Grand Challenges in Biology and Biotechnology

Pabulo H. Rampelotto
Antonio Trincone *Editors*

Grand Challenges in Marine Biotechnology

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Pabulo H. Rampelotto • Antonio Trincone
Editors

Grand Challenges in Marine Biotechnology

Editors
Pabulo H. Rampelotto
Federal University of Rio Grande do Sul
Porto Alegre, Rio Grande do Sul, Brazil

Antonio Trincone
Istituto di Chimica Biomolecolare
Consiglio Nazionale delle Ricerche
Pozzuoli, Naples, Italy

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Foreword

Marine biotechnology (MBT) gives different associations and meanings depending on where you are in a value chain and what your aims are. To approach this area in a coherent manner, MBT should be understood by the enabling role biotechnology has in making it possible to realize value creation from marine, biological resources. For defining MBT, the OECD has recommended to use their generic definition of biotechnology by introducing "marine" in the text, and this has been the ERA-NET Marine Biotechnology project's (2013–2017) common ground for the development of this area. Then it applies to and involves all stakeholders from basic science to developers of industrial products and services. However, to a limited degree, MBT is a separate business sector in its own right. This concept is illustrated in Fig. 1, showing that the application of biotechnology early in the value chain enables innovations for industries within diverse sectors like pharma, food, nutraceuticals, cosmeceuticals and process industries to develop products and services. The book chapters illustrate this principle well, highlighting the importance of basic applications of the biotechnology toolbox and infrastructure to enable different industrial sectors to develop products and values from marine bioresources. This also applies to the farming of fish and shellfish where MBT has a significant role in breeding, health, feeding and environmental matters.

The book describes how the marine environment has delivered, and is expected to deliver, biological molecules and principles that can only be discovered from this unique biosphere. Economic estimates have been made on how MBT will contribute to societal matters, and it is evident that utilization of the marine biological resources and principles through the application of biotechnology will contribute substantially to the development of the emerging circular bioeconomy.

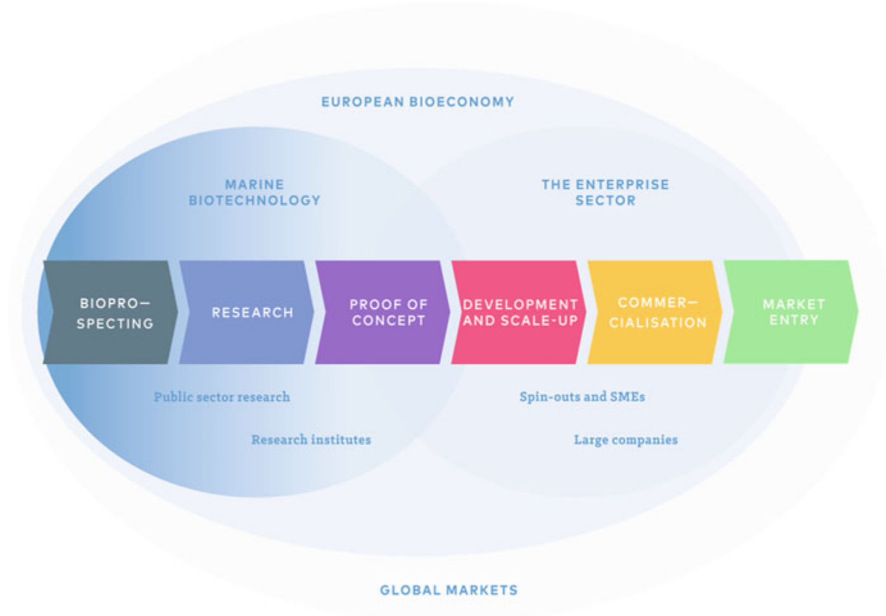

Fig. 1 The contribution of marine biotechnology to business areas along the value chain (Hurst et al. 2016)

The *Marine Biotechnology Strategic Research and Innovation Roadmap*, published by the ERA-NET MBT project in September 2016[1], emphasized the need to continuously develop the biotechnology toolbox and infrastructure in a broad sense to be able to work efficiently and sustainably with exploration of marine bioresources. In addition to the technology development, there is a need to sustainably produce and process marine biomass within a frame of political support, as well as product innovations and differentiations as illustrated in Fig. 2.

Taken together, the book pulls these aspects well together in a coherent way, emphasizing the need to continue to develop MBT as an enabler for the discovery of valuable and innovative marine products and services to be refined in their respective value chains. The legacy from the ERA-NET MBT project is taken further in a new European ERA-NET project developing the Blue Bioeconomy. It is a pleasure to observe that regional involvement, industrial engagement as well as open science

[1]Hurst D, Børresen T, Almesjö L, De Raedemaecker F, Bergseth S (2016) Marine biotechnology strategic research and innovation roadmap: insights to the future direction of European marine biotechnology. Marine Biotechnology ERA-NET, Oostende

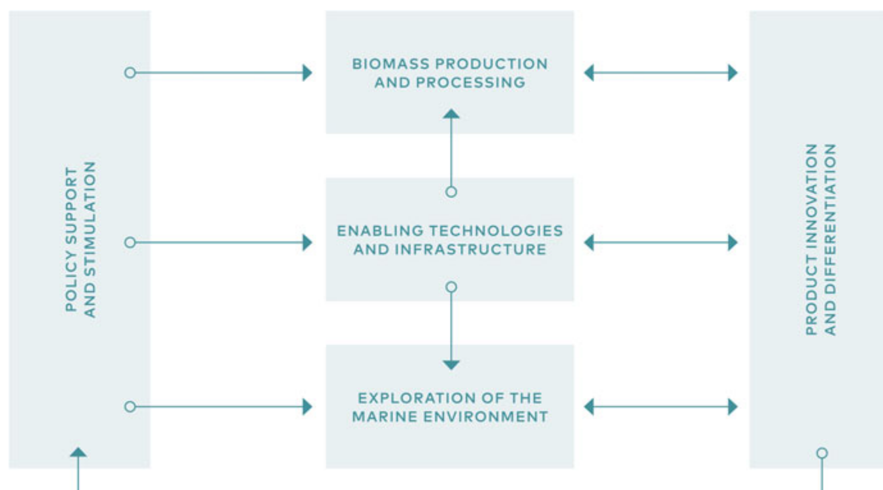

Fig. 2 The five thematic areas of the MBT roadmap and their interconnections (Hurst et al. 2016)

and data management incorporating all stakeholders through workshops and conferences will secure increased literacy and developments based on marine biological resources with value for our societies.

Marine Biotechnology ERA-NET
Department for Enabling Technologies
Division for Innovation
The Research Council of Norway
Lysaker, Norway

Steinar Bergseth

Preface

The idea of this book is primarily a fruit of our nice cooperation in the last years. We both have been involved as editors and guest editors in several editorial projects highlighting the potential of marine biotechnology. As a natural consequence, by late 2015, we found ourselves discussing the interesting possibility of joining our efforts towards a landmark project in the field, i.e. a book within the Grand Challenges in Biology and Biotechnology series. As part of this series, the book would have a unique perspective on the subject by exploring how marine biotech can contribute to the achievement of major national and international policy goals, such as economic growth and job creation, public health, environmental protection and sustainable development. In general, it is expected that the knowledge made available through this reliable reference will be used by leading authorities in their efforts towards the development of a blue bioeconomy.

The 15 chapters that compose the book are organized in three sections. The first one discusses how marine may provide new solutions to some of the grand challenges of the twenty-first century. The second section explores the economic potential of marine biotechnology, while the third and last section focuses on how to unleash this enormous potential by presenting major national and international strategies, policies and programmes designed to support the development of marine biotechnology.

In developing this book, we recognize that advances in marine biotechnology may foster the much-needed source of innovation and economic growth, closely aligned with the sustainable use of marine bioresources in ways that establish new markets, generates revenue and increases employment. It is our hope that this fine collection of chapters will be a valuable resource for scientist and decision-makers and will stimulate further research and development (R&D) into the vibrant area of marine biotechnology.

Porto Alegre, Brazil — Pabulo H. Rampelotto
Pozzuoli, Italy — Antonio Trincone

Introduction

The marine environment is considered Earth's last frontier of exploration. In fact, a common belief is that just less than 5% of the vast and rich marine environment has been explored. Our seas and oceans represent a very unknown resource for the discovery of novel organisms, (bio)products, (bio)processes, and for the development of bioinspired synthetic drugs.

Recent advances in genetics and other (bio)molecular techniques are providing all necessary tools to access these still-untapped marine resources on a larger scale and, consequently, enabling exploitation of the true promise of the blue biotechnology.

Acknowledgment of the potential of marine biotechnology is in due course by many governments in the world to provide solutions to some of global Grand Challenges of the twenty first Century such as sustainable supply of food and energy, development of new drugs and health treatments, and providing new industrial materials and processes. For this reason, the advances in marine biotechnology may foster the much-needed source of innovation and economic growth in many countries and pave the way towards the development of a global blue economy, i.e., a new economic model based on the sustainable exploration of our ocean ecosystems. They can create jobs and wealth by contributing to the development of greener and smarter economies.

The Promise of the Blue Biotechnology

Marine biotechnology can play an important role towards meeting challenges of the new century and can greatly contribute to economic recovery and growth worldwide by delivering new knowledge (scientific knowledge of marine life and ecosystems) and facilitating access to products and services by cutting-edge technologies.

In 2011, it was estimated that 20,000 marine natural products were discovered in the previous five decades, and preclinical and clinical pipelines for new pharmaceutics contained almost 1500 molecules, specially related to anticancer research. However, in

marine biotechnology field, significant contributions for new antibiotics, anticancer, and immune system modulator can be accounted. Due to widespread use of known molecules (i.e., antimicrobial resistance), new antibiotics are necessary, and this is one of the point of worldwide interest in applied marine pharmacology. In this context, more than in others, marine microbes are still seen as untapped resource.

Other metabolites from marine organisms with nutraceutical applications are considered within food lines of research, as well; this field requires relatively less economical efforts compared to the pharmaceutics above indicated. Nutrients, enzymes, and other metabolites (essential fatty acids) are the key molecules considered within this aspect.

Another great challenge relates to the application of marine biotechnology for algal biofuels since algae biomass is used to generate biodiesel, bioethanol, biogasoline, biomethanol, biobutanol, and other biofuels. Absence of lignin, present in cellulosic biomass, and cultivation that hardly impacts on food production are the two major positive points for marine biofuel revolution. In this context, cultivation of microalgae may have a smaller footprint in comparison to land biomass. This energy sector is positively marked by microalgae, macroalgae, and bacteria showing their utility in microbial fuel cells, i.e., systems that harvest the electricity generated by microbial metabolism. However, an overall opinion is that marine biorefining is in his infancy compared to biorefineries for terrestrial biomass.

The first part of this book discusses how marine biotechnology can realize these increasing contributions, highlighting the most important topics at the current state of the art in these scientific sectors. Seven chapters, compiled by worldwide leader groups, identify cutting-edge areas that may benefit from a greater attention by governments supporting policies to general field of marine biotechnology. The pharmaceutical sector is very present embracing marine originating antibiotics, seaweeds with health promoting activities, sustainable food supply of their bioactive materials, and an overview of the literature with a special focus on structural features and mechanisms of action of many lead compounds. The present decline in the development of antibiotics over the past few decades and the decrease in the development of new antimicrobial agents, with increasing number of bacteria showing multiresistance to the existing antibiotics, have raised an important problem in this respect as above mentioned. A need to find new molecules is clear. Marine nature environment is an enormous source of biodiversity and may provide us with new molecules from plants, fungi, and other macro- and microorganisms. Seaweed bioactives possess a wide spectrum of biological actions, including antioxidant, anti-inflammatory, antivirus, anticancer, antihypertensive, fat-lowering, and neuroprotective activities and have gained much importance in pharmaceutical sector, in particular with their polysaccharides, pigments, phlorotannins, peptides, minerals, and vitamins. Current research is focused on intensive efforts for isolation and identification of these new ingredients. This section of the book includes other three key topics such as marine fungi, marine eubacteria with the highest hydrogen production yield, and the use of gasification and anaerobic digestion of seaweed for the production of biofuels. As for fungal organisms, not only obligate marine fungi but also a multitude of terrestrial fungi (whose occurrence at sea has been considered incidental) are increasingly regarded as an

evidence of marine ecological flexibility. Thus, special physiological adaptations of these organisms are considered in view of their possible biotechnological exploitation. There are many examples of the production of bioenergy from marine organisms; however, the potential of algal biomass as a source of liquid and gaseous biofuels is still a highly topical theme. The last two chapters in this section focused on energy theme: a summary of the fermentative pathways related to hydrogen production in the thermophilic microorganisms of the genera Thermotoga and Pseudothermotoga (marine species with the highest hydrogen yields among eubacteria), and a detailed report on major projects in the UK on gasification and anaerobic digestion of seaweed, suggesting promises of these technologies for exploiting bioenergy.

The Economic Potential of Marine Biotechnology

Understanding socioeconomic contributions of marine biotechnology is important for a number of reasons and is necessary to drive correctly the future developments. Bioeconomy has a political priority because of its potential for economic growth and social benefits and in almost every continent, the planning of related strategies started as governmental actions. Marine biotechnology can make important contributions to bioeconomy through the construction or the greening of a number of industries with flourishing of innovative products and processes, and the consequential creation of new jobs. It is within the concepts under the term bioeconomy that this point can be discussed, marine biotechnology being fully framed within those economic sectors that are founded on bioscience and biotechnology innovation.

Market value of marine biotechnology products and services is difficult to estimate; important elements are the tracking of the range of products and services across different sectors and precise identification of the roles and contributions of marine biotechnology, separating them out from other factors. In recent reports evaluating statistically its value, the market of marine biotechnology is traditionally separated in different sectors such as pharmaceutical products for the marine-derived drugs, biotechnology itself, including all other related bioproducts, fish and shellfish for aquaculture industry, and the biomass-related markets, for other specialized compounds including those derived from agar, alginates, and carrageenan. All these sectors were estimated at 2.8 billion EUR in 2010 with an annual growth of 4–5%.

The second part of this book explores the economic potential of marine biotechnology. In the first chapter of this section, a thorough description of the firms and of organizations belonging to the marine industries is reported, with geographical distribution, dimension, main markets served, and production activities. Through the construction of an original database as the result of a multiple phases methodology, based on the use of a wide number of sources, a first investigation of the organizations that belong to the marine industrial sectors is reported. Some case studies of marine firms are also presented. It is evidenced that the absence of a shared definition of the blue industries translates into a lack of thorough analysis of the global sector. The survey offers a general idea on the characteristics that are shaping this young sector. A

focus is given, in the second contribution, on compounds that successfully reached the market; particularly, the approaches employed by the nutraceutical, pharmaceutical, and cosmeceutical companies in marketing their products are described as found in novel and/or ongoing marine natural products development programs. As for highly promising marine bioactives, in order to improve their market entry success rates, suggestions are provided highlighting what can be done in novel and/or ongoing development programs. This section of the book is concluded by a report on the activity of the European Marine Biological Research Infrastructure Cluster, with a reflection about major issues such as lack of connectivity, practical and cultural difficulties in connecting science with industry, and different regional developments throughout Europe. Managing these multi-country and multi-stakeholder collaborations characterizing international partnerships is extremely important to better address benefits of marine biotechnology. A common opinion is that there is a need to look for synergies among supported projects to reduce the likelihood of duplication. Conclusions drawn outlined lack of connectivity between research services, some practical and cultural difficulties in connecting science with industry, including uneven regional development and innovation policies throughout Europe.

Supporting the Development of Marine Biotechnology

Despite significant progress, marine biotechnology is yet far from fulfilling its potential. How to unleash this enormous capability is the grand challenge that this field is facing. Moreover, marine biotechnology brings up a number of ethical, legal, and social issues connected to the harvesting of marine bioresources, thus raising questions of trans-boundary approach for benefit sharing and intellectual property rights. This very aspect cannot be neglected. Moreover, due to the global presence of marine bioresources, R&D infrastructures are the most important keys to drive innovation.

This last section is devoted to investigating on real supports that marine biotechnology field have had, discussing national and international strategies, policies, and programs. Four chapters in this section give a great overview with the last one about legal landscape regulating the access to and utilization of marine genetic resources. SeaBiotech, BluePharmTrain, and ChiBio are specifically covered in single contributions while many other research programs such as BAMMBO, Bluegenics, GIAVAP, LIPOYEASTS, MaCuMBA, MAMBA, MAREX, MARINEBIOTECH, PharmaSea, Polymode, SeaBioTech, Sunbiopath, EMBRIC, INMARE, NOMORFILM, and TASCMAR are collectively analyzed in detail. A large number of libraries are mentioned as research activity products: sequences, active species, extracts, active fractions, and compounds composing a massive quantity of data that needs to be deeply studied considering all possible fields. The effort of the EU Marine Biotechnology ERA-NET (ERA-MBT) in preparing a roadmap that identified an optimistic future for marine biotechnology is also reported, summarizing the five thematic areas: (1) exploration of the marine environment, (2) biomass production and processing, (3) product innovation

and differentiation, (4) enabling technologies and infrastructure, and (5) policy support and stimulation. Long-term actions of the marine biotechnology field are identified in building research networks and international collaborations and implementing global agreements on access to resources.

A clear implication for scientists working on marine biotechnological research as well as any user of genetic resources along the biodiscovery pipeline is the legal landscape regulating the access to and the utilization of genetic resources. Recent changes with the entry into force of the Nagoya Protocol in 2014, and the adoption of the related EU Regulation on user compliance in 2014, are discussed in the last chapter of this section. This chapter informs users on their legal obligations regarding access and benefit-sharing with a focus on marine genetic resources.

Conclusions and Perspectives

To realize the potential that the oceans offer in terms of new products and processes requires the concerted effort of many parties. Effective academic–industrial collaboration is essential to bring any novel marine biotechnological outputs to the market. Key to this success is the establishment of formal and informal agreements with stakeholders and institutions to enhance the commercialization of marine biotechnologies through education, market research, and business development activities. That will provide the parties with access to new tools and research capabilities which would otherwise be unavailable to them on an individual basis.

Stimulating the development of research strategies and programmes for marine biotechnology research and aligning these at the national and international level is also of primary interest, as well as the development of local and major networks, which allows productive face-to-face interaction between academic and industrial partners that can help to break down last barriers from applied to commercially exploited science.

As such, this book highlights the relevance of collaborative industry–academia approach and uses the "Grand Challenges" as the basis for a logical analysis of the current and possible future development of marine biotechnology, set against its capacity to deliver products and processes to address high-level societal needs and opportunities in the areas of food, energy, environmental health, human health and well-being, as well as industrial products and processes.

Center of Biotechnology and PPGBCM
Federal University of Rio Grande do Sul
Porto Alegre, RS, Brazil
Pabulo Henrique Rampelotto

Istituto di Chimica Biomolecolare
Consiglio Nazionale delle Ricerche
Pozzuoli, Naples, Italy
Antonio Trincone

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About the Editors

Pabulo Henrique Rampelotto is the founder and editor-in-chief of the Springer book series Grand Challenges in Biology and Biotechnology. He is also editor-in-chief, associate editor, senior editor, guest editor and member of the editorial board of several scientific journals on the field of life sciences and biotechnology. Furthermore, Pabulo is member of four Scientific Advisory Boards of Lifeboat Foundation, alongside several Nobel Laureates and other distinguished scientists, philosophers, educators, engineers and economists. Most of his recent work has been dedicated to the editorial process of several scientific journals in life science and biotechnology, as well as to the organization of special issues and books in his fields of expertise. In his special issues and books, some of the most distinguished team leaders in the field have published their work, ideas and findings, including Nobel Laureates and several of the highly cited scientists according to the ISI Institute.

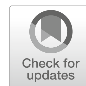
"When he is not working, Pabulo enjoys spending time walking in the woods, in the mountains, and near the sea. . .thinking, always thinking". (Lifeboat Foundation, 2016)

Antonio Trincone is author of more than 140 scientific publications (articles, reviews, book contributions) that appeared in international leading scientific journals. He has been a senior researcher working since 1983 in Naples at the Istituto di Chimica Biomolecolare belonging to Consiglio Nazionale delle Ricerche, Italy. He has been professor of organic chemistry in charge for several years at the University of Salerno, Italy. His research activity focused on biomolecular aspects, many regarding stereochemistry and bioorganic

chemistry of enzymes as biocatalysts. Recent achievements are directed to practical interest in the biorefinery pipeline context using thermophilic and/or marine enzymes and to the biocatalyzed synthesis of bioactive compounds. Antonio has been the editor of different scientific books and special issues dedicated to marine biotechnology. He is specialty chief editor of marine biotechnology (Frontiers in Marine Science), editing different research topics for this journal, and he is part of Editorial Board of MDPI journals *Marine Drugs* and *Molecules* and guest-edited different special issues for these journals. Most of his recent work is dedicated to the editorial process and review activity for scientific projects, published articles and scientific positions.

Part I
The Promise of the Blue Biotechnology

Chapter 1
The Marine Ecosystem as a Source of Antibiotics



Yuly López, Virginio Cepas, and Sara M. Soto

1 Introduction

In spite of the remarkable impact on health that the antimicrobials have achieved in the 1960s and 1970s, 40 years later infectious diseases remain the second-leading cause of death worldwide [1].

Nowadays, one of the most important health problems is the increase, emergence, and spread of antimicrobial resistance among the different microorganisms (bacteria, fungi, virus, and parasites). In the case of bacteria, resistance to antibiotics is increasing in both community and hospital settings in association with an increase in mortality and morbidity. As shown in Fig. 1.1, the discovery of new antibiotics with new mechanisms of action slowed in the year 1968 after the discovery of cephalosporins [2]. After that, most of the antibiotics developed belonged to the existing classes and were considered as "new generations."

Unfortunately, the development of an antibiotic has, sooner or later, been followed by the emergence of bacterial strains resistant to these antibiotics. Figure 1.1 shows several examples of this [3]:

– In the 1940s penicillin was introduced into the clinical setting. Yet, in the mid-1940s, the first *Staphylococcus aureus* strains producing penicillinases resistant to penicillin were identified.
– In the 1950s, aminoglycoside, chloramphenicol, tetracycline, and macrolides were developed, with multiresistant strains of *S. aureus* emerging within the same decade.

Y. López · V. Cepas · S. M. Soto (✉)
ISGlobal, Barcelona Centre for International Health Research (CRESIB), Hospital Clínic - Universitat de Barcelona, Barcelona, Spain
e-mail: sara.soto@isglobal.org

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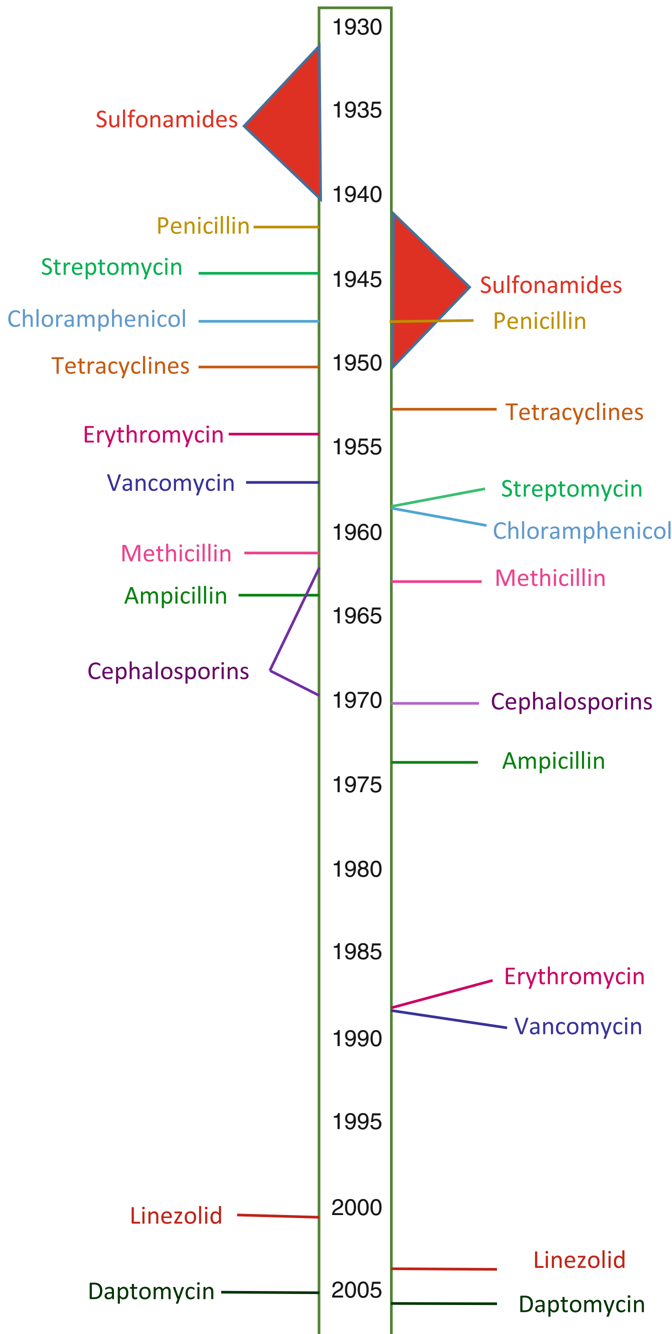


Fig. 1.1 Emergence of resistance to the antibiotics

– In 1960, methicillin was synthetized, and only 1 year later, the first methicillin-resistant *S. aureus* strain (MRSA) appeared, constituting an important health problem today.
– In 1980, the third generation of cephems was developed, followed by the emergence of the first extended-spectrum beta-lactamases (ESBL) Gram-negative-producing strains only 3 years later.

Antimicrobial resistance has an impact not only on health, but it also has an important economical impact. Thus, it has been estimated that the annual cost due to antimicrobial-resistant *S. aureus* infections is about $4.6 billion taking in account both hospital and nosocomial infections [4].

The high impact of antimicrobial resistance on healthcare and the economy has led to an urgent need to develop novel compounds with new mechanisms of action to treat infectious processes. However, the demand for new antibiotics is not in parallel with the development of new antimicrobial agents. Thus, since 2000 only three new classes of antibiotics have been marketed for human use, one of which was only for topical use.

At present, the following classes of new antibiotics have been approved and/or are under development: monocyclic beta-lactams, pleuromutilins, quinolones, carbapenems, polymyxins, cephalosporins, cephalosporin+becta-lactamase inhibitors, ketolides, glycopeptides, tetracyclines, beta-lactamase inhibitors, aminoglycosides, and oxazolidinoses [5].

The question is whether the need for new antibiotics is really urgent or not, and what problems do their development entail? There are three main problems to address [6]:

1. Regulations for drug approval by each government.
2. Economic factors affecting the price, demand, and availability of a product or market forces. The cost associated with the development of a new drug is estimated to be $400–$800 million, with the market for antimicrobials being estimated at between $26 and 45 billion per year [2].
3. Problems encountered by scientists working on this subject. In this regard, scientists are encouraged to find [1]:

   – New molecules active against multiple bacterial species and active against multiple types of infections
   – New molecules that do not generate resistance
   – New compounds acting in targets found thanks to genomics

In addition, antimicrobial agents are less economically attractive than other drug classes for several reasons [1]:

– Their use in short-course therapies in a given patient.
– The development of new agents generates high competitivity among the pharmaceutical companies.
– There is a preference for broad-spectrum antimicrobial agents that are very rare to find.

In spite of all these reasons, the development of new antimicrobial agents has several advantages in the pharmaceutical industry: the time needed for the clinical development of these agents is shorter compared with other pharmaceutical products aimed at other pathologies, and antibiotics have the highest approved success rates due to the variety of “in vitro” assays and the current animal model tests [2].

In this sense, nature is an important source of molecules demonstrating different activities. The most studied natural products are those produced by bacteria and fungi as demonstrated by 9 of the 12 antibiotic classes derived from a natural product. Seventy-four of the 88 antibacterials marketed from 1981 to 2005 present structures taken from nature [7]. Nonetheless, at present, synthetic products are the most commonly used in medicine.

Several approaches have been undertaken or are currently under development in the search for new molecules from natural organisms. The platform most commonly used is the screening of microorganisms from soil by growth in different culture media followed by bioactivity assays and the identification of the compound responsible for this activity (Wasksman platform). Other approaches involve the study of microorganisms isolated from extreme environments such as in the Antarctica to test combinations between known antibiotics and newly found molecules and/or to search for antivirulence compounds.

In all these approaches, the screening of enormous natural extract libraries has led to several problems which have been solved with advances in science. It is important to know the structure of the new active compound. Thus, a process called dereplication has been developed, involving the purification and characterization of metabolite(s) of interest from among a large mixture of metabolites present in the whole extract [6]. The isolation of active compounds requires multiple steps of extraction and HPLC, followed by structure identification using magnetic resonance (MR) and mass spectral (MS) protocols.

Another possible problem is the amount of active molecule available. These metabolites are usually found in small quantities, and changes in metabolism can lead to the loss of the capacity of these organisms to produce these metabolites. In addition, it must be taken into account that these metabolites are often toxic for humans or must first be chemically modified to improve their activity as an antibiotic.

However, we must also [8]:

– Explore other ecological niches such as marine environments.
– Explore peptides and compounds produced by animals and plants.
– Mimick the natural lipopeptides of bacteria and fungi.

As mentioned previously, the most important platform for the screening of new molecules is soil as part of the terrestrial environment. However, the ability of marine habitats to produce antibiotic metabolites remains largely unexplored due to the enormous diversity of potentially attainable species.

To date the following metabolites from marine environments have been found [7]:

- **Alkaloids** including 8-hydroxymanzamine (active against *Mycobacterium tuberculosis*), marinopyrrole 1 (active against MRSA), and cribrostatin 6 (active against *Streptococcus pneumoniae*).
- **Polyketides** constructed by polyketide synthases, such as abyssomicin C (active against MRSA), pestalone (active against MRSA), and ariakemicin A (active against *S. aureus*).
- **Terpenes** including axisonitrile 3 (active against *M. tuberculosis*), haliconadin C (active against *M. luteus*), and bromosphaerone (active against *S. aureus*).
- **Ribosomal peptides** which are antimicrobial peptides synthetized by ribosomes and include arenicin-1 (active against Gram-negative and Gram-positive microorganisms), halocidin (active against *Pseudomonas aeruginosa*), hadistin (active against Gram-negative microorganisms), and clavanin A (active against *Escherichia coli* and *Listeria monocytogenes*).
- **Non-ribosomal peptides** constructed by large multifunctional protein complexes named non-ribosomal peptide synthetases. Among these peptides we can find bogorol 1 and emericellamide A (both active against MRSA) and thiocoraline (active against *S. aureus* and *Bacillus subtilis*).

We have to consider that these molecules could have new targets such as virulence, type III secretion system, quorum sensing, bacterial metabolism, cell division, biosynthesis of aminoacyl-tRNAs, two-component bacterial systems, native proton force, and efflux pumps [8] as well as the use of phages. Indeed, the marine environment could be an important source of quorum-sensing inhibitors due to the multiple associations between the eukaryotic and prokaryote organisms that live in this habitat.

In the last years, Europe is funding projects related to the search of bioactive compounds. Thus, several projects have been developed in the search for new molecules with antibiotic activity. **BAMMBO** (sustainable production of Biologically Active Molecules of Marine Based Origin-FP7-265896) deals with innovative solutions to overcome existing bottlenecks associated with culturing marine organisms for sustainable production of HAVPs for the pharmaceutical, cosmetic, and industrial sectors. **MICRO B3** (Biodiversity, Bioinformatics, Biotechnology) involves a bioinformatic platform with experts in plankton ecosystems and oceanography for developing techniques for the analysis of molecular, genetic, and environmental data from oceanic expeditions. **MACUMBA** (Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications-FP7-311975) exploits diverse marine microorganisms for the production of HAVPs.

Currently, the **NOMORFILM** project (H2020-Grant Agreement 634588) is searching for new antimicrobial molecules from microalgae. Microalgae are a source of secondary metabolites useful as new bioactive compounds. The activity of these compounds against bacterial pathogens and biofilm formation has yet to be determined. Biofilm formation is especially important in infections and tissue inflammation related to implants and catheters which may lead to implant rejection and subsequent removal and replacement with a new device, with an increase in antibiotic consumption, together with a health costs of about 50,000–90,000 € per infectious episode. Considering these complications, the search for new antimicrobial agents

that are effective against bacteria in their two media, planktonic and biofilm stage, is a priority in clinical practice. For this reason, the global objective of the NOMORFILM project is to search for antibiofilm compounds isolated from microalgae that will be useful in the treatment of this kind of infections and can be incorporated into the manufacturing of medical prosthetic devices.

Briefly, the oceans and seas are environments that offer us an enormous source of bioactive compounds. Indeed, their inhabitants have survived depredators in these habitats for millions of years. Some of these interesting marine organisms are described in this chapter.

2 Marine Cyanobacteria and Bacteria

Antibacterial agents of marine origin have been widely studied in the last decade, with microalgae and cyanobacteria constituting two of the most promising sources of novel bioactive molecules.

2.1 Cyanobacteria

Cyanobacteria belong to the Eubacteria kingdom and the division Cyanophyta. They are the oldest fossils identified, dating from 3.5 billion years ago. Cyanobacteria are aquatic and perform photosynthesis like plants, but they are prokaryotic organisms [9]. They constitute a rich nutrient source, producing chlorophyll, amino acids, minerals, and carotenoids, among others.

In the last decades, novel bioactive compounds have been isolated from cyanobacteria. In this regard, these fossils are considered one of the most promising groups of organisms thanks to the diversity of the secondary metabolites that they secrete. Among these compounds different proportions of toxins, lipopeptides, amino acids, fatty acids, macrolides, and amides are the most frequently found depending on the marine environment they inhabit [9].

Some highly valued metabolites of cyanobacteria showing antibacterial activity are cryptophycin and lipopeptides. Lipopeptides isolated from cyanobacteria have been extensively studied, showing antitumor, cytotoxic, antiviral, antibacterial, antimalarial, and antimycotic activity.

2.2 Bacteria

Nature has been a source of medicinal agents for centuries, and an impressive number of modern drugs have been isolated from microorganisms, many based on their use in traditional medicine [10].

The main source of obtaining these antagonistic molecules corresponds to soil bacteria belonging to the order *Actinomycetales*, which have historically shown great benefits in the discovery of therapeutic agents such as streptomycin, another aminoglycosides, macrolides, and tetracyclines. Similarly, secondary metabolites of various chemical structures and biological activities have been extracted from some species of *Bacillus* genus; some examples of these antibiotics used in medical treatments are bacitracin, gramicidin S, polymyxin, and tyrothricin [11], which demonstrate their effectiveness in treatment of infections caused by Gram-positive and even Gram-negative anaerobes [12].

Indeed, nearly 50,000 natural compounds have been discovered, being a good source of new families of antibiotics, antitumoral and antiviral agents [13, 14].

Globally, there are about 3×10^8 km^3 of ocean sediment saturated with 8×10^7 km^3 of porewater inhabited by an estimated 3×10^{29} microbial cells [15]. The genetic and biochemical diversity of these marine microbes is immense, with these microbes having developed complicated biochemical and physiological systems with which they can adapt to the extreme habitats and unfavorable conditions of marine environment. Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to their much more complex living circumstances and greater diversity of species and bioactivity, making them a likely rich source of novel effective drugs [16, 17] (Table 1.1).

Several strains were isolated from marine environment with antimicrobial activity against wide variety of pathogenic bacteria (Table 1.2). The marine bacteria live in a biologically competitive environment for space and nutrients, under unique conditions of salinity, pressure, temperature, light, oxygen, and pH. They have therefore developed mechanisms of defense against competitors and predators for their own survival, synthesizing secondary metabolites of great value in pharmaceutical and biotechnological applications [19, 20].

Several molecules that have been isolated from marine microorganisms mainly correspond to secondary metabolites that are chemically divided into **peptides, saponins, terpenoids, alkaloids, nucleosides polycyclic ethers, sterols, amino acids,** etc. (Table 1.3; Fig. 1.2) [18]. However, a critical point in the search for

Table 1.1 Important compounds isolated from marine algae

Secondary metabolite class	Mechanism of action	Examples
Phlorotannins	Inhibition of oxidative phosphorylation	Phloroglucinol
	Binding to bacterial proteins	Phlorofucofuroeckol
Polysaccharides	Increasing permeability of bacterial cell	Laminarin
		Fucoidan
Peptides	Binding bacterial cytoplasmic membranes	Lectins
Terpenes	Inhibiting bacterial growth	Xanthophylls
		Fucoxhanthin
Lactones	Inhibiting bacterial quorum-sensing	Furanones

Table 1.2 List of antibacterial activity of marine bacteria against some pathogenic organisms (adapted from [18])

Marine bacteria with antimicrobial activity	Test strain
Pseudomonas putida	*Bacillus subtilis*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Serratia marcescens*, *Aeromonas hydrophila*, *Rothia* sp., *Staphylococcus aureus*, MRSA[a]
Actinomycetes	*S. aureus*, *Bacillus subtilis*, *E. coli*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, *Pseudomonas aeruginosa*
Pseudomonas Aeruginosa	*Aeromonas punctate*, *Kokoris marina*, *Rothia* Sp., *Vibrio* sp., *S. aureus*, *Staphylococcus epidermidis*, *E. coli*, MRSA[a], *Proteus vulgaris*, *Bacillus thuringiensis*, *B. subtilis*, *Enterococcus faecalis*
Pseudoalteromonas sp.	*S. aureus*, MRSA[a]
Pseudomonas sp.	K*lebsiella pneumoniae*, *S. aureus, Shigella flexneri*, *P. aeruginosa*, *B. subtilis*, MRSA[a]/ORSA[b]
Bacillus sp.	*Kokoris marina*, *Rothia* sp., *Aeromonas punctata*, *Rothia* sp., *Vibrio* sp., *S. aureus*
Brevibacterium frigoritolerans	*Rothia* sp., *Vibrio* sp., *S. aureus*

[a]Methicillin resistant *S. aureus*
[b]Oxacillin-resistant *S. aureus*

Table 1.3 Natural compounds and peptides with antimicrobial activity isolate from marine bacteria

Metabolite	Isolated from	Chemical scaffold	Activity against	References
7-methylcoumarin	*Streptomyces* spp.	Phenolic	Gram-positive bacteria (*Staphylococcus aureus)*	[21]
Macrolactin (D, S, S)	*Bacillus marinus*	Macrolide	*S. aureus* and two species of fungi (*Pyricularia oryzae* and *Alternaria solani*)	[22]
Macrolactin (T, B O)			*E. coli, S. aureus*, *B. subtilis* and several soil bacteria	
Tropodithietic acid (TDA)	Rosobacter clade (*R. gallaeciensis*, *Pheobacter* sp., *Silicibacter* sp. and *Ruegeria mobilis*)		Pathogens in aquaculture as *Vibrio anguillarum*, *V. coralliilyticus* and *V. shiloi*	[23, 24]
Tauramamide	*Brevibacillus laterosporus*	Lipopeptide	*Enterococcus* spp.	[25]
TP1161	*Nocardiopsis* spp.	Thiopeptide	Vancomycin resistant *Enterococcus faecalis* and *E. faecium*	[26]

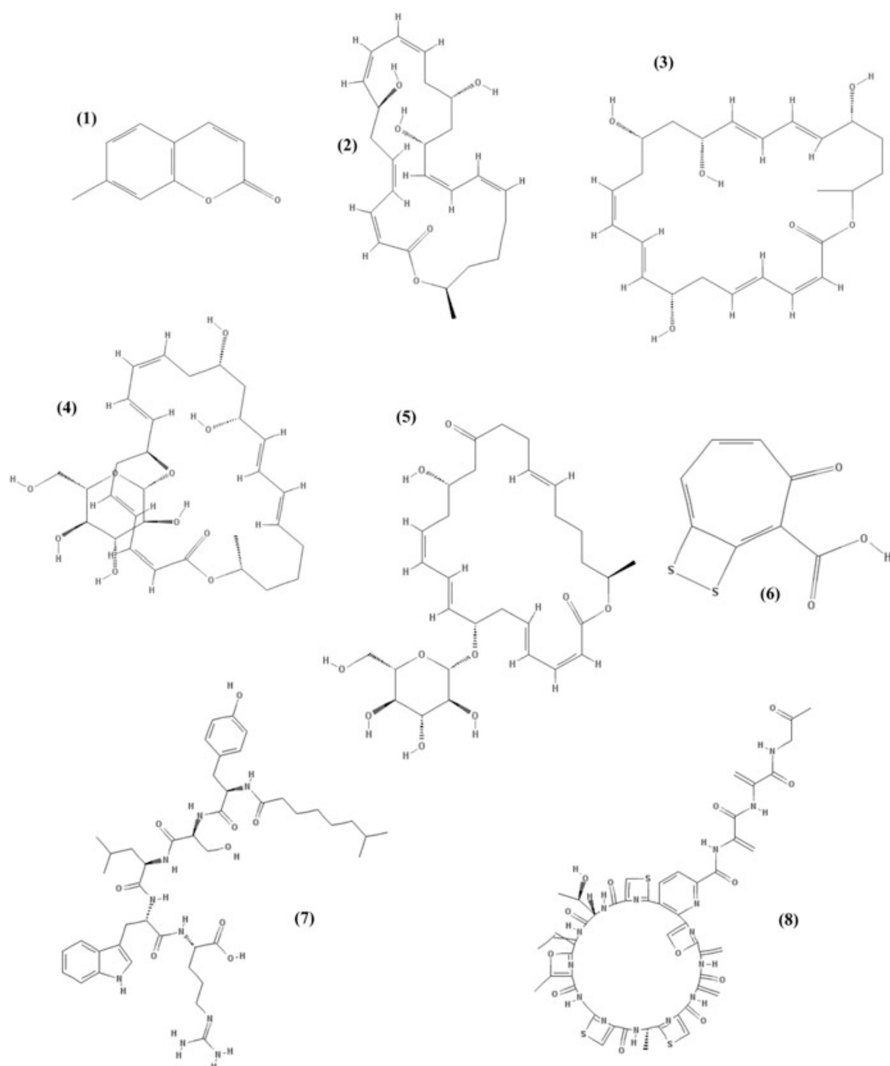


Fig. 1.2 Structures of secondary metabolites with antimicrobial activity isolate from marine bacteria. (**1**) 7-Methylcoumarin, (**2**) macrolactin A, (**3**) macrolactin T, (**4**) macrolactin B, (**5**) macrolactin O, (**6**) tropodithietic acid, (**7**) tauramamide, and (**8**) TP1161

new molecules is the extraction and identification of these metabolites to test against clinically relevant bacteria and to find the active compound to be followed by clinical trials.

Two different procedures are followed for the separation of the bioactive fractions of different groups. In the first step, the fractions of low or medium polarity contain organic lipophilic compounds that can generally be separated by standard normal or

reverse phase column chromatography and high-performance liquid chromatography (HPLC) to obtain the individual components. Another method is based on high-polarity fractions that contain water-soluble organic compounds [18]. These chromatography techniques are necessary to fractionate and concentrate the pure active molecule.

Some phenolic compounds from marine bacteria have also been described, such as **4,4′,6-tribromo-2,2′-biphenol** (CMMED 290) which was isolated from an extract of the marine bacteria *Pseudoalteromonas* spp. and displays significant antimicrobial activity against MRSA [27]. Another *Pseudoalteromonas* species, the marine bacterium *Pseudoalteromonas phenolica* O-BC3 0 T [28], produces **2,2′,3-tribromo-biphenyl-4,4′- dicarboxylic acid**. This compound has antimicrobial activity against MRSA with minimum inhibitory concentrations (MIC) between 1 and 4 μg/ml. In addition, this bacterium shows high activity against *B. subtilis*, *Enterococcus serolicida*, and some fungi species. On the other hand, El-Gendy et al. [21] have isolated three phenolic compounds, **7-methylcoumarin (1)** and the flavonoids **rhamnazin** and **cirsimaritin**, from the marine bacteria *Streptomyces* spp., but only **7-methylcoumarin** showed a potent antibacterial activity, principally against Gram-positive bacteria. The compounds isolated were reported to be antimicrobial products. Other antimicrobial phenolic compounds of marine origin include **ammonificins A** and **B**, which are chroman derivatives from the marine hydrothermal vent bacterium *Thermovibrio ammonificans* [29]. A new 24-member macrolide, **macrolactin T**, and a new polyene δ-lactone, **macrolactin U**, together with **macrolactins A (2)**, **B**, **D**, **O**, and **S** were isolated from the culture broth of the bacterium *Bacillus marinus*, which was isolated from *Suaeda salsa* collected on the coastline of the Bohai Sea of China. Macrolactins are a large group of macrolide antibiotics, e.g., macrolactins T **(3)**, B **(4)**, and O **(5)** show inhibitory activity against *S. aureus* and two species of fungi, *Pyricularia oryzae* and *Alternaria solani* [22]. In addition, it has increasingly been reported that macrolactins, along with the microbes producing it, are being used to control soil-borne pathogen diseases in agricultural production [30].

A **bromophenyl** compound has been isolated from the marine bacteria resistant to a methicillin *Pseudoalteromonas haloplanktis* INH strain. Cetina et al. [31] detected seven bioactive compound producer strains and observed the presence of a likely association between pigments and toxicity in several marine heterotrophic bacteria with pigmentation. However, a study of pigment synthesis in *P. tunicate* determined that the pigment had no antimicrobial activity against the target bacteria. Lu et al. [32] found that **diketopiperazine** and **macrolide** are the two most important secondary bioactive metabolites from marine microorganisms.

Strains affiliated with *Roseobacter* clade as *Roseobacter gallaeciensis*, *Phaeobacter* sp., *Silicibacter* sp., and *Ruegeria mobilis* were isolated from the German Wadden Sea. These microorganisms have a "biphasic swim-or-stick lifestyle" that enables their symbiosis with phytoplankton. Several reports showed organisms have been of particular interest due to their ability to form the antibacterial compound named tropodithietic acid (TDA) **(6)** [23, 33, 34]. The new antibiotic showed strong inhibiting properties against marine bacteria and microalgae, being

considered a potent probiotic against the most important pathogens in aquaculture as *Vibrio anguillarum*, *V. coralliilyticus*, and *V. shiloi* [24, 35].

Several studies have described the isolation of antimicrobial peptides from marine bacteria. Thus, two new cyclic lipopeptides, **maribasins A** and **B**, have been isolated from the fermentation broth of the marine microorganism *Bacillus marinus* B-9987 [36]. These compounds exhibit broad-spectrum activity against phytopathogenic fungi. Another *Bacillus* species, *B. amyloliquefaciens* SH-B10, isolated from deep-sea sediments, produces two antifungal **lipopeptides** purified by bioactivity-guided fractionation. Both compounds show significant inhibitory activities against five plant fungal pathogens in a paper-agar disk diffusion assay [37]. A new lipopeptide named **tauramamide (7)** was isolated from *Brevibacillus laterosporus* PNG276 obtained from Papua New Guinea. This peptide shows a potent and relatively selective inhibition of pathogenic *Enterococcus* spp. [25]. Other antimicrobial peptides from the marine bacterium *Nocardiopsis* spp. **TP-1161(8)** are **thiopeptides** and **depsipeptides**. Structure elucidation revealed that these compounds are new thiopeptide antibiotics with an unusual aminoacetone moiety. The "in vitro" antibacterial activity of these thiopeptides against a panel of bacterial strains showed a MICs values ranging from 0.25 to 4 μg/ml against Gram-positive strains and showed a strong activity against vancomycin-resistant bacterial strains represented by *Enterococcus faecalis* and *E. faecium* 569, with a MIC of 1 μg/ml [26]. **Unnarmicine A** and **C** are new antibacterial depsipeptides synthesized by the marine bacterium *Photobacterium* MBIC06485. Both compounds selectively inhibit the growth of two strains belonging to the genus *Pseudovibrio*, one of the most prevalent genera in marine environments [38]. Other unusual peptides are the **ariakemicins A** and **B**. These peptides are linear hybrid polyketide-non-ribosomal peptides isolated from a marine gliding bacterium of the genus *Rapidithrix* spp. [39]. The ariakemicins are composed of threonine, two Ω-amino-(Ω-3)-methyl carboxylic acids with diene or triene units, and δ-isovanilloylbutyric acid. These antibiotics selectively inhibit the growth of Gram-positive bacteria, specifically antistaphylococcal activity.

3 Marine Fungi

The fungi kingdom is a large eukaryotic group including yeast and molds. Fungi are widespread in marine water as they are major decomposers of woody and herbaceous substrata. For this reason, marine fungi may be established in mangroves, algae, plants, plankton, sands, and many other ecological niches. Marine fungi may be classified into two groups:

– Obligate marine fungi: All fungi that complete their life cycle in the sea or in an estuarine habitat [40].
– Facultative marine fungi: These are fungi that can grow in marine environments, but one part of their cycle is developed in a terrestrial ambience or freshwater [41].

Marine fungi include about 10,000 species. Nevertheless, this number may differ from the real number since many ubiquitous species have been included in marine fungi [42]. The predominant species among marine fungi are *Aspergillus*, *Penicillium*, and *Alternaria* [43].

Several molecules with antibacterial activity have been isolated from marine fungi, with 195 strains isolated from beach, estuarine, and mangrove habitats [44]. The species isolated from mangroves have shown the strongest antibacterial activity against *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *E. coli*. Further activities from other fungi species are shown in Table 1.4 and Fig. 1.3.

Chevalone E is an example of an active compound obtained when the fungi *Aspergillus similanensis* is associated with other marine organism such as sponges. This metabolite does not present activity against *E. coli*, *S. aureus*, and *E. faecalis* but does show synergism with the antibiotic oxacillin against MRSA [45].

Deep-sea conditions (over 1000 m below the surface) are extreme, being characterized by complete darkness, low temperature, or high pressure, among other extreme factors. Nevertheless, marine fungi such as *Aspergillus candidus* grows in these conditions. This specie secretes the compound **terphenyl** that is active against *S. aureus*, *B. subtilis*, and *Vibrio* spp. showing antibacterial activity with 83.9–100% of inhibition [46].

Other compounds from deep-sea habitats include the prenylxanthones **emerixanthones A–D (1–4)**, isolated from *Emericella spand*, which show activity against *E. coli*, *K. pneumoniae*, *S. aureus*, *E. faecalis*, *Acinetobacter baumanii*, and *Aeromonas hydrophila*. The diameters of inhibition are between 1 and 3 mm. Compound D also shows mild antifungal activity against pathogens such as *Fusarium* spp., *Penicillium* spp., *Aspergillus niger*, *Rhizoctonia solani*, *Fusariumoxy sporium f.* sp. *niveum*, and *Fusariumoxy sporium f.* sp. *cucumeris*, with diameters of inhibition between 3 and 4 mm [47].

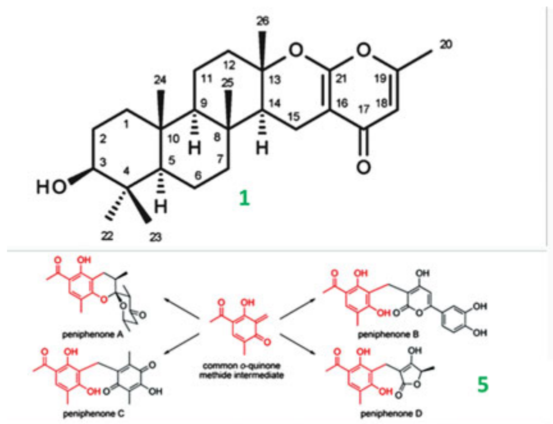
A few compounds show antibiofilm activity as in the case of **flavipesins A** from *Aspergillus flavipes*. The activity of this compound is comparable to that of penicillin in *S. aureus* biofilms. Flavipensis A can penetrate the biofilm matrix and decrease the number of living cells inside the mature biofilm from 390.6 to 97.7 μg/mL [48].

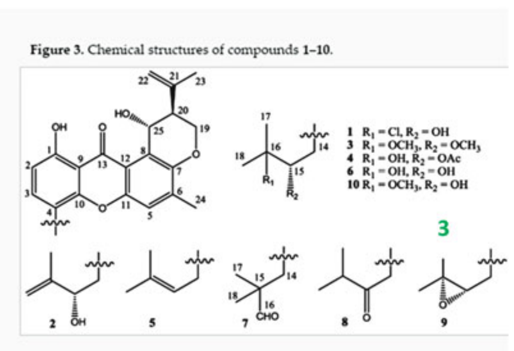
Antituberculosis agents have also been obtained from marine fungi. An example of these agents is **peniphenones A−D** from the mangrove fungus *Penicillium dipodomyicola* HN4-3A. The antituberculosis activity of these compounds against *Mycobacterium tuberculosis* is reflected by their LD50 values of between 0.16 and 1.37 μM [49].

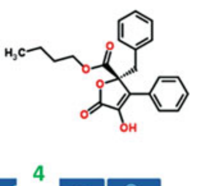
Zopfiella marina is a facultative marine ascomycete that synthetizes two antimicrobial compounds, **zopfiellamide A (5)** and **zopfiellamide B**. Zopfiellamide A has greater antibacterial activity than zopfiellamide B. Nevertheless, the antibacterial activity of compound A shows MICs of 2–10 μg/ml against *Acinetobacter calcoaceticus* and moderate inhibitory effects against Gram-positive microorganisms such as *Arthrobacter citreus*, *Bacillus brevis*, *B. subtilis*, *B. licheniformis*, *Corynebacterium insidiosum*, *Micrococcus luteus*, *Mycobacterium phlei*, and *Streptomyces* spp. [50].

Table 1.4 Natural compounds with antimicrobial activity isolate from marine fungi

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Chevalone E	*Aspergillus similanensis*		*Escherichia coli, Staphylococcus aureus and Enterococcus faecalis*	[45]
Terphenyl	*Aspergillus candidus*	Aromatic hydrocarbon	*S. aureus*, *Bacillus subtilis* and *Vibrio* spp.	[46]
Emerixanthones A–C	*Emericella spand*		*E. coli*, *Klebsiella pneumoniae*, *S. aureus*, *E. faecalis*, *Acinetobacter baumanii*, and *Aeromonas hydrophila*	[47]
Emerixanthones D	*Emericella spand*		*Fusarium* spp., *Penicillium* spp., *Aspergillus niger*, *Rhizoctonia solani*, *Fusariumoxy sporium f.* Sp. *Niveum* and *Fusariumoxy sporium f.* Sp. *cucumeris*	[47]
Flavipesins A	*Aspergillus flavipes*		*S. aureus*	[48]
Peniphenones A–D	*Penicillium dipodomyicola*		*Mycobacterium tuberculosis*	[49]
Zopfiellamide A	*Zopfiella marina*	Alkaloid	*Acinetobacter calcoaceticus, Arthrobacter citreus*, *Bacillus brevis*, *B. subtilis*, *Bacillus licheniformis*, *Corynebacterium insidiosum*, *Micrococcus luteus*, *Mycobacterium phlei*, and *Streptomyces* spp.	[50]







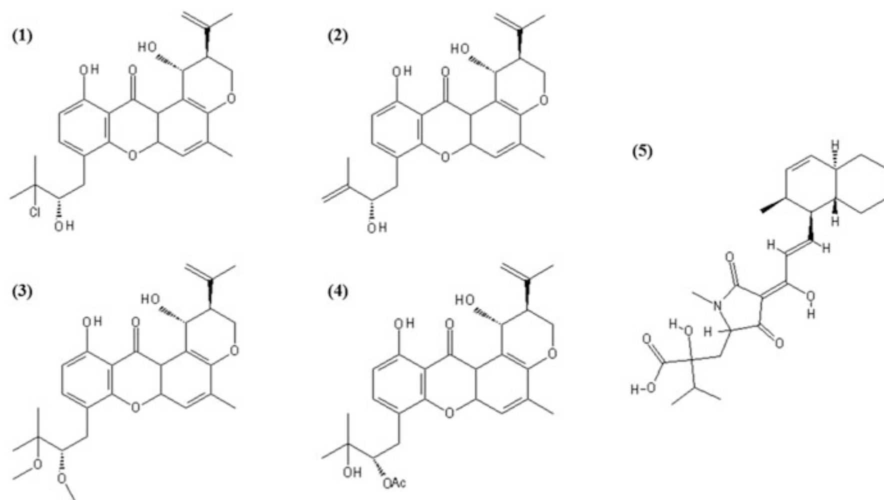


Fig. 1.3 Chemical structures of emerixanthones A (**1**), B (**2**), C (**3**), D (**4**), and zopfiellamide A (**5**). Emerixanthones A–C showed moderate antibacterial activity and emerixanthone D only antifungal activity. Zopfiellamide A showed a great antibacterial activity

Overall, there seems to be some evidence to indicate that new compounds from fungi that live in the sea or in estuarine or mangrove habitats are a promising source of new marine compounds. Nonetheless, it should not be forgotten that more than 10,000 species with promising metabolites remain in the oceans.

4 Sponge

Sponges (phylum Porifera) are sessile aquatic organisms, filter feeders, and the oldest multicellular animals. There are more than 8700 species including marine and nonmarine species according to the World Porifera Database [51]. They are located in all the seas and at all marine depths, adapting multiple forms and playing an important role in biogeochemical cycling [52]. In addition, sponges contribute to carbon flow from pelagic to benthic environments due to their feeding activity.

The body structure of a sponge consists in a set of specialized cells but is not organized into tissues or organs. There are two different layers: pinacoderm, the external layer formed by pinacocyte cells, and a gelatinous matrix called mesohyl, the inner layer or connective tissue. Sponges possess an aquiferous system with external pores (ostia) that allows the entry of food and oxygen. Once the fluid is filtered, waste is discarded by the oscula, the exit porus. All this water movement is facilitated thanks to choanocytes, flagellated collar cells which generate constant water flow through the sponge. The skeleton is made up of two types of skeletal

elements: the spicules, formed by siliceous or calcareous elements, and protein fibers called spongins.

The phylum Porifera is divided into four different groups:

- Calcarea or Calcispongiae sponges formed by calcium carbonate with simple one-, three- or four-rayed spicules. They are of limited size and live in shallow waters and coral reefs.
- Hexactinellida, glass sponges or Hyalospongiae made of siliceous spicules with six-rayed spicules arranged in three planes at right angles to each other. They live in deep water and areas of difficult access.
- Homoscleromorpha, the last to be added, is differentiated in a new clade separated from the Demospongiae and is composed of two families, Oscarellidae and Plakinidae [53].
- Demospongiae which may be supported by either siliceous mineral spicules (never six-rayed) or spongin protein fibers, although some sponges might have none or both. This class is the largest group with more than 95% (7300 species) of known species and includes large populations in freshwater and bath sponges.

Marine sponges are sessile organisms, and their defense mechanism against bacteria, eukaryotic organisms, or viruses is based on the production of a diverse range of secondary metabolite products, allowing efficient chemical protection. Most of these compounds are the result of either the sponge or the microorganisms that live on it [54]. The relevance of marine sponges and their importance in the discovery of new compounds has been demonstrated in the literature. Scientists are actively seeking potential molecules, and only in the last 7 years, more than 620 novel molecular structures have been identified [55]. Even though most of those compounds have shown biological activity in the laboratory, their pharmacological use and synthesis might be expensive due to their chemical complexity.

Sponges are the richest marine phylum with biologically active secondary metabolites [56]. These secondary metabolites have a vast applicability against different diseases such as cancer, malaria, and tuberculosis, among others [57]. The range of action and chemical structure of the sponges already described as antimicrobial agents differ, with the most common being the **alkaloids**, **terpenoids**, and **peptides**.

Axinellamines (Fig. 1.4) **A (1)** and **B (2)** are alkaloids with a complex polycyclic skeleton isolated from the sponge *Axinella* sp. Their biological activity was first described in 1999. They exhibit activity against several Gram-positive and Gram-negative bacteria. The antibacterial activity of compound A shows MICs of 0.5–16 μg/ml and compound B of 1–32 μg/ml. Moreover, axinellamines cause membrane destabilization in *E. coli* [58]. Axinellamines A and B have recently been chemically synthetized using a new asymmetric synthesis method [59].

Alkaloids such as **oroidin** (isolated from *Agelas oroides*) have been described activity against *S. aureus* and *E. faecalis* [60]. **Sceptrin**, **dibromosceptrin**, and **bromoageliferin** (from *Agelas conifera*) show antimicrobial activity against *S. aureus* and *A. baumannii* (Table 1.5). This evidence has encouraged scientists

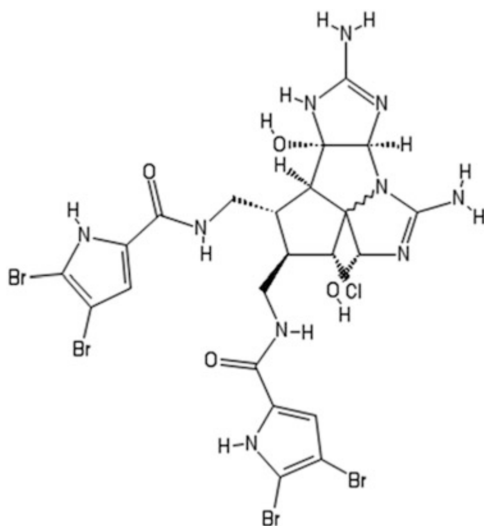


Fig. 1.4 Structure of axinellamines. Axinellamines showed activity against several Gram-positive and Gram-negative bacteria

Table 1.5 Natural compounds with antimicrobial activity isolate from sponge

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Axinellamines A and B	*Axinella* sp	Alkaloid	*Escherichia coli*, *Pseudomona aeruginosa*, MRSA[a], *Staphylococcus. epidermidis*, *Enterococcus faecalis*, *Corynebacterium efficiens*, *Yersinia pestis*	[58]
Oroidin	*Agelas oroides*	Alkaloid	*S. aureus* and *E. faecalis*	[60]
Sceptrin, Dibromosceptrin and Bromoageliferin	*Agelas conifera*	Alkaloid	*S. aureus* and *Acinetobacter baumanii*	[61]

[a]Methicillin resistant *Staphylococcus aureus*

to continue with their research on these alkaloids and to perform assays against Gram-negative and Gram-positive bacteria [61].

In the search for new metabolites with antibacterial properties, sponges are promising organisms, and continued investigation of these organisms will hopefully lead to the discovery of powerful new biomolecules to improve the fight against multidrug resistance.

5 Cnidaria

The phylum Cnidaria includes more than 10,000 species which are widespread throughout the seas, with only a few species having been found in freshwater [62]. These organisms are usually found in shallow warm waters. They have two embryonic cell layers, ectoderm and mesoderm (diploblastic organisms), that form the epidermidis and gut cavity, respectively, in adult organisms. Cnidarians are organisms with radial symmetry although they can also exhibit directional asymmetry or bilateral symmetry. These organisms play an important role in inorganic carbon precipitation in reef-building corals [63]. This phylum has been divided into five classes: Anthozoa (including coral), Cubozoa (cube jellyfishes), Hydrozoa (the most variable class), Scyphozoa (true jellyfishes), and Staurozoa (the most recently characterized class) [64].

Cnidarians are an interesting group because of their venomous properties. Their chemical products have been reported to cause mainly local damage, but some species, such as Australian species, produce several cardiac or neurological problems. Some proteins are considered to be responsible for the hemolytic effects of these organisms due to alterations in cell permeability resulting in ion transport, pore formation, oxidative stress, or osmotic lysis [65].

Cnidarians lack an external structure to protect them from other organisms. Thus, secondary metabolites, which can be used against bacteria, have been developed from the need for self-protection. Among these excreted products are **sesquiterpenes, diterpenoids, steroids**, **terpenes**, and **peptides** that present antimicrobial activity.

The chemistry of the cnidarians is dominated by metabolites derived from terpene biosynthesis. **Terpenes** are a large diverse class of organic compounds biosynthetically derived from isoprene units. Some authors have described terpenes and terpenoids as the same compound. Nevertheless, terpenoids can be considered as modified terpenes. Terpenes may be derived from terpenoids, diterpenoids, sesquiterpenoids, and cembranoids.

Bipinnapterolide B is a terpenoid that has been isolated from the octocoral *Pseudopterogorgia bipinnata.* The activity of bipinnapterolide B has been tested "in vitro" against *Mycobacterium tuberculosis* showing inhibition in 66% of the samples at a concentration of 128 mg/mL [66].

Xeniolide I is a diterpenoid that has been isolated from the cnidarian *Xenia novaebrittanniae.* It has shown antibacterial activity in *E. coli* ATCC and *B. subtilis* at a concentration of 1.25 mg/mL [67]. Other diterpenes isolated from *P. elisabethae* and their posterior derivatives have shown activity against the resistant *M. tuberculosis* H37Rv strain [68]. Another four diterpenoids (**elisabethin E**, **elisabethin F**, **pseudopterosin P**, and **pseudopterosin Q**) have been described, but only P and Q exhibit selective activity against three Gram-positive bacteria (*Streptococcus pyogenes*, *S. aureus*, and *E. faecalis* [69].

One diterpene, a compound composed of two terpene units, has been related to antimycobacterial activity. This compound is the **homopseudopteroxazole** that induces 80% of *M. tuberculosis* inhibition with a MIC value of 12.5 μg/mL [70]. The diterpene **pseudopterosins U**, isolated from *P. elisabethae*, exhibits activity against *S. aureus* and *E. faecalis* with an IC50 of 2.97 μM and 3.19 μM, respectively [71].

Three new phenolic bisabolane-type **sesquiterpenoids** from *Dichotella gemmacea* have been isolated and show moderate antimicrobial activity against *S. aureus* and MRSA [72].

Soft corals also yield a variety of **steroids**. **Litosterol** and **nephalsterols B** and **C** have been isolated from Red Sea *Nephthea* spp. Litosterol and nephalsterols C inhibit the growth of *M. tuberculosis* with MICs of 3.13 and 12.5 mg/mL, respectively [73].

The cembranoid **sarcophytolide** was isolated from soft corals of the genus *Sarcophyton.* This cembranoid shows activity against *S. aureus* with a MIC of 125 mg/mL [74].

Finally, cnidarians are able to synthetize peptides. Thus, **aurelin**, a new peptide with 40 residues purified from the mesoglea of the jellyfish *Aurelia aurita* (order Semaeostomeae), is considered a promising peptide. Its activity consists in its structural similarity with the defensins and K+ channel blockers of sea anemones. This compound shows activity against Gram-positive (*L. monocytogenes)* and Gram-negative (*E. coli*) bacteria [75].

The cnidarian *Hydra magnipapillata* produces a large number of peptides (**hydramacin-1**, **periculin-1**, **arminin**, and **kazal-2**), which show activity against a wide range of Gram-positive and Gram-negative bacteria. **Hydramacin-1** (Fig. 1.5) has demonstrated the greatest activity, with concentrations less than 1 μM and killing 99.9% of bacteria against Gram-negative species such as *E. coli*, *K. pneumoniae*, *K. oxytoca*, *S. typhimurium*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Yersinia enterocolitica*. On the other hand, the highest activity against Gram-positive pathogens was observed in *Staphylococcus haemolyticus* with a concentration of 1.8 μM. Its structure is quite similar to the scorpion oxin-like superfamily [76].

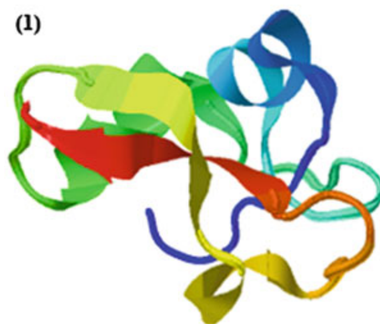


Fig. 1.5 Hydramacin-1 peptide with the greatest antibacterial activity isolated from *Hydra magnipapillata*

The peptide **periculin-1** has shown antimicrobial activity against *Bacillus megaterium* at concentrations ranging between 0.2 and 0.4 μM [77].

Arminin is another peptide synthetized by cnidarians and consists of a strongly positively charged C-terminal region and a highly negatively charged N-terminal region. This peptide has shown antimicrobial activity against *E. coli*, *Bacillus megaterium*, and *S. aureus* at concentrations of less than 0.5 μM. In addition, MRSA is inhibited at concentrations of 0.4–0.8 μM and a LD 90 of 0.2 μM. Arminins tested under physiological conditions did not show adverse effects to human erythrocytes. All of these properties make arminin an interesting antibacterial agent [78].

Finally, the peptide **kazal-2**, a serine protease inhibitor, has shown antimicrobial activity against *S. aureus* at concentrations of 0.7–0.8 μM [79]. A summary of the activity and derived species of the aforementioned compounds is illustrated in Table 1.6.

6 Bryozoa

The Bryozoa or Ectoprocta phylum includes 5869 species which are found to be widespread in marine water, brackish-water, and freshwater [80]. They live in sessile colonies, formed by several individual units called zooids, and are colonial filter feeders. The colonies provide an external skeleton or zoecium that may be formed by quitin and gelatin and might include calcium.

Ectoprocta are one of the most abundant and diverse members of Antarctic benthos. Approximately 55% of all bryozoan species are found below a depth of 40 m, and approximately 27% of these species live in very deep water (>700 m) [81]. To date, the ecology of the Antarctica remains poorly studied because of few groups that have investigated the Antarctic benthos [82].

In recent years, there has been increasing interest in secondary metabolites of the Bryozoa species.

The majority of bryozoan metabolites isolated to date are **alkaloids**, **ceramides**, and **sterols**, being alkaloids the group with the most active compounds discovered (Table 1.7).

Some examples of alkaloids from Bryozoa include the **amathaspiramides** (isolated from *Amathia wilsoni*), **euthyroideones** (from *Euthyroides episcopalism*), and **pterocellins** (from *Pterocella vesiculosa*). Amathaspiramide A has shown modest antibacterial activity against *B. subtilis* but has not been tested against marine bacteria which may be a more effective indicator of its activity [83]. A new alkaloid, **5-bromo-8-methoxy-1-methyl-carboline**, was isolated from the bryozoan *Pterocella vesiculosa*. This new alkaloid has demonstrated antimicrobial activity against *B. subtilis* (MIC = 2–4 μg/mL) and *C. albicans* and *Trichophyton mentagrophytes* (MIC of 4–5 μg/mL) [84].

Other important metabolites with antibacterial activity extracted from bryozoan are terpenes. Thus, **eusynstyelamide F** was isolated from *Tegella* cf. *spitzbergensis*

Table 1.6 Natural compounds and peptides with antimicrobial activity isolate from Cnidaria

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Bipinnapterolide B	*Pseudopterogorgia bipinnata*	Terpenoid	*Mycobacterium tuberculosis*	[66]
Xeniolide I	*Xenia novaebrittanniae*	Diterpenoid	*Escherichia coli* and *Bacillus subtilis*	[67]
21-((1*H*-imidazol-5-yl) methyl)-pseudopteroxazole	*Pseudopterogorgia elisabetha*	Diterpenoid	*M. tuberculosis*	[68]
Elisabethin P, Q	*Pseudopterogorgia elisabethae*	Diterpenoid	*Streptococcus pyogenes, Staphylococcus aureus* and *Enterococcus faecalis*	[69]
Homopseudopteroxazole	*Pseudopterogorgia elisabethae*	Diterpenoid	*M. tuberculosis*	[70]
Pseudopterosins U	*Pseudopterogorgia elisabethae*	Steroids	*S. aureus* and *E. faecalis*	[71]
Litosterol and Nephalsterols B–C	*Nephthea* spp.		*M. tuberculosis*	
Sarcophytolide	Genus *Sarcophyton*	Cembranoid	*S. aureus*	[74]
Aurelin	*Aurelia aurita*	Peptide	*Listeria monocytogenes* and *E. coli*	[75]
Hydramacin-1	*Hydra magnipapillata*	Peptide	*E. coli, Klebsiella pneumoniae, K. oxytoca, Salmonella typhimurium, Citrobacter freundii, Enterobacter cloacae, Yersinia enterolitca and Staphylococcus hemolyticus*	[76]
Periculin-1	*Hydra magnipapillata*	Peptide	*Bacillus megaterium*	[77]
Arminin	*Hydra magnipapillata*	Peptide	*E. coli*, *B. megaterium* and *S. aureus*[a]	[78]
Kazal-2	*Hydra magnipapillata*	Peptide	*S. aureus*	[79]

[a]Methicillin resistant *Saphylococcus aureus*

Table 1.7 Natural compounds with antimicrobial activity isolate from Bryozoa

Metabolite	Isolated from	Chemical scaffold	Activity	References
Amathaspiramide A	*Amathia wilsoni*	Alkaloid	*Bacillus subtilis*	[83]
5-Bromo-8-methoxy-1-methyl-carboline	*Pterocella vesiculosa*	Alkaloid	*B. subtilis, Candida albicans* and *Trichophyton mentagrophytes*	[84]
Eusynstyelamide F	*Tegella* cf. *spitzbergensis*	Alkaloid?	MRSA[a], *Escherichia coli*, *Pseudomona aeruginosa* and *Corynebacterium glutamicum*	[85]

[a]Methicillin resistant *Staphylococcus aureus*

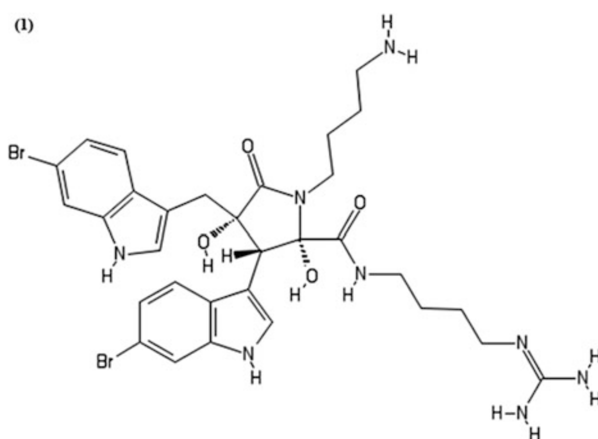


Fig. 1.6 Structure of eusynstyelamide F, isolated from *Tegella* cf. *spitzbergensis*, exhibited stronger antibacterial activity

together with three other compounds (**eusynstyelamides D** and **E** and **ent-eusynstyelamide B**) (Fig. 1.6). However, eusynstyelamide F showed higher activity compared to the others. Their range of action was tested in *S. aureus*, *E. coli*, *P. aeruginosa*, *Corynebacterium glutamicum*, and MRSA, with eusynstyelamide F being more active against *S. aureus* and *C. glutamicum* with a MIC of 6.25 μg/mL and 12.5 μg/mL, respectively [85].

Nowadays, only a few compounds are in experimental study phases compared to other phylum such as sponges. Some reasons for this may be the problems associated with working with these compounds. To the naked eye, phylum Bryozoa is commonly confused with hydroids and other species. Moreover, they are located in places of difficult accessibility since they live in deep areas, in which there is a lack of biomass available for extraction.

7 Mollusca

The phylum Mollusca is one of the most attractive invertebrate phyla, and they are widely distributed worldwide, having many representatives in the marine and estuarine ecosystems including slugs, whelks, clams, mussels, oysters, scallops, squids, and octopods [86–88]. Molluscs are a highly diverse group in size, anatomical structure, behavior, and habitat. Around 10,000 extant species and another 70,000 fossil molluscs have been described. However, many species have not yet been identified, making these the largest marine phylum with about 23% of all named marine organisms. Representatives of this phylum live in an enormous range of habitats including marine, freshwater, and terrestrial environments [89].

Taxonomically, there are three well-known types of molluscs, including clams and mussels (Bivalvia, 20,000 species), snails and slugs (Gastropoda, 70,000 species), and squids and octopuses (Cephalopoda, 900 species). In addition, there are four other classes: Chitons (Polyplacophora, 1000 species), tusk shells (Scaphopoda, 500 species), *Neopilina* and its relatives (Monoplacophora, 25 species), and the vermiform and primitive Aplacophora (200 species). It is of note that the members of these seven classes are phenotypically very different but remarkably similar in terms of their organizational model [86, 88, 90].

The many relationships between humans and molluscs mainly involve them as sources of food, money, jewels, and art, but molluscs play an important role in science as model organisms in the study of neurobiology and evolutionary biology and as a source of bioactive metabolites [90].

Among marine invertebrates, many classes of bioactive compounds from different species of molluscs have exhibited antitumor, antileukemic, antibacterial, and antiviral activity [87, 91]. Another important feature of some species of molluscs, including gastropod and bivalves, is that they are widely used as biomarkers of environmental pollution by infection and heavy metal exposure [92, 93].

Concerning the antibacterial activity related to this phylum, several molecules have been studied as shown in the Table 1.8 and Fig. 1.7. Thus, **hexadecylglycerol**, which is isolated from the digestive gland and skin of *Archidoris montereyensis* (nudibranch), has shown "in vitro" antibacterial activity against *S. aureus* and *B. subtilis* [94, 95]. The same compound obtained from *Perna viridis* (bivalve) extracts showed the highest activity against *E. coli* K1, *A. baumannii*, and *P. aeruginosa* [96]. Furthermore, there have been many studies on antimicrobial compounds from gastropods, using different samples including hemolymph, egg masses, or whole body extracts [87, 89].

Other molecules, such as **diemensin A–B**, **kelletinin I–II (1, 2)**, and **chromodorolide A (3)**, have been isolated from extracts of the gastropod species *Siphonaria diemenensis*, *Kelletia kelletii*, and *Chromodoris* sp., respectively. All these molecules inhibit the growth of Gram-positive and Gram-negative bacteria as well as the cell division of sea urchin eggs and the growth of L1 leukemia [94, 96–98].

Interesting compounds active against human pathogens have been isolated as a result of the relationship between a coral and *Trochus tentorium* [99]. Crude whole

Table 1.8 Natural compounds with antimicrobial activity isolate from Mollusca

Metabolite	Isolate from	Chemical scaffold	Activity against	References
Hexadecylglycerol	*Archidoris montereyensis*		*Staphylococcus aureus* and *Bacillus subtilis*	[94–96]
	Perna viridis		*Escherichia coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*	
Diemensin A–B	*Siphonaria diemensis*		Gram-positive and Gram-negative bacteria	[94, 96–98]
Kelletinin I–II	*Kelletia kelletii*		Gram-positive and Gram-negative bacteria	
Chromodorolide A	*Chromocloris* sp.		Gram-positive and Gram-negative bacteria	[94, 96–98]
Crude whole body extracts	*Trochus tentorium*		*S. pneumoniae* and *K. pneumoniae,* and lower activity against *E. coli*, *S. pneumoniae*, *S. aureus* and *V. cholerae*	[99]
Polysaccharides	*Sepia aculeata* and *Sepia brevi*		*B. subtilis*, *E. coli*, *K. pneumoniae*, *V. cholerae*, *V. parahaemolyticus*, *S. aureus*, *P. aeruginosa*, *Salmonella* spp. and four fungal species (*Candida* sp., *Rhizopus* sp., *Aspergillus flavus,* and *A. fumigatus*)	[100, 101]

body extracts using solvents such as acetone, ethyl acetate, dichloromethane, and methanol presented high activity against *S. pneumoniae* and *K. pneumoniae* and lower activity against *E. coli*, *S. pneumoniae*, *S. aureus*, and *V. cholerae*.

The marine mollusc *Melo melo* is a potential source of bioactive antibacterial and antifungal compounds. Kanagasabapathy et al. [102] observed that methanol extract of mucus, nerve tissue, body tissue, and kidney showed antimicrobial activity against *K. pneumoniae*. Protein studies such as thin-layer chromatography (TLC) and SDS-PAGE were used to determine the presence of peptides or amide groups. Thus, four proteins of 14, 17, 22, and 45 kDa were found to provide antimicrobial activity. Similar results were reported by Periyasamy et al. [87] who found many proteins between 2 and 110 kDa in weight from *B. spirata* muscle presenting antibacterial activity against pathogenic microbial forms. However, some novel and uncharacterized mechanisms of action that might ultimately benefit from the ongoing global search for clinically useful antimicrobial agents need to be explored to explain the antimicrobial activity of *B. spirata*.

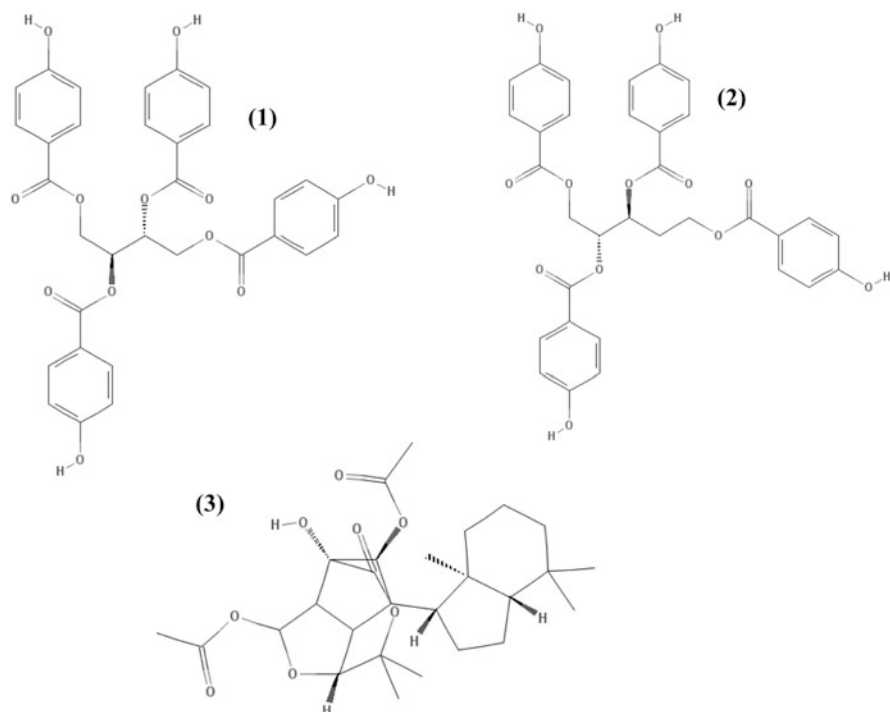


Fig. 1.7 Structures of secondary metabolites with antimicrobial activity (**1**) kelletinin I, (**2**) kelletinin II, and (**3**) chromodorolide A isolate from *Siphonaria diemenensis*, *Kelletia kelletii*, and *Chromodoris* sp., respectively. (Gastropod, Mollusca)

Four novel antimicrobial peptides from *Rapana venosa* have been detected. This gastropod of Asiatic origin is a successful invasive organism, which represents a serious threat to the malacological resources of marine waters worldwide. However, it has also demonstrated to be an interesting source of antimicrobial peptides. **Proline-rich peptides**, with molecular masses of between 3 and 95 kDa, have been isolated from hemolymph samples, showing strong antimicrobial activity against *S. aureus* and *K. pneumoniae* [103, 104].

Cephalopods are another interesting group to study. This group has the highest number of extinct species (~4000). For this reason, the living species are considered to be true survivors [90, 105]. These species have developed interesting defense mechanisms including high melanin production that can be distributed by ejected water as jet propulsion. The ejected cloud of melanin is usually mixed, upon expulsion, with mucus forming a thick cloud and resulting in visual and chemosensory impairment of the predator, like a smoke screen, avoiding their predation [88, 106]. In addition, cephalopods are important as a food source as well as animal models in scientific investigations, being a storehouse of many biologically important substances [107, 108].

Most studies on cephalopods are focused on the potential use of **polysaccharides** as potent antibacterial molecules. Shanmugam et al. [101] investigated the antibacterial and antifungal activity of extracts from the cuttle of two cephalopod species, *Sepia aculeata* and *Sepia brevimana*, against eight species of bacteria (*B. subtilis*, *E. coli*, *K. pneumoniae*, *V. cholerae*, *V. parahaemolyticus*, *S. aureus*, *P. aeruginosa*, *Salmonella* spp.) and four fungal species (*Candida* sp., *Rhizopus* sp., *Aspergillus flavus*, and *A. fumigatus*). They found that their potent activity is due to the presence of polysaccharides, and that this activity is directly related to the concentration of the extracts. In addition, similar results were found using methanolic extracts and fractionated polysaccharides from other cephalopod species such as *Loligo duvauceli* [100]. This same research group reported that **glycosaminoglycans** (GAGs) or **mucopolysaccharides** from a small cephalopod species named *Euprymna berryi* showed very good activity against five pathogenic bacteria and four fungal strains including *Shigella* spp. and *E. coli*. In addition, the mucopolysaccharides showed potent antifungal activity against *C. albicans* and *A. fumigatus* [109].

8 Annelida

The Annelida phylum is made up of bilaterally symmetrical animals with bodies that consist of three regions. The body is divided into parts or similar segments also called metamerisms, which are arranged in a linear series along the anteroposterior axis of the body. Annelids are of particular phylogenetic interest, because they are the first coelomates with a complete digestive tract, a closed circulatory system with hemoglobin in the plasma to carry oxygen and carbon dioxide, and their nervous system is developed and they have an excretory structure [86, 88].

The diversity of annelids comprises ringed or segmented worms including rag worms, earthworms, and leeches, with over 17,000 living species ranging in size from less than 1 mm to well over 3 m (Gippsland earthworm and *Amynthas mekongianus*, respectively) [88, 110].

Annelids have been divided into three taxonomic classes (Polychaeta, Oligochaeta, and Hirudinea). However, more recent phylogenetic investigations only consider two taxonomic classes, Polychaeta and Clitellata, with the latter being subdivided into the subclasses Oligochaeta and Hirudinea [88]. The Polychaeta class is the largest and most diverse group, with more than 12,000, mainly marine, species having been described. The Clitellata class (of about 5000 species) includes the subclass Oligochaeta, which is composed of freshwater annelids, earthworms and a variety of marine species, and the subclass Hirudinea which the best-known members are leeches. Marine species are mostly blood-sucking parasites, mostly of fish, while most freshwater species are predators [86, 111].

The annelids have successfully invaded all habitats where sufficient water is available. They are important players in the benthic communities as they are critical for the biomass of seashore, estuary, freshwater, and terrestrial soils. Moreover, they

occupy the central position in the trophic networks and are a major food source for fishes, birds, and terrestrial fauna [112]. The importance of this group of invertebrates not only lies in their great diversity and evolutionary success but also as a source of novel molecules of biological interest, highlighting the potential use of biomarkers to monitor the influence of environmental perturbation and as a potential source of new molecules with antimicrobial activity, synthesized in response to defense mechanisms to fight pathogens [112–114].

The immunity system of annelids is an attractive object of study for comparative immunologists, because, despite being primitive, they have developed both cellular and humoral inmate response against pathogens [115]. This immune system is very conserved throughout the animal kingdom and is the most ancient first line of immune protection for invertebrates. Numerous components participate in innate immune response, including important humoral factors such as antimicrobial proteins and antimicrobial peptides (AMPs) as well as cells that kill invasive pathogens using phagocytic or cytotoxic systems [112, 115, 116].

In the last years, great emphasis has been given to the study of compounds with antimicrobial activity obtained from marine annelids, especially from Polychaete worms. In this sense, the most studied antimicrobial proteins are **lysozymes** [112]. The first bacteriolytic molecules were identified as **lysozyme-like** of 14 kDa from *Nereis diversicolor* produced by a type of cells named G1 and detected in the coelomic fluid about 24 h after infection with bacteria. This protein has bacteriostatic activity, cleaving the B-1-4 bonds between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan and providing access of the latter to the bacterial cell wall of Gram-positive bacteria, being mostly active against this bacterial type [112].

Other antimicrobial factors have been isolated from coelomic fluid from *Glycera dibranchiata* (Polychaeta, Glyceridae), probably as part of the organisms defense against bacterial infection. They show very good activity against Gram-negative bacteria, including *S. marcescens*, *P. aeruginosa*, and *E. coli* strains [117].

On the other hand, numerous studies on the effectors of the innate immune system have demonstrated the contribution of antimicrobial peptides (AMPs) to host defense [112, 114] (Table 1.9; Fig. 1.8). **Antimicrobial peptides** are small well-conserved molecules among strains. Based on their structural features, five major classes have been defined:

1. Linear α-helical peptides without cysteines. The prototypes of this family are the cecropins.
2. Loop-forming peptides containing a unique disulfide bond. These are mainly isolated from amphibian skin.
3. Open-ended cyclic cysteine-rich peptides, being defensins the most widespread.
4. Linear peptides containing a high proportion of one or two amino acids such as indolicidin.
5. Peptides derived from larger molecules known to exert multiple functions [112, 114, 121].

The principal source of AMPs in annelids has been found in three species of marine Polychaetes, *Arenicola marina*, *Nereis diversicolor*, and *Perinereis aibuhitensis*. **Perinerin** is the bioactive compound isolated from the clam worm

Table 1.9 Peptides with antimicrobial activity isolate from Annelida

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Perinerin	*Arenicola marina*	Peptide	Gram positive and negative bacteria	[118]
Arenicins (**1**–**2**)	*Arenicola marina*	Peptide	*E. coli*	[112, 119]
Hedistin	*Nereis diversicolor*	Peptide	Gram-positive bacteria, gram-negative bacteria (marine bacteria as *Vibrio alginolyticus)*	[114, 120]

Fig. 1.8 Structures of two novel antimicrobial peptides isolate from lugworm *Arenicola marina* (marine Annelida). (**1**) Arenicin 1 and (**2**) arenicin 2

Perinereis aibuhitensis [118]. This peptide consists of 51 amino acids, including 4 cysteine residues presumably implicated in 2 disulfide bridges. Antimicrobial assays show high activity against Gram-negative and Gram-positive bacteria and fungi at physiological concentrations. Moreover, perinerin has shown rapid bactericidal activity against *Bacillus megaterium* during the exponential phase, suggesting a pore-forming activity [112, 113, 122]. It has also been reported that the coelomic fluid of *Perinereis cultrifera* presents potent antibacterial and antifungal activity [123].

Two novel AMPs of interest have been described in the lugworm *Arenicola marina* [119]. **Arenicin-1 (1)** and **arenicin-2 (2)** are amphipathic peptides formed by 21 residues, and each isoform possesses 2 cysteine residues implicated in 1 disulfide bond. Both isoforms show equal activity against fungi, as well as Gram-positive and Gram-negative bacteria [112]. Thus, the use of concentrations of about 5 μM of arenicin kills *E. coli* in 5 min, producing a rapid membrane permeabilization accompanied by peptide intercalation into the bilayer and the release of cytoplasmic material [113, 115, 119].

The most important AMP isolated from marine annelids is **hedistin**. It is purified from the rag worm *Nereis diversicolor*. Hedistin is a linear peptide of 22 amino acids that contains bromotryptophan residues. Moreover, the primary structure of hedistin includes a C-terminal amidation that could increase the net charge and, consequently, the electrostatic attraction to target membranes like the negatively charged bacterial membrane. This suggests that this C-terminal amidation of hedistin might be implicated in its bactericidal properties [113, 120, 124]. Hedistin is active against

a large spectrum of Gram-positive bacteria, but, interestingly, it is also very active against Gram-negative bacteria, especially the marine bacteria *V. alginolyticus* which is a causative agent of episodes of mass mortality of larvae of bivalves in commercial hatcheries. This could be attributable to the capacity of *Vibrio* to degrade the native cuticle collagen of *Nereis*. Vibrial collagenase helps bacteria to enter the worm body, making the mechanical defense barrier of the cuticle inefficient against *Vibrio* invasion [114]. No cytotoxicity of either hedistin forms was observed against *Nereis cœlomocytes* [120].

9 Echinodermata

Echinoderms are deuterostome invertebrates with a phylogenetic position closely related to chordates and hemichordates [125]. The phylum contains about 7000 extant species, including sea lilies, feather stars, brittle stars, starfish, sea urchins, sand dollars, and sea cucumbers, as well as about 13,000 extinct species with a fossil registry from the early Cambrian period [86, 88]. These animals have a unique morphology that includes a water vascular system and a pentamerously symmetrical body shape in adult invertebrates that have an endoskeleton consisting of magnesium calcite [88, 126, 127].

Taxonomically, the phylum Echinodermata is categorized into two subphyla, the Pelmatozoa, including the class of Crinoidea (sea lilies and feather stars, 625 recent species), and the Eleutherozoa which comprises the classes of Asteroidea (starfish, 1500 recent species), Echinoidea (sea urchins, sand dollars, and sea biscuits, 950 recent species), Holothuroidea (sea cucumbers, 1150 recent species), and Ophiuroidea (brittle stars, 2000 recent species) [86, 88, 127, 128].

Echinoderms are exclusively marine organisms. They are generally benthic animals found in shallow water and occupy habitats from the intertidal zone to the deep sea. The size of the echinoderms ranges from small sea cucumbers and brittle stars of 1 cm to starfish that surpass 1 m in diameter and sea cucumbers of up to 2 m in length [86, 88].

The environment where the echinoderms live is exposed to relatively high amount of bacteria, fungi, viruses, and parasites, many of which are potentially pathogenic. The survival of these organisms relies on the production of efficient antimicrobial components to defend themselves against microbial infections and fouling [126, 129, 130]. As invertebrates in general, echinoderms have an innate immune system, but as all other invertebrates, they lack a vertebrate-type adaptive immune system [126, 131]. Principally, the immune response occurs in the coelomic fluid mediated by coelomocytes, in which a series of cells are activated for their defense (phagocytes, vibratile cells, colorless and red spherule cells). In addition, compounds like complement factors, lectins, lysozymes, and AMPs have been identified as participants in the host defense system [126, 129, 132, 133].

In this regard, there are many studies searching for new antimicrobials in a great variety of echinoderm species. For example, antimicrobial activity has been reported

Table 1.10 Natural compounds and Peptides with antimicrobial activity isolate from Echinodermata

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Echinochrome-A	*Echinus esculentus*	Quinona pigments	Gram positive and negative bacteria	[139, 140]
Spinochromes D	*Echinus esculentus*	Quinona pigments	Gram positive and negative bacteria	[139, 140]
Strongylocins	*Strongylocentrotus droebachiensis*	Peptide	Marine fish pathogens (*Listonella anguillarum*)	[141]
Centrocins 1- 2	*Strongylocentrotus droebachiensis*	Peptide	Gram positive and negative bacteria	[142, 143]
EeCentrocins	*E. esculentus*	Peptide	Gram-negative bacteria, gram-positive bacteria and against fungi	[130]
5-cc	*Paracentrotus lividus*	Peptide	Antistaphylococcal bacteria *S. epidermidis* (inhibitor of formation young and mature biofilm)	[144, 145]

in several species of echinoderms collected from the Gulf of California, Mexico, the Caribbean, and the coast of Norway [62, 134]. A variety of antimicrobial factors, including **steroidal glycosides**, **polyhydroxylated sterols**, **naphthoquinone pigments** (Service and Wardlaw 1984), **lysozymes** [135, 136], **complement-like substances** [137], and **antimicrobial peptides** [138] (Table 1.10; Fig. 1.9), have been isolated from sea cucumbers. Additionally, six new **cytotoxic triterpene glycosides** from *Mensamaria intercedens* lampert, displaying a broad range of antibacterial, antifungal, and cytotoxic activity, have been identified by extensive spectroscopic analysis (NMR and ESIMS) and chemical methods [146].

Various extracts from the gut and gonads of the sea urchin *Tripneustes gratilla* have shown antimicrobial activity. Compared to chloroform extracts, methanol extracts exhibit higher activity against Gram-positive and Gram-negative bacteria as well as against selected fungal species such as *Penicillium* spp. Furthermore, tissues tested in different reports showed high antibacterial activity [147] and, interestingly, did not exhibit hemolytic activity against human erythrocytes, being a very good early indicator of potentially low toxicity toward mammalian/human cells [134]. In contrast, other parts of the body of sea cucumbers such as the Cuvierian organs did not present activity [132].

Other important metabolites with biological activity from echinoderms are **saponins**. These compounds from sea stars and sea cucumbers have been extensively studied, but were not found to be useful as drugs because of their tendency to cause cell lysis [17]. On the other hand, **asterosaponins** are other metabolites that could play an important role in chemical defense to protect the starfish from parasites and predators. In addition, they have hemolytic, antineoplastic, cytotoxic, antitumor,

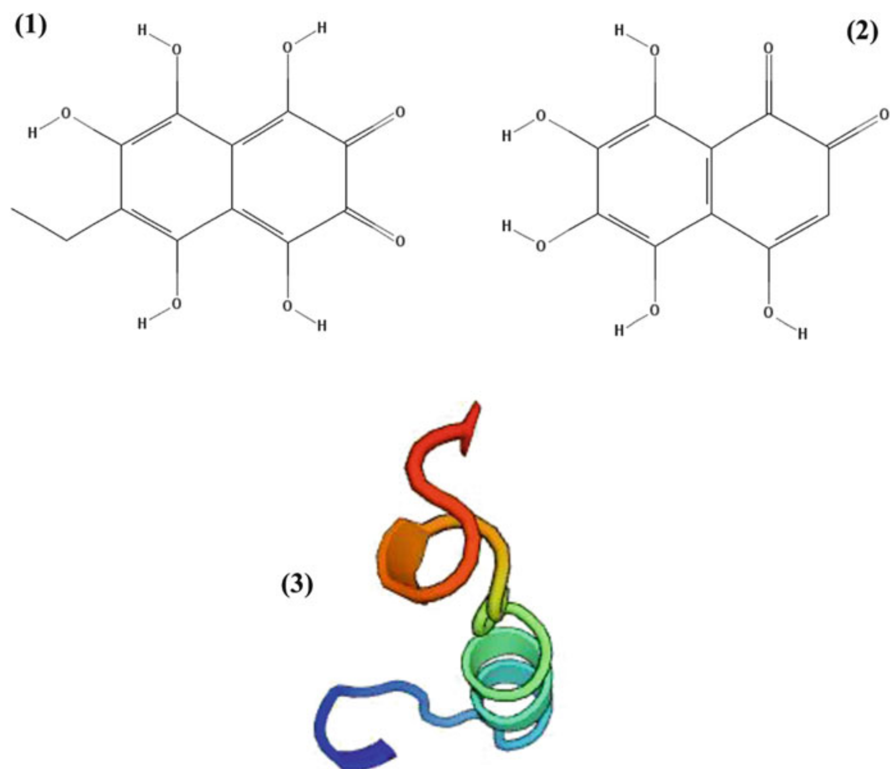


Fig. 1.9 Structures of two quinone pigments (**1**) echinochrome and (**2**) spinochromes D with antimicrobial activity and novel antimicrobial peptide (**3**) EeCentrocins isolate from *Echinus esculentus* (sea urchin, Echinodermata)

antiviral, antifungal, and anti-inflammatory activity. However, further studies on the activities of asterosaponins have been hampered by their poor accessibility [17, 148].

Interestingly, some **quinone pigments** from *Echinus esculentus* (sea urchin) contain antimicrobial compounds, in which **echinochrome-A (1)** and **spinochromes D (2) and E** have been identified [139]. Both compounds may play decisive roles in the regulation of lipid peroxidation and in immune defense. Specifically, echinochrome is synthesized by sea urchin pigment cells and possesses a strong bactericidal effect during embryonic and larval development [140, 149]. Another important drug isolated from sea urchin pigment cells is **histochrome** (Moscow, Russia) which presents cardiological and ophthalmological activity [150].

A large number of antimicrobial peptides (AMPs) have previously been found in the coelomic fluid of echinoderms [49, 129, 130]. The first completely sequenced AMPs in echinoderms were the **strongylocins** that are expressed in the coelomocytes of the green sea urchin *S. droebachiensis* [141]. This family includes two isoforms, the **SdStrongylocins 1b** and **2b.** Both are cysteine-rich peptides containing three disulfide bonds with MW in the 5.6–5.8 kDa range and display

strong activity against the marine fish pathogens such as *Listonella anguillarum* with a MIC of 1.3–2.5 μM [126]. Another peptide family identified from the coelomic fluid of *S. droebachiensis* is **centrocins 1** and **2** [142]. The centrocins are a family of heterodimeric AMPs ranging between 4.4 and 4.5 kDa in mass and are composed of two peptide chains: a 30-amino acid residue heavy chain (HC) and a 12-amino acid residue light chain (LC) connected by a single disulfide bond. Studies of bioactivity have shown that the cationic HC displays potent activity against both bacteria and fungi [143].

An interesting AMP of 5 kDa with antistaphylococcal biofilm properties has been isolated from *Paracentrotus lividus* [144, 145]. The **5-CC** peptide inhibits the formation of young biofilm (6-h old) from *S. epidermidis* 1457 as well as the formation of mature biofilm (24-h old) in the same clinical strain. This antibiofilm activity could be due to interference of 5-CC peptide with microbial surface proteins (adhesins, autolysins) that facilitate attachment to plastic surfaces in the first step of staphylococcal biofilm formation. However, further studies are needed to explain the mechanism of action of 5-CC in the prevention of adhesion and biofilm formation [151].

Novel peptides of 5–6 kDa were first characterized from coelomocyte extracts of the edible sea urchin, *E. esculentus*, collected from sub-Arctic waters. These AMPs are novel members of the centrocin and strongylocin families. The **EeCentrocins (3)** have a heterodimeric structure composed of a HC and a LC connected by a single disulfide bond. Studies on bioactivity show that both chains seem to be necessary for maintaining antibacterial activity. The secondary structure and the three-dimensional conformation of EeStrongylocin 2 as dictated by its three disulfide bonds remains unknown but should be explored [130].

Interesting peptides have been isolated from other species of echinoderms. For example, two peptides of about 2 kDa have been isolated from the starfish *Asterias rubens* and identified as fragments of the histone H2A molecule [130]. In addition, two other new peptides were found in *A. rubens*, corresponding to fragments of actin and filamin A. Additionally, these peptides showed that peptides can agglutinate Gram-positive and Gram-negative bacteria, exhibiting strong antibacterial activity in "in vivo" and "in vitro" conditions [152].

10 Tunicate

The Urochordata or Tunicata phylum frequently includes marine animals. Most are sessile and their body is covered by a complex exoskeletal robe. They have a highly developed perforated pharynx, but in the adult, the notochord and nerve cord usually disappear, and only the larval stages that look like microscopic tadpoles have the distinctive characteristics of chordates. Currently, about 1250 species have been described, and the earliest probable species of tunicate appears in the fossil record in the early Cambrian period [86, 88, 153, 154].

Taxonomically, members of the Tunicata phylum are divided into four classes: (1) Appendicularia, also known as larvaceans, are solitary, pelagic, and the free-swimming; (2) Thaliacea or pelagic tunicates are planktonic, solitary, or colonial and include three orders of free-swimming (Salpida, Pyrosomida, and Doliolida); (3) Sorberacea are organisms with great similarity to ascidians. They are benthic, live at great depths, have a dorsal nerve cord in the adult state, and are carnivorous; and (4) Ascidiacea or sea squirts are benthic, solitary, or colonial forms, with a cuticle made of polysaccharide [86, 88, 154, 155].

Ascidia is the group of organisms mainly studied because of the greater diversity of species that they possess and their interesting chemistry and physiology [155]. The way of life of ascidians and their bright colorful exterior make them more vulnerable to predation and more attractive to predators. Since they do not possess an escape mechanism, these organisms must rely on the production of chemical toxins for their survival [156]. Most of the ascidians and tunicates generally do not possess endobionts and have a well-developed immune system, which is activated against the presence of pathogenic species. However, a variety of studies have shown that these organisms appear to be good hosts of Cyanobacteria or even bacteria, which favor the production of toxins and molecules with biological interest as novel antimicrobials, according to a mechanism that has yet to be elucidated [155, 157].

Many antibacterial compounds have previously been isolated from tunicates or tunicate-associated marine bacteria/algae (Table 1.11 and Fig. 1.10). Thus, the **rubrolides (1)** are a family of approximately 20 polysubstituted butenolides isolated from an African ascidian *Synoicum globosum*, and they display highly sought-after biological properties including, anticancer, antidiabetic, anti-inflammatory, and antiviral activity. In addition, some of their synthetic analogs have shown potent

Table 1.11 Natural compounds and peptides with antimicrobial activity isolate from tunicate

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Rubrolides A	*Synoicum globosum*	Butenolides	*Staphylococcus aureus*, *Bacillus subtilis*	[158, 159]
Cystodimine A–B	*Cystodytes dellechiajei*	Pyridoacridine alkaloids	*Escherichia coli and Micrococcus luteus*	[160]
Synoxazolidinone A	*Synoicum pulmonaria*	Pyridoacridine alkaloids	*Corynebacterium glutamicum*	[161]
Styelin D	*Styela clavate*	Peptides	*Staphylococcus aureus* and MRSA[a]	[162]
Halocidin	*Halocynthia aurantium*	Peptides	MRSA[a]	[163]
Peptidolipins	*Trididemnum orbiculatum*	Lipopeptide	MRSA and MSSA[b]	[164]

[a]Methicillin resistant *Staphylococcus aureus*
[b]Methicillin susceptible *Staphylococcus aureus*

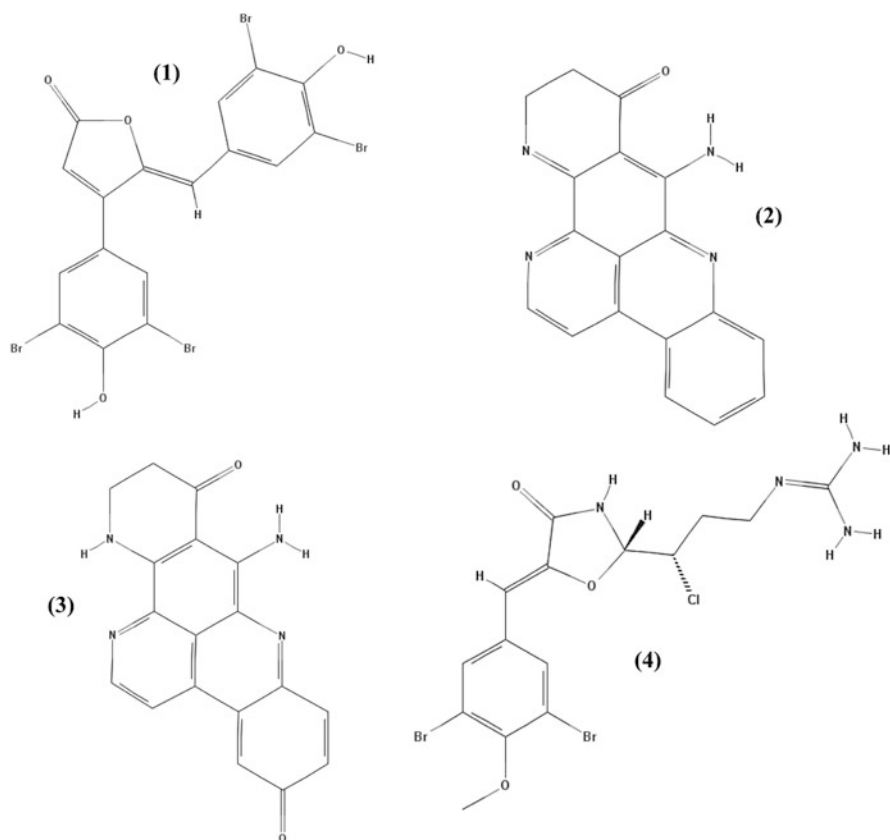


Fig. 1.10 Structures of antimicrobial compounds isolate from ascidians (tunicate). (**1**) Rubrolide A isolates from *Synoicum globosum*, and (**2**) cystodimine A, (**3**) cystodimine B, and (**4**) synoxazolidinone A isolate from *Cystodytes dellechiajei*

antimicrobial activity against *S. aureus* and *B. subtilis* and also possess significant herbicidal and biofilm inhibitory activities (Sikorska et al. 2012; [159]).

Mediterranean and Norwegian ascidians (*Cystodytes dellechiajei* and *Synoicum pulmonaria*, respectively) are sources of interesting alkaloids. **Pyridoacridine alkaloid**s (N-deacetylshermilamine B, cystodimine A **(2)**, and cystodimine B **(3)**) from *C. dellechiajei* are active against *E. coli* and *Micrococcus luteus* [160], and **synoxazolidinone A (4)** from *S. pulmonaria* is active against *Corynebacterium glutamicum* [161].

Several antimicrobial peptides have been isolated from ascidian. The peptide **Styelin D** has been isolated from *Styela clava* and corresponds to a peptide of 32 residues that contains a 6-bromotryptophan residue in its sequence. This native peptide showed antibacterial activity against *S. aureus* and MRSA at any range of pH and salinity. However, the non-brominated version showed decreased activity at low pH and/or high salinity [162]. **Halocidin peptide** (3443 Da) was isolated from

hemocytes of *Halocynthia aurantium*. This peptide presents strong activity against a wide variety of pathogenic bacteria including MRSA [163]. Another peptide isolated from hemocytes of the tunicate *Halocynthia aurantium* is **di-cynthaurin** that contains unpaired cysteine and forms a covalent homodimer, with each monomer consisting of 30 amino acid residues with similar biological characteristics to halocidin.

The symbiotic association between the ascidian *Trididemnum orbiculatum* and the bacteria *Nocardia* spp. has been studied. The compound **peptidolipins** produced by *Nocardia* spp. shows activity against methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA [164]. On the other hand, the relationship between cyanobacteria and ascidians has also been studied, mainly between *Synechocystis* or *Prochloron* species and the ascidian family Didemnidae [155]. Ascidians provide protection to cyanobacteria that live inside the tissues, tunic, or on the surface of the ascidians (for this reason the tunic is often transparent), and the cyanobacteria provide organic materials and cytotoxic constituents for protection to ascidians against depredator organisms. For example, the protein **didemnin B** prevents translocation by stabilizing aminoacyl-tRNA bound to the ribosomal A-site, similar to the antibiotic kirromycin. This molecule could be an interesting antibiotic to study. Furthermore, there are a substantial number of algal metabolites isolated from "free-living" algae which are similar or identical to ascidian metabolites [156, 165]. However, a better understanding of the mutual relationships of these organisms and biosynthetic studies are necessary to firmly establish whether these biologically interesting compounds are produced by the tunicate, the alga, or through a combined effort of both organisms.

11 Marine Algae

Marine algae, including microalgae, have been submitted to modern screening methods in order to identify secondary metabolites showing antibacterial activity [166]. Algae are a large group of photosynthetic and autotropic organisms distributed extensively throughout the aquatic environment, with some terrestrial species in extremophile environments. Morphologically they can be unicellular cells (diatoms) or large multicellular algae. Their size can range from microscopic organisms to large macroscopic structures of up to several meters in length. The great power adaptation of algae species in all media (terrestrial or aquatic) and their ability to live in extremophile conditions (osmotic stress, salinity, oxygen, and high UV radiation) and constant competition with seawater bacteria make them species of interest to study their secondary metabolites.

Microalgae are microscopic and unicellular algae that have the ability to perform photosynthesis, producing about half of the atmospheric oxygen. The biodiversity of microalgae is enormous, and only 50,000 species have been described among 200,000–800,000 species estimated to exist.

Microalgae such as diatoms have developed precise systems to fight pathogenic bacteria that coexist in the marine environment. The secretion of secondary metabolites is one of these systems. Among these metabolites, fatty acids, peptides, polysaccharides, aromatic organic acids, alcohols, aldehydes, terpenes, sterols, phlorotannins, polyketides, and hydroquinones, among others, are produced by marine algae [55, 167]. About 15,000 novel compounds from microalgae have been chemically characterized, including carotenoids, toxins, fatty acids, enzymes, polymers, antioxidants, and sterols [168].

Some of the most studied compounds from marine algae with antibacterial activity are shown in Table 1.12.

Fatty acids act against bacteria inhibiting their electron transport chain and oxidative phosphorylation in cell membranes. One of the most studied fatty acids

Table 1.12 Natural compounds with antimicrobial activity isolate from Bryozoa

Metabolite	Isolated from	Chemical scaffold	Activity against	References
Cyclopentaneacetic acid	Sargassum spp.	Fatty acid	*Staphylcoccus aureus* and *Klebsiella pneumonia*	[166]
Algae extract	*Solieria filiformis*	Lectin	*Pseudomona aeruginosa, Enterobacter aerogenes, Serratia marcencens, Salmonella typhi, K. pneumoniae* and *Proteus* sp.	[169]
Phlorofucofuroeckol-A	*Eisenia bicyclis*	Phlorotannin	*S. aureus*[a]	[170]
Fucoidan	*Cladosiphon ocamuranus*, *Fucus evanescens*, and *F. vesiculosus*	Polysaccharide	*Helicobacter pylori*	[171]
Laminarin	*Ascophyllum nodosum* and *Laminarina hyperborea*	Polysaccharide	*S. aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*	[172]
Fucoxanthin	*Himanthalia elongate, Turbinaria triquetra* and *Laurencia obtusa*	Terpene	*L.monocytogenes, E. coli, Bacillus cereus, Bacillus subtilis, K. pneumoniae, S. aureus* and *P. aeruginosa*	[173, 174]

[a]Methicillin resistant *Staphylococcus aureus*

is **cyclopentaneacetic acid** that is able to perforate cell walls causing a rupture of the chromatin and changing the cell shape and size in microorganisms including *S. aureus* and *K. pneumoniae* [166].

Peptides are another important active group. The particularity of the peptides that makes them effective against bacteria is their amphipathic conformation. This property enables them to bind to both polar and nonpolar sites of the bacteria cytoplasmic membrane, causing changes in the cellular processes [175]. Among these peptides, **lectins** are very promising as antibacterial agents. Thus, lectin isolated from the red algae *Solieria filiformis* has shown "in vitro" antibacterial activity against Gram-negative and Gram-positive microorganisms, including *P. aeruginosa*, *E. aerogenes*, *S. marcescens*, *S. typhi*, *K. pneumoniae*, and *Proteus* sp. [169].

Phlorotannins also have important antibacterial activity. The mechanisms used by phlorotannins to inhibit bacterial growth are by the inhibition of oxidative phosphorylation and by binding with bacterial proteins and cell membrane, leading to cell lysis. They have been shown to be more effective against Gram-positive microorganisms. One phlorotannin, **phlorofucofuroeckol-A** (with low molecular weight), has high antibacterial activity against MRSA. It suppresses the expression of the *mec*I, *mec*R1, and *mec*A genes, responsible for resistance to methicillin. The repression of these genes causes a suppression of penicillin-binding protein 2a production [170].

Polysaccharides are compounds formed by repeated monosaccharide units linked by glycosidic bonds. Their antibacterial activity consists of increasing the permeability of the cytoplasmic membrane, protein leakage, and binding of bacterial DNA [176]. Among these polysaccharides, **fucoidan** and laminanin are able to inhibit the growth of *S. aureus* and *E. coli* as well as the biofilm formation of *Helicobacter pylori* [171, 172].

Terpenes are compounds formed by repeated isoprene units. Among them, **xanthophyll** is produced by diatoms and has been reported to be active against bacteria. One of the most important terpenes of this group is **fucoxanthin** (Fig. 1.11) [173, 174] which inhibits the growth of *L. monocytogenes*, *E. coli*, *Bacillus cereus*, *B. subtilis*, *K. pneumoniae*, *S. aureus*, and *P. aeruginosa*.

Finally, **lactones** are cyclic esters of hydroxycarboxylic acids. The most frequently studied group of lactones are furanones. The red alga *Delisea pulchra* produces **furanones** that are delivered to the surface at concentrations able to regulate bacterial colonization. Thus, furanones have been used as inhibitors of bacterial- and macro-fouling. Furanones inhibit bacterial colonization and biofilm development through interference with a key bacterial quorum-sensing pathway, the acylated homoserine lactone regulatory system in Gram-negative bacteria. They also interfere with the alternative AI-2 signaling system in Gram-negative and Gram-positive bacteria.

Some of these secondary marine metabolites enhance the antibacterial activity of antibiotics used in clinical practice to fight against pathogenic bacteria. Thus, fucoidan, together with ampicillin or gentamicin, decreases the MIC and the minimal bactericidal concentration (MBC) of both antibiotics fourfold. Marine alginate-derived

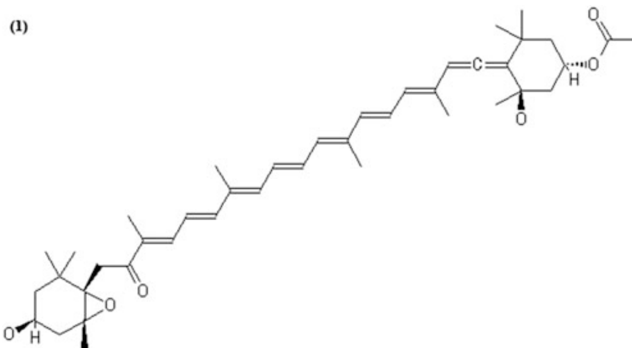


Fig. 1.11 Chemical structure of fucoxanthin, antimicrobial agent which inhibits a large range of Gram-positive and Gram-negative bacteria

oligosaccharide (ADO) together with azithromycin decreases the MIC of the azithromycin 2.8-fold, from 152.6 μg/ml to 54.2 μg/ml [92, 93].

12 Conclusions

Despite the enormous diversity of our oceans and seas and the different bioactivities found, very few new antibiotics obtained from marine organisms are currently on the market.

Much research remains to be done in this regard if we are to find new antibiotics from among marine microorganisms such as microalgae. The integration of microbiology with chemistry will be an important tool to advance in the successful search for new marine drugs.

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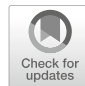
Chapter 2
Seaweeds: Valuable Ingredients for the Pharmaceutical Industries



Evi Amelia Siahaan, Ratih Pangestuti, and Se-Kwon Kim

1 Introduction

Substantial research efforts are being devoted to the identification and characterization of novel bioactive materials with versatile properties and function that might help prevent chronic disease or optimize health. The marine ecosystem has the potential to supply high added value ingredients that exhibit multiple activities, and algae are promising organisms for providing both essential compounds for human nutrition and novel bioactive substances with medicinal and pharmaceutical value [1], suited for developing nutraceuticals, functional foods, and ingredients in pharmaceutical industries [2].

Marine macroalgae or sometimes referred as seaweeds are known for their richness in bioactive substances like polysaccharides, pigments, polyphenols, peptides, minerals, and certain vitamins. Hence, seaweed or seaweed-derived bioactive substances possess great potential as valuable ingredients in pharmaceuticals. As an example, since the last few decades, seaweed-derived polysaccharides have increasingly been used as biomaterials for various pharmaceutical purposes.

E. A. Siahaan
Research and Development Division of Marine Bio-Industry, Indonesian Institute of Sciences (LIPI), North Lombok-NTB, Republic of Indonesia

R. Pangestuti
Research Center for Oceanography, Indonesian Institute of Sciences (LIPI), Ancol-Jakarta, Republic of Indonesia

S.-K. Kim (✉)
Department of Marine-Bio Convergence Science, Specialized Graduate School Science and Technology Convergence, Pukyong National University, Busan, Republic of Korea
e-mail: sknkim@pknu.ac.kr

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2 Polysaccharides of Seaweeds

Polysaccharides are multiple sugar molecules that linked together by glycosidic bonds, and they have numerous commercial applications in food, cosmetic, and biomedical products [3–6]. Polysaccharides have many unique properties and are well known as the most extensively exploited chemicals extracted from seaweeds. Seaweeds have low lipid, high carbohydrate, and more dietary fibers. Seaweeds can be classified into three main groups based on pigmentation: brown algae, red algae, and green algae. Botanists refer to these groups as *Phaeophyta, Rhodophyta, and Chlorophyta* [7]. They contain high amounts of polysaccharides ranged from 4 to 76% of their dry weight [2], notably cell wall structural, also mucopolysaccharides and storage polysaccharides [8, 9]. The cell wall polysaccharides of seaweeds mainly consist of cellulose, hemicelluloses, and neutral polysaccharides, which are concluded to physically support the thallus in water. The cell wall and storage polysaccharides are species specific. The major cell wall sulfated polysaccharides in green seaweeds are ulvans, those in red seaweeds are agarans and carrageenans, and those in brown seaweeds are alginates and fucoidan as well as the storage polysaccharide laminaran [9–12]. Seaweeds polysaccharides are unbranched polysaccharides obtained from the cell membranes of some species of seaweeds, largely used as gelatin and thickener in the food industry and as a gel for electrophoresis in molecular biology and biochemistry [13]. Chemically, it is constituted by galactose sugar molecules; it is the primary structural support for algae's cell walls. The field of natural polysaccharides of seaweeds is already large and expanding. Over the past few years, medical and pharmacological industries have shown an increased interest in seaweed-derived polysaccharides. Due to the wide variations in their molecular weights, structural parameters, and physiological characteristics, seaweeds polysaccharides show diverse pharmacological activities. Exploration of seaweeds polysaccharides for drug delivery applications is still in its infancy. Further, seaweed-derived polysaccharides can be converted into nanoparticles and exhibit suitable particle size, high drug encapsulation, and sustained drug release with high biocompatibility, thereby demonstrating their high potential for safe and efficient drug delivery [14, 15]. Further, it has been reported that bioactive compounds present in seaweeds can act as both metal-reducing agents and capping agents during the formation of stable nanoparticles [16].

2.1 Ulvan

Among polysaccharides isolated from green algae, ulvans are the greatest component of the cell walls and the most attracted constituent for its physiochemical and biological features. The name of ulvan was actually derived from the original terms of ulvin and ulvacin introduced by Kylin with reference to different fractions of *Ulva lactuca* water-soluble sulfated polysaccharides. Nowadays, it is being used to refer

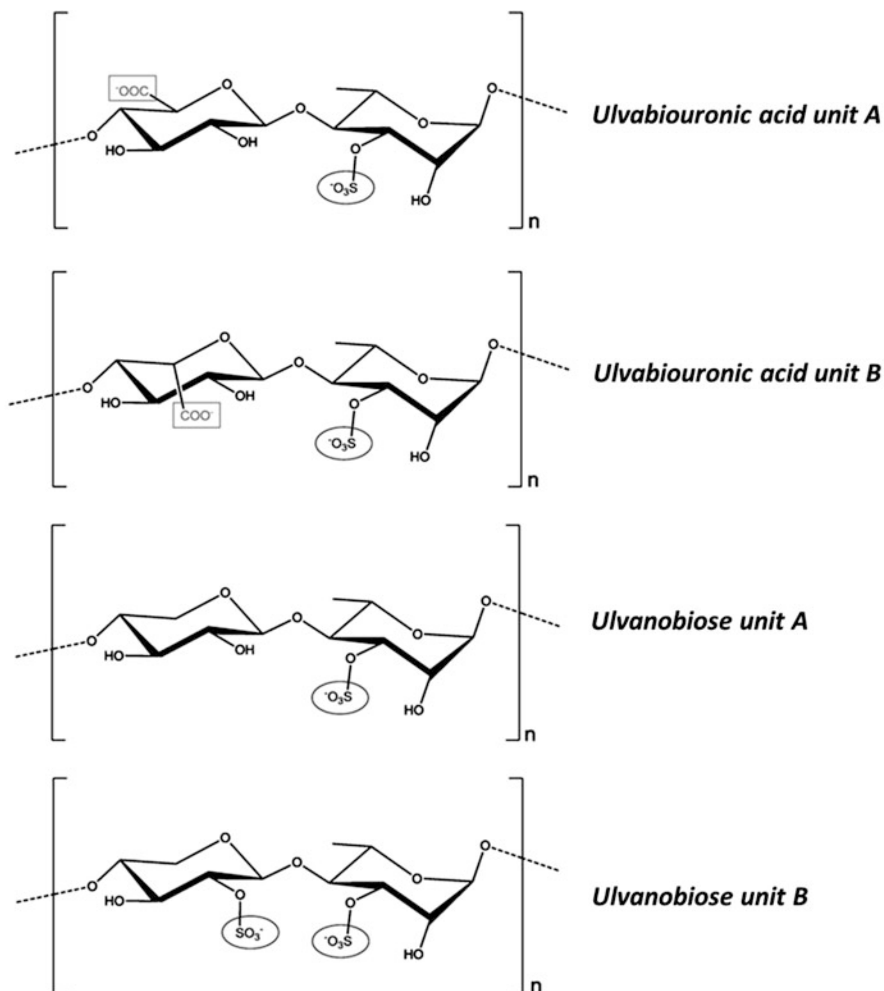

Fig. 2.1 Chemical structure of disaccharide units of ulvabiouronic acid and ulvabiose in ulvans

to polysaccharides from members of the Ulvales, mainly, *Ulva* sp. The isolated yield of ulvan represents 8–29% of algae dry weight [17].

Ulvans are constituted by a central backbone of disaccharide units formed by an L-rhamnose 3-sulfate linked to (1) a D-glucuronic acid residue (ulvabiouronic acid unit A), (2) an L-iduronic acid residue (ulvabiouronic acid unit B), (3) a D-xylose 4-sulfate residue (ulvabiose unit A), and (4) a D-xylose residue (ulvabiose unit B) (Fig. 2.1). The average molecular weight of ulvans ranges from 189 to 8200 kDa [18]. Generally, ulvans are a source of sugars for the synthesis of fine chemicals. Several studies reported that sugar composition of ulvans is extremely variable, being the most frequent rhamnose (16.8–45.0%), xylose (2.1–12.0%), glucose (0.5–6.4%), glucuronic acid (6.5–19.0%), and iduronic acid (1.1–9.1%). Mannose,

galactose, and arabinose have also been found in ulvan from some *Ulva* species [19–27]. In particular, they are a potential source of iduronic acid, the only occurrence of this rare uronic acid in plants [28].

Rhamnose, a major component of ulvans, is a rare sugar, used as a precursor for the synthesis of aroma compounds. Combinatorial libraries in glycopeptide mimetics are another example of the use of L-rhamnose in the pharmaceutical industry. The production of rhamnose from *Monostroma* sp, a Japanese species of Codiales, has been patented. Rare sulfated sugars such as rhamnose 3-sulfate and xylose 2-sulfate are also of interest [17].

The variant sugar of ulvan can have methodological, taxonomic, and/or ecophysiological origins. Yet, it is difficult to accurately specify the sugar constituents of ulvan for the aldobiouronic linkage is particularly resistant to acid hydrolysis and iduronic acid is partially destroyed during acid hydrolysis [25, 29–31].

2.2 Agar and Carrageenan

Agars and carrageenans are abundant galactans obtained by water extraction from cell walls and intercellular matrix of red seaweeds. These sulfated polysaccharides are great commercial products that have been widely used in the food industry because of their rheological features as gelling and thickening agents. Red seaweed galactans have a linear backbone consisting of disaccharide repeating fragments with alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues. Based on their stereochemistry, specifically galactans with 4-linked α-galactose residues of the L-series are termed agar, and those of the D-series are termed carrageenans [32].

Agar is hot water-soluble and gel-forming galactan at concentrations as low as 0.5% which could be obtained from algae species of the Rhodophytan families Gelidiaceae and Gracilariaceae [33]. It is a mixture of polysaccharides containing agaropectin and agarose, with similar structure and function properties as carrageenan [34, 35]. Agaropectin is a sulfated nongelling fraction [36]. Agarose is a major gelling fraction that is composed of repeating D-galactose and 3,6-anhydro-L-galactose units in few combinations [37]. It is a high quality of agar, an essential material in genetic engineering and immunology. Another one of the best studied agarans is porphyran, a highly agarose substitute [38]. Porphyran can be isolated from *Porphyra* species of Rhodophyta and shows a linear backbone consisting of the 3-linked β-D-galactose and 4-linked α-L-galactose-6-sulfate or 3,6-anhydro-α-L-galactose units. The precise composition of porphyran shows seasonal and environ mental variations [39].

Carrageenans are major sulfated galactans of red seaweed cell walls exhibiting 30 to 75% of dry weight [40]. They are water-soluble polysaccharides mostly containing more sulfates than agars, and their gel-forming properties are cation-dependent [41]. Their chemical structure is a linear sulfated polysaccharide constituted by a central backbone built up of repeating disaccharide fragments with

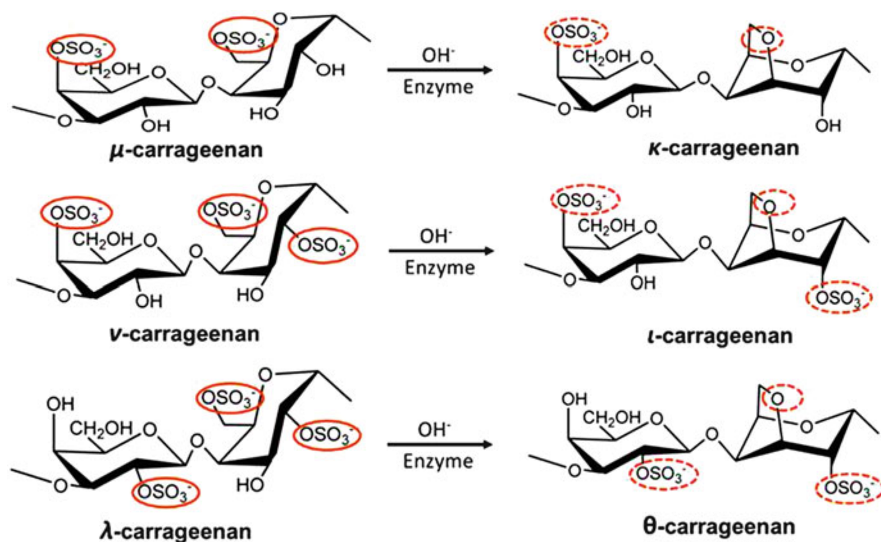

Fig. 2.2 Chemical structure of repeating disaccharide units of different types carrageenans

alternating 3-linked β-D-galactopyranose (G-fragments) and 4-linked *α*-galactopyranose (D-fragments) or 3,6-anhydro-*α*-galactopyranose (AnGal-fragments) [10]. Carrageenans are classified based on their structures which are usually designed by Greek letters as shown in Fig. 2.2. There are six forms of carrageenans most commonly used in industry, namely, λ-carrageenan, ι-carrageenan, κ-carrageenan, μ-carrageenan, *ν*-carrageenan, and θ-carrageenan. The main commercial carrageenans are κ- and ι-carrageenans that being well known as gel-forming substances; μ- and *ν*-carrageenans, respectively, as biogenetic precursors of κ-and ι-carrageenan; λ-carrageenans are as the polysaccharides that give aqueous solutions of very high viscosity; and θ-carrageenans are formed from λ-carrageenans by alkaline elimination [42–44]. Carrageenan is widely used than agar as emulsifier and stabilizer in a number of food products especially dairy products.

2.3 Alginate, Fucoidan, and Laminaran

Alginates, also called alginic acids or algins, are abundant polysaccharides in brown seaweeds mainly extracted from *Laminaria hyperborea*, *L. digitata*, *L. japonica*, *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Ecklonia maxima*, *Lessonia nigrescens*, *Durvillaea antarctica*, and *Sargassum* sp. by treatment with aqueous alkali solutions, typically with NaOH, and comprise up to 40–44% of dry matter [45, 46]. They are complex, linear block copolymers of two (1-4)-linked uronic acid residues, β-D-mannuronic (M) and α-L-guluronic (G) acids. The monomers can occur

in homopolymeric M blocks, homopolymeric G blocks, or heteropolymeric random MG blocks [47]. The M/G content and block sequence/length of alginate depend on the species, the part of the thallus, and the harvest period of algae [48, 49]. Alginate is present in the cell walls and intercellular matrix as mixed salt with different cations, such as Na^{+}, K^{+}, Mg^{2+}, and Ca^{2+}. The calcium and magnesium salts do not dissolve in water; the sodium salt does [50]. The composition (i.e., M/G ratio), sequence, G-block length, and molecular weight are thus critical factors affecting the physical properties of alginate and its resultant hydrogels. It reported that only the G-blocks of alginate are believed to participate in intermolecular cross-linking with divalent cations to form hydrogels [51]. The functionalities and applications of alginates depend on their compositions and chemical structures.

Fucans are sulfated polysaccharide group that are constituted of a fucose backbone with less than 10% of other monosaccharides, such as galactose, mannose, xylose, uronic acids, and acetyl groups. One of the best studied fucans from brown algae is fucoidan, which was first isolated by Kylin in 1913 [52]. Fucoidans 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 [50, 53]. Fucoidan is viscous in very low concentrations and susceptible to breakdown by diluted acids and bases. Fucoidans are present complex, heterogeneous polysaccharides of algal origin and can vary even within the same species. The heterogeneity in structures, substituents, sulfatation, and type of linkages is affected by the species, the part of the thallus, and the harvest period [54–56]. Fucoidan isolated from brown algae *Fucus vesiculosus*, *Ascophyllum nodosum*, *Sargassum kjellmanianum*, *Sargassum thunbergii*, *Cladosiphon okamuranus*, *Undaria pinnatifida*, *Laminaria saccharina*, *L. digitata*, and *Analipus japonicus*, in which the percentage of L-fucose ranged from 12.6 to 36.0% and the percentage of sulfate content from 8 to 25% [57].

Laminaran is the storage polysaccharides of brown seaweeds comprising up to 35% of dry biomass [58] and can be isolated in huge numbers from species belonging to *Laminaria/Saccharina* and *Alaria* to a lesser extent from species belonging to *Ascophyllum*, *Fucus*, and *Undaria*. Their content in the algae is highly dependent on the species and environmental conditions. Laminaran is a small glucan of 5 kDa and presents either insoluble or water-soluble form. Structurally, this glucan is a linear polysaccharide containing large amounts of sugars and a low fraction of uronic acids linked by β-1,3-glycosidic bonds and β-1,6-glycosidic bonds. Two types of polymeric chains are present in laminaran, G-chains with glucose at the end and M-chains with mannitol as the terminal reducing end [59].

2.4 *Bioactivity and Pharmaceutical Value of Seaweeds Polysaccharides*

Over the past two decades, various sulfated polysaccharides extracted from seaweeds have gained much attention in functional food, cosmeceutical, and

pharmaceutical application due to their biological and physicochemical features. Nowadays the field of polysaccharide biotechnology is becoming larger and expanding steadily regarding to the positive health effects of sulfate groups in the polysaccharides [60]. Numerous studies have been reported about in vitro and in vivo beneficial biological effects of seaweed-derived sulfated polysaccharides as antiviral, antibacterial, anticoagulant, anticancer, anti-inflammatory, and their immunomodulating activities [61–65]. Table 2.1 presented a summary of bioactivities of seaweed-derived sulfated polysaccharides according to the group of macroalgae, Phaeophytes, Rhodophytes, and Chlorophytes.

2.4.1 Antiviral and Antibacterial Activities

A review on the antiviral activity of seaweed sulfated polysaccharides against several kinds of virus was well recorded by Wijesekara [60] and Ahmadi [155]. The potential antiviral activity of seaweeds polysaccharides was first shown by Gerber [156] who investigated that the polysaccharides isolated from *Gelidium cartilagineum* (Rhodophyceae) protected the embryonic eggs against influenza B or mumps virus. The ability of seaweed sulfated polysaccharides to inhibit enveloped virus multiplication including herpes simplex virus (HSV), human immunodeficiency virus (HIV), human cytomegalovirus, dengue virus, and respiratory syncytial virus is well established [95, 157–159]. Another way of exerting their activity is by blocking the attachment of virions to the host cell surfaces [160]. The chemical structure including the degree of sulfation, molecular weight, constituent sugars, conformation, and dynamic stereochemistry seems to play an important role in the antiviral properties of seaweeds sulfated polysaccharides [10, 161]. In addition, both the degree of sulfation and the distribution of sulfate groups on the constituent polysaccharides play an important role in the antiviral activity of these sulfated polysaccharides. Seaweeds polysaccharides with low degrees of sulfation are generally inactive against viruses [157]. It was discovered that carrageenans are selective inhibitors of several enveloped and non-enveloped viruses and act predominantly [162, 163]. In addition, they are also extremely effective against a range of sexually transmitted human papillomavirus (HPV) types that lead to cervical cancer and genital warts [164, 165]. Carlucci [166] found that λ-type carrageenan is active against the replication of HSV upon its firm interaction that leads to inactivation of HSV virion. They also discovered that the λ-carrageenan and moderately cyclized μ-/ι-carrageenan isolated from *Gigartina skottsbergii* showed promising antiviral activities toward diverse strains of HSV-1 and HSV-2 during virus attachment stage [167, 168]. Similar work has been conducted by Neushul. He observed carrageenans, sulfated polysaccharides extracted from red algae, are co-internalized into infected cells with the HSV, inhibiting the virus. They also interfered with fusion (syncytium formation) between cells infected with the HIV and inhibited the specific retroviral enzyme reverse transcriptase [169]. Surprisingly, similar results were reported by different groups of researchers, who analyzed the chemical structure and antiviral activity of carrageenan (*lambda*, *kappa*, and *iota*) against HSV-2 infection [170, 171]. Moreover, other investigation was reported that

Table 2.1 Summary of bioactivities of seaweed-derived sulfated polysaccharides

Algae group	Source	Sulfated polysaccharides	Health benefit effects	References
Chlorophytes	*Caulerpa* spp.	Galactan	Antioxidant, anticoagulant, antithrombotic, antiviral, anti-proliferative, anti-inflammatory	[66–68]
	Codium spp.	Arabinogalactan	Anticoagulant, antithrombotic, antiviral	[66, 69]
	Monostroma nitidum	Rhamna	Antithrombotic, anticoagulant, antitumor, hepatoprotective, immunomodulator	[70–73]
	Enteromorpha prolifera	Ulvan	Immunomodulator, antioxidant, hypolipidaemic	[74–77]
	Ulva spp.	Ulvan	Anti-adhesive, antiproliferative, hepatoprotective	[17, 78]
	Ulva lactuca	Galactan	Antioxidant, anti-prolliferative, hypocholesterolaemic, hepatoprotective, antitumor, antiviral, anti-inflammatory, antinociceptive	[64, 79–85]
	Ulva pertusa	Ulvan	Antioxidant, hypotriglyceridaemic, decrease LDL-cholesterol, and increase HDL-cholesterol, immunostimulatory	[64, 70, 71, 86, 87]
Rhodophytes	*Porphyra* spp.	Galactan porphyran	Antitumor, hypotensive, regulates, blood cholesterol	[88, 89]
	Porphyra yezoensis	Porphyran	Antitumor, immunomodulatory, hypolipidaemic	[88, 90–92]
	Gloiopeltis complanata	Agaran	Antiviral	[89]
	Aghardiella tenera	Agaran, galactan	Antiviral	[93, 94]
	Chondrus crispus	λ-carrageenan	Antiviral, anticoagulant, antithrombotic	[95–97]
	Euchema cottonii	Galactan	Antioxidant	[66]
	Euchema spinosa	κ-carrageenan	Anticoagulant, antithrombotic	[11, 96, 97]
	Gracilaria corticata	Agaran, galactan	Antiviral	[98]
	Schizymenia pacifica	λ-carrageenan	Antiviral	[96, 97, 99]
Phaeophytes	*Dictyota cervicornis*	Fucan	Anticoagulant, antioxidant, anti-proliferative	[66, 100]
	Dictyopteris delicatula	Heterofucan	Anticoagulant, antioxidant, antitumor, anti-proliferative	[66, 101]
	Cladosiphon okamuranus	Fucan	Anti-proliferative, antiviral, anti-inflammatory, antiadhesive, antitumor, immunomodulator, angiogenic,	[102–110]

			gastroprotective, cardioprotective, restenosis preventive	
	Ascophyllum nodosum	Fucan, laminaran	Immunomodulatory, anti-inflammatory, anticoagulant, antithrombotic, anti-metastatic, antitumor, antiadhesive, restenosis preventive, angiogenic, antibacterial	[60, 102, 111–120]
	Fucus spp.	Fucan	Immunostimulant, antiviral, antitumor, antiproliferative, antiadhesive, anticoagulant, antioxidant, anti-metastatic, anti-inflammatory, anti-angiogenic	[66, 102, 121–129]
	Ecklonia cava	Fucan	Anti-proliferative, antitumor, anticoagulant, antioxidant, antithrombotic, anti-inflammatory	[130–136]
	Fucus sp.	Laminaran	Antitumor, decreases liver triglyceride, cholesterol and phospholipid levels; serum hypocholesterolaemic, hypotensive, antibacterial, immunomodulator anticoagulant	[115, 118, 120]
	Saccharina sp.	Laminaran	Antitumor, anticoagulant, decreases liver triglyceride, cholesterol and phospholipid levels; serum hypocholesterolaemic, hypotensive, antibacterial, immunomodulator	
	Eisenia bicyclis	Laminaran	Antitumor	[79, 131, 137, 138]
	Sargassum horneri	Fucoidan	Antitumor, antiviral	[121, 139]
	Sargassum patens	Fucoidan	Antiviral	[140]
	Saccharina japonica	Galactofucan	Anti-lipidaemic, increases HDL, antiviral, antitumor, immunomodulator, antioxidant neuroprotective	[102, 141–147]
	Undaria pinnatifida	Fucoidan, galactofucan	Antiviral, anticoagulant, antitumor, anti-proliferative, immunomodulatory, anti-inflammatory	[102, 128, 141, 148–154]

polysaccharides isolated from two red algae *Sphaerococcus coronopifolius* (Gigartinales, Sphaerococcaceae) and *Boergeseniella thuyoides* (Ceramiales, Rhodomelaceae) containing λ-carrageenans inhibited in vitro replication of the HIV at 12.5 μg/mL and HSV-1 on Vero cell values of EC_{50} of 4.1 and 17.2 μg/mL, respectively. The sulfated polysaccharides from *S. coronopifolius* and *B. thuyoides* inhibit HSV-1 and HIV-1 replication in vitro at concentrations that have no effect on cell viability [172].

Fucoidans extracted from marine brown seaweeds possess some biological activities, and fucoidans show the antiviral activity against many RNA and DNA viruses both in vivo and in vitro, including important human pathogens such as HIV, HSV1-2, dengue virus, and cytomegalovirus [159, 173]. Fucoidans have the ability to block the interaction of viruses to the cells so as to inhibit viral-induced syncytium formation [157]. Fucoidan isolated from several seaweed species, *Undaria pinnatifida*, Splachnidium rugosum, *Gigartina atropurpurea*, *Plocamium cartilagineum*, *Adenocytis utricularis*, *Stoechospermum marginatum*, and *Cystoseira indica*, exhibited potential antiviral effects against HSV-1 and HSV-2 [67, 68, 149, 161, 174–177]. Furthermore, fucoidan from the marine alga *Cladosiphon okamuranus* (Phaeophyceae) potently inhibits dengue virus type 2 infection [173], and it was confirmed that virus particles bound exclusively to fucoidan, indicating that fucoidan interacts directly with envelope glycoprotein on the virus. Hence, this could be developed as a potential inhibitory agent against the dengue virus. Ulvan has also been studied for antiviral activity in vitro against a number of human and avian influenza viruses. This work has been reported by Ivanova [178]. Ulvan polysaccharides isolated from green algae had good inhibitory effect on influenza A virus, the inhibition effect being dose-dependent and strain-specific. Likewise, ulvan has been shown to have high and specific activity against herpes simplex virus [179].

Sulfated polysaccharides from seaweeds, such as ulvan, alginates, fucoidans, and laminaran, have been confirmed to have antibacterial activity against *Escherichia coli* and species from *Staphylococcus*. A fucoidan from *L. japonica* and sodium alginate were found to inhibit *Escherichia coli* [180]. Laminaran from *Fucus*, *Laminaria*, and *U. pinnatifida* demonstrated to have an effect on pathogenic bacteria [115].

2.4.2 Anti-inflammatory and Immunomodulatory Activities

Seaweed-derived sulfated polysaccharides possess immunomodulatory activities. These activities may be potential to stimulate the immune response or to control immune cell activity to mitigate associated negative effects such as inflammation [181]. The immunostimulating effect of sulfated polysaccharides is mainly based on macrophage modulation. Macrophages are the residence of immune system which play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines. Therefore, seaweed-derived sulfated polysaccharides are also known to have promising anti-inflammatory activities [182]. One of

the interests in seaweed-derived sulfated polysaccharides as anti-inflammatory agents is the growing body of evidence illustrating their ability to interfere with the migration of leukocytes to sites of inflammation. For example, in a rabbit model of bacterial meningitis, leukocyte rolling was markedly reduced by intravenous infusion of fucoidan isolated from brown algae *F. vesiculosus* [183]. Fucans from other brown seaweeds including *Laminaria* spp., *Fucus* spp., *A. nodosum*, and *C. okamuranus* also inhibit leukocyte recruitment to the abdominal cavity during acute peritonitis in rats [102].

Some other works described the interaction of the anti-inflammatory activity with the immunomodulatory ability of seaweeds sulfated polysaccharides. They suggest that they may have utility in influencing innate immunity to reduce the pro-inflammatory state. This seems to be the case in the work by Li [184, 185] who confirmed the anti-inflammation mechanism in vivo via the immunomodulatory system in vivo, since the fucoidan from *L. japonica* reduced the inflammation of rats' myocardium damaged cells, by inactivating the cytokines HMGB1 and NF-κB, two groups of proteins secreted by the immune cells during inflammatory diseases. These protective and regenerative effects of fucoidans (from *A. nodosum*), via the immunomodulatory system, were also verified in the destruction/proteolysis of connective tissue by Senni [186].

Sulfated polysaccharides collected from red algae, carrageenan, are also known to be a potent inflammatory agent in rodents and prime mice leukocytes to produce tumor necrosis factor-α (TNF-α) in response to bacterial lipopolysaccharides [187]. Sulfated polysaccharides extracted from marine green alga *Ulva rigida* have induced a more than twofold increase in the expression of several chemokines and interleukins and also induced nitrite production. *U. rigida* sulfated polysaccharides can stimulate macrophage secretion of prostaglandin E2 (PGE2) and induce an increase in cyclooxygenase-2 (COX-2) and nitric oxide synthase-2 (NOS-2) expression. This suggested that their potential in clinical applications for modifying certain macrophage activities in diseases where macrophage function is impaired or needs to be boosted. However, sulfated polysaccharides may have potential biomedical applications in stimulating the immune system or in controlling macrophage activity to reduce associated negative effects [62].

2.4.3 Anticoagulant and Antithrombotic Activities

Blood coagulation (clotting) is a process that converts specific circulating elements of the blood system into a gel, forming a blood clot in order to stop the flow of blood through the injured vessel wall whenever. As endogenous or exogenous anticoagulants interfered with the coagulation factors, the blood coagulation can be stopped [188, 189]. Heparin (glycosaminoglycan) has been familiar and the most commonly used as a commercial anticoagulant/antithrombotic medicine. However, heparin treatment has been reported to cause serious side effects such as development of thrombocytopenia, hemorrhagic effect, ineffectiveness in congenital or acquired antithrombin deficiencies, and incapacity to inhibit thrombin bound to fibrin

[190]. Due to several side effects of heparin, discovering alternative sources of safer anticoagulant/antithrombotic drugs is needed. Investigation of blood anticoagulant properties from various marine brown algae has been carried out. As resulted, sulfated polysaccharides derived from seaweeds are identified to be alternative sources in producing a new anticoagulant/antithrombotic drug [191]. It has been reported that two types of sulfated polysaccharides possess high anticoagulant activity including sulfated fucoidans from marine brown algae and carrageenan from marine red algae [112, 167]. However, some other sulfated polysaccharides from green seaweeds also showed potent anticoagulant properties, but their mechanisms of action are associated not only to a direct increase in the clotting time by inhibiting the contact activation pathway (intrinsic pathway) but also by inhibiting the heparin cofactor II-mediated action of thrombin [192, 193], this showing a potent antithrombotic bioactivity. In addition, Maeda and colleagues [194] have revealed that the anticoagulant sulfated polysaccharides from the green alga *Monostroma nitidum* yielded a sixfold higher activity than that of heparin. In comparison, marine brown algae extracts exhibit higher anticoagulant activity than red and green algae extracts [53, 112].

2.4.4 Anticancer Activities

Many studies have reported that seaweed-derived polysaccharides have antiproliferative activity in cancer cell lines in vitro, as well as inhibitory activity of tumor growth in mice. Seaweed-derived polysaccharides also possess antimetastatic activity by blocking the interactions between cancer cells and the basement membrane. Seaweed-derived polysaccharides inhibit tumor cell proliferation and tumor cell adhesion to various substrates. Most recently, Palanisamy et al. [195] purified fucoidan from *Sargassum polycystum*. Fucoidan possesses antiproliferative effect on human breast cancer cell lines (MCF-7). Anticancer activity of fucoidans has been reported to be closely related to their sulfate content and molecular weight. It is suggested that anticancer activity of fucoidans could be significantly enhanced by lowering their molecular weight only when they are depolymerized at mild conditions [60]. Furthermore, polysaccharides from seaweeds are known to be important free radical scavengers and antioxidants for the prevention of oxidative damage, which is an important contributor in carcinogenesis.

3 Pigments of Seaweeds

In recent years, seaweeds have served as an important source of bioactive natural substances. Among functional ingredients identified from seaweeds, pigments have received particular attention. Pigments are widespread, not only in seaweeds but also

in all living matters such as mammals, fungi, and invertebrates [196]. However, seaweeds are potential sources of non-animal pigment. Algae contain different types of pigments, which are specific to particular groups as previously described. Four basic classes of pigments found in seaweeds are chlorophylls, carotenoids, xanthophylls, and phycobilins. The green algae include primarily chlorophylls a and b and the brown algae are characterized by a high content of fucoxanthin and the red algae by a high content of phycoerythrin and phycocyanin (phycobilins).

Chlorophylls occur in algae in the highest concentrations among other pigments and are able to absorb light in the red and blue regions and, as a result, to emit a green color. Therefore, these compounds are used as natural coloring agents [197]. There are four kinds of chlorophyll found in seaweeds; the first and most important is chlorophyll a which absorbs most energy from wavelength of violet-blue and orange-red light [2]. These pigments are essential molecules for photosynthesis, by passing their energized electrons onto molecules which will manufacture sugars. The second and third chlorophylls are chlorophylls b and c, and the fourth type of chlorophyll is chlorophyll d which was found in red algae [198].

Nowadays, most of the studies involving seaweeds pigments are focused on carotenoids as commercially relevant bioactive compounds. They represent photosynthetic pigment in red, orange, or yellow wavelength. Carotenoids are lipophilic isoprenoid molecules, which are classified as carotenes (β-carotene, α-carotene, lycopene) and xanthophylls (lutein, astaxanthin, zeaxanthin, violaxanthin, loroxanthin, fucoxanthin) [199]. Carotenoids play an important role as an essential constituent of photosynthetic apparatus, in the reaction center of photosystems where they act as (1) accessory pigment for light harvesting processing during photosynthesis, (2) structural stabilizers for protein assembly in photosystems, and (3) inhibitor for photo or free radical oxidation by excess light exposure [200].

The most abundant carotenoid with provitamin function in seaweed is carotene. It can be cleaved by a β-carotene-15,15′-dioxygenase, resulting in the formation of retinal [201, 202]. Fucoxanthin, β-carotene, and violaxanthin are found in brown seaweed. The main carotenoids of red seaweed are α- and β-carotene and their derivates such as zeaxanthin and lutein. Green algae have a similar composition of carotenoids in comparison with higher plants. The main part of carotenoids of brown seaweed is formed by β-carotene, lutein, violaxanthin, anteraxanthin, zeaxanthin, and neoxanthin [203]. Different seaweeds have different carotenoid pigments like green seaweed species have β-carotene, lutein, violaxanthin, neoxanthin, and zeaxanthin, while red seaweeds contain mainly α- and β-carotene, lutein, and zeaxanthin. In brown seaweeds β-carotene, violaxanthin, and fucoxanthin are present [204]. One very visible carotenoid in algae is the brown pigment which colors kelps and other brown seaweeds as well as the diatoms. Fucoxanthin is one of the most abundant carotenoids contributing around 10% estimated total production of carotenoids in nature [205]. Besides their role in photosynthetic and pigmentation effects, pigments have also been reported to provide health benefits such as antioxidant, anticancer, anti-inflammatory, anti-obesity, and neuroprotective activities.

3.1 Antioxidant Activity of Seaweeds Pigments

The propagation of (per)oxidative reactions spread out by the reaction of the new formed products or free radicals with other chemical species, including peroxyl and alkoxyl radicals, which in turn can go on attacking lipids and other biomolecules. Free radicals are generated during cell metabolism, and their levels may be increased by pro-oxidants. They need to receive an electron to pair with the unpaired electron. When these important chemical reactive species are overproduced in vivo, they can cause an imbalance between ROS (reactive oxygen species) and RNS (reactive nitrogen species) and their elimination by the endogenous antioxidant, including enzymes (e.g., catalase) and glutathione (GSH), which are part of the cell defense mechanisms. Many studies reported that ROS can attack lipids, proteins, and DNA, forming lipid radicals, amino and thiyl radicals, and sugar- and base-derived radicals that, in turn, can be oxidized into peroxyl radicals (Table 2.2) [206]. It is known that ROS are associated with a wide range of diseases, such as aging, Alzheimer's, Parkinson's, atherosclerosis, cardiovascular disease (CVD), cancer, inflammatory and neurological diseases, diabetes, and obesity, among others [207–209]. RNS are also involved in the peroxidation of lipids and nitration of proteins, as well as in the formation of many other reactive species, such as malondialdehyde (MDA), a marker of oxidative stress. Therefore, antioxidants have been widely used to conquer oxidation and peroxidation process, especially natural antioxidant which has no potential health hazards. Recently, there is a considerable interest in the food as well as pharmaceutical industry for the development of antioxidants from natural sources, such as marine flora and fauna. Among marine resources, seaweeds represent one of the richest sources of natural antioxidants. One of the attractive antioxidant features in algae is their pigments [210–212].

Pigments not only function as colorants but they also contribute to the antioxidant activity of seaweeds. Antioxidant activities of algae pigment have been determined by various methods such as ferric thiocyanate (FTC), thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,20-azinobis-3 ethylbenzothiazoline-6-sulfonate (ABTS) radical scavenging effect, singlet oxygen quenching activity, lipid peroxide inhibition, superoxide radical, and hydroxyl radical scavenging assays.

Some research studies reported that chlorophyll a and related compounds derived from brown algae have antioxidant activities in methyl linolenate systems and exhibit antioxidant activity in the dark because of its porphyrin ring structure

Table 2.2 Free radical species causing oxidative stress

Group of free radical species	Species
ROS	Superoxide ($O_2^{\bullet -}$), Hydrogen peroxide (H_2O_2), Hydroxyl radical ($HO^{\bullet}$), Singlet oxygen ($^1O_2$), Ozone (O_3)
RNS	Nitrous oxide ($NO^{\bullet}$), Peroxynitrate ($ONOO^{\bullet}$), Nitrogen dioxide (NO_2^{-})
Lipid hydroperoxides	Lipid radical ($L^{\bullet}$), Lipid peroxyl radical ($LO_2^{\bullet}$)

[213–215]. Recently, Cho et al. [152, 153] suggested that strong antioxidant activity of the *Enteromorpha prolifera* was caused by chlorophyll a derivatives, pheophorbide a, rather than phenolic compounds. According to Cahyana et al. [216], pyropheophytin a of *Eisenia bicyclis* is a more potent antioxidant than commercial antioxidants such as α-tocopherol and butylated hydroxytoluene (BHT).

Carotenoids have been implicated as important dietary nutrients having antioxidant potential, being involved in the scavenging of reactive oxygen species (ROS), singlet molecule oxygen ($^1O_2$), and peroxyl radicals generated in the process of peroxidation. Further, they are effective deactivators of electronically excited sensitizer molecules which are involved in the generation of radicals and singlet oxygen [217, 218].

Yan et al. [219] identified that fucoxanthin was the major antioxidant of Hizikia fusiformis, and the radical scavenging activity was investigated. It showed that fucoxanthin has a strong radical scavenging activity. The potential involvement of fucoxanthin in radical scavenging activity may correlate to the presence of unusual double allenic bonds at C-7′ position. These findings were confirmed in a recent study by Sachindra et al. [220] which isolated fucoxanthin from *Undaria pinnatifida* and prepared two fucoxanthin metabolites, fucoxanthinol and halocynthiaxanthin. The antioxidant activities of these three carotenoids were assessed by DPPH, hydroxyl radical scavenging activity, and singlet oxygen quenching activity.

Phycobilin pigments are used as antioxidants, anti-inflammatory, and radical scavenging bioactive constituents. Furthermore, phycobiliproteins are applied as food and cosmetic colorants and as fluorescent markers in biomedical research (C-phycocyanin and R-phycoerythrin) [57, 221]. Previous work has demonstrated antioxidant activity of phycoerythrobilin derived from *Porphyra* sp. [222].

These evidences suggest that seaweeds pigments prove to be one of the useful candidates in search for effective, nontoxic substances with potential antioxidant activity and could be used as a rich source of natural antioxidants with potential application in the food industry as well as cosmetic and pharmaceutical areas.

3.2 Anti-inflammatory Activity of Seaweeds Pigments

The anti-inflammatory effect of pigments is mainly based on modulation of macrophage function. Macrophages are the residents of immune cells in the innate immune system which plays an important role in the maintenance of homeostasis by changing their function according to the tissue. As the residents of the immune system, macrophages are a predominant source of pro-inflammatory mediators including nitric oxide (NO), prostaglandin E2 (PGE2), pro-inflammatory cytokines (tumor necrosis factor-α (TNF-a), interleukin-6 (IL-6), and interleukin-1b (IL-1b), and ROS [223].

Secondary metabolites derived from seaweeds are known to have promising anti-inflammatory activities [182]. However, the scientific analysis of anti-inflammatory activity of seaweed-derived pigments has been poorly carried out, and until now

only few studies were reported. For example, pheophytin a isolated from *E. prolifera* has been reported to suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide radical and inflammatory responses in mouse macrophages [224]. Fucoxanthin is recently known to be a potent anti-inflammatory agent in vitro and in vivo in responses to bacterial lipopolysaccharides (LPS). Shiratori et al. [225] reported that anti-inflammatory effect of fucoxanthin is comparable with prednisolone, a commercially available steroidal anti-inflammatory drug. Supporting those findings, Heo et al. [226] demonstrated an anti-inflammatory effect of fucoxanthin isolated from *Myagropsis myagroides* in LPS-stimulated RAW264.7 cells. Fucoxanthin treatment attenuates the productions of NO and PGE2 by inhibiting inducible NO synthase (iNOS) and cyclooxigenase-2 (COX-2) expressions. The anti-inflammatory activities of fucoxanthin were due to the suppression of nuclear factor-jB (NF-jB) and the phosphorylation of mitogen-activated protein kinases (MAPKs) [227].

3.3 Neuroprotective Activity of Seaweeds Pigments

Neurodegenerative diseases are estimated to surpass cancer and be the second most common cause of death among elderly by the 2040s [228, 229]. Many categories of natural and synthetic compounds have been reported to possess neuroprotective activities. However, these synthetic neuroprotective agents are believed to have certain side effects such as dry mouth, tiredness, drowsiness, anxiety or nervousness, difficulty to balance, etc. [230, 231]. For this reason, many investigations of discovering a safe and effective neuroprotection have been conducted by scientists. Several studies have provided insight into neuroprotective properties of seaweed-derived pigments [211, 212]. Okuzumi [232] found that fucoxanthin isolated from *H. fusiformis* inhibited N-myc expression and cell cycle progression of GOT0 cells, a human neuroblastoma cell line. Fucoxanthin at a concentration of 10 mg/ml reduced the growth rate of GOT0 cells to 38%, but its exact mechanisms of action are not yet completely understood. Ikeda et al. [233] found that wakame was able to attenuate the development of hypertension and its related diseases in stroke-prone spontaneously hypertensive rats (SHRSP). Furthermore, they isolated fucoxanthin from wakame and showed that fucoxanthin amazingly attenuated cell damage in cortical neurons during hypoxia and oxygen reperfusion [234]. Since ROS generation is considered to occur after hypoxia and reoxygenation, whereby free radicals damage neurons; it may assume that neuroprotective activity of fucoxanthin is mainly based on their scavenging activity. Neurite outgrowths are fundamental neuronal features which play an important role in neuronal development during embryogenesis and in the adult brain. Pheophytin a and its analog, vitamin B12 derived from *S. fulvellum*, have been reported to promote neurite outgrowth in pheochromocytoma (PC12) cells [235, 236]. Neurite outgrowth-promoting activity of pheophytin a has been reported to be closely related to their low molecular weight. The rationale for this is that low-molecular-weight pheophytin may incorporate into

the cells more efficiently and therefore promote neurite outgrowth effectively. Based on several findings, it may be concluded that pigments are a valuable source of neuroprotective agents and could be introduced for the preparation of novel functional ingredients in functional foods and pharmaceuticals as a good approach for the treatment and or prevention of neurodegenerative disease. Further, it can be suggested that pigments are an alternative source to synthetic ingredients that can contribute in neuroprotection. Until now, neuroprotective activities of pigments have been observed in vitro. Therefore, further researches are needed in order to investigate pigment neuroprotective activities in vivo and in human subject.

4 Phlorotannin

Phlorotannins are the only polyphenols abundantly found in brown algae and are different from terrestrial plant polyphenols which typically contained a range of different polyphenols [237]. Generally, brown algae contain phlorotannin up to 25–30% of thallus dry weight, but this varies across location and season [238]. Most of them have been extracted from species belonging to Lessoniaceae, Fucaceae, Alariaceae, Sargassaceae, Cystoseiraceae, Laminariaceae, and Ishigeaeceae. A variety of low-, intermediate-, and high-molecular-weight phlorotannins (range of 126 Da to 650 kDa) have been reported from seaweeds but more commonly found in the range of 10–100 kDa. These soluble phlorotannins are stored in membrane-bound cell organelles in the cytoplasm called physodes. When physodes fuse with the cell walls, phlorotannins form a complex with the structural polysaccharides, alginate in particular [239, 240]. These phlorotannins help to protect algae from stress conditions and herbivores, such as abalone and sea cucumber.

Phlorotannins are formed by polymerization of phloroglucinol units (1,3,5-trihydroxybenzene). The phloroglucinol subunits are linked with hydroxyl groups on the aromatic skeletons forming six major subgroups of phlorotannins (Fig. 2.3): eckols, fuhalols, fucophlorethols, phlorethols, fucols, and isofuhalos [241]. A group of fucols is formed when aromatic rings are connected purely by aryl-aryl bonds. Phlorethols are formed solely by aryl ether bonds. Fuhalols are constructed of phloroglucinol units that are connected with para- and ortho-arranged ether bridges containing one additional OH group in every third ring. When there exists at least one three-ring moiety with a dibenzodioxin element substituted by a phenoxyl group at C-4, the group is named eckols. They are usually of low molecular size and thus far have only been found in the Alarieae. Carmalols are further derivatives of phlorethols containing a dibenzodioxin moiety. Endofucophlorethols and isofuhalols are small, distinct, specialized groups.

Investigative studies of health beneficial effects from phlorotannin have been reported extensively. It has been proved that phlorotannin-rich extracts possess certain biological activities such as antioxidant, bactericide, anticancer, antidiabetic, and antiallergic.

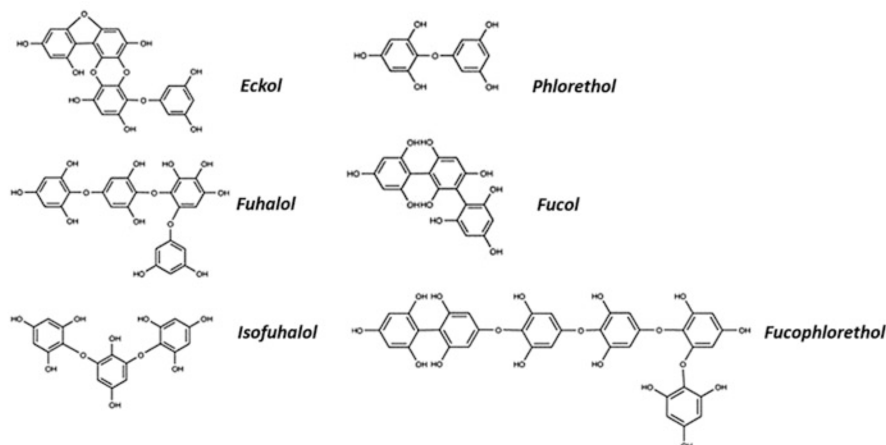

Fig. 2.3 Chemical structures of six different classes of phlorotannins

4.1 Antioxidant Activity of Phlorotannins

Numerous studies have reported that phlorotannins derived from marine brown algae have strong antioxidant activities against free radical-mediated oxidation damage [242–245]. The antioxidant activities of phlorotannin are mainly attributed to scavenging activity against superoxide and hydroxyl radicals and metal-chelating ability [246]. Phloroglucinol was found to scavenge reactive oxygen species (ROS) in hydrogen peroxide-treated HT1080 cells in a time-dependent manner [247]. It was also found to decrease the oxidation of membrane proteins of HT1080 cells (preexposed to hydroxyl radical produced by Fenton's reaction) and inhibited the oxidative damage of DNA [247]. The brown alga *Ecklonia stolonifera* collected from South Korea yielded a new phlorotannin, eckstolonol, which possessed a potent DEPP radical scavenging activity [248]. Li et al. [180] and Shibata et al. [249] demonstrated phlorotannin against 2,2-diphenyl-1,2-picrylhydrazyl (DPPH) and hydroxyl, superoxide, and peroxyl radicals using electron spin resonance method while tocopherol and ascorbic acid as positive control. The significant results showed phlorotannin presents greatly radical scavenging activities against superoxide and DPPH radicals compared to tocopherol and ascorbic acid. Several phlorotannins (phloroglucinol, eckol) which purified from brown seaweeds such as *E. cava*, *E. kurome*, *E. bicyclis*, and *H. fusiformis* are responsible for potent antioxidant activities and have shown protective effects against hydrogen peroxide-induced cell damage [250, 251]. Similarly, another research group has reported the potential antioxidant activities of three phlorotannins (phloroglucinol, eckol, and dieckol) purified from *E. cava*. Their results suggest that eckol samples scavenged around 93% of DPPH at 0.25, 0.5, and 1 mg/ml of concentrations and were higher than the other phlorotannins. Moreover protective effects of the phlorotannins against H_2O_2-mediated DNA damage increased with increased concentrations of the samples in the mouse T-cell lymphoma cell line (L5178Y-R) [252].

4.2 Bactericidal Activity of Phlorotannins

It has been identified that some synthetic preservatives and additives used in the food industry promote tumors and mutagens over long-term use [253, 254]. Therefore, exploration of novel natural antibiotics has been increased greatly, and these studies have shown that phlorotannin possesses potential antimicrobial agents that can be used in the food industry and pharmaceutical industry [255–257]. The isolated phlorotannins, such as phloroglucinol, eckol, fucofuroeckol-A, phlorofucofuroeckol-A, dioxinodehydroeckol, 8,80-bieckol, 7-phloroeckol, and dieckol, are effective against some pathogenic foodborne bacteria, antibiotic-resistant bacteria, and human tinea pedis fungus. Dieckol and 8,8-bieckol from *Ecklonia kurome* have been successfully reduced the growth of *Campylobacter jejuni* and *Vibrio parahaemolyticus* [258]. Similar work showed that phlorotannins in hexane fraction of *E. stolonifera* have effectively inhibited the growth of *Staphylococcus aureus* [259]. In addition, the MIC (minimum inhibitory concentration) values for eckol from *E. cava* indicate potent antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) in the range of 125–250 lg/mL [255]. Moreover, a phlorotannin-containing extract of brown seaweed *Ascophyllum nodosum* has been shown to reduce the prevalence of *Escherichia coli* O157:H7 in bovine feces [260]. Another research group showed that dieckol purified from *E. cava* has fungicidal activity [261]. It has shown a potent antifungal activity against *Trichophyton rubrum* associated with dermatophytic nail infections in humans.

In general, the bactericidal effect tends to increase with the degree of polymerization of phloroglucinol. The interactions between bacterial proteins and phlorotannins were considered to play an important role in the bactericidal action of phlorotannins [258, 262, 263]. Therefore it is thought that phlorotannins from brown algae could be very useful in the food and pharmaceutical industries as antibiotic agents.

4.3 Anticancer Activity of Phlorotannins

Cancer is a dreadful pathological condition indicated by abnormal cell growth and spread to other parts of the body. Cancer remains one of the high-ranking causes of death in the world though there are considerable therapeutic approaches for its management. It is considered as the second life-taking disease which is preceded by heart diseases. The formation of cancer cells in the 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. Phlorotannins from brown algae have been suggested to possess anticarcinogenic effects. Seaweeds anticarcinogenicity may involve effects on cancer cell proliferation and antioxidant activity [264]. Phlorotannin extract from *Laminaria japonica* has been reported to inhibit the growth of the human hepatocellular carcinoma cell (BEL-7402) and on murine leukemic cell (P388) effectively in a dose-dependent manner [265]. Cho et al. [266] reported that methanol extracts

of *L. japonica* dose-dependently inhibited the growth of human gastric (AGS) and HT-29 colon cancer cells. Similarly, Lin [267] reported that the anticancer activity of phlorotannins from *Sargassum thunbergii* Kuntze inhibited the growth of BEL-7420 liver cancer cells and A549 lung cancer cells. Phlorotannin composition of different *Sargassum muticum* samples collected along the European Atlantic coast has been investigated. Among other species from different locations, *S. muticum* extract from Norway which contains the richest phlorotannins showed the highest anticancer activity and a good cytotoxic potential at concentrations in the medium micromolar range [268]. Thus, it may suggest that potential involvement of *S. muticum* extracts in anticancer activity may correlate to the phlorotannin contents.

Phlorotannin derivative dioxinodehydroeckol isolated from *E. cava* has shown an excellent result on reducing the growth of MCF-7 (human breast cancer cells) via induction of the apoptosis [269]. Moreover, phlorotannin derivatives such as fucodiphloroethol G, dieckol, eckol, and phlorofucofurofuroeckol from *E. cava* have shown potent cytotoxic effect on human cancer cell lines such as human cervical adenocarcinoma cell (HeLa cell), HT1080, A549, and HT-29 [184, 185]. It has been reported that the total polyphenolic content in the algae is the key for their antiproliferative ability [270]. Green synthesis of silver nanoparticles (AgNPs) was synthesized by using extracts of marine algae *E. cava* with phlorotannins as the main components. AgNPs greatly induced apoptosis and led to the consequent anticancer effect against human cervical cancer cells [14, 15]. Collectively, these scientific reports clearly suggest the importance and the ability of phlorotannins in reducing the risk of cancer and its related diseases.

4.4 Antidiabetic Activity of Phlorotannins

Diabetes mellitus is an abnormal metabolism of glucose, due in part to resistance to the action of insulin in peripheral tissues. Diabetes mellitus is a complex disorder that is characterized by hyperglycemia. It is one of the world's most serious chronic diseases that is developing prominence amidst an increasingly obese and aging world population. Diabetes mellitus is classified into two types, type I (insulin-dependent diabetes mellitus) and type II (non-insulin-dependent diabetes mellitus). The most prevalent form of diabetes is type II which contributes for more than 90% of the diabetic population [271, 272]. The control of blood glucose levels is the key to preventing or reversing diabetic complications and improving the quality of life for hyperglycemic patients. The enzymes α-glucosidase and α-amylase play a significant role in the digestion of dietary complex carbohydrates. Inhibition of these two enzymes can prevent the progression of diabetes [273]. Several studies have been reported that phlorotannin and its derivatives have exhibited promising antidiabetic effects. These studies were conducted by investigating in vivo antidiabetic effect on diabetic mouse after feeding phlorotannin extracts. The methanolic extracts of *E. stolonifera* that are rich in phlorotannin have shown strong inhibition of α-glucosidase in type II diabetic mice [274]. Derived phlorotannin dieckol and diphlorethohydroxycarmalol (DPHC) isolated from *E. cava* and *Ishige*

okamurae have resulted in reduction of the plasma glucose level and improve insulin resistance on genetically diabetic mice [275, 276]. Moreover, Heo et al. [277] evaluated the inhibitory effect of DPHC against α-glucosidase and α-amylase on streptozotocin-induced diabetic mice. Further, DPHC may delay the absorption of dietary carbohydrates in the intestine, leading to suppression of an increased blood glucose level after a meal. Thus suggesting DPHC as a capable therapeutic candidate for treating diabetes [270]. The potent antidiabetic activities of seaweeds phlorotannins show an interesting advance in the search for novel functional compound in many industrial uses such as functional foods and pharmaceuticals.

4.5 Antiallergic Activity of Phlorotannins

Allergic diseases are affecting approximately one-third of a generation in the world, and due to environmental changes and food habits, the prevalence and incidents of allergies are increasing. Allergic diseases are caused by the immunological activation of mast cells and the release of endogenous mediators such as leukotrienes, prostaglandins, and histamine, which are considered as a marker of degranulation in cells and a wide variety of other inflammatory mediators including eicosanoids, proteoglycans, proteases, and several pro-inflammatory and chemotactic cytokines (tumor necrosis factor-α interleukins) [278, 279]. Mast cell degranulation occurs in response to immunological stimuli in which the antigen-IgE antibody reaction predominates on the cell membrane [280–283]. Hyaluronidase (HAase, EC 3.2.1.35), an enzyme which cleaves the polysaccharides hyaluronic acid in the extracellular matrix of connective tissue, is mainly known to be involved in allergic reaction [284, 285]. In relation to antiallergic properties, many phlorotannins from brown algae were considered as potential natural inhibitors of allergic reactions. Ethanolic extract of phlorotannin from *Sargassum tennerimum* has shown a strong inhibitory effect on the activation of hyaluronidase [286]. This work examined antihyaluronidase activity of crude phlorotannin extracted from brown seaweed and used antiallergic drug DSCG and a natural polyphenol catechin as their positive controls. It is resulted that inhibition of hyaluronidase using phlorotannin content in the ethanolic extract was superior compared to DSCG and catechin. Phlorotannins of fucodiphloroethol G, eckol, dieckol, 6,6′-bieckol, and phlorofucofuroeckol-A purified from *Ecklonia cava* were evidenced to be significant antiallergic activity by inhibiting histamine release by modulating the binding between IgE and FcεRI receptors which mediate the allergen release in human basophilic leukemia (KU812) and rat basophilic leukemia (RBL-2H3) [287, 288]. Similarly, phlorotannins of dioxinodehydroeckol and phlorofucofuroeckol-A obtained from *Ecklonia stolonifera* showed a suppressive effect on cell surface expression of FcεRI and total cellular protein and mRNA levels of the FcεRI a chain in KU812 cells [289]. Further, both of these compounds exerted inhibitory effects against the elevation of intracellular calcium level and histamine release from anti-FcεRI α chain antibody (CRA-1)-stimulated cells. Moreover, Sugiura et al. [290] isolated a new phlorotannin from *Eisenia arborea* called phlorofucofuroeckol-B and evaluated

its inhibitory effect on histamine release from RBL cells. The investigation showed that phlorotannin of phlorofucofuroeckol-B resulted strong effect on inhibition histamine release from the allergy model rat in a dose-dependent manner. Therefore, it can be suggested that phlorotannin derivatives isolated from marine brown algae may be used as potential antiallergic agents.

4.6 Anti-inflammatory Activities of Phlorotannins

Inflammation is a part of the nonspecific protective response of the body to harmful stimuli (i.e., damage to tissues, pathogens, specific disease conditions, and harmful chemicals) [291]. Seaweed-derived bioactive compounds such as phlorotannins are known to have promising anti-inflammatory activities. The anti-inflammatory effect of phlorotannins is mainly based on macrophage modulation. Macrophages are the residents of immune cells in the innate immune system which plays an important role in the maintenance of homeostasis by changing their function according to the tissue. As an example, Lee et al. [292] reported anti-inflammatory activities of fucofuroeckol-A from *Eisenia bicyclis* in lipopolysaccharide-induced mouse macrophages. Fucofuroeckol-A suppressed the production of the production of pro-inflammatory mediators and downregulated the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs). Other phlorotannins including phloroglucinol, eckol, dieckol, 7-phloroeckol, phlorofucofuroeckol-A, dioxinodehydroeckol, phlorofucofuroeckol-B, catechol, vidalols A and B, diphlorethohydroxycarmalol, and octaphlorethol A have also been reported to possess anti-inflammatory properties [291].

5 Seaweed Bioactive Peptides

Recently, research has been focused on algae as a source of natural bioactive components, and much attention has been paid to bioactive peptides due to their health functionalities [189, 293]. Bio-functionalities or bioactivities of peptides have been described as mimic hormones or showing drug-like activities. In addition, they could alter the physiological functions or raise a positive impact through binding to specific receptors and interact on target cells or inhibition by enzyme actions [294]. Nowadays, there is an enormous interest in using marine alga proteins as a source of bioactive peptides [75, 189, 295]. Red and green seaweeds have relatively high protein concentrations, with an average value of 10–30% dry matter [203, 296, 297], while brown seaweeds are low, with an average of 3–15% of dry weight [203, 298]. Bioactive peptides usually contain 2–20 amino acid residues and present a variety of biological activities. Generally, bioactive peptides can be obtained from inner food-protein sources by three ways: (1) hydrolysis by digestive enzymes from

animals; (2) hydrolysis by proteolytic enzymes, harvested by microorganisms or plants; and (3) hydrolysis by proteolytic microorganisms during fermentation. However, due to the lack of residual chemicals in the final peptide products, enzymatic hydrolysis is preferred, especially in the pharmaceutical and food industries [299]. Further, the use of biocatalytic processes using marine enzymes has become an important and useful natural product for biotechnological applications. Bioprocesses using biocatalysts like marine enzymes have advantages such as hyperthermostability, salt tolerance, barophilicity, cold adaptability, chemoselectivity, regioselectivity, and stereoselectivity [300]. Depending on the amino acid sequence, seaweed-derived biopeptides may be involved in various biological functions [301], including antioxidant, antihypertensive [302], and immunomodulatory effects [303].

5.1 Antioxidant Activity of Seaweed-Derived Peptides

Seaweeds have been considered as a rich source of natural antioxidants due to the presence of various secondary metabolites with antioxidant effect [304]. Especially, the antioxidant activities of some protein hydrolysates or peptides from seaweeds have been investigated. One of the first pronounced antioxidative effects was revealed from water-soluble, protease enzymatic extracts of seven species of marine edible brown seaweeds, including *Ecklonia cava*, *Scytosiphon lomentaria*, *Ishige okamurae*, *Sargassum fullvelum*, *Sargassum horneri*, and *Sargassum thunbergii* around Jeju-Do coasts in South Korea [305, 306]. Enzymatic extracts of *E. cava* have scavenged DPPH free radicals more effectively than other seaweed extracts. The highest inhibitory capacity of lipid peroxidation in linoleic acid was observed in Alcalase and Neutrase extracts of *E. cava* and of *S. lomentaria*, respectively [305, 306]. The protease extracts from brown seaweeds *S. lomentaria* and *I. okamurae* displayed high hydrogen peroxide scavenging activities. Moreover, the concentration of protease extracts (140 μg/mL) including Alcalase, Flavourzyme, Kojizyme, and Protamex was shown with remarkably high scavenging activities of 91.62, 93.41, 96.27, and 93.71%, against hydrogen peroxide, respectively. Kojizyme extract of *I. okamurae* showed a prominent cytoprotective effect against H_2O_2-induced DNA damage on human lymphocytes in the dose-dependent manner [307]. In another work, a brown seaweed *S. lomentaria* was shown strong ROS scavenging activities after being hydrolyzed by proteases [262, 263]. In addition, two antioxidant peptides, carnosine and glutathione, also have been found in macroalgae. However, these peptides are present in high concentrations in animal muscles. Furthermore, the red seaweed *Ancanthophora delilei* has been described as a source of carnosine (β-alanyl-L-histidine), a histidyl peptide with an antioxidant activity as well as is associated with the ability to chelate transition metals [308].

5.2 Antihypertensive Activity of Seaweed-Derived Peptides

Hypertension is recognized as a key target for controlling cardiovascular disease-related mortality including arteriosclerosis, stroke, myocardial infarctions (MI), and renal disease in later stage as well [309, 310]. Diet therapy and lifestyle modifications are the most desirable tool that effectively reduces the blood pressure. Angiotensin-I-converting enzyme (ACE-I) has long been revealed as a key part of the rennin-angiotensin system (RAS), which plays an important role in regulating blood volume and is responsible for the control of blood pressure and fluid balance in humans. There are two ways to control blood pressure by modulating RAS: one is direct inhibition of angiotensin-I generation from angiotensinogen by rennin, and another is blockage of conversion from angiotensin-I to angiotensin-II by ACE. This stated that rennin and ACE inhibitory factors are considered as the way to treat hypertension. Therefore, natural sources of ACE-I inhibitors raise the possibilities that could be formulated with dietary intake [311].

In recent years, there has been a great interest to search novel ACE inhibitors from seaweeds as alternatives for synthetic drugs [189]. ACE inhibitory activities of enzymatic hydrolysates from seven brown algae species, namely, *E. cava*, *I. okamurae*, *S. fulvellum*, *S. horneri*, *S. coreanum*, *S. thunbergii*, and *S. lomentaria*, have been reported [312]. In their experiment, five commercial proteases including Kojizyme, Flavourzyme, Neutrase, Alcalase, and Protamex were employed to obtain respective hydrolysates from the selected seaweeds. Thus, *E. cava* was exhibited the most potent ACE inhibitory effect among the species tested. Moreover, Cha [313] reported that five different enzymatic digests from a brown seaweed *E. cava* exhibited potent angiotensin-converting enzyme (ACE) inhibitory effects with IC_{50} values of 2.33−3.56 μg/mL. Suetsuna and colleagues have characterized ten kinds of dipeptides from *U. pinnatifida*. Four of them significantly decreased the blood pressure in spontaneously hypertensive rats [302]. In another experiment, an edible red algae species, *Porphyra yezoensis*, has been hydrolyzed by seven commercial proteolytic enzymes, and then Alcalase was selected as the effective hydrolysate among them. Furthermore, glutelin was isolated as the major protein (77.1%) from *P. yezoensis* as high extraction yield (28.3%) than other reported proteins such as albumin and gliadin [314]. In another work, Suetsuna [315] showed that *P. yezoensis* has potent ACE-I inhibitory activity against spontaneously hypertensive rats (SHR).

Many studies have shown that seaweed-derived bioactive peptides possess remarkable activities relevant toward many diseases [316]. The possibilities of designing new functional foods, nutraceuticals, and pharmaceuticals derived from seaweed bioactive peptides are promising. While much information is available on biological activities of seaweed-derived bioactive peptides, future studies should be directed toward evaluation of bioavailability in human subjects as well as clinical trials. In addition, safety and quality standards of marine-derived peptide-based products should be evaluated prior to commercialization.

5.3 Immunomodulatory Effects of Seaweed-Derived Peptides

Seaweeds proteins and peptides have gained an increasing demand not only as nutrition but also as its potential for its health-promoting effects. In recent years, in vitro and in vivo studies have revealed that bioactive secondary metabolites may induce and promote the human health factors [317]. It has been evaluated that bioactive peptides from seaweeds possess immune-stimulant activity. *E. cava* hydrolysate was shown to have the immune-stimulating effect on murine splenocytes in vitro. Additionally, in ICR mice treated with *E. cava* hydrolysate, the proliferation of splenocytes was dramatically enhanced; the numbers of CD4+ T cells, CD8+ T cells, and CD45R/B220+ B cells were markedly increased; TNF-α and IFN-γ were downregulated, and IL-4 and IL-10 were upregulated [318]. Further, Cian [319] found that enzymatic hydrolysates from a phycobiliprotein by-product of *Porphyra columbina* exhibited immunosuppressive effects on rat splenocytes by enhancing IL-10 production and inhibiting the production of TNF-α and IFN-γ (pro-inflammatory cytokines). The above works suggest that the immunomodulatory mechanism of seaweed-derived peptides is primarily involved in cytokine regulation and T-cell and B-cell proliferation pathways.

6 Seaweed Vitamins

Vitamins are organic essential compounds needed in the human body in trace amounts for different chemical and physiological processes. Based on their solubility, vitamins are classified into two groups: water-soluble vitamins (vitamins B_1, B_2, B_{12}, and C) and fat-soluble vitamins (provitamin A carotenoids with vitamin A activity, vitamins E, D, and K). Seaweeds are a good source of water-soluble vitamins (vitamins B_1, B_2, B_{12}, and C) and fat-soluble vitamins (provitamin A carotenoids and vitamins E) [320]. Seaweed vitamins are important not only because of their biochemical functions and antioxidant activity but also due to other health benefits such as decreasing blood pressure (vitamin C), preventing cardiovascular diseases (β-carotene), or reducing the risk of cancer (vitamins E and C, carotenoids) [2]. The compositions of seaweed vitamins vary and are affected by seaweed species and environmental condition [296].

The water-soluble vitamins are needed as enzyme cofactors. Several B vitamins serve as coenzymes for enzymes with functions in the catabolism of foodstuffs to produce energy for the body. Some of them are fundamental for their antioxidant activity and other health benefits. Thus low levels of some B group vitamins (B_2, B_6, B_{12}) can result in reduced levels of DNA methylation and therefore in some kinds of cancer [321]. Vitamin B_1 and B_2 are present in sufficient amount especially in brown and red seaweeds. The highest amount of both vitamins was detected in wakame and kombu, 0.3 and 0.24 mg B_1/100 g dry weight and 1.35 and 0.85 mg B_2/100 g dry

weight, respectively [322]. Lower levels of these vitamins are present in arame (0.06–0.12 and 0.65–0.92 mg/100 g dw, respectively), *Caulerpa lentillifera*, and *Ulva reticulata* [323, 324]. The highest vitamin B_{12} content in seaweed is present in the red alga *P. umbilicalis* and *S. muticum*, 10–20 ppm and 10 ppm, respectively [325].

Vitamin C is present in all brown, green, and red seaweeds. The levels of vitamin C average between 500 and 3000 mg/kg dry matter for the green and brown algae, whereas the red algae contain vitamin C levels of around 100–800 mg/kg [326]. According to McDermid and Stuercke [327], vitamin C was determined in abundant amounts in green seaweeds *Enteromorpha flexuosa* and *Ulva fasciata* of 3000 and 2200 mg/kg dry matter and in red seaweed *Eucheuma denticulatum* of 2000 mg/kg dry matter.

Vitamin A, retinal, is important in the vision process. Besides the maintenance of epithelial tissue and prevention of its keratinization, vitamin A also presents important systematic functions in the growth and reproductive efficiency [202]. However, plant food as algae does not contain intrinsic vitamin A but contains provitamins, carotenoid, and linear polyenes with a cyclic structure which possesses a ring [328]. Vitamin E (tocopherols and tocotrienols) is an important liposoluble antioxidant and prevents oxidation of polyunsaturated fatty acids absorbed from the diet. The brown seaweeds contain higher levels of vitamin E than green and red seaweeds. Among the brown algae, the highest levels are observed in the *Ascophyllum* and *Fucus* sp., which contain between 200 and 600 mg of tocopherols/kg of dry matter [329]. Brown algae contain α-, β-, γ-tocopherol, while the green and red algae contain only α-tocopherol. It was shown that the γ- and α-tocopherol increase the production of nitric oxide and nitric oxide synthase activity (cNOS) and also play an important role in the prevention of cardiovascular disease [330]. Despite the low lipid content in seaweed, the presence of vitamin E is relevant as it acts as a strong antioxidant which prevents the formation of free radicals. α-Tocopherol, the most important member of the tocol group, is capable of fixing free radicals via its phenol group in the structure and thus is considered to play an important role in the oxidation of biological membranes, lipoproteins, and fat deposits, controlling or reducing lipid peroxidation. Moreover, α-tocopherol is heat and acid stable and could be used for dietary purposes and supplementation [331].

In many cases, light is an important regulator of vitamin biosynthesis in seaweeds as most seaweeds spend large amounts of time exposed to direct sunlight in an aqueous environment. As a result, seaweeds contain many forms of antioxidant, including vitamins and protective pigments; thus plants growing in bright light have higher ascorbate content [332]. Moreover, algae growing in the littoral zone or on the surface tend to have higher levels of vitamin C than algae harvested from depths between 9 and 18 m [333].

Loss of vitamins can be induced by storage conditions such as the influence of light and oxygen. Moreover, there is a negative influence on vitamin content caused by technological processing such as drying (sun-, oven-, freeze-drying) and sterilization, as well as culinary processes such as cooking, roasting, or baking, which could decrease the vitamin content due to water extrusion and high temperatures during these prose procedures [333].

There are significant differences of certain vitamin contents caused by seasonal variations, for example, in *Eisenia arborea*. It was observed that the highest content of some vitamin (A, B1, B2, and partly also vitamin C) was in spring, while vitamin E showed the lowest value in this season [323]. Moreover, seasonality was also observed as the primary factor of carotene content in *Palmaria palmata*, with the highest content of carotenes found in summer and the lowest in winter [2].

Ortiz reported that [334] 100 g of seaweed provides more than the daily requirements of vitamin A, B2, B12, and two-thirds of the vitamin requirement. Most of the red seaweeds (*Palmaria, Porphyra*) contain large amounts of provitamin A and significant quantities of vitamins B1, B2, and B12 which are also present in green seaweeds. The vitamin content of brown seaweeds (*Undaria, Saccharina,* and *Laminaria*) appears to be less remarkable, but brown seaweeds have high vitamin C and E content [296, 335]. Collectively, usage of seaweed vitamins as nutraceuticals or ingredients for functional food shows prospective possibilities for developing food and pharmaceutical industry.

7 Seaweed Minerals

Minerals are structural components and an essential part of the human body as they perform many necessary functions in the living body, such as building tissue, cell transport, and a wide range of metabolic processes serving as various catalytic metalloenzyme cofactors. More than 95% of mineral intake originates from food. Thus, the mineral level in human population depends on their concentration in vegetable or meat raw material for food products [336–341].

Seaweeds are known to be high in mineral content and are used as feed and food supplements to supply minerals. Seaweeds have a 10–100 times greater mineral content than traditional vegetables [320, 342, 343]. Mineral compositions of seaweeds are varied according to many exogenous and endogenous factors and also correspond to the concentration of minerals in seawater [344, 345]. In addition, the distribution and storage of minerals in seaweed may be influenced by several factors such as different environmental conditions (geographic location, wave exposure, seasonal effects) and condition of seaweed such as age [346–348].

The capability of seaweed to absorb inorganic substances from the environment is regarded with the presence of polysaccharides in seaweed cell walls and also is able to predestine a place of mineral storage in different parts of seaweed tissue [346, 349]. The presence of different polysaccharides in seaweed cell walls is the reason why diverse groups of seaweed have different ability to uptake minerals. Brown seaweeds have higher absorption rate than red and green seaweeds; this can be explained due to the presence of alginic acid, alginate, and salt of alginic acid. These polysaccharides, which are included in seaweed cell walls mostly like calcium, magnesium, sodium and potassium salts, have strong ion exchange properties [350].

Adsorption studies have been conducted mostly in the connection with recognition of uptake mechanism of toxic metal ions by different species of seaweed [351–353]. In addition, cell walls of brown seaweeds form by cellulose, and its carboxyl groups can participate in the accumulation of metals [352]. The mechanism of an ion-exchange process has been found responsible for cationic metal sorption into brown seaweed *Sargassum hemiphyllum* according to Tsui [354] and also into brown seaweed *Ecklonia maxima* according to Williams and Edyvean [355]. Brown seaweed, especially genus *Laminaria*, is a significant source of iodine. It was reported that Laminariales have the great capacity to accumulate iodine by more than 30,000 times the iodine concentration in the seawater [346]. The concentration of iodine in different genera of brown, red, and green seaweed from diverse authors is presented in Table 2.3.

The polysaccharides of red seaweed are represented by sulfated galactans such as agar and carrageenan. The level of sulfatation in carrageenan molecules and also the presence of hydroxyl and carboxyl groups might be responsible for binding metals [357]. It was reported that the extent of cadmium accumulation in red seaweed *Gracilaria tenuistipitata* depends on the light, and cadmium uptake in darkness proceeds mainly by passive diffusion across the cell wall along the concentration gradient [352].

Table 2.3 Iodine contents in some species of seaweed

Seaweed	g/kg Dry matter				
	Saenko et al. [343]	Kolb et al. [322]	Teas et al. [347]	Hou and Yan [356]	Dawczynski et al. [298]
Brown seaweed					
Ascophyllum nodosum			0.65		
Cymathaere japonica	2.0–4.5				
Ecklonia maxima			2.12		
Eisenia bicyclis			0.59		
Fucus evanescens	0.2–0.61				
Hizikia fusiformis					0.26
Laminaria angustata			2.35		
Laminaria digitata			2.00		
Laminaria digitata japonica		1.7			
Laminaria japonica	5.6			3.04	
Laminaria sp.	1.1–3.4				2.93
Sargassum myabei	0.43				
Undaria pinnatifida		0.26	0.02		0.16
Red seaweed					
Palmaria palmata			0.07		
Porphyra sp.	0.03–0.34				0.04
Green seaweed					
Ulva fenestrate	0.04				
Ulva pertusa				0.013	

An adequate intake of minerals is essential for a high nutritional quality of the diet and contributes to the prevention of chronic nutrition-related diseases and degenerative diseases including cancer, cardiovascular disease, Alzheimer's disease, and premature aging [358, 359]. However, too high intakes of trace elements could cause toxicity and too low intakes of trace elements may result in nutritional deficiencies [360]. Dietary Reference Intakes (DRIs) are used quite a lot and refer to a set of four nutrient-based reference values that represent the approach to provide quantitative estimates of nutrient intakes. For the evaluation of contribution of mineral contents in seaweed on daily intake, the amounts of minerals were expressed in an 8 g sample of seaweed matter. This value was calculated as an average mean from reported seaweed consumption ranging from 5 to 12 g adults per day in Asian countries [361, 362]. In addition, these values were compared with recommended daily intake (RDI) according to Velisek [363] and expressed in the percentage proportion to RDI of minerals. Mineral content of few seaweed genera is well documented as the trace elements and macroelements in brown, red, and green seaweed are shown in Tables 2.4 and 2.5. The participation of macroelements on RDI is presented in Table 2.4 and is also different across diverse seaweed genera.

Table 2.4 Trace element contents (mg/8 g) in some species of seaweed and their contribution to RDI

	Fe	Zn	Mn	Cu	Fe	Zn	Mn	Cu	
Seaweed	mg in 8 g	mg in 8 g	mg in 8 g	mg in 8 g	% RDI	% RDI	% RDI	% RDI	References
Laminaria digitata japonica	0.1	0.07	0.20	0.2	1.0	0.7	0.7	0.9	[322]
Undaria pinnatifida	0.1	0.08	0.03	0.02	1.2	0.8	0.9	0.9	
Padina australis	3.57	0.10		0.04	35.7	1.0		1.8	[364]
Sargassum polycystum	2.22	0.03		0.02	22.2	0.3		0.9	
Kappaphycus alvarezii	0.56	0.14		0.04	5.6	1.4		1.8	
Caulerpa racemosa	6.50	0.08		0.06	65.0	0.8		2.7	
Ulva reticulata	2.24	0.14		1.43	22.4	1.4		63.6	
Eisenia bicyclis	0.51	0.22	0.03	0.03	5.1	2.2	0.9	1.3	[365]
Hizikia fusiformis	0.45	0.13	0.05	0.02	4.5	1.3	1.4	0.9	
Laminaria japonica	0.59	0.15	0.04	0.01	5.9	1.5	1.1	0.4	
Undaria pinnatifida	0.57	0.18	0.06	0.03	5.7	1.8	1.7	1.3	
Palmaria palmata	5.74	0.30	0.04	0.04	57.4	3.0	1.1	1.8	
Porphyra tenera	14.66	0.16	2.88	0.13	146.6	1.6	82.3	5.8	
Hizikia fusiformis	5.43	0.13	0.20	0.02	54.3	1.3	0.6	0.9	[298]
Laminaria sp.	2.11	0.08	0.09	0.01	21.1	0.8	2.6	0.4	
Undaria pinnatifida	1.47	0.26	0.06	0.01	14.7	2.6	1.7	0.4	
Porphyra sp.	3.07	3.07	0.30	0.08	30.7	30.7	8.6	3.6	
RDI (reference daily intake) for adult in mg per day [363]					10	10	3.5	2.3	

Table 2.5 Macroelement contents (mg/8 g) in some species of seaweed and their contribution to RDI

Seaweed	Ca mg in 8 g	Mg mg in 8 g	P mg in 8 g	Ca % RDI	Mg % RDI	P % RDI	References
Laminaria digitata japonica	70.4	44.0	24.0	8.8	13.6	2.0	[322]
Undaria pinnatifida	76.0	32.4	36.0	9.5	10.0	3.0	
Padina australis	266.4	32.0		28.3	9.9		[364]
Sargassum polycystum	149.6	45.6		18.7	14.0		
Kappaphycus alvarezii	22.4	23.2		2.8	7.2		
Caulerpa racemosa	148.0	30.4		18.5	9.4		
Ulva reticulata	143.2	172.0		17.9	53.0		
Eisenia bicyclis	54.3	52.4	6.2	6.8	16.1	0.5	[365]
Hizikia fusiformis	51.9	54.8	8.2	6.5	16.9	0.7	
Laminaria japonica	45.9	53.8	38.1	5.7	16.6	3.2	
Undaria pinnatifida	39.5	96.0	48.3	4.9	29.6	4.0	
Palmaria palmata	16.6	27.7	39.8	2.1	8.5	3.3	
Porphyra tenera	45.8	324.8	16.2	5.7	100.0	1.3	
Hizikia fusiformis	106.4	53.4	23.4	13.3	16.5	1.9	[298]
Laminaria sp.	59.4	45.6	45.6	7.4	14.0	3.8	
Undaria pinnatifida	71.9	69.4	21.3	9.0	21.4	1.8	
Porphyra sp. Seaweeds: macroelement contents.	26.4	27.9	41.8	3.3	8.6	3.5	
RDI (reference daily intake) for adult in mg per day [363]				800	325	1200	

8 Conclusions

Seaweeds are a potentially good source of functional ingredients including polysaccharides, pigments, phlorotannins, peptides, mineral, and vitamins. In this chapter, some of the different seaweed-derived functional ingredients and biological activities have been described. This is an area of actual significance as new functional ingredients with biomedical applications are intensively pursued by the scientist, as well as investigations of their biological activities. Seaweed-derived bioactive materials have shown potent biological activities including antioxidant, immunomodulatory, antiviral, antidiabetic, anticancer, etc. However, understanding the specific structures and bioactivities relationship of seaweed-derived bioactive materials is still a great challenge; up to now there is a considerable gap in this area compared with isolation rate of new compounds. In the future, it is expectable that seaweed-derived functional ingredients may thrive as active substance in pharmaceuticals to fight common diseases and that their industrial uses as functional foods and nutraceuticals will also increase. Up to now, most of the biological activities of seaweed-derived materials have been observed in vitro or in vivo. Therefore, adequate clinical trials are needed in development of seaweed bioactive materials in pharmaceutical industries.

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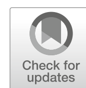
Chapter 3 Anti-infective Compounds from Marine Organisms



Elena Ancheeva, Mona El-Neketi, Georgios Daletos, Weaam Ebrahim, Weiguo Song, Wenhan Lin, and Peter Proksch

Abbreviations

Anti-HCMV	Anti-human cytomegalovirus
BCG	Bacille Calmette-Guérin
CH_2Cl_2	Dichloromethane
DKPs	Diketopiperazines
DOPA	2-Amino-3-(3′,4′-dihydroxyphenyl) propionic acid
EMA	European Medicines Agency
EtOH	Ethanol

Both the authors Elena Ancheeva and Mona El-Neketi have contributed equally for this chapter. E.A. contributed to the years 2014–2016, Introduction, and Conclusion. M.E. contributed to the years 2010–2013 and combining the chapter in the final form.

E. Ancheeva · G. Daletos (✉) · P. Proksch (✉)
Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany
e-mail: georgios.daletos@uni-duesseldorf.de; proksch@uni-duesseldorf.de

M. El-Neketi
Faculty of Pharmacy, Department of Pharmacognosy, Mansoura University, Mansoura, Egypt

W. Ebrahim (✉)
Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

Faculty of Pharmacy, Department of Pharmacognosy, Mansoura University, Mansoura, Egypt
e-mail: weaam.ebrahim@uni-duesseldorf.de

W. Song
Shouguang City, People's Republic of China

W. Lin
State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, People's Republic of China

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EtOAc	Ethyl acetate
FDA	Food and Drug Administration
HSV	Herpes simplex virus
HAT	Human African trypanosomiasis
HCMV	Human cytomegalovirus
HMPV	Human metapneumovirus
HRV2	Human rhinovirus serotype 2
HRV3	Human rhinovirus serotype 3
HIV-1	Human immunodeficiency virus type 1
Human APOBEC3G	An innate intracellular antiviral factor
H1N1	Influenza A virus subtype H1N1 = hemagglutinin type 1 and neuraminidase type 1
H5N1	Influenza A virus subtype H5N1 = hemagglutinin type 5 and neuraminidase type 1
ICL	Isocitrate lyase
IC_{50}	Half maximal inhibitory concentration
MeOH	Methanol
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MS/MS	Tandem mass spectrometry
MSSA	Methicillin-sensitive *S. aureus*
MIC	Minimum inhibitory concentration
NA	Neuraminidase activity (NA)
NMR	Nuclear magnetic resonance
PIV	Parainfluenza virus
PCR	Polymerase chain reaction
RSV	Respiratory syncytial virus
ROESY	Rotating frame Overhauser effect spectroscopy
SAR	Structure-activity relationship
SARS	Severe acute respiratory syndrome
USA	United States of America
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*
VCD	Vibrational circular dichroism
Vif	Viral infectivity factor of HIV-1
WHO	World Health Organization

1 Introduction

Natural products play a pivotal role in drug discovery of anti-infectives, as highlighted by the fact that nearly two-thirds of all drugs currently available on the market for treatment of infectious diseases are natural products or derivatives thereof [1]. It is commonly accepted that the success of natural products as a prolific source

of lead structures is due to metabolome evolution of macro- and microorganisms, which resulted in the accumulation of antimicrobial compounds as a response toward antagonistic environmental conditions.

Historically, most of the currently used anti-infectives of natural origin were derived from soil-dwelling microorganisms, such as fungi and actinomycetes, and in limited cases from terrestrial plants (antimalarial agents) [2, 3]. A few notable examples include the β-lactam antibiotic penicillin G from the fungus *Penicillium notatum*, the macrolide erythromycin from the actinomycete *Saccharopolyspora erythraea*, and the antimalarial compound artemisinin from the plant *Artemisia annua* [4]. Most of these agents were introduced between the 1940s and 1960s, during the so-called 'golden era' of antibiotic discovery; however, the development of novel antibiotics dramatically dropped over the past several decades, partially based on the misleading notion that the battle against microbial infections has been won. Moreover, in recent years, the emerging microbial drug resistance against many clinically used anti-infective drugs has jeopardized their treatment efficacy. In particular, according to the World Health Organization (WHO), resistant strains of Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus* sp.), Gram-negative bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*), *Mycobacterium tuberculosis*, *Candida* sp., *Plasmodium falciparum*, or HIV, among others, have become a global threat [5]. This antibiotic resistance crisis may lead to extremely severe public health consequences unless adequate global responses, including development of new antimicrobials, are achieved [6]. Thus, the discovery of new antimicrobial agents from nature, preferably with new mechanisms of action, higher selectivity, and less sensitivity to drug-resistance development, remains an important investigation area of natural products research.

Marine-derived natural products occupy a special place in delivering new drugs into the pharmaceutical market, as a rich source of numerous novel chemical entities with pronounced biological activities [7]. This is exemplified by the fact that in the last two decades the number of FDA-/EMA-approved marine drugs (ziconotide, 2004; omega-3-acid-ethyl esters, 2004; eribulin mesylate, 2010; brentuximab vedotin, 2011; and trabectedin, 2015) substantially increased since the discovery of the unusual nucleosides spongothymidine and spongouridine from the sponge *Tethya crypta*, leading to the first marine drugs ara-A (vidarabine) and ara-C (cytarabine) by the late 1950s [8, 9] (Fig. 3.1). Literature data have shown that the majority of these metabolites, including those entering clinical trials, are mainly being developed in the areas of analgesia and cancer [10]. However, following the success of marine-derived natural products in drug discovery, marine organisms have captured wide attention as a promising source for antimicrobial bioprospecting, as highlighted by the considerable rise of publications in recent years dealing with the discovery of anti-infective agents [1, 11].

The present chapter provides an overview of key publications on marine anti-infective compounds from various marine sources between 2010 and 2016, classified primarily according to the spectrum of antimicrobial activity (antibacterial, antifungal, antiviral, antiprotozoal compounds) with further subdivision based on

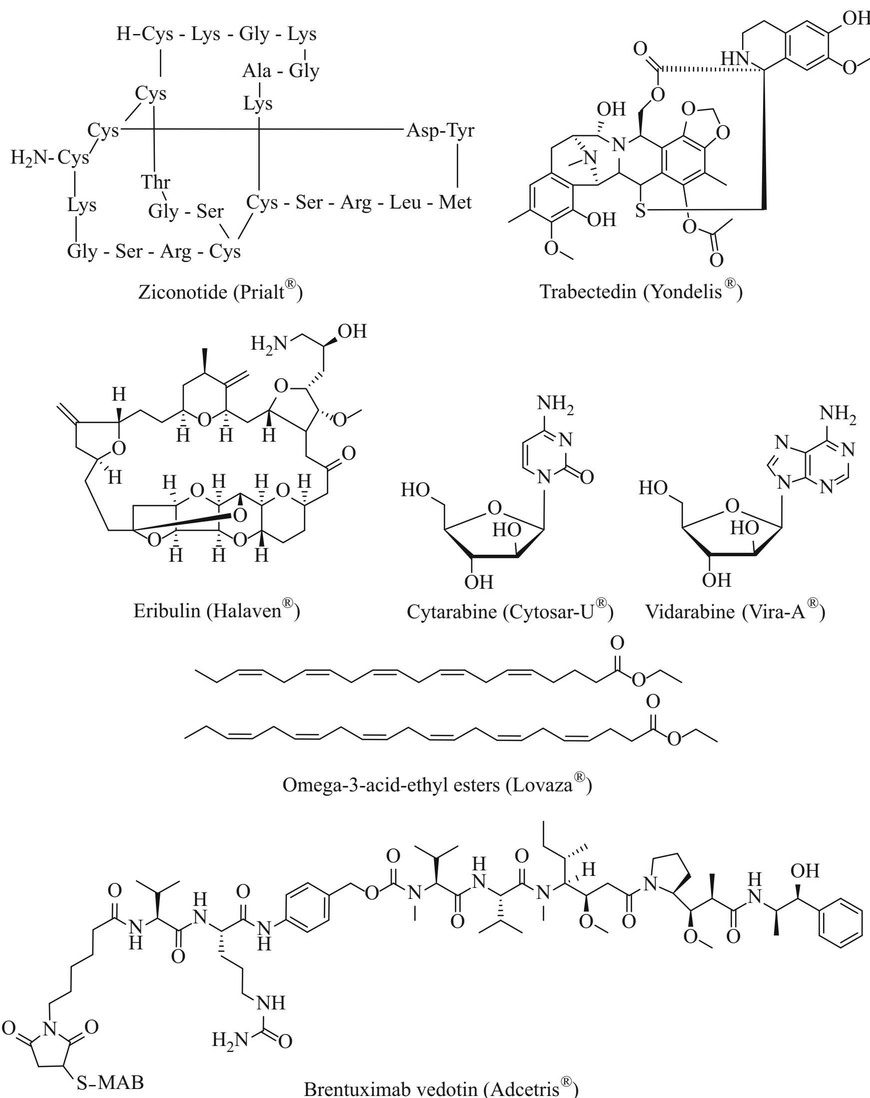


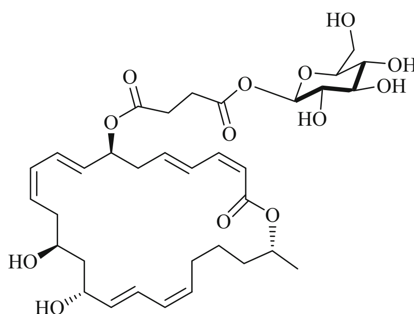
Fig. 3.1 Structures of marine-inspired drugs approved by FDA/EMA

their natural sources including marine microorganisms (bacteria/fungi) and macroorganisms (algae, invertebrates). The examples of new antimicrobials were compiled with focus on their structural features, structure-activity relationships, and mode-of-action studies.

2 Antibacterial Compounds

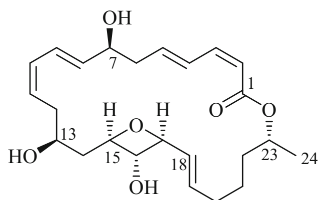
2.1 From Bacteria

Marine microbial metabolites are well known for their chemically diverse structures and for their broad biological activities. Twenty-four-membered macrolactins are frequently produced by *Bacillus* strains and possess antibacterial, anticancer, and antiviral activities. The culture broth of bacterium *Bacillus* sp. 09ID194, isolated from a marine sediment sample collected from Ieodo in Korea's southern reef, was found to produce three macrolides including macrolactins A, Q, and W. Macrolactin W (**1**) showed antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria: *Bacillus subtilis* (KCTC 1021), *Staphylococcus aureus* (KCTC 1916), *Escherichia coli* (KCTC 1923), and *Pseudomonas aeruginosa* (KCTC 2592) with minimum inhibitory concentration (MIC) value = 64 μg/ml in the serial dilution assay. However, no cytotoxic activity was recorded for **1** against different cancer cell lines, emphasizing its importance as a selective antibacterial drug candidate [12].

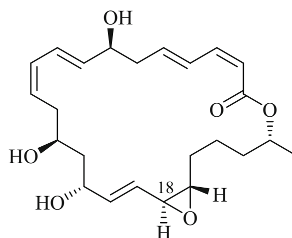


Macrolactin W (**1**)

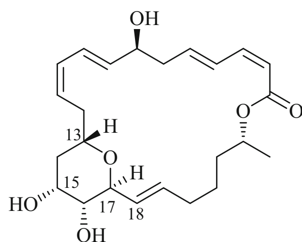
Further investigation of the same bacterial strain afforded three additional new bioactive macrolactins including 15,17-epoxy-16-hydroxy macrolactin A, 18,19-epoxy macrolactin A, and 13,17-epoxy-16-hydroxy macrolactin A (**2**−**4)** [13]. These macrolides feature an oxetane ring in **2**, an epoxide moiety in **3**, and a tetrahydropyran ring in **4**. The configurations of **2**−**4** were assigned by calculating the coupling constants, through analysis of ROESY spectra, and by performing the modified Mosher's method. Compounds **2**−**4** exhibited MICs of 0.06–0.07 μg/ml, against *B. subtilis* (KCTC 1021) and *E. coli* (KCTC 1923), and 0.009–0.07 μg/ml against *Saccharomyces cerevisiae* (KCTC 7913) in the broth dilution assay. The presence of an oxygen at C-15 was mandatory for the antibacterial activity [13].



15,17-Epoxy-16-hydroxy macrolactin A (**2**)

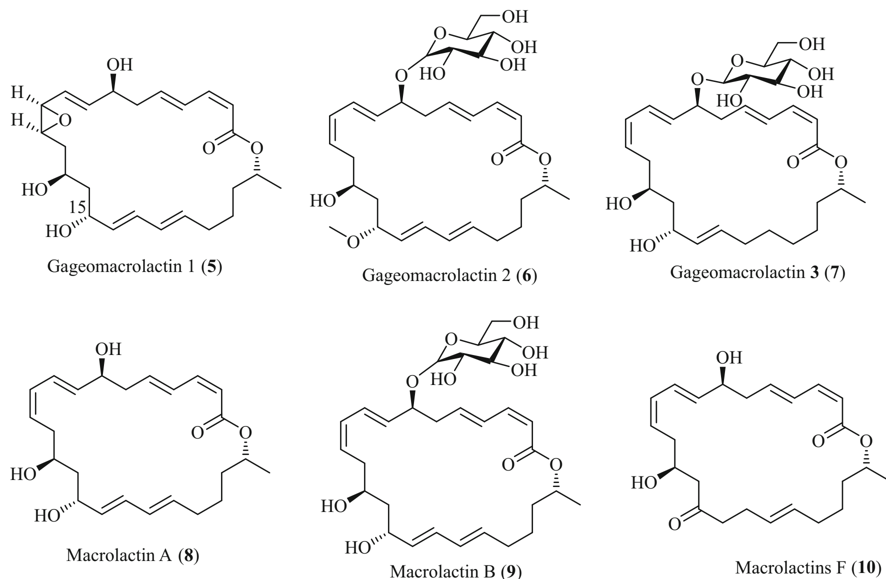


18,19-Epoxy macrolactin A (**3**)



13,17-Epoxy-16-hydroxy macrolactin A (**4**)

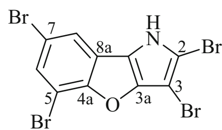
Further macrolactin analogues were obtained from the culture broth of *B. subtilis* strain 109GGC020 isolated from a marine sediment sample obtained from Gageocho, in Korea's southern reef. The new compounds gageomacrolactins (**5**–**7**) and the known macrolactins A (**8**), B (**9**), F (**10**), and W (**1**) exhibited significant broad-spectrum antibacterial and antifungal activities. Compounds **5**–**7** revealed antibacterial activities against Gram-positive (*S. aureus*, *B. subtilis*, and *Bacillus cereus*) and Gram-negative (*E. coli*, *Salmonella typhi*, and *P. aeruginosa*) bacteria with MIC values in the range of 0.008–0.03 µg/ml vs. azithromycin (positive control, MIC 0.008 µg/ml). All isolated compounds inhibited also the mycelial growth of the fungi *Aspergillus niger*, *S. cerevisiae*, *Candida acutatum*, and *Candida albicans* with MIC values ranging from 0.016 to 0.204 µg/ml vs. amphotericin B (positive control, MIC 0.018 µg/ml). Structure activity relationship (SAR) studies revealed that the hydroxyl function at C-15 of the macrolactone moiety is essential for antimicrobial activity. These results emphasize the impact of macrolactins as potential antibacterial/fungicidal drugs [14].



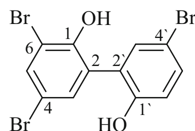
Gageomacrolactin 1 (**5**) Gageomacrolactin 2 (**6**) Gageomacrolactin **3** (**7**)

Macrolactin A (**8**) Macrolactin B (**9**) Macrolactins F (**10**)

Proteobacteria that are derived from the marine environment are prolific producers of many new and bioactive metabolites. The crude extract of *Pseudoalteromonas* sp. CMMED 290, associated with a nudibranch collected in shallow waters of Kaneohe Bay, Oahu (Hawaii), displayed significant broad-spectrum antibiotic activity against *S. aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (ATCC 43300), and *E. coli* (ATCC 25922). Chromatographic workup afforded compounds **11** and **12**, which showed potent antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) with MIC values in the low μM range [15]. 2,3,5,7-Tetrabromobenzofuro[3,2-b]pyrrole (**11**) exhibited activity against MRSA (MIC 0.90 μg/ml). The mechanism of antimicrobial activity of this compound was suggested to be due to its ability to disrupt the bacterial cell wall membrane, whereas human erythrocytes were not lysed when the compound was applied at the same dose [15].

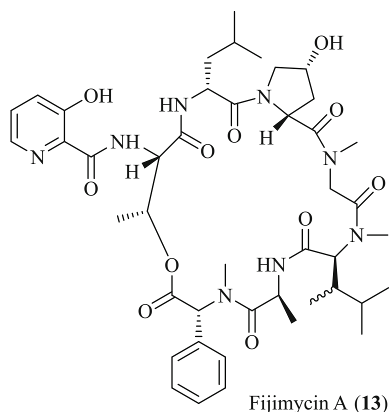


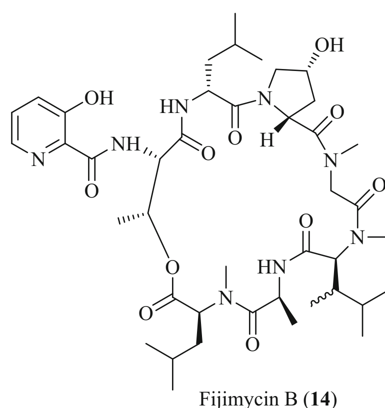
2,3,5,7-Tetrabromobenzofuro[3,2-b]pyrrole (**11**)

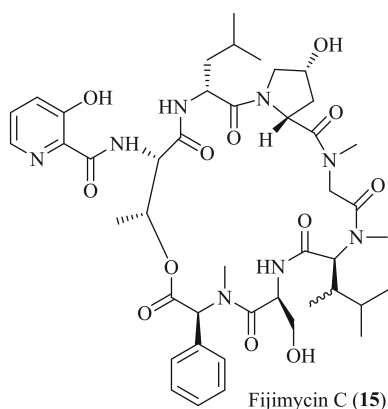


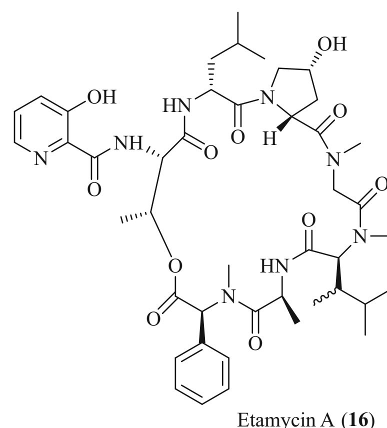
4,4',6-Tribromo-2,2'-biphenol (**12**)

MRSA-caused infections are challenging problems in both community and hospital settings. An extract of the strain *Streptomyces* sp. CNS-575, isolated from a marine sediment sample collected at ca. 0.5 m depth from Nasese shoreline, Viti Levu, Fiji, exhibited potent antibiotic activity against MRSA. Chromatographic workup afforded fijimycins A–C (**13–15**), in addition to etamycin A (**16**). The antibacterial activities of these metabolites were assessed against three MRSA strains, the hospital-associated strain (ATCC33591), the sequenced hospital-associated strain (Sanger 252), and the community-associated strain (UAMS1182). Fijimycins A (**13**), C (**15**) and etamycin A (**16**) exhibited strong to medium antibiotic activities against the three MRSA strains with MIC values ranging from 4–32 μg/ml. The weak activity of fijimycin B (**14**) against both ATCC33591 and UAMS1182 indicated that the α-phenylsarcosine unit is essential for antibacterial activity. The similar antimicrobial activities of the stereoisomers fijimycin A (**13**) and etamycin A (**16**) suggest that substituting D- for L-α-phenylsarcosine has only negligible effects with regard to the anti-MRSA activities [16].

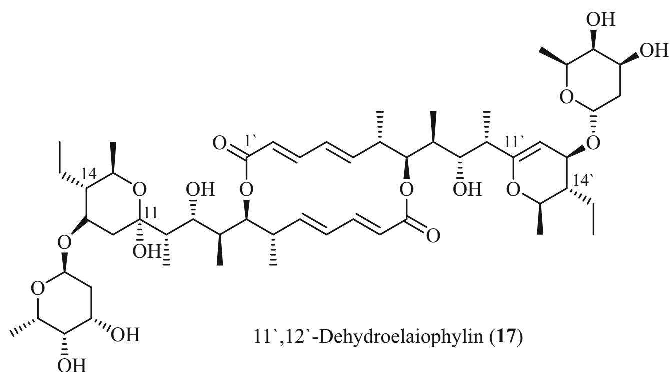

Fijimycin A (**13**)


Fijimycin B (**14**)

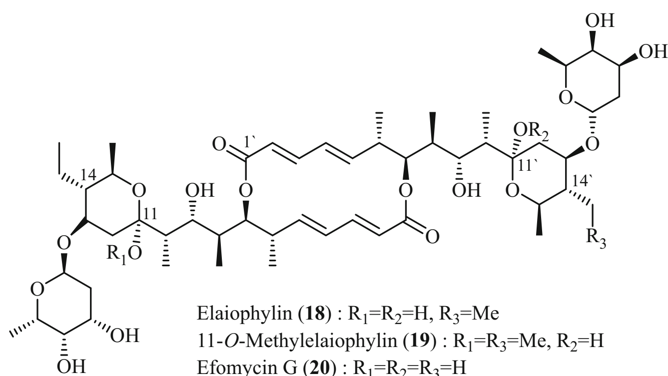

Fijimycin C (**15**)


Etamycin A (**16**)

Streptomyces sp. 7-145 was isolated from a marine sediment sample obtained from Heishijiao Bay, Dalian, People's Republic of China (P.R. China). Wu et al. selected this strain for further investigation based on polymerase chain reaction (PCR) screening, targeted for strains that may produce glycosylated antibiotics. Investigation of the culture broth of the studied *Streptomyces* sp. afforded two new elaiophylins-6-deoxyglycoside derivatives along with four known ones [17]. Among the purified compounds, 11′,12′-dehydroelaiophylin (**17**), elaiophylin, 11-*O*-methylelaiophylin, and efomycin G (**18–20**) were the most potent ones against different MRSA strains and vancomycin-resistant enterococci (VRE) with MIC values ranging from 1 to 4 μg/ml [17]. In addition, **18** and **20** were also active against methicillin-resistant *Staphylococcus epidermidis* (MRSE) with MICs ranging from 2 to 16 μg/ml [17]. No cross-resistance was observed between **17** and **20** and erythromycin or azithromycin (14- and 15-membered macrolides) confirming that the activity of elaiophylins (16-membered lactone) is not affected by the macrolide resistance mechanism in Gram-positive bacteria. SAR studies revealed that the hemiketal moiety at 11 and 11′ positions is important for the antibacterial activity, whereas substitution of the 14′ ethyl group with a methyl group reduces the activity, thereby proving the essential role of the alkyl group at positions C-14 or C-14′.

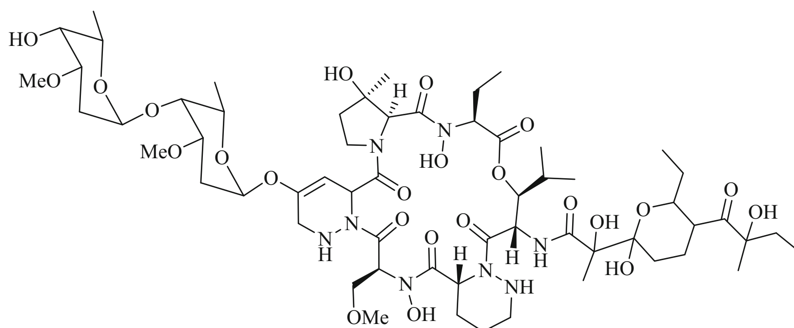


11`,12`-Dehydroelaiophylin (**17**)



Elaiophylin (**18**) : R_1=R_2=H, R_3=Me
11-*O*-Methylelaiophylin (**19**) : R_1=R_3=Me, R_2=H
Efomycin G (**20**) : R_1=R_2=R_3=H

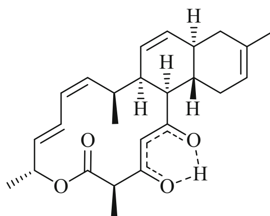
Chemical investigation of a marine bacterium *Streptomyces* sp. (CMB-M0244) derived from a sediment sample collected off South Molle Island, Queensland, Australia, led to the isolation of a first-in-class glyco-hexadepsipeptide-polyketide mollemycin A (**21**) [18]. Mollemycin A (**21**) showed pronounced activity against a panel of Gram-positive bacteria, including *S. aureus* (strains ATCC 25293 and ATCC 9144), *S. epidermidis* ATCC 12228, and *B. subtilis* (strains ATCC 6051 and ATCC 6633), as well as against the Gram-negative bacteria *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 with IC_{50} values ranging from 13 to 65 ng/ml. Moreover, compound **21** was exceptionally potent against drug-sensitive 3D7 and multidrug-resistant Dd2 clones of the Malaria parasite *P. falciparum* with IC_{50} values of 12 and 9 ng/ml, respectively, whereas it exhibited at least 20-fold lower cytotoxicity toward human neonatal foreskin fibroblast cells, verifying this compound as a potential lead antimalarial agent for further investigation [18].



Mollemycin A (**21**)

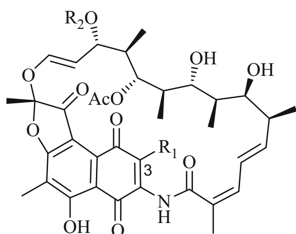
Bioassay-guided fractionation of the EtOAc extract of *Streptomyces* sp. CNH365, isolated from a sediment sample collected off Gaviota State Beach, California, USA, yielded a structurally unique 14-membered macrolide, termed anthracimycin (**22**). Compound **22** exhibited potent activity against a panel of Gram-positive pathogens, including *Bacillus anthracis*, *E. faecalis*, *S. pneumoniae*, as well as methicillin- and vancomycin-resistant strains of *S. aureus* with MIC values less than 0.25 μg/ml [19, 20]. Moreover, **22** showed marked effects on the growth rate of MRSA even at sub-MIC concentrations (1/16 MIC; 0.0078 μg/ml), compared to the antibacterial agent vancomycin. Notably, **22** exhibited low mammalian cell cytotoxicity with an IC_{50} value of 70 μg/ml and was shown to exert synergistic effects with the human cathelicidin LL-37 on MRSA growth. Further investigation employing an optimized macromolecular synthesis assay indicated disruption of nucleic acid synthesis from **22** at concentrations near the MIC, thus suggesting that this effect might be related to its mode of action. Following these intriguing results, a murine peritonitis model of infection was employed to ascertain in vivo efficacy of **22**. Interestingly, anthracimycin (**22**) at a single i.p. injection dose of 1 or 10 mg/kg, even one hour after MRSA infection, provided sufficient protection against mortality and was well

tolerated by mice, thus suggesting its potential as a novel lead scaffold against MRSA [19].

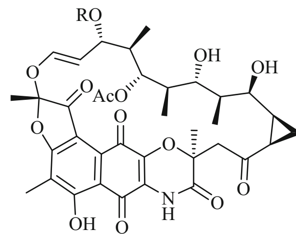


Anthracimycin (**22**)

Four new rifamycins (ansa macrolides), 3-amino-27-demethoxy-27-hydroxyrifamycin S (**23**), 3-amino-rifamycin S (**24**), and sporalactams A (**25**) and B (**26**), were isolated from the culture broth of the marine-derived bacterium *Micromonospora* sp. RJA4480, derived from a sediment sample collected in Barkley Sound, British Columbia (Canada) [21]. Interestingly, compounds **23** and **24** are the first natural rifamycins to bear a 3-amino group. Moreover, the structures of compounds **25** and **26** feature an unprecedented heterocyclic core connected with a 14-membered ansa bridge, thus forming a novel ansa macrolide template. All isolated compounds (**23**–**26**), together with two reference compounds 27-demethoxy-27-hydroxyrifamycin S (**27**) and rifamycin S (**28**), both lacking the 3-amino substituent, were evaluated for their antimicrobial activity against MRSA, *E. coli*, and *M. tuberculosis*. Among the isolated compounds, rifamycins **23** and **24** displayed exceptional antibacterial activity (MIC_{90} values ranging from 0.07 to 0.63 ng/ml), which was higher than that of the reference compounds **27** and **28** (up to 700-fold). Moreover, sporalactam B (**26**) showed selective activity against *M. tuberculosis*, both in broth and in macrophages, with MIC_{90} values of 44 ng/ml and 2.2–6.5 μg/ml, respectively, comparable to those of **27**. These data implied that the 3-amino substituent as well as 27-O-methylation plays an important role in the antimicrobial potency of these metabolites, probably by favoring their binding to RNA polymerase [21].



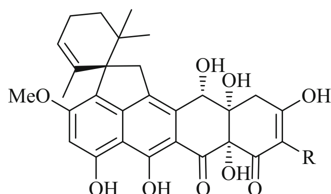
3-Amino-27-demethoxy-27-hydroxyrifamycin S (**23**): R_1=NH_2, R_2=H
3-Amino-rifamycin S (**24**): R_1=NH_2, R_2=Me
27-Demethoxy-27-hydroxyrifamycin S (**27**): R_1=H, R_2=H
Rifamycin S (**28**): R_1=H, R_2=Me



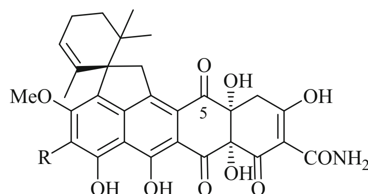
Sporalactam A (**25**): R=H
Sporalactam B (**26**): R=Me

2.2 From Fungi

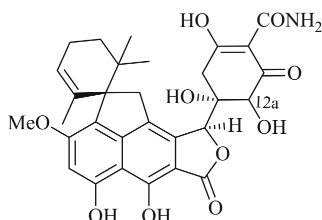
Four new viridicatumtoxins C–F, together with four known metabolites, viridicatumtoxins A (**29**) and B (**30**), spirohexaline (**34**), and previridicatumtoxin (**35**), were reported from the fungus *Paecilomyces* sp. (CMB-MF010), isolated from the inner tissues of a mollusk of the genus *Siphonaria* collected near Shorncliffe, Australia [22]. Viridicatumtoxins are a rare family of fungal polyketides structurally related to the tetracycline class of antibiotics. Notably, viridicatumtoxins A–E (**29**–**33**), as well as compounds **34** and **35**, showed potent activity against MRSA and VRE with IC_{50} values less than 5.5 μg/ml. Among them, the 5-oxo analogue viridicatumtoxin B (**30**) was found to be the most active compound with IC_{50} values of 85 and 23 ng/ml, respectively, possessing significantly higher activity than the positive control oxytetracycline ($IC_{50} = 0.23$ and 5.1 μg/ml, respectively). Moreover, viridicatumtoxin A (**29**) was shown to be remarkably stable when subjected to different acid degradation protocols, suggesting that the viridicatumtoxin framework could potentially be utilized to optimize oral bioavailability and efficacy of tetracycline antibiotics. In a subsequent study, viridicatumtoxin A (**29**) and spirohexaline (**34**) were discovered as inhibitors of recombinant undecaprenyl pyrophosphate (UPP) synthase from *S. aureus* with IC_{50} values of 2.3 and 5.1 μg/ml, respectively [23]. Moreover, these molecules showed only weak inhibitory activities toward catalytically related enzymes, such as octaprenyl pyrophosphate (OPP) synthase of *E. coli* and dehydrodolichyl pyrophosphate (DedolPP) synthase of the yeast *S. cerevisiae*, thus suggesting that these compounds selectively inhibit UPP synthase, which is in agreement with their anti-*S. aureus* activity. In addition, molecular docking studies revealed that **29** and **34** bind to the active site of this enzyme and compete with the natural substrate farnesyl pyrophosphate (FPP), thus corroborating their mode of antibacterial action via inhibition of UPP synthase activity [23].



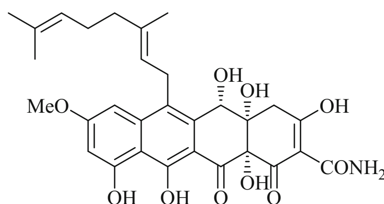
Viridicatumtoxin A (**29**): R=$CONH_2$
Spirohexaline (**34**): R=$COCH_3$



Viridicatumtoxin B (**30**): R=H
Viridicatumtoxin C (**31**): R=OH

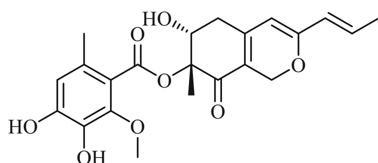


(*R*) 12a: Viridicatumtoxin D (**32**)
(*S*) 12a: Viridicatumtoxin E (**33**)

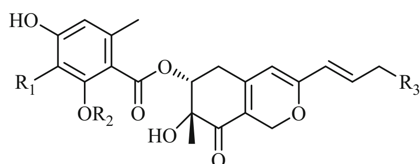


Previridicatumtoxin (**35**)

Azaphilones are produced by several fungal genera including *Aspergillus*, *Monascus*, and *Penicillium*. Six unprecedented azaphilones, comazaphilones A–F, were obtained from extracts of *Penicillium commune* QSD-17, a fungus derived from a marine sediment sample obtained at 210 m depth from the Southern China Sea [24]. Comazaphilone C (**36**) was shown to be active against MRSA, *P. fluorescens*, and *B. subtilis* with MIC values of 16, 64, and 32 μg/ml, respectively. Comazaphilone D (**37**) was active only against MRSA and *P. fluorescens* with MIC values of 32 and 16 μg/ml, respectively, while comazaphilone E (**38**) was active against *P. fluorescens* and *B. subtilis* with MIC values of 32 and 16 μg/ml, respectively. The MIC values of the positive control, ampicillin, ranged from 4 to 8 μg/ml. SAR studies showed that the position of the double bond at C-10 and of the orsellinic acid moiety at C-6 in the azaphilone core structure affects the antibacterial activity of the respective compounds.

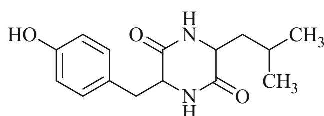


Comazaphilone C (**36**)



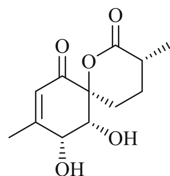
Comazaphilone D (**37**) : R_1=R_2=R_3=H
Comazaphilone E (**38**) : R_1=OH, R_2=Me, R_3=H

Infections by pathogenic bacteria are often due to the formation of microbial biofilms. Medical devices are often contaminated with *Staphylococcus epidermidis* biofilms which are involved in the transmission of infections. Scopel et al. succeeded in isolating the dipeptide *cis-cyclo* (Leucyl-Tyrosyl) (**39**) from culture broth of *Penicillium* sp. F37, a fungal strain obtained from the sponge *Axinella corrugata* collected in South Brazil. *Cis-cyclo* (Leucyl-Tyrosyl) (**39**) selectively inhibits up to 85% of biofilm formation, thus pointing to **39** as a promising broad-spectrum drug candidate [25].

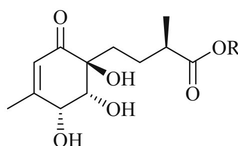


cis-cyclo (Leucyl-Tyrosyl) (**39**)

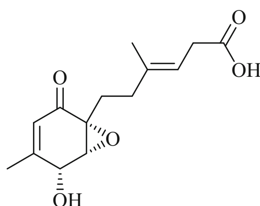
The deep-sea fungus *Penicillium* sp. F23-2, obtained from a deep ocean sediment sample collected at a depth of 5080 m, afforded five new ambuic acid derivatives, penicyclones A–E (**40**–**44**) [26]. Interestingly, compound **40** possesses an unprecedented spiro-δ-valerolactone moiety. Bioactivity screening of **40**–**44** revealed significant antibacterial activity against *S. aureus* with MIC values in the range between 0.3 and 1.0 μg/ml, whereas no cytotoxic effects were detected against a panel of different cancer cell lines (HeLa, BEL-7402, HEK-293, HCT-116, and A549) at similar concentrations (IC_{50} > 12 μg/ml), suggesting their selective antibacterial activity.



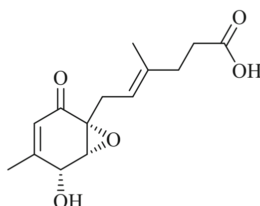
Penicyclone A (**40**)



Penicyclone B **(41):** R = H
Penicyclone C **(42):** R = Me



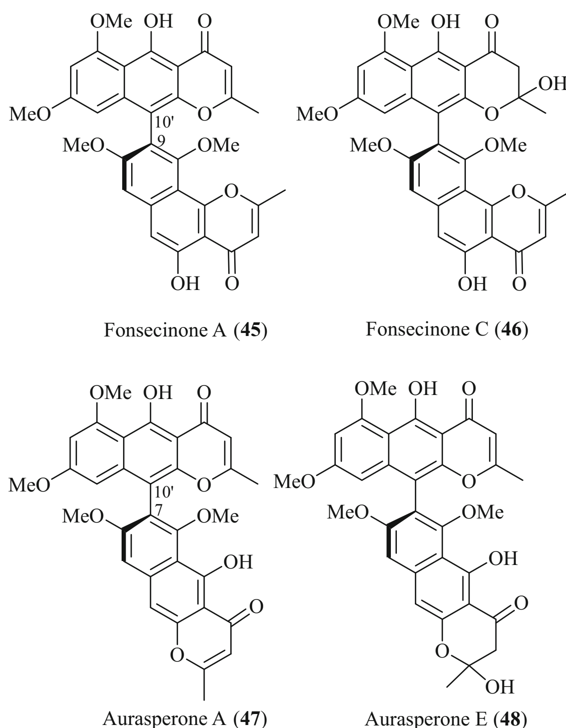
Penicyclone D (**43**)



Penicyclone E (**44**)

With the purpose to identify novel antibiotics from microbial sources, a series of naphtho-γ-pyrones were isolated from the fungus *Aspergillus* sp. Z120 that was obtained from the Marine Culture Collection of China [27]. Among them, the

asperpyrone-type metabolites fonsecinones A (**45**) and C (**46**), formed via a C-10′–C-9 linkage, displayed significant antibacterial activity toward extended spectrum beta-lactamase (ESBL)-producing *E. coli* (MIC = 4.3 μg/ml), followed by the dimeric naphtho-γ-pyrones aurasperones A (**47**) and E (**48**) with C-10′–C-7 linkages (MIC = 8.5 μg/ml), equipotent to the inhibitory effects of the positive controls amikacin and ceftriaxone, respectively. Subsequent molecular docking-based target identification studies were employed revealing the bacterial enoyl-acyl carrier protein reductase (FabI), a key enzyme in the bacterial fatty acid synthesis, as a possible antibacterial target for compounds **45–48**. To further confirm these results, all compounds were subjected to a FabI inhibition assay and were shown to inhibit FabI in a concentration-dependent manner, thus indicating that this effect is likely related to the mode of action of these metabolites [27].

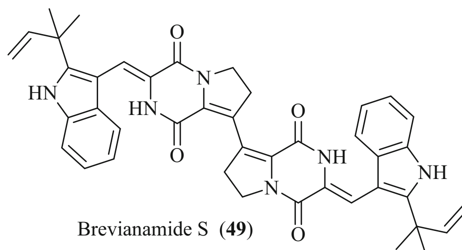


Fonsecinone A (**45**) Fonsecinone C (**46**)

Aurasperone A (**47**) Aurasperone E (**48**)

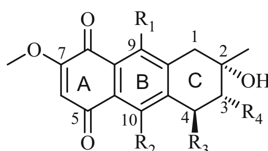
Diketopiperazines (DKPs) are widespread in nature and show diverse biological activities, such as disruption of biofilm formation [28]. *Aspergillus versicolor* MF030 was isolated from the Bohai Sea sediment (P.R. China). Fermentation of the strain yielded brevianamide S (**49**), a rare natural dimeric DKP having antitubercular activity, and three monomeric DKPs brevianamides T, U, and V, along with the known brevianamides N and K and deoxybrevianamide E. Among the isolated metabolites, **49** exhibited antibacterial activity against bacille Calmette-Guérin (BCG) with a MIC value of 6.25 μg/ml, versus the positive control isoniazid (MIC, 0.05 μg/ml). In spite of the moderate effect of **49**, its selectivity toward BCG

makes brevianamide S a potential new drug candidate as a next-generation antibiotic for the treatment of *Mycobacterium tuberculosis* [29].



Brevianamide S (**49**)

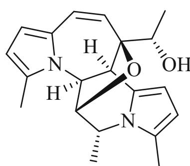
The fungus *Nigrospora* sp. isolated from fresh tissue of an unidentified sea anemone, collected from the Weizhou coral reef in the South China Sea (P.R. China), afforded two new hydroanthraquinone derivatives 4a-*epi*-9 α-methoxy-dihydrodeoxybostrycin and 10-deoxybostrycin together with seven known anthraquinones nigrosporin B, α-hydroxydihydrodesoxybostrycin, α-hydroxyhalorosellinia A, bostrycin, 4-deoxybostrycin, 3,5,8-trihydroxy-7-methoxy-2-methylanthracene-9,10-dione, and austrocortirubin as well as ten further acetylated derivatives. All isolated compounds were evaluated for their antibacterial activity against different bacterial strains. 10-Deoxybostrycin (**50**) and nigrosporin B (**51**), along with the acetylated compound 3-acetoxy-4-deoxybostrycin (**52**), displayed pronounced antibacterial activity. Compound **52** showed the strongest activity against *B. cereus* (ACCC 11077) and *Vibrio anguillarum* (ATCC 19019) with MIC values of 17.7 and 35.5 ng/ml, respectively. Nigrosporin B (**51)** revealed high antibiotic activity against *B. subtilis* (ATCC 6633) and *B. cereus* (ACCC 11077), with MIC values of 94.9 ng/ml against both bacteria. The MIC value of the positive control ciprofloxacin amounted to 414.2 ng/ml. SAR studies revealed that the cycloaliphatic ring C is essential for the activity [30].



10-Deoxybostrycin (**50**) : $R_1 = R_3 = R_4 =$ OH; $R_2 =$ H
Nigrosporin B (**51**) : $R_1 = R_3 =$ H, $R_2 = R_4 =$ OH
3-Acetoxy-4-deoxybostrycin (**52**) : $R_1 = R_2 =$ OH, $R_3 =$ H, $R_4 =$ OAc

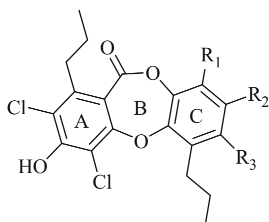
A structurally unprecedented alkaloid, curvulamine (**53**), was reported from the fungus *Curvularia* sp. IFB-Z10, isolated from the fish *Pennahia argentata* [31]. Isotope-feeding and enzyme inhibition studies suggested that the unique carbon framework of **53** is derived from two unprecedented oligoketide extender units that are formed via decarboxylative condensation between a tetraketide acyl-CoA substrate and the amino acid alanine. Notably, curvulamine (**53**) exhibited potent antibacterial activity toward a panel of patient-derived pathogens, including

Veillonella parvula, *Streptococcus sp.*, *Peptostreptococcus sp.*, and *Bacteroides vulgatus* with MIC values of 0.12 μg/ml, being more active than the antimicrobial agent tinidazole, which was used as a positive control (MICs, 0.12–0.5 μg/ml).

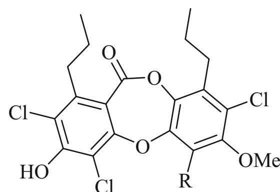


Curvulamine (**53**)

Chemical investigation of the fungus *Spiromastix* sp. MCCC 3A00308 that was isolated from deep-sea sediment collected in the South Atlantic Ocean (2,869 m depth) led to the discovery of two new classes of compounds with antimicrobial properties, the antibacterial chlorodepsidones, spiromastixones A–O, and the antiviral aromatic lactones, spiromastilactones A–M (see Sect. 4.1) [32, 33]. Spiromastixones F–J (**54**−**58**) displayed potent inhibitory effects against MRSA (MIC range from 1.92 to 14.9 μg/ml) as well as against methicillin-resistant *Staphylococcus epidermidis* (MRSE) strains (MIC range from 0.86 to 14.9 μg/ml). Likewise, **54** showed activity against vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* with MICs of 1.9 μg/ml. In addition, spiromastixones G (**55**), J (**58**), K (**59**), and L (**60**) showed pronounced antibacterial effects against the Gram-positive strains *S. aureus*, *Bacillus thuringiensis*, and *B. subtilis* with MIC values less than 2 μg/ml, comparable to those of the positive control penicillin G. However, all compounds were found to be inactive against the Gram-negative bacterium *E. coli* (MIC > 128 μg/ml), suggesting their selective antibacterial action. Moreover, comparison of the antibacterial properties of the isolated spiromastixones revealed that increase of chlorine substitution as well as O-methylation in ring C are essential structural features for the antibacterial activity of these compounds [32]. Thus, the pronounced growth inhibition effects of **54–60** toward several Gram-positive bacteria, including drug-resistant clinical isolates, render these metabolites attractive for further antibiotic development.



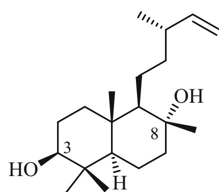
Spiromastixone F (**54**): R_1=H, R_2=OH, R_3=Cl
Spiromastixone G (**55**): R_1=H, R_2=OMe, R_3=Cl
Spiromastixone H (**56**): R_1=Cl, R_2=OH, R_3=H
Spiromastixone I (**57**): R_1=R_3=Cl, R_2=OH
Spiromastixone J: (**58**): R_1=R_3=Cl, R_2=OMe



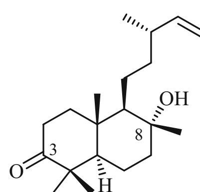
Spiromastixone K (**59**): R=H
Spiromastixone L (**60**): R= Cl

2.3 From Algae

Green algae are a prolific source of bioactive constituents. A prominent member of this group is *Ulva fasciata* Delile (family Ulvaceae), commonly known as "sea lettuce," which grows in coastal regions of Asia-Pacific [34]. Numerous compounds such as terpenes, polyphenolic compounds, and steroids were reported from *U. fasciata* [35]. These compounds include two potent antibacterial agents, labda-14-ene-3α,8α-diol (**61**) and labda-14-ene-8α-hydroxy-3-one (**62**) [36]. Both compounds were active against *Vibrio parahaemolyticus* and *Vibrio harveyi* (MIC, 30 µg/ml each). SAR studies revealed that electronegative hydroxyl or carbonyl group(s) are essential for proton exchange reactions with basic amino acyl residues in the active sites of virulent enzymes of these pathogenic bacteria.

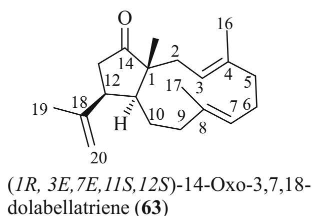


Labda-14-ene-3α,8α-diol (**61**)

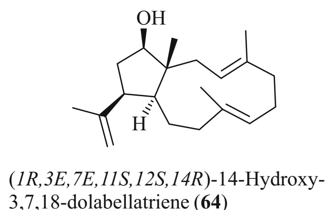


Labda-14-ene-8α-hydroxy-3-one (**62**)

Brown algae are commonly found in the tropical and subtropical waters, especially in the Atlantic, Pacific, and Indian Oceans, the Caribbean and Mediterranean Sea, and in the Sea of Japan. Secondary metabolites that are frequently isolated from brown algae are sesquiterpenes and diterpenes which have been shown to exhibit antibacterial, antiviral, cytotoxic, algicidal, antifouling, antifeedant, and/or ichthyotoxic activities [37]. Chromatographic separation of the CH_2Cl_2/MeOH extract of the brown alga *Dilophus spiralis* (collected at Elafonisos Island, Greece) yielded seventeen diterpenes featuring a dolabellane skeleton. These compounds showed weak to moderate activity against six strains of *S. aureus* including a standard laboratory strain (ATCC 25923), two epidemic MRSA strains (EMRSA-15 and EMRSA-16), a macrolide-resistant variant (RN4220), and two multi-drug-resistant effluxing strains (SA1199B and XU212). Reduction of (1*R*,*3E*,7*E*,11*S*,12*S*)-14-oxo-3,7,18-dolabellatriene (**63**) which was devoid of antibacterial activity using $NaBH_4$ yielded the C-14 epimeric alcohol (1*R*,3*E*,7*E*,11*S*,12*S*,14*R*)-14-hydroxy-3,7,18-dolabellatriene (**64**). Interestingly, the semisynthetic compound **64** showed antibacterial activity against all tested strains of *S. aureus*, with MIC values in the range of 2–4 µg/ml. The hydroxyl group at C-14 was crucial for antibacterial activity, whereas the presence of the ketone functionality at C-14 as found in the natural product rendered the compound inactive [38].



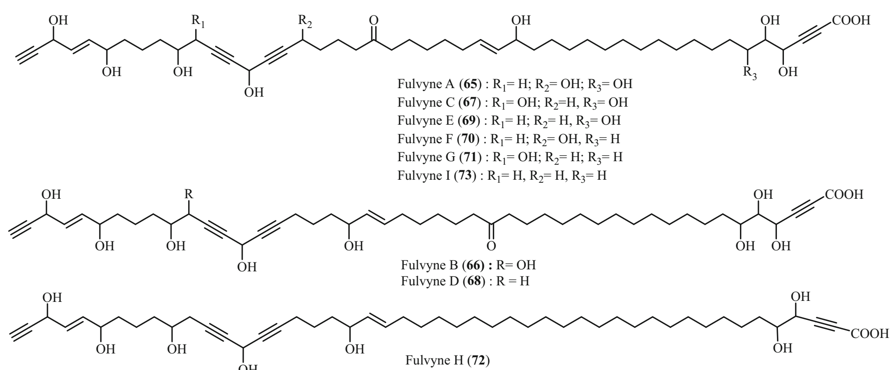
(*1R, 3E,7E,11S,12S*)-14-Oxo-3,7,18-dolabellatriene (**63**)



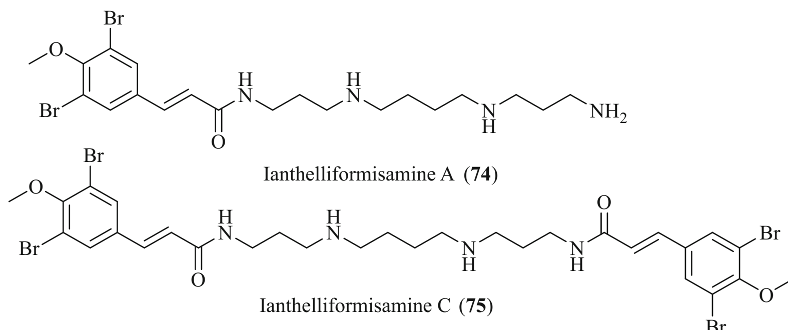
(*1R,3E,7E,11S,12S,14R*)-14-Hydroxy-3,7,18-dolabellatriene (**64**)

2.4 From Invertebrates

Haliclona sponges are well-known sources of polyacetylenic lipids. *Haliclona fulva* is a Mediterranean sponge, collected at the Gulf of Naples (Italy). Chromatographic workup of its butanolic extract revealed nine highly oxygenated acetylenes fulvynes A–I (**65**–**73**), whose structures include long linear alkyl chains with a rare propargylic acid terminal moiety and poly-oxygenated carbons. All isolated compounds exhibited potent antibacterial activity against a chloramphenicol-resistant strain (PY79) of *B. subtilis* with MICs in the range of 9.7–49.7 μg/ml [39].



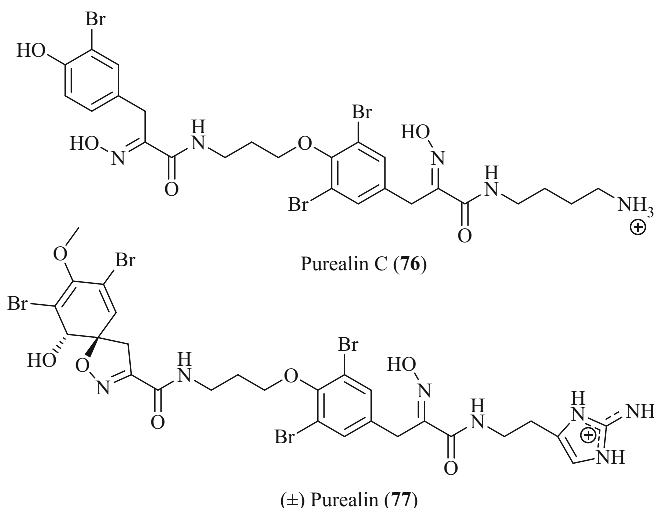
Three new bromotyrosine analogues ianthelliformisamine A–C along with the known aplysamine I and araplysillin I were isolated from the sponge *Suberea ianthelliformis* collected at North Stradbroke Island, Australia. Only ianthelliformisamines A and C (**74, 75**) exhibited antibacterial activity against the Gram-negative bacterium *P. aeruginosa* with MICs of 18.2 and 14.7 μg/ml, respectively, while **75** showed activity against *S. aureus* with a MIC of 7.33 μg/ml. The spermine nucleus in **74** and **75** is essential for the antibiotic activity against *P. aeruginosa* [40].



Ianthelliformisamine A (**74**)

Ianthelliformisamine C (**75**)

Further bromotyrosine derivatives were isolated from two marine sponges of the genus *Pseudoceratina* collected off Port Campbell, Victoria, southern Australia. Chromatographic workup of the first sponge afforded twelve bromotyrosine derivatives, including the new derivatives aplysamine-7, (−)-purealin B, purealin C (**76**), and purealin D and two new enantiomers (−)-purealidin R and (−)-aerophobin-2 along with five known compounds, while the second *Pseudoceratina* sponge yielded the first reported racemic bromotyrosine-analogue (±)-purealin (**77**) as a new compound along with the known purealidin A. Among the isolated compounds, only purealin C (**76**) and (±)-purealin (**77**) revealed significant broad-spectrum antibacterial activity against different *S. aureus* and *B. subtilis* bacterial strains with MIC values ranging from 0.6 to 5.4 μg/ml [41].

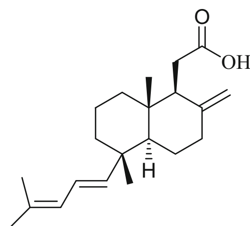


Purealin C (**76**)

(±) Purealin (**77**)

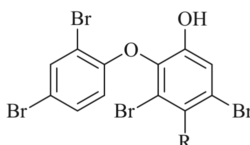
The new antibacterial bicyclic terpene derivative clathric acid (**78**) was obtained from the methanolic extract of the sponge *Clathria compressa*, collected at Panama City, Florida (USA), along with two new *N*-acyl taurine derivatives clathrimides A and B. Only clathric acid (**78**) exhibited antibacterial activity against different Gram-positive bacterial strains including *S. aureus* (ATTC 6538P), MRSA

(ATTC 33591), and vancomycin-resistant *S. aureus* (VRSA) with MIC values of 32, 64, and 64 μg/ml, respectively [42].



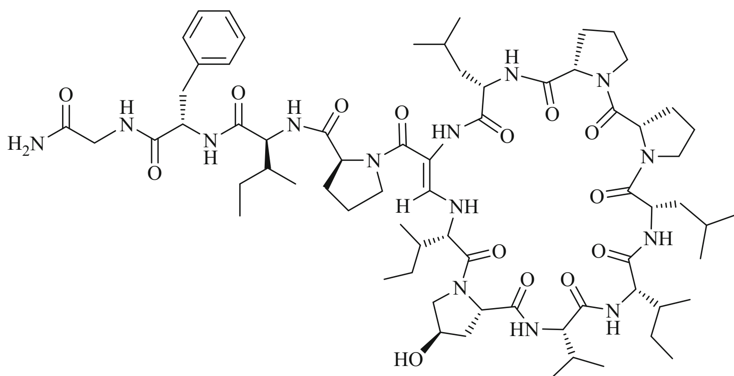
Clathric acid (**78**)

Bioassay-guided isolation of the MeOH extract of the Indo-Pacific marine sponge *Dysidea granulosa* afforded the polybrominated diphenyl ethers 2-(2′,4′-dibromophenoxy)-3,5-dibromophenol (**79**) and 2-(2′,4′-dibromophenoxy)-3,4,5-tribromophenol (**80**) [43]. The in vitro antibacterial potency of **79** and **80** was evaluated toward a panel of 14 foodborne and clinical human pathogens. Notably, both compounds were found to be potent inhibitors of the Gram-positive bacteria *B. cereus*, *Listeria monocytogenes*, and MRSA with MIC values of 0.1 μg/ml. Moreover, **79** exerted a broader spectrum of activity, effectively inhibiting the Gram-negative bacterium *Klebsiella pneumoniae* (MIC = 0.1 μg/ml), comparable or even more active than the antimicrobial agents ciprofloxacin (MIC = 0.125 μg/ml), cefoxitin (MIC = 0.25 μg/ml), and imipenem (MIC = 0.25 μg/ml), which were used as positive controls. These results suggested that the 3,5-dibromophenol substitution of **79** plays an important role in mediating antimicrobial activity, and thus this metabolite represents a potential lead scaffold for drug development against MRSA- and *K. pneumoniae*-associated infections, which are difficult to treat with currently available antibiotics [43].



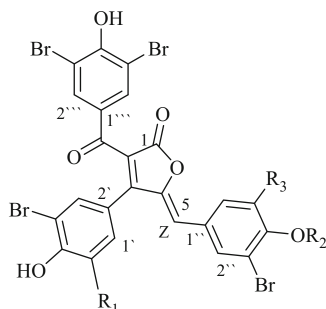
2-(2',4'-Dibromophenoxy)-3,5-dibromophenol (**79**): R=H
2-(2',4'-Dibromophenoxy)-3,4,5-tribromophenol (**80**): R=Br

A unique class of proline-rich peptides, callyaerins A–M, were obtained from the Indonesian marine sponge *Callyspongia aerizusa* [44]. Callyaerins feature an unusual core structure comprising a cyclic peptide part and a linear peptide side chain, which are connected through a rare, non-proteinogenic (Z)-2,3-diaminoacrylic acid (DAA) moiety. Among the isolated compounds, callyaerin A (**81**) exhibited the strongest activity against *M. tuberculosis* with a MIC_{90} of 2.7 μg/ml. In addition, **81** was found to be inactive against THP-1 (human acute monocytic leukemia) and MRC-5 (human fetal lung fibroblast) cells (IC_{50} >10 μg/ml), highlighting its potential as a promising antitubercular lead compound.



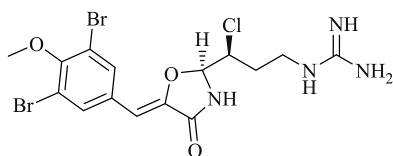
Callyaerin A (**81**)

Marine tunicates are a prolific source for biologically active secondary metabolites such as peptides and alkaloids having anticancer, antiviral, and antifungal or antibacterial activities [45, 46]. Lyophilized specimens of the colonial tunicate *Pseudodistoma antinboja* collected at a depth of 10−15 m off the shore of Tong-Yeong City, South Sea, Korea, were extracted with 50% MeOH/ CH_2Cl_2 yielding nine butenolides. Only cadiolides C–G (**82–86**) displayed antibacterial activity against a panel of Gram-positive strains including methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains with MIC ranging from 0.13 to 12.5 μg/ml. Among the tested compounds, cadiolide C (**82**) was the most potent one, showing equal or even stronger activity compared to the well-known antibiotic drug vancomycin (MIC, 0.5–1 μg/ml). The location of bromine atoms was found to be important for the antibiotic activity of the investigated compounds. The free phenolic group enhances the activity, whereas its methylation reduces the activity as shown for **85** which is less active than **82** while permethylation resulted in a complete loss of activity [47].

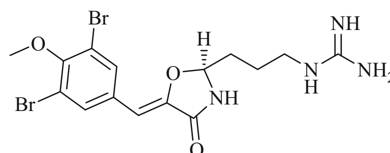


Cadiolide C (**82**) : R_1=R_2=R_3=H
Cadiolide D (**83**) : R_1= Br, R_2=R_3=H
Cadiolide E (**84**) : R_1=R_2=H, R_3= Br
Cadiolide F (**85**) : R_1=H, R_2=Me, R_3= H (*E/Z*)
Cadiolide G (**86**) : R_1=Br, R_2=H, R_3=Br

The colonial ascidian, *Synoicum* sp., is a prominent source of numerous secondary metabolites showing a variety of biological activities. Examples include cytotoxic palmerolide A, ecdysteroids, a tetrahydrocannabinol derivative, prunolides A–C, a number of rubrolides, the antidiabetic tiruchanduramine, and the anti-inflammatory halogenated furanone *E*/Z-rubrolide O. *S. pulmonaria* is abundant in the Arctic-boreal waters of the North Sea and in the deep cold waters of West Scotland and Northern Ireland [48]. The sub-Arctic ascidian *S. pulmonaria*, obtained off the Norwegian coast, afforded novel brominated guanidinium oxazolidinones named synoxazolidinones A and B (**87** and **88**) [49]. The 4-oxazolidinone ring present in compounds **87** and **88** is rare in natural products. Synoxazolidinone A (**87**) showed promising antibacterial activity against the Gram-positive *S. aureus* as well as against MRSA with MIC values of 10 µg/ml in both cases. Moreover, **87** featured a MIC value of 6.25 µg/ml when tested against the Gram-positive bacterium *Corynebacterium glutamicum*. Synoxazolidinone B (**88**) displayed lower activities against MRSA (MIC of 30 µg/ml). This weaker activity of **88** compared to **87** corroborates the importance of the chlorine substituent for the antibacterial activity [49].

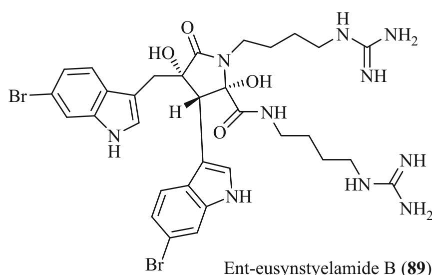


Synoxazolidinone A (**87**)

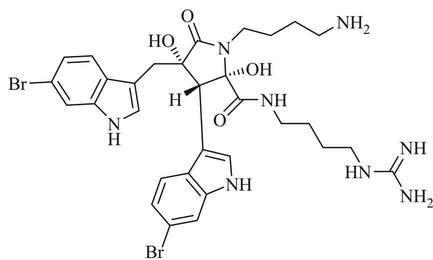


Synoxazolidinone B (**88**)

The Arctic bryozoan *Tegella cf. spitzbergensis* yielded four brominated eusynstyelamide alkaloids including ent-eusynstyelamide B and eusynstyelamides D–F. Ent-eusynstyelamide B (**89**) is the enantiomer of the known brominated tryptophan metabolite eusynstyelamide B [50]. These compounds were tested against several bacteria including *S. aureus*, *E. coli*, *P. aeruginosa*, *C. glutamicum*, and MRSA. Ent-eusynstyelamide B and eusynstyelamide F (**89** and **90**) exhibited antibacterial activity with MIC values as low as 6.25 µg/ml against *S. aureus* (ATTC 9144) and MIC values of 12.5 and 6.25 µg/ml against *C. glutamicum* (ATTC 13032), whereas the MIC value against *E. coli* (ATCC 25922) was 12.5 µg/ml. Moreover, (**89**) exhibited a MIC value of 20 µg/ml against MRSA (ATCC 33591).



Ent-eusynstyelamide B (**89**)



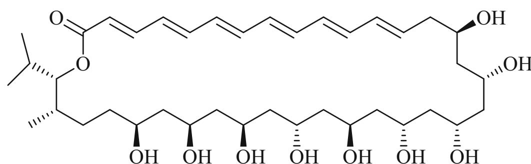
Eusynstyelamide F (**90**)

3 Antifungal Compounds

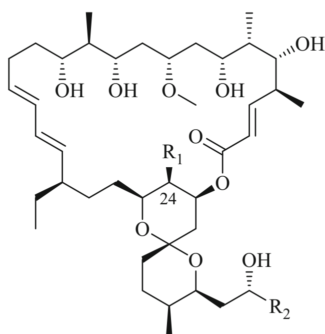
3.1 From Bacteria

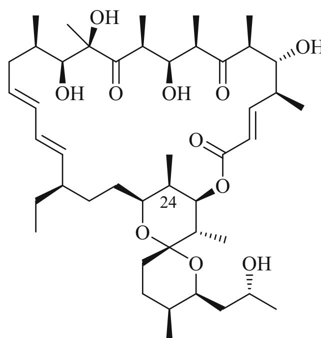
Actinobacteria are prolific sources for many important antibiotics such as aminoglycosides, erythromycin, tetracyclines, or vancomycin [51]. *Streptomyces* sp. CN Q3 43 was isolated from a sediment sample collected at North Cat Cay at the Bahamas and cultivated on seawater-based medium. Chromatographic workup of the extract yielded two polyene polyol (36-membered) macrocyclic lactones bahamaolides A and B. Bahamaolide A (**91**) showed antifungal activity against various pathogenic fungi with a MIC value of 12.5 μg/ml and exhibited strong inhibitory activity against *C. albicans* isocitrate lyase (ICL) with an IC_{50} value of 7.65 μg/ml which is almost equal to that of 3-nitro-propionate ($IC_{50} = 6.84$ μg/ml) which was used as a positive control [52].

Chromatographic workup of the culture broth of *Actinoalloteichus* sp. NPS702, obtained from sediment of Usa Bay, Kochi Prefecture, Japan, afforded nine new 26-membered macrolides of the oligomycin class including neomaclafungins A–I (**92–100**) along with oligomycin A (**101**). Compounds **92–100** revealed potent antifungal activity against *Trichophyton mentagrophytes* (ATCC 9533), with MIC values ranging from 1 to 3 μg/ml, whereas oligomycin A (**101**) was the least potent compound encountered (MIC = 10 μg/ml) [53].



Bahamaolide A (**91**)

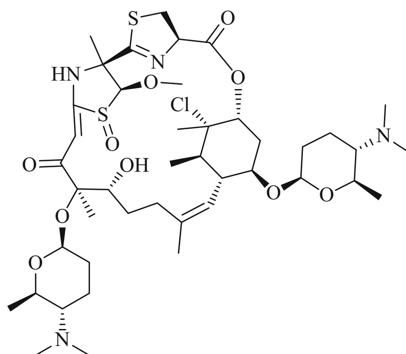




Oligomycin A (**101**)

	R_1	R_2
Neomaclafungin A (**92**):	$CH_2CH_2CH_2OH$	CH_3
Neomaclafungin B (**93**):	$CH_2CH_2CH(CH_3)OH$	CH_3
Neomaclafungin C (**94**):	$CH_2CH_2CH_2OH$	CH_2CH_3
Neomaclafungin D (**95**):	$CH_2CH_2CH(CH_3)OH$	CH_2CH_3
Neomaclafungin E (**96**):	CH_2CH_3	CH_3
Neomaclafungin F (**97**):	$CH_2CH_2CH_3$	CH_3
Neomaclafungin G (**98**):	$CH_2CH_2CH_2CH_3$	CH_3
Neomaclafungin H (**99**) :	$CH_2CH_2CH_3$	CH_2CH_3
Neomaclafungin I (**100**) :	$CH_2CH_2CH_2CH_2CH_3$	CH_3

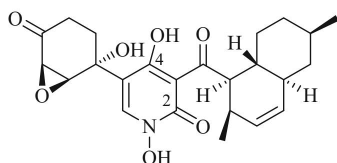
Chemical investigation of a marine-derived actinobacterium *Actinomadura* sp., obtained from the ascidian *Ecteinascidia turbinata*, led to the isolation of a novel polyketide forazoline A (**102**) [54]. Interestingly, **102** exhibited in vivo efficacy in a murine model of infection with *C. albicans* at concentrations ranging from 0.125 to 2.5 mg/kg, comparable to those of amphotericin B, without any apparent toxicity. A chemical genomic approach with the yeast *S. cerevisiae* indicated that **102** possibly dysregulates phospholipid homeostasis, thus affecting the cell membrane integrity. To further evaluate this hypothesis, membrane permeability studies were employed, which showed that **102** causes permeabilization of fungal membranes in a dose-dependent manner, corroborating its putative mode of action. Moreover, **102** was found to exert a synergistic effect with the clinically used antifungal agent amphotericin B and thus represents a potential lead scaffold for further development of anti-*C. albicans* agents [54].



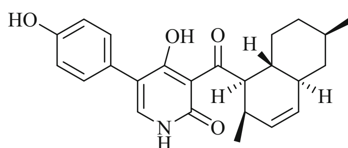
Forazoline A (**102**)

3.2 From Fungi

Deep-seated candidiasis is a fungal infection that is commonly treated with azoles. However, recent years have seen an increasing number of reports on resistance of *Candida* strains against azole derivatives [55]. This calls for the discovery of new antifungal drugs targeted against azole-resistant *C. albicans* strains. Four new alkaloids featuring a 4-hydroxy-2-pyridone moiety including didymellamides A–D were obtained from the fungus *Stagonosporopsis cucurbitacearum*, isolated from an unidentified sponge collected at Atami-shi, Shizuoka Prefecture, Japan. Didymellamide A (**103**) exhibited broad antifungal activity against azole-resistant strains as well as against azole-sensitive *C. albicans*, C. *glabrata*, and *Cryptococcus neoformans* with MIC values of 1.6–3.1 μg/ml, respectively, while didymellamide B (**104**) was only activity against *C. neoformans* with a MIC value of 6.3 μg/ml [56].



Didymellamide A (**103**)

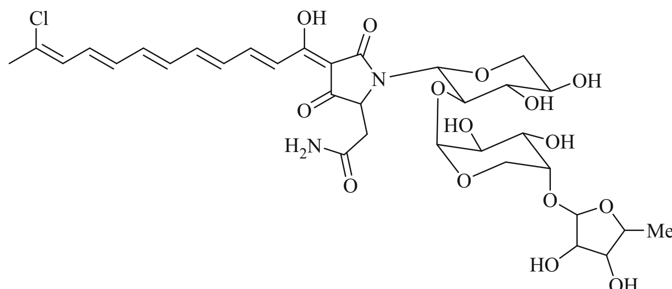


Didymellamide B (**104**)

3.3 From Invertebrates

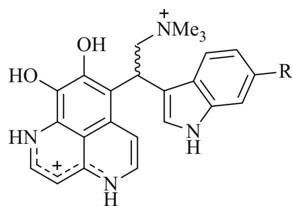
Sponges of the genus *Melophlus* are well-known sources for chemically diverse bioactive secondary metabolites such as tetramic acid derivatives [57]. The unprecedented tetramic acid glycoside aurantoside K (**105**) was isolated from *Melophlus* sp. collected at the Fiji Islands, Melanesia. The compound showed pronounced

antifungal activity against amphotericin-resistant *C. albicans* and wild-type *C. albicans* with MIC values of 31.25 and 1.95 μg/ml, respectively [58].

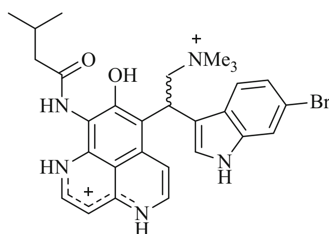


Aurantoside K (**105**)

Aaptamine-type alkaloids are heteroaromatic alkaloids with a 1*H*-benzo[de][1,6] naphthyridine ring system showing a wide range of bioactivities including antimicrobial, antiviral, antioxidant, and cytotoxic activities [59, 60]. Seven new compounds including six new aaptamine alkaloids were isolated from an Okinawan marine sponge *Suberites* sp. collected from Unten Port, Nakijin, Okinawa, Japan. Among the purified compounds, nakijinamine A (**106**) displayed antifungal activity against *C. albicans*, *C. neoformans*, and *T. mentagrophytes* with MIC values of 0.25, 0.5, and 0.25 μg/ml, respectively, as well as antibacterial activity against *S. aureus*, *B. subtilis*, and *M. luteus* with MIC values of 16, 16, and 2 μg/ml, respectively. Nakijinamines B (**107**) and F (**108**) exhibited antifungal activity against *C. albicans* (MIC value of 8 μg/ml, for both compounds) [61].

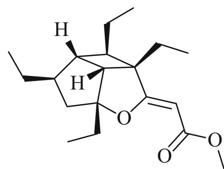


Nakijinamine A (**106**) : R= Br
Nakijinamine B (**107**) : R= H



Nakijinamine F (**108**)

The sponge *Hippospongia lachne* collected in the South China Sea (P.R. China) is a promising source for antifungal drugs. Bioassay-guided fractionation of the EtOH extract of *H. lachne* yielded the polyketide hippolachnin A (**109**), featuring a rare four-membered carbon ring. Hippolachnin A exhibited potent antifungal activity against the pathogenic fungi *C. neoformans*, *Trichophyton rubrum*, and *Microsporum gypseum*, with MIC values of 0.13 μg/ml, respectively [62].

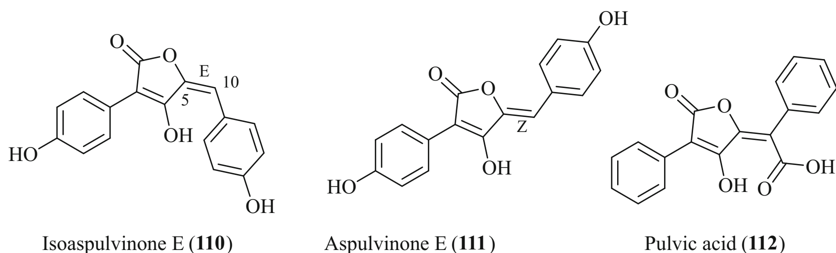


Hippolachnin A (**109**)

4 Antiviral Compounds

4.1 From Fungi

Influenza type A, caused by the virus subtype H1N1, is a life-threatening disease which leads to 20−50 million deaths worldwide [63]. Until now, very few drugs are available to treat H1N1 viral infections [64]. Gao et al., in 2013, succeeded in obtaining three antiviral butenolides including isoaspulvinone E (**110**), aspulvinone E (**111**), and pulvic acid (**112**) from the fungus *Aspergillus terreus* Gwq-48, isolated from mangrove rhizosphere at the coast of Fujian province (P.R. China). The compounds exhibited significant anti-influenza A H1N1 activities with IC_{50} values of 32.3, 56.9, and 29.1 μg/ml, respectively, compared to the positive controls ribavirin (IC_{50} 24.6 μg/ml) or zanamivir (IC_{50} 28.4 ng/ml). In addition, compound **110** showed anti-H1N1 viral neuraminidase activity (NA). Docking of **110** and **111** into NA active sites indicated that the *E* double bond $\Delta^{5(10)}$ is essential for activity [65].



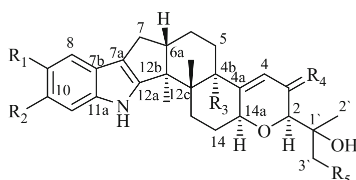
Isoaspulvinone E (**110**) Aspulvinone E (**111**) Pulvic acid (**112**)

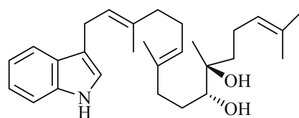
Further anti-H1N1 metabolites were isolated from the culture broth of the fungus *Penicillium camemberti* OUCMDZ-1492, obtained from marine sediment at mangrove roots of *Rhizophora apiculata* collected from Hainan Province (P.R. China). Chromatographic workup yielded six new indole-diterpenoids along with five known compounds. Among the isolated compounds, 3-deoxo-4b-deoxypaxilline (**113**), 4a-demethylpaspaline-4a-carboxylic acid (**114**), 4a-demethylpaspaline-3,4,4a-triol (**115**), 9,10-diisopentenylpaxilline (**116**), (6*S*,7*R*, 10*E*,14*E*)-16- (1*H*-indol-3-yl)- 2, 6, 10, 14- tetramethylhexadeca-2,10,14-triene-6,7-diol (**117**), emindole SB (**118**), 21-isopentenylpaxilline (**119**), paspaline (**120**), and paxilline (**121**) revealed significant activity against the H1N1 virus with IC_{50} values ranging

from 3.32–32.8 μg/ml compared to the positive control ribavirin 27.6 μg/ml. The most potent compound was **119**, while **120** was the least active one. SAR studies indicated that:

1. The anti-H1N1 activity of hexacyclic indole-diterpenoids increased due to the presence of a methylene group as in **113**, a carboxy/or hydroxy group at position 4a as in **114** and **115**, a hydroxy group at position 4b as in **121,** and an isopentenyl group at position 9 as in **119**.
2. The anti-H1N1 activity decreased by phenyl isoprenylation as in **116**.
3. The pronounced activity of **117** with the aliphatic diterpene moiety and of **118** with the pentacyclic indole-diterpene proved that the cyclic diterpenoid nucleus has a limited effect on the activity.

Moreover, the presence of 3-oxo and 4b-hydroxy, together with 9-isopentenyl substituents in **119**, was responsible for the highest anti-H1N1 activity among the separated compounds [66].

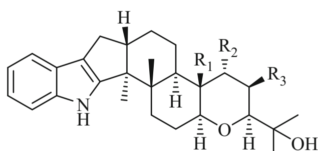


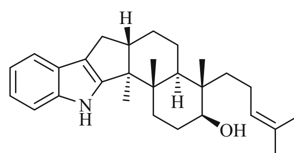


3-Deoxo-4b-deoxypaxilline (**113**) : R_1=R_2=R_3=R_5=H, R_4= H_2
9,10-Diisopentenylpaxilline (**116**) : R_1=R_2= [isopentenyl], R_3=OH, R_4=O, R_5=H

(6*S*,7*R*, 10*E*,14*E*)-16- (1H-Indol-3-yl)-2, 6, 10, 14-tetramethylhexadeca-2,10,14-triene-6,7-diol (**117**)

21-Isopentenyl-paxilline (**119**) : R_1= [isopentenyl], R_2=R_5=H, R_3=OH, R_4=O
Paxilline (**121**) : R_1=R_2=R_5=H, R_3=OH, R_4=O

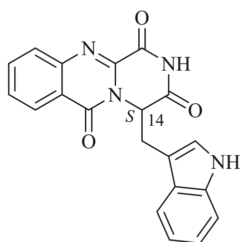




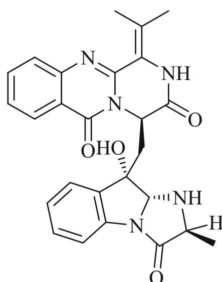
4a-Demethylpaspaline-4a-carboxylic acid (**114**) : R_1=CO_2H, R_2=R_3=H
4a-Demethylpaspaline-3,4,4a-triol (**115**) : R_1=R_2=R_3=OH
Paspaline (**120**) : R_1=CH_3,R_2=R_3=H

Emindole SB (**118**)

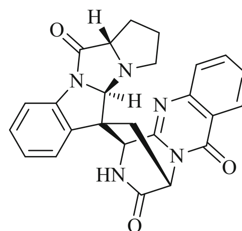
The fungus *Cladosporium* sp. PJX-41 was isolated from mangrove sediment in Guangzhou (P.R. China). Chromatographic workup afforded six new indole alkaloids, five of them being glyantrypine analogues. Six alkaloids including (14*S*)-oxoglyantrypine (**122**), norquinadoline A (**123**), deoxynortryptoquivaline (**124**), deoxytryptoquivaline (**125**), tryptoquivaline (**126**), and quinadoline B (**127**) showed antiviral activity against influenza virus A (H1N1), with IC_{50} values ranging from 30.4 to 48.6 μg/ml comparable to the positive control ribavirin that exhibited an IC_{50} value of 21.2 μg/ml [67].



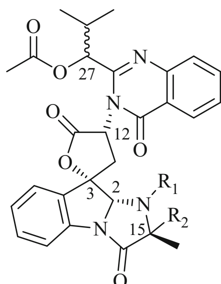
(14-*S*) Oxoglyantrypine (**122**)



Norquinadoline A (**123**)

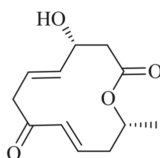


Quinadoline B (**127**)



Deoxynortryptoquivaline (**124**) : R_1=H, R_2=H; (2*R*, 3*S*, 12*R*, 15*S*, 27*S*)
Deoxytryptoquivaline (**125**) : R_1=H, R_2=CH_3; (2*R*, 3*S*, 12*R*, 27*S*)
Tryptoquivaline (**126**) : R_1=OH, R_2=CH_3; (2*S*, 3*S*, 12*R*, 27*S*)

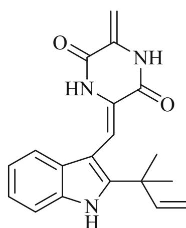
The potent antiviral metabolite, balticolid (**128**), was isolated from an unidentified fungus collected at the coast of the Greifswalder Bodden, Baltic Sea, Germany [68]. Balticolid (**128**) displayed strong activity against influenza A virus and herpes simplex virus (HSV) type I with an IC_{50} value of 0.10 μg/ml but was nontoxic to eukaryotic cells when investigated at similar concentrations.



Balticolid (**128**)

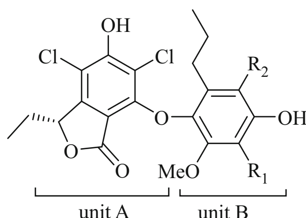
Bioassay-guided fractionation of a crude extract of the deep-sea-derived fungus *Eurotium rubrum* F33, isolated from sediment collected from the South Atlantic Ocean (2067 m depth), afforded a series of prenylated indole diketopiperazine alkaloids with antiviral activity toward the influenza virus strain A/WSN/33 (H1N1) [69]. All compounds were tested against H1N1 propagated in Madin-Durby canine kidney (MDCK) cells and the known compound neoechinulin B (**129**) exhibited the strongest antiviral activity with an IC_{50} value of 8.8 μg/ml. Moreover, **129** was found to exert high potency against oseltamivir- or amantadine- and ribavirin-resistant

influenza clinical isolates with IC_{50} values ranging from 5.4 to 7.1 μg/ml, respectively. However, **129** showed lack of cytotoxicity against MDCK cells, highlighting its selective antiviral activity. Subsequent mode-of-action studies, employing hemagglutination inhibition, surface plasmon resonance, and polykaryon assays, revealed that **129** binds to the HA1 subunit of hemagglutinin glycoprotein, blocking its interaction with the sialic acid receptor, and thus inhibits the attachment of H1N1 virus to host cells. Interestingly, **129** was found to inhibit influenza virus (strain A/WSN/33) propagation without causing viral resistance, as shown in a multi-passage experiment, employing a plaque formation assay (even after a fifth passage), in contrast to clinically used anti-influenza drugs, such as amantadine [69]. Hence, these results demonstrated that **129** may serve as a useful lead compound for the development of new anti-influenza agents.



Neoechinulin B (**129**)

Further investigation of the fungus *Spiromastix* sp. MCCC 3A00308 (see Sect. 2.2) afforded an array of new aromatic lactones with various chlorine atom substituents, spiromastilactones A–M [33]. Bioactivity assays of these compounds revealed that most of the derivatives, bearing a pseudo-depsidone skeleton, possess antiviral activity. Spiromastilactones D (**130**) and E (**131**) showed the strongest antiviral activity against A/WSN/33 (H1N1) influenza virus propagated in MDCK cells with IC_{50} values of 2.8 and 5.3 μg/ml, respectively, similar to those of the positive controls oseltamivir (3.1 μg/ml) and amantadine (1.97 μg/ml). These findings allowed to conclude that monochlorination of ring B, in contrast to dichlorination, is important for expression of the strong antiviral activity of these metabolites. Moreover, formation of the pseudo-depsidone scaffold is essential for mediating antiviral properties, since the respective monomers spiromastilactone A (unit A) and divaric acid (unit B) were inactive. The most active compound, spiromastilactone D (**130**), exhibited a broad antiviral spectrum toward influenza A and B viruses, including oseltamivir- or amantadine-resistant clinical isolates, and therefore was chosen for further mode-of-action and molecular docking studies. Accordingly, **130** was shown to potentially bind to the hemagglutinin protein (HA1) subunit, adjacent to the sialic acid receptor binding pocket, thus disrupting the HA-sialic acid receptor interaction, which is crucial for the attachment of influenza viruses to host cells. Moreover, **130** was found to inhibit viral genome replication, probably via interfering with the viral ribonucleoprotein complex [33], verifying this metabolite as a potential anti-influenza lead structure for further development.

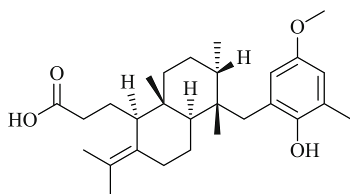


Spiromastilactone D (**130**) : $R_1 = H, R_2 = Cl$
Spiromastilactone E (**131**): $R_1 = Cl, R_2 = H$

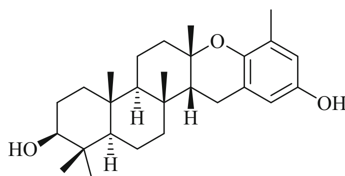
4.2 From Algae

Paramyxoviridae type viruses include both human metapneumovirus (HMPV), respiratory syncytial virus (RSV), and parainfluenza virus (PIV) [70]. Among them, HMPV is the most dangerous one as it can cause significant morbidity especially in infants and in elderly patients [71]. The brown seaweed *Stypopodium zonale* which is common along the Brazilian coast accumulates mainly meroditerpenes, with several of them exhibiting antiviral activity. Two meroditerpenoids atomaric acid[1] (**132**) [72] and epitaondiol (**133**) were obtained from *S. zonale*, and the methyl ester of atomaric acid (**134**) was prepared by semi-synthesis [73]. Compounds **132–134** exhibited antiviral activity against HMPV replication and showed selectivity indices of >56.81, 49.26, and 12.82, respectively. Epitaondiol (**133**) exhibited potent anti-HMPV activity ($IC_{50} = 1.01$ μg/ml) compared to atomaric acid methyl ester (**134**; IC_{50}= 2.66 μg/ml) and its parent compound atomaric acid (**132**) (IC_{50}= 3.52 μg/ml). Each compound exerted its effect by a unique mechanism: atomaric acid interacts with viral particles outside of the host cells thus preventing infection of the cell cultures. The compound has no effect on cellular receptors or on viral penetration. The meroditerpene epitaondiol (**133**) inhibits the penetration of viral particles into cells but without affecting the post-penetration stages or interacting with cellular receptors. The methyl ester of atomaric acid inhibits penetration of viral particles without exhibiting any effect on the post-penetration events and cellular receptors. These compounds showed selective antiviral activity with low cytotoxic activity against LLC-MK2 cells, thus highlighting their potential to inhibit HMPV in vitro [73].

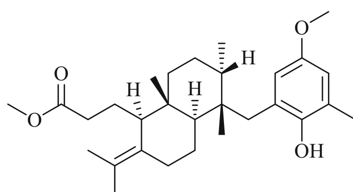
[1]The structures of atomaric acid is drawn incorrectly in the original manuscript [72], and it is drawn correctly in this chapter.



Atomaric acid (**132**)

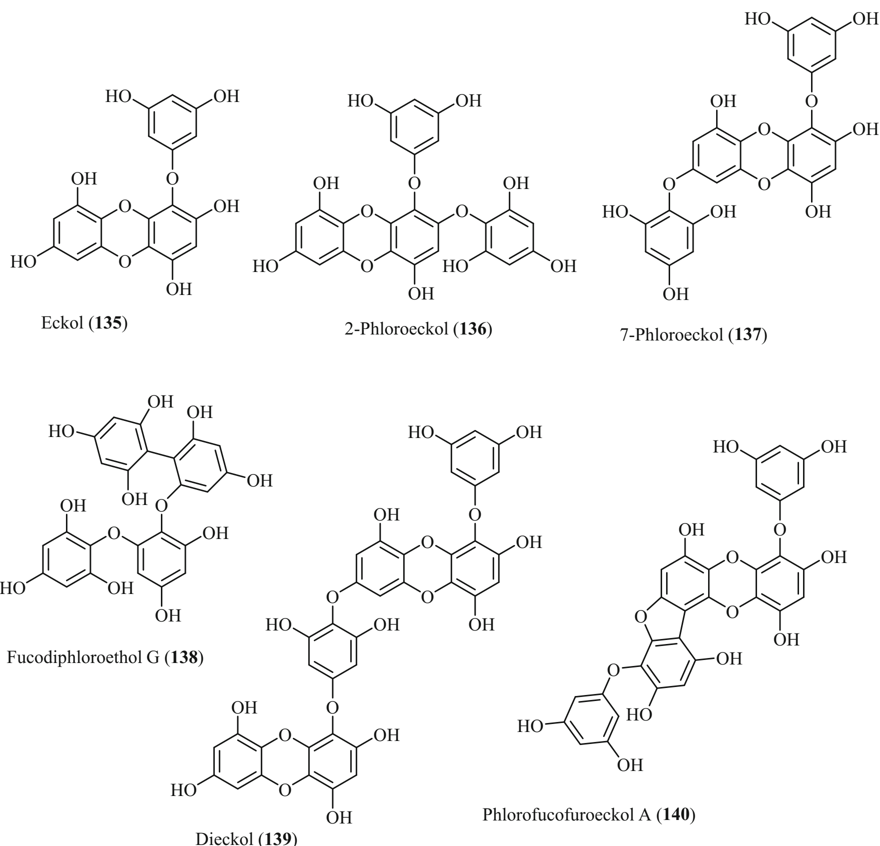


Epitaondiol (**133**)



Methyl ester of atomaric acid (**134**)

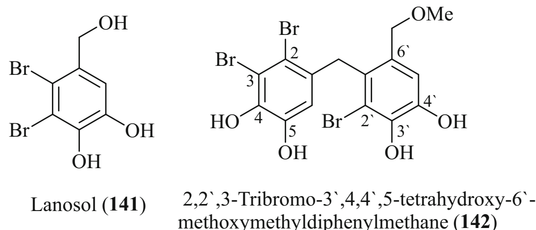
Severe acute respiratory syndrome (SARS) is a severe pneumonia caused by a novel human coronavirus (SARS-CoV); its SARS-CoV 3CL^{pro} plays a crucial role in viral replication [74]. Betulinic acid [75], indigo, aloe emodin [76], the biflavonoids amentoflavones [77], and quinone-methide triterpenoids [78] are among its naturally occurring inhibitors. Park et al. [79], succeeded in isolating nine phlorotannins from the ethanol extract of *Ecklonia cava*, an edible brown alga. Six of the isolated compounds showed anti-SARS-CoV 3CL^{pro} activity including the phlorotannin derivatives eckol (**135**), 2-phloroeckol (**136**), 7-phloroeckol (**137**), fucodiphloroethol G (**138**), dieckol (**139**), and phlorofucofuroeckol A (**140**) with IC_{50} values of 2.0–20.9 μg/ml. Dieckol (**139**) was the most potent derivative encountered, with an IC_{50} value of 2.0 μg/ml, compared to 18.1 μg/ml for the positive control hesperetin [79].



Eckol (**135**) 2-Phloroeckol (**136**) 7-Phloroeckol (**137**)

Fucodiphloroethol G (**138**) Dieckol (**139**) Phlorofucofuroeckol A (**140**)

Chromatographic workup of the red alga *Neorhodomela aculeata* collected at the port of Namae, South Korea, afforded the new compound lanosol along with five known polybromocatechols. Two of the isolated compounds exhibited antiviral activity: 2,3-dibromo-4,5-dihydroxybenzyl alcohol (lanosol, **141**) and polybromocatechol (2,2′,3-tribromo-3′,4,4′,5-tetrahydroxy-6′-methoxymethyl-diphenylmethane, **142**). Compound **141** exhibited antiviral activity against HRV2 with an IC_{50} value of 2.50 µg/ml, while compound **142** revealed anti-HRV2 activity, with an IC_{50} value of 7.11 µg/ml, and anti-HRV3 activity with an IC_{50} value of 4.69 µg/ml. The positive control ribavirin exhibited IC_{50s} of 2.15 and 5.09 µg/ml against HRV2 and HRV3, respectively. Compounds **141** and **142** were not cytotoxic making them potential drug candidates for new antiviral drugs against two different human rhinoviruses [80].

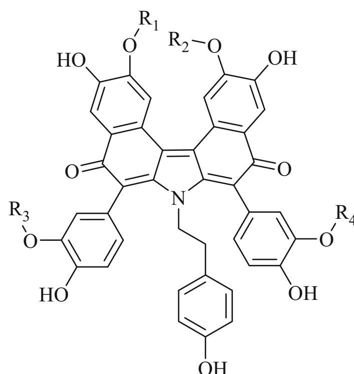


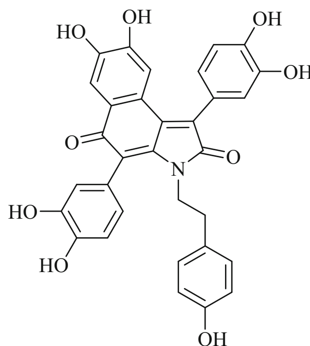
Lanosol (**141**) 2,2`,3-Tribromo-3`,4,4`,5-tetrahydroxy-6`-methoxymethyldiphenylmethane (**142**)

4.3 From Invertebrates

DOPA (2-amino-3-(3′,4′-dihydroxyphenyl) propionic acid) is a key biogenetic precursor for many structurally unique alkaloids. Naturally occurring alkaloids with pyrrole core structures are examples of DOPA-derived alkaloids and are characterized by different substitution patterns [81]. Based on the cyclic condensation and substitution pattern of the pyrrole ring, these compounds are classified as lamellarins, lukianols, polycitrins, polycitons, storniamides, and ningalins. DOPA-derived alkaloids were found to exert several pharmacological activities, such as cytotoxicity, HIV-1 integrase inhibition, and multidrug resistance reversal activity in addition to immunomodulatory activity [82]. Fifteen new DOPA-derived pyrrole alkaloids, baculiferins A–O, were isolated from the marine sponge *Iotrochota baculifera* collected in the South China Sea (P.R. China) [82]. All compounds were found to feature one to three *O*-sulfate units. Among the isolated compounds, baculiferins C, E–H, and K–N (**143**–**151**) exhibited potent inhibitory activity against HIV-1 IIIB virus with IC_{50} values ranging from 1.4 to 8.6 μg/ml. Interestingly, these compounds showed weak to moderate activity against the tumor cell lines HCT-8, Bel-7402, BGC-823, A549, and A2780.

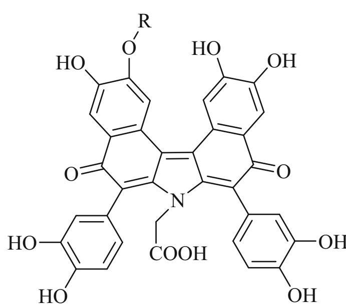
The binding activities of these compounds to HIV-1 targets including recombinant gp41 (a transmembrane protein of HIV-1), Vif (viral infectivity factor of HIV-1), and human APOBEC3G (an innate intracellular antiviral factor) were evaluated to elucidate the mechanism of their anti-HIV activities. Baculiferins containing *N*-acetyl groups (**149** and **150)** were found to show the highest binding affinities toward both Vif (RU >1800) and APOBEC3G (RU >2170). The binding activity of these compounds suggested that the antiviral activity of the compounds is due to their interaction with the targets Vif, APOBEC3G, and/or gp41 [82].

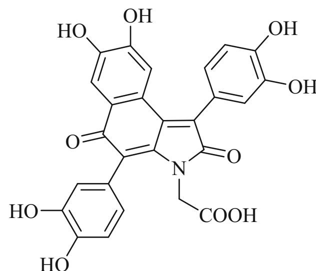




Baculiferin C (**143**) : $R_1=R_2=H$, $R_3=R_4=SO_3H$
Baculiferin E (**144**) : $R_1=R_3=SO_3H$, $R_2=R_4=H$
Baculiferin F (**145**) : $R_1=R_4=SO_3H$, $R_2=R_3=H$
Baculiferin G (**146**) : $R_1=R_3=R_4=SO_3H$, $R_2=H$
Baculiferin H (**147**) : $R_1=R_2=R_3=SO_3H$, $R_4=H$

Baculiferin K (**148**)

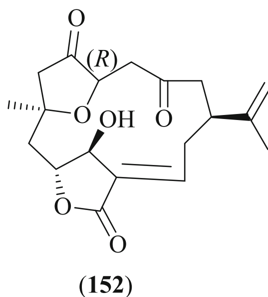




Baculiferin L (**149**) : R=H
Baculiferin M (**150**) : $R=SO_3H$

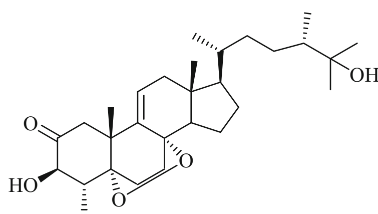
Baculiferin N (**151**)

Soft corals of the genus *Sinularia* (Alcyoniidae) are well-known sources of macrocyclic norcembranoids. Many of these macrocyclic norcembranoids were found to exhibit a wide array of bioactivities such as antifungal and cytotoxic properties. *Sinularia gyrosa* collected along the coast of the Dongsha Atoll off Taiwan was shown to yield several of these metabolites [83]. Purification of the acetone extract of this soft coral gave rise to seven new norcembranoids, gyrosanoids A–G, and in addition to known norcembranoids [84]. Among these metabolites, compound (**152**) was found to show significant antiviral activity against human cytomegalovirus (HCMV) with an IC_{50} value of 1.9 μg/ml.

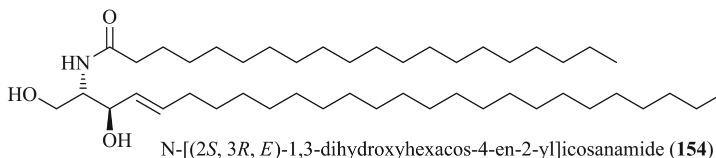


(**152**)

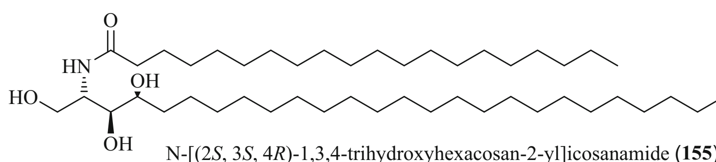
Sinularia candidula, collected in the Egyptian Red Sea, afforded an unprecedented polyhydroxylated sterol, 3β-25-dihydroxy-4-methyl-5α,8α-epidioxy-2-ketoergost-9-ene (**153**) together with three new ceramide derivatives *N*-[(2*S*,3*R*,*E*)-1,3-dihydroxyhexacos-4-en-2-yl]icosanamide (**154**), *N*-[(2*S*,3*S*,4*R*)-1,3,4-trihydroxyhexacosan-2-yl]icosanamide (**155**), and (*R*)-2′-hydroxy-*N*-[(2*S*,3*S*,4*R*)-1,3,4-trihydroxypentacosan-2-yl] nonadecanamide (**156**). All isolated compounds (**153–156**) exhibited selective antiviral activity against H5N1 avian influenza strain using the plaque inhibition assay possessing virus titer reductions of 55.16%, 48.81%, 10.43%, and 15.76%, respectively, at concentrations less than or equal to 1 ng/ml [85].



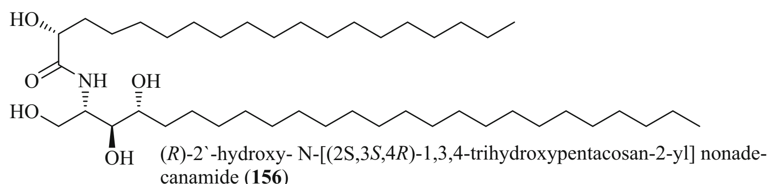
3β-25-dihydroxy-4-methyl-5α,8α-epidioxy-2-ketoergost-9-ene (**153**)



N-[(2*S*, 3*R*, *E*)-1,3-dihydroxyhexacos-4-en-2-yl]icosanamide (**154**)

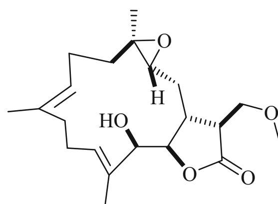


N-[(2*S*, 3*S*, 4*R*)-1,3,4-trihydroxyhexacosan-2-yl]icosanamide (**155**)



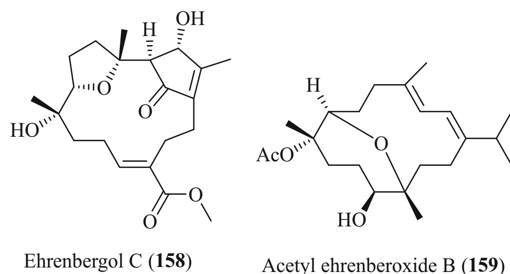
(*R*)-2`-hydroxy- N-[(2S,3*S*,4*R*)-1,3,4-trihydroxypentacosan-2-yl] nonadecanamide (**156**)

Further anti-HCMV compounds were obtained from species of the soft coral genus *Lobophytum* collected at the Dongsha Atoll (Taiwan) which is well known for producing a large variety of bioactive secondary metabolites including cytotoxic, antibacterial, anti-inflammatory, and HIV-inhibitory compounds [86, 87]. Chromatographic workup of an extract of *L. durum* gave rise to five new cembranolides durumolides M–Q, of which durumolide Q (**157**) exhibited significant antiviral activity against HCMV with an IC_{50} value of 5.2 μg/ml [88].



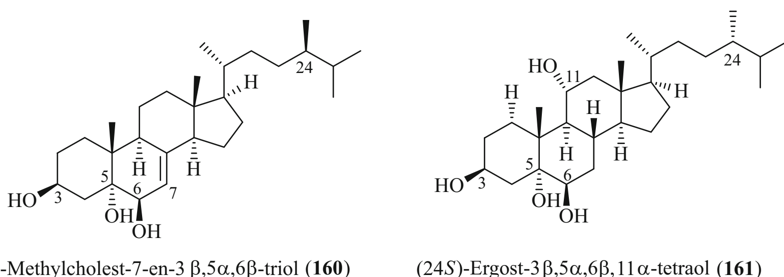
Durumolide Q (**157**)

Further anti-HCMV metabolites were obtained from the soft coral *Sarcophyton ehrenbergi* collected at San-hsian-tai, Taiwan. Purification of its acetone extract yielded two new antiviral diterpenoids, ehrenbergol C and acetyl ehrenberoxide B (**158** and **159**). The compounds exhibited selective antiviral activity toward HCMV, with IC_{50} values of 20 and 8.0 μg/ml, respectively [89].



Ehrenbergol C (**158**) Acetyl ehrenberoxide B (**159**)

Chromatographic workup of a *Sarcophyton* sp. collected in the South China Sea (P.R. China) yielded three new polyhydroxylated steroids along with seven known steroid derivatives. The isolated compounds featured a 3β, 5α, 6β-trihydroxylated steroidal moiety. Compounds **160** (24*R*)-methylcholest-7-en-3β,5α,6β-triol and **161** (24*S*)-ergost-3β,5α,6β, 11α-tetraol displayed antiviral activity against H1N1 (Influenza A virus) with IC_{50} values = 19.6 and 36.7 μg/ml, respectively, which is equal to or even more active than those of the positive control ribavirin /IC_{50} value of 24.6 μg/ml [90].



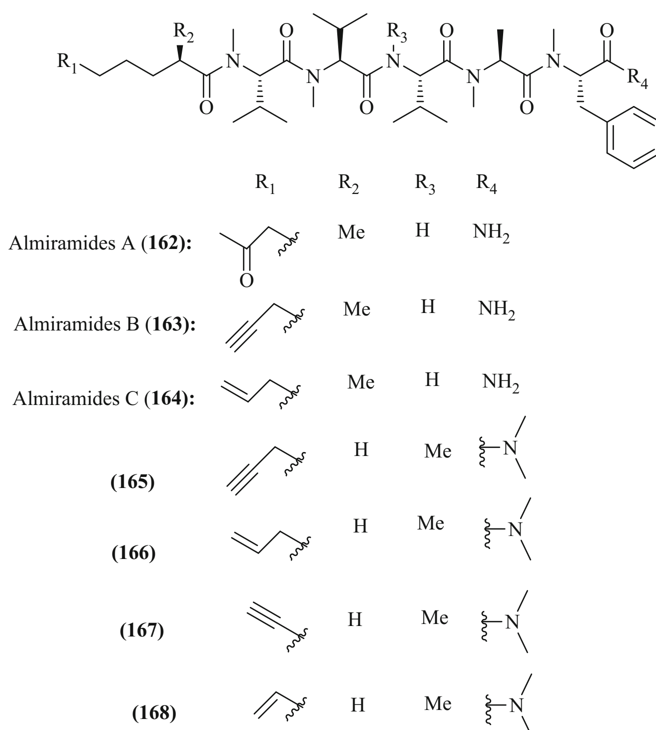
(24*R*)-Methylcholest-7-en-3 β,5α,6β-triol (**160**) (24*S*)-Ergost-3 β,5α,6β,11 α-tetraol (**161**)

5 Antiprotozoal Compounds

5.1 From Bacteria

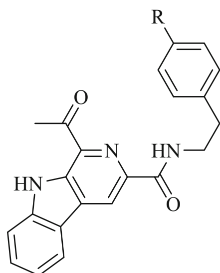
Leishmaniasis (also known as Leishmaniosis) is one of the most dangerous diseases in tropical regions such as India, Sudan, and Brazil. The disease is caused by more than twenty species of taxonomically related intracellular parasites of the genus *Leishmania* and is characterized by cutaneous, mucosal, and visceral manifestations. Main symptoms include ulcerative skin lesions and the destruction of the mucous membranes of the nose, mouth, and throat [91]. An extract of the cyanobacterium

Lyngbya majuscula obtained from mangrove roots in the Bocas del Toro National Marine Park, Bocas del Toro Province, on the north coast of Panama exhibited strong in vitro activity in two complementary screens against the tropical parasite *Leishmania donovani*, the causative agent of visceral leishmaniasis. Chromatographic workup of the extract yielded the *N*-methylated linear lipopeptides almiramides B–C (**162–164**), which showed potent anti-leishmanial activity with IC_{50} values in the range of 1.4–1.7 μg/ml, respectively. The semisynthetic products (**165–168**) showed IC_{50} values in the range of 2.0–4.9 μg/ml with improved therapeutic indices compared to the natural products [92].

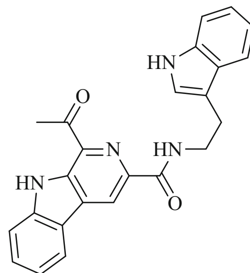


Malaria is a serious infectious disease especially in Central and South America, Southeast Asia, Sub-Saharan Africa, the Middle East, and India which is caused by *Plasmodium* parasites. It is estimated that 0.8−1.2 million people die every year due to malaria [93]. Four new β-carboline alkaloids, including marinacarbolines A−D (**169**−**172**), and two new indolactam alkaloids, 13-*N*-demethyl-methylpendolmycin (**173**) and methylpendolmycin-14-*O*-*α*-glucoside (**174**), were obtained from the culture broth of marine actinomycete *Marinactinospora thermotolerans* SCSIO 00652 isolated from sea sediment sample in the northern South China Sea (P.R. China) obtained at a depth of 3865 m. The new compounds **169**−**174** exhibited

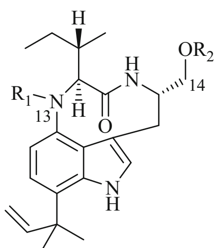
antiplasmodial activities against the malaria parasite *P. falciparum* lines 3D7 and Dd2, with IC_{50} values ranging from 0.74 to 14.0 μg/ml [94].



Marinacarboline A (**169**) : R=OMe
Marinacarboline B (**170**) : R=OH
Marinacarboline C (**171**) : R=H



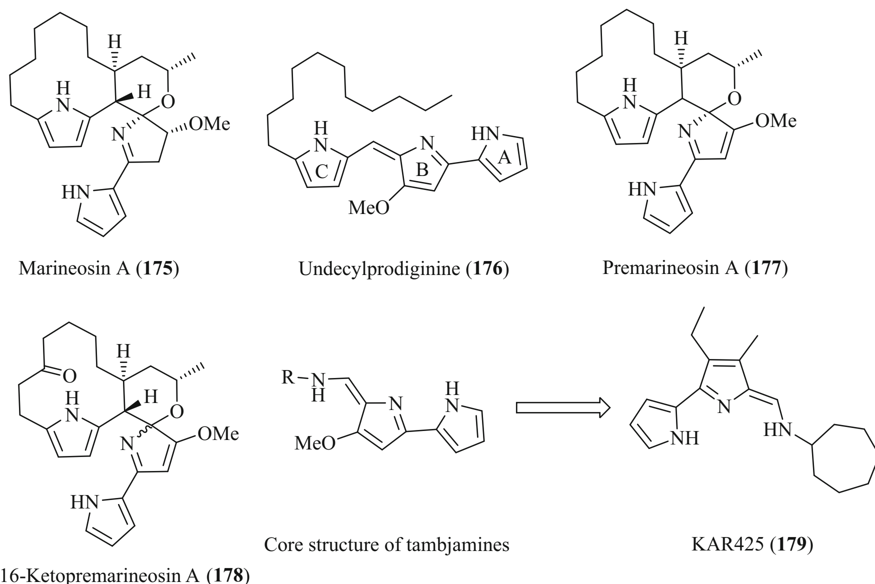
Marinacarboline D (**172**)



13-N-Demethyl-methylpendolmycin (**173**) : R_1=H, R_2=H
Methylpendolmycin-14-*O*-α-glucoside (**174**) : R_1=Me, R_2=Glu

In 2008, an intriguing prodigiosin-related compound, marineosin A (**175**), was isolated from the actinomycete *Streptomyces* sp. CNQ-617, derived from sediment collected off shore of La Jolla, California [95]. Biosynthetic studies assisted to identify the corresponding *mar* gene cluster, leading to **175** that appeared to be homologically similar to the *red* gene cluster described for the soil-dwelling *S. coelicolor*, responsible for the formation of undecylprodigionine (**176**) with additional steps requiring the spiroaminal functionality formation [96]. During further investigation, the antimalarial potency of **175** as well as of two further biosynthetic intermediates, premarineosin A (**177**) and 16-ketopremarineosin A (**178**), isolated from a mutant strain of *S. venezuelae*, was observed. Accordingly, the IC_{50} values of **175**, **177**, and **178** against *P. falciparum* (strains D6, Dd2, and 7G8) were detected at the nanomolar range between 0.6 and 84 ng/ml, being comparable or even more potent than those of the positive control chloroquine. Notably, the most active compound, premarineosin A (**177**), demonstrated high SIs (347 and 2779) against chloroquine-resistant *P. falciparum* strains Dd2 and 7G8, respectively. The remarkable antimalarial activity of this fascinating group of secondary metabolites inspired extensive medicinal chemistry and SAR studies. Accordingly, Kancharla and coworkers

screened a library of B-ring functionalized prodiginins and 94 new synthesized tambjamine derivatives [97]. Naturally occurring tambjamines are found almost exclusively in marine invertebrates (i.e., nudibranchs, bryozoans, and ascidians), and their structures resemble those of prodigiosins, bearing an enamine moiety instead of a pyrrole moiety (ring C) as in the latter [98]. This study revealed that the bipyrrole scaffold of tambjamines has improved bioactivity profiles with regard to in vitro/in vivo antimalarial efficacy and selectivity. In particular, the analogue KAR425 (**179**) was shown to be an excellent antimalarial lead, being active against the multidrug-resistant strains Dd2 and 7G8 with IC_{50} values of 16 and 18 ng/ml, respectively, while possessing significantly lower cytotoxicity toward HepG2 cells ($IC_{50} = 5711$ ng/ml; SI = 348 and 321, respectively). Moreover, in a subsequent in vivo study, **179** exhibited 100% protection of mice infected with *Plasmodium yoelii* until day 28 when administered orally during 4 days at doses of 25 and 50 mg/kg or after a single-administration dose of 80 mg/kg, whereas no signs of toxic effects were observed. It is also worth noting that the proposed synthesis of KAR425 (**179**) includes the use of inexpensive and accessible precursors, such as cycloheptanamine, and does not involve challenging chemical reactions [97]. Thus, tambjamines and prodiginines have proven to be privileged scaffolds for library design and for development of novel antimalarial agents.

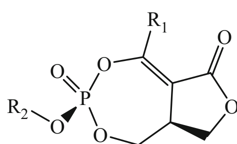


Marineosin A (**175**) Undecylprodiginine (**176**) Premarineosin A (**177**)

16-Ketopremarineosin A (**178**) Core structure of tambjamines KAR425 (**179**)

A novel antimalarial compound, salinipostin A (**180**), was discovered by Schulze et al. as a result of a screening program directed toward the search for lead compounds among bacterial marine natural products [99]. This unusual derivative was isolated from *Salinispora* sp., obtained from a marine sediment sample, collected near Keawekaheka Bay, Hawaii, at a depth of 15 m. Salinipostin A (**180**) exhibited potent activity against the chloroquine-resistant W2 strain of

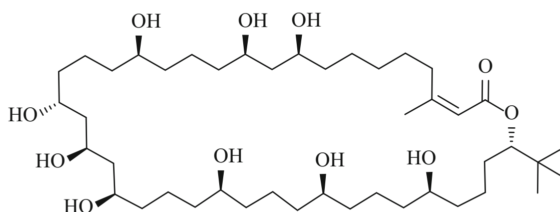
P. falciparum with an EC_{50} value of 0.024 ng/ml, demonstrating a remarkable selectivity index (SI > 1000). Furthermore, a series of related derivatives salinipostins B–K (**181–190**) were isolated during chemical investigation of *Salinispora* sp., which allowed to explore the structure-activity relationships of this new intriguing class of antimalarials. The chemical structures of **180–190** were unambiguously elucidated by detailed MS/MS as well as by 1D and 2D NMR analysis, including ^{31}P NMR, and their absolute configuration was deduced by means of VCD. Evaluation of the antimalarial activity of **180–190**, which share a common bicyclic phosphotriester motif, revealed a broad range of EC_{50} values between 0.024 (for **180**) and 21.4 μg/ml (for **189**), hinting at the important impact of the side chains (R_1 and R_2) on the antiparasitic effect of these compounds. Accordingly, derivatives with longer vinyl and/or branched R_1 chains as well as with a pentadecyl moiety at R_2, such as salinipostins A, F, and I (**180**, **185**, and **188**, respectively), showed significantly higher activity than the rest of the analogues. Interestingly, none of the compounds displayed cytotoxicity toward mammalian HEK293T cells, indicating their high selectivity against *P. falciparum*. Further biological studies on *P. falciparum* revealed that morphological and developmental changes in the parasite culture, caused by the treatment with the most potent compound salinipostin A (**180**), differ from those that appear as a result of the treatment with chloroquine, suggesting a novel mode of action for **180**. Moreover, growth stage specificity of **180** was evaluated with the respective wash in/wash out experiments that indicated early stages of the parasite life cycle, especially the ring stage, to be the most sensitive ones. A further attempt to generate resistant parasite populations toward **180** failed, thus suggesting that this compound is less susceptible to drug-resistance development than other currently available antimalarial drugs, possibly by targeting fundamental processes in *Plasmodium* growth [99].



Salinipostin A (**180**): R_1 = Bu, R_2 = $C_{15}H_{31}$
Salinipostin B (**181**): R_1 = Bu, R2 = $C_{14}H_{29}$
Salinipostin C (**182**): R_1 = Bu, R_2 = $C_{13}H_{27}$
Salinipostin D (**183**): R_1 = *iso*Bu, R_2 = $C_{14}H_{29}$
Salinipostin E (**184**): R_1 = *iso*Bu, R_2 = $C_{13}H_{27}$
Salinipostin F (**185**): R_1 = Pr, R_2 = $C_{15}H_{31}$
Salinipostin G (**186**): R_1 = Pr, R_2 = $C_{14}H_{29}$
Salinipostin H (**187**): R_1 = Pr, R_2 = $C_{13}H_{27}$
Salinipostin I (**188**): R_1 = Et, R_2 = $C_{15}H_{31}$
Salinipostin J (**189**): R_1 = Et, R_2 = $C_{14}H_{29}$
Salinipostin K (**190**): R_1 = Et, R_2 = $C_{13}H_{27}$

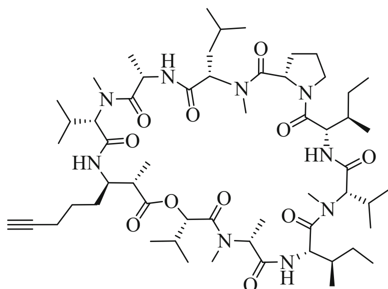
An unprecedented polyhydroxylated macrolide with a 40-membered lactone ring, bastimolide A (**191**), was isolated from the recently described tropical marine cyanobacterium *Okeania hirsute*, collected from the Caribbean coast of Panama [100]. Chemical conversion of **191** to the respective nona-*p*-nitrobenzoate and subsequent X-ray analysis allowed the assignment of its absolute configuration. Bioassay screening against several causative agents of neglected parasitic tropical diseases revealed significant antimalarial activity for **191** at nanomolar

concentrations toward a broad spectrum of drug-resistant *P. falciparum* strains (TM90-C2A, TM90-C2B, W2, and TM91-C235). The respective IC_{50} values were between 63 and 213 ng/ml, whereas only moderate cytotoxicity against Vero and mammalian MCF-7 cells was observed (IC_{50} = 1.7 and 2.5 μg/ml, respectively), with the respective SIs ranging from 7.8 to 56 depending on the tested strain. In addition, **191** exhibited antiparasitic activity against *Trypanosoma cruzi* and *Leishmania donovani* with IC_{50} values of 5.1 and 2.4 μg/ml, correspondingly [100].

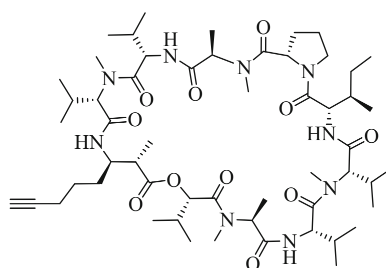


Bastimolide A (**191**)

Two new cyclic depsipeptides, companeramides A (**192**) and B (**193**), were isolated from a cyanobacterial assemblage collected from Coiba Island, Panama [101]. Interestingly, the structures of **192** and **193** contain the unusual amino acids 3-amino-2-methyl-7-octynoic acid (Amoya) and hydroxy isovaleric acid (Hiva), rarely encountered in nature. Both compounds exerted in vitro activity against *P. falciparum* strains D6, Dd2, and 7G8 at low (sub)micromolar concentrations (IC_{50} values between 0.23 and 1.19 μg/ml). Compounds **192** and **193** were also tested for cytotoxicity against several human cancer cell lines and were found to be nontoxic at concentrations of 1.08 and 1.06 μg/ml, respectively. The cytotoxicity and selectivity of these compounds at higher concentrations is yet to be studied.



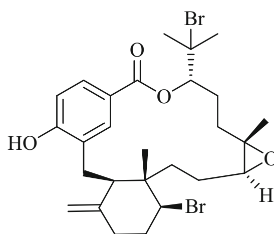
Companeramide A (**192**)



Companeramide B (**193**)

5.2 From Algae

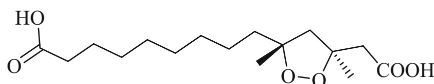
Callophycus serratus is a marine red alga that was found to produce antimalarial metabolites [102]. It is found at depths of 3–20 m throughout the tropical and subtropical Pacific Ocean. Four new bromophycolides, R–U, were obtained from the Fijian red algae *C. serratus* [102]. These natural products feature a diterpene-benzoate macrolide core structure. Bromophycolide S (**194**) showed antimalarial activity against the malaria parasite *P. falciparum* at submicromolar concentrations (IC_{50} value of 0.52 μg/ml).



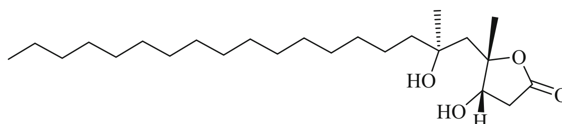
Bromophycolide S (**194**)

5.3 From Invertebrates

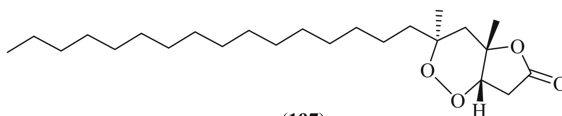
Sponges of the family Plakinidae are known for the accumulation of polyketides featuring cyclic peroxides. Several sponge-derived peroxides showed activity against the protozoan parasites *P. falciparum*, *Leishmania chagasi*, *Trypanosoma brucei brucei*, and *Trypanosoma cruzi* [103]. The Puerto Rican sponge *Plakortis halichondrioides* was found to yield 5-membered-ring polyketide endoperoxides. Antimalarial assessment against *P. falciparum* revealed that epiplakinidioic acid (**195**) showed potent activity with a MIC value of 0.3 μg/ml. Semisynthetic derivatives **196** and **197** were found to show comparable antimalarial activity with MIC values of 0.6 and 0.3 μg/ml, respectively [104].



Epiplakinidioic acid (**195**)

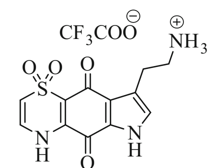


(**196**)

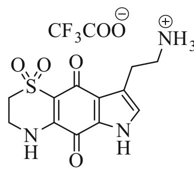


(**197**)

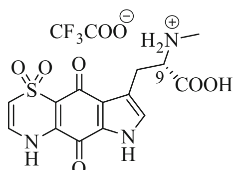
Further investigation of sponges from the genus *Plakortis* resulted in the isolation of unprecedented antimalarial compounds. *Plakortis lita* was collected from the Tydeman Reef, Queensland, Australia, and subjected to bioassay-guided fractionation, thus affording four unprecedented thiazine-derived alkaloids, thiaplakortones A–D isolated as trifluoroacetic acid salts. Thiaplakortone A (**198**) was the most potent compound showing inhibition of chloroquinone-sensitive (3D7) and chloroquinone-resistant (Dd2) *P. falciparum* lines with IC_{50} values of 1.9–20.0 ng/ml, respectively. The weak cytotoxicity of **198** against the HEK293 human cell line corresponds to selectivity indices of 76 and 591 against the 3D7 and Dd2 strains. Moreover, thiaplakortones B–D (**199–201)** showed activity against chloroquinone-sensitive (3D7) and chloroquinone-resistant (Dd2) *P. falciparum* lines with IC_{50} values ranging from 27.1 to 91.7 ng/ml for **199**, from 60.0 to 108.4 ng/ml for **200**, and from 55.8 to 97.9 ng/ml for **201**. Thus, the selectivity of thiaplakortone A (**198**) as well as its high efficacy against *P. falciparum* emphasizes its possible role as a novel antimalarial drug candidate. The structure-activity relationship revealed that (1) the thiazine moiety does not influence the antimalarial activity and (2) the 2-methylaminopropanoic acid chain decreases the antimalarial activity as indicated in **200** and **201**, while the ethylamine side chain increases the activity as seen in **198** [105].



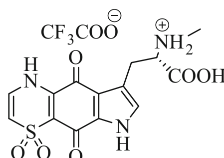
Thiaplakortone A (**198**)



Thiaplakortone B (**199**)

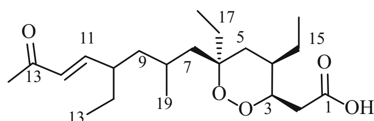


Thiaplakortone C (**200**)

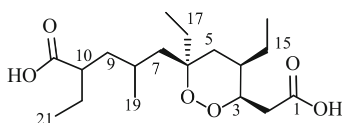


Thiaplakortone D (**201**)

Bioassay-guided fractionation of the Australian marine sponge *Plakortis* sp. [106], previously reported as a prolific source for antimalarial drugs [104, 105], yielded two new cyclic polyketide peroxides, 11,12-didehydro-13-oxo-plakortide Q (**202**) and 10-carboxy-11,12,13,14-tetranorplakortide Q (**203**). Antitrypanosomal assay showed potent activity of **202** against *T. brucei brucei* with IC_{50} values of 18.04 ng/ml. Compound **203**, which features a carboxyl group in the side chain, showed a 20-fold decrease in activity when compared to **202**.

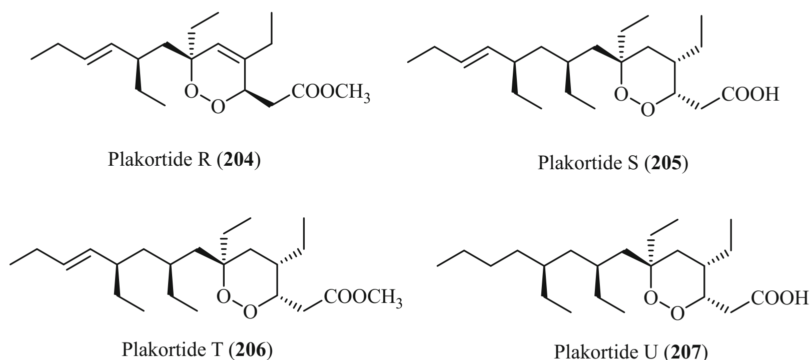


11, 12-Didehydro-13-oxo-plakortide Q (**202**)

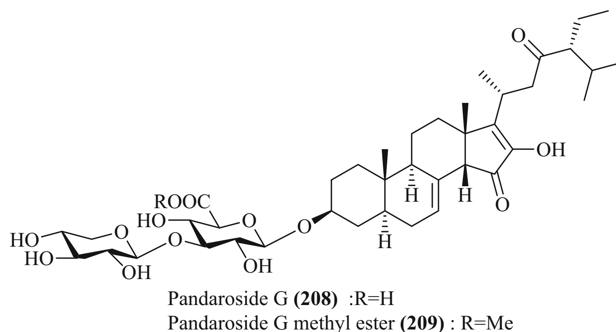


10-Carboxy-11,12,13,14-tetranorplakortide Q (**203**)

Plakortides R–U (**204-207**) were isolated from the sponge *Plakinastrella mamillaris* collected at the Fiji Islands, Melanesia, South Pacific Ocean. Plakortide U (**207**) was the most potent compound encountered and exhibited antiplasmodial activity against the chloroquine-resistant FcM29 *P. falciparum* strain with an IC_{50} value of 0.3 μg/ml, while plakortides R–T (**204–206**) showed moderate activity with IC_{50} values of 1.62–19.1 μg/ml. No cytotoxic activity was recorded for the isolated compounds [107].



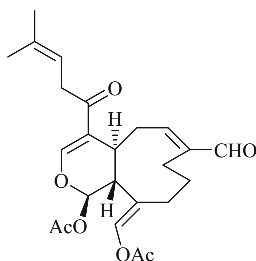
Human African trypanosomiasis (HAT) or African sleeping sickness is caused mainly by two subspecies of protozoan parasites, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. The disease if not being treated is usually fatal. More than 60 million people in poor regions of Africa are endangered by this disease as stated by the WHO [108]. The Caribbean sponge *Pandaros acanthifolium* yielded twelve steroidal glycosides, including pandarosides E–J along with their corresponding methyl esters. The metabolites feature an unusual oxidized D-ring and a cis C/D ring junction. Pandaroside G (**208**) and its methyl ester (**209**) strongly inhibited the growth of *T. brucei rhodesiense* with IC_{50} values of 0.6 and 0.03 μg/ml, respectively, and of *L. donovani* with IC_{50} values of 0.99 and 0.04 μg/ml, respectively [109].



Pandaroside G **(208)** :R=H
Pandaroside G methyl ester **(209)** : R=Me

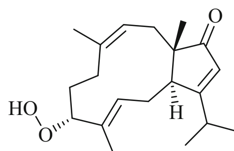
Cristaxenicin A (**210**) is a diterpenoid, isolated from the deep-sea Gorgonian *Acanthoprimnoa cristata* collected from Yakushima-Shinsone, Kagoshima Prefecture, southern Japan. Cristaxenicin A (**210**) exhibited antiprotozoal activity against both *Leishmania amazonensis* and *Trypanosoma congolense* with IC_{50} values of

0.11–0.04 μg/ml, respectively, versus that of the positive control amphotericin B, which showed IC_{50} values of 0.019 and 0.813 μg/ml, respectively [110].



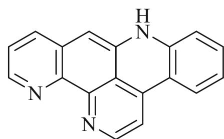
Cristaxenicin A (**210**)

Gorgonian octocorals of the genus *Eunicea*, which are abundant in the Caribbean Sea, is considered to be a rich source for antimalarial compounds. Numerous natural products have been isolated from members of this genus, and most of them exhibit unique structural features and pronounced biological activities. Chromatographic workup of a Colombian gorgonian coral of the genus *Eunicea* afforded eighteen diterpenes of the dolabellane type. Testing the inhibitory activity of these diterpenoids toward *P. falciparum* W2 (chloroquine-resistant) revealed that most of these compounds were active with IC_{50} values ranging from 9.4 to 59.6 μM. In particular, dolabellanone 9 (**211)** showed a pronounced activity with an IC_{50} value of 9.4 μM (2.99 μg/ml) [111].

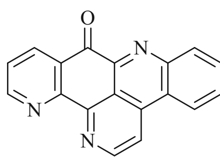


Dolabellanone 9 (**211**)

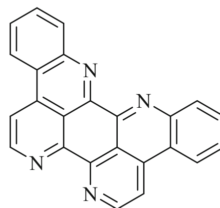
Chromatographic workup of the Australian ascidian *Polysyncraton echinatum* led to the isolation of three pyridoacridine alkaloids, including 12-deoxyascididemin (**212**), ascididemin (**213**), and eilatin (**214**). Compounds **212–214** are potent inhibitors of *T. brucei* with IC_{50} values of 0.02, 0.009, and 0.47 μg/ml, respectively [108].



12-Deoxyascididemin (**212**)

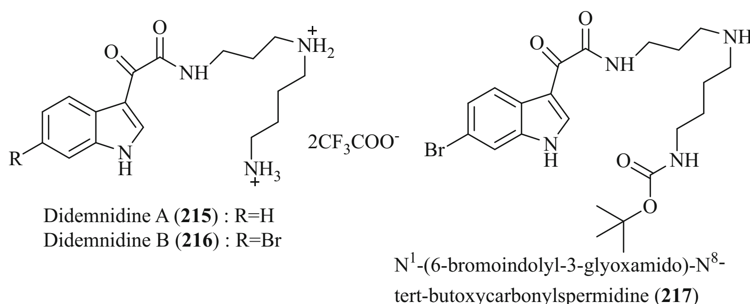


Ascididemin (**213**)



Eilatin (**214**)

Two new alkaloids named didemnidines A (**215**) and B (**216**) were isolated from the methanol extract of the marine organism, *Didemnum* sp. ascidian, obtained from Tiwai Wharf at 7 m depth, Tiwai Point, Southland, New Zealand. Compounds **215** and **216** feature an indole-3-glyoxylamide moiety connected to the N-1 position of spermidine which is rare in marine organisms. Didemnidine B (**216**) was active against the malaria parasite *P. falciparum* (IC_{50} 5.9 μg/ml). The semisynthetic compound N^1-(6-bromoindolyl-3-glyoxamido)-N^8-tert-butoxycarbonylspermidine (**217**) showed an IC_{50} value against *P. falciparum* of 4.2 μg/ml and against *T. brucei rhodesiense* of 4.9 μg/ml, respectively [112].

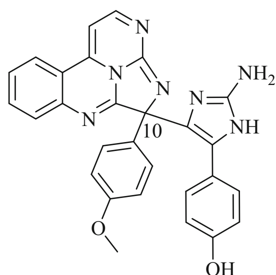


Didemnidine A (**215**) : R=H
Didemnidine B (**216**) : R=Br

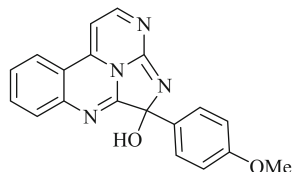
N^1-(6-bromoindolyl-3-glyoxamido)-N^8-tert-butoxycarbonylspermidine (**217**)

Chemical investigation of the marine ascidian *Eudistoma* sp. led to the isolation of three unusual related alkaloids, eudistidines A–C [113]. Among them, eudistidine C (1:1 mixture of **218a** and **218b**) represents a highly complex alkaloid, bearing fused pyrimidine and imidazole rings, a guanidine, as well as amidine moieties. The proton-deficient nucleus of eudistidine C was unambiguously assigned with the help of a new sensitive NMR pulse sequence LR-HSQMBC (long-range heteronuclear single-quantum multiple-bond correlation), allowing the detection of $^4J_{CH}$ and $^5J_{CH}$ correlations [114]. Total synthesis of eudistidine C confirmed the structure of this alkaloid, and a series of aryl-substituted (at C-10) derivatives were obtained. Eudistidine C epimers (**218a** and **218b**), eudistidine B (**219**), and their synthetic congeners (**220–224**) exhibited significant antiparasitic activity against chloroquine-sensitive D6 and chloroquine-resistant W2 *P. falciparum* strains with IC_{50} values in the range from 0.26 to 3.4 μg/ml [113]. The antimalarial potency of these compounds was investigated employing a plasmodial lactate dehydrogenase activity assay with artemisinin and chloroquine as positive controls. Interestingly, **218a** showed an up to 1.7-fold stronger antimalarial activity toward both *P. falciparum* strains in comparison with its epimer **218b**. Among the tested compounds, **219** and its synthetic derivative **224** exhibited the most potent antiplasmodial effect against D6 and W2 strains with MIC values of 0.46 and 0.36 μg/ml (for **219**) and 0.48 and 0.26 μg/ml (for **224**), respectively. Moreover, a subsequent cytotoxicity assay against Vero cells (monkey kidney fibroblasts) revealed no apparent activity for **218a,b–224**, suggesting their selective antimalarial activity. Additionally, compounds **218b**, **220**, **221**, and **224** were found to inhibit the binding of the oncogenic transcription factor HIF-1α (hypoxia-inducible factor 1) to p300 [113]. Thus,

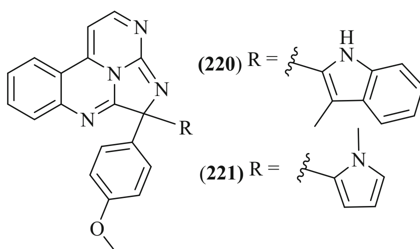
eudistidines C (**218a,b**) and B (**219**), as well as their synthetic analogues, carrying the tetracyclic eudistidine-like scaffold, represent a novel class of lead structures for the development of potent and selective antimalarial agents.

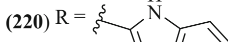


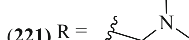
Eudistidine C (+)-*R* (**218a**)
Eudistidine C (-)-*S* (**218b**)

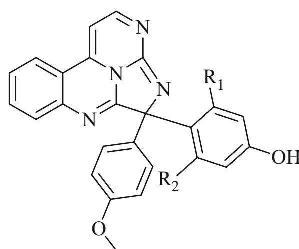


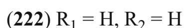
Eudistidine B (**219**)

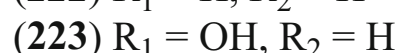


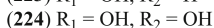




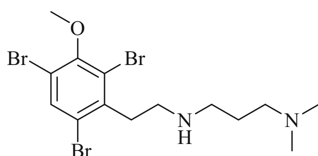




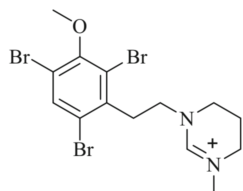




Convolutamines I (**225**) and J (**226**) were obtained from the marine bryozoan *Amathia tortuosa* collected at the Bass Strait, Tasmania, Australia. Compounds **225** and **226** were shown to be active against the parasite *T. brucei brucei* with IC_{50} values of 0.52 and 6.44 μg/ml, respectively [115] (Table 3.1).



Convolutamines I (**225**)



Convolutamines J (**226**)

Table 3.1 Summary of promising marine anti-infective compounds with identified/postulated modes of action (or molecular targets)

Source	Compound name	Mode of action/ molecular target	Spectrum of activity	Reference
Pseudoalteromonas sp. CMMED 290, associated with a nudibranch	2,3,5,7-Tetrabromobenzofuro [3,2-b]pyrrole (**11**)	Disrupts the bacterial cell wall membrane whereas human erythrocytes were not lysed upon application of the same dose of **11**	Antibacterial; against MRSA (ATCC 43300)	[15]
Streptomyces sp. CNH365, isolated from a sea sediment sample	Anthracimycin (**22**)	Disrupts nucleic acid synthesis	Gram-positive bacteria, including MRSA	[19]
Fungus *Paecilomyces* sp. (-CMB-MF010), associated with a mollusk *Siphonaria* sp.	Viridicatumtoxin A (**29**), Spirohexaline (**34**)	Inhibits recombinant undecaprenyl pyrophosphate (UPP) synthase from *S. aureus*	MRSA and VRE	[22 23]
Marine fungus *Aspergillus* sp. Z120	Fonsecinones A (**45**) and C (**46**), Aurasperones A (**47**) and E (**48**)	Enoyl-acyl carrier protein reductase (FabI), a key enzyme in the bacterial fatty acid synthesis (postulated antibacterial target)	ESBL-producing *E. coli*	[27]
Streptomyces sp. CN Q3 43, isolated from a sea sediment sample	Bahamaolide A (**91**)	Inhibits the activity of *Candida albicans* isocitrate lyase (ICL)	Anti-fungal; especially *Candida albicans*	[52]
Actinobacterium *Actinomadura* sp., obtained from the ascidian *Ecteinascidia turbinata*	Forazoline A (**102**)	Dysregulates phospholipid homeostasis (postulated)	Anti-fungal; *C. albicans*	[54]
Aspergillus terreus Gwq-48, isolated from mangrove rhizosphere	Isoaspulvinone E (**110**)	Anti- H1N1 viral neuraminidase activity (NA)	Anti-viral; especially anti-influenza A H1N1	[65]
Deep-sea-derived fungus *Eurotium rubrum* F33	Neoechinulin B (**129**)	Binds to the HA1 subunit of hemagglutinin glycoprotein	Anti-influenza A H1N1	[69]
Deep-sea-derived fungus *Spiromastix* sp. MCCC 3A00308	Spiromastilactone D (**130**)	Binds to the HA1 subunit of hemagglutinin glycoprotein	Anti-influenza A H1N1	[33]

(continued)

Table 3.1 (continued)

Source	Compound name	Mode of action/ molecular target	Spectrum of activity	Reference
Brown seaweed *Stypopodium zonale*	Atomaric acid (**132**)	Interacts with viral particles outside of the host cells thus preventing infection of the cell cultures; inhibits post-penetration stage. The compound has no effect on cellular receptors or on viral penetration	Antiviral; HMPV	[73]
	Epitaondiol (**133**)	Inhibits the penetration of viral particles into cells without affecting the post-penetration stages or interacting with cellular receptors		
	Methyl ester of atomaric acid (**134**)			
Marine sponge *Iotrochota baculifera*	Baculiferins L (**149**) and M (**150**)	Binding activities to HIV-1 targets including both Vif (viral infectivity factor of HIV-1) and human APOBEC3G (an innate intracellular antiviral factor)	Antiviral; HIV-1 IIIB virus	[82]

6 Conclusions

Natural products have been the most successful source of lead compounds for the treatment of infectious diseases since the dawn of the antibiotic era. In recent years, the emergence of resistant microbial strains against virtually all major classes of known anti-infectives poses a worldwide health threat, and thus further mining of natural sources for novel anti-infective agents is eminent [116]. Marine macro- and microorganisms have not been systematically investigated as a source of novel anti-infective agents, despite the fact that more than three-quarters of the Earth's surface is covered by the seas and oceans. Nevertheless, continued interest in the quest for structurally unprecedented bioactive secondary metabolites in combination with recent advances in sampling techniques has opened new avenues for the exploration of this fascinating ecological niche [7, 117]. Indeed, over the last decade, increased marine bioprospecting efforts were observed, particularly aimed at the deep-sea

environment, providing novel leads [118]. As a matter of fact, the potent proteasome inhibitor salinosporamide A, from the marine sediment actinomycete *Salinispora tropica*, has recently entered phase I clinical trials as an anticancer agent [119]. Undoubtedly, the marine environment represents an untapped source of bioactive chemical entities, many of which featuring new carbon frameworks without any terrestrial counterparts. This structural richness may give rise to potential scaffolds for the exploration of new biological targets, thus offering the advantage of the discovery of anti-infective leads with novel mechanisms of action tackling the global challenge posed by antibiotic resistance [120]. Several metabolites from various marine sources (i.e., sponges, bacteria, and fungi), described in this review, have already been identified as lead structures against both drug-sensitive and drug-resistant pathogens, such as the antiviral spiromastilactones (**130** and **131**), the antibacterial anthracimycin (**22**), or the antimalarial thiaplakortones (**198–201**) and salinipostins (**180–190**) possessing activities at (sub)micromolar concentrations [20, 33, 99, 105]. Hence, given the enormous potential of marine natural products in drug development, as demonstrated by the success stories of several marketed marine-derived drugs (i.e., Halaven®, Adcertis®, Yondelis®), it becomes increasingly apparent that the oceans hold great promises as the next source of novel anti-infective agents.

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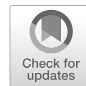
Chapter 4
The Marine-Derived Filamentous Fungi in Biotechnology



Rosario Nicoletti and Anna Andolfi

1 Introduction

Since the ancient ages, progress of mankind was accompanied by an ongoing improvement in the nutritional conditions, which was supported at some extent by the unaware employment of fungi in food manufacturing. In modern times the biotechnological importance of these microorganisms has remarkably increased after their exploitation in many different industrial processes, as well as in biofuel production and bioremediation. Hence, the term 'mycotechnology' has been introduced, subtending an even more widespread impact by selected fungi in the near future [1].

As known for species occurring in terrestrial environments, marine fungi basically play an ecological role as decomposers of organic materials [2], but unlike the former, so far they have not been adequately considered for an employment in biotechnological processes. However, it is a general opinion that the unique physico-chemical properties of the marine environment could have induced special physiological adaptations in these microorganisms, deserving to be exploited in biotechnology. Besides easily accessible contexts, the marine environment includes a number of unexplored habitats characterized by extreme conditions, such as the sea depths, the hydrothermal vents and the anoxic sediments from the ocean trenches, where novel strains and their valuable genes and gene products await to be discovered. Starting from this diversity, the present review examines the perspectives for a

R. Nicoletti (✉)
Council for Agricultural Research and Agricultural Economy Analysis, Rome, Italy

Department of Agriculture, University of Naples 'Federico II', Portici, Italy
e-mail: rosario.nicoletti@crea.gov.it

A. Andolfi
Department of Chemical Sciences, University of Naples 'Federico II', Naples, Italy

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biotechnological exploitation of marine fungal strains, on account of reports from over 250 papers dealing with their characterization.

2 General Outline of Marine Fungi

The cultural antinomy between land and sea that dominates in many fields of human knowledge has somehow influenced mycologists in a problematic attempt to separate the marine from the terrestrial fungal species. Unlike other eukaryotes, the definition of marine fungi only makes sense under an ecological viewpoint, without a consistent taxonomic foundation. In fact, most filamentous fungi recovered at sea are facultative since they are also known from fresh water or terrestrial contexts, while a relatively small number of obligate marine species have been reported to grow and sporulate exclusively in the sea water. Besides a few *Basidiomycota*, these species mostly belong to the *Ascomycota* and their anamorphs. They sometimes represent separate phylogenetic lineages which define new genera [3], or even new orders, such as the Halosphaeriales [4, 5], the Lulworthiales [6] and the Koralionastetales [7]. Increasing species numbers of obligate marine fungi are reported by recent systematic revisions: 467 species belonging to 244 genera [2], 530 species in 321 genera [8] and 549 species in 337 genera [3]. However, the actual number of accepted taxa has to be continuously updated, also considering that it is affected by several issues, such as mistaken isolation, incomplete species description, duplication, loss of pure cultures, existence of cryptic species, misidentification or unaccomplished identification and lack of sporulation. Particularly, species ascription of many strains which only produce sterile mycelium in pure culture often results problematic even with the help of DNA sequencing [9]. Actually, in studies concerning fungal diversity after isolations carried out from several marine substrates, percentage of identification at the species level ranged from 11.2 to 80.5% [10]. And recent metagenomic studies have disclosed a large diversity of fungi in marine habitats, particularly the deep sea, including a good proportion of novel taxa/lineages [11–16].

However, the semantic problem of how defining fungi recovered from the marine environment seems now on the way to find a solution, based on the distinction of the marine-derived fungi as a category which includes, but does not identify, the marine fungi *sensu stricto* that exclusively carry out their life cycles in marine habitats [17]. Finally, a more comprehensive definition has just been contrived by eminent experts, covering any fungus that is repeatedly recovered from marine habitats by reason of its ability to grow and/or sporulate in marine environments, to form symbiotic relationships with other marine organisms or to adapt and evolve at the genetic level and is metabolically active in marine environments [18]. The latter aspect is actually more relevant than usually assumed and underlines a factual adaptation of many 'terrestrial' species which overcomes any hypothesis of their casual underwater occurrence. In this regard it is quite meaningful to consider data resulting from an investigation concerning mycoflora associated with Neptune grass (*Posidonia oceanica*), where about two-thirds of a sample of 107 strains were not

affected by increasing salt concentrations or even showed their enzyme production to be triggered in such conditions, implying their nature of true marine fungi [19].

The concept of marine fungi is generally extended to strains recovered from mangroves. Although they thrive in a habitat which is dominated by salt water, mangrove plants are not considered marine organisms in a strict sense, and this concept of independence should be extended to their endophytes and other associated fungi, which have not been included as a subject of this review. On the other hand, mangrove debris settling at the bottom in the intertidal zone, as well as wood and other plant parts floating at sea after having been dispersed from every kind of terrestrial sources, are known to harbour many fungi able to develop in the submerged context, which have been particularly characterized for their ligninolytic activities [10].

The term 'fungi' is itself controversial, considering that since the beginning of the modern systematics of the living organisms, it has been used to designate heterogeneous entities. However, the affirmation of molecular biology as a tool for assessing phylogenetic relationships in view of a correct taxonomy has established a deep furrow of separation between the true filamentous fungi and the so-called straminipilan fungi (kingdom Chromista), which are not a subject in this review. Although currently classified as a division of Fungi, and widespread at sea, the *Chytridiomycota* are also excluded by reason of lacking a true hyphal organization. Conversely, despite the absence of an organized mycelium, yeasts are rightfully considered in the following pages, since they derived from multicellular ancestors and represent lineages pertaining to the *Ascomycota* or *Basidiomycota* [20, 21].

3 Habitats and Diversity of Marine Fungi

Microorganisms living at sea present a physiological adaptation to a series of conditions characterizing such a peculiar environment, that is, high salinity, sodium concentration and pH, oligotrophic nutrient availability, low oxygen and hydrostatic pressure increasing with depth. As a consequence of high salinity, they are forced to maintain a lower water potential in their cells in order to realize water uptake, which is usually obtained through the accumulation of osmolytes such as trehalose, mannitol, glycerol or other polyols [22]. The toxic effects of high levels of sodium ions are controlled by means of their compartmentation into vacuoles or through the action of sodium-efflux ATPase [23]. High salinity of sea water reflects subalkaline pH values, which are thought not to be optimal for fungi. However, facultative marine fungi have demonstrated a good adaptation to higher pH values [24], and most species display the so-called *Phoma* pattern, that is, tolerance to salinity increasing with incubation temperature [25].

The oligotrophic condition also greatly influences the adaptation and occurrence of fungi in different marine habitats. Considering their strategies for nutrient uptake, these species can be ascribed to the main functional groups of saprotrophs and symbionts, with the latter including both mutualists and parasites of other organisms.

By reason of their rigid cell walls, whose chitin structure contrasts the osmotic pressure during osmotrophic feeding, fungi can essentially access nutrients through the release of depolymerizing enzymes, enabling the assimilation of the digested monomers. In the end, this reliance on osmotrophy represents an ecological constrain for fungi, which can only thrive in environments where nutrients from organic substrates may be available, such as plant and animal hosts, sediments and organic debris [2]. Conversely, the lower concentration of products in the pelagic and surface waters is unfavourable to osmotrophic nutrition since the secreted enzymes would be quickly lost by diffusion in the liquid environment, the reason why the microbial component of plankton is dominated by free-floating unicellular organisms performing primary production or phagotrophy. This is confirmed by data resulting from a biomolecular investigation on mycoplankton carried out in Hawaiian coastal waters, which showed a limited fungal diversity dominated by basidiomycete yeasts, with about 60% of new phylotypes [26]. In the lower water column, where particulate matter is overlapped to sediments on and on, there is increased niche availability for saprotrophs, so that fungi represent the dominant eukaryotic microbes [11]. In fact figures concerning seabed sediments are definitely indicative of a higher relative occurrence and diversity of fungi. As an example, results of a recent investigation based on a biomolecular approach carried out in the Chinese subtropical seas yielded 816 fungal taxa, this time with predominance of ascomycetes, including more than 20% unknown species [27].

Despite the extreme environmental conditions, characterized by absence of light, minimal oxygen concentrations, temperatures around 2 °C and high hydrostatic pressures, recent investigations have disclosed an unexpected fungal diversity in sediment samples from sea depths [11, 28, 29]. Besides known ubiquitous species, these sources have disclosed the existence of novel fungal phylotypes, such as the so-called deep-sea fungal group 1 [14]. Presumptively novel fungal groups were also evidenced in samplings from methane hydrate-bearing sediments in the South China Sea [30] and oxygen minimum zones of the Arabian Sea [31]. Moreover, significant fungal diversity has resulted from the unusual ecosystem of the deep-sea hydrothermal vents [12], again with evidence for putative new taxonomic groups [32]. A notable incidence of new phylotypes (33%) also resulted in a more specific investigation concerning yeasts from the mid-Atlantic ridge hydrothermal fields [33]. More in general, yeasts have confirmed to represent the dominant fungal component in deep sea in several oceanic locations [11, 15, 34–36]. Reports concerning deep-sea fungi seem to confirm that these species may have undergone specific physiological adaptations to the extreme conditions of this particular environment. This is the case of a strain of *Aspergillus terreus*, whose conidia were able to germinate under high pressure (200 bar), unlike other strains of the same species of terrestrial origin. Moreover hyphae ascribed to this species were directly detected in the sediments through immunofluorescence staining [28]. Investigations on deep-sea sediment cores have also enabled to culture fungi from a past of different environmental conditions. For example, Raghukumar et al. [37] cultured fungi that had been presumably buried for nearly 420,000 years at about 365 cm below the seafloor at 5000 m depth in the Central Indian Ocean.

The overt anoxic conditions characterizing sea depths are another clue for possible physiological adaptations. This resulted for fungi isolated from anoxic zones in the Arabian Sea, which proved to be able to grow under oxygen-deficient conditions by performing anaerobic denitrification. Again, analysis of this biological material carried out by both culturing and biomolecular methods showed that many known species can actually proliferate in these extreme conditions and that a few lineages possibly represent novel taxonomic groups [31].

As anticipated above, tolerance to salinity is probably the prominent aspect characterizing physiological adaptation of marine organisms. This tolerance can reach extreme levels in fungi inhabiting salt pans, even if their inclusion in the range of the marine species is somehow questionable [10]. Although there is an indication that most halophilic species do not require salt and can grow in a wide range of osmotic conditions [38], salterns represent a privileged source for the isolation of obligate halophilic fungi [39, 40]. Originally isolated from a saltern on the Adriatic coast of Slovenia, *Wallemia ichthyophaga* is the most halophilic eukaryote known so far. This species proved to be unable to grow in media with NaCl concentrations lower than 1.5 M and was reported to cluster together with the congeneric *W. sebi* and *W. muriae*, which show preference for high-sugar environments, in an isolated lineage at the base of the *Basidiomycota* tree, to which taxonomists have assigned the rank of a separate class, the Wallemiomycetes [41, 42].

Under the metabolic viewpoint, the ability to develop in high salinity conditions depends on a more general aptitude to challenge low water activity, which also occurs in other extreme environments characterized by dryness or temperatures low enough to keep water in the solid state, where a somehow similar composition of fungal populations has been observed [43]. However, the real mechanisms responsible for adaptation to salinity can change according to the species. As an example, the extremely tolerant black yeast *Hortaea werneckii* presents melanized cell walls, which reduce permeability to glycerol and increase the content of this compatible solute [44]. Mannitol and other polyols can be accumulated by other species for performing the same function of maintaining high osmotic conditions [45]. Whatever the biochemical mechanism, the adaptation of marine fungi to salinity implies the existence of an extensive genetic basis that can be relevant for biotechnological applications. For instance, possible future exploitation has been conceived for the creation of plant genotypes with some extent of salt tolerance/resistance, in a way similar to what is accomplished in melon and tomato with the introduction of the gene HAL1 from *Saccharomyces cerevisiae* [46].

Besides the extreme environmental conditions outlined above, a large fraction of marine fungi reside as symbiotic forms in marine plants and animals. The increasing number of reports from these hosts that are in progress worldwide requires a more detailed examination.

Seagrasses represent one of the pioneering fields of investigation which introduced the notable diversity of marine fungi. In fact, preliminary data were gathered in the 1980s from the underwater meadows of *P. oceanica* in the isle of Ischia (Naples Bay, Italy) displaying the occurrence of an assemblage of ligninolytic fungi such as *Corollospora maritima*, *Papulaspora halima* and *Lulworthia* spp. [47]. More recently,

the composition of the mycoflora associated to different parts of *P. oceanica* was systematically investigated at a site in the Ligurian Sea, with a total of 88 identified taxa. Their capability to produce laccases, peroxidases and tannases was particularly evaluated [19]. In another investigation on the large seagrass *Enhalus acoroides* carried out in Southern Thailand, 42 fungal species were identified. Most of them were *Ascomycota* (98%), belonging to the *Sordariomycetes* (36%), the *Eurotiomycetes* (33%) and the *Dothideomycetes* (24%), while the *Hypocreales* represented the predominant order, followed by the *Eurotiales* and the *Capnodiales* [48]. Other species which have been investigated at some extent for the occurrence of endophytic fungi are the turtle grass (*Thalassia testudinum*) [49–51], the common eelgrass (*Zostera marina*) [52, 53], the dugong grass (*Halophila ovalis*) [54] and the shoalweed (*Halodule wrightii*) [49]. Leaves and rhizomes of more species in the Cymodoceaceae (*Cymodocea serrulata*, *Halodule beaudettei*, *Halodule uninervis*, *Syringodium* sp.), together with *E. acoroides* and *Thalassia* spp., have been investigated along the coast of Tamil Nadu (India), yielding as many as 305 isolates representing 32 morphospecies [55]. A partial list of known saprobic and endophytic fungi recovered from seagrasses is reported by Jones [3].

A widespread occurrence of fungal symbionts has been also documented in seaweeds, with many reports concerning saprobic and parasitic fungi associated with species in the Chlorophyta, Rhodophyta and Phaeophyta [56–62]. The occurrence of algal endophytic strains has been particularly reviewed by Suryanarayanan [63]. Besides true endosymbionts, mostly belonging to the *Ascomycota* and their related anamorphs, these species include latent pathogens and casual residents which nevertheless deserve consideration for their biological properties. A few species have been described for their mutualistic adaptation to seaweeds (mycophycobioses), such as *Halosigmoidea* (=*Sigmoidea*) *marina* on *Fucus serratus* [61] and *Mycophycias ascophylli* on *Ascophyllum nodosum* and *Pelvetia canaliculata* [64, 65]. Relationships of these fungi with their hosts can be so strong that in the new species *Acremonium fuci*, isolated from wracks (*Fucus distichus* and *F. serratus*), conidial germination only occurs in the presence of *Fucus* tissue or aqueous tissue homogenates, but not in sea water alone [66].

From seagrass and other marine plants, the concept of endophytism has been extended to fungi inhabiting the inner tissues of sponges, corals, ascidians and other animal organisms which live anchored at the seabed. However, this extension is basically incorrect under the semantic viewpoint, and the more appropriate, even though seldom used [67, 68], concept of endozoism will be used throughout this manuscript.

In corals fungi represent a lesser-known symbiotic community whose functions and taxonomic composition deserve to be studied more in depth. Actually, they can be found within the polyps and in the calcium carbonate skeleton, where they penetrate to form endolithic structures [69], in coral mucus and reef sediments, in a quite systematic occurrence that is considered as a clue of either a mutualistic or a parasitic behaviour [70]. Several investigations carried out in different reefs worldwide have demonstrated that fungal infections by *Aspergillus sydowii*, and possibly other species, may cause decline of sea fans (*Gorgonia* spp.) [71–74] and impair

corals by affecting their dinoflagellate photosymbiont *Symbiodinium* [75]. Whatever the specific role of every single species, the basic function of the fungal endolithic community of corals is attested by an investigation on the microbial metagenome of *Porites astreoides*, which showed that up to 38% of the genome of the coral holobiont is of fungal origin (with 93% of this referable to the *Ascomycota* and 77% to the *Sordariomycetes*), including a wide diversity of fungal genes involved in carbon and nitrogen metabolism, particularly in the conversion of nitrate and nitrite to ammonia [76]. A high species diversity has been also reported from gorgonians [77]. Another fundamental investigation carried out on the species *Acropora hyacinthus* by means of a DNA-based phylogenetic analysis showed a high diversity of *Basidiomycota* and *Ascomycota*, including several novel clades separated from known fungal taxa by long and well-supported branches. Four taxa were present in all coral colonies sampled and may represent obligate associations. Messenger RNA sequenced from a subset of these same colonies contained an abundance of transcripts involved in metabolism of complex biological molecules [78]. Fungi from corals and other cnidarians have displayed interesting properties for biotechnological applications concerning bioremediation [79].

A possible pathogenic role of fungi, alone or in combination with bacteria [80], has been also observed in sponges. Actually, culture-based studies have revealed a large diversity of fungi associated with sponges with different extents of symbiotic relationships [81–91]. Considering the known difficulties in the isolation of ecologically obligate species, the recently expanding molecular approach can disclose an even larger diversity [92, 93]. On a more specific ground, 23 and 21 species were respectively identified in an investigation concerning *Suberites zeteki* and *Mycale armata* [92], while one genus of *Basidiomycota* and at least 25 genera of *Ascomycota* were reported from *Gelliodes fibrosa*, *Haliclona caerulea* and *M. armata* [94]. In the case of demosponges of the genus *Chondrilla*, it has been shown that associated endosymbiotic yeasts are maternally transmitted through fertilized eggs [95]. The relationships of endozoic fungi with their sponge hosts can be so close that a horizontal gene transfer has been demonstrated based on the presence of introns in the mitochondrial DNA of *Tetilla* sp. [96], introducing intriguing scenarios on the real foundations of the mutualistic relationships between fungi and their hosts.

4 Secondary Metabolites

The issue of the symbiotic relationships is undoubtedly more relevant when considered in view of the chemical products mediating these interactions. In fact, an increasing number of reports worldwide have pointed out that many bioactive compounds first extracted from marine plants and animals are actually produced by their associated fungi [97]. Even if in some symbiotic fungi the biosynthetic abilities depend on the interaction with the host organism and are not fully expressed in axenic cultures [88], the general convenience to exploit the fermentative aptitudes of microbial strains instead of relying on a problematic collection from the natural

environment has further boosted the search for endophytic/endozoic fungal strains to be investigated for the production of secondary metabolites. More particularly, the necessity to face the increasing problem of microbial resistance has generated a huge demand for novel antibiotics, resulting in the finding/characterization of interesting prospects [98, 99]. In the period 2010–2015, as many as 285 antibacterial and antifungal compounds, 116 of which are new, have been reported from marine-derived fungi [100]. Besides remarkable direct achievements, this systematic search has resulted to be propulsive for the finding of products possessing other fundamental bioactive properties, such as antiviral, antitumour, antioxidant, antifouling, enzyme inhibitors, etc. And more new bioactive molecules can be obtained in the laboratory by coculturing marine-derived fungi with other microorganisms (e.g. the libertellenones [101]), a condition which often brings to the activation of silent gene clusters. Actually, as reflected by the occurrence of a high number of dedicated gene clusters in their genome, diversity in secondary metabolite production by many facultative marine fungi has been introduced as a possible determinant of their capacity to adapt to very different environmental conditions [102].

The list of secondary metabolites produced by marine-derived fungi is far too long for any attempt to resume it in this review, also considering that the unremitting addition of novel products would make it obsolete quite soon. A review issued in 2011 reports 272 metabolites discovered from marine fungi until 2002 and a progressively increasing number in the following years which brought to a total of over a thousand novel structures in 2010 [103]. The last available update has added about 150 new compounds in the past 3 years [104]. Again these figures are influenced by the difficulty to qualify many producing strains as real marine fungi and in some cases by the simultaneous finding of the same product by both a marine and a terrestrial strain [17]. Some research work on methods has been also carried out in the aim to improve the ability to detect new compounds [105–107].

A few more or less recent reviews can be useful to draw an idea of the biosynthetic capacities of marine-derived fungi, ordered according to criteria concerning their origin, taxonomy or functions. In this respect, partly updated data are available on the secondary metabolites produced by fungal symbionts of sponges [97], while a more recent systematic revision concerning algicolous fungi considers 366 new compounds, the two-thirds of which exhibited interesting antimicrobial, cytotoxic, antioxidative and/or enzyme-modulatory activities [108]. Potential anticancer and antiviral agents obtained from marine fungi of any origin have been, respectively, treated by Gomes et al. [109] and Moghadamtousi et al. [110]. Five hundred and fifty compounds or compound families, half of which are new, have been listed as products of about 150 marine-derived strains of *Penicillium* and *Talaromyces* [111]. Secondary metabolites produced by *Trichoderma* strains of marine origin and their bioactivities and potential use have been revised by Ruiz et al. [112]. A recent review particularly focuses on the new metabolites reported by marine strains sensu stricto [17], while other papers concern specific categories of bioactive compounds, such as the terpenoids [113] and the anthraquinones [114]. Along with other pigments, the latter class of compounds has been also treated in a review concerning fungal colouring products for the food industry [115].

Table 4.1 Examples of prospect drugs originally extracted from marine-derived fungi

Product	Producing fungus	Basic bioactivities	Reference
Aigialomycin D	*Aigialus parvus*	Antimalarial	[120]
Aquastatin A	*Cosmospora* sp.	Antidiabetic	[121]
Ascosalipyrrolidinone	*Ascochyta salicorniae*	Antimalarial	[122]
Avrainvillamide	*Aspergillus* sp.	Antitumor	[123]
7-Chlorofolipastatin	*Aspergillus unguis*	Anticholesterolemic	[124]
Chromanone A	*Penicillium* sp.	Antitumor, antioxidant	[125]
Corollosporine	*Corollospora maritima*	Antibiotic	[126]
Gymnastatins	*Gymnascella dankaliensis*	Antitumor	[127]
Halovirs	*Scytalidium* sp.	Antiviral	[128]
Halymecin A	*Fusarium* sp.	Antialgal	[129]
Leptosins	*Leptosphaeria* sp.	Antitumor	[130]
Myrothenone A	*Myrothecium* sp.	Tyrosinase inhibitor	[131]
Nigrospoxydon A	*Nigrospora* sp.	Antibiotic	[132]
Pestalone	*Pestalotia* sp.	Antibiotic	[133]
Phenylahistin	*Aspergillus ustus*	Antitumor	[118]
Sansalvamide A	*Fusarium* sp.	Antiviral	[134]
Scopularides A–B	*Scopulariopsis brevicaulis*	Antitumor	[135]
Sorbicillactone A	*Penicillium chrysogenum*	Antitumor, antiviral	[136]

Whatever the point of view chosen for presenting such an extraordinary multitude of compounds, the conclusion is that the current knowledge is rather limited if compared to the real extent [116]. However, besides the dated and well-known case of cephalosporin C [117], this disclosed chemodiversity has not yielded a remarkable number of new drugs yet [99]. Basically, this is by reason of the time required for the development of pharmaceutical products. As an example, the cyclic dipeptide plinabulin, a synthetic derivative of phenylahistin discovered 20 years ago from a marine strain of *Aspergillus ustus* [118], is still undergoing clinical trials for the treatment of non-small cell lung cancer [119]. A short list of prospect drugs reported from marine-derived fungi is given in Table 4.1, not including a few notable products which were first extracted from terrestrial strains, such as gliotoxin, mycophenolic acid and griseofulvin [137].

In the end of this chapter, a short mention is due for a few compounds which, although not being typical fungal metabolites, may introduce the opportunity for a biotechnological exploitation of the producing strains. This is the case of citric acid used in many different processes, such as in water softening for breaking down metal ions, in the cosmetic industry and as a food additive based on its properties as a flavouring or preservative agent. Some strains of *Yarrowia lipolytica* have been characterized as high citric acid producers, and this property further improved through genetic manipulation [138–140]. 2-Hydroxyphenylacetic acid is an aromatic compound used in the pharmaceutical industry as intermediate in the preparation of antihypertensive agents. Eight spongicolous strains belonging to the species *A. sydowii*, *Penicillium decaturense*, *Penicillium raistrickii* and *Bionectria* sp. were

able to synthesize this compound when phenylacetonitrile was added in the culture medium. The transformation occurred through hydrolysis of the nitrile group, followed by hydroxylation of the aromatic ring [141].

Yeasts are known for their nutraceutical effect and importance in supplying beneficial products, such as γ-aminobutyric acid, which plays a regulatory role in neural transmission in the mammalian central nervous system and in muscle tone. As a result of an investigation concerning marine yeasts carried out in Japan, a strain named Hachinohe No. 6 was selected for its content in this product, seven to ten times higher than commercial bread yeasts [142].

5 Polysaccharides

Besides small molecules, in vitro culturing of marine-derived fungal strains has led to the discovery of some novel extracellular polysaccharides with interesting bioactive properties. One of these products from a strain of *Phoma herbarum*, named YCP, proved to be able to increase mice phagocytic activity in vitro and in vivo, indicating immunomodulatory effects [143]. Similar effects have been reported for glucans produced by strains of *Candida tropicalis* and *Debaryomyces hansenii*, which could find application in aquacultures [144, 145], and strains of *Candida sake* and *Candida aquaetextoris*, conferring protection against the white spot syndrome virus (WSSV) in prawns (*Fenneropenaeus indicus* and *Penaeus monodon*, respectively) [146, 147].

More strains have yielded exopolysaccharides displaying antioxidant properties. An isolate of *Keissleriella* sp. from sediments collected in the Yellow Sea was found to produce EPS2, composed of galactose, glucose, rhamnose, mannose and glucuronic acid. This polyose possesses free radical scavenging activities and is considered for possible pharmaceutical applications in the treatment of atherogenesis, Alzheimer's disease and cancer [148, 149]. A deep-sea strain of *Penicillium* sp. produces three polysaccharides (PS1-1, PS1-2, PS2-1) which consist of mannose and variable amounts of glucose and galactose, displaying good scavenging effects on superoxide and hydroxyl radicals [150]. Moreover, a strain of *Aspergillus versicolor* from the depths of the South China Sea releases in culture a mannoglucogalactan (N1) with a molecular weight of about 20.5 kDa, displaying high radical scavenging activity [151]. The same bioactivity was observed for AVP, a mannoglucan with a molecular weight of about 7 kDa released in the fermentation broth by another strain of *A. versicolor* recovered from the coral *Cladiella* sp. in Hainan Island [152], as well as for YSS, a branched galactomannan produced by a strain of *A. terreus* from a barracuda fish from the Yellow Sea [153]. Finally, the polyextremotolerant black yeast *Aureobasidium pullulans* [154], well represented in marine contexts, is exploited for the extraction of pullulan, an exocellular polysaccharide consisting of maltotriose units with several applications in the food and medical industries [155]. A strain providing higher yields of this product in an optimized medium was isolated from a mud sample collected in the Eastern China Sea [156].

6 Enzymes

Microorganisms represent the fundamental source for most enzyme complexes employed in biotechnology, and a huge research activity is in progress worldwide to improve the effectiveness of the products currently in use and to exploit novel sources in view of increasing efficiency of processes.

Marine-derived fungi stand out as producers of biocatalysts able to operate in the unusual conditions characterizing their habitats, such as high hydrostatic and osmotic pressure, salinity and low temperature [157]. Particularly, salinity is known to adversely affect protein structure and function, the reason why many strains from salterns and other hypersaline contexts have been selected as a source for enzymes able to operate in the conditions of alkaline pH and high salt content which characterize several industrial effluents (Table 4.2).

In addition, fungal strains recovered from different substrates in cold seas are particularly studied as a source for enzymes displaying remarkable catalytic activities at low temperatures. Not surprisingly, good results in this direction have been obtained from strains collected within the many investigations carried out in recent years in the Antarctic environment [174, 180–183].

There are several industrial sectors that employ enzymes and/or their biological sources to lead more or less complex processes. Although there are evident interconnections deriving from the multipurpose usage characterizing some enzyme classes, the market for these products is usually divided into several segments [184], which are considered below for a more circumstantial overview concerning biotechnological applications.

6.1 Enzymes Used in the Pharmaceutical, Cosmetic and Food Industries

Within the marine sources of catalytic activities pertaining to this miscellaneous category, a strain of *Cryptococcus* sp. isolated from a deep-sea sediment sample collected in the Japan Trench was reported to produce superoxide dismutase, which is used in the medical and the cosmetic industries for curing inflammation and reducing damage caused to human skin by free radicals [185, 186]. A strain of *Beauveria bassiana* was characterized for the production of a salt-tolerant L-glutaminase, an exoenzyme finding applications in the food and pharmaceutical industries [187]. A psychrotolerant strain of *Guehomyces pullulans* from a sediment sample collected in Antarctica was selected as a producer of β-galactosidase, which is particularly employed for the pretreatment of milk destined to lactose-intolerant consumers [188].

Gallic acid is used as a substrate for production of an antibacterial drug (trimethoprim) and as an antioxidant in the food industry, while catechin gallates are employed in manufacturing of coffee-flavoured soft drinks and of instant tea, to

Table 4.2 Short list of salt-tolerant enzymes produced by marine-derived fungi

Enzymes	Producing fungi	References
Amylase	*Aureobasidium pullulans*	[158]
	Aspergillus gracilis	[159]
	Aspergillus penicillioides	[160]
Cellulase	Many spongicolous strains	[161]
	Aspergillus niger	[162]
	Arthrinium saccharicola	[163]
	Many strains from *Agarum cribrosum*	[164]
Chitinase	*Corollospora maritima*	[165]
	Beauveria bassiana	[166]
β-Glucosidase	*A. niger*	[167]
Laccase, Li/Mn-peroxidase	*Aspergillus sclerotiorum*, *Mucor racemosus*, *Cladosporium cladosporioides*	[168]
	Many strains from *Posidonia oceanica*	[19]
Lipase	*Geotrichum marinum*	[169]
	Nine yeast strains from several sources	[170]
	Yarrowia lipolytica	[171]
	A. pullulans	[172]
	Aspergillus awamori	[173]
	Many strains from Antarctica	[174]
Protease	Many strains from deep sea	[24]
	A. pullulans	[175, 176]
	Many strains from Antarctica	[174]
Tannase	*A. awamori*	[177, 178]
	Many strains from *Posidonia oceanica*	[19]
Xylanase	*A. niger*	[179]
	Many strains from Antarctica	[174]

enhance antioxidant activity of green tea, for flavour improvement in wines, and in fruit juice clarification. This compound is commonly obtained from tannins through enzymatic hydrolysis of ester and depside bonds, and a tannic acid hydrolase to be exploited for this purpose has been characterized from a strain of *Aspergillus awamori* from sea water [177, 178].

Other enzymatic activities finding application for the synthesis of several organic compounds have been reported for Brazilian spongicolous strains of *Penicillium myczinskii*, *Bionectria* sp., *Trichoderma* sp. and *A. sydowii* able to catalyze the stereoselective reduction of chloroacetophenone [189], of *A. sydowii* performing the transformation of bromoacetophenone derivatives [190] and of *Trichoderma* sp. and *A. sydowii* realizing the stereoselective hydrolysis of benzyl glycidyl ether, which is a possible intermediate in the synthesis of β-adrenergic blockers [191].

Phytase is used in animal feed as a supplement to enhance the nutritive value of grains and oil seeds after the release of inorganic phosphate from hydrolysis of

phytic acid. A strain of *Kodamaea ohmeri* isolated from the gut of a marine fish was selected as a producer of this enzyme [192].

Besides animal feed, where they are used for increasing the digestibility of several kind of forages, xylanases find application in the food industry, particularly in bakery, and in juice and beer manufacturing to degrade arabinoxylans for beverage clarification [193]. However, the search for marine fungal strains producing xylanase is mainly driven on account of application in the paper industry, which will be examined later on.

Inulinases (β-fructan fructanohydrolases) catalyse the hydrolysis of inulin contained in plant tissues to yield fructose and fructooligosaccharides, which are known as prebiotics and find wide application in the food industry for the production of milk derivatives, desserts, ice creams, sauces, bakery and confectionery [194]. These enzymes have been particularly reported from yeast strains collected in Chinese sea contexts, belonging to the species *Meyerozyma* (*Pichia*) *guilliermondii* [195] and *Cryptococcus aureus* [196].

6.2 Biofuels

The ability of marine-derived yeasts to produce inulinase has been considered for a possible exploitation in biodiesel production based on the transformation of inulin or raw plant materials such as extract of Jerusalem artichokes and hydrolysate of cassava starch, to synthesize lipids. The list includes strains of *Rhodotorula mucilaginosa* from the edible fish *Synechogobius hasta* [197, 198] and sea water [199] and strains of unspecified marine origin belonging to the species *Y. lipolytica* [200], *Rhodosporidium toruloides* [201] and *M. guilliermondii* [202]. Hydrolytic properties of inulinase by the latter species have been successfully enhanced through laboratory manipulation and the selection of a mutant strain [203]. Another strain of this yeast has been used as donor of the inulinase gene (*INU1*) for its successful expression in a strain of *Pichia pastoris* [204] and in a strain of *Saccharomyces* sp. performing a higher inulinase activity [205]. Properties of the latter strain as an ethanol producer were also improved after transformation with the inulinase gene from *Candida membranifaciens* subsp. *flavinogenie* [206]. Finally, similar results were obtained through the expression of the exo-inulinase gene from *C. aureus* in *P. pastoris* [207].

The use of biomasses for biofuel production is fundamentally based on the conversion of their cellulose content to ethanol. In this respect, cellulosolytic properties are assumed to be widespread among marine-derived fungi, and a study addressed to this specific subject showed that as much as 89% of the collected strains were able to produce cellulase [208]. In addition, more than 70% of a sample of 133 strains belonging to 27 *Penicillium* spp. collected in several sites in South Korea displayed endoglucanase and β-glucosidase activities [209]. On a more specific ground, production of cellulase has been evidenced in strains recovered from sediments [210, 211], salterns [212] and water samples [162, 213]. Hydrolytic

properties of cellulase have been successfully enhanced through laboratory manipulation in transformants of a strain of *Aspergillus niger* [214]. Cellulase from a strain of *Cladosporium sphaerospermum* has also displayed a potential for saccharification of seaweed biomass in solid state fermentation [215]. Finally, a strain of *Candida* sp. has been considered for the production of bioethanol through alcoholic fermentation starting from carrageenan contained in a seaweed hydrolysate [216]. Indeed, yeasts are renowned for their aptitude to perform alcoholic fermentation, and a study has been specifically addressed with the aim to improve the capacity to isolate these fungi from marine contexts and to select them for their efficiency in the production of bioethanol from marine biomasses [217].

6.3 Enzymes Used in the Textile and Paper Industries

Cellulases, as well as other β-glucanases such as the laminarinases, can be also used in bio-fertilizer processing, as bio-textile auxiliaries, for the treatment of cotton and linen products and more in general for the degradation of any kind of plant-derived material. This enzymatic complex is widespread among both obligate and facultative marine fungi recovered from submerged wood, algae and other plant detritus in coastal waters [218]. These strains are also considered as a source of hemicellulolytic xylanases and other enzymes, such as feruloyl esterases, manganese peroxidases and laccases, to be employed in the above fields [219]. In the paper and pulp industry, the use of xylanases can improve the lignin dissolution rate and reduce the usage of chlorine compounds for bleaching, thereby improving pulp properties and reducing pollution. Marine-derived fungi represent a valuable source for this enzyme. In fact, an above-mentioned study reported 84% of the strains within a selected sample of marine ascomycetes to perform xylanolytic activity [208], while even the totality of another sample from Antarctic sponges was effective in this respect [180]. Moreover, half of the strains within a sample of endophytes from seagrasses and seaweeds collected along the coast of South-eastern India displayed this catalytic activity [220]. Within the many marine-derived strains reported for xylanase production, a special mention is deserved by Egyptian isolates of *Aspergillus* sp. from the inner tissue of a soft coral (*Rhytisma* sp.), displaying a high xylanase activity in the presence of the antimetabolite 2-deoxyglucose [221] and of *Aspergillus flavus*, *C. sphaerospermum* and *Epicoccum purpurascens* from decayed submerged wood, whose coculturing remarkably increased their good catalytic activities [222].

6.4 Enzymes for Environmental Applications

Effluents from the paper and pulp industry contain large quantities of black liquors with toxic chlorinated lignin-degradation products, namely, chloroaliphatics, chlorolignins, and chlorophenols [223]. Besides the direct use of purified enzymes,

a method has been developed for the treatment of paper mill effluents based on a co-immobilization system consisting of two selected strains from sediments (*Penicillium janthinellum* and *Pestalotiopsis* sp.), by which the insoluble fine fibres can be successfully biodegraded and removed from the wastewater. The association of these two fungi exhibited more stable and stronger biocatalytic activity on a broader spectrum of substrates [224].

A by-product of sugar mills and alcohol distilleries, molasses spent wash, contains dark brown recalcitrant compounds defined as melanoidins, which are also toxic to many microorganisms involved in the wastewater treatment processes. Anaerobic digestion of these effluents is usually made by distilleries in order to obtain a dark brown sludge to be used as fertilizer [225]. Similar problems arise with coloured effluents released by textile industries, which contain mixtures of dyes resistant to degradation [226].

Wastewaters from the textile industry represent a major source of pollution in many countries, containing consistent amounts of dyes, metals/metalloids, salts and other organic pollutants. The biotechnological approach for remediation is based on the use of microbial strains which can perform the degradation and decolourization of textile effluents through the concurrent action of enzymes such as azoreductases, peroxidases and laccases [227]. By reason of lack of substrate specificity, peroxidases and laccases can degrade or modify a wide range of aromatic and other recalcitrant xenobiotics occurring in industrial effluents. Moreover, these enzymes are reported to be consistently induced in effluents containing such environmental pollutants [228]. Due to their oxidoreductive action, ligninolytic complexes have found practical application for the treatment of several kinds of industrial effluents, and many marine-derived fungi have been characterized as a source for these enzymes, as a result of investigations involving single [229–231] or multiple strains/species [208, 228, 232–238].

Chitinases are involved in the degradation of chitin, which is considered as the second most widespread polysaccharide and is the dominant component of residues of the food industry treating crustaceans. Therefore, these enzymes may have multiple applications in the treatment of these biomasses. However, although chitinases are widespread among fungi, there are few reports from marine-derived strains. Besides citations in Table 4.2, chitinolytic activity has been evidenced in strains of *Trichoderma* spp. recovered from Mediterranean sponges (*Psammocinia* sp.) [239] and in a strain of *A. terreus* from sediments [240].

7 Bioremediation

Besides representing a source of enzyme complexes to be used for the treatment of industrial effluents, marine-derived fungi may find direct employment in several bioremediation issues. Nowadays, one of the environmental problems causing major concern worldwide is represented by pollution caused by the polycyclic aromatic hydrocarbons, which basically derives from incomplete combustion of fossil fuels or

arises in consequence of oil spills from pipelines, tanker ships and offshore platforms. The evidence is strong that the resident mycoflora can readily adapt and proliferate in coastal areas impacted by oil spills, representing a fundamental resource to be exploited for remediation from these frequent accidents [241, 242]. Tar balls transported to the shore from the open sea are also colonized by fungi [243]. The process of petroleum biodegradation is quite complex, depending on the amount and the structural complexity of the hydrocarbons and their availability to microorganisms. Actually, fungal strains of marine origin have been reported to effectively degrade highly dangerous pollutants such as phenanthrene, also by reason of their capacity to adsorb these molecules on the mycelial surface [244]. Among several reports concerning marine-derived fungi potentially useful in this respect, a strain related to *Aspergillus silvaticus* from marine sediments in China proved to be able to degrade benzo[a]pyrene by using this compound as the sole carbon source [245]. Even more effectively, a strain of *Aspergillus sclerotiorum* isolated from the scleractinian coral *Mussismilia hispida* in Brazil was effective in depleting pyrene and benzo[a]pyrene. These aromatic products were converted into the corresponding pyrenyl sulphates through a supposed metabolic pathway where conjugation with sulphate ions is preceded by hydroxylation mediated by the cytochrome P-450 monooxygenase system [246]. More common are fungi able to degrade the aliphatic component of crude oil, such as a series of yeast strains (*Y. lipolytica*, *Candida* spp.) recovered in the Mumbai Port (India) [247, 248]. Another strain of *Y. lipolytica* was particularly effective in the degradation of bromoalkanes [249].

Additional perspectives for application in bioremediation from oil spills may result with reference to the ability by some fungi to release biosurfactants promoting oil-water emulsification. Compounds with such properties are known to be produced by yeast strains of *Y. lipolytica* from India [250] and Brazil [251] and of *Pseudozyma hubeiensis* recovered from a deep-sea clam in Japan, synthesizing an erythritol derivative [252]. Moreover, in a recent investigation carried out on a sample of 100 marine-derived fungi in Italy, strains of the species *Acremonium sclerotigenum*, *Myceliophthora verrucosa*, *Arthopyrenia salicis* from the green alga *Flabellia petiolata*, *Penicillium roseopurpureum* from the brown alga *Padina pavonica* and *Penicillium chrysogenum* from *P. oceanica* were selected for their ability to produce hydrophobins, which represent the most powerful surface-active proteins known so far [253].

Another field of bioremediation concerns unexploded ordnance, leading to contamination of marine environments by 2,4,6-trinitrotoluene (TNT) or hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). An Indian strain of *Y. lipolytica* was found to be able to reduce the nitro groups in TNT to aminodinitrotoluene through a dual pathway involving either the formation of amino derivatives or denitration to 2,4-dinitrotoluene, which in turn can be metabolized by other microbes [254]. Four fungal strains recovered from sediments in Oahu (Hawaii), tentatively identified as members of *Rhodotorula*, *Bullera*, *Penicillium* and *Acremonium*, were found to be able to perform mineralization of this compound. Particularly, the latter strain promoted a biotransformation pathway involving compounds such as methylene dinitramine, nitrous oxide and formaldehyde, together with trace amounts of a few nitro-triazine derivatives [255].

Nitrile-hydrolyzing biocatalysts find application for the treatment of wastes containing nitrile and cyanide compounds. A strain of *A. sydowii* was characterized as a promising biocatalyst for the preparation of carboxylic acids based on its ability to transform phenylacetonitrile compounds into phenyl acetate derivatives [256].

Remarkable work has been done in recent years by Brazilian researchers concerning degradation of pesticides by spongicolous fungi. Strains of *A. sydowii*, *Penicillium miczynskii* and *Trichoderma* sp. were selected for their ability to degrade DDD (1,1-dichloro-2,2-bis-(4-chlorophenyl)ethane) [257], while strains of *A. sydowii*, *P. raistrickii*, *Cladosporium* spp., *Microsphaeropsis* sp., *Acremonium* sp. and *Westerdykella* sp. were able to grow in a medium containing the pyrethroid esfenvalerate and its main degradation metabolites and to decompose them at some extent [258]. Moreover, strains of *A. sydowii* and *P. decaturense* were able to perform the biodegradation/bioconjugation of methyl parathion and its degradation product *p*-nitrophenol [259]. The same compounds were decomposed by strains of *Penicillium citrinum* and *Fusarium proliferatum* recovered from the ascidian *Didemnum granulatum* in Brazil [260]. Finally, a strain of *P. miczynskii* performed the biotransformation of dieldrin with high conversion rates (90%) [261].

Besides their well-documented biotechnological use as antibiotic producers, fungi can be exploited in view of their ability to degrade these substances. Salmon farming requires the use of antibiotics, such as oxytetracycline, for the control of fish bacterial diseases. High quantities of this wide spectrum antibiotic are used and dispersed in the coastal areas where these farms are located, with a negative impact on the environment. By obvious evidence, in this case the remediation can only occur as a result of an eventual action of naturally spread agents capable to degrade the molecules. Investigations carried out in Chile showed that five fungal strains from marine sediments belonging to the species *Penicillium crustosum*, *R. mucilaginosa*, *Talaromyces atroroseus*, *Trichoderma harzianum* and *Trichoderma deliquescens* were effective for this purpose [262].

As a result of technological and socio-economic progress, pollution by metals has drastically increased over the years, leading to bioaccumulation at levels exceeding the normal limits in every step of the food chain and ensuing serious ecological and medical problems. All the biological components of an ecosystem can be effective in removing heavy metals from the surrounding environment through their metabolic activity. Particularly, the metal-sequestering properties of the fungal cell and the absorption potential of the dead fungal biomass have been pointed out in bioremediation of pollution by these toxic products [263]. Such an opportunity is also to be considered with reference to the marine environment. In fact, the experimental evidence that in marine-derived fungi halophilism is correlated to tolerance to heavy metals qualifies these microorganisms among the best sources to be exploited in this respect. Several *Penicillium* strains recovered from coastal waters and salterns around Goa (India) disclosed high tolerance to cadmium, copper and lead in their sulphate and nitrate forms [264]. High tolerance to $CuSO_4$ correlated to increased superoxide dismutase activity was displayed by an above-mentioned strain of *Cryptococcus* sp. [185, 186]. Two strains of the species *Corollospora lacera* and *Monodictys pelagica* were able to accumulate lead extracellularly and to tolerate

high concentrations of this metal in the growth medium; conversely, they were remarkably affected by cadmium, which was detected in higher amounts in the mycelium of the latter strain [265]. A strain of the yeast *Rhodosporidium diobovatum* recovered from deep-sea sediments in the Indian Ocean proved to be an efficient intracellular accumulator of lead sulphides, thereby potentially interesting in the bioremediation of lead-contaminated soils [266]. High tolerance to lead was also observed in another strain of *Penicillium* sp. from deep-sea sediments collected near the Mariana Islands, which was able to accumulate the metal either on the outer cell wall or in vacuoles, and even in the form of large particles in the cytoplasm [267]. Dead biomass of a strain of *A. niger* of unspecified marine origin was found to be effective for chromium biosorption, at a rate which increased together with acidity of the medium [268]. The same properties were displayed by strains of *Y. lipolytica* [269]. Strains of *A. niger* and *A. flavus* associated to a seaweed (*Eucheuma* sp.) showed luxuriant growth at concentrations of hexavalent chromium up to 100 ppm, with *A. flavus* exhibiting higher accumulation potential [270]. A strain of *Aspergillus candidus* grew very well when exposed to arsenic at different concentrations, reflecting a remarkable tolerance to both the trivalent and pentavalent forms of this element. The capacity to bioaccumulate these toxic compounds qualifies the above strain/species as a candidate for bioremediation of arsenic-contaminated substrates [271]. Many *Aspergillus* strains recovered from different marine substrates, particularly those belonging to the species *A. versicolor*, *A. flavus* and *A. niger*, exhibited consistent tolerance to lead and copper whose cations were sequestrated through biosorption [272]. Finally, biosorption is also responsible for removal of nickel dispersed in aqueous solutions by a strain of *Y. lipolytica* [273].

8 Nanotechnologies

Last but not the least, exploitation of marine-derived fungi is in progress even as producers of metallic nanoparticles, which have found application in diverse fields, such as electronics, coatings, cosmetics, packaging and medicine. Many microorganisms have been reported for their ability to synthesize these nanoparticles, but fungi are especially considered for their tolerance and metal accumulation ability [274]. Silver nanoparticles are particularly employed with reference to their antibacterial properties [275]. The ability to produce silver nanoparticles following administration of $AgNO_3$ to the growth substrate has been pointed out by strains recovered from mangrove sediments belonging to the species *Penicillium fellutanum* [276], *Pichia capsulata* [277], *A. niger* [278], *A. flavus* [279] and *Trichoderma gamsii*. Besides antimicrobial activity, nanoparticles from the latter strain exhibited antioxidant effects and cytotoxicity against the tumour cell line HEp2 [280]. Moreover, the synthesis of golden nanoparticles following administration of chloroauric acid has been reported by strains of *Y. lipolytica* [281, 282] and *A. sydowii* [283]. Finally, intracellular synthesis of stable lead sulphide nanoparticles has

been documented by an already-mentioned strain of *R. diobovatum*, whose growth remarkably increased after supplementing non-protein thiols [266].

9 Future Perspectives

The recent findings concerning biosynthesis of metallic nanoparticles represent a meaningful case of how the biotechnological exploitation of marine-derived fungi is on the point to make a quantum leap and may lead to the creation of biofactories with considerable perspectives. Novel applications are on the way particularly in the biomedical field, where these products have been the subject of fine studies on cerebral ischemia [284]. Indeed, the enlargement of the range of possible applications is in turn conducive for further investigations in diverse environmental contexts, which result in the finding of strains displaying novel properties.

However, the future perspectives for biotechnological applications concerning marine-derived fungi also rely on further progress in our capacity to explore in depth their biochemical properties and the biomolecular basis of the latter. Particularly, more circumstantial genetic studies are desirable to unravel the complex relationships existing between endophytic/endozoic fungi and their hosts, which represent a fertile evolutionary ground in view of the possibility of horizontal gene transfer [285, 286]. Moreover, implementation of metagenomic investigations may allow to access the biosynthetic potential of non-culturable species/strains [287, 288].

And even the material collected so far may deserve to be further investigated, considering that a multipurpose biotechnological aptitude has been disclosed in a few cases. In this respect, a meaningful example is represented by strain NCIM 3589 of *Y. lipolytica*, recovered from mud in the Mumbai Port in India, which has been characterized for applications in the field of bioremediation towards different kinds of marine pollutants [248, 249, 254, 269, 289] and for the production of lipase [171], citric acid [139] and golden nanoparticles [281].

Within a general context characterized by an increasing pressure of the human society on the environment, with the urgent need for an effective remediation to pollution and a reduction of carbon dioxide emissions, the incentive is powerful for the development of more effective processes in view of a rational use of wastes and biomasses. And the challenge is great for the development of research programmes aiming at a more comprehensive appraisal of the marine biological sources finalized to the selection and exploitation of fungal strains which may help humanity in meeting those needs.

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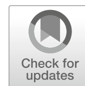
Chapter 5
Aplysinopsins as Promising Marine Natural Product Drug Leads: Recent Developments



Kevin Lewellyn and Jordan K. Zjawiony

1 Introduction

Natural products (NPs) have been a source of new drug leads since the dawn of medicine. A significant percentage of new small-molecule drug leads are derived from or based on natural products, with 25% of new small-molecule drugs launched in 2014 being natural products or derived from natural products [1]. NPs often possess unique chemical scaffolds that are not found in synthetic chemical libraries. Many other NPs are in clinical development at the moment, and there are a number of reviews that cover NPs that are currently on the market or in various stages of clinical development [2].

Initially, most natural product-derived medicines were of terrestrial origins. However, the last few decades have seen SCUBA and other technologies make exploration of marine environments easier. As a result, many new marine natural products (MNPs) have been identified. Furthermore, these MNPs often occupy new chemical spaces that were not represented by terrestrial NPs. A study by Kong et al. found that 71% of the molecular scaffolds of MNPs were found exclusively in marine organisms [3]. However, even with the aforementioned advanced SCUBA techniques and increased exploration, the ocean still remains a largely unexplored territory [4]. Scientists estimate that less than 5% of deep oceans have been explored. To date, it is estimated that roughly 25,000 marine natural products have been

K. Lewellyn
School of Pharmacy, University of Mississippi, Oxford, MS, USA

J. K. Zjawiony (✉)
Department of BioMolecular Sciences, Division of Pharmacognosy, School of Pharmacy, University of Mississippi, Oxford, MS, USA
e-mail: jordan@olemiss.edu

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Table 5.1 FDA-approved marine natural products

Name	Natural source	Treatment
Brentuximab vedotin (SGN-35)	Cyanobacterium (*Symploca hydnoides*)	Cancer [6]
Trabectedin (ET-743)	Tunicate (*Candidatus Endoecteinascidia frumentensis)*	Cancer [7]
Cytarabine (Ara-C)	Sponge (*Cryptotheca crypta*)	Cancer [8]
Eribulin mesylate (E7389)	Sponge (*Halichondria okadai)*	Cancer [9]
Ziconotide	Cone snail (*Conus magus*)	Pain [10]
Omega-3-acid ethyl esters	Various fishes	Hypertriglyceridemia [11]
Arabine (Ara-A)	Sponge (*Tethya crypta)*	Antiviral [12]

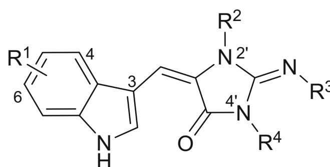
described [5]. There are currently seven marine natural products that are FDA approved for use in the United States, as shown in Table 5.1, most of which are anticancer agents. Several others are in various stages of clinical trials. For a thorough review of the current status of MNPs in clinical development, please see the 2016 review by Newman and Cragg [13]. Beyond clinical development of MNPs, there are several excellent reviews covering the most up-to-date MNP discoveries of new compounds and biological activities. Please see the reviews of Blunt et al. and Motuhi et al. for recent reviews of MNP research efforts [14, 15].

One class of NPs that have received significant interest from the biomedical community is the marine indole alkaloids. Many of them share structural similarities to endogenous neurotransmitters, thus their attractiveness as drug leads to possibly treat central nervous system (CNS) disorders such as anxiety and depression. Several indole alkaloids are on the market to treat CNS disorders. Perhaps the most well-known are the triptans such as zolmitriptan and rizatriptan, which are used to treat migraines [16]. In most cases these drugs act as agonists of serotonin receptor subtypes 5-HT_{1B} and 5-HT_{1D}.

2 Aplysinopsins

One specific group of marine indole alkaloids to pique the interest of MNP researchers are the aplysinopsins. Aplysinopsins are tryptophan-derived MNPs that were first isolated in 1977 from Indo-Pacific sponges from the genera *Thorecta* [17]. Those species were subsequently reassigned as the *Aplysinopsis* genera, thus the name of the parent compound aplysinopsin (**1**). In addition to sponges, aplysinopsin analogs have been isolated from a variety of marine sources: corals [18], mollusks [19], and sea anemones [20].

The general structure of aplysinopsins is shown in Fig. 5.1. The basic structure consists of an indole moiety and an imidazolidinone. As shown in Fig. 5.1, there are



General Structure of Aplysinopsin Analogs

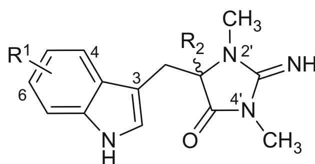
CMPD	Name	R^1	R^2	R^3	R^4
1	aplysinopsin	H	CH_3	H	CH_3
2	5,6-dibromo-2′-demethylaplysinopsin	4,6-Br	H	H	CH_3
3	2′-de-*N*-methyl-aplysinopsin	H	H	H	CH_3
4	6-bromo-2′-de-*N*-methylaplysinopsin	6-Br	H	H	CH_3
5	6-bromoaplysinopsin	6-Br	CH_3	H	CH_3
6	6-bromo-4′-de-*N*-methylaplysinopsin	6-Br	CH_3	H	H
7	methylaplysinopsin	H	CH_3	CH_3	CH_3

Fig. 5.1 Molecular structures of natural aplysinopsin alkaloids **1**–**7**

several common structural variations in naturally occurring aplysinopsin analogs. The following section details the structural variations in naturally occurring aplysinopsin analogs; synthetic derivatives will be addressed in subsequent sections.

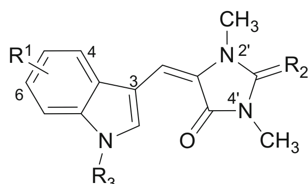
First is the configuration of the double bond between C8 and C1′. Initial reports of aplysinopsins typically described the *E* isomer of the analog; however, it is not clear in many of these early reports if the configuration was actually determined. In 1988, Guella et al. published a straightforward NMR method for differentiating between aplysinopsin geometrical isomers [21]. Examining $^{1}H,^{13}C$ heteronuclear coupling constants between H-C(8) and C(5′) allows for the determination of the predominant isomer. Further work by Guella indicated that analogs without an alkyl group at N (2′) will exist predominantly in the *Z* configuration, while those with an alkyl group at N(2′) will exist predominantly in the *E* configuration [22]. In the same report, Guella et al. also describe the ability to enrich the mixture of aplysinopsin *Z/E* isomers via irradiation with 350 nm light. Using this methodology, they were able to convert compound **3**, which was initially synthesized with a *Z/E* ratio of >95:5, to a mixture of *Z/E* of approximately 3:1. Furthermore, crystallography of several aplysinopsin analogs by Johnson et al. [23] revealed that analogs substituted at N (2′) exist and can be crystallized in the *Z* configuration.

In addition, the bromination pattern on the indole moiety can vary from non-brominated to monobrominated at C6, to dibromo derivatives which are



CMPD	Name	R^1	R^2
8	1′,8-dihydroaplysinopsin	H	H
9	6-bromo-1',8-dihydroaplysinopsin	6-Br	H
10	6-bromo-1'-hydroxy-1',8-dihydroaplysinopsin	6-Br	OH
11	6-bromo-1'-methoxy-1',8-dihydroaplysinopsin	6-Br	OCH_3
12	6-bromo-1'-ethoxy-1',8-dihydroaplysinopsin	6-Br	OCH_2CH_3

Fig. 5.2 Structures of aplysinopsins **8–12**



CMPD	Name	R^1	R^2	R^3
13	3′-deimino-3′-oxoaplysinopsin	H	O	H
14	6-bromo-3′-deimino-3′-oxoaplysinopsin	6-Br	O	H
15	N-propionylaplysinopsin	H	NH	$OCCH_2CH_3$
16	N-methylaplysinopsin	H	HH	CH_3

Fig. 5.3 Structures of aplysinopsins **13–16**

halogenated at positions C5 and C6 (Fig. 5.1). Further structural variation is seen in 1′,8-dihydroaplysinopsins and the related C1′ substituted aplysinopsin analogs (analogs **8–12,** Fig. 5.2). These analogs were isolated from Indo-Pacific sponges [24]. Also, analogs with substitutions on N-1 (**13–16**) were identified from sponges [18] and anthozoans [6] (Fig. 5.3).

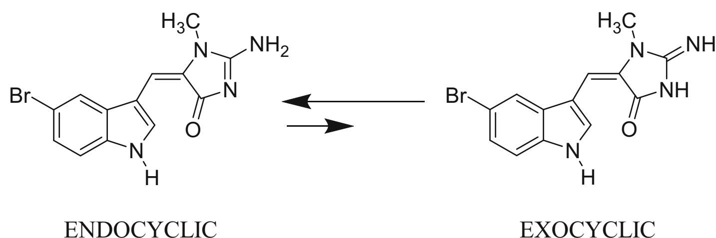


Fig. 5.4 5-Bromo-2′-de-*N*-methylaplysinopsin (**17**)

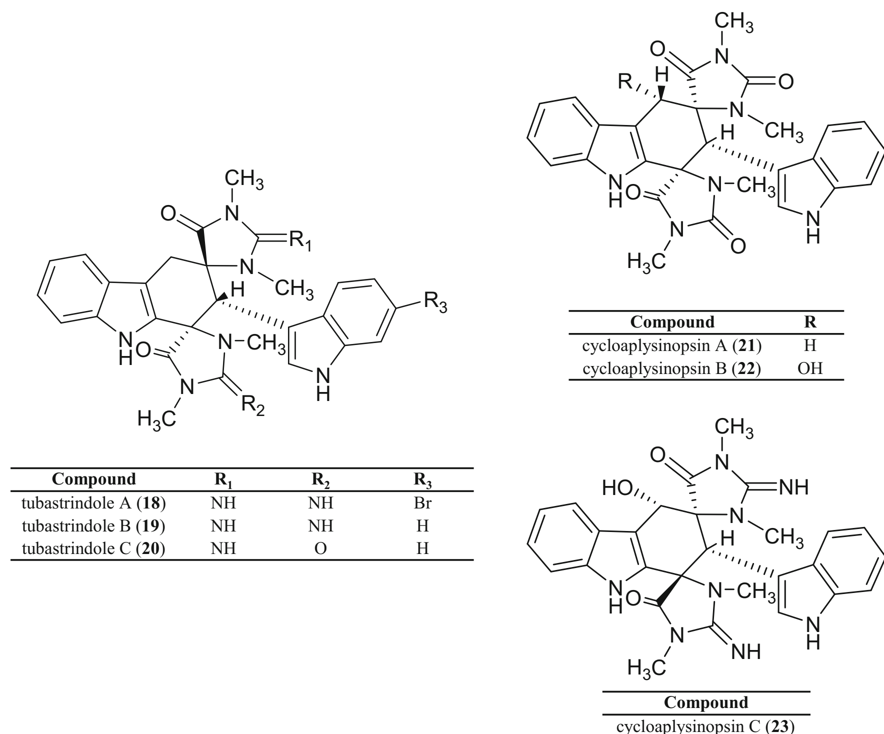
The methylation pattern of the imidazolidinone ring can vary widely, as shown in the analogs listed in Fig. 5.1. There have been naturally occurring analogs isolated with mono-, di-, and tri-methylation patterns along the ring. The tautomeric form of the imidazolidinone ring is another source of variation among naturally occurring analogs. The imine double bond can be endo- or exocyclic as shown in Fig. 5.4. The aforementioned crystallography work of Johnson et al. addressed this concern using 5-bromo-2′-de-*N*-methylaplysinopsin (**17**). Their work showed this analog exists in the endocyclic form in both the solid state and in d_6-DMSO solution [23].

Aplysinopsin dimers (Fig. 5.5) were first isolated from the coral *Tubastraea faulkneri* in 2000 [25]. The first full spectroscopic descriptions of aplysinopsin dimers were published in 2003 as tubastrindoles A–C (**18–20**) were isolated from extracts of *Tubastraea* sp. [26]. Additionally, cycloaplysinopsins A (**21**) and B (**22**) were isolated from dendrophylliid coral and fully characterized [27]. The related cycloaplysinopsin C (**23**) was described in 2009 by Meyer et al. [28]. For a more thorough review of aplysinopsin dimers and their possible biosynthetic origins, please see the work of Boyd and Sperry [29].

3 Synthesis of Aplysinopsin Analogs

Further clinical development of MNPs often suffers from the fact that supplying enough material for in vitro and in vivo assays, much less human trials, can be challenging and has devastating effects on the ecosystems they are harvested from. For example, it is not feasible or reasonable to harvest thousands of kilograms of marine sponges regularly. Therefore, supply issues are often times the pinch points in drug development efforts for MNPs.

Aplysinopsins are no exception. Initial isolation efforts possessed low overall yields, but fortunately the aplysinopsin scaffold is relatively easy to access via synthetic methodologies. The earliest synthetic efforts proceeded with the base-catalyzed condensation of an appropriate 3-formylindole derivative with the designated imidazolidinone that possesses the requisite α-methylene carbonyl functionality as shown in Fig. 5.6. The imidazolidinones were typically hydantoin or creatinine derivatives [21, 22, 30].



Compound	R_1	R_2	R_3
tubastrindole A (**18**)	NH	NH	Br
tubastrindole B (**19**)	NH	NH	H
tubastrindole C (**20**)	NH	O	H

Compound	R
cycloaplysinopsin A (**21**)	H
cycloaplysinopsin B (**22**)	OH

Compound
cycloaplysinopsin C (**23**)

Fig. 5.5 Aplysinopsin dimers

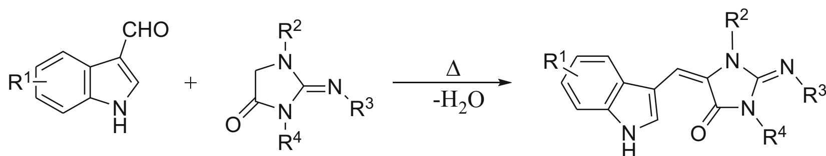


Fig. 5.6 Condensation of indole-3-carbaldehydes with hydantoins leading to aplysinopsin analogs

Throughout the 1990s, chemists sought more efficient methodologies and several new synthetic routes to aplysinopsins were developed. Stanovnik and Svete authored a comprehensive review of the synthesis of aplysinopsins in 2005 that covered all methodologies reported from the late 1980s up to 2005 [31]. The following section here will review what new synthetic methodologies have been applied to aplysinopsins since that time, in chronological order.

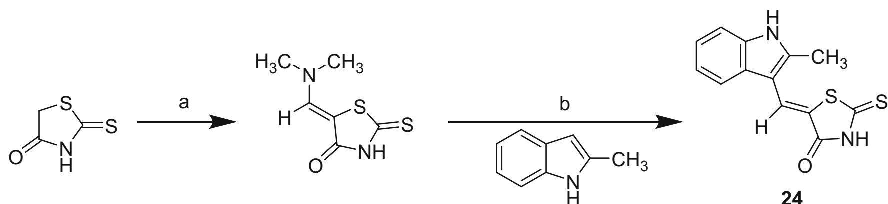


Fig. 5.7 Reagents and conditions for **24**: (a) Bredereck's reagent, DMF, reflux 3 h; (b) AcOH, reflux

3.1 Thioaplysinopsin Analogs

The aforementioned group of Branko Stanovnik continued their innovative work on aplysinopsins with the work of Jakse et al. in 2007 [32]. This group has led the way in regard to the incorporation of heterocycles into the aplysinopsin scaffold to generate synthetic aplysinopsin analogs, such as the thioaplysinopsins. Here they described the stereoselective synthesis of thioaplysinopsin analogs, as shown in Fig. 5.7. As an example, let us examine the synthesis of (*Z*)-5-[(2-methylindol-3-yl)methylidene]-2-thioxo-1,3-thazol-4-one (**24**). The first step in the synthesis is the conversion of rhodanine to the dimethylaminomethylidene. Following that, 2-methylindole was added, the mixture refluxed in acetic acid for several hours and the resulting product **24** purified via crystallization. The overall yield for this particular analog was 40%, and the average for all thioaplysinopsin analogs prepared was approximately 50%. This methodology provides easy access to novel thioaplysinopsin analogs.

3.2 Pentamidine-Aplysinopsin Synthesis

In an effort to discover antileishmanial compounds, the group of Porwal et al. synthesized a group of pentamidine-aplysinopsin hybrid molecules in 2009 [33]. The synthesis of these molecules presents an interesting reaction pathway that is useful not only for the intended pentamidine-aplysinopsin analogs but also potentially for other aplysinopsin hybrid molecules. Their initial efforts at synthesizing compound **25** via a more traditional route of synthesis of the appropriate aplysinopsin analog, then coupling with the desired pentamidine fragment, were unsuccessful. They observed poor yields and a mixture of *E* and *Z* isomers. Ultimately, their successful route, as shown in Fig. 5.8, began with commercially available indole-3-carbaldehyde, which was stirred with *p*-cyanophenoxypentylbromide in toluene for 1.5 hours to generate the pentamidine portion of the molecule. The resulting crude solid was decanted and then recrystallized from 1% MeOH in chloroform. This yielded intermediate **26** at 71% yield. Following that, **26** was stirred with 2-thiohydantoin in ethanolamine at

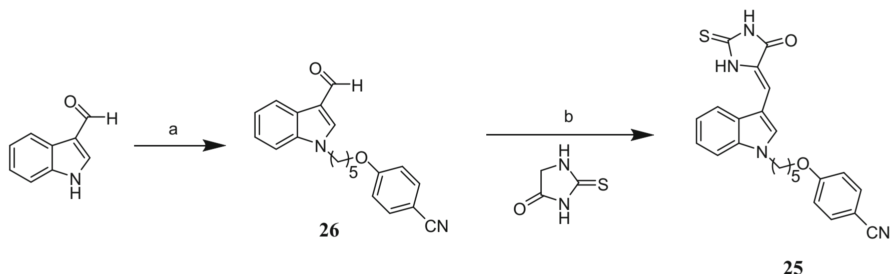


Fig. 5.8 Reagents and conditions for **25**: (a) p-cyanophenoxypentylbromide, toluene, NaOH, tetrabutylammonium bromide (TBAB), rt.; (b) ethanolamine, ethanol (abs), 60 °C

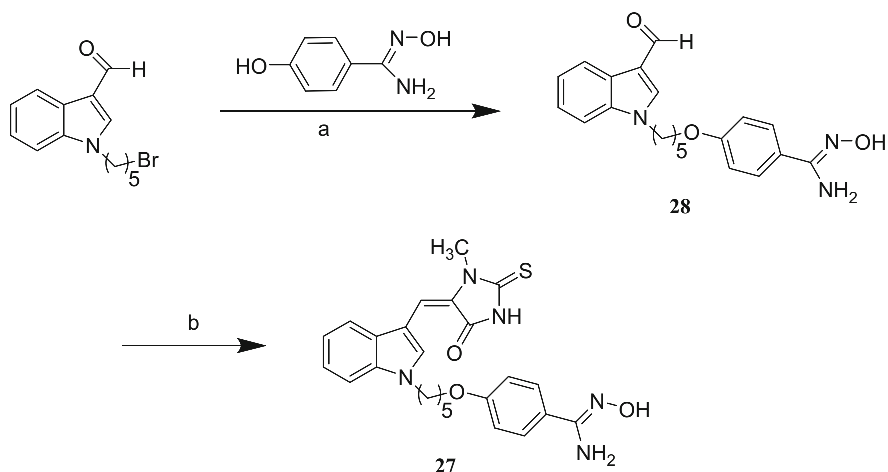
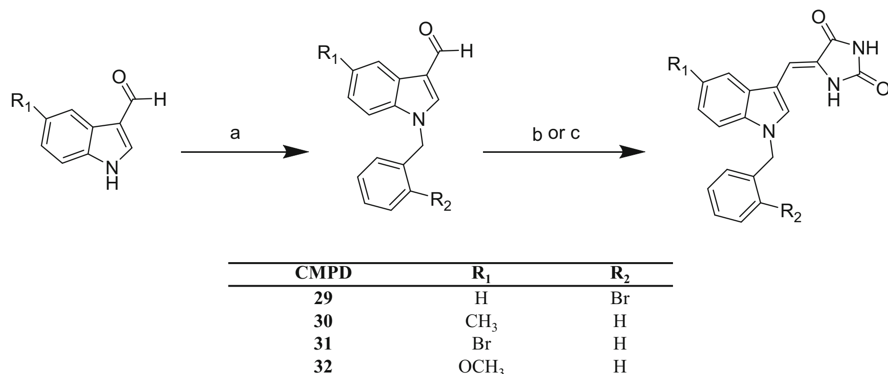


Fig. 5.9 Reagents and conditions: (a) MeCN, K_2CO_3, 60 °C; (b) 1-methyl-2-thiohydantoin, ethanolamine, 60 °C

60 °C for 2–3 h. The product was precipitated out and analog **25** recrystallized from methanol for a yield of 82%.

Seeking to optimize compound **25**'s antileishmanial activity, a series of new analogs was designed and synthesized, including the potent analog **27**, whose biological activity is discussed in Sect. 4.1. In their efforts to improve the lead compound **25**, they converted the cyano function of **25** into various other functionalities including various amides. They found that the most potent of these new analogs was compound **27**, which replaces the cyano functionality with an amidoxime functionality. Synthetically, this was achieved by synthesizing *p*-amidoximophenol by combing hydroxyl amine hydrochloride and *p*-cyanophenol in methanol and heating with the base K_2CO_3 for 8 h at 60 °C. The product was purified via column chromatography, and then the *p*-amidoximophenol was reacted with 1-(5-bromopentyl)indole-3-carbaldehyde in acetonitrile, as shown in Fig. 5.9. The mixture was heated with K_2CO_3 for 5–6 h at 60 °C. The intermediate **28** was



CMPD	R_1	R_2
29	H	Br
30	CH_3	H
31	Br	H
32	OCH_3	H

Fig. 5.10 Reagents and conditions: (a) appropriate benzyl halide, NaOH, benzyltriethylammonium chloride (TEBA), dichloromethane, rt.; (b) hydantoin, NaOAc, AcOH, microwave irradiation, 40–60 s; (c) hydantoin, NaOAc, AcOH, 115–116 °C, 8–12 h

recrystallized from ethyl acetate in hexane (5%) following workup. Intermediate **28** was then stirred with 1-methyl-2thiohydantoin in ethanolamine at 60 °C for 2–3 h. The crude product was recrystallized from methanol for a yield of 67%.

3.3 Microwave-Assisted Synthesis of Aplysinopsins

Beginning in 2009, Crooks et al. published several papers describing the potential for aplysinopsins to be used as anticancer drug leads, as discussed in Sect. 4.3. During their work, they also developed a more efficient method for the aldol condensation of substituted indole-3-carboxaldehydes with creatinine [34]. Overall, their reaction scheme (Fig. 5.10) began with the synthesis of the appropriately substituted *N*-benzylindole-3-carboxaldehydes. These were generated in good yields (85–90%) by treating the indole-3-carboxaldehyde with substituted benzyl halides under phase-transfer catalytic conditions. Following that, the *N*-benzylindole-3-carboxaldehydes were heated with hydantoin for the aldol condensation, yielding compounds **29–32**. This was done using two methods: the traditional reflux in the presence of NaOAc and AcOH for 7–10 h or via microwave irradiation for 30–60 s. Furthermore, the group found that, in general, the yields were greater when using the microwave irradiation method as well. The Crooks group would follow this up with two more reports of anticancer activity from aplysinopsin analogs that were all synthesized using this methodology [35, 36]. It represents and efficient and fast synthetic method to quickly access a large number and variety of *N*-benzyl aplysinopsin analogs.

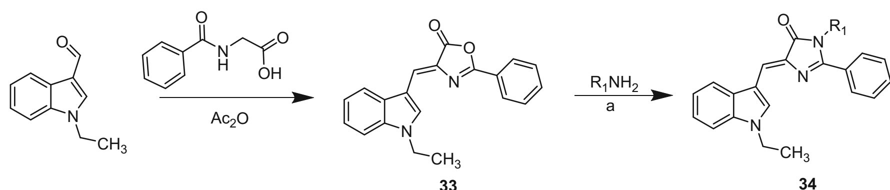


Fig. 5.11 Reagents and conditions: R1 = 5-flurotryptamine; (a) ethylene glycol, reflux, 30 min

3.4 Recyclization of Oxazolones into Aplysinopsins

Another unique approach to access the aplysinopsin scaffold was published in 2014 by Suzdalev and Babakova, which demonstrated the ability to access the aplysinopsin scaffold via recyclization of readily available oxazolones [37]. Their synthetic strategy involves generating aplysinopsin analogs via heating oxazolones with primary amines in ethylene glycol or DMF. The first step, synthesis of the oxazolone, proceeds via the Erlenmeyer-Plochl reaction, as outlined in Fig. 5.11. They were able to produce the desired oxazolones with a largely predominating *Z* isomer in most cases. In the example shown, where the starting indole-3-carbaldehyde is substituted with an ethyl group at the indole nitrogen, the resulting oxazolone (**33**) was actually found to be exclusively *Z* configured.

Once the desired oxazolones are obtained and purified via recrystallization, they are refluxed in DMF or ethylene glycol to force an ANRORC-type recyclization and generation of the imidazolone derivative aplysinopsin analogs as shown in Fig. 5.11. The reaction to generate analog **34** was also reported as exclusively the *Z* isomer is formed.

4 Biological Activities of Aplysinopsins

The various aplysinopsin analogs have been reported to possess a multitude of biological activities over the years. The namesake analog, aplysinopsin (**1**), was isolated using bioassay-guided fractionation techniques, and it was found to possess an inhibitory activity against P388 lymphocytic leukemia at a dose of 200 mg/kg [38]. In a separate study, several aplysinopsin analogs, notably aplysinopsin (**1**) and methylaplysinopsin (**7**), showed moderate cytotoxicity against the murine lymphoma cell line LH-1210, with half maximal inhibitory concentration (IC_{50}) values of 2.3 and 3.5 μg/mL, respectively.

MNPs have previously been a good source for antimicrobial drug leads [39]; therefore aplysinopsins were also evaluated for such activity. 6-Bromoaplysinopsin (**5**) was found to inhibit the growth of *Trichophyton mentagrophytes* [40], and a mixture of 6-bromoaplysinopsin (**5**) and 6-bromo-4′-de-*N*-methylaplysinopsin (**6**) possessed inhibitory activity toward *Bacillus subtilis* [41]. In the same study, MNPs

which inhibit the growth of *Staphylococcus aureus* were investigated, and compounds **1** and **5** were found to have moderate activity. For a more nuanced look at all of the reported biological activities prior to 2009, please see the review in *Marine Drugs* by Bialonska [42]. The rest of this section will be devoted to reviewing the biological activities of aplysinopsin analogs from 2009 to present.

4.1 Antiplasmodial and Antileishmanial Activity

Prior to 2009, several aplysinopsin-related compounds were reported to have antiplasmodial activity [43]. Since that time, two more studies have also found aplysinopsin analogs which possess antiplasmodial activities. In a 2009 report from Meyer et al. [28], three aplysinopsin analogs were isolated from the stony coral *Tubastraea* sp. One of the analogs, cycloaplysinopsin C (**23**, Fig. 5.5), was evaluated for the ability to inhibit the growth of *Plasmodium falciparum* and was found to inhibit two strains (chloroquine-sensitive F32/Tanzania and chloroquine-resistant FcB1/Colombia), with IC_{50} values of 2.7 and 1.8 μM, respectively, compared to reference values of 68 and 174 nM from the positive control chloroquine.

Following that, in 2011, aplysinopsin (**1**) was identified in an in vitro antiplasmodial screen as having an IC_{50} of 0.43 μg/mL [44]. This work was part of a large screening effort in which 184 natural product compounds were selected at random and screened for their antiplasmodial and antifungal activity. Interestingly, the group from France found that despite a wide array of chemical scaffolds in the set of 184 compounds, all of the compounds which showed activity against *P. falciparum* were indole derivatives. These studies suggest that aplysinopsins may be valuable drug leads to fight malaria, a disease that the World Health Organization estimates kills more than 600,000 annually [45].

A unique aplysinopsin-pentamidine molecule was described by Porwal et al. in 2009 [33]. This hybrid molecule was a result of the group's efforts to synthesize a new antileishmanial drug lead that could improve on activity but at the same time decrease the toxicity associated with known antileishmanial agents such as antimonials [46], pentamidine [47], and miltefosine [48]. Using the notion that small-molecule natural products are inherently more suitable as drug leads due to their biosynthetic origins, they sought to incorporate an aplysinopsin moiety into the pentamidine scaffold. Aplysinopsins were chosen due to their possessing a cyclic guanidine functionality and had a high potential for activity on two biological targets of interest: plasmepsin II and the serotonin receptor system. Based on this, they designed and synthesized several molecules that replaced one of the amidinophenoxy groups of pentamidine with an aplysinopsin analog. Their initial efforts yielded the aplysinopsin-pentamidine hybrid **25** (Fig. 5.8), which displayed a 62% growth inhibition of amastigotes at 12.5 μg/mL and, perhaps more importantly, showed no toxicity. Compound **25** also showed moderate activity in vivo, with an average parasite reduction in hamsters of 62%. Again, in the in vivo experiment, no adverse reactions were observed.

Hoping to improve on the activity of analog **25**, several new leads were synthesized with variations in key structural features such as the chain length between the pentamidine and aplysinopsin, changes in geometry, and addition of functional groups such as thiocarbonyl, cyano, and phenolic moieties. This second group of analogs yielded compound **27**, which was found to be ten times more active than pentamidine, and significantly less toxicity was observed when compared to pentamidine as well.

4.2 CNS Activity

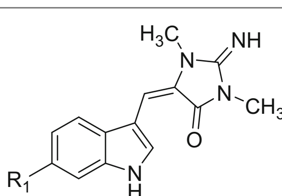
As mentioned previously, several neuromodulatory activities of aplysinopsins have been reported over the years [42]; however, of special interest are their affinity for serotonin receptors and inhibitory activity of monoamine oxidases (MAO).

4.2.1 Serotonin Receptor Activity

There is considerable interest in the serotonin receptor modulation activities of aplysinopsin analogs. The first report of such activity was in 2002, with analogs isolated from the Jamaican sponge *Smenospongia aurea* shown to possess affinity for subtypes 5-HT$_{2A}$ and 5-HT$_{2C}$ [43]. Their structure activity relationship (SAR) study showed that halogenation of the indole, as well as the pattern of *N*-alkylation, was key structural features for improving K_i values and enhancing selectivity indexes among 5-HT$_2$ subtypes 2A and 2C. Increasing selectivity among these subtypes remains a challenge due to their highly conserved sequence homologies. The work by Cummings et al. in 2010 screened a library of 20 aplysinopsin analogs for their affinities to 3 serotonin receptor subtypes: 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ [49]. They found four compounds that possessed significant selectivity for either 5-HT$_{2A}$ or 5-HT$_{2C}$. Of note, they found that analogs with fluorine substitution at C6 or non-halogenated analogs possessed higher affinity for the 5-HT$_{2A}$ receptor subtype. Conversely, analogs with chlorine or bromine substitution at C6 showed a preference for 5-HT$_{2C}$, suggesting that the size of the substituent at C6 may play a key role in receptor interaction. Recall that fluorine and hydrogen are similar in size of their van der Waals radii (1.35 Å for fluorine vs. 1.17 Å for hydrogen), whereas chlorine and bromine are much larger, 1.80 and 1.95 Å, respectively [50]. Furthermore, the bond length of carbon to fluorine is 1.26–1.41 A, similar to the 1.08–1.10 A of hydrogen to carbon bonds. These factors result in fluorine being an acceptable hydrogen mimic in serotonin receptor sites. Their work found that compounds **35** and **1** showed a clear preference for 5-HT$_{2A}$ over 5-HT$_{2C}$ and that the opposite was true for compounds **36** and **5** as shown in Table 5.2.

The authors' own work from 2013 further expanded on the selectivity between serotonin receptor subtypes by screening a library of 50 aplysinopsin analogs against

Table 5.2 Affinities for serotonin subtypes 5-HT2A and 5-HT2C



Compound	R1	Binding affinity (K_i nM)	
		5-HT2A ± SEM	5-HT2C ± SEM
35	F	235 ± 42	2114 ± 203
1	H	598 ± 53	14,451 ± 5893
36	Cl	N/A	166 ± 55
5	Br	N/A	2202 ± 674

N/A = no detectable affinity at highest concentration tested

Table 5.3 Serotonin receptor subtypes screened against

5-HT_{1A}	5-HT_{2A}	5-HT_4
5-HT_{1B}	5-HT_{2B}	5-HT_{5A}
5-HT_{1D}	5-HT_{2C}	5-HT_6
5-HT_{1E}	5-HT_3	5-HT_7

Table 5.4 Additional CNS receptors evaluated

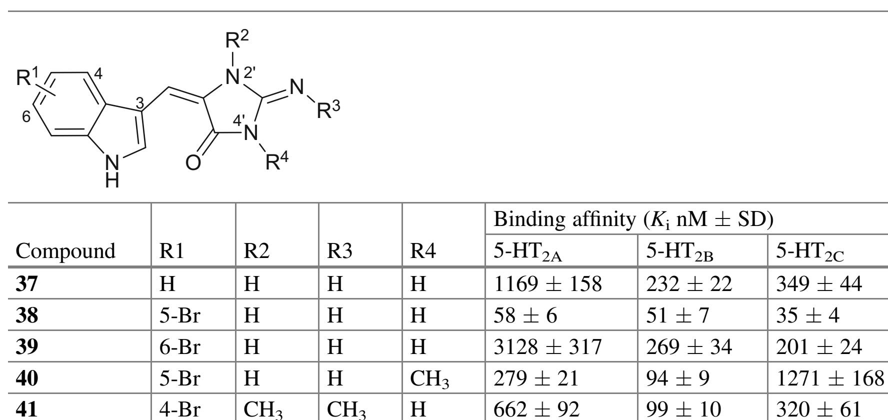
Alpha1A	Beta2	D5	H4	MOR
Alpha1B	Beta3	DAT	KOR	NET
Alpha1D	BZP rat brain site	DOR	M1	SERT
Alpha2A	D1	GABAA	M2	Sigma1
Alpha2B	D2	H1	M3	Sigma2
Alpha2C	D3	H2	M4	
Beta1	D4	H3	M5	

12 serotonin receptor subtypes, listed in Table 5.3, and 34 other CNS receptors, listed in Table 5.4 [51].

Of the 12 serotonin receptors screened against, the highest affinities were found for the 5-HT_{2B} and 5-HT_{2C} subtypes, as shown in Table 5.5. The control values for these screens were as follows: 5-HT_{2A} (chlorpromazine, 13 nM), 5-HT_{2B} (methysergide, $K_i = 26$ nM), and 5-HT_{2C} (chlorpromazine, $K_i = 26$ nM). This is the only report of aplysinopsin analogs being evaluated for their affinity to 5-HT_{2B}, which is important due to the side effects of 5-HT_{2B} agonist activity, such as cardiac insufficiency and pulmonary hypertension [52]. These side effects led to patient deaths in the fenfluramine/phentermine case [53], so evaluation of any 5-HT_{2B} activity is a requirement for compounds which are found to act on the serotonin receptor system.

This SAR study revealed that halogenation, specifically bromination, at C4 and C5, of the indole led to K_i values as low as 35 nM. These results were somewhat complementary of the previous work done by Cummings et al., in that the 6-bromo derivative did show a preference for 5-HT_{2C} over 5-HT_{2A} as expected. However, here the non-halogenated analog **37** showed a preference for 5-HT_{2C} over 5-HT_{2A},

Table 5.5 Selected serotonin receptor subtype binding affinities



Compound	R1	R2	R3	R4	Binding affinity (K_i nM ± SD)		
					5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$
37	H	H	H	H	1169 ± 158	232 ± 22	349 ± 44
38	5-Br	H	H	H	58 ± 6	51 ± 7	35 ± 4
39	6-Br	H	H	H	3128 ± 317	269 ± 34	201 ± 24
40	5-Br	H	H	CH_3	279 ± 21	94 ± 9	1271 ± 168
41	4-Br	CH_3	CH_3	H	662 ± 92	99 ± 10	320 ± 61

which would seemingly contradict their result. However, both reports are unable to draw any solid conclusions regarding the number and position of *N*-methylations on the imidazolidinone ring. Clearly these in vitro receptor binding assays indicate that the aplysinopsin scaffold may have the potential to be used as a "tunable" probe of serotonin receptors; however, the exact structure activity relationships are still not known and will require further exploration. For example, what structural feature of analog **38** allows it to have such nonexclusive binding affinity for all three receptor subtypes?

4.2.2 Monoamine Oxidase Inhibition

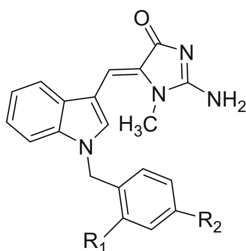
One of the first reported biological activities of aplysinopsin analogs was their ability to inhibit monoamine oxidase (MAO) [54]. Monoamine oxidase inhibitors (MAOIs) have been used to treat depressions for decades; however they were often plagued with serious side effects, and thus their use declined [55]. Since the isolation of the two isoforms of MAO (A and B), there has been a renewed interest in drugs leads that can selectively inhibit one isoform over the other [56]. Since both indoles [57] and imidazolidinones [58] are known to possess MAO inhibitory activities, it was a logical step to investigate aplysinopsins for their MAOI activities.

In 2012, the work of Lewellyn et al. evaluated a library of 50 aplysinopsin analogs for their MAO-A and MAO-B inhibitory activities [59]. In general, it was found that the aplysinopsin analogs showed more inhibitory activity toward MAO-A than MAO-B, with IC_{50} value for MAO-A inhibition ranging from 0.0056 to 26.12 μM. One lead compound, 6-bromoaplysinopsin (**5**), possessed the lowest IC_{50} value for MAO-A inhibition (0.0056 μM) and also possessed a selectivity index of 80.24 for MAO-A over MAO-B. Its IC_{50} value was lower than that of the positive control clorgyline, which had an IC_{50} value of 0.0067 μM.

The SAR revealed that two structural features were crucial for MAO-A inhibitory activity and selectivity over MAO-B. First was the *N*-methylation of the imidazolidinone. Multiple *N*-methylations are required, with one required to be at N-2′. When N-2′ is methylated, the condensation of the indole aldehyde and imidazolidinone will produce the *E*-configured isomer. If N-2′ is non-methylated, the resulting double bond will be *Z* configured. The most potent and selective MAO-A inhibitors were those which were methylated at N-2′, and thus were *E* configured, indicating that one, or both, of those factors plays a key factor in the SAR of aplysinopsins and MAOI activity. Furthermore, bromination at position C5 or C6 resulted in improved IC_{50} values among all analog subgroups. These results were in agreement with a study that examined indole analogs' MAOI activities and found substitution at C5 resulted in increased potency [60].

4.3 Anticancer Activities

Beginning in 2010, the Crooks group from the University of Kentucky published several reports regarding the antiproliferative activity of a group of aplysinopsin analogs. More specifically, they were investigating *N*-1-benzyl aplysinopsin analogs. Their initial report detailed the synthesis (Sect. 3.3) and evaluation of eight *N*-benzylaplysinopsin analogs in the NCI 60 human tumor cell line panel [34]. Of those eight, two *Z*-configured analogs (**42** and **43**, Fig. 5.12) showed enough activity in the preliminary growth inhibition screen to be further screened in dose-response cytotoxicity studies against the 60 human tumor cell lines. In the secondary screen, compound **43** was shown to be the more potent of the two, showing inhibitory effects against nearly every single cell line, with LC_{50} values less than 10 μM for



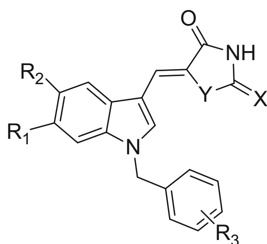
CMPD	R_1	R_2
42	Br	H
43	H	$COOCH_3$

Fig. 5.12 Structure of *N*-benzyl aplysinopsin analogs **42** and **43**

Table 5.6 Antiproliferative activity of *N*-benzyl aplysinopsin analogs **29–32**

Compound	Breast cancer cell lines (IC_{50} μM)	
	MCF-7	MDA-231
29	4.4	21.8
30	5.2	20.1
31	7.6	12.4
32	7.2	7.0
5-Fluorouracil	15.2	3.5

Table 5.7 Structure of *N*-benzyl aplysinopsin analogs **44** and **45**



Compound	R1	R2	R3	X	Y
44	COOCH3	H	*p*-CN	O	NH
45	H	Cl	*p*-F	NH	S

19 of the 60 cancer cell lines examined. Compound **42** was less promising, showing similar <10 μM LC_{50} (the concentration required to kill 50% of cells) values in only the following four of the cell lines: colon HT29, CNS SF-539, melanoma LOX IMVI, and ovarian OVCAR-3. This initial report clearly indicated that the aplysinopsin scaffold possessed utility as a lead for designing small molecules as anticancer drugs.

Later the same year, the Crooks group reported evaluating another set of 13 *N*-benzyl aplysinopsin analogs and evaluating them for their antiproliferative activities against MCF-7 and MDA-231 breast cancer cell lines, as well as the A549 and H460 lung cancer cell lines [36]. These cell viability assays revealed that the 13 analogs had moderate to weak antiproliferative effects against the two lung cancer cell lines. Overall, the analogs were more effective against the H-460 cell line, with best activity shown by compound **30**, which had an IC_{50} value of 14.6 μM. In contrast, the analogs showed more promising antiproliferative activity against the two breast cancer cell lines. Specifically, four compounds (**29–32**) showed greater activity than the positive control 5-fluorouracil, as shown in Table 5.6.

The last report for the Crooks group regarding anticancer activity of *N*-benzyl aplysinopsin analogs was published in 2011 [35]. This study screened 15 analogs against the NCI 60 human tumor cell line panel. Only two compounds (**44** and **45,** Table 5.7) passed the initial screen threshold of 60% growth inhibition to advance the second level of five dose-response screening. In the single dose-response studies, compound **44** displayed >60% growth inhibition in 50 of the 60 cell lines used, compared to compound **45**, which inhibited growth in 14 of the 60 lines. In the

multidose response studies, compounds are evaluated for both their GI_{50} (the concentration required to cause 50% growth inhibition) and their LC_{50}. Compound **44** showed promising results in regard to GI_{50} values, inhibiting all but two of the cell lines tested. Its GI_{50} values ranged from 0.30 to 6 μM, with submicromolar level inhibitory values against several lines including SK-MEL-2 melanoma (GI_{50} = 307 nm), RXF 393 renal cancer (GI_{50} = 585 nM), and IGROV1 ovarian cancer (GI_{50} = 667 nM). Compound **45** did not possess quite as broad or potent GI_{50} values; however it still inhibited roughly half of the cell lines tested against, with GI_{50} values ranging from 0.19 to 98.8 μM. Of note, it possessed low nanomolar GI_{50} values against two cell lines: UACC-257 melanoma (13.3 nM) and OVCAR-8- ovarian cancer (19.5 nM).

4.4 Glycine-Gated Chloride Channel Receptor Modulation

While the bulk of biological activities reported for aplysinopsins since 2009 focus on either CNS receptors or anticancer agents, there are several other activities reported. In a 2013 report, Balansa et al. reported the isolation of several aplysinopsin-related compounds from the sponge *Ianthella* cf. *flabelliformis* [61]. The group from Australia found five previously described aplysinopsins, including oxoaplysinopsin derivatives, as well as the aplysinopsin dimer tubastrindole B (**19**, Fig. 5.5) in the EtOH extract from the sponge. Crude extract of the sponge was found to possess glycine-gated chloride channel receptor (GlyR) modulatory activity. GlyR modulators are potentially useful as both probes to further understand GlyR functionality differences among different GlyR isoforms, as well as leads for the development of drugs to treat several disorders: breathing disorders [62], epilepsy [63], and movement disorders [64]. During the screening of more than 2500 marine invertebrate and algae, this crude extract was found to express some GlyR modulatory activity [65]. Further isolation efforts resulted in the identification of aplysinopsin analogs, and in vitro assays were able to attribute all of the GlyR activity to the aplysinopsins present. Specifically, the mixture of the *E*/*Z* mixture of 3’-deimino-3’- -oxoaplysinopsin compounds (**46** and **47**, Fig. 5.13) and tubastrindole B was found to possess the GlyR activity. The geometric isomer mix of **46/47** was found

8*E*-3'-deimino-3'-oxoaplysinopsin (**46**)
8*Z*-3'-deimino-3'-oxoaplysinopsin (**47**)

8*E*-3'-deimino-4'-demethyl-3'-oxoaplysinopsin (**48**)
8*Z*-3'-deimino-4'-demethyl-3'-oxoaplysinopsin (**49**)

Fig. 5.13 Structures of oxoaplysinopsin analogs

Table 5.8 GlyR modulatory activities of aplysinopsin analogs

Compound	α1 GlyR IC_{50} (μM)	α3 GlyR IC_{50} (μM)
46/47	>200	67 ± 16
Tubastrindole B (19)	25.9 ± 5.3	>300
48/49	8.8 ± 0.5	33.9 ± 7.0

to be a weak GlyR antagonist, whereas tubastrindole B was found to be a strong GlyR antagonist, as shown in Table 5.8. Of further special interest, the two antagonists each show a preference for different GlyR isoforms. **46/47** showed a preference for α3 over α1, whereas the tubastrindole B dimer showed a strong selectivity for α1 GlyR. Following up on this discovery, the group synthesized several aplysinopsin derivatives in hopes of clarifying the extent to which GlyR molecular probes based on aplysinopsins can be tuned to select for a particular GlyR isoform. The results of their SAR study showed that introducing a 3′-oxo functionality in place of the 3′-imino results in a significant increase in potency, as shown in Table 5.8. Furthermore, *N*-methylation also appears to play a key role in selectivity between isoforms, as un-*N*-methylated derivatives showed no activity, whereas the 2′-*N*-methylated **48/49** showed selectivity for α1 GlyR, and **46/47** which are dimethylated showed selectivity for α3 GlyR. Finally, the previously mentioned dihydroaplysinopsin (**8**) isolated was found to possess no GlyR activity at all, indicating that the double bond (and subsequent geometry) plays a crucial role. It should be noted that while the mixtures of **46/47** and **48/49** were screened as such, they were able to be isolated in a darkened laboratory and rapidly analyzed to confirm their characterization. However, as previously noted, exposure to light results in rapid isomerization and leads to the mixtures reported.

4.5 In Vivo Studies

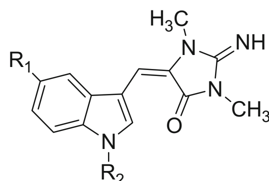
There have been a limited number of in vivo studies of aplysinopsin analogs. An early study conducted in 1982 used an ex vivo experimental design to evaluate MAO inhibition [54]. The results showed that the MAOI activity was reversible and relatively short in time, indicating that the analog tested, methylaplysinopsin (**7**), was quickly cleared from the plasma. As mentioned previously, 6-bromoaplysinopsin (**5**) showed promising IC_{50} values against *P. falciparum*; however, in subsequent in vivo testing, it was found to be inactive [66]. In a 2008 study, Kochanowska et al. isolated multiple aplysinopsin analogs from three different Florida sponges and evaluated one of them, aplysinopsin (**1**), in the Porsolt forced swim test. Unfortunately, the compound showed no significant antidepressant activity in the in vivo test [67]. These examples represent the few, limited in scope, in vivo studies that have been done with aplysinopsin analogs that were conducted prior to 2009.

In 2009 there was finally a positive report regarding in vivo activity of aplysinopsin analogs. The previously discussed aplysinopsin-pentamidine hybrid molecule (**25**, Sect. 3.2) was found to retain its in vitro antileishmanial activity when evaluated in an in vivo hamster model [33]. Not only did it maintain the same level of efficacy, there were also no reported side effects. The authors' own work in 2013 evaluated three lead compounds (**1**, **5**, and **38**) in the chick anxiety-depression model. Of the three compounds, two (**1** and **5**) were chosen for evaluation due to their nanomolar level MAOI activity toward MAO-A. Compound **38** was selected for evaluation based on its potent, if not selective, serotonin receptor binding affinities. The chick anxiety-depression model was selected due to its ability to evaluate both behavioral despair model and depression-like states [68] but also simultaneously evaluate anxiolytic conditions [69]. Despite excellent in vitro activity profiles, the three compounds tested in the chick model were unable to reproduce their in vitro efficacy. Compound **38** did exhibit a moderate antidepressant activity near the end of the depression evaluation phase, indicating a possible later onset of its effects on serotonin receptors.

Following this, in unpublished work, the authors sought to improve the in vivo efficacy of the aplysinopsin analogs and increase their bioavailability, as poor bioavailability is one of the leading causes of drug development failures [70]. In order to improve the physiochemical properties and ADME (absorption, distribution, metabolism, and extraction) characteristics of lead aplysinopsin compounds, they used Schrodinger's QikProp program to evaluate potential analogs. Specifically, they examined the properties which might be most important or a drug to be able to cross the blood-brain barrier and reach its therapeutic target. In addition to this in silico method, they also evaluated the susceptibility to phase I metabolism as well as MDR-1 efflux pumps, as they are both potential causes of low in vivo efficacy.

Careful analysis of the QikProp data leads to the proposal of 12 new aplysinopsin analogs (**50–61**, Fig. 5.14). Structurally, the QikProp data showed that alteration to the substitution pattern of the imidazolidinone had little to no effect on the pertinent parameters. Therefore, that substitution was kept consistent in all of the new proposed analogs: *N*-methylation on N-2′ and N-4′. The rest of the new analogs were substituted either on the indole ring or on the indole nitrogen, as shown in Fig. 5.14.

Evaluation of the susceptibility of the aplysinopsin scaffold to phase I metabolism was examined using a rat liver microsomal study. 5-Bromoaplysinopsin was found to have an intrinsic hepatic clearance rate of 23 μL/min/mg and a $T_{1/2}$ of 61 min. The clearance rate is similar to other antidepressant drugs, indicating that metabolic stability is not like the source of aplysinopsin's lack of in vivo efficacy. Also, its $T_{1/2}$ of 61 min is nearly identical to that of imipramine (66 min), which is the positive control used in the aforementioned chick anxiety-depression assay. Efflux pump susceptibility was evaluated using three aplysinopsin analogs: **52**, **57**, and **58**. The initial assays suffered from limited signal windows from the positive control; however, based on the small signal window available, none of the tested compounds appeared to be MDR-1 efflux pump substrates. Obviously, further work in this area



Compound	R1	R2	Compound	R1	R2
50	H	H	**56**	H	$COCH_3$
51	H	*p*-Cl-benzyl	**57**	F	H
52	H	*o*-Cl-benzyl	**58**	OCH_3	H
53	H	*p*-NO_2-benzyl	**59**	H	CH_3
54	H	*o*-NO_2-benzyl	**60**	H	CH_2CH_3
55	CN	H	**61**	H	$CH_2CH_2CH_3$

Fig. 5.14 Structures of second-generation aplysinopsin analogs **50–61**

will need to be done to rule out this cause of loss of efficacy, but the preliminary results are promising.

The 12 new analogs were first evaluated for their MAO inhibitory potential. An SAR revealed that compounds substituted at C5 showed micromolar level IC_{50} values for inhibitory activity at MAO-A. Specifically, compounds **57** and **58** had IC_{50} values of 0.204 and 0.547 μM, respectively. In addition, they also had a high selectivity index for MAO-A over MAO-B, with SIs of 99 and 53, respectively. This SAR complements the authors' earlier SAR study of 50 aplysinopsin analogs that found substitution at C5 or C6 results in an increased MAO-A inhibitory activity and selectivity [59]. Interestingly, the enhanced potency was observed when the substitution was either electron withdrawing (halogens Br and F) or electron donating (methoxy group). Regarding the serotonin receptor binding affinities of these 12 analogs, this group of analogs did not possess the low nanomolar level affinities as the previous work by the authors [51]. However, in much the same fashion, the most potent of these analogs, compound **52**, showed little selectivity among the 5-HT_2 receptor subtypes B and C, with K_i values of 787 nM for 5-HT_{2B} and 493 nM for 5-HT_{2C}. Of note, several of the *N*-benzylaplysinopsin analogs displayed nanomolar level affinity for the kappa opioid receptor (KOR). Compounds **51**, **52**, and **53** exhibited K_i values of 384, 932, and 666 nM, respectively. This is the first such report of KOR affinities by aplysinopsin analogs. Opioid receptor ligands are of interest to researchers, as they look for ligands that have less side effects when compared to mu opioid agonists like morphine. Of particular interest regarding aplysinopsins is a recent report describing how the kappa opioid receptor systems may be involved in regulation of the serotonin receptor systems in the brain [71]. It should be noted that these aplysinopsin compounds did not show significant affinity for any of the other opioid receptor subtypes screened against.

Finally, based on the above in vitro findings, three lead compounds were selected for further in vivo evaluation. Compounds **52**, **57**, and **58** were evaluated in the chick anxiety-depression model. Compound **52**, the *p*-chloro-*N*-benzylaplysinopsin analogs, showed a moderate antidepressant effects at 10 mg/kg in the later phases of the assay, just as compound **38** had shown previously [51]. Compound **58**, the 5-methoxyaplysinopsin analog, displayed a potent antidepressant activity across all doses. However, the 5-fluoroaplysinopsin analog (**57**) showed no activity across all doses. These results are promising and show that proper application of in silico ADME predictors can help guide the design and synthesis of analogs that may possess more in vivo efficacy.

5 Summary

Herein we have summarized the biological activities reported for aplysinopsins and related analogs since 2009. Of note, we have focused on their antiplasmodial and antileishmanial activities, CNS activity, potential as anticancer agents, as well as glycine-gated chloride channel receptor modulators. Regardless of the fact that aplysinopsins were first described four decades ago, we are still discovering potential new uses for them in the human health arena. Despite being a relatively simple chemical scaffold, it is an easily modifiable scaffold that can be used to generate large compound libraries for screening efforts. While many synthetic endeavors still use methodologies developed in the 1980s, several groups have developed exciting new synthetic strategies to quickly and easily generate new aplysinopsin analog libraries. We have summarized those methods which were published after 2005. For example, the work of Porwal et al. may be especially useful for generating aplysinopsin hybrid molecules, which opens up a plethora of options in terms of new aplysinopsin analogs. Furthermore, the work of the Crooks group has provided an extremely fast method to generate analogs via microwave irradiation, which is just as, if not more, efficient than traditional base-catalyzed condensation methods. Overall, the recent reports in the last decade signal that the aplysinopsin scaffold is still very much worth exploring, and there may be new biological activities yet to be discovered. In addition, medicinal chemists will also continue to push the boundaries of structural variation for aplysinopsin analogs.

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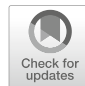
Chapter 6
Potential of Hydrogen Fermentative Pathways in Marine Thermophilic Bacteria: Dark Fermentation and Capnophilic Lactic Fermentation in *Thermotoga* and *Pseudothermotoga* Species



Laura Dipasquale, Nirakar Pradhan, Giuliana d'Ippolito, and Angelo Fontana

1 Introduction

The steadily increasing use of renewable forms of energy is envisaged to mitigate climate risks, but effective reduction of the concentration of greenhouse gases entails carbon sequestration strategies and transition to fuels that are not carbon based. Hydrogen (H_2) is commonly considered a secure and environmentally safe alternative to traditional energy vectors based on fossil fuels. Utilization of hydrogen by combustion engine or fuel cell produces heat and electricity with pure water as only waste. Nevertheless, hydrogen production requires manufacturing by processes that are currently energy-consuming and polluting. The access to this hydrogen does not solve the environmental issue, and it is clear that only production of the energy vector from renewable sources can be sustainable in the long term.

Hydrogen gas is also the side product of metabolism of a few microorganisms through biological transformations that include photobiological water splitting and fermentation of biomass. The latter process, which comprises photo- and dark fermentation, is particularly interesting because it is entitled of high productivity and allows use of residual material such as agro-food waste. Hyperthermophilic and extremely thermophilic bacteria and archaea are promising candidates for the

L. Dipasquale · G. d'Ippolito · A. Fontana (✉)
Bio-Organic Chemistry Unit, Consiglio Nazionale delle Ricerche—Institute of Biomolecular Chemistry, National Research Council of Italy, Pozzuoli (Naples), Italy
e-mail: angelo.fontana@cnr.it

N. Pradhan
Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Cassino, FR, Italy

Bio-Organic Chemistry Unit, Consiglio Nazionale delle Ricerche—Institute of Biomolecular Chemistry, National Research Council of Italy, Pozzuoli (Naples), Italy

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development of dark fermentation (DF)-based technologies because the rate and yield are higher than in mesophilic processes. In the last years, we reported that substitution of N_2 with CO_2 in the headspace of a standard culture of *Thermotoga neapolitana* DSMZ 4359 induces shift from DF to an unprecedented process that we named capnophilic lactic fermentation (CLF) after the mandatory presence of CO_2 and increase of lactic acid production [1, 2]. Differently from DF, CLF shows a so far unexplained deviation from the Thauer rule [3] with simultaneous synthesis in good yields of hydrogen and lactic acid [2, 4]. Very recently we noticed the acquisition of a novel phenotype and genotype of our lab strain of *T. neapolitana* that we have proposed as a new subspecies named *T. neapolitana* subsp. *capnolactica* with regard to the improved ability to produce lactic acid under capnophilic conditions [5].

T. neapolitana is a marine, organotroph eubacterium that ferments sugars to hydrogen under strictly anaerobic conditions. The microorganism belongs to the order *Thermotogales* that embraces a family of extremely thermophilic eubacteria that have been mostly isolated from marine geothermally heated environments across the globe, including shallow and deep-sea marine hydrothermal systems [6]. The order comprises the emended genus *Thermotoga* and the new genus *Pseudothermotoga* gen. nov. [7]. The aim of the present study is to give an overview of the previous research on *T. neapolitana* and to report unpublished results on the occurrence of CLF and DF in the other members of the *Thermotoga* and *Pseudothermotoga* genera maintained under standard (*Tn*N_2) and capnophilic (*Tn*CO_2) conditions.

2 Species and Culture Conditions

2.1 Taxonomy

Thermotogales (phylum *Thermotogae*, class *Thermotogae*) are anaerobic, Gram-negative, rod-shaped bacteria encapsulated by a unique "toga"-like outer membrane. These bacteria are strict organotrophs, fermenting preferentially simple and complex carbohydrates or complex organic matter. Genomic data from 17 members of the phylum support the division of the current genus *Thermotoga* in two evolutionary distinct taxonomic genera. On the basis of genome sequence data and genome-derived characteristics, Bhandari and Gupta [7] have proposed a reclassification of the genus *Thermotoga*. According to this work, the former genus *Thermotoga* should retain only the species *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermotoga petrophila*, *Thermotoga naphthophila*, *Thermotoga* sp. EMP, *Thermotoga* sp. A7A, and *Thermotoga* sp. RQ2, while the other *Thermotoga* species (*Thermotoga lettingae*, *Thermotoga thermarum*, *Thermotoga elfii*, *Thermotoga subterranea*, and *Thermotoga hypogea*) are moved to a new genus, *Pseudothermotoga* gen. nov. [7]. According to molecular analysis [8], the recently reported *Thermotoga caldifontis* and *Thermotoga profunda* should be also included in the *Thermotoga* genus.

The genus *Thermotoga* embraces bacteria that have an optimal growth temperature in the range of 77–80 °C [7]. Together with members of the order *Aquificales*, these bacteria show the highest growth temperatures known so far. The genus *Pseudothermotoga* includes species that generally grow in the range of 65–70 °C. All members of the order produce hydrogen, but extent can change from one strain to another. The biogas is formed as final product and is released to protect the microorganisms [9].

2.2 Strain Origin

Thermotoga and *Pseudothermotoga* species have been isolated from geothermally heated environments across the globe, including continental solfatara springs of low salinity, shallow and deep-sea marine hydrothermal systems, and high-temperature marine and continental oil fields, and have been extensively studied for insights into the basis for life at elevated temperatures and for biotechnological opportunities (e.g., biohydrogen production, biocatalysis). Indeed the species *T. maritima* had been originally isolated from a geothermally heated, shallow marine sediment at Vulcano, Italy [10]. The second species of this genus, *T. neapolitana*, was obtained from a submarine hot spring at Lucrino near Naples, Italy [11, 12]. Members of the marine *T. maritima*-*T. neapolitana* group are widespread within high-temperature ecosystems, such as shallow submarine hydrothermal systems. The isolates from deep-subsurface petroleum reservoirs were *T. petrophila* and *T. naphthophila* isolated from the Kubiki oil reservoir production fluid in Niigata (Japan; [13]). *T. caldifontis* and *T. profunda* are the newest members of the family [8]. Both species were isolated from terrestrial hot springs in Japan. The optimum growth conditions for strain *T. profunda* were 60 °C at pH 7.4, and those for strain *T. caldifontis* were 70 °C at pH 7.4. Further *Pseudothermotoga* isolates from oil production wells were described as *P. elfii* [14], *Pseudothermotoga subterranea* (East Paris Basin; [15]), and *Pseudothermotoga hypogea* (Africa; [16]) and represent a third ecological group originating from subsurface ecosystems and adapted to levels of salinity intermediate between those of marine species and those of terrestrial species. *Pseudothermotoga thermarum* was isolated in 1989 from continental solfataric springs with low ionic strength in Lake Abbe, Djibouti [17]. Despite the terrestrial origin, the bacterium is able to grow at low levels of salinity. *Pseudothermotoga lettingae* was isolated from a sulfate-reducing bioreactor, where methanol was the only carbon source [18].

2.3 Culture Conditions

The following strains of *Thermotoga* and *Pseudothermotoga* genera used in this study *T. maritima* DSMZ 3109^T, *T. neapolitana* DSMZ 4359^T, *T. petrophila* DSMZ 13995^T, *T. naphtophila* DSMZ 13996^T, *T. caldifontis* DSMZ 23272^T, *T. profunda*

DSMZ 23275^T, *P. thermarum* DSMZ 5069^T, *P. elfii* DSMZ 9442^T, *P. subterranea* DSMZ 9912^T, *P. hypogea* DSMZ 11164^T, and *P. lettingae* DSMZ 14385^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *T. neapolitana* subsp. *capnolactica* was originally bought as *T. neapolitana* DSMZ 4359^T and maintained in our laboratory under saturating concentrations of CO_2 for several years [5].

Except for *T. neapolitana* subsp. *capnolactica*, all the other strains were reactivated from lyophilized samples in *Tn* medium [19] supplemented with 28 mM (0.5% w/v) glucose and 0.4% (w/v) yeast extract/tryptone by using 120 mL sealed flasks with working volume of 30 mL, without agitation and with pH adjustment to optimal value for each bacterium by means of 1 M NaOH. Anaerobic cultures were grown at optimal temperature for each bacterium (60 °C for *T. profunda*; 70 °C for *T. caldifontis*, *P. subterranea*, *P. hypogea*, *P. lettingae*, *P. elfii*; 80 °C for *T. neapolitana*, *T. neapolitana* subsp. *capnolactica*, *T. maritima*, *T. naphtophila*, *T. petrophila*, and *P. thermarum*) under N_2 atmosphere. Fermentation test batches were inoculated (6% v/v) at room temperature with precultures grown overnight. Before each experiment, culture media were degasified by N_2 or CO_2 bubbling. Samples were regularly taken (1.5 mL every 24 h) from the cultures to monitor the main fermentation parameters including optical density at $\lambda = 540$ nm (O.D.), dry cell weight (mg mL^{-1}), glucose consumption, production of organic metabolites (acetic acid, lactic acid, alanine, valine, ethanol, 2,3-butanediol), hydrogen, and CO_2. After each sampling, hydrogen was removed by flushing into culture media pure nitrogen or carbon dioxide for 3 min, and pH was adjusted to optimal value for each bacterium by means of 1 M NaOH [1]. All the cultures were performed in triplicate.

3 Fermentative Hydrogen Production by *T. neapolitana*

Originally isolated from shallow submarine hot spring near Lucrino in the Bay of Naples in 1986 [11, 12], *T. neapolitana* is a Gram-negative bacterium that grows between 55 and 90 °C with an optimal growth temperature of 80 °C. An overview on *T. neapolitana* has been reported in [20]. The species is well-recognized producer of hydrogen from sugar fermentation. In the following paragraphs, we briefly summarize the production of hydrogen by the two main fermentation pathways, dark fermentation and capnophilic lactic fermentation, that are currently known to operate in this eubacterium and in its subspecies, *T. neapolitana* subsp. *capnolactica*.

3.1 Dark Fermentation (DF) by T. neapolitana

In analogy with the other members of the genus, *T. neapolitana* displays a wide metabolic ability to use a large variety of substrates, including sulfur compounds [14]. Conversion of glucose to pyruvate is only due to Embden-Meyerhof-Parnas

(EMP) glycolytic pathway, but it has been calculated that about 15% of hydrogen is due to fermentation of protein source [19]. According to the classical model of dark fermentation, glucose oxidation to acetate can theoretically produce 4 mol of hydrogen per mole of sugar [3]. As fermentative hydrogen production is a mean to dispose of electrons, there is a direct relationship between the biogas yield and the type of the organic products that are concurrently released during the process. As shown in Fig. 6.1, yield is optimized only when all glucose is converted to acetate, because there is no leak of electrons in other reactions. The entire pathway is thermodynamically driven by formation of ATP, but when hydrogen accumulates and consumption of NADH stops, pyruvate is diverted away from acetate production. In this case lactic acid or alanine is a common side product of the process [6]. According to the mechanism of Fig. 6.1, no hydrogen is produced when these metabolites are the only products of the fermentation.

Synthesis of lactate is under control of lactate dehydrogenase (LDH) and requires concomitant reoxidation of NADH to NAD^+. Lactate levels reported during fermentation by *Thermotoga* species vary from trace amounts up to levels rivaling those of acetate [21–24]. On the other hand, the bacterium lacks the key genes necessary for further reduction of acetyl-CoA to ethanol and butyrate [25–27].

Evolution of hydrogen is an efficient way to dissipate excess reductant as a diffusible gas during microbial fermentation and photobiological processes. Synthesis of the gas by *T. neapolitana* is mostly due to the characteristics of the heterotrimeric [FeFe]-hydrogenase. Hydrogenases (H2ase) constitute a family of metalloenzymes that reduce protons to hydrogen by low-potential electrons. These enzymes play a central role in energy metabolism of many bacteria and archaea but are also found in a few eukaryotes [28]. Hydrogenases are traditionally classified in

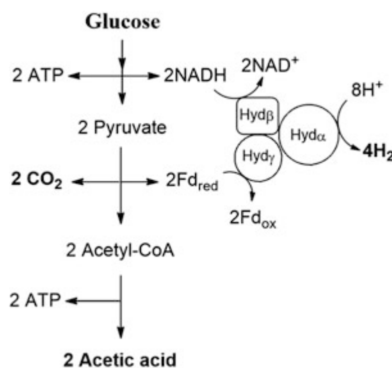

Fig. 6.1 Dark fermentation (DF) in *T. neapolitana*. The marine bacterium operates synthesis of hydrogen from sugar fermentation. Pyruvate is the key intermediate of the process and derives from Embden-Meyerhof-Parnas glycolytic pathway. Hydrogenase (Hyd) is represented by trimer composed of Hydα, Hydβ, and Hydγ. The final organic product of dark fermentation is acetate even if other minor compounds are also released in the medium. The scheme illustrates the direct correlation between acetate synthesis and hydrogen production. The largest yield of hydrogen cannot exceed 4 mol/mol of glucose (Thauer limit) and can be achieved only when acetate is the product of the process (see main text). Fd_{red} = reduced ferredoxin; Fd_{ox} = oxidized ferredoxin. Protons and other side products and cofactors are omitted for simplicity

three groups according to the structure of the catalytic site: [FeFe]-hydrogenases, [NiFe]-hydrogenases, and [Fe]-hydrogenases [28–30]. Sequence analysis on the three proteins that form the hydrogenase of *T. maritima*, which has more than 90% homology with that of *T. neapolitana*, suggests a [Fe-Fe]-heterotrimer. The α subunit (HydA) is a protein that contains the catalytic site, which is termed H cluster, composed of three 4Fe-4S and two 2Fe-2S clusters [23]. This is one of the most complex iron-containing structures characterized to date and requires the specific action of three highly conserved proteins to be assembled [31, 32]. Other three 4Fe-4S clusters and one 2Fe-2S cluster are also present in the β-subunit (HydB), whereas the hydrogenase γ-subunit (HydC) contains one 2Fe-2S cluster. According to the model suggested by Schut and Adams [23], HydA transfers electrons to H^+ from NADH through the β-subunit and from reduced ferredoxin through the γ-subunit. However, despite the detailed proposal of the active site, how the endergonic reaction of hydrogen production is accomplished under physiological conditions has been vague for a long time.

Reduction of H^+ is an energetically unfavorable reaction that is typically influenced by environmental conditions such as pH, cell growth rate, and hydrogen partial pressure. In 2008, a new mechanism of driving endergonic redox reactions was proposed for the synthesis of butyric acid from crotonyl-CoA in *Clostridium kluyveri* by simultaneous transfer of electrons from NADH ($E^{0'} = -320$ mV) to ferredoxin ($E^{0'} = -450$ mV) and crotonyl-CoA ($E^{0'} = -10$ mV) [33]. The process is thermodynamically favored because the flux of electrons is “bifurcated” toward two acceptors with different redox potentials in order to conserve the total energy of the system [34]. Analogously, the hydrogenases of *T. maritima* and *T. neapolitana* are suggested to be electron-bifurcating enzymes that couple the endergonic reduction of H^+ to hydrogen by NADH with the exergonic reduction of H^+ to hydrogen by reduced ferredoxin [23]. The trimeric hydrogenase complex of these bacteria is not able to produce hydrogen with the oxidation of reduced Fd or of NADH when each was used as the sole electron donor. Only, the presence of both biological reductants promoted efficient hydrogen production with yield close to the Thauer limit. Because the hydrogenase of *T. neapolitana* uses both NADH and reduced ferredoxin as electron donors, hydrogen production may be influenced by factors that affect both reductants. Furthermore, the composite mechanism of this hydrogenase is consistent with the complexity of the trimeric structure, which is much greater than that of the typical Fd-dependent, single subunit [FeFe]-hydrogenase found in *Clostridium* spp.

3.2 Capnophilic Lactic Fermentation (CLF) by T. neapolitana

A continuous inflow of gases, mainly nitrogen (N_2), is the most commonly reported method for removing hydrogen from the reactor headspace and increasing productivity [35, 36]. Sparging pure cultures of *T. neapolitana* with CO_2 significantly

increases the rate of both glucose consumption and hydrogen production even if there is no improvement of the overall productivity and molar yield that remained substantially unchanged in comparison with standard N_2 conditions [1]. Paradoxically, CO_2 stimulated also synthesis of lactic acid. Feeding experiments with labeled precursors clearly proved that at least part of exogenous CO_2 was biologically coupled with acetyl-CoA to give lactic acid when the cultures were stripped by CO_2 gas or enriched in sodium bicarbonate. The process recycled both glycolysis-derived acetyl-CoA and exogenous acetate. In this latter case, the overall outcome was a conversion of equimolar concentration of acetate and CO_2 into lactic acid. The fermentative CO_2-dependent synthesis of lactic acid and hydrogen was named capnophilic lactic fermentation (CLF), and as suggested in Fig. 6.2, it put forward the possibility to fully convert sugar to lactic acid (or other reduced derivatives of pyruvate) without affecting hydrogen synthesis by means of an additional consumption of reducing equivalents deriving from other cellular processes [2].

The transition from acetate production (dissimilation) to acetate utilization, also named acetate switch, is a common facet in the metabolism of many bacteria [37]. It is noteworthy that, according to acetyl-CoA synthetase (ACS) mechanism, acetate assimilation is an endergonic process that requires one mole of ATP and one mole of CoA per each mole of acetate that is imported and utilized in the bacterial cell. Assimilation of CO_2 in *T. neapolitana* is committed to synthesis of lactic acid and,

Fig. 6.2 Capnophilic lactic fermentation (CLF) in *T. neapolitana*. The process is an anaplerotic pathway that is activated by increase of the concentration of CO_2 in the reactor. As depicted in the scheme, acetyl-CoA deriving from sugar catabolism (see Fig. 6.1) undergoes to reductive carboxylation by reverse catalysis of pyruvate:ferredoxin oxidoreductase (PFOR). Newly generated pyruvate is then reduced to lactic acid by lactic dehydrogenase. The pathway is able also to use exogenous acetate. Hydrogen production is not affected by the capnophilic route, thus allowing simultaneous synthesis of lactic and hydrogen in deviation of the Thauer limit. Fd_{red} = reduced ferredoxin; Fd_{ox} = oxidized ferredoxin. Protons and other side products and cofactors are omitted for simplicity

like in acetogenic bacteria and methanogens [38, 39], is dependent on pyruvate: ferredoxin oxidoreductase (PFOR). The experiments established the presence of PFOR in strict occurrence with pyruvate synthase activity and production of lactic acid. Labelling of lactic acid during sugar fermentation unambiguously demonstrates that the catabolic and biosynthetic reactions can occur at the same time. However, pyruvate synthase levels were detectable for a longer time in cultures exposed to CO_2 thus suggesting that the two processes could be independently regulated by either glucose or CO_2. This is consistent with the apparent absence of cross talking between hydrogen production and capnophilic lactic fermentation under CO_2-enriched conditions [2].

In *Thermotogales* reductive carboxylation of acetyl-CoA (Ac-CoA) likely requires the pool of ferredoxin that is also involved in hydrogen production. The role of ferredoxin as efficient reductant in pyruvate synthesis has been demonstrated in vitro with *Clostridium thermoaceticum* [40] and suggested in vivo for methanogenic archaea, such as *Methanosarcina barkeri* [41]. It is noteworthy that the sequence of pyruvate oxido-reductase of this last organism has a good relation to those of *T. neapolitana* and *T. maritima* [2]. CLF is an example of biological sequestration of CO_2 by coupling with an exogenous substrate (acetate, glucose, etc.) and release of the end product (lactic acid) outside of the cell. Incorporation of radioactivity from ^{14}C-glucose into cells proved that little of this carbon is used in primary metabolic pathways. Analogously, no carbon deriving from CO_2 or HCO_3^- is apparently fixed in the microbial biomass. This is not too surprising but makes a difference between CLF and mechanisms of fixation known in other anaerobes. In fact, unlike autotrophic [42, 43] and heterotrophic [44] pathways for CO_2 assimilation, the capnophilic metabolism of *T. neapolitana* implies complete excretion of CO_2 after "incorporation" in lactic acid and no synthesis of primary metabolites (e.g., proteins, sugars, or lipids). Recently, the first mathematical model of this process has been reported to describe the kinetics of the formation of lactate and hydrogen [4]. The model can effectively predict that about 90% of lactate was produced via the CLF pathway.

4 Anaerobic Sugar Fermentation in *Thermotoga* and *Pseudothermotoga* Genera

4.1 Bacterial Growth and Tolerance to CO_2

The lyophilized strains of *Thermotoga* and *Pseudothermotoga* genera were reactivated and grown on glucose in *Tn* medium enriched with yeast extract and tryptone [19]. Growth was measured as density at $\lambda = 540$ nm (O.D.) and also monitored as wet and dry cell weight. The choice to use the same medium for the

different species reflects our decision to make homogeneous the response. This condition is probably not optimal for every strain, and the resulting results do not indicate the best fermentation performance.

As reported in Fig. 6.3, the bacteria showed the common ability to growth on glucose under both N_2 (*Tn*N_2, standard DF) and CO_2 (*Tn*CO_2, CLF) sparging. Stimulatory effect of CO_2 on the growth of obligate anaerobes is widely recognized [45] even if CO_2 is considered an inhibitor of biochemical processes, and, with this aim, the gas is used as a bactericide in food packaging and carbonated beverages.

Stripping with CO_2 significantly increases hydrogen yields in mixed cultures due to the removal of hydrogen [46]. A major complication with using CO_2 as stripping gas is the formation of bicarbonate accompanied by acidification of the medium and increased osmotic pressure. Acidification has been shown not to be necessarily the primary origin for growth inhibition by CO_2 since analogous reduction of the pH in N_2 atmospheres did not have the same inhibitory effect. However, higher solute concentrations can lead to growth inhibition and cell lysis. Bicarbonate and carbonate can also react with amino groups forming carbamates, which can affect the function of proteins. In addition, at low external pH, CO_2 can diffuse into the cell and lowers the intracellular pH by forming bicarbonate and thus reducing the growth rate. Finally, elevated partial CO_2 pressure might inhibit catabolism by reversing the biological decarboxylating reactions.

CO_2 atmosphere did not inhibit fermentation and growth of *Thermotoga* and *Pseudothermotoga* species despite previous studies report acidification of the medium and increase of the osmotic pressure [47]. This was not unexpected since other hyperthermophilic bacteria, e.g., *Pyrococcus furiosus*, can grow optimally in the presence of high salt concentrations for the ability to produce osmoprotectants, such as mannosylglycerate, di-myo-inositol phosphate, and glutamate [48].

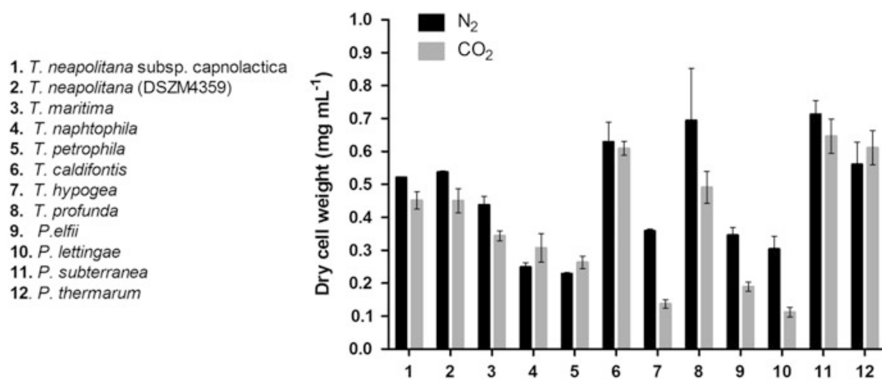

Fig. 6.3 Dry cell weight (mg mL^{-1}) comparisons in *Thermotoga* and *Pseudothermotoga* genera after glucose fermentation in *Tn* medium under N_2 or CO_2 atmosphere

4.2 Glucose Consumption and Hydrogen Yield Under N_2 and CO_2 Conditions

Response in *Thermotoga* and *Pseudothermotoga* species to CO_2-enriched atmosphere was monitored by glucose consumption and production of hydrogen and organic metabolites (acetic acid, lactic acid, and alanine; valine, ethanol, and 2,3-butanediol) according to the methods described by Dipasquale et al. [1].

T. neapolitana subsp. *capnolactica*, *T. neapolitana* DSMZ 4359, *T. maritima*, *T. profunda*, *P. thermarum*, and *P. subterranea* showed the highest consumption rate of glucose and fermented almost completely the sugar under N_2 and CO_2 conditions (Table 6.1). Except for *T. maritima*, *P. hypogea*, and *P. lettingae*, use of CO_2 improved the efficiency of glucose consumption even if the effect was quantitatively different from one species to another. The most evident increase was recorded with *T. naphtophila*, *T. petrophila*, *T. caldifontis*, *T. profunda*, and *P. subterranea*, which are all species isolated from oil deposit or deep hot springs where presumably CO_2 concentration is higher than in other habitats [8, 13, 15]. All strains also produced hydrogen, but the molar yield (mol hydrogen/mol glucose) in these experimental conditions was significantly higher than 2 only with *T. neapolitana* subsp. *capnolactica*, *T. petrophila*, and *T. caldifontis* (Table 6.1).

Table 6.1 Glucose consumption and hydrogen yield (mean ± standard deviation, $n = 3$) in *Thermotoga* and *Pseudothermotoga* genera after glucose fermentation in N_2 (*Tn*N_2) or CO_2 (*Tn*CO_2) atmosphere

	Glucose consumption (mM)		Hydrogen yield (mol H_2/mol consumed glucose)	
Genus *Thermotoga*	*Tn*N_2	*Tn*CO_2	*Tn*N_2	*Tn*CO_2
T. neapolitana subsp. *capnolactica*	25.7 ± 0.1	28.3 ± 1.0	2.5 ± 0.06	2.9 ± 0.1
T. neapolitana DSMZ 4359	21.7 ± 0.6	20.8 ± 2.3	2.5 ± 0.03	1.9 ± 0.1
T. maritima DSMZ 3109	23.2 ± 1.0	19.9 ± 0.6	1.9 ± 0.06	2.0 ± 0.1
T. naphtophila DSMZ 13996	13.3 ± 1.1	20.8 ± 1.7	2.2 ± 0.2	1.6 ± 0.2
T. petrophila DSMZ 13995	9.2 ± 1.3	14.2 ± 0.6	3.0 ± 0.4	1.9 ± 0.1
T. caldifontis DSMZ 23272	10.9 ± 1.1	15.2 ± 0.9	2.6 ± 0.1	1.8 ± 0.03
T. hypogea DSMZ 11164	8.8 ± 1.1	4.3 ± 0.1	1.1 ± 0.3	0.5 ± 0.1
T. profunda DSMZ 23275	18.1 ± 0.4	22.6 ± 1.7	1.5 ± 0.2	0.7 ± 0.04
Genus *Pseudothermotoga*				
P. elfii DSMZ 9442	7.0 ± 0.9	6.7 ± 0.2	2.0 ± 0.2	2.1 ± 0.1
P. lettingae DSMZ 14385	9.3 ± 0.5	8.1 ± 0.7	1.2 ± 0.1	1.3 ± 0.3
P. subterranea DSMZ 9912	23.1 ± 2.1	27.0 ± 1.4	1.8 ± 0.2	1.4 ± 0.1
P. thermarum DSMZ 5069	Complete	Complete	1.8 ± 0.02	1.5 ± 0.1

Initial hexose concentration was 28 mM in 120 mL sealed flasks with working volume of 30 mL, incubated without agitation and with pH adjustment to optimal value for each bacterium by means of 1 M NaOH. Anaerobic cultures were grown at optimal temperature for each bacterium (60 °C for *T. profunda*; 70 °C for *T. caldifontis*, *P. subterranea*, *P. hypogea*, *P. lettingae*, *P. elfii*; 80 °C for *T. neapolitana*, *T. maritima*, *T. naphtophila*, *T. petrophila*, and *P. thermarum*)

Despite the increase of glucose consumption, almost all species lowered hydrogen production under *Tn*CO_2 conditions, and only *T. maritima*, *P. elfii*, and *P. lettingae* showed substantially unvaried yield of the energy vector (Table 6.1). *T. neapolitana* subsp. *capnolactica* was the only species to increase H_2 yield moving from N_2 to CO_2. In a few species, production of hydrogen did not reach 50% of the theoretical Thauer limit [3], but it is noteworthy that these experiments were not carried out under optimized culture conditions.

4.3 Organic Metabolite Production Under N_2 and CO_2 Conditions

^{1}H-NMR analysis of the fermentation broths from *Tn*N_2 and *Tn*CO_2 experiments revealed that all the tested strains produced a mixture in which acetic acid, lactic acid, and alanine were the predominant and most recurring metabolites (Table 6.2). Despite our expectation on CLF metabolism, CO_2 atmosphere did not induce a generalized increase of lactic acid to the detriment of acetic acid. Only *P. subterranea* showed a significant change in the fermentation products, but this was mostly driven by synthesis of L-alanine. Production of this amino acid is a trait that *Thermotogales* share with members of the archaeal order Thermococcales. Therefore, it has been proposed that L-alanine production from sugar fermentation is a remnant of an ancestral metabolism that these microbes have inherited from their common ancestor [9]. The species *P. hypogea*, *P. elfii*, *P. lettingae*, and *P. subterranea* showed a significant increase of alanine production under CO_2-enriched atmosphere (Table 6.2). This process was associated to reduction of hydrogen in *P. hypogea* as probably consequence of a shift of glucose fermentation from acetate to pyruvate and then alanine. The other three species maintained a constant level of hydrogen from N_2 to CO_2, but we did not observe any reduction in acetate synthesis, thus suggesting that CLF does not operate in these bacteria. It is to note that the constant level of acetate in comparison with N_2 cultures suggests that synthesis of alanine is probably independent of DF. The investigation of the mechanism for the biosynthesis of alanine is behind the scope of this work, but the most plausible explanation is derivation of this amino acid from protein catabolism. Amino acids and peptides of the medium, such as the hydrolates of yeast extract and tryptone, have been already shown to contribute to total hydrogen production and biomass formation in *T. neapolitana* [19, 49].

In addition to acetic acid, lactic acid, and alanine, *P. hypogea*, *P. elfii*, and *P. lettingae* produced valine, while the broth of the other strains contained also ethanol and 2,3-butanediol. *T. neapolitana* subsp. *capnolactica*, *T. neapolitana* DSMZ 4359, *T. profunda*, *P. subterranea*, and *P. thermarum* were the major producers of these products, whereas the highest valine level was found in *T. profunda*. However, increase of organic acid production under CO_2 conditions was found only in *T. naphtophila*, *T. caldifontis*, *P. elfii*, and *P. subterranea* (Table 6.3). A few species responded to CO_2 by reducing the fermentation process.

Table 6.2 Main organic metabolite productions (mean ± standard deviation, $n = 3$) in *Thermotoga* and *Pseudothermotoga* genera after glucose fermentation in N_2 (*Tn*N_2) or CO_2 (*Tn*CO_2) atmosphere

	Acetic acid (mM)		Lactic acid (mM)		Alanine (mM)	
	TnN_2	*Tn*CO_2	*Tn*N_2	*Tn*CO_2	*Tn*N_2	*Tn*CO_2
Genus *Thermotoga*						
T. neapolitana subsp. *capnolactica*	27.3 ± 0.8	22.1 ± 0.9	8.6 ± 0.2	11.3 ± 0.1	2.5 ± 0.2	3.0 ± 0.3
T. neapolitana DSMZ 4359	30.2 ± 0.4	20.8 ± 0.1	2.2 ± 0.02	1.2 ± 0.06	1.9 ± 0.3	2.4 ± 0.3
T. maritima DSMZ 3109	25.5 ± 0.5	18.3 ± 0.3	5.3 ± 0.8	1.6 ± 0.2	2.4 ± 0.06	2.3 ± 0.3
T. naphtophila DSMZ 13996	15.7 ± 0.1	19.2 ± 0.1	1.4 ± 0.06	5.0 ± 0.02	0.8 ± 0.1	1.8 ± 0.05
T. petrophila DSMZ 13995	13.1 ± 0.05	12.6 ± 0.1	2.0 ± 0.01	3.8 ± 0.02	0	0.3 ± 0.1
T. caldifontis DSMZ 23272	16.7 ± 3.6	15.6 ± 1.5	2.2 ± 0.5	2.3 ± 0.4	3.2 ± 0.9	6.6 ± 0.7
T. hypogea DSMZ 11164	6.4 ± 0.1	3.1 ± 0.2	0.1 ± 0.004	0.1 ± 0.004	2.9 ± 0.1	3.4 ± 0.3
T. profunda DSMZ 23275	15.9 ± 0.4	5.6 ± 0.2	5.7 ± 0.1	2.3 ± 0.04	1.4 ± 0.06	2.6 ± 0.3
Genus *Pseudothermotoga*						
P. elfii DSMZ 9442	8.3 ± 0.06	7.8 ± 0.3	0.2 ± 0.03	0.1 ± 0.01	4.2 ± 0.3	10.0 ± 0.3
P. lettingae DSMZ 14385	5.1 ± 0.05	4.4 ± 0.1	0.2 ± 0.003	0.05 ± 0.01	2.7 ± 0.05	3.7 ± 0.2
P. subterranea DSMZ 9912	30.6 ± 6.9	31.9 ± 7.9	16.2 ± 4.6	10.7 ± 4.0	9.5 ± 0.4	20.0 ± 8.0
P. thermarum DSMZ 5069	30.0 ± 2.2	24.8 ± 0.7	6.5 ± 0.2	5.6 ± 0.6	1.1 ± 0.07	2.2 ± 0.2

Table 6.3 Total main organic metabolite productions (mean ± standard deviation, $n = 3$) in *Thermotoga* and *Pseudothermotoga* genera after glucose fermentation in N_2 (*Tn*N_2) or CO_2 (*Tn*CO_2) atmosphere

	Total acetic acid, lactic acid, and alanine productions (mM)	
Genus *Thermotoga*	*Tn*N_2	*Tn*CO_2
T. neapolitana subsp. *capnolactica*	38.4 ± 1.1	36.4 ± 1.3
T. neapolitana DSMZ 4359	34.3 ± 0.7	24.4 ± 0.4
T. maritima DSMZ 3109	33.2 ± 1.3	22.2 ± 0.8
T. naphtophila DSMZ 13996	17.8 ± 0.3	25.9 ± 0.1
T. petrophila DSMZ 13995	19.0 ± 0.2	16.7 ± 0.2
T. caldifontis DSMZ 23272	22.1 ± 5.1	24.4 ± 2.7
T. hypogea DSMZ 11164	11.1 ± 0.5	8.3 ± 0.9
T. profunda DSMZ 23275	23.0 ± 0.5	10.6 ± 0.5
Genus *Pseudothermotoga*		
P. elfii DSMZ 9442	14.1 ± 0.6	18.3 ± 0.9
P. lettingae DSMZ 14385	8.0 ± 0.1	8.2 ± 0.3
P. subterranea DSMZ 9912	49.6 ± 12.0	62.6 ± 19.8
P. thermarum DSMZ 5069	37.7 ± 2.5	32.6 ± 1.5

Analysis of the single components made evident that in most species the acetic acid yields decreased under *Tn*CO_2 condition in comparison with *Tn*N_2, but only in *T. neapolitana* subsp. *capnolactica*, *P. hypogea* and *T. naphtophila*, there was a positive effect on lactic acid. Oddly lactic acid production resulted to be lower under capnophilic conditions than *Tn*N_2 in the cultures of *T. neapolitana* DSMZ 4359, *T. maritima*, *T. profunda*, *P. subterranea*, and *P. lettingae*. The ratio of lactic acid versus acetic acid (LA/AA) showed the highest values with *T. neapolitana* subsp. *capnolactica*, *T. naphtophila*, *T. petrophila*, *T. profunda*, and *T. hypogea* under capnophilic conditions (Fig. 6.4), whereas it remained unchanged or even slightly decreased in comparison with *Tn*N_2 in the other strains.

Taking in consideration also glucose consumption and hydrogen yield, *T. neapolitana* subsp. *capnolactica*, *T. naphtophila* and, at a lesser extent, *T. petrophila* were the only species to give increase of LA/AA ratio with no or little detriment of hydrogen production. Apparently the original strain of *T. neapolitana* DSZM 4359 does not show the same performance. Our finding of *T. neapolitana* subsp. *capnolactica* is rather recent, but we cannot exclude that the mutation with the consequent increase of lactic acid production occurred earlier. Thus, in consideration of the present results, it is very likely that most of our previous reports [1, 4, 19, 50] were due to this new subspecies instead of the original DSZM 4359 strain.

According to the CLF model [1, 2], the key enzymatic step of the pathway is the reductive addition of CO_2 to acetyl-CoA catalyzed by an enzyme of the PFOR family. Sequence analysis of the four proteins that compose PFOR showed a very high identity in the three species of *Thermotoga* genus (Table 6.4). This was in agreement with the results in production of lactic acid in these species under CO_2 and indirectly supported the assumption about the role of this enzyme in the regulation of the CLF pathway.

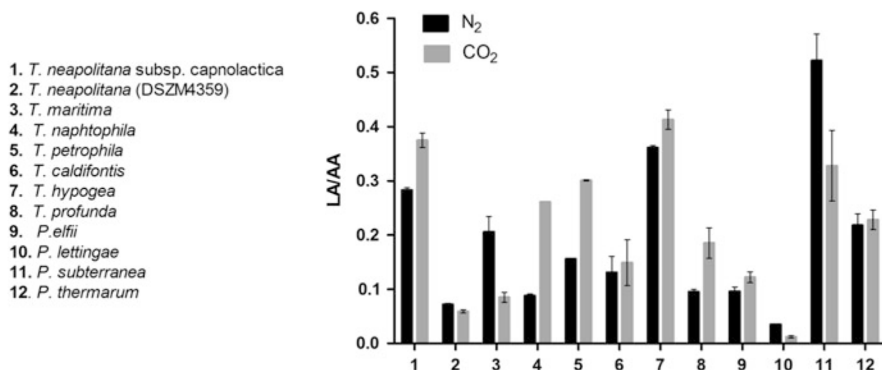

Fig. 6.4 Comparison between LA/AA ratio in *Thermotoga* and *Pseudothermotoga* genera after glucose fermentation in *Tn* medium under N_2 (*Tn*N_2) or CO_2 (*Tn*CO_2) conditions

Table 6.4 Identity (%) obtained by National Center for Biotechnology Information (NCBI)-BLAST between amino acidic sequences of *T. neapolitana* porA, porB, porC, and porD and those from *T. naphtophila* and *T. petrophila*

	Identity (%)	
T. neapolitana	*T. naphtophila*	*T. petrophila*
porA	97	97
porB	98	98
porC	96	93
porD	97	97

5 Challenges

Dark fermentation can achieve a maximum H_2 conversion of 33%, i.e., four molecules of H_2 per molecule of glucose. Based on thermodynamics, the process with mesophilic fermentation requires almost double the amount of feedstock compared to operation with thermophilic bacteria to produce the same amount of hydrogen. In general, only extreme thermophilic bacteria and archaea are able to use most of the reducing equivalents formed during glycolysis for the production of hydrogen, thus approaching the theoretical maximum of 4 mol H_2 per mol of hexose [3]. Recently, we reported that a combined process based on *T. neapolitana* under CLF conditions and *Rhodopseudomonas palustris* 42OL produces 9.4 ± 1.3 mol H_2 per mol of glucose [50], which is the highest value reported so far by combining dark and photofermentation.

In addition to the thermodynamic factor, other important aspects do favor the fermentation yields in thermophilic cultures [51]. In particular, due to higher hydrolysis activity, better pathogenic destruction, and less risk of biological contamination, extreme thermophilic fermentation seems also attractive for complex feedstock, such as lignocellulosic residues or agricultural and food residues. However, development of these processes at industrial level is so far hampered by inherent drawbacks, such inoculum production, low cell density, and tolerance.

Capnophilic lactic fermentation (CLF), as previously reported in *T. neapolitana* subsp. *capnolactica* and here described in *T. naphtophila* and, at a lesser extent, *T. petrophila*, may become the cornerstone for economically attractive biotechnological production of lactic acid from carbohydrate-rich feedstocks such as agro-food waste. However, this process needs optimization of upstream and downstream processes including the use and treatment of different substrates, the definition of the operational parameters, the reactor design, and the separation of the fermentation products. CLF process can be more economically sustainable if the reactors can handle with high organic loading rate (OLR) and with a short hydraulic retention time (HRT). These issues can be targeted either by using immobilized biomass or by applying other bioreactor configurations such as packed bed reactor (PBR) or fluidized bed reactor (FBR).

Hydrogen yield approaching the theoretical maximum value (i.e., Thauer limit) of 4 mol hydrogen/mol glucose can be achieved only if the flux electrons produced by glucose oxidation are directed to reduce protons (Fig. 6.1). Nevertheless, in practice, these reducing equivalents are also used for biosynthetic purposes and the formation of other fermentation products, i.e., lactate. Manipulation of the redox balance has been demonstrated to push the metabolism of mesophilic bacteria toward formation of organic acids. Evidence is that ferredoxin-NADH knob plays an important role for lactic production under CLF condition. Therefore, targeting the redox balance of *Thermotoga* and *Pseudothermotoga* species might constitute a promising metabolic strategy to improve the fermentation yields.

CLF pathway represents a first example of biological sequestration of CO_2 by coupling with an exogenous substrate (acetate, glucose, etc.) and release of the end product (lactic acid) outside of the cell. Since *T. neapolitana* does not convert CO_2 to the reduced organic compounds required for cell metabolism, the above mechanism is not related to the autotrophic fixation known in other anaerobes. The process appears to be unprecedented, but its regulation is largely unknown to date. However, the pathway offers the possibility to convert directly CO_2 into chemicals, which could become an economically feasible option after further improvement. Recently this approach has been explored for production and secretion of isobutyraldehyde and isobutanol directly from CO_2 by the genetically modified cyanobacterium *Synechococcus elongatus* [52]. The application of CLF and *Thermotoga* and *Pseudothermotoga* species in biotechnological processes will depend on the full elucidation of the molecular and biochemical characteristics of the process, as well as on selection and engineering of the productive species.

Finally, production of lactate independent of hydrogen suggests that CLF may trigger or be related to metabolic pathways other than glycolysis. One of the possibilities is the contribution of peptides and the effects of sparged CO_2 on peptide catabolism. Accurate redox and carbon balances are necessary to provide convincing data about the role of peptide in the lactate synthesis from acetate. Fermentation of *Thermotoga* and *Pseudothermotoga* strains is a challenging system in this regard, due to the dependence on peptides in the medium that makes it difficult to determine just how much acetate, hydrogen, or lactate is made as part of peptide versus glucose metabolism. New culture conditions must be designed to allow these measurements and to perform studies committed to estimate the energy and stoichiometry of the fully coupled reactions.

6 Conclusions

Thermotoga and *Pseudothermotoga* genera embrace a small but heterogeneous group of thermophilic and hyperthermophilic bacteria. To date the scientific and biotechnological attention for these microorganisms has been driven by the capability to produce hydrogen and, at lower extent, to the possibility to clean sites contaminated with petrochemicals and industrial wastes. The recent discovery of taxonomically related mesophilic members (*Mesotoga prima* and *Mesotoga infera*) [53, 54] and the finding of an apparently unprecedented pathway for synthesis of lactic acid from acetate and CO_2 clearly opened the use of these organisms in other biotechnological sectors. In particular, after further improvement, the fermentative transformation of agro-food residues combined with hydrogen production seems to be suitable to design a platform for the valorization of waste and CO_2, with recovery of both energy and value-added products. As outlined in this study, only a few members of the two genera show the capability to perform capnophilic lactic fermentation. However, all the species produce energy carriers and fuels, such as hydrogen and ethanol, and chemicals like acetic acid, butyric acid, and lactic acid, among others. In this context, these microorganisms appear as an attractive solution that will reduce those residues and increase the global efficiency of biomass-based production of energy and valuable chemicals, interlinked in a biorefinery concept.

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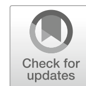
Chapter 7
Anaerobic Digestion and Gasification of Seaweed



John J. Milledge and Patricia J. Harvey

1 Introduction

Global utilisation of macroalgae is a multibillion dollar industry [1, 2] with world production of seaweed increasing, between 1970 and 2010 from <2 million to 19 million tonnes fresh weight [3]. The volume of seaweed commercially produced has increased by 8% per annum in the last decade with seaweed production reported at 27 million tonnes in 2014 [4], and the commercial seaweed market is projected to grow to US$17.6 billion in 2021 [5]. The current uses of seaweeds include human foods, fertilisers, phycocolloids and cosmetic ingredients [6], with Asia being the main market [7, 8], and the macroalgal nonfuel industry being currently 100 times bigger globally in wet tonnage terms than the microalgal industry [9–11]. However, seaweed is still considered an underutilised resource worldwide [12].

Algae, unlike terrestrial crops, do not require agricultural land for cultivation, and many species grow in brackish or salt water avoiding competition for land and fresh water required for food production [9, 13]. The potential biomass yield of algae per unit area is also often higher than that of terrestrial plants with, for example, brown seaweeds grown 'under cultured conditions' having yields of ~13.1 kg dry weight (dw) m^{-2} per year compared to ~10 kg dw m^{-2} per year from sugarcane [14, 15]. This high potential biomass yield and growth systems that do not compete for land or freshwater with agricultural crops have led to research interest in the use of algae as a source of biofuel [16, 17], but much of the research on algal biofuels has been focused on micro- rather than macroalgae (Fig. 7.1). Nevertheless, despite their

J. J. Milledge (✉) · P. J. Harvey
Algae Biotechnology Research Group, University of Greenwich, Kent, UK
e-mail: j.j.milledge@gre.ac.uk

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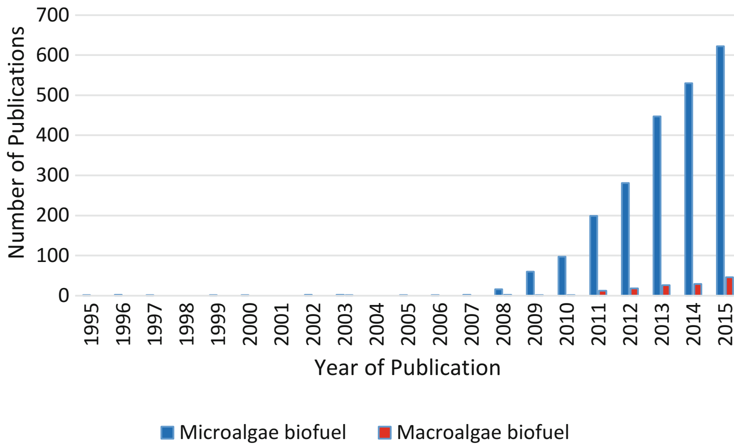

Fig. 7.1 Number of publications per year in Web of Science for the search terms microalgae biofuel and macroalgae biofuel

obvious potential and considerable research, there are no economically viable commercial-scale quantities of fuel from either micro- or macroalgae [1].

The process operations used for algal-derived fuel production can be grouped into four main areas:

1. Cultivation (including seedling production)
2. Harvesting
3. Postharvest treatments including cleaning, size reduction, preservation and storage
4. Energy extraction

Any future successes of macroalgal-derived fuel will be dependent on achieving an optimised, energy efficient process in each of these four areas. This chapter focuses on the methods of energy extraction from macroalgae and in particular anaerobic digestion and gasification of macroalgae.

2 Method of Converting Seaweed to Biofuels

The methods of extraction of energy from macroalgae can be categorised according to whether an initial drying step is required or not. This leads to two distinct groups of processes [1, 13]:

1. Energy extraction methods requiring dry macroalgae:

   a. Direct combustion
   b. Pyrolysis
   c. Gasification (conventional)
   d. Transesterification to biodiesel

Table 7.1 Methods of energy extraction from macroalgal biomass

	Utilises entire organic biomass	Requires biomass drying after harvesting	Primary energy product
Direct combustion	Yes	Yes	Heat
Pyrolysis	Yes	Yes	Primarily liquid by fast pyrolysis
Gasification	Yes	Yes[a] (conventional)	Primarily gas
Biodiesel production	No	Yes[b]	Liquid
Hydrothermal treatments	Yes	No	Primarily liquid
Bioethanol production	No[c]	No	Liquid
Biobutanol production	No[c]	No	Liquid
Anaerobic digestion	Yes	No	Gas

[a]Supercritical water gasification (SCWG) an alternative gasification technology can convert high moisture biomass
[b]No current commercial process for the wet transesterification of wet macroalgal biomass
[c]Polysaccharides require hydrolysis to fermentable sugars. Some of the sugars produced from the breakdown of seaweed polysaccharides are not readily fermented

2. Energy extraction methods for wet macroalgae:
   a. Hydrothermal treatments
   b. Fermentation to bioethanol or biobutanol
   c. Anaerobic digestion (AD)

A summary of the potential methods of energy extraction is given in Table 7.1, detailing the primary energy products and two major process parameters, the need for drying prior to processing and the potential to utilise the entire organic biomass feedstock, both of which have significant impacts on the overall energy balance of macroalgal biofuel.

2.1 Seaweed as a Feedstock

The composition of a biomass feedstock will influence the processing methods to produce biofuel. The water content of macroalgae (80–90%) is generally higher than that of many terrestrial crops (sugarcane ~75%, grain maize 14–31%) [15, 18–20] and thus may be better suited to wet processing methods. The elemental analysis and higher heating value (HHV) for a number of seaweeds are presented in Table 7.2. The HHV of seaweed is generally lower than terrestrial energy crops (17–20 kJ g^{-1} dw) due to their high ash content [11]. Sulphur content of seaweed is generally higher than terrestrial plants with the sulphated polysaccharides of seaweeds also being chemically very different from those of land plants; those in brown seaweed being mainly sulphated fucans (fucoidans), with other sulphated polysaccharides containing galactose, xylose, glucose and other simple sugars also being found [24, 25]. The growth in

Table 7.2 Compositional and higher heating value (HHV) data for some species of seaweed being considered as potential biofuels

	Ash	Carbon	Hydrogen	Oxygen	Nitrogen	Sulphur	HHV
	% dw	% dw	% dw	% dw	% dw	% dw	MJ kg^{-1} dw
Ascophyllum nodosum[a]	21.1	37.3	5.2	31.0	3.0	2.5	15.6
Chorda filum[b]	11.61	39.14	4.69	37.23	1.42	1.62	15.6
Enteromorpha prolifera[c]	30.1	28.75	5.22	32.28	3.65	0	12.2[e]
Fucus serratus[b]	23.36	33.5	4.78	34.44	2.39	1.31	16.7
Fucus vesiculosus[b]	22.82	32.88	4.77	35.63	2.53	2.44	15.0
Laminaria digitata[b]	25.75	31.59	4.85	34.16	0.9	2.44	17.6
Laminaria hyperborea[b]	17.97	34.97	5.31	35.09	1.12	2.06	16.5
Laminaria saccharina[c,d]	24.2	31.3	3.7	36.3	2.4	0.7	11.1[e]
Macrocyctis pyrifera[b]	38.35	27.3	4.08	34.8	2.03	1.89	16.0

[a] [1]
[b] [11]
[c] [20]
[d] [21]
[e] Calculated using version of the DuLong equation [22, 23]

a marine environment also results in the salt (sodium chloride) content of seaweed being higher than terrestrial plants with salt being 15% of dried weight of unwashed *Sargassum muticum* [26]. The higher moisture, ash, salt and sulphur content of seaweed has considerable implications for the energy extraction from seaweed.

2.2 Dewatering and Drying Macroalgae

Many processes for the manufacture of fuels from biomass, such as direct combustion, pyrolysis, gasification and current commercial biodiesel production, require a dry feedstock, and drying is required prior to energy extraction. The inclusion of a drying stage in macroalgae-to-fuel processes will have a significant impact on energy return on investment (EROI). The energy to heat water from 20 to 100 °C and evaporate it at atmospheric pressure requires an energy input of approximately 2.6 MJ kg^{-1} or over 700 kWh m^{-3} [13]. The removal of water from the algal biomass by evaporation can, therefore, be very energy intensive, and finding a more controllable and cost-effective method of large-scale seaweed drying, compared to that of sun-drying, is clearly key to establishing a viable seaweed-to-fuels processing industry [27].

Sun-drying is the main method of drying seaweed [27–29]. Clearly this approach does not require fossil fuel energy, but is both weather and volume dependent. Sun-drying in tropical locations may take 2–3 days in sunny weather and could take up to 7 days in rainy seasons [27]. Despite these limitations, solar methods are the least expensive drying option [30], but large areas are required as only around

100 g of dry matter can be produced from each square metre of sun-drier surface [31].

Coal-fired driers have been used in Ireland for the production of seaweed meal products to achieve a moisture content ~10%, but this approach is uneconomic for biofuel production [32]. The energy to dry a seaweed with a moisture content of 88% has been calculated to be 0.7 MJ kg^{-1}, higher than the lower heating value (LHV) of dry seaweed [32].

The dewatering (the removal of water by mechanical methods from the algal biomass, such as pressing and centrifugation) generally uses less energy than evaporation to remove water and may be a useful step prior to drying to reduce overall energy input. However, screw-pressing brown seaweed has been found to be infective unless the seaweed is pretreated with hydrochloric acid [33] adding cost to mechanical dewatering. Reducing the moisture content of the seaweed is also beneficial as it prevents the growth of spoilage-causing microorganisms and slows down detrimental enzymatic reactions [27], but certain species of seaweed are inherently more resistant to degradation, for example, brown seaweed is more stable than green seaweed, attributed to the presence of polyphenols, and can be stored at ambient temperature for hours or even days without starting to deteriorate [32]. The reduction of seaweed biomass moisture content to 20–30% not only increases 'shelf life' but also reduces transportation costs [32, 33].

2.3 *Direct Combustion*

Direct combustion is, historically and currently, the main method by which energy from dry biomass resources is realised, providing heat or steam for household and industrial uses or for the production of electricity [34]. Macroalgal combustion does not appear to have been greatly explored [35, 36]. However, the high energy required to dry seaweed, the relatively low thermal values and high ash and sulphur content, which can cause fouling and corrosion of boiler and unacceptable emissions, could preclude direct combustion as an economic method of exploiting seaweed [35–38].

2.4 *Biodiesel*

The higher lipid content of some microalgae compared to macroalgae has focused much of the published research work on the production of biodiesel from the microalgal lipids via transesterification [13, 39, 40]. Macroalgal biomass typically has lower lipid content, 0.3–6% compared to microalgae which can have over 70% [41–44]. Macroalgae would, therefore, not appear to be a suitable feedstock for the production of biodiesel via transesterification.

2.5 Bioethanol

First-generation bioethanol, such as that produced from corn in the USA and sugarcane ethanol in Brazil, is now widely produced and used and currently is the liquid biofuel with the highest production volume (>90 GL) [45, 46]. Bioethanol can be readily used in current supply chains, with 86% of cars sold in Brazil in 2008 capable of using ethanol or a mixture of ethanol and fossil fuel petroleum [47]. Brown, green and red algae have all been fermented to ethanol, but brown algae are suggested as the principal feedstock for bioethanol production because they have high carbohydrate contents and can be readily mass-cultivated [48]. Although polysaccharides are the predominant component of macroalgae making up to 76% of the total dry weight, and typically ~50% [49], the polysaccharide composition of brown seaweed is different to that of terrestrial plants with the major polysaccharides of brown algae being laminarin, mannitol, alginate and fucoidan [48]. These algal polysaccharides have been found to be difficult to ferment using conventional bioethanol technology and require considerable pretreatment for the production of bioethanol [50, 51]. Wargacki et al. [52] have concluded that the full potential of ethanol production from macroalgae cannot currently be realised because of the inability of industrial microbes to metabolise alginate polysaccharides. Research is currently being undertaken to increase bioethanol yield by using organisms that produce alginate lyases, such as *Vibrio splendidus* and engineered *Escherichia coli*, to produce ethanol from alginate by expressing the requisite metabolic, transporter and lyase genes from *V. splendidus* [52, 53]. Using organisms engineered with alginate lyases, yields of ~80% of the maximum theoretical yield from macroalgae have been achieved [52].

Large seaweed ethanol production facilities have been proposed in both Denmark [54] and Japan, 'Ocean Sunrise Project' [55], but the economic and energy feasibility of these schemes is unknown, and as yet there appears to be no large-scale production of ethanol from macroalgae [54]. Horn et al. [56] concluded that a commercial industrial seaweed bioethanol process will require higher ethanol yields to be viable, and research is being carried on selection and genetic modification of microorganisms to increase bioethanol yield that may permit future commercial production of ethanol from seaweed.

2.6 Biobutanol

Whilst seaweed cultivation for bioethanol is being explored in Asia, Europe and South America, it is biobutanol from macroalgae that is attracting research interest and investment in the USA [57]. Butanol has been explored as a transportation fuel for around 100 years, and has been suggested as a biofuel with the potential, not only to augment but even replace ethanol as a gasoline additive due to its low vapour pressure and higher energy density [58]. Although biobutanol has been produced on

a pilot scale from algal sugars [58], it has been concluded that significant improvements in yield and process costs are still needed to make industrial-scale butanol from the fermentation of seaweed economically feasible [59].

2.7 Hydrothermal Processing

Hydrothermal processing is a high pressure process where 'wet' biomass is converted into primarily a stable liquid hydrocarbon fuel (bio-oil) in the presence of a catalyst [34, 38, 60]. The ability of hydrothermal liquefaction to handle wet biomass makes it one of the most interesting methods of producing biofuel from algae [61], and hydrothermal treatment of algae has attracted research interest [38, 62–64]. Hydrothermal liquefaction of biomass with a moisture content above 90% is believed to have an unfavourable energy balance [65], and reviews of thermal treatments for biofuel production have concluded that commercial interest in liquefaction is low due to the more complex feed systems and higher costs compared with those for pyrolysis and gasification [34, 60, 66, 67]. The production of biofuel from seaweed via hydrothermal treatment, thus, will require considerable more research to reduce process costs.

3 Gasification and Anaerobic Digestion

Both gasification and anaerobic digestion (AD) have been suggested as promising methods for exploiting bioenergy from biomass [68], and two major projects have been funded in the UK on the gasification and anaerobic digestion of seaweed, MacroBioCrude and SeaGas. A recent study that analysed four methods of microalgal bioenergy production found that anaerobic digestion produces more net energy than supercritical gasification, the latter requiring higher energy input and having a negative return on energy investment [13, 69]. This conclusion is supported by a related study that has demonstrated that anaerobic digestion of 'algal residues' can have a higher net energy return and much lower GHG emissions than gasification [70]. Despite the energy benefits from anaerobic digestion processes, gasification is a significantly more rapid process, which is a clear operational benefit, and if higher yields of combustible gas can be achieved through gasification, then this may lead to a more favourable energy balance.

3.1 Gasification

Gasification is the conversion of organic matter by partial oxidation at high temperature (800–1000 °C) mainly into a combustible gas mixture (syngas) [34, 60, 71, 72]. The

gasification processes involves a number of stages: initially pyrolysis occurs in a reaction producing char, which is then gasified in the presence of a gasifying agent such as O_2 or H_2O to produce syngas. Importantly, the amount of syngas produced through further gasification of the char is considerably greater than that produced through conventional pyrolysis at 800–900 °C [73]. Syngas can be produced from biomass with and without the presence of a catalyst; however, non-catalytic processes require a higher temperature than catalytic processes [74]. Nickel compounds, olivine and dolomite have typically been employed as cracking catalysts to enhance gasification.

The syngas has a calorific value of 4–6 MJ m^{-3} [60] and is a mixture of hydrogen (30–40%), carbon monoxide (20–30%), methane (10–15%), ethylene (1%), nitrogen, carbon dioxide and water vapour [34, 72]. The gas can be burnt to produce heat or converted to electricity and heat in combined gas turbine systems [34, 60]. Syngas from gasification of biomass can be converted catalytically into hydrocarbons and water through the Fischer-Tropsch process, a catalytic chemical reaction in which carbon monoxide (CO) and hydrogen (H_2) in the syngas are converted into hydrocarbons of various molecular weight [75]. The condition of the Fischer-Tropsch process is usually chosen to maximise the formation of higher molecular weight hydrocarbon liquid drop-in fuels which can be used in current combustion engines and infrastructure. The syngas produced from gasification can also be used to produce methanol and hydrogen as a fuel for transport and other uses [60, 72], but the cost of methanol produced from methane from biomass has been estimated at 1.5–4 times higher than from fossil fuel gas [76].

The gasification of dry lignite and woody biomass can have high yields with up to 90% of the original chemical energy in the biomass being recovered as energy in syngas [77], with the net energy return, including energy inputs, for pyrolysis operation of dry land agricultural biomass waste ranging from 42 to 53% [78].

Conventional biomass gasification processes require dry feedstock [38, 79], and the energy required for drying may make it unviable energetically, but integration of drying and gasification processes could reduce overall energy input [80], and the use of solar drying could allow gasification to be net energy positive [28]. Supercritical water gasification (SCWG) is an alternative gasification technology for the conversion of high moisture biomass, and hydrothermal gasification is seen as a potential processing method for wet carbohydrate-rich biomass, such as macroalgae [81]. It is suggested that SCWG processes can be net energy positive in well-engineered systems [82], but the presence of water can alter the composition of the gases produced [83]. Catalytic supercritical water gasification of *Ulva lactuca* has produced a methane-rich gas [84]. A study of SCWG of *S. latissima* harvested during various months found that seaweed harvested in July produced gas with the highest calorific value, due to the lower ash content and the higher carbohydrate content [85]. Upon addition of NaOH in the SCWG of the macroalgae, S*accharina,* there was a threefold increase in H_2 production, along with an increase in methane, decrease in C_2-C_4 yields and the elimination of CO and CO_2 from the syngas

[86]. The origins of these observations are believed to be due to the removal of CO_2 through reactions that form Na_2CO_3, a process that disrupts the water-gas shift equilibrium together with a similar scenario for increased methane production through alkaline catalysed decarboxylation of acetate groups of the primary sugar components of seaweed. Similarly, the addition of alumina-supported ruthenium (Ru/Al_2O_3) catalysts was found to double the H_2 and CH_4 yields from SCWG of *S. latissima* compared to the uncatalysed reaction with an increase of the gasification efficiency from 58% to 92% [85]. CO and C_2-C_4 yields correspondingly decreased, whilst CO_2 yield increased. However, subsequent reuse of the regenerated catalyst led to a decrease in the yields of H_2 and CH_4, although, after reuse of the catalyst, three times the H_2 and CH_4 yields were still above those from the uncatalysed processes. Analysis of the spent catalysts showed the build-up of surface sulphur and calcium. The addition of CO_2 during the steam gasification of *S. japonica* at 700 °C has been shown to increase the yield of CO in the resulting syngas by 20% [87].

Pretreatment of macroalgae with water and acid may be employed to remove Mg, K, Na and Ca salts and other mineral matter by up to 90% [88] which can lead to high char levels, but conversely alkaline species can catalytically aid the steam gasification of macroalgae to H_2 at temperatures above 700 °C [89]. Higher syngas yields from steam gasification of macroalgae are possible due to their high content of inorganic elements compared to land-based biomass such as Japanese cedar and apple branches; however, the latter materials can be co-gasified with seaweed in a biorefinery, leading to enhanced syngas yields [89, 90].

A recent review has concluded that there is little data available on the gasification of algae and in particular on the energy balance and the need for drying of algae prior to gasification [30]. If gasification of macroalgae can be achieved using wet biomass, it may be more economic and energetically attractive than traditional dry methods of gasification. The enthalpy change needed to take ambient temperature liquid water to a low-density supercritical state (400 °C and 250 bar) is similar to that required to vaporise liquid water at ambient temperature, but the advantage of the SCWG process is that much of the energy invested in reaching a supercritical state can be captured and used again, with the hot effluent from the gasification reactor being used to preheat the wet biomass feed stream [79]. However, supercritical water macroalgae gasification still faces a variety of engineering and scale-up issues, and considerable further research and development is required. Both gasification and anaerobic digestion have been suggested as promising methods for exploiting bioenergy from biomass in India [68], but despite the energy benefits from anaerobic digestion processes, gasification is a significantly more rapid process, which is a clear operational benefit, and if higher yields of combustible gas can be achieved through gasification, then this may lead to a more favourable energy balance. Rowbotham et al. [91] have suggested that thermochemical processing methods, such as gasification and hydrothermal liquefaction, are more applicable and versatile treatment options than anaerobic digestion and fermentation, due to the technological

difficulties associated with treatment and refining to liquid fuels of complex, heterogeneous, multicomponent feedstocks, such as seaweed. [80], However, Smith and Ross [38] reported that the high chlorine, ash and alkali content, low calorific value and high moisture content make macroalgae not only unattractive option for combustion but also gasification without extensive pretreatment, and as a consequence, the majority of research into utilising macroalgae as a biofuel has focused on the production of biogas by anaerobic digestion.

3.2 Anaerobic Digestion

Anaerobic digestion (AD) consists of a series actions by different groups of bacteria that convert organic compounds to methane, carbon dioxide and bacterial cells. The biogas produced from the AD of seaweed typically contains methane 50–70%, carbon dioxide 30–45%, hydrogen <2% and hydrogen sulphide <3.5% [49, 92, 93]. AD consists of four stages [94–97] (Fig. 7.2):

1. Hydrolysis: carbohydrates, proteins and fats are decomposed into monosaccharides, disaccharides, amino acids and fatty acids.
2. Acidogenesis: acidifying bacteria convert hydrolysis products to short-chain organic acids.

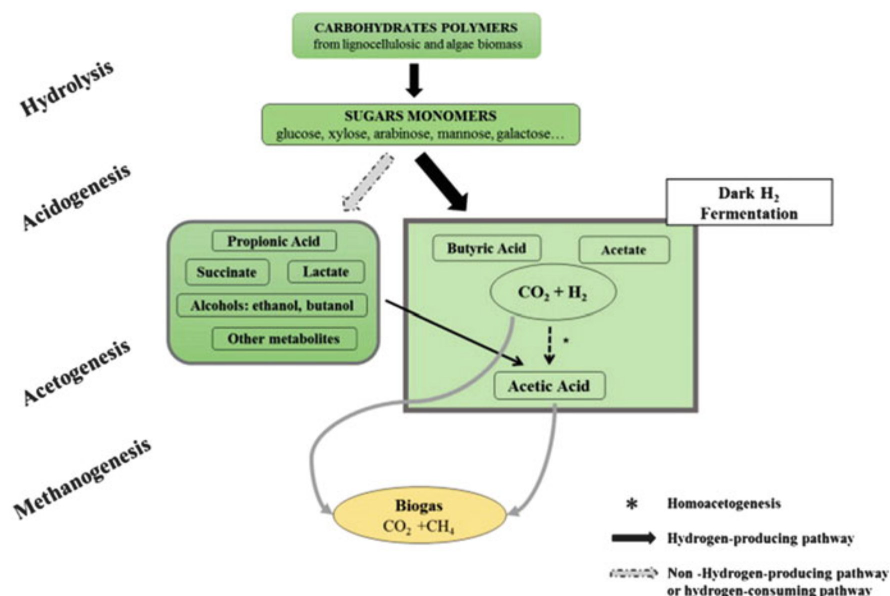

Fig. 7.2 Scheme of carbohydrate polymers degradation through anaerobic digestion [96]

3. Acetogenesis: acetogenic bacteria produce acetic acid, H_2 and CO_2 from fermentation products (dark fermentation is the fermentative conversion of organic substrate to hydrogen). The acetogens fall into two main groups:

   a. Hydrogen-producing acetogens break down volatile fatty acids to CO_2 and H_2 (butyrate: $CH_3CH_2CH_2COOH + 4H_2O \rightarrow CH_3COOH + 2CO_2 + 6H_2$)
   b. Homoacetogens: $4H_2 + 2CO \rightarrow CH_3COOH + 2H_2O$

4. Methanogenesis: end of the degradation chain; two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide:

   a. Acetoclastic methanogenesis ($CH_3COOH \rightarrow CH_4 + CO_2$)
   b. Autotrophic or hydrogenotrophic methanogenesis ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$)

Anaerobic digestion is generally the process of choice for biomass with a high water content such as seaweed [28, 98], and various groups assessing the suitability of seaweed for anaerobic digestion (AD) from the 1970s through to the 1990s generally found that seaweeds were mostly a suitable biomass for AD [99]. Seaweed-derived biogas was used industrially in the nineteenth century, and more recently Tokyo Gas demonstrated that 20 m^3 of methane can be produced from 1 tonne of seaweed which when blended with natural gas was used to power a 9.8 kW electrical generation plant [54]; currently biofuel from seaweed AD is perhaps the closest to industrial exploitation [37, 100]. Not only is it a relatively simple process from an engineering/infrastructure stance, but it has the potential to exploit the entire organic carbon content of macroalgae and can readily tolerate high moisture content without incurring additional process energy penalties. It is likely to play a leading role in combination with other methods and could be the major method of biofuel production from macroalgae.

It has been suggested that the use of biogas from seaweed could reduce GHG emissions by 42–82% compared to the use of natural gas [101]. Moreover, the digestate (material remaining after the anaerobic digestion) contains both nitrogen- and phosphorus-containing compounds, which makes it a possible seaweed-derived fertiliser or biological feedstock, and could add additional income streams to seaweed AD processes [102].

The bacteria involved in the production of methane by anaerobic digestion are sensitive to the chemical composition of the feedstock [103–105]. The proportions of carbohydrates, proteins and lipids affect the potential of algae as a substrate for anaerobic digestion [106] with lipids yielding higher volumes of biogas per gram of feed material than either carbohydrate or protein [23, 97, 107]. It has been suggested that the low lipid content of macroalgae (Table 7.3) make them 'especially suitable' for biogas production using anaerobic fermentation [42, 43]. The theoretical yield of biogas, calculated from the chemical composition of macroalgae ($C_cH_hO_oN_nS_s$) using the 'Buswell equation' [108, 109], can be high.

Table 7.3 Lipid, protein and carbohydrate composition of various macroalgae % dw [96]

% composition (dw)	Lipids	Proteins	Carbohydrates
Green algae			
Codium fragile	1.8	10.9	32.3
Enteromorpha linza	1.8	31.6	37.4
Ulva lactuca	6.2	20.6	54.3
Red algae			
Gelidium amansii	0–3.1	15.6–16.3	61–67.3
Porphyra tenera	4.4	38.7	35.9
Gracilaria verrucosa	3.2	15.6	33.5
Brown algae			
Laminaria japonica	1.8–2.4	9.4–14.8	51.9–59.7
Hizikia fusiforme	0.4–1.5	5.9–13.9	28.6–59
Saccharina japonica	0.5	19.9	44.5
Sargassum fulvellum	1.6	10.6	66
Ecklonia stolonifera	2.4	13.6	48.6
Undaria pinnatifida	1.8–2.0	15.9–18.3	40.1–52
Sargassum fulvelum	1.4	13	39.6

However, practical yields of biogas from the anaerobic digestion of macroalgae are considerably below the theoretical maximum at typically ≤50% of the calculated maximum yield [26, 110]. The destruction of organic volatile solids from microalgae was found to be only 60–70% of that found in raw sewage [110, 111]; however, the methane yield (0.271 $m^3\ kg^{-1}$) from *Ulva lactuca* was found to be similar to that from cattle manure and land-based energy crops, such as grass-clover [84, 112]. Methane yields from the anaerobic digestion of macroalgae have been reported in the range of 0.14–0.40 $m^3\ kg^{-1}$ of volatile solids [42] but are typically 0.2 $m^3\ CH_4\ kg^{-1}$ [113]. There is considerable conjecture about the reasons for the relatively low practical methane yields from seaweed compared to their theoretical values [37, 99, 114–116]. Potential causes of the recalcitrance of seaweed in AD are (a) seaweed structure and cell wall structure, (b) seaweed polysaccharides, (c) polyphenols, (d) organic sulphur compounds, (e) other antimicrobial and toxins, (f) C:N ratio and (g) heavy metals [95, 98]. The Consortium for Algal Biofuel Commercialization (CAB-Comm), established to conduct research to enable commercial viability of alternative liquid fuels produced from algal biomass, found in a sensitivity analysis that increasing CH_4 yield from anaerobic digestion from seaweed was the most important factor in improving processs energy balance and reducing greenhouse gas emissions, and thus further research on the factors reducing practical methane yields is vital [117].

The hydrolysis of seaweed-derived polysaccharides, particularly alginates, is considered the rate-limiting step in the AD of seaweed [99, 118]. Typical inocula for anaerobic digesters are from municipal sewage sludge and animal manure slurry, but inocula containing higher proportions of bacteria capable of fermenting marine phycocolloids have been shown to increase methane production [99]. The addition

of bacteria from the rumen of Ronaldsay sheep, which had a diet almost entirely of seaweed, was found to increase the methane yield (0.253 l CH_4 g^{-1} VS) and volatile solid utilisation (67%) from anaerobic digestion of *Laminaria hyperborea* [99].

Brown seaweed contains high levels of phenolics with levels up to 14% dw being reported [119] with *Sargassum muticum* containing 0.7–6% [120–122] and *Ascophyllum nodosum* 0.2–5% [115]. Polyphenols are suggested as one of the elements in low yields of methane from brown seaweeds [98, 115, 116, 123, 124]. Tabassum et al. [115] found that methane yield decreased with seasonal increases in phenolic content of *Ascophyllum nodosum*, and Moen et al. [118] found that methane yield from *Ascophyllum nodosum* increased when polyphenols were fixed with low concentrations of formaldehyde. Hierholtzer et al. [123] found that there was a significant effect from the presence of phloroglucinol and phlorotannins extracted from *L. digitata* (2–200 mg L^{-1}) on the methane production from the AD of sodium acetate. Gallic acid at concentration of 10 mg L^{-1} has been shown to inhibit biogas production from starch by up to 75% [125]. However, recent research at the University of Greenwich has found no significant effect of lower gallic acid concentrations of 7% of volatile solids (0.18 mg L^{-1}), more typical of seaweed, on methane yields from three substrates. López et al. [126] have suggested that mixtures of phenolics can act either synergistically or antagonistically, and with phenolic concentration also appearing to have an effect on methane yield, there is a need for considerable more research on the effect of concentration of individual phenolics and mixtures of phenolics on methane yield from compounds typically found in seaweed.

AD methane yields from brown algae are generally higher than those from green algae [99]. The high sulphate concentration typical for green macroalgae can also lead to the formation of H_2S which results in inhibition of methane production, foul odours, sulphur dioxide emissions on combustion of the biogas and a corrosive environment [42, 127]. Biogas from AD of *Ulva* contained up to 3.5% H_2S, making it unsuitable for energy recovery without treatment [92]. The emission of H_2S can be controlled by the addition of metal ions such as iron or by gas scrubbers, but both add to the cost of biogas production [42, 127]. The upgrading of biogas is beyond the scope of this chapter, but upgrading of biogas typically uses ~11% of the energy content in the biogas [128] and has been extensively reviewed by Petersson and Wellinger [129], Ryckebosch et al. [130] and Bauer et al. [131].

One of the advantages of growing macroalgae for biofuel is that they grow in seawater and do not compete for limited fresh water resources. Low salt concentrations can stimulate microbial growth, but high salt concentrations ($\geq$10 g L^{-1}) are known to inhibit anaerobic systems through an increase of osmotic pressure or dehydration of methanogenic microorganisms [132, 133]. The toxicity of salt is predominantly determined by the sodium cation and other light metal ions, such as potassium, have also been found to be toxic to methanogens at high levels [134]. An optimal sodium concentration for mesophilic methanogens in waste treatment processes of 230 mg Na L^{-1} has been recommended [135]. Mesophilic methanogenic activity is halved at 14 g Na L^{-1} [135, 136], the approximate level of sodium found in sea water [137]. Adams et al. [138] found that washing *Laminaria digitate* with

freshwater tended to increase methane yields due to the loss of salt, despite >50% of laminarin being lost during washing. However, in a study of *Ulva lactuca*, it was found that washing of algae had no effect on methane yield [84]. Anaerobic digesters can be acclimatised to higher salt levels if they are continuously exposed to gradually increasing salt concentration rather than salt shock [133, 139]. Adaptation of methanogens to high concentrations of sodium over prolonged periods of time can allow the anaerobic digestion of high salt concentration wet biomass, with the sodium concentration to halve methanogenic activity increasing to 37.4 g Na L^{-1} after acclimation [135]. It therefore appears possible to produce biogas from macroalgae without the use of fresh water, but the high salt concentration could also be mitigated by mixing algal biomass with other types of biomass to 'dilute' the salt [42].

Size reduction of macroalgae biomass, as with direct combustion, may be required prior to AD as the reduction in size of the algal fronds prior to anaerobic digestion has been shown to significantly increase the yield of methane from *Ulva lactuca* from 174 to 271 m^3 kg^{-1} [84] and from Baltic beach-cast seaweeds by up to 53% [140].

It has been suggested the cost of production of biogas from seaweed is high with estimates suggesting that it could be 7–15 times more expensive than natural gas [57]. A survey by Bruton et al. [32] found seaweed AD to be the closest process to commercialisation, but the cost of the raw material must be reduced by at least 75% over current levels to be economically viable. Roesijadi et al. [8] in an economic assessment of the production of gasoline from methane from seaweed AD found that it was not economic, but assumed a biogas yield of 0.17 m^3 kg^{-1} VS at the lower end of the literature methane yield. However, recent studies on energy return of the production of biogas from microalgae have shown it to be potential energetically viable with an EROI of over 3 [141, 142]. Anaerobic digestion of seaweed has been proven to be technically feasible at scale, and it has been suggested that it could be a cost-competitive with anaerobic digestion of terrestrial biomass and municipal solid waste [54].

4 Conclusions

The production of biofuel from seaweed is economically, energetically and technically challenging at scale. It is probably too early, at the current stage of biofuel development, to select definitively what method or combinations of methods for exploiting energy from macroalgae will be commercial exploited. Processes that exploit the entire algal biomass such as AD or gasification appear to offer the best chances of success. However, currently anaerobic digestion is closest to industrial exploitation. Not only is it a relatively simple process from an engineering/infrastructure stance, but it has the potential to exploit the entire organic carbon content of macroalgae and can readily tolerate high moisture content without incurring additional process energy penalties. It is likely to play a leading role in combination with other methods and could be the major method of biofuel production from macroalgae.

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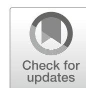
Part II
The Economic Potential of Marine Biotechnology

Chapter 8 The Global Market for Marine Biotechnology: The Underwater World of Marine Biotech Firms



Gaia Raffaella Greco and Marco Cinquegrani

1 Introduction

3.8 billion years of evolution have produced a very rich library of natural products, and scientists have really only started exploring and understanding these in the last 50 years. Paul Wender, Stanford University[1]

The exploration of the seas began with the expeditions endowed by the *Royal Society* in the eighteenth and nineteenth centuries and steered by legendary captains and scientists such as James Cook and Robert Fitzroy. Charles Darwin, in 1831, sailed out on the *HMS Beagle* to circumnavigate the world. His journey lasted almost 5 years and changed the history of science through the theory of evolution and natural selection he developed at the time.

In 2004, the man who raced the US government to sequence the human genetic code, Craig Venter, was circling the globe by his luxury yacht *Sorcerer II* hoping to update the scientific voyages of his renowned predecessors. Venter took the decision after a pilot project led in the Sargasso Sea, the aim of which was to apply the whole-genome shotgun method to an entire ecosystem—and not to an individual genome only. His team discovered, during the *Sorcerer II* expedition, at least 1800 new

[1]Cited in Elvidge S. [1], "Bioprospecting at the poles", *Chemistry and Industry, Natural products*, pp. 17–19.

G. R. Greco (✉)
General Services, Stazione Zoologica Anton Dohrn, Naples, Italy

Istituto di Calcolo e Reti ad Alte Prestazioni, Consiglio Nazionale delle ricerche, Naples, Italy
e-mail: gaia.greco@icar.cnr.it

M. Cinquegrani
Istituto di Calcolo e Reti ad Alte Prestazioni, Consiglio Nazionale delle ricerche, Naples, Italy

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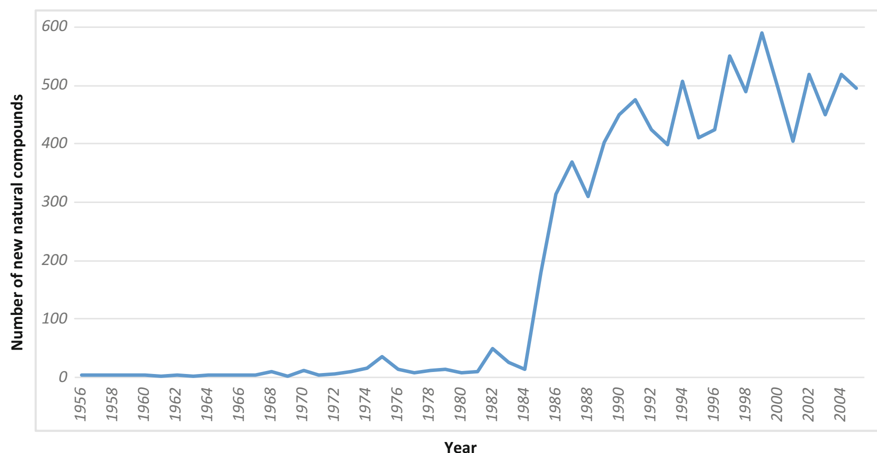

Fig. 8.1 The discovery of marine natural products between 1950 and 2008. Source: Hu G.-P., Yuan J., Sun L., She Z.-G., Wu J.-H., Lan X.-J., Zhu X., Lin Y.-C., Chen S.-P. (2011), "Statistical Research on Marine Natural Products Based on Data Obtained between 1955 and 2008," Marine Drugs, n. 9, pp. 514–25

species and more than 1.2 million new genes, doubling the number of genes previously known—considering all the species in the world.[2]

In May 2015, a special issue of *Science* was dedicated to the investigations led by the European team of scientists who had been mapping the biodiversity of a wide range of planktonic organisms all over the world on the schooner *Tara*.[3] During the 2009–2013 expeditions, they collected 35,000 samples that gave birth to a catalogue of several millions of new genes.

Even if several assessments exist, it is estimated that approximately 80% of all marine life in today's oceans remain unexplored [3]. Despite the long history of marine exploration, therefore, significant scientific progresses only occurred during those last years, and a huge amount of work still needs to be done.

Hu et al. [4] performed a quantitative analysis on three major biological classes between 1950 and 2008: marine microorganisms (including phytoplankton), marine algae, and marine invertebrate. In particular, they investigated how the trend in the discovery of new compounds of marine origin has changed in that specific lapse of time (see Fig. 8.1).

It is interesting to analyze how and why the trend changed so evidently in the mid-1980s and then again, at the beginning of the 1990s. Hu et al. [4] found that the fast growth of marine chemistry is mainly the result of the invention and development of high-resolution nuclear magnetic resonance (NMR) spectrometers. Several studies [5–7] contemplate a broader set of variables—basically belonging to two

[2]Shreeve J. [2], "Craig Venter's Epic Voyage to Redefine the Origin of the Species", *Wired*, August.

[3]http://science.sciencemag.org/content/348/6237

classes of reasons: on the one side, the technological evolution of scuba diving and underwater exploration and, on the other side, the scientific breakthroughs that shaped biology and biotechnology research at the end of the 1970s. Molecular biology first, and "omic" sciences later, changed the way scientists led their research. At the same time, the scuba diving progresses of the 1970s and the introduction of remotely operated underwater vehicles (ROVs), unmanned vehicles, and gliders generated new tools for the exploration of the seas, allowing the investigation of inaccessible areas and entailing a research shift: from large creatures (sponges, soft corals, red algae) to microorganisms (microalgae, cyanobacteria, fungi).

In the *Blue Growth Strategy* of the European Union Framework Program for Research and Innovation *Horizon 2020*, the authors highlight that "marine biodiversity and biotechnology research have a huge potential to contribute to new knowledge for high value products and processes and increase marine resources and biodiversity understanding".[4]

Marine organisms have revealed an incredible genetic, biological, and chemical variety and find their best application in the biotechnology industry [6]. Recently, marine compounds have been used, as it will be better described later, for the development of new pharmaceuticals and new cosmetic ingredients, for the production of enzymes and nutritional additives, and for the supply of a wide set of scientific services [8].

Despite the very powerful development of blue biotechnologies in the near future (and their potential), the state of the art of the business and the firms engaged in the industrial sectors are not well documented yet. How many companies are active in marine biotechnology today? Are marine compounds still an underutilized resource? In which industries do they operate? Were they born from academic institutions? Do they patent their research? Are these firms young?

Leary et al. [5] claim that the commercially sensitive nature of the information concerning blue biotechnology inhibits a global and comprehensive vision of the firms. Probably a clear picture is not available, and this is because information is so scattered in patent databases, PROs, and secrecy.

This study aims first to describe the marine biotechnology sector as it appears from the most recent analysis on the subject. After the definition of "blue" and "biotechnology" in both institutional and scientific literature, the main reports on the marine sectors will be presented. As it will be evidenced, the absence of a shared definition of the blue industries translates into a lack of thorough analysis of the global sector.

The authors will then analyze fundamental market trends in those industries where marine biotechnology has major commercial applications. The purpose is to comprehend how blue compounds could contribute to the development of the pharmaceutical, energy, cosmetic, and biochemical sectors, understanding if there

[4]EU, DG Internal Policies, "Ocean Research in Horizon 2020: The Blue Growth Potential", 2015. http://www.europarl.europa.eu/RegData/etudes/STUD/2015/518775/IPOL_STU(2015)518775_EN.pdf

is space for further research and industrial growth processes. Following the analysis of the dynamics typifying productive sectors at a global level, the authors will present an investigation on blue biotechnology industry.

The drawing up of an original dataset of firms is the result of a multiple-phase methodology based on the use of a wide number of sources, as it will be explained later in the text. The descriptive and qualitative analysis of the sample of organizations inspired the authors to analyze the temporal and geographical distribution of them, their dimension, and the main markets served.

Concluding, the authors will describe the research and productive activities, the role firms take along the value chain of the sector, and the mechanisms of collaboration with other organizations (both public and private).

Although it could be considered as a starting point, the survey offers interesting foods for thought and gives a general idea of the characteristics that are shaping this young sector. The main evidences of this analysis shall be presented in the conclusions.

2 Blue Biotechnology: A Definition

Van Beuzekon and Arundel [9] in the *OECD Biotechnology Statistics* define a biotechnology organization as "a firm that is engaged in biotechnology by using at least one biotechnology technique to produce goods or services and/or to perform biotechnology R&D. Some of these firms may be large, with only a small share of total economic activity attributable to biotechnology." This is the mostly accepted definition in the international literature on economics. It is relevant to keep it in mind, because the authors stress the wide meaning that biotechnological activities and processes hold. Biotechnology techniques can be mainly used for production reasons or to conduct research and development activities, but they can either represent the biggest part of processes or just a small fraction.

The industries in which biotechnology finds use are defined by the different colors usually adopted to classify their sector of activity: red for pharmaceuticals, white for industrial processes, green for agriculture fields, and yellow for food and feed sectors. DaSilva [10] finds 10 areas of biotech activities normally used to promote public perception and understanding and underlining how the removal of bottlenecks in science and technology could be achieved through programs that make use of the synergies existing between them.

Blue biotechnology (or marine biotech), differently from other biotech sectors, is not classified by its use in specific industries, but is defined by the resources involved in the offer of products and services. In facts, they are labeled for the marine or freshwater bio-resources as a target or source of biotechnological applications (Cordis, European Commission[5]; [11]). Blue biotech organizations could belong

[5] cordis.europa.eu/programme/rcn/11852_en.html

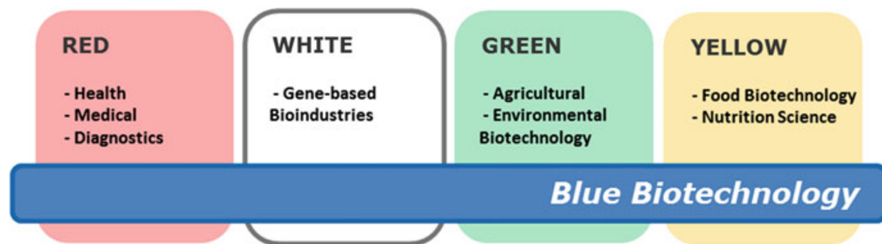

Fig. 8.2 The colors of blue biotechnology

to all the subsectors concerned with biotechnologies (Fig. 8.2). It is an important evidence, because it makes clear why it is so hard to evaluate the dimension of marine markets.[6]

Although an institutional literature on marine organizations and networks does exist [11–14], the economic reports on biotechnology industries describing main actors and trends such as *Ernst and Young* [15] "Beyond borders: Reaching new heights" or *Deloitte* "[16], Global life sciences outlook" still don't make reference blue biotechnology in their classifications. Anyway some companies using marine compounds in research or production activities are mentioned in the text (*BioMarin Pharmaceuticals*, *Jazz Pharmaceuticals*, *Gentium*, *Allergan*). This could be consequence of the very young age and the early stage of development of the sector. Of course, it could cast doubts on the need to analyze the specificity of blue biotech and its independence as a field of study.

Boyen et al. [11] point out two characteristics of blue biotechnology: the first one is biodiversity; life was born in the oceans more than 3 billion years ago, and only 2 billion years later, life developed on earth. This means that most marine organisms do not have a terrestrial counterpart. The discovery of new compounds over the last 30 years, as highlighted earlier in the text, has been constant—and significant scientific innovations are probably yet to come [4].

The other peculiarity of blue biotechnology is the specific knowledge related to seas and aquatic environments: from intertidal zones to deep ocean. The long history of evolution gave marine organisms strong peculiarities to the way they live, defend themselves, and communicate. Only marine scientists and researchers could interpret the dynamics characterizing these habitats and can contribute to the development of knowledge, both from a scientific and industrial point of view.

Among the first products of marine origin reaching the market, you can find *cytarabine*, a compound originally isolated from the Caribbean sponge, *Cryptotheca crypta*. This drug was approved by FDA (*Food and Drug Administration*) in 1969 for the treatment of myeloid leukemia, non-Hodgkin's lymphoma, and meningeal leukemia (Fig. 8.3). Despite all research efforts, after 40 years the pharmaceutical

[6]It is to underline that the term "blue" has a wider meaning with respect to "marine," as it considers industrial applications also of freshwater organisms. It is to stress, because often blue biotechnology is considered in reports on maritime policies or marine research centers.

Fig. 8.3 Some of the first marine product to reach the market (and the original sources)

developed by the *Bedford Laboratories* remains the best way to treat these diseases. It was estimated that in 2007 the global revenues for cytarabine (and vidarabine) amounted to $93 million [6].

Still in the pharmaceutical industry, Yondelis (trabectedin, ET-743)—a drug used in the treatment of soft tissue sarcoma and ovarian cancer—reached the market in 2007. *Pharmamar*, a Spanish company, developed the product together with *Johnson & Johnson Pharmaceutical R&D*. It took a long time of process (almost 30 years) to understand the structure of the active compound, so to find a way for total synthesis (first) and semisynthetic route (later). Mariculture and aquaculture gave the companies the chance to carry on clinical trials, but those systems were too expensive to ensure industrial manufacturing [17].

In the cosmetic industry, *Estée Lauder* put *Resilience* on the market in the early 2000. The product contains pseudopterosin, an extracellular extract taken from the Caribbean Sea whip (a soft coral). The company, *OsteoArthritis Science Inc.*, was developing the compound for the pharmaceutical industry, meaning it has an anti-inflammatory and wound-healing agent. But the product was delivered for skin care first, since its way through commercialization in the cosmetic sector was quite faster [18].

The development of the green fluorescent proteins (GFPs) discovered by Shimokura, Chalfie, and Tsien—who received the Nobel Prize for Chemistry in 2008—is nowadays used in the biomedical sector as a marker. The protein originally described from the jellyfish *Aequorea victoria* emits light in the infrared region. This allows the visualization of processes that would be otherwise invisible in a normal light [19]. Once again, the applications in these last years have widened their range

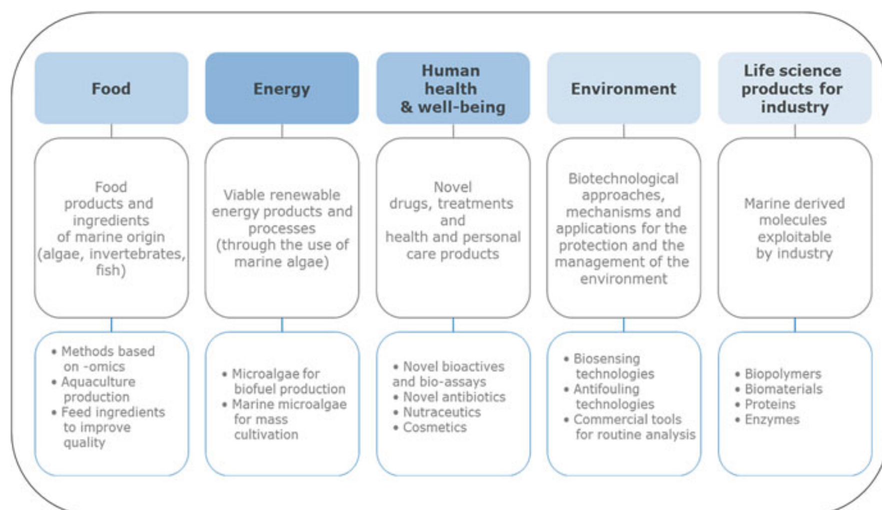

Fig. 8.4 The main sectors of blue biotechnology. Source: [3]

from molecular biology R&D to other industrial sectors, such as food and cosmetics.[7]

Several reports and scientific papers describe "business cases": cases of firms that overcame the many barriers of harvesting and chemical synthesis and reached the market through the development of natural compound of marine and freshwater origin. "Champions of innovations" are, as evidenced, the products and services that arrived successfully to the final consumers, getting a steady percentage of the total market. While the interest in the development of marine compounds remains high, the description of the single cases does not give a general idea of the dimensions of the industry, or of its main actors, and not even on the trends shaping the productive sectors where blue biotechnology could play a pivotal role.

The *Marine Board* of the *European Science Foundation* in its Position Paper n. 15, "Marine Biotechnology: a New Vision and Strategy for Europe" [12], tries to describe the achievements, challenges, and opportunities of blue biotechnology investigating all the industries concerned with marine sciences. The report still represents the wider description of the products and sectors overall. In Fig. 8.4, you shall find the schematization of the industrial areas of interest represented in the *EMBRC Business Plan* [3].

As it can be noticed, many are the industries that could see a growth process of products and services of marine origin. Not only the pharmaceutical and cosmetic fields are mentioned but also the nutraceutical sector, aquaculture, environmental monitoring activities, and the production of biomass for energy reasons all the way to the industrial exploitation of enzymes and biomaterials. It is essential to understand

[7]http://biolume.net/entertainment/

how in those last years marine sciences have affected each production sector, bringing to evidence the state of commercial development of blue biotechnologies.

3 The Blue Biotechnology Industries

In 2012, OECD (Organization for Economic Cooperation and Development) organized the first global forum on marine biotechnology in Vancouver, Canada. In 2013, in the report "Marine Biotechnology. Enabling Solutions for Ocean Productivity and Sustainability," OECD describes industries, applications, and new challenges that governments and international organization will have to face, but does not give an evaluation of the dimension of the field [20].

In the "Study in support of Impact Assessment work on Blue Biotechnology" [21], a conservative estimate values blue biotechnology in percentage of the EU bioeconomic sector as a whole. In 2013, marine biotechnology would represent the 2–5% of the sector (between around 300 and 750 millions €), the growth rate being slightly below of the biotech sector (around 4–5%).

Global Industry Analysts, a market research agency that describes 85 companies in the annual report on the marine sector, forecasts for blue biotechnologies an annual growth rate of 4–5%, envisaging the market to reach a value of 3.5 billion € by 2018.[8] The report underlines that less conservative estimates predict an annual growth in the sector of even up to 10–12% in the coming years, considering the huge potential and high expectations for further development of this sector on a global scale.

Still, *Smithers Rapra*, in the report "The Future of Marine Biotechnology for Industrial Applications to 2025," highlights how the boundaries of the marine biotechnology market are not well defined yet, since some of the marine biotechnology applications involve the use of nonmarine bio-resources. The rubber and plastics consulting company, subject to these premises, values the global market of marine biotechnology at $4.1 billion in 2015, having the potential to reach $4.8 billion by 2020 and $6.4 billion by 2025. The key drivers of the market would be the new applications of marine-derived enzymes in the cosmetic industry and the use of macro- and microalgae in biofuel production. The authors stress that the largest market for marine biotechnology would be North America, mainly focused on the production of algae-derived bioenergy. Asia-Pacific countries would represent the fastest growing market, mainly due to the aquaculture and hydrocolloid segments. Europe would be one of the leading regions contributing to the growth of the market,

[8] http://www.slideshare.net/GlobalIndustryAnalystsInc/marine-biotechnology-a-global-strategic-business-report

poised to emerge as a major regional market, thanks to its as-yet unexplored and underexploited marine resources.[9]

Concluding, for *Conference Series*, the global market for marine biotechnology products and processes is currently estimated at 2.8 billion € (2010) with a cumulative annual growth rate of 4–5%. The global marine biotechnology market is expected to reach $5.9 billion by 2022 growing at a CAGR (Compound Annual Growth Rate) of 6.2% from 2014 to 2022. The demand for natural products has increased and consequently the growth factor for marine biotechnology.[10]

In the Study in support of Impact Assessment work on Blue Biotechnology [21], it is underlined that blue biotechnology has three major areas of growth: Far East Asia, the European Union, and North America. Particularly, the main countries where marine resources are being developed are the USA, Brazil, Canada, China, Japan, Republic of Korea, and Australia. Other promising countries where marine biotechnologies are increasing their importance are Thailand, India, Chile, Argentina, Mexico, and South Africa.

The comparison of the different reports tells us that there is not agreement in the views concerning both definition and dimension of the industry. Still, there are a few studies analyzing how many companies are involved in each subsector, the geographical distribution of these organizations, their average dimension, and the relationships among them and with their academic partners.

DG Maritime Affairs and Fisheries, in its report on blue biotechnology (2014), found 97 firms: a population formed by 71 SMEs and 26 MNCs (multinational corporation). The authors led a stakeholder analysis that included companies, research centers, funding agencies, and networks (286 stakeholders representing 236 institutions in 25 countries).[11] With a mirroring exercise based on the total number of biotechnology firms in Europe in 2013 (1799), representing blue biotechnology 2–5% of the sector, the authors expect a number of companies ranging between 36 and 90 (maybe more, considering new start-ups and new spin-offs). The organization of the European Commission underlines the importance of the European research infrastructure and knowledge, while the USA would hold a leadership in the biofuel sector and Asia would establish supremacy in bioinformatics.

BCC Research, a provider of market research reports and consulting firm, attempts to define the value of the marine-derived drugs. The marine pharmaceutical market is expected to rise by 2016, reaching a total value of 8.6 billion € at a compound annual growth rate of 12.5% for the 5-year period from 2011 to 2016.[12]

Vigani et al. [22] of the *Joint Research Centre of European Commission* present an investigation of the microalgae-based food and feed sector. They found

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[9]http://www.smithersrapra.com/market-reports/biomaterials-industry-market-reports/the-future-of-marine-biotechnology-for-industrial

[10]http://naturalproducts.pharmaceuticalconferences.com/events-list/marine-biotechnology

[11]Ibidem.

[12]http://www.bccresearch.com/market-research/pharmaceuticals/marine-derived-pharma-markets-phm101a.html

50 companies mainly distributed in North America, Europe (10 organizations), and Asia and evidenced how the commercial production of microalgae would still be in its infancy.

Moreover, the authors do underline that data on algae-based food and feed products are mostly firm-specific, only estimated studies seem to be available by now, and there is no information about the products offered. In particular, the European countries show a high level of professionals in engineering, in the biofuel sector and great potentials in the agro-food sector—which is supported by a solid tradition.

After introducing the main institutional and commercial reports concerning the marine biotechnology industry, in the followings, the authors will briefly describe those trends globally affecting the industries in which blue biotechnology finds application.

3.1 Cosmetics

The *US Food and Drug Administration* defines cosmetics as "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body… for cleansing, beautifying, promoting attractiveness, or altering the appearance" (FD&C Act, 1938). Among the products included in this definition, there are skin care, hair care, fragrance, personal hygiene, and makeup, which in turn could be classified in face makeup, lipstick, eye makeup, and nail products [23].

Ernstv & Young (*EY* [24]) elaborated the annual report "Seeking sustainable growth" on the luxury and cosmetic industries. In 2014, the consulting firm values, for cosmetics, a global market of almost 181 billion € at a growing rate of 3.6% (Fig. 8.5a). Principally, skin care holds the highest market share (35%), followed by hair care (23%), makeup (17%), fragrances (13%), products for hygiene (11%), and others (1%) (Fig. 8.5b). The long-term growth is previewed as constant and this is quite relevant, as the industry has demonstrated to be resilient to economic crises.

The industry seems to be strongly concentrated, with few companies operating on a global level. Major players carried out in those last years (including 2014) strategic acquisitions to foster growth in emerging countries and to broaden their offer into innovative segments. In the USA, first players in the cosmetic sector for market share are *P&G* (14.2%), *L'Oréal* (13.6%), *Estée Lauder* (7.8%), *Unilever* (7.4%), *Limited Brands* (4.6%), *J&J* (4.5%), *Coty* (3.3%), *Mary Kay* (3.1%), *Avon* (3%), and *Revlon* (2.3%) (Statista, 2011[13]). In 2015, *EY* values at world level the first seven companies for market capitalization: *L'Oréal* confirm his position as a cosmetic giant (93 billion €) followed by *Estée Lauder* (B 29€), *Beiersdorf* (B 18€), *Coty* (B 7.5€), *Shiseido* (B 6.7€), *L'Occitane* (B 3.6€), and *Natura* (B 3.2€).

[13]http://www.statista.com/statistics/243953/market-share-of-the-leading-10-beauty-companies-in-the-us/

Fig. 8.5 The global cosmetics industry market growth from 2005 to 2014 (**a**); the global cosmetics market segmentation in 2014 by products (**b**) and by geographies (**c**). Source: EY ([24])

Many are the trends that have portrayed the cosmetic industry in those last years. They have mainly to do with diverse distribution channels and especially with the launch of the online market, with the consumers' changing identity and consumption patterns, globalization of the industry, and, as said before, the waves of mergers and acquisitions [25]. The authors will analyze briefly the two dynamics more closely influencing the relationship between cosmetics and blue biotechnology: the use of *natural* and environmentally friendly *products* and the importance of *innovation* as a *growth driver of the sector*. Of course, the two variables are also deeply intertwined.

The worries of consumers for what concerns the long-term effects of synthetic products usage is pushing companies to a broader use of organic products. There is a need for natural and environment-friendly products as consumers become more and more suspicious about chemical ingredients. In the following, there is an extract of the *EY* report (2015) on natural products:

Fig. 8.6 Examples of marine dermocosmetics actually on the market

> The product portfolio of industry players has expanded into natural and green products, replacing commonly used chemicals and synthetic ingredients with organic inputs, responding to changes in consumer tastes and preferences.
>
> Initially introduced into the skin-care product segment, the natural product shelf has now expanded to other segments of the industry.
>
> The new product lines have created new markets for the industry, and new companies have entered the industry to satisfy this demand.
>
> The demand for research and development personnel has increased significantly, which is key to remaining competitive and staying on top of the latest industry trends.

The "Study in support of Impact Assessment work on Blue Biotechnology" [21], which investigates the European databases for patents in the field of blue biotechnology, seems to confirm the strong innovation capacity grown by the firms of the cosmetic sector. They found 1774 patent families with marine origin and developed a chart of the top assignees. First organization for number of patents in the cosmetic field is *Henkel* with 144 (on 153) marine patents. It follows *Procter&Gamble*, *L'Oréal*, *Unilever*, *Kao Germany*, *Sederma*, among others.

Besides *Estée Lauder*'s face and neck crème *Resilience*, many are the products that have recently reached the global market (Fig. 8.6). *Shu Uemura* launched his deep seawater line in 1998, becoming a pioneer of the marine cosmetic sector.[14] *Biotherm*, in collaboration with Stanford University and the Roscoff Marine Biological Station, developed "blue therapy," a set of skin care products based on a cocktail of different marine organisms: an extract of *Alaria esculenta*, a deep-water algae native to Greenland's glacial waters, a yellow alga (*Laminaria ochroleuca*), and elements of marine plankton.[15] Still *Solazyme* antiaging beauty line "Algenist" is the result of the research developed in the San Francisco Bay Area (the product is distributed by *Sephora*). The scientists, initially investigating for alternative

[14] http://www.shuuemura.com/_en/_ww/discover-shu-uemura/shu-uemura-timeline.aspx

[15] http://www.biotherm.it/landing?fdid=activeIngredients

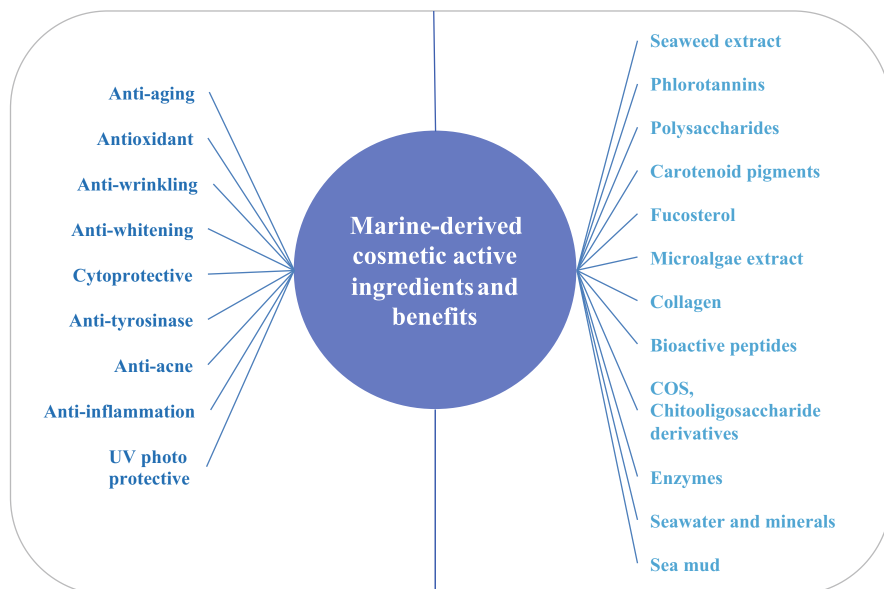

Fig. 8.7 The active ingredients and the benefits of marine compounds. Source: adaptation on Kim [28]

renewable energy from microalgae, discovered (and patented) the many properties of the *alguronic acid*.

Cosmetics are unceasingly developing products composed by active ingredients with the aim to get a better body look and to protect skin's health. Marine ingredients, especially the ones extracted from microalgae and microorganisms (bacteria and fungi), became more and more popular among industrial groups working on cosmetic and skin products, for they offer a range of benefits: they are technically more sophisticated than they used to be and they can be eco-friendly [5, 26].

Wang et al. [27] describe macro- and microalgae properties against skin aging (photoprotectivity, antioxidant capacity, and the reduction of collagen degradation) by analyzing the use of different algal compounds in cosmetics (moisturizing, thickening, pigmenting agents). It is interesting to note that marine algae could play a role in skin whitening—which is a common practice all over the world, but especially in Asia.

Figure 8.7 appears to be a comprehensive picture: the authors gave a representation of the different marine compounds that are used as active ingredients in the cosmetic sector and the many scientifically acknowledged properties of these compounds as described by Kim [28].

The authors need to point out, when speaking about bioactive ingredients, that the word "cosmeceutical" is commonly used (in the scientific literature): it's a truncation of the two words "cosmetics" and "pharmaceuticals." The term is not new, as it was coined by Raymond Reed, founding member of the US Society of Cosmetic

Chemistry, in 1961 [27]. FDA guidelines affirm that products claiming to offer medical benefits or physiological effects are over-the-counter (OTC) drugs. So even if there is a thin red line between cosmetics and drugs, there is not a specific regulation for cosmeceuticals. The consequence is that producers have to choose which products to offer, following different of rules sets. Norms about OTC drugs are of course more restrictive, and all the claims have to be clinically demonstrated.

Concluding, there are not specific reports describing the dimension of blue biotechnology in cosmetics. Main information could be found in studies on macro- and microalgal biofuels and on bio-based chemicals of marine origin.

3.2 Pharmaceuticals

The long-term outlook for the pharmaceutical sector confirms to be positive, previewing an average annual growth of 4.3% during 2015–2019 and reaching by 2019 a global sale of about 1.4 trillion dollars. Brazil, Russia, and China represent nowadays 50% of the global pharma revenue. For these countries, however a reduction of health-care budgets is foreseen. Main factors driving the growth of the global markets remain the aging population, the rise of chronic diseases, and the use of innovative and expensive treatments. *Deloitte* report “2016, Global life sciences outlook” highlights how the global pharmaceutical market has been resilient to past economic recessions, although many variables that affect industry dynamics such as the economic uncertainty, the ever-changing regulatory systems, and the increased demand for innovation could still undermine the evolution of the sector. The development of the life sciences industry depends heavily on the general economic trend and on the level of health-care spending decided by each nation, factors that may strongly vary—from developed to developing regions. While the spending in pharmaceuticals, medical technologies, and biotechnology is expected to continue its positive trend, thanks to the increasing demand, the industry margins are being eroded because of evolving clinical, regulatory, and competitive dynamics.

Biotech drugs represent in 2014 the majority of the top 10 pharma products by sale, including monoclonal antibodies and recombinant compounds. Biotech drugs are projected to grow by 2019, reaching 445 billion dollars in value [29].

The falling R&D productivity and the “patent cliff” tend to affect the revenue of research-based companies, even though the results among firms are uneven. The forecast for R&D expenditures previews a steady growth from 2013 to 2020 (about 2.3%). The total expenditure for research and development activities will reach the total value of 162 billion dollars. While the USA and European countries still represent the largest investors in research and development activities, the rapid growth of emerging markets such as Brazil and China is contributing to the relocation of research activities in those countries [30]. Particularly, China is likely to become the next hotspot for innovation. Many are the global companies that decided to establish research units there: *AstraZeneca*, *Eli Lilly*, *GSK*, *JNJ*, *Novartis*, *Roche*, *Sanofi*, *Bayer*, and *Merck*, among others [31].

Gautam and Pan [31] analyze the key trends that impacted pharmaceutical companies in the period 1995–2015, in order to understand the main changes that characterized the sector in the last 20 years. In the second half of the 1990s, an intense wave of mergers and acquisition reshaped the competitive environment in the drug industry (*Astra* and *Zeneca*, *Ciba-Geigy* and *Sandoz*, and *Sanofi* and *Aventis* are some examples). Although the process of mergers is still going on today, some companies decided to reorganize their activities: *Abbott* split in two (*Abbott* and *AbbVie*); *Novartis* and *GSK* cut consumer health and vaccines branches; *Bristol-Myers* Squibb divested the medical devices, nutrition, and multiple therapy areas to focus on oncology, cardiology, and virology. The purpose was to invest on their knowledge strengths, outsourcing to other organizations all noncore activities.

Another trend highlighted by Gautam and Pan [31] is the shift of big pharmas: from developing primary care and small-molecule medicines to specialty drugs and biologics targeted for high-unmet medical needs. While large companies missed the biologics wave at the end of the 1990s, targeted acquisitions in the period 2005–2015 allowed the transit to the antibody, protein, and cell therapies (such as Sanofi-Genzyme, Roche-Genentech, Pfizer-Wyeth):

> Pharma and biotech industries have traditionally been wary of natural products, seeing them as complex, unstable, difficult to isolate and characterize, and having variable potency. New and growing interest from Big Pharma is a route to lower cost drugs as they are a potential source of more effective therapeutics because they may provide a greater interaction with cells and pathways than the traditional small molecule "lock and key" mechanisms, where one molecule targets one receptor. Andrew Mearns Spragg, Aquapharm Chief Technical Officer[16]

In marine biotechnology, Molinski et al. [32] highlight how big pharmaceutical companies declined their participation during the 1990s, missing research and development niches, then exploited by entrepreneurial scientists, mainly in collaboration with companies. The development in analytical technology, spectroscopy, and high-throughput screening (and the partial failure of combinatorial chemistry in delivering new drugs) raised a new interest on blue biotechnology in those recent years.

According to the last update published by professor Mayer of Midwestern University (April, 2016), seven molecules of marine origin were approved by FDA (cytarabine, vidarabine, ziconotide, mega-3-acid ethyl esters, eribulin mesylate, brentuximab, and trabectedin, ordered after their approval date). Four compounds are in *phase III* trials, while 8 are in *phase II* and 12 candidates in *phase I*.[17] No estimate on the status of compounds in preclinical testing is given. Mayer et al. [33] found in the period 1998–2006 in the global marine preclinical pipeline 592 compounds that showed antitumor and cytotoxic activity and 666 additional chemicals demonstrating a variety of pharmacological activities. This leads to an increase in the

[16]Cited in Elvidge S. [1], "Bioprospecting at the poles", Chemistry and Industry, Natural products, pp. 17–19.

[17]http://marinepharmacology.midwestern.edu/clinPipeline.htm

number of natural products in the clinical pipeline (phases I–III), from 13 to 31, in the period from 2010 to 2016, testifying an important increase and a promising trend for the years to come. Glaser and Mayer [34] describe this phenomenon as "marine pharmacology renaissance," asking major opinion leaders which variables might have hampered or enhanced the development of natural products. Driven by the academic community and the sporadic involvement of pharmaceutical companies, the more collaborative approach between industrial sector and academic world, together with scientific and technological advancements, paved the way for a rebirth of natural and specifically marine compounds. The topic will be further analyzed in the "discussion" section, highlighting the important number of firms that entered the marine pharmaceutical industry.

Box: "Converting Compounds Derived from Nature to New Drugs. The Scientist Profile of Phil Baran"

Scripps Research Professor Phil Baran is driven by the desire to create, the exhilaration of discovery, and ultimately saving lives. At an age when most researchers' careers are just ramping up, the 32-year old is already a standout in the field of organic chemical synthesis, making great strides in developing innovative ways to synthesize chemicals well. His laboratory's work has already advanced an array of biomedical studies by synthesizing promising potential drugs and creating new chemicals.

As the candidate drug pipelines at major pharmaceutical companies are drying up, it could be increasingly important to explore new compounds derived from nature as potential new drugs. A range of drugs from aspirin to the widely used cancer treatment *Taxol* have been discovered in nature, but the complexity of producing natural products has made some companies reluctant to focus on them.

"Natural products have an unparalleled track record in the betterment of human health," said Phil. "In fact, 9 of the top 20 best-selling drugs were either inspired or derived from natural products. Even the best-selling drug of all time, Lipitor, was based on a natural product lead—and it has helped prolong many lives."

Phil and his team have developed new techniques that dramatically reduce the time, complexity, and cost of synthesizing natural products with pharmaceutical potential. Their work dislodges previously entrenched beliefs in the organic chemistry field about how such products must be produced and could help to advance and expand the use of natural products in drug discovery programs.

"There is this far-ranging and damaging perception that natural products are too complex to be used in a drug discovery setting despite their overwhelming track record in medicine," said Phil. "I think if our work has helped in even a small way to revive the use of natural products, then we've served our purpose."

(continued)

"We focus on inventing new science and exploring uncharted realms of chemistry," said Phil. "That's the idea. That is our passion."

Target molecules often show great promise but come from sources that would be difficult or impossible to collect in sufficient quantities to support initial studies of their efficacy as disease treatments, much less in the quantities needed to support clinical trials. Short of decimating a marine ecosystem, researchers can typically only isolate milligrams of a given natural product from marine species. In contrast, Phil's work often leads to the production of gram quantities of these products (Fig. 8.8). In some cases, other research groups have been able to reveal a product's medical bioactivity only because he has produced the synthetic product.

Phil has collaborated with *Genentech* on compounds isolated from a sea squirt, or marine tunicate, showing potential for killing colon cancer cells in laboratory experiments. The natural supply of these molecules, known as the *haouamines*, was drained, and only through total synthesis could the biological activity be explored.

He and his colleagues, through some inventive steps, also established the first technique to synthesize *kapakahines*. These marine-derived natural products isolated from a South Pacific sponge in trace quantities have shown antileukemia potential. Phil's group synthesized *kapakahines* in large quantities, more than a decade after their discovery. With supplies now in hand, and unlimited production potential established, research on the compound can proceed and may eventually lead to new drug treatments. This is currently being pursued with pharma giant *Bristol-Myers Squibb*.

His team of scientists has also developed an inexpensive and in many ways astonishing new method for economically producing a pharmaceutical steroid. The molecule, called *cortistatin A*, which was isolated in 2006 from a marine sponge discovered over 100 years ago, has shown huge promise for treating conditions ranging from macular degeneration to cancer. The achievement marked the finish line in a race that saw numerous research laboratories working to accomplish the feat. Synthetic chemistry, says Phil, is something like mountain climbing. Except rather than simply reaching a new peak that nobody has climbed before, you are also inventing new harnesses, ropes, and climbing tools as you go. And you are finding ways to ascend with fewer steps than anyone has ever done before.

"It's very difficult and very rewarding," said Phil. "You have to develop new tools as the dynamics are constantly fluctuating. Invention is a prerequisite for success."

Source: Extract of "Scientist Profile—Phil Baran. Converting compounds derived from Nature to new drugs," Scientist Profile, The Scripps Research Institute, www.scripps.edu.

Fig. 8.8 Sirenas bringing in samples from the deep sea (The activity of sampling was the result of a joint expedition with *Chapman Expeditions* and the *Carmabi Research Station*. Source: Barry Brown/ Coral Reef Photos. Available at: http://www.smithsonianmag.com/innovation/will-next-big-cancer-drug-come-ocean-180961354/)

3.3 Food, Feed, and Nutraceuticals

KPMG, one of the largest global consulting companies, in [35] released an investigation on the nutraceutical sector: *Nutraceuticals: The future of intelligent food*. The purpose of the report was to describe the emerging industry, to present main products, and to explain the dynamics the sector will have to face and their basic logics. The firm defines nutraceuticals as food products that have a medicinal benefit. Specifically, KPMG considers functional foods (such as vitamin-enriched products), nutritional supplement, sport drinks, and medically formulated foods. A wide range of products that spans from sports supplements to prescription drugs for patient with serious medical conditions.

As underlined in the report, nowadays, functional foods represent a big opportunity, being the global market predicted to reach a value of 250 billion dollars in 2018. The market has surged in recent years and is today (2015) five times larger than the total market for nutraceuticals in 1999.

One main factor influencing the development of the sector would be, according to the *Frost and Sullivan* Report of 2011 (together with Indian Federation of Chambers of Commerce and Industry), the increased life expectancy at a global level. The trend has led to an increase in the incidence of lifestyle (age related) diseases such as

diabetes, high blood pressure, high cholesterol and obesity, among others. As a result, there has been a significant increase in the deaths due to lifestyle diseases worldwide. Consumers are seeing nutraceuticals as a path to a healthy lifestyle that will keep these diseases at bay, leading in turn to an increase in nutraceutical consumption. Still, *Frost and Sullivan* Report [36] underlines that consumers' preference is shifting from synthetic ingredients to natural and organic foods, beverages, and supplements. Consumers are moving toward food products obtained from natural extract, non-GMO (genetically modified organism). Specifically, the average consumer is described as more informed, looking for the latest scientific breakthroughs in health and wellness.

Microalgae, in particular, despite their higher production costs, show better quality compared to synthetic and traditional alternatives. Enzing et al. [37] underline that in the short run, microalgae do not represent an effective competitor in the market of carbohydrates and proteins. The microalgae sector would need higher production volumes and a substantial reduction of production costs in order to get such a level. Mature markets for microalgae refer to products such as *spirulina* and c*hlorella*, sold as dietary supplements by a relatively large number of producers. Those algae do not go through a real production process; they are simply dried. All main production countries are concentrated in Asia and the USA. A separate argument can be developed for high-value components from microalgae. Concluding, in the DG Maritime Affairs and Fisheries report [21], the authors stress that, although macro- and microalgae are the primary sources for biotechnological applications in the food sector, there are many other functional ingredients of marine origin such as cyanobacteria, fungi, crustaceans, and fish wastes. Enzing et al. [37], by analyzing high-value food additives, found out that Martek's (now DSM) algae-derived DHA can be found in 99% of all food products made for the infant segment. As it will be highlighted later on in the discussion section, many of the companies mentioned in the KPMG report are now producing or developing products of marine or freshwater origin.

Box: "*DSM* and *Evonik*: A Joint Venture for Omega-3 Fatty Acids from Natural Marine Algae for Animal Nutrition"

Royal DSM and *Evonik* announced their intention to establish a joint venture for omega-3 fatty acid products from natural marine algae for animal nutrition. This breakthrough innovation will enable the production of omega-3 fatty acids for animal nutrition without using fish oil from wild caught fish, a finite resource. *Evonik* and *DSM*'s alternative omega-3 source is the first to offer both EPA and DHA and will be aimed at initial applications in salmon aquaculture and pet food.

DSM Nutritional Products and Evonik Nutrition & Care will each hold a 50% share in the joint venture and co-own the production facility, which will be built at an existing site of *Evonik* and is expected to come on stream in 2019.

(continued)

The joint venture plans to invest around US$200 million in the facility (USD 100 million by each party over circa 2 years). The initial annual production capacity will meet roughly 15% of the total current annual demand for EPA and DHA by the salmon aquaculture industry. The setup of the joint venture, to be named *Veramaris* and headquartered in the Netherlands, will be finalized subject to regulatory approvals and other customary closing conditions.

Evonik's and *DSM*'s highly concentrated algal oil is a high-value and pure source that will enable the animal nutrition industry to keep up with the increasing demand for these two essential omega-3 fatty acids without endangering fish stocks, contributing to healthy animal nutrition as well as to the ecological balance and biodiversity of the oceans.

The joint venture follows the joint development agreement, signed in July 2015. Under this agreement, *Evonik* and *DSM* have jointly worked on the development of products and the manufacturing process and explored opportunities for commercialization. Both companies achieved positive results in the development of the product while extensively working with the entire value chain, including fish feed producers, fish farmers, and retailers.

Under the joint development agreement, *DSM* and *Evonik* have successfully produced pilot-scale quantities of the algal oil at *DSM*'s production facility in Kingstree, South Carolina (USA). The successful product and process development was only possible thanks to the complementary competencies that *Evonik* and *DSM* bring to the collaboration: *DSM* has expertise in the cultivation of marine organisms including algae and long-established biotechnology capabilities in development and operations; *Evonik*'s focus has been on developing industrial biotechnology processes and operating competitively large-scale manufacturing sites for fermentative amino acids.

The algal oil from *DSM* and *Evonik* means that the vision of salmon farming without using fish-based resources is—for the first time—becoming realistic. By replacing fish oil in salmon feed with this EPA and DHA rich alternative, the fish-in-fish-out ratio could be reduced significantly. This alternative will enable the aquaculture industry to continue to grow sustainably. *DSM* and *Evonik* are also pursuing applications of their algal oil for other aquatic and terrestrial animal species.

Source: extract from https://www.dsm.com/corporate/media/informationcenter-news/2017/03/2017-03-08-dsm-and-evonik-establish-joint-venture-for-omega-3-fatty-acids-from-natural-marine-algae-for-animal-nutrition.html

3.4 Energy

> It is interesting to see how development has progressed. Unfortunately, I don't think a lot has happened in the bioreactor field in 10 years. There is a lot of development work to be done and many companies have gone bankrupt in the process. I think there is a lot to learn from them. Aina Charlotte Wennberg, NIVA[18]

The US Energy Information Administration (EIA) forecasts an important increase in oil demand in the near future, passing form 90 million barrels per day in 2014 to almost 120 barrels per day by 2040. It is to underline that the price of petroleum has constantly declined from August 2014. The drop in oil price created great uncertainty about the fossil fuel market, for reasons spanning from geopolitical questions to technical and environmental factors. While global petroleum reserves continue to be depleted and environmental issues keep emerging (global warming and ocean acidification due to greenhouse gas emissions), oil consumption heavily depends on nonrenewable resources [38]. The future energy needs of developing countries such as India and China, driven by changes in lifestyles and by an increase of the middle class, represent the primary motivation for the increasing demand for fuels.

The International Energy Agency [38] underlines how, despite the incredible improvements reached in algae production in those last years, commercial algae-based bioenergy or biofuels production is more challenging nowadays than it was in 2010. The constant reduction of the oil price, in fact, made algae-based energy sources uncompetitive—compared to other sources (primary fossil fuels). Energy companies living at the edges of science, as a consequence, diversified their production starting to face other sectors. One of them is high-value food, feed, and specialty products. Probably, in the near future, only the bull increase in oil price could open the way to commercialization of algal biofuels. Even though algae-based technology demonstrated good results at laboratory scale, industrial processes still need to be developed.

Even if several assessments exist, it is estimated that there are more than 30,000 species of algae in the world. While around 100 are well-known, only 15–20 algae species are actually used for fuel or other substances production [39]. This is interesting evidence; it makes clear the vastness of competition dynamics among producers along with the wide possibility of choosing different algal strains for different production conditions. Runge [39] underlines that in 2009 there were about 100 algae biofuel start-ups in the USA and 200 algae companies were estimated at a global level:

> When the biotechnology revolution began, big pharmaceutical companies understood nothing about biology. They began their research and development projects and all failed. They could never invent new products, because they were not entrepreneurial. Nimble biotech firms such as Genentech showed how to do that (before it was purchased by Roche). As

[18]Quoted in "Thinking Blue: Addressing Today's Challenges with Marine Biotechnology" (2012), BlueBio Project Report.

yesterday, companies of the bio-energetic sector will show giant companies the way to produce earnings from alternative fuels. Ed Legere, co-founder of Algenol Biofuels[19]

3.5 *Bio-Based Chemicals*

The *International Energy Agency* (IEA) Bioenergy report [40] estimates the global bio-based chemical production (excluding biofuels) at 50 million tons. The bio-based products in the report are defined as chemicals and polymers produced from renewable resources, biomass from dedicated production, or residues from forestry, agriculture, aquaculture, and other industry and domestic sources.

The production of bio-based chemicals could generate, according to the authors of the report, a 10–15 billion dollar revenue for the global chemical industry. OECD [41] underlines that biochemicals will constitute 10% of the chemical market by 2015. Particularly, polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) are commercialized polymers with an annual growth of about 10–30% [40]. Still OECD [41] previews that specialty chemicals such as adhesives, surfactants, and solvents would constitute 60% of the total value of all biotech-based chemical production by 2025.

Examples of bio-based products are nonfood starch, cellulose fibers and derivatives, tall oils, fatty acids, and fermentation products as ethanol and citric acid (see Fig. 8.9). The US Department of Agriculture defines bio-based products as both commercial and industrial products that are composed entirely, or in significant part, by biological products or renewable domestic agricultural materials or forestry products (USDA 2006).

While the majority of organic chemicals and polymers are still derived from fossil-based feedstock (oil and gas predominantly), the report brings up that all industrial materials, from a technical point of view, could be substituted by their bio-based counterparts. Of course, the greatest problem is still the production cost of bio-based products, which still exceeds the cost of petrochemical production. Main drivers of the sector would be the oil price trend and the consumer demand for environmental-friendly products. While biochemicals and materials can be produced in single-product processes, the report emphasizes the efficiency of any integrated biorefinery process, this approach endorsing a sustainable valorization of biomass resources in the production of both bio-based products and secondary energy carriers (fuels, power, heat). The algae oil technology platforms show several advantages:

1. Higher productivity as a result of the use of the entire biomass in contrast to plants
2. No land constraints, resulting from the possibility to grow algae in seawater or nonarable land (not competing with conventional agriculture)
3. Freely available elements for the growth of the biomass (sunlight, water, CO_2)

[19]"Going Commercial. Algenol boosts yields, cuts costs for biofuel production", Jerry Perkins, Biofuels Journal, 2° trimester 2014.

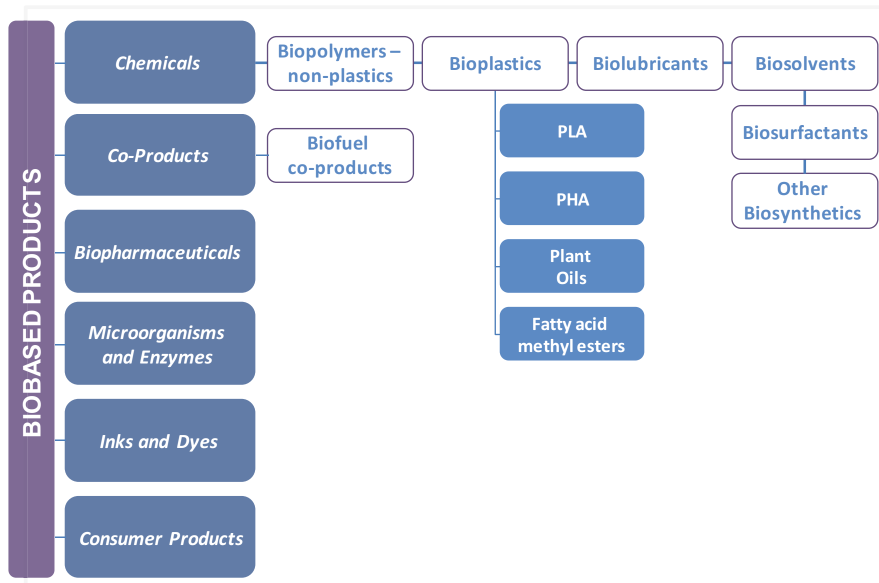

Fig. 8.9 A bio-based products representation. Source: adaptation on [42]

Today the production capacity is still too limited in comparison to land-based energy crops. The microalgae production is mainly devoted to the extraction of high-value products. Technology needs to be improved for both cultivation and biorefinery (in Table 8.1 a representation of main products from algae biochemical components).

In the *Study in support of Impact Assessment work on Blue Biotechnology* [21], the wide range of potential applications that bio-based chemicals could play is stressed, also in the context of protection of the marine environment. Applications include biofouling control, environmental monitoring, marine habitat restoration, bioremediation, and natural resource and environment management. The authors highlight the infancy stage of development of blue biotechnologies in the marine environment and the huge potential of products that could definitely be used to improve the environmental health of marine ecosystems.

4 The Analysis of a Sample of Firms

4.1 Methodology

The authors developed a dataset of blue biotechnology firms. They considered the firms with research and production processes based on marine and limnic organisms and compounds, according to the OECD [41] definition previously discussed.

Table 8.1 Bio-derived products from algae biochemical components

Feedstock	Wt (%)	Product	Market size
Fatty acids	10–45	Hydrocarbon fuel products	5,000,000
Omega-3 fatty acids	3–6	Polyols	11,000,000
	3–6	Polyurethane	11,000,000
	3–6	Nutraceuticals	22,000
Hydroxy fatty acids	≈1	Surfactants, fuel additives	3,500,000
Branched chain fatty acids	≈1	Surfactants, fuel additives	3,500,000
Fatty alcohols	≈1	Surfactants, fuel additives	3,500,000
Sterols	2–4	Surfactants/emulsifiers	2,000,000
	2–4	Hydrocarbon fuel products	5,000,000
	2–4	Phytosterol nutra/pharmaceuticals	25,000
Phytol	3–4	Raw material for vitamin E, fragrance	1
	3–4	Surfactants, fuel additives	3,500,000
Polar lipids	10–35	Ethanolamine	600,000
	10–35	Phosphatidylcholine, phosphoinositol, and phosphatidylethanolamine (lecithin)	20,000–30,000
Glycerol	2–6	Di-acids for nylon production	2,500,000
	2–6	Feed, pharmaceuticals	25,000
Fermentable sugars (glucose, mannose)	10–45	Polylactic acid (PLA) polymers	300,000
	10–45	Di-acids (e.g., adipic acid)	2,500,000
	10–45	Ethanol	68,000,000
Mannitol	3–6	Polyether polyols	2,300,000
Alginate	≈3–5	Alginate additives	12,000
Starch	5–40	Polysaccharide-derived bioplastics	2,000,000
Protein	19–40	Thermoplastics	5,000,000
Amino acids/ peptides	19–20	Polyurethane	11,000,000
Amino acids/ peptides	19–20	Biobutanol, mixed alcohol fuels	40,000,000

Source: IEA Bioenergy Report [38]
Biomass composition shown as wt% of dry biomass ranges, based on observed, literature-reported, or measured. Market size based on HIS report on sorbitol. For more information: http://www.ieabioenergy.com/wp-content/uploads/2017/01/IEA-Bioenergy-Algae-report-update-20170114.pdf

Specifically, the authors chose not to include in the analysis the companies concerned in aquaculture, for in that case there is not an effective raw material transformation. The aim of this investigation is to draw up a list of firms that can give an overlook of the blue biotechnology fields: on the one side, geographically widening the spectrum of analysis and, on another side, trying to consider all the industries interested in the commercial applications of marine and limnic biotechnologies.

Greco and Cinquegrani [8] presented a first investigation of the organizations related to blue biotechnology. The dataset of companies described in that analysis, along with the sources needed for its development, has been improved and updated to January 2017.

The authors used a desk-based research approach, as the data were built mainly on secondary sources of information [43]. Purposely, they followed a three-step process to gather the different information on the organizations involved in the study.

In the initial phase, the authors start an investigation on the scientific and institutional literature concerning marine natural products. The aim was to have several reliable sources in order to build a list of companies interested by this analysis.

They analyzed several studies on single organisms and bioactive compounds; they collected reports describing the blue biotech sectors at a national and local level; they examined the marine pharmaceutical clinical pipeline as described by the Midwestern University (see [44–49]; Atlanpole BlueCluster, 2009; [12, 50–60]; IEA Bioenergy Report [38][20,21,22,23,24,25,26,27,28]).

As many of the investigated companies participated to international financed programs, the authors considered also the firms cited as partners in research projects related to marine biotechnology in the Sixth and Seventh Framework Programs of the European Union (see Table 8.2).

The need to include companies based in non-European countries pushed the authors to search for private organizations participating to International Marine Associations. In particular, they collected the business members of EABA (European Algae Biomass Association), Algae Biomass Organization, NAABB (National Alliance for Advanced Biofuels and Bioproducts), Japanese Society of Marine Biotechnology, Pan-American Marine Biotechnology Association, and ESMB (European Society for Marine Biotechnology).

Another meaningful source of information relies upon international trade fairs and international organizations summit participants. Trade fairs always host companies which are active on different markets, looking for new clients, new partnerships, and

[20] La bolla delle alghe, 9 aprile 2009, Nova, Sole24Ore. http://marinepharmacology.midwestern.edu/clinPipeline.htm

[21] Defined by the sea: Nova Scotia's Ocean Technology Sector present and future (2010).

[22] Thinking Blue: Addressing Today's Challenges with Marine Biotechnology (2012), Blue Bio Report.

[23] Strategy for the marine biotechnology cluster in Tromso 2012–2015, Biotech North.

[24] Seeing Purpose and Profiting Algae, New York Times.

[25] BiomassAlternativeEnergy.Availableonlineat:www.prezi.com

[26] Keywords were marine biotechnology, marine bioactive compounds, and marine biomaterial (firm, company).

[27] What Happened to Biofuels (2013). The Economist, Technology Quarterly n.3, 7 September.

[28] http://marinepharmacology.midwestern.edu/clinPipeline.htm

Table 8.2 European Framework Programs related to marine biotechnology

EU Sixth Framework Program	EU Seventh Framework Program	
• FISH&CHIPS	• SeaBiotech	• Sunbiopath[a]
• MARBEF[a]	• PharmaSea	• Marine Fungi
• Marine Genomics[a]	• BlueGenics	• SPLASH
• HERMES	• Macumba	• Bioclean
• SPONGES	• MicroB3	• ULIXES
• AquaBreeding	• AtSea	• NatPharma
• Aquafunc[a]	• MyOcean2	• CoreShell
• Biodiversa[a]	• Jerico	• BEADS (SME)
• ERATS	• Moose	• ERA -NET MB
• EurOceans[a]	• NetAlgae	• GIAVAP
	• Perseus	• MABFUEL
	• Pegasos	• MAMBA
	• Gateways	• ColorSpore
	• ALGADISK	• Special
	• Meece[a]	• FishPopTrace
	• CSA Marine Biotech[a]	• BioWalk4BioFuels
	• KEOPS[a]	• Marex
	• MARMED[a]	• JPI Oceans[a]
	• Bammbo[a]	• SWAFAX[a]
	• Polymode	• HYFFI

[a]Just *Public Research Organizations* (PROs) as partners

new collaborations. Principally, the authors considered organizations participating to BIT's 4th and 5th Annual World Congress of Marine Biotechnology (2015 and 2016), Algae Biomass Organization Summit 2014, Biomarine International Cluster Business Convention 2014, and CSA Marine Biotech 2013 ERA-NET.

A free research on the Google search engine concluded the first part of the investigation.[29] The activity led the authors to consider different companies lists associated with producers, association, and online magazines.[30]

All the companies mentioned in reports, scientific papers, trade fairs list, and other sources were considered for the analysis. Of course, different organizations were cited by more than one source. In the second phase of the research, the authors tried to understand if the firms collected in the first list belonged effectively to the blue biotechnology sector as it has being portrayed earlier in the text. Through the investigation of all the official websites, they meant to understand if and how those companies operate inside the marine biological and biotechnological sector. Many companies, in fact, work in related fields and support sectors, but do not have research or production units using marine or freshwater organisms. Here are some

[29]Keywords were marine biotechnology, marine bioactive compounds, and marine biomaterial (firm, company). The authors checked all the results of the first 15 pages.

[30]www.environmental-expert.com, www.biomarine-resources.com, Algae fuel producers on en.wikipedia.org, www.cleantick.com/companies, www.ethanolproducer.com, biopharmguy.com, biomarine-resources.blogspot.it, www.oilgae.com, www.seao2.com/algaebiofuels, www.algaeu.com

examples: tools for the aquaculture industry, research instruments, marketing consulting, distribution of goods and services, and technology and products developed originally in other organizations. When the information was not easy to find (for poor description of the activities, for the wide content of the products and services offered by the company), the authors relied on other sources, mainly newspaper articles and press releases.

In the third and last phase of the analysis, the authors decided which variables to consider for the investigation and collected the data for every single company. Specifically, information gathered is:

- *Business name*
- *Head Office* (city, state)
- *Websites URL*
- *Year of foundation*
- *Number of patents*
- *Short description of the products or services offered*
- *Markets served*
- *Multinational corporation (MNC)*
- *Spin-off*
- *General notes*

The data concerning the firms found their primary source on the official website of the company. The information on the number of patents has been obtained through *Espacenet*, a service offered by the *European Patent Office*. The online system of research gives free access to more than 90 million patent documents (mainly applications)—since 1836 to date.[31] The authors structured their research by applicant (the one who applies for a patent and holds the rights deriving from it), trying to get an idea of the dimension of patents' portfolios of each company.[32]

The markets served have been classified following the industries classification of the European Science Foundation of 2010 and represented by Thorndyke in the EMBRC Business Plan 2013 (Fig. 8.4, previously explained). The "human health and well-being" entry, anyway, has been subdivided into pharmaceutical, nutraceutical, and cosmetic sector, considering both the significant dimension and the different characteristics of the companies of each industry. Finally, the aquaculture industry has not been entirely considered: only research services and products conceived for the industrial sector were taken into account.

The companies' dynamics are not easy to follow, as many firms went through acquisition, merge, spin-off, and other financial and organizational processes. The authors tried to keep information updated, even though it was not the central goal of

[31] https://worldwide.espacenet.com/

[32] Patents' portfolios could be, in some cases, undervalued. For Asian companies this could happen because of the non-perfect dialogue between the patent systems. In the case of start-ups and spin-offs, this could happen because the patents could be registered with the name of the inventor, not by the firm itself (so you lose the information).

this investigation. Furthermore, many of the firms have a multinational field of production and competition and quite a noticeable size, so the authors used the classifications concerning the world's largest companies made by *Forbes* (2016) and *Fortune* (2014) to convey the idea of the global players included in the dataset.[33] Finally, several organizations of the sample were born within scientific institutions or private research centers, so the authors also kept record of firms defining themselves spin-offs (both academic and industrial).

Without certified statistical classifications and other knowledge bases (national and international), there are several limits to the data collection. As the survey has been developed in English language, information concerning small firms, organizations having an initial local market, and, above all, companies from China and other Asian countries has to a large extent been omitted (or was extremely difficult to gather). Although the research attempts to be inclusive and to consider all different production realities, it was mainly based on reports and documents realized for countries belonging to the European Union.

4.2 Discussion

Through a multiphase methodology, the authors built a sample of 544 companies using organisms of freshwater and marine origin in both research and production activities. The size and the extent of the output is a first result of this kind of investigation. As underlined earlier, in fact, there is no evidence about the dimension of the blue biotechnology sector.

Although the sample makes mention of several different types of organizations, a wider spectrum of firms emerges than initially hypothesized. Of course, the research wants to represent a first point of departure, helping to create a new basis for the comprehension of the main dynamics that affect the marine industries.

Specifically, the countries represented in the investigation are 41. Of those, 24 nations show more than three marine organizations in the national economic systems. As it can be noticed in Fig. 8.10, European countries with *275* companies represent more than one half of the total sample. The USA with 32% (171) is today the largest country for number of firms, followed by Canada with *23* companies and India, Israel, and Japan with *12*.

In the USA, specifically, an important percentage of firms (23%, representing 39 companies) chose to establish their headquarters in California, while 11% (18 companies) are based in Massachusetts. At least 10 other states show a number of firms ranging between 5 and 11 (Colorado, Illinois, Hawaii, Texas, New Jersey, Arizona, Ohio, New York, Florida, and Washington).

[33] http://www.forbes.com/sites/steveschaefer/2016/05/25/the-worlds-largest-companies-2016/#e3465fdc5524 and http://beta.fortune.com/global500/list

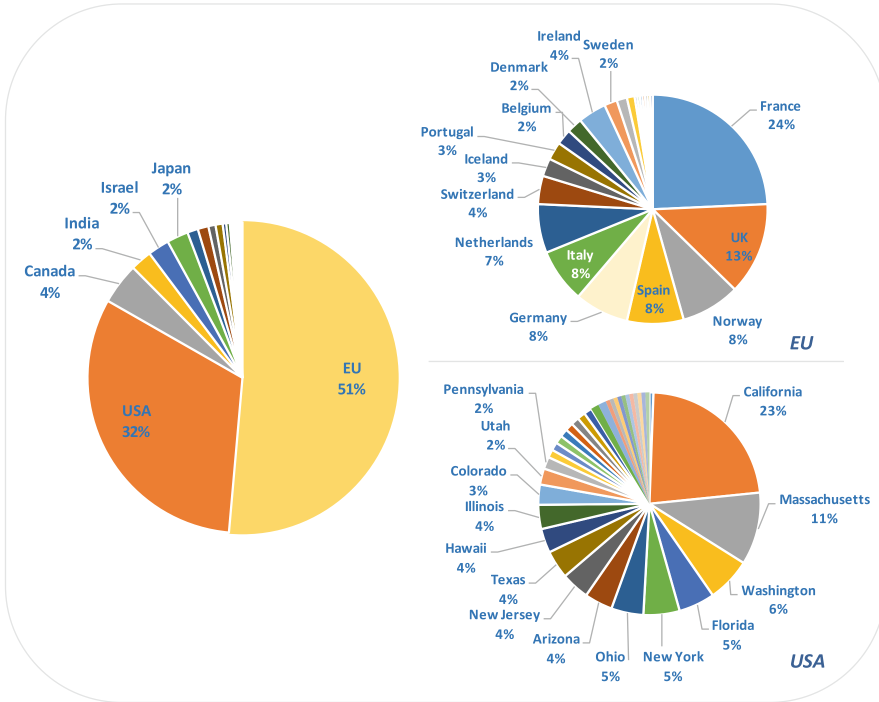

Fig. 8.10 The geographical dimension of the sample (Australia, China, New Zealand, Taiwan, Korea, Mexico, Argentina, Russia, Malaysia, Singapore, South Africa, Thailand, and Uruguay are not represented, having a total percentage equal or lower than 1%)

Among the European countries, France holds 24% of all EU firms (67 companies), followed by the UK with 36 organizations (13%). Norway, Spain, Germany, Italy, and the Netherlands have almost the same number of companies (23, 22, 21, 21, 19 each), followed by Ireland, Switzerland, Portugal, and Iceland (11, 10, 7, 7 each). As stated earlier in the text, following the organizational and financial history of the single companies wasn't an easy task for the authors. The marine industrial sectors seem to be characterized by a certain degree of instability, due to the overweight of small and young companies. Out of 544 organizations, 60 companies (about 11%) are no longer active on the markets. Sixteen of those have been acquired by other organizations; in other cases mergers occurred or reorganization of the activities entailing company name change. For instance, *Ocean Nutrasciences* acquired in 2010 *ABK-Gaspesie*, a spin-off of the University of Québec; in 2013, *Aquapharm Bio-discovery* (under bankruptcy) has been acquired by *Lallemand*; in 2010, *BASF* became the owner of *Cognis*, while in 2013 they bought *Pronova Biopharma* and *Verenium Biosciences*. There are also several cases of joint ventures: *British Petroleum* and *DuPont* gave birth in 2009 to *Butamax Advanced Biofuels*; *Royal Dutch Shell* and *HR Petroleum* established *Cellana* in 2007.

As evidenced in the description of the trends shaping the single industries, it is clear how firms follow the paths of evolution of the industry to which they belong, like it occurs in the pharmaceutical and cosmetic sectors.

Another distinctive feature of many companies is their association with national and international groups. The authors accounted 75 organizations belonging to financial and industrial groups (almost 14%). That's worth to mention—as it could explain the availability of financial resources faced by certain firms and their efficacy in finding markets for the technology they develop. The companies that were born into or became part of an industrial group have a market power that newborn companies will need a long time to build:

> With a few notable exceptions, most industrial contributions to Marine Biotechnology in Europe are generated through specialized Small and Medium-sized Enterprises (SMEs). These small companies assume most of the risks inherent in RTD in a highly unstable economic environment and are characterized by a rapid turn-over. ESF ([61], p. 31)

In the in-depth analysis of the dataset, the authors found 50 companies that, according to the classification of *Forbes* (2016) and *Fortune* (2014), can be defined as global players in their specific business (see Table 8.3). The multinational corporations in the dataset are active in all the different industry segments here considered: energy, cosmetic, food, environment, industrial, and pharmaceutical. Particularly, 28 operate in the pharmaceutical sector (more than one half), 17 in the energy business, 18 in food or nutraceuticals, and 13 in cosmetics. In the cosmetic sector, the authors find about all the biggest players of global level: *Estée Lauder*, *L'Oréal*, *Johnson & Johnson*, *Henkel*, *Unilever*, and others. These companies not only are market leaders but also represent the peak of enormous industrial groups managing an extensive number of brands.

The multinational pharmaceutical companies directly or indirectly engaged in marine biotechnology are *Pfizer*, *Eisai*, *Novartis*, *Eli Lilly*, *BASF*, and *Boehringer Ingelheim*. In the energy sector, giant firms such as *Statoil*, *Total*, *UOP*, and *Royal Dutch Shell* have projects based on macro- and microalgae for energy production purposes. *ExxonMobil* began research collaborations with Synthetic Genomics in 2009, and in 2013 the project was labeled as "basic science research program." The agreement has recently been extended as it can be deducted from the quotations below:

> Together with ExxonMobil, we have made significant strides to identify and enhance algal strains capable of high oil production while still maintaining desirable rates of growth. The extension of our agreement reflects the tremendous progress made to date, and the promise in using our core synthetic biology technologies to build cell production systems capable of

Table 8.3 The multinational corporations of the dataset

MNCs	Country	Year of est.	Patents (N)	Markets served					General notes
3M	USA	1902	53,059	Industrial					
Abbott Lab	USA	1888	25,840	Pharma	Nutra				
AbbVie	USA	2013	5186	Pharma					Industrial spin-off of the Abbott Laboratories
Allergan inc	USA	1948	11,567	Pharma					
Astellas pharma	USA	2005	3994	Pharma					Owner of Agensys
BASF	Germany	1865	100,000	Industrial	Pharma				Kelpak producer (agribusiness)
Beiersdorf	Germany	1882	8464	Cosmetic					LA PRAIRIE is the top brand of the company
BioMarin Pharma	USA	1997	464	Pharma					Industrial spin-off of Glyko Biomedical Ltd. First collaboration with Genzyme for the commercialization of a drug
L'Oreal	France	1909	294	Cosmetic					Biotherm is a brand of the group
Boehringer Ingelheim	Germany	1885	37,712	Pharma					
BP BiofuelS	UK	1908	11,860	Energy	Industrial	Food			
Celanese	USA	1912	24,217	Nutra	Cosmetic	Energy	Industrial		Owner of Nutrinova
Du pont	USA	1802	100,000	Industrial	Pharma	Food	Energy	Cosmetic	Several marine bio-based firms are in the ownership of the company
Eisai	Japan	1941	10,079	Pharma					
Eli Lilly	USA	1876	45,925	Pharma					

(continued)

Table 8.3 (continued)

MNCs	Country	Year of est.	Patents (N)	Markets served					General notes
Estée Lauder	USA	1946	319	Cosmetic					In 2008, the company bought AGI dermatics USA. La Mer is the top brand of the firm
ExxonMobil	USA	1870	22,055	Energy	Industrial				
FMC Corp	USA	1883	22,429	Pharma	Food	Industrial			FMC Corporation of Chicago, Illinois, purchased Marine Colloids Inc. in 1977, creating Marine Colloids Division. Litex A/S manufactured carrageenan
Fuji Chemicals	Japan	1946	4234	Pharma	Nutra				Life Science Division
Genentech/ Roche	USA	1976	19,740	Pharma					A member of the Roche group since 2009
General Atomics	USA	1955	705	Energy	Environment	Industrial			
Genzyme Corporation	USA	1981	3338	Pharma					Sanofi Aventis bought the company in 2011
GlaxoSmithKline	UK	2000	6835	Pharma	Cosmetic	Nutra			The company was born with the merge of different organizations
Henkel KGAA	Germany	1876	59,729	Cosmetic	Industrial				
Jazz Pharma	UK	2003	18	Pharma					
Johnson & Johnson	USA	1886	9556	Pharma	Cosmetic	Nutra	Industrial		Orthobiotech is a member of the group
Kao Germany GMBH	Japan	1887	48,876	Cosmetic	Industrial				

Mead Johnson Nutrition	USA	1905	25	Nutra	Food				Split off Bristol Meter Squibb in 2009
Millennium	USA	1993	6241	Pharma					Takeda Oncology Company bought the company in 2008
Neste Oil Corp	Finland	1948	538	Energy	Environment				Partnership with Cellana
Nestec SA (Nestlè)	CH	1866	35,839	Food					
Novartis	CH	1990	45,445	Pharma					Sandoz is a Novartis Division
Novozymes a/s	Denmark	2000	8339	Energy	Pharma	Food	Industrial		
Perrigo	Ireland	1887	416	Nutra	Pharma	Industrial			The company bought the Irish firm Elan
Pfizer	USA	1849	67,579	Pharma	Nutra				Pharmacia & upjohn and Wyeth owner
Procter and Gamble	USA	1837	100,000	Cosmetic	Nutra	Pharma			
Raytheon company	USA	1922	16,295	Energy	Industrial				
Repsol	Spain	1927	646	Energy	Industrial				
Roche Group	CH	1896	86,115	Pharma	Industrial				
Royal DSM	Netherlands	1902	21,470	Food	Nutra	Pharma	Energy	Industrial	Owner of Ocean Nutrition and Martek Biosciences
Sanofi Aventis	France	1999	19,700	Pharma	Cosmetic				
Royal Dutch Shell	Netherlands	1907	89,572	Energy	Industrial	Environment			
Solvay	Belgium	1863	25,269	Pharma	Cosmetic	Energy	Environment	Industrial	Owner of Rhodia
Statoil	Norway	1972	2344	Energy	Industrial	Environment			In October 2007, Statoil merged with the oil and gas divisions of Norsk Hydro

(continued)

Table 8.3 (continued)

MNCs	Country	Year of est.	Patents (N)	Markets served					General notes
Suez Environnement	France	1880	152	Energy	Environment				
Thermo Fisher Scientific	USA	2006	924	Pharma	Industrial				The company was created in 2006 by the merger of Thermo Electron and Fisher Scientific
Total	France	1924	11,088	Energy	Industrial				
Unilever	Netherlands	1929	65,571	Cosmetic	Food				
UOP	USA	1914	15,285	Energy	Industrial	Environment			Honeywell Group
Veolia	France	1853	915	Environment	Energy	Industrial			

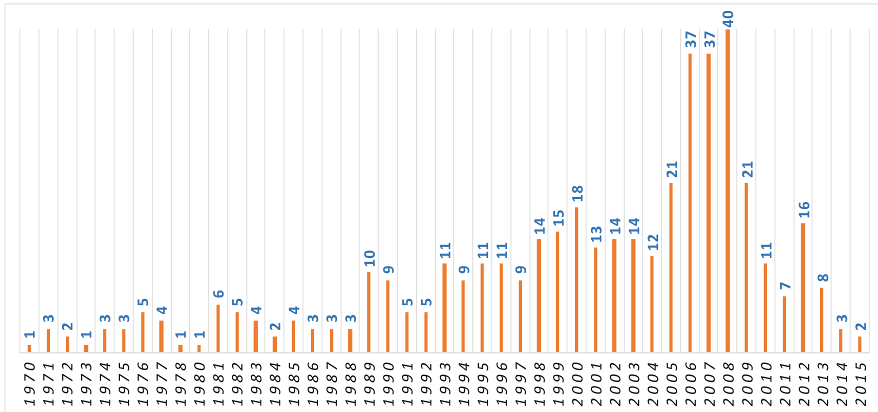

Fig. 8.11 The year of establishment of the firms of the sample

> reshaping industries. Oliver Fetzer, Ph.D., Chief Executive Officer of Synthetic Genomics[34]

> Synthetic Genomics and ExxonMobil remain committed to advancing the scientific fundamentals of algal biofuels. We know this will be a long-term endeavor and are optimistic based on the results we have seen to date. Vijay Swarup, Vice President for Research and Development at ExxonMobil[35]

The investments needed for the upscaling of marine biofuels are, nowadays, massive. Experts of the industry do highlight how MNCs are the sole companies that can afford development projects, from both a financial and temporal point of view.

In those last years, marine compounds received much attention from MNCs. Not only in the pharmaceutical sector blue biotechnology has attracted interest from global companies, since it represents a new technology wave and a new competition ground.

Figure 8.11 shows the trend that shaped the establishment of companies in the marine sector since 1970 to date. Of course, the sample represents a wide variety of firms, where incumbent companies cohabit with newcomers. The organizations founded before 1970 are, actually, 84 (almost 15%), the oldest being *DuPont* and *Lankhorst Yarns*, established in 1802 and 1803.

The biotechnology boom of the 1980s does not seem to have influenced directly the evolution of the blue sector. A consistent process of growth is visible by the end of the 1990s. Anyway, it is only in the second half of the years 2000s that the marine industry reached his peak, with the formation of a consistent number of firms. The years 2006, 2007, and 2008 marked the rise of 37, 37, and 40 new organizations,

[34]18th January 2017, Press Release, available at: http://news.exxonmobil.com/press-release/synthetic-genomics-and-exxonmobil-renew-algae-biofuels-research-agreement

[35]Ibidem.

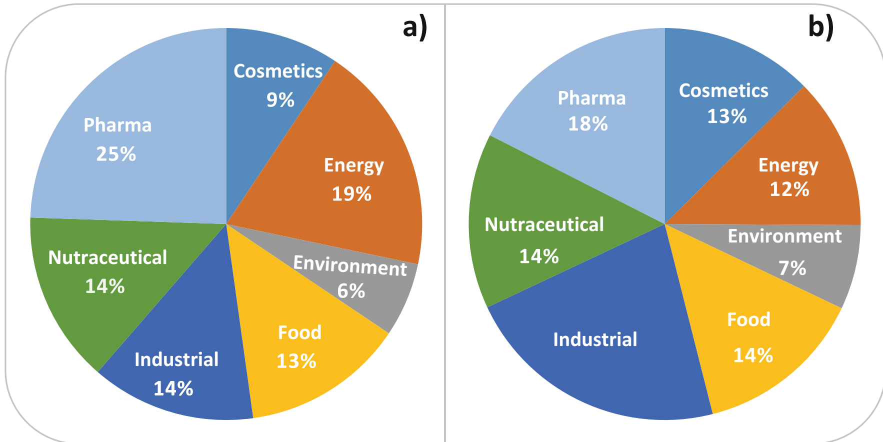

Fig. 8.12 The industries served

respectively (21% of the sample). It is not difficult to link these dynamics to the evolution of molecular biology in that same period of time.

Figure 8.12 clarifies in which industries the companies effectively operate. In particular, Fig. 8.12a represents the first market served by the companies, while in Fig. 8.12b, all the productive sectors mentioned by the firms are considered. It is evident how slightly the dimensions change.

The percentages stay almost constant for nutraceutical, food, and environmental industries. The "industrial" sector reveals an important increase in Fig. 8.12b (peaking to 22%), testifying that many companies do not reach the final consumer, but work on a B2B (business-to-business) dimension. The pharmaceutical industry maintains an important share (18–25%) of the blue markets, although all the sectors are quantitatively significant.

The authors analyzed specifically the industry distribution in the USA and in the European Union (for the first market declared by each firm). As Fig. 8.13 shows, marine biotechnology companies differ in terms of product specialization. While in Europe there is a strong presence of organizations in the cosmetic, in the food industry, these percentages decrease considerably in the USA, reaching 2 and 6% of the total, respectively. More significant percentages, instead, exemplify the pharmaceutical sector—passing from 21 to 32%—and, above all, the energy industry, which in the USA represents 32% of the companies (versus the EU 11%).

The stakeholder analysis led for the Study in support of Impact Assessment work on Blue Biotechnology [21] evidenced that every organization taken into analysis basically tends to focus on more than one specific subsector of blue biotechnologies. Firms and academic groups involved in biological diversity are also simultaneously investigating several application fields (and so it is also for the patents). The investigation of the number of markets served by the companies mentioned in the dataset reinforces the observations expressed by the authors of *DG Maritime Affairs and Fisheries*. As displayed by Figs. 8.14, 29% (151) develop their activities in one

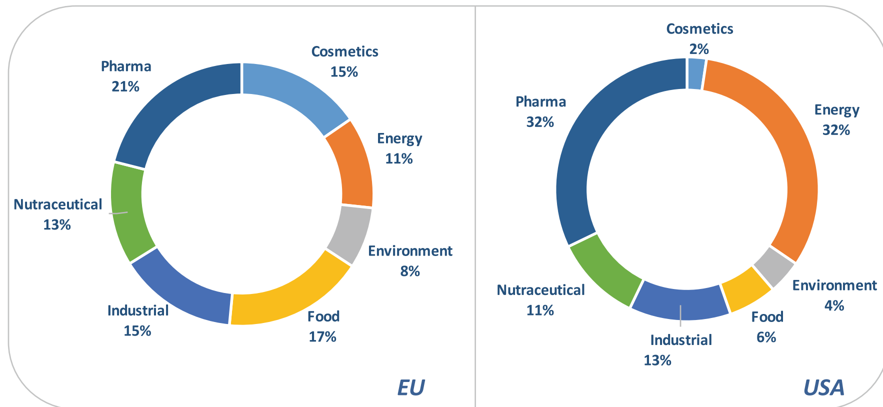

Fig. 8.13 A focus on the industries of the EU and USA

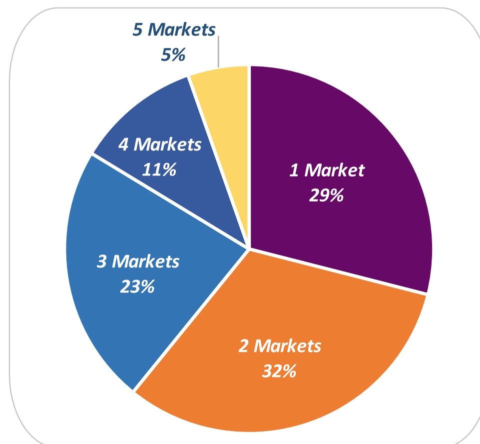

Fig. 8.14 The number of markets served by the companies of the sample

industry, while 71% of the companies work for more than one industrial sector. This is an interesting finding, since it entails that it would be of little relevance to analyze single industries or specific marine compounds, as you would end up excluding a significant part of the dataset. Undoubtedly, in this early phase of development of blue biotechnologies, all extensions of analysis are necessary, in order to understand the dynamics typical of each single business. As stressed in the quotations below, the use of certain compounds could be really wide and stretch across different industries:

> BioLume's business strategy has two broad components; (1) developing and commercializing the medical imaging applications covered by its patents, (2) developing and commercializing the food, beverage, personal care, and cosmetic applications covered by its patents.
>
> These strategies are complementary as the data needed for development of either will benefit both components.[36]
>
> Amadéite, the Human Care Division of Olmix Group, develops algae-based natural ingredients for the food, nutraceutical and human health industries. Amadéite is developing high-value natural organoleptic solutions to meet growing consumer demand for healthier and more natural products. Algae are an outstanding source for food supplements, given their great diversity and rich content in natural ingredients with proven health benefits. With its unique pragmatic approach to exploring new mechanisms of action, Olmix has identified different algal extracts with potential for use in central nervous system (CNS) diseases, oncology, immunity and infection. After only 3 years of pre-clinical and toxicological studies, Olmix started its first clinical study on human CNS disorders in June 2015.[37]

The companies taken into account in the dataset make a variety of strategic choices. While some organizations choose to operate in niche markets as dietary or cosmetic supplements, other firms seem to try to benefit of the research and production advantages related to marine compounds (above all the algae sector). Of course, this is also the result of different levels of development of organism use in those industries. In the energy sector, well-invested companies were born with the aim of producing biofuels. As the way through industrialization and commercialization was long, they also used their research and development achievements to improve products and services for cosmetics and nutraceuticals (among others). In reaching the market, the companies could get several benefits: knowledge about looked-for products and services, visibility, credibility, new funds to invest (securing survival), competitor analysis, and opportunities for collaborations.

Furthermore, the investigation of the firms' activities substantiates the extensive number of companies offering services—mainly consulting—to other organizations. Access to library of marine organisms, laboratory services, harvesting systems for sustainable production, mitigation strategies studies, IPRs, and marketing are some of the service activities deployed by marine organizations. Still, biochemistry emerges as one of the much promising fields of marine compound application: coatings, biocides, cleaners, natural foams, medical biomaterials, and bioplastics are only a few examples of the potential use of blue biotechnologies.

The companies' record also shows the different levels of specialization of their production and service operations. While several firms focus on discoveries or science-based phases, other organizations build their know-how on development, upscaling, and commercial activities. Companies marked by a strong degree of differentiation, therefore, coexist with others, which might be specialized in peculiar kinds of research or specific production phases. In the Study in support of Impact Assessment work on Blue Biotechnology [21], small and medium enterprises (SMEs) emerge as single-focused marine bioactive companies, mainly operating at the initial stages of the product development. The investigation of the companies in

[36] http://www.biolume.net/

[37] http://www.olmix.com/human-care

the dataset reveals a broader universe of firms and brings up how SMEs can be active in all the phases shaping the value chain of marine biotechnology.

Leary et al. [5] document that there is no evidence that private organizations conduct their own in-house bioprospecting with the purpose of industrial research and development. If in the past the collection of marine organisms was evidently supplied by research institutions, the analysis of the core activities of the companies in object suggests now a change in this trend. Many firms are offering access to libraries, collection of marine organisms or cultured species (along with research and consulting services) as core activities.

Still, blue biotechnology companies can choose to develop all the processes composing the value chain, or they can just focus on a specific stage. In the cosmetic industry, for example, some marine firms decided to realize bioactive compounds selling patents and consulting to bigger firms (for the development, production, and commercialization of the products), while others opted for the production of marine compounds for the companies of the beauty sector. Still, some firms keep developing all the phases, reaching the final market with their products; *Algenist* and *Maressentia* could be cited as examples. It is evident that the production of an intermediate product doesn't change the representation of the activities of the firm, but it simply implies that the value chain of the blue biotechnology company precedes the value chain of a downstream firm.

The authors found 81 organizations defining themselves spin-offs and representing approximately 15% of the sample. In economic literature, spin-offs are defined as firms whose products or services are the result of scientific knowledge or know-how developed in the academic environment or within research departments of the firms of origin. Professors, researchers, and PhDs decide sometimes to quit their mother organizations and to find new venture where to implement the acquired knowledge, developing a new product or service for the market. So academic spin-offs but also industrial spin-offs are considered. Specifically, the academic ones play a crucial role within the research institutions, as they are perceived as harmless for the role carried out by research centers. Spin-offs represent a virtual bridge between academic and industrial world, as they offer an alternative to the commercialization of science [62].

The authors accounted the companies defining themselves as spin-offs on the official webpage (history page or press releases), but it is clear that the academic origin typifies many other firms of the sample (see Table 8.4).[38]

[38]Many companies have professors, scientists, and researchers that come from different public research centers, for example, and cannot be defined as spin-offs (of one organization). Still, several academics participate in the boards of direction of the firms.

Table 8.4 The spin-offs of the sample

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
AbbVie (MNC)	USA	2013	5186	We target specific difficult-to-cure diseases where we can leverage our core R&D expertise to advance science. We're constantly working to create solutions that go beyond treating the illness to have a positive impact on patients' lives, on societies, and on science itself	Abbott Group
Abcam	UK	1998	4	Antibodies, biochemical	University of Cambridge, UK
ABK-Gaspesie	Canada	1995	0	Manufacturing, food and kindred products, fresh or frozen packaged fish	University of Québec—acquired by Ocean Nutrasciences in 2010
AerBio	Ireland	2006	1	AER BIO is an industrial biotechnology company. Our unique enzyme-based products deliver a revolutionary "wet" extraction process for manufacturing bio-based oils, proteins, and other valuable products from algae biomass	National Institute for Bioprocessing Research and Training (NIBRT)
Agensys	USA	1997	675	Agensys specializes in developing a pipeline of therapeutic fully human monoclonal antibodies (mAbs) to treat cancer	UCLA—acquired by Astellas Pharma in 2007
AlgaeFuel—A4F	Portugal	2008	2	A4F develops microalgae production units	Necton SA
Alga-labs	Canada	2006	0	Water treatment with microalgae	AirScience Technologies

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (n)	Mission	Organization of origin
Algen	Slovenia	2010	1	AlgEn is currently involved in diverse activities including AlgaeBioGas project, wastewater treatment, biogas digestate, preliminary testing as on algae nutrient, photobioreactor control system development, Algal Bank maintenance	University of Ljubljana
Algenics	France	2008	7	Pharma biotech	IFREMER (The French Research Institute for Exploitation of the Sea)
AlghItaly	Italy	2013	1	AlghItaly produces microalgae for the nutraceutical industry	Algain Energy
Amyris	USA	2003	538	Synthetic biology to produce target molecules	University of California
Applied Enzyme Technology—AET	UK	1994	0	Applied Enzyme Technology is a company with core expertise in enzyme stabilization	University of Leeds
Arbonova	Finland	2003	3	Chemistry—pure bioactive substances from nature	Åbo Akademi University
Arterra Bioscience	Italy	2004	8	Arterra Bioscience srl is an Italian research-based biotech company with a strong know-how in the discovery of active molecules	Arena Pharmaceuticals
Artes Biotechnology	Germany	2002	6	R&D contract service for the development of targets of customer's choice as well as the transfer of own, in-house developed cell lines and processes for vaccines, bio-pharmaceuticals, biosimilars, and enzymes	Rhein Biotech Group

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
ArcticZymes	Norway	2009	1	Bioprospecting activities in the marine Arctic to be a leader in the commercialization of novel cold-adapted enzymes for molecular research and diagnostics	Biotec Pharmacon
Astareal	Sweden	1990	3	AstaReal offers a superior range of bulk and retail natural astaxanthin products that support human health and nutrition	Uppsalla University
Avantium	Netherlands	2000	240	Avantium is a leading technology company specialized in the area of advanced catalytic research, with ground-breaking innovation as our primary goal	Royal Dutch Shell
Axxam	Italy	2001	13	Axxam is a leading provider of integrated discovery services for the entire life sciences industries as pharmaceutical, crop protection, animal health, cosmetics, and nutrition	Bayer Group
BioActor	Netherlands	2005	2	BioActor develops proprietary health ingredients for use in functional foods, medical nutrition, and dietary supplements	Maastricht University
Bio-Iliberis	Spain	2007	3	Bio-Iliberis offers the opportunity to develop and merchandise advanced biotechnological solutions for environmental restoration	CSIC—Spanish National Research Council

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
Biomar microbial technologies	Spain	1996	0	Pharma biotech. Many sectors covered, including services—very large collection of marine organisms	PharmaMar (Zeltia Group)
BioMarin Pharma (MNC)	USA	1997	464	Pharma biotech	Glyko Biomedical
Biotec Pharmacon	Norway	1990	103	Biotec Pharmacon develops, manufactures, and markets immune modulating compounds for the human health sectors and marine enzymes used in molecular biology	University of Tromsø
Bitop AG	Germany	1993	49	Innovative medical devices for the application fields: allergy, dermatology, cough and cold, respiratory disease, and dry eyes/dry nose	Private University of Witten/Herdecke
Cellectis SA	France	1999	357	Cellectis is a gene-editing company focused on developing immunotherapies based on gene edited engineered CAR-T cells (UCART)	Institute Pasteur
Cognetix	USA	1996	120	Cognetix is a drug discovery and development company focuses on commercialization of conopeptide-based pharmaceuticals	University of Utah
Coral Biome	France	2009	0	Highest-quality cultured corals—Our primary focus is on Caribbean soft corals, especially zoanthids, *Ricordea, and other rare corallimorphs*	University of Aix-Marseille
Direvo Industrial Biotechnology	Germany	2008	109	Emerging biomass conversion industry	Direvo AG

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
Eco2capture	USA	2011	0	Technology to enhance the capture of CO2 for use in the production of microalgae products for multiple end use applications	Ohio University
Ecotechsystems	Italy	2003	0	Consultancy on the design, management, and execution of oceanographic and environmental surveys and studies in transitional environments and inland waters	Polythechnic University of Marche
Evocatal	Germany	2006	11	Evocatal develops and produces enzymes and fine chemicals for the chemical and pharmaceutical industries	University of Düsseldorf
FeyeCon	Netherlands	2001	23	We are a true process and product development company, focusing in food, water, health, and energy	Deft Technical University
Fotosintetica e Microbiologica srl—F&M	Italy	2004	3	F&M supplies photobioreactors and provides expertise on microalgae cultivation for production of functional foods, feeds, natural drugs and cosmetics, and renewable fuels	University of Florence
Gelyma	France	1997	9	Gelyma is an independent French company involved in the research, development, manufacturing, and marketing of specialty ingredients for novel blue cosmetic strategies	University of Marseilles
Genomatica	USA	1998	390	Green chemicals	University of California

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
Giotto Biotech	Italy	2011	0	Technologies associated with nuclear magnetic resonance (NMR), providing biomolecules, organic synthesis, metabolomics, NMR access, and information technologies	University of Florence
Glycomix	UK	2007	0	Glycomix offers in-depth consultancy, laboratory services, and a number of specialities	Dextra Laboratories and National Centre for Macromolecular Hydrodynamics
Greenaltech	Spain	2009		To advance human health and well-being by unleashing the power of microalgae	Oryzon Genomics
Greensea	France	1988	0	Greensea is a company specialist in the large-scale culture of marine and freshwater microalgae, in the development of marine vegetation compounds (algae and marine plant life) with medium and high added value	GreenTech
GTP Technology	France	2000	0	Consulting on recombinant proteins	University Paul Sabatier
H2ope Biofuels	USA	2008	1	Commercial production of molecular hydrogen from light, water, and photosynthetic algae	University of Delaware
Hemarina	France	2007	8	Research and development of marine oxygen carriers for therapeutic and industrial applications	University of Brest
Herboreal	UK	2007	0	Herboreal offers the largest range of naturally occurring coumarin and furocoumarin standards for analysis	University of Edinburgh

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
Horizon Discovery	UK	2005		Research tools to organizations engaged in genomics research and the development of personalized medicines	University of Turin
Hypha Discovery	UK	2004	4	Microbial technology company in pharma-ceutical and agro-chemical R&D through the discovery and production of microbial and mam-malian metabolites	Brunel University
I-Tech AB	Sweden	2000	17	I-Tech markets and sells a marine biocide	University of Gothenburg and Chalmers University of Technology
Madep	Switzerland	2002	0	Isolation and cultiva-tion of new bacteria and fungi for pollution degradation and site remediation without generation of new pollutants	Changins agricultural research station
Manros therapeutics	France	2007	1	New treatments against cancers and neurodegenerative diseases	Spin-off CNRS e Université Paris Descartes
Marinomed	Austria	2006	12	Therapies against respiratory diseases using its innovative antiviral respiratory technology platform	Veterinary University of Vienna
Matrix Genetics	USA	2007	8	Biotechnology company focused on producing renewable fuel and specialty chemicals derived from cyanobacteria (blue-green algae)	Targeted Growth
Mead Johnson Nutrition (MNC)	USA	1905	25	Safe, high-quality, innovative products that help meet the nutritional needs of infants and children	Bristol Meter Squibb

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
Mizon (Nabion)	Korea	2000	40	Nabion develops nature base beauty cosmetics	Amorepacific (MNC)
Muradel Pty	Australia	2011	0	Manufacture of biofuels, by growing and harvesting halophytic microalgae and converting it and other carbonaceous materials to fuel	Joint venture between Murdoch University, Adelaide University, and SQC
NanotecMARIN	Germany	2007	9	The formation of biomaterials by aquatic invertebrates and their biotechnological application in biomedicine. Another focus is the development of therapies for prevention of osteoporosis and other bone disorders	BIOTEC-MARIN, University of Mainz
Necton (Phytobloom)	Portugal	1997	2	Necton S.A. has developed an extensive know-how in growing various microalgae using specialized cultivation techniques	Catholic University of Portugal
Neurotrope Bioscience	USA	2012	1	Neurotrope BioScience is focused on developing new therapies with Bryostatin 1 for the treatment of neurodegenerative diseases and developmental disorders	Blanchette Rockefeller Neuroscience Institute
OceanBasis	Germany	2001	0	Extracts from marine organisms for cosmetic industry, R&D of medical products against cancer, collagen for wound healing and orthopedics	Coastal Research & Management (and Zoological at the University of Hamburg)
Omnia molecular	Spain	2005	1	Anti-infectives targeted at difficult-to-treat infections	Barcelona Institute for Research in Biomedicine

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (n)	Mission	Organization of origin
OP Bio Factory	Japan	2006	4	Libraries, marine life, land plants, marine and land microorganisms (actinomycetes, filamentous fungi, bacteria, yeast, lactic acid bacteria)	Ocean Planning Ltd.
Photon8	USA	2008	1	Genetically improved algae for fuel, food, and nutraceutical	University of Texas
Phytolutions	Germany	2008	3	Research-intensive procedures and technologies for the efficient use of marine algae as a source for biofuels, chemicals, animal feed, and building materials	Jacob's University
Prokazymes ehf	Iceland	2006	0	Enzymes for research, diagnostic, and industrial testing purposes	Matis (former Prokaria)
Remedios Limited	UK	1999	1	Environmental services including tailored solutions to the investigation, risk assessment, management and sustainable remediation of contaminated land	University of Aberdeen
Rosetta Green ltd	Israel	2010	8	Improved plant traits for the agriculture and biofuel industries	Rosetta Genomics
SBAE Industries	Belgium	2006	8	Products of microalgae for various applications, including nutritional supplements for fish farms, aquaculture, cosmetic products, additives for food industry, and biofuel production	University of Ghent
Scandinavian Biogas	Sweden	1996	2	Industrial level production of biogas	Stockholm Vatten
Sea4Us	Portugal	2013	0	Services which include the collection of marine specimens and their	Technical University of Lisbon

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
				identification, processing, and storage for biotechnology research purposes	
Seaweed energy solutions—SES	Norway	2006	1	Development of large-scale cultivation of seaweed	Norwegian University of Life Sciences
Shellfish Hatchery Systems Ltd	UK	1995	0	Consultancy on marine biotoxins, shellfish diseases, carrying capacities, development of new species for aquaculture, development of hatchery techniques for a range of species, development of new materials, and design for aquaculture holding systems	Northbay Shellfish Ltd
Simris Alg AB	Sweden	2010	0	Natural food products and health supplements for people and animals	Royal Institute of Technology and Lund University
Sirenas marine discovery	USA	2011	1	Preclinical leads for a broad range of diseases	Scripps Research Institute
Solix biofuels	USA	2006	13	Algal growth system (AGS®)	Colorado State University
Stemmatters	Portugal	2012	0	Regenerative medicine and tissue engineering	European Institute of Excellence for Tissue Engineering
Subitec	Germany	2000	0	Consultancy on biomass-refineries and production of algal biomass	IGB—Fraunhofer Institute
Tequesta biosciences	USA	2003	0	Production process that is independent of the need to harvest and extract the molecule from marine soft corals	Scripps Research Institute
Tere Group	Italy	2012	0	L’azienda è specializzata nella	T.M. (IT)

(continued)

Table 8.4 (continued)

Spin-off company	Country	Year of est.	Patents (*n*)	Mission	Organization of origin
				produzione e commercializzazione di sistemi per la filtrazione della CO2 e per la produzione Olio e di biodiesel da alghe	
The National Collection of Industrial, Marine	UK	1982	1	Microbiology and chemical analysis company that houses the biggest reference collection of industrially and environmentally valuable microorganisms in the UK	Aberdeen University
Triphase Accelerator Corporation	Canada	2010	2	Consultancy on clinically enabled oncology assets	Ontario Institute for Cancer Research—OICR
VESO (Akvaforsk, AFGC)	Norway	1992		Consultancy on vaccine distribution and contract research for Norwegian authorities	Center for Contract Research
Zymetech	Iceland	1999	3	Research, development, and manufacturing of marine-derived enzymes	University of Iceland

5 Conclusions

Scientific and technological breakthroughs from the early 1970s to the early 2000s gave new life to marine biology and biotechnology applications. Academic sciences have strongly highlighted chemical and genetic diversity of marine organisms (both macro and micro). Some limitations in the efficient supply of blue compounds in the development stage of research and industrialization processes have certainly limited the growth of the sector. The first products conquering the markets in the pharmaceutical, chemical, food, and cosmetic industries, along with scientific advancements in marine compounds, definitely put blue biotechnology on the political agenda. The first phase was characterized by a sort of indifference of the private sectors, followed by an approach phase triggered by numerous dynamics. Undeniably, in several productive sectors, the need for natural products increased consistently. In the cosmetic sector, consumers look for safe and effective products for both body and skin. In the energy industry, companies look for environmental-friendly alternatives to petroleum, both to reduce the long-term effect of depletion of oil reserves and to

find new ways around public environmental measures adopted by governments (green policies that limit oil consumption). Still, in the aquaculture sector, the use of antibiotics to feed fish has been long opposed, and firms have been forced to find alternative solutions (e.g., probiotics).

Another relevant industrial trend concerns the market globalization, which not only generated a wave of mergers and acquisitions but also hampered competition among companies, MNCs, and groups of firms. Organizations look now for new ways to satisfy the markets through innovation and differentiation of both products and processes. Moreover, in the pharmaceutical industry, as said before, research and development efficacy dropped, and the failure of combinatorial chemistry in the delivery of new products pushed big companies, once again, toward natural and marine biodiversity, in order to find new active compounds.

Despite the efforts of the international economic and political organizations (and the many market research and consulting), it is evident that a global picture of the marine biotechnology industry is difficult to get. Several analysis and reports have tried different dimensions and diverse levels of specialization, which is likely to be attributable to the quite blurred definition of blue biotechnology[39] and to the wide variety of the industries involved.

The investigation on the dimension and trends portraying each industry projects both light and shadow on the issue. In the cosmetic sector, traditional firms seem to have no research competencies, so the development of bioactive compounds would need to be outsourced to organizations holding a proper scientific know-how. At the same time, big companies are developing innovative research centers that some experts consider to be at the frontier of science, as it is happening in the pharmaceutical industry. In the energy sector, as previously highlighted, by-products have taken the lead, becoming the core activities of the majority of the investigated firms. Those organizations do work now for the cosmetic, pharmaceutical, nutraceutical, and feed sectors also. Big pharma, although more focused, is trying to earn (and not to lose) new markets, investing in preventive medicine with the production of nutraceuticals.

The analysis of the dataset makes clear how the different sectors are linked to each other, with a big percentage of the firms producing for more than one market. The investigation of the dataset widens the geographical perspective of blue biotechnology, considering a reliable distribution of firms. Many countries show a significant percentage of companies operating in the blue biotechnology industry. The USA holds the biggest share, followed by European countries. Main industries for the USA are the pharmaceutical and the energy sector, whereas European nations offer a bigger share of industrial businesses. The year of establishment testifies the presence of companies of long tradition and some newborn firms. Companies founded before 2000 represent about 50% of the total number.

From the analyses of the firms in the dataset, it is not possible to fully understand the relationships over time among companies. The presence of a significant (and

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[39] An official statistical definition is not still available (NACE codes).

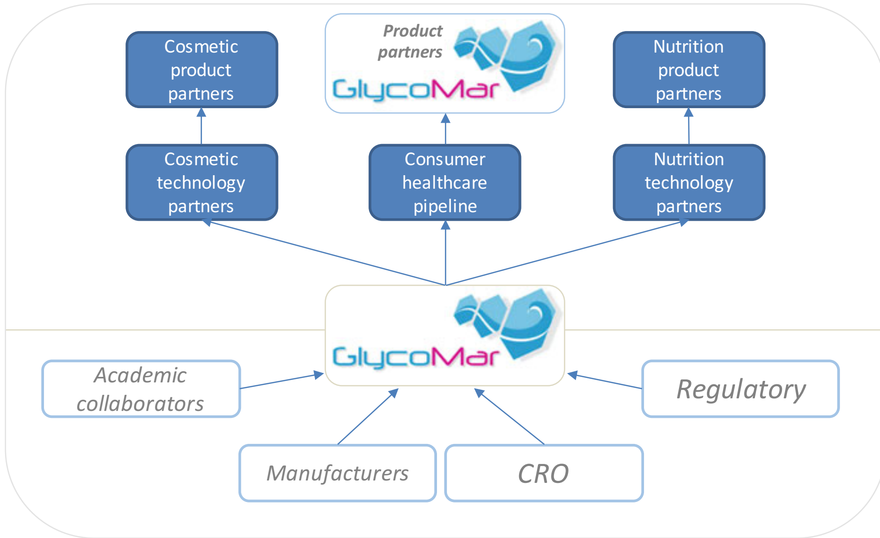

Fig. 8.15 The collaboration model of Glycomar. Source: http://www.atlanticstrategy.eu/sites/all/themes/clean_theme/doc/events/brest/presentations/2B_BAVINGTON.pdf

probably underestimated) number of spin-offs of academic origin demonstrates the pivotal role played by the academic institutions. Martins et al. [6] emphasize that industry-academia partnerships value both participants in a win-win collaboration system. Academia get closer to what is called "the market issue"—having more funds to manage and learning to address very important questions ever since the early stages of their development programs—while industries get greater chances to offer new natural products to both the final market and the clients:

> Collaboration with large companies, who may also be your target customers or licensees, can fulfill a number of requirements: validate your technology, provide a potential route to market, provide early collaboration fees/milestone revenue, provide commercialization expertise you lack, and can be an 'image enhancer'[40] (see Fig. 8.15).
>
> Making marine biotechnology effective for the generation of new ideas, processes, and products requires the bringing together of skills and equipment from a host of disciplines, including taxonomy (a dying art), marine invertebrate biology, marine microbiology, molecular biology, genetics, chemistry, biochemistry and biomedicine—in addition to which many other disciplines should be included as possible end users (biomaterials, nutrition, etc.). The daily interaction among these scientists, when adequately resourced and focused on a common goal, will create novelty in many fields. Michael J. Allen and Marcel Jaspars [63].
>
> Technologies can come into being only if they are commercially viable. They are often the end result obtained jointly by academia and industry. Quite often, neither of these players can fully develop every one of the numerous steps involved in achieving success in technology development. In their research relating to organisms, their applications and the

[40] http://www.glycomarblog.com/

processes, academia and industries participate at the different levels of established, emerging or exploratory technologies. [64]

Probably, as stressed by Allen and Jaspars [63] and proven by the dynamics of all the above-mentioned pharmaceutical industries, there are too many forms of knowledge needed for the evolution of blue biotechnologies—from discovery to final product—for each actor on his own. Only collaboration and creation of fruitful partnerships could lead to advancement in science and ultimate progress for society. Scientists from various fields could contribute to the development of the blue sector, and, at the same time, marine scientists could explore a wider set of alternatives in their research paths (not only in the pharmaceutical industry).

In the report of the European Science Foundation [61], the need to participate to interdisciplinary projects is strongly supported, and the research analysis and the production activities of the companies seem to corroborate this thesis:

> The compartmentalization among education branches (medicine, pharmacology, engineering, life sciences) is not suitable to the multidisciplinary demand of the industrial sector. In medical sciences, teaching is oriented to foster therapy and not the industrial path. In life sciences, the long paths of academic research are promoted at a disadvantage to the applied carrier. There are few possibilities to have mixed paths. Boyen et al. [11]

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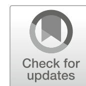
Chapter 9
How to Succeed in Marketing Marine Natural Products for Nutraceutical, Pharmaceutical and Cosmeceutical Markets



Ricardo Calado, Miguel Costa Leal, Helena Gaspar, Susana Santos, António Marques, Maria Leonor Nunes, and Helena Vieira

1 Introduction

Oceans play a fundamental role in life on Earth, not only regulating the weather and climate and providing most of the oxygen but also supporting society's well-being and sustainable economic growth due to their wide range of valuable ecosystem services (food, raw materials, water, medicinal resources, renewable energy, etc.). A

R. Calado
Departamento de Biologia & CESAM & ECOMARE, Universidade de Aveiro, Aveiro, Portugal

M. C. Leal
Department of Fish Ecology and Evolution, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Centre for Ecology, Evolution and Biogeochemistry, Kastanienbaum, Switzerland

MARE – Centro de Ciências do Mar e do Ambiente, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

H. Gaspar
MARE – Centro de Ciências do Mar e do Ambiente, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

Centro de Química e Bioquímica (CQB), Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

S. Santos
Centro de Química e Bioquímica (CQB), Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

A. Marques
Division of Aquaculture and Seafood Upgrading, Portuguese Institute for the Sea and Atmosphere, IPMA, Lisboa, Portugal

CIIMAR, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal

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substantial fraction of Earth's biodiversity is present in marine ecosystems, which harbours 2.2 million species, more than a quarter of the ~8.7 million species on Earth [1]. Indeed, only 1 out of the 35 animal phyla (phylum Onychophora) exists out of the marine ecosystems that harbour 15 exclusive animal phyla [2].

This high and unique marine biodiversity has been universally recognized as an important and rather untapped source of chemical diversity, with new marine natural products (MNP) being continuously discovered [3–5] and providing valuable leads for the development of new drugs [6]. Despite the raising number of molecules entering into the marine drug discovery pipeline, the number of marine drugs approved in the past decade has not been increasing at the same rate [7, 8]. MNPs have been unanimously acknowledged as the "blue gold" to face the urgent need for new drugs [6, 9]. The great advantage for the successful development of new marine-based drugs [10] lies not only on the high marine chemical diversity but also on the fact that marine organisms show primitive versions of human genetic systems, which renders them good models to tackle human medical issues.

Since the metabolites produced by the marine organisms are intimately connected with local environment [11], the discovery of marine chemical diversity has been driven by bioprospecting organisms in their one habitat. Biological material has been sourced from coastal areas, sea floor and extreme environments, such as hydrothermal vents and transition environments where freshwater inputs create osmotic stress (e.g. estuaries and underwater springs). More recently, researchers have been focusing on more sustainable sources of biological material, such as waste fractions or by-products from marine food or feed production. Other interesting and highly promising sources of untapped chemical diversity are associated with the microbiome of marine organisms and genetic resources that are not often expressed by marine organisms but show promising leads for drug discovery. The diverse nature of MNP led to several studies that summarized the biodiscovery of MNP from a chemical, taxonomical and geographical perspective [4, 5, 12]. This information can provide important suggestions for future bioprospecting efforts and, ultimately, improve the success rate of finding new chemical entities that can potentially be introduced into the marine drug discovery pipeline. It is also important to highlight that identifying the taxa and regions where most marine chemical diversity has been discovered is crucial for nations worldwide to acknowledge their marine bioresources and estimate the potential that their marine ecosystems hold for the Blue Bioeconomy [13].

M. L. Nunes
CIIMAR, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal

Universidade Lusófona de Humanidades e Tecnologias, Lisboa, Portugal

H. Vieira (✉)
Faculdade de Ciências, Biosystems and Integrative Sciences Institute (BioISI), Universidade de Lisboa, Edificio TecLabs, Lisboa, Portugal
e-mail: hmvieira@fc.ul.pt

1.1 Marine Natural Products: A Historical and Taxonomic Perspective

Marine organisms have historically been valued by humans, providing a multitude of compounds that contribute to our general well-being. The most obvious contribution is their pivotal role in our nutrition. Fish, for instance, has been part of human diet since immemorial times. The same is valid for several groups of marine invertebrates (namely, shellfish) and macroalgae (popularly known as seaweeds), which have also been used as food for millennia. This historic use of marine organisms, or their derived products, particularly as food and/or food ingredients, has been associated to a variety of benefits for human health, as they provide high-quality proteins, essential amino acids and fats, vitamins and minerals, among others. While the functional properties of marine-derived products were once associated to the organism itself, scientific breakthroughs allowed the identification of the specific marine molecules with desired positive effects to human health. Numerous of these marine molecules have been isolated and characterized due to their toxic effects, which would be subsequently employed for "positive" use as pharmaceuticals, for instance, as painkillers. It was therefore not a surprise to verify that along with the mounting scientific evidence on the benefits promoted by marine products to human's health came a significant increase in the efforts to investigate and isolate bioactive compounds present in marine organisms that promote such benefits. From an overall interest in marine products' biomass and/or class of compounds, secondary metabolites (hereafter referred to as MNP) have become the main focus of such research efforts, as they hold the potential to give origin to the highest-value products in different value chains.

Since 1940, when the first MNP reported was extracted from a sea anemone [14], the discovery of new marine molecules has been increasing rapidly. Although it was only in the 1970s that a notable boost in MNP discovery occurred, the pattern currently observed shows an exponential increase in the number of new marine molecules reported, with the number of new MNP being discovered per year steadily increasing (Fig. 9.1). Indeed, in the end of 2015, a total of 26,722 MNPs were already documented (Royal Society of Chemistry 2016).

The most rapid increase in the discovery of MNP was observed between the 1970s and 1980s, with an increase of about 305% in the number of new marine molecules reported. The discovery of MNP continues to steadily increase, which is likely associated with modern sampling and analytical techniques that allowed collecting organisms from deeper environments and thoroughly covering a wider range of chemical diversity. Nevertheless, and despite the "premium" label that deep sea habitats have as a bioprospecting target, the number of MNP reported from areas deeper than 50 m is still insignificant when compared to the whole MNP library [16].

The large number of MNP currently known originates from a great variety of organisms that range from microorganisms, such as bacteria, to macroorganisms, such as fish. Nevertheless, MNP discovery has been mostly focused on particular branches of the phylogenetic tree of life, particularly animals, which account for

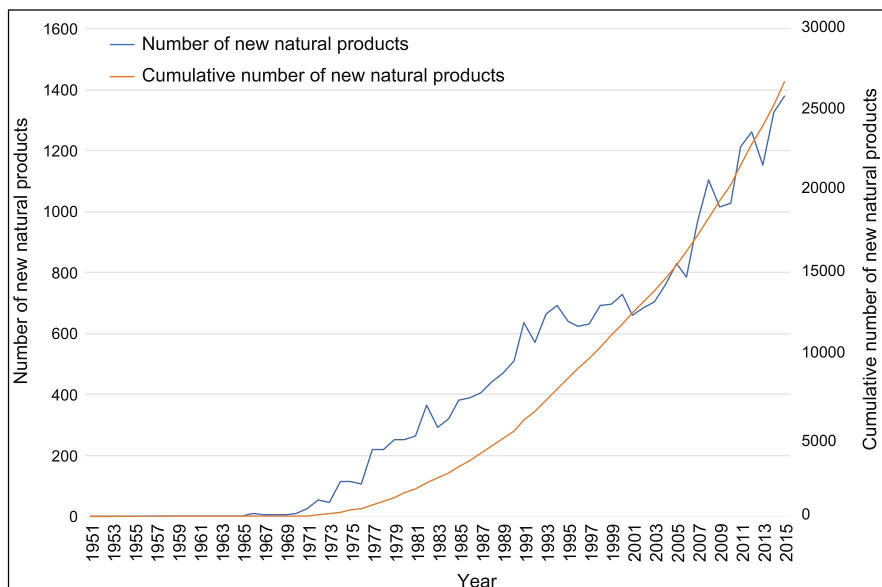

Fig. 9.1 Number of new marine natural products discovered between 1940 and 2015. Data source: Marin Lit (Royal Society of Chemistry [15])

approximately 70% of all MNP known so far (Fig. 9.2). Animals and macrophytes altogether encompass 81% of all MNP, suggesting that bioprospecting efforts have been notably biased towards macroorganisms. Microorganisms, which have been popular terrestrial sources of new chemical diversity [17], are still a relatively minor source of MNP compared to other marine organisms (Fig. 9.2). It is also interesting to note that bioprospecting has been biased within each major group (animals, macrophytes and microorganisms). For instance, most MNP from macrophytes were mostly discovered from red algae (Rhodophyta), while most MNP from animals were retrieved from sponges (Porifera) and most MNP from microorganisms were associated with ascomycetes (Ascomycota) (Fig. 9.2).

High marine chemical diversity has often been associated with the remarkable biodiversity found in oceans. Nevertheless, about 10% of all MNP currently known were discovered from solely four genera (Fig. 9.3; *Aspergillus*, *Laurencia*, *Sinularia* and *Streptomyces*). These genera represent macrophytes (*Laurencia*, 854 MNP), soft corals (*Sinularia*, 730 MNP), fungi (*Aspergillus*, 708 MNP) and bacteria (*Streptomyces*, 593 MNP). As observed in Fig. 9.3, this information suggests that marine bioprospecting has been focused on a small group of organisms. Indeed, half of all MNP currently known were discovered in approximately 61 genera. This does not necessarily mean that the remaining marine organisms do not display interesting and rich chemical diversity, and it is probably a consequence of the few genera that are exceptionally rich in diverse MNP.

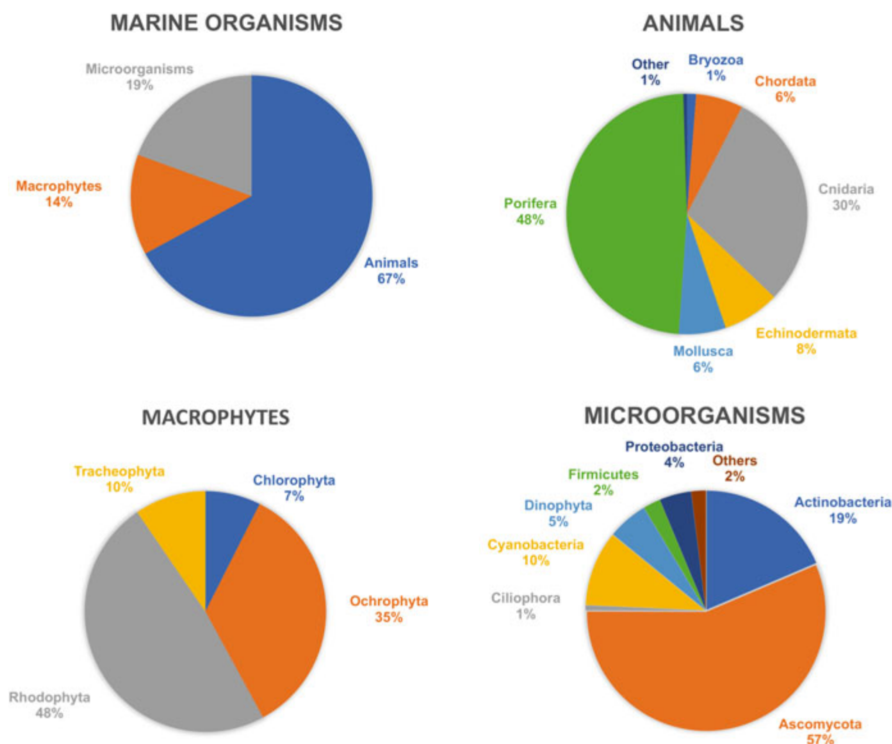

Fig. 9.2 Distribution of marine natural products among marine organisms (animals, macrophytes and microorganisms) and within each group. Data retrieved from MarinLit (Royal Society of Chemistry [15])

It is clear that marine biodiversity plays a key role in the discovery of new molecules with potential bioactivity. In general terms, while the value associated with marine biodiversity for the society is already well perceived, its potential for innovative biotechnological applications arising from the bioprospecting of new molecules is still somehow underestimated. In order to match bioprospecting interests of marine biota with conservation efforts, it is necessary that the society clearly understands the importance that chemical marine diversity already plays, and will continue to play, in the pathway towards a social, ecological and economical sustainable development supported by blue growth.

The bioprospecting of marine chemical diversity has been steadily increasing over the last decades, although the overwhelming majority of marine taxa remain to be screened. Additionally, not only the biodiscovery of MNP has not been evenly distributed across different marine taxa (Fig. 9.2); it is also extremely biased towards certain invertebrate groups, namely, sponges and cnidarians. This bias can be due to the fact of these two phyla representing a high number of species, with ~8500 being recognized within Porifera and ~11,500 in Cnidaria [18]. While researchers have been mostly targeting Porifera and Cnidaria to reveal untapped chemical diversity,

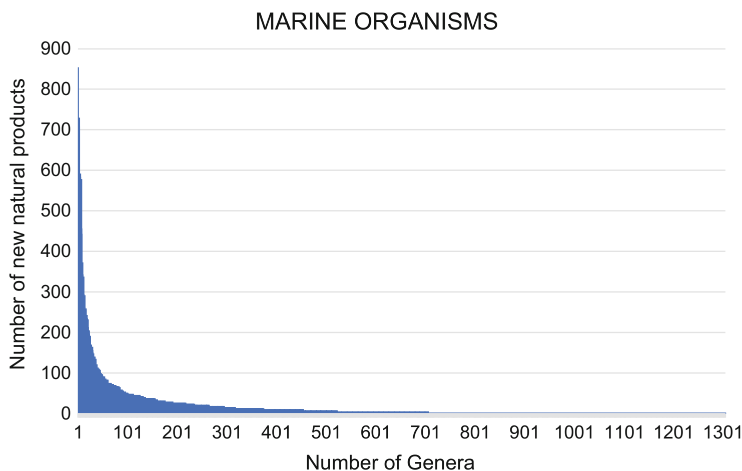

Fig. 9.3 Number of new natural products discovered sorted by marine genera yielding the highest number of natural products. Each bar corresponds to a single genus (total of 1305 genera). Data retrieved from MarinLit (Royal Society of Chemistry [15])

other groups of marine invertebrates also display a large number of species, namely, Annelida (~12,900 species), Mollusca (~46,500 species) and Arthropoda (~56,200 species) [18]. Therefore, it is likely that these groups may soon experience an increase in screening efforts targeting their biomolecules. Nonetheless, it is worth highlighting that the lower number of MNP described from annelids, molluscs and arthropods, when compared with those already reported from sponges and cnidarians, is likely associated with life traits. In other words, a number of marine invertebrates, namely, those within the Porifera and Cnidaria, are sessile and/or soft-bodied and therefore must rely on an array of chemical defences developed along the evolutionary history of these organisms to dissuade predators and competitors and/or stun their preys [19]. This chemical richness is more conspicuous in tropical reefs, where sponge and cnidarian species dominate the seascape, with chemical "warfare" playing a key role in intra- and interspecific competition for space.

Nowadays, it is accepted that MNP once assumed to originate from several invertebrate species may rather be synthetized by their associated microbiome [8]. Symbiotic microorganisms colonizing marine invertebrates, such as sponges and corals, exhibit a diverse array of bioactivities (e.g. cytotoxic and antibiotic activity) and already yielded promising leads for drug discovery [7]. If most MNP currently associated with marine invertebrates are produced by their symbiotic microbiota, this may create additional problems for marine drug development, particularly associated with biomass supply [20]. The development of new technology to overcome hurdles associated with understanding symbiotic relations and, more generally, the microbiota is critical. For instance, new omic techniques, such as metagenomics, may provide new insights on gene pools that have not been

addressed thus far. The further development of molecular instrumentation generating increasing volume of data, together with more powerful bioinformatics to handle such large amount of data, will certainly provide powerful tools that could pave the way for the discovery of new MNP.

On the other hand, the increasing bioprospecting efforts towards marine microorganisms and their different groups, like cyanobacteria, extremophiles or other marine bacteria and fungi not commonly protected by a host is an open avenue. This approach displays two key advantages: (1) a higher sustainability regarding future supply, as they can be replicated in laboratories, and (2) a lower impact on the ecosystem due to their collection from the ocean. Furthermore, for bioactive targeting markets where chemical or artificial synthesis is not considered, such as nutraceuticals or cosmeceuticals, these sources may be even more relevant [20].

1.2 MNP Discovery and Development

With the recent technological development, marine organisms can now be used by food industry as natural preservatives, pigments, stabilizers, gelling agents and others, while others exhibit beneficial effects and can be used as functional food ingredients, nutraceuticals, dietary supplements and prebiotics. In some cases the use of the same ingredients for several purposes is blurring the limit between sectors such as food and drug industries. Examples of that blending can be found in alginates, carraghenates and other bioderived ingredients isolated from algae, or sea-related products, like fish, shellfish or molluscs, or, even more recently, hydrolysate protein mixtures prepared from fish coproducts and functional ingredients like omega-3 fatty acids, fucoidan and many others. Due to their broad panel of bioactivities, including antitumour, anti-microtubule, anti-proliferative, photoprotective, antibiotic, anti-oxidant and anti-infective [21], MNPs are also exceptionally interesting high-value ingredients for applications in the pharmaceutical industry and constitute a growing field in the sector. Following the same trend, the cosmetic industry is progressively turning to the sea searching for new ingredients due to their sustainability, innovation and diversity. Here too, lines between markets are blurring. The cosmetic industry has introduced a special class of products, cosmeceuticals, a combination of cosmetics and pharmaceuticals, that incorporates bioactive ingredients in creams, lotions and ointments [22].

Feeding the nutraceutical, pharmaceutical and cosmeceutical industries with a continuous pipeline of new leads is a laborious endeavour, where biodiscovery of new MNP can play a key role. In the most traditional process for bioactive discovery, a natural product is firstly extracted from the source, screened against a specific target or targets, isolated through a bioassay-guided procedure, fractionated and purified using chromatographic techniques, yielding essentially a single biologically active compound that needs then further validation and testing according to complex regulatory demands of each sector. Despite its widespread use, this traditional method of natural product bioactive discovery is slow, labour-intensive, barely efficient and provides no guarantee of success. This initial step of MNP biodiscovery

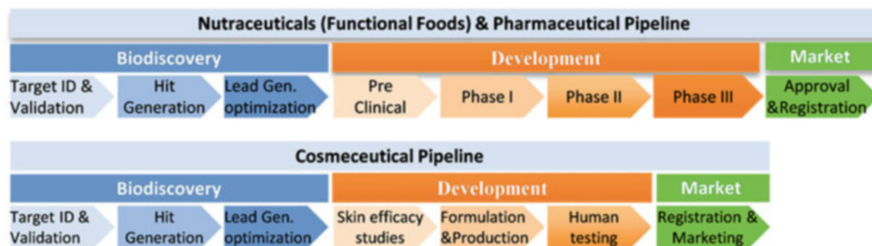

Fig. 9.4 Nutraceutical, pharmaceutical and cosmeceutical discovery and development general pipelines

is common to most market applications, but according to the specific market segment, the subsequent steps needed to launch a bioactive or preparation on the market must obey specific methodologies. Accordingly, it is easily understandable that some MNP development phases are specific, in terms of market needs, regulations, target consumers and types of products (Fig. 9.4).

The most well-known biodiscovery and development pipeline is the pharmaceutical one. It has clear divisions and comprises a biodiscovery stage, where the first step is the identification of the molecular target disease and the last one the isolation and optimization of the lead compound in order to initiate safety and efficacy studies. The development stage comprises the preclinical phase, where efficacy, toxicity and pharmacokinetic studies are performed in relevant in vitro (test tube or cell culture) or in vivo (animal) and the clinical phases. The clinical phases are sequentially ordered to test first safety in humans, in a small non-patient and healthy group, Phase I; then the efficacy and side effects are evaluated in a medium-sized patient group against a placebo or a standard approved drug for the condition under study, Phase II; and finally, if both phases are passed, the compound enters clinical Phase III, where a large group of patients are treated with the formulated compound and compared against a control group, for a longer period of time. Once completed, these clinical trial phases will lead to a compilation of a large set of data relating to safety, toxicity windows, efficacy levels and dosages, secondary effects and formulation processes, among many others, and comprise the final New Drug Approval (NDA) dossier that is submitted to regulatory agencies. This development pipeline is shared with one of the nutraceutical industries, mainly in what concerns functional foods that wish to claim specific health benefits. In fact, they share the same regulating and approval administrative bodies, like the Food and Drug Administration (FDA) in the USA and the European Medicine Agency (EMA), as well as the European Food Safety Authority (EFSA), in European countries, with local and national agencies also having a role in market introduction approval in each country. However, for nutraceuticals, due to the long timelines and heavy costs of such developments, manufacturers and developers often tend to skip this route and select easier paths. The reuse of old ingredients known to be safe, as well as to validate in vitro and in small cohorts of animals new efficacy claims, are common practice. Introducing new functional ingredients into the market with a smaller pool of data

collection and without any specific health claim is another practice that takes advantage of public's acceptance of the benefits of nutraceuticals and from the power of a particular brand.

On the other hand, the cosmeceutical development pipeline (Fig. 9.4) is clearly shorter than the pharmaceutical and nutraceutical ones, with less time and costs involved, since not only animal studies are generally abolished (especially in Europe where they are actually forbidden) but also human testing phases are much shorter and involve less people. For a cosmeceutical to reach the market, a bioactive compound, extract or fraction needs to be isolated and validated in the screening models used to discover them. Once this is accomplished, the major concerns for a cosmeceutical developer are the constant supply of that product and ensuring its safety and efficacy to consumers. As animal testing is abolished in cosmetics, a larger set of efficacy and safety trials are performed using cell-based models, as well as full 3D skin models to evaluate penetration, bioavailability and efficacy, as well as general toxicity. Toxicity towards other human organs besides skin is also analysed such as the eye, liver and heart. Once a cosmeceutical is considered safe, the following step is to test and develop several formulations and to characterize them physically, chemically and biochemically. Finally, the product is tested into a small group of volunteers that are given either the formulated product or a placebo and a prescription regime (e.g. apply once or twice a day, drink or eat, etc.). These human tests usually occur in a short period of time, between 14 and 52 days, and evaluate the performance and efficacy of the product using the most appropriate technologies for the condition under study. With the completion of such studies, specific benefit claims can be made, and the product formulation is registered by the owner and introduced in the market. The blur between cosmeceutical ingredient developers and cosmetic formulators and marketing companies is increasing, as most of the first need to go through this entire path to demonstrate efficacy and safety of their ingredients, in order to licence or sell them to the latter. Due to the fact that cosmeceutical development is less money and time-consuming, an ever-increasing number of pharmaceutical and nutraceutical companies are currently operating in this space, with recent year's boom in cosmeceutical ingredients clearly demonstrating this trend.

Despite the differences between pharmaceutical, nutraceutical and cosmeceutical segments, they are all mined with serious bottlenecks that can be divided into three major areas: biodiversity access, supply and technological issues and market barriers [7, 8]. The initial biodiscovery process is common to these market segments, and so they share the same bottlenecks, such as biodiversity and biomass access limitations, compound screening and isolation technological hurdles and commercial supply and feasibility constrains. Animal studies may not be performed for cosmeceuticals as they are for pharmaceuticals, but safety and efficacy testing is still needed, as well as a special attention to market entry prices and manufacturing to commercial scale feasibility. However, there is still much in common in these pipelines, and MNP development can benefit from deep knowledge of each pipeline specificities and commonalities to devise solutions for bottlenecks that have already been identified. This is detailed in Sect. 3 of this chapter.

2 Marine Natural Products and Value Chains

The ERA-NET of Marine Biotechnology has just recently launched “The Marine Biotechnology Strategic Research and Innovation Roadmap” [23], a document supported on 3 years of research and data collection that sets out highlights of research and innovation spanning scientific, technological, economic and societal challenges and sets a marine biotechnology research and innovation agenda until 2030. The roadmap is pillared on specific challenges in the MNP bioactivity discovery and development and identifies five thematic areas of action to overcome MNP hurdles; the first three enable exploration of the marine environment, support biomass production and processing and contribute to product innovation and differentiation. The remaining two themes provide the foundation to support growth in the Blue Bioeconomy, i.e. policy support and stimulation, and the provision of enabling technologies and infrastructure. This document is particularly relevant when it comes to fostering the development of MNP into concrete marketable solutions and products. MNP can be used in a variety of applications from food feed, agriculture, chemical industry, drugs and medical devices, beauty, biomaterials and bioplastics. While these are very different markets, with distinct value chains, some steps are common and so are some hurdles. Despite the obvious differences in these market segments and their development pipelines, MNP can penetrate all markets, and sometimes, the same MNP can be used and/or formulated for two or more different applications. Carragens are examples of MNP used in more than one application/market, as they are used both as a nutraceutical and food, as well as in pharmaceutical applications; other examples are chitosan, which is used in the three segments previously referred, and derivatives of astaxanthin, which are used in nutraceuticals and cosmeceuticals too.

In the next sections, this chapter will focus in the nutraceutical, pharmaceutical and cosmeceutical markets, the most appealing and dynamic now, providing an in-depth analysis of the current state of the art in each sector regarding MNP discovery and development, and examples of successful MNP and their paths to the market. With this approach, major lessons learned will be drawn to foster the development of more products derived from marine biota.

2.1 Nutraceutical Marine Natural Products

Aquatic ecosystems offer diverse nutrients valuable for human diet, well-being and health. Marine animals, in addition to being a rich source of easily digested, high-quality proteins containing all essential amino acids, provide essential fats, omega-3 polyunsaturated fatty acids (n3-PUFAs), vitamins (e.g. A, B and D) and minerals (including calcium, iodine, zinc, iron and selenium). Macroalgae also provide a great variety of food ingredients and nutraceutical molecules, characterized by a highly variable composition, depending on species, collection time and habitat. For

example, red and brown seaweeds are alternative sources of vitamins, minerals and proteins and are good sources of essential fatty acids [24, 25]. They are also rich providers of insoluble and soluble dietary fibres [26, 27]. Microalgae, the most primary and simply organized members of marine plant life, are as well rich sources of food ingredients, such as β-carotene; vitamins C, A, E, H and complex B; astaxanthin; polysaccharides; and polyunsaturated fatty acids. Some MNPs with their biochemical diversity are recognized as functional foods or functional ingredients, given their positive effect in the regular physiological functioning of the human organism and, even, in disease prevention. Some of those compounds can be used by the food industry as natural preservatives, pigments, stabilizers, gelling agents and others, while others exhibit beneficial effects and can be used as functional food ingredients, nutraceuticals, dietary supplements and prebiotics. An interdisciplinary approach is required to increase our knowledge, explore the potential of marine environment and produce value-added food. Hence, isolation and investigation of novel bioactive ingredients with biological activities from some discards, auction withdrawals and processing by-products (including all raw materials, edible or inedible) have attracted great attention. Moreover, many studies have reported that functional materials derived from such raw products can be used as nutraceuticals due to their potential in the prevention and/or treatment of various diseases [28]. For the purpose of this work, “nutraceutical”, coming from the words “nutrition” and “pharmaceutical”, is a product that is generally sold in medicinal forms and provides health benefits. “Functional foods” are those that can give a specific medical or physiological benefit, other than a purely nutritional effect. Functional foods usually contain ingredients with known bioactive compounds in defined amounts and provide a clinically proven health benefit [29, 30].

This section provides an overview of health benefits and potential utilization of polyunsaturated fatty acids, proteins, peptides, pigments, polysaccharides, chitin, chitosan and its derivatives, which are often used as natural additives in foods, nutritional supplements and cofactors enhancing health and general well-being [31].

2.1.1 Marine Lipids

Lipids from fish, some marine mammals, algae, marine plants and microalgae have been identified as relevant sources of polyunsaturated fatty acids [32]. In fact, fish both from fisheries and aquaculture are richer in n3-PUFAs, mainly eicosapentaenoic acid (20:5n3, commonly called EPA) and docosahexaenoic acid (22:6n3, DHA). Additionally, the docosapentaenoic acid (22:5n3, DPA) is gaining attraction among both scientists and consumers in recent years due to its biological effects [33]. In seaweeds and marine plants, n6-fatty acids (n6-PUFAs), such as linoleic acid (LA) (18:2n6) and arachidonic acid (AA) (20:4n6), are usually the most abundant, although in some species the n6:n3 ratio is of about 1.0. The levels and profiles of fatty acids in microalgae are quite variable, but in most cases they synthesize long-chain fatty acids in the form of triacylglycerols, such as α-linolenic acid (ALA) (18:3n3), EPA, DHA, LA, γ-linolenic acid (GLA) (18:3n6) and AA. The amount and variety of fatty acids in marine lipids

vary from one species to another and also with the biological stage, geographical area, temperature, condition and spawning state, etc. [34].

Most marine n3-PUFAs originate from fish flesh or whole body (e.g. mackerel and menhaden), by-products from the fish processing industry (namely, filleting and portioning processes) and special internal organs (such as the liver of the lean white fish). To a lesser extent, DPA has been found in most marine fish species (100–200 mg per 100 g of edible portions) but is particularly present in seal meat and blubber [35].

However, it is noteworthy that the original source of these PUFAs is not the fish itself but marine algae and phytoplankton. Other important sources of marine oils are fungi (*Phycomycetes*), thraustochytrids (large-celled marine heterokonts), extremophiles and krill. To a lesser extent, docosapentaenoic acid (22:5n3, DPA) is also present in different proportions in blubbers.

Due to the increasing body of evidence on the potential health beneficial roles of n3-PUFAs in humans, there has been an increasing interest on seafood and on marine lipids for human consumption (Table 9.1). The outcomes are thoroughly documented in many scientific opinions and papers highlighting their benefits for (1) cardiovascular system [40]; (2) cardiovascular disease risk factors associated with obesity such as hypertriglyceridemia [41]; (3) anti-inflammatory effects [42]; (4) onset delay of some cancers, namely, colon [43], breast [44] and prostate [45]; (5) foetal development [46]; and (6) positive influence on psychiatric/psychological disorders such as schizophrenia and depression [47].

Based on several studies, national and international bodies have recommended dietary intakes for n3-PUFAs, namely, EPA and DHA, for different segments of the population [48]. However, available data is not yet sufficient to establish a tolerable upper intake level of n3-PUFAs, DHA, EPA and DPA, individually or combined for any population group [48].

Stability and safety of fish oils are a major concern, since its oxidation generates chemically and functionally undesired products that reduce its palatability and are health hazards linked to ageing, cancer and heart diseases. Consequently, fish oils have to be suitably extracted, preserved and protected with appropriate primary and secondary antioxidants and films or coatings. Encapsulation offers effective protection against photo oxidation and free radical autoxidation by protecting from light and oxygen, leading to sensory stabilization [49]. Thus, micro-, nano- and coencapsulation, as well as edible nanoemulsions, to protect fish oils can fulfil the nutritional and physical requirements for enrichment of a variety of foods, such as milk, bakery products, salad dressings, bread and juices, and comply with the nutraceutical market demands [50]. Fish oil encapsulation is achieved using polymers such as chitosan, gelatin, maltodextrin, starch, whey proteins and plant gums via spray drying, coacervation, ultrasonication and membrane emulsification techniques, and each method has its own merit and shortcomings [51].

For therapeutic or even food enrichment purposes, common fish oils may not provide the necessary amounts of n3 fatty acids, and production and use of concentrates may be required [52]. Available methods suitable for large-scale production of concentrates include low-temperature crystallization, fractional or molecular distillation, chromatography, supercritical fluid extraction, urea complexation and enzymatic splitting [53]. The

Table 9.1 Marine bioactive molecules: sources, food applications and health effects

Bioactive molecules	Major marine sources	Food applications	Health effects
Omega-3 fatty acids	Fish, microalgae and fungi Seafood processing by-products	Fortification of food products, feeds and infant formulas n3 concentrates	Numerous benefits (e.g. neurodevelopment, reducing risk of cardio-vascular diseases, hypertension)
Proteins (e.g. collagen, gelatin) Fish protein hydrolysates Bioactive peptides Amino acids (e.g. taurine)	Fish, crustaceans, and molluscs Seafood processing by-products	Edible coatings Stabilizing, whipping, gelling, suspending, texturizing and thickness agents Milk and protein replacers Energizing drinks	Antioxidant, antihypertensive, anticoagulant, antibacterial and antiskin ageing activities Blood pressure reduction
Chitin, chitosan and derivatives	Crustaceans and cephalopods Seafood processing by-products	Gelling, emulsifying and food technological agents Food preservatives Edible protective films Dietary fibre providers	Antitumour, antibacterial, anti-Alzheimer's and anti-fungal activities Dietary fibre absorption Lipid absorption reduction
Polysaccharides	Red and brown algae Sea cucumbers Cyanobacteria/extremophiles	Food colour, food gel strength improver and antioxidant agents Food coatings Food gums	Anti-HIV, anticoagulant, antiviral, antithrombotic and anti-inflammatory activities
Carotenoids	Crustacean shells *Dunaliella salina*, *Sarcina maxima*, *Chlorella protothecoides*, *C. vulgaris* and *Haematococcus pluvialis*	Natural food colourings Food technological agents	Vitamin A precursors, antioxidant, anticarcinogenic and anti-inflammatory activities
Vitamins and minerals	Almost all marine sources	Food technological agents	Many essential functions in the body (e.g. transport inside cells and cofactors during metabolic processes)
Phlorotannins	Marine brown algae	Food technological agents	Antioxidant, anti-HIV and antiproliferative activities

Adapted from [36–39]

n3 concentrates may be produced in the free fatty acid, simple alkyl ester and acylglycerol forms.

Structured lipids, triacylglycerols containing combinations of short-chain fatty acids, medium-chain fatty acids and long-chain fatty acids located in the same glycerol molecule, have been developed to fully optimize the benefits of their fatty acid varieties

in order to affect metabolic parameters such as immune function, nitrogen balance and lipid clearance from the bloodstream. These specialty lipids may be produced via direct esterification, acidolysis and hydrolysis or interesterification.

A broad variety of marine oils have been approved as GRAS (generally recognized as safe) food ingredients, including oils from menhaden, salmon, tuna, anchovy, microalgae and krill. The chemical binding form, i.e. free fatty acids, ethyl esters, triacylglycerols or phospholipids, will also make a difference to the bioavailability of n3 fatty acids. In general, it has been reported that triacylglycerol forms are more effective than ethyl esters of long-chain n3 fatty acids; however, some authors continue to indicate that more information about the absorption mechanisms, stability and oxidation of both forms are still needed. Antarctic krill and squid oils contain mostly phospholipid n3 fatty acids, with krill oil offering a potent antioxidant-astaxanthin [54].

The market of n3-PUFA nutraceutical products from all sources is currently well established with a market share around 45%, 25%, 25% and 5%, respectively, for North America, Asia-Pacific, Europe and the rest of world. The key players of the n3-PUFA market include Royal DSM (The Netherlands), Epax AS (Norway), Aker BioMarine ASA (Norway), Napro Pharma AS (Norway), BASF SE (Germany), Croda International PLC (UK) and Ocean Nutrition Canada (Canada) [55]. The EPA/DHA ingredient market size is expected to register US$19 billion by 2022, growing at a compound annual growth rate (CAGR) of 9.1% from 2015 to 2022 [55, 56]. Although the market is not yet sufficiently stabilized, some nutraceutical companies can be highlighted: Neptune Technologies & Bioresources Inc. (Canada) teamed with Marine Life Sciences (USA) that market a blend of Antarctic krill oil and concentrated marine algae; Aker BioMarine (Norway), the world's largest krill oil producer, is the exclusive supplier for Valensa International (USA), which markets a krill oil-based supplement for joint and eye health; and Enzymotec (Israel) is a global supplier of refined krill oil and Parma Marine (Norway) that markets phospholipid-bound n3fatty acids. Algal oil with DHA is currently produced by DSM-Martek Biosciences (USA), through heterotrophic culture of *Crypthecodinium cohnii* (DHA > 30%) for infant nutrition formula in combination with fungal arachidonic acid and *Schizochytrium* spp. (DHA > 33% with 13.5% of DPA) for general nutrition supplements. Lonza Group (Switzerland) produces microalgal oil (DHA > 43%) from *Ulkenia* spp., and Photonz Corp. (New Zealand) has developed pharmaceutical grade EPA oil from cultured diatom *Nitzschia laevis* [57].

Food and beverage enrichment with n3-PUFAs is a foremost opportunity for manufacturers, being, however, required that the sensory attributes are not changed and the associated costs are not significantly increased. Presently, a wide range of n3-PUFAs products are available for various applications, and a high number of enriched foods can be found in the market (Table 9.2). Unfortunately, fish oils and its derivatives may be contaminated with a series of toxic pollutants like organic mercury, polychlorinated dibenzodioxins (PCDD/Fs) and polychlorinated biphenyls (PCBs). Accordingly, several methods have been developed (adsorption, deodorization, packed column stripping and a combination of processes) for removing unwanted compounds and at the same time preserving the nutritional quality of

Table 9.2 Different food products enriched with n3-PUFAs

Class	Food item	Main market countries
Dairy	Milk, milk fortified, yogurt, yogurt-based drinks, butter, buttery spreads, ice cream	Argentina, Indonesia, Italy, Spain, UK
Grain-based	Bread/hard bread, cereals, crackers, noodles, pasta	Australia, Denmark, France, Germany, Korea, Ireland, Taiwan, UK
Confectionary	Sweets, candies, cakes, bars	USA
Spreads	Margarines, spreads, toppings	Ireland, Japan, UK
Dressings	Salad dressings, mayonnaise, sauces	Korea
Juices	Fruit, orange, pineapple	Brazil, Germany, Japan, Spain
Meat and seafood	Red meat, fish and poultry products, nuggets	UK, USA
Infant formulas	Milk for different ages	Australia, Brazil, Japan, New Zealand, Taiwan, UK
Other	Eggs, supplements	Japan, USA

Adapted from [58]

fish oil. Activated carbon at 0.5% dosage is efficient in adsorbing PCDD/Fs and non-ortho PCBs, but only 58% of the mono-ortho PCBs are usually removed.

2.1.2 Fish Proteins

The health benefits of seafood consumption have been mainly attributed to marine lipids, and those of proteins, peptides and other water-soluble nitrogen components have received less attention. In fact, some of the effects of these compounds may even have been in part attributed to n3 fatty acids in intervention studies. However, in vitro and in vivo animal studies, carried out over the last 30 years, showed that proteins and peptides derived from marine sources have bioactive properties that may promote human health [59, 60]. Moreover, fish provide more than 3.1 billion people with almost 20% of their average per capita intake of animal protein [61].

Very often the amount of essential amino acids of fish proteins is greater than in standard protein, and protein efficiency ratio is slightly above that of casein, the major milk protein [62]. In terms of health, data collected from the large Nurses' Health Study (a prospective study following more than 84,000 women during a 26-year period) demonstrated significant reduction in cardio diseases from choosing fish or poultry as major dietary protein source [63]. Some epidemiological studies suggested that fish proteins (e.g. cod proteins) could contribute to prevent type 2 diabetes [64] and that the presence of taurine could also improve insulin sensitivity [62]. A diet rich in seafood proteins has been suggested to be advantageous in weight management mediated through increased satiety and increased thermogenesis [65]; however long-term clinical trials are still needed.

The isoelectric extraction and isolation of relevant proteins can be a viable approach [66] for obtaining protein isolates with increased functionality and oxidative stability over conventional protein concentrates/isolates or surimi [67–69].

Gelatin is a valuable protein derived from skeleton, fins and skin of fish and seafood by-products through partial hydrolysis of collagen. At present, fish gelatin production is close to 3000 tons/year, accounting for about 1–2% of the annual world production [70]. However, factors such as the outbreak fear of mad cow disease (BSE) and increasing demand for no mammalian gelatin for halal and kosher food markets have revived the interest in this compound [70]. Its main applications are as an ingredient in processed functional foods (e.g. edible and active coatings), as well as in nutraceuticals [71] (Table 9.1). Some present producers are located in France (Rousselot, Copalis and Weishardt Group), Italy (Lapi Gelatine), Spain (Junca Gelatines), Norway (Seagarden), Canada (Norland Products), Japan (YSK, Nitta Gelatin), Taiwan/Japan [Jellice (from fish scales)], Korea (Amicogen, Geltech Co.) and China (several small producers).

Other bioactive proteins obtained from marine organisms include albumin and protamine. The former has antioxidant and anticoagulatory activities, and protamine, derived from fish milt and roe, is a promising antibacterial preservative for the food industry and also shows potential health effects as antioxidant [36]. In most marine animals and some algae, there is also a nonprotein nitrogen fraction mainly constituted by volatile bases, free amino acids, nucleotides, purine bases and urea in the case of cartilaginous fish species. Within free amino acids, taurine has received great attention, and seafood is considered a good source.

2.1.3 Fish Protein Hydrolysates

Fish protein hydrolysates (FPHs) are obtained by hydrolysis of marine proteins with acids, alkalis or enzymes and have functional characteristics to be used not only as animal feed ingredients but also for human consumption as food ingredients and nutraceuticals. Chemical hydrolysis causes the damage of some amino acids and the formation of undesirable compounds, such as sodium chloride and lysinoalanine, whereas enzymatic hydrolysis allows a hydrolytic process more controllable. Enzymatic proteolysis via proteolytic enzymes already present in the fish (endogenous proteases) or added enzymes from other sources have been extensively studied and described by several authors over the last 60 years [72, 73]. Presently, most FPHs are obtained by digestion under controlled conditions of pH, time and temperature required by the added enzymes to give industrially acceptable products of envisaged quality and functionality, taking as well into consideration the protein substrate concentration and the enzyme-substrate ratio. The enzymes used for this procedure can be from plant (papain and ficin), animal (trypsin and pancreatin) or microbial (pronase, flavourzyme and alcalase) sources. Alcalase, produced from *Bacillus licheniformis*, and flavourzyme, a fungal protease/peptidase complex produced by submerged fermentation of a selected strain of *Aspergillus oryzae*, not genetically modified, have been proven to be some of the best enzymes in the preparation of

FPHs with good functional properties [74, 75]. Bitterness, the major problem affecting the sensory acceptability of FPHs, is due to the exposure of hydrophobic side chains of some amino acids (e.g. valine, isoleucine, phenylalanine, tryptophan, leucine and tyrosine) that normally reside in the interior portions of proteins [76]. Both Kojizyme™ and Flavourzyme™, exopeptidase enzyme mixtures, can decrease the bitter taste of FPHs, giving them attributes to be used as a flavour supplement in various food products [77].

Proximate composition and the balanced mixture of free amino acids, di-, tri- and oligopeptides of FPHs, give them excellent solubility and functional characteristics (e.g. emulsifying and foaming properties, fat absorption/fat binding/water holding capacities) for human functional foods due to the positive effect on gastrointestinal absorption [78]. Currently, they are used as milk replacers in young animal feeding, production of fermented fish sauces and fish peptone broth as a microbiological growth media and, namely, as food binding and gelling agents for several products, such as fish fillet blocks (Table 9.1). Recently some successful attempts have been made to use FPHs as substitutes of sodium chloride to avoid drip losses (weight decrease owing to dripping away of food tissue juices) in vacuum-packed seafood products.

There is a great potential for FPHs to also be used as nutraceuticals, as all FPHs exert a weak to moderate in vitro inhibition of angiotensin I-converting enzyme (ACE), suggesting that hydrolysis of fish proteins yields hypotensive peptides. FPHs also demonstrate calcitonin-like activities for calcium and phosphorus metabolism for the treatment of hypocalcaemia or osteoporosis and gastrin and cholecystokinin-like activities for stimulation of digestive enzyme secretion, which restrict stomach emptying, thus giving the feeling of satiety. Commercial available products containing FPHs for nutraceutical applications are Stabilium 200, an Atlantic fish autolysate (www.yalacta.com), and Protizen®, a white fish hydrolysate (www.copalis.fr), both carrying relaxing effect; Nutripeptin®, a cod hydrolysate, for lowering glycemic index (www.copalis.fr); Seacure®, a fish fillet hydrolysate obtained by fermentation using a marine microorganism, mainly composed of dipeptides and tripeptides, for improving gastrointestinal healthy (www.propernutrition.com); and Fortidium Liquamen®, a fish autolysate of white fish (*Molva molva*) (www.biothalassol.com) exhibiting multi-effects such as reducing oxidative stress, lowering glycemic index and antistress [79].

2.1.4 Marine Bioactive Peptides

Marine bioactive peptides, which are specific protein fragments isolated from protein hydrolysates of algae, fish, molluscs, crustaceans and by-products of seafood processing, have shown numerous bioactivities, namely, antioxidative, antihypertensive, antithrombotic, immunomodulatory, osteoprotective, antiviral, antilipemic, antitumour/cytotoxic, opiate, antimicrobial, antidiabetic, analgesic, appetite suppressing, antiobesity, immunomodulatory and neuroprotective [80].

Such peptides usually contain about 3–40 amino acid residues, but longer residues have been found, and their activities are based on amino acid composition and

sequence. Bioactive peptides that are inactive or latent within the parent protein sequence can be naturally occurring biomolecules, produced by microbial fermentation or generated with a variety of different enzymes, such as commercially available enzymes or, for instance, gastrointestinal enzymes. The biologically active peptides or functional peptides (genuine or generated) can, in addition to their nutritional value, exert several physiological effects, mainly the antihypertensive by inhibiting the angiotensin-converting enzyme (ACE; EC 3.4.15.1) [81]. This capacity is strongly influenced by the presence of specific amino acids, such as tyrosine, phenylalanine, tryptophan and arginine [82, 83]. Positively charged amino acids are associated with the activity of antimicrobial peptides [84]; histidine, leucine, tyrosine, methionine and cysteine residues are linked with radical scavenging activity, whereas hydrophobic amino acids such as proline and hydroxyproline participate in the inhibition of lipid peroxidation [85, 86]. Recent studies have also linked seafood peptides to antiobesity effects mediated through stimulation of hormones that regulate satiety. The complexity of the functions they perform results from the fact that they are able to act in the digestive tract, either on the intestinal epithelium or after absorption into the systemic circulation [87].

Different peptides can be generated by both acid and alkaline hydrolysis although this approach is generally not compatible with food additive manufacturing strategies. In general, a range of different processes are used to isolate and purify bioactive peptides from marine protein hydrolysates, through mild hydrolysis conditions with proteolytic enzymes. The physicochemical conditions (temperature, time and pH) of the reaction media must be adjusted to optimize the activity of the enzyme used and the peptide molecular weight [88]. Membrane separation techniques, such as nanofiltration, ultrafiltration and electro-membrane filtration, provide a potentially suitable industrially relevant technology for the enrichment of marine peptides within specific molecular weight ranges [89, 90]. In addition, membrane bioreactor technology, which combines enzymatic hydrolysis of marine proteins and peptide separation by ultrafiltration, is being considered as a potential method for the generation and fractionation of marine-derived bioactive peptides [60]. The application of active peptides has been mostly as functional ingredients in food and feed industries and as nutraceuticals (Table 9.1). Some of the commercial products in the market include Levenorm® produced by Ocean Nutrition (Canada), PeptACE® from Natural Factors (USA and Canada) and Bonito Peptide® from Ortho Molecular Products (UK) [57]. Other products containing hydrolysed proteins able to reduce blood pressure have been approved, such as by Japanese authorities in the case of the Katsuobushi oligopeptide® which is prepared through the hydrolysis of bonito muscle (a thermolysin digest: Leu-Lys-Pro-Asn-Met) [57].

2.1.5 Chitin, Chitosan and Related Compounds

Among the bioactive macromolecules obtained from marine by-products, chitin (β(1→4)-linked 2-acetamido-2-deoxy-β-D-glucose (N-acetylglucosamine)) and its derivative chitosan (α(1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose) have

great importance [91]. Their main sources are the processing streams of crustaceans. The preparation of purified chitin involves the deproteinization with hot concentrated alkaline solutions, the removal of calcium carbonate with concentrated hydrochloric acid followed by a treatment with ethanol and ethyl ether. The resulting chitin may be deacetylated giving origin to chitosan.

One of the major reasons for the use of chitosan as a functional additive is its hypocholesterolemic activity, explained on the basis of decreased cholesterol absorption and interference with bile acid absorption and antioxidant activity, given their protection from free radicals; this last property depends on chitosan molecular weight and viscosity, as shown in fish model systems [92]. Chitosan oligomers also show scavenging activity on hydroxyl and superoxide radicals [93]. This property makes them not only potential additives for lipid oxidation inhibition in food but also prevention of pathological processes associated with free radical modification of cellular compounds, such as atherosclerosis, arthritis, diabetes, inflammatory and neurological disorders [80].

Chitin and chitosan polymers have a wide range of applications (Table 9.3) in several fields from chemistry and biotechnology to nutraceuticals and functional food [80, 95]. Chitosan has been also successfully used for the inhibition of microbial spoilage growth, since it has been proven to be a broad-spectrum antimicrobial agent against both gram-positive and gram-negative bacteria and fungi [80]. Chitosan is approved for use as a food additive or dietary supplement in countries such as Japan, England, the USA, Italy, Portugal and Finland [96] and in 1992 was accepted as a functional food ingredient by Japan's health department. The main producing countries are Japan and the USA with smaller operations in India, Italy and Poland [97]. Chitin and chitosan have a variety of nutraceutical applications, including immune enhancement, anti-inflammatory properties, disease recovery and as dietary fibre (Table 9.1). Their ability to be useful in these areas is largely dependent on factors such as molecular weight and degree of N-acetylation, which can greatly influence solubility and interactions with other biomolecules. Recently, chitooligosaccharide (COS) has been studied in the nutraceutical field for its antidiabetic and hypocholesterolemic properties and adipogenesis inhibition. In the

Table 9.3 Main applications of chitin and chitosan

Foods and feeds	Additives	Health
Clarifying agent in wines and juices Dietary fibre, edible films Protein flocculation, tannin removal Food coatings Controlled release of nutrients	Oxidation inhibition Thickening and stabilization Texture modulation Matrix for the slow release of additives	Hypocholesterolemic effect Wound treatment Dental products Drug transport Nutraceutical encapsulation Anti-inflammatory agent Nutraceutical applications

Adapted from [38, 94]

food industry, COS has been used as dietary food additive and as dietary supplements to decrease body weight and serum lipids.

2.1.6 Marine Polysaccharides

Some important algal species belonging to Phaeophyceae (brown algae), Chlorophyta (green algae) and Rhodophyta (red algae) are important sources of polysaccharides of nutritional health benefit interest, giving them the potential to serve as valuable bioactive ingredients in functional foods and nutraceuticals. Their possible edibility is an attractive characteristic feature for using them in medicinal foods by direct consumption through the diet and indirect consumption through their extracted nutraceuticals and functional food molecules.

Typical polysaccharides present in brown algae are fucoidan, laminarin, cellulose, alginates and mannitol, while the fibres are mainly cellulose and insoluble alginates. Alginates represent the most important seaweed colloid in terms of volume and have a strong role in the manufacture of several processed foods and as nutraceutical. Green algae, considered an important food source in many parts of the world as a marine vegetable, is a rich natural source of carbohydrates, vitamins, essential amino acids, minerals and dietary fibres. Ulvan, the most relevant sulphated polysaccharide present in green algae, is currently receiving a great deal of attention, owing to physicochemical and biological properties of potential interest for nutraceutical applications. These properties are highly dependent on the chemical composition, charge density and molecular weight. The main polysaccharides in red algae species are floridean starch, cellulose, xylan and mannan, while the water-soluble fibre fraction is formed by sulphur-containing galactans, such as agar, agarose and carrageenan [36]. The wide practical uses of these polysaccharides are based on their ability to form strong gels in aqueous solutions. Gelling polysaccharides usually have molecules built up of repeating disaccharide units with a regular distribution of sulphate groups, but most of the red algal species contain more complex galactans devoid of gelling ability because of various deviations from the regular structure. Moreover, several red algae may contain sulphated mannans or neutral xylans instead of sulphated galactans as the main structural polysaccharides.

Most polysaccharides from algae are not digestible by the human gastrointestinal tract and, therefore, can be regarded as dietary fibres [98]. Human consumption of algal fibre has been proven to be health promoting, and its benefits are well documented [26]. Storage polysaccharides, such as agar, carrageenans and alginates, are the most commercially exploited components from seaweeds. Marine polysaccharides are used in a multitude of food applications, such as in the thickening of aqueous solutions, formation of gels, water-soluble films and as stabilizing agents [99]. Fucoidans, a complex series of sulphated polysaccharides, are reported to display numerous physiological and biological properties, including anticoagulant, antiviral, antithrombotic, antitumour and antioxidant activities, as well as having an effect on the inflammatory and immune systems [99]. Porphyran, another sulphated polysaccharide, has been reported as a gelling agent, nutritional supplement and

antioxidant [100]. Laminarin, the second major storage glucan in brown algae, has been identified as a modulator of intestinal metabolism through its effects on mucus composition, intestinal pH and short-chain fatty acid production [101]. Oligosaccharides can be beneficial to health when added to the diet to enhance the growth of prebiotic bacteria [102]. For instance, xylooligosaccharides and fructooligosaccharides, nondigestible oligomers, are used as a preferential substrate by anaerobic bacteria, such as bifidobacteria and lactobacilli [103]. Algal polysaccharides have numerous health benefits, giving them the potential to serve as valuable bioactive ingredients. Within the most relevant beneficial health effects of algal polysaccharides antioxidant, anticancer, anti-arteriosclerosis and antitumour activities may be discriminated [104, 105].

Polysaccharides from microalgae also have wide applications in food industry, for example, as gelling, emulsifying, stabilizers, flocculant and binding agents. They have also great potential to be used in films and coatings of food. The polysaccharides from microalgae can also be used as nutraceuticals due to their fibre content, ability of acid binding and cation exchange, and they have also potential as prebiotics [106]. In some cases they have bioactive potential similar to polysaccharides from seaweeds as hypolipidemic and hypoglycaemic agents.

Additionally, algins and exopolysaccharides from cyanobacteria can also be used for the stabilization of emulsions or as bioflocculants.

Edible algae used as foodstuffs, food supplements and derived nutraceuticals may be contaminated by several inorganic and organic arsenic species. Some of the organic species seem to be toxicologically relevant, but until now no health-based guidance values are available for risk characterization. Some other elements such as cadmium, lead and mercury have also been reported to be present in edible algae. Thus, the utilization of most algal species requires the monitoring and characterization of both the levels and the chemical species present.

2.1.7 Marine Carotenoids

Marine animals and algae (macro and micro) accumulate various carotenoids, exhibiting diverse chemical structures and biological features [107]. Within marine organisms, astaxanthin is the most abundant carotenoid, and its consumption can prevent or reduce the risk of various disorders in humans and animals [108, 109]. The US Food and Drug Administration (USFDA) has approved the use of astaxanthin as a food colourant in feeds [110], and the EU considers natural astaxanthin as a food dye. Astaxanthin produced from *Haematococcus pluvialis*, a green microalga, is the main source for human consumption [111] and as pigment in farmed fish feeds.

Astaxanthin and fucoxanthin, a carotenoid commonly found in edible seaweeds, are considered the most interesting from a nutraceutical perspective. Fucoxanthin, a specific carotenoid in brown algae, has gained much attention for its antiobesity and antidiabetic effects attributable to a unique mechanism. Fucoxanthin induces uncoupling protein 1 expression in white adipose tissue. That inner membrane mitochondrial protein, UCP1, can dissipate energy through oxidation of fatty acids and heat production. Furthermore, fucoxanthin improves insulin resistance and

ameliorates blood glucose levels through downregulation of adipocytokines related to insulin resistance in white adipose [112]. Some of these compounds, in addition to provitamin A activity, are involved in other physiological functions such as immune response and cell communication.

Astaxanthin is used for preventing bacterial infection, inflammation, vascular failure, cancer, cardiovascular diseases and lipid peroxidation inhibitor, reducing cell damage and body fat and improving brain function and skin thickness.

2.1.8 Other Relevant Compounds

Macroalgae are rich sources of polyphenolic antioxidants, such as catechins, flavonols and phlorotannins [113], and have been explored as functional food ingredients [114]. Several potential health benefits have been endorsed for the dietary intake of marine polyphenols, but such effects still need additional confirmation by epidemiological and interventional studies.

Phlorotannins can be used as functional ingredients and nutraceuticals, since they have several health beneficial biological activities including antioxidant [115], anti-HIV [116] and antiproliferative [117] activities (Table 9.1).

Vitamins A, D and E are present in important amounts both in the muscle and liver of marine lean fish species. The nutritional aspects and health benefits of vitamins A, D and E are already well known and its consumption strongly encouraged (Table 9.1).

Most types of marine organisms are a reasonable source of minerals such as phosphorus, potassium, iodine and selenium. Some shellfish, such as clams and oysters, are a good source of iron, zinc, magnesium, copper and other trace minerals. Particularly selenium and iodine are more abundant in marine organisms than in land-based foods. As a dietary source, seaweeds are particularly high in iodine. Increased dietary intake of selenium has been linked to protection against various cancers and cardiovascular and neurological diseases. Thus, a higher intake of seafood and marine ingredients could potentially contribute to an improved iodine and selenium status in some regions.

Squalamine (an aminosterol), squalene (an alkylglycerol) and its derivative squalane (a saturated derivative of squalene) are found in shark liver oils and may deliver immune enhancement, anti-angiogenic activity and skin conditioning. Up to date, anticancer, antioxidant, drug carrier, detoxifier, skin-hydrating and emollient activities of these substances have been reported both in animal models and in vitro environments. According to promising results from recent studies, squalene and squalane are considered important substances in practical and clinical uses, also displaying a huge potential for the nutraceutical and pharmaceutical industries [118]. Because of the demand of shark products (i.e. fins) and the exploitation

rates of some shark species, concern has steadily grown regarding the status of many shark stocks and their non-sustainability. There is also an international concern about the presence of environmental pollutants in the shark livers. Both aspects contribute for limiting squalene production from shark livers [119, 120].

Phytosterols (sterols and stanols) and the respective fatty acid esters are found especially in marine invertebrates and some algae and microalgae [121–123]. Some studies have demonstrated that phytosterol ingestion may reduce LDL cholesterol levels by 10–16%.

2.2 Pharmaceutical Marine Natural Products

As pointed earlier, natural products and their derivatives play an important role in drug discovery. An assessment of all FDA-approved new chemical entities (NCE) since the early 1931 revealed that one third of all NCEs were NPs, NP derivatives or synthetic mimetics related to NPs [124]. Although the great majority of these compounds are derived from plants, mammals and microorganisms, MNP had also an important contribution to drug discovery. Traditionally, research on MNP was focused mainly on macroorganisms, i.e. algae, sponges, corals and other marine invertebrates, but there was a significant trend in the recent years towards research on microorganisms.

However, despite the large number of compounds isolated from marine organisms, many of them with pronounced biological activity, those that have either been marketed or are under development, are relatively few.

Besides the usual drawbacks in any drug discovery process, the industrial development of many promising MNP was hampered by additional difficulties, such as the sustainable supply of the active compounds, the ecological impact on natural populations associated with the harvest of target organisms, issues related to structural complexity and scale-up, along with insufficient investment by pharmaceutical companies. Different blockades had to be overcome by pharmaceutical companies in order to develop and market these marine drugs, being the sustainable supply on an industrial scale on top of all issues. The aim of this section is to describe the strategies taken by pharmaceutical companies and/or academia in order to succeed in marketing their products. Based on the several excellent literature reviews with comprehensive discussion on marine drug discovery [7, 125–128], an update was done on the marine-derived drugs in the market. At the moment, the Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved ten drugs from marine origin to treat several diseases, with a stronghold in the area of anticancer chemotherapy (Table 9.4).

Table 9.4 Marine natural products (NP) or derivatives (D) thereof approved for use by the FDA [129] or EMA [130]

Compound (trademark)	NP/D	Original NP/source organism	Company (country)	Target area	Approval date
Cytarabine (Cytarabine; Cytosar-U®; Depocyte®)	D	Spongouridine and spongothymidine/sponge *Cryptotethya crypta*	Bedford (USA); Enzon (USA)	Cancer	FDA 1969 EMA
Vidarabine (Vira-A®)	D	Spongouridine and spongothymidine/sponge *Cryptotethya crypta*	King Pharma (USA)	Antiviral	FDA 1976 EMA
Fludarabine Phosphate (Fludara®)	D	*ibidem*	Genzyme Corp (USA)	Cancer	FDA 1991 EMA
Nelarabine (Arranon®)	D	*ibidem*	GlaxoSmithKline (UK)	Cancer	FDA 2005 EMA2007
Ziconotide (Prial®)	NP	ω-Conotoxin/marine snail *Conus magus*	Elan Corporation (Ireland)	Pain	FDA 2004 EMA 2005
Omega-3-acid ethyl esters	D	Omega-3-fatty acids/fish	GlaxoSmithKline (UK)	Hypertriglyceridemia	FDA 2004 EMA 2001
Trabectedin (Yondelis®)	NP	Ecteinascidin 743/tunicate *Ecteinascidia turbinata*	PharmaMar (Spain)	Cancer	FDA 2016 EMA 2009
Eribulin mesylate (Halaven®)	D	Halichondrin B/sponge *Halichondria okadai*	Eisai (Japan)	Cancer	FDA 2010 EMA 2011
Brentuximab vedotin (Adcetris®)	D	Dolastatin 10/sea hare *Dolabella auricularia*	Seattle Genetics (USA); Takeda (Japan)	Cancer	2011 FDA 2012 EMA
Iota carrageenan (Carragelose®)	NP	Iota carrageenan/red algae *Eucheuma*/*Chondrus*	Marinomed (Austria); Boehringer (Germany)	Antiviral	OTC

2.2.1 Marketed Marine Drugs and Close Analogues in Clinical Trials

Cytarabine (Cytosar-U®; Depocyte®), Vidarabine (Vira-A®), Fludarabine Phosphate (Fludara®) and Nelarabine (Arranon®/Atriance®)

Cytarabine (Cytosar-U®, Ara-C) has been, for more than three decades, one of the cornerstone drugs in the treatment of cancers of white blood cells such as acute myeloid leukaemia [131] and non-Hodgkin lymphoma [132]. By 2015 generic cytarabine accounted for 15.6% market share, with a revenue of US$0.07 billion [133].

The development of cytarabine, inspired by two C-nucleoside-derived compounds, spongouridine and spongothymidine isolated from the Caribbean sponge *Cryptotethya crypta* in 1951, marked the beginning of the era of "drugs from the sea" [134]. These compounds were unique nucleosides that possessed an arabinose moiety rather than the traditional ribose isomer, and, unlike their analogues found in DNA and RNA, they were found in the free state and could be readily extractable by alcohol or acetone. By the 1950s, traditional nucleosides composed by deoxyribose units were being examined for anticancer activity, and the fact that these arabinosyl nucleosides were found in the free state piqued the interest of scientists, who hypothesized that they were used as a defence mechanism for *Cryptotethya crypta*. This understanding of the role of those compounds in the sponge was the key feature that inspired the synthesis of several ara-nucleosides that steered the development of the marked drugs cytarabine, FDA approved in 1969 for cancer, and vidarabine, FDA approved in 1976 as antiviral (Fig. 9.5) [131].

Cytarabine, an antimetabolite antineoplastic agent that inhibits the synthesis of DNA, was chemically synthesized in 1959. It is metabolized intracellularly into its active form, the cytosine arabinose triphosphate, which competes with deoxycytidine triphosphate, the physiological subtract of DNA polymerase. This metabolite damages DNA by multiple mechanisms, including inhibition of DNA polymerase and incorporation into DNA and RNA. Rapidly dividing cells, such as those in some cancer, which require DNA replication for mitosis, are therefore the most affected [135]. However, cytarabine suffers from several limitations, such as short plasma half-life, low stability and limited bioavailability, which restrict its clinical utility [135]. Due to these drawbacks, therapeutic regimens consist in continuous intravenous infusion for 7 days, the three first ones in combination with other drugs, which diminishes patients' tolerability and increases side effects. Cytarabine has low rates of passive diffusion across membranes and enters into the cells acting as a mimetic substrate (antimetabolic agent) for specialized nucleoside transporter proteins. Various prodrug strategies that improved cytarabine delivery systems have been extensively exploited in order to achieve optimal pharmacokinetic and pharmacodynamic properties. The inability of cytarabine to cross the blood-brain barrier and exert its action in the brain is very limited, which is a limitation to treat patients with meningeal leukaemia. This weakness was surpassed by the formulation of a slow-release liposomal form of cytarabine (DepoCyte®) which enables its gradual release, thus ensuring prolonged cytotoxic drug concentrations in cerebrospinal fluid [136]. DepoCyte® was approved in the USA (1999) and in Europe (2001). Trying to

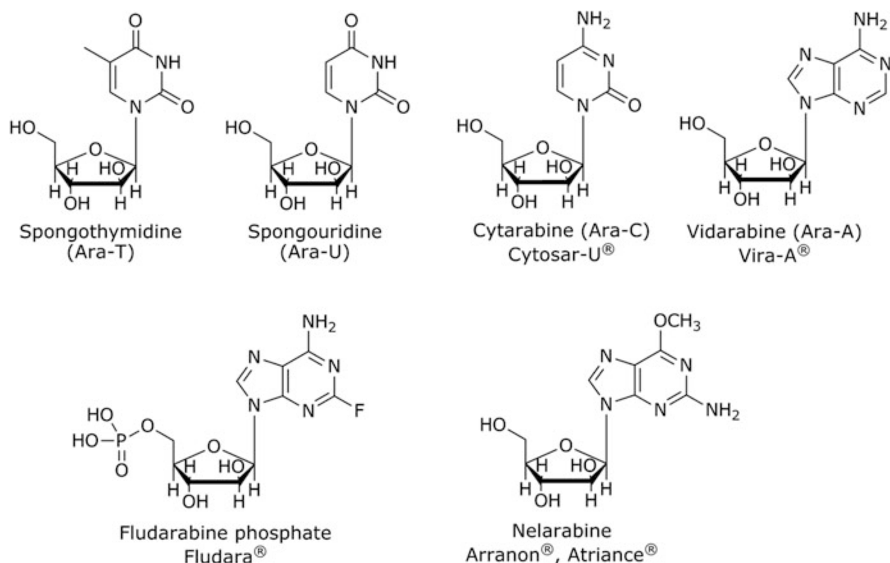

Fig. 9.5 Structures of the marine natural products spongouridine and spongothymidine from the sponge *Cryptotethya crypta* and the synthetic drugs cytarabine, vidarabine, fludarabine phosphate and nelarabine

synthesize a prodrug capable of crossing the cellular membrane without the help of transport proteins, the Oslo-based Clavis Pharma (now Weifa ASA, formerly known as Aqualis ASA, Clavis Pharma ASA) has developed elacytarabine (CP-4055), the lipophilic 5′-elaidic acid ester of cytarabine. Having the elaidic acid residue, the new drug molecules enter directly into the cells via membrane integration, which results in prolonged intracellular retention of the active nucleoside cytarabine. However CP-4055 failed to go beyond phase III clinical trials [137], and the company suspended all ongoing development work, reporting that its experimental drug failed to register a significant improvement in overall survival for late-stage patients with acute myeloid leukaemia [138].

Despite the improvement of the cure rates brought by the use of cytarabine in patients with haematological cancers, some of them did not respond to this therapy, having a poor prognosis, and so there was a need for novel drugs. A series of purine nucleoside analogues was synthesized and introduced in clinical trials [139].

Vidarabine was one of those nucleosides designed as a potential anticancer drug, but its rapid deamination by adenosine deaminase made it unfeasible as an anticancer agent. However, it was later discovered that it possesses a broad spectrum of antiviral activity mainly against herpes viruses, poxviruses and certain rhabdoviruses, hepadnaviruses and RNA tumour viruses. It is more toxic and less metabolically stable than other current antivirals such as acyclovir and ganciclovir; further it is poorly soluble with low oral bioavailability [140]. Although vidarabine (Ara-A) was first obtained by total synthesis, it was later isolated from the gorgonian *Eunicella cavolini* [141], but obviously, this

source is not an alternative to the supply due the extraction yield of solely 0.04%. Currently this drug is obtained from *Streptomyces antibioticus* [134]. Vidarabine was discontinued in the USA by June 2001 possibly due to its restricted antiviral therapeutic window [134] though it is still in use in EU for ophthalmologic applications.

Fludarabine (F-Ara-A), a derivative of vidarabine having a fluorine atom at the C-2 position of the purine base, was synthesized in 1969. The objective of this substitution was to avoid the problem of deamination while keeping the anticancer activity. Indeed, the fluorouracil synthesized earlier had anticancer activity, although it could not be developed as a drug due to its high toxicity. On the other hand, it was known that 2-fluoroadenosine was not deaminated by adenosine deaminase [142]. The limited solubility of fludarabine and difficulties in its formulation led to the synthesis of the prodrug fludarabine 5-monophosphate [143]. After entering into the cells by facilitated transport, fludarabine appears to be converted by intracellular kinases into its triphosphate ester, which is the actual cytotoxic metabolite. This drug seems to inhibit ribonucleotide reductases and ultimately interfere with the DNA synthesis [144]. Fludarabine is currently to treat patients suffering of chronic lymphocytic leukaemia who were refractory to prior treatment with alkylating agents [145].

Nelarabine was developed based on the structure of the guanosine nucleoside (Ara-G) synthesized in 1964 that due to its low water solubility and difficulty of synthesis did not follow for clinical studies. Later on subtle structure modifications on the core structure of Ara-G overcome the aforementioned drawbacks and led to the invention of nelarabine [146]. In fact nelarabine is a prodrug that is demethoxylated in vivo by adenosine deaminase to form the active compound Ara-G [143, 145]. After entering the cell, Ara-G is phosphorylated at C-5′ by cellular kinases, and the resulting active Ara-GTP is misincorporated into DNA, leading to the inhibition of its synthesis and cell death. In 2005 the FDA granted accelerated approval for nelarabine (Arranon®) for the treatment of T-cell acute lymphoblastic leukaemia and T-cell lymphoblastic lymphoma that has not responded to or has relapsed after treatment with at least two chemotherapy regimens [147]. This drug was approved in Europe by EMA in 2007.

Ziconotide (Prialt®)

Ziconotide is a nonnarcotic pain reliever used to treat severe chronic pain in people who cannot use, or do not respond to, standard pain-relieving agents. It was the first and, until now, the only analgesic drug of marine origin to obtain approval from the FDA and EMA, in 2004 and 2005, respectively. The drug is the synthetic equivalent of the naturally occurring ω-conotoxin MVIIA found in the marine snail *Conus magus* (Fig. 9.6). It was developed in 1987 by the Irish company Elan Corporation under the trade name Prialt® [148]. It is now commercialized by the Japanese Eisai

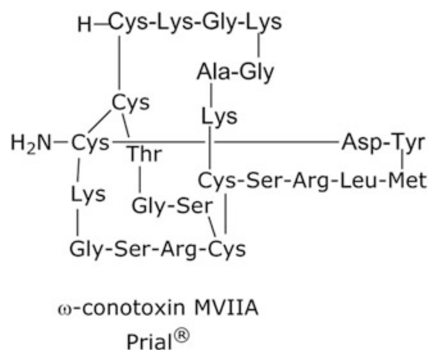

Fig. 9.6 Structure of ziconotide (ω-conotoxin MVIIA) from the marine snail *Conus magus*

Company that, in 2006, acquired from Elan Corporation the strategic production Elan's Prialt® in Europe [149].

Again, as for cytarabine, the starting point to the development of this drug was the comprehension of the interaction of cone snails with their environment. Indeed, scientists were driven to study the composition of the snail venoms enticed by the fact that cone snails are predatory gastropods that prey on fish, worms or molluscs and capture their prey by injecting lethal or paralyzing venoms [150]. In addition there were records of about three dozen people being killed by cone snails and apparently with little pain [151]. Over 700 species from the genus *Conus* have been described, with the venom of each containing a unique and diverse mixture of over 100 pharmacologically active compounds, mainly neurotoxic peptides, named conotoxins [152]. Conotoxins are structurally defined peptides that range in size from 8 to 30 amino acid residues and typically have one or more disulphide bonds; in particular ω-conotoxins contain 24–27 amino acid residues, 6 of them from cysteine and 3 disulphide bridges.

The systematic isolation and purification of *Conus* venoms in the early 1970s led to the discovery of ω-conotoxin MVIIA as a new analgesic drug. This achievement was only possible because of the advances of technology of those years: first, the introduction of high-performance liquid chromatography (HPLC) for purification and second, a new assay for following biological activities of venom peptides [153]. Because ω-conotoxin MVIIA could only be obtained in tiny quantities from the natural source, which limited its availability for research and medical applications, it was necessary to accomplish its chemical synthesis, which Elan Corporation achieved by solid-phase peptide synthesis [148]. There are a few examples of successful use of recombinant technology to produce other ω-conotoxins but thus far have provided only low yields and are not suitable for industrial production [154]. Ziconotide, the synthetic ω-conotoxin MVIIA, targets the *N*-type voltage-sensitive calcium channel, which was not identified, at the time, as an analgesic drug target [153], a fact that stresses the importance of using natural compounds as scaffolds for new drugs. This new mechanism of action has provided relief for patients with severe neuropathic pain, but because ziconotide does not easily cross the blood-brain barrier, it must be administrated directly in the central nervous

system by intrathecal administration. In addition, ziconotide-treated patients experience neuropsychiatric adverse side effects and elevation of creatine kinase levels. Therefore, drug discovery researchers are considering various approaches to identify and develop novel orally active *N*-type calcium channel-selective blockers that have the potential to be superior to ziconotide. As such, other ω-conotoxins are being investigated. However, difficulties in collecting the biological material and bioconservation issues require approaches based on chemical synthesis or a molecular engineering approach to expand access to the peptide constituents of most *Conus* venoms. A peptidomimetic approach may also be attempted in order to surpass the problem of short half-life, poor proteolytic stability and low oral bioavailability of this kind of compound [155]. In order to address this issue, a European consortium from five countries (France, Belgium, Spain, Portugal, Denmark) ran the European project VENOMICS, with a 6 million euros budget. The aim of the project was to investigate the structural and pharmacological diversity of venom peptides through the development of innovative "omics" technologies. Over 200 animal samples from different species were studied and analysed by transcriptomic and proteomic methods, and a toxin bank of sequences was constructed. More than 3600 most interesting sequences were set for production by chemical synthesis or recombinant expression and screened against several therapeutic targets allowing identification of active drug candidates [156]. This innovative approach has opened a new avenue in venom exploration without the limitation issues associated with the availability of natural products.

Fish n-3 Fatty Acid Derivatives (Omacor®/Lovaza®)

Omacor® is the brand name, in Europe, of an anti-hypertriglyceridemia highly purified preparation with high concentration of ethyl esters of n-3 fatty acids sourced from fish oils. The major constituents of this lipid-regulating agent are ethyl esters of eicosapentaenoic acid (52%) and docosahexaenoic acid (41%) [157] (Fig. 9.7). In the USA, this preparation is marketed under the trade name Lovaza®.

In contrast with other over-the-counter fatty acid preparations, Omacor/Lovaza® is a prescription drug launched in the European market by 2001 and in the USA by 2004 and is indicated as an add-on therapy to treatment with statins to reduce triglyceride levels in adult patients with severe hypertriglyceridemia [114]. It is also prescribed as adjuvant therapy for secondary prevention in post-myocardial infarction in Europe and the Middle East countries, although clinical trials report divergent conclusions concerning the role of n-3 fatty acid derivatives in these conditions [158]. These fish oil drugs are similar to fish oil dietary supplements, but their production is regulated in a different manner and has been tested in clinical trials. Prescription pharmaceutical products have to be manufactured according to standards of regulatory entities (FDA or EMA, for instance). This is of relevance in the case of polyunsaturated fatty acids, which can become oxidized resulting in degradation products that have a suspected harmful action. The manufacturing of Omacor/Lovaza® involves transesterification of fish triglycerides with ethanol in the

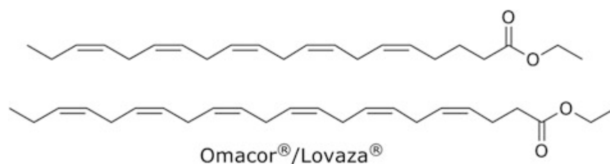

Fig. 9.7 Structure of the major ethyl esters of n-3 fatty acids from Omacor®/Lovaza®

presence of a lipase specific for the transesterification of the saturated and monounsaturated fatty acids and further separation of n-3 fatty acid ethyl esters from their saturated counterparts [159]. The procedure of transesterification has often been misunderstood by stating that it represents a refinement of ethyl esters of EPA and DHA, and the drug has been incorrectly referred as highly purified fish oil. Indeed, these ethyl esters are truly semisynthetic compounds, which are not present in fish or fish oils, and only after intestinal absorption breakdown into the fatty acids naturally produced in the body. The approach to this drug development came from the observation that certain ethnic populations, such as the native Alaskans, who had diets with a high content in polyunsaturated fatty acids, had low mortality rates from cardiovascular diseases [160]. Several clinical trials have been conducted in order to evaluate the efficacy and safety of Omacor/Lovaza® on hypertriglyceridemia [157]. Patients treated with this drug have shown a marked reduction in triglyceride levels [161]. Omacor/Lovaza® has also been shown to be safe and generally well tolerated. The precise way in which Omacor/Lovaza® works, as well as other n-3 fatty acids preparations, is not clear. However evidences from preclinical and clinical trials suggest that the n-3 fatty acids induce the decrease of the serum triglycerides concentration through several possible mechanisms: (a) reducing hepatic triglyceride synthesis by suppressing the expression of regulatory enzymes such as acyl-CoA:1,2-diacylglycerol acyltransferase; (b) increasing the β-oxidation of fatty acids, which in turn results in a reduction of the available substrate necessary for triglyceride and very-low-density lipoprotein (VLDL) synthesis; (c) reducing the incorporation of triglycerides into VLDL and triglyceride secretion; and (d) enhancing triglyceride clearance from VLDL particles [162].

Trabectedin (Yondelis®) and Lurbinectedin

Trabectedin is an antineoplastic drug from marine origin, sold under the brand name Yondelis® (Fig. 9.8). It is currently approved in Europe (EMA 2009) and the USA (FDA 2016) for the treatment of patients with unresectable or metastatic liposarcoma or leiomyosarcoma, which have received a prior anthracycline-containing regimen. It is also approved, in combination with pegylated liposomal doxorubicin, for the treatment of patients with relapsed platinum-sensitive ovarian cancer. Trabectedin has a complex mechanism of action that is not completely understood, but it appears that it affects key cell biology processes in tumour cells and acts as a microenvironmental modulator. Trabectedin binds to specific selected triplets of the DNA minor groove, interfering with cell division, genetic transcription processes and DNA

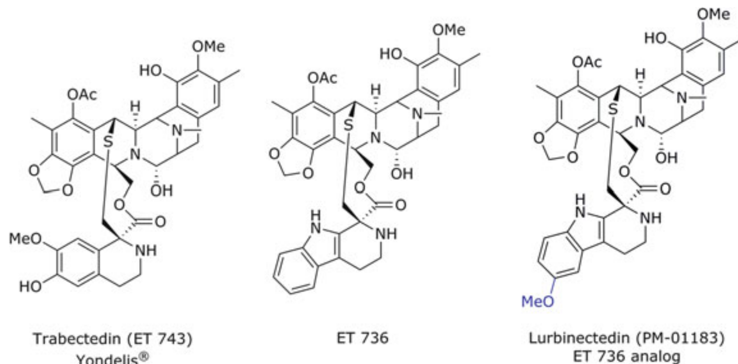

Fig. 9.8 Structure of natural ecteinascidins (ET 743, ET 736) from *Ecteinascidia turbinata* and their synthetic analogue lurbinectedin

repair machinery, including transcription-coupled nucleotide excision repair and homologous recombination repair [163, 164]. Besides these molecular mechanisms, trabectedin also has host-modulating properties that appear to be central for its therapeutic action. Data from preclinical and clinical evidence exposed the ability of this drug to decrease the number of tumour-associated macrophages and to modify the tumour microenvironment and angiogenesis [165]. Thus, it is reasonable to assume that trabectedin has multiple mechanisms of action, which can vary according the specific target tumour.

Trabectedin is an alkaloid originally isolated from the Caribbean ascidian *Ecteinascidia turbinata* under the support of a National Cancer Institute screening program, during the 1950s and 1960s, of plants and marine organisms [166]. In 1969 crude aqueous ethanol extracts of the ascidian were reported to possess notable in vivo anticancer activity, but, due to the minute amount of the active compounds in the extract, attempts to isolate the compounds responsible for the activity were unsuccessful [167]. It took almost 30 years until the structure of the active alkaloids, of which trabectedin was the most abundant representative (0.0001% yield) [168], could be completely elucidated simultaneous by the Rinehart [169] and Wright groups [170]. Ecteinascidins are highly sophisticated alkaloids formed by a monobridged pentacyclic skeleton composed of two fused tetrahydroisoquinoline rings (subunits A and B) linked to a 10-member lactone bridge through a benzylic sulphide bond. Additionally, ecteinascidins may have a tetrahydroisoquinoline or a tetrahydro-β-carboline ring (subunit C) attached to the rest of the structure through a spiro ring. In the case of trabectedin, subunit C contains a tetrahydroisoquinoline ring (Fig. 9.8). It took 1 ton of animals to isolate 1 g of trabectedin and about 5 g were believed to be needed for a clinical trial. To provide more material for clinical trials, a first enantioselective synthesis of the compound was achieved by Corey in 1996, using an approach with more than 30 steps and a 0.75% yield [171]. In 2000, a more effective synthesis was implemented in order to attain a more efficient, reproducible and economical route to work in the multigram scale [172]. In parallel

with chemical efforts for a synthetic pathway to assure the production of sufficient quantities of trabectedin to support clinical needs, an ambitious program of mariculture and aquaculture of *Ecteinascidia turbinata* was launched by PharmaMar in 1997 [168]. This approach provided enough quantity of trabectedin to carry on the clinical development but revealed to be impractical for commercialization. The cost of aquaculture and deep freezing facilities for biomass from one side, and the low extraction and isolation yield for the other, turned the process economically unfeasible. The problem was finally solved with the development of a semi-synthetic industrial viable route to produce trabectedin. This procedure uses safracin B, an antibiotic obtained by fermentation of *Pseudomonas fluorescens* on a kilogram scale, which is transformed in its cyanoderivative, providing a robust and cheap starting material for the synthesis of trabectedin [173]. Nevertheless the synthetic transformation of cyanosafracin B in trabectedin is accomplished with a yield of only 1.14% in a 20-step route [148]. The process to obtain trabectedin combined refined molecular and chemical tools. On the one hand, it took advantage of biomolecular tools that made possible to identify and clone the gene cluster responsible for the biosynthesis of safracin, which improved and manipulated the productivity of *Pseudomonas* sp. and used genetic engineering for synthesizing safracin [174]. Using the same semi-synthetic approach [175], PharmaMar developed another derivative of ecteinascidins, lurbinectedin (PM01183), for the treatment of various types of solid tumours, which is now in phase III clinical trials. Lurbinectedin (Fig. 9.8) is a novel synthetic tetrahydroisoquinoline designed after natural ecteinascidins containing a tetrahydro-β-carboline ring in the subunit C [176], being this last unit the only difference from trabectedin.

Eribulin Mesylate (Halaven®)

Eribulin mesylate gained FDA's approval in 2010 and EMA's in 2011, for metastatic breast cancer in patients who have received at least two prior chemotherapeutic agents (including an anthracycline and a taxane) [177]. In January 2016, FDA granted the Halaven® application priority review status and also the designation of orphan drug, in order to facilitate and expedite its application for the treatment of liposarcoma that cannot be removed by surgery or is metastatic [178].

Eribulin mesylate is an analogue of the marine natural product halichondrin B (Fig. 9.9), a large polyether macrolide isolated for the first time in 1986 from a rare sponge collected in Japan, *Halichondria okadai*, along with other macrolides, and subsequently found in unrelated sponge species such as *Phakellia carteri*, *Lissodendoryx* sp. or *Axinella* spp. [148]. Early studies on the cytotoxic activity of these compounds disclosed that they behaved as potent antimitotic agents which interacted with tubulin, a protein component of the cytoskeleton [179]. Halichondrin B and its synthetic analogue eribulin exert their pharmacologic effects, through a mechanism of action distinct from other tubulin-targeted drugs, either binding directly to the plus ends of microtubules or inducing tubulin aggregates by competing with soluble tubulin in addition to the growing ends of the microtubule. Unlike

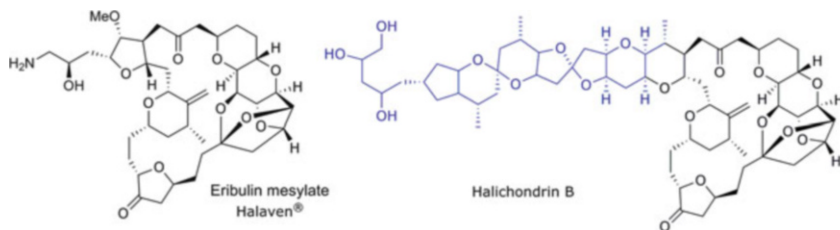

Fig. 9.9 Structures of the marine natural product halichondrin B from the sponge *Halichondria okadai* and the synthetic eribulin mesylate analogue

vinca alkaloids, eribulin destabilizes microtubules by blocking tubulin polymerization and inhibiting microtubule growth, but not shortening [180]. As in the case of other active natural products, the difficulty in collecting sufficient material and the extremely low yield of the active compound has hampered, for years, the development of halichondrin B for clinical application. In order to run the first preclinical trials, 10 g of compound were needed, and collection from the wild was obviously ruled out. The supply issue for the start of preclinical studies was partially solved by aquaculture of *Lissodendoryx* sp., which afforded 310 mg of halichondrin B from 1 ton of sponge [181]. However, the survival and growth of aquaculture sponges were highly dependent on climacteric conditions, and the yield of halichondrin B was lower than the one obtained from the wild typo sponge collected off the east coast of New Zealand's South Island [167]. Although aquaculture could partially address the supply, it became soon evident that the only viable alternative to obtain halichondrin B would be a synthetic pathway. The first total synthesis of halichondrin B was achieved in 1991 [182], but this route could not be regarded as cost-effective for industrial application. Subsequent studies identified the macrocyclic lactone C-1-C-38 moiety as the pharmacophore [183]. This discovery led the researchers from Eisai Research Institute in collaboration with Kishi and co-workers, from Harvard University, to synthesize a simplified macrocyclic ketone analogue (eribulin mesylate, former E7389), equally active, through a 62-step synthetic pathway, enabling the first clinical trials [184].

The development of eribulin mesylate was a significant paradigm shift in the pharmaceutical industry as it concerns natural product synthesis. Indeed, for some important anticancer drugs such as taxol or trabectedin, the supply problem was solved through semisynthesis, but in the case of eribulin, the lack of naturally occurring sources to support the hemi-synthetic strategy rendered the total synthesis the only feasible option to solve the material supply problem. The total synthesis of a compound with halichondrin's structural and stereochemical complexity on a marketable scale was unprecedented and currently stands alone [184]. Although eribulin has a structure considerably less complicated than halichondrin B, with 19 stereogenic centres instead of 32, as well as a shorter carbon backbone (36 carbons instead of 54), some very difficult issues had to be addressed in order to achieve a sustainable supply of the drug. Several synthetic routes for the preparation of eribulin have been disclosed, each of which utilizes the same strategy described by

Kishi and co-workers for the total synthesis of halichondrin B [185]. The development and launch of eribulin, considered the most complex non-peptidic synthetic drug on the market today [181], is a demonstration that organic synthesis has now the knowledge and the tools to surpass the structural complexity of natural molecules on an industrial scale and that a close partnership between academic and industry scientists is likely the future of drug development.

Brentuximab Vedotin (Adcetris®) and Close Analogues

Brentuximab vedotin (SGN-35) was approved by FDA (2011) and EMA (2012) for the treatment of relapsed or refractory CD30-positive lymphoproliferative disorders such as Hodgkin lymphoma and anaplastic large cell lymphoma [186]. Adcetris® has a mechanism of action completely different from Cytosar®, Halaven® and Yondelis®. It belongs to a special class of drugs called antibody drug conjugate (ADC), which are monoclonal antibodies, that specifically recognize a tumour-associated antigen, attached to a cytotoxic, small-molecule payload by chemical linkers with labile bonds. The antibody portion of the ADC serves as a transport vehicle that recognizes and binds to a protein antigen expressed in specific tumour tissues, allowing sensitive discrimination between healthy and diseased tissues [187]. Brentuximab vedotin is an ADC, containing an antitubulin drug, monomethyl auristatin E (MMAE), linked to the anti-CD30 monoclonal antibody cAC10 (Fig. 9.10). After binding specifically on the cell surface of CD30-positive malignant cells, brentuximab delivers the cytotoxic agent inside the cell via lysosomal degradation [188]. The development of this drug was particularly challenging and combined biotechnological innovations and chemistry.

The cytotoxic agent of brentuximab vedotin is monomethyl auristatin E, a derivative of dolastatin 10, a pentapeptide present in the extracts of the marine mollusc *Dolabella auricularia*, that showed outstanding antineoplastic activity back to 1972 [189]. As in the case of trabectedin, due to the very small percentage of the active compounds in crude sea hare extract, it took 15 years until the structure of the most potent compound could be elucidated: 1 ton of mollusc biomass was collected from the wild to isolate just 29 mg of dolastatin 10 [190]. Only 2 years later, Pettit's group achieved the total synthesis of dolastatin 10 [191], enabling the determination of its absolute stereochemistry and to tackle effectively the supply issues, in order to pursue with a broad assessment of cytotoxic studies. Later on it was found that dolastatins are in fact produced by the cyanobacteria *Symploca hydnoides* and *Lyngbya majuscula*, which are part of the sea hare's diet [192]. Despite the remarkable in vitro activity, dolastatin 10 was not suitable as a good drug candidate because appreciable in vivo activity was only attained at dosages where toxic side effects were significant. Taking advantage of Pettit's synthesis of dolastatin 10 that allowed structural and chiral modifications, many synthetic analogues, named auristatins, could be prepared in large quantities, MMAE being one of them. Senter et al. [193] from the American company Seattle Genetics had a remarkable idea that MMAE having high potency, water solubility

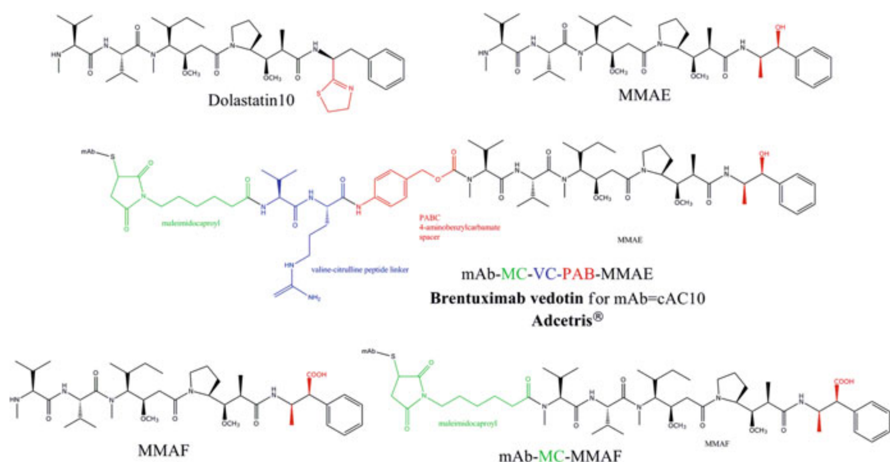

Fig. 9.10 Structures of the natural dolastatin 10, its derivatives MMAE, MMAF and brentuximab vedotin

and stability under physiological conditions, together with a structure that had a functionality able to be attached to a linker and subsequently conjugated to monoclonal antibodies, could be developed as a ADC. Brentuximab vedotin was designed by linking MMAE to the anti-CD30 monoclonal antibody brentuximab cAC10 that had been used in an unconjugated form in other clinical trials [193], through a protease-cleavable dipeptide linker. An accelerated FDA approval of this drug was granted only 5 years after the beginning of its clinical development, but it took nearly 40 years from the initial bioactive extract to the approved drug. The success of brentuximab vedotin was a major milestone for ADCs as a whole and prompted the clinical development of novel ADCs derived from dolastatin 10 analogues, namely, monomethyl auristatin E or F, for oncological diseases (Table 9.5, Fig. 9.10). There are currently 16 drugs in clinical trials using monomethyl auristatin E or F as payloads and different monoclonal antibodies according to the cancer target [125].

Iota Carrageenans (Carragelose®)

Carragelose is the brand name of a sulphated galactose polymer with unique antiviral properties (Fig. 9.11). It acts as a protective physical barrier in the nasal cavity, binding directly to the virus particles, thereby preventing adsorption to cellular receptors and subsequent internalization and reducing viral replication [196].

Carragelose® is a type of carrageenan, a family of linear sulphated polysaccharides that are extracted from red edible seaweeds. Carrageenans are widely used in the food industry, for their gelling, thickening and stabilizing properties. There are three main varieties of carrageenan, designated as iota, kappa and lambda carrageenan, according to the number and location of the sulphate groups on the hexose

Table 9.5 Antibody-drug conjugates based on dolastatin 10 derivatives undergoing clinical development, according FDA [194] and EMA [195]

Compound	Cytotoxic agent	Antibody target	Developer	Status EMA 2016	Status FDA 2016
Depatuxizumab mafodotin ABT-414	MMAF	EGFR	AbbVie	Phase II	Phase II
Glembatumumab vedotin CDX-011	MMAE	GNMB	Celldex Therapeutics; NCI (USA)	Phase II	Phase II
AGS-16C3F	MMAF	ENPP3	Agensys & Astellas	Phase II	Phase II
DNIB0600A Lifastuzumab vedotin RG-7599	MMAE	NaPi2b	Genentech Roche	Phase II	Phase II
Pinatuzumab vedotin DCDT2980S	MMAE	CD22	Genentech Roche	Phase II	Phase II
Polatuzumab vedotin DCDS-4501A	MMAE	CD79b	Genentech Roche	Phase I/II	Phase I/II
Tisotumab vedotin GEN701 HuMax®-TF-ADC	MMAE	TF (CD142)	Genmab Seattle Genetics	Phase I/II	Phase I/II
Denintuzumab mafodotin SGN-CD19A	MMAF	CD19	Seattle Genetics	None	Phase II
MLN-0264 Indusatumab vedotin	MMAE	GUCY2C	Takeda	None	Phase II
PSMA ADC	MMAE	PSMA	Progenics	None	Phase II
GSK2857916	MMAF	BCMA	GlaxoSmithKline	None	Phase I
ABBV-399 Telisotuzumab vedotin	MMAE	cMet	AbbVie	None	Phase I
AGS67E	MMAE	CD37	Agensys & Astellas	None	Phase I
ASG-15ME	MMAE	SLITRK6	Agensys & Astellas Seattle Genetics	None	Phase I
ASG-22ME Enfortumab vedotin	MMAE	Nectin-4	Agensys & Astellas Seattle Genetics	None	Phase I
SGN-LIV1A	MMAE	LIV1A	Seattle Genetics	None	Phase I

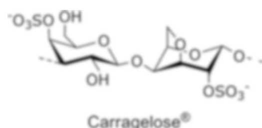

Fig. 9.11 Structure of the major carrageenan from red seaweeds present in Carragelose®

moiety [197]. Carragelose® is an iota carrageenan, which means that it has two sulphate groups per disaccharide repeating unit. Polysaccharides, such as carrageenans, were known to possess antiviral properties since 1985, but the antiviral effects of these compounds as drug leads were not explored because having high-molecular-weight components, they cannot pass across cell membranes, rendering difficult their use as drugs [198]. The vision of Marinomed Biotechnologie GmbH in using Carragelose® to block viral attachment to the host cells has overcome the difficulty, or even inability, of Carragelose® to penetrate in virus particles. In turn, this enabled the launch into the market of an innovative product that is effective against a broad spectrum of respiratory viruses.

2.2.2 Marine Natural Products and Close Analogues in Development

Besides the ten drugs from marine origin currently in the market, there is a robust and active pipeline of compounds in different stages of clinical trials. As of December 2016, the global marine pharmaceutical clinical pipeline included 25 drugs: 16 ADC (Table 9.5, Fig. 9.11), 6 MNP and 3 synthetic compounds based on MNP scaffolds (Table 9.6, Figs. 9.8 and 9.12).

Table 9.6 Marine natural products (NP) or derivatives (D) thereof in clinical trials according FDA [194] and EMA [195]

Candidate	NP/ D	Developer	Target area	Status EMA 2016	Status FDA 2016
Plitidepsin Dehydrodidemnin B Aplidin®	NP	PharmaMar	Cancer	Phase III	Phase III
Squalamine OHR-102	NP	Ohr Pharmaceutical	Neovascular AMD[a]	Phase III	Phase III
Lurbinectedin PM01183	D	PharmaMar	Cancer	Phase III	Phase III
Plinabulin NPI-2358	D	BeyondSpring Pharmaceuticals	Cancer	None	Phase III
Tetrodotoxin Tectin®	NP	WEX Pharmaceutical	Chronic pain	None	Phase III
DMXBA GTS-21	D	UF, USA VA Office R&D, USA CU, USA	CNS disorders Obesity	None	Phase II Phase II Phase I
Bryostatin 1	NP	Neurotrope BioScience NCI, USA	Alzheimer Cancer	None	Phase II Phase II
PM060184	NP	PharmaMar	Cancer	Phase II	Phase I
Marizomib NPI-0052 Salinosporamide A	NP	Triphase	Cancer	None	Phase I/II

[a]Neovascular age-related macular degeneration

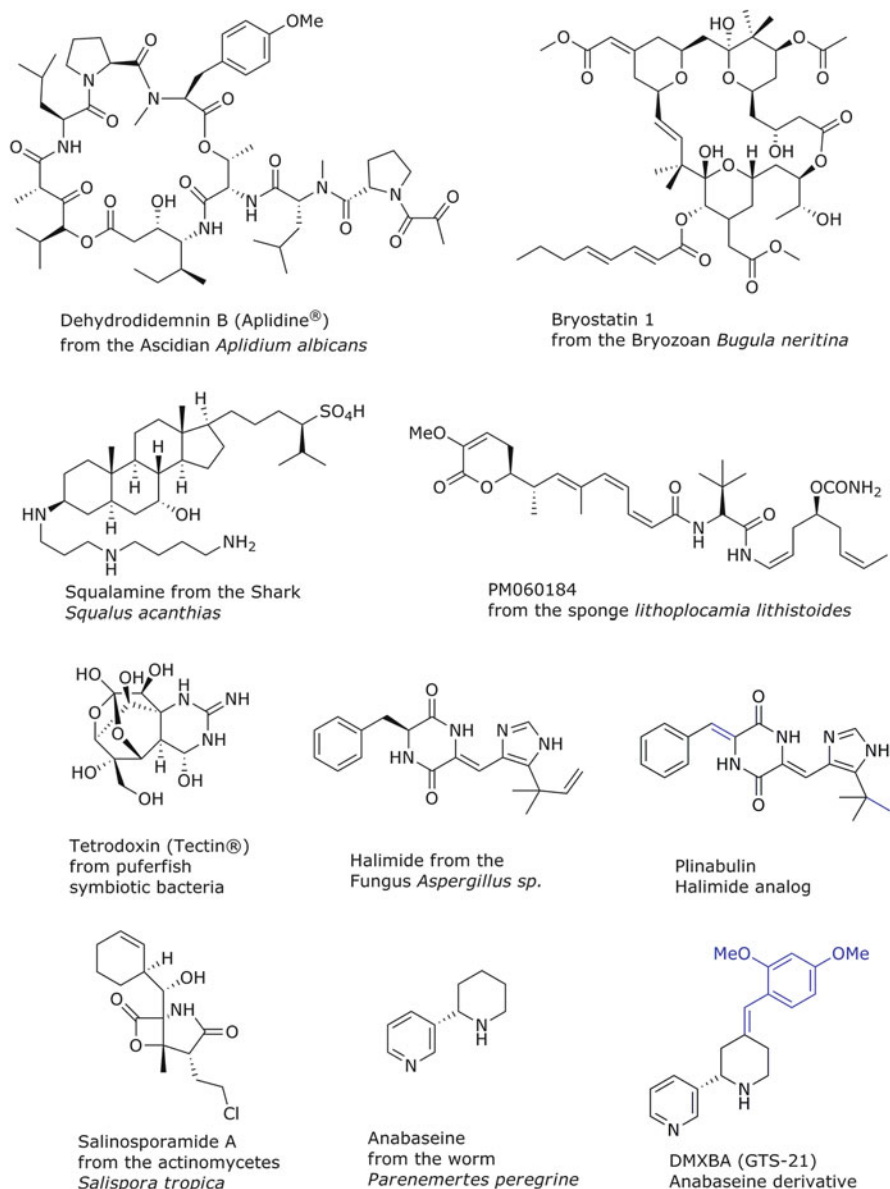

Fig. 9.12 Structures of MNP and their synthetic analogues under clinical trials

Dehydrodidemnin B [166], squalamine [199], tetrodotoxin [200], bryostatin 1 [201], PM060184 [202] and salinosporamide A [203] are synthetic equivalents of the metabolites isolated from the biological source. Lurbinectedin is an alkaloid synthesized based on the structure of natural ecteinascidins [127], plinabulin [127] is

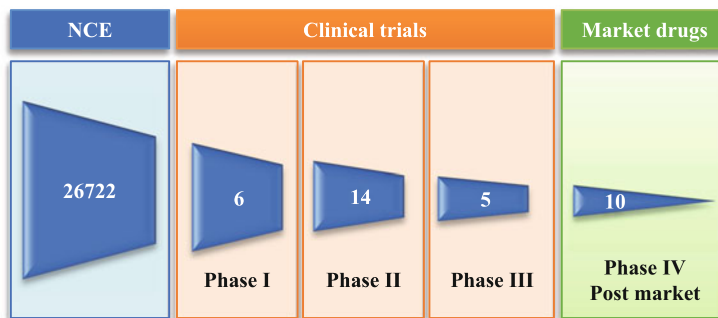

Fig. 9.13 Marine drug discovery and development pipeline [15]. *NEC* new chemical identities

a synthetic *terc*-butyl analogue closely related to the naturally occurring metabolite halimide [204], and DMXBA has a structure inspired by the natural anabaseine [205]. The leading therapeutic area is cancer (88%), but other targets such as Alzheimer's, schizophrenia or obesity are also being evaluated.

Figure 9.13 illustrates the evolution of marine-derived NCE through the several stages of clinical trials until they reach the market. At the moment 26722 NCE are listed in the MarinLit database. An update of literature data and Mayer's marine pharmaceutical clinical pipeline [126, 128] with information from FDA and EMA allowed to detect 6 compounds in phase I, 14 in phase II, 5 in phase III and 10 marketed drugs. For the purpose of this review, as represented in Tables 9.4–9.6, only compounds in their latest stage of clinical trials were accounted.

2.3 *Cosmeceutical Marine Natural Products*

The cosmeceutical market is the third market segment discussed in this chapter and one of the fastest growing applications for MNP. According to the FDA, a cosmetic is defined as "any article intended to be applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance without affecting the body's structure or functions". This broad definition includes also any material intended for use as a component of a cosmetic product too. Cosmetic preparations that are intended to prevent a disease or alter the structure or function of the human skin are considered drugs, like sunscreens or antidandruff shampoos. A cosmetic can have many formats like creams, oils, gels, ointments and lotions, but also oral forms and combinations are within this category. If these compositions contain active ingredients, they can be called cosmeceuticals (although sometimes we can call cosmeceutical to the specialty ingredient itself) and if in the ingestible form are denominated nutracosmetics or functional foods, further blurring the lines that separate these market segments. There are currently many cosmetic ingredients

and components, derived mostly from chemical sources but also from plants, minerals and, more recently, marine sources. In the past decades, a great deal of interest has been developed by consumers towards favouring novel natural sources for bioactive ingredients in cosmetics (but not only) due to their numerous beneficial effects against synthetic ingredients. The recent and continuous rising market share of organic- and natural-based cosmetics is just a proof of this trend. Natural sources of cosmetic ingredients and actives have long been used and are widely accepted by consumers, but the industry has been mostly focused on plants as sourcing materials. More recently, the cosmetic industry is increasingly turning to the sea and oceans in a search for new ingredients and is moving fast to fostering the use of marine biotechnology as a key tool in such development. Algae have been widely and anciently exploited for skincare applications but only more recently have other marine bioresources, such as phytoplankton and microbials, been sourced for new active and specialty ingredients for cosmetics and cosmeceuticals [206].

Table 9.7 summarizes all known cosmetic bioactives for which the authors could find publically available information.

Marine organisms have evolved biochemical and physiological mechanisms that include the production of bioactive metabolites for purposes such as reproduction, communication and protection against predation, infection and competition. Therefore, many marine-derived metabolites will have a major role and application in human systems, be it skin or internal organs. Interestingly, an increasing number of suppliers of the cosmetic industry are being pushed to include extracts made from coastal plants, seaweeds, algae, microbial organisms and biotechnology derived, as well as sea minerals, into cosmetic ingredients. These extracts contain vitamins and minerals and other actives, and they show ultraviolet and anti-oxidant protection and general anti-ageing benefits [22, 206–209]. In fact, activities such as antioxidant, antiwrinkle, anti-tyrosinase and antiacne are among the most usual activities of marine cosmetic ingredients for skin health [9, 22]. Biotechnology-derived ingredients have been booming in cosmeceuticals mostly due to the perfect fit between this technology and cosmetic market demands. Biotechnological ingredients offer versatility in brand positioning and trends; both high-tech cosmeceutical and natural/organic brands use them. Furthermore, new launches already include bacterial and yeast derivatives, being 78% in the facial and neck treatment categories. In skincare, biotechnology had a compound annual growth of 4.5% between 2010 and 2015, being one of the fastest growing segments within the cosmeceutical ingredients [210]. Hence, an entire new paradigm of beauty care, combining cosmetics and pharmaceutical properties into novel products with biologically active ingredients, is emerging, and these new specialty actives, also called cosmeceuticals (cosmetics + pharmaceuticals) or nutracosmetics, will be the hallmark of the next decades. In fact, the borderline between pharmaceutical and cosmetics is getting thinner, and the customer's demand for innovative, sustainable and truly efficacious products has brought the cosmetic industry to a whole new molecular and subcellular level of development. Moreover, it has opened new avenues for typical pharmaceutical companies to go faster to the so-called "low hanging fruit" markets, where development is faster and, yet, cheaper than a full drug, contributing also to this boom in

Table 9.7 List of cosmeceuticals with publically available information

Name of bioactive	Marine source	Claimed cosmetic activity
3M3.WHITERIG G®	Concentrate of *Dictyopteris membranacea*	Anti-ageing, anti-dullness, brightener, whitening
Abyssine® (EPS HYD657)	*Alteromonas* spp. extract	Anti-inflammatory, anti-UVB
ACB Wakame Bioferment Advanced	*Undaria pinnatifida* cell culture ferment extract	Anti-ageing, anti-ox, mitochondrial metabolism activator
Actigym®	*Bacillus* sp. extract	Slimming and toning
Actiporine 8G®	Microalgae *Jania rubens* active	Anti-ageing, antipollution, detox and slimming
Alariane AD®	*Alaria esculenta* extract	Hair care
Ambre Oceane®	*Pelvetia canaliculata* extract	Anti-ageing, plumping
Antarcticine®	*Pseudoalteromonas antarctica* extract	Cryo-protective, antiwrinkles, restructuring
ANTILEUKINE 6®	*Laminaria ochroleuca* extract	Photoageing protection, suncare
BIOENERGIZER P BG PF®	*Pelvetia canaliculata* + *Laminaria digitata* extracts	Hair care
BIOEXTENDER SPE®	Hydrolysed Rhodophycea sp. extract	Hair care
BIOPLASMA®	*Scenedesmus obliquus* extract	Anti-ageing, firming, antiwrinkle, hydrating, energizing
BIORESTORER PF®	*Hypnea musciformis* extract	Hair care
BLUE SEAKALE SC®	*Crambe maritima* extract	Photoageing protection, suncare
Brighlette®	Plankton extract	Whitening agent
Cellynkage®	*Halomonas eurihalina* EPS	Menopausal rejuvenator, collagen inducer
CELTOSOME®	*Crithmum maritimum* extract	Firming, antiwrinkle, anti-ageing, whitening
CHONDRUS CRISPUS FLAKES®	*Chondrus crispus* powder	Spa treatments
CITYGUARD +®	Sea water + microalgae oligoalginate	Anti-ageing, antipollution, detox
CODIVAVELANE BG PF®	*Codium tomentosum* extract	Moisturizing, hydration, skin barrier
Coraline Concentrate®	*Corallina officinalis* extract	Anti-hunger, slimming
COSTALENE®	*Skeletonema costatum* extract	Anti-inflammatory, suncare
DEPOLLUTINE®	*Phaeodactylum tricornutum* extract	Antistress, antipollution
DERMOCHLORELLA D®	Amino acid concentrate from *Chlorella vulgaris*	Healing, refirming, eye contour and anti-dullness
DICTYOPTERIS OIL®	*Dictyopteris membranacea* supercritical extracted oil	Plumping, filler

(continued)

Table 9.7 (continued)

Name of bioactive	Marine source	Claimed cosmetic activity
EarlyBoost®	*Jania rubens* taurine	Anti-ageing, anti-pollution
EPHEMER®	*Undaria pinnatifida* extract	Anti-ageing, colourant and suncare
ESCULANE®	*Laminaria digitata* extract	Hair care
EXO-H®	Exossine EPS extracted from Kopora (*Alteromonas* spp.)	Hydrating, skin barrier
EXO-P®	Exossine EPS extracted from Kopora (microorganism mat)	Anti-pollution, anti-oxidant
EXO-T®	Exossine EPS extracted from Kopora (*Vibrio alginolyticus*)	Anti-stretch marks, skin texture
Eyedeline®	*E. crustaceum* extract	Anti-eye bags, dark circles and wrinkles
GELALG®	*Chondrus crispus* extract	Tightening, anti-ageing, marine silicone, structural component, emulsifier
GREVILLINE®	*Skeletonema costatum* extract	Anti-inflammatory, suncare, anti-ageing
HOMEO-AGE®	*Ascophyllum nodosum* extract	Anti-ageing, antiwrinkle
HOMEO-SHIELD®	*Fucus serratus* extract	Moisturizing, hydrating, skin barrier
HOMEO-SHOOTE®	*Ascophyllum nodosum* extract	Shooting, sensitive skin
Hyadisine®	*Pseudoalteromonas* sp. extract	Hydration and water balance
Hyanify®	*Pseudoalteromonas* sp. extract	Antiwrinkle and rejuvenator
Hydranov®	*Furcellaria lumbricalis* oligofurcellaran	Hydration
HYDROXAN CH®	Chitin derived	Moisturizing, skin barrier
INULA®	*Inula chrithmoide* leaf/flower extract	Firming, antiwrinkle, anti-ageing
JUVEFOXO®	Jellyfish-derived peptide	Anti-ageing, DNA damage repair, suncare
JUVENESSENCE AD®	*Alaria esculenta* extract	Anti-ageing, antipollution
KALPARIANE AD®	*Alaria esculenta* extract	Plumping, anti-ageing
LANABLUE®	*Aphanizomenon flos-aquae* extract	Anti-ageing, antiwrinkles, cell turnover
LANACITYN PF®	*Alteromonas* ferment extract + *Chrysanthellum indicum* extract	Detox, antipollution
LEX®	Salmon egg extract	Antiwrinkles, nutrition
Marine Collagen	Animal origin	Anti-ageing
Matmarine®	*Pseudoalteromonas* sp. extract	Antiacne, oil control
MATRIGENICS 14G®	Fertile bases of *Undaria pinnatifida*	Anti-ageing, antipollution
MDI Complex®	Marine glycosaminoglycans	Dark circles, puffiness, sensitive skin

(continued)

Table 9.7 (continued)

Name of bioactive	Marine source	Claimed cosmetic activity
MICRO ALGUE 80®	*Solum diatomeae* powder	Spa treatments
NAIVE ESSENCE®	*Crithmum maritimum* extract	Anti-ageing, antipollution
NeuroGuard®	*Laminaria hyperborean* + *Lessonia nigrescens* oligosaccharides	Anti-ageing (neuroageing), antipollution
Nocturshape®	*Halomonas* sp. EPS	Circadian rhythm enhancer, slimming and anticellulite
OLIGOGELINE PF®	*Chondrus crispus* extract	Moisturizing, hydration, marine silicone, structural component, emulsifier
OLIGOPHYCOCORAIL SPE®	*Corallina officinalis* extract	Energizing, anti-ageing
PHEOHYDRANE®	*Laminaria digitata* biotechnology-derived polysaccharide + amino acids from microalgae *Chlorella vulgaris*	Hydration
Phormiskin Bioprotech G®	Microalgae *Phormidium persicinum* biotechnology-derived thioredoxin and pigments	Anti-ageing, oil control, detox
PHORPHYRALINE®	*Porphyridium cruentum* extract	Moisturizing, anti-ageing
PHYCOBOREANE 2C®	*Laminaria hyperborea* extract	Body shape, slimming
PHYCOCORAIL®	*Lithothamnion calcareum* extract	Photoageing protection, suncare
PHYCOJUVENINE®	*Laminaria digitata* extract	Anti-ageing, antipollution
Phycosaccharide AC®	*Laminaria* sp. symbiotic micro-organism oligosaccharide	Healing, antiacne, anti-dullness
Phycosaccharide AI®	*Laminaria digitata* biotechnology-derived oligosaccharide	Healing, antiwrinkles and anti-dullness
PYRENOINE PF®	*Fucus spiralis* extract	Anti-oxidant, anti-ageing
QT-40®	Macroalgae *Ulva lactuca* biotechnology-derived oligosaccharide	Plumping, reconstructive, collagen and hyaluronic acid synthesis inducer
Resilience® (Pseudopterosins)	*Pseudopterogorgia elisabethae* extract	Wound healing, anti-inflammatory, analgesic
RHODOFILTRAT PALMARIAG®	*Palmaria palmata* carrageen concentrate	Slimming, glow
RHODYSTEROL S®	*Gelidium cartilagineum* extract	Spa treatments
SCOPARIANE®	*Sphacelaria scoparia* concentrate	Firming, slimming
SCULPTOSANE®	*Scenedesmus obliquus* extract	Body shape, firming
SEA LAVANDER SG®	*Limonium gerberi* extract	Plumping, anti-ageing
SEA SATIN®	*Beta vulgaris* root extract	Hair care
SEA STARWORT BG/OIL®	*Aster maritima/tripolium* extract	Photoageing protection, suncare

(continued)

Table 9.7 (continued)

Name of bioactive	Marine source	Claimed cosmetic activity
SeaCode®	*Pseudoalteromonas* sp. extract	Antiwrinkles, lip filler
SEAMAYWEED SC®	*Tripleurospermum maritimum* extract	Photoageing protection, suncare
SEAMOSS BG PF®	*Chondrus crispus* extract	Moisturizing, hydration
SEASHINE®	*Alaria esculenta* + *Undaria pinnatifida* extract	Moisturizing, whitening
SILIDINE®	Microalgae *Porphyridium cruentum* extract	Shooting, anti-inflammatory, anti-Rosacea, heavy legs (endothelin-I stimulator)
THALASSINE 2G®	*Tripleurospermum maritimum* extract	Firming, antiwrinkle, anti-ageing
WAKAMINE®	*Undaria pinnatifida* extract	Moisturizing
XCELL-30®	*Halymenia durvillei* extract	Anti-ageing

the cosmeceutical industry. Business projections anticipate that the global cosmeceutical market will reach US$61 billion by 2020, and revenues are expected to grow at two digits in the next decades [211].

This growth is also driven by the rising desire of people to maintain healthy skin without using harsh chemicals. Capitalizing on the growing need to look younger, but at the same time using only natural and organic powerful agents, the need for marine bioactives is increasing as the marine environment is perceived as safe and innovative fulfilling current customer's trends. Further advances in technology, namely, marine biotechnology, are just another factor counting in to industries in the cosmetic field (and R&D labs and centres in MNP fields) answering to that demand. This has led to an unprecedented search for safe, effective and inexpensive natural bioactive ingredients from marine sources worldwide.

After pioneer Shu Uemura first incorporated deep-sea water into its skincare and make-up products, the cosmetic industry's interest in marine ingredients has finally followed the path to the ocean in search for innovation, and it looks far from ceasing. In fact, there is currently a thriving marine trend in cosmetics in recent years. An example clearly supporting this trend can be observed by performing a simple Google search for "sea water cosmetics", "ocean cosmetics" or "marine ingredient cosmetics", as it generates approximately 15,000,000 entries. Selected marine-derived actives have started to appear in new prestige skincare launches, including Elemis (The Steiner Group, London, UK), La Prairie (Beiersdorf, Montreux, Switzerland), Crème de la Mer (Estée Lauder, New York, NY, USA) and Blue Therapy (Biotherm, Tours, France), among many others. This high demand for marine ingredients is leading to several new raw materials, as well as specialty biotech firm's suppliers focusing on such products. The Norwegian firm Aqua Bio Technology has developed a novel range of ingredients derived from salmon hatcheries, while US-based Heliae is using new strains of algae for cosmetic applications. Other companies like Lipotec, CODIF and BiotechMarine are using biotechnology to harvest actives from marine sources. Mergers and acquisitions

(M&A) are happening fast in this sector too, with recent examples such as the SEPPIC acquisition of BiotechMarine or Lubrizol acquisition of Lipotec, demonstrating a savvy appetite for innovation in MNP for cosmetics and personal care industries.

This section will focus on reviewing current marine cosmetic active ingredients and drawing conclusions on what can foster further discovery and development of successful new products for this industry.

2.3.1 Seaweed-Based Ingredients

Classical Seaweed-Based Ingredients

Seaweeds have long been used as food, as healer in traditional remedies and as skin rejuvenator. Among marine organisms, sea algae have been identified as a source of cosmeceuticals due to their high levels of vitamins and minerals [7, 206]. However, many still consider that seaweeds are underexploited and their full potential as a cosmetic ingredient source is yet to be uncovered. Seaweeds are rich in vitamins and minerals and said to be very tolerant to desiccation, temperature variation and extreme harsh saline conditions. Additionally, the components of several species of seaweeds may make up a mixture product that could be very promising for skincare healing and maintenance in current stressful lifestyles [206]. Many commercial cosmetic components using a vast array of seaweed extracts are available, and *Laminaria* sp. extracts are probably the most abundant ones. *Laminaria japonica* is known to store marine minerals in a highly concentrated form and has been used to generate special algae-based ingredient active as ultraviolet radiation damage protectors. In addition, this species is also known for the production of effective moisture-binding agents that protects against dehydration, a feature that may prove to be harnessed in the future. Another example is the use of extracts of *Undaria* which are known to improve the condition of the extracellular matrix that keep the skin dense and plump while circulating water throughout epidermis. Recently other seaweeds and marine plants have started to be exploited. An Italian company called Lacote has in fact a vast range of skincare products using Guam algae (LACOTE 2017). Also, the German company OceanBasis possesses a huge portfolio of algae-based cosmeceuticals derived from their aquaculture farm in the Baltic Sea. The certified farm provides a sustainable source of active ingredients for its Oceanwell line (OCEAN BASIS 2017).

Other bioactive components of seaweed have been isolated and tested as cosmetic bioactives, like phlorotannins, diverse polysaccharides and carotenoid pigments.

Phlorotannins (Fig. 9.14) are a highly hydrophilic component of many marine organisms but are especially abundant in brown algae like kelps. They exist in many forms and have several intermediate configurations too. Studies have demonstrated that phlorotannins can have antidiabetic, anticancer, anti-oxidation, antibacterial, radioprotective and anti-HIV properties. However, in vivo studies on the effects of these compounds are lacking, most of the research having so far been performed in vitro [212, 213].

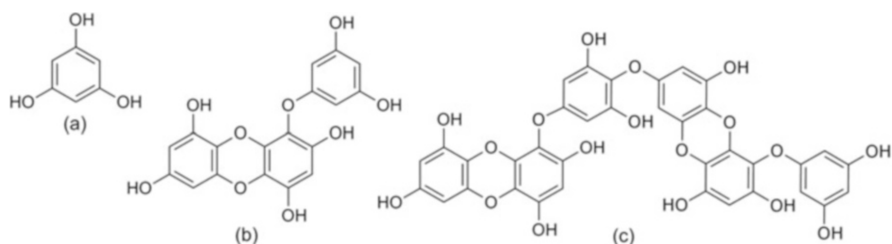

Fig. 9.14 Some phlorotannins derived from marine algae. (**a**) Phloroglucinol, (**b**) eckol and (**c**) dieckol

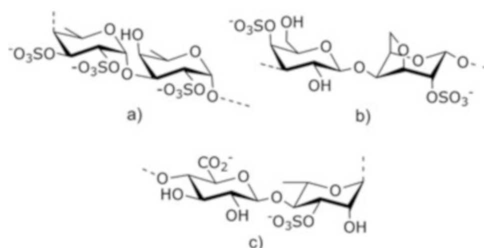

Fig. 9.15 Sulphated polysaccharides derived from marine algae (**a**) fucoidan, (**b**) carrageenan and (**c**) ulvan

Algae are very rich in polysaccharides (Fig. 9.15) as their cell walls have plenty in their constitution, but they vary slightly depending on the species. For example, brown algae are rich in fucoidans, red algae in carrageenans and green algae in ulvans. These components have been shown to have several cosmetic benefits, but fucoidan is by far the most widely used; several studies have already demonstrated its effects, ranging from anti-ageing and whitening to anti-inflammatory [214], and these components are already used by brands in Europe and Japan.

Carotenoids are a group of pigments produced by plants, fungi, algae and microorganisms or present via diet in animals. Several seaweed-derived carotenoids (Fig. 9.16) have been described, but fucoxanthin and astaxanthin have been known to be major cosmetic ingredients. These components are described by many brands as super ingredients for their plethora of effects and studies have supported their activity as fat metabolizers, anti-inflammatory, antinociceptive and anticancer [215].

Biotechnology-Based Seaweed Ingredients

Companies such as SEPPIC (BiotechMarine—SEPPIC Group) or Lipotec are strategically using novel biotechnology-based seaweed ingredients to gain a high-end market positioning. Herein, biotechnology-based seaweed ingredients are intended to describe commercial products that are obtained via laboratory manipulation of growth, induction of metabolites, extraction, separation and/or combination of

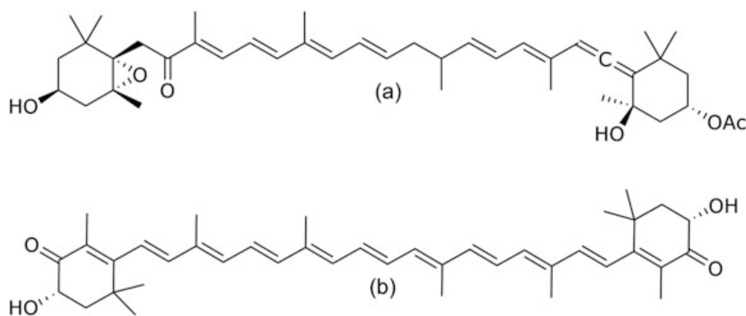

Fig. 9.16 Carotenoids derived from marine algae (**a**) fucoxanthin and (**b**) astaxanthin

compounds or mixtures of compounds derived from seaweed using molecular biology and biotechnology tools in their processing. Examples include products such as Alariane AD®, an *Alaria esculenta* extract originated from the Artic, that has an anti-frizz effect and is commercialized for hair care, or EPHEMER®, a gametophyte extract from *Undaria pinnatifida* that reinforces the skin anti-oxidant capacity by contributing to mitochondrial DNA protection via a 63% increase in aconitase activity and protecting cells from free radicals by increasing 19% in catalase activity [216]. CODIF, a Brittany French marine company is also very active in developing cosmetic active ingredients from macroalgae, with its product PHEOHYDRANE®, a combination ingredient of Kelp extract and *Chlorella* microalgae cell wall extracts, promoting hydration of skin via water retention. According to the manufacturer, PHEOHYDRANE® is obtained biotechnologically from the membrane polysaccharide of the brown algae combined with amino acids extracted from the microalgae, *Chlorella vulgaris*, and minerals obtained from sea water. The elements in this product, complexed to the polysaccharide, are essential to water retention in skin layers. Thanks to their strong hygroscopic potential, they have a restructuring effect on the hydrolipid film and the skin barrier, thus preventing dehydration phenomena and allowing the active bringing of water to the skin epidermis layers [217]. Clinical trials revealed that after applying this product twice a day for 7 days, moisture levels increased by 215% but remained unchanged with the placebo. Additional tests using the transient thermal transfer (TTT) and the Hydrascan confirmed increased hydration levels at the deepest and outermost layers of the skin. Another example, from the same company, is MATRIGENICS 14G®, a concentrate of fertile bases (reproducing containing parts of the algae) from *Undaria pinnatifida* with restructuring and rejuvenating facial properties [217]. Within this product there is wakamic ester, a sulphated galactofucan able to activate the genes involved in the synthesis and organization of the main components of the extracellular matrix: collagen, elastin and hyaluronic acid. In vitro studies have demonstrated that this MNP activates genes like COL1, COL4, COL8 or LUM involved in collagen synthesis and assemble; ELAST, FBN1 or LOX involved in elastin synthesis and assemblage or even HS or CD44 involved in acid hyaluronic synthesis and receptor activity [217]. These results were confirmed with clinical studies demonstrating that

MATRIGENICS 14G® significantly decreases volume and area of forehead wrinkles by, respectively, −23.8% and −14.4% on average versus placebo and has an impact in nasal furrows or mouth wrinkles too [217].

2.3.2 Microalgae Biotechnology-Based Ingredients

Marine microalgae cosmetic applications are a fast and growing trend among specialty cosmetic producers. Microalgae-derived products and functional ingredients have several advantages for the cosmetic market as they can be branded as plant-derived, natural and sustainable and are the source of some of the most innovative solutions in our products today. Their importance is so evident that some cosmetic brands have their own microalgae manufacturing units. Microalgae are microscopic unicellular organisms that can live in marine or fresh water and are capable of converting solar energy to chemical energy via photosynthesis. They also produce a wide range of bioactive metabolites such as proteins, lipids, carbohydrates, carotenoids and vitamins that can be exploited for commercial use in the food or cosmetic industry. Some microalgae species are established in the skincare market with the main ones being *Arthrospira* and *Chlorella* [218]. Microalgae extracts are incorporated in many face and skincare products (e.g. anti-ageing cream, refreshing or regenerating care products, emollient and anti-irritant in peeler masks), sun protection and hair care products. Microalgae-derived new polysaccharides, especially if sulphated, are particularly interesting because they can offer a promising and innovative alternative to the existing seaweed-derived ones and underlie the development of new functional cosmeceutical products.

Some of the most recent advances in novel microalgae functional bioactives derived from marine biotechnology include Actiporine 8.G®, Dermochlorella DG®, XCELL-30®, Alguronic Acid® and Alguard®.

Actiporine 8.G® and Dermochlorella DG® are both from CODIF Recherche & Nature (Britany, France). The first is derived from a *Jania rubens* extract produced in photobioreactors and has been shown to have an anti-ageing effect via Actiporine 8 channel activation. It has been shown that Aquaporin 8 (AQP8), a membrane channel, already known for its role in the transport of urea, is also capable to ensure the passage of H_2O_2 through the mitochondrial membrane. The anti-ageing strategy developed by Actiporine 8.G® is based on an increase in the number of AQP8 to promote H_2O_2 evacuation, mitochondrial detoxification and therefore the activation of collagen synthesis by fibroblasts. As for the second one, it is a *Chlorella* sp. extract containing oligopeptides that act as restructuring actives on the dermal-epidermal junction to increase firmness and skin tone. The active substance also acts on the epidermis to erase vascular imperfections as it boosts collagen production into blood vessels (dark circles and small vascular imperfections) [217].

XCELL-30® from Greensea (Mèze, France) is developed from microalgae endemic to Madagascar and specifically acts on cellular turnover in the basal layer of the epidermis, thus allowing the preservation of the youthful characteristics of the skin [219].

Alguronic Acid® from Algenist (San Francisco, CA, USA), a novel and powerful microalgae compound, is responsible for regenerating and protecting the microalgae cell in harsh environments and conditions. When scientifically tested, Alguronic Acid® demonstrated significant anti-ageing properties, helping to rejuvenate the skin for a more youthful appearance [220].

Alguard® is a natural sulphated polysaccharide compound isolated from a red microalga (*Porphyridium* sp.) that acts as a shield, creating a thick protective layer around the cell and protecting it in its intertidal extreme environment. Research has revealed a wealth of biological activities, demonstrating that Alguard® is not merely a physical barrier but an active protection against photo damaging, ageing and microabrasion of the skin [221].

2.3.3 Specialty Biotechnology-Derived Active Ingredients

Many are the cosmetic ingredients not necessarily derived from algae but from other marine organisms such as corals, sponges or microorganisms. This type of ingredients tends to be specialty active ingredients due to their complex nature (usually they are isolated compounds, mixtures of known compounds or simpler fractions derived from original extracts), their chemical profile (novel molecules and/or complex macromolecules) and even the amount of data usually produced around them before market introduction. Previously some were already mentioned for macroalgae, but a growing number of these ingredients derived both from microorganisms or marine biotechnology are emerging. The following are some of the most well-known or already established specialty active ingredients of marine origin and/or marine biotechnology.

The First Commercial Success: Abyssine® by Unipex (New York, NY, USA)

Abyssine® was one of the first specialty ingredients introduced successfully in the cosmetic market, by Unipex, in the late 1980s. Exopolysaccharides (EPS) are one of the most common molecular classes of compounds used in the personal care bioactive ingredient sector. Various microorganisms, including proteobacteria, cyanobacteria and archaea, produce EPS. These EPS-producing bacteria abound in marine ecosystems and can be isolated from the water column, sediments, animals, etc. Bacteria-producing polymers with novel structures and innovative properties have been isolated in atypical environments, including extreme environments [222, 223]. The ocean has plenty of those, such as deep-sea hydrothermal vents. These are areas of plate tectonics with diverse physicochemical characteristics, prone to the development of microorganisms with chemical defence systems against harsh environments. Many bacteria living near hydrothermal vents have several types of associations with other organisms (shrimp, worms or molluscs), and some can actually produce EPS under laboratory conditions. These bacteria are mostly found to be part of the genera *Vibrio*, *Alteromonas* or *Pseudoalteromonas* [224–229]. Marine bacterial EPS have

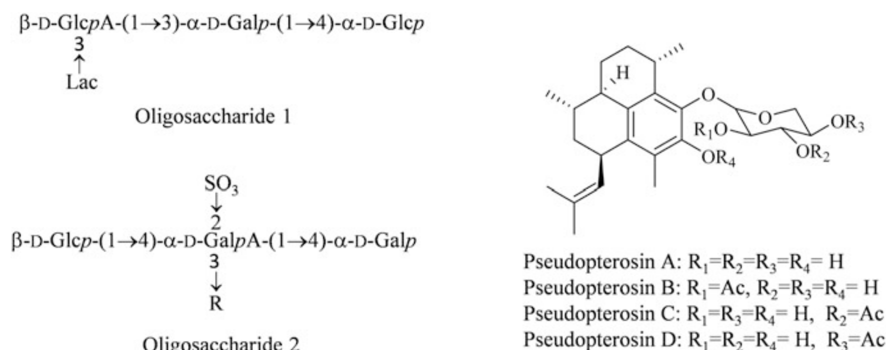

Fig. 9.17 Chemical structures of *deepsane* oligosaccharide 1, oligosaccharide 2 and pseudopterosins

various physiological roles: they are involved in responses to environmental stress, in recognition processes and cell-to-cell interactions and also in adherence of biofilms to surfaces [230].

Abyssine®, introduced in the market by Lucas Meyers, is an *Alteromonas* ferment extract containing the EPS HYD657, named *deepsane*, and it is produced and secreted by the strain *Alteromonas macleodii* subsp. *fijiensis biovar* deepsane [224]. This strain was collected in 1987, close to a hydrothermal vent located on the East Pacific Rise at 2600 m depth, from the annelid polychaete *Alvinella pompejana* [231]. Although discovered in the late 1980s, only in 2012 some light was shed into the chemical structure of deepsane [232], which has been concluded to be a high-molecular-weight polymer of 1.1×10^6 g/mol constituted by two different oligosaccharides (Fig. 9.17).

Several biological screenings were performed in order to evaluate the bioactivity of this particular EPS, and it was shown that it effectively protects keratinocytes from inflammatory agents, such as interferon gamma (INF-γ) and intercellular adhesion molecule 1 (ICAM-1). Protective effects have also been demonstrated in cells sensitive to ultraviolet attacks and play a major role in the human cutaneous immune defence system [233]. *Deepsane* has found its market route via applications in cosmeceuticals and is commercially available under the name of Abyssine® for soothing and reducing irritation of sensitive skin against chemical, mechanical and ultraviolet B (UVB) aggression. This bioactive ingredient is commercialized as a ferment extract, and therefore Abyssine® is also rich in minerals, proteins, organic and inorganic compounds as well as amino acids.

A Very Strong Brand Successful Marine Line: Resilience® by Estée Lauder (New York, NY, USA)

Resilience® is derived from a special extracellular extract from the Caribbean Sea whip (gorgonian) *Pseudopterogorgia elisabethae* (*Gorgoniidae*) and is a line of

skincare products from the Estée Lauder Companies. This extract is mainly composed by pseudopterosins, which are tricyclic diterpene glycosides (Fig. 9.17). As with many other natural compounds, its initial development was aimed at a pharmaceutical application, but, having in mind potential skin applications and the faster cosmetic development timelines, it reached the skincare market utilization first. The pseudopterosins are potent anti-inflammatory and analgesic agents that inhibit eicosanoid biosynthesis by inhibition of both phospholipase A2 (PLA2) and 5-lipoxygenase. Intriguingly, these compounds are found to inhibit only human neutrophils-PLA2, but not PLA2 from other sources [234]. The pseudopterosins have been originally licenced to a small and medium enterprise (SME) pharmaceutical company, OsteoArthritis Sciences Inc. (Cambridge, MA, USA), for medical use as potential anti-inflammatory drugs. Preclinical tests for one of the pseudopterosins, a potent topical anti-inflammatory compound, were undertaken, and an Investigational New Drug (IND) application filed with the FDA. Additionally, a derivative of a natural pseudopterosin, methopterosin, has completed Phase I and II clinical trials as a wound healing agent. It was however discontinued due to the lack of strong effect and drugability. The high lipophilicity and lack of aqueous solubility have hindered the study of the pseudopterosins as drugs for many years. The amphiphilic structure of pseudopterosins would be assumed to impart some aqueous solubility, but in fact, the pseudopterosins have little to none, and this limits the efficacy of the drug in biological model systems. Several methods have been explored to alter their physical properties (e.g. their solubility) and bioavailability in biological systems including the synthesis of pseudopterosin succinate salts and the production of alternative formulations [126, 235].

The original extract of pseudopterosins has found its place into the marketplace via the personal care route, as the regulatory framework for cosmeceuticals allows the use of complete extracts once the efficacy and safety of it as a whole is demonstrated. It was introduced as an additive to prevent irritation caused by exposure to the sun or to chemicals in the Resilience® Estée Lauder cosmetic skincare product [236].

Recent Innovations and Marine Biotech Role: Noctürshape®, Cellynkage® and SeaCode® by Lipotec (Barcelona, Spain)

Nocturshape® by Lipotec is one of the most recent market launches of marine biotechnology-derived products for the cosmetic market [210]. It is a marine biotechnology-derived exopolysaccharide (EPS) that acts specifically on nocturnin, a circadian deadenylase, mainly active in humans during the night period, which plays an important role in post-transcriptional regulation of metabolic genes under circadian control. It degrades poly(A) tails of specific target mRNAs leading to their degradation and suppression of translation. It also exerts a rhythmic post-transcriptional control of genes necessary for metabolic functions, including nutrient absorption, glucose/insulin sensitivity, lipid metabolism, adipogenesis, inflammation and osteogenesis. This protein plays an important role in favouring adipogenesis

over osteoblastogenesis and acts as a key regulator of the adipogenesis/osteogenesis balance. Nocturnin activation promotes adipogenesis by activating the peroxisome proliferator-activated receptor gamma (PPARG) transcriptional activity in a deadenylase-independent manner by facilitating its nuclear translocation [237, 238]. Given its action, Nocturshape® was introduced into the market as a blue sliming and anticellulite ingredient for body contour products.

Cellynkage® by Lipotec is another recently launched marine natural product ingredient, specific for menopausal skincare. It is also a biotechnology-derived exopolysaccharide whose main action is to improve both the direct communication among neighbouring skin cells and the signalling exchange between epidermal and dermal cells. The manufacturer Lipotec claims that in vitro testing demonstrated it improved skin thickness, collagen density and microrelief structure, as well as increased skin surface smoothness (56 days, 2%); it was launched as a complete menopausal skin rejuvenation ingredient [210].

Another example of an EPS with tremendous market impact is SeaCode® by Lipotec, a mixture of extracellular glycoproteins (GPs) and other glucidic exopolymers produced by biotechnological fermentation of a *Pseudoalteromonas* sp. isolated in the intertidal coasts of Antarctic waters [210]. GPs are essential molecules in any living organism, appearing in nearly all biological processes. They consist of polypeptides covalently bounded to oligosaccharide chains (glycans), where the carbohydrate may represent from 1% to 80% of the total mass and can be either O-linked or N-linked. Consistent with this, GPs possess a large diversity of properties and functions, playing a key role in cellular protein maintenance, cell-to-cell communication, stress recovery and as constituents of cell walls and therefore much sought after for skincare and reconstitution dermal and epidermal purposes. These macromolecules are often significant integral membrane proteins, where they influence cellular interaction. GPs mediate the adhesion between cells, which is essential for the development of functional tissues, as well as cell-substrate unions where they act as receptors for adhesion ligands, as it occurs with fibroblasts and fibronectin. This capacity has structural effects when binding cells with proteins like collagen, for example, as it offers strength and support to the matrix [239]. Moreover, GPs can act as vehicles (e.g. for vitamins and hormones), as key hormones (erythropoietin), as enzymes (transferases, oxidoreductases and hydrolases), as protecting and lubricating agents (mucins or elements secreted by the lachrymal and sweat glands), as cryoprotecting molecules that guard from freezing by modifying or avoiding crystal formation and as vital elements of the immune system (surface compounds of B or T cells with bacteria-binding properties or immunoglobulins) [240]. The combination of GPs and EPS has therefore the potential to provide not only complete protection of the skin but also reconstruction effects as well.

To date, there is no available data on the structural composition of SeaCode®. However, on the company page, it is stated that SeaCode® is a bioactive ingredient containing EPSs isolated from *Pseudoalteromonas* bacteria that proved to ameliorate skin properties by highly enhancing the in vitro synthesis of essential dermal proteins (like type I collagen) in human dermal fibroblast, adult (HDFa) cells, maintaining cutaneous tonicity and firmness of those fibroblasts. In vivo tests on a

panel of volunteers have demonstrated that SeaCode® offers a statistically significant effect in improving skin roughness by 16.5% and 25.1% after 1 and 4 weeks, respectively, mostly at the upper lip wrinkles, due to its replenishing effect [210].

Besides these ingredients, Lipotec has developed other interesting and potent marine-derived cosmetic ingredients, such as Hyadisine®, Antarcticine® or even Hyafin®, mostly isolated from libraries of microorganisms constructed from samples originating from extreme condition locations. In parallel, CODIF, Greensea or SEPPIC (Marine Biotech) are also actively launching novel specialty ingredients derived from marine biotechnology using microorganisms or microalgae and seaweed, or even, more recently combination products, supporting the predictions of a strong boost in marine natural products for cosmeceutical markets.

2.3.4 Other Non-premium-Derived Marine Bioactive Ingredients

In addition to the specialty ingredients mentioned above, the marine environment contains a source of polyunsaturated fatty acids (PUFAs), polysaccharides, essential minerals and vitamins used as cosmeceuticals. Furthermore, many have been using hyaluronic acid, collagen or elastin from several different marine origins as a substitute for synthetic or land-based animals that are less well accepted by consumers worldwide, either due to infectious and/or contamination reports or religious constraints. Potassium alginate and fucoidan from brown algae, aluminium silicate from sea mud, chitin from crustaceans and shell powder from oysters are some more examples of less differentiated but widely used marine active ingredients. Sea mud contains various nutrients and minerals which make it recognized for its therapeutic properties against psoriasis and other skin-related disorders [209]. Another example is marine phytoplankton that is known to contain more than 65 amino acids, essential fats, vitamins and key minerals, among other active ingredients with cellular regeneration capacities. New trends are pointing towards the use of food sources such as marine fish-derived collagen and gelatine as excellent functional ingredients for the cosmetic industry [241]. In fact, the high moisturizing properties of these compounds make them suitable as novel ingredients for cosmetic creams and gels.

Other marine-derived bioactive peptides are being explored. These peptides are inactive in the sequence of their parent protein and can be released by enzymatic hydrolysis of marine organisms or seafood by-products. These 3–20 amino acid peptides have bioactivities that are based on their sequence and composition and several have been described in the literature like chitooligosaccharide (COS) derivatives [22].

It is interesting to note that some of the best within the class of marine skincare ingredients originated from pharmaceutical programs, and most of them failed in their development as drugs, not necessarily by the lack of function but because the pharmaceutical development has many and several other constraints, as already discussed. Consequently, some of these scientific discoveries found their space in the market via cosmetic science and product development and represent interesting business cases, as well as demonstrate that there is a great need for innovation in

skincare sciences. This track record also illustrates the opportunity for increasing MNP success rates via exploring alternative, nondrug routes. Hence, due to the ability to create such interesting ingredients with scientifically demonstrated activities and impacts, marine skincare is truly a wave of the future in beauty and marine product development.

3 Major Challenges and Suggestions for Success in MNP Development

MNP discovery and development faces a series of hurdles and bottlenecks along its pipeline from ocean to the market. MNP can be used in a variety of market applications, and each segment has its own bottlenecks and specificities, but there are common challenges, and solutions, to all markets. The greatest hindrance to the development of MNP in any sector is, with no doubt, its cost-effective production, which is closely related with a series of specific challenges. In this section, challenges will be analysed in the nutraceutical, pharmaceutical and cosmeceutical segments providing not only a specific overview for each segment but also outlining lessons learned from common challenges and using past experiences and strategies from one segment to overcome bottlenecks in another segment. A deep analysis to all challenges has allowed their mapping and distributing to the different market segment (Fig. 9.18), namely, biodiversity, supply and technological and market challenges.

A macroanalysis of this map (Fig. 9.18) clearly distinguishes common and specific market challenges in each group and allows the identification of potential general and specific suggestions for success in each challenge group.

Despite the singularities of each market segment, they share the same challenges in terms of the biodiversity group. In all market segments using MNP, access to raw material to initiate a discovery program, and for later supply, is crucial. This is a first and yet determinant hurdle to overcome, as the ocean is still very hard place in which to bioprospect, and there is an immense amount of knowledge not yet available about the deep sea, extreme habitats and remote sea areas. As it was pointed earlier, the search for biologically active compounds from marine sources has occurred either exploring unexploited taxonomical groups and geographical sources or exploring new taxa and/or regions of confirmed chemical diversity, or even combining both of these strategies [12, 242]. In order to improve the success rate of discovery of new active MNPs, it is important to focus on optimizing sampling strategies in order to ensure fully accessibility to the ocean biodiversity [243]. This biodiversity is governed under the host of the Convention on Biological Diversity (CBD) and its Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits, and pharmaceutical and cosmeceutical industries, particularly, will profit from the access to a larger range of marine genetic resources (MGR). However, despite the advances on the marine agenda, there are some issues

Fig. 9.18 Major challenges and tips for success in MNP development for nutraceutical, pharmaceutical and cosmeceutical development. Numbers correlate among each box colour. *NGS* new-generation sequencing, *USP* unique selling proposition

that remain unclear that can hold back the access to MGR, e.g. their distribution in areas involving several states or in international high seas and deep seabed areas [244]. Therefore, harmonization of Nagoya Protocol in different countries, with tighter control supervision by national jurisdictions, and a fair, transparent and simple access scheme to this genetic biodiversity by non-locals, is needed to boost industry interest and developments in this arena.

Using advanced submersible technologies, such as manned submersibles and, more recently, remote-operated vehicles (ROVs), remarkable new deepwater ecosystems have been discovered, many of them in harsh environments devoided of

light and under crushing pressure, which have modulated the structures and properties of metabolites from organisms inhabiting these zones [245]. Nevertheless, more technology is needed to allow the development of more sophisticated vehicles that can go deeper in the ocean. This way, the collection in locations never reached before will enable the identification of novel organisms and potentiate findings of novel structures with new market applications. At the same time, it is important to make robotics more accessible and to democratize its access to a wider range of users. Indeed, these amenities are available only in wealthy laboratories, a fact that is particularly critical because the majority of biological diversity is located in underdeveloped countries from tropical and subtropical regions [246]. This is one more reason for why a good implementation of the Nagoya Protocol is crucial, to overcome the legal aspects to access and utilize MGR by scientific groups with more developed engineering and technological knowhow.

To learn more about the original environment of organisms, the physicochemical conditions of their habitat needs to be studied and this knowledge integrated into potential uses and applications. Such a strategy has been taken by several successful programs aiming to identify potential novel marine bioactives. Efforts are also needed to improve marine taxonomic expertise, employing both classical and molecular methods, to speed up the identification of species diversity, gaining competitiveness and making marine biodiscovery more efficient. On the other hand, the development of omics technologies enables the analysis of uncultured, unknown and hidden biodiversity in ocean samples. Metagenomics, single cell isolation and amplification methods, as well as whole or partial community new-generation sequencing (NGS) tools, are fostering the knowledge of marine biodiversity to a new degree, opening new avenues to circumvent these bottlenecks. Finally, considering alternative and yet unexploited sources of raw materials, such as waste raw products of marine industries (like seafood processing industries, including canning industries) and by-catch wastes, can be a good and sustainable alternative source to biodiversity access and novel MNP development totally aligned with current trends towards zero waste and circular economy concepts.

Globally, independently from the market segment, MNP discovery is on high demand for novel rapid screening tools, hit identification and hit-to-lead faster development processes, with the production of commercially viable yields at the centre of this challenge; it is thus mandatory to explore new approaches to compete successfully with other alternative bioactive discovery sources and methods.

If having access to biodiversity is a sine qua non requisite to a successful MNP development, it is not sufficient per se. The development of strategic and market-oriented screening programs is a must for shorter market entry and higher success chances, and this is also valid for any MNP market segment. Within the supply and technological challenge group, many are the common challenges too, like the need to streamline the biodiscovery tools and pipelines, the need to match separation and purification processes, as well as production technologies and supply demands to specific market requisites and consumer trends. More meaningful and productive screening programs are the key to find new hits and leads in natural product libraries that can generate a new product. Appropriate targets, library

format and deconvolution tools and use of faster and sophisticated high-throughput screening (HTS) technologies are paramount to overcome this bottleneck in all markets. However, when it comes to manufacturing and sustainable supply of bioactive and/or MNP, this is even more difficult, being heavily dependent of each market segment regulations and requirements. Still, common solutions are related to the source of MNP used and the growth and/harvest methods employed. Without doubt, supply issues have been the main shortcoming that development of bioactives has faced. This has only been partly overcome when synthetic procedures or biotechnological production reached a high level of progress. Another major route being pursued to surpass the limitation of raw biological material needed for a successful product development is the increased focus on marine microorganisms, since fermentation and scale-up of production processes using cultured microorganisms can eliminate supply problems. Nevertheless, further improvements still must be made regarding the identification, cloning, genetic manipulation and expression of biosynthetic pathways in order to apply these methods for production of identified NPs. In addition, metagenomic techniques can also be applied so that MNPs can be identified and used as leads, even when deriving from unculturable microorganisms. At the current state of the art, choosing microorganisms is therefore a good strategy for a faster commercial success. In the case of macroorganisms, culture methods such as aquaculture, integrated multitrophic aquaculture (IMTA) or mariculture are some routes being used for sustainable and continuous supply. Some novel laboratory cloning tools for cnidarians and ascidians are also being deployed to the same end. Synthetic or semisynthetic approaches are almost always undertaken in the pharmaceutical segment but cannot be deployed to other market segments.

Finally, when it comes to market hurdles, MNP development teams tend to disregard them until very late in their natural product development programs. At the very early stages of MNP development programs, several very important market-related questions must be addressed by the team in charge and carefully planned ahead of starting development programs such as (1) what are the potential industry applications and the market need of that particular activity, (2) is there space for that bioactive and is it innovative, (3) what is the target price/kg of the final bioactive, (4) what is the formulation desired and the route of administration, (5) what is the manufacturing process and how sustainable is the supply and (6) via what routes and channels can the product reach the market value chain. The high number of NP hits and leads coming of the HTS technologies and their much lower market introduction rate stresses out the need for a focused strategy on this field. SMEs have a commercialization goal and, therefore, introduce very early on their discovery and development programs the issues indicated above. It is crucial for them to have a clearly defined strategy; otherwise the risk of failing and running out of financial resources is very high. It is important to be aware that the cost of technology and manufacturing processes, sometimes with poor yields, raises the market cost per kilogram and may render these products economically unviable. Market challenges are common to all segments, but answers to each bottleneck are market specific as consumers and their needs are different, regulations are specific and overall market demand is

substantially variable too. These issues are specifically addressed in each market subsection below.

3.1 Nutraceutical-Specific Challenges

Marine resources, micro- and macroalgae, vertebrate and invertebrate animals and marine by-products, offer an important source of important nutrients and natural bioactive compounds having technological and health benefits, with potential use in the food industry, as well as nutraceuticals. The full utilization of such materials and nutrients requires new integrated approaches (e.g. screening of extraction conditions, in vitro measuring of functional activities and exhaustive chemical characterization to discover new bioactive compounds) to help the further design of processes to obtain such products in the most green, sustainable, and efficient way possible, complying with the rules of green chemistry and green engineering. An analysis of the main challenges in the nutraceutical market segment is presented next (Table 9.8).

Marine functional food and marine nutraceuticals are exciting clusters whose market is expected to grow. A major emerging issue is biomass availability. Maintaining consistency of the supply chain and ensuring security of supply have to be addressed reflecting the present concerns for the environment and the concept of sustainable production. In 2014 the global capture fisheries and aquaculture supplied 167.2 million tons [61]. From the production utilized for direct human consumption (around 146 million tons), 67 million tons were destined to direct human consumption, and the rest (approximately 80 million tons) was conveyed to be preserved or processed into different forms (e.g. dried, salted, smoked, prepared, canned and frozen), generating huge amounts of offal, secondary products and by-products that can constitute 30–70% of raw materials. Such materials (e.g. heads, filleting frames, scales, guts, shells, dark flesh, bones and skin) have gained attention with a growing focus on handling and preserving in a controlled, safe and hygienic way. Thus, they serve other purposes, ranging from products for human consumption (e.g. fish heads, fish sauces) to recycling into marketable products (e.g. bioactive). Regarding seaweed production, a continuous growth based on farming has been registered. In 2014, about 28.5 million tons of seaweeds were harvested for direct consumption (traditionally in Japan, the Republic of Korea and China) or further processing for food, fertilizers, nutraceuticals, pharmaceuticals, cosmetics, etc. [61]. Regarding microalgae, the production is poorly reflected in available aquaculture statistics worldwide, but, for example, *Spirulina* spp. production is reported by a few countries without production data being reported to FAO [61].

Recent advances in food technology related to marine microorganisms have opened novel avenues to develop natural substances as food, functional food ingredients or nutraceuticals. Even though dozens of research articles demonstrate the immense potentials of marine microbial metabolites as leading compounds for food

Table 9.8 Nutraceutical MNP development challenges: this table identifies specific challenges for nutraceutical market sector (based on those identified in Fig. 9.18) and the degree of difficulty currently felt to overcome that challenge (low, moderate or high) and connects those to a tip/tips to overcome such challenge

Challenge	Type (market, biodiversity, supply and technical)	Difficulty to overcome challenge (low, moderate or high)	Tips for success
Inventory of available marine resources	Biodiversity	Moderate	Definition of a concerted road map and the relevant actors to assess the availability of unused fishery biomass, by-products derived from processing activities and cultured biomass
			Creation of dedicated information portals
			Development of dedicated support actions
Efficient harvest, storage, separation and purification processes	Biodiversity + supply and technical	Moderate	Development of processes to support the culture and harvesting of biomass
			Establishment of separation protocols for marine raw materials or derived parts, its classification and storage
			Development of processes to control the culture of marine biomass at sea and on land
			Generation of efficient transformation and refining processes, including concepts of multistream inputs and mixed biomass
Technical improvement of the biodiscovery pipeline	Biodiversity + supply and technical	Moderate	Search of sustainable methods for new raw materials supply
			Definition of quality criteria for new raw material acceptance
Increasing the potential for success in finding novel bioactives	Biodiversity + supply and technical	Moderate	Advancement of new strategies to produce biomolecules for food and nutraceutical applications
			Definition of suitable protocols combining techniques for suitable extraction, separation and biochemical characterization
			Unequivocal identification of bioaccessibility and bioavailability by using in vitro methods

(continued)

Table 9.8 (continued)

Challenge	Type (market, biodiversity, supply and technical)	Difficulty to overcome challenge (low, moderate or high)	Tips for success
Developing food/feed products and ingredients marketable for nutrition and health	Supply and technical	Moderate	Development of functional food products and ingredients of marine origin with optimal nutritional properties for human health
			Development of new, low environmental impact feed ingredients to improve quality of farmed products and human health benefits
Developing certification schemes for marketable products and ingredients	Supply and market	High	Collection of existing information about authenticity
			Development of reference materials and markers
			Delivery of certification schemes specifically for functional foods and nutraceuticals
Determining market needs	Market	Moderate	Preparation of suitable and harmonized regulations
Distribution in value chain	Market	High	Dissemination of adequate information about benefits for consumers
			Introduction of digital traceability and labelling solutions
Competition	Market	Moderate	Establishment of strong interaction between research and industry to ensure that technological advances are integrated to correspond to market opportunities

and nutraceutical developments, the potential production rates are still poorly or not even addressed at all.

In such circumstances, an inventory of existing wild or cultured sources and the subsequent biomass, useful fractions, amounts and biochemical characteristics is necessary. The inputs to this inventory have to be obtained based on sustainable productions and without adverse impacts on marine ecosystems.

The successful implementation of this strategy will require a joint effort and engagement of relevant actors, including researchers, the private sector (e.g. individual companies, associations, technology platforms), policy-makers and advisors at national and international level. Additionally, the mobilization of the necessary support is needed in terms of funding, human resources and research infrastructures.

Marine raw materials or derived parts for food purposes are carefully handled and stored according to applicable regulations, including hygiene good practices and monitoring of pathogenic, marine biotoxins and environmental chemical contaminants. Oftentimes, less emphasis is placed on the quality of harvest and storage of raw materials, resulting in ingredients that do not meet this standard. Nonetheless, it is already well known that the way of handling such materials determines the types of products that they can give origin to. Therefore, optimizing processes and strategies to harvest, store and separate MNP ensures that raw and intermediate materials achieve the necessary quality for various products. It is also important to emphasize that animal by-products have special regulation regarding management and that such co-streams are divided in three categories according to the potential risks towards human, animal and environment health. Thus, the efficient use of marine raw materials, derived parts and cultured biomass for functional or nutraceutical purposes require the definition of new strategies, protocols, processes and treatments to support the culture and harvest of biomass, classification and storage, including concepts of multi-stream inputs and mixed biomass, to ensure high quality and best price.

Over the last decade, evidences of diversity of marine bioactive compounds were gathered, ranging from n3 fatty acids to peptides and enzymes to be used as ingredients in functional food and nutraceuticals. However, technological and societal developments enabled the utilization of existing molecules in new products and an increased demand towards more complex bioactive metabolites. Still, marine biodiscovery faces several challenges, to reduce barriers for successful industrial exploitation of marine biodiversity, including the clarification of legal aspects to facilitate the access to marine resources, their sustainable use and secure exploitation, as well as the access to marine biotechnology data and innovative research materials.

Physical and chemical processes currently applied to produce functional food ingredients and nutraceuticals from marine sources have limitations in what regards functionality and quality of final products, while enzymatic processes appear to be more useful for obtaining tailored molecules/products. For instance, to produce marine oils, efforts should focus on research for specific lipases and proteases to control the production of tailor-made products. On the other hand, FPHs and active peptides are currently prepared by enzymatic processes under controlled conditions, but optimization of existing enzymatic processes is needed (e.g. specific enzymes and models to forecast hydrolysis degree and biological activities) to produce reproducible enriched and tailored hydrolysates or peptides for specific foods or nutraceuticals with increased activity (antioxidant, antihypertensive, etc.). Further research is also needed to understand extraction processes in heterogeneous media and reproducibility of customary processes. In addition, it is required to develop genetic markers for accurate identification of marine raw materials used for novel bioactives and define strategies for its storage stability. Finally, suitable good manufacturing and hygienic practices have to be elaborated to ensure the quality and safety of new molecules.

Besides, the characterization of novel functional or nutraceutical structures using chemical approaches remains a major bottleneck, limiting the capacity to uncover the next generation of bioactive molecules. Therefore, increased focus on the developing of new isolation and separation methods, analytical chemistry techniques associated to genetic databases and unequivocally identification of bioaccessibility and bioavailability of such biomolecules by using in vitro methods is necessary. For instance, mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques offer wide possibilities for predicting the structural composition of novel molecules, and the combined use of these techniques with HPLC in tandem or triplet (LC-MS, LC-MS/MS or LC-MS/NMR) is highly effective to establish the structure of new bioactives from complex samples. The integration of biochemical characterization of novel marine molecules with genetic, bioinformatics, synthetic biology, bioinformatics and nanobiotechnology may enable development that conforms with next generation of solutions.

World population growth, food security, health and well-being are the prime drivers of global food/feed industries. Thus, at present, the relevance of marine sources is greater than ever before. The likelihood that marine bioresources will form the basis of new food and non-food products is leading to enhance the knowledge of its biochemical diversity to ensure product differentiation. Additionally, the issue of the impact of the technological processes on marine active molecules is still open. Thus, suitable developments in food ingredients, nutraceuticals, functional foods and food and feed supplement areas are opportunities to add value and develop new markets. Therefore, it is crucial to ensure that marine food and related industries are closely aligned with knowledge providers and that sufficient funding continues to be available.

Globalization and the growing complexity of food and feed chains, combined with recent food alarmism, have raised consumer and market awareness regarding quality and authenticity of functional foods and nutraceuticals. Authenticity tools must assure that the product purchased by consumers and industrials matches its description, origin, production method, processing technologies, environmental footprint, quality control procedures and certification and that it conforms with set standards. In general, informed consumers are prepared to pay extra for certified products and are increasingly demanding understandable and reliable information on labels. These trends have led to harmonizing standards and the development of accurate tools to verify that marketable products match their description and to detect fraud. In addition, there is a need to coordinate and harness transnational capacity and resources, especially databases, reference materials, training and research capabilities. Thus, it is of utmost importance to define the adequate characteristics of functional molecules and nutraceuticals, make available reference materials and markers to confirm the quality and/or authenticity (or potential adulterants) and develop, validate and standardize verification methods to support certification strategies.

Production of functional foods and nutraceuticals is very important for some food sectors and nutraceutical industries. However, technical challenges have to be addressed to improve its success in the market, namely, the correct identification of

active compounds and the regulatory uncertainty and credibility of labelling claims [247]. Despite nutraceuticals currently experiencing a growing interest from consumers and health professionals, a shared regulatory definition has not yet been assessed. The USA developed the Dietary Supplement and Health Education Act that allowed flexibility and blurred boundaries between food and medicines. Yet, current European regulations consider nutraceuticals as food supplements (Regulation (EC) N° 1924/2006 2006), updated by Regulation (EC) N° 2283/2015 (2015). This regulation, focused on "novel foods", defines food categories and completes the definition of food supplements; nonetheless, the term "nutraceutical" is still not officially recognized or mentioned. Regarding labelling claims, the Regulation (EC) N° 1924/2006 (2006) establishes rules for the labelling, presentation and advertising of food, whereas the Regulation (EC) N° 353/2008 (2008) implemented rules for health claims. The European legislation on this subject aims to ensure that claims for food components and nutraceuticals are properly justified and scientifically substantiated [248]. The EU legal framework referred above enables the use of "health claims" and "reduction of disease risk claims" for food outside the scope of medicinal law. Health claims are only approved for use on labelling, presentation and advertising of food in the EU market after a scientific evaluation without any distinction between food supplements and nutraceuticals. Such evaluation is made by the European Food Safety Authority. Thus, the term nutraceutical is accepted even if not officially recognized, indicating a lack of regulation. Such a situation requires the preparation of suitable and harmonized regulations to ensure food safety, respectability and truthful and not misleading labelling to support market needs.

There are a series of requirements to make a successful distribution operation of functional food and nutraceuticals, from a consumer and a supplier perspective, including safety, affordability, availability and acceptability. Target products must be safe and healthy at the point of delivery to the population, since unsafe and unhealthy compounds may have harmful effects that offset any benefits from its consumption. This is particularly important during processing, storage, distribution and/or preparation, where losses may occur due to spoilage, adulteration or inappropriate handling. Prices are the fundamental determinant of buying practices. So, appropriate mechanisms should be implemented based on the principle that consumers must be economically able and willing to buy the product. Functional food and nutraceuticals must be available in physically and socially acceptable locations, and such availability should be accessed through the distribution of information at market level and from consumer data. Apart of their functional and nutraceutical value, products must be acceptable to consumers in different perspectives including physical appearance, simplicity of preparation and social/cultural habits, prevailing tastes, consumption patterns and preparation practices. Thus, for different functional or nutraceutical products, suitable information must be provided and different requirements established based on price, risk and uncertainties faced by consumers and suppliers. Understanding how consumers perceive functional foods, nutraceuticals and the factors that influence their purchasing behaviour and the implementation of digital traceability systems are the key to a successful commercialization over the value chain.

The global functional food and nutraceutical market is expected to grow at a steady CAGR of nearly 8% during the next 10 years. However, the majority of bioactive food ingredients and nutraceuticals are of plant origin. Nevertheless, several active molecules are also found in animal products (e.g. dairy products, meat and marine organisms). In this context, it is important to anticipate how competition among molecules from different sources will take place based on their availability, chemical structure, mechanisms of action and proven physiological properties. To make marine-based functional foods and nutraceuticals more competitive and successful, strong research, responsible stakeholder relationships and adequate regulatory structures are needed. Additionally, companies will have to invest on technological advances to develop a powerful portfolio of technologies for new compound delivery (with known lifecycles and benefits/risks) and work closely with governments and research centres to correspond to market opportunities.

3.2 Pharmaceutical-Specific Challenges

As it can be easily deduced from the overview of MNP currently in the market or in development, for many years MNP were solely on the radar of a few pharmaceutical companies. Reasons for this included difficulty in sourcing/harvesting samples, lead compound isolation only in extremely small quantities, lengthy and poor yield synthetic routes, impracticality of scale-up and difficulties in the isolation and/or purification procedures. High toxicity of the active compound, ecological and legal considerations, government policies, lack of infrastructure and insufficient capital investment have also inhabited the development of MNPs by the pharmaceutical industry [249].

As stressed earlier, biodiversity challenges cross all market segments where MNPs are the raw material. The main issues connected with the access to biodiversity were pointed out together with some tips to overcome them. However, as the bottom line, biodiversity access is a key factor to unlock the total potential of the marine environment, and serious work must be done by governmental politics and academic and industry researchers in order to improve bioprospecting activities.

However, besides access to biological material, the pharmaceutical industry faces specific challenges, mainly technical: reliable HTS assays, fast and robust dereplication processes, resupply of the active compound by a sustainable, moderate cost and viable scaled-up synthetic pathway (Table 9.9).

Following the process of target identification and validation, the next step of a drug discovery process is the development of the screening assays. A variety of screening paradigms exist to identify hit molecules [250] being HTS the most widely used in the case of NP. The key to successfully apply HTS methodology to NPs is constructing robust libraries that, ideally, should be composed by crude extracts, simplified extract fractions and pure compounds [251]. Screening simultaneously crude extract and pre-fractionated libraries is an effective strategy to avoid false

Table 9.9 Pharmaceutical MNP development challenges: this table identifies specific challenges for cosmeceutical market sector (based on those identified in Fig. 9.18) and the degree of difficulty currently felt to overcome that challenge (low, moderate or high) and connects those to a tip/tips to overcome such challenge

Challenge	Type (market, biodiversity, supply and technical)	Difficulty to overcome challenge (low, moderate or high)	Tips for success
Biodiversity access	Biodiversity	Moderate	Increase focus on new taxa, new habitats; increase focus on marine microbiota
Discovery of new active molecules	Biodiversity + supply and technical	Moderate	Improve HTS assays and dereplication processes; genomic and metabolomic profiling
Sustainable supply	Supply and technical	High	Synthesis of the active compound or analogues; semi-synthesis; prodrugs; genetic engineering
Adequate production strategies and technologies	Supply and technical	High	Effective academia/industry/collaboration to assure reliable technological developments
Market needs	Market	Low	To bet on chronic diseases, especially on those resistant to current drugs
Competition—Me too companies	Market	High	To bet on diseases with no effective drugs and on drugs with new and more efficient MoA

results and to improve the success rate of hit identification [252, 253]. Although HTS has experienced significant improvements in both quality and quantity of information obtained, new technologies and developments in instrumentation and robotics could be useful to increase the discovery hit rate.

Once the process of bioassay-guided fractionation has selected a particular bioactive marine natural product as a lead structure, the issues of short-term small-scale and long-term manufacturing-scale supply become the decisive factors in its further progress towards development. Indeed, supply issue has been, without any doubt, the main shortcoming that development of marine bioactives has faced that was only overcome when remarkable synthetic procedures or biotechnological production reached a high level of progress. Usually, the compound of interest is present only in low amounts and/or can be very difficult to isolate, a fact that is per se a major drawback [167]. Additionally, the problem related to the variability of the organism itself also poses an extra level of difficulty. When dealing with secondary metabolites, the environment is a key factor for their biosynthesis: stress factors such as predation, overgrowth by fouling organisms or competition for space may dramatically affect the secondary metabolism pathway of a certain organism. As environmental conditions are not static, it is possible that a resupply of the same organism will not provide the same target metabolite. Therefore, it is advisable to

select species that are less susceptible to environmental influence or more prone to laboratory reproduction under more controlled conditions, in order to assure a sustainable supply of the target product.

The realistic approach to the supply problem has to pass through total synthesis and/or by semi-synthesis. However this is a difficult endeavour, especially because MNPs are complex and exquisite molecules possessing, almost always, one or several stereocentres, a fact that renders mimetic synthesis hard to achieve. However, significant advances have been achieved in this area, and there are cases of successful total and semi-synthetic approaches. The industrial production of eribulin mesylate, a complex molecule inspired by a MNP, is totally synthetic [184]. Semi-synthesis is, in some cases, a good solution for compound supply. This process involves harvesting a biosynthetic intermediate from the natural source, rather than the lead itself, and converting it into the lead. This approach has two advantages. First, the intermediate may be more easily extracted in a higher yield than the final product itself. Second, it may allow the syntheses of analogues of the final product. To design simpler analogues, keeping the pharmacophore moiety is another approach that allows to overcome the complexity of the synthetic process and improve lead's properties [254]. However, either for synthetic or semi-synthetic pathways, it is advisable to integrate early in the development strategy the concept that a commercial viable synthesis must be as short as possible; thus a balance must be achieved between the yield and the steps needed to complete the process, and the need for a high number of intermediate purification steps must be avoided.

Marine product scale-up and resupply have been attempted by mariculture and aquaculture in order to solve the problem of sustainable supply of macroorganisms. However, the unique and sometimes exclusive conditions of the sea make cultivation or maintenance of isolated samples very difficult and often impossible. For example, sponges and their microbiota are generally not suitable for cultivation; hence, the compound of interest may need to be extracted and purified from the specimens collected in the wild [12]. Even in the case where mariculture is possible, the costs associated with the entire method are not compatible with an industrial application. PharmaMar has attempted this approach in order to obtain enough biomass of trabectedin to run clinical tests [168]. However, due to the complex process of isolation and purification on a large scale and to the low yield, this methodology was revealed to be economically non-viable. In the case where the actual producers of the bioactive metabolites are the associated microbiota and these are able to be cultured independently, scale-up can be a possibility, but there are still many cultivation challenges in this area. Further improvements still have to be made regarding the identification, cloning, genetic manipulation and expression of biosynthetic pathways in order to apply these methods for production of identified natural products.

Another approach is to use genetic engineering of cells, particularly microorganisms, for the development of strains dedicated to the overproduction of a certain metabolite, since microbial fermentations are very easy to transpose at the industrial level. The metabolites obtained through this method can be the final product or an intermediate in the semi-synthesis. A success case of combination of fermentation

methodologies and semi-synthesis is trabectedin that is now produced beginning with a precursor obtained by fermentation of *Pseudomonas fluorescens*, followed by chemical transformation [168].

Besides the supply problem, two technical challenges have to be addressed correctly in order to improve the success in the marketing of marine natural drugs: the correct identification of the active compound and the dereplication process. In the field of identification, NMR spectroscopy continues to be the vital element for structure elucidation, and recent advances that enable identification on a nano- or even picomole-scale [6] gave a significant contribution to a more efficient chemical characterization of marine compounds. These advances also allow the exploration of marine species existing in very low biomass in nature, such as thinly encrusting invertebrates or microalgae slimes [126]. Nevertheless, it is very important to improve structure elucidation using the most rigorous and unequivocally methods available and cross-checking of the structure in light of current biosynthetic knowledge.

The performance of MNP screening programs depends strongly on fast dereplication strategies (identification of already known compounds), which are vital for prioritization of which extracts, strains or even pure compounds are to be explored further. In the simpler process, dereplication can be used for the identification of the major compounds in an extract, with no target associated. This first strategy can also be combined with biological assays to accelerate bioactivity-guided fractionation protocols. Another approach integrates dereplication in untargeted metabolomic studies for the chemical profiling of natural extract collections or in targeted identification of a certain class of metabolites. Finally, for microbial strains, a specific dereplication approach is by using taxonomic identification based on gene sequence analysis [255].

Reliable, robust, rapid and sensitive analytical methods are required in order to improve the dereplication process. Hyphenated technics, such as liquid chromatography with ultraviolet detection (LC-UV), liquid chromatography-mass spectrometry (LC-MS, LC-MS/MS) or LC-NMR and more recently LC-MS/NMR, are valuable tools for a rapid dereplication process [256], especially if used early in the prefractionation stage [257]. Access to suitable databases is essential for the rapid dereplication of crude extracts in natural product research. A recent review on strategies for the dereplication of MNP highlighted the relevant databases in this field [253, 256]. An extended free access to natural compound databases should be encouraged in order to speed up MNP programs. When using pure natural compound libraries, in silico dereplication is also a possibility that must be emphasized, which can also be used to browse databases in the quest for new active molecules. The advantages of this approach are high capacity, no need for physically isolating the compounds and less time and money. Additionally, early evaluation of absorption, distribution, metabolism and excretion/toxicity in pharmacokinetics (ADMET) properties is also possible [258]. Because virtual screening is only a predictive tool, in the case of NPs, it is important to integrate in silico screening with traditional avenues, gathering information from bioassay-guided fractionation, online analytical activity profiling, ethnopharmacological screening, and, if it is

the case, chemoinformatics, in order to achieve an optimization of drug lead discovery [258].

An analysis of the development history of MNP drugs that reached the market clearly shows that, because of market-driven business reasons, the initial research was, mostly undertaken by academia or small biotech that do not face the intense profit pressures that large pharmaceutical companies do. This was a consequence of all the difficulties that were pointed out earlier, but that were greatly overcome in the last decade, due to technological breakthroughs. At present, the marine pipeline has a stronghold on anticancer clinical and preclinical agents, a fact that should be harnessed by pharmaceutical companies that prioritize their resources based on the likelihood of revenues, which are more likely to be achieved when targeting chronic diseases such as cancer and lipid metabolism. However, it is important that academia and marine biotech find ways of reducing the risks, time and cost of research, bridging the gap between innovation and business models.

3.3 Cosmeceutical-Specific Challenges

Most of the specific challenges faced by cosmeceutical MNP are related to market and technical bottlenecks (Table 9.10), despite sharing the same common challenges in biodiversity access and knowledge and technological and supply constrains with the markets mentioned in the previous subsections. This is no surprise given that development of a cosmeceutical, albeit seen by many as an easier avenue than pharmaceutical, has its own specificities and those are mostly related to market demands and consumer behaviours.

To solve the biodiversity challenges, most of the cosmeceutical MNPs have been focused on phytoplankton and algae—macro and micro—and, more recently, microorganisms that are either cultivable in the lab or where biotechnology can help to overcome harvest and/or production challenges. In fact, this later trend has experienced an immense growth in the past 5 years, judging by the number of novel cosmeceutical launches derived from bacteria, fungi and/or marine biotechnology. Even algae-based products have been transforming into sophisticated biotechnology-derived products, as already referred in the previous section.

Regarding supply and technological challenges, the limited number of screening tools and technologies designed and used in cosmeceutical MNP may be hindering innovation and development in this field as they are not target specific in most cases and not many have been developed, and shared, to screen for specific molecular level targets. In fact, only in recent years have we witnessed a rise in the number of truly innovative and with well-identified MoA products. Some good examples are Nocturshape® [210] and Actiporine 8G® [217], which target nocturnin and Actiporine 8G, respectively, to control specific skin problems.

Novel screening cellular models for discovering marine bioactives with new modes of action and using novel skin targets have started to boom, and an additional level of sophistication in these systems is to be expected in the next years

Table 9.10 Cosmeceutical MNP development challenges: this table identifies specific challenges for cosmeceutical market sector (based on those identified in Fig. 9.18) and the degree of difficulty currently felt to overcome that challenge (low, moderate or high) and connects those to a tip/or tips to overcome such challenge

Challenge	Type (market, biodiversity, supply and technical)	Difficulty to overcome challenge (low, moderate or high)	Tips for success
Biodiversity access and knowledge	Biodiversity	Moderate	Increase focus on microorganisms usage and/or lab cultivable organisms; metagenomics and single cell isolation and enrichment; NGS and taxonomic studies
Discover innovative molecules/fractions	Biodiversity + supply and technical	Moderate	Better screening assays, diversify types of marine organisms screened, prescreen samples for novel compounds; metagenomics and other omics tools
Sufficient quantity supply	Supply and technical	Low	Increase focus on microorganism usage and/or lab cultivable organisms; metagenomics and single cell isolation and enrichment; evaluate biotechnological production routes; perform preliminary yield production tests
Adequate production strategies and technologies	Supply and technical	Moderate	Secreted metabolites or easy nonsolvent-based, aqueous or mechanical extractions, biotechnological production routes, correct final formulation studies, reproducibility of manufacturing process determined early on
Determination of MoA	Supply and technical	High	Improve models of 3D skin testing, develop cutting edge cellular models for MoA, increase clinical trial outputs in cosmeceuticals
Adequate marketable format and delivery formulation	Market + supply and technical	Low	Perform desired market format study before formulating, evaluate potential formulation and delivery routes at early stages
Accurate market need determination	Market	Moderate	Compare competitive products and MoA, determine their market share and what is the USP or VP of new MNP, envisage marketing story
Compliance with target market pricing and cost of production	Market + supply and technical	High	Evaluate comparable MNP market pricing, and determine early on your target market price; predict MNP yields early on and evaluate final production costs versus target market pricing

(continued)

Table 9.10 (continued)

Challenge	Type (market, biodiversity, supply and technical)	Difficulty to overcome challenge (low, moderate or high)	Tips for success
Distribution in value chain	Market	High	Identify key players and distributors of specialty ingredients in your market; establish early stage mutually beneficial partnerships
Competition—Me too companies	Market	Moderate	Avoid copying existing products; develop novel MoA or source MNP; prefer compelling marketing stories

MoA mode of action, *NGS* new-generation sequencing

contributing to a bigger infusion of scientifically supported claims into the cosmeceutical world. On the other hand, employing and improving HTS assays and platforms that target or are mechanism specific for skin conditions (and skin processes modulation too) will foster faster cosmeceutical MNP development.

Another issue to consider is the type of MNP used in cosmeceuticals. If the majority of cosmeceutical products continue to either be extracts, fractions or combination of bioactives, another significant hurdle is quantity for testing, as well as the low bioavailability of the active component in these mixtures. Coupling screening programs with sensitive and robust tools to allow usage of limited quantities, while eliminating poorly performing samples, can truly be the answer to handle low concentration actives and thousands of samples simultaneously. On the other hand, pure cosmeceutical MNP compounds are rare, and this may be due to the difficulty and cost of isolating such compounds, as market price constraints can limit this endeavour. Pure natural product libraries are considerably more expensive and difficult to obtain but offer the advantage of a straightforward hit detection. Increasing the number of such libraries, while working on novel ways and technologies to reduce the cost of pure MNP isolation, is also a good strategy for the field as further aid can come at later stages via marine biotechnology tools.

The supply of MNP for cosmeceutical purposes needs to be reproducible and constant, at a level capable of responding to market volume demand. However this is a major challenge for MNPs and still persists as a major hurdle for this market segment. This issue should be given serious consideration at the beginning of any natural product discovery program, as choosing between micro- and macroorganisms can alter dramatically the elected development strategy. Here too, technologies such as fermentation, election of only secreted products or even aquacultured or maricultured algae are some of the answers to this challenge. Furthermore, marine biotechnology tools can be used, and have been, unlocking this bottleneck, and we expect to see much more of this biotechnology-derived MNPs entering the cosmeceutical space.

Still, most of the challenges faced by a MNP in the cosmeceutical path are market-based ones. A cosmeceutical has a less demanding regulatory path when compared to a drug, involving also efficacy and safety testing like in any consumer product but without involving animal testing (in fact, in Europe it is actually forbidden to test cosmetics in animals). However, cosmeceutical companies and developers are only willing to put in the market completely secure and effective products. Consequently, more technology and models will be expected in these phases of the cosmeceutical MNP value chain. More accessible and additional cellular and three-dimensional skin models are also needed to fasten the cosmeceutical development timeline. These can help in delivering increased amount of data for safety and efficacy analysis in this not-animal tested era.

Likewise, a cosmeceutical has a very tight and difficult route to market, with price constraints and distribution channel control at the top of the most challenging tasks to overcome. At the centre of this challenge, market rules and willingness to pay of the final consumer drive the ultimate price definition for a new cosmeceutical. As the cosmetic value chain is a very disperse one, with several key players and intermediates, ranging from the commodity ingredient suppliers to the specialty ingredient developers and suppliers, as well as from the formulator and manufacturer to the final distribution company, this dispersion lowers the margins for each player, in order to successfully introduce a new product aiming to meet the defined target price range. This is particularly true in the cosmetic industry, where the search for organic, natural and nonhazardous claims is in high demand by an ever-more conscious consumer. In this new consumer trend, recombinant technologies or chemically derived products are not acceptable, and profit margins are yet too small to introduce very expensive ingredients per pack.

On the other hand, some of the supply and technical challenges mentioned before for cosmeceutical products arise in relation to its market demands—natural, nonchemical or recombinant derived and tight price range—limiting the possible production routes. Fermentation (for microbial-derived active ingredients), preferred secreted bioactives (like EPS), clonal growth (for some species of sponges and cnidarians), sustainable harvesting (macroorganisms) or eco-aqua-/mariculture coupled with mechanical or aqueous base extraction methods to obtain the final ingredient are all solutions to these market bottlenecks. No new MNP for this industry can be achieved via traditional pharmaceutical routes like synthetic or solvent-based, as these no longer have space in the market.

Furthermore, the demand for innovative activities and molecules is driving the search for novel sources and actions of cosmeceutical, and here, marine habitats with its extreme options have been an increasing source. However, with such price level constraints and high volumes required for successful market commercialization, an increasing focus on microbial and microalgae-based actives, as well as novel development technologies like marine biotechnology and production tools, such as fermentation and high-throughput extraction strategies, has been seen. Additionally, metagenomic tools and NGS technologies have facilitated the speed of discovery of novel MNP among uncultivable communities of marine samples that can later be obtained by alternative biotechnological routes.

In the past 2 years, we have witnessed a tremendous amount of novel biotechnology-derived ingredients, mostly from bacterial sources, as highlighted in the previous cosmeceutical section. This current trend is likely to continue, and more cosmeceuticals are expected to arise, as more and more companies and researchers are finding novel and faster routes to this savvy market. Novel MPNs derived from marine bacteria, *archaea* or fungi are emerging as innovative yet sustainable, reproducible and easier-to-manufacture solutions. These novel types of MNPs are appealing to a larger group of cosmeceutical manufacturers that see in this product a potential solution to many of the hurdles encounters in macroorganism- or plant-derived cosmetic ingredients.

The repositioning of old or nonsuccessful pharmaceutical compounds is also an important novel avenue for MNP development in cosmeceuticals. Finally, when launching a new MNP development program, special attention needs to be given to competing products and developments underway. The cosmeceutical market is being flooded with novel ingredients, and sometimes groups are developing similar source MNP or targeting similar/same family targets, and this will raise the level of difficulty to successfully penetrate the market. Therefore, attention should be focused on developing cosmeceutical MNPs that are innovative either based on novel sources/types of organisms or based on novel skin/metabolic targets. Alternative innovation can also be obtained when focusing on developing MNPs that outperform existing ingredients on potency or even, alternatively, are capable of being manufactured at amazing final prices due to production technology improvements or new formulations. In either case, a compelling marketing story needs to be devised from day 1 of development.

4 Conclusions

Looking at MNP discovery and development as a whole, a clear picture is formed integrating the type of sources used, the number of marketed products from each source originated so far, as well as market penetration levels and value delivered in each market segment (Fig. 9.19). This figure demonstrates the unquestionable need to source novel genera and types of marine materials for fostering the discovery of new and innovative MNP and to couple this development with clear value and pipeline constraint knowledge to increase chances of market success.

As highlighted throughout the current chapter, nutraceutical, pharmaceutical and cosmeceutical value chains targeting marine products display specific features and face unique bottlenecks that may hamper their development in the years to come. Nonetheless, these value chains also share common needs and transversal challenges to foster the development of an integrative framework that enables synergies for the sustainable growth of the world's Blue Bioeconomy. The smart valorization of marine biological resources through these distinct value chains might be possible if a biorefinery-like approach is implemented allowing populations to gain access to safe and secure seafood while simultaneously maximizing the use of any non-food organisms, as well as resting raw products that may accumulate over consecutive

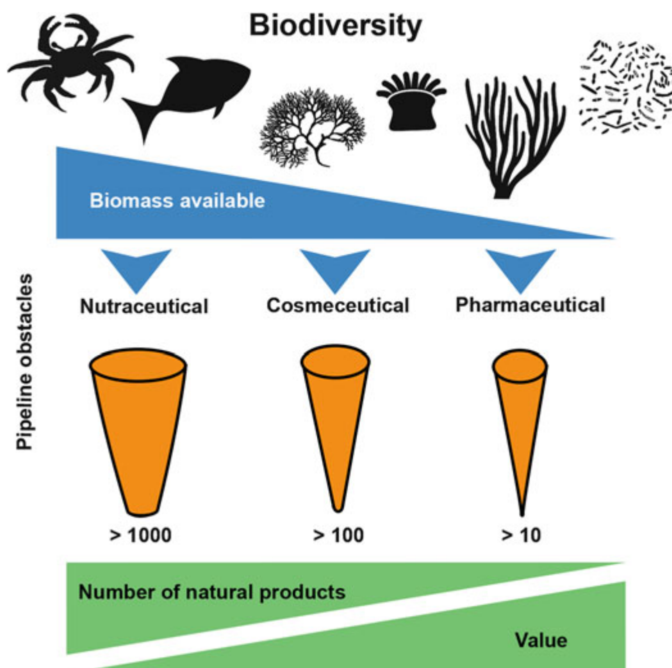

Fig. 9.19 Integrated view of MNP development based on sources, number of products and market value for nutraceutical, pharmaceutical and cosmeceutical segments

steps of present and future processing lines. As an example, it is possible to envisage that smart processing of a fin fish captured/cultured to secure the production of high-quality food proteins for human consumption will also take into account the potential valorization of its trimmings holding potential molecules of interest for nutraceuticals, its scales and bones with potential for cosmeceutical products, as well as unique secondary metabolites in its blood and viscera with pharmaceutical applications. However, it is important to highlight that at some stage of the valorization process of marine resources, several logistic challenges may arise. Due to the perishable nature of some of the biomass targeted for valorization, a smart integration of processes with available facilities and logistic channels (e.g. cold storage, drying tunnels, collection and distribution fleets) from other value chains maybe required to tackle logistic and financial constraints. The scale-up of industrial processes to a level where they become economically feasible may also pose a challenge to biomass suppliers, as they may need to pool batches from different players/origins. This practice adds up to the logistic challenges referred above and put at risk issues associated with sanitary certification. Moreover, traceability frameworks may be seriously compromised when different batches of biomass are combined to secure the supply of a given production pipeline. Suppliers may also no longer be able to secure biomass replicability, in terms of quality and composition, if batches from different origins need to be combined.

While the days of smart valorization may still appear to be far away, marine-based products that have long been traded as commodities (e.g. fish meal and fish oil) are already becoming less available to value chains targeting their use in low-value products. The ever-increasing demand for marine products, that is unlikely to be met by a corresponding increase in supply due to the state of the world's fisheries and generalized biodiversity crisis, will ultimately benefit from smart valorization strategies. Ultimately, this mismatch between supply and demand for marine products will favour value chains aiming to supply high-value products. Nutraceuticals, pharmaceuticals and cosmeceuticals are certainly well-ranked among such high-value products. However, these industries will need to face the challenge of integrating the common steps shared by their development pipelines. More than sharing success stories on products developed by each value chain, sharing pitfalls and failures impairing the advance of a target organism biomass and extract of molecules for the development pipeline will be paramount to identifying opportunities and avoiding research "dead ends".

Regulatory boundaries may easily turn value chains into "value drains" when considering the biotechnological valorization of marine products. Indeed, either by omission or "overregulation", enterprises developing innovative products inspired by the sea may easily become tangled in legal frameworks. When developed products fit within a category yet to be regulated by competent authorities, their marketing may be negatively affected, or even impaired, as consumers may perceive such products as unsafe. On the other side, the existence of stringent regulatory frameworks may even constrain the initial steps of the discovery pipeline. If the biotechnological use of most land-based organisms is already a legal conundrum, the use of marine organisms raises this challenge to a whole new level. National and international laws, regulations, conventions and bans are just a few legal aspects to which one needs to pay attention when aiming to explore marine biodiversity for biotechnological purposes. While the market being targeted by such products is global, there is no international regulatory body that may contribute to the harmonization of regulations between countries and assure investors that products being developed in a pipeline will indeed be suitable for the global market. Given the complex governance of exploration rights targeting marine genetic resources and biodiversity, the legal framework constraining blue biotechnology is unlikely to be simplified in the years to come.

While the challenges ahead for nutraceutical, pharmaceutical and cosmeceutical value chains will certainly put a number of enterprises in a "make or break" situation, opportunities for success are also exciting in light of the technological breakthroughs already achieved. At present, it is possible to screen more, better and faster than ever anticipated in previous years. The advent of the omics approach broadened the scope of opportunities to turn marine chemical diversity into innovative products inspired by the sea. One may point that there is an apparent overall positive perception of consumers towards marine-based products and that there has never been a more favourable environment to the development of value chains positioned in these markets. Nonetheless, consumers are also growingly aware on the need to see product safety and benefit claims solidly supported by scientific-based information

and more conscious than ever on issues related to sustainability and the circular economy. Decision-making by the end consumer is not always made easy and at times may be biased by media "disinformation". Indeed, the publication of reports (often with little or even no scientific support) that contradict the claims made by suppliers of marine-based products may represent a constraint to their acceptance in competitive markets. Consumers may decide to stay with more familiar land-based products and choose not to risk using a more "exotic" marine-based counterpart. Several industries (and countries), whose economic activity is heavily dependent on their agricultural production and already have solid trading channels on raw or processed land-based products, may become fierce opponents of any "blue solutions" that may ultimately decrease their market share. As the volume of land-based production is by several orders of magnitude higher than that of marine-based products, some market segments may not be available in the short term. Altogether, these issues will lead the role marine biotechnology, and other similar integrating tools, is expected to have on MNP development in the future.

As with any new endeavour, turning to the sea requires careful consideration to challenges identified here, particularly if the goal is to commercialize a MNP. Designing a MNP program from the start to incorporate feasible alternatives to all pointed bottlenecks is a strategy that can lead to success in unlocking novel blue solutions to today's societal challenges. The potential behind marine resources comprise, undoubtedly, a huge economic powerhouse for the world and can deliver smart, sustainable and inclusive growth, in line with today's modern and sophisticated societal goals.

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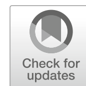
Chapter 10
The European Marine Biological Research Infrastructure Cluster: An Alliance of European Research Infrastructures to Promote the Blue Bioeconomy



Mery Piña, Pierre Colas, Ibon Cancio, Annie Audic, Lucas Bosser, Adelino Canario, Philip Gribbon, Ian A. Johnston, Anne Emmanuelle Kervella, Wiebe H. C. F. Kooistra, Matthieu Merciecca, Antonios Magoulas, Ilaria Nardello, David Smith, Nicolas Pade, Douglas Robinson, Antoine Schoen, Fanny Schultz, and Bernard Kloareg

1 Marine Biotechnology: An Emerging Field

The marine environment accounts for over 90% of the biosphere and harbours immense biodiversity. Marine organisms have historically been difficult to access and study, but through the foundation of marine laboratories and more recently biological Research Infrastructures in Europe, a wider range of marine biodiversity can now be examined in greater detail than ever before.

M. Piña · P. Colas · A. E. Kervella · B. Kloareg (✉)
Sorbonne Université, UPMC Univ Paris 06 et CNRS, Station Biologique de Roscoff, Roscoff, France
e-mail: kloareg@sb-roscoff.fr

I. Cancio
CBET Research Group, Dept. of Zoology and Animal Cell Biology, Fac. Science and Technology and Research Centre for Experimental Marine Biology and Biotechnology (PiE-UPV/EHU), University of the Basque Country (UPV/EHU), Bilbao, Basque Country, Spain

A. Audic
Conseil régional de Bretagne, Rennes, France

L. Bosser
Conférence des Régions Périphériques Maritimes, Brussels, Belgium

A. Canario
Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, Faro, Portugal

P. Gribbon
Leibniz-Institut für Molekulare Pharmakologie (FMP) im Forschungsverbund Berlin e.V., Berlin, Germany

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Biotechnology, i.e. the application of biological knowledge to develop novel products and other benefits for the environment and mankind, is of growing importance for Europe and will increasingly contribute to shape the future of our society. Marine (blue) biotechnology, which involves the use of marine bioresources either as the source or the target of applications, is increasingly an important component of the global biotechnology sector [1, 2]. This development largely stems from the need to meet growing demands for bioproducts and food that cannot be satisfied from terrestrial sources alone.

Marine biotechnology is the key to unlocking the potential of the unique diversity of marine organisms [3]. It is rooted in basic research bringing together marine biology, microbiology, physiology, toxicology, analytical chemistry, *omics* technologies, bioinformatics and systems biology. The result is new applications and services in fields such as drug discovery, diagnostics, nutrition and food ingredients, aquaculture and agriculture, bioremediation, biomaterials, cosmetics and bioenergy. It also leads to unique insights into the causative underpinnings of ecosystem functioning/services and an unprecedented potential for monitoring global changes and instruct policies.

In its analysis of the potential, hindrances and opportunities in the field of marine biotechnology, the Organisation for Economic Co-operation and Development [4] states: *Advances in genomics and computer science have transformed earlier views of the ocean. It is no longer simply a source of food, but a vast reservoir of genetic potential and a means of achieving a wide range of socio-economic benefits.*

I. A. Johnston
School of Biology, Scottish Oceans Institute, University of St Andrews, St Andrews, UK

W. H. C. F. Kooistra
Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy

M. Merciecca
Ministère de l'Education Nationale, de l'Enseigement Supérieur et de la Recherche, Paris, France

A. Magoulas
Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research Crete, Heraklion, Greece

I. Nardello
Sorbonne Université, UPMC Univ Paris 06, EMBRC, Paris, France

D. Smith
Centre for Biosciences and Agriculture International (CABI), Egham, UK

N. Pade
Marine Biological Association of the UK, The Laboratory, Plymouth, UK

D. Robinson · A. Schoen
Université Paris-Est, ESIEE—IFRIS, Noisy Le Grand, France

F. Schultz
Sorbonne Université—DR&I—Bureau de développement de l'activité contractuelle et de transfert, Paris, France

Genome sequencing is no longer the barrier it was a decade ago and our understanding of marine bioresources has improved significantly. (However) new Infrastructures are needed, with new models, new culture systems and new bioinformatics-based approaches to visualize genomics and other types of data.

In its Blue Growth strategy, the European Commission states that seas and oceans are drivers of the European economy and have great potential for innovation and growth. In order to achieve the goals of the Europe 2020 Strategy for Smart, Sustainable and Inclusive Growth, five different sectors are envisaged, two of which are related to the exploitation of marine biological resources: aquaculture and marine biotechnology. The global ocean economy, measured in terms of the contribution of ocean-based industries to economic output and employment, is very significant. Preliminary calculations on the basis of the OECD's Ocean Economy Database value the ocean economy in 2010 at USD1.5 trillion or approximately 2.5% of world gross value added (GVA). The projections suggest that, between 2010 and 2030 on a "business-as-usual" scenario basis, the ocean economy could more than double its contribution to the world global value added, reaching over USD 3 trillion [5]. In this same report, the OECD holds that *marine biotechnology has the potential to address a raft of major challenges (such as sustainable food, human health, energy, security and environmental remediation) and to make a significant contribution to green growth in many industrial sectors*.

2 The European Marine Biological Resource Centre (EMBRC), a Pan-European Research Infrastructure in Marine Biology and Ecology

2.1 The Genesis and Scope of EMBRC

Europe led the way in the creation of marine stations, in the second half of the nineteenth century. These undertakings happened within a short period of ca. 30 years in a number of European countries, prompted by the urge to study the evolution of life on Earth, which originated and evolved in the oceans, and to better understand the diversity of marine life.

With the onset of genomics and the related post-genomics approaches, marine biology and ecology are becoming as sophisticated as "terrestrial" biological and ecological sciences. This fundamental shift, which brings marine life into the forefront of biological research, widens the scientific scope of marine model organisms, and, more than ever perhaps, marine biodiversity today is a major target for fundamental science.

Marine biodiversity is also an increasingly important resource for industrial applications in the health, food, energy and environment remediation sectors and therefore constitutes the focus of a diverse Research, Development and Innovation (RD&I) community.

The European Marine Biological Resource Centre (EMBRC) originated from the perception of the increased demand for marine biological resources to meet the needs of this emerging community, as well as of the untamed challenges that such a demand poses to a fragmented marine research. EMBRC is a distributed Research Infrastructure (RI), which reunites the main marine biological laboratories in Europe. In 2008, it was included on the Roadmap of the European Strategy Forum on Research Infrastructures (ESFRI), supporting the view that marine biological stations are perceived again as strategic for European science and industry.

A Memorandum of Understanding for the establishment of EMBRC as a European Research Infrastructure Consortium (ERIC) entered into force on December 10, 2013. It has now been signed by seven EU member states (Belgium, France, Greece, Italy, Portugal, Spain and UK) and two associated countries (Israel and Norway). France was chosen to host EMBRC-ERIC, and the Core Office is established in Paris.

Based on the current configuration of EMBRC national nodes, the distribution of EMBRC laboratories is shown in Fig. 10.1. These marine laboratories feature in-house developed research communities and infrastructure, with staff ranging between ca. 50–300 full-time employees. They share similar typologies, notably (1) access to unique marine ecosystems and biological resources, including access to wet labs and culture collections, and (2) on-site support for a broad range of biological and ecological research activities, including genomics, post-genomics, bioimaging and bioinformatics.

The services currently offered by EMBRC are the following:

- Access to marine ecosystems, including associated historical time-series data
- Access to marine model organisms for evolutionary and developmental biology, physiology, ecosystem functioning, gene discovery, molecular farming, biogeochemistry and biotechnology
- Access to logistics for ex situ maintenance and experiments, including wet labs and up-to-date equipment for biological research (*omics*)
- Access to rare or unique specialist research capacity, e.g. bioreactors, micro- or meso-cosms and marine mammal facilities
- Access to biological and environmental data and bioinformatics
- Access to teaching/training laboratory space and conference facilities
- Access to logistics for hosting and catering visiting scientists and conferences

A core mission of EMBRC is to acquire sufficient understanding of marine ecosystems to allow for the sustainable exploitation of marine biological resources [6]. The EMBRC user community covers a wide panel of scientific fields. Application sectors range from gene and cell engineering (molecular farming, cell factories), biorefineries, biostatistics, software development and nutrition, to medicine and health care, aquaculture, crop disease control and environmental remediation, bioenergy and the development of biomaterials.

Fig. 10.1 Distribution of EMBRC laboratories in Europe

2.2 Links of EMBRC with Maritime Regions

By their very nature, EMBRC laboratories are located in peripheral maritime regions, often far from the major knowledge centres. A number of maritime regions in Europe now fully recognize in their Smart Specialization Strategies (S3) the potential of marine biotechnologies to help them reduce the gap with socio-economic mainstream regions. These features have been fully endorsed by the Conference of Peripheral Maritime Regions (CPMR). CPMR operates as both a think tank and a lobby group for maritime regions. It focuses mainly on social, economic and territorial cohesion, maritime policies and accessibility. Maritime regions often lack major concentrations of industry. Yet, traditional activities as fisheries or shipbuilding offer synergistic opportunities based on bioresource

availability. The capacity of EMBRC to support these developments was highlighted in a brokerage event in 2012.[1]

3 The Rationale for the European Marine Biological Research Infrastructure Cluster (EMBRIC)

3.1 Contribution of Other Pan-European Research Infrastructures to the Development of the Blue Bioeconomy

Marine biology and ecology are experiencing a scientific revolution, notably in relation to the implementation of new *omics* techniques. It follows that, as in terrestrial research, marine biologists are becoming more and more specialized, resulting in greater complexity for individual scientists to achieve highly significant results. Marine biotechnology cannot therefore take full advantage of the most recent advances in scientific knowledge, a significant weakness in the fast-moving, knowledge-based bioeconomy. The need for interconnectivity between a variety of disciplines, such as biology, ecology, genomics, bioanalysis and structural and analytical chemistry, supported by computational and mathematical developments, is more critical than ever before.

Connectivity, however, is often lacking between EMBRC laboratories and the other RI excellence centres located inland (Fig. 10.2). A range of other factors also hinders knowledge and technology transfer from science to industry including cultural differences between the science and business communities, lack of incentives for public researchers, legal barriers and fragmented markets for knowledge and technology [7]. In addition, the emerging marine biotechnology sector mainly involves SMEs, which lack the resources to engage in significant medium- and long-term R&D activities. For example, 90% of the aquaculture companies in Europe have fewer than 10 employees [8] and could benefit from the cutting-edge technologies delivered by RIs.

Accelerating the development of the blue bioeconomy requires RI pipelines through which workflows lead rapidly to marketable products. A number of other RIs provide the required complementarity to contribute to the development of such marine biotechnological pipelines.

The European Marine Biological Research Infrastructure Cluster (EMBRIC) is a 4-year project funded by the European Commission through the Research Infrastructures Work Programme of the EU Framework Programme for Research and Innovation, Horizon 2020. This European project is a consortium of 27 institutions, receiving a total funding of 9,041,611 €.

[1]"The Potential Role of Regions in the Development of European Research Infrastructures: The Example of the European Marine Biological Resources Centre (EMBRC)": https://webgate.ec.europa.eu/maritimeforum/en/node/2715

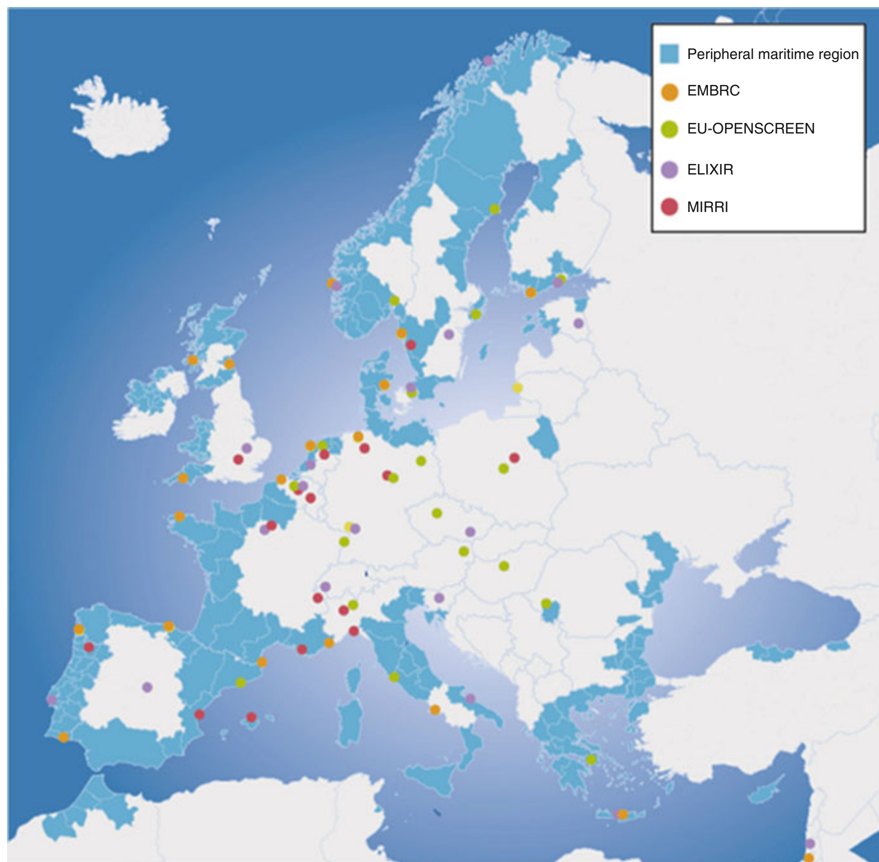

Fig. 10.2 Laboratory distribution of the RIs involved in EMBRIC. The map illustrates the geographic separation of marine stations (EMBRC laboratories), mostly located in areas with direct access to marine habitats, from the laboratories of other ESFRI RIs such as EU-OPENSCREEN, MIRRI and ELIXIR, which are mainly concentrated inland. The maritime regions shaded in blue are affiliated with the CPMR

The idea for EMBRIC originated as early as at the beginning of 2014. It was initiated by EMBRC, which quickly enlisted the participation of the RIs referred to as ELIXIR, EU-OPENSCREEN and MIRRI as well as the Infrastructure Initiatives known as AQUAEXCEL and RISIS.

ELIXIR, the European infrastructure for biological information, manages data for all of European biological sciences, including such fields as crop science and forestry, human data, rare diseases as well as marine metagenomics. EU-OPENSCREEN, the European infrastructure for chemical biology, also supports life science research and its translation to medicine and agriculture. MIRRI facilitates access to high-quality microorganisms, associated data and expertise. AQUAEXCEL integrates key aquaculture research facilities in Europe, covering all fish culture systems. RISIS, the infrastructure for research and innovation policy,

provides platforms and data sets for researchers dealing with science and innovation studies. All of these RIs recognized the need for more connectivity between RIs, between science and industry, and between European regions.

3.2 Objectives and Scope of EMBRIC

The overarching objective of EMBRIC is to build interconnectivity along three dimensions: science, industry and regional RD&I policies. The expected outcome is a sustainable alliance of RIs that foster innovation in marine biotechnologies.

The specific objectives of EMBRIC are to:

1. Develop coherent chains of high-quality services for access to biological, analytical and data resources by connecting cognate ESFRI and other RIs (EMBRC, MIRRI, EU-OPENSCREEN, ELIXIR, AQUAEXCEL, RISIS) and deploying common underpinning technologies and practices.
2. Strengthen the connection between science and industry through company forums and collaborative projects.
3. Geographically defragment public and private sector communities in the domain of marine biotechnology by involving regional RD&I policymakers in the construction of EMBRIC.

EMBRIC is designed to propose integrated multidisciplinary value chains of services for the exploration and sustainable exploitation of marine bioresources as sources of biomolecules and/or as whole organisms for food. The cluster integrates RIs that provide access to the full spectrum of marine diversity (EMBRC) or are specialized in the provision of specific groups of organisms (MIRRI, prokaryotes and fungi; AQUAEXCEL, finfish). Using these biological resources as raw materials, the cluster is developing service-oriented pipelines for natural product discovery and genetic selection for aquaculture (Fig. 10.3). EU-OPENSCREEN contributes its services and expertise in the area of natural product discovery, and AQUAEXCEL contributes in the aquaculture domain. ELIXIR provides cross-cutting expertise on data services and management. RISIS specializes in the quantitative analysis of research and innovation through organized data sources. Members of RISIS are developing indicators to estimate socio-economic impact of EMBRC marine research centres on local innovation ecosystems across Europe, to understand the socio-economic benefits stemming from research projects and innovation activities.

In practice, this amounts to (1) enlisting the participation of relevant RIs and organizing workflows that cover complete value chains, from the capture or culture of marine organisms to technology transfer; (2) building an Infrastructure alliance which will be capable of fulfilling the needs of its users throughout various dimensions and scales (science and technology, technology transfer, subsidiarity across Europe); and (3) mobilizing regional, national and EU policymakers to develop and finance a sustainable alliance.

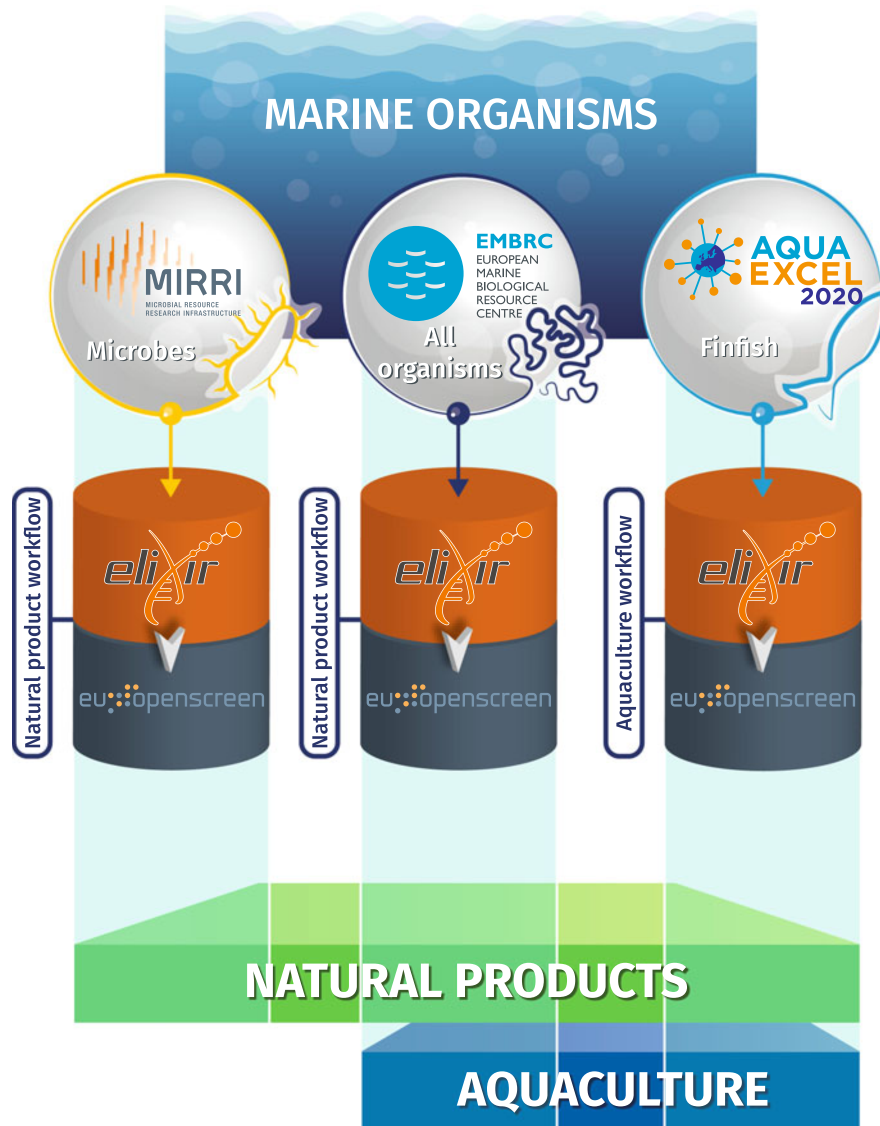

Fig. 10.3 General organization of EMBRIC workflows for value creation. Marine bioresources are provided by EMBRC, AQUAEXCEL or MIRRI. Involvement of ELIXIR and EU-OPENSCREEN expert centres further streamlines the study of these bioresources in view of the delivery of active biomolecules (natural product workflow) or aquaculture products (aquaculture workflow)

The main assumptions associated to this model are:

1. For the scientific dimension: The establishment of and provision of access to integrated workflows will create synergies within the alliance and with its user communities that will accelerate the development of key enabling technologies, for the benefit of the scientific community as a whole.

2. For the technology transfer dimension: The fostering of synergies between regional innovation ecosystems and the emergence of a community of practice for technology transfer [9, 10] in marine biotechnology will lead to more effective and efficient development of innovative products.
3. For the policy dimension: The demonstration of a positive incentive/risk ratio in EMBRIC will convince a critical mass (>50%) of its participating legal entities to commit to providing resources along with a regulatory framework to grow and sustain the alliance.

The EMBRIC project focuses on two specific sectors of marine biotechnology, namely, (1) discovery and development of marine natural products and (2) genetic selection of finfish and shellfish for aquaculture. Many opportunities, such as those dealing with the utilization of macroalgae (seaweeds) or animal-algal symbioses (corals and molluscs), are not covered by EMBRIC at present. As the project develops, it will undertake to reach out to other sectors.

3.3 Organization of Workflows at EMBRC Facilities

European RIs are seen as an indispensable hardware to fully exploit and ensure long-lasting effects of research programming, i.e. RIs collectively mobilize the necessary resources to drive integration at the scientific, technological and geographical levels.[2]

EMBRC facilities are situated in locations with unique yet complementary assets, covering most of the marine biodiversity in Europe. These maritime territories differ in ecological conditions and natural bioresources. At each facility, different blends of research, higher education and technology transfer are present, somewhat depending on the socio-economic forces that make use of these bioresources, e.g. seaweeds in Brittany, salmon in Scotland and Norway, sea bass and sea bream in Crete and microalgae in Campania and Occitanie.

With a few exceptions, however, EMBRC facilities lack the critical mass in key science areas or technologies such as bioinformatics, chemical biology or microbiology. EMBRIC enables fuller experimental workflows and virtually extends our investigation capacity. Involving infrastructure elements from ELIXIR, EU-OPENSCREEN and MIRRI, these pipelines are geared to fast-track bio-discovery (bioactives, biorefineries) processes and to develop new production systems (from cell factories to aquaculture). The EMBRIC vision is not only to foster an alliance between several pan-European Research Infrastructures but also to partner in the materialization of discovery pipelines for research on specific bioresources at specific locations, with each RI operating different parts of the pipelines (Fig. 10.4).

[2]cf. Declarations at the 2nd International Conference on Research Infrastructures: http://www.icri2014.eu/

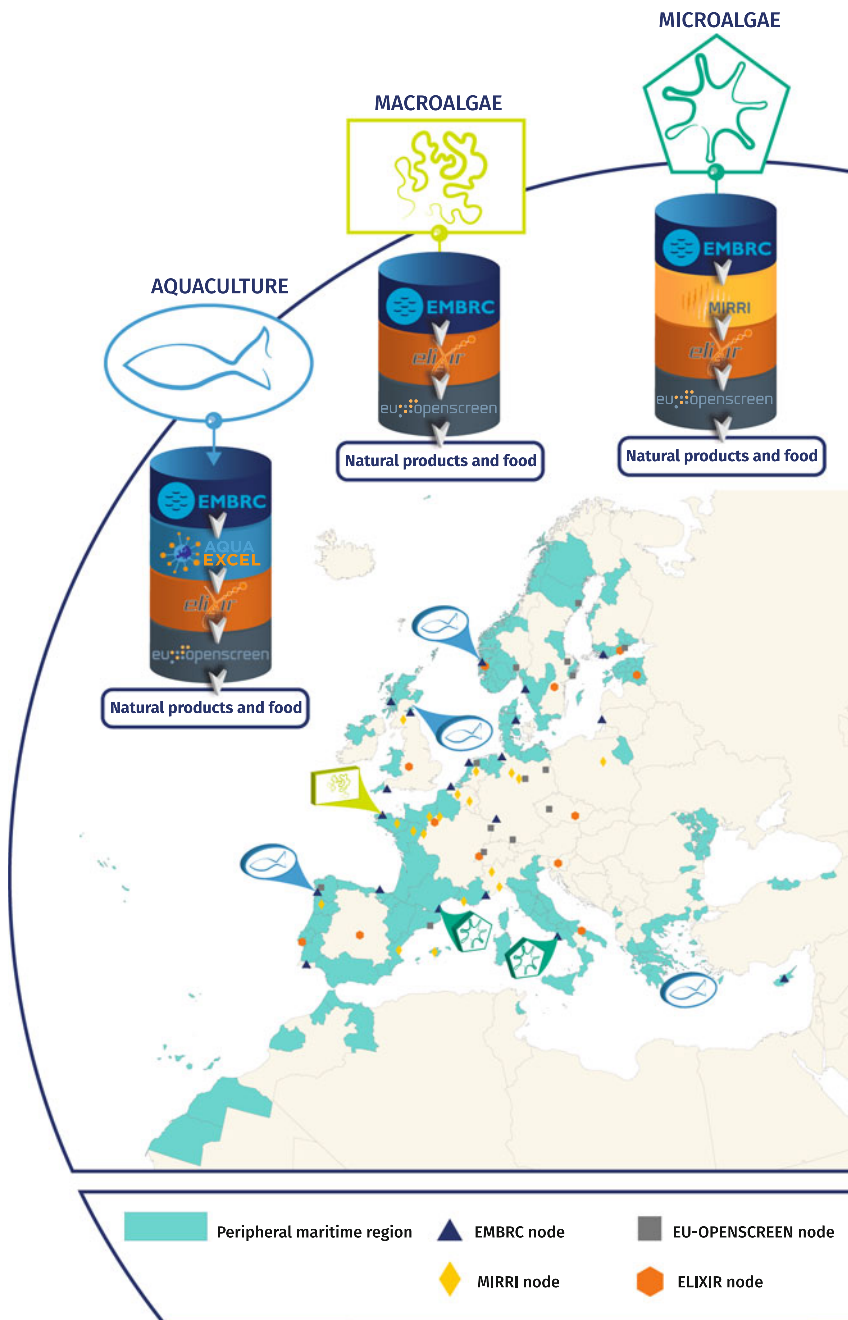

Fig. 10.4 Examples of EMBRIC innovation clusters. The map highlights the RI workflows in relation to the marine bioresource they stem from: (1) macroalgae (Brittany), (2) fish and shellfish (Scotland, Crete, Basque Country, Galicia), (3) microbes (bacteria and/or microalgae, Occitanie, Campania)

4 EMBRIC: An Instrument to Promote the Blue Bioeconomy

4.1 Organization of Innovation Clusters at EMBRC Facilities

The term “innovation ecosystem” best describes the matrix of interactions between scientific, industrial, economic and political stakeholders which enable technological development and innovation. Top scientific and technical talent, an entrepreneurial drive, a profound sense of community and strong cooperation and coordination among stakeholders are the pillars of a successful innovation ecosystem. Innovation ecosystems typically involve a number of elements, ranging all the way from basic scientific discovery, knowledge and technology transfer, technological and economic maturation, intellectual property (IP) brokerage, prototyping and demonstration for the development of new products and services, and receptive companies. These are key elements of innovation chains.

The distance from the generation of knowledge to the delivery of products or services to the market can be measured according to a Technology Readiness Level (TRL) scale, spanning from the observation of basic principles in the laboratory (TRL 1) to the actual system proven in industry (TRL 9).[3]

As excellence foci, the EMBRC expert centres are able to attract private research users. Since EMBRC contributes to research, while companies mainly undertake development, the pooling of resources and skills at EMBRC facilities will potentially lead to more innovative products and services in reaching the marketplace. Basically at EMBRC facilities, the private partners will have access to research activities best performed by academia (TRL 1–3/4) which complement private sector research (TRL 4/5–9), reducing unnecessary duplication. In this process and with the input of other RIs, EMBRIC is accelerating the maturation of technologies in EMBRC expert centres, thereby accelerating knowledge and technology transfer to companies and potentiating innovation.

Such innovation clusters can range from the implementation of good practices to genuine science parks with the possibility of attaining TRL as high as 8–9 (Table 10.1). Stage 1 is achievable by all EMBRC laboratories. Stage 4 involves integration of strategic research, initial and long-life training, and technology transfer into a separate legal entity. Science parks allow the clustering of companies that can mutualize services and facilities, but also engage in collaborations and joint ventures that boost local development and favour the circular economy.[4]

[3]See the full description of TRLs in the Horizon 2020 programme here: https://ec.europa.eu/research/participants/data/ref/h2020/wp/2014_2015/annexes/h2020-wp1415-annex-g-trl_en.pdf

[4]The circular economy describes the idea of “closing the loop” of product life cycles through increased recycling and reuse of resources that overall benefits the environment and the economy. See the EU Action Plan for the Circular Economy: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52015DC0614

Table 10.1 From expert centres to innovation clusters in EMBRC facilities

Stage	TRL	Organization criteria	Impact
1. EMBRC laboratory	1–2	Signing the EMBRC charter	Local
2. Technological platform	3–4	In-house facilities	Regional
3. Incubator	5–7	Separate facility	National
4. Science park	8–9	Physically and legally independent entity	European

An example for such an initiative is provided by "Blue Valley", a science park under development next to the facilities of the Station Biologique de Roscoff (SBR). Blue Valley will combine into a common governance local and regional authorities, the science and higher education operators of SBR and a variety of private companies. The objective of this science park is to promote territorial economic development based on the sustainable exploitation of the marine bioresources in Brittany. Blue Valley will involve a number of enterprises, covering the whole value chain, from production systems to biotransformation as well as monitoring the environment.

4.2 The Need for Public and Private Investments at Various Scales

The Regional Scale The main drivers of the blue bioeconomy in Europe are the territories, for two reasons: (1) technological production is anchored in regional development; and (2) the production systems of marine bioresources are essentially located next to the sea, in maritime regions. Regions and local authorities are hence relevant dimensions to organize and promote the knowledge-based blue bioeconomy.

Yet, the capacity to retain or attract human and financial capital for investment in the development of the maritime economy is far from assured, even in those regions where favourable pre-existing socio-economic forces are present. Developing the emerging sector of marine biotechnologies will require significant investments before returns can be expected. Based on arguments for technology push and market pull combined with indicators of positive socio-economic impact, EMBRIC regional innovation clusters can showcase ground-breaking research and technologies, to attract public and private funds.

The National Scale Science production is a global process, which requires steady funding to reach and maintain excellence as well as competitiveness. Most Member States and Associated Countries in Europe have recognized that progress in research and innovation is based on three pillars: human capital, research programming and research infrastructures. In coordination with ESFRI, they have launched the establishment of national roadmaps for RIs.

In France, this roadmap contains as many as ca. 100 entries, including 23 National Infrastructures in Biology and Health, among them the Centre National de Ressources Biologiques Marines (EMBRC France).

In Spain, the map of Spanish Unique Scientific and Technical Infrastructures is composed of 29 RIs. This roadmap has been used to take decisions on the investment priorities of the European Regional Development Fund in coordination with the regional S3. The Spanish map aligns with the ESFRI plans, and Spain participates in more than 30 ESFRI infrastructures, among them EMBRC.

The Portuguese national roadmap was adopted in 2013 after peer evaluation by an international panel. It comprises 40 RIs aligned to 23 ESFRI RIs, including EMBRC Portugal with 4 regional facilities. The Portuguese RIs will receive major investments in personnel, building renovation and instrumentation between 2018 and 2021, totalling 100.5 million euros, financed (66%) by national and European Structural and Investment Funds (ESIF) in alignment with the S3 of the regions where the RIs are implanted.

In Greece, the National Roadmap for Research Infrastructures was adopted after international evaluation and released in December 2014. It includes a list of 26 RIs, most of them linked with ESFRI RIs. CMBR (Centre for the study and sustainable exploitation of Marine Biological Resources), which is an EMBRC-based RI, is included in the list. Recently 20 of the RIs, among them CMBR, were evaluated and judged as aligned with the priorities of the S3 at the national and regional level and thus eligible for support through structural funds. The General Secretariat for Research and Technology (GSRT) released in 2016 a call for the support of these RIs totalling 73 M€, and the evaluation of the proposals is currently in progress. A new call of approximately equal total financial support is expected in 2017.

The European Scale Regional innovation clusters are expected to specialize according to their main bioresources (Fig. 10.4). Competition between these regional clusters will certainly occur to some extent, e.g. to attract scientists, companies and jobs. Yet, complementarities and alliances can be promoted and supported, leading to the integration of innovation ecosystems in marine biotechnology all the way to a multiregional pan-European ecosystem fostering cohesive and inclusive growth. A powerful incentive for integration is collaboration and subsidiarity at the European level.

Regions have the political power to help in this process, using European Structural and Investment Funds as instruments to promote the development of innovation clusters. They can also foster the establishment of regional capital funds, combined with guaranties from the European Investment Bank to promote the development of the marine biotechnologies in their territories. In this respect, the European Fund for Strategic Investments (EFSI or Juncker Plan) is an interesting opportunity.[5] EFSI

[5]EFSI platforms are referred to as platforms that consist in *Special purpose vehicles, managed accounts, contract-based co-financing or risk-sharing arrangements or arrangements established by any other means by which entities channel a financial contribution in order to finance a number of investment projects* (Regulation (EU) 2015/1017 of the European Parliament and of the Council of 25 June 2015 on the European Fund for Strategic Investments, the European Investment Advisory Hub and the European Investment Project Portal and amending Regulations (EU) No

can either be geographically focussed (regional scale or national) or thematically focussed (marine biotechnology, for instance).

5 Conclusions

Generation of basic scientific knowledge in the marine domain is primarily carried out by academic operators, while translational research is principally the domain of the private sector, and resulting economic development is largely supported and accompanied by regional authorities. EMBRIC hence faces the need of marrying scientific excellence, a national and European challenge, with territorial development and cohesion, a multiregional challenge. A recent study by EMBRIC partners [11], based on the pilot case study of the Station Biologique de Roscoff and to be extended to other EMBRC facilities, shows that the two challenges can be reconciled to promote the blue bioeconomy.

EMBRIC addresses the necessity of scientifically integrating the marine biological research community with other disciplines. The construction of EMBRIC workflows will enable more precise and complete matching of user demands with service supply. Such an alliance between the EMBRIC RIs in the implementation of expert centres at the EMBRC facilities will mobilize entire innovation chains in their regions, promoting socio-economic development.

There are a number of other Research Infrastructures or Integrated Infrastructure Initiatives that are relevant to the EMBRIC alliance, such as LifeWatch (biodiversity data), Euro-Bioimaging (advanced microscopy), Instruct (structural biology) or Eurofleet (oceanographic vessels). Indeed, several EMBRC laboratories already combine elements from these latter infrastructures as well as from those of the current EMBRIC alliance, a testimony of the growing importance of the marine bioresources in fundamental and strategic research in biology and ecology.

A top priority of EMBRC also is to embed with the maritime regions of Europe, directly and via their pan-European representation (CPMR). This will be achieved by identifying common incentives between the regional innovation ecosystems to overcome any regional tendencies for isolation. Alignment of regional with national funds as well as convergence between H2020 and structural funds should encourage maritime regions to build on complementarities and synergies. One promising idea worthy of consideration would be the creation of a Maritime Investment Fund under the responsibility of the European Investment Bank, to support the development of innovation ecosystems and to foster the growth of companies involved with the development of the marine biotechnology sector.

As EMBRIC develops, we can foresee an extended alliance to sustain the project legacy (Fig. 10.5). For example, the mission of LifeWatch is to advance biodiversity

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1291/2013 and (EU) No 1316/2013—the European Fund for Strategic Investments, Official Journal of the European Union, L 169, 1 July 2015).

Fig. 10.5 Potential alliances to sustain the EMBRIC legacy

research and to provide major contributions to addressing the big environmental challenges, including knowledge-based solutions for environmental management. A closer interaction between the current EMBRIC consortium and LifeWatch would serve well the preservation of marine habitats and of marine bioresources.

We believe that given the need of establishing synergies between research and innovation grants, on the one hand, and structural funds, on the other hand, regional, national and EU policymakers can be convinced to support EMBRIC activities beyond the lifetime of the project. We suggest that this policy, the clustering of RIs to resolve upcoming bottlenecks and to promote regional economies, is extended to address other societal Grand Challenges.

In conclusion, the RI EMBRC-ERIC and the RI cluster project EMBRIC recommend the implementation of two main strategies to promote the blue bioeconomy: (1) the concrete combination of diverse RI elements into EMBRIC innovation clusters in peripheral maritime regions and (2) the convergence of regional, national and European policies at these focal points. These initiatives are essential to

integrating European peripheral maritime regions into the mainstream of the global knowledge-based bioeconomy and to giving Europe a leadership in marine biotechnologies.

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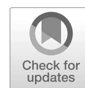
Part III
Supporting the Development of Marine Biotechnology

Chapter 11
Grand Challenges in Marine Biotechnology: Overview of Recent EU-Funded Projects



Chiara Lauritano and Adrianna Ianora

1 Introduction

The EU FP7 programme funded several projects from 2007 until 2017. Theme 2 'Food, Agriculture and Fisheries, and Biotechnologies' played a key role to support marine-related research projects and covered sustainable production and management of fisheries and aquaculture, quality and safety in food products as well as marine biotechnologies (http://ec.europa.eu/research/bioeconomy/pdf/interim_catalogue_of_marine_projects-2012_en.pdf). Theme 2 included the following activities: (2.1) sustainable production and management of biological resources from land, forest and aquatic environments; (2.2) fork to farm, food (including seafood), health and well-being; and (2.3) life sciences, biotechnology and biochemistry for sustainable non-food products and processes. The projects belonging to Theme 2 are reported in Table 11.1. In this chapter, we focus only on projects related to activity 2.3 and, in particular, on KBBE-3-2 on 'Marine and fresh-water biotechnology (blue biotechnology)' projects since these are the ones that are most related to drug discovery. In addition, we also focus on the few ongoing projects funded under the topic 'Blue Growth' of H2020 aimed at improving the exploitation of marine organisms for drug discovery and other industrial applications. Exploring the potential of marine biodiversity has increased and will further increase also, thanks to advancements in sampling and cultivation technologies and in molecular biology techniques. The number of potential compounds isolated from marine organisms now exceeds 28,000 with hundreds of new compounds being discovered every year [1]. However, those that have either been marketed or are under development are relatively few

C. Lauritano · A. Ianora (✉)
Biotechnology Laboratory, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy
e-mail: adrianna.ianora@szn.it

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Table 11.1 The table reports Theme 2 EU FP7 activities: (2.1) sustainable production and management of biological resources from land, forest and aquatic environments; (2.2) fork to farm, food (including seafood), health and well-being; and (2.3) life sciences, biotechnology and biochemistry for sustainable non-food products and processes. For each activity, Knowledge-Based Bio-Economy (KBBE) and relative projects are reported

Activity 2.1 Sustainable production and management of biological resources from land, forest and aquatic environments		
KBBE-1-1 Enabling research		
WildTech		
KBBE-1-2 Increased sustainability of all production systems (agriculture, forestry, fisheries and aquaculture), plant health and crop protection		
AquaTrace	ECOKNOWS	PRO-EEL
ARIMNet	EUROSHELL	PROMICROBE
ARRAINA	FACTS	REPROSEED
BENTHIS	FishPopTrace	SARNISSA
BIVALIFE	LIFECYCLE	SELFDOTT
BRIGHTANIMAL	MADE	SOCIOEC
COEXIST	MYFISH	TARGETFISH
COPEWELL	PREVENT ESCAPE	TXOTX
KBBE-1-3 Optimized animal health production and welfare across agriculture, fisheries and aquaculture		
EMIDA	STAR-IDAZ	
KBBE-1-4 Socio-economic research and support to policies		
AFSPAN	COMFISH	MEFEPO
AQUAINNOVA	CREAM	PEGASUS
AQUAMED	DEEPFISHMAN	TAPSIM
ASEM-AQUACULTURE09	EcoFishMan	TRANSDOTT
BECOTEPS	JAKFISH	
KBBE-1-5 The Ocean of Tomorrow: joining research forces to meet the challenges in ocean management		
ACCESS	VECTORS	MERMAID
COCONET	PERSEUS	H2OCEAN
MICRO B3	TROPOS	
Activity 2.2 Fork to farm: Food (including seafood), health and well-being		
KBBE-2-3 Food processing		
AFTER	COLORSPORE	
KBBE-2-4 Food quality and safety		
BASELINE	ECsafeSEAFOOD	PERFOOD
CONFFIDENCE	NAFISPACK	PROMETHEUS
KBBE-2-5 Environmental impacts and total food chain		
GMSAFOOD	SECUREFISH	
SEAT	SENSE	
KBBE-2-7 Coordinated call with India (Department of Biotechnology—DBT)		
NAMASTE		
Activity 2.3 Life sciences, biotechnology and biochemistry for sustainable non-food products and processes		

(continued)

Table 11.1 (continued)

KBBE-3-1 Novel sources of biomass and bioproducts		
AQUATERRE		
KBBE-3-2 Marine and fresh-water biotechnology (blue biotechnology)		
BAMMBO	MaCuMBA	PharmaSea
BlueGenics	MAMBA	PolyModE
GIAVAP	MAREX	SeaBioTech
LIPOYEASTS	MarineBiotech	SUNBIOPATH
KBBE-3-3 Industrial biotechnology: novel high added value bioproducts		
MARINE FUNGI	MG4U	SPECIAL
MetaExplore	RADAR	
KBBE-3-4 Biorefinery		
APROPOS	CHIBIO	SPLASH
KBBE-3-5 Environmental biotechnology		
BIOCLEAN	MAGICPAH	
KILL●SPILL	ULIXES	
KBBE-3-6 Emerging trends in biotechnology		
MEM-S		

[2, 3]. To date, there are seven approved pharmaceuticals in clinical use, four of which are anticancer drugs, and about 26 natural products in Phase I to Phase III clinical trials, 23 as anticancer agents, 2 for schizophrenia and Alzheimer's and 1 for chronic pain (http://marinepharmacology.midwestern.edu/clinPipeline.htm).

The harsh, and sometimes extreme, chemical and physical conditions in the oceans have favoured the synthesis of a great variety of compounds by marine organisms. These compounds are often unique in terms of diversity and structural and functional features with respect to compounds isolated from terrestrial plants representing a reservoir of new bioactives with great pharmaceutical potential [4]. Oceans are areas of the planet once thought of as environments with insurmountable physical and chemical barriers to life. These are now known to be niche habitats populated by 'extremophiles', organisms that require extreme environmental conditions for survival. Such environments range from marine hot springs (temperatures > 100 °C), polar (high latitudes, low temperatures) and deep sea (depths > 1000 m, high pressures) to the deep biosphere (sub-seafloor, extremely low in nutrients). Recent advances in exploration and analytical techniques have allowed for the discovery of numerous extremophile organisms, mainly bacteria that produce novel bioactive compounds because of their physiological adaptation to extreme environmental conditions. Opportunities for the use of these ocean bioresources in markets for industrial enzymes, functional foods, cosmeceuticals, biomaterials, bioprocessing, pharmaceutical products and medical devices are destined to rapidly grow in the years ahead [5].

In order to speed up the marine drug discovery pipeline, several EU projects have focused on implementing new sampling and cultivation technologies, massively

Table 11.2 Starting and ending dates of the EU projects and their relative websites discussed in this review

Project name	Starting date	Ending date	Website
BAMMBO	01 March 2011	01 March 2014	www.bammbo.eu, http://cordis.europa.eu/project/rcn/97837_en.html
BlueGenics	01 August 2012	31 July 2016	www.bluegenics.eu
GIAVAP	01 January 2011	31 December 2013	http://cordis.europa.eu/project/rcn/97420_en.html
LIPOYEASTS	01 August 2008	31 July 2011	http://www.lipoyeasts.ugent.be/
MaCuMBA	August 2012	July 2016	www.macumbaproject.eu
MAMBA	01 July 2009	30 June 2013	http://mamba.bangor.ac.uk/media.php
MAREX	01 August 2010	31 July 2014	https://www.marex.fi/
MARINE BIOTECH	01 December 2013	30 November 2017	www.marinebiotech.eu
PharmaSea	October 2012	March 2017	www.pharma-sea.eu
PolyModE	01 May 2009	30 April 2013	http://polymode.eu
SeaBioTech	01 August 2012	31 July 2016	http://spider.science.strath.ac.uk/seabiotech/
SUNBIOPATH	01 January 2010	28 February 2013	http://cordis.europa.eu/project/rcn/92954_en.html
EMBRIC	01 June 2015	31 March 2019	www.embric.eu
INMARE	01 April 2015	31 March 2019	www.inmare-h2020.eu
NoMorFilm	01 April 2015	31 March 2019	www.nomorfilm.eu
TASCMAR	01 April 2015	31 March 2019	http://www.tascmar.eu/

sequencing genomes and transcriptomes of new species, studying the metabolome and compounds synthetized by marine organisms and enhancing scientific and technological research infrastructures. The EU-funded projects under the topic 'Marine and freshwater biotechnology (blue biotechnology)' were BAMMBO, BlueGenics, GIAVAP, LIPOYEASTS, MaCuMBA, MAMBA, MAREX, MarineBiotech, PharmaSea, PolyModE, SeaBioTech and SUNBIOPATH, while examples of ongoing H2020-funded projects are EMBRIC, INMARE, NoMorFilm and TASCMAR (Table 11.2). Here we discuss the scientific ambitions and efforts of these EU projects to find new molecules for the prevention and treatment of diabetes, cancer, age-related diseases and bacterial infections, as well as new products for industrial applications. Often with a focus on underexploited marine microorganisms, essentially photo- and chemosynthetic bacteria together with fungi and microalgae, these projects have aimed to achieve optimized and sustainable production of relevant biomass and high added value compounds for pharmaceutical, nutraceutical and cosmeceutical applications (Fig. 11.1).

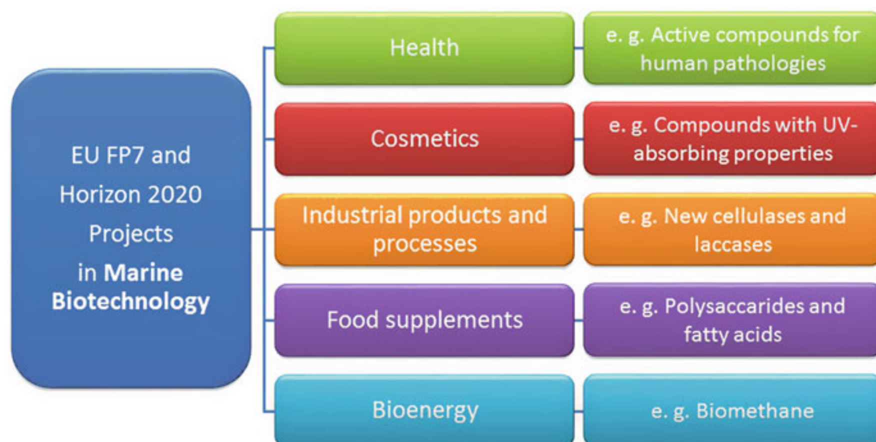

Fig. 11.1 EU FP7 and H2020 marine biotechnology projects can be described as the use of marine micro- and macroorganisms to provide products, technologies and processes in different fields: health, cosmetics, industrial products and processes, food supplements and bioenergy. Examples of products discussed in this chapter are reported as well

Major efforts have focused on the isolation and cultivation of marine organisms (including species considered uncultivable), in genetically modifying strains in order to produce high yields of the desired products, as well as in massively screening both micro- and macroorganisms for prevention or treatment of several human diseases and/or industrial applications. This chapter compares the most significant findings and advances of these projects and how they have contributed to setting the ground for the implementation of new programmes on ocean bioproducts for the future.

2 BAMMBO and MaCuMBA

The use of marine species is often hindered by the low success rate for isolating novel microorganisms and by poor growth efficiency. Consequently, the vast majority of marine microorganisms have not been cultivated and are often considered as 'unculturable'. Today, one of the main challenges for microbiologists is to develop strategies to cultivate the uncultured majority of marine microorganisms. BAMMBO and MaCuMBA were two EU FP7 projects whose aim was to improve the isolation rate and/or growth efficiency of marine microorganisms in order to sustainably produce high yields of value-added products for the pharmaceutical, cosmetic and industrial sectors.

The BAMMBO (sustainable production of biologically active molecules of marine-based origin) (http://www.bammbo.eu/) project, which ended in 2014, aimed to improve laboratory methods for culturing both macro- and microalgae and created and tested several new prototype reactors for sustainable algal

production [6]. BAMMBO partners screened massive numbers of marine organisms (i.e. algae, sponges, fungi, bacteria and yeast) for bioactivity such as antioxidant, anticancer or antimicrobial effects [7–9]. Discoveries included antioxidants, antimicrobial compounds, enzymes to prevent skin ageing and an extract that prevents barnacles attaching to surfaces ([10], http://cordis.europa.eu/result/rcn/163827_en.html). BAMMBO researchers also created a digital repository (biobank) of the organisms screened for their bioactivities and studied methods to detect algal toxins [11–14]. They developed immortalized algal cell lines to limit the harvesting of natural resources. For example, immortalized macroalgal cell tissue cultures of *Fucus spiralis* (for polyphenol production) and *Sphaerococcus coronopifolius* (for halogenated terpene production) were created, but regeneration was evident only in initial trials, and these macroalgal cell tissue cultures lost their viability after 20 days (*S. coronopifolius*) and 2 months (*F. spiralis*) (http://cordis.europa.eu/result/rcn/163827_en.html). Overall, the project led to 37 scientific publications; 23 IP items documented including categories of new discoveries/compounds, patents, novel inventions, know-how and new and/or modified production processes; eight invention disclosure forms filed and registered with the coordinator; 4 patent application intentions; and eight technology package offerings (as reported in *http://cordis.europa.eu/result/rcn/163827_en.html*).

On the other hand, the MaCuMBA (www.macumbaproject.eu/) project, which ended in July 2016, was specifically designed to explore the diversity of marine microorganisms from conventional and extreme habitats (e.g. cold environments) using cultivation-dependent strategies and to improve their isolation rate and growth efficiency by applying innovative methods and automated high-throughput procedures. Several strains were isolated as a result of MaCuMBA activities and were housed in four major collections belonging to partners of the MaCuMBA project: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Roscoff Culture Collection (RCC), Université de Bretagne Occidentale Culture Collection (UBOCC) and Culture Collection Yerseke (CCY). UBOCC is divided into two subcollections: UBO-Extremophiles Laboratoire de Microbiologie des Environnements Extrêmes (LM2E) and UBO-Fungi Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne (LUBEM). CCY also deposited all strains of cyanobacteria at DSMZ. Several microorganisms, until now considered as uncultivable, have been collected and sampled. For example, as reported in the results section in the project website (http://www.macumbaproject.eu/macumba-results/macumba-deliverables), 21 hyperthermophilic autotrophic methanogens have been isolated from deep hydrothermal vent sites known at the Cayman Trough (depth 4965 m, Beebe Vent Field). They have been cultured, and the analyses of their optimal growth conditions (temperature, pH, salinity) are presently ongoing. Similarly, around 100 mesophilic aerobic bacterial strains off the coast of Brittany in France were isolated and cultivated. There are ongoing studies to culture them using different sources and organic matter concentrations in order to fully characterize some very interesting new isolates. Approaches used included the cocultivation of microorganisms, as well as gradient cultures and other methods mimicking the natural environment, with the objective of obtaining sustainable production and

replicability of culture conditions. Several studies focused on viruses, with new viruses discovered possessing novel characteristics in their genomes [15, 16]. For example, metagenome sequencing allowed the description of the complete genomes of 208 new marine phages (viruses infecting prokaryotic cells) in a sample off the coast of Alicante, Spain [15]. Other studies were performed to identify and characterize the abundance and diversity of known or new species of bacteria and microalgae by using various techniques, such as flow cytometry sorting (FCS) and genome and 16S/18S sequencing [17–27]. Transcriptomes and metatranscriptomes were generated to better characterize the sequenced species and/or to understand gene expression changes depending on environmental variability [28–31]. Other studies focused on microalgal parasites, consisting primarily of chytrid fungi. For example, the novel parasitoids, *Parvilucifera rostrata* sp. nov. (Perkinsozoa) [32] and *Dinomyces arenysensis* gen. et sp. nov. [33], infecting planktonic dinoflagellates were identified. Finally, efforts were applied to improve protocols for flow cytometry analysis of phytoplankton cultures and natural samples [34]. Overall, the project led to almost 100 scientific publications as reported in http://cordis.europa.eu/project/rcn/104389_en.html.

3 GIAVAP and SUNBIOPATH

Other projects focused on improving microalgal culturing by also engineering modifying strains (e.g. GIAVAP and SUNBIOPATH). The GIAVAP (Genetic Improvement of Algae for Value Added Products; http://cordis.europa.eu/project/rcn/97420_en.html) project, which ended in 2013, focused on genetic modifications to make microalgae better suited for industrial applications. GIAVAP successfully performed genomic and transcriptome sequencing of two high-value microalgal species (*Parietochloris incisa* and *Haematococcus pluvialis*) and analysed quantitative transcriptomics in these species under various conditions of nitrogen starvation [35, 36]. The consortium adapted genetic engineering techniques to seven algal strains (*Chlamydomonas reinhardtii*, *Chlorella* sp., *Haematococcus pluvialis*, *Nannochloropsis oceanica*, *Ostreococcus tauri*, *Phaeodactylum tricornutum* and *Parietochloris incisa*) of economic interest focusing on long-chain polyunsaturated fatty acids, carotenoids and the overexpression of peptides of commercial value [37–41]. Hamilton et al. [37], for example, investigated the scalable production of omega-3 long-chain polyunsaturated fatty acid (LC-PUFA) (EPA) and docosahexaenoic acid (DHA) from a transgenic strain of the diatom *Phaeodactylum tricornutum*. In addition, they evaluated the different EPA concentrations obtained culturing the selected strain in various culturing volumes (from 1 L flasks to a 550 L closed photobioreactor and a 1250 L raceway pond with artificial illumination). A significant production of DHA over EPA, equivalent to 6.4 μg/mg dry weight DHA in a mid-exponentially growing algal culture, was observed in cells grown in the photobioreactor. Transgenic strains grown in raceway ponds, for example, produced the highest levels of EPA (12.8%) incorporated in neutral lipids. Overall, these

results clearly demonstrated the potential for the development of the transgenic *P. tricornutum* as a platform for the commercial production of EPA and DHA. In addition, these data unequivocally showed that the production of the compounds of interest changed using different culturing volumes (e.g. flasks, bioreactors and raceway ponds). In parallel, the project developed (or adapted pre-existing) cultivation technologies for a replicable and sustainable production of bioproducts, as well as improved harvesting and extraction methods for lipids, carotenoids and proteins. For example, Pal et al. [42] investigated the effects of osmotic downshift induced by the transfer of *Nannochloropsis oceanica* from artificial seawater medium to the same medium without NaCl or freshwater modified BG-11 medium as a function of photosynthetically active radiation. Alterations in growth, total fatty acid (FA) content and composition and in relative contents of metabolites relevant to osmotic adjustments were studied. For instance, they found that the highest EPA contents were determined in the cultures grown in BG-11 medium. Products identified by the GIAVAP partners were tested for energy, pharmaceutical, nutritional or medical applications for economic evaluation of the production processes and their economic exploitation (e.g. recent GIAVAP achievements by using modified strains may provide new approaches for improving the economic value of algal biomass production). The project led to 5 patents in algal genetic engineering and at least 32 peer-reviewed publications (some of them available via OpenAIRE at http://cordis.europa.eu/project/rcn/97420_en.html).

Similarly, SUNBIOPATH ('towards a better sunlight to biomass conversion efficiency in microalgae'; http://cordis.europa.eu/project/rcn/92954_en.html), which ended in 2013, aimed at improving culturing biomass yields and valorization of biomass from two chlorophycean photosynthetic microalgae, *C. reinhardtii* and *Dunaliella salina*. Biomass yields were improved both by modifying culturing conditions and by genetically engineering the strains (for references see http://cordis.europa.eu/project/rcn/92954_en.html). For instance, researchers studied the photosynthetic apparatus changes in *C. reinhardtii* upon acclimation to different light intensities and found that, when grown in high light, cells had a faster growth rate and higher biomass production compared with low and control light conditions [43]. In addition, cells acclimated to high light showed an increased carotenoid content, particularly zeaxanthin. However, cells acclimated to low light intensity were indeed able to produce more biomass per photon available as compared with high light-acclimated cells, since a large part of the light absorbed dissipated as heat. SUNBIOPATH researchers studied the photochemistry and sunlight capture processes in light-harvesting complexes (Lhcs) present in the chloroplasts of these algae. Significant progress was made in the development of chloroplast genetic engineering of microalgae (e.g. *Chlamydomonas*; [44]); however the commercial exploitation of this technology still requires additional research. The consortium demonstrated that LhcSR3, a light-harvesting protein, is able to sense the luminal pH of the chloroplast and to act as a quencher of excitation energy [45]. The data demonstrated that LhcSR3 is a key protein involved in biomass production. The consortium also showed that induction of LhcSR3 by high light depends on a Ca++ sensor protein (CAS), an important finding, which opens new insights into the regulation of the high light

response of *Chlamydomonas* [46]. Other SUNBIOPATH studies focused on identifying modified strains to obtain high concentrations of triacylglycerols (TAG; [47]). Acyl-CoA diacylglycerol acyltransferases (DGATs) have been demonstrated to play an important role in the accumulation of TAG compounds in higher plants. La Russa et al. [47] investigated the three most promising type 2 DGAT candidate genes potentially involved in TAG lipid accumulation by constructing overexpression strains. For each of the genes, three *Chlamydomonas* strains with enhanced DGAT mRNA levels were found, identifying useful strains for TAG production. Optimal, replicable and sustainable growth of the engineered microalgae was determined by SUNBIOPATH researchers in photobioreactors of different sizes (up to 250 L), some of which were designed and built during the project (http://cordis.europa.eu/result/rcn/58522_en.html). Biomethane production, reduction in carbon dioxide (CO_2) emission and biomass yields were some of the parameters studied. Compared to other biofuels, biomethane is attractive because the yield of biomass to fuel conversion is higher. The project led to at least 47 publications, some available via OpenAIRE at http://cordis.europa.eu/project/rcn/92954_en.html.

4 BlueGenics, LIPOYEASTS, MAMBA and PolyModE

Other EU FP7 projects were more focused on molecular approaches for drug discovery, as well as to produce various bioproducts and/or biodiesel, e.g. BlueGenics, LIPOYEASTS, MAMBA and PolyModE. The BlueGenics project (www.bluegenics.eu) was an industry-driven project that aimed to combine the knowledge obtained from marine genomics, chemo-genetics and advanced chemistry to produce recombinantly prepared novel secondary metabolite (lead) compounds and bring them up to preclinical studies. BlueGenics successfully tested more than 400 sponge and bacterial extracts and set up a protein kinase platform (an Alzheimer's disease cellular screening platform) and a screening platform for antiprotozoan activity. Regarding the culturing of marine organisms, researchers established the SustainCycle system for sustainable and reproducible sponge culturing, as well as the successful scale-up of a sponge primmorph culture and the implementation of a protocol to culture the bacterium *Rhodothermus marinus* in bioreactors in order to reach high productivities. This bacterium is used in the saccharification and fermentation of brown macroalgal carbohydrates to yield commercially valuable compounds, such as thermostable enzymes and glycosidic carotenoids (https://ria.ua.pt/handle/10773/14921?mode=full). Molecular investigations mainly focused on polyketide synthases (PKS), genes involved in the synthesis of bioactive compounds in many species [48, 49]. Swf, a new group of mono-modular type 1 PKS/FAS, which appears to be specifically associated with sponge symbionts, was identified for the first time. BlueGenics team also identified >50 gene clusters from uncultivated symbionts of marine sponges and cloned the first poriferan laccase from *Suberites domuncula* (a copper-containing enzyme involved in the antibacterial defence system of sponges; http://cordis.europa.eu/result/rcn/193365_en.html). Regarding preclinical results, a novel microparticulate material with morphogenetic activity consisting

of amorphous Ca2+-polyphosphate (Ca-polyP; see preliminary results in Wang et al. [50]) was developed, as well as Leucettines, a family of marine sponge-derived 2-aminoimidazolone alkaloids for the treatment of Alzheimer's disease and Down syndrome in mice models (http://cordis.europa.eu/result/rcn/193365_en.html). Finally, the BlueGenics team isolated several compounds from marine sponges such as isoswinholide B and swinholide K, potent cytotoxic dimeric macrolides [51] and new tridecapeptides from *Theonella swinhoei* [52]. Novel adociaquinone derivatives from *Xestospongia* sp. [53] and new incisterols A5 and A6 from *Plakortis cfr. lita* [54] were also isolated, as well as barrettides from *Geodia barretti* [55]. Examples of compounds isolated from other marine organisms include phallusiasterols A and B [56] and phallusiasterol C [57] from the Mediterranean tunicate *Phallusia fumigata* and conithiaquinones A and B, tetracyclic cytotoxic meroterpenes from the Mediterranean ascidian *Aplidium conicum* [58]. The project led to the publication of more than 100 papers (http://cordis.europa.eu/project/rcn/104248_en.html), 7 patent applications, an enzyme website (http://prokazyme.com/), a marine natural product database (initially named BG-DNPT (BlueGenics Database of Natural Products and Taxonomy); see details in http://cordis.europa.eu/result/rcn/193365_en.html) and a signature database (an EST-expressed sequence tag sponge database named 'SpongeBase').

LIPOYEASTS is a project on 'Mobilising the enzymatic potential of hydrocarbonoclastic bacteria and the oleaginous yeast *Yarrowia lipolytica* to create a powerful cellular production platform for lipid-derived industrial materials' which ended in 2011 (http://www.lipoyeasts.ugent.be/articles.htm; [59]). It proposed to develop the oleaginous yeast *Yarrowia lipolytica* into a microbial factory by directing its versatile lipid metabolism towards the production of industrially valuable compounds like wax esters (WE), polyhydroxyalkanoates (PHAs), free hydroxy fatty acids (HFAs) and isoprenoid-derived compounds (carotenoids, polyenic carotenoid ester) [60]. Conversion of lipid intermediates into these products was achieved by introducing heterologous enzyme functions isolated from marine hydrocarbonoclastic bacteria into *Y. lipolytica.* This allowed to obtain biopolymers, waxes and other chemicals in high demand in chemical and biotechnological industries, potentially replacing the need for a very expensive chemical synthesis and also contributing to the elimination of oil spills via an environmentally friendly approach. The main results of the project included the generation of recombinant *Yarrowia* strains with a modified pool of acyl-CoA and 3-hydroxyacyl-CoA intermediates with desired acyl chain lengths; conversion of lipid intermediates into PHA in *Yarrowia*, recombinant strains for the production of HFAs and WE; and conversion of lipids into carotenoids and carotenoid esters in the yeast (http://cordis.europa.eu/result/rcn/54174_en.html). For example, the project created genetically modified yeast, converting lipids into PHA with a 20–25% yield, the highest level ever achieved by any yeast strain. Similarly, the same strategy was applied to generate yeast strains making wax esters, carotenoids and biodiesel. Beopoulos et al. [60] described the coordinated pathways of lipid metabolism, storage and mobilization in this yeast, focusing in particular on the roles and regulation of the various enzymes and organelles involved in these processes, as well as the production of biosurfactants. They also discussed the culture conditions that enhanced the

accumulation of lipids with a specific composition and presented examples of the potential use of *Y. lipolytica* in fatty acid bioconversion, substrate valorization and single-cell oil production. LIPOYEASTS researchers have also licenced their wax ester/biodiesel yeast production platform to a major biotechnology company in the United States and are submitting a manuscript describing the invention (http://cordis.europa.eu/result/rcn/85730_en.html).

The MAMBA ('Marine metagenomics for new biotechnological applications'; http://mamba.bangor.ac.uk/media.php) project, which ended in 2013, aimed to use new microbial strains to produce fine chemicals, antioxidants and/or anticancer drugs. Microorganisms, and particularly bacteria, are a promising and still mostly untapped source of enzymes for commercial use. However, scientists cannot grow most of these organisms in laboratory conditions, thus limiting the access to the enzymes they produce. In this project, researchers focused on microorganisms taken from various extreme marine locations and used them to create 'expression libraries' that were then screened directly for enzyme activity on specific enzyme targets (substrates). New active enzymes were hyperexpressed and crystallized in order to elucidate their structures. Using this method to screen for enzyme activity, MAMBA researchers identified more than 1100 potential new enzymes, 600 of which were selected for more in-depth studies. Half of these were isolated to be further investigated, and more than 40 new enzyme structures were elucidated [61–65]. MAMBA researchers effectively doubled the number of known enzymes that operate at extreme environments (temperatures, high salinity and extreme pH values). For instance, new cellulases, nicotinamidases and pyrazinamidases were identified [61–65]. Thies et al. [64], for example, created a metagenomic library in *Escherichia coli* with environmental DNA (eDNA) isolated from a microbial community of a slaughterhouse drain biofilm. By functional screening of this library, they identified several lipases, proteases and two clones with biosurfactant and haemolytic activities: putative N-acyl amino acid synthases. The protocols and screening platform used in the project have been commercialized, and several of the enzymes identified have been earmarked for commercial development (http://cordis.europa.eu/result/rcn/141319_en.html). The project led to at least 27 publications of which some are available via OpenAIRE at http://cordis.europa.eu/project/rcn/91262_en.html.

The PolyModE project (http://polymode.eu), which also ended in 2013, aimed to identify, characterize and optimize novel polysaccharide-modifying enzymes and to develop robust fermentation strategies for their large-scale production. The aim was to exploit the potential of polysaccharides for food, pharmaceuticals, cosmetics and technical applications. Polysaccharides are in fact extensively used in Europe for these applications (e.g. as hydrocolloids in the food industry). However, many of these sugars need to be modified via chemical means with extremely complex, time-consuming and expensive methods. The six complex carbohydrates with the highest current market share or expected future market potential were selected: alginate, carrageenan, chitosan, glycosaminoglycan, pectin and xanthan gum. For each of these, the industrial partners involved in the PolyModE project identified the enzymes offering the most promising potential for improved production of polysaccharides with novel physico-chemical properties and biological functionalities. These enzymes were

alginate epimerases, carrageenan sulfatases, chitosan deacetylases, glycosaminoglycan sulfatases, pectin deacetylases and xanthan gum deacetylases (http://cordis.europa.eu/project/rcn/90529_en.html). For each of these enzymes, details are reported in the final report summary available at http://cordis.europa.eu/result/rcn/143656_en.html. For example, Thomas et al. [66] characterized two marine alginate lyases from the bacterium *Zobellia galactanivorans* revealing two distinct modes of action and exquisite adaptation to their natural substrate. Genicot et al. [67] found a novel carrageenan sulfatase in the marine bacterium *Pseudoalteromonas carrageenovora*. In addition, partners optimized several expression and fermentation systems. For example, they successfully expressed several enzymes such as sulphatases and chitin-deacetylases in *Hansenula polymorpha* (http://cordis.europa.eu/result/rcn/143656_en.html). The PolyModE project led to more than 20 publications, some of which are available via OpenAIRE at http://cordis.europa.eu/project/rcn/90529_en.html.

5 SeaBioTech

The EU FP7 SeaBioTech project (http://spider.science.strath.ac.uk/seabiotech/) was designed and driven by SMEs to create innovative marine biodiscovery pipelines to convert the potential of marine organisms into novel industrial products for the pharmaceutical (human and aquaculture), cosmetic, functional food and industrial chemistry sectors (http://spider.science.strath.ac.uk/seabiotech/index.php). It aimed to collect species from previously unexplored habitats, including geothermal intertidal biotopes in Iceland, hydrothermal vent fields and deep-sea oligotrophic basins of the Eastern Mediterranean Sea and unsampled areas off the Scottish coast. SeaBioTech team combined metabolomics assisted by system biology and functional bioassays to increase the ability to uncover positive hits and to increase the yield of bioactive metabolites through fermentation technology at the industrial scale to deliver promising enzymes, polymers and small molecules for industrial needs. For details, see chapter 'From Seabed to Test-Bed: Harvesting the Potential of Marine Biodiversity for Industrial Biotechnology' by Edrada-Ebel et al. of this volume.

6 MAREX and PharmaSea

The EU FP7 MAREX project (http://cordis.europa.eu/project/rcn/95006_en.html), which ended in 2014, brought together both industrial and academic partners in order to isolate, characterize and sustainably exploit new compounds from extracts prepared from marine organisms. The organisms of interest belonged to pre-existing culture collections or were newly harvested or acquired from institutional, local, national and commercially available collections. They were micro- and macroalgae, cyanobacteria, sea anemones, tunicates and fishes from the Atlantic, Pacific and Indian

Oceans and Mediterranean, Baltic and Arabian Seas. MAREX innovations were targeted for industrial product development (related to pharmaceutical, nutraceutical, cosmetic, agrochemical, food processing and material and biosensor applications) in order to improve the growth and productivity of European marine biotechnology. MAREX aimed at a better understanding of environmentally conscious sourcing of marine biotechnology products and increased public awareness of the potential of marine biodiversity. Raw extracts, fractions and purified compounds of these organisms were studied for several therapeutically and industrially significant biological activities, including anticancer, anti-inflammatory, antiviral, antifouling and anticoagulant activities, as well as for ion channel/receptor modulation and plant growth regulation. Additional objectives were also to develop suitable and sustainable cultivation and harvesting processes for selected organisms, as well as to determine chemical structures and stereochemistry of the bioactive compounds, to design and synthesize lead compounds, to design and synthesize mimetics of the most promising compounds and to select an aptamer against a bioactive compound. Over 600 marine organisms were collected from the Atlantic, Indian and Pacific Oceans and from the Adriatic, Arabian, Baltic and Mediterranean Seas, including cyanobacteria, microalgae, sponges, sea anemones, tunicates and sea cucumbers. Partners developed methods and techniques for the cultivation of several algal and bacterial species. Several species showed activity; for some of them, raw extracts were fractionated, and active fractions were used for the identification of the active compounds [68–71]. The project also contributed to the design, synthesis and evaluation of around 400 new synthetic compounds based on natural products involved for possible cancer, bacterial infection and inflammation treatment [72–75]. For instance, new polyethers, such as iubol, 22-hydroxy-15 (28)-dehydrovenustatriol, 1,2-dehydropseudodehydrothyrsiferol and secodehydrothy rsiferol, were isolated and characterized from the red seaweed *Laurencia viridis*, a rich source of secondary metabolites derived from squalene [76]. These new polyethers exhibited significant anti-proliferative activity against a panel of cancer cell lines. The structures of these new polyethers were determined through the interpretation of NMR spectroscopic data, and the relative configuration was proposed on the basis of NOESY spectrum and biogenetic considerations. Interesting examples are also studies on sponges. In particular, Sepe et al. [73] studied a series of 4-methylenesteroid derivatives isolated from *Theonella* marine sponges as novel Pregnane-X-receptor (PXR) modulators. PXR is a member of a superfamily of nuclear receptors that activates gene transcription and is involved in the metabolism of endobiotics including bilirubin, bile acids, glucose and lipids, as well as xenobiotics. Thus, developing PXR ligands represents a good opportunity for the treatment of various pathologies (e.g. diabetes, obesity, dyslipidemias and liver disorders). The project investigated the effects of different modifications on ring A and on the side chain of 4-methylenesteroid derivatives towards PXR modulation, giving the basis for designing novel PXR modulators [73]. Zidar et al. [71] studied the marine alkaloids, clathrodin and oroidin, which were originally isolated from sponges of the genus *Agelas*. They were prepared and evaluated for possible antimicrobial activity against three bacterial strains (*Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli*) and one fungal strain (*Candida albicans*). Oroidin showed promising Gram-positive antibacterial activity. Using

oroidin as a scaffold, 34 new analogues were designed, prepared and screened for their antimicrobial properties. Of these compounds, 12 exhibited >80% inhibition of the growth of at least 1 microorganism, and the most active derivative was found to be 4-phenyl-2-aminoimidazole against the Gram-positive bacteria but also *E. coli*. In addition, several synthetic compounds showed UV-absorbing properties that might be potentially interesting to further explore cosmetic applications of these compounds (http://cordis.europa.eu/result/rcn/159271_en.html). Overall, the MAREX project led to the publication of 62 papers, plus another 21 submitted (as reported in the final report summary on the CORDIS website (http://cordis.europa.eu/result/rcn/159271_en.html).

The EU FP7 PharmaSea project (http://www.pharma-sea.eu/), which ended in 2017, also brought together a broad interdisciplinary team of academic and industrial researchers to produce new products for development in three accessible market sectors, health (infection, inflammation, neurodegenerative diseases), personal care and nutrition. Within the project a wide variety of marine microorganisms (mainly bacteria and microalgae) were analysed, including collections held by some partners and new collections of strains collected in extreme environmental conditions (deep, hot and cold).Overall the PharmaSea project cultivated more than 13,000 microbial strains, dereplicated more than 15,000 extracts, biologically screened more than 130,000 samples and identified about 700 validated bioactive extracts. Ninety structural chemical families were identified, of which 17 have new structural features and 5 compound families have novel skeletons.

PharmaSea delivered five drug lead compounds that have already entered early-stage animal studies (for central nervous system diseases), thereby achieving its objective to produce two compounds at large-scale and promote these in preclinical evaluations. Compounds identified from both *Streptomyces* [77] and *Spongionella* [78] indicate that they could be interesting lead candidates in drug development for neurodegenerative diseases, such as Parkinson's, Alzheimer's, Friedreich ataxia or amyotrophic lateral sclerosis.

PharmaSea partners have also improved sampling and culturing techniques, sequenced a large number of microorganism genomes and transcriptomes and chemically investigated and identified several interesting bioactives. Many studies focused on microorganisms isolated from deep or very cold environments (e.g. Antarctica) that are unique in terms of biodiversity and metabolite production [79–81]. Tedesco et al. [82] evaluated the antimicrobial activity of monoramnholipids produced by bacterial strains isolated from the Ross Sea (Antarctica). Bioassay-guided purification brought to the identification of three rhamnolipids, two of which are new, with unreported antimicrobial activity against Bcc strains.

Other studies evaluated the bioactivity of warm water strains. Gnavi et al. [83] tested the antimicrobial activity of nine sterile mycelia isolated from the green alga *Flabellia petiolata* and identified several sphingosine bases, including a compound previously unreported from natural sources. Lauritano et al. [84] showed that three diatom species, i.e. *Cylindrotheca closterium*, *Odontella mobiliensis* and *Pseudonitzschia pseudodelicatissima*, displayed specific anti-inflammatory activity, the diatom *Skeletonema marinoi* showed anticancer (blocking human melanoma cell proliferation) activity, while the diatoms *Leptocylindrus aporus* and *Leptocylindrus*

danicus exhibited anti-biofilm activity (against the bacteria *Staphylococcus epidermidis*). The chemical nature of these bioactive compounds is currently under investigation.

7 ERA-NET MarineBiotech

The EU FP7 ERA-NET MarineBiotech (ERA-MBT; www.marinebiotech.eu) project ended in November 2017. The project was designed to deliver better coordination of relevant national and regional Research, Technology Development and Innovation (RTDI) programmes in Europe. The aim was to reduce fragmentation and duplication and pave the way for common programmes and cooperation in the provision and use of research infrastructures. ERA-MBT is a consortium of national funding bodies seeking complementarities between national activities to pool resources to undertake joint funding of transnational projects in the area of marine biotechnology. It involves 19 partners from 14 countries which work with stakeholders from industry and organizations to identify needs and gaps in the value chain from research to development. ERA-MBT recognizes that Europe's marine ecosystems and organisms are largely unexplored, understudied and underutilized and aimed to support Europe's marine biotechnology community to participate in a lasting enterprise-driven network that adds value to marine biological resources in ways that nurture and sustain the lives of European citizens (http://www.marinebiotech.eu/marine-biotechnology-era-net). While the field of marine biotechnology represents a large potential for European added value, the current level of collaborative research is not sufficient. Interdisciplinary cooperation and networking are necessary. This includes basic research bringing together marine biology, physiology, taxonomy, microbiology, biotechnology, nanotechnology, systems biology, bioinformatics, toxicology, -omics technologies and chemistry. The result will be new applications in fields such as drug discovery, novel foods and food ingredients, bioremediation, biomaterials, aquaculture, diagnostics, production processes and bioenergy. No single country or region has the necessary capacity, knowledge or resources to fully exploit research and innovations from the marine environment. Advanced infrastructures both at sea and in the laboratories will be needed, and trans-European collaboration can be a solution. The activities proposed by the ERA-MBT project included (1) the launching of three thematic calls; (2) arranging a number of stakeholder events promoting dialogue between science, industry and policy; (3) the launching of a questionnaire asking stakeholders to identify the main challenges for successful development within the area; (4) establishing a communication forum to highlight opportunities for interlinkage and collaboration; (5) performing an outlook analysis of the future of marine biotechnology; (6) establishing a Strategic Research and Innovation Agenda (SRIA); (7) directing funds towards transnational teams to engage in the identified SRIA; (8) performing outreach activities for seeking complementarities and avoiding overlap with other activities sharing common interests with ERA-MBT; (9) developing a lasting

network of funding agencies and stakeholders; and (10) providing relevant information in the field of marine biotechnology in an online and open-access portal (http://www.marinebiotech.eu/activities). As reported in the periodic report summaries (http://cordis.europa.eu/project/rcn/111289_it.html), ten projects were funded with a budget of about 15 m€ during the first two transnational calls, while a third call has just closed and communication of the funding recommendations will be in July 2017 (http://www.marinebiotech.eu/third-transnational-call). Stakeholder meetings have been organized, such as one on 'Integrating National Efforts to Build the Future of Marine Biotechnology', held in Lisbon (Portugal) on 28–29 October 2014, and another on 'Marine Biotechnology—enabling future innovations', held in Brussels (Belgium) on 13–14 October 2016. An updated website is available at http://www.marinebiotech.eu/ where all activities are reported.

The ERA-MBT has produced a 'Marine Biotechnology Strategic Research and Innovation Roadmap', which presents and discusses five core areas needed for the successful development of marine biotechnology. The identified thematic areas are (1) exploration of the marine environment, (2) biomass production and processing, (3) product innovation and differentiation, (4) enabling technologies and infrastructure and (5) policy support and stimulation. These include the exploration of target environments and hotspots; development of new sampling and taxonomic, chemical and biochemical methods to identify new bioactive species/compounds; optimization of the culturing conditions sustaining also the health of the cultured species; creation of material depositories and related datasets; and development of new biocompatible materials and new bioremediation strategies. Overall, the project efforts should lead to greater integration of research actors and activities across Europe and towards selected areas beyond Europe (e.g. Canada). These collaborations will establish a self-sustained and long-lasting network of programme managers, scientific communities and enterprises in order to further develop marine biotechnology after the ERA-MBT project has ended.

8 Horizon 2020 Projects

More than 13,000 projects have already been funded under the EU Horizon 2020 programme (2014–2020). Of these, very few are on marine biotechnology (e.g. EMBRIC, INMARE, NoMorFilm and TASCMAR), all of which will end in 2019. Other marine projects are mainly focused on ecology, conservation, aquaculture and fisheries (e.g. CERES, Fishtimator, NEMAQUA and PrimeFish).

The EU H2020 project EMBRIC (European Marine Biological Research Infrastructure Cluster; http://www.embric.eu/) aims to form a permanent cluster of research institutes (RIs) which will foster innovation in marine biotechnology. The project focuses on (1) discovery and development of marine natural products and (2) marker-assisted selection in aquaculture. For EMBRIC details, see chapter 'EMBRC and EMBRIC: Two European Instruments to Promote the Blue Bioeconomy' by Kloareg et al. in this volume.

INMARE (Industrial Applications of Marine Enzymes, http://www.inmare-h2020.eu/) aims to shorten the pipeline for marine enzyme discovery in view of the increasing demand for new enzymes for industrial applications. The driver is to target samples from extreme geographically different sites and address the development and demonstration of innovative technologies for high-throughput enzyme screening and/or for the expression of marine enzymes and proteins through dedicated hosts. The project focuses on key research challenges such as identification of sequences encoding potential enzymes of interest and identifying critical parameters for purification and scale-up processes. Academic and industry cooperation and/or agreements on issues related to property rights and intellectual property are being considered in order to maximize exploitation potential. Until now, researchers involved in this project have published the genome of a sulphur-reducing and acetate-oxidizing haloarchaeon isolated from the deep-sea hypersaline anoxic Lake Medee, i.e. *Halanaeroarchaeum sulfurireducens* M27-SA2 [63], and two reviews on enzymes with biotechnological potential from marine organisms: one on enzymes used in fish and seafood processing [85] and another on the α-/β-hydrolase fold superfamily of esterases-lipases discovered in metagenomes [86].

The NoMorFilm project (Novel marine biomolecules against biofilm, http://www.nomorfilm.eu/) aims to search for anti-biofilm compounds isolated from microalgae (4000 microalgae species) that will be useful in the treatment of infections and that can be incorporated in the manufacturing of medical prosthetic devices. Biofilm formation by bacterial pathogens is important in infections and tissue inflammation related to implants and catheters [84, 87, 88]. The search for new antimicrobial agents that will be effective against biofilm-forming bacteria is a key priority in clinical practice. The project also addresses the biosynthesis of targeted bioactive compounds in sustainable microalgal cocultures, diminishing cultivation costs by mimicking natural aquatic ecosystems. The most industrially interesting anti-biofilm molecules will be incorporated into nanoparticles to develop manufacturing methodologies able to incorporate these compounds into real prosthetic device matrixes. Marketing of results are assured by the presence of several SMEs involved in the manufacture and distribution of prosthetic devices. Pietrocola et al. [89] is the first paper from this project which studies the molecular interactions of human plasminogen with fibronectin-binding protein B (FnBPB), a fibrinogen-/fibronectin-binding protein from the bacterium *Staphylococcus aureus*.

Finally, the EU H2020 TASCMAR (Tools And Strategies to access original bioactive compounds by Cultivating MARine invertebrates and associated symbionts; http://www.tascmar.eu/project) project aims to develop new tools and strategies to overcome existing bottlenecks in the discovery and industrial exploitation of marine-derived biomolecules (secondary metabolites and enzymes) with applications in the pharmaceutical, nutraceutical and cosmeceutical industries. Exploitation of neglected and underutilized marine invertebrates and symbionts from the mesophotic zone will be combined with innovative approaches for the cultivation and extraction of marine organisms, including the prototypes Platotex™ and Zippertex™. These unique improvements will ensure sustainable supply of biomass and promote the production of high added value bioactive marine compounds. A

focused panel of in vitro, cell-based, in ovo and in vivo bioassays will be combined with a metabolomic approach in order to discover metabolites with anti-ageing and/or angiogenesis modulating activity. The catalytic potential of mesophotic symbionts and deriving enzyme candidates will be evaluated in the fine chemicals and bioremediation industries. The project also aims to develop higher standards for bioprospecting in areas of rich marine biodiversity. Overall, a panel of libraries (marine organisms, extracts, pure metabolites and biocatalysts) will be constructed and exploited throughout the project. One publication related to this project is available until now that is a review on dysfunctions of mitochondria during ageing, cancer and neurodegeneration [90].

9 Conclusions

The position paper published by the Marine Board in 2010 on marine biotechnology [91] presented a shared vision for European marine biotechnology whereby by 2020:

> an organized, integrated and globally competitive European Marine Biotechnology sector will apply, in a sustainable and ethical manner, advanced tools to provide a significant contribution towards addressing key societal challenges in the areas of food and energy security, development of novel drugs and treatments for human and animal health, industrial materials and processes and the sustainable use and management of the seas and oceans.

Many of these objectives have been achieved also, thanks to the strong EU funding support during the FP7 programme. There have been major achievements in sampling extreme deep, cold and acidified environments (e.g. BAMMBO, MaCuMBA, PharmaSea), optimizing cultivation methods by creating ad hoc systems such as bioreactors/raceway ponds (BAMMBO, MaCuMBA), speeding up the production of a specific product by genetically modifying microorganisms (GIAVAP, LIPOYEASTS, SUNBIOPATH) and sequencing genomes/transcriptomes of marine species to identify enzymes of industrial interest or gene clusters responsible for the synthesis of bioactives (GIAVAP, BlueGenics, MAMBA, PolyModE). Several enzymes, new metabolites, new compounds and new materials have been found allowing for important discoveries and new products for development in different market sectors: health, personal care, cosmetics and nutrition.

There are now a large number of libraries: sequences, active species, extracts, active fractions and compounds. The massive quantity of data now in our hands need to be deeply studied considering all possible fields, already covered or new topics, including new and rare human pathologies.

Notwithstanding all these positive results, very few projects have been funded until now on marine biotechnology during Horizon 2020, the biggest EU research and innovation programme ever with nearly 80 billion EUR of funding available over 7 years (2014–2020). More specifically, during the 2007–2013 FP7 programme, 164 million EUR was spent on 28 projects in the blue biotechnology sector compared to 26 million EUR during Horizon. In particular there have been very few projects

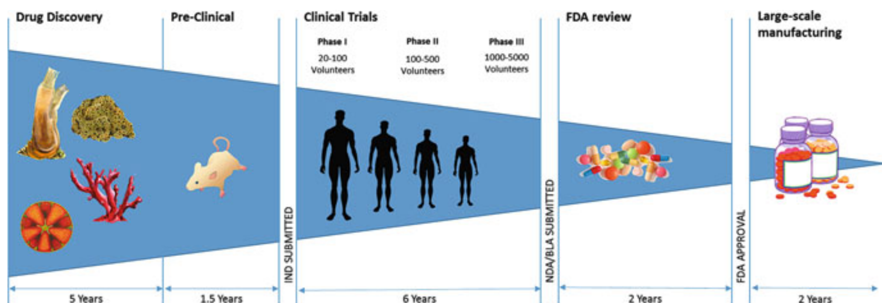

Fig. 11.2 The marine drug development pipeline. BLA, biologic license application; FDA, Food and Drug Administration; IND, investigational new drug; NDA, new drug application

funded on drug discovery and on the lead compounds identified until now to cure important human pathologies such as cancer and neurodegenerative diseases. These candidate molecules now need to be subjected to preclinical testing (in the laboratory and/or in animals) to assess chemical, biological and toxicological properties (Fig. 11.2).

But for now there are no projects in the final programming period of H2020 that foresee preclinical testing of lead molecules that are critical in order to advance successful compounds into a clinical trial programme which involves evaluation of the drug in people with the disease. To strengthen Europe's competitive position in the emerging bio-economy, the European Maritime and Fisheries Fund has decided to support the set-up of a marine bio-economy forum in 2017. The forum will bring together industry, public authorities, academia and finance to propose concrete solutions for bringing marine biotech products sooner to the market (https://ec.europa.eu/maritimeaffairs/sites/maritimeaffairs/files/swd-2017-128_en.pdf).

In order to implement new strategies for future marine biotechnology research and innovation, the ERA-MBT recently prepared a roadmap that identified an optimistic future for marine biotechnology with the sustainable utilization of marine bioresources in ways that establish new markets, generating revenue and increased employment [5]. This is also foreseen in a recent report from Smithers Rapra, The Future of Marine Biotechnology to 2025 (http://www.efibforum.com/news/2015/global-market-for-marine-biotechnology), which estimates that the global market of marine biotechnology at $4.1 billion in 2015 has the potential to reach $4.8 billion by 2020 and $6.4 billion by 2025. There is now a strong momentum to drive progress in European marine biotechnology, but if Europe does not act now through increasing its support with targeted funding and coordinated research, also regarding preclinical and clinical development of new compound leads, it will begin to lose ground on other global leaders in this field such as the United States, Japan and China. This would allow marine biotechnology to reach its full potential and thereby support the further development of the Blue Biotechnology economy in Europe allowing it to grow and become more competitive worldwide.

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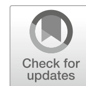
Chapter 12
SeaBioTech: From Seabed to Test-Bed: Harvesting the Potential of Marine Biodiversity for Industrial Biotechnology



RuAngelie Edrada-Ebel, Arnthor Ævarsson, Paraskevi Polymenakou, Ute Hentschel, Daniele Carettoni, John Day, David Green, Guðmundur Óli Hreggviðsson, Linda Harvey, and Brian McNeil

1 Introduction

The 48-month SeaBioTech project was designed and driven by SMEs to convert the huge potential from as yet underdeveloped marine biotechnology into novel bioactive pharmaceuticals (anticancer, antiparasitic, antibiotic and against metabolic diseases), cosmetic and food (antioxidant) as well as industrial chemistry (biocatalysts, reagents) sectors. The project made use of the biodiversity from marine extreme environments. Such environments are characterized by geochemical and physical conditions at the edges of the compatibility with life, and they are colonized by highly adapted organisms called extremophiles. These can provide unique chemicals and

R. Edrada-Ebel (✉) · L. Harvey · B. McNeil
Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland, UK
e-mail: ruangelie.edrada-ebel@strath.ac.uk

A. Ævarsson
PROKAZYME, Reykjavik, Iceland

P. Polymenakou
Hellenic Centre of Marine Research, Heraklion, Crete, Greece

U. Hentschel
Marine Microbiology Research Unit, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

D. Carettoni
AXXAM, San Raffaele Biomedical Science Park, Milan, Italy

J. Day · D. Green
Scottish Association for Marine Science, Scottish Marine Institute, Dunbeg, Argyll, UK

G. Ó. Hreggviðsson
MATIS, Reykjavik, Iceland

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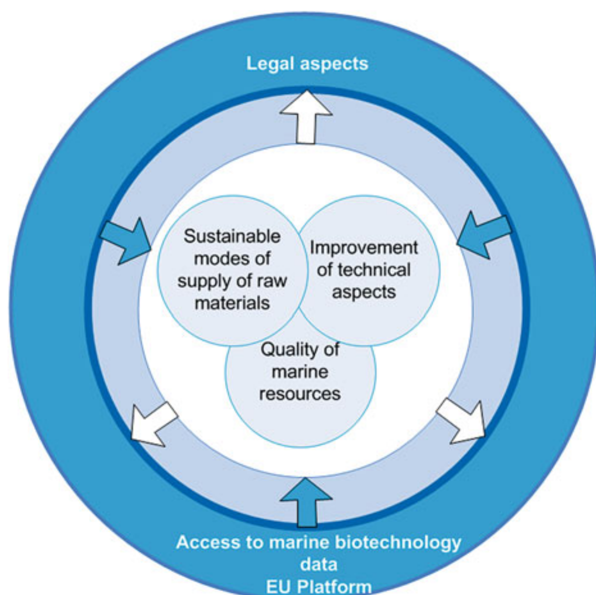

Fig. 12.1 Concept of SeaBioTech, showing the interactions between the five key challenges to be faced in order to improve marine biodiscovery pipelines. The first three challenges in the inner most circle concerns the primary goals of the project that includes: (1) the quality of marine resources, (2) the improvement in technical aspects, and (3) a sustainable mode of supply of raw materials for the industries. The transversal activities involving challenges on (4) the legal aspects and (5) the access to marine biotechnology data are the second level represented on the outer circle

novel enzymes that have enormous potential because they maintain their performance even in harsh industrial process conditions. However, there are significant bottlenecks that presently restrict the marine biodiscovery pipelines relating to:

- Limited availability of collections of marine extremophiles and little knowledge of their potential use in biotechnology (lack of qualitative and quantitative data with respect to the application performance)
- Limited transfer of knowledge from fundamental research into technically realizable and cost-effective products and technologies
- Technical hurdles with methods and processes, including in the cultivation and storage of organisms and in extraction, isolation and characterization of bioactive components
- Lack of industrial-scale production techniques for marine substances, based on the limited understanding of the process physiology of the native producer microorganism

To develop efficiently marine biodiscovery pipelines and provide access to sustainable and economical production methods, SeaBioTech has tackled five key challenges (Fig. 12.1) with an integrated approach combining access to unique marine biodiversity, innovative culturing approaches, genomic and metagenomics analyses coupled with metabolomics, natural product chemistry, bioactivity evaluation and industrial bioprocessing along with legal aspects, market analysis and transfer of knowledge. SeaBioTech has not only increased the number of marine-based products but also their success rate for future commercialization. SeaBioTech's research and technological progress was completely within the framework provided

by the participating SMEs relating to their definition of product opportunities and proof-of-concept demonstration activities.

1.1 SeaBioTech Has Put Together a Marine Biodiscovery Pipeline Using an Integrated Approach

The project's innovation plan corresponded to the following scientific, technical and technological challenges as shown in Fig. 12.1.

Challenge 1 The quality of marine resources—the approach to resource quality begun by standardizing the sampling process from unique and previously untapped habitats, which included geothermal intertidal biotopes in Iceland, hydrothermal vent fields and deep-sea oligotrophic basins of the Eastern Mediterranean Sea, and unsampled areas of Scottish coasts that are likely to be highly productive sources of new bioactive compounds. The marine resources also included the partners' existing biobanks (UK's Culture Collection of Algae and Protozoa, MATIS's Icelandic collection, Eastern Mediterranean Sea collections) as well as new in situ sampling. The SeaBioTech sampling process guaranteed the quality of marine resources for further industrial development, including identification of marine microorganisms and their variability based on genomics and metagenomics. This project also integrated the critical aspect of the maintenance of the sampled species with their intrinsic quality and their secondary metabolites, by developing special cultivation media and storage conditions.

Challenge 2 The improvement in technical aspects—to improve marine biodiscovery and reassure industries about its feasibility, SeaBioTech perfectly combined metabolomics assisted by systems biology and functional bioassays to increase the ability to disclose positive hits with an economical and faster approach; it is an affordable, innovative and efficient method to separate, elucidate the structure and identify the bioactive metabolites.

Challenge 3 Sustainable modes of supply of raw materials for the industries—the last technical brick for industries is the sustainability of these newly discovered raw materials not only at lab scale but also at industrial scale. Thus, SeaBioTech benefited from the power of well-controlled metabolic engineering of interesting organisms (bacteria, microalgae, cyanobacteria) increasing the yield of bioactive metabolites at lab scale and multiply this yield through fermentation technology at industrial scale to deliver promising enzymes, polymers and small molecules as industries need.

The second level embraces the last two challenges as transversal activities: challenge 4 refers to the legal framework necessary to secure the access to marine resources, their sustainable use and their exploitation process; and challenge 5 refers to the access to a marine biotechnology database and biobank.

Challenge 4 The whole biodiscovery process was completed by the clarification of all legal aspects to gain visibility and efficiency for industry. SeaBioTech coordinated the legal procedures with national, European and international authorities/ stakeholders to propose harmonization of the legal process related to marine bioprospecting, biodiscovery and marine biotechnology for commercial purposes.

Challenge 5 To crystalize this innovative approach, SeaBioTech created a centralized tool to describe the whole marine biodiscovery pipeline including available biobanks, the identified marine organisms, compounds and extracts, the cutting-edge methods in identification and elucidation and metabolic engineering to be further used for industrial purposes with all related procedures on legal process for companies, academia and legal authorities.

1.2 SeaBioTech Is an Industry-Driven Project

Contrary to previous approaches, SeaBioTech commenced by defining industry needs—more specifically SMEs'—across marine biodiscovery pipelines. To achieve the overall goal of making sustainable marine-based compounds more attractive for industries along with shortened time to market, the specific objectives of SeaBioTech are to:

- Provide a pipeline of commercially viable products based on relevant bioactivity screening of samples of marine origin
- Develop efficient standardized processes and methods across the biodiscovery pipeline
- Introduce industrial bioprocessing methods suitable for commercial production of marine-sourced materials
- Clarify, harmonize and potentially simplify the legal aspects related to marine biodiscovery processes
- Create a central EU platform and biobank based on an integrated approach to biodiscovery pipelines for future use by other consortia, academia and companies

1.3 Identification of Industry Needs: Providing an Industry-Driven Project

It is easier to put the key challenges for marine biotechnology into an addressable context by defining what the concrete output from marine biodiscovery pipelines might be. Therefore, SeaBioTech started with a thorough market analysis of the various industrial sectors that are relevant to the partners, particularly the SMEs. This clearly indicated where there is a need for products that go beyond the current state of the art. This in turn provided the perspective for the technical challenges and

highlighted the needs for improvements. All SMEs within SeaBioTech were committed to the concept of marine bioprospecting as a strategy to provide them with key future products that are beyond the state of the art and enhanced their business competitiveness. The SeaBioTech project was specifically designed to deliver to the SMEs' progress, eliminating or dramatically reducing bottlenecks to allow the SMEs to develop innovative products for the world market. It is important to note that the success of the project must show other SMEs and industries what is technically feasible and economically attractive from marine biotechnology—SeaBioTech will represent a sustainable and reproducible model for the European biotech industry. The companies involved in SeaBioTech are focused on the biodiscovery pipelines of three compound categories: (1) polymers having bioactivities such as wound healing, optic turgor in lenses and pharmaceutical additives driven by marine biopolymers (MBL) and MATIS; (2) enzymes having bioactivities such as transaminases, reductases, etc. driven by Ingenza, Prokazyme, Lund and MATIS; and (3) small molecules having bioactivities as therapeutics (antibiotic, anticancer, etc.) driven by AXXAM, HDL, PHARMAQ, SIPBS and UWUERZ. Each step of the biodiscovery pipeline related to these three types of compounds and the related target application explored to provide innovative ingredients for novel industrial products. The whole SeaBioTech biodiscovery process was informed by Rothwell's coupling model of innovation [1] so that there is a regular interplay between understandings of market needs and technical "pull".

2 Methodology and Overall Strategy

Work packages (Fig. 12.2) were set up to organize a schematic flow of materials and data between partners. In order to achieve the desired outcomes of a greatly improved pipeline of products from marine biotechnology, the first step is to identify clearly the market opportunities for the companies involved in SeaBioTech and the precise bottlenecks they have to solve to target their respective markets. The target applications of the consortium involved pharmaceuticals, fish health, food, cosmetic, chemical and industrial in *WP1 (Definition of product opportunities*, *dissemination*, *exploitation plan and IPR*). WP1 formed the basis of the subsequent research activities in WP3–WP7. Through an understanding of market and technical requirements, several partners each contributed to the definition of demand statements for their own industrial sectors. In addition, WP1 integrated the IPR management. In parallel, *WP2* (*sampling process*) created a huge collection of novel microbes and microbial consortia for genome and metagenomic analyses and to facilitate their biotechnological exploitation. WP2 led by HCMR collected information from all culture collections available from partners, isolated novel microbes from several diverse environments and organisms and facilitated their exploitation at WP3/WP4 for bioactivity screening and genomic analyses. WP2 also prepared samples that can be screened in *WP3* (*Detection and characterisation of commercially relevant bioactivities*) led by AXXAM. WP3 was responsible for detecting bioactivities that

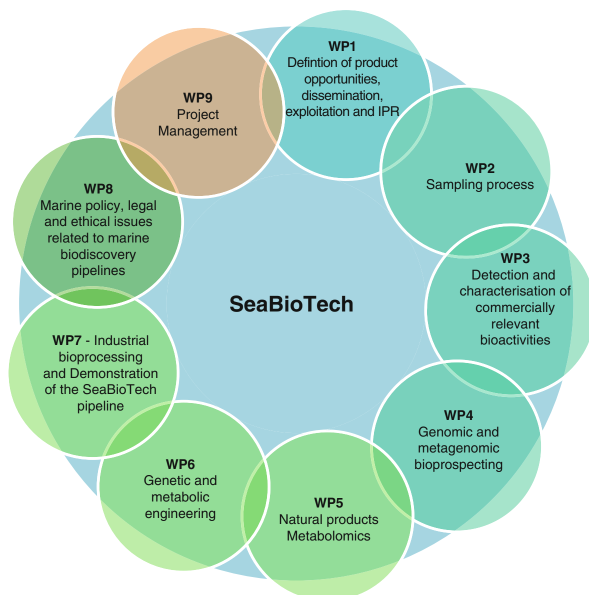

Fig. 12.2 The SeaBioTech work packages

were selected as priority commercial targets in WP1 by testing samples provided by WP2 and WP4. WP3 provided the detailed bioactivity assessments to guide isolation of substances with commercial potential. *WP4 (Genomic and metagenomic bioprospecting)* led by the University of Wuerzburg used molecular techniques to pinpoint novel enzymes of commercial interest and isolated the genes for synthetic pathways for novel small molecules for testing in WP3. This also allowed structural variations to be prepared as a mean to improve bioactivities. A number of high-throughput solid-phase screening in vitro and direct selection methods in vivo were applied in this work package to identify novel enzymatic activities of interest from metagenomic libraries constructed from genomic DNA derived from marine micro-organisms. *WP5 (Natural products metabolomics)* led by SIPBS was the analytical arm of the consortium, undertaking dereplication studies on microbial extracts of interesting isolates from WP2 and those screened for the presence of biosynthetic clusters from WP4. WP5 isolated and structure elucidated the bioactive natural products determined in WP3. Along with WP6, their sustainable production by the microbial cultures was optimized through metabolomics tools. When interesting metabolites were confirmed in WP5 in collaboration with WP3, organisms could be engineered to guarantee sustainability of the interesting metabolites in *WP6 (Genetic and metabolic engineering)* also led by SIPBS. WP6 undertook the research that will allow organisms producing targeted substances to be maintained at the laboratory scale. It also performed genetic manipulation to produce structural variants of the target substances as a means to improving their commercial properties. When lab scale is validated, it is essential to integrate at industrial scale. *WP7 (Industrial bioprocessing)* led by MATIS focused upon developing rapid and robust

methods for the industrial exploitation of microbial- and enzyme-based marine products. In order to achieve this, WP7 tasks will link very closely to those in WP5 (*Metabolomics*) and WP6. A subtask in WP7 consisted of a series of projects conducted to test the viability of the outputs from earlier WPs as the basis for new commercial products relevant to various partners, including pharmaceutical, functional foods, novel enzymes and research tools. *WP8 (Marine policy, legal and ethical issues)* ensured that the SeaBioTech project develops in accordance with all relevant national and international legislation governing bioprospecting and the marine environment. The WP's main goal is to contribute to the ongoing development of the legal framework for marine bioprospecting and ensure dissemination of the project results to the scientific community and to public and political stakeholders. The key task was the creation of an EU platform allowing access to data from SeaBioTech and to physical samples in SeaBioTech's biobank. *WP9 (Project management)* deployed and implemented management best practices through a clear focus on both strategic and operational administration.

3 The SME Partners and Their Activities

In this section, we present the roles and contributions of SeaBioTech's partners from the industry as well as the SME's mutual gain from the consortium. With the analysis of market opportunities and the generation of an initial exploitation plan, the respective SMEs defined specific commercial goals and strategies to reach these goals within SeaBioTech project. The exploitation plan also further underlined the importance of collaboration between the company and RTD partners as a key to the successful exploitation of the opportunities and potential of the SeaBioTech project. Through this EU-funded partnership, the SMEs made agreements with academic and research institutions in the consortium for the licensing of products that will be offered by the respective companies with shared revenues according to specific agreements. The strategy was to exploit potential collaborations with academic groups in SeaBioTech as a new business scheme for increased portfolio of products for the research laboratory market in Europe and elsewhere. SeaBioTech brought together significant members of the fields of marine biotechnology and biocatalysis experts for the first time and delivered industrially useful novel biocatalysts by developing highly innovative and powerful screening and selection technologies and novel, high yielding, scalable and economic enzyme production systems. Some of the SMEs had taken steps to further develop the successful strategy of alliance with its partners in the SeaBioTech project with continued collaboration that will extend well beyond the lifetime of the SeaBioTech project. Very good collaboration with both academia and SMEs that will continue after the end of the project is one of the main and high-impact results for the SMEs. SME partners have communicated with various potential end users and current market producers to develop collaborations for the future development of the compounds. As a next step to support the potential commercialization of bioactive compounds, further funding will be

required to undertake the studies to better understand mechanism of action, develop a suitable patient stratification strategy and assess tractability for conventional medicinal chemistry. However, one disadvantage for the academic partners is that a SME will not release any publication on the compounds for reasons of commercial sensitivity especially if patents are to be filed in the coming years.

3.1 Prokazyme (PKZ)

PKZ has been engaged in commercializing enzymes that have advantageous biochemical properties over competing products. In this project, their work was focused on developing enzymes from extremophiles that were recognized as being potentially valuable in many applications. However, very few novel enzymes from the hundreds reported in the literature reach the market. Generally, the limitations in this area are difficulties in obtaining large enough supplies in a sustainable way and challenges in producing the enzyme to a high standard of purity in an economically attractive manner. PKZ saw opportunities in bioprospecting the unique genomic resources it had access to through the SeaBioTech gene banks and in the enormous, untapped marine biodiversity sampled through SeaBioTech. The SeaBioTech project offered significant progress above the state of the art on new marine compounds (particularly oligosaccharides), as well as enzymes, increasing PKZ existing offerings of specialized extremophilic enzymes for the R&D market. PKZ has made strategic plans for future commercial production of enzymes on an economical large scale. As part of this future strategy, it is the intention that the production of enzymes shall be transferred from PKZ to a subsidiary company. PKZ and MATIS have initiated a large research proposal with a consortium consisting of 15 partners in Europe. The research proposal, "Virus-X: Viral Metagenomes for Innovation Value", has secured a 8 million EUR funding from the European Union under the Horizon 2020 framework. PKZ will coordinate the project and within the project extend its collaboration with specific partners from the SeaBioTech project. A grant agreement was made during this period with the European Union for the funding, and the project started on 1 April 2016, and will be continued until 30 March 2020.

3.2 PHARMAQ

PHARMAQ specializes in vaccines and therapeutics for farmed fish. A key need for aquaculture is to have effective antiparasitic agents that are potent and selective against the target parasite while having no damaging effects on the environment. Most of the antiparasitic products available within aquaculture today are derived from known pesticides developed for terrestrial applications, and some of these are limited by their toxicities.

Hence, SeaBioTech offered a unique opportunity to search for bioactives from novel and unexplored sources, particularly by uncovering potential new therapeutics for aquaculture applications and defining their suitability for commercial development. The SeaBioTech project yielded good results for PHARMAQ. SeaBioTech developed an HTS (high-throughput screening) assay directed against a target special for salmon lice. The assay was very valuable in screening large libraries in the search for new actives against one of the most devastating parasites in aquaculture. Some compounds with effect against salmon lice have been identified. Although the effect has so far only been identified at relatively high concentrations, the compounds were worthy of further examination.

3.3 Marine Biopolymers Ltd (MBL)

MBL supplies chemicals derived from marine sources, including alginates and polyphenols. While the potential value of compounds such as alginate and fucoidan is well recognized, their widespread use is limited by technical problems: (1) low quality and low yields from existing extraction methods, (2) lack of higher-performance purification approaches to provide products at the "fine chemical" standard and (3) incomplete analysis and characterization of isolated components. MBL sees huge product opportunities arising out of the collaborative work in the SeaBioTech project. During the project's lifetime, MBL focused on defining the company's interests on polysaccharide compounds and their growing market demand. In addition to the polysaccharides, the sampling events of macro- and micro-epiphytes have presented interesting and new chemistry and bioactivity across a range of compounds. MBL continued sampling of key macroalgal species to develop seasonal metabolomic data. It has achieved commercially valuable improvements arising from the SeaBioTech consortium. An initial market analysis was also explored defining the potential market size/demand and market areas the compounds could feed into, whether that be as a stand-alone product or as an ingredient in a current or new formulation. Although MBL initially had a strong focus on polysaccharides, it was clearly observed that there are additional compounds that MBL now plans to commercialize over the coming years subject to the availability and success of appropriate follow-up funding mechanisms.

3.4 Ingenza (IGZ)

SeaBioTech provided opportunity to discover new biocatalysts with industrially relevant substrate specificities for integration with IGZ's current bioprocesses for the manufacture of enantiopure chiral amines, unnatural amino acids and other chiral chemical platforms. The most promising identified biocatalysts were developed using economic and scalable fermentation and bioprocess systems. Further development and

implementation of inABLE®, which is IGZ's combinatorial genetics technology for the efficient and selective assembly of DNA expression vectors, took place in the project. These technologies were key tools for improvement of strain construction and screening and have been used and developed through SeaBioTech, and the technology is of core importance to all of IGZ's commercial interests.

The screening of both alternative metagenomics libraries and those of the work package partners for new and novel enzymes of commercial interest to IGZ was carried out. This allowed expression constructs to be made and screens to be developed in WP6, which led to subsequent production processes in WP7. These generic fermentation protocols which had been developed previously were then implemented to test the growth and expression of positive hits which were highlighted in the subsequent screening of the work package partner's databases. These novel marine enzymes were cloned into an industrially relevant *Escherichia coli* strain using inABLE® compatible parts. Further optimization of the expression of these strains has been carried out in shake flasks followed by activity assays of the successfully expressed enzymes. Based on these results, fermentation development has been implemented, linking into the deliverables required for WP7. A production process of the most successful enzymes was implemented and scaled up during the course of SeaBioTech.

3.5 Horizon Discovery Ltd (HDL)

HDL has been developing new drug discovery opportunities in the cancer field through its creation of unique cell lines that are engineered to represent particular forms of cancers. HDL saw great application in screening marine-derived natural products from the project to therapeutically "deorphan" the cancer genome. HDL's expertise on cell-based screens using genetically defined human disease models represented the ideal approach to directly find unexpected uses for naturally bioactive molecules from the project to such "orphan targets", where the full complexity of cell biology was screened in a rational manner to find novel cancer-selective agents. Ultimately, HDL was able to show that several fractions containing single compounds had a marked ability for specifically killing cancer cells via inducing apoptosis. This is proof of principle that bioactive compounds isolated from these particular classes of marine organisms may have at least some of the required characteristics for exploitation in the oncology arena.

3.6 AXXAM

AXXAM is a lead compound discovery company that services the pharmaceutical, agrochemical and life science sectors. AXXAM was SeaBioTech's link to the mainstream of pharmaceutical development and marketing companies. AXXAM

provided a panel of functional assays that detect activities relevant to key diseases (infections, inflammatory diseases, chronic pain, etc.). AXXAM supported the hit discovery programmes of SeaBioTech by performing in total 11 screening campaigns on a comprehensive number of 927 crude samples of marine origin on an array of cell-based and enzymatic assays, which was refined based on the obtained results to 7 assays (TRPA1, TRPM8, TRPV1, PPARα, EL, HDAC6, HDAC2) suitable for high-throughput screening of complex extracts. These functional assays were developed to measure the activity of validated targets in three main disease indications: cancer (HDAC6 and HDAC2), metabolic syndrome (EL, PPARα) and pain (TRPA1, TRPM8, TRPV1). At the end of the primary screening activity, 287 crude extracts were confirmed as primary hits, distributed as follows: TRPA1 (12), TRPM8 (37), PPARα (36), HDAC6 (81), HDAC2 (3) and EL (118). In collaboration with WP2–WP5, 31 crude extracts derived from 17 marine microorganisms were prioritized and included in the SeaBioTech pipeline. A subset of 15 crude extracts was fractionated by WP5, and 629 fractions were subjected to screening against the primary assays TRPM8, TRPA1, PPARα, HDAC6 and EL, respectively. The support to dereplication activities led to the identification of 148 fractions containing the sought bioactivity against the following primary targets: TRPA1 (9), TRPM8 (5), PPARα (5), HDAC6 (76) and EL (53). Remarkably, one series of 27 fractions derived from the crude extract SBT0541 (*Algoriphagus marincola*) was confirmed to contain negative modulators of the catalytic activity of endothelial lipase (EL). Among them, 8 fractions contained pure compounds which were identified by WP5, which allowed the definition of a preliminary structure-activity relationship. This finding appeared consistent with the targeted enzyme endothelial lipase (EL), which physiologically releases fatty acids from phospholipids in HDL particles. The compounds displayed a dose-dependent inhibition on EL, with partial inhibition at the highest compound concentrations tested. The negative modulation of the EL activity identified by AXXAM has never been reported in literature.

In addition, the collaboration between SIPBS, AXXAM and PHARMAQ has been reinforced throughout the SeaBioTech project to promote an integrated hit discovery programme for the identification of marine compounds with antiparasitic activity directed against *Lepeophtheirus salmonis*, a major threat for aquaculture. Three high-throughput assays made available by AXXAM (TRPA1, TRPV1 and voltage-gated Na channel) were applied as preselection tools for the prioritization of crude extracts and fractions to be tested by PHARMAQ with the low-throughput phenotypic assay on living parasites. In total, AXXAM screened over 750 crude extracts for this purpose, which generated a list of 135 hits prioritized for testing at PHARMAQ. A number of these hits were confirmed for their parasiticidal activity on *L. salmonis*, and further characterization is ongoing at PHARMAQ on a subset of fractions to identify the pure compounds responsible for the sought bioactivity.

Newly discovered and underexplored species of marine microorganisms were demonstrated to be effective sources of novel therapeutics to be progressed to address unmet medical needs and threatening parasitic infections for aquaculture. Thus, the availability of novel therapeutics for human health and aquaculture will

directly contribute towards improving quality of life, health, employment and economic strength. In addition, the knowledge gained through SeaBioTech concerning the assay development and screening of complex marine extracts may directly or indirectly translate into new opportunities for the CROs to expand their potential market and for pharmaceutical and life science companies to undertake novel R&D projects.

4 Addressing the Challenges Through Scientific Breakthroughs

4.1 Challenge 1: Access, Sampling, Storage and Quality Maintenance of Marine Resources Present in Extreme Environments and Sponge Symbionts

The characterization of natural microbial communities in extreme environments has been a major challenge for microbial ecology. Considering that 71% of the earth's surface has an average depth of 3800 m, deep-sea environments have attracted much interest as niches of microbial life with considerable exploitation potential. Extreme environments are characterized by geochemical and physical extremes, at the edges of the compatibility with life. Many diverse extreme environments have been described, and they are colonized by highly adapted organisms called *extremophiles* [2]. These organisms fall into a number of different classes that include thermophiles, acidophiles, alkalophiles, psychrophiles, barophiles (piezophiles), etc., depending on their ecological niche [3]. Because of their unique metabolic adaptations to their environment, the extremophiles are considered to have an enormous potential for unique biotechnological applications because they allow the performance of industrial processes even in harsh conditions, under which conventional proteins are denatured or inefficient [2, 4]. Consequently, these unique properties have resulted in several novel applications of enzymes in industrial processes. Similarly, the novel biochemistry of extremophiles is predicted to generate novel chemicals that are distinct in structure from those from more conventional organisms. Hence, such compounds are likely to be useful in drug discovery applications. However, only a minor fraction of extremophile organisms has been exploited. Very few sources have been explored to date so that there was a rich potential for SeaBioTech to go beyond the state of the art so long as it is possible to obtain samples of a suitably high quality.

The first aspect of the "quality of marine resources" challenge was simply to obtain access to extremophile samples from the marine environment. Companies seeking a wide range of biodiversity from extreme marine environments would struggle because such sources are not commercially available currently. The Australian Institute of Marine Sciences is no longer supporting access to its collections; MarBank in the University of Tromsø in Norway has a limited collection, which is not openly available; the National Cancer Institute in the USA provides

access to a small number of marine-derived samples. There appears to be a single commercial source, Magellan Bioscience in the USA, which works collaboratively with other companies through offering access to its collection of marine microbes. However, few of these are extremophiles. Beyond that are the scattered "ad hoc" collections found in some university departments and research institutes. The SeaBioTech project had access to several extreme environments that have not yet been explored for commercially relevant bioactivities and had capitalized on untapped resources associated with some of the participants, notably microbial symbionts from sponges and the UK's Culture Collection of Algae and Protozoa (CCAP). The benefits of these sources are explained below.

4.1.1 Geothermal Intertidal Biotopes in Iceland

Intertidal biotopes harbour a large diversity of ecologically and biotechnologically interesting organisms. This is a highly dynamic environment subject to constant periodic disturbances with steep gradients of temperature, mineral composition and salinity. The organisms need to tolerate periods of dryness and even exposure to harsh UV radiation during low tide. Temperature gradients are manifested most clearly in the contrast between the hot fluid in geothermal coastal hot areas and the cold seawater, and the hot spring water may have high levels of sulphur compounds and toxic metals. These habitats have rich invertebrate fauna and often covered by a profusion of algal vegetation containing various complex recalcitrant polysaccharides that may be utilized by a variety of microbes, factors influencing the microbial diversity. Photosynthetic microbial mats are abundant in these areas, and many hot springs may have both chemolithoautotrophic and photosynthetic organisms as primary producers, adapted microbes to unique conditions. Rare species in these areas include various obligate heterotrophs, but their presence may be masked by the dominant primary producers, and therefore they are not easily studied or accessible for biotechnological exploitation. The unique geothermal environments on the coasts of Iceland sustained a relatively high diversity of microorganisms and unique organisms not previously exploited as a resource for bioactive microbial metabolites or enzymes of industrial interest. Past studies revealed a great number of novel organisms indicating that geothermal habitats harbour an enormous diversity still to be isolated, characterized and exploited [5–8]. Within the SeaBioTech project, a total of 49 samples were collected from coastal geothermal sites in Iceland, primarily from photosynthetic microbial mats and also from polysaccharide enrichments in situ, and a total of 194 strains were isolated: 122 from Laugarvík, 47 from Yngingarlindir and 25 from Reykhólar. Numerous strains representing novel species and genera were isolated, especially from Yngingarlindir. Alginate-degrading anaerobic isolates from Reykhólar were close to the genus *Clostridium*, and five of them were selected for whole genome sequencing and genome annotation analyses in WP4. A preliminary study of the species composition of cyanobacteria from the clone sequences from the YL samples was performed, and the largest taxon contained several species representing distant (88–95% 16S rDNA similarity) relatives of *Geitlerinema*

sp. within the *Oscillatoriales*. A similar study on the composition of cyanobacteria in four of the Laugarvík biomat samples revealed the majority of sequences belonged to a filamentous *Leptolyngbya* sp. highly related to a *Leptolyngbya* sp. found in arctic hot springs in Greenland. Results from culture-independent biodiversity studies in Yngingarlindir and Laugarvík indicated novel species of cyanobacteria. Seven cyanobacteria strains were (M24–M36) isolated from mat samples and identified. Strains of interest (32) were selected for extractions in WP3. The extracts (62) and relevant control samples (6) were labelled and sent to the relevant partners for bioactivity screening. Based on novelty, 39 strains were selected for whole genome sequencing and annotations in WP4 and WP6. From the total of 39 strains, 38 strains were sequenced and their genomes annotated.

4.1.2 Deep-Sea Oligotrophic Basins and Hydrothermal Vent Fields in the Eastern Mediterranean Sea

The Eastern Mediterranean Sea is a dynamic region with unique hydrographic and geomorphologic features (e.g. the Mediterranean Ridge, Hellenic Volcanic Arc, deep abyssal plains, seamounts, deep anoxic hypersaline basins, hydrothermal vent areas, submarine volcanoes and mud volcanoes, methane and hydrogen sulphide cold seep sites, etc.). The subduction of the African plate below Europe has resulted in the formation of the Mediterranean Ridge and deep basins as well as volcanism in the Hellenic Volcanic Arc. Major hydrothermal systems are found along the Hellenic Volcanic Arc at Methana, Milos, Santorini and Nisiros islands [9]. Venting gases in these areas contain substantial amounts of CO_2, H_2 and H_2S, thus providing the chemical environment for chemolithoautotrophic primary production [10]. Steep chemical and temperature gradients [11] create diverse niches for numerous microbial populations. Initial screening studies of microbial diversity indicated a high spatiotemporal variation in microbial community structure [12] combined with highly diverse bacterial communities, with less than 33% of 16S rDNA sequences being related at a 90%, or higher, level to cultivated organisms [13].

The deep eastern basin of the Mediterranean Sea is one of the world's most oligotrophic areas and is characterized by an overall nutrient deficit [14]. As a result, only small amounts of organic matter reach the seafloor through the water column, resulting in low bacterial community growth and abundance [15]. Previous studies on the composition of microbial communities in these environments have shown that they are highly diverse, and the estimated total sequence richness has been found to be comparable to estimates for microorganisms inhabiting terrestrial ecosystems [16, 17]. Thus, these highly oligotrophic environments harbour a unique prokaryotic diversity, different from that described among other oxic and pristine marine sediments, and thus they can be considered as "bacterial hotspots" that deserve further investigation to assess their biotechnological potential.

For the first time, SeaBioTech was able to launch bioprospecting activities on organisms from these Mediterranean sources which were led by the Hellenic Centre for Marine Research (HCMR). Samples were collected in Santorini volcanic

complex (Santorini caldera including the newly discovered Kallisti lakes, Kolumbo volcano, Aegean Sea, Greece) and in the deep-sea oxic Ierapetra basin, South Crete. Santorini volcanic complex is a part of the Hellenic Volcanic Arc characterized by a unique convergent setting and by a unique enrichment of polymetallic spires in As, Sb, Zn, etc. Two major sampling events were organized by HCMR in September 2013 and in May 2014 in this volcanic complex with the Research Vessel Aegaeo and the remote operated vehicle of HCMR from which a large number of water samples (>100), polymetalic active and inactive gas chimneys (>30 samples and subsamples) from the submarine Kolumbo volcano and microbial mat samples from Santorini caldera and Kolumbo volcano (>30) were collected and used for microbial strain isolation, community characterization and metagenomic libraries construction. In total, 280 microbial strains were finally isolated from the Kolumbo/Santorini samples for the other tasks and WPs, belonging to different species mainly within the *Bacillales* of *Firmicutes* phylum and within the *Pseudomonadales* of *Gammaproteobacteria*. Several novel species were also identified, whereas additional strains isolated from the Milos sampling event of May 2013 are available also in MATIS strain collection. In addition a series of physicochemical parameters (e.g. gas analysis of the active vents, nutrients, organic carbon, metals, chloropigments, etc.) were also estimated in order to explain microbiological results and further evaluate the potential risks of the active submarine volcanoes of the Hellenic Volcanic Arc [18]. HCMR has created a collection of 280 strains from the extreme environments of the Hellenic Volcanic Arc.

4.1.3 Coastal Sites in Scotland with Extreme Conditions

The west coast of Scotland and its outer islands provide a wide variety of extreme ecological niches including rock pools, which undergo major shifts in osmotic potential and temperature; unusual niches such as the stratified, anoxic microzone at the head of Loch Etive; and highly polluted sites on the River Clyde estuary. These sources have not yet been explored for bioprospecting, but within SeaBioTech examination of the microbial diversity in these sites was undertaken. SAMS (Scottish Association for Marine Science) has created a unique collection of strains encompassing of a wide range of taxa including: a range of heterotrophic eubacteria, cyanobacteria and eukaryotic microalgae. In total 480 biological isolates have been identified in the project and processed down the biodiscovery pipeline by SAMS, with 116 of these being identified by 18S rRNA gene sequence NCBI blast results in Period 3. Of these 310 biological isolates were processed down the biodiscovery pipeline. Of the 310 samples processed, 246 were of bacteria identified in this project by molecular barcoding (16S rRNA gene), and 64 were algal, with identity confirmed by 18S rRNA gene sequence NCBI blast results. All the live microorganisms identified are held in the bacterial and protistan collections at SAMS. All bacterial isolates are held as frozen/cryopreserved master stock cultures at −80 °C, with glycerol (5% in medium) as cryoprotectant. The algal isolates are maintained by serial transfer, and where practicable they are also held as cryopreserved master cultures and stored at −196 °C in the CCAP Cryostore.

In addition, MBL has created a collection of 165 strains over 4 sampling sessions from Culzean Bay and Oban. Of those strains which were isolated, the dominant members were affiliated within the class of *Gammaproteobacteria* and the phylum of *Firmicutes*.

4.1.4 Microbial Symbionts from Sponges

Marine sponges often harbour dense and diverse microbial communities, with many of the microorganisms being specific to sponge hosts. These microbes, which can include bacteria, archaea and single cell eukaryotes, comprise up to 40% of sponge volume and may have a profound impact on host biology. For example, photosynthetically fixed carbon from cyanobacterial symbionts provides >50% of the energy requirements of certain tropical sponges, while other microorganisms may contribute to host defence via the production of biologically active metabolites. The latter also hints at the pharmacological potential of sponge-associated microorganisms. The group of Professor Ute Hentschel at the University of Wuezburg (UWUERZ) has a long experience in marine sponge microbiology, many of which have been collected from the Mediterranean Sea [19]. Samples and background knowledge were made available to SeaBioTech from two collection efforts to the Greek islands yielded the following biomaterial: 64 unique actinomycetes were isolated from 12 different marine sponge species, which were affiliated to 23 genera representing 8 different suborders based on nearly full-length 16S rRNA gene sequencing; 4 putatively novel species belonging to the genera *Geodermatophilus*, *Microlunatus*, *Rhodococcus* and *Actinomycetospora* were identified based on a sequence similarity <98.5% to validly described 16S rRNA gene sequences; and 13 isolates showed antioxidant, antimicrobial and antitrypanosomal activities.

4.1.5 Existing Collections

The marine resources exploited under SeaBioTech also included all culture collections available from partners. MATIS had amassed large strain collections of extreme organisms and also recently set up facilities and pipeline for eukaryotic microalgae collection and analysis. The Culture Collection of Algae and Protozoa (CCAP), located at SAMS, holds a uniquely diverse range of marine, freshwater and terrestrial protists (algal and protozoan) as well as prokaryotic cyanobacteria. Additionally, SAMS has collections of marine bacteria that are not replicated in any accessible Biological Resource Centre. HCMR has an existing microbial collection from deep-sea sediments and from submarine volcanic sites in the Eastern Mediterranean. The Natural Products Metabolomics group at the Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS) also has a collection of marine microbes from the Northern Scottish coastlines of Orkney and Shetland. These collections have not been previously investigated for their potential to produce bioactive secondary metabolites and provided biotechnologically exploitable metabolites within SeaBioTech.

4.1.6 Advances in the Sampling and Collection of Extremophiles

From existing collections from different partner institutions, isolates have also been additionally generated from the following sources: Scottish sponge isolates (~150), Scottish and Antarctic sediment cores (~100 of which 54 have been processed) and polar Antarctic and Arctic sediment cores (~150). SeaBioTech partners shared their expertise in the successful sampling of extremophiles and developed a common and efficient strategy to optimize the useful access to marine biodiversity. Targeted scientific and technological tools (ROV-based technology) for deep-sea sites and scientific diving for shallow sites for observing and sampling submarine ecosystems and collecting sponge samples were deployed to explore the series of diverse habitats described above. MATIS focused on geothermal coastal areas around Iceland and developed various methods isolating psychrophilic and thermophilic microbes relevant to the project. Specialized techniques were developed in the project for accessing rare species in order to increase the overall "phylogenetic depth" of the obtained strain collection. In addition to direct production of samples through cultivation methods (described in the next section), SeaBioTech also employed molecular genetics, particularly a metagenomic sampling approach vastly increasing its access to relevant DNA from marine samples. The advent of molecular genetics in the 1970s prompted a major revelation in microbiology [20]. A huge pool of microbiota was discovered that had been previously missed because of their lacks of growth on laboratory media [21]. Several dozens of phyla have been discovered since then, encoding many novel metabolic functions and pathways [22]. Because of the sheer numbers of microorganisms in environmental samples, the limits of discovery have clearly not yet been reached: in addition to the 10^5–10^6 bacteria per ml seawater, an unimaginable number of microorganisms are associated with algal and animal surfaces, residing as commensals in the intestines of animals, or as symbionts in highly specialized organs, such as the cellulose-degrading symbionts of wood-boring bivalves or the symbiotic microbial consortia of marine sponges. In order to access this largely untapped resource of marine microorganisms, metagenomic strategies were employed in the project. Metagenomics (or "environmental genomics") involves the direct extraction of community high-molecular-weight DNA from an environmental sample and the cloning of the resultant DNA pool (called the "metagenome") into suitable vectors [23–25]. The cloning vectors have been designed to hold small, medium or large insert sizes. These vectors (fosmid, cosmid) are then propagated in surrogate host strains, such as *E. coli*, or specialized overexpression strains, such as *Streptomyces albus*, and others. With the generation of large libraries consisting of tens to hundreds of thousands of clones, the genomic complexity of the original microbial community can be maintained. These libraries were then screened, in what has been termed a "functional metagenomics" approach, for phenotypic activities, and the responsible operon structures are sequenced. In doing so, a number of enzymes (including esterases, lipases, cellulases, amidases, amylases), ribosomal operons, antibiotics and pigments have been recovered from environmental microbial communities whose large uncultured fraction would

otherwise have been inaccessible [26–28]. Owing to the environment from which the enzymes had been isolated, they may have novel properties, such as increased stabilities under alkaline, acidic or low- or high-temperature conditions. Functional metagenomics is thus a highly promising strategy for the recovery of biotechnologically relevant enzymes from the marine environment.

Another strategy used by SeaBioTech to tap into the environmental DNA pool is by "sequence-driven metagenomics". This approach has been undertaken by Venter and colleagues to yield a global genomic inventory of the oceans [29]. Other studies have employed sequence-driven metagenomics, for example, to characterize the genomic repertoire of the microbial consortia of marine sponges [30, 31], of whale fall carcasses [32] and the deep sea [33]. The main outcomes of sequence-driven metagenomics are predictions on the metabolic repertoire of a given sample, to delineate metabolic pathways and to assess the potential of an environmental sample to perform specific, sought-after tasks. Single-cell genomics based on whole genome amplification (WGA) is an emerging technology in the field of environmental microbiology, which is complementary to metagenomics [34, 35]. Owing to the experimentation and manipulation of single microbial cells, this technique allows promising genomic insights into complex environmental microbial consortia whose members are frequently resistant to cultivation [36]. Importantly, functional assignments of primary and secondary metabolism genes to specific bacterial genes of known phylogenetic identity are possible [37]. Metagenomics and other omics methods have opened new avenues for the sustainable production of marine enzymes/drugs that would otherwise be inaccessible by conventional microbiology techniques. By merging the scientific disciplines of molecular genetics, microbiology, chemistry and biochemistry, the promise that marine microorganisms hold for industry is becoming a manageable task. The advent of massive parallel DNA sequencing techniques has set the stage for the next level of genomic and metagenomic bioprospecting. This methodology provided the means for isolating genes directly from environmental DNA without cloning. In the SeaBioTech consortium, high-throughput pyro-sequencing technology from Roche (the 454 genome sequencing platform) was the key instrument for metagenomics mining which was complemented upon demand by other sequencing technologies (e.g. Illumina). Importantly, sequence read lengths on the average of 700 bases were obtained with the 454 FLXplus platform, which resulted in higher numbers of informative sequences. The advantages of sequence-based metagenomics are many: this gave enzyme leads at least of an order of magnitude greater than other currently used screening techniques. A large number of genes were predicted to be detected that do not turn up using activity screening due to expression problems or the use of suboptimal substrates. And, as the genomic/metagenomic enzyme/gene discovery methodology is sequence-based, gene redundancy was eliminated very early in the process, which minimized the downstream analysis work. This was especially important for large-scale metagenomic sequencing projects as the sequence capture method reduced the need for a high coverage of sequencing for complete gene retrieval. The SeaBioTech methodology took the metagenomic mining out of the domain of large specialized companies and brought it into the field of small companies, universities

and institutions. Hence, one of the most important contributions of SeaBioTech project was "affordable metagenomics". Samples for metagenome libraries were made available from the project which included strains from Yngingarlindir water samples in Iceland, microbial mats and sponges from Milos Island and Santorini volcanic complex in Greece, strains from Kallisti lakes water samples and strains collected from Kolumbo microbial mats covering the ocean floor and the polymetallic chimneys.

4.1.7 Metagenomic Bioprospecting

UWUERZ employed a metagenomic bioprospecting approach to unravel the differences in the functional gene repertoire between three Mediterranean sponge species, *Petrosia ficiformis*, *Sarcotragus foetidus* and *Aplysina aerophoba*, and seawater, collected during a SBT sampling expedition (WP2). Microbial diversities were compared to those of other sponges within an EMP global sponge microbiome effort and contributed to the largest microbiology survey in sponges so far conducted [31].

With respect to gene function, different signatures were observed between sponge and seawater metagenomes with regard to microbial community composition, GC content and estimated bacterial genome size. The analysis showed further a pronounced repertoire for defence systems in sponge metagenomes. Specifically, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), restriction modification, DNA phosphorothioation and phage growth limitation systems were enriched in sponge metagenomes [38]. These data suggest that the "defensosome" is an important functional trait for an existence within sponges that requires mechanisms to defend against foreign DNA from microorganisms and viruses.

With respect to secondary metabolism, the most abundant marker genes in the microbial metagenomes belonged to the groups of saccharides, bacteriocins, terpenes and fatty acids. Other indicator genes of secondary metabolism—linaridin, lantipeptides, ectoines, phosphonates, proteusin, polyketide synthases, nucleosides, microcins, siderophore or homoserine lactones—were found only in low copy numbers. Interestingly, while siderophores and homoserine lactone hits were only identified in seawater, lantipeptides, linaridines and type I polyketide synthases were exclusively found in the sponge metagenomes. A total of 120 type I PKS genes in the three sponge metagenomes were further identified. Phylogenetic analysis assigned the majority (109/120) to the symbiont ubiquitous *supA*-type PKS group. Most similar sequences from the sponge metagenomes were derived from bacterial symbionts of other sponge species. Most of the polyketide synthases in the *supA* clade of the tree resulted in a hit to epothilone with low-to-moderate sequence identities. Despite the variance of possible products in the FAS-like PKS clade, the order of the genes surrounding the polyketide synthase was highly conserved.

MATIS sequenced 34 novel bacterial strains from geothermal intertidal areas in Iceland, assembled and annotated for bioprospecting. An additional four strains that had been sequenced before SeaBioTech were also annotated at the beginning of SeaBioTech to allow bioprospecting to start. Of the 38 sequenced strains, 13 (34%)

belong to the α-*Proteobacteria*, 10 (26%) to *Bacteroidetes*, 7 (18%) to Firmicutes, 6 (16%) to γ-*Proteobacteria* and one strain each to *Actinobacteria* and *Chloroflexi*. All strains are thermophiles or moderate thermophiles.

HCMR generated two metagenomic libraries from the Kallisti lakes in Santorini caldera characterized by high concentrations of metals and differences in pH, temperature and nutrient concentrations. HCMR also generated another three metagenomic libraries from a polymetallic spire located within the submarine Kolumbo volcano of the Hellenic Volcanic Arc. Each library has been constructed from different microbial mat layers of the spire characterized by differences in metal concentrations.

4.1.8 Genome Mining of Bacterial Isolates

UWUERZ provided draft genomes of three selected actinomycetes [39]. Metabolomic analysis in WP5 has shown the chemical richness of the sponge-associated actinomycetes *Streptomyces* sp. SBT349, *Nonomuraea* sp. SBT364 and *Nocardiopsis* sp. SBT366 that had been isolated from sponges during a SBT sampling expedition. The genomes of these three actinomycetes were subsequently sequenced, and draft genomes were mined using antiSMASH and NaPDoS. *Streptomyces* sp. SBT349 displayed the most diverse read-out. A total of 108 potential secondary metabolite gene clusters were predicted, encoding for 23 type I polyketide synthases (PKS), 11 non-ribosomal peptide synthetases (NRPSs), 2 terpenes, 21 saccharides, 3 siderophores, 3 lantipeptides, 1 butyrolacetone, 1 bacteriocin, 1 phenazine, 1 ladderane and 1 linaridin, as well as 26 unidentified putative clusters. Furthermore, NaPDoS predicted the presence of natural products such as nystatin, rapamycin, rifamycin, epothilone and tetronomycin. For *Nonomuraea* sp. SBT364, NaPDoS predicted the presence of gene clusters encoding for rifamycin, avermectin, avilamycin, concanamycin and tetronomycin. Thirdly, for *Nocardiopsis* sp. SBT366, gene clusters encoding for pikromycin, alnumycin, amphotericin and mycinamicin were predicted. In summary, UWUERZ efforts provided new insights into the genomic underpinnings of actinomycete secondary metabolism, which may deliver novel chemical scaffolds with interesting biological activities for the drug discovery pipeline.

An extremely high level of novelty was presented by this panel of novel strains. Based on 16S rRNA gene sequencing of the 38 genomes, 19 strains (50%) shared less than 94% similarity with their closest relative and are therefore considered novel species and novel genera. Ten (26%) shared between 94% and 97% similarity and are considered novel species, and the remaining 9 strains (24%) shared more than 97% similarity with their closest relative. Strain MAT4553, which has 90% similarity with its closest relative *Rhodothermus marinus* (16S rRNA gene), was selected for further characterization carried out by MATIS. It has been assigned the species name *Rubrimicrobum thermolitorum*, and characterization is still currently ongoing.

All 38 strains were annotated using subsystem annotation servers (RAST and MG-RAST), the genomes mined for novel genes of interest and analysed by antiSMASH for putative secondary metabolite gene clusters. A total of 2432

putative gene clusters were predicted, including 20 non-ribosomal peptide synthetase clusters and a total of 30 polyketide synthase clusters of types I, II or III. A total of 64 genes encoding novel enzymes for applications in marine macroalgal biorefineries were identified and delivered for cloning, expression and functional analysis in WP6 including 51 carbohydrate-active enzymes (CAE) 3 enzymes (oxidases) putatively active on polyphenols, 5 alcohol dehydrogenases, a sulfatase and 4 proteases. A total of 58 genes encoding novel enzymes including thioesterase, cyclic peptide-related genes and (3) lysine exporters, for application in synthesis of added value chemical and pharmaceutical, were identified and delivered to IGZ for cloning, for expression in their proprietary inABLE® system and for further analysis and selection in WP6.

SAMS undertook whole genome sequencing of five bacterial strains and delivered a total of four draft whole bacterial genomes. The fifth bacterial genome was to be of the filamentous cyanobacterium, *Nodularia harveyana* CCAP 1452/2. This was advanced to the point of achieving an axenic culture (WP2) and development of a useable DNA extraction protocol based on mechanical tissue disruption without predigestion of the cell walls using the lysozyme and purification using the quaternary ammonium detergent cetyl trimethyl ammonium bromide. However, significant quantities of polysaccharide were found to contaminate the DNA preparations, and refinements to the protocols were not successful in removing this. This meant that the genome sequencing centre was unable to prepare the DNA library required for PacBio RSII genome sequencing.

All genome data was mined for enzymatic and secondary metabolite potential. In terms of carbohydrate-active enzymes and xenobiotic degradation potential, *Colwellia* and *Rhodococcus* (SBT017), respectively, had the greatest potential of the four organisms. The *Colwellia* genome data will serve as an important resource for the scaling-up and commercialization of the gel-forming biopolymer this organism produces (WP7) during a PhD studentship working in conjunction with the multinational company, Unilever. The *Rhodococcus* genome is undergoing further analysis to link the secondary metabolite clusters identified with the metabolome of this organism fermented under different conditions (WP5 and WP7).

The *Acidobacteria* (*Holophagales*) genome showed an especially high number of novel secondary metabolite gene clusters belonging to the non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) classes. Metabolomic analysis (WP5) did not identify production of any secondary metabolites putatively linked with these clusters, nor was any bioactivity identified (WP3). The lack of novel secondary metabolite production by the *Acidobacteria* is hypothesized to be a failure to induce the many cryptic secondary metabolite operons. This hypothesis is given some support by the observation that many signal transduction systems were found within or immediately adjacent to these clusters. This suggests that these clusters are tightly regulated and are part of a signal transduction relay activated by specific signalling molecules or environmental stressors. In conclusion, this organism holds significant potential for secondary metabolite production. But, to achieve this though, further funding is required to try to activate the cryptic secondary metabolite

clusters, as well as continue to isolate and genome sequence new marine *Acidobacteria* from the environment.

Vibrio splendidus SBT027 produced a range of bisindoles, including the compound turbomycin. Several putative genes were identified that may be linked with turbomycin production. First, the biosynthetic pathway for the assumed precursor, L-tryptophan, was identified. Second, the enzyme 4-hydroxyphenylpyruvate dioxygenase had previously been identified as a part of turbomycin production, and this was identified in this genome. Third, inosine-5′-monophosphate dehydrogenase has been shown to be important in bisindole production previously, and this gene was also identified. However, as these genes are not organized in an apparent gene cluster, it is uncertain how these genes are involved in turbomycin production by this *Vibrio*. Moreover, the above genes are all highly conserved and syntenic in all other *Vibrio splendidus* genome sequenced isolates. This suggests either that all *V. splendidus* are capable of turbomycin production or that the main pathway for bisindole and/or turbomycin production in *V. splendidus* SBT027 has not been correctly identified. Clearly, further work is required to identify this pathway.

The second aspect of the "quality of marine resources" challenge is cultivation. Traditional techniques are often inadequate for accessing the microbial diversity of any given habitat. Studies from many extreme areas including MATIS' current and ongoing work in Iceland have demonstrated that cultivation of microorganisms living under extreme conditions is particularly difficult. The main reason for the low ratio of presently cultivatable microbial species is that their isolation takes place under both space and time limited by laboratory conditions. Other factors that explain the low ratio of cultivated species include unknown conditional or nutritional requirements or other important chemical components supplied to the species in their natural environment and missing in laboratory media. There could also be requirements for interdependent cocultivation of two or more different species. A nutritionally rich laboratory medium is not a natural medium for many environmental microbes. Copiotrophic organisms therefore gain a competitive edge and outcompete oligotrophic species, although they may be more abundant in the habitat. Furthermore, rich medium may be growth inhibiting for oligotrophic species. Classical resource-competition theory maintains that highest diversity occurs when many resources are limiting. In addition, high species diversity can be maintained by periodic disturbance or by environmental fluctuations (i.e. nutrients, pH and temperature) [40]. Laboratory conditions that allow microbes to grow to high density in a short time are "unnatural" for many natural microorganisms that normally grow slowly, at very low and steady-state concentrations of nutrients [41]. Re-creation of "nature-like" or natural, low-nutrient or oligotrophic conditions has been attempted in a few cases but can only be done on a small scale and with great effort [42]. Growing many oligotrophs in the laboratory on a large enough scale would be practically impossible. Different modifications of the enrichment concept have been developed in order to culture more novel organisms. These included serial dilutions or pretreatment of the sample. The purpose is to kill or dilute out numerically less numerous copiotrophic fast-growing organisms in the sample before inoculating the enrichment medium [43, 44]. Still another attempt towards

"nature-like" enrichments is the technique of in situ enrichment or substrate colonization, which has been used in several environments. In situ enrichment is based on the principle of introducing one or few new factors into an existing "natural" environment. Techniques of in situ enrichments have been of interest to microbiologists ever since bacteria were found to colonize microscope slides submerged in aquatic environments [45, 46]. Such techniques have been used in hot springs to obtain specific groups of microorganism, by using specific substrates such as cellulose. These techniques may be of special value for isolating or enriching species utilizing polysaccharides unique to the marine organism found in coastal areas. In the SeaBioTech project, the consortium developed selective enrichment methods and serial dilutions for accessing rarer and potentially more interesting members of bacterial and protistan communities. The consortium was able to increase the overall diversity and phylogenetic depth of obtained strain collection for consequent screening for bioactive microbial metabolites and thus maximized the likelihood of obtaining novel bioactive lead compounds. Enrichment methods were developed targeted towards certain metabolic types belonging to heterotrophic actinomycetes, thermophilic bacteria, marine and extremophilic cyanobacteria and rare coastal psychrophilic heterotrophs by using various cultivation methods and enrichment procedures. Special substrates such as complex recalcitrant polysaccharides or single carbon sources of predetermined type and structures were used often in conjunction with group specific inhibitory substances.

A third aspect of the "quality of marine resources" challenge is accurate identification. Sampling of marine microbes from a range of environments was explored by 16S rRNA and/or other candidate genes in order to assess the potential of the communities for industrial purposes and redirect new sampling. By 16S rRNA gene sequencing followed by phylogenetic tree construction analyses, the taxonomic identity of the bacterial isolates was determined. Colony lysates were amplified by PCR (polymerase chain reaction) using the universal bacterial primers 27f and 1492r, while PCR products were sequenced directly. DNA extraction protocols (i.e. for cells with hardy cell walls) and PCR conditions were optimized where necessary. Full 16S rRNA sequences of selected candidates were provided and phylogenetically isolated. Strain descriptions of novel species or genera were also undertaken. The genomic potential of bacterial isolates was assessed by PCR screening for genes indicative of secondary metabolism such as polyketide synthases, non-ribosomal encoded peptide synthetases, halogenases and other genes of relevance for secondary metabolism. Likewise, metagenomics approaches were employed to assess the genomic potential of previously uncultivated marine microbial consortia. Biotechnologically relevant gene clusters were cloned into cosmid/fosmid vectors, sequenced and analysed with bioinformatic prediction tools. Full genome sequencing was performed for isolates of special interest using deep sequencing (454/Illumina).

4.2 Challenge 2: Improvement of Technical Aspects of the Biodiscovery Pipeline

Once samples from marine bioresources were obtained, they were explored for the presence of useful bioactivities. When activity was found, the component responsible was identified and characterized. The SeaBioTech project developed systems to enhance the efficiency and effectiveness of both bioactivity detection and compound isolation and characterization. SeaBioTech focused on discovering useful marine components with enzyme activity, as biopolymers or with drug-like properties. The enzyme activities were predicted from analysis of metagenomic data followed by functional expression [47]. Biopolymers were identified, quantified, as well as extracted and isolated. Development of biopolymers included progressive pharmaceutical screening as well as investigating the potential role of (isolated) algal endophytes in improving polymer and cultivated macroalgae resources, which are a well-established, but not fully developed, source of natural polymers. The next sections discussed the present state of "drug hunting" and how SeaBioTech enhanced this process through improvements in screening and natural product chemistry.

4.2.1 Bioactivity Screening

The biodiscovery pipelines focused on the following categories: polymers, enzymes and small molecules used for drug discovery, functional foods or cosmetics. Drug discovery programmes seeking new bioactive compounds are driven by the existence of unmet therapeutic needs. In recent decades, advances in the understanding of the molecular basis of diseases and sequencing of the human genome and of pathogenic hosts have expanded the number of plausible therapeutic targets for the development of innovative drugs [48, 49]. Therefore, a wealth of new technologies and paradigms has been established since the mid-1990s, with the initial expectation of generating novel drugs in a greater number and in a shorter time. Among others, cardinal roles were played by molecular biology, combinatorial chemistry and high-throughput screening [50]. First, genetic manipulation of expression host cells using molecular biology allowed the development of target-based functional assays, in place of the traditional phenotypic systems [50]. In parallel, improvements in organic synthesis through combinatorial chemistry exponentially expanded the size of small-molecule compound collections [51]. Consequently, natural products (which had been the basis of most previous drug discovery programmes) were progressively neglected. To confront the massive effort required to test the large number of newly identified molecular targets with huge chemical libraries, multiple areas of biology, chemistry, engineering, robotics, statistics and information technology were integrated to create high-throughput screening (HTS). Hence, HTS has been established in large pharmaceutical companies as the technological platform able to screen compound collections containing over 1,000,000 molecules on

biochemical and cellular assays in an automated manner and miniaturized format [52, 53]. Subsequently, prominent academic institutions decided to exploit the potential of these technological advancements through initiatives to assemble centralized compound collections and screening facilities with the aim of identifying molecular probes with prospective applications in basic and applied biomedical research [54].

Although this pioneering approach to drug discovery has been successful in delivering innovative clinical candidates and marketed drugs [55, 56], it is undoubtedly true that the original expectations in terms of overall performance are far from being met and unlikely to be achievable. Rather, the increasing costs associated with the infrastructural and technological investments have contributed (within a framework of tackling more challenging diseases, higher scientific risks, increasing safety requirements and larger clinical trials) to the so-called productivity gap in pharmaceutical R&D, which has been posing major issues for the sustainability of drug development in the private and public sectors [57]. Therefore, while the main technological improvements are still considered essential cornerstones for R&D, the basic paradigms of the process are currently under debate [55, 58–60]. In particular, phenotypic screenings have been currently reconsidered as valid options along with target-based molecular assays, particularly for certain therapeutic areas (e.g. pathogenic infections, cancer and others) [60, 61]. Moreover, emphasis has been given to highly validated targets, i.e. targets whose activity has been proven to be modulated by a chemical compound and with a direct causative link to the disease to be addressed. Therefore, highly innovative but poorly characterized targets were deprioritized [62]. More recently, attention has been focused on the quality of the compounds in the chemical libraries, rather than on the number of compounds. In fact, retrospective analysis unequivocally clarified that early combinatorial chemistry produced large libraries with very limited diversity [55, 56]. At present, investments in compound collections are not aimed at a numerical size increase, but at ensuring a constant stream of new chemotypes, meaning that natural products and mimetic derivatives are back into consideration [56, 58]. This implies that drug discovery has to face well-known problems inherent to natural products, like supply at screening scale, purification, identification and structural complexity [63, 64]. However, technical solutions have been rapidly developing to overcome these bottlenecks and in order to gain access to the potential of this valuable source of chemical diversity [65–67]. Under this developing scenario, the SeaBioTech consortium integrated some of the most advanced technological applications with state-of-the-art expertise in drug discovery research to identify bioactive compounds from libraries of marine origin. To increase the chances of a positive outcome of the screening campaigns, the assay types applied in SeaBioTech comprised a wide array of target-based and phenotypic assays.

Some were configured in HTS-suitable formats to ensure a high processivity of large compound collections and of hit profiling; some will be performed as low-throughput assays to achieve a high level of information directly from primary screening (e.g. assays against sea lice affecting farmed salmon). Most importantly, all assays within SeaBioTech represented functional assays designed to provide unambiguous responses concerning their relevance for biomedical and

biotechnological applications (e.g. isogenic X-MAN human disease models from HDL). Having no pre-existing knowledge on the bioactivities present in the extract/compound collections obtained from underexplored marine sources, SeaBioTech members screened a very wide set of assays with relevance to diverse therapeutic areas, including cancer (AXXAM; HDL; MATIS), bacterial, viral and parasitic infections (SIPBS; AXXAM; UWUERZ), inflammation (SIPBS; MATIS), cardiovascular diseases (AXXAM; MATIS), metabolic disorders (SIPBS) and pain (AXXAM). Besides human health, SeaBioTech sought bioactive compounds to treat parasitic infections in aquaculture (PHARMAQ) and for food and cosmetics industry (MATIS). It is worth noting that discovery programmes in these fields are encouraged by the successful outcome of research projects using compounds of marine source, which have recently yielded molecular probes, preclinical candidates and therapeutic drugs in several clinical areas, including cancer [68–70]; bacterial, viral and parasitic infections [71]; inflammation [71, 72]; Alzheimer's disease [73]; and pain [71, 74].

Since the final aim of SeaBioTech was the exploitation of the value of the new compounds, participants did not limit their investigation to the identification of hit compounds through primary screening, but also employed their competencies in more advanced stages of the drug discovery process, including studies on selectivity, mechanism of action, early toxicology and proof of principle in animal models. This guaranteed that the outcome of the bioactivity assessments was not just be compounds that "hit" particular targets but an activity profile of a bioactive substance and its drug-like properties. Such compounds represented potential development candidates, a critical step towards new medicines.

The organizational aspects of SeaBioTech also provided progress beyond what is normally achieved in drug discovery programmes in individual SMEs or in academic institutes. For SMEs, the successful outcome of large-scale drug discovery projects entails on extensive collaborations and partnership with public academic institutions. On the other side, access to advanced technological platforms, cost-sustainable exploitation of the results and interrelation between specific expertises, knowledge and competences were considered essential prerequisites to identify and progress novel molecular entities for biotechnological and biomedical applications. Hence, SeaBioTech was structured to promote and implement synergistic collaboration at two levels. First, extracts and compounds of marine origin collected and isolated by public research institutes will be made accessible to private companies, which in turn will make available their technological platforms and market-oriented approach to develop innovative products for human health and life sciences. In addition, SeaBioTech represented a valuable opportunity to synergistically link the public and the private sectors, offering the possibility to progress within an integrated partnership and providing common objectives through mutual connections.

Second, SeaBioTech inherently enhanced the collaboration among different SMEs contributing at different stages of the project (identification of hit compounds, hit-to-lead phase, characterization of lead compounds), in order to define the chemical and pharmacological properties of the products. Thus, participation in SeaBioTech epitomized a valid opportunity for SMEs to establish collaborative

partnerships with companies with contiguous expertise and complementary technologies. In parallel, the strategy adopted in SeaBioTech for bioactivity detection embodied an impressive improvement in terms of potential exploitation of the chemical diversity of the marine compound collections. Indeed, libraries of marine origin were subjected to screening campaigns against a panel of more than 20 assays covering over 10 different therapeutic areas or biotechnological applications. This approach increased the probability that bioactive compounds are retrieved as positive hits, thus predicting a superior success rate compared to traditional screening on a few assays. In practice, the adopted strategy places SMEs and research institutes within.

The screening method in SeaBioTech closely resembled a large pharmaceutical company, in which a proprietary compound collection was routinely screened against a series of disease-relevant assays. However, in SeaBioTech, costs and risks are shared among different participants, making the overall process more sustainable. In addition, as the consortium has access to an underexplored chemical diversity and the project focused also on products for aquaculture, food industry and cosmetics, in which a lower attrition rate is usually experienced, it then gave a remarkably high productivity for SeaBioTech. The central goal of the entire SeaBioTech consortium was the isolation and pharmacological characterization of novel lead candidates of marine origin. This goal was achieved through an integrated effort between WP2 and WP5 with the 6 members of WP3 (SIPBS, AXXAM, HDL, PHARMAQ, UWUERZ, MATIS), who have made available comprehensively an array of 41 functional assays with relevance to 12 therapeutic and life science indications. The screening process and the bioactivity-assisted dereplication of crude extracts and fractions have led to the isolation and characterization of 35 pure compounds with promising therapeutic properties. Notable examples are the following: (1) SBT0345 from *Streptomyces* sp. was fractionated by UWUERZ to yield three novel natural products, namely, strepthonium A, ageloline A and strepoxazine A. Strepthonium A inhibited the production of Shiga toxin produced by enterohemorrhagic *E. coli* at a concentration of 80 μM, without interfering with the bacterial growth [75]. Ageloline A exhibited antioxidant activity and inhibited the inclusion of *Chlamydia trachomatis* with an IC_{50} value of 9.54 ± 0.36 μM without cytotoxicity towards human kidney 2 cells [76]. Strepoxazine A displayed antiproliferative property towards human promyelocytic HL-60 cells with an IC_{50} value of 16μg/mL [77]. Moreover, SBT0345 from *Streptomyces* sp. was yielded also the known compound phencomycin, which displayed cytotoxicity against colon cancer cell line SW48 at 30 μg/mL, and tubermycin B, which showed cytotoxicity against colon cancer cell lines DLD-1 and HCT116 at 30μg/mL. (2) SBT0348 from *Streptomyces* sp. was fractionated by UWUERZ to yield one novel compound, petrocidin A, exhibiting significant cytotoxicity towards the human promyelocytic HL-60 and the human colon adenocarcinoma HT-29 cell lines, with IC_{50} values of 3.9 and 5.3 μg/mL, respectively. (3) SBT0961 from *Polysiphonia lanosa* yielded three fractions, which were identified by HDL as active and selective for rapidly dividing cancer cells, with antiproliferative properties strongly correlated with the induction of cell death via apoptosis. (4) MATIS identified from microorganisms

collected from the Icelandic coastline 11 hits displaying high antioxidant activity, 9 hits that inhibited cell viability of breast cancer cell line and 13 hits that inhibited viability of intestine cancer cell line. (5) SIPBS isolated 13-methyltetradecanoic acid (SBT2309) from *Muricauda ruestringensis*, a compound with activity against PTP1B, a target to treat diabetes and metabolic syndrome. Remarkably, SIPBS isolated the same compound showing comparable activity against PTP1B at the end of an independent bioactivity-assisted screening campaign from extracts of another microorganism, *Algoriphagus marincola.* (6) SIPBS isolated a series of structurally related fatty acids from extracts of *Algoriphagus marincola*, which showed activity against PTP1B and allowed the definition of a preliminary structure-activity relationship on the basis of the relative potency. Remarkably, AXXAM isolated with an independent screening campaign for inhibitors of endothelial lipase, a validated target for atherosclerosis, a series of fatty acids derived from *Algoriphagus marincola* partially overlapping with the hits showing activity against PTP1B at SIPBS. This finding appears consistent with the targeted enzyme EL, which physiologically releases fatty acids from phospholipids in HDL particles. (7) SBT1997, a pure compound isolated by SIPBS from *Polysiphonia lanosa* as active against α-glucosidase, was identified as a known compound termed lanosol. Lanosol was documented in literature as an α-glucosidase inhibitor. (8) A series of bromophenyl homologous compounds have been identified by PHARMAQ from *Polysiphonia lanosa* extracts and fractions having a potent parasiticidal activity against *Lepeophtheirus salmonis*, a major threat for farmed salmon in aquaculture.

4.2.2 Metabolomics Approach: Improving Isolation and Identification of Target Compounds

SeaBioTech used the state-of-the-art approaches to isolation of bioactive compounds from extracts and microbial broths coupled with appropriate NMR (nuclear magnetic resonance) spectroscopy and mass spectrometry to elucidate chemical structures. Moreover, SeaBioTech pioneered the use of metabolomics as a new means to guide strain selection and the isolation of compounds [78, 79], as well as to help improve the productivity of downstream fermentation methods. Metabolomics is relatively a new field of "omics", adopting to the system biology approach, with the goal of qualitatively and quantitatively analysing all metabolites contained in an organism at a specific time and under specific conditions. The metabolome is the complete set of small molecules found in a cell, tissue or organism at a certain point in time. Metabolomics is considered as the most functional approach in monitoring gene function and identifying the biochemical status of an organism [80]. Metabolomics was utilized to confirm the results of the presence of biosynthetic gene clusters involved in the production of the biologically active components. This was accomplished with bioactive strains which, included the anti-mycobacterial *Vibrio splendidus* SBT-027 (MacIntyre et al. unpublished data) and *Rhodococcus* sp. SBT-017, found to be active against metabolic diseases (Hislop et al. unpublished data). Metabolomics in combination with genomics enhanced the

production of important secondary metabolites which is one of the expressed phenotype in a living organism. Literature has shown that gene clusters are involved in every step of a biosynthetic pathway as in the production of biologically active polyketides [81]. With genomics, gene clusters can be manipulated to control a biosynthetic pathway. The procedure of employing metabolomics together with genomics to optimize a biosynthetic pathway to selectively produce biologically active secondary metabolites was explored during the project's lifetime. To identify and quantify the metabolites in natural product extracts is a massive job [82, 83]. This is due to the fact that secondary metabolites have diverse atomic arrangements which results in variations in chemical and physical properties. They can also be found in wide range of concentrations. Reliable, robust, selective and high-resolution analytical methods are therefore required in identifying and quantifying multiple chemical groups of natural products. Mass spectrometry and NMR spectroscopy were the complementary analytical methods and were commonly employed in tandem as metabolomics tools. Mass spectrometry is sensitive even at femtogram levels but may not be reproducible between instrument types and ionization capability of the metabolites. While NMR data is reproducible, it may not be sensitive enough to detect metabolites at lower concentrations. Efficient high-throughput gradient flash and/or medium-pressure chromatography, where gram quantities of a microbial extract can be loaded in a column, will be employed to isolate the bioactive natural products from microbial extracts. High-throughput gradient medium-pressure chromatography is capable of delivering reproducible isolation schemes with high product yield, which is optimum in the purification of marine microbial extracts obtained from multiple batches and has great advantage over conventional column chromatography [82, 83]. Structure elucidation was accomplished utilizing pulse field gradient 2D NMR that would be able to provide high-resolution data to determine the structure of complex molecules with multiple chiral centres as well higher-molecular-weight peptides and oligosaccharides [84].

Metabolomics provided statistical and computational tools to this standard approach of rapid HPLC (high-performance liquid chromatography) fractionation, which identified the active entities at an earlier stage [78, 85]. The goal of HPLC fractionation is to get to higher purity of active components which, however, is not achievable in the initial chromatographic isolation work. With metabolomics tools, it will be possible to pinpoint the active components at the first fractionation step as well as identify the functional groups involve in the bioactivity which would be present in a series of fractions as implied by the bioactivity screening results. This can be chemometrically achieved by such metabolomic/PCA approaches (principal component analysis) as shown in an example presented in Fig. 12.3. The use of metabolomics aided in prioritizing the fractions that will go further for purification work, which should save time and resources in isolating the target compounds.

Within SeaBioTech, metabolomics was used for quality control of the natural products and isolates to monitor the manifestation of a different metabolic profile between individuals, environmental alterations during growth and harvesting, postharvesting treatment, extraction and method of isolation, all of which can affect the efficacy of natural products.

Fig. 12.3 (**a**) PCA scoring plot based on mass spectrometry data from all extracts. (Taxonomic identification data and bioactivities of the outliers (red dots) are shown in the plot.). (**b**) Heat map based on mass spectrometry data. Outliers in PCA analysis plot are annotated (asterisk)

Through the metabolomics approach, the link between chemical profile and bioactivity pattern of the secondary metabolites is correlated. Metabolomics was widely applied in the bioactive screening of natural products although it has several advantages over the reductionist approach. Metabolomic tools enhanced the identification and dereplication steps, as in bioassay-guided isolation work [80]. Metabolomics was applied to dereplicate the biosynthesis of the natural product at different development stages of their biological source as well as simultaneously screen for the bioactivity. By using combinations of different analytical methods, the bioassay-guided isolation

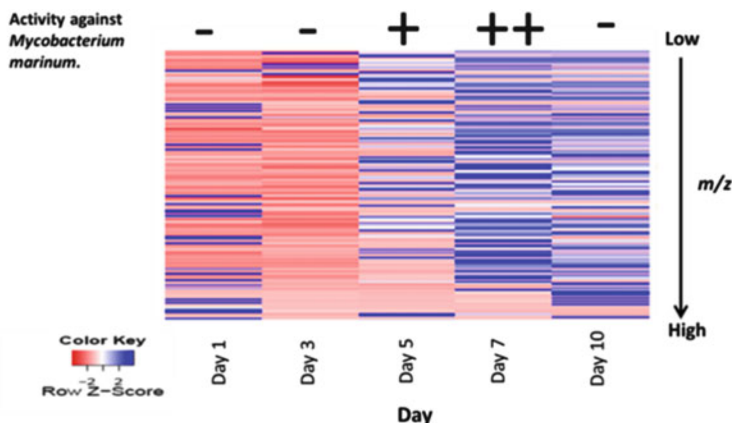

Fig. 12.4 Significant increase in bioactive metabolite production in *Vibrio splendidus* starts in Day 5 which steadily increases to Day 10. However, bioactivity against *Mycobacterium marinum* was only observed for the Day 7 extract

route was shortened, and rapid dereplication of known activities was rapidly delivered [82]. The SeaBioTech consortium encompasses the expertise in metabolomics. VTT (Technical Research Centre of Finland) together with SIPBS developed MACROS and modified the algorithms of MZmine (version 2.10), a web-based differential expression analysis software [86] to efficiently detect the production of interesting secondary metabolites during the cultivation and production processes that would assist in maintaining or enhancing biosynthesis of the desired compounds [79, 87]. An example is shown in Fig. 12.4. The results were integrated and coupled to an in-house database that includes DNP (Dictionary of Natural Products) and Antibase, a database of microbial secondary metabolites to further identify microbial secondary metabolites. Figure 12.4 presents the HRMS heat map data as processed by MZmine. The experiment analysed the production of anti-mycobacterial metabolites from a *Vibrio* sp. collected from the Atlantic coastline of the Northern Scottish Isles.

Within the SeaBioTech project, metabolomics was applied at two levels: first, to identify and track active compounds highlighted by screening assays and second, to optimize the biotechnological production of active compounds in the later stages of the pipeline. Dereplication of secondary metabolites from promising isolates was achieved by HRFTMS (high-resolution Fourier transform mass spectrometry) using the LTQ Orbitrap and high-resolution NMR. Through multivariate analysis, this enabled Fourier transformation of FID (free induction decay) data of multiple samples to statistically validate the parameters in the production of pharmacologically interesting secondary metabolites. Metabolomes were identified with the aid of existing high-resolution MS and NMR records from in-house databases like DNP and Antibase. MZmine is a software that was utilized to perform differential analysis on the mass spectral data from a vast number of sample populations to find significant expressed features of complex biomarkers between parameter variables. This would be further validated through available reference standards and

two-dimensional homonuclear NMR, e.g. TOCSY (total correlation spectroscopy) and *J*-resolved NMR, experiments to classify unknown by-products or degradants which may affect the quality of the desired product. The NMR metabolomic software from MNova was employed for metabolome recognition as well as to statistically validate the occurrence of metabolic by-products at the different physiological states. VTT optimized the much-needed algorithms to analyse the huge dataset generated by the dereplication study as well as metabolomic profiling in monitoring and exploring the relationship between culture methods, diversity, bioactivity, and metabolome evolution in selected marine isolates. Efficient cultivation and production processes at a small volume scale fermenter are developed through real-time metabolomic-assisted optimization. Samplings were done in real time for detailed metabolome analysis to fully characterize intermediates, by-products and degradants. Applying metabolomics for real-time analysis will in parallel check the stability of the production of the desired components when changing certain fermentation parameters prior to scale up. In addition, a chemometric study was accomplished to support and develop algorithms that was adapted and optimized to target the bioactive secondary metabolites. Metabolomics has become a powerful tool in systems biology, and it allowed SeaBioTech to gain insights into the potential of natural marine isolates for synthesis of significant quantities of promising new agents and guide the manipulation of the environment within fermentation systems in a rational manner to select the desired metabolome. Dereplication work was finalized for samples originating from Milos, Crete, and the geothermal vents of Iceland as well as those covering Scottish coastline and additional sample strains from the Antarctica region. Seventy-seven (77) bacterial samples were dereplicated from the NPMG-Orkney archive. A total of 34 bacterial extracts from Milos and Crete were analysed, yielding SBT348 and SBT687 as the candidate strains for further compounds isolation and purification. While based on mass spectrometry profiles of strains from the Scottish coastline and the Antarctica region, three isolates revealed distinct patterns, KP130 (an unidentified bacteria isolated from Maud Rise, Antarctica), KP044 (a *Streptomyces* strain isolated from St. Andrews sediment) and KP121 (a *Bacillus* strain from Bransfield Strait, Antarctica). The metabolites responsible for these unique profiles were identified using principle component analysis (PCA) and found to be a series of polymers *m/z* 363-1911 with spacing of 86 Da (KP130), a series of piscicides and antimycins known to be produced by *Streptomyces* spp. (KP044). These PCA outliers were also identified in the molecular network, demonstrating their complementary nature of metabolomics tools for secondary metabolite discovery. Metabolomic profiles have been documented into the SeaBioTech database. Metabolomes were dereplicated for priority strains, while biosynthetic gene-based screening explored the presence of the genes for the respective secondary metabolite (WP4). However, bioactivity was used to prioritize strains for the WP7 pipeline (WP3). Extracts of priority strains were prepared from scale-up for further fractionation and isolation of bioactive secondary metabolites. Metabolomic-guided targeted isolation work was done in parallel to and in support of the bioassay resulting to a quick identification of the active metabolites. A total of 65 natural products have been elucidated and have been documented in the

SeaBioTech database which has been linked to ChemSpider and PubChem databases (http://spider.science.strath.ac.uk/seabiotech/pure_compounds_show.php).

At VTT, an axenic *Euglena gracilis* microalga was introduced as a model organism for metabolic profiling. It was cultivated in 2 L stirred glass tank bioreactors in the presence of glucose under constant light or in the dark. The analyses showed that in light, the glucose intake was delayed, while the culture generated more biomass suggesting the contribution of photosynthesis. Lipidomic profiling by UPLC-QTof-MS in ESI+ mode (VTT) indicated that phosphatidylcholines were the prior lipid species, but in light cells accumulated large amounts of galactosyldiacylglycerols and ether-bonded lipids, while in dark medium-chain wax esters were typically formed. LTQ Orbitrap-based metabolomic profiling (SIPBS), on the other hand, showed the richness of metabolites formed in dark especially, and numerous spectral library suggestions for terpenoids of marine origin were obtained. Bioactivity testing (AXXAM) was also indicating some HDAC6 and PPARα activities for the ethyl acetate extract of cells cultivated in the dark.

4.3 Challenge 3: Sustainable Modes of Supply of Raw Materials

Marine organisms have provided many promising bioactive compounds with exciting therapeutic potential. However, their development has been severely curtailed because of the difficulties in obtaining adequate amounts. Examples include anticancer agents, ecteinascidin-743 and bryostatin. Ecteinascidin 743 (trabectedin, marketed as Yondelis®) was first isolated from the sea squirt *Ecteinascidia turbinata* in 1984. However, yields from the sea squirt were extremely low, and for further drug development, 1 tonne of animals was needed to isolate 1 g of trabectedin. It was only after 15 years that the supply problem was resolved by a semisynthetic process of starting with safracin B, which was obtained by fermentation of the bacterium *Pseudomonas fluorescens*. In the case of bryostatin, laboratory colonies of the bryozoan *Bugula neritina* exhibited a reduced number of symbionts and a reduction of bryostatin content thus implicating those bacterial symbionts as the true sources of the bryostatins. When some macro-organisms were placed in aquaculture in attempts to scale up production of a bioactive compound, the active material was lost, almost certainly because of loss of associated microbial species in the transfer from the wild to the cultured environment.

SeaBioTech's goal is to avoid such problems by basing much of its scale-up on the knowledge gained through its genomic and metagenomic studies of the gene clusters involved in synthesis of bioactive small molecules. There is extensive information on manipulating genes for non-ribosomal peptides and for polyketides. In addition, SeaBioTech explored the biosynthesis of marine polysaccharides, for which much less is known. This aspect of the project, its background and the advances made by SeaBioTech will be explained in detail in the following section.

4.3.1 Sustainable Production of Macromolecules at the Lab Scale by Metabolic Engineering

Microbes in extreme environments often adapt through production of extracellular polysaccharides (EPS). They are highly hydrated, which helps to deter desiccation, and they mediate adhesion to inert surfaces or living tissues, which is important for colonization of host organisms and the formation of biofilms. Often these polysaccharides have novel and unusual characteristics and [88, 89] that can be exploited in various fields—in the food, pharmaceutical and cosmetics industries as emulsifiers, stabilizers, gel agents, coagulants, thickeners and suspending agents and in high-value medical applications such as in tissue engineering and drug delivery [90]. Due to low production levels, few of these organisms have been exploited commercially. It follows that they are therefore basically untapped as a genetic resource for these activities. SeaBioTech explored the possibility of accessing these sources by developing a platform for production of tailor-made polysaccharides and oligosaccharides. While bioprospecting platforms have proven their value in mining natural genetic resources, the exploitation of promising leads is often hampered by low production yields. This is especially true as regards complex carbohydrate molecules—oligosaccharides, polysaccharides and glycosides. These limitations can in theory be overcome by pathway engineering of the source organism. Biosynthetic pathways can be influenced at three different levels: synthesis of sugar nucleotide precursors, assembly of the repeating unit and polymerization with concomitant export. By modifying the expression of single genes or groups of genes, the conversion efficiency of the chemical entities involved can be increased and, therefore, enhance EPS yield [91]. While highly justifiable in many cases, such an approach necessitates the time-consuming work of developing genetic tools, selectable markers, transformation methods and ideally species-specific expression vectors for each organism. An alternative approach to make use of these genetic resources is to develop a versatile polysaccharide production microbe with suitable genetic tools for hosting genes from other organisms. Such genes could exert their effects in the biosynthetic pathway in variety of ways depending on the gene, by introducing novel monosaccharide components and/or other substituents and by forming new linkages. Metabolic engineering of platform organisms for producing novel oligosaccharides and polysaccharides derivatives presents substantial challenges. Microbial polysaccharides are species-specific, highly heterogeneous polymers. These glycans include many unusual sugars not found in vertebrates, such as variously modified hexoses, noncarbohydrate substituents and an oligosaccharide sequence-repeating unit that can vary in size depending on the degree of polymerization. Besides requiring very complex synthetic machinery, the cellular context also matters, e.g. interference with energy metabolism, for synchronization/co-regulation of synthesis pathways, post-synthesis modifications and secretion mechanisms. Successful metabolic engineering of EPS-producing strains has been reported. In *Acetobacter xylinum*, the disruption of the diguanylate cyclase gene led to enhanced production of bacterial cellulose with altered structural properties

[92], and it has been shown that inactivation of the C5 epimerase in *P. fluorescens* led to the production of the homopolymer polymannuronate. It is expected that continued effort in this field will open up an enormous potential for the biotechnological production of biopolymers with tailored properties suitable for various high-value applications [93]. The assembly of extracellular polysaccharide involves a set of genes that are often clustered in the bacterial chromosome in separate regions. This arrangement allows a simple mechanism for changing capsule types by merely swapping different gene cassettes. Genes in one particular region can encode enzymes involved in nucleotide sugar formation and capsule-specific transferases, whereas genes in other regions encode type-independent transport activities required for movement of the polysaccharides across the inner membrane and periplasm. In other instances, synthases have formed membrane pores through which the polysaccharide is transported concomitantly with synthesis. This has been shown, for example, in hyaluronate synthetase in *Streptococcus* [94] and suggested by MATIS as the most plausible model on synthesis/transport mechanism of periplasmic beta glucan oligosaccharides involving a multidomain glucosyltransferases in Proteobacteria [95]. Besides a region coding for a Leloir glucosyltransferase of family GT2, another region codes for a domain belonging to a non-Leloir glucosyltransferase of family GH17; a transmembrane domain is predicted to form a membrane pore through which the newly synthesized glucan chain, product of GT2, is transported. The periplasmic GH17 enzyme domain then further modifies the nascent β-glucan leading to the formation of branched and cyclic OPGs. The specific features of polysaccharide synthesis suggest that alteration of polysaccharide structure can be achieved by region, gene and even domain swapping in their synthetic pathways and individual glucosyltransferases.

Glucosyltransferases are key enzymes in the anabolic polysaccharide biosynthesis, and microbial genome sequencing gives unprecedented access to this type of enzymes. They can now be systematically identified and compared with various bioinformatic tools. Valuable targets can be rationally selected, and by appropriate molecular techniques, they can be inserted into target production organisms and their effect on the biosynthesis pathway analysed by various methods. Glucosyltransferases catalyse the transfer of a glycosyl group from a high-energy donor or oligosaccharide to an acceptor. The Leloir type of enzymes utilize nucleotide phosphosugars (NP-sugar-dependent) as donors producing a nucleotide and saccharide as reaction products, and it has been shown that microbial glucosyltransferases are more versatile than their eukaryotic counterparts.

A number of Leloir multigene families have been identified, and an important focus of the bioprospecting effort was analysing and selecting an array of glucosyltransferase genes for cloning into a platform polysaccharide producing thermophile for expression and structural and functional studies of the resultant effects on source oligosaccharide. The source polysaccharide was analysed for structural alteration including changes in monosaccharide composition linkage types, repeat structure and the degree of branching. Relevant accessory enzymes was also defined and co-expressed with selected transferases if needed. Of these, enzymes for generation of activated sugars are most critical. Their requirement is

dependent on which glucosyltransferase will be selected for expression in the hosting system and the inherent capabilities of the host.

The platform species envisaged for polysaccharide production needed to fulfil certain criteria. It should (a) produce polysaccharides in high quantities; (b) be able to import a variety of sugars to be used as acceptors; (c) produce a great variety of activated sugars, at least many important ones; and (d) produce few and low amounts of side products. The ideal strain chosen for such polysaccharide production is the thermophilic marine bacterium *Rhodothermus marinus*, which served as the "model organism" in this project. Under certain conditions, it produces large quantities of EPS. The organism has a broad substrate range, degrading a large variety of polysaccharides and growing on their constituent uronic acids, hexoses and pentoses. *R. marinus* showed diverse metabolic activity and is easily cultivated. The genome has been fully sequenced, and various genetic tools and selectable markers have been developed in previous projects of the MATIS group.

R. marinus belongs to the phylum *Bacteroidetes*, and it was first isolated from the coastal geothermal area in Iceland. It is an aerobic heterotroph that grows at temperatures of up to 77 °C [96]. It has been subject of considerable research much of which has been devoted to its thermostable enzymes on account of their biotechnological potential, particularly polysaccharide degrading enzymes. Interestingly, several of these enzymes are secreted and exhibit optimum activities at 80–100 °C, which far exceeds the optimum for growth. Examples are cellulose, xylan and mannan degrading enzymes, some of which have been studied in great detail [97–105]. The work by MATIS has focused on developing gene transfer and genetic selection for the genetic engineering of *R. marinus* [106–110]. *R. marinus* was considered suitable for genetic studies because of its aerobic nature, competence growth in the defined media. Importantly, it exhibited reproducible growth on solid media, and clonal populations were easily obtained. Restriction negative host strain has been established, and expression vectors and selectable markers have been developed. Selectable markers, initially, biochemical and genetic properties of the species were poorly known and mainly restricted to single characterized proteins and genes, none of which could serve as a selective marker. The preferred antibiotic selection for thermophiles was based on the thermos-adapted kanR determinant, which was unsuitable for *R. marinus* because of its natural resistance to aminoglycosides. In continuing work, two selective markers were identified, *trpB* and *purA*, which encode proteins of the tryptophan and adenine biosynthetic pathways, respectively. A restriction deficient *R. marinus* isolate was chosen as a recipient for gene transfer experiments [106, 108]. The endogenous *trpB* and *purA* were deleted from the chromosome of the recipient, making it compatible with both Trp+ and Ade+ selection. Moreover, the deletions prevented both the development of spontaneous revertants and unintended marker integration. *Expression vectors*, a small, cryptic *R. marinus* plasmid, pRM21, of 2935 bps [110] served as the starting point for constructing *R. marinus—E. coli* shuttle vectors [109]. They contained the *R. marinus trpB* gene expressed from the promoter of the *R. marinus groESL* operon. These vectors served as basis for the construction of cloning vectors and allowed for the cloning and expression of foreign genes as well as induced expression in

R. marinus following temperature shifts. Two reporter genes were also identified, allowing for the investigation of *R. marinus* promoter activities in vivo [107]. Both random and site-directed inactivation of *R. marinus* genes have been implemented. Unmarked deletions were generated resulting in a double mutant with the genotype Δ*trpB*Δ*pyrA*. Here, the marker carried by the vector, outside homologous sequences, is lost through resolution of cointegrate. Subsequently, in-frame deletions using the *trpB* and the *purA* marker genes have been introduced. The selection efficiency of the strain was, e.g., demonstrated by insertional mutagenesis of the carotenoid biosynthesis genes *crtBI*. The resulting Trp+ and CrtBI mutants were colourless rather than orange-red [106, 108]. Also, marked deletions were obtained by performing gene replacements with linear molecules, which yielded double-crossover recombinants in a single step [106, 108]. The existence of selective markers and expression vectors enables rational genetic manipulation of *Rhodothermus*, which can result in altered metabolic pathways and novel products.

The extensive recombinant techniques available for *R. marinus*, existing genome sequence data, as well as broad substrate range and saccharide conversion features makes *R. marinus* feasible for metabolic engineering and eventually a versatile platform organism for production of structurally modified polysaccharide derivatives. By using metabolic engineering approaches, *R. marinus* was streamlined for production of complex molecules by eliminating the formation of side products by increasing gene dosages of critical genes, eliminating and/or modifying regulation mechanisms. By hosting appropriate genes from other organisms, synthetic pathways can be modified, and consequently structure and properties of a target molecule can be altered.

4.3.2 Sustainable Production of Secondary Metabolites at the Industrial Scale

In traditional biotechnology, all industrial manufacturing processes began (and begin) with plate cultivation, followed by scale transfer to liquid culture and further scale-up [111–116]. These steps, for some marine isolates, can be problematic and can be associated with a loss or reduction in the synthesis of desired metabolites or formation of unwanted by-products [117]. The immense biodiversity apparent in the marine environment is a potentially rich source of novel antibiotics, other secondary metabolites and metabolic potential [117], but in order to fully exploit this potential, we must take interesting activities often noted under lab conditions and transfer them to industrial-scale production. However, biomanufacturing using marine microorganisms presents several unusual challenges distinct from those encountered when manufacturing bioproducts from conventional terrestrial microorganisms. In part, these reflect the origins of the isolates themselves (source microbes). They include the use of media containing salt at moderate-to-high levels (0.43 M to 2.5%) [36, 118, 119], which can present corrosion and wear issues on seals and bearings of fermenters. The range of temperature optima for cultivation of marine microbes also presents challenges to the biotechnology industry, with interesting bioactivities

noted in marine microbes with psychrophilic (4 °C) [120] to thermophilic optimal temperatures (85 °C) [121]. Since the biotechnology industry basically uses processes and fermenters designed for organisms with temperature optima from around 25 to 40 °C [122], these unusual temperature requirements required significant redesign of plant in terms of heat removal and mass transfer (low O_2 solubility as temperature rises). Even when isolation of interesting fungal microbes from marine sources using agar brine plate cultures rather similar to the industrial workhorse *Aspergillus* is successful, this does not easily lead to new industrial products due to some of the barriers discussed above [123]. Other hurdles to rapid industrial exploitation include the use of unusual energy sources (H_2) [124] which are unfamiliar to the mainstream fermentation industry, and dangerous, or unusual substrates or toxic by-products (e.g. H_2S [119]) also unfamiliar to the bioprocess industries and with significant safety implications. Such isolates usually are exposed to low levels of dissolved oxygen due to the sparing solubility of oxygen in seawaters, whereas the modern fermentation industry is geared up to deliver products largely from mesophilic cultures in highly aerated and agitated fermenters [122]. On occasions, the early treatment or storage of natural isolates leads to loss or reduction in metabolite synthesis on scale-up. The perception of strain instability is a critical barrier. Despite the above, there is no fundamental reason why marine isolates should be inherently less stable than terrestrial, and even those from extreme environments have been shown to be amenable in some cases to cultivation under non-extreme conditions [117]. Overall, these hurdles and bottlenecks contribute to a less than certain and lengthier path to market for marine products when compared to terrestrial-derived products arising from a narrower ecological range and may well inhibit any further exploitation of an activity. The challenge is to match huge biodiversity in growth characteristics with a bioprocessing industry, which is largely based upon a very limited range of optimized processes to effectively and efficiently scale up interesting activities from bench scale to industry volumes. One approach to this is simply to move novel activities from less tractable marine isolates to industrial workhorse organisms, which the bioprocessing industry is familiar with and accustomed to scaling up. Pathway and metabolic engineering is widely used in the biotechnology industry [125], and this may well overcome some of the challenges noted. Further, the path to industrial production for both source microbe-derived and novel construct-derived products can be made more certain and faster, by applying a combination of best industrial manufacturing practice for new fermentation products, together with novel in process real-time monitoring and multivariate analysis techniques [111, 126]. These techniques would enhance the flow of process data in early development phase and put the physiology of these marine microbes and constructs on a sounder basis, hence ensuring the acceleration of industry exploitation [111, 126], faster delivery of marine products to markets and safer and more predictable scale-up.

4.4 Challenge 4: Legal Aspects Relating to Access to Marine Bioresources

Bioprospecting can be defined as commercially focused research and development that uses naturally occurring compounds. It includes steps from first discovery, through patenting, improvement, development and commercialization. A simple breakdown of bioprospecting is as follows: phase 1, on-site collection of samples; phase 2, isolation, characterization and culture of specific compounds; phase 3, screening for potential uses, such as pharmaceutical or other uses; and phase 4, product development and commercialization, including patenting, trials, sales and marketing [127]. Bioprospecting using a country's genetic resources is covered by the United Nations Convention on Biodiversity (the CBD) [128]. This will extend to a coastal country's exclusive economic zone (EEZ) and its continental shelf, as defined by the United Nations Convention on the Law of the Sea (UNCLOS—article 56(1) and article 77(1)). However, there is no international treaty that regulates bioprospecting in the water column above the continental shelf or in areas beyond national jurisdiction ("the deep sea", UNCLOS article 87(1)). Instead, each state is required to regulate the activities of its nationals in those areas, particularly with concern to avoid environmental damage. Aspects of the regulatory framework may distinguish between bioprospecting (as defined above) and the undertaking of scientific research without commercial motive [127]. To summarize the present legal position in relation to marine bioprospecting:

Coastal states have the sovereign right to allow, prohibit and regulate marine bioprospecting and/or scientific research in the water column of their EEZ and on the seabed (including the subsoil) until the farther of either the limits of their EEZ or the outer edge of their continental shelf.

State regulation is subject to a number of international obligations incumbent upon coastal states, including in relation to the protection and preservation of the environment and to the conservation and sustainable use of marine genetic resources. Significantly, such regulation may also be impacted by access and benefit-sharing mechanisms established pursuant to the CBD.

All states enjoy free access to marine genetic resources located seaward of other states' EEZs and continental shelf. They have jurisdiction to allow, prohibit and regulate marine research and bioprospecting activities conducted by their nationals and/or vessels flying their flags; free access is subject to a number of international obligations incumbent upon coastal states, including in relation to the protection and preservation of the environment and to the conservation and sustainable use of marine genetic resources. Significantly, such free access is also subject to the duty of states to cooperate for the conservation of marine genetic resources. The mechanisms of benefit sharing and the related legal aspects of research on marine bioresources are a very important aspect of this project in collaboration with other marine biotechnology programmes that include PharmaSea and MicroB3. Many marine ecosytems are still little studied, but

their vast and novel biodiversity offers many possibilities for the discovery and development of novel industrial products.

In spite of considerable previous work, particularly the CBD, many aspects remain unresolved. The discussion of equitable benefit sharing among interested parties often gets stuck because it tends to focus on percentages of a future income from possible blockbuster products. Another equally important aspect is to evaluate and discuss the mechanisms that can be used for more short term, more secure and non-monetary ways of benefit sharing from bioprospecting activities, as is highlighted in the Nagoya Protocol to the CBD. These are particularly important and relevant when it comes to sampling and research on novel ecosystems and unusual natural phenomena, particularly in the world oceans since they are still more underexplored than on land. SeaBioTech addressed the legal aspects in a concise way doing a direct evaluation of the legal and access issues connected to sampling in the project itself. Another task was to find and study some key cases of this sort that have come up recently, in particular in relation to novel marine ecosytems. Two such examples are the smectite geothermal cones north of Iceland and the Tufa columns in Greenland. SeaBioTech worked with other marine biotechnologically oriented projects to assist in the interpretation and application of best practice and conforming to current national, European and international legislations as well as the most recent Nagoya Protocol.

In addition to the close liaison maintained with the other KBBE Bioprospecting projects, SAMS, acted as a link between SeaBioTech and the ESFRI road map Research Infrastructures (RIs): EMBRC and MIRRI (MIcrobial Resource Research Infrastructure). This has involved relevant CBD related input to the development of the H2020 EMBRIC project. SAMS has also been responsible for providing advice to the government of the Republic of the Seychelles on building a Blue economy, including the need for managing access to MGR.

4.5 Challenge 5: Improving Access to Marine Biotechnology Data Through an EU Platform

As highlighted in the recent position paper "Marine Biotechnology: A New Vision and Strategy for Europe" (European Science Foundation, September 2010), there is a need for a "central European information portal, which provides a one-stop-shop for state-of-the-art reports on novel discoveries and success stories, challenges and applications". Currently, there are few sources of comprehensive information relevant to marine biotechnology. The Coordination and Support Action Project under FP7, Marine 4Genomics Users, created a "single entry-point to marine genomics knowledge". However, this did not encompass information relating marine samples to bioactivity test results, comparable to the USA's NIH Roadmap initiative with results being openly available in the PubChem BioAssay database.

For general information on marine biodiversity, there is the National Ocean Service, which is run by the US government agency, the National Oceanic and Atmospheric Administration, and there is MarineBio in the USA, which is a non-profit organization that tries to provide a broad range of information relating to marine conservation and science. However, neither covers details of species in particular environments or bioprospecting information. For extremophiles, there is a developing resource hosted by the Indian organization, the Institute of Genomics and Integrative Biology, although this is not focused on marine species and does not cover bioprospecting. As described earlier, under challenge 1 (quality of marine resources), there is also very limited access to physical samples from marine environments. Hence, SeaBioTech developed and established both an information portal and a physical repository of samples for further genetic analysis and for use in additional bioactivity testing. SeaBioTech activity complemented other EU-funded projects such as FP5 MarGenes, FP6 Diatomics, FP6 Marine Genomics Network of Excellence, FP7 Micro B3 (Biodiversity, Bioinformatics, Biotechnology) and FP7 MAREX. It also linked to other projects funded under the present call. In that way, SeaBioTech provides a major contribution in achieving another recommendation of the ESF's position paper on marine biotechnology towards the creation of a virtual European Marine Biotechnology Institute.

SeaBioTech has provided input to the PharmaSea case studies: Role of biorepositories and impact of proposed EU regulation on ABS; the European blue biotech community's preparedness and response to the implementation of the Nagoya Protocol.

5 Conclusion: Impacts and Future Insights

In this section, we summarize the project's achievements to answer the challenges set by the consortium. The achieved milestones along with the encountered confrontations and some strategies used to yield to the challenges set by the SeaBioTech consortium are presented on Table 12.1.

5.1 A Reproducible Quality of Marine Resources

Addressing the first challenge on quality of marine resources collected during the project's lifetime, the consortium was given the opportunity to investigate some of the unique environments/habitats on earth, isolate/characterize microbial species living there and create large strain collections for biotechnological exploitation. Some of the isolated strains were characterized by high novelty and biotechnological potential as they showed very low similarity with any other previously characterized bacteria. New knowledge was gained about gene diversity in extreme environments, as well as valuable information about environmental microbial functioning through

Table 12.1 Achieved milestones along with the encountered confrontations and some strategies used to yield to the challenges set by the SeaBioTech consortium

Milestones achieved to support project challenges	WP	Encountered confrontations and some strategies used to yield to the challenge
1. A reproducible quality of marine resources		
Forty bacterial extremophiles were prioritized from a collection of *ca.* 3000 strains	WP2, WP5	Prioritized isolates were recollected at the same seasonal period of the initial collection for replication purposes for the repository
Five best positive hits were identified during primary screening	WP3	Variation of chemical composition was encountered due to subtle changes in the laboratory conditions, which was monitored by metabolomic profiling
2. An improved and integrated technology for drug discovery		
Availability of SOP for fraction dereplication, metabolomic profiling and purification	WP5	Metabolomic and bioactivity profile preceded isolation work on prioritized extracts for the pipeline
Construction of insertion modules and expression plasmids finished	WP4	Alternative expression systems or other systems for refolding of the proteins were used
Small-to-medium scale cultivation optimized	WP5, WP6	Culture of each organism was cultivated under a variety of conditions that is metabolomic-guided to ensure replication of the original chemical profile or improvement in the concentration of the active constituents
Biologically active compounds isolated and identified	WP5	If the selected targets were not affordable in the project time frame, suitable alternatives were selected as biology-driven construction of simpler assay models
3. A sustainable mode of supply of raw materials for the industries		
Industrial-scale cultivation optimized	WP7, WP10	Mitigation of risk by metabolomics analysis and re-prioritization of strains, i.e. selection of alternative lead strains
Carbohydrate structure data from mutants	WP4, WP6	Targeted gene transfer ensured close link between genetic changes to strains and subsequent polymer structure and function
4. A harmonized legal position on marine bioprospecting		
Legal aspects harmonized	WP8	The availability of additional academic expertise was enlisted
Central EU platform	WP1	A common board with BlueGenics, PharmaSea, Macumba and SeaBioTech was set up
5. A centralized biobank repository and database of information		
Establishing a metabolomics and metagenomics database. The repository contains 3209 strains, 1140 crude samples and 606 fractions plated in ready-to-screen format and 63 pure compounds	WP2, WP5, WP4	Genomic/metagenomics mining is iterative in nature: further rounds of sequencing generated leads were supported by metabolomic and bioactivity profile

the application of modern metagenomic deep-sequencing techniques. Genomic sequence data by UWUERZ has revealed the presence of a large fraction of putatively silent biosynthetic gene clusters in the genomes of actinomycetes that encode for secondary metabolites that remain silent under standard fermentation conditions. Our work has provided here novel insights into actinomycete biodiversity as well as into the effects and consequences of elicitation of secondary metabolism in actinomycetes. Huge metagenomics datasets were created and used as a source for bioprospecting. WP2 served as the foundation of SeaBioTech discovery pipeline. By focusing on previously unexplored environments, WP2 attempted to increase the odds of discovering novel bacterial species that would contain novel bioactive compounds of potential economic interest. Indeed, WP2 supplied the other work packages with novel cultivable strains holding a great potential for the discovery of novel natural products of high-added value. In addition, through SeaBioTech sampling campaigns, knowledge on the activity of the extreme environments of the Hellenic Volcanic Arc was exploited demonstrating the need of a monitoring programme for this dangerous environment [18].

5.2 An Improved and Integrated Technology for Drug Discovery

For the improvement in technical aspects, SeaBioTech integrated metabolomics-assisted methodology with systems biology and functional bioassays increasing the ability to divulge positive hits that proved to be affordable, innovative and efficient method [79] to separate, elucidate the structure and identify the bioactive metabolites. Novel and underexplored species of marine microorganisms were investigated for the first time as potential sources of novel therapeutics, and they provide positive indications that lead compounds can be isolated and progressed to address significant unmet medical needs (e.g. cancer, infections against, metabolic syndrome and inflammation) and threatening parasitic infections for aquaculture. WP3 partners in charge of the screening activities improved the performance and throughput of the assays, to comply with the requirement to process a remarkably high number of extracts, fractions and compounds of marine origin. Major improvements were obtained for the development of automated, high-throughput screening platform to provide cell-based assays for the detection of hits with anticancer activities, in particular for cell proliferation (HDL). Moreover, assay systems were modified to achieve a suitable robustness to screen complex marine extracts and subsequently to produce more accurate and reliable results (SIPBS, AXXAM).

The personalized medicine market worldwide is estimated to be over 400 billion euros, and the core diagnostic and therapeutic segment of the market are estimated at over 40 billion euros. The need to address this market and the benefit of doing so are supported by many facts, including a 75% increase in personalized medicine investment over the last 5 years, and 30% of all pharma companies now require compounds

in R&D to have patient-relevant treatments. The potential novel marine products identified through the SeaBioTech consortium may enable such therapeutics to progress through the R&D process. In particular, prospective lead compounds have been isolated with a potential to address therapeutic indications for human health such as cancer, bacterial infections and metabolic syndrome and to develop an effective treatment against the fish parasite *L. salmonis*, which represent a major threat for aquaculture. In addition, the knowledge gained through SeaBioTech concerning assay development and screening of complex marine extracts may directly or indirectly translate into new opportunities for the CROs to expand their potential market and for pharmaceutical and life science companies to undertake novel R&D projects. In addition, the phenotypic assay performed on the fish parasite of aquaculture plants *Lepeophtheirus salmonis* was also optimized to increase its capacity and processivity, thereby expanding the possibility to screen extracts and fractions of marine source (PHARMAQ). The lead compounds isolated at the end of the SeaBioTech collaboration have the potential to be evolved into novel therapeutics. The availability of novel therapeutics for human health and aquaculture will directly contribute towards improving quality of life, health, employment and economic strength.

Automated dereplication and chemical profiling aid screening for diversity and novelty were established in WP5. Marine invertebrate-associated symbiotic bacteria produce a plethora of novel secondary metabolites, which may be structurally unique with interesting pharmacological properties. Selection of strains usually relies on literature searching, genetic screening and bioactivity results, often without considering the chemical novelty and abundance of secondary metabolites being produced by the microorganism until the time-consuming bioassay-guided isolation stages. The development of a comprehensive metabolomics workflow pathway including an in-house developed Excel macro embedded with a database made it possible to rapidly dereplicate higher number of strains, providing putative identities of known metabolites in each extract. It is also shown that the dereplication results can also be correlated with bioassay screening results to support drug discovery efforts with the objective of both finding a bacterial isolate that has a unique diverse chemistry and is biologically active. Overall, this shows that metabolomics approaches are worthwhile for the selection of strains for the isolation of novel natural products and that this methodology reduces redundancy in drug discovery programmes. Additionally, we have shown through PCA and heat map analysis that strains with nearly identical 16S rRNA sequences do not necessarily produce the same secondary metabolites.

Metabolomic-assisted isolation of target compounds efficiently improved the purification of the bioactive secondary metabolites. Multivariate analysis that included principal component analysis (PCA), hierarchical clustering (HCA) and orthogonal partial least square-discriminant analysis (OPLS-DA) were used to evaluate the HRFTMS and NMR data of crude extracts from different fermentation approaches. Statistical analysis identified the best culture one-strain-many compounds (OSMAC) condition and extraction procedure, which was used for the isolation of novel bioactive metabolites. As a result, new natural products can be isolated from cultivated broth cultures (described under Sect. 4.2.2). New natural products with novel mechanisms of actions were isolated. Biologically active

compounds were isolated and purified from prioritized strains. SBT345 (*Streptomyces* sp.) showed antioxidant, anticancer cell lines (DLD-1, HCT116) activities and some activities in the enzymatic reactions. Compounds SBT1620 (phencomycin), SBT1621 (tubermycin B), SBT1186 (benzethonium) and SBT1187 (ageloline A, new compound) were isolated from SBT345. SBT1877 showed antioxidant and anti-*Chlamydia trachomatis* activities. SBT017 (*Rhodococcus* sp.) yielded 16 pure compounds after scale-up, one of which was elucidated as isohalobacillin B. SBT0027 (*Vibrio splendidus*) yielded 27 pure compounds, 7 of which are bisindole analogues with strong to medium potency against *Mycobacterium marinum*. Three analogues are new. Other pure compounds from SBT0027 consisted of diketopiperazines, long chain amines and hydroxylated fatty acids, the activities of which still need to be determined. SBT167 (*Polysiphonia lanosa*), an algal macro-epiphyte, yielded the di-bromo-dihydroxylated-benzaldehyde as its major component. SBT167 was found to be active against parasitic sea lice and in several enzymatic assays against metabolic diseases. From the Icelandic collection, new BHA congeners bioactive against metabolic diseases were isolated.

5.3 A Sustainable Production of Raw Materials

The last technical brick for the industry is the sustainability of the production of raw materials not only at lab scale but also at industrial scale. The programme has developed standard operating protocols for the growth and exploitation of resources from both natural isolates and construct microorganisms, developed by identifying, isolating the genes of interest from marine species and inserting them into organisms which are regarded as industry work horses, e.g. *E. coli.* Scale-up predictions for processes developed in WP10 were formulated by the fermentation group in SIPBS. Accelerated process development has been achieved either by utilizing powerful gene technologies to create construct organisms or by utilizing bioprocessing techniques with metabolomics with source microorganisms to identify bottlenecks in the relevant catabolic pathways. Both of these techniques resulted in successful bioprocess intensification of the relevant target compounds or enzymes. Industrial partners identified appropriate target compounds, which allowed us to selectively mine the gene pool of the marine organisms for useful enzymes. Suitable protocols were then generated for the bioprocess and put together in a process manual.

Combining the novel gene technologies, metabolomics and ability to rapidly scale processes, using clearly defined standard operating procedures, is the unique aspect of the programme. This is of particular interest to industrial partners and significantly benefits both the companies involved in SeaBioTech and the scientific community in general. Many of the techniques can now be regarded as generic and could be exploited elsewhere on other projects and processes. Genes from source organisms, which express novel enzymes, have been successfully inserted into industry workhorse organisms and have been successfully scaled up. Such enzymes have novel capabilities and are successfully utilized by some industry partners. In

particular generating new construct microorganisms has allowed the exploitation of enzymes, e.g. alginate lyases and thioesterases, to name but two, capable of utilizing different kinds of feedstocks and which allow processes which previously suffered from bottlenecks to work effectively and efficiently. This is a significant scientific breakthrough as the potential for industry is great. A novel polymer was isolated from *Colwellia* sp. The organism has been successfully grown at scale in WP7, and a spin-off project has developed between SAMS and Unilever. New bioactive compounds have been identified (WP3) and tested at scale in WP7. Initial trials have shown the organisms from which the bioactives are isolated can be grown at scale but research to improve the productivity of the bioactives continues.

The generation of new enzymes and polysaccharides will have considerable influence on the economies of the consortium partner companies and on the economy of the EU and also on global markets. The enzymes in particular have significant industrial capability, and applications will be numerous. The ability to use new substrates, previously un-useable either because it was not scientifically possible or because process economics were not favourable, will have significant impact on increased process efficiency, improved supply chains (substrate choice increases) and reduction in upstream costs. As seen above, impact will not just be industrial as IGZ sees significant potential in the healthcare market where opportunities in drug discovery from marine-derived biocatalysts are highly relevant to the biosynthesis of compounds for the treatment of disease. The market share for companies who use SeaBioTech-derived enzymes and compounds could expand rapidly.

5.4 A Harmonized Legal Position on Marine Bioprospecting

SeaBioTech liaised closely with, and contributed to, common areas of activity dealing with legal/ethical aspects being undertaken in the parallel EU-funded projects: MICROB3, BlueGenics and PharmaSea. An overarching group of experts was formed, i.e. the Advisory Panel of Policy and Legal Experts (APPLE). APPLE, an advisory board, brought together the breadth of experience, legal, scientific and commercial, necessary to address the critical policy and legal barriers which currently hinder progress in innovative marine biotechnology in Europe. The projects have worked together on these aspects to avoid duplication of effort and enable a wider-reaching and more global approach of benefit to these consortia and beyond. During the lifetime of the project, the legal implications to bioprospecting have changed status with the implementation of the Nagoya protocol, which became legally binding from 12 October 2014. An overarching, generic Material Transfer Agreement (MTA), conforming to the requirements of the Nagoya Protocol, has been developed by Microbio3. This has, with minor adjustments, been applied across the projects. SeaBioTech contributed to the development, structure and content of the PharmaSea deliverable on development of web-based, interactive, toolkit to assist marine genetic resource (MGR) practitioners in navigating the

different legal and policy regimes involved in access to MGR and associated benefit sharing. This area has rapidly developed, and online resources associated with the CBD Clearing House are available to users/potential users of biological resources. Work undertaken by APPLE, particularly the PharmaSea legal team, has resulted in considerable progress with respect to the developing of possible solutions to the implications of the collection of materials in areas beyond the economic exclusive zone (EEZ), i.e. in areas beyond national jurisdiction (ABNJ). These were presented at the UN HQ, New York, on 16–20 June 2014 for consideration for possible future proposed changes to the UN Convention on the Law of the Sea (UNCLOS).

5.5 A Centralized Biobank Repository and Database of Information

SeaBioTech created a centralized tool to organize the marine biodiscovery pipeline through a biobank repository and database of information for marine strains which included names of the identified marine organisms, compounds and extracts, their bioactivities, the cutting-edge methods in identification and elucidation and metabolic engineering to be further used for industrial purposes with all related procedures on legal process for companies, academia and legal authorities. The assembly of a centralized repository of marine extract and compounds of marine origin was among the major legacy of SeaBioTech. The centralized repository contains at the end of the project 3209 samples of marine origin, including 1140 crude samples and 606 fractions plated in ready-to-screen format and 63 pure compounds. In addition, the repository contains samples which were received in a too small amount for general screening. Thus, they were stored and annotated in case further sample is obtained to ensure sufficient material is available for assaying. The annotation of samples, fractions and pure compounds stored in the centralized repository was managed through a database implemented by SIPBS and accessible in a secure manner through the SeaBioTech Portal to all partners involved in sampling, screening and dereplication activities (http://spider.science.strath.ac.uk/seabiotech/index.php). The SeaBioTech database sample submission portal ensures tracking of samples and transfer of data between partners ensuring CPD compliance. The detailed mechanisms to ensure access to the biological resources, and their associated data, beyond the lifetime of the project will be agreed and implemented over the next 6–10 months. Each sample was assigned a unique SeaBioTech code, and all information associated to each sample related to parental microorganism, genomics, LCMS, NMR data, bioactivity results and pharmacological profiling generated during the SeaBioTech collaboration was entered into the database. In addition, each sample was connected to its relevant negative control sample (e.g. culture media) that enabled validation and correct analysis of potentially active entities during bioactivity screening. The database played an essential role on the prioritization of samples, fractions and compounds for the SeaBioTech pipeline and

represented a valuable asset for the prospective exploitation of the results obtained by SeaBioTech. The repository of extracts, fractions and pure compounds derived from underexplored marine microorganisms and the related information managed by the centralized database represents a valuable infrastructure for future R&D projects in diverse life science areas.

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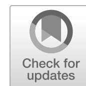
Chapter 13
BluePharmTrain: Biology and Biotechnology of Marine Sponges



Georg Steinert, Carla Huete Stauffer, Nele Aas-Valleriani, Erik Borchert, Agneya Bhushan, Alexandra Campbell, Maryam Chaib De Mares, Margarida Costa, Johanna Gutleben, Stephen Knobloch, Robert Gregory Lee, Stephanie Munroe, Deepak Naik, Eike Edzard Peters, Ellen Stokes, Wanlin Wang, Eydís Einarsdóttir, and Detmer Sipkema

1 Introduction

1.1 BluePharmTrain

BluePharmTrain is a multidisciplinary alliance of 17 European academic and industrial partners working in collaboration to train young scientists in a multitude of aspects of blue biotechnology (www.bluepharmtrain.eu). It has been funded as a Marie Curie Initial Training Network that started on 1 September 2013 and will end

Authors "Georg Steinert" and "Carla Huete Stauffer" contributed equally to this work.

G. Steinert · J. Gutleben · D. Sipkema (✉)
Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands
e-mail: detmer.sipkema@wur.nl

C. H. Stauffer
Marine Ecology Department, Centre d'Estudis Avançats de Blanes (CEAB), Consejo Superior de Investigaciones Científicas (CSIC), Blanes, Spain

N. Aas-Valleriani
Tallinn University of Technology, Tallinn, Estonia

E. Borchert
School of Microbiology, University College Cork, National University of Ireland, Cork, Ireland

A. Bhushan · E. E. Peters
Institute of Microbiology, Eidgenössische Technische Hochschule Zürich, Zürich, Switzerland

A. Campbell
School of Biosciences, Cardiff University, Cardiff, UK

M. C. De Mares
Microbial Ecology Group, Genomic Research in Ecology and Evolution in Nature (GREEN), Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands

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on 31 December 2017. The focus of BluePharmTrain is using marine sponges as source for the development of new pharmaceuticals. We focus on marine sponges since they have been long recognised as the most prolific source of novel secondary metabolites in the marine environment. More than 7000 bioactive molecules have been reported to date, and additional compounds are being discovered every year [1]. In addition, sponges are known to host extremely diverse microbial communities that, in some cases, can account for up to half of the sponge's body mass [2]. Given that many of the secondary metabolites, which have been found in sponges, are believed to be made by symbiotic microbes, the development of a variety of methods to obtain substantial quantities of sponge-derived bioactives will be important to get more of these bioactives into clinical trials (Fig. 13.1).

Therefore, BluePharmTrain has the following key objectives:

- Establish routines for the isolation of sponge-specific microorganisms by integrating novel high-throughput cultivation strategies and state-of-the-art genomics and transcriptomics
- Establish sponge cell cultures of target species by translating ecology into technology
- Develop heterologous expression tools for sponge-derived bioactives

In order to achieve these goals, the BluePharmTrain research and training network provides a complementary set of experimental and conceptual network-wide training modules and workshops to 15 young researchers. The training includes both scientific content related to subjects in cell biology, microbiology, natural product chemistry, (meta)genomics and (meta)transcriptomics (omics) and socio-economics, which are complemented with transferable skills, such as entrepreneurship and scientific ethics. In the following sections, we describe the state of the art in sponge biotechnology including the BluePharmTrain achievements.

M. Costa
Faculty of Pharmaceutical Sciences, University of Iceland, Reykjavík, Iceland

S. Knobloch
Department of Research and Innovation, Matis ohf., Reykjavik, Iceland

R. G. Lee · E. Stokes
Birmingham Law School, University of Birmingham, Birmingham, UK

S. Munroe
Division of Bioprocess Engineering, Wageningen University, Wageningen, The Netherlands

Harbor Branch Oceanographic Institute, Florida Atlantic University, Boca Raton, FL, USA

D. Naik
Department of Microbial Ecology and Diversity Research, Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

W. Wang
Sustainable Place Research Institute, Cardiff University, Cardiff, UK

E. Einarsdóttir
Pharma Mar, S.A., Madrid, Spain

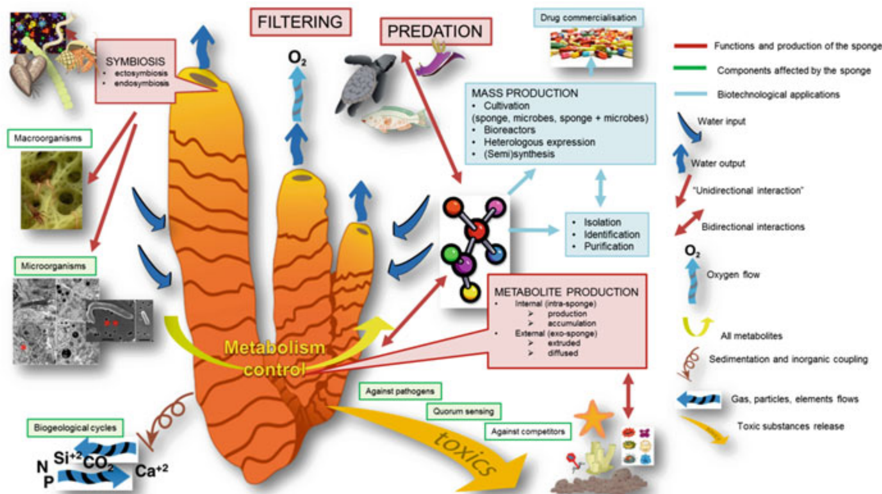

Fig. 13.1 Schematic vision of the sponge holobiont roles in the ecosystem and the metabolite production detailing which parts the BluePharmTrain project addresses. Sponges filter the surrounding water; they can shelter macro- and microorganisms which may become symbionts; they participate in biogeochemical cycles; they harbour symbionts that produce metabolites of interest as a result of metabolism control or for defence purposes. Adapted from Maldonado [3], Ahn et al. [4]

1.2 Origin and Structure of Sponges

Sponges (Phylum Porifera) are a broad taxonomic group of animals comprising approximately 8500 species that are classified into four distinct groups, the Hexactinellida (glass sponges), the Demospongiae (common sponges), the Calcarea (calcareous sponges) and the Homoscleromorpha, whose phylogenetic position was settled as a separate class only recently [5]. Over 80% of the described sponge species belong to the Demospongiae [6, 7]. Sponges have a critical role in the search for the origins of multicellular animals (Metazoa), as they are generally recognised as the simplest and one of the oldest surviving metazoan lineages. The phylogenetic relationship of sponges to other animals has been a source of long-standing debate [8, 9]. Phylogenetic analyses have placed sponges as the earliest diverging animal lineage (Fig. 13.2), but recent reports have instead suggested Ctenophora (comb jellies) as the earliest diverging animal lineage [10, 11]. However, it is clear that at least 640 million years ago sponges branched off from other metazoans [12] and that the evolution of Metazoa from unicellular/colonial organisms occurred some 1300–600 million years ago in the pre-Ediacaran period [8].

Sponges have two different lifestyles. Their larvae are motile, but they fix to a surface and develop as adults in a sessile (non-motile) form [13]. Sponges are filter feeders and use small pores (ostia) in their outer walls (pinacoderm), through which surrounding seawater is drawn and ejected from the larger opening at the top

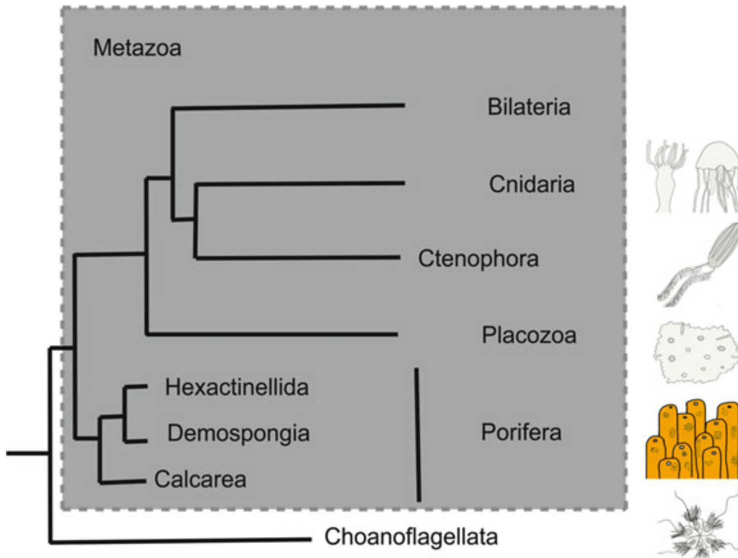

Fig. 13.2 Simplified phylogenetic tree showing where sponges are in the tree of life, showing also their closest related animals

(osculum) [14]. Specific types of sponge cells perform particular tasks and functions within the sponge (Fig. 13.3). For example, the water is flowing in one direction through the sponge through the coordinated movement of the flagella of the choanocytes (flagellated cells) in each of the chambers connected by a series of aquiferous canals. Once passing through the choanocyte layer, the particulate matter is engulfed by the amoebocyte cells. Microorganisms that pass this filter remain in the mesohyl, the inner sponge tissue. It is in the mesohyl that intact microbial cells potentially engage in symbiotic interactions with the sponge [15] (Fig. 13.4).

1.3 Microbial Associations with Sponges

Marine sponges belong to the most ancient living Metazoa holding a basal position in the metazoan phylogenetic tree [16, 17]. Sponge-like body fossils from ~580 to 600 million years ago place the earliest evidence of Precambrian sponges into the Ediacaran period, well before the Cambrian radiation event [12, 18]. Moreover, immunological evidence based on shared immunological responses of bacteria from different sponges also suggests that the origin of bacterial symbiosis dates back ~600 million years ago [19], which would make sponge-microbe associations one of the most ancient relationships between animals and microorganisms known to date [2]. It is now established that marine and freshwater sponges harbour dense and

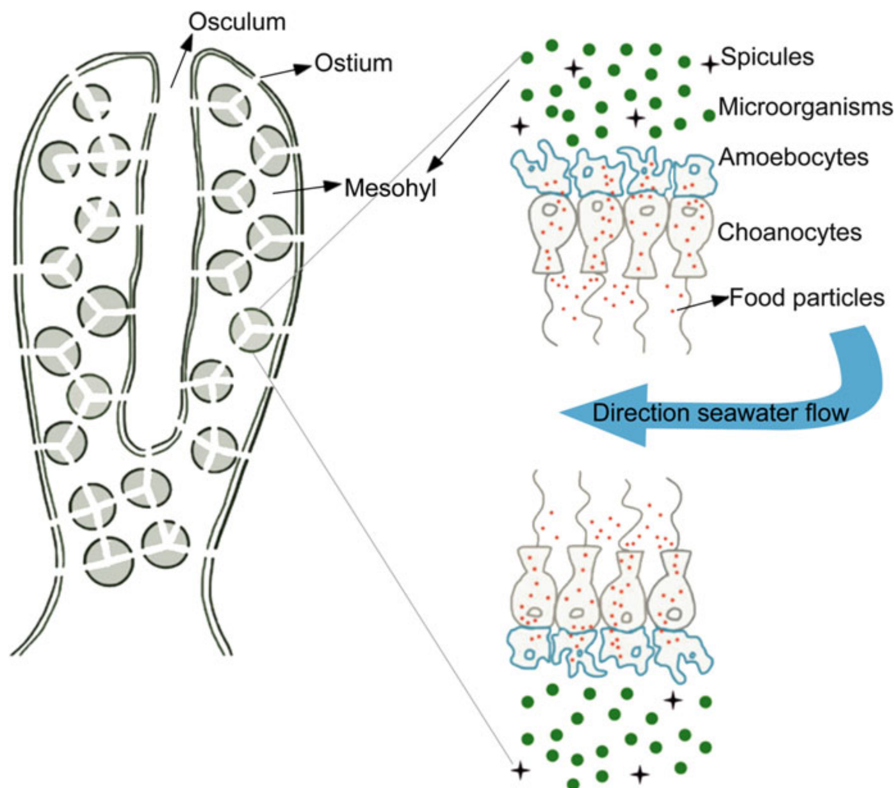

Fig. 13.3 General body structure and main cell types of a syconoid sponge. Detail of the water flow through the sponge channels on behalf of the flagella of choanocytes that are in the ostia (plural of ostium) and oscula (plural of osculum) together with a simplified description of the cell layers. Microorganisms can be embedded in the mesohyl of the sponge together with other structures of the sponge as spicules

diverse microbial communities, encompassing all three domains of life including Bacteria, Archaea and Eukarya [14].

Hitherto, sponges were found to associate with up to 47 prokaryotic phyla, including *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Crenarchaeota*, *Cyanobacteria*, *Firmicutes*, *Nitrospirae*, *Proteobacteria* (Alpha-, Beta- Gamma, Delta-), *Spirochaetes* and several candidate phyla such as *Poribacteria*, PAUC34f, or the sponge-associated unclassified lineage (SAUL) as the most abundant taxa [14, 20, 21].

Despite the circumstance that prokaryotic microorganisms are a major food source for the filter-feeding sponges [22], early electron microscopy and bacterial cultivation studies indicated that sponges feature distinctive permanent bacterial populations within their extracellular mesohyl matrix (Fig. 13.4), which could either harbour relatively dense bacterial assemblages making up to up to 38% of the sponge biomass or were lacking large aggregations of bacteria within the sponge host [23–25]. In

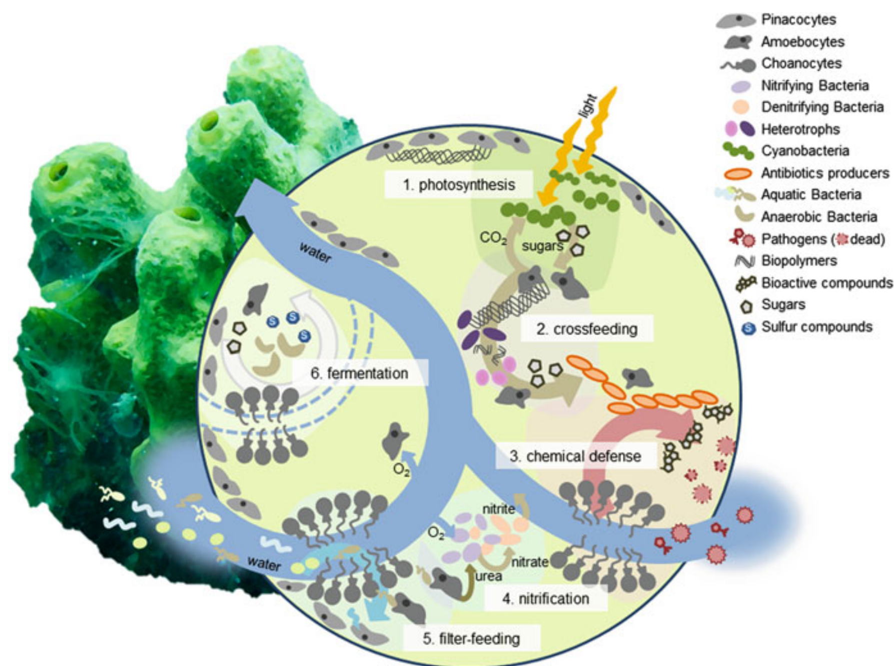

Fig. 13.4 Diagram of the components and their possible functions in a marine sponge microbial-associated community, showing as where the production of secondary metabolites can take place. 1. Photosynthesis: some associated microorganisms as cyanobacteria or microalgae can apport important carbon sources to the sponge as well as increase the oxygen concentration in certain areas of the sponge, creating as well microhabitats. 2. Cross feeding: not all microorganisms use the same sources of food, so the excreted substances of some may be the source of others, including the sponge cells. 3. Chemical defence: to avoid the expansion of pathogens, to control and balance the associated community, to eliminate or combat pathogenic components and many more, the associated community can produce and secrete all sort of antibiotics that are those of interest in the search of novel drugs. 4. Nitrification: the sponge is a per se combination of habitats on behalf of the channels and cell layers, and some of the associated microbes can utilise the ammonia from the sponge as a source for the transformation into nitrogen-based compounds and make them available. 5. Filter feeding: sponges filter water in high volumes, and not all what is filtered is a source of food or becomes a symbiotic partner; in any case, the sponge eliminates from the surrounding water a large number of microorganisms. 6. Fermentation: it is an anaerobic process, carried out in certain compartments of the sponge that remain in detriment of oxygen

some sponge species, the density of bacterial populations reach 10^8–10^{10} bacteria/g of sponge wet weight, whereas other sponge species comprise bacterial densities between 10^5 and 10^6 bacteria/g of sponge wet weight, which mirrors roughly the bacterial concentrations in seawater [26]. This apparent disparity of microbial population densities amongst different sponge species led to the empirical division into ‘bacteriosponges’ or high-microbial-abundance (HMA) and low-microbial-abundance (LMA) sponges [26, 27]. Recent quantitative approaches demonstrated the differential distributions of certain prokaryotic taxa that belong to ‘sponge-associated

phyla' such as *Chloroflexi*, *Actinobacteria*, *Cyanobacteria* and *Poribacteria* between HMA and LMA sponges [28–31]. Apparently, this division into high- and low-microbial-abundance (HMA and LMA) sponges does not follow any discernible phylogenetic patterns [32].

One key characteristic of sponge-microbial associations is that specific prokaryotic taxa are enriched in sponges, while they are absent or only present in low abundances in the surrounding seawater [2, 33]. In fact, phylogenetic reconstructions based on more than 7500 sponge-derived 16S rRNA gene sequences suggested that a certain proportion of the sponge-associated microbiota can be divided into 173 globally distributed sponge-specific monophyletic clusters [34]. Nonetheless, screenings of short 16S rRNA gene sequence reads derived from various different marine environments (e.g. sediment, seawater or hydrothermal vents) against the 173 sponge-specific clusters revealed that parts of the categorised as 'sponge-specific' prokaryotes can be found in very low abundances within those various marine ecosystems [21, 35]. These results underlined the general perception that the taxonomic composition of sponge-associated microbial communities can be clearly differentiated from the communities present in the surrounding seawater and sediment (e.g. [23–25, 33, 36–38]).

Besides the prevalence of certain taxa and the presence of sponge-enriched or sponge-specific clusters, another major characteristic of sponge-prokaryotic associations is the influence of host identity in the composition of such assemblages, being the identity of the symbionts presumably determined by the identity of the sponge host (e.g. [21, 39–41]). Sponge host phylogeny appears to determine in some way the diversity of the prokaryotic community [21]. In general, the composition of sponge symbiont communities seems to be characterised by prevalent specialists (i.e. present only in one or a few sponge species) and generalists (i.e. found amongst many sponges). The latter group of generalist or cosmopolitan prokaryotes are primarily present in the core sponge microbiome (i.e. the group of prokaryotes consistently present amongst all individual sponge hosts), while specialists are underrepresented [21].

The intriguing specificity and stability of sponge-prokaryotic relationships raises the question about how those associations are established and maintained. The two main modes of symbiont transmission from one sponge generation to another are vertical and horizontal transmission [2] (Fig. 13.5). Horizontal transmission is the mode where the sponge host acquires the free-living symbiont from the environment, hence the surrounding seawater. Evidence for the existence of horizontal transmission in sponges occurs in the form of phylogenetically highly related sponge-associated bacteria which were found in geographically distant sponges [26, 42]. Moreover, sponge-specific sequences were also found in the surrounding seawater [35, 37]. This indicates that sponge-specific microbes in the marine environment provide a 'seed bank' for a later uptake by sponges and subsequent colonisation. In fact, members of the candidate phylum *Poribacteria* appear to be rare and inactive in the surrounding seawater [30]. Conversely, vertical transmission represents the transfer of the symbiont via the gametes or larvae of the sponges. Evidence for this mode of transmission derives from a large number of studies that

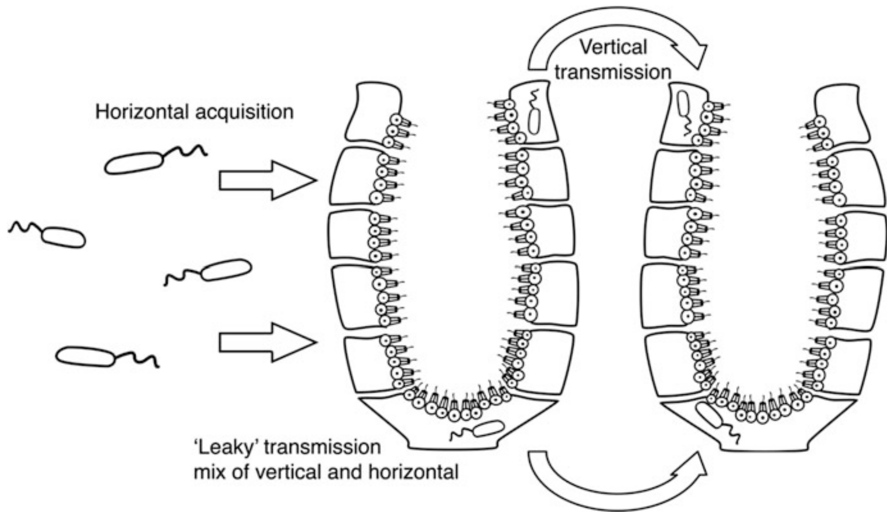

Fig. 13.5 The three transmission modes for the establishment and maintenance of sponge-associated microbes. Horizontal transmission concerns an uptake from the environment, vertical transmission refers to that passed through offspring's and 'leaky' transmission has a shared vertical transmission with an uptake from the environment that may or may not be transferred amongst generations or could be modified between generations

found bacteria and also archaea in various sponge reproductive stages (e.g. [43–49]). Finally, the mixture of both processes, vertical and horizontal transmission, is the so-called leaky vertical transmission [50, 51]. The first molecular evidence for this mixed transmission mode was observed in the Mediterranean sponge *Petrosia ficiformis* [52].

The functions of the sponge microbiome apparently comprise a large variety of interactions, beneficial or harmful, which range from being a source of food, over parasitism and microbial pathogenesis, to mutualism and commensalism [51]. The knowledge on symbiont functions is still limited to date, since most of the highly abundant sponge symbionts remain still uncultivated [2]. Bacteria inside sponges seem to harvest and distribute precious nutrients from the water column and distribute them in bioavailable forms to other members of the reef community in the postulated 'sponge loop' [53, 54]. Thus, sponge-microbe symbioses contribute to resolve the so-called reef paradox, which debates how the world's most diverse and productive ecosystems can exist in such extremely nutrient poor waters. For example, the nitrification rates of some sponges and their symbionts can result increased compared to those of the surrounding environment [55, 56], as *Nitrospirae* and *Thaumarchaeota* which are known as nitrifiers. Metabolism measurements and metagenomics studies point out that nitrogen cycling processes [57, 58], sulphur cycling [59] and vitamin biosynthesis (e.g. [21, 60–63]) can be mediated by bacterial sponge symbionts (Fig. 13.4). In general, symbiont 'quality' rather than quantity seems to be determining the advantageous effect for the host [64]. Metabolically

versatile microbiomes may allow seemingly competing sponge species to exploit unique physicochemical niches within marine ecosystems [65]. In many cases, convergent evolution of symbiont functions is becoming evident, i.e. identical functions such as nitrification and dissolved organic carbon uptake rates are mediated by different, sponge host-specific microbial communities in phylogenetic divergent sponge species [21, 66].

To conclude, symbiosis between sponges and their associated microbes is ancient and extremely intricate (Fig. 13.4). The combination of the sponge (macroorganism) and the symbiotic associated microbes (microorganisms), acting as essential parts of a larger whole and that can't survive out of that larger whole, is known as holobiont. Holobiont was termed by Margulis and Fester [67] who defined all the organisms participating in a symbiosis were 'bionts', and thus the complex organism resulting from the combination of 'bionts' was a 'holobiont'. To date, knowledge on the precise interactions within the sponge holobiont is limited due to the inherent complexity of the system and the continuous lack of model sponges and cultured sponge symbionts [68]. However, recent insights from advancements in the field of metagenomics help deciphering the genetic foundations of sponge-microbe interactions and unravel in silico physiology and adaptation mechanisms of these ancient animals (e.g. [60, 69–71]).

1.4 Humans and Sponges

Most sponges have a hard silica or calcite skeleton for structural support giving them a rough and brittle texture. However, some sponge genera, such as *Spongia* and *Hippospongia*, possess only a soft sponging skeleton with elastic and durable fibres. These sponges, today often referred to as 'bath sponges', have been used by humans for thousands of years and for various purposes [72]. The use of sponges is mentioned in the Bible, as well as in Homer's Iliad and Odyssey, and played a significant role in ancient Egyptian, Greek and Roman civilisation [73, 74]. Their texture and natural absorbance ability made them ideal as bathing and cleaning tools or as water filter and portable drinking devices. Roman legionaries relied on them as padding for their helmets and other uses as contraceptives or as painting tools have also been documented [75].

An early method for finding bath sponges was by collecting beach cast sponge skeletons at the shore. Later, sponge collection was carried out at sea by fishermen diving to sponge grounds and harvesting wild sponges [75]. Having a long history in the Mediterranean, commercial sponge fisheries only spread to other tropical and sub-tropical regions over the past century. By the beginning of the twentieth century, sponge fishing was widely practiced in the West Atlantic, most notably the Caribbean Sea and Mexican Gulf, where sponge fisheries became a lucrative industry [76].

Through the high demand for bath sponges and the introduction of more sophisticated sponge diving equipment over the past century, it repeatedly came to

overfishing of sponge grounds. Occasional disease outbreaks with devastating effects on natural sponge populations further led to the collapse of many natural sponge stocks and a fluctuation of bath sponge supply [74, 77, 78]. The introduction of synthetic sponges by the 1950s replaced natural sponges in everyday use, and although there is still a high demand for natural bath sponges today, they are mostly sold as 'speciality' face and bath sponges in cosmetic stores or as tools for sponge painting [75, 79, 80]. It is estimated that over 300 tonnes of bath sponges are still being produced annually, with about 15 species having economic importance ([81]; www.fao.org).

Apart from their useful material properties, sponges have been valued for medicinal purposes since the Greek Antiquity. The Greek physician Hippocrates described the use of sponges as remedy for illnesses in several of his medical works. Remarkably, physicians at that time had identified the anti-inflammatory properties of sponges which were applied to wound healing and similar uses [82]. However, the real pharmaceutical potential of sponges has only come to light during the twentieth century with modern techniques sparking a renewed interest in sponges as producer of novel bioactive compounds [83].

2 Bioactive Compounds

2.1 Bioactive Metabolites Isolated from Sponges

Amongst marine organisms, sponges are one of the richest sources of structurally diverse, novel bioactive natural products, with more and more natural products being discovered every year, contributing to 29% of the total marine natural products discovered between 2000 and 2010 [84]. Sponges are sessile organisms and use chemical defence systems to ward off predators and compete for surface area (Fig. 13.1). The bioactive compounds found have been shown to exhibit a wide range of activities such as anticancer activity by halichondrin B [85], the thrombin inhibitor nazumamide A [86], the antifungal aurantoside A [87] and the anti-inflammatory and antipsoriatic compound avarol [88]. These diverse bioactivities have generated considerable interest in sponge compounds as novel drug leads for human biomedical research. However, the pharmaceutical pipeline is very long, it has a big opening and a narrow end and from discovery to pharmacy store and an enormous amount of time and money is spent. During its residence in the pharmaceutical pipeline of approximately 15 years, an estimated number of 10,000 new natural compounds have been discovered, and only very few reach the stage of clinical trials (Fig. 13.6). Cytosar-U® used for treatment of acute myelogenous leukaemia and Vira-A® for treatment of herpes infections were the first drugs on the market that were developed based on marine natural compounds and originally isolated from marine sponges.

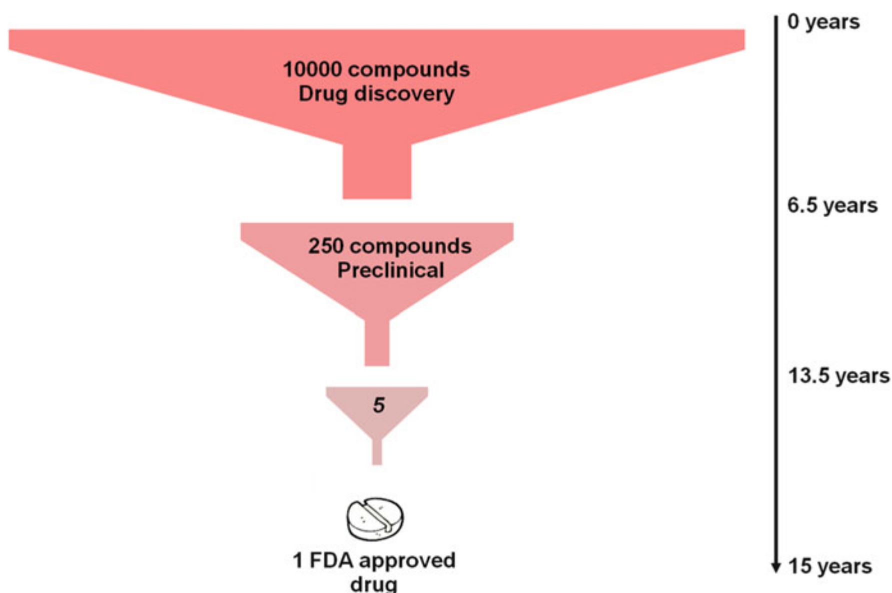

Fig. 13.6 Timeline illustrating how prolonged and selective is the process from the discovery of a compound until its approval as a drug. Some of the selection criteria concern the mass production possibility, the toxicity against specific cell types or the expense of the processing of the compound

2.2 Isolation and Purification of Sponge Secondary Metabolites

Despite the huge improvements in chromatographic and spectroscopic techniques during the last few decades, the isolation and structure elucidation of compounds from natural sources is still a very challenging and time-consuming task [89]. The first step for the isolation of pure secondary metabolites, both produced by the sponge or by the associated microbes, involves the submersion of the sponge material into organic and/or aqueous solvents. The sponge material can be immediately submersed into the solvents, or it can be freeze-dried first. However, in some cases, the remaining seawater absorbed by the sponge may cause degradation of the compounds by intracellular enzymes that are also released during the extraction [90]. Maceration or pre-cutting into small pieces also facilitates the solvent penetration and a more efficient extraction. The solvents used for extraction depend on the characteristics of the targeted compounds. Ethanol, ethyl acetate, methanol or chloroform can be used as extraction solvents, but a mixture of ratio one-to-one dichloromethane and methanol (CH_2Cl_2:MeOH, 1:1) is the most common, as it covers a broad range of polarities [91–93]. Aqueous extractions can also be performed [94, 95]. Subsequent evaporation of the solvents results in a crude extract.

Due to the cosmopolitan occurrence of many bioactive compounds, most natural product extracts contain previously characterised compounds. This inevitably leads

to a high rediscovery rate of active compounds [96]. De-replication at an early stage of the drug discovery process allows prioritisation of samples with potentially new active molecules, saving time and money [97]. One way of prioritising at the start is the use of bioassay-guided isolation. This method follows the separation of an active compound by screening for activity against a specific target activity, such as anticancer, antimicrobial, antifungal or antiparasitic activity [98]. The next step is then to analyse the crude extracts to evaluate the chemical content and search for new interesting compounds. As the crude extracts are complex mixtures, containing neutral, acidic, basic, lipophilic and hydrophilic compounds, a crude fractionation is usually carried out as a first separation step. Liquid-liquid partition [99], solid phase extraction (SPE) [100] and size exclusion chromatography (SEC) [99, 101] are commonly used, as they represent low-resolution separation techniques and allow an initial and unspecific separation of the different compounds. The final separation usually includes high-resolution separation techniques, such as preparative and semi-preparative HPLC [93]. When connected to a diode array detector (DAD), the compounds can be followed based on the retention time and ultraviolet (UV) spectrum. Additionally, an evaporative light scattering detector (ELSD) may allow the detection of the compounds that do not absorb UV light [102].

The structural elucidation of the isolated compounds is the final step to identify the isolated chemical entities. This is a complex part of the process that requires detailed chemistry expertise as well as modern techniques and equipment. Commonly, different analytical techniques, such as UV, infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometric (MS) methods, are used to elucidate the structure of a given molecule. Integrated analysis of results obtained through these different techniques then allows for a detailed molecular structure to be predicted [103].

Mass spectrometers have been used as powerful tools for de-replication and structural elucidation as it provides an idea about the molecular formula [104]. A great advantage of MS is that it is very sensitive, capable of detecting compounds in microgram quantities. Despite the importance of MS and other spectroscopic techniques, one-dimensional (D) and 2D NMR are the most powerful techniques for structural elucidation as they provide detailed information about the structural components and the way they are organised, the dynamics and the 3D disposition of the molecule [105]. 1D experiments (^{1}H, DEPT, ^{13}C, ^{15}N, ^{19}F, ^{31}P and others) give information about the atoms present in the molecule, allowing a first indication of the molecular structure. 2D experiments (HSCQC, COSY, TOCSY, HMBC, NOESY, ROESY and others) provide more detailed information and show correlations between the different atoms. The length of the correlations seen in correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) spectra usually does not exceed three bonds; however longer distances can be seen with other experiments, such as total correlated spectroscopy (TOCSY) [106].

The last step for complete structural elucidation is the determination 3D disposition of the molecule. Well-defined conformations, as in the case of small cyclic compounds, can be easily accomplished from proton-proton *J*-coupling and/or nuclear Overhauser effect (NOE) intensities. However, bigger and more complex

compounds require a computational approach, which, many times, coupled to experimental NMR data allow decoding of the 3D molecule disposition [107].

2.3 Identification of Secondary Metabolite Producers

Sponges are an important source of bioactive metabolites, with >250 novel compounds isolated from sponges each year (e.g. [1, 108]). These compounds, which comprise alkaloids, polyketides, terpenoids and other bioactive products, are of great commercial interest due to their vast potential pharmacological properties [83, 109–111]. Bioactive capabilities of sponge natural products span a broad spectrum, from antibacterial and antifungal to antitumour and antiviral applications [112–115]. For almost 40 years, the origin of the secondary bioactive metabolites found in marine sponges has been widely discussed. However, multiple lines of evidence have led to the widely accepted axiom that the majority of compounds isolated from sponges are of microbial origin rather than sponge-derived (e.g. [110, 116–118]). In particular, the structural similarity of some of the natural products from sponges to complex polyketides and non-ribosomal peptides, two groups of metabolites exclusively known to microbes, suggests the bacterial origin of these compounds. In addition, many compounds isolated from sponges share structural elements which can also be found in compounds from cultured microbes. One prominent example is the pederin family (see Fig. 13.7), a group of compounds that almost all show high antiviral and antitumour activity [119, 120]. Pederin was originally isolated in 1919 from the dermestid beetle *Paedarus fuscipes*, but it took almost 50 years before until its chemical structure was elucidated due to the low concentration of the compound produced in its natural host: with approximately 25 million specimens being required for structural elucidation [121]. Pederin was unique with respect to group of natural products to which it belonged until the late 1980s, when two marine natural products were isolated which closely resemble the structure of pederin. Mycalamide A was from a marine sponge from the genus *Mycale* [122], and onnamide A was from a sponge of the genus *Theonella* [123]. In the following years, the pederin family grew further with the isolation of mycalamide B, further onnamides and theopederins, icadamides and psymberin (irciniastatin A), all of which were isolated from different marine sponges (reviewed in [124]). Until now, two further examples of pederin-like compounds of bacterial origin have been identified. The only cultivable producer of a pederin family compound was recently discovered as a symbiont (*Nostoc* sp.) of the lichen *Peltigera membranacea*, which produces the compound nosperin [125]. The other example, diaphorin, was isolated from a betaproteobacterium (‘*Candidatus* Profftella armature’), a symbiont from the Asian citrus psyllid *Diaphorina citri* [126].

Initial studies to determine the origin of natural products in marine sponges were all based on tissue dissociation and subsequent cell sorting into individual cell population based on different physical parameters of the cells and specific staining by fluorescent dyes. The extraction and analysis of individual cellular populations

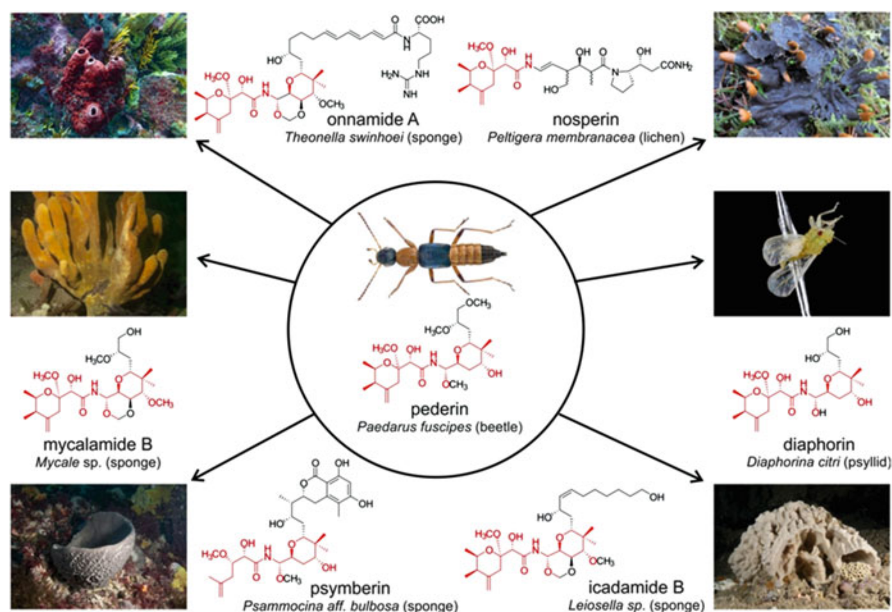

Fig. 13.7 Pederin family compounds. The first isolated pederin was isolated from a beetle, but since then several other pederin compounds have been isolated in sponges and in a lichen, showing broad distribution of these compounds. Image of *Theonella swinhoei* courtesy of Toshiyuki Wakimoto, University of Tokyo; image of *Mycale* sp. courtesy of Mark Norman, Museum Victoria (Melbourne, AUS), license: CC-BY; image of *Psammocinia* sp. courtesy of Mark Norman, Museum Victoria (Melbourne, AUS), license: CC-BY; Image of *Paedarus fuscipes* courtesy of Udo Schmidt (www.flickr.com), license: CC-BY-SA; image of lichen *Peltigera membranacea* courtesy of Richard Sullivan (www.mushroomobserver.org), license: CC-BY-SA; image of *Diaphorina citri* courtesy of USGS Native Bee Inventory and Monitoring lab (www.flickr.com), license: public domain; Image *of Leiosella* sp. courtesy of Mark Norman, Museum Victoria (www.portphillipmarinelife.net.au), license: CC-BY

revealed the presence of the antifungal glycopeptide theopalauamide in the filamentous bacterial cell population of the marine sponge *Theonella swinhoei*, whereas the cytotoxic polyketide swinholide A was mainly present in the unicellular bacterial fraction [116]. But with the recent advantages in whole-genome sequencing and the development of improved bioinformatic tools for gene annotation and function prediction, sequencing of individual bacterial genomes or even metagenomes has become the method of choice. Using single-cell genomics, Wakimoto et al. [127] could identify '*Candidatus Entotheonella*' as the producer of the cytotoxic compound calyculin A in the marine sponge *Discodermia calyx*. Research within BluePharmTrain has elaborated on this work and showed that also the metabolites cyclotheonamide A and onnamide A are localised in *Candidatus Entotheonella* members [128].

However, not all bioactive compounds discovered in sponges are (suspected to be) of bacterial origin. Metabolites known to be produced by the sponge are, for

example, avarol, found in *Dysidea avara* [129], stevensine from *Axinella corrugata* [130], crambesicidins have been linked to spherulous cells of *Crambe crambe* [131] and an array of cytotoxic brominated isoxazoline alkaloids [132] has been located inside sponge cells and are therefore presumed to be produced by the sponges themselves and not by their associated microbes. Moreover, a large proportion of the bioactivities observed from sponge extracts has not yet been linked to chemical structures or suspected producers. In addition, a number of enzymes with unique properties with potential therapeutic applications have been ascribed to sponges. Amongst the enzymes degrading 2-5A oligomers as components of the antiviral 2-5A system in mammals, a novel 2-5A degrading enzyme with different catalytic properties than their mammalian orthologs was found in the marine sponges *Tethya aurantium*, *Thenea muricata* and *Chondrilla nucula* [133]. This enzyme could be a representative of the group of 2′,5′-specific ribonucleases that primarily control the cellular level of 2′,5′-oligoadenylates [133]. Another example of a sponge-specific enzyme is the ATP N-glycosidase that converts adenosine-5′-tri-phosphate into adenine and ribose-5-triphosphate. This unusual enzymatic activity was first found in marine sponge *Axinella polypoides* but subsequently in several other sponges and could be used in the development of anti-infectives and anticancer drugs [134]. The sponge origin of these novel enzymes has been verified by genomic analyses (unpublished BluePharmTrain data).

2.4 The 'Supply Problem'

To date, there are only a few marine natural products known that have made their way into clinical trials, despite the extraordinarily high discovery rate of new highly active compounds. The reason for this discrepancy is that the supply of sufficient pure compound must be guaranteed, which is in many cases a huge problem due to the minute concentration of marine natural products in their animal sources sometimes less than 1 ppm of the wet weight [135]. Besides the technical problem in processing immense amounts of material for isolation, removal of large quantities of biomass from its natural habitat can have major ecological implications. Therefore, alternative strategies have to be found in order to fulfil the needs for drug development. In this respect, some progress has been made in the aquaculture of sponges as previously described, but none of the systems have been implemented so far in the production of clinically relevant marine natural products. Total chemical synthesis or semi-synthetic approaches are alternate strategies to solve the supply problem. A very prominent example is Halaven®, a highly structurally synthetic analogue of the even more complex sponge-derived natural product halichondrin B, which was recently approved for the treatment of late-stage metastatic breast cancer [136]. Despite the impressive achievement with Halaven®, involving 62 synthetic steps [137], the synthesis of these highly complex natural molecules still remains a great challenge for chemists.

The main scientific challenge of BluePharmTrain is to develop a robust technology platform for generating sufficient bioactive compounds from sponges and their microbes, to bridge the gap between discovery of metabolites of pharmaceutical interest and having enough to initiate clinical trials. BluePharmTrain will focus on key sponge species that vary with respect to (1) chemical structure class of the bioactive compound, (2) symbiont load, (3) actual (putative) producer in the sponge-microbe conglomerate, (4) sponge morphology and (5) geographical distribution of the species. Novel and comprehensive cultivation techniques will be applied to key species. These would include, inter alia, growth in sea by mariculture, cocultivation of sponge cells and microbial symbionts and cultivation of individual constituents of the sponge-microbe holobiont (sponge cells, bacteria, fungi and archaea). We have selected this multiphasic cultivation approach to close the gap between the natural environment and fully controlled cell cultures in small steps. Alternatively, we will also target cultivation-independent approaches, such as heterologous expression, to get access to secondary metabolites of sponge-associated microorganisms. This approach leads to diverse strategies for producing sponge-derived bioactive compounds, which can be picked out for designing tailor-made processes for other marine sponges, as well as other marine invertebrates.

3 Sponge Biotechnology

3.1 Legal Aspects of Sponge Biotechnology

Different potential strategies to obtain sponge-derived compounds are related to a number of legal aspects that need to be considered before scientific research and biotechnological application. They are related to (1) mapping out international legal protection of marine environment and identifying regulatory triggers and potential gaps related to marine biopharmaceutical research and development, (2) analysing opportunities for and challenges to anticipatory governance and responsible innovation and (3) identifying intellectual property issues arising from this area of research. The access and benefit-sharing (ABS) regime under the Nagoya Protocol on the Convention on Biological Diversity (CBD) affords a contemporary example of a governance framework for responsible research and innovation and governs the access and use of genetic resources by researchers and industries. Genetic resources have been considered as ‘common heritage of mankind’ for decades and conflicts between commercialisation and inappropriate distribution of benefits arise from biopiracy that caused common concerns for the international community [138]. Against this background, the Nagoya Protocol was adopted in 2010 and entered into force in 2014. It aims to establish a clear, legally binding framework to determine how researchers and companies can access the genetic resources and the traditional knowledge associated with those resources. It also aims at the fair and equitable sharing of the benefits arising from the use of genetic resources and the associated traditional knowledge. ‘Genetic resources’ under the Protocol refers to

materials from plants, microorganisms and animals, including sponges, which are utilised for their genetic materials rather than other attributes (article 2 of the CBD). The 'benefits' shared under the Protocol can include profits from products developed from genetic resources, from transfer of relevant technologies and from appropriate funding (article 1 of the CBD). Potential users include companies and researchers from commercial industries (e.g. pharmaceutical, biotechnology, cosmetic sectors), the public sector and non-profit organisations engaging in such research (e.g. universities, museums, botanical gardens, zoos, etc.). Parties to the Protocol are obliged to facilitate access to these resources for environmentally sound uses (e.g. signatory states must designate at least one competent national authority on ABS). Users may gain access to genetic resources by prior informed consent or mutually agreed terms, which is usually effected by contract.

However, the complexities and uncertainties arising from the Protocol and the capacity gaps in countries make the implementation work extremely challenging. For example, the relationships between the Nagoya Protocol and other ABS-related international instruments, such as the United Nations Convention on the Law of the Sea (UNCLOS) and the Agreement on Trade-Related Aspects of Intellectual Property (TRIPS), have yet to be reconciled [139]. Gaps in legislation and institutional capacity across different countries do not help in this regard. To date, relatively little attention has been devoted to the implementation of the Nagoya Protocol, even in the European Union (EU) where legislation has been introduced to bring EU law in line with international obligations on ABS (see Regulation (EU) No 511/2014). Although the EU took an active part in regulatory development, major user countries like China and United States of America (USA) are still not parties to the Convention. Another major problem that hampers the effectiveness of the Protocol includes the lack of legal clarity, certainty and transparency in both international and domestic ABS legal frameworks. The difficult, time-consuming and bureaucratic regulations, together with problems of legal uncertainty in provider countries, have contributed to the shutdown of natural product programmes in many large pharmaceutical companies [140]. Unfortunately, neither the Protocol nor the Convention provides enough incentives for the users. Besides, many controversial issues covered by the Protocol (e.g. sovereign rights, rights of indigenous and local communities, transfer of technology, Intellectual property rights) render the interpretation of the Protocol challenging.

An economic analysis of ABS regime and its effectiveness in biodiversity conservation was conducted, following a legal review of the relevant law and policy. Many economists consider biodiversity loss to be the result of both market failure and policy failure, as negative environmental externalities mean that the market fails to reflect the true value of biodiversity, so that policy fails to correct the market failure [141]. The ABS introduces in effect a market-driven approach to conservation of genetic resources, which involve developed countries in the conservation activities within developing countries and which suggest that protection might be better achieved by acknowledging the commercial opportunities presented by genetic resources and internalising these into pricing mechanisms [142]. However, 20 years after the adoption of CBD, changes in market trends and technology

development have significantly reduced the demand for genetic resources. For example, pharmaceutical industry has shifted its interest to microorganisms and away from plants; meanwhile, scientific discovery suggests that microorganisms share genes worldwide, so one compound collected in one country can often be found in others [140]. With these developments, the balance between the users and providers of genetic resources has changed significantly; therefore, policies and institutional arrangements may have to be revised accordingly.

3.2 Marine Sponge Cultivation

For a long time, harvesting of natural sponge populations was the only means of collecting sponges. During the nineteenth century, first attempts were made to farm bath sponges in the sea, using the knowledge that sponge tissue could be divided into smaller fragments that regrow into larger ones [73]. With the advent of modern biotechnology and the discovery of thousands of novel bioactive compounds in marine sponges, new methods were required to obtain large quantities of sponge biomass for extraction of the target compounds. Inspired by successes in bath sponge cultivation and the growing field of aquaculture, first trials were conducted with pharmaceutically relevant sponge species [135, 143–145]. To date, three different production techniques, in-sea aquaculture, cultivation in bioreactors and cell cultures, have been studied and continue to be in the focus of researchers looking to overcome the 'supply issue' in sponge biotechnology.

Growth trials in the sea have the advantage of using environmental conditions similar to those found in natural sponge habitats. However, concerns have been raised about the consistency of production in an environment with naturally fluctuating water conditions [146]. Despite these concerns, moderate growth and bioactive compound production could be demonstrated for several sponge species leading to continued research into this production technique [147–151].

In order to fully control the cultivation conditions, closed aquaculture systems or bioreactors were designed that mimic the natural environment of the sponge and create stable, optimised growth conditions [152–156]. Despite many attempts, sustaining sponge growth in a bioreactor remains to be difficult due to the extent of environmental factors that need to be considered and controlled [157]. Interestingly, sponges can be found in large public aquaria, where the water conditions can sustain sponge growth [158], suggesting that this technique carries the potential to produce sponge biomass on a larger scale, albeit requiring further research. Although large scale production of marine sponges in the sea or in bioreactors (Fig. 13.8) remains to be demonstrated, much progress has been made over the past decades, leading to an increased understanding of the role that environmental conditions, such as water currents, nutrient availability and suitable substrates play in sponge cultivation (see [72, 146, 157] for reviews).

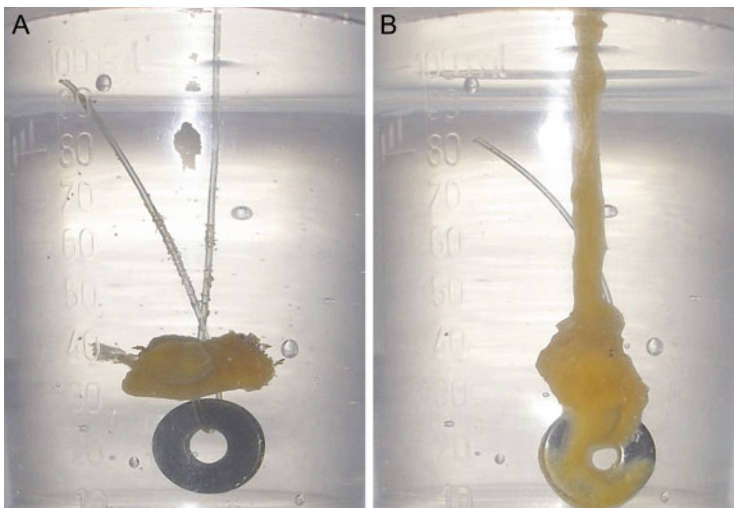

Fig. 13.8 Example of a sponge explants (*Halichondria panicea*) kept in an experimental bioreactor, in the beginning (**a**) and after several weeks (**b**) of a growth trial. Suitable substrate, feed, water currents and many other parameters play a crucial role in sustaining sponge growth in bioreactors. Small-scale growth trials can therefore give valuable information on suitable cultivation conditions needed to upscale sponge production

3.3 Sponge Cell Culture

Sponges are organised at the cellular level, and although the cells differentiate to perform different functions, they lack true tissues [159]. As a result, sponges can be dissociated into individual cells, and the cells will reaggregate to form a functional sponge, i.e. with differentiated cells that form canals and chambers through which water is pumped [160]. This property formed the basis for sponge cell cultures used as models to study developmental biology [161] and immunology [162] and also forms the basis for development of in vitro production methods for sponge-derived bioactive molecules [163, 164].

Sponges are dissociated into single cells or cell aggregates to establish cell lines that can be optimised to produce sufficient biomass for extraction of the compounds and/or to over-express production of the bioactive compounds [163]. While primary sponge cell cultures have been demonstrated to increase in cell number (biomass) and overexpress production of a bioactive compound [165, 166], there are no immortalised cell lines of marine invertebrates [167], including sponges [168]. The main challenges in creating sponge cell lines are gaps in our knowledge about nutritional requirements to optimise sponge cell metabolism, growth and division [164, 169, 170]. Another persistent problem is contamination and overgrowth of microbes that under normal conditions, in an 'intact' sponge, are kept under control but divide rapidly in sponge cell culture [164]. It has also been reported that once sponge cells are dissociated, they begin to rapidly lose telomerase

activity that is necessary for proliferation [171]. And finally, when sponge cells are dissociated, a large percentage of the cells become apoptotic [172].

Current advances in sponge cell culture are focusing on applying findings from '-omics' research [173] to immortalise cells by editing the genome to up- or down-regulate genes of interest (e.g. tumour promoters, oncogenes, tumour suppressors) [174]. Once a cell line is established, it can be used as a fusion partner in sponge-sponge hybridomas to fuse a metabolite-producing but not dividing sponge cell with a rapidly dividing, but not metabolite-producing, cell line. Pomponi et al. [175] have demonstrated proof of concept by fusing cells of different sponge species. Another approach to in vitro production of sponge cell biomass is immobilisation of cells to create three-dimensional cultures [176].

3.4 Isolation and Cultivation of Sponge-Associated Microorganisms

Inside a sponge, bacteria exist as complex multispecies communities, and the first step in isolation is to separate the bacteria of interest from the rest of the community. Traditionally, this is done by plating serial dilutions of homogenised sponge tissue on media containing various carbon sources using agar as a solidifying agent to obtain pure colonies of bacteria [177, 178]. However, conventional microbiological techniques like agar plating often result in isolation of copiotrophs/microbial weeds (fast growing bacteria that grow in high nutrients conditions), which usually overgrow the oligotrophs (slow growing bacteria that grow in nutrient deficient conditions). The key to isolate oligotrophic bacteria from a sponge bacterial community is to eliminate these 'microbial weeds'. There is no universal solution for this problem, but few strategies have been adopted and will be discussed further.

Growth of most copiotrophic bacteria can be suppressed with the use of nutrient deficient media. Using such media should provide a selective advantage for isolation of oligotrophs and enable longer incubation periods for enrichment of slow growers. However, some copiotrophs have adapted to grow in oligotrophic conditions, for example, bacteria from the genus *Pseudovibrio*, often isolated from marine sponges are capable of growing in ultra-oligotrophic seawater [179]. Kurtböke [180] demonstrated the use of bacteriophages isolated from sponges to eliminate the fast growing copiotrophs, which led to the successful isolation of a novel *Micromospora* species. The use of phages in combination with the use of oligotrophic media is very promising, but maintenance of phage library for individual sponge species is tedious, and a universal phage collection for sponge-associated copiotrophs is essential. The dilution-to-extinction method by Rappe et al. [181] which relies on the principle of diluting the natural community to a minimal concentration of one viable cell per well has been previously successful in isolating the dominant marine bacterioplankton SAR11, while copiotrophs present at lower densities can be effectively removed. Toledo et al. [182] used dilution to extinction and diluted the cells to one cell per ml

and encapsulated each cell in agarose microcapsules. These microcapsules were maintained into mini fermentation columns, thereby creating an artificial sponge environment. None of the above methods however were successful in either isolating or enriching interesting sponge-specific bacteria. The failure of these methods can be attributed to the fact that bacteria inside a sponge are embedded in a mesohyl matrix often associated with complex microbial communities, and some of these microbial associations might be obligatory for their growth.

Most approaches on cultivation of sponge bacteria rely on emulating an artificial sponge environment in the laboratory. Steinert et al. [183] attempted to culture these bacteria in situ employing a diffusion growth chamber. Briefly, a *Rhabdastrella globostellata* sponge homogenate was mixed with cultivation media and injected in a diffusion chamber constructed by combining two centrifuge microfilter sections (pore size 0.2 μm). This chamber was later implanted into *R. globostellata* tissue and incubated for 16 weeks. Subsampling from the growth chamber and dilution agar plating led to the successful isolating of a sponge-specific *Bacteroidetes*. However, it is noteworthy that the approach was not successful in all the tested sponges used in the study. The method serves best to enriching novel bacteria rather than isolating pure bacterial cultures from the enrichments.

For successful isolation of sponge bacteria, it is essential to identify the interaction and dependencies between the bacteria and their host. These bacteria interact with their host to modulate phagocytosis by using eukaryotic-like proteins [184] which have a widespread occurrence in sponge bacteria amongst distantly related sponges from different ecological niches indicating convergent evolution within sponges. Understanding the overall host-symbiont recognition mechanism and the molecules which mediate these mechanisms may be important to develop universal protocols for isolation. Secondly, in most of the studies, solid cultivation on agar resulted in higher cultivation success, but previous reports [185] and cultivation data from our lab (unpublished BluePharmTrain data) show that agar as a solidifying agent has detrimental effects and a solidifying agent, such as gellan gum, is more suitable for isolating novel taxa. Thirdly, it is important to relate to environmental conditions, such as oxygen concentration during isolation, as sponges can stop pumping water by closing the osculum and maintain a total anoxic state for days during which anaerobic processes like denitrification take over [186]. Interestingly, the only *Chloroflexi* isolate reported from sponges was enriched under anaerobic conditions, and the phylogeny of most *Chloroflexi* from sponges suggests that they are facultative/strict anaerobes [187]. Draft genomes from sponge-specific bacteria suggest that these microbes have regulatory mechanisms for growth, and hence isolating these bacteria, which have remained recalcitrant to growth, may be resolved. In conclusion, a multidimensional approach is necessary to crack this enigma of uncultivable sponge bacteria, which hide the secrets of the basic concepts of invertebrate-bacterial symbiosis.

3.5 Heterologous Expression of Biosynthetic Gene Clusters

Finding gene clusters that encode for the enzymes responsible for the production of the biochemical compounds is another alternative for natural compound production. As a result of rapid developments in sequencing technology, synthetic biology and bioinformatics, an increasing number of genome sequences from marine microorganisms have become available, making genome mining an attractive tool for the discovery of novel compounds. Bioinformatic tools are typically employed to screen for and subsequently identify novel gene clusters, many of which are responsible for the production of the novel natural products. Once these natural product gene clusters have been identified, it is in principle possible to heterologously express these gene clusters using genetic engineering and manipulation of suitable hosts in order to study their characteristics and activity or finding similar clusters in cultivable bacteria. For example, Ueoka et al. [188] were able to isolate the compounds tolytoxin and luminaolide from cultivable cyanobacteria, while sequences from the cluster responsible for the production of the highly similar misakinolide A were first obtained from the sponge *T. swinhoei*.

The transfer of natural product pathways into a heterologous host is not only a promising tool to generate the parent natural product but also to produce homologues by biotechnological engineering yielding products with better pharmacokinetic characteristics. This seemingly simple concept of transferring the gene cluster into an artificial host faces several significant challenges posed by the nature of these molecules, which are synthesised by multimodular enzyme mega-complexes. In addition, the heterologous host must also possess the cellular machinery that is necessary for successful and stable protein production. Polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) proteins also need to be post-translationally modified [189] to be active and need a specific substrate pool within the heterologous host (coenzyme-A-activated short-chain carboxylic acids and specific non-proteinogenic amino acids). Taking these challenges into account, it is not surprising that only a few bacterial host strains have been identified (*Escherichia coli*, streptomycetes, pseudomonads and bacilli) for successful heterologous expression.

In recent years, many advances have been made in expression strategies, including DNA assembly methods for large biosynthetic gene clusters and technologies enabling better regulation and host engineering leading to an increased number of heterologously expressed secondary metabolites (reviewed in [190]). Considering the immense repertoire of natural product biosynthetic gene clusters that are becoming accessible through the advances in high-throughput sequencing, there is still a need for the establishment of more efficient modification, transfer and expression systems. By using these systems, even the biosynthetic potential of ‘silent’ pathways could be activated.

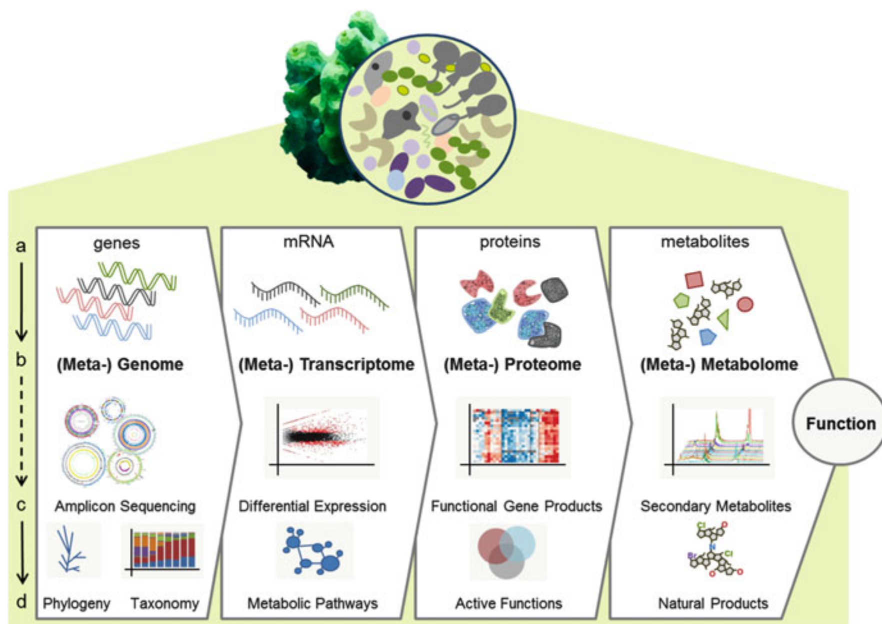

Fig. 13.9 Schematic explanation of the process to apply high-throughput sequencing to obtain the '-omic' outputs from the various biomolecules extracted from the holobiont for further analysis to understand the complex sponge holobiont—sponges and their associated microbial community. The different lines show the various steps of the process with the involved molecules: (a) single biomolecules implied, (b) the collection of all the biomolecules implied, (c) the technique applied to analyse the biomolecules and (d) the expected outputs from the applied '-omic' technique. Black arrows indicate consequential relationship; dotted arrows indicate which technique to apply for each biomolecule

3.6 *Genes, Genomes and Beyond: Molecular Methods in Sponge Biotechnology*

The search of natural products has greatly benefited from technological innovations and molecular tools, resulting in next-generation high-throughput sequencing (HTS) and big data creation [128, 191–193]. For example, these techniques have increased the knowledge in putative pathways of secondary metabolite production but also in which are the requirements for certain cultivation adaptations or even aided in the understanding of symbiotic relationships amongst the host and the associated microbial communities (e.g. [194–197]).

In general, the combination of these techniques uses the neologism '-omics' (Fig. 13.9), referring to the fact that all the fields of study end in '-omics' as genomics, transcriptomics, proteomics or metabolomics [198]. The fields of study derive from the suffix '-ome', which identify which part of the biological pool of molecules it deals with: genes in the genome, transcripts from the transcriptome, proteins in the proteome and metabolites in the metabolome. In these fields, we also

find the prefix '-meta', referring to the fact that the analysis not only considers a single study object (e.g. an organism) but also what is beyond it (e.g. the associated communities within the organism).

These different technologies have been applied in many disciplines referred to sponges and the sponge associates, not only in the search of natural products but also in the understanding of the biology of the organism(s) [199]. Identifying the target organism or the producer(s) of the secondary metabolites, describing the composition of the community it lives in and the produced molecules, inferring a function and explaining the interactions amongst them, are just some of the aims in sponge biotechnology. Altogether, here we present a series of molecular approaches, from DNA barcoding to the various fields of '-omic' applications, for marine biotechnology concerning sponges and the sponge-associated microbes (Fig. 13.9).

3.6.1 DNA Barcoding

The concept of DNA barcoding is based on the premise that the genetic profile of each species is unique and that short standardised sequences can be used as a taxonomic marker to distinguish individuals of a species [200]. This barcode principle is applied to identify unknown samples compared to a pre-existing classification as a complementary measure to thorough phylogenetic analyses based on the use of short genetic markers as, for example, 16S rDNA for prokaryotes, 18S and 28S rDNA for eukaryotes or ITS rDNA for fungi [201–203].

The phylum Porifera contains around 8500 validly described species (not including the recently described cryptic species), distributed globally in aquatic ecosystems [204]. Sponge taxonomists can identify a range of species based on morphology such as in- and outside colour, tissue texture, body shape and spicule composition [205]. However, sponges are infamously difficult to identify due to their morphological plasticity, and many species remain poorly or ambiguously classified. As the result of the evolutionary differences that are conserved in the genetic code, certain genes can assist traditional taxonomic methods such as morphological characterisation or aid, for instance, in identifying cryptic sponge species [206]. For sponges, the mitochondrial cytochrome c oxidase subunit I (COI) gene is the most commonly used reference gene for barcoding [204, 207]. Further barcoding genes in use for sponge identification include gene fragments of mitochondrial cytochrome oxidase subunit 2 (cox2), mitochondrial ATP synthase (ATP6), and the nuclear ribosomal 28S rRNA gene (C2-D2) [208]. The sponge barcodes of approximately 500 species together with morphological annotations can be found in the Sponge Barcoding Project database (www.spongebarcoding.org) [209].

For prokaryotes, with the majority of organisms being uncharacterised and uncultivated, taxonomic identification using molecular genetic information plays an important role in identifying species and assigning them to certain taxonomic lineages [210, 211]. The most widely used reference gene for bacteria and archaea is the 16S rRNA gene [212, 213]. The first culture-independent studies to describe the microbial communities associated with sponges used 16S rRNA gene denaturing

gradient gel electrophoresis (DGGE) to determine diversity and 16S rRNA gene clone libraries to assign taxonomic affiliations (e.g. [33, 214–216]). These studies gave the first insights into the microbial diversity of sponges previously unattainable through culture-based methods. In addition, by applying 16S rRNA gene fluorescence in situ hybridisation (FISH) to sponge histological cuts, the prokaryotic community within sponges could be identified and localised [217–219].

3.6.2 High-Throughput Amplicon Sequencing

With the introduction of HTS techniques, the capacity of researchers has greatly improved to simultaneously analyse large amounts of samples by generating large datasets of selected amplified gene fragments (i.e. amplicons) as outcomes. The capacity of simultaneous sequencing of environmental samples and target genes has allowed comparing marine taxonomy, diversity and richness of entire communities through DNA barcoded amplicons.

Up to date metabarcoding studies have been carried out amongst different marine environments, from marine coastal sediments [220] to ballast water [221], and different marine communities from zooplankton [222] to invertebrates [223]. Moreover, the deep sequencing of fragments of the small subunit 18S rRNA gene and the COI gene has been applied to metabarcoding investigations of diverse marine communities, also including sponges [220, 223, 224].

Correspondingly, the advancements of HTS technologies enabled also the comprehensive analysis of the diversity and taxonomic compositions of natural prokaryotic communities based on the 16S rRNA genes found in environmental samples. In a mainly uncultured prokaryotic world, this was the first method to grant a broad and cost-efficient access to the bacterial multiplicity from natural communities. The first HTS 16S rRNA gene amplicon study on marine sponges detected 23 bacterial phyla and described that bacterial diversity as unmatched in other marine invertebrates [37]. Since then, numerous deep-sequencing surveys highlighted the remarkable stability of sponge-associated prokaryotic communities in time and space, with the host identity as the key driver of symbiont composition (e.g. [20, 21, 39, 41, 50]).

3.6.3 Genomics and Metagenomics

The genome is the full set of DNA in an organism and thus includes not only all the nuclear genes but also mobile elements, pseudogenes, repetitive elements, intergenic sequences, promoters and other regulatory sequences, as well as the mitochondrial- and chloroplast DNA of eukaryotic organisms [225]. Therefore, the field of genomics entails the study of some or all of the genomic elements mentioned above through comparative analysis of several sequences of the genome [198].

The first and only available draft genome sequence of the demosponge, *Amphimedon queenslandica*, shed some light on how sponges fit into the picture of eukaryotic genome content, structure and genome organisation, in other words,

into the evolution of animal complexity [173]. The *Amphimedon* genome revealed that gene structure and order remained conserved in other animals. The authors identified six aspects that appeared with the metazoa: (1) regulated cell cycling and growth, (2) programmed cell death, (3) cell-cell and cell-matrix adhesion, (4) developmental signalling and gene regulation, (5) allorecognition and innate immunity and (6) specialisation of cell types.

Srivastava et al. [173] also addressed host-microbe symbiosis based on the genomic features of *A. queenslandica*. This genome includes an expanded repertoire of innate immunity genes that regulate host-microorganism interactions, for example, inter-kingdom signalling gene families that encode receptor tyrosine kinases (cell surface receptors involved in signalling cascades) and guanine nucleotide-binding protein-coupled receptors (transmembrane receptors involved in the activation of intracellular signalling motivated by extracellular inputs).

To date, there are only two studies available describing mobile genetic elements in Porifera. The first description is the long terminal repeat retrotransposon Baikalum-1 in the freshwater sponge *Lubomirskia baicalensis* (Demospongiae; Ceractinomorpha) from Lake Baikal (Siberia, Russia) [226]. The authors proposed that retrotransposon Baikalum-1 is involved in adaptation of sponges to the freshwater habitat, by inducing duplication and diversification of the ancestral silicatein gene of marine species. The second study [227] described the presence of 51 non-protein-coding RNAs in ribosomal protein genes of three sponge species from the genus *Suberites* as well *as A. queenslandica*.

The vast majority of sponge symbionts remain uncultured despite extensive efforts to cultivate them. Present HTS techniques and novel bioinformatics algorithms have assisted in the recovery of uncultivated sponge symbiont genomes. The first available sponge symbiont genome was *Cenarchaeum symbiosum*, obtained by fosmid library sequencing [61]. This genome includes evidence of the use of ammonia and urea as a nitrogen source; *C. symbiosum* genes encode proteins for chemolithotrophic ammonia oxidation, including ammonia monooxygenase, ammonia permease, urease, a urea transport system, putative nitrite reductase and nitric oxide reductase accessory protein.

Not surprisingly, nitrogen metabolism has been one focus area in sponge microbiology. Sponges excrete ammonia as a metabolic waste product and therefore constitute an oasis of nitrogen sources in the poor-nitrogen seawater. Nitrogen cycling is known to occur both aerobically (nitrification) and anaerobically (denitrification) in sponges (e.g. [57, 228, 229]). For instance, genomes from the candidate phylum *Poribacteria*, obtained through single-cell sorting and multiple displacement amplification [63], revealed several important genes for denitrification. Interestingly, the *Poribacteria* genomes also contain genes that imitate those of their hosts: proteins containing eukaryotic-like motifs such as ankyrin repeats (ANKs), tetratrico peptide repeats (TPRs), leucine-rich repeats (LRRs), fibronectin type III (Fn3), laminin G and immunoglobulin-like domains [63].

Metagenomics has been defined as the analysis of the total DNA isolated from environmental samples [230] and is aimed mostly to prokaryotic communities and their symbiotic relationships. Shotgun metagenomic sequencing is applied to obtain

short DNA sequences directly from the sampled community without prior amplification [231]. It is possible to obtain genomic sequences of prokaryotes present in the environment from multiple metagenomes by genome binning based on differential coverage, similar GC content and tetranucleotide frequencies across a set of overlapping DNA segments (i.e. contigs). These tools have been used in sponges, and contig binning resulted in the reconstruction of draft genomes of a sponge-associated sulphur-oxidising bacterium [232], genomes of '*Candidatus Synechococcus spongiarum*' [233, 234] and two prominent symbionts of *A. queenslandica* [235].

As already mentioned, sponges are amongst the richest known sources of biologically active secondary metabolites. Therefore, metagenomics have also been applied more specifically to identify potential drug candidate gene clusters. Metagenomics techniques have been used not only to derive the genomes of prokaryotic symbionts but also for the analysis of the entire holobiont [236].

Metagenomic datasets can be screened for gene clusters which encode enzymes that catalyse molecules of the classes alkaloids, terpenoids, polyketides and small peptides [237]. These molecular classes and hybrid combinations thereof exhibit an almost infinite range of bioactive properties. In 2004, the Piel group discovered a gene cluster in the highly complex metagenome of the sponge *T. swinhoei* that strongly resembled the polyketide pederin cluster [238]. This sponge appears to be a rich source of a whole group of polyketides that structurally resemble pederin [239].

Functional clone library screening, without prior sequence information, can also lead to successes: antibacterial proteins from fosmid clones were obtained from a metagenome of the sponge *Cymbastela concentrica* [240] and *D. calyx* [241] identification of such complex secondary metabolite gene clusters from even more complex metagenomic datasets is the first crucial step in the potential drug development pipeline. Sequence-based screening, for example, with the antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) webserver [242], can be applied to further mine metagenomic datasets. However, one must keep in mind that novel gene clusters have to be related to already known sequences and that not necessarily the complete gene pathway can be retrieved [98].

3.6.4 Transcriptomics and Metatranscriptomics

A transcriptome is the full arrange of transcribed RNA produced in a cell or population of cells at any one time [198]. The transcriptome is not stable but dynamic; it is altered under circumstances in response to an external change (e.g. environmental or signalling) modifying the gene expression pattern. Measuring the level of expression of certain genes, under defined conditions, has aided in understanding differences amongst experimental parameters in the absence of a complete genome to refer to. For years, techniques to understand gene expression profiles were based on a gene by gene sort of method, where once the gene was identified the produced copies were quantified, as in quantitative polymerase chain reactions (qPCRs), for example [243]. In addition, the metatranscriptome shows the complete set of transcriptomes of interacting organisms. Besides understanding the

gene expression pattern of a given condition, transcriptomics are also being used for genotyping and describing novel genes as a more cost-effective alternative to genome sequencing.

Backsides of techniques dealing with expression patterns involve the instability of RNA transcripts which is a pure technological issue; for these techniques, sampling and conservation play a crucial role in having a good quality transcriptome. The methods to improve collections modes and post-sequencing analysis are improving continually [244–246]. Transcriptomics applied to marine microbes, sponges and to the sponge holobiont are at their initial stages, but some examples have been successful.

Although marine prokaryotic communities have benefit largely from metagenome analysis to describe them, the application of transcriptomics to 'free-living' marine prokaryotic communities has shown further interesting results: linkage of bio-geological cycles and geographical connections amongst basins at sea [247, 248] showed transcriptional networks, and Frias-Lopez et al. [249] described the naturally occurring transcription patterns in the ocean to further compare it to altered conditions as could be global warming or acidification responses of the whole community.

The application of transcriptomics and metatranscriptomics to sponges is not broad though increasing, especially as an alternative to genome sequencing and to address the high variability of sponge (e.g. [250–253]). At the moment, there is no real reference or model sponge since they are so variable, and thus there has not been consensus up to date [68]. Groups working with sponges are mostly applying these techniques to explain evolutionary traits [254, 255] as well as sponge life cycles and their benthic-pelagic coupling [256], but the search of secondary metabolite pathways is also of interest. At the moment, and due to large gaps in knowledge, the transcriptome studies are work-in-progress or limited to a description of the profile. Understanding the transcriptomes, interpreting the changes and the implications in sponges, at the moment are still far from complete.

Symbionts and endosymbionts are always challenging to study, but metatranscriptomes have found their way successfully in this field when considering the sponge holobiont. Symbionts in sponges are not only limited to bacteria, but also archaea, eukaryotes and larger organisms have been described [257]. Many groups are tackling the symbiotic relationships through transcriptomic techniques: Richardson et al. [258] tested the application of antibiotics to 'remove' certain prokaryotic partners to study the response of the community. Riesgo et al. [259] compared the symbiotic (*Symbiodinium* sp.) and aposymbiotic conditions of *Cliona varians* determining how different the transcriptional profiles are when the host differentially uptakes symbionts. Díez-Vives et al. [260] have found eukaryotic-like proteins in the microbiome of sponges, which could be explaining patterns of coevolution.

3.6.5 Proteomics and Metaproteomics

Proteomics describes the mass scale investigation of the proteome; therefore, of all proteins in a given sample [261, 262], the term proteomics was first used in 1997 [263]. Proteomes are far more complex than genomes, as genomes are rather stable, but the proteome describes a response to environmental factors and differs from cell to cell in a given sample (the consequence of the transcriptome). Therefore, proteomes can be used to investigate, for example, the flexibility of (micro)organisms in respect to changing environmental conditions [264].

Metaproteomics (i.e. proteomics undertaken at the level of environmental samples) was used for the first time in sponges in conjunction with metagenomics to investigate the interactions between the sponge *C. concentrica* and its microbial community [62]. This study was able to prove a shuffling of metabolites between the sponge and its microbiome, therefore providing proof for the assumption that part of the microbiome is symbiotic to the sponge. The analysis provided expression evidence for specific transport functions for halogenated aromatics, typical sponge metabolites. In addition, the authors find the nitrogen metabolism of bacterial and archaeal symbionts to be closely linked to the sponge host, which secretes and accumulates ammonium. The metaproteome showed presence of phagocytosis resistance genes, eukaryotic-like proteins and bacteria-eukaryote mediators, supporting the standing hypothesis that sponge symbionts might use these proteins to escape phagocytosis and/or control their symbiotic relationship with their hosts. The data also revealed specific protein expression by a Phyllobacteriaceae bacterium and a *Nitrosopumilus*-like crenarchaeon, thus linking particular functions to uncultured phylotypes.

3.6.6 Metabolomics

The metabolome of a given organism comprises all of its synthesised metabolites, which are usually defined as intermediates and products of the metabolism with a size smaller than 1 kDa [265, 266]. Metabolomic data is usually gathered by mass spectrometry and stored as mass-to-charge ratios of individual metabolites in databases, as for most 'omics' approaches the metabolome is quite dynamic. Nonetheless 'metabolic fingerprints' can be generated and used like DNA markers for phylogenetic comparisons.

In sponge biology, metabolomics can be used, for example, to distinguish between sponge sister species [267]. Furthermore, this technique can be applied to assess the relationship between bacterial isolates and its host organism, as described in Viegelmann et al., the metabolic fingerprints of a *Haliclona simulans* sponge and a *Streptomycete* sp. isolate from that sponge were compared, and similarities between the two fingerprints were found, implying a possible symbiotic relationship [268].

3.6.7 Metabonomics

Metabonomics measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation [269] in terms of diversity profiles, to try to understand the whole systemic change in complex samples (e.g. living systems). Metabolomics pursue the analytical description of complex biological samples, quantifying and characterising the small molecules of the given sample. The difference between Metabolomics and metabonomics relies on a the practical aim: global living system seeking clues on the effects of foreign compounds for metabonomics and single organisms or tissues for metabolomics [269–273].

Most of the metabonomic applications have been orientated to disease, pathological responses and clinical outputs [269, 273–275] as it has been proven to identify clinical conditions through a profile of metabolites and their response in time. Boroujerdi et al. [276] profiled the metabolites produced by symbiotic corals under the infection of *Vibrio corallilyticus* during bleaching events. However, to date, there are no studies done on sponges or sponge symbionts using metabonomics.

4 Outlook

All the techniques and advances up to now described in this chapter, and addressed by the BluePharmTrain Project, are mostly based on sponges and sponge-bacteria interactions or metabolic linkages. In addition, most of the sponge specimens are or have been collected from shallow waters (above ~40 m depth) by self-contained underwater breathing apparatus (SCUBA) diving or similar methods; this allows and simplifies repetitive sampling. Technological innovation in sampling techniques and the increase of information in reference databases have allowed the search of new drugs in previously inaccessible fields, as the 'deep sea' or the search of biological and symbiotic roles of archaea and fungi but also of viruses.

4.1 The 'Deep Sea'

Sponges are well distributed in the oceans and play a pivotal role in the ecosystem as mentioned previously; more strikingly, the oceans are the biggest habitat in the world and approximately account for 71% of the surface of our planet. This huge area divides into two main parts the coastal or shallow water regions and the 'deep sea', roughly anything deeper than 200 m respective to the sea level. This classification makes the 'deep sea' the biggest continuously connected habitat on the whole planet [277]. The 'deep sea' was long time assumed to be very hostile to living beings, because of the huge pressure, low temperature and absence of sunlight. Controversially, a number of expeditions with remotely operated vehicles (ROV)

and a few manned specialised submarines have shown that there is indeed life in the 'deep sea' and that it is much more diverse than ever believed [278]. Interestingly we have send 12 people to the moon already: from 1969 to 1972, six manned moon missions were carried out by the USA, but only three people have been to the Marianas Trench, the deepest part of the ocean. In 1960, Don Walsh and Jacques Piccard reached the bottom of the Marianas Trench aboard the *Triste* and lastly James Cameron aboard the *Deepsea Challenger* in 2012. However, most parts of the oceans are 'unexplored'. Especially hydrothermal vents are hotspots for living beings and biological diversity even within the 'deep sea' and are one of the few well-described habitats in the vast 'deep sea' [279].

As mentioned previously, most sponges in shallow waters pump huge amounts of seawater through their body and filter it as food source, the same accounts for their 'deep sea' counterparts as well as harbouring a diverse community of microorganisms with a multitude of possible functions, like chemical defence, nitrogen, silica, sulphur and carbon cycling [280]. Next-generation sequencing studies have shown similarities in the overall microbial community structures between sponges from shallow and 'deep sea' waters (most abundant phyla are *Proteobacteria* and *Actinobacteria*) but also remarkable differences, for example, in the abundance of Archaea (some microbial communities of 'deep sea' sponges comprise of up to 70% Archaea) [281, 282]. Nonetheless, as sponges are one of the oldest extant metazoans and have relied on (or even co-evolve) for at least millions of years with their endosymbiotic communities, it is reasonable to expect also a divergent evolution of the metagenome (all genes from the sponge and its inhabiting microbes) from shallow water sponges or terrestrial habitats. Therefore, 'deep sea' sponges possibly harbour a treasure of 'novel' genes and gene products (or are at least considerably different), which may be of interest for new antibiotics or enzymes of biotechnological application [191, 283]. The lack of accessibility to adequate amount of samples and prolonged studies of the 'deep sea' environment itself result in little knowledge about the possible role of sponges in the overall 'deep sea' ecosystem, though they appear to be abundant in patches [284]. So far, it can only be stated that 'deep sea' sponges are found throughout the 'deep sea' with possible aggregations at certain areas as hydrothermal vents or deep sea volcanoes. At the moment, the general assumption is that their role might be comparable to functions of shallow water sponges, though some rare behaviour has been observed as carnivorism [285, 286].

4.2 Archaea, Fungi and Viruses

Currently the main focus, in order to utilise the biotechnological potential of marine sponges, is put on the sponge itself and on their bacterial symbionts. As has been alluded to earlier, the abundant and diverse bacteria found in sponges are of great interest for the marine blue biotechnology research community, while the sponge-

associated archaea are still much less studied in the search for new bioactive compounds and drug discovery.

Archaea are mostly missing in the marine natural products literature. However, hyper-thermophilic sulphur-metabolising *Thermococcus* spp. strains from marine hydrothermal system yielded cyclic polysulfides that demonstrated antifungal and anthelmintic activities [287]. The highly temperature-stable enzymes of such marine hyperthermophiles are making them interesting for novel biotechnological applications. For instance, the hyperthermophilic enzyme isolated from the archaeal species *Pyrococcus furiosus* is now commercially utilised as *Pfu* DNA polymerase [288]. Besides these perspectives, human methanogenic archaea belonging to the phylum Euryarchaeota appear to be highly resistant to antibiotics that inhibit either only bacteria or only eukaryal, but not to antibiotics that were effective against both groups [289]. This feature makes them good candidates for novel antimicrobial activity screenings focusing only molecules affecting archaeal members.

The first studies on the sponge-associated microbiome found that the archaeal phyla Thaumarchaeota and Euryarchaeota are frequently present across various sponge species. Additionally, the established application of high-throughput sequencing of the 16S rRNA gene in sponge-microbe community studies revealed that Archaea are predominant in many different sponge taxa and environments, from the tropical regions to the deep sea [29, 281, 290]. While the latter Euryarchaeota can often be found in similar abundance and diversity in the surrounding seawater, research has highlighted the possible physiological importance of Thaumarchaeota for the sponge hosts. For example, in the two cold-water sponges *Geodia barretti* and *Phakellia ventilabrum* from Norway, several 16Sr RNA gene sequences belonging to Thaumarchaeota were found. These archaea are potentially involved in nitrification processes and, by quantitative PCR amplification of the ammonia-monooxygenase gene, were shown to have high transcriptional activity [57, 228]. These findings led to the overall assumption that ammonia-oxidising archaea are one of the main drivers of the nitrification processes present in many sponges. Concurring results from studies of the microbiomes associated with coral and ascidia support this general assumption [291–293].

Although certain archaea seem to serve important physiological functions within particular sponges and are often observed in high abundances within their hosts, a large-scale cultivation of sponge-associated archaea was not successful so far [164]. The only exception are a few Thaumarchaeota strains obtained from enriched cultures [294], thus highlighting the importance of sophisticated cultivation approaches, such as cocultivation and long-term cultivation experiments, in order to access these still underexplored microbes.

However, there is more than just the prokaryotic realm populating and interacting with the sponge host. Considering the already known symbiotic prokaryotic relationships, it is tempting to presume that also microalgae, fungi, protists and even viruses are important parts of the sponge holobiont, with putatively important roles as well.

For instance, another sponge-associated group of organisms with large biotechnological potential are eukaryotic fungi. A broad range of biological activities have been determined for bioactive compounds produced from sponge-derived fungi,

which comprise antibacterial, antiviral, anti-inflammatory and anticancer activities [98, 295]. Up to date, phylogenetic analyses of the 18S rRNA gene from fungi have uncovered 3 phyla, 32 orders and more than 120 genera associated with several sponges [295]. It is noteworthy that 18S rRNA gene analyses are usually accompanied by contamination from other eukaryotic sponge symbionts, which often impedes a comprehensive molecular screening of all sponge-derived DNA extracts. Compared to bacteria and archaea, the cultivation of sponge-associated fungi has been more successful, with hundreds of fungi being obtained by different cultivation approaches [164]. For example, the phylogenetic analysis of 80 cultured fungi derived from the sponge *H. simulans* yielded 19 genotypes, with some of these isolates showing antimicrobial activities.

Although the identification of sponge-associated fungi seems to be feasible and the culture-dependent results are promising, it is still not clear how extensive and specific the symbiotic relationship between fungi and sponges is. In fact, many fungi isolated from sponges are closely related to terrestrial relatives. Some sponge-associated fungi may be only temporary inhabitants of the sponges as a result of non-selective filtering of brackish, marine or terrestrial fungi and fungi-spores from the surrounding water column, in a similar fashion to bacteria. Moreover, the presence of fungi in sponges seems to vary considerably within the same sponge species. For example, contradictory results described the marine sponge *Myxilla incrustans* as either a rich [296] or a poor [297] source for fungal isolates. In this specific case, geographically different sampling locations of *M. incrustans* may have played a role regarding these contrasting results [164], which hints to a bigger influence of geography or local factors on the fungal community as compared to the relatively stable prokaryotic relationships. Even though there have been inconclusive results, transmission electron microscopy (TEM) investigations of sponge oocytes, results from culture in vitro of sponge primmorphs and single cells and the analysis of bioactive compounds support the general view that many sponge-derived fungi are truly associated with the sponge [295].

The abundance of viruses in the oceans is high and ranges from approximately ~3×10^6 viruses ml^{-1} in the deep sea to 10^8 viruses ml^{-1} in productive coastal waters, with an even higher abundance in marine sediments [298]. Marine viruses have great effect on community structures from a bacterial up to a eukaryotic point of view and are a major force of biogeochemical cycles [299, 300]. To date, viruses have not been extensively studied in sponges, but due to the filter-feeding nature of most sponges and the tremendous abundance of viruses in the oceans, there is a high chance of to date hidden interactions between sponges and viruses. A study by Claverie et al. [301] had at least shown that sponges can also be infected by mimiviruses, giant DNA viruses. First attempts in facilitating this research are made by constructing metagenomic analysis pipelines, like HoloVir [302], MetaVir2 [303], VIROME [304] and VMGAP [305] to decipher diversity and function of viruses in sponges and the marine environment in general. Investigating this vast mass of viral diversity is important for the development of new antimicrobial treatment strategies. It was very recently shown that genomic information of

viruses infecting multidrug-resistant *Klebsiella pneumonia* strains can be used to identify novel antimicrobials effective against these emerging pathogens [306].

5 Conclusions

Over a period of 4 years, from 2013 till 2017, an international network of young scientists has studied/is studying the multidisciplinary aspects related to sponge biotechnology. The research comprised a multitude of biotechnological approaches and techniques, such as the cultivation of sponges and sponge-associated symbionts, structure elucidation of sponge-derived secondary metabolites and the application of a vast range of (meta)genomic tools to explore the biology and pharmaceutical potential of the sponge holobiont. The applied research, which is connected to BluePharmTrain, has already resulted in the elucidation of previously unknown molecules and discovery of novel enzymes and gene clusters of secondary metabolites and identified and localised the actual producers of some of those compounds in the sponge holobiont by microscopy or metagenomic binning tools. In addition, a number of bacteria that belong to so-called ‘sponge-specific’ clusters have been isolated (unpublished BluePharmTrain data), and a number of metabolites, structurally very similar to sponge-derived metabolites, have been found from culturable free-living bacteria (i.e. [98, 128, 188, 191, 251, 307]). Thus, at this point, most of our breakthroughs are related to sponge-associated microbiota rather than to the sponges themselves. This is aligned with the developments in the pharmaceutical industry, which has shifted interest to microorganisms, and away from plants. This also has socio-economic consequences as microbes share genes worldwide, so one compound collected in one country can often be found in others according to Baas Becking’s famous quote: ‘Everything is everywhere, but the environment selects’. This will have significant impact on policies and institutional arrangements concerning the access and benefit-sharing regime under the Nagoya Protocol on the Convention on Biological Diversity.

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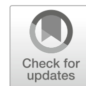
Chapter 14
ChiBio: An Integrated Bio-refinery for Processing Chitin-Rich Bio-waste to Specialty Chemicals



Volker Sieber, Michael Hofer, Wolfram M. Brück, Daniel Garbe, Thomas Brück, and Catherine A. Lynch

1 Marine Chitin: Sources, Structures, and Properties

Chitin is the second most abundant biopolymer in the world next to cellulose, with 10^{10}–10^{11} tons available [1, 2]. Chitin is a main constituent of fungal, yeast, and algal cell walls [3–5] as well as of cuticles of arthropods and such of crustacean shells. Worldwide more than 13,000,000 tons of crustaceans are caught from marine habitats each year giving rise to a substantial source of chitin of marine origin. Up to 50% of this catch is shell waste [6] and consists of 30–40% protein, 30–50% calcium carbonate, and 20–30% chitin, depending on the species and with seasonal variations [7]. Chitin itself is a linear polysaccharide and is composed of two subunits:

V. Sieber (✉)
Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Straubing branch, Straubing, Germany

Technical University Munich, Munich, Germany
e-mail: sieber@tum.de

M. Hofer
Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Straubing branch, Straubing, Germany

W. M. Brück
Institute of Life Technologies, University of Applied Sciences Western Switzerland, Sion, Switzerland

D. Garbe · T. Brück
Industrial Biocatalysis Group, Technical University Munich, Munich, Germany

C. A. Lynch
Letterkenny Institute of Technology, Letterkenny, Ireland

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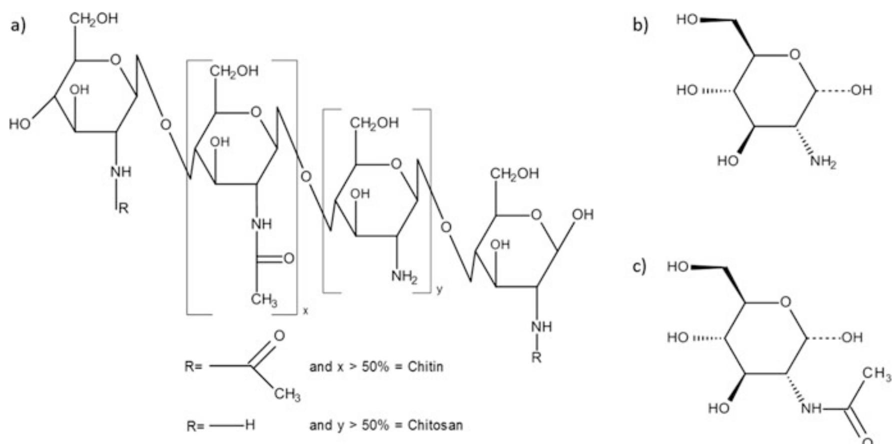

Fig. 14.1 Chemical structures of (**a**) chitin/chitosan, (**b**) glucosamine, and (**c**) N-acetylglucosamine

D-glucosamine and *N*-acetyl-D-glucosamine (Fig. 14.1). In general chitin has a ratio of 90:10 of *N*-acetyl-D-glucosamine to D-glucosamine, which varies depending on the chitin source [8–10]. Due to its high acetylation degree, chitin is hydrophobic and therefore insoluble in water and most organic solvents [11, 12]. The acetylation degree is responsible for the physicochemical properties. By deacetylation these properties can be changed. In general acetylation degrees above 50% are responsible for the insolubility of chitin, while acetylation degrees below 50% make the material generally soluble in acidic aqueous solvents and are used to differentiate chitosan from chitin. Besides its solubility the deacetylation degree is also influencing flexibility, polymer conformation, chemical reactivity, viscosity, and bioactivity [13–15]. Structurally chitin is similar to cellulose but closely associated with proteins, calcium carbonate, lipids, and pigments in crustacean shells [16]. As cellulose in plants, chitin is responsible for the structural integrity in crustaceans. Based on the crystal structure, chitin occurs in three distinct polymorphic forms: alpha-, beta-, and gamma-chitin. The difference is in the arrangement of the molecular chains. Alpha-chitin, the by far most abundant form, has an antiparallel chain arrangement, while in beta-chitin the chains are arranged in parallel. Gamma-chitin is a mixture of the alpha and beta form [17–19].

Chitin and chitosan have attracted huge interest for a wide range of different applications due to their excellent properties as biocompatible material. Both are vulnerable to enzymatic hydrolysis by lysozyme, and it was also shown that lipases in human fluids are able to hydrolyze chitosan [20]. These activities lead to nontoxic degradation products, showing the biodegradability of chitin and chitosan, which is an important factor for applications in medicine and pharmacy.

2 Chitin and Chitosan Applications

Chitin and chitosan attracted huge interest for a number of applications due to their unique properties such as biodegradability, biocompatibility, and nontoxicity especially in the field of medicine and pharmacy. Mainly chitosan- and chitin-derived products, such as oligosaccharides or glucosamine and *N*-acetylglucosamine, are used. In 2015 the chitosan market had a volume of USD 63 billion, and the amount of chitosan on the market was 13,700 tons [21], which is expected to increase to 124,000 tons and USD 4.2 billion [22] in 2020.

2.1 Applications in Medicine and Pharmacy

Chitin but mainly chitosan has attracted huge attention in this field. As investigations have shown, chitosan is able to interact with plasma proteins and blood cells through its free amino groups, leading to the formation of clots [23] and activating the complement and blood coagulation system [24–26] as polymeric contact material. Water-soluble chitosan and chitosan oligomers show this activity only after their sulfatation. In combination with the biocompatibility, biodegradability, and low toxicity, chitin and chitosan are therefore very attractive materials for the treatment of wounds and burns [27]. The adhesive properties of chitosan in combination with its antimicrobial effect and oxygen permeability led to numerous patents and products on the market [28].

In pharmacy, chitosan is widely used for the preparation of drug delivery systems. Although alone chitosan films showed only a limited use due to the low release control of these systems, in combination with hydrophilic polymers, membranes with excellent properties for drug release can be prepared [29]. Therefore, chitosan was mixed with pectin, alginate, or polyacrylate to form polyelectrolyte complexes for the development of controlled release systems [30]. Chitosan-xanthan microspheres have been described for the delivery of drugs to the gastrointestinal tract, according to their biodegradability and pH sensitivity [31]. Nanoparticles of chitosan have been developed as drug delivery system for the nasal mucosa, increasing drug penetration into the human body [32]. Plenty of other reports describe the usage of chitosan as well as chitin in film, gel, or powder form for drug delivery systems, showing the huge potential of these molecules [33, 34].

2.2 Applications in Agriculture

Chitin and chitosan are already widely used in agriculture for protecting plants from infections by pathogenic fungi, bacteria, and viruses but also for the improvement of soil. Especially, chitin and chitosan oligomers have been shown to have a direct anti-

pathogenic effect on all types of microbes and are potent inducers of the plant protection system [35]. In the soil chitin supports the growth of symbiotic plant partners and chitin-degrading microorganisms [36]. Through their chitin-degrading activity, these microorganisms reduce the growth of pathogenic fungi and nematodes [37]. In addition, chitin and chitosan have a beneficial effect on plant growth by improving plant metabolism and resulting in higher yields.

2.3 Application in Wastewater Treatment

Chitosan is widely used as water treatment agent. Due to its polycationic nature, it has excellent flocculation properties and can also be used as chelating agent. It binds a variety of metal ions like Hg^{2+}, Cu^{2+}, or Zn^{2+} [38], dyes [39], hydrocarbons [40], and organic residues in wastewater and effluent sludge [41]. Organic- as well as mineral-contaminated water can be cleared, and in combination with its biodegradability, chitosan is often the method of choice.

2.4 Application in Food and Cosmetics

The *N*-acetylglucosamine monomer from chitin is present in human milk and improves the growth of *Bifidobacterium* sp. in the human gut. Disturbance of the bifidobacterial flora leads to lactose intolerance, because they are responsible for the lactase production. Investigations showed that the addition of chitinous material to food increases the tolerance toward whey-containing products [29]. Chitin and chitosan are also used for the preservation of food [42] or as antioxidants [43]. Chitosan blocks the absorption of dietary fat and cholesterols, reducing the overall cholesterol value [44]. Additionally, it has been shown that chitosan contributes weight and body fat loss in human [45]. Therefore, chitosan has the potential to increase the nutritional value of food. Chitosan is also employed as additive in creams and lotions due to its antimicrobial activity and as thickening agent [46]. It was also tested as additive for nail lacquers.

3 Chitin and Chitosan Production

Up to 50% of the total weight of crustacean, such as shrimps, crabs, or lobsters, consists of shell material. This waste product of the fishery industry is currently the most important source for chitin and chitosan production. For the extraction of the valuable chitin from the shell materials, harsh conditions are necessary to break up the complex protein-chitin-calcium carbonate structure, which guarantees the exoskeleton stability. Over the last decades, several chemical as well as biological

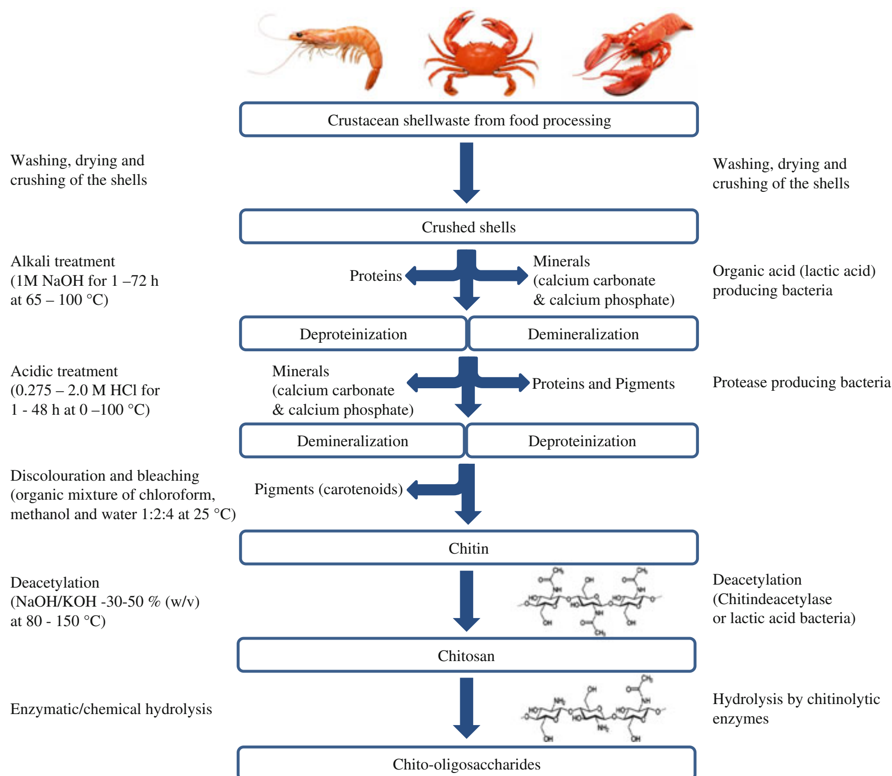

Fig. 14.2 Extraction of chitin from crustacean shell waste by chemical and biological methods

processes were developed to prepare pure chitin and chitosan from shell waste material and are commercially used (Fig. 14.2). The processing steps determine the properties of the final product, such as purity, acetylation degree, and molecular weight. Depending on the process parameters, the resulting products have different properties which have a strong impact on their applicability. All processes follow in general the same route from deproteinization (DP) and demineralization (DM), discoloration (DC), and in the case of chitosan production to deacetylation (DA).

3.1 Classical Chemical Processing of Crustacean Shells for Chitin and Chitosan Production

DP is performed by alkaline treatment using high amounts of NaOH and increased temperatures. DM is performed by acid treatment using HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH, with HCl as preferred agent [8, 47]. Although NaOH and HCl treatment results in nearly complete removal of proteins and minerals, these

harsh conditions lead to undesired side reactions such as polymer hydrolysis and deacetylation, impairing the final product quality [48–51]. Different chitin qualities can therefore be explained by the used chemicals in their corresponding processing steps. Pigments, mainly melanin and carotenoids, are finally removed by treatment with potassium permanganate, hydrogen peroxide, sodium hypochlorite, or organic solvents resulting in a white colorless product. The final step in chitosan preparation is performed either by a harsh alkaline hydrolysis treatment or by enzymes. Chitosan with different deacetylation degrees can be obtained by the variation of process temperature and incubation time. The most important parameters in chitin and chitosan synthesis are the final product quality in terms of molecular weight, acetylation/deacetylation degree, and polydispersity. As already mentioned, the usage of harsh chemicals such as HCl and NaOH in the processing of chitin can have a negative impact on the product quality, limiting its use in applications. Quality improvements can be achieved by using stirred reactors to increase the contact between the material and the chemicals and therefore reducing reaction times and needed reaction temperature [13]. Besides the product quality impacts of the used chemicals, they also create a waste problem, since neutralization and decontamination of the wastewater are necessary. Therefore, the chemical processing of crustacean shell waste is neither sustainable nor environmentally friendly. In view of these disadvantages, eco-friendly, efficient, and economically viable technologies are needed.

3.2 Biotechnological Processing of Crustacean Shells for Chitin and Chitosan Production

An alternative to the chemical extraction route for chitin is to use biological approaches. The biological methods for chitin extraction follow the same route as the chemical ones (Fig. 14.2). By using enzymes and microorganisms instead of harsh chemicals, the biological processing of crustacean shell waste has the advantages of being more eco-friendly, safe, technologically flexible, and economically feasible [52, 53]. Ensiling of these waste materials is the oldest method using lactic acid-producing microorganisms [54]. By using low-cost substrates, such as lignocellulose, an economically feasible processing is possible. The lactic acid produced by the microorganisms during ensiling is responsible for the demineralization by reacting with calcium carbonate forming calcium lactate, which can be precipitated and removed. Through the lowering of the pH, proteases are activated, which then catalyze the deproteinization, leading to a liquefaction of the nonsolid compounds of crustacean shell waste. The liquor is rich in proteins, amino acids, fats, and carotenoids [55]. Additionally, the low pH suppresses the growth of spoilage microorganisms. Besides these pure microbial processes, several studies examined the usage of commercial enzymes for deproteinization [56, 57] or demineralization [58] alone

or in combination with microbial agents [59, 60]. Also the combination of lactic acid fermentation with chemical steps has been investigated [61–63]. In general, three different strategies for the biological deproteinization and demineralization of crustacean shell waste were developed: (1) lactic acid fermentation, (2) non-lactic acid fermentation, and (3) co-fermentation. Table 14.1 gives a comprehensive overview of studies in these fields. In the following section, only selected examples are presented.

3.2.1 Lactic Acid Fermentation

Starting from shrimp waste, Rao et al. [66] used *L. plantarum 541* and controlled the pH during fermentation. By using acetic acid at a pH of 6 and a surplus of glucose, approximately 75% DP and 86% DM could be achieved. In the absence of pH control, only 68% DP and 64% DM were reached. Fermentation of minced scampi waste (*Nephrops norvegicus*) using *Lactobacillus paracasei A3* and added glucose resulted in 77.5% DP and 61% DM after 5 days at 30 °C, while the solid fraction contained 17.5% chitin (dry mass) [88]. Crayfish waste was also used in fermentation with *L. paracasei* and dextrose yielding 94% DP and 97% DM [89]. A mixture of proteolytic enzyme-producing bacteria (*L. plantarum, L. salivarius, S. faecium*, *and P. acidilactici*) was used for the anaerobic fermentation of prawn [90]. This showed to be an effective method to break down shell waste. 91% DM efficiency was reached on average in combination with nearly unmodified chitin which was identified through elemental analysis measuring the nitrogen content of the resulting material.

3.2.2 Non-lactic Acid Fermentation

The amount of acid and proteases produced by *Bacillus subtilis* during fermentation allowed shell demineralization as well as deproteinization. Using shrimp shell waste (*Metapenaeopsis dobsoni*), the fermentation process yielded 84% DP and 72% DM [91]. *Pseudomonas aeruginosa* strain K-187 produces proteases as well as chitinases and lysozyme during cultivation with shrimp and crab shells. After 5 days DP reached 82% in a solid-state process [92]. Using *P. aeruginosa F722* with crab shell wastes at 30 °C, 92% DM and 63% DP after 7 days were reported [93]. The deproteinization and demineralization of crab shell waste using *Serratia marcescens FS-3* reached 47% and 84% after 7 days. In combination with 1% Delvolase®, the deproteinization could be increased from 47 to 90%. For the degradation of shrimp shell waste, *Bacillus cereus* and *Exiguobacterium acetylicum* were used in a fermentation at 37 °C [94]. Deproteinization yielded 97 and 93% and demineralization 95 and 92%, showing the huge potential of these strains for chitin-rich waste processing.

Table 14.1 Fermentation-mediated extraction of chitin from crustacean shell wastes

Waste source	Strains and/or proteolytic enzymes	DP	DM	Refs.
Lactic acid fermentation				
Penaeus sp.	*Lactobacillus* spp. B2	85	87.6	[64]
Demineralized *Nephrops norvegicus*	Stabisil: *Streptococcus faecium* M74, *L. plantarum, Pediococcus acidilactici*	40	n.d.	[61]
Nephrops norvegicus	Sil-All$^{4 \times 4}$: *L. plantarum, L. salivarius, S. faecium, P. acidilactici*	n.d.	90.99	[65]
Nephrops norvegicus	*L. paracasei* A3	77.5	61	[63]
One-step shrimp fermentation	*L. plantarum* 541	75	86	[66]
Pretreated *Procambarus clarkii* (crayfish)	*L. paracasei* A3	94	97.2	[67]
Procambarus clarkii	Immobilized *Lactobacillus pentosus* 4023	81.5	90.1	[68]
Chionoecetes japonicus	*L. paracasei* ssp. *tolerans* KCTC-3074	54.7	55.2	[69]
Parapenaeus longirostris	*L. helveticus*	91	44	[70]
Shrimp shell	*Lactobacillus plantarum* PTCC 1058	–	82% (date syrup) 75% (glucose) 71% (sucrose)	[71]
Shrimp bio-waste	Non-amylolytic strain *L. plantarum* 541	59.8	81.4	[72]
	Amylolytic strain *L. plantarum* A6	52.2	65.5	[72]
Crab shell (CS) waste	*L. paracasei* subsp. *tolerans* KCTC-3074	–	89–92	[73]
The teguments of white shrimp, *Parapenaeus longirostris*	*L. helveticus* strain Milano	76	60	[70]
Non-lactic acid fermentation				
Metapenaeus dobsoni	*Bacillus subtilis*	84	72	[74]
Shrimp and crab shell	*Pseudomonas aeruginosa* K-187	82	–	[75]
Shrimp and crab shell powder	Proteases of *P. aeruginosa* K-187	72	–	[76]
Natural shrimp shells	Immobilized proteases of *P. aeruginosa*	78	–	[76]
Acid-treated natural shrimp shell	Immobilized proteases of *P. aeruginosa*	45	–	[76]
Shrimp and shell crab powder	Immobilized proteases of *P. aeruginosa*	67	–	[76]
Crab shell powder	*P. aeruginosa* F722	63	92	[74]

(continued)

Table 14.1 (continued)

Waste source	Strains and/or proteolytic enzymes	DP	DM	Refs.
Chionoecetes opilio (natural crab shell waste)	*Serratia marcescens* FS-3	47	84	[77]
	Delvolase®	90	–	[77]
	Combination of Delvolase® and *Serratia marcescens* FS-3	85	–	[77]
	S. marcescens FS-3 supernatant culture	81		[77]
Shrimp shell waste	*Bacillus cereus*	97.1	95	[78]
	Exiguobacterium acetylicum	92.8	92	[78]
Squid pen	*Bacillus sp*. TKU 004	73	n.d	[79]
Penaeus monodon	*Pediococcus acidilactici* CFR2182	97.9 ± 0.3	72.5 ± 1.5	[52]
Shrimp shells	*Pediococcus sp*. L1/2	n.d.	83	[80]
Fresh shrimp waste (FSW) or shrimp waste powder (SWP)	*Pseudomonas aeruginosa* A2	56% and 85% for SWP and FSW	–	[81]
Shrimp waste	Crude alkaline proteases extract from the viscera of the striped seabream (*Lithognathus mormyrus*)	79	–	[82]
Shrimp waste	*Bacillus cereus* SV1	88	–	[83]
Co-fermentation				
Two-step fermentation of *Penaeus monodon* and *Crangon crangon*	First step: anaerobic deproteinization by autochthonous flora of Indonesian shrimp shells and/or proteolytic bacteria	97.4	99.6	[84]
	Second step: *L. casei* MRS1	90.8	99.7	[84]
Prawn waste	*Lactobacillus lactis*	66.5	78.8	[85]
	Teredinibacter turnerae	77.8	23.3	[85]
	Co-fermentation of both species	95	95	[85]
Red crab shell waste	One-step fermentation: *L. paracasei* ssp. *tolerans* KCTC-3074 and *S. marcescens* FS-3	52.6	97.2	[86]
	Successive two-step fermentation	94.3	68.9	[86]
	Two *Bacillus licheniformis* strains with treatment of the final fermentation product with 0.9% lactic acid	99	98.8	[87]

3.2.3 Co-fermentation

For the extraction of chitin from prawn waste, lactic acid-producing bacterium *L. lactis* and a protease-producing bacterium *Teredinibacter turnerae* were combined. Both bacteria were cultivated separately and used in combination for fermentation. *L. lactis* alone reached a DP of 66% and DM 79%, while *T. turnerae* alone reached 78% DP and 23% DM [95]. The co-fermentation of *L. paracasei* ssp.

tolerans KCTC-3074 with *S. marcescens FS-3* yielded a DM level of 97% but only a DP of 53% after 7 days with red crab shell waste [71]. By using both strains in a successive two-step fermentation process, the DP efficiency was nearly constant at 94% and the DM efficiency was increased to 69% [96]. Two from shrimp shell waste isolated *B. licheniformis* strains were used for the fermentation of shrimp shells [87]. After optimization of fermentation parameters and a subsequent demineralization step with 0.9% lactic acid after fermentation, 99% DP and 99% DM could be reached.

4 ChiBio: A Chitin Bio-refinery

In general a bio-refinery is the sustainable processing of biomass into a spectrum of marketable products. At the beginning of the development of bio-refineries, all kind of biomass was used, especially for the production of biofuels, raising the food or fuel discussion [97]. Nowadays, novel-developed bio-refinery concepts only use biomass waste streams, e.g., straw, as starting material [98]. Within the EU-funded project ChiBio, a bio-refinery on the basis of crustacean shell waste was developed. It aims at the development of a novel bio-refinery process for a sustainable, waste-free, low energy conversion route of negative value crustacean shell waste streams into high-value, high-performance chemical intermediates and products for the polymer industry. As stated before, crustacean shells are a waste product of food production from shrimps, prawns, or lobsters all over the world. Most of this shell waste is dumped into the sea, which is a main pollutant to coastal areas. In Europe, sea dumping is forbidden, and a cost-intensive proper waste management is a burden for the fishery industry [99]. There are more than 300,000 t/a of shell waste material available in Europe alone, showing the potential of this yet under-explored biomass. As processing of crustacean shell wastes is currently mainly chemically based with all the negative environmental and ecological impact, the ChiBio project targeted the development of a bio-refinery concept using state-of-the-art biotechnological methods for the processing of crustacean shell waste in a sustainable and eco-friendly manner (Fig. 14.3). Starting from the raw material, an effective biological treatment of the shell waste was developed yielding chitin, calcium carbonate, and a protein- and lipid-rich liquid. Chitin is further processed into its basic building blocks glucosamine and *N*-acetylglucosamine by enzymatic degradation. Both molecules were used as substrates for the production of novel bio-based monomers, which were finally evaluated in polymer materials to reveal novel applications in the material sector. All by-products of the ChiBio bio-refinery, e.g., calcium carbonate and the protein- and lipid-rich liquid, are valuable side products for their usage in the construction and the biogas industry. In connection with a life cycle analysis, ChiBio showed that a sustainable bio-refinery concept for crustacean shell waste is feasible and sustainable.

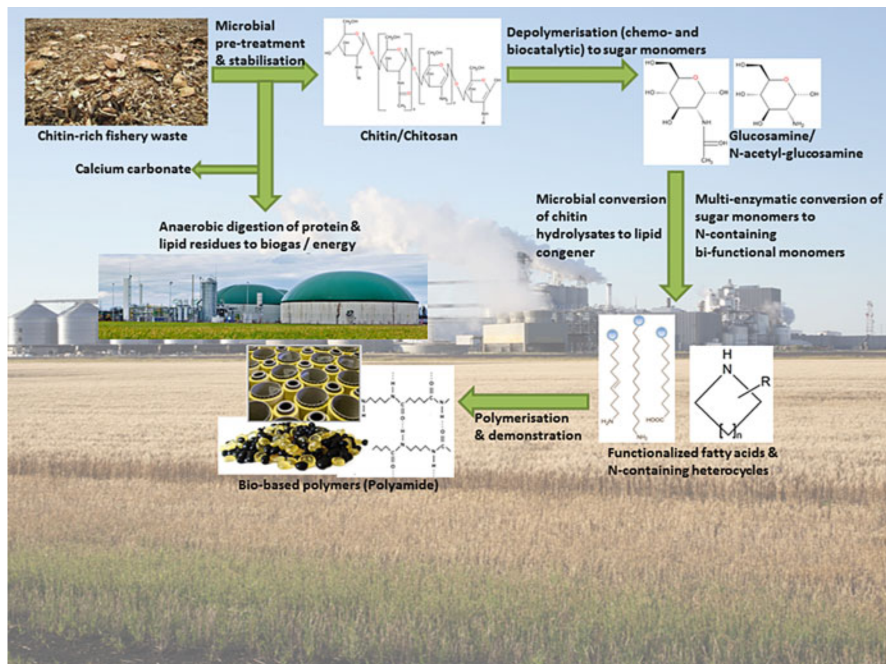

Fig. 14.3 Schematic overview of the ChiBio bio-refinery process. Chitin-rich crustacean shell waste is deproteinized and demineralized by microorganisms to chitin/chitosan. As by-products calcium carbonate and a liquid rich in protein and lipids are obtained, which are raw materials for the construction industry and for the production of biogas. Chitin and chitosan are further depolymerized to their basic building blocks glucosamine and N-acetylglucosamine. Through microbial and enzymatic conversion technologies, these building blocks are transformed into functional fatty acids and N-containing heterocycles, which are suitable building blocks for the production of novel bio-based polymers

4.1 Pretreatment

For the pretreatment step developed within ChiBio, we compared the demineralization and deproteinization of brown crab shell using conventional chemical methods and optimized biological methods using commercial *Lactobacillus* spp*., Pseudomonas aeruginosa,* and *Serratia marcescens* strains in addition to proteolytic and acid-producing bacterial isolates from crab shell waste.

An initial survey of the mineral content of obtained brown crab shell waste showed that calcium in the form of $CaCO_3$ was by far the most abundant mineral.

In 5-day fermentations utilizing the mixed exogenous microbiota present on the shell, ash mineral content and FTIR results showed that self-fermented crab shells were neither fully decalcified nor deproteinized. At the end of the fermentation, a pH

of 5.7 ± 0.4 was observed with a yield of chitinous material of 31.80 (±0.07) %, an ash content of 45.00 (±1.20) %, and a calcium content of 3.4 (±0.5) g per 100 g. Untreated crab, in comparison, contained 66.30 (±0.06) % ash and 4.3 (±0.1) g per 100 g calcium. Chemically produced chitin from brown crab treated using 1 mol/l hydrochloric acid and 1 mol/l sodium hydroxide yielded 15.19 (±0.04) % chitinous material with 4.50 (±0.05) % ash and 0.94 (±0.36) g per 100 g calcium [100]. During a 5-day fermentation using 1×10^6 cfu ml^{-1}, each of a combination of *Bacillus cereus* and *Pseudomonas spp.* isolated from crab shell, resulted in 98.8% demineralization with an overall yield of chitinous material of 15.4 (±1.56)%. The degree of acetylation was 81.9 (±1.0) % as calculated from FTIR analysis [101]. Experiments were performed using either 5 or 10 g of shell waste in 100 ml of a 10% (w/v) glucose solution. All of these initial experiments were performed in a shaking incubator at 175 rpm and 30 °C using a shell particle size ranging from 250 to 750 μm.

One-step fermentations at 30 °C using 1×10^6 cfu per ml of commercial strains *Serratia marcescens* (DSMZ 30121), *Pseudomonas aeruginosa* (DSMZ 8924), or *Pseudomonas aeruginosa* (DSMZ 7232) yielded unsatisfactory results for either deproteinization or demineralization. Subsequently, further trials using sequential fermentations incorporating 1×10^6 cfu ml^{-1} of the lactic acid bacterium *Lactobacillus plantarum* subsp. *plantarum* (DSMZ 20174) to further reduce the overall pH of the reaction were performed. The most effective fermentation was obtained in a sequential two-step fermentation using *S. marcescens* (DSMZ 30121) for 5 days and *L. plantarum* subsp. *plantarum* (DSMZ 20174) for 7 days, both at 30 °C in a shaking incubator at 180 rpm. The use of these organisms successfully decreased the pH rapidly from pH 6.07 ± 0.2 from the first (*S. marcescens*) fermentation to 4.6 ± 0.3 using *Lactobacillus plantarum* subsp. *plantarum*. No difference in acid production was observed between 8% (w/v) and 10% (w/v) glucose. The decrease in pH was rapid enough to inhibit the endogenous microbiota as observed by plate culture. The overall yield of chitinous material from the whole brown crab by the two-step fermentation was 19 (±0.02) %, an ash content of 7.0 (±0.1) %, and a calcium content of 2.1 (±1.0) g per 100 g. The degree of acetylation was 82 (±11) % as calculated from FTIR analysis [100]. This was comparable to the yield obtained with native microorganisms isolated from crab shell and from other studies [102]. Like the traditional chemical treatment using 1 mol/l hydrochloric acid and 1 mol/l sodium hydroxide, the production of acids using the facultative heterofermentative and aero-tolerant *L. plantarum* also allowed for successful removal of $CaCO_3$ by predominantly producing D- and L-lactate by stereospecific lactate dehydrogenase enzymes [103]. In literature, demineralization efficiencies of 94.3% and >99% were commonly achieved by lactic acid-producing bacteria and with ±10% glucose as a carbon source [56, 84]. Demineralization efficiency, as seen by FTIR spectroscopy, of 93% using 8% glucose was achieved using the methods established by ChiBio. *S. marcescens* was used to effectively remove residual proteins from the shells through the production of extracellular proteases without affecting the degree of acetylation from endogenous chitinases [86]. A successive two-step fermentation *using S. marcescens* followed by *L. plantarum* was required to achieve optimal

results for the removal of residual proteins and minerals from brown crab since co-fermentations were ineffective due to differing acid tolerances and generation times under prescribed conditions. The order of the fermentative organisms applied was important as demineralization proved more effective when the shell was already deproteinized. Fermentation times were comparable to other studies demonstrating that the use of proteolytic organisms and lactic acid fermentation could provide a viable alternative to chemical treatments for the extraction and recovery of chitinous materials even though the calcium content is still higher than in commercial samples available today. However, the process costs for microbial chitin recovery as demonstrated here do not support the recovery of chitin from crustacean waste using a mainstream carbon source such as glucose even though cost analysis revealed similar expenditures to chemical production (approx. USD20/kg [100]). Other low-cost carbon sources such as molasses, whey, corn steep liquor, lignocellulose, and other waste streams could be potentially substituted and could in turn significantly reduce production costs.

4.2 Enzymatic Chitin Depolymerization

The microbial processed chitin was used as starting material for the further degradation into its basic building blocks glucosamine and *N*-acetylglucosamine. Two strategies were followed to yield enzyme systems for the targeted degradation of chitin: (1) chitinolytic enzyme cocktails from chitin-degrading microorganisms and (2) cloning and heterologous expression of single chitin-degrading enzymes for their usage in defined enzyme reactions.

4.2.1 Chitinolytic Enzyme Cocktails

Chitin-degrading microorganisms are well-known, and in-depth analysis of their enzymatic machinery has been done for some microorganisms [104, 105]. *Serratia marcescens* is one of the best known organisms, with well-defined chitinolytic enzyme machinery. It includes four enzymes, ChiA and ChiB (EC 3.2.1.14), which are in opposite direction working chitinases; ChiC (EC 3.2.1.14), an endo-acting non-processive chitinase; and CBP21 (EC 1.14.99.B7), a lytic polysaccharide monooxygenase that acts through oxidative cleavage. These enzymes degrade the chitin polymer into short-chain oligomers, which are then processed by the enzyme chitobiase, *N*-acetylhexosaminidase, into monomeric *N*-acetylglucosamine [106]. Besides *S. marcescens*, also *Cellvibrio japonicus*, *Amantichitinum ursilacus* (*A. ursilacus*), and *Andreprevotia ripae* were analyzed for their chitinolytic potential within ChiBio [107, 108]. In a first step methods for the production of chitinolytic enzyme cocktails of the natural strains were developed, which were then used for the development of a chitin degradation process. The strains were cultivated under standard conditions using M9 media with 2% chitin (w/v) at 37 °C under shaking

for 5 days. The broth was then centrifuged to remove cells and chitin, and the supernatant was filtered through 0.22 μM filters and used as chitinolytic enzyme cocktail. The final aim was to generate hydrolysates containing the chitin monomers glucosamine and *N*-acetylglucosamine from the pretreated crustacean shell waste. The best results were obtained with the chitinolytic enzyme cocktail from *S. marcescens*, hydrolyzing up to 77% of the used chitin at 50 °C and pH 6 after 24 hours. To reach this degree of depolymerization, it was necessary to mill the material to a fine powder with an approximate particle size of 0.2 mm, larger particles reduced the efficiency. In addition the used chitin was from a chemical pretreatment step. Using biologically derived chitin reduced the yield to 20% under the same conditions.

4.2.2 Chitin-Degrading Enzymes

All described enzymes from *S. marcescens* were overexpressed in *E. coli* and purified by affinity chromatography [109]. For optimization of the mono-component enzymes, the amount of ChiA, ChiB, ChiC, and CBP21 was varied from 0 to 90% and analyzed in combination with varying process parameters (pH, temperature, and incubation time). Depending on the chitin source and the pretreatment method, different enzyme cocktails yielded optimal conversion rates (Table 14.2). For chemically pretreated brown crab shell chitin, a monomer yield of 57.3% could be achieved (ChiA 30.4%, ChiB 28.1%, ChiC 10.6%, CHB 10.1%, and CBP21 20.8%). On the other hand, biologically pretreated brown crab shell chitin only yielded 45.9% monomer (ChiA 39.6%, ChiB 28.9%, ChiC 21.0%, CHB 10.0%, and CBP21 0.5%). For comparison commercially available chitin yielded 61.7% monomer (ChiA 38.1%, ChiB 29.7%, ChiC 14.0%, CHB 10.2%, and CBP21 8%). The data shows the potential of the enzymatic toolbox for the targeted degradation of chitin. In addition this data clearly reveals that independent on the chitin composition, different enzyme mixtures are necessary to maximize the monomer yields.

Although neither chitinolytic enzyme cocktails nor mono-component enzyme mixtures gave full conversion of chitin into its monomers, these tools open the route for the production of glucosamine and *N*-acetylglucosamine. The combination

Table 14.2 Summary of optimization of chitin active enzymes for maximal degradation of benchmark substrates

Chitin source	Pretreatment	ChiA (%)	ChiB (%)	ChiC (%)	CHB (%)	CBP21 (%)	Yield (%)
Commercial chitin	Chemical	38.1	29.7	14	10.2	8	61.7 ± 3.5
Brown crab shells	Chemical	30.4	28.1	10.6	10.1	20.8	57.3 ± 1.7
Brown crab shells	Biological	39.6	28.9	21.0	10.0	0.5	45.9 ± 1.5

of both systems might be a way to realize the full conversion of chitin into its basic building blocks.

4.3 Monomer Synthesis

The demand for plant- and animal-based lipids for the food and pharmaceutical industry and for the production of biofuels has driven the search for new sources of relevant lipids, such as the very-long-chain omega-3 (ω-3) polyunsaturated fatty acids (VLC-PUFAs), eicosapentaenoic acid (EPA; 20:5 Δ5,8,11,14,17), and docosahexaenoic acid (DHA; 22:6 Δ4,7,10,13,16,19). To date, these PUFAs are derived primarily from fish and crustaceans, which results on a negative impact on the marine food chain and related ecosystems [110, 111]. The need for more environmentally friendly alternatives of VLC-PUFAs has become more economically competitive driving forward the establishment of new technologies focused on providing ω-3 VLC-PUFAs from plant, algae, and yeast biomass. In particular, advances in process and metabolic engineering of oleaginous yeasts such as *Yarrowia lipolytica* take advantage of fast growth rates and high yields of designer lipids in combination with waste biomass feedstocks to offer a sustainable production approach for non-food lipid production [112, 113]. However, most *Y. lipolytica* are ex novo lipid producers and thus require fatty acids in their growth medium.

In the course of the ChiBio project, a new de novo lipid-producing yeast from a crab shell waste disposable site was isolated, which by 18S rDNA and ITS taxonomic identification was classified as *Trichosporon oleaginosus* (phylum *Basidiomycota*, order *Tremellales*). Initial physiological characterization showed that this strain could metabolize *N*-acetylglucosamine as well as a range of other pentose and hexose sugars without any metabolic preferences, making it ideal for fermentative high-value lipid production on cost-efficient waste biomass hydrolysates or alternative biotechnological waste streams [114]. Enzymatically liquefied crude crab shell hydrolysate proved to be a sufficient growth substrate for the yeast and de novo lipid accumulation. *N*-acetylglucosamine, a monomeric product of the enzymatic degradation of chitinous materials, was used as a carbon and energy source without adverse effects on de novo lipid biosynthesis, indicating high tolerance of higher nitrogen concentrations. However, due to the nitrogen content of *N*-acetylglucosamine, only phosphate limitation could be utilized for the induction of lipogenesis. Nonetheless, lipid accumulation was 35% (w/dcw) in *Trichosporon* fermentations after 72 hours and remained constant up to 168 hours. At 24 hours about 50% of the final lipid content was already present. However, with fermentation substrates where *N*-limiting conditions could be applied, *Trichosporon* sp. was able to accumulate up to 60% (w/dcw) triglycerides. The lipid fraction based on crude crab shell hydrolysate as a feedstock consisted of 47% (w/w) oleic acid as principal fatty acid component which is a suitable building block in the polymer and lubricant industry. For the production of VLC-PUFAs such as alpha-linoleic and eicosadienoic acid, genetic engineering approaches of fatty acid biosynthesis

pathways were surveyed. With no detailed genome information and molecular biology tools at hand for *Trichosporon*, a random genome integration protocol was developed [115]. Initial gene transfer methods using conventional techniques, such as electroporation, were unsuccessful. However, in the course of the ChiBio project, an *Agrobacterium tumefaciens*-mediated DNA transfer protocol used in plant engineering was adapted for *Trichosporon* applications. Existing plasmid systems were modified and utilized reported promoters from related *Basidiomycetes*. These strategies allowed random genomic integration of recombinant genes into *Trichosporon* sp. However, the functional expression of the genetic elements was inefficient. Whole genome sequencing of *Trichosporon oleaginous* recovered the full-length sequence of native, constituent promoter systems [116]. The native *Trichosporon* promoter regulating the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase finally provided for strong, constitutive expression of recombinant genes [117]. Subsequently, various *Trichosporon* mutants that produce enzymatically modified fatty acid profiles could be generated. A detailed GC-MS-based fatty acid profiling of the *Trichosporon* mutants showed that in vivo desaturation of linoleic acid to yield alpha-linoleic acid and its congener eicosatrienoic (ETE) and eicosadienoic acid (EDA) was particularly successful.

4.4 Novel Bio-based Polymers from Chitin-derived Monomers

ChiBio aims to develop novel value chains by producing bio-based monomers for the polymer industry on the basis of chitin-derived building blocks. Therefore, ChiBio-derived building blocks were used for initial technical trials to produce polyamide test bars as demonstrators and to characterize their mechanical properties. From the two monomers, being 1.19-nonadecane dicarboxylic acid and an aromatic dicarboxylic acid (ADCA), respectively, the latter was chosen for such demonstrator activities. The co-polyamide 6.12 doped with 3% of the ChiBio ADCA had been polymerized at pilot-plant scale. The granulate polymer as obtained was then used to manufacture testing bars by injection molding as the demonstrator production series. It could indeed be molded under such common industrial conditions; however most machine parameters had to be adjusted to quite uncommon values. The demonstrator production series yielded a good number of test bars. With this batch of demonstrator parts, the physical mechanical properties of this ChiBio-influenced polyamide had then been scrutinized. These demonstrator specimens exhibited a sharp drop of ductility features compared to neat homo-PA6.12 (Table 14.3). Nevertheless, the co-polyamide could be processed under standard technical conditions, in the present case on common injection-molding equipment, albeit with processing parameters unusual for thermoplastic polymers. Toward future applications and developments, using ADCA as the comonomer in bulk machined parts appears unlikely, considering the lack of ductility performance. However, it might likely be fruitful when looking at

Table 14.3 Characteristic physical mechanical data of the bulk-produced polyamide (PA) 6.12, containing 3% of one of the ChiBio key monomers, ADCA

Properties	Measurement values	
E-modulus [MPa]	2900 ± 100	
Tension at break [MPa]	6.5 ± 0.1	
Elongation at break [%]	0.2 ± 0.1	
Impact strength (+23 °C and −30 °C, resp.; unnotched)	1.7 ± 0.1	1.3 ± 0.1
Impact strength (+23 °C and −30 °C, resp.; notched)	0.8 ± 0.1	0.5 ± 0.1

surface applications, given that this monomer would render a co-polyamide equipped with a high degree of heteroaromatic and NH functional chemical moieties.

4.5 Life Cycle Analysis

The utilization of crustacean waste for the production of value-added products is a viable option for the European fishery industry as about 60 w/w-% of the crustacean catch is accumulating as waste. Besides potentially profiting from selling value-added products, the saving of disposal costs which range from about 60 €/t for landfilling to 160 €/t for incineration could create an additional boost for the concept, and illegal ocean dumping could be avoided.

The main cost factors identified in the economic process analysis are the stirred tank reactors for the pretreatment, the *Lactobacillus* seed, the enzymatic depolymerization, and especially the monomer synthesis. Summarized, at this early state the process is not cost-efficient enough. The presented estimations are not integrating effects of experience and learning curves. As approximation learning rates decrease the unit costs of technologies by constant percentage for each doubling of experience. This process runs over time and leads to a decrease of unit costs [118]. A progress ratio of about 10–15% seems to be appropriate for a couple of technologies. A massive process development and further intense research on the process time and particularly enzyme efficiency could eventually result in a marketable price.

International reported prices range between USD6/kg for plain chitin and USD20/kg for pharma grade chitosan as a benchmark for the pretreatment section and raw material supply. Consequently the pretreatment of the raw material to yield the chitin is a key step in the process starting from a material with negative to low input price resulting to a significant price of pure chitin/chitosan. This cost structure and the various competing application of chitin/chitosan derivatives require an integrative bio-refinery approach including cost-effective biotechnological pretreatment as substitute for the harsh conditions and high chemical load in the chemical processing route. Despite positive results presented in the literature [68, 119, 120], an effective biotechnological pretreatment procedure could not be established within the project. Future research effort has to address this process step within the bio-refinery process chain.

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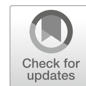
Chapter 15
Utilisation of Marine Genetic Resources (GRs): The Access and Benefit-Sharing Legal Framework



Arianna Broggiato, Laura E. Lallier, Aysegül Sirakaya, and Thomas Vanagt

Acronyms

ABNJ	Areas beyond national jurisdiction
ABS	Access and benefit-sharing
CBD	Convention on biological diversity
CNA	Competent national authority
EEZ	Exclusive economic zone
EU	European Union
GRs	Genetic resources
IRCC	Internationally recognised certificate of compliance
LOSC	Law of the sea convention
MAT	Mutually agreed terms

For further information related to the use of marine genetic resources for research and industry, please consult Leary, David et al. (2009) "Marine Genetic Resources (GRs): A Review of Scientific and Commercial Interest". Marine Policy 33(2), 183–194. Web. 14 June 2017.

A. Broggiato · T. Vanagt (✉)
eCOAST Marine Research, Oostende, Belgium

ABS-int, Sint Martens Latem, Belgium
e-mail: thomas.vanagt@ecoast.be

L. E. Lallier
eCOAST Marine Research, Oostende, Belgium

Department of European, Public and International Law, Faculty of Law, University of Ghent, Ghent, Belgium

A. Sirakaya
Department of European, Public and International Law, Faculty of Law, University of Ghent, Ghent, Belgium

ABS-int, Sint Martens Latem, Belgium

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MTA Material transfer agreement
NP Nagoya Protocol
PIC Prior informed consent

1 Introduction

One of the major challenges marine biotechnology deals with nowadays is compliance with the legal requirements related to access to genetic resources (GRs) and fair and equitable benefit-sharing (ABS) arising from the utilisation of these resources. The majority of scientists, practitioners, research institutions and biotechnology companies seem to agree on the fact that they are not prepared to be ABS compliant (PharmaSea Deliverable 6.5, Presentation on the preliminary results of survey and interviews). Therefore, this chapter that focuses on introducing the main aspects of ABS and its effect on the daily activities of R&D practitioners is of critical importance.

Genetic resources have many areas of application in both commercial and non-commercial R&D. Commercial users (such as pharmaceutical companies, biotech companies, companies specialised in agricultural products, cosmetics companies) often use genetic resources to develop specialty enzymes, for gene enhancement or for small molecules. Genetic resources are also used for non-commercial research (i.e. academic research) such as taxonomic research or when analysing ecosystems [1].

The initial step the practitioners need to take is to question whether or not they, their organisation or their activities have any implications related to ABS. They, for instance, need to check their ABS compliance when:

- They are returning from field trips, in their country or abroad, with genetic resources (plants, animals or microbes) or derivatives.
- They are receiving or transferring genetic resources from/to other research groups within their company or from/to other institutions/companies.
- Genetic resources they handle are deposited into collections.
- The company they work for is performing research and development, product development and commercialisation using genetic resources.
- The genetic resources maintained or used for research and development in their company are further used in in-house development or by a third party.

In all of these instances, practitioners and the organisations they work for must comply with the international, European and national ABS rules illustrated in this chapter. In this regard, particular attention should be given to the Convention on Biological Diversity (CBD) [2] and the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (Nagoya Protocol) [3], as well as the United Nations Convention on the Law of the Sea (LOSC) [4] considering the marine dimension of this book.

The present chapter is divided into three sections: in the first section the international and European legal instruments regulating ABS are introduced and their

main principles described, together with the relevant obligations under the law of the sea. The second section contains a schematic and simple step-by-step guide for practitioners willing to access marine genetic resources. The third section describes in more detail the users' compliance requirements adopted by the European legal framework dealing with ABS, containing important steps that practitioners have to fulfil when proceeding downstream in the R&D pipeline toward the development of a product derived from marine genetic resources. Since the international as well as the EU legal framework on ABS does not make a difference about the application of the framework between the terrestrial or marine genetic resources, the authors opt for explaining the user obligations related to genetic resources as a whole. However, the authors do provide insights on the special considerations related to marine genetic resources in particular.

2 Access and Benefit-Sharing Legal Framework and the Law of the Sea

2.1 Convention on Biological Diversity and the Nagoya Protocol

The Convention on Biological Diversity entered into force in 1993. The Convention addresses biodiversity through three main objectives: biodiversity conservation, sustainable use of biodiversity components and the fair and equitable sharing of the benefits arising out of the utilisation of GRs (article 1 CBD). The CBD explicitly recognises the sovereign rights of states to exploit their own resources in accordance with their own environmental policies. Thus, in the context of marine GRs, it is up to the coastal state where the GR would be sampled to choose whether or not to regulate access to it and under which terms.

The Nagoya Protocol entered into force on 12 October 2014. The Protocol provides a legal framework on the implementation of one of the three objectives of the CBD, which is the fair and equitable sharing of benefits arising out of the utilisation of genetic resources, thereby contributing to the conservation and sustainable use of biodiversity.

Since the adoption of the Nagoya Protocol, the topic of the use of genetic resources and access and benefit-sharing obligations related thereto has rapidly grown in terms of importance. This is due to the fact that the Nagoya Protocol obliges both the user and the provider. While the user of GRs must abide by the law of the provider country, the latter must ensure certainty and clarity of the requirements and measures for granting access to GRs. Below is the summary of the obligations enshrined in the Nagoya Protocol:

– *Access obligations*, if enacted, must be clear, transparent and fair and should provide rules and procedures for prior informed consent (PIC) and mutually agreed terms (MAT).

– *Benefit-sharing obligations* must be subject to MAT between provider and user, include research and development, subsequent applications and commercialisation, and may be monetary and/or non-monetary.
– *Compliance obligations* must be ensured through the adoption by user countries of legislative and regulatory measures, the respect of contractual obligations contained in MAT and the monitoring of the utilisation of GRs by user countries, including by the means of checkpoints.

2.1.1 Key Elements of Access and Benefit-Sharing

This section aims to provide clarity on the scope-related definitions found in the CBD and the Nagoya Protocol, as well as the EU ABS Regulation.

Definitions

As legal terminology is a language of its own, this part gives more precision on the meaning of terms used in the framework for ABS of marine GRs, which does not necessarily correspond to the scientific world's lexicon. In international law, it is common practice to find a provision relating to the definition of terms used throughout the text of a treaty, for clarity and interpretation purposes. The object (GRs), their use (utilisation and biotechnology) and the players (provider and user) are the main components of the regime hereby introduced.

Genetic Resources
Before the Nagoya Protocol, the CBD defined GRs in rather broad terms. The Nagoya Protocol and the EU ABS regulation kept the CBD definition as a basis.

[1]According to the CBD, the term "genetic resources" means genetic material of actual or potential value, where "genetic material" includes "any material of plant, animal, microbial or other origin containing functional units of heredity".

Utilisation and Biotechnology
The notion of utilisation of GRs was introduced under the Nagoya Protocol. This was done to provide a clearer view on when the ABS obligations begin. Article 2 of the Nagoya Protocol reads:

1. "'Utilization of genetic resources' means to conduct *research and development* on the *genetic and/or biochemical composition* of genetic resources, including through the application of *biotechnology* [. . .]".
2. "'Biotechnology' as defined in Article 2 of the [CBD] means any *technological application* that uses *biological systems, living organisms or derivatives*, thereof, to make or modify *products or processes for specific use*."

[1]Article 2 CBD.

3. "'Derivative' means a naturally occurring *biochemical compound* resulting from the *genetic expression or metabolism* of biological or genetic resources, even if it does not contain functional units of heredity".

The definitions are the result of the work of an expert group that was established to address terms and concepts during the negotiations of the Nagoya Protocol which is the Group of Legal and Technical Experts on Concepts, Terms, Working Definitions and Sectoral Approaches. The Group provided a list of possible "specific uses" of GRs as referred to in the definition of "biotechnology" [5]:

- Genetic modification
- Biosynthesis (use of genetic material as a "factory" to produce organic compounds)
- Breeding and selection
- Propagation and cultivation in the form received
- Conservation
- Characterisation and evaluation
- Production of compounds naturally occurring in genetic material (i.e. extraction of metabolites, synthesis of DNA segments and production of copies)

Provider and User

In the context of ABS of GRs, the two main players involved are the provider and the user. The provider is to be understood as the *country* that is "the country of *origin* of such resources or the party that *has acquired* the genetic resources in accordance with the CBD".[2] The user of GRs was not precisely defined until the EU ABS Regulation (see following paragraph) defined it as "a natural or legal person that *utilizes* genetic resources or traditional knowledge associated with genetic resources".[3]

The relationship between a provider and a user is very much based on negotiations in good faith to ensure the equitable sharing of benefits arising from the utilisation of GRs. The Protocol states that such sharing shall be made via mutually agreed terms (MAT). This clearly means that although a provider country is perfectly entitled to regulate access to its GRs and require benefit-sharing arrangements, it cannot impose such arrangements to the user in an arbitrary manner. Figure 15.1 shows the process, from targeting the GRs to their possible uses, may it be commercial or not. In between, the relationship of the provider and the user is tagged with PIC and MAT (see below for further details). Those are two steps that need to be taken by practitioners when they wish to become "users" of GRs in the meaning of the ABS regime. Those elements are further explained below. Please note that it is a country's sovereign right to *not* regulate access to its GRs, in which case obtaining PIC and MAT is not necessary, and the use of GRs from that country is free and unrestricted as far as the ABS framework is concerned (other restrictions might come from other legislations).

[2] Article 5(1) Nagoya Protocol.

[3] Article 3(4) EU Regulation 511/2014.

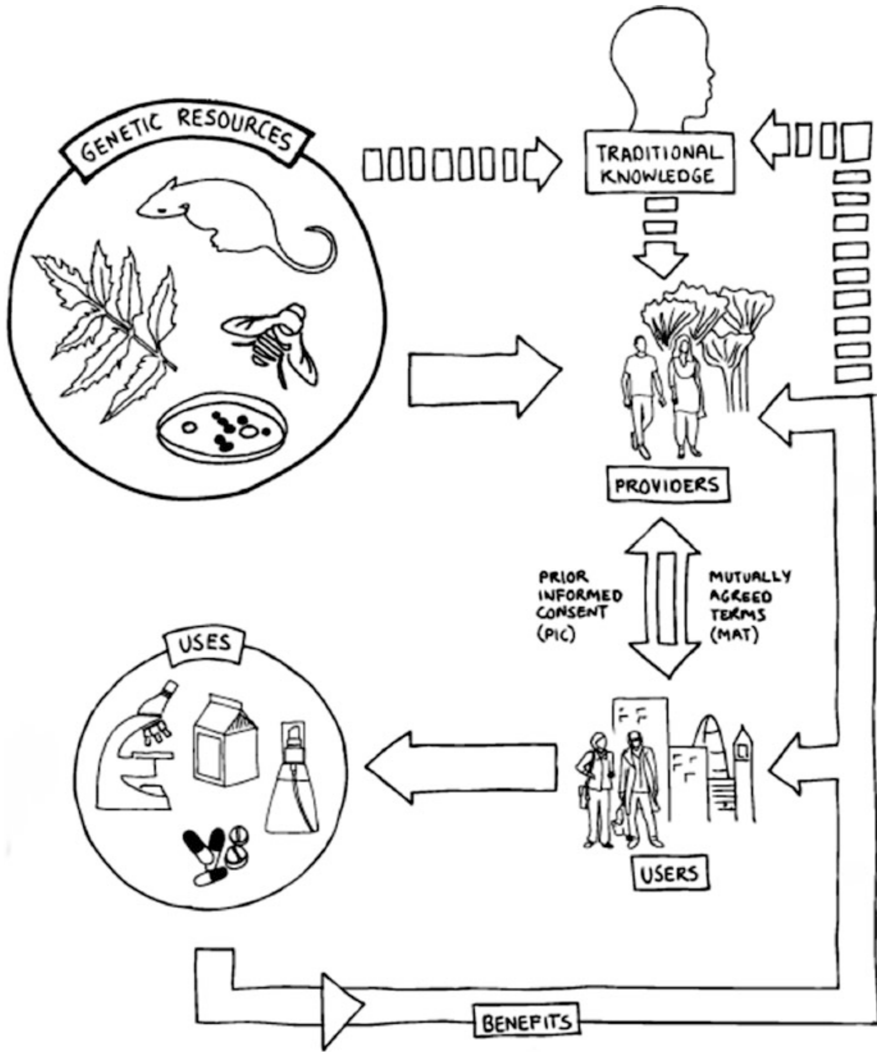

Fig. 15.1 Access and benefit-sharing framework in the Nagoya Protocol. Source: Introduction to Access and Benefit-sharing, factsheet produced and published by the CBD secretariat (2011), p. 4. Available at: www.cbd.int/abs/infokit/revised/web/all-files-en.pdf (Accessed 14 March 2017)

2.1.2 Access and Benefit-Sharing Obligations

The core of ABS obligations lays in two distinct but intertwined elements: the user must seek the PIC from the provider and negotiate MAT with regard to benefit-sharing.

Prior Informed Consent

According to the Nagoya Protocol, "access to genetic resources for their utilization *shall be subject to the prior informed consent* acquired from the Party providing such resources".[4] In fact, PIC may be seen as the formality that crystallises the agreement on the content of MAT. PIC should be issued in a written decision by the competent national authority (CNA) of the provider country, which can take the form of a permit or its equivalent. Once the PIC is registered in a database called the ABS Clearing House, it will constitute an internationally recognised certificate of compliance (IRCC).[5]

- *Competent national authorities:* The Nagoya Protocol requires from the parties to designate a CNA that will be responsible for granting PIC.[6] Parties also have to designate a national focal point to help applicants (future users) be better informed about the specifics of a provider's legal and administrative procedures related to ABS.
- Internationally recognised certificate of compliance: The issuance of an IRCC is an important aspect of the ABS regime, as it will later serve, in case of dispute, as evidence regarding the user's access to GRs, as well as his compliance with the provider's PIC and MAT rules and procedures. IRCCs also help the EU Member States to monitor the utilisation of GRs by users within the EU (see Sect. 3).

Mutually Agreed Terms

The MAT solidifies the terms and conditions regarding the utilisation of GRs. The three elements presented below are the most common in MAT, although this list is non-exhaustive.

- *Benefit-sharing arrangements:* The benefit-sharing conditions are palpably the key elements of the ABS relationship between the provider and the user. However, benefits are not necessarily monetary. For instance, sharing of results, participation of nationals in product development, contributions to education and training in the country of origin, technology and capacity transfer are some examples of non-monetary benefits that can be offered by the user.[7] The types of benefit-sharing will of course depend on the negotiation between the user and the provider country; however, the Nagoya Protocol and the Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits

[4]Nagoya Protocol, Article 6.

[5]Article 14 and 17 Nagoya Protocol.

[6]Article 13 Nagoya Protocol.

[7]A non-exhaustive list of possible monetary and non-monetary benefits is provided in Annex I of the Nagoya Protocol.

Arising out of their offer guidance to both user and provider countries for the types of benefits inherent to ABS.[8] Nevertheless, the user should carefully inspect the provider country's ABS legislation (where exists), in order to see whether the law has pre-set benefit-sharing conditions. In such cases, the user will not have a leeway for negotiation.

– *Subsequent third-party transfer:* As there are many more players than the provider and the user in the biodiscovery pipeline [7], the transfer of the GRs and/or the research results associated to a "third party" who was not involved in the PIC and MAT process is a possibility that should be anticipated in MAT. Some provider states will require the user to come back to its CNA for consent prior to any transfer.
– *Change of intent:* In many cases, the GR is first accessed for basic research purposes and will not entail monetary benefit-sharing arrangements upfront. Nevertheless, there is a chance that any associated discovery might lead to applied research and raise commercial interests. It has been recommended, therefore, to leave room in MAT for the negotiation of new terms further down the line, e.g. in a "commercial development agreement" [8, 9].

2.2 European Union ABS Regulation on Compliance

Currently, many implementing (regional and national) laws on ABS have been adopted or are under discussion. On 12 October 2014 [10], the EU Regulation 511/2014 on compliance measures for users from the Nagoya Protocol in the Union entered into force. It is one of the first regulations on compliance with ABS requirements to come into existence. It is also the most comprehensive compliance system so far put in place. The authors therefore lay down the EU ABS compliance system in detail as an example of a comprehensive implementation of the Nagoya Protocol. In this first section only the main principles of the EU ABS Regulation and the Commission Implementing Regulation (EU) 2015/1866 of 13 October 2015 [11] laying down detailed rules for the implementation of Regulation (EU) No 511/2014[9] are introduced. Their legal requirements applicable to practitioners and organisations dealing with GRs in the EU will be described in details in the third section of the chapter, dealing with users' compliance duties.

The EU Regulation 511/2014 Article 4 introduces the due diligence obligation as well as the obligation to check if all the legal documents are in place for a genetic resource that will be subject to a third-party transfer.

[8]Secretariat of the Convention on Biological Diversity [6]. Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization. Montreal: Secretariat of the Convention on Biological Diversity.

[9]Commission Implementing Regulation (EU) 2015/1866 of 13 October 2015 laying down detailed rules for the implementation of Regulation (EU) No 511/2014 of the European Parliament and of the Council as regards the register of collections, monitoring user compliance and best practices, http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32015R1866

The EU ABS Regulation states that "users shall exercise *due diligence* to ascertain that genetic resources [...] which they utilise have been accessed in accordance with applicable ABS legislation or regulatory requirements". This obligation of due diligence means that the onus is on the user, when endeavouring research on GRs, to make sure that the applicable ABS legislation of the provider country is respected and that the GRs are acquired with the appropriate MAT and IRCC, if this is required by the provider country. This will have an impact on research funding, for instance, as applications for EU or national research grants will need to be accompanied by a declaration of due diligence as evidence that all the ABS obligations have been fulfilled and the relevant permit has been obtained. The EU ABS Regulation does not apply to previously obtained GRs accessed from collections (i.e. material obtained by the collections before the entry into force of the Nagoya Protocol—12 October 2014).

Legal Framework for Activities in the Oceans: United Nations Convention on the Law of the Sea Due to the fact that the sovereignty of a state can extend to the sea, the CBD and the Nagoya Protocol also apply in the maritime jurisdiction of a coastal state that has adopted ABS measures. However, at sea, this ABS framework overlaps with the one of the LOSC addressing marine scientific research in general. The LOSC was adopted in 1982 [4] and entered into force in 1994. It can be described as a "global and general framework setting the boundaries of states' jurisdiction and regulating the activities taking place there, including marine scientific research" [12].

When collecting the GRs that will be the object of the research project on site, there are two distinct regimes to take into consideration. More precisely, when the research project is based on GRs sampled at sea, the regimes set by the LOSC and the CBD overlap, yet complement each other. The LOSC indeed addresses marine scientific research in general and refers to the act of sampling. The CBD refers to the utilisation of the sampled GRs. However, when research is based on material obtained from a collection, thus not needing sampling expedition, the only framework that applies to the situation is the one of the CBD.

2.2.1 Marine Scientific Research

The LOSC addresses marine scientific research on many occasions. Depending on the specific maritime area where the activity occurs, the act of scientific research can either be a freedom or a right and can be subject to regulations or not. The maritime spaces are delimited in the LOSC as shown in Fig. 15.2. Up to the exclusive economic zone's (EEZ) 200 nautical miles from the coastline for the water column, and up to 350 nautical miles for the continental shelf,[10] the maritime space is under the jurisdiction of the coastal state. Within these limits, all third states—including

[10]The distances mentioned are a maximum allowed by the LOSC. Depending on each coastal states maritime features and/or specific claims, the extent of the maritime zones under their jurisdiction may vary.

their researchers—have the right to conduct marine scientific research provided that they respect the requirements of the coastal state.[11] Beyond the 12 nautical miles of the territorial sea, however, the coastal state does not have full sovereignty but rather exclusive rights relating to specific activities only. In particular, coastal states have the right to regulate and authorise marine scientific research as they wish, as long as it respects the basic rights of third states. The LOSC gives further details on the minimum requirements to organise research campaigns in the waters of the coastal states.[12] For instance, notification should occur at least 6 months prior to a scientific project, and participation of national observers to the campaign is a prerogative of the coastal state. The authorisation and conditions are often granted in the form of a research permit, the authority responsible being different in each case depending on the administrative organisation of the coastal state. However, under ordinary circumstances, the coastal state *must* grant its consent to scientific activities, since the objective of the LOSC is clearly to promote marine scientific research rather than restraining it [13].

Beyond the limits of the EEZ and/or the continental shelf, two distinctive legal principles apply: the freedom of the high seas and the "common heritage of mankind" for the Area (the seabed and ocean floor and subsoil thereof, beyond the limits of national jurisdiction) and its (mineral) resources. Pursuant to Part XI, marine scientific research in the Area is encouraged and promoted, and it shall be carried out exclusively for peaceful purposes and for the benefit of mankind as a whole. The freedoms of the high seas[13] are proclaimed by the LOSC in Part VII and include the freedom of scientific research.

2.2.2 Marine Genetic Resources in Areas Beyond National Jurisdiction

In 2006, the United Nations started discussing issues related to the conservation and sustainable use of marine biological diversity beyond areas of national jurisdiction (BBNJ Working Group), including marine genetic resources. Over time, the status of marine GRs in areas beyond national jurisdiction (ABNJ) was vividly discussed in the BBNJ Working Group, not without divergences of opinions as to whether they should be subject to freedom of the high seas or to the common heritage of mankind principle.

In 2014, the General Assembly requested the BBNJ Working Group to make recommendations on the scope, parameters and feasibility of an international instrument under the LOSC to regulate marine biodiversity beyond national jurisdiction.[14] In the recommendations thereby issued by the BBNJ Working Group in February

[11] Article 238 LOSC.

[12] Articles 245 to 257 LOSC.

[13] Highs seas are determined as the surface and water column beyond national jurisdiction (Article 86 LOSC).

[14] UNGA Resolution 69/245, A/69/L.29 & Add.1.

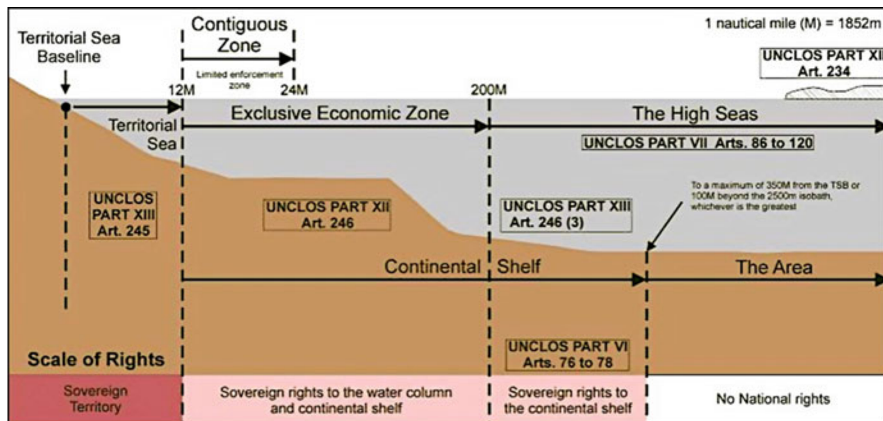

Fig. 15.2 Maritime boundaries in the Law of the Sea Convention. Source: Alan Evans, National Oceanography Centre, UK

2015, the United Nations General Assembly [14] decided to launch the development of an international legally binding instrument under the LOSC on the conservation and sustainable use of marine biological diversity of areas beyond national jurisdiction (ABNJ). This new binding instrument is expected to address, amongst others, the topic of "the conservation and sustainable use of marine biological diversity of ABNJ, in particular, together and as a whole, marine GRs, including questions on the sharing of benefits".[15]

In other words, the LOSC might be, depending on the outcome of the negotiation process, completed by an implementing tool with the binding force of a treaty that will regulate access to and use of GRs in ABNJ, in a similar way to the Nagoya Protocol being a supplementary agreement to the CBD.[16] While marine scientific research on GRs is currently free in ABNJ, it is therefore important to keep in mind that it will likely be subject to specific ABS requirements in a near future.[17]

[15]Resolution 69/292, A/69/L.65 & Add.1.

[16]As the scope of the CBD is limited to areas under the jurisdiction of States, neither the CBD or the Nagoya Protocol can address the matter of access to and use of GRs in ABNJ.

[17]A first draft text of the international legally binding instrument is expected by the end of 2017 (A/RES/69/292 par. 1(a)).

3 Step-By-Step Approach for ABS Compliance in Accessing GRs[18]

3.1 Preliminary Considerations

As already seen, marine scientific research activities need to be organised in full respect of international and national legal commitments related to ABS and the Law of the Sea Convention .

This section of the chapter illustrates the steps to be followed in order to guarantee that legal compliance and certainty is achieved. This is beneficial for the research community, the provider countries and also the possible private stakeholders. Scientists will need to take the following steps in order to comply with the ABS requirements set out in the CBD, the Nagoya Protocol as well as the EU ABS Regulation, *and* other requirements according to the LOSC. Please note that additional national or regional laws might exist that require a scientist to get additional permits, for instance, to sample in national parks for example.

First of all, when a scientist is planning to undertake a research project on GRs (notwithstanding the instances where the source of the GR is the country of registration of the research institution where he/she works), the first necessary step is to refer the matter and inform the legal representative of the research institution and the legal department or the technology transfer office of the institution (if any exists[19]). The only person who is entitled to sign legal documentation on behalf of a research institution is the legal representative of the institution, or any other member who has been invested of such competence. Therefore, it is of fundamental importance that employed scientists do not sign the legal documentation described in this section, unless they are entitled to do so. Receiving professional legal advice and support by private consultancies is the best option in case the research institution does not have in-house counsel or competences to deal with ABS.

This section illustrates the steps to follow in order to identify what kinds of permits are necessary in relation to ABS in the country where a sampling is planned and how to obtain them. Figure 15.3 sums up the relationship between the provider

[18]This step-by-step approach has been developed by adapting the guidelines illustrated by ten Hoopen et al. [15]. The main author of this chapter was a contributor to that Handbook.

[19]If no legal department nor technology transfer office exists, it is recommended to look for an external legal consultant or consultancy private company with outstanding experiences in providing legal support on ABS. ABS-int (http://www.abs-int.eu/en/home), for example, is a multidisciplinary team that consists of professionals with different backgrounds, including science, law and regulatory with experience in providing advice to national and international companies and institutions, including in the fields of sustainable resources management, biodiscovery, biosafety regulations, stewardship, environmental law and intellectual property law. GeoMedia is also a company providing such kinds of support (http://www.geo-media.de/consulting.html?&L=1).

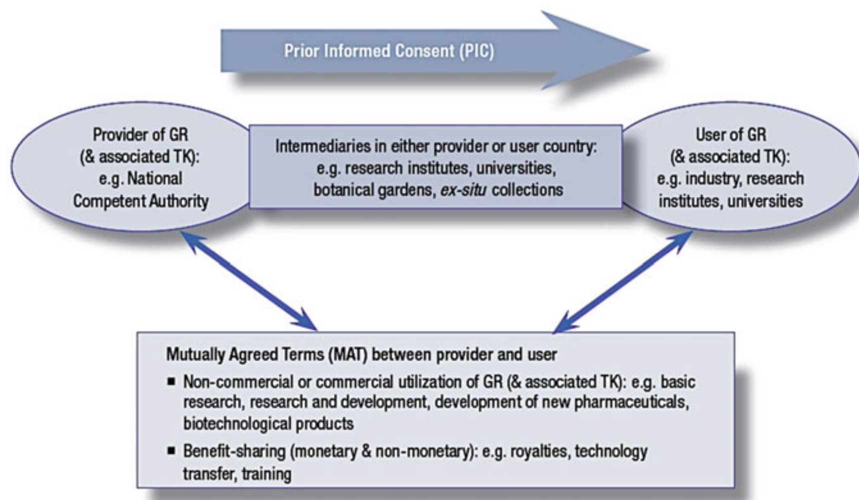

Fig. 15.3 Process and content of mutually agreed terms. Source: CBD Secretariat, Frequently Asked Questions on Access and Benefit-Sharing, at: www.cbd.int/doc/programmes/abs/factsheets/abs-factsheet-faqs-en.pdf

and the user. Let us recall that the user needs to obtain the prior informed consent (PIC) of the provider country, and both provider and user need to agree on mutually agreed terms (MAT) that include benefit-sharing provisions.

In the cases where the research that includes utilisation of a GR takes place in the EU, scientists should be able to foresee what kind of ABS issues they might be facing and how to handle compliance issues related to the EU ABS Regulation as well as the national and international obligations. This should start from the moment of *the drafting phase of a project*. As a matter of fact, this is required by the EU ABS Regulation through the due diligence declaration system. The initial checkpoint foreseen under the EU Regulation is at the stage of external funding. At this checkpoint, the users have to submit a declaration of due diligence to the competent national authority of their country. Certain funding programmes of the EU, such as Horizon 2020, already impose an ABS self-evaluation within the ethical self-assessment part of the proposal.

Therefore, scientists need to undertake a preliminary analysis in order to demonstrate that they will be able to exercise due diligence This means that they need to be sure that the GRs they accessed or will access for utilisation is or will be accessed in accordance with the national ABS legislation of the provider country. Once they ensure that all required legal permits are in place prior to sourcing the desired GRs, they will then need to undertake the steps described further below.

3.2 Access to In Situ Marine Genetic Resources

As step 0, scientists need to check in which maritime zone the sampling is planned:

– It can be in waters under national jurisdiction (case A: internal waters, territorial seas, exclusive economic zones).
– In areas beyond national jurisdiction (case B: the high seas and the Area, the deep seabed beyond national jurisdiction).
– In the Antarctic Treaty area (case C: the Antarctic).

3.2.1 Case A: Sampling in Internal Waters, Territorial Seas or Exclusive Economic Zones

If sampling is planned to take place in internal waters, territorial seas and/or exclusive economic zones, regardless of whether in a foreign country or the country of the research institution, the following steps need to be undertaken:

Step 1: First of all, users need to verify if the country from where they want to source their GRs is a party to the CBD and the Nagoya Protocol.[20] If this is the case, as soon as possible, notify the primary national focal point (NFP) for CBD, and the NFP and competent national authorities of the Nagoya Protocol of the country where sampling is planned[21] (provider state) and ask if any additional steps need to be taken.[22] It is often useful, and sometimes even required by ABS national legislation, to undertake such research project with a local partner, who might know about the national procedure and might help in negotiating benefit-sharing terms (see intermediaries in Fig. 15.3).

In the particular context of marine GRs, the CNA is sometimes different from the one competent for terrestrial GRs.

Step 2: Ask for advice on the specific requirements to be fulfilled prior to sampling activities according to the provider state's legislation on ABS.[23] Basic information on national ABS legislations and measures can be found on the website of the ABS Clearing House.[24]

Step 3: If the provider state has ABS legislation in place requiring prior informed consent (PIC) and mutually agreed terms (MAT), contact the provider state's

[20] List of parties can be found here: https://www.cbd.int/information/parties.shtml

[21] If this country is party to the Convention on Biological Diversity and to the Nagoya Protocol.

[22] Contact details of the CBD NFP of the Provider State can be found at www.cbd.int/information/nfp.shtml and more detailed information on national ABS legislations and procedures can be found in this website; the country is part of the CBD only www.cbd.int/abs/measures/ or in the website of the ABS clearing House https://absch.cbd.int/countries if the country is also a Party to the NP.

[23] Such requirements may include a simple notification of the sampling and/or an ABS agreement.

[24] ABS Clearing House Mechanism: https://absch.cbd.int

competent authority for ABS and start negotiating an ABS agreement.[25] Legal support will be needed for such negotiation.[26]

Step 4: Access and use the material only in accordance with the requirements set out in the permit and/or the ABS agreement.

Step 5: Transfer the sampled material and/or the associated knowledge (which includes data and scientific results) to a third party only in accordance with the requirements set out in the permit/ABS agreement[27] and upon the signature of a material transfer agreement . The MTA sets out the conditions on use (if any) of the sample material and/or associated knowledge by the third party, in accordance with the original ABS agreement/permit negotiated with the provider country. Together with the MTA, users need to transfer to subsequent users the IRCC and the information on the MAT. In case the IRCC does not exist, the following information has to be passed on:

– Date and place of access to GR
– Description of the GR
– The source
– The presence or absence of rights and obligations related to ABS
– The access permit and the MAT.

The EU Regulation also obliges the users to keep this information 20 years after the end of the utilisation.[28] Finally, if this information is not complete or if there is uncertainty, the subsequent user should either obtain an access permit or discontinue utilisation, according to the EU ABS Regulation..

Step 6: If a user wants to use the material and/or associated knowledge for other purposes than the ones agreed upon in the ABS agreement/permit obtained (change of intent), then the user needs to go back to the relevant authorities of the provider state and renegotiate, as should be specified in the MAT.

As already underlined, complying with the ABS requirements does not account for compliance with the requirements of marine scientific research in the LOSC.

[25]One template ABS agreement targeting exactly marine microorganisms has been developed by the EU-funded project Micro B3 (https://www.microb3.eu): Model Agreement on Access to Marine Microorganisms and Benefit-Sharing (Micro B3 ABS Model Agreements). The text including the commentary can be found at https://www.microb3.eu/work-packages/wp8

The Micro B3 ABS Model Agreement can be adapted to different needs: to public domain, hybrid and full commercial use at the point of access. It has been used as basis for ABS negotiations during the Ocean Sampling Day Campaign (an https://www.microb3.eu/osd), and it is now available as an example of best practices in the website of the CBD secretariat. It has been subsequently endorsed by the EU-funded project PharmaSea (http://www.pharma-sea.eu)

[26]The ABS agreement will not be needed in the drafting phase of a project, but it is advisable to start making contact with the competent authorities already in the drafting phase.

[27]Users need to be absolutely sure that they have the right to transfer the material and/or associated knowledge before sending the material and data to a third party. If the permit/agreement is silent on this, it does not mean that you have the right to transfer. In the latter case, a clarification with the Provider State might be necessary.

[28]Article 4(6) EU Regulation 511/2014.

According to the LOSC, a research permit is needed from the coastal state in order to undertake marine scientific research in the territorial seas and exclusive economic zones of that coastal state.

If scientists are planning to sample in:

(a) Their own state's national waters (meaning the research institution's country), they need to contact the authority which is competent to release such a permit (it is usually the Ministry of Environment or the Ministry of Research or the Ministry of Transports) and provide full details on the research project.
(b) In a foreign country's waters, they need to contact the embassy of the provider state at least 6 months in advance of the expedition and provide full details on the research project. The embassy should assist in obtaining the necessary consent and permits from the competent authority.

3.2.2 Case B: Sampling in Areas Beyond National Jurisdictions

There is neither notification nor permits required so far. As previously highlighted, there is currently an undergoing process at the United Nations level to discuss an implementing agreement to the LOSC addressing marine biodiversity in ABNJ: the negotiation of such an intrument are starting in 2018. The instrument is expected to have implications for accessing and/or utilising GRs coming from ABNJ in the future. It is advisable to keep a record of the sampling provenance for a period of 20 years, in order to be able to demonstrate the source, in case this is questioned.

3.2.3 Case C: Sampling in the Antarctic Treaty Area

Any activity undertaken in the Antarctic Treaty area (south of 60° latitude) is subject to prior notification. Moreover, the national legislation of the country undertaking the research might require from the researchers to apply for and to obtain a permit.[29]

3.2.4 Access to Ex Situ Marine Genetic Resources

The users have to exercise due diligence according to the EU ABS Regulation, which means that they have to seek either the IRCC related to the materials they want to access in the collection (if it exists) or all the relevant ABS information related to the materials (see Step 5 above). When this information is insufficient or uncertainties exist about the legality of access, the users have to obtain an access permit from the provider country or discontinue utilisation. Of course, this is only applying to materials deposited in the collections after the entry into force of the EU ABS

[29] In case of entering and sampling in a "Special Protected Area" a special permit is needed: basic information can be found here http://www.ats.aq/e/ep_protected.htm

Regulation. Given the immense efforts collections will have to undertake in order to adjust their management system to the new rules, this is going to be challenging, both for collections and users. However, the EU ABS Regulation prescribes the possibility for collections to apply for being registered (either as a whole or in part) in the Register of Collections established by the European Commission. This will have the advantage for the users to assert that, when accessing materials in a registered collection, they have exercised due diligence.

4 Compliance Under the EU Regulation

The EU ABS Regulation only applies to access that occurred after 10 December 2014 to the genetic resources that are obtained from provider countries that are parties to the Nagoya Protocol. Therefore, if access happened before 10 December 2014 or if the provider country is not a party to the Nagoya Protocol, the user does not have obligations regarding the compliance measures foreseen under the EU Regulation 511/2014. Additionally, the EU Regulation only applies when the GR is brought within the EU for R&D purposes. Hence, if R&D happens outside of the EU, the EU Regulation does not apply.[30]

Regarding user compliance, the initial step to take once a GR is to be accessed is to look into the national ABS law of the provider country. If a GR is obtained from other sources than the provider country, and if the user is unsure of the documentation regarding the country of origin, the EU Regulation advises the user to seek a permit from the provider country or stop utilisation. If there is no national ABS legislation of that provider country, or if it operates on free access principle, then the user does not have to obtain PIC and MAT but should keep records of obtaining this information and how as well as where it is obtained. National focal points are the most dependable sources regarding the existence of national obligations on ABS.

If the provider country has ABS legislation in place that requires PIC and MAT, then the user should obtain the PIC which will serve as permit for access and negotiate MAT with the provider.

As previously mentioned, EU compliance on ABS operates by several checkpoints. However, users' everyday activity has to be tweaked in order to be compliant at all times. Furthermore, the competent authorities of EU Member States will highly likely be conducting inspections within the facilities of the users in order to check whether the users have been compliant. Therefore, it is not possible to be compliant only for the sake of checkpoints as the declarations required during those checkpoints oblige the user to be compliant in a day-to-day manner.

One of the checkpoints is the receipt of an external research funding. The receipt of funding does trigger due diligence obligation under Article 7(1) of the EU Regulation. The Implementing Regulation 2015/1886 Article 5(2) states that the

[30]Article 2 EU Regulation 511/2014.

declaration is to be made to the competent authority of the country where the user conducting R&D activities is based, after receiving the first instalment of the funding and obtaining the genetic resource. If the R&D is undertaken by a consortium, then the declaration is to be made to the competent authority of the country where the consortium is led. In any case, the declaration must be made before the final report or the end of the project. The due diligence declaration must be made in the form set out in Annex III to the Implementing Regulation 2015/1886.

Another checkpoint occurs when the user transfers research results together with the GR to a party outside the EU. According to the Implementing Regulation Article 6(2)(e), the transferring party is obliged to make a due diligence declaration when it transfers the results and the material outside the EU. The due diligence declaration has to be made to the national competent authority of the country where the research activities are based.

When the research results, together with the GR, are transferred to a third party within the EU for further development, the transferring user has to be due diligent. It does need to transfer to the subsequent user the relevant ABS information (date and place of access, description, the source from which GRs were directly obtained and subsequent users, rights and obligations related to ABS, access permits MAT) or the IRCC if in place. Nevertheless, it is advised to the subsequent user to enter into a material transfer agreement with the transferring party. The transferring party should verify, before that, that he/she is authorised by the PIC or MAT to transfer the GR to a third party.

If the previous user only established an MTA on the basis of research and not commercialisation, and if the receiving party intends to commercialise a product by utilising the GR (change of intent), then the receiving party has to renegotiate the terms of PIC and MAT with the provider country to ensure that he/she is authorised to commercialise.

The last checkpoint for making a due diligence declaration is when the user is seeking market approval for the product created by utilising the GR. According to the Implementing Regulation Article 6(2), the user would be obliged to make a due diligence declaration prior to seeking market approval for a product created by utilising the GR. This declaration is to be made to the competent national authority of the EU Member State where the user who seeks for market approval is based in.

Once the last user is done with the utilisation of GR, he/she is obliged to keep all the records related to the access, benefit-sharing, utilisation, third-party transfer, IRCC and so on for 20 years after the end of utilisation.[31] Therefore, the users are advised to establish a highly effective and precise data management system in order not to have any issues related to compliance.

Even though the intermediate entities, which are the ones that operate between institutions of basic research and industrial users, are not the users that seek market approval, they may have obligations under the EU Regulation 511/2014. If the intermediate user is transferring GR to a user established within the EU, then the

[31] Article 4(6) EU Regulation 511/2014.

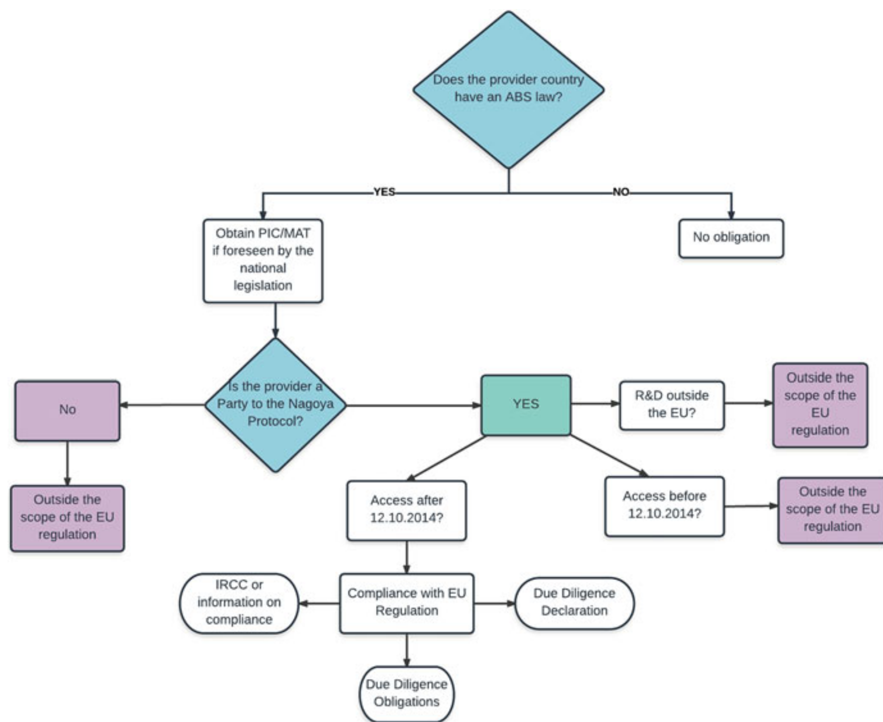

Fig. 15.4 Flowchart on compliance under the EU Regulation 511/2014. Illustrated by the authors, 2017

intermediate has to be due diligent and transfer the abovementioned necessary information to a subsequent user. Likewise, for transfers outside the EU, the intermediate user would have to perform a due diligence declaration. The only obligation an intermediate user would not be bound by is the due diligence declaration that is to be made by the end-user prior to seeking market approval.

Article 7 of the EU Regulation 511/2014 authorises national competent authorities to monitor user compliance; however, the EU Regulation leaves it to the Member States to further regulate how this will be enforced. It is therefore crucial for users to check or seek external legal help on the applicable national ABS legislation regarding the enforcement issues: what they can expect from the inspections and what provisions related to administrative and/or criminal sanctions and charges are applicable.

Below is a flowchart prepared by the authors summarising the steps that need to be taken in order to be compliant under the EU Regulations (Fig. 15.4):

The flowchart based on the obligations laid out under the EU Regulation 511/2014 demonstrates that the timing and the status of the provider country under the Nagoya Protocol define the user obligations regarding access.

5 Conclusions

This chapter introduced the implication for users of GRs of the legal ABS framework associated to the utilisation of marine GRs. These are requirements to be respected starting from the early stages of a research project involving GRs until the end of utilisation of GRs. Compliance with ABS requirements is crucial at the time of accessing, collecting or acquiring the GRs that will be the object of a given project, and there is a need for a continuous follow-up through the monitoring mechanisms put in place by state parties, particularly in the EU.

This chapter aimed at informing practitioners of the marine biotechnology pipeline working with GRs on the new regulatory framework brought by the CBD, the Nagoya Protocol as well as the EU ABS Regulation on compliance. At the same time, this chapter strived to raise awareness on the potential overlap with permit requirements due to sampling GRs at sea, according to the law of the sea. Compliance with all ABS requirements ensures that the value of interesting discoveries at the non-commercial research level is safeguarded for further development and commercialisation, by utilising only GRs acquired with the necessary documentation, which in turn provides the legal certainty sought by subsequent parties further down the value chain. It is equally important to practitioners to comply with ABS requirements to avoid being accused of committing biopiracy.

ABS is not only important to avoid legal and ethical issues while conducting research. The ultimate goal of this legal framework is to encourage the conservation of biological diversity, through the promotion of research on GRs and the fair and equitable sharing of the benefits arising out of their utilisation. As mentioned in this chapter, a great majority of such benefits are non-monetary and are meant to enhance capacity-building, training and the sharing of knowledge with the developing world that is, in many cases, where the most biodiversity-rich countries are.

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