoms linked by characteristic single and double bonds. Astaxanthin has a number of geometric (Z) isomers, optically active and also have three possible stereoisomers [99]. In nature, astaxanthin exist as conjugated proteins or esterified fatty acids [100]. Synthetic astaxanthin are algal-based and yeast-based [101]. Synthetic astaxanthin and their three significant natural sources (*Haematococcus pluvialis*, carotenogenic yeast *Xanthophyllomyces dendrorhous* and crustacean shells) are used widely as feed add-ons [101, 102].

10.4.4.1 Antioxidant Potential of Astaxanthin

Astaxanthin has been evidenced the strong antioxidant activity in various *in vitro* studies [103–105]. Astaxanthin is a potent quencher of singlet oxygen [106, 107], a functionally effective inhibitor of peroxyl radical-dependent lipid peroxidation [108–110]. Synthetic astaxanthin and *Haematococcus* algae extract are excellent scavengers of hydroxyl radicals and superoxide anions [104].

Antioxidant potential of astaxanthin has been examined in rodent models and cultured animal cells. Astaxanthin and several other carotenoids have shown inhibition of peroxyl radical-mediated lipid peroxidation in liposomal [110, 111]. Ahead of β-carotene and lutein, astaxanthin have shown better ability to protect rat kidney fibroblasts oxidative stress [112]. Antioxidant effects have been detected in vitamin E-deficient rats which are fed with astaxanthin-rich diet and have shown significant progress, which includes inhibition of peroxidation of erythrocyte membranes [113]. In another study, astaxanthin fed rats (control diet) treated with carbon tetrachloride have shown significant inhibition of lipid peroxidation in the serum and liver [105].

10.4.4.2 Potential Cancer Preventative Activity

Astaxanthin is an important constituent of the traditional diets of Eskimos and several coastal tribes in North America. Low cancer incidence has been attributed in these populations as their diet contains high levels of astaxanthin and it plays a

protective role in cancer chemoprevention among these people as well [114]. Regardless, the currently existing data on the potential of astaxanthin in cancer prevention is limited to *in vitro* cell culture studies and *in vivo* studies with rodent models.

In vitro Cell Culture Studies

Several investigations reported the possible preventive effects of astaxanthin on various types of cancers [115–118]. UVA radiation is the primary causative agent in skin tumour pathogenesis. Astaxanthin- rich algal extract conferred significant protection against UVA-induced DNA damage to melanocytes, human skin fibroblasts, and intestinal CaCo-2 cells [115]. Significant inhibition of androgen-induced proliferation of human prostate cancer cells has been demonstrated in the presence of astaxanthin [119, 120]. Astaxanthin have also shown inhibitory effect on murine mammary tumor cell proliferation by up to 40 %, in a dose-dependent manner [116]. Methylcholanthrene-induced (Meth-A) mouse tumor cells cultured in an astaxanthin supplemented medium have reportedly shown reduced cell numbers and lower DNA synthesis rates prior to 2 days post-incubation than control cultures [118]. In addition, of eight carotenoids tested, Astaxanthin is the most effective among the eight carotenoids, which are invariably inhibited the invasion of rat ascites hepato cellular carcinoma cells in culture [117]. Two human colon cancer cell lines treated with astaxanthin showed significantly reduction in viablility than control cultures, although a stronger effect has been reported from α-carotene, β-carotene or canthaxanthin [121].

In vivo Rodent Model Studies

Dietary astaxanthin inhibited the growth of transplanted Meth-A tumor cells in a dose-dependent manner in BALB/c mice [118]. Meth-A tumor cell growth has been inhibited when dietary astaxanthin was supplemented at first and third week prior to tumor inoculation, but no such inhibitory effect was evidenced when supplementation was begun at the same time as tumor inoculation [122]. These results clearly proposed that astaxanthin inhibit tumor development in the early stages of progression but not in the later stages [122]. In another study, astaxanthin supplementation significantly reduced transplanted mammary tumor growth and suppressed spontaneous liver carcinogenesis [123]. The accumulation of potential tumor promoting polyamines in the skin of vitamin A-deficient nude mice exposed to UVA and UVB irradiation has been inhibited by dietary astaxanthin [124].

A series of studies on cancer chemoprevention by natural and synthetic compounds revealed that several carotenoids, including astaxanthin, as an effective antitumor agents [115, 116, 117, 122, 125]. In earlier investigations, the possible preventive effects of astaxanthin and canthaxanthin was found to significantly reduced the proliferation of on N-butyl-N(4-hydroxybutyl)nitrosamine (OH-BBN)—induced mouse urinary bladder carcinogenesis. In two other related studies, 4-NQO-induced

rat oral carcinogenesis and azoxymethane (AOM)-induced rat colon carcinogenesis, cell proliferations were significantly reduced in astaxanthin-supplemented rats relative to control rats [126, 127]. Astaxanthin, canthaxanthin and β-carotene reduced the number of DNA single-strand breaks and the number and size of liver pre-neoplastic cell lesions induced in rats by aflatoxin B1 [128].

Even though many studies reported the potent anti-carcinogenic effects of astaxanthin in *in vivo*, a few reports have extended less compelling results. Similarly, activation of pim-1 gene expression (involved in regulation cell differentiation and apoptosis) was stimulated in lutein-fed mice but not in astaxanthin-fed mice [63]. In an *in vivo* study, dietary astaxanthin has reportedly shown negative results; dietary supplementation with either β-carotene or astaxanthin aggravated carcinogenic expression in the skin of nude mice after UV irradiation [129].

10.5 Conclusion

Chemopreventive and anticancer activity of various seaweed carotenoids have been described in many epidemiological studies. From the better scientific understanding the mechanisms of action of seaweed carotenoids and strategy to reduce the risk of cancer incidence and mortality rate, it would include increased consumption of marine product and seaweeds as a part of a healthy, balanced diet. Hence, seaweed carotenoids found many pharmacological importances in a wide range of pharmaceutical industry, because of the reason for which carotenoids enriched seaweeds are suddenly becoming an attractive source for cancer drug discovery in scientific community. In addition, the molecular mechanism, action and interaction of the anticancer seaweed carotenoids have to be determined through further research.

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Chapter 11
Amelioration Effect of a Tertiary Sulfonium Compound, Dimethylsulfoniopropionate, in Green Sea Algae on Ehrlich Ascitic-tumor, Solid Tumor and Related Diseases

Kenji Nakajima

Abstract A tertiary sulfonium compound, dimethylsulfoniopropionate (DMSP), has been studied intensively in bacteria, plankton, algae, and halophytic plants in estuarine, coastal, and oceanic waters. Moreover, DMSP is well known to be contained in large amounts in green sea algae. However, there is no report on the physiological roles except for the functions of an osmoregurant and a cryoprotectant. Therefore, we have investigated the physiological function of DMSP in the terrestrial animals suffered from inflammatory disorders, especially free cell and solid cancers. As a result, we have found that DMSP exerts significant healing effects on a wide range of immune-deficient diseases: cancer, stress-induced gastric ulcers, various symptoms with aging in senescence-accelerated mice and neurodegenerative disorders in central nervous systems (Alzheimer's and Parkinson's diseases) in rodents. Of great interest is that administration of DMSP cures chronic 3'-methyl-4-dimethylaminoazobenzene (MeDAB)-induced solid cancers in rats and crucial free cell cancers of Ehrlich ascites carcinoma with an unavoidable rapid death in mice with no toxicity. These results demonstrate that DMSP plays a pivotal role for immunotherapy in these inflammatory disorders at the precancer state, in particular cancers.

Keywords Anti-cancer effects · Dimethylsulfoniopropionate · Ehrlich ascites carcinoma · Solid cancer · Aging · Ulcers · Neurogenesis

11.1 Introduction

Cellular senescence is exposed to nonlethal intrinsic or extrinsic stress that results in persistent growth arrest with a distinct morphological and biochemical phenotype. The engagement of senescence may represent a key component for therapeutic

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intervention in the eradication of cancer occurring with ageing. Normal cells in the body follow an orderly path of growth, division, and death. When this process breaks down, cancer begins to form. Unlikely, regular cells, cancer cells do not experience programmatic death and instead continue to grow and divide. This leads to a mass of abnormal cells that grow out of control. Accordingly, the cellular senescence in anti-cancer therapy may be particularly important in aging because of the age related-changes down-regulation at the level of both cancer suppressor genes and immune functions [1–3]. Accordingly, 65 year old- and further older-people bear higher risk suffering from cancer compared to younger peoples [4]. Cancer has remained a major cause of death and the number of individuals with various cancers and has continued to expand over a long period. Furthermore, cancer in brain elicits neural retardation and destruction of central nervous systems, that is, destroy a principle character of humans. Since several decades, a number of chemical anticancer drugs have been developed but they prove to give serious side effects to patients at present [5–7]. In contrast, chemoprevention for cancers of products and purified compounds originating from plants, especially herbal plants, has been recently found in abundant numbers [5–7]. However, naturally-occurring compound with the potent ant-cancer effect without side effect has not been detected [5–7]. In this chapter, I report amelioration effect of DMSP in green sea algae on stress and aging closely related to cancer, solid and free cell cancer, and neural degeneration caused by brain cancer with model animals of Alzheimer' and Parkinson' s diseases.

11.2 Materials and Methods

Dimethylsulfonioacetate (DMSA) and dimethylsulfoniopropionate (DMSP) were synthesized by refluxing dimethylsulfide and 2-bromoacetic acid or 3-bromopropionic acid and purified to 99.9 and 99.8 % purification (from the element analysis) by washing with ethyl ether and crystallizing from methanol [8]. The structures of DMSA and DMSP were identified by NMR, IR, elemental and mass analysis or X-ray or thermal-degradation. 2-Bromoacetate and 3-bromopropionate were purchased from Tokyo Kasei Chemicals Co. Ltd. 1-Amino-1-carboxy-3-dimethylsulfoniopropionate (MeMet chloride), dimethylsulfide (DMS), 3′-amino-4-dimethylamonoazobenzen (MeDAB), vitamin E (α-tocopherol) and ferulic acid, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-pyridinium chloride (MPP^+), and Wright, Türk and Giemsa solutions, were purchased from Wako Pure Chemicals Co. Ltd., Japan. MeMet was further washed with cold methanol and ethyl ether before use. All other chemicals used were of best available quality. The strains (in particular, R1 with slight aging-properties (progenitor of SAM P series), P1 with early aging, and P8 with deficit of learning and memory, and generally with immune deficit) of the male and female senescence-accelerated mice (SAM mice) [9–12] were kindly provided by Takeda Pharmaceutical Co., Ltd., Japan. Ehrlich ascites carcinoma (EAC) cells were kindly donated by Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University and maintained

in vivo in mice by weekly intraperitoneal (i.p.) transplantation. Pheochomocytoma (PC-12) cells were obtained from Dai-nippon Sumitomo Pharm, Co, Ltd., Japan. Nerve growth factor (NGF) (R & D Systems, Inc., USA), Polystyrene Petri dishes (3.5 ϕ cm) (Iwaki Div. Asahi Scitechno Glass, Co. Ltd., Japan) coated with collagen, RPMI 1640 culture medium (Sigma Co., Ltd., USA), horse serum and fetal bovine serum (Gibco Co., Ltd., USA) were used. The dry green sea algae (moisture content 15.8 ± 0.02 % (mean ± SD, n = 5)) was obtained from Gokasho-Bay in the Mie Prefecture in Japan and stored in a freezer (− 20 °C). Jcl-SD male rats and ICR/Jcl male and female mice were purchased from the Oriental Yeast Co., Ltd., Japan. C57BL6 and SD-Wister male mice were purchased from CLEA Co., Ltd., Japan. All the experiments with rats and mice had free access to distilled water and solid diets (MF, Oriental Yeast Co. Ltd. Japan) for one week (for acclimation). Moreover, housing of all the test animals were reared at a temperature of 22 ± 2.0 °C and relative humidity of 60 ± 3.0 %, and at dark and light cycles (12/12 h) in the pathogen free rearing room, unless otherwise stated. Body weights of all test animals were measured at specified times during the experimental period.

11.2.1 Preparation of Stress-Induce Gastric Ulcer in Rats [13]

SD-Wister male rats at the age of 6 weeks had free access to the commercial solid diets and either the distilled water or one of the DMSP and the vitamin U (MeMet) solution (5 mM each). The test rats were provided by water inversion methods [13]. In brief, after 12 days, the rats were deprived of food and drink for 24 h. Each starved rat was tightly fixed in an aluminum frames and wire net cage and was then vertically immersed to the height of the rat's xiphoid in a water bath at 23 °C for 21 h in darkness. Ulcerous rats were cut open along a greater curvature and areas of ulcerous portions were calculated totaled. Ulcer indexes were expressed as the ratio of the sum of area of ulcerous parts of test rats against that of the control rats.

11.2.2 Preparation of Alloxan-Induced Diabetes Mellitus in Rats [14]

Thirty male SD-JCL male rats at the age of 6 weeks (body weight 113.6 ± 1.0 g (mean ± SD) were divided into 5 groups in which the distilled water was given to two groups, 2.5 mM DMSP solution to one group, and 5 mM DMSP solution to two groups (each group 6 rats). All the rats had free access to commercial diets during the experimental period. After 10 days, all the rats were starved with no drink and food for one day. On the next day, the alloxan saline solution (mg/10 g body weight) were i.p.-injected into the rats in the distilled water group, the 2.5 mM DMSP solution group and in rats of one of the 5 mM DMSP solution groups for preparation of the acute diabetes rats.

11.2.3 Estimation of Total Grading Score [10, 15]

Each grading score of the aging mice was counted based on the individual senescence scores (numbered for each item of reactivity, passivity, glossiness, coarseness, loss of hair (further erosion and ulcers of skin), periophthalmic lesion, cornea opacity, cornea ulcers, cataract and lordokyphosis) from 1 (best) to 5 or 6 (worst) and totaled, then expressed in terms of the total grading score.

11.2.4 Estimation of Loss of Learning and Memory [15, 16]

Loss of learning and memory with aging was estimated by the step-through experiment (passive avoidance test) with the equipment (DC-6V 300 mA, Sony AC Adapter AC-9) [16], in which the test mouse is electrically stimulated just when the mouse moves in small distance from the copper mesh plate in the remaining light compartment into the same plate in the neighboring dark compartment. In the experiments, the five mice were simultaneously measured by the modified methods. The time (s) of the test mouse remaining in the light compartment after removing the wall between the light and the dark compartments is expressed as the criterion for assessing the learning and memory. Before the test, preliminary experiments were performed once a day for three days.

11.2.5 Representative Figures of Skin Ulcers in SAM P1 Mice [16]

SAM mice are accompanied by various aging symptoms compared to those in normal mice. Representative figures in male and female SAMP 1 mouse suffered from ulcer under the necks and legs were taken. Ulcerous mice were individually detected and thereafter given with 5×10^{-4} M DMSP solution until their complete healing. Ulcerous portions were individually detected and their area was calculated to assess amelioration of diseased mice.

11.2.6 Treatment of Aged SAM P8 Mice with DMSP, Vitamin E and Ferulic Acid [17]

Twenty aged SAM P8 mice at the age of 50 weeks were divided into four groups (five mice each). One milliliter of the saline solution (control), 2% DMSP, vitamin E or ferulic acid solution dissolved or miscible in saline solution was employed as the test solutions. The solutions were heated to body temperature and directly added to the stomachs of the test mice twice a week using a sterilized zonde (ϕ 0.5 mm×8 cm) and a syringe (1-ml volume), except that 0.02 g of oily authentic

vitamin E solution and then 0.98 ml of saline solution were injected for the addition of the vitamin E solution.

11.2.7 Long Term-Treatment of SAM R1 and P8 Mice with DMSP [18]

Ten male and female SAM R1 and P8 mice to the age of 4 weeks were fed distilled water (control) and either one of the 0.25 or 0.5 mM DMSP solution for a long time, respectively. Effect of oral administration of DMSP solutions on growth, total grading score, loss of memory, and survival rate were examined for up to about 72 weeks.

11.2.8 Treatment of SAM P8 Mice with Powdery Dry M Nitidum [19]

SAM P8 mice control at the age of 43 weeks were divided into two groups, one was fed the pulverized diets and the other was fed the test diets with algal powder at 5 % (w/w) in the control diets. The diets and drinking solution were freely fed and freshly changed. The body weights and loss of leaning and memory were measured and counted for about 30 days.

11.2.9 Preparation of MeDAB-Induced Liver Cancers [20]

Jcl-SD male rats at the age of 4 weeks beard free access to the distilled water and 10 and 20 mM DMSP solution and the control and test powdery diets containing 0.06 % MeDAB (w/w). The solutions and diets were given singly or in combination in the control and test solution and the control and test diets groups during the experimental period, respectively. The body weights were measured at specified times for up to 28 weeks. After 30 weeks, the serum was taken by cutting the tail ends of the rats with the MeDAB-induced liver cancers with and without DMSP at specified times and γ-glutamyltranspeptidase (γ-GTP) [E.C.2.3.2.1] activity in serum was automatically measured with a Spotchem (SP-4410, Kyoto Daiichi Science Co., Ltd.) and test paper (Kyoto Daiich Science) for determination of γ-GTP activity).

11.2.10 Estimation of Delayed-Type Hypersensitive Immune (DTH) Reaction on Ear Skins and Footpad

Jcl-SD male rats treated with MeDAB at the age of 30 weeks were anesthetized with 0.2 ml of pentobarbital (50 mg/ml) (Dainihon Seiyaku Co., Ltd., Japan). An

aliquat (0.05 ml) of the phytohem–agglutinin (PHA) (Japan Wellcome Co., Ltd., Japan) solution (10 μg/ml) was injected into right inner skins in rat ears of rats, and the color of ear skin portions was measured with a Color Measuring System (Σ80, Nippon Denshoku Ind., Co., Ltd., Japan) through a square hole (5 × 5 mm) of the black thick paper 24 h after the injection [20]. Furthermore, to examine DTH immune activity on footpad, 18 Jcl-SD male mice at the age of 4 weeks were divided into three groups (n = 6) and subjected to the injection of the saline solution, 10 and 20 mM DMSP solutions and EAC cells at the same time in the same way as mentioned below. After 20 weeks, the mixture (0.2 ml) of equal volume of bovine serum albumin saline solution (10 mg/ml) and Freuind incomplete adjuvant solution were subcutaneously injected into the center of dorsal portions in three group mice. After 3 weeks, the saline solution (3.5 ml, pH 6.3) containing bovine serum albumin (20 mg) was freshly mixed with 1 ml of 10% aluminum sulfate (K^+) and centrifuged, which were repeated three times. The mixture (0.02 ml) of thus treated bovine serum albumin (0.5 mg/ml) was injected into the skin of rear foot arch in test mice. After 24 h, the vertical height from the foot instep to foot arch was measured by the slide- and outside-calipers (5 times each) and the swelling in the foot arch was expressed as the ratio (%) (mean ± SD, n = 10) of the height of the swelling portion versus the initial height to the foot arch from foot instep [21].

11.2.11 Treatment of EAC Bearing-Mice with Preliminary Administration of DMSP [21]

Four-week old ICR/Jcl male mice were divided into 5 groups (8 animals in each group). Thereafter, the saline solution, 5, 10 and 20 mM DMSP solutions (1 ml in each) were i.p.-injected every other day into two control and three DMSP group mice for two weeks. Then, 0.5 ml mixture (5×10^5 cells/ml) of EAC cells which were washed twice with and suspended in Ham's 12 F medium were i.p.-injected into one control group (Carcinoma group) mice and DMSP group (DMSP-Carcinoma group) mice, respectively, except for another control group (Control group) mice. Thereafter, the body weight and number of alive mice in all the groups were measured and counted with increasing rearing time up to 300 d after the injection of EAC cell suspension. Moreover, incubation of 10, 20, and 30 mM DMSP solution with EAC cells was performed on a Harm's medium for up to 5 h as follows. EAC cells freshly taken from the ascites fluid in EAC-bearing mice were suspended in Ham's medium containing 8% fetal bovine serum and centrifuged. The washed EAC cells were suspended in the Ham's medium containing DMSP at indicated concentrations (2.5×10^4 cells/ml) and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 5 h in a closed and sterilized box (Ch-16, Hitachi Co., Ltd., Japan). One ml was drawn from the incubation mixture at indicated times. Then, dead cells were counted in a Neubauer haematocytometer using trypan blue dye and expressed in terms of the rate (%) (mean ± SD, n = 5) of dead cells versus all the EAC cells in each group.

11.2.12 Treatment of EAC Bearing-Mice with DMSP, DMSA, and MeMet [22]

Sixty four ICR/Jcl male mice at the age of 4 weeks were divided into 8 groups (8 animals in each group). Then, 0.5 ml of the saline solution and the MeMet, DMSA or DMSP solution at 10 mM were i.p.-supplemented singly into two mice each (two series of four test solution groups). Thereafter, an appropriate volume of ascites fluid containing EAC cells was withdrawn from EAC-bearing mice, centrifuged, suspended in Ham's 12F medium and centrifuged. The EAC cells were suspended in the medium containing 8% fetal bovine serum. The suspension (0.5 ml) of EAC cells (2×10^6 cells) was further i.p.-supplemented to the four group mice treated with the saline, MeMet, DMSA, and DMSP solution which were mentioned above. The test groups thus consisted of eight groups of the Control, DMSA, DMSP, MeMet, Carcinoma-Control, -DMSA, -DMSP or -MeMet group in total and were then subjected to raising for 10 days. After the test solutions was injected singly or in combination with EAC cells, the body weights in all the group mice were measured at specified times up to 10 days. The ascites fluid in the peritoneal cavity was carefully collected into plastic tubes first from the hole opened with a syringe on abdominal site and then from the opened abdominal cavity by a scissors under deep ether anesthesia on the 10th day, then kept in an ice-cold bath. Then, the blood was simultaneously drawn from the portal vein by the heparinized syringe and the hematological experiments with the fresh blood were immediately performed as follow. In brief, to estimate the red blood cells (RBCs), the blood and saline solution were absorbed and enough shaken in the melangeur for the RBC estimation. The numbers of RBC were counted onto the Neubauer haematocytometer under magnification (× 100–400). For the determination of white blood cells (WBCs), the blood was shaken with Türk solution in the melangeur. The numbers of WBCs were counted in the same way as mentioned above. For the preparation of blood smear, an appropriate amount (about 0.05 ml) of blood was equally spread in a layer on the slide glass and dried in an air. The slide glass was fixed by immersion into the methylalcohol. For the dyeing with the Wright solution, 10–15 drops of the Wright solution were put on the slide glass with the thin-laid blood and kept for 2 min. The residual solution was removed and several drops of phosphate buffer (pH 6.4) were put on the surface on the slide glass, which was then submitted to washing with distilled water and drying. For the dyeing with Giemsa solution, about 3 ml of the Giemsa solution (3–3.5%) were dropped on the slide glass stained by Wright dyes and kept for 10–20 m. Then, the glass was washed with distilled water and dried. Moreover, the glass was immersed into xylene solution and mounted. The RBCs, WBCs and differential WBCs (lymphocytes, neutrophils and monocytes) were carefully detected and their numbers were counted by the characteristic colors, size and shape of the cells, nucleus and cytoplasm after dyeing with a microscopic observation (× 100–400). Furthermore, incubation of DMSA, DMSP, MeMet with EAC cells was conducted as follows. The EAC cells obtained in the same way as mentioned above were suspended in the Ham's medium containing 8% fetal bovine serum (2.5×10^4 cells/ml) and incubated with the DMSA, DMSP or MeMet solution

at concentrations of 5 mM (in final) in a humidified atmosphere of 5 % CO_2 in air at 37 °C for 5 h. One milliliter was drawn from the incubation mixture at indicated times. Then, dead cells were counted in a Neubauer haematocytometer using trypan blue dye and expressed in terms of the ratios (%) (mean ± SD, $n = 5$) of the dead cells versus all the EAC cells in each group.

11.2.13 Preparation of MPTP-Induced Parkinson's Disease in Normal Mice [23]

Twelve C57BL6 male mice at 15 weeks of age were divided into two groups, one was fed the distilled water (the control group) and the other was fed the DMSP solution at 5×10^{-4} M (the DMSP group) for 2 weeks. Thereafter, the MPTP saline solution (0.5 ml) was i.p.-injected into all the test mice in the control and DMSP groups at the ratio of 20 mg/kg body wt as a daily dose from 5:00 p.m. for three consecutive days from the day before the start of the experiments. From the next day (the first determination day) after the first injection of MPTP, the determination of the moving ability (running power) of the mice was initiated at 10:00 a.m. at 0, 1, 2 and 5 days after the first MPTP injection (four times determinations), using a Wheel Running Instrument (15 ϕ cm) (motor: Kato Power Pack Standard Model No. 22–011) made in-house which electrically turns at the constant turning rate of 10 rpm and expressed in terms of the continuous turning numbers. The estimation of running number in the mice was begun at 2:00 p.m. in the same days. The running numbers and the duration time of immobility were determined 1, 2, 3 and 5 days after the initial MPTP injection and the values obtained are shown on the vertical axes at 0, 1, 2 and 5 day, respectively.

To estimate the amounts of catecholamines in the brains without the cerebellum of the mice, all the mice were subjected to decapitation 2 days after the last injection of MPTP, and their brains and then cerebellums were immediately removed under the cold (0 °C) conditions and stored in a freezer (−20 °C) before use. After thawing, an appropriate sample of the whole brain without cerebellum was suspended in a mixed solution (0.1 wet g/0.5 ml) containing ethylendiaminetetraacetate (EDTA-2Na) at 0.1 M in a 0.2 M perchloric acid solution, which was homogenized while cold (0 °C) using a Kinematic Polytron (PT10–35) and an Aggregate (PCU-11) (Kinematica Ag Littau (Switzerland)) for 30 s. The mixture was centrifuged at 4 °C and 26,000 rpm for 10 min using a Beckman T-100. The supernatant was filtered through a Chromatodisc (pore size 0.45 μm, Kurabou Co., Ltd., Japan). The filtrate (10 μl) was used for the analyses of the catecholamines by high performance liquid chromatography (LC-6A) using a Chromatopack (SCL-6A) (Shimadzu Co., Ltd., Japan) and an electric chemical detector (Shiseido Nanospace S1–2. Shiseido Co., Ltd., Japan) under the stated conditions (column; Type 120 (highly purified silica gel) (4.4 ϕ mm×15 cm), mobile solution; mixed solution containing 13.6 g KH_2PO_4, 0.15 g octanesulfonic acid-Na, 65 ml of 2 % H_3PO_4, 10 mg EDTA-2Na, 60 ml acetonitrile in 1 l of ultrapure water, flow rate; 0.3 ml/min, attenuation; 8, detection sensitivity; 700 mA).

11.2.14 Preparation of MPTP-Induced Parkinson's Disease in SAM P8 Mice [24]

Twenty SAM P8 mice at the age of 15 weeks were divided into two groups; one fed distilled water (the control group) and the other DMSP solution at 5×10^{-4} M (the DMSP group) for 2 weeks. Thereafter, the control and DMSP groups were divided into two groups. One group was further subjected to an intraperitoneal injection of MPTP saline solution (0.5 ml) at 20 mg/kg body wt as a daily dose for 3 consecutive days from the day before the start of the experiment and then at 10 mg/kg body wt only on the first day of every weeks after the 2nd to the 5th week in the control and DMSP groups with MPTP. The body weights of mice in the *a, b, c* and *d* groups at the start of the experiments were 28.1 ± 0.67 g (mean ± SD, $n = 5$). The frequency of immobility duration was determined using a polygraph (RMP-6008M, Nihon Koden Co., Ltd., Japan) under the stated determination conditions (high and low cut; 30 and 0.3 Hz, sensitivity; 0.5 and 2 mV, determination time; 6 min), in which the test mouse was placed upside down and the tail of the mouse was suspended by connecting it to the bottom edge of a vertical spiral wire tied to a horizontal thin-iron bar of a round sensor box (CP-2US10, Midori Precisions Co., Ltd., Japan) 37 cm from the floor in order to record the moving stimuli (newly developed tail suspension test). The duration of the immobility (total numbers/6 min) after the determination of the moving ability was calculated using a computer (Think Pad 2656–41J, IBM Co., Ltd., USA) and software (Bimutas II. Kissei Comtec. Co., Ltd., Japan). The determination of the catecholamines in the brain without cerebellum of the mice was conducted by high performance liquid chromatography and an electric chemical detector [23].

11.2.15 Estimation of Outgrowth of Neurites from PC 12 Cells Treated with DMSP and NGF [25]

The basal incubation medium contained the RPMI 1640 medium (85 ml), horse serum (10 ml), fetal bovine serum (5 ml), streptomycin (5 mg) and penicillin 5000 U (100 ml in total), which were gently mixed and filtered through a membrane filter (pore size 0.20 μm, Advantec Co., Ltd., Japan). In the experiments with NGF and/or DMSP, NGF and/or DMSP was dissolved into the basal medium without increasing the volume of the medium to the indicated concentrations and then filtered. The filtrate was subjected to the following experiments.

The freshly maintained PC-12 cells were initially incubated in the collagen-coated Petri dishes containing the basal medium in a closed and sterilized box (CH-16, Hitachi Co., Ltd., Japan) containing the specified concentration of 5% carbon dioxide at 37 °C for three days and then centrifuged at 800 rpm. The same experiments were repeated three times. Thereafter, the same experiments were performed two times using the Petri dishes without a collagen-coating. Whenever the incubation medium was mixed with the new basal medium (1:1, v/v), the cells adhering to the

dishes were mechanically dislodged from the dishes and mixed to raise the homogeneous population of the cells by aspiration and expulsion of the cell mixtures through a Pasteur pipet. For the trial, the final lower layer after the centrifugation was suspended in the new medium to prepare the cell mixtures (6×10^4 cells/6 ml), which was divided into three collagen-coated Petri dishes (2.0 ml each) for one test solution and incubated as a function of time at 37 °C for 4 days. All the dishes were surveyed and confirmed to have over 10 sights/dish (initially scoring about 5×10^2 cells/10 sights) using an inverted microscope (Model CK × 41, Olympus Co., Ltd., Japan). The number of cells, which have the neurites being the same as or longer than the diameter of the cells, was then counted at the indicated times for 4 days and expressed in terms of the ratio (%) (mean ± SD, $n = 3$) of the number of neurites-bearing cells versus that of the total cells at the indicated times. The appearance of the neurites was also confirmed by photographs. All the procedures were conducted in a room sterilized by ultraviolet light and not containing any other experimenters. The statistical analyses were performed using the ANOVA and Scheffe's tests.

11.2.16 Estimation of Outgrowth and Elongation of Neurites from PC Cells Treated with MPTP and Recovery by DMSP in the Presence of NGF [26]

The incubation medium and transplantation of the PC 12 cells were preliminarily repeated on the basal medium in collagen-coated or non-coated Petri dishes in an atmosphere containing specified concentration of 5% carbon dioxide. The treated cells were used to examine singly or in combination the effects of DMSP, MPTP and NGF on the outgrowth of neurites from PC 12 cells. The incubation medium initially consisted of the cell mixtures (2×10^{-4} cells/dish (2 ml)) (three collagen-coated dishes for one test solution) and were incubated at 37 °C for 5 days. All the dishes were surveyed, confirmed and counted under magnification (× 200 (400 for confirmation)) using an inverted microscope. The neurite-bearing cells were expressed as the cells having neurites which are the same as or longer than the diameter of the cells (25). The figures of neurite-bearing cells were taken under magnification (× 200) on the 3rd day. The statistical analyses were performed using ANOVA and Sheffe's test.

11.2.17 Care and Treatment of the Experimental Animals

Care and treatment of the experimental animals were performed in accordance with the National Institute of Health Guide (NIH Publication No. 80–23, 1996) and also with Koshien University Guide for the Care and treatment of Laboratory Animals. The area of ulcer portions in skin of SAM P1 mice and stomach in SAM pP8 mice caused by stresses were calculated carefully using Image J software (1.45 m, Scion Image, Set

Scale). Statistical analyses and two-dimensional structures of test compounds were performed using ANOVA and the Tukey–Kramer, Mann–Whitney U, Fisher's PLSD and Sheffe's tests and CSC ChemDraw (StatView-SAS Institute, Inc., version 5 and Cambridge Scientific Computing, Inc., version 3.1, software), respectively.

11.3 Results

11.3.1 Effect of DMSP on Stress-Induced Gastric Ulcer in Mice [13]

Effect of addition of 5 mM DMSP and MeMet solution to gastric ulcer in SD-Wister male rats caused by stresses was examined for 12 days. Relative ulcer index reduced markedly in the order of Control, MeMet and DMSP solutions on the 12th day. In particular, administration of DMSP solution reduced the ulcer index by about one third compared to Control solution on the 12th day (data not shown).

11.3.2 Alloxan-Induced Diabetes Mellitus (IDDM) in Rats [14]

Effect of oral administration of 0.5 mM DMSP solution on alloxan-induced diabetes mellitus (1-type diabetes mellitus) SD-JCL male rats at the age of 4-weeks were examined for up to 50 days. The growth of the control, and the 10, 20 mM DMSP solution group with and without alloxan normally increased with increasing incubation times showing no significant difference among them ($p < 0.05$, by Tukey–Kramer test). On the contrary, administration of 0.5 mM DMSP solution reduced the amounts of glucose in plasma by about 95% and those in urine by about 92% based on the mean amounts comparing to those in rats with liver cancer induced by alloxan for 50 days among them (data not shown).

11.3.3 Representative Figures of Aging (Ulcers) and Eradication Process in SAM P1 Mice [15, 16]

Representative figures indicate occurrence of ulcers under the necks and legs in male and female SAM P1 mice with aging and complete recovery of these symptoms following oral administration of 0.5 mM DMSP solution (Fig. 11.1). Moreover, results of amelioration of ulcers in SAM P1 male and female mice by administration of DMSP solution is shown in Fig. 11.2. The results demonstrate that administration of low concentration of DMSP completely heal all the ulcers for up to 36 days. Total grading score of Control, ferulic acid, vitamin E and DMSP in male and female SAM P8 mice in this order were significantly decreased for up to 30 days. Results are given in Fig. 11.3. In particular, supplementation of DMSP

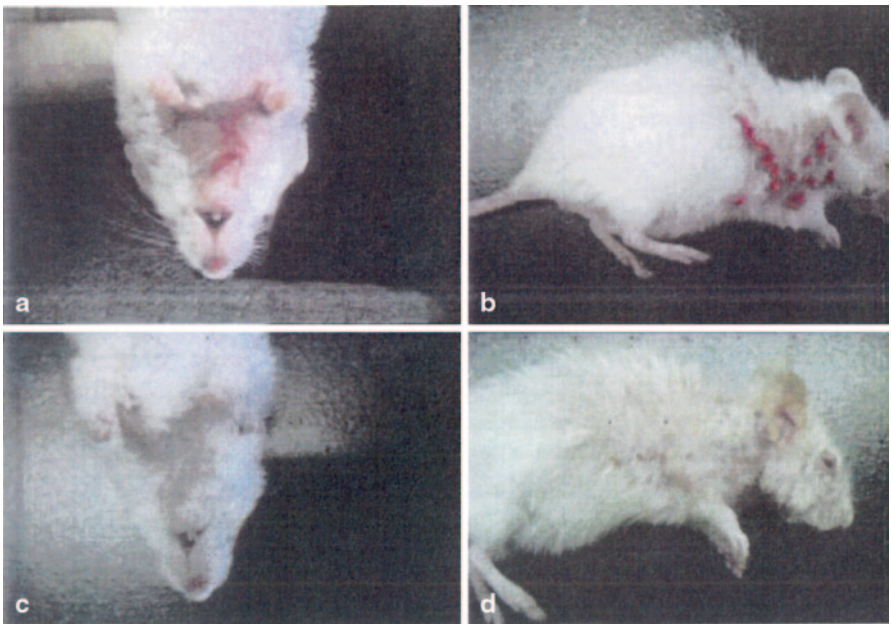

Fig. 11.1 Representative figures of male and female SAM P1 mice suffered from ulcer. **a**, **b** typical ulcerous mice. **c**, **d** ameliorated mice

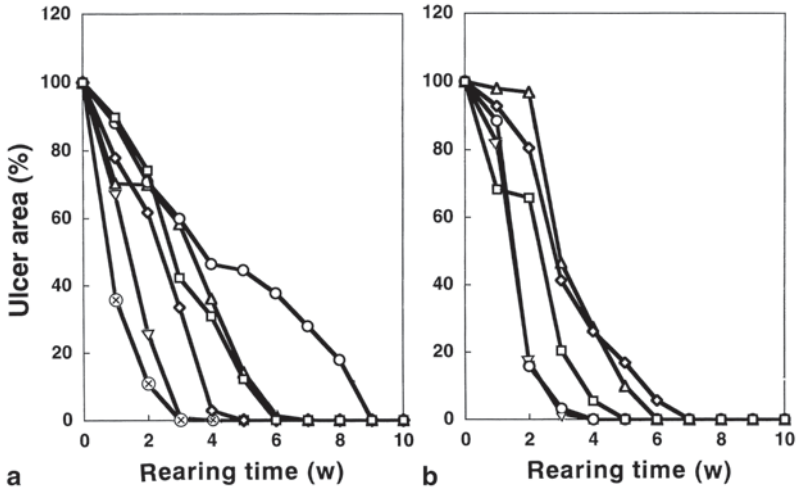

Fig. 11.2 Ulcers of SAMP1 male and female mice and their complete recovery. **a** □, ◇, ○, △, ▽, ⊕; ulcers under the necks and on the legs at 15-, 16-, 18-, 21-, 28-, and 42-week-old male SAM P1 mice. **b** □, ◇, ○, △, ▽; ulcers at 16-, 28-,52-, 58-, and 75-week-old female SAM P1 mice. Ulcerous SAM P1 mice were individually detected and thereafter recovered by administration of 0.5 mM DMSP solution

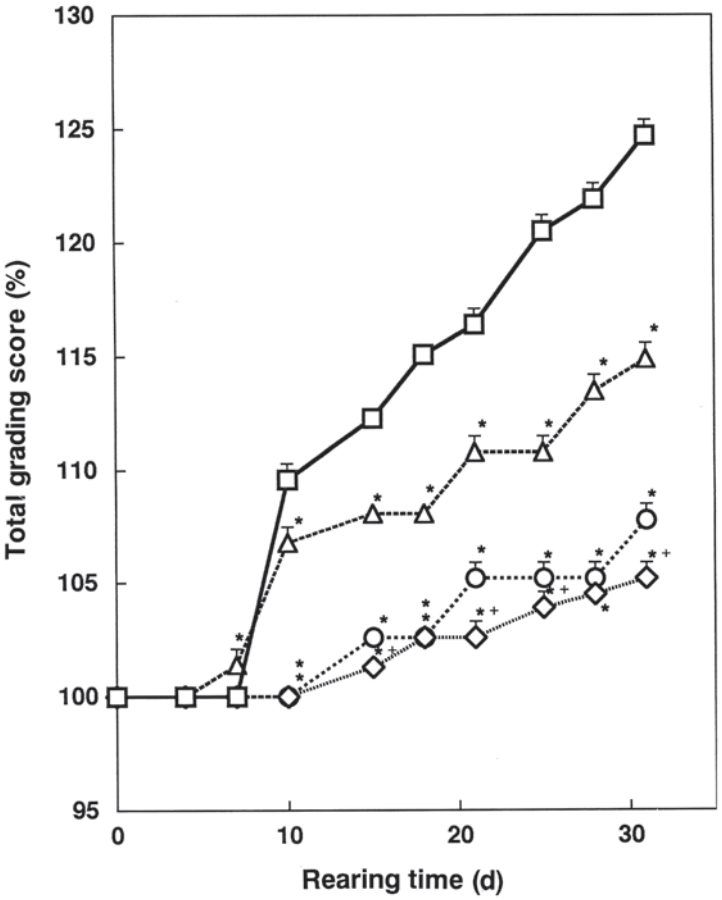

Fig. 11.3 Effect of DMSP, vitamin E and ferulic acid on the growth of aged male SAM P8 mice. Mean ± SD ($n = 5$) $^{*,+}p < 0.05$ (vs control at the indicated times, start values). □, Δ, ○, ◇: The control (distilled water), ferulic acid, vitamin E and DMSP. One ml of 2% DMSP, vitamin E or ferulic acid solutions was directly put twice a week into the stomachs of the aged male SAM P8 mice at the age of 4 weeks and reared for 28 days

remarkably restricted the values for up to 30 days. The loss of learning and memory of male SAM P8 mice given with these compounds, especially DMSP and vitamin E, was remarkably ameliorated comparing to that of Control for up to 28 days (data not shown).

11.3.4 Long Term Effects of DMSP on SAM R1 and P8 Mice [18]

Effect of 0.5 mM DMSP solution on growth, total grading score, loss of learning and memory, and survival rate of SAM R1 and P8 mice was examined from 4 to 68

weeks, from 16 to 72 weeks, from 24 to 64 or 96 weeks, and 0 to 96 weeks, respectively. Oral administration of 0.5 mM DMSP solution to male and female SAM R1 and P8 mice at the age of 4 weeks effectively recovered similarly their growth and significantly reduced total grading scores in both strains during the experimental period, accompanied by reduction of total grading score by about 27% in male SAMP8 mice and by about 31% in female SAM P8 mice compared to those in control SAM R1 and P8 mice without DMSP solution on the 72nd week. In contrast, the total grading score in male and female SAM R1 and P8 mice without DMSP were the same during experimental period without significant difference ($p < 0.05$, by Tukey–Kramer test). Moreover, effect of 0.5 mM DMSP solution on learning and memory of male SAM P8 mice was examined for up to 64 weeks. Learning and memory in male SAM R1 and P8 mice with and without DMSP decreased gradually for up to 64 weeks. However, the values of learning and memory of SAM R1 and P8 mice with DMSP left higher from 40 weeks to 64 weeks and from 32 to 64 weeks, respectively. In particular, those of SAM P8 mice with DMSP left high by about two fold those of SAM P8 mice without DMSP solution on the 48th week (data not shown). In contrast, mortality of male and female SAM R1 and SAM P8 mice gradually reduced with increasing rearing times. All the SAM P8 mice without DMSP solution died for up to 80 weeks. However, oral administration of 0.5 mM DMSP solution to the male and female SAM P8 mice maintained survival rates at the rates of 30% in the former strains and 60% in the latter strains from 80 weeks to 96 weeks (for at least 16 weeks), respectively (data not shown).

11.3.5 Effect of Green Sea Alga, M. nitidum, on Body Weight and Learning and Memory of SAM P 8 [19]

Male and female SAM P8 mice at the age of 43 weeks had free access to the distilled water and either the control powder diets or the algal diets containing powdery *M. nitidum* at 5% (w/w) for about one month. The growth of male and female SAM P8 mice given with the control diets linearly decreased whereas the growth of male and female SAM P8 mice with the algal diets linearly increased for up to 20 days at several percentages, respectively (data not shown). In contrast, loss of learning and memory in male and female SAM P8 mice with algal diets was fairly ameliorated as compared with that in the control mice for up to 28 days with significant difference ($p < 0.05$, by PLSD test) (data not shown).

11.3.6 Effect of DMSP on MeDAB-Induced Liver Cancer in Rats [20]

The effect of the 10 and 20 mM DMSP solutions on the growth of rats with cancerous liver induced by MeDAB was examined for up to 28 weeks. The growth in all groups rapidly increased up to 8 weeks and thereafter gradually increased

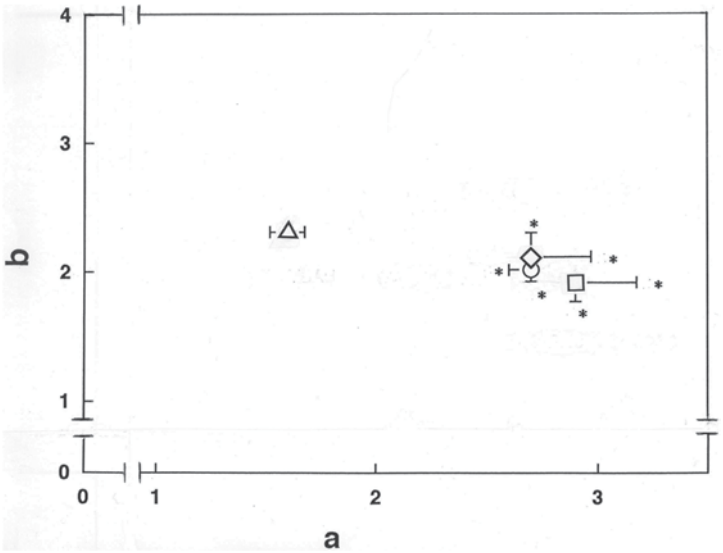

Fig. 11.4 Delayed-type hypersensitive immune reaction in the ears of four group rats. Mean ± SD ($n = 6$) * $p < 0.05$ (vs Carcinoma, toward yellow and red color). *a* red color, *b* yellow color (in LAB system). ○, Δ, □, ◊: Control, Carcinoma-Control, 10, 20 mM DMSP-Carcinoma groups. An appropriate volume of phytohemagglutinin solution was used as the antigen. The red turned color on the injection portion of the antigen was directly measured 24 h after the injection with a color difference instrument and expressed in terms of "Lab" systems

up to 28 weeks. However, the growth of all the groups with and without MeDAB diets almost unchanged for up to 28 weeks without significant difference ($p < 0.05$, Tukey–Kramer test) (data not shown). The effect of DMSP on DTH immune activity in rats with the MeDAB-induced liver cancer was examined in the ear skin of test rats with the PHA injection. Results are shown in Fig. 11.4. The rats in the control solution group with the control diets and the rats in the 10 and 20 mM DMSP solution groups with MeDAB-diets afforded almost the same red-yellow color, showing the occurrence of DTH immune reaction ($p < 0.05$, by Tukey–Kramer test).

11.3.7 Effect of Preliminary Administration of DMSP on EAC Bearing-Mice [21]

The effect of administration of DMSP solutions at the concentrations of 5, 10 and 20 mM on the body weight of EAC bearing-mice was examined with increasing rearing times up to 300 days. The results are given in Fig. 11.5. The body weight appeared to rapidly increase in the order of the mice in the Carcinoma, 5, 10 and 20 mM DMSP-Carcinoma group, especially in former two group mice, and in this order reached maxima on the 40th, 44th, 44th and 48th day. Thereafter, the body weight of other group mice rapidly decreased except for the Control and 5 mM DM-

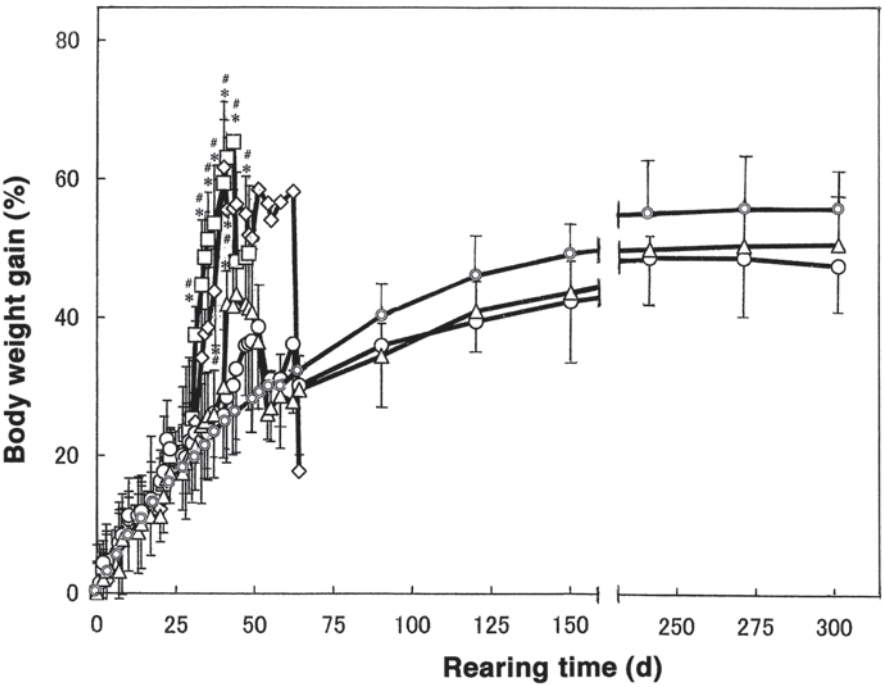

Fig. 11.5 Effect of preliminary administration of DMSP on the body weights of EAC bearing-mice. Mean ± SD ($n = 8$~4). $^{*, \#}$ $p < 0.05$ (vs Control, 20 mM DMSP-Carcinoma group at indicated times). ⊙, □, ◇, ○, △: Control, Carcinoma, 5, 10, 20 mM DMSP-Carcinoma groups. The body weights at the start of the experiments were 34.5 ± 0.21 g. Saline and DMSP solutions at 5, 10 and 20 mM were preliminarilly i.p.-injected every second day to five group mice for 2 week. Thereafter, EAC cell suspension was peritoneally injected into the mice in one control group (Carcinoma group) and DMSP-groups (DMSP-Carcinoma groups) but not another control group (Control group)

SP-Carcinoma group mice. However, the decline of body weights in the Carcinoma group mice ceased on the 47th day and that in the 10 and 20 mM DMSP-Carcinoma group mice simultaneously stayed on the 54th day but not the Control and 5 mM DMSP-Carcinoma group mice. Thereafter, the 10 and 20 mM DMSP-Carcinoma group mice exhibited almost the same growth curve as that in the Control mice in DMSP-Carcinoma and continued to 300 days (63 % survival rate) (Fig. 11.6). The effect of 5 and 10 mM on the DTH immune reaction in EAC bearing-mice were examined by the footpad test. The results are given in Table 11.1. The preliminary administration of saline and 5 and 10 mM DMSP solutions, especially the last solution, proved to activate the DTH immune system of the diseased mice. Moreover, the effect of DMSP at the concentrations of 5 to 30 mM on EAC cells was examined on the synthetic medium for 5 h. The results are given in Table 11.2, which indicated that the dead cells reduce in the dose-dependent manner of DMSP among these groups with significant difference ($p < 0.05$, Tukey–Kramer test).

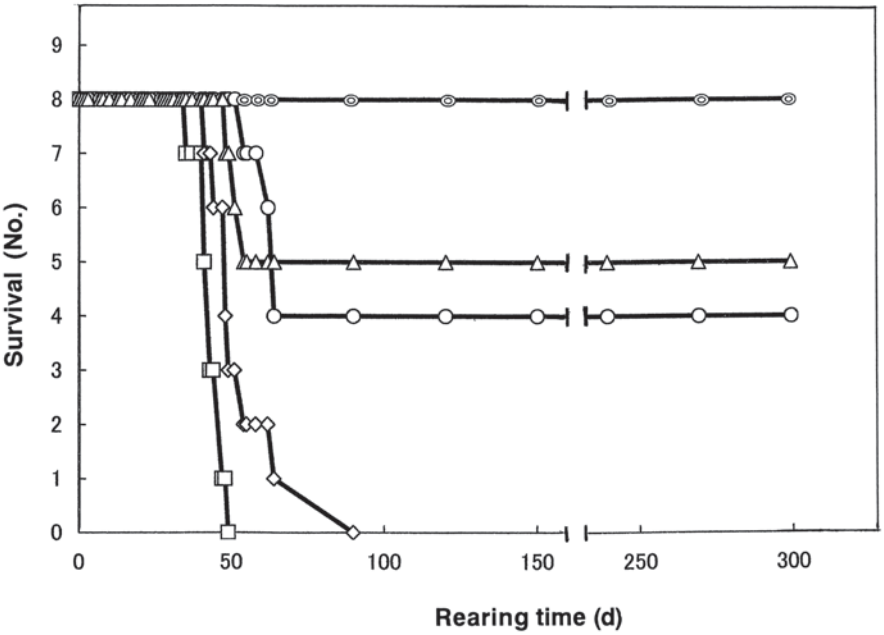

Fig. 11.6 Effect of DMSP on the survival time of EAC bearing-mice. ⊙, □, ◊, ○, Δ: Control, Carcinoma, 5, 10, 20 mM DMSP-Carcinoma groups. The experimental conditions were the same as those in Fig. 11.5. The values were expressed in terms of the number of alive mice at indicated times

Table 11.1 Effect of 5 and 10 mM DMSP solution on the delayed-type hypersensitive immune reaction of EAC bearing-mice

Group	Delayed-type hypersensitivity
	Swelling ratio (%)
Control	0.11 ± 0.12
DMSP (5 mM)	0.52 ± 0.23*
(10 mM)	2.49 ± 0.45**

Mean ± SD of six mice ($n = 10$, each)
*$p < 0.05$ vs control; **$p < 0.05$ vs 5 mM DMSP group

11.3.8 Effect of DMSP, DMSA, and MeMet Solutions on EAC-bearing mice [22]

An aliquot (0.5 ml) of the saline solution and the 10 mM MeMet, DMSA, DMSP solutions were i.p.-supplemented singly or in combination with EAC cells (5×10^5 cells/ml) into two mice each (two series of four test-solution groups), as mentioned in detail in Methods. Dimensional structures of DMSA, DMSP, and MeMet and their effects on the body weights of EAC bearing-mice were given in Fig. 11.7a and b. The body weights of all the mice increased with the increasing rearing-times up to 10 days to a varied extent. Especially, the body weights of the Carcinoma-MeMet group very rapidly increased after 6 days and thereafter maintained a plateau region

Table 11.2 Effect of several concentrations of DMSP on EAC cells on the synthetic culture medium

Incubation time (h)				
Group				
	0	1	3	5
Dead cells (%)				
Control	0	2.04 ± 0.52	4.26 ± 0.43	5.31 ± 0.52
DMSP (5 mM)	0	0.78 ± 0.35*	1.65 ± 0.44*	1.74 ± 0.52*
(10 mM)	0	0.58 ± 0.28*	1.31 ± 0.21*	1.45 ± 0.42*
(20 mM)	0	0.35 ± 0.26*	1.13 ± 0.30*	1.13 ± 0.61*
(30 mM)	0	0.34 ± 0.19*	0.45 ± 0.32#	0.48 ± 0.43***

Means ± SD ($n = 5$)
*$p < 0.05$ vs the control at indicated times; **$p < 0.05$ vs 5 mM DMSP groups at the indicated times; ***$p < 0.05$ vs 10 mM DMSP groups at the indicated times; # $p < 20$ mM DMSP groups at the indicated times

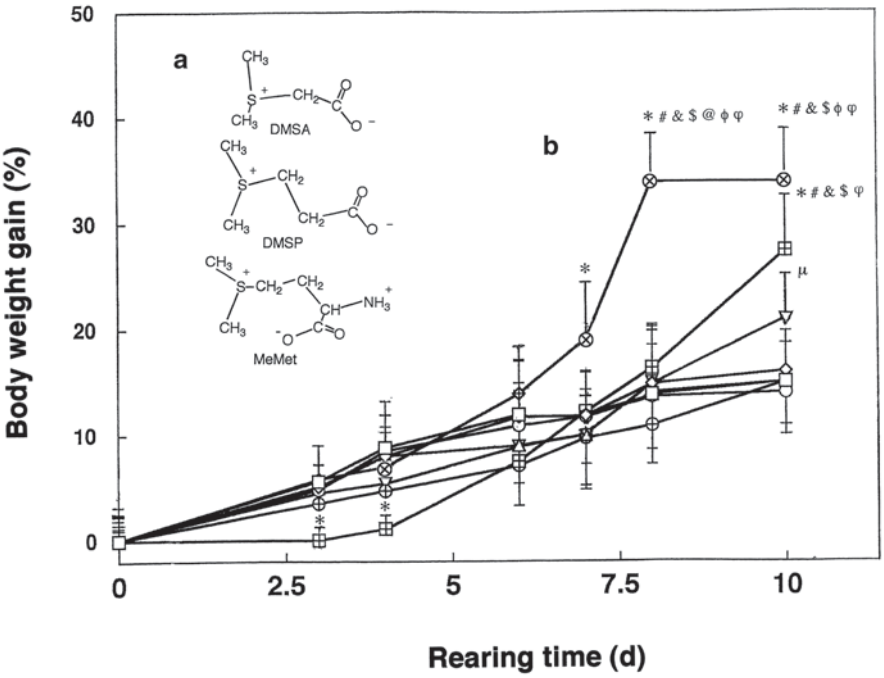

Fig. 11.7 Effect of MeMet, DMSA, and DMSP on the body weights of EAC bearing-mice. Means ± SD (n-8). *, #, &, $, @, φ, ϕ, μ $p < 0.05$ (vs control, DMSA, DMSP, MeMet, Carcinoma-Control, -DMSA, -DMSP, and -MeMet, groups at indicated times). □, ○, Δ, ◊, ⊠, ⊕, ∇, ⊗: Control, DMSA, DMSP, MeMet, Carcinoma-control, -DMSA, -DMSP and -MeMet. The solutions at the concentration of 10 mM were i.p.-injected singly or in combination with EAC cells to eight group mice ($n = 8$ in each group). Thereafter, the body weights were determined at indicated times up to 10 days

Table 11.3 Effect of DMSA, DMSP, and MeMet on the hematological parameters of EAC bearing-mice on the 10th day

Group	Asites fluid (ml)	RBC ($\times 10^9$ cells/μl)	WBC ($\times 10^6$ cells/μl)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
Control		6.4±0.12	4.0±0.10	79.8±2.2	18.2±2.1	1.8±0.03
MeMet		6.2±0.11	4.1±0.11	79.7±2.3	18.3±2.2	1.8±0.05
DMSA		6.3±0.14	3.9±0.07	79.9±2.2	18.1±2.4	1.8±0.06
DMSP		6.0±0.13	4.0±0.04	79.9±2.2	18.1±1.4	1.8±0.04
Carcinoma-control	6.0±0.3	3.9±0.16*	22.3±8.16*	40.7±4.1*	58.3±3.1*	1.3±0.06*
Carcinoma-MeMet	5.7±0.7	4.2±0.13*	9.1±0.96**	53.6±6.5**	45.4±6.5*	1.3±0.05*
Carcinoma-DMSA	1.9±0.8**	5.7±0.12***	5.4±0.72***	80.3±3.5**	18.1±3.4**	1.8±0.04**
Carcinoma-DMSP	2.3±0.7**	5.9±0.13***	5.8±1.20***	79.0±4.5**	19.0±3.5**	1.8±0.03**

Mean ± SD ($n=8$)
RBCs: red blood cells, WBCs: white blood cells, negligible
*$p<0.0$ vs control groups; **$p<0.0$ vs DMSA groups; ***$p<0.0$ MeMet groups

from 8 to 10 days. The Carcinoma-Control group tended to show the lowest values up to 6 days but then rapidly continued to increase up to 10 days. The body weights of the Carcinoma-DMSA and -DMSP group gave almost the same values as those in the Control, MeMet, DMSA and DMSP group without significant differences among them ($p<0.05$, Tukey–Kramer test). Especially, the body weights of the Carcinoma-DMSA group showed the favorable values, significantly distinguishing from those in the Carcinoma-Control and -MeMet group. The effects of MeMet, DMSA and DMSP on the accumulation of ascites fluid, the amounts of RBC and WBC and the ratio (%) of lymphocyte, neutrophil and monocyte of EAC bearing-mice were examined on the 10th day. The results are given in Table 11.3. The volume of the ascitic fluid in the Control, MeMet, DMSA and DMSP group was negligible. However, the volume of the fluid in the Carcinoma-Control and -MeMet group were largest among all the groups. In contrast, the volume of the fluid in the Carcinoma-DMSA and -DMSP group nearly approached to one-third of the volume in the Carcinoma-Control and -MeMet group on the 10th day. However, all other hematological values in the Carcinoma-DMSA and-DMSP group showed almost the same values as those in the Control group. The amounts of RBC in the Carcinoma-Control and -MeMet group were lowest among all the groups. The amounts of WBC in the Carcinoma-Control and -MeMet group were much higher and fairly higher than those in the Control group, respectively. The values of lymphocyte in the Carcinoma-Control and -MeMet group were lowest and lower when compared to those in the Control group, respectively. The values of neutrophil in the Carcinoma-Control group were much higher than those of the Control group, which were followed by the Carcinoma-MeMet group. The values of monocyte in the Carcinoma-Control and-MeMet group were lowest among these groups. The body weights, the volume of ascites fluid and hematological values in the Control, MeMet, DMSA and DMSP group exhibited the same values during the experimental period, respectively.

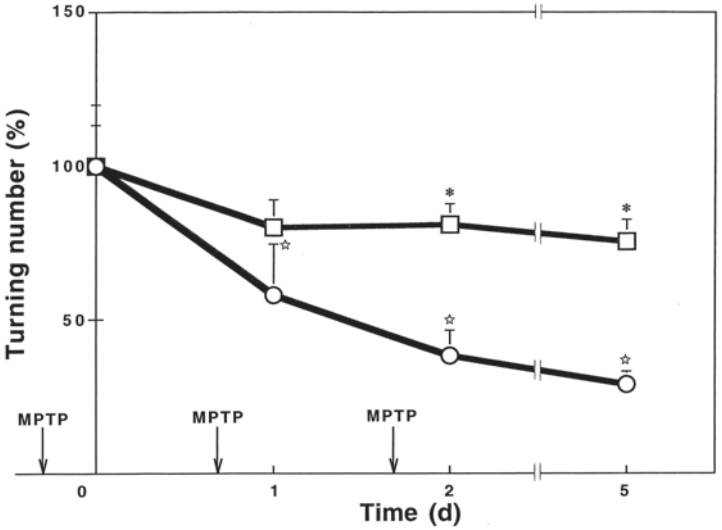

Fig. 11.8 The moving ability of the mice with or without DMSP after the injection of MPTP. Mean ± SD ($n = 6$). *,* $p < 0.05$ (vs control at incdicated times, the corresponding start values). ○, □: the moving ability in the control and DMSP groups with MPTP. The DMSP solution at 5×10^{-4} M and the distilled solution were given *ad libitum* to two groups of test mice for two weeks. An appropriate amount of the MPTP solution was i.p.-injected into all the mice once a day initially for 3 days. The moving ability (running power) of the mice in both groups was determined by a Wheel Running Instrument and expressed in terms of turning numbers. The values at the start of the experiments were 7.6 ± 1.51 and 10.5 ± 1.41 of the control and DMSP groups

The EAC cells obtained in the same way as mentioned above were suspended in the Ham's medium containing 8% fetal bovine serum (2.5×10^4 cells/ml) and incubated with the DMSA, DMSP, and MeMet solutions at a concentration of 5 mM (final) under a humidified atmosphere of 5% CO_2 in air at 37 °C on the synthetic medium for 5 h. One milliliter was drawn from the incubation mixture at indicated times. Then, dead cells were counted in a Neubauer haematocytometer using trypan blue dye and expressed in terms of the ratios (%) (mean ± SD, $n = 5$) of the dead cells versus all the EAC cells in each group. The ratios of dead cells in all the groups increased with increasing incubation times up to 5 h but the ratios at the specified times decreased in the order of the Control, MeMet and DMSA, and DMSP, respectively (data not shown).

11.3.9 Effect of DMSP on MPTP-induced Parkinson's Disease in Normal Mice [23]

The moving ability (running power) of the mice in the control and DMSP groups was estimated after the injection of MPTP. Results are shown in Fig. 11.8. The moving ability of the mice in the DMSP group with MPTP was evidently stronger than that in the control group with MPTP and showed normal levels during the

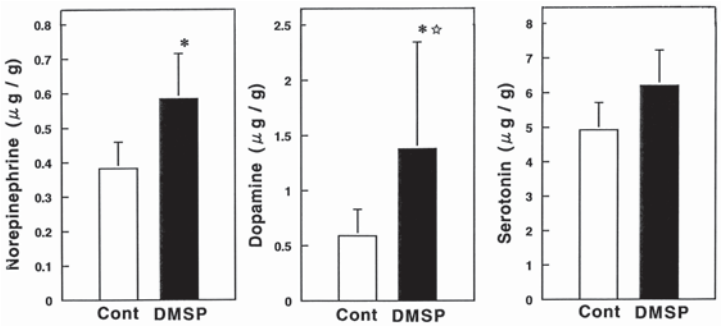

Fig. 11.9 The amounts of norepinephrine, dopamine and serotonin in the brains of the mice in the control and DMSP groups were estimated after the last MPTP injection. Mean ± SD (n=6), *,* $p < 0.05$ (vs control significant differences; U-test of Mann-Whitney or ANOVA and Fisher's PLSD-test). The experimental conditions are the same as shown in Fig. 11.8

experimental period ($p < 0.05$, PLSD and Tukey–Kramer tests) The statistical analyses indicated that the mice with DMSP and MTPT have fairly higher values comparing to the mice with MTPT.

The amounts of catecholamines (dopamine, norepinephrine, serotonin) in the brains of the mice in the control and DMSP groups were examined 3 days after the last injection of MPTP. These results are shown in Fig. 11.9. The amounts of dopamine and norepinephrine, especially dopamine, in the DMSP group mice were clearly higher than those in the control group mice, showing more than 2 fold those of the mice in the control group.

11.3.10 Effects of DMSP on MPTP-Induced Parkinson' Disease in SAM P8 Mice [24]

The immobility and tremor duration of the mice was determined by the newly developed tail suspension test and expressed in terms of the frequency (total numbers/6 min) (mean ± SD, n = 5) of immobility and tremor duration. The body weights of mice in the a, b, c and d groups at the start of the experiments were 28.1 ± 0.67 (means ± SD, n = 5) g, respectively. The frequency of immobility duration and of tremor duration in all the groups was estimated at the start, the 1st and 2nd and the start, the 4th and 5th week, respectively. The results of frequency of immobility duration are shown in Fig. 11.10. The frequency of immobility- and tremor-duration (data not shown) in all the test groups was much the same at the start of the experiments, respectively. Thereafter, the frequency of immobility and tremor duration in all the groups exhibited almost the same trends. The frequency of immobility and tremor duration in the *b* group proved to be much greater than that in the *a* group, but the supplementation of DMSP decreased the frequency of immobility and tremor duration in the *b* group by that in the *d* group at indicated times. However, the frequency of the immobility duration in the *c* group was comparable to that

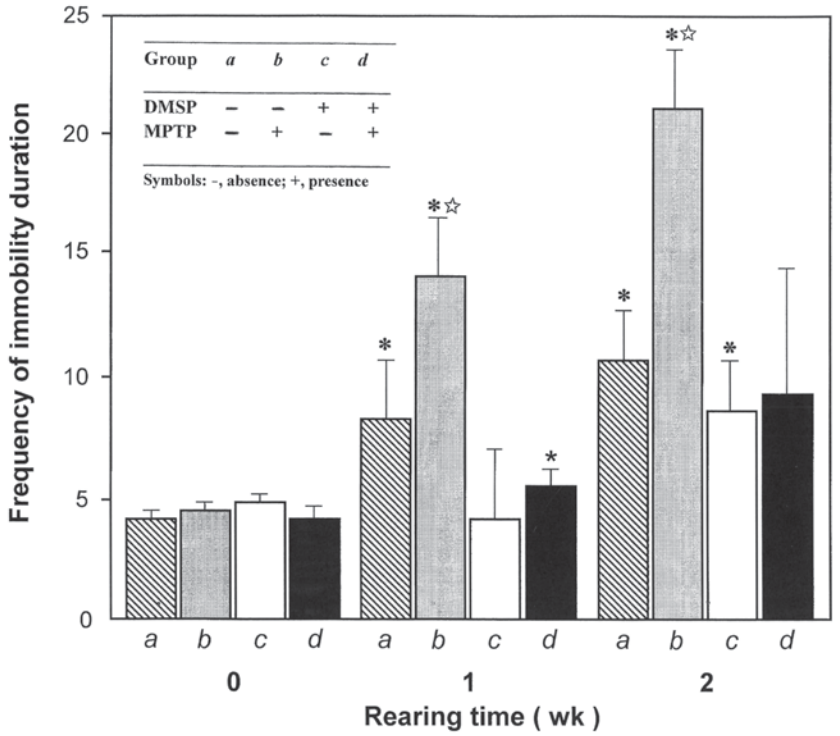

Fig. 11.10 Frequency of immobility duration of SAMP8 mice in combination of DMSP and MPTP. Mean ± SD ($n = 5$). *,* $p < 0.05$ (vs corresponding start values and the *a* group at the indicated times). The DMSP solution and the distilled solution were given ad libitum to two groups (ten mice in each) for two weeks. An appropriate amount of the MPTP solution was then i.p.-injected into one of the two groups, forming the four groups. The immobility duration was determined by a new Tail suspension test and expressed in terms of the frequency (total numbers/6 min) of immobility duration

in the *d* group at indicated times. In contrast, the amounts of cathecholeamine were measured in the same way as described above [23]. The amounts of norepinephrine, dopamine and DOPAC in the brains without cerebellum of the mice in the c and d groups were estimated on the 5th week. The results are shown in Fig. 11.11, in which the amounts of norepinephrine, dopamine and DOPAC in the *d* group proved to be significantly higher than those in the *c* group.

11.3.11 Effect of DMSP and NGF on the Outgrowth of Neurites of PC 12 Cells [25]

The effects of various concentrations (0.05, 0.5, 5, 20, 50 and 500 ng/ml) of NGF on the number of the neurite-bearing cells were examined on the basal medium in the collagen-coated dishes on the 4th day. The number of neurite-bearing cells increased

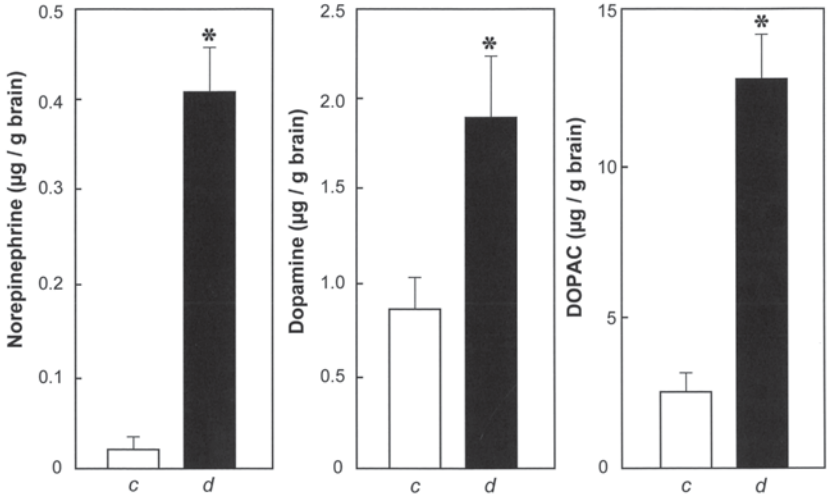

Fig. 11.11 The amounts of norepinephrine, dopamine and DOPAC in the brains of SAMP8 mice in the control and MPTP groups with DMSP. Mean ± SD ($n = 5$) * $p < 0.01$ (vs the corresponding control values). The amounts of norepinephrine, dopamine and DOPAC in the brains without cerebellum of the mice in the two groups: *c*, [MPTP (+)- [MPSP(−)] and *d*, [DMSP (+)- MPTP (+)], were estimated immediately after the determination of tremer duration on the 5th week [10]

with the increasing concentrations of NGF and reached maxima at concentrations of more than 0.5 ng/ml, at which the statistical analyses showed no significant differences ($p < 0.05$, Scheffe's test) over the concentrations from 0.5 to 500 ng/ml (data not shown). The combined effects of the various concentrations (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M) of DMSP with NGF (5 ng/ml) on the number of neurite-bearing cells were examined on the basal medium in the collagen-coated dishes up to 4 days. These results are shown in Fig. 11.12. The number of neurite-bearing cells in all the groups rapidly increased up to 3 days and thereafter showing almost plateaus up to 4 days. The number of the neurite-bearing cells showed high values in the order of the combinations of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} DMSP groups with NGF whereas the NGF group showed the lowest values and the control group exhibited negligible values on the 3rd day. The cell growth (cell number) in all the groups was almost the same except for the largest growth in the control group under the same experimental conditions (data not shown).

11.3.12 Combination Effect of Various Concentrations of DMSP with MPTP and NGF on the Number of Neurite-Bearing PC-12 Cells [26]

Effect of different concentrations of DMSP with MPTP and NGF on the outgrowth on neurites from PC 12 cells was examined for up to 5 h. Results are given in Fig. 11.13. All the neurite-bearing cells rapidly increased with increased incubation times up to 3 days.

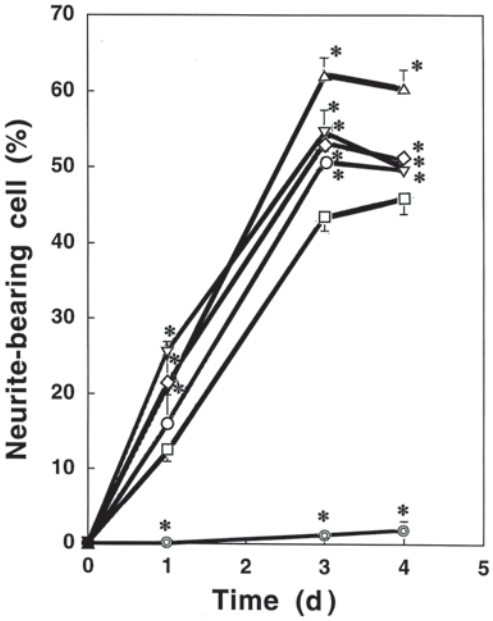

Fig. 11.12 Combined effect of various concentrations of DMSP with NGF on the suspended PC-12 cells. Mean ± SD ($n=3$). $^{*}p<0.05$ (vs corresponding the NGF group at the indicate time) ⊙, □, Δ, ∇, ◊, ○: the control, the NGF and the DMSP groups at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M with NGF. The combined effect of the various concentrations of DMSP with NGF (5 ng/ml) on the suspended cells was examined on a basal medium in the collagen-coated dishes with increasing incubation times up to 4 days. The suspended cells are expressed in terms of the ratio (%) of the number of their cells versus that of the total cells at the indicated times

Especially, those in the NGF and the combination group of DMSP (10^{-3} M) with NGF and MPTP indicated maxima on the 3rd day among the groups. The values were followed in the order of the combination group of the DMSP (10^{-3} M) with NGF and MPTP, the DMSP (10^{-4} M) with NGF and MPTP, the DMSP (10^{-5} M) with NGF and MPTP, and also decreased up to 4 days except for the NGF group whereas the NGF and MPTP group gave the lowest values up to 3 days. In particular, the number of neurite bearing-PC 12 cells in the NGF group and the DMSP (10^{-3} M) with NGF and MPTP group, of great interest, showed the similar values on the 3rd days. The values in the control group very slightly increased up to 5 days. The values in the 10^{-3} M DMSP group with MPTP and NGF were, of great interest, coincident with those in the NGF group on the 3rd day. However, the decline of the values in the former group after 3 days is likely to be ascribable to accelerated formation of MPP^{+} from MPTP with the increasing incubation times. This is likely true for values in other DMSP groups with NGF and MPTP. Representive figures in the NGF group, the DMSP (10^{-3} M) with NGF and MPTP group, and the NGF and MPTP group in this order are shown on the 3rd day (Fig. 11.14). On the contrary, the cell growth in all the groups was almost the same except for the largest growth in the control group under the same experimental conditions (data not shown).

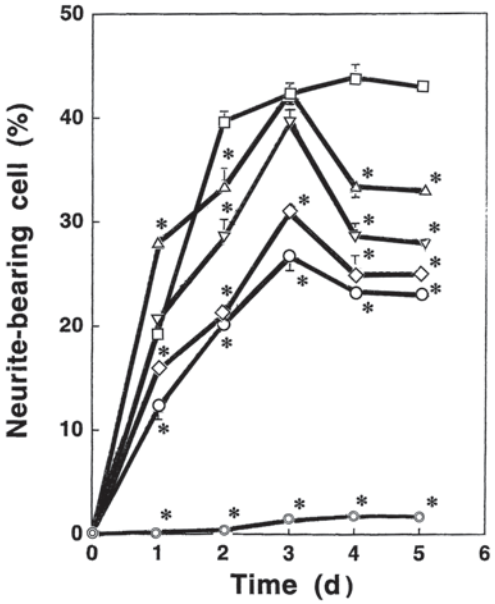

Fig. 11.13 Effect of various concentrations of DMSP with MPTP and NGF on the outgrowth of neurites from PC12 cells. Mean ± SD ($n = 3$) * $p < 0.05$ (vs the NGF group at the indicated times). ⊙, ○, □, Δ, ∇, ◊: the control, the MPTP and NGF, the NGF, the DMSP (10^{-3} M), MPTP and NGF, the DMSP (10^{-4} M), MPTP and NGF, and the DMSP(10^{-5} M), MPTP and NGF groups. The effect of DMSP at the concentrations of 10^{-3}, 10^{-4} and 10^{-5} M with MPTP (5 ng/ml) and NGF (5 ng/ml) on the outgrowth of neurites from PC 12 cells were examined in combination in the collagen-coated dishes with increasing incubation times up to 5 days. The outgrowth of neurites was expressed in terms of the ratio (%) of the neurite-bearing cells versus the total cells at the indicated times

11.3.13 Effects of DMSP and MPTP or MPP^+ on the Neurite Bearing-PC12 Cells

The effect of DMSP (10^{-3} M), MPTP and MPP^+ (5 ng/ml, each) with NGF (5 ng/ml) on the number of neurite-bearing cells was examined on the 1st and 3rd day under the same experimental conditions as above. The growth of neurites bearing-cells in the three groups was not restricted by administration of MTPT and MPP^+ at 1 and 3 days whereas supplementation of MPTP strongly and that of MPP^+ very strongly inhibited the number of neurites-bearing cells with NGF but without DMSP on the 3rd day (unpublished data).

11.4 Discussion

DMSP was found at high cellular concentrations of approximately 100–400 mM in ocean-dwelling green micro- and macro-algae [27, 28]. DMSP is degraded chemically and enzymatically to a smelly compound, dimethylsulfide (DMS), by marine

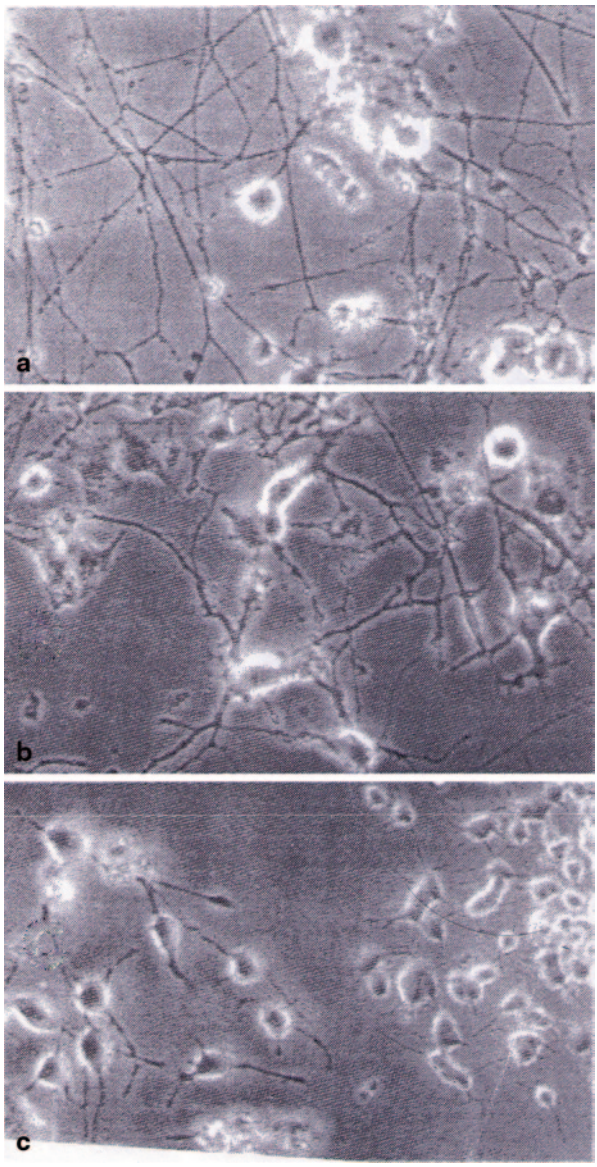

Fig. 11.14 Representative figures of the neurite-bearing cells in different conditions. **a** NGF (5 ng/ml). **b** MPTP (5 ng/ml) and 10^{-3} M DMSP. **c** NGF (5 ng/ml) and MPTP (5 ng/ml). Figures of neurites-bearing cells were taken in the collagen-coated dishes under (× 200 (× 40 for confirmation)) with an inverted microscope on the 3rd day and a representative one among them is shown for each group

bacterial and algal enzymes [29–31]. The role of DMSP and DMS in sulfur cycles has been studied extensively with focus on global climate regulation through DMSP/DMS- and DMS/dimethylsulfoxide (DMSO)-coupling [29, 32] A large number of studies on the metabolism of DMSP and its derivatives have also been reported in bacteria, plankton, algae, and halophytic plants in estuarine, coastal, and oceanic waters [27–29, 32]. However, there is no report on the physiological roles of DMSP in diseased terrestrial animals.

Only initial ip administration of 20 mM DMSP solutions to EAC-bearing mice resulted in same body weights as those in the control group for up to 10 days [22]. Moreover, single ip supplementation of a large amount of DMSP (7 g net weight/kg body weight in aqueous paste (about 3.2 M, 1 ml)) to rats, oral and sequential administration of high concentration of 20 mM DMSP solution (208 mg/day/rat) for up to 33 weeks to young rats [20] or a single ip injection of DMSP (180 mM, 1 ml (39.6 mg/mouse)) to juvenile mice did not result in toxicity for a long time. These findings indicate that it is possible to administer orally and intraperitoneally high and more frequent doses of DMSP to animals suffering from acute and chronic cancer without side effects *in vivo*, which strongly suggests that DMSP mitigates and heals different lines of cancers in different types of animals.

Various psychiatric, physiological and physical stresses elicit a wide range of disorders, in particular, cancer [33–38]. Especially at present, people are exposed to various kinds of stresses from environments. Stress proves to decline the host mediated-immune system in animals [33–38]. Therefore, elevation of immune system prevents and heals various diseases containing cancer caused by stresses.

In fact, we found that oral preliminary administration of low concentration (0.5 mM) of DMSP to test rats for 12 days effectively ameliorated gastric ulcers caused by "fears"; starvation of drink and foods for 24 h, fixing rats into wire-net box, immersion of rats into water in the dark for 24 h [13]. The facts indicate that administration of DMSP recovered injury and wound cells and tissues caused by stresses in stomach of rats to normal ones to a great extent, probably resulting in elevation of immune systems and in repair functions of damaged cells.

Aging is also involved in high risk of cancers [39–41]. Accordingly, substantial increase in the number of elderly people in the populations of developed countries in the coming years is a great and contemporary problem. Aged parsons older than 65 years are at higher risk of cancer in developing countries compared to younger peoples [4].

In contrast, test animals for investigating aging, senescence accelerated mice (SAM mice) derived by gene mutation, bear a great validity for cancer therapy because SAM mice bear all the properties with proceeding aging (appearances of aging, delayed growth, early death, dysfunction of learning and memory, erosion and ulcer, immune deficiency), as mentioned in details in "total grading score" [10]. Therefore, strains of SAM mice have been frequently used as a valuable aging-model animal with early aging except for manifestation of nonthymic lymphomas and histiocytic sarcoma [9].

Aging is closely related to incidence and proceeding of cancer, accompanied by down-regulation of immune systems as described above. Here, we found that oral and i.p. administration of low concentration (0.5 mM) of DMSP solution completely cure characteristic symptoms of aging [15–19] and, interestingly, heals dysfunction of learning and memory [16] in SAM P1 mice. In addition, we found that skin ulcers in male and female SAM P1 mice which individually occurred over the ages from 15 to 42 weeks also cures completely for up to 9 weeks after oral administration of 0.5 mM DMSP solution [15], as clearly shown in Fig. 11.1. Furthermore, we found that administration of DMSP lengthens almost to lost- mustachios of SAM P1 mice

at the same time for about one week (data not shown). Complete recovery of lost-hairs and their area in normal rats at the age of 15 days to normal length and area were also confirmed by oral administration of 20 mM DMSP solution for 13 days (data not shown). We further found that delayed growth, early aging (total grading score) and dysfunction of learning and memory of aged SAM P8 male and female mice are effectively ameliorated by administration of 0.5 mM DMSP solution for a short period (about 30 days) [17]. In addition, the experiments with male and female SAM R1 and P8 mice indicate that oral administration of DMSP (0.5 mM) for a long time (life span) significantly ameliorates aging-phenomena of these mice and prolongs, of great interest, their life in two strains, to further 16 weeks from 80 weeks at least, at which all the control SAM P8 mice died [18]. This amelioration of loss of learning and memory in male and female SAM P8 mice proves to be also attained by administration of pulverized green sea algae, *M. nitidum,* in foods for about 30 days [19]. The single injection of 1 ml of 10 mM solution (1.7 mg DMSP) is equivalent to ingestion of about 0.04 g dry algae (41.2 mg/g) per 40 g body wt. (mouse) per day. Therefore, the ingestion of diets containing 5% dry algae in food once a day by mouse is considered to be enough for amelioration of various stresses, various symptoms of aging and repairs of damaged cells and tissues. This is true for humans [21].

The recovery from these symptoms and diseases caused by stress and aging is clearly ascribable to enhancement of immune system functions following administration of DMSP. The findings are clearly confirmed by the facts that 1-type diabetes mellitus (IDDM) in pancreas of rats caused by alloxan, which is ascribable to autoimmune disease, is greatly ameliorated following oral administration (10 and 20 mM) of DMSP solution [14]. Namely, the results indicate that decrease and destruction of β cells in Langerhans's island of pancreas caused by alloxan was restored by administration of DMSP.

All the facts indicate that amelioration of all the diseased animals results in restoration of deficit immune systems caused by stress and aging, simultaneously accompanied by amelioration of injury and wound cells and tissues to lineage ones. The findings show likely that enhancement of host mediated-immune systems also ameliorates and heals cancer by administration of DMSP [3], also as clearly described below.

The activity of γ-GTP in the control group given with MeDAB diets showed much higher activity when compared to the activity of the control group given with normal diets and of the 10 and 20 mM DMSP solution group fed with MeDAB diets with significant difference ($p < 0.05$, by Tukey–Kramer test) [20]. Moreover, the liver weights in the control group with MeDAB diets significantly enlarged by about 2.5 fold those of the control group with normal diets and the 10 and 20 mM DMSP solution group with MeDAB diets, as shown in Fig. 11.15, although the weights in to latter three groups were the same ($p < 0.05$, Tukey–Kramer test) [20]. These findings demonstrate that administration of 10 and 20 mM DMSP solutions to MeMAB-induced cancerous rats keeps structure and function of cancerous livers under the normal conditions. Whereas DTH activity in ear skin in the control and MeDAB-induced liver cancer group with DMSP showed same high values comparing to the activity in cancerous rats without DMSP with significant

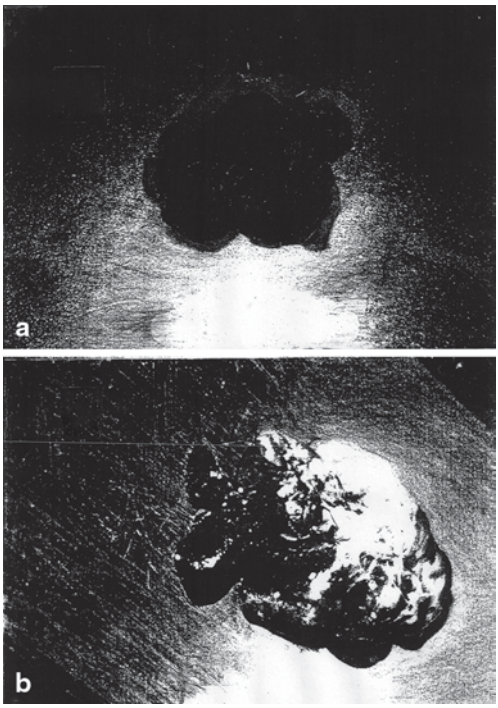

Fig. 11.15 Representative figures of MeDAB-induced liver cancer. The figures were taken on 33rd week. **a** Liver in rats given with MeDAB diets following oral administration of 20 mM DMSP solution. **b** Liver cancers in rats caused by MeDAB diets without DMSP (the whitish portions on the liver enlarged by cancers show cancers)

difference ($p < 0.05$, Tukey–Kramer test) [20]. Elevation of DTH activity in footpad is also obtained by supplementation of DMSP to EAC bearing-mice [20].

Furthermore, we further found that single ip administration (0.5 ml) of the control and the DMSP (in marine), DMSA, and MeMet (in terrestrial animals and plants) solution (10 mM in each) to EAC bearing-mice in this order increased the body weight in the form of ascites fluid (Fig. 11.16) but administration of these compounds without EAC cells to normal mice provided no affect on their growth for up to 10 days [22]. The accumulation of ascitic fluid (about 2.0 and 2.4 ml in alive mice) by initial administration of DMSA and DMSP (10 mM, each) [22] and accumulation of ascitic fluid (about 7.7 and 4.1 ml in dead mice) by preliminary supplementation of DMSP (10 and 20 mM) [21] to EAC bearing-mice at early rearing time were compared, which indicate likely that EAC bearing-mice under the former conditions also underlie a long life (longevity) of a majority of EAC bearing-mice. In particular, administration of DMSA and DMSP solutions to EAC bearing-mice restored the values of RBCs, WBCs, and differential WBC cells (lymphocyte, neutrophil and monocyte) to normal levels along with normal growth on the 10th day, significantly distinguished from those in EAC bearing-mice and EAC-bearing mice with MeMet with significant difference ($p < 0.05$, Tukey–Kramer test). Moreover, incubation of these compounds with EAC cells rather proves to increase the number of EAC cells for up to 5 h [22]. Preliminary ip administration of 10 and 20 mM DMSP solution to EAC bearing-mice ($n = 8$, each) prolonged their

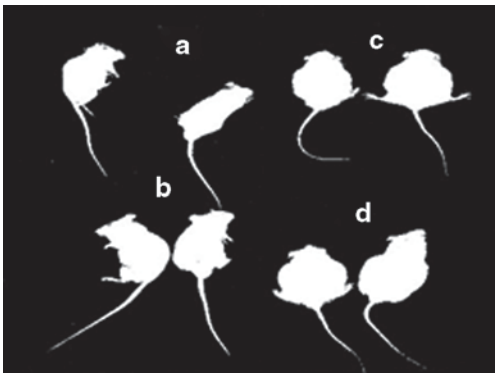

Fig. 11.16 Representative figures of EAC bearing-mice with increased incubation times. Figures of EAC bearing-mice ($n=5$ in each) after injection (0.5 ml) of EAC cells (2×106 cells/ml) to 4-week-old mice were taken at the increasing rearing times. **a** 0-week-old mice ($n=5$, actively move under anesthesia), **b** 6-day-old-mice ($n=5$, can move under anesthesia), **c** 12-day-old mice ($n=5$, can slowly move in a ball shape due to accumulation of ascitic fluid), **d** 18-day-old mice (Residual two alive mice can't move in a ball form by accumulation of ascitic fluid in peritoneal cavity

life span of residual three and four mice in the former and the latter group up to 300 days [21]. However, direct incubation of DMSA and DMSP with EAC cells rather increased proliferation of EAC cells *in vitro*. Furthermore, the administration of DMSA and DMSP also activated DTH activity in footpad. These facts show likely that only preliminary administration of DMSA and DMSP to EAC bearing-mice destroy not directly but indirectly EAC cells by suppression of aging and elevation of immune systems.

Moreover, aggressive cancer cells are "parasites" that use oxidative stress as a "weapon" to extract nutrients from surrounding stromal cells. However, DMSP has been shown to be a weak antioxidant at low concentrations (5–50 mM) but not at high concentrations (100–500 mM). This radical scavenging activity was assessed by diphenyl-2-picrylhydrazyl as a radical and α-tocopherol as a measure of the *in vitro* antioxidant effect in our preliminary experiments.

Accordingly, the anti-cancer effect of DMSP is likely to not depend on anti-oxidation functions but on up-regulation of immune systems and repair mechanisms of damaged cells and tissues without any upward side effects.

Therefore, these findings indicate that healing effect of EAC bearing-mice by DMSP is ascribable to enhancement of host mediated-immune systems and to newly formed-cells and -tissues from resided or recruited multipotent progenitor stem cells in or to local microenvironments in injury and wound cells and tissues in these diseased, especially cancerous mice and rats. Furthermore, DMSA, of great interest, involves probably in healing of EAC-bearing mice in the same way as that of DMSP.

Monocytes and macrophages are reported to increase at early rearing time of EAC bearing-mice [42–44]. Moreover, macrophages play a significant role at early times for preventing and curing not only EAC but also various cancers [42–44]. We find that administration of 20 mM DMSP solution to EAC bearing-mice accumulates macrophages in peritoneal cavity at early rearing time by about three fold those in normal mice in our preliminary experiments (data not shown).

In brief, monocytes and macrophages originate from common myeloid progenitor stem cells in the bone marrow, a complex process that is mediated by the cytokine-cascade and chemokines [42, 43, 45–47]. The increase of macrophages result from inflammation or cell death of viscera caused by EAC cells in the peritoneal cavity and from enhancement of the recruitment of newly-formed bone marrow macrophages, produced by the cytokine-cascade system without a cytokine storm [20, 22], to all tissues and viscera in the body [43–48].

In support of damaged-cell repairs, we further found that oral administration of 0.5 mM DMSP solution completely cured Parkinson's disease in normal- [23] and senescence-accelerated-mice [24], a condition caused by a dopaminergic neuron-destructive agent MPP^+ formed from MPTP (10–20 mg/Kg body wt for three days) in the substantia nigra of the brain. Injury and destruction of neurons in central nervous systems occur also in incidence and proceeding of cancer. However, we demonstrated that supplementation of 10 mM DMSP solution (1 mM) actively promotes, singly or in combination with MPTP (10 ng/2 ml) or with NGF and MPTP (10 ng/2 ml each), outgrowth and elongation of neurites in nerve model cells, Pheochromocytoma cells (10^4 cells/2 ml), on the RPMI 1640 medium containing serums and antibiotics *in vitro* [25, 26]. These findings indicate likely that DMSP mitigates and heals neurodegerative diseases: brain and peripheral cancer, Alzheimer's disease, Parkinson's disease, Lewy body disease, Amyotrophic lateral sclerosis (ALS or Lou Gehring's disease), etc., regardless of genetic (like SAM P strains) or non-genetic diseases.

Taken together our results and other findings suggest it is likely that inflamed and damaged cells and tissues in diseased animals and humans [21, 47, 49] in Japan, who customarily eat green sea algae [4, 21], are regenerated to lineage cells from multipotent stem cells in a similar way as explained above [22, 49–52], which is accompanied by activation of the innate immune system [3, 47, 53, 54] by DMSP signaling. Recently we found the significant facts that i.p administration of much higher concentration of DMSP (70 mM, 0.5 ml) to EAC bearing-mice completely eradicate crucial EAC and accumulated large amounts of activated macrophages in the peritoneal cavity in mice with no inflammation and without any toxicity. The findings strongly support all the above insight [55].

11.5 Competing Interests

The authors declare that they have no competing interests.

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Chapter 12
The Current Status of Novel Anticancer Drugs from Marine Actinobacteria

Panchanathan Manivasagan and Se-Kwon Kim

Abstract Marine actinobacteria are emerging as a valuable resource for bioactive substances encompassing a variety of unique structural classes. Marine actinobacteria are one of the most efficient groups of secondary metabolite producers and are very important from an industrial point of view. Chemotherapy is one of the main treatments used to combat cancer. A great number of anticancer compounds are natural products or their derivatives, mainly produced by microorganisms. In particular, actinobacteria are efficient producers of a large number of bioactive natural products that show a range or biological activities including antimicrobial, anticancer and enzyme inhibition. Marine actinobacteria have attracted special attention in the last ten years for their ability to produce interesting pharmacological lead compounds.

Keywords Marine actinobacteria · Anticancer · Natural products · Bioactive compounds

12.1 Introduction

Marine microorganisms are widely recognized as rich sources of novel natural products [1, 2]. In recent years, numerous novel bioactive compounds discovered from marine actinobacteria have been reported [3–6]. They are responsible for the production of about half of the discovered bioactive secondary metabolites notably antibiotics, antitumor agents, and immunosuppressive agents [7–10].

Marine actinobacteria also constitute an important and potential source of novel bioactive compounds. They produce different types of antibiotics, because the environmental conditions of the ocean differ greatly from terrestrial conditions [11]. Novel actinobacteria with biopharmaceutical potential have been increasingly iso-

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lated from marine habitats [12–15]. A new major marine *Streptomyces* genus have recently been described and shown to produce biological activities, including antibiotics [16, 17]. The antibiotics are entirely new and unique when compared to those from the terrestrial ones.

Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by actinobacteria, representing 45% of all bioactive microbial metabolites discovered [18]. Among actinobacteria, around 7600 compounds are produced by *Streptomyces* species [18]. Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry [18]. Members of this group are producers, in addition, of clinically useful drugs such as anthracyclines, peptides, aureolic acids, enediynes, antimetabolites, carzinophilin, mitomycins and others [19]. However, the search for novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to multiple chemotherapeutic drugs. In addition, the high toxicity usually associated with cancer chemotherapy drugs and their undesirable side effects increase the demand for novel anticancer drugs active against untreatable tumors, with fewer side effects and/or with greater therapeutic efficiency [20]. This book chapter discusses the current status of novel anticancer drugs from marine actinobacteria

12.2 Actinobacteria from Marine Environment

Actinobacteria are Gram-positive, often filamentous, bacteria known for their unsurpassed capacity for the production of secondary metabolites with diverse biological activities. Extensive screening of terrestrial actinobacteria, started in the early 1950s, has yielded many important drug leads, later developed into antimicrobial (amphotericin B, erythromycin, vancomycin), anticancer (daunorubicin, bleomycin, mitomycin) and immunosuppressive (rapamycin) drugs. Despite this apparent success, most of the actinomycete-based screening programs at big pharmaceutical companies have been abandoned in the recent years due to several reasons. One of the reasons was high costs of the internal screening programs, combined with the low number of new drug leads and relatively low profit on such drugs as new anti-infectives [21]. Another reason has been frequent re-discovery of the same compounds, mostly due to the redundancy of the samples, as well as strain isolation and screening technologies [22].

In the recent years, actinobacteria isolated from the marine environment (sediments, sponges, tunicates, neuston, etc.) have attracted considerable attention [23]. True marine actinobacteria are usually considerably more difficult to culture compared to their terrestrial relatives, most likely due to the special growth requirements. However, development of both sampling and cultivation techniques allowed isolation of representatives of several true marine actinomycete genera producing novel compounds with interesting biological activities [12].

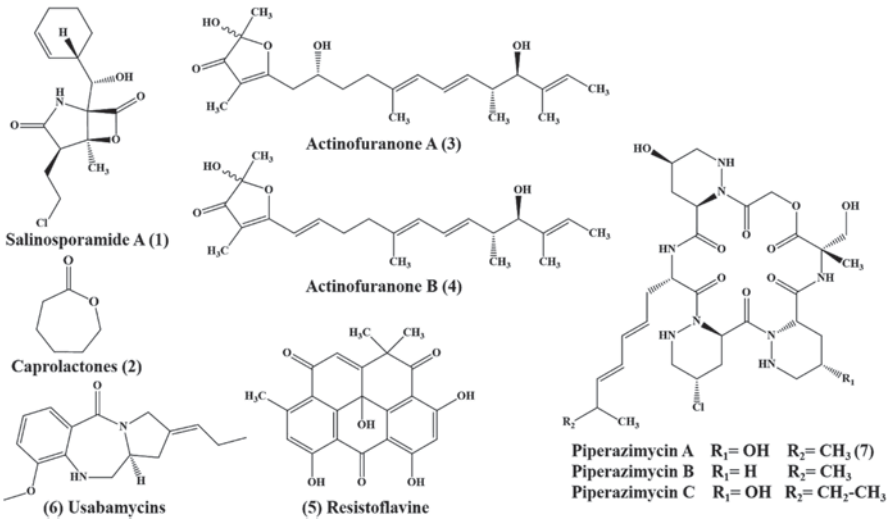

Fig. 12.1 Chemical structures of salinosporamide A, caprolactones, actinofuranone A, actinofuranone B, resistoflavine, usabamycins and piperazimycins

12.3 Anticancer Activity

Cancer still remains one of the most serious human health problems and breast cancer is the second most universal cause of cancer deaths in women. Therapeutic methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy and these techniques are individually useful in particular situations and when combined, they offer a more efficient treatment for tumor. Many of the antitumor compounds from marine drugs are derived from marine actinobacteria and these metabolites play an important role in identification of pharmaceutical compounds. Currently, it appears that there have been only a few studies focusing on finding bioactive compounds derived from marine actinobacteria to be used as anticancer agents, as well as agents against infectious organisms.

Pure active compounds extracted from the marine actinobacterium, *Salinispora tropica* have shown inhibitory effects in many malignant cell types [24]. In particular, Salinosporamide A **(1)** (Fig. 12.1) is a novel rare bicyclic beta-lactone gamma-lactam isolated from an obligate marine actinobacterium, *Salinispora tropica* [25, 26]. Salinosporamide A is an orally active proteasome inhibitor that induces apoptosis in multiple myeloma cells with mechanisms distinct from the commercial proteasome inhibitor anticancer drug Bortezomib [27]. It is being developed by Nereus Pharmaceuticals, Inc. (as NPI-0052) and was scheduled to enter clinical studies for treatment of cancer in humans in 2006. NPI-0052 is currently being evaluated in multiple phase I trials for solid tumors, lymphoma and multiple myeloma (http://www.nereuspharm.com/NPI-0052.shtml). NPI-0052 represents the first clinical candidate for the treatment of cancer produced by saline fermentation of an obligate marine actinobacterium [28].

Prudhomme et al. [24] tested Salinosporamide A for its utility as an anticancer and antimalarial drug. It was shown to have inhibitory activity against parasite development *in vitro* (*Plasmodium falciparum*) and *in vivo* (*P. yoelii*). The exact mode by which salinosporamide A inhibits *Plasmodium* erythrocytic development is unknown; however, it is likely due to the inhibition of the proteasome complex. It is interesting to note that chloroquine resistant strains are still sensitive to Salinosporamide A. Targeting the proteasome system has a huge therapeutic implication as it can restrain growth and survival of most cell types [24]. These attributes, taken with the fact that it is already in phase I clinical trials as an antitumor agent, make it an excellent candidate for alternative therapies, such as antibacterial, antiparasitic, antifungal or antiviral treatments.

Caprolactones **(2)** (Fig. 12.1) are new antibiotics isolated from *Streptomyces* sp. showing moderate phytotoxicity and promising activity against cancer cells with concomitant low general cytotoxicity [29]. Two new polyketides, actinofuranones A **(3)** and B **(4)** (Fig. 12.1), were isolated from the culture extract of a marine-derived *Streptomyces* strain, designated as CNQ766. It showed weak *in vitro* cytotoxicity against mouse splenocyte T-cells and macrophages with IC_{50} values of 20 μg/mL and were inactive against human colon carcinoma HCT-116 cells [30]. Resistoflavine **(5)** (Fig. 12.1) is a cytotoxic compound, isolated from *S. chibaensis* $AUBN_1/7$. It showed cytotoxic activity against human gastric adenocarcinoma HMO2 and hepatic carcinoma HePG2 cell lines [31]. Usabamycins **(6)** (Fig. 12.1) are new anthramycin-type analogues isolated from *Streptomyces* sp. NPS853. Usabamycins show weak inhibition of HeLa cell growth and selective inhibition of serotonin (5-hydroxytrypamine) $5\text{-}HT_{2B}$ uptake [32].

Piperazimycins (**7**) (Fig. 12.1) are cyclic hexadepsipeptides isolated from the fermentation broth of a *Streptomyces* sp. strain CNQ-593, isolated from marine sediments at a depth of approximately 20 m near the island of Guam. Cytotoxic activities of piperazimycins were initially evaluated *in vitro* against the human colon carcinoma HCT-116 cell line. All compounds exhibited significant cytotoxicity with an average GI_{50} of 76 ng/mL for each. Piperazimycin A also showed potent biological activity when evaluated against the NCI's cancer cell line panel, with mean GI_{50}, TGI and LC_{50} values for all the cell lines of 100 nM, 300 nM and 2 μM, respectively. Overall, piperazimycin A exhibited a nearly 3-fold more potent activity against solid tumors (average LC_{50} of 13.9 μM) than against the leukemia cell lines tested (average LC_{50} of 31.4 μM). It was most active against the melanoma (average LC_{50} of 0.3 μM), central nervous system (average LC_{50} of 0.4 μM) and prostate cell lines (average LC_{50} of 0.6 μM) cancers [33].

Neomarinones **(8)** (Fig. 12.2) are sesquiterpenoid naphthoquinones with a mixed polyketide-terpenoid origin [34]. Neomarinone, isomarinone, hydroxydebromomarinone and methoxydebromomarinone were produced by the actinobacterial isolate CNH-099 obtained from sediments at 1 m depth in Batiquitos Lagoon, North of San Diego, California. These compounds showed moderate *in vitro* cytotoxicity, (IC_{50} of 8 μg/mL) against human colon carcinoma HCT-116 cells. In addition, neomarinone generated a mean IC_{50} value of 10 μM in the NCI's 60 cancer cell line panel [35, 36]. Nonactin **(9)** (Fig. 12.2), a cyclic polyether also known as macrotetrolide,

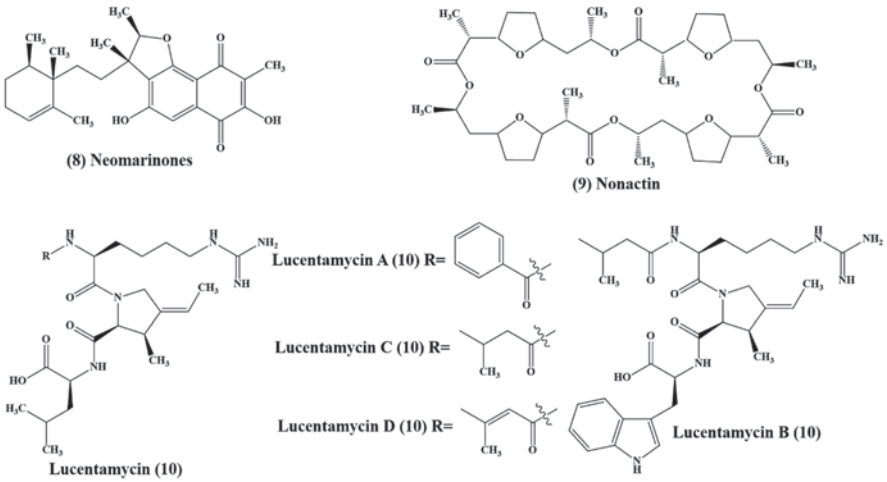

Fig. 12.2 Chemical structures of neomarinones, nonactin and lucentamycins

has been isolated from cultures of *Streptomyces* sp. KORDI-3238, isolated from deep-sea sediments collected at Ayu Trough in the Western Pacific Ocean [37]. Biosynthesis of gene cluster of nonactin has previously been isolated and characterized from *Streptomyces griseus* DSM40695 [38], revealing that it is synthesized by a non-iteratively acting type II PKS that involves five ketosynthases and lacks the acyl carrier protein. Nonactin exhibited significant cytotoxicity against the multidrug-resistant human erythroleukemia cell line K-562 [39].

Lucentamycins **(10)** (Fig. 12.2), 3-methyl-4-ethylideneproline-containing peptides, are produced by *Nocardiopsis lucentensis* strain CNR-712, isolated from the sediments of a shallow saline pond from the island of Little San Salvador, in the Bahamas. Lucentamycins A and B showed significant *in vitro* cytotoxicity against human colon carcinoma HCT-116 cell line with IC_{50} values of 0.20 and 11 μM, respectively. However, lucentamycins C and D were not cytotoxic in the same assay, suggesting that the presence of an aromatic ring is essential for the biological activity of this class of compounds [40].

Mansouramycin C **(11)** (Fig. 12.3) is an isoquinolinequinones antibiotic isolated from *Streptomyces* sp. Cytotoxicity profiling of the mansouramycins in a panel of up to 36 tumor cell lines indicated significant cytotoxicity of several derivatives, with pronounced selectivity for non-small cell lung cancer, breast cancer, melanoma and prostate cancer cells [41]. Four new polyketides, salinipyrones A **(12)** (Fig. 12.3) and B **(13)** (Fig. 12.3), and pacificanones A **(14)** and B **(15)** (Fig. 12.3) have been isolated from cultures of the obligate marine actinobacteria *Salinispora pacifica* CNS-237, found in the sediments collected from the Palau island, Western Pacific Ocean. Biological activity of these compounds is currently being examined in diverse bioassays. In the initial screening, salinipyrones and the pacificanones displayed no significant activity in a cancer cytotoxicity assay using HCT-116 human colon cancer cells. In an isolated mouse splenocyte model of allergic inflam-

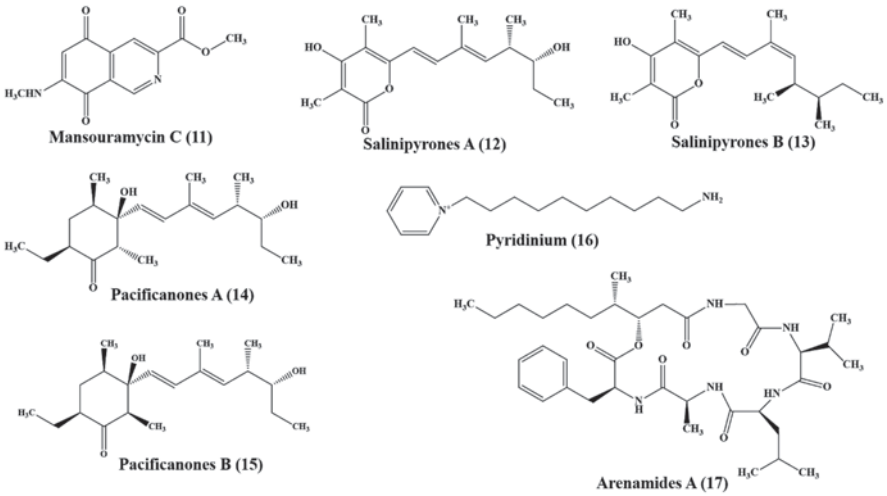

Fig. 12.3 Chemical structures of mansouramycin C, salinipyrones A, salinipyrones B, pacificanones A, pacificanones B, pyridinium and arenamides A

mation, salinipyrone A displayed moderate inhibition of interleukin-5 production by 50 % at 10 μg/mL without measurable human cell cytotoxicity [42].

Pyridinium **(16)** (Fig. 12.3) is a salt antibiotic isolated from *Amycolatopsis alba.* The compound showed potent cytotoxic activity against cancer cell lines of cervix (HeLa), breast (MCF-7) and brain (U87MG) *in vitro* and also exhibited antibacterial activity against Gram-positive and Gram-negative bacteria [43]. This new α-pyrone containing secondary metabolite was detected by HPLC–DAD analysis in a culture filtrate extract of *Streptomyces* sp. NTK 227, a strain isolated from the Atlantic Ocean sediments and found to be a member of the *Streptomyces albidoflavus* 16S rRNA gene clade.

Three new cyclohexadepsipeptides, arenamides A-C **(17)** (Fig. 12.3), were isolated from the fermentation broth of a marine actinobacterial strain identified as *Salinipora arenicola* CNT-088 which was obtained from the marine sediments at a depth of 20 m off the Great Astrolab Reef, in the Kandavu Island chain, Fiji. Arenamides A and B exhibited weak *in vitro* cytotoxicity against human colon carcinoma HCT-116 with IC_{50} values of 13.2 and 19.2 μg/mL, respectively [44]. In addition, arenamides have been associated to chemoprevention of carcinogenesis by suppression of NF*κ*B activation. NF*κ*B regulates the expression of a number of genes, the products of which are involved in tumorigenesis [45, 46]. Effect of arenamides on NF*κ*B activity was studied with stably transfected 293/NF*κ*B-Luc human embryonic kidney cells, induced by treatment with tumor necrosis factor (TNF). Arenamides A and B blocked TNF-induced activation in a dose- and time dependent manner with IC_{50} values of 3.7 and 1.7 μM, respectively [44].

Albidopyrone **(18)** (Fig. 12.4) showed moderate inhibitory activity against protein-tyrosine phosphatase B [47]. Two new cytotoxic antibiotics, piericidins C7 and C8 **(19)** (Fig. 12.4), were isolated from a marine *Streptomyces* sp. [48].

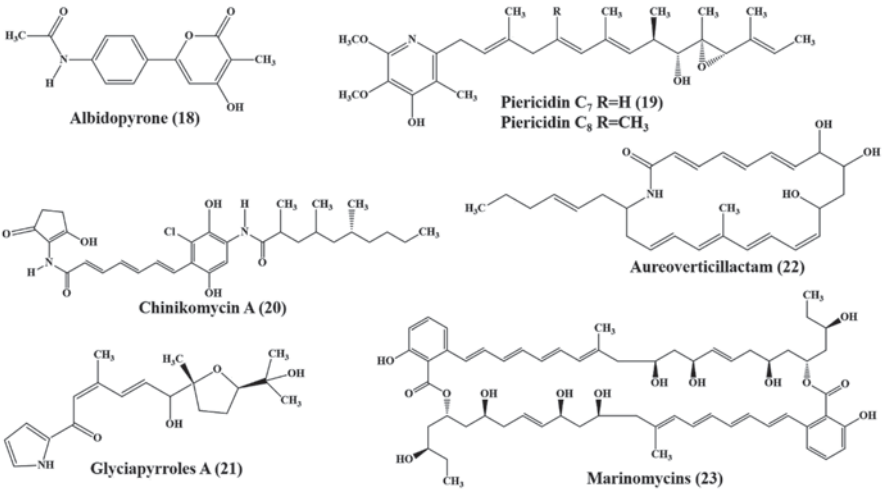

Fig. 12.4 Chemical structures of albidopyrone, piericidins, chinikomycin A, glyciapyrroles A, aureoverticillactam and marinomycins

Biological activity of piericidins was examined using rat glial cells transformed with the adenovirus E1A gene (RG-E1A-7), Neuro-2a mouse neuroblastoma cells, C6 rat glioma cells and 3Y1 rat normal fibroblast. Adenovirus E1A gene product inactivated the retinoblastoma tumor suppressor protein that plays an important role in cell-cycle and apoptosis control in mammalian cells and is inactivated during the development of a wide variety of cancers [49]. Piericidins C7 and C8 showed selective cytotoxicity against RG-E1A-7 cells (IC_{50} of 1.5 nM and 0.45 nM, respectively), and inhibited the growth of Neuro-2a cells (IC_{50} of 0.83 nM and 0.21 nM, respectively) without cytotoxic cell death. On the other hand, C6 rat glioma cells and 3Y1 rat normal fibroblast were not affected by piericidins [50].

Chinikomycins **(20)** (Fig. 12.4) are two novel antitumor antibiotics isolated from *Streptomyces* sp. They exhibited antitumor activity against different human cancer cell lines, but were inactive in antiviral, antimicrobial and phytotoxicity test [51]. Glyciapyrroles A **(21)** (Fig. 12.4) is a new pyrrolosesquiterpenes antibiotic isolated from *Streptomyces* sp. (NPS008187). Glyciapyrroles A possesses potent antitumor activity against the pair tumor cell lines at concentration up to 1 mM [17]. Aureoverticillactam **(22)** (Fig. 12.4) is a 22-membered macrocyclic lactam produced by *Streptomyces aureoverticillatus* NPS001583 isolated from marine sediments. Aureoverticillactam was found to possess moderate growth inhibitory activity against human colorectal adenocarcnioma HT-29, Jurkat leukemia and mouse melanoma B16F10 cell lines [4]. Marinomycins **(23)** (Fig. 12.4) are new antitumor antibiotics isolated from *Marinispora* sp. Marinomycins show significant antimicrobial activities against drug resistant bacterial pathogens and demonstrate impressive and selective cancer cell cytotoxicities against six of the eight melanoma cell lines in the National Cancer Institute's 60 cell line panel. The discovery of these new compounds from a new, chemically rich genus further documents that marine actinobacteria are a significant resource for drug discovery [52].

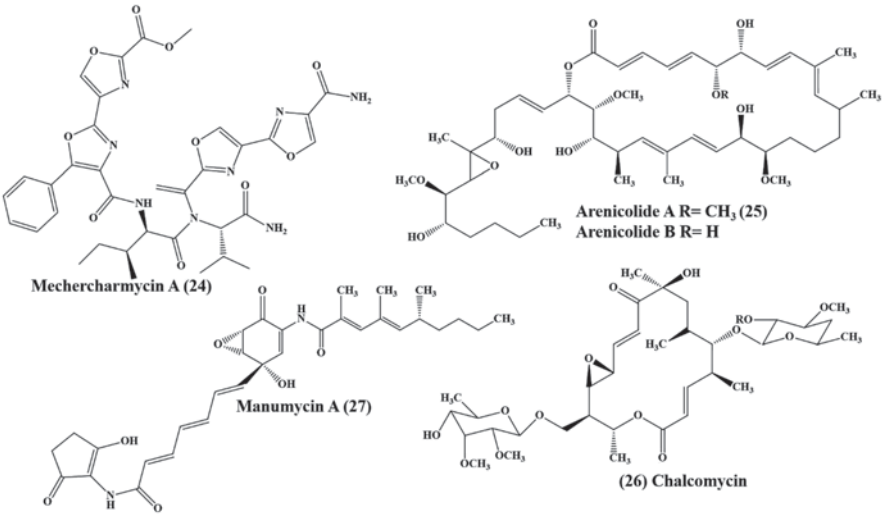

Fig. 12.5 Chemical structures of mechercharmycin A, arenicolides, chalcomycinand manumycin A

A new cytotoxic substance named mechercharmycin A **(24)** (Fig. 12.5) was isolated from marine-derived *Thermoactinomyces* sp. YM3-251. Mechercharmycin A exhibited relatively strong antitumor activity, whereas mechercharmycin B exhibited no such activity [53]. A higher number of type I polyketide derived compounds with antitumor activity have been isolated from marine actinobacteria. Once such compound is arenicolides **(25)** (Fig. 12.5), 26-membered polyunsaturated macrolactones, produced by the obligate marine actinobacteria *S. arenicola* strain CNR-005, isolated from the marine sediments, at a depth of 20 m from the coastal around the island of Guam. In particular, arenicolide A was found to exhibit moderate cytotoxicity toward the human colon adenocarcinoma cell line HCT-116 with an IC_{50} of 30 μg/mL [54]. Chalcomycin **(26)** (Fig. 12.5), a 16-membered macrolide, is produced by *Streptomyces* sp. M491 isolated from the Qingdao coast (China). Chalcomycin has been found to inhibit protein synthesis in HeLa human cervix carcinoma cell line [55, 56].

Manumycins constitute a class of compounds with antibiotic, cytotoxic, and other biological activities. It has been reported that manumycin A **(27)** (Fig. 12.5) and its analogues inhibit Ras farnesyl transferase and the growth of *Ki*-ras-activated murine fibrosarcoma in mice [57]. The side chains in manumycins appear to be a typical polyketide-derived moiety, differing with respect to their combinations of starter and elongation units. The central cyclohexene ring may be derived from the polyketide as in the case of manumycins or from some modified amino acid like 3-amino-5-hydroxybenzoic acid. Manumycin A and chinikomycins A and B (the quinone form of chinikomycin A) were isolated from *Streptomyces* sp. M045 derived from the sediments of Jiaozhou Bay in China [51].

Aureolic acid **(28)** (Fig. 12.6) (Chromomycin B, A_2 and A_3) are a new antitumor antibiotics isolated from *Streptomyces* sp. WBF16. These compounds showed

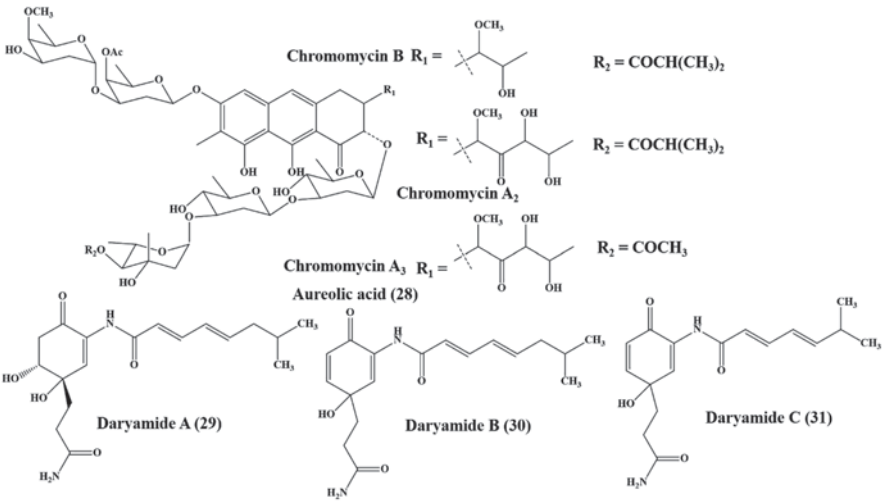

Fig. 12.6 Chemical structure of aureolic acid, daryamides A, daryamides B and daryamides C

strong cytotoxicity against SGC7901, HepG2, A549, HCT116 and COC1 and HUVEC [58]. Daryamides are new antitumor-antibiotics isolated from marine-derived *Streptomyces* strain CNQ-085. Daryamides A **(29)** (Fig. 12.6), B **(30)** and C **(31)** (Fig. 12.6) were subjected to cytotoxicity evaluation against the human colon carcinoma cell line HCT-116. Daryamide A exhibited significantly more potent cancer cell cytotoxicity, with an IC_{50} of 3.15 μg/mL, than daryamides B and C and very weak antifungal activity against *Candida albicans* [59].

Diazepinomicin **(32)** (Fig. 12.7) is an unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain [3]. It possesses antibacterial, anti-inflammatory and antitumor activity. It has a broad spectrum of *in vitro* cytotoxicity and has demonstrated *in vivo* activity against glioma, breast and prostate cancer in mouse models. Chlorinated dihydroquinones **(33)** (Fig. 12.7) are novel antibiotics produced by a new marine *Streptomyces* sp [15]. The compounds formally possess new carbon skeletons, but are related to several previously reported metabolites of the napyradiomycin class. Structures of the new molecules possess significant antibacterial and cancer cell cytotoxicities. Caboxamycin **(34)** (Fig. 12.7) is a new benzoxazole antibiotic and was detected by HPLC-diode array screening in extracts of *Streptomyces* sp. NTK 937, another strain which was isolated from the sediments collected from the Canary Basin. The compound, caboxamycin was named after the first letters of the collection site from where the organism was isolated and from letters drawn from its chemical structure. Caboxamycin showed inhibitory activity against both Gram-positive bacteria and against the tumor cell lines gastric adenocarcinoma (AGS), hepatocellular carcinoma (Hep G2) and breast carcinoma cells (MCF7). The antibiotic also showed an inhibitory activity against the enzyme phosphodiesterase [60].

Chandrananimycin A **(35)** (Fig. 12.7) is a novel antibiotic isolated from *Actinomadura* sp. Chandrananimycin A possesses potent antifungal activity against

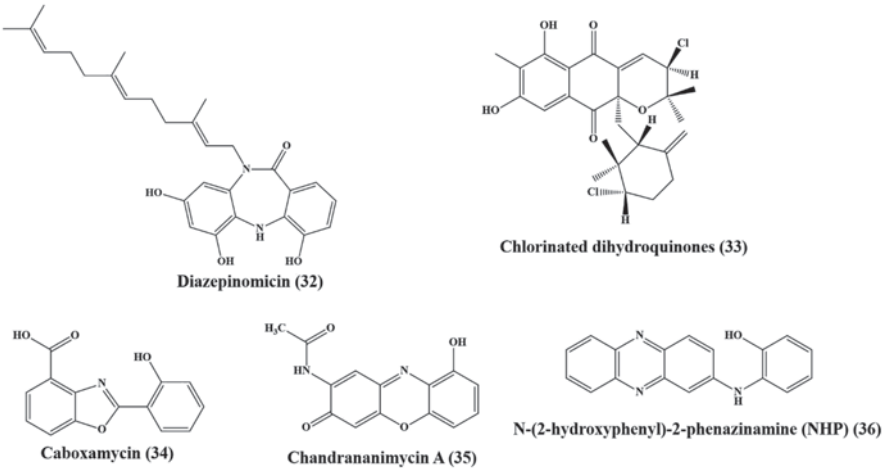

Fig 12.7 Chemical structures of diazepinomicin, chlorinated dihydroquinones, caboxamycin, chandrananimycin A and N-(2-hydroxyphenyl)-2-phenazinamine (*NHP*)

Mucor miehei. It also exhibits antialgal activity against the microalgae *Chlorella vulgaris* and *C. sorokiniana* and antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis,* along with anticancer activity [61]. N-(2-hydroxyphenyl)-2-phenazinamine (NHP) **(36)** (Fig. 12.7) is a new antibiotic isolated from *Nocardia dassonvillei*. The new compound showed significant antifungal activity against *Candida albicans,* with a MIC of 64 μg/mL and high cancer cell cytotoxicity against HepG2, A549, HCT-116 and COC1 cells [62].

12.4 Conclusions

Actinobacteria and, in particular the genus *Streptomyces*, have been well known during the last seventy years as prolific producers of novel bioactive compounds, anticancer drugs included. With the increasing development of oceanographic studies leading to the isolation of new actinobacteria from marine sources, new prolific genera in the production of useful compounds have been found, such as *Salinispora*. However, the Ocean, without any doubt, is keeping a myriad of new actinobacteria providing novel structural diversity to be discovered and used. In addition, the continuous effort for unravel the biosynthesis of the already known compounds and the isolation and characterization of their biosynthesis gene clusters will lead to the development of new anticancer compounds, hopefully with improved therapeutic properties, by using combinatorial biosynthesis approaches.

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Chapter 13
Natural Products with Anticancer Activity from Marine Fungi

Valliappan Karuppiah, Fengli Zhang and Zhiyong Li

Abstract Cancer is one of the major diseases, which require the improved drugs with fewer side effects. Until now, several marine natural products have been accessed for the anticancer property and few of them are in clinical trials too. Marine fungi are taxonomically diverse, largely productive, biologically active, and chemically unique offering a great scope for discovery of new anticancer drugs. The natural products isolated from the marine fungi are possibly inhibiting the processes such as inflammation, cell differentiation and survival, and metastasis of various signal transduction pathways and their by reducing the risk of cancer. In this chapter, we have discussed about the anticancer, anti-inflammatory and cytotoxic activities of marine derived fungi.

Keywords Marine fungi · Natural products · Anticancer

13.1 Introduction

Death due to cancer is the second highest amongst all diseases [1–3]. Due to the shortcoming of chemotherapy, dose limits, side effects, and low selectivity for cancer cells, detection of much more efficient, harmless and highly selective antitumor drugs are required in urgent. Recently, research has been focused towards the development of novel anticancer drugs from the natural products. Recent reviews on drug discovery have shown that more than two thirds of the anticancer drugs are either natural products or developed based on the knowledge gained from natural products [4, 5]. Marine microbes have fascinated enormous interest in the pharmaceutical community as they generate a wide range of metabolites that are structurally unique and pharmacologically active [6, 7]. Due to the potential bioactivity of the natural products isolated marine microorganism, they represent a hopeful resource for discovering new anticancer drugs [8, 9].

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Almost all forms of life in the marine environment such as algae, sponges, corals, ascidians have been explored for their natural products [10, 11]. The large numbers of structurally and pharmacologically significant natural products have been isolated with novel antibiotic, antitumor and anti-inflammatory properties [6, 12–16]. As interests have shifted towards the marine microorganisms, the fungi have begun to be renowned as a potential source for bioactive compounds. Fungi growing in the marine environment can be classified into obligate and facultative marine fungi. Obligate marine fungi are defined as the fungi that grow and sporulate exclusively in a marine or estuarine habitat and the facultative marine fungi are defined as the fungi from freshwater or terrestrial areas are able to grow in the natural marine environment [17]. Most of the fungi grow in unique and extreme environs and therefore they have the ability to generate unique and unusual secondary metabolites. It is clearly understood that the secondary metabolites produced by the fungi probably act as a chemical defense due to their adaptation and substrate competition [18, 19].

Marine fungi have proven to be untapped resources for the rich and promising source of novel anticancer, antibacterial, antiplasmodial, anti-inflammatory and antiviral agents [20–27]. Bioactive compounds such as sargassamide, halimide and avrainvillamide isolated from a marine fungus have shown selective inhibition against cancer cell lines, and shown in vivo activity in preclinical models (P-388 lymphocytic leukemia). Among them, halimide and avrainvillamide have been authorized by the pharmaceutical industry and are in preclinical development [17].

13.2 Antitumor Compounds from Marine Fungi

13.2.1 MMP Inhibitors

Carcinogenesis is a multistep process regulated by a range of signal transduction pathways such as inflammation, cell differentiation and survival, and metastasis. Most of the cellular functions are interconnected and abnormalities in the normal cellular function lead to tumor generation and progression. Few of these abnormalities have been targeted for cancer therapy and some inhibitors from marine fungi have been isolated and studied to neutralize tumor progression and carcinogenesis.

Matrix metalloproteinases (MMPs) are protein-degrading zinc dependent endopeptidases, which degrade the extracellular matrix (ECM) [28]. The expression and activity of MMPs in adult tissues are normally quite low, but significant increase in various pathological conditions leads into unwanted tissue destruction, such as inflammatory diseases, tumor growth and metastasis. It has been paying wide attention due to their apparent role in carcinogenesis and cellular invasion by degrading the extracellular matrix [29]. Apart from its major role, MMPs are also important for cancer cell transformation, growth, apoptosis, signal transduction and immune regulation [30, 31]. The secondary metabolites namely, chrysophanol, physcion, and emodin isolated from the marine fungal species of *Microsporum* showed the potent anticancer activity. All these compounds considerably inhibit the matrix

metalloproteinase MMP-2 and MMP-9 expressions in a dose-dependent manner. The MMP-2 and MMP-9 have been inhibited by suppressing the expression of JNK and ERK signaling pathways [32].

13.2.2 Topoisomerase Inhibitors

Topoisomerases play an important role in retaining the integrity of the DNA helix during replication, transcription, and chromosome condensation during mitosis [33]. It has been noted that topoisomerase levels have been increased in several cancers. DNA topoisomerases are important for cell proliferation. Hence, DNA topoisomerases (topo) I and II are established as a molecular target of anticancer drugs [34–37]. In order to identify the effective anticancer drug numerous topoisomerase inhibitors have been isolated from various natural sources [38]. These drugs either prevent the formation of covalent bonds between topoisomerase and DNA or stabilize the intermediate topoisomerase-DNA covalent binary complex thus preventing DNA relegation.

Leptosins (Leps) F and C, isolated from a marine fungus, *Leptoshaeria* sp. showed the potent cytotoxic activity. Lep F inhibited the activity of topo I and II, whereas Lep C inhibited topo I *in vitro*. Interestingly both of the compounds have been found to be catalytic inhibitors of topo I. Cell cycle analysis of Lep C-treated cells showed that Lep C appeared to inhibit the progress of cells from G1 to S phase. Furthermore, Leps F and C inhibited the Akt pathway based on dose-dependent and time-dependent dephosphorylation of Akt (Ser473) [39]. Secalonic acid D (SAD) isolated from the fermentation broth of marine lichen-derived fungus *Gliocladium* sp. T31 is an excellent inhibitor of topo I. SAD displays a considerable inhibition of topo I in a dose-dependent manner with the minimum inhibitory concentration (MIC) of 0.4 μM. SAD inhibits the binding of topo I to DNA but does not induce the formation of topo I-DNA covalent complexes [40].

13.2.3 PKC Inhibitors

Members of the protein kinase C (PKC) consist of a number of serine-threonine kinases. It can be classified into three groups based on their activating factors. PKCs are known to be a major player in carcinogenesis and maintenance of the malignant phenotype. Potentiation of malignant phenotype may be arbitrated by activation of selective PKC isoenzymes or through altered isoenzyme expression profiles compared to the originating tissue. Activation of PKCα and β isoenzymes has been often linked to malignant phenotype while PKCδ is thought to mediate anti-cancer effects [41]. One of the isoforms, PKCε, has been demonstrated to increase proliferation, motility, and invasion of fibroblasts or immortalized epithelial cells. It has been proven in xenograft and transgenic animal models that overexpression of PKCε is tumorigenic, resulting in metastatic disease [42]. Protein kinase inhibitor

exclusively inhibits the action of one or more protein kinases which subsequently block the downstream signaling. Especially, inhibition of tyrosine kinase prevents the growth of cancer cells. A number of protein kinase inhibitors have been isolated from marine fungi.

A marine fungus, *Chaetomium* sp. produce a novel benzonaphthyridinedione derivative, chaetominedione. The screening of biological activity revealed that chaetominedione considerably inhibits the activity of the p56lck tyrosine kinase (93.6% enzyme inhibition at 200 log/ml) [43]. Ulocladol is another $p56^{lck}$ tyrosine kinase inhibitor which has been isolated from a culture of the *Ulocladium botrytis*. Ulocladol inhibit the enzyme activity up to7% at 0.02 ug ml^{-1} [44]. An undescribed fungus of the genus *Microsphaeropsis*, isolated from the Mediterranean sponge *Aplysina aerophoba*, produces two new batman derivatives and three new 1,3,6,8-tetrahydroxyanthraquinone congeners. These compounds are the inhibitors of PKC-*€*, CDK4, and EGF receptor of tyrosine kinases [45]. 1403C is a novel anthraquinone derivative isolated from cultures of the marine-derived mangrove endophytic filamentous fungus *Halorosellinia* sp. Due to its potent inhibition of protein kinase B (PKB), the secalonicound has been identified as an anticancer drug candidate. A recent study showed that marine fungal strain of *Halorosellinia* sp. pulse fed with glucose solutions yielded 4.5 g/l of 1403C compound [46]. This strategy is valuable for fermentation scale-up of *Halorosellinia* sp. (No. 1403) for 1403C production which could solve the supply problem of 1403C for clinical studies.

13.2.4 Apoptosis Inducing Metabolites

Apoptosis represents cell death, which is accomplished through a highly ordered intrinsic cellular suicide program. It is essential for the cellular processes such as fetal development, tissue homeostasis, immune response, aging, etc. Deregulations of apoptosis (either up- or down-regulation) leads to one-half of the major diseases [47]. Mutations cause uncontrolled cell growth and lead to inadequate cell death, which lead to the development of cancer. Apoptosis has been concerned in a number of circumstances that are essential to the pathogenesis and progression of tumors, that include: (1) a permissive environment that promotes oncogene activation and tumor suppressor gene inactivation; (2) excessive cell accumulation due to the failure of programmed cell death; (3) tumor cells acquire the ability to evade elimination by the immune system; (4) enhanced tumor cell survival under environmental stresses such as the lack of oxygen (hypoxia) and nutrients; (5) tumor cell survival in a suspended state that is required for metastasis; and (6) resistance to chemotherapy and radiation treatment [48, 49].

Apoptosis can also be triggered by extracellular cues such as DNA damage, removal of growth factors, exposure to cytokines, environmental stress (such as treatment with chemotherapeutic agents or radiation), and detachment from the extracellular matrix [50]. Apoptotic cells are differentiated by morphological criterion such as the breakdown of the nuclear membrane, chromatin condensation, DNA fragmentation, cell shrinkage and the formation of apoptotic bodies. Caspases are

a group of cysteine proteases that play a key role in the regulation and execution of apoptosis [50]. These enzymes are initially produced as inactive zymogens. Upon activation by apoptotic signals, the initiator caspases cleave and activate the effector caspases, which then either directly cleave cytoskeletal and nuclear substrates or activate other downstream degradation enzymes. Cellular apoptotic machinery is regulated by a group of proapoptotic and antiapoptotic molecules at nearly every level along the signaling pathway.

Anthracenedione derivatives isolated from mangrove endophytic fungi *Halorosellinia* sp. and *Guignardia* sp. are potent inhibitors of mitochondrial function of cancer cell KB and thereby lead to apoptosis [51]. A new oxepin-containing diketopiperazine-type marine fungal metabolite, namely protuboxepin A isolated from *Aspergillus* sp. SF-5044 showed antiproliferative activity in several cancer cell lines. Protuboxepin A induces cancer cell growth inhibition by round-up morphology, M phase arrest, and an increase in the $subG_1$ population in tumor cells in a dose dependent manner. Protuboxepin A directly binds to α,β-tubulin and stabilizes tubulin polymerization thus disrupting microtubule dynamics. This disruption leads to chromosome misalignment and metaphase arrest which induces apoptosis in cancer [52]. Phomopsidin, an inhibitor of microtubule assembly have been isolated from a *Phomopsis* sp. obtained from a submerged mangrove branch [53]. Phomopsidin inhibit microtubule assembly with an IC_{50} of 5.7 μM and is similar in potency to colchicine (IC_{50} = 10 μM) and rhizoxin (IC_{50} = 4 μM).

Wentilactone B (WB), a tetranorditerpenoid derivative isolated from the marine algae- derived endophytic fungus *Aspergillus wentii* EN-48, has been shown to trigger apoptosis and inhibit metastasis in HCC cell lines. WB induces G2/M phase arrest and apoptosis in human hepatoma SMMC-7721 cells via the Ras/Raf/ERK and Ras/Raf/JNK signaling pathways [54]. Mycoepoxydiene (MED) is a polyketide isolated from a marine fungus associated with mangrove forests. MED induces the reorganization of cytoskeleton in actively growing HeLa cells by promoting formation of actin stress fiber and inhibiting polymerization of tubulin. MED could induce cell cycle arrest at G2/M in HeLa cells. MED-associated apoptosis have been characterized by the formation of fragmented nuclei, PARP cleavage, cytochrome c release, activation of caspase-3, and an increased proportion of sub-G1 cells [55].

A new ubiquitin-activating enzyme (E1) inhibitor, himeic acid A, has been isolated from a culture of marine-derived fungus, *Aspergillus* sp. Ubiquitin-activating enzyme plays important role in cell cycle progression. Himeic acid A inhibits the E1-ubiquitin intermediate formation in a dose-dependent manner [56]. Marine fungal metabolite 1386A, a newly identified small molecular compound extracted from the mangrove fungus 1386A in the South China Sea showed cytotoxity to cancer cells. A class of DNA-cleaving antitumor antibiotics, spiroxins A–E, have been isolated from an unidentified fungus (LL- 37H248) associated with an unidentified soft coral [57]. Spiroxin A showed potent cytotoxicity (IC_{50} = 0.09 μg ml^{-1}) against a panel of 25 diverse cell lines. Additionally, spiroxin A showed *in vivo* activity against an ovarian carcinoma in nude mice (59% growth inhibition after 21 days). Spiroxin A appears to act *via* multiple mechanisms and it nick plasmid DNA under reducing conditions. Furthermore, spiroxin A react with thiol-type reducing agents

suggesting an oxidative stress mechanism of action involving thiol conjugates. 1386A (17.1 μmol/l) significantly altered the global miRNA expression profile of the MCF-7 cells at 48 h. Forty-five miRNAs have been differentially expressed in MCF-7 cells. Target prediction suggests that these miRNAs potentially target many oncogenes and tumor-suppressor genes associated with cancer development, progression and metastasis [58]. Physcion (11.8 mg) isolated from the culture broth extract (1710 mg) of marine derived fungi significantly induce cell apoptosis through down-regulating of Bcl-2 expression, up-regulating of Bax expression, and activating the caspase-3 pathway. Furthermore, physician induces the formation of reactive oxygen species (ROS) in HeLa cells [59].

13.2.5 Anti-Inflammatory Metabolites

Cancers are having a tendency to rise at place of chronic inflammation [60]. Later, it turned out that acute inflammation is contributed to the regression of cancer [61]. However, epidemiologic studies support that chronic inflammatory diseases are commonly associated with increased risk of cancers [60–62]. The study intending at the association between inflammation and cancers first led to the determination whether the reactive oxygen and nitrogen species generated by inflammatory cells, such as leukocytes recruited to the inflammatory foci to kill infectious agents, may cause mutagenic assaults and result in tumor initiation [63]. Now, it has been understood that the growth of cancers from inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish an inflammatory microenvironment [62]. Although this host response may suppress tumors, it may also facilitate cancer development via multiple signaling pathways [64].

Chaetoglobosin Fex (Cha Fex) is a cytochalasan-based alkaloid isolated from the marine-derived endophytic fungus *Chaetomium globosum QEN-14*. The mechanism of Cha Fex on inflammation is mediated by Toll-like receptor 4 (TLR4) signaling in macrophages. Cha Fex considerably inhibit the LPS-induced production of tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) in peritoneal macrophages and murine macrophage cell line RAW264.7. Moreover, Cha Fex significantly attenuated the LPS-stimulated degradation of inhibitory kappa B-alpha and the subsequent translocation of the p65 subunit of nuclear factor-kappa B (NF-kB) to the nucleus. Chou Fex also reduces the phosphorylations of extracellular-signal-related kinase (ERK1/2), p38, and c-Jun N-terminal kinase (JNK1/2). Furthermore, Cha Fex didn't affect LPS binding to the RAW264.7 cells and human monocytes, while Cha Fex has able to inhibit the increase of membrane-associated CD14 (mCD14) expression both on RAW cells and human monocytes induced by LPS to a certain degree. These results suggest that the anti-inflammatory property of Cha Fex may be attributed to NF-kB inhibition as well as the negative regulation of ERK1/2, p38, and JNK1/2 phosphorylations [65].

Two diketopiperazine type indole alkaloids, neoechinulins A and B have been isolated from the marine fungus *Eurotium* sp. SF-5989. Neoechinulin A markedly

suppressed the production of nitric oxide (NO) and prostaglandin E2 (PGE2) and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX -2) in a dose dependent manner ranging from 12.5 to 100 μM without affecting the cell viability. On the other hand, neoechinulin B affected the cell viability at 25 μM although the compound displayed similar inhibitory effect on NO production to neoechinulin A at lower doses. Furthermore, neoechinulin A decreased the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Neoechinulin A also blocked the activation of nuclear factor-kappaB (NF-κB) in LPS-stimulated RAW264.7 macrophages by inhibiting the phosphorylation and degradation of inhibitor kappa B (IκB)-α. Moreover, neoechinulin A decreased p38 mitogen-activated protein kinase (MAPK) phosphorylation. A small-molecule, bis-N-norgliovictin isolated from marine-derived fungus, significantly inhibited lipopolysaccharide (LPS, ligand of TLR4)-induced TNF-α production in RAW264.7 cells [66]. Asperlin isolated form marine-derived fungus *Aspergillus* sp. significantly suppressed the iNOS expression, iNOS-derived NO production, COX-2 expression, and PGE_2 production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Molecular investigation revealed that asperlin induced heme oxygenase (HO)-1 expression through nuclear translocation of nuclear factor E2-related factor 2 and increased HO activity in RAW264.7 macrophages [67]. Moreover, many more potent anti-inflammatory compounds with unique inhibitory mechanism of inflammatory signals have been identified from marine fungi. These metabolites represent new class anti-inflammatory compounds that do not follow the mechanisms of which exert many side effects [27].

13.2.6 Cytotoxic Compounds

Marine-derived filamentous fungi are a source of new and potentially biologically active secondary metabolites [68–70]. The structurally strange cytotoxic gymnastatins A, B, and C have been isolated from the *Gymnascella dankaliensis* associated with the sponge *Halichondria japonica* [71]. Gymnastatins A, B, and C exhibited potent cytotoxicity in a P388 lymphocytic leukemia with ED_{50} values of 18, 108 and 106 ng ml^{-1}, respectively. Gymnastatins D and E, which lack ketone showed weak cytotoxicity with ED_{50} values of 10.5 and 10.8 μg ml^{-1}, respectively [72]. The presence of a Michael acceptor in gymnastatins A and B might act as potent cytotoxins. The unusual ergostanoids gymnasterones A and B [73] as well as dankasterone [74] have been isolated from *G. dankaliensis* have shown to be weakly cytotoxic. Asperazine, isolated from the *Aspergillus niger* associated with the sponge *Hyrtios proteus* showed selective cytotoxicity against leukemia cells [75]. Trichodenones A–C and harzialactones A and B have been isolated from *Trichoderma harzianum* derived from *Halichondria okadai* [76]. Trichodenones A, B, and C showed modest cytotoxicity against a leukemia P388 cell line with ED_{50} values of 0.21, 1.21, and 1.45 μg ml^{-1}, respectively. Harzialactone B showed weak activity (ED_{50}=60 μg ml^{-1}) and harzialactone A was inactive. Insulicolide A have been isolated from the mycelia of *Aspergillus insulicola* strain MD10-2 derived from

the sponge *Cinachyrella australiensis* in the South China Sea exhibits cytotoxic activity against human lung cancer cell line H-460 with an IC_{50} of 6.9 μM, approximately to the positive control paclitaxel (IC_{50} 4.94 μM) (data not published). (+)-Terrein isolated from the marine sponge-derived *Aspergillus terreus* PF-26 shows cytotoxic activity against human hepatoma Bel-7402 cell line with IC_{50} value 11.63 μM ± 0.02. It was found that (+)-Terrein was able to arrest cell cycle of Bel-7402 at the G2/M phase. Cell cycle-related genes such as CCND2, CCNE2, CDKN1C, CDKN2B, ANAPC5, PKMYT1, CHEK2 and PCNA were down-regulated in the 10 μM of (+)-terrein treated Bel-7402 cells (data not published).

Leptosin is one of the largest classes of cytotoxic fungal metabolites produced by *Leptosphaeria* sp. OUPS-4, derived from the marine alga *Sargassum tortile*. The leptosins can be divided into five subclasses or groups based on structural similarity and biological activity, which includes leptosins A-E, G, G1, G2, H-K, K1, K2, M, M1, N and N1 [77–81]. The leptosins showed the wide range of activity against a P388 leukemia cell line. Pyrenocine E isolated from the *Penicillium waksmanii* associated with brown alga *Sargassum ringgoldianum* showed moderate cytotoxicity with an ED_{50} of 1.30 μg ml^{-1} against P388 leukemia cells [82]. Novel cytotoxic sesquiterpenoid nitrobenzoyl esters have been isolated from a culture of *Aspergillus versicolor* derived from the surface of the Caribbean green alga *Penicillus capitatus* [83]. Compound, 9α,14-dihydroxy-6β-p-nitro- benzoylcinnamolide, showed a mean LC_{50} of 1.1 μg ml^{-1} in the National Cancer Institute's 60 cell-line panel. An unusual amino acid-derived communesins A and B have been isolated from the *Penicillium* sp. (OUPS-79) associated with marine alga *Enteromorpha intestinalis* [84]. Communesins A and B represent a novel carbon skeleton. Communesin B showed potent activity against P388 lymphocytic leukemia with ED_{50} of 0.45 μg ml^{-1}. While, communesin A exhibited only moderate cytotoxicity (ED_{50} 3.5 μg ml^{-1}).

Additionally, *Penicillium* isolate OUPS-79 produced two unrelated classes of cytotoxic compounds, penochalasins A-H [85, 86] and the penostatins A-I [87–89]. Cytotoxic activities of the penochalasins were evaluated against P388 leukemia cells, among them penochalasins A-C are the most potent. The ED_{50} values for penochalasins A-H are 0.4, 0.3, 0.5, 3.2, 2.1, 1.8, 1.9, and 2.8 μg ml^{-1}, respectively. Penostatins A-I [87–89] represent the third distinct class of new metabolites from *Penicillium* isolate OUPS-79. The cytotoxicity for penostatins A-I ranged from 0.5 to 11.0 μg ml^{-1}. *Penicillium* sp. (#CNC-350) isolated from the Caribbean green alga *Avrainvillea longicaulis* produced two active diketopiperazine dimers, 11,11′-dideoxyverticillin A (**137**) and 11′-deoxyverticillin A as well as a noncytotoxic diketopiperazine [90]. Compounds 11,11′-dideoxyverticillin A and 11′-deoxyverticillin A showed potent cytotoxicity against HCT-116 human colon carcinoma cells with IC_{50} values of 30 ng ml^{-1}.

A *Fusarium* sp. derived from a driftwood sample in a mangrove habitat of the Bahamas produced two related classes of sesterterpenes, neomangicols A-C [91] and mangicols [92]. Neomangicols A and B exhibited moderate cytotoxicity against a panel of cell lines. Neomangicol A was most active against an MCF-7 human breast carcinoma (IC_{50} = 4.9 μM) while neomangicol B was less active (mean IC_{50} = 27 μM). Magical A-G was screened in the NCI's 60-cell line panel and did

not exhibit selectivity, but showed growth inhibition in the low μM range. Two new polyphenols, expansols A and B, and two new phenolic bisabolane sesquiterpenoids, isolated from marine-derived fungus *Penicillium expansum* 091006 derived from mangrove plant *Excoecaria agallocha*. Expansol A exhibited moderate cytotoxicity against the HL-60 cell line with an IC_{50} value of 15.7 μM, and expansol B inhibited the proliferation of A549 and HL-60 cells with IC_{50} values of 1.9 and 5.4 μM [93]. A series of new derivatives of deoxybostrycin derived from the marine mangrove fungus *Nigrospora* sp. and has potential to be a lead for new drugs because of its various biological properties. Most of the compounds exhibit strong cytotoxicity with IC_{50} values ranging from 0.62 to 10 μM against MDA-MB-435, HepG2 and HCT-116 cancer cell lines [94].

A new member of the roridin family, 12,13-deoxyroridin E isolated from the *Myrothecium roridum* derived from a submerged woody sample [95]. The roridin class of trichothecenes is potently cytotoxic and showed IC_{50} values of 25 and 15 ng ml^{-1} in HL-60 and L1210 cell lines, respectively. The ethyl acetate extract of a new deuteromycete, *Acremonium neocaledoniae* derived from driftwood sample exhibited potent cytotoxicity, which was attributed to the known metabolites verrucarin A, isororidin A, and the new verrol 4-acetate. Verrol 4-acetate showed fairly potent cytotoxic activity against a KB cell line (IC_{50} = 400 ng ml^{-1}) [96]. Isomeric peptide, aspergillamides A isolated from a sediment-derived strain of *Aspergillus* sp. exhibited moderate cytotoxicity against HCT-116 cells (IC_{50} = 16 μg ml^{-1}) [97].

Sansalvamide isolated from a *Fusarium* sp. derived from the surface of the seagrass *Holodule wrightii* [98]. Sansalvamide showed the cytotoxicity with IC_{50} of 27.4 μg ml^{-1} against the NCI's 60-cell line panel, with some selectivity against a colon cancer cell line COLO 205 (IC_{50} = 3.5 μg ml^{-1}) and a melanoma cell line SK-MEL-2 (IC_{50} = 5.9 μg ml^{-1}). *N*-methylsansalvamide was subsequently isolated from a different strain of *Fusarium* obtained from the Caribbean green alga *Avrainvillea* sp. [99]. *N*-methylsansalvamide showed growth inhibition with a GI_{50} of 8.3 μM against NCI's cell line panel, while sansalvamide had a GI_{50} of 3.6 μM. Fellutamides A and B were isolated from the mycelium of *P. fellutanum* derived from the gastrointestinal tract of the marine fish *Apogon endecataenia* [100]. Fellutamides A and B showed cytotoxicity against several cell lines, but showed the most activity against P388 murine leukemia exhibiting IC_{50} values of 0.2 and 0.1 μg ml^{-1}, respectively. 11- and 4′-epichaetomugilin A isolated from a strain of *Chaetomium globosum* has originally isolated from the marine fish *Mugil cephalus*. 11-Epichaetomugilin A weakly inhibited the growth of cultured P388 cells and HL-60 cells with IC_{50} of 88.9 and 66.7 μm, respectively [101].

Coriolin B and three new chlorinated cyclic sesquiterpenes have been isolated from marine fungi species. Biological studies revealed that these metabolites strongly inhibited human breast and central nervous system cancer cell lines with IC_{50} values of 0.7 μg (breast) and 0.5 μg (neuroblastoma) [102]. An ethanol extract of a static culture of *Aspergillus niger* isolated from the Mediterranean sponge *Axinella damicornis* yielded a new secondary metabolites bicoumanigrin A, which have *in vitro* anti-proliferative activities on human cancer cell lines [103]. Further, *Aspergillus versicolor* isolated from a marine sponge lead to isolate a cytotoxic lipopeptide

which have been tested against five human tumor cell lines (A549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF 498, human CNS cancer; and HCT15, human colon cancer). The lipopeptide significantly acts against XF498 and HCT15 cell lines [104]. Another two new cyclodepsipeptides which have strong anti-proliferative activity against pancreatic tumor cells have been isolated from a marine sponge-derived fungus, *Scopulariopsis brevicau*. These compounds have been patented as a potential anti-tumor drug [105]. Epoxyphomalin A and B, two new prenylated polyketides with unusual structural features have been isolated from the marine-derived fungus *Phoma* sp. Epoxyphomalin A showed superior cytotoxicity at nanomolar concentrations toward 12 of a panel of 36 human tumor cell lines [106]. However, detail studies to be conducted to confirm the molecular mechanism by which compound exhibited their cytotoxicity.

13.3 Conclusion and Future Perspectives

The biggest challenges in the field of cancer biology are to discover an antitumor drug which has the ability to kill the cancer cells without any side effects to the surrounding cells. The marine fungus is having the ability to produce chemically unique bioactive molecules, which is supported by the identification of new antitumor metabolites through the application of classical screening and isolation techniques. Here we revealed that cancer initiation, developments and curing process could be effectively mediated with marine-derived fungal metabolites. Although, these metabolites show potent anticancer activity, efforts are required to gain deeper knowledge regarding the mechanism of action by which the compound inhibits the various signal transduction pathways linked to cellular processes such as inflammation, cell differentiation and survival, carcinogenesis, and metastasis. Thus, comprehensive *in vitro* as well as *in vivo* studies have to be conducted to reveal the mechanism of action of isolated compounds. Furthermore, the large scale productions of these compounds are required to meet the demand for clinical trials and drug development. Combining genetic and metabolic engineering will be the future solution for commercial production of these compounds. Integration between combinatorial biochemistry and computer based molecular modeling designs along with post genomic technologies could be used for sustainable production of these metabolites. Combinatorial biosynthesis involving introduction of novel biosynthesis genes into microorganisms will result in the synthesis of the novel metabolites due to the effect of new enzymes on the metabolic pathways. Thus modern microbial genetics and bioinformatics help to overcome supply and sustainability issues from the past and to promote marine fungal secondary metabolites to a well-recognized alternative for future drug discovery programs.

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Chapter 14
Toluquinol, A Marine Fungus Metabolite, Inhibits Some of the Hallmarks of Cancer

Melissa García-Caballero, Miguel Ángel Medina and Ana R. Quesada

Abstract Ten general hallmarks of cancer have been proposed so far: sustaining proliferative signaling, evading growth suppressors, resisting apoptosis, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability and mutation, tumor promoting inflammation, avoiding immune destruction and deregulating cellular energetic. Targeting the mentioned "hallmarks" in a tumor can block cancer's ability to grow and metastasize. Thus, the better understanding of cancer biology has allowed the development of targeted therapies and numerous patients have been benefited so far. The best strategy would be the use of drugs or drug combinations that can target multiple hallmarks at the same time. The chemical and biological diversity of the marine environment is being exploited aiming to discover new anticancer drugs. Toluquinol is an example of a marine compound with antitumor properties. Isolated from the marine fungus *Penicillium* sp. HL-85-ALS5-R004, this compound inhibits the proliferation of actively growing tumor cells, blocks angiogenesis in vitro and in vivo, and induces apoptosis in tumor and endothelial cells. Taken together, these data indicate that toluquinol inhibits several hallmarks of cancer, essential for tumor progression and invasion, underscoring its potential pharmacological utility for new cancer therapies.

Keywords Toluquinol · Angiogenesis inhibitor · Antiproliferative · Apoptosis · Cancer · Marine *Penicillium*

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14.1 Hallmarks of Cancer

Cancer and tumor metastasis are the main cause of death worldwide in both men and women claiming over 7 million lives each year [1]. In the US, for example, lifetime probability of developing cancer is ~44% for men or ~38% for women, respectively [2]. In most cases, localized tumors can be successfully treated by surgery, radiotherapy and other more selective methods. In contrast, medicine is often helpless in the face of metastatic cancer. For this reason, current cancer research mainly focuses on the identification of specific agents with a potential to suppress tumor growth and prevent the processes that cause benign tumors to gain metastatic competence and begin to spread to distant organs [3].

There are over 200 different types of cancers that affect virtually every organ. In spite of the extreme diversity of oncologic diseases, they share some fundamental features. In 2000, Douglas Hanahan and Robert Weinberg published a review in which they suggested that all cancers share six common phenotypes or hallmarks [4]. This article appeared in the Millennium issue of the journal Cell, and it went on to influence a generation of cancer researchers. The article has been referenced by others over 10,000 times, and was downloaded 20,000 times a year between 2004 and 2007 (http://scienceblog.cancer-researchuk.org/2010/11/10/-ncri-conference-the-hallmarks-of-cancer/). Nowadays, their ideas are regarded as established facts and new hallmarks of cancer have been included in this list. In 2011 Hanahan and Weinberg published in the same journal the revised work "Hallmarks of cancer: the next generation", in which they refined and extended the concept of cancer hallmarks to provide a useful conceptual framework for understanding the complex biology of cancer [5].

The ten hallmarks of cancer so far described are depicted in Fig. 14.1. The first six hallmarks of cancer [4] are commented in sections 1.1 to 1.6 and the four "new" ones [5] are discussed in sections 1.7 to 1.10.

14.1.1 Self-Sufficiency in Relation to Growth Signals

The cells of our bodies are members of a huge support group. They constantly supply their neighbors with encouraging messages, and when stimulatory signals such as extracellular matrix (ECM) molecules, diffusible growth factor and signaling molecules implicated in the cellular adhesion/interaction are bound by transmembrane receptors, normal cells can acquire an active proliferative status from a quiescent state. In contrast, tumor cells show a reduced dependence on external stimulating signals from their microenvironment. There are different ways to sustain proliferative signaling in cancer cells: they may elevate the number of surface receptors; they can induce structural alterations in the receptor molecules that facilitate ligand-independent firing; they may generate growth factor themselves or send signals to activate normal cells in the tumor-microenvironment; and cancer cells can induce a constitutive stimulation of the cytoplasmic circuitry involved in different signaling pathways, among others [6–9].

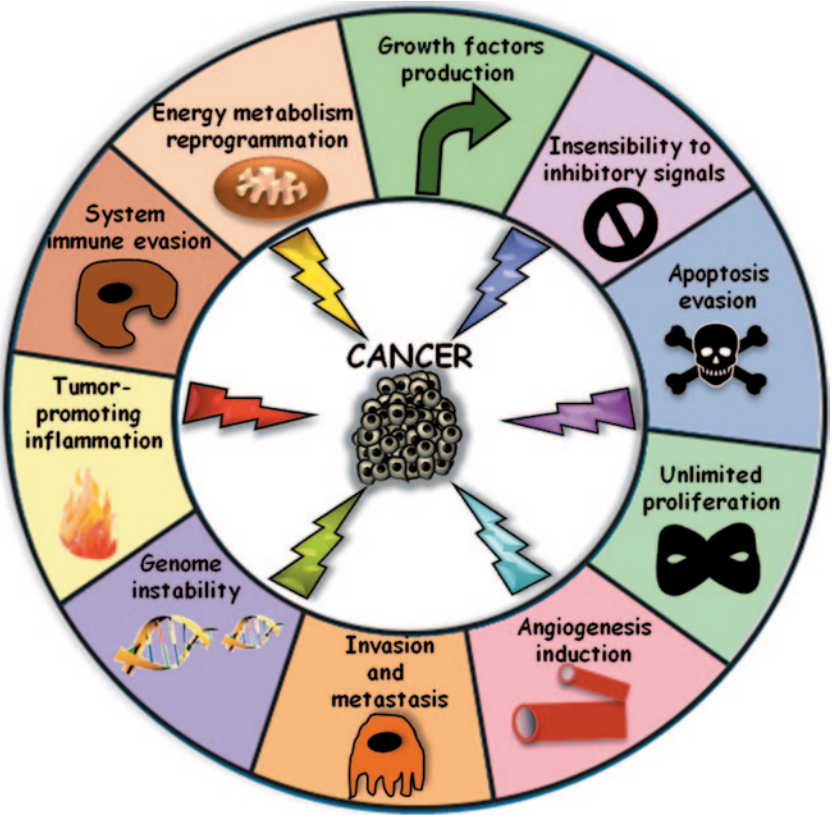


Fig. 14.1 The Hallmarks of cancer. (Adapted from Hanahan and Weinberg (2011) Cell 144:646–674)

14.1.2 Insensitivity to Growth Suppressors

In normal conditions, antiproliferative factors are produced to maintain cellular quiescence and tissue homeostasis. The signals block proliferation by inducing cells to enter in a reversible quiescent (G_0) state. Tumor suppressor genes act avoiding cell proliferation but sometimes their inactivation leads to an unregulated cell growth. Retinoblastoma-associated (RB) and TP53 proteins have been described as two tumor suppressors which play key roles in the mechanisms that help cells to decide between their proliferation and their senescence and apoptotic way [10–12].

It has been demonstrated that cell contacts in a dense populations inhibit cell growth progression. In this phenomenon both, Merlin, the product of the NF2 gene [13, 14] and the LKB1 epithelial polarity protein [15–17] are key players. Other mechanism that confers antiproliferative properties to cells is the TGF-β pathway [18–20]. Cancer cells must evade the mentioned programs that exert a negative regulation on cell progression in order to growth uncontrollably.

14.1.3 Apoptosis Evasion

The ability of tumor cells to increase in number is not only due to their high proliferation rate but also to their capability to resist cell death. “Apoptosis”, also called “programmed cell death” or “cell suicide” is a physiological phenomenon that occurs throughout life during development. The apoptotic machinery is composed of both upstream regulators and downstream effector components [21].

There are factors involved in the reception and processing of extracellular death-inducing signals (the extrinsic apoptotic pathway) like Fas ligand/Fas receptor or TNF ligands/TNF receptor, and others involved in the monitoring and integration of intracellular signals (the intrinsic apoptotic pathway) in which the mitochondria is involved. The most important apoptosis effectors are the caspases, a family of cysteine-aspartic proteases that can be classified into two groups, initiator and executioner caspases, according to their function [22]. Caspases-8 and -9 are initiator caspases responsible for the activation of the cascade of proteolysis. This cascade culminates in the activation of effector caspases that carry out the execution phase of apoptosis. In this phase cell suffers morphological and biochemistry changes such as chromatin condensation, DNA damage, externalization of phosphatidylserine residues in membrane bilayers, cell cycle alterations, mitochondrial transmembrane potential reduction, intracellular acidification, reactive oxygen species production and cellular proteins proteolysis [23], among others. Finally cell is disassembled and then consumed by the surrounding cells and by phagocytic cells. Apoptosis is controlled by a dialogue between the pro- and the anti-apoptotic members of the Bcl-2 family of regulatory proteins, which include Bcl-xL, Bcl-w, Mcl-1, A1 and cytochrome *c* as inhibitors; and Bax, Bim, Puma and Bak as activators [21, 24].

Tumor cells prevent their self-destruction circumventing normal apoptotic mechanisms. Several abnormality sensors that play key roles in tumor development have been identified [21, 25, 26]: the loss of TP53 tumor suppressor function, the upregulated expression of Bcl-2, Bcl-xL, the downregulated expression of Bax, Bim and Puma, and a significant loss or inactivation of lead members in the caspase family.

Other two cell death phenomena should be mentioned, autophagy and necrosis. Autophagy plays a central role in regulating important cellular functions such as cell survival during starvation and cellular stress deficiency [27, 28]. In this process cells break down cellular organelles, such as ribosomes and mitochondria, recycling the resulting catabolites for biosynthesis and energy metabolism. Intersections between the regulatory circuits governing autophagy, apoptosis and cellular homeostasis have been observed [27, 29, 30]. Although autophagy seems to offer another barrier that needs to be eluded during tumor progression, the functional relevance of autophagy in cancer remains controversial [31]. On the other hand, necrosis is the cell death that leads to cell destruction and release of its intracellular content into the surrounding tissue microenvironment. This process appears under a genetic control and it probably helps to tumor progression and invasion [32]. The necrotic process attracts immune inflammatory cells, which can produce growth factors and stimulate tumor proliferation, angiogenesis and invasion [32–35].

14.1.4 Unlimited Replicative Potential

Normal cell lineages have a limited number of cell division cycles and this limitation is associated with two different processes that block cell proliferation: senescence (an irreversible, viable but quiescent, unproliferative state) and crisis (a state that induces cell death) When cells escape from a crisis state and start to proliferate unlimitedly they are denominated as immortalized cells. This property is acquired by cancer cells. Senescence is a mechanism against neoplasia and it functions as a protective barrier to tumor expansion [36–41].

Related to the replicative potential are the telomeres, regions of repetitive nucleotide sequences located at the end of each chromatid, which protect the chromosomal DNA from deterioration or from fusion with neighboring chromosomes. Telomeres are shortened during each cell generation and this progressive reduction provokes the loss of their ability to protect the ends of chromosomes and consequently, the DNA instability and the entrance into a crisis stage [41, 42]. Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in non immortalized or somatic cells, but expressed at functionally significant levels in germ cells and in the majority of spontaneously immortalized cells, including human cancer cells. This fact allows them to maintain their telomere length and proliferative potential, avoiding the events that trigger senescence and crisis/apoptosis. Frequently, cancer cells undergo a substantial number of successive telomere-shortening cell divisions during their evolution from normal cells-of-origin, and consequently, the development of some human neoplasias may be prevented by telomere-induced crisis before they become a macroscopic tumor [43, 44]. However, the delayed acquisition of telomerase function serves to generate tumor-promoting genomic alterations and its subsequent activation stabilizes the mutations and confers the unlimited replicative capacity that cancer cells require in order to generate clinically apparent tumors [45]. Other functions of telomerase, and in particular of its protein subunit TERT, are telomere-independent and they are related with the capability to amplify signal by the Wnt pathway, the contribution as a cofactor of the β-catenin/LEF transcription factor complex [46], the induction of cell proliferation and/or apoptosis evasion [47], the contribution in DNA-damage repair [48] and RNA-dependent RNA polymerase function [49]. These additional telomerase properties could collaborate with the tumorigenesis process.

14.1.5 Angiogenesis Induction

During embryogenesis, the development of the vasculature involves the creation of new blood vessels from endothelial precursors by a process termed vasculogenesis [50]. Once the primary vascular network is formed the sprouting of new blood vessels from pre-existing ones occurs by angiogenesis. Endothelial cells become activated in response to an angiogenic stimulus and undergo a series of phenotypic changes, leading them to release proteases. Such proteases will allow endothelial

cells to degrade the extracellular matrix, migrate, proliferate, avoid apoptosis, that could be triggered by the loss of survival signals, and, finally, differentiate to form new vessels. Angiogenesis is a highly regulated process, very active in embryos but largely quiescent in adults, limited to some processes related to reproductive cycles, wound healing and bone repair. Nevertheless, a deregulated and persistent activation of the "angiogenic switch" is related to diseases such as proliferative retinopathies, psoriasis and rheumatoid arthritis and is essential for tumor growth and metastasis [51]. Like normal tissues, tumors require sustenance in the form of nutrients and oxygen, as well as an ability to remove metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by angiogenesis, provide the sustenance in the form of nutrients and oxygen as well as an ability to remove metabolic wastes and carbon dioxide that tumors need to their growth. Moreover, the new blood vessels present in the tumor microenvironment help in the neoplastic growth [52].

There are many proangiogenic factors. Vascular endothelial growth factor-A (VEGF-A) is the prototypic pro-angiogenic factor and the major regulator of physiological and pathological angiogenesis. VEGF-A is bound by tyrosine kinase receptors VEGFR-1, -2 and -3 [53]. VEGF gene expression can by upregulated both by hypoxia and by oncogene signaling [54–56]. Opposing the activities of angiogenic factors, a large number of endogenous anti-angiogenic factors have been functionally characterized [57]. Angiogenesis results as an imbalance of pro- over anti-angiogenic factors.

Angiogenesis is stimulated in an early stage of cancer development, but tumor vasculature differs from the normal vasculature showing abnormal characteristics, including fragility, chaotic arrangements and imperfect vessel walls due to discontinuous endothelial cell lining and weak investiture with vascular smooth muscle cells. Additionally, pericytes, the supporting cells that are closely linked to the outer surfaces of the endothelial tubes in normal tissue vasculature, present poor connections in tumor-vasculature, although their presence is necessary to maintain a functional tumor vasculature [58, 59]. On the other hand, endothelial cells in tumor vasculature are usually irregularly shaped, forming an uneven luminal layer with loose interconnections and focal intercellular openings [60]. These characteristics allow macromolecule diffusion and aid the metastatic process by facilitating the entrance of tumor cells into the bloodstream [61]. However, different gradations of the angiogenic switch have been observed in cancers because the switching mechanism can vary in its form, even though the net result is a common inductive signal (e.g., VEGF) [62, 63]. Finally, it is necessary to emphasize the role of different cell types originated in the bone marrow such as neutrophils, mast cells, macrophages and myeloid progenitors, which infiltrate in primary lesions and turn on the angiogenic switch to stimulate the pathological angiogenesis in the tumor microenvironment [64–68].

Besides tumor-associated blood vessels, it is well known that tumor lymphatic vasculature serves as a major route for cancer cell spreading from primary sites to lymph nodes and, further, to distant tissues. Several human solid tumors, such as breast cancer, melanoma or prostate cancer, are now considered to be

lymphangiogenic because they have the ability to induce their own lymphatic vessels to establish metastasis [69].

14.1.6 Invasion and Metastasis

The process of invasion and metastasis can be summarized as a succession of subsequent discrete steps, often termed the invasion-metastasis cascade [70, 71]: local invasion, intravasation by cancer cells into nearby blood and lymphatic vessels, pass of cancer cells through the blood and lymphatic systems, escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), formation of small nodules of cancer cells (micrometastases), and finally, growth of micrometastatic lesions into macroscopic tumors.

Alterations in molecules involved in the cell-to-cell and cell-to-ECM adhesion promote the invasion-metastasis cascade steps. For example, the loss, mutation or inactivation by tumor cells of E-cadherin, contribute to the ability of cancer cells to invade and metastasize [72]. *N*-cadherin appears upregulated in many invasive carcinomas [73]. Furthermore, transcription factors like Snail, Slug, Twist, and Zeb1/2, can induce the developmental regulatory program "epithelial–mesenchymal transition" (EMT), and therefore the acquisition of migratory, invasion an apoptosis resistance abilities by epithelial cells [74–80]. However, this program offers certain plasticity because it is possible the reverse process, named the mesenchymal–epithelial transition (MET), in which carcinoma cells, that have undergone an EMT during initial invasion and metastatic dissemination, acquire a non-invasive state in their new niche [81].

Metastasis involves interactions between cancer cells and the local microenvironment. Metastatic cells rely on extrinsic signals from a supportive microenvironment to establish themselves as new colonies at a distant site. In an autonomous way, cancer cell could not have the malignant and metastatic phenotype in the acquired capability for invasive growth and metastasis [64, 82]. For example, mesenchymal stem cells (MSCs) present in the tumor stroma secrete the chemokine CCL5 that acts reciprocally on cancer cells to stimulate invasive behavior [83]. In addition, tumor-associated macrophages (TAMs) can produce matrix-degrading enzymes, such as metalloproteinases and cysteine cathepsin proteases, growth factors and chemokines, which facilitate the metastatic dissemination of the cancer cells [84]. Cancer cell invasion can be carried out by different strategies and depends on the type of cancer. The EMT program regulates a particular type of invasiveness that has been termed "mesenchymal." Other two mechanisms are the "collective invasion" and the "amoeboid invasion". The first one involves nodules of cancer cells advancing en masse into adjacent tissues and is characteristic of, for example, squamous cell carcinomas (this invasion mechanism is rarely metastatic). In the second one, cancer cells show morphological plasticity, finding their way through the interstices in the extracellular matrix [85]. Therefore, the facilitation of cancer cell invasion by inflammatory cells can be considered a new concept in oncology [86].

At least initially, the most disseminated cancer cells are poorly adapted to the foreign microenvironment of the tissue in which they have landed. Therefore, each type of disseminated cancer cell may need to develop its own set of ad hoc solutions, resulting in a huge variety of distinct colonization programs in the foreign tissue [87]. Although cancer cells can clearly disseminate from pre-neoplastic lesions and seed the bone marrow and other tissues, when and where cancer cells develop the ability to colonize foreign tissues as macroscopic tumors and develop into pathologically significant macrometastases, remains unproven [88]. Some studies describe genes that act as metastatic indicators linked to the capability to establish macroscopic metastases in specific tissues [89]. However, the colonization is dependent not only on the metastatic cells, but also the stromal components in the colonized tissue.

14.1.7 Genome Instability and Mutations

Genomic instability is a characteristic present in all cancers and it is clearly an enabling characteristic that is causally associated with the acquisition of hallmark capabilities. There are various forms of genomic instability. Most cancers have chromosomal instability (CIN), which refers to the high rate by which chromosome structure and number changes over time in cancer cells compared with normal cells. Microsatellite instability (MSI; also known as MIN) is a form of genomic instability characterized by the expansion or contraction of the number of oligonucleotide repeats present in microsatellite sequences [90]. In addition, the accumulation of mutations can be accelerated by compromising the surveillance systems that normally monitor genomic integrity and force genetically damaged cells into either senescence or apoptosis [91].

Genomic instability in tumor lesions has been specially attributed to the mutations in caretaker genes, that is, genes that primarily function to maintain genomic stability. The classical caretaker genes are DNA repair genes and mitotic checkpoint genes. The tumor suppressor gene TP53 could also be considered as caretaker gene because of its function in the DNA damage response [92]. The repertoire of alterations in these caretaker genes includes those whose products are involved in (1) detecting DNA damage and activating the repair machinery, (2) directly repairing damaged DNA, and (3) inactivating or intercepting mutagenic molecules before they have damaged the DNA [93]. Thus, these caretaker genes can lose their properties as tumor suppressor genes in the course of tumor progression, either through inactivating mutations or via epigenetic repression [94]. It is widely accepted that defects in genome maintenance and repair systems are selectively favorable and therefore, instrumental for tumor progression. They accelerate the rate at which evolving premalignant cells can accumulate advantaged genotypes.

The loss of telomeric DNA in many tumors generates karyotypic instability and associated amplification and deletion of chromosomal segments [95]. Moreover, telomerase is more than an enzyme related to the unlimited replicative potential hallmark and must also be added to the list of critical caretakers responsible for maintaining genome stability and integrity.

In the past few years, several consortia have begun sequencing the genomes of human cancers taking advantage of the advances in the DNA-sequencing technologies. Currently, thousands of mutations have been identified. These studies have revealed distinctive patterns of DNA mutations in different tumor types [96–98].

14.1.8 Tumor-Promoting Inflammation

Solid tumors consist of neoplastic cells, non-malignant stromal cells, and migratory hematopoietic cells, including cells from the innate and adaptive immune system. Complex interactions between these cell types in this microenvironment regulate tumor growth, progression, angiogenesis and metastasis [99, 100].

Cells and mediators of inflammation form a major part of the tumor microenvironment in all types of cancers. Although it initially was accepted the fact that the immune response was carried out to eradicate tumors, now the paradigm has changed and it is accepted that immune cells can enhance tumorigenesis and progression, helping incipient neoplasias to acquire hallmark capabilities [34, 64]. In some cancers, inflammatory conditions precede development of malignancy; in others, oncogenic change drives a tumor-promoting inflammatory milieu. The catalog of tumor-promoting inflammatory cells now includes macrophage subtypes, mast cells, and neutrophils, as well as T and B lymphocytes [66, 67, 82, 100–103].

Besides differentiated immune cells, partially differentiated myeloid progenitors can also be found in cancer [66], representing the link between circulating cells from bone marrow origin and the differentiated immune cells typically found in normal and inflammed tissues. These cells also stimulate angiogenesis and tumor progression [64]. In contrast to a normal lesion or infection, where the immune inflammatory cells appear transiently and then disappear, in neoplasias they are persistent. This inflammatory status is associated with tumor progression [104].

Cytokines, such as macrophage migratory inhibitory factor (MIF), TNF-α, interleukin (IL)-6, IL-17, IL-12, IL-23, and IL-10 are major mediators of communication between cells in the inflammatory tumor microenvironment. However it is known that neoplastic cells often overexpress proinflammatory mediators including growth factors, such as VEGF, fibroblastic growth factor (FGF)-2 and epidermal growth factor (EGF); proteases, such as matrix metalloproteinases (MMPs), cysteine cathepsin proteases and heparanase; eicosanoids and chemokines [99].

14.1.9 Evasion of Immune Destruction

Cancer is characterized by the accumulation of a variable number of genetic alterations and the loss of normal cellular regulatory processes [105]. These events have long been known to result in the expression of neoantigens, differentiation antigens, or cancer testis antigens, which can lead to presentation of peptides, bound to major histocompatibility class I (MHCI) molecules on the surface of cancer cells,

distinguishing them from their normal counterparts [106]. Thus, the immune system plays a crucial role in resisting or eradicating formation and progression of incipient neoplasias, late-stage tumors, and micrometastases. Cells and tissues are continuously monitored by an ever-alert immune system, and such immune surveillance is responsible for recognizing and eliminating the vast majority of incipient cancer cells and thus emerging tumors.

For an anticancer immune response to lead to effective killing of cancer cells, a series of stepwise events must be initiated and allowed to proceed and expand iteratively. These steps have been called as the "cancer-immunity cycle" [106]: first, neoantigens created by oncogenesis are released and captured by dendritic cells (DCs) for processing an anticancer T cell response; next, DCs present the captured antigens on MHCI and MHCII molecules to T cells, promoting the priming and activation of effector T cell responses against the cancer-specific antigens that are viewed as foreign, or against which central tolerance has been incomplete. The nature of the immune response is determined at this stage, with a critical balance representing the ratio of T effector cells versus T regulatory cells, being crucial to the final outcome. Finally, the activated effector T cells traffic to and infiltrate the tumor bed, specifically recognize and bind to cancer cells through the interaction between its T cell receptor (TCR) and its cognate antigen bound to MHCI, and kill their target cancer cell.

Following this reasonable cycle of responses, nobody would develop tumors. However, solid tumors appear because either they avoid their detection by the various arms of the immune system, or they are able to limit the extent of immunological killing, thereby evading eradication. The role of defective immunological monitoring of tumors would seem to be validated by the striking increases of certain cancers in immunocompromised individuals [107]. However, the majority of these cancers are induced by viruses, suggesting that much of the control of this class of cancers normally depends on reducing viral burden in infected individuals, in part through eliminating virus-infected cells. The immune system acts as an important barrier to tumor formation and progression, at least in more than the 80% of non-virus induced cancer [108].

Epidemiologic studies have supported the existence of antitumor immune responses in some forms of human cancer [109–111]. Thus, patients with colon and ovarian tumors with cytotoxic T lymphocytes (CTLs) and natural killer cells (NK) infiltrations have a better prognosis than those that lack such abundant killer lymphocytes [111, 112]. Furthermore, some immunosuppressed organ transplant recipients have been observed to develop donor derived cancers, suggesting that in the ostensibly tumor-free donors, the cancer cells were held in a dormant state by a fully functional immune system [113].

Research in this topic has demonstrated that cancer cells can evade immune destruction by disabling immunological components that have been designed to eliminate them. Thus, deficiencies in the development or function of CD8+CTLs, CD4+Th1 helper T cells, or (NK) cells have increased tumor incidence [114], or the myeloid-derived suppressor cells and regulatory T cells recruitment can suppress the functions of cytotoxic lymphocytes [115].

14.1.10 Energy Metabolism Reprogramming

Under aerobic conditions, normal cells consume glucose which is transformed into pyruvate via glycolysis in the cytosol and thereafter to carbon dioxide in the mitochondria. Under anaerobic conditions, glycolysis is favored and relatively little pyruvate is dispatched to the oxygen-consuming mitochondria. Otto Warburg observed in the 1920s an anomalous characteristic of cancer cell energy metabolism due to cancer cells predominantly produced ATP/energy through the glycolytic pathway rather than through the tricarboxylic acid (TCA) cycle, even in the presence of adequate oxygen [116]. This phenomenon is known as the "Warburg Effect" or the "aerobic glycolysis" and is characterized by increased glucose uptake and reliance on glycolysis for ATP production despite available oxygen source [117]. This characteristic seems to be a general property of highly malignant tumors, independent of their carcinogenic origin, in fact, significant activation of glycolysis is observed in human cancer tissues through the use of a leading-edge metabolomic profiling method based on positron emission tomography (PET) with a radiolabeled analog of glucose (18F-fluorodeoxyglucose, FDG) as a reporter.

Since complete oxidation of glucose carbons through the TCA yields 15–16 times more ATP than glycolysis, the question of why cancer cells use glycolysis in spite of its inefficient energy production arises. It is believed that the glycolysis rate increases in order to match the increased anabolic needs of the rapidly proliferating cells for precursor metabolites, including nucleosides and amino acids, among others, to biosynthesize macromolecules and organelles required for assembling new cells [118]. In addition, aerobic glycolysis in cancer cells excrete lactate which does not contribute to the production of precursor metabolites, but it is used by cells present in the tumor microenvironment. Interestingly, some tumors have been found to contain two subpopulations of cancer cells working symbiotically that differ in their energy-generating pathways. One of them consists of glucose-dependent ("Warburg-effect") cells that live under hypoxic conditions and secrete lactate, whereas the other cell subpopulation, better-oxygenated, that preferentially import and utilize the lactate produced by their neighbors as their main energy source, employing part of the citric acid cycle to do so [119]. Intratumor oxygenation, ranging from normoxia to hypoxia, is not static in tumors and it fluctuates temporally and regionally [120], likely as a result of the instability and chaotic organization of the tumor-associated neovasculature.

But not only glucose metabolism is changed in cancer. In fact, there is an overall metabolic reprogramming, also affecting glutamine and lipid metabolism [121, 122].

Current literature shows that mutation and other alterations in several transcription factors and metabolic enzymes are crucial in mediating the aberrant metabolic behavior of tumor cells. Some examples of these alterations promoting the metabolic reprogramming are: glucose transporters upregulation, notably GLUT1, which substantially increases glucose import into the cytoplasm [123]; increase of HIF1α and HIF2α transcription factors levels, which in turn upregulate glycolysis [124, 125]; isocitrate dehydrogenase 1/2 enzymes activating (gain-of-function) mutations

[126]; succinate dehydrogenase and fumarate hydratase mutations which increase the accumulation of succinate and/or fumarate, two metabolites that can inhibit the degradation of HIF1α, and increase the expression of some specific genes involved in glycolysis, angiogenesis, and cell proliferation [127]. The majority of these aberrations are related to activated oncogenes (e.g., RAS, MYC), mutant tumor suppressors (e.g., TP53) and the hypoxia conditions [123].

Kareva and Hahnfeldt have recently suggested that metabolic reprogramming and immune evasion are two linked hallmarks [128]. Through upregulation of glycolysis and the consequent lowering of pH in the tumor microenvironment, tumors can take advantage of a pH control system to gain control of the immune system and suppress both cytotoxic and antigen-presenting cells.

Finally, new studies have proposed to increase the list of hallmarks including these others.

- –*Aberrant alternative splicing:* The vast majority of human genes are alternatively spliced, and, not surprisingly, aberrant alternative splicing is increasingly linked to cancer. Splice isoforms often encode proteins that have distinct and even antagonistic properties. The abnormal expression of splice factors and splice factor kinases in cancer changes the alternative splicing of critically important pre-mRNAs. Therefore, the aberrant alternative splicing should be added to the growing list of cancer hallmarks [129].
- –*Long non-coding RNAs (lncRNAs):* Up to 70% of our genome is transcribed into RNA that does not serve as templates for proteins. Long ncRNAs are deregulated in several human cancers and show tissue-specific expression. In addition, some of these functions are associated with the stimulation of cell proliferation, new vessels formation or apoptosis evasion [130].

14.2 Targeted Cancer Therapies: Fighting with the Hallmarks of Cancer

In order to develop and evolve towards a malignant and invasive status, cancer cells must acquire modifications in almost each of the 10 processes described above. This underlines the complex cellular and molecular nature of cancer, resulting from a number of coordinated and complementary functional changes in multiple pathways. Moreover, only cells with a high developmental plasticity may sustain the complex situations supporting the coordinated perturbation of multiple hallmarks [131].

A major challenge for the development of effective anticancer therapies is the complexity of the disease. The action of most cancer drugs is directed against a limited number of well-established targets, reflecting the difficulty, time and cost involved in the identification and validation of new key molecular targets that underlie the pathways that are crucial for the disease development [132]. Advances in technologies such as RNA interference (RNAi) are among those developments

that have allowed for an extension of therapeutic strategies from cytotoxic and hormonal therapies to a broader range of molecular targets.

An optimal anticancer strategy would be the one that selectively kills tumor cells while sparing normal cells. Although the two traditional cancer treatments, chemotherapy and radiation, were not specifically designed to target tumor cells [133], they take advantage of the enhanced sensitivity to either DNA damage or cell cycle arrest due to the inherent replication stress in cancer cells [134], seeking an optimal dose and schedule to kill tumor cells while minimizing the damage to normal cells. Nevertheless, the goal of targeted cancer therapies is to block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. They are also called "molecular targets therapies" or "biological therapies" of cancer. By focusing on molecular and cellular changes that are specific to cancer cells, targeted cancer therapies may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells. Targeted cancer therapies are being studied for being used alone, in combination with other targeted therapies, and in combination with other traditional cancer treatments, such as chemotherapy or radiotherapy.

Targeted cancer therapies interfere with cancer cell division (proliferation) and spread in different ways. Some of them focus on proteins that are involved in cell signaling pathways, which form a complex communication system that governs basic cellular functions and activities, such as cell division, migration, responses to specific external stimuli, and even cell death. By blocking signals that tell cancer cells to grow and divide uncontrollably, targeted cancer therapies can help stop cancer progression and may induce cancer cell death through apoptosis. Other targeted therapies can cause cancer cell death directly, by specifically inducing apoptosis, or indirectly, by stimulating the immune system to recognize and destroy cancer cells and/or by delivering toxic substances directly to the cancer cells. The development of targeted therapies, therefore, requires the identification of key targets playing a crucial role in cancer cell growth and survival. For this reason, targeted therapies are often referred to as the product of a "rational drug design" [133].

Once a target has been identified, a therapy must be developed. Most targeted therapies are either small-molecule drugs or monoclonal antibodies. Small-molecule drugs are typically able to diffuse into cells and can act on targets that are found inside the cell. Most monoclonal antibodies cannot easily cross cell membranes and are directed against targets that are outside the cell or on the cell surface.

Many targeted cancer therapies have been approved by the US Food and Drug Administration (FDA) for the treatment of specific types of cancer, others are being studied in clinical trials and many more are in preclinical testing. Some targeted therapies block specific enzymes and growth factor receptors involved in cancer cell proliferation (e.g., Trastuzumab-Herceptin®-, Erlotinib-Tarceva®- or Cetuximab-Erbitux®-); others modify the function of proteins that regulate gene expression and other cellular functions (e.g., Romidepsin-Istodax®- or Alitretinoin-Panretin®-); some induce cancer cells to undergo apoptosis (e.g., Pralatrexate-Folotyn®- or Bortezomib-Velcade®-); others block the growth of tumor-angiogenesis (e.g., Bevacizumab-Avastin®-, Sunitinib-Sutent®- or Pazopanib-Votrient®-); or

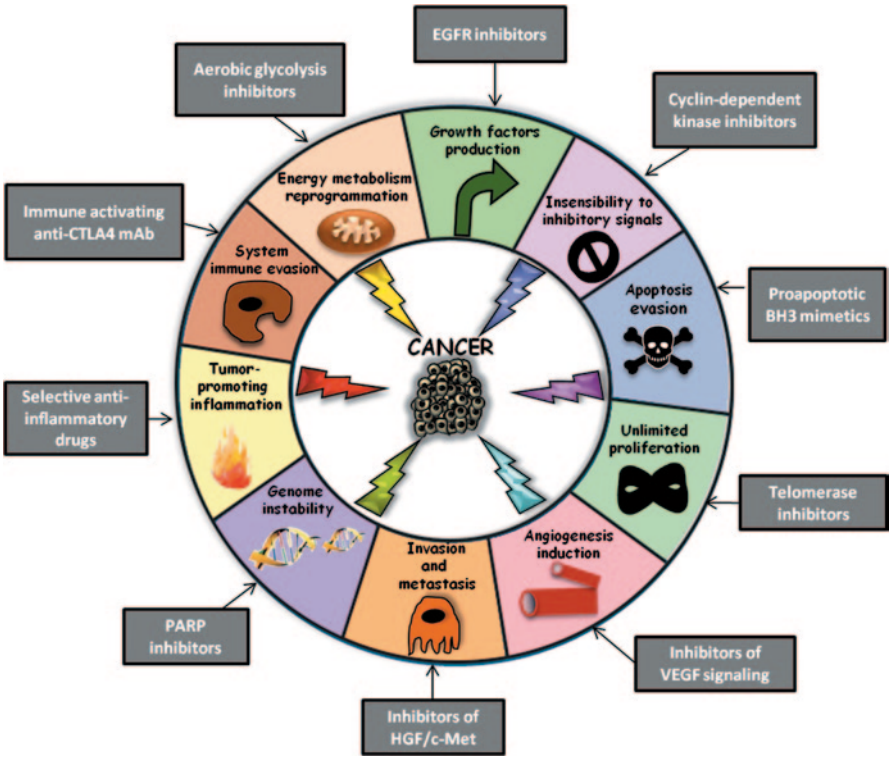


Fig. 14.2 Therapeutic Targeting of the Hallmarks of Cancer. Examples of drugs that can interfere with each of the hallmarks of cancer. The targeted therapies have been developed to inhibit the acquired capabilities necessary for tumor growth and progression. (Adapted from Hanahan and Weinberg (2011) Cell 144:646–674)

help the immune system to destroy cancer cells (e.g., Ipilimumab-Yervoy™- or Rituximab-Rituxan®-), and others includes monoclonal antibodies that specifically deliver toxic molecules to cancer cells (Tositumomab and 131I-tositumomab-Bexxar®- or Denileukin diftitox-Ontak®-) (http://www.cancer.gov/cancertopics/factsheet/Therapy/-targeted). Nevertheless, targeted therapies have some limitations, including the potential for cancer cells to develop resistance to them. This adaptation can be accomplished by mutation, epigenetic reprogramming or remodeling of the stromal microenvironment, allowing tumor cells to reestablish the functional capability. In addition, in response to therapy, cancer cells may reduce their dependence on a particular hallmark capability, becoming more dependent on another.

The future of cancer therapies may lie in simple drugs targeting the communication routes interconnecting the hallmarks of cancer processes and, specifically, modulating critical factors in a way that enables them to express their protective potential (Fig. 14.2). For this reason targeted therapies may work best in combination, either with other targeted therapies or with traditional anticancer therapies.

14.3 Marine Environment as Source of New Antitumor Compounds

Although advances in the field of chemo-preventive and therapeutic medicine have been made regularly over the last 10 years, the search for novel anticancer treatments continues. For many years, research has essentially focused on plants and terrestrial microorganisms, mainly because these specimens are easily available and folk traditions have described beneficial effects from their use for the treatment of many diseases. In contrast, the first marine bioactive compounds were isolated in the early 1950s and the focus on the discovery of marine molecules only began in the mid-twentieth century.

The ocean, considered "the mother of life", covers over 70% of the Earth's surface and is an unexplored area of opportunity. Oceans comprise some areas like coral reefs with a huge biodiversity. A recent census of marine life, carried out with the participation of 2700 scientists from over 80 nations, in which the diversity, distribution and abundance of marine organisms was taken into account, yielded the discovery of over 6000 potentially novel species [135–137]. As a consequence of those research efforts, it is clear that the marine environment represents an important and rich source of unknown natural compounds whose medicinal potential must be evaluated. Extreme conditions like low temperatures and high pressures, present in the marine habitat, support the production of secondary metabolites or bioactive compounds and the ability of marine organisms to adapt to the difficult life conditions. This adaptation has resulted in the development of unique biochemical features which may find their application in virtually all industries. Moreover, marine organisms exhibit a variety of molecules with unique structural characteristics, not found in terrestrial natural products. The development of culture techniques and media formulations for the culture in the laboratory of marine microorganisms has fueled their use by pharmaceutical industries [138].

According to the records of the FDA and those of the European Agency for the Evaluation of Medicinal Products (EMA), from 1940 to 2010, among the 113 drugs (including natural compounds and their derivatives) approved in cancer treatment, three were of marine origin (cytarabine, trabectedin and eribulin) [139]. In 2012 it was estimated that 118 marine anticancer molecules were presently in pre-clinical trials and 22 other molecules were undergoing clinical trials [140]. In addition, pharmaceutical companies including PharmaMar, Biomar, Bedford, Enzon, Eisai Inc., Novartis, Aventis, Eli Lilly, Abbott Inflazyme, Pfizer and Taiho Pharmaceuticals Co., have therapeutic compounds of marine origin under development.

Blunt and colleagues in 2004 made a list about the major sources of biomedical compounds from a marine environment: sponges (37%), coelenterates (21%) and microorganisms (18%) as the major producers, followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%) bryozoans (1%), etc. [141]. The bioactive compounds can work in different ways and some of them act as MMP, HIF, Nuclear Factor-ƙβ (NF- ƙβ), topoisomerase or protein kinase C inhibitors, among others [142].

Although the number of natural products increases every day, only a few marine compounds are currently being used in the clinic. Some reasons for this low success rate include the cytotoxicity that some marine derived compounds show on normal human cell lines, the lack of sufficient amount of the bioactive natural product, troubles to access to the source of the samples, difficulties in the processes related to isolation, purification or synthesis of the compound or poor infrastructure and insufficient capital investment [142]. In order to solve some of the described problems and enhance marine nature compounds, a more integrated approach comprising high-throughput screening (HTS) methods, computational biology and bioinformatics might be useful. The semisynthetic approach, consisting on the change of certainly functional groups in natural compounds to create structural analogues with greater pharmacological activity and with fewer side effects, could be one of the possible alternatives. Genomics and implication of combinatorial biosynthesis can also be applied for discovery and modification of natural marine products [142].

14.4 Toluquinol, A Marine Compound, Inhibits Certain Cancer Cell Capabilities

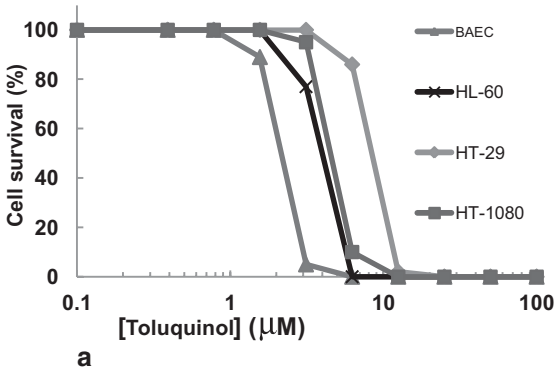
Toluquinol, also denominated 2-methylhydroquinone or 2,5-toluenediol, isolated from the fermentation broth of a marine fungus is an example of a marine-derived compound acting on several of the hallmarks of cancer. In the course of a blind screening, it was initially selected by means of its ability to inhibit the endothelial cell differentiation in vitro [143], as a part of our ongoing research efforts aimed at exploring the biosynthetic potential of rare marine microorganisms.

Although terrestrial fungi have been a rich source of relevant bioactive compounds, produced as secondary metabolites, and used for the treatment of a number of human diseases [144, 145], the fungi species present in the marine environment have been largely unknown in the past. More recently, they have been started to be exploited, revealing themselves as prolific producers of a plenty of structurally unique and biologically active secondary metabolites with potential use as antitumor drugs [146, 147]. In the case of toluquinol, it was extracted from the broth of the marine fungus *Penicillium* sp. HL-85-ALS5-R004, and purified by means of a series of extractions and chromatographic techniques guided by the biological activity assay [144].

With the aim to analyze the biological activities of toluquinol, a battery of in vitro and in vivo experimental procedures were performed [144]. The obtained results clearly showed that this compound interferes with some cellular and molecular mechanisms in tumor and endothelial cells.

14.4.1 Toluquinol Inhibits the Growth of Tumor Cells

Toluquinol interferes with one of the hallmarks of cancer described by Hanahan and Weinberg by impairing the unlimited replicative potential, characteristic of



	BAEC	HL-60	HT-29	HT-1080
IC50 (μM)	2.3 ± 0.7	3.2 ± 0.9	8.6 ± 0.3	5.8 ± 2.3

b

Fig. 14.3 Proliferation inhibition on tumor and endothelial cells by toluquinol. Effect of the incubation for 3 days with toluquinol on the in vitro growth of BAECs (▲), HL60 (X), HT29 (♦), and HT1080 (■). Data are expressed in terms of percentage of growth of toluquinol-treated cells with respect to control cells. The figure illustrates a representative experiment performed with quadruplicate samples. Each point represents the mean of quadruplicates; SD values were typically lower than 10% of the mean values and are omitted for clarity. (B) Half-maximal inhibitory concentration (IC50) values calculated from dose-response curves as the concentration of compound yielding 50% of control cell survival. They are expressed as means ± SD of three independent experiments with quadruplicate samples each. (Adapted from García-Caballero et al. (2013) Biochem Pharmacol 85:1727–1740, with permission from Elsevier)

tumor cells. Figure 14.3 shows that toluquinol represses the proliferative capability of the following three cultured human tumor cell lines: promyelocytic leukemia HL60 cell line, fibrosarcoma HT1080 cell line and colon adenocarcinoma HT29 cell line. The IC_{50} values, which represent the concentrations of toluquinol yielding a 50% of cell growth, were lower than 10 μM in the three cell lines. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT dye reduction assay in 96-well microplates, which depends on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue formazan product, was the method used to analyze tumor growth in presence of toluquinol [144]. The fact that toluquinol could also inhibit the endothelial cells proliferation indicates that it is a non-specific cell growth inhibitor affecting both tumor and endothelial cells.

14.4.2 Toluquinol Blocks the Angiogenic Process

The endothelial cell morphogenesis on Matrigel assay is a quantitative and reliable in vitro angiogenesis assay that can be adapted for high throughput use [148]. In vitro,

endothelial cells plated on a gelled basement matrix (Matrigel) align themselves to form cords, which are already evident a few hours after plating. The minimal concentration of compound yielding a complete inhibition of bovine aortic endothelial cells (BAECs) alignment and cord formation was 5 μM toluquinol, with partial inhibition observed at 2.5 μM (Fig. 14.4a) The treatment with toluquinol, at the concentrations used to inhibit the differentiation of BAECs, did not affect their viability along the assay time, indicating that this compounds was inhibiting one of the functions of activated endothelial cells at non toxic concentrations. This suggests that toluquinol could behave not only as a potential anti-tumor compound but also as a potential anti-angiogenic drug.

Angiogenesis involves the acquisition by endothelial cells of the capability to migrate and invade through ECM, degrade the basement membrane and, in general, to remodel the ECM. Thus, we decided to study the effect of toluquinol on both processes. To study the effect of the compound on endothelial cells migration, the so called “wound healing” assay was used. Results shown in Fig. 14.4b show that 10 μM toluquinol exerted a significant inhibition of the migratory capability of BAECs in this assay. Similar results were obtained when toluquinol was added at a concentration of 10 or 20 μM to the upper well of a Boyden chamber invasion assay, resulting in a significant inhibition of the ability of BAECs to invade through Matrigel-coated filters. Among the enzymes involved in the ECM remodeling, the MMPs play a key role in angiogenesis. Results obtained with gelatin zymographies of conditioned media of toluquinol-treated BAECs demonstrated that toluquinol inhibited the MMP-2 secretion by endothelial cells, whereas no effect on the MMP-2 and MMP-9 levels secreted by the HT1080 tumor cells to their conditioned media was observed after treatment with toluquinol up to 20 μM (Fig. 14.4c). All these data led us to conclude that toluquinol inhibits the migratory, invasive and proteolytic capabilities of endothelial cell [144].

Additionally, in order to gain insight into the molecular mechanism of the toluquinol-induced inhibition of angiogenesis, the effects of this compound on the FGF2 or VEGF-activated BAECs was investigated. Previous analysis had discarded a direct effect of this compound on the kinase activity of the receptors of the main angiogenic factors at the concentrations needed to inhibit angiogenesis in vitro (toluquinol, tested at 2 or 20 μM in an in vitro radiometric protein kinase assay, did not inhibited the kinase activity of the VEGFR1, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3 or FGFR4 purified human recombinant proteins). This made us think that the molecular target of toluquinol could be acting downstream the growth factors receptors. Among the network of pathways governing angiogenesis, the PI3K/Akt pathway plays a central role, controlling essential cellular functions, such as proliferation, migration, differentiation, survival and tumor growth [149], making this pathway an exciting target for molecular therapeutics [150]. Akt plays an important role in regulating normal vascularization and pathological angiogenesis [151]. The phosphorylation of the kinase domain of Akt is stimulated by a wide variety of growth factors and mitogens, including FGF2 and VEGF [149].

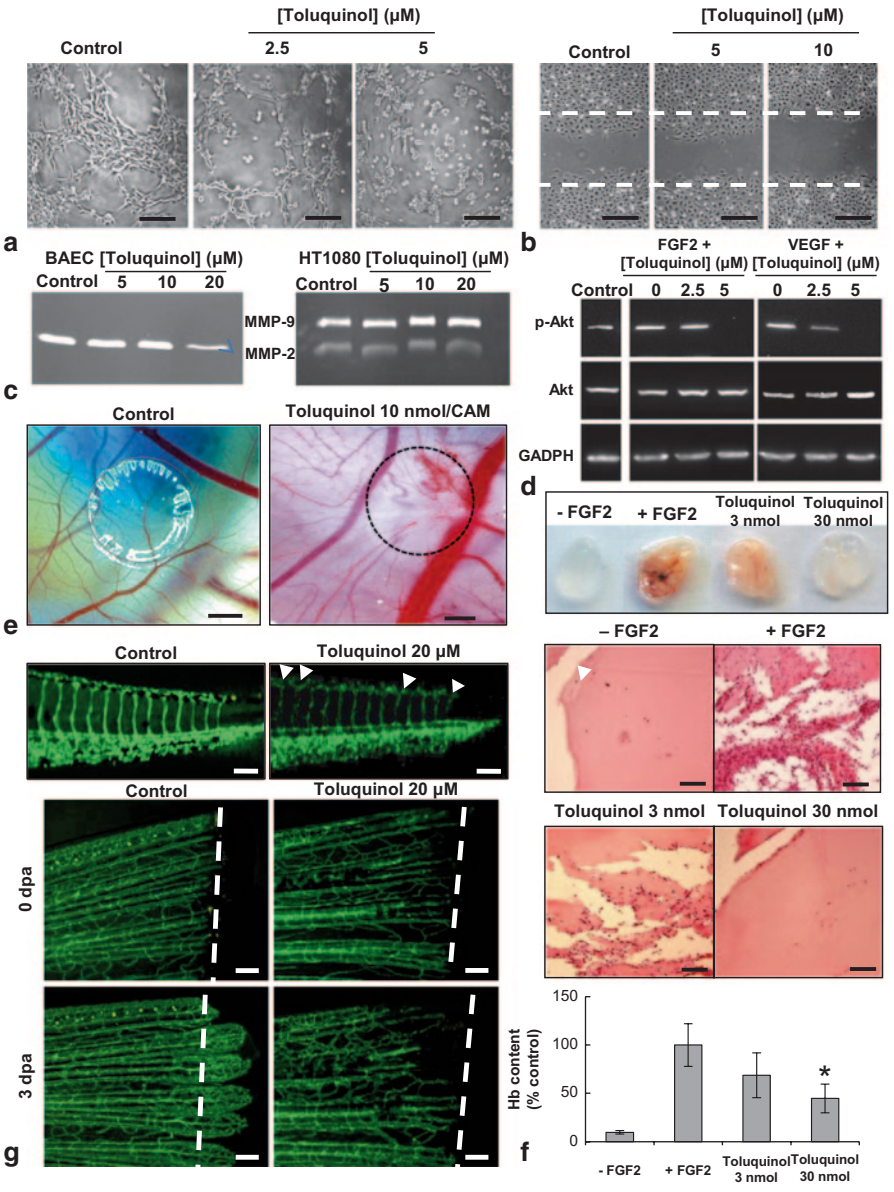


Fig. 14.4 Toluquinol inhibits angiogenesis in vitro and in vivo. Effect of toluquinol on tubulogenesis assay (**a**), on migratory (**b**) and proteolytic capabilities of endothelial cells (**c**) Representative western blot showing the effects of VEGF, FGF2 and toluquinol on the content of phosphorylated Akt and total Akt in protein extracts from BAECs (**d**) In vivo angiogenesis inhibition in CAM assay (**e**), Matrigel plug assay (**f**), and zebrafish models (**g**). (Adapted from García-Caballero et al. (2013) Biochem Pharmacol 85:1727–1740, with permission from Elsevier)

Phosphorylation of Akt is an important signaling component involved in both FGF2 and VEGF mediated angiogenesis. Therefore we examined the effect of toluquinol on the FGF2 and VEGF-induced phosphorylation of Akt in BAECs. Although a basal phosphorylation of Akt was observed in unstimulated cells, exposure of BAECs to VEGF (100 ng/mL) or FGF2 (100 ng/mL) resulted in an activation of this intracellular target protein, as demonstrated by the significant increase in pAkt/total Akt ratio. In contrast, Akt phosphorylation was abrogated in BAECs by the presence of 5 μM toluquinol (Fig. 14.4d) [144].

The in vitro antiangiogenic effect of toluquinol was further validated by using different angiogenesis in vivo models. In the choriallantoic membrane (CAM) assay toluquinol exhibited its antiangiogenic activity as low as 5 nmol per CAM, where 70% of the eggs scored positive. This antiangiogenic effect was observed as an inhibition of the ingrowth of new vessels in the area covered by the methylcellulose discs containing the drug (Fig. 14.4e) Moreover, the peripheral vessels (relative to the position of the disc) grew centrifugally, avoiding the treated area, where a decrease in the vascular density could be observed. The toluquinol in vivo antiangiogenic activity was confirmed with the intradermal Matrigel plug model (Fig. 14.4f). Our results showed that this compound caused a strong inhibition of the FGF2-mediated cell invasion in the Matrigel plug. The Matrigel plugs without FGF2 were colorless and showed the absence of vasculature, whereas the Matrigel plugs containing FGF2 were apparently red, due to neovascularization. Matrigels containing FGF2 with 3 or 30 nmol toluquinol were only partially red, indicating a decreased blood vessel formation, also confirmed by histological analysis. Furthermore, we measured the hemoglobin content in the plugs as indicator of neovascularization. 30 nmol toluquinol significantly inhibited the FGF2-induced angiogenesis in the Matrigel plug in vivo assay (Fig. 14.4f) [144].

Two different assays based on the transgenic (TG(fli1:EGFP)y1) zebrafish line were used to confirm the previously observed in vivo antiangiogenic activity of toluquinol. In these animals, enhanced green fluorescent protein (EGFP) expression is driven under the 15-kb promoter of the transcription factor friend leukemia virus integration-1 (fli-1). This promoter is ubiquitously activated in endothelial cells along the complete embryo, young and adult zebrafish. This ubiquitous expression leads to green in vivo fluorescence in all endothelial cells, and permits observation of bright blood vessels at all stages of embryogenesis [152]. Results shown in Fig. 14.4g demonstrate that 20 μM toluquinol added to water reduced the number of caudal intersegmental vessels at late tail bud stages of Fli-EGFP transgenic zebrafish. This inhibitory effect was observed in a dose dependent manner.

Zebrafish caudal fin can be used as a model for regenerative angiogenesis, sensitive to chemical inhibition by antiangiogenic compounds [153]. In fin regeneration experiments, zebrafish caudal fins are amputated at mid-fin level, and then allowed to recover. Amputated blood vessels heal their ends by 1 day post-amputation (dpa) and then reconnect arteries and veins via anastomosis, to resume blood flow at wound sites by 2 dpa [154]. By 3 dpa, networks of endothelial cells in the regenerated tissue formed a vascular plexus that extended to the fin tip. When

20 μM toluquinol was added to water, a prevention of new vessel formation, accompanied to both fin regeneration arrest and vessel lumen enlargement, was observed (Fig. 14.4g). The fin regeneration arrest led to the absence of fin blastema. Varicosities were mostly observed in inter-ray regions. In some instances, reduction of stump vasculature could be detected, suggesting distal vascular degeneration.

All these results show that toluquinol represses angiogenesis, which is considered to be a hallmark of cancer, playing a pivotal role in the progression and malignization of tumors.

14.4.3 Toluquinol Provokes Endothelial and Tumor Cell Apoptosis

The induction of endothelial apoptosis is a common mechanism exhibited by a number of angiogenesis inhibitors and has been postulated to contribute to the anti-angiogenic potential of these compounds [155–159]. As a first approach to study a possible apoptogenic activity of toluquinol, the effect of different concentrations of this compound on the nuclear morphology was investigated in BAECs, HL60, HT-29 and HT1080 cells after 14 h treatment. Figure 14.5a shows that toluquinol concentrations of 5 μM or higher, induced chromatin condensation in endothelial, leukemia, colon adenocarcinoma and fibrosarcoma cells. Similar results were obtained by using the TUNEL assay to detect the DNA fragmentation induced by this compound, indicating that toluquinol induces apoptosis in tumor and endothelial cells.

To further confirm the apoptogenic activity of toluquinol, cell cycle studies were carried out. The cell cycle distribution of toluquinol-treated BAECs was evaluated by flow cytometric analysis after propidium iodide staining. As shown in Fig. 14.5b, untreated BAECs showed a classic pattern of proliferating cells distributed between the G1 (59%), S (19%) and G2/M (19%) phases. Nevertheless, incubation with toluquinol significantly increased the apoptotic sub-G1 cell subpopulation in a concentration-dependent pattern, confirming that the growth inhibitory effect produced by this compound on activated endothelial cells could be due, at least in part, to an induction of apoptosis. Moreover, toluquinol, when added at 5 or 10 μM, resulted in an appreciable decrease of BAECs in the G1 phase of cell cycle, accompanied by a concomitant increase in cell number in the G2/M phases. This pattern is consistent with a G2/M cell cycle arrest after incubation with toluquinol and may underlie the growth inhibitory activity observed for this compound. Similar G2/M arrests have been described for esophageal cancer cells after being treated with marine tripenyl toluquinones and toluhydroquinones, and they have been related to an irreversible cellular damage mediated by the Reactive Oxygen Species (ROS) generation [160]. There is growing evidence that ROS are important for the induction of apoptosis under both physiologic and pathologic

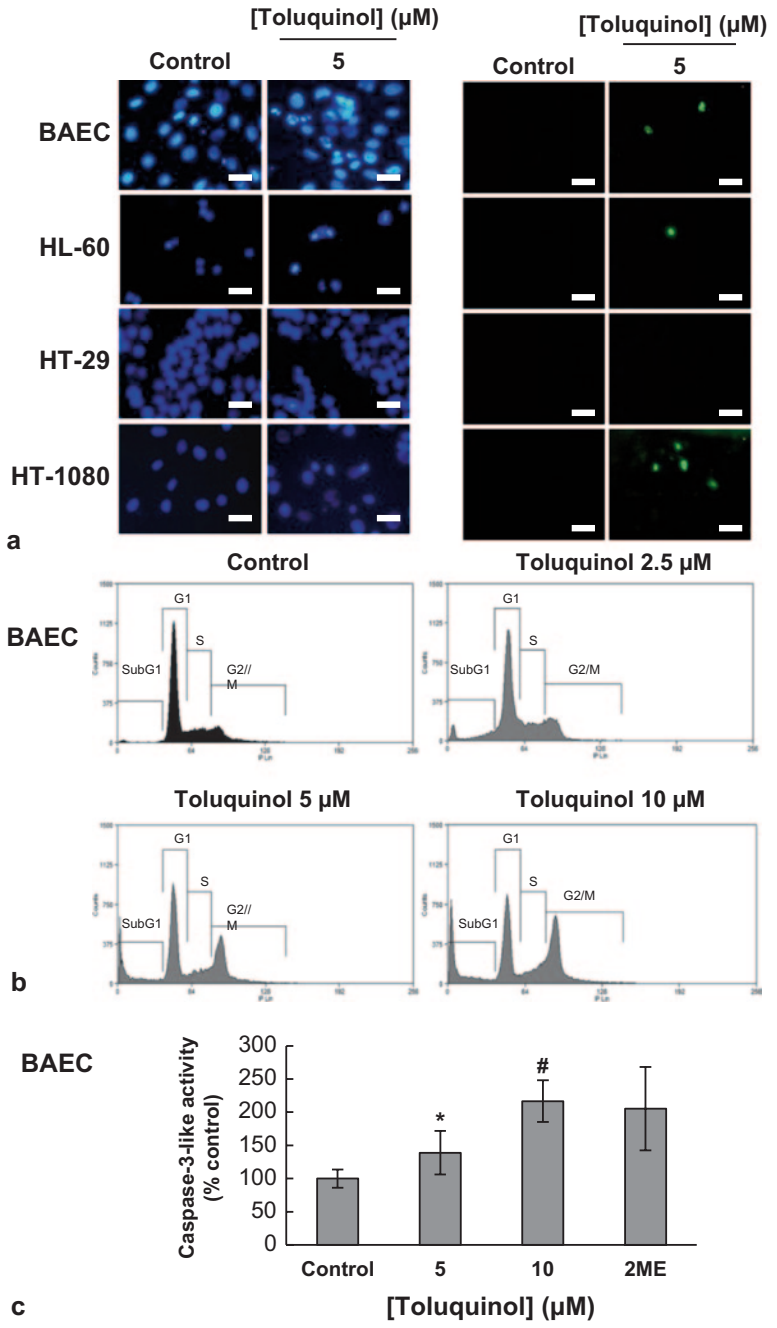


Fig. 14.5 Apoptosis induction in tumor and endothelial cells by toluquinol. **a** Results obtained with Hoechst staining (*left* panel) and TUNEL assay (*right* panel) after treatment with toluquinol. **b** Cell cycle analysis in BAEC treated with toluquinol. **c** Caspase-3 activity in BAE cells incubated in presence of toluquinol. (Adapted from García-Caballero et al. (2013) Biochem Pharmacol 85:1727–1740, with permission from Elsevier)

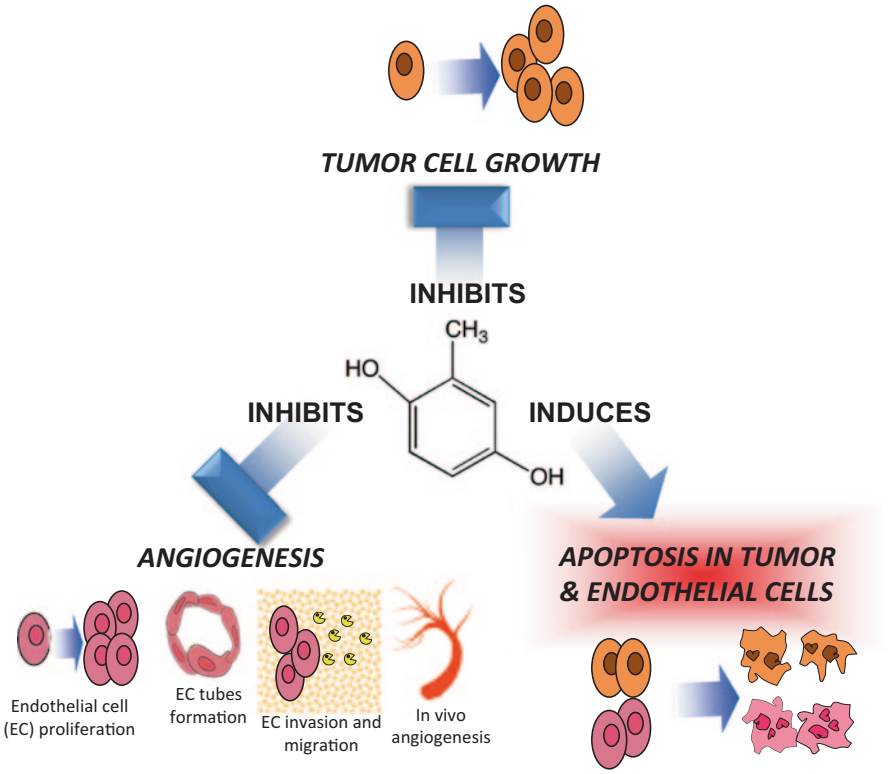


Fig. 14.6 Toluquinol interferes with three hallmarks of cancer

conditions [161]. Toluquinol and some structurally related marine tripenyl toluquinones and toluhydroquinones have been reported to induce an increase of DNA damage by generation of ROS [160, 162] that could contribute to the observed pro-apoptogenic activity of toluquinol. Moreover, given that cancer cells are often more susceptible to ROS, those compounds have been proposed to present plausible leads for new anticancer treatments [160].

Activation of caspases is considered to be the prerequisite to define apoptotic cell death. The induction of endothelial apoptosis by toluquinol was confirmed using the caspase3/7 fluorogenic substrate DEVD-AMC. Measurement of the activity of the effector caspase 3, showed that toluquinol promotes an activation of the caspase proteolytic cascade after treatment of BAECs with toluquinol, what suggests that caspase-dependent mechanisms could play a role in the apoptosis induction within those cells (Fig. 14.5c).

All these results indicate that toluquinol could interfere with the "apoptosis evasion" hallmark of cancer by inducing apoptosis by different mechanisms in tumor and endothelial cells.

Results regarding the biological activity of toluquinol are summarized in Fig. 14.6, showing that this compound interferes at least with three hallmarks of

cancer, by inhibiting the replicative potential of tumor cells, blocking angiogenesis and inducing apoptosis in tumor and endothelial cells.

14.5 Conclusions

The description of the hallmarks of cancer by Hanahan and Weinberg has provided a conceptual framework that can be used to understand the complexity of the biology of cancer, promoting the development of molecular targeted therapies at present and may increasingly do so in the future. The identification of new drugs with pharmacological potential for the treatment of cancer from marine source is a hot topic, with a continuously increasing number of marine derived compounds showing therapeutic properties for the treatment of cancer and other human diseases. Experimental data, showing that toluquinol interferes with several hallmarks of cancer, including increased tumor cell proliferation, angiogenesis induction and apoptosis evasion, suggest the potential of this compound as a new antitumor drug. This fact reinforces the possibility of utilizing compounds derived from marine organisms as potential new sources of tumor inhibitors.

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Chapter 15
Anticancer Diketopiperazines from the Marine Fungus

Zhan-Lin Li and Hui-Ming Hua

Abstract Diketopiperazines (DKPs) of marine resources, especially those isolated from marine-derived fungi, have been paid increasing attention for their diversity in chemical structure and bioactivity. In this chapter, the marine-fungi derived DKPs with cytotoxic, cell cycle progression inhibiting, or apoptosis-inducing activities, are displayed with the classification according to their functionalities into three groups including prenylated DKPs, thio-DKPs, and simple DKPs, each of which is subsequently sorted referring to their structural features or composing in amino acid residues.

Keywords Diketopiperazine · Marine-derived fungus · Anticancer · Prenylated diketopiperazine · Tryprostatin · Notoamide · Echinulin · Thio-diketopiperazine · Gliotoxin · Luteoalbusin

15.1 Introduction

Diketopiperazines (DKPs), with the scaffold of 2,5-diketopiperazine formed via the condensation of two amino acid residues, are an unique class of natural products of great importance for their structural novelty and diverse bioactivities. Marine organisms-derived DKPs [1], of which 76 % were isolated from marine microorganisms, exhibit various activities such as cytotoxicity, antibacterial, antifungal, antifouling, etc. Marine-fungi derived DKPs account for the largest number (85 %) of those from marine microorganisms and are talked about in this chapter, with special attention to those displaying anticancer effects.

According to the striking functionalities on the skeleton of cyclic peptides, which are critical for the bioactivities of DKPs, all of the molecules mentioned in the following sections are classified into prenylated diketopiperazines (Type A), thio-diketopiperazines (Type B), and simple diketopiperazines (Type C).

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The distribution of marine-fungi derived DKPs and the origins of the fungi are listed in Table 15.1.

15.2 Prenylated Diketopiperazines

(-)-phenylahistin (halimide), is a representative fungal prenylated DKP which was isolated from *Aspergillus ustus* NSC-F038 [67] and arrested the cell cycle of P388 in the G2/M phase. Its synthetic analogue, NPI-2358, was approved for the phase-II clinic trial in the treatment of lymphoma or solid lung cancer as a vascular disrupting agent, attracting the attention on the anticancer marine fungi-derived prenylated DKPs, which are categorized into 7 sub-types according to their compositions in amino acid residues and structural features.

15.2.1 Tryprostatin Derivatives

These marine fungi-derived prenylated DKPs, taking brevianamide F consisting of L-tryptophan and L-proline as the scaffold, were firstly isolated by Cui CB in 1995 [10]. The aromatic ring in tryptophan was unsubstituted or substituted by hydroxyl or methoxyl. A prenyl group was attached to C-2 of tryptophan, making the formation of the six-membered or spiro ring between the indole moiety and the diketopiperazine skeleton possible. Tryprostatin derivatives came from *Aspergillus* and *Pseudallescheria* fungi and exhibited anticancer activities via cytotoxic, cell cycle inhibiting, and microtubule assembly inhibiting mechanisms.

15.2.1.1 Tryprostatins

Tryprostatins A (**1**) and B (**2**) were firstly obtained from the fermentation extract of *Aspergillus fumigatus* BM939 isolated from a 760 m deep sea sediment sample, completely inhibiting cell cycle progression of mouse tsFT210 cells in the M phase with IC_{50} values of 78.7 and 18.8 μM, respectively [10, 11]. Succeeding experiments uncovered the mechanism of tryprostatin A involved inhibition of microtubule assembly via interfering with the interaction between microtubule-associated protein 2 (MAP2) and the C-terminal domain of tubulin [68]. Additionally, tryprostatin A was found to reverse a mitoxantrone-resistant phenotype at 10 ~ 50 μM and inhibit the cellular breast cancer resistance protein (BCRP)-dependent mitoxantrone accumulation in human gastric carcinoma and human breast cancer cell lines [69].

Subsequently, 6-hydroxytryprostatin B (**3**) and 18-oxotryprostatin A (**4**) were isolated from *A. fumigatus* YK-7 [21] and *A. sydowi* PFW1-13 [33], respectively. **4** Showed weak cytotoxicity against A-549 cell with IC_{50} of 1.28 μM, while the IC_{50} values of **3** against U937 and PC-3 cells were > 100 μM.

Table 15.1 Existence of diketopiperazines isolated from marine-derived fungi

Genus	Strain	Origin	DKP type[a]	Ref.
Acrostalagmus	*Acrostalagmus luteoal bus* SCSIO F457	Deep sea sediment	B, C	[2]
Aspergillus	*Aspergillus* 16-02-1	Deep sea sediment	C	[3]
	Aspergillus aculeatus CRI322-03	Sponge *Stylissa flabelliformis*	A, C	[4]
	Aspergillus effuses H1-1	Mangrove rhizosphere soil	A	[5, 6]
	Aspergillus flavus C-F-3	Alga *Enteromorpha tubulosa*	C	[7]
	Aspergillus fumigatus	Holothurian *Stichopus japonicus*	A, C	[8]
	Aspergillus fumigatus 030402d	>33 m sea sediment	A, B	[9]
	Aspergillus fumigatus BM939	760 m deep sea sediment	A	[10–15]
	Aspergillus fumigatus Fres	Sea sediment	B, C	[16, 17]
	Aspergillus fumigatus H1-04	Sea mud	B	[18]
	Aspergillus fumigatus KMM4631	Superficial mycobiota of soft coral *Sinularia* sp.	A	[19, 20]
	Aspergillus fumigatus YK-7	Sea mud of intertidal zone	A, B	[21]
	Aspergillus niger	Provided by AIMS	C	[22]
	Aspergillus niger EN-13	Inner tissue of brown alga *Colpomenia sinuosa*	C	[23]
	Aspergillus sp. #MFA212	Surface of marine red alga *Lomentaria catenata*	A, C	[24]
	Aspergillus sp. CNC688	Brown alga *Sargassum* sp.	C	[25]
	Aspergillus sp. KMD-901	1198 m sea sediment	B	[26]
	Aspergillus sp. MF297.2	Mussel *Mytilus edulis galloprovincialis*	A	[27–31]
	Aspergillus sp. SF-5044	Intertidal sediment	C	[32]
	Aspergillus sydowi PFW1-13	Sea driftwood	A, B	[33]
	Aspergillus taichungensis	Mangrove plant *Acrostichum aureum*	C	[34]
	Aspergillus versicolor MF030	60 m sea sediment	A	[35]
Asteromyces	*Asteromyces cruciatus* F156	Unkonwn	B	[36]
Aureobasidium	*Aureobasidium pullulans*	Sponge unidentified	C	[37]
Cephalospo rium	*Cephalosporium* sp. (2090#)	Mangrove plant	C	[38]
Chromocleista	*Chromocleista* sp. R721	70 m sea sediment	C	[39]
Cladosporium	*Cladosporium* sp. F14	Mangrove sea water	C	[40]
Eurotium	*Eurotium cristatum* KUFC7356	Sponge unidentified	C	[41]
	Eurotium repens	Sponge *Suberites domuncula*	A	[42]

Table 15.1 (continued)

Genus	Strain	Origin	DKP type[a]	Ref.
	Eurotium rubrum	Inner tissue of stems of mangrove plant *Hibiscus tiliaceus*	A	[43]
Gliocladium	*Gliocladium* sp. YUP08	Sea mud	A	[44]
Nectria	*Nectria inventa* 049207c	>600 m deep sea sediment	B	[9]
Nigrospora	*Nigrospora* sp. PSU-F12	Sea fan (*Annella* sp.)	B	[45]
Oidiodendron	*Oidiodendron truncatum*	Antarctic soil	B, C	[46]
Paecilomyces	*Paecilomyces* cf. *javanica*	Sponge *Jaspis* cf. *coriacea*	B	[47]
Penicillium	*Penicillium bilaii* MST-MF667	Boat ramp	B, C	[48]
	Penicillium chrysogenum MTCC 5108	Leaves of mangrove plant *Porteresia coarctata*	A	[49]
	Penicillium griseofulvum	2481 m deep sea sediment	A	[50]
	Penicillium sp.	Sea mud	C	[51]
	Penicillium sp. 386#	Sea mud	C	[52]
	Penicillium sp. F1	5080 m deep sea sediment	A	[53]
	Penicillium sp. F23-2	5080 m deep sea sediment	A	[54, 55]
Plectosphaerella	*Plectosphaerella cucumerina*	100 m sea sediment	B	[56]
Pseudallescheria	*Pseudallescheria* sp.	Surface of drift wood	A, C	[57]
Unidentified	Unidentified 1356	Mangrove plant	C	[58]
	Unidentified 1893	Mangrove plant *Kandelia candel*	C	[59]
	Unidentified 2534	Mangrove plant *Avicennia marina*	C	[60]
	Unidentified A-f-11	Sea mud	A, C	[61]
	Unidentified B4	Mangrove plant	C	[62]
	Unidentified CRIF2 (order of Pleosporales)	Sponge unidentified	B	[63]
	Unidentified M-3	Laver (*Porphyra yezoensis*)	A	[64]
	Unidentified ZSU-H26	Mangrove plant	C	[65]
	Unidentified ZZF41	Mangrove plant	C	[66]

[a] *Type A* Prenylated DKPs, *Type B* Thio-DKPs, *Type C* Simple DKP

N-prenylbrevianamide F (**5**) [8, 61] and a series of 1,1-dimethylallyl (reverse prenyl) substituted brevianamide F derivatives including deoxybrevianamide E (**6**) [27, 35], brevianamide K (**7**), brevianamides T-V (**8**-**10**), and a dimer brevianamide S (**11**) which were isolated from *A. versicolor* [35], are also included in this group (Fig. 15.1) for their structural similarity. **5** Inhibited the growth of K562 cells by

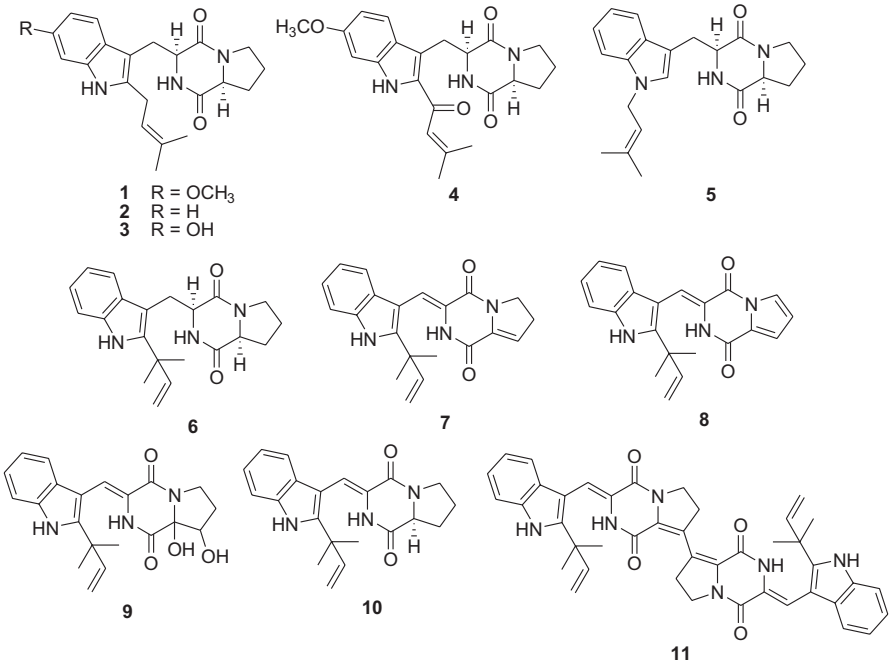


Fig. 15.1 Structures of marine fungi-derived tryprostatin analogues

17.4% at 10 μg/ml [61], while the bioactivities of the others were not significant except for the antitubercular activity of **11** [35].

15.2.1.2 Fumitremorgins

Fumitremorgins (Fig. 15.2) are downstream products of tryprostatins via cyclization between the methylene on the prenyl group and the nitrogen atom belonging to tryptophan on the 2,5-diketopiperazine ring.

Fumitremorgins B (**12**) and C (**13**), demethoxyfumitremorgin C (**14**), and 12,13-dihydroxyfumitremorgin C (**15**) were isolated from *A. fumigatus* BM939 with cell cycle inhibiting activities [11] and then obtained from the other *Aspergillus* strains [8, 21, 33, 61]. **13** And **15** were also found in *Pseudallescheria* fungus with mild antibacterial activity [57]. Cyclotryprostatins A (**16**), B (**17**), and C (**18**) were isolated from *A. fumigatus* BM939 and inhibited the cell cycle of tsFT210 cells at G2/M phase with IC_{50} values of 5.6, 19.5, and 23.4 μM, respectively [15]. Prenylcyclotryprostatin B (**19**) and 9-hydroxyfumitremorgin C (**20**) were separated from *A. fumigatus* YK-7 with antiproliferative activities against U937 and PC-3 cells [21].

The *β*-carbon of tryptophan moiety in fumitremorgins could be oxidized into carbonyl to afford cyclotryprostatin D (**21**) [15]and 13-oxofumitremorgins B (**22**) [8]. Prenyls in fumitremorgins underwent oxidation to afford **23**, **24** [8], 20-hydrox-

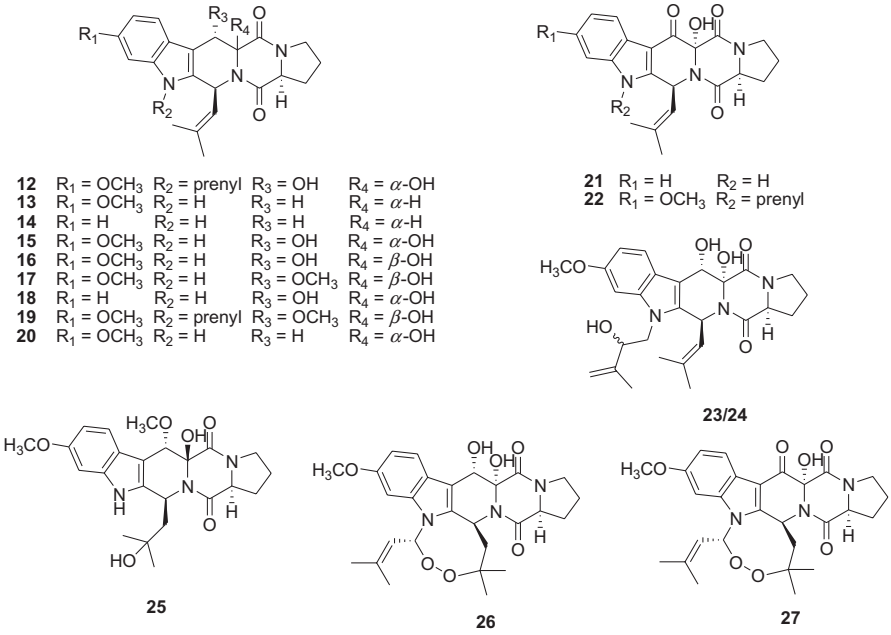


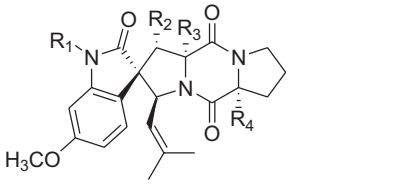
Fig. 15.2 Structures of marine fungi-derived fumitremorgin analogues

ycyclotryprostatin B (**25**) [21], and further verruculogen (**26**) and 13-oxo verruculogen (**27**) [8]with a peroxide bridge between two prenyls. **21** Inhibited the cell cycle of tsFT210 cells [15] and **23**, **24**, and **27** were reported to have antitumor activities against MOLT-4, HL-60, A-549, and BEL-7402 cells [8].

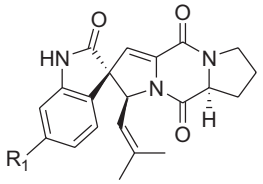
15.2.1.3 Spirotryprostatins

Spirotryprostatins A (**28**) and B (**29**) were firstly isolated from *Aspergillus fumigatus* BM939 in 1996 [13, 14] inhibiting cell cycle of tsFT210 cells at G2/M phase with IC_{50} values of 197.5 and 14.0 μM, respectively, followed by spirotryprostatins C-F (**30**–**33**) isolated from *A. fumigatus* [20, 8] and 6-methoxyspirotryprostatin B (**34**) isolated from *A. sydowi* [33]. Spirotryprostatins C-E exhibited moderate cytotoxic activities against four tumor cell lines [8]and **34** showed cytotoxicity against A-549 and HL-60 cells with IC_{50} values of 8.29 and 9.71 μM, respectively [33]. Spirotryprostatin F was reported to have phytoregulating activity at low and ultralow concentrations [20].

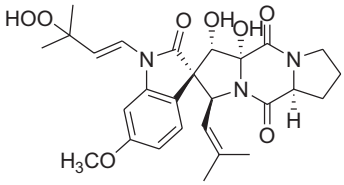
Although there was no report on the biosynthesis of spirotryprostatins (Fig. 15.3), these molecules, with a unique spiro ring system composed of a γ-lactam fused to a benzene ring and a pentacyclic enamine fused to a 2,5-diketopiperazine moiety, can



28 R_1 = H R_2 = H R_3 = H R_4 = H
30 R_1 = prenyl R_2 = OH R_3 = OH R_4 = H
31 R_1 = prenyl R_2 = OH R_3 = OH R_4 = OH
33 R_1 = H R_2 = OH R_3 = OH R_4 = H



29 R_1 = H
34 R_1 = OCH_3



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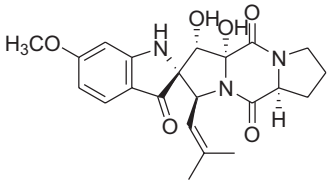

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Fig. 15.3 Structures of marine fungi-derived spirotryprostatin analogues

be considered to be the downstream products of fumitremorgins via bond cleavage, cyclization, and oxidation.

What's more interesting is the discovery of a novel spirotryprostatin derivative (**35**) with a different spiro system and weak cytotoxic activity from two *A. fumigatus* strains [8, 19].

15.2.2 Notoamides

Notoamides A-R (**36**–**53**), with the same skeleton, brevianamide F as tryprostatin derivatives, along with sclerotiamide (**54**), stephacidin A (**55**), and (−)-versicolamide B (**56**) were separated from an *Aspergillus* strain isolated from the mussel *Mytilus edulis galloprovincialis* [27–31]. The attractive feature in the structure of these analogues was the 2,2-dimethyl-2*H*-chromone moiety in the L-tryptophan residue, except for notoamide J (**45**) and the novelty existed in that the 1,1-dimethylallyl attached at the *ortho*-carbon (**41**–**42**, **44**, **53**, **55**) to nitrogen atom in indole moiety or that on the *meta*-carbon (**36-37**, **43**, **49**, **54, 56**) could fuse with the oxidized DKP ring by intramolecular Diels-Alder reaction to afford the complicated scaffold [30]. The mechanism of the transfer of 1,1-dimethylallyl from *ortho*-carbon to *meta*-carbon was interpreted as pinacol rearrangement [30]. Furthermore, this scaffold underwent oxidation, decarboxylation, and cyclization to produce the

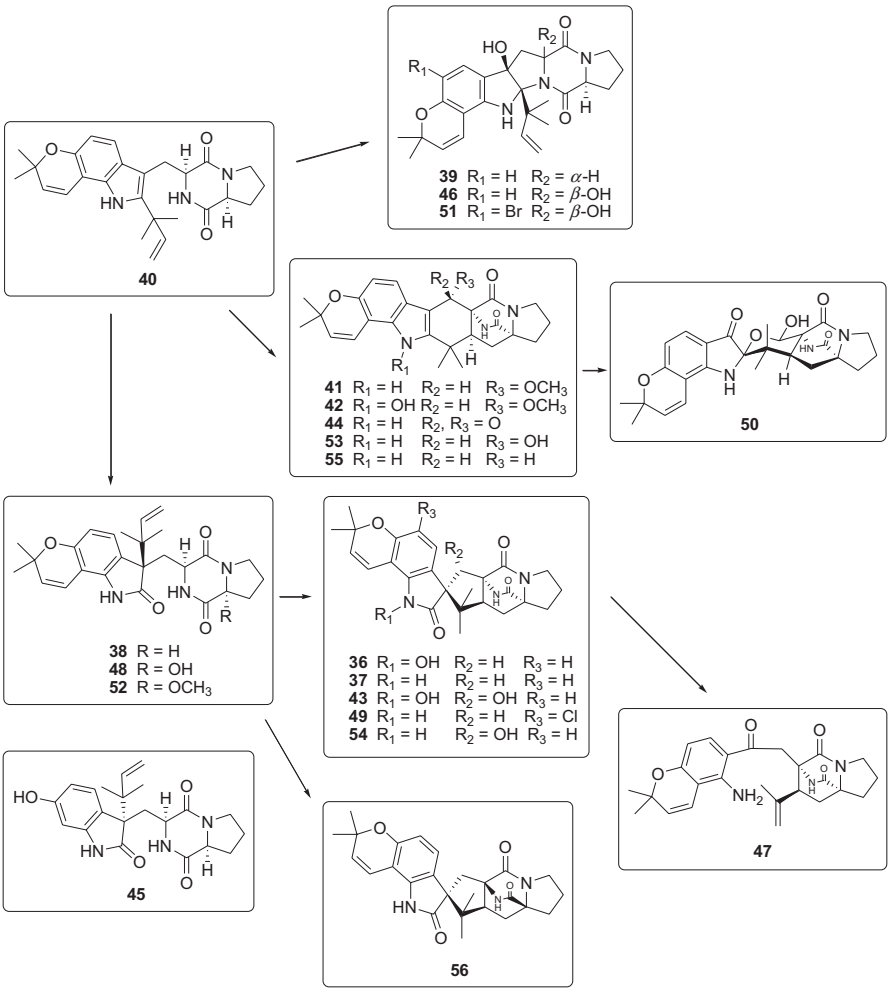


Fig. 15.4 Structures of marine fungi-derived notoamide analogues

terminal product like notoamides L (**47**) and O (**50**). The structures of notoamide analogues are arranged according to their biogenetic relationship in Fig. 15.4.

Compared to the intensive investigations into the structure, biosynthetic pathway, and total synthesis [70] of notoamides, there were few reports on their bioactivity. Notoamides A-C (**36**–**38**), exhibited moderate cytotoxicity against HeLa and L1210 cells with IC_{50} values in the range of 22–52 μg/ml and notoamide C (**38**) induced G2/M cell cycle arrest at 6.3 μg/ml [27]. Notoamide I (**44**) showed weak cytotoxicity against HL-60 cell with an IC_{50} value of 21 μg/ml, while the IC_{50} values for notoamides F (**41**), J (**45**), and K (**46**) were more than 50 μg/ml [28].

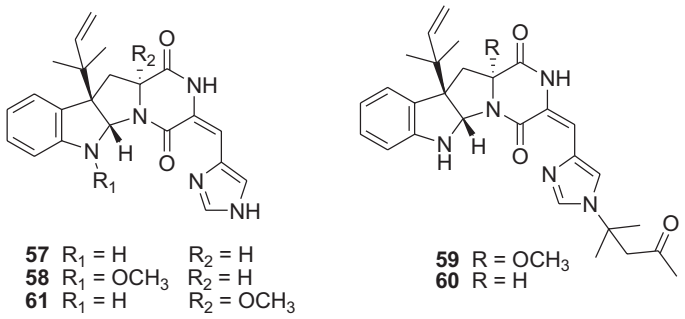
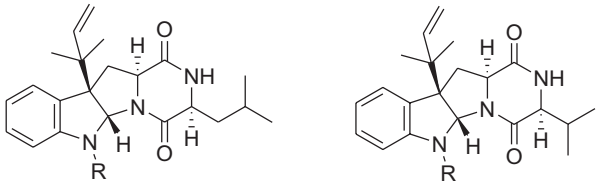


Fig. 15.5 Structures of marine fungi-derived roquefortin analogues

Fig. 15.6 Structures of marine fungi-derived brevicompanine analogues



15.2.3 Roquefortins

Marine-derived roquefortins (Fig. 15.5) were reported from a *Penicillium* fungus isolated from a 5080 m deep sea sediment sample, including roquefortins C (**57**) and F-I (**58**–**61**) [54, 55]. Biogenetically, requefortins serve as the precursor of meleagrin derivatives and originated from cyclo(L-tryptophan-L-histidine) via cyclization, oxidative dehydrogenation, and the substitution of modified 1,1-dimethylallyls.

Roquefortins F (**58**) and G (**59**) exhibited cytotoxicities against A-549, HL-60, BEL-7402, and MOLT-4 cells [54], while the IC_{50} values of roquefortins H (**60**) and I (**61**) against A-549 and HL-60 cells were more than 100 μM [55].

15.2.4 Brevicompanines

Brevicompanines (Fig. 15.6) have the similar structure to requefortins in the L-tryptophan moiety except for the functionalities at nitrogen atom and α-carbon. While, the other amino acid residue of brevicompanines is L-leucine or L-valine. From a

deep sea sediment derived fungus *Penicillium* sp., brevicompanines D-F (**62**–**64**), fructigenine B (**65**), and *allo*-brevicompanine B (**66**) composed of L-leucine, as well as brevicompanines G-H (**67**–**68**) composed of L-valine were isolated, inhibiting lipopolysaccharides (LPS)-induced nitric oxide production in BV2 microglial cells [53].

15.2.5 Echinulins

Echinulins have the skeleton composed of L-alanine and L-tryptophan with the substitutions of prenyl and 1,1-dimethylallyl. The α- and *β*-carbons of L-tryptophan can undergo dehydrogenation to form a *Z*-double bond and those of L-alanine experience the same reaction to from a terminal double bond which can condense with polyketides to afford a novel spiro-ring system enriching the structural diversity of echinulins.

14-Hydroxyterezine D (**70**) exhibited weak cytotoxicity against A-549 cells with IC_{50} of 7.31 μM [33], and **74** (dihydroneoechinulin B) and **75** (didehydroechinulin B) showed cytotoxicities against HL-60, BEL-7402, and A-549 cells [6] While, **77** (variecolorin O) and variecolorins M and N (**79**–**80**) did not show cytotoxic activity against these three cells [50].

Interestingly, these spiro-echinulins (**84**–**90**) showed significant cytotoxicities, e.g. **84** (dihydrocryptoechinulin D) [5] and **85** (cryptoechinulin D) [6]showed potent activity on P388 cells with an IC_{50} values of 1.83 and 3.43 μM, respectively, and the (+)-enatiomer of **85** (IC_{50} 2.50 μM) was more potent than the (−)-enatiomer [6]. Further investigation into the mechanism of **84** found that its molecular target was topoisomerase I and the (+)-enatiomer showed moderate activity against topoisomerase I than the racemic **84**, while the (−)-enatiomer was almost inactive [5]. Variecolortides A-C (**88**∼**90**) which were isolated from *Eurotium rubrum*, together with 7-*O*-methylvariecolortide A (**87**) [43], exhibited weak cytotoxic activities against K562 human leukemia cells with IC_{50} values of 61, 69, and 71 μM, respectively [71].

The molecules left in Fig. 15.7 are echinulin (**69**) [42, 50], terezine D (**71**) [33], preechinulin (**72**) [50], tardioxopiperazine A (**73**) [50], neoechinulin A (**76**) [49, 24, 50], variecolorin H (**78**) [50], neoechinulin B (**81**) [6, 50], isoechinulin B (**82**) [50], golmaenone (**83**) [24], and effusin A (**86**) [5].

15.2.6 Others

Besides these prenylated DKPs above which can be assigned to a certain group, a new diketopiperazine, indole-*N*-prenyl-cyclo(tryptophan-valine) (**91**) (Fig. 15.8) was isolated from an unidentified marine fungus with antifungal activity [64]. Additionally, PJ-147 (**92**) and PJ-157 (**93**), a pair of geometric isomers, were isolated from marine fungus *Gliocladium* sp. YUP08 [44] and exhibited significant cytotoxic activity against six tumor cell lines with the least IC_{50} of 1.37 μM [72].

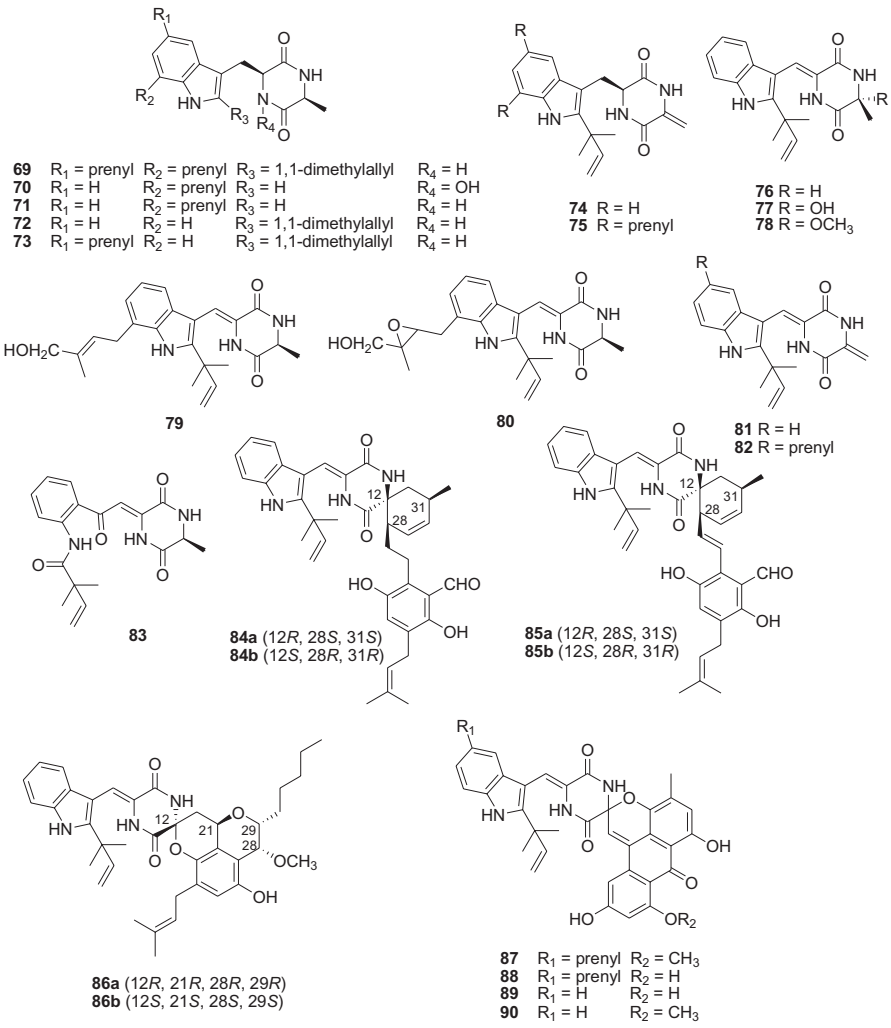


Fig. 15.7 Structures of marine fungi-derived echinulin analogues

15.3 Thio-diketopiperazines

Thio-diketopiperazines, taking gliotoxin as the firstly isolated and representative compound, exhibited diverse bioactivities including cytotoxic, antiviral, antifungal, and antibacterial activity. Especially, epipolythio-diketopiperazines, characterized by a bridged polysulfide DKP structure, has become the spotlight of research on anticancer drugs for that it directly inhibited the interaction between hypoxia-inducible factor-1α (HIF-1α) and the transcriptional coactivator p300 [73, 74]. Marine-fungus

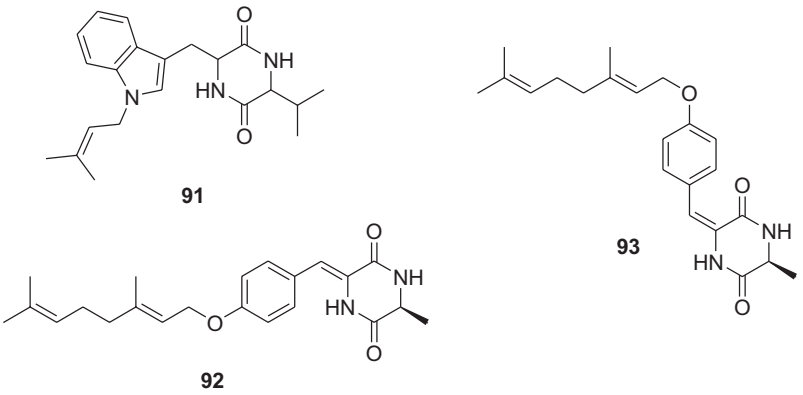


Fig. 15.8 Structures of other marine fungi-derived prenylated DKPs

derived thio-diketopiperazines are divided into the following five groups according to their composition of amino acid residues.

15.3.1 Gliotoxins

L-phenylalanine and L-serine constitute the skeleton of gliotoxin (**94**), and the disulfide linkage is critical for its cytotoxic activity. Progress on the biosynthesis of **94**, as well as its role in the virulence of *Aspergillus fumigatus* was summarized recently [75]. Gliotoxin completely inhibited the proliferation of tsFT210 and A549 cells at the concentration of 0.1 μM [18] and its IC_{50} values against U937 and PC-3 cells were 0.20 and 0.39 μM, respectively [21]. After the cleavage of the disulfide bond, these derivatives only showed weak cytotoxicity and **95** [bisdethiobis(methylthio) gliotoxin], retaining the same structure as gliotoxin in the phenylalanine moiety, exhibited the highest cytotoxic activity with IC_{50} value of 0.52 μM against U937 cells [21]. While, the IC_{50} values of the novel spirogliotoxin (**101**) against U937 and PC-3 cells were more than 100 μM [21].

For its *in vivo* toxicity, gliotoxin (**94**) could not be directly applied as anticancer drug and seeking for its analogues with lower toxicity and higher selectivity is still ongoing.

The structures left in Fig. 15.9 correspond to didehydrobisdethiobis(methylthio)gliotoxin (**96**) [16, 18, 21], gliovictin (**97**) [36, 63], bis-*N*-norgliovictin (**98**) [16, 18], (*Z*)-6-benzylidene-3-hydroxymethyl-1,4-dimethyl-3-methylsulfanylpiperazine-2,5-dione (**99**) [63], and a new gliotoxin analogue (**100**) [16].

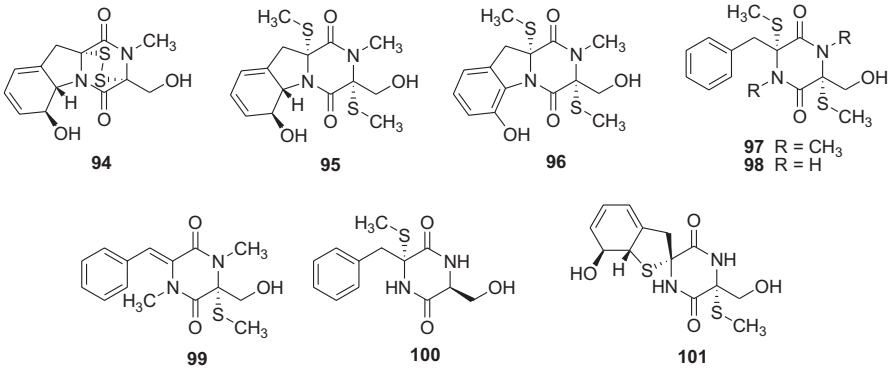


Fig. 15.9 Structures of marine fungi-derived gliotoxin analogues

15.3.2 Thio-cyclo(tryptophan-serine)

The skeleton of this group of thio-DKPs (Fig. 15.10) is composed of D-amino acids and their structural novelty also existed in the cyclization, dimerization, and substitution by indole moiety and cinnabarinic acid.

15.3.2.1 Chetoseminudins

Chetoseminudins B and C (**102**–**103**) with a cleaved sulfide bridge were isolated from marine-derived *Acrostalagmus* [2] and *Oidiodendron* [46] with nearly no cytotoxic activity.

15.3.2.2 Chetracins

Epithio-cyclo(tryptophan-serine) underwent cyclization and dimerization to afford chetracins B and C (**104**–**105**), melinacidin IV (**106**) from *Oidiodendron* fungus [46] and chaetocin (**107**) from *Nectria* fungus [9], and further cleavage of sulfide bridge to give chetracin D (**108**) [46]. **104**, **105**, **106** And **108** exhibited significant cytotoxicities against five human cancer cells lines with the least IC_{50} of 0.003 μM from **104** against BEL-7402 cells [46].

15.3.2.3 Luteoalbusins

Luteoalbusins have an additional indole moiety attached to the γ-carbon of tryptophan residue in the cyclo(tryptophan-serine) skeleton. Luteoalbusins A (**109**) and B (**110**), T988A (**111**), and T988C (**112**), as well as two derivatives with the cleaved

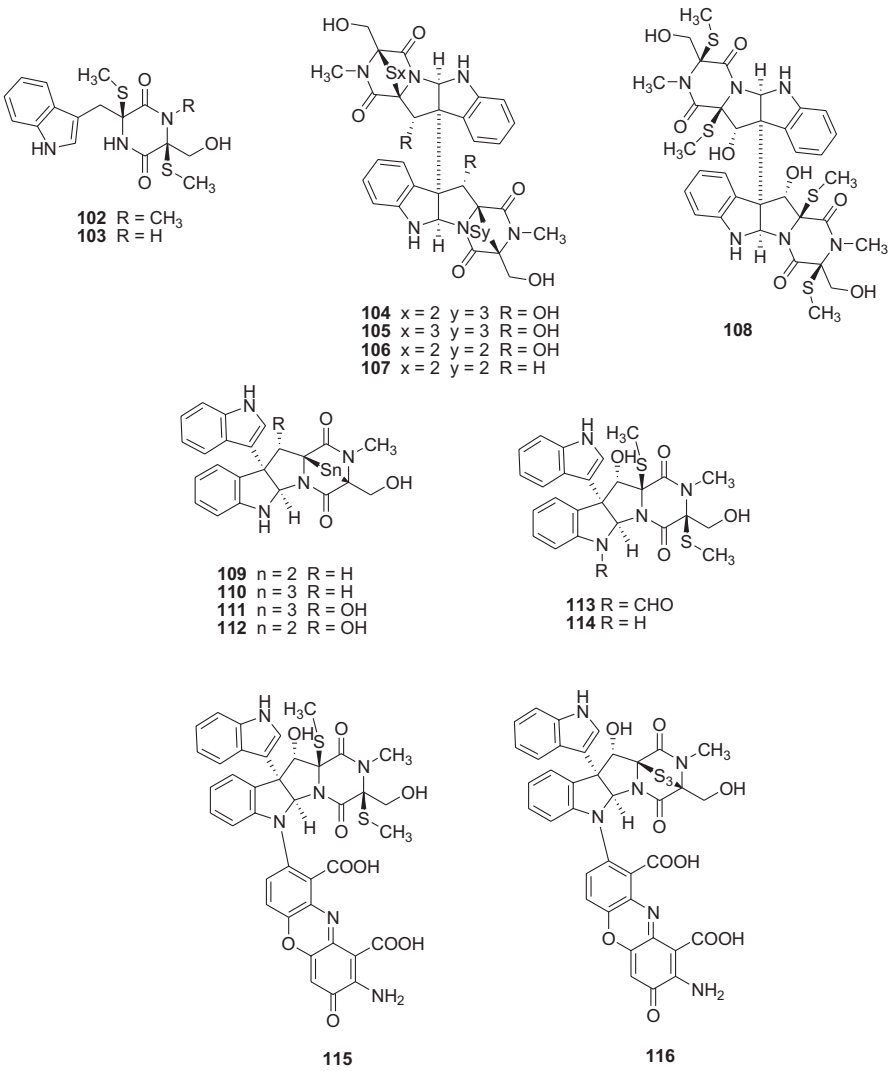


Fig. 15.10 Structures of marine fungi-derived thio-cyclo(tryptophan-serine) derivatives

sulfide bridge, oidioperazine A (**113**) and T988B (**114**), were isolated from *Acrostalagmus* [2] and *Oidiodendron* [46]fungi. **109**–**112** Showed lower cytotoxicity than **104**–**106** but comparable activity to **108**, while the cytotoxicity decreased remarkably when the polysulfide bridge was broken (**113**–**114**) [2, 46].

Furthermore, T988B (**114**) and T988A (**111**) coupled with cinnabarinic acid to afford two novel thio-diketopiperazines plectosphaeroic acids A (**115**) and C (**116**) from a marine isolate of the fungus *Plectosphaerella cucumerina* [56].

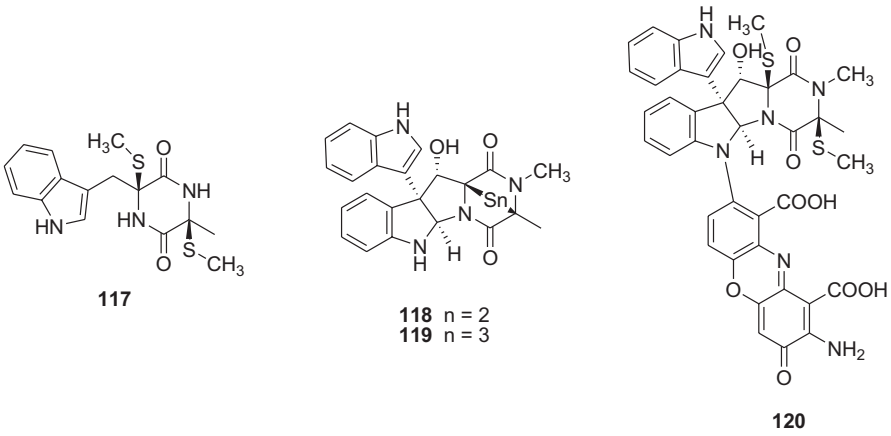


Fig. 15.11 Structures of marine fungi-derived thio-cyclo(tryptophan-alanine) derivatives

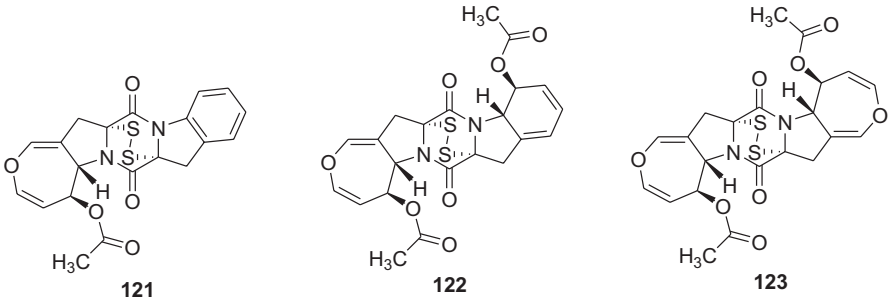


Fig. 15.12 Structures of marine fungi-derived apoaranotins

15.3.3 Thio-cyclo(tryptophan-alanine)

Four thio-cyclo(tryptophan-alanine) derivatives (**117**–**120**) (Fig. 15.11) were isolated from *Nectria* [9], *Acrostalagmus* [2], and *Plectosphaerella* [56] fungi. Significant cytotoxic activities were observed for **118** and **119** (gliocladines C and D) [2].

15.3.4 Apoaranotins

Three apoaranotin derivatives (Fig. 15.12), deoxyapoaranotin (**121**), acetylapoaranotin (**122**), and acetylaranotin (**123**) were isolated from an *Aspergillus* strain with cytotoxic activities against six tumor cell lines. Furthermore, **121**–**123** were found induce apoptosis in HCT116 cells and **122** exhibited *in vivo* inhibitory effects on tumor proliferation in a xenograft model [26].

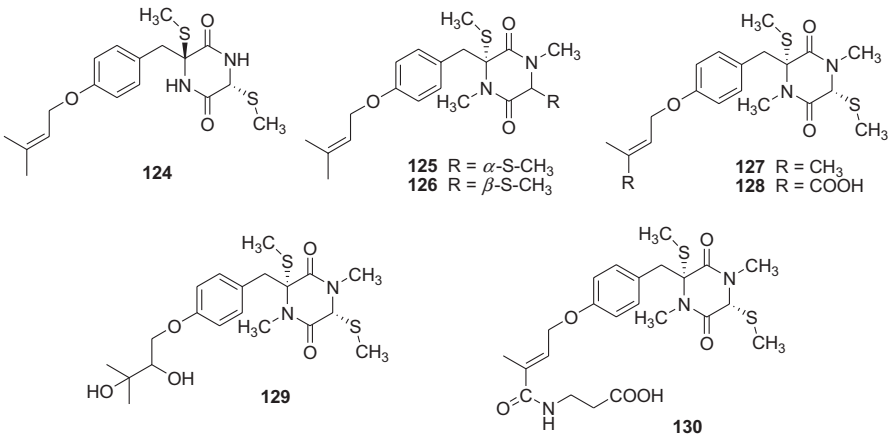


Fig. 15.13 Structures of marine fungi-derived thio-cyclo(phenylalanine-glycine) derivatives

Biogenetically, apoaranotins are derivatives of cyclo(L-phenylalanine-L-phenylalanine) and are structurally related to gliotoxin.

15.3.5 Thio-cyclo(phenylalanine-glycine)

Seven cyclo(phenylalanine-glycine) derivatives (Fig. 15.13) with the substitutions of thiomethyls and prenyls were isolated from *Nigrospora* (Sch54796, **124**) [45], *Paecilomyces* (**125**–**126**) [47], and *Penicillium* (**127** and bilains A-C, **128**–**130**) [48] fungi. Only **127** displayed weak cytotoxicity against NS-1 cells with LD_{99} of 0.15 μM [48].

15.4 Simple Diketopiperazines

Simple diketopiperazines, which are also called cyclic dipeptides when there is no functionality attached to the diketopiperazine ring and the amino acid residues, are always regarded as secondary metabolites of little pharmaceutical value from the microorganisms but the precursors of the novel prenylated or thio-diketopiperazines. While, novel simple diketopiperazines were continuously isolated from marine-derived fungi, with the rare amino acid residues or dimerized structures.

In this section, the author gives a brief description on these novel molecules (Fig. 15.14) and the existence of the known cyclic dipeptides can be referred in the Introduction section.

Two new DKPs (**131**–**132**) with a D-*cis*-4-hydroxyproline residue were isolated from the marine yeast *Aureobasidium* [37]. Mactanamide (**133**) with a rare D-2,6-dihydroxyphenylalanine, were isolated from a marine *Aspergillus* strain [25]. Two

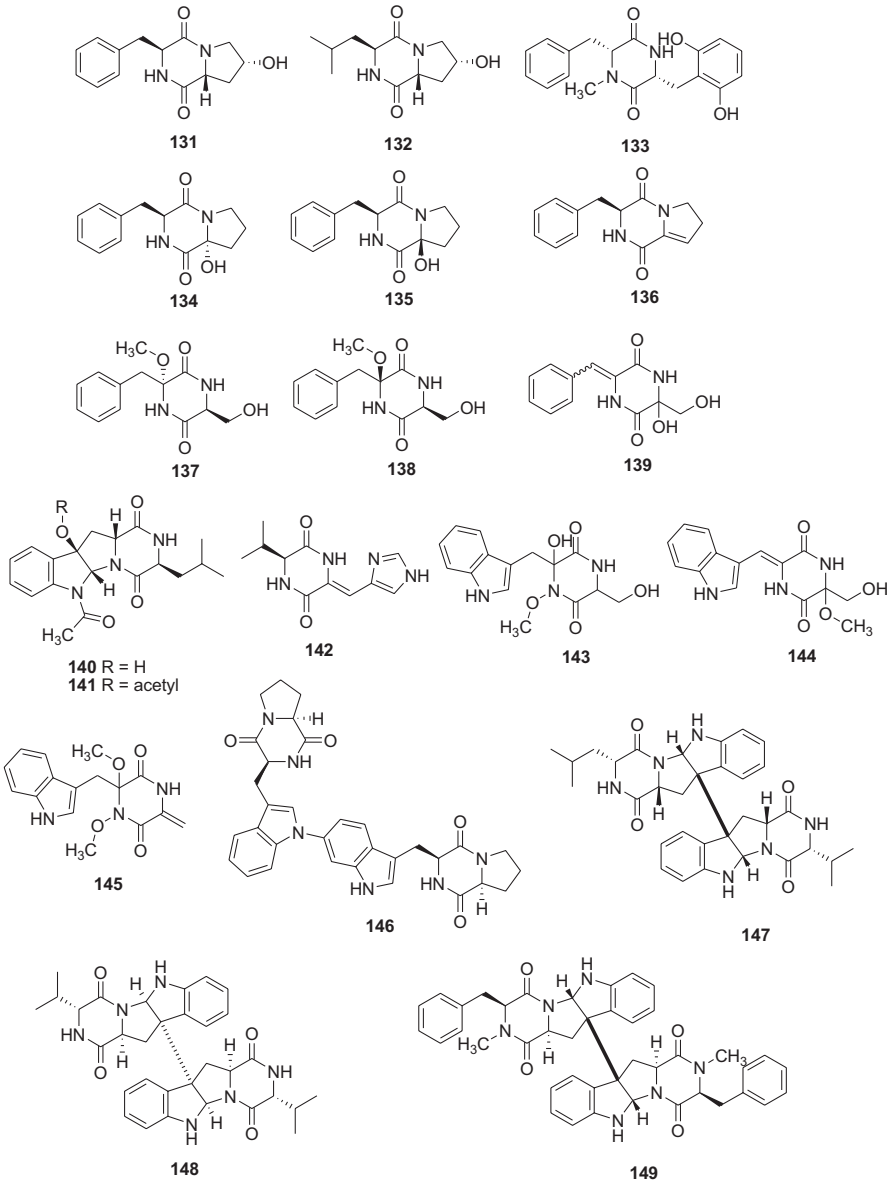


Fig. 15.14 Structures of novel marine fungi-derived simple diketopiperazines

new DKPs (**134**–**135**) composed of hydroxylated proline residue were obtained without cytotoxicity against P388, A549, PANC-1, and NCI-ADR-RES cells at a concentration of 5 μg/ml and they decomposed to **136** in the NMR tube at room temperature [39]. Three new DKPs (**137**–**139**) which failed to display cytotoxic

activity against K562 cell line, were isolated from a marine-derived *Aspergillus fumigatus* [17]. Two new DKPs with cyclized tryptophan residue (protubonines A and B, **140–141)** were separated from an *Aspergillus* fungus and no cytotoxicity against five cell lines was observed for them at 250 μM [32]. Pre-aurantiamine (**142**), containing histidine residue, was identified by X-ray crystallographic analysis [4]. Oidioperazines B-D (**143–145**) with tryptophan residue were isolated from *Oidiodendron truncatum* [46].

Four dimerized tryptophan-containing simple diketopiperazine, aspergilazine A (**146**) [34], **147** [22], eurocristatine (**148**) [41], and ditryptophenaline (**149**) [7] were isolated from marine-derived fungi without the report on their anticancer activity.

15.5 Summary

The natural marine fungi-derived diketopiperazines with anti-cancer activities were mainly isolated from *Aspergillus* and *Penicillium* genera. Prenylated and thio-DKPs account for the majority of this group and are the most potential to be developed as clinically applicable anticancer drugs with 2,5-diketopiperazine scaffold. The thio-DKPs exhibited much higher activities than the prenylated one, while significant cytotoxicity plays as a double-edged sword and restricts their application.

The upsurging functional genomics remarkably accelerated the research progression on the biosynthesis of marine fungi-derived diketopiperazines. The thirteen gene clusters involved in the biosynthesis of gliotoxin (**94**) in *Aspergillus fumigatus* have been organized [76] and the key gene clusters include one coding for a two-module non-ribosomal peptide synthetase (NRPS), GliP responsible for the construction of 2,5-diketopiperazine skeleton, two coding for a cytochrome P450 monooxygenase, GliC and a glutathione *S*-transferase, GliG introducing two sulfur atoms into the molecule, and one coding for gliotoxin oxidase, GliT catalyzing the formation of the disulfide bridge. These results disclose the biosynthetic mystery of natural DKPs, and stimulate us to think over the application of these gene clusters by gene engineering techniques, throwing light on the chemoenzymatic synthesis of prenylated DKPs of tryprostatins [77, 78] and fumitremorgins [79]. Additionally, the One Strain-Many Compounds (OSMAC) approach termed by Zeeck [80], by which novel and interesting secondary metabolites were obtained by a tiny change of the culture medium, is still enriching the chemical diversity of fungi-derived natural products including diketopiperazines.

It is foreseeable that diketopiperazines, especially those isolated from marine-derived fungi, are of great significance to be investigated in the discovery of natural anticancer drugs.

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Chapter 16
Meroterpenoids from Marine Microorganisms: Potential Scaffolds for New Chemotherapy Leads

Nelson G. M. Gomes, Suradet Buttachon and Anake Kijjoa

Abstract Meroterpenoids, including several biologically active metabolites from marine microorganisms mainly fungi and actinomycete bacteria, represent promising structural scaffolds with not only diverse biological activities such as antimicrotubule, cytotoxic and antiproliferative but also different mechanisms of action. In this chapter, an overview on structural diversity and anticancer activity of mixed biogenesis terpenoid derivatives (meroterpenoids) from marine microorganisms is presented with highlight on individual examples of the most promising candidates in cancer chemotherapy and prevention.

Keywords Anticancer activities · Cytotoxicity · Marine bacteria · Marine-derived fungi · Marine-derived actinomycetes · Marine microorganisms · Meroterpenoids · Terpenyl alkaloids · Terpenyl glycosides · Terpenyl polyketides

16.1 Introduction

Natural products are widely recognized as valuable sources for drug discovery which provide several lead compounds and drug agents for use in human Medicine. In the last decades, Oceans and marine life forms have emerged as an important source of bioactive natural products with promising biological activities that could lead to the development of alternatives for cancer therapy [1]. Even though only four marine-derived natural products have been approved as anticancer agents so far, an increas-

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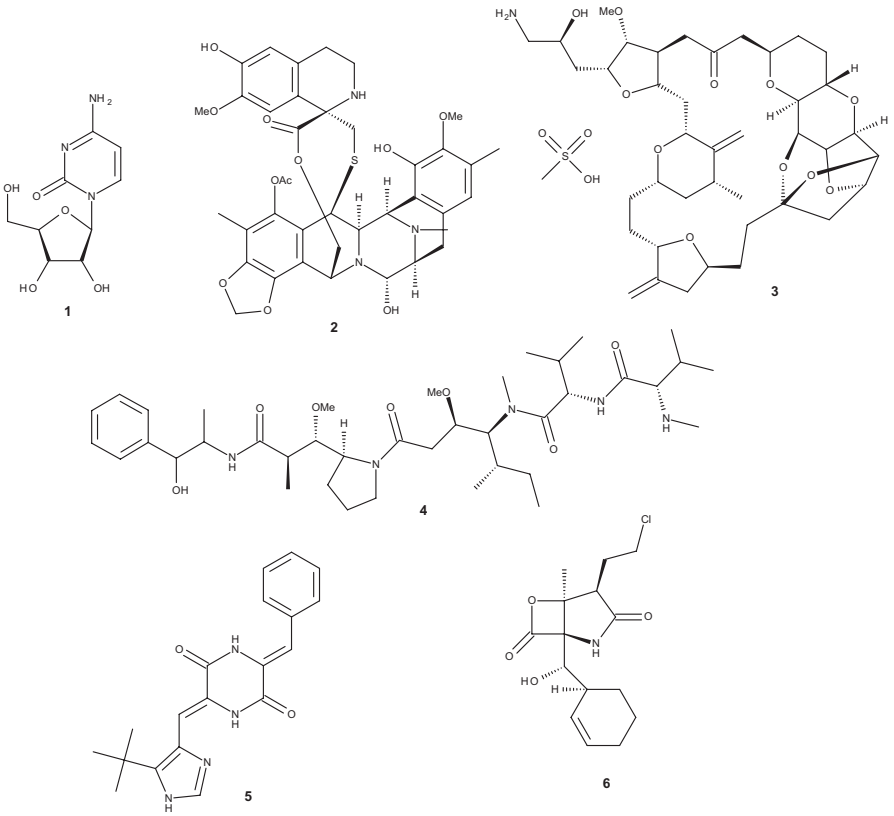
ing number of substances under clinical trials reveal the potential of the marine environment [2]. The classical example of marine natural products as a template for drug development is the nucleosides spongothymidine and spongouridine, isolated from the marine sponge *Tethya crypta*, whose synthetic analog cytarabine (**1**) became the first marine-derived drug that received a FDA approval in 1969. This drug is currently used in combination therapy for the treatment of acute lymphoblastic leukemia, acute myeloid leukemia and chronic myelogeneous leukemia, or alone for prevention and treatment of meningeal leukemia ([3]; http://www.cancer.gov/cancertopics/druginfo/cytarabine, Accessed October 3, 2013). Following this, three additional marine-derived drugs have been approved for use in chemotherapy. In 2001, trabectedin (Yondelis®), also known as ecteinascidin-743 (ET-743) **(2)**, was developed by PharmaMar. This drug was approved in Europe for the treatment of soft tissue sarcoma, and later for the treatment of relapsed platinum-sensitive ovarian cancer (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Assessment_Report_-_Variation/human/000773/WC500059175.pdf, Accessed October 3, 2013). Ecteinascidin-743 (**2**) is a tetrahydroisoquinolone alkaloid, originally extracted from the tunicate *Ecteinascidia turbinata*. Due to the supply problem and high costs associated with its chemical synthesis, nowadays this drug is produced by hemisynthesis starting from cyanosafracin B, a fermentation product of the bacteria *Pseudomonas fluorescens* [4, 5, 6, 7]. Another marine anticancer agent with success story resulted from the discovery of the antimicrotubule macrolide halichondrin B. This compound was isolated from the marine sponge *Halichondria okadai* in the late 1980s. Its analogue eribulin mesylate (**3**) had been developed by Esai Pharmaceuticals [8, 9, 10] and was approved in USA, EU and Asia under the trade name Halaven® for the treatment of metastatic breast cancer. Eribulin mesylate (**3**) is currently under clinical trials for the treatment of other types of cancer (http://www.cancer.gov/cancertopics/druginfo/fda-eribulinmesylate, Accessed October 3, 2013). Another interesting example of marine-derived drug is brentuximab vedotin (**4**) (Adcentris®), an anti-CD30 antibody conjugate, consisting of monomethyl auristatin E, which was approved by FDA for the treatment of Hodgkin lymphoma and systemic anaplasic large cell lymphoma ([11]; http://www.cancer.gov/cancertopics/druginfo/fda-brentuximabvedotin, Accessed October 3, 2013). Monomethyl auristatin E is a synthetic analog of dolastatin 10, a compound originally isolated from the marine mollusk [12].

Although the previously approved compounds are described as originally produced by marine invertebrates, marine microbiota is also considered as a major source of antitumor drug leads [13, 14, 15]. For example, Nereus Pharmaceuticals has developed plinabulin (**5**), a synthetic analog of halimide, as a vascular disrupting agent which is currently under a Phase II clinical trial for the treatment of solid tumors and lymphoma [16, 17, 18, 19]. Halimide itself is a potent cytotoxic compound produced by a marine-derived fungus *Aspergillus ustus*. Another interesting marine-derived compound is salinosporamide A (**6**), a proteasome inhibitor originally isolated from the marine bacterium *Salinispora tropica* [20]. Nereus Pharmaceuticals has licensed this compound under the name Marizomib®, which is now

in Phase I clinical trials as a single agent in patients with advanced solid tumors, and also in combination with vorinostat, a histone deacetylase inhibitor, for the treatment of advanced pancreatic carcinoma, non-small cell lung carcinoma and melanoma [21, 22].



Despite a limited number of marine microbial antitumor agents currently on the market or in clinical trials, there are strong evidences that some promising marine natural compounds in clinical trials as well as some approved marine-derived anticancer agents, supposedly produced by invertebrates, are in fact metabolic products of their associated microorganisms, or derived from a diet of prokaryotic microorganisms [14, 23]. For example, dolastatin 10 which was first reported as a metabolite of the Indian Ocean sea hare *Dolabella auricularia* [12], was later found to be produced by the gastropod's diet cyanobacteria of the genera *Simploca* and *Lyngbya* [24, 25]. A recent report by Rath and co-workers on metagenomic sequencing of total DNA from *Ecteinascidia turbinata* and associated microorganisms, suggested

that the individual genes responsible for trabectedin production has microbial origin from the associated-γ-proteobacterium *Candidatus Endoecteinascidia frumentensis*. Therefore, this finding can eventually lead to a renewable path that could overcome trabectedin supply issue [26].

Literature surveys provide a clear notion that an increasing number of naturally occurring meroterpenoids, especially those of new structural types with relevant biological activities, have been reported in the past few years. Although some reviews on meroterpenoids, mostly concerning only fungi, have been published [27, 28], this chapter deals with meroterpenoids, especially those with anticancer activity, produced by all types of marine-derived microorganisms.

16.2 Meroterpenoids

Meroterpenoids are a class of secondary metabolites in which the terpenoid moieties are linked to molecules from different biosynthetic pathways, exhibiting a pronounced richness of structural diversity and a broad range of biological and pharmacological activities. Cornforth [29] has coined the term "meroterpenoids" as "*Compounds containing terpenoid elements along with structures of different biosynthetic origin*". Due to the predominant number of reported natural meroterpenoids of mixed polyketide-terpenoid origin, the most recent definition of meroterpenoids refers nearly exclusively to this group of metabolites [30]. However, we prefer to stick to Cornforth's definition since it covers all types of terpenoid-containing secondary metabolites. Terpenoid moieties can be appended to such diverse molecules such as ribosomal and non-ribosomal peptides, phenazines, pyrroles, however predominantly polyketides. Prenylation, a chemical or enzymatic addition of an isoprenoid to an accepting molecule and frequently leading to an increase in biological activity, is controlled by several prenyltransferase enzymes which can lead either to the addition of a simple linear side chain or highly rearranged terpenic moieties [31−33].

16.2.1 Terpenyl Polyketides

Polyketides represent a diverse family of secondary metabolites usually produced by polyketide synthases through repeated decarboxylative condensation of malonyl-CoA derived extender units, although they are often derivatized and modified. Orsellinic acid terpenoid-derived compounds are the most representative class of polyketide-derived terpenoids, mainly represented by γ- and α-pyrones which arise directly from a tetraketide precursor or from the methylated analogues derived from *S*-adenosylmethionine (SAM). 3-Methylorsellinic acid, 5-methylorsellinic acid and 3,5-dimethylorsellinic acid (**7**) can react with a terpenyl moiety to form the

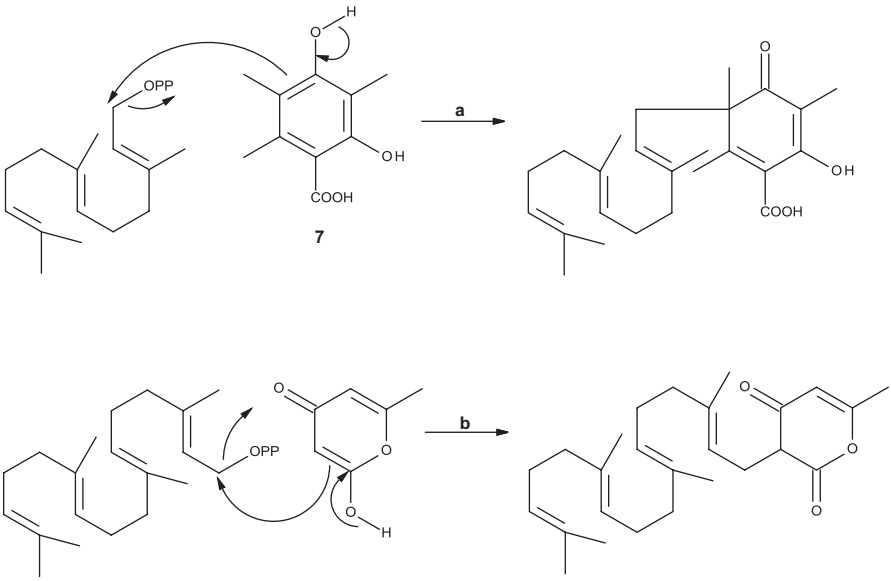


Fig. 16.1 Biogenesis of terpenyl tetraketide

meroterpenoid structure that can undergo additional structural modifications (Fig. 16.1a). Triketide-derived pyrones are also relatively common in fungi, presenting a similar addition mechanism through the reaction of GGPP with the pyrone moiety, with subsequent different modes of cyclization that may lead to distinctive cyclic structures (Fig. 16.1b) [27].

Meroterpenoids containing quinones are also widespread in marine microorganisms, with prenylated naphtoquinones and reduced hydroquinone analogues being mainly reported from marine microorganisms, especially fungi and actinomycetes [34]. These compounds are originated either from the polyketide pathway or from the shikimate route while the position of the prenyl sidechain and other substituents on the quinone ring system seems to be related to the source organism.

16.2.2 Terpenyl Alkaloids

Prenylated alkaloids are very common secondary metabolites from both terrestrial and marine sources, and the most common representatives are prenylated indole alkaloids produced by fungi of the genera *Penicillium* and *Aspergillus*. Although *N*-prenylation is the most widespread in Nature, C-prenylation on the indole/indoline ring is also found depending on the prenyl substituent. In nature, the second amino acid can be commonly found to extend the indole nucleus, leading to the formation of a diketopiperazine core or derivatives, with a typical substitution pattern with

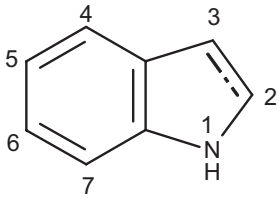


Fig. 16.2 Structure of the indole ring system

reverse prenyl moieties usually attached to N-1, C-2 or C-3 of the indole or indoline ring, however the regular prenyl moieties can be also connected to C-5, C-6 or C-7. In indole alkaloids derived from only one L-tryptophan molecule, prenylation at C-4 may also occur (Fig. 16.2) [35].

16.2.3 Terpenyl Glycosides

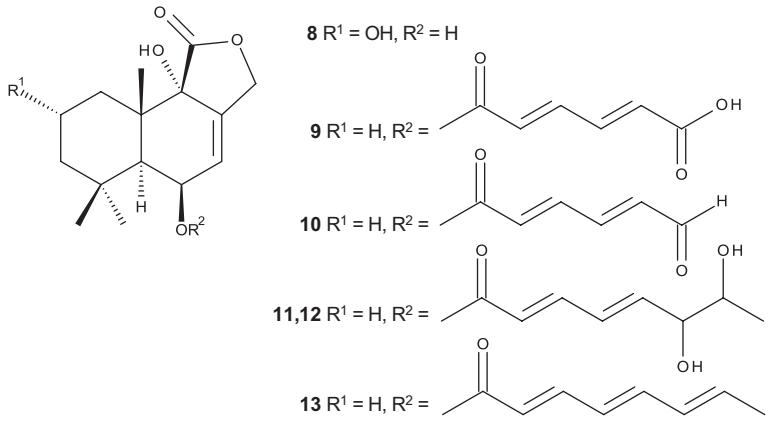
Terpene glycosides, predominantly with a triterpene or sterol core, are another class of meroterpenoids. Despite their rare occurrence in Nature, this class of meroterpenes has been reported from marine sources, especially from the marine-sponges and sea cucumbers, and in lesser extent from the marine fungi [36, 37].

16.3 Naturally Occurring Meroterpenoids from Marine Microorganisms

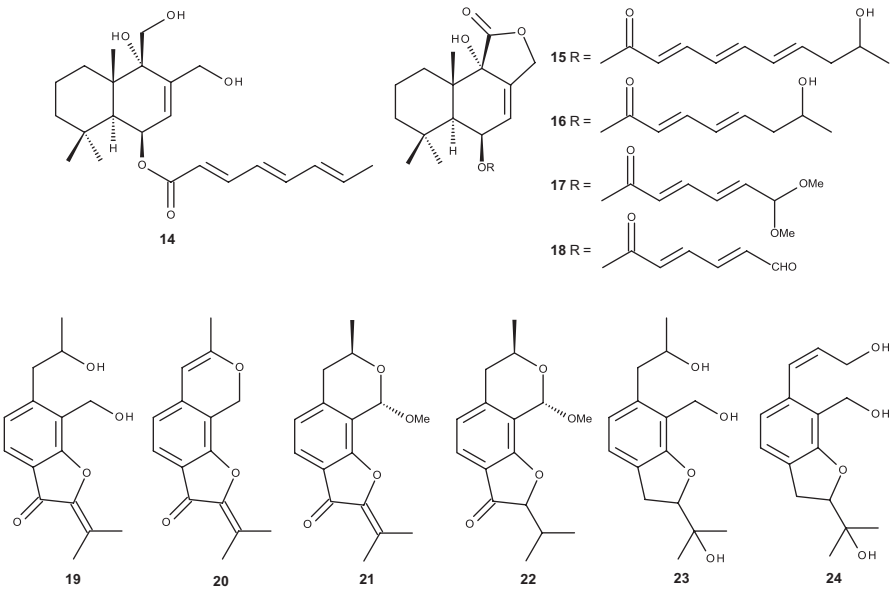
Although *Aspergillus* and *Penicillium* are the main representative genera of marine fungi which produce meroterpenoids analogously to their terrestrial counterparts [27, 38, 39], the Gram-positive Actinomycete bacteria are also prolific sources of these compounds, being classified as one of the most promising resources for new bioactive metabolites including meroterpenoids [40–42].

16.3.1 From Marine Fungi

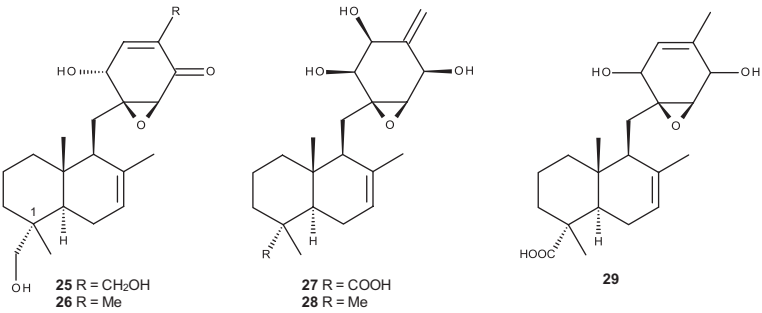
Aspergillus ustus (strain 8009), isolated from the marine sponge *Suberites domuncula* collected from the Adriatic Sea, was found to produce strobilactone B (**8**) together with four new strobilactone B derivatives (**9–12**) and RES-1149-2 (**13**). Compounds **9**, **10** and **13** were found to be active against the murine lymphoma cell line L5178Y with EC_{50} values ranging from 0.6 to 5.3 μM, and compound **10** was found to be the most active congener for this cell line. Additionally, compounds **10** and **13** also displayed a strong cytotoxic activity against HeLa cells, with EC_{50} values of 5.9 and 7.5 μM, respectively [43].



Ustusolates A-E (**14–18**), the drimane sesquiterpenes related to strobilactone B, and six new isochromane derivatives, ustusoranes A-F (**19–24**), were later reported from another *Aspergillus ustus* strain (O94102), isolated from the mangrove plant *Bruguiera gymnorrhiza* collected in Wenchang, Hainan Province of China. Using the sulforhodamine B (SRB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) methods, ustusorane E (**23**) was found to display strong cytotoxic activity against HL-60 cells. On the other hand, ustusolates C (**16**) and E (**18**) were found to exhibit moderate inhibitory activity against A549 and HL-60 cells, with IC_{50} values of 10.5 and 9.0 μM, respectively, while ustusolate A (**14**) displayed only weak cytotoxicity with IC_{50} value of 20.6 and 30.0 μM, respectively [44].



Epoxyphomalins A-E (**25–29**), the new sesquiterpene epoxycyclohexenones with an unprecedented and distinctive substitution and oxidation pattern, were isolated from the culture of the fungus *Paraconiothyrium* cf *sporulosum* isolated from the Caribbean marine sponge *Ectyplasia perox* Duch. & Mich. 1864, collected at Lauro Club Reef, Dominica. All metabolites were tested for cytotoxic activity profiles against a panel of 36 human tumor cell lines. Epoxyphomalins A (**25**), B (**26**) and D (**28**) were found to exhibit an *in vitro* growth inhibitory activity in 12 and 8 of the 36 tested tumor cell lines with IC_{50} values ranging from 0.01 to 0.038 μM and 0.251 to 0.402 μM, respectively [45]. On the contrary, epoxyphomalins C (**27**) and E (**29**) were found to be devoid of activity against the cell lines tested while epoxyphomalin D (**28**) exhibited moderate activity with mean IC_{50} value 6.12 μM and displayed selectivity toward PC3M (prostate cancer) and BXF 1218 L (bladder cancer) cell lines with IC_{50} = 0.72 and 1.43 μM, respectively.

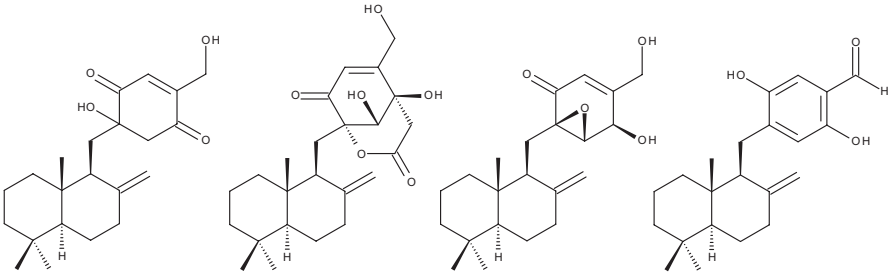


The absence of activity of epoxyphomalins C (**27**) and E (**29**), when compared to epoxyphomalin D (**28**), suggested that the substituents at C-1 are critical components of epoxyphomalins pharmacophore, with a large and polar carboxylic acid functionality leading to a pronounced decrease in cytotoxicity [46]. Strikingly, epoxyphomalin A (**25**) displayed not only remarkable cytotoxicity at nanomolar concentrations but also an intriguing activity profile in COMPARE analyses that did not correlate with those of reference anticancer agents. This result suggested that epoxyphomalin A (**25**) might have a different mode of action from that of the reference compounds. Furthermore, it was found also that incubation of purified human 20S proteasome with epoxyphomalins A and B (**25**, **26**) led to a dose-dependent inhibition of chymotrypsin-, caspase-, and trypsin-like proteasome activities, indicating a potent inhibition of 20S proteasome. Consequently, epoxyphomalins could be considered as new promising chemotherapeutic alternatives for the treatment of several tumors due to their capacity to inhibit the proteasome complex which plays a preponderant role in cell proliferation, apoptosis and differentiation [46].

Penicilliumin A (**30**), a new drimenyl cyclohexenone derivative from the *Penicillium* sp. strain F00120, isolated from a deep sea sediment sample collected in the northern South China Sea, was found to display moderate inhibitory activity

against the proliferation of B16 (mouse melanoma), A375 (human melanoma), and HeLa (human cervical carcinoma) cells with GI_{50} values of 27.37, 22.88 and 44.05 μM, respectively [47]. Two new sesquiterpene quinones related to penicilliumin A **(30)**, purpurogemutantidin and purpurogemutantin (**31**), together with the known macrophorin A (**32**), were later reported from a bioactive mutant BD-1-6 of a marine-derived wild-type *Penicillium purpurogenum* G59 strain, isolated from a soil sample collected at the tideland of Bohay Bay, Tianjin, China. Purpurogemutantidin and purpurogemutantin (**31**) significantly inhibited K562 (human chronic leukemia K562), HL-60 (acute leukemia), HeLa (cervical carcinoma), BGC-823 (gastric adenocarcinoma) and MCF-7 (breast adenocarcinoma) cells, with inhibition rates (IR%) ranging from 62.8–88.0% at 100 μM, while macrophorin A (**32**) displayed significant growth inhibitory activity against K562 and HL-60 cells with IC_{50} values of 1.48 and 0.85 μM, respectively [48]. Interestingly, macrophorin A (**32**) had been previously reported from the plant pathogenic fungus which caused *Macrophoma* fruit rot of apple, and exhibited cytotoxic activity against murine L-5178Y cells [49]. Fraga [50], after verifying that penicilliumin A (**30**) and purpurogemutantidin had identical NMR spectra, has proposed that purpurogemutantidin and penicilliumin A (**30**) are the same compound.

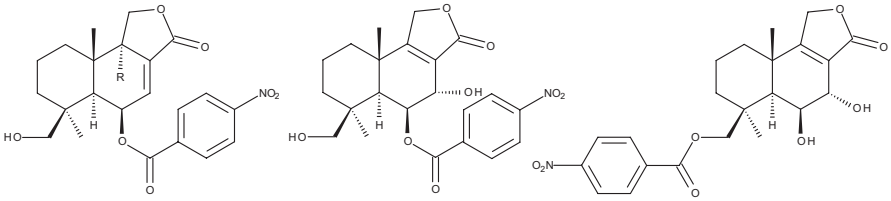
Chen et al. [51] reported isolation of the drimane sesquiterpene hydroquinone (**33**) from the deep sea sediment-derived fungus *Phialocephala* sp. (strain FL30r). This compound was found to exhibit strong cytotoxic activity against P388 and K562 cancer cell lines, with IC_{50} values of 0.16 and 0.05 μM, respectively.



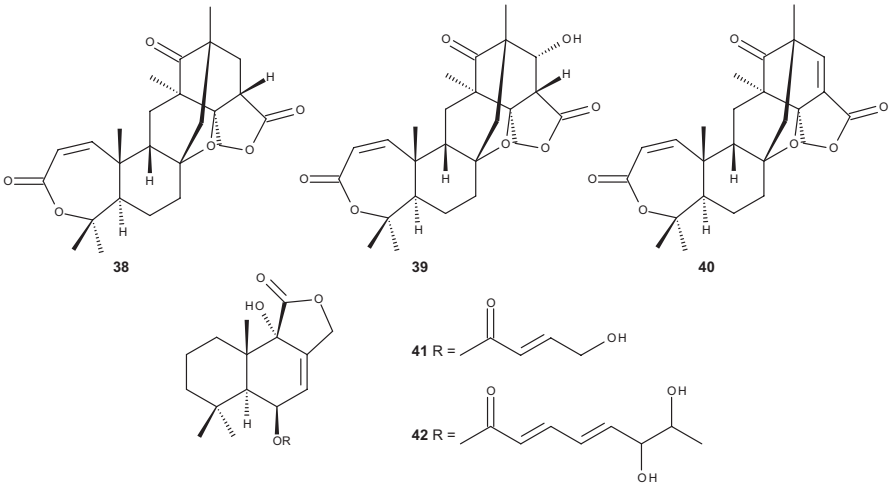
30 **31** **32** **33**

Belofsky et al. [52] reported isolation of four new sesquiterpenoid nitrobenzoyl esters, 9α,14-dihydroxy-6β-*p*-nitrobenzoylcinnamolide (**34**), 7α,14-dihydroxy-6β-*p*-nitrobenzoylconfertifolin (**35**), 14-hydroxy-6β-*p*-nitrobenzoylcinnamolide (**36**) and 6β,7α-dihydroxy-14-*p*-nitrobenzoylconfertifolin (**37**), from an organic extract of the culture broth and mycelia of the algicolous fungus *Aspergillus versicolor* (CNC 327), isolated from the surface of the green calcareous alga *Penicillus capitatus* collected at Eleuthera Point, Bahamas Islands. Compound **34** was evaluated for its antitumor activity in the National Cancer Institute's 60 cell line panel, and showed a mean LC_{50} of 1.1 μg/mL. It also exhibited good cytotoxicity toward colon

cancer cell lines HCC-2998, HCT-I 16, and CNS cancer cell line SNB-75, with LC_{50} values of 0.53, 0.44 and 0.44 μg/mL, respectively. This degree of cytotoxicity approximately mimics that of the antitumor agent etoposide (LC_{50} = 0.98 μg/mL vs. HCT-116). They have also found that, among all the cell lines tested, compound **34** exhibited the most potent activity toward the breast cancer cell line BT-549, with LC_{50} = 0.27 μg/mL. Interestingly, compound **34** showed selective toxicity against five renal cancer cell lines (786-0, ACHN, CAK-1, TK-10, and UO-31), with a mean LC_{50} of 0.51 mg/mL (range = 0.47–0.57 μg/mL).

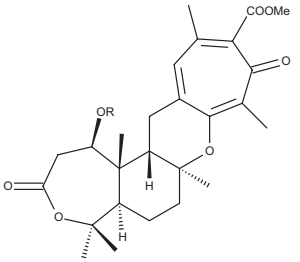


Cohen et al. [53] reported isolation of the sesquiterpene-derived insuetolides A-C (**38–40**) and the new strobilactone B derivatives (**41** and **42**), from the marine fungus *Aspergillus insuetus*, isolated from the Mediterranean sponge *Psammocinia* sp. collected in Israel. Insuetolide C (**40**), **41** and **42** were found to inhibit the *in vitro* proliferation of a MOLT-4 cell line by 51%, 55% and 72%, respectively, at a concentration of 50 μM.



Tropolactones A-C (**43–45**), the heptacyclic lactones containing a substituted 2,4,6-cycloheptatriene-2-one ring which is presumably derived from an oxidative

ring expansion of the 2,5-cyclohexadiene-2-one ring of tropolactone D (**46**), were reported as metabolites of the marine-derived *Aspergillus* sp. (strain CNK-371), isolated from an unidentified sponge collected at Manele Bay, Hawaii. Tropolactones A-C (**43**–**45**) displayed weak cytotoxic activity against human colon adenocarcinoma cells (HCT-116) with IC_{50} values of 13.2, 10.9 and 13.9 μM, respectively [54].



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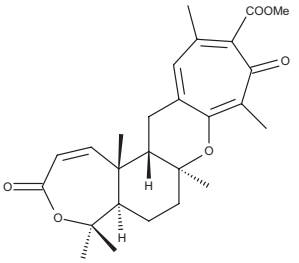

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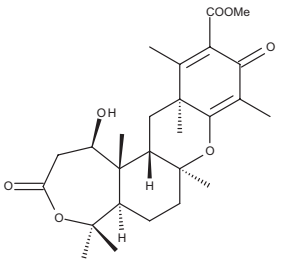
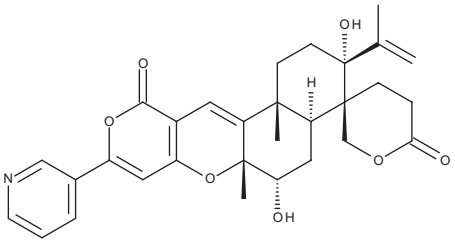


46

Kitano et al. [55] reported isolation the pyridino α-pyrone sesquiterpene, pileotin A (**47**) along with oxalicine B (**48**) from *Aspergillus fumigatus* strain OUPS-N138, isolated from the sea urchin *Toxopneustes pileolus*. Oxalicine B (**48**) was proved to be cytotoxic against murine P388 leukemia cell line with moderate activity (IC_{50} = 55.9 μM).



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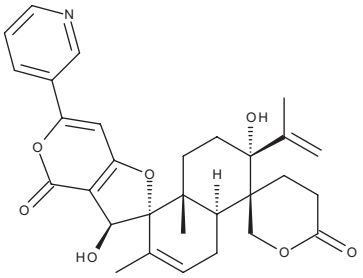
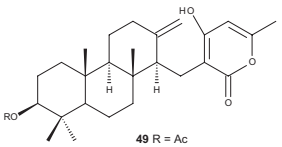


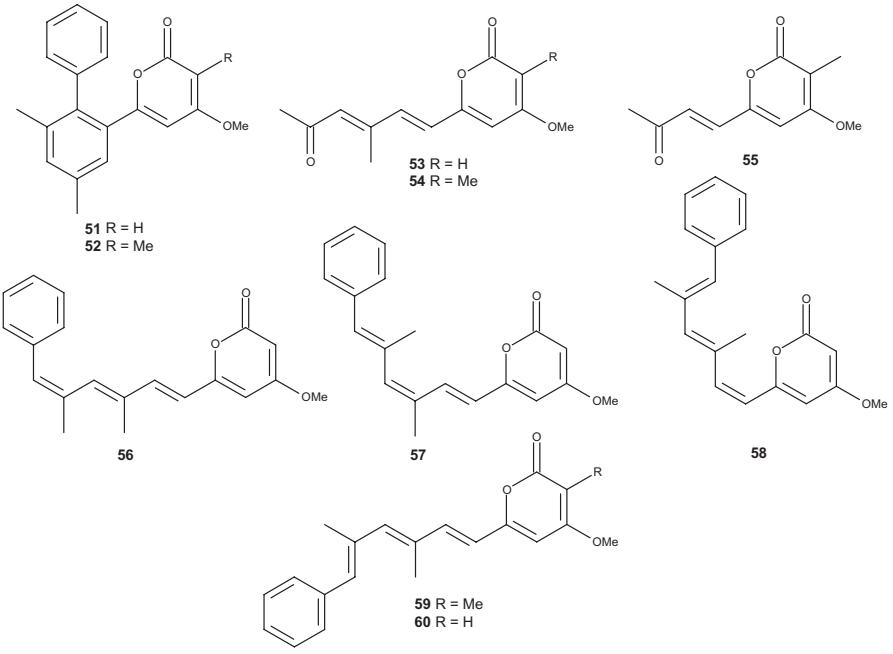
48

The culture of the marine fungus *Neosartorya laciniosa* (KUFC 7896), isolated from a diseased coral (*Porites lutea*) collected at Ao Nuan Lan Island, Chonburi Province, the Gulf of Thailand, yielded the known α-pyrones aszonapyrones A (**49**) and B (**50**). Both compounds were tested for their *in vitro* growth inhibitory activity against MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines and it was found that only aszonapyrone A (**49**) exhibited strong cytotoxic activity, with GI_{50} values of 13.6, 11.6 and 10.2 μM, respectively [56].



49 R = Ac
50 R = H

The endophyte *Aspergillus niger* MA-132, obtained from the mangrove plant *Avicennia marina* collected in Dongzhai Harbor in Hainan, China, yielded eight new α-pyrone derivatives, nigerapyrones A-H (**51–58**), along with the two analogs asnipyrones A (**59**) and B (**60**). While nigerapyrone E (**55**) showed modest to weak cytotoxicity against several cancer cell lines with IC_{50} values ranging from 38 to 105 μM, nigerapyrone B (**52**) showed selective inhibitory activity against the HepG2 cell line with an IC_{50} of 62 μM. Nigerapyrone D (**54**) also displayed moderate to weak cytotoxicity against MCF-7, HepG2, and A549 cell lines, with IC_{50} values of 121, 81, and 81 μM respectively, while asnipyrone A (**59**) exhibited moderate activity against the A549 cell line (IC_{50} = 62 μM) [57].



51 R = H
52 R = Me

53 R = H
54 R = Me

55

56

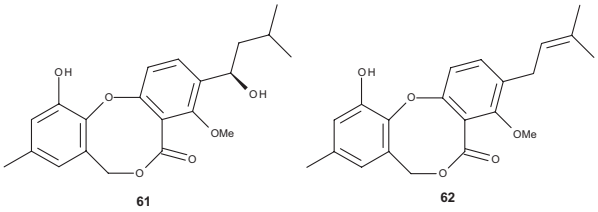
57

58

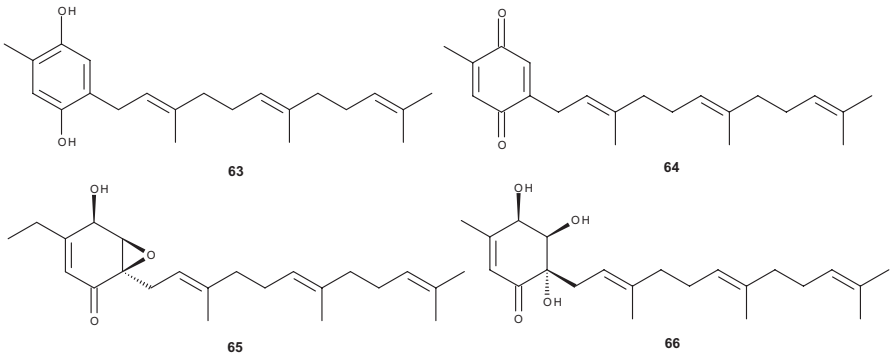
59 R = Me
60 R = H

Penicillide (**61**) and its new analog, prenpenicillide (**62**), were isolated from a strain of *Penicillium* sp., collected from sediments in Jiaozhou Bay, China. Both

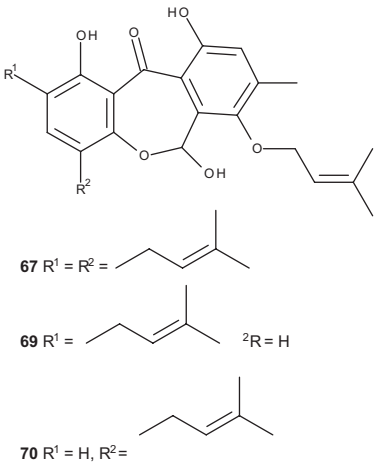
compounds exhibited selective growth inhibitory activity against HepG2 cell line, with IC_{50} values of 9.9 and 9.7 μM, respectively [58].

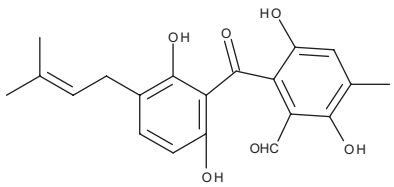


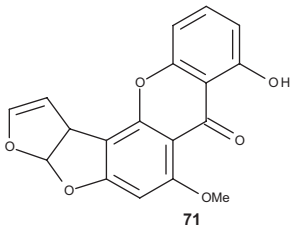
Four farnesyl quinones (**63–66**) were isolated from another *Penicillium* sp. strain, isolated from the surface of a polymeric cord collected in Bijin Island, Gyeongnam Province of South Korea. Farnesylcyclohexenones deacetoxyyanuthone (**65**) and the 2,3-hydro derivative (**66**) were proposed as oxidized derivatives of farnesylhydroquinone (**63**). Farnesylhydroquinone (**63**) and deacetoxyyanuthone (**65**) displayed moderate *in vitro* cytotoxicity against A-549 (human lung cancer), SK-OV3 (human ovarian cancer), SK-MEL-2 (human skin cancer), XF498 (human central nervous system cancer) and HCT15 (human colon cancer) tumor cell lines, with ED_{50} values ranging from 4.73 to 10.07 μM [59, 60].



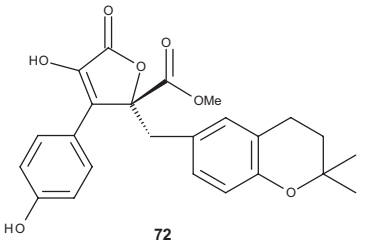
The benzophenone derivatives, arugosins G (**67**) and H (**68**) and the previously reported arugosins A (**69**) and B (**70**) were isolated, together with the biosynthetically related potent cytotoxic agent sterygmatocystin (**71**) [61], from the endophytic fungus *Emericella nidulans* var. *acristata* isolated from a Mediterranean green alga. In a panel of 36 human tumor cell lines, arugosins A (**69**) and B (**70**) were found to exhibit cytotoxicity against seven cell lines at the concentration of 10 μM [62].

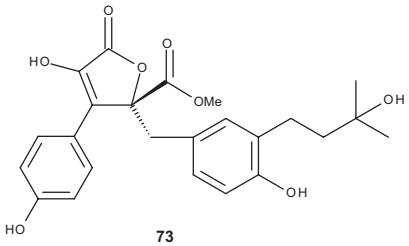


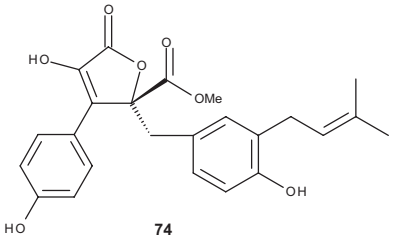




The culture of an *Aspergillus terreus* strain, isolated as an epiphyte from the soft coral *Sinularia kavarattiensis* collected from the coast of Mandapam, Tamil Nadu, India, produced the novel prenylated aromatic butelonides, aspernolides A (**72**) and B (**73**), along with the known butyrolactone I **(74)**. Aspernolide A (**72**) was found to exhibit weak cytotoxic activity against five tumor cell lines, NCI-H460, ACHN, Calu, Panc1 and HCT-116 [63].

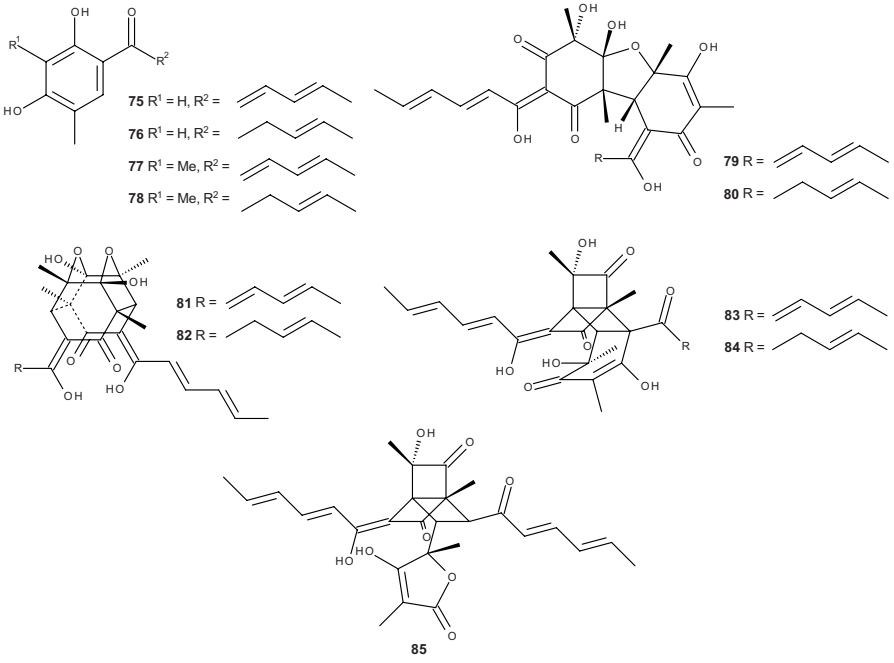






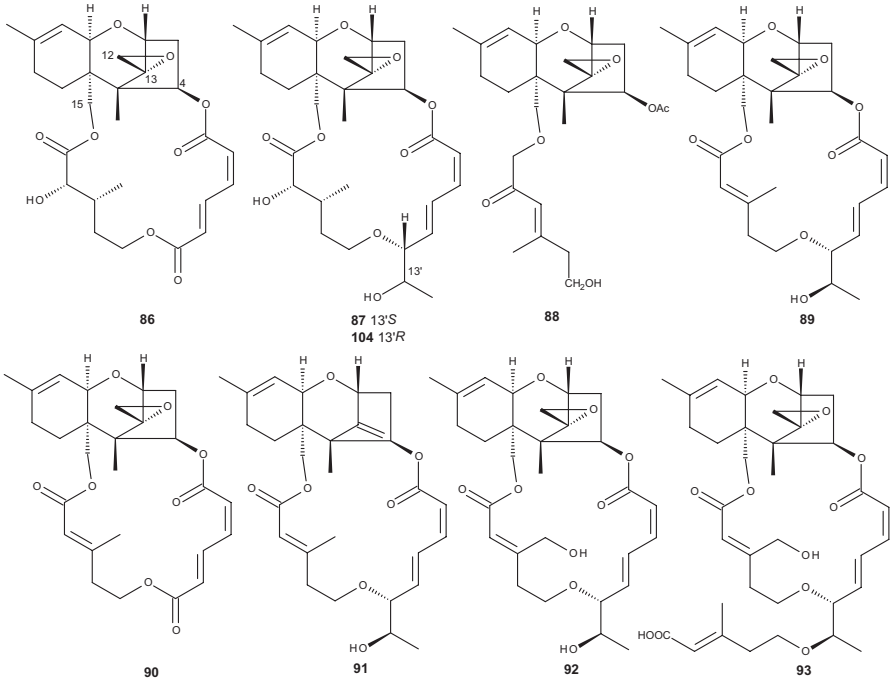
The fungal genus *Trichoderma* is shown to be a source of several monomeric (sorbicillinoids) and dimeric sorbicillin-related natural products (bisorbicillinoids).

Four sorbicillinoids, sohirnone A (**76**), sorbicillin (**77**), 2′,3′-dihydrosorbicillin (**78**) and the new 6-demethylsorbicillin (**75**) were isolated, together with seven dimeric sorbicillin derivatives, bisvertinolone (**79**), trichodimerol (**81**), dihydrotrichodimerol (**82**), bisorbicillinol (**83**), bisvertinoquinol (**84**), bisorbibutenolide (**85**) including the new 10,11-dihydrobisvertinolone (**80**), from the fungus *Trichoderma* sp., isolated from the marine sediment collected in Fujian Province, China. Compounds **75–85** were evaluated for their cytotoxic properties against HL-60 cell line by SRB method; however only birvertinolone (**79**) and trichodimerol (**81**) displayed potent cytotoxicity with IC_{50} values of 5.3 and 7.8 μM, respectively, while the rest of the compounds exhibited scarcely any activity at 50 μM. These results led to the suggestion that the conjugated double bonds in the sidechain might favor the cytotoxic activity of both sorbicillinoids and bisorbicillinoids [64].

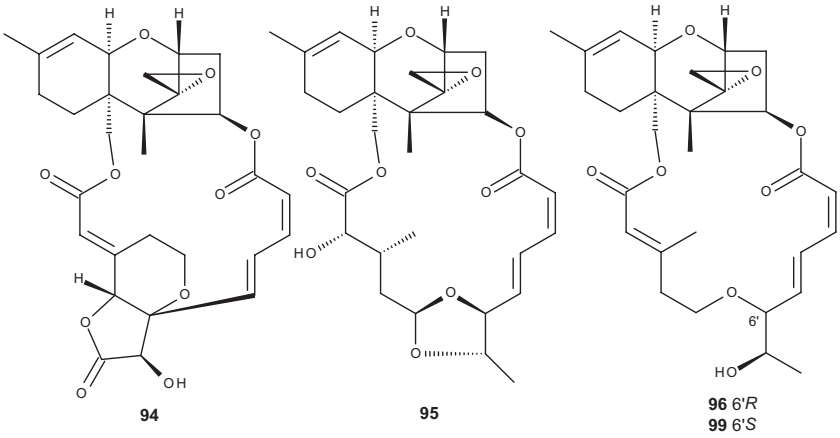


Another major group of compounds produced by fungi is trichothecenes. Trichothecenes are sesquiterpene mycotoxins produced by a large number of terrestrial as well as some marine fungal species, mainly from the genera *Myrothecium* and *Acremonium*. In addition to the highly rearranged sesquiterpenoid-derived moiety, trichothecenes usually carry two additional polyketide-derived hydroxyl acid moieties at C-4 and C-15 which is linked through an ether or ester bridge to give rise to a macrocycle. To date, more than 200 trichothecenes have been reported, and many of which were found to display a broad range of biological activities,

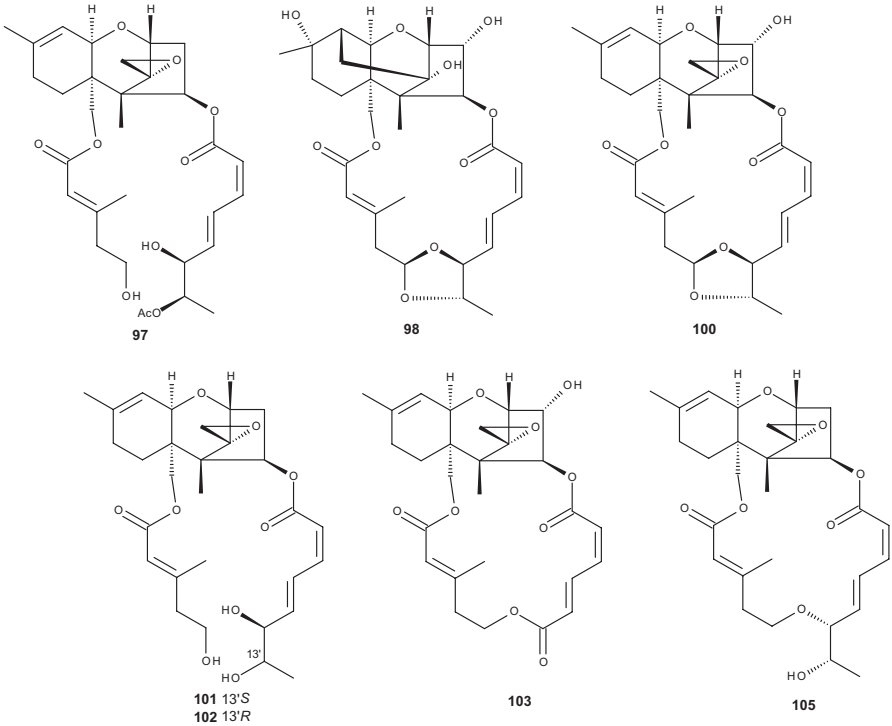
including antitumor. Verrucarin A (**86**), isororidin A (**87**) and the new congener verrol 4-acetate (**88**) were isolated from the cytotoxic deuteromycete *Acremonium neo-caledoniae*, collected from New Caledonia [65]. Namikoshi et al. [66] reported isolation of, besides the known roridin E (**89**), verrucarin A (**86**) and verrucarin J (**90**), a new macrocyclic trichothecene possessing a double bond at C-12 and C-13 which they have named 2,13-deoxyroridin E (**91**), from the marine-derived fungus *Myrothecium roridum*, isolated in Palau. As compound **91** showed reduced cytotoxicity about 80 folds less than that of roridin E (**89**), the authors suggested that the epoxide at C-12 and C-13 would, therefore, affect the cytotoxicity of macrocyclic trichothecenes.



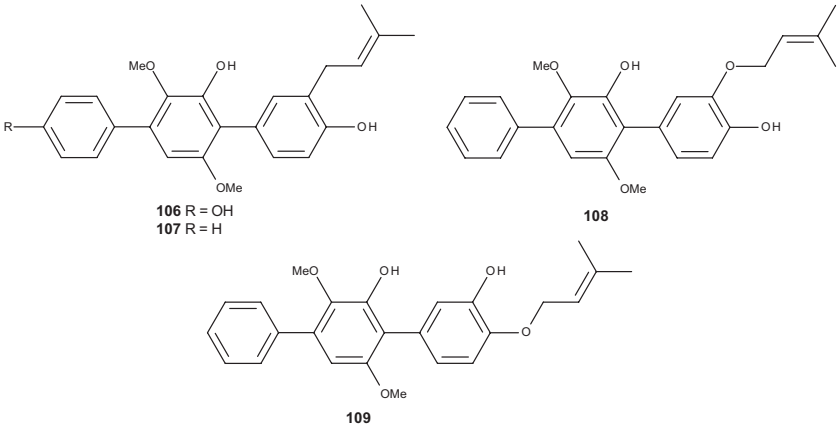
Xu et al. [67] reported isolation of three new macrocyclic trichothecenes, 12′-hydroxyroridin E (**92**), roridin Q (**93**) and 2′, 3′-deoxyroritoxin D (**94**) from the marine-derived fungus *Myrothecium roridum* TUF 98F42, as well as another new macrocyclic trichothecene named roridin R (**95**) from *Myrothecium* sp. TUF 02F6. 12′-Hydroxyroridin E (**92**) and roridin R (**95**) were found to display strong cytotoxic activity against the murine leukemia cell line L1210, with IC_{50} values of 0.19 and 0.45 μM, respectively while roridin Q (**93**) exhibited only moderate activity (IC_{50} = 31.2 μM).



Amagata et al. [68] also reported isolation of three new trichothecenes, 3-hydroxyroridin E (**96**), 13′-acetyltrichoverrin B (**97**), and miophytocen C (**98**), together with nine known macrolide analogs including roridins L (**99**) and M (**100**), trichoverrin A (**101**) and B (**102**), verrucarin A (**86**), verrucarin M (**103**), roridin A (**104**), isororidin A (**87**) and epiroridin E (**105**) from the salt water culture of *Myrothecium verrucaria* strain 973023, isolated from a marine sponge *Spongia* sp. collected off the coast of Maui, Hawaii. Apart from miophytocen **(98)** which lacks the C-12,C-13 epoxide function, all the isolated compounds displayed significant cytotoxicity against murine and NCI-60 panel of human tumor cell lines [68].

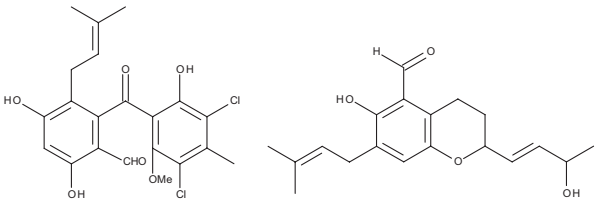


Prenylated *tris*-phenyl derivatives are another group of fungal metabolites. Wei et al. [69] reported isolation of three novel terphenyllin derivatives, prenylterphenyllin (**106**), 4″-deoxyprenylterphenyllin (**107**) and 4″-deoxyisoterprenin (**108**), together with the known 4″-deoxyterprenin (**109**), from a marine-derived fungus *Aspergillus candidus* IF10, isolated from a marine sediment collected at Gokasyo Gulf, Japan. Compounds **106–109** exhibited cytotoxic activity against human epidermoid carcinoma KB cells (KB3–1) with IC_{50} of 8.5, 3.0, 2.5, and 4.5 mg/mL, respectively.

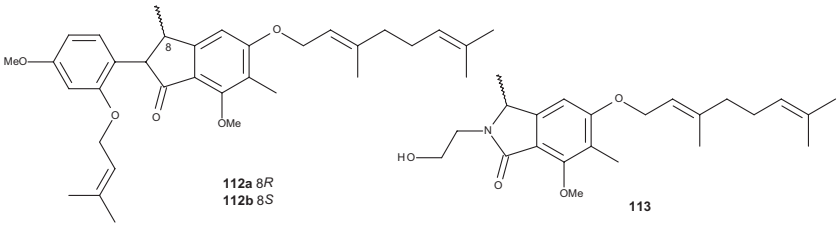


In their effort to develop new methods to enhance secondary metabolite production by using mixed fermentation for the discovery of antibiotics that show efficacy against drug-resistant pathogens, Cueto et al. [70] have isolated pestalone (**110**), a new prenylated chlorinated benzophenone antibiotic, from the mixed fermentation of a marine-derived fungus *Pestalotia* sp. strain CNL-365, isolated from a sample of the brown alga *Rosenvingea* sp. collected in the Bahamas Islands, and an unidentified antibiotic-resistant marine bacterium strain CNJ-328. They have found also that pestalone (**110**) was not produced in control experiments in which the fungus and bacterium were cultured individually, suggesting that the production of pestalone (**110**) is induced by bacterial competition. Experimental evidences have clearly demonstrated that pestalone (**110**) is a product of fungal biosynthesis in response to an external trigger, and is not produced by a mixed biosynthesis in which the fungus transforms a bacterial metabolite. Although pestalone (**110**) was found to exhibit moderate *in vitro* cytotoxicity in the National Cancer Institute's human tumor cell line screen (mean GI_{50} = 6.0 μM), it showed potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MIC = 37 ng/mL) and vancomycin-resistant *Enterococcus faecium* (MIC = 78 ng/mL).

Wang et al. [71] reported isolation of chaetopyranin (**111**), a prenylated benzaldehyde derivative, from the culture of the endophytic fungus *Chaetomium globosum*, isolated from the inner tissue of the marine red alga *Polysiphonia urceolata*, collected from the Qingdao coastline, China. Chaetopyranin **(111)** was found to exhibit moderate to weak cytotoxicity against three tumor cell lines: HMEC (human microvascular endothelial cells), SMMC-7721 (hepatocelular carcinoma cells) and A549 (human lung epithelial cells), showing IC_{50} values of 15.4, 28.5 and 39.1 μM, respectively.

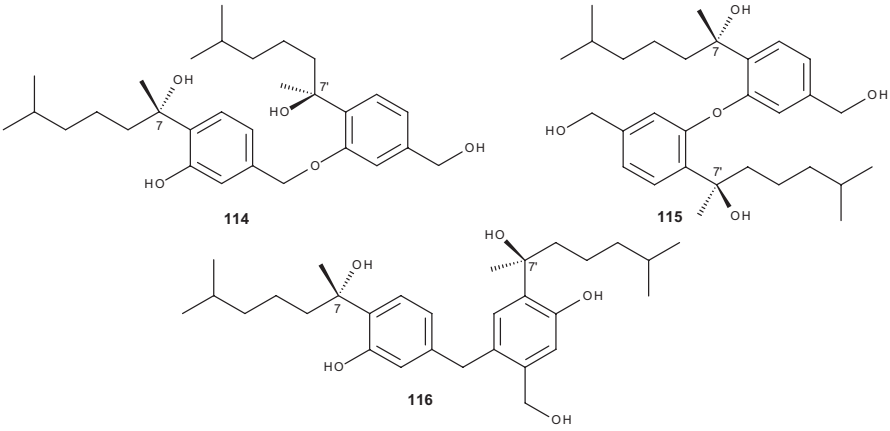


During the search for new natural products produced from the marine-derived fungus *Stachylidium* sp., Almeida et al. [72] have isolated three novel prenylated phthalimidine derivatives: marilines A_1 (**112a**), A_2 (**112b**) and B **(113)** from the culture of the marine-derived fungus *Stachylidium* sp., isolated from the marine sponge *Callyspongia* cf. *C. flammea*. Marilines A_1 (**112a**) and A_2 (**112b**) are enantiomers, differing only in the absolute configuration of C-8. Using the combination of electronic circular dichroism (CD) spectroscopy and quantum-chemical CD calculations, the configuration of marilines A_1 (**112a**) and A_2 (**112b**) was determined as 8*R* and 8*S*, respectively. Mariline A_1 (**112a**) was tested against five cancer cell lines and exhibited a mean GI_{50} of 24.4 μM, while mariline A_2 (**112b**) was tested against 19 cancer cell lines and showed a GI_{50} of 11.02 μM. Interestingly, the observed antiproliferative pattern of mariline A_2 (**112b**) did not correlate with that of any of the standard antiproliferative compounds with known mechanism of action as deduced by COMPARE analysis. Moreover, both marilines A_1 (**112a**) and A_2 (**112b**) were found to inhibit human leukocyte elastase (HLE), a serine protease considered to be a primary source of tissue damage associated with inflammatory diseases such as chronic obstructive pulmonary disease, cystic fibrosis, and adult respiratory distress syndrome, with the same potency (IC_{50} = 0.86 μM) [72].

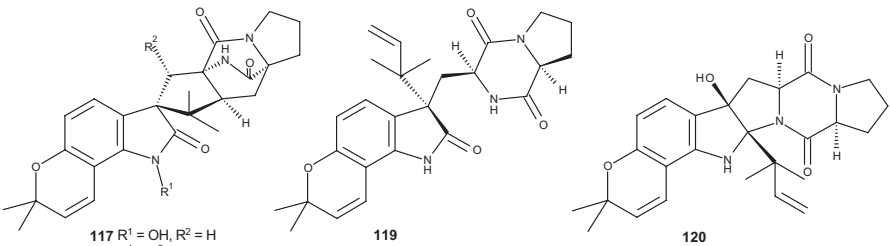


Sun et al. [73] reported isolation of three new phenolic bisabolane sesquiterpenoid dimers which they have named disydonols A-C (**114–116**), from the fermentation broth of a marine-derived fungus *Aspergillus* sp., isolated from the marine sponge *Xestospongia testudinaria* collected from the South China Sea. Compounds **114–116** were tested for their *in vitro* cytotoxic activity against HepG-2 human hepatoma cell line and Caski human cervical cell line by MTT assay. Compound **114** displayed moderate *in vitro* cytotoxicity toward these two cell lines (IC_{50} = 9.31 and 12.40 μg/mL, respectively) while compound **116** showed selective activity against these two cell lines with IC_{50} of 2.91 and 10.20 μg/mL, respectively. However, compound **115** was found to be relatively non-cytotoxic (IC_{50} > 100 μg/mL) against these two tumor cell lines. Since compounds **114** and **116**, which possess the 7*S* and

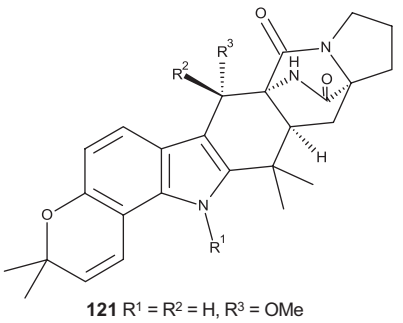
7′*S* configuration, displayed more potent cytotoxicity toward the tumor cell lines than **115** which possesses the 7*S* and 7′*R* configuration, it was suggested that the cytotoxic activity might be weakened due to the mesomeric effect, and the activity of these compounds is stereoselective.



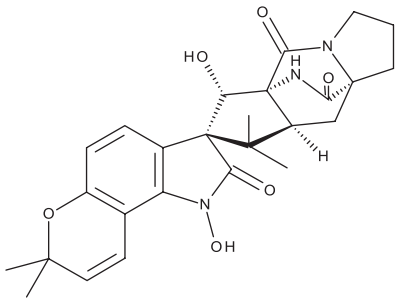
Prenylated diketopiperazine alkaloids are also produced by many fungal species. Kato et al. [74] reported isolation of four doubly prenylated diketopiperazine alkaloids, notoamides A-D (**117–120**), from the culture of a marine-derived fungus *Aspergillus* sp., isolated from a common mussel *Mytilus edulis* collected off Noto Peninsula in the Japan Sea. Notoamides A-C (**117–119**) were found to exhibit moderate cytotoxicity against HeLa and L-1210 cells, with IC_{50} values in the range of 22–52 μg/mL. In contrast, the IC_{50} value of notoamide D (**120**) was greater than 100 μg/mL. Since notoamide D (**120**) contains a pyrroloindole ring instead of a dihydroxypyrano-2-oxindole ring system, which is common to notoamides A-C **(117–119)**, this variation is thought to be responsible for the marked differences in their cytotoxicity. Furthermore, they have also found that notoamide C (**119**) was able to induce G2/M-cell cycle arrest at a concentration of 6.3 μg/mL [74, 75].



Later on, Tsukamoto et al. [76] reported isolation of six new notoamides, notoamides F-K (**121–126**), from the culture of the same fungus. They have found also that only notoamide I (**124**) showed weak cytotoxicity against HeLa cells with an IC_{50} value of 21 μg/mL, whereas IC_{50} values for notoamides F (**121**), J (**125**), and K (**126**) were more than 50 μg/mL [76, 77].



121 $R^1 = R^2 = H$, $R^3 = OMe$
122 $R^1 = OH$, $R^2 = H$, $R^3 = OMe$
124 $R^1 = H$, $R^2 = R^3 = O$



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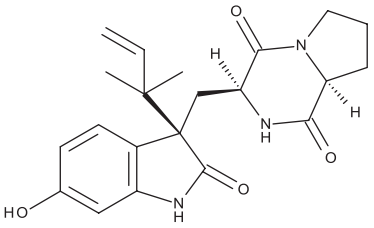

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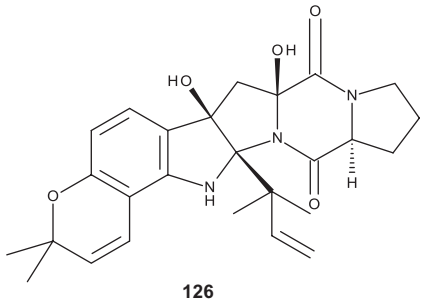
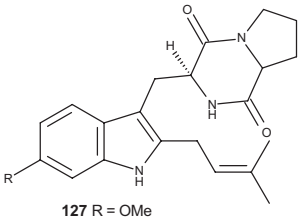
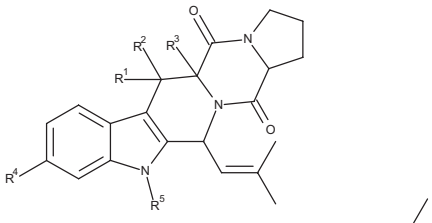


126

Cui et al. [78−83], have described isolation of a series of prenylated indole diketopiperazine alkaloids, including tryprostatins A (**127**) and B (**128**), fumitremorgins B (**129**) and C (**130**), 12,13-demethoxyfumitremorgin C (**131**), 12,13-dihydroxyfumitremorgin C (**132**), cyclotryprostatins A-D (**133–136**), spirotryprostatins A (**137**) and B (**138**), and verruculogen (**139**), from the culture of *Aspergillus fumigatus* BM939. All compounds caused a complete cell cycle progress inhibition of tsFT210 cells (mouse mammary carcinoma cell line) in the G2/M phase. Moreover, spirotryprostatin B (**138**) was found to display cytotoxic activity on the growth of human chronic myelogenous leukemia K562 and human promyelocytic leukemia HL-60 cells, with MIC values of 35 and 10 μM, respectively [80].

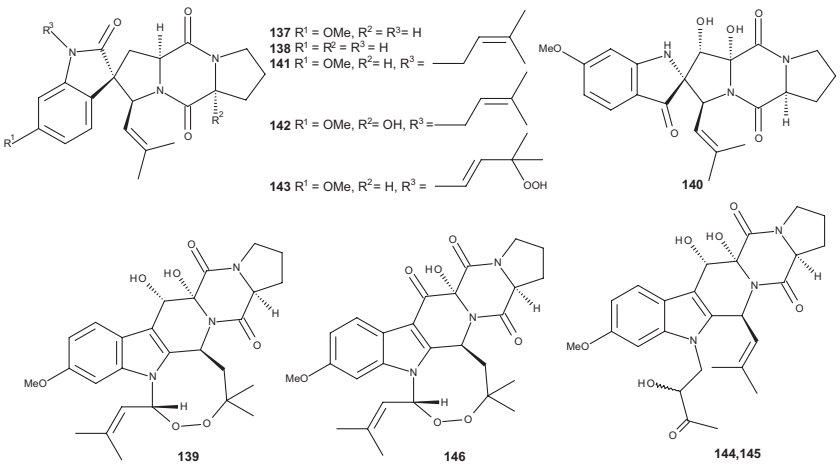


127 R = OMe
128 R = H

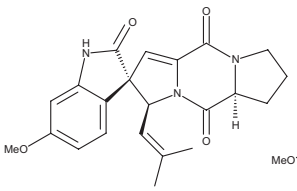


129 R^1 = β -H, R^2= α -OH, R^3= α -OH, R_4= OMe, R^5 =
130 $R^1 = R^2 = H$, R^3 = β -H, R^4= OMe, $R_5 = H$
131 $R^1 = R^2 = H$, R^3 = α -H, $R^4 = R^5 = H$
132 R^1 = β -H, $R^2 = R^3$ = α -OH, R^4 = OMe, $R^5 = H$
133 R^1 = β -H, R^2= α -OH, R_3= β -OH, R^4 = OMe, $R^5 = H$
134 R^1 = OMe, $R^2 = H$, R^3 = α -OH, R^4= OMe, $R^5 = H$
135 R^1 = β -H, $R^2 = R^3$ = α -OH, $R^4 = R^5 = H$
136 $R^1 = R^2 = O$, R^3 = α -OH, $R^4 = R^5 = H$

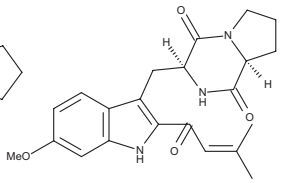
Due to the promising antitumor activities demonstrated by tryprostatins A (**127**) and B (**128**), synthesis of several analogs and their subsequent study on the inhibition of microtubule assembly were carried out [84−86]. Wang et al. [87] described isolation of another series of prenylated diketopiperazine derivatives, including the prenylated indolo-diketopiperazine **140**, spirotryprostatins C-E (**141–143**), two derivatives of fumitremorgin B (**144, 145**) and 13-oxoverruculogen (**146**), from the culture of the fungus *Aspergillus fumigatus*, isolated from the holothurian *Strichopus japonicus* collected from Lingshan Island, Qingdao, China. All the isolated compounds, except **140** (compound **140** was evaluated only for HL-60 and A549), were evaluated for their cytotoxicity against MOLT-4, HL-60, A549, BEL-7402 cell lines, and were found to exhibit the selected activity to the four cancer cell lines. Further analysis of the activity data suggested that compounds **143–145** showed better susceptibility to MOLT-4, HL-60, and A549 (with IC_{50} values ranging from 3.1 to 11.0, 2.3 to 5.4 and 3.1 to 11.6 μM, respectively) than those of compounds **140–142** and **146** [87].



Zhang et al. [88] isolated, among other fungal metabolites, three indolo- diketopiperazine alkaloids: 6-methoxyspirotryprostatin B (**147**), 18-oxotryprostatin A (**148**) and 14-hydroxyterezine D (**149**) from the ethyl acetate extract of a marine-derived fungal strain *Aspergillus sydowi* PFW1-13, isolated from a driftwood sample collected from the beach of Baishamen, Hainan, China. 6-Methoxyspirotryprostatin B (**147**), 18-oxotryprostatin A (**148**) and 14-hydroxyterezine D (**149**) displayed weak cytotoxic activity against A-549 cells with IC_{50} values of 8.29, 1.28, and 7.21 μM, respectively. 6-Methoxyspirotryprostatin B (**147**) was also found to be active against HL-60 cells (IC_{50} = 9.71 μM).



147



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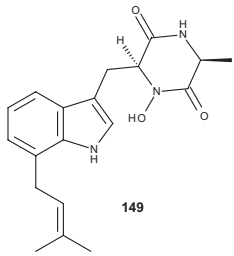
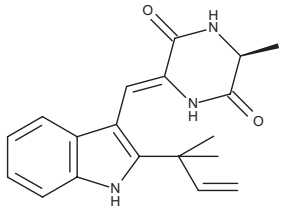


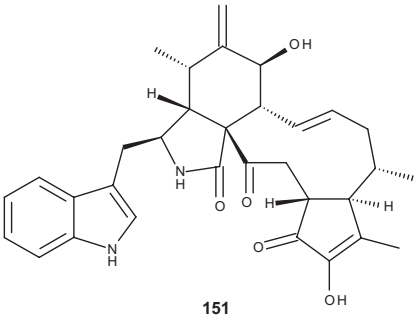
149

Neoechinulin A **(150),** another prenylated indolo-diketopiperazine alkaloid, has been reported from several marine-derived fungi, especially from the genus *Eurotium* [89, 90]. Recently, Wijesekara et al. [91] have investigated the cytotoxic effect of neoechinulin A **(150)** isolated from a marine-derived fungus *Microsporum* sp. strain MFS-YL, isolated from the surface of a marine red alga *Lomentaria catenata*, collected at Guryongpo, Nam-Gu, PoHang in Republic of Korea, on human cervical carcinoma HeLa cells and the apoptosis induction in HeLa cells. By using Western blot analysis, they have found that neoechinulin A **(150)** could induce cell apoptosis through down-regulating of Bcl-2 expression, up-regulating of Bax expression, and activating the caspase-3 pathway.

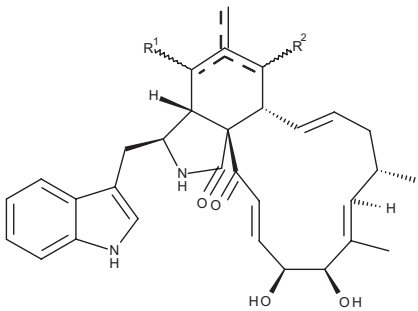


150

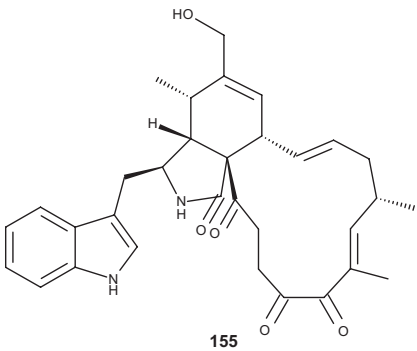
Cui et al. [92] described isolation and structure elucidation of seven new cytochalasan derivatives, cytoglobosins A-G (**151–157**), together with two structurally related known compounds, isochaetoglobosin D (**158**) and chaetoglobosin F_{ex} (**159**), from the cultures of *Chaetomium globosum* QEN-14, an endophytic fungus derived from the marine green alga *Ulva pertusa*. Cytoglobosins A-E (**151–155**) and G (**157**) were evaluated for their cytotoxicity against P388, A549, and KB cell lines. It was found that only cytoglobosins C (**153**) and D (**154**) exhibited cytotoxicity toward the A-549 cell line ($IC_{50} = 2.26$ and 2.55 μM), while cytoglobosins A (**151**), B (**152**), E (**155**), and G (**157**) were found to be inactive ($IC_{50} > 10$ μM).



151



152 R^1 = α-Me, R^2 = β- OH, $\Delta^{6(12)}$
153 R^1 = Me, R^2 = β- OH, Δ^5
154 R^1 = α-Me, R^2 = H, Δ^6



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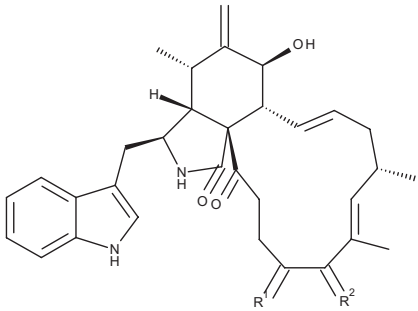
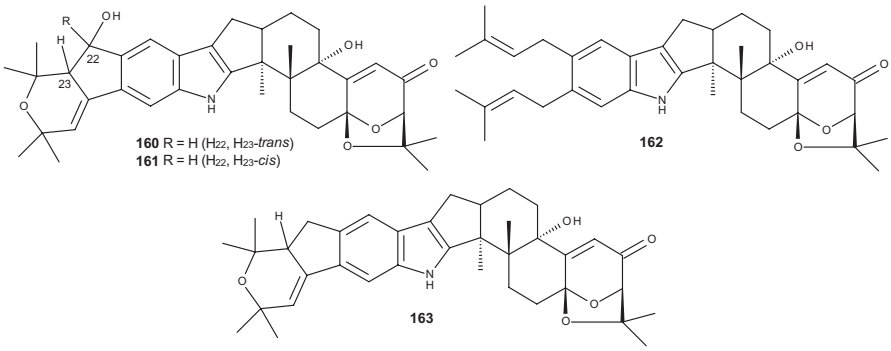
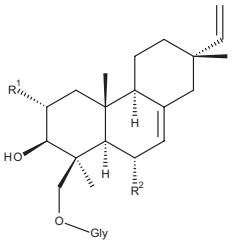


156 R^1 = O, R^2 = H, β-OH
157 R^1 = R^2 = H, β-OH
158 R^1 = R^2 = O
159 R^1 = H, β-OH, R^2 = O

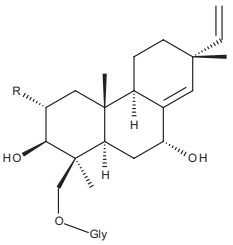
The culture of the fungus *Penicillium janthinellum* Biourge, isolated from marine bottom sediments collected in Amursky Bay in Japan, was found to produce three new indole diterpenoid alkaloids, shearinines D-F (**160–162**), together with the known shearinine A (**163**). All of the isolated compounds did not show any cytotoxicity up to 200 μM against mouse epidermal JB6 P^+ Cl 41 cells. However, shearinine E (**161**) was found to display cancer-preventive properties inhibiting EGF-induced malignant transformation of JB6 P^+ Cl 41 cells in a soft agar with $INCC_{50}$ (inhibition of number of the colonies) value of 13 μM. Additionally, shearinines A (**163**), D (**160**) and E (**161**) were found to induce apoptosis in human leukemia HL-60 cells at 100 μM concentration by 10, 39 and 34% of the apoptotic cells when compared to control cells, respectively [93].



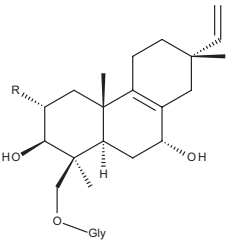
Afiyatullov et al. [102, 103] have first reported isolation of three new isopimarane diterpene glycosides, virescenosides M (**167**) and N (**168**), together with the known virescenosides A (**164**), B (**165**) and C (**166**), from the culture of the marine-derived fungus *Acremonium striatisporum*, isolated from superficial microbiota of the sea cucumber *Eupentacta fraudatrix* collected from the Sea of Japan. Interestingly, virescenosides A (**164**), B (**165**) and C (**166**) have been previously reported as constituents of the terrestrial strain *Acremonium luzulae* [96–98]. Further investigation of the secondary metabolites from this fungal strain led the same authors [99] to isolate more three new isopimarane glycosides which they have named virescenosides O (**169**), P (**170**) and Q (**171**). It is interesting to note that the sugar moiety in virescenoside Q (**171**) is *β*-D-mannopyranose instead of *β*-D-altropyranose of other virescenosides. In continuation of their work of this fungal strain, they also isolated four more new isopimarane glycosides, namely virescenosides R (**172**), S (**173**), T (**174**) and U (**175**) [100]. All these diterpenic glycosides were found to exhibit *in vitro* cytotoxic activity against tumor cells of Ehrlich carcinoma with IC_{50} values ranging from 10–100 μM. Later on, another three new virescenosides have been isolated, virescenosides V (**176**), W (**177**) and X **(178)**, however no cytotoxic activity have been reported by Afiyatullov et al. [101].



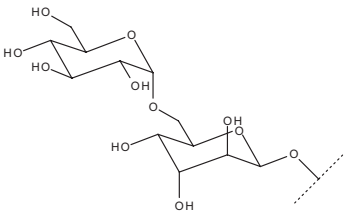
164 R^1 = OH, R^2 = H, Gly = Alt
172 R^1 = R^2 = H, Gly = Alt
165 R^1 = R^2 = H, Gly = Alt, 3-ketone
171 R^1 = R^2 = H, Gly = Man
166 R^1 = OH, R^2 = H, Gly = Alt-Glc
177 R^1 = R^2 = OH, Gly = Alt



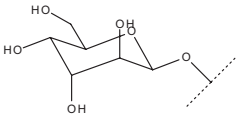
168 R = OH, Gly = Alt
176 R = H, Gly = Alt
169 R = H, Gly = Alt, 3-ketone
173 R = H, Gly = Alt, 3,7-diketone
175 R = OH, Gly = Alt, 7-ketone



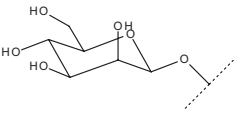
170 R = OH, Gly = Alt, 7-ketone
174 R = H, Gly = Alt, 7-ketone
178 R = H, Gly = Alt, 3,7-diketone
167 R = OH, Gly = Alt



Alt-Glc = α -D-glucopyranosyl (1-6)-β-D-altropyranosyl-

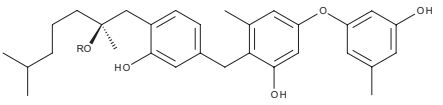


Alt = β-D-altropyranosyl-

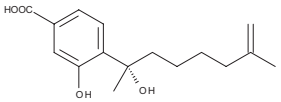


Man = β-D-mannopyranosyl-

Lu et al. [102] have isolated two new polyphenols containing both phenolic bisabolane sesquiterpenoid and diphenyl ether units, expansols A (**179**) and B (**180**), and two new phenolic bisabolane monoterpenoids, (*S*)-(+)-11-dehydrosydonic acid (**181**) and (7*S*,11*S*)-(+)-12-acetoxysydonic acid (**182**), from the culture of the marine-derived fungus *Penicillium expansum* 091006, endogenous with the mangrove plant *Excoecaria agallocha* collected in Wenchang, Hainan Province, China. Expansols A (**179**) and B (**180**) were found to exhibit moderate cytotoxic activity against HL-60 cell line with IC_{50} values of 15.7 and 5.4 μM, respectively, while expansol B **(180)** also displayed strong inhibitory activity against A549 cell line (IC_{50} = 1.9 μM).



179 R = Me
180 R = H



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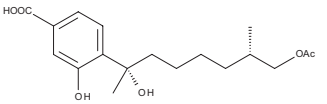
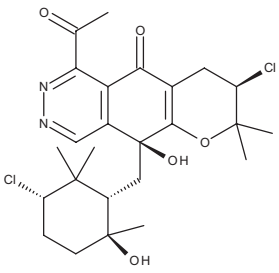


182

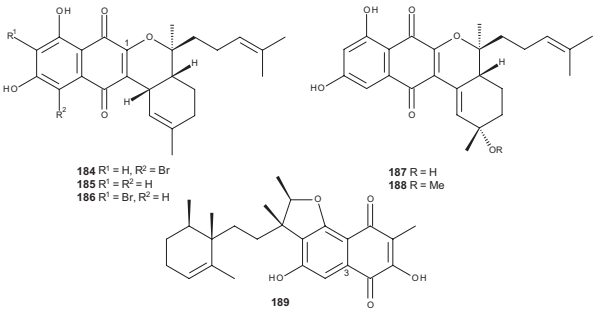
16.3.2 From Marine Bacteria

Cho et al. [103] described isolation and structure elucidation of azamerone (**183**), an unusual meroterpenoid having an unprecedented chloropyranophthalazinone core with a 3-chloro-6-hydroxy-2, 2, 6-trimethylcyclohexylmethyl sidechain, from the culture of a new marine-derived bacterium related to the genus *Streptomyces* (strain CNQ766). Azamerone (**183**) displayed weak cytotoxic activity against mouse splenocyte populations of T-cells and macrophages (IC_{50} = 40 μM), possibly due to topoisomerase inhibition.



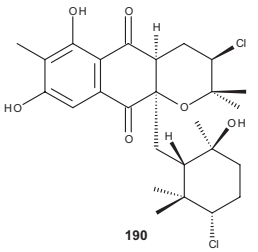
183

The marinones are a group of a new structural class of sequiterpenoid naphtoquinones antibiotic, isolated from marine actinomycetes. Pathirana et al. [104] first reported isolation of marinone (**184**) and its debromo analog, debromomarinone (**185**), from the organic extract of the liquid culture of a marine actinomycete isolate CNB-632. Hardt et al. [105] reported isolation of three additional derivatives, isomarinone (**186**), hydroxydebromomarinone (**187**) and methoxydebromomarinone (**188**), together with the structurally related novel compound neomarinone (**189**), from the fermentation broth of a taxonomically-novel marine actinomycete (strain #CNH-099) found in a sediment sample taken at -1 m in Batiquitos Lagoon, North of San Diego, CA. It is interesting to note that while marinone (**184**), debromomarinone (**185**), isomarinone (**186**), hydroxydebromomarinone (**187**) and methoxydebromomarinone (**188**) possess a non-rearranged sesquiterpenoid residue, formally attached via C-1 of farnesyl pyrophosphate (FPP) to the naphthoquinone core, neomarinone (**189**) possesses a highly rearranged sesquiterpenoid residue attached to the naphthoquine core through C-3 of FPP. Later on, Kalaitzis et al. [106] used feeding experiments with ^{13}C-labeled acetate and administration of the general precursor [U-^{13}C6]glucose to revise the structure of neomarinone (**189**) previously reported by Hardt et al. [105].



The marinones are cytotoxic compounds, presenting moderate *in vitro* inhibitory activity against human colon adenocarcinoma HCT-116 cells with a mean IC_{50} = *ca.* 8 μM. Neomarinone (**189**) was found to generate a mean IC_{50} of 10 μM in assays with the NCI-60 panel of cancer cell lines [105]. Suárez et al. [107], in their attempt to determine the configuration of the methyl groups of the cyclohexenyl ring relative to the methyl groups in the furyl residue of neomarinone (**189**), have carried out an efficient stereoselective synthesis of its sidechain while Peña-López et al. [108] have succeeded in performing a total synthesis of (+) neomarinone.

The fungal metabolites napyradiomycins are a group of meroterpenes consisting of the semi-naphthoquinone core with prenyl (normally cyclized) and monoterpenyl substituents on C-4a and C-10a, respectively. The monoterpenoid moiety in the napyradiomycin A series is linear while that of napyradiomycin B and C series is cyclized to a 6- or 14-membered ring, respectively [109]. In a pursuit of bioactive secondary metabolites from marine–derived actinomycete bacteria, the group of Fenical [110, 111] has succeeded in isolating three new chlorinated dihydroquinones (**190–192**) and one previously reported analog (**193**), from the culture of the obligate marine actinomycete strain CNQ-525 from a sediment sample obtained near La Jolla, California. Although these compounds possess new carbon skeletons, their structures are related to the napyradiomycin class of antibiotics. Compounds **190**, **191** and **193** showed strong cytotoxic activity toward HCT-116 cells with IC_{50} of 2.4, 0.97 and 1.84 μM, respectively [110, 111]. Winter and Moore [112] have shown that the napyradiomycin biosynthetic gene cluster from *Streptomyces* sp. CNQ-525 was wholly responsible for the synthesis of these chlorinated meroterpenoids in which the putative V-ClPOs may be responsible for the chlorination and cyclization of these compounds. Bernhardt et al. [113] have reported the first biochemical characterization of a bacterial vanadium dependent chloroperoxidase, NapH1 from *Streptomyces* sp. CNQ-525, which catalyzes a highly stereoselective chlorination-cyclization reaction in the biosynthesis of napyradiomycin antibiotics, including of compound **190** and **191**.



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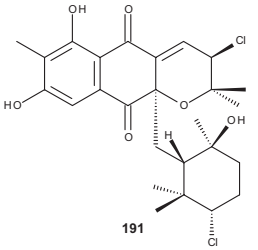

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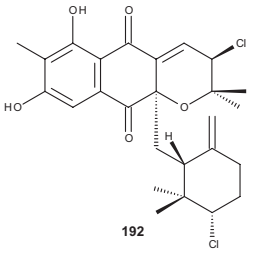

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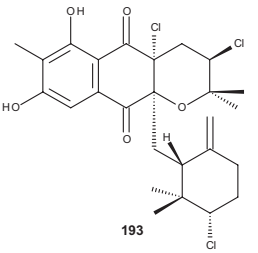
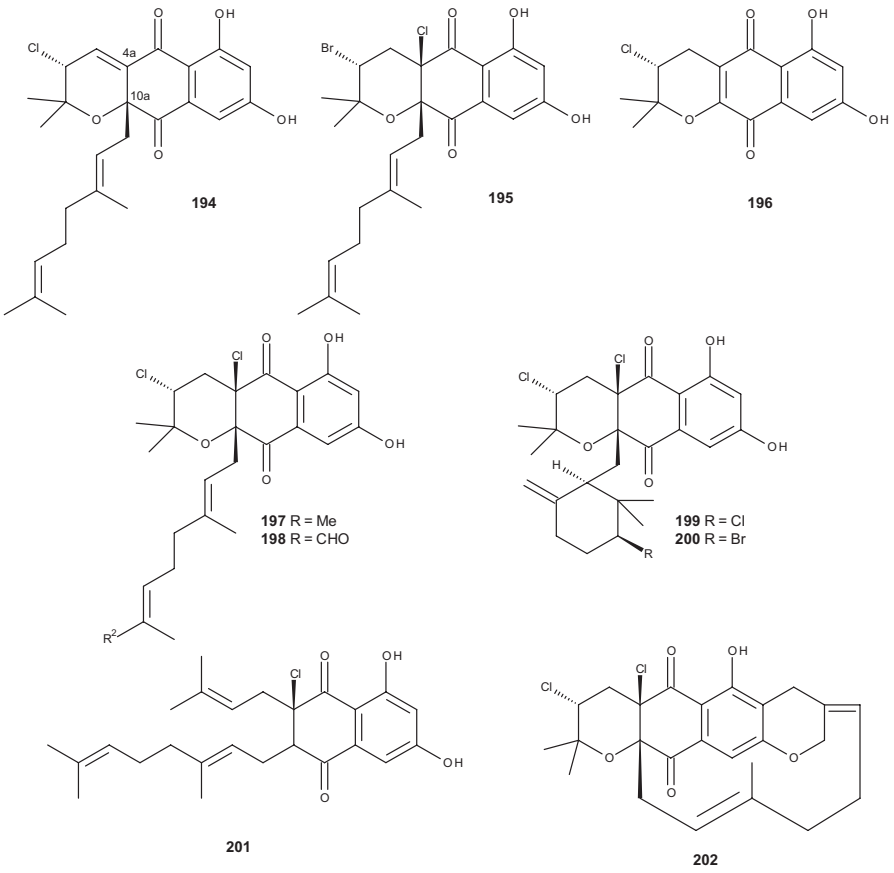
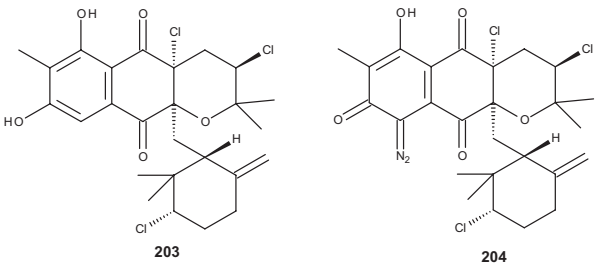


193

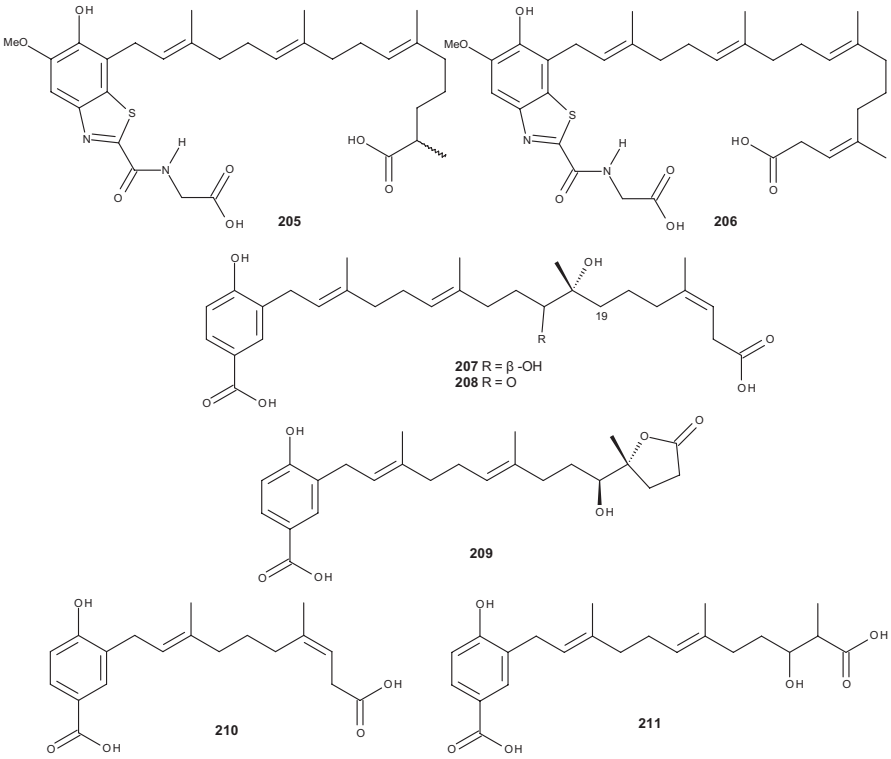
Wu et al. [114] have recently reported isolation of another three new napyradiomycin derivatives: 4-dehydro-4a-dechloronapyradiomycin A1 **(194)**, 3-dechloro-3-bromonapyradiomycin A1 (**195**) and 3-chloro-6,8-dihydroxy-8-α-lapachone (**196**), together with the six known related analogs napyradiomycin A1 (**197**), 18-oxonapyradiomycin A1 (**198**), napyradiomycin B1 (**199**), napyradiomycin B3 (**200**), naphthomevalin (**201**), and napyradiomycin SR (**202)** from the marine-derived *Streptromyces* sp. strain SCSIO 10428, isolated from a sediment sample collected from Xieyang Island, Guangxi Province, China. Napyradiomycins **197–202** have been previously isolated from the culture of *Streptomyces antimycoticus* NT17 [115]. Compounds **195**, **197**, **199** and **200** were found to exhibit moderate cytotoxicity against four human cancer cell lines: SF-268, MCF-7, NCI-H460, and HepG2 with IC_{50} values below 20 μM [114].



The antibacterial napyradiomycin derivatives A80915A (**203**) and A80915B (**204**), which were previously reported from *Streptomyces aculeolatus* [116], were isolated from the culture of a marine-derived actinomycete, *Streptomyces* sp. strain CNQ-525 from a marine sediment, collected at a depth of 152 m near La Jolla, California, USA. Besides their potent and rapid bactericidal activity against contemporary MRSA strains, they also displayed strong antiproliferative activity (with IC_{50} values of 3 μM and 500 nM for **203** and **204**, respectively) against HCT-116 human colon adenocarcinoma cells [117].

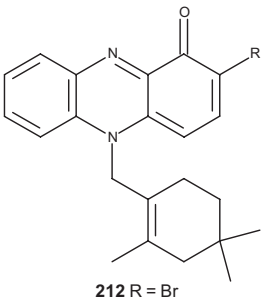


The erythrazoles are another mixed biosynthetic origin fungal metabolites containing a tetrasubstituted benzothiazole, an appended diterpene side chain, and a glycine unit. By using high-throughput screening for the Locus Derepression Assay (LDR), Hu and MacMillan [118] have isolated erythrazoles A (**205**) and B (**206**) from the extract of the marine-derived bacterium *Erythrobacter* sp. strain SNB-035 from a sediment sample, collected from Trinity Bay Galveston, Texas, USA. Erythrazole B (**206**) was found to exhibit cytotoxicity against the non-small cell lung cancer (NSCLC) cell lines H1395 (IC_{50} = 1.5 μM), H2122 (IC_{50} = 2.5 μM) and HCC366 (IC_{50} = 6.8 μM) while erythrazole A **(205)** did not exhibit any activity at concentration up to 20 μM against these three cell lines. In a continuation of their work, Hu et al. [119] have also isolated five derivatives of 4-hydroxybenzoic acid appended with a modified terpene side chain which they have named erythrolic acids A-E (**207–211**) from the same strain of the marine-derived bacterium. Erythrolic acid D (**210**) was found to be the only molecule that showed activity, with IC_{50} value of 2.4 μM against the NSCLC cell line HCC44 and 3.4 μM against HCC366.

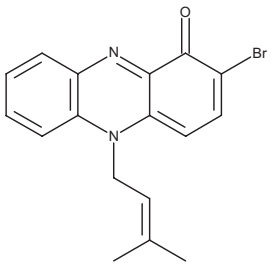


Kondratyuk et al. [120] reported isolation of two new brominated phenazine monoterpenoid derivatives (**212**) and (**213**), and the known lavanducyanin (**214**) [121], from a marine-derived *Streptomyces* sp. strain CNS284. These compounds were evaluated for their anti-inflammatory activity as well as for their *in vitro* cytotoxicity against HL-60 cells. It was found that treatment of HL-60 cells with **212–214**

resulted in accumulation in the subG1 phase of the cell cycle, indicating that apoptosis is partly responsible for cell killing of these compounds.

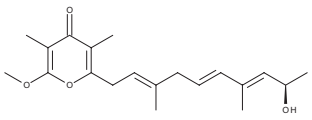


212 R = Br
214 R = H



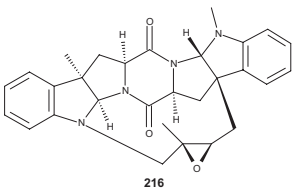
213

Kalkipyrone (**215**), a novel α-methoxy-*β*,*β*′-dimethyl-*γ*-pyrone possessing an alkyl sidechain isolated from an assemblage of the marine cyanobacteria *Lyngbya majuscule* and *Tolypothrix* sp., collected from the splash zone at Playa Kalki in the Caribbean Island Curaçao, was the first γ-pyrone-derived natural product reported from a cyanobacterium. Kalkipyrone (**215**) was evaluated in the NCI's 60 human-tumor cell line *in vitro* assay, but was found only modestly inhibitory to several renal and melanoma cell lines [122].



215

Nocardioazines A (**216**) and B (**217**), members of a new class of prenylated diketopiperazine alkaloids containing a bridged diketopiperazine scaffold, were produced by a non-saline liquid culture of *Nocardiopsis* sp. (CMB-M0232), isolated from a sediment sample off South Molle Island, Australia. Although nocardioazines were non-cytotoxic to human cancer cell lines, a P-gp overexpressing colon cancer cell line (SW620Ad300) assay revealed that nocardioazine A (**216**) was capable of reversing doxorubicin resistance in a P-gp overexpressing drug resistant colon cancer cell line [123].



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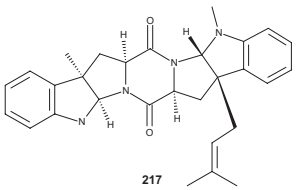
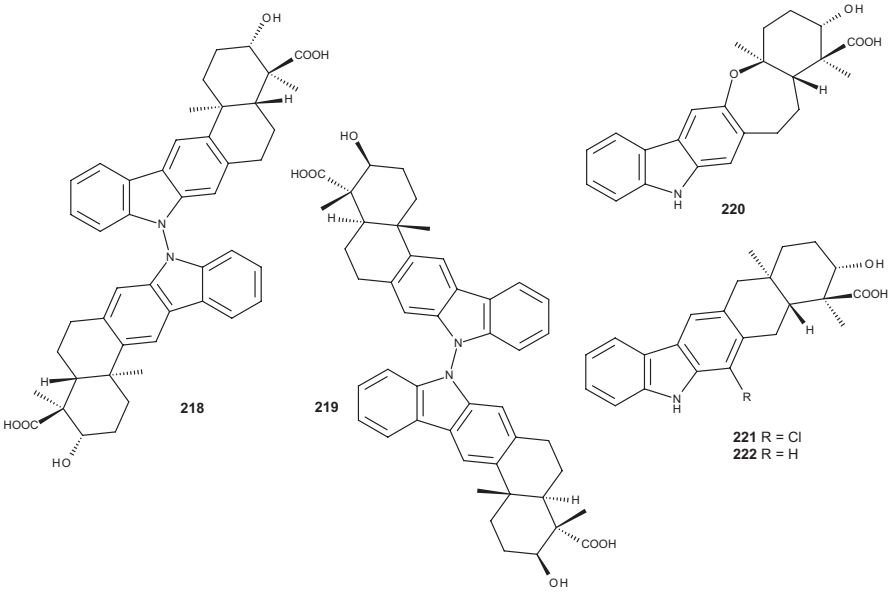
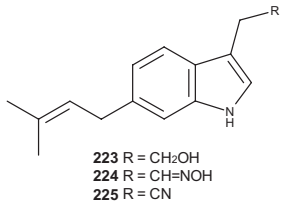


217

Zhang et al. [124] described isolation of four new dimeric indolo-sesquiterpenes: dixiamycins A (**218**) and B (**219**), oxiamycin (**220**) and chloroxiamycin (**221**), together with the previously reported xiamycin A (**222**) [125, 126], from the marine-derived actinomycete *Streptomyces* sp. SCSIO 02999, isolated from a sediment collected from South China Sea. Dixiamycins A (**218**) and B (**219**) were the first examples of atropisomerism of naturally occurring N-N-coupled atropo-diastereomers with a dimeric indolo-sesquiterpene skeleton and a stereogenic N-N axis between sp^3-hybridized nitrogen atoms. All the compounds were found to exhibit weak cytotoxicity against four human tumor cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), SF268 (glioma) and HepG2 (hepatocellular carcinoma) [124].

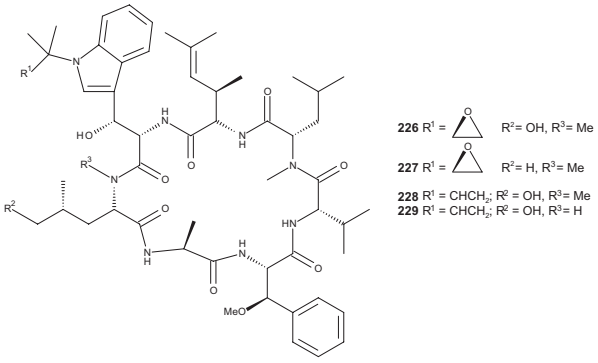


Bioassay-guided fractionation of *Streptomyces* sp. strain BL-49-58-005, isolated from an unidentified marine invertebrate collected in Mexico, yielded three isoprenoid indole-alkaloids (**223**–**225**). The isolated compounds were evaluated for their cytotoxicity against a panel of 14 different tumor cell lines. Compound **223** was found to exhibit best activity (GI_{50} = 8.46 μM) against human leukemia K562 cell line while the aldoxime mixture **(224)** displayed cytotoxic activity in micromolar range against several tumor cell lines including LN-caP (prostate cancer), HMEC1 (endothelial cancer), K562 (leukemia), Panc1 (pancreas cancer), and LOVO and LOVO-DOX (colon cancer) [127].

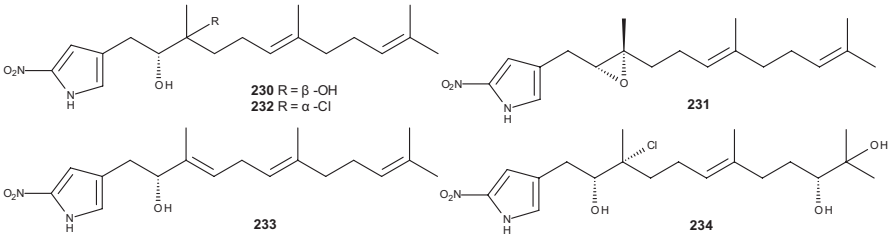


Renner et al. [128] first described isolation of three unusual prenylated 21-membered cyclic heptapeptide alkaloids, cyclomarins A-C (**226–228**), from the culture of *Streptomyces* strain CNB-982, isolated from a sediment sample collected in Mission Bay, California, in saline culture condition. Cyclomarin A (**226**), the major metabolite, was found to be cytotoxic *in vitro* against a panel of human cancer cell lines with a mean IC_{50} = 2.6 μM. Interestingly, cyclomarin A (**226**), one of the most active anti-inflammatory agents discovered in Nature, was also obtained by chemical synthesis in the named Marinovir®. This compound was also developed as an anti-herpetic agent [129].

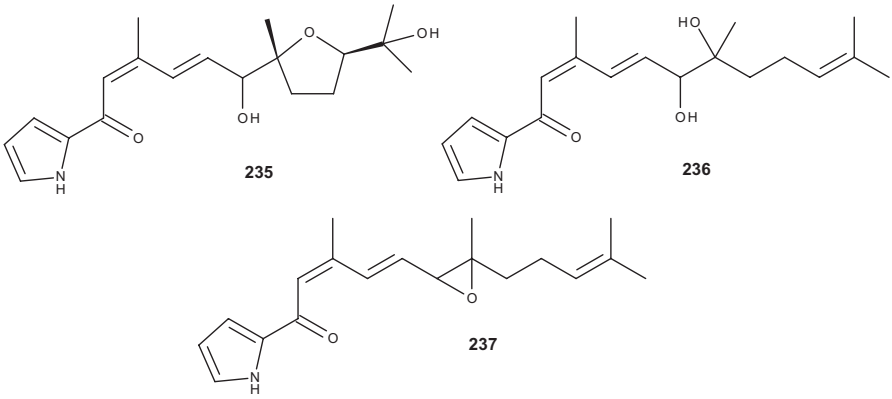
Schultz et al. [130], in their efforts to explore the biosynthesis of the cyclomarin peptides in *Streptomyces* sp. CNB-982 with stable isotopes, have isolated, besides the previously described cyclomarins A (**226**) and C (**228**), a new cyclomarin analog named cyclomarin D (**229**), from the marine-derived actinomycete *Salinispora arenicola* CNS-205, collected in Palau. This compound was found to exhibit strong cytotoxic effect against human colon adenocarcinoma cell line HT-116 with IC_{50} value of 2.0 μM.



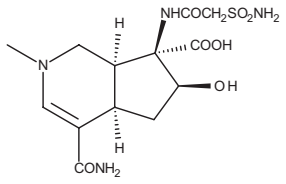
Five new farnesyl α-nitropyrroles, nitropyrrolins A-E (**230–234**), were isolated from the saline culture of a marine-derived bacterium actinomycete strain CNQ-509, isolated from a marine sediment sample collected off La Jolla, California. Nitropyrrolins A-E (**230–234**), as well as several synthetic derivatives, were evaluated for their cytotoxicity against HCT-116 cells. Nitropyrrolin D (**233**) was found to be the most active with IC_{50} = 5.7 μM, while the other nitropyrrolins and the synthetic analogs displayed moderate activities ranging from 10 to 30 μM [131].



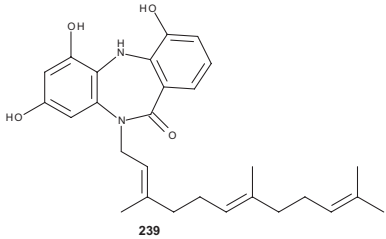
Chemical analysis of the *Streptomyces* sp. (NPS008187), isolated from a marine sediment collected in Alaska, resulted in the discovery of three new pyrrolosesquiterpenes: glyciapyrroles A-C (**235–237**). The three compounds were tested for their cytotoxic activity but only glyciapyrrole A (**235**) displayed inhibitory activity against both colorectal adenocarcinoma HT-29 and melanoma B16-F10 tumor cell lines with IC_{50} value of 180 μM [132].



Altemicidin (**238**) produced by the strain *Streptomyces sioyaensis* SA-1758, isolated from sea mud collected at Gamo, Miyagi Prefecture, Japan, was the first microorganism-derived monoterpene alkaloid with a 6-azaindene skeleton. Altemicidin (**238**) exhibited remarkable growth inhibition of murine lymphoid leukemia L1210 and carcinoma IMC cells (IC_{50} = 0.84 μM and 0.82 μM, respectively) [133, 134].

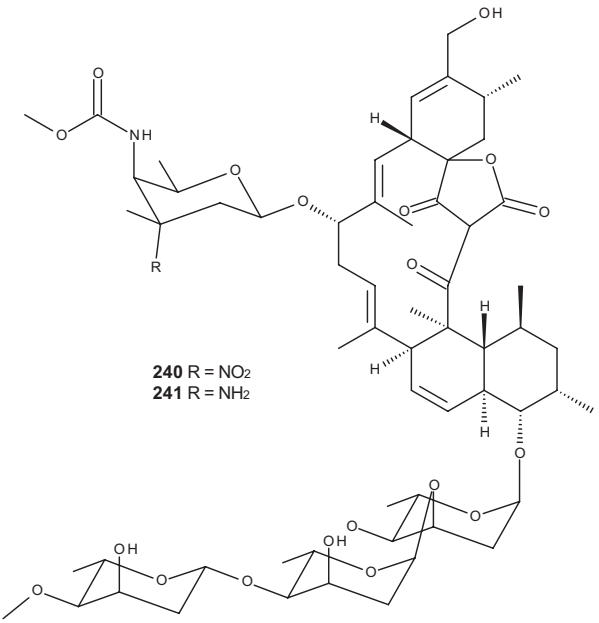


The marine-derived actinomycete *Micromonospora* strain DPJ12, isolated from the ascidian *Didemnum proliferum*, collected at Shishijima Island, Japan, furnished the antibiotic diazepinomicin (ECO-4601/TLN-4601) (**239**), a unique dibenzodiazepinone alkaloid comprises of a dibenzodiazepine core with a farnesyl sidechain [135].



Diazepinomicin (**239**) displayed a broad spectrum of *in vitro* cytotoxicity in the low micromolar range when tested in the NCI 60 cell line panel, and significant antitumor activity against rat glioma tumors. Furthermore, the highly significant antitumor activity in human hormone-independent breast (MDA-MB-231) and prostate (PC-3) xenografts led to the initiation of a Phase I clinical trial against solid tumors [136]. Phase I/II studies revealed a dual anticancer activity through a selective binding to the peripheral benzodiazepine receptor (PBR) leading to specific accumulation of TLN-4601 in PBR positive tumors, and Ras-MAPK pathway inhibition [137, 138]. Consecutive studies indicated that diazepinomicin (**239**) may cause the inhibition of Ras-mitogen-activated protein kinase-signaling pathway by Raf-1 protein depletion, resulting in antitumor activity and decreased tumor Raf-1 protein levels in MIA-PaCa-2 tumor-bearing mice. Furthermore, the inhibition of mutationally activated K-Ras-MAPK signaling and a decrease in the *in vitro* contact-dependent and -independent growth of pancreatic cells, coupled with activation of apoptotic cascades, supported further clinical development of diazepinomicin (**239**) in mutated K-Ras-mediated cancers [139, 140]. A Phase II clinical trial in patients with glioblastoma multiforme (GBM) was initiated in 2008 by Thallion Pharmaceuticals, but was soon concluded due to lack of efficacy [141]. Curiously, structure of the antitumor antibiotic BU-4664 L produced by a terrestrial strain of *Micromonospora* sp., reported in 1996 by Bristol-Meyers Squibb Co. group [142], was later revised to diazepinomicin [143]. The preclinical development of ECO-4601 **(239)** as an anticancer agent has been completed in 2006 [138].

Bioassay-guided fractionation of the culture extract of *Streptomyces carnosus* strain AZS17, isolated from a *Hymeniacidon* sp. sponge collected from coastal waters of the East China Sea, yielded lobophorins C (**240**) and D (**241**). Both compounds were tested for their *in vitro* cytotoxic activity against a human breast cancer cell line 7402 and human breast cancer cell line MDA-MB 435. Lobophorin C (**240**) displayed strong cytotoxic activity (IC_{50} = 0.6 μM) against 7402 hepatoma cells while lobophorin D (**241**) showed significant inhibitory effect on MDA-MB 435 cell line (IC_{50} = 7.5 μM) [144].



240 R = NO_2
241 R = NH_2

16.4 Conclusion

The chemical diversity of marine-derived microbial metabolites is immeasurable and can be considered as a new frontier for new chemical entities for the discovery of new pharmaceutical leads. Cultivation of chemically prolific microorganisms, such as marine-derived fungi and bacteria, represents one of the most promising methods for natural products discovery, suggesting that efforts to cultivate new marine microorganism taxa are a high priority. However, interdisciplinary approaches are necessary to untap this potential since the marine environments are highly complex. Due to the high degree of structural diversity allied with different mechanisms of cytotoxicy, the potential of meroterpenoids as new scaffolds for anticancer agents has attracted the interest from synthetic chemists and pharmaceutical industry alike, and therefore represents a logical target for drug discovery in cancer chemotherapy and/or chemoprevention.

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Chapter 17
Antitumor Compounds from Actinomycetes in Deep-sea Water of Toyama Bay

Yasuhiro Igarashi

Abstract Actinomycetes are the richest source of bioactive small molecules with high structural complexity and diversity which have been an inspiration to drug development. After the intensive screening efforts from soil-derived actinomycetes over a half century, it became necessary to exploit new microbial resources for discovering new drug leads. The deep-sea water in Toyama Bay is known as the 'specific deep-sea water of Japan Sea' because Sea of Japan is almost enclosed from the Pacific Ocean and the water exchanges very slowly with the neighboring seas. Furthermore, high dissolved oxygen concentration in this sea water gives rise to high biological productivity. As a part of investigation on the deep-sea water in Toyama Bay, we undertook the isolation of actinomycetes from deep-sea water for evaluation of bioactive compound production. This chapter summarizes the chemistry and biology of antitumor compounds produced by actinomycetes collected from the deep-sea water in Toyama Bay.

Keywords Actinomycetes · *Micromonospora* · Deep-sea water · Antitumor compounds

17.1 Introduction

Toyama Bay is located in the northern shores of Honshu, Japan, and faced to Sea of Japan. The sea water present deeper than 300 m is called 'deep-sea water', which occupies about 60 % of the total volume of Toyama Bay. The deep-sea water of Toyama Bay has a low temperature, annually stable around 2 °C. The salinity is also stable at 34.0–34.1 g/kg, higher than that of surface sea water (32–33 g/kg). Utilization of the deep-sea water is being studied for commercial/industrial purposes such as aquatic culture of cold-water shrimps. As a part of investigation on the deep-sea water in Toyama Bay, we undertook the isolation of actinomycetes from

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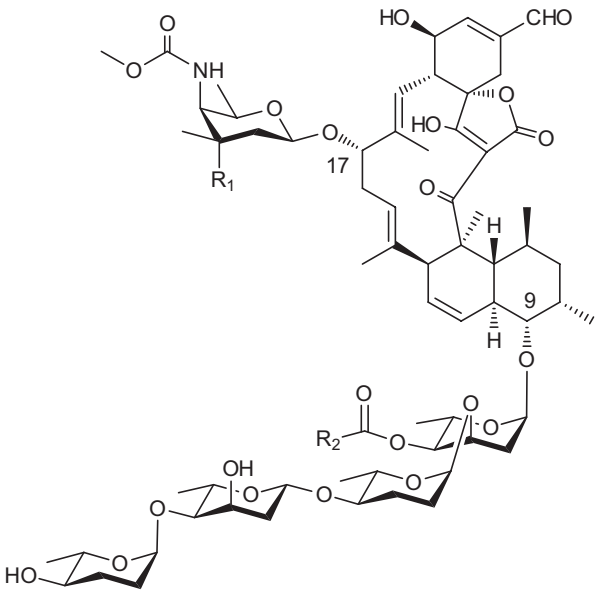
deep-sea water collected at −321 and −700 m for evaluation of antibiotic production. Microorganisms were isolated by membrane filter method: (1) deep-sea water was filtered through a cellulose membrane filter (0.2 μm pore size); (2) the filter was placed on an isolation agar plate; (3) the plate was incubated for 2–3 weeks; (4) growing colonies on the medium were transferred to a fresh medium and purified. Isolated strains were cultured in a liquid medium and the culture extracts were subjected to antimicrobial and cytotoxic bioassays to select the strains for the isolation chemistry. In this article, four chemically different classes of antitumor compounds produced by the deep-sea water-derived actinomycetes are described from the view point of structure, bioactivity and biosynthesis.

17.2 Antitumor Compounds from Actinomycetes in Deep-sea Water

17.2.1 Arisostatin A, an Apoptosis Inducer from Micromonospora sp.

In the search for bioactive compounds from actinomycetes collected from the deep-sea water in Toyama Bay, two new glycosylated polyketides were isolated from the culture extract of *Micromonospora* sp. TP-A0316. Guided by the antimicrobial activity against *Bacillus subtilis*, chromatographic fractionation of the culture extract using HP-20 resin and silica gel, followed by HPLC purification, gave arisostatin A (**1**, 27.1 mg) and arisostatin B (**2**, 6.6 mg), respectively from 3 L of culture ([1]; Fig. 17.1). These compounds were named after '*ariso-umi*', an ancient name of Toyama Bay. Arisostatins are the new members of tetrocarcin-type cytotoxic compounds of which structures were determined by extensive analysis of 1D- and 2D-NMR spectra [2]. The structural difference between tetrocarcin A (**3**, [3]) and arisostatin A lies in the acyl group of the digitoxose unit connecting directly to the aglycon: arisostatin A has an isobutyl group instead of an acetyl group in tetrocarcin A. In arisostatin B, the nitro group in the deoxysugar attached at the 17-OH group is reduced to an amino group.

Arisostatin A showed a potent cytotoxic effect on human cancer cells with IC_{50} values in submicromolar range (Table 17.1). Mode of action study using human AMC-HN-4 cells (AMC-head and neck-4 cells) indicated that arisostatin A activates caspase 3, a key effector protease responsible for apoptosis induction [4]. In addition, arisostatin A induced the loss of mitochondrial membrane potential and release of cytochrome *c* into cytosol in association with the production of reactive oxygen species. All these cellular responses are the typical marker of apoptosis triggered by mitochondrial function loss. Tetrocarcin A is known to inhibit the function and transcription of Bcl-2, leading to the disruption of mitochondrial membrane homeostasis [5]. Bcl-2 is an anti-apoptotic protein, overexpressed in a wide range of cancer cells. In case of arisostatin A, the expression levels of Bcl-2 and related



arisostatin A (**1**): $R_1=NO_2$, $R_2=CH(CH_3)_2$
B (**2**): $R_1=NH_2$, $R_2=CH(CH_3)_2$
tetrocarcin A (**3**): $R_1=NO_2$, $R_2=CH_3$

Fig. 17.1 Structures of arisostatins and tetrocarcin A

Table 17.1 *In vitro* cytotoxicity of arisostatin A to human cancer cells

Cell line	IC_{50} (μM)
SF-539 (brain)	0.21
HCC2998 (colon)	0.22
NCI-H522 (lung)	0.22
DMS114 (lung)	0.23
HBC-4 (breast)	0.26
HBC-5 (breast)	0.06

anti-apoptotic proteins were not affected, suggesting that the mode of action of tetrocarcin A and arisostatin A is different.

Tumor metastasis is the process by which a tumor cell leaves the primary tumor, disseminates via the blood or lymphatic vessels, grows at a distant site, and establishes a secondary tumor. In this process, translocation of tumor cells across extracellular barriers, namely invasion, is a crucial step to accomplish the metastasis. *In vitro* inhibition of tumor cell invasion can be evaluated using transwell chamber with Matrigel/fibronectin-coated filters [6]. Tetrocarcin A and arisostatin A suppressed the invasion of murine colon carcinoma 26-L5 cells to *ca*. 30% at 80 nM, comparing to the non-treated cells (unpublished result, Fig. 17.2). Arisostatin derivative lacking a sugar moiety at C-17 (**4**, Fig. 17.3) and the aglycone (**5**, Fig. 17.3) were less potent but still showed potent inhibitory activity at 80 nM.

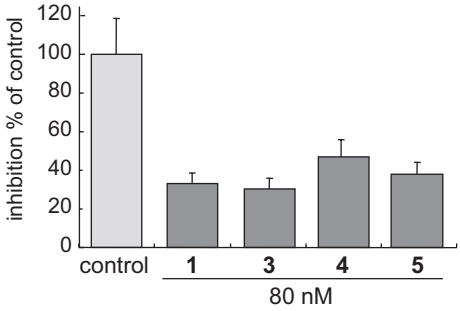


Fig. 17.2 Inhibition of tumor cell invasion by tetrocarcin A and arisostatin A

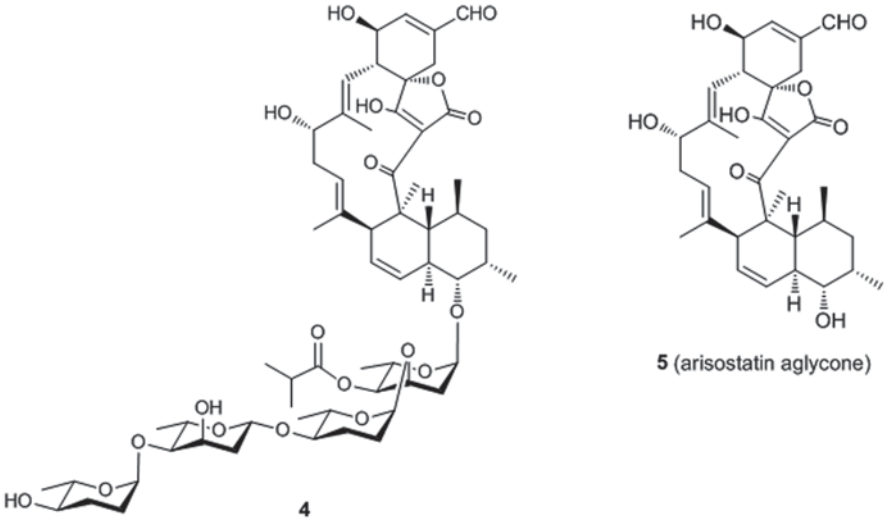


Fig. 17.3 Structures of arisostatin derivatives 4 and 5

Tetrocarcin A and arisostatin A belong to a family of spirotetronate antibiotics. This family includes structurally and biologically diverse compounds (Fig. 17.4): kijanimicin (**6**, [7]), an antitumor antibiotic; chlorothricins (**7**, [8]), cholesterol biosynthesis inhibitors; versipelostatin (**8**, [9]), a down-regulator of molecular chaperon GRP78. These compounds are known to be assembled by the polyketide pathway, specifically type I PKS (polyketide synthase) system featured by multifunctional megaenzymes [10]. This multi-catalytic enzyme system is feasible for combinatorial biosynthesis by genetic engineering of the biosynthetic pathway to produce novel analogues [11]. Biosynthetic genes for tetrocarcin A was characterized and its biosynthetic pathway has been proposed ([12]; Fig. 17.5), which will open up future prospects of combinatorial biosynthesis of new leads based on the spirotetronate antibiotics.

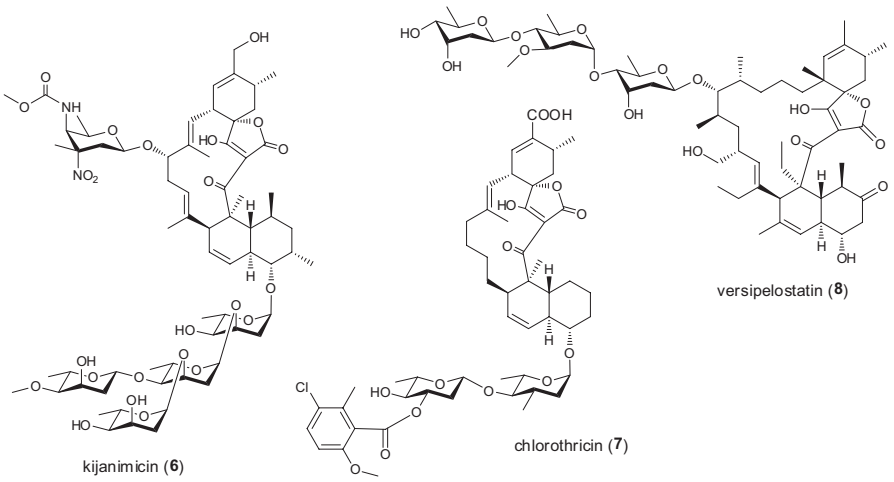


Fig. 17.4 Spirotetronate antibiotics from actinomycetes

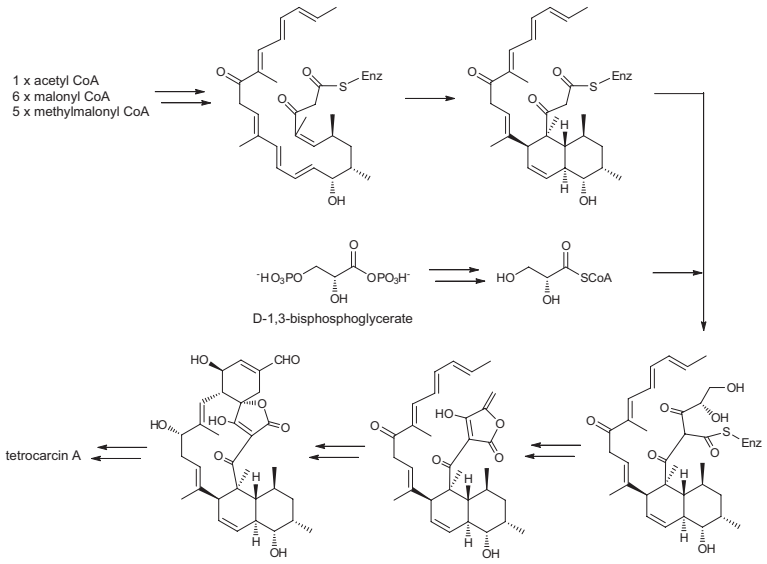


Fig. 17.5 Biosynthetic pathway for tetrocarcin A proposed by Liu et al.

17.2.2 Kosinostatin, a DNA-targeted Agent from Micromonospora sp.

In the same period when arisostatins were discovered, another *Micromonopora* strain TP-A0468 of deep-sea water origin was found to produce antimicrobial substances active against Gram-positive bacteria. The strain was cultured in a liquid medium and the ethyl acetate extract of the whole culture broth was subjected to liquid-liquid partition between *n*-hexane and methanol. The methanol layer was concentrated and subjected to an ODS column chromatography, yielding 60 mg of pure yellow powder from 3 L of culture as an active principle, designated kosinostatin (**9**) after '*koshi-no-kuni*' which was anciently used to refer to the province including the present Toyama prefecture [13]. The structure of kosinostatin was determined by extensive spectroscopic analysis using NMR and MS as a glycosylated anthraquinoid tetracycle in which a pyrrolopyrrole was spirally conjugated to the aglycon ([14], Fig. 17.6). This structurally uncommon compound belongs to the quinocycline antibiotics originally isolated from *Streptomyces aureofaciens*, comprising four congeners, quinocyclines A and B and isoquinocyclines A (**11**) and B (**10**) [15]. Quinocyclines A and B were reported to be unstable and spontaneously isomerized to isoquinocyclines A and B. The structure of isoquinocycline A was determined by X-ray crystallographic analysis [16] while the structure of quinocyclines remained unknown for many years. According to the results from precise NMR analysis, we confirmed that kosinostatin is a diastereoisomer of isoquinocycline B regarding to the spirocenter connecting the pyrrolopyrrole and anthraquinoid moieties and kosinostatin and quinocycline B are identical based on the observation of the spontaneous isomerization from kosinostatin to isoquinocycline B. Isolation of kosinotatin from a soil-derived *Streptomyces* was also reported later [17].

Kosinostatin displayed potent cytotoxicity to human cancer cells with IC_{50} values ranging from 0.02 to 0.98 μM (Table 17.2). Mode of action prediction by COMPARE program [18] indicated the closest similarity to DNA intercalaters such as

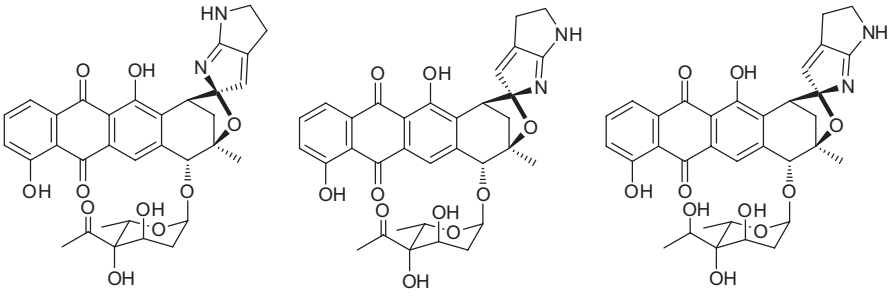


Fig. 17.6 Structures of kosinostatin and quinocyclines

Table 17.2 Cytotoxicity of kosinostatin against human cancer cells

Cell lines	IC_{50} (μM)	Cell lines	IC_{50} (μM)	Cell lines	IC_{50} (μM)
Breast		Colon		Lung	
HBC-4	0.02	HCC2998	0.05	NCI-H23	0.13
BSY-1	0.06	KM-12	0.09	NCI-H226	0.02
HBC-5	0.10	HT-29	0.07	NCI-H522	0.04
MCF-7	0.05	HCT-15	0.98	NCI-H460	0.04
MDA-MB-231	0.21	HCT-116	0.03	A549	0.09
				DMS273	0.40
Central nervous system		Stomach		DMS114	0.10
U251	0.12	St-4	0.15		
SF-268	0.08	MKN1	0.04	Ovary	
SF-295	0.12	MKN7	0.04	OVCAR-3	0.09
SF-539	0.04	MKN28	0.05	OVCAR-4	0.10
SNB-75	0.55	MKN45	0.02	OVCAR-5	0.05
SNB-78	0.15	MKN74	0.03	OVCAR-8	0.06
				SK-OV-3	0.25
Kidney		Prostate			
RXF-631L	0.18	DU-145	0.11	Melanoma	
ACHIN	0.11	PC-3	0.11	LOX-IMVI	0.03

doxorubicin and epirubicin. Kosinostatin actually inhibited the human DNA topoisomerases I and IIa with IC_{50} of 10–30 and 3–10 μM, respectively.

The most striking feature of quinocycline antibiotic is its unprecedented pyrrolopyrrole substructure fused to the type II PKS-derived aromatic core. It was extremely difficult to predict the biosynthetic origin of this bicyclic system according to the known biosynthetic route to pyrroles. In addition, the spirocyclic linkage between the tetracyclic aglycone and the pyrrolopyrrole is connected via an unusual *N, O*-spiro center, for which linkage an unknown C-C bond formation mechanism should be involved. Very recently, the biosynthetic origin of the pyrrolopyrrole was finally identified and a unique biosynthetic pathway was proposed for the quinocycline ([19], Fig. 17.7). Quite unexpectedly, nicotinic acid is the precursor for the pyrrole ring and two additional carbons for another nitrogen-containing ring are derived from D-ribofuranose. Pyrrolopyrrole formation is proposed to be started with the incorporation of nicotinic acid and enzymes similar to the tryptophan biosynthetic pathway are likely involved in the construction of the bicyclic structure.

In addition to the antitumor activity, quinocyclines have significant *in vitro* antibacterial activity against *Mycobacterium tuberculosis*, a causative agent of tuberculosis [20]. *In vivo* tuberculosis mice experiments indicated a significant prolongation of survival time but severe toxicity was also indicated. Further exploration of the biosynthetic enzymes is expected to provide a platform for the development of kosinostatin analogues with non-toxic antitumor and/or anti-tuberculosis drugs.

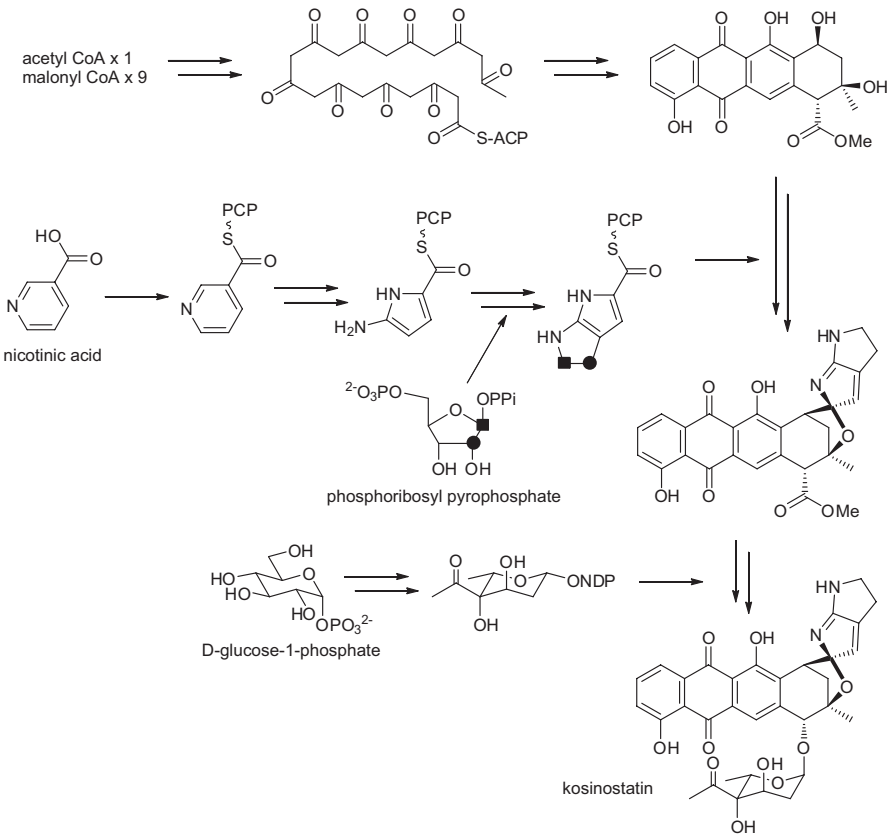


Fig. 17.7 Biosynthetic pathway for kosinostatin proposed by Tang et al.

17.2.3 BU-4664L, a Cell Migration Inhibitor from Micromonospora sp.

BU-4664L is a secondary metabolite of *Micromonospora* first described in a patent from Bristol-Myers Squibb [21]. This compound is featured by the uncommon heterocyclic core, dibenzo [b, e] [1,4] diazepinone, modified with a farnesyl carbon chain. According to the patent, BU-4664L inhibits 5-lipoxygenase (IC_{50} 1.7 μM), a key enzyme of leukotriene production. Overproduction of leukotrienes causes inflammation in asthma and is associated with the production of inflammatory mediators such as histamine and prostaglandins. In addition, BU-4664L was shown to display *in vitro* cytotoxicity to cancer cells and *in vivo* therapeutic effects in tumor-bearing mice. Owing to these pharmacological potential, together with its naturally rare structural feature, BU-4664L has been recognized as a unique lead compound for anti-inflammatory and antitumor drugs.

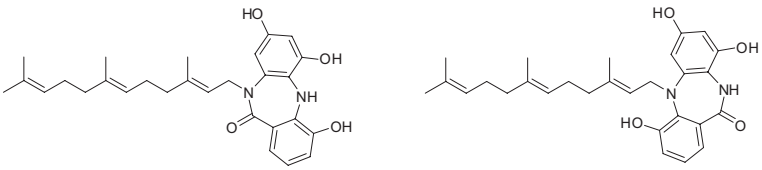


Fig. 17.8 Structure of BU-4664L

In the course of our screening program for novel inhibitors of tumor cell invasion/migration from microbial metabolites, BU-4664L (**12**) was isolated from the culture extract of *Micromonospora* collected from the deep-sea water in Toyama Bay (Fig. 17.8). The structure of BU-4664L was proposed in the patent in which the farnesyl side chain is attached to the amino group [21], but our analysis based on the 2D NMR data as well as careful comparison of the spectral data revealed that the farnesyl group is located on the amido-nitrogen atom [22]. Just before our report on the structural revision of BU-4664L, diazepinomicin was reported as a new compound from *Micromonospora* sp. isolated from a marine ascidian [23]. To this compound, two more code names, ECO-4601 and TLN-4601, are given. In this article, the name 'BU-4664L' is used to refer to this compound for clarification. BU-4664L-producing strains likely inhabit in a wide range of marine environments. In our laboratory, production of this compound was observed in the culture extract of *Micromonospora* strains from marine samples including sea water and sediment collected at various sites (unpublished results).

BU-4664L inhibited the invasion of murine colon 26-L5 carcinoma cells with an IC_{50} value of 1.0 μg/mL (2.2 μM) in a concentration dependent manner without showing cytotoxic effect [24]. BU-4664L suppressed the migration of the same cell line (IC_{50} 0.63 μg/mL) and inhibited the proteolytic activities of the matrix metalloproteinases (MMPs) MMP-2 and MMP-9, gelatinases responsible for degradation of the basement membrane, with IC_{50} of 0.46 and 0.60 μg/mL, respectively. Angiogenesis is another crucial step for metastasis and tumor development. In the process of angiogenesis, endothelial cells migrate into the surrounding extracellular matrix where they form a capillary blood vessel. BU-4664L inhibited the vascular formation of HUVECs (human umbilical vein endothelial cells) with an IC_{50} value of 0.72 μg/mL by inhibiting the migration of endothelial cells (IC_{50} 0.43 μg/mL). The antitumor effects of BU-4664L might be attributed not only to its anti-proliferative property but also to anti-invasive and anti-angiogenic properties.

Several derivatives (compounds **13–16**, Fig. 17.9) were prepared from BU-4664L for structure-activity relationship study [24]. The derivatives showed lower or no cytotoxicity comparing to the parent compound (Table 17.3). Compounds **14** and **15** inhibited angiogenesis more potent than BU-4664L with IC_{50} values of 0.11 and 0.23 μg/mL, respectively. In addition, these derivatives displayed an extremely potent inhibitory activity against cell migration of HUVECs with IC_{50} values of 7.6 ng/mL (15 nM) and 2.4 ng/mL (5.2 nM), respectively.

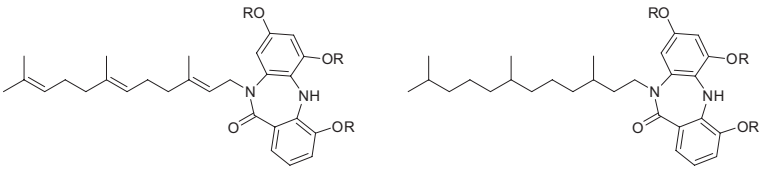


Fig. 17.9 Structures of semi-synthetic derivatives of BU-4664L

Table 17.3 Inhibition of invasion, migration, and angiogenesis by BU-4664L and derivatives

	IC_{50} (μg/mL) (colon 26L5 cells)		IC_{50} (μg/mL) (HUVEC)		
	Invasion	Cytotoxicity	Angiogenesis	Migration	Cytotoxicity
BU4664-L	1.0	7.8	0.72	0.43	2.0
13	>1.0	7.6	>1.0	NT	>10
14	1.0	>10	0.11	0.0076	>10
15	>1.0	10	0.23	0.0024	>10
16	1.0	>10	>1.0	NT	>10

Potent therapeutic effects of BU-4664L (diazepinomicin) in human breast cancer MDA-MB-231 and prostate cancer PC-3 xenograft mouse experiments have been reported [25]. Additionally, BU-4664L was indicated to inhibit the Ras-MAPK (mitogen activate protein kinase) signaling pathway [26] which is responsible for cell growth, differentiation and survival. Ras is the most common oncogene in human cancer and Ras mutation is frequently associated with tumorigenesis. Ras is also involved in the regulation of cell motility via PI3K (phosphoinositide 3-kinase) pathway which might explain the mode of action of BU-4664L in cell migration inhibition.

On the basis of the precursor feeding experiments and in silico analysis of the biosynthetic genes, biosynthetic pathway for BU-4664L is proposed in which three precursor components are coupled to assemble this unique structure ([27], Fig. 17.10). The dibenzodiazepine core would be constructed from the two aromatic precursors, 3-hydroxyanthranilate from the shikimic acid pathway and 2-amino-6-hydroxy[1,4] benzoquione derived from erythrose-*O*-4-phosphate and phosphoenolpyruvate via 3-amino-5-hydroxybenzoic acid. Farnesyl group separately provided from the mevalonate pathway could be transferred onto the amido nitrogen to complete the biosynthesis. More than 40 enzymes are proposed to be involved in the biosynthesis, highlighting the enzymatic complexity necessary for the construction of this uncommon natural product.

As BU-4664L accumulates in implanted gliomas by binding to the peripheral benzodiazepine receptor, it is expected as a new anticancer drug for glioblastoma, although the initial phase II trial was unsuccessful [28]. Based on the proposed biosynthetic pathway, precursor directed biosynthesis has been attempted to produce unnatural BU-4664L analogues [29]. Future study will be focused on the chemical/biochemical production of new analogues for the development of antitumor drugs from BU-4664L.

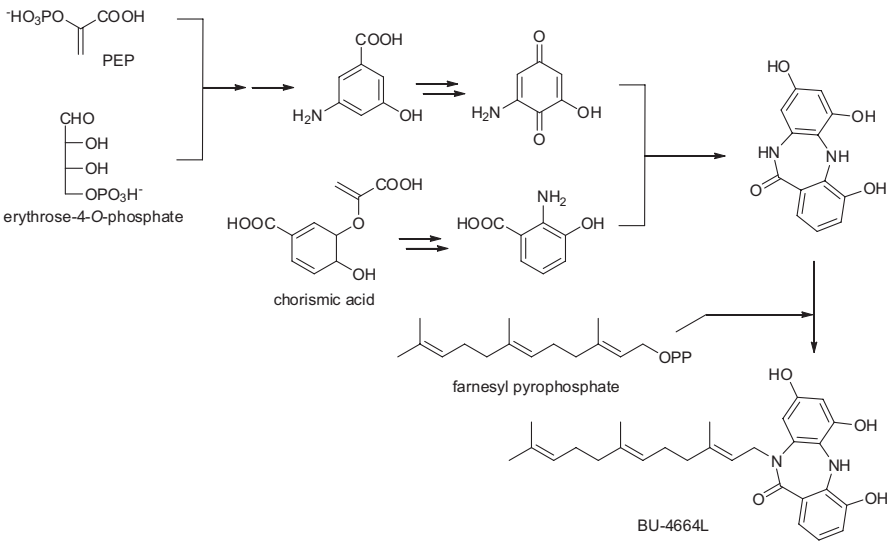


Fig. 17.10 Biosynthetic pathway for BU-4664L proposed by McAlpine et al.

17.2.4 Rakicidin, a Hypoxia Selective Cytotoxic Agent from Micromonospora sp.

Rakicidins are the 15-membered depsipeptides consisting of three amino acids and a 3-hydroxyfatty acid (Fig. 17.11). To date, four congeners, rakicidins A (**17**) and B (**18**) from *Micromonospora* and rakicidins C (**19**) and D (**20**) from *Streptomyces*, have been reported. All these compounds share the common amino acid components, 4-amino-2,4-pentadienoate, glycine and hydroxyaspargine (or glutamine in rakicidin C), and a 3-hydroxyfatty acid unit which varies in the chain length and the methylation pattern. Rakicidins A and B were first isolated from soil-derived *Micromonospora* at Bristol-Myers Squibb as cytotoxic compounds that displayed IC_{50} values of 40 and 200 ng/mL, respectively, against murine lung carcinoma cells [30]. Rakicidin C is a shorter chain congener isolated from a soil-derived *Streptomyces* in which the hydroxyaspargine moiety is replaced with glutamine [31]. This compound was reported to have no antimicrobial or no cytotoxic activity. Rakicidin D was isolated from *Streptomyces* collected from marine sediment in Thailand [32]. It also has a shorter side chain and exhibits anti-invasive activity against colon 26L5 cells with an IC_{50} of 6.7 μM. Rakicidins A and B are frequently co-produced with BU-4664L. In our laboratory, we have isolated several *Micromonospora* strains which produce both BU-4664L and rakicidins A and B from marine samples such as deep-sea water and sediment (unpublished results).

After more than twenty years since the first isolation, rakicidin A is highlighted as a potential inhibitor of cancer cell growth under hypoxic conditions. In solid tumors, oxygen supply is insufficient to support rapid tumor growth and angiogenesis, leading to the development of hypoxic regions inside the tumor. Cancer cells

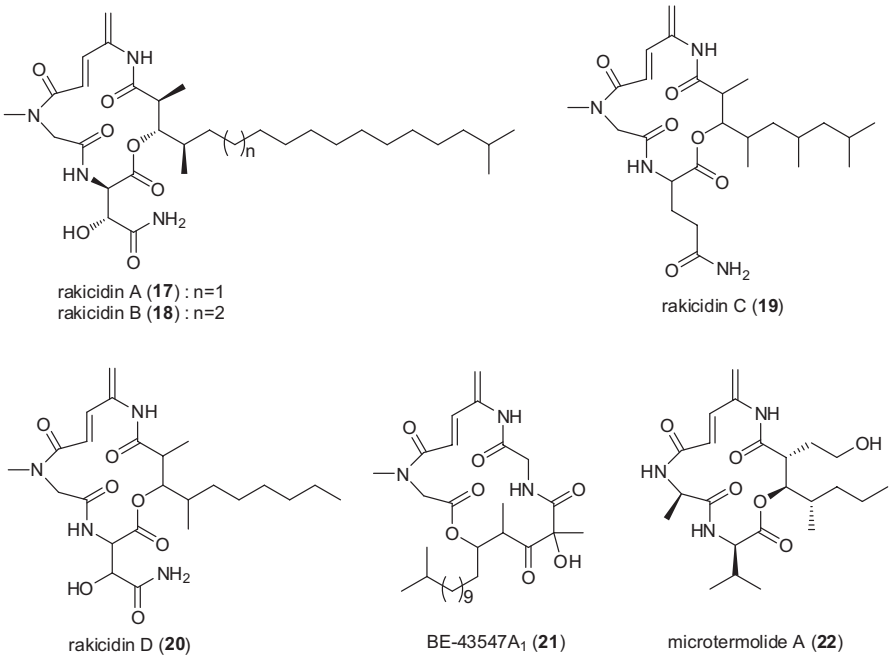


Fig. 17.11 Rakicidins and related microbial metabolites

adapted to hypoxic conditions become resistant to antitumor drugs and acquire stem cell-like, primitive phenotype, rendering these cells escape and survive from various environmental stresses. In the screening for hypoxia selective cytotoxic agents, rakicidin A was rediscovered from *Micromonospora* sp. ML99–43F1, which was screened from 20,000 extracts of microbial cultures. Rakicidin A displayed a selective cytotoxicity to hypoxic cancer cells approximately 17.5-fold more potent than to normoxic cancer cells [33]. More recently, rakicidin A was found to induce apoptosis in hypoxia-adapted leukemic cells with stem cell-like characteristics [34]. In addition, rakicidin A exhibited synergistic cytotoxicity in combination with imanitib against hypoxia-adapted leukemic cells which are resistant to Abl tyrosine kinase inhibitors. Owing to these promising results, rakicidin A is thought to be a potential clinical candidate for cancer chemotherapy. However, the chemistry of rakicidin has not been explored yet because its absolute configuration had not been elucidated. Therefore, we undertook the stereochemical assignment of rakicidin A.

Rakicidin A can be obtained from 1-butanol extract of the *Micromonospora* culture by several steps of chromatography as a film-like solid. This solid material is hardly soluble to many of laboratory solvents including DMSO, but barely soluble in DMF and most soluble in pyridine. Initially, we attempted various chemical transformations such as lactone-opening for rakicidin A but we were unable to obtain products with satisfactory yields. We assumed that this unfavorable result was due to the conjugated double bond in the 4-amino-2,4-pentadienoyl moiety which might react with nucleophiles. Hydrogenation of the C-C double bonds proceeded

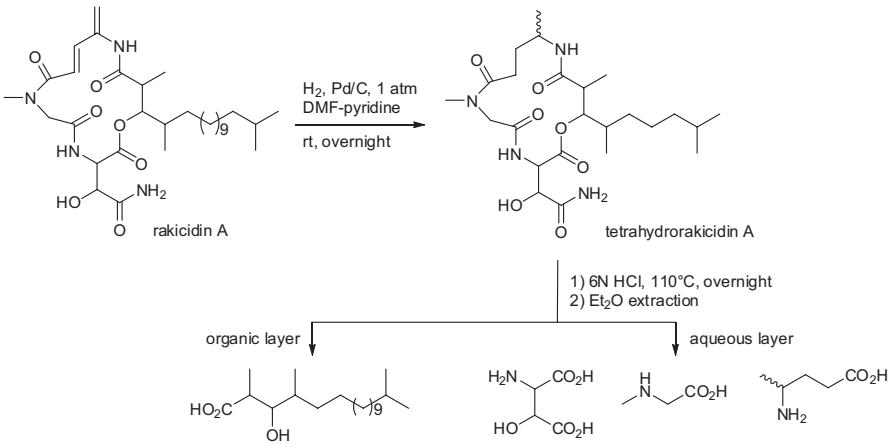


Fig. 17.12. Degradation of rakicidin A for stereochemical analysis

smoothly by a catalysis of Pd/C in a mixture of DMF and pyridine to give tetrahydrorakicidin A, which was highly soluble to organic solvents and amenable to various transformations. Alkaline hydrolysis of this reduced derivative to the seco-acid was successful, but the following MTPA derivatization was unsuccessful. Then, we hydrolyzed the reduced derivative under acidic condition, followed by a diethyl ether extraction which gave the branched fatty acid in the organic layer and the amino acid residues remained in the aqueous layer. The relative configuration of the two methyl and one hydroxyl substituents in the fatty acid moiety was determined on the basis of the $^{3}J_{HH}$ coupling constants as 2,3-*anti*, 3,4-*syn*, in comparison with the reported values for all possible isomers of 2,4,6-trimethyl-3-hydroxyheptanoic acids. The β-hydroxyaspargine was determined to have 2*R*,3*R*-configuration on the basis of TLC and Marfey's analysis in comparison with standard amino acid samples (Fig. 17.12, unpublished results).

Biosynthesis of rakicidins, including the biosynthetic origin of 4-amino-2,4-pentadienoyl moiety, remains to be elucidated. The unusual amino acid is rare in natural products: in addition to rakicidins, only two classes of peptidic metabolites, BE43547 (**21**, [35]) and microtermolide A (**22**, [36]), both of which are produced by *Streptomyces*, are known to contain this unusual amino acid. Identification of the gene cluster for rakicidin biosynthesis is currently investigated with a *Micromonospora* strain isolated from the deep-sea water.

17.3 Future Prospects

Microbial community composition in the deep-sea water has not been well studied [37]. The deep-sea water in Toyama Bay is called the 'specific deep-sea water of Japan Sea' because Sea of Japan is almost enclosed from the Pacific Ocean and the

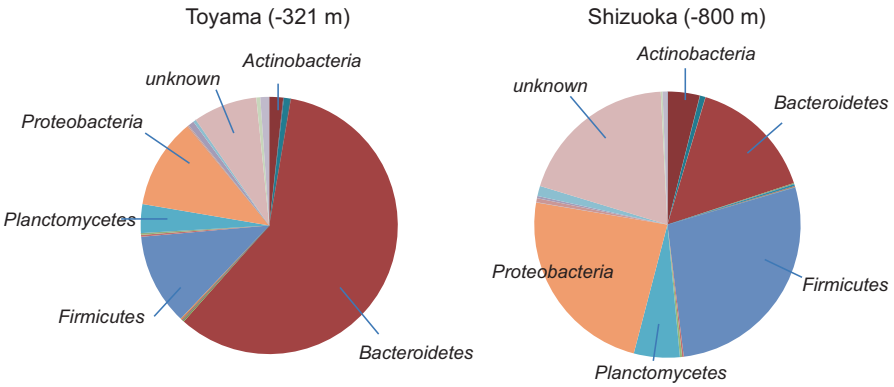


Fig. 17.13 Phylum-level microbial population in the deep-sea water

water exchange with the neighboring seas is very slow. In addition, dissolved oxygen concentration in the deep-sea water of Toyama Bay is higher than that in the Pacific Ocean, resulting in high biological productivity. This specific character might be reflected in the microbial community. The 16S rRNA gene-based metagenomic analysis indicated that the bacterial community composition in the deep-sea water of Toyama Bay (−321 m) and the deep-sea water collected in Shizuoka (−800 m) which is faced to the Pacific Ocean is largely different (Fig. 17.13, unpublished results). Further analysis of microbial communities in the deep-sea water collected at different sites in Japan is currently under investigation, which will provide insight into this untapped reservoir of drug candidates.

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Chapter 18
Tunicates: A Vertebrate Ancestral Source of Antitumor Compounds

Edwin L. Cooper and Ralph Albert

Abstract An alarming increase in cancer deaths around the globe has sparked a quest for new effective antitumor drugs developed through biological screening of both terrestrial and marine organisms. Recently, analyses of marine-derived alkaloids isolated from tunicates, a close relative to vertebrates, reveal various anti-cancer activities including anti-angiogenic action, anti-proliferative activity, inhibition of key cellular events like topoisomerase function and tubulin polymerization with respect to cytotoxicity and apoptosis. Vast numbers of potential anticancer molecules in the marine biosphere cannot be underestimated. Tunicate compounds have already entered the market and shown significant success. Moreover, improved technology has enabled synthesis of these molecules by a diverse cadre of professionals including career biologists, immunobiologists, invertebrate immunologists, and marine biologists who use an amazing array of procedures. Although there are no guarantees of consistently reliable success, identifying and comprehending the basic nature of these compounds is a promising first step towards unique pharmaceutical designs that promise to provide a therapeutic solution.

Keywords Antitumor compounds · Tunicates · Bioprospecting · Cytoxicity · Aplidine · Trabectedin

18.1 Introduction

18.1.1 Tunicates and Immunity

Tunicates, also known as urochordates, belong to the subphylum Tunicata or Urochordata. They are a marine group of saclike filter feeders with two siphons

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and are classified within the phylum Chordata. Tunicate larvae exhibit chordate characteristics as they possess a notochord, a dorsal nerve cord, pharyngeal slits, and a post-anal tail; however most tunicates lose their tails and ability to move, while retaining only a vestigial nervous system. Many tunicates, commonly known as "sea squirts", live on the ocean floor or on the sides of wharfs and boats, but others known as salps live in the pelagic zone and retain the ability to move [1].

During evolution, the immune system has resulted in a dichotomy between an innate and adaptive immune system (Fig. 18.1). Both systems possess cells that first recognize an antigen in order to destroy it. The innate immune system is natural, nonspecific, non anticipatory, non-clonal, germ line, and not memory related, while the adaptive immune system has the following almost exactly opposite characteristics: acquired, specific, memory of past infections, anticipatory of future attacks, clonal, and somatic [2]. Urochordates may represent the transitory phase from innate to adaptive immunity. Because of their inability to move, tunicates rely on an innate humoral vs. cellular immune system as well as a variety of natural toxins. These compounds have a surprisingly large amount of structural and functional diversity, allowing for potential cross-application to mammalian cell targets [3], including those that are cancerous. Another intriguing characteristic of tunicates is tumor immunity despite the presence of oncogenes such as *ras* and *src*, which are genes that code for key proteins in cancer signal transduction pathways [4, 5].

Tunicates have repeatedly been shown as a primitive model organism to study immunodefense since the innate immune system has been hypothesized as an important functional component that may partially explain the lack of metastatic tumors in invertebrates [7]. Tunicates exhibit cell mediated immunity (cytotoxicity, histo-incompatibility) and humoral immunity (lectins, defensins, cytokine molecules) [8]. These same mechanisms make tunicate compounds potentially effective against human cancer cells due to their antitumor activity [3].

The growing global cancer casualty list has fueled redoubled efforts to develop antitumor drugs. Cancer research has resulted in some notable successes in targeting specific tumor lines, although there is still much to be done. The traditional Western approach has focused on treatment through chemotherapy, irradiation, hemostasis, and immunotherapy [4]. While this avenue is still being explored, it is important to recognize that other less-traveled paths offer exciting new alternative treatments. Natural sources offer many medicinal molecules that have been used throughout history in ancient practices like Indian Ayurveda [9], traditional Chinese medicine (TCM) [10], and Japanese Kampo [11]. One potential stumbling block concerns the specificity of the administered drugs. Avoiding or circumventing pleiotropic effects is essential.

These sources have not been fully explored under the Western medicinal model, but further investigation can reveal promising new compounds [12] for both traditional Western models and complementary and alternative medicine (CAM) [13]. A source of constant, although incremental, progress in the search for antitumor compounds over the last 30 years, tunicates have yielded many potential anticancer compounds. This review summarizes the general antitumor mechanisms that are effected by tunicate compounds, presents a list of different compounds discovered in tunicates, focuses on potential pharmaceutical solutions, and looks to the future of this unique and therefore important line of research inquiry.

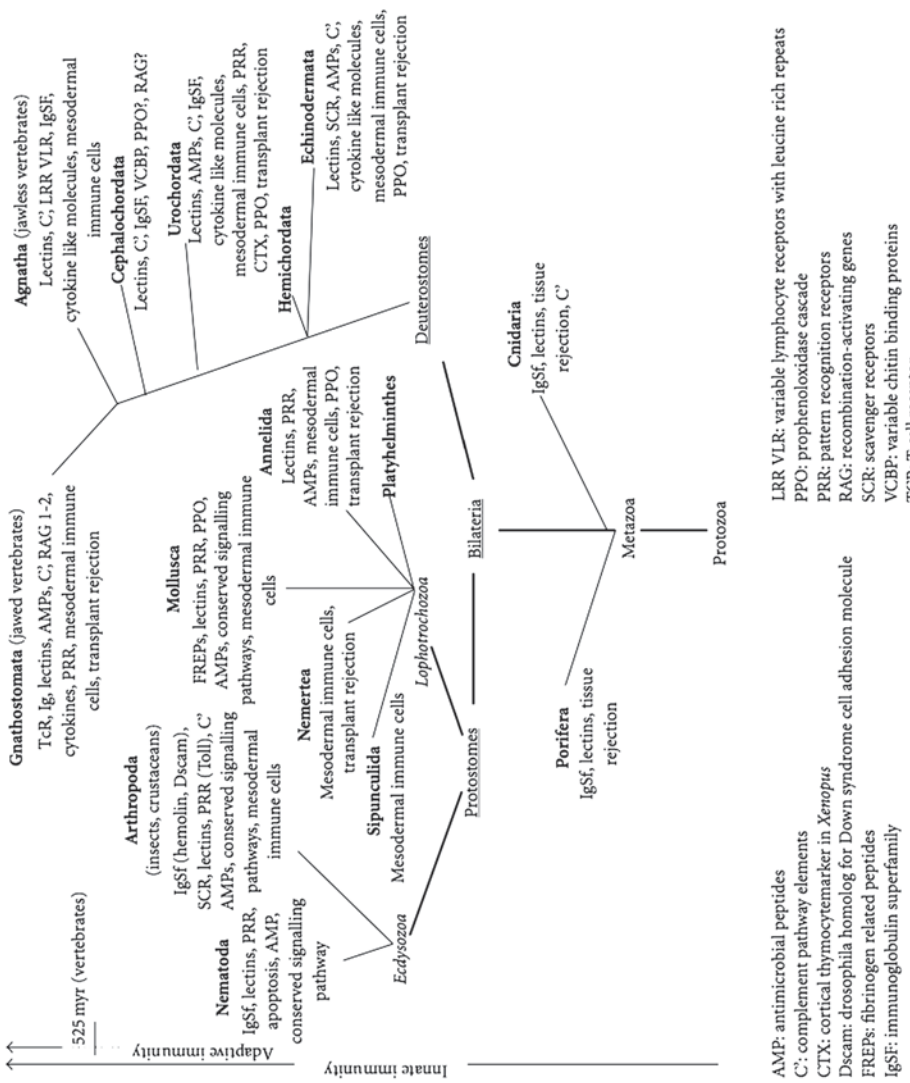

Fig. 18.1 Phylogenetic tree of the animal kingdom highlighting the evolution of key immunological elements. Two *arrows* on the *left side* of Fig. 18.1 indicate possible appearance of the two branches of immunity. Innate immunity may be observed along the entire animal kingdom. Traditionally accepted adaptive immunity appeared only in vertebrates, while certain adaptive immune mechanisms may have appeared early at the level of arthropods and molluscs illustrated by dots (*below* the arrow). (Figure reproduced from [6])

18.2 Bioprospecting in the Sea

18.2.1 Why Dive for Drugs?

Bioprospecting is the search for useful natural products from a vast array of organisms [13]. A specific focus has been anticancer compounds in marine organisms, with a comprehensive fifteen year study by the U.S. National Cancer Institute finding that approximately 4% of marine species possessed potential antitumor compounds. This percentage is comparable to that of terrestrial organisms, indicating a vast realm of possibilities [14]. Researchers have cast a wide net, deriving potential anticancer compounds from other marine invertebrates including the Bryozoa ("moss animals" like *Cristatella mucedo*), Mollusca (sea slugs, etc.), and Echinoderms (sea stars, etc.) [15, 16]. Some of the major findings of bioactive anticancer molecules in marine invertebrates have been presented in website format [17].

Many investigators, however, question the reasoning behind the pursuit of natural products for new drugs in the modern age of technology. Why search the marine biosphere for new products against human disease when a quicker and perhaps more focused and reliable method exists due to the availability of pharmaceutical chemical libraries? The answer to this question lies in the immense structural and functional diversity natural inhibitors exert on biological targets. This number is much greater than potential drug targets encoded by the human genome [18]. In addition, many molecular mechanisms that are targeted by these novel drug candidates are highly conserved. In other words, the structures of drugs we use today more closely resemble those of natural products. These compounds can act as structural scaffolds and starting points for chemical modification [19]. Thus, such an untapped pool of diverse marine molecules makes bioprospecting in search of marine natural products an exciting new source with the promise to yield novel drugs and treatments.

18.2.2 Tunicates as Food

Sea squirts have been consumed in many different countries, most notably in Korea and Japan. *Halocyntia roretzi* is a prominent food item, with aquaculture being the preferred method for producing tunicate [20]. In addition to being a tasty seafood delicacy, tunicates may also provide a promising anticancer protection after having being eaten. In fact, the solitary tunicate (*Styela clava*) contains the enzyme alcalase, which is antiproliferative on AGS, DLD-1, and HeLa cells [21]. Milk, soy, and fish proteins are known to have health-enhancing components [22], and tunicates can also provide anticancer value as a part of a diet [23]. Such opportunities exist to use molecules derived from existing cuisines and to apply them to a Western approach to medicine through research inquiry [24]. Since we consume both animal and plant products as nutritious sources, at the same time, immunosuppression and/or anticancer activity may be operation [25].

18.3 Anticancer Mechanisms (Fig. 18.2)

18.3.1 Cytoxicity

Cytotoxic anticancer compounds induce cell death through apoptosis or necrosis to prevent cancerous cell growth. Apoptosis is triggered to cause genetically-determined programmed cell death, while necrosis is the premature death of cells caused by external factors. It may be essential to coordinate drug activity with effective assays. Such an approach requires countless trial and error attempts. Many of the antitumor compounds discovered have been tested for antitumor activity using a quantitative MTT/MTS assay. This relatively simple colorimetric assay determines the cytotoxic potential of compounds by measuring cell proliferation and viability through the use of various dyes [4]. Viable cells are marked by purple formazan; thus, cytotoxicity can be measured by the lack of purple coloring. Unfortunately, this test fails to provide much insight into specific antitumor mechanisms. Further tests are necessary for more specific details regarding how compounds actually work. However, the MTT/MTS assay is still a valuable tool to use to first, filter the numerous potential anticancer molecules, and second, determine which ones should be tested further.

Many potential anticancer compounds derived from numerous different tunicate species have shown signs of cytotoxicity against various cancer cell lines. For example, Jimenez et al. [26] found that dichloromethane extract from *Eudistoma vannamei* exerts significant cytotoxic effects. This extract was tested on HL-60 promyeloblastic leukemia cells, with results suggesting that cytotoxicity is related to initiation of apoptosis in these cells. Takeara et al. [27] demonstrated that compounds found in the methanol extract of *Didemnum psammatodes* show cytotoxic activity and antileukemic effects. Interestingly, these molecules also exhibited antiproliferative activity. Furthermore, Sung et al. [28] analyzed the cytotoxicity of 7-beta-hydroperoxycholesterol and 7-alpha-hydroperoxycholesterol (a stereoisomer) from the lipophilic extracts of a Formosan (Taiwanese) tunicate, against various cancer cell lines. Fedorov et al. [29] reported the cancer-preventive properties of 3-demethylubiquinone Q2. They provided evidence that all tested quinones inhibited JB6 Cl41 cell transformation and p53 activity (an important tumor suppressor protein) and induced apoptosis, AP-1 (an important transcription factor for various genes that may be mutated to cause cancer), and NF-κβ activity (a protein complex that controls the transcription of DNA and is linked to cancer formation).

Other molecules have also shown promise against human cancer cells as assayed by cytotoxicity. Rashid et al. [30] provided evidence of three new beta-carboline alkaloids from *Eudistoma gilboverde* that exhibited varying levels of cytotoxicity. Oda et al. [31] analyzed the cytotoxicity of lissoclibadins and lissoclinotoxins from the tropical ascidian *Lissoclinum cf. badium* against nine different human cancer lines to determine their potential anticancer efficacy. Given that the compounds from different ascidians function through similar pathways, bioprospecting these marine species could yield more antitumor compounds that target specific tumor or cancer cell lines and types.

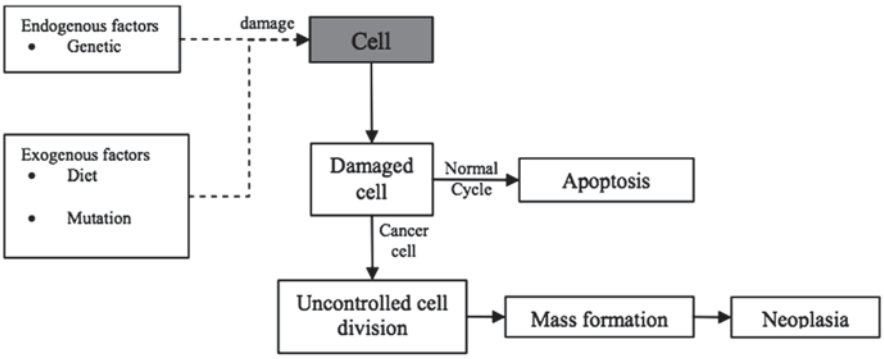

Fig. 18.2 Flowchart showing events that lead to cancer. (Figure reproduced from [23]) (This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).)

18.3.2 Antiproliferative Activity

In addition to causing cell death, anticancer compounds can prevent cancer cells from spreading through antiproliferative activity such as inhibition of topoisomerase (a protein that is essential to DNA replication and transcription) function and tubulin (an important protein for cell movement and structure) polymerization. Manzanares et al. [32] emphasize that the potent antiproliferative activity of compounds from ecteinascidins against tumor cells has raised them to the level of prospective anticancer agents. The implications of this physiological effect are far-ranging, as Takeara et al. [27] prove by demonstrating the antileukemic effects of constituents derived from *Didemnum psammatodes*. They investigated the chemical nature of the methanolic extract, which led to the identification of fourteen known compounds. Further studies on their mode of action suggest that these activities are connected to inhibition of DNA synthesis and induction of necrosis and apoptosis. Urdiales et al. [33] revealed the antiproliferative effect of dehydrodidemnin B, as results from their experiment show that its daily administration decreases the total number of tumor cells in Ehrlich carcinoma cultures by 70–90 %. They also discovered that dehydrodidemnin B inhibited the myc-oncogenic pathway, another important signal transduction pathway, which hindered protein synthesis that was necessary for cancer cells to replicate and avoid apoptosis.

18.3.3 Anti-Inflammatory Pathways

The goal of analyzing anti-inflammatory pathways is to understand how to prevent angiogenesis: the recruitment of blood vessels that enhance unwanted tumor growth by metastasis [4]. Inhibiting and minimizing these effects can be a beneficial approach in the endless fight against cancer. Although research into this anticancer

mechanism is still ongoing for urochordate compounds, some promising compounds do warrant further scrutiny. Appleton et al. [34] note that Kottamides A-D, bioactive alkaloids derived from the New Zealand ascidian *Pycnoclavella kottae* exhibits anti-inflammatory activity as revealed by analysis with 15N natural 2-D NMR. In addition, Xu et al. [35] discovered that chondroitin sulfate extracted from *Styela clava* suppressed inflammatory factors and enhanced the effect of treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). As explained, anti-inflammatory properties are often discovered for tunicate compounds when testing for cytotoxicity or other characteristics. Thus, searching specifically for anti-inflammatory features, a key component of the innate immune system, may be a promising avenue of future research.

18.4 Pharmaceutical Solutions: Need for More Research

Antitumor compounds derived from tunicates have been proven to be effective in the laboratory, but are they are effective as pharmaceutical treatments when administered to patients? Results have been decidedly mixed, but certain molecules do show promise as revealed by results of clinical trials in the United States and Europe. Didemnin B, Aplidine (also known as Dehydrodidemnin B), and Trabectedin are three of the successful tunicate compounds that have been tested over the years. Didemnin B is derived from Caribbean tunicate *Tridemnum solidum* in the early 1980s [14]. While Didemnin B did enter clinical trials, side effects that included neuromuscular toxicity and anaphylaxis derailed the process. However, the antitumor properties associated by Didemnin B inspired further investigation of tunicate compounds. Aplidine was the next molecule discovered. Derived from the Mediterranean tunicate *Aplidium albicans*, it was much more potent against human tumors and smaller in size than Didemnin B [36]. Trabectedin was another successful anticancer molecule derived from tunicates. It has gone through clinical trials and is on the market as Yondelis® [37]. Table 18.1 summarizes the function and clinical progress of these three therapeutic solutions.

18.4.1 Didemnin B

Didemnin B (DB) was the first tunicate extract to undergo clinical trials for its antitumor properties. Although it did not reach the market, Didemnin B's early successes and viability as a potential pharmaceutical product inspired further investigation into other tunicate compounds. Thus, its legacy is that of a stepping stone to future successes. Rinehart [14] provides a review of the structure, function, and previous clinical status of Didemnin B. It was the first marine-derived compound to enter phase I and II clinical trials, but neuromuscular toxicity as a side effect derailed its chances of progressing further. The closely related dehydrodidemnin

Table 18.1 Summary of clinical progress (Phases I–III) and function of tunicate-derived anticancer drugs

Compound name	Summary of function	Clinical progress
Didemnin B	Didemnin B (DB) was the first tunicate extract to undergo clinical trials for its antitumor properties. Although it did not reach the market, Didemnin B's early successes and viability as a potential pharmaceutical product inspired further investigation into other tunicate compounds [14]	It was the first marine-derived compound to enter phase I and II clinical trials, but neuromuscular toxicity as a side effect derailed its chances of progressing further [14]
Aplidine	Also known as Plitidepsin and Dehydrodidemnin B, it is structurally identical to Didemnin B except for a pyruvyl group at the N-lactyl side chain [4]. It is more potent than Didemnin B [14]	Phase I and II trials were successful in targeting various cancer lines [38]. It is currently undergoing Phase III trials for Relapsed/Refractory Myeloma (NCT01102426)
Trabectedin	Trabectedin, also known as ET-743, binds to the minor groove of DNA giving rise to a covalent adduct with the exocyclic amino group at position 2 of a guanine in a fashion similar to antibiotics called saframycin [32]	Trabectedin is effective against advanced or metastatic non-gastrointestinal stromal tumour soft tissue sarcoma (STS) whose disease progresses during or after chemotherapy with doxorubicin or ifosfamide in Phase II trials [42]
		Phase III trials have revealed that trabectedin is effective against ovarian cancer [39]
		Phase III trials are ongoing to test effectiveness against soft-tissue sarcomas [47]

B (DDB, Aplidine) was isolated in 1988 from *Aplidium albicans*. Aplidine is more active than DB and does not result in cardiotoxicity like Didemnin B. Urdiales et al. [33] studied the antiproliferative effect of Didemnin B and Aplidine, which was analyzed against Ehrlich carcinoma growing *in vivo* and in primary cultures. When taken in dose-limited quantities, mice receiving DDB or DB showed an overwhelming decrease in tumor cells and significant increase in vitality relative to controls.

18.4.2 Aplidine

Perhaps the most important consequence of the discovery of Didemnin B was the subsequent discovery of the closely related dehydrodidemnin B. This compound, also known as Aplidine, is ten times as active as Didemnin B. Le Tourneau et al. [38] review the marine plitidepsin aplidine derived from the Mediterranean tunicate *Aplidium albicans*—a synthetic antitumor compound structurally related to didemnins. Aplidine's mechanism of action involves several pathways: cell cycle arrest, inhibition of protein synthesis and antiangiogenic activity. The previous

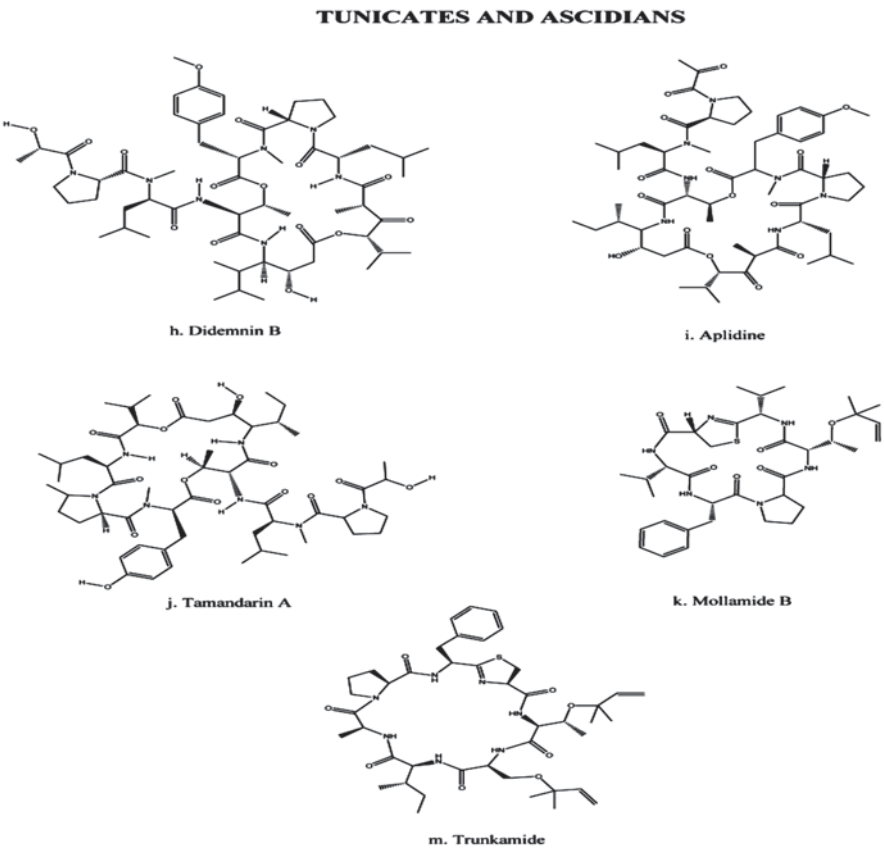

Fig. 18.3 Chemical structures of some bioactive peptides and depsipeptides from tunicates. (Figure reproduced from [23].) (This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/))

Urdiales et al. [33] experiments suggested that Aplidine is particularly potent when delivered during the lag phase of growth. It is able to halt the progression of certain components of the myc-oncogenic pathway by inhibiting protein synthesis and enzymatic activity needed for these cancer cells to replicate and bypass apoptosis. Among the clinical Phase I trials, aplidine exerted antitumor activity in many tumor lines, with particular efficacy against advanced medullary thyroid carcinoma. Based on these results, aplidine has undergone phase II tests. Like Didemnin B, aplidine is not without its dose-limiting toxicities, including neuromuscular and dermal toxicity, diarrhea and asthenia. However, the neuromuscular toxicity was reversed by L-carnitine, a common treatment for the adult form of the genetic disease carnitine palmitoyl transferase deficiency type 2 [38]. With clinical trials still ongoing, Aplidine is a promising treatment option that warrants further investigation and refinement (Fig. 18.3).

18.4.3 Trabectedin

Trabectedin, derived from *Ecteinascidia turbinate*, is the most promising tunicate pharmaceutical agent thus far. Trabectedin, also known as ET-743, binds the minor groove of DNA, much like antibiotics called saframycin. Analysis by a variety of biochemical, spectroscopic, and computer-based procedures has revealed that binding of ET-743 to DNA is accompanied by minor groove widening and DNA bending towards the major groove [32]. Twelves et al. [40] showed that ET-743 interferes with DNA binding proteins and transcription factors. With regards to phase I and II clinical trials studying antitumor activity in multiple tumor types, they explain that dose-limiting toxicities were hematological, including neutropenia and thrombocytopenia. Significant liver toxicity was observed in specific instances. Antitumor activity in phase I and phase II trials has been observed in multiple tumor types, including soft tissue sarcomas, melanomas and breast cancer [40]. ET-743 displayed significant antitumor activity against sensitive and resistant human xenografts. There were dosage limitations in animal models, but patterns of reversibility in monkeys have assuaged some of these concerns [41].

18.5 Tunicate Anticancer Molecules: What Does the Future Hold?

18.5.1 Biosynthesis: A Possible Solution?

Schöffski et al. [42] discuss recent clinical impacts of ET-743 in advanced/metastatic soft tissue sarcoma, while suggesting that a link between specific translocations underlying this disease and the drug's mechanism of action is being explored. Furthermore, they insist that the potential of trabectedin should be further explored in soft tissue sarcomas in general and in specific subtypes, both in combination with other cytotoxic agents and with modulators of intracellular signaling. Poveda [43] supports this view as well. Given these promising results, trabectedin seems poised to make the biggest investigative splash in the market. Setbacks during the FDA approval process have prevented trabectedin from reaching the United States, but it has still entered the market in Europe [37].

Although tunicate anticancer compounds have the potential to be extremely successful, a lack of adequate numbers of naturally occurring tunicates in the marine biosphere to provide these molecules looms as an enormous obstacle to pharmaceutical implementation [44]. Aquaculture has been presented as a potential solution to this roadblock [45], but sufficient tunicate compounds cannot be harvested through this method. Many of the potential antitumor molecules analyzed up to now are available in only miniscule quantities in most ascidians [44], so aquaculture would only be a band-aid solution to a gaping wound of a dilemma [46]. All is not lost, however, as strong work at the lab bench has yielded promising results that when analyzed could increase the meager supply of tunicate compounds.

Instead of trying to completely rebuild the complex cyclic depsipeptide structure through chemical synthesis or attempting to inefficiently farm tunicates through aquaculture, a better solution appears to be a focus on synthetically producing these same compounds in associated microorganisms [46]. In fact, aplidine has been produced by the marine α-proteobacteria *Tistrella mobilis* and *Tistrella bauzanensis* via a unique post-assembly line maturation process [44]. Xu and his colleagues revealed a potential alternative to the current inability to produce sufficient tunicate anticancer compounds by converting didemnin X and Y precursors to didemnin B. More investigation into potential methods to expand supply of these molecules should be a priority.

18.5.2 Where Do We Go Next? A Plan for the Future

Bioprospecting, as a planned search, for novel marine antitumor molecules, as illustrated, appears to be an important source of raw materials for pharmaceutical discovery. Translational medicine, which unites marine natural products chemists and pharmacologists who discover the compounds with medical investigators and pharmaceutical companies, will be essential to converting the basic molecules into effective treatment options. This collaboration could then lead to mutual benefits for both sides. Pharmaceutical companies acquire alternative sources of pharmaceutical building blocks, while academic programs gain access to technologies and knowledge that corporations possess. Government funding can also increase the efficacy of this search.

It is imperative to focus on producing new drugs to help implement the many discoveries made in cancer research. With untapped marine biodiversity waiting to be bioprospected, a focus on tunicates and other marine organisms may yield therapeutic solutions. Already, tunicate anticancer compounds have undergone clinical trials and reached the market. While there are no guarantees of success, investigating these molecules can be a stepping stone towards use as practical pharmaceuticals that can significantly assist the fight against cancer.

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Chapter 19
Trabectedin (ET-743) from Marine Tunicate for Cancer Treatment

Harika Atmaca and Emir Bozkurt

Abstract Trabectedin (Yondelis; ET-743) is a DNA binding agent that was originally derived from the marine tunicate *Ecteinascidia turbinata* and is currently prepared synthetically. It's mechanism of action is different from the conventional alkylating agents used in cancer chemotherapy. Two of the three fused rings of trabectedin molecule are involved in the minor groove binding to DNA; forming DNA adducts on N2 position of guanine, and bends DNA towards the major groove, while the third is known to interact with different transcription factors and DNA-binding proteins. Trabectedin has been found to inhibit the proliferation of various tumor cells *in vitro* and *in vivo*. It is the first anticancer marine derived drug that has been approved by the European Union, Russia and South Korea for the treatment of advanced or metastatic soft tissue sarcoma. Several Phase II clinical trials are also going on for several cancer types including breast and prostate.

Keywords Trabectedin (Yondelis; ET-743) · *Ecteinascidia turbinata* · DNA binding · Apoptosis · Cell cycle · Tumor microenvironment

19.1 Trabectedin (ET-743) from Marine Tunicate for Cancer Treatment

Ocean is the largest habitat of our planet covering 71 % of the Earth's surface and yet we haven't explored more than 95 % of it. There are numerous marine species and they have to adapt their environment to maintain their life and to cope with their predators. For this aim some of them developed physical defense systems such as sharp teeth and thorns whereas the others developed biologically active molecules. Since the chemical and biological diversity of the marine environment is immeasurable, they are promising sources of potential cancer drugs.

In 1950's, the first worthy marine source in cancer treatment, a Caribbean sponge, *Cryptotheca crypta (Tethya crypta)* was discovered. Within the next 5 years, spon-

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Fig. 19.1 Source organism of Tratebectedin; *Ecteinascidia turbinata*

gouridine and spongothymidine were successfully isolated from this sponge and led to the synthesization of first marine-derived drug in cancer treatment, cytarabine (cytosine arabinoside, Ara-C), in 1959. Cytarabine received FDA approval as an anticancer drug in June 1969 [1].

Trabectedin (Yondelis®, Ecteinascidin-743, ET-743) was isolated from the marine tunicate *Ecteinascidia turbinata* which is a Caribbean Sea squirt belonging to the Ascidiacea class within the subphylum Tunicata (Fig. 19.1). The first *in vitro* cytotoxic activity of ethanolic extracts of *Ecteinascidia turbinata* was reported against various cancer cell lines in 1969. Later, six potential active compounds called ecteinascidins (ET-729, ET-729A, ET-743, ET-745, ET-759B, and ET-770) were successfully isolated by Kenneth L. Rinehart [2]. ET-743 was the most abundant compound thus; it was selected for further development. Unfortunately, isolation technologies were not improved to acquire enough ET-743 that will be used in clinical studies. To isolate 1 g ET-743, 1 t animal was needed [3]. Then, *Ecteinascidia turbinata* has been successfully grown in aquaculture facilities and the active ingredient was purified by a Spanish company PharmaMar. In parallel with PharmaMar, chemists were also tried to find out a way to produce ET-743 synthetically. First total synthesis of ET-743 was done by Corey et al. using a multistep enantiocontrolled process in 1996 [4]. Three years later, Martinez et al. were found a synthetic analogue of ET-743 (Phthalascidin) showing the same *in vitro* activity as ET-743 [5]. Although Martinez and Corey were continued to improve the total synthesis of ET-743, it was still not enough for industrial manufacturing [6, 7]. In June 2000, Cuevas et al. from PharmaMar, was successfully developed a semi-synthetic process starting from cyanosafracin B [8]. Cyanosafracin B is an antibiotic obtained through fermentation of the bacteria *Pseudomonas fluorescens*. This was the first process providing a way to produce ET-473 as semi-synthetically, on a multigram scale. From 2002 to 2006, four additional synthetic processes have been described by the groups of Fukuyama, Zhu, Danishefsky, and Williams [1]. Now, ET-743 is produced synthetically and commercially available under the name of Yondelis® [9].

Trabectedin is the first anticancer marine derived drug that has been approved by the European Union, Russia and South Korea for the treatment of advanced or metastatic soft tissue sarcoma. Phase II trials are also going on for several cancer types including breast and prostate.

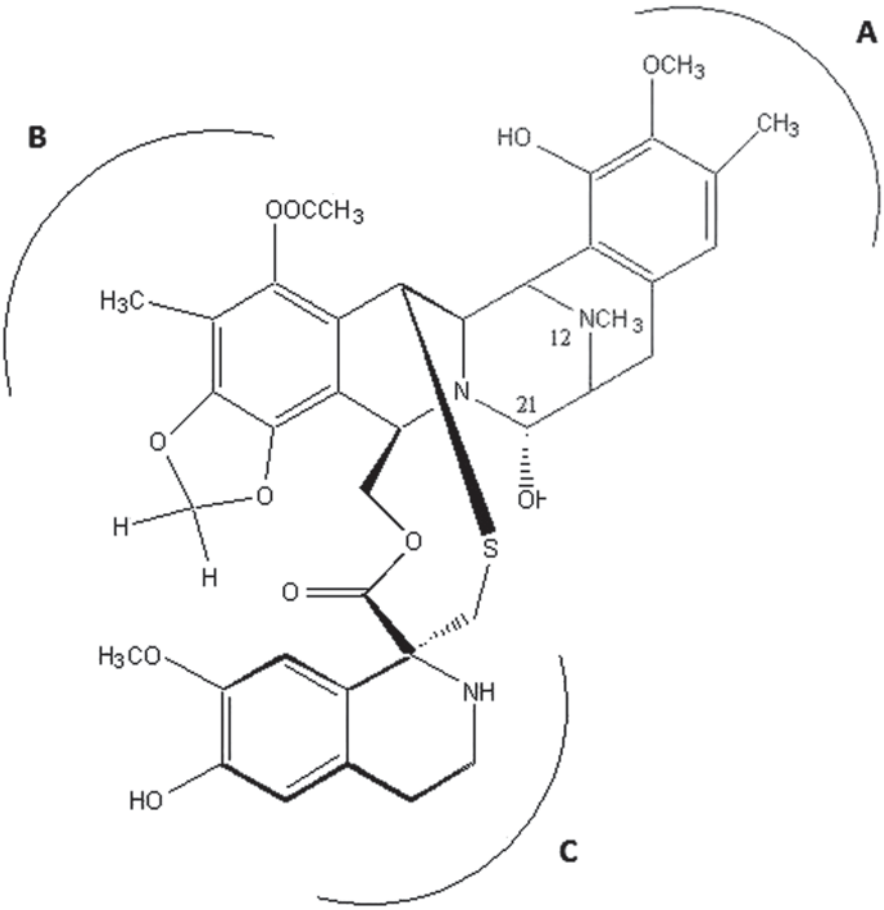

Fig. 19.2 Chemical structure of Trabectedin. It consists of three subunits, A, B and C, of which A and B bind to DNA and C interacts with transcription factors

19.1.1 Chemical Properties

IUPAC name of Trabectedin is [6R-(6α, 6aβ, 7β, 13β, 14β, 16α, 20R*)]-5-(acetyloxy)—3, 4, 6, 6a,7, 13, 14, 16,-octahydro-6,8,14-trihydroxy-7,9-dimethoxy-4,10,23-trimethylspiro [6,16 (epithiopropanoxymethano)-7,13-imino-12H-1,3-dioxolo [7, 8]-isoquino [3, 2-b] [3] benzazocine-20, 1 (2H)-isoquinolin]-19-one. Molecular formula is $C_{39}H_{43}N_3O_{11}S$ (Fig. 19.2). Molecular weight is 761.837 g/mol, and half-life is between 33 and 50 h. It is a hydrophobic molecule and is soluble in DMSO, methanol, ethanol, chloroform, ethyl acetate, and acetone. Solubility increases with the acidic pH. Trabectedin is metabolized by cytochrome P (CYP) 450 3A4, 2C9, 2C19, 2D6, 2E1 [1, 10, 11].

19.1.2 Mechanism of Action

Trabectedin is a DNA binding agent and transcription is a major target of it but accumulating evidence suggests that Trabectedin has a complex mechanism of action and diverse range of molecular targets.

19.1.2.1 DNA Binding

Trabectedin differs from other DNA alkylating agents presently used in cancer chemotherapy by binding to the DNA minor groove non-covalently. It has three subunits (Fig. 19.2); two of them are responsible for binding to DNA. The binding of Trabectedin to DNA changes the natural conformation of the DNA by bending it towards the major groove. In addition, through its C21 carbon, Trabectedin alkylates the N2 position of the guanine residue, preferentially flanked by guanine or cytosine on the 3 side and a pyrimidine on the 5 side. When alkylation occurs, DNA strands are cross linked and cannot replicate, causing cell death. Two of the three subunits of Trabectedin bind to DNA while the third does not have contact with DNA. It protrudes out from the DNA minor groove and effects transcription via interfering with DNA binding proteins. Trabectedin strongly inhibits the binding of Nuclear Transcription Factor Y Subunit Alpha (NF–Y), which is one subunit of a trimeric complex, forming a highly conserved transcription factor that binds to CCAAT motifs in the promoter regions in a variety of genes controlling the cell cycle [12]. It also inhibits *Heat shock protein 70 (HSP70)* gene containing two CCAAT boxes activating by NF-Y whereas it does not affect the promoters lacking CCAAT boxes. It also inhibits the binding of *p21 (CIP1)*, a cell cycle regulatory gene, *c-Fos and c-Jun,* oncogenes functioning as transcription factors, *MDR1* gene, encoding for the P glycoprotein (P-gp) which are under dependence of a promoter containing CCAAT boxes [13]. These studies and the fact that the expression of genes which are target of Trabectedin is under the control of transcription factor Sp-1 implicate that Trabectedin is a transcription factor dependent, CCAAT box dependent inhibitor of transcription activation. It has been shown that induction of the Sp1-regulated *p21* gene by Trichostatin A (TSA), a promoter activator, was blocked by Trabectedin at concentrations that had minimal effect on uninduced expression [14].

19.1.2.2 DNA Repair

Not only does Trabectedin interfere directly with transcription, but it also targets the transcription-coupled nucleotide excision repair (TC-NER) [15, 16]. It blocks TC-NER by trapping XPG-DNA complexes and prevents further processing; causing DNA single-strand breaks [16–18]. Thus, TC-NER and XPG deficient cells are resistant to Trabectedin [15, 16, 19]. In the absence of an intact XPG-DNA complex, these toxic lesions do not occur, and the single-strand breaks caused by Trabectedin, though not repaired by the NER pathway, are less toxic to cells.

The roles of homologous recombination (HR) and non homologous end joining (NHEJ) pathways which are involved in double-strand DNA breaks were also investigated in the mechanism of action of Trabectedin. It has been shown that the HR system is required to repair the double-stranded DNA breaks that are produced by Trabectedin and the recruitment of the TC-NER. Cells deficient in HR were approximately 100 times more sensitive to the drug [20–22]. In contrast, these differences were not observed in NHEJ-deficient cells.

19.1.2.3 Cell Cycle

In vitro cell cycle studies revealed that Trabectedin slows the rate of progression of cells through S phase toward G2 and blocks cells in G2-M phase[15, 23]. This activity in G2 phase of the cell cycle is unique and contrasts to other alkylating agents that are generally active in S phase.

Cells whose DNA has been damaged by adduct formation or UV or γ irradiation undergo a G1/S arrest, a process requiring p53. Treatment of various cells with Trabectedin indeed increases p53 protein levels, but p53 −/− mouse embryo fibroblasts are as sensitive as their p53 +/+ counterparts as p53 −/− colon carcinoma A2780 cells and an isogenic line expressing a dominant-negative p53 [24]. Thus, p53 does not involve in cell-cycle arrest mediated by Trabectedin [23, 24].

Studies with microarray analyses have allowed the identification of genes that are involved in cell cycle arrest after treatment with Trabectedin in different sarcoma cell lines. These genes[*calcineurin-binding protein calsarcin 1 (CS-1), Activating transcription factor 3 (ATF3), spermidine/spermine N1-acetyltransferase (SAT), Transcription factor Jun-B (JUNB), Growth arrest and DNA-damage inducible beta (GADD45B), and DNA bindng protein inhibitor 2 (ID-2)*] have a common role as transcription regulators, leading to cell cycle arrest in G2-M and apoptosis, which can be correlated with the cell cycle arrest and apoptosis induced after treatment of Trabectedin in sarcoma cell lines [7, 15, 18, 23, 25].

In addition, significant changes were also detected in the expression of a number of cell cycle-related genes in human leukemia cell line HeLa after Trabectedin treatment. Histones (H1 histone family, member X, H4 histone family, member G, H4 histone family, member E) and minichromosome maintenance protein 5 (MCM5) were found to be down-regulated in HeLa cells [25]. Histones play important roles during the G2/M transition and reduced MCM5 protein levels induce a delay in S phase and block cell cycle progression. Trabectedin treatment up-regulated cyclin G2, growth arrest and DNA-damage inducible alpha (GADD45A) and cyclin-dependent kinase inhibitor 1 (p21) transcripts in HeLa cells. Cyclin G2 has been reported to be up-regulated during late S phase and GADD45A and p21 induce G2 cell cycle arrest. Based on the changes in the expression of these cell cycle-related genes, Gajate et al. attributed the observed effects of Trabectedin on cell cycle, namely a delay in S phase and an arrest in G2/M phase [25].

19.1.2.4 Apoptosis

Trabectedin is known to induce apoptosis in various tumor cells; however, there are limited studies according to the apoptotic mechanisms of it. Apoptotic effect of Trabectedin was firstly investigated on Ewing's sarcoma and osteosarcoma cells because of its strong antiproliferative activity on sarcoma cells. Trabectedin shows a significant induction of apoptosis in Ewing's sarcoma cells, not in osteosarcoma cells [26]. Apoptotic effect of Trabectedin alone and in combination with other drugs in HT1080 human fibrosarcoma cells were also demonstrated [27]. Later, it is found that Trabectedin induces PARP fragmentation in A549 human lung adenocarcinoma, Jurkat, Raji, K562 and HL-60 human leukemia cells lines whereas do not induce PARP fragmentation in p53 mutant, Burkitt CA46 cell line. At concentrations ranging from 5 to 10 nM, it is shown that Trabectedin induces a significant cleavage of caspases-8, 9, and PARP inA549 cells. In addition, it induces a dose-dependent increase in the protein levels of FLIP (L), p53 and Bcl-2 (100 nM) and a dramatic downregulation in the phosphorylation level of s473-AKT, after 72 h [28]. Strong *in vitro* antimeningioma activity of Trabectedin was observed and involvement of deregulated expression of cell death-regulatory genes was reported by Preusser M et al. [29].

The apoptotic effect of Trabectedin on HL-60 and HeLa human leukemia cells were also investigated in detail by another group. They reported that Trabectedin induces DNA fragmentation at concentrations of 10 or 100 ng/ml in human leukemia cells and the apoptotic pathway involves mitochondrial cytochrome c release, JNK activation and caspase-3 activation in these leukemia cells [25].

Another detailed study was carried out by using human breast cancer cells. It is shown that Trabectedin induces apoptosis in MCF-7 and MDA-MB-453 breast cancer cells in a time and concentration-dependent manner. The expression levels of the death receptor pathway molecules, TRAIL-R1/DR4, TRAIL-R2/DR5, FAS/TNFRSF6, TNF RI/TNFRSF1A, and FADD were significantly increased in MCF-7 cells. However, in MDA-MB-453 cells, the mitochondrial pathway related pro-apoptotic proteins Bax, Bad, Cytochrome c, Smac/DIABLO, and Cleaved Caspase-3 expressions were induced, and the expression levels of anti-apoptotic proteins Bcl-2 and Bcl-XL were reduced in MDA-MB-453 cells. These data indicates that Trabectedin causes selective activation of extrinsic and intrinsic apoptotic pathways in two genotypically different breast cancer cells [30].

19.1.2.5 Tumor Microenvironment

Besides its anti-tumor effects, it is revealed that Trabectedin has modulatory effects on the tumor microenvironment. It inhibits the production of several inflammatory mediators such as CCL2, CXCL8, IL-6, vascular endothelial growth factor (VEGF), and the matrix binder protein pentraxin 3 (PTX3), in primary cultures or cell lines of myxoid liposarcomas and patient-derived myxoid liposarcoma xenograft mouse model [31].

The effects of Trabectedin on leukocytes, angiogenesis and on the expression of inflammatory mediators were investigated on different mouse tumor models. It is

Table 19.1 *In vitro* activity of Trabectedin on different human carcinoma cell lines

Tumor type	Cell line	Exposure time (h)	IC_{50}(nM)	Reference
Fibrosarcoma	HT-1080	72	0.0002; 0.01	[23, 37]
Mesenchymal chondrosarcoma	HS-16	72	0.02	[[32]
Liposarcoma	HS-18	72	0.06; 0.27	[23, 37]
	SW872	96	0.5	[38]
	1455	96	0.1	[38]
Mixed mesodermal sarcoma cell line	HS-42	72	0.004	[23]
Malignant hemangiopericytoma	HS-30	72	0.3	[23]
Rhabdomyosarcoma	RD	48	4	[39]
Alveolar rhabdomyosarcoma	RH30	48	1.5	[39]
Colon carcinoma	HCT-116	72	0.50	[5]
	HCT-116	96	0.16	[40]
	SW480	96	0.68	[40]
Breast carcinoma	MCF-7	48	4.8	[30]
	MCF-7	96	1.5	[36]
	MDA-MB-453	48	2.5	[30]
	MX-1	96	0.1	[36]
Ewing's sarcoma	TC-71	72	0.23	[26]
	A673		1.0	[38]
	EW8	48	0.5	[39]
Osteosarcoma	Hos	48	6	[39]
	U-2 OS	48	4	[39]
	U-2 OS	96	0.42	[26]
	Saos-2	48	4	[39]
	Saos-2	96	0.15; 0.11	[26, 38]
Lung carcinoma	A-549	72	1.0	[5]
Prostate carcinoma	PC-3	72	0.70	[5]
Malignant melanoma	A375	72	0.15	[5]
Hepatic carcinoma	Hep G2	48	0.36–1.07	[11]

found to be highly cytotoxic to mononuclear phagocytes, including tumor macrophages, but not cytotoxic to other leukocyte subsets such as neutrophils and lymphocytes. Besides its selective cytotoxic effect, Trabectedin also reduced the vessel network [9, 31, 32]. Similar results were also shown by using human soft tissue sarcoma patients receiving Trabectedin as neo-adjuvant therapy.

19.1.3 Preclinical Studies with Trabectedin

The activity of Trabectedin has been investigated in a wide range of cell lines including leukemia, sarcoma, breast, non-small cell lung, ovarian and melanoma and found to be potent cytotoxic against these cell lines (Table 19.1). *In vitro*, the cytotoxic effect of Trabectedin is in the nanomolar range, and in the case of some very sensitive cell lines such as sarcomas, in the picomolar range.

In vivo effect of Trabectedin was investigated using human TE-671 rhabdomyosarcoma xenografts. It (0.05 and 0.15 mg/kg) was given intravenously (i.v.) to mice either a single 0.2 mg/kg injection or one 0.1–0.2 mg/kg injection three times every 4 days. 0.2 mg/kg injection of Trabectedin caused 62% tumor weight inhibition [33].

Effect of Trabectedin was also investigated on human ovarian carcinoma xenografts. At the maximum tolerated dose of 0.2 mg/kg using an intermittent schedule of one i.v. injection every 4 days, ET-743 was highly active against HOC22-S (sensitive to cisplatinum), inducing long-lasting, complete regressions, and against HOC18 (marginally sensitive to cisplatinum), inducing partial tumor regressions. Moreover, significant growth delay was observed in mice bearing late-stage HOC18 tumor (400-mg tumor weight; nonresponsive to cisplatinum). However, Trabectedin was not active against MNB-PTX-1, a tumor that is highly resistant to chemotherapy [34].

Later, Trabectedin was tested on human melanoma and non-small-cell lung cancer xenografts. It was very active at the maximum tolerated dose (MTD) in the chemo-sensitive melanoma MEXF 989 and non-small-cell lung cancer LXFL 529 xenografts. In the responding xenografts, it caused complete remissions. The compound was inactive in the chemo-resistant xenograft melanoma MEXF 514 and non-small-cell lung cancer LXFA 629 [35].

Anti-tumor effect of Trabectedin on human breast cancer xenografts were tested by injecting 50 μg/kg Trabectedin (q3d × 3). The average tumor volume was decreased by 82.3% by day 21, relative to the control without toxic deaths [36].

19.1.4 Mechanisms of Trabectedin Resistance

Trabectedin is known to be highly selective for myxoid liposarcoma, characterized by the translocation t(12;16)(q13; p11) leading to the expression of FUS-CHOP fusion gene. Thus, mechanisms of Trabectedin resistance are comprehensively studied in these cells. It is revealed that 3083 genes, 47 miRNAs and 336 proteins differentially expressed between sensitive (402–91) and resistant (402–91/ET) myxoid liposarcoma cell lines. Among those three miRNAs (miR-130a, miR-21 and miR-7) containing CHOP binding sites in their promoter region, are found to be differentially expressed in these cells. Moreover, the central role of genes such as CCDN1, RB1, E2F4, TNF, CDKN1C and ABL1 in both pre- and post-transcriptional regulatory network was demonstrated. All the hallmarks of cancer were altered in the resistant cell line indicating that the mechanism of resistance is acting with more strength on the same molecular markers triggered by cancer development [41].

Molecular aberrations between the parent and resistant cell line related to Trabectedin resistance were investigated on a human chondrosarcoma cell line, CS-1. There were no significant differences in the levels of membrane transporters such as P-glycoprotein or multidrug resistance protein 1 (MRP1) but the cell migratory ability was reduced in ET-743-resistant cell variant. Moreover, Type I collagen α1 chain mRNA levels were significantly lower in the ET-743-resistant CS-1 cell line. Thus,

it is suggested that prolonged exposure to ET-743 may cause changes in cell function through cytoskeleton rearrangement and/or modulation of collagen levels [42].

Resistance to Trabectedin is also shown in ER5 colorectal carcinoma cells and is found to be associated with an insertion of adenine at codon 240 which resulted in a stop codon at position 243 in XPG gene [43].

19.1.5 Clinical Studies

The drug is approved in Europe for adults with advanced soft tissue sarcoma after failure of, or intolerance to, anthracyclines and ifosfamide. Phase II studies identified 1.5 mg/m^2 of Trabectedin via 24-h continuous intravenous (IV) infusion every 3 weeks as the optimal regimen [44]. It is also currently being studied in phase II clinical trials for ovarian, endometrial, breast and prostate cancer.

19.1.5.1 Sarcomas

First preliminary clinical study investigating the antitumor effects of Trabectedin in advanced pretreated sarcoma patients was published in 2001. Delaloge et al. analyzed 29 patients, 12 of them were in phase 1 trial and 17 were in compassionate use program. Trabectedin was given as a 24-h infusion every 3 to 4 weeks. The median duration of response was found to be 10.5 months. Hematologic toxicity was observed in six patients and transaminitis was observed in one patient. This study showed that 24-h continuous Trabectedin infusion is effective and feasible in advanced sarcoma patients [45].

Demetri et al. examined 270 advanced sarcoma (liposarcoma, 34.4%; leiomyosarcoma, 65.6%) patients after failure of prior conventional chemotherapy. Two different schedules were carried out in this randomized phase II study. While 136 patients were assigned to 1.5 mg/m^2 24-h intravenous infusion once every 3 weeks (q3 weeks 24-h), 134 were assigned to 0.58 mg/m^2 3-h IV infusion every week for 3 weeks of a 4-week cycle (qwk 3-h). They showed that both Trabectedin regimens demonstrated antitumor activity in patients. Moreover, it was indicated that q3 weeks 24-h infusion was significantly beneficial when compared to qwk 3-h. While the median OS was found 13.9 months in patients treated with Trabectedin q3 weeks 24-h, it was found 11.8 months in qwk 3-h treated patients. Most common toxicities were observed in this study are fatigue, neutropenia and thrombocytopenia [46]. Ploner et al. were investigated the antitumor effects of Trabectedin in 101 STS patients. Median PFS and OS were found 3.9 and 11.6 months, respectively [47]. Schur et al. were retrospectively analyzed 60 STS patients treated with Trabectedin. Median PFS and OS were found 2.2 and 11.8 months, respectively [48].

Isabelle Ray-Coquard was pointed out that Trabectedin may be more efficient in uterine sarcomas [49]. Six months later, Monk et al. were published confirmatory results. They evaluated the effects of Trabectedin in 20 advanced uterine leiomyosarcoma patients. Median PFS and OS were found 5.8 and 26.1 months, respec-

tively [50]. However, further studies are needed to investigate whether Trabectedin is more effective in gynecological sarcomas.

Le Cesne et al. indicate that approximately 20% of soft tissue sarcomas have subtype-specific chromosomal translocations, which can act as abnormal transcription factors. They published an interesting retrospective analysis of Trabectedin effects including 81 patients had translocation related sarcomas. Median PFS and OS were found 4.1 and 17.4 months, respectively. This study suggested that Trabectedin shows antitumor activity in STS with particularly interesting levels of disease control in TRS subtypes [51].

Another intriguing study investigating the relationship between Trabectedin and ERCC5, ERCC1, and BRCA1 gene status was published by Italiano et al. They were analyzed 113 STS patients and indicated that high ERCC1 expression was associated with better clinical benefit from Trabectedin. They observed that high expression of the ERCC5 gene is strongly associated with improved outcome in patients with STS. This finding was observed only in patients who had tumors that have homozygous for the common allele of the ERCC5 Asp1104H is polymorphism. They suggested that specific BRCA1 haplotype (AAAG) may be a relevant clinical marker of response to Trabectedin. Moreover, they concluded that the status of ERCC5, ERCC1, and BRCA1 represents a potential DNA repair signature for predicting the clinical response to Trabectedin in patients with advanced STS [52].

A Phase I study of Trabectedin and Doxorubicin were investigated in patients with STS, by Blay et al. They found that the combination of doxorubicin 60 mg/m^2 followed by Trabectedin 1.1 mg/m(2) every 21 days is safe and active in patients with soft-tissue sarcoma [53].

Baruchel were published a report from the Children's Oncology Group. Trabectedin was given over 24 h every 3-weeks to children with recurrent rhabdomyosarcoma, Ewing sarcoma, or nonrhabdomyosarcoma soft tissue sarcomas. Forty two patients were evaluable and they found that Trabectedin is safe when administered over 24 h at 1.5 mg/m^2. It was clearly concluded that Trabectedin did not demonstrate sufficient activity as a single agent for children with relapsed pediatric sarcomas [54].

In conclusion, Trabectedin strongly considered being a new important treatment option for advanced sarcoma patients with a previous history of treatment with standard drugs. Moreover, its side effects are easily manageable and it is suitable to combine other chemotherapeutics.

19.1.5.2 Ovarian Cancer

Trabectedin as a single agent was studied in a number of phase I studies including patients with solid tumors. The results of these studies with Trabectedin monotherapy revealed that maximum tolerated doses changes with the schedule and found to be 0.65–1.9 mg/m^2 [44, 55–58].

A Phase II study by Krasner et al. was done with single-agent Trabectedin, with platinum (P)-sensitive ($n=62$) or -resistant ($n=79$) ovarian cancer patients.

Trabectedin was given at a dose of 0.58 mg/m^2 weekly for 3 weeks and repeated every 28 days. The overall response rate (ORR) was 29.0% and median progression-free survival (PFS) was 5.1 months in P-sensitive patients. In P-resistant patients ORR was 6.3% and median PFS was 2.0 months [59]. Similar results were obtained in another study [60]. Trabectedin was administered at a dose of 1.65 mg/m^2 over 3 h every 21 days. The overall RR was 43% in 23 P-sensitive patients compared with 7% in P-refractory patients ($n=28$).

Patients previously treated with less than two or two previous chemotherapy lines were randomized to receive Trabectedin 1.5 mg/m^2 24 h (arm A, $n=54$) or 1.3 mg/m^2 3 h (arm B, $n=53$). ORR was 38.9% and median PFS was 6.2 months in arm A whereas ORR was 35.8% and median PFS was 6.8 months in arm B [61].

A large phase III study (OVA-301) comparing pegylated liposomal doxorubicin (PLD) alone with a combination of PLD and Trabectedin in 672 patients with recurrent ovarian cancer was done by Monk et al. [62]. The results from OVA-301 showed that the combination of Trabectedin and PLD improves progression-free survival and overall response rate over PLD alone with acceptable tolerance in the second-line treatment of recurrent ovarian cancer. Based on the recently reported results of OVA-301 study in 2009 the European Commission granted marketing authorization for Trabectedin combined with PLD for the treatment of patients with relapsed platinum-sensitive ovarian cancer.

19.1.5.3 Other Solid Tumors

A phase II trial revealed that Trabectedin can induce response and tumor control in advanced breast cancer patients, who have failed after previous therapy with anthracyclines or taxanes, with manageable toxicity. Twenty-seven patients were treated with the recommended dose of Trabectedin (1.5 mg/m^2 as a 24-h i.v. continuous infusion every 3 weeks) and 22 (81.5%) of them were considered to be evaluable for response. They observed that Trabectedin-induced tumor shrinkage in the target measurable lesion in three patients, who responded to treatment, was found 86, 76 and 100%, respectively [63].

In a phase I study, Sessa et al. were observed that one patient who treated with increasing doses of Trabectedin (starting with 0.75 mg/m^2) and cisplatin (75 mg/m^2) had stable disease [64]. In another phase I study, Gore et al. were observed that 3 breast cancer patients treated increasing doses of Trabectedin (starting with 0.4 mg/m^2) in combination with capecitabine (2000 or 1600 mg/m^2/day) have maintained stable disease for at least 6 months [65].

The results of a multicenter phase II trial evaluated the efficacy and safety of Trabectedin in metastatic castration-resistant prostate cancer patients. In this study, two schedules were evaluated in three cohorts: weekly as 3-h i.v. infusion at 0.58 mg/m^2 for 3 out of 4 weeks (Cohort A, $n=33$), and every 3 weeks (q3wk) as 24-h infusion at 1.5 mg/m^2 (Cohort B1, $n=5$) and 1.2 mg/m^2 (Cohort B2, $n=20$). Trabectedin resulted in PSA declines ‡50% in 12.5% (Cohort A) and 10.5% (Cohort B2) of patients. Among men pretreated with taxane-based chemotherapy, PSA response

was 13.6% (Cohort A) and 15.4% (Cohort B2). The dose of 1.5 mg/m^2 which was approved for soft tissue sarcoma was not tolerable in these patients (given as 24-h infusion q3wk) [66]. Future studies in prostate cancer should include molecular characterization of tumors that might have enhanced sensitivity to Trabectedin.

Previous studies in sarcoma revealed that patients with high expression of nucleotide excision repair (NER) genes (XPG and/or ERCC1) and low expression of homologous recombination repair (HR) genes (BRCA1) are highly sensitive population of patients with significantly improved outcome to Trabectedin. An exploratory phase II clinical trial evaluated the efficacy and safety of Trabectedin in patients with NSCLC, who have over expression of NER genes (XPG and/or ERCC1), and under expression of HR genes (BRCA1), after the failure of standard platinum-based treatment. Patients were selected according to their mRNA expression (elevated XPG and/or ERCC1, with low BRCA1). Trabectedin was administered as a 1.3 mg/m^2 3-h intravenous infusion every 3 weeks (q3wk). Two of 18 evaluable patients achieved progression-free survival rate at 3 months. The primary efficacy objective (at least 3 of 18 patients being progression-free at 3 months) was not met, and therefore the trial was early finalized. Therefore, further clinical trials with Trabectedin as single agent in this indication are not warranted [67].

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Chapter 20
Anti-Cancer Effects of Chitin and Chitosan Derivatives

Mustafa Zafer Karagozlu and Se-Kwon Kim

Abstract Despite considerable progress in medical research, cancer is still one of the high-ranking causes of death in the world. It is the second most common cause of death due to disease after heart disease and according to World Health Organization it will be cause of death more than 10 million people in 2020. Therefore one of the main research goal for researchers investigating new anticancer agents. But the major complication for the cancer cure without surgeries is side effects. Especially, cytotoxic anti-cancer chemotherapeutic agents generally produce severe side effects, while reducing host resistance to cancer and infections. Therefore, it is important to find new, powerful anti-cancer agents that are highly effective, biodegradable and biocompatible. Chitin and chitosan are biopolymers which have unique structural possibilities for chemical and mechanical modifications to generate novel properties, functions. These biopolymers are biocompatible, biodegradable and non-toxic and their chemical properties allow them easily processed into gels, sponges, membranes, beads and scaffolds forms also. Due to their unique properties, they are excellent candidate for cancer cure or cancer diagnosis.

Keywords Chitosan · Chitosan derivatives · Anti-cancer · Anti-tumor

20.1 Introduction

Chitin is a natural polysaccharide which has been firstly identified in 1821. Henri Braconnot who is the director of the botanical garden in France, observed a material in mushrooms which did not dissolve in sulfuric acid. Braconnot named it as

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fungine. In the late 1830s, it was isolated from insects, and in 1859, chitosan, a derivative of chitin, was produced [1].

Chitin is synthesized by an enormous number of living organisms and it is the second most abundant polymer after cellulose. Especially it occurs in nature as ordered crystalline microfibril forming structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast. It is also produced by a number of other living organisms in the lower plant and animal kingdoms, serving in many functions where reinforcement and strength are required.

Studies on chitin and chitin derivatives have been intensified since 1990 because these polysaccharides show excellent biological properties such as biodegradation in the human body [2, 3], and immunological [4, 5], antibacterial [6, 7] and wound-healing activity [8–10].

On the other hand, chitosan is a natural nontoxic heteropolysaccharide composed of β-1,4-linked-D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc) in varying proportions. These polysaccharides have been widely studied and applied in different fields, Even chitosan is a derivative of chitin it has own unique functions such as support material for gene delivery [11], cell culture [12] and tissue engineering [13, 14]. Especially in the biomedical and pharmaceutical industries chitosan and chitosan derivatives have been widely used due to their various biological functions to their various biological functions such as anti-microbial [15], anti-bacterial [16], antioxidant [17] and immunostimulating [18] effects. Besides they have antitumor [19], anti-diabetic [20] and anti-viral [21] activities. Unfortunately, poor solubility of chitosan is principal limiting factor for its wide application. Thereupon recent studies on chitosan have attracted interest in converting it to more soluble form like chitooligosaccharides (COS). COS are the degraded oligomer of chitosan, which can be obtained by either chemical [22–24] or enzymatic [25] hydrolysis of chitosan. It has been reported that lower oligomers of chitosan are not only water-soluble but also exhibit versatile biological activities similar to chitosan [26]. The biological activity of COS is known to depend on their structure and molecular weight [27]. Beside, investigators mentioned that properties of COS, such as degrees of polymerization (DP), degrees of acetylation (DA), charge distribution and nature of chemical modification to the molecule strongly influence its observed biological activities [28].

20.2 Anti-Cancer Activity as a Therapeutic Agent

A main goal of cancer research is to completely prevent recurrence following surgery and to increase life time of the patient. But the major problem of the cure without surgeries is side effects. Especially, cytotoxic anti-cancer chemotherapeutic agents generally produce severe side effects, while reducing host resistance to cancer and infections. Therefore, it is important to find new, powerful anti-cancer agents that are highly effective, biodegradable and biocompatible. Chitosan and chitosan derivative anti-cancer agents is known to be a favorable pharmaceutical material because of its biocompatible and biodegradable properties [29].

Several chitin derivatives were investigated their anti-tumor activity [30]. Murata et al. [30] reported that 6-O-sulfated chitin significantly inhibited the lung tumor

colonization in proportion to the degree of sulfation. Furthermore, 6-O-sulfated carboxymethylated chitin (SCM-chitin) with a high degree of sulfation caused a marked decreased of number of lung tumor colonies in the spontaneous lung metastasis model. SCM-chitin also significantly inhibited the arrest of B16–Bl6 cells in lungs after co-injection with radiolabeled tumor cells.

A large amount of literature exists regarding the effects of anti-tumor activities of chitosan and its derivatives [31, 32]. Suzuki et al. [31] found that N-acetyl chitosan oligomer, particularly the hexane and heptamer, display notable anti-tumor activity against sarcoma 180 solid tumors in BALB/C mice as well as in MM-46 solid tumor implanted in C3H/HC mice. These results indicated that the effect was not by direct cytodial action on the tumor cells and was indeed host-mediated. Tokoro et al. [32] showed that hexameric chitosan oligomer had growth inhibitory effect against Meth-A solid tumor transplanted into BALB/C mice. The anti-tumor mechanism was assumed to be involve in increased production of lymphokines including interleukins 1 and 2, sequentially, leading to manifestation of anti-tumor effect through proliferation of cytolytic T-lymphocytes. In addition, the anti-tumor activity of low-molecular weight chitosan with the higher than hexamer were investigated. Qin et al. [26]. Reported that low-molecular weight chitosan (LMWC) were prepared by enzymatic hydrolysis using cellulose and hemicellulose, and investigated the inhibition of growth of sarcoma 180 tumor cells in mice. Maeda and Kimura [33] prepared various molecular weight such as 21, 46 and 120 kDa chitosans by enzymatic hydrolysis, and examined the anti-tumor activity in sarcoma 180-bearing mice. The anti-tumor activity of various molecular weight of chitosans showed that 21 kDa chitosan significantly reduced the tumor growth and final tumor weight. Moreover, 21 and 46 kDa chitosans enhanced the natural killer (NK) activity in intestinal intraphelial lymphocytes or splenic lymphocytes. Harish et al. [34] generated LMWC by depolymerization induced by potassium persulfate under nitrogen atmosphere. Moreover, Jeon, Park and Kim [16] also carried out a study to identify the correlation between molecular weight of chitooligosaccharides and their anti-tumor activity. In their research different molecular weight COSs has been prepared by UF membrane reactor system. The researchers suggested that, medium molecular weight molecular COS ranging 1.5–5.5 kDa could effectively inhibit the growth of Sarcoma 180 solid (S180) or Uterine cervix carcinoma No. 14 (U14) tumor in BALB/c mice. Hasegawa et al. [35] reported the growth inhibitory effect of chitosan on bladder tumor cells. They observed DNA fragmentation, which is characteristic of apoptosis, and elevated caspase-3-like activity in chitosan treated cancer cells. In addition, modified chitosans were reported to display the growth inhibitory effect on tumor cells [36], and this property was employed by Ouchi et al. [37] by conjugating chitosan or chitosanaminooligosaccharide to 5-fluorourcil (5FU) in order to provide a macromolecular system with strong anti-tumor activity and reduced side effects. Indeed, the strong anti-tumor activity exhibited by 5FU is accompanied by undesirable side effects. *In vivo* studies demonstrated that chitosan-5FU conjugate exhibited a strong survival effect against lymphocytic leukemia in mice. Furthermore, chitosan-5FU and COS-5FU conjugates showed remarkable growth inhibitory effects on Met-A fibrosarcoma and MH-134Y hepatoma. Both conjugates displayed no acute toxicity, even in high dose ranges. Therefore, they reported that chitosan-5FU and COS-5FU are expected to act clinically as macromolecular prodrugs of 5FU.

Furthermore, studies on anti-tumor activity of chitosan and COSs revealed that partially deacetylated chitin and carboxymethyl chitin with an adequate degree of substitution were effective toward controlling various tumor cells Nishimura et al. [4]. Unlike many other biological molecules, COSs could exert their biological activities following oral administration and effects are more or less similar to those of intraperitoneal injection. Moreover, Qin et al. [26] have demonstrated that water soluble COSs prepared with a mixture of tetramer and pentamer could inhibit growth of S180 tumor cells in mice after oral and intraperitoneal administration. Therefore, COSs and their N-acetylated analogues that soluble in basic physiologic environments could be considered good candidates to develop potential nutraceuticals.

The anti-tumor mechanism of these chitooligosaccharides was probably related to their induction of T-cell proliferation to produce the tumor inhibitory effects. Through analysis of the splenic cell changes in cancerous mice, Suzuki et al. [31] proved that the anti-tumor mechanism of chitooligosaccharides is to enhance acquired immunity by accelerating T-cell differentiation to increase cytotoxicity and maintain T-cell activity. Besides *in vitro* researches demonstrated that charge properties of the chitosan is also important for anti-cancer activity. Karagozlu et al. [38] and Huang et al. [18] studied the anti-cancer activities of differently charged chitooligosaccharide (COS) derivatives using four cancer-cell lines: HeLa, Hep3B, SW480 and AGS. Neutral red and MTT cell-viability studies suggested that highly charged COS derivatives could significantly reduce cancer-cell viability, regardless of their positive or negative charge. Furthermore, fluorescence microscopic observations and Western blotting studies confirmed that the anti-cancer effect of these highly charged COS derivatives were triggering of intrinsic apoptotic pathway.

Laminin are basal proteins in basal lamina and are known to correlation with metastasis of tumor cells. A peptide containing the Tyr-Ile-Gly-SerArg (YIGSR) sequence, corresponding to a partial sequence of laminin, inhibited angiogenesis and thus depressed tumor growth. Nishiyama et al. [39] prepared YIGSR-chitosan conjugate and assayed antimetastatic activity. The conjugate proved to have higher inhibitory activity against experimental lung metastasis of B16BL6 melanoma cells in mice than did the parent peptide.

Kong et al. [15] also investigated the MMP inhibition of chitin, water soluble chitosan and their carboxymethylated derivatives. In the research, chitosan and chitin, carboxymethyl-chitosan (CM-chitosan) and carboxymethyl-chitin (CM-chitin), were synthesized by means of carboxymethylation reaction. Their antioxidative and matrix metalloproteinase-2 and -9 (MMP-2 and -9) inhibitory effects were investigated in HT1080 human fibrosarcoma cells. The research suggests that, CM-chitosan and CM-chitin as a potent antioxidant and MMP inhibitor via alleviations of radical induced oxidative damage.

20.3 Anti-Cancer Activity as a Carrier

Chitosan and chitin are also used as a drug carrier to provide anti-cancer and anti-tumor chemotherapy which can improve drug absorption, stabilize drug components to increase drug targeting and enhance drug release. As a gene carrier, chitosan can

be used for DNA protection and effect the expression period of genes. It has been reported that the conjugates of some kinds of anti-cancer agents with chitin and chitosan derivatives display good anti-cancer effects with a decrease in side effects over the original form due to a predominant distribution in the cancer tissue and a gradual release of free drug from the conjugates. For instance, Doxorubicin is one of the most used anti-cancer agents whish can load in various polymeric or natural hydrogels [40, 41]. Cho et al. [57] prepared Doxorubicin hydrogel containing COS–DOX to obtain sustained-release profiles of doxorubicin from thermo-responsive and photo-crosslinkable hydrogels and examined its anti-cancer activity on human lung cancer adenocarcinoma cell line *in vitro* and *in vivo*. The research demonstrated that released fraction composed of doxorubicin and chitosan–doxorubicin oligomers showed comparable *in vitro* cytotoxicity to free doxorubicin. Besides, Doxorubicin hydrogels containing chitosan–doxorubicin conjugates showed superior *in vivo* anti-cancer effects in human solid tumors compared to free doxorubicin or hydrogel containing free doxorubicin after 3 weeks.

Moreover, doxifluridine and 1-β-D-,-D-arabinofuranosylcytosine (Ara-C) is a typical time-dependent anti-tumor agent. But major problem of Ara-C large dose required because of its resistance in the body. It can be quickly eliminated or inactivated [42]. Thus, various derivatives of Ara-C have been developed in attempts to improve efficacy. The one of the derivatives of Ara-C modified with cytidine deaminase. This modification catalyzes the transformation from an amino group to a hydroxyl group [43]. But the major problem of usage of the glu-Ara-C in cancer treatment is the release time of the drug. The prolonged release and inhibition of cytidine deaminase play an essential role in enhancement of the antitumor effect of Ara-C [44–46]. Chitin can conjugate with glu-Ara-C (Chi-glu-Ara-C) to extend the release time of the drug. The antitumor effect of Chi-glu-Ara-C was investigated by intraperitoneal administration to mice intraperitoneally inoculated with P388 leukemia [47].

Carboxymethyl chitin (CMC) is also used for drug delivery application. The hydrophobic anticancer drug 5FU was loaded into CMC nanoparticles via emulsion cross-linking method. Drug release studies showed that the CMC nanoparticles provided a controlled and sustained drug release at pH–6.8 [48]. Moreover CMC is a promising biopolymer for cancer diagnosis application also. Manjusha et al., developed novel folic acid conjugated CMC coordinated to manganese doped zinc sulphide (ZnS:Mn) quantum dot (FA-CMCS-ZnS:Mn) nanoparticles. The system can be used for targeting, controlled drug delivery and also imaging of cancer cells. The biocompatible FA-CMCS-ZnS:Mn was used on breast cancer cell line MCF-7 to study the imaging, specific targeting and cytotoxicity of the drug loaded nanoparticles. The results showed that the bright and stable luminescence of quantum dots can be used to image the drug carrier in cancer cells without affecting their metabolic activity and morphology [48].

For gene delivery to cancer cells, several polymers have been used as non-viral vectors [49–52]. Even if low solubility and transfection efficiency is limiting the usage of chitosan in gene therapy applications, chitosan is a promising candidate as a vector for gene delivery to cancer cells. Therefore, researchers have modified this polymer in order to get an effective transfection. For instance, Germershaus et al.

[53], Kean et al. [54] and Thanou et al. [55] quaternize the chitosan. According to their researches on quaternized chitosan derivatives properties such as their charge, solubility, plasmid interactions and transfections were increased. In 2011, Safari et al. [56] prepared N,N-diethyl N-methyl chitosan (DEMC) for gene delivery to human pancreatic cancer cells. According to their biological research and the mathematical modeling results both showed that after DEMC transfection, cancer cell fluorescence intensity and size has been changed.

20.4 Conclusion

Although the surgical methods are still promising and widely accepted treatments against defined cancer, non-surgical treatments against cancer have also received much attention with an aim to reduce and eliminate complications after surgical treatments. Therefore overcome side effect complication of the anti-cancer agent is the main scope of the cancer researchers. Recent studies of the chemical modification of chitin and chitosan are discussed from the viewpoint of biomedical applications because of their excellent biological properties such as biodegradation and biocompatibility in the human body. Such properties can be considered as valuable extensions of the use of chitin and its derivatives. These natural biological properties allows them to be valuable biomaterial for both anti-cancer therapy of human solid tumors and cancer diagnosis applications in various ways.

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Chapter 21
Meroterpenes from Marine Invertebrates: Chemistry and Application in Cancer

David M. Pereira, Patrícia Valentão and Paula B. Andrade

Abstract Nowadays alternative sources of new drugs for human therapeutics have been pursued. In the particular case of natural products, there is a trend for exploiting non-conventional sources of molecules, of which marine micro and macroorganisms are the best example. Regarding the marine environment, the majority of bioactive molecules are terpenes, which constitute a family of natural products with high chemical diversity. Among terpenes, the group of meroterpenes, molecules derived from mixed biosynthesis that include both a terpene and quinone/hydroquinone moiety, are particularly interesting because of their anticancer activity.

In this chapter we will address the chemistry, distribution and application of meroterpenes in cancer. The most relevant sponges, nudibranchs and ascidians-derived meroterpenes will be addressed and the underlying mechanism of action in cancer cells will also be discussed. Particular attention will be given to compounds that exert their effect by displaying pro-apoptotic properties.

Keywords Meroterpenes · Terpenes · Marine invertebrates · Ascidians · Sponges · Nudibranchs · Apoptosis · Anti-cancer · Prenylated quinone · Prenylated hydroquinone

Abbreviations

AIF	Apoptosis-inducing factor
APAF-1	Apoptotic protease activating factor 1
cAMP	Cyclic adenosine monophosphate
CHOP	C/EBP homology protein
COLO-205	Human colon adenocarcinoma cell line
DCF-DA	Dichlorofluorescein diacetate

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DISC	Death-inducing signaling complex
FADD	Fas-associated protein with death domain
H460	Human non-small cell lung carcinoma
Hep3B	Human hepatoma cell line
HL-60	Human promyelocytic leukemia cells
HT-29	Human colon adenocarcinoma cell line
HUVEC	Human umbilical vein endothelial cells
JNK	c-Jun *N*-terminal kinases
K562	Human chronic myelogenous leukemia cell line
KB	Mouth epidermal carcinoma cell line
L1210	Mouse lymphocytic cell line
L5178Y	Mouse lymphoma cell line
LnCaP	Human prostate carcinoma cell line
LTB4	Leukotriene B4
MCF-7	Human breast cancer cell line
miRNA	Micro RNA
NF-κB	Nuclear factor kappa B
OMM	Outer mitochondrial membrane
P-388	Murine lymphocytic leukemia cell line
PC-3	Human prostate cancer cell lines
PKC	Protein kinase C
ROS	Reactive oxygen species
SAR	Structure–activity relationship
SF-268	Human glioblastoma cell line
tBid	Truncated Bid
TNF	Tumor necrosis factor
TXB2	Thromboxane B2
U937	Human leukemic monocyte lymphoma cell line
Urb	Under-phosphorylated Rb protein
WHCO1	Human esophageal cancer cell line

21.1 The Sea as a Source of Bioactive Molecules

Nature has been a source of natural medicines since immemorial times. In recent History, the development of medicine resulted in the convergence to the use of isolated molecules within a formulation rather than chemically complex mixtures [1].

Marine environment remains to this day the most diversified ecosystem on Earth and also the least studied. Marine organisms have to cope with the several challenges that marine life represents, including low temperature and light availability and high pressures. If we consider that many marine organisms have primitive immune systems and soft bodies, it is understandable that what they lack in physical defenses is frequently balanced with remarkable chemical defenses. In this regard, it is

known that organisms with lower physical defenses, such as sponges and mollusks, are usually the ones with the most bioactive molecules. Marine natural products have proved to be an amazing source of chemical diversity and, consequently, many sea-derived molecules were shown to exhibit a number of different pharmacological activities and are currently under clinical trials to treat various pathological conditions, such as cancer, inflammation and allergy. Among the several classes of marine natural products described, the most relevant in a biological context are terpenes, alkaloids, peptides, sterols, among others. We will focus our attention in one particular case of terpenes derivatives: meroterpenes.

21.2 Chemistry of Meroterpenes

Meroterpenes are a class of natural products that exhibit a remarkable chemical diversity. This rich chemistry is a consequence of their mixed biosynthesis, as they are composed of an aromatic moiety/carbohydrate residue and also a terpenoid portion that can range from one to nine isoprene units. Prenylated quinone/hydroquinone derivatives are amongst the most numerous and widespread in marine environment. Their carbon skeletons originate from intra- and intermolecular cyclisation and/or rearrangement of terpene chains, to give unique polycyclic or macrocyclic structures, often having diverse functional groups [2, 3].

Quinones are a class of natural products widespread in Nature and often essential to life due to their pivotal role in oxidative processes in biological systems. Several subclasses of polyprenylated 1,4-benzoquinones and hydroquinones are known, including ubiquinones, plastoquinones and tocopherols. Ubiquinones include coenzyme Q10 and play an important role in the electron transport chain in mitochondrion [4, 5]. In photosynthetic organisms a similar role is played by plastoquinone, which is involved in the electron transport chain in photosynthesis [6, 7]. Finally, tocopherols are a group of antioxidant compounds present in most organisms, in which they are part of several defense mechanisms. In the particular case of animals, many tocopherols are used due to their vitamin E activity [8, 9]. Natural products displaying a terpene moiety and quinone/hydroquinone and that are not included in the above-mentioned classes are generically called meroterpenes [3].

From a structural point of view, meroterpenes range from simpler molecules with a prenyl unit linked to the hydroquinone unit to unique chemical scaffolds, which result from intra- and intermolecular ring closures and/or rearrangements of the terpene chains [3]. For example, many terpenylquinones can be sorted in distinct groups according to their clerodane, labdane or halimane-type decalin rings (Fig. 21.1). In the case of labdane-type decalin ring meroterpenes, in some molecules the ring can be further cycled, through a carbon or oxygen bridge between the decalin and benzo(hydroquinone) moiety, thus originating a fourth ring. Such is the case of corallidyctals A–D, metabolites isolated from the sponge *Aka corallifagum* [10] that display a 5-carbon fourth ring and whose biological properties will be discussed later. Compounds with a fourth six-membered ring are also known and in-

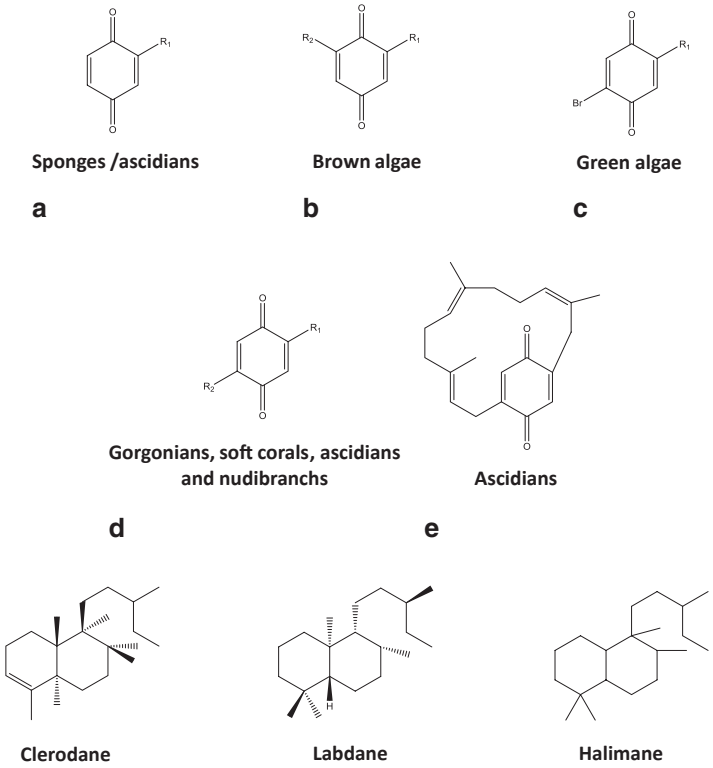

Fig. 21.1 Ring patterns according to taxonomical origin and clerodane, labdane and halimane-type decalin rings

clude ent-chromazonarol, 8-epichromazonarol, puupehenol and its cyanide ketone derivative, cyanopuupehenone [11–14]. These last two have been investigated for their anticancer activity [13, 15].

The rich chemistry of quinones/hydroquinoes has great influence in the biological properties of these molecules, being largely responsible for their anticancer capacity. Molecules with quinone/hydroquinone moieties are frequently involved in Michael-type 1,4-addition reaction, as well as redox cycling. In this way, reactive oxygen species (ROS) are produced, which, in turn, can exert a pro-oxidant effect in several biological targets and hence contribute to the anticancer activity of some of these molecules [16–18].

As it has been reviewed recently [19], until 2011 around 600 metoterpenes had been described, sorted as prenylated quinone/hydroquinone, tuloquinones/tulohydroquinones and naphthoquinones.

21.3 Distribution of Meroterpenes

Meroterpenes are not exclusive to marine organisms, being found also in many terrestrial species.

In the marine environment, the main sources of meroterpenes are brown algae, microorganisms, soft corals and marine invertebrates, such as sponges or ascidians [3].

Although natural products chemistry will never be a predictable area, the growing body of proof available today allows us to draw some trends regarding the chemistry of marine-derived meroterpenes. As it has been reviewed recently by Sunassee and Davies-Coleman [19], some common chemical traits can be found in marine meroterpenes according to their source. As so, compounds with the A ring substitution pattern are characteristic of sponges and ascidians, in opposition to rings B and C, which are almost exclusively found in brown and green algae, respectively (Fig. 21.1).

The discovery of anticancer compounds from ascidians is a perfect example of integrated disciplines in drug discovery and has resulted from a very simple observation: neoplasms had not been described in these organisms. The interest of ascidians as a source of bioactive molecules with application in cancer is easily pictured by the fact that, nowadays, 50 % of all compounds that reached clinical trials were from Ascidians, one of which has already entered the market (Trabactadin) [19].

21.4 Anticancer Activity of Meroterpenes

21.4.1 What to Target?

In the continuous search of anticancer activity, regardless of the source, one must bear in mind the biological targets that these compounds are expected to modulate in order to exert their effect. In the particular case of anticancer capacity, this means that both processes that constitute the onset of cancer, as well as the therapeutic targets, must be known.

Cancer is a complex genetic disease involving changes in metabolic pathways and gene expression. In the earlier years of cancer research, carcinogenesis has been primarily attributed to abnormalities in oncogenes and tumor-suppressing genes. However, lately other factors, such as micro RNA (miRNA), have been recognized for their importance in cancer onset and progression [20].

Carcinogenesis, a process by which normal cells are transformed into cancer cells, is a multi-step process that includes initiation, promotion and progression. In all cases, a number of epigenetic and genetic alterations affecting both oncogenes and tumor suppressor genes take place [21].

While the initiation stage can be inhibited by agents that either prevent the action of/inactivate carcinogens or act as antioxidants, later stages are prone to inhibition by drugs that suppress tumor growth or stimulate apoptosis. This is further sup-

ported by the fact that defects in apoptosis are common traits found across several types of cancer, being one of the critical steps in tumor genesis and resistance to therapy. For this reason, apoptotic pathways are important targets in cancer therapy [22]. Other mechanisms of action are important in cancer chemotherapy, including tubulin-targeting agents, vascular disruptive agents and protein kinase modulators; however, in this work we will focus in apoptosis.

When addressing apoptosis, two classic apoptotic pathways are usually considered: the intrinsic/mitochondrial pathway, in which mitochondrion plays a pivotal role, and the extrinsic/death receptors pathway that involves the activation of death receptors.

In the mitochondrial pathway, disruption/permeabilization of mitochondrial membranes takes place, thus causing the leakage of pro-apoptotic molecules, including cytochrome *c*, Smac/DIABLO, HtrA2/Omi, apoptosis inducing factor (AIF) and endonuclease G. In the cytoplasm, cytochrome *c* joins the apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9, thus forming a complex called apoptosome, which induces activation of the initiator caspase-9 and, subsequently, the effector caspase-3 [23]. The latter triggers some of the above referred hallmarks of apoptosis, like oligonucleosomal DNA fragmentation, blebbing and apoptotic bodies. At this point it is also important to refer the Bcl-2 proteins family, which is fundamental to the apoptotic process. Members of the Bcl-2 family can act either as anti-apoptotic or as pro-apoptotic factors. Proteins with anti-apoptotic activity are predominantly found within outer mitochondrial membrane (OMM) or in the endoplasmic reticulum, while the pro-apoptotic exist in the cytoplasm, endoplasmic reticulum or loosely bound to the OMM as monomers. The major anti-apoptotic factors are Bcl-2, Bcl-2-xL, Bcl-2-related gene A1 (A1) and Mcl-1 [24]. In what concerns to pro-apoptotic proteins, one further division includes effector proteins Bak, Bax and BH3-only proteins Bid, Bim, Bad. Unbalance of the amounts of pro-apoptotic/apoptotic proteins, either by increase of the former or decrease of the latter, can trigger the apoptotic process.

In the extrinsic pathway, mitochondrion is not the major player. Instead, pro-apoptotic membrane receptors belonging to the TNF superfamily are activated by ligands that include FasL, TRAIL and TNF-α, which activate Fas, DR4/DR5 and TNFR, respectively [25]. When activated, these receptors undergo conformational changes that lead to the assembly of death-inducing signaling complex (DISC). After this point, the receptors recruit Fas-associated death domain (FADD) and procaspase-8. The association of procaspase-8 to DISC leads to its autoproteolytic cleavage and hence the two subunits that form the active enzyme are released [26]. After this event, two different routes can be taken according to the cell type: in type I cells, caspase-8 activity *per se* is enough to activate effector caspase-3, -6 and -7, which subsequently triggers the apoptotic response. In type II cells, caspase-8 cleaves Bid, thus originating truncated Bid (tBid), which, in turn, promotes the oligomerization of Bax/Bak present in the mitochondrial membrane. These proteins will then cause the release of cytochrome *c* and smac/DIABLO that activate the intrinsic pathway and increase even more the active caspase-8 by a positive feedback [25–27].

21.4.2 Meroterpenes from Ascidians

When addressing bioactive molecules from Ascidians, a chemical criterion can be taken: nitrogen-bearing molecules or non-nitrogen-bearing ones, with prenylated quinones/hydroquinones being the most expressive manifestation of the latter. In a general way, most of the secondary metabolites found in ascidians are nitrogen-bearing molecules, either alkaloids or peptides. However, in some taxonomic groups of Ascidians, such as those of the genus *Aplidium*, several meroterpenes have been found, namely diprenyl-quinone/hydroquinone, chromene/chromane derivatives [28] and cyclofarnesylated hydroquinones/quinones [29, 30]. Diprenylquinones or diprenylhydroquinones constitute the majority of compounds and display a linear side chain of neryl derivatives, such as glabruquinone B and verapliquinone B/D.

The first compound described was geranylhydroquinone (Fig. 21.2), a rather simple compound isolated from an unknown species of the genus *Aplidium* and later found across several species of the genus. This metabolite was shown to be effective against several leukemia cell lines and tumor development models in animals. After this discovery, several linear diprenylquinones/hydroquinones have been described [31–33].

Methoxyconidiol (Fig. 21.2) has been isolated from *Aplidium* aff. *densum* [34]. The antimitotic effect of methoxyconidiol in eukaryotic cells was evaluated by using sea urchin early embryos [35]. Fertilized eggs from two species, *Sphaerechinus granularis* and *Paracentrotus lividus*, were used. Results showed that at a concentration of 20 μM methoxyconidiol was able to disrupt M-phase progression and block cytokinesis with no effect on DNA replication nor S phase being found. Mitotic spindle establishment was compromised, an effect probably resulting from a perturbation of microtubule dynamics. From a mechanistic point of view, methoxyconidiol-treated eggs had cyclin B/Cdk1 activated, thus affecting the outcome of M-phase. This result unveils the potential use of this compound in cancer cells, although further studies are required.

Cordiachromene A (Fig. 21.2), a chromenol isolated from *Aplidium antillense*, was evaluated for its capacity to affect P-388 leukemia cell line and KB mouth carcinoma cell line. Although the mechanism of action was not pursued, strong cytotoxicity was found (IC_{50} values of 0.14 and 2 μM, respectively).

Longithorone A (Fig. 21.2), obtained from *Aplidium longithorax* [36], is a very interesting compound due to its unusual macrocyclic nature, which comprises a farnesyl moiety that bridges the C2 and C5 positions present in the benzoquinone moiety. The result is a macrocycle with two units that yield the carbocycle. Apart from this remarkable chemistry, the anticancer activity of longithorone A has been shown to be moderate, at least against the cancer cell lines tested until now, namely the P-388 murine leukemia cell line, with an ED_{50} around 16 μM. Another compound of this series, the chemically simpler longithorone J (Fig. 21.2), has been isolated from the same species, but collected from a distinct geographical location [37]. This less complex compound displays lower toxicity (IC_{50} value c.a. around four times higher), providing an important information regarding structure–activity relationship (SAR) for future studies.

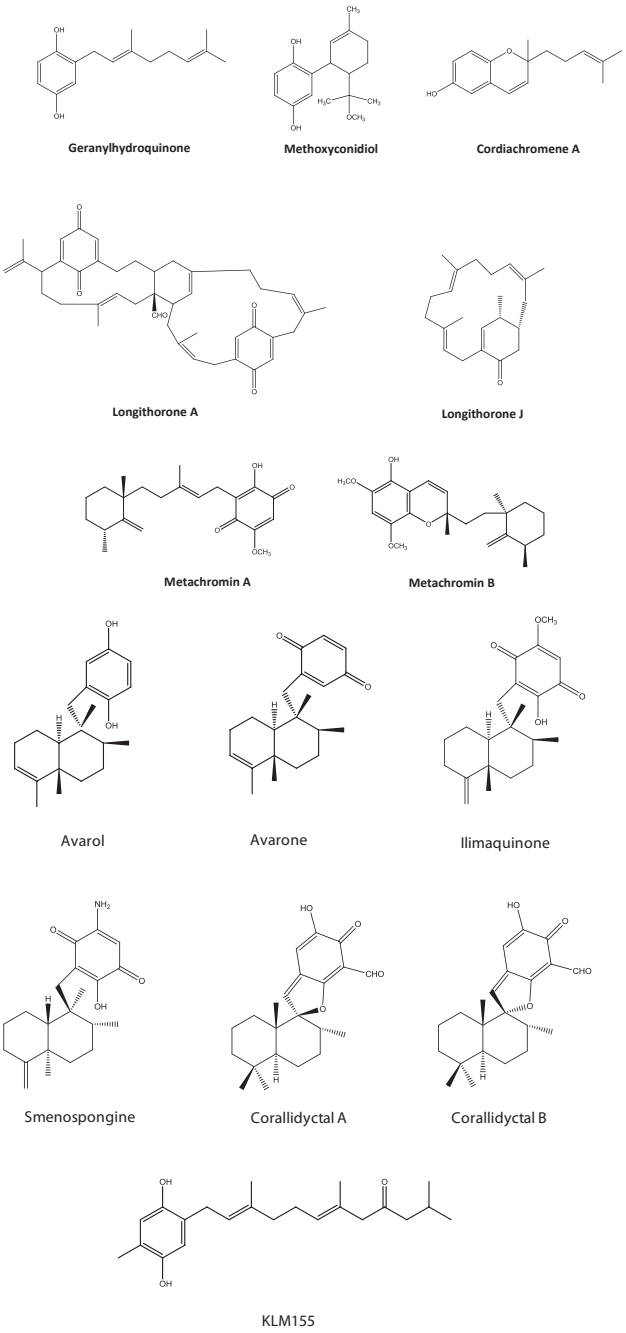

Fig. 21.2 Some of the meroterpenes discussed in this work

21.4.3 Meroterpenes from Sponges

Although brown algae rank first when it comes to the number of described meroterpenes, sponges surpass them concerning to meroterpenes whose anticancer activity has been reported.

Sponges of the *Ircinia* genus are a remarkable source of polyprenylated benzoquinones and benzohydroquinones. *Ircinia spinulosa*, *Ircinia muscarum* and *Spongia officinalis* are the source of the compounds 2-heptaprenylhydroquinone-4-sodium sulfate, 2-octaprenylhydroquinone-4-sodium sulfate, 2-tetraprenylhydroquinone, 2-hexaprenylhydroquinone, 2-heptaprenylhydroquinone and 2-octaprenylhydroquinone. These compounds were evaluated for their ability to inhibit CDC25A phosphatase, a cyclin-dependent kinase that plays a pivotal role in cell cycle progression. All the compounds revealed to be able to inhibit this enzyme, 2-hepta and 2-octaprenylhydroquinone-4-sodium sulfates being the most potent ones, with IC_{50} values of 5 μM [38]. More recently, 2-heptaprenylhydroquinone and 2-octaprenylhydroquinone, together with the new compound hydroxy-nonaprenylhydroquinone, were isolated from the Mediterranean sponge *Sarcotragus spinosulus* and displayed cytotoxicity against the chronic myelogenous leukemia cell line K562. Effect on cell viability and proliferation was demonstrated, however the precise mechanism of action is yet to be addressed [39].

Although meroterpenes from sponges can, in a general way, be sorted in groups sharing some chemical traits, some exceptions can be pointed out. Such is the case of metachromins A and B (Fig. 21.2), a series of compounds isolated from *Hippospongia metachromia*. When tested against the mouse lympohocytic cell line L1210 these compounds displayed anti-tumor activity (IC_{50} values of 6.7 and 4.5 μM, respectively) [40]. In subsequent studies, metachromins C–H were evaluated against the same cancer cell line, IC_{50} values corresponding to 5.6, 7.5, 0.56, 1.4, 2.8 and 4.7 μM, respectively [41, 42]. More recently, metachromins I–T were described, but their activity against cancer cells was only moderate [43–46]. In 2011, metachromins U–W were reported, the V compound being the most potent, exhibiting IC_{50} values against human glioblastoma (SF-268), human breast cancer (MCF-7), human non-small lung carcinoma (H460) and human colon adenocarcinoma (HT-29) cell lines of 5.1, 3.2, 5.1 and 10 μM, respectively [47]. In subsequent studies by Shen et al., the related compounds hippochromin A and B were isolated from a Taiwanese sponge, *Hippospongia metachromia* [48]. The derivatives hippochromin A diacetate and metachromin B monoacetate exhibited potent anti-proliferative effect against human colon (COLO-205) and KB tumor cells, although the mechanism of action was not investigated [48].

Avarol is a sesquiterpene hydroquinone isolated from the sponge *Dysidea avara*, avarone being the corresponding quinone compound (Fig. 21.2). Anticancer activity of avarol was addressed for the first time using the L5178Y mouse lymphoma cell system *in vitro*, where the compound reduced cell growth to 50% at a concentration of 0.9 μM. The authors also showed that incubation with avarol interfered with mitosis, an effect attributed to the prevention of telophase. Alterations of the permeability properties of the cell membrane were also suggested [49]. In subsequent

studies the same group demonstrated that the main mechanism responsible for the biological activity of avarol was related with its ability to generate ROS, thus leading to DNA break and subsequent apoptosis [50]. It was also shown that these compounds were not mutagenic agents, being even able to reduce the mutagenic effect of benzo[a]pyrene, an activity related with their ability to inhibit benzo[a]pyrene monooxygenase [51, 52].

Ilimaquinone (Fig. 21.2) was one of the compounds isolated from *Dactylospongia elegans*. In a panel consisting of four cancer cell lines and one normal cell line, ilimaquinone was the compound displaying highest activity. Earlier studies showed an interesting activity of this compound, namely its ability to induce vesiculation and breakdown of Golgi membranes, thus being an inhibitor of protein synthesis [53]. In fact, this mechanism has been extensively studied and, nowadays, ilimaquinone is a well-known inhibitor used in biochemical studies [54–59]. Curiously, it seems that the anticancer activity of ilimaquinone is not related with its capacity to induce Golgi vesiculation.

Proliferation assays showed that ilimaquinone induced a concentration-dependent anti-proliferative effect in several types of cancer cell lines, including the prostate cancer cell lines PC-3 and LNCaP, non-small cell lung cancer cell line A549 and human hepatoma cell line Hep3B. From a mechanistic point of view, ilimaquinone induced a time-dependent increase of G1 phase arrest and a subsequent increase of hypodiploid sub-G1 phase of the cell cycle. Incubation with ilimaquinone resulted in up-regulation and nuclear translocation of growth arrest and C/EBP homology protein (CHOP), which is nowadays a well-known pro-apoptotic stimulus that frequently arises from endoplasmic reticulum stress [60, 61]. This was confirmed by showing that ilimaquinone-mediated anti-proliferative effect was significantly reduced in antisense CHOP overexpressing cells. Ilimaquinone also inhibited DNA binding of nuclear factor κB (NF-κB), though this effect was not related to the anticancer effect. In summary, it is suggested that ilimaquinone has anti-proliferative effect through the G1 arrest of the cell cycle and the up-regulation and nuclear translocation of CHOP [62].

Smenospongine (Fig. 21.2) is a sesquiterpene aminoquinone isolated from the sponge *D. elegans* [63]. Initial studies showed that the compound induced G1 phase arrest in the human chronic myelogenous leukemia cell line K562, an effect that was not found in the human promyelocytic leukemia cell line HL-60 and human leukemic monocyte lymphoma cell line U937 after incubation with the compound for 24 h. Instead, HL60 and U937 cells presented a sub-G1 accumulation, a trait compatible with apoptotic cells. In K562 cells, smenospongine up-regulated p21, a protein important in cell cycle regulation. In addition to this effect, exposure to smenospongine at 15 μM inhibited phosphorylation of Rb in K562 cells after 48 h of incubation, which resulted in increased levels of under-phosphorylated Rb protein (URb) [63]. Rb is located downstream of p21 and has been proved to regulate the G1/S progression.

Recently, further studies were developed in solid tumors. Smenospongine was able to inhibit proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC). In a panel consisting of several cancer cell lines, smenospongine inhibited the growth of 39 human solid cancer cells *in vitro*, with a

mean Log GI_{50} value of −5.55 [64]. The precise mechanism and molecular targets involved leading to growth inhibition remain unknown.

Corallidyctals display the above-mentioned labdane ring. Corallidyctals A–D (Fig. 21.2) are metabolites isolated from the sponge *A. corallifagum* [10] with a 5-carbon fourth ring. Compounds A and B revealed to inhibit PKC with IC_{50} values below 30 μM, an activity that was not extended to other cAMP-dependent kinases, thus showing the selectivity of these molecules. Other studies, aiming to evaluate the effect of these compounds in other molecular targets, could further confirm the potential application of these compounds.

21.4.4 Meroterpenes from Nudibranchs

The Arminacea species *Leminda millecra* is the source of several triprenylated toluquinones and toluhydroquinones that received the names KLM153, KLM154, KLM155 (Fig. 21.2), KLM156, KLM157 and KLM159 [65]. These compounds were studied for their ability to affect the viability of the esophageal cancer cell line WHCO1 [18], with preliminary results showing a range of IC_{50} values from 9.5 μM in the case of KLM155 to 83 μM for KLM154. For this reason, the former was selected for further mechanistic studies. Morphological traits of apoptosis were found and the presence of this process of cell death was confirmed by evaluating the activity of caspase-3/7. A G2/M cell cycle arrest was also found by flow cytometry. Incubation with KLM155 was shown to increase the intracellular generation of ROS, as assessed by the dichlorofluorescein diacetate (DCF-DA) assay. When several concentrations were tested, a positive correlation between the compound's ability to generate ROS and its cytotoxicity was found [18]. Due to the well-established connection between the presence of ROS and activation of mitogen-activated protein kinase pathways, the authors pursued the effect of KLM155 at several concentrations in the levels of phosphorylated p38, phosphorylated ERK, phosphorylated JNK and phosphorylated c-Jun. Phosphorylated ERK levels were increased in a concentration-dependent manner, while a minor induction of phosphorylated p38 was detected. Phosphorylated JNK levels increased only at the highest concentration, while a marked increase in c-Jun levels was detected [18]. The role of ROS and mitogen-activated protein kinase pathways in KLM155-mediated pro-apoptotic effect was further demonstrated by showing that ROS scavengers and a JNK inhibitor blocked KLM155-induced apoptosis [18].

21.5 Future Directions

The most optimistic numbers suggest that we have explored less than 5% of sea environment [66]. Given the exciting discoveries already made, particularly regarding chemical diversity and biological potential, the future seems promising. However, from a biomedical point of view, several questions are yet to be answered.

One of the topics in discussion nowadays is the true origin of many of the metabolites found in marine organisms. Across several taxa, notably Porifera, microorganisms can account to as much as 40 % of sponging biomass and many metabolites have already been proved to be, in fact, of microbial origin. This is believed to be a very common trait in marine organisms and the next few years will probably highlight the importance of symbiosis in hotspots of bioactivity.

Another important question that should be addressed is related with the degree of detail that we have regarding the biological effects displayed by marine-derived molecules. Although screening assays are commonly employed to screen for bioactive molecules, future trends should include more detailed studies addressing the underlying mechanism and cellular and molecular targets.

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Chapter 22
Marine Sponge Sesterpenoids as Potent Apoptosis-Inducing Factors in Human Carcinoma Cell Lines

Giuseppina Tommonaro, Salvatore De Rosa, Rosa Carnuccio, Maria Chiara Maiuri and Daniela De Stefano

Abstract Cancer is a leading cause of death in industrialized countries. Although mortality rates have declined in recent years owing to earlier detection and more options in treatment, most cancers remain incurable. Mutations and epigenetic alterations of cancer genes promote the malignant transformation of cancer progenitor cells by disrupting key processes involved in normal growth control and tissue homeostasis. In addition, tumor development and progression are also dependent on the microenvironment surrounding the malignant cell. Conventional chemotherapy for cancer utilizes cytotoxic agents that elicit their therapeutic effect partly through apoptosis induction. Moreover, overexpression of anti-apoptotic proteins in cancer cells can inhibit programmed cell death and engender chemoresistance. Therefore, chemotherapeutic interventions fail to determine complete health in patients. Conversely, drugs developed more recently, known as 'targeted therapy', may show less unwanted toxicity, although they are generally cytostatic. Thus, there is an urgent need to develop new effective drugs. Natural products play a dominant role in the discovery of lead compounds for the development of drugs to treat human diseases. Terpenoids are by far the largest class of natural products. Within this class of compounds, the sesterterpenes form a rare group of isoprenoids, which occur in widely differing source. Particularly, marine organisms have provided a large number of sesterterpenoids, possessing novel carbon skeleton and a wide variety of biological activities. It has been largely reported that the anti-inflammatory activity is the most relevant among the biological activities observed for marine sesterterpenoids.

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Herein, we describe the link between chronic inflammation and cancer, and the more significant biologically active sesterterpenoids from marine organisms, grouped in a biogenetic sequence. Moreover, natural products that do not contain 25 carbon atoms but are obviously sesterterpene derivatives are also included. The high potential for some of these products suggested that they could be developed as drugs for the treatment of inflammation and cancer-related inflammation.

Keywords Cancer · Chronic inflammation · Marine sponges · Terpenoids · Sesterterpenoids

22.1 Introduction

22.1.1 Cancer and Inflammation: Advances on an Old New Link

Cancer remains a global health problem and a major cause of death worldwide. Statistical analysis published by the International Agency for Research on Cancer from the World Health Organization reveals that if the estimated trends continue, the incidence of all cancer cases will raise from 12.7 million new cases in 2008 to 21.2 million by 2030 [1]. Cancer is a multistep process characterized by the progression of a transformed cell through the acquisition of cancer fitness traits that are the signature of the malignant cells. Hanahan and Weinberg [2] have identified the six hallmarks of cancer. The first four, (a) sustaining proliferative signaling, (b) evading growth suppressors, (c) resisting cell death, and (d) enabling replicative immortality, represent intrinsic modifications of the cell replicative ability, depending in part on successive somatic mutations or epigenetic modifications in the evolving tumors. The other two, (e) angiogenesis induction and (f) invasion and metastasis activation are based on the ability of a successful tumor to allow growth and dissemination of the malignant cells by modifying the tumor microenvironment, providing the conditions for accessing to nutrients and inducing tissue rearrangement. Moreover, they indicate new emerging hallmarks associated to the capability of cancer cells to evade immunological destruction as well as to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. In addition, the cancer cells present also two enabling characteristics: the development of genomic instability (which generates mutations that can orchestrate hallmark capabilities); and the tumor-promoting consequences of inflammatory responses [2]. These considerations point the attention on the environment in which the tumor develops [3]. At first, the malignant lesion, keeps the functional and morphologic characteristics of the tissue from which it is originated [4]. However, to successfully grow the malignant cells need to escape the microenvironment restriction on cancer progression or profit of the surrounding environment to develop [5].

There is accumulating evidence that although resistance to apoptosis is a hallmark of cancer and can cause resistance to drug treatment, cancer cells are typically 'addicted' to a fairly small number of anti-apoptotic proteins for their survival [6]. Most prominent among these are the anti-apoptotic BCL-2 family members, inhibitor of apoptosis proteins (IAPs) and the caspase 8 inhibitor FLIP. Mutations, amplifications, chromosomal translocations and overexpression of the genes encoding these proteins have been associated with various malignancies and linked to resistance to chemotherapy and targeted therapies. Moreover, these genes are transcriptional targets for prosurvival transcription factors such as nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3). During tumorigenesis, these transcription factors are activated by oncogenic mutations in kinases that regulate upstream prosurvival signalling pathways [7]. Complex molecular and cellular mechanisms are involved in each of the cancer development step. Just as inactivation of the p53 pathway may be universal in the neoplasia, the activation of the NF-κB pathway may, conversely, be frequent in carcinogenesis, and a requirement for inflammation and promotion [8]. Among the key orchestrators at the intersection of the intrinsic and extrinsic pathways include transcription factors is NF-κB, modulating the inflammatory response through soluble mediators (cytokines, chemokines) and cellular components (e.g. tumor-associated macrophages), promoting tumorigenesis. NF-κB aids in the proliferation and survival of malignant cells, promotes angiogenesis and metastasis, subverts adaptive immunity, and alters responses to hormones and chemotherapeutic agents. Importantly, however, in all malignancies, NF-κB acts in a cell type-specific manner: activating survival genes within cancer cells and inflammation-promoting genes in components of the tumor microenvironment [9].

22.1.2 Natural Anti-tumoral Drugs

Natural products play a dominant role in the discovery of leads for the development of drugs to treat human diseases. It should be realised that the bioactive compounds, which are synthesised in nature to protect a given organism, had been selected from a wide variety of possibilities and were under the pressure of evolution for several hundreds of million years to reach an optimal activity.

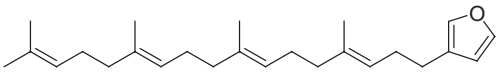
The terpenoids (isoprenoids) are a class of secondary metabolites that may be formally considered to be constructed from the five-carbon isoprene unit [10]. The terpenes have been classified primarily on the basis of their number of isoprene units (monoterpenes C_{10}, sesquiterpenes C_{15}, diterpenes C_{20}, sesterterpenes C_{25}, triterpenes C_{30} and carotenoids C_{40}) and then on their carbon skeleton. The monoterpenes, sesquiterpenes, diterpenes and sesterterpenes contain the isoprene units linked head to tail, while the triterpenes and carotenoids contain two C_{15} and C_{20} units, respectively linked in the middle tail to tail. Several thousand terpenes have been isolated and they are by far the largest class of natural products. Within this class of compounds,

the sesterterpenes form a rare group of isoprenoids, which occur in widely differing source and have been isolated from terrestrial fungi [11], plants [12] and insects [13] as well as from marine organisms [14, 15], mainly from sponges and nudibranches. Marine organisms have provided a large number of sesterterpenoids, possessing novel carbon skeletons different from those present in terrestrial species. Several sesterterpenoids isolated from marine organisms have shown a wide variety of biological activities.

The aim of this contribution is to review the more significant sesterterpenoids from marine organisms, which show biological activities, emphasising those compounds with a potential industrial application. In this review the structures will be covered in a biogenetic sequence and also include natural products that do not contain 25 carbon atoms but are obviously sesterterpene derivatives, such as degraded sesterterpenes with 21–24 carbon atoms, and alkylated sesterterpenes with 26–27 carbon atoms. In addition, some of our own results on anti-inflammatory activity of marine metabolites have been reported. Likewise, some data on the different directions that can be taken to obtain secondary metabolites have been included in the final section to suggest alternative production of marine metabolites and to highlight the possible future importance of marine biotechnology in the production of large quantities of marine secondary metabolites.

22.2 Linear Sesterterpenoids

Marine organisms are a rich source of acyclic sesterterpenoids. Furanosesterterpenes are a prominent class of secondary metabolites mainly isolated from marine sponges of the family Thorectidae. The basic component of this interesting group is furospinosulin-1 (**1**), first isolated from *Ircinia spinosula* [16] and later from several Dictyoceratida species, including *Fasciospongia* sp. (South Africa) [17], the *Thorecta* sp. (Australia) [18] and *Spongia idia* (California) [19] that contains also the oxidized derivative, idiadione (**2**).



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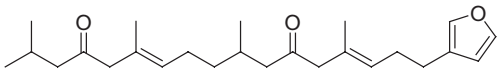

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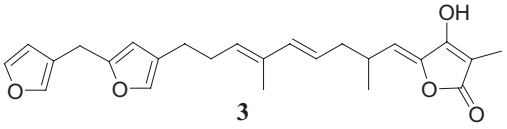
Compounds **1** and **2** showed activity at 10 µg/ml [19] in the *Artemia salina* bioassay, which gives results that correlate well with cytotoxicity in cancer cell lines such as human epidermoid carcinoma KB, murine lymphoma P388 [20], mouse lymphoma L5178y and murine lymphoma L1210 [21]. Fusetani et al. [22] reported the isolation of two compounds, dehydroderivative of ircinin (**3**) and an isomer of variabilin (**4**) from the sponge *Cacospongia scalaris* (Japanese). Both compounds inhibited the cell division of fertilised starfish eggs at a concentration of 1.0 µg/ml. This assay is a variation on the test with sea urchin embryos, which can detect DNA and RNA synthesis inhibitors, microtubule assembly and protein synthesis inhibitors, the common leads for the development of anticancer drugs [23].



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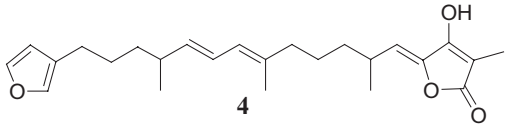

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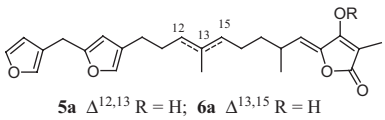
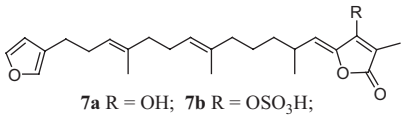
From sponges of the genus *Ircinia*, (Northern Adriatic Sea—Italy), were isolated ircinin-1 (**5a**), ircinin-2 (**6a**), variabilin (**7a**), and the corresponding sulphates **5b–7b**. The sulphated derivatives **5b–7b** showed greater activity in the *A. salina* bioassay (LC_{50}: 1.72 and 1.22 µg/ml, ircinins and variabilin sulphated, respectively), than the corresponding non-sulphated compounds **5a–7a** (LC_{50}: 2.38, 2.73 and 2.10 µg/ml), being less toxic in the fish (*Gambusia affinis*) lethality test (LC_{50}: **5b–6b** 5.09, **7b** 9.50, **5a** 3.35, **6a** 3.03 and **7a** 3.15 µg/ml) [24]. It has been demonstrated that ircinin-1 inhibited human melanoma cell growth, through a G1 cell cycle arrest associated with a marked decrease in the protein expression of D-type cyclins. Ircinin-1 treatment also resulted in induction of p53-independent apoptosis, characterized by DNA fragmentation, alternated ratio of Bax/Bcl-2, cleavages of poly(ADP-ribose) polymerase, cytochrome c release, cleavage activations of caspase-3 and -9, upregulation of Fas and Fas-L, and decrease expression of the inhibitor of apoptosis protein (IAP)-1 [25].

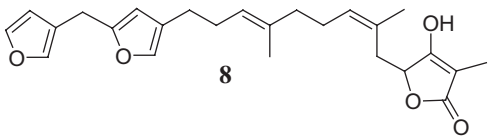
Moreover, three metabolites of this class of compounds, spongionellin (**8**), dehydrospongionellin (**9**) [26] and okinonellin B (**10**) [27], were isolated from a sponge of genus *Spongionella* (Japan) and were shown to inhibit the cell division of fertilised starfish eggs at 2.0–5.0 µg/ml.



5a $\Delta^{12,13}$ R = H; **6a** $\Delta^{13,15}$ R = H
5b $\Delta^{12,13}$ R = SO_3H; **6b** $\Delta^{13,15}$ R = SO_3H



7a R = OH; **7b** R = OSO_3H;



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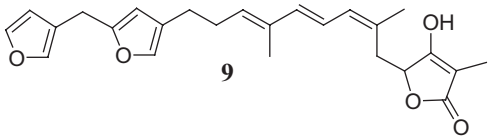

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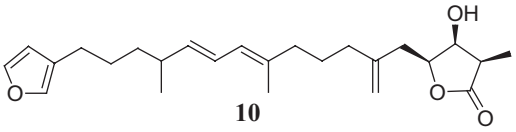

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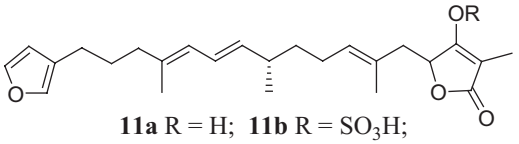
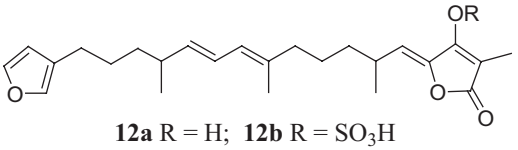


11a R = H; **11b** R = SO_3H;



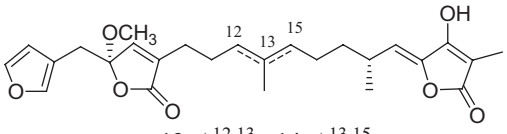
12a R = H; **12b** R = SO_3H

Palinurin (**11b**) and fasciculatin (**12b**) sulphates, together with palinurin (**11a**) and fasciculatin (**12a**), were isolated from the Tyrrhenian sponges *I. variabilis* and *I. fasciculata,* respectively. The sulphated derivatives were more active in *A. salina* bioassay (LC_{50}: **11b** 3.04, **12b** 1.44, **11a** 7.56, **12a** 2.03 μg/ml) and less toxic in the fish lethality test (LC_{50}: **11b** 2.30, **12b** 2.20, **11a** 1.67, **12a** 1.04 μg/ml) [28]. Recently, it has been demonstrated that palinurin is capable of inhibiting the activity of glycogen synthase kinase 3 beta (GSK-3β) through a non-ATP/substrate competitive mechanism, probably by binding to an allosteric site located at the N-terminal lobe of the enzyme [29]. This novel allosteric mechanism of action confers palinurin a high degree of selectivity

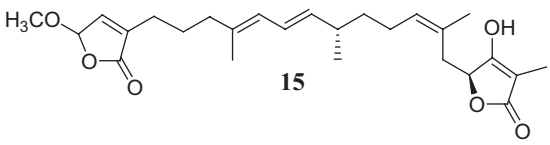
towards GSK-3β, which is implicated in the regulation of cell cycle, proliferation, apoptosis and its de-regulation has been described in various human cancers [30].

Sponges of the genus *Sarcotragus* are a rich source of sesterterpenes with both conjugated and nonconjugated tetronic acid moieties [29, 31]. Sarcotins G (**13**) and H (**14**) are closely related to ircinin-1 (**5a**) and -2 (**6a**), except that a furan ring is replaced by a 5-methoxy-2(5H)-furanone moiety. These compounds showed cytotoxic activity with IC_{50} values 5.0–10.0 μg/ml against five human tumour cell lines (lung carcinoma A549, ovarian carcinoma SK-OV-3, skin carcinoma SK-MEL-2, central nervous system carcinoma XF498 and colon carcinoma HCT15) [31], while sarcotin F (**15**), an oxidised derivative of palinurin (**10a**) showed less cytotoxic activity (IC_{50} 7.6–24.1 μg/ml) in the same panel of cell lines [31].

From the sponge *Fasciospongia cavernosa*, collected in the bay of Naples (Italy), were isolated two linear sesterterpenes, cacospongionolide D (**16**) with a γ-hydroxybutenolide moiety, and luffarin-V (**17**) with two γ-butenolide functionalities in the molecule. Cacospongionolide D showed a potent activity (LC_{50} 0.1 μg/ml) [32] in the *A. salina* bioassay, and a moderate ichthyotoxicity to *G. affinis* (LC_{50} 2.54 μg/ml) in the fish lethality assay. Luffarin-V was less active (LC_{50} 1.72 μg/ml) in the *A. salina* bioassay. Cacospongionolide D is an isomer of cacospongionolide (**136**) that has been shown to inhibit significantly cell proliferation as well as to induce apoptosis in several human cancer cell lines [33].



13 $\Delta^{12,13}$; **14** $\Delta^{13,15}$



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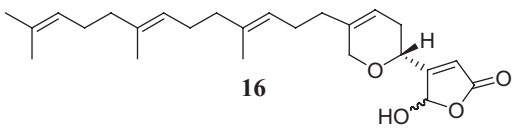

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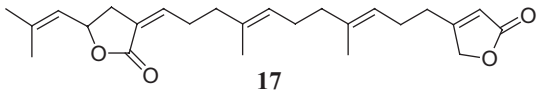

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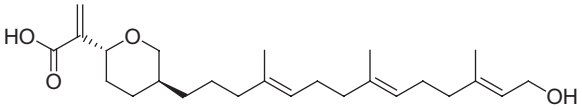
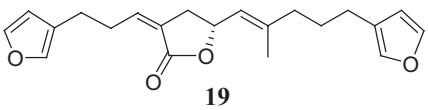


18

An unusual sesterterpenoid acid (**18**) with a tetrahydropyran ring was isolated from the Indonesian sponge *Hippospongia* sp. that inhibited the human Ras-converting enzyme (hRCE), with an IC_{50} value of 10 μg/ml [34]. The Ras signalling pathway has emerged as an important target for the development of anticancer drugs. Ras is a membrane bound G protein controlling cell differentiation and proliferation. Mutated Ras genes, encoding activated Ras proteins, have been identified in approximately 30% of all human cancers. The approaches to therapeutic intervention in the Ras signalling have focussed on the development of inhibitors that block the lipid modification needed for proper Ras membrane localisation (farnesyl transferase inhibitors) or to find inhibitors of proteolytic processing of Ras (RCE protease inhibitors) [35].

In the sponge of the genus *Spongia* have been recovered a large amounts of linear, closely related difuranoterpenes containing 21 carbon atoms. The first two C_{21} compounds, ninetin (**19**) and dihydroninetin (**20**) isolated from *S. nitens* [36] possess a γ-lactone ring in the central part of the chain. *S. officinalis* and *Hippospongia communis* contain several C_{21} furanoterpenes in large amounts [37, 38]. From a specimen of *S. officinalis*, collected in the Northern Adriatic Sea, together with furospongin-2 (**21**), previously reported from the same sponge collected in the Tyrrhenian Sea [39], its three isomers (**22–24**) were isolated. Compounds **21–24** showed high activity (LC_{50} 0.09–1.60 μg/ml) in the *A. salina* bioassay [40].



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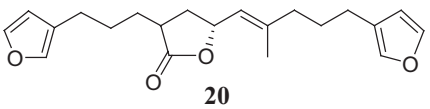

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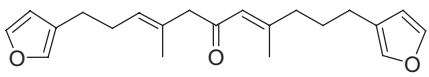

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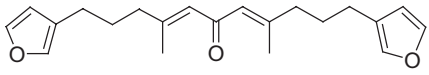

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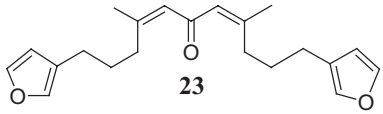

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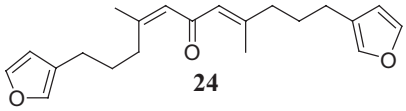
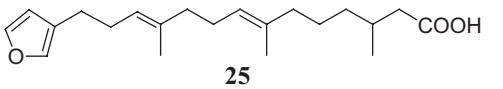


24

The C_{21} furanoterpene acid **25** related to variabilin (**7a**) showed antiviral (HSV and PV1) and cytotoxic activities comparable with that of variabilin [41]. Three chlorinated C_{24} norsesterterpenes (**26–28**), closely related to ircinin-1 (**5a**) and ircinin-2 (**6a**), were isolated from the sponge *I. oros*, collected in the Northern Adriatic Sea [59]. The norsesterterpenes, **26–28**, showed less cytotoxic activity (LC_{50} 8.2–8.6 μg/ml) than ircinin-1 (**5a**) and ircinin-2 (**6a**) (LC_{50} 2.4 and 2.7 μg/ml), in the *A. salina* bioassay [42].

The norsesterterpenoids, sarcotins N (**29**), O (**30**) and *ent*-kurospongin (**31**), isolated from *Sarcotragus* sp., showed moderate cytotoxicity, with IC_{50} values 3.0–30.0 μg/ml, against a panel of five human tumour cell lines (A549, SK-OV-3, SK-MEL-2, XF498 and HCT15) [43].



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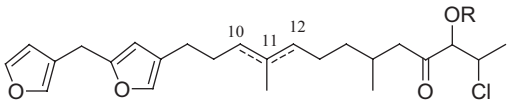
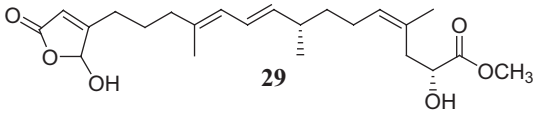


26 $\Delta^{10,11}$ R = H; **27** $\Delta^{10,11}$ R = Ac;
28 $\Delta^{11,12}$ R = H



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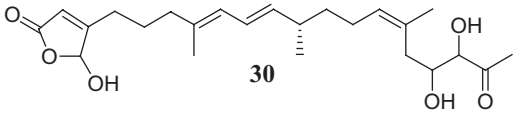

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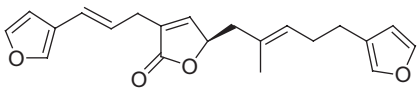
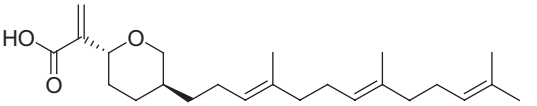


31

Three unusual norsesterterpenes, rhopaloic acids A–C (**32–34**), isolated from a species of *Rhopaloeides* (Japan) selectively inhibited the gastrulation of fertilised eggs of the Starfish *Asterina pectinifera* showing a MIC (minimum inhibitory concentrations) values of 0.5, 0.4 and 0.2 μM, respectively [44]. Rhopaloic acid A, exhibited also potent cytotoxicity against human myeloid K562 cells (IC_{50} 0.04 μmol/l), human leukaemia MOLT4 cells (IC_{50} 0.05 μmol/l), and L1210 cells (IC_{50} 0.10 μmol/l) [45]. Furthermore, rhopaloic acids A–C together with rhopaloic acids D–G (**35–38**) were isolated from an Indonesian *Hippospongia* sp. Rhopaloic acids A–E showed a RCE protease inhibitory activity with IC_{50} values of ≈10 μg/ml [45]. Compounds **32–36** were more active in the cell-based assay against colon tumour LoVo cells

($IC_{50} \approx 1$ µg/ml) than in the enzyme assays, suggesting that the cytotoxic effect of the compounds might result from hitting more than one molecular target [45].

Muqubilone (or aikupikoxide A) (**39**), a norsesterterpene peroxide acid, isolated from the sponge *Diacarnus erythraeanus* (Red Sea) showed cytotoxic activity with an $IC_{50} > 1$ µg/ml, against two type of human cancer cells (A549 and HT29) [46].



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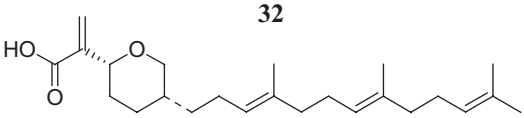

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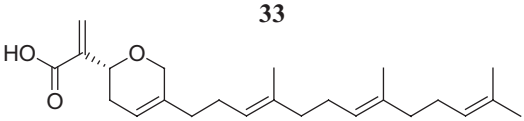

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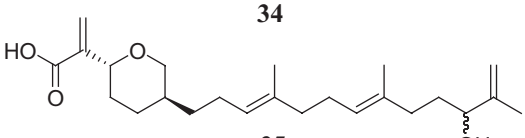

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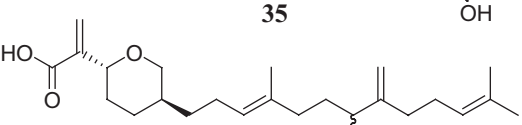

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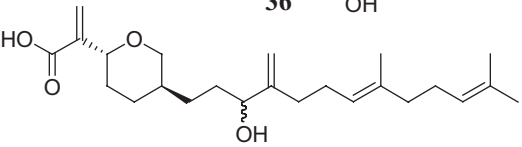

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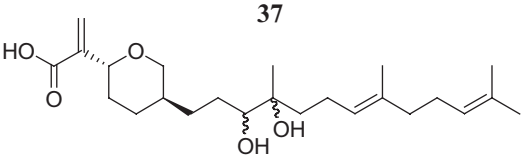

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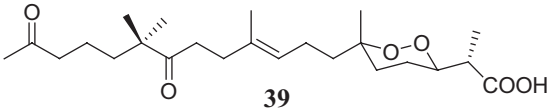
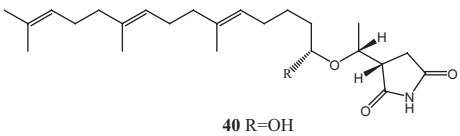
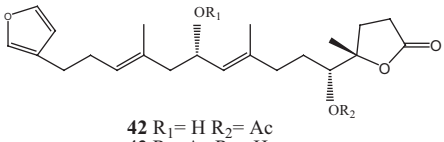


39

Eight sesterterpenoids, hippolides A–H were isolated from the marine sponge *Hippospongia lachne* (China). Compound **40** showed cytotoxicity against A549, HeLa, and HCT-116 cell lines with IC_{50} values of 5.22×10^{-2}, 4.80×10^{-2}, and 9.78 μM, respectively. Compound **41** exhibited moderate cytotoxicity against the HTC-116 cell line with IC_{50} value of 35.13 μM [47]. Two C22 furanostesterterpenoids, 15-acetylirciformonin B (**42**) and 10-acetylirciformonin B (**43**) were isolated from the sponge *Ircinia* sp. Both compounds exhibited cytotoxic activity against K562, DLD-1, HepG2, and Hep3B cancer cell lines [48].



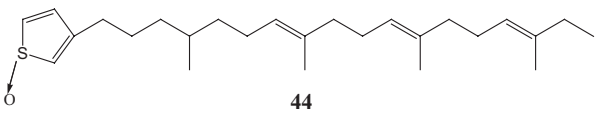
40 R=OH
41 R=Me



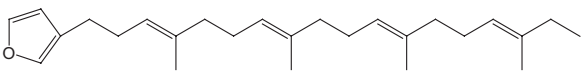
42 R_1= H R_2= Ac
43 R_1=Ac R_2= H

Compound **44**, a thiophene-S-oxide acyclic sesterterpenoid, isolated from a marine sponge *Xetospongia* sp., showed significant cytotoxicity against Vero cells with an IC_{50} of 31 μM [49].

Furospinosulin-1 (**45**), a marine sponge-derived furanosesterterpenoid, showed selective antiproliferative activity against DU145 human prostate cancer cells under hypoxic conditions in concentration ranging from 1 to 100 μM. It also exhibited antitumor activity at a level of 10–50 mg kg^{-1} after oral administration to a mouse model inoculated with sarcoma S180 cells. Analogues of **45** were prepared, and among all the demethyl analogue showed excellent hypoxia-selective inhibitory activity like that of **45** and showed *in vivo* anti-tumor activity after oral administration [50].



44

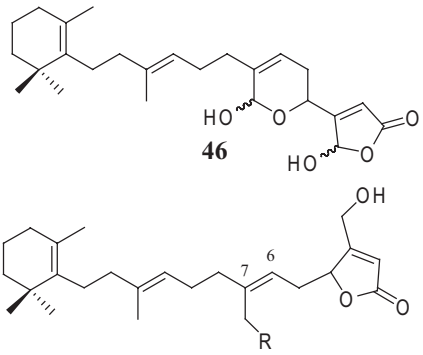


45

22.3 Monocarbocyclic Sesterterpenoids

Marine sponges of genus *Luffariella* (Thorectidae; Dictyoceratida) are a rich source of monocarbocyclic sesterterpenoids and most of them possess interesting cytotoxic activity.

Kobayashi et al. [51] described, from the sponge *Luffariella* sp. (Okinawa), the isolation of manoalide (**46**), (6E)- (**47**) and (6Z)-neomanoalide (**48**) that showed cytotoxic activity against L1210 cells (IC_{50} 0.032, 9.8 and 5.6 μg/ml for **46**, **47** and **48**, respectively), and only manoalide was active against KB cells with an IC_{50} value of 0.3 μg/ml [51, 52].

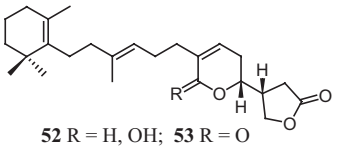


46

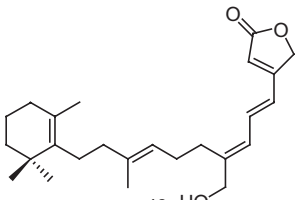
47 $\Delta^{6,7}E$, R = OH
48 $\Delta^{6,7}Z$, R = OH;

Kobayashi et al. [51] also reported, from the same sponge *Luffariella* sp., the isolation of several sesterterpenoids related to manoalide, named luffariolides A–J (**49–57**). All luffariolides showed cytotoxic activity against L1210 cells (IC_{50} 1.1–4.5 μg/ml) and only luffariolides F (**54**) and G (**55**) exhibited weak activity also against KB cells [51–53].

Tasnemoxides A–C (**58–60**) were isolated from *Diacarnus erythraeanus* (Red Sea) and showed moderate cytotoxicity ($IC_{50} > 1$ μg/ml) against three cancer cell lines including P388, A549 and HT29 [54]. Further sesterterpenes (**61** and **62**) and two norsesterterpene (**63** and **64**), related to hippospongin, were isolated from the Okinawan sponge *Ircinia* sp. The norsesterterpenes **63** and **64** were more cytotoxic ($IC_{50} < 1$ μg/ml) than the sesterterpenes **61** and **62** ($IC_{50} \geq 1$ μg/ml) against KB cells [55].



52 R = H, OH; **53** R = O



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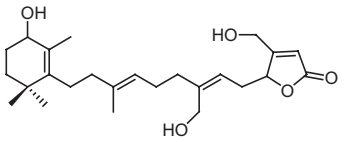

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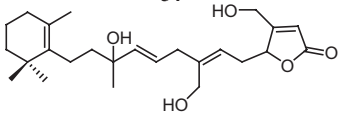

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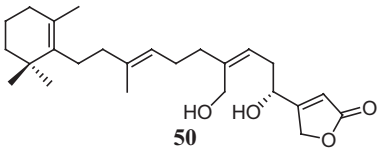

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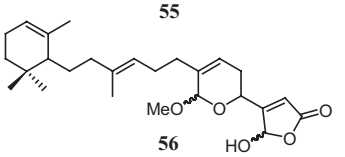

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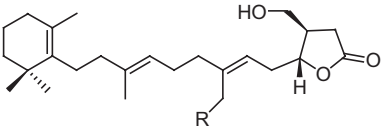
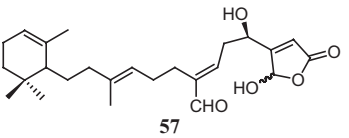


51a R = OH; **51b** R = OAc



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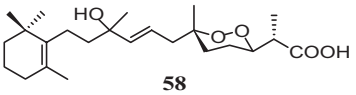

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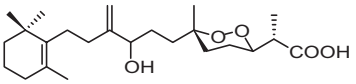

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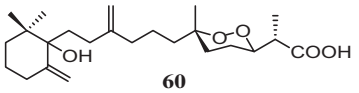

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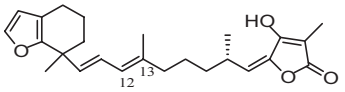
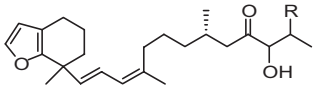


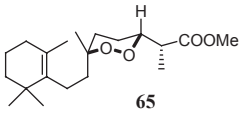
61 $\Delta^{12,13}E$; **62** $\Delta^{12,13}Z$



63 R = H; 64 R = Cl

Eight cyclic peroxide norterpenoids, compounds **65–72**, have been isolated and characterized from the Red Sea sponge *Diacarnus erythraeanus*. Among these metabolites, (−)-muqubilin A (**69**) (tested on Hs683, U373, U251, SKMEL28, A549, MCF-7, PC-3, LoVo and B16F10 human cancer cell lines) and the new compounds **67** and **68** (tested on Hs683, U373, U251, SKMEL28, A549, MCF-7 and PC-3 human cancer cell lines) displayed mean IC_{50} growth inhibitory concentrations in vitro of < 10 μM, while the remaining compounds (**65**, **70–72**) were inactive in these cancer cell lines. Compound **69** displayed no selectivity between normal and cancer cells in terms of in vitro growth inhibition. Quantitative video microscopy analysis carried out on (−)-muqubilin A-treated cells validated the data obtained by means of the MTT colorimetric assay, while flow cytometry analysis revealed ROS production but no induction of apoptosis in cancer cells [56].

As part of an ongoing research program to discover natural products that suppress the hypoxia-activated tumor survival pathways, the lipid extract of the Papua New Guinea marine sponge *Diacarnus levii* was found to suppress hypoxia induced HIF-1 activation and hypoxic tumor cell survival. Bioassay-guided isolation of *D. levii* yielded four new norsesterterpene peroxides, diacarnoxides A–D (**73–76**). Diacarnoxide B exhibits a significantly enhanced ability to suppress the growth of tumor cells under hypoxic conditions [57].



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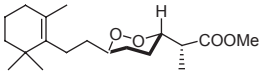

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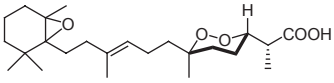

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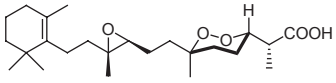
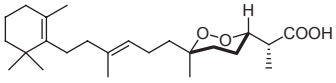
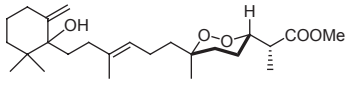


68



69



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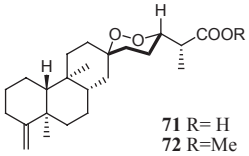
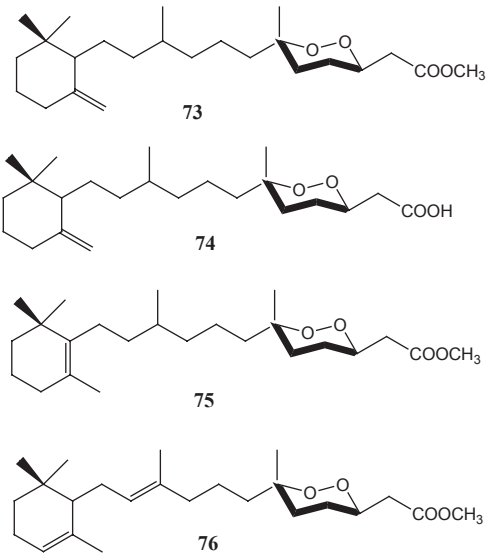


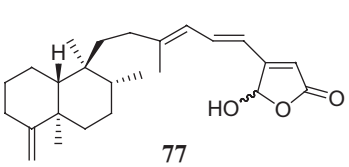
71 R= H
72 R=Me



22.4 Bicarbocyclic Sesterterpenoids

Sesterterpenoids with a bicarbocyclic skeleton in many instances show structures reminiscent of the clerodane and labdane diterpenoids. From the Palauan sponge *Thorectandra* sp. were isolated palauolide (**77**), palauolol (**78**) together with their derivatives, named thorectandrols A–E (**79–83**). Compounds **77–83** were tested for antiproliferative and cytotoxic activities against 12 human tumour cell lines originated from breast, CNS, colon, lung, ovarian and renal carcinoma, leukaemia and melanoma. Palauolol (**78**) was active in all the cell lines with IC_{50} in the range 0.5–7.0 μg/ml, while palauolide (**77**) showed less activity in all the cell lines with IC_{50} 7.7–53 μg/ml. Thorectandrols A–D were weakly active with IC_{50} over 30 μg/ml, whereas thorectandrol E was not cytotoxic to any of the cell lines at the maximum dose tested [58, 59].

From a specimen of *Fasciospongia cavernosa* was isolated an isomer of cacospongionolide, named cacospongionolide B (**84**) that showed a high cytotoxicity (LC_{50} 0.25 μg/ml), in the *A. salina* bioassay and a moderate ichthyotoxic to *G. affinis* (LC_{50} 1.05 μg/ml) [60]. There are two varieties of *F. cavernosa*, one is massive, and the second is encrusted. From specimen of the massive form were isolated only one or two correlated metabolites, while from specimen of the encrusted form were isolated a complex mixture of cacospongionolides: cacospongionolides D (**16**) [32], E (**85**) [61] and F (**86**) [62] that was also synthesised [63], and related metabolites, such as 25-deoxycacospongionolide B (**87**) [64] and cavernosolide (**133**) [65].



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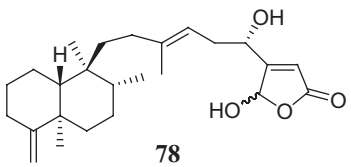
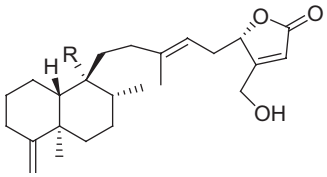
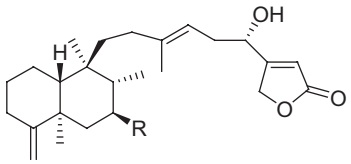


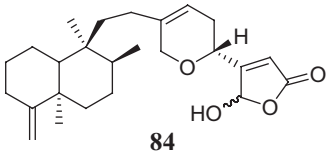
78



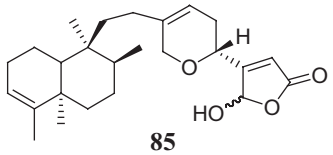
79 R = H; **80** R = OAc



81R = OAc;**82** R = H
83 R = OH



84



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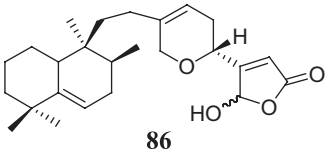

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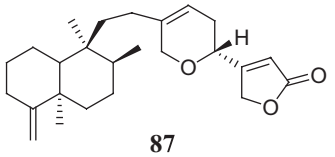
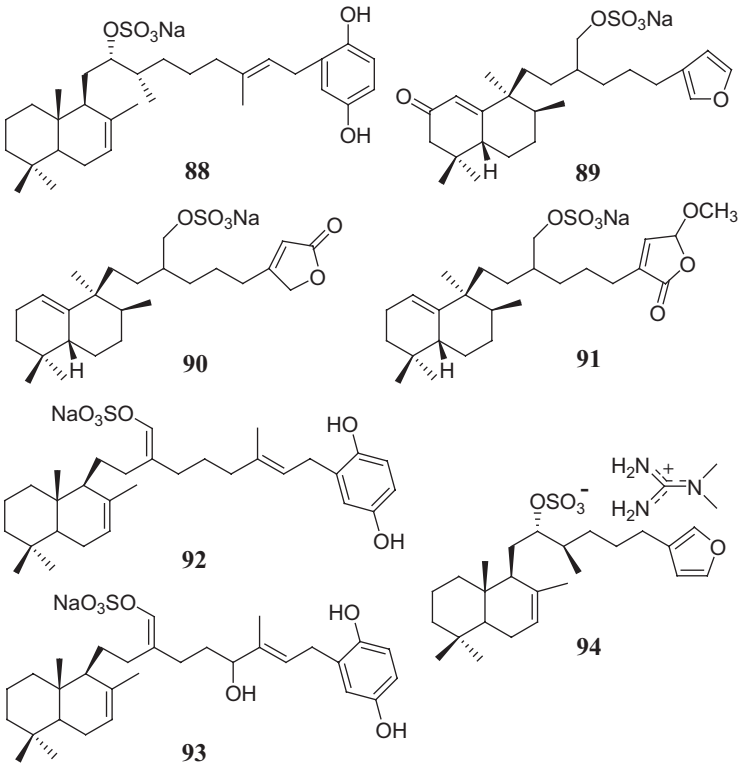
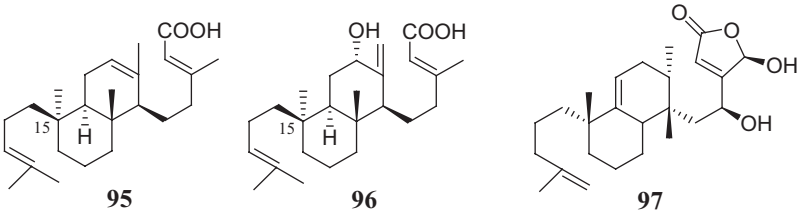


87

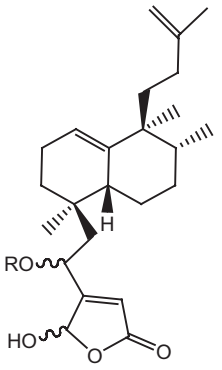
A number of carbobicyclic sesterterpenoid sulphates were found, including halisulfate 1 (**88**), isolated from *Halichondria* sp. [66]; halisulfates 8–10 (**89–91**), isolated from the Australian sponge *Darwinella australensis* [67]; hipposulfates A (**92**) and B (**93**), isolated from the Okinawan *Hippospongia metachromia* [68] and sulfircin (**94**) that was isolated as its N, N-dimethylguanidinium salt, from a deep-sea member of the genus *Ircinia* [69]. Halisulfate 1 (**88**) is an inhibitor of human 12-lipoxygenase (12-HLO) (IC_{50} 1.0 μM) and 15-HLO (IC_{50} 0.9 μM) [70]. 12-HLO is involved in the development of psoriasis and controlling cancer cell proliferation, while 15-HLO in the development of atherosclerosis and tumourigenesis. Halisulfates 9 (**90**) and 10 (**91**) inhibited cell division of the fertilised eggs of the sea urchin *Strongylocentrotus intermedius* (IC_{50} 50 and 35 μg/ml for **90** and **91**, respectively) [67]. Hipposulfates B (**93**) showed cytotoxic activity with an IC_{50} of 2.0 μg/ml against several human tumour cell lines [68].



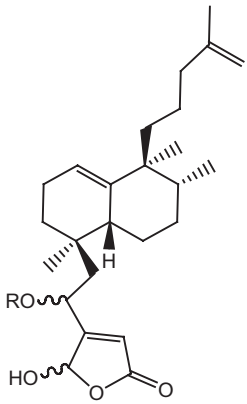
Kohamaic acids A (**95**) and B (**96**) were isolated from the Okinawan *Ircinia* sp. They exhibited cytotoxicity against P388 cells, with IC_{50} values of > 10 (32 %) and 2.8 μg/ml, respectively [71]. Dysidiolide (**97**), isolated from the Caribbean sponge *D. etheria*, is a potent inhibitor of the human cdc25A protein phosphatase (IC_{50} 9.4 μM), a potential target for anticancer therapy. Moreover, dysidiolide inhibited growth of the A549 (IC_{50} 4.7 μM) and P388 (IC_{50} 1.5 μM) cells [72].



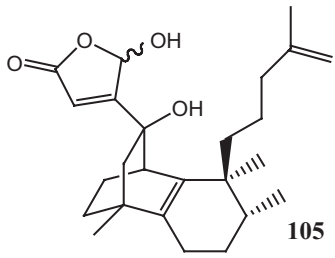
Sesterterpenolides analogues of dysidiolide (**98–105**) have been synthesized and their in vitro antitumoral activity against human HeLa, A549, HT-29 and HL-60 carcinoma cells is presented. The proliferation inhibition data showed a significant antitumour activity of the compounds **98**, **99**, **100**, **101**, **102**, **103**, **104**, **105**, inhibiting proliferation of distinct cancer cell types with an IC_{50} in the low micromolar range. Results showed that the presence of the γ-hydroxybutenolide moiety is essential for this activity. Moreover, compound 101 with a tricyclic framework showed the highest activity against leukaemic HL-60 cells. This could be related to the fact that HL-60 cells divide more rapidly than the solid tumour cells HeLa, A549 and HT-29, and dysiodiolide has been reported to inhibit cdc25A causing cell cycle arrest [73].



98 R= Ac 4*R*
99 R= H 4*S*
100 R= H 4*R*

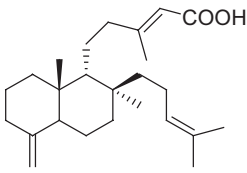


101 R= Ac 4*S*
102 R= Ac 4*R*
103 R= H 4*S*
104 R= H 4*R*



105

From *D. cinerea* were isolated two new inseparable metabolites, bilosespens A (**106**) and B (**107**). The mixture of both bilosespens showed cytotoxic activity with an IC_{50} of 2.5 μg/ml against four human tumour cell lines (A549, P388, MEL28 and HT29) [74].



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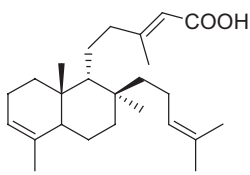
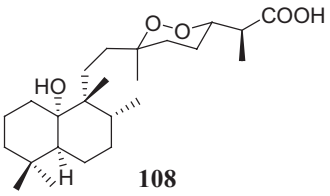


107

Carbobicyclic norsesterterpenoids, containing cyclic peroxides were isolated from four sponge genera, *Mycale, Latrunculia, Sigmosceptrella* and *Diacornis*. From a Thai *Mycale* sp. were isolated two related norsesterterpenoids 1,2-dioxanes, mycaperoxides A (**108**) and B (**109**), which showed significant cytotoxicity (IC_{50} 0.5–1.0 μg/ml) against the cell lines P388, A549 and HT29 [75].

Two sesterterpenoids, **110** and **111**, were isolated from the sponge *Coscinoderma* sp. Both compounds exhibited moderate cytotoxicity against the K562 cell line and inhibitory activities against isocitrate lyase, sortase A, and Na^+/K^+-ATPase [76].



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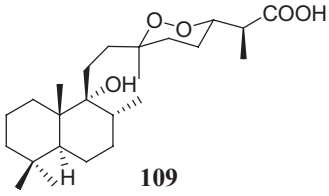

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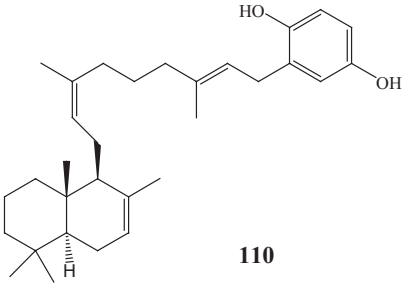
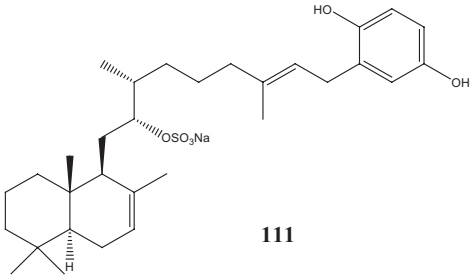


110

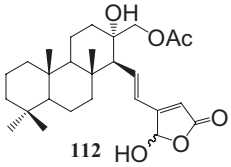


111

22.5 Tricarbocyclic Sesterterpenoids

Marine sponges are a rich source of tricarbocyclic sesterterpenoids with a cheilanthane skeleton, which seems to be derived from geranylfarnesol by a cyclisation initiated at the isopropylidene group that is typical of triterpenes.

Spongianolides A–F (**112–117**) possessing a γ-hydroxybutenolide moiety, were isolated from a *Spongia* sp. [77]. The absolute stereochemistry of spongionolide A was established by its total synthesis [78]. Spongianolides A–E inhibited protein kinase C (PKC) at IC_{50} 20–30 μM, moreover, compounds **112–115** potently inhibited (IC_{50} 0.5–1.4 μM) the proliferation of the mammary tumour cell line MCF7 [77]. Simultaneously, from the Caribbean sponge *Cacospongia linteiformis* were isolated the spongianolides C and D (**114** and **115**) designated as lintenolides A and B and lintenolides C–G (**119–122**) [79, 80]. All lintenolides A–G inhibited the growth of murine fibrosarcoma WEHI 164, murine monocyte/macrophage J774, bovine endothelial GM7373 and murine leukemia P388 cell lines [81].



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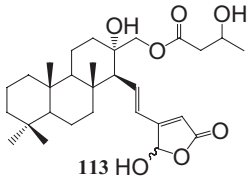

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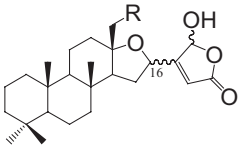
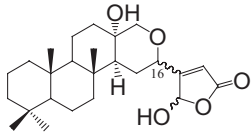
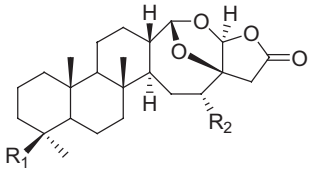


114 16R, R = $OCOCH_3$
115 16S, R = $OCOCH_3$
116 16R, R = $OCOCH(OH)CH_3$
117 16S, R = $OCOCH(OH)CH_3$
118 16R, R = H
121 16R, R = OH
122 16S, R = OH

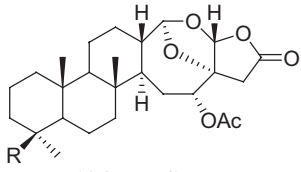


119 16R
120 16S

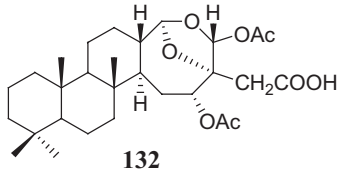
From the New Caledonian sponge *Petrosaspongia nigra* were isolated several tricarbocyclic sesterterpenoids petrosaspongiolides A–J (**123–132**) [82, 83]. Petrosaspongiolides A–J exhibited cytotoxicity (IC_{50} 0.5–14.8 μg/ml) against human bronchopulmonary non-small-cell-lung carcinoma cell line (NSCLC-N6) [83]. Furthermore, petrosaspongiolide M (PM) was able to inhibit NF-κB activation in mouse peritoneal macrophages [84]. In addition, PM has been described as a potent inhibitor of the proteasome machinery [84]. Due to their ability to induce apoptosis and interfere with cell cycle regulation, gene expression, carcinogenesis and DNA repair, proteasome inhibitors have recently been gaining greater attention as novel pharmacological tools in cancer drug discovery [84].



123 $R_1 = CH_3$, R_2 = OAc
125 $R_1 = CH_2OAc$, R_2 = OAc
126 $R_1 = CH_2OH$, R_2 = OAc
127 R_1 = CHO, R_2 = OAc
128 R_1 = COOH, R_2 = OAc
129 $R_1 = CH_2OH$, R_2 = OH
130 R_1 = COOH, R_2 = OH



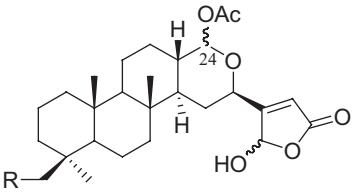
124 R = CH_3
131 R = CH_2OAc



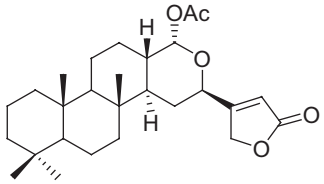
132

Cavernosolide (**133**), isolated from the Tyrrhenian sponge *Fasciospongia cavernosa* showed high cytotoxicity (LC_{50} 0.37 µg/ml) in the *A. salina* bioassay and a moderate ichthyotoxicity (LC_{50} 0.75 µg/ml) to *G. affinis* [65].

Inorolide C (**134**) was isolated from the nudibranch *Chromodoris inornata*. It was shown to inhibit the proliferation of KB (IC_{50} 6.4 µg/ml) and L1210 (IC_{50} 1.9 µg/ml) cells [85].

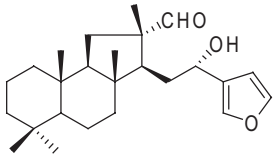


133 24R, R = H



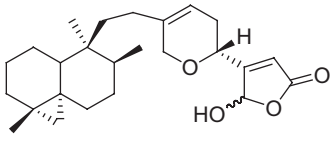
134

From the Okinawan sponge *Hyrtios erectus* was isolated hyrtiosal (**135**), possessing a novel rearranged tricarbocyclic skeleton (hyrtiosane) [86]. Its structure was confirmed by total synthesis [87]. This compound exhibited *in vitro* antiproliferative activity against KB cells with an IC_{50} of 3.0 µg/ml [86].



135

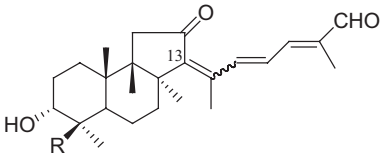
A new tricarbocyclic sesterterpene, cacospongionolide (**136**), bearing a γ-hydroxybutenolide moiety, was isolated from the Dictyoceratide sponge, *Fasciospongia cavernosa*, erroneously classified as *Cacospongia mollior* [88]. Cacospongionolide was reported as a potent inhibitor of human synovial and bee venom PLA_2 [61]. Besides, cacospongionolide showed high cytotoxic activity (LC_{50} 0.1 µg/ml), in the *A. salina* bioassay, very high inhibition (75 %) in the crown-gall potato disc assay, an antitumoural like test [33].

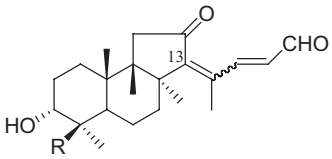


136

From the New Caledonian sponge *Rhabdastrella globostellata* were isolated two isomalabaricane sesterterpenes, aurorals 1 and 2 (**137** and **138**) and the

corresponding trinor-sesterterpenes aurorals 3 and 4 (**139** and **140**) [89]. From the Okinawan sponges *Rhabdastrella (Jaspis) stellifera* were isolated the corresponding oxidised compounds jaspiferals C–F (**141–144**) [90]. Since jaspiferals C–F were isolated together with the related triterpenes stelliferins A–F [91] and nortriterpenes jaspiferals A–B [90], we can suppose that also aurorals 1–4 and jaspiferals C–F are degraded triterpenoids. Aurorals, which differ from jaspiferals by the presence of a primary alcohol group at C-4 position, exhibited higher cytotoxic activity on the KB cells. The mixtures of aurorals 1–2 (**137** and **138**) and jaspiferals C–D (**141** and **142**) showed ID_{50} values of 0.2 and 5.5 μg/ml, respectively. The mixtures of aurorals 3–4 (**139** and **140**) showed moderate activity on KB cells with an IC_{50} of 8.0 μg/ml, while jaspiferals E–F (**143** and **144**) were inactive until 10 μg/ml [89]. Furthermore, the mixtures of jaspiferals C–D, and jaspiferals E–F exhibited cytotoxicity against L1210 cells with IC_{50} values of 4.3 and 3.1 μg/ml, respectively [90].

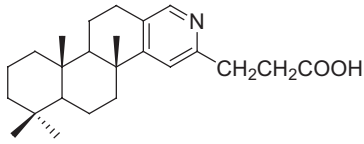




137 R = CH_2OH, $\Delta^{13} = Z$
138 R = CH_2OH, $\Delta^{13} = E$
141 R = COOH, $\Delta^{13} = Z$
142 R = COOH, $\Delta^{13} = E$

139 R = CH_2OH, $\Delta^{13} = Z$
140 R = CH_2OH, $\Delta^{13} = E$
143 R = COOH, $\Delta^{13} = Z$
144 R = COOH, $\Delta^{13} = E$

From the New Caledonian *Petrospongia nigra*, together with the previously reported petrosaspongiolides A–J (**123–132**) was isolated a pyridium alkaloid 23-norsesterterpene named petrosaspongiolide L (**145**) that showed cytotoxic activity against NSLC-N6 cells with IC_{50} value of 5.7 μg/ml [68].

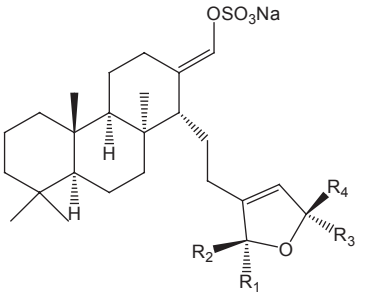


145

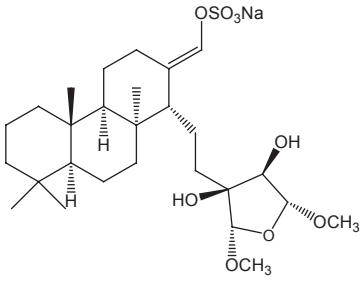
Six sesterterpenoids **146–151** were isolated from the sponge *Coscinoderma* sp. (Micronesia). They showed moderate cytotoxicity against the K562 cell line and inhibitory activities against isocitrate lyase, sortase A, and Na^+/K^+-ATPase [61].

From the fungus *Aspergillus ustus* isolated from the Mediterranean sponge *Suberites domuncula* were isolated five ophiobolin-type sesterterpenoids **152–156**. All compounds were tested (at a concentration of 10 μg/ml) for their cytotoxicity against the murine lymphoma cell line L5178Y [92]. None of the compounds reduced survival of cells by more than 10–20% compared to controls. In a recent study, it is reported the anti-proliferative effect of ophiobolin O in human breast cancer MCF-7 cells. Results showed that ophiobolin O induced cycle G_0/G_1 phase

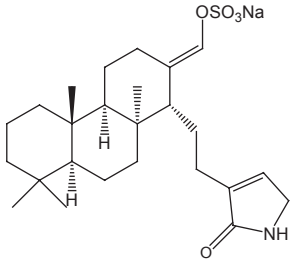
arrest in MCF-7 cells and it reduced the viability of MCF-7 cells in a time- and concentration-dependent manner inducing apoptosis through activation of the MAPK signalling pathways [93].



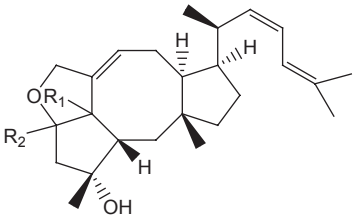
146 R_1=H, R_2= H, R_3,R_4=O
147 R_1,R_2=O, R_3=H, R_4=OMe
148 R_1,R_2=O, R_3= OMe, R_4=H
149 R_1=OMe,R_2=H, R_3= OMe, R_4= H



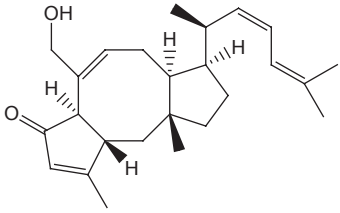
150



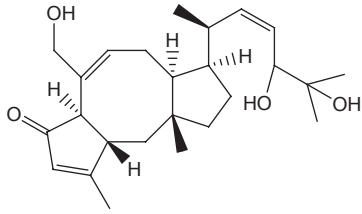
151



152 R_1= H R_2= OH
153 R_1= H R_2= OMe
154 R_1= H R_2= OMe



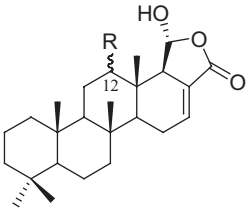
155



156

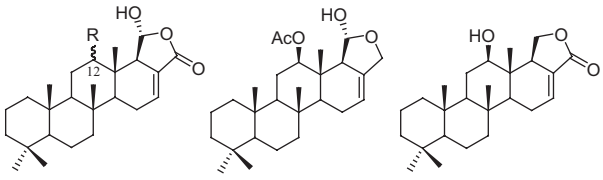
22.6 Tetracarbocyclic Sesterterpenoids

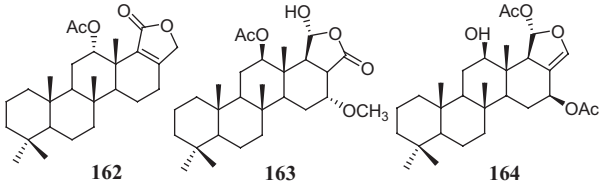
The main group of marine tetracarbocyclic sesterterpenoids is of those with a scalarane skeleton, which appears to be of the same origin as cheilanthane and is formed by closely biosynthetic process involving additional cyclisation. Metabolites of this class have been reported from marine sponges of the order Dictyoceratida and their predator nudibranches [14, 15]. The first example of this group was scalarin (**157**), isolated from the sponge *Cacospongia scalaris* bearing a γ-hydroxybutenolide moiety [94].



157 R = α-OAc

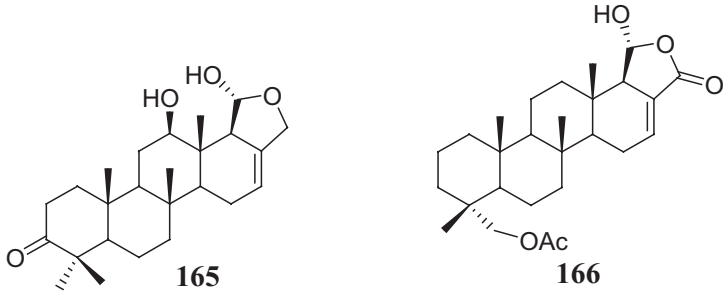
A number of 19-deoxy, 20-deoxo, 12-*O*-deacetyl and 12-epimers were isolated [14, 15]. From the Japanese *Spongia* sp. were isolated 12-*epi*-scalarin (**158**), 12-*O*-deacetyl-12-*epi*-scalarin (**159**), 12-*epi*-deoxoscalarin (**160**) and 12-*O*-deacetyl-19-deoxyscalarin (**161**) [95]. These compounds exhibited selective cytotoxicity against four tumour cell lines, being more active on L1210 cell line (IC_{50} 13.2, 2.3, 2.1 and 1.6 μg/ml for **158–161**, respectively) and less active on A549, KB and HeLa cell lines with an IC_{50} in the range of 14.3–29.4 μg/ml [95]. 12-*O*-deacetyl-19-deoxyscalarin (**161**), first isolated from the sponge *Hyrtios erecta*, showed also cytotoxicity against P388 cells with IC_{50} of 2.9 μg/ml [96]. Moreover, compound **161** showed antitumour activity *in vivo* on sarcoma-180-implanted mice with an increase of lifespan (ISL) of 50.3% at 5 mg/kg intraperitoneal administrations. This activity is more potent than of a positive control, 5-fluorouracil (ISL: 32.9%) at the same dose [95]. 12-*Epi*-acetylscalarolide (**162**), isolated from the Spanish *C. scalaris*, showed significant cytotoxic activity towards a panel of four tumour cell lines (P388, A549, HT29 and MEL28 cells) [97]. 12-*O*-acetyl-16-*O*-methylhyrtiolide (**163**), with an additional methoxy group at C-16 exhibited cytotoxicity against L1210, A549, KB and HeLa cell lines with IC_{50} values of 2.2, 5.3, 15.6 and 5.3 μg/ml, respectively [95].





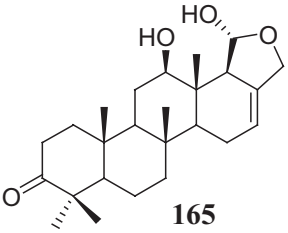
Salmahyrtisol B (**164**) isolated from the Red Sea *Hyrtios erecta* showed significant cytotoxicity to murine leukemia (P-388), human lung carcinoma (A-549), and human colon carcinoma (HT-29) [98].

Generally, scalarane sesterterpenoids are not functionalised on A- and B-rings. A structure–activity study showed that an oxygen-bearing substituent at C-3 of scalaranes, together with the presence of hydroxyl groups at C-12 and C-19, leads to increase of antitumour activity [99]. Accordingly, salmahyrtiol C (3-oxo-12-*O*-deacetyl-12-*epi*-deoxyscalarin) (**165**), first isolated from the Japanese *H. erecta* [99] and subsequently from the Red Sea *H. erecta* [98], exhibited potent cytotoxicity against P388 (IC_{50} of 14.5 ng/ml) and human gastric carcinoma MNK-1 (IC_{50} of 57.7 ng/ml), MNK-7 (IC_{50} of 56.0 ng/ml) and MNK-74 (IC_{50} of 36.8 ng/ml) cells. Intraperitoneal administration of **165** (0.5–8.0 mg/kg) on mice with P388 leukaemia increased the mean survival time (10.7–15 days) and ISL (24.4–74.4%) dose-dependently [99]. 12-Deacetoxy-21-acetoxyscalarin (**166**), isolated from the Japanese *H. erecta,* showed cytotoxic activity against P388 cells with IC_{50} value of 0.9 μg/ml [100].

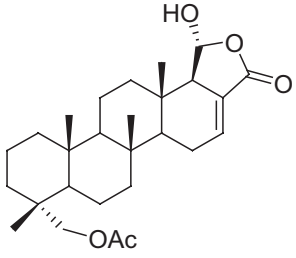


From the Maldivian *H. erecta* were isolated sesterstatins 1–3 (**167–169**) that showed cytotoxic activity against P388 cells with IC_{50} value of 0.46, 4.2 and 4.3 μg/ml, respectively [101]. Additional 3- (**170** and **171**) and 19-oxygenated

scalaranes (**172** and **173**) were isolated from the nudibranch *Chromodoris inornata* that showed cytotoxic activities against L1210 (IC_{50} 6.6, 0.95, 4.1 and 0.35 μg/ml for **170–173**, respectively) and KB (IC_{50} 22.8, 5.2, 21.0 and 3.1 μg/ml for **170–173**, respectively) cell lines [85]. Scalaradial (**174**) and its 12-deacetoxy derivative (**175**) are two classical examples of compounds with a 1,4-dialdehyde moiety. Scalaradial (**174**) was isolated from two species of *Cacospongia, C. mollior* [102] and *C. scalaris* [97]; 12-deacetoxyscalaradial (**175**) was isolated from *C. mollior* [103]. The majority of terpenoids, containing an unsaturated 1,4-dialdehyde functionality, are intensely pungent [104] and generally are very versatile repellents [105]. This activity was explained by their interaction with vanilloid receptors [106].



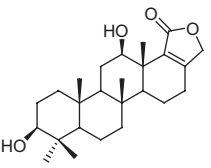
165



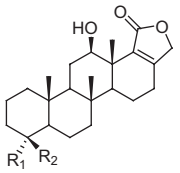
166

Scalaradial has been shown to inhibit significantly cell proliferation as well as to induce apoptosis in several human cancer cell lines [33]. From the Japanese *C. scalaris* was isolated deacetylscalaradial (**176**) that showed interesting cytotoxic activity against L1210 cells with an IC_{50} value of 0.58 μg/ml [107]. Scalaradial (**174**) and deacetylscalaradial (**176**) were shown to act on both R- and C-type vanilloid receptors [106]. From the *C. scalaris*, collected in the Southern Coast of Spain, were isolated 18-*epi*-scalaradial (**177**) and 19-dihydroscalaradial (**178**). Both compounds showed significant cytotoxicity towards four tumour cell lines (P388, A549, HT29 and MEL28 cells) [97].

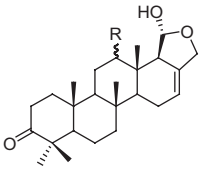
From the Japanese *H. erecta* were isolated two sesterterpenoids (**179** and **180**) [100] related to scalarolbutenolide (**181**), isolated from the Mediterranean *Spongia nitens* [108]. Compounds **179** and **180** were cytotoxic against P388 cells with IC_{50} values of 0.4 and 2.1 μg/ml, respectively [100]. These compounds cannot strictly be considered as scalarane, because they show different arrangements of the carbons C-24 and C-25. 16-Acetylfuroscalarol (**182**), with moderate cytotoxicity, isolated from the Spanish *C. scalaris* [97] and 12-*O*-acetyl-16-*O*-deacetyl-12,16-episcalarolbutenolide (**183**), cytotoxic against L1210 (IC_{50} 2.4 μg/ml) and KB (IC_{50} 7.6 μg/ml) cell lines, isolated from the nudibranch *C. inornata* [85], showed the same carbon skeleton of scalarolbutenolide. From the Indonesian *Phyllospongia* sp. were isolated two sesterterpenes (**184** and **185**), which exhibited cytotoxicity against KB cells at 10 μg/ml [109].



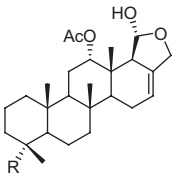
167



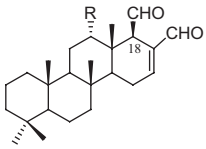
168 $R_1 = CH_3$, $R_2 = CH_2OH$
169 $R_1 = CH_2OH$, $R_2 = CH_3$



170 R = β-OAc
171 R = α-OAc

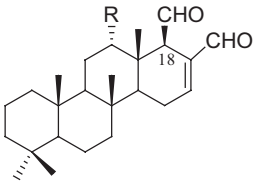


172 $R = CH_2OH$
173 $R = CH_2OAc$

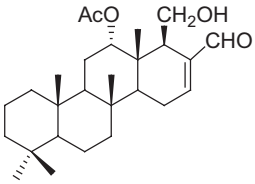


174 R = OAc
175 R = H

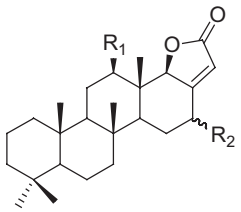
Tetracarbocyclic norsesterterpenoids are extremely rare and are only isolated from sponge of subclass Dictyoceratida. Hyrtial (**186**), isolated from *H. erecta*, was the first 25-norscalarane to be reported. It showed anti-inflammatory activity at 50 μg/ml close to the activity of indomethacine [110]. From the Okinawan sponge *H. erecta* were isolated 12-deacetylhyrtial (**187**) and its Δ^{17} isomer (**188**) that showed cytotoxic activity against KB cells with IC_{50} values of 10.0 and 2.82 μg/ml, respectively [111]. Norscalarals A–C (**189–191**) isolated from the Spanish *C. scalaris* showed cytotoxicity against P388, A549, HT29 and MEL28 tumour cell lines [97]. Petrosaspongiolide K (**192**), isolated from the New Caledonian *Petrosaspongia nigra*, was the first reported 23-norscalarane. Petrosaspongiolide K showed cytotoxic activity (IC_{50} 1.3 μg/ml) against NSCLC-N6 cells [83].



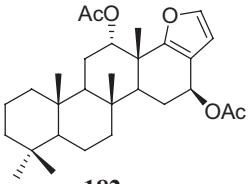
176 R = OH
177 R = OAc, 18-*epi*



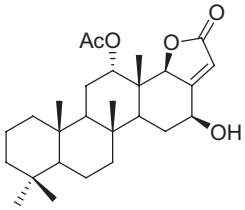
178



179 R_1 = OH, R_2 = βOH
180 R_1 = OAc, R_2 = βOH
181 R_1 = OH, R_2 = αOAc



182



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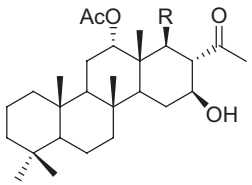
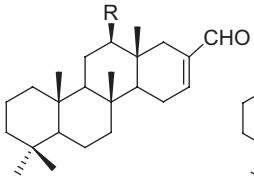
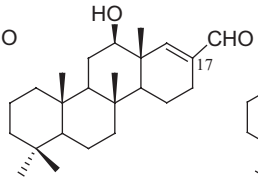


184 R = H
185 R = OH

Four 24-homoscalaranes (**193–196**) that exhibited 30–95% inhibition of the growth of KB cells at 10 μg/ml were isolated from the Indonesian *Phyllospongia* sp. [109].



186 R = OAc
187 R = OH



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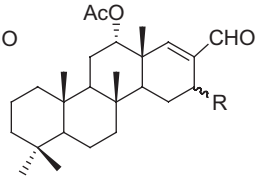
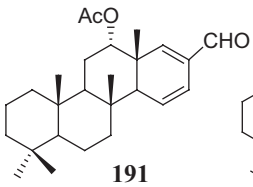


189 R = 16β-OH
190 R = 16α-OH



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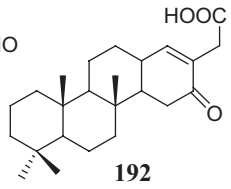
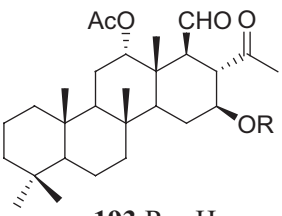


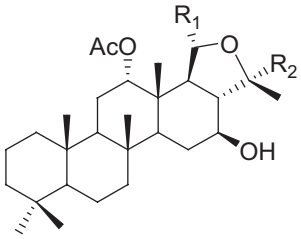
192

From the Madagascan sponge *Carteriospongia* sp. were isolated several antiproliferative homoscalarane-type sesterterpenoids (**197–206**) [112]. Compounds **197**,

199 and **201** displayed submicromolar antiproliferative activity against the A2780 ovarian cell line with IC_{50} values of 0.65, 0.26 and 0.28 μM, respectively, while compounds **202** and **203** showed moderate activity (4.5 and 8.7 μM, respectively). Compounds **199** and **201** also displayed anti-proliferative activity against the H522-T1 non-small cell lung and A2058 human melanoma cancer cell lines.

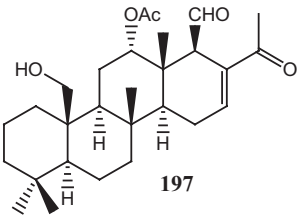


193 R = H
194 R = Ac

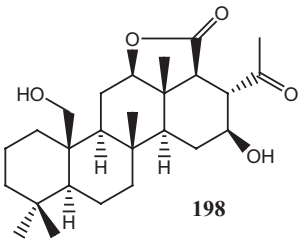


195 R_1 = OH, R_2 = H
196 R_1 = OMe, R_2 = OMe

Two new sesterterpenoids named flabelliferins A (**207**) and B (**208**) were isolated from the lipophilic extract of the sponge *Cateriospongia flabellifera*, collected in the South Pacific near Vanuatu. The structure and absolute configuration of these two compounds were assigned by a combination of one- and two-dimensional NMR spectroscopy and by Mosher's ester analysis. Flabelliferin A (**207**) has a rare 25-homocheilanthane carbon skeleton, while flabelliferin B (**208**) is a 24-nor-25-homoscalarane sesterterpenoid. Both compounds were tested against the human colon tumour cell lines KM12 and COLO205. They did exhibit growth inhibitory effects, as compound **207** had IC_{50} values of 15 and 20 μM, respectively, while compound **208** had IC_{50} values of 18 and approximately 20 μM, respectively [113].



197



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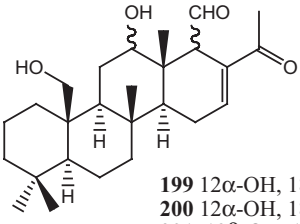
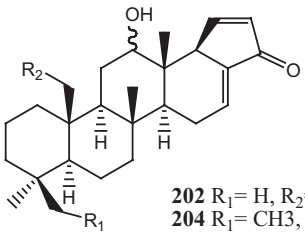
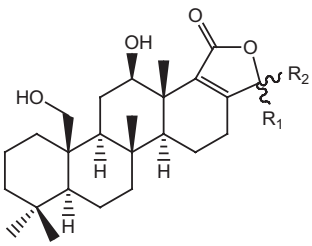


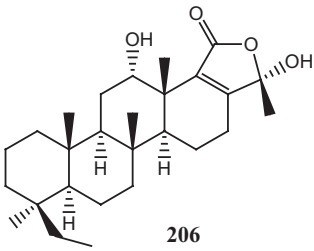
199 12α-OH, 18α-CHO
200 12α-OH, 18β-CHO
201 12β-OH, 18β-CHO



202 R_1= H, R_2= OH, 12β-OH
204 R_1= CH3, R_2= H,12α-OH

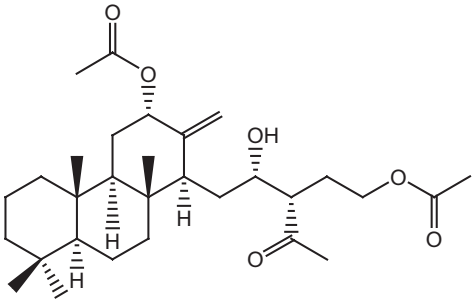


203 R_1= OH, R_2= CH_3
205 R_1= α-H, R_2= β-CH_3

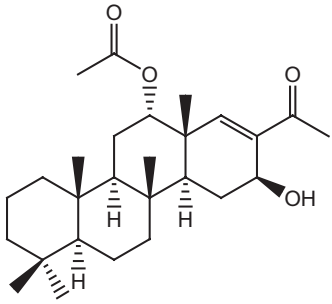


206

Several bishomosesterterpenoids were isolated from *P. foliascens*, collected in different seas. From the Neo Guinean sponge *C. foliascens* were isolated several bishomosesterterpenoids, but only compounds **209–211** showed ichthyotoxic effects towards *L. reticulatus* at LD_{50} of 5, 20 and 40 mg/l, respectively [114]. Phyllactones A (**212**) and B (**213**), with moderate cytotoxicity against KB cells (IC_{50} 20.0 μg/ml), were isolated from the Chinese *P. foliascens* [115]. From the Indonesian *Phyllospongia* sp. were isolated two 20,24-dihomoscalaranes (**214** and **215**) that showed cytotoxicity against KB cells at 10 μg/ml [109]. From the Australian *Strepsichordaia lendenfeldi* were isolated four different acyl derivatives (**216–219**) and three esters with the same skeleton and different acyl groups (**220–222**). All these compounds exhibited potent cytotoxicity against both P388 and A549 cell lines [116].



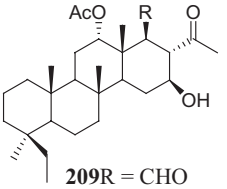
207



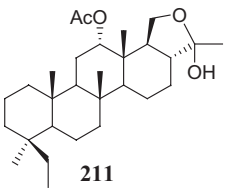
208

From the Red Sea *Hyrtios erecta,* together with hyrtiosal (**135**), previously reported [86], was isolated salmahyrtisol A (**223**), a furan sesterterpene with a new tetracarbocyclic skeleton. The coexistence of the unusual sesterterpenes **223** and **135** is noteworthy from the biosynthetic viewpoint and maybe hyrtiosal is the logical biosynthetic intermediate for salmahyrtisol A. Salmahyrtisol A showed cytotoxic activity with an with $IC_{50} \geq 1$ μg/ml against three type of cancer cells including P388, A549 and HT29 [98].

From the Japanese nudibranch *Chromodoris inornata* were isolated two sesterterpenes, inorolides A (**224**) and B (**225**) with a new carbon skeleton. Both compounds exhibited cytotoxic activity against L1210 (IC_{50} 1.9 and 0.72 μg/ml for **224** and **225**, respectively) and KB (IC_{50} 3.4 and 2.2 μg/ml for **224** and **225**, respectively) cell lines [85].



209 R = CHO
210 R = H



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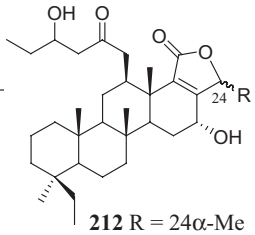
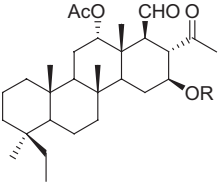
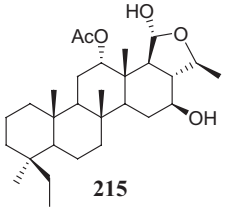
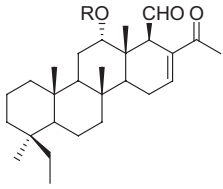


212 R = 24α-Me
213 R = 24β-Me





215

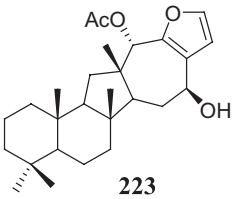


214 R = H
216 R = $CH_3CHOHCH_2CO^-$
217 R = $CH_3CH_2CHOHCH_2CO^-$
218 R = $CH_3CH_2CO^-$
219 R = CH_3CO^-

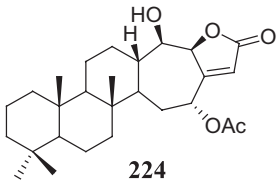
220 R = $CH_3CH_2CH(OCOCH_3)CH_2CO^-$
221 R = $CH_3CH_2CH(OCOCH_2CH_3)CH_2CO^-$
222 R = $CH_3CH(OCOCH_3)CH_2CO^-$

From the marine fungus *Fusarium heterosporum* were isolated two groups of sesterterpenes, neomangicols A–C (**226–228**) [117] and mangicols A–G (**229–235**) [118], both with unusual carbon skeleton that constitutes two new classes of rearranged sesterterpenes. Neomangicols A (**226**) and B (**227**) were found to be active against a variety of cancer cell lines. Neomangicol A was most active against MCF7 and human colon carcinoma CACO2 cell lines, displaying IC_{50} values of 4.9 and 5.7 μM, respectively. Neomangicol B was less active having a mean IC_{50} value of 27 μM across the entire panel (versus 10 μM for neomangicol A). Mangicols A–G (**229–235**) showed weak cytotoxicity with IC_{50} values ranging from 18 to 36 μM in the 60 cell lines panel.

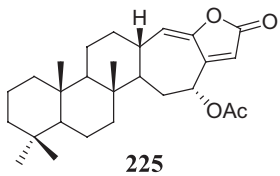
Aspergilloxide (**236**), a sesterterpene epoxide diol with a new carbon skeleton was isolated from the marine fungus of the genus *Aspergillus*. It showed little cytotoxicity towards HCT116, but its acetate derivative (**237**) inhibited HCT116 cell line at 61 μM [119].



223

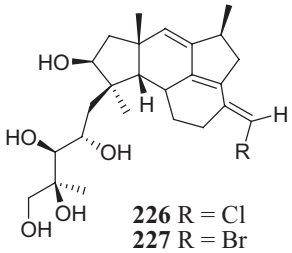


224

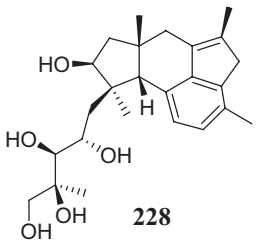


225

Hyatella intestinalis (S. E. Queensland, Australia) yielded the cytotoxic norsesterterpenoid mooloolabenes A–E **238–242** and sesterterpenoid mooloolaldehyde **243** [120]. All compounds were cytotoxic toward the P388 mouse leukemia cell line, with IC_{50} values in the range 3–10 µg/ml. *Phyllospongia papyracea* (Papua New Guinea) yielded acylated scalarane-type sesterterpenoids, [121] while the same sponge collected in Hainan, China, yielded very similar metabolites, of which **244** was cytotoxic against the leukemia P388 cancer cell line, with an IC_{50} value of 5 µg/ml. The cytotoxic scalarane-type sesterterpenoids **245** and **246** were obtained from *Hyrtios erectus* (Kavieng, Papua New Guinea). Compounds were tested in the antimitotic assay against human breast cancer MCF-7 cells, showing a toxicity at concentration of 50 µg/ml [122]. A series of cytotoxic scalarane sesterterpenoids **247–256** were obtained from a *Smenospongia* sp. (Soheuksan Is., Korea). Compounds showed significant cytotoxicity against a K562 cell line, and some were even more potent than doxorubicin [123].



226 R = Cl
227 R = Br



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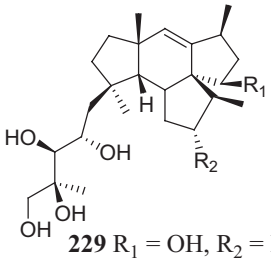
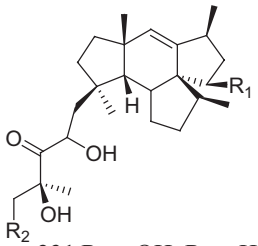
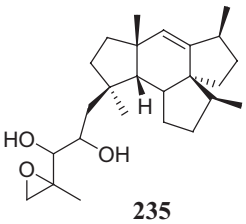


229 R_1 = OH, R_2 = H
230 R_1 = H, R_2 = OH
232 R_1 = R_2 = H



231 R_1 = OH, R_2 = H
233 R_1 = H, R_2 = OH
234 R_1 = R_2 = H



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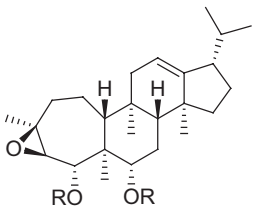
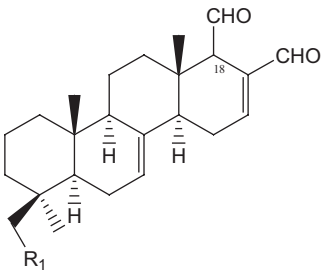
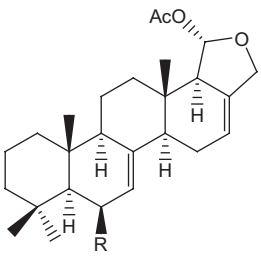


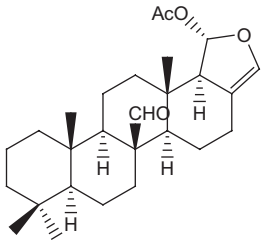
236 R = H
237 R = Ac



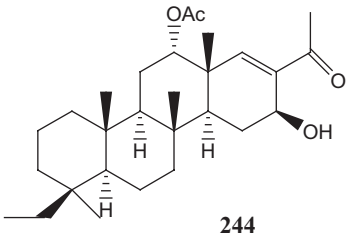
238 (18*R*) R_1= H
239 (18*S*) R_1= H
240 (18*R*) R_1= OAc



241 R= OAc
242 R= H



243



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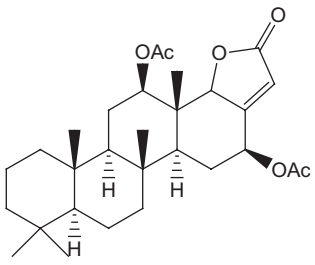

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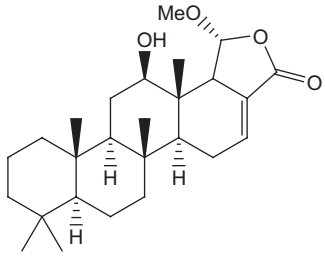
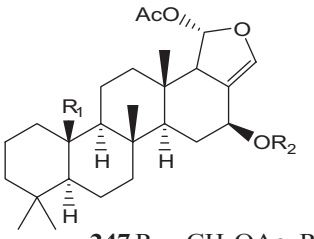


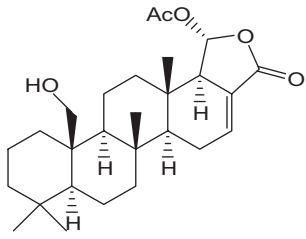
246

Cytotoxic phyllofenones D **257** and E **258** were isolated from *Phyllospongia foliascens* (Yongxing Is., China). Compound **257** showed cytotoxic activity against the P388 leukemia cell line with an IC_{50} value of 6.5 μg/ml [124]. Five sesterterpenoids **259–263** were isolated from the sponge *Hyatella* sp. (Soheuksan-do, Korea). Compounds **259–263** exhibited moderate cytotoxicity against a K562 cell line, antibacterial activity, and weak inhibitory activity against isocitrate lyase [125]. The scalarane sesterterpenoid hippospongide B **264** was isolated from a

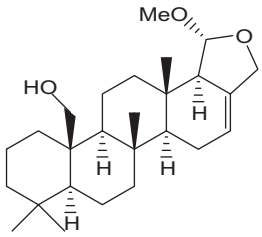
sponge *Hippospongia* sp. (Taitung, Taiwan). Hippospongide B **264** exhibited significant cytotoxicity against DLD-1, HCT-116, T-47D, and K562 cancer cell lines [126]. Heteronemin **265**, a spongean sesterterpenoid isolated from the sponge *Hyrtios* sp., was able to affect several cellular processes, including cell-cycle, apoptosis, mitogen-activated protein kinases (MAPKs) pathway and the NF-κB activation cascade. The potential ability of the compound to potently inhibit NF-κB pathway and affect cell viability was confirmed by *in vitro* studies, thus showing heteronemin as a potent and promising inhibitor of TNFα-induced NF-κB activaton as well as an apoptosis inducer [127, 128]. It has been shown that heteronemin induces apoptosis as shown by annexin V-FITC/propidium iodide-staining, nuclear morphology analysis, pro-caspase-3, -8 and -9 and poly(ADP-ribose) polymerase (PARP) cleavage as well as truncation of Bid on chronic myelogenus leukemia cells [129]. Furthermore, heteronemin inhibited important mechanisms contributing to tumour intravasation in vitro [130].



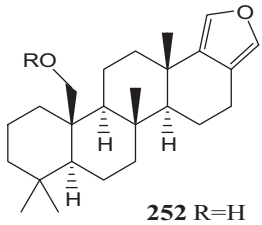
247 $R_1 = CH_2OAc$, R_2=Ac
248 R_1 = Me, R_2= H
249 R_1 = CHO, R_2= Ac



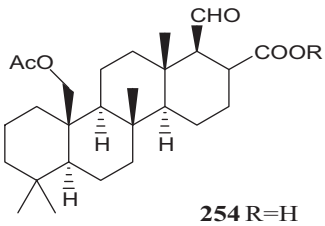
250



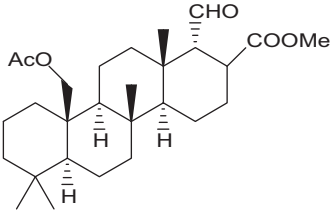
251



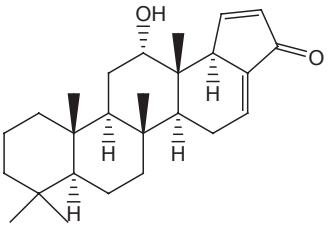
252 R=H
253 R=Ac



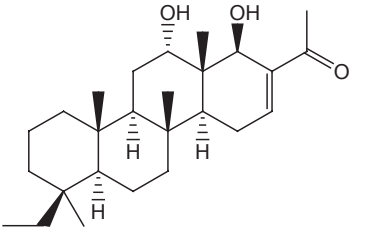
254 R=H
255 R= Me



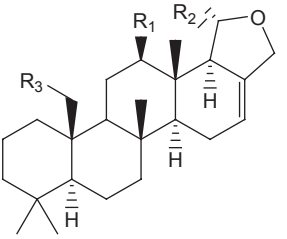
256



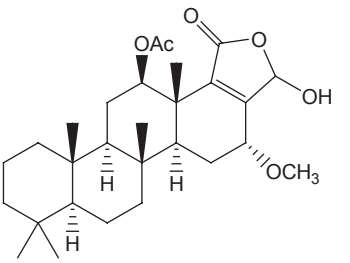
257



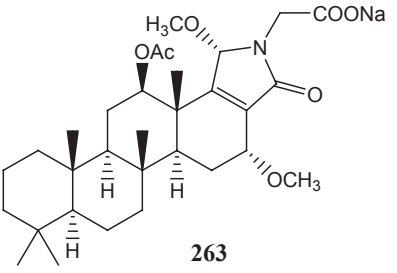
258



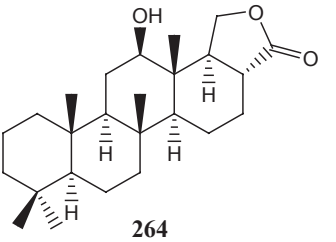
259 R_1= OAc, R_2= OH, R_3= OAc
260 R_1= OH, R_2= OH, R_3= OAc
261 R_1= OAc, R_2= OMe, R_3= H



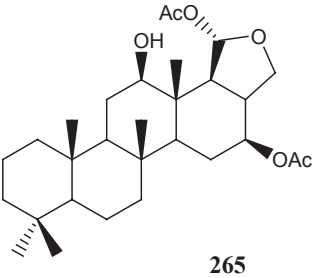
262



263



264

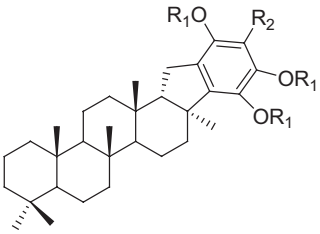


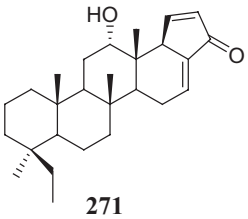
265

22.7 Pentacarbocyclic Sesterterpenoids

Although numerous marine sesterterpenoids have been found, only a few sesterterpenoids possessing a pentacarbocyclic skeleton have been isolated. Disidein (**266**) and two halogenated related derivatives (**267, 268**) were isolated from the Mediterranean sponge *Dysidea pallescens* [131, 132]. The stereochemistry of disidein was determined by X-ray analysis of the acetyl derivatives of bromo-disidein (**270**), which shows the same carbon skeleton of scalarane. The triacetyl disidein (**269**) showed moderate analgesic activity [133].

Phyllofenone B (**271**), an additional bishomoscalarane derivative with a pentacarbocyclic skeleton was isolated from *P. foliascens*. It showed cytotoxicity against P388 cells with IC_{50} value of 5.0 μg/ml [134].





271

266 $R_1 = R_2 = H$; **269** $R_1 = Ac$, $R_2 = H$
267 $R_1 = H$, $R_2 = Br$; **270** $R_1 = Ac$, $R_2 = Br$
268 $R_1 = H$, $R_2 = Cl$

22.8 Conclusions

Cancer remains a global health problem and a major cause of death worldwide. Facing such an alarming disease progression, varieties of therapies have been developed but expected efficiency has still not been reached. Thus the need to develop innovative strategies is crucial. Progress in the knowledge about tumor biology and molecular aspects of cancer has facilitated the design of new therapies aiming to overtake the limitations faced by research during the past decades. The main goal in anti-cancer approaches is to maximize efficacy of cancer treatments and to minimize systemic toxicity. The actual knowledge about natural compounds from marine sponges, such as sesterterpenoids represent a powerful tool, although there is still the need to deepen the effects of such molecules in preclinical studies.

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Chapter 23
Advances of Microtubule-Targeting Small Molecular Anticancer Agents from Marine Origin

Xiaobo Wang, Lun Yu, Zhiguo Liu, Pengfei Xu, Huilong Tan, Tao Wu and Wenbin Zeng

Abstract The unparalleled effectiveness of microtubule-targeting drugs has been validated by the successful use of vincristine, vinblastine, and paclitaxel for the treatment of a wide variety of human cancers. However, drug-resistance, both inherent and acquired, is common because of constant usage of anticancer agents. A worldwide search for compounds with similar mechanisms of action but improved characteristics has been urgent for wide clinical use. The largely unexplored marine world that presumably harbors the most biodiversity has provided inspiration for the development of new chemical classes of anticancer drugs. As such, they have also received considerable attentions from both academic industrial researchers who are interested in their bioactivity and chemistry. In this paper, several classes of typical microtubule-targeting marine natural products with diverse structure and unique bioactivity are summarized, focusing on their chemical and bioactive properties. Particularly, the preclinical and clinical trials of microtubule-targeting marine natural products are also discussed.

Keywords Microtubules · Anticancer agents · Marine natural products · Small molecular

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23.1 Introduction

Microtubules have fibrillar structures that play an important role in cellular processes including transport, signaling and mitosis [1]. They are composed of tubulin which is a heterodimer of closely related and tightly linked globular α- and β-tubulin proteins. There is a dynamic equilibrium between free and polymerized tubulin [2]. The dynamic process can be a target of anticancer drugs, which inhibits tubulin through polymerization (taxanes, epothilones etc.) or depolymerization (vinca alkaloids etc.) [3]. The chemical and biological diversity of the marine environment is immeasurable and therefore is an extraordinary resource for the discovery of new small molecular anticancer agents [4]. The widespread use of the marine natural products has provided unparalleled validation of microtubules as a high-value molecular target for the development of new anticancer drugs. A number of antimitotic marine natural products or synthetic analogs have cleared the multiple hurdles of the drug development pathway to reach phase I and phase II clinical trials, and there is the real hope that one or more of these will become clinically useful drugs alongside Vinca alkaloids and the taxanes [5]. Herein, several classes of typical microtubule-targeting marine natural products are outlined. These bioactive marine natural products are isolated from different organism include invertebrate animals, algae, fungi and bacteria. In addition, the structures and dynamics properties of microtubule are also discussed.

23.2 Microtubules as a Novel Anticancer Target

23.2.1 Biological Characteristic of Microtubules in Mitosis

Microtubules are highly dynamic polymeric protein fibers that have important cellular roles in mitosis, transport, positioning of organelles, and determination of shape [5]. They have specific structures composed of α- and β-tubulin heterodimer subunits that are changing their conformation dynamically by chaperones as a heterodimer complex [6]. A single microtubule is comprised of 10–15 protofilaments that further polymerized to give rise to the characteristic hollow cylinder with external diameters of 24 nm [7]. Because of the head-to-tail association of the α- and β-heterodimers, microtubules have polar structures and different polymerization rates at two ends. This final structure is organized in a polar manner such that the α-tubulin subunit is exposed at one end (the minus end), while the β-tubulin subunit is exposed at the other (the plus end). In each of protofilament, the α- and β-heterodimers oriented with α-tubulin subunit is exposed at the slower-growing end (the minus end), while the β-tubulin subunit is exposed at the faster-growing end (the plus end). Meanwhile, there is another tubulin isoform, γ-tubulin, which has functions as a template for the correct assembly of microtubules [8]. GTP binding and the process of hydrolysis on β-tubulin mainly dictate the stability of the

microtubule polymer at the more dynamic plus end. There are two GTP binding sites on tubulin, a hydrolyzable site on the β-subunit and a non-hydrolyzable site on the α-subunit. For assembly into microtubules, the β-tubulin subunit must be bound to GTP at the hydrolyzable site, shortly after which the GTP is hydrolyzed to GDP. Thus, the majority of β-tubulin is in the GDP-bound form and "capped" with GTP-bound tubulin at the plus end. When this GTP cap is stochastically lost, the protofilaments break up and the microtubules rapidly depolymerize. Soon after polymerization, the tubulin subunits hydrolyse their bound GTP and become non-exchangeable. Thus, the microtubule lattice is mainly composed of GDP-tubulin, which has the function for depolymerization characterized by the rapid loss of GDP-tubulin subunits from the microtubule plus end.

23.2.2 Mechanisms of Microtubule-Targeting Agents

Microtubules are dynamic filamentous cymentous cytoskeletal proteins that are responsible for cellular integrity and architecture, mitosis, intracellular transport, cell signaling, and gene expression. Tubulin exists in the cell as dimmers of α and β subunits, which are associated with regulatory proteins such as microtubule-associate proteins. There is a dynamic equilibrium between free and polymerization tubulin causing a state called "dynamic instability". Disrupting this equilibrium either through inhibiting tubulin polymerization or depolymerization can arrest cells at G2/M and induce apoptosis [3]. So, marine natural products that target the microtubule can be classified into two main groups based on their mechanism of action. Microtubule-destabilizing agents promote depolymerization and prevent polymerization of tubulin, and microtubule-stabilizing agents promote polymerization of tubulin and stabilize the polymer, preventing depolymerization [9].

23.3 Marine Agents with Microtubule-Depolymerizing Activity

23.3.1 Halichondrins and Halistatins

In 1985, a polyether macrolide, Norhalichondrin A (**1**), was first found in a series of potent antitumour compounds from the Japanese sponge (*Halichondria okadai* Kadota) [10]. Subsequently, the isolation of the same Japanese sponge afforded further seven halichondrins [11]. Three typical classes of halichondrins, norhalichondrins A (**1**), B (**2**) and C (**3**), halichondrins B (**4**) and C (**5**), and homohalichondrins A (**6**), B (**7**) and C (**8**), could be characterized by the degree of oxidation at C12 and C13. Among them, halichondrin B (**4**) is the most cytotoxic because it has an IC_{50} in the 0.1 ng/mL range in vitro against B-16 melanoma cell line and %T/C values (157–244) in vivo against the same cell line [11].This study ascertained that it was

of very high potency. In vitro evaluation of halichondrin B (**4**) and homohalichondrin B (**7**) indicated that both of them are noncompetitive inhibitors of the binding of vinblastine to tubulin [12]. Further studies with [^{3}H]-labeled halichondrin B demonstrated that halichondrin B (**4**) was bound to and dissociated from tubulin rapidly at one binding site per α/β-heterodimer, with an apparent Kd of 0.31 μM [13]. Thus, it becomes a clear candidate for clinical development.

The promising antimitotic activity of halichondrin B and the limited supply posed a dilemma and then stimulated its total synthesis [14]. As one of the witnesses, researchers of Eisai Research Institute made and screened more than 180 analogs of halichondrin, and they found that eribulin (**9**), only the macrolide fragment of halichondrin B, showed just the same antimitotic activity as its natural product in vitro and in vivo [15–17]. The truncated halichondrin B analogue, eribulin mesylate (E7389) (**9**), was tested in phase I trials as a single agent or for combination drug therapy with carboplatin, cisplatin or gemcitabine against solid tumors. The maximum tolerated dose, toxicity profile, preliminary anticancer activity and pharmacokinetics were evaluated by Goel and Tan, respectively [18, 19]. Jordan and his colleague further demonstrated that different from other antimitotic drugs such as vinblastine and paclitaxel, eribulin (**9**) works by an end-poisoning mechanism that results predominantly in inhibition of microtubule growth, but not shortening [20], and also generats tubulin aggregates [21]. Shortly, eribulin (**9**) has a novel mechanism of action involving modulation of microtubule dynamics and is greatly promising in clinical development. Currently, eribulin (**9**) has entered a series of phase II trials for the solid tumors, such as sarcomas, head and neck tumors, non-small cell lung cancer, and breast cancer [22–24] and phase III trials for the treatment of prostate, sarcoma, breast, NSCL, bladder, head and neck and ovarian cancers [25, 26]. The isolation of halistatin 1 (**10**), an isomer of halichondrin C, was isolated from the sponge *Phakellia carteri* collected in the Republic of Comoros [27]. Later, they described the structure of halistatin 2 (**11**) [28], isolated from *Axinella* cf. *carteri* also collected in the Comoros, and halistatin 3 (**12**) isolated from a *Phakellia* sp. collected in the Federated State of Micronesia [29]. Furthermore, halistatin 1, halistatin 2 and halichondrin B were evaluated in the NCI's human tumour primary in vitro screen. The overall mean GI_{50} values were 7.1×10^{-10}, 6.8×10^{-10} and 2.3×10^{-10} M, respectively [28], suggesting that these products are of high anticancer activity (Figs. 23.1 and 23.2).

23.3.2 Rhizoxin Analogs

Rhizoxin (**13**), a 16-membered macrolide, was firstly isolated from *Rhizopus chinesis*, the pathogen of rice seedling blight. Further studies demonstrated that it exhibits antimitotic activity in many eukariotic cells by inhibition of microtubule polymerization [30] and is active against vincristine- and adriamycin-resistant tumor cell lines in vitro and in vivo [31]. In 2000, Roberge and his colleague isolated and reported the structure of rhizoxin analog WF-1360 C (**14**) and the new seco acid (**15**)

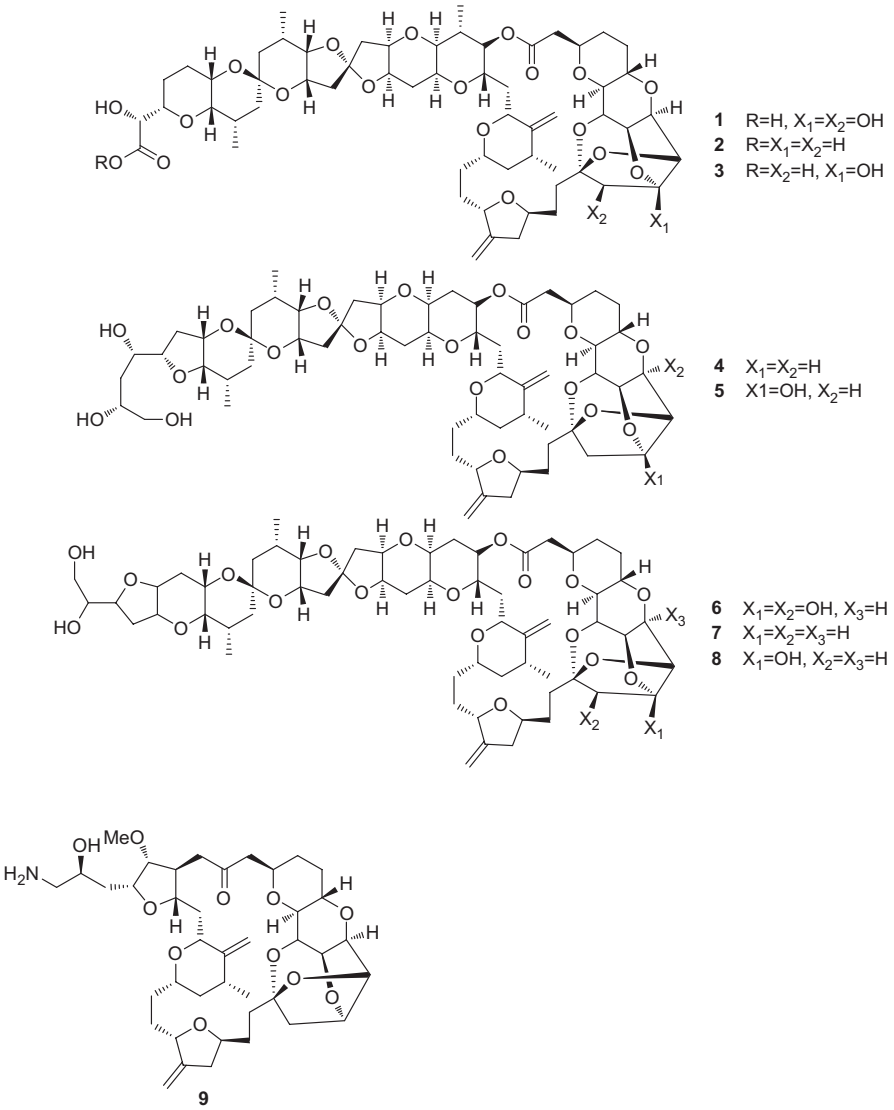


Fig. 23.1 The chemical structures of halichondrins and their analogues

from laboratory cultures of a marine bacterial *Pseudomonas sp.*, collected off the coast of British Columbia [32]. It is assumed that they inhibit tubulin polymerization in a manner similar to rhizoxin (**13**) [33]. A schematic model for the interaction of rhizoxin with β-tubulin at the rhizoxin-maytansine binding site was proposed by Iwasaki and his co-workers [34]. It suggested that, upon binding to β-tubulin, the bicyclic portion of rhizoxin was incorporated into a hydrophobic pocket. Furthermore, Roberge's cell-based assay for antimitotic agents showed **14** and **15** had an

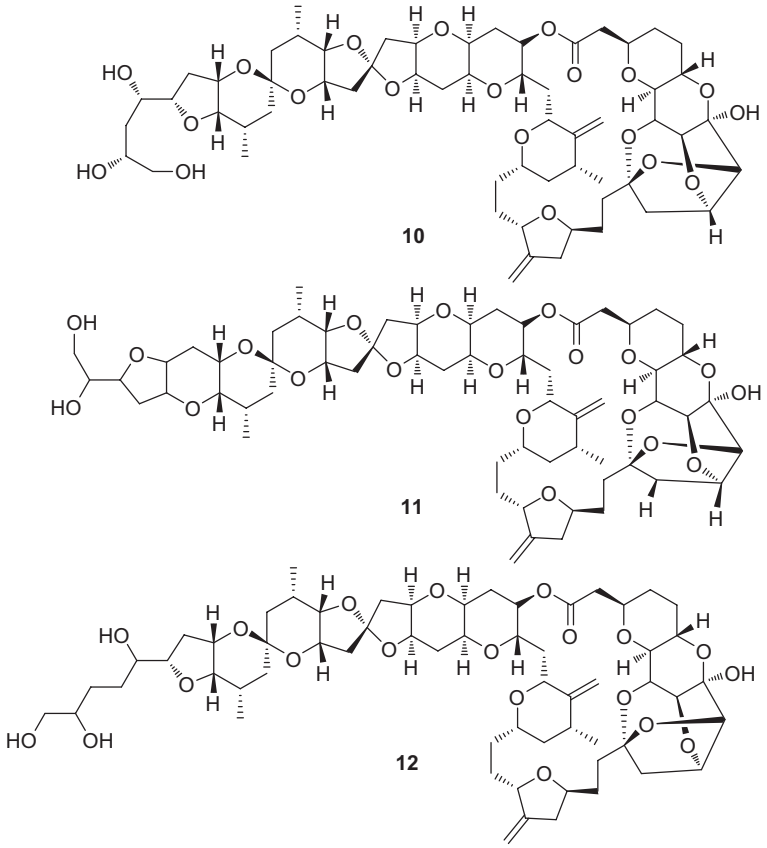


Fig. 23.2 The chemical structures of halistatins

IC_{50} of 52 nM and 8 nM, respectively [32]. The potent biological activity, potential as chemotherapeutic agents and unique structure of rhizoxins prompted scientists to undertake the total synthesis of this class of compounds. In 1993, Ohno and co-workers reported the first total synthesis of rhizoxin (**13**) itself from three subunits with Horner-Emmons and Julia olefinations as coupling strategies [35]. It was synthesized with 8% overall yield by a 52 steps synthetic procedure. Later, Kende and co-workers reported the first total synthesis of natural didesepoxyrhizoxin using a triply convergent strategy with a 1.7% overall yield by a 39 steps synthetic procedure [36]. In addition, there have been a great deal of efforts towards total synthesis of this class of compounds [37–39]. Preclinical and phase I studies with rhizoxin by Graham and his colleagues described the preclinical and clinical pharmacology of rhizoxin with a pharmacokinetically guided dose-escalation (PGDE) strategy. The evaluation of phase I found a maximum tolerated rhizoxin dose of 2.6 $mg \times m^{-2}$, with reversible, but dose-limiting, mucositis, leucopenia and diarrhoea [40]. Multicentre phase II clinical trials were then initiated by the EORTC ECSG in melanoma,

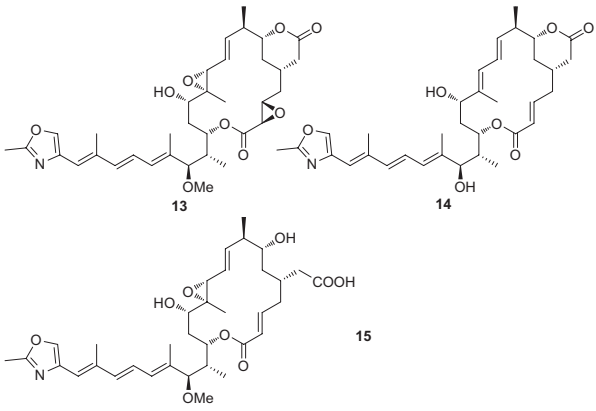


Fig. 23.3 The chemical structures of rhizoxin analogs

breast, head and neck, and non-small-cell lung cancers. These studies demonstrated the rapid and variable elimination of rhizoxin from the systemic circulation. The presence of pharmacodynamic relationships and the low level of systemic toxicity suggested that future trials of rhizoxin with alternative dosage or treatment schedules may be warranted [41] (Fig. 23.3).

23.3.3 Dolastatins

The dolastatins were originally isolated from the sea hare, *Dolabella auricularia*, by Pettit et al in the 1970s [42]. Later, it was identified as secondary potent antimitotic and cytostatic bioactive metabolites, which is resulted from consumption of marine cyanobacteria of the genus *Symploca*. The extremely small quantities of dolastatins present in the sea hare extracts make the assay-guided isolation and structure elucidation exceptionally challenging. As part of a 15-year sustained effort for elucidating syntagmatic relation, the total synthesis and complete absolute configuration of dolastatin 10 (**16**), dolastatin 15 (**17**) were reported by Pettit's group [43,44]. The dolastatins, essentially a class of small oligopeptides, contain four unique non-protein amino acids dolavaline (Dov), dolaisoleucine (Dil), dolaproline (Dap) and dolaphenine (Doe). In this family, the linear peptide dolastatin 10 (**16**) and a seven-unit depsipeptide, dolastatin 15 (**17**), were chosen for research in view of potent and promising anticancer activities at picomolar or low nanomolar concentrations [45]. Mechanistically, Bai and his coworkers have shown that they cause cell cycle arrest in metaphase by strongly inhibiting microtubule assembly, tubulin-dependent GTP hydrolysis, and binding of vinca alkaloids to tubulin [46–48]. Dolastatin 10(**16**) showed more potent activity in a variety of mouse models of cancer than paclitaxel or vinblastine. It has been evaluated in several phase I and phase II anticancer clinical trials [49–52]. Therefore, the novel mechanism of action, high potency, and positive therapeutic index in preclinical and clinical models accelerated the development of more effective analogues. Miyazaki and coworkers synthesized a series

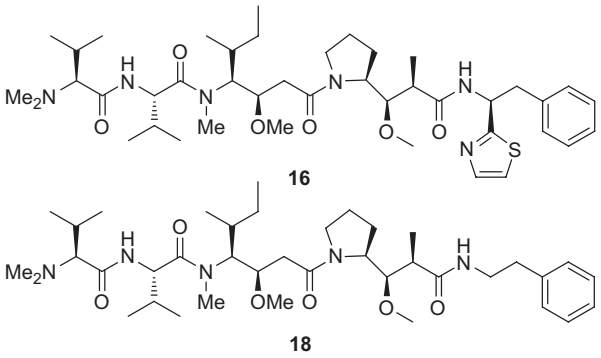


Fig. 23.4 The chemical structures of dolastatin 10 and its analogues

of dolastatin 10 (**16**) analogs, and found that TZT-1027 (Soblidotin, **18**) without the thiazole ring, also showed excellent antimicrotubule activity against P388 leukemia in mouse models [53, 54]. The antimicrotubule activities of TZT-1027 were superior or comparable to those of the reference agents such as paclitaxel, vincristine. In experiments with drug-resistant P388 leukemia, TZT-1027 showed good activity against drug-resistant P388 leukemia [54]. Furthermore, TZT-1027 could attack the well-developed vascular system of advanced tumors by a putative protein kinase-dependent mechanism, and then blocked tumor blood flow. It was proposed that it kills tumors by exerting a combination of indirect anti-vascular effects and direct cytotoxic effects, making it a potentially powerful in clinical cancer therapy [55]. TZT-1027 has been evaluated in both phase I and phase II clinical trials [56–58]. A second derivative in clinical trials is tasidotin (ILX-651) (**19**), an analogue of dolastatin 15. It is also a tubulin-interactive drug, weakly inhibiting tubulin polymerization to microtubules but strongly suppressing the dynamic instability of microtubules. It has completed phase I trials and is currently in phase II trials under Genzyme. Further details of the discovery and development of the dolastatins are available in a comprehensive review [59]. Cematodin (LU-103793, **20**), the third derivative of dolastatin 15 (**17**), which is the hydrochloride salt of a pentapeptide containing some of the unusual amino acids found in dolastatin 15, was selected for clinical trials. The cytotoxicity of cemadotin was caused by binding at a novel site on tubulin to result in the suppression of spindle microtubule dynamics [60]. Unfortunately, the clinical trials of cemadotin have not gained expected activity in patients with advanced breast cancer given as third-line chemotherapy as expected [61] (Figs. 23.4 and 23.5).

23.3.4 Cryptophycins and Arenastatin

In 1994, Kitagawa and his colleague isolated arenastatin A (**21**) from specimens of the Okinawa sponge *Dysidea arenaria* [62]. Arenastatin A (**21**) is a cyclic didepsipeptide and exhibited extremely potent cytotoxicity against KB cells with IC_{50}

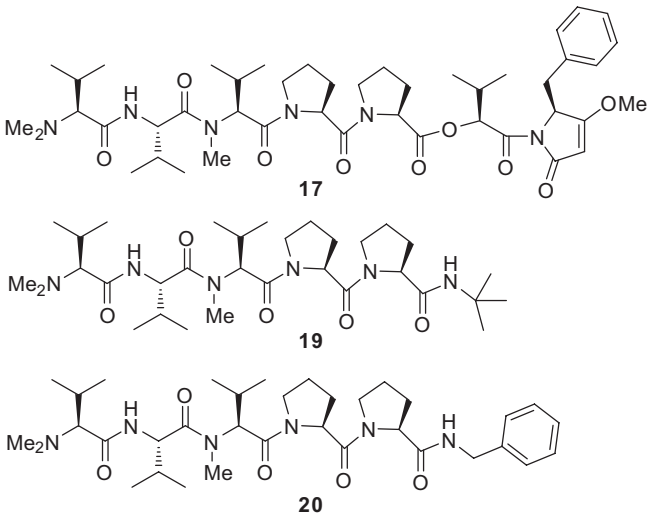
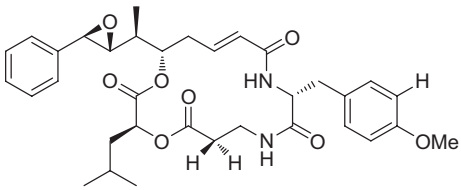


Fig. 23.5 The chemical structures of dolastatin 15 and its analogues

of 5 pg/mL. Also in 1994, cryptophycins 1 (**22**) to 4 (**25**) was isolated by Moore's group from laboratory cultures of the freshwater cyanobacterium *Nostoc* sp. (GVS 224). Subsequently, they discovered the potent and selective antitumor characteristics of the cryptophycin class of agents, which resuscitated interest in these novel depsipeptides [63]. Up to now, more than 28 cryptophycins have been reported. These natural compounds cause hyper-phosphorylation of Bcl-2, triggering the apoptotic cascade, arrest tumor cells at the G1/M phase, inducing a block in cellular proliferation, and inhibit the growth of tumor cells [64]. Cryptophycins are also potent chemotherapeutic agents because they are poorer substrates for p-glycoprotein pumps and are conferred an advantage in the chemotherapy of multidrug-resistant tumors cell lines [65]. Among these cryptophycins, cryptophycin 1 (**22**) is 100–1000 times more potent than vinblastine or paclitaxel as an antiproliferative agent. It disrupts microtubule dynamics, inhibits nucleotide exchange, causes tubulin to aggregate into small ring-shaped oligomers, and binds to the Vinca domain to lead to noncompetitively inhibiting the binding of vinblasine but not colchicine to tubulin [66–68]. The lack of large scale isolation methods for cryptophycin 1 and its high potency accelerated the development of more effective analogues. Hundreds of synthetic analogues had been produced for the structure-activity relationship (SAR) studies in search for more powerful and drugable compounds [69, 70]. Among these compounds, Cryptophycin-52(LY355703, **26)**, a synthetic analog of cryptophycin 1, is in advanced development. It has demonstrated very potent antiproliferative activity against human tumor cell lines in vitro (IC_{50}s ranging from 12 to 40 pM). The mechanism of action includes arresting cells in the G2-M phase of the cell cycle by binding to microtubules and suppressing their dynamics [71–73]. It also had a broad range of antitumor activity against both murine and human tumors alone and in combination regimens [74, 75]. Cryptophycin 52 (**26**) has been undergone phase

Fig. 23.6 The chemical structure of arenastatin A



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I clinical trials [76, 77].In addition, "clicktophycin-52" (**27**), a bioactive cryptophycin-52 triazole analogue, was synthesized via 1,4-disubstituted 1H-1,2,3-triazole ring replacing an endocyclic trans-amide linkage within the macrocyclic antitumor agent cryptophycin-52 (**26**). Compared to cryptophycin-52, in vitro cytotoxicity against the multidrug resistant human cancer cell line KB-V1 is only slightly reduced [78] (Figs. 23.6 and 23.7).

23.3.5 Curacins

In 1994, Curacin A (**28**) was originally isolated by Gerwick and his colleague from extracts of a field sample of Caribbean cyanobacterium *Lyngbya majuscula* collected in Curacao. Curacin A (**28**) showed potent brine shrimp toxicity and

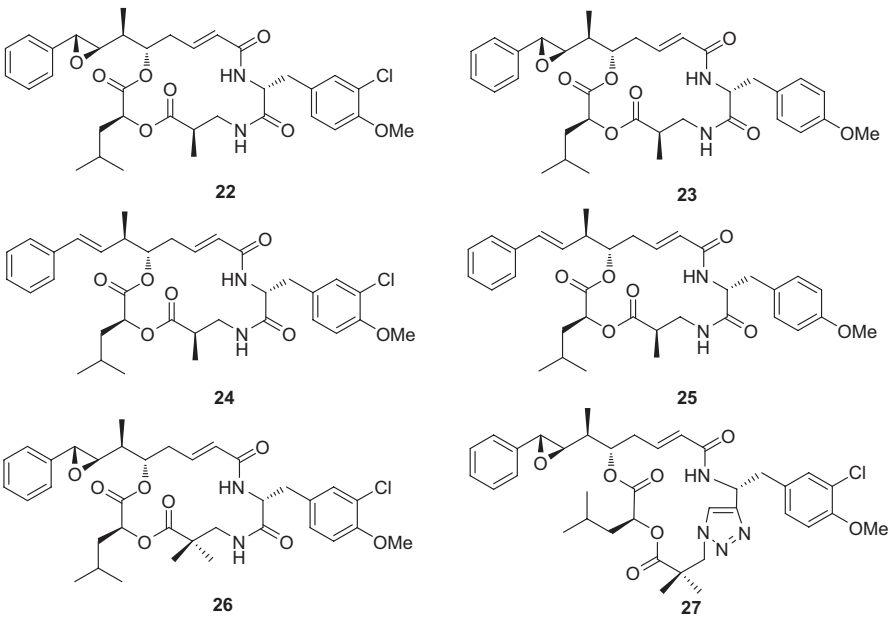

Fig. 23.7 The chemical structures of cryptophycins and their analogues

anti-proliferative activity in vitro [79].They further examined the initial Curacao collection of L. *majuscula* and isolated the minor analogs curacins B(**29**) and C(**30**) [80], while a Virgin Islands collection yielded curacin D (**31**) [81]. Subsequently, Nagle and his coworker compared the degradation products obtained from the natural product with the same compounds prepared enantiospecifically via synthesis to establish the absolute configuration of curacin A (**28**) [82]. Then the absolute configuration was confirmed by White's asymmetric total synthesis [83]. Evaluation of curacin A (**28**) using NCI cell line panel and COMPARE algorithm analysis provided the first indication that the compound was of tubulin-binding activity [79]. It was observed to bind selectively to the colchicine site of tubulin but not vinblastine/vincristine site, ascertaining that it belongs to the colchicine site family of antitubulin agents [84]. Further studies demonstrated that it was a competitive inhibitor of colchicine binding. Due to its extremely large association constant, its binding rate to tubulin is rapid while its dissociation rate from tubulin is extremely slow [85, 86]. Curacin A (**28**) exhibited an IC50 value of 0.72 ± 0.2 μM for inhibition of tubulin polymerization. Its inhibition of colchicine binding was found to be 94 ± 2 % at 5.0 μM. The natural product was also tested against MCF-7 breast cancer cells and found to have a GI50 (50 % growth inhibitory) value of 0.038 ± 0.01 μM.

All these studies demonstrated that Curacin A (**28**) is a potent in vitro cytotoxin that inhibits the growth of cancer cells at low nM concentrations. However, traditional chemical analogue of curacin A has met with limited success [87]. While many natural congeners with closely related structures showed comparable activity to curacin A (**28**), most synthetic derivatives were greatly inferior. Structural characters such as the presence of a readily oxidized thiazoline heterocycle, four double bonds including a conjugated diene, and, especially, high lipophilicity are strong deterrents from a therapeutic evaluation of curacin A and its analogues. The currently available data indicated that the greatest losses of activity occured in dramatic changes in structure, such as the introduction of large groups, the loss of substantive portions of the parent structure, or the replacement of the side chain with much simpler lipids. Nevertheless, the combination of the promising biological activity of the curacins with their novel structures stimulated extensive synthetic efforts toward making drug-like analogs [88] (Fig. 23.8).

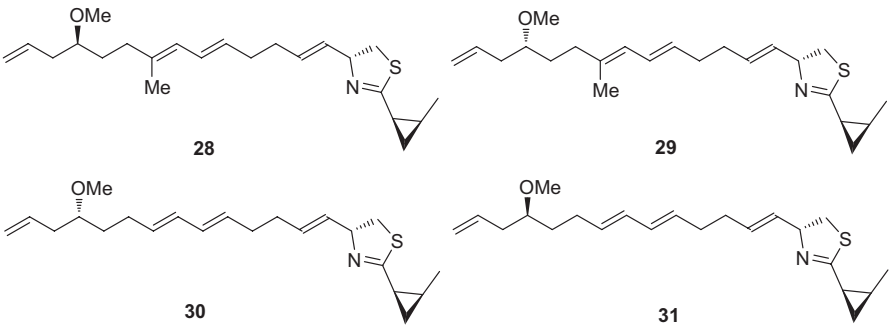

Fig. 23.8 The chemical structures of curacins

23.3.6 Methoxyconidiol and Stypodiols

Methoxyconidiol (**32**) is a meroterpene previously isolated by Banaigs and coworkers in 2005 from the ascidian *Aplidium aff. densum* collected off the coast of Masirah Island, Oman [89]. Primary biological screening showed that it was a moderate antiproliferative activity against bacteria but inactive at milimolar concentrations against mammalian tumor cell lines. Further investigation in sea urchin embryos revealed that methoxyconidiol shows arrested cells in M phase, completely cytokinesis inhibitation, and microtubule-disruptive activities without having any effect on DNA replication [90]. Moreover, while the cell cycle regulatory kinase cyclin B/CDK1 is activated, cyclin B proteolysis is inhibited, impeding the output of M-phase. This characteristic cell cycle arrest induced by methoxyconidiol in sea urchin eggs emphasized the interest for this drug as a putative antiproliferative agent for tumor cells. The discovery of stypotriol (**33**) from the marine brown algae *Stypopodium zonale* was reported by Fenical and his colleague. It was found to have cytotoxicity, and also showed anti-fish feeding activities [91]. Initial studies showed that this marine natural product inhibits beef brain microtubule assembly in vitro [92, 93]. Further biological investigations revealed that this compound disrupts the establishment of the mitotic spindle, blocks cytokinesis, and arrests cells in the G2 stage of development [94, 95]. In addition, Maccioni and his coworker reported the isolation of 14-keto-stypodiol diacetate (**34**) from the algae *Stypopodium flabelliforme* collected off the coast of Easter Island. They also found that the activity against microtubules of 14-keto-stypodiol diacetate (**34**) is similar to that of stypotriol (**33**) [96] (Figs. 23.9 and 23.10).

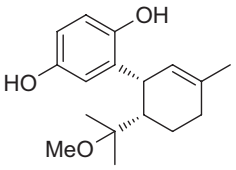

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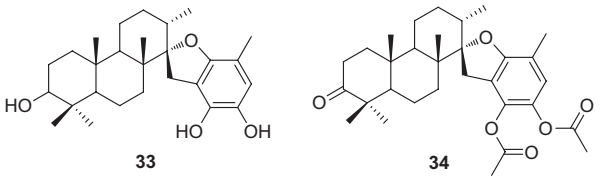
Fig. 23.9 The chemical structure of methoxyconidiol



33 **34**

Fig. 23.10 The chemical structures of stypodiols

23.4 Marine Agents with Microtubule-Stabilizing Activity

23.4.1 Discodermolides

Discodermolide (**35**), a unique polyketide, was first isolated from the Caribbean deep-sea sponge *Discodermia Dissoluta*. [97]. Years later, the isolation of a series of Discodermolide analogues including 2-epi-discodermolide (**36**), 2-desmethyldiscodermolide (**37**), 19-desaminocarbonyldiscodermolide (**38**), 5-hydroxymethyldiscodermolate (**39**), and 9-cyclodiscodermolide (**40**), were reported [98]. These compounds were collected from samples of sponges in the genus *Discodermia* around the Bahamian archipelago waters. The gross structure of discodermolide was determined by extensive spectroscopic studies and the single crystal X-ray crystallography helped to establish its relative configuration. Later, Hung and coworkers synthesized both (+) and (−) optical antipodes of **35**, and it turned out that the natural product was the (+) enantiomer [99, 100] (Fig. 23.11).

Initially reported to be a potent immunosuppressive agent, (+)-discodermolide was later recognized to possess significant antiproliferative activity in vitro [101]. Years after, researches to investigate the biological screening of a variety of human and murine cell lines revealed pronounced cytotoxicity, causing cell cycle arrest at the G2/M phase with IC_{50} values ranging from 3 to 80 nM. It was disclosed that in a similar fashion to Taxol, discodermolide functions by stabilising microtubule, halting mitosis and causing cell death by apoptosis [102]. Subsequently, Kowalski reported that discodermolide exhibited the inhibition effect of in vitro proliferation of P388 murine leukemia cells with an IC_{50} of 0.5 mg/mL. Also, it inhibited the growth of *Candida albicans*, suppressed the two-way mixed lymphocyte response of both murine splenocytes and human periferal blood lymphocytes at 0.5 and 5 mg/mL respectively, with greater than 85 % viability of the splenocyte cells [103]. In vivo experiments on ovarian tumor xenograft bearing mice have shown that the

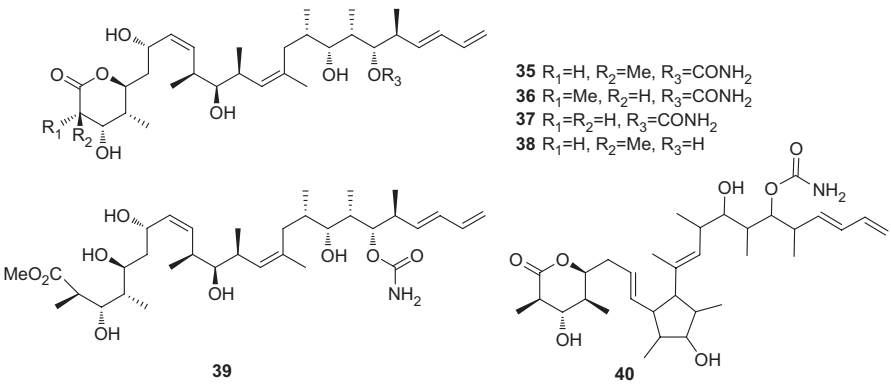


Fig. 23.11 The chemical structures of discodermolides

synergy activity of discodermolide with Taxol was observed, inducing tumor regression (not suppression alone as observed with a single treatment of either discodermolide or Taxol) without toxicity to the mouse [104]. Extensive NMR experiments allowed the detection of binding of discodermolide to unassembled tubulin α/β-heterodimers. Moreover, the combination of experimental TR-NOE and STD NMR data with CORCEMA-ST calculations indicated that discodermolide targets an additional binding site at the pore of the microtubules, which is different from the internal binding site at the lumen previously determined by electron crystallography [105]. The novel structure of discodermolide combined with its highly encouraging biological profile makes it an extremely attractive candidate for clinical development. In order to supply material for clinical trials, several groups worked on the total synthesis of discodermolide, represented by Smith group and Paterson group in which several generations of total synthesis were developed respectively [106, 107]. Markedly, Novartis Pharma AG licensed discodermolide from the Harbor Branch Oceanographic Institution and launched an impressive large-scale total synthesis campaign with 36 steps. Sixty grams of (+)-discodermolide were obtained. Then discodermolide was progressed into phase I clinical trials in patients with advanced solid malignancies in 2004. However, research on the agent was abruptly stopped when some indications of pneumotoxicity (3 of 32 patients) appeared [108]. In 2006, by using elegant NMR studies, Sanchez et al. elucidated the bioactive conformation of discodermolide bound to soluble tubulin and proposed a common pharmacophore with the epothilones [109]. More recently, Smith extended discodermolide analog program, wherein the metabolically less stable lactone portion of the molecule and C14–C15 olefin were replaced with isosteres either individually or together. Pleasingly, these analogs preserved the cytotoxicity at a level similar to (+)-discodermolide [110]. In addition, its analogues keeping the 'U' shape conformation were synthesized by Elsa and coworkers, and bioevaluations showed that such derivatives may be a clinical candidate in the near future [111].

23.4.2 Peloruside A

Peloruside A (**41**), a cytotoxic 16-membered macrolide, was isolated from extracts of the sponge *Mycalehentscheli* collected in Pelorus Sound on the north coast of the South Island, New Zealand [112]. It was initially found to be ten-fold less potent inhibitor of proliferation of P388 murine leukemic cells ($IC_{50} = 18$ nM) than the two other natural products isolated from the same sponge, mycalamide A and pateamine. Later, the absolute configuration was established since Liao and coworker fulfilled the total synthesis of (+)-peloruside A, which turned out to be the enantiomer of the natural product **41** [113] (Fig. 23.12).

With structure similar to the epothilones (Epo), Peloruside A is cytotoxic at nanomolar concentrations. In vitro evaluations demonstrated that Peloruside A inhibits microtubule dynamics at concentrations near those needed to block progression through the cell cycle, but much lower than those needed to cause tubulin assembly [114]. Like paclitaxel, peloruside A arrests cells in the G2/M phase of the cell cycle

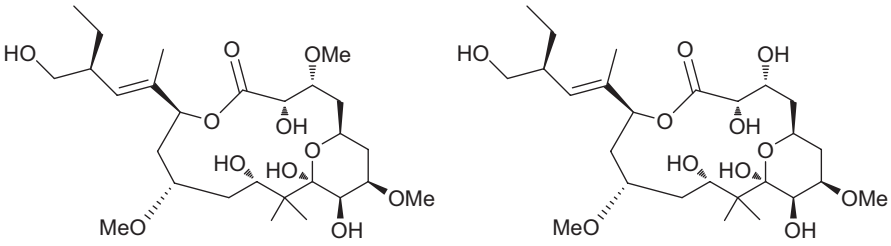


Fig. 23.12 The chemical structures of Peloruside A (**41**) and Peloruside B (**42**)

and induces apoptosis [115]. Gaitanos found that Peloruside A has a unique binding site on tubulin that differs from the taxoid site. Subsequent studies elucidated that peloruside A is not a good substrate for the P-glycoprotein efflux pump, but is active against drug-resistant cell lines with point mutations in the paclitaxel binding site on β-tubulin, and is unable to displace the fluorescent paclitaxel derivative Flutax-2 from tubulin [116]. Wilmes confirmed that peloruside A, when added in combination with other microtubule stabilizing agents, acts synergistically to cause tubulin polymerization in vitro and enhance the antimitotic action of the drugs [117]. The intriguing structure, very low natural abundance, and clinical potential of peloruside A have led to a great rise of synthetic interest. In 2010, McGowan described a convergent total enantioselective synthesis of peloruside A, which was featured by a chiral-catalyst-controlled diastereoselective hetero-Diels–Alder reaction [118]. Furthermore, some modifications have also been carried out. By a reduction reaction with sodium borohydride, Hood obtained pyranose ring opening analogue of peloruside A. Biological studies indicated that the IC50 for cell growth inhibition, compared with peloruside A, was increased 26-fold in a murine leukocyte cell line [119]. The activity of the side chain analogs was also under investigation by the laboratories of Drs Miller, Northcote, Diaz, and Paterson [120]. At the almost same time, Singj and coworkers synthesized the 3-des-O-methyl variant of peloruside A, named peloruside B (**42**). It was found that peloruside B possesses similar bioactivity to peloruside A, promotes microtubule polymerization and arrests cells in the G2/M phase of the cell cycle, as does paclitaxel [121]. Meanwhile, synthesis and biological evaluation of structural variants of peloruside B are the subjects of ongoing research in Ghosh laboratories. In a short summary, the relatively simple structure of peloruside makes it suitable for the design and synthesis of analogues with improved tumor targeting and reduced tumor cross-resistance.

23.4.3 Laulimalides (Fijianolides)

In back-to-back papers published in 1988, the structures of the cytotoxic polyketide macrolides laulimalide (fijianolide B) (**43**) and isolaulimalide (fijianolide A) (**44**)

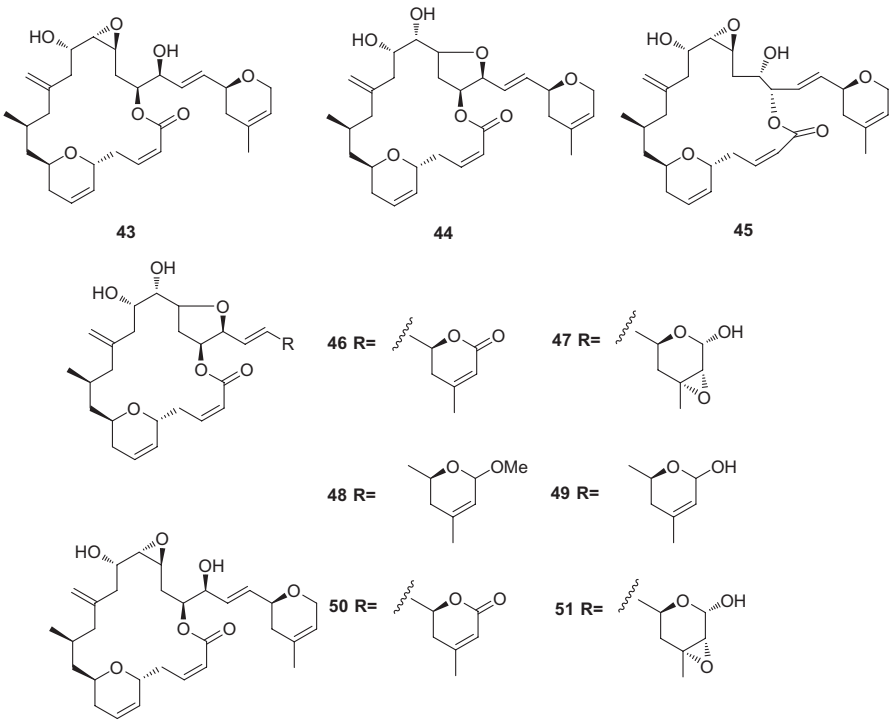
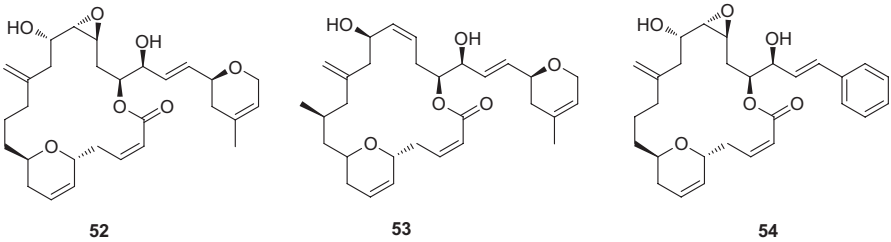


Fig. 23.13 The chemical structures of laulimalides (Fijianolides)

were reported jointly by two different groups [122, 123]. The biological tests revealed that laulimalideand isolaulimalide showed modest cytotoxicities (IC_{50}s of 0.5–11 mg/mL) for HT-29 human colon tumor cells and P388 murine leukemia, and against the KB cell line with IC_{50} > 200 ng/mL. Later, a third isomer named neolaulimalide (**45**) was isolated along with (**43**), (**44**) and other cytotoxic compounds from the sponge *Fasciospongia rimosa* [124]. Reisolation from *Cacospongia mycofijiensis* unearthed, six additional fijianolides [D(**46**)–I(**51**)], each varying with respect to the oxidation state and/or substitution pattern of the C_{20} sidechain [125] (Figs. 23.13 and 23.14).

As a new class of microtubule-stabilizing agents, Laulimalides act in a mechanism of microtubule stabilization and disruption of mitotic spindle formation, leading to apoptosis [122]. In 1999, Mooberry reported that laulimalide induced tubulin polymerization and stabilization with a mechanism of action similar to paclitaxel. However, unlike paclitaxel, laulimalide retained activity in P-glycoprotein (PgP)-overexpressing multidrug-resistant (MDR) cells [126]. It appeared to have a different binding site on tubulin compared with paclitaxel. This was also supported by the finding that laulimalide retained activity against cell lines containing tubulin mutations which showed to promote resistance to paclitaxel and epothilones [127]. These results were consistent, and indicated that the laulimalides in combination with paclitaxel or 2ME2 caused significant synergistic effects [128]. However, the recent research results by Bajaj M



52 53 54

Fig. 23.14 The chemical structures of laulimalides analogues

and coworkers indicated that, in C. elegans embryos, laulimalide acted as a microtubule stabilizing agent at concentrations of 200–1 μM, and a microtubule depolymerizer at concentrations of 50–100 nM, presenting a dose-dependent modulation of microtubule behaviour [129]. Such promising biological profiles highlight that laulimalide may be a highly attractive lead for the development of new anticancer agents. This sparked several total syntheses of laulimalides aimed at providing materialsupply for further biological evaluation. In 2009, Mulzer's group described a fully stereoselective and flexible approach for the synthesis of neolaulimalide (**45**) in 21 steps along the longest linear sequence in 3 % overall yield [130]. This group also made further improvements towards the preparation of larger amounts of laulimalide (**43**) [130]. The novel mechanism of action, high potency accelerated the development of more effective analogues too. Paterson and coworkers synthesized a series of analogues of laulimalide by simplifications in the macrocycle (deletion of the 11-methyl group) and the side chain (truncation or substitution), and found that the most potent analogue with low nanomolar IC_{50} values was 11-desmethyl-laulimalide (**52**) [131]. However, truncation or substitution of the side chain led to significant loss of activity, which clearly proved that the side chain must be a critical pharmacophore region for laulimalide. Wender and coworkers also described the syntheses of five laulimalide analogues, among which the des-epoxy laulimalide (**53**) was tested to be the most potent analogue with IC_{50} values averaging 0.11 μM. [132]. Despite the synthesis efforts mentioned above, the in vivo experiment disappointedly revealed that synthetic laulimalide exhibited only minimal tumor growth inhibition, whereas severe toxicity and mortality was observed. The results led to the conclusion that due to the unfavorable efficacy to toxicity ratio in vivo, laulimalide may be a poor candidate for clinical development [133].

23.4.4 Ceratamines

Ceratamines A (**55**) and B (**56**), the novel heterocyclic alkaloids, were isolated from extracts of the marine sponge *Pseudoceratina* sp. collected in Papua New Guinea in 2003. In cell-based assay, Ceratamines A and B both displayed promising antimitotic activity, with IC_{50}s of 10 μg/mL [134]. When discovered, the imidazo[4,5, d]azepine core heterocycle in the ceratamines appeared to have no precedent at any oxidation level among known natural products or synthetic compounds [135].

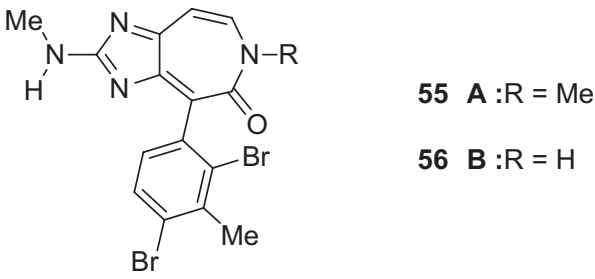


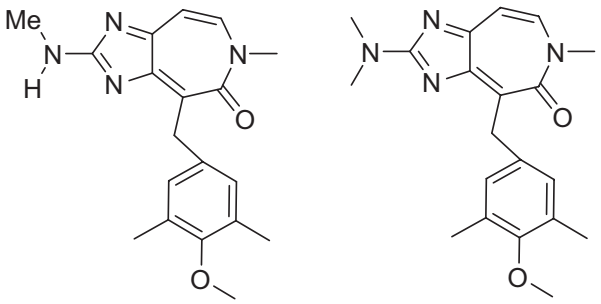
Fig. 23.15 The chemical structures of ceratamines

Importantly, they represented the first examples of a novel family of antimitotic heterocyclic alkaloids. In vitro evaluation further confirmed that the ceratamines do not compete with paclitaxel when binding to microtubules [136]. They stabilize microtubules but do not bind to the taxol binding site, and they produce a novel mitotic arrest phenotype characterized by the presence of multiple pillar-like structures of tubulin. These facts uncovered that the ceratamines affect the microtubule network of interphase and mitotic cells in ways that are different from those described for other microtubule-stabilizing agents (Fig. 23.15).

Unlike other microtubule-stabilizing agents identified thus far, the ceratamines are structurally simple due to no chiral centers, thus promoting the interest of the synthesis of ceratamines and their analogues. Coleman and coworkers reported the first synthetic route to ceratamines A (**55**) and B (**56**) that has the potential to produce sufficient quantities of the natural products for preclinical evaluation [137]. The synthesis of ceratamines analogues and their biological evaluation were mainly conducted by Nodwelland coworkers. At their initial research, they completed the synthesis of desbromoceratamine, which helped to provide information of the natural product structure and illustrated the importance of the bromine atoms for the remain of full potency and efficacy for antimitotic activity [138, 139]. Based on their previous work, Nodwell synthesized the ceratamine A analogue (**57**) and (**58**). The subsequent biological evaluations showed that replacing the bromine atom in the natural product with methyl groups generates analogues that are more active than natural ceratamine A, with IC_{50}s about 3.5 and 3.0 μg/mL, respectively. Meanwhile, the role of imidazoazepine heterocycle played in ceratamine was also been investigated. The analogue lacking aromatic imida-zoazepine heterocycle showed no antiproliferative activity, indicating that aromatic imidazoazepine heterocycle is an indispensable antimitotic pharmacophore in ceratamine. To our best knowledge, though ceratamine analogues into clinical trials hasn't been reported, efforts to explore new and bioactive compounds based on ceratamines for drug potential are still ongoing (Fig. 23.16).

23.4.5 Nigricanosides

Nigricanosides A (**59**) and B (**60**), potently cytotoxic glycolipids, were bioassay-guided isolated from extracts of the green alga *Avrainvillea nigricans* [140]. The finding was prompted by the fact that crude extracts of A. *nigricans* showed strong



57 **58**

Fig. 23.16 The chemical structures of ceratamine analogues

antimitotic activity in Roberge's cell-based assay. The methyl ester of nigricanoside A (**61**) was found to stimulate the polymerization of pure tubulin in vitro at 10 mM, arrest human breast cancer MCF-7 cells in mitosis with an IC_{50} of 3 nM, and inhibit the proliferation of both MCF-7 and HCT-116 cancer cells with IC_{50}s of 3 nM. Further biological evaluations were not reported due to the short supply of material. It was mainly because that the total synthesis of Nigricanosides was impeded by the lack of the information about the relative or absolute configurations of the stereogenic centers in the nigricanosides. The fact was acknowledged that nigricanosides are the first examples of a new class of ether-linked glycoglycerolipids. They are unique oxylipin derivatives including two oxygenated fatty acids and a galactosyl glycerol moiety that are connected to each other by ether bonds. Despite limited conformational information, the unique structure, strong bioactivity, and natural scarcity have prompted researchers to attempt its full stereochemical assignment and total synthesis of nigricanosides. Espindola et al applied a combination of MDEC and the 1D-TOCSY-MDEC to obtain the necessary coupling information from the synthetic C_{16} fatty acid to assign the C_9/C_{10} anti stereochemistry. This could help to resolve the configurational details of the nigricanosides [141]. Recently, Kinashi and coworkers developed an effective method for the stereoselective construction of the C8′–O–C6″ ether bond of Nigricanosides A. The method involved connecting the galactose moiety to the C20 fatty acid chain based on chirality transferring Ireland–Claisen rearrangement [142]. Such work together pushed forward the development of methodologies towards the total synthesis of nigricanosides (Fig. 23.17).

23.4.6 Eleuthosides/Sarcodictyins

Eleutherobin (**62**), a potently cytotoxic diterpene glycoside, was isolated in from extracts of a rare soft coral identified as an *Eleutherobia* sp collected from near Bennett's Shoal in Western Australia [143]. Subsequent in vitro biological study showed that it inhibited the proliferation of a panel of tumor tissue cell lines with IC_{50}s 10–15 nM. The tumor tissue selectivity of eleutherobin, determined in the

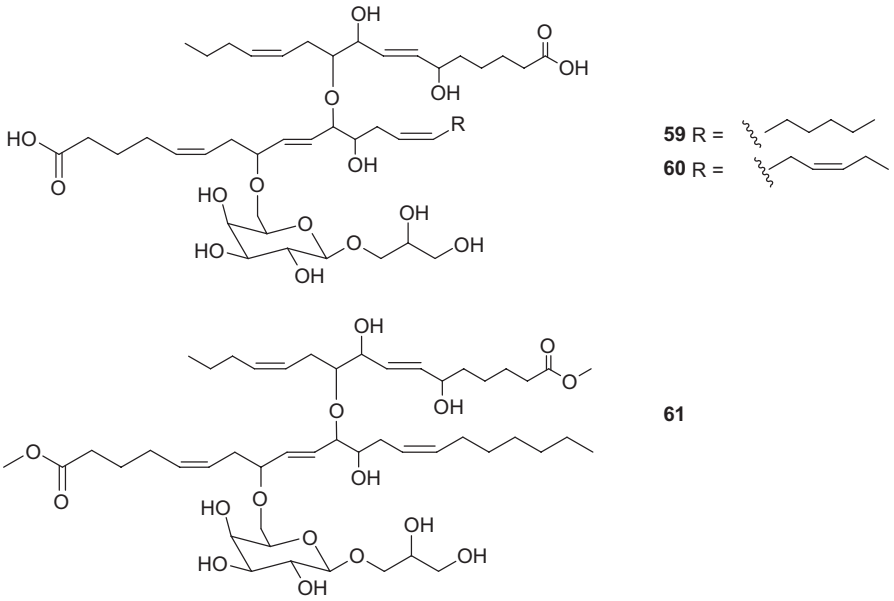


Fig. 23.17 The chemical structures of nigricanosides

National Cancer Institute's 60 cell panel, showed an approximate 100-fold increased potency (over the mean cytotoxicity) toward selected breast, renal, ovarian, and lung cancer cell lines [144]. A morphological examination of HCT116 cells incubated with eleutherobin at moderate concentrations (EC_{50} = 525 nM) showed mitotic arrest while those incubated with lower concentrations (3 to 10 nM) exhibited micronuclei. Treatment of these cells with doses of eleutherobin ranging from 1 to 5 mM resulted in the formation of microtubule bundles similar to those obtained with taxol [145]. More importantly, when investigated mechanistically, eleutherobin was found to stabilize microtubules by competing for the paclitaxel (Taxol) binding site on the microtubule polymer [143]. Sarcodictyins A (**63**) to F (**68**), structurally related to eleutherobin(**62**), were isolated by the same Pietra's group in 1987 [146] and 1988 [147] from the Mediterranean soft coral *Sarcodictyon roseum*. Another two natural structure-like eleutherobin analogues, the eleuthosides A (**69**) and B (**70**), were isolated by Kashman's group from the South African soft coral *Eleutherobiaaurea* [148]. Hamel and co-workers employed synthetic sarcodictyins A and B (*vide infra*) and confirmed many of these findings, including induction of tubulin polymerization and competitive inhibition of taxol binding [144]. Researchers at Pharmacia-Upjohn reported that sarcodictyins A, B, C, and E exhibited cytotoxicity against L1210 murine leukemia cells with IC_{50} values in the range of 408.5 ± 21.3 nM to 911.7 ± 393.5 nM versus taxol at 16.6 ± 5.2 nM [149]. All of the compounds were found to compete with 3H-taxol in tubulin binding assays, suggesting that they bind at the same or at over-lapping sites. They also discovered the cytotoxicities of both sarcodictyin A and B in several other neoplastic cell lines,

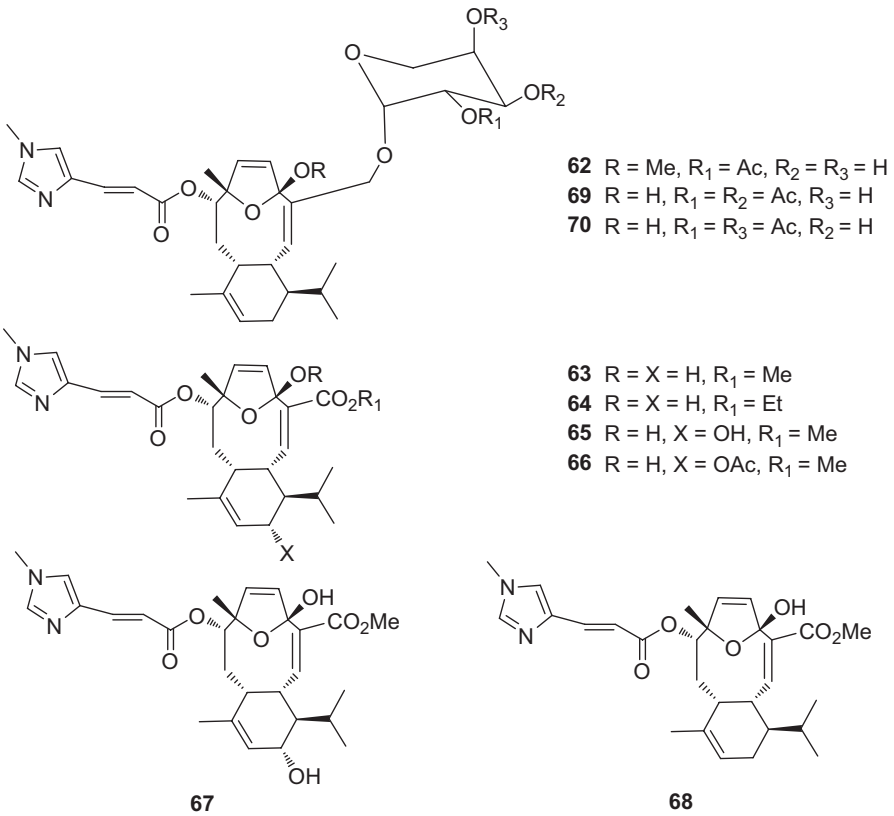


Fig. 23.18 The chemical structures of eleuthosides/sarcodictyins

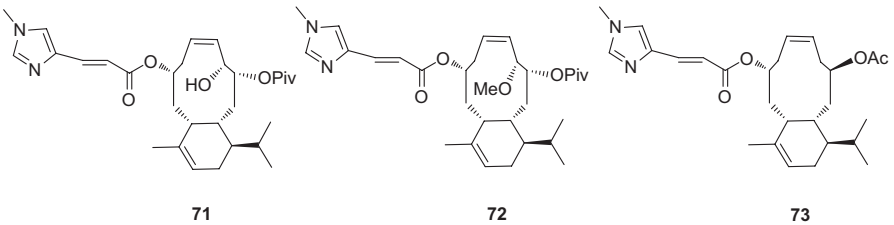


Fig. 23.19 The chemical structures of eleuthosides analogues

and indentified IC_{50} values in the range of 200–500 nM against prostate carcinoma, melanoma, breast carcinoma, and ovarian carcinoma [144] (Figs. 23.18 and 23.19).

Unfortunately, the relative scarcity of these rare marine natural products hampered further in vitro and in vivo biological testing. Accordingly, numerous synthetic groups have taken up the challenge of developing a total synthesis of one or more

members. Nicolaou reported the first total synthesis of sarcodictyin A [150], eleutherobin [151], and Danishefsky's group reported the second total synthesis of eleutherobin a year later [152]. Ojima and Danishefsky incorporated the eleuthosides into one of the first proposals for a common pharmacophore for microtubule-stabilizing natural products [153]. Chemical transformations of natural eleutherobin revealed that the $\Delta^{2',3'}$ olefin in the N -methylurocanic ester moiety is essential for antimitotic activity [154]. Lots of work have been accomplished with an aim to develop potent eleutherobin analogs. A series of synthetic sarcodictyins analogues coupled with their biological evaluations have made the SAR (structure-activity relationships) for natural sarcodictyin products possible in Nicolaou's labtorary [155]. Several important clues were obtained as follows. First the α,β-unsaturated hetero-aromatic side chain is essential for both tubulin binding and antiproliferative activities. Moreover, the natural (N)-methylimidazole hetero-aromatic displays optimal activity, whereas substitution with pyridine, thiazole, or oxazole resulted in a decrease in activity. In addition, ketal substitutions at C4 are well tolerated and tend to improved activity against several cell lines. Finally, esters provide the optimal activity while amines, amides or alcohols show reduced activity. Such information has guided many rational designs of structurally simpler sarcodictyins analogues. For example, Beumer et al described the synthesis of a number of novel simplified eleutheside analogs with potent tubulin-assembling and microtubule-stabilizing properties, using ring closing metathesis as the key-step for obtaining the 6–10 fused bicyclic ring system. These simplified analogs of the natural product (lacking inter alia the C-4/C-7 ether bridge, considered important determinant of antimitotic activity), represented by compound (**71**) and (**72**), retained potent microtubule-stabilizing activity. Cytotoxicity was observed against three common tumor cell lines (human ovarian carcinoma and human colon carcinoma cell lines, IC50 in the μM range) [156]. With the same tactic, Telser also reported the simplified analogue **73**, which was turned out to retain microtubule stabilizing properties [157].

23.5 Microtubule-Targeting Marine Agents in Clinical Trails

Since 1984, there have been many ocean organisms for drug discovery collected from worldwide. The Harbor Branch Oceanographic Institution (HBOI) in Fort Pierce, FL housed about 30,000 samples and published the structures of over 200 compounds with biological activities, including discodermolide [158]. Nowadays, the NCI-DTP's (National Cancer Institute-Developmental Therapeutic Program) Natural Products Repository holds 10,000 marine organisms in storage collected from more than 25 countries [159]. The intense attempts to more effectively explore the rich diversity of biology and chemistry offered by marine life promote the emergence of an exciting ‘‘marine pipeline’’ of new anticancer clinical and preclinical agents. It was estimated that currently there have been 118 marine natural products (MNPs) in preclinical trials, 22 MNPs in clinical trials and 3 MNPs on the market.

These compounds either came from marine sources directly, or were synthesized as a result of knowledge gained from a prototypical compound [160]. In conclusion, there are significant numbers of very interesting molecules either in or approaching Phase II/III clinical trials in cancer and a substantial number of other potential agents following in their wake in preclinical trials in these or other diseases. Table 23.1 reports some of the names of the original compounds with the producers, the chemical class they belong to, the organisms where the drug was originally isolated and the principal mechanism of action accompanied by their clinical status.

The unicity of marine natural products among anticancer drugs can be attributed to their extreme structural diversity including intricate carbon skeletons, different and particular mechanisms of action, and the compatibility of the human body with compounds from the marine environment [161]. Often potent antitumor marine natural products were the "lead compound" to more potent synthetic analogues. These synthetic molecules trend to be cost-effective and have less impact on the marine environment. For example, Eribulin mesylate (E7389, Halaven®, Eisai Europe, Ltd.), a structurally simplified, synthetic analogue of Halichondrin B, is

Table 23.1 Some of Microtubule-targeting Agents in Clinical Trials

Compound name	Chemical class	Organism	Mode of action	Company	Status
Eribulin Mesylate (E7389), Halaven®	Fully synthetic macrocyclic ketone, Halichondrin B analogue	Sponge: *Halichondria okaday*, *Axinella carteri* (Halichondrin B)	Microtubule interfering agent	Eisai Inc.	FDA-EMA Approved for metastatic breast cancer
Soblidotin®, Auristatin PE, (NSC-654663, TZT-1027)	Synthetic dolastatin 10 derivative peptide	Opisthobranchs: *Dolabella auricularia,* Marine cyanobacterium: *Symploca* sp.	Microtubule interfering and vascular disrupting agent	Daiichi Pharmaceutical (Licensee)	Phase I–II
Tasidotin, Synthadotin® (ILX-651)	Third- generation Dolastatin-15 analogue. Sevensubunit depsipeptide	Mollusc: *Aplysidae*	Microtubule interfering agent	Genzyme Corporation	Phase II
Hemiasterlin (E7974)	Tripeptide	Sponge: *Siphonochalina*	Microtubule interfering agent	Eisai Inc.	Phase I
Taltobulin (HTI-286)	Hemiasterlins analogue. Tripeptide	Sponge: *Siphonochalina* (Hemiasterlins)	Microtubule interfering agent (depolarizing)	Wyeth	Phase I
Discodermolide	Polyhydroxylated lactone	Sponge: *Discodermia dissoluta*	Microtubule stabilizing agent	Novartis Pharma AG	Phase I

characterized by a unique mode of interaction with tubulin [20]. European Agency for the Evaluation of Medicinal Products (EMA) and the U.S. Food and Drug Administration (FDA) both granted drug status to Eribulin mesylate for treatment of metastatic breast cancer.

23.6 Conclusion and Perspective

In eukaryotic cells, microtubules (MTs) have numerous key roles that are important in cell proliferation, trafficking, signaling and migration [162, 163]. The critical role that microtubules play in cell division makes it a very suitable target for the development of chemotherapeutic drugs against cancer cells. The unparalleled effectiveness of microtubule-targeting drugs has been validated by the successful use of vincristine, vinblastine, and paclitaxel for the treatment of a wide variety of human cancers. However, natural microtubule-targeting agents have been used in the clinic for years, and drug-resistance, both inherent and acquired, is common [164]. A worldwide search for compounds with similar mechanisms of action but improved characteristics has been urgent for wide clinical use. Over the past 50 years of war against cancer, there have been many anticancer agents used in the clinic that are either natural products or derived form natural sources. These natural products have saved or prolonged the lives of millions of patients with cancer. The initial discoveries from the marine led to the belief that true marine-derived drugs would not be overly long in reaching the market. Since then, the largely unexplored marine world has provided inspiration for the development of new chemical classes of anticancer drugs. As such, considerable attentions from both academic and industrial researchers who are interested in their diversity of bioactivity and chemistry have been paid. New microtubule-targeting agents are derived from marine sources, such as soft corals (eleutherobin and sarcodictyin) or sponges. Marine sponges, however, remain the most prolific source of MTAs [165–167]. These marine natural products are complex compounds with specific stereochemistry. Thus, they are well adapted to bind to the complex three-dimensional surface of tubulin. Meanwhile, in contrast to paclitaxel, some of them present anticancer activities at picomolar or low nanomolar concentrations. Therefore, a number of antimitotic marine natural products or synthetic analogs have cleared the obstacles and reached clinical trials in new drug development, and there has been one of these used in clinic.

Since 1970s, scientists across the world have been devoted to the discovery, isolation, biochemical/pharmacological characterization, pre-clinical and clinical trials of the marine products and their derivatives. Nevertheless, few marine derived drugs are available on the market to date. Several hurdles, including the supply problem and target identification, may be accountable for this shortfall. Can the challenges associated with successful drug development of these oftentimes complex structures be overcome? The answer is yes, because many technologies (including sampling strategies, structure elucidation, total chemical synthesis, and bioengineering) have been greatly improved with the increase of attention on marine environment, which

are all crucial to the success of marine natural products as drug leads. Furthermore, the maturity of genomics and proteomics will provide us a routine method to predict biosynthetic and drug potential, and application of molecular imaging technologies will accelerate the new drug development process. Consequently, generate grounds for our belief that microtubule-targeting small molecular anticancer agents from marine origin will form a new "pipeline" of antitumor drugs that flow into the market and pharmacies in the future.

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Chapter 24
Cytotoxic Triterpene Glycosides from Sea Cucumbers

Valeria P. Careaga and Marta S. Maier

Abstract The class Holothuroidea (sea cucumbers) belonging to the phylum Echinodermata is characterized by the production of triterpene glycosides with sulfate groups attached to the monosaccharide residues in a great majority of the saponins isolated so far. Due to their toxicity and membranotropic action, these polar compounds have attracted the attention of chemists and pharmacologists and a wide spectrum of biological activities has been found for these secondary metabolites. The purpose of this communication is to review the structural characteristics and the cytotoxic properties of triterpene glycosides isolated from sea cucumbers in the last five years, focusing on structure-activity correlations and research on their mechanisms of action.

Keywords Triterpene glycosides · Sea cucumbers · Echinodermata · Cytotoxic activity

24.1 Introduction

Echinoderms belonging to the class Holothuroidea (sea cucumbers) produce complex mixtures of triterpene glycosides (holothurins) that are responsible for their general toxicity and may play a defensive role due to their membranotropic action [1, 2]. Most of these saponins contain an aglycone based on a "holostanol" skeleton [3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone] (**1**) (Fig. 24.1) and a sugar chain of two to six monosaccharide units linked to the C-3 of the aglycone. Two main series of aglycones can be distinguished in their structures: glycosides based on a 3β-hydroxy-holost-9(11)-ene aglycone and those containing a 3β-hydroxy-holost-7-ene skeleton. Usually aglycones that have a $\Delta^{9,11}$ double bond are

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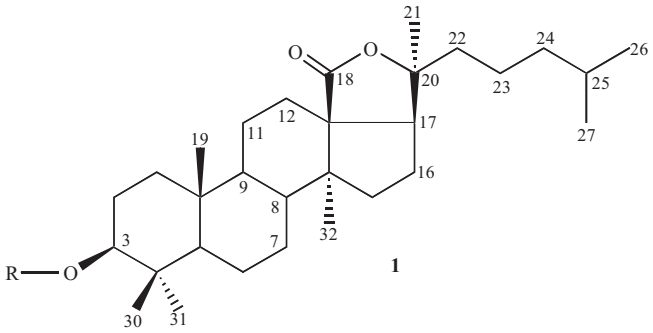

Fig. 24.1 Structure of hypothetical holostanol

characteristic of sea cucumbers belonging to the order Aspidochirota, while those with a Δ^7 unsaturation were generally isolated from animals of the order Dendrochirotida.

Sea cucumber triterpene glycosides differ in the composition and number of the sugar units, the number and positions of the sulfate groups and the structural characteristics of the aglycone. Some examples of holothurins having non-holostane aglycones have been found in seven species of sea cucumbers belonging to the order Dendrochirotida. The majority are monosulfated at the glucose or xylose units [1].

Most of the triterpene glycosides are tetra- or pentaglycosides. Carbohydrate units include quinovose, glucose, 3-*O*-methylglucose, xylose and sometimes 3-*O*-methylxylose, 3-*O*-methylquinovose, 3-*O*-methylglucuronic acid and 6-*O*-acetylglucose. The first monosaccharide unit is always xylose, while 3-*O*-methylglucose and 3-*O*-methylxylose are always terminal. Sixty percent of the triterpene glycosides isolated so far from holothurians have sulfate groups linked to the monosaccharide units of the oligosaccharide chain. Although most of them are monosulfated oligoglycosides, a number of di- and trisulfated glycosides have been isolated, mainly from the order Dendrochirotida. Most tetra- and pentasaccharides are sulfated at C-4 of the xylose unit. Additional sulfate groups at C-6 of the 3-*O*-Me-glucose and glucose units have been found in trisulfated tetraglycosides. The majority of tetrasaccharides show a linear chain with the most common 3-*O*-Me-Glc-(1 → 3)-Glc-(1 → 4)-Qui-(1 → 2)-Xyl structure. The few disaccharides that have been isolated show a Qui-(1 → 2)-4-OSO_3Na-Xyl chain attached to C-3 of the triterpenoid aglycone. Some hexasaccharides have been isolated from sea cucumbers of the order Aspidochirota: *Stichopus japonica*, *Stichopus chloronotus*, *Parastichopus californius* and *Bohadschia bivittata* [1]. They are non-sulfated glycosides with a linear 3-*O*-Me-Glc-(1 → 3)-Glc-(1 → 4)-Xyl chain and a branching of a linear trisaccharide at C-2 of the xylose unit.

Several holothurins are specific for different taxonomic groups of sea cucumbers and structural characteristics of triterpene glycosides have been used to resolve taxonomic problems in the class Holothuroidea [3].

Triterpene glycosides are produced in the skin and in the Cuvier's tubules of sea cucumbers and are ejected when the animals are disturbed. This behavior may be

associated to a defensive function due to the ability of holothurins to form complexes with cholesterol and other Δ^5-sterols that lead to the development of single ion channels and larger pores which cause significant changes in the physico-chemical properties of cell membranes [4–6]. Sea cucumbers are resistant to their own toxins due to the presence of Δ^7-, 14α-methyl- and 14α-dimethyl-$\Delta^{9,11}$-sterols as well as their conjugated forms such as steryl sulfates and steryl xylosides [7].

Sea cucumbers are important as human food source and they have long been utilized in folk medicine in Asia. Their triterpene glycosides exhibit a wide spectrum of biological effects: antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory activities [5]. These biological activities are a consequence of their membranotropic action against any cellular membrane containing Δ^5-sterols.

Several monographs concerning the structures and biological activities of holothurins have been published [1–3, 5, 8–10]. The aim of the present review is to report the most recent findings in the field, focusing on the structural characteristics and cytotoxic activities of these glycosides from 2009 to 2013.

24.2 Chemical Structure and Cytotoxic Activity

24.2.1 Glycosides with 3β-hydroxy-holost-9,11-ene Aglycones

Two new triterpene glycosides with hydroxyl groups at positions 12α and 17α of the holostanol skeleton, scabrasides A (**2**) and B (**3**) (Fig. 24.2), were isolated from the sea cucumber *Holothuria scabra* [11]. This holothurian is widely distributed in the South China Sea and is used as a tonic in China. The in vitro cytotoxicity of both glycosides was evaluated against human leukemia (HL-60, MOLT-4), human lung cancer (A-549), and human hepatoma (BEL-7402) cells. Compounds **2** and **3** showed high activities towards HL-60 and MOLT-4 with IC_{50} values of 0.05 and 0.09 μM (for compound **2**) and of 0.25 and 0.08 μM (for compound **3**), respectively. Both compounds displayed lower activities towards A-549 and BEL-7402 cells.

The new sulfated triterpene glycoside scabraside D (**4**) (Fig. 24.2) showed significant cytotoxicity against A-549 (IC_{50} = 1.72 μM), mouse leukemic cell (P-388) (IC_{50} = 0.96 μM), gastric cancer cell (MKN-28) (IC_{50} = 1.27 μM), human colorectal cancer cell (HCT-116) (IC_{50} = 1.72 μM), and human breast cancer cell (MCF-7) (IC_{50} = 1.80 μM) [12].

Two sulfated tetraglycosides, hemoiedemosides A (**5**) and B (**6**), (Fig. 24.3) isolated from the Patagonian sea cucumber *Hemioedema spectabilis* [13] were evaluated for *in vitro* cytotoxicity and antiproliferative activity on human lung cancer (A-549) and cervical cancer (HeLa) cell lines [14]. Compounds **5** and **6** differ only in the degree of sulfation of the oligosaccharide chain. Trisulfated glycoside **6** contains an additional sulfate group at C-6 of the terminal 3-*O*-methyl glucose unit. Glycoside **6** exhibited high antiproliferative activity towards Hela and A-549 with IC_{50} values of 2.15 and 3.16 μM, respectively. Compound **5** was less active than **6** with IC_{50} values of 7.43 μM in A-549 and 9.95 μM in HeLa cell lines. Besides, the

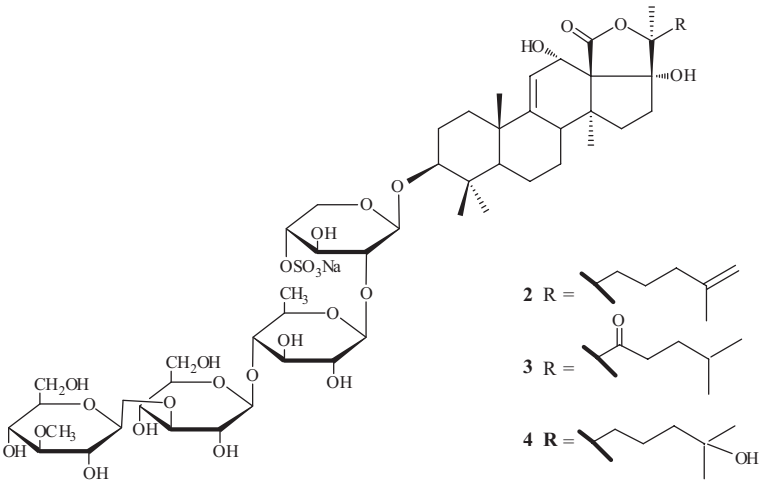

Fig. 24.2 Chemical structures of scabrasides A (**2**), B (**3**) and D (**4**)

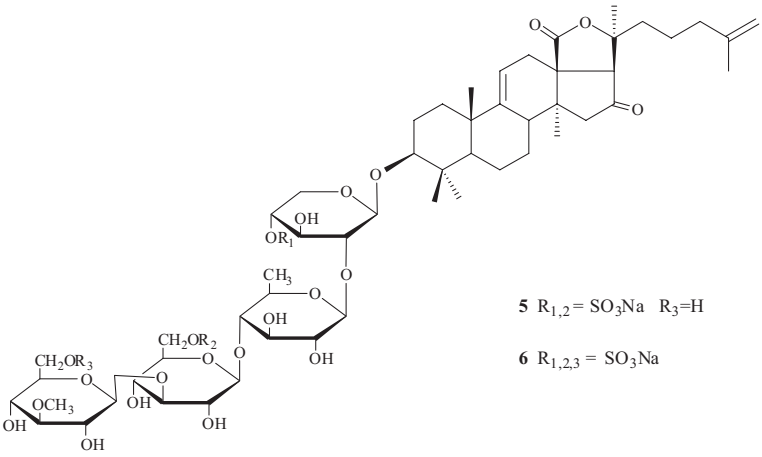

Fig. 24.3 Chemical structures of hemoiedemosides A (**5**) and B (**6**)

higher cytotoxicity of glycoside **6** on both cell lines with CC_{50} values of 2.80 μM for Hela and 5.96 μM for A-549 may be related to the presence of a third sulfate group in the carbohydrate chain. Similar results were observed for okhotosides B_1, B_2 and B_3. Monosulfated okhotoside B_1 showed lower cytotoxicity than the disulfated analogs B_2 and B_3 in HeLa cell line [15].

One new sulfated diglycoside, leucospilotaside B (**7**) (Fig. 24.4) was isolated from the sea cucumber *Holothuria leucospilota* [16]. Glycoside **7** exhibited significant cytotoxicity against four tumor cell lines (lung cancer A-549, leukocythemia

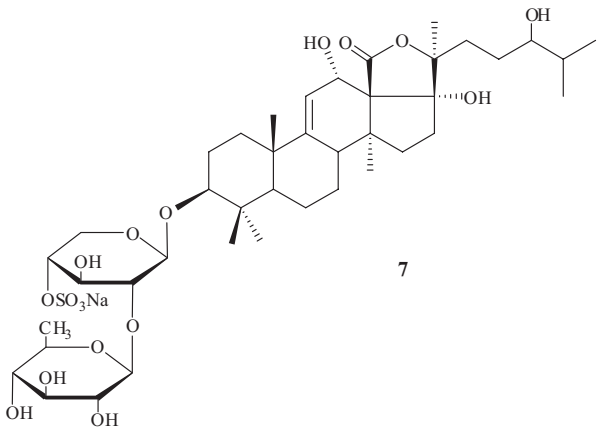

Fig. 24.4 Chemical structure of leucospilotaside B (**7**)

HL-60 and MOLT-4, and liver cancer BEL-7402) with IC_{50} values ranging from 0.44–2.62 μg/ml.

Two new disulfated holostane glycosides with an acetyl group at position 16β of the aglycone, pentactasides B (**8**) and C (**9**) (Fig. 24.5), were isolated from the sea cucumber *Pentacta quadrangularis* [17]. Both saponins differ only in the side chain of the triterpene aglycone. The *in vitro* cytotoxicity of both glycosides was evaluated against five human tumor cell lines (P-388, A-549, HCT-116, MCF-7 and MKN-28). Compounds **8** and **9** showed significant cytotoxicities against all tumor cell lines with IC_{50} values between 0.09 and 2.30 μM. Interestingly, the activity of **8** against HCT-116 (IC_{50} = 0.09 μM) and of **9** against A-549 (IC_{50} = 0.58 μM) was significantly higher than that of the positive control, 10-hydroxycamptothecine (IC_{50} of 0.14 and 0.74 μM, respectively).

Two sulfated triterpene glycosides, holothurin A_1 (**10**) and 24-dehydroechinoside A (**11**) (Fig. 24.6), isolated from the sea cucumber *Pearsonothuria graeffei*, possess an identical carbohydrate chain and differ in their side chains [18]. Glycoside **10** has a hydroxyl group at C-22, while **11** possesses a 24(25)-double bond. Both glycosides were evaluated for their effects on metastasis *in vitro* and *in vivo*. Both compounds strongly inhibit tumor metastasis but **10** had more potent antimetastatic activity than **11**, suggesting that the 24(25)-double bond in the side chain of **11** could promote antimetastasic activity. In addition, only glycoside **10** markedly suppressed the expression of NF-*κ*B indicating that a hydroxyl group at C-21 may relate to the targeting of NF-*κ*B in tumor metastasis. Recently, 24-dehydroechinoside A (**11**) was isolated from the sea cucumber *Holothuria scabra* [12] and showed significant cytotoxicity (IC_{50} = 0.41–1.21 μM) against five tumor cell lines (P-388, A-549, MKN-28, HCT-116 and MCF-7).

Ds-echinoside A (**12**) (Fig. 24.7), a non-sulfated triterpene glycoside, was isolated for the first time as a natural product from the sea cucumber *P. graeffei* [37]. Previously, it had been obtained by desulfation of echinoside A [19]. Compound **12** inhibited the proliferation of human hepatocellular liver carcinoma cells HepG2

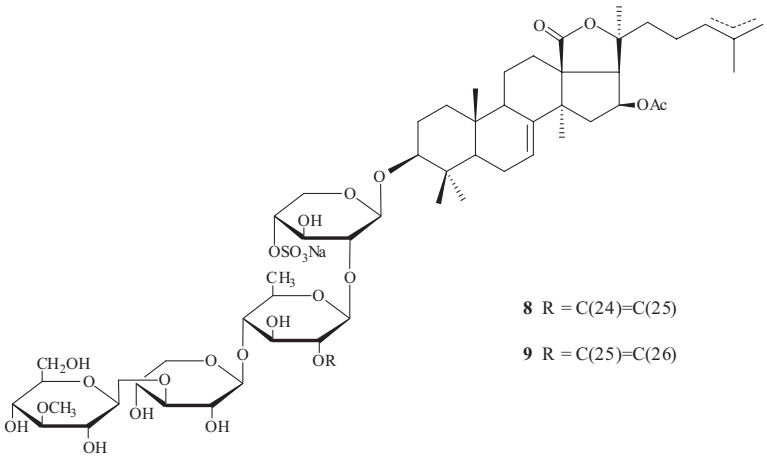

Fig. 24.5 Chemical structures of pentactasides B (**8**) and C (**9**)

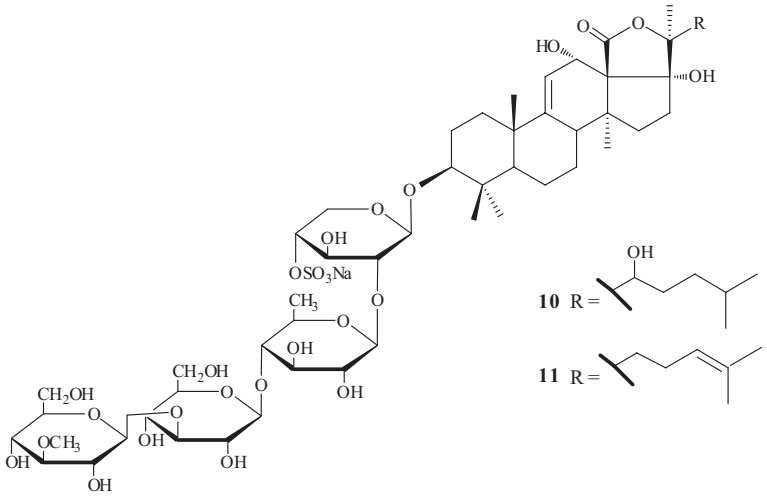

Fig. 24.6 Chemical structures of holothurin A_1 (**10**) and 24-dehydroechinoside A (**11**)

(IC_{50} = 2.65 μM) and suppressed HepG2 cell adhesion, migration and invasion in a dose-dependent manner. It exhibited a significant antimetastatic activity through the specific inhibition of NF-*k*B-dependent matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) expressions.

Recently, echinoside A (**13**) (Fig. 24.7) was isolated from *P. graeffei* [21]. Glycoside **13** and its desulfated analog **12** exhibited marked anti-cancer activity in HepG2 cells by blocking cell-cycle progression and inducing apoptosis through the mitocondrial pathway.

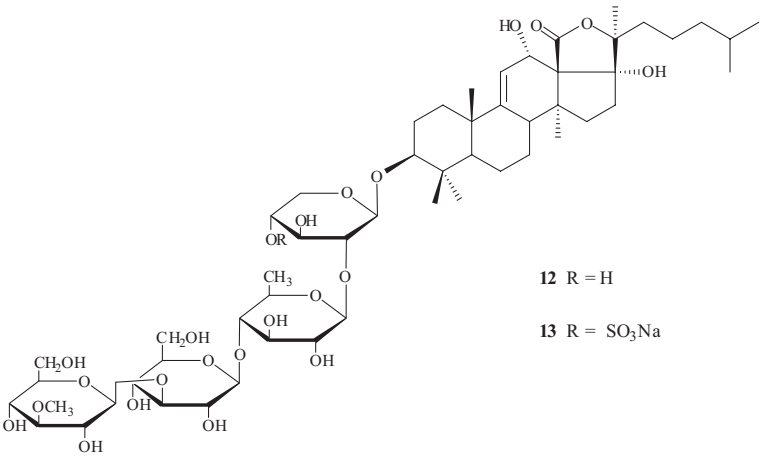

Fig. 24.7 Chemical structures of ds-echinoside A (**12**) and echinoside A (**13**)

24.2.2 Glycosides with 3β-hydroxy-holost-7-ene Aglycones

Two monosulfated glycosides (**14** and **15**) (Fig. 24.8) with an oxygenated function at C-16 and differences in the third unit of the carbohydrate chain were evaluated for their effects on caspase activation and apoptosis of human leukemia (HL-60) cells [22]. Frondoside A (**14**), a major triterpene glycoside isolated from sea cucumber *Cucumaria frondosa*, has an acetoxy group at C-16 of the aglycon and xylose as the third monosaccharide residue [23], while cucumarioside A_2-2 (**15**), isolated from *Cucumaria japonica,* depicts a 16-keto group and a glucose residue as the third monosaccharide unit in the carbohydrate chain [24].

Both glycosides strongly induced apoptosis of HL-60 cells, but frondoside A (**14**) had more potent effects than cucumarioside A_2-2 (**15**) on cytotoxicity, cell cycle changes and apoptosis. This study indicated that the acetyl group at C-16 of the aglycon in frondoside A may play a significant role in frondoside's cytotoxicity and caspase activation since glycoside **14** led to no caspase activation before early apoptosis while cucumarioside A_2-2-induced apoptosis was caspase-dependent. New studies on cucumarioside A_2-2 have shown that this glycoside affects mouse cell immunity *in vitro*, which is reflected in significant changes in immune cell morphology and behavior [25]. A new immunomodulatory lead named cumaside was created on the basis of a mixture of monosulfated triterpene glycosides isolated from *C. japonica* (mainly cucumarioside A_2-2) with cholesterol in an approximate molar ratio of 1:2. Cumaside showed antitumor activity against different forms of experimental mouse Ehrlich carcinoma *in vivo* both independently and in combination with cytostatics [26].

Recently, it has been demonstrated that frondoside A inhibited the migration of human breast cancer cell line MDA-MB-231 in a wound healing assay [27] as well as the metastasis *in vivo* from a gland-implanted tumor [28]. Frondanol-A5P,

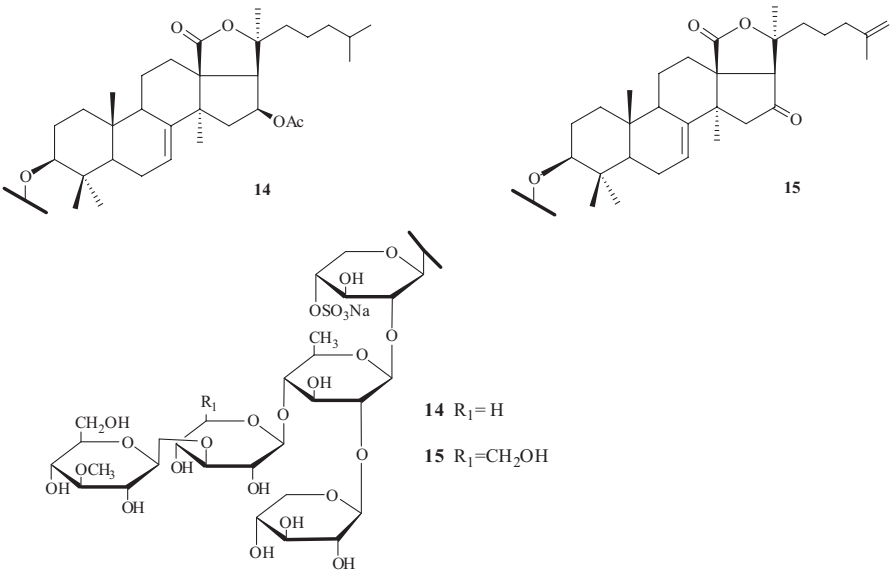

Fig. 24.8 Chemical structures of frondoside A (**14**) and cucumarioside $A_{2-}2$ (**15**)

a parent compound containing *C. frondosa*-derived glycosides, inhibited growth of S2013 and AsPC-1 pancreatic cancer cells [29].

Four new triterpene glycosides, cucumariosides H_5 (**16**), H_6 (**17**), H_7 (**18**) and H_8 (**19**) along with the known cucumarioside H (**20**) (Fig. 24.9) were isolated from the Far Eastern sea cucumber *Eupentacta fraudatrix*. These glycosides have a rare branched pentasaccharide carbohydrate moiety with one sulfate group at C-4 of the first xylose unit and 3-*O*-methyl-D-xylose as the terminal monosaccharide unit. Glycosides **16–18** and **20** differ from each other in the side chains of the aglycones, while cucumarioside H_8 (**19**) has a novel aglycone with an unprecedented 16(22)-epoxy group [30]. The cytotoxic activities of glycosides **16–18** and **20** against mouse spleen lymphocytes and hemolytic activity against mouse erythrocytes were studied. Glycoside **17** having a 24(25)-double bond in the aglycone side was less active, while cucumariosides H (**20**) and H_5 (**16**) having diene-systems and H_7 (**18**) with a saturated side chain in the aglycone moiety showed a more potent cytotoxic and hemolytic activities. The new cucumarioside H_2 (**21**) with a 25-hydroxyl group in the side chain showed low activity against mouse spleen lymphocytes and Ehrlich carcinoma cells [31]. Evaluation of the cytotoxicity of new minor cucumariosides A_1 (**22**), A_3 (**23**), A_4 (**24**), A_5 (**25**), A_6 (**26**), A_{12} (**27**) and A_{15} (**28**) showed that glycosides **22** and **26** were the most active [32]. These results demonstrate that the structure of aglycone side chains influences significantly the cytotoxic action of these glycosides.

Patagonicoside A (**29**), the major triterpene glycoside of the sea cucumber *Psolus patagonicus* [33], and its desulfated analog (**30**) (Fig. 24.10) were tested for their antiproliferative activity in human hepatocellular (Hep3B), human mammary

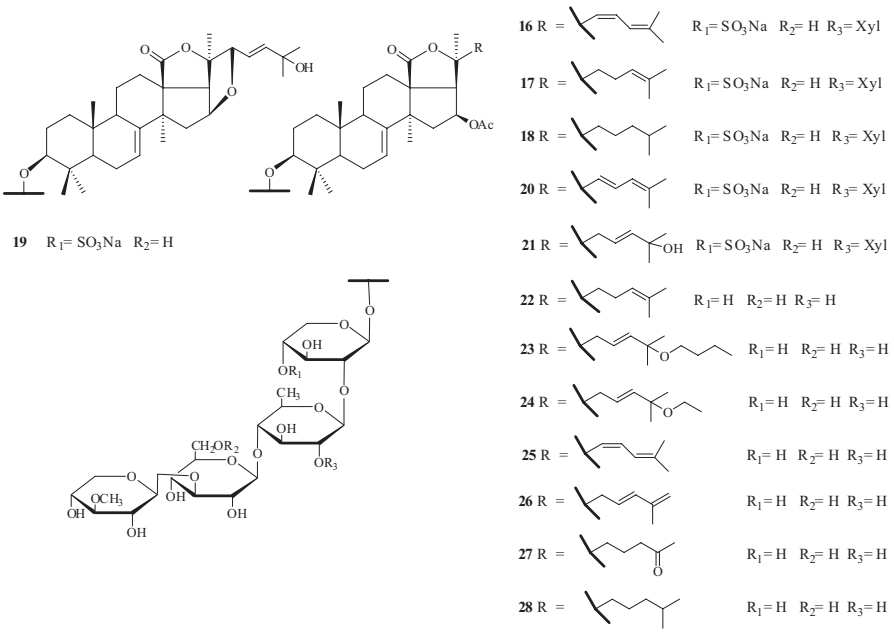

Fig. 24.9 Chemical structures of cucumariosides H_5 (**16**), H_6 (**17**), H_7 (**18**), H_8 (**19**), H (**20**), H_2 (**21**), A_1 (**22**), A_3 (**23**), A_4 (**24**), A_5 (**25**), A_6 (**26**), A_{12} (**27**) and A_{15} (**28**)

(MDA-MB231), and human lung (A-549) carcinoma cells. Both compounds were able to suppress the growth of the three tumor cell lines. Compound **30** exhibited a slightly stronger antiproliferative activity for Hep3B (IC_{50} = 5.09 μM) and MDA-MB231 (IC_{50} = 5.39 μM) while the effect for A-549 was similar to that of Patagonicoside A with IC_{50} values of 16.53 and 15.04 μM, respectively. Studies on the structure-activity relationship for sea cucumber glycosides revealed that their biological activities depend on both the aglycone and the carbohydrate structures. An 18(20)-lactone in the aglycone moiety is an important requirement for membranotropic action, together with the presence of a linear tetrasaccharide fragment in the carbohydrate chain. Besides, glycosides containing quinovose as the second monosaccharide unit are the most active. Patagonicoside A (**29**) and its desulfated analog (**30**) contain these favorable structural features. Nevertheless, both glycosides showed low hemolytic activity (82 and 87 μM, respectively) in comparison with sea cucumber triterpene glycosides containing a linear tetrasaccharide chain [5]. This could be assessed to the uncommon presence of two 12α- and 17α-hydroxyl groups and a Δ^7 double bond in the aglycone moiety and may be related to their lower level of cytotoxicity. In order to approach to the mechanism of antiproliferative action of these compounds, the effect of both glycosides on nuclear factor-κB (NF-κB) activation was studied. Patagonicoside A and its desulfated analog promoted NF-κB translocation to the nucleus of tumor cells A-549, in absence and in presence of

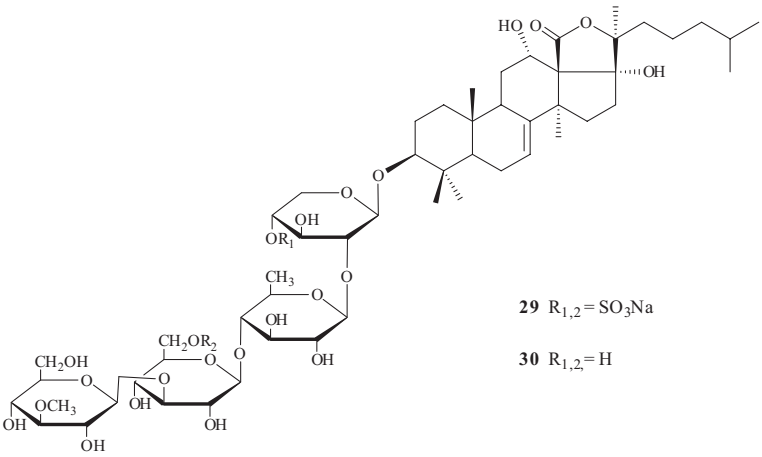

Fig. 24.10 Chemical structures of Patagonicosides A (**29**) and its desulfated analog (**30**)

the tumor necrosis factor-α (TNF-α). Hence, their antiproliferative action would be related to their membranolytic activity [34].

Chemical examination of the sea cucumber *Pseudocnus dubiosus leoninus* led to the isolation of a new disulfated tetraglycoside Pseudocnoside A (**31**) (Fig. 24.11) [14]. Compound **31** was evaluated for *in vitro* cytotoxicity and antiproliferative activity on two human tumor cell lines (A-549 and HeLa) and these activities were compared to those of two structurally related triterpene glycosides, patagonicosides B (**32**) and C (**33**) (Fig. 24.11) isolated from *Psolus patagonicus* [35]. The antiproliferative activity of **31** in A549 cells (IC_{50} = 14.53 μM) was considerably higher than in HeLa cells (IC_{50} = 57.36 μM). Patagonicosides B (**32**) and C (**33**) exhibited similar antiproliferative and cytotoxic activities in HeLa and A549 with IC_{50} values of 7.94 and 9.73 μM (for compound **32**) and of 3.57 and 5.56 μM (for compound **33**), respectively. This study indicated that hydroxyls at C-12α and C-17α may play an important role in the cytotoxicity of these glycosides in comparison to a keto group at C-16 as in compound **31**.

Stichoposide C (**34**) (Fig. 24.12) is a quinovose-containing hexaoside that was isolated from the sea cucumbers *Stichoposus chloronotus* [36] and *Thelenota ananas* [37].

Glycoside **34** induced apoptosis in mouse CT-26 subcutaneous tumor and HL-60 leukemia cells in a dose-dependent manner and increased ceramide generation *in vivo* through activation of acid and neutral sphingomyelinases [38].

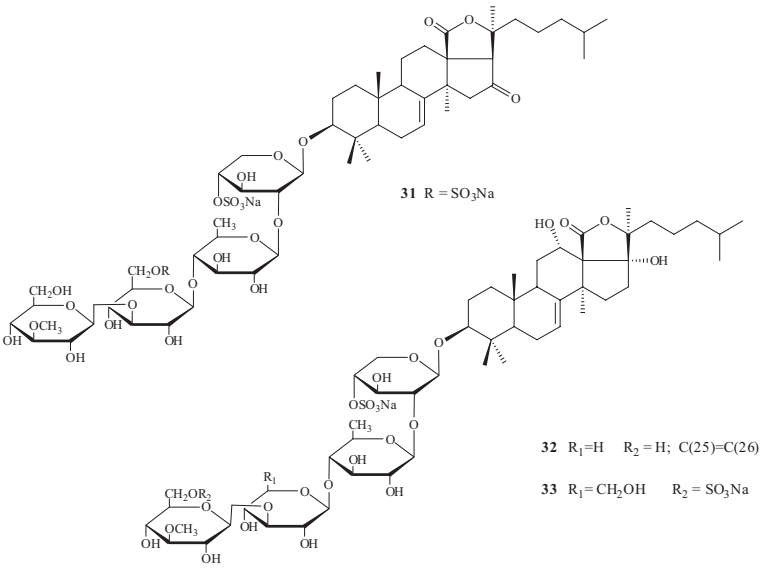

Fig. 24.11 Chemical structures of Pseudocnoside A (**31**) and Patagonicosides B (**32**) and C (**33**)

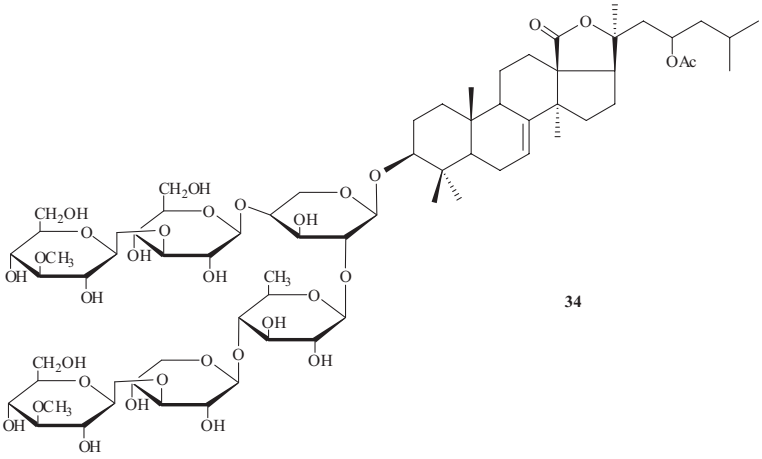

Fig. 24.12 Chemical structure of Stichoposide C (**34**)

24.3 Concluding Remarks

During the last five years many new examples of sea cucumber glycosides were isolated, in particular those containing a 3β-hydroxy-holost-7-ene aglycone. Notably, the sea cucumber *Eupentacta fraudratrix* is a rich source of triterpene glycosides

containing this type of aglycone and displaying differences in their side chains. An increased interest in the evaluation of the cytotoxicities of these polar metabolites has allowed establish structure-activity correlations as well as investigation on their mechanisms of action. Frequently, different tumour cell lines exhibit a differential sensitivity to the cytotoxic effects of sea cucumber glycosides, which can be related to their chemical structures.

Usually, sea cucumber triterpene glycosides occur as complex mixtures of structurally related compounds that are present in low amounts in the organisms. Therefore, their isolation requires a combination of chromatographic procedures to obtain the pure compounds. Recently, new strategies based on the isolation of partially purified mixtures of bioactive triterpene glycosides and their admixture with cholesterol, such as cumaside, pose new alternatives to the development of pharmaceutical drugs. The growth of sea cucumbers by aquaculture would contribute to the supply of triterpene glycosides preventing the overexplotation of these echinoderms.

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Chapter 25
Targeting Cellular Proapoptotic Agents from Marine Sources

Ming Liu, Xiukun Lin and Lanhong Zheng

Abstract Marine organisms are an important source for the discovery of anticancer agents. In recent years, an increasing number of lead compounds have been isolated and some of them possess potent apoptosis-inducing activities in cancer cells. Apoptosis is a form of programmed cell death and is a critical defense mechanism against the occurrence of cancer. There is a long list of pro- or anti-apoptotic molecules that can trigger apoptosis. Therefore, searching agents that target these pro- or anti-apoptotic molecules has become an important strategy for the anticancer agent developments. This review summarizes some of marine-derived agents with pro-apoptotic activities and discusses the existing challenges and our perspectives on the development of anticancer agents from marine source.

Keywords Apoptosis · Anticancer · Marine organisms · Natural

25.1 Introduction

Natural products or small molecules synthesized based upon natural product leads represent more than 60% of the cancer chemotherapeutic agents currently used in the clinics [1, 2], as exemplified by the plant-derived compounds vincristine, irinotecan, etoposide, taxanes and camptothecines. Increasing evidence suggests that marine environment contains different classes of biologically active compounds with strong anticancer or cytotoxic properties. Potent anticancer compounds,

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including alkaloids, steroids, terpenes, peptides, macrolides, polyketides, etc, have been isolated from different marine organisms [3–10]. The most potent class of compounds has entered the clinical trials and attracts much attention owing to their high efficiency and low toxicity, and some clinical trials have been finished with promising results [11]. These compounds exhibit anticancer activity through multiple mechanisms, of which those that effect via induction of cancer cell apoptosis are of particular interests to us. In this primer, we first briefly introduce the apoptosis and the common targets for marine pro-apoptotic products and then summarize those agents from marine source that can induce apoptosis of cancer cells, since there still lack sufficient reviews on this line of studies.

25.2 Apoptosis and Common Targets for Marine Pro-apoptotic Products

Apoptosis is a naturally occurring and evolutionarily programmed cell death [12]. Apoptosis plays an indispensible role in development, physiology and homeostasis [13–16]. Its deregulation, i.e., either loss of pro-apoptotic signals or gain of anti-apoptotic signals, can lead to a variety of pathological conditions including cancer initiation, progression and treatment failure [17, 18]. Modulation of apoptotic pathways and selective induction of apoptosis by chemical agents are a promising approach for cancer therapy [15, 19–22]. In general, apoptosis is mediated by the activation of different caspase cascades [23, 24]. In mammals, there are mainly two major signaling systems that result in the activation of caspases, i.e., the extrinsic death receptor pathway [25–28] and the intrinsic mitochondrial pathway [23, 29, 30]. Increasing evidence indicates that these two pathways are not independent systems but, instead, there are many crosstalks between them. The disturbance of kinases and growth receptor signaling pathway (such as PI3K/AKT pathway, JNK and p38 MARK pathways) could also stimulate apoptosis [31]. There is a long list of pro- or anti-apoptotic molecules that can trigger apoptosis. Therefore, developing anticancer agents that target these pro- or anti-apoptotic molecules has become an important strategy for cancer chemotherapy.

Growing evidence indicates that most marine anticancer agents with cytotoxicity may trigger apoptosis by targeting many cellular proteins (Fig. 25.1), and the induced apoptotic process includes both intracellular and extracellular pathways. As aforementioned, apoptosis occurs primarily through two well-characterized pathways in cells, both of which are initiated by caspase activation via mechanisms involving the intrinsic or mitochondrial-mediated effectors. Some of marine anticancer products can disturb the growth receptor signaling pathway and lead to the apoptosis. The balance between Bcl-2 and Bax plays a key role in maintaining cell viability. Therefore, inhibition of Bcl-2 or induction of Bax becomes a good strategy for triggering an apoptotic process [32]. Since caspases are involved in intrinsic or extrinsic apoptosis pathway, identification of caspase activators becomes another approach for the discovery of novel anticancer agents [33, 34]. In short, apoptosis is a very complicated process and involves a huge number of signaling molecules,

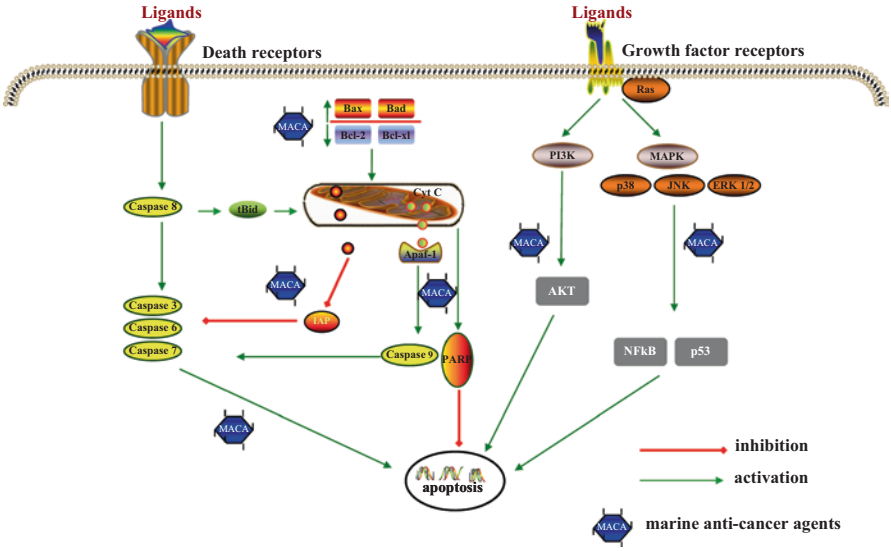

Fig. 25.1 Shematic diagram of common target genes for marine pro-apoptotic products

and failure in apoptosis activation is one of the major impediments to the treatment of cancer. Therefore, a good strategy for the development of new anticancer agents is to identify or develop such agents that can target multiple apoptosis-regulating molecules.

25.3 Compounds from Marine Organisms with Pro-apoptotic Activity

25.3.1 Compounds That Alter Bcl-2/Bax Ratios

Proteins in the Bcl-2 family contain both pro-apoptotic and pro-survival members. Of this family, Bcl-2 and Bcl-xL have emerged as major targets for anticancer agents [35]. In the intrinsic mitochondrial pathway, at least 18 pro- and anti-apoptotic proteins of the Bcl-2 family are pivotal regulators of apoptosis [32, 36], all of which may be targets for marine compounds. Moreover, these proteins may directly or indirectly antagonize each other's functions and are important in delivering afferent signals of the extrinsic pathway.

Many natural products with cytotoxic activity from marine source have been found to induce cell death by regulating the balance of Bcl-2 family members. Bryostatin 1, derived from a marine invertebrate bryozoa, *Bugula neritina,* is a member of the bryostatins family that possesses the same macrocyclic lactone structural motif with varying side chains [37]. In addition to stimulating the activity of protein kinase C (PKC) [38, 39], bryostatin 1 can induce apoptosis through

the mitochondrial pathway. Bryostatin 1 increases the pro-apoptotic Bax protein level and decreases the anti-apoptotic Bcl-2 protein levels, leading to a direct up-regulation of the Bax/Bcl-2 ratio [40–42].

Several other compounds from marine source induce apoptosis also by down-regulation of the Bcl-2 expression. Dolastatins isolated from a sea hare *Dolabella auricularia* belong to family of peptides and depsipeptides. These natural compounds are highly cytotoxic to a variety of cancer cells through multiple mechanisms, including G_2/M phase cell cycle arrest, apoptosis induction and microtubule inhibition [43, 44]. Apoptosis induced by dolastatins is associated with a decrease in Bcl-2 level and an increase in P53 expression [44]. The discovery of Dolastatin 10 has lead to the development of brentuximab vedotin, which has been recently approved by the US Food and Drug Administration for the treatment of Hodgkin's lymphoma and systemic anaplastic large cell lymphoma [45]. Carotenoid and fucoxanthinol isolated from the sea squirt *Halocynthia roretzi* inhibit the growth of a number of cancer cells including human leukaemia and breast and colon cancer cells. This inhibitory activity is mediated by the induction of apoptosis via mechanisms involving a decrease in the expression levels of Bcl-2 [46].

25.3.2 Compounds That Activate Caspases

Caspases are a family of cysteinyl aspartate-specific proteases involved in apoptosis and are subdivided into groups of initiators (Caspase 8, 9, 10) and executioners (Caspase 3, 6, 7). Many anticancer drugs have been developed from marine source with the effects of caspase activation. For instance, ascididemin is a pyridoacridine alkaloid isolated from the Mediterranean ascidian, *Cystodytes dellechiajei*. Ascididemin and its analogues exhibit significant cytotoxic activities against a variety of tumor cells by multiple mechanisms. Ascididemin and its analogues cause DNA breakage by mechanisms independent of the topoisomerase I or II [47, 48]. Ascididemin induces robust apoptosis in leukaemia cells via a mitochondrial pathway that requires activation of caspase 2, which can be inhibited by a specific caspase 2 inhibitor. JNK activation by ascididemin is a step upstream of mitochondria *via* reactive oxygen species, whereas a specific JNK inhibitor can partially inhibit caspase 2 and 9 activation, cytochrome c release and DNA fragmentation, indicating that JNK contributes to, but is not essential for, ascididemin induced apoptosis [49]. Another compound, spisulosine (ES285) isolated from the clam *Mactromeris polynima,* has anticancer effects through several mechanisms [50]. It triggers apoptosis by activating caspase 3 and 12 and modifying phosphorylation of p53 without affecting other survival/apoptosis pathways such as those initiated by JNK, Erks or Akt [50, 51]. However, SZ-685C, an anthracycline analogue isolated from marine-derived mangrove endophytic fungi, could induce apoptosis through the Akt/FOXO pathway, as well as by both caspase 8 and caspase 9 dependent apoptotic pathways [52].

In cell culture, marine alkaloid naamidine A isolated from marine sponges potently induces apoptosis of cancer cells by activation of caspases 3, 8, and 9 but independent of extracellular signal-regulated kinase (ERK) 1/2 and does not re-

quire functional p53 [53]. Similarly, the proapoptotic activity of 3- and 10-bromofascaplysins is mediated by caspase 8, 9, and 3 activaition [54]. Ircinin-1 isolated from the marine sponge *Sarcotragus sp*. can inhibit the growth of human melanoma cell line *in vitro* by G_1 phase cell cycle arrest and induction of apoptosis. Ircinin-1 induced apoptosis involves the release of cytochrome c, activations of caspase 3 and 9, upregulation of Fas and Fas-L and decrease in the cellular inhibitor of apoptosis protein cIAP-1 [55]. Porphyrans are sulfated polysaccharides and the main components of *Porphyra*. In AGS human gastric cancer cells, they show potential apoptotic activities with a marked increase in caspase 3 activation and ensuing poly (ADP-ribose) polymerase (PARP) cleavage. The caspase 3 activation is possibly due to a negative regulation of phosphorylation of the type I insulin-like growth factor receptor (IGF-IR) in the AGS gastric cancer cells [56]. A marine carotenoid, Siphonaxanthin, isolated from green algae, could effectively induces apoptosis in human leukemia (HL-60) cells through caspase-3 activation and accompanied with the enhanced expression of GADD45α and DR5 (TRAIL receptor-2), and the suppressed expression of Bcl-2 [57].

Bis (2-ethylhexyl) phthalate (BEHP), isolated from Marine *Bacillus pumilus* MB 40, was able to induce apoptosis by activation of caspases family proteins such as caspase-8, caspase-9 and caspase-3. Moreover, an increase in the expression of Bax mRNA and a decrease in mRNA of Bcl-2 in was also observed in BEHP treated K562 cells [58]. Pardaxin, an antimicrobial peptide isolated from the Red Sea Moses sole, triggers apoptosis in HT-1080 cells. Pardaxin-treated cells showed elevation of caspase 3, 7 activities, disruption of the mitochondrial membrane potential, and accumulation of reactive oxygen species (ROS) production. Inhibition of ROS production and caspase 3, 7 activities reduced pardaxin-induced effects, indicating that the pardaxin induced apoptosis is caspase-dependent [59]. Acetylapoaranotin is a diketopiperazine disulfide that was isolated from marine *Aspergillus sp*. KMD 901. It induces apoptosis in human colon cancer cells (HCT116) as demonstrated by caspase 3, 8, 9 cleavages. This compound also significantly inhibits tumor growth in mice *in vivo* [60].

25.3.3 Compounds That Trigger Mitochondrial Cytochrome C

Cytochrome c normally attaches to the outer surface of the inner mitochondrial membrane and locates in the cristae where it functions in the electronotransport system. Its release from the cristae into the cytosol is a pivotal step of apoptosis initiation [61]. Once released to the cytoplasm, cytochrome c binds to apoptotic protease activating factors (Apaf-1) and then activates caspase 9, an initiator caspase [62]. LAQ824, a HDAC inhibitor originally isolated from marine sponge, *Psammaplysilla sp.,* has bee shown to induce cell cycle arrest and cell death which preferentially occurs in cancer cells over normal cells [63]. Recent study shows that LAQ824 potentially induces apoptosis by inducing the release of several mitochondrial related pro-apoptotic factors in cancer cells including cytochrome c. The expression of cytochrome c, AIF, Smac and Endo G are all enhanced in A549 cancer cells after

treatment with LAQ824 [64]. Moreover, LAQ824 is found to induce activities of caspases 3, 8 and 9 [64].

Lamellarin D isolated from the sea slug *Elysia ornate* is one of lamellarins that are a group of polyaromatic pyrrole alkaloids. Lamellarin D has the most potent cytotoxicity against many tumor cell lines [65, 66] by targeting the topoisomerase I and mitochondria. It causes S and G_2/M cell cycle arrest and promotes apoptosis by inducing mitochondrial permeability and disrupting the inner mitochondrial transmembrane potential and mitochondrial swelling, leading to the cytochrome c release [67].

Some marine derived polypeptides also could induce the release of cytochrome c and induces the mitochondrial-mediated apoptosis. For example, the anticancer polypeptide (Mere15) was purified from *Meretrix meretrix Linnaeus* and the anticancer mechanism was associated with a mitochondrial-mediate pathway apoptosis [68, 69]. A novel polypeptide (CS5931) extracted from *Ciona savignyi* induces apoptosis through a mitochondrial-mediated pathway in human colorectal carcinoma cells could also induce the release of cytochrome c from the mitochondria [70].

25.3.4 Compounds That Disturbs the Growth Receptor Signaling Pathway

Jun N-terminal kinases (JNKs) and p38 mitogen-activating protein kinases (MAPKs) have important roles in the signaling mechanisms that orchestrate cellular responses to many types of cellular stress, in addition to regulation of proliferation, differentiation, survival and migration of certain cell types [71, 72]. Activation of JNK and p38 MAPK pathways can trigger cytochrome c release and subsequently the activation of caspase cascades [71]. Many anticancer compounds from marine source display their pro-apoptotic activity by activating the JNK and p38 MARK pathways.

Aplidine is a cyclic depsipeptide isolated from a sea squirt, *Aplidium albicans* [73]. It shows strong potency against Ehrlich carcinoma *in vivo* and induces apoptosis of colonic, breast and prostate cancer cell lines via the mitochondrial pathway. Aplidine induces early oxidative stress and results in the activation of JNK and p38 MAPK, which in turn results in cytochrome c release, followed by activation of the caspase cascades and protein kinase Cδ (PKC-δ) [74–76]. Aplidine also shows inhibitory effects on spontaneous angiogenesis *via* blocking VEGF secretion and production of the matrix metalloproteinases (MMP-2 and MMP-9) [77, 78]. Aplidine, also called plitidepsin in clinical trials, is well tolerated with minor toxicity in finished Phase I clinical trials [79, 80].

Ecsteinascidins are a group of tetrahydroisoquinolone alkaloid compounds isolated from the marine tunicate *Ectenaiscidia tubinata*. Ectenaiscidia 743 (ET-743), now called trabectedin, is the most potent one for its high cytotoxic activity and is abundant in the tunicate [81]. ET-743 shows dose-dependent cytostatic and pro-apoptotic effects through activation of two different signaling pathways, namely a transcription-dependent pathway leading to cell cycle arrest and a transcription-independent pathway leading to rapid apoptosis that involves JNK, caspase-3 and

mitochondria [82–84]. It has been shown that ET-743 induces persistent JNK activation before the onset of apoptosis. No JNK activation was observed in the presence of curcumin, an inhibitor of the JNK signaling pathway, indicating that ET-743 induced apoptosis is mediated by persistent JNK activation. Bcl-2 seems to be involved as well in ET-743 induced cell apoptosis, since overexperession of Bcl-2 abrogates ET-743 induced apoptosis [85, 86]. Many other marine compounds display pro-apoptotic activities via activating JNK pathway as well. For example, onnamide A and theopederin B, which are heterocyclic compound isolated from the marine sponge *Mycale sp.*, inhibit the protein synthesis in Mv1Lu cells and trigger apoptosis of HeLa cells at nanomalor concentrations. These compounds induce apopotosis *via* sustained activation of the p38 kinase and JNK pathways [87].

Upregulation of Akt in cancer cells is a common event, and the inhibition of the PI3K/Akt pathway could inhibit the development and progression of several tumors. For example, bostrycin is a novel anthracene analog isolated from marine fungi and reported as promoter of apoptosis in several cancer cells by down-regulation of PI3K/AKT pathway proteins [88]. Doxorubicin analogue 1403P-3, isolated from the mangrove endophytic fungus, is an anthracenedione derivative with potent anticancer activities, recenty researches showed that fact that blockon the Akt activation may contribute the anticancer activity of 1403P-3 [89]. FBA-TPQ, synthetic analog of the natural makaluvamines from sponges of the genera *Zyzzya,* activates PI3K/Akt pathways in OVCAR-3 cells and exhibits significant anticancer activity against ovarian cancer [90].

Fascaplysin was identified as an antimicrobial pigment from the marine sponge *Fascaplysinopsis sp*. and recent studies showed that fascaplysin is an inhibitor of Cdk4, with an IC_{50} about 0.55 μM, and induce apoptosis in a variety of cancer cells [91]. Since decrease in the Cdk4 activity, fascaplysin has also been revealed to be capable of inhibiting angiogenesis [92]. Based on the structure of fascaplysin, many compounds have been developed and some of them are more specific for, or more potent in, inhibiting Cdk4 activity, thus having a better potential to be new chemotherapeutic agents [93–95].

Other kinase inhibitors of marine origin have been recently reviewed by Sandip B. Bharate, et al. [96]. These marine derived kinase inhibitor include serine/threonine kinase inhibitors, cyclin-dependent kinase inhibitors, glycogen synthase kinase-3 inhibitors, check-point kinase inhibitors, casein kinase inhibitors, mitogen-activated protein kinase inhibitors, and polo-like kinase inhibitors. These marine-derived small-molecule inhibitors of protein kinases offer a fertile source for the discovery of anti-cancer agents.

25.3.5 Compounds That Targets the Nuclear Factor (e.g., NF-κB, STAT 3)

NF-κB (nuclear factor κB) transcription factors are key regulators of programmed cell death. NF-κB often antagonizes apoptosis by activating the expression of anti-apoptotic proteins and antioxidant molecules, but it can also promote apoptosis un-

der certain conditions. Numerous findings have emphasized the importance of the NF-κB signaling pathway as a promising target for cancer prevention and treatment [97, 98]. One of the phloroglucinol derivatives, dioxinodehydroeckol, is isolated from brown alga *Ecklonia Cava.* Treatment of MCF7 human breast cancer cells with dioxinodehydroeckol induces apoptosis through NF-κB family and NF-κB dependent pathway [99]. Cytotoxic Arenamides (A-C) from the fermentation broth of a marine bacterial strain Salinispora arenicola blocked TNF-induced activation of NF-κB in a dose- and time-dependent manner [100]. It is conceivable that some other marine derived chemicals may have inhibitory effects on NF-κB as well, such as heteronemin, a marine sesterterpene isolated from the sponge *Hyrtios* sp., inhibits NF-κB activation and activates both initiator caspases-8 and-9, respectively, in chronic myelogenous leukemia cells [101].

MESP (cholest-1-en-3-one-20(R)-oic acid methyl ester,) is a marinely derived steroid from the Sanya soft coral *Spongodes sp* and may serve as an important candidate drug lead for Hepatocellular carcinoma therapy. MESP inhibited the phosphorylation of signal transducers and activators of transcription 3 (STAT3), a critical survival signaling factor that reduced the expression of the antiapoptotic protein x-linked inhibitor of apoptosis protein but enhanced the expression of the proapoptotic protein Bax, thus promoting caspase-dependent apoptosis [102].

25.3.6 Compounds That have Multiple Targets on Apoptotic Pathways

Many anticancer agents from marine source can affect different steps along apoptotic pathways. For instance, ascididemin not only activates caspases but also affects the JNK pathway, as discussed above. LAQ824 induces the release of several mitochondria related pro-apoptotic factors in cancer cells and also activates caspase activity. ET-743 induced apoptosis involves not only activation of the JNK pathway but also participation of Bcl-2. Ircinin-1 induced cell demise also involves both intrinsic and extrinsic pathways.

Some of the marine anticancer agents induce apoptosis by both alteration of Bcl-2 expression and activation of caspases. Cephalostatin 1, isolated from the marine worm *Cephalodiscus gilchristi*, induces apoptosis of cancer cells using Smac/DIABLO as a mitochondrial signaling molecule. Cephalostatin 1 selectively triggers the mitochondrial release of IAP antagonist Smac/DIABLO, and this release can be delayed by over-expression of Bcl-xL, an anti-apoptosis protein [103]. Further study reveals that cephalostatin 1 also inactivates the Bcl-2 *via* hyperphosphorylation by JNK [104]. Like cephalostatin 1, Jaspamide induces apoptosis by activation of caspase-3 and decrease of Bcl-2 protein levels, but it also involves increased Bax level *via* a caspase-independent pathway as an additional mechanism [105]. Another small compounds bromophenol derived from algae, bis(2,3-dibromo-4,5-dihydroxybenzyl) ether, induces mitochondrial apoptosis in K562 cells *via* activation of caspases and changes the Bcl-2/Bax *in vitro* [106].

In some cases, activation of caspase and JNK pathways as well as modulation of Bcl-2 are all involved in the induction of apoptosis by marine products. Cryptophycin 52 initiates apoptosis more rapidly than paclitaxel and vinblastine and may be the most potent inhibitor of microtubule dynamics identified hitherto. Cryptophycin 52 induced apoptosis of prostate cancer cells is androgen status independent, cell type specific and caspase required. It is also modulated by the Bcl-2 family members and linked to, but not dependent of, p53, in addition to a strong correlation with phosphorylation mediated by the c-Jun NH2-terminal kinase [107]. Dideoxypetrosynol A, a polyacetylene isolated from a sponge, *Petrosia sp.*, indcues apoptosis in several human tumor cell lines through apoptotic mechanisms. Apoptosis induced by dideoxypetrosynol is associated with up-regulation of Bax expression, down-regulation of Bcl-2, activation of caspase 3 and caspase 9, cleavage of PARP and selective down-regulation of cIAP-1 [108].

Jaspine B, an anhydrophytosphingosine derivative isolated from the marine sponge *Jaspis sp.*, induces apoptosis in melanoma cells with cytochrome c release and caspase activation [109]. Jaspine B treatment also leads to an increase in cellular ceramide level before the onset of apoptosis. Jaspine B inhibits the activity of sphingomyelin synthase (SMS) and in turn affects the ceramide formation and metabolism. Inhibition of the enzymes for ceramide clearance such as SMS and ceramidases has been a novel approach for cancer chemotherapy [109].

Somocystinamide A (ScA) stimulates apoptosis in tumor cell lines and angiogenic endothelial cells *via* both the intrinsic and extrinsic pathways, but the more effective mechanism is the activation of caspase 8 and the extrinsic pathways [110]. Palmitic acid shows selective toxicity via apoptosis in leukaemic cell lines [111, 112]. Lipotoxicity in PC12 and rat cortical cells induced by Palmitic acid was apoptotic as evidenced by nuclear morphology, caspase 3 activation as well as lamin B and PARP cleavage. Palmitic acid can also increase the expression of the mRNAs coding for the cell death associated proteins BNIP3 and the Fas receptor, suggesting the involvement of the extracellular pathway of apoptosis [112].

Zalypsis demonstrated potent in vitro and in vivo inhibitory effects against both human solid and hematologic neoplasms, it induced apoptosis via both the intrinsic and extrinsic pathways through activation of caspases 3, 8, 7 and 9 and PARP cleavage [113]. In phase I clinical trial, Zalypsis showed good tolerance and preliminary evidence of its antitumor activities against several solid tumors [114].

25.3.7 Compounds Lead to Apoptosis with Unclear Mechanisms

Although a huge effort has been put on the development of anticancer agents from marine source, this area is still a virgin field and much fewer compounds have been identified so far compared with the researches on, and the products isolated from, other natural sources such as Chinese herb medicines. Nevertheless, many marine compounds have been isolated by screening for cytotoxicity and some of them, as aforementioned, are known to induce cell apoptosis. However, there are still many

other compounds that are still unknown for their mechanisms of induction of cytotoxicity, because researchers isolated hundreds of novel compounds every year and these potential anticancer compouds possibly target multiple apoptosis-regulating molecules and thus involve complicated mechanisms. For instance, Didemnin B inhibits the synthesis of RNA, DNA, and proteins and binds to palmitoyl protein thioesterase [115, 116], and induces apoptosis of a variety of cancer cells. However, the exact mechanism for these effects has yet to be illustrated [117]. Compounds from marine source, like chondropsins [118, 119], mycalamides (mycalamide A, B, C, & D) [120–122], peloruside A [123–125] and philinopside A [126], have been found to induce apoptosis in different cancer cells but the mechanisms have not yet been investigated.

Both philinopside A and bastadin 6 have antiangiogenic activity and proapoptotic activity. Phlinopside A is a novel sulfated saponin isolated from the sea cucumber, *Pentacta quadrangulari* and induces apoptosis both *in vitro* and *in vivo* [126]. Bastadin 6, a macrocyclic and tetrameric bromotyrosine derivative isolated from the marine sponge *Lanthella basta*, inhibits angiogenesis and neovascularization *in vivo*. The induction of apoptosis may be an underlying mechanism but the details are still lacking and further investigation are needed [127]. Similarly, several other marine anticancer agents, including sansalvamide [128, 129], turbinamide [130], jaspolide B [131], Erythrazoles B [132], also show apoptotic activity *via* unknown mechanisms. Sansalvamide, an inhibitor of topoisomerase I, induces cell death with only some characteristics of apoptotic cells [128], which implies that it may not effect *via* targeting canonical apoptosis-regulating molecules or it has additional targets.

25.4 Conclusions, Challenges, and Perspectives

Development of novel chemotherapeutic agents from marine source is a research still at its neonatal age due in part to the lack of an analog of a long ethno-medical history for finding clues, as compared with terrestrial habitats. Technical difficulty in collecting marine organisms and the preparation of large amount of leading compounds is another hurdle. Although we reviewed the apoptosis-related molecules as the target of the marine-derived compounds, we should keep in mind that: Are the molecules really the targets of these compounds? Are the changes of these molecules the results of the apoptosis or otherwise the reasons? If it is the results, then what is the real molecule target? To date, only a relatively small number of marine derived compounds that effect by targeting pro-apoptotic molecules have been advanced to clinical trials. However, some marine derived compounds as described herein are already in preclinical development or early-phase clinical trials. There are reasons to be optimistic, since compounds from marine sources attract more and more interests because of their anticancer potential, which will eventually yield excellent lead compounds and clinical useful agents for the pharmaceutical pipelines.

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Chapter 26
Discovery and Computer-Aided Drug Design Studies of the Anticancer Marine Triterpene Sipholanes as Novel P-gp and Brk Modulators

Ahmed I. Foudah, Asmaa A. Sallam and Khalid A. El Sayed

Abstract Sipholane triterpenes are marine natural products isolated from the Red Sea sponge *Callyspongia siphonella*. Based on their structure similarity to the marine polyepoxysqualene terpenoids sodwanones, sipholanes were tested for various anticancer activities. Sipholenone A showed cytotoxicity and anti-angiogenic activity against human and mouse breast cancer cells. Sipholenols A and L, sipholenone E, and siphonellinol D showed potential activity in multi-drug resistant (MDR) tumors overexpressing P-glycoprotein (P-gp) and their in-silico binding mode justified their activity order. Recently, a kinase assay profiling platform was used to identify the breast tumor kinase, Brk (also known as protein tyrosine kinase 6, PTK6) as a potential target for 19,20-anhydrosipholenol A 4-*β*-benzoate (**28**), a semisynthetic ester analog of sipholenol A. Brk has recently emerged as an attractive therapeutic target for controlling breast cancer proliferation and migration. Additional semisynthetic modifications afforded sipholenol A 4*β*-4′,5′-dichlorobenzoate as a potent breast cancer migration inhibitor, with an IC_{50} of 1.3 μM in the wound-healing assay, without any cytotoxicity to the non-tumorigenic breast cells MCF10A. Pharmacophore modeling and 3D-QSAR studies highlighted the important pharmacophoric features responsible for the antimigratory activity and Brk phosphorylation inhibition. Those features are restricted to rings A and B (perhydrobenzoxepine) together with the substituted aromatic ester moiety, thus resulting in a much simpler structure and eliminating rings C and D ([5,3,0] bicyclodecane system). This will open new horizons for the future design and synthesis of novel sipholane-inspired active compounds with perhydrobenzoxepine-aromatic cores, both feasibly and cost-effectively. These results demonstrate the potential of marine natural products for the discovery of novel scaffolds for the control and management of metastatic breast cancer.

Keywords Breast cancer · Breast tumor kinase (Brk) · Computer-aided drug design · P-glycoprotein · Marine natural products · Proliferation and migration · Protein tyrosine kinase 6 (PTK6) · Sipholane triterpenes

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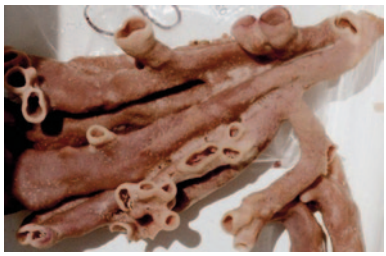


Fig. 26.1 The Red Sea sponge *Callyspongia siphonella*

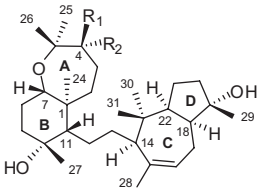
26.1 Sipholane Triterpenes

Thirty sipholane triterpenes have been isolated from the Red Sea sponge *Callyspongia siphonella* (Fig. 26.1). They possess a perhydrobenzoxepine (rings "A" and "B") and a [5,3,0] bicyclodecane (rings "C" and "D") ring system, which are linked together by an ethylene bridge. Based on their skeletons, sipholane triterpenes are divided into four different classes: sipholane, siphonellane, neviotane and dahabane (Fig. 26.2) [1–6]. The sipholanes are the most abundant class. Recently, sipholanes were reported as novel P-gp modulators (capable of reversing multidrug resistance (MDR) in P-gp-overexpressing tumors) and inhibitors of Brk phosphorylation.

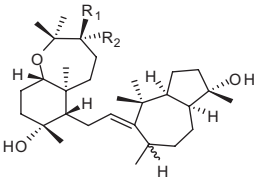
26.2 P-gp Modulation and Cancer Chemotherapy

About 50 % of men and 33 % of women in the US will develop cancer during their lifetimes [7, 8]. The development of multidrug resistance (MDR) in cancer cells is one of the major causes leading to failure of chemotherapy [1, 9]. Various cellular mechanisms responsible for development of MDR include increased drug efflux, reduced drug uptake, activation of detoxifying proteins, DNA repair, and failure to undergo apoptosis [9, 10]. Anticancer drug delivery can be impaired by either reduced drug uptake or increased drug efflux. Decreased drug uptake can give rise to resistance to water-soluble drugs that enter the cells by transporters and carriers or by endocytosis, e.g., methotrexate, 5-FU, 8-azaguanine, and cisplatin [11, 12]. Increased drug efflux is the most common mechanism of MDR and results from increased expression of ATP binding cassette (ABC) transporters that function as efflux pumps [9, 11]. P-gp-mediated drug efflux is generally responsible for the development of classical MDR in acute myelogenous leukemia, colon, kidney, adrenocortical, hepatocelluar, breast, and lung cancers [9, 10]. Like other ABC transporters, P-gp utilizes the energy of ATP hydrolysis to transport drug molecules out of the cell [11]. ATP hydrolysis occurs in the ATPase domain of P-gp which is stimulated in the presence of various drug substrates [11–13]. Examples of drugs transported by P-gp include Vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes. Inhibition of P-gp-mediated drug efflux may resensitize MDR cancer cells to chemotherapeutic drugs, improving chemotherapy for patients with MDR tumors [1, 9].

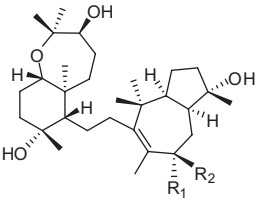
Sipholanes



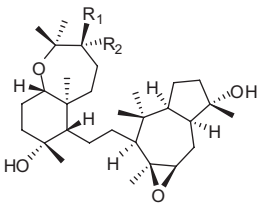
Sipholenol A (**1**) (R_1 = OH, R_2 = H)
Sipholenone A (**2**) (R_1, R_2 = O)



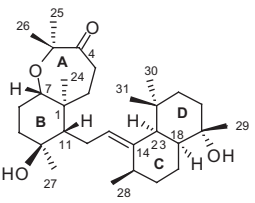
Sipholenol C (R_1 = OH, R_2 = H)
Sipholenone D (R_1, R_2 = O)



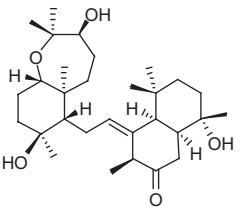
Sipholenol D (R_1, R_2 = O)
Sipholenol E (R_1 = OH, R_2 = H)
Sipholenol H (R_1 = H, R_2 = OH)



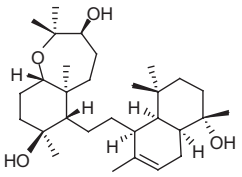
Sipholenol G (R_1 = OH, R_2 = H)
Sipholenone B (R_1, R_2 = O)



Sipholenone E (**3**)

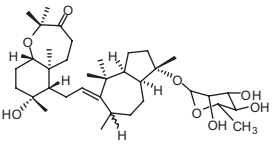


Sipholenol J (**6**)

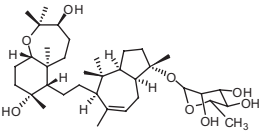


Sipholenol L (**4**)

Sipholane Glycosides

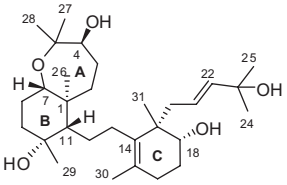


Sipholenoside A

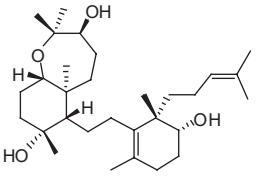


Sipholenoside B

Siphonellanes

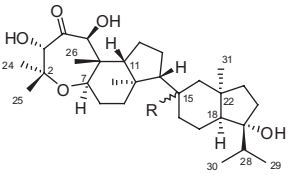


Siphonellinol C

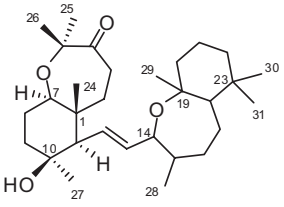


Siphonellinol D (**5**)

Neviotanes



Neviotine A (R = β-OH)
Neviotine B (R = α-OH)



Dahabinone A

Fig. 26.2 Representative triterpenes of the sponge *C. siphonella*: Sipholanes and sipholane glycosides, siphonellanes, neviotanes, and dahabanes [1–6]

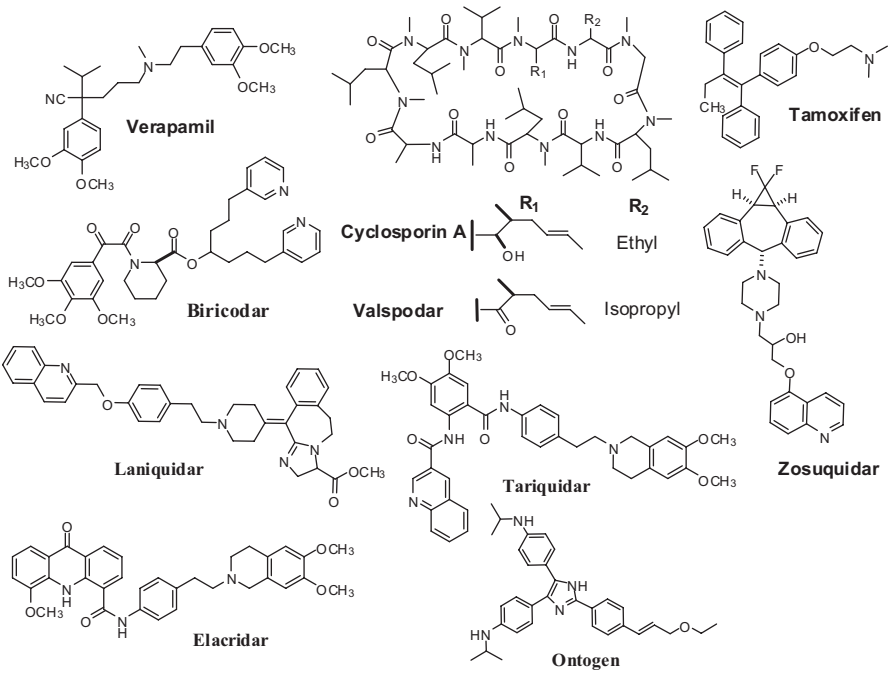


Fig. 26.3 Structures of various first, second and third-generation P-gp modulators

Since the discovery of verapamil as a P-gp modulator, various agents have been investigated for their ability to reverse P-gp-mediated MDR in cancer patients (Fig. 26.3) [9, 11–13]. First-generation modulators, like verapamil, cyclosporin A, quinine and tamoxifen are weak inhibitors and require doses greater than those used therapeutically to reverse P-gp-mediated MDR, resulting in serious toxic effects [11–14]. Second-generation modulators, like valspodar (an analog of the first generation P-gp modulator cyclosporin A) and biricodar, are more potent and considerably less toxic. However, they significantly increased plasma levels of certain standard chemotherapeutic drugs by inhibiting their metabolism, resulting in undesirable effects [15, 16]. Third-generation modulators are the most potent and have been developed using combinatorial chemistry and structure-activity relationship (SAR) studies [15, 16]. They have nanomolar (nM) affinity for P-gp and have minimal pharmacokinetic interactions with chemotherapeutic drugs [14–16]. They include tariquidar (XR9576), laniquidar (R101933), zosuquidar (LY335979), elacridar (GF120918), and ontogen (OC144-093, Fig. 26.3) [13–16]. Tariquidar, for example, binds with high affinity and is highly specific to P-gp [13–16]. However, its phase III clinical trial for the treatment of lung cancer was suspended due to unfavorable toxicity [15, 16]. Other third-generation P-gp modulators are still in clinical trials, and their efficacy is still under evaluation but their fate is unclear [15, 16]. Hence, the so-called “ideal P-gp-modulator” has yet to be discovered and there remains an urgent need to discover and develop efficacious and well tolerated

P-gp modulators. The search for natural product-derived MDR-reversal compounds is currently an active area due to the uniqueness and chemical diversity of natural products [9]. Novel naturally-derived P-gp inhibitors include pervilleine A, tetrandrine, FG020326 and derivatives, ONO-1078, 5-*O*-benzoylated taxinine, agosterol A, and short hairpin RNA (shRNA) [17–24].

26.3 PTK6-BRK as a Valid Cancer Target

The intracellular protein kinase, Brk (breast tumor kinase, also known as protein tyrosine kinase 6, PTK6) has been implicated in the development and progression of a number of different tumor types [25]. Brk is one of the Src family tyrosine kinases, which was cloned from metastatic breast tumor samples and cultured human melanocytes [26, 27]. Brk is overexpressed in up to 86% of invasive human breast tumors, prostate and colon carcinomas [28]. Brk was detected in approximately two-thirds of breast tumors analyzed, where approximately a third of these showed Brk overexpression by levels ranging from five- to forty three-fold compared to normal tissue [25]. Brk is normally expressed in differentiating epithelial cells of the intestine, skin, prostate, and oral cavity, where it has been shown to promote cellular differentiation, apoptosis, and more recently to mediate migration and wound-healing [26]. However, it is not detected in normal mammary tissues or fibroadenomas, nor is it detected at various stages of mammary development. Overexpression of Brk in tumor samples relative to the restricted levels in normal or differentiating tissues suggest that Brk may have a role in the processes underlying tumorigenesis, such as promotion of cancer cell proliferation, migration, and survival [27]. Recent studies suggest that Brk can promote breast cancer cell migration through multiple mechanisms and in response to a number of different ligands [25, 27]. Its expression levels increase in association with the carcinoma content of breast tumors, tumor grade, and invasiveness [26–30]. These observations strongly suggest that high levels of *de novo* expression of Brk make it an attractive therapeutic target in breast cancer. Furthermore, inhibition of Brk kinase activity may provide a potentially novel approach to sensitize the response of tumor cells to other chemotherapeutics and to prevent or inhibit metastasis of cancer with enhanced therapeutic windows [30].

26.4 Marine Environment: A Rich Resource of Unique Bioactive Natural Products

Oceans cover over 70% of Earth and possess 80% of Earth's animal life [31–34]. The marine environment is an excellent source for anticancer drug discovery because of its vast chemical and biological diversity [15–23]. Marine chemodiversity can be largely attributed to the great number of species in the oceans (sources can range from the marine actinomycetes and cyanobacteria to the seaweeds, sponges,

corals, and mollusks) as well as the distinct marine environment [35]. Cytarabine (Cytosar-U, Ara-C, Depocyt, Bedford Laboratories-Enzon Pharmaceuticals; anticancer), Vidarabine (Ara-A, Vira-A, King Pharmaceuticals; antiviral) and Ziconotide (Prialt; Elan Pharmaceuticals; pain treatment) are all marine-derived drugs approved for use by the FDA [36]. In 2007, trabectedin (Yondelis; PharmaMar) was approved in the European Union for advanced soft tissue sarcoma [36–40]. The macrolide eribulinmesylate and the peptide soblidotin are also marine-derived compounds currently in phase III clinical trials for cancer [36]. Ten other marine natural products are currently in phase I/II clinical trials [36]. This highlights the important role of marine natural products in the discovery and development of novel therapeutics agents.

26.5 Sipholane Triterpenes as Novel P-gp Modulators

Sipholenol A (**1**), sipholenone E (**3**), sipholenol L (**4**) and siphonellinol D (**5**) were reported to potentiate the cytotoxicity of several P-gp substrate anticancer drugs, including colchicine, vinblastine, and paclitaxel, but not the non-P-gp substrate cisplatin [2]. They significantly reversed MDR in the cancer cell lines KB-C2 and KB-V1 in a concentration-dependent manner [1–3, 9]. However, they had no effect on the response to cytotoxic agents in cells lacking P-gp expression or expressing MRP1 or BCRP [2, 3, 12, 31]. Sipholenol A showed no cytotoxicity in numerous cell lines, regardless of their membrane transporter status [3, 11, 12]. Sipholenol A significantly inhibited the function of P-gp through direct interaction, and sipholane triterpenes were proposed as a novel class of potential P-gp inhibitors for the treatment of MDR in P-gp-overexpressing tumors [11, 12]. [^{3}H]-Paclitaxel accumulation and efflux studies demonstrated that these three sipholanes produced a time-dependent increase in the intracellular accumulation of [^{3}H]-paclitaxel by directly inhibiting P-gp-mediated drug efflux. Sipholanes **3-5** dose dependently enhanced P-gp ATPase activity and inhibited its photolabeling with [^{125}I]-iodoarylazidoprazosin, a known ATPase substrate but had no effect on the expression of P-gp as determined by Western blot analysis [9]. The above discussion strongly suggests that sipholane triterpenes represent a novel class of P-gp modulators.

26.6 Identification of the Binding Mode of the Sipholanes in P-gp

The in-silico binding mode of the sipholanes was studied utilizing the advantage of recent P-gp crystal structure availability [41]. Three binding sites were identified in the P-gp crystallographic structure; QZ59-RRR, QZ59-SSS, and verapamil binding sites [41]. Sipholenone E (**3**), sipholenols L (**4**) and J (**6**), and siphonellinol D (**5**) were docked at each of these binding sites [9]. The docking scores in the binding site of QZ59-RRR were consistent with the P-gp reversal activity of sipholenols [9].

26.7 In-silico Sipholane Binding Modes

Figure 26.4 A shows the binding mode of sipholenone E (**3**), aligned with the crystallographic structure of QZ59-RRR, as predicted by Surflex-Dock simulation [42, 43]. Rings B and C of sipholenone E (**3**) were aligned with the isopropyl moieties of QZ59-RRR [9, 41] and occupied the hydrophobic pockets (Phe 724, Phe 974, and Val 978) and (Phe 766, Phe 833, Gly 985, and Met 982), respectively [9]. Although it has not been reported that QZ59-RRR formed hydrogen bonds (HB) with the internal cavity of P-gp, the docked pose of sipholenone E (**3**) formed a HB (Its C-10 hydroxyl group with the Gln 721), potentially explaining its potent activity and higher binding score compared to QZ59-RRR (Table 26.1) [9]. This interaction was not observed with the less active sipholenols L (**4**) and J (**6**).

The ligand binding pocket of P-gp consisted primarily of hydrophobic and aromatic residues [41]. Of the 73 solvent accessible residues in the internal cavity, 15 were polar and only two (His 60 and Glu 871) were charged or potentially charged [41]. Therefore, it was reasonable to predict that hydrophobic ligands would be more likely to interact with the ligand binding pocket [41]. This fact can partly

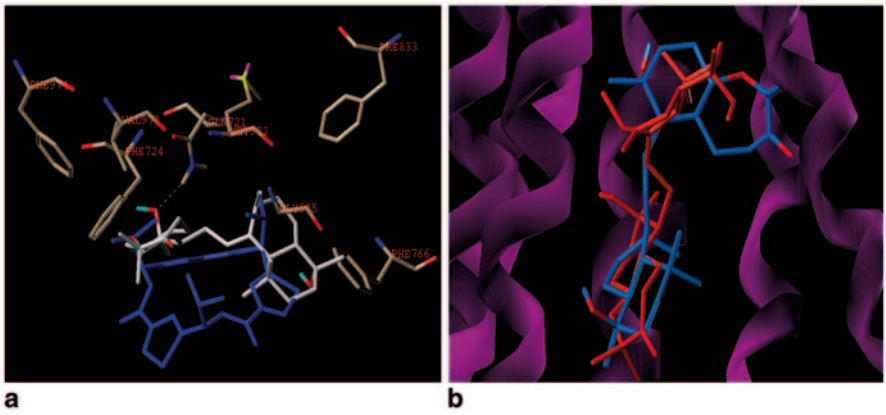


Fig. 26.4 Docked poses of sipholenone E (**3**), sipholenol J (**6**) and QZ59-RRR in the QZ59-RRR binding site of P-gp. **a** The docked pose of **3** (*white*) as predicted by Surflex-Dock compared to the crystallographic structure of QZ59-RRR (*blue*). **b** The alignment of docked poses of **3** (*red*) and **6** (*blue*) [41]

Table 26.1 Binding scores of **3–6** and QZ59-RRR as predicted by Hammerhead Scoring Function of Surflex-Dock [9]

Compound	Total score (-logK_d)
Sipholenone E (*3*)	6.43
Siphonellinol D (*5*)	5.78
Sipholenol L (*4*)	5.55
Sipholenol J (*5*)	4.62
QZ59-RRR	6.03

explain the lack of activity of sipholenol J (**6**), the more hydrophilic C-16 ketone analog of sipholenone E (**3**). The ketone group at C-16 in ring C of **6** proved sterically clashing with the aromatic ring of Phe 833, thereby drastically changing sipholenol J's position into an inactive orientation (Fig. 26.4b), where rings C and D are projected upward.

26.8 Pharmacophore Modeling

P-gp is one of the best characterized ABC transporters in terms of substrate specificity and modulators, including anticancer compounds, calcium channel blockers, immunosuppressants, antipsychotics, antimalarials, antiarrhythmics, antifungals, and antibacterial agents [44]. P-gp has a pseudosymmetrical structure, with twelve transmembrane domains (TMD) and two nucleotide-binding domains (NBD) [45]. The hydrophobic drug substrates bind to the TMD, whereas ATP binds to the NBD and is hydrolyzed, allowing the transport of substrate molecules across the cell membrane [15, 45]. The delay of the discovery of a high-resolution, 3D structure of P-gp till 2009 has impeded the development of new P-gp modulators to a certain extent [41]. Therefore, pharmacophore mapping and QSAR studies have emerged as important tools for the development of P-gp modulators using ligand-based approaches [46]. A QSAR study of various P-gp substrates and modulators suggests that a highly effective P-gp modulator should possess the following features: log $P > 2.92$, an 18-atom long or longer molecular axis, nucleophilicity, and at least one tertiary basic nitrogen atom [47]. Based on the comparison of various P-gp substrates, inducers, and non-substrates, Seelig et al. proposed that two types of structural elements, Types I and II, are required for the substrates to interact with P-gp [48]. The proposed pharmacophore models contain two electron donor groups either 2.5 ± 0.3 Å (type I) or 4.6 ± 0.6 Å (type II) apart, or three electron donor groups, with the outer two groups 4.6 ± 0.6 Å (type II) apart [48]. Pajeva and Wiese proposed a general pharmacophore model of two hydrophobic centers, three H-bond acceptors and a H-bond donor for P-gp substrates and modulators that bind to the verapamil binding site of P-gp [44].

A pharmacophore model (Fig. 26.5) was generated for the P-gp modulating sipholanes (**1**, **3-5**) using DIStanceCOmparison technique (SYBYL's DISCOtech, SYBYL 8.0 software) which basically maps the essential pharmacophoric features required to bind the most active sipholanes [10]. The highest-scoring model reflects maximum structural overlap and maximum number of pharmacophoric features (Fig. 26.5). The model has three hydrophobic points (HY-1 to HY-3) that are 2.97 to 3.93 ± 0.25 Å apart, and two H-bond acceptors (AL-1 and AL-2) that are 11.11 ± 0.25 apart Å [10]. AL-2 can also function as H-bond donor (DL) [10]. This model can be useful for future virtual screening of databases to discover new P-gp modulators, which can be subsequently tested *in-vitro* to confirm their activity.

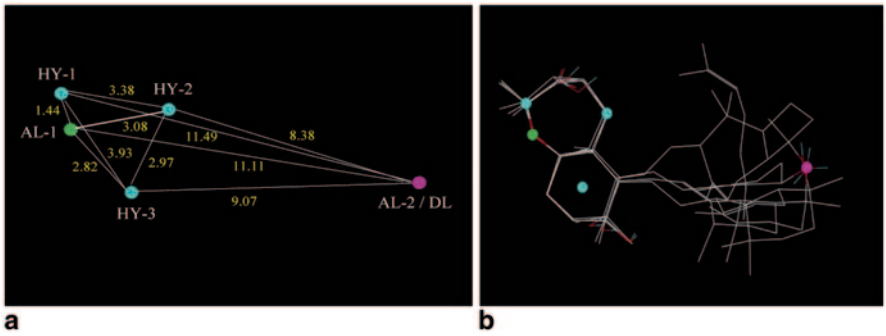


Fig. 26.5 DISCOtech pharmacophore model generated for sipholanes **1**, **3-5**. Pharmacophore features are color-coded: *blue*, hydrophobic point; *green*, H-bond acceptor; *pink*, H-bond acceptor and/or donor. **a** Pharmacophore features with separation distances in Å with a tolerance of 0.25 Å: *AL* H-bond acceptor ligand; *HY* hydrophobic point; *DL* H-bond donor ligand. **b** Pharmacophore model overlapped with compounds **1**, **3-5** [10]

26.9 Sipholanes as Novel Inhibitors of Migration, Proliferation and Brk Phosphorylation

The screening of an in-house marine-derived natural products library in the wound-healing assay (WHA) allowed for the identification of sipholanes **1** and **2** as inhibitors of the migration of the highly metastatic MDA-MB-231 breast cancer cells at mid-μM concentrations [49]. This finding encouraged the reinvestigation of other sipholanes from the source sponge *C. siphonella* (**3-17**), as well as a number of previously reported products of sipholenol A biocatalysis [3] (**18-22**), for antimigratory activity in order to establish a preliminary structure-activity relationship (SAR) profile. Sipholenol A (**1**), sipholenones A (**2**), sipholenol E (**3**), and 16-*α*-epoxy-22-hydroxysipholenol A (**18**) were the only active sipholanes with, IC_{50} values in the mid-μM range (28–37 μM, Table 26.2), suggesting that the various elaboration and structural variations at rings C and D do not play a critical role in determining the antimigratory activity of this class of natural products. The good natural abundance of sipholanes **1** and **2** encouraged their selection for semisynthetic modifications, which afforded a number of structurally diverse analogs (Scheme 26.1). Five different reactions types, including acid-catalyzed elimination, esterification, etherification, carbamoylation and oxime formation, were used to give 20 analogs **25-44**, which were evaluated in the WHA (Table 26.2). Ester analogs of **1** were more active than ether, oxime and carbamate analogs. This illustrates the important role of the ester carbonyl group as well as the distance limit between the aromatic side chain and C-4 oxygen for optimal antimigratory activity [49]. Longer distances, as represented by oximes or carbamates, significantly decreased the antimigratory activity. Aromatic esters showed a better activity profile than aliphatic esters; for example, the benzoate **28** was twice as active as the acetate **25** (Table 26.2). The aromatic ester was tolerant to bioisosteric substitution as evident from the comparable activity of isonicotinate ester (**29**) to the benzoate ester **28** (Table 26.2) [49].

Table 26.2 Antiproliferative and antimigratory activities of compounds **1-59** against human breast cancer cell lines

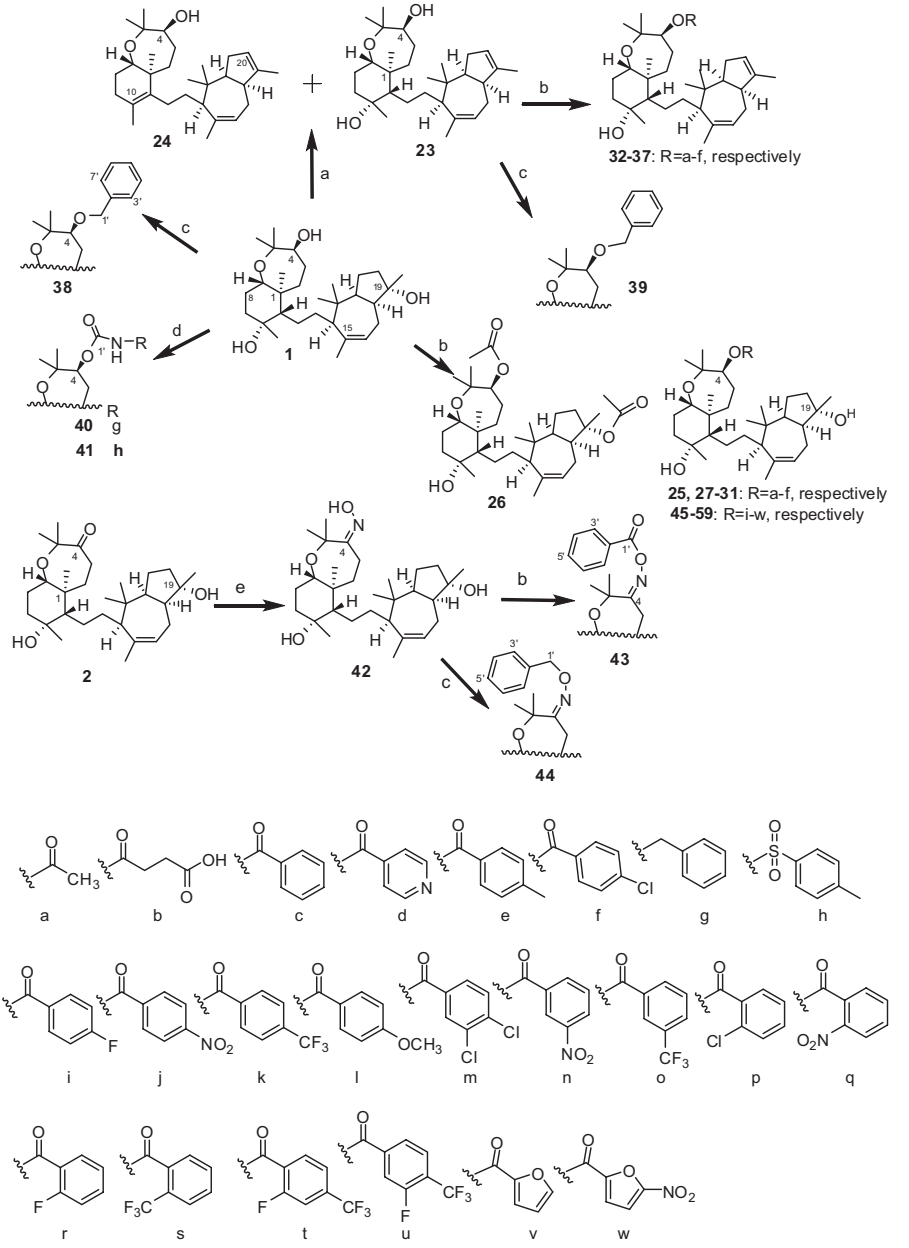
Compound	Antiproliferative activity (IC_{50}, μM)		Antimigratory activity (IC_{50}, μM)
	MCF-7	MDA-MB-231	MDA-MB-231
1	44.5	>50	37.5
2	>50	>50	28.1
3	>50	>50	28.0
4	>50	>50	42.0
5	>50	>50	>50
6	>50	>50	>50
7	31.2	>50	39.0
8	39.8	>50	45.0
9	>50	>50	48.0
10	27.4	>50	>50
11	21.8	>50	>50
12	>50	>50	>50
13	>50	>50	>50
14	>50	>50	>50
15	>50	>50	>50
16	>50	>50	42.6
17	>50	>50	>50
18	>50	>50	33.0
19	>50	>50	38.3
20	>50	>50	47.5
21	>50	>50	>50
22	>50	>50	>50
23	>50	>50	35.3
24	>50	>50	46.7
25	47.9	38.0	16.8
26	39.2	26.2	20.3
27	>50	27.9	18.0
28	21.0	16.1	7.9
29	25.3	18.0	10.6
30	>50	33.4	11.8
31	24.1	10.3	5.3
32	43.5	39.2	16.0
33	>50	29.6	21.0
34	23.1	18.4	7.2
35	>50	19.1	9.5
36	>50	32.7	12.3
37	22.0	12.7	5.9
38	>50	29.1	12.2
39	>50	35.7	13.4
40	24.5	40.3	13.9
41	>50	47.1	18.0
42	46.8	42.7	>50
43	25.6	27.4	28.5

Table 26.2 (continued)

Compound	Antiproliferative activity (IC_{50}, μM)		Antimigratory activity (IC_{50}, μM)
	MCF-7	MDA-MB-231	MDA-MB-231
44	>50	38.2	31.4
45	33.5	11.3	2.4
46	21.6	8.9	3.4
47	23.4	19.5	7.8
48	41.7	30.6	17.9
49	11.9	8.1	1.3
50	36.0	34.7	5.9
51	37.9	36.3	6.1
52	12.1	24.3	14.1
53	5.2	38.7	18.6
54	11.3	28.7	22.7
55	19.2	31.5	34.5
56	>50	36.3	26.4
57	>50	22.2	11.9
58	25.7	14.0	9.3
59	21.3	11.1	3.5

In order to identify a potential target, KINOME scan profiling was performed to quantitatively measure interactions between the most active ester analog **34** and a diverse kinase panel [49]. Analog **34** was screened against 451 human kinases using an ATP site competition-binding assay at a single concentration of 10 μM. In the KINOME scan panel, **34** caused more than 65% displacement of Brk kinase from an immobilized ligand, indicating the selectivity of this analog as a Brk kinase inhibitor with a selectivity score (S_{35}) < 0.01 (Fig. 26.7) [49]. Brk has an established role in promoting cancer cell proliferation, migration and survival, and is an attractive therapeutic target in breast cancer [27]. Accordingly, second-generation substituted aromatic esters were prepared (**45-59**) and evaluated for Brk phosphorylation inhibition as well as inhibition of proliferation and migration of human breast cancer cells (Scheme 26.1).

The antimigratory activity of aromatic esters appears to be strongly influenced by *ortho*-, *meta*- and *para*-substitutions (C-3′, C-4′ and C-5′, respectively, Table 26.2). An electron-donating group (such as OCH_3 and CH_3) at the *para*-position (C-5′) reduced the antimigratory activity in the following order: **48** < **30** < **28** (Table 26.2). On the other hand, an electron-withdrawing group (EWG) at the *para*-position, as in **45**, enhanced the antimigratory activity by seven-fold when compared to an electron-donating group (EDG), as in **48**, and three-fold when compared to the unsubstituted aromatic moiety, as in **28**. However, the influence of the electron-withdrawing group on activity appeared to have a limit. EWG of moderate hydrophobicity (according to Craig's plot) was associated with better antimigratory activity: **45** > **31** > **47** (Table 26.2). Moreover, an EWG at the *meta*-position (for example, **50** and **11**) was also associated with a better antimigratory activity when compared to the unsubstituted benzoate **28**. On the contrary, analogs with an EWG at the



Scheme 26.1 Semisynthetic transformations of sipholenol A (*1*) and sipholenone A (*2*). Reagents and conditions: **a** *p*-toluenesulfonic acid, $CHCl_3$, reflux, 3 h; **b** acid anhydride, DMAP, DCM (anhyd.), reflux, 12 h; **c** benzyl bromide, DMF, NaH, room temperature, 12 h; **d** isocyanate, toluene, Et_3N, reflux, 3 h; **e** hydroxylamine, pyridine, ethanol, reflux, 4 h

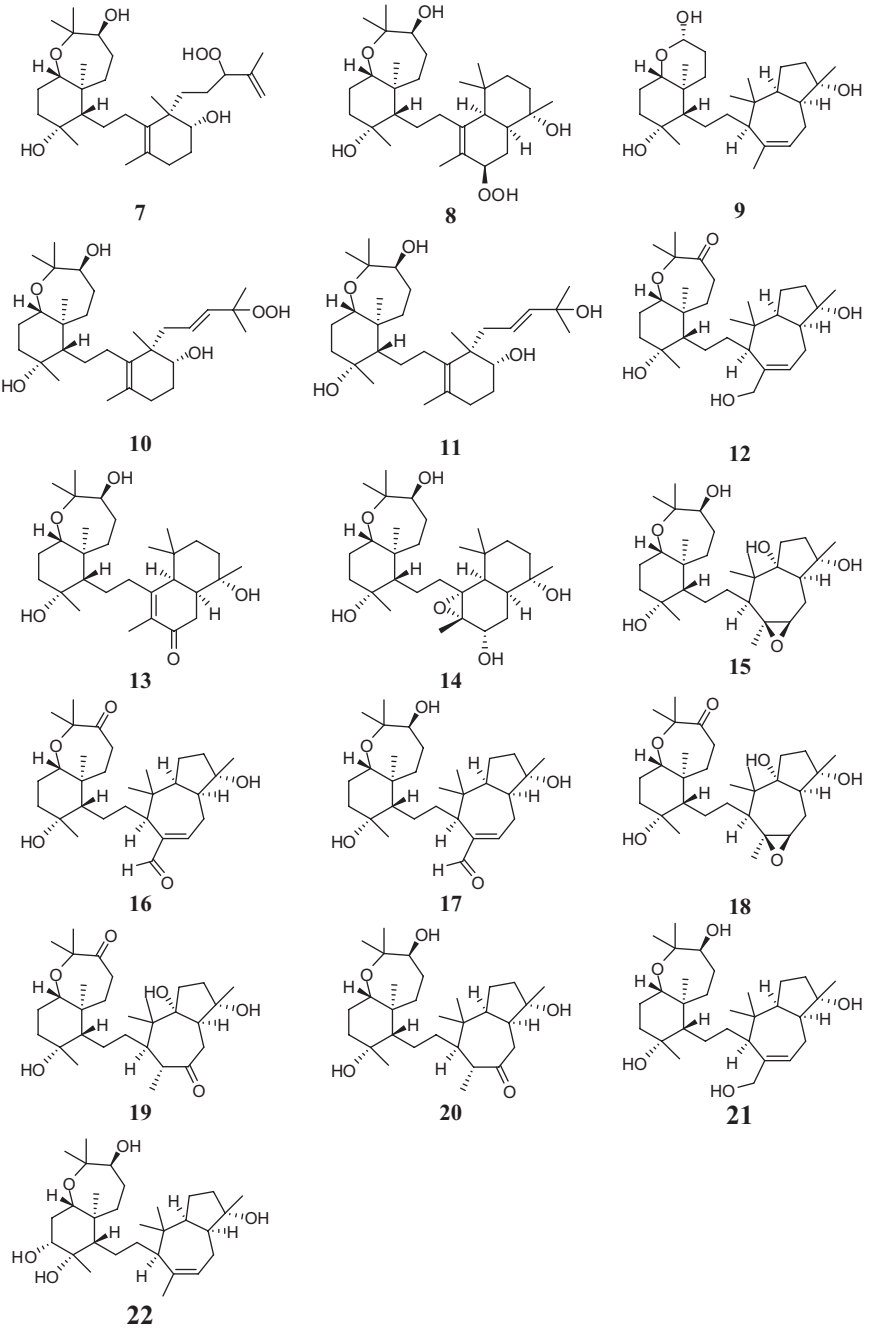


Fig. 26.6 Sipholane triterpenes isolated from the sponge *C. siphonella* (**7-17**) and sipholenol A biotransformation products **18-22** [3, 10, 11]

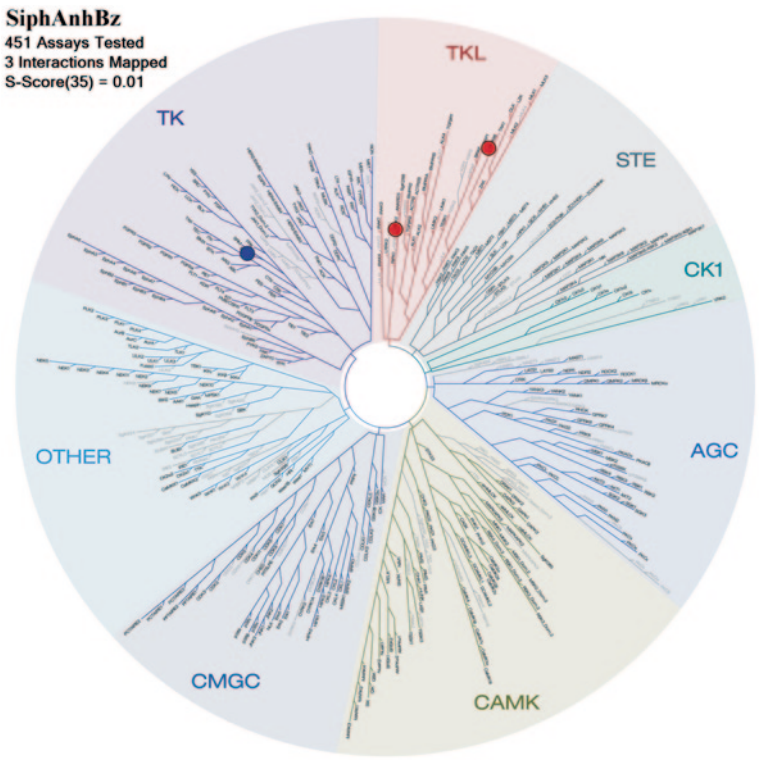


Fig. 26.7 Kinome dendrogram of compound **34**'s selectivity profiling at 10 μM. PTK6 is colored *blue*, and other target kinases are colored *red*. Dendrogram was generated using TREE*spot*™ Software tool with 35 % cutoff. Dendrogram reprinted with permission from KINOME*scan*™

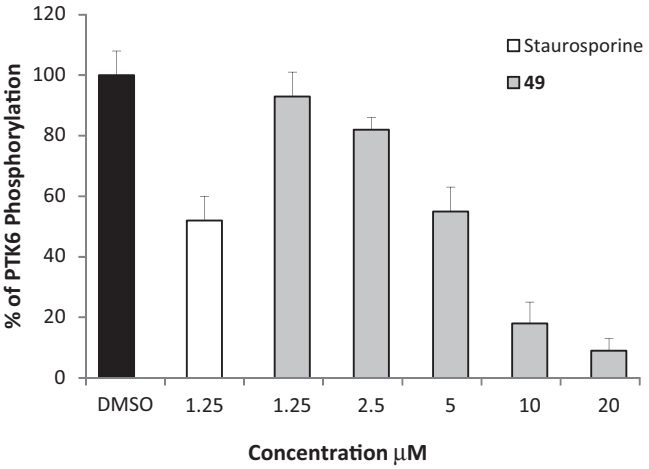


Fig. 26.8 PTK6 phosphorylation inhibition of the most active analog **49** at various concentrations. Results were obtained using a Z′-LYTE assay kit; error bars indicate the SEM of $n = 3$/dose; staurosporine was used as a positive control

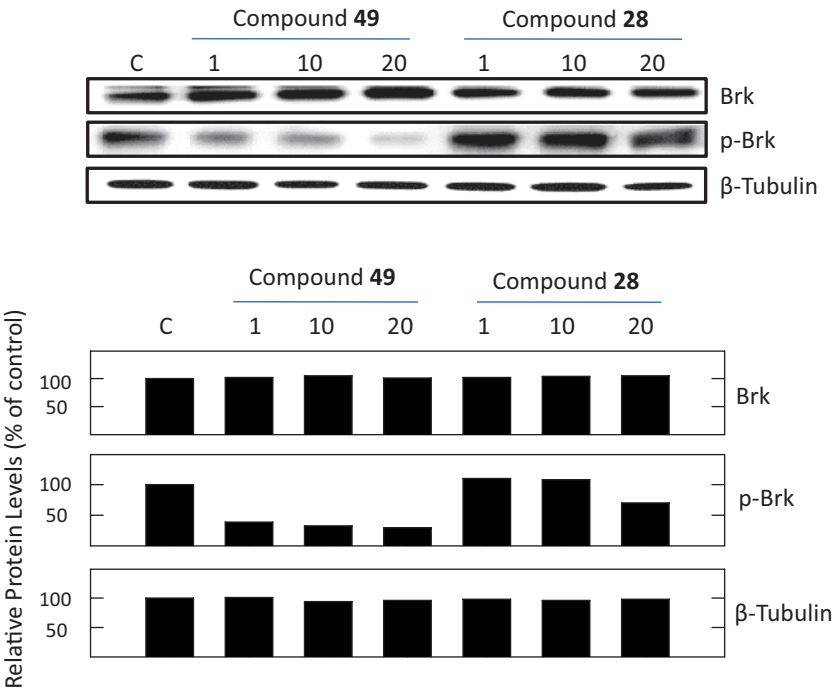


Fig. 26.9 Western blot and densitometric analysis of Brk and p-Brk after exposure of MDA-MD-231 cells to 1, 10, and 20 μM treatments of analogs **28** and **49** for 72 h [50]. β-Tubulin was used as a loading control

ortho-position (**52** vs. **31**, **53** vs. **50**, **54** vs. **45**, **55** vs. **51**) were less active than the unsubstituted benzoate ester (**28**). Having a moderate EWG at both the *ortho*- and *para*-positions imparted the highest activity: 4′,5′-dichlorobenzoate analog (**49**) proved to be the most active analog with an IC_{50} of 1.3 μM (Table 26.2) [50].

In the proliferation assay, sipholenol A (**1**) and most of the natural sipholanes (**2** and **3-22**) showed moderate to high μM antiproliferative activity against MCF-7 and the highly metastatic MDA-MB-231 breast cancer cell lines (Table 26.2).

In general, all semisynthetic analogs showed a better antiproliferative activity than parent compounds sipholenol A (**1**) and sipholenone A (**2**) as well as most of the natural sipholanes, indicating the importance of introducing various bulkier substitutions at the C-4's secondary alcohol group [49].

Inhibition of Brk phosphorylation by **49** was evaluated *in vitro* using cell-free Z'-LYTE kinase assay (Fig. 26.8) and cell-based Western blotting analysis (Fig. 26.9). In the former assay, analog **49** was able to inhibit PTK6 phosphorylation with an IC_{50} value of 5.3 μM (Fig. 26.8). In contrast, parent compounds **1** and **2** showed weak inhibition even at a high concentration (25 μM), which is consistent with their moderate antimigratory activity [50]. Western blotting analysis showed a relatively large, dose-dependent inhibition of Brk phosphorylation after treatment with **49**

for 72 h as compared to the vehicle-treated control group, without affecting total Brk levels (Fig. 26.9). Moreover, the effect of **28** on Brk phosphorylation was only detected at a higher dose (20 μM, Fig. 26.9). These results are consistent with the superior antimigratory activity observed for **49** compared with **28**.

Selectivity to cancer cells is an important factor for determining the success of a potential anticancer agent. The cytotoxicity of sipholanes and semisynthetic analogs towards non-tumorigenic human breast cells was assessed using MCF10A cells [50]. All active analogs were nontoxic at concentrations equal to their IC_{50} values or higher in both WHA and proliferation assays, suggesting their good selectivity towards malignant cells.

26.10 Pharmacophore Modeling and 3D-QSAR

26.10.1 Pharmacophore Model

PHASE module of Schrödinger molecular modeling software was used to build pharmacophore models for sipholanes and their semisynthetic analogs in order to rationalize their chemical structure with their antimigratory activity [50]. Each of these models contained a maximum of six of the following features: hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic group (H) and aromatic ring (R). Ten pharmacophore models with three different variant combinations (AAADHR, AAAHHR and AADHHR) survived the scoring process (Table 26.3) [51, 52]. These represented top-scoring hypotheses with the best alignment of actives

Table 26.3 Various pharmacophore hypotheses generated by PHASE

Hypothesis	Survival Score	Survival-inactive Score	Post-hoc Score	# Matches[a]	Energy[b]	Activity[c]
AAADHR.348	3.500	2.181	6.430	19	0.012	5.886
AAADHR.389	3.497	1.992	6.428	19	0.012	5.886
AAAHHR.7687	3.472	2.095	6.402	19	0.012	5.886
AAAHHR.5876	3.470	1.907	6.401	19	0.012	5.886
AADHHR.3334	3.483	2.328	6.413	19	0.012	5.886
AADHHR.7559	3.481	2.214	6.411	19	0.012	5.886
AADHHR.3400	3.475	1.991	6.405	19	0.012	5.886
AADHHR.308	3.471	2.437	6.101	19	0.313	5.886
AADHHR.290	3.464	2.337	6.094	19	0.313	5.886
AADHHR.305	3.460	2.129	6.090	19	0.313	5.886

[a] The number of active ligands that matched the hypothesis
[b] Energy of the reference conformer relative to the lowest-energy conformer
[c] Reference ligand activity expressed as pIC_{50}

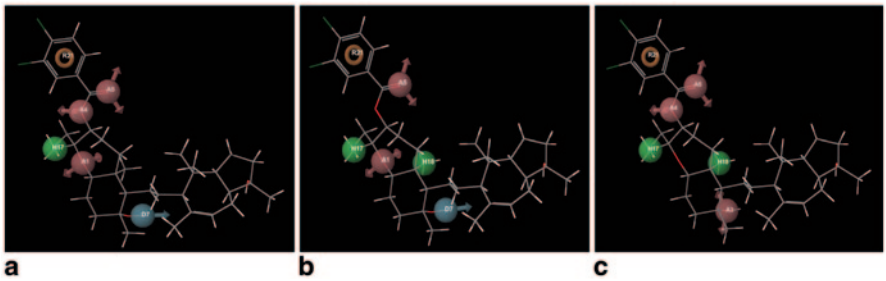


Fig. 26.10 Most active compound **49** aligned with the top scoring hypothesis of variant **a** AAADHR; **b** AADHHR; and **c** AAAHHR. *Red* spheres represent hydrogen bond acceptors (A), *green* spheres represent hydrophobic groups (H), *cyan* spheres represent hydrogen bond donors (D) and *brown ring* represents aromatic (R) groups

[51, 53–55]. In general, a good hypothesis should provide superior alignment with active compounds, discriminate between active and inactive compounds and display the lowest possible relative conformational energy values [51]. Figure 26.10 shows the alignment of the most active compound **49** with the top scoring hypothesis from each of the three variants. The generated hypotheses successfully identified the most important structural features implicated in the antimigratory activity of sipholanes: C-1′ carbonyl oxygen, aromatic ring and C-10 hydroxyl group. The absence of any of these features compromised the antimigratory activity as previously discussed. For example, the C-4-*O*-benzoyl ester **28** is about twice as active as both the C-4-*O*-phenyl ether **38** and the C-4-*O*-acetate ester 2**5**. Moreover, sipholenol A (**1**) is almost as active as its anhydro analog **3** but is significantly more active than the dianhydro analog **4**, indicating the importance of the C-10 hydroxyl, but not the C-19 hydroxyl, to the antimigratory activity. The pharmacophore models further supported the notion that rings C and D are not major determinants of the antimigratory activity of sipholanes since they had no contribution to the pharmacophoric elements. This allowed for structural simplification and would make the future synthesis of antimigratory sipholane-based analogs more feasible.

26.10.2 3D-QSAR

A 3D-QSAR model was generated in order to statistically establish a relationship between the 3D-spatial arrangement of pharmacophoric features and the antimigratory activity of sipholanes. The surviving pharmacophore hypotheses (Table 26.4) were used to align analogs for building an atom-based 3D-QSAR model by partial least square (PLS) analysis [50, 51, 53]. Analogs were divided into training and test sets. The latter was used to validate the generated models [54]. Several models containing

Table 26.4 Statistical parameters of the best atom-based 3D-QSAR model generated by PHASE

Hypothesis	PLS[a]	SD[b]	r^{2} [c]	F[d]	P[e]	RMSE[f]	q^{2}[g]	Pearson-R[h]
AADHHR.3334	1	0.302	0.650	51.9	7.711e-008	0.194	0.664	0.847
	2	0.253	0.763	43.3	3.673e-009	0.135	0.836	0.953
	3	0.237	0.799	34.5	3.264e-009	0.151	0.795	0.940
	4	0.211	0.847	34.7	7.219e-010	0.134	0.840	0.938
	5	0.166	0.910	48.4	9.229e-012	0.128	0.853	0.943

[a] Number of factors in the partial least squares (PLS) regression model
[b] Standard deviation (SD) of the regression
[c] Value of r^2 for the regression
[d] Variance ratio. Large values of F indicate a more statistically significant regression
[e] Significance level of variance ratio. Smaller values indicate a greater degree of confidence
[f] Root-mean-square error of the test set
[g] Value of q^2 for the predicted activity of the test set
[h] Value of Pearson-R for the predicted activities of the test set

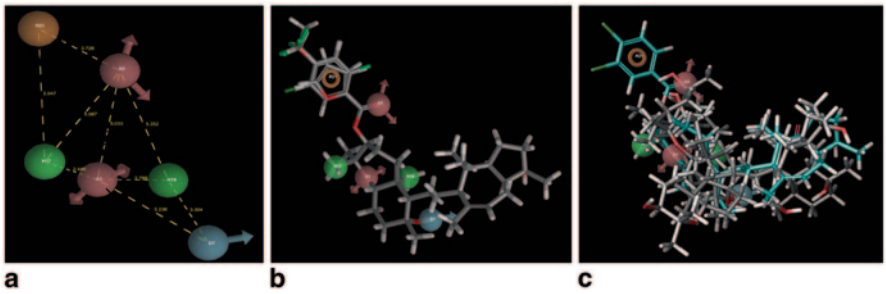


Fig. 26.11 Alignment based on the pharmacophore hypothesis AADHHR.3334 generated the best 3D-QSAR model. (**a**) Common pharmacophoric features identified by this hypothesis. All distances are in Å unit. Alignment of some of the most active (**b**) and least active (**c**) sipholane A analogs to this pharmacophore. The most active analog **49** is shown in *cyan*

up to five PLS factors were generated and the best 3D-QSAR model was based on the AADHHR.3334 pharmacophore hypothesis (Fig. 26.11, Table 26.4). This is a five-PLS factor 3D-QSAR model with good statistics and predictive ability as reflected from r^2 (0.910), q^2 (0.853) and Pearson-R (0.943) values (Table 26.4). The model offered a good correlation between predicted and actual antimigratory activity for both training and test set compounds (Fig. 26.12).

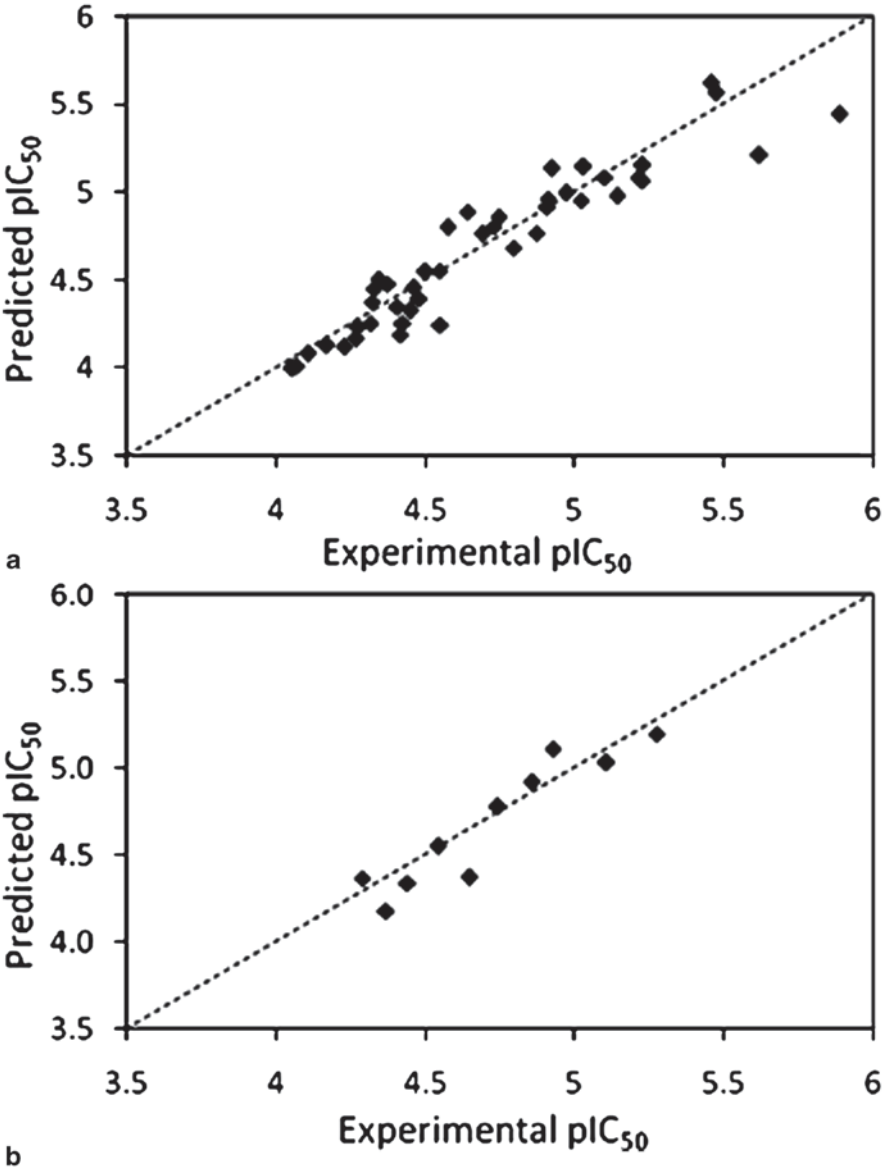


Fig. 26.12 Scatter plots for the predicted and experimental pIC_{50} values as calculated by the 3D-QSAR model for the (**a**) training set, and (**b**) test set analog members

26.11 Conclusions

Sipholane triterpenes represent a novel marine natural product class of P-gp modulators and Brk phosphorylation inhibitors with tremendous potential for further development and optimization. Substituted aromatic sipholenol A esters showed enhanced antimigratory activity, compared to sipholenol A. Pharmacophore modeling and 3D-QSAR studies of sipholanes provided the foundation for future design of novel antimigratory entity based on simplified sipholane structure, including rings A and B (perhydrobenzoxepine) connected to substituted aromatic esters with the elimination of rings C and D, the [5,3,0] bicyclodecane system. This will make future synthesis of new active sipholane-based hits more feasible and cost-effective. These results demonstrate the contribution of the marine environment towards the discovery of novel P-gp modulators and Brk phosphorylation inhibitors appropriate for use to control metastatic breast cancer.

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Chapter 27
Molecular Targets of Anticancer Agents from Filamentous Marine Cyanobacteria

Lik Tong Tan and Deepak Kumar Gupta

Abstract The prokaryotic marine cyanobacteria, especially the filamentous forms, are known to produce a plethora of structurally unique natural products. A majority of these molecules are nitrogen-containing, belonging to the hybrid polyketide-polypeptide structural class. Various activities of clinical significance have been attributed to these molecules, ranging from anticancer, neuromodulating to antiprotozoal properties. Particularly in the area of cancer therapy, a number of potent marine cyanobacterial compounds, including dolastatins 10, 15, and largazole, have been identified as anticancer drug leads and are being further developed synthetically for clinical usage. The high potencies of these compounds are due to their exquisite interactions or interference with cellular pathways, specific macromolecules, or enzymes, such as the JAK-STAT signaling pathway, histone deacetylase, proteasome, protein kinase C, actin and microtubule filaments. This mini review covers more than 90 references and features more than 40 anticancer compounds, consisting of marine cyanobacterial natural products and their synthetic analogues. Biological data of these compounds are discussed based on their molecular targets.

Keywords Marine cyanobacteria · Polyketide-polypeptide · Anticancer · Drug targets

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27.1 Introduction

Filamentous marine cyanobacteria are found in diverse communities, such as epiphytes and microbial mats in coral reef ecosystems. Since the 1970s, natural products research on these marine photosynthetic microbes has revealed its chemical richness in terms of unique structures. The high success rate of marine cyanobacteria in colonizing different habitats could due to the ecological importance of these natural products, including UV-protection, feeding deterrence, allelopathy, as well as resource competition [1]. Currently, almost 500 natural products have been isolated from various cyanobacterial genera, particularly *Lyngbya*, *Symploca*, and *Oscillatoria* [2–4]. A majority of these compounds are products of the biosynthetic enzymes, NRPS (Non-Ribosomal Polypeptide Synthetase) and PKS (Polyketide Synthase), giving rise to hybrid type structures consisting of amino acids as well as acetate-derived units. Moreover, research focusing on the characterization of marine cyanobacterial biosynthetic gene clusters carried out in the past 10 years has revealed unusual features relating to mechanisms and enzymatic reactions [5].

A number of pharmacological trends have been observed amongst the various marine cyanobacterial compounds that have been evaluated for biological activities. A significant number of these molecules have been reported to possess potent anticancer properties. More importantly, these compounds are known to inhibit/interfere with validated cellular targets important in cancer therapy. These cellular targets include cytoskeletal structures, e.g., microtubules and actin filaments, as well as enzymes, such as proteasome, and histone deacetylases. Another pharmacological trend observed is the preponderance of compounds having neuromodulating properties. These neurotoxic compounds are known to either activate (e.g., antillatoxin) or block (e.g., kalkitoxin and jamaicamides) voltage-gated sodium channels [6–8]. It is only recently that marine cyanobacteria are known to be an important source of antiprotozoal (e.g., coibacins) and anti-inflammatory (e.g., honaucins) agents [9, 10].

This mini review features a selection of important marine cyanobacterial compounds with potential use in cancer therapy. The selection includes molecules that have been used as drug leads for further development of anticancer agents as well as recently discovered molecules with potent activities. In addition, emphasis is placed on molecules with known mode of actions, particularly those interfering with medically relevant drug targets. Moreover, these compounds are selected to reflect current research trends in natural products research on marine cyanobacteria and they are presented based on their interference with molecular targets, such as actin and microtubule filaments, apoptotic/cellular signaling pathways, histone deacetylase, proteasome, and protein kinase C.

27.2 Actin and Microtubule Filaments Disruptors

27.2.1 Dolastatins 10/15 and Related Compounds

One of the earliest examples of potent microtubule disruptors reported from marine cyanobacteria are the dolastatins, such as dolastatins 10 (**1**) and 15 (**2**) (Fig. 27.1; [11]). The dolastatins were originally reported from the Indian Ocean sea hare, *Dolabella auricularia*. However, these compounds were initially speculated to be

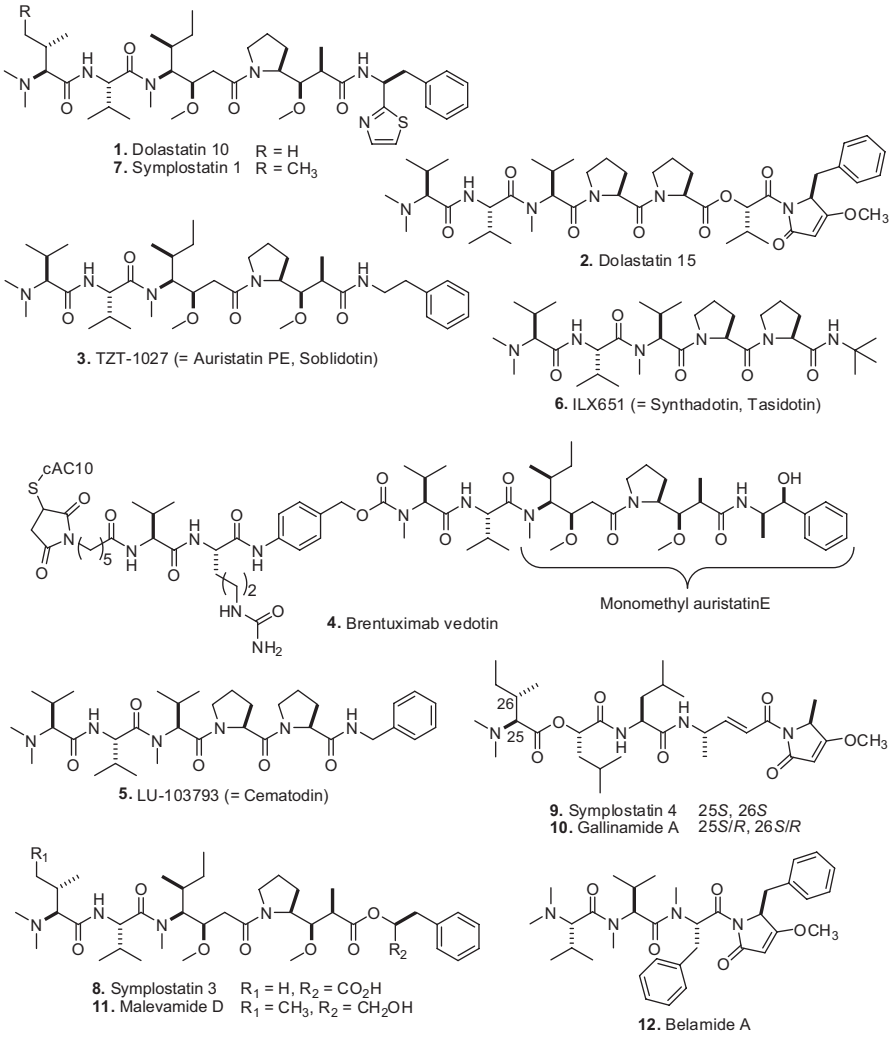


Fig. 27.1 Natural and synthetic microtubule filaments disruptors

diet-derived due to minute quantities obtained from the invertebrates. Subsequently, a number of dolastatins and related molecules were isolated from filamentous marine cyanobacteria, which are the natural diet of the sea hares [12].

Dolastatin 10 (**1**) is a linear pentapeptide consisting of a valine unit along with four unique residues including dolavaline, dolaisoleucine, dolaproline, and dolaphenine. Amongst the dolastatins, **1** is the most active molecule in inhibiting cancer cell growth [13]. The GI_{50} values (in μg/mL) of dolastatin 10 when tested in a panel of human tumor cell lines, including OVCAR-3, SF-295, A498, NCI-H460, KM20L2, and SK-MEL-5, were 9.5×10^{-7}, 7.6×10^{-6}, 2.6×10^{-5}, 3.4×10^{-6}, 4.7×10^{-6}, and 7.4×10^{-6}, respectively. The potent nature of dolastatin 10 was subsequently attributed to its binding to tubulin at a distinct site near to the *Vinca* alkaloid site [13]. The binding resulted in the disruption of normal tubulin function as well as the exchangeable guanosine triphosphate site leading to cell cycle arrest in the metaphase. In the 1990s, dolastatin 10 entered phase I clinical trials through the National Cancer Institute and subsequently progressed to phase II trials. However, further clinical trials were discontinued due to significant side effects, including the development of peripheral neuropathy reported in 40% of patients as well as poor efficacy in patients with hormone refractory metastatic adenocarcinoma and recurring platinum-sensitive ovarian carcinoma [14].

Several dolastatin 10 analogues were subsequently synthesized for further SAR studies which led to the development of a simplified derivative, TZT-1027 (**3**) (=Soblidotin; Auristatin PE) (Fig. 27.1), with reduced toxicity and it proceeded to several clinical trials in USA, Europe, and Japan. In addition to its excellent pharmacological profile, TZT-1027 possesses antivascular property by inducing damage of vascular endothelial cells [14]. Furthermore, TZT-1027 was shown to have antiangiogenic activity in an *in vivo* chick chorioallantoic membrane assay and in an *in vitro* tube formation assay using human umbilical vein endothelial cells [15]. Such dual activities make TZT-1027 a useful agent for the treatment of solid tumors [16]. Moreover, this synthetic analog was found to enhance the antitumor effect of radiation in H460 cells in *in vitro* studies as well as in *in vivo* system using nude mice [17].

Various phase I and II clinical trials using TZT-1027 were carried out in USA, Europe, and Japan. Phase I clinical trials were focusing mainly on the efficacy of TZT-1027 in patients with either solid tumor, non small-lung cancer, advanced refractory cancer, or soft tissue sarcoma [18–22]. It was found that the recommended dosage for phase II studies were generally higher in the European studies (2.4 to 4.8 mg/M^2) as compared to those in Japan (1.5 to 1.8 mg/M^2) and the most common dose limiting toxicity was neutropenia. Due to promising data from phase I studies of TZT-1027 against soft-tissue sarcoma, phase II trials were conducted in patients with advanced or metastatic soft-tissue sarcomas. Phase II study revealed that TZT-1027 was safe and well tolerated [23]. However, in another phase II study conducted on patients with advanced non-small cell lung cancer after treatment with platinum-based chemotherapy, TZT-1027 was found inactive as an anticancer drug [24].

In another development, variants of auristatin PE, such as auristatins E and F, have been formulated as antibody drug conjugates (ADCs). ADCs make use of monoclonal antibodies (mAbs) for the delivery of cytotoxic drugs into tumor cells. Cytotoxic drugs are covalently tethered to mAbs via peptide linker and these ADCs target tumor cells by the recognition of specific antigens expressed on the surfaces of tumor cells. Recently an antibody–drug conjugate, brentuximab vedotin (**4**) (Fig. 27.1), which contain the anti-mitotic agent, monomethyl auristatin E, has been approved by US Food and Drug Administration in August 2011 for the treatment of systemic anaplastic large cell lymphoma and Hodgkin lymphoma [25]. In Phase II trials, when brentuximab vedotin was administered to patients with refractory or relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma, the objective response was observed in 75 and 86 % of patients, respectively. Currently there are at least two auristatin-based ADCs, SGN-35 and CRO11-vc-MMAE, in clinical trials for the treatment of patients with Hodgkin's lymphoma and melanoma [26]. Preliminary phase I studies using SGN-35 indicated that patients responded well to the treatment with 23 % showing complete regression and 45 % indicating objective responses [26].

Dolastatin 15 (**2**) is a linear peptide consisting of seven amino acid or hydroxyl acid units. Preliminary biological data of this molecule indicated that it is on average about seven times less active than dolastatin 10 [11]. Similar to dolastatin 10, the potent cytotoxic nature of dolastatin 15 is due to its binding with the β-subunit of the dimeric form of tubulin. Although dolastatin 15 did not enter clinical trials, two simplified synthetic analogues, LU-103793 (**5**) (=Cematodin) and ILX651 (**6**) (=Synthadotin; Tasidotin) (Fig. 27.1), were developed as drug candidates for clinical testing. LU-103793 (**5**) was developed as a water soluble analog and it entered phase I trials in USA and Europe in the 1990s. Preliminary results from phase I clinical studies on patients with advanced solid malignancies were favorable to warrant its continuation into phase II clinical trials. Unfortunately, LU-103793 did not perform well in phase II clinical testing and its clinical evaluation was eventually discontinued.

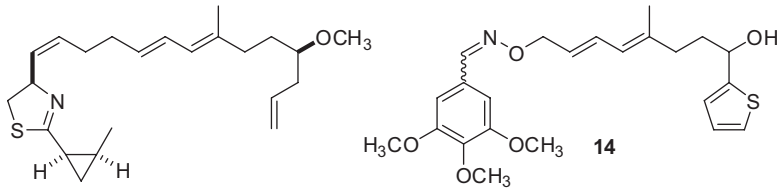
ILX651 (**6**) was developed as a third generation water soluble dolastatin 15 analog with superior metabolic stability and bioavailability compared with LU-103793. Phase I clinical studies of ILX651 on patients with advanced solid tumors indicated that the compound is well tolerated without any observed cardio toxicities as displayed by cemadotin [27–29]. ILX651 just completed in at least three phase II clinical trials on patients with hormone-refractory prostate cancer and advanced or metastatic non-small lung carcinoma. However, published data on the outcome of these phase II studies are currently not available.

Since the discovery of dolastatins 10 (**1**) and 15 (**2**), several related analogues, such as symplostatins 1 (**7**, a dolastatin 10 analog), 3 (**8**, a dolastatin 10 analog), and 4 (**9**, a dolastatin 10/15 hybrid), gallinamide A (**10**, a dolastatin 15 analog), malevamide D (**11**, a dolastatin 10 analog), and belamide (**12**, a dolastatin 15 analog) (Fig. 27.1) have been reported from various *Symploca* species [30–35]. A number of these analogues possess potent cytotoxic activity against various cancer cell lines in the nanomolar range and were shown to interfere with the normal function of microtubules.

Of the various cyanobacterial-derived dolastatin analogues, symplostatins 1 (**6**), 3 (**7**), and malevamide D (**10**) are the most cytotoxic. The IC_{50} values reported for symplostatins 1 and 3 against KB and LoVo cells were 0.15–0.20 nM and 0.34–0.50 nM and 3.9 nM and 10.3 nM, respectively. This contrasted with the IC_{50} values for the more potent dolastatin 10 at 0.052 nM and 0.076 nM when tested against KB and LoVo cells, respectively [31, 36]. At a concentration of 2.5 nM, symplostatin 1 caused the complete loss of cellular microtubules in A-10 cells and its EC_{50} was observed at 0.6 nM. However, a higher concentration of 1 μg/mL was required for symplostatin 3 to induce complete depolymerization of cellular microtubules. Similar to dolastatin 10, symplostatin 1 is highly toxic due to symptoms such as necropsy and liver toxicity when injected intravenously into mice at single doses of 1.0 or 1.4 mg/kg. Malevamide D is another potent dolastatin 10 analog with IC_{50} values of 0.3–0.7 nM when evaluated against P-388, A-549, and HT-29 cell lines. Even though it was not tested in A-10 cells, it is speculated that it targets microtubules due to structural similarity with symplostatin 3.

27.2.2 Curacin A

The curacins are a class of unique thiazoline-containing lipopeptides possessing exquisite antimitotic activities. Reported in 1994, curacin A (**13**) (Fig. 27.2) is the most potent molecule and was isolated as a major component (8–10%) from the organic extract of the marine cyanobacterium, *Lyngbya majuscula*, collected from Curacao [37]. Biological screening of this molecule in the NCI 60 cell line revealed curacin A as a potent anti-tubulin agent with IC_{50} value of 0.72 μM in tubulin polymerization inhibition. Specifically, curacin A binds selectively to the colchicine site of tubulin and the inhibition of colchicine binding was about 94% when tested at 5 μM. Due to its unique structural features and high potency as an anti-tubulin agent, curacin A was a target of at least seven total synthesis and numerous synthetic analogues were also generated for SAR studies [38]. The most promising synthetic analog to date is the simplified compound **14** (Fig. 27.2), which is 3 times more potent when compared to curacin A in inhibiting GTP/glutamate-induced polymerization of tubulin with IC_{50} value at 0.17 μM [39].



13. Curacin A

Fig. 27.2 Natural and synthetic microtubule filaments disruptors

27.2.3 Dolastatins 11/12 and Related Compounds

Dolastatins 11 (**15**) and 12 (**16**) (Fig. 27.3) are potent cytotoxic cyclic depsipeptides originally isolated from the sea hare, *Dolabella auricularia* [40]. These molecules

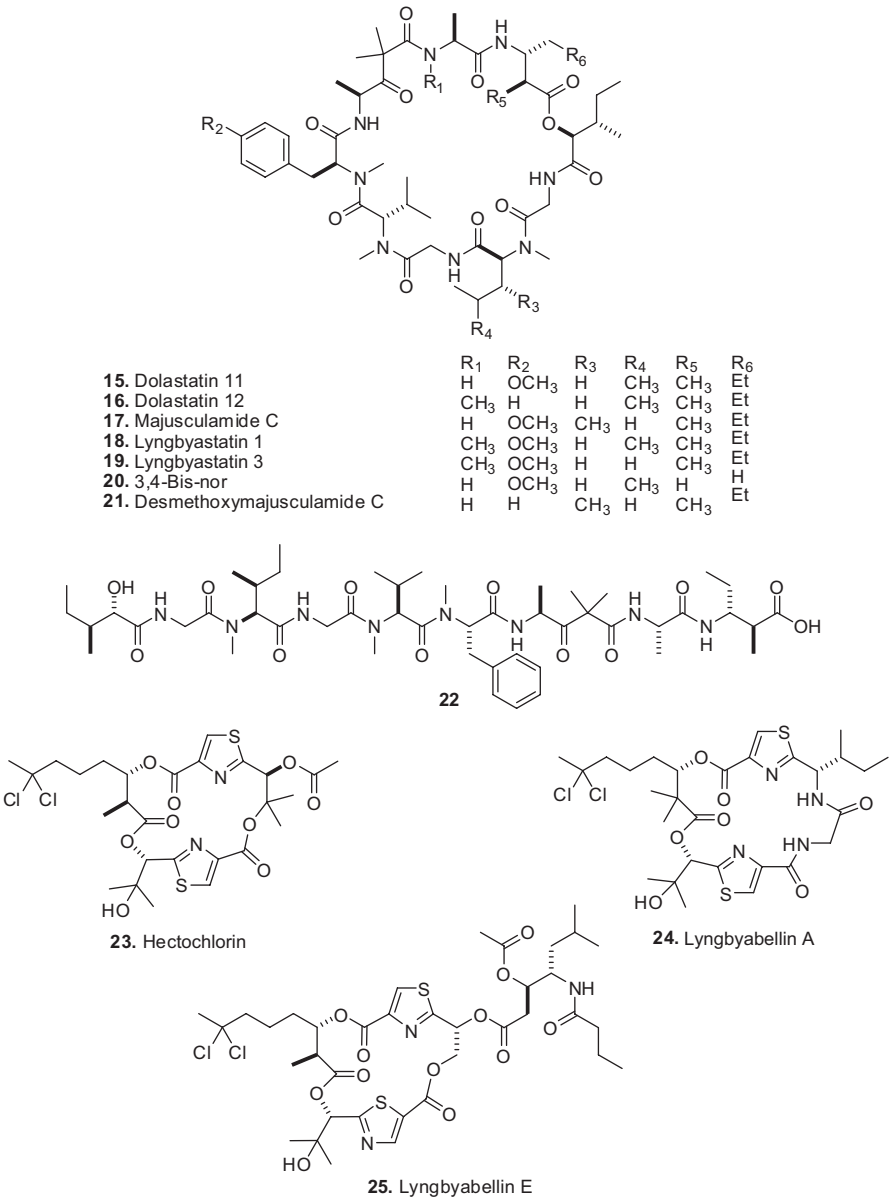


Fig. 27.3 Natural and synthetic actin filaments disruptors

belong to the hybrid polyketide-polypeptide structural class, comprising of eight amino acid units and one hydroxyl acid residue. Dolastatin 11 is highly toxic with GI_{50} values in the nanomolar range when tested against a panel of different cell lines including NCI-H460, OVCAR-3, SF-295, and KM20L2 [41]. Overall, dolastatin 11 is 10–30 times more cytotoxic than dolastatin 12 in several cell lines [42]. Its cytotoxicity was due to involve the disruption of microfilaments by inducing hyperpolymerization of actin in PtK1 cells resulting in the inhibition of cytokinesis [42]. Subsequently, it was shown that dolastatin 11 stabilize microfilaments by the connection of two long-pitch strands in F-actin using X-ray fiber diffraction diagrams from oriented filament sols [43]. However, dolastatin 11 has not been pursued further for clinical development due to reports of other analogues displaying toxic effects such as pulmonary hemorrhage in mice [30]. Ali and co-workers synthesized a total of 20 dolastatin 11 analogues for SAR studies using a panel of different cell lines as well as actin binding properties. Of these 20 synthetic analogues, the 3,4-bis-nor analog (**20**) (Fig. 27.3) was found to have reduced cytotoxicity but still maintain relatively strong anti-actin activity [41].

Since the discovery of dolastatins 11 and 12, various isolates of filamentous marine cyanobacteria have been reported to produce related compounds, such as majusculamide C (**17**), lyngbyastatins 1 (**18**), and 3 (**19**) (Fig. 27.3). A recent isolation of a dolastatin 11 analog, desmethoxymajusculamide C (**21**) (Fig. 27.3), from a Fijian marine cyanobacterium, *Lyngbya majuscula*, was found to be significantly cytotoxic in both cyclic and ring-opened form (**22**) (Fig. 27.3) [44]. Furthermore, both cyclic and ring-opened forms displayed similar effects in the actin microfilament disruption assays. At 52 nM, both forms induced loss of filamentous F-actin when tested against A-10 cells.

27.2.4 Hectochlorins/Lynbyabellins

Another class of marine cyanobacterial cyclic depsipeptides having potent actin-disruption activity is the lyngbyabellins/hectochlorins. These are unique bithiazole-containing compounds having the unusual dichlorinated β-hydroxyl acid residue, 7,7-dichloro-3-hydroxy-2-methyl-octanoic acid. In this series of related compounds, hectochlorin (**23**) (Fig. 27.3) is the most potent in promoting actin polymerization [45]. Hectochlorin was initially reported from an isolate of *Lyngbya majuscula* obtained from Hector Bay, Jamaica, and its complete structure was determined using 1D, 2D NMR, as well as X-ray crystallography method. The pharmacological target for hectochlorin was suggested to be actin microfilaments based on the observed accumulation of CA46 cells in the G2/M phase of the cell cycle demonstrated using flow cytometry. Subsequently, it was shown that hectochlorin induce actin polymerization in PtK2 cells with EC_{50} value at 20 μM. In addition, compound **23** was unable to displace a fluorescent phalloidin analog from the polymerized actin suggesting a different binding site for this molecule. Biological evaluation of **23** in the NCI 60 cancer cell lines showed significant cytotoxicity against a number of cancer cell lines, including colon melanoma, ovarian, and renal cells. Furthermore,

the dose-response curve of hectochlorin gave a flat shape, suggesting that the molecule is antiproliferative instead of cytotoxic.

A number of hectochlorin-related compounds with potent actin polymerization activity are lyngbyabellins A (**24**) and E (**25**) (Fig. 27.3), reported from a Guamanian and Papua New Guinea strain of the marine cyanobacterium, *Lyngbya majuscula*, respectively [46, 47]. Both compounds displayed moderate cytotoxicity against various cell lines. At concentrations of 0.01–5.0 μg/mL, lyngbyabellin A disrupted cellular actin microfilaments in A-10 cells. However, in *in vivo* testing, it was shown to be lethal to mice when injected at 2.4 to 8.0 mg/kg [46]. Lyngbyabellin E had similar actin polymerization activity and at 60 nM, it caused complete loss of cellular microfilament network giving rise of binucleated cells in A-10 cells [47].

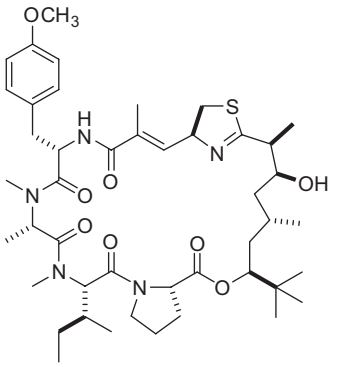
27.3 Induction and Interference of Apoptotic/Cellular Signaling Pathways

27.3.1 Apratoxins

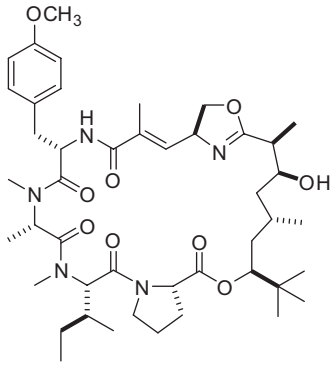
The apratoxins are a novel class of potent cytotoxic cyclic depsipeptides isolated from strains of *Lyngbya* sp. collected from different geographical locations. They possess significant biological activities in the nanomolar range when tested against a panel of various cancer cell lines, including HT29, HeLa, and U2OS. Pharmacological studies conducted on apratoxin A (**26**) (Fig. 27.4) revealed its ability to interfere with specific signaling pathways as well as protein interactions involved with the formation and maintenance of cancer cells [48]. For instance, studies by Liu and co-workers found apratoxin A to be a potent inhibitor of the JAK (Janus kinase)-STAT (Signal Transducer and Activator of Transcription) signaling pathway by down regulating interleukin 6 signal transducer [49]. In another study using a synthetic oxazoline analog of apratoxin A, it was proposed that compound **27** (Fig. 27.4) stabilizes the Hsp90 (heat shock protein 90) client proteins-Hsc70/Hsp70 interaction, thereby inhibits the function of Hsp90 [50]. By inhibiting the function of Hsp90, it resulted in the promotion of Hsp90 client proteins via chaperone-mediated autophagy. The chaperon Hsp90 has been an important target for the development of anticancer drugs due to its role regulatory roles in transformed cell types. Recently, Chen and co-workers revealed apratoxins to exert its apoptotic effect by down regulating receptors and associated growth ligands in cancer cells [51]. SAR studies were subsequently conducted and based on the structural features of apratoxins A and E, a hybrid synthetic molecule, **28** (Fig. 27.4), with improved *in vivo* antitumor properties was identified [51].

27.3.2 Aurilides/Lagunamides

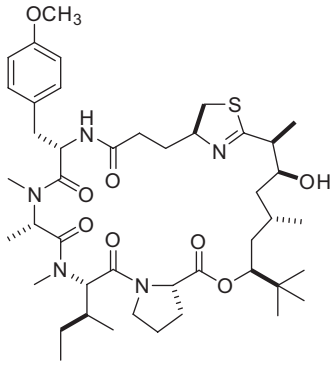
Aurilide (**29**) (Fig. 27.4) and related compounds, such as the lagunamides, are potent cytotoxic cyclic depsipeptides having activities in the pico to nanomolar range.



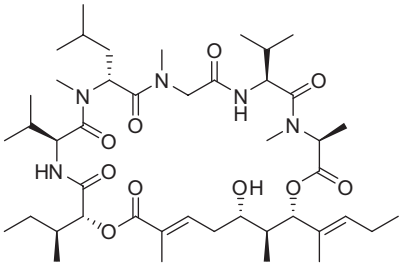
26. Apratoxin A



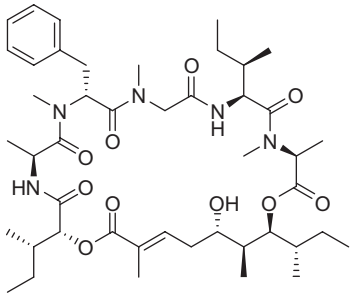
27. Oxapratoxin A



28. Synthetic hybrid apratoxin A/E molecule



29. Aurilide



30. Lagunamide A

Fig. 27.4 Natural and synthetic modulators of apoptotic/cellular signaling pathways

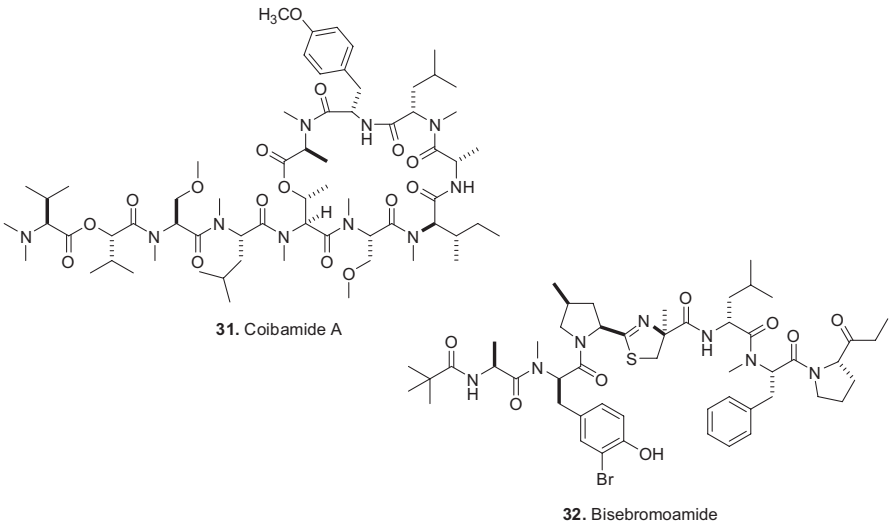


Fig. 27.5 Natural modulators of apoptotic/cellular signaling pathways

Similar to the dolastatins, aurilide was first isolated from the Japanese sea hare, *Dolabella auricularia* [52, 53]. However, the microbial biogenesis of this molecule is implicated due to reports of related analogs purified from marine cyanobacterial strains [54]. Pharmacological investigation conducted by Sato and co-workers showed aurilide (**29**) to be a potent inhibitor of mitochondrial prohibitin 1 [55]. The inhibition of prohibitin 1 resulted in the activation of the proteolytic process of optic atrophy 1, which further led to the mitochondria-induced apoptosis. Prohibitin 1 is an attractive drug target due to its role in disease pathogenesis, such as cancer, inflammation, diabetes, and obesity [56].

Selective growth inhibitory activity was observed in another aurilide-analog, lagunamide A (**30**) (Fig. 27.4), when tested in a panel of cancer cell lines, including P388, A549, PC3, HCT8, and SK-OV3 cells, with IC_{50} values ranging from 1.6 to 6.4 nM [57]. Furthermore, the anti-proliferative effect of this molecule is suggested to operate via the mitochondrial mediated apoptosis based on studies using HCT8 and MCF7 cell lines. There is currently growing interest in developing aurilide-class of molecules as potential anticancer agents as indicated by several reports on its total syntheses and SAR studies [58–61].

27.3.3 Coibamide A

Coibamide A (**31**) (Fig. 27.5) is a potent antiproliferative cyclic depsipeptide purified from the Panamanian marine cyanobacterial strain, *Leptolyngbya* sp. [62]. The discovery of this molecule stemmed from the International Cooperative Biodiversity Groups (ICBG) program, involving scientists from the US and Panama. Complete chemical structure of coibamide A was deduced by extensive 2D NMR

spectroscopic experiments including COSY, TOCSY, multiplicity-edited HSQS, HSQC-TOCSY, HMBC, H2BC, ^{1}H-^{15}N gHMBC, and ROESY, mass spectroscopic data as well as chemical manipulation. This molecule possesses a high degree of *N*-methylation where eight out of 11 residues are *N*-methylated. More importantly, coibamide A displayed potent cytotoxicity against NCI-H460 lung cancer cells and mouse neuro-2a cells, with LC_{50}s less than 23 nM. In addition, the compound was evaluated in the NCI's panel of 60 cancer cell lines and it exhibited significant activities against MDA-MB-231, LOX IMVI, HL-60(TB), and SNB-75 at2.8 nM, 7.4 nM, 7.4 nM, and 7.6 nM, respectively. Furthermore, "COMPARE-negative" analysis indicated that coibamide A might inhibit cancer cell proliferation via a novel mechanism [62]. Mode of action studies on coibamide A were recently conducted using human U87-MG and SF-295 glioblastoma cells and mouse embryonic fibroblasts (MEFs). Through a series of biochemical investigations, it was revealed that the apoptotic activity of coibamide A is due to its induction of the mTOR-independent autophagy in cancer cells [63].

27.3.4 Bisebromoamide

Recently, a linear peptide, bisebromoamide (**32**) (Fig. 27.5), having potent cytotoxic properties, was reported from an Okinawan marine cyanobacterium, *Lyngbya* sp. [64]. This novel peptide contained a unique *N*-methyl-3-bromotyrosine, a modified 4-methylproline, a 2-(1-oxo-propyl)pyrrolidine, and a *N*-pivalamide unit. Based on the total synthesis of bisebromoamide, reported by Gao and co-workers [65], the stereochemistry of the molecule has been revised to compound **32**. The revised structure of bisebromoamide was also confirmed through synthesis by Sasaki and co-workers [66]. In addition, a highly convergent method of synthesizing bisebromoamide and other simplified analogues was recently reported by Li and co-workers [67].

Bisebromoamide (**32**) possessed exquisite cytotoxic property against HeLa S_3 cells with an IC_{50} value of 0.04 μg/mL. When tested against a panel of 39 human cancer cell lines, the molecule showed an average GI_{50} value of 40 nM. Furthermore, biochemical data suggested that the ERK (extracellular signal regulated protein kinase) signalling pathways could potentially be a target for this compound. SAR studies on bisebromoamide and synthetic analogues revealed that the stereochemistry of the methylthiazoline moiety and methyl group at the 4-methylproline unit did not influence the cytotoxicity activity significantly [67]. Bisebromoamide was subsequently identified as an actin filament stabilizer based on cell morphological profiling analysis [68]. A recent report by Suzuki and co-workers revealed bisebromoamide to inhibit phosphorylation of extracellular signal-regulated kinase and AKT (protein kinase) when tested in renal cell carcinoma [69]. In addition, based on studies using two renal cancer cell lines, 769-P and 786-O, it was shown that this peptide induces apoptosis through ERK and mTOR inhibitions [70].

27.4 Histone Deacetylase Inhibitors

27.4.1 Largazole

Largazole (**33**) (Fig. 27.6) is a novel cytotoxic 16-membered depsipeptide isolated from the Floridian marine cyanobacterium, *Symploca* sp. [71]. Largazole has a number of unusual structural features, such as a 3-hydroxy-7-mercaptohept-4-enoic acid unit and the linkage of a 4-methylthiazoline unit to a thiazole. In addition, the presence of a thioester functional group in largazole is unprecedented in marine cyanobacterial secondary metabolism. The compound is a potent inhibitor on the growth of transformed human mammary epithelial cells (MDA-MB-231) with GI_{50} of 7.7 nM and it was less susceptible against non-transformed murine mammary epithelial cells (NMuMG) at GI_{50} of 122 nM [71]. Furthermore, compound **33** showed exquisite antiproliferative activity against transformed fibroblastic osteosarcoma U2OS cells (GI_{50} 55 nM) over non-transformed fibroblasts NIH3T3 (GI_{50} 480 nM) when compared to paclitaxel, actinomycin D, and doxorubicin [71].

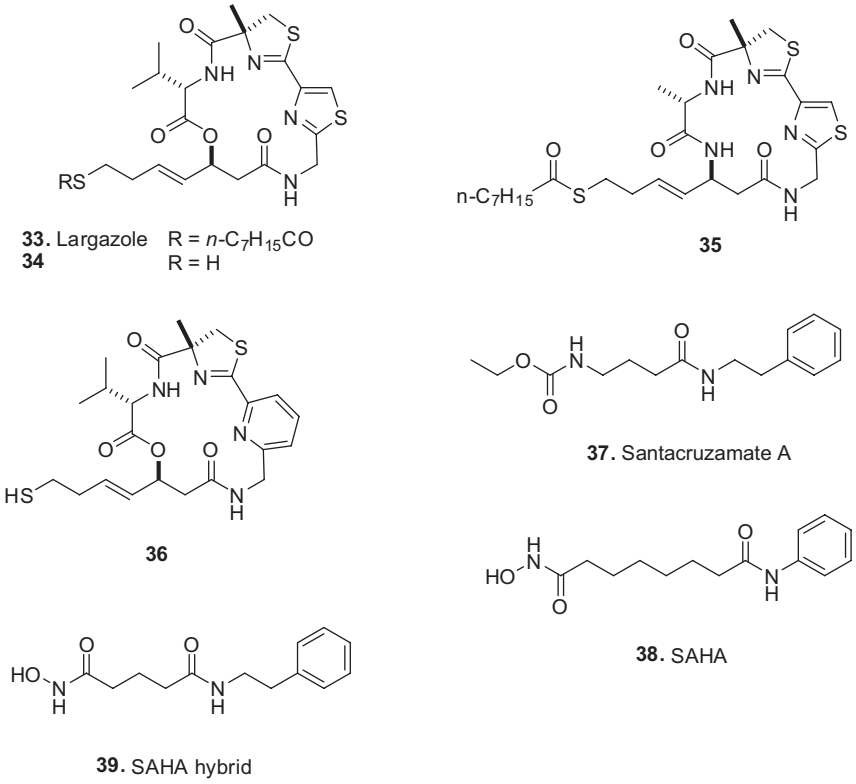


Fig. 27.6 Natural and synthetic histone deacetylase inhibitors

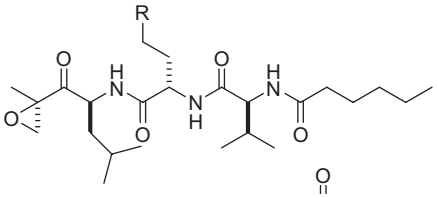
Due to the phenomenal biological activity of largazole, several organic synthetic research groups have accomplished its total synthesis [72–76]. Through these synthetic efforts, it was revealed that largazole is a potent class I histone deacetylase (HDAC) inhibitor [72, 73]. It has been observed that the over expression of class I HDAC enzymes, such as HDAC1 and HDAC3, occurs in colon and prostate tumors, respectively [77, 78]. The specific inhibition of such enzymes is therefore crucial in anticancer chemotherapy. Further biological studies by Ying et al. [72] and Bowers et al. [73] revealed the natural product as a pro-drug and it is activated by cleavage of the octanoyl residue from the 3-hydroxy-7-mercaptohept-4-enoic acid unit to provide the active free thiol molecule, **34** (Fig. 27.6).

It has been established that the (*S*)-3-hydroxy-7-mercaptohept-4-noic acid moiety in largazole is an essential structural motif which is also present in other known HDAC inhibitors, such as FK228, FR901375, and spiruchostatin. This common moiety constitutes one of three essential pharmacophores for an efficient HDAC inhibitor. The other two pharmacophores include a hydrophobic region that allows binding to the rim of the active site and a metal-binding domain which coordinates with the zinc ion in the active site. Extensive SAR studies conducted on largazole derivatives have shown that the four-atom linker joining the macrocycle and the octanoyl unit in the side chain and the *S* stereochemistry at C-17 are important for HDAC inhibitory activity [79]. In addition, modification of the Val unit to a Ala unit in largazole (refer to compound **35** in Fig. 27.6) did not show significant change in biological activity [72]. It was demonstrated that the free thiol molecule, **34**, showed significant activity against class I HDAC enzymes (HDAC1, HDAC2, and HDAC3) in the picomolar range [73]. In another study by Bowers and co-workers [80], largazole analogue having a pyridine unit (**36**) (Fig. 27.6) showed enhanced potency with IC_{50} in the subnanomolar range against class I HDAC enzymes [80]. This synthetic molecule, **36**, is reported to be three to four times more active than largazole and is biochemically the most potent class I HDAC inhibitor.

In a span of 5 years since the initial report of largazole, a myriad of significant *in vitro* and *in vivo* activities have been attributed to this molecule, including anti-tumor, anti-osteogenic, and anti-fibrotic activities [81–83]. Recent studies by Law and co-workers showed the potential use of largazole in combinatorial drug therapy for cancer treatment. When the natural product was used together with dexamethasone, a synthetic glucocorticoid steroid, it caused induction of E-cadherin to be localized at the plasma membrane in triple-negative breast cancers as well as suppressing cancer invasion *in vitro* [84]. E-Cadherins are transmembrane proteins involved in cell adhesion and maintenance of tissue architecture. Disruption in E-cadherin expression or function will result in loss of cell adhesion and possible tumor progression. In another study by Ghosh and co-workers, largazole was shown to be effective in sensitizing EBV + (Epstein-Barr virus +) lymphoma cells to the antiherpes viral drug, ganciclovir, at nanomolar concentrations [85]. Recently, Ungermannova and co-workers revealed largazole and related analogues to be novel inhibitors of ubiquitin activating enzyme (E1) and are useful as potential molecular tools for understanding the ubiquitination process [86].

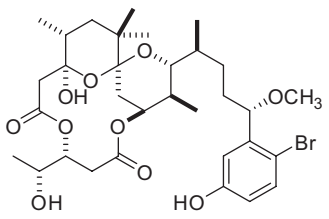
27.4.2 Santacruzamate A

A picomolar histone deacetylase inhibitor, santacruzamate A (**37**) (Fig. 27.7), was recently purified from the extracts of a tuft-forming marine cyanobacterium collected from Coiba National Park, Panama [87]. The morphology of this cyanobacterial strain was reported to be similar to the genus *Symploca* with 4.5% divergent from the type strain for *Symploca* based on 16S rRNA analysis. Due to structural similarities of santacruzamate A with clinically approved HDAC inhibitor, such as suberoylanilide hydroxamic acid (=SAHA, **38**) (Fig. 27.7), the molecule was tested in a series of anti-HDAC assays. This led to the discovery of santacruzamate A as a potent inhibitor of HDAC2, a Class I HDAC, with IC_{50} of 119 pM. In addition, compound **37** showed cytotoxicity against HCT116 and Hut-78 cancer cell lines with GI_{50} of 28.3 μM and 1.4 μM, respectively. Total synthesis, achieved in two steps, of the natural product as well as a synthetic hybrid compound, **39** (Fig. 27.7), was reported. However, the synthetic hybrid compound was about 30 times less potent compared to santacruzamate A when tested against HDAC2. Further work on generating analogues of santacruzamate A and its mechanism of HDAC inhibition is currently underway.

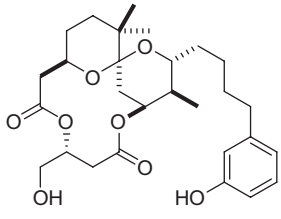


40. Carmaphycin A R =

41. Carmaphycin B R =



42. Aplysiatoxin



43. Aplog-1

Fig. 27.7 Natural and synthetic proteasome inhibitors and activators of protein kinase C

27.5 Proteasome Inhibitors

27.5.1 Carmaphycins

Recently, carmaphycins A (**40**) and B (**41**) (Fig. 27.7) having potent anti-proteasome properties were isolated in small amounts from *Symploca* sp. obtained from CARMABI beach, Curacao [88]. These compounds are structurally related to the known proteasome inhibitor, epoxomicin, in having a terminal α,β-epoxyketone moiety and a methionine sulfoxide in **40** or methionine sulfone in **41**. As part of the drug development process, their total synthesis was also accomplished using an efficient and scalable convergent method [88]. The inhibitory properties of carmaphycins A and B were evaluated against *Saccharomyces cerevisiae* 20S proteasome and found to possess comparable IC_{50} values of 2.5 nM and 2.6 nM, respectively. The inhibitory activities of these compounds are similar with those reported for epoxomicin and the marine-derived salinosporamide A with IC_{50} values of 2.7 nM and 1.4 nM, respectively. Additional cytotoxic assay showed carmaphycins to be particularly active against solid tumor cell lines, such as human lung adenocarcinoma and colon cancer cell lines. Preliminary structural biology investigation of the carmaphycins suggested distinct binding site compared to epoxomicin, salinosporamide A, and bortezomib. The SAR studies of these potent cyanobacterial compounds are currently underway.

27.6 Protein Kinase C Activator

27.6.1 Aplysiatoxins

Aplysiatoxin (**42**) (Fig. 27.7) and related analogues, e.g., oscillatoxins and nhatrangins, are distinct polyketide classes of marine toxins isolated from several cyanobacterial species, including *Lyngbya majuscula*, *Schizothrix calcicola*, and *Oscillatoria nigro-viridis* [89–91]. They are commonly known for their dermatoxic property causing inflammation of the skin. Several aplysiatoxin-related analogues are known to possess potent tumor-promoting properties through the activation of protein kinase C (PKC) [92]. PKC belongs to a family of serine/threonine kinases and plays important roles in cell proliferation, differentiation and apoptosis [93]. Due to its importance in cellular signal transduction pathways, PKC is a potential drug target for the treatment of various diseases, particularly in cancer therapy [94].

Researches from Japan recently developed new simplified analogues based on aplysiatoxin class of molecules, e.g., aplog-1 (**43**) (Fig. 27.7), with potent anti-proliferative properties as potential anticancer agents [95–103]. These synthetic analogues have been shown to inhibit the action of tumor promotors as well as prevent growth of cancer cells in similar ways to bryostatin 1. For instance, the

synthetic analogue, aplog-1 (**43**) exhibited growth inhibitory properties when tested against nine cancer cell lines (e.g., HBC-4, MDA-MB-231, SF-295, HCC2998, and NCI-H460 etc.) with a log GI_{50} ranging from −4.95 to −6.33 [95]. In addition, compound **43** significantly inhibited the Epstein-Barr virus early antigen induction by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate. Aplog-1 also displayed binding and activation of PKCδ as well as the induction of the activated PKCδ to translocate to the nuclear membrane in CHO-k1 cells. The discovery of synthetic aplysiatoxins having PKC activating property could be a significant therapeutic lead not just for cancer but also other diseases, such as Alzheimer's disease and Acquired Immune Deficiency Syndrome.

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Chapter 28
P-gp Inhibitory Activity from Marine Sponges, Tunicates and Algae

Xiao-cong Huang, Priyank Kumar, Nagaraju Anreddy, Xue Xiao, Dong-Hua Yang and Zhe-Sheng Chen

Abstract The only effective therapy for metastasis in cancer patients is chemotherapy, which all too frequently fails due to innate or acquired multi-drug resistance (MDR). Historically, ATP binding cassette (ABC) transporters, such as P-glycoprotein (P-gp), are recognized as the major culprits responsible for MDR. Over-expressing of P-gp in cancer cells, can lead to premature efflux of clinical chemotherapeutic agents and correlate with poor chemotherapeutic outcome and relapse of some cancers. The most likely strategy to overcome MDR is to search for inhibitors from natural products. With unique and novel chemical structures, marine-derived metabolites are an attractive new resource, to prime the search for new P-gp inhibitors. This chapter summarizes P-gp inhibitory activity in marine natural products (MNPs) and validates that MNPs can deliver new ABC transporter inhibitor scaffolds.

Keywords ABC transporter · P-glycoprotein · Inhibitor · Multidrug resistance · Sponges · Tunicates · Algae · Agosterol A · Sipholane triterpenoids · Lamellarins · Parguerenes

28.1 Introduction

Cancer has emerged as one of the most devastating diseases in the world. Although surgery and radiation therapy are efficacious for many solid tumors, these two treatments are limited to patients who suffer from localized disease. Unfortunately,

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cancer cells can strike any of the tissues in the body, invade into surrounding tissues or organs, and spread to other sites of the body through the bloodstream or lymphatic system in a process known as metastasis. Many cancer patients are in the metastatic phase of a disease prior to the treatment of surgery or radiation therapy. The only effective therapy for systemic metastasis is chemotherapy, which accesses the systemic circulation and can treat primary tumors and metastatic diseases by anticancer drugs. However, chemotherapeutic regimens have limited success in many cancers due to fundamental causes such as innate (primary) resistance to anti-cancer drugs, or acquired resistance in cancer cells after several treatment courses. Based on an analysis for randomized clinical trials, it was reported that the 5-year survival benefit attributable solely to cytotoxic chemotherapy in adult malignancies was only 2.3 and 2.1 % in Australia and the U.S.A., respectively [1]. Of various mechanisms, multidrug resistance (MDR) is the main factor that contributes to the poor effectiveness of antineoplastic therapy and was considered to be responsible for the failure of chemotherapy in more than 90 % of patients at metastatic stages [2].

It has been postulated that there are three major mechanisms contributing to MDR [3]. First, the down-regulation of uptake (influx) proteins is responsible for resistance to some antineoplastic drugs. For example, decreased mRNA of organic anion transporting polypeptide 1B3 (OATP1B3/SLCO1B3) was found to be associated with lower intracellular paclitaxel in a human hepatoma cell line (HepG2) [4]. Another example is the impaired expression of reduced folate carrier (RFC) that is attributable to the intrinsic resistance to methotrexate in osteosarcoma [5]. Second, alternation of cancer cells, such as evasion of apoptosis, increased repair of DNA damage and alternation of drug targets, can drastically decrease the cytotoxicity of anticancer drug [6]. Last but not least, the overexpression of a group of energy-dependent transporters, known as adenosine triphosphate binding cassette (ABC) transporters, leads to the efflux of a variety of mechanistically and structurally unrelated drugs, such as vinca alkaloids, anthracyclines, taxanes and epipodophyllotoxins, and induce MDR phenotype [3].

Since permeable glycoprotein (P-gp/ABCB1), the first member of ABC transporters, was described by Dano in 1973 [7] and Ling et al. in 1976 [8], 48 representatives from this transporter family have been identified. In 2001, based on sequence homology and domain organization, these ABC transporters were organized into seven distinct subfamilies, designating from ABCA through ABCG [9].

Of these 48 proteins, P-gp is the most commonly encountered and extensively studied in the laboratories. While it plays a pivotal physiological and pharmacological role in the body on detoxification and extrusion of xenotoxins [10], the overexpression of P-gp in cancer cells can confer to cross-resistance against some of the most popular but structurally different anticancer drugs and result in decreased intracellular accumulation of anticancer drugs. Consequently, this impact can significantly lead to the development of MDR and failure in chemotherapeutic treatment [11–13].

The sea covers more than 70% of the Earth's surface and represents greater than 95% of the biosphere. It has been conservatively estimated that more one million animal species are living in the world's oceans [14], which is considered to be a more stable and uniform environment than terrestrial zones [15]. Having survived over eons in such an environment, marine organisms, such as invertebrates and microbes, have developed biochemical and physiological defensive mechanisms based on the production of numerous bioactive secondary metabolites [16, 17]. With unique structural features, these innovative metabolites possess the great potential to become leads for multiple pharmacological purposes targeting at malignant tumors, bacteria, viruses and inflammatory diseases, etc. Indeed, marine natural products have attracted the attention of chemists and biologists keen to evaluate and exploit their medicinal properties.

Metabolites with property of reversing MDR mediated by P-gp have been extracted from different marine organisms such as sponges, tunicates and algae, and in lesser extent from bryozoan [18], or gorgonian [19, 20]. In this chapter, we provide a chemical and biological view on compounds initially isolated from sponges, tunicates and algae. We highlight the P-gp inhibitory activity from these metabolites, which may be valuable in the combination with other anti-cancer drugs to improve the effect of chemotherapy for cancer patients.

28.2 Overview of P-glycoprotein (P-gp)

P-gp is a member of ABCB subfamily and coded as ABCB1. Like most of ABC transporters, this 170 kDa cell membrane glycoprotein has a "typical" ABC transporter structure (Fig. 28.1), which is a core full functional unit consisting of two nucleotide-binding domains (NBD1 and NBD2) and two trans-membrane domains (TMD1 and TMD2) [21]. Each TMD, also known as polytropic membrane spanning domain (MSD), classically holds six trans-membrane helices with numbers ranging between 5 and 10 [22]. The role of TMDs is believed to mediate vectorial efflux of substrate using energy that is released when ATP is bound and hydrolyzed by NBDs [23]. In 2009, the high-resolution crystal structure of P-gp was released and has been used to interpret the phenomenon of ligand-binding in a cavity between TMDs [24, 25]. P-gp is the most studied and characterized ABC transporter since it was discovered in 1970s [7]. Using different drug-selected or transfected human cell lines, most researchers in 1980s focused on the molecular mechanism underneath the MDR phenotype from P-gp. The link of this protein to MDR was not clinically recognized until in 1985 when P-gp was first detected with higher level in ovarian samples from two patients by Ling et al. [26]. This finding greatly broadened the understanding of failure to chemotherapy in a variety of cancer patients [26].

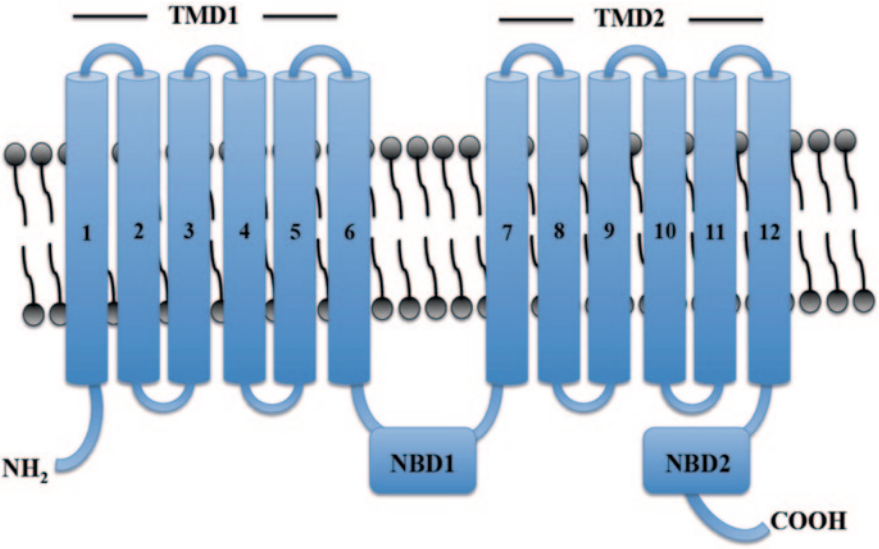


Fig. 28.1 Membrane topology model of P-gp

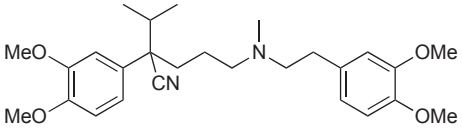
Over the past three decades, crucial functional and mechanistic studies towards P-gp intensively extended to identification of its substrates, localization in normal tissues and cells [27], physiological activity, and measurement of ATPase activity [28]. Based on its location on the gastrointestinal tract, important blood barriers and compartments in the body, P-gp focuses on the prevention of xenotoxins from entering the vital organs including brain, placenta and testes. It is also a crucial key on elimination of toxins by preventing their absorption on gut mucosa and increasing their excretion from liver and kidney. While P-gp is universally accepted as a protective role in human, it should be noted that, on the other hand, the physiological activity of P-gp also limits the oral bioavailability of a number of drugs and increases the difficulty to deliver therapeutic agents to target areas in the brain. Moreover, the up-regulation of its expression in some cancers, especially at relapse stage, is closely related to the failure of chemotherapy [29]. Given that P-gp transports a vast number of chemotherapeutic drugs including those clinically use as first and second line anticancer agents [30], clinical trials with application of inhibitors to reverse MDR caused by P-gp [31] is under evaluation [3].

28.3 Strategies to Overcome P-gp Mediated Drug Resistance

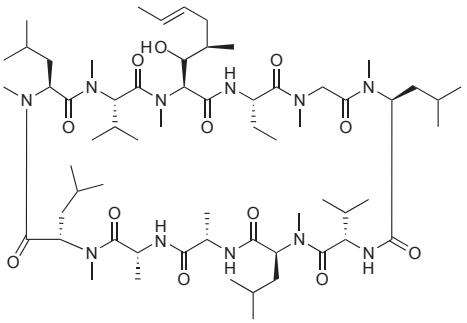
The disappointing effectiveness from chemotherapeutic agents in cancer patients is preferentially believed to correlate with overexpression of ABC transporters, especially the most characterized P-gp [32]. Various approaches have been applied experimentally to overcome MDR caused by this drug pump. Unfortunately, while some approaches have been involved in different stages of clinical trials, no satisfactory clinical solution to MDR has been achieved.

28.3.1 Three Generations of P-gp Inhibitors

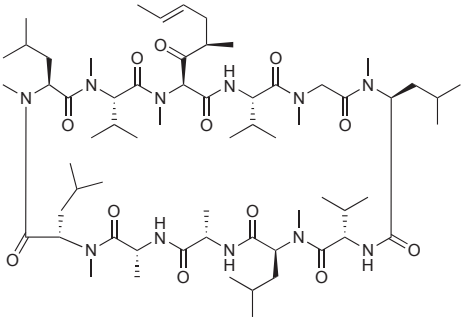
Verapamil (**28.01**) is the first reported chemo-sensitizer to restore drug sensitivity in MDR cancer cells by blocking ABC transporters-mediated drug efflux. Being a non-cytotoxic calcium channel blocker, verapamil (**28.01**) increased the cytotoxicity of vincristine (VCR) either in VCR-resistant leukemia cell line P388/VCR or P388/VCR-bearing mice [33]. The experimentally overcoming of VCR resistance *in vitro* and *in vivo* led to a phase I clinical trial of combination of VCR and verapamil (**28.01**), which was failed due to unachievable reversal effect from verapamil (**28.01**) at the highest tolerable level in patients [34]. In addition to verapamil (**28.01**), another P-gp inhibitor cyclosporin A was found (**28.02**). Cyclosporin A is a natural product isolated from the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidium booth* [35]. Due to its high effectiveness and low cytotoxicity [36], it has been regarded as another benchmark inhibitor for P-gp inhibitors in *in vitro* assays. Similar to verapamil (**28.01**), several clinical trials (phases I and II) carried out for cyclosporin A (**28.02**) were not successful in patients with incurable malignancies because of the pharmacokinetic interactions between cyclosporin A (**28.02**) and the concurrent anticancer agents [37, 38]. Following the discovery of these two first-generation inhibitors of P-gp, verapamil (**28.01**) and cyclosporin A (**28.02**), reversal agents with more potent modulatory activity have been generated by structurally modification and optimization with combinatorial chemistry approaches. The major reversal agents, such as valspodar (PSC-833, **28.03**) [39], zosuquidar (LY335979, **28.04**) [40] and tariquidar (XR9576, **28.05**) [41], were claimed as second or third generation, of which some showed promising results in clinical trial phases [42–44].



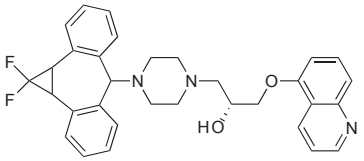
verapamil (**28.01**)



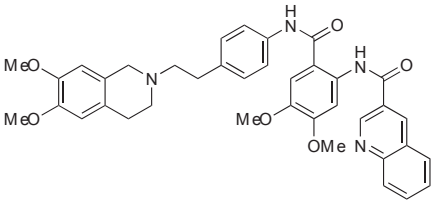
cyclosporin A (**28.02**)



valspodar (PSC-833, **28.03**)



zosuquidar (LY335979, **28.04**)

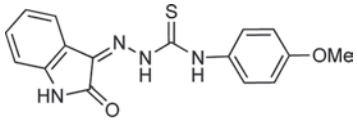


tariquidar (XR9576, **28.05**)

28.3.2 Cytotoxicity Agents Specifically Targeting P-gp

Recognizing the limitation of known inhibitors of P-gp, some research groups developed molecules that can exploit P-gp function to induce cytotoxicity, rather than

suppression of P-gp. Szakacs et al. in 2004 [45] and Ludwig et al. in 2006 [46] reported that a thiosemicarbazone derivative (NSC73306, **28.06**) was a selective cytotoxic agent for P-gp expressing cells but not for parental cells, and that cytotoxicity was potentiated by P-gp, instead of being antagonized. Without the selective cytotoxicity towards cancer cells overexpressing other ABC transporters, NSC73306 (**28.06**) is currently in preclinical assessment for combating MDR caused by P-gp [47]. Currently at least more than 15 compounds have been discovered specifically targeting at P-gp overexpressing cancer cells [48]. Mechanisms behind this hypersensitivity or "collateral sensitivity" are still under investigation.



NSC 73306 (**28.06**)

28.3.3 Other Approaches to Overcome P-gp Mediated MDR

As MDR still remains major obstacle in cancer chemotherapy and success from reversal or specific killing agents against ABC transporters have not been achieved, several other strategies by different mechanisms have been investigated as means to circumvent MDR. These approaches include but not limit to (i) applying P-gp specific monoclonal antibodies, such as MRK16 [49, 50] or UIC2 [51] to bind P-gp and block efflux of anticancer drugs, (ii) embedding drugs within liposomal membranes to improve the delivery or release in target tissues, or directly inhibit P-gp [52], (iii) blocking the signal transduction pathway to abrogate expression of ABC transporters [53], (iv) using small-interfering RNA (siRNA) [54], short-hairpin RNA [55], ribozymes [54], or antisense oligodeoxyribonucleotides [56] to down-regulate MDR genes and inhibit their expression, (v) encapsulating anticancer drugs with poly(ethylene oxide)-modified poly(ε-caprolactone) nanoparticle (PEO-PCL) to more effectively kill drug-resistant cancer cells [57], (vi) using fatty acid-polyethylene glycol-fatty diesters to prevent expression of P-gp and its phenotype [58], (vii) screening cytotoxic drugs that can evade efflux by P-gp, BCRP or MRP1, and (viii) designing drugs that are substrates of solute carriers (SLC) to increase their intake by SLC and avoid MDR.

Despite various strategies that have developed in the past several decades to overcome P-gp mediated drug efflux, none of them has been successfully applied in clinic. Since direct blocking of P-gp is still considered as the most economic and effective method to resolve this obstacle, intentions have been turned back into development of P-gp inhibitors, of which some utilize natural products [59]. As noted above, the benchmark inhibitor cyclosporin A (**28.02**) was originally from a natural product. Indeed, natural products are increasingly recognized as a source of inhibitors of disease-linked MDR pumps. Such natural products, sourced from

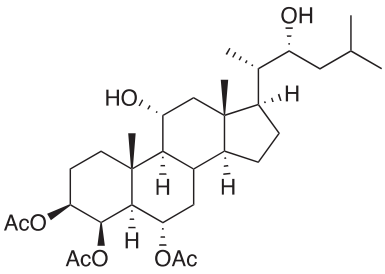
plants, fungi and marine organisms, represent a diverse resource of novel chemical structures, some with inhibitory properties against P-gp. Compared with three earlier generations of P-gp inhibitors, modulators originating from natural sources, sometimes referred as the "Fourth Generation Inhibitors" [69], account for 70% of reported MDR reversal agents discovered in the past 15 years [30]. While P-gp inhibitors extracted from marine sponges, tunicates and sponges are highlighted here, detailed analysis of inhibitors isolated from terrestrial plant and fungi can be sourced in a series of reviews [30, 59, 60].

28.4 P-gp Inhibitory Activity from Metabolites Isolated from Marine Sponges

In courses of screening P-gp inhibitors from marine organisms, several research groups have isolated agosterol A, sipholane triterpenoids, irciniasulfonic acid B, kendarimide A and 9α,11α-epoxycholest-7-ene-3*β*,5α,6α,19-tetrol-6 acetate from marine sponges as agents that can reverse P-gp mediated drug efflux.

28.4.1 Agosterol A (AG-A), Sterol from Sponge

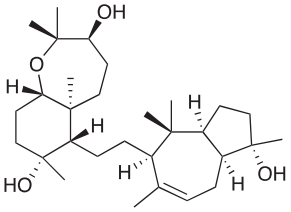
Aoki et al. [61] in 1998 reported that agosterol A (AG-A **28.07**), a polyhydroxylated sterol acetate, isolated from marine *Spongia* sp., reversed ABC transporters mediated MDR. Agosterol A (**28.07**) at 3 μM reversed the P-gp-mediated MDR by sensitizing the drug selected P-gp overexpressing KB-C2 cells to colchicine, vincristine, doxorubicin and etoposides [61, 62]. Interestingly, agosterol A (**28.07**) has a unique structure that is unrelated to known MDR reversal agents. Structure and activity relationship of agosterol A (**28.07**) demonstrated that acetoxyl groups in agosterol A (**28.07**) might contribute to its ability to reverse the P-gp-mediated MDR [62]. Moreover, agosterol A (**28.07**) is the first example of an agent that inhibits the function of both P-gp and multidrug resistance associated protein (MRP1) by direct interaction [63].



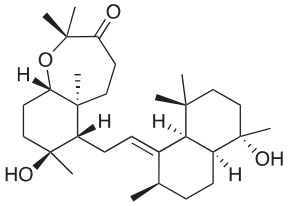
agosterol A (**28.07**)

28.4.2 Sipholane Triterpenoids: Triterpenoids from Sponge

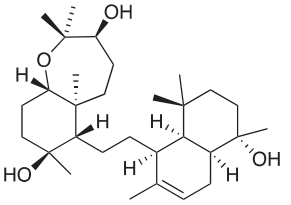
Sipholane triterpenoids, isolated from red sea sponge *Callyspongia siphonella*, contain perhydrobenzoxepine (rings A and B) and a bicyclodecan system (rings C and D) linked by an ethylene bridge [64]. Thus far only 30 triterpenoids have been isolated from this sponge that consist four skeletons viz. sipholane, siphonellane, neviotane and dahabane [64]. Out of these the most important triterpenoids are sipholanes that include sipholenol A (**28.08**), sipholenone E (**28.09**), sipholenol L (**28.10**) and siphonellinol D (**28.11**) (Fig. B) [64, 65]. Shi Z et al. reported that sipholenol A (**28.08**) reversed the P-gp-mediated MDR in KB-C2 and KB-V1 cells [66]. Sipholenol A (**28.08**) increased the accumulation of paclitaxel by directly inhibiting the P-gp-mediated efflux and photolabelling of P-gp with its transport substrate [^{125}I]-iodoarylazidoprazosin. Sipholenol A (**28.08**) treatment for 72 h did not affect the expression of P-gp in KB-C2 and KB-V1 cells [66]. Other members from the sipholane triterpenoid family including sipholenone E (**28.09**), sipholenol L (**28.10**) and siphonellinol D (**28.11**) were also reported to sensitize KB-C2 cells to anticancer drugs by inhibiting the function of P-gp, similar to that of sipholenol A (**28.08**) [65]. Furthermore, *in silico* molecular docking analysis identified the ligand binding sites of these compounds [64].



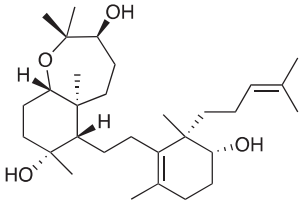
sipholenol A (**28.08**)



sipholenone E (**28.09**)



sipholenol L (**28.10**)

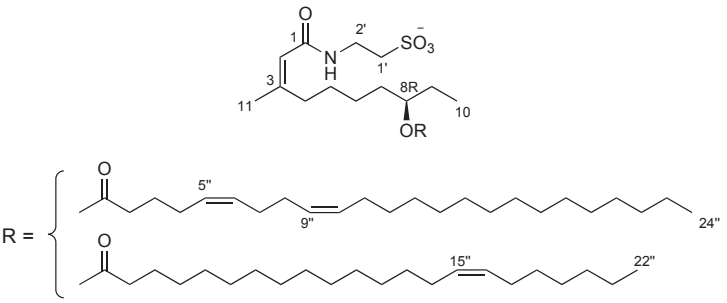


siphonellinol D (**28.11**)

28.4.3 Irciniasulfonic Acid B, A Taurine Conjugated Fatty Acid Derivative from Marine Sponge

Since the recognition of verapamil (**28.01**) as a reversal agent against P-gp-mediated MDR, many compounds have been reported to reverse MDR. Several compounds,

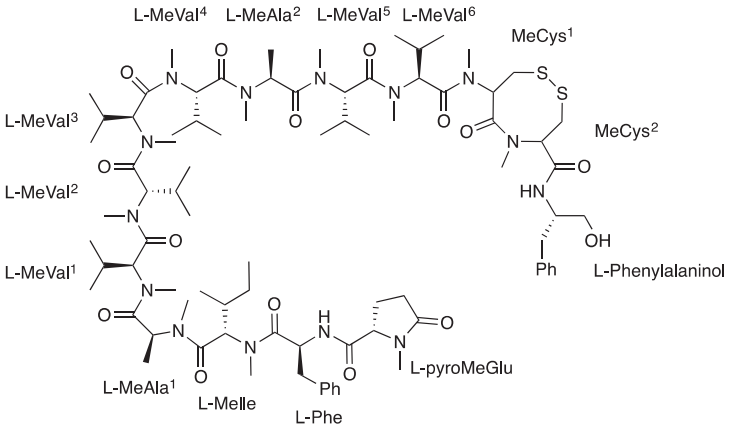
such as agosterol A (**28.07**) or sipholane triterpenoids, have been isolated from marine sponges that can modulate the ABC transporter-mediated MDR [61, 66]. In a screening for other MDR modulators in marine invertebrates, Chisato et al. in 2006 isolated a novel taurine conjugated fatty acid analogue named irciniasulfonic acid B (**28.12**) as an active constituent in Japanese marine sponge, *Ircinia* sp. [67, 68]. Irciniasulfonic acid B (**28.12**) at 100 μM reversed the MDR in P-gp overexpressing KB/VJ-300 cells [68].



Irciniasulfonic acid B (**28.12**)

28.4.4 Kendarimide A, A Linear Peptide from Marine Sponge

Several researchers have screened marine sponges to discover novel P-gp inhibitors. Aoki et al. isolated agosterol A (**28.07**) from marine sponge of *Spongia* sp. and reported its ability to reverse both P-gp- and (MRP1)-mediated MDR [61]. Kendarimide, isolated from another marine sponge of *Haliclona* sp. collected at Sulawesi Island, Indonesia, has also been shown to reverse P-gp-mediated MDR [69]. A methanol soluble extract of the dried *Haliclona* sp. was first found to have reversal activity against P-gp-mediated MDR. This methanolic extract was then separated and purified by reverse-phase HPLC to obtain kendarimide A (**28.13**), which was shown to completely reverse the resistance to colchicine in P-gp overexpressing human epidermoid carcinoma KB-C2 cells at a concentration of 6 μM. The structure of kendarimide A (**28.13**) was characterized to be a linear peptide composed of N-methylpyroglutamic acid (pyroMeGlu), N-methylated eight membered cysteinyl-cysteine (ox-[MeCys-MeCys]), together with many N-methyl amino acid residues. The amino acid sequence of kendarimide A (**28.13**) was determined by 2D NMR and FAB MS analysis. Interestingly, cyclosporin A (**28.02**), a potent MDR modulator of P-gp, is a peptide composed of 11 amino acid residues including many N-methyl residues. Thus MDR reversal activity of kendarimide A (**28.13**) and cyclosporin A (**28.02**) may be related to their structural similarity.

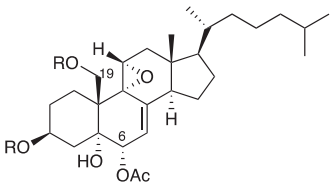


kendaramide A (**28.13**)

28.4.5 9α,11α-epoxycholest-7-ene-3β,5α,6α,19-tetrol 6-acetate, A Sterol from Marine Sponge

Over the past few decades, HIV-infected patients have been maintained on low-dose prophylactic fluconazole therapy to prevent opportunistic fungal infections as a result of azole-resistant *Candida albicans* (*C. albicans*) isolates. These fungi are resistant to the azole class of antifungal agents, mainly imidazoles and triazoles. Azoles, such as fluconazole, ketoconazole, and itraconazole, block fungal ergosterol biosynthesis by inhibiting lanosterol 14 α-demethylase. Patients undergoing bone marrow transplants are seen to be more susceptible to infection by azole-resistant *C. albicans*. Molecular mechanisms of fluconazole resistance have been identified to result from point mutations in the *ERG11* gene that encodes for the fluconazole target enzyme lanosterol demethylase, and also as a result of overexpression of *ERG11* or modifications of other genes involved in ergosterol biosynthesis. In addition, a decreased intracellular accumulation of fluconazole by membrane-associated efflux pumps was also identified as a mode of resistance in *C. albicans* [70]. Two families of efflux pumps were identified in *C. albicans*, which include the major facilitators of MDR, one, which utilizes a proton gradient and the other being a classical active efflux transporter, which require hydrolysis of ATP for its efflux function [71]. Fluconazole resistance can be effectively com-batted by inhibiting the efflux transporter. Jocab et al. screened several natural extracts to identify the natural products that reverse the efflux pump mediated azole resistance [72]. They found that 9α,11α-epoxycholest-7-ene-3*β*,5α,6α,19-tetrol 6-acetate (**28.14**) isolated from the lipophilic extract of an Australian collection of *Dysidea arenaria* cf. Bergquist (family Dysideidae), reversed MDR efflux pump mediated resistance of Fluconozole in *C. albicans*. The IC_{50} of fluconazole was reduced from 300 to 8.5 μM (35-fold enhancement) when combined with **28.14** (3.8 μM). **28.14** is the

first marine natural product that has been shown to reverse azole resistance and the first possible inhibitor of the MDR1-type efflux pump of *C. albicans*. **28.14** has structural similarities with agosterol A (**28.07**) which is a known inhibitor of tumor cell MRP1- and P-gp-mediated MDR at 5 μM. **28.14** was treated with Ac2O/pyridine to prepare 9α,11α-epoxycholest-7-ene-3*β*,5α,6α,19-tetrol-3,6,19 triacetate (**28.15**). **28.15** was spectroscopically identical with **28.14**. However, **28.15** failed to reverse MDR1-mediated resistance at concentrations up to 90 μM.



R= H, 9α,11α-epoxycholest-7-ene-3β,5α,6α,19-tetrol 6-acetate (**28.14**)
R= Ac, 9α,11α-epoxycholest-7-ene-3β,5α,6α,19-tetrol-3,6,19 triacetate (**28.15**)

28.5 P-gp Inhibitory Activity from Metabolites Isolated from Marine Tunicates

Various marine natural products isolated from tunicates are capable of reversing multi-drug resistance encoded by MDR1 gene. Of these natural products, lamellarins, ecteinascidin 743 and patellamides represent metabolites from tunicates with remarkable activity.

28.5.1 Lamellarins: Pyrrole Derivatives from Marine Tunicates

The first four lamellarins, A–D (**28.16**, **28.32**, **28.17** and **28.33**) [73], were isolated from a Pacific Ocean marine prosobranch mollusk, *Lamellaria* sp. by Raymond J. Andersen et al. in 1985. Their structures were assigned by X-ray crystallographic study and interpretation of spectral data. Following this discovery, more than fifty lamellarins have been found from other marine organisms such as tunicates [74–79] or sponges [80, 81].

Classically, the lamellarins, which share a central pyrrole ring, are categorized into two groups [82]. With a fused central pyrrole ring, the first group (Type I) could be further divided into two sub-sections [82]. The difference between these two sub-sections is the saturation status of the olefin at C-5 and C-6 [82] (Type Ia in Table 28.1 and Type Ib in Table 28.2). The second group (Type II) with a non-fused central pyrrole, due to the lacking of P-gp inhibitory activity, is not discussed here.

The lamellarins are capable of exhibiting a broad spectrum of biological activities, including cytotoxicity towards tumor [83–86], and a series of enzymes such as

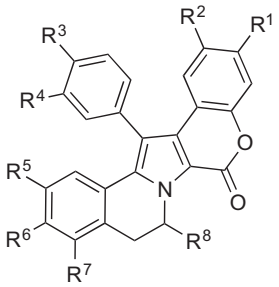
Table 28.1 Natural lamellarins from marine tunicates (Type Ia, **28.16–28.31)** [91, 92]

No.	Lamellarins	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
28.16	A	OH	OMe	OH	OMe	OMe	OMe	OMe	OH
28.17	C	OAc	OMe	OAc	OMe	OMe	OMe	OMe	H
28.18	E	OH	OMe	OMe	OH	OMe	OMe	OH	H
28.19	G	OMe	OH	OMe	OH	OMe	OH	H	H
28.20	I	OH	OMe	OMe	OMe	OMe	OMe	OMe	H
28.21	K	OH	OMe	OH	OMe	OMe	OMe	OH	H
28.22	L	OH	OMe	OMe	OH	OMe	OH	H	H
28.23	S	OH	OH	OH	OH	OMe	OH	H	H
28.24	T	OH	OMe	OMe	OH	OMe	OMe	OMe	H
28.25	Z	OMe	OH	OH	OH	OMe	OH	H	H
28.26	χ	OH	OMe	OH	OMe	OMe	OH	H	H
28.27	A1	OH	OH	OH	OMe	OMe	OH	H	H
28.28	A2	OH	OH	OH	OMe	OMe	OMe	OH	H
28.29	A3	OH	OMe	OH	OMe	OMe	OMe	H	H
28.30	A4	OH	OH	OH	OH	OH	OH	H	H
28.31	A6	OMe	OH	OH	OMe	OMe	OH	H	H

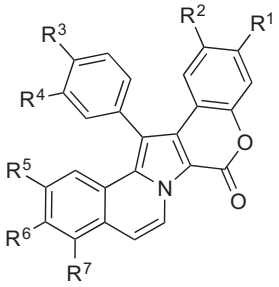
Table 28.2 Natural lamellarins from marine tunicates (Type Ib, **28.32–28.38**) [91, 92]

No.	lamellarins	R^1	R^2	R^3	R^4	R^5	R^6	R^7
28.32	B	OH	OMe	OH	OMe	OMe	OMe	OMe
28.33	D	OH	OMe	OH	OMe	OMe	OH	H
28.34	D-triacetate	OAc	OMe	OAc	OMe	OMe	OAc	H
28.35	M	OH	OMe	OH	OMe	O Me	OMe	OH
28.36	N triacetate	OAc	OMe	OMe	OAc	OMe	OAc	H
28.37	X	OH	OMe	OMe	OH	OMe	OMe	OH
28.38	A5	OH	OH	OH	OMe	OH	OH	OH

HIV-1 integrase [79], protein kinases (i.e. GSK-3β and CDK5/p25) [87], together with cell division inhibition [73], immunomodulation [75] andantioxidant activity [73]. Apart from the above biological activities, a series of lamellarin members, initially isolated from the marine ascidian *Didemnum* sp. (tunicate) [88], have exhibited activity to reverse P-gp mediated drug efflux. Lamellarin I (**28.20**) was the first member reported as P-gp inhibitor [89]. At 2 μM, lamellarin I (**28.20**) not only increased the accumulation of rhodamine 123 in P-gp-positive Lo Vo/Dx cells to a higher level than verapamil, but also sensitized the MDR cells (P-gp over-expressing P388/Schabel) to doxorubicin at the level of the parental cells, suggesting that lamellarins could potentially be used to treat multi-drug resistant tumors. In addition to lamellarin I (**28.20**), lamellarin A (**28.16**), B (**28.32**), D-triacetate (**28.34**) K (**28.21**), L (**28.22**), M (**28.35**) and N-triacetate (**28.36**), isolated from marine ascidians [88], have been found to be non-cytotoxic inhibitors of P-gp [90]. It was noted that lamellarin D-triacetate (**28.34**), at 30 nM greatly increased the cytotoxicity of doxorubicin against CHOC5 cells (Chinese Hamster Ovary cells over-expressing P-gp) by 1000-fold [90], suggestive of lamellarin D-triacetate (**28.34**) as the most potent P-gp inhibitor in lamellarin class.

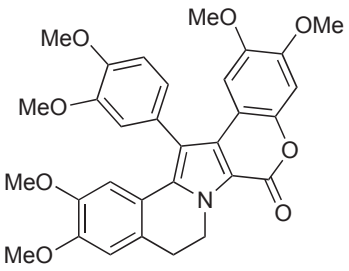


Structure of lamellarins (Type Ia)



Structure of lamellarins (Type Ib)

Recently, Capon group isolated sixteen lamellarins from two extracts of marine ascidian *Didemnum* sp., collected at Wasp Island, Duras, New South Wales and Northern Rottnest Shelf, Western Australia, Australia respectively [91]. These metabolites included lamellarins, A1 (**28.27**), A2 (**28.28**), A3 (**28.29**), A4 (**28.30**), A5 (**28.38**), A6 (**28.31**), C (**28.17**) [73], E (**28.18**) [74], K (**28.21**) [75], M (**28.35**) [75], S (**28.23**) [76], T (**28.24**) [77], Z (**28.25**) [93], G (**28.19**) [74], X (**28.37**), and χ (**28.26**) (of which the latter has previously been first described as an acetylated derivative [84] and synthesized in 2006 [93]). Access to these lamellarins provided a rare opportunity for investigation of the relationship between the structure and P-gp inhibitory activity (SAR), probing interactions between lamellarins and P-gp with a view to reversing multi-drug resistance in a human colorectal carcinoma cell line (SW620 Ad300). The investigation on P-gp inhibitory activity from these marine metabolites revealed that lamellarin X (**28.37**), E (**28.18**), M (**28.35**), χ (**28.26**), K (**28.21**), A3 (**28.29**) and A6 (**28.31**) are P-gp inhibitors, and that lamellarin E (**28.18**) was more active than verapamil in Hoechst 33342 accumulation and efflux assay using flow cytometry. The SAR studies, which were expanded to include the semi-synthetic lamellarin A7 (**28.39**) and a series of lamellarin-inspired synthetic analogues, revealed that P-gp inhibitory activity correlated with higher levels of lamellarin methylation, and that the molecular basis behind lamellarin P-gp inhibitory activity requires the intact molecular architecture of thenatural product scaffold.



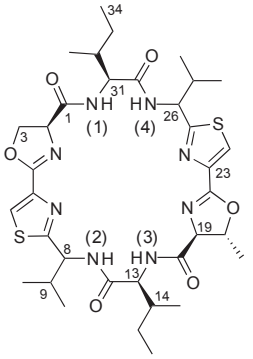
lamellarin A7 (**28.39**)

Capon group also revealed that P-gp over-expressing SW620 Ad300 showed resistance to three lamellarins (Type Ia), A1 (**28.27**), A2 (**28.28**) and S (**28.23**) (fold resistance: 10.6, 5.2 and 10.7, respectively) and that the resistance of MDR cells to A1 (**28.27**), A2 (**28.28**) and S (**28.23**) was abolished in the presence of 3 μM cyclosporin A (dose-modifying factor >4-fold, 6.5-, 5.2- and 19.1-folds respectively), indicating that lamellarins A1 (**28.27**), A2 (**28.28**) and S (**28.23**) are probably substrates of P-gp [91].

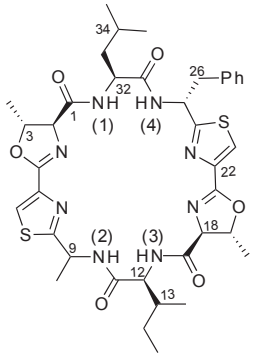
28.5.2 Patellamides: Cyclic Peptides from Marine Tunicate

Patellamides were produced by one species of tunicate, *Lissoclinum patella* collected from various sites. As members of the cyanobatin superfamily [94], patellamides belong to a group of lipophilic cyclic peptides containing an unusual fused oxazoline-thiazole unit. Patellamide A–C (**28.40**–**28.42**) were initially isolated from the tunicate *Lissoclinum patella* collected at Eil Malk Island, Palau Islands by Ireland et al. [95]. These three metabolites showed moderate cytotoxic activities against L1210 murine leukemia cells with IC_{50} values of 2.9, 1.6 and 2.4 μM, respectively [95]. The potential anticancer activity from this class of cyclic peptides inspired the identification of other four members of patellamides, including D (**28.43**), E (**28.44**), F (**28.45**) and G (**28.46**) from the same tunicate collected at Great Barrier Reef, Australia [96, 97], Pulau Salu, Singapore [98], northwestern Australia [99] and Pohnpei, Federated States of Micronesia [100]. Compared to A–C (**28.40**–**28.42**), D–G (**28.43**–**28.46**) exerted much less cytotoxicity against different cell lines, with IC_{50} values ranging from 11 to 125 μM [96, 98, 99]. The ability of patellamide D (**28.43**) to restore sensitivity of a series of anti-proliferative agents was compared with verapamil in a human leukemic cell line CEM/VLB100 by Williams et al. [101]. In comparison with its parental cell line CCRF-CEM, CEM/VLB100 was highly resistant to the cytotoxic effect of vinblastine, doxorubicin and colchicine (≥100-fold) due to the overexpression of P-gp. When co-administered with patellamide D (**28.43**, 3.3 μM), CEM/VLB100 resistance to vinblastine was significantly reduced by 67-fold, comparable with that observed for 5.1 μM verapamil (**28.01**, 48-fold). When CEM/VLB100 cells were co-exposed to patellamide D(**28.43**) or verapamil (**28.01**), cytotoxicity of doxorubicin was increased with IC_{50} values from >1 to 110 and 160 ng/mL, respectively. However, resistance to colchicine could

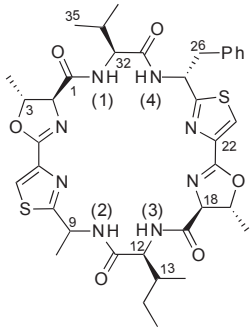
not be completely circumvented by patellamide D (**28.43**, 2.8-fold) or verapamil (**28.01**, 1.4-fold). These results successful reveal that patellamide D (**28.43**) selectively antagonizes the transport mediated by P-gp. During a continuation of search for anti-MDR agents from marine organisms, the same group confirmed that patellamide B (**28.41**) and C (**28.42**) were capable of reducing resistance to vinblastine in CEM/VLB100 (10-fold) and that patellamide G (**28.46**) did not alter sensitivity of vinblastine [100]. Overall, these data suggest that these lipophilic cyclic peptides from tunicate are potentially useful to overcome multi-drug resistance in cancer cells overexpression of P-gp.



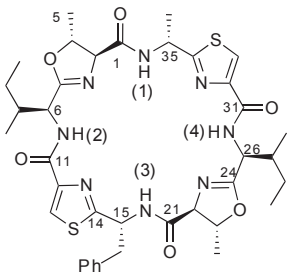
patellamide A (**28.40**)



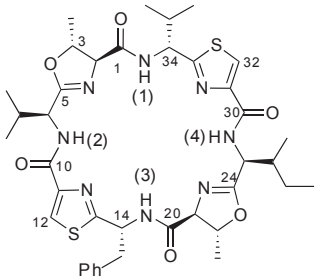
patellamide B (**28.41**)



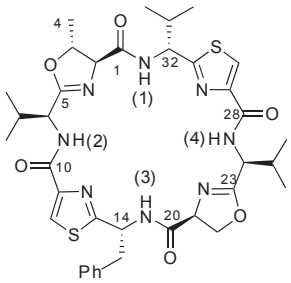
patellamide C (**28.42**)



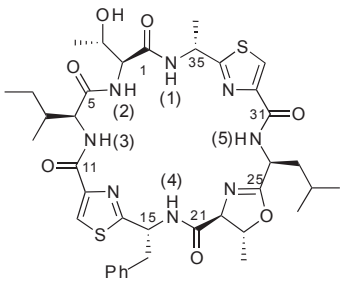
patellamide D (**28.43**)



patellamide E (**28.44**)



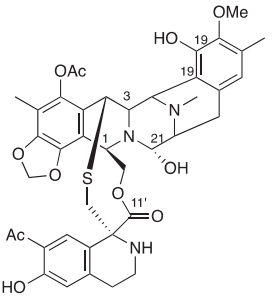
patellamide F (**28.45**)



patellamide G (**28.46**)

28.5.3 Ecteinascidin 743: A Tetrahydroisoquinolone from Marine Tunicates

Ecteinascidin 743 (ET-743, **28.47**), a marine tetrahydroisoquinolone alkaloid, was first isolated from aqueous extracts of the Caribbean tunicate *Ecteinascidia turbinata* [102, 103]. With remarkable cytotoxic activity both *in vitro* and *in vivo* in a variety of tumors [104], ET-743 (**28.47**) has been marketed for treatment of advanced soft tissue sarcoma or relapsed platinum-sensitive ovarian cancer with authorization from the European Commission [105, 106]. Currently, ET743 (**28.47**) is under clinical trials for other solid neoplasms such as breast and prostate cancer [105]. In contrast to traditional DNA-interacting agents, through binding to the minor groove of DNA [107], ET-743 (**28.47**) triggers DNA damage and results in the alternation of DNA repair as well as the transcription processes [108], which induces cell death.



ecteinascidin 743 (ET-743, **28.47**)

In 2002, Kanzaki et al. investigated the MDR-reversal activity of ET-743 (**28.47**) on P-gp overexpressing cancer cells [109]. It was reported that pretreatment with 0.1 nM (non-cytotoxic concentration) ET-743 (**28.47**) for 2 days partially reversed the resistance of P-gp overexpressing KB-8-5 or KB-C2 cells against vinblastine and doxorubicin, and that such an effectiveness was higher than that from the pretreatment with 3 μM cepharanthine, a known reversing agent of P-gp. The reversal activity from ET-743 (**28.47**) was also observed in cellular accumulation assay, in which 0.1 nM ET-743 (**28.47**) increased the accumulation of vinblastine and doxorubicin by 1.9-fold in both KB-C-2 and KB-8-5 cells. However, the effect of ET-743 (**28.47**) was not attributed to the direct inhibition of P-gp activity. Instead, it was demonstrated that this marine alkaloid could probably down-regulate expression of P-gp, therefore reduce the efflux mediated by P-gp.

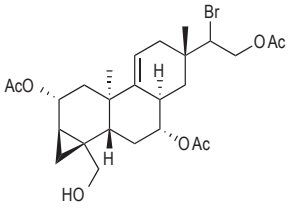
In general, these results reveal that ET-743 (**28.47**), a marine-derived alkaloid from tunicate, not only can be an anti-tumor drugs, but also can be an agent to overcome P-gp mediated drug resistance in MDR cancer cells.

28.6 P-gp Inhibitory Activity of Metabolites Isolated from Marine Algae

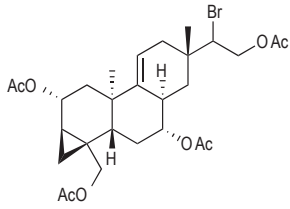
Among marine organisms, algae are one of the rich resources of metabolites with P-gp inhibitory activity.

28.6.1 Parguerenes: Bromoditerpenes from Marine Red Alga

In 1982 Schmitz et al. [110] reported five brominated diterpenes, parguerol and other analogues, isolated from a sea hare *Aplysia dactylomela*, collected near La Parguera, Puerto Rico. Following this discovery, more than twenty parguerenes were isolated from various marine organisms, mostly red algae (*Laurencia obtuse*, *Laurencia filiformis*, *Laurencia saitoi*, *Laurencia nipponica* and *Jania rubens* Lamx) and sea hares (*Aplysia dactylomela, Aplysia kurodai* and *Aplysia fasciata*), sourced from many regions (i.e., U.K., Japan, Egypt, China or Spain [111–119]). In addition, synthetic efforts from Schmitz [110] and Takeda [115] contributed to knowledge of parguerenes, which exhibit a diverse range of bioactivities including cytotoxicity [110, 115] and antitumor activity [119], anthelmintic activity [119], chemical defense [117] and neurotrophic effects [112].



pargeuerene I (**28.48**)



pargeuerene II (**28.49**)

The interaction between parguerenes and P-gp has not been reported until recently. 15-Bromoparguer-9(11)-ene-2,7,16,19-tetrol 2,7,16-triacetate (parguerene I, **28.48**) and 15-bromoparguer-9(11)-ene-2,7,16,19-tetrol 2,7,16,19-tetraacetate (parguerene II, **28.49**), sourced from a southern Australian collection of the Red alga *Laurencia filiformis*, were initially reported by Huang et al. [120] as P-gp inhibitors through a series of detailed studies. Parguerene I (**28.48**) was first isolated in 1996 by Capon et al. [116] from a collection of the southern Australian marine red alga *Laurencia filiformis*. Unlike parguerene I (**28.48**), parguerene II (**28.49**) was initially reported as a synthetic compound by Schmitz et al. [110] in 1981. The natural occurred parguerene II (**28.49**) was not discovered until it was isolated in 1989 by Suzuki et al. [111] from the marine red alga *Laurencia obtusa* (Hudson) Lamouroux, collected

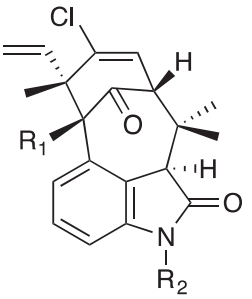
at Teuri Island, Hokkaido, Japan. This rare bromoditerpene was later isolated from another marine red algae *Laurencia filiformis*, collected at Bells Beach, Victoria, Australia, or *Laurencia saitoi*, collected at Isoyake areas in the Sea of Japan and Coast of Yantai, China respectively.

In a screening program for new inhibitors of P-gp from a structurally diverse library of ~1200 marine natural products, collected from southern Australian and Antarctic marine invertebrate, algae and microbes, Huang et al. [120] identified parguerenes I (**28.48**) and II (**28.49**) as hits capable of increasing the accumulation of a P-gp substrate, calcein acetoxymethyl ester (calcein AM) in P-gp overexpressing SW620 Ad300 cells. The P-gp inhibitory activity of these two parguerenes was supported by a flow-cytometry assay, demonstrating that both of them decreased P-gp mediated efflux of Hoechst 33342, another substrate of P-gp. Moreover, parguerenes I (**28.48**) and II (**28.49**) increased the cytotoxicity of known clinically significant cancer chemotherapeutic agents (P-gp substrates) such as vinblastine, doxorubicin or paclitaxel, and reversed P-gp mediated MDR in human colon cancer SW620 Ad300 cells, leukemia lymphoblast CEM/VLB100 cells or P-GP transfected HEK293/P-GP cells. The radio-ligand accumulation and efflux assays confirmed that their MDR reversal effect was achieved by enhancement of the accumulation and decrease of efflux of anticancer drugs. It was noted that parguerenes did not alter the sensitivity of cisplatin (non-P-gp substrate) in P-gp overexpressing cells and that co-incubation of HEK293/P-GP cells with parguerene I (**28.48**) or II (**28.49**) has no effect on P-gp expression. It has been determined that parguerenes interacted with and altered the extracellular antibody binding epitope of P-gp in a manner that differed from that of the known inhibitors verapamil (**28.01**) and cyclosporin A (**28.02**), suggestive of a different mechanism of action. Structure activity relationship observations between parguerenes I (**28.48**) and II (**28.49**) suggest scope to further manipulate and optimize the parguerene inhibitory pharmacophore. In summary, the discovery and characterization of the interaction between parguerenes and P-gp reveal a new inhibitory pharmacophore isolated from red alga, deserving of ongoing investigation.

28.6.2 Welwitindolinones: Indole Derivatives from Marine Blue-Green Alga

A screening for potential useful natural products led by Stratmann et al. in 1994 resulted in the discovery of a lipophilic extract of the marine blue-green alga (cyanobacteria) *Hapalosiphon welwitschii* W. & G.S. West collected in Queensland, Australia, as a collection of MDR reversing agents [121]. N-methylwelwitindolinone C isothiocyanate (**28.50**), the major indole alkaloid isolated from the lipophilic extract, was confirmed as a fraction associated with the ability to reverse actinomycin D and daunomycin resistance in a vinblastine-selected P-gp over-expressing human ovarian adenocarcinoma cell line SK-VLB [121, 122]. Another two analogues

welwitindolinone C isothiocyanate (**28.51**) and N-methylwelwitindolinone C isonitrile (**28.52**), isolated from the same blue-green alga, together with N-methylwelwitindolinone C isothiocyanate (**28.50**) belong to a family of 3,4-bridged oxindole alkaloids. In 1995, Smith et al. [122] examined the ability of these welwitindolinones to interact with P-gp in another multi-drug resistant breast cancer cell line MCF-7/ADR, which was drug-selected from sensitive MCF-7 cell line [123]. As expected, exposure of MCF-7/ADR cells with 2.5 μM N-methylwelwitindolinone C isothiocyanate (**28.50**) increased their sensitivity to vinblastine by approximately 200-fold, a level similar to that of MCF-7 cells. N-methylwelwitindolinone C isothiocyanate (**28.50**) was revealed as the most active compound that was superior to verapamil. At as low as 0.1 μM, it significantly increased the cytotoxicity of vinblastine, paclitaxel and actinomycin D by 40–90-fold. By contrast, N-methylwelwitindolinone C isonitrile (**28.52**), which was different from N-methylwelwitindolinone C isothiocyanate (**28.50**) by replacing the isothiocyanate group with an isonitrile group, was ineffective with any of these anti-cancer drugs. The results from enhancement of intracellular of [^{3}H]-vinblastine and [^{3}H]-paclitaxel in SK-VLB-1 cells or competition with binding of [^{3}H]-azidopine to P-gp in MCF-7/ADR, reinforce the proposition that the isothiocyanate group plays an essential role in affinity of P-gp and reversal activity. Welwitindolinone C isothiocyanate (**28.51**), the third member in this study, is structurally similar to other two analogues. At 0.1 μM, welwitindolinone C isothiocyanate (**28.51**) only showed modest effect on reversing resistance to vinblastine (4-fold) or actinomycin D in MCF-7/ADR. It was reported that welwitindolinone C isothiocyanate (**28.51**) inhibited the polymerization of purified tubulin *in vivo*, suggesting that it could be served as a new anti-microtubule compound with equal cytotoxic efficacy against sensitive cancer cells and multi-resistant cancer cells overexpressing P-gp [124].



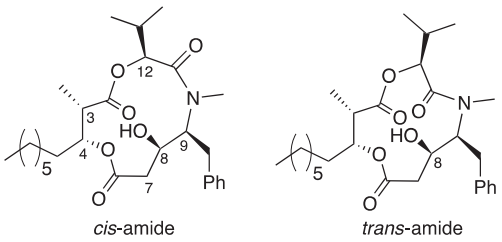
R_1 = SCN, R_2 = CH_3, N-methylwelwitindolinone C isothiocyanate (**28.50**)

R_1 = SCN, R_2 = H, welwitindolinone C isothiocyanate (**28.51**)

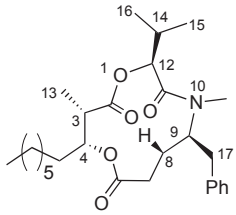
R_1 = NC, R_2 = CH_3, N-methylwelwitindolinone C isonitrile (**28.52**)

28.6.3 Hapalosin, A Cyclic Depsipeptide from Marine Blue-Green Alga

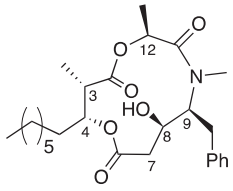
Hapalosin (**28.53**), a 12-membered cyclic depsipeptide, was isolated from the same lipophilic extract of the blue-green alga (cyanobacteria) *Hapalosiphon welwitschii* W. & G.S. West as N-methylwelwitindolinone C isothiocyanate in 1994 [125]. The discovery from Stratmann et al. [125] confirmed that hapalosin was the major component responsible for the MDR-reversal activity of the lipophilic extract. It was found that this molecule (20 μM) was 1.6-fold more potent than verapamil to increase the accumulation of [^{3}H]-paclitaxel in SKVLB1 cells. Interestingly, there was no difference between hapalosin (**28.53**) and verapamil for enhancement of [^{3}H]-vinblastine in the same cell line. Moreover, while 2.5 μM hapalosin (**28.53**) has no effect on the cytotoxicity of 5-fluorouracil or cisplatin (non-substrates of P-gp), it abolished the resistance in P-gp overexpressing MCF-7/ADR cells to a panel of natural anti-cancer drugs such as vinblastine, daunomycin, actinomycin D, paclitaxel and colchicine (substrates of P-gp). The reversal activity of hapalosin hapalosin (**28.53**) supports a value of this compound for the development of new anti-MDR agents. Recognizing that hapalosin hapalosin (**28.53**) is an inseparable mixture of conformers (*cis*-/*trans*- = 3:1) [126–128], a series of single (*cis*- or *trans*-) or mixtures of hapalosin derivatives have been synthesized for structure–activity relationship study. However, in light of the observations from these isomers, it is doubtful that the *s*-*cis* amide conformation is the sole factor accounting for their MDR-reversing activities. Instead, the lack of reversing activity of 8-deoxyhapalosin (**28.54**) in K562 R and S/Adriblastine against human erytholeucemic cell lines determined that active hapalosin-type compounds require C-8 hydroxy group [129]. In addition, the finding that the C12-methyl derivative (**28.55**) of hapalosin displayed higher reversal activity than that of verapamil and hapalosin, has evidenced an essential role of hydrophobic interaction of the C12-substituent with the receptor site [128]. Overall, to provide a clinically successful compound, different routes of synthesis to achieve a potent MDR-reversing analogue of hapalosin by modification of various factors to the marcocycle conformation and the pattern of substituents were proposed [130–132].



hapalosin (**28.53**)



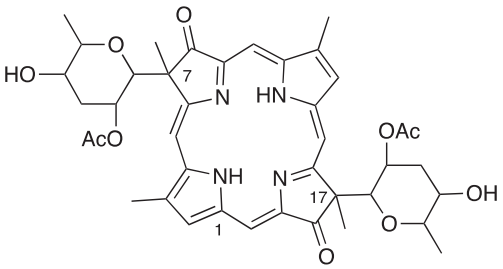
8-deoxyhapalosin (**28.54**)



C12-methyl derivative (**28.55**) of hapalosin

28.6.4 Tolyporphin, A Prophyrin from Blue-Green Alga

Tolyporphin (**28.56**) was previously isolated from the lipophilic extract of the blue-green alga *Tolypothrix nodosa*, colleted at Nan Madol, Pohpei [133]. In 1994, Smith et al. found that this unusual prophyrin significantly increased the cytotoxicity of daunomycin or paclitaxel in P-gp overexpressing human ovarian SKVLB1 cells at 0.5 μM [134]. Tolyporphin (**28.56**) did not alter the sensitivity of SKVLB1 and MCF-7/ADR cells to non-P-gp-transported cisplatin and melphalan, but rather prompted the accumulation of [^{3}H]-vinblastine in these two cell lines. The photoaffinity-label and radio-ligand binding study in membranes isolated from SKVLB1 indicated that the reversal effect from tolyporphin (**28.56**) was attributable to the competitive binding to P-gp and thus the inhibition of efflux of cytotoxic P-gp substrates. Seven other porphyrin-containing compounds isolated from *Tolypothrix nodosa* were under investigation by the same research group to reveal potential structural features for drug binding to P-gp.



tolyporphin (**28.56**)

28.7 Conclusion

There were numerous efforts to overcome MDR caused by P-gp in the past three decades. Of these approaches, direct inhibition is believed to be the most efficient way. Unfortunately, although three generations of P-gp inhibitors have been developed, none have proved to be clinically useful. Despite these disappointing results, there remains a compelling cause to continue to search for and develop inhibitors from other sources, such as natural products.

With their unique chemical structures, marine-derived metabolites are an attractive new resource to prime the search for new P-gp inhibitors. Indeed, the research has focused on bioactivity-guided fractionation and analysis of small molecules extracted from marine organisms. Metabolites capable of overcoming P-gp transport function are mainly isolated from marine sponges, tunicates and algae. Of these compounds, some exerted significant activity on reversal of P-gp mediated drug efflux, sometimes at very low concentrations. For example, lamellarin D-triacetate (**28.34**) at 30 nM increased the cytotoxicity of doxorubicin in P-gp overexpressing ovary cells by 1000-fold. Although there is still no successful application of marine-derived compounds as P-gp inhibitors in clinic, with the provision of a variety of reversal agents of P-gp, it is believed that marine natural products can continue to deliver new P-gp inhibitor scaffolds, of which some could be led for further development.

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Chapter 29
Marine Cyanobacteria Compounds with Anticancer Properties: Implication of Apoptosis

Maria do Rosário Martins and Margarida Costa

Abstract Marine cyanobacteria have been proved to be an important source of potential anticancer drugs. Although several compounds were found to be cytotoxic to cancer cells in culture, the pathways by which cells are affected are still poorly elucidated. For some compounds, cancer cell death was attributed to an implication of apoptosis through morphological apoptotic features, implication of caspases and proteins of the Bcl-2 family, and other mechanisms such as interference with microtubules dynamics, cell cycle arrest and inhibition of proteases other than caspases.

Keywords Marine cyanobacteria · Anticancer compounds · Apoptose

29.1 Introduction

Cyanobacteria have been long recognized as an important component of the microbial community of a wide range of environments. These organisms are found in both terrestrial and aquatic ecosystems, in a vast array of ecological conditions, including extremes that often exclude other organisms [10]. In the marine environment cyanobacteria occur as free living planktonic organisms in the open ocean and near shores, as benthic forms along the maritime coasts and as symbionts with invertebrates such as sponges [84]. Furthermore, in this environment cyanobacteria have a substantial ecological importance through their involvement in the global carbon cycle and nitrogen fixation, contributing significantly to the global primary productivity [9].

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The extensive fossil record of this Gram-negative photosynthetic prokaryotes point them as the most primitive photosynthetic organisms on earth [93]. As being the first photosynthetic autotrophs, it is hypothesised their role in generating the oxygenic atmosphere that we depend on, during the Archaean and Proterozoic Era [40]. The ancient origin of cyanobacteria and its long evolutionary history is reflected in a high degree of biological adaptation and tolerance to environmental stress, which is in turn a reflection of its morphological, biochemical and physiological diversity.

Cyanobacteria diversity, ubiquity, dominance and tolerance to environmental pressures have long attracted investigation on their potential as a source of secondary metabolites, with interesting biomedical properties. While much of the early research on cyanobacteria secondary metabolites was focused on the production of toxins hazards to animals and humans by freshwater and brackish water strains [119], marine cyanobacteria emerged as a promising resource for the discovery of new drugs with potential pharmacological applications, namely in the field of cancer treatment. In fact, in recent years a plethora of natural compounds were isolated and identified from marine cyanobacteria, mainly from benthic forms, revealing a great scope for the discovery of novel anticancer compounds or lead structures for the development of new drugs [76].

The chemically diverse nature of compounds isolated from marine cyanobacteria is also a reflection of a long evolutionary history, and seems to be due to a richness in enzymes responsible for methylations, oxidations and tailoring [44], and for both non-ribosomal peptide synthethases and polyketide synthases biosynthetic pathways. The result is the production of a large chemical variety of compounds such as linear peptides [44], cyclic peptides [99], linear lipopeptides [82], depsipeptides [36], cyclic depsipeptides [103], fatty acid amides [12], swinholides [2], glicomacrolides [110] and macrolactones [91], which have been found to exhibit a wide range of pharmacological interesting bioactivities such as antibacterial, antifungal, antiviral, cytotoxic and anticancer [98]. Concerning cancer as a major cause of death and morbidity within the human population, increasing with changing life style, we have been witnessed an enormous increase in research concerning anticancer drug discovery [25]. A vast array of marine cyanobacteria compounds is described as potential anticancer [16]. Some, such as the linear pentapeptide Dolastatin 10 reached the clinical trials phase. Unfortunately, in that case, the compound dropped from trials due to the development of neurological complications [87]. Although several compounds were found to induce cytotoxicity in cancer cell lines, the mechanisms undergoing cell cytotoxicity are, in most of the cases, not elucidated. In cases where cell cytotoxicity pathways were explored the implication of apoptosis as the mechanism of cell death has been the most explored, since apoptosis is crucial in controlling cell proliferation and therefore in controlling tumor progression.

Considering the role of marine cyanobacteria for anticancer drug discovery, we aim with this chapter to provide an overview of the implication of apoptosis as a mechanism of cell death, induced by marine cyanobacteria compounds in cancer cell lines.

29.2 General Cytotoxicity of Marine Cyanobacteria Compounds in Cancer Cell Lines

The traditional approach in screening the anticancer potential of natural compounds is based on its cytotoxicity towards mammalian cancer cell lines, measured by assessing cell growth or viability. Two major assays for testing cytotoxicity have been used, the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay and the sulforhodamine (SRB) assay [47]. The MTT assay is a viability assay based on the conversion of the tetrazolium salt MTT into formazan crystals by living cells. MTT is metabolized in active mitochondria enzymes. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the *in vitro* cytotoxicity of compounds. The SRB assay relies on the uptake of the negatively charged SRB by basic amino acids in the cells. Since the amount of SRB taken by the cells depends in the amount of cells, this assay in broadly used to estimate cell density. Both MTT and SBR assays have been extensively applied to assess the cytotoxic potential of compounds isolated from marine cyanobacteria. From our literature review published in 2012 [16], 70 compounds isolated from marine cyanobacteria were found as potentially anticancer based mainly in the MTT and SBR assays. Here we complete this previous review and present in Table 29.1 a compilation of 103 compounds. Since this last review 33 compounds are new, which represents a considerable effort in screening marine cyanobacteria compounds for anticancer properties.

It is striking that most of the bioactive compounds from marine cyanobacteria have been isolated from filamentous forms of the genus *Lyngbya* or, as recently found, *Moorea* for some strains [23, 24, 27]. However this is not entirely surprising since these cyanobacteria are found to occur in high densities in maritime ecosystems of tropical and sub-tropical regions, which makes the harvest of biomass accessible directly from the field. Due to their slower growth in environmental conditions, other cyanobacteria such as the picoplanktonic *Cyanobium*, *Synechocystis* and *Synechococcus* and the filamentous *Leptolyngbya* and *Pseudanabaena* have been largely overlooked. In the last years, we have been focused on the production of bioactive compounds from marine cyanobacteria isolated from the Portuguese coast. We have been found cytotoxic and antimicrobial effects of extracts of strains of the genus *Aphanotece*, *Cyanobium*, *Synechocystis* and *Synechococcus* [18, 66, 67, 94] and we isolated Hierridin B from a *Cyanobium* strain, which showed selective toxicity towards a colon cancer cell line [53]. More recently we screened twenty eight marine cyanobacteria strains, belonging to the genera *Cyanobium*, *Synechocystis* and *Synechococcus* and the filamentous genera *Nodosilinea*, *Leptolyngbya*, *Pseudanabaena* and *Romeria*, in eight human tumour cell lines for anticancer potential [17]. 8.9% of the strains revealed strong cytotoxicity, 17.8% showed moderate cytotoxicity and 14.3% showed low toxicity. The results obtained revealed that the studied genera of marine cyanobacteria are also a promising source of novel compounds with potential anticancer activity and highlights the interest to explore also the smaller filamentous and picoplanktonic genera of cyanobacteria.

Table 29.1 Cytotoxicity revealed by cell viability and proliferation assays towards cancer cells by marine cyanobacteria compounds

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Ankaraholide A	*Geitlerinema*	Glycosilated swinholide	MTT assay	NCI-H460 lung tumor	119.0 nM	[2]
			SRB assay	MDA-MB-435 breast carcinoma	8.9 nM	
Apratoxin A	*Lyngbya majuscula*	Cyclic depsipeptide	SRB assay	KB oral epidermoid cancer	0.52 nM	[58]
				LoVo colon cancer	0.36 nM	
			MTT assay	U2OS osteosarcoma	10.0 nM	[69]
				HT-29 colon adenocarcinoma	1.4 nM	
				HeLa cervical carcinoma	10.0 nM	
			Non-specified	HeLa cervical carcinoma	2.2 nM	[62]
Apratoxin A Sulfoxide	*Moorea producens*	Cyclic depsipeptide	MTT assay	NCI-H460 lung tumor	3.4 nM	[111]
Apratoxin B	*Lyngbya sp.*	Cyclic depsipeptide	MTT assay	KB oral epidermoid cancer	21.3 nM	[60]
				LoVo colon cancer	10.8 nM	
Apratoxin C	*Lyngbya sp.*	Cyclic depsipeptide	MTT assay	KB oral epidermoid cancer	1.0 nM	[60]
				LoVo colon cancer	0.73 nM	
Apratoxin D	*Lyngbya majuscula; Lyngbya sordida*	Cyclic depsipeptide	MTT assay	H-460 lung cancer	2.6 nM	[34]
Apratoxin E	*Lyngbya bouilloni*	Cyclic depsipeptide	MTT assay	U2OS osteosarcoma	59.0 nM	[69]
				HT-29 colon adenocarcinoma	21.0 nM	
				HeLa cervical carcinoma	72.0 nM	
Apratoxin F	*Lyngbya bouilloni*	Cyclic depsipeptide	MTT assay	H-460 lung cancer	2.0 nM	[112]
			Hemocytometer counting	HCT-116 colorectal cancer	36.7 nM	
Apratoxin G	*Lyngbya bouilloni*	Cyclic depsipeptide	MTT assay	NCI-H460 lung cancer	140 nM	[112]
Apratoxin H	*Moorea producens*	Cyclic depsipeptide	MTT assay	NCI-H460 lung tumor	89.9 nM	[111]
Aurilide B	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	NCI-H460 lung tumor	0.04 μM	[38]
Aurilide C	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	NCI-H460 lung tumor	0.13 μM	[38]
Belamide A	*Symploca* sp.	Linear tetrapeptide	Non-specified	HCT-116 colon cancer	0.74 μM	[97]
Bisebromoamide	*Lyngbya* sp.	Peptide	SRB assay	HeLa S_3 epithelial carcinoma	39.2 nM	[109]
				39 cancer cell lines	13.0–100.0 nM	

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Biselyngbyaside	*Lyngbya* sp.	Glicomacrolide	SRB assay	HeLa S3 epithelial carcinoma	0.16 μM	[110]
				SNB-78 central nervous system cancer	0.036 μM	
				NCI H522 lung cancer	0.067 μM	
Calothrixin A	*Calothrix*	Pentacyclic indolophenanthridine	^{3}H-thymidine incorporation into DNA	HeLa epithelial carcinoma	1.6 μM	[13]
			MTT assay	Leukemia CEM cells	0.20 μM	[48]
Calothrixin B	*Calothrix*	Pentacyclic indolophenanthridine	MTT assay	HeLa epithelial carcinoma	0.25 μM	[7]
				Leukemia CEM	1.05 μM	[48]
Carmaphycin A	*Symploca* sp.	Peptide	MTT assay	H-460 lung cancer	9.0 nM	[85]
				HCT-116 colon tumor	19.0 nM	
Carmaphycin B	*Symploca* sp.	Peptide	MTT assay	H-460 lung cancer	6.0 nM	[85]
				HCT-116 colon tumor	43.0 nM	
Caylobolide A	*Lyngbya majuscula*	Macrolactone	Non-specified	HCT-116 colon tumor	9.9 μM	[63]
Caylobolide B	*Phormidium* spp.	Macrolactone	MTT assay	HT-29 colorectal adenocarcinoma	4.5 μM	[91]
				HeLa cervical carcinoma	12.2 μM	
Coibacin A	cf. *Oscillatoria* sp.	Polyketide lactone	MTT assay	H-460 lung cancer	31.5 μM	[3]
Coibacin B	cf. *Oscillatoria* sp.	Polyketide lactone	MTT assay	H-460 lung cancer	17.0 μM	[3]
Coibacin C	cf. *Oscillatoria* sp.	Polyketide lactone	MTT assay	H-460 lung cancer	21.3 μM	[3]
Coibacin D	cf. *Oscillatoria* sp.	Polyketide lactone	MTT assay	H-460 lung cancer	11.4 μM	[3]
Coibamide A	*Leptolyngbya* sp.	Cyclic depsipeptide	MTT assay	Astrocytoma SNB75	7.6 nM	[74]
				Lung NCI-H460	23.0 nM	
				Breast MDA-MB-231	2.8 nM	
				Melanoma LOX IMVI	7.4 nM	
				Leukemia HL-60	7.4 nM	
				U87-MG glioblastoma	28.8 nM	[41]
				SF-295 glioblastoma	96.2 nM	

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Cryptophycin 1	*Nostoc* spp.	Cyclic depsipeptide	Cell morphology examination	SKOV3 ovarian carcinoma	Tested at 50 pM	[79]
Dolastatin 10	*Symploca* sp.	Linear Pentapeptide	MTT assay	Lung A549 carcinoma	< 1.0 nM	[11]
				NCI-H69 lung cancer	0.059 nM	[46]
				NCI-H82 lung cancer	0.032 nM	
				NCI-H446 lung cancer	0.048 nM	
				NCI-H510 lung cancer	0.184 nM	
				DU-145 prostate cancer	<1.0 nM	[116]
Dragonamide	*Lyngbya majuscula*	Lipopeptide	Non-specified	A-549 lung epithelial adenocarcinoma	>1 μM	[43]
				HT-29 colon adenocarcinoma		
				MEL-28 melanoma		
Dragonamide C	*Lyngbya polychroa*	Linear lipopeptide	MTT assay	U2OS osteosarcoma	56.0 μM	[32]
				HT-29 colorectal adenocarcinoma	22.0 μM	
				IMR-32 neuroblastoma	49.0 μM	
Dragonamide D	*Lyngbya polychroa*	Linear lipopeptide	MTT assay	U2OS osteosarcoma	59.0 μM	[32]
				HT-29 colorectal adenocarcinoma	32.0 μM	
				IMR-32 neuroblastoma	51.0 μM	
Hierridin B	*Cyanobium* sp.	Glycolipid	MTT assay	HT-29 colorectal adenocarcinoma	82.3 μM	[53]
Hoiamide A	Assemblage of *Lyngbya majuscule* and *Phormidium gracile*	Cyclic depsipeptide	Non-specified	H-460 lung cancer	11.2 μM	[14]
Hoiamide B	Cyanobacterial sample	Cyclic depsipeptide	Non-specified	H-460 lung cancer	8.3 μM	[14]
Homodolastatin 16	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	WHCO1 esophageal cancer	4.82 μM	[19]
				WHCO6 esophageal cancer	11.32 μM	
				ME180 cervical cancer	9.3 μM	
Isomalyngamide A	*Lyngbya majuscula*	Fatty acid amide	MTT assay	MCF-7 breast cancer	4.6 μM	[12]
				MDA-MB-231 breast cancer	2.8 μM	

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Isomalyngamide A-1	*Lyngbya majuscula*	Fatty acid amide	MTT assay	MCF-7 breast cancer	<20 μM	[12]
				MDA-MB-231 breast cancer	12.7 μM	
Jamaicamide A	*Lyngbya majuscula*	Polyketide-Peptide	MTT assay	H-460 lung cancer	~15 μM	[21]
Jamaicamide B	*Lyngbya majuscula*	Polyketide-Peptide	MTT assay	H-460 lung cancer	~15 μM	[21]
Jamaicamide C	*Lyngbya majuscula*	Polyketide-Peptide	MTT assay	H-460 lung cancer	~15 μM	[21]
Kalkitoxin	*Lyngbya majuscula*	Lipopeptide	Trypan blue dye exclusion assay	HCT-116 colon	2.7 mM	[118]
Lagunamide A	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	A549 lung cancer	2.9 nM	[114]
				HT-29 colon adenocarcinoma	2.5 nM	
				HCT8 ileocecal colorectal adenocarcinoma	1.6 nM	
				SKOV3 ovarian carcinoma	3.8 nM	
Lagunamide B	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	HCT8 ileocecal colorectal adenocarcinoma	5.2 nM	[114]
Lagunamide C	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	Adenocarcinoma A549 cancer	2.4 nM	[113]
				Ovary SK-OV cancer	4.5 nM	
				Ileocecal colorectal HCT8 cancer	2.1 nM	
				Prostate PC-3 cancer	2.6 nM	
Largazole	*Symploca* sp.	Cyclic depsipeptide	MTT assay	MDA-MB-23I breast cancer	7.7 nM	[108]
				U2OS osteosarcoma	55 nM	
				A549 lung cancer	0.32 μM	[129]
				HCT-116 colorectal carcinoma	0.08 μM	
Lyngbyabellin A	*Lyngbya majuscula*	Cyclic depsipeptide	Non-specified	KB nasopharyngeal carcinoma	0.435 nM	[55]
				LoVo colon adenocarcinoma	7.25 nM	
				HT-29 colon adenocarcinoma	47.0 nM	[73]
				HeLa cervical carcinoma	22.0 nM	
27-deoxylyngbyabellin A	*Lyngbya bouillonii*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	12.0 nM	[73]
				HeLa cervical carcinoma	7.3 nM	
Lyngbyabellin B	*Lyngbya bouillonii*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	1.1 μM	[73]
				HeLa cervical carcinoma	0.71 μM	

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Lyngbyabellin E	*Lyngbya majuscula*	Lipopeptide	MTT assay	H-460 lung cancer	0.4 μM	[37]
Lyngbyabellin F	*Lyngbya majuscula*	Lipopeptide	MTT assay	H-460 lung cancer	1.0 μM	[37]
Lyngbyabellin G	*Lyngbya majuscula*	Lipopeptide	MTT assay	H-460 lung cancer	2.2 μM	[37]
Lyngbyabellin H	*Lyngbya majuscula*	Lipopeptide	MTT assay	H-460 lung cancer	0.2 μM	[37]
Lyngbyabellin I	*Lyngbya majuscula*	Lipopeptide	MTT assay	H-460 lung cancer	1.0 μM	[37]
Lyngbyabellin J	*Lyngbya bouillonii*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	54.0 nM	[73]
				HeLa cervical carcinoma	41.0 nM	
Lyngbyabellin N	*Moorea bouillonii*	Lipopeptide	MTT assay	H-460 lung cancer	0.0048–1.8 μM	[15]
				HCT-116 colorectal carcinoma	40.9 nM	
Lyngbyaloside	*Lyngbya bouillonii*	Macrolide	MTT assay	HT-29 colon adenocarcinoma	37.0 μM	[73]
				HeLa cervical carcinoma	35.0 μM	
Lyngbyaloside B	*Lyngbya* sp.	Macrolide	Non-specified	KB nasopharyngeal carcinoma	4.3 μM	[59]
				LoVo colon adenocarcinoma	~15 μM	
2-*epi*-lyngbyaloside	*Lyngbya bouillonii*	Macrolide	MTT assay	HT-29 colon adenocarcinoma	38.0 μM	[73]
				HeLa cervical carcinoma	33.0 μM	
18E-lyngbyaloside C	*Lyngbya bouillonii*	Macrolide	MTT assay	HT-29 colon adenocarcinoma	13.0 μM	[73]
				HeLa cervical carcinoma	9.3 μM	
18Z-lyngbyaloside C	*Lyngbya bouillonii*	Macrolide	MTT assay	HeLa cervical carcinoma	53.0 μM	[73]
Majusculamide C	*Lyngbya majuscula*	Cyclic depsipeptide	Non-specified	OVCAR-3 ovarian carcinoma	518 nM	[86]
				A498 kidney cancer	58.9 nM	
				NCI-H460 lung cancer	3.25 nM	
				KM20L2 colorectal cancer	1.32 nM	
				SF-295 glioblastoma cancer	132 nM	
Malevamide D	*Symploca hydnoides*	Peptide	Non-specified	A-549 lung cancer	0.3–0.7 nM	[42]
				HT-29 colon cancer	0.3–0.7 nM	
				MEL-28 melanoma cancer	0.7 nM	
Malyngamide 2	*Lyngbya sordida*	Fatty acid amine	MTT assay	H-460 lung cancer	27.3 μM	[64]

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Malyngamide 4	*Moorea producens*	Lipopeptide	SRB assay	A-549 lung cancer	44.0 μM	[95]
				HT-29 colon cancer	40.0 μM	
				MDA-MB-23I breast cancer	50.0 μM	
Malyngamide A	*Lyngbya majuscula*	Fatty acid amine	SRB assay	A-549 lung cancer	75.0 μM	[95]
				HT-29 colon cancer	88.0 μM	
				MDA-MB-23I breast cancer	70.0 μM	
Malyngamide B	*Lyngbya majuscula*	Fatty acid amine	SRB assay	A-549 lung cancer	52.0 μM	[95]
				HT-29 colon cancer	45.0 μM	
				MDA-MB-23I breast cancer	60.0 μM	
Malyngamide C	*Lyngbya majuscula*	Fatty acid amine	MTT assay	H-460 lung cancer	3.07 μM	[31]
Malyngamide J	*Lyngbya majuscula*	Fatty acid amine	MTT assay	H-460 lung cancer	178 nM	[31]
Malyngamide K	*Lyngbya majuscula*	Fatty acid amine	MTT assay	H-460 lung cancer	2.59 μM	[31]
Malyngolide dimmer	*Lyngbya majuscula*	Cyclodepside	MTT assay	H-460 lung cancer	<55 μM	[35]
Nostocyclopeptide A1	*Nostoc* sp.	Cyclic heptapeptides	Non-specified	KB oral epidermoid cancer	~1 μM	[28]
				LoVo colon cancer	~1 μM	
Nostocyclopeptide A2	*Nostoc* sp.	Cyclic heptapeptides	Non-specified	KB oral epidermoid cancer	~1 μM	[28]
				LoVo colon cancer	~1 μM	
Obyanamide	*Lyngbya confervoides*	Cyclic depsipeptide	Non-specified	KB oral epidermoid cancer	0.97 μM	[120]
				LoVo colon cancer	3.14 μM	
Palauamide	*Lyngbya* sp.	Cyclic depsipeptide	Non-specified	HeLa cervical carcinoma	39 nM	[130]
				A549 lung adenocarcinoma	19 nM	[130]
				BGC gastrocarcinoma	26 nM	[130]
				KB oral epidermoid cancer	13 nM	[124]
Palmyramide A	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	H-460 lung cancer	39.7 μM	[105]
Pitipeptolide A	*Lyngbya majuscula*	Cyclic depsipeptide	Non-specified	LoVo colon cancer	278 nM	[57]
			MTT assay	HT-29 colon adenocarcinoma	13.0 μM	[77]
				MCF- 7 breast cancer	13.0 μM	
Pitipeptolide B	*Lyngbya majuscula*	Cyclic depsipeptide	Non-specified	Human LoVo colon cancer	2.4 μM	[57]
			MTT assay	HT-29 colon adenocarcinoma	13.0 μM	[77]
				MCF- 7 breast cancer	11.0 μM	

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Pitipeptolide C	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	67.0 μM	[77]
				MCF- 7 breast cancer	73.0 μM	
Pitipeptolide E	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	75.0 μM	[77]
Pitipeptolide F	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	87.0 μM	[77]
				MCF- 7 breast cancer	83.0 μM	
Pitiprolamide	*Lyngbya majuscula*	Cyclic depsipeptide	MTT assay	HCT116 colorectal carcinoma	33.0 μM	[78]
				MCF- 7 breast cancer	33.0 μM	
Pseudodysidenin	*Lyngbya majuscula*	Lipopeptide	Non-specified	A-549 lung epithelial adenocarcinoma	>1.83 μM	[43]
				HT-29 colon adenocarcinoma cell line	>1.83 μM	
				MEL-28 melanoma	>1.83 μM	
Somocystinamide A	*Lyngbya majuscula*	Lipopeptide	XTT assay	Jurkat leukemia	3 nM	[127]
				CEM leukemia	14 nM	
				A549 lung carcinoma	46 nM	
				Molt4 T leukemia	60 nM	
				MCF-7 breast cancer	210 nM	
				NB7 neuroblastoma	810 nM	
				PC-3 prostate carcinoma	970 nM	
				M21 melanoma	1.3 μM	
				U266 myeloma	5.8 μM	
Symplocamide A	*Symploca* sp.	Cyclic peptide	Non-specified	H-460 lung cancer	40.0 nM	[54]
Symplostatin 1	*Symploca hydnoides*	Linear Pentapeptide	SRB assay	MDA-MB-435 melanoma	0.15 nM	[80]
				SK-OV-3 adenocarcinoma	0.09 nM	
				NCI/ADR ovarian carcinoma	2.90 nM	
				KB oral epidermoid carcinoma	1.25 nM	[39]
Tasiamide	*Symploca* sp.	Cyclic peptide	Non-specified	KB oral epidermoid cancer	578 nM	[121]
				LoVo colon cancer	4.18 μM	
Tasiamide B	*Symploca* sp.	Peptide	Non-specified	KB oral epidermoid cancer	0.8 μM	[123]
Tasipeptin A	*Symploca* sp.	Cyclic depsipeptide	Non-specified	KB oral epidermoid cancer	0.93 μM	[122]

Table 29.1 (continued)

Compound	Source	Class of compound	Cytoxicity assay	Human cancer cells	IC50	Reference
Tasipeptin B	*Symploca* sp.	Cyclic depsipeptide	Non-specified	KB oral epidermoid cancer	0.82 μM	[122]
Ulongapeptin	*Lyngbya* sp.	Cyclic depsipeptide	Non-specified	KB oral epidermoid cancer	0.63 μM	[125]
Veraguamide A	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	H-460 lung cancer	141 nM	[75]
				HT-29 colon adenocarcinoma	26.0 μM	[92]
				HeLa cervical carcinoma	21.0 μM	
Veraguamide B	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	30.0 μM	[92]
				HeLa cervical carcinoma	17.0 μM	
Veraguamide C	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	5.8 μM	[92]
				HeLa cervical carcinoma	6.1 μM	
Veraguamide D	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	0.84 μM	[92]
				HeLa cervical carcinoma	0.54 μM	
Veraguamide E	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	1.5 μM	[92]
				HeLa cervical carcinoma	0.83 μM	
Veraguamide F	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	49.0 μM	[92]
				HeLa cervical carcinoma	49.0 μM	
Veraguamide G	*Symploca* cf. *hydnoides*	Cyclic depsipeptide	MTT assay	HT-29 colon adenocarcinoma	2.7 μM	[92]
				HeLa cervical carcinoma	2.3 μM	
Viqueamide A	*Rivularia* sp.	Cyclic depsipeptide	MTT assay	H-460 lung cancer	60 nM	[8]
Wewakazole	*Lyngbya majuscula*	Cyclic dodecapeptide	MTT assay	H-460 lung cancer	10.1 μM	[64]
Wewakpeptin A	*Lyngbya semiplena*	Depsipeptide	MTT assay	H-460 lung cancer	0.65 μM	[36]
Wewakpeptin B	*Lyngbya semiplena*	Depsipeptide	MTT assay	H-460 lung cancer	0.43 μM	[36]
Wewakpeptin C	*Lyngbya semiplena*	Depsipeptide	MTT assay	H-460 lung cancer	5.9 μM	[36]
Wewakpeptin D	*Lyngbya semiplena*	Depsipeptide	MTT assay	H-460 lung cancer	3.5 μM	[36]

MTT 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, *XTT* 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, *SBR* Sulforhodamine

29.3 Apoptosis Involvement in the Cytotoxicity of Marine Cyanobacteria Compounds

Apoptosis is an active cellular process of cell death triggered by a variety of stimuli such as the exposure to cytotoxic drugs. Apoptotic cell death is characterised by typical morphological and biochemical features that are in the base of methods and protocols applied to evaluate the implication of this mechanism in the cytotoxicity induced by natural compounds.

Two main apoptotic pathways are involved in the apoptotic cell death, both converging in the same executioner pathway. The extrinsic pathway is triggered by the activation of a family of death receptors at the cell surface, whereas the intrinsic pathway is triggered by the release of proteins from the mitochondria to the cytosol such as cytochrome c. In both cases families of proteins are involved, namely the cysteine proteases caspases in both the extrinsic and intrinsic pathway [50] and the Bcl-2 family, via intrinsic pathway predominantly [30]. Both pathways culminate in the activation of the caspases 3, 6 and 7, which in turn activate endonucleases and proteases, leading to a degradation of both nuclear and cytoskeleton proteins [100]. As a final consequence of the process, cell fragmentation into membrane-enclosed vesicles, known as apoptotic bodies, occurs. Since there is no release of the cellular constituents and apoptotic bodies are engulfed by macrophages and neighbouring cells, there is essentially no inflammatory response [29]. In fact, apoptosis is the preferred form of cancer cell death in cancer treatments due to the scarcity of inflammatory response. Thus, compounds that act as a stimulus that trigger this type of cell death are an attractive approach for cancer therapy.

Several marine cyanobacteria compounds were found to act as apoptotic inducers. In the following sections we present a compilation of those compounds grouped according to the apoptotic marker in focus.

29.3.1 Apoptosis by Apoptotic Morphological Features

Cells undergoing apoptosis show typical morphological changes. Cell shrinkage, blebbing of plasma membrane, chromatin condensation, nucleolus segregation, nuclear fragmentation, and formation of apoptotic bodies are considered morphological hallmarks of apoptosis [22]. Considering marine cyanobacteria, the compounds biselyngbyaside, cryptophycin 1, calothrixin A, dolastatin 10, lagunamide A and somocystinamide A were found to induce morphological features of apoptosis in cancer cell lines (Table 29.2). From the compounds biselyngbyaside, cryptophycin 1, lagunamide A and somocystinamide, only one type of morphological change is described, although other apoptotic markers were studied such as activation of caspases, which emphasise their potential as anticancer drugs [13, 116, 120, 75]. From these six compounds, calothrixin A and dolastatin 10 were subjected to a more detailed and comprehensive morphological study, that revealed several typical morphological markers of apoptosis such as cell shrinkage, plasma membrane

Table 29.2 Morphological apoptotic features induced by marine cyanobacteria compounds on cancer cell lines

Compound	Source	Class of compound	Tested cells	Cell effect	Concentration	Reference
Biselyngbyaside	*Lyngbya* sp.	Glicomacrolide	Mouse RAW264 macrophage cells differentiated into osteoclast-like cells	Nuclear shrinkage and fragmentation	100 nM	[128]
Cryptophycin 1	*Nostoc* spp.	Cyclic depsipeptide	Human MDA-MB-435 mammary adenocarcinoma	DNA fragmentation	50 pM	[79]
Calothrixin A	*Calothrix*	Pentacyclic indolophenanthridine	Human Jurkat T cells	Cell shrinkage; chromatin condensation; plasma membrane blebbing	1 μM	[13]
Dolastatin 10	*Symploca* sp.	Linear Pentapeptide	NCI-H69 lung cancer cells	Cell shrinkage; cytoplasmic vacuolization; chromatin condensation; nuclear fragmentation into apoptotic bodies. DNA fragmentation	1.3 nM	[46]
			NCI-H82 lung cancer cells			
			NCI-H446 lung cancer cells			
			NCI-H510 lung cancer cells			
			A549 lung carcinoma		0.97 nM	[11]
Lagunamide A	*Lyngbya majuscula*	Cyclic depsipeptide	HCT8 ileocecal colorectal adenocarcinoma	Cell shrinkage	100 nM	[114]
Somocystinamide A	*Lyngbya majuscula*	Lipopeptide	A-549 lung cancer cells	Plasma membrane blebbing	100 nM	[127]

blebbing, chromatin condensation, DNA fragmentation and nuclear fragmentation into apoptotic bodies [13, 46].

29.3.2 Apoptosis by Caspases Involvement

The family of cysteine aspartic proteases termed caspases is tightly associated with the regulation of apoptosis. Since all caspases exist as inactive precursors, which are activated to generate active enzymes [96], induction of apoptosis is almost always associated with the activation of these proteases, and therefore measurement of its activity is a convenient way to assess whether the cells are undergoing apoptosis. Caspases involved in the execution phase of apoptosis are divided into initiators (mainly 8 and 9) and effectors (3, 6 and 7) depending on the timing of activation. Effector caspases, once activated, mediate the cleavage of cellular proteins and other caspases, in a cascade of reactions that cause typical cellular morphological changes, including nuclear condensation and DNA and cell fragmentation [89]. The activation of caspases, namely caspase 8 as an initiator caspase and caspase 3 as an effector is a commonly studied endpoint to assess the implication of apoptosis in cell cytotoxicity. Several marine cyanobacteria compounds were found to induce apoptosis by activating caspases (Table 29.3). Biselyngbyaside, coibamide A, cryptophycin 1, curacin A and dolastatin 10 were found to induce caspase 3 activation at the nanomolar range [11, 41, 79, 128] and lagunamide A induced caspase 9 activation in an colorectal adenocarcinoma cell line at a concentration of 100 nM [114]. Somocystinamide A is a potent inductor of apoptosis, through a mechanism involving the activation of caspase 8 [4]. This particular aspect of somocystinamide is quite interesting, since numerous malignant tumors maintain expression of caspase 8, thus being an attractive target for tumor suppression [4].

29.3.3 Apoptosis by Alterations in the Bcl-2 Protein Family

The Bcl-2 family includes both pro- and anti-apoptotic proteins being the ratio between these two crucial to determine the susceptibility of cells to a death stimuli [83]. Pro-apoptotic proteins regulate the mitochondrial outer membrane permeabilization resulting in cytosolic release of cytochrome c. In this group are included the Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1 are antiapoptotic proteins and avoid the release of cytocrome c. Cytocrome c in the cytosol is crucial to the following steps of apoptosis due to the formation of the apoptosome that activates caspase 3 [126].

Concerning marine cyanobacteria compounds, dolastatin 10 and symplostatin 1 were found to be able to modulate the apoptotic process of cells (Table 29.4). By reducing the Bcl-2 protein content in cells in a process that may be related to Bcl-2 phosphorylation [46, 117], and increase the pro-apoptotic Bad proteins by inhibiting Bad phosphorylation [11], dolastatin 10 was found to be able to trigger the apoptotic process. Also symplostatin 1 can be considered a potential candidate as an antican-

Table 29.3 Marine cyanobacteria compounds involved in the dynamic of caspases

Compound	Source	Class of compound	Tested cells	Concentration	Effect	Reference
Biselyngbyaside	*Lyngbya* sp.	Glicomacrolide	Mature osteoclasts derived murine RAW264 macrophage cell line	30 nM	Caspase-3 protein activation	[128]
Coibamide A	*Leptolyngbya* sp.	Cyclic depsipeptide	U87-MG and SF-295 glioblastoma	300 nM	Caspase-3,7 activation	[41]
Cryptophycin 1	*Nostoc* spp.	Cyclic depsipeptide	Human MDA-MB-435 mammary adenocarcinoma	50 pM	Caspase-3 protein activation	[79]
Curacin A	*Lyngbya majuscula*	Lipopeptide	Human A549 lung carcinoma	0.58 nM	Caspase-3 protein activation	[11]
Dolastatin 10	*Symploca* sp.	Linear Pentapeptide	Human A549 lung carcinoma	0.97 nM	Caspase-3 protein activation	[11]
Lagunamide A	*Lyngbya majuscula*	Cyclic depsipeptide	HCT8 ileocecal colorectal adenocarcinoma	100 nM	Caspase-9 protein activation	[114]
Somocystinamide A	*Lyngbya majuscula*	Lipopeptide	Jurkat leukemia cancer cells	100 nM	Decrease in Caspase-3 and -9 levels	[127]
				100 nM	Increased in Caspase-8 levels	[127]
Symplostatin 1	*Symploca hydnoides*	Linear Pentapeptide	Human MDA-MB-435 breast carcinoma	1.5 nM	Caspase-3 protein activity increased	[80]

Table 29.4 Marine cyanobacteria compounds involved in the dynamic of Bcl-2 protein family

Compound	Source	Class of compound	Tested cells	Concentration	Cell effect	Reference
Dolastatin 10	*Symploca* sp.	Linear Pentapeptide	Human Reh lymphoblastic leukemia cells	0.13 nM	Reduction Bcl-2 total content protein	[117]
			Human lung cancer cells: NCI-H69 and -H510	1.3 nM	Bcl-2 protein phosphorylation	[46]
			Human A549 lung carcinoma	0.97 nM	Increase Bad protein levels	[11]
				0.97 nM	Bad phosphorylation inhibition	
Symplostatin 1	*Symploca hydnoides*	Linear Pentapeptide	Human MDA-MB-435 breast carcinoma	1.5 nM	Bcl-2 protein phosphorylation	[80]

cer agent due to its ability to increase Bcl-2 phosphorylation as observed with the breast human carcinoma cell line MDA-MB-435 [80].

29.3.4 Other Anticancer Involved Mechanisms

Other mechanisms where marine cyanobacteria compounds were found to act and that are related to cancer, involve de dynamics of microtubules and cell cycle (Table 29.5) and the inhibition of several proteases, other than caspases (Table 29.6).

Microtubules are one of the major cytoskeletal components in eukaryotic cells. This tubulin made structures maintain cell shape, are involved in intracellular transport of vesicles and organelles, and during cell division form the mitotic spindle, crucial for the alignment of chromosomes to the equatorial plane and its subsequent segregation to daughter cells. The role that microtubules play in cell division makes them highly attractive targets for anticancer drugs. In fact, microtubules are among the most successful targets for anticancer therapy [45]. Several anticancer compounds disrupt microtubule function by supressing microtubules dynamics or inducing microtubule depolymerisation, in an action that leads to mitotic arrest and apoptosis [45]. Microtubule-disrupting agents are thought to arrest cells in mitosis by triggering the mitotic checkpoint, in a series of biochemical reactions that ensure proper attachment of chromosomes to the mitotic spindle [1]. Most microtubule-targeted compounds have been discovered in large-scale screens of natural products. Considering marine cyanobacteria, several compounds were found to induce the disruption of microtubules and cell cycle arrest namely in the G2/M phase (Table 29.5) such as

Table 29.5 Marine cyanobacteria compounds involved in the dynamics of microtubules and cell cycle arrest

Compound	Source	Class of compound	Tested cells	Effect on cell cycle	Concentration	Reference
Apratoxin A	*Lyngbya majuscula*	Cyclic depsipeptide	Human HeLa cervical carcinoma	G_1 phase cell cycle arrest	50 nM	[62]
Belamide A	*Symploca* sp.	Linear peptide	Rat aorta A-10 cells	Microtubule disruption	20 μM	[97]
Calothrixin A	*Calothrix*	Pentacyclic indolophenanthridine	Human leukemia CEM cells	G_2/M and S phases cell cycle arrest	1 μM	[48]
			Human cancer Jurkat cells	G_2/M phase cell cycle arrest	1 μM	[13]
Calothrixin B	*Calothrix*	Pentacyclic indolophenanthridine	Human leukemia CEM cells	G_1 phase cell cycle arrest	0.1 μM	[48]
Coibamide A	*Leptolyngbya* sp.	Cyclic depsipeptide	Human NCI-H460 lung cancer	G_1 phase cell cycle arrest	25 nM	[74]
Cryptophycin 1	*Nostoc* spp.	Cyclic depsipeptide	Human MDA-MB-435 mammary adenocarcinoma	G_2/M phase cell cycle arrest	50 pM	[79]
Curacin A	*Lyngbya majuscula*	Lipopeptide	Chinese hamster cells	G_2/M phase cell cycle arrest	100 ng/mL	[26]
Dolastatin 10	*Symploca* sp.	Linear peptide	Human A549 lung carcinoma	G_2/M phase cell cycle arrest	Non-specified	[11]
			NCI-H69 lung cancer	G_2/M phase cell cycle arrest	1.3 nM	[46]
			NCI-H82 lung cancer	G_2/M phase cell cycle arrest	1.3 nM	
			NCI-H446 lung cancer	G_2/M phase cell cycle arrest	1.3 nM	
			NCI-H510 lung cancer	G_2/M phase cell cycle arrest	1.3 nM	
			DU-145 prostate cancer	G_2/M phase cell cycle arrest	10.0 nM	[116]
			Four different human lymphoma cell lines	G_2/M phase cell cycle arrest	1.27 nM	[6]
			Rat aorta A-10 cell line	Microtubule depolymerization	0.75 nM	[56]

Table 29.5 (continued)

Compound	Source	Class of compound	Tested cells	Effect on cell cycle	Concentration	Reference
Hectochlorin	*Lyngbya majuscula*	Lipopeptide	CA46 Burkitt lymphoma cell line	G_2/M phase cell cycle arrest	0.2 μM	[65]
Symplostatin 1	*Symploca hydnoides*	Linear peptide	Human MDA-MB-435 mammary adenocarcinoma cell line	G_2/M phase cell cycle arrest	1.5 nM	[80]
			Purified bovine brain tubulin	Extent of tubulin assembly inhibition	2–10 μM	
			Rat aorta A-10 cell line	Formation of abnormal spindles	1.0 nM	
			Rat aorta A-10 cell line	Microtubule depolymerization	2.5 nM	[56]
Symplostatin 3	*Symploca* sp.	Linear peptide	Rat aorta A-10 cell line	Microtubule depolymerization	1.34 μM	[61]

Table 29.6 Marine cyanobacteria compounds involved in the dynamic of proteases other than caspases

Compound	Source	Class of compound	Model tested	IC50	Reference
Bisebromoamide	*Lyngbya* sp.	Peptide	ERK (extracellular signal regulated protein kinase) in NRK rat kidney epithelial cell	0.1–10 μM	[109]
Bouillomide A	*Lyngbya bouillonii*	Depsipeptide	Elastase	1.9 μM	[90]
			Chymotrypsin	0.17 μM	
Bouillomide B	*Lyngbya bouillonii*	Depsipeptide	Elastase	1.9 μM	[90]
			Chymotrypsin	9.3 μM	
Grassystatin A	*Lyngbya confervoides*	Linear depsipeptide	Cathepsin D	26.5 nM	[51]
			Cathepsin E	886 pM	
Grassystatin B	*Lyngbya confervoides*	Linear depsipeptide	Cathepsin D	7.27 nM	[51]
			Cathepsin E	354 pM	
Kempopeptin A	*Lyngbya* sp.	Cyclic depsipeptide	Bovine pancreatic α-chymotrypsin	2.6 μM	[107]
			Porcine pancreatic elastase	0.32 μM	
Kempopeptin B	*Lyngbya* sp.	Cyclic depsipeptide	Trypsin	8.4 μM	[107]
Largamide A	*Lyngbya confervoides*	Cyclic depsipeptide	Porcine pancreatic elastase	1.41 μM	[71]
Largamide B	*Lyngbya confervoides*	Cyclic depsipeptide	Porcine pancreatic elastase	0.53 μM	[71]
Largamide C	*Lyngbya confervoides*	Cyclic depsipeptide	Porcine pancreatic elastase	1.15 μM	[71]
Largamide D	*Oscillatoria* sp.	Cyclic depsipeptides	α-chymotrypsin	10 μM	[88]
Largamide E	*Oscillatoria* sp.	Cyclic depsipeptides	α-chymotrypsin	10 μM	[88]
Largamide F	*Oscillatoria* sp.	Cyclic depsipeptides	α-chymotrypsin	4.0 μM	[88]
Largamide G	*Oscillatoria* sp.	Cyclic depsipeptides	α-chymotrypsin	25.0 μM	[88]
Lyngbyastatin 4	*Lyngbya confervoides*	Cyclic depsipeptide	Bovine pancreatic α-chymotrypsin	0.30 μM	[68]
			Porcine pancreatic elastase	0.03 μM	
Lyngbyastatin 5	*Lyngbya* spp.	Cyclic depsipeptide	Porcine pancreatic elastase	3.2 nM	[106]
			Bovine pancreas α-chymotrypsin	2.8 μM	

Table 29.6 (continued)

Compound	Source	Class of compound	Model tested	IC50	Reference
Lyngbyastatin 6	*Lyngbya* spp.	Cyclic depsipeptide	Porcine pancreatic elastase	3.3 nM	[106]
			Bovine pancreas α-chymotrypsin	2.5 μM	
Lyngbyastatin 7	*Lyngbya* spp.	Cyclic depsipeptide	Porcine pancreatic elastase	8.3 nM	[106]
			Bovine pancreas α-chymotrypsin	2.5 μM	
Lyngbyastatin 8	*Lyngbya semiplena*	Cyclic depsipeptide	Porcine pancreatic elastase	123 nM	[52]
Lyngbyastatin 9	*Lyngbya semiplena*	Cyclic depsipeptide	Porcine pancreatic elastase	210 nM	[52]
Lyngbyastatin 10	*Lyngbya semiplena*	Cyclic depsipeptide	Porcine pancreatic elastase	120 nM	[52]
Molassamide	*Dichothrix utahensis*	Depsipeptide	Bovine pancreatic α-chymotrypsin	0.032 μM	[33]
			Porcine pancreatic elastase	0.234 μM	
Pompanopeptin A	*Lyngbya confervoides*	Cyclic peptide	Porcine pancreatic trypsin	2.4 μM	[70]
Somamide B	*Lyngbya majuscula* and *Schizothrix* sp. assemblage	Cyclic depsipeptide	Porcine pancreatic elastase	9.5 nM	[106]
			Bovine pancreas α-chymotrypsin	4.2 μM	
Symplocamide A	*Symploca* sp.	Cyclic peptide	Chymotrypsin	0.38 μM	[54]
Tiglicamide A	*Lyngbya confervoides*	Cyclic depsipeptide	Porcine pancreatic elastase	2.14 μM	[72]
Tiglicamides B	*Lyngbya confervoides*	Cyclic depsipeptide	Porcine pancreatic elastase	6.99 μM	[72]
Tiglicamides C	*Lyngbya confervoides*	Cyclic depsipeptide	Porcine pancreatic elastase	7.28 μM	[72]

the linear peptide belamide A, isolated from the marine cyanobacteria *Symploca* that was found to induce microtubule disruption in rat aorta A-10 cells, displaying classic tubulin destabilizing antimitotic characteristics [97]. In the same cell line, also dolastatin 10, symplostatin 1 and symplostatin 3 were found to induce microtubule depolymerisation [56, 97], making these attractive compounds for cancer therapies.

Proteases are enzymes, which in addition to hydrolysis of peptide bonds of proteins are also considered important signaling molecules in processes such as blood coagulation, cell cycle and neurodegenerative disorders [115]. Examples of proteases, other than caspases, which dynamics was found to be disturbed by marine cyanobacteria compounds, are elastase, chymotrypsin, trypsin and catherpsin (Table 29.6). Elastase is a protolythic enzyme that disintegrates matrix proteins such as elastin [5]. Although some evidences have linked elastases to apoptosis [102], the main aims concerning these proteases are related to its role in facilitating cell dissemination, invasion and metastasis, since elastase catalyse the disruption of the elastic fibers barrier of tissues [81]. Several marine cyanobacteria compounds were found to inhibit elastases (Table 29.6) such as the depsipepides bouillomides A and B [90] and molassamide [33] and the cyclic depsipeptides largamides A-C [71], lyngbyastatins 4-10 [68, 106], somamide B [106] and tiglicamide A-C [72]. Trypsin and chymotrypsin have been found in a variety of tumours, such as ovarian and colorectal carcinomas [101]. Such as elastase, these enzymes appear to be involved in cancer cells invasion and metastasis, through the disruption of proteins in tissues matrixes. Several marine cyanobacteria compounds were also found to act as trypsin and chymotrypsin protease inhibitors such as the depsipeptides bouillomides A-B [51, 90], the cyclic depsipeptides kempopeptins A-B [107] and also the cyclic depsipeptides largamides [88]. Protease inhibitors are ubiquitous in nature and were found to inhibit tumor invasion and metastasis [49]. By acting as protease inhibitors, marine cyanobacteria compounds my have an interesting role in preventing invasion and metastasis of cancer.

Cathepsins are noncaspase proteases that have been most closely linked with apoptosis. Cathepsins are lisossomal proteases that in response to certain signals are released from the lysosomes into the cytoplasm. Once in the cytoplasm these proteases were found to trigger apoptotic cell death via various pathways, including the activation of caspases or the release of proapoptotic factors from the mitochondria [20, 104]. Concerning marine cyanobacteria compounds grassystatins A and B, two linear depsipeptides isolated from *Lyngbya confervoides* were found to inhibit cathepsins D and E [51]. Such involvement in protease inhibition may be used as powerful tools to probe catherpsin function, in this case, or proteases in general, leading to new insights into their potential in cancer therapies.

29.4 Conclusions and Future Remarks

Marine cyanobacteria are undoubtedly a rich source of potential anticancer drugs. These organisms have shown us an arsenal of compounds that may act at different levels of the metabolic pathways involved in the apoptotic cell death, being thus

valuable tools for cancer therapies. However, for a great majority of compounds, only the cytotoxic potential, revealed by standard cytotoxicity assays, was evaluated. In this sense, future challenges in marine cyanobacterial compounds as anticancer drugs lie in increasing the understanding of their basic cytotoxic mechanisms on cells. Furthermore, much of the cytotoxicity screenings of marine cyanobacteria compounds were performed in cancer cell lines. Because the potential utility of the compounds as anticancer drugs depends on the degree of selective toxicity on tumor cells sparing normal cells, evaluation of cytotoxicity should also be extended to normal cells.

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Chapter 30
Cytotoxic Cembrane Diterpenoids

Bin Yang, Juan Liu, Junfeng Wang, Shengrong Liao and Yonghong Liu

Abstract Cembrane-type diterpenoids are a large and structurally varied group of natural products isolated from both terrestrial and marine organisms. From a biomedical perspective, cytotoxicity is the most remarkable property of this class of diterpenoids, respresented by sarcophytol A. The present paper reviews all the cytotoxic metabolites of cembrane diterpenes, reported up to 2013. The natural products discussed in this review can be divided into several different structural families featuring a variety of ring sizes and oxidation patterns. The currently hypothetical biosynthesis and total syntheses of some representative cembrane diterpenoids will be presented as well.

Keywords Cembrane-type diterpenoids · Cytotoxicity · Biosynthesis · Total syntheses

30.1 Introduction

Cancer is a group of diseases that can affect various organs of the body, and is characterized by the uncontrolled growth of abnormal cells and invasion into normal tissue. Cancer cells can also spread to other parts of the body and produce new tumors. If the spread of cells becomes uncontrolled, it can lead to death. In this way, the development of new, more potent and selective cytotoxic drugs is needed. Secondary metabolites (natural products) have played an important role in the discovery and development of medicinal agents [1, 2]. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural/chemical features not found in terrestrial natural products. Marine organisms have evolved biochemical and physiological mechanisms that include the production of anticancer compounds [3–5].

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Cembrane-type diterpenoids are a large and structurally varied group of natural products isolated from both terrestrial and marine organisms [6]. Cyclisation of a geranylgeraniol derived precursor between carbon 1 and 14 generate a 14-membered diterpenoid, named cembrane or thumbergane. In 1962, the first cembranoid diterpene, (+)-cembrene (**1**), was reported from pine oleoresin [7]. In 1979, Kobayashi and his co-workers reported a new cembranoid diterpene, sarcophytol A (**2**), from the Okinawan soft coral *Sarcophyton glaucum* [8]. It has attracted the most attention because of its strong inhibitory activity against tumor promoter. This was followed by the isolation of hundreds of cytotoxic cembranoids from plants and insects as well as of marine origin [6, 9–17]. In the marine environment, coelenterates of the orders Alcyonacea and Gorgonacea are recognized as the most prominent source of cembranoids, which usually exhibit cyclic ether, lactone, or furane moieties around the cembrane framework [18–20]. Moreover, diterpenoids of the cembrane family have been shown to play an ecological role in the chemical defense against predator or competing reef organisms. From a biomedical perspective, cytotoxicity is the most remarkable property of this class of diterpenoids [21]. They also have been reported to possess multiple biological activities such as neuroprotective, anti-inflammatory, antiarthritic, calcium-antagonistic, and antimicrobial effects.

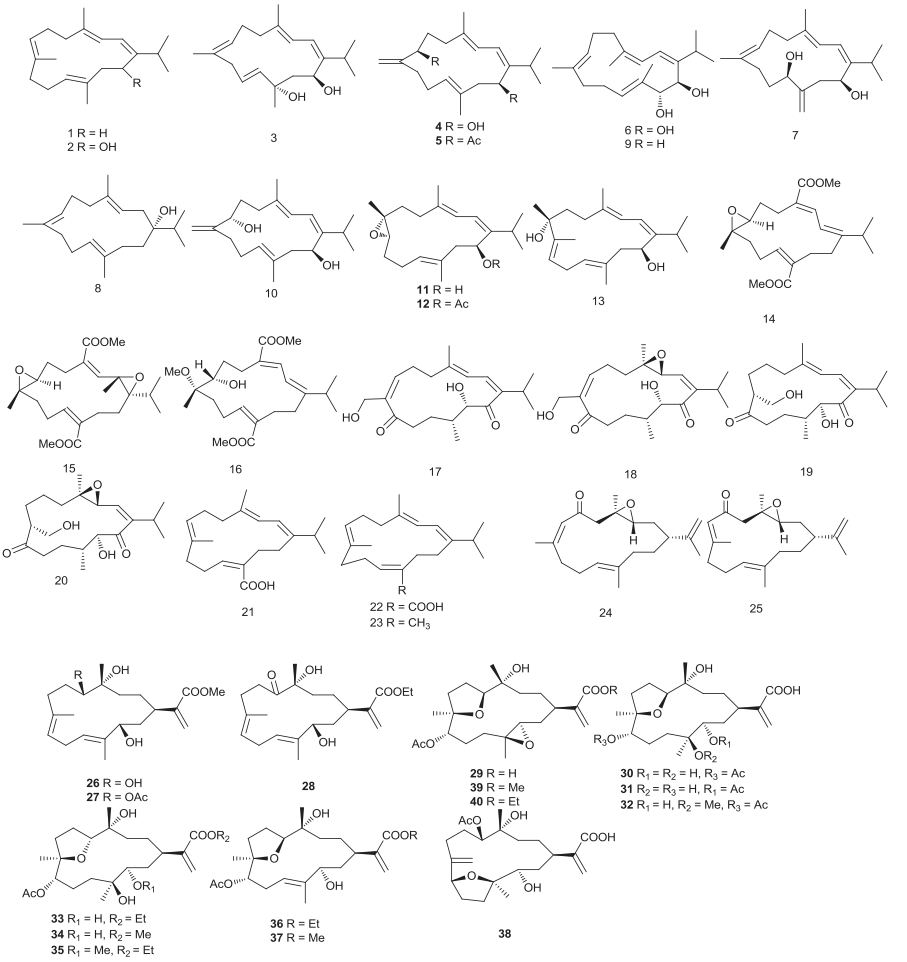
30.2 Classification of Cembrane Type Diterpenoids

30.2.1 Simple Cembrene

Soft corals have proven to be a rich source of a variety of diterpenes, of which cembranoids that exhibit a range of biological activities are the most commonly encountered ones [22]. *Sarcophyton* is among the most abundant soft coral genera on many coral reefs, and it tends to form large monospecific “carpets” of up to several square meters [23]. *Sarcophyton* is one of the more studied organisms.

Sarcophytol A (**2**) is a cembranoid isolated in 1979 from the Okinawan soft coral *Sarcophyton glaucum* [8]. It has been reported to have antitumor activity and also potent inhibitory activities against the various classes of tumor promoters. Moreover, it was demonstrated that **2** in diet inhibited the spontaneous tumor development in organs such as the mamma, liver, and thymus, besides inhibiting chemical carcinogenesis in the colon. In those experiments **2** did not show any toxic effect on animals [24–31]. Recently, inhibition of TNF-α release from cells was proposed as a mechanism of the anticarcinogenesis of **2**. Sarcophytol A has potential as a new cancer chemopreventive agent. A series of analogues, sarcophytol G (**3**), sarcophytol H (**4**) and its diacetate (**5**), sarcophytol J (**6**), sarcophytol I (**7**), sarcophytol M (**8**), sarcophytol N (**9**), and sarcophytol O (**10**) were also reported by Kobayashi and his coworkers from the soft coral *Sarcophyton glaucum* [32]. Three similar cembranoid diterpenes, namely (7*R*, 8*R*, 14*S*, 1*E*,3*E*,11*E*)-7,8-epoxycembra-1,3,11-trien-(14)-ol (**11**), (7*R*,8*R*,14*S*,1*E*,3*E*,11*E*)-14-acetoxy-7,8-epoxycembra-

1,3,11-triene (**12**) and (7*R*,14*S*,1*E*,3*E*,8*E*,11*E*)-cembra-1,3,8,11-tetraene-7,14-diol (**13**), were isolated from the soft coral *Sarcophyton trocheliophorum*. Both **11** and **12** showed similar cytotoxic profiles with IC_{50} values of 2.5 μg/mL against P388 (murine leukaemia), 5 μg/mL against A549 (human lung carcinoma), 10 μg/mL against HT29 (human colon carcinoma) and 10 μg/mL against MEL28 (human melanoma) cell lines in culture. Sarcrassins A–C (**14**–**16**) have been isolated from the soft coral *Sarcophyton crassocaule* collected from the Bay of Sanya, Hainan Island, China. Compounds **14**, and **16** exhibited cytotoxic activities against KB cell lines with IC_{50} values of 19.0, and 5.0 μg/mL, respectively [33].



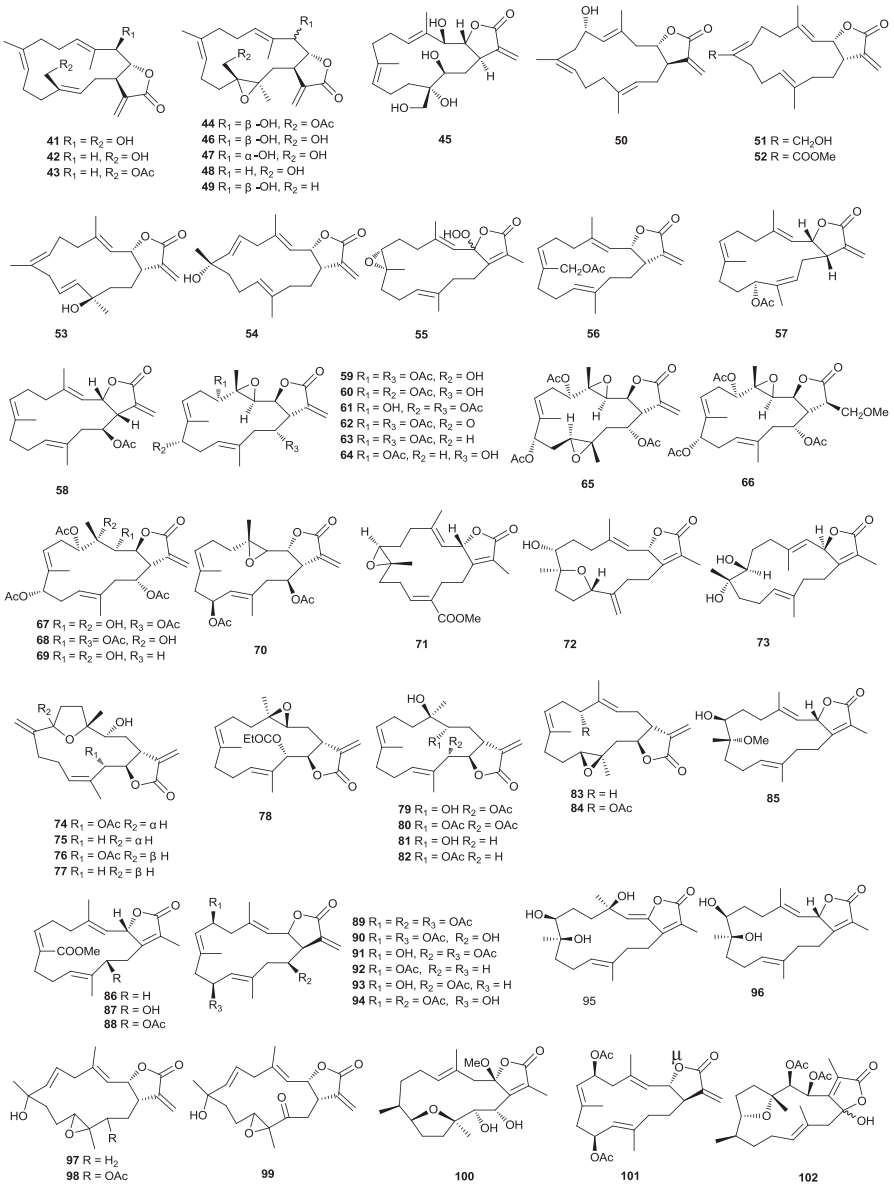
Flabellatenes A (**17**), and B (**18**) have been isolated from the extract of the Antarctic sponge *Lissodendoryx flabellata.* Compound **17** was cytotoxic on mouse neuroblastoma cells (N18-T62) at low concentrations (0.16 μM), whereas significantly reduced cell proliferation of human tumoral cells (DU-145 and MCF-7) in a

dose-depending manner [34]. Two new cembrane-type diterpenoids have been isolated from the 2-propanol extract of the sea pen *Gyrophyllum sibogae* collected in South Africa: 7,8-dihydroflabellatene A (**19**) and 7,8-dihydroflabellatene B (**20**). Compound **19** showed the activity against all of the lines tested. GI_{50} values (μM) for some of the lines evaluated are: DU-145 (82.0), IGROV (90.3), K-562 (62.7), and HT-29 (14.5) [35]. Three new cembranoids, crotocembraneic acid (**21**) and neocrotocembraneic acid (**22**), neocrotocembranal (**23**), were isolated from the stem bark of *Croton oblongifolius*. Compound **23** inhibited platelet aggregation induced by thrombin, with an IC_{50} value of 47.21 μg/mL, and exhibited cytotoxicity against P-388 cells in vitro, with an IC_{50} value of 6.48 μg/mL [36, 37].

The new cembrane diterpenes leptodienones A (**24**) and B (**25**) have been isolated from the gorgonian *Leptogorgia laxa* collected in the Gulf of California. Compound **24** showed growth inhibitory activity of MDA-MB-231 and HT-29 cells with GI_{50} values of 16.2 and 14.9 μM, respectively. Compound **25** was more potent and inhibited the growth of the three cell lines with GI_{50} values of 6.3, 5.6, and 10.9 μM against MDA-MB-231, A-549, and HT-29, respectively [38]. A systematic examination of the Chinese soft coral *Dendronephthya* sp. resulted in the isolation and characterization of 15 new cembranoid diterpenes, namely, dendronpholides C–F, I–P, R(**26**–**38**), sinulaflexiolides E, and F(**39**, **40**). Dendronpholides C(**26**), and J(**31**) showed a selective and significant inhibition against BGC-823 (IC_{50}: 0.05, and 0.20 μg/mL, respectively) [39, 40].

30.2.2 Cembranolides

Cembranolides are a class of diterpenes containing a 14-membered carbocyclic nucleus, commonly fused to a five-, six-, or seven-membered lactone ring. There is a wide range of structural complexity within the series [41]. A large number of highly functionalized cembranoid diterpenes, containing an α-methylidene-γ-lactone moiety, have been isolated and identified from marine soft corals, especially from the genera *Lobophytum*, *Sarcophyton*, and *Sinularia*. All of which belong to the family Alcyoniidae within the order of Alcyonacea. Four new cembranoids, presinularolide B (**41**), 3-dehydroxylpresinularolide B (**42**), 3-dehydroxyl-20-acetylpresinularolide B (**43**), and 20-acetylsinularolide B (**44**), together with five analogues, sinularolides B–E (**46**–**49**) were isolated from a South China Sea soft coral *Lobophytum crassum*. Sinularolides A–E (**45**–**49**) were firstly isolated from the soft coral *Sinularia gibberosa*. 3-Dehydroxylpresinularolide B (**42**) showed significant cytotoxicity against both A-549 and P-388 cell lines with 95.7 and 100% inhibition at 10 μM, Moderate antitumor activities were observed for presinularolide B (**41**) against A-549 cells and for sinularolide C (**46**) against P-388 cells with 64.0 and 63.8% inhibition at a concentration of 10 μM, respectively [42–44]. The soft coral *Lobophytum crassum* continues to provide novel cembranoids with γ-lactone, including crassumolides A–D, F (**50**–**54**) [45], 2-hydroperoxysarcophine (**55**) [46], lobocrassolide (**56**) [47], and two unnamed diterpenes (**57** and **58**) [48]. Crassumolide C (**52**) was isolated for the first time from a natural source. Compounds **50**, and **52** were cytotoxic toward Ca9–22 cancer cells, moreover, they were found to inhibit the accumulation of the pro-inflammatory proteins iNOS and COX-2 at 10 μM [45]. Eleven new cytotoxic cembranolides,

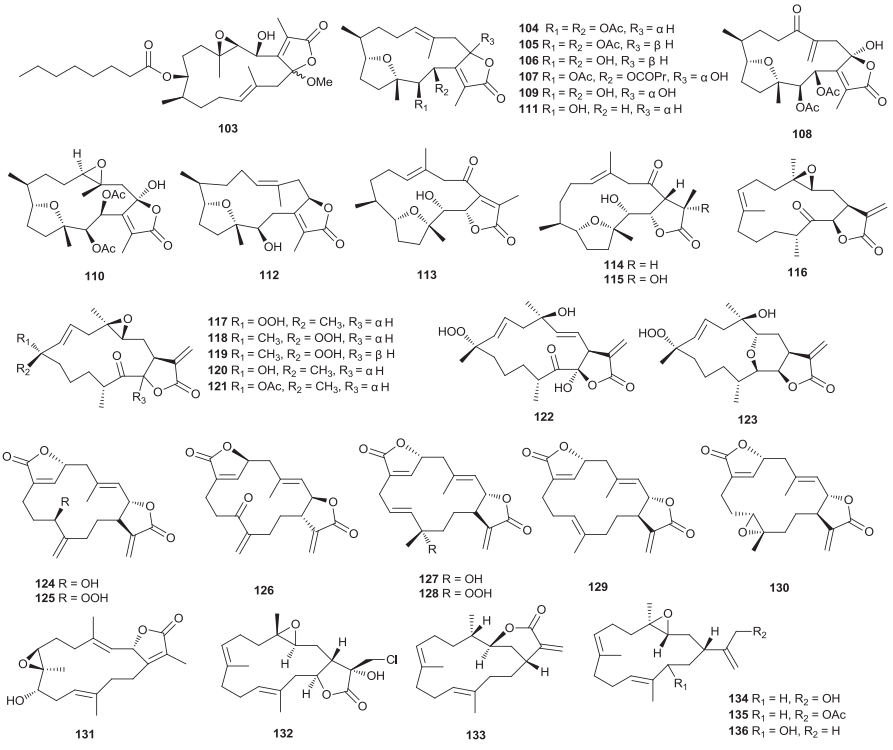


michaolides A–K (**59**–**69**) were isolated from the CH_2Cl_2 extract of the Formosan soft coral *Lobophytum michaelae* [49]. Bioactivity-guided fractionation of a $CHCl_3$ extract of the soft coral *Lobophytum micbaeh* afforded a new cytotoxic cembranolide, lobomichaolide (**70**) [50].

The soft coral of genus *Sarcophyton* are rich sources of cembranoid diterpenes, possessing an α-methylidene-γ-lactone moiety, including (+)-12-methoxycarbonyl -11*Z*-sarcophine (**71**) [51], trocheliophorol (**72**) [52], (7*R*,8*S*)-dihydroxydeepoxy-ent-sarcophine (**73**) [53], sarcocrassocolides A–E (**74**–**78**) [54], crassocolides A–F (**79**–**84**) [55], (−)-7β-hydroxy-8α-methoxydeepoxysarcophine (**85**) [56], 13-dehy-

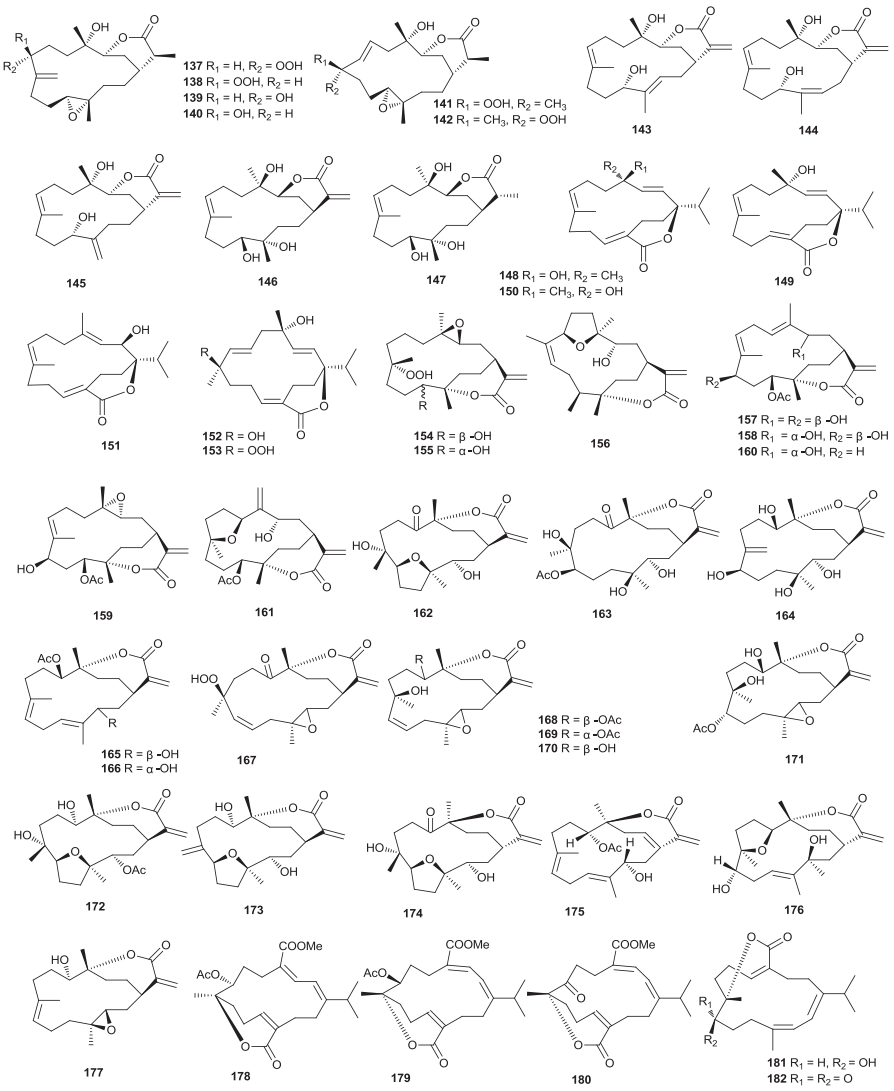
droxysarcoglaucol-16-one, sarcoglaucol-16-one (**86**, **87**) [57], 16- oxosarcoglaucol acetate (**88**) [23]. Crassocolide A (**77**) was cytotoxic against the four cancer cell lines, being significant (IC_{50} 3.1 μg/mL) to moderate (IC_{50} 8.6–11.9 μg/mL) against Hep G2, and MCF-7, MDA-MB-231, and A549, respectively [55].

Six new marine diterpenoids (**89**–**94**) were isolated from the Okinawan soft coral *Clavularia koellikeri*. Compound **89** showed cytotoxic activity against human colorectal adenocarcinoma cells (DLD-1, IC_{50} 4.2 μg/mL) and strong growth inhibition against human T lymphocytic leukemia cells (MOLT-4, IC_{50} 0.9 μg/mL) [58]. Some other analogues were isolated from *Sinularia polydactyla* (**95**, **96**) [56], *Sinularia mayi* (**97**–**99**) [59], *Briareum violacea* (**100**) [60], *Clavularia violacea* (**101**) [61]. Pachyclavulariolides E–R (**102**–**115**) have been isolated from specimens of *Pachyclavularia violacea* collected in Papua New Guinea [62–64]. Among these compounds, pachyclavulariolide F (**103**) showed in vitro cytotoxicity against murine leukemia P388 with an IC_{50} 1.0 μg/mL [62]. Compound **108** exhibited significant cytotoxicity against P-388 and HT-29 tumor cells with ED_{50}'s of 2.8 and 3.3 μg/mL, and moderate cytotoxicity against KB and A-549 tumor cell with ED_{50}'s of 7.6 and 6.7 μg/mL, respectively. Metabolites **106**, and **107** also were found to exhibit significant cytotoxicity against P-388 cells with ED_{50}'s of 1.3, and 2.5 μg/mL, respectively [63]. Compound **111** has been shown to exhibit significant cytotoxicity toward P-388 cancer cells (ED_{50} 3.2 μg/mL) [64].



The first chemical investigation of the gorgonian octocoral *Eunicea pinta*, lead to the isolation and structure determination of eight new cembranolides, including 12-epieupalmerone (**116**) and uprolides H–M (**117**–**123**). Compound **116** showed strong growth inhibition against non-small cell lung cancer cells (NCI-H322M, IC_{50} 0.90 μg/mL) and renal cancer cells (TK-10, IC_{50} 0.13 μg/mL), whereas compound **117** displayed strong growth inhibition against human T lymphocytic leukemia cells (MOLT-4, IC_{50} 0.01 μg/mL; SR, IC_{50} 0.07 μg/mL) [65]. Bioassay-directed fractionation led to the isolation of five new cembrane-type diterpenoids (**124**–**128**), along with two known compounds, ovatodiolide (**129**) and 4,5-epoxovatodiolide (**130**) from a methanol extract of *Anisomeles indica* [66–68]. Compound 129 exhibited moderate cytotoxicity against all of the lung (A-549), breast (MDA-MB-231 and MCF-7), and liver (Hep G2 and Hep 3B) cancer cell lines. Compounds **127** and **129** exhibited selective activities toward collagen with IC_{50} values of 41.9 ± 7.1 and 19.7 ± 6.7 μM, respectively. In contrast to **124** and **129**, compounds **127**, **128**, and **130** showed selective activities toward thrombin with IC_{50} values of 20.0 ± 6.2, 11.9 ± 5.3, and 4.8 ± 0.4 μM, respectively. Sarcophine (**131**), isolated in good yield from the Red Sea soft coral *Sarcophyton glaucum*, was found to serve as an effective inhibitor of JB6 cell transformation. Five new cembrane-type diterpenoids, lobocrassins A–E (**132**–**136**), were isolated from the soft coral *Lobophytum crassum*. Lobocrassin A (**132**) is the first cembranoid possessing an α-chloromethyl-α-hydroxy-γ-lactone functionality and is the first chlorinated cembranoid from soft corals belonging to the genus *Lobophytum*. Lobocrassins B (**133**) and C (**134**) were found to be the stereoisomers of the known cembranes, 14-deoxycrassin and pseudoplexaurol, respectively. Lobocrassin B (**133**) exhibited modest cytotoxicity toward K562, CCRF-CEM, Molt4, and HepG2 tumor cells (IC_{50}: 2.97, 0.48, 0.34, 3.44 μg/mL) and displayed significant inhibitory effects on the generation of superoxide anion and the release of elastase by human neutrophils [69, 70].

A large number of cembrane-type diterpenoids possessing a *δ*-lactone ring were isolated from the soft coral. Manaarenolides A–I (**137**–**145**) have been isolated from the ethyl acetate extract of the Taiwanese soft coral *Sinularia manaarensis*. Among these metabolites, diterpenes (**137**, **138**, **139**, and **140**) were discovered for the first time as the hydroperoxycembranolides, and compounds **143** and **144** exhibited moderate cytotoxicities against the tested cell lines (ED_{50}'s 7.2, 8.7, 10.9, and 13.4 μg/mL for **143**, and 7.4, 7.6, 9.3, and 5.8 μg/mL for **144**, against the growth of Hepa59 T/VGH, KB, Hela, and Med cells, respectively) [71]. Two new cytotoxic cembranoid diterpenes, sinuflexolide (**146**), dihydrosinuflexolide (**147**), have been isolated from the soft coral *Sinularia flexibilis*. Compound **146** exhibited cytotoxicity toward the growth of A549, HT-29, KB, and P-388 cells, and compound **147** exhibited cytotoxicity toward the growth of P-388 cells [72]. Six new cembranoids, laevigatlactones A–F (**148**–**153**) were isolated from leaves of *Croton laevigatus*. Among these compounds, the hydroxy of C-1 and the carboxy of C-12 are connected to form a six-membered lactone. Compound **149** exhibited modest cytotoxicity against HeLa cells, with an IC_{50} value of 38.4 μM [73].



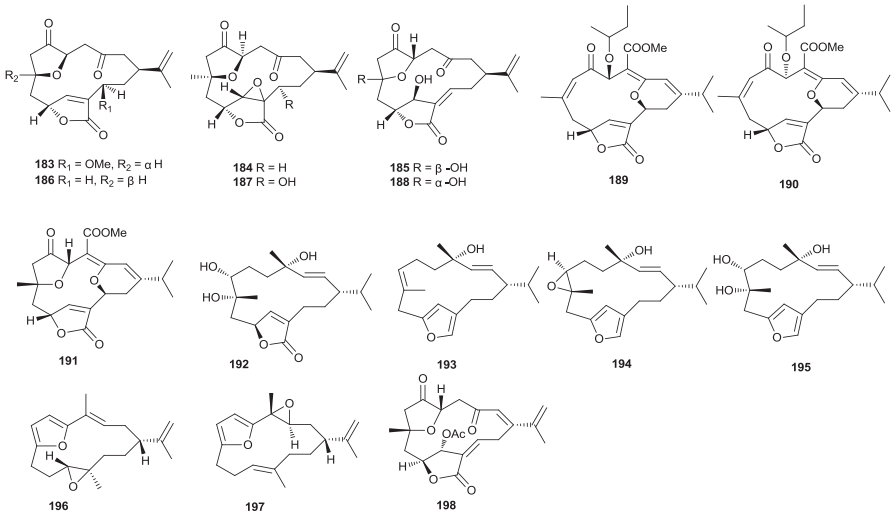
An abundance cembrane diterpenes of containing a *ε*-lactones ring were identified from the soft coral especially from the genus *Sinularia*. Chromatographic investigation of the octocoral *Sinularia flexibilis* afforded eight new diterpenes, sinuladiterpenes A–H (**154**–**161**), Compound **155** exhibited in vitro cytotoxic activity against human colon adenocarcinoma (WiDr) cell line with ED_{50} 8.37 μg/mL [74, 75]. Moreover, a series of analogues were also isolated from *Sinularia flexibilis*, including sinulaflexiolides B–D, J, and K (**162**–**166**) [41], flexilarins D–J (**167**–**173**) [76], sinulariolone (**174**) [77], sandensolide monoacetate (**175**) and flexibolide (**176**) [78], and sinulariolide (**177**) [79]. Sinulaflexiolides D (**164**) showed selective inhibitory activity against the gastric gland carcinoma cell line BGC-823 at 8.5 μM. Four

new cembrane diterpenes (**178**–**182**), which contain an intriguing seven-membered lactone moiety were isolated from the genus *Sarcophyton*. Sarcrassins D (**179**), and E (**180**) exhibited cytotoxic activities against KB cell lines with IC_{50} values of 13.0, and 4.0 μg/mL, respectively [33, 80, 81].

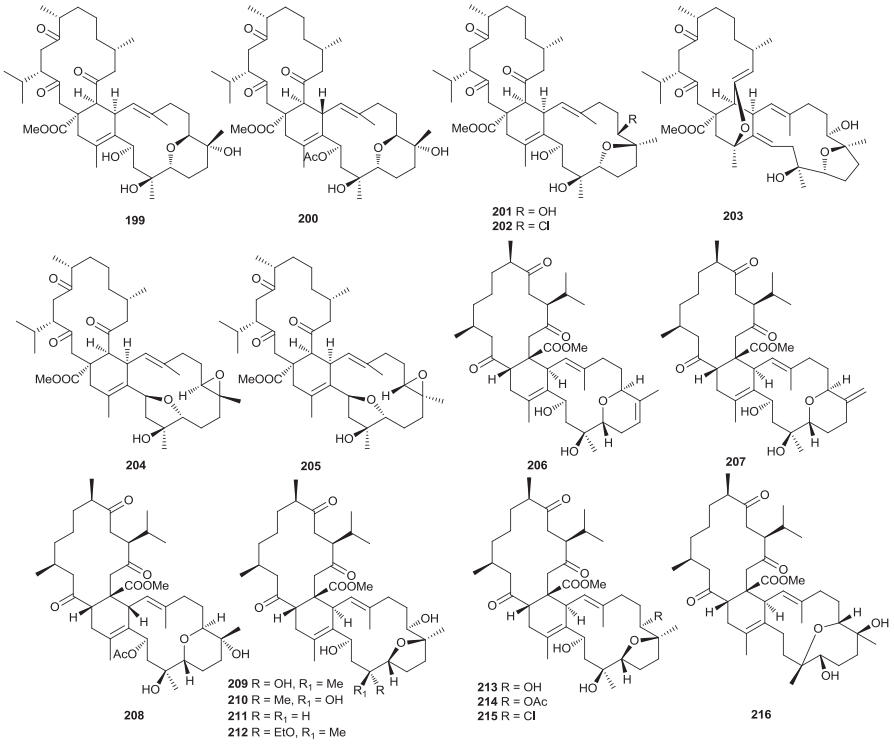
30.2.3 Furanocembranoids

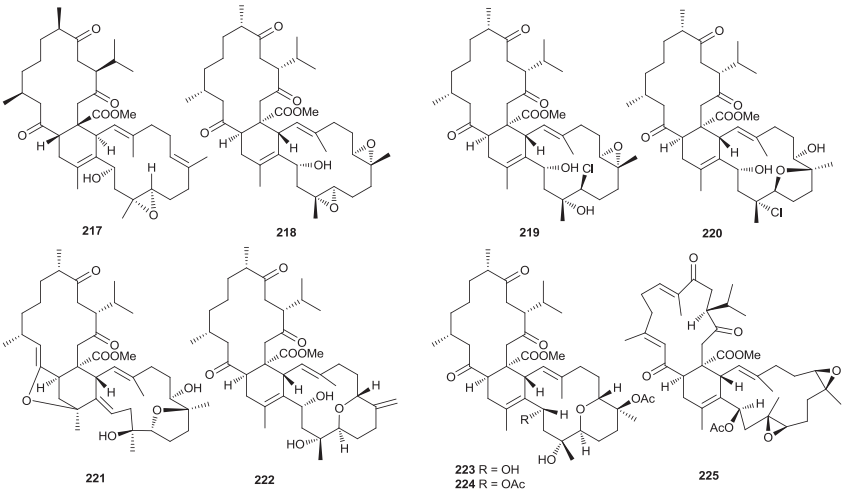
Furanocembranoids feature a canonical cembrane skeleton and a 14-membered carbocyclic ring as well as a furan heterocycle. In addition, these natural products typically feature a butenolide moiety encompassing C10–C12, as well as C20, which is why they have been sometimes referred to as "furanocembranolides" in the older literature [82]. Investigation of the chemical constituents of a Taiwanese soft coral *Sinularia scabra* has afforded five new norditerpenoids, scabrolides C–G (**183**–**186**), which were found to be analogues with those of sinuleptolide (**187**) and 5-*epi*-sinuleptolide (**188**) also isolated previously from the same species and other *Sinularia*. Metabolite **184** was found to exhibit significant cytotoxicity against the growth of Hepa59T/VGH and KB cell lines (ED_{50}'s 0.5 and 0.7 μg/mL, respectively) [83–85]. Chemical investigation of the hybrid soft coral *Sinularia maxima* × *Sinularia polydactyla* yielded three new cembranolide diterpenes (**189**–**191**). Compound **191** shows strong cytotoxicity on the breast cancer SK-BR3 cell line and cervical cancer HeLa and HeLa-Apl cell lines with GI_{50} values of 0.039, 0.48, and 0.56 μM, respectively [86]. A novel cembranoid (**192**) was isolated from the stem bark of *Croton oblongifolius* and it showed broad cytotoxic activity against five further cell lines (BT474, CHAGO, Hep-G2, KATO-3, and SW-620) [87].

Three novel furanocembranoids (**193**–**195**) were isolated from the stem bark of *Croton oblongifolius*. Compounds **193**, and **195** showed broad cytotoxic activity against five further cell lines (BT474, CHAGO, Hep-G2, KATO-3, and SW-620) [87].



Two new furanocembranoid diterpenes, crematofuran (**196**) and isocrematofuran (**197**), have been isolated from the Dufour gland secretion of the Brazilian ant *Crematogaster brevispinosa rochai*. The toxicity of **196** towards other ants is on the same level as that of nicotine [88]. 5-Episinuleptolide acetate (**198**), a cytotoxic norcembranoidal diterpene recently identified from the Formosan soft coral *Sinularia sp*., exhibited potent activity against the K562, Molt 4 cells, HCT-116, DLD-1 cells, t-47D, MDA-MB- 231 cells K562, Molt 4 and HL 60 cancer cell lines, with IC_{50} values of 0.67, 0.59, 4.09, 0.92, 3.09, 2.95, 4.09, 3.21 and 2.53 μg/mL, respectively. The antiproliferative assay, as well as the annexin V-FITC/propidium iodide (PI) apoptotic assay, indicated that the HL 60 cell line is the most sensitive one towards 5-episinuleptolide acetate. This diterpenoid led to caspases-3, -8, and -9 activation as well as PARP cleavage. It also induced ROS generation, calcium accumulation and disruption of mitochondrial membrane potential. Additionally, the expression levels of Hsp90 protein and several client proteins were downregulated in response to 5EPA treatment. These results suggest that 5-episinuleptolide acetate's cytotoxic effect on HL 60 cells may be attributed to the inhibition of Hsp90 as well as the induction of mitochondrial stress which finally results in apoptotic cell death.



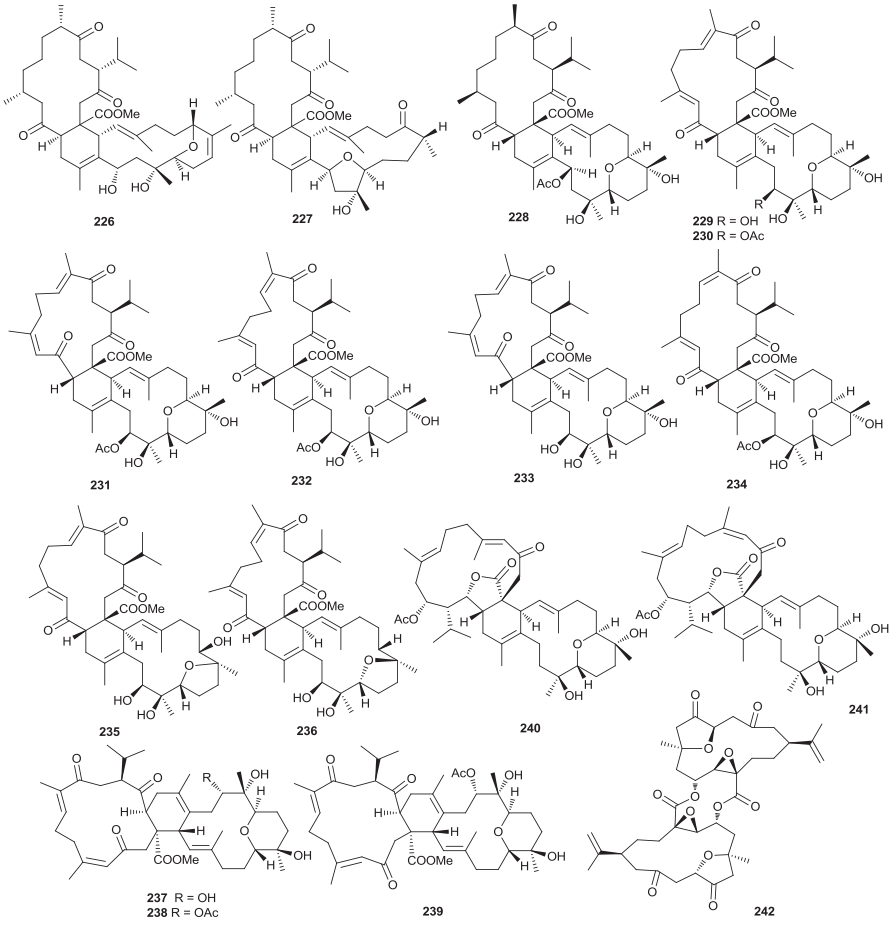


30.2.4 Biscembranoids

Biscembranoids are a family of marine natural products with unusual structure pattern mainly found from marine soft corals, featured by a **14**–**6**-14-membered tricyclic backbone of tetraterpenoids. Their structures varied mainly in ring C where high oxygenation and tri-, penta-, and hexa-epoxy cyclic moieties are frequently observed. The plausible biogenetic pathway of biscembrane analogues was assumed to be derived by a Diels–Alder cycloaddition of two "mono" cembranoids, representing cembranoid-diene and cembranoid- dienophile. This depiction was late on supported by the natural occurring monomeric cembranoids, such as dienophiles methyl sarcoate and methyl tetrahydrosarcoate (methyl tortuosoate), which were regarded to be the precursors to form left part of biscembranoids [89, 90].

Biscembranoid diterpenoids were discovered in two species of the genus *Lobophytum pauciflorum* yielded lobophytones A–S (**199**–**217**) [89–91], while *Sarcophyton* producted other analogues, ximaolides A–G (**218**–**224**) [92, 93], methyl neosartortuate acetate (**225**) [94], methyl tortuoate A (**226**), methyl tortuoate B (**227**) [22], nyalolide (**228**) [23], bisglaucumlides A–K (**229**–**239**) [95, 96]. Compound **209** showed significant inhibition toward LPS-induced nitric oxide (NO) in mouse peritoneal macrophage with IC_{50} values of 4.70 μM [89–91]. Compounds **226**, and **227** exhibited in vitro cytotoxicity against the human nasophyringeal carcinoma CNE-2 cell line, with IC_{50} values of 22.7 and 24.7 μg/mL, and the murine P-388 tumor cell line, with IC_{50} values of 3.5 and 5.0 μg/mL, respectively [22]. Two

biscembranes with an unprecedented fused carbon skeleton, bislatumlides A (**240**) and B (**241**), were isolated from the Hainan soft coral *Sarcophyton latum*, and they exhibited mild cytotoxicity toward several cell lines. IC_{50} values of 7 μg/mL were determined in assays against A-549 lung carcinoma and HT-29 colon adenocarcinoma human tumor cell lines, and 5.8 μg/mL against the P388 murine lymphocytic leukemia cell line [97]. Study of *Sinularia gardineri* (Pratt) (Alcyoniidae), collected in the Red Sea, revealed a new heptacyclic norcembranoid dimer singardin (242). Compound **242** show cytotoxicity to murine leukemia (P-388), human lung carcinoma (A-549), human colon carcinoma (HT-29), and human melanoma cells (MEL-28) [98].

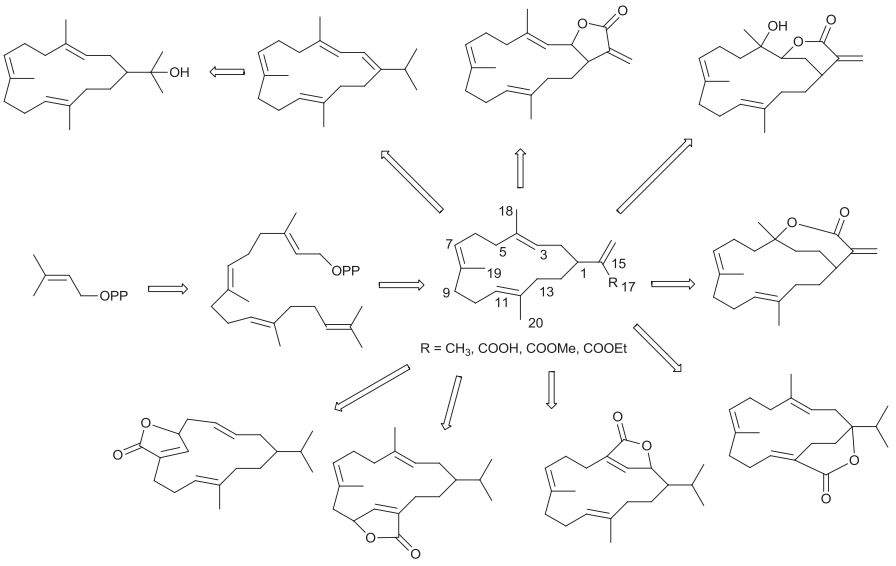


30.3 Biosynthesis and Total Syntheses of Cembrane Diterpenoids

30.3.1 Hypothetical Biosynthesis of Cembrane Diterpenoids

The simple cembrane generally contained a varying 14-membered carbocyclic ring backbone and an isopropyl group, isopropenyl group, or isopropyl/isopropenyl acid group at C-1 [99]. Their biogenesis involves intramolecular cyclisation from geranylgeranyl diphosphate leading to the 14-membered ring hydrocarbon neocembrene, followed by selective enzymatic oxidations at the 14-membered ring (Scheme 30.1) [100].

Cembranolides are a class of diterpenes containing a 14-membered carbocyclic nucleus, commonly fused to a five-, six-, or seven-membered lactone ring. These compounds involve new ring formations as well as skeletal rearrangements. Many of the newly formed rings result from nucleophilic attacks of epoxide moieties by alkoxide or carboxylate anions. The more oxidised CO_2H groups at C-17, could be connected with C-2, C-3, or C-4 to form a lactone ring. C-18 and C-2, C-19 and C-6, C-20 and C-10, and C-20 and C-1 could also be connected to the same oxygen atom to form a lactone ring (Scheme 30.1).

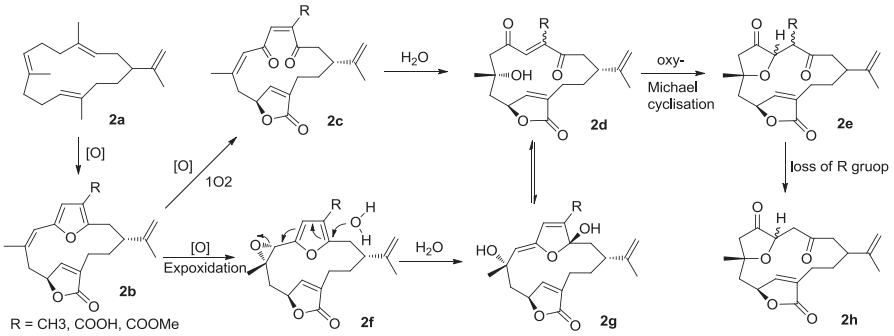


Scheme 30.1 Exploring biosynthetic relationships among cembranoids

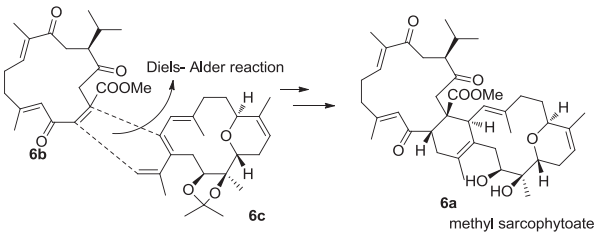
30.3.2 Hypothetical Biosynthesis of Furanocembranoids

Furanocembranoids feature a 14-membered carbocyclic ring backbone which accommodates a furan ring across C3 and C6, and a butenolide ring across C10 and C20. Their biogenesis involves intramolecular cyclisation from geranylgeranyl diphosphate leading to the 14-membered ring hydrocarbon neo-cembrene, followed by selective enzymatic oxidations at C3, C6, C10 and C20, and further ring closures, to produce the furan and butenolide rings in **2b** (Scheme 30.2) [82]. In the furanobutenolide-based cembranoids **2b** that have been characterized from Nature the C18 R-substituent is either CH_3 or the more oxidised CHO, CO_2H and CO_2Me groups, with the degree of oxidation varying from one genus of coral to another.

It seems likely that the 3(2H)-furanone rings in macrocyclic norcembranoids emerge from initial oxidations and hydrolyses of the alkenylfuran units in the furanocembranoids **2b** (Scheme 30.2) [101]. These oxidation/hydrolysis processes most likely involve sunlight-generated singlet oxygen or monooxygenases, and would lead to the enedione intermediates **2d** by either: (i) oxidative cleavage of the furan ring in **2b** [102, 103], followed by facial selective hydration of the *Z* or *E* C7–C8 alkene bond in the resulting dienediones **2c** [18, 68, 104–106]; or (ii) facial selective epoxidation of the C7–C8 alkene bond in **2b**, leading to **2f** [107–110], followed by epoxide ring opening and in situ hydration of **2f** producing the cyclic hemi-ketal structures **2g** [111], which tautomerise to the enediones **2d** (Scheme 30.2). The 3(2H)-furanone ring in the penultimate precursor **2e** to the norcembranoid **2h** is then produced from **2d** by cyclic ether ring formation (which we will refer to as an oxy-Michael cyclisation). Loss of the C18 R-group in **2e** finally produces **2h**. Although cembranoid enediones of constitution **2c** are found alongside the furanocembranoids **2b** in corals, only those where R = Me have been isolated and characterised. Correspondingly, methyl ethers of the cyclic hemi-ketal structures **2g** have also been characterised in corals, but so far only where R = CHO or CO_2Me.



Scheme 30.2 Proposed biosynthetic origin of furanocembranoids



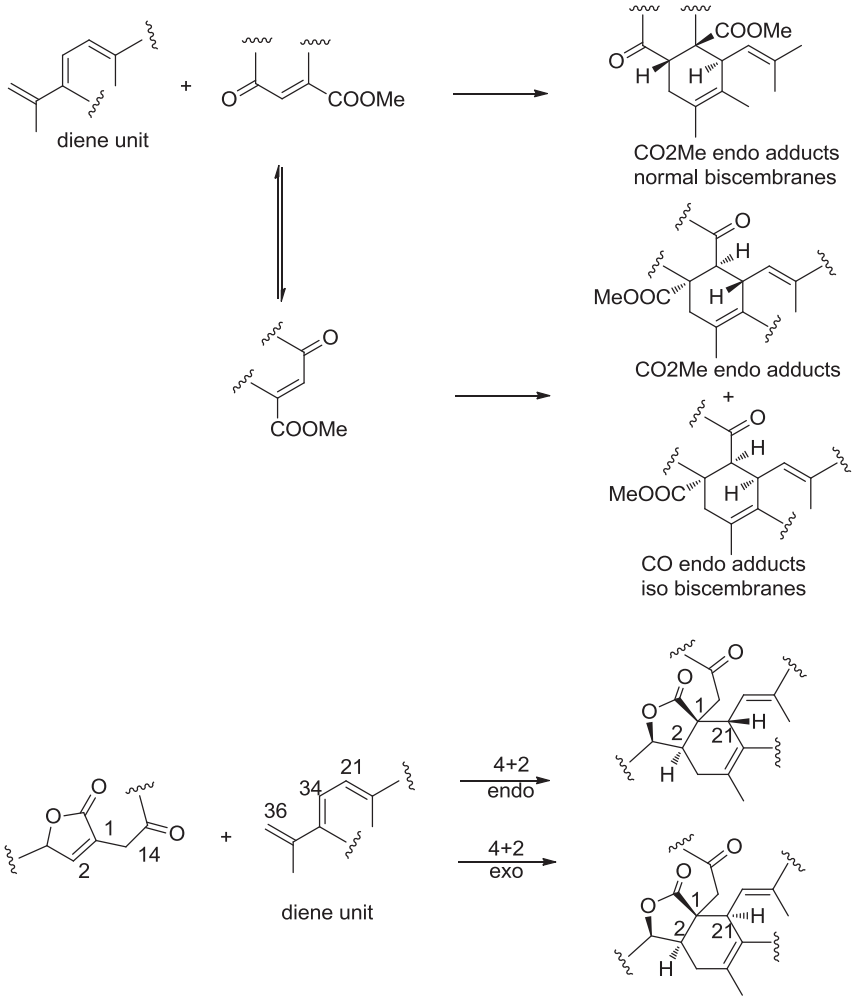
Scheme 30.3 Hypothetical biosynthesis of methyl sarcophytoate

30.3.3 Hypothetical Biosynthesis of Biscembranoids

Biscembranoids represent an emerging group of natural products from soft corals of the genera *Sarcophyton*, *Sinularia*, and *Lobophytum* (family Alcyoniidae). In 1986, Su, and her co-workers isolated methyl isosartortuoate from the Chinese soft coral *Sarcophyton tortuosum* as the first member of the biscembranoids. Up to now, more than 60 biscembranoids have been isolated from the three genera to date [112].

The biscembranoids are considered to be biogenetically synthesized by the Diels–Alder reaction between two different cembranes: the 14-membered dienophile and diene units (Scheme 30.3). Evidence for such a biogenetic hypothesis is the isolation of the dienophile unit from the original coral; i.e., methyl sarcoate, methyl tortuosoate (methyl tetrahydrosarcoate), and isosarcophytonolide D have been isolated along with their biscembranoids [113, 114]. In contrast, probably because of its highly reactive nature the diene unit has been isolated only from the soft coral which produces methyl neosartortuate acetate.

Three types of Diels−Alder cycloaddition have been found to form the biscembranoid framework, including cycloaddition between the trisubstituted conjugated $\Delta^{21(34)}/\Delta^{35(36)}$-butadiene moiety of one monomer and the double bonds of $\Delta^{1(14)}$, antipodal $\Delta^{14(1)}$, and $\Delta^{1(2)}$, of the other monomer, respectively (Scheme 30.4) [13]. Most of the reported biscembranoids exhibited the coupling between the $\Delta^{1(14)}$ double bond activated by a 20-carboxymethyl group and a trisubstituted conjugated $\Delta^{21(34)/\Delta35(36)}$-butadiene moiety, as suggested first for methyl isosartortuoate and then by many other articles. It is noteworthy that this Diels–Alder reaction proceeded in high site-, endo/exo-, π-face-, and regioselectivities except for the $E \rightarrow Z$ isomerization at the C4-position. Plausible explanations for these selectivities are as follows. The $\Delta^{1(2)}$ doubly activated double bond is more reactive than the other double bonds. The $\Delta^{34(21)}$ and $\Delta^{22(23)}$ double bonds do not have the *s-cis* conformation because of the steric repulsion between the 38-methyl and 33-methylene groups, whereas the $\Delta^{21(34)}$ and $\Delta^{35\,(36)}$ double bonds easily reside in the *s-cis* conformation under the given reaction conditions. The CO_2Me *endo* transition states are more favorable than the CO *endo* transition states because both reactants in the latter reside in a more crowded position. The other type of

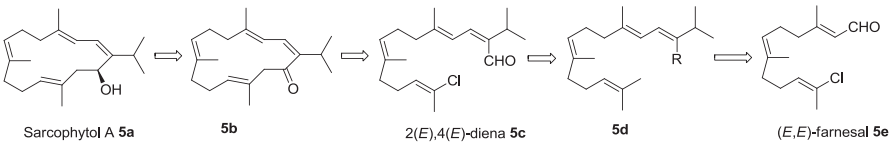


Scheme 30.4 Plausible Diels–Alder reactions of Bislatumlides

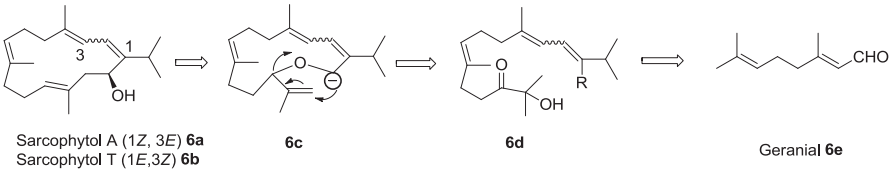
Diels−Alder cycloaddition have been found to form the biscembranoid framework, by an undescribed coupling pattern between the $\Delta^{1(2)}$ double bond involving an α, β-unsaturated γ-lactone ring as a dienophile group and a trisubstituted conjugated $\Delta^{21(34)}/\Delta^{35(36)}$-butadiene moiety, respresented by bislatumlides A−F (Scheme 30.4) [13, 113, 114].

30.3.4 Total Syntheses of Sarcophytol A and Analogues

Sarcophytol A: a cembranoid isolated in 1979 from the Okinawan soft coral *Sarcophyton glaucum*, has been reported to have antitumor activity and also potent



Scheme 30.5 Synthetic strategy for sarcophytol A



Scheme 30.6 The retrosynthetic route of sarcophytol A

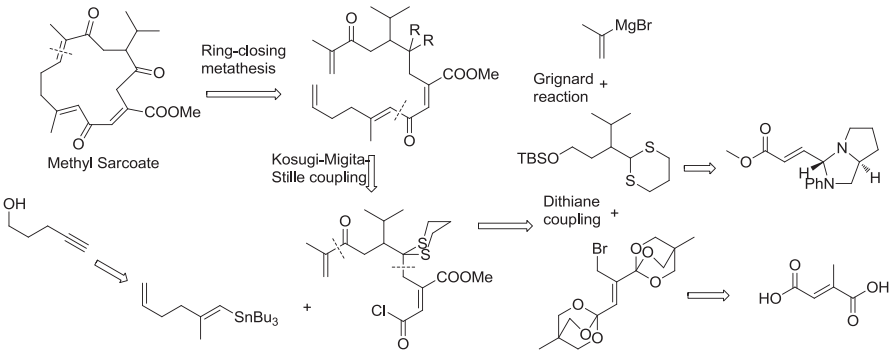
inhibitory activities against the various classes of tumor promoters [27, 30, 31, 115]. In 1988, the geometrical structure and absolute configuration of sarcophytol A were finally confirmed to be 2*Z*, 4*E*, 8*E*, 12*E*, and 1*S*, respectively, and since then efforts to synthesize this cembranoid have used a variety of methodologies [115–124].

Takayanagi, and his co-workers have achieved the first total synthesis of sarcophytol A (Scheme 30.5) [122]. Their strategy started from (*E, E*)-farnesal (**5e**) as outlined in Scheme 30.5, and involved three key steps: (1) stereoselective synthesis of the acyclic precursor for the macrocyclization, conjugated 2(*Z*),4(*E*)-dienal **5c**; (2) macrocyclization of **5c** using a modified protected cyanohydrins procedure; and (3) final enantioselective reduction of the resulting 14-membered ketone **5b** to **5a**.

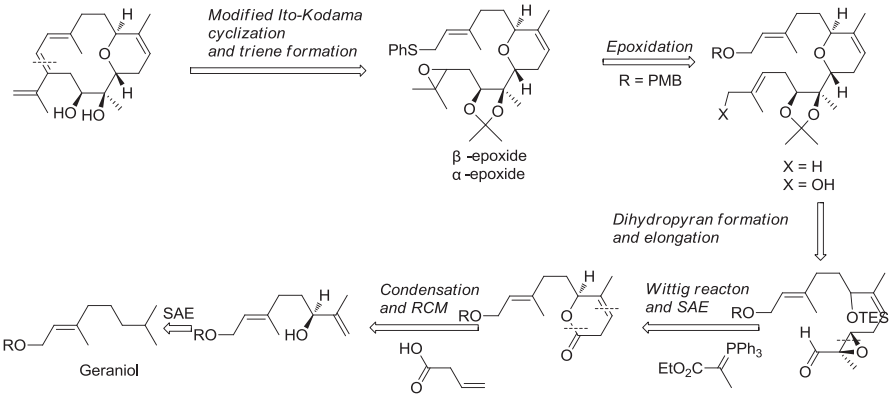
Kodama, and his co-workers reported a new type of synthesis of sarcophytol A(**6a**) and also sarcophytol T (**6b**) as well as their enantiomers (Scheme 30.6) [118]. The two key steps in their synthesis are the stereospecific [2, 3] Wittig rearrangement of chiral bis-ally1 ether **6c**, which allows us to, simultaneously, construct the macro-carbocycle and introduce the 14-hydroxyl group and the enantioselective baker's yeast reduction 4 of an o-hydroxy ketone such as **6d** which is accessible from geranial (**6e**).

30.3.5 Total Syntheses of Methyl Sarcophytoate, A Marine Natural Biscembranoid

The complex and intriguing structures of the biscembranoid have attracted great interest as targets for total synthesis [113, 125–127]. The total synthesis of methyl sarcophytoate (**3a**), a marine natural biscembranoid, has been achieved by the thermal Diels–Alder reaction between the 14-membered dienophile unit, methyl sarcoate



Scheme 30.7 Retrosynthetic analysis of methyl sarcoate



Scheme 30.8 Retrosynthetic analysis of the new synthesis of the diene unit

(**3b**), and the 14-membered diene unit **3c** (Scheme 30.3). Methyl sarcoate (**3b**) was prepared using *n*-BuLi-Bu2Mg-mediated dithiane coupling, Kosugi–Migita–Stille coupling, and Grubbs ring-closing metathesis (Scheme 30.7). The diene unit **3c** was prepared using Sharpless asymmetric epoxidation, Grubbs ring-closing metathesis, 6-exo-tet epoxide opening, and *n*-BuLi-Bu2Mg-mediated Ito-Kodama cyclization (Scheme 30.8). The final Diels–Alder reaction between **3b** and **3c** proceeded with high site, endo/exo, π-face, and regioselectivities. During this reaction, partial $E \rightarrow Z$ isomerization at the C4 position was observed [126–128].

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Chapter 31
Anti-cancer Effects of Triterpene Glycosides, Frondoside A and Cucumarioside A_2-2 Isolated from Sea Cucumbers

Chang Gun Kim and Jong-Young Kwak

Abstract Very low toxicity and various *in vitro* effects of triterpene glycosides make it a suitable agent for cancer prevention and treatment. The molecular mechanisms of action of marine triterpene glycosides, including Frondoside A and Cucumariosides on the apoptosis of cancer cells are not fully investigated. Frondoside A from sea cucumber *Cucumaria frondosa* and Cucumariosides from *Cucumaria japonica* are pentaosides and the main structural difference between Frondoside A and Cucumarioside A_2-2 is in the functional group at C-16 of the aglycone (acetoxy or keto group) and the third carbohydrate unit in the carbohydrate chain. They strongly induce apoptosis of leukemic cells but Frondoside A-induced apoptosis is more potent and rapid than Cucumarioside A_2-2-induced apoptosis. The effects of Frondoside A and A_2-2 for cell death-inducing capability can be compared with attention paid to structure-activity relationships. In this review, the differential effects of Frondoside A and Cucumarioside on the apoptosis of leukemic cells are discussed.

Keywords Triterpene glycosides · Frondoside A · Cucumarioside · Apoptosis · Caspase · Anti-cancer

31.1 Introduction

The suppression of tumor cell growth by sea cucumber glycosides were discovered in 1952 by Nigrelli [1]. Glycosides, substances consisting of a sugar moiety (carbohydrate chain) and triterpene or steroid aglycone, are widely distributed in plants. Triterpene glycosides were also found in marine invertebrates belonging to the class sea cucumber (Holothurians) in the animal kingdom and in some sponges [2, 3]. Sea cucumbers are one of the marine animals which are important as human food source, particularly in some parts of Asia [2]. In the United States and Canada, sea cucumber extracts have been used for over-the-counter dietary health supplements [4].

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Frondoside A and Cucumariosides are derived from the Atlantic edible sea cucumber *Cucumaria frondosa* and Far-eastern edible sea cucumber *Cucumaria japonica*, respectively [2, 5]. Especially, *Cucumaria japonica* is a variety source of different Cucumariosides [2, 6–8]. Thus, food supplements containing cucumaria extracts are currently used to treat cancer patients in Korea, Japan, China and other countries.

Triterpene glycosides from sea cucumbers demonstrate a wide spectrum of biological effects, such as anti-fungal, anti-tumor, hemolytic, cytostatic, pro-apoptotic and immunomodulatory activities [2, 3]. Frondoside A and Cucumariosides showed cancer preventive activities, mainly on *in vitro* level and suppressed tumor growth *in vivo* [9–12]. They have anti-tumor activity by inducing apoptosis of cancer cells although the precise mechanisms underlying the anti-tumor activity of the compounds have not been fully characterized [11, 13, 14]. Recently, our results revealed that Frondoside A and Cucumariosides both possess anti-leukemic properties by inducing apoptosis [13]. The present review discusses the possibility of marine triterpene glycosides, Frondoside A and Cucumariosides as marine anti-cancer agents and the relationship between the structure and function of them. Furthermore, this review also evaluates whether *in vivo* results as well as *in vitro* findings are likely to be applicable for the development of Frondoside A and Cucumariosides as marine anti-cancer drugs.

31.2 Chemical Structure of Triterpene Glycosides, Frondoside A and Cucumarioside A_2-2

Triterpene glycosides are characteristic secondary metabolites of some marine invertebrates, especially echinoderms, octocorals and sponges. They are usually of the lanosterol-type with an 18(20)-lactone and the sugar chain of up to six monosaccharide units is generally linked to the C-3 of the aglycone [15–17]. Carbohydrate chains of triterpene glycosides have two to six monosaccharide residues including xylose, quinovose, glucose and 3-O-methylglucose and sometimes 3-O-methylxylose, 3-O-methylquinovose, 3-O-methylglucuronic acid and 6-O-acetylglucose [17, 18]. The chemical compositions of two triterpene glycosides, Frondoside A and Cucumarioside A_2-2 are shown in Fig. 31.1.

Frondoside A is a major triterpene glycoside isolated from sea cucumber *Cucumaria frondosa* and has a sulfate, acetoxy group at C-16 of the aglycon, pentasaccharide chain, xylose at the third monosaccharide residue and 3-O-methylglucose as terminal monosaccharide residue [5]. Cucumarioside A_4-2, a glycoside isolated from *Cucumaria japonica*, has monosulfated pentaoside having 16-keto group in aglycone and glucose residue as a third monosaccharide unit in the carbohydrate chain [19, 20]. The substance A_2-2 is probably biogenetically connected with A_4-2 and has 3-O-methylglucose instead of glucose as the terminal monosaccharide unit (Fig. 31.1) [16, 19, 20]. Therefore, the main structural difference between Frondoside A and A_2-2 is in the functional group at C-16 of the aglycone (acetoxy or keto group) and the third carbohydrate unit in the carbohydrate chain.

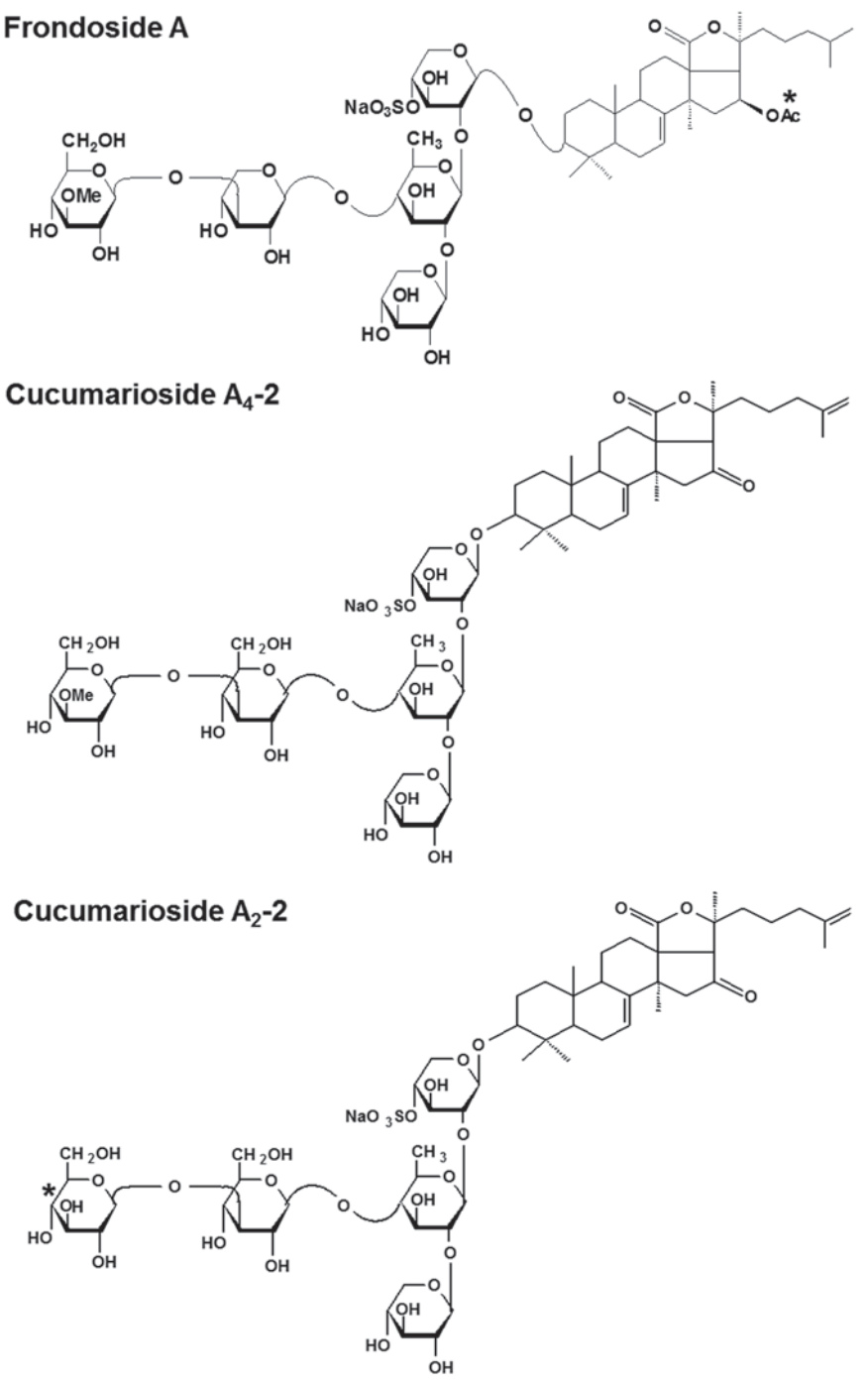

Fig. 31.1 Chemical structure of triterpene glycosides, Frondoside A, Cucumarioside A_2-2 and Cucumarioside A_4-2

31.3 Cytotoxic Effects of Frondoside A and Cucumariosides

Sea cucumber glycosides have a strong membranolytic action through formation of molecular complexes with sterols in biomembranes and subsequent generation of solitary ion channels and large aqueous pores [3, 21, 22]. Recently, Pislyagin et al. have shown that the glycosides effectively increase the microviscosity of the lipid bilayer of splenocyte membranes [23]. Other groups have investigated the membranolytic properties of Cucumariosides from *C. japonica* and the mechanism underlying haemolysis [20]. It was suggested that cytotoxicity to tumor cells and hemolytic actions correlate with each other [20]. In addition, the structures of the aglycone and carbohydrates seem to confer membranolytic activity [2]. Mikami et al. also reported that the presence of acetyl groups usually increases cytotoxic potency [24].

Triterpene glycosides contain different numbers of sulfate groups bounded with sugars. It was demonstrated that Cucumarioside A_2-2 having a sulfate group at C-4 of the first xylose residue is significantly more active than its desulfated derivative [20]. Cucumarioside A_2-2 was shown to have hemolytic activity with EC_{50} of 5 μg/ml [25]. On the other hands, EC_{50} for cucumarioside A_2-2 in *in vitro* cytotoxic activity on sea urchin embryos was demonstrated to be 0.45 μg/mL [12]. The hemolytic activity of Cucumarioside A_6-2 having an additional sulfate group at C-6 of the terminal 3-O-methylglucose residue was quite lower than that of Cucumarioside A_2-2 [20]. Moreover, Cucumarioside A_3 having an additional sulfate group at C-6 of the third monosaccharide unit was less active than Cucumarioside A_2-2. Therefore, cytotoxic activity may be dependent on the positions of sulfate groups attached to the carbohydrates. Similar results were reported by Miamoto et al. They demonstrated that the desulfated derivatives inhibit more significantly tumor cell growth than glycosides with C-6 sulfates in monosaccharide units [26]. In comparison to anti-cancer activity, the macrophage lysosomal activity and increase of intracellular Ca^{++} concentrations were also demonstrated to be related to chemical structures of Cucumariosides and be influenced by the number and positions of sulfate groups in the carbohydrate moiety of the molecules [27, 28]. On the other hands, Cucumarioside A_2-2 was the most active in inducing a rapid increase in cytosolic Ca^{++} content, when compared to the polysulfated derivative of A_2-2 [28].

The LD_{50} of Frondoside A and Cucumarioside A_2-2 for mice was 9.9 mg/kg and 10 mg/kg, respectively after intraperitoneal injection [27, 29]. This suggests that acute toxicity for these two compounds is similar and generally low. Frondoside A, Cucumarioside A_2-2 and Cumarioside A_4-2 induced apoptosis in human leukemia cells HL-60, THP-1 and NB-4 *in vitro* in cytotoxic doses [13, 30]. Cytotoxicity test of Frondoside A on leukemia cell viability, using neutral red release assay with different concentrations of this agent for 24 h, showed that it has a dose-dependent influence on HL-60 viability and totally inhibits cell viability at concentrations at or above 2 μM [13]. The LD_{50} of Frondoside A in HL-60 cells was approximately 5–10 folds lower than LD_{50s} for Cucumariosides A_2-2 and A_4-2 [13]. Frondoside A showed potent cytotoxicity against HeLa tumor cell lines with IC_{50} values of

2.1 μg/mL [31]. Other groups demonstrated that Frondoside A causes concentration-responsive (0.01–5 μM) decreases in viability of LNM35, A549, and NCI-H460-Luc2 lung cancer cells [32]. Frondoside A had a stronger apoptotic effect on HL-60 and NB4 cells than on THP-1 cells [13]. This result suggests that anti-tumor activity of Frondoside A may depend on the cancer cell type. Moreover, Frondoside A enhanced the inhibition of lung tumor growth induced by the chemotherapeutic agent cisplatin [32].

When the cytotoxicity of two holoturians against various leukemia cells is compared, hemolytic activity of Frondoside A is three times more potent than A_2-2, which is consistent with the data on anti-tumoral effects obtained. The structures of both the aglycone part and the carbohydrate chain are very important for anti-tumor activity. However, the some changes in the carbohydrate residues may not play a significant role in the cytotoxicity of Frondoside A and Cucumariosides since the A_2-2 and A_4-2 differ only in the structure of their terminal monosaccharide residue (glucose and methylglucose, respectively). Silchenko et al. have investigated a possible mechanism in the differences of cytotoxicity between triterpene glycosides. They showed that the presence of 25-hydroxy group in aglycone moiety of triterpene glycoside significantly decreases cytotoxic activities [33]. This result suggests that water solubility may affect the potency of triterpene glycosides in cytotoxicity. Our study also indicated that the acetyl group at C-16 of the aglycone in Frondoside A may play a significant role in Frondoside A cytotoxicity since Frondoside A has more potent effects than Cucumarioside A_2-2 on cytotoxicity, cell cycle changes and apoptosis [13]. Similarly, monosulfated Cucumariosides A_2-2 and A_4-2 were among the most active compounds for stimulating peritoneal macrophage lysosomal activity, while desulfation of their carbohydrate moiety completely abolished this activity [27]. However, they demonstrated that lysosomal activity and cytoxicity of cucumariosides depends on structures of both aglycone and carbohydrate chain but there is no direct correlation between two activities of the glycosides [34].

31.4 Apoptotic Activity of Triterpene Glycosides

Silchenko et al. demonstrated that Frondoside A decreases the UVB-, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- and EGF-induced AP-1-dependent transcriptional activities in JB6-LucAP-1 cells [31]. Therefore, they suggested that Frondoside A influences signal transduction in living cells in the same manner as some anti-cancer and cancer chemopreventive agents. Induction of apoptosis is the most frequently observed mechanism of anti-tumor agents. A major pathway of apoptosis has been shown that it is controlled at the mitochondrial level and caspase-3 is an effector caspase and is involved in the later stages of cell death [35, 36]. Caspases are cystein-dependent aspartate-directed proteases, which can be divided into two groups, initiator and its downstream effector caspases [37, 38]. Initiator caspases, including caspase-2, -8, -9 and -10, are self-activated in response to apoptotic signals, whereas activation of effector caspases, including caspase-3 and -7, requires

cleavage by activated initiator caspases [39]. In addition, there have been growing evidences that apoptotic pathways also can be activated without participation of caspases, through mechanisms involving activation of other classes of proteases such as calpains (calcium-dependent proteases), cathepsins, serine proteases, granzymes, etc. [40]. These proteases often cooperate with caspases, but they also can provoke characteristic apoptotic changes in a caspase-independent manner [41].

It was shown that treatment of human pancreatic cancer cells with low concentration of Frondoside A induces apoptosis through caspase-9, -3 and -7, increased bax, decreased bcl-2 and mcl-1, and arrested cell cycle and upregulated p21 [11]. In addition, Attoub et al. demonstrated that Frondoside A causes concentration-dependent reduction in viability of lung cancer cells LNM35, A549 and NCI-H460-Luc2, melanoma MDA-MB-435, breast cancer cells MCF-7 and hepatoma cells HepG2 over 24 h and increases the activities of caspase-3 and -7 in LNM35 cells [32]. However, the effects of caspase inhibitors on the Frondoside A-induced apoptosis of cancer cells have not been investigated. We observed that mitochondrial membrane permeability is not changed and the accumulation of cytochrome c in the cytosolic fraction is not observed in HL-60 cells treated with Frondoside A, Cucumarioside A_2-2 and Cucumarioside A_4-2 [13]. More interestingly, the levels of procaspase-3, -8, and -9 proteins in lysates from Frondoside A-treated HL-60 cells were not changed, whereas, Frondoside A-induced apoptosis in 50–70% of cells. The cleavages of procaspase-3 and PARP but not procaspases-8, -9, and -12 were significantly increased in Cucumarioside A_2-2 or Cucumarioside A_4-2-treated HL-60 cells. Furthermore, the annexin-V positivity was not inhibited by zVAD-fmk, whereas both the annexin-V positivity and cleavage of caspases induced by Cucumariosides were efficiently blocked by caspase inhibitors. These results suggest that Frondoside A initiates apoptosis in a caspase-independent manner.

The acetyl group at C-16 of the aglycon in Frondoside A may play a significant role in Frondoside A-induced caspase activation. Frondoside A had more potent effects than Cucumarioside A_4-2 on apoptosis but did not have caspase activation before early apoptosis, whereas Cucumarioside A_2-2 and Cucumarioside A_4-2 showed similar effects on procaspase cleavage and mitochondrial permeability [13]. Recently, mitochondria-mediated and caspase-independent apoptotic pathways were described [42]. The release of apoptogenic molecules such as apoptosis-inducing factor (AIF) or cytochrome c is followed by caspase-independent or -dependent nuclear degradation [43].

31.5 Effects of Frondoside A on the Invasion and Metastasis of Cancer Cells

Tumor-bearing animal models are commonly utilized to study the effects of therapeutic interventions on cancer metastasis. A substantial number of animal studies have been conducted for the treatment of cancer using Frondoside A. The cancer inhibitory effect of Frondoside A in tumor-bearing mice may be partly due to its

anti-angiogenic and anti-metastatic effects as well as apoptotic effects [11, 32, 44, 45]. Frondoside A, at concentrations that are not cytotoxic to the cells, was demonstrated to exert a strong inhibitory effect on the migratory and invasive properties of MDA-MB-231 breast cancer cells [44]. In addition, Attoub et al. demonstrated that Frondoside A (0.01 and 1 mg/kg/day, i.p. for 25 days) significantly decreases the growth, the angiogenesis and lymph node metastasis of LNM35 tumor xenografts in athymic mice [32]. Similar to *in vivo* results, *in vitro* experimental results revealed that Frondoside A also impairs lung cancer cell migration and invasion [45]. These results suggest that Frondoside A treatment suppresses the tumorigenesis and formation of metastases. Moreover, these results support the feasibility of anti-cancer drug development from Frondoside A. On the other hands, Ma et al. conducted basic studies on the mechanism of *in vivo* action of Frondoside A. The ability of Frondoside A to inhibit metastasis was by blocking EP4 activities and by protecting natural killer (NK) cells from PGE2-mediated immune suppression [45]. They have shown that Frondoside A antagonizes the prostaglandin E receptors EP2 and EP4 [45]. Holt et al. have investigated the role of Frondoside A on NK cells. They demonstrated that PGE2 and the EP4 agonist PGE1-OH suppress the secretion of interferon-γ (IFNγ) from NK cells by 95 and 100 %, respectively but Frondoside A restores the capacity of NK cells to secrete IFNγ in the presence of PGE2 or PGE1-OH [46]. Other groups also demonstrated that Frondoside A suppresses TPA-induced matrix metalloproteinase (MMP)-9 enzymatic activity, secretion and expression in MBA-MB-231 human breast cancer cells, leading to inhibition of invasion and migration of the cells [47]. Frondoside A also significantly inhibited phosphoinositide-3-kinase (PI3K/Akt), extracellular signal-regulated kinase (ERK1/2) and p38 mitogen activated protein kinase (MAPK) activation which is involved in the expression of MMP-9 in breast cancer cells [47, 48]. These results suggest that common signaling pathways in apoptosis and MMP-9 expression are regulated by Frondoside A. In comparison, *in vitro* treatment of peritoneal macrophages with Cucumarioside A_2-2 stimulated cell adhesion as well as the spreading reaction and motility velocity [49]. Therefore, it seems to be important to compare the efficacy of Cucumarioside A_2-2 on anti-metastatic effects to that of Frondoside A in tumor-bearing mice.

31.6 Conclusion

Sea cucumbers, including edible species, contain physiologically active triterpene glycosides. Thus, these compounds may be considered to be both anticancer and cancer preventive agents. Frondoside A and Cucumarioside A_2-2 both possess anti-leukemic properties by inducing apoptosis. Moreover, anti-cancer effects of Frondoside A and Cucumarioside A_2-2 might be through inhibition of tumorigenesis and metastasis. The mechanism of this action is uncertain and will be explored in future studies. Caspase-independent anti-leukemic agents, such as Frondoside A will be useful to overcome chemoresistance, where is associated with defects in both the

extrinsic and intrinsic pathways of apoptosis. As a next step, determination of the structural characteristics responsible for the *in vivo* anti-cancer activities will be essential for their use as a marine drug. Furthermore, in near future, phamacokinetic analysis after oral administration of Frondoside A and Cucumariosides is recommended for further clinical trials.

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Chapter 32
Pederin, Psymberin and the Structurally Related Mycalamides: Synthetic Aspects and Biological Activities

Zbigniew J. Witczak, Ajay Bommareddy and Adam L. VanWert

Abstract Pederin, psymberin, and mycalamides are related members of a relatively new family of potent natural antiviral and antitumor compounds originally isolated from marine sponges in 1988. This natural family of chemicals is of great interest to medicinal chemists and biologists, stemming from its extremely low abundance in source organisms and strikingly potent biological activity. They have clearly emerged as promising new synthetic targets, and are the focus of quite an interdisciplinary approach to molecular characterization. In this chapter we review diverse synthetic approaches to this family of natural products that has been demonstrating remarkable biological activity. We discuss relevant history, biological origins with the latest information on source organisms and their hosts, in-depth synthetic approaches, and biological data supporting their potential as therapeutic compounds.

Keywords Mycalamides · Psymberin · Psympederin · Irciniastatin A · Pederin · Onnamide · Marine · Natural products · Anticancer activity · Antineoplastic · Marine sponge · Paederus fuscipes · Polyketides · PC3 · Hela · T98G · KM12

32.1 Introduction

In 1988, an extract from the sponge genus *Mycale* in New Zealand's Otago Harbour yielded a small quantity of brown oil (307 mg from 200 g of sponge) whose bioactive components exhibited the unusual ring system which is now known as the mycalamides [1–7]. Structurally, mycalamides A and B are remarkably similar to the insect toxin pederin, and exhibit potent and comparable cytotoxicity and antitumor activity.

Despite their production in highly distinct species, the commonalities between these mycalamides, pederin [8], and the subsequently discovered onnamide A, an antiviral compound from a Japanese sponge [9], indicate that they likely have related modes of biosynthesis. Pederin is found in some beetles, e.g., *Paederus fuscipes*,

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and structurally is considered a polyketide that bears vesicant and potent antitumor properties. Although pederin has weak antibacterial properties, it is severely toxic to eukaryotic cells. This property likely stems from its ability to block protein synthesis and cell division. There is significant evidence indicating that pederin is synthesized by a bacterial symbiont of host beetles, perhaps a close relative to *P. aeruginosa* [10]. This finding raises the possibility of culturing bacteria to generate a large quantity of pederin for therapeutic use or chemical modification to enhance pharmacodynamics/pharmacokinetics. Currently, however, isolating a practical quantity of pederin from biological sources remains challenging. Approximately 220 lb, or 25 million insects, were needed to extract enough pederin for structural analysis (Scheme 32.1).

Comprehensive characterization of the pharmacodynamic, toxicological, and therapeutic potential of all these congeners has been limited by their paucity. Therefore, alternative and practical synthetic approaches should prove to be highly useful for this remarkably potent set of compounds. Developing new and efficient synthetic strategies should facilitate the production of new lead modifications. Ideally, such derivatives of the natural compounds will have more suitable pharmacodynamic and pharmacokinetic properties, and most importantly, tissue targeting may become a realistic goal.

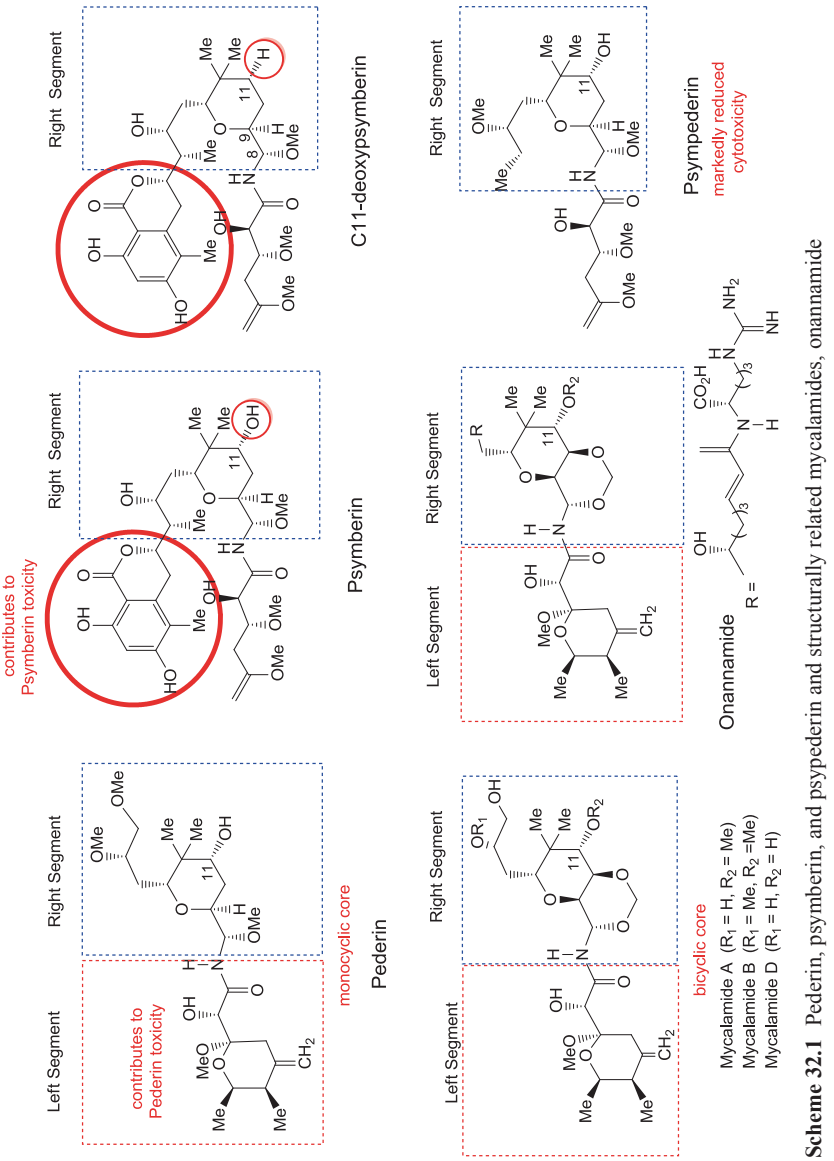
32.2 Synthetic Methodologies

An account of the major synthetic strategies for producing compounds from the mycalamide, pederin, and psymberin families is presented in separate sections below. All presented methods reflect a clear mastery of modern synthesis techniques and reagents by the investigators.

32.3 Synthetic Methodologies for Mycalamides

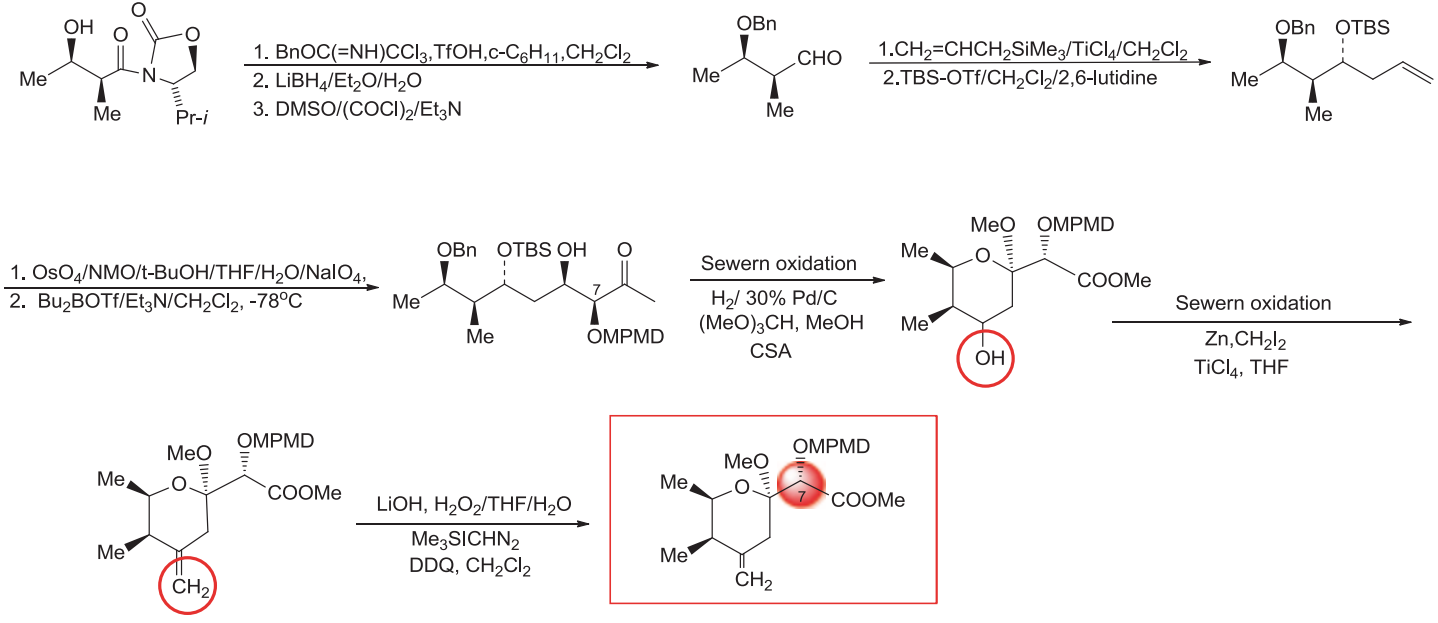
Several synthetic strategies for producing mycalamides have been published. All proposed methods to date have been complex strategies with low yields. Adding to the complexity of these strategies is the requirement for stereospecificity at several steps. Thus, development of more efficient synthetic strategies is critical for realizing the full potential of this class of compounds.

A synthetic approach to produce the right half of mycalamides was demonstrated using Kishi's method. This technique utilizes α-D-glucopyranoside as the starting material. In the early 1990s a multi-step sequence for developing this significant portion of the mycalamide structure was published [11, 12]. The basic synthetic strategy involves an alteration of the previously published methodology [13, 14]. The conjugation method to produce total mycalamide required the activation of pederate, the left portion of the molecule, with *p*-toluenesulfonyl chloride/DMAP/dichloromethane at room temperature followed by treatment with the amines produced by hydrogenation of azides. In Roush's method, an excellent chiral precursor, methyl



Scheme 32.1 Pederin, psymberin, and psypederin and structurally related mycalamides, onannamide

7-benzoylpederate, is used to effectively control the stereoselectivity of the coupling strategy for generation of the amide bridge [15–18]. An alternative approach to synthesize methyl pederic acid involves 7-*O*−(3,4-dimethoxybenzyl) pederate, an intermediate in Kishi's synthesis of mycalamides A and B and onnamide A [11, 12]. Scheme 32.2 depicts this significantly diastereoselective synthesis. Incorporation of the *exo* methylene moiety involves use of the very important Takai-Nozaki protocol (CH_2I_2, $TiCl_4$, Zn, THF).



Scheme 32.2 Roush's synthesis of 7-O-(3,4-dimethoxybenzyl)

A strategy for producing pederic acid derivatives was employed in the synthesis of 7-epi-mycalamide [15–19]. One key step in the method is the mismatched aldol reaction of the imide and aldehyde. This step yielded a ca. 5:4 mixture of two isomeric aldols, with incorrect C-7 stereochemistry. Enrichment of the isomeric mixture to mycalamide A required epimerization of C-7 at the beta-keto imide step. A divergent and effective approach involves Swern style oxidation of the isomeric mixture of aldols. This approach proceeds under reaction conditions that minimize C-7 epimerization, selectively yielding 7-*epi*-mycalamide.

A strategy for producing (+)-methyl 7-benzoylpederate, a mycalamide intermediate, has been reported by Trotter et al. in the Nakata group [20]. This effective strategy employs the well-known Nakata's [20] precursor, that has been utilized in similar methods, resulting in generation of the basic framework of the system. One limitation of the approach is that the conjugation strategy employs the original technique that yields an isomeric mixture of target products. These products are challenging to purify.

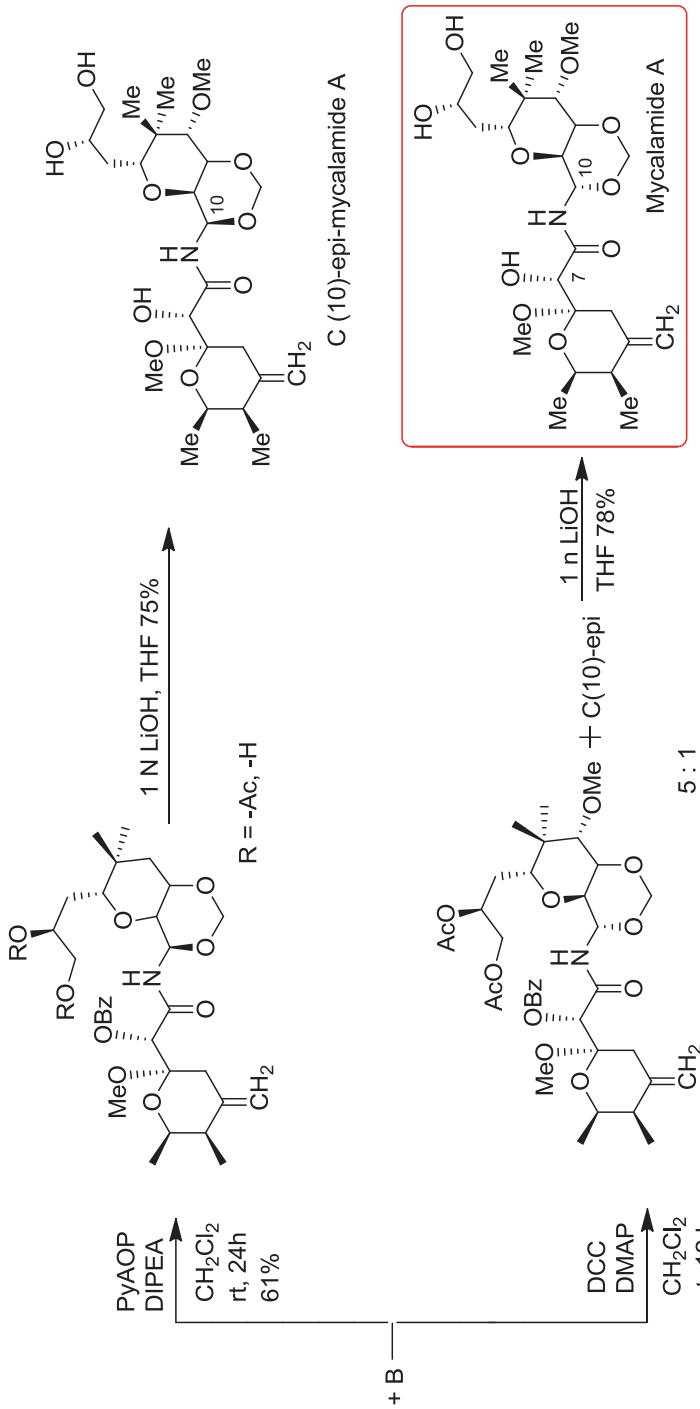
Stereoselectivity is a focus of Toyota's/Ihara's method for mycalamide synthesis. In this procedure D-mannitol is a useful chiral precursor for eventual production of the right half of the mycalamide structure. In this strategy, a Lewis-acid-catalyzed intermolecular aldol reaction and oxypalladation are critical steps [21–24]. This approach yields an effectively protected right half of the mycalamide structure that is primed for functionalization and conjugation with the remaining half of the structure.

For the total synthesis of (+)-mycalamide A, Kagawa and Toyota produce an α, β-unsaturated ester from D-mannitol as described above [21]. The ester is then used to synthesize an intermediate, and the left and right portions are joined by transmethylation of the intermediate. Subsequently, conjugating the resultant vinyl anion to the ester group regioselectively via nucleophilic addition effectively produces the final precursor in 50 % yield. Modifications of the functional group of this precursor can produce (+)-mycalamide A [25].

Trost and Probst demonstrated an approach for the synthesis of (−)–mycalamide A. In this method they synthesized (−)-7-benzoylpederate (left-half of mycalamide) from (2*S*,3*S*)-2, 3-epoxybutane and the right-half of mycalamide from (R)-pantolactone [26]. A multi-step sequence was used to form a terminal azide on the right half that was suitable for conjugation with the left half, yielding the total mycalamide.

Sohn and Rawal's approach to mycalamide A synthesis employed a convergent coupling of pederic acid (Structure A, Scheme 32.3) with mycalamine (structure B) [27].

In this approach the left portion, (+)-7-benzoylpederic acid, was produced through 7 steps with ~35 % overall yield. A major step in this method is the palladium (II)-catalyzed tandem Wacker/Heck cyclization reaction to form a tetrahydropyran ring (Scheme 32.3) prior to coupling to the right half of mycalamide. The right half, mycalamine (structure B), was synthesized from diethyl D-tartarate in 21 steps with 10.5 % yield. Joining of the two halves stereoselectively was accomplished *via* (1) DCC/DMAP catalysis with 56 % yield of mycalamide A, or (2) reaction with



Scheme 32.3 Rawal's coupling approach to mycalamide A

benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluoro-phosphate/Diisopropyl-ethyl-amine (PyAOP/DIPEA) to produce *C* (10)-*epi*-mycalamide A (61 % yield).

32.4 Synthetic Methodologies for Pederin

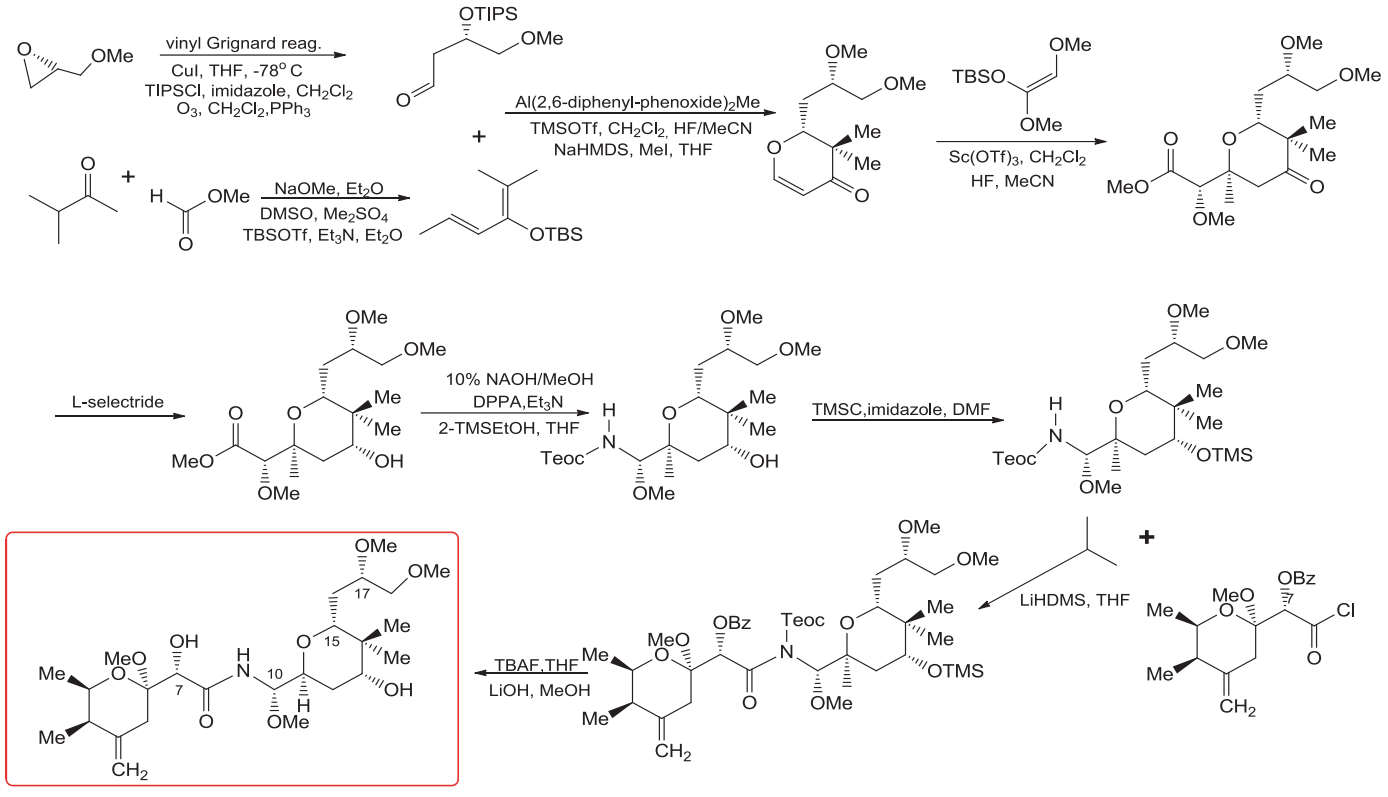
From earlier years to more recent times many literature reports [13, 14, 28–44] describe total synthesis of pederin, precursors, or related structures. The total synthesis of pederamide, a hydrolysis product of pederin, was first reported by Tsuzuki and co-workers in 1976 [41]. Subsequently, Nakata et al. reported the first synthetic method for production of total pederin in 1985 [13, 14]. In this method they stereoselectively produced (+)-benzoylselenopederic acid via the novel reducing agent, $Zn(BH_4)_2$. Two major steps in their procedure were (1) construction of an aminal linker using a metallateddihydropyran; and (2) production of an N-acyl aminal via a rhodium-catalyzed reductive hydroboration of an acyl amine.

Kocienski's strategy [45] for the right portion template, originally described in 1987 [28], is one of the most useful synthetic methods for efficient production of an important precursor of the whole family of mycalamides. A relatively new approach, published by Floreancig et al. [42, 43] employs the Paterson's pinene-derived boron enolatealdol strategy. This effective strategy proceeds with a highly stereoselectivealdol reaction. This reaction is a pivotal step for ensuring appropriate orientation of bonds at C-15.

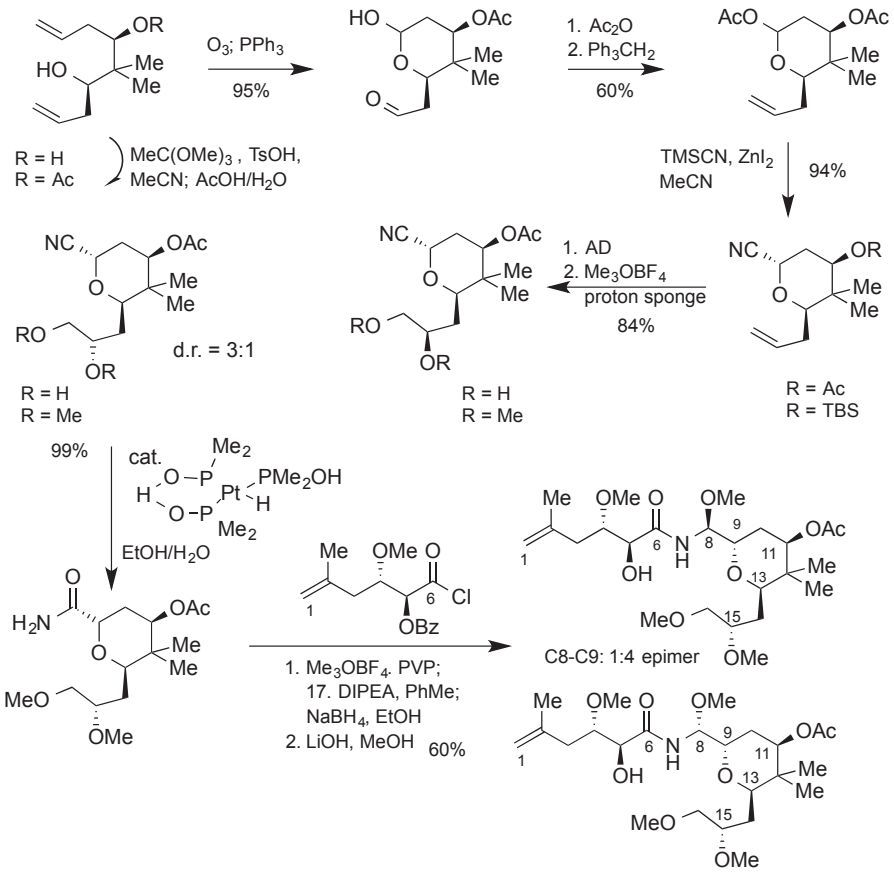
In 2007, another approach for the total synthesis of pederin was reported by Jewett and Rawal [30]. The group achieved direct coupling of the left and right halves using pederic acid chloride with the lithium anion of carbamate in a toluene/pyridine solution. This approach was highly effective. The deprotection steps to complete the approach required the use of *t*-butyl ammonium fluoride (TBAF) in a tetrahydrofuran solution followed by a hydrolytic quench with lithium hydroxide in methanol. This strategy resulted in formation of total pederin with a marked 88 % yield (Scheme 32.4). Various other diverse methods for the production of pederic acid esters, which serve as specific templates for coupling approaches, have also been published [37–40].

32.5 Synthetic Methodologies for Psymberin

Psymberin was first isolated by Crews et al. in 2004 from the Marine Sponge *Psammocinia* using a "bioassay-guided fractionation" method [46]. Irciniastatin A, isolated by Pettit et al. in the same year from the marine sponge *Ircinia ramose*, was found to be identical to psymberin [47]. The amide side chain of psymberin was a focus of Williams et al. in a 2005 report [48]. Their group employed X-ray crystallography and synthetic model compounds for comparison with natural psymberin.



Scheme 32.4 Rawal's sysnthesis of pederin

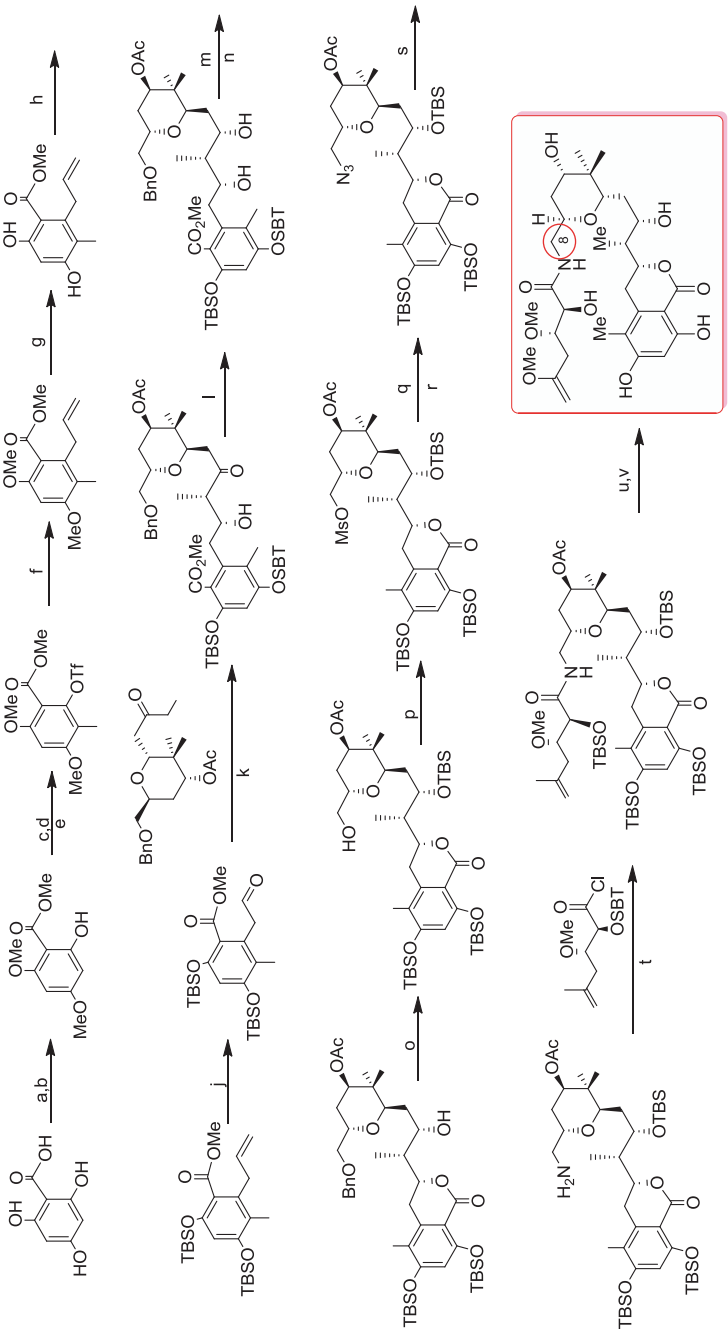


Scheme 32.5 De Brabander's sysnthesis of psymberin

They determined that the *anti* configuration of the side chain is the natural form [48]. Crews and coworkers [46] also reported assignment of absolute stereochemistry of Psymberin as 5*S*, 8*S*, 9*S*, 11*R*, 13*R*, 15*S*, 16*R*, 17*R* and with multiple NOE enhancements. Psymberin was assessed for *in vitro* activity against an impressive 60 human cancer cell lines [46].

Generation of psymberin stereoisomers was reported by De Brabander et al. [49]. In the following year the group reported on the synthesis of a novel compound sharing characteristics with psymberin and pederin [50]. They referred to the new compound as "psympederin". This hybrid is depicted in Scheme 32.5, and starts with a C_2-symmetrical diol that is conveniently acetylated by acid catalysis into cyclic orthoformate [50]. Hydrolysis of the monoacetate, followed by reduction with triphenylphosphine, resulted in production of a lactol with a yield of 95%. Subsequently, the lactol intermediate was functionalized with multiple steps to the amide. In the final step of the pathway, the amide was converted into "psympederin" (Scheme 32.5).

Several diverse methodologies for the synthesis of psymberin and functionalized congeners have been reported in the past 10 years [51–60]. Konopelski et al. and



Conditions:
(a) Me_2SO_4, K_2CO_3, acetone, RT, 91%. (b) BCl_3, CH_2Cl_2,-78° C, 1h, then to RT, 91%. (c) $POCl_3$, DMF, 0° C-RT, 75%. (d) H_2, Pd/C, EtOH/EtOAc, RT 99%. (e) Tf_2O, Py, CH_2Cl_2, 0° C-RT, 96%. (f) $[Pd(PPh_3)_4]$, LiCl, allyltributylstannane, THF, reflux, 84%.(g) BBr_3, CH_2Cl_2,-78° C -RT, 58%. (h) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0° C-RT, 80%. (i) $K_2OsO_4.2H_2O$, $NaIO_4$, 2,6-lutidine, 1,4-dioxane/H_2O, RT 80%. (k) $PhBCl_2$, DIPEA, CH_2Cl_2, -78° C-RT, 63%. (l) Et_2BOMe, $NaBH_4$,THF/MeOH 4:1, -78° C 84%. (m) CSA, CH_2Cl_2, 0° C-RT, 99%. (n) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0° C-RT, 89%.(o) $Pd(OH)_2$, H_2, THF, RT, 99%. (p) MsCl, Et_3N,CH_2Cl_2, 0° C-RT, 94%. (q) $(nBu)_4NN_3$, toluene, RT, 50%. (r) TBSOTf, 2,6-lutidine, CH_2Cl_2, -15° C-RT, 73%. (s) $Pd(OH)_2$/C, H_2,THF, RT,(t) DIPEA,CH_2Cl_2, 0° C-RT. (u) TASF,DMF, 50° C. (v) LiOH, THF, 0° C-RT, 35%.

Scheme 32.6 Pietruszka's sysnthesis of 8-desmethoxy psymberin

Shao and Huang et al. [51, 57], in addition to Pietruszka and co-workers [58–60], have designed methods for synthesizing new analogues of psymberin, including putative intermediates in the natural biosynthetic pathway. Pietruszka's synthetic approach to 8-desmethoxy psymberin is depicted in Scheme 32.6.

32.6 Biological Activity

32.6.1 Activity of Mycalamides

Evaluation of the biological and potential therapeutic activity of this class of compounds has been difficult, stemming from their very low concentration in host organisms. Mycalamide-A exhibited promising *in vivo* anti-viral activity against the A59 coronavirus in mice [2]. Furthermore, mycalamides A and B and onnamide showed *anti-cancer* properties *in vitro* and *in vivo* in mice and human tumor cells at concentrations ranging from 5 to 200 nM and 2.5 to 10 μg/kg [61]. All three compounds were shown to inhibit protein synthesis, some at low nanomolar concentrations [61, 62]. Mycalamides A and B exhibited the ability to convert transformed rat kidney epithelial cells (NRK) back to their normal morphology, possibly through selective inhibition of protein synthesis [63]. More recently, Dyshlovoy et al. showed that mycalamide A was able to block epidermal growth factor-induced cancerous transformation of a murine epidermal cell line (JB6 Cl41 P(+)), and induce apoptosis at subnanomolar concentrations, possibly through inhibition of NF-kB and AP-1 nuclear transcription factors [64]. Richter et al. showed that synthetic congeners of mycalamide-B, i.e., 18-O-methyl mycalamide-B and 10-epi-18-O-methyl mycalamide-B, exhibited anti-proliferative effects against carcinomas [65]. Specific cell lines that demonstrate susceptibility to mycalamide A, mycalamide B, or onnamide include murine lymphoma P388 cells, HL-60, HT-29, and A549 human tumor cells, and leukemia cells [61–63, 65]. Mycalamide-A is also active against B16 melanoma, Lewis lung carcinoma, M5076 ovarian sarcoma, colon26 carcinoma, and the human MX-1, CX-1, and Burkitt's lymphoma tumor xenografts [61].

In addition to its antitumor properties, mycalamide-A has demonstrated powerful immunosuppressive action, with a potency at inhibiting $CD4^+$ T cells of approximately 1000 times that of cyclosporin A on a molar basis [66]. This places mycalamides amongst the most powerful immunosuppressive compounds discovered to date. Mycalamide A has also been shown to induce apoptosis with a preference for 32 D myeloid cells that have Ras or Bcr/abl alterations [67].

The mycalamides appear to have a unique mechanism of action, which is of great interest for studying the immunobiology of T cells and potentially for developing agents that can modulate T-cell function. The strong immunosuppressive ability of these compounds and their unique mechanism of cytotoxicity makes them promising, both as potential biochemical tools (e.g., FK506 and rapamycin have already led to several advances in cell biology), and as potential therapeutic agents for preventing graft-vs.-host and host-vs.-graft disease in post-transplant surgery patients. In addition, these agents have potential in alleviating patients with autoimmune disorders. It is also of potential utility that mycalamide A does not appear to be a substrate for the major drug efflux pump, P-glycoprotein (MDR1/ABCB1). Therefore, P-gp is not expected to be a limiting factor in its cytotoxicity [68].

Table 32.1 Differential sensitivities (LC_{50}) of various cell lines to Psymberin as identified in the NCI developmental therapeutics in Vitro screening program [59]

Cell line	LC_{50} (M)	Cell line	LC_{50} (M)
Breast cancer		*Colon cancer*	
MCF7	$>2.5\times10^{-5}$	HCC-2998	3.76×10^{-7}
HS578T	$>2.5\times10^{-5}$	HCT-116	$<2.5\times10^{-9}$
MDA-MB-435	$<2.5\times10^{-9}$	HT29	$>2.5\times10^{-5}$
NCI/ADR-RES	1.9×10^{-5}	SW-620	$<2.5\times10^{-5}$
T-47D	1.36×10^{-5}		
Melanoma		*Leukemia*	
LOX IMVI	$>2.5\times10^{-5}$	CCRF-CEM	$>2.5\times10^{-5}$
MALME-3M	$<2.5\times10^{-9}$	HL-60 (TB)	$>2.5\times10^{-5}$
SK-MEL-2	$>2.5\times10^{-5}$	K-562	$>2.5\times10^{-5}$
SK-MEL-28	$<2.5\times10^{-9}$	MOLT-4	$>2.5\times10^{-5}$
SK-MEL-28	1.41×10^{-5}	RPMI-8226	$>2.5\times10^{-5}$
UACC-257	$>2.5\times10^{-5}$	SR	$>2.5\times10^{-5}$
UACC-62	$<2.5\times10^{-9}$		

32.6.2 Activity of Psymberin

Psymberin also known as Irciniastatin A is a naturally occurring cytotoxic agent which was independently isolated by Pettit et al. [47] from marine sponge *Psammocina* species and Crews et al. [46] from Ircinia ramose species in 2004. It has been recently documented that psymberin is rather produced by the symbiotic bacteria associated with the marine sponges and not by marine sponges themselves [10]. Psymberin structural features closely resemble that of other pederin family members including mycalamide A. In general, psymberin lacks the commonly present acetal-containing pederate side chain, but is identified with a dihydroisocoumarin unit that is not widely found with other members of pederin family. The activity of pysmberin on various human cancer cell lines is listed in Table 32.1. Out of the 60 cell lines tested, psymberin displayed a differential cytotoxicity profile and was more potent against colon cancer, melanoma and breast cancer cell lines. Several research groups have attempted to synthesize this fascinating marine drug owing to its complex structure, limited natural abundance and biological properties [59]. Huang et al. reported several analogues of psymberin which were constructed by modifying the tetradhydropyran ring core of psymberin. All the analogues (**1, 1A, 1B, 1C**) were biologically tested using major human cancer cell lines to identify their IC_{50} values (Table 32.3). Compound 1 (C11-Deoxypsymberin) was found to be most potent of all the analogues and its potency was 3 to 10 fold more compared to psymberin or its epimer [57]. Modifications of **1** further revealed high potency against the HOP62 human lung cancer cell line [57] (Fig. 32.1).

De Brabander et al. [69] synthesized psymberin and its analogues whose biological activity against major human cancer cell lines is listed in Table 32.2 [70]. Psymberin and its two epimers 8-epipsymebrin and 4-epipsymberin exhibited

Table 32.2 Mycalamide A, Psymberin and its analogues cytotoxicity against different human cancer cell lines [69–71]

IC_{50} (nM)					
Structure	PC-3	SK-MEL-5	Hela	T98G	KM 12
Mycalamide A	2.5 ± 0.2	–	–	2.87 ± 0.07	0.95 ± 0.02
Psymberin	0.98 ± 0.12	2.29 ± 0.13	0.64 ± 0.14	1.37 ± 0.06	0.45 ± 0.01
4-*epi*-psymberin	346.5 ± 102.8	762.8 ± 70	618.6 ± 267	186.7 ± 51.3	126.08 ± 8.6
8-*epi*-psymberin	200.2 ± 27.6	352.0 ± 2.1	> 1000	85.8 ± 48.4	37.1 ± 5.5
Psympederin	821.8 ± 89.1	> 1000	> 1000	> 1000	710.9 ± 35.8
c-8-*epi*-psympederin	255.5 ± 11.4	> 1000	> 1000	> 1000	> 1000

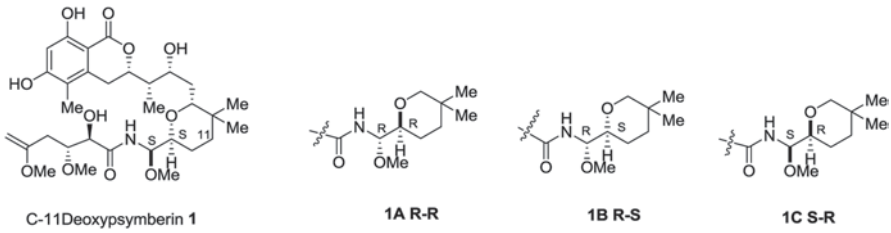


Fig. 32.1 Structural variations at the tetrahydropyran core of C11-Deoxypsymberin and its Epimers

Table 32.3 Activity of Psymberin, C-11 Deoxypsymberin 1 and its Epimers, 1A, 1B, 1C [53]

IC_{50} (nM)							
Tissue type (human)	Cell line	Psymberin	1	1A R/R	1B R/S	1C S/R	*epi*-Psy
Normal	NHDF	0.84	0.066	n. d.	n. d.	3.8	n.d.
Kidney	ACHN	0.76	0.265	n. d.	n. d.	8.7	6800
Prostate	DU145	0.30	0.149	n. d.	n. d.	5.9	3800
Prostate	PC3	0.19	0.073	n. d.	n. d.	2.9	3100
Lung	H226	0.18	0.034	n. d.	n. d.	1.6	2400
Lung	HOP62	0.42	0.055	177	46	3.0	4600
Breast	MB231	0.27	0.142	n.d.	n.d.	5.3	4200
Gastric	MKN45	0.28	0.076	n.d.	n.d.	3.9	5200
Colon	SW620	0.82	0.160	n.d.	n.d.	6.1	4800

meaningful IC_{50} values whose values were relatively lower when compared to the two hybrids that were synthesized with a dimethoxy unit instead of dihydroisocoumarin unit. The structural variations of psymberin and its epimers when compared with the two hybrids indicate that retention of dihydroisocoumarin unit is essential for the biological activity of psymberin [70]. Furthermore, it was revealed that

structural alterations of psymberin could result in decreased cellular uptake of these compounds in HeLa cells compared to psymberin [70]. After incubation of HeLa cells with 100 nM of psymberin and its epimers, the intra-cellular concentration of psymberinepimers (4-epi & 8-epi) was about 20-fold less than pysmberin and the intra-cellular concentration of psympederin was below the limit of detection [70]. The same study [70] employing forward genetic screen in *C. elegans* identified that ribosome was the primary target for psymberin and also demonstrated that in contrast to other pederins, psymberin does not exhibit blistering activity due to the absence of acetal group. Most recently, Pietruszka group synthesized 8-desmethoxy pysmberin, a putative biosynthetic precursor of psymberin in 25 steps whose biological activity is yet to be determined [58].

Psymberin exhibits very potent antiproliferative activity against a wide selection of human tumor cell lines including KM12, PC3, SK-MEL-5, and T98G with IC_{50} at range 0.45–2.29 nM [46]. The data pertinent to differential sensitivities of various cell lines to psymberin is summarized in Table 32.2. Crews and co-workers [46] also reported that psymberin exhibits phenomenal activity against a human colon cancer cell line HCT-116 with IC_{50} at range 2.5×10^{-9} M. It is interesting to note that according to the authors [46] ring A is not essential for activity, but in contrast *N*-acylaminal functionality is absolutely crucial for cytotoxicity.

32.7 Summary and Conclusions

The mycalamides and their congeners, which were initially isolated from various natural sources, have garnered significant attention from the scientific community in recent years. Owing to their limited supply from the natural sources, several research groups have focused on their biosynthesis and biological evaluation. The various schemes presented in this chapter highlight the selected synthetic approaches employed for preparing structurally unique motifs and their analogs. In addition, recent advancements in the synthetic approaches to these compounds have improved their total synthesis. These compounds and their derivatives have been evaluated for their biological activities and are proven to be very effective in extremely low concentrations. Potent cytotoxicity was observed when various cancer cell lines were treated with mycalamides, psymberin, and their analogs in the lower nanomolar range. Similarly, mycalamides have also demonstrated antiviral properties and T-cell-modulating potential. Hence, advances with this class of natural products exemplify the power of an interdisciplinary approach to drug discovery. The design of newly functionalized analogs, with potent anticancer, antiviral, and immunosuppressive activity, can now be realized.

While much remains to be revealed about the biological properties of psymberin, pederin, mycalamides, and analogs, it is reasonable to conclude that the novel synthetic approaches discussed here and in recent reviews [71, 72] will evolve to yield more potent and more highly-targeted compounds in the near future.

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Chapter 33
Antitumor Effects of Sea Hare-Derived Compounds in Cancer

Masaki Kita and Hideo Kigoshi

Abstract Sea hares (family Aplysiidae) are a rich source of bioactive substances. Especially, over the past 40 years, the genera *Aplysia* and *Dolabella* have afforded numerous bioactive secondary metabolites that exhibit antitumor activity. For example, the depsipeptide dolastatin 10 and its analogue are currently in cancer clinical trials. Meanwhile, the chemical probe approach has revealed that the antitumor macrolide aplyronine A inhibits microtubule assembly in association with actin. This article highlights the recent findings regarding the chemical biology of antitumor and antineoplastic compounds from sea hares, as well as molecules that have been discovered and characterized after 2000.

Keywords Antitumor · Sea hare · *Aplysia* · Antitumor · Actin-deplymerization · Cytotoxicity · Tubulin

33.1 Introduction

Sea hares, which belong to the opisthobranch group of mollusks (clade Aplysiomorpha), are shell-less, slow-moving benthic marine mollusks [1]. The order Anaspidea includes two families, Akeridae and Aplysiidae [2]. Almost all chemical studies have focused on Aplysiidae specimens. The family Aplysiidae includes nine genera, *Aplysia* (Linnaeus 1767), *Bursatella* (Blainville 1817), *Dolabella* (Lamarck 1801), *Dolabrifera* (Gray 1847), *Notarchus* (Cuvier 1817), *Petalifera* (Gray 1847), *Phyllaplysia* (Fischer 1872), *Syphonota* (Adams 1854), and *Stylocheilus* (Gould 1852). Sea hares are herbivorous, and are typically found on seaweed in shallow water. They are not eaten by other marine animals and have been postulated to contain chemical defense substances. The poisonous properties of sea hare secretions were known in Roman times. In addition, sea hares release ink from their ink glands, which deter predators. This ink acts as a screen, while at the

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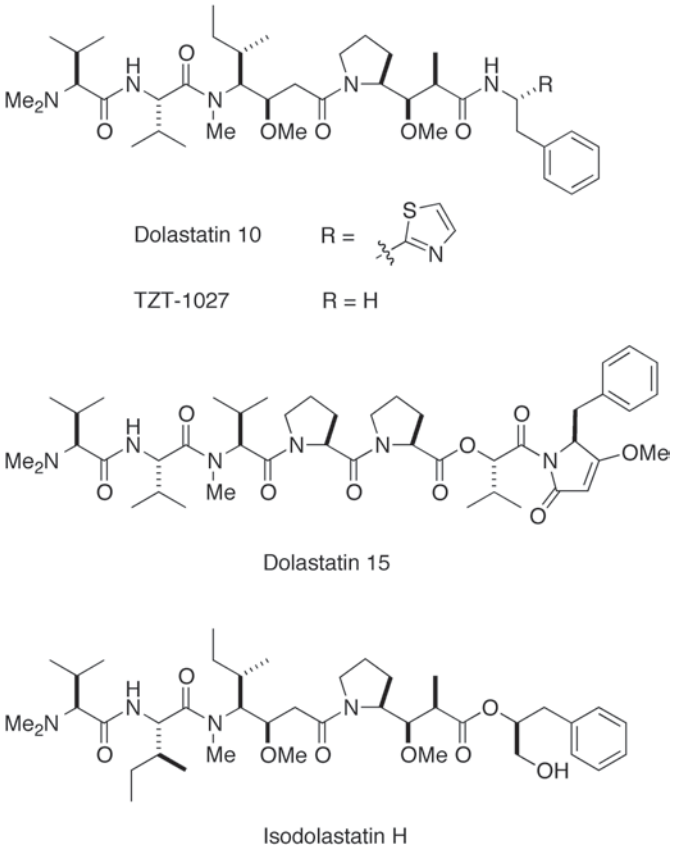
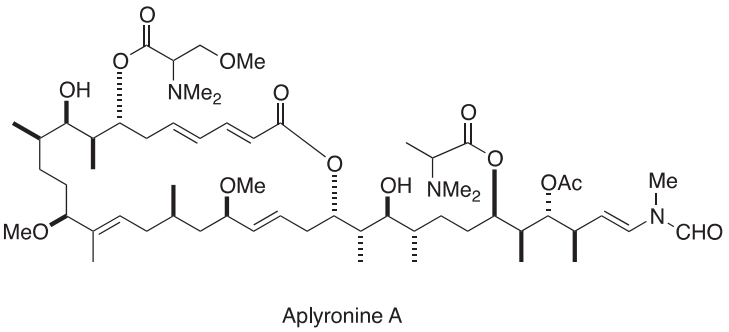


Fig. 33.1 Antitumor compounds and its derivatives from *Dolabella* sp

same time adversely affecting the smell sensors of predators [3]. Several defense substances have been investigated, such as a cytolytic factor from the purple fluid of the sea hare *Aplysia kurodai*, and antineoplastic glycoproteins from their eggs or albumen gland [4, 5].

Sea hares are known to be a rich source of bioactive substances. Especially, over the past 40 years, the genera *Aplysia*, *Dolabella*, and *Stylocheilus* have afforded numerous bioactive secondary metabolites. Pettit and co-workers have investigated the bioactive constituents of the Indian Ocean sea hare *Dolabella* sp., and have isolated novel peptide- and depsipeptide-type compounds called dolastatins 1–18 [6–8]. These mixed peptide–polyketide backbones are derived from a polyketide synthase/non-ribosomal peptide synthetic pathway (PKS/NRPS), and most of them are known to have antineoplastic properties. Dolastatin 10 has been shown to have unprecedented potency in experimental antineoplastic and tubulin assembly systems (Fig. 33.1). Dolastatin 10 and three structural modifications are currently in human cancer phase II and III clinical trials [9, 10]. For example, a synthetic derivative,



Aplyronine A

Fig. 33.2 Antitumor compound from *Aplysia* sp

TZT-1027 (= auristatin PE), has completed a phase II evaluation in advanced or metastatic soft-tissue sarcomas [11]. Dolastatin 15 is another potent antiproliferative metabolite of *D. auricularia*. Recently, it was shown that dolastatin 15 induces apoptosis and G2/M cell cycle arrest in human multiple myeloma cells [12]. Two derivatives of dolastatin 15 are also in cancer clinical trials (phase I–II) [13]. For a general review of dolastatins and related marine peptides and depsipeptides for clinical trial, see the references [14, 15].

On the other hand, Yamada and co-workers intensively examined the cytotoxic constituents of two species of sea hares, *A. kurodai* and *D. auricularia*, which were collected off the coast of Shima peninsula, Japan. Structurally and functionally diverse secondary metabolites have been discovered, and some have been shown to have antitumor effects against xenograft in mice, including isodolastatin H from *D. auricularia* and the polyketide macrolide aplyronine A from *A. kurodai* (Fig. 33.2). Isodolastatin H has been shown to exhibit antitumor activity against P388 leukemia with a T/C value of 141 % [16]. Yamada and co-workers reviewed the chemical and biological aspects of the antitumor compounds from two sea hare species [17–20]. Another review in 2009 [21] focused on the chemistry and biology of aplyronines as actin-depolymerizing agents. Kamiya [22] and Miyamoto [23] also reviewed the secondary metabolites from Japanese sea hare.

Recent progress in chemical biology research has accelerated identification of the targets of bioactive natural products as well as observations of their dynamic behaviors in living systems. In addition, photoaffinity labeling combined with mass spectrometric approaches has been developed as a useful proteomics tool. In early studies, aplyronine A was characterized as an actin-depolymerizing agent. Through the use of fluorescence microscopy observations and photoaffinity-tag experiments, we recently showed that aplyronine A synergistically binds to tubulin in association with actin, inhibits tubulin polymerization, and prevents spindle formation and mitosis in tumor cells [24]. Tubulin-targeting agents have been widely used in cancer chemotherapy [25–27]. To our knowledge, however, aplyronine A is the first microtubule inhibitor that also binds to actin and affects microfilament dynamics.

It is generally believed that most of these bioactive secondary metabolites are not actually produced by the sea hare itself, but rather are instead produced by cyanobacteria, which are first consumed by the sea hare while it grazes on algae and seaweed, and then concentrated in its digestive glands. For example, several analogues of the dolastatins, originally derived from *D. auricularia*, have been found in the marine cyanobacteria *Lyngbya majuscula* and *Symploca hydnoides* [28, 29]. Conceivably, these sequestered compounds may function as a chemical defense for the sea hare against predators. Several reviews have discussed the structural and functional resemblance of marine cyanobacterial metabolites in marine algae, sponges, and mollusks [30–33]. Fermentation methods using marine cyanobacteria may eventually be competitive with total syntheses for the scale-up production of new anticancer drugs in this family.

This article highlights recent findings in studies on the chemical biology of aplyronine A, as well as various sea hare-derived antitumor and antineoplastic compounds, with emphasis on promising compounds that have been recently discovered or characterized (especially after 2000).

33.2 Chemistry and Biology of Aplyronines

33.2.1 Isolation and Structure

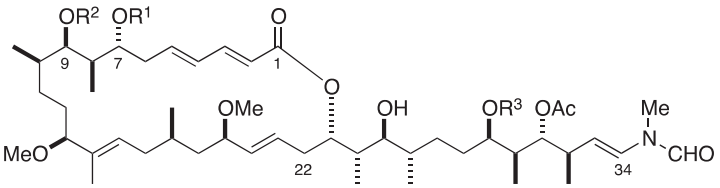
Aplyronine A was originally isolated by eight-step chromatographic separation of the lipophilic extract of the sea hare *A. kurodai* guided by a cytotoxicity assay against HeLa S3, a human cervical carcinoma cell line [34]. Seven minor congeners, aplyronines B–H have also been isolated (Fig. 33.3) [35].

The absolute stereostructure of aplyronine A, which has 17 asymmetric centers, was determined by NMR analysis and the asymmetric synthesis of five fragments obtained from chemical degradation [34–38]. Aplyronine A was revealed to be an inseparable mixture of four diastereomers with respect to two amino acid esters (ca. 1.1:1 and 3:1 for the *N*,*N*,*O*-trimethylserine and *N*,*N*-dimethylalanine ester moieties, respectively, and the *S*/*R* ratios varied among the animal specimens examined): this was confirmed by the asymmetric synthesis of aplyronine A as a diastereomeric mixture of amino acid esters with the same ratios as in the natural compound [39–41].

The structures of aplyronines B–H were elucidated by means of spectral analysis, as with aplyronine A. While aplyronine E was considered to be 22-methylaplyronine A, aplyronines D and F–H were deduced to contain two amino acid residues, one of which was different from both of those in aplyronine A.

33.2.2 Asymmetric Synthesis of Aplyronines A–C

In 1994, Yamada and co-workers achieved the asymmetric synthesis of aplyronine A (Fig. 33.4), by a convergent route in 0.39% overall yield based on the longest



	R^1	R^2	R^3	IC_{50} (ng/mL)
Aplyronine A	TMSer	H	DMAla	0.48
Aplyronine B	H	TMSer	DMAla	3.11
Aplyronine C	H	H	DMAla	21.1
Aplyronine D	TMSer	H	DMGly	0.075
Aplyronine E	= 22-methylaplyronine A			0.201
Aplyronine F	TMSer	H	MMAla	0.201
Aplyronine G	DMSer	H	DMAla	0.127
Aplyronine H	H	DMSer	DMAla	10.4

Fig. 33.3 Structure and cytotoxicity of aplyronines. TMSer, *N*,*N*,*O*-trimethylserine; DMSer, *N*,*O*-dimethylserine; DMAla, *N*,*N*-dimethylalanine; DMGly, *N*,*N*-dimethylglycine; MMAla, *N*-methylalanine. Cytotoxicity data for HeLa S3 cells were taken from the references [18] and [41]

linear sequence (47 steps); the average yield for each step was 89 % [39]. This synthesis included Julia olefination between the C5–C20 segment and the C21–C34 segment, followed by the Horner–Wadsworth–Emmons (HWE) reaction and Yamaguchi macrolactonization as key steps. The total syntheses of aplyronines B and C were also accomplished by the same group in 1996 [41, 42].

Recently, Paterson and co-workers accomplished a highly stereocontrolled total synthesis of aplyronine C [43]. This route exploits an aldol methodology to install the requisite stereochemistry and features a boron-mediated aldol coupling of an *N*-methyl enamide-bearing methyl ketone with a macrocyclic aldehyde to introduce the full side chain.

33.2.3 Cytotoxicity and Antitumor Activity

Aplyronines A–H exhibit cytotoxicity against HeLa S3 cells in vitro. Five of the eight aplyronines A, D–G are particularly strong cytotoxins (Fig. 33.3). The structural feature that is common to these five aplyronines is the presence of the methylated serine ester moiety at C7 of the macrolide part of the molecule: aplyronine A is more cytotoxic (ca. 50 times) than aplyronine C, which lacks the methylated serine ester group. Furthermore, the location of the methylated serine ester group in the molecule affects the cytotoxicity: aplyronine A is considerably more cytotoxic than aplyronines B and H.

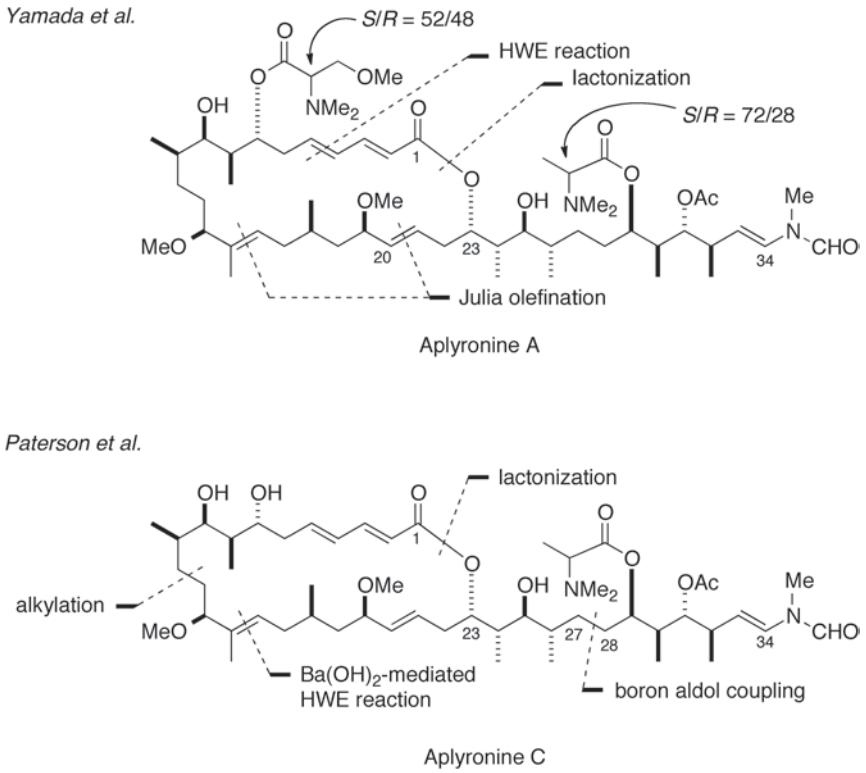


Fig. 33.4 Outline of the asymmetric synthesis of aplyronines

Aplyronine A has been shown to exhibit potent antitumor activity in vivo against five tumor cell lines [18, 21, 34] (Table 33.1). Its antitumor activity against P388 leukemia, Lewis lung carcinoma and Ehrlich carcinoma is particularly noteworthy.

33.2.4 Interaction with Actin

In 1996, the primary target molecule of aplyronine A was shown to be actin [44]. Actin is the most abundant protein in the eukaryotic cytoskeleton and is essential for the regulation of various cellular functions, such as muscle contraction, cell division, and the migration of tumor cells. Aplyronine A forms a 1:1 complex with globular actin (G-actin, monomer), and inhibits the polymerization of G-actin to fibrous actin (F-actin, polymer). Mycalolide B [45, 46], a cytotoxic tris-oxazole macrolide from the marine sponge *Mycale* sp., also depolymerizes F-actin by severing it to form a 1:1 complex with G-actin (Fig. 33.5) [47]. The side-chain portion of aplyronine A was deduced to participate in its binding to actin, based on the structural similarity of the side-chains in the two compounds.

Table 33.1 Antitumor activity of aplyronine A

	Dose (mg/kg/day)[a]	Test/Control (%)
P388 leukemia	0.08	545
Colon 26 carcinoma	0.08	255
Lewis lung carcinoma	0.04	556
B16 melanoma	0.04	201
Ehrlich carcinoma	0.04	398

[a] On days 1, 2, 3, 4, 5, intraperitoneally (i.p.)

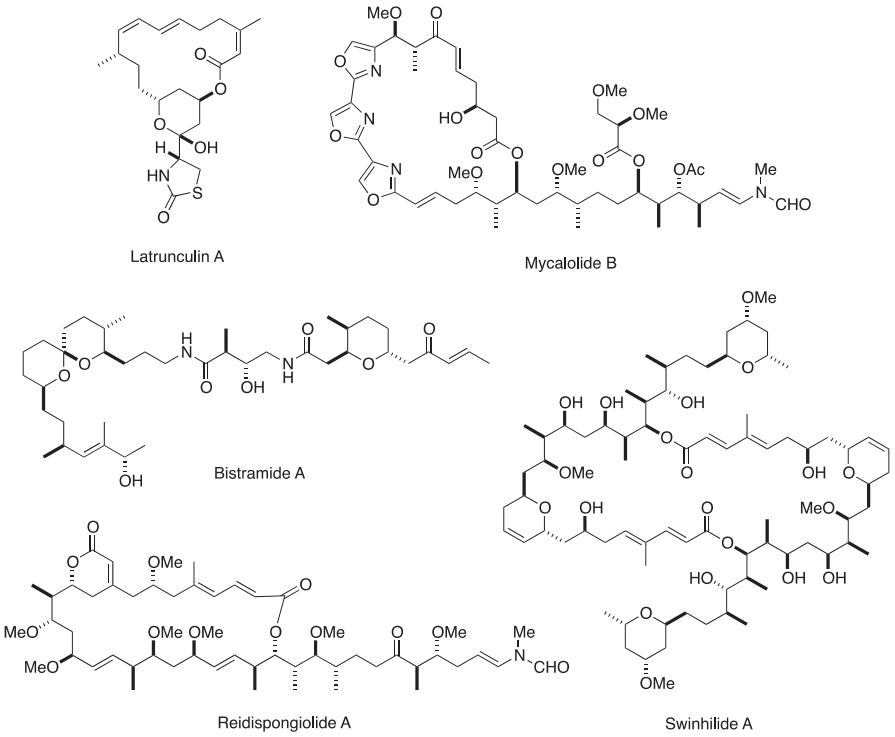


Fig. 33.5 Representing actin-depolymerizing marine natural products

In the 1980s, the Kashman group identified latrunculins of marine origin and investigated their biological properties [48, 49]: latrunculins were the first actin-binding substances to be isolated from a marine source. To date, various actin-depolymerizing agents have been found in marine invertebrates, such as ulapualides [50], mycalolides [45–47, 51, 52], kabiramides [53, 54], sphinxolides/reidispongiolides [55, 56], swinholides [57, 58], and bistramides [59–61], and they generally show potent cytotoxicity. They are considered to be useful for the development of novel pharmacological tools for analyzing actin-mediated cellular functions. Thus, a great deal of attention has been paid to the synthesis and function of actin-targeting

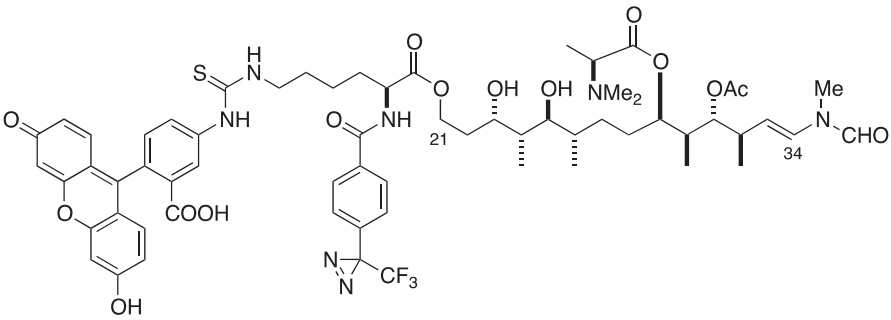


Fig. 33.6 Aplyronine side chain photoaffinity probe

compounds and their use as effective molecular probes in chemical biology. Excellent reviews are available for comprehensive details regarding actin-targeting natural products [62–65].

33.2.5 Structure-Activity Relationships

After the total synthesis of aplyronines, studies of the structure-activity relationship in aplyronine derivatives, including both cytotoxicity and actin-depolymerizing activities, have been performed [66, 67]. For aplyronine A to reveal strong cytotoxicity, the combination of the macrolide ring part and the side-chain part was found to be essential, since both the macrolactone analogue (C1–C23) and the side-chain moiety (C21–C34) alone showed extremely weak cytotoxicity. Furthermore, the presence of the *N*,*N*,*O*-trimethylserine ester, the conjugated diene and two hydroxyl groups is important, while the C14 methyl, the *N*-methyl enamide and the *N*,*N*-dimethylalanine ester group are not essential.

As for the structure-actin depolymerizing activity relationship, the side-chain moiety of aplyronine A was shown to play a key role in the actin-depolymerizing activity, since a synthetic side-chain analogue revealed relatively strong activity while the macrolactone moiety was totally inactive. Interaction between the side-chain part of aplyronine A and actin was also demonstrated by photo-affinity labeling experiments (Fig. 33.6) [68]. Since both aplyronine A and mycalolide B competitively inhibited the binding of the side-chain probe to actin, this probe was revealed to bind to actin specifically at the same binding site in these macrolides: these results also supported the notion that aplyronine A and mycalolide B bound to actin at the same site.

As described above, the side-chain part of aplyronine A is essential for both its cytotoxicity and actin-depolymerizing activity. On the other hand, its cytotoxicity is markedly influenced by the trimethylserine ester, the conjugated diene and two hydroxyl groups, whereas these functional groups only affect actin-depolymerizing activity to a quite small extent.

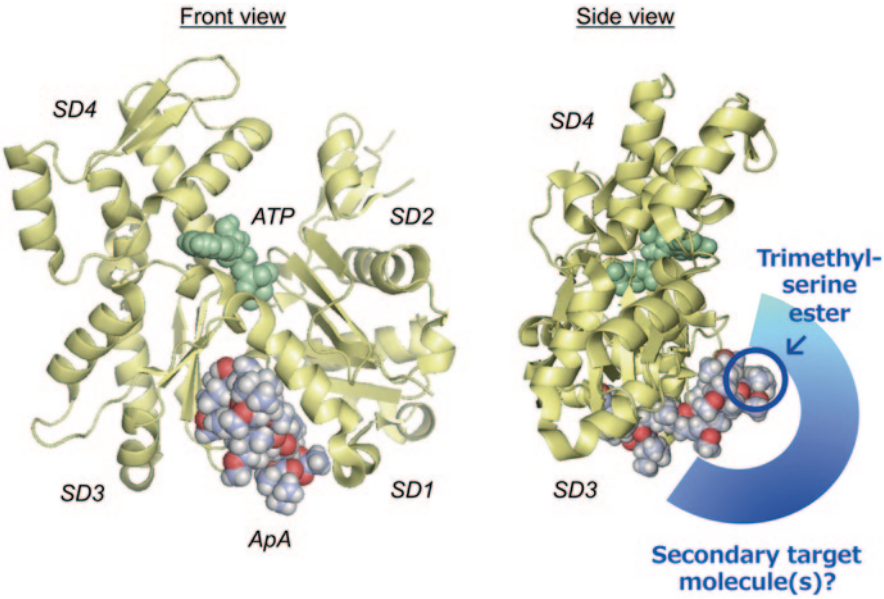


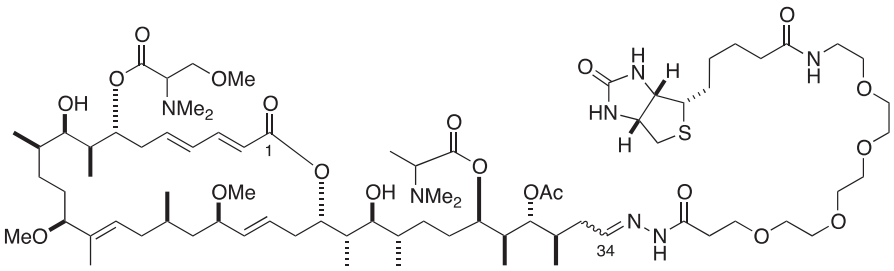
Fig. 33.7 Structure of actin–aplyronine A complex. [PDB: 1WUA]

33.2.6 Structure of the Actin–Aplyronine A Complex

In 2006, X-ray analysis of the actin–aplyronine A complex was performed at a resolution of 1.45 Å, and showed that aplyronine A binds to a hydrophobic cleft between subdomains (SD) 1 and 3 of actin (the barbed end) by intercalating its side-chain part (Fig. 33.7) [69]. X-ray crystal structures of the complexes of actin with other agents, such as kabiramide C [70], sphinxolide B [71], reidispongiolides A and C [72], swinholide A [72], and bistramide A [73], have also been reported. The contacts between actin and each macrolide are similar to those observed in the actin–aplyronine A complex. Consequently, the hydrophobic interactions between the cleft of actin and aplyronine A play a key role in the actin-depolymerizing activity. Meanwhile, the C7 trimethylserine moiety protrudes toward the bulk solvent region of the actin–aplyronine A complex [69]. The above findings suggest that aplyronine A first binds to actin to form the actin–aplyronine A complex, which then binds to another biomolecule to exhibit cytotoxicity: the protruding trimethylserine ester group of aplyronine A would play an important role in the second stage.

33.2.7 Preparation of Aplyronine Chemical Probes and Observation of Localization in Tumor Cells

To identify the hypothetical secondary target molecules of aplyronine A and to further investigate its mode of action, chemical probes were prepared. According to



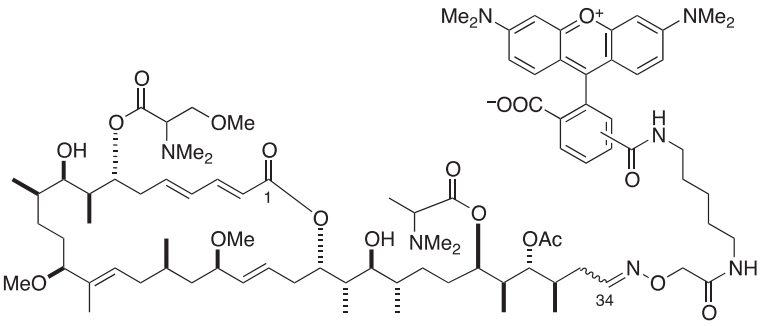


Fig. 33.8 Aplyronine A chemical probes

synthetic studies on biocytinylated mycalolide and kabiramide derivatives [52], acidic hydrolysis of the *N*-methyl enamide moiety of natural aplyronine A followed by condensation with hydrazide gave a PEG-linked aplyronine A biotin derivative (ApA–Bio) (Fig. 33.8) [74]. Similarly, aplyronine A C34 aldehyde was condensed with an oxyamine to afford tetramethylrhodamine (TAMRA)-conjugated aplyronine A (ApA–FL) [75]. The cytotoxicity of ApA–Bio was only ca. 10-fold less than that of aplyronine A (IC_{50} 96 pM). ApA–FL also showed potent cytotoxicity against HeLa S3 cells (IC_{50} 370 pM). Both derivatives exhibited actin-depolymerizing activity in vitro, comparable to that of natural aplyronine A.

Fluorescent derivatives of aplyronines A and C were similarly incorporated into HeLa S3 cells, and were well-retained and distributed throughout the cytoplasm, suggesting that aplyronines A and C have similar cellular permeability and accumulation levels [75]. Due to the high concentration of intracellular actin and the high affinity of aplyronines for actin, they might be trapped within cells as an actin complex. Furthermore, ApA–FL caused rapid and prominent disassembly of the actin cytoskeleton in HEp-2 cells, as in the case of HeLa S3 cells treated with aplyronine A or ApA–Bio [74]. These results suggested that aplyronine A and its derivatives might disrupt some cell-adherent systems or signaling pathways.

33.2.8 Dephosphorylation of FAK and Caspase-Dependent Apoptosis in Tumor Cells Caused by Aplyronine A

In general, protein tyrosine phosphorylation, which is important for cell growth and survival, is widely up-regulated in tumor cells. Among such survival signals, treatment with 1 μM aplyronine A induced the dephosphorylation of focal adhesion kinase (FAK, Tyr576/Tyr577) in HeLa S3 cells [75]. This effect was mostly identical to that caused by the apoptogenic agent staurosporine (1 μM). FAK provides critical survival signals for cells to resist apoptosis and is important for cell motility, invasion, metastasis, and angiogenesis [76]. Since caspase-mediated FAK dephosphorylation/proteolysis has been implicated in focal adhesion disassembly during apoptosis in endothelial cells [77, 78], aplyronine A might affect similar signaling pathways during apoptosis in tumor cells. In fact, it significantly reduced cellular viability and induced DNA fragmentation at 1 nM in HeLa S3 and human leukemia HL60 cells [79]. In addition, treatment with aplyronine A at 1 nM for 24 h led to potent caspase 3 activation in HeLa S3 cells, while treatment with aplyronine C did not, even at 100 nM [80]. These significant differences in apoptosis-related activities were consistent with their cytotoxicity, which highlighted the importance of the C7 trimethylserine moiety for the potent activities of aplyronine A.

To date, various actin-polymerization-stimulating or -blocking molecules have been shown to cause apoptosis, which induces potent cytotoxicity; examples include cytochalasin D (100 nM against T-cells) [81], jasplakinolide (100 nM against HL-60 cells [82] and T-cells [83]), latrunculin A (1–10 μM against MKN45 or NUGC-4 cells) [84], and mycalolide B (100 nM against HL-60 cells) [79]. It is unclear whether the dephosphorylation of FAK in tumor cells caused by aplyronine A directly induces caspase-dependent apoptosis, or is a consequence of caspase 3 activation. Still, the apoptogenic effect of aplyronine A (1 nM) is much stronger than those of other actin-targeting molecules, and thus might be very important in terms of its potent antitumor activity.

33.2.9 Target Identification of Aplyronine A Using Biotin Derivatives

Next, the interactions of biotinylated aplyronine A with whole cellular proteins were investigated. To minimize interference with intrinsic biotin-binding molecules, an excess amount of ApA–Bio was pre-treated with NeutrAvidin agarose, onto which the lysate of HeLa S3 cells was loaded. Two proteins, actin-related proteins 2/3 (Arp2 and Arp3), were specifically purified with abundant actin from the cell lysate [74]. Arp2 and Arp3 form the Arp2/3 complex, which binds to the sides of an existing actin filament and initiates the growth of a new actin filament to form branched-actin-filament networks [85–87]. Due to the high structural similarity between G-actin and actin-related proteins, especially around the hydrophobic clefts between subdomains 1 and 3, we expected that aplyronine A might bind to Arp2 or

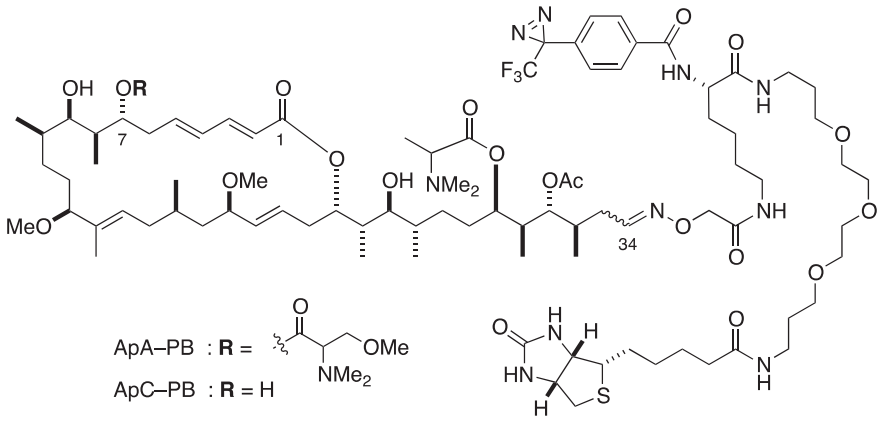


Fig. 33.9 Phoroaffinity biotin probes of aplyronines

Arp3 to give 1:1 complexes, and inhibit the ability of the Arp2/3 complex. These properties might enhance the potent disassembly of actin filaments caused by aplyronine A.

33.2.10 Photoaffinity Biotin Probes of Aplyronines and Their Interactions with Arp2/3

While Arp2 and Arp3 have been identified as presumed targets of aplyronine A, the detailed molecular mechanism and binding modes of aplyronine A with actin and actin-related proteins are still unclear due to their polymerization properties and instability as a complex in vitro. Thus, based on the finding that the C34 *N*-methyl enamide moiety of aplyronine A can be replaced with hydrogen bond acceptors (i.e., imines and hydrazones) without a significant loss of activity, an aplyronine A photoaffinity PEG-linked biotin derivative (ApA–PB) and its aplyronine C analogue (ApC–PB) were synthesized from natural aplyronines (Fig. 33.9) [88]. ApA–PB showed potent cytotoxicity against HeLa S3 cells (IC_{50} 1.2 nM) and inhibited actin polymerization, as with aplyronine A. In contrast, ApC–PB was ~260-fold less cytotoxic than ApA–PB. As mentioned above, aplyronine C, which lacks the C7 trimethylserine ester moiety, exhibits 44-fold less cytotoxicity, but just as much actin-depolymerizing activity, as aplyronine A. Therefore, we expected that ApC–PB would serve as a useful negative probe.

With the use of the photoaffinity biotin derivatives of aplyronines A and C, Arp2 and Arp3 were specifically purified as binding proteins along with actin from HeLa S3 cell lysate. As in the experiments with the side-chain photoaffinity derivative, actin was specifically photolabeled with ApA–PB, and this interaction was competitively inhibited by excess aplyronine A. However, Arp2 and Arp3 did not covalently bind to either aplyronine photoaffinity derivative. Thus, we concluded that

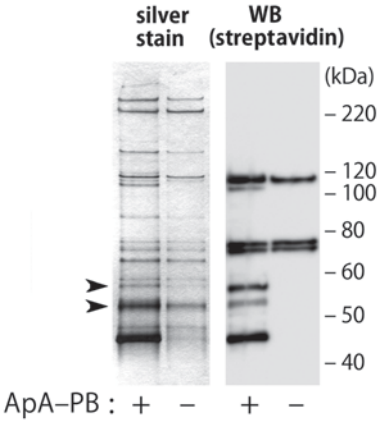


Fig. 33.10 *In situ* photolabeling experiments. HeLa S3 cells were treated with or without ApA–PB and then irradiated with UV (365 nm). Labeled proteins were affinity-purified with NeutrAvidin agarose, subjected to 10% SDS-PAGE, and detected with silver stain or HRP-conjugated streptavidin. The arrowheads show the proteins that were identified as α/β-tubulin. (Data were taken from the reference [24])

actin-related proteins might indirectly bind to aplyronine A as ternary adducts of the actin–aplyronine A complex or through oligomeric actin, and were not critical target proteins of aplyronine A that are essential for its potent antitumor activity.

33.2.11 Aplyronine A Binds to Tubulin by Interaction with Actin in Cells and in vitro

Next, photolabeling experiments with living tumor cells were carried out. Notably, actin (43–45 kDa) and several proteins (52–55 and 58 kDa) were detected, following *in situ* photoreaction with ApA–PB in HeLa S3 cells and subsequent affinity purification using NeutrAvidin agarose and silver staining (Fig. 33.10) [24]. Blotting analysis with streptavidin-HRP conjugate revealed biotinylated bands at 45, 55, and 58 kDa, consistent with the results of silver staining. Mass analysis of tryptic peptide fragments established that the silver-stained bands at 52–55 and 58 kDa included α- and β-tubulin. Western blotting analysis revealed that the 58 kDa band was β-tubulin that was covalently bound to ApA–PB. In contrast, only actin was photolabeled to the ApC–PB control. Together, these results suggest that aplyronine A interacts with both actin and tubulin.

While actin and aplyronine A alone each had little effect on tubulin polymerization in vitro, their 1:1 complex delayed nucleation and growth phases, and reduced the final polymer mass of tubulin [24]. Formation of a 1:1:1 complex of actin–aplyronine A–tubulin heterodimer (145 kDa) was demonstrated by gel permeation HPLC analysis. In contrast, the actin–aplyronine C complex did not attenuate microtubule growth or interact with tubulin.

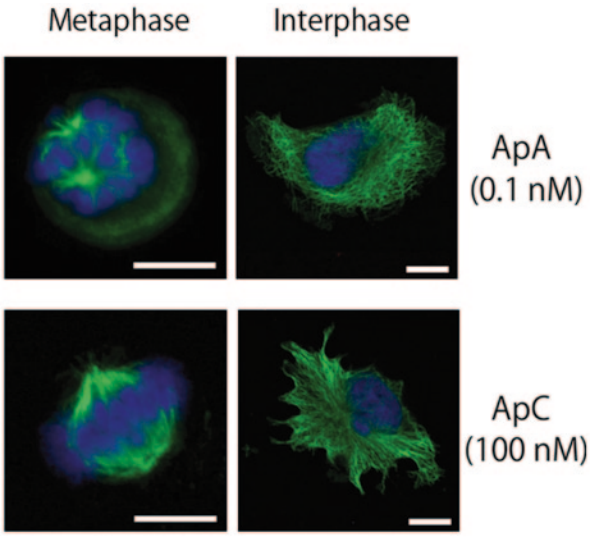
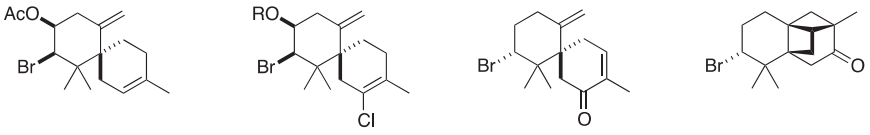


Fig. 33.11 Observation of HeLa S3 cells treated with aplyronines. Cells were immunostained with anti-α-tubulin (green) and co-stained with DAPI (blue). Scale bars = 10 μm. (Data were taken from the reference [24])

33.2.12 Aplyronine A Inhibits Spindle Formation and Mitosis in Tumor Cells

Immunostaining experiments showed that HeLa S3 cells treated with 100 pM aplyronine A had irregular, multipolar spindle structures with unaligned chromosomes (Fig. 33.11) [24]. The same treatment inhibited cell-cycle progression in M-phase. In contrast, treatment with ApC had no detectable effects on spindle formation, and inhibited cell-cycle progression in M-phase only at 100 nM. Since aplyronines A and C have almost the same effects on actin assembly in vitro, the significant differences in cytotoxicity, mitosis inhibition, and apoptogenic effects of these two compounds are likely to be due to the tubulin-binding properties of aplyronine A.

Microtubule–actin interactions underlie many fundamental cellular processes, such as cell motility, neuronal pathfinding, cell division, and cortical flow [89]. A variety of proteins mediate microtubule–actin interactions and regulate their dynamics. It is possible that aplyronine A modulates the coordination between microtubules and actin, and affects cytoskeleton dynamics. Actin is one of the most abundant cytoplasmic proteins, and thus it is possible that a variety of actin-targeting agents interact with multiple cellular targets via protein–protein interactions. Our findings regarding aplyronine A should provide further insights into the molecular mechanisms of structurally diverse natural products that regulate cytoskeletal dynamics.



Acetyldeschloroelatol | Acetylelatol R = Ac, Elatol R = H | Dactylone | aplydactone

Fig. 33.12 Halogenated marine sesquiterpenoids

33.3 Various Emerging Antineoplastic and Antitumor Metabolites from Sea Hares

Here, we focus on recent developments from chemical and biological studies on antineoplastic and antitumor compounds from sea hare. Several general reviews are available regarding the bioactive compounds obtained from marine organisms including sea hare [90–103]. In addition, several interesting reviews of natural product-derived compounds in clinical trials are also available [104–106].

33.3.1 Terpenoids

A variety of halogenated sesquiterpenoids have been isolated from the genus *Aplysia* since the 1970s. They exhibit various biological activities including anticancer, anti-HIV, alguicidal, ichthyotoxic, nematicidal, antiplasmodial, and antibacterial activities. Two chamigrenes, acetyldeschloroelatol and acetylelatol, were isolated from the sea hare *A. dactylomela* from the Canary Islands (Fig. 33.12) [107, 108]. While elatol shows moderate cytotoxicity against HeLa (human cervix carcinoma) and Hep-2 (human larynx carcinoma) cells (IC_{50} 1.3–2.0 μM), acetyldeschloroelatol and acetylelatol are inactive at 58 μM. These results support the hypothesis that the acetate derivatives decrease the toxicity of the corresponding alcohols in a strategy to store toxic metabolites acquired through the diet. Further investigation of dactylone, a known compound that was purified from A. *dactylomela*, established that this metabolite, at non-toxic doses, acted as a cancer-preventive agent by inhibiting the expression of cyclin D3 and cdk4, followed by cell cycle arrest and p53-independent apoptosis [109].

Despite the lack of biological evaluations, a unique sesquiterpenoid containing two fused four-membered rings, aplydactone, was isolated from the same *Aplysia* species [110]. Its structure was determined by extensive NMR and X-ray analyses. A biosynthetic pathway for the formation of the unprecedented carbon skeleton in aplydactone was suggested to be the result of [2+2]-cycloaddition in dactylone.

A variety of squalene-derived metabolites have been isolated from red alga. Among them, tetracyclic polyethers bearing the 6,6,6,5-oxacyclic ring system, such as thyrsiferol [111], venustatriol [112], and thyrsiferyl 23-acetate [113], have been investigated due to their strong biological activities, such as antiviral, cytotoxic and

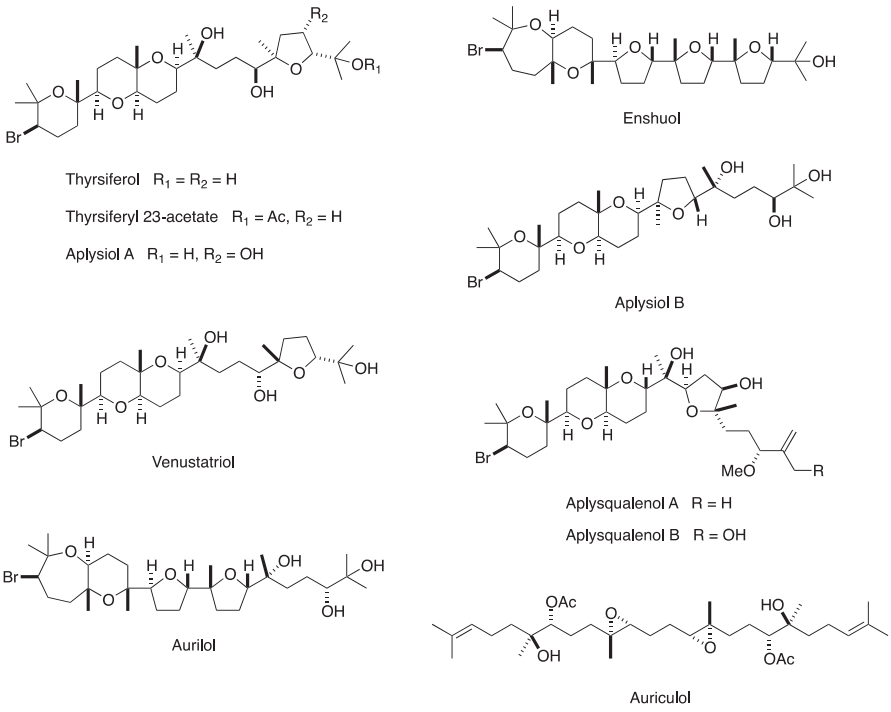
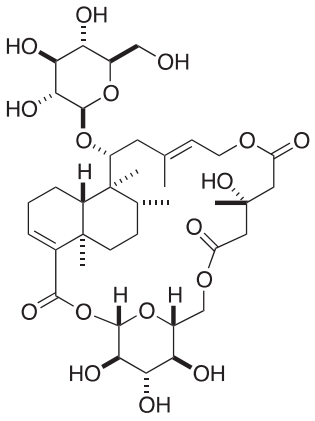


Fig. 33.13 Squalene-derived marine triterpenoids

protein phosphatase inhibitory effects (e.g., the ED_{50} of thyrsiferyl 23-acetate was 0.3 ng/mL against P388 cells) (Fig. 33.13) [114, 115].

Along with other secondary metabolites, sea hares have been shown to accumulate toxic terpenoids of algal origin. Aurilol is a tetracyclic oxasqualenoid from the sea hare *Dolabella auricularia* with novel a 7,6,5,5-oxacyclic ring system that exhibits moderate cytotoxicity against HeLa S3 cells (IC_{50} 4.3 μg/mL) [116]. Aurilol shares the A–D rings of enshuol, which was isolated from the Japanese red algae *Laurencia omaezakiana* [117]. Morimoto and co-workers elucidated the complete stereostructures of both aurilol [118] and enshuol [119] by asymmetric total synthesis.

Two triterpenoids bearing a bromine-containing pyran and a dioxabicyclo[4,4,0] decane ring, named aplysiols A and B, have been isolated from a South China Sea collection of the anaspidean sea hare *Aplysia dactylomela* [120]. Their structures were proposed mainly based on spectroscopic NMR techniques, Mosher's method, biogenetic considerations, and an integrated NMR-QM (Quantum Mechanical) approach. The selective presence of aplysiols on the mantle of the sea hare *A. dactylomela* suggested that these molecules may be involved in the chemical defense mechanisms of the mollusk. Recently, aplysiols C–E were isolated from the red alga *Chondria armata* [121], and aplysiols B and C were isolated from the red alga *Laurencia viridis* [122]. Further spectroscopic analysis and derivatization studies led to a revision of the stereochemistry for aplysiol B.



Syphonoside

Fig. 33.14 A macrocyclic glycoterpenoid syphonoside

In 2009, two bromotriterpene polyethers, aplysqualenol A and B, were isolated from the Caribbean sea hare *Aplysia dactylomela* [123]. Their chemical structures and relative configurational assignments were established from spectroscopic analysis supported by QM calculations. Aplysqualenol A exhibited potent inhibitory activity against SNB-19 CNS cancer and T-47D breast cancer, with IC_{50} values of 0.4 μM, as well as a range of antiviral properties. With the use of forward and reverse affinity purification methods, the target protein of aplysqualenol A was identified as dynein light chain (DYNLL1) [124]. Dynein is a motor protein that plays a major role in motion by regulating cell movement along microtubules and guiding the positioning of organelles within the cell, but the function of its light chain part is less understood [125]. The discovery of aplysqualenol A as a ligand to DYNLL1 suggests the possibility of small-molecule cytoplasmic dynein motor regulators.

In addition, auriculol, a highly oxygenated squalene, was isolated from the sea hare *D. auricularia*, and its stereostructure was determined by spectroscopic and chemical methods [126]. Auriculol exhibits moderate cytotoxicity against HeLa S3 cells (IC_{50} 6.7 μg/mL). Based on its oxidation sites, it is expected that aurilol is a biosynthetic precursor for a variety of squalene-derived polyether compounds.

A novel macrocyclic glycoterpenoid syphonoside was isolated from both the sea hare *Syphonota geographica* and the sea grass *Halophila stipulacea*, two Indo-Pacific species that migrated to the Mediterranean Sea through the Suez Canal (Fig. 33.14) [127, 128]. Structure determination and assignment of the absolute stereochemistry of this compound were accomplished through a combination of spectroscopic techniques, degradation experiments, and conformational analysis methods. Syphonoside did not exhibit cytotoxicity against human and murine cell lines at 400 μM, but did decrease the number of floating cells, especially in A431 and P19 cells at 440 and 360 nM, respectively. Therefore, it was suggested that syphonoside might suppress high-density-induced apoptosis, which may result in a prolonged viability and maximum cell density.

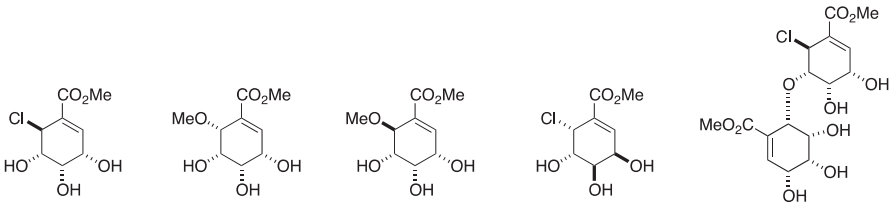


Fig. 33.15 Pericosines

33.3.2 Shikimate Derivatives

Pericosines A–E are shikimate-related metabolites of the fungus *Periconia byssoides*, which was found in the gastrointestinal track of the sea hare *A. kurodai* (Fig. 33.15) [129]. The stereochemistry of pericosine A was confirmed by its total synthesis from shikimic acid [130]. Pericosines A, B, and D inhibit the growth of murine P388 lymphocytic leukemia cells (ED_{50} 0.1, 4.0, 3.0 μg/mL, respectively) [131]. Pericosine A was evaluated in a disease-oriented panel of human cancer cell lines at the Japanese Foundation for Cancer Research (JFCR). Notably, it showed selective inhibition against the human breast cancer cell line HBC-5 (log GI_{50} −5.22) and the human brain cell line SNB-75 (log GI_{50} −7.27). Pericosine A also showed significant in vivo anti-tumor activity against P388 cells, and inhibited protein kinase EGFR (100 μg/mL) and topoisomerase II (100–300 μM) [132].

33.3.3 Peptides and Depsipeptides

Along with dolastatins 10 and 15 in clinical trials, the Pettit group has examined the structural modification of dolastatin 10 [133]. Replacement of the dolaphenine subunit of dolastatin 10 with aminoquinoline or tyramine phosphate groups led to the identification of analogues that exhibited potent cytotoxicity, and, in the case of phosphate derivatives, enhanced water-solubility (Fig. 33.16).

Several peptides and depsipeptides have been isolated from the sea hare *Dolabella auricularia*. Recent representative progress in studies on the mode of action includes studies with dolastatins 11 [134]. While the linear dolastatins 10 and 15 are known to target microtubules and inhibit mitosis, dolastatin 11 arrests cytokinesis in vivo and induces the hyperpolymerization of actin to stabilize F-actin in vitro, like phalloidin and jasplakinolide [135]. In 2003, the position of bound dolastatin 11 in F-actin was determined by the use of X-ray fiber diffraction analysis [136]. In the complex structure, dolastatin 11 was located in the gap between the two long-pitch F-actin strands to stabilize microfilaments.

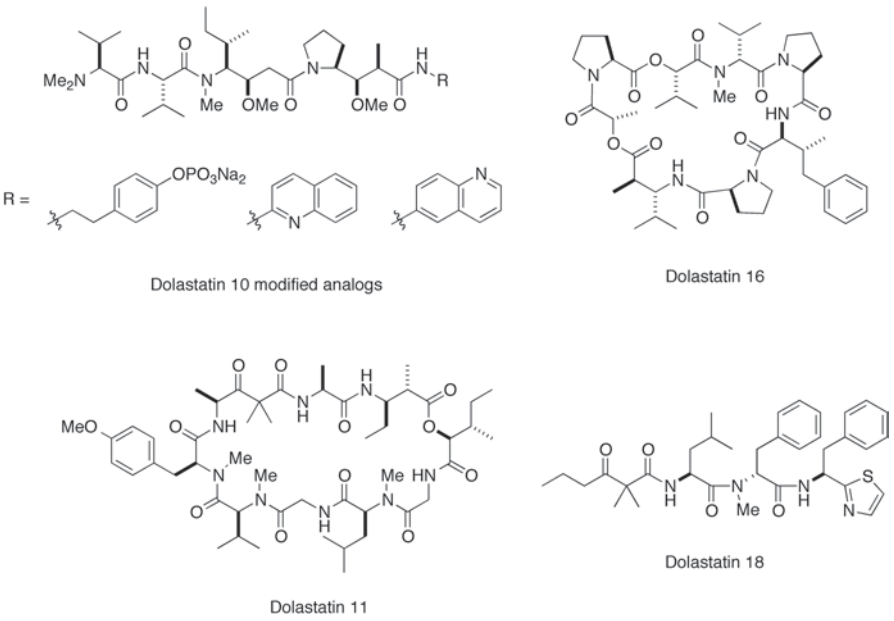


Fig. 33.16 Dolastatins and their modified analogs

With regard to structural studies, the X-ray crystal structure of dolastatin 16 was reported in 2011 [137], and stereochemistries of its two amino acid units, dolamethylleucine (Dml) and dolaphenvaline (Dpv), were established by synthesis [138]. Dolastatin 16 strongly inhibited the growth of the lung (NCI-H460, GI_{50} 0.96 ng/mL), colon (KM20L2, GI_{50} 1.2 ng/mL), brain (SF-295, GI_{50} 5.2 μg/mL), and melanoma (SKMEL-5, GI_{50} 3.3 μg/mL) cell lines. Dolastatin 18 was isolated in a small quantity, but a preliminary anticancer evaluation revealed that it significantly inhibited the growth of several human cancer cell lines [139]. Pettit and co-workers synthesized dolastatin 18 and reported its X-ray crystal structure in 2004 [140], which established its complete stereochemical assignment. Cancer cell-line evaluations using synthesized dolastatin 18 have also been reported (P388: ED_{50} 7.11 μg/mL; human tumors: GI_{50} 2.7–4.1 μg/mL).

Aurilide is a 26-membered cyclodepsipeptide that was isolated as a minor constituent of the sea hare *D. auricularia* (Fig. 33.17) [141]. It belongs to the mixed polyketide–polypeptide structural class and consists of five amino acids (Val, *N*-Me-D-Leu, *N*-Me-Gly, Val, and *N*-Me-Ala), *allo*-D-isoleucic acid, and a unique extended polyketide moiety. Its absolute stereochemistry was confirmed by analysis of its hydrolysis products and enantioselective synthesis [142, 143]. This molecule induces apoptosis to result in a potent antiproliferative effect against human cancer cells at the pM to nM range (e.g., IC_{50} 11 ng/mL against HeLa S3 cells). In the NCI human cancer cell panel, aurilide showed a high level of cytotoxicity (mean panel GI_{50} 0.12 μg/mL), and was selectively active against lung, ovarian, renal and

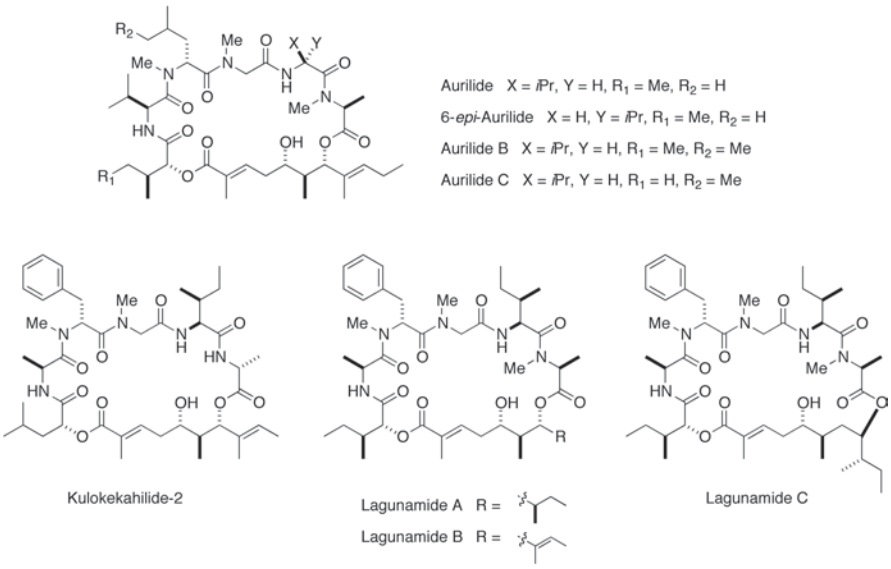


Fig. 33.17 Aurilides and their congeners

prostate cancer cell lines [143]. Aurilide showed unusually high in vivo antitumor activity in the NCI's hollow-fiber assays, and is expected to be a promising candidate for cancer treatment. Solid-phase library synthesis of aurilide and its derivatives was reported in 2003 [144]. Further structure-cytotoxicity relationship studies revealed that 6-*epi*-aurilide was almost non-cytotoxic (IC_{50}>4 μg/mL against HeLa S3 cells) [145]. With the use of affinity matrix chromatography for both aurilide and its 6-epimer conjugates, Uesugi and co-workers recently established that aurilide selectively binds to prohibitin 1 in mitochondria [146]. This study was the first to demonstrate that a small molecule induces apoptosis through a pathway that involves the remodeling of mitochondrial cristae controlled by prohibitin and dynamin-like GTPase optic atrophy 1 (OPA1), leading to permeabilization of the outer membrane and cell death.

Structural congeners of aurilide have been isolated from different marine organisms. Aurilides B and C were isolated from a strain of the marine cyanobacterium *Lyngbya majuscula* collected from Papua New Guinea, and their stereostructures were determined by spectroscopic analysis in 2006 [147]. From the same cyanobacterium, dolabellin and lyngbyabellins E–I were also isolated. As with aurilide, aurilides B and C showed significant in vivo antitumor activities based on NCI's hollow fiber assay. Kulokekahilide-2, a cytotoxin that is structurally related to aurilide, was isolated from the Hawaiian cephalaspidean mollusk *Philinopsis speciosa* [148, 149]. The total synthesis of kulokekahilide-2 was completed, and SAR studies revealed that the chirality at the L-Ala and *N*-Me-D-Phe residues is essential for its potent cytotoxicity [150]. Lagunamides A–C were isolated from the filamentous marine cyanobacterium *Lyngbya majuscula*, from Pulau Hantu, Singapore [151,

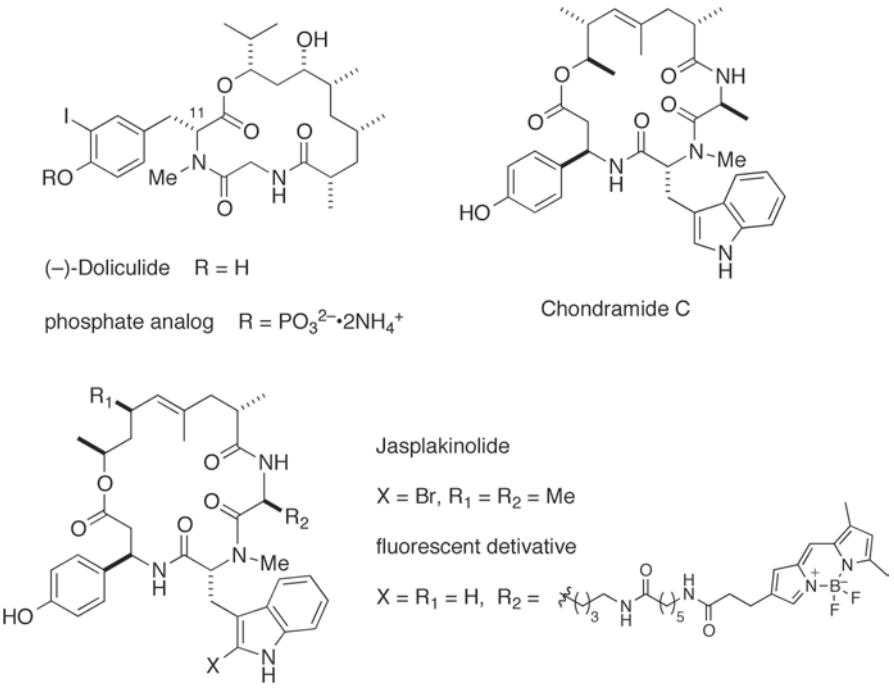


Fig. 33.18 Marine depsipeptides that enhance actin assembly

152]. Lagunamide C has a 27-membered ring system due to an additional methylene carbon in the polyketide moiety. Lagunamides A and C exhibited selective growth-inhibitory activity against a panel of cancer cell lines at nM order, including the cell lines P388, A549, PC3, HCT8, and SK-OV3. The isolation of aurilide congeners from marine cyanobacteria indicates that sea hares and other mollusks accumulate aurilides from their diet of marine cyanobacteria.

Doliculide was isolated as a minute constituent from the visceral organ of *Dolabella auricularia* (Fig. 33.18) [153]. Doliculide is a 16-membered depsipeptide with an iodotyrosine moiety and a dilactam-lactone ring. The asymmetric total synthesis of doliculide was achieved to establish its absolute stereostructure [154, 155]. Doliculide exhibits potent cytotoxicity against HeLa S3 cells with an IC_{50} value of 1.0 ng/mL. A synthetic analogue, 11-*epi*-doliculide, was shown to be 1000-fold less cytotoxic than doliculide. Meanwhile, a doliculide phosphate analogue showed an antitumor effect against Lu-61 xenograft in nude mice. Total syntheses of doliculide were reported in 2001 [156], 2004 [157], and 2012 [158].

With regard to biochemical studies, Hamel, Ghosh and co-workers reported that doliculide arrested cells at the G2/M phase by enhancing actin assembly [159]. Doliculide is structurally related to cyclodepsipeptides such as jasplakinolide (jaspamide) from the marine sponge *Jaspis* sp. [160, 161], and to chondramides from a terrestrial myxobacterium, *Chondromyces crocatus* [162, 163]. These depsipeptides are known as potent actin binders with higher cell-membrane permeability than the

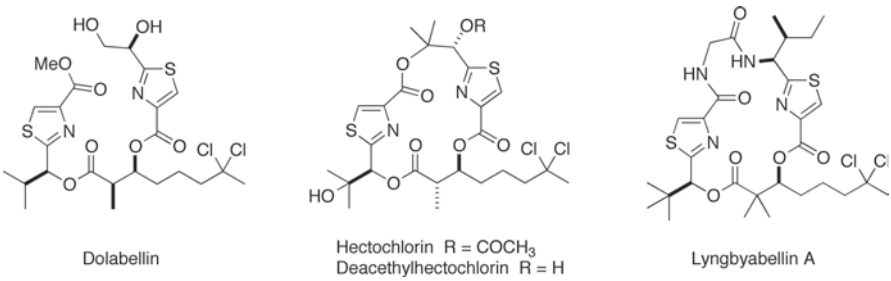


Fig. 33.19 Dolabellin and thiazole-containing depsipeptides

fungi-derived peptide phalloidin [164–166]. Recently, non-toxic fluorescent derivatives of jasplakinolide were synthesized, which selectively image static, long-lived actin filaments against dynamic F-actin and monomeric G-actin populations in living cells, without the disruption of actin dynamics [167]. These derivatives could be prominent imaging tools for cytoskeleton studies, especially in cases where genetic manipulation is either not feasible or unavailable.

Various peptides and depsipeptides with thiazole amino acids are known. Dolabellin is a bisthiazole substance that was isolated from the Japanese sea hare *D. auricularia* in 1995 (Fig. 33.19). Its structure was elucidated by spectral analysis and subsequent asymmetric synthesis [168]. Dolabellin showed moderate cytotoxicity against HeLa S3 cells (IC_{50} 6.1 μg/mL). Two structural congeners of dolabellin, hectochlorin and deacetylhectochlorin, were also isolated from the Thai sea hare *Bursatella leachii* [169]. Deacetylhectochlorin showed cytotoxicity against the human epidermoid carcinoma of the nasopharynx cell line KB, and the human small cell lung cancer cell line NCI-H187 with ED_{50} values of 0.31 and 0.32 μM. Hectochlorin was originally found in the marine cyanobacterium *Lyngbya majuscula* from Jamaica and Panama in 2002 [170]. It showed potent cytotoxicity against CA46 cells, a human Burkitt lymphoma line, and was as potent as jasplakinolide (IC_{50} 20 nM). Hectochlorin is equipotent to jasplakinolide in its ability to promote actin polymerization, but cannot displace a fluorescent phalloidin analogue from polymerized actin. In 2007, a hectochlorin biosynthetic gene cluster was cloned and its biological function was characterized [171]. Other bisthiazole substances that are structurally related to dolabellin and hectochlorin, lyngbyabellins A–I, were also isolated from the marine cyanobacterium *L. majuscula* from Guam, Parau, and Papua New Guinea [28, 172–176]. Lyngbyabellin A interferes with microfilament formation in cultured cells, and exhibits moderate cytotoxicity against the cell lines KB (human nasopharyngeal carcinoma) and LoVo (human colon adenocarcinoma) (IC_{50} 0.03 and 0.50 μg/mL, respectively). In vivo trials revealed that lyngbyabellin A is toxic to mice, and at sublethal doses (i.e., 1.2–1.5 mg/kg) there was no antitumor activity against the murine colon adenocarcinoma cell line C38 or the mammary adenocarcinoma cell line M16 [28].

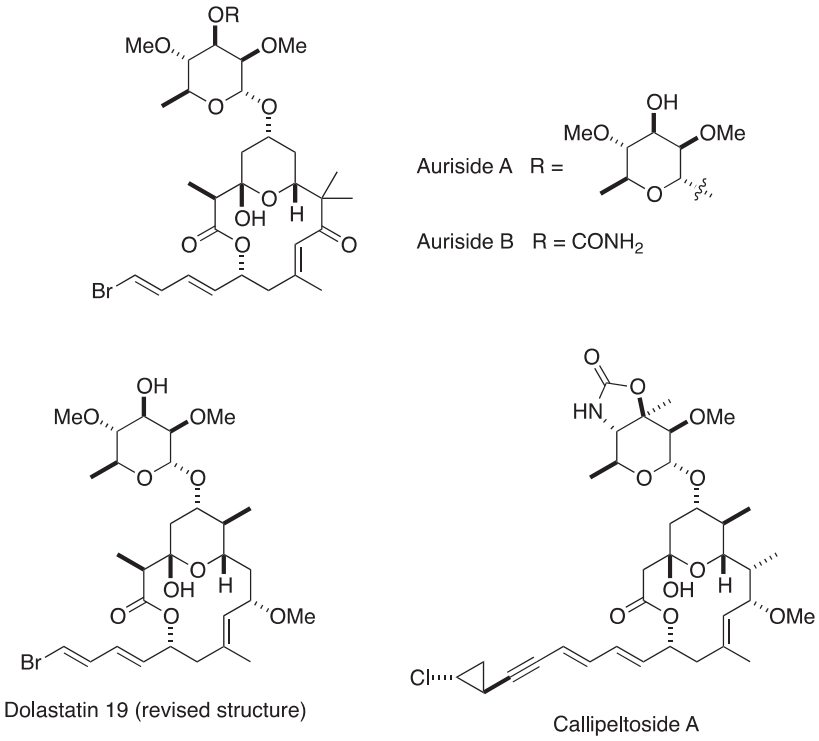


Fig. 33.20 Aurisides and their congeners

33.3.4 Polyketides

Bioassay-guided fractionation of the cytotoxic constituents of the Japanese sea hare *D. auricularia* by Yamada and co-workers led to the isolation of aurisides A and B (Fig. 33.20) [177]. NMR analysis and degradation experiments revealed that they are 14-membered macrolide glycosides with bromine-substituted conjugated diene, cyclic hemiacetal, and 2,4-di-*O*-methyl-L-rhamnopyranoside moieties. Aurisides A and B show cytotoxicity against HeLa S3 cells with IC_{50} values of 0.17 and 1.2 μg/mL, respectively. Two enantioselective total syntheses of aurisides A and B were reported in 2005 and 2006 [178, 179].

The Pettit group also isolated an auriside analogue, designated dolastatin 19, from the Gulf of California shell-less mollusk *D. auricularia* as a very minute constituent (0.5 mg from 600 kg *D. auricularia*) in 2004 [180]. Initial screening indicated that it significantly inhibits cancer cell growth (GI_{50} values of 0.72 and 0.76 μg/mL for breast MCF-7 and colon KM20L2 cells). In 2007, Paterson and co-workers accomplished the total synthesis of dolastatin 19 and revised its stereochemistry [181, 182]. Callipeltoside A, a structurally related cytotoxin from the sponge *Callipelta* sp., was also synthesized.

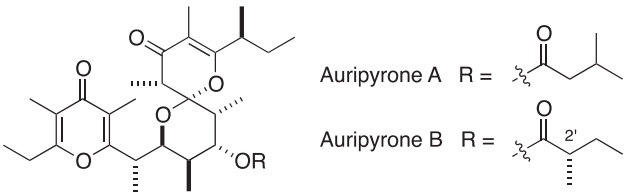


Fig. 33.21 Auripyrones

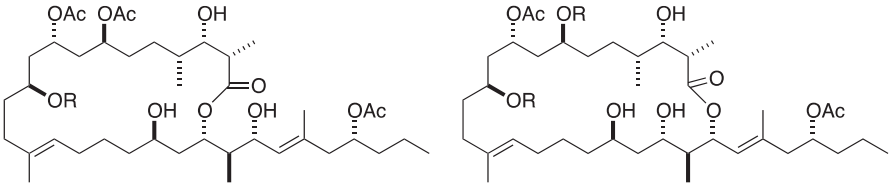


Fig. 33.22 Dolabelides

Two closely related polypropionates, auripyrones A and B possessing a γ-pyrone ring and a spiroacetal moiety, were isolated as minute constituents from the sea hare *D. auricularia* (Fig. 33.21) [183]. Auripyrones A and B exhibited cytotoxicity against HeLa S3 cells with IC_{50} values of 0.26 and 0.48 μg/mL, respectively. The relative stereochemistries of the two compounds, except for the configuration of C2' in auripyrone B, were deduced using detailed spectroscopic analysis. The first asymmetric total synthesis of auripyrone A was reported in 2006, and that of auripyrone B in 2009, which confirmed their absolute configurations [184–187]. The JFCR39 human cancer cell panel assay indicated that auripyrones might inhibit cancer cell proliferation through a novel mechanism [188].

Dolabelides A–D are 22- or 24-membered macrolides, and their stereostructures were determined by a combination of chemical and spectroscopic methods (Fig. 33.22) [189, 190]. While the detailed biological mechanisms that underlie the activities of dolabelides A–D have not been characterized, they exhibit moderate cytotoxicity against HeLa S3 cells (IC_{50} 6.3, 1.3, 1.9 and 1.5 μg/mL, respectively). Leighton and co-workers achieved the asymmetric synthesis of dolabelide D in 2006 [191], and this was followed by the synthesis of dolabelide C by Hanson's group in 2011. [192]

Aplysiatoxin, a polyketide bislactone with a spirocyclic acetal structure, was originally isolated from the digestive gland of the sea hare *Stylocheilus longicauda* [193], and shows irritant and tumor-promoting effects by activating protein kinase C (PKC) (Fig. 33.23). Meanwhile, bryostatin-1, a unique PKC activator that was isolated from the marine bryozoan *Bugula neritina*, shows little tumor-promoting activity, and is currently in clinical trials for the treatment of cancer. However,

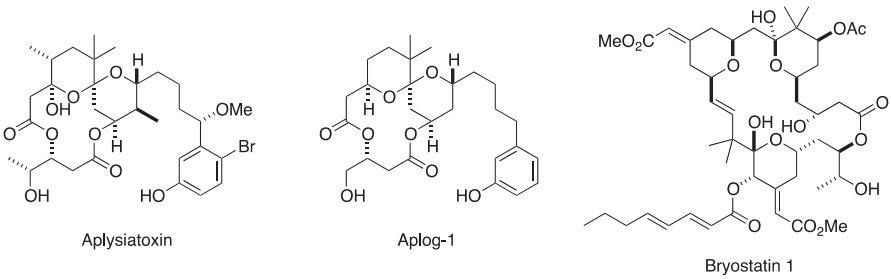


Fig. 33.23 Aplysiatoxin and bryostatin 1

its limited availability from natural sources and its synthetic complexity have hampered studies on its mode of action and development as a therapeutic agent. Recently, Irie and co-workers developed a simple and less-lipophilic analogue of tumor-promoting aplysiatoxin, and designated it aplog-1 [194]. Aplog-1 was found to be a novel PKC activator with anticancer and anti-tumor-promoting activities. Aplog-1 and bryostatin-1 bind selectively to PKC isozymes δ, η, and θ [195], and the unique biological activities of aplog-1 and bryostatin-1 are suggested to be derived from their ability to bind to PKCδ, an isozyme that is involved in apoptosis and which plays a tumor-suppressor role.

Malyngamides are structurally characterized as *N*-substituted amides of long-chain methoxylated fatty acids with a broad spectrum of bioactivities. To date, more than 30 congeners of malyngamides have been identified, and some of them display potential activity as immunosuppressive, anti-inflammatory, anticancer and anti-HIV agents [196, 197]. Recently, malyngamide C and its 8-*epi*-isomer were shown to inhibit bacterial quorum sensing [198, 199]. Although malyngamides were primarily isolated from marine cyanobacteria *Lyngbya* sp., some are derived from sea hares, such as *Stylocheilus longicauda* (malyngamides O and P) [200], and *Bursatella leachii* (malyngamides S [201] and X [202]), which are known to accumulate a wide variety of secondary metabolites from their diet (Fig. 33.24). Malyngamide O has a standard C_{14} fatty acid linked to acyclic amines with an enol methyl ether moiety. It exhibits moderate cytotoxicity against P388 mouse lymphoma, A549 human lung carcinoma, and HT29 human colon carcinoma with IC_{50} values of 2 μg/mL. Malyngamide S is a combination of the shorter 12-carbon fatty acid side chain and a highly substituted cyclohexane ring with diketo-functionality. It moderately inhibits the proliferation of HL-60 human leukemia cells (IC_{50} 6–8 μM), and exhibits NCI human tumor activity (panel average values: GI_{50} 16.6 μM). Malyngamide X is the first lyngbic acid connected to a new tripeptide backbone, and shows moderate cytotoxicity against KB oral human epidermoid carcinoma and NCI-H187 human small cell lung cancer (ED_{50} 8.2 and 4.1 μM, respectively). Progress in the synthesis of malyngamides was recently reviewed [203].

Almost all chemical studies have considered Aplysiidae specimens, whereas there have been few studies of Dolabriferidae mollusks. In 1996, Gavagnin and co-workers reported the first chemical study of the tropical opisthobranch *Dolabrifera*

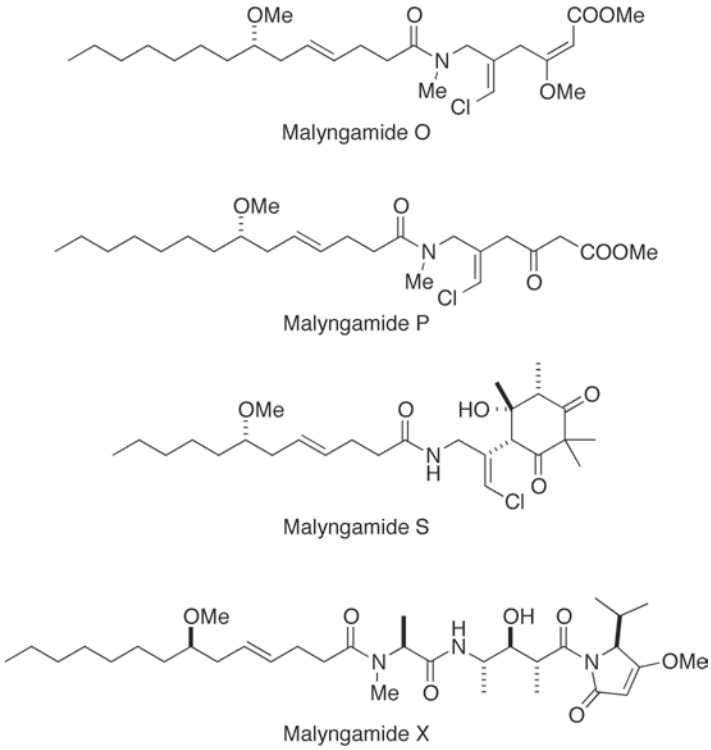


Fig. 33.24 Malyngamides

dolabrifera (Rang 1828), which led to the isolation of dolabriferol, a propionate-derived compound [204]. (Note: *D. dolabrifera* is currently classified as the family Aplysiidae.) Its gross and relative stereochemistry was established by X-ray crystallographic analysis. The absolute configuration of (−)-dolabriferol was confirmed by asymmetric total synthesis [205]. In addition, the Caribbean mollusk *D. dolabrifera* from Puerto Rico was shown to contain two congeners, dolabriferols B and C [206]. Their absolute configurations were determined by chemical degradation and X-ray crystallographic analysis. Biological screening of dolabriferols indicated no significant in vitro cytotoxicity toward A2058 melanoma or DU145 prostate cancer cells at 10 μM. Dolabriferol C showed weak inhibitory activity against *Mycobacterium tuberculosis* H37Rv at 128 μg/mL (Fig. 33.25).

33.3.5 Sterol Derivatives

Several highly degraded sterols have been reported as constituents of the genus *Aplysia*. Aplykurodin A and B were isolated from the Japanese sea hare *A. kurodai* [207], and related isoprenoids, 4-acetylaplykurodin B and aplykurodinone B, were also identified as ichthyotoxic compounds from the anaspidean mollusk *A. fasciata* collected in the Bay of Naples, Italy (Fig. 33.26) [208]. In 1997, a reinvestigation of

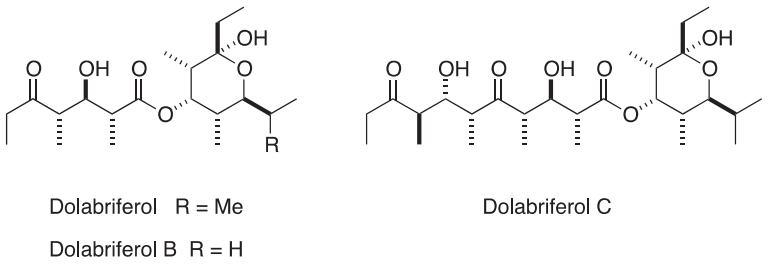


Fig. 33.25 Dolabriferols

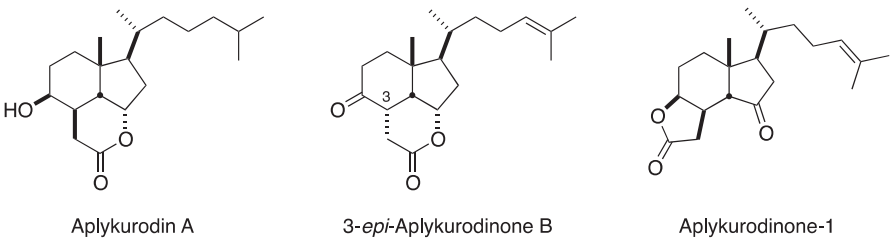


Fig. 33.26 Marine sterol derivatives

the bioactive substances from *A. fasciata* afforded 3-*epi*-aplykurodinone B, which was shown to be cytotoxic against P388 mouse lymphoma, A549 human lung carcinoma, HT29 human colon carcinoma, and MEL28 human melanoma tumor cell lines in vitro (ED_{50} values of 2.5 μg/mL in all cases) [209].

In 2005, two degraded sterols, aplykurodinones-1 and -2, were isolated from the skin of the sea hare *Syphonota geographica* collected along the coast of Greece [210]. The structure and absolute stereochemistry of aplykurodinone-1 were elucidated through a combination of spectroscopic methods, X-ray crystallography, and chemical correlation. Aplykurodinone-1 possesses a *cis*-fused C–D ring and an unsaturated side-chain. The total synthesis of aplykurodinone-1 was accomplished as a racemic form [211], followed by an enantiopure form [212].

33.3.6 Alkaloids

Several alkaloids have been isolated from sea hares and their digestive glands. Epidithiodioxopiperazine toxins are a class of fungal metabolites. Gliocladins A–C are fungal-derived marine alkaloids from a strain of *Gliocladium* sp., which were originally separated from the sea hare *A. kurodai* (Fig. 33.27) [213]. They contain rare dioxo- or trioxopiperazine structures. Gliocladins A–C exhibit moderate cytotoxicity against P388 lymphocytic leukemia in cell culture, with gliocladin C being the most potent (IC_{50} 2.4 μg/mL). Their stereostructures were established by NMR spectroscopic analysis, and synthetic studies established their absolute stereostructures [214–216].

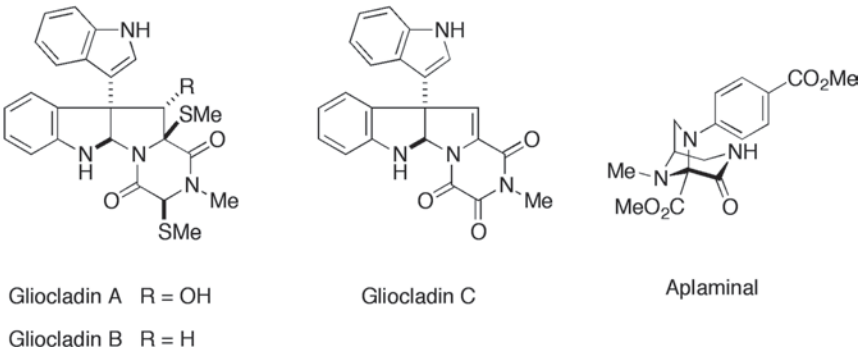


Fig. 33.27 Marine alkaloids

Aplaminal, a new triazabicyclo[3.2.1]octane framework metabolite, was isolated from the sea hare *A. kurodai* in 2008 [217]. Its structure was determined by the analysis of NMR data and confirmed by an X-ray crystallographic analysis. Aplaminal exhibits cytotoxicity against HeLa S3 cells (IC_{50} 0.51 μg/mL). In the same year, (−)-aplaminal was synthesized in 9 steps in 19% overall yield and its absolute stereochemistry was confirmed [218].

33.4 Conclusion

From sea hares, and especially from the genera *Dolabella* and *Aplysia*, a variety of new cytotoxic substances have been isolated, such as dolastatin 10 and aplyronine A, and their chemical and biological properties have been evaluated. Synthesis, derivatization and mode of action studies using chemical probe approaches have contributed to their clinical trials as antitumor agents. Meanwhile, due to the scarcity of available natural sources, the biological properties of most of sea hare-derived compounds have rarely been clarified, and there have been particularly few studies on in vivo antitumor effects. Further precise and practical chemical syntheses of sea hare-derived compounds and their derivatives, identification of their true producers, and biosynthesis studies should enable us to discover and develop a new class of pharmacological tools and therapeutic agents.

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Chapter 34
Marine Sponge Derived Actinomycetes and Their Anticancer Compounds

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Abstract Chemotherapy is one of the main treatments used to combat cancer. A great number of anticancer agents are natural products or their derivatives, mainly produced by microorganisms and the actinomycetes are prolific producers of pharmacologically important compounds, accounting for about 70 % of the naturally derived antibiotics that are currently in clinical use. In addition, actinomycetes yield a large number of natural substances with different biological activities, including anticancer activity. In this context, marine sponge derived actinomycetes have attracted special attention in the recent past for their ability to produce novel pharmacological lead compounds.

Keywords Marine actinomycetes · Natural products · Anticancer compounds · Sponge

34.1 Introduction

Microbial natural products continue to provide raw materials and design for the majority of pharmaceutical lead discovery and drug development [1]. However, after decades of fruitful bio-prospecting and drug discovery, traditional sources of natural products (e.g., plants and terrestrial actinomycetes) are realizing the 'Law of diminished returns' [2]. In the face of declining antibiotic and anticancer drug discovery, marine environment has emerged as an important source of bioactive

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natural products. There are, for example, several exciting marine derived molecules currently in the pharmaceutical market, and dozens more are progressing through the development [3]. In many cases, source organisms are as diverse as the molecular structures but yet, bacteria living in close association with the larger 'host' organisms, often produce novel metabolites [4].

Marine sponges (Porifera), in particular, harbor extremely rich and diverse populations of microorganisms, and have yielded many bioactive natural products [4] and novel bioactive compounds [5]. Presence of a large number of microorganisms within the mesophyl of many demosponges has been well-documented [6]. Bacteria can contribute up to 40 % of the sponge biomass (equal to about 10^8–10^9 bacteria/g of tissue) and are probably permanently associated with the host sponge unless, they are disturbed by external stress factors [7]. Number of bacteria and cyanobacteria associated with the marine sponges have been found to be the sources of antibiotics and other bioactive compounds and it has been reported that the wider biosynthetic capabilities of sponges are associated with their symbiotic microorganisms [8]. The marine bacterium, *Pseudomonas* isolated from its host sponge *Suberea creba,* collected from the Coral sea of New Caledonia, produced strong antibiotic quinines [9]. Although many bioactive compounds have been discovered in sponges [10, 11], only a few of them have been commercialized [10]. A serious obstacle to the ultimate development of most marine natural products that are currently undergoing clinical trials or preclinical trials is the problem of supply. Therefore, the research has been focused to disclose the mechanism of secondary metabolites synthesis [12].

Marine actinomycetes are the most economically and biotechnologically valuable prokaryotes. Albeit, marine actinomycetes are considered as a rich source of novel bioactive compound, they have not been fully explored. Though their genomic and metabolomics diversity remains unknown, many promising bioactive compounds including antimicrobial, anticancer, antitumour and immunosuppressive agents and enzymes have been discovered from them [13]. Recently, actinomycetes associated with the marine sponges have been reported as the richest sources of potential bioactive substances [14]. In this book chapter, we focus attention on the marine sponge derived actinomycetes and their anticancer compounds.

34.2 Marine Actinomycetes as a Potential Source of Bioactive Substances

Marine actinomycetes have been recognized as potential sources of bioactive compounds, and the works done earlier have also shown that these microbes are the richest sources of secondary metabolites. They hold a prominent position as targets in screening programs due to their diversity and their proven ability to produce novel metabolites and other molecules of pharmaceutical importance [15]. Since the discovery of actinomycin [16], actinomycetes have been found to produce many commercial bioactive compounds and antitumor agents in addition to enzymes of

industrial interest [17]. Approximately, two-thirds of the thousands of naturally occurring antibiotics have been isolated from these organisms [18]. Of them, many have been obtained from *Streptomyces* [19] and these natural products have been an extraordinary source for lead structures in the development of new drugs [20, 21].

Marine actinomycetes are the best sources of secondary metabolites and the vast majority of these compounds which are of commercial interest are derived from the genus *Streptomyces*, whose species are distributed widely in the marine and terrestrial habitats [22]. In fact, the genus *Streptomyces* alone accounts for a remarkable 80% of the actinobacterial natural products reported to date and its biosynthetic capacity surpasses others in the microbial world [23].

Marine actinobacteria are widely distributed in the biological sources such as fishes, molluscs, sponges, seaweeds and mangroves, besides seawater and sediments [24]. These organisms are gaining importance not only for their taxonomic and ecological perspectives, but also for their production of novel bioactive compounds like antibiotics, antitumor agents, immunosuppressive agents, enzymes, enzyme inhibitors and pigments [25].

34.3 Novel Secondary Metabolites from Marine Actinomycetes

Actinomycetes represent one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria, including five subclasses and 14 suborders [26]. Among the five subclasses, actinobacteria—bacteria belonging to the Order *Actinomycetales* (commonly called actinomycetes)—account for approximately 7000 of the metabolites reported in the Dictionary of Natural Products. Actinomycetes have a high GC content in their deoxyribonucleic acid (DNA) and grow as aerial mycelia [27]. They are responsible for the production of about half of the discovered secondary metabolites [28, 29], notably antibiotics [30], antitumour agents [31], immunosuppressive agents [32] and enzymes [33]. A large number of actinomycetes have been isolated and screened from the soil in the past few decades. Recently, the rate of discovery of new metabolites from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased [34]. Thus, it is crucial that new groups of actinomycetes from unexplored or underexploited habitats are to be pursued as sources of novel secondary metabolites.

34.4 Marine Sponge-Associated Actinomycetes

The marine environment, particularly with sponges, is a rich source of novel bioactive metabolites; 287 novel metabolites were isolated from the marine sponges in 2008 [35]. The availability of microbial biomass is a limiting factor for isolating

marine natural products. The wide spread isolation of typical microbial metabolites from sponges leads to the hypothesis that these metabolites are in fact the products of microbial metabolism [36]. Isolation of secondary metabolite-producing bacteria from the sponges and the microbial secondary metabolism gene clusters from the metagenome of sponges have led to the general understanding that these metabolites are, in many cases, the products of microbial symbionts and are not derived from the microbial diet of sponges [37]. Thus, the marine organism-associated microbes have been attracting increasing interest as potential sources of marine natural products in order to solve the supply shortage.

Marine sponges (Phylum Porifera) are sessile marine filter feeders that can filter large volumes of surrounding water through a unique aquiferous system [38]. Many bacteria, microalgae, and other organic particles in the seawater can thus be retained and digested by phagocytosis as foods [39]. The microorganisms that are not digested may survive and inhabit the host sponges. As a result, marine sponges become a rich reservoir of diverse, highly concentrated marine bacteria, some of which may not have been cultured yet. It has been reported that the sponges *Aplysina cavernicola* and *Ceratoporella nicholsoni* harbor a large number of bacteria that can amount to 38% and 57% of the biomass volume, respectively [40, 41]. The bacterial concentration in sponges is estimated to exceed those present in seawater by about two to four orders of magnitude [42]. In the recent years, there has been a growing interest in the study of bacteria associated with the marine sponges as sources of bioactive natural products [43, 44] and there are evidences to support that these bacteria are the real producers of bioactive metabolites originally isolated from their host sponges [45, 46].

Abundance of actinomycetes in marine sponges is variable but it can make up over 20% of the total microorganisms in some marine sponges [47, 48]. Over 30 sponge genera have been reported to be the hosts of actinomycetes [49, 50]. Among the nearly 10,000 sponge-derived microbial sequences submitted to the public databases, about one-sixth belong to *Actinobacteria* [51], indicating that this is an important group among sponge-associated microorganisms.

Recent studies have shown a novel and abundant actinomycetes assemblage in the marine sponge *Rhopaloeides odorabile*, as assessed by both the culture-independent molecular approach and culture-based method [52]. Several earlier studies have also found strains of actinomycetes from the marine sponges [43, 53]. However, our understanding of the sponge-associated-actinomycetes community is still inadequate as isolation and exploitation efforts have just begun. Further systematic investigations covering more sponge species are required. There are evidences for the presence of biosynthesis genes encoding polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) in marine sponge-derived actinomycetes and they are the useful indicators for the selection of strains to isolate new natural products [54].

Novel actinomycete groups have been found in the Great Barrier Reef sponges *Rhopaloeides odorabile, Pseudoceratina clavata* and *Candidaspongia flabellate*,

and the Mediterranean sponges, *Aplysina aerophoba* and *Theonella swinhoei* [6, 55, 56]. Unusual actinomycetes, belonging to *Micrococceae, Dermatophilaceae* and *Gordoniaceae*, have also been isolated from sponges [57]. Jensen et al. (2005) reported on the isolation of actinomycetes from algal and sponge samples and observed different rates of recovery of actinomycetes. Marine sponge-associated bacterial communities include the following taxa: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Proteobacteria*, *Spirochaetes* and *Verrucomicrobia* [49, 50, 52, 58, 59]. Among the bacterial associates, members of *Actinobacteria* are often sponge-specific [60] and have been identified as dominant producers of biologically active compounds [61, 62].

34.5 Sponges and Associated Actinomycetes Involved in Drug Production

Class: *Demospongiae*
Order: *Dendroceratida*
Family: *Darwinellidae*

Dendrilla nigra is a rich source of cultivable marine actinomycetes. Investigations on this sponge specimen collected from the Vizhinjam coast (West coast of India) revealed that the *Micromonospora, Saccharomonospora and Streptomyces* was the major cultivable actinobacterial group found in the sponge [60]. A number of actinomycete strains were also obtained from *Dendrilla nigra*, collected from the southwest coast of India. Among the eleven heterotrophic actinobacteria isolated from one specimen, *Nocardiopsis dassonvillei* MAD08 was prominent in its antibacterial and anticandidal activity against the multidrug resistant pathogenic microbial strains. The antibacterial activity was assigned to the presence of 11 compounds and the anticandidal activity, to a single protein. Uniqueness of this strain was reflected in the expression of both organic solvent (antibacterial) and water soluble (antifungal) antimicrobial compounds. In future, this may lead the way towards large scale profitable production of antimicrobials from *Nocardiopsis dassonvillei* MAD08 [63].

Streptomyces dendra sp. nov. MSI051 isolated from *Dendrilla nigra* from the same coast exhibited a broad spectrum of antibacterial activity. The host sponge, as well as the associated bacterial symbiont MSI051, contained high levels of PLA2 (Phospholipase A2) [64]. Since PLA2 is a well-established antibacterial protein in the defense system of higher animals, its presence in the sponge-associated bacteria might indicate an integrated functional role in the host defense system [65]. Another strain, *Streptomyces* sp. BLT7 isolated from *Dendrilla nigra* obtained, from Kanyakumari (Southeast coast of India) also showed potential antibacterial activity [12].

Order: ***Haplosclerida***
Family: ***Chalinidae***

The marine sponge, *Haliclona* has been extensively examined, and at least 190 metabolites exhibiting anti-fouling, antimicrobial, antifungal, antimalarial and cytotoxic activities have been isolated from it [66]. From *Haliclona simulans* of the west coast of Ireland, 52 bacteria were isolated and they belonged to the genera, *Pseudoalteromonas*, *Pseudomonas*, *Halomonas*, *Psychrobacter*, *Marinobacter*, *Sulfitobacter*, *Pseudovibrio*, *Salegentibacter*, *Bacillus*, *Cytophaga*, *Rhodococcus* and *Streptomyces* [67]. These were found to be rich sources of biological activities with over 50%, exhibiting antimicrobial activities. Twelve *Streptomyces* sp. were found to produce substances active against drug-resistant pathogenic bacteria. PKS (polyketide synthase) and NRPS (nonribosomal peptide synthetase) genes found in actinobacteria, suggest a high potential for secondary metabolite production by these organisms. Detection of a wide spectrum antibiotic activities in the *Streptomyces* isolates SM2 and SM4 is an another evidence to support that culturable sponge microbiota is an important source of biologically active compounds.

Family: ***Petrosiidae***

Genus *Petrosia* has been recognized as a source of diverse metabolites [68, 69]. An anti-infective alkaloid, Manzamine A was isolated from the culture of the actinobacterium *Micromonospora* sp. obtained from the deep water Indonesian sponge *Acanthostrongylophora* sp. [70]. Manzamine A, initially described as an antitumor agent against mouse leukemia cells [71] has recently been shown to possess antimalarial properties that inhibit *in vivo* growth of the rodent malarial parasite, *Plasmodium berghei* [72]. Large scale culture of the sponge derived *Micromonospora* sp. has been achieved in 20-litre fermentations, maintaining manzamine production [73].

Order: ***Poecilosclerida***
Family: ***Mycalidae***

An actinomycete strain, *Saccharopolyspora* sp. nov. associated with the sponge, *Mycale plumose* collected from the Qingdao coast (China) showed cytotoxic activities against temperature sensitive mutant cell lines of mouse (tsFT210). This has led to the isolation of two prodigiosins analogs, metacycloprodigiosin and undecylprodigiosin, which belong to a family of naturally occurring polypyrrole red pigments produced by a specific group of microorganisms including *Streptomyces* and *Serratia* strains. Both the above mentioned prodigiosin analogs have exhibited potent *in vitro* cytotoxic activity against the cancer cell lines such as P388, HL60, A-549, BEL-7402 and SPCA4 [74].

Order: ***Spirophorida***
Family: ***Tetillidae***

A chitinase exhibiting antifungal activity was isolated from the marine *Streptomyces* sp. DA11 associated with the south China sponge *Craniella australiensis*. Compared with the chitinase derived from the terrestrial organisms, marine chitinase with higher pH and

salinity tolerance may contribute to special biotechnological applications and therefore, novel marine chitinase could be of great importance [75].

Order: ***Verongida***
Family: ***Aplysinellidae***

Ten strains of marine actinomycetes belonging to the genus *Salinospora* were isolated from the Great Barrier Reef sponge *Suberea clavata* (formerly *Pseudoceratina clavata*) [55]. *Salinospora* strains previously isolated from marine sediments showed significant cancer cell cytotoxicities as well as antifungal and antibiotic activities [76]. *Salinospora* has been found to be a potential new source of rifamycins and polyketide synthesis gene clusters specific to rifamycin synthesis. *Salinospora* isolate from *Suberea clavata* was found to produce compounds of the rifamycin class, including rifamycin B and rifamycin SV [77].

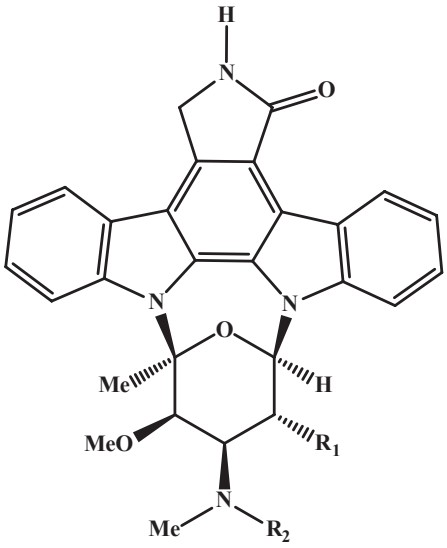
34.6 Anticancer Compounds from the Marine Sponge Derived Actinomycetes

Cancer is one of the most important causes of mortality in the modern world, with more than 10 million new cases reported every year [78]. It is well established that cancer is a multifactorial disease caused by a complex mixture of genetic and environmental factors [79–81], where considerable advances have led to a more comprehensive understanding of cancer at the genetic, molecular, and cellular levels, providing with new targets and strategies for therapy [82]. Nevertheless, these advances have yet to be effectively translated into functioning diagnostics and therapy. For example, effectiveness of many anticancer drugs is limited due to their inability to reach the target site in sufficient concentrations and efficiently exert the pharmacological effect without causing irreversible unwanted injury to the healthy tissues and cells [83, 84].

IB-96212, a 26-membered macrolide that contains a spiroketal lactone structure, is produced by the actinomycete, *Micromonospora* sp. L-25-ES25-008, isolated from a sponge, collected from the Indian Ocean near the coast of Mozambique [85]. This compound showed cytotoxic activity against mouse leukemia P-388 and human lung nonsmall cell A-549, colon adenocarcinoma HT-29 and melanoma MEL-28 cell lines. The activity against P-388 cell line was four orders of magnitude higher than the activity against A-549, HT-29 and MEL-28 cell lines [86].

Two new indolocarbazole alkaloids, 4′-*N*-methyl-5′-hydroxystaurosporine **(2)** and 5′-hydroxystaurosporine **(3)** were isolated together with the known staurosporine **(1)** (Fig. 34.1) from the culture broth of a marine *Micromonospora* sp. L-31-CLCO-002 obtained from a homogenate of the sponge *Clathrina coriacea* collected from the coast of Fuerteventura Island in the Canary Islands archipelago [87].

Two known compounds of the prodigiosin family have been isolated from the cultures of *Saccharopolyspora* sp. nov. actinomycete, isolated from the sponge, *Mycale plumose,* collected along the coast of Qingdao, China [74]. The compounds

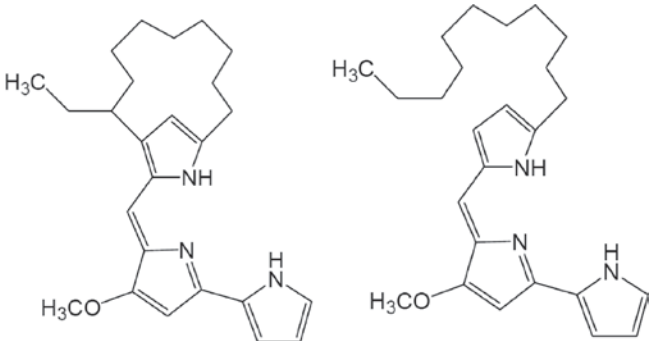


Staurosporine **(1) R1 = H R2= H**

4' - *N*-methyl-5'-hydroxystaurosporine **(2) R1 = OH R2= Me**

5 ' - hydroxystaurosporine **(3) R1 = OH R2= H**

Fig 34.1 Chemical structure of staurosporine, 4′-*N*-methyl-5′-hydroxystaurosporine and 5′-hydroxystaurosporine



Metacycloprodigiosin (4) **Undecylprodigiosin (5)**

Fig 34.2 Chemical structure of metacycloprodigiosin and undecylprodigiosin

were identified as metacycloprodigiosin **(4)** and undecylprodigiosin **(5)** (Fig. 34.2) and they exhibited significant cytotoxic activities *in vitro*, [88], against five cancer cell lines: mouse lymphoma P388, human peripheral blood promyeloblast HL60, lung carcinoma A-549 and SPCA4, and hepatic carcinoma BEL-7402 with IC_{50}

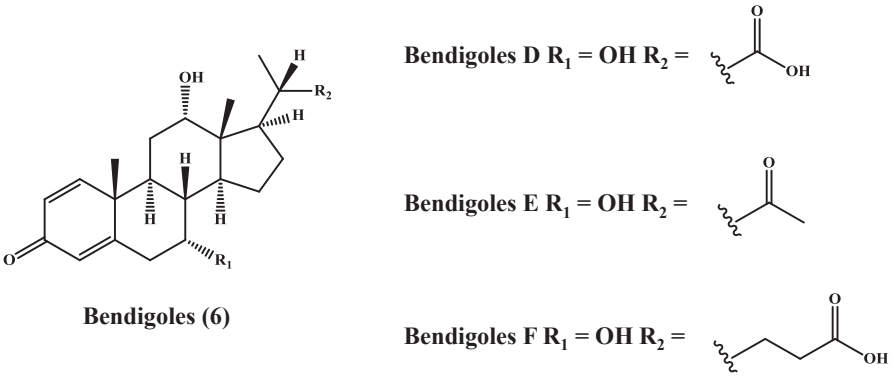


Fig 34.3 Chemical structure of bendigoles D-F

values range between 0.007 and 7.52 μM for metacycloprodigiosin and 0.013–0.11 μM for undecylprodigiosin [74].

Apart from the anticancer compounds produced by the marine actinomycetes depicted above, there are several additional compounds with antitumor activity like the topoisomerase I inhibitors cyclopropane and 14-methylhexadecanoic fatty acids produced by *Streptomyces* sp. strain KM86-913, isolated from a marine sponge collected from the seashore of Keomun Island, Korea [43]. In other cases, compounds identified are yet to be characterized as is the case of light-activated cytotoxic compounds produced by different microorganisms, including actinomycetes isolated from marine sponges collected from various places along the coast of Peninsular Malaysia [89].

Bendigoles D–F **(6)** (Fig. 34.3) are bioactive sterols isolated from the new marine sponge derived actinobacterium, *Actinomadura* sp. SBMs009. Isolation of these compounds was guided by a novel high-content screen for NF-κB and glucocorticoid receptor (GR) activity, and cytotoxicity assays. Interestingly, D displayed cytotoxicity against the L929 (mouse fibroblast) cell line with an IC_{50} approximated to 30 μM and was the most active inhibitor of GR-translocation, while D–F was the most effective inhibitor of NF-κB nuclear translocation with an IC_{50} of 71 μM [90].

Two new anthracyclines, tetracenoquinocin **(7)** and 5-iminoaranciamycin **(8)**, together with the known compounds aranciamycin **(9)** (Fig. 34.4) and antibiotic SM 173B were isolated from the culture of *Streptomyces* sp. Sp080513GE-26 associated with a marine sponge, *Haliclona* sp. These compounds were evaluated for cytotoxicity against two cancer cell lines. Cytotoxic activities of these compounds against human cervical carcinoma HeLa cells and human acute myelogenous leukemia LH-60 cells were examined. Aranciamycin showed cytotoxicity with IC_{50} values of 2.7 and 4.1 μM against HeLa and HL-60 cells, respectively, while tetracenoquinocin exhibited weaker cytotoxicities with IC_{50} values of 120 and 210 μM, respectively, and 5-iminoaranciamycin was inactive to these cancer cells (IC_{50}>200 μM). On comparing the cytotoxic activity of these compounds, it was found that the ketone functional group at C-5 was essential for the cytotoxicity against the cancer cells [91].

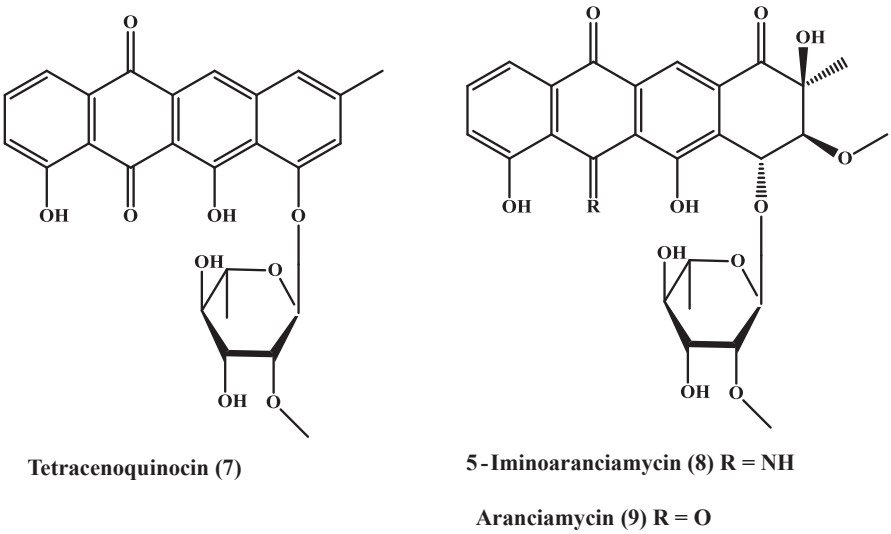


Fig 34.4 Chemical structure of tetracenoquinocin, 5-iminoaranciamycin and aranciamycin

34.7 Conclusion

Recent advances made in understanding the marine sponge derived actinomycetes have dramatically enriched our knowledge on the diversity, population ecology and biotechnology applications of sponge-associated actinomycetes. However, more information on the diversity of the sponge-associated actinomycetes is needed, and many aspects of the sponge–actinomycetes associations remain to be explored. Exploration of biotechnological potentials of actinomycetes associated with the sponges is limited due to difficulty to cultivate these actinomycetes associated with sponges. Environmental genomic approaches could be useful to explore the uncultivable actinomycetes populations for the purpose of deriving novel bioactive compounds. Compounds with novel structures and biological activities can also be identified from the actinomycetes consortia associated with sponges in the near future, using new microbiological cultivation methods, molecular techniques, and analytical tools. Particularly, coordination between microbiologists, mycologists, bioengineers and natural product chemists to investigate biodiversity and biotechnological potential of sponge associated microbes will contribute significantly to the pharmaceutical industries. There is immense scope in the marine actinomycetes for the exploration of anticancer compounds.

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Chapter 35
Cytotoxic Terpene-Purines and Terpene-Quinones from the Sea

Marina Gordaliza

Abstract The cytotoxic and antiproliferative properties of many natural terpene-purines and terpene-quinones/hydroquinones offer promising opportunities for the development of new drugs. Some of these marine natural products or derivatives, displayed broad spectrum cytotoxic activity against several cancer cells.

Keywords Terpene-purine · Terpene-quinone · Terpene-hydroquinone · Cytotoxicity

35.1 Introduction

Natural products have played a significant role in drug discovery. Over the past years, natural product derived compounds have led to the discovery of many drugs to treat human disease. Over 60 % of the current anticancer drugs have their origin in one way or another from natural sources. Nature continues to be the most prolific source of biologically active and diverse chemotypes, and it is becoming increasingly evident that associated microbes may often be the source of biologically active compounds originally isolated from host macro-organisms. While relatively few of the actual isolated compounds advance to become clinically effective drugs in their own right, these unique molecules may serve as models for the preparation of more efficacious analogues using chemical methodology such as total or combinatorial (parallel) synthesis, or manipulation of biosynthetic pathways. In addition, conjugation of toxic natural molecules to monoclonal antibodies or polymeric carriers specifically targeted to epitopes on tumors of interest can lead to the development of efficacious targeted therapies. The role played by natural products in the discovery and development of effective anticancer agents, and the importance of multidisciplinary collaboration in the generation and optimization of novel molecular leads from natural product sources are essential [1].

Drugs developed from marine sources give us this hope and also give us novel mechanisms to fight some of the most debilitating diseases encountered today,

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including: HIV, osteoporosis, Alzheimer's disease and cancer [2–5]. Although, the costs associated with developing drugs from marine sources have been prohibitive in the past, the development of new technology and a greater understanding of marine organisms and their ecosystem are allowing us to further develop our research into this area of drug development [4].

Marine floras are potential source of anticancer compounds, but they are least explored. Of the anticancer compounds extracted so far, the marine algae contribute 65.63 %, the mangroves 28.12 %, and the bacteria 6.25 %. Owing to a diverse chemical ecology, the marine organisms, especially marine flora, have a great promise for providing potent, cheaper, and safer anticancer drugs, which deserve an extensive investigation [6]. A program focused on the discovery of anticancer drugs from sponge extracts is being carried out by Valeriote and Crews Research Groups. An *in vitro* disk diffusion, solid tumor selective assay, was used to examine 2036 extracts from 683 individual sponges. The bioassay-directed fractionation discovery component led to the identification of active pure compounds from many of these sponges. In most cases, pure compound was prepared in sufficient quantities to both chemically identify the active compounds as well as pursue one or more of the biological development components [7].

The first marine-derived compound to go into clinical trials as an antitumor agent was a cyclic peptide known as didemnin B, isolated from the encrusting ascidian *Tridemnum solidum*. This failed at the Phase II level due predominately to the methodology used for dosing in the early 1980s, viz a single bolus of the compound at the maximum tolerated dose. Subsequently investigators at the Spanish company PharmaMar were able to clinically investigate a very closely related substance, aplidine or didehydrodidemnin B, which only differed from the original by the conversion of a lactyl side chain to a pyruvyl sidechain [8, 9]. Trabectedine, launched in Europe in 2007, was the first anti-cancer drug from a marine source [10, 11]. Trabectedine was developed by PharmaMar and approved for treatment of sarcoma in the EU in 2007 and for ovarian carcinoma again in the EU in 2009. Many countries, but not the USA, have approved the compound since then. The compound was originally produced by very large-scale aquaculture of the nominal producing organism, the tunicate *Ecteinascidia turbinata* and ultimately by semi-synthesis from a precursor, cyanosafracin B, produced by fermentation of a marine pseudomonad [9, 11]. In 2011, the US FDA approved the monoclonal antibody-auristatin E combination (Adcetris®) as a treatment for Hodgkins lymphoma. Auristatin E is a semi-synthetic analogue of dolastatin. Dolastatin was produced by a cyanobacterium (also known as a blue–green alga) [10, 12].

The common problems in the isolation of marine natural products are their low concentration in the producing organisms and the complexity of their structures. Thus, Nature has provided special structures around which talented chemists can practice their art. Among the natural compounds that are receiving an increasing interest, we can find the terpenylpurines and the terpenylquinones from marine sources [13, 14]. In literature a large number of these compounds have been found described, however in this chapter will only include those compounds having some toxicity.

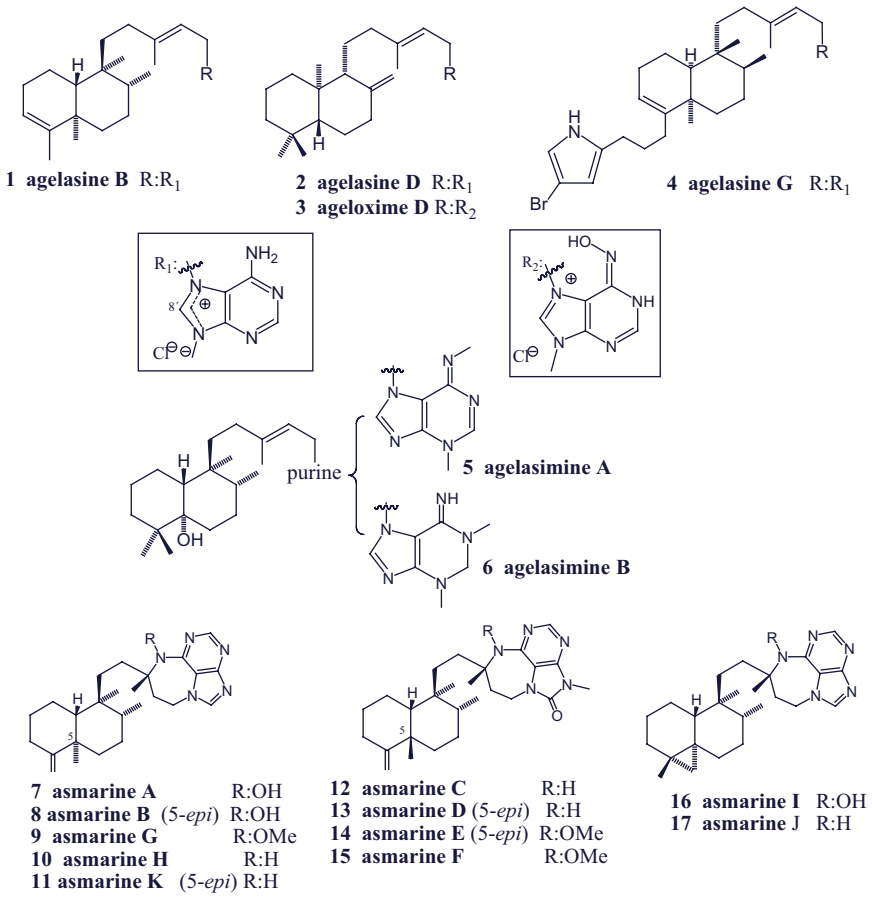


Fig. 35.1 Agelasines B, D and G; ageloxime D; agelasimines A and B; and asmarines

35.2 Terpene-Purines

Terpenylpurines are hybrid substances in which a terpene moiety (usually a diterpene moiety) is generally found as a substituent of position 7 of a 9-methyladeninium ion.

Agelasines and ageloximes (Fig. 35.1) are bicyclic diterpenoids having a 9-methyladeninium chromophore, and which are quaternary adenine salts. Agelasimines are adenine-related bicyclic diterpenoids which are not quaternary adenine derivatives of a bicyclic diterpene. Agelasine G **4** have the general agelasine structure with a pyrrole heterocycle at the diterpene moiety. The asmarines are alkaloids with a unique tetrahydro [1, 4] diazepino [1,2,3-*g, h*] purine (THDAP) structure.

Agelasines have been isolated from Pacific *Agelas* sea sponges species and asmarines have been isolated from *Raspailia* marine sponge species. Agelasines B

and D have been isolated from the Okinawan sea sponge *Agelas nakamurai* and from the Pacific sea sponge *Agelas* sp. Agelasine G was isolated from *Agelas* sp. collected off Konbu, Okinawa. Agelotoxime D has been isolated from Indonesian *Agelas* sponges. Agelasimines A and B were isolated from the Solomon Islands orange marine sponge *Agelas mauritiana* [15, 16]. Asmarines A–F were isolated from the marine sponge *Raspailia* sp. collected near Nakora Island, Dahlak Archipielago, Eritrea. Asmarines A, F, G and H were isolated from the Kenyan sponge *Raspailia* sp. Asmarines A, F, I, J and K were isolated from the Nosy Be Islands (Madagascar) sponge *Raspailia* sp. The wide range of biological activity makes these compounds attractive targets for synthesis [17, 18].

Some agelasine and agelasimine analogues displayed profound cytotoxic activity against several cancer cell lines, including a drug resistant renal cell line [17]. Agelasine B **1** in MCF-7 cells induced the activation of apoptosis in response to a sustained increase in the Ca^{2+} concentration after blocking SERCA activity. Agelasines D **2** and ageloxime D **3** exhibited cytotoxicity against L5178Y mouse lymphoma cells. Agelasine G **4** exhibited cytotoxic activity against lymphoma L1210. Simplified agelasine analogues modified in the purine 2-position exhibited activity against several cancer cell lines (U-937, GTB, RPMI 8226/s, CEM/s, ACHN, VERO and MCR-5 cells. Asmarines A **7** y B **8** have cytotoxic activity against cell cultures of P-388 murine leukemia, HT-29 human colon carcinoma and MEL-28 human melanoma. Moreover, were tested for cytotoxic activity against prostate DU-145 prostate, IGROV-ET ovarian, A-549 NSCL, PANC1 pancreas and LOVO colon cancer cell lines [18, 19].

35.3 Terpene-Quinones

The terpenylquinones constitute an interesting group of marine natural product for which a wide variety of biological activities have been described, including anti-inflamatory, antifungal, anti-HIV and most often antitumor activities.

Avarol **18**, a sesquiterpene hydroquinone, and its quinone derivative avarone **23** (Fig. 35.2) are the most representative compounds of bioactive terpenequinones [20, 21]. Avarol and avarone were isolated from the marine sponge *Dysidea avara*. Both compounds were first discovered as anti-leukaemia agents *in vitro* and *in vivo*, and later it was found that they had an *in vitro* inhibitory capacity against HIV-1. Additionally, the potent T-lymphotropic cytostatic activity shown by avarol, and its low toxicity in mice, its ability to cross the blood–brain barrier and its ability to stimulate the synthesis of interferon make both these compounds optimum candidates for transformations aimed at improving their cytostatic and antiviral activity. Some monophenyl thioavarol derivatives have been described as lacking cytotoxicity, which could point to promising UVB photoprotective agents through the potent inhibition of NF-kappaB activation with a mild antioxidant pharmacological profile. Various formulations with high avarol content have been used for the prevention and treatment of skin cancer, tumors in the gastrointestinal tract and urinary tract [20, 21].

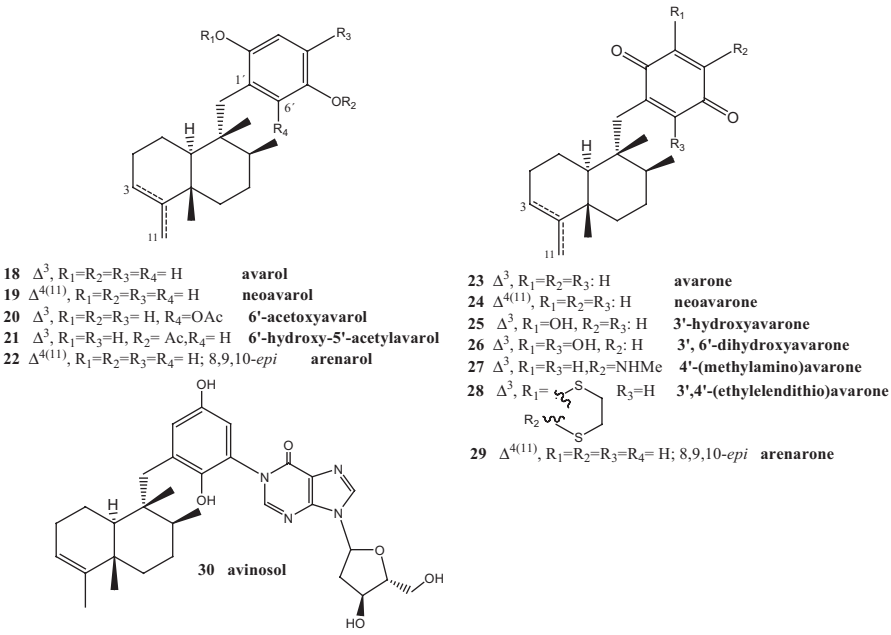


Fig. 35.2 Avarol and related compounds

Several analogues, included in Fig. 35.2, showed cytotoxicity.6′-acetoxyavarol **20**, 3′-hydroxyavarone **25** and 3′,6′-dihydroxyavarone **26,** were isolated from *Dysidae cinera* (collected in the Red Sea) showed cytotoxicity against P-388 mouse lymphoma indicated high potency for 6′-acetoxyavarol **20**, 3′-hydroxyavarone **25**, and 3,6′-dihydroxyavarone **26**, with IC_{50} of 0.6, <0.6 and 1.2 μg/mL, respectively. 4′-(methylamino)avarone **27** and 3′,4′-(ethylenedithio)avarone **28**, which were more active against melanoma cells, although overall the latter compound showed low activity. The most active compound was 4′-(methylamino)avarone **27**, with an IC_{50} value of 2.4 μM against melanoma Fem-X cells, and no cytotoxicity against normal lymphocytes. An *in vitro* cytotoxicity assay against U937 human histiocytic lymphoma cells determined the order of cytotoxic potency ((−)-neoavarone>(+)-avarone>(+)-aureol>(+)-avarol>(−)-neoavarol) and some aspects of their structure-activity relationships [20–22].

Avinosol **30** (Fig. 35.2), a merotepenoid isolated from the marine sponge *Dysidea* sp. collected in Papua New Guinea, appeared to be the first example of a naturally occurring meroterpenoid-nucleoside conjugate, and showed anti-invasion activity in cell-based assays [23]. Two sesquiterpenoids with a quinone and hydroquinone moiety were isolated respectively from the marine sponge *Dysidea arenaria*: arenarol **22** and arenarone **29**. These compounds showed cytotoxic activity when assayed against P-388 leukaemia cells, with ED_{50} = 17.5 μg/mL for arenarol and ED_{50} = 1.7 μg/mL for arenarone.

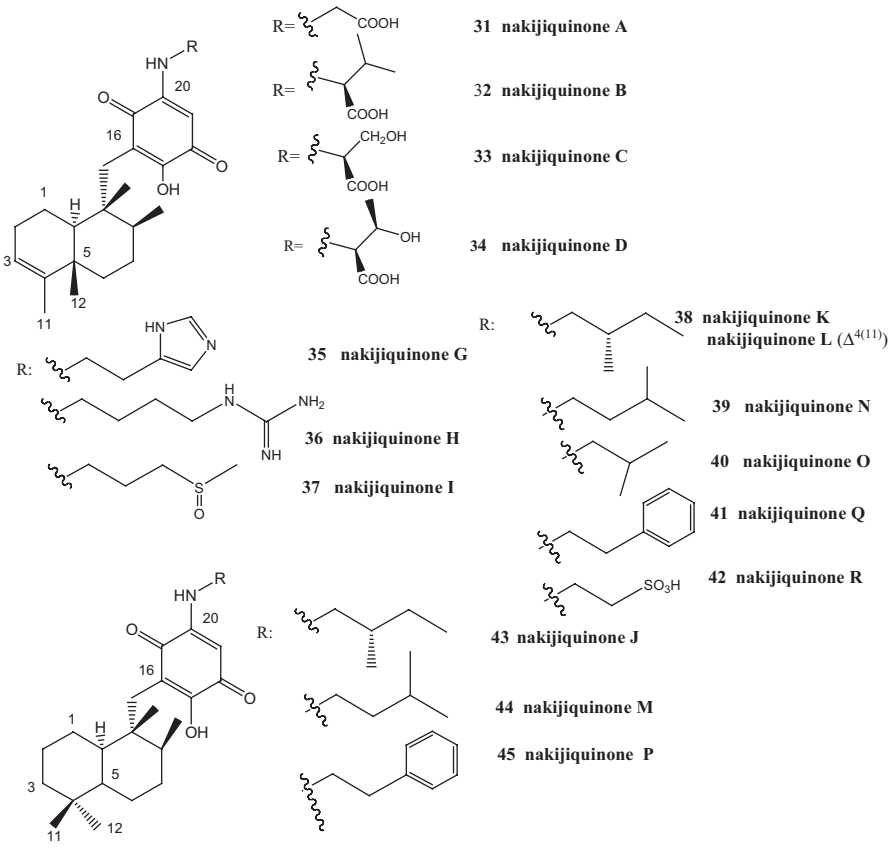
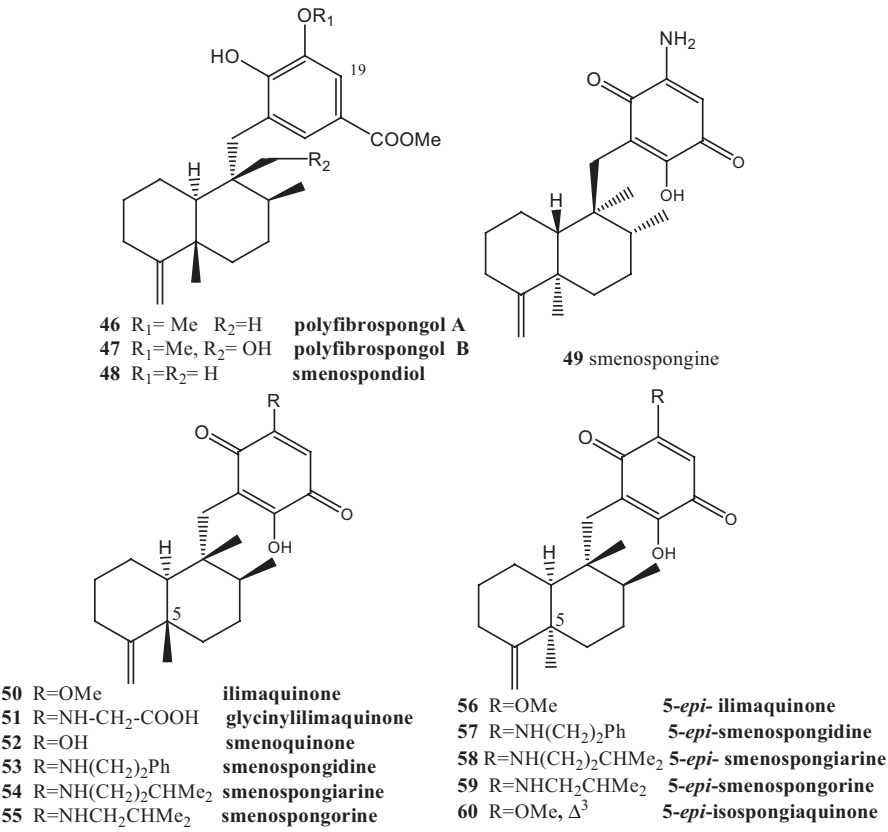


Fig. 35.3 Nakijiquinones

Nakijiquinones A–D (Fig. 35.3) showed *in vitro* cytotoxicity against L-1210 (IC_{50} values between 2.8 and 8.1 μg/mL) and KB (IC_{50} values between 1.2 and 7.6 μg/mL).

Nakijiquinones G–I, containing a different amino group derived from amino acids, were isolated from Okinawan marine sponges of the family Spongiidae, and showed modest cytotoxicity and inhibitory activity against HER2 kinase. Nakijiquinones J–R, were tested for inhibitory activities against EGFR and HER2 tyrosine kinases. Among them, nakijiquinones P and R exhibited inhibitory activities against EGFR (76 and > 99 % inhibition, respectively), while nakijiquinones N and R showed inhibitory activities against HER2 (66, 59 and 52 % inhibition, respectively). The HER2/Neu receptor tyrosine kinase is hugely overexpressed in about 30 % of primary breast, ovary, and gastric carcinomas. Nakijiquinones are the only naturally occurring inhibitors of this important oncogene [24, 25].

Another type of compound with bioactive properties includes those whose benzo(hydro)quinone ring is substituted by a methoxycarbonyl group, as is the case



46 R_1= Me R_2=H **polyfibrospongol A**
47 R_1=Me, R_2= OH **polyfibrospongol B**
48 R_1=R_2= H **smenospondiol**

49 smenospongine

50 R=OMe **ilimaquinone**
51 R=NH-CH_2-COOH **glycinylilimaquinone**
52 R=OH **smenoquinone**
53 R=NH$(CH_2)_2$Ph **smenospongidine**
54 R=NH$(CH_2)_2$CHMe$_2$ **smenospongiarine**
55 R=NHCH$_2$CHMe$_2$ **smenospongorine**

56 R=OMe **5-*epi*- ilimaquinone**
57 R=NH$(CH_2)_2$Ph **5-*epi*-smenospongidine**
58 R=NH$(CH_2)_2$CHMe$_2$ **5-*epi*- smenospongiarine**
59 R=NHCH$_2$CHMe$_2$ **5-*epi*-smenospongorine**
60 R=OMe, Δ^3 **5-*epi*-isospongiaquinone**

Fig. 35.4 Polyfibrospongols, ilimaquinones and smenospongines

of polyfibrospongols and smenospondiol (Fig. 35.4). Extracts of the marine sponge *Polyfibrospongia australis*, collected in Taiwan, were used to isolate polyfibrospongols A **46** and B **47**, compounds showing cytotoxic activity against different tumour lines. Also showing cytotoxic, as well as antibacterial, activity is smenospondiol **48**, isolated from dichloromethane extracts of several species of the genus *Smenospongia* and with a very similar structure. These compounds showed interesting levels of cytotoxicity when assayed against the P-388 (mouse lymphoma), KB-16 (human nasopharyngeal carcinoma) and A-549 (human lung carcinoma) cells, their CI_{50} values lying between 0.6 and 2.0 μg/mL.

Bioassay-guided isolation from the marine sponge *Hippospongia sp*. collected at Palau led to the isolation of three sesquiterpene quinone metabolites: ilimaquinone **50**, 5-*epi*-ilimaquinone **56** and 5-*epi*-isospongiaquinone **60** (Fig. 35.4). The cytotoxicity against the NCI-H460, HepG2, SF-268, MCF-7, HeLa, and HL-60 human tumour cell lines, the inhibitory effects on the maturation of starfish oocytes, and cell cycle arrest in the HepG2 cell line were evaluated [26]. 5-*Epi*-ilimaquinone **56**

showed cytotoxic activity (IC_{50}) against P-388 leukaemia cells (2.2 μg/mL) and different solid tumours: A-549 (0.9 μg/mL), HT-29 (3.4 μg/mL) and B16/F10 (1.1 μg/mL). It has been suggested that ilimaquinone **50** induces a concentration-dependent antiproliferative effect in several types of cancer cell lines, including PC-3 and LNCaP prostate cancer, A549 non-small cell lung cancer and Hep3B hepatocellular carcinoma cells. The anticancer mechanism of ilimaquinone in the representative PC-3 cells was identified. Ilimaquinone **50** induced a time-dependent increase in G1 phase arrest and a subsequent increase in the hypodiploid sub-G1 phase (apoptosis) of the cell cycle [26].

One compound closely linked to the above ones is glycinylilimaquinone **51**, a metabolite isolated from a specimen from the genus *Fasciosponia* from the Phillipines. This structure displayed cytotoxic activity, with IC_{50} = 7.8 μg/mL, against a human carcinoma tumour cell line (HT-29). Other compounds with free hydroxyl and/or amino functions on the benzo(hydro)quinone ring have been isolated from the genus *Smenospongia*. Bioassays performed on the dichloromethane extract of different species of this genus revealed both cytotoxic and antibacterial activities. From this extract, smenosquinone **52**, smenospongidine **53**, smenospongiarine **54** and smenospongorine **55** were isolated. The cytotoxicity of some of these compounds was assessed against L-1210 leukaemia cells. The results for smenoquinone, and smenospongiarin were IC_{50}: 2.5 and 4.0 μg/mL, respectively.

Smenospongine **49** from genus *Smenospongia* and *Dysidea*, showed promising biological activities: cytotoxicity against L-1210 leukaemia cells with a LD_{50} = 1 μg/mL. Smenospongine induced erythroid differentiation and G1 phase arrest of K562 chronic myelogenous leukemia cells. Smenospongine induced dose-dependent apoptosis in HL60 and U937 cells. Smenospongine treatment increased the expression of p21 and inhibited the phosphorylation of Rb in K562 cells, suggesting the p21-Rb pathway plays an important role in G1 arrest in K562 cells. *Dactylospongia elegans*, from Papua Nueva Guinea and Thailand, contains a total of 17 merosesquiterpenoids, among which are (+)-*epi*-smenospongidine **57** and (+)-*epi*-smenospongiarine **59**. These compounds were assayed *in vitro* against solid tumour models (A-549, HT-29 and B16/F10) and leukaemia cells (P-388), (+)-*epi*-smenospongiarine **58**, with IC_{50} values between 0.6 and 0.9 μg/mL being of particular interest. The structure-activity relationship study of these compounds revealed that the quinone skeleton is indispensable and the amino group plays an important role for their differentiation-inducing activity to K562 cells into erythroblast [27].

Corallidictyals and chromazonarols (Fig. 35.5) are compounds with a fourth six-membered oxygen ring. Corallidyctals A–D were isolated from the marine sponge *Aka (Siphonodyctio) corallifagum*. Both corallidytal A and B inhibit PKC with an IC_{50} = 28 μM, while assays addressing another cAMP-dependent kinase did not afford inhibition at concentrations of 300 μM, indicating its selectivity. Further, the assays revealed selectivity against the α isoform of PKC. Corallidyctals C and D were tested in antiproliferative assays using cultures of mouse fibroblasts and activity was linked to the presence of the ortho-hydroquinone moiety [28]. Assays

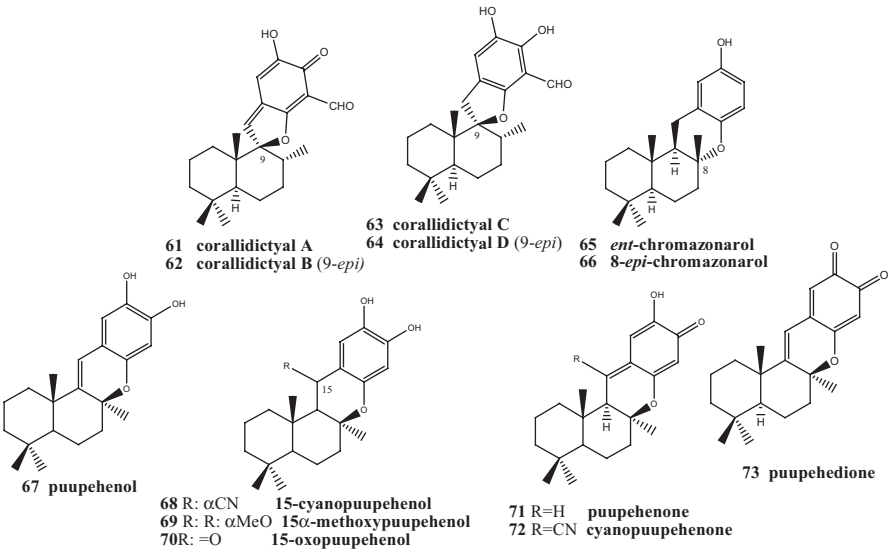


Fig. 35.5 Corallidictyals, chromazonarols and puupehenols

on cytotoxic activity were performed against P-388, A-549, HT-29 and MEL-28 cells and against the KB, Bel-7402, PC-3M, Ketr 3 and MCF-7 human tumour cell lines. Cytotoxicity tests showed generally low cytotoxicity of corallidictyals and chromazonarols.

From one species of the genus *Verongida* two metabolites, also with with a fourth six-membered oxygen ring, were obtained: 15-cyanopuupehenol **68** and 15-cyanopuupehenone **72**. Activity assays performed on puupehenone **71** revealed cytotoxic activity against different neoplastic lines, with an interesting IC_{50} = 0.5 μM against A-549 and HT-29, and even better inhibition values against the synthesis of DNA and RNA (0.3 and 0.4 μg/mL, respectively) [29].

Mamanuthaquinone **74** (Fig. 35.6), a cytotoxic metabolite of *Fasciospongia* sp. collected in the Fiji islands [30]. Activity assays revealed a certain toxicity, especially against HCT-116 human colon carcinoma (IC_{50} = 2 μg/mL). Strongyline A **75**, a metabolite isolated from the marine sponge *Strongylophora hartmani*, showed cytotoxic activity against p-388 leukaemia cells (IC_{50} = 13 μg/mL) [31].

More recently, neopetrosiquinones A and B (Fig. 35.6), sesquiterpene benzoquinones have been isolated from the deep-water sponge *Neopetrosia* cf. *proxima*, of the Petrosiidae family. Neopetrosiquinones A **76** and B **77** inhibit the *in vitro* proliferation of the DLD-1 human colorectal adenocarcinoma cell line with IC_{50} values of 3.7 and 9.8 μM, respectively, and the PANC-1 human pancreatic carcinoma cell line with IC_{50} values of 6.1 and 13.8 μM, respectively. Neopetrosiquinone A also inhibited the *in vitro* proliferation of the AsPC-1 human pancreatic carcinoma cell line with an IC_{50} value of 6.1 μM [32].

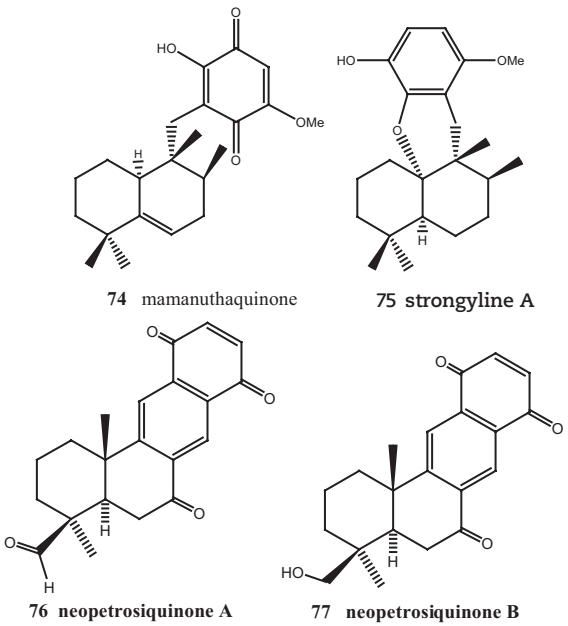


Fig. 35.6 Mamanuthaquinone, stronglyline A and neopetrosiquinones

Bolinaquinone **78** (Fig. 35.7) is a cytotoxic sesquiterpene from the genus *Dysidea*, whose quinone moiety is located on an unusual carbon of the decalin. This compound showed cytotoxic activity against HCT-116 human colon carcinoma (IC_{50} = 1.9 μg/mL). The cytotoxicity studies carried out suggest that this compound acts by interfering with or damaging DNA. 21-dehydroxybolinaquinone **79**, isolated from the Hainan sponge *Dysidea villosa,* showed moderate PTP1B inhibitory activity and cytotoxicity, with IC_{50} values of 39.5 and 19.5 mM, respectively [33].

Paniceins A_2 and F_1(Fig. 35.7), with an open decalin B ring, were obtained from *Reniera mucosa,* They were assayed against the P-388, A-549, HT-29 and MEL-28 cell lines with a view to determining their *in vitro* cytotoxicity. The results were for panicein A_2 ED_{50} = 5 μg/mL against all the lines assayed and for panicein F_1 ED_{50} = 5 μg/mL against all cell lines except HT-29; the latter showing medium potency in additional DHFR inhibition tests (ED_{50} = 3 μg/mL) [34].

Other related compounds, formed by two subunits, were isolated from species of the genus *Hyrtios* (New Caledonia), such as dipuupehenone **82** and bispuupehenone **83**, the latter also found in the species *Hyrtios eubamma* (Tahiti). Bioactivity assays of these compounds unveiled the cytotoxic activity of dipuupehedione 82 against KB cells (ED_{50} = 3 μg/mL) [35]. Popolohuanone E **82**, a potent topoisomerase II inhibitor with selective cytotoxicity against the A549 non-small cell human lung cancer cell line, was isolated from *Dysidea* sp. Pohnpei sponges [36].

Regarding the mechanism of action of terpenylquinones, the accumulated data about the biological activity of quinone moieties suggest redox processes and/or

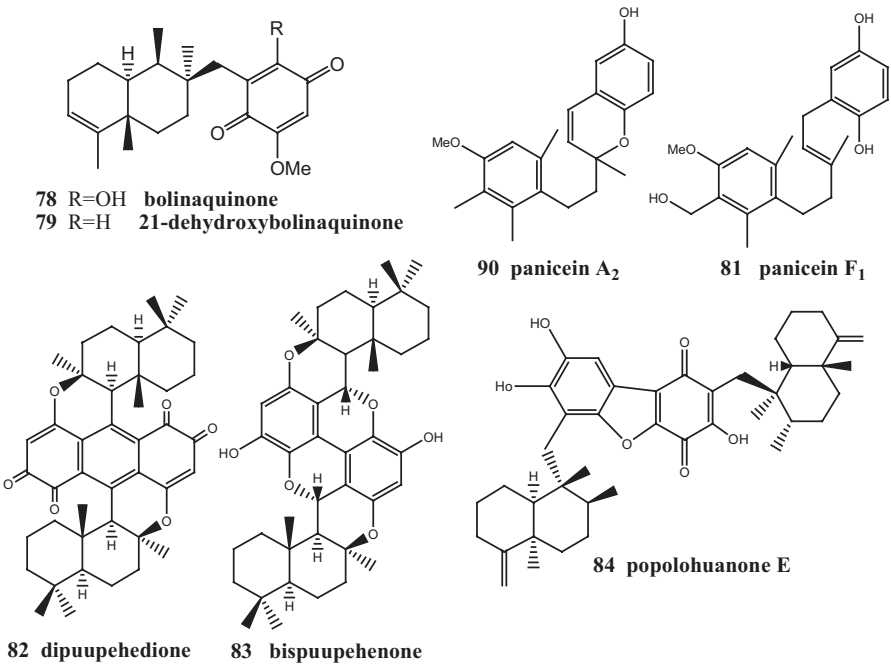


Fig. 35.7 Bolinaquinones, paniceins and other compounds

Michael-type addition–elimination reactions. Their cytotoxicity has been explained in terms of their ability to undergo redox cycling and the generation of reactive oxygen species, which would damage tumor cells. The results obtained with avarol **18** and avarone **23** supported the mechanism of antitumor action via the reactive oxygen radicals but there were also indications of the relevance of arylation of biomolecules, such as proteins [20].

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Chapter 36
Pyridoacridine Alkaloids from Marine Origin: Sources and Anticancer Activity

Anake Kijjoa

Abstract The pyridoacridines are a group of highly colored, polycyclic aromatic natural products isolated from marine invertebrates, especially marine sponges and tunicates. As a class, the pyridoacridines are considered DNA binding molecules and have been characterized mainly on the basis of their cytotoxicity, even though they may possess other interesting biological activities such as antibacterial, antifungal, antiviral, antiparasitic and insecticidal activities. Recent reports have demonstrated that the individual pyridoacridines can vary dramatically in their molecular mechanism of cell killing. It was suggested that both core structure and substituents can have significant influences on the electronic distribution and topology of the molecule of these compounds, which ultimately affect the mechanism underlying their biological activities. This chapter covers structures and sources of the isolated marine pyridoacridine alkaloids, as well as the mechanisms underlying the cytotoxicity of certain naturally occurring marine pyridoacridines.

Keywords Anticancer activities · Apoptosis · Caspase-3 activation · Cytotoxicity · DNA intercalator · Iminoquinone · Pyridoacridine alkaloids · ROS generation · Topoisomerase II poisoning · Ubiquitylation inhibitor

36.1 Introduction

Cancer is a constant and major burden on the human population, and epidemiological evidence shows that current treatment of cancer with chemotherapy and surgery are still far from optimal. Therefore, further research is required to look for alternative drugs that are more effective and have less side-effect than the drugs currently used in chemotherapy. One of these avenues is to search for new chemical entities, *via* genetic mining from the marine environment, which can be potential for the development of arsenals in cancer chemotherapy [1].

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Marine organisms constitute an important source of bioactive compounds with continuing interest in the pharmaceutical in biotechnological sectors. In recent years, the chemistry of natural products derived from marine organisms, along with the development of new *in vitro* bioassay tools, has become the focus of a much greater research effort. Currently there are around 15 marine natural products in various phases of clinical development, mainly in the oncology area, with more on the way and several products already on the market [2]. Among the myriad of the secondary metabolites from marine sources, marine alkaloids represent a major group of bioactive marine natural products. Specifically, the pyridoacridines, with most of the compounds being isolated from the marine sponges and tunicates, constitute the largest group of the marine alkaloids which exhibit relevant biological activities such as antibacterial, antifungal, antiviral, antiparasitic, insecticidal and anti-tumor activities [3]. Almost all natural marine pyridoacridines have been reported to possess significant cytotoxicity against cultured cells, and the family as a whole seems to be of great interest as a source of new lead structures for the development of future generation of therapeutic agents [4]. Due to the importance and the volume of work published for the pyridoacridine alkaloids, this chapter will focus only on the structures of the naturally occurring marine pyridoacridines and their sources, as well as the mechanisms underlying the cytotoxicity of some relevant compounds. Although excellent reviews on chemistry and biological activities of the pyridoacridine alkaloids have been previously published by Molinski [5], Ding et al. [6], Delfourne and Bastide [4] and Marshall and Barrows [3], this chapter covers the pyridoacridine alkaloids isolated from the marine sources and the mechanisms underlying the cytotoxicity of certain naturally occurring marine pyridoacridines, reported until October 2013.

36.2 Pyridoacridine Alkaloids

Pyridoacridines are planar polyheterocyclic compounds possessing a common 11H-pyrido[4, 3, 2-*mn*]acridine skeleton (**1**) (Fig. 36.1), even though the majority are more accurately described as “pyridoacridone”, derivatives of the hypothetical iminoquinone, 8H-pyrido[4,3,2-*mn*]acridone (**2**) (Fig. 36.1). Among marine alkaloids, the pyridoacridines seem to be the largest group, with most of the compounds being isolated from marine sponges and ascidians and, in lesser extent, from certain mollusks and one coelenterate [5]. While the pyridoacridine alkaloids found in the mollusk *Chelynotus semperi* were undoubtedly produced from an ascidian dietary source, their distribution across three other phyla led to the suggestion that the alkaloids might be produced by the same or similar organisms associated with the different hosts [7].

The structures of the pyridoacridine alkaloids vary depending on the nature and position of the side chains on ring C and on the acridine nitrogen, as well as on the presence of the rings fused with ring C. The oxidation states of the rings can also vary, with partial saturation frequently observed in the ring attached to ring

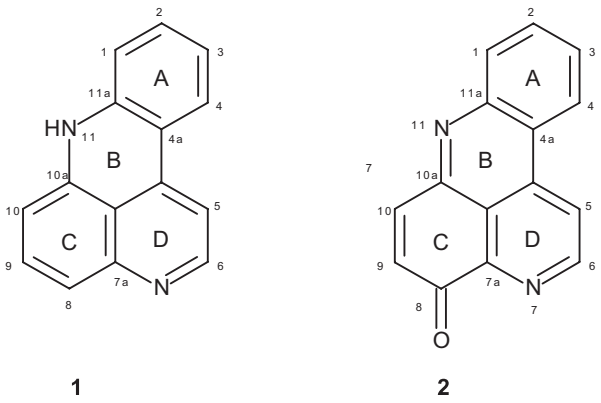


Fig. 36.1 Structures of 11H-pyrido[4, 3, 2-*mn*]acridine (**1**) and 8H-pyrido[4,3,2-*mn*] acridone (**2**) skeleton

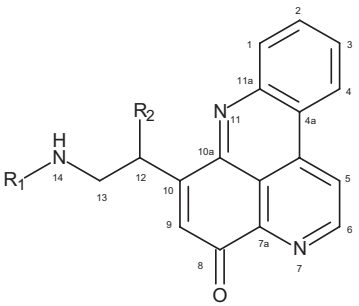
C and, occasionally in ring D [5]. Based on the number of rings attached to the 11H-pyrido[4, 3, 2-*mn*]acridine (**1**) or 8H-pyrido[4,3,2-*mn*]acridone (**2**) skeleton, pyridoacridines can be divided into tetracyclic, pentacyclic, hexacyclic, heptacyclic and octacyclic alkaloids.

36.2.1 Tetracyclic Alkaloids

The first group of the pyridoacridines possesses the oxidized ring C, i.e., an iminoquinone subunit (**2**). Kobayashi et al. [8] reported isolation of cystodytin A–C (**3–5**) (Fig. 36.2), the first members of the tetracyclic pyridoacridine alkaloids, from the Okinawan marine tunicate *Cystodytes dellechiajei*. Later on, the same group isolated cystodytin D–I (**6–11**) (Fig. 36.2), also from the same tunicate [9]. McDonald et al. [10] reported isolation of another cytodytin analogue which they have named cytodytin J (**12**), in addition to cytodytin A (**3**), from the methanol extract of a Fijian *Cystodytes* ascidian (Fig. 36.2). Appleton et al. [11] reported isolation of cystodytins J (**12**) and cystodytin K (**13**), from the ascidian *Lissoclinum notti*, collected in New Zealand (Fig. 36.2).

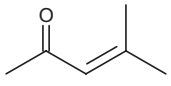
Halogenated tetracyclic pyridoacridine alkaloids have been also reported from the marine sources. Kim et al. [12] reported isolation of pantherinine (**14**), a brominated pyridoacridine, from the ascidian *Aplidium pantherinum*, collected at Stenhouse Bay, South Australia. Pantherinine (**14**) has, in addition to a bromine substituent on C-3 of the aromatic ring A, the amino group on C-9 of the quinone moiety (Fig. 36.3).

The thiomethyl substituent is also common in the pyridoacridine alkaloids. Lissoclins A and B (**15**, **16**), iminoquinones with the thiomethyl substituent on C-9, were isolated from *Lissoclinum sp*., collected from the Great Barrier Reef [13]. Lissoclin A (**15**) differs from lissoclin B (**16**) only in the side chain at C-10, with isovaleryl in the former and tigloyl in the latter (Fig. 36.3).

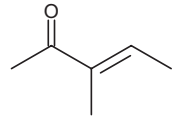


3, R_1 = H, R_2 = X
4, R_1 = H, R_2 = Y
5, R_1 = H, R_2 = Z
6, R_1 = OH, R_2 = X
7, R_1 = OH, R_2 = Y
8, R_1 = OMe, R_2 = X
9, R_1 = OMe, R_2 = Y

X =



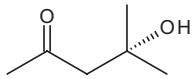
Y =



10, R_1 = O-C-$(CH_2)_7CH$=$CH(CH_2)_7CH_3$, R_2 = X

11, R_1 = O-C-$(CH_2)_7CH$=$CH(CH_2)_7CH_3$, R_2 = Y

Z =



12, R_1 = CHO, R_2 = H

13, R_1 = CHO, R_2 = OMe

Fig. 36.2 Structures of cystodytins A–K (**3–13**)

Another group of structurally related thiomethyl substituted iminoquinones are diplamine (**17**), isodiplamine (**18**) and diplamine B (**19**) (Fig. 36.3). Diplamine (**17**) was first isolated as a cytotoxic alkaloid from the tunicate *Diplosoma* sp. [14], and later, together with isodiplamine (**18**), from the New Zealand ascidian *Lissoclinum notti* [11] while diplamine B (**19**) was reported from the ascidian *Lissoclinum* cf. *badium*, collected in the Coral Sea, Papua New Guinea [15].

The second group of the tetracyclic alkaloids possesses the 11H-pyrido[4, 3, 2-*mn*]acridine skeleton (**1**). The most simple tetracyclic pyridoacridines of this group are styelamines A–D (**20–23**) (Fig. 36.4), which were isolated from an extract of the ascidian *Eusynstyela latericius*, collected from Ujung Pandang, Indonesia [16]. The common feature of these compounds is the presence of the hydroxyl group at C-8 of the aromatic ring C. Styelamines C (**22**) and D (**23**) were later isolated from the purple morph of the ascidian *Cystodytes dellechiajei*, collected in Catalonia [17]. The structurally related varamines A (**24**) and B (**25**) (Fig. 36.4), with the methoxyl group at C-8 and the thiomethyl substituent at C-9, were reported as metabolites of the Fijian ascidian *Lissoclinum vareau* by Molinski and Ireland [18], and norsegoline (**26**) (Fig. 36.4) was reported as a metabolite of the marine tunicate *Eudistoma* sp. [19, 20].

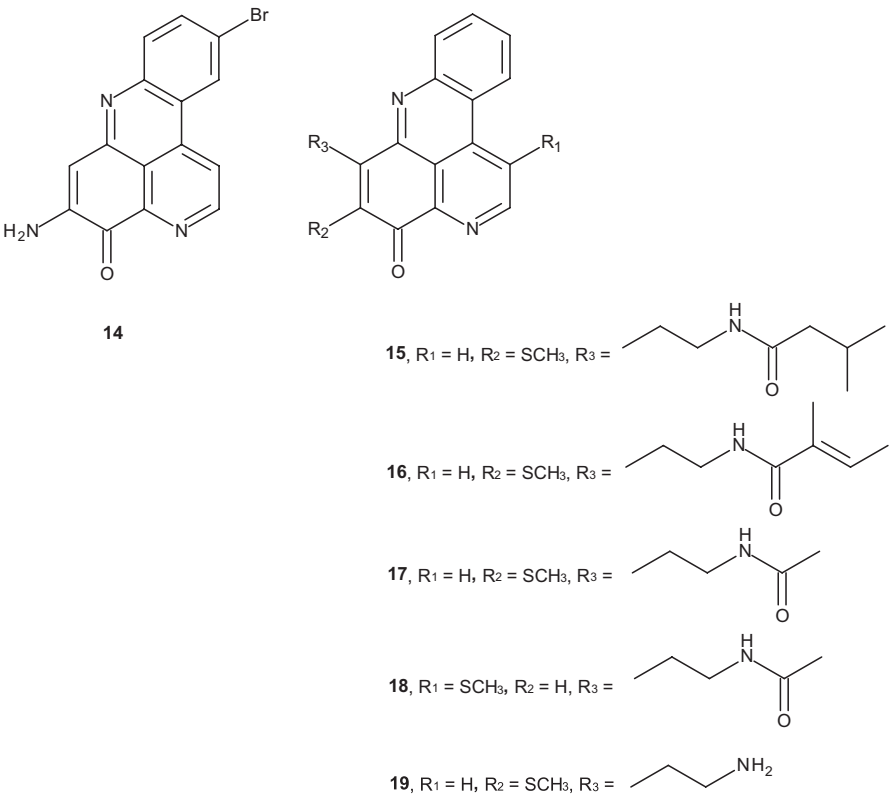


Fig. 36.3 Structures of pantherinine (**14**), lissoclin A (**15**) and B (**16**), diplamine (**17**), isodiplamine (**18**) and diplamine B (**19**)

36.2.2 Pentacyclic Alkaloids

Amphimedine (**27**) (Fig. 36.5), the first marine-derived pentacyclic pyridoacridine alkaloid, was isolated from a Pacific sponge *Amphimedon* sp. [21]. It was later isolated, together with neoamphimedine (**29**) (Fig. 36.5), from the marine sponge *Xestospongia* sp., collected from Surigao, Phillipines [22]. Tasdemir et al. [23] reported isolation of amphimedine (**27**), neoamphimedine (**29**), and deoxyamphimedine (**31**) (Fig. 36.5), from two specimens of the marine sponge *Xestospongia* sp., collected from the Philippines and Palau. Thale et al. [24] described isolation of neoamphimedine (**29**) and 5-methoxyneoamphimedine (**30**) (Fig. 36.5) from the marine sponge *X.* cf. *exigua*, collected from Indonesia, as well as of neoamphimedine (**29**), 5-methoxyneoamphimedine (**30**), neoamphimedine Y (**35**) and neoamphimedine Z (**36**) (Fig. 36.5), from *X.* cf. *carbonaria,* collected from Papua New Guinea. Wei et al. [25] isolated three new analogues of amphimedine, i.e., 1-hydroxy-deoxyamphimedine (**32**), 3-hydroxy-deoxyamphimedine (**33**), debromopetrosamine (**37**), along with amphimedine (**27**), neoamphimedine (**29**) and deoxyamphimedine (**31**)

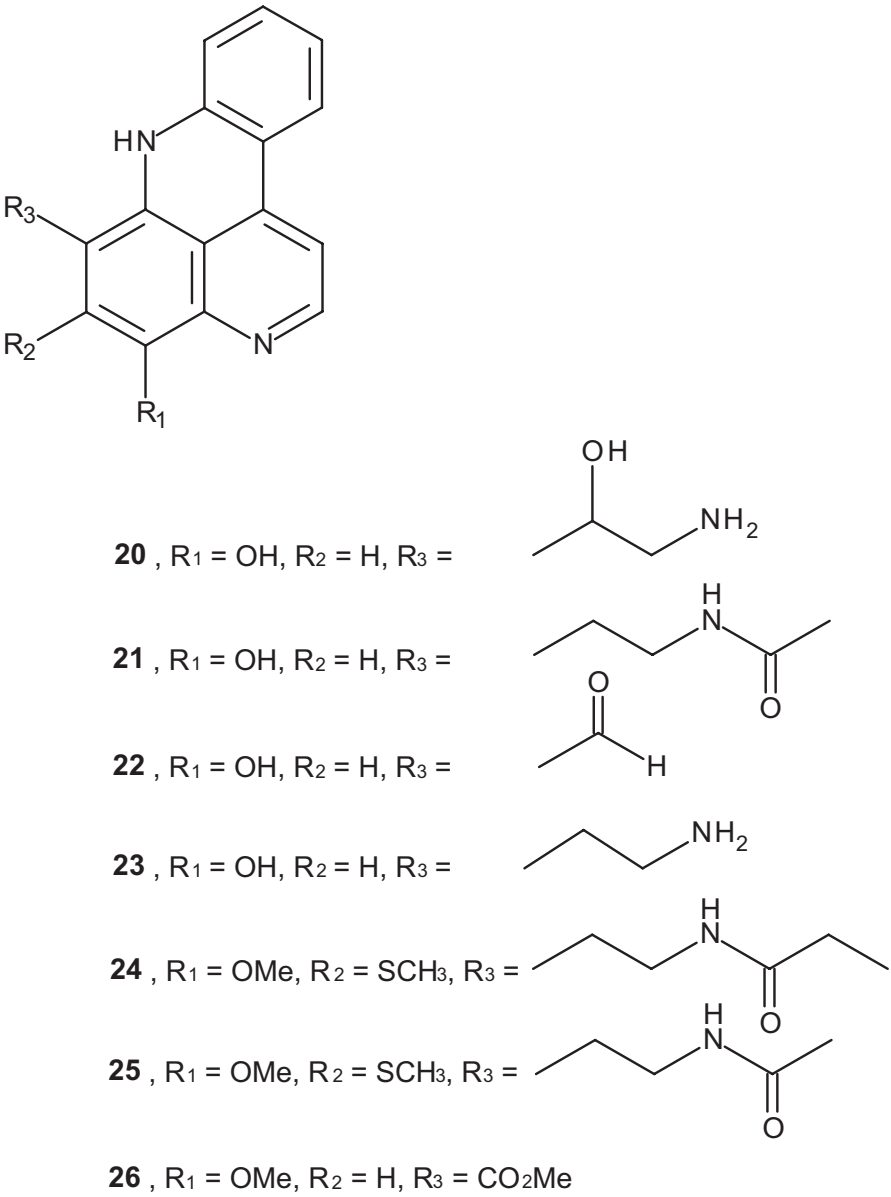
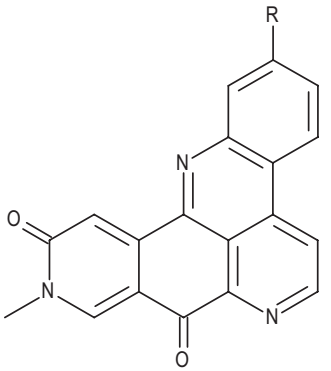
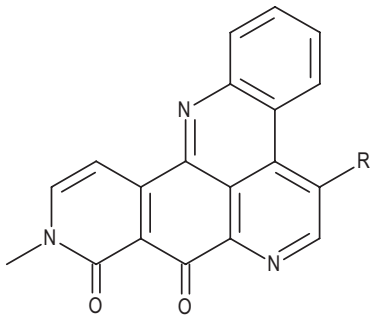


Fig. 36.4 Structures of styelamines A–D (**20**–**23**), varamines A (**24**) and B (**25**), and norsegoline (**26**)

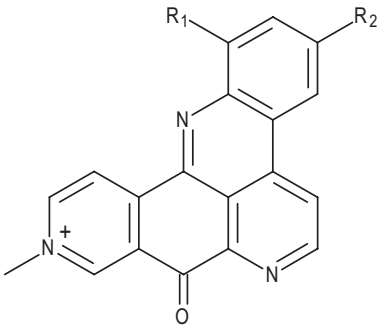
(Fig. 36.5), from the marine sponge *X.* cf. *carbonaria*. Recently, Bry et al. [17] reported isolation of, among other pyridoacridine alkaloids, demethyldeoxyamphimedine (**34**) (Fig. 36.5), from the purple morph of the Mediterranean ascidian *Cystodytes dellechiajei*.



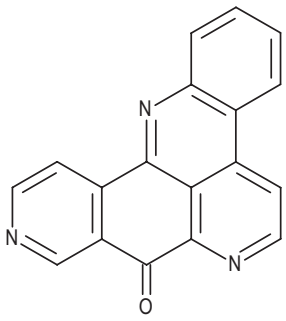
27, R = H
28, R = Br



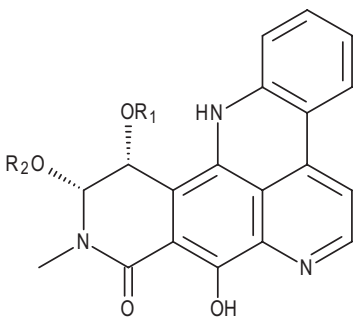
29, R = H
30, R = OMe



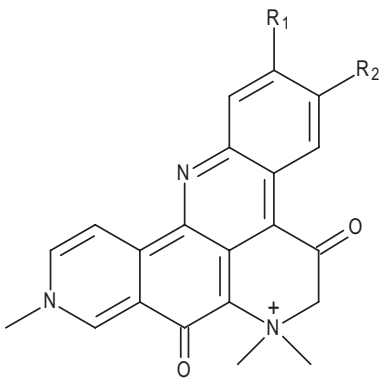
31, $R_1 = R_2 = H$
32, $R_1 = OH$, $R_2 = H$
33, $R_1 = H$, $R_2 = OH$



34



35, $R_1 = R_2 = H$
36, $R_1 = R_2 = Me$



37, $R_1 = R_2 = H$
38, $R_1 = Br$, $R_2 = H$
39, $R_1 = H$, $R_2 = Br$

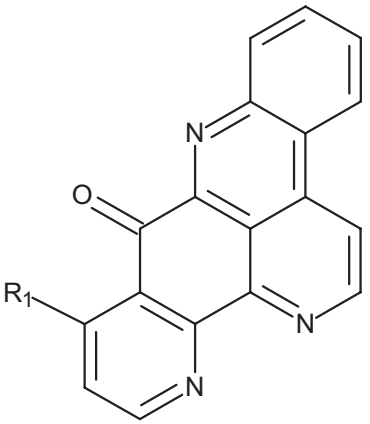
Fig. 36.5 Structures of amphimedine (**27**), 2-bromoamphimedine (**28**), neoamphimedine (**29**), 5-methoxyamphimedine (**30**), deoxyamphimedine (**31**), 1-hydroxyamphimedine (**32**), 3-hydroxy-deoxyamphimedine (**33**), demethyldeoxyamphimedine (**34**), neoamphimedine Y (**35**), neoamphi-medine Z (**36**), debromopetrosamine (**37**), petrosamine (**38**) and petrosamine B (**39**)

Another interesting pentacyclic pyridoacridine alkaloid is petrosamine (**38**) (Fig. 36.5). This compound was first isolated from the methanol extract of the marine sponge *Petrosia* sp., collected in Belize [26]. In a crystalline form, petrosamine (**38**) exists as a diketone with the carbonyl groups on C-5 and C-8. However, in solution, the C-5 carbonyl exists in an *enol* form.

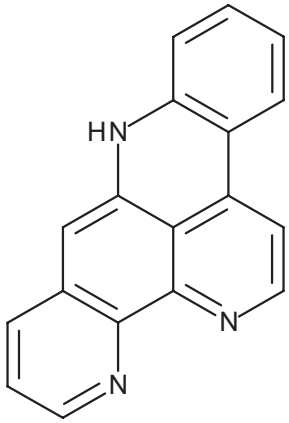
In a search for compounds with anticholinesterase activity, Nukoolkarn et al. [27] reported isolation of petrosamine (**38**) and 2-bromoamphimedine (**28**), from the marine sponge *Petrosia* n. sp., collected from the intertidal zone of Phuket Island, Thailand. They have found that only petrosamine (**38**) exhibited strong anti-acetylcholinesterase activity approximately six times higher than that of the reference galantamine A. Computational docking study of petrosamine (**38**) with the enzyme from the electric eel *Torpedo californica* (TcAChE) revealed that the major contribution to the petrosamine-TcAChE interaction was due to the quaternary ammonium group of petrosamine (**38**). Using the *Helicobacter pylori* enzyme aspartyl semialdehyde dehydrogenase (ASD) to detect antibacterial activity for bioassay-guided fractionation, Carroll et al. [28] reported isolation of petrosamine B (**39**) from the methanol extract of the marine sponge *Oceanapia* sp., collected from North Queensland, Australia. Petrosamine B (**39**) is isomeric with petrosamine (**38**); the only difference between the two compounds is the position of the bromine atom. Petrosamine B (**39**) was found to be weak inhibitor of ASD with an IC_{50} of 306 μM.

Ascididemin (**40**) (Fig. 36.6), a member of the pentacyclic pyridoacridines with a pyridine ring fused with C-8 and C-9 of ring C of the iminoquinone moiety, was reported as a potent antileukemic pentacyclic aromatic alkaloid from the Okinawan tunicate *Didemnum* sp. [29]. It was later isolated from various sources such as the Mediterranean ascidian *Cystodytes dellechiajei* [30], a previously undescribed red *Didemnum* sp. and *Didemnum rubeum*, collected from Indonesia [31], an unidentified Singaporean ascidian [32], and together with 12-deoxyascididemin (**42**) (Fig. 36.6) from the Australian ascidian *Polysyncraton echinatum* [33]. In contrast to ascididemin (**40**), meridine (**43**) (Fig. 36.6) possesses a pyridine ring fused with C-9 and C-10 of ring C of the iminoqunone moiety. Meridine (**43**) was isolated, together with its relatively stable tautomer (**44**) and 11-hydroxyascididemin (**41**) (Fig. 36.6), from the ascidian *Amphicarpa meridiana*, collected from South Australia [34], and later from the marine sponge *Ecionemia geodides*, also collected from Australia [35]. It is interesting to mention also that 11-hydroxyascididemin (**41)** was also isolated from the Mediterranean ascidian *Cystodytes dellechiajei*, however its structure was wrongly assigned as cystodamine [36, 37].

Shermilamines are pentacyclic pyridoacridines whose ring C of the 11H-pyrido[4, 3, 2*mn*]acridine skeleton is fused with the 3-thiomorpholinone ring through C-8 and C-9. While different analogues of aminoethyl substituents can be found at C-10, C-2 can have bromine substituent. The group of Sheuer has first reported isolation of shermilamines A (**45**) and B (**46**) (Fig. 36.7) from a purple colonial tunicate *Trididemnum* sp., collected in Guam [38, 39]. Shermilamine B (**46**) was later isolated from several sources, including an unidentified purple colonial tunicate and its prosobranch mollusk predator *Chelynotus semperi*, also by Sheuer's group [7], and together with *N*-deacetylshermilamine B (**50**) (Fig. 36.7), from the Mediterranean



40, R = H
41, R = OH



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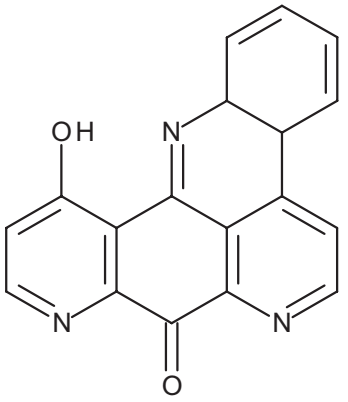

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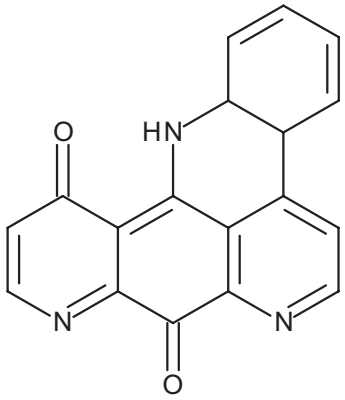

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Fig. 36.6 Structures of ascididemin (**40**), 11-hydroxyascididemin (**41**), 12-deoxyascididemin (**42**), meridine (**43**) and meridine tautomer (**44**)

purple morph of the ascidian *Cystodytes dellechiajei* [17, 40], as well as from the Fijian *Cystodytes* sp. ascidian, together with shermilamine C (**47**) (Fig. 36.7) [10]. Koren- Goldshlager et al. [41] reported isolation of shermilamines D (**48**) and E (**49**) (Fig. 36.7) from the Indian Ocean tunicate *Cystodytes violatinctus*, collected at the Mayotte Lagoon, Comoros Islands, northwest of Madagascar.

The kuanoniamines are members of the pentacyclic pyridoacridine alkaloids whose ring C is fused with a 1,3-thiazole ring through C-8 and C-9. The name kuanoniamine is derived from the Hawaiian word "*kuanoni*" meaning change when

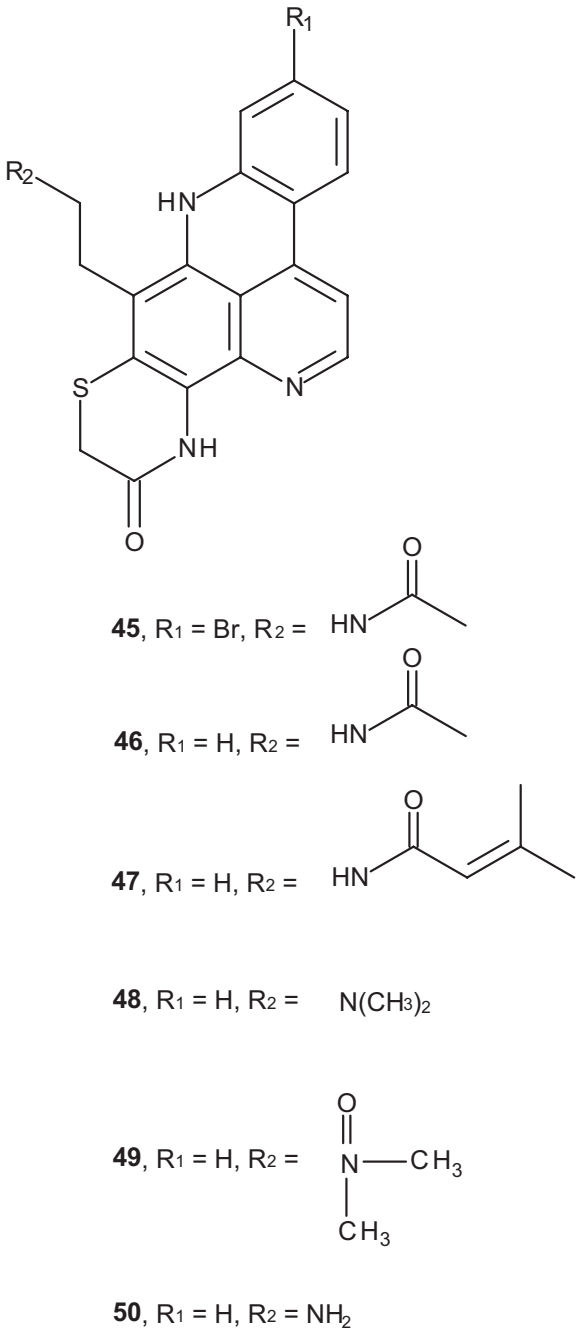


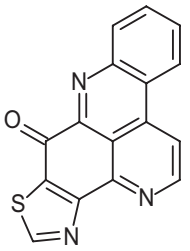
Fig. 36.7 Structures of shermilamines A–E (**45–49**) and *N*-deacetylshermilamine (**50**)

pertaining to color since the colors of these compounds are extremely sensitive to changes in pH. They are yellow in neutral and basic solution while in acidic solution they change to brilliant purple [7]. Kuanoniamines have been isolated from both marine sponges, mostly from the genus *Oceanapia*, and ascidians. Carrol and Sheuer [7] first described isolation of kuanoniamines A–D (**51–54**) (Fig. 36.8) from both the lamellariid mollusk *Chelynotus semperi* and an unidentified purple colonial tunicate, collected from Mante Channel, Pohnpei. Kuanoniamines C (**53**) and D (**54**) were later isolated from the purple tunicate, tentatively identified as *Cystodytes* from Pohnpei, by Gunawardana et al. [42]. McDonald et al. [10] isolated, among other pyridoacridine alkaloids, kuanonamine D (**54**) and dehydrokuanoniamine B (**55**) (Fig. 36.8) from a Fijian *Cystodytes* sp. ascidian. Eder et al. [43] described isolation of kuanoniamine C (**53**) and *N*-deacetylkuanoniamine D (**56**) (Fig. 36.8) from the marine sponge *Oceanapia* sp., collected from Truk Lagoon, Micronesia. The new kuanoniamines, kuanoniamines E (**57**) and F (**58**) (Fig. 36.8) were isolated, together with kuanoniamines A (**51**), C (**53**) and D (**54**), from an ascidian collected in Singapore [32]. During a search for bioactive compounds from marine sponges from the Gulf of Thailand, Kijjoa et al. [44] also isolated kuanoniamines A (**51**) and C (**53**), from the marine sponge *Oceanapia sagittaria*. Kuanoniamine D (**54**) and *N*-deacetylkuanoniamine D (**56**) were also isolated from the purple morph of Mediterranean ascidian *Cystodytes dellechiajei* [17, 40].

A structurally related pentacyclic pyridoacridine dercitin (**61**) (Fig. 36.8) was isolated from the deep-water marine sponge *Dercitus* sp., collected from Bahamas by Gunawardana et al. [45]. The same group also reported isolation of nordercitin (**59**), dercitamine (**60**) (Fig. 36.8) and dercitamide from the marine sponges *Dercitus* sp. and *Stelletta* sp. [46]. Later on, the same group has revised the structure of dercitamide to kunoniamine C (**53**) [42]. Lissoclinidine (**62**) (Fig. 36.8), a pentacyclic alkaloid containing a 1,3-oxathiolane ring fused with C-8 and C-9 of ring C, was first isolated from the ascidian *Lissoclinum notti*, collected in New Zealand [11] while its *N*14-deacetyl derivative, lissoclinidine B (**63**) (Fig. 36.8) was isolated together with diplamine B (**19**) and isolissoclinotoxin B, from *Lissoclinum* cf. *badium*, collected of the coast of Papua New Guinea [15]. Solomon and Faulkner [47] also reported isolation of dercitamide (kuanoniamine C), as a major metabolite, and another structurally related sagitol (**64**) (Fig. 36.8) as a minor metabolite, from the marine sponge *Oceanapia sagittaria* in Palau.

Sebastianine A (**65**) (Fig. 36.9), a pentacyclic alkaloid comprising of a pyrole ring fused with C-9 and C-10 of the ring C of a pyridoacridine system, was isolated from the methanol extract of the deep blue morphs of the ascidian *Cystodytes dellechiajei*, collected near São Sebastião Island, Brazil [48]. Another pyrole containing pentacyclic pyridoacridines are arnoamines A (**66**) and B (**67**) (Fig. 36.9), however in these compounds the pyrole ring is fused with C-10 of ring C and N-11 of ring B. Arnoamines A (**66**) and B (**67**) were also reported from the ascidian *Cystodytes* sp, collected in the vicinity of Arno Atoll, Republic of Marshall Islands [49].

Besides the iminoquinone moiety, 1,4-diimine moiety can be found in the pyridoacridines, although they are rare for the pyridoacridine structure class. Examples are ecionines A (**68**) and B (**69**) (Fig. 36.9), which were isolated from the marine sponge *Ecionemia geodides*, collected from Tasmania, Australia [35].



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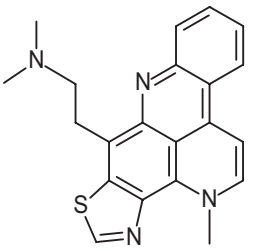
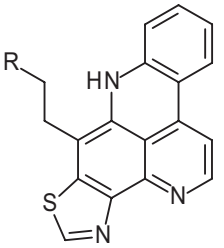
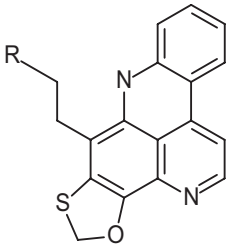
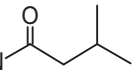


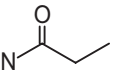
61



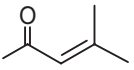


62, R = NHCOMe
63, R = NH_2

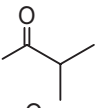
52, R = 

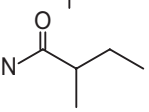
53, R = 

54, R = 

55, R = 

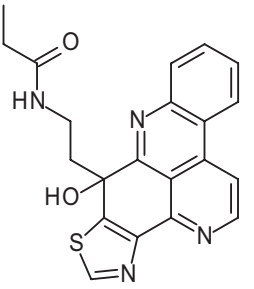
56, R = NH_3

57, R = 

58, R = 

59, R = $N(CH_3)_2$

60, R = $NHCH_3$



64

Fig. 36.8 Structures of kuanoniamines A–D (**51–54**), dehydrokuanoniamine B (**55**), *N*-deacetylkuanoniamine D (**56**), kuanoniamines E and F (**57**, **58**), nordercitin (**59**), dercitamine (**60**), dercitin (**61**), lissoclinidine (**62**), lissoclinidine B (**63**) and sagitol (**64**)

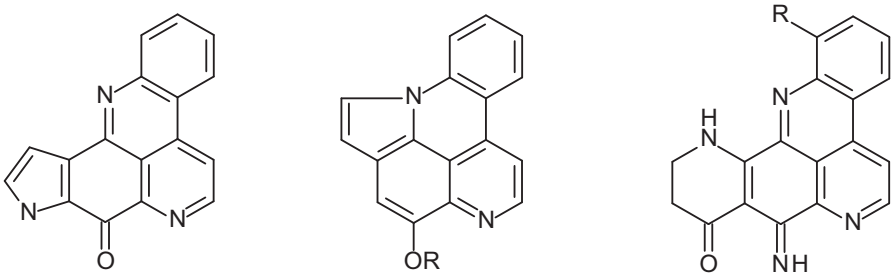


Fig. 36.9 Structures of sebastianine A (**65**), arnoamines A (**66**) and B (**67**), ecionines A (**68**) and B (**69**)

36.2.3 Hexacyclic Alkaloids

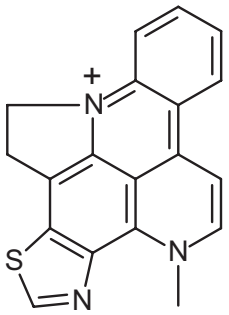
So far, only few hexacyclic pyridoacridine alkaloids have been isolated from marine sources. These alkaloids can be considered as derived from cyclization of the side chain on C-8 or C-10 of the pentacyclic pyridoacridine derivatives. Thus, while cyclodercitin (**70**) (Fig. 36.10) was isolated, together with nordercitin (**59**), dercitamine (**60**) and kuanoniamine C (**53**), from the marine sponges *Dercitus* sp. and *Stelletta* sp. [46], a structurally related stellettamine (**71**) (Fig. 36.10) was isolated from a *Stelletta* sp. [42].

Similarly, cycloshermilamine D (**72**) (Fig. 36.10) and shermilamine D (**48**) were isolated from the same tunicate (*Cystodytes violatinctus*) [50]. On the other hand,13-didemethylaminocycloshermilamine D (**73**) (Fig. 36.10) was isolated, together with shermilamine B (**46**) and *N*-deacetylshermilamine B (**50**), from the purple morph of the Mediterranean ascidian *Cystodytes dellechiajei* [17]. In the same way, sebastiamine B (**74**) (Fig. 36.10) was isolated together with sebastiamine A (**65**) from the deep blue morphs of the ascidian *Cystodytes dellechiajei*, collected near São Sebastião Island, Brazil [48].

A group of hexacyclic pyridoacridine alkaloids possessing the benzo 1,6-phenanthroline ring system are the segolines. Segoline A (**75**), isosegoline A (**76**) and segoline B (**77**) (Fig. 36.11) were isolated, together with norsegoline (**26**), from the marine tunicate *Eudistoma* sp. [19, 20]. Later on, Viracaoundin et al. [51] reported isolation of segoline A (**75**) and segoline C (**78**) (Fig. 36.11) from the purple tunicate *Eudistoma bituminis*, collected from the Mayotte lagoon, in the Comoros Islands, northwest of Madagascar.

36.2.4 Heptacyclic Alkaloids

Eilatin (**79**) (Fig. 36.12) is the only known marine heptacyclic pyridoacridine alkaloid. It was first isolated from the tunicate *Eudestoma* sp. by Rudi et al. [20]. Later



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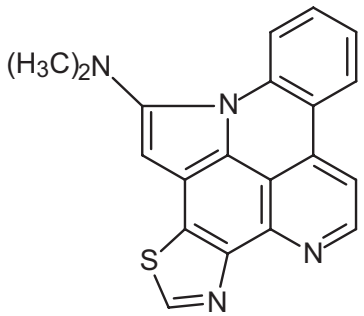

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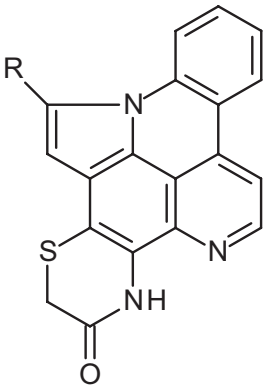
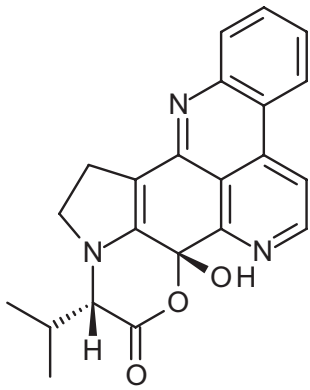


72, R = N(CH$_3$)$_2$
73, R = H



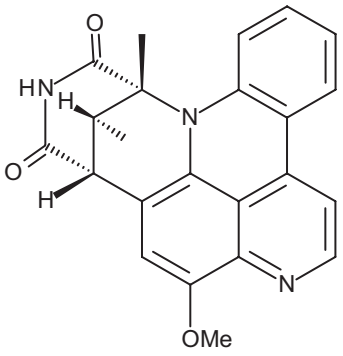
74

Fig. 36.10 Structures of cyclodercitin (**70**), stellettamine (**71**), cycloshermilamine D (**72**), 13-dedimethylaminocycloshermilamine D (**73**) and sebastianine B (**74**)

on, the same group described isolation of eilatin (**79**), together with norsegoline (**28**), segoline A (**75**), isosegoline A (**76**), segoline B (**77**), and debromoshermilamine A, from the same organism [19]. Eilatin (**79**) was also isolated from a Fijian *Cystodytes* sp. ascidian [10] and the Australian ascidian *Polysyncraton echinatum* [33].

36.2.5 Octacyclic Alkaloids

Up to date, eudistones A (**80**) and B (**81**) (Fig. 36.12) are the only described marine-derived octacyclic pyridoacridine alkaloids. They were both isolated, together with ascididemin (**40**), from the Seychelles tunicate *Eudistoma* sp. [52].



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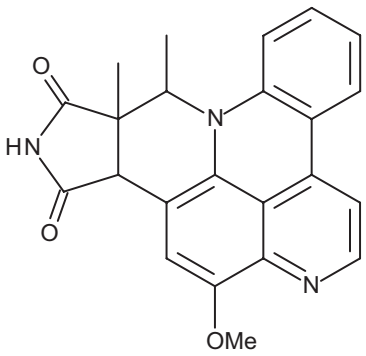

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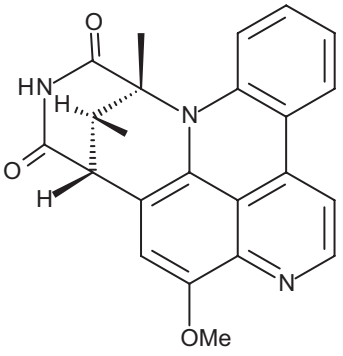

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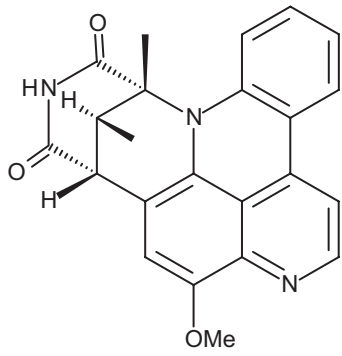
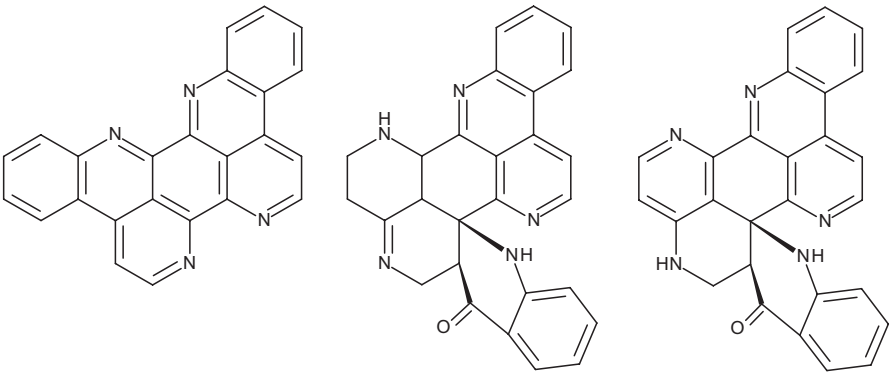


78

Fig. 36.11 Structures of segoline A (**75**), isosegoline A (**76**), segolines B (**77**) and C (**78**)

36.3 Anticancer Activities of Pyridoacridine Alkaloids

As a class, the pyridoacridines are considered DNA binding molecules and have been characterized mainly on the basis of their cytotoxicity. Consequently, their cytotoxicity is often attributed to their ability to intercalate DNA and thereby interact with, or inhibit, DNA metabolizing enzymes. Some pyridoacridines which are cytotoxic to cultured mammalian tumor cell lines have demonstrated excellent antitumor activity in various models, while others have proven too toxic to be useful. Recent work has demonstrated that the individual pyridoacridines can vary dramatically in their molecular in cell killing [3]. Although some excellent reviews on antitumor activity of naturally occurring pyridoacridine alkaloids from marine sources and their synthetic analogues have been previously published [3, 4, 6], there is still



79 80 81

Fig. 36.12 Structures of eilatin (**79**), eudistones A (**80**) and B (**81**)

no review that covers recent developments of the studies of mechanisms of the antitumor activity of particular groups of these marine-derived alkaloids. The fact that almost all of the pyridoacridine alkaloids, isolated from the marine sources, have been tested for their *in vitro* cytotoxicity on human tumor cell lines but only a few of them have been further investigated for their mechanism of action, has prompted us to focus on relevant groups of these alkaloids whose mechanisms of cytotoxicity have been thoroughly investigated.

36.3.1 Mechanism for Apparent Intercalator-Induced Inhibition of Topoisomerase II by Pyridoacridine Alkaloids from a Cystodytes sp. Ascidian

One of the early works on the mechanisms of cytotoxicity of the marine pyridoacridine alkaloids was carried out by the group of Chris Ireland. McDonald et al. [10] have evaluated the *in vitro* cytotoxic activity of cystodytin A (**3**), cystodytin J (**12**), diplamine (**17**), shermilamine B (**46**), shermilamine C (**47**), kuanoniamine D (**54**), dehydrokuanoniamine D (**55**) and eilatin (**79**), against the human colon tumor cell line HCT-116. The result showed that all compounds were active to varying degree. Diplamine (**17**, $IC_{50} < 1.4$ μM) and cystodytin J (**12**, $IC_{50} = 1.6$ μM) were the most potent; both inhibited HCT-116 replication with IC_{50} values less than 2 μM, while shermilamine B (**46**, $IC_{50} = 13.8$ μM) and shermilamine C (**47**, $IC_{50} = 16.3$ μM) were the least active. DNA intercalation studies using an ethidium bromide displacement assay showed that diplamine (**17**, $K_{disp} = 21$ μM) and cystodytin J (**12**, $K_{disp} = 51$ μM) were the most efficient intercalators. Interestingly, shermilamine C (**47**), the least cytotoxic pyridoacridine, was the poorest intercalator ($K_{disp} > 100$ μM) followed shermilamine B (**46**), which was the second to last in term of cytotoxicity and intercalative ability ($K_{disp} > 100$ μM). These pyridoacridines were also evaluated for

their capacity to inhibit topoisomerase II catalytic activity. All compounds were found to be capable of inhibiting decatenation. Like the classic cleavable complex-stabilizing etoposide, diplamine (**17**) was able to effect dose-dependent inhibition of topoisomerase II-catalyzed kinetoplast DNA (kDNA) decatenation *in vitro*. Interestingly, diplamine (**17**, IC_{90} = 9.2 μM) was found to be more potent than etoposide (IC_{90} = 68 μM). Cystodytin J (**12**) and diplamine (**17**) were found to be the most potent (IC_{90} = 8.4 and 9.2 μM, respectively) inhibitors while shermilamine C (**47**) and shermilamine B (**46**) were among the least potent (IC_{90} = 138 μM and 118 μM, respectively). Furthermore, these compounds were also tested for their ability to exert enhanced toxicity toward the double strand break repair-deficient xrs-6 Chinese hamster ovary (CHO) mutant cell line relative to the repair-proficient BR1 cell line, and none of them showed significant BR1/xrs-6 differential toxicity. Since enhanced toxicity toward the DNA double strand break repair-deficient CHO xrs-6 cell line *versus* the repair-competent BR1 line indicates "cleavable complex" mediated toxicity, they suggested that no cleavable complex formation had occurred and that these compounds did not cause double strand breaks in DNA. On the other hand, cystodytin J (**12**) and diplamine (**17**) were found to cause dramatic effects on RNA and DNA synthesis. However, no effect was observed on protein synthesis during the 9 h exposure period. On the basis of the observed correlations between HCT-116 cytotoxicity, topoisomerase II inhibition, and DNA intercalation, they hypothesized that these pyridoacridines brought about cell death by inhibiting DNA interactive protein (e.g., topoisomerase II) following intercalation and as a result of intercalation, the pyridoacridines inhibited proliferation of HCT-116 cells by interfering with nucleic acid structure and function. This interference was brought about partly by disruption of the function of topoisomerase II. They also suggested that by intercalating into DNA, the pyridoacridine interrupted the interaction between topoisomerase II and DNA, and consequently, the enzyme could not carry out its normal function during replication. The fact that these compounds showed neither enhanced toxicity toward DNA double strand break repair-deficient CHO cell lines nor produced cleavable complexes led the authors to suggest that these alkaloids inhibit topoisomerase II catalytic activity not by producing a cleavable complex but by interacting with DNA itself. They argued that since the pyridoacridines intercalated into DNA with high affinity and may change the topology of the molecule, it is likely that they inhibit other DNA binding enzymes necessary for replication. Therefore, other enzymes such as polymerases or topoisomerase I may be unable to bind or function properly due to the presence of intercalator molecules in the DNA. Based on the relative cytotoxic, intercalative, and topoisomerase II inhibitory activities of the pyridoacridines tested, the authors suggested the structural characteristics for these activities. The tetracyclic alkaloids cystodytin J (**12**) and diplamine (**17**), which possessed the iminoquinone portion, were found to be the best intercalators and topoisomerase II inhibitors and also the most potent growth inhibitors of the series. They suggested also that the diminished potencies of the other pyridoacridines may result, in part, from steric effects of the additional ring (s), although electronic effect of the iminoquinone may also be important since the pentacyclic alkaloids shermilamine B (**46**) and shermilamine C (**47**) which lack the

iminoquinone moiety, were among the least active in all the assays. The authors also proposed that the heptacyclic alkaloid eilatin (**79**) may cleave DNA by producing hydroxyl radical, which can damage DNA, following intercalative binding with DNA. Structurally, eilatin (**79**), a phenantroline possessing a double nitrogen bay region to chelate metal, is able to facilitate redox cycling to generate ROS. Other pyridoacridines may have less hydroxyl radical-generating ability due in part to a diminished ability to chelate metal ions and are less able to nick DNA in a manner similar to eilatin (**79**).

36.3.2 Influence of N-14 Substitution on the Observed Antitumor Activity of Cystodytins and Styelsamines

Although most of naturally occurring cystodytins and styelsamines have been evaluated for their cytotoxicity, exhibiting a range of potency (IC_{50} = 0.12–2.9 μM), only the DNA binding ability of cytodytin J (**12**) has been reported [10]. The fact that the antitumor activity of the pyridoacridine alkaloids is often attributed to DNA binding, Marshall and others have noted that such correlation is compound specific [3]. Consequently, Fong and Copp [53] have explored the influence of N-14 substitution on the antitumor activity of the cystodytin and styelsamine alkaloids by preparing library of natural and non-natural analogues to evaluate their DNA affinity, by ethidium bromide displacement assay, and cytotoxicity towards a panel of human tumor cell lines. The ethidium bromide displacement assay revealed that styelsamines B (**21**) and D (**23**) exhibited the highest affinity for calf thymus (CT) DNA within the styelsamine compound library (Fig. 36.13), with the apparent binding constant (K_{app}) 5.33×10^6 and 3.64×10^6 M^{-1}, respectively, while other styelsamine analogues showed mild to low affinity for CT-DNA. These results prompted them to suggest that various sidechains could hinder DNA binding. For the cystodytin library (Fig. 36.13), they have found that cystodytins A (**3**) and J (**12**) exhibited slightly higher apparent binding constants than their non-natural analogues.

Using the preliminary one dose (10 μM) testing against 57 human tumor cell lines, they have found that, of the styelsamine analogues, N^{14}-3-phenylpropanamide (**84**) (Fig. 36.13) was the most active, having the 10 μM dose resulting in mean cell killing (negative growth), and the remaining analogues were considered either mildly active, or inactive in the case of palmitamide (**85**) (Fig. 36.13). Of the four cystodytin analogues tested (**3**, **12**, **86**, **87**), cystodytin J (**12**) was considered to be inactive, while the 3-phenylpropanamide (**87**) (Fig. 36.13) was observed to be the most active. Interestingly, sub-panel selectivity was observed for both natural and some synthetic analogues of styelsamine. Thus, styelsamine B (**21**) was found to be more selective towards melanoma, non-small cell lung cancer and ovarian panels; styelsamine D (**23**) towards non-small cell lung cancer, CNS and renal; styelsamine-N^{14}-3-methylbut-2-enamide (**82**) towards leukemia and renal; styelsamine-N^{14}-benzamide (**83**) towards melanoma and renal, and styelsamine-N^{14}-3-palmitamide (**85**) towards colon and renal cancer cell lines. In contrast, cytodytins

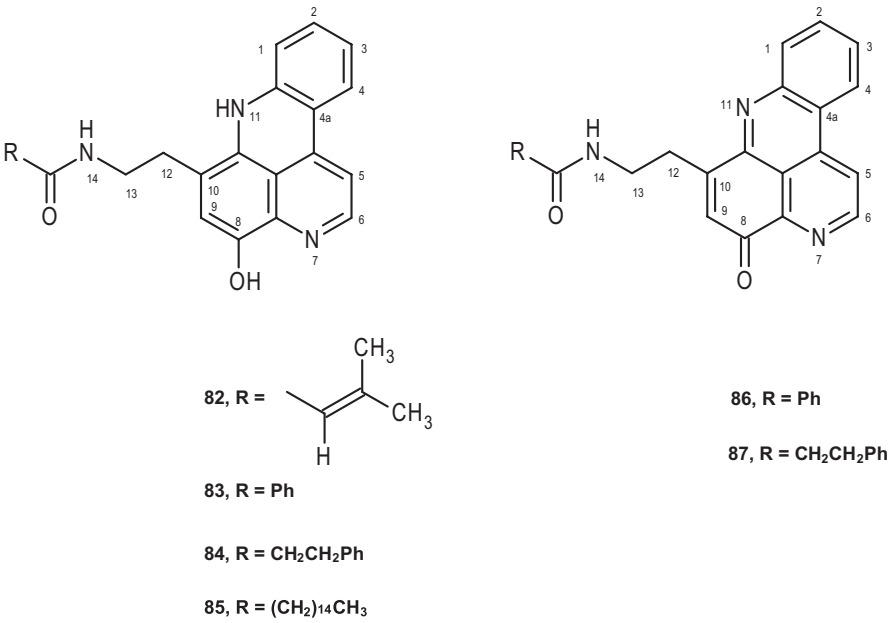


Fig. 36.13 Structures of styelsamine-N^{14}-3-methylbut-2-enamide (**82**), styelsamine-N^{14}-benzamide (**83**), styelsamine N^{14}-3-phenylpropanamide (**84**), styelsamine-N^{14}-3-palmitamide (**85**), cystodytin-N^{14}-benzamide (**86**), cystodytin-N^{14}-phenylpropanamide (**87**)

A (**3**), J (**12**) and cystodytin-N^{14}-benzamide (**86**) were essentially non-selective, while cystodytin-N^{14}-phenylpropanamide (**87**) exhibited selectivity towards colon, melanoma and renal cancer cell line sub-panels. They have found also that, in general, most compounds exhibited poor cytotoxicity, unable to reach LC_{50} (50% lethality) or TGI (total growth inhibition, cytostatic) levels of activity. Interestingly, they also found that there was no correlation between DNA affinity (K_{pp} values) and cytotoxicity (GI_{50} values) for these compounds since styelsamines B (**21**), D (**23**) and styelsamine-N^{14}-3-methylbut-2-enamide (**82**), all exhibited almost the same level of growth inhibition (GI_{50} = 3.2–4.0 μM), whereas styelsamines B (**21**) and D (**23**) bound approximately ten times more strongly to DNA than styelsamine-N^{14}-3-methylbut-2-enamide (**82**). Similarly, styelsamine-N^{14}-3-palmitamide (**85**) and cystodytin-N^{14}-phenylpropanamide (**87**), which exhibited approximately the same level of DNA affinity, showed different levels of antiproliferative activity (**85** was inactive; GI_{50} of **87** was 0.32 μM). More importantly, they have suggested that lipophilicity was an important determinant of cell based antiproliferative activity since cell penetration is clearly a requisite condition for molecules that exert biological activity by targeting DNA. By plotting one dose mean cell growth inhibition activities against clog P, they have verified that, for styelsamine and cytodytin series, the optimal activity occurred with alkaloid having clog P around 4.0 to 4.5. Consequently, they concluded that sidechain modified analogues of styelsamines and/or cystodytins may have potential as new classes of antitumor agents.

36.3.3 Mechanism of Antitumor Activity of Ascididemin

Among the pyridoacridine alkaloids isolated from marine sources, ascididemin (**40**) was the most extensively investigated for the mechanism of its antitumor activity. It was found to be highly toxic to human colon (HCT-116) and breast (MCF-7) cancer cell lines [54] as well as to different leukemic cell lines [30]. Most importantly, ascididemin (**40**) was equally toxic to drug-sensitive and multidrug-resistant cell lines. Ascididemin (**40**) was also found to be able to intercalate DNA, preferentially at GC-rich sequences [30]. Dassonneville et al. [55] have investigated the effect of ascididemin (**40**) on the activity of topoisomerases and have found that, in the presence of ascididemin (**40**), a band corresponding to linear DNA could be clearly seen proving that ascididemin (**40**), like etoposide, inhibited the relegation of DNA once the double helix had been cleaved by the enzyme; however, the intensity was much weaker than that caused by etoposide. These data suggested that ascididemin (**40**) is a weak inhibitor of topoisomerase enzymes. By using the radiolabelled DNA substrate, they have found that ascididemin (**40**) effectively functioned as a topoisomerase II poisoning, stabilizing DNA-topoisomerase II covalent complex. Furthermore, ascididemin (**40**) was proved to be less toxic to P338 (sensitive to camptothecin) cells than to P388CPT5 (resistant to camptothecin) cells, suggesting that topoisomerase I was not a cellular target for ascididemin (**40**). Moreover, ascididemin (**40**) was found to be equally toxic to both human leukemia sensitive (HL-60) and resistant (HL-60/MX2) to mitoxantrone cell lines, and since HL-60/MX2 cells display atypical multidrug resistance with the absence of P-glycoprotein overexpression and altered topoisomerase II catalytic activity and reduced levels of topoisomerase IIα and IIβ, they suggested that topoisomerase II did not contribute to the cytotoxic action of ascididemin (**40**). This finding was corroborated by the immunoblot assay which showed the absence of DNA-topoisomerase II covalent complexes in HL-60 leukemia cells, in the presence of ascididemin (**40**). They have also investigated the effects of ascididemin (**40**) on the cell cycle of HL-60 leukemia cells and have found that the increase of the sub-G1 population (apoptotic cells) was up to 70% in the ascididemin-treated HL-60 leukemia cells whereas it represented only 3% in the cell in the control. Interestingly, the increase in the sub-G1 population was concomitant with the decrease of the G1 population (from 45 to 10%). Additionally, they have shown also that ascididemin (**40**) was able to cleave PARP (poly(ADP-ribose) polymerase), an enzyme involved in DNA repair by caspase-3. Thus, they concluded that the induction of apoptosis is associated with an activation of caspase-3 by ascididemin (**40**).

In order to clarify the conflicting data about the mechanism of antitumor activity of ascididemin (**40**), Matsumoto et al. [56] attempted to investigate the mechanism responsible for DNA damage of ascididemin (**40**), together with two structurally related synthetic alkaloids **88** and **89** (Fig. 36.14). It is interesting to note that these compounds share two common structural motifs, i.e., a double nitrogen bay region and a reducible, iminoquinone heterocyclic ring.

The results showed that ascididemin (**40**), **88** and **89** significantly inhibited topoisomerase II catalytic activity and generated some minor topoisomerase II dependent

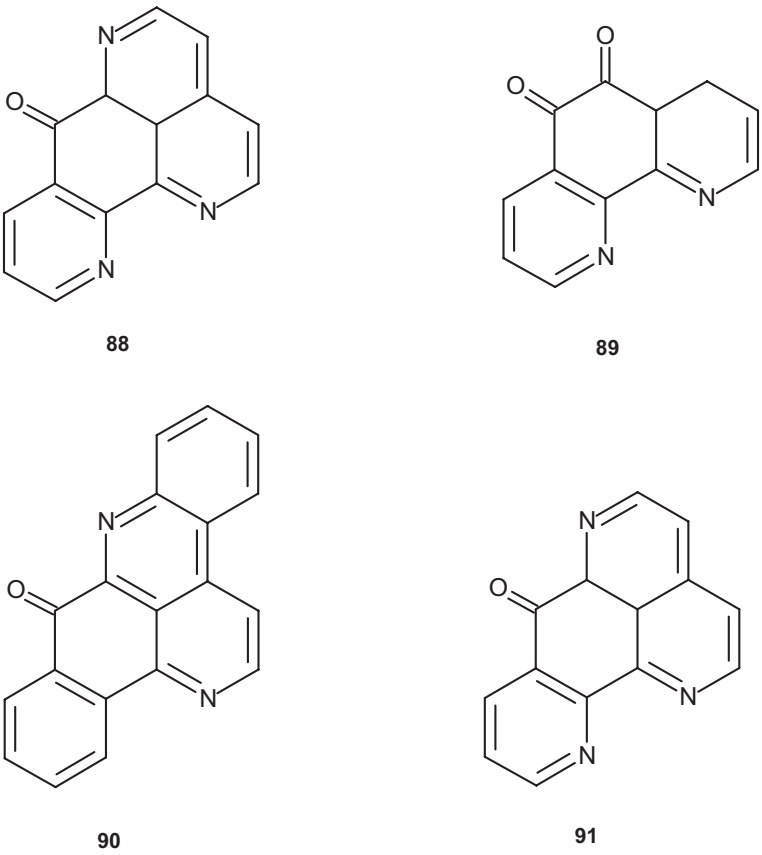


Fig. 36.14 Structures of DNA cleaving (**88**, **89**) and DNA non-cleaving (**90**, **91**) synthetic alkaloids

DNA cleavage, while no cleavage was found in the presence of **90** and **91**. However, it was shown that only 5 mM dithiothreitol (DDT) and 91 μM of ascididemin (**40**), **88** or **89** in H_2O was required to stimulate maximal DNA cleavage, independent of topoisomerases. Moreover, they have observed that DNA cleavage by these compounds was both concentration and time dependent. Assessment of DNA cleavage under anoxic conditions revealed that oxygen was a necessary component for *in vitro* DNA cleavage stimulation by these compounds. Consequently, they hypothesized that the DNA cleavage was caused by reactive oxygen species (ROS), which was substantiated by the electron paramagnetic resonance (EPR) study. Additionally, they have found also that addition of metal ions ($FeSO_4$ or NiCl) did not facilitate DNA cleavage by these compounds, and addition of chelators did not protect against the cleavage, thus excluding Fenton-type reaction as the primary ROS generation. The fact that the anti-oxidative enzyme catalase was able to extensively protect against DNA cleavage by ascididemin (**40**), **88** or **89**, it was suggested that H_2O_2 could be the possible reactive species initially generated. Thus, it was

proposed that production of ROS likely occurs through reduction of the iminoquinone moiety. This hypothesis was supported by the experiments that used cyclic voltammetry to measure reductive potential of these compounds, which showed that the three cleaving analogues, i.e., ascididemin (**40**), **88** and **89**, were marginally more easily reduced than the two non-cleaving compounds (**90** and **91**). As the reductive potentials of ascididemin (**40**), **88** and **89** are comparable to that of NADPH (−280 mV), it is consistent with bioreduction in the cell. They have also found that the capacity of these compounds did not correlate with the DNA cleavage activity since ascididemin (**40**) and **91** were found to completely displace ethidium bromide at 10 and 20 μM concentrations, whereas **88**, **89** and **90** did not at 20 μM concentrations. Consequently, the authors proposed that the ability of ascididemin (**40**) and its cleaving analogues (**88**, **89**) to interact closely with DNA increases the likelihood that ROS formed as a result of their reduction would directly affect DNA. Although the analogues **90** and **91** can intercalate DNA, they cannot produce ROS, thus fail to cleave the DNA. The ROS-mediated mechanism was also corroborated by the fact that antioxidants and antioxidant enzymes were able to protect DNA against ascididemin-induced DNA cleavage. Mechanism-based cytotoxicity screening revealed the capacity of ascididemin (**40**), **88** and **89** to predominantly cause single strand DNA breaks since the mutant of Chinese hamster ovary (CHO) cell line EM9 was particularly sensitive to the lethality of these molecules as compared to the wild-type AA8 cell line. Since the EM9 cell has a defective XRCC1 protein, which plays a critical role in DNA ligase III activity, DNA polymerization and repaired of DNA single strand breaks, this deficiency makes it hypersensitive to compounds that cause ROS, or single strand breaks *via* topoisomerase I. Finally, they have found also that glutathione not only afforded strong protection of DNA cleavage induced by ascididemin (**40**), **88** and **89** but also from the cytotoxicity of these compounds to AA8 cells. Interestingly, they have shown that heme oygenase-1 (OH-1), an oxidative stress protein, was induced in the AA8 cells by ascididemin (**40**), **88** and **89,** thus linking cellular oxidative stress with cell damage by these compounds.

Following the work of Dassonneville et al. [55] which demonstrated that ascididemin (**40**) was a potent inducer of apoptosis in the HL-60 leukemia cells, probably associated with caspase-3 activation, Dirsch et al. [57] have attempted to investigate the molecular pathway utilized by this pentacyclic pyridoacridine alkaloid to trigger cell death. They have found that treating the leukemia Jurkat T cells with ascididemin (**40**) produced all signs of apoptosis, including in cell shrinkage, membrane blebbing, chromatin condensation and fragmented nuclei. They have also shown that the ascididemin-triggered apoptosis was concentration and time-dependent. That this apoptosis did not involve protein synthesis was substantiated by their finding that pre-incubation of Jurkat cells with cycloheximide had no influence on ascididemin-mediated cell death. Also, the anti-CD95 antibody ZB4 was found to be unable to reduce ascididemin-triggered apoptosis, thus ruling out the involvement of CD5 death receptor. Additionally, ascididemin (**40**) was found to induce outer (cytochrome *c* release) and inner (loss of electrochemical gradient) mitochondrial membrane permeabilization; and the release of cytochrome *c* was found to be kinetically parallel a decrease of mitochondrial potential. These results led to the

conclusion that ascididemin (**40**) was able to induce mitochondrial dysfuntion. By treating Jurkat cells overexpressing mitochondria-protecting Bcl-2 or Bcl-x_L with 5 μM ascididemin (**40**), it was found that these cells were protected against ascididemin (**40**) compared to control. The results suggested that ascididemin (**40**) induces a mitochondria-dependent pathway of apoptosis. They also compared the kinetics by which the initiator caspase-2, -9 and -8 and the effector caspase-3 are activated by ascididemin (**40**). It was found that the activation of caspase-8 correlated well with activation of the effector caspase-3, which corroborated the findings that ascididemin-triggered apoptosis occurs independent of death receptor. Interestingly, caspase-2 processing was found to be more pronounced at 4 h after treatment with ascididemin (**40**) than caspase-9, and pre-treatment of Jurkat T cells with the specific caspase-2 inhibitor zVDVADfmk was found to completely prevent ascididemin-mediated apoptosis. Thus, these data suggested an initial and relevant role for caspase-2 in ascididemin-triggered apoptosis. As caspase-2 was found to process in Jurkat/*bcl-*X_L cells incubated with ascididemin (**40**), it was concluded that caspase-2 activation occurs upstream of mitochondria events. On the other hand, caspase-8 was found to process only in Jurkat/*neo* and not in Jurkat/*bcl-*X_L cells, confirming that caspase-8 acts as effector caspase downstream of mitochondria. Moreover, they have shown that pre-treatment with the caspase-2 inhibitor zVDVADfmk resulted in strong reduction of the cleavage of caspase-9, corroborating that caspase-2 acts upstream of caspase-9. Moreover, ascididemin (**40**) was also found to induce Bid cleavage, and this cleavage is inhibited by the caspase-2 inhibitor zVDVADfmk, suggesting that caspase-2 activation lies upstream of ascididemin-induced mitochondria activation and that Bid cleavage by caspase-2 may contribute to mitochondrial perturbation. Finally, they hypothesized that ascididemin (**40**) might activate c-Jun N-terminal protein kinase (JNK), which might be involved in ascididemin-induced apoptosis signaling. In order to prove this hypothesis, they have set up experiments whose results revealed that ascididemin (**40**) activated/phosphorylated JNK after 4 h, paralleling caspase-2 activation. Since Bcl-x_L was unable to abrogate JNK phosphorylation, it was concluded that activation of JNK occurs upstream of mitochondria. That ascididemin (**40**) triggered ROS generation which contributed significantly to JNK activation was substantiated by the fact that anti-oxidant *N*-acetylcystein (NAC) almost completely abrogated JNK phosphorylation, and since caspase-2 and -9 processing was reduced by JNK inhibitor SP00125, it was suggested that JNK acts upstream of both caspase-2 and -9. It was found also that pre-treatment with the JNK inhibitor SP00125 caused a decrease of cytochrome *c* release from the mitochondria as well as ascididemin-induced DNA fragmentation. These data led Dirsch et al. to conclude that ascididemin triggers JNK activation *via* ROS generation and active JNK contributes *via* non identified target to caspase activation and cytochrome *c* release from mitochondria.

G-quadruplexes are a family of secondary DNA structures that consists of four-stranded structure stabilized by G-quartets [58]. It is well recognized that telomeres protect chromosomal ends from fusion events and provide a mean for complete replication of chromosome, and telomere repeats are added by a specialized enzyme, telomerase, which is overexpressed in most tumor cells. It is also known that

the 3'-terminal region of the G-rich strand of human telomeres is single-stranded and may adopt a G-quadruplex conformation [59, 60]. As this structure has been shown to directly inhibit telomerase elongation *in vitro* [61], ligands that selectively bind to G-quadruplex structures may interfere with telomere structure and telomere elongation and replication of cancer cells [62, 63]. Base on this concept, Guittat et al. [64]) have investigated the capacity of ascididemin (**40**) and meridine (**43**) to stabilize G-quadruplexes and to inhibit telomerase. Using several methods such as equilibrium dialysis, mass spectrometry and fluorescence melting experiments to study the interaction of ascididemin (**40**) and meridine (**43**) with unusual DNA structures, they have found that ascididemin (**40**) and meridine (**43**) were able to bind G-quadruplexes, however their binding affinities are relatively modest, especially ascididemin (**40**). Furthermore, they have demonstrated that ascididemin (**40**) and meridine (**43**) significantly preferred the human telomeric intramolecular quadruplex over the parallel one. Using TRAP (Telomeric Repeat Amplification Protocol) assay, ascididemin (**40**) was found to inhibit telomerase with an IC_{50} of 87 μM, while meridine (**43**), which has higher affinity for quadruplexes, was more potent showing an IC_{50} of 11 μM.

36.3.4 Mechanism of Antitumor Activity of Neoamphimedine and Deoxyamphimedine

Although the structures of amphimedine (**27**), neoamphimedine (**29**) and deoxyamphimedine (**31**) are closely related, their biological activities and mechanisms of action differ significantly. Amphimedine (**27**) is relatively inactive compound compared to neoamphimedine (**29**) and deoxyamphimedine (**31**). Amphimedine (**27**) does not significantly intercalate DNA while neoamphimedine (**29**) and deoxyamphimedine (**31**) were found to displace ethidium bromide from DNA, although deoxyamphimedine (**31**) was found to be more effective than neoamphimedine (**29**). Furthermore, neoamphimedine (**29**) and deoxyamphimedine (**31**) are cytotoxic, although by different mechanisms, amphimedine (**27**) is not.

De Guzman et al. [22] have shown that neoamphimedine (**29**) was cytotoxic to normal CHO AA8 cells with an IC_{50} = 2 mg/ml, and quantitative DNA cleavage assays revealed it stimulated topoisomerase II dependent cleavage 3% above control compared to etoposide (which stimulated 38% cleavage at the same concentration) while no DNA cleavage was detected with amphimedine (**27**) in the presence of topoisomerase II. They have also found that neoamphimedine (**29**) was able to stimulate topoisomerase II to catenate DNA to a high molecular weight complex. By using Rad 52+/− yeast strains that express normal or elevated topoisomerase levels to test the cytotoxicity of amphimedine (**27**) and neoamphimedine (**29**), with etoposide (a topoisomerase II poison) as positive control and topotecan (a topoisomerase I poison) as a negative control, Marshall et al. [65] have found the enhanced cytotoxicity for neoamphimedine (**29**) and etoposide but not for amphimedine (**27**) and topotecan. Furthermore, they have tested both amphimedine

(**27**) and neoamphimedine (**29**) in mammalian cell lines: HCT-116 (human colon tumor), SK-mel-5 (human melanoma), KB (human epidermoid nasopharyngeal tumor), MCF-7 (human breast cancer), A2780wt (human ovarian tumor wild type), A2780AD (human ovarian tumor multi-drug resistant), AA8 (CHO wild type), xrs-6 (CHO double strand break repair), and have found that while neoamphimedine (**29**) was cytotoxic in every of these cell lines, amphimedine (**27**) did not show any toxicity at doses tested. Interestingly, neoamphimedine (**29**) was found to retain its cytotoxicity in the MDR-expressing A2780AD cell line while doxorubicin, M-AMSA and taxol all had significantly reduced toxicity compared to the A2780wt. Since the results obtained from the yeast cell cytotoxicity assays suggested a topoisomerase II dependent mechanism, they have tested amphimedine (**27**) and neoamphimedine (**29**) in a panel of mutant CHO cell lines. In contrast to all clinical topoisomerase II drugs tested in the xrs-6 cell line, which showed significant enhanced toxicity [66], no significant enhancement of cytotoxicity was detected at IC_{50} for amphimedine (**27**) and neoamphimedine (**29**) in the xrs-6 cells, although a small increase was observed in the xrs-6 cell line treated with neoamphimedine (**29**) compared to the AA8 cell line. Since this result was inconsistent with the yeast data, Marshall et al. [65] hypothesized that neoamphimedine (**29**) is a topoisomerase II-dependent drug, whose primary mechanism of action is not the stabilization of cleavable complexes. In order to investigate the mechanism underlying its cytotoxicity, the ability of neoamphimedine (**29**) to interfere with the function of purified human topoisomerase II was investigated *in vitro*. It was found that neoamphimedine (**29**) induced minimal DNA cleavage *via* formation of cleavage complex. In experiments using 84 ng topoisomerase II, up to 8.9% DNA cleavage (50 μM neoamphimedine) was detected. They have found also that the percentage of cleavage decreased as the concentrations of neoamphimedine (**29**) increased, and cleavage was not detected with 140 ng topoisomerase II. Using gel electrophoresis, they have confirmed that the cleavage was due to single strand DNA nicking of the plasmid substrate. Interestingly, during the experiments they have found that in the reactions containing active topoisomerase II and supercoiled substrate DNA routinely appeared as a high molecular weight (HMW) complex upon electrophoresis analysis, and this activity was concentration-dependent and was apparent whether the substrate plasmid was relaxed or supercoiled. However, this HMW complex formation was not observed with amphimedine (**27**) nor any other pyridoacridines tested in their laboratory. Further experiments have allowed them to confirm that the HMW complex was not formed by a protein-DNA aggregation or chemical cross linking but, it was in fact a catenated complex of plasmid DNA. In the experiments using SDS or TE wash, neoamphimedine (**29**) was found to induce topoisomerase to catenate DNA in a time and concentration-dependent manner. Additionally, by filter-binding assay to quantify DNA aggregation, they have shown that DNA aggregation increased in proportion to the concentration of neoamphimedine (**29**). Since KB tumors respond well to topoisomerase II drugs, including doxorubicin and etoposide, neoamphimedine (**29**) was evaluated for its *in vivo* anticancer activity in nude mice bearing human KB tumors, together with amphimedine (**27**) and etoposide. The results showed that neoamphimedine (**29**) was as effective as etoposide while amphimedine (**27**),

a topoisomerase II-inactive isomer, did not exhibit any antitumor activity. In addition, neoamphimedine (**29**) was shown to have an antitumor activity in mice bearing HCT-116 cell tumors and there was no difference between neoamphimedine (**29**) and 9-aminocamptothecin, a drug effective in this system.

The structure of deoxyamphimedine (**31**) differs from that of neoamphimedine (**29**) in the substituents on C-8 and C-9 of ring C of the 8H-pyrido[4,3,2-*mn*] acridone (**2**). While deoxyamphimedine (**31**) has a positively charged 1-methylpyridinium ring attached to C-8 and C-9 of ring C, neoamphimedine (**29**) has a 1-methylpyridin (2H)-one ring on the same position. Marshall et al. [67] have shown that deoxyamphimedine (**31**) was cytotoxic to every human tumor cell lines previously tested with amphimedine (**27**) and neoamphimedine (**29**); however its IC_{50} observed values were slightly lower than those reported for neoamphimedine (**29**) [65]. Furthermore, deoxyamphimedine (**31**) was found to be almost equally toxic to the A2780wt (IC_{50} = 0.3 μM) and its paired multi-drug resistant cell line AD2780wt. Thus, they concluded that the lack of significant fold difference for deoxyamphimedine (**31**) in both of these cell lines indicated that it was not likely a substrate for multi-drug resistant pump that often impedes the efficacy of cancer drugs in chemotherapy. Furthermore, they have found also that deoxyamphimedine (**31**) was selectively more toxic to the mutant CHO cell lines (EM9, IC_{50} = 3.8 μM and xrs-6, IC_{50} = 4.2 μM), than the wild type (AA8, IC_{50} = 13.7 μM), thus confirming DNA strand breakage as a contributing mechanism for its cytotoxicity. Ethidium bromide displacement assays revealed that deoxyamphimedine (**31**) was a potent DNA intercalator, displacing 50 % EtBr at 1 μM (while neoamphimedine required approximately 100 μM to displace 50 % EtBr), suggesting that deoxyamphimedine (**31**) had a higher affinity for DNA than its analogues. They have also carried out the topoisomerase I and II DNA cleavage assays and found that deoxyamphimedine (**31**) was a potent cleaver of DNA *in vitro* and this activity was independent on the presence of topoisomerase I or II. Like ascididemin (**40**), deoxyamphimedine (**31**) could fully cleavage DNA under aerobic conditions in the experiments carried out with DDT and DNA. It was found also that the amount of cleaved DNA observed was greatly increased with the increasing amounts of deoxyamphimedine (**31**) and DTT, but the amount of induced DNA cleavage was attenuated under hypoxic conditions. Additionally, higher temperature and high salt conditions did not reverse deoxyamphimedine-induced DNA damage, suggesting that the DNA damage was ROS-induced. As addition of metals ($FeSO_4$) and chelators did not alter the DNA cleaving ability, it was concluded that the ROS generation was not a Fenton-type reaction. Extensive protection against DNA cleavage by catalase suggested H_2O_2 as a likely ROS intermediate. Additionally, since antioxidants and ROS scavengers such as glutathione, benzoic acid and NAC were found to be able to protect against DNA damage, it was suggested that the generation of the hydroxyl radical most likely occurred. Thus they hypothesized that the iminoquinone portion of deoxyamphimedine (**31**) is responsible for the redox cycling and ROS generation. For this, direct reduction of the iminoquinone portion of deoxyamphimedine (**31**) to the semi-iminoquinone species seems to likely facilitate the production of H_2O_2 and DNA damaging free radicals.

36.3.5 Mechanism of Antitumor Activity of Lissoclinidine B and Diplamine B

Lissoclinidine B (**63**) and diplamine B (**19**) were isolated together from the ascidian *Lissoclinum* cf. *dadum,* and were tested in the Hdm2 (Human double minute 2) electrochemiluminescence assay by Clement et al. [15]. The structures of these compounds are quite distinct since the pentacyclic lissoclinidine B (**63**) has an extra 1,3-oxathiolane ring fused to ring C of the 11H-pyrido[4, 3, 2-*mn*]acridine (**1**), while the tetracyclic diplamine B (**19**) contains the iminoquinone portion.

Lissoclinidine B (**63**) and diplamine B (**19**) were tested for their effects on cellular p53 and Hdm2 in the tert-immortalized human retinal pigment epithelial (RPE) cells, and the cellular p53 and Hdm2 levels were determined by immunoblotting. They have shown that lissoclinidine B (**63**) and diplamine B (**19**) were able to increase p53 and Hdm2 in a dose-dependent manner, and at 10 μM exhibited the greatest increase in p53 and Hdm2, similar to 50 μM of the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN). Furthermore, it was found that lissoclinidine B (**63**) and diplamine B (**19**) displayed more potent activity in cells (10 μM) than in free cell assay system (IC_{50} values 98.1 ± 6 μM and 101.3 ± 4 μM, respectively). They have also treated the $p53^{-/-}mdm2^{-/-}$mouse embryo fibroblasts (MEFs) transiently transfected with plasmid encoding Hdm2 under the control of a p53-independent CMV (cytomegalovirus) promotor, with 10 μM lissoclinidine B (**63**) and diplamine B (**19**). After 8 h of incubation, both compounds were found to increase Hdm2 in MEFs, demonstrating that lissoclinidine B (**63**) and diplamine B (**19**) functioned at least in part by stabilizing Hdm2. Since diplamine B (**19**) was quite unstable and degraded over time, only lissoclinidine B (**63**) was further investigated. Using U2OS (a human osteosarcoma) cells transiently co-transfected with plasmids encoding p53 and Hdm2 prior to incubation with ALLN or lissoclinidine B (**63**) to directly investigate whether lissoclinidine B (**63**) blocked Hdm2-mediated p53 ubiquitylation, they have found that ALLN caused an accumulation of p53, including higher molecular weight forms of protein indicative of ubiquitylation, while incubation with lissoclinidine B (**63**) similarly blocked p53 degradation in a dose-dependent manner. They have also confirmed the results in an additional experiment with HCT-116 colon carcinoma cells expressing wild-type p53 which also showed that lissoclinidine B (**63**) prevented the accumulation of ALLN-induced ubiquitylated p53, following treatment with both lissoclinidine B (**63**) and ALLN. Thus, they concluded that lissoclinidine B (**63**) inhibits both Hdm2-mediated ubiquitylation and degradation of p53. Furthermore, they have also used U2OS-pG13 cells that stably express endogenous wild-type p53 and p53-responsive luciferase reporter (reporter pG 13) to examine whether lissoclinidine B (**63**) activates p53-dependent transactivation. Since treatment with lissoclinidine B (**63**) produced a dose-dependent increase in luciferase activity, they suggested that stabilization of p53 resulted in activation of p53-dependent transcription. Finally, they have shown that treatment of *p53*-deficient (A9) and wild-type *p53* MEFs (C8) with lissoclinidine B (**63**) produced a marked increase in cell death in C-8 cells while *p53*-deficient A9 cells were relatively resistant. They have also observed cleavage of PARP in

response to lissoclinidine B (**63**) specifically in the p53-expressing C-8 cells but not in A-9 cells, which is consistent with apoptotic cell death. Thus, these data indicated that lissoclinidine B (**63**) induces cell apoptosis in a p53-dependent manner which is consistent with a primary role in inhibiting Hdm2 and thereby activating p53.

It is interesting to point out that despite the structural difference between lissoclinidine B (**63**) and diplamine B (**19**), both of them were found to be equally active (IC_{50} values 98.1 ± 6 and 101.3 ± 4 μM, respectively). Thus, it was suggested that the functionality required for their activity lies in the aromatic ring system and sidechain, and may not be affected by substituent differences at C-8, C-9 or N-11.

36.3.6 Anticancer Activity Evaluation of Kuanoniamines A and C

Kijjoa et al. [44] have investigated the effects of kuanoniamines A (**51**) and C (**53**), isolated from the marine sponge *Oceanapia sagittaria* collected from the Gulf of Thailand, on the growth of five human tumor cell lines: MCF-7 (breast adenocarcinoma, estrogen-dependent ER+), MDA-MB-231 (breast adenocarcinoma, estrogen-independent ER−), SF-286 (glioma), NCI-H460 (non-small cell lung cancer), UACC-62 (melanoma), and a non-tumor human cell line MRC-5 (diploid embryonic lung fibroblast), by SRB method. Kuanoniamine A (**51**) was found to potently inhibit the growth of all five human tumor and the non-tumor MRC-5 cell lines, exhibiting its GI_{50} (concentration that causes 50% of cell growth inhibition) values less than 5 μM. Although kuanoniamine C (**53**) also inhibited the growth of all these tumor cell lines, its effect was at least ten times weaker than that observed for kuanoniamine A (**51**) for MDA-MB-231, SF-268, NCI-H460, and UACC-62. Specifically, the low GI_{50} value of kuanoniamine C (**53**) on MCF-7 (0.81 ± 0.11 μM) suggested a high selectivity of this compound for this estrogen dependent (ER+) breast cancer cell line. Using [^{3}H] thymidine incorporation assay, they have found that the DNA synthesis of the MCF-7 cells was dramatically affected by treatment with kuanoniamine A (**51**), and the DNA synthesis was found to be dependent on its concentration rather than the exposure time. In contrast, kuanoniamine C (**53**) exhibited a stimulatory effect at low concentrations and inhibitory effect at high concentrations. This biphasic effect of kuanoniamine C (**53**) on DNA synthesis of the MCF-7 cells resembled that of phytoestrogens on the estrogen dependent breast cancer cells [68]. The MTT-reducing capacity assay revealed that the effect of kuanoniamine A (**51**) on cell viability of MCF-7 cells was much more potent than that of kuanoniamine C (**53**), and the loss of cell viability by kuanoniamine A (**51**) was in agreement with its drastic effect on DNA synthesis. Flow cytometric analysis of the DNA content revealed that kuanoniamine A (**51**) caused an extensive reduction of the MCF-7 cells in G2/M phase with a concomitant increase in G1 phase and a cellular fraction in S phase. In contrast, kuanoniamine C (**53**) exhibited no significant effect on these cells. The TUNEL assay showed that both kuanoniamines A (**51**) and C (**53**) caused an increase in apoptotic cells, suggesting that their antiproliferative effects on the MCF-7 cells could be in part due to the phenomenon of apoptosis.

36.4 Conclusion

The pyridoacridines represent an interesting group of marine alkaloids which exhibit interesting biological activities. However, much attention has focused on their anticancer activity since nearly most of the compounds of this class exhibit cytotoxicity in the cultured human tumor cells. Although many of the naturally occurring marine pyridoacridines tested so far are too toxic to be considered viable for development of anticancer drugs, the study on the mechanisms underlying their cytotoxicy has shed light to the relationship between the structure and the mechanism responsible for their cytotoxicity. The progress in synthetic chemistry combined with a rapid advance in bioassays, which can allow scientists to identify molecular targets of this class of compounds, will lead to the optimization of the molecules with retain or increase the activity while diminishing the toxicity.

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