Tomás G. Villa
Patricia Veiga-Crespo *Editors*

# Antimicrobial Compounds

## Current Strategies and New Alternatives

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# Preface

Since the very beginning, the relationship between humans and microorganisms has moved between love and hate. People have used bacteria and yeast to manufacture foods, even when they did not know what a microorganism was. Thanks to bacteria and fungi, we can produce wine, yogurt, beer, or bread. However, microorganisms have been also sources of illness, chaos, and destruction. For example, the bubonic plagues during the Middle Ages caused a dramatic decrease in the European population. Also, for centuries, doctors, physicians, and even, wise men treated patients and fought against infections without knowing their enemies or without having the right weapons.

The development of the initial microscopes allowed to identify the enemies; the bacteria and fungi could be observed, but not destroyed. Some researchers were able to find out how microorganisms spread between people and then, they could isolate the ill from the healthy individuals. The first clear and efficient step against infections was the development of vaccines. But, what did they have to do with the ill?

The twentieth century brought light into the darkness. The first drug against syphilis was developed, the salvarsan was the first active principle able to attack a microorganism, *Treponema pallidum.* Its unique problem was that salvarsan had arsenic inside and this could originate several side effects in the patients.

When Sir Alexander Fleming was able to understand what was happening in a contaminated Petri dish, he discovered penicillin, and the Antibiotic Era began officially. Then someone had the idea that the end of the infection illnesses was coming. A century and several active principles later, deaths by infections are almost 25 % of the total deaths per year around the world.

What did happen in the middle? The irrational and bad use of antimicrobial drugs exerted a selective pressure over microorganisms. Bacteria and fungi developed different mechanisms to avoid the antimicrobials, and humans with their bad behavior just selected them.

In the last 20 years, the number of new antimicrobials has been lower and lower, while the number of resistant microorganisms has become higher and higher. The classic techniques to develop new antimicrobials became less and less effective and pharmaceutical companies tried to put their efforts in other fields with a better ratio of profits, such as stroke injuries, hypertension, and so on. However, we still need new antimicrobials.

The search for new sources of antimicrobials, the design of more efficient research policies, and the use of new technologies are now mandatory. This is the goal of this book. During the different chapters, the reader will be able to find out the latest advances in the development of new antimicrobial drugs together with a recapitulation of new potential sources of drugs.

The aim of this book is twofold. It tries to be an accurate and extensive review of the actual state of the art in the field of antimicrobial research, but, on the other hand, it also tries to be the initial point for developing new alternatives and strategies in the fight against resistant microorganisms.

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# Contents


**1 Strategies for the Design and Discovery of Novel Antibiotics using Genetic Engineering and Genome Mining** . . . . . 1
Carlos Olano, Carmen Méndez and José A. Salas

**2 X-Ray and Neutron Scattering Foundations for the Research in Antimicrobials** . . . . . 27
Daniel Piso

**3 Antibacterial, Antiviral and Antifungal Activity of Essential Oils: Mechanisms and Applications** . . . . . 51
Karola Böhme, Jorge Barros-Velázquez, Pilar Calo-Mata and Santiago P. Aubourg

**4 New Antimicrobial Agents of Plant Origin** . . . . . 83
Javier Sampedro and Elene R. Valdivia

**5 Advances in Beta-Lactam Antibiotics** . . . . . 115
José-Luis Barredo, Gulay Ozcengiz and Arnold L. Demain

**6 The Cornerstone of Nucleic Acid-Affecting Antibiotics in Bacteria** . . . . . 149
M. Gacto, M. Madrid, A. Franco, T. Soto, J. Cansado and J. Vicente-Soler

**7 Genetic Analysis and Manipulation of Polyene Antibiotic Gene Clusters as a Way to Produce More Effective Antifungal Compounds** . . . . . 177
Michal Letek, Luis M. Mateos and José A. Gil

**8 Enzybiotics: The Rush Toward Prevention and Control of Multiresistant Bacteria (MRB)** . . . . . 215
Patricia Veiga-Crespo, Angeles Sanchez-Perez and Tomás G. Villa

**9 New Cell Wall-Affecting Antifungal Antibiotics** . . . . . 237
Juan Carlos Ribas, Ángel DurÁn and Juan Carlos G. Cortés

**10 Perspectives in the Research on Antimicrobial Peptides** . . . . . 269
Miguel Viñas, Francesc Rabanal, Roland Benz,
Teresa Vinuesa and Ester Fuste

**11 Glycopeptides and Bacterial Cell Walls** . . . . . 285
Fernando Santos-Beneit, Juan F. Martín and Carlos Barreiro

**Index** . . . . . 313

# Introduction

New tools to look within microbial genomes potentially hiding a rich array of pharmaceutical surprises has been developed in recent years and this has led to the discovery of a novel antibiotics that could highlight new treatments for serious diseases caused by multi-drug-resistant microorganisms.

Undoubtedly, the availability of whole microbial genomes and related information has opened up many of these new ways for antibiotic hunting. The greatest benefit obtained from these new approaches lies in the ability to examine several genomes simultaneously, so a potential target can be evaluated against another, and the effects compared across species. This issue was first addressed as early as 1998 by Allsop (1998), and two years later by Mendez et al. (2000) (for more information see Chap. 1). In 2002 the topic was again reviewed by Haney et al. concluding that *taken together, these technologies, overlaid within an established drug discovery program, now affords the opportunity for the identification, validation, and process design for high-throughput target mining at unprecedented volumes and timeframes* (Haney et al. 2002).

In this same year Chaker et al. (2002) revisited this topic in a comprehensive paper that concludes with two ideas worth of being quoted in this introduction; one is that in general, it takes a total of 5–8 years to progress antibiotic candidates through drug development to regulatory approval, with a significant probability of failure at each stage and two, that concerns the diversity of both target enzymes and inhibitory molecules. If there are *ca* 300 broad-spectrum essential potential target genes, it may be an achievable goal to identify and screen all of them in a relatively short period of time.

As pointed out by Demain and Sanchez (2009) microbes have made a great contribution to the well-being of mankind. In addition to producing many primary metabolites, such as amino acids or vitamins, they are capable of producing secondary metabolites that has saved millions of lives in the last 80 years, after introduction of B-lactam antibiotics such as penicillins in the market. Despite the fact that companies are reluctant to invest in the seeking of new antibiotic substances from a variety of sources, we need to move on and try to keep up with this old tradition plus the designing of new drugs, supported by the pure force of organic chemistry.

Also, the use of unusual bacterial components as targets for new antibiotics is starting to produce good results. This is the case of Lipid II, a membrane-anchored

cell-wall precursor essential for bacterial cell-wall biosynthesis, and that can be the target for vancomycin, lantibiotics, mannopeptimycins, and ramoplanin; it may therefore be worth to exploit their potential as new treatments for bacterial infections (Breukink and de Kruijff 2006). As for the exploitation of old microbial groups known since the very beginning of the antibiotic era, one has to keep in mind the marine actinomycetes that constitute a fabulous group for the discovery of a pleyad of new antibiotics. Quoting Fenical and Jensen (2006) *Members of the genus Salinispora have proven to be a particularly rich source of new chemical structures, including the potent proteasome inhibitor salinosporamide A, and other distinct groups are yielding new classes of terpenoids, amino acid–derived metabolites and polyene macrolides.*

An example of the above is shown in the work of Haste and co-workers (Haste et al. 2010). Their work highlighted the discovery of the streptogramin etamycin produced by an actinomycete species isolated from the coast of Fiji that demonstrated potent activity against HA- and CA-MRSA with MICs as low as 1–2 mg $l^{-1}$ against HA- and CA-MRSA strains and was found to be non-cytotoxic at concentrations more than 20-fold above MIC. By all means this new antibiotic was comparable to the mighty vancomycin and also conferred significant protection from mortality in a murine model of systemic lethal MRSA infection. These data emphasize once more the utility of the marine environment as a yet-to-be-discovered source of antibiotics.

The combinatorial synthesis of antibiotics has to be necessarily addressed here (Weissman and Leadlay 2005) since the bacterial multienzyme polyketide synthases (PKSs) produce a diverse array of products that have been developed into medicines, including antibiotics and anticancer agents. So far and as indicated by these authors "directed engineering of modular PKSs has resulted in the production of more than 200 new polyketides, but key challenges remain before the potential of combinatorial biosynthesis can be fully realized."

The search for healing principles in plants is an ancient idea and people ever since the very beginning of civilization have dug into all kind of plants to get relief from their pains by means of infusions and the like. In fact, there is evidence that Neanderthals living 60,000 years ago already used plants for the treatment of diseases. It is estimated that there are up to 500,000 species of plants on Earth (Borris 1996; Murphy 1999). Only a small percentage (*ca* 9 %) of these are used as foods while the rest are either kept for their pharmaceutical potential or are poisonous to humans or animals. Hippocrates mentioned up to 300–400 medicinal plants and many of them have passed to our use thanks to the Arabs and monks throughout the Middle Age.

Of particular interest are the so-called bioenhancers of plant origin. They are phytomolecules capable of enhancing bioavailability of a given drug with which it is combined. The term bioavailability enhancer was first coined by Indian Scientists at the Regional Research Laboratory, Jammu (RRL, now known as Indian Institute of Integrative Medicine) discovered and scientifically validated piperine as the world's first bioavailability enhancer in 1979 (for a comprehensive review see (Dudhatra et al. 2012)). There are many ways by which bioavailability may be

enhanced, such as by promoting directly the absorption of the drug, by enhancing the binding of the drug to specific receptors, by increasing the microorganism permeability to the drug even when intracellularly located, by inhibiting the effluxing pumps, and so on. Several topics need to be addressed: (i) mechanism of action of the bioenhancer, (ii) pathway(s) of entrance into the general metabolism, and (iii) interaction of the bioenhancer with drugs other than the one it was intended for. Piperine is at present a good candidate for bioenhancing and has been studied in relation to a large variety of active compounds and also it has been reported to show a plethora of different effects that include anti-inflammatory activity (Kumar et al. 2007), antipyretic activity (Parmar et al. 1997), fertility enhancement (Piyachaturawat and Pholpramool 1997), antifungal activity (Navickiene et al. 2000), antimutagenic activity (Wongpa et al. 2007), and many other activities (see the review (Dudhatra et al. 2012)). In addition to piperine other natural compounds such as *Zingiber officinale*, niaziridin, glycyrrhizin, *Cuminum cyminum*, *Carum carvi*, allicin, lysergol, *Aloe vera*, *Stevia rebaudiana*, curcumin, sinomenine, genistein, *Ammannia multiflora*, capsaicin, quercetin, naringin may show positive bioenhancing activity.

Essential oils have been long known for exhibiting antibacterial, antifungal, antiviral activities and they are regularly used in medicine and in the food industry (Bassole and Juliani 2012). These essential oils are worth being studied here and many have been reported as having either of these activities, for example eight eucalyptus species essential oils (Elaissi et al. 2012), the one from *Marrubium vulgare* L (Zarai et al. 2012), from *Pelargonium graveolens* (Ghannadi et al. 2012), or from *Thymus vulgaris* as anti *Helicobacter pylori* (Esmaeili et al. 2012), among others. Recent ones such as the one prepared from *Hypericum triquatrifolium* is worth being cited here on account of its activity as antibacterial, antifungal although it did not show antiviral activity but exhibited low cytotoxic effects (Rouis et al. 2013).

Viruses causing serious hemorrhagic fevers belong basically to four families of ssRNA-viruses (namely *Arenaviridae, Bunyaviridae, Filoviridae*, and *Flaviviridae*), present on all continents except Antarctica and having high risk of being used as a weapon in bioterrorism. Thus, the approach of using antiviral drugs from a vast variety of plants either directly or as essential oils is being deeply investigated in recent years as the use of ribavirin-based therapies show low activity on such viruses, besides the side effects (Joniec et al. 2012).

The old and most important group of antibacterial compounds (B-lactam group) is still the source of active research in order to obtain new products with different properties in terms of broader action pattern, biodisponibility, and the like. These antibiotics have been serving mankind for many years and despite this and as pointed out by Ozcengiz and Demain (2013) *they continue to provide health to the world population by virtue of industrial production and discoveries of new secondary metabolite molecules with useful activities. Sales of these remarkable compounds have reached over $20 billion dollars per year. They include penicillins, cephalosporins, cefoxitin, monobactams, clavulanic acid and carbapenems. Strain improvement of the penicillin-producing species of* Penicillium *has been truly*

*remarkable, with present strains producing about 100,000 times more penicillin that the original* Penicillium notatum *of Alexander Fleming.* Following this line of reasoning, the developments of the biotechnological applications of penicillin acylases merit to be pointed out. This group of enzymes is involved mainly in the industrial production of 6-aminopenicillanic acid and the synthesis of semisynthetic beta-lactam antibiotics, or in the peptide synthesis and the resolution of racemic mixtures of chiral compounds (Arroyo et al. 2003). Penicillins are the most important antibiotics as far as annual production, but as resistant microorganisms they have steadily increased through the years, so the natural penicillins G and V, produced in bulk by fermentation have almost no use for direct therapy. The majority of it is used for the production of 6-amino penicillanic acid (6-APA), being penicillin acylases the key enzymes for producing a vast variety of semisynthetic penicillins which not only exhibit better properties such as increased stability, easier absorption, and fewer side effects than penicillin G or penicillin V, but also address the problem of microbial resistance to antibiotics (Rajendhran and Gunasekaran 2004). Interestingly, penicillin acylase activity is present in a variety of microorganisms including gram-negative and gram-positive bacteria, filamentous fungi, and yeast although its role in bacteria is not clear; it has been suggested to be involved in the degradation of certain phenylacetylated compounds when the bacterium is dwelling in a free-living mode (Valle et al. 1991).

Unusual or diverse semisynthetic B-lactam antibiotics may have quite unexpected applications that would justify all the efforts being made to conduct the basic research. This is the case for a number of established agents such as these antibiotics that have recently been re-investigated for their potential as neuroprotective agents for the treatment of amyotrophic lateral sclerosis (Nirmalananthan and Greensmith 2005). Also, there is recent evidence that upregulation of one of the glutamate transporters, GLT-1, with beta-lactam antibiotics attenuates the damage observed in models of both acute and chronic neurodegenerative disorders (Sheldon and Robinson 2007).

Biotechnology has been since the very beginning on the seeking for accelerated findings of new drug molecules, and many of them will be addressed in the present book. As many of them suffer from sensitivity to degradative processes or low bioavailability, renewed strategies have been designed to overcome these drawbacks. So, liposome technology or nanoparticles have proven to be good examples. In this sense the use of polyacrylate emulsions containing nanoparticles measuring 30–50 nm in diameter, and onto which an antibiotic molecule can be covalently attached represents a new approach (Sampath et al. 2008; Sheldon and Robinson 2007). This methodology has been positively used against β-lactamase-producing microbes such as methicillin-resistant *Staphylococcus aureus* (MRSA). These glycosylated nanoparticles have been also employed to "study carbohydrate interactions in the field of chemical glycobiology, but their potential in drug delivery is relatively unexplored particularly in the context of antibacterials research" (Sampath et al. 2008).

The update of a book like the present one in which a variety of topics are issued, must include those concerning inhibition of bacterial transcription by the appropriate

antibiotic, actinomycins, rifamycins and streptovaricin, streptolydigin and tirandamycin, microcin J25, sorangicins, myxopyronins, corallopyronin, ripostatins, and fidaxomicin. It must also review those involved in the interference of the DNA synthesis such as novobiocin and related aminocoumarins, cyclothialidine, and particularly those antibiotics affecting DNA and with antitumor activities including mitomycins or griseofulvin.

The discovery of penicillin by Fleming, and its subsequent industrial exploitation marked the beginning of the antibiotic era and the forgetfulness of bacteriophages as a way of biologically fighting microbial pathogens; in other words, chemistry prevailed over biology at those early times in controlling pathogenic bacteria. It is true that antibiotics have changed the practice of medicine and has reduced the number of cases throughout the world of a variety of diseases, but at the same time the number of antibiotic-resistant bacteria has increased to such an extent that old-time antibiotics are entirely useless. These resistance phenomena represent not only an important healthcare issue but also an economic problem, with an estimated cost of several million dollars per year. So, methicillin-resistant Staphylococcus aureus (MRSA) caused nearly 60 % of nosocomial infections in 2001 and more than 58 of the new isolated in Europe were MRSA (ECDC 2009); 30 % of Streptococcus pneumoniae strains are penicillin-resistant and vancomycin-resistant Enterococcus faecium and Enterococcus faecalis are constantly increasing. The enzybiotics may represent without a doubt a new way of treating the diseases caused by these recalcitrant multiresistant bacteria (for a comprehensive review (Villa and Veiga-Crespo 2010)).

As regards polyene antibiotics and due to their importance as potent antifungal plus antiparasitic activity (i.e. Leishmania, sp) together with their action against enveloped viruses or prions (Hartsel and Bolard 1996). The actual state of the art has to be addressed, although their therapeutic use is limited by serious side effects. Recent years have seen the publication of several break-through papers concerning the genetic analysis and manipulation of Streptomyces species with the ability to produce high levels of nystatin, amphotericin B, or pimaricins and related polyenes (Caffrey et al. 2008). As it is known the general polyene biosynthesis involves a rather complex assembly as well as oxidative and glycosylation modifications, all of them controlled by different gene clusters. Their study and manipulation in the appropriate way may lead to the obtention of new polyenes with lower side effect, more potent, or of broader spectrum. These answers will be found doubtless in the years ahead.

As for antibiotics able to interact with microbial membranes thus interfering with their physiology, they must be addressed here. Peptide molecules exhibiting antimicrobial activity are found in Nature, sometimes as by-products in animals, plants, or microbes. Additionally, peptide molecules can be synthesized in chemical laboratories covering in principle an unlimited extension of chemical structures, sequences, and spectra of action. The well-known peptidic compounds lantibiotics and bacteriocins from lactic bacteria have been used for many years now as food preservatives and in the last few years the eventual application of such molecules to fight against infectious diseases is finally increasing. Lantibiotics are post-translationally modified peptides, of which nisin A is the best studied example, so

biochemical manipulations of nisin A has resulted in the obtention of a variety of them with increased activity against Gram-positive bacteria. Of these, nisin V (Met21Val) is the most relevant by virtue of its exhibiting enhanced antimicrobial efficacy against several clinical and food-borne pathogens, including *Listeria monocytogenes* (Campion et al. 2013).

Besides these exogenous antibacterial peptides, endogenous host defense peptides do constitute one of the first line of defenses against pathogens. These peptides are small (ca 100 aminoacids) and with amphipathic properties. In eukaryotes they include disulfide-stabilized (e.g., defensins) and α-helical or extended (e.g., cathelicidins) peptides. In prokaryotes they are known as indicated before bacteriocins and lantibiotics. One target for prokaryotic and eukaryotic antibacterial peptides is the bacterial cell wall (Yount and Yeaman 2013), and therefore the primary component of the cell wall that is the peptidoglycan. Knowing the complexity of its synthesis, one can easily envisage a vast variety of points where these peptides may be developed to act, avoiding simultaneously the unwanted side effects of their massive use.

In addition, new results support the idea of a two-state model for septal and peripheral PG synthesis (in pneumococcus and related Gram-positive bacteria) in such a way that the proteins involved have already turned up as promising vaccine candidates and targets of antibiotics (Sham et al. 2012). Also and as pointed out by Ashford and Bew (2012) *the vancomycin family of glycopeptide antibiotics has been inspiring research in the field of synthetic chemistry since the 1980s. Recent studies have moved away from the focus of total synthesis into new territory: the design and evaluation of novel compounds based on the natural products which exhibit improved antibacterial activity.*

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# Chapter 1
# Strategies for the Design and Discovery of Novel Antibiotics using Genetic Engineering and Genome Mining

**Carlos Olano, Carmen Méndez and José A. Salas**

**Abstract** Most bioactive natural products currently known are synthesized by members of the *Actinomycetales* order. The development of genetic engineering provides novel genetic tools for the modification of known antibiotics and other bioactive compounds to generate derivatives with improved therapeutic properties. This new technology, named *combinatorial biosynthesis*, is able of introducing structural modifications in bioactive compounds not easily accessible by chemical means. Furthermore, progress in genome sequencing in this group of microorganisms shows that actinomycetes have a greater potential of synthesizing bioactive compounds than was anticipated. Each genome sequenced shows the presence of 18–37 gene clusters potentially directing the biosynthesis of bioactive compounds that have not been previously identified. Novel strategies are being developed to activate these *cryptic* or *silent* gene clusters in these microorganisms, allowing the identification of novel potentially bioactive compounds. This chapter will revise the state of the art in this field of research.

## 1.1 Introduction

Since the discovery of penicillin in 1928 and its subsequent clinical development from 1940, natural products, in particular antibiotics, have provided great benefits to mankind. In these 85 years, natural products have resulted to be indispensable in the clinic field as antibacterial, antifungal, antiparasitic, anticancer, and immunosuppressive agents, or highly essential in the veterinary and agriculture fields as feed additives or plant- and livestock-protecting agents such as herbicides, pesticides, or antiparasitic agents. As a result, the impact of infectious diseases has

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decreased, and several neoplastic and viral diseases became controllable, leading to a significant increment in the population average lifetime (Bérdy 2012). However, infectious diseases are even today the second leading cause of death globally, responsible of more than 9 million deaths (16.2 %) per year. Lower respiratory infections, diarrheal diseases (cholera and dysentery), HIV/AIDS, and tuberculosis are among the top 10 leading causes of death worldwide (WHO 2011). In addition, microbes continuously fight to survive, and there is an increasing resistance toward antibiotics derived from the widespread and uncontrolled application of them (Read et al. 2011). Antibiotic resistance is widespread in nature, conforming what has been denominated *intrinsic resistome* of bacterial organisms. This resistome is composed of more than 20,000 potential resistance genes of nearly 400 different types and varied phylogenetic origins that act or might act as resistance genes only in the presence of antibiotics (Davies and Davies 2010). Some of these resistance genes have been detected in 30,000-year-old permafrost sediments, confirming that antibiotic resistance is an ancient, naturally occurring phenomenon highly distributed in the environment that predates in the modern selective pressure of clinical antibiotic use. This is consistent with the rapid emergence of resistance in the clinic (D'Costa et al. 2011). As a consequence, more than 70 % of pathogenic bacteria are resistant to most antibiotics on the market, and the mortality of some multiresistant infections has reached rates up to 80 % (Bérdy 2012). For these reasons, there is a consensus regarding the urgent need of new and potent antimicrobial compounds (Baquero et al. 2011; Carlet et al. 2012), not only by generating novel derivatives of known antibiotics but also by exploiting the living organisms' chemical world, consisting of the myriads of small molecules that compose what has been defined as *parvome* (Davies 2011).

Considering the source of all known bioactive natural products, 47 % (33,000) are microbial metabolites and from these 84 % (28,000) have antibiotic activity. Historically, the most prolific producers of antibiotics have been actinomycetes, Gram-positive bacteria belonging to the phylum *Actinobacteria* that represents one of the largest taxonomic groups among the 18 major lineages currently recognized within the domain bacteria (Gao and Gupta 2012). In particular, the filamentous actinobacteria, especially *Streptomyces* species, produce 39 % of all microbial metabolites, 73 % of them showing antibiotic activity. Among microbial bioactive natural products, some structural types such as polyene macrolides, large-membered macrolides, anthracyclines, polyethers, cyclopolylactones, aminoglycosides, streptothricins, actinomycins, and quinoxaline peptides are produced almost exclusively by actinomycetes. Glycopeptides and orthosomycins are mainly produced by non-*Streptomyces* species (Bérdy 2012).

One-half of the drugs commonly used today were discovered in the *golden age* of antibiotics, expanding approximately from the clinical development of penicillin in 1941 to the discovery of lincomycin in 1964, which was introduced into the clinic as the semi-synthetic derivative clindamycin in 1968. After this golden age, the efficiency of classical screening methods failed and rediscovery was more frequent, leading to the discovery of only a few new chemical structures such as

clavulanic acid or daptomycin in 1976 and 1986, respectively (Fernandes 2006; Demain and Sanchez 2009). In the following period, the improvement of isolation and dereplication methods increased the identification of new bioactive metabolites, but the introduction of new compounds into therapy considerably decreased, even considering the development of novel synthetic and semisynthetic agents (Donadio et al. 2010; Buttler and Cooper 2011). During the last decade, research efforts were refocused, and classical screening methods have been changed by high-throughput screening (HTS) methods, the extensive use of combinatorial chemistry, and the synthetic modification of natural products (Singh 2011; Bérdy 2012). HTS technologies have led to the discovery of novel antibiotic entities produced by actinomycetes such as platensimycin (Wang et al. 2006), kibdelomycin (Phillips et al. 2011), and merochlorin A (Sakoulas et al. 2012), all highly active against multidrug-resistant methicillin-resistant *Staphylococcus aureus* (Fig. 1.1a). Combinatorial chemistry has also led to the development of novel antibiotics against *S. aureus* such as 3-aminoquinazolinediones (Hutchings et al. 2008) or 6-fluoroquinolones (Ghosh and Bagchi 2011; Minovski et al. 2012) active against *Mycobacterium tuberculosis* (Fig. 1.1b). In addition, the synthetic modification of natural products has had a great success, resulting in novel drugs, in particular active against *S. aureus*, such as new carbapenems, cephalosporins (Long 2003), erythromycins (Mutak 2007), and glycopeptides (Jeya et al. 2011) (Fig. 1.1c).

Other sources of novel antibiotics are the reinvestigation of metabolites previously considered inactive or that were developed for other uses and the exploitation of microorganisms isolated from traditionally neglected or extreme environments (Bérdy 2012). Over the past few decades, there are numerous examples of old or inactive compounds, the last ones representing close to 60 % of known metabolites, which after reinvestigation with modern screening methods have been found to show other useful activities (Cunha 2006; Chen and Kaye 2011). Regarding the investigation of new sources of bioactive compound producers, only a small fraction of the living species has been identified. In particular, a detailed investigation of the marine biosphere was only initiated in the last 25 years, and only recently, it has become clear that actinobacteria are well adapted to live in symbiosis with terrestrial and marine invertebrates, where they are considered to play a protective role by producing antibiotics (Imhoff et al. 2011; Seipke et al. 2012). At least 30,000 compounds have been isolated from marine organisms (Hu et al. 2011). In addition, it has been estimated that only 1 % of existing actinobacteria and not more 5 % of fungi are cultivable and can be identified using conventional methods (Singh and Macdonald 2010). As a consequence, at least 10 million microbial species may exist in hidden or encrypted forms in nature (Bérdy 2012).

Furthermore, new fields for the generation and identification of novel antibiotics and other bioactive drugs have blossomed at the end of the last century. Since the development of recombinant DNA technology, an increasing number of bioactive metabolite biosynthesis gene clusters have been isolated and characterized form different microorganisms, in particular actinomycetes, leading to the

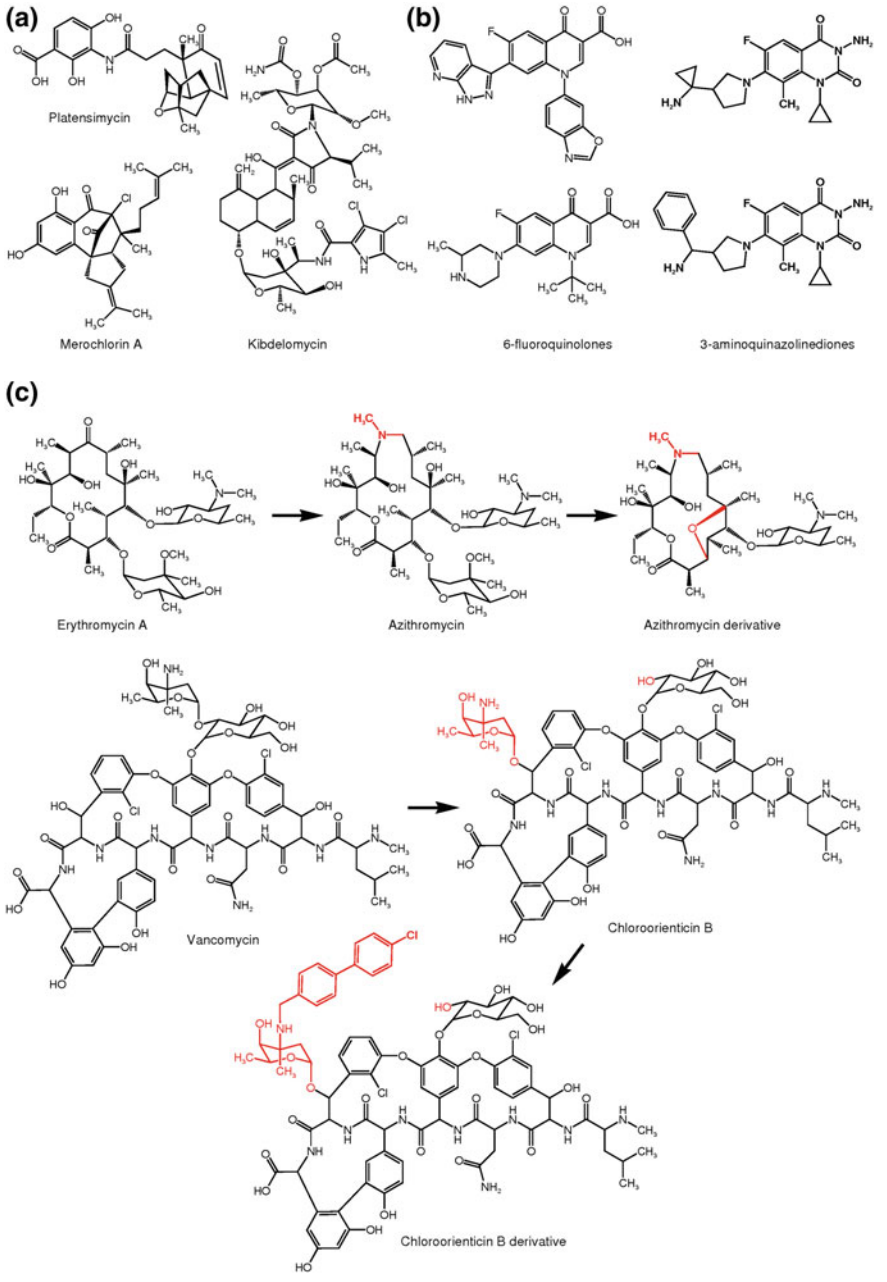


**Fig. 1.1** Novel antibiotics obtained by different methods. **a** screening using HTS methods, e.g., platensimycin, kibdelomycin, and merochlorin, **b** combinatorial chemistry, e.g., 6-fluoroquinolones, and 3-aminoquinazolinediones, **c** semi-synthetic approaches, e.g., erythromycin and vancomycin derivatives showing the modified moieties in *red*

development of genetic engineering approaches to develop new bioactive compounds (Olano et al. 2011). These strategies, known as *combinatorial biosynthesis* (Fig. 1.2), aimed to create novel genetic combinations of structural biosynthetic genes. Combinatorial biosynthesis methods consist in gene inactivation for the generation of mutant strains that might accumulate novel compounds, expression of specific genes or combination of genes in wild type, mutant strains or heterologous hosts, and mutasynthesis where a mutant strain is fed with alternative precursors for the biosynthesis of the desired compound (Olano et al. 2010; Kwon et al. 2012; Winter and Tang 2012). Some of these approaches have been also used for improving the production of bioactive metabolites produced by actinomycetes (Olano et al. 2008a). In addition, the increased efficiency and reduced cost of DNA sequencing has prompted researchers to join whole prokaryotic genome sequencing projects (Loman et al. 2012; Lasken 2012). At the present time, 2,428 microbial genomes are available, corresponding 300 of them to actinomycetes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). This, together with the improvement of bioinformatics annotation (Wood et al. 2012; Torrieri et al. 2012) and biosynthesis gene cluster search tools (Fedorova et al. 2012), has shown that the metabolic capabilities of microorganisms known to produce bioactive

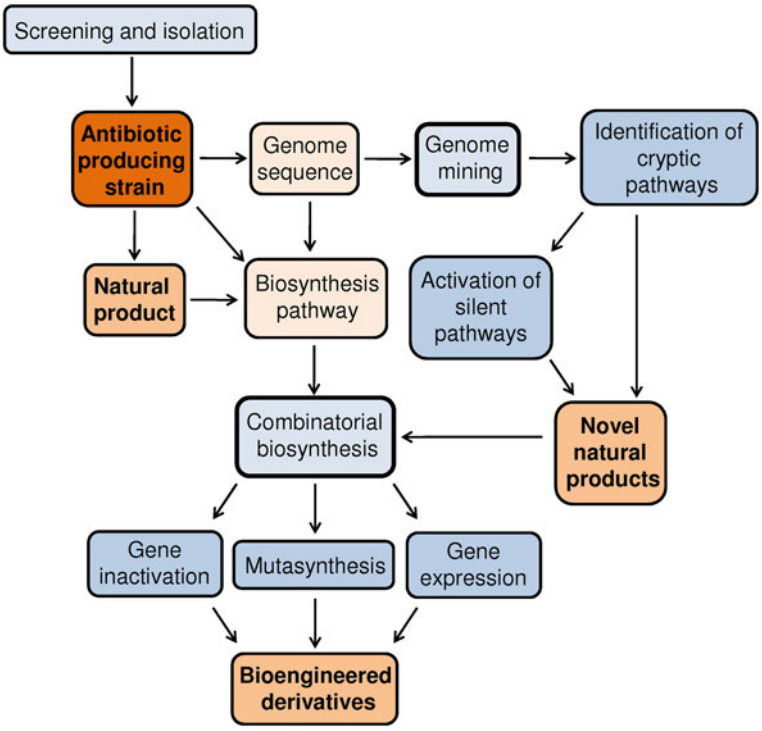


**Fig. 1.2** Scheme representing the different strategies used for generating novel antibiotics using genetic engineering and genome mining of microbial producers

compounds have been clearly underestimated (Nett et al. 2009). However, the production of secondary metabolites is not equally distributed within microorganisms, and only those having genomes higher than three mega bases are normally capable of producing such compounds (Donadio et al. 2007). This fact has been uncovered by genome mining for fungi (Brakhage and Schroeckh 2011), mycobacteria (Wenzel and Muller 2009), pseudomonads (Gross and Loper 2009), cyanobacteria (Kalaitzis et al. 2009), uncultivable bacteria (Brady et al. 2009), and in particular for actinomycetes. In the last case, genome sequencing of several antibiotic producers has revealed the presence in their chromosomes of 18–37 clusters involved in the biosynthesis of known or predicted secondary metabolites belonging to different structural classes (Nett et al. 2009). The fact that such elevated number of clusters involved in the biosynthesis of unknown metabolites (*cryptic* pathways) are present in microbial genomes has prompted the search for strategies to confirm the function of these pathways identifying the corresponding produced compounds (Zerikly and Challis 2009; Winter et al. 2011). This approach quite often requires the activation of those pathways that are not expressed, known as *silent* pathways (Fig. 1.2).

In the next following sections, we will highlight the different approaches used for the development of novel derivatives of known compounds through combinatorial biosynthesis as well as those used in genome mining for the identification of novel bioactive compounds from actinomycetes.

## 1.2 Generation of Novel Derivatives by Combinatorial Biosynthesis

During the last 28 years, since the identification of the *Streptomyces coelicolor* biosynthesis gene cluster involved in the production of blue-pigmented antibiotic actinorhodin in 1984, a large number of clusters from actinomycetes have been characterized (Olano 2011). This has allowed unraveling the biochemical steps leading to the production of different structural classes of bioactive compounds. In addition, the characterization of these clusters represents a great source of genetic tools for the generation of novel derivatives of known bioactive compounds by combinatorial biosynthesie approaches (Olano et al. 2010; Kwon et al. 2012; Winter and Tang 2012).

Actinomycete biosynthesis gene clusters are characterized by the presence in close vicinity of all genetic elements required for the production of the secondary metabolite. These elements normally include pathway-specific regulatory genes to control the expression of the cluster and resistance elements to avoid self-destruction of the producer of the bioactive compound. In addition, the most important elements in terms of combinatorial biosynthesis are those encoding enzymes directly involved in the biosynthetic process, known as structural genes. These participate in the assembling of precursors to generate the structural core,

the biosynthesis of specific precursor units, and tailoring modification by attachment of different structural elements such as deoxysugars or by the introduction of simpler chemical moieties (Olano 2011).

### *1.2.1 Targeting Precursor Condensing Steps*

Polyketides, nonribosomal peptides, and isoprenoids (e.g., erythromycin A, vancomycin, and platensimycin, Fig. 1.1) are large families of natural products widely produced by actinomycetes and therefore highly represented among biosynthetic gene clusters found in these microorganisms (Nett et al 2009). The structure of these compounds is characterized by the presence of a core backbone, produced by sequential sets of condensing steps involving different precursors, which is finally decorated by the addition of different chemical moieties. The carbon skeleton of polyketides is synthesized by polyketide synthases (PKS) catalyzing stepwise decarboxylative Claisen-type condensation of acyl-CoA precursors that are further reduced and modified. There are three types of PKSs, but the most complex one corresponds to type I PKSs, large multifunctional enzymes harboring a varying number of modules consisting ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains that catalyze sequential condensations (Fig. 1.3a). After each condensation, the extended $\beta$-keto thioester can be reduced by a combination of the following reduction domains: ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) (Fig. 1.3a). The number of modules present in a PKS normally reflects the size of the carbon chain (Hertweck 2009). Nonribosomal peptides are synthesized using proteinogenic and nonproteinogenic amino acids and carboxylic acids by the activity of nonribosomal peptide synthetases (NRPS). The NRPS assembly line follows the same chemical logic as PKS does for chain elongation, with several domains (adenylation, condensation, and peptidyl carrier protein) organized into modules and additional domains with epimerization, methyltransferase, reductase, or oxidase activity for the corresponding amino acid modification (Koglin and Walsh 2009). On the other hand, isoprenoids are generated by the condensation of five carbon unit precursors, isopentenyl diphosphate and dimethylallyl diphosphate, to produce linear polyprenyl diphosphates with specific chain lengths. Geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate synthases yield C10, C15, and C20 polyprenyl chains, respectively. These chains are most often processed by cyclases to produce the parent skeleton of monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20) (Daum et al. 2009).

The inactivation of genes encoding PKSs, NRPSs, or isoprenoid synthases leads to abrogation of the biosynthesis of the corresponding compound. For this reason, any attempt to modify the core structure of these natural products must involve more delicate approaches such as site-directed mutagenesis (Reeves et al. 2001; Eppelmann et al. 2002; Nakano et al. 2011) or in-frame deletion of modules or domains in the case of PKSs or NRPSs (Brautaset et al. 2002; Mootz et al. 2002).

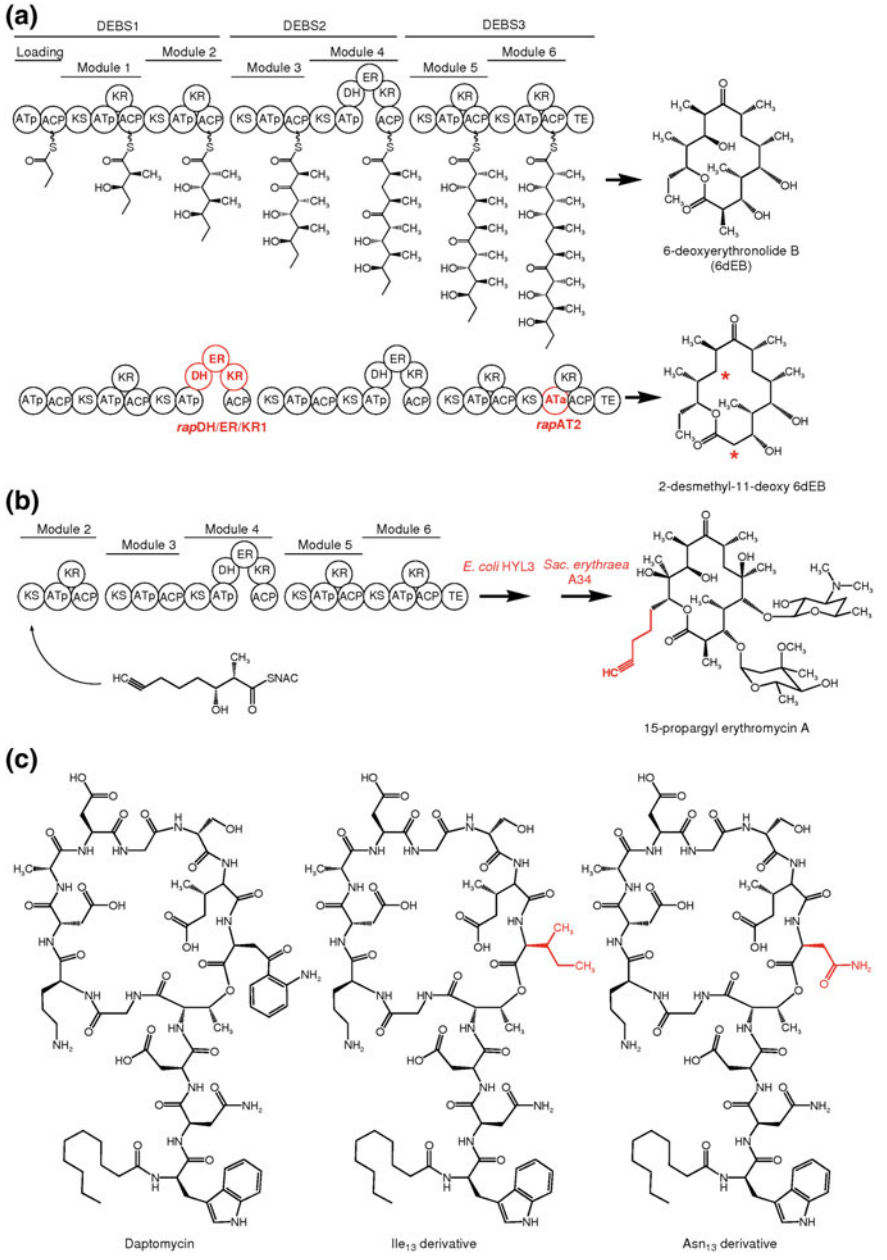


**Fig. 1.3** Derivatives of 6-deoxyerythronolide B, erythromycin, and daptomycin generated by combinatorial biosynthesis by altering the multidomain enzymes involved in the biosynthesis of the structural core. **a** 6-deoxyerythronolide B and derivative generated by DEBS (erythromycin PKS) and rapamycin PKS domain swapping, **b** erythromycin and analog generated by precursor-directed biosynthesis, **c** daptomycin and some analogs generated by NRPS module swapping between DptD and LptD (Ile13 derivative) or DptD and LptC (Asn13 derivative)

In addition, modular enzymes such as Type I PKSs or NRPSs can be engineered by other approaches such as domain or module swaps using as donors the same kind of elements from enzymes involved in the biosynthesis of structurally related natural products. Taking advantage of the colinearity between modules and the final structure, the new elements inserted will be translated into structural modifications present in the novel derivative. Two good examples of this approach are the generation of erythromycin A and daptomycin derivatives (Fig. 1.3).

Erythromycin A is a macrolide antibiotic produced by *Saccharopolyspora erythraea* by the activity of a Type I PKS (DEBS) consisting of one loading and six extension modules. The product of the PKS, 6-deoxyerythronolide B (6dEB) (Fig. 1.3a), is then further modified by the introduction of two hydroxyl groups and two deoxysugar moieties to generate the final antibiotic (Rawlings 2001). A combinatorial library of 6dEB derivatives was generated by substituting the ATs and $\beta$-carbon-processing domains of DEBS modules 2, 5, and 6 with counterparts from the rapamycin PKS (RAPS) that encode alternative substrate specificities and $\beta$-carbon reduction/dehydration activities. The engineered DEBS versions containing single, double, and triple catalytic domain substitutions were expressed, using a single plasmid, in the heterologous hosts *S. coelicolor* CH999 or *Streptomyces lividans* K4-114 where they catalyzed the production of 6dEB derivatives with the corresponding single, double, and triple modifications such as 2-methyl-11-deoxy 6dEB (Fig. 1.3a). Thus, by generating 21 DEBS versions, 60 novel 6dEB derivatives were generated, and many of them have also been processed further to erythromycin analogs by a DEBS-blocked mutant of *Sac. erythraea* (McDaniel et al. 1999). A further improvement of this approach consisted in the coexpression of the library containing the genetically modified DEBS PKS with a plasmid containing a set of nine desosamine biosynthetic and auxiliary genes that included the desosaminyl transferase. The resulting *S. lividans* strains produced desosaminylated 6dEB derivatives (Tang and McDaniel 2001). Clearly, the preparation of very large libraries by this approach would be laborious, and a refinement of this method was developed. In the new system, each individual DEBS gene or its modified version was expressed on a separate, but compatible plasmid, then the separate genes are coexpressed in a heterologous host. The combination of 5 DEBS1, 2 DEBS2, and 9 DEBS3 genes (including the wild-type gene and versions containing swapped RAPS domains, modules eliminated by in-frame deletion, or domains inactivated by site-specific mutagenesis) led to 59 6dEB derivatives (Xue et al. 1999).

In a Type I PKS such as DEBS, all modules are susceptible to be swapped by a module from a different PKS, including the loading domain. Swapping DEBS loading domain by broad substrate specificity avermectin PKS counterpart, 12 novel erythromycins were generated containing a wide variety of different starter units (Marsden et al. 1998). To obtain such kind of starter-unit-modified derivatives, a more straightforward method was developed consisting in the removal of DEBS loading domain. Then, precursor-directed biosynthesis was used by feeding the strain expressing the truncated form of the PKS with chemically synthesized alternative precursors in the form of N-acetylcysteamine thioesters (SNAC)

(Ward et al. 2007). Following this method, the novel antibiotic 15-propargyl erythromycin A was recently generated (Fig. 1.3b). To achieve this goal, the loading-domain-deleted DEBS was expressed in *Escherichia coli* HYL3, engineered for improved polyketide production, and fed with the corresponding SNAC precursor. The resulting 6-dEB derivative was converted into the final antibiotic by feeding *Sac. erythraea* A34, mutant with a greatly diminished capacity for 6-DEB production but with all the tailoring modification enzymes functional (Harvey et al. 2012).

As mentioned above, NRPSs are also modular enzymes following the same logic for chain elongation as PKSs but mainly condensing amino acids. As well as PKSs, the number of modules present in a NRPS reflects the size of the final peptide product. For these reasons, NRPSs have also been engineered by module swapping. For example, several derivatives of lipopeptide antibiotic daptomycin have been generated using this method (Doekel et al. 2008; Nguyen et al. 2010). Daptomycin is a 13-membered peptide cyclized to form a 10-membered ring and a 3-membered exocyclic tail, to which is attached a decanoic acid side chain through the N-terminus of L-Trp1 (Fig. 1.3c). The thirteenth amino acid corresponds to kynurenine (Kyn13). When the corresponding module in daptomycin NRPS DptD is substituted by its counterpart involved in the biosynthesis of lipopeptide antibiotic A54145 (LptD) that introduces an isoleucine residue, a daptomycin Ile13 was generated. When DptD module 13 was swapped by LptC module 11, an asparagine (Asn13) derivative was obtained (Doekel et al. 2008). Following the same approach, A54145 derivatives have been recently engineered (Nguyen et al. 2010).

### *1.2.2 Targeting Precursor Biosynthesis Steps*

Secondary metabolites are built using, as scaffold elements, precursors supplied by primary metabolism. However, in some biosynthesis clusters, there are genes dedicated to produce specific precursors not provided by primary metabolism or when supplied an extra amount is required at due time for the production of the secondary metabolism. These precursors serve normally as starter units for PKSs or NRPSs but occasionally are used as extender units. The inactivation of genes involved in the biosynthesis of these precursors, in particular if acting as starter units, leads to nonproducing mutants. These mutant strains can be used for precursor-directed biosynthesis of novel compounds in an approach known as mutasynthesis or mutational biosynthesis. This method has been used to generate novel derivatives of numerous secondary metabolites including aminocoumarin antibiotics (Heide et al. 2008) and macrolide antibiotics such as erythromycin (Toscano et al. 1983) or borrelidin (Moss et al. 2006). In the case of borrelidin, a *borG* mutant (Olano et al. 2004a) affected in the biosynthesis of PKS starter unit, cyclopentane-*trans*-1,2-dicarboxylic acid, was used. Eight derivatives were obtained by feeding the mutant strain with different dicarboxylic acids. In

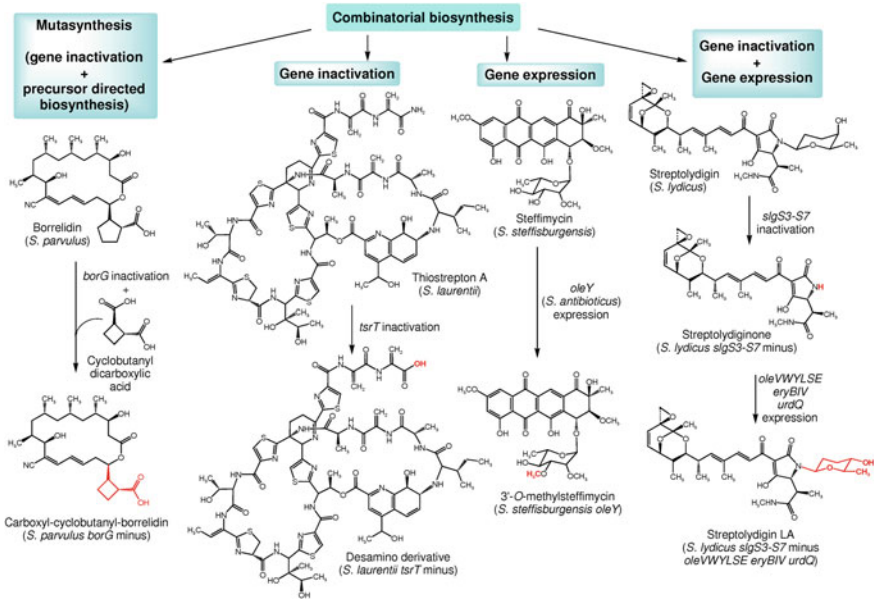


**Fig. 1.4** Derivatives of different *Streptomyces* natural products using combinatorial biosynthesis approaches: gene inactivation, mutasynthesis, and gene expression

particular, carboxyl-cyclobutanyl-borrelidin was generated using as starter unit cyclobutane-*trans*-1,2-dicarboxylic acid (Fig. 1.4).

Occasionally, the inactivation of genes involved in the biosynthesis of precursor elements can lead to novel derivatives, in particular if the precursor is used as an extender unit. Streptolydigin (Fig. 1.4), a hybrid polyketide-peptide antibiotic, is biosynthesized by the activity of a Type I PKS, composed by one loading domain and seven extension modules, followed by the incorporation of an amino acid by the activity of an NRPS system (Olano et al. 2009). The amino acid precursor is glutamate which is converted to 3-methylaspartate by a glutamate mutase and then, after loaded to the NRPS, transformed into 3-methylasparagine by an asparagine synthetase and methylated to generate the final scaffold *N*-methyl-3-methylasparagine. All these enzymes are encoded by genes present in the biosynthesis gene cluster. The inactivation of either the glutamate mutase or the asparagine synthetase abrogated the production of streptolydigin since *N*-methyl-3methylasparagine was no longer available. The NRPS was flexible enough to incorporate glutamate instead, leading to the production of two analogs: streptolydigin B in the asparagine synthetase mutant (Horna et al. 2011) and streptolydigin B and C in the glutamate mutase mutant (Gómez et al. 2011).

### 1.2.3 Targeting Tailoring Modification Steps

Final steps in the biosynthesis of secondary metabolites are performed by tailoring modification enzymes. These enzymes introduce chemical modifications into the structural core and include group transferases such as glycosyl-, methyl-, acyl-, prenyl-, carbamoyl-, or aminotransferases. Other tailoring modifications introduce oxygen-containing functionalities such as hydroxyl, epoxide, aldehyde, and keto groups or modify these moieties by addition or removal of hydrogen atoms. These reactions, catalyzed by oxygenases, oxidases, peroxidases, reductases, and dehydrogenases, can provide a base for additional modifications introduced by the group transferases mentioned above. In addition, the introduction of chlorine and bromine atoms in secondary metabolites is frequently performed by halogenating enzymes known as flavin-dependent halogenases. Due to such variety of activities, genes encoding tailoring modification enzymes are the most prolific in terms of novel compounds produced by combinatorial biosynthesis approaches (Walsh et al. 2001; Olano et al. 2010).

The inactivation of genes involved in tailoring modification leads to intermediate or novel natural product derivatives. That is the case of the disruption of a gene encoding an amidotransferase involved in the biosynthesis of antibiotic thiostrepton (Fig. 1.4), which abrogates thiostrepton production and results in the detection of a new metabolite (Kelly et al. 2009). All types of enzymatic activities have been targeted by this method. In particular, the inactivation of genes encoding enzymes participating in oxygenation reactions or in the production and attachment of deoxysugars has been highly prolific in the generation of novel compounds. Oxygenases such as cytochrome P450 s, FAD-dependent oxygenases, dioxygenases, epoxidases, or hydroxylases have been targeted in order to obtain derivatives from several antibiotics, e.g., erythromycin (Weber et al. 1989), borrelidin (Olano et al. 2004b), or tirandamycin (Carlson et al. 2011). On the other hand, inactivation of genes involved in the production of deoxysugars or in glycosylation leads to mutants accumulating compounds with a different pattern of glycosylation such as those derived from erythromycin (Dhillon et al. 1989), mithramycin (Fenández et al. 1998; Remsing et al. 2002), or hygromycin A (Palaniappan et al. 2006).

Tailoring enzymes show, in general, a broad substrate specificity, being able to modify not only their particular substrate but also any other structurally related or containing a portion of the structure close to the natural substrate. According to this, heterologous expression of genes encoding such enzymes in strains producing natural products can result in novel derivatives containing alternative decorations. In particular, the substrate flexibility of glycosyltransferases has been widely exploited (Salas and Méndez 2007; Méndez et al. 2008; Luzhetskyy et al. 2008). This has been possible by developing a family of *sugar biosynthesis plasmids* cloning PCR-amplified sugar biosynthesis genes, flanked by unique restriction sites, into a bifunctional vector (*E. coli-Streptomyces*) under the control of constitutive promoters. These constructs, like a *plug and play* cassette system, allow genes to be easily removed and replaced by others coding for different enzymatic functions. This

resulted in the generation of a series of plasmids, each directing the biosynthesis of a different sugar and, when introduced into an appropriate host, endowing the host with the capability of synthesizing novel deoxysugars (Rodríguez et al. 2000; Lombó et al. 2004; Pérez et al. 2005, 2006). Novel mithramycin derivatives were generated containing alternative deoxysugars and different glycosylation patterns introducing these constructs into wild-type *S. argillaceus* (Pérez et al. 2008; Baig et al. 2008). The OleY *O*-methyltransferase is another good example of substrate flexibility. During the biosynthesis of oleandomycin, the L-oleandrose moiety is biosynthesized and transferred to the aglycon as L-olivose. OleY methylates L-olivosyl-oleandolide to render the oleandomycin intermediate L-oleandrosyl-oleandolide. In addition, OleY can also methylate L-rhamnosyl- and L-mycarosyl-erythronolide B (Rodríguez et al. 2001). Furthermore, the expression of *oleY* into anthracycline steffimycin producer *S. steffisburguensis* leads to 3′-*O*-methylsteffimycin (Olano et al. 2008b) harboring an additional methyl group in the naturally occurring 2-*O*-methylrhamnose moiety (Fig. 1.4).

Tailoring modification coding genes can be expressed not only in wild type but also in mutant strains generated by gene inactivation. The expression of sugar biosynthesis plasmids into an *S. argillaceus* mutant specifically blocked in the biosynthesis of D-oliose conducted to mithramycin derivatives lacking the D-oliose residue while containing instead several other deoxysugars. Some of those compounds showed additional substitutions in the sugar profile (Pérez et al. 2008). Derivatives of the antibiotic streptolydigin have also been generated by expressing several sugar biosynthesis plasmids into a mutant strain having all genes involved in the biosynthesis of L-rhodinose deleted and consequently producing the streptolydigin aglycon (Fig. 1.4). The novel derivatives obtained carry L-amicetose, L-digitoxose, D-amicetose, or D-olivose instead of the naturally occurring L-rhodinose (Olano et al. 2009).

As we have mentioned before, the development of 6dEB derivatives has been mainly addressed by expressing the precursor condensing enzymes in various heterologous hosts such as *S. lividans*, *S. coelicolor*, or *E. coli*. These and other hosts are also suitable to express whole biosynthesis gene clusters or combinations of core biosynthesis and tailoring modification genes from different clusters (Baltz 2010). The combination of both elements has conducted to the production of novel compounds derived from several natural products in different heterologous hosts. For example, derivatives of rebeccamycin and staurosporine (Salas et al. 2005; Sánchez et al. 2005, 2009), oviedomycin (Lombó et al. 2009), elloramycin and tetracenomycin (Lombó et al. 2004; Pérez et al. 2005, 2006), or steffymycin (Olano et al. 2008b) have been obtained by expressing genes in *S. albus*. In addition, aminoglycoside (Thapa et al. 2008; Park et al. 2011) and anthracycline (Han et al. 2011) derivatives have been generated in *S. venezuelae*.

## 1.3 Identification of Novel Natural Products by Genome Mining

Whole-genome sequencing of bacterial genomes has become a routine task based on the recent advances in DNA sequencing (Loman et al. 2012; Lasken 2012) and the development of improved bioinformatic tools for gene annotation (Wood et al. 2012; Torrieri et al. 2012). Actinomycete genome sequencing has been prompted by the capability of these microorganisms to produce bioactive natural products. Some of the first sequenced actinomycete genomes corresponded to *S. avermitilis*, *S. coelicolor*, *Sac. erythraea*, *Salinispora tropica*, or *S. griseus*, producers of avermectin, actinorhodin, erythromycin, salinosporamide, and streptomycin, respectively (Nett et al. 2009). The analysis of these genome sequences has revealed the presence of numerous biosynthetic gene clusters that might be involved in the biosynthesis of additional secondary metabolites belonging to different structural classes (Nett et al. 2009). These biosynthesis gene clusters are defined as cryptic or orphan since the natural product they originate from is unknown. The putative structure of these metabolites can be predicted using bioinformatic tools that consider the function of the genes present in the cluster (Ziemert and Jensen 2012). In addition, the identification of the natural product requires experimental approaches that we will discuss in this section.

### *1.3.1 Identification of Cryptic Pathways and Their Products*

A good number of cryptic clusters contain gene coding for multimodular PKSs and NRPSs. As mentioned above, these enzymes follow a colinearity rule for assembling precursors in the same order of the modules that compose them. The type of building blocks incorporated into the final product can also be predicted based on the catalytic centers of the AT or adenylation domains present in each module. In addition, the modification of each building block depends upon the presence of additional domains in each module such as DH, ER, KR, epimerase, methyltransferase, or oxidase (Hertweck 2009; Koglin and Walsh 2009).

Structural predictions are a valuable help for the identification of new natural products based on its deduced physicochemical properties. Genomic analysis of *Streptomyces* sp. SPB78 draft genome led to the identification of a hybrid PKS-NRPS with great similarity to that involved in the biosynthesis of polycyclic tetramate macrolactam dihydromaltophilin. Extracts of *Streptomyces* sp. SPB78 grown on different media were screened for the production of polycyclic tetramate macrolactams that exhibit a UV maximum around 220–323 nm. This approach led to the identification of frontalamide A (Fig. 1.5), a new member of this family of compounds (Blodgett et al. 2010). The *genomoisotopic* approach relies also in bioinformatic analysis of a NRPS or hybrid PKS–NRPS pathway in order to predict the amino acids that will be used as precursors. The administration of

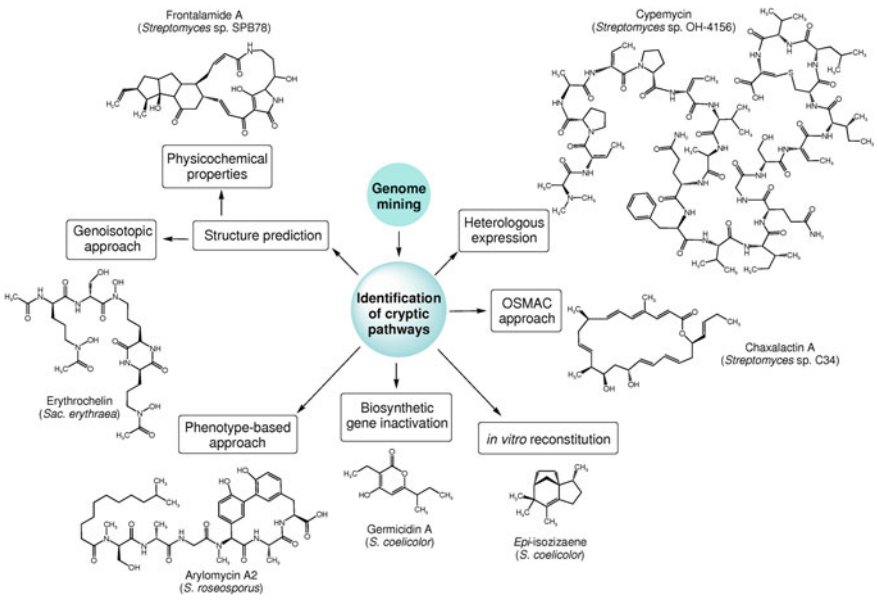


**Fig. 1.5** Bioactive compounds identified by genome mining followed by the characterization of cryptic pathways

radioactively labeled precursors to the producer organism allowed the identification of the final radioactive products. In such a way, the hydroxamate-containing siderophore antibiotic erythrochelin (Fig. 1.5) was isolated and characterized from *Sac. erythraea* (Robbel et al. 2010). The same natural product was purified and then characterized by a bioassay-based fractionation following its antibiotic activity against *Micrococcus luteus* or *Bacillus subtilis* (Lazos et al. 2010). Another recently developed phenotype-based approach has been used for the identification of novel peptide anti-infective agents such as arylomycin (Fig. 1.5) produced by *S. roseosporus* (Liu et al. 2011). This approach connects phenotype (e.g., growth inhibition of *Sthaphylococus epidermidis* cocultured with *S. roseosporus*) with chemotype (anti-infective agent) using MALDI imaging mass spectrometry. Then, a peptidogenomic mining approach connects the chemotype of a peptide natural product to its biosynthetic gene cluster by matching the mass spectrometry structures to predicted genome-based structures. Only one or two amino acid residues are required for NRPS-derived peptides to identify the correct gene, while for ribosomal encoded peptides, five to six consecutive amino acids are needed.

When there is not an available bioassay/phenotype-based or a physical–chemical-based prediction approach to afford the characterization of the cryptic biosynthesis cluster, approaches based on molecular biology can be applied. One of these is the inactivation of structural genes in order to generate a mutant strain where the biosynthesis gene cluster turns inactive, thus abrogating the metabolite

production. The comparison of the mutant versus the wild-type strain metabolic profiles by high-performance liquid chromatography and mass spectrometry might reveal the natural product that is absent in the mutant strain. The corresponding natural product can be isolated and structurally characterized from the wild-type strain. Following this approach, germicidin A (Fig. 1.5) biosynthesis cluster was identified from *S. coelicolor* after inactivation of a structural gene encoding a Type III PKS (Song et al. 2006). The opposite approach to gene inactivation is the expression of the entire biosynthesis gene cluster, cloned in a suitable vector, into a heterologous host. The comparison of metabolic profiles between the heterologous host carrying and lacking the cloned cryptic cluster might provide the evidence of the new product accumulated. Linear peptide cypemycin (Fig. 1.5) produced by *Streptomyces* sp. OH-4156 was identified following the expression of its cluster cloned in a cosmid vector and expressed in *S. venezuelae* (Claesen and Bibb 2010). Another method involving molecular biology technologies is the in vitro reconstitution approach. In this system, a biosynthetic enzyme encoded by the cryptic cluster is expressed and purified. The pure enzyme is incubated with the putative predicted substrate to identify the product generated during the reaction. The expression and purification from *E. coli* of a *S. coelicolor* sesquiterpene synthase followed by its incubation with different putative substrates led to the identification of *epi*-isozizaene (Fig. 1.5), biosynthetic intermediate of sesquiterpene albaflavenone (Lin et al. 2006).

Other methods involve the enhancement of the biosynthesis gene cluster expression in order to identify the natural product. This can be achieved by several systems such as the one strain/many compounds (OSMAC) approach. This method is based on the systematic alteration of the culture media composition or cultivation parameters to force the expression of cryptic clusters. This method led to the discovery of chaxalactin A (Fig. 1.5) and two additional members of a novel class of 22-membered macrolides produced by *Streptomyces* sp. C34 (Rateb et al. 2011). In addition, any system that deregulate secondary metabolism can be used to enhance the production of secondary metabolites, leading to the discovery of novel bioactive compounds. Several of these approaches have been recently reported such as the treatment with chemicals known to deacetylate eukaryotic histone proteins (widespread in bacteria) that change chromatin structure and in turn up-regulate biosynthetic pathways (Moore et al. 2012) or the use of small molecules that remodel and up-regulate secondary metabolism by inhibiting fatty acid biosynthesis (Craney et al. 2012). The production of secondary metabolites can be also magnified by inducing mutations conferring resistance to different antibiotics by *ribosome engineering*. In this case, the overproduction of secondary metabolites correlates with an enhanced protein synthesis (Wang et al. 2008).

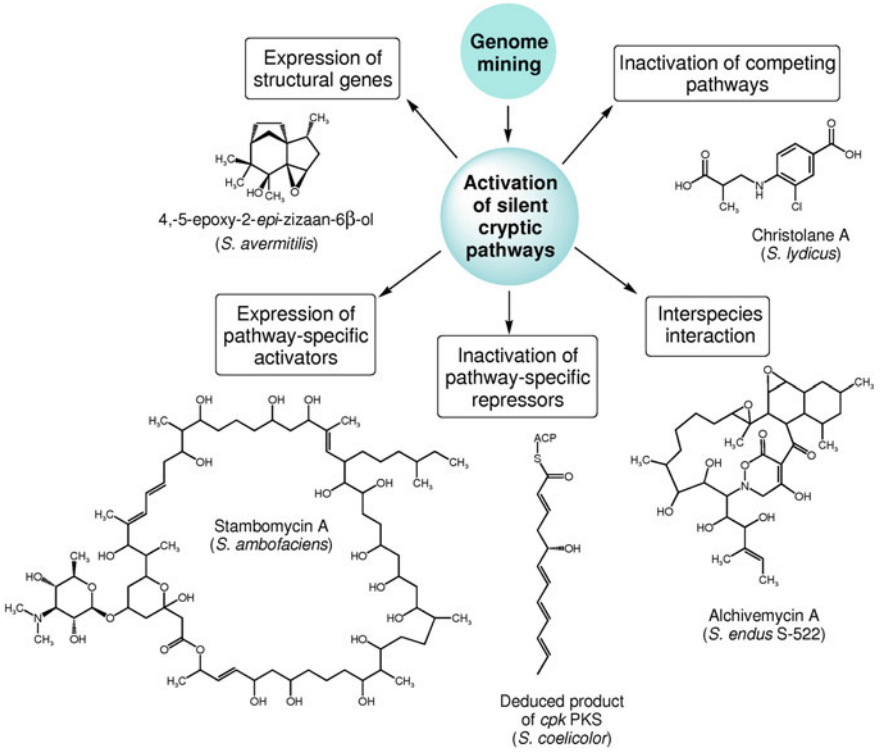


**Fig. 1.6** Bioactive compounds identified by genome mining followed by the activation of silent cryptic pathways

## *1.3.2 Activation of Silent Cryptic Pathways*

Some cryptic biosynthesis clusters are, in addition, not expressed (silent) under standard fermentation conditions and might be activated in order to identify the product they originate. The first approach to activate the expression of a particular silent cluster is the deregulation of its expression. This can be achieved by several methods such as the expression of structural genes under the control of a constitutive promoter. The expression of a sesquiterpene synthase (*sav3032*) from *S. avermitilis* under the control of the native constitutive promoter *rpsJp* led to *epi*-isozizaene. Furthermore, *epi*-isozizaene, its oxidized derivatives, albaflavenol, and albaflavenone, as well as a the previously unknown doubly oxidized derivative 4b,5b-epoxy-2-*epi*-zizaan-6$\beta$-ol (Fig. 1.6) were produced in *S. avermitilis* by coexpressing the sesquiterpene synthase with the cytochrome P450-encoding *sav*3031 (Takamatsu et al. 2011).

The activation of silent clusters can also be addressed by targeting pathway-specific regulatory genes, expressing activators, or inactivating repressors. The

constitutive expression of *samR0484*, encoding a putative large ATP-binding regulator of the LuxR family (LAL) protein in *S. ambofaciens*, switched on the expression of a biosynthesis gene cluster involved in the production of 51-membered glycosylated macrolides (Laureti et al. 2011). This led to the identification of stambomycin A (Fig. 1.6) and three additional compounds of the same family. On the other hand, inactivation of *scbR2*, encoding a putative $\gamma$-butyrolactone receptor acting as transcriptional regulator from *S. coelicolor*, led to the discovery of a novel antibacterial activity and a yellow-pigmented secondary metabolite. Both the antibacterial activity and the pigment are linked to the Type I PKS gene cluster *cpk,* being the antibacterial compound converted into the yellow pigment (Gottelt et al. 2010). At the moment, no structure has been reported for any of these compounds being only available the predicted structure of the linear PKS product (Fig. 1.6) (Pawlik et al. 2007). The apparently simple task of deregulating a silent biosynthesis cluster, following the methods described in this paragraph, could be further complicated. In occasions, it can be difficult to correctly define a particular regulator as activator or repressor. In fact, regulators are normally considered as repressors such as the TetR family (Ramos et al. 2005) have been shown to have opposite effects. Two highly similar TetR proteins such as SlgR1 (Gómez et al. 2012a) and TrdK (Mo et al. 2011), each controlling the production of related compounds streptolydigin and tirandamycin, activate or repress their respective biosynthesis gene clusters.

In addition to the genetic engineering-guided activation of specific silent biosynthesis gene clusters, serendipity might play an important role to activate silent clusters, leading to the discovery of novel bioactive natural products. It has been reported that by abrogating the biosynthesis of a particular compound, another pathways in the same organism can increase the production of known natural products by using the additional nonconsumed precursors (Sun et al. 2002; Ou et al. 2009). Furthermore, this approach can lead to the activation of silent clusters, remaining dormant because of the lack of precursors available. Such is the case of the production of christolane A (Fig. 1.6) and two related analogs by *S. lydicus*. The biosynthesis of these compounds can only be detected when streptolydigin biosynthesis has been interrupted by, for example, the inactivation of a structural gene (Gómez et al. 2012b). Another method where serendipity is an important player is the exploration of interactions occurring between species, either microorganisms sharing the same ecological niches (Seyedsayamdost et al. 2012) or microorganisms living as symbionts with insects, plants, or marine animals (Seipke 2012). The proposed biological function of many natural products, especially secondary metabolites, is to be antibiotics to fight against competitors but also signal molecules involved in intra- or inter-species interactions (Romero et al. 2011). Thus, the influence between species is expected to turn on the expression of cryptic clusters in response to the threat of a foe or in response to molecular signals, leading to the appearance of novel natural products. An example of this kind of approach is the discovery of antibiotic alchivemycin A (Fig. 1.6) produced by *S. endus* S-522 only in the presence of mycolic acid-containing bacteria such as *Tsukamurella pulmonis* (Onaka et al. 2011).

## 1.4 Conclusion and Future Perspectives

Infectious diseases have been one of the main concerns of public health for the last century. Enormous efforts have been and are applied to fight against infective agents leading in some cases to their eradication. However, there is still an urgent need for novel anti-infective agents due to the increasing resistance toward known antibiotics and the rapid worldwide distribution of vectorborne and waterborne infectious diseases due to climate change.

Microorganisms, especially actinomycetes and fungi, are the main sources of anti-infective agents and have an inexhaustible capability to produce bioactive compounds that are the main warheads to fight against infection diseases and other illnesses. Bioactive natural products have unique structural features, which rarely occur among common synthetic compounds and for that reason are also good leads for the structural modification aimed to increase or change their therapeutic activity. In this task, combinatorial biosynthesis is playing an important role that will lead to successful therapeutics in the future. Considering that the most prescribed antibiotic drugs nowadays are semisynthetic or modified antibiotics, and the youth of genetic engineering, in the near future novel anti-infective agents generated by combinatorial biosynthesis approaches are expected to boost.

Combinatorial biosynthesis feeds on the identification of any natural product biosynthesis gene cluster in order to use the genetic information to modify the structure of the compound. The source of these natural products has been recently expanded by whole-genome sequencing projects and the use of genome mining. This has unveiled the presence of previously unknown biosynthesis gene cluster that determines the biosynthesis of novel compounds often overlooked under standard fermentation and detection conditions. Once each biosynthesis cluster is associated to its corresponding compound, the use of combinatorial biosynthesis approaches will generate further structural diversity, increasing the arsenal of novel drugs. In addition, the biosynthesis gene clusters contain elements that can be used to increase the production of both natural and engineered products that will facilitate the reduction in production costs and in turn their commercial use.

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# Chapter 2
# X-Ray and Neutron Scattering Foundations for the Research in Antimicrobials

**Daniel Piso**

**Abstract** Scattering is becoming a very important alternative for research in biology. X-rays and neutrons permit to obtain information about the molecular processes, determine biological structures and even be helpful in the design of new drugs. Research in antimicrobials can also be benefited from instruments using these techniques. The history, physical foundations and theoretical apparatus that enable the extraction of information from scattering are explained. Big scientific facilities that play major role in scattering investigation are also mentioned. Some of them are already in operation, and others will provide the scientific community with powerful instruments in the short or medium term. Also, a basic description of the two most representative scattering techniques for biology is included. Finally, some success case of antimicrobials and biology investigations are included to illustrate the effectiveness of these experimental tools.

## 2.1 Introduction

During the last decades, neutron and X-ray scattering based techniques have become major alternatives in the research of static and dynamic biological structures. These techniques have proven to be two of the most powerful structural determination techniques, especially, for the study of membranes, which is one of the central axes in antimicrobials research.

The X-ray crystallography began when Max von Laue and his colleagues discovered that the phenomenon of interference of light could also be applied to X-rays. The distance between atoms in macromolecules is comparable to the radiation wavelength. This fact made von Laue come to the conclusion that a crystal would act as a three-dimensional interference lattice for X-ray radiation (Friedrich et al. 1912).

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The next step was the determination of the structure of the compounds by using the information obtained from X-ray photographs. This accomplishment was done by Lawrence Bragg for the first time. What Lawrence Bragg did was to calculate the intensities of all reflections (interference points) in an X-ray diffraction photograph and to compare these values with the observed intensities. In 1915, W. L. Bragg and W. H. Bragg (son and father) shared the Nobel Prize in Physics, the son, W. L. Bragg, for his work on diffraction and crystal structures and his father, W. H. Bragg, for studies on the origin and properties of X-rays. Bragg's calculations were used by R. J. Havighurst to calculate the electron density distribution in a crystal of sodium chloride along cubic edges and cube diagonals (Havighurst 1927). Then, in 1934, J. D. Bernal published in Nature the photograph of a protein (pepsin) using X-ray crystallography (Bernal and Crowfoot 1934).

Particularly, X-ray crystallography has been successfully applied to solve the crystal structures of proteins, beginning in the late 1950s with the structure of the myoglobin and haemoglobin (Kendrew et al. 1958). From the calculated electron density maps, a model of the myoglobin molecule to 6 Å resolution was constructed. The model contained a number of dense rod-like features that had the dimensions of α-helices and made up the bulk of the polypeptide chain.

Early in the 1970s, Tardieu et al. (1973) published a study based on X-ray scattering describing the structure of a variety of lecithin-water phases observed below the "melting" temperature of the hydrocarbon chains. All the lamellar phases assumed by pure lipid/water systems were identified. The results emphasize the clear-cut difference between the liquid-like and the other types of partly ordered conformations, as well as the correlations which exist between the chemical composition and the structure of the lipids below the melting temperature of the chains. Since then, more than 60 K structures of proteins and other biological molecules have been determined with X-rays.

X-rays have wavelengths much shorter than the visible light, so they are useful to find atomic positions, mainly for crystalline materials. However, this technique is much more efficient when it comes to atoms with a high number of electrons. Because of that, the visibility for atoms involved in biological structures (oxygen and hydrogen) it is not so good. This is the main reason that led the scientist to consider neutrons to determine the matter structure.

Regarding neutrons, it was Ernest Rutherford who, in 1920, suggested that the difference between the atomic number and the atomic mass could be explained by the existence of a neutrally charged particle in the atomic nucleus (Rutherford 1920). Finally, James Chadwick proved the existence of the neutrons by analysing the radiation obtained when α particles fell on a beryllium layer (Chadwick 1932).

Neutrons have no charge and their electric dipole moment is almost zero, so they have a bigger penetration depth in matter. They interact with atoms via nuclear forces, which are very short range ($10^{-15}$ m). So, the matter appears not very dense to neutrons and they can travel long distances through most materials without any interaction. For example, the attenuation of neutrons by aluminium is about 1 % per millimetre compared to 99 % in the case of X-rays.

Neutrons have a unique sensitivity to hydrogen atoms. This capability enables an accurate determination of molecular structures, which is important for the design of new therapeutic drugs. The bad part is that they are slightly scattered, and to obtain significant data, high flux of particles is needed. Because of this, they are more difficult to produce. In that sense, a big effort is being put in constructing high-performance facilities to obtain high-energy and flux neutron beams to operate different instruments that will be the most powerful ones to study a wide range of biological structures (Danared 2012; White 2002). There is also a great variety of instruments that make possible the extraction of useful information from different aspects of the neutron scattering.

The first neutron spectrometer was mounted at a port on the Oak Ridge graphite reactor in the mid-1940s. Most of the work with neutrons was focused on crystal lattices (Brockhouse and Hurst 1952). In 1955, Brockhouse and Stewart (Brockhouse and Stewart 1955) were the first people to observe the interaction of thermal neutrons with lattice phonons. Neutrons travelling through a crystal of aluminium both lost and gained energy through scattering. This changes in energy provided new information in interatomic forces and the normal modes of vibration.

The scattering of neutrons from hydrogen has driven an important experimental activity for the determination of the structure of biologically important compounds during the last decades. Biological experiments can be classified into two categories: high resolution and low resolution. The first technique determines the position of hydrogen atoms in biological molecules. The second one exploits the difference in sensitivity of neutron radiation to hydrogen and to deuterium in order to determine the location of molecular compounds in large structures.

Nowadays, there are big expectations when it comes to scattering techniques. Multiple applications that will help to understand the complexity of matter and nature are gathering big scientific efforts. A central part is the understanding of the molecular basis of the biological processes (Peggs 2013). Biological systems such as enzymes, tissues, organs rely on the interplay of large molecules such proteins and nucleic acids. Scattering methods are highly powerful probes for life sciences. The final goal is to decipher these molecular mechanisms through structural and dynamical analysis of complexes (Pieper et al. 2008; Wood et al. 2007). Antimicrobial research, as relying heavily in cell membrane structures and drug discovery, can also get benefit of scattering techniques, as shown later. Multiresistant bacterial strains threatening public health, individualized and preventive treatments approach are paradigmatic challenges to be addressed.

Even though there exist (especially in the X-ray world) some commercial instruments, the investigation that uses scattering is relying more and more in big facilities (accelerators). The second section of this chapter is devoted to accelerators: synchrotrons for producing X-rays and spallation sources for producing neutrons.

## 2.2 Accelerators

### *2.2.1 Synchrotrons and Storage Rings*

Oliphant invented the synchrotron principle in 1943 (Oliphant 1943): *Particles should be constrained to move in a circle of constant radius thus enabling the use of an annular ring of magnetic field…, which would be varied in such a way that the radius of curvature remains constant as the particles gain energy through successive accelerations by an alternating electric field applied between coaxial hollow electrodes.*

By means of an injected short pulse, the field rises and the particles are accelerated until they have energy enough to hit a target or to be extracted out of the synchrotron. The particle acceleration is achieved by using fields in a RF resonator, fed by a radio transmitter. Particles return to the resonator at each turn of the synchrotron. The guiding field is provided by a ring of magnets.

Later on, Veksler (1944) and McMillan (1945) completed the concept by means of the *Phase Stability Principle*. If the particles are arranged to ride in the rising edge of the field wave in the accelerator cavity, they receive more energy when they are late and less energy when they are early. So, the particles end up oscillating about the stable synchronous phase.

The synchrotron acceleration was demonstrated by first time in 1946 by Goward and Barnes (Goward and Barnes 1946) in a refurbished machine at Woolwich Arsenal, UK. Only 2 months later, Elder et al. (1947) operated a 70 MeV machine built by them at General Electrics Laboratories in USA.

The acceleration of particles to very high speeds in synchrotrons produces radiation. Particularly, this is the technique used in accelerators to produce X-rays. The speed of particles in synchrotrons is highly relativistic, and the power radiated is proportional to the fourth power of the particle speed and is inversely proportional to the square of the radius of the path. It becomes the limiting factor on the final energy of particles accelerated in electron synchrotrons:

$$P \approx \frac{2\mathrm{K}e^2\gamma^4 v^4}{3c^2 r^2}. \tag{2.1}$$

For an accelerator like a synchrotron, the radius is fixed after construction, but the inverse dependence of synchrotron radiation loss on radius argues for building the accelerator as large as possible.

Nowadays, more than 50 synchrotron light sources big facilities exist in the world (Lightsources). Here, in Table 2.1, some of the more outstanding installations are summarized. All of them operate accelerating particles at high energies in very big rings. Almost any of these facilities are putting big effort in the investigation in structural biology and new drugs design as we will show later.

**Table 2.1** Example of facilities using high-energy synchrotrons

| Name | Accelerator energy (GeV) | Ring diameter (m) |
|---|---|---|
| Diamond light source (Suller 2002) | 3 | 561 |
| ESRF (Revol et al. 2013) | 6 | 844 |
| APS (Borland et al. 2007) | 7 | 1104 |
| MAX IV (Curbis et al. 2013) | 3 | 528 |

### *2.2.2 Spallation Sources*

As mentioned before, high flux of neutrons is required to keep on developing more and more advanced instruments for neutron science. This can be achieved by using spallation sources. They produce neutrons by means of the interaction of high-energy protons (GeV) with a heavy metal target (Broome 1996) (Fig. 2.1).

The protons interact with the target, and a few energetic particles are produced. The nucleus transitions to a highly excited state. While it comes back to the ground state, it emits neutrons, protons, deuterons, tritons and alpha particles (evaporation). The energetic particles go on to interact with other target nuclei producing more excited nuclei and more neutrons in a nuclear cascade.

For some materials, the excited nuclei will undergo fission to leave two fragments which will come to ground state by evaporation. All these processes generate a lot of heat in the target, and it has to be refrigerated.

The energy of neutrons suitable for scattering ranges from neV to eV. The number of produced neutrons depends on the proton energy. Neutron scattering is an intensity limited field because its interaction with the matter is weak. Because of this, there is a strong interest in more powerful neutron sources. Table 2.2 shows the evolution of the main characteristics of neutron sources. The upper facilities were the first neutron sources designed. Meanwhile, the last one (ESS) is

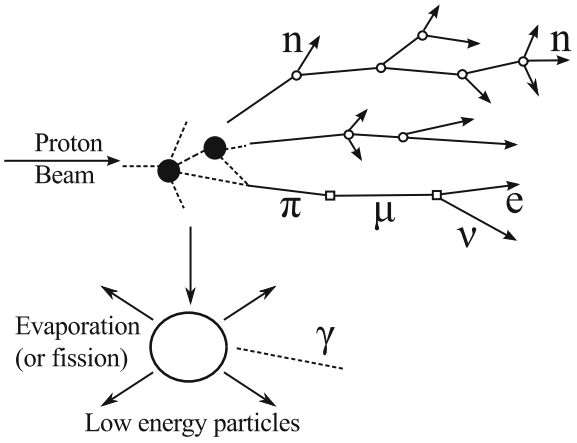


**Fig. 2.1** The spallation process

**Table 2.2** Main parameters of different facilities producing neutrons

| Name | Accelerator energy | Average beam power (MW) | Repetition rate (HZ) | Protons per pulse ($10^{13}$) | Pulse length at target ($\mu$s) |
|---|---|---|---|---|---|
| IPS, ANL (Brow 1995) | 50 meV Linac 500 meV RCS | 0.0075 | 30 | 0.3 | 0.1 |
| ISIS, RAL (Gardner 1994) | 70 meV Linac 800 meV RCS | 0.16 | 50 | 2.5 | 0.45 |
| SINQ, PSI (Bauer et al. 1997) | 590 meV Cyclotron | $\leq$0.9 | C. W. | – | – |
| LANSCE, LANL (Garnett et al. 2011) | 800 meV | 1 | 30 | – | 1,200 |
| SNS, ORNL (Plum 2010) | 2.5 GeV | 1.4 | 60 | 15 | 1,000 |
| ESS (Eshraqi and Danared 2011) | 2.5 GeV | 5 | 14 | $9.10^5$ | 2.87 |

still to be constructed in the following months. The energy delivered and the number of protons produced is much bigger nowadays, which severely affects to the neutrons production rates.

## 2.3 Foundations of X-ray Scattering

The interatomic distances in crystals and molecules amount to 0.15–0.4 nm which correspond to the wavelength of the electromagnetic spectrum for X-rays having photon energies between 3 and 8 keV. Accordingly, phenomena like constructive and destructive interference should become observable when crystalline and molecular structures are exposed to X-rays.

There are three types of interactions when a sample is illuminated with X-rays (Cullity 1967; Warren 1969):

- Electrons may be liberated from their bound atomic states in the process of photoionization. This is an inelastic scattering process.
- A second type of inelastic scattering is the so-called Compton scattering. Energy is transferred to an electron, which proceeds, however, without releasing the electron from the atom.
- Finally, X-rays may be scattered elastically by electrons (Thomson scattering). The electron oscillates as a dipole at the frequency of the incoming beam and becomes a source of radiation. The wavelength $\lambda$ of X-rays is conserved for Thomson scattering. This is the process used for structural investigations by X-ray diffraction.

Figure 2.2 shows elastic scattering for a single free electron of charge e, mass $m$ and at position $R_0$. The incoming beam is accounted for by a plane wave:

$$E_0 \cdot \mathrm{e}^{-i\left(\vec{K}_0 \cdot \vec{R}_0\right)}. \tag{2.2}$$

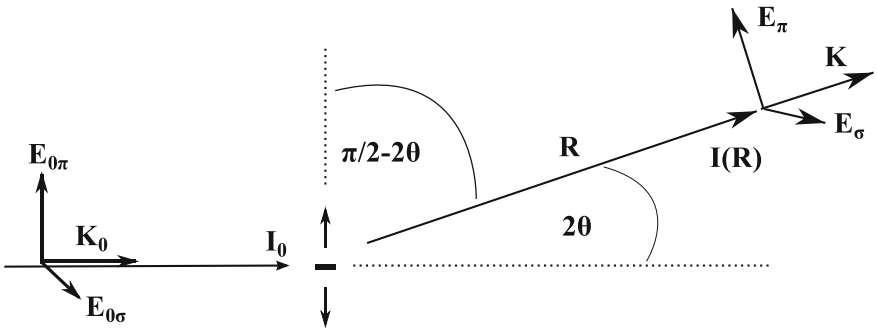


**Fig. 2.2** Single electron scattering centre

$E_0$ is the electrical field vector and $K_0$ the wave vector.

The dependence of the field on time will be neglected throughout. The wave vectors $K_0$ and $K$ describe the direction of the incoming and exiting beam and both are of magnitude $2\pi/\lambda$. The plane defined by them is denoted as the scattering plane. The angle between $K$ and the prolonged direction of $K_0$ is the scattering angle that will be denoted by $2\theta$:

$$2\theta = \arccos \frac{\langle \vec{K},\, \vec{K}_0 \rangle}{K \cdot K_0}. \tag{2.3}$$

The oscillating charge e will emit radiation of the same wavelength $\lambda$ as the primary beam. If the amplitude of the scattered wave $E(R)$ is considered at a distance R, the mathematical expression is

$$E(R) = E_0 \frac{1}{4\pi\varepsilon_0 R} \frac{\mathrm{e}^2}{\mathrm{mc}^2} \sin \angle(E_0,\, R) \mathrm{e}^{-\mathrm{i}\vec{K}\cdot\vec{\mathrm{R}}}. \tag{2.4}$$

where $\varepsilon_0$ and $c$ are the vacuum permittivity and velocity of light. The field vector E and wave vector K are oriented perpendicular to each other as is usual for electromagnetic waves. The field vectors in both cases will be denoted by $E\pi$ and $E\sigma$. The angle between $E\sigma$ and $R$ is always 90°, and the sin term will equal unity. For the case of $\pi$ polarization, however, it may be expressed by virtue of the scattering angle according to $\sin \angle(E_0, R) = |\cos\, 2\theta|$. Since the intensity is obtained from the sum of the square of both field vectors, the expression

$$\left(\frac{1}{4\pi\varepsilon_0}\right)^2 \left(\frac{\mathrm{e}^2}{\mathrm{mc}^2}\right) \left(\mathrm{E}_\sigma^2 + \mathrm{E}_\pi^2 \cos^2 2\theta\right) \tag{2.5}$$

in a non-polarized beam both polarization states will have the same probability of occurring

$$\bar{E}_\sigma^2 = \bar{E}_\pi^2 = \frac{I_0}{2}. \tag{2.6}$$

Then, the intensity of the scattered beam at distance $R$ is finally obtained:

$$I(R) = I_0 \frac{r_e^2}{R^2} \frac{1 + \cos^2 2\theta}{2}. \tag{2.7}$$

The intensity of the scattering scales with the inverse of $R^2$ and $r_e^2$. For distances $R$ of the order of $10^{-1}$ m, the probability of observing the scattering by a single electron tends to zero. In this analysis, only the scattering from electrons is taken into account. From Eq. (2.5), it can also be derived that the interaction for atomic nuclei is $10^{-6}$ times smaller and then neglectable. Therefore, from the point of view of scattering, the atoms are modelled by the number of electrons within them. $r_e$ is substituted by $Zr_e$ (Z is the number of electrons) in this development to reach a quantitative description for the X-ray elastic scattering from an atom.

Atomic positions are described by the lattice vector $r_{n_1,n_2,n_3} = n_1 a c_1 + n_2 a c_2 + n_3 a c_3$ with $c_1$, $c_2$ and $c_3$ being the unit vectors of the three orthogonal directions in space. The task is to quantify the strength of the scattered fields at a point R when elastic scattering occurs according to Eq. (2.4) at all atoms. The reference point of R is chosen such that it starts at the origin of the crystal lattice $r_{000}$. The wave vector of the primary beam $K_0$ is assumed to be parallel to the [100] direction of the crystal. The scattering plane defined by $K_0$ and $K$ may coincide with one of the (010) planes. The wavefronts of the incoming plane waves, which are the planes of constant phase, are then oriented parallel to (100) planes. An atom on the position $r_{n_1,n_2,n_3}$ would then cause a scattering intensity to be measured at $R$ (assuming $r_{n_1,n_2,n_3} \ll R$—Fraunhofer diffraction)

$$E_0 \frac{Zr_e}{R} e^{-iKR} \sum_{n_1,n_2,n_3} e^{-i(K-\vec{K}_0) r_{n_1,n_2,n_3}}. \tag{2.8}$$

The strength of the total scattered field depends on the spatial orientation of the wave vectors $K_0$ and $K$ with respect to the crystal reference frame $\{c_i\}$.

The quantity $Q = K - K_0$ is called the *scattering vector*.

The geometric construction of vector Q in Fig. 2.3 shows that the scattering vector magnitude is

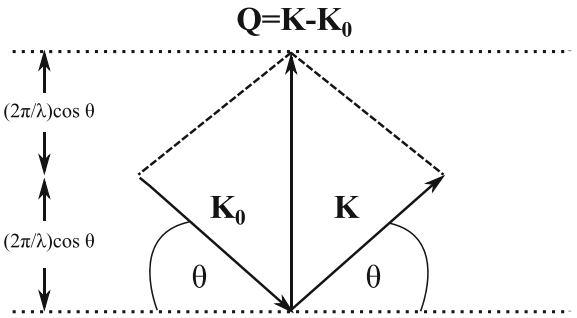


**Fig. 2.3** Scattering vector geometry

$$|Q| = \frac{4\pi}{\lambda} \sin\theta. \tag{2.9}$$

The scattering vector $Q$ is a physical quantity fully under the control of the experimentalist. The orientation of the incident beam $(K_0)$ and the position of the detector $(K)$ decide the direction in which the scattering vector $(Q)$ of X-rays proceeds. And the choice of wavelength determines the amplitude transfer to which the sample is subjected. From these considerations, it is possible to understand the collection of a diffraction pattern as a way of scanning the sample's structure by scattering vector variation.

If the summation factor of Eq. (2.8) is expanded into three individual terms and the geometry of the simple lattice is used, it is found that the field amplitude of the scattered beam is proportional to

$$\sum_{n_1=0}^{N_1-1} \sum_{n_2=0}^{N_2-1} \sum_{n_3=0}^{N_3-1} \mathrm{e}^{-iQ(n_1ac_1+n_2ac_2+n_3ac_3)}. \tag{2.10}$$

From this equation, it is possible to reach the interference function. It describes the distribution of scattered intensity in the space around the crystal.

$$\Im(Q) = \frac{\sin^2(N_1aQc_1/2)}{\sin^2(aQc_1/2)} \frac{\sin^2(N_2aQc_2/2)}{\sin^2(aQc_2/2)} \frac{\sin^2(N_3aQc_3/2)}{\sin^2(aQc_3/2)}. \tag{2.11}$$

For large values of $N1$, $N2$ and $N3$, the three factors in $\Im$(Q) only differ from zero if the arguments in the $\sin^2$ function of the denominator become integer multiples of $\pi$. Let us name these integers $h$, $k$ and $l$ in the following:

$$\Im(Q) \to \max \Leftrightarrow aQc_1 = 2\pi h,\ aQc_2 = 2\pi k,\ aQc_3 = 2\pi l. \tag{2.12}$$

This equation yields as condition for maximum intensity

$$I(R) \to \max \Leftrightarrow \frac{|Q|}{2\pi} = \frac{\sqrt{h^2+k^2+l^2}}{a} \tag{2.13}$$

which can be rewritten by inserting the magnitude of the scattering vector (Eq. 2.9).

$$I(R) \to \max \Leftrightarrow 2\frac{a}{\sqrt{h^2+k^2+l^2}} \sin\theta \tag{2.14}$$

The distance between two adjacent planes is called the *interplanar spacing*

$$d_{hkl} = \frac{a}{\sqrt{h^2+k^2+l^2}} \tag{2.15}$$

Keeping this meaning of integer triples in mind, Eq. (2.14) tells us that to observe the maximum intensity in the diffraction pattern of a simple cubic crystal, and the following equation must be satisfied:

$$2d_{hkl} \sin\theta = n\lambda. \tag{2.16}$$

The equation is called *Bragg equation* and was applied by W.H. Bragg and W.L. Bragg in 1913 to describe the position of X-ray scattering peaks in angular space.

A sample with crystallographic lattice planes with distances $d_{hkl}$ is irradiated by plane wave X-rays impinging on the lattice planes at an angle $\theta$. The relative phase shift of the wave depends on the configuration of atoms.

Having arrived at this point, it can be stated that we have identified the positions in space where constructive interference for the scattering of X-rays at a crystal lattice may be observed. It has been shown that measurable intensities only occur for certain orientations of the vector of momentum transfer $Q$ with respect to the crystal coordinate system $\{c_i\}$. Various assumptions were made that were rather crude when the course of the intensity of Bragg reflections is of interest. It has been assumed, for instance, that the atom's electrons are confined to the centre of mass of the atom. In addition, thermal vibrations, absorption by the specimen, etc., were neglected.

## 2.4 Foundations of Neutron Scattering

The best way of understanding neutron scattering is looking at how a neutron is scattered by a single nucleus. Then, if the effects of different nuclei are accounted, it is possible to describe phenomena like neutron diffraction, inelastic neutron scattering, etc. The physics of neutron scattering is a quantum mechanical process (Liang et al. 2009). Therefore, it is necessary to bear in mind the wave-particle duality to understand its fundamentals. Essentially, this means that the square modules of the wave function of a neutrons gives the probability of finding that neutron in a particular point in space.

The speed of neutrons and their wavelength are inversely proportional. Normally, a wave vector $\vec{k}$ pointing along the neutrons' trajectory is defined as having magnitude $2\pi/\lambda$. It can also be related to the neutron velocity through the equation.

$$\left|\vec{k}\right| = 2\pi m v/h \tag{2.17}$$

$h$ is the Planck's constant, and $m$ is the neutron's mass.

If a neutron passes close to a nucleus within an effective area, called *cross section* $\sigma$, it is scattered in any direction with equal probability (Fig. 2.4).

This scattering is isotropic because the range of the nuclear interaction between the neutron and the nucleus is tiny compared to the wavelength of the neutron. As shown in Fig. 2.4, the wave of an incident neutron is represented as a plane wave $\left(e^{ik\cdot x}\right)$, without loss of generality. Additionally, the modulus of this wave is 1, so

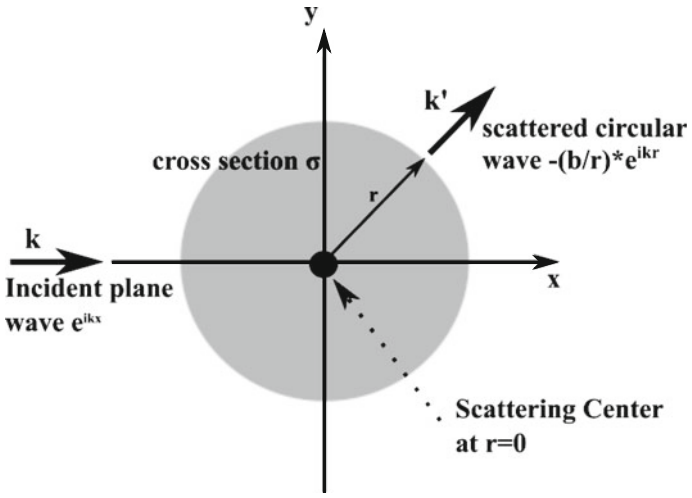


**Fig. 2.4** Single neutron scattering centre

the probability of being found anywhere is the same but has a definite momentum $mv = h\upsilon/2\pi$.

The wave of the scattered neutrons depends on the interaction between the neutron and the nucleus. When the nucleus is considered to be at the origin of the coordinate system, the wave of the scattered neutron is $-(b/r) \cdot e^{ik \cdot r}$ and it is a circular wave. The square of this function gives the intensity of the neutron beam, which decreases with the term $1/r^2$. $b$ gives the strength of the interaction between the nucleus and the proton (*scattering length*). And the minus means for repulsive interaction.

The strength of the interaction is not directly related with the atomic number (Diagnoux and Lander 2003). For example, hydrogen and deuterium, which are very important in the study of biological structures, have scattering lengths that are relatively large and very different from each other. This difference is used to label biological molecules with different isotopes in such a way that boosts the amount of information obtained from neutron scattering experiments.

To reproduce how matter scatters neutrons, the addition of the contribution of all individual scattering centres has to be performed. Regardless of the type of collision (elastic or inelastic), the energy and momentum are conserved. If the neutron losses energy $E$, the same amount is gained by the scattering nucleus.

From Eq. (2.17), it could be easily derived the momentum lost by the neutron in the collision (*momentum transfer*).

$$m \cdot \Delta\vec{v} = \frac{h}{2\pi}\vec{Q} = \frac{h}{2\pi}(\vec{k} - \vec{k}') \tag{2.18}$$

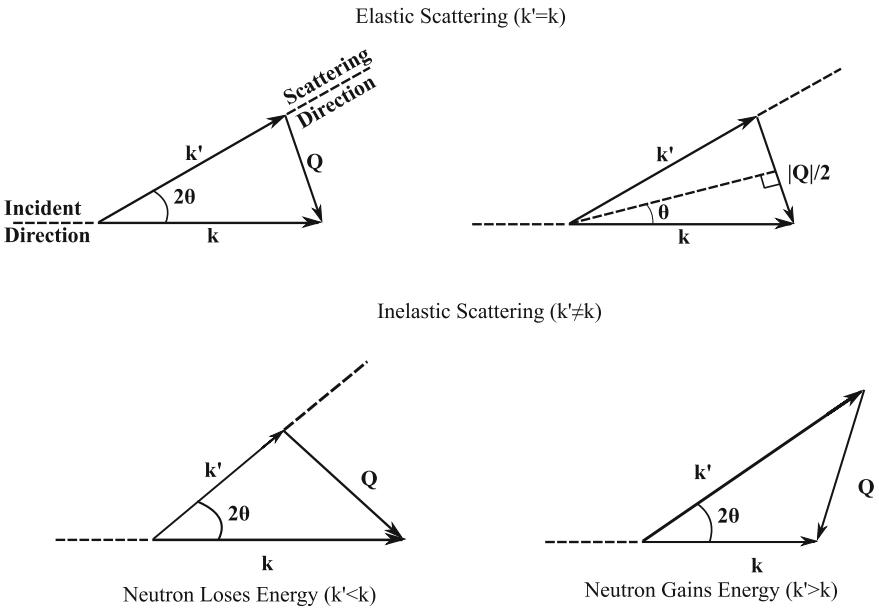


**Fig. 2.5** Scattering triangles for elastic and inelastic scattering

(see also Fig. 2.5) $\vec{k}$ is the wavevector of the incident neutron and $\vec{k}'$ of the scattered one. $\vec{Q}$ is called the *scattering vector*. Finally, the angle $2\theta$ is the angle between the trajectory of the incident neutron and the scattered one (*scattering angle*).

In the case of the elastic scattering ($\left|\vec{\mathrm{k}}\right| = \left|\vec{\mathrm{k}}'\right|$), with simple trigonometric rules, we can obtain:

$$\sin\theta = \frac{Q/2}{K} \tag{2.19}$$

and then,

$$Q = 2 \cdot \mathrm{k} \cdot \sin\theta = \frac{4 \cdot \pi \cdot \sin\theta}{\lambda} \tag{2.20}$$

For all neutron experiments, the most important magnitude is the intensity of neutrons as a function of the scattering vector $\vec{Q}$ and the energy $E$, $I(\vec{Q}, E)$.

Van Hove showed in 1954 (van Hove 1954) that the intensity of neutrons can be written in terms of time-dependent correlations between the positions of pair of scattering atoms. This result is crucial for the scattering problem because it permits to represent the intensity as proportional to a Fourier transform, which gives the probability of finding two atoms separated by a certain distance. This is the key, because if the intensity of neutrons is known, it is possible to derive the atoms positions in the sample and, hence, the sample structure.

The probability of a neutron with wavevector $\vec{k}$ being scattered by a potential $V(\vec{r})$ and becoming a new outgoing wavevector $\vec{k}'$ (Born approximation (Gottfried and Yan 2003)) is proportional to the module of the following probability function:

$$\left| \int \mathrm{e}^{i\vec{k}\vec{r}} V(\vec{r}) \mathrm{e}^{i\vec{k}'\vec{r}'} d\vec{r} \right|^2 = \left| \int \mathrm{e}^{ik\vec{Q}\vec{r}} V(\vec{r}) d\vec{r} \right|^2 \tag{2.21}$$

This probability is integrated over the whole sample. $V(\vec{r})$ is the so-called *Fermi pseudo-potential*, which for an assembly of nuclei situated at positions $\vec{r}_j$ is given by

$$V(\vec{r}) = \frac{2\pi\hbar}{m} \sum_j b_j \delta(\vec{r} - \vec{r}_j) \tag{2.22}$$

$m$ is the neutron's mass. $\delta(\vec{r})$ is a Dirac delta that takes unity value at the atoms positions. $b_j$ is the scattering length mentioned before. Taking all this into account, the scattering law $I(\vec{Q}, E)$ can be written as (Squires 2012):

$$I(\vec{Q}, E) = \frac{1}{h} \frac{k'}{k} \sum_{i,j} b_i b_j \int_{-\infty}^{\infty} \left\langle \mathrm{e}^{-i\vec{Q}\cdot\vec{r}_i(0)} \mathrm{e}^{-i\vec{Q}\cdot\vec{r}_j(t)} \right\rangle \mathrm{e}^{-i(E/\hbar)t} dt. \tag{2.23}$$

Equation (2.23) is a sum over all of the positions of the nuclei in the sample. The angular brackets indicate that a thermodynamic average over all possible configurations of the sample is needed. In order to better understand the physical meaning of these mathematical expressions, it is possible to simplify (2.23) treating vectors as classical (not quantum mechanical) quantities:

$$\sum_{i,j} b_i b_j \left\langle \mathrm{e}^{-i\vec{Q}\{\vec{r}_i(0) - \vec{r}_j(t)\}} \right\rangle = \sum_{i,j} b_i b_j \int_{\text{sample}} \delta(\vec{r} - \vec{r}_i(0) + \vec{r}_j(t)) \, \mathrm{e}^{-i\vec{Q}\cdot\vec{r}} d\vec{r} \tag{2.24}$$

Another useful simplification is to assume that in the sample, all the nuclei have the same scattering length $b_i = b_j = b$. The right hand of the previous equation becomes

$$\mathrm{N}b^2 \int_{\text{sample}} G(\vec{r}, t) \mathrm{e}^{-i\vec{Q}\cdot\vec{r}} \tag{2.25}$$

where

$$G(\vec{r}, t) = \frac{1}{N} \sum_{i,j} \delta(\vec{r} - [\vec{r}_i(0) - \vec{r}_j(t)]) \tag{2.26}$$

$N$ is the number of nuclei in the simple. $G(\vec{r}, t)$ is the so-called the *time-dependent pair correlation function*, which describes how the correlation between the positions of nuclei evolves with time.

Finally, the Van Hove's scattering law is proportional to the space and time Fourier transforms of the time-dependent correlation function. Inverting Eq. (2.26), we can obtain information of the sample structure from neutron scattering information.

However, generally the scattering lengths are not the same. Even if a sample for the same isotope is considered, the interaction between the matter and the neutron depends on the spin state. Conversely, it is possible to average them over all spin states. The average interaction is denoted by angular brackets:

$$\sum_{i,j} \langle b_i b_j \rangle A_{ij} = \sum_{i,j} \langle b \rangle^2 A_{ij} + \sum_i \left( \langle b^2 \rangle - \langle b \rangle^2 \right) A_{ij} \tag{2.27}$$

$A_{ij}$ is the integral in Eq. (2.26).

The first term in (2.27) is *coherent scattering* and depends on the distance between nuclei. The *elastic coherent scattering* produces information about the equilibrium structure, and *inelastic coherent scattering* produces information about the collective motion of the atoms.

The second term is the *incoherent scattering* and is the same for all atoms, independently of others. *Incoherent elastic scattering* does not provide useful information and is considered as undesired background in neutron scattering experiments. *Incoherent inelastic scattering* provides information about atomic diffusion.

## 2.5 Two Important Scattering Techniques for Antimicrobial Research

X-rays and neutrons have properties of plane waves, that is, amplitude and wavelength, and as they pass through matter, secondary wavelets are generated by interactions with individual atoms, and the resulting coherent scattering can constructively or destructively interfere.

Small-angle scattering arises from these coherent secondary wavelets that are scattered by atoms within a single molecule, and as a result, it is observed for molecules in crystals or in solution. This is a very useful technique, and it is considered the most important application for the study of biological macromolecules. It is able to characterize the shapes and dispositions of components within bimolecular complexes.

Although small-angle scattering solution is often described as a low-resolution technique (as it does not provide information on atomic coordinates), it is more appropriate to describe it as a technique capable of providing high-precision information with respect to size and shape. It is the rotational averaging of the molecules in solution that limits the information content of small-angle scattering more than the resolution limits of the experiments.

To interpret scattering data in terms of accurate structural parameters, the scattering signal must be measured from a sample of monodisperse, identical particles. Sample preparation is therefore a critically important.

A highly collimated X-ray or neutron beam is used to illuminate the sample, usually a protein or macromolecular complex in solution (typically >1 mg m $L^{-1}$ in 5–30 $\mu$L for X-ray scattering and >3 mg m $L^{-1}$ in 150–300 $\mu$L for neutron scattering). Traditionally, the radiation is of a single wavelength (or narrow band of wavelengths), although the development of pulsed neutron sources has led to time-of-flight neutron scattering instruments that can use white radiation to maximize flux. The scattered radiation is recorded on a detector, while the direct beam is usually absorbed by a beam stop; the size and position of which are key factors determining the minimum angle measured in an experiment. New developments in solid-state devices have led to detectors that can absorb an intense direct X-ray beam without incurring damage.

### 2.5.1 Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) is a fundamental method for structure analysis of condensed matter. The applications cover various fields, from metal alloys to synthetic polymers in solution and in bulk, biological macromolecules in solution, emulsions, porous materials, nanoparticles, etc. First X-ray applications date back to the late 1930s when the main principles of SAXS were developed in the seminal work of Guinier and Fournet (1955). This technique provides structural information on inhomogeneities of the electron density with characteristic dimensions between one and a few hundred nm.

The colloidal dimensions (tens or thousands of Å) are very large compared to X-rays wavelength. This fact makes the scattering angle very small when a sample is illuminated. So, X-ray scattering is only observed when there are electron density inhomogeneities of colloidal size in the sample. That means that this technique is not able to reveal the atomic structure of the materials. However, it is able to measure the shapes and sizes of nanoparticles and large molecules.

SAXS works in liquids or solids and has short response times, so it can be used to follow biological processes in real time. This makes SAXS an ideal complement to time-consuming analytical techniques such as electron microscopy and X-ray diffraction.

In particular, if a usual wavelength of 1 Å is used, the scattering radiation pattern is limited to a scattering angle of one or two degrees. In general, there are two restrictions that simplify this technique significantly: the system is statistically isotropic and there is no correlation between point separated wide enough. Then, the radiation pattern (of an electron density distribution $\bar{\rho}^2$) depends only on the

distance ($r$). Any phase factor $e^{-ihr}$ of an incident plane wave can be replaced by its average over all r directions.

$$\left\langle e^{-ihr} \right\rangle = \frac{\sin hr}{hr}. \tag{2.28}$$

Then, the intensity is reduced to

$$I(h) = \int_0^\infty 4\pi r^2 \bar{\rho}^2(r) \frac{\sin hr}{hr} dr. \tag{2.29}$$

At large $r$, the respective electron densities should become independent and might be replaced by the mean value of $\rho$. In this case, for extremely small angles, the constant value across the whole volume acts like a blank object. Therefore, it makes no contribution to the diffraction pattern. It is then convenient to drop this background from the beginning.

The simplest case is that particles show spherical symmetry. As all orientations in space are equivalent, considering an sphere of radius $R_0$ and volume $V$ of uniform density, we obtain

$$I_s(h) = (\Delta\rho)^2 V^2 \left[ 3 \frac{\sin hR_0 - hR_0 \cos hR_0}{(hR_0)^3} \right]^2 \tag{2.30}$$

For particles of non-spherical shape, the intensity can only be calculated by numerical methods. If we consider some symmetry orientation and we expand the expression for intensity in a power series, we obtain the Guinier approximation, which is a universal approximation for all particles.

$$I(h) = I(0) e^{-\frac{h^2 R^2}{3}} \tag{2.31}$$

R is the only parameter of this equation and is called *radius of gyration*. This Guinier formula holds incredibly well in the majority of the cases. Only in very anisometric particles, it should be replaced by another approximation.

High concentration of inhomogeneities reduces the intensity at low scattering angles. There are well-developed commercial instruments providing this technique. But if higher intensities are required, there exist highly specialized and powerful instruments (radiation from synchrotrons), as mentioned before. All modern synchrotrons provide SAXS beamlines. These instruments offer the possibility of performing time-resolving studies or observation of transient states in macromolecular assemblies. Moreover, in many problems, particularly the determination of density inhomogeneities within a particle, neutron techniques are the only solution.

### 2.5.2 Small-Angle Neutron Scattering

The theoretical apparatus used for small-angle neutron scattering (SANS) is very similar to that developed for X-rays. We have seen already in our discussion of diffraction that elastic scattering at a scattering vector $Q = (4\pi/\lambda)\sin\theta$ results from periodic modulations of the neutron scattering lengths with period $d = 2\pi/Q$. Combining these two expressions gives us Bragg's Law (Eq. 2.16). In the case of a crystal, d was interpreted as the distance between planes of atoms. To measure structures that are larger than typical interatomic distances, we need to arrange for Q to be small, either by increasing the neutron wavelength, $\lambda$, or by decreasing the scattering angle. Because it is not know how to produce very high fluxes of very long wavelength neutrons, it is always needed to use small scattering angles to examine larger structures such as polymers, colloids, or viruses. For this reason, the technique is known as small-angle neutron scattering, or SANS.

For SANS, the scattering wavevector, $Q$, is small, so the phase factors in do not vary greatly from one nucleus to its neighbour. For this reason, the sum in Eq. (2.23) can be replaced by an integral, and the intensity for SANS can be written as

$$S(\vec{Q}) = \left| \int_{\text{sample}} \rho(\vec{r}) \mathrm{e}^{i\vec{Q}\cdot\vec{r}} d\vec{r} \right|^2 \tag{2.32}$$

where $\rho(\vec{r})$ is a spatially varying quantity called the *scattering length density* calculated by summing the coherent scattering lengths of all atoms over a small volume (such as that of a single molecule) and dividing by that volume. In many cases, samples measured by SANS can be thought of as particles with a constant scattering length density, $\rho$, that are dispersed in a uniform medium with scattering length density $\rho_m$. Examples include pores in rock, colloidal dispersions, biological macromolecules in water and many more. The integral in Eq. (2.32) can, in this case, be separated into a uniform integral over the whole sample and terms that depend on the difference, $(\rho_p - \rho_m)$, a quantity often called the contrast factor. If all of the particles are identical and their positions are uncorrelated, Eq. (2.32) becomes

$$S(\vec{Q}) = N(\rho_p - \rho_m)^2 \left| \int_{\text{sample}} \rho(\vec{r}) \mathrm{e}^{i\vec{Q}\cdot\vec{r}} d\vec{r} \right|^2 \tag{2.33}$$

where the integral is now over the volume of one of the particles, and $N_p$ is the number of particles in the sample. The integral of the phase factor $\mathrm{e}^{-i\vec{Q}\cdot\vec{r}}$ over a particle is called the form factor for that particle. For many simple particle shapes, the form factor can be evaluated analytically, whereas for complex biomolecules, for example, it has to be computed numerically.

Usually, the intensity one sees plotted for SANS is normalized to the sample volume so that $S(Q)$ is reported in units of $cm^{-1}$ (as compared with Eq. (2.33), which has units of length squared).

Equation (2.33) allows us to understand an important technique used in SANS called contrast matching. The total scattering is proportional to the square of the scattering contrast between the particle and the matrix in which it is embedded. If we embed the particle in a medium whose scattering length density is equal to that of the particle, the latter will not scatter—it will be invisible.

The form factor in Eq. (2.33) will be evaluated by integrating over this central region only. The scattering patterns will be different in the external and central regions, and from two experiments, we will discover the structure of both the coating and the core of the particle. Variation of the scattering length density of the matrix is often achieved by choosing a matrix that contains hydrogen (such as water). By replacing different fractions of the hydrogen with deuterium atoms, a large range of scattering length densities can be achieved for the matrix. Both DNA and typical proteins can be contrast matched by water containing different fractions of deuterium. Isotopic labelling of this type can also be applied to parts of molecules in order to highlight them for neutron scattering experiments.

## 2.6 Antimicrobial and Biological Research Using Scattering

One of the major troubles in the design of new antimicrobials is how to attack the lipid bilayer of cell membranes. Some antimicrobial peptides exert their activity directly in the plasmatic membrane but the question is, how? Antimicrobial peptides secretion is a part of a natural immune response of many living organisms, acting as a rapid response to infection by diverse bacterial species, attacking directly the lipid bilayer or across a receptor (Latal et al. 1997; Lohner and Prenner 1999).

He and co-workers (He et al. 1995) were able to describe how alamethicin, an antimicrobial peptide, was able to create aqueous pores in the cell layer using the neutron in-plane scattering. Also, they were able to determinate that the pore formation was concentration-dependent process and a critical lipid value had to be reached to form a 18 Å in diameter pore. This pore formation was the molecular mechanism of the antimicrobial action. Thanks to this technique, it was possible, also, to determine the water dependence of the process (He et al. 1996). Similar research was done to determinate the pore structure originated by magainin (Ludtke et al. 1996).

The antimicrobial peptide protegrin-1 (PG-1) interacts with membranes in a manner that strongly depends on membrane composition: different lipids, different speed of integration in the lipid matrix. This integration is responsible of the cell membrane destabilization and, consequently, the cell death. In the case of the gram-negative bacteria, the integration occurs primary in the outer membrane

of the cell wall, thus allowing the AMP to reach the inner membrane. Thanks to X-ray scattering technique, it was possible to differentiate how PG-1 interact different kind of lipid bilayers (Gidalevitz et al. 2003).

The lipid A is one of the major components of the outer membrane of gram-negative bacteria. The X-ray scattering techniques of X-ray reflectivity (XR and grazing incident X-ray diffraction coupled to pressure-area isotherm methods were crucial to elucidate the disposition of these lipids in the outer membrane of bacteria at the level of air–aqueous interface (Neville et al. 2006). The analysis let also determinate that the hydrocarbon-associated chains are responsible of rigidity.

Not only the structure of the new potential drugs is important, but also it is mandatory to establish the relationship between some molecular structures and the resistance phenomena. For example, one the major resistance factor to almost aminoglycoside antibiotics is the amino(6′)-acetyltransferase-Ie/aminoglycoside(2″)-phosphotransferase-Ia [AAC(6=)-Ia/APH(2″)-Ia]. During almost three decades, this bifunctional enzyme structure remained practically unknown. However, the X-ray scattering was the tool to model its structure and the analysis of the impact of substrate binding on the enzyme (Caldwell and Berghuis 2012). The knowledge of the rigid structure, now, could permit the design of more specific therapies able to evade the action of this enzyme or design new molecules that would be able to interfere with the enzyme, letting the aminoglycosides keep their antibacterial activity (Shi and Berghuis 2012; Shi et al. 2013).

The neutron scattering technology can help us to elucidate the secondary side effects of antimicrobial drugs. In a series of experiments conducted in the Institut Laue-Langevin in Grenoble, Barlow and his colleagues from King's College London described by neutron diffraction experiments how the amphotericin B (AmB) interacts with fungal and animal cells membrane. These experiments were the first evidence for the theoretical model of leaky holes resulting from the development of Barrel-like structures, formed in both membranes upon the introduction of AmB. The difference between human and fungal cells was penetration of the barrels at the same AmB concentration, deeper in fungal membranes, explaining in this way, why AmB attack mainly the fungal cells. However, when the dose of drug was increased, the effects reached the animal cells, generating the secondary site effects (Foglia et al. 2012). The atomic details of drug binding have been largely unknown due to the lack of key information on specific hydrogen atom positions and hydrogen binding between the drug and its target enzyme. Acetazolamide (AZM) is a sulphonamide used since a long time ago to treat a wide spectrum of diseases such as glaucoma and epilepsy. When the wrong isomer of AZM is binding to the target enzyme, unpleasant side effects are provoked. The neutron scattering technology gave the precise information about the interactions between carbon anhydrase II and AZM (target enzyme and drug, respectively) (Fisher et al. 2012).

The cell membrane constitutes a significant hydrophobic barrier. This implies the presence of transmembrane proteins responsible to form transmembrane polar pore, to transport the substances in/out. One exception to these carriers is some bacterial toxins, able to insert and cross the lipid bilayer itself. The neutron

scattering helped to study the interaction of colicin N and its outer membrane receptor, the OmpE, that is member of the Omp proteins family, also evolves in the antimicrobial resistance (Clifton et al. 2012). The pH-dependent membrane insertion of the diphtheria toxin T domain in lipid bilayers could be described by specular neutron reflectometry and solid-state NMR spectroscopy (Chenal et al. 2009).

The communication between cells is fundamental in the pluricellular organisms. One part of these interactions is made using vesicles: the vesicles are responsible of the transport of neurotransmitters between nerve cells and also the uptake of glucose from blood in response in insulin signalling. The responsible of mediate in vesicles formation are proteins members of the superfamily protein SNARE. Christie and co-workers used small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) to investigate the interactions between the SNARE—superfamily protein Syntaxin and its regulatory protein Munc18 (Christie et al. 2012).

Other important mechanism of communication between cells is mediated by adhesion proteins, which are the responsible of the formation of intercellular junctions and the control of intermembrane spacing. Neural cell-adhesion molecule (NCAM) is expressed in both developing and adult vertebrate organisms and is a cell surface glycoprotein, which mediates cell adhesion, signalling, migration and plasticity in the central system (Doherty and Walsh 1996). The combination of the neutron and X-ray specular reflectivity helped to elucidate the structure of the ectodomain of NCAM (Clifton et al. 2012).

Cholesterol is an important structural component of most cell membranes, evolved in their organization, dynamics and so on. This lipid is also related in molecular pathways and intracellular cascades (Ikonen 2008; Maxfield and Tabas 2005; Maxfield and Wustner 2002). The healthy cells maintain a cholesterol gradient along the exotic pathways from the endoplasmic reticulum to the plasmatic membrane, suggesting an important role of the homoeostasis of cholesterol in the cells. The importance of cholesterol in the transport is reflected in the severe disorders that its disorder generates, such as the neurodegenerative diseases—Niemann–Pick type-C or Alzheimer's disease. The use of the non-invasive time-resolved small-angle neutron scattering to measure cholesterol intermembrane exchange and intramembrane flipping rate showed a more precise measurement of the transport and the differences with classic techniques, less sensitive and precise (Garg et al. 2011).

There are a huge number of biological applications based in protein arrays based in the interaction between antibodies, scaffold proteins and a surface. The array-based techniques assume the perpendicular disposition of the structure. Polarized neutron reflection was used to probe the perpendicular structure between the IgG, a gold surface and the scaffold protein, concretely a fusion protein based on the porin outer membrane OmpA from *Escherichia coli* and the Z domain of *Staphylococcus aureus* protein A.

## 2.7 Conclusion

Antimicrobials research faces many challenges regarding the solution of growing threats, even to the collective health and safety. The fight against multiresistant bacterial strains, for instance, implies better knowledge of different molecular processes, new drugs, the development of individualized and preventive clinical approaches, as well as new regimes in drug design and delivery.

Scattering is a very valuable tool for exploring molecular interactions, to further the understanding the mechanisms of different diseases. Development, formulation into effective pharmaceuticals and manufacturing of drugs are fields that can also be very strongly benefited from the scattering techniques.

Apart from the more commercial instruments available, there have been intensive efforts developing big facilities in different countries to provide biologists (and other scientists) with extremely powerful tools to support their investigations. At this moment, more powerful facilities providing a wider range on instruments are planned and will be constructed in the near future. These facilities are providing the ideal environment for multidisciplinary investigations and for pushing forward the frontiers of the scientific knowledge.

This chapter tries to give an overview of the possibilities of using these techniques in the world of antimicrobial research, what opportunities may arise for this discipline by using scattering. From the basic foundations, that give a flavour of the mechanisms involved, to the more suitable instruments for biology and success cases, a comprehensive overview of scattering was provided. The main purpose is to encourage the antimicrobials research community to consider scattering as one of the more powerful experimental alternatives to enhance their investigations.

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# Chapter 3
# Antibacterial, Antiviral and Antifungal Activity of Essential Oils: Mechanisms and Applications

**Karola Böhme, Jorge Barros-Velázquez, Pilar Calo-Mata and Santiago P. Aubourg**

**Abstract** Essential oils are natural products which combine antimicrobial and antioxidant activity, thus providing natural protection against microbial pathogens and other undesirable agents. Among the essential oils extracted from aromatic plants, oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) oils have been proposed for different biomedical and industrial applications. The antimicrobial mechanisms found in these essential oils have been explained on the basis of their content in natural compounds such as carvacrol, thymol, p-cymene and c-terpinene, among others. Although these two essential oils have received much attention, scientists working in the fields of biomedicine and food science, among others, are paying increasing attention to a wider variety of aromatic natural oils in an effort to identify novel and natural applications for the inhibition of microbial pathogens. Accordingly, a detailed revision of the main essential oils and their applications in biomedicine, food science and other industrial fields is presented. The review not only focuses on the main antibacterial applications reported to date, but also in the current and future developments for the inhibition of virus and fungi.

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## 3.1 Introduction

Essential oils (EOs) are oily, aromatic and volatile liquids that can be harvested from plant material. Usually, EOs are formed in specialised cells or groups within stems or leaves and are concentrated in particular regions of the plant, such as the bark, leaves or fruit. The composition of EOs is very complex, and many EOs contain 20–60 individual volatile compounds. The main components are hydrocarbons (pinene, limonene and bisabolene), alcohols (linalool and santalol), acids (benzoic acid and geranic acid), aldehydes (citral), cyclic aldehydes (cuminal), ketones (camphor), lactones (bergapten), phenols (eugenol), phenolic ethers (anethole), oxides (1,8 cineole) and esters (geranyl acetate) (Sell 2006; Miguel 2010).

Essential oils are multifunctional and exhibit a wide spectrum of activities, such as antiphlogistic, spasmolytic, antinociceptive, immunomodulatory, psychotropic, acaricidal, expectorative and cancer-suppressing activities. Furthermore, EOs and their components possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties. These activities can be mediated by single compounds or groups of compounds, and these secondary metabolites have biological functions in the plants from which they originate, such as protection against predators and microbial pathogens, as well as involvement in defence mechanisms against abiotic stress (Bassolé and Juliani 2012; Lang and Buchbauer 2012).

Due to the broad range of antimicrobial and other beneficial effects, EO-producing plants have been used as medicinal plants over thousands of years. In total, 3,000 EOs are known, of which approximately 300 are commercially important, primarily in the flavouring and fragrances industries. In medicine, only a few EOs are used in aromatherapies and some components of EOs are used for flavouring in the food industry. However, the recent trend of using natural compounds in medicines and food preservation has led to an increasing interest in EOs in both research and industrial settings.

Food safety and foodborne illnesses are an increasingly important public health issues. Thus, the growth and metabolism of microorganisms in food products present a global problem. Microbial toxins cause serious foodborne illnesses, and each year millions of people die from diarrhoeal disease caused by the consumption of contaminated food (WHO 2002). Additionally, food represents a good growth medium for microorganisms, and microbial spoilage of food products result in high economic losses for the food industry. Thus, effective methods of reducing or eliminating foodborne pathogens or microbes that cause food spoilage are needed.

At the same time, an increasing demand from the consumer side for products with fewer synthetic food additives exists, and thus, a strong interest in using natural substances for food preservation as an alternative for the broad spectrum of synthetic chemical additives has appeared. Several methods have been suggested

for the biopreservation of food products, such as the use of bifidobacteria or natural plant extracts. In addition, new methods of applying antimicrobial substances to foods, such as on packaging films, have been implemented in order to apply high concentrations of preservatives to food surfaces and increase the shelf life.

Thus, plants like oregano, thyme, garlic, bay leaf, rosemary and clove or their extracts, known as EOs, can be used alone or in combination with other preservation methods, such as irradiation or modified atmosphere packaging (MAP), to improve the shelf life of food products (Mejlholm and Dalgaard 2002; Mahmoud et al. 2004; Miguel et al. 2004; Wong and Kitts 2006; Kykkidou et al. 2009; Yerlikaya and Gokoglu 2010). In many studies, the application of EOs resulted in a considerable extension of the food's shelf life. For example, a new active packaging, consisting of a label containing cinnamon EO attached to plastic packaging, was used to extend the shelf life of late-maturing peaches.

An important consideration when using EOs as preservation agents in food is the impact on sensory acceptability. In general, a relatively high concentration of EOs is required to achieve a significant antimicrobial and/or antioxidant activity. However, the high aromatic potential of EOs can affect the organoleptic quality of the corresponding food, resulting in unacceptable odour and taste during consumption (Gutierrez et al. 2008). Therefore, research should focus on optimising EO formulations and their applications to food products to facilitate the successful use of EOs in the food industry. The main objective is to obtain an effective antimicrobial and antioxidant activity at low concentrations without negatively affecting the organoleptic quality of food.

However, the antioxidant and antimicrobial activities of EOs have been shown to depend not only on the plant species but also other factors such as the number of samples, the method used to extract active compounds and the method employed for measuring antioxidant and antimicrobial capacity. In addition, the chemical composition of EOs can vary according to geo-climatic location, growing conditions (season, soil type, amount of water) and the plant's genetics. Consequently, the antioxidant activities and antimicrobial activities of EOs from the same plant can vary considerably according to numerous studies, and realistic comparisons between different types and sources of EOs can be difficult (Sangwan et al. 2001; Burt 2004). Additionally, most research focuses on the EO in its entirety, while information concerning the antioxidant activity of individual major constituents is scarce.

In the following sections, the antioxidant, antibacterial, antifungal and antiviral activities of several EOs are reviewed. Additionally, some applications of EOs in the medical field are discussed with a special focus given to the application of EOs as preservative agents in food products. Studies concerning the determination of the antioxidant and antimicrobial activity of some EOs in food products are summarised. Table 3.1 gives an overview of some commercially important EOs, their main active compounds and known antimicrobial and/or antioxidant activity.

**Table 3.1** Plants whose essential oils contain bioactive compounds

| Plant | Bioactive compound | Activity |
|---|---|---|
| Nutmeg (*Myristica fragrans*) | Sabinene, 4-terpineol, myristicin | Antifungal, antioxidant |
| Cedar (*Cedrus libani*) | Limonene | Antifungal |
| Garlic (*Allium sativum*) | Diallylle disulphide | Antibacterial, antiviral, antifungal, antioxidant |
| Clove (*Syzygium aromaticus*) | Eugenol and eugenyle acetate | Antiviral, antibacterial, antifungal |
| Coriander (*Coriandrum sativum*) | Linalool, E-2-decanal | Antifungal, antibacterial |
| Cinnamon (*Cinnamomum cassia*) | Cinnamaldehyde | Antibacterial, antiviral, antifungal, antioxidant |
| Eucalyptus (*Eucalyptus globulus*) | 1,8-cineole | Antibacterial, antiviral |
| Peppermint (*Mentha piperita*) | Menthol, menthone | Antiviral, antioxidant |
| Lavender (*Lavandula officinalis*) | Linalool, linalyle acetate | Antibacterial, antiviral |
| Tea tree (*Melaleuca alternifolia*) | Terpinen-1-ol-4 | Antibacterial, antifungal, antiviral |
| Lemon (*Citrus limonum*) | Limonene | Antibacterial, antiviral, antifungal, antioxidant |
| Oregano (*Origanum vulgare*) | Carvacrol, thymol, terpinene, cymene | Antibacterial, antifungal, antiviral, antioxidant |
| Rosemary (*Rosmarinus officinalis*) | Pinene, bornyl acetate, camphor, 1,8-cineole | Antibacterial, antifungal, antiviral, antioxidant |
| Sage (*Salvia officinalis*) | Camphor, pinene, 1,8-cineole, tujone | Antibacterial, antifungal, antiviral, antioxidant |
| Thyme (*Thymus vulgaris*) | Thymol, carvacrol, terpinene, cymene | Antibacterial, antifungal, antiviral, antioxidant |

## 3.2 Antioxidant Effects of Essential Oils

### *3.2.1 General Aspects and Antioxidant Behaviour of EOs*

An antioxidant is a substance that is capable of inhibiting specific oxidising enzymes, reacting with oxidising agents prior to the damage of other molecules, sequestering metal ions or repairing components of antioxidant systems such as iron transport proteins. Antioxidants (namely, vitamins, enzymes or $Fe^{+2}$) have the ability to protect cells from free radical damage and serve as chemopreventive agents by inhibiting free radical generation and play important roles in neutralising oxidative damage caused by these free radicals. The presence of antioxidants in the human diet has generated great interest due to their positive effects on human health due to their ability to neutralise free radicals and protect cells from oxidant damage.

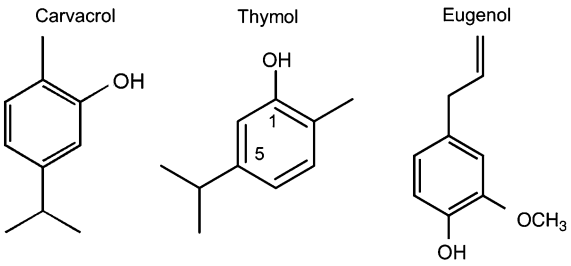


**Fig. 3.1** Chemical structure of carvacrol, thymol and eugenol bioactive components of essential oils

Recently, interest in research into the role of plant-derived antioxidants in food and human health has grown. Plants are known to produce a wide variety of molecules with strong antioxidant effects such as vitamins (ascorbic acid, vitamin C; α-tocopherol, vitamin E; β-carotene, vitamin A precursor) and phenolic compounds. Among the main phenolic compounds identified in plant extracts, phenolic acids (e.g., p-coumaric acid, caffeic acid, rosmarinic acid and gallic acid), phenolic diterpenes (e.g., carnosic acid and epirosmanol) and flavonoids (e.g., aromatic compounds) possess antioxidant activity (Shan et al. 2005).

A large pool of studies describing the antioxidant activity of EOs exists (Adorjan and Buchbauer 2010; Miguel 2010). In preliminary studies, EOs from *Rosmarinus officinalis, Salvia fruticosa*, *Foeniculum dulce*, *Thymus vulgaris* and *Laurus nobilis* inhibited lipid oxidation; these results can be explained by the presence of phenolic compounds (carvacrol, thymol and eugenol) (Zygadlo et al. 1995; Özcan et al. 2001) (Fig. 3.1).

In 2007, the antioxidant and free radical scavenging activities of EOs from flowers and fruits of *Otostegia persica* have been investigated (Sharififar et al. 2007). By using GC–MS analysis, α-pinene, 1-octen-3-ol and cubenol were identified as the major constituents of the EO in flowers (EOFLs), while the most prominent in EO in the fruit (EOFR) was hexadecanoic acid. These results show that EOFLs possessed greater antioxidant and radical scavenging activity attributed to the high amount of oxygenated monoterpenes.

The antioxidant properties of some popular and commercially available EOs from lemon (*Citrus limon*), pink grapefruit (*Citrus paradisi*), coriander (*Coriandrum sativum*), clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), lavender (*Lavandula angustifolia*), peppermint (*Mentha. piperita*) and frankincense (*Boswellia carteri*) have been reported. The results show that clove EO exhibited the highest amount of total phenols (eugenol, eugenyl acetate, β-caryophyllene and 2-heptanone) and had the highest antioxidant activity, as well as the highest DPPH radical scavenging activity and the highest FRAP value. Lavender EO and limonene also exhibited a high DPPH radical scavenging activity. Furthermore, peppermint EO possessed the highest radical scavenging activity against ABTS radicals, while lavender oil was most effective for inhibiting linoleic acid peroxidation after 10 d. It has been shown that all EOs tested were capable of chelating iron (II), but the greatest effect was achieved by the rosemary EO (Chaieb et al. 2007; Misharina and Samusenko 2008; Viuda-Martos et al. 2010; Yang et al. 2010b).

In another study, the chemical composition and antioxidant effects of the EO from *Mentha piperita* were investigated. The main constituents found were menthol, menthone, menthyl acetate, 1,8-cineole, limonene, β-pinene and β-caryophyllene (Schmidt et al. 2009). As a result, *M. piperita* exhibited anti-radical activity on DPPH and hydroxyl radicals. Likewise, EO and methanol extracts of *Psammogeton canescens* were tested in vitro and showed significant antioxidant activity. The EO was a more effective radical reducer than the methanol extract at all concentrations tested. The chemical composition analysed by GC–MS showed that the main constituents of the oil were β-bisabolene, apiole, α-pinene and dill apiole (Gholivand et al. 2010).

A separate study demonstrated that antioxidant activity can differ significantly depending on the location where the plant was collected, as shown for Tunisian cultivated sage (*Salvia officinalis*) EO (Ben Farhat et al. 2009). Furthermore, the antioxidant potential of *Ruta montana* EO possessed anti-radical activity in a concentration-dependent manner (Kambouche et al. 2008).

### *3.2.2 Antioxidant Behaviour of EOs and Health Advantages*

Free radical-mediated oxidation is an important energy-producing biological process in all living organisms. When oxygen-derived free radicals are overproduced, they can induce oxidative damage to biomolecules such as lipids, proteins and nucleic acids that produce oxidative stress. This oxidative stress may lead to atherosclerosis, ageing, cancer, diabetes mellitus, inflammation and several degenerative diseases in humans (Adorjan and Buchbauer 2010; Miguel 2010).

Including antioxidants in the diet has been found to have beneficial effects on human health because they protect biologically important cellular components, such as DNA, proteins and membrane lipids, from reactive oxygen species attacks. The use of naturally occurring antioxidants in daily life has been regarded as an effective way of promoting human health. Synthetic antioxidants have fallen out of favour because of their carcinogenicity, and thus natural antioxidants from plant products have become an increasingly promising alternative.

Among plant-derived products, EOs have been reported to have a strong potential for antioxidant, therapeutic and pharmaceutical purposes. EOs are known to scavenge free radicals, and this property makes them important in health maintenance and disease protection. Thus, volatile phenolic compounds found in EOs have been determined to be the main active ingredients in most herbs, e.g., menthol (in mint), carvacrol (in oregano and rosemary), thymol (in thyme) and eugenol (in clove). Meanwhile, the main components of EOs such as terpenoids, specifically monoterpenes (C10) and sesquiterpenes (C15), as well as a variety of low molecular weight compounds, have been found to be profitable (Anthony et al. 2012).

### 3.2.3 Antioxidant Effects of EOs on Food Preservation

Lipid oxidation is very important in the food industry. In addition to the effects on human health in the form of oxidative stress or oxidative damage, major concerns about lipid oxidation used in food technology exist because of the formation of oxidation products such as fatty acid hydroperoxides and secondary degradation products. These autoxidation products of fats and oils are responsible for off-flavours and characteristic rancid odours and are responsible for the decrease of both the nutritional quality and the safety of foods (Fasseas et al. 2008; Anthony et al. 2012).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) have been used to retard or minimise the oxidative deterioration of foods. Recently, consumers have rejected synthetic antioxidants because of their carcinogenicity, and in recent decades, increasing interest in natural antioxidants, especially plant-derived antioxidant compounds, has appeared. Many herbs, spices and their extracts have been added to a variety of foods to improve their sensory characteristics and extend shelf life. Herbs of the Lamiaceae family, mainly oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*), have been reported to have significant antioxidant capacity (Shan et al. 2005). Thus, numerous studies have shown that EOs are an abundant source of compounds exhibiting strong antioxidant activity and that these compounds can be used as natural antioxidants in the food industry. The effect of oregano EO on meat quality has been studied the most, whereas less information about other plants is available. Additionally, EO use has been especially important in meat products, although they have also been useful in seafood that has high polyunsaturated fatty acid content. Select applications of EOs as antioxidants on different foods will now be described.

#### 3.2.3.1 Meats

The short shelf life of refrigerated, packed meat makes its commercialisation more difficult than other types of foods. Most related research advocates the addition of EOs to meat products in post-slaughter stages or their inclusion in active packaging.

Oregano (*Origanum vulgare*)-based films have been shown to stabilise lipid oxidation in beef muscle slices for 7 days at 4 °C (Oussalah et al. 2004). Likewise, oregano EO added to chitosan coating successfully protected dry fermented sausages from lipid oxidation (Krkić et al. 2012). Differences in the fatty acid profiles (myristic, oleic and linoleic acids) between coated and control sausages were observed after 2 months of storage, but after 7 months of storage, there was no difference. The content of most aldehydes was significantly lower in coated sausage than in the control after 7 months of storage, and odour and flavour were better for the coated sausage.

The use of basil (*Ocimum basilicum*) and coriander (*Coriandrum sativum*) EOs as antioxidants in Italian salami was investigated. The formulation containing 0.75 mg/g basil EO exhibited antioxidant activity on lipids but not the protein in the Italian salami during processing and after 30 days of storage at 18–25 °C (Cichoski et al. 2011). Likewise, salami with coriander EO exhibited reduced lipid oxidation, increasing the shelf life of the product. In addition, salami treated with the commercial antioxidant BHT had less antioxidant activity than those treated with coriander EO (Marangoni and Moura 2011).

De Oliveira et al. (2011) have shown the possible benefits of combining EOs and minimal amounts of sodium nitrite in cured meat products. Winter savoury (*Satureja montana* L.) EO was added at concentrations of 7.80, 15.60 and 31.25 μl/g in mortadella sausages formulated with different sodium nitrite levels (0, 100 and 200 mg/kg) and stored at 25 °C for 30 days. The effect on colour development and lipid oxidation (TBARS) was analysed, and the 100 mg/kg nitrite concentration appeared to be sufficient for the formation of the characteristic red colour. The use of EO at concentrations exceeding 15.60 μl/g adversely affected the colour of the product by reducing redness and increasing yellowness. A significant effect on lipid oxidation was observed in samples containing EO plus reduced amounts of sodium nitrite (De Oliveira et al. 2011).

The effects of thyme EO on the lipid stability of vacuum-packaged (VP) and refrigerated (4 °C) chicken liver were studied by (Papazoglou et al. 2012). Lipid oxidation was low, as determined by malondialdehyde (MDA) values, during the entire storage period (>12 d).

Incorporating EOs and other natural antioxidants in animal diets is another strategy to obtain lipid stability in food products. Several studies have been carried out to evaluate this strategy. The antioxidant effects of oregano EO and vitamin E were evaluated in chicken (Botsoglou et al. 2003a; Avila-Ramos et al. 2012). The results showed that α-tocopheryl acetate supplementation was more effective than dietary incorporation of oregano EO to extend the lipid stability of chicken. However, there was a synergistic effect between dietary EO and α-tocopheryl acetate supplementation in retarding lipid oxidation in raw and cooked turkey during refrigeration (Botsoglou et al. 2003b). Feeding swine with oregano EO also had positive effects, resulting in a lower oxidation of pork lipids than that of control samples throughout storage (Alarcon-Rojo et al. 2013). However, further studies did not find an effect with EO compounds (carvacrol and cinnamaldehyde) that were added to the diet of growing lambs on the sensory characteristics of the meat product (Chaves et al. 2008). Likewise, the studies on the effects of dietary oregano EO supplementation on finishing pig meat suggested a lack of an antioxidant effect (Simitzis et al. 2010).

#### 3.2.3.2 Seafood

The effect of different EOs (bay leaf, thyme, rosemary, black seed, sage, grape seed, flax seed or lemon oils) on lipid oxidation of chub mackerel (*Scomber*

*japonicus*) was studied during 11 months of frozen storage at −20 °C (Erkan 2012). Thyme oil treatment was found to be particularly effective in delaying lipid oxidation. However, the other EO treatments resulted in lower TBA and free fatty acid values in the fish than those of control samples throughout storage. The addition of oregano EO at 0.2 % to fresh salted, packaged rainbow trout (*Oncorhynchus mykiss*) fillets stored for a period of 21 days at 4 °C also showed that lipid oxidation, as determined by thiobarbituric acid values (TBA), did not occur during the refrigerated storage period (Pyrgotou et al. 2010).

The efficacy of oregano and thyme EOs in quality retention of refrigerated (4 °C) squid (*Loligo vulgaris*) ring ready-to-eat (RTE) product was studied by (Sanjuás-Rey et al. 2012). Both EOs had inhibitory effects on lipid oxidation, as determined by the presence of peroxide and thiobarbituric acid-reactive substances and the formation of interaction compounds, was observed, with oregano oil being more effective at lower concentrations.

Successful inhibition of lipid oxidation was also achieved using *Zataria multiflora* EO, either separately (0.2/0.4 %) or in combination with a coating, on fresh silver carp (*Hypophthalmichthys molitrix*) fillets during storage at 4 °C (Zabol 2012). Likewise, laurel (*Laurus nobilis*) and cumin (*Cuminum cyminum*) EOs induced a decrease in lipid oxidation by ca. 40 % of TBA value on fresh vacuum-packed (VP) wild and farmed sea bream (*Sparus aurata*) fillets evaluated during storage on ice (Attouchi and Sadok 2010).

A mixture of 0.25 % turmeric and 0.25 % lemongrass EOs showed synergistic effects on the retardation of lipid oxidation of green mussel (*Perna canalicula*) stored at 4 °C (Masniyom et al. 2012). However, samples treated with 0.5 % lemongrass oil exhibited a higher likeness score for odour and flavour compared to samples treated with other EOs, making lemongrass EO the most promising agent to prevent deterioration and maintain the odour and flavour attributes of mussel during prolonged refrigerator storage.

Another strategy to delay lipid oxidation of fish products is the reduction of stress during transportation. *Lippia alba* EO has shown to be effective for reducing the formation of peroxides and thiobarbituric reactive substances compared to control fillets of silver catfish (*Rhamdia quelen*). It can be concluded that *L. alba* EO used as a sedative in the water to transport silver catfish can delay the lipid oxidation of fillets during frozen storage (Veeck et al. 2012).

#### 3.2.3.3 Other Foods

Oregano and laurel EOs have been shown to protect fried-salted peanuts against lipid oxidation, considerably increasing their shelf life, whereas rosemary EO was less effective (Olmedo et al. 2008). It was concluded that these EOs could be used as natural antioxidants in foods with high lipid contents. In another study, these authors evaluated the antioxidant effect of aguaribay (*Schinus areira*) and cedron (*Aloysia triphylla*) EOs on fried-salted peanuts and found that these EOs were also

effective against lipid oxidation. However, these EOs could affect the sensory profile and consumer acceptance of the product (Olmedo et al. 2012).

Asensio et al. (2011) evaluated the preservative effect of 0.05 % oregano EO on extra virgin olive oil during storage. Chemical indicators of lipid oxidation were measured, and in general, olive oil samples with added oregano EO had lower peroxide, conjugated dienes and p-anisidine values and higher chlorophyll and carotenoid contents during storage (Asensio et al. 2011). Likewise, the antioxidant effects of EOs from rosemary, clove and cinnamon were determined on hazelnut and poppy oils (Özcan and Arslan 2011). These EOs were added at concentrations of 0.25 and 0.5 % to the oils and stored at 50 °C in the dark for 14 days. All tested EOs showed an antioxidant effect compared to control groups, and cinnamon oil was the most effective at retarding lipid oxidation of crude oils followed by clove and rosemary oils. Microwave heating induces severe quality and composition losses in soybean oil. To protect the oil, spike lavender (*Lavandula latifolia*) EO has been added and was shown to counteract the oxidation compared to control oils (Rodrigues et al. 2012).

In a further study, coriander EO (0.05, 0.10 and 0.15 %) was successfully applied as a natural antioxidant in cake during 60 days of storage at room temperature. Interestingly, the sensory properties of cakes containing 0.05 % CEO were not different from those of controls ($P < 0.01$) (Darughe et al. 2012).

## 3.3 Antibacterial Effects of Essential Oils

### *3.3.1 General Aspects*

Antibacterial activity can be either bacteriostatic (inhibition of the bacterial growth) or bactericidal (destruction of bacterial cells). However, differentiating between these two actions is sometimes difficult. Antibacterial activity in relation to these two different modes of action is measured as the minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (MBC) (Burt 2004). The antibacterial activity of EOs has been evaluated by adapting standard tests for antibiotic resistance. This process is especially difficult for EOs due to the volatility of some active substances, their high viscosity and their water insolubility. However, most studies that focus on the antibacterial activity of EOs are aimed at the designation of a possible inhibitory effect against bacteria, with special interest on health, environment and food. To rapidly screen for antibacterial activities of many EOs against a broad range of bacteria, the agar diffusion method is the most widely used. The EOs are applied to the inoculated agar using reservoirs, such as filter paper disks or holes punched in the agar. After incubation, the diameters of a possible inhibition zone are measured (Faleiro 2011).

The inhibitory action of EOs against bacteria is linked to the high hydrophobicity that causes higher cell permeability and results in the loss of cellular

components, such as ions, ATP, glucose, etc. Depending on the quantity and strength of the EO, an irreversible damage of the cell membrane induces the lysis of the bacterial cells (cytolysis) and therefore death. The difference in the cell wall structure of Gram-negative and Gram-positive bacteria explain the differences in antibacterial action, with Gram-positive strains much more sensitive to EOs. Another suggested mechanism is the inhibition of amylase and protease production, which stops toxin production and electron flow and results in coagulation of the cell content (Smith-Palmer et al. 2004; Di Pasqua et al. 2007; de Souza et al. 2010).

Essential oils and their components are known to exhibit inhibitory activity against a variety of bacteria. The oils of plants of the family *Lamiacae* are among the EOs that have been demonstrated to have a strong antimicrobial activity. Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) oils are the most studied EOs exhibiting antibacterial activity against a broad range of Gram-positive and Gram-negative bacterial species. Additionally, a number of further *Origanum*, *Salvia* and *Mentha* species showed effective inhibition of bacterial growth (Hammer et al. 1999; Dorman and Deans 2000; Moreira et al. 2005; Gutierrez et al. 2008).

The antibacterial effect of EOs is attributed to a small variety of phenolic compounds and terpenoids, which have antibacterial activity in their pure form (Aureli et al. 1992). Carvacrol and thymol, the main components of EOs from *Lamiacae* family plants, have the most well-studied effect against bacteria. Other components with antibacterial activity are 1,8-cineole, citral, eugenol, geraniol, α-pinene, perillaldehyde and terpinen-4-ol (Kim et al. 1995b; Cosentino et al. 1999; Lambert et al. 2001). When applying all substances present in an EO, the antimicrobial effect of the individual compounds can be combined (additive effect), reduced (antagonist effect) or enhanced (synergetic effect).

The increasing interest in EOs as antibacterial agents resulted in a huge number of studies that analysed the antibacterial potential of many EOs against a broad range of bacterial species. Nevertheless, the great variability of plants that produce EOs, the differences in composition of EOs of even closely related plant species and the diversity of testing methods make generalisation very difficult. The antibacterial activities of a number of EOs against a huge number of bacterial species are listed in the book "Handbook of Essential Oils: Science, Technology, and Applications" (Baser 2010). Furthermore, reviews covering the antimicrobial activity of EOs summarise the large amount of screens performed to determine the antibacterial activity of specific EOs against a number of bacterial species, mainly pathogens (Kalemba and Kunicka 2003; Burt 2004; Lang and Buchbauer 2012). As mentioned before, some oils inhibit only Gram-positive strains, whereas EOs with a stronger antibacterial activity can inhibit both Gram-positive and Gram-negative strains due to the higher sensitivity of Gram-positive bacterial strains to EOs. The two Gram-negative species *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, both human pathogens that cause serious infections in clinics, exhibited a special robustness against EOs. Nevertheless, the oils of *Rosmarinus officinalis*, *Nepeta cataria*, *Mentha longifolia*, *Mentha viridis* and *Monticalia*

*andicola* showed a great inhibitory effect against *K. pneumoniae,* and oils from *Salvia rubifolia*, *M. andicola*, *Eugenia beaurepaireana* and *Pituranthos chlorantus* strongly affected *P. aeruginosa*. In contrast, *Aeromonas* species were very susceptible to most EOs.

### 3.3.2 Antibacterial Effects of EOs and Biomedical Applications

EO-producing plants have always been used as medicinal plants and antimicrobial agents in traditional medicine long before the discovery of microorganisms. Currently, the popularity of EOs is increasing because of the rejection of synthetic drugs whenever possible due to their unwanted side effects. Furthermore, the increasing drug resistance of clinically relevant bacterial strains represents a global problem. Thus, one of the main research aims in this field is the search for new antibacterial agents, especially natural compounds.

>EOs from organisms such as *Cleistocalyx operculatus*, *Eucalyptus globulus*, *Lavandula stoechas*, *Lavandula angustifolia*, *Lavandula luisieri*, *Melaleuca alternifolia*, *Salvia rosaefolia*, *Tanacetum parthenium*, *Thymus vulgaris* and *Zataria multiflora* have shown an inhibitory effect against methicillin-resistant *Staphylococcus aureus* strains (MRSA) (Dung et al. 2008; Roller et al. 2009; Polatoglu et al. 2010). *Z. multiflora* showed the highest antibacterial activity against MRSA, with an MIC of 0.25–1.0 μl/ml (Mahboubi and Ghazian Bidgoli 2010), followed by *T. vulgaris* that had an MIC of 18.5 μg/ml (Tohidpour et al. 2010). The activity of these two EOs is linked to their high thymol contents. The other mentioned active EOs contain mainly 1,8-cineole and terpinen-4-ol. *Helichrysum italicum* exhibited antibacterial activity against a number of Gram-negative drug-resistant strains, such as *Acinetobacter baumanii*, *Enterobacter aerogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* (Lorenzi et al. 2009). McMahon et al. (2008) made the important observation that some staphylococci have adapted to the EOs of *M. alternifolia*, indicating that bacterial strains can develop resistance to EOs similarly to antibiotics (McMahon et al. 2008). Thus, it is important to apply the EOs in a high concentration to achieve the irreversible damage of cells and avoid the evolution of resistance.

Limonene-containing EOs, such as those from several *Citrus* species, *Abies koreana* and *Fortunella japonica*, showed a high efficacy against *Propionibacterium acnes* and *Staphylococcus epidermidis*, both of which cause serious skin infections. The MICs were between 0.3 and 10 μl/ml, and these EOs could be applied in the treatment of skin infections, as well as in cosmetics to prevent infections (Baik et al. 2008; Kim et al. 2008; Yoon et al. 2009; Yang et al. 2010a). In general, EOs are suitable for use as bio-preservatives in cosmetic formulations, inhibiting bacterial growth and at the same time providing further beneficial effects and aroma.

The EOs of *Achillea ligustica*, *Mentha longifolia*, *Hyptis pectinata*, *Mentha piperita* and *Rosmarinus officinalis* effectively inhibit the growth of the dental

bacteria *Streptococcus pyogenes* and *Streptococcus mutans* more than chlorohexidine, thus making them possible substrates for dental formulations to avoid caries (Nascimento et al. 2008; Maggi et al. 2009).

The bacterium *Helicobacter pylori* colonises the human stomach and can cause ulcers and gastritis, thus making it a major target of antibacterial studies. However, few EOs have been tested against this bacterial species, although inhibitory effects on *H. pylori* have been shown for the EOs of *Apium nodiflorum*, *Plinia cerrocampanensis* and *Thymus caramanicus*, which have MICs of 12–60 μg/ml (Menghini et al. 2010; Vila et al. 2010). The EO of *Dittrichia viscosa* subsp. *revoluta* also has high antibacterial activity with an MIC of 0.33 μl/ml and could be applied for the treatment or prevention of *H. pylori* infections (Miguel et al. 2008).

### 3.3.3 Antibacterial Effects of EOs and Applications in Food Preservation

Historically, EOs have been used for flavour in foods and beverages. Due to their variable antimicrobial compound contents, they have a great potential as natural agents for food preservation.

Different EOs have been tested against foodborne pathogenic and spoilage bacteria. The oil from *Artemisia incana* showed considerable inhibitory effects against 26 foodborne pathogenic bacteria (Cetin et al. 2009). Likewise, *Magnolia liliflora* and *Allium schoenoprasum* (chives) EOs inhibited a number of food spoilage and foodborne pathogenic bacteria (Bajpai et al. 2008; Rattanachaikunsopon and Phumkhachorn 2008). *Artemisia echegarayi* EO exhibited antibacterial activity against the seven foodborne pathogens (*Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* serovar e*nteritidis* and *Salmonella enterica* serovar *typhimurium)*, but not against *Proteus mirabilis.* Two terpenes, thujone and camphor, were identified from this essential oil as the principal constituents responsible for antibacterial activity (Laciar et al. 2009). The leaf EOs from seven Himalayan Lauraceae species (*Neolitsea pallens*, *Lindera pulcherrima*, *Dodecadenia grandiflora*, *Persea duthiei*, *Persea odoratissima*, *Persea gamblei* and *Phoebe lanceolata*) had potent antibacterial activities against three Gram negative (*E. coli*, *S. enterica* and *Pasteurella multocida*) and one Gram positive (*S. aureus*) foodborne bacterial species, with MIC values between 3.90 and 31.25 μl/ml (Joshi et al. 2010). Additionally, laurel oil (*Laurus nobilis*) inhibited the microbial growth of *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Enterococcus faecalis* and *Listeria monocytogenes* at concentrations from 0.02 to 2.5 % (vol/vol) but was inactive against *Escherichia coli* and *Salmonella typhimurium* (Erkmen and Özcan 2008). In a further study, the effects of garlic, bay, black pepper, origanum, orange, thyme, tea tree, mint, clove and cumin EOs on *L. monocytogenes*, *E. coli*, *Salmonella enteritidis*, *P. mirabilis* and *B. cereus* were analysed, showing that thyme, origanum, clove and orange EOs have strong

antibacterial activities against these bacterial species (Irkin and Korukluoglu 2009). The effect of EOs on the important foodborne pathogenic species *Salmonella* spp. has been evaluated in several studies. Citrus EOs had antibacterial activity against 11 serotypes/strains of *Salmonella*, with the most active compound being terpenes from orange essence (MIC = 0.125 % − 0.5 %) that is composed principally of d-limonene (94 %) and myrcene (3 %) (O'Bryan et al. 2008). Carvacrol, citral and geraniol showed potent antibacterial activities against *Salmonella typhimurium* and its rifampicin-resistant strain. The most potent compound in this EO was carvacrol, with an MIC value of 250 μg/ml for both strains (Kim et al. 1995a). Similarly, 63 *Campylobacter jejuni* isolates were screened for their resistance and susceptibility to cinnamaldehyde and carvacrol, the main constituents of plant-derived cinnamon and oregano EOs. The results showed that both substances exhibited a rapid antimicrobial activity against both antibiotic-resistant and non-resistant *C. jejuni* strains at concentrations of ~0.1 % and higher (Ravishankar et al. 2008).

However, even if a number of EOs showed inhibitory effects of the growth of a number of food pathogenic and spoilage species, it should be mentioned that these studies were carried out with pure bacterial cell cultures. The antibacterial action of EOs for the use in a complex food matrix can be very different and requires separate studies in the corresponding food of interest. In general, a much higher concentration is needed in food compared to the MIC determined for pure bacterial cultures. This is most likely due to the higher nutrient content in foods that enables the bacterial cells to repair cell damage (Gill et al. 2002). In addition, the high fat and protein content protects the bacterial flora against the EO activity. This assumption has been confirmed by various studies because EOs had a higher antibacterial effect in low-fat food than in high-fat food (Tassou et al. 1995; Smith-Palmer et al. 2001; Burt 2004).

It should be mentioned that for an effective application in the food sector, only EOs with a strong antibacterial potential are suitable, enabling application of very low concentrations that do not greatly influence the organoleptic properties of the corresponding food product. Additionally, for the safe application as preservative agents in foods, the activity against probiotic bacteria of the intestinal flora should be low to prevent negative side effects (Cetin et al. 2010). EOs or compounds isolated from EOs are applied directly to the food product or combined with further preservation methods, such as vacuum or MAP (Irkin and Esmer 2010). Thus, the preservative effects of increasing the salt content and decreasing the pH, storage temperature and amount of oxygen has a synergetic effect on the antibacterial activity of EOs (Skandamis and Nychas 2001). To achieve a thorough interaction of the EOs with the product, layers or films of the EOs are being applied.

Because the antibacterial effect and influence on the odour and flavour can vary significantly between the different food matrices, every EO has to be tested in the corresponding food product. In a number of studies, EOs have been tested in specific foods or in food models for their antibacterial activity in studies that are reviewed in the following sections.

#### 3.3.3.1 Meats

In various meats, oils from coriander, clove, oregano and thyme exhibited elevated inhibitory effects against *L. monocytogenes* and *A. hydrophila* at concentrations of 5–20 μl/g (Tsigarida et al. 2000; Menon and Garg 2001; Burt 2004). When screening the alcohol extracts of angelica root, banana purée, bay, caraway seed, carrot root, clove (eugenol), marjoram, pimento leaf and thyme, only clove and pimento extract significantly inhibited the growth of *A. hydrophila* and *L. monocytogenes* in refrigerated, cooked, ready-to-eat meat (cooked chicken, refrigerated cooked beef and beef slices prepared from roasted whole sirloin tips). *A. hydrophila* was more sensitive to the EOs (Hao et al. 1998a, b). Cilantro EO was not suitable to inhibit the growth of *L. monocytogenes* strains on vacuum-packed ham. Ham disks were inoculated with a cocktail of five *L. monocytogenes* strains, treated with 0.1, 0.5 and 6 % cilantro oil diluted in sterile canola oil or incorporated into gelatine that included lecithin to enhance the incorporation of the cilantro oil, then vacuum-packed and stored at 10 °C. An inhibitory effect was observed only for the highest concentration of cilantro oil (Gill et al. 2002).

The inhibitory effect of oregano EO on autochthonous spoilage microflora on minced meat packed under aerobic or modified atmosphere (MA) and stored at 5 °C has also been studied. In all packaging conditions, only concentrations of 0.5 and 1 % oregano oil were effective. In addition to the microbial growth analysis, microbial metabolite formation has been evaluated. The results indicated that oregano EO delayed glucose and lactate consumption under aerobic and MA conditions. Furthermore, under aerobic storage, proteolysis was significantly inhibited, and the production of acetate under the MA was inhibited (Skandamis and Nychas 2001). The oil of mustard reduced the growth of aerobic mesophilic bacteria, as well as lactic acid bacteria, in an acidified chicken meat model stored for 2 weeks at 22 °C (Lemay et al. 2002).

An increase of the preservative effect of MAP and vacuum packaging with bay (*Laurus nobilis*) EO has been shown in ground chicken breast meat stored at 4 °C. Total viable counts (TVC), as well as the growth of two important foodborne pathogens, *L. monocytogenes* and *E. coli,* were notably reduced (Irkin and Esmer 2010).

In further studies, a synergetic effect has been observed for EOs combined with nisin against foodborne pathogens in mince meat products during storage at 4 and 10 °C for 12 days. Thus, the bactericidal effect of oregano EO against Salmonella *enteritidis* in minced sheep meat and the antibacterial effect of thyme EO against *L. monocytogenes* and *E. coli* in minced beef meat were enhanced by the addition of nisin. In all of these studies, the inhibitory effect was higher when stored at 10 °C than at 4 °C (Solomakos et al. 2008a, b; Govaris et al. 2010).

The combination of thyme oil and chitosan was applied to ready-to-cook chicken-pepper kebab stored under aerobic conditions at 4 °C for a period of 12 days. Treatments reduced the microbial growth, with lactic acid bacteria (LAB), *Brochothrix thermosphacta* and Enterobacteriaceae being highly sensitive against this combination, while Pseudomonads were the most resistant. The products' shelf lives were extended by ca. 4 and 6 days (Giatrakou et al. 2010).

The use of antibacterial films as wrappings has been demonstrated to be an effective technology for controlling surface contamination by foodborne pathogenic microorganisms in meat and poultry products. Edible films containing cinnamaldehyde or carvacrol (0.5, 1.5, 3 %) showed antibacterial effects against *S. enterica* and *E. coli* O157:H7 artificially inoculated onto chicken breasts and *L. monocytogenes* artificially inoculated onto ham. Carvacrol films were more active against all three pathogens than cinnamaldehyde films (Ravishankar et al. 2009).

#### 3.3.3.2 Seafood

Food products of marine origin are known to spoil rapidly due to the growth and metabolism of bacteria because fish and other seafood represent an optimal growth medium for spoilage bacteria. Thus, the enhancement of seafood shelf life has special interest in the fishing and food industries. However, only a few studies have examined the application of EOs to avoid microbial spoilage of seafood. The oregano EO is the most studied EO in this context and exhibits the best antibacterial activity against seafood spoilage bacterial species. As in meat products, the antibacterial effects of EOs are influenced by fat content, with high fat content causing less efficacy (Burt 2004). The application of EO-coatings and spreading the EOs on the surface of fish has been demonstrated to be effective in enhancing the shelf life of seafood products (Ouattara et al. 2001; Harpaz et al. 2003).

Thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) were added at 0.05 % (vol/vol) as preservatives for the cold storage of Asian sea bass (*Lates calcarifer*). Both EOs considerably slow the spoilage process, with the fish treated with these oils being adequate for consumption after 33 days of storage (Harpaz et al. 2003).

*Photobacterium phosphoreum* is one of the main bacterial spoilage species in vacuum-packed or MAP fish products. Of nine tested EOs, oregano and cinnamon EOs had the strongest antimicrobial activity on *P. phosphoreum,* and oregano oil (0.05 %, v/w) reduced the growth of *P. phosphoreum* in naturally contaminated MAP cod (*Gadus morhua*) fillets and extended shelf life from 11–12 to 21–26 d at 2 °C (Mejlholm and Dalgaard 2002).

The combination of low-dose gamma irradiation and EO coating on the shelf life of pre-cooked shrimp (*Penaeus* spp.) stored at 4/−1 °C has been studied. Antimicrobial coatings were prepared by incorporating various concentrations of thyme oil and trans-cinnamaldehyde in coating formulations prepared from soy or whey protein isolates. The results showed that gamma irradiation and EO coating had synergistic effects, reducing the total aerobe counts and inhibiting the growth of *P. putida*, leading to an extension of shelf life of at least 12 days (Ouattara et al. 2001).

The antimicrobial effect of oregano and thyme EOs on a refrigerated (4 °C) squid (*Loligo vulgaris*) ring ready-to-eat (RTE) product has been studied by Sanjuás-Rey et al. (2012). Oregano EO exhibited an inhibitory effect on the microbial activity of aerobic, anaerobic and psychrotrophic bacterial species, as

well as of Enterobacteriaceae. EOs were added at different concentrations to the coating medium during processing. Oregano EO showed a more pronounced effect at higher concentrations. The application of oregano EO was combined with MAP, resulting in an enhancement of the quality of the squid rings (Sanjuás-Rey et al. 2012). Likewise, oregano EO combined with MAP and storage at 4 °C inhibited the microbial growth on salted sea bream (*Sparus aurata*) (Goulas and Kontominas 2007), sword fish (*Xiphias gladius*) fillets (Giatrakou et al. 2008) and salted rainbow trout (*Oncorhynchus mykiss*) fillets (Pyrgotou et al. 2010). The studies in rainbow trout showed significantly reduced growth of Lactic acid bacteria (LAB), followed by $H_2S$-producing bacteria (including *Shewanella putrefaciens*), *Pseudomonas* spp. and *Enterobacteriaceae* when treated with salt and oregano EO and stored under MAP conditions (Frangos et al. 2010).

However, the addition of thyme EO did not lead to any inhibitory effects on the microbial activity of the refrigerated RTE squid (Sanjuás-Rey et al. 2012). In contrast, the application of thyme EO extended the shelf life of fresh fish, such as gilthead sea bream (*Sparus aurata*) (Attouchi and Sadok 2010) and sea bass (*Dicentrarchus labrax*) fillets when combined with MAP and swordfish (*Xiphias gladius*) (Kykkidou et al. 2009). Two further studies compared the antibacterial effects of oregano and thyme EO on cod (*Gadus morhua*) fillets (Mejlholm and Dalgaard 2002) and Asian sea bass (*Lates calcarifer*) (Harpaz et al. 2003). The oregano EO exhibited a stronger antibacterial activity in the first study, but no differences between both types of oils were observed in slowing the spoilage in the second study.

The effect of *Zataria multiflora* EO on the quality of fresh silver carp (*Hypophthalmichthys molitrix*) fillets has been studied, showing an inhibition of microbial growth when used alone or in combination with coating (Zabol 2012). Furthermore, turmeric and lemongrass EOs lowered the microbial deterioration of green mussels (*Perna canaliculus*). The best results were obtained by applying a mixture of both EOs: 0.25 % turmeric EO and 0.25 % lemongrass EO (Masniyom et al. 2012).

The preservative effect of laurel EO (*Laurus nobilis*) and cumin EO (*Cuminum cyminum*) has also been demonstrated on fresh vacuum-packed sea bream (*S. aurata*) fillets. The treatment of wild and farmed sea bream fillets with laurel or cumin EOs induced a decrease in bacterial growth by ca. 0.5–1 log cfu/g, extending the shelf life of fish fillets by approximately 5 days of ice storage (Attouchi and Sadok 2010).

#### 3.3.3.3 Dairy Foods

Most studies of the application of EOs in dairy products were aimed at the inhibition of the human pathogens *L. monocytogenes*, *S. enteritidis* and *E. coli O157:H7*.

In cheese, the growth of *L. monocytogenes* and *S. enteritidis* was restricted by clove, bay, cinnamon and thyme EOs when they were applied at concentrations of 1 % and stored at 4 and 10 °C. Only clove oil was effective against these two pathogens in cheese (Smith-Palmer et al. 2001; Vrinda Menon and Garg 2001).

In a subsequent study, feta cheese was inoculated with E. coli O157:H7 or *L. monocytogenes* and was then stored under MAP at 4 °C. The addition of oregano and thyme EOs at doses of 0.2 ml/100 g resulted in a decrease of the bacterial populations of both species (Govaris et al. 2011).

A synergetic inhibitory effect on *L. monocytogenes* was observed in skimmed milk, when HHP treatment was combined with carvacrol (Karatzas et al. 2001).

The inhibitory effect of a mixture of plant EOs (DMC) against a broad range of foodborne pathogens and spoilage species was tested in Spanish soft cheese. The EO mixture showed inhibitory activity against Gram-positive species such as *L. monocytogenes*, *Listeria innocua*, *Staphylococcus aureus*, *Lactobacillus brevis* and *Micrococcus luteus.* The EOs were less effective against Gram-negative species, such as *Salmonella choleraesuis*, *E. coli* O157:H7, *Enterobacter cloacae* and *Pseudomonas aeruginosa*. No inhibitory effects were observed on *Pseudomonas fluorescens* (Mendoza-Yepes et al. 1997).

#### 3.3.3.4 Other Foods

The antibacterial effects of natural volatile compounds against *Salmonella* have been studied on alfalfa seeds and sprouts. Only acetic acid, cinnamic aldehyde and thymol caused significant reductions in *Salmonella* populations after treatment for 7 h (Weissinger et al. 2001).

Likewise, the antibacterial activity of carvacrol on the foodborne pathogen *Bacillus cereus* was studied on rice. Carvacrol caused a growth inhibition of this pathogen at concentrations of 0.15 mg/g and higher. Due to the intense smell and taste of carvacrol at high concentrations, the treatment was combined with cymene, resulting in a synergistic effect (Ultee et al. 2000).

In a subsequent study, the effects of carvacrol and cinnamic acid on microbial spoilage of fresh-cut fruit were studied. Fresh-cut kiwifruits and fresh-cut honeydew were dipped in carvacrol or cinnamic acid solutions at 1 mM and resulted in a reduction of TVC during storage for 5 days at 4 and 8 °C, without adverse sensory consequences (Roller and Seedhar 2002).

Additionally, a positive inhibitory effect of thyme oil against *L. monocytogenes* was found in apple–carrot juice stored at 4 °C, at a concentration of 0.5 % and against E. coli O157:H7 on shredded lettuce and baby carrots stored at 4 °C after washing with a suspension of the EO (1.0 ml/l for 5 min) (Singh et al. 2002).

## 3.4 Antifungal Activity of Essential Oils

### *3.4.1 General Aspects*

When analysing the chemical composition of EOs that exhibit strong antifungal activity against moulds, no obvious patterns become apparent. Some EOs contained predominantly non-phenolic terpenes, while others contained a high

percentage of phenolic monoterpenes, such as thymol and carvacrol. The phenylpropanoid eugenol was detected often in EOs with strong activity against moulds. In particular cases, the non-phenolic bicyclic monoterpenes camphor and α-pinene exerted noteworthy antifungal activities. EOs that exert strong antimicrobial activity against yeasts possess high thymol, carvacrol, cymene, linalool or α-pinene contents. Remarkably, many EOs with inhibitory effects against yeasts were found in genera of the Lamiaceae family including *Thymus* spp., *Origanum* spp., *R. officinalis, O. sanctum* and *Z. multiflora*. All of these EOs had at least one of the previously mentioned substances as the primary component.

Fungicidal mechanisms can involve the destruction of existing mycelia, as well as the inhibition of new mycelia development. Therefore, *Citrus sinensis* EO, which was noticed to be rich in limonene (84.2 %), exerted a marked antifungal activity against *Aspergillus niger* by destroying its mycelial cell walls (Sharma and Tripathi 2008). Moreover, EOs have been found to be capable of inhibiting the formation of spores. Thus, chamazulene was found to be the lead molecule in *Achillea millefolium* EO constituting 42.2 % of the whole oil. This EO was found to exert genotoxic effects against the fungal cells and suppress the development of spores (Sant'Anna et al. 2009).

Many moulds are able to produce toxic molecules, so-called mycotoxins, which represent a threat to human health because some of them (e.g., aflatoxin) act as carcinogens. In a preliminary study, the inhibitory effect of various concentrations of ground mint, sage, bay leaves, thyme, aniseed and red pepper and citrus peel oils on the growth and toxin production of an aflatoxin-producing strain of *Aspergillus parasiticus* was studied (Karapinar 1985). Of the different herbs tested, thyme was found to be a highly effective antifungal agent. Growth and aflatoxin formation were depressed by a 10-day incubation with orange and lemon EOs at a 1.6 % concentration. In the same manner, EOs of dill, coriander, basil, marjoram, rosemary, mint and thyme demonstrated antifungal activities against *Aspergillus flavus* and inhibited aflatoxin B1 production in vitro (El-Habib 2012). Dill EO was the most effective against aflatoxin production, while the EO of thyme and basil delayed the growth of *A. flavus*. In a recent study, the antifungal activity of cinnamon EO was evaluated on *A. flavus,* and the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined in direct contact with the mould by macrodilution. A strong activity was observed with an MIC of 0.05–0.1 mg/ml and an MFC of 0.05–0.2 mg/ml. Furthermore, polyethylene terephthalate films containing cinnamon EO were tested in vapour phase, without direct contact with the mould, and produced total inhibition at 4 % CIN EO (Becerril et al. 2013).

In another study, the antifungal properties of 16 EO constituents were tested against five *Aspergillus* spp., four *Penicillium* spp. and two *Fusarium* spp. that are widely reported to contaminate foods. The strongest antifungal activity was caused by o- or p-alkyl substituted phenols; most potent compounds were isoeugenol, cinnamaldehyde, carvacrol, eugenol and thymol. The most resistant organism was *Penicillium verrucosum* var. *cyclopium* followed by *Penicillium roqueforti*, whereas *Penicillium. viridicatum* was the most sensitive (Knobloch et al. 1989).

Oregano (*Origanum hypericifolium*) EO was active against 14 fungi isolated from hazelnut and walnut and completely inhibited (100 %) hyphal growth when in direct contact and in headspace assays after 3 and 6 days, respectively. This high inhibitory effect correlates with the presence of aromatic components, such as monoterpenes, carvacrol, thymol and p-cymene (Ocak et al. 2012).

### 3.4.2 Inhibition of Fungal Activity and Health Aspects

Essential oils have exhibit marked antifungal activities. These inhibitory effects have been observed on different types of fungi such as dermatophytic fungi, moulds, phytopathogenic fungi and yeasts (Lang and Buchbauer 2012). Various dermatophytic fungi are responsible for the generation of fungal infections on human skin, nails and hair. Dermatophytes such as *Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum* are more susceptible to EOs compared to other types of fungi such as *Aspergillus* species and yeasts. The prevalent substances in the EOs that have been reported to have activity against dermatophytic fungi cannot be assigned to one particular chemical group, but it seems that the presence of phenylpropanoids (e.g., estragole, eugenol) and the monocyclic sesquiterpene alcohol α-bisabolol correlates with strong antifungal effects against fungi that cause skin infections.

Moreover, thymol, carvacrol and geraniol were shown to inhibit the development of *Candida* biofilms.

### 3.4.3 Inhibition of Fungal Activity and Food Preservation

In a preliminary attempt, the fungistatic properties of EOs from lavender, marjoram, mint, basil, sage, savoury, thyme and verbena were tested against a number of food spoilage and pathogenic fungi. The greatest fungistatic activities were shown by thyme, savoury and verbena oil (Pellecuer et al. 1979).

Several EOs that exhibited antimicrobial activity against phytopathogenic fungi belong to the Lamiaceae family. Furthermore, the existence of carvacrol, α-pinene and p-cymene in the EOs was related to a high antifungal effect. Additionally, the phenylpropane derivate eugenol is of a particular importance because it showed antifungal activity against a wide range of different phytopathogenic fungi. EO from cymbopogon, ajowan and dill seed exhibited high antifungal activity against plant and food roots. Cymbopogon oil was proposed to serve as a broad-spectrum fungistatic compound for the control of phytopathogenic fungi. The bioactive ingredients were identified as the simple monoterpenes geraniol, thymol and carvone, which have minimum inhibitory concentrations of 160, 200 and 225 mug/ml, respectively. It has been suggested that synthetic modifications can be applied to these molecules, especially to geraniol, to obtain an optimal antifungal product with lipophilicity (Sridhar et al. 2003). Amiri et al. (2008) isolated eugenol from *Syzygium*

*aromaticum* EO, and an eugenol-lethicin combination was subsequently tested for antifungal activity against phytopathogens. The presence of eugenol effectively diminished fungal infections in stored apples, underscoring their potential use as biofungicide of this combination (Amiri et al. 2008).

Antifungal activities of EOs from thyme, summer savoury and clove were evaluated in culture medium and tomato paste (Omidbeygi et al. 2007). The results showed that all EOs inhibited the growth of *A. flavus*, while the thyme oil and summer savoury showed the strongest inhibition at 350 and 500 ppm, respectively. Taste panel evaluations were carried out in a tomato ketchup base, and the 500 ppm thyme oil sample was accepted by panellists. The growth of *A. flavus* was also entirely impeded by applying savoury EO (thymol and carvacrol) on lemons 1 week before they were exposed to pathogens (Dikbas et al. 2008). In addition, this EO effectively suppressed the growth and the aflatoxin B1 and G1 synthesis of *A. parasiticus* (Razzaghi-Abyaneh et al. 2008). Likewise, the EOs from rosemary and ajowan inhibited the growth of *A. parasiticus* and its aflatoxin production. Ajowan EO was more effective in inhibiting growth, and rosemary EO was more effective against aflatoxin production. It was concluded that both EOs could be safely used as preservatives on some types of foods to protect them from toxigenic fungal infections (Rasooli et al. 2008). In a further study, antifungal effects of thyme and rosemary EOs on two aflatoxigenic *A. flavus* strains previously isolated from hazelnuts were investigated (Ozcakmak et al. 2012). Fungal growth was almost completely inhibited after a 90 min application of thyme EO concentration (250 and 125 μl/ml), while rosemary EO caused only a slight growth inhibition. Similarly, the prevalent substances in *Lippia alba* EO (neral, geranial), as well as the entire EO, were shown to inhibit both aflatoxin B1 production and *A. flavus* growth (Shukla et al. 2009). Moreover, the growth of other *Aspergillus* species and *Fusarium* strains was greatly impaired; thus, this EO was considered suitable for food preservation.

The effects of the EOs from lemon (*Citrus lemon*), Mandarin orange (*Citrus reticulata*), grapefruit (*Citrus paradisi*) and orange (*Citrus sinensis*) on the growth of moulds commonly associated with food spoilage (*A. niger*, *A. flavus*, *Penicillium chrysogenum* and *Penicillium verrucosum*) were studied (Viuda-Martos et al. 2008). All the oils had antifungal activity against all the moulds. Orange EO was the most effective against *A. niger*, and mandarin EO was most effective at reducing the growth of *A. flavus*, while grapefruit was the best inhibitor of the *P. chrysogenum* and *P. verrucosum* mould. Thus, citrus EOs can be considered a suitable alternative to chemical additives for use in the food industry.

The antifungal activity of cinnamon EO on pathogens such as *Rhizopus nigricans*, *A. flavus* and *Penicillium expansum* was also investigated. The results revealed that cinnamon oil had the potential to be employed as a natural antifungal agent for fruit applications, as cinnamaldehyde is its main constituent (Xing et al. 2010).

Despite its high antibacterial activity, oregano EO was also demonstrated to be a very competent antifungal agent. The EO of oregano has strong anti-*Aspergillus* activity and completely inhibited radial mycelial growth of *A. flavus*, *A. fumigatus*

and *A. niger*, during 14 days of interaction with MIC values between 80 and 20 μl/ml. In addition, this EO had a significant inhibitory effect on further assayed *Aspergillus* spp. and was able to inhibit fungal spore germination (Carmo et al. 2008; Mitchell et al. 2010).

Furthermore, oregano EO also showed inhibited yeast growth with MIC values of 20 and 0.6 μl/ml (Souza et al. 2007).

In further studies, a comparison of the antifungal activities of various EOs against a variety of food-spoiling yeasts was carried out in has been reviewed (Kunicka Styczyńska 2011). EOs from basil, garlic, marjoram, onion, peppermint, thyme, lemon and grapefruit were tested against yeasts that frequently cause food spoilage (*Candida rugosa, Debaryomyces hansenii, Kluyveromyces marxianus, Rhodotorula glutinis, Rhodotorula minuta, Saccharomyces cerevisiae, Trichosporon cutaneum, Yarrowia lipolytica* and *Zygosaccharomyces rouxii*) because they are the most popular spices and flavouring agents used in the food industry. The ranking of oils by their fungistatic activity against all these yeasts was as follows: thyme > marjoram > peppermint > basil > lemon > grapefruit > onion, garlic oils.

In recent studies, EOs were combined with coating and the ability to inhibit fungal growth was tested. Inhibition by vapour contact with *A. niger* and *Penicillium digitatum* by Mexican oregano (*Lippia berlandieri*), cinnamon (*Cinnamomum verum*) or lemongrass (*Cymbopogon citratus*) EOs added to amaranth, chitosan, or starch edible films was studied. It was postulated that edible chitosan edible that incorporate Mexican oregano or cinnamon EO could improve the quality of foods by the action of the volatile compounds on surface growth of moulds (Avila-Sosa et al. 2012). In another study, chitosan coatings, containing bergamot, thyme and tea tree EOs were applied to oranges (cv. Navel Powell), after inoculation with *Penicillium italicum* (Cháfer et al. 2012). Preventive antimicrobial treatments with coatings containing tea tree EO were the most effective with a reduction of the microbial growth of 50 %, compared to the uncoated samples. The results of this study provided a useful tool for the development of new environmentally friendly and healthier commercial applications in the control of the main postharvest fungal decay of citrus fruits.

## 3.5 Antiviral Activity of Essential Oils

A virus is a small infectious particle (20–300 nm), which is able to infect cells of another living organism and replicate itself. Viruses cannot reproduce on their own because they are composed only of genes and a protein coat and are sometimes surrounded by a lipid envelope. Viral infections provoke an immune response that usually eliminates the infecting virus.

Many EOs have been used for centuries in folk medicine, and in recent years, the biological properties of various EOs have been demonstrated by a number of studies (Adorjan and Buchbauer 2010; Elizaquível et al. 2012). To combat viral infections with EOs or their constituents seems to be a promising treatment

considering that many viral infections cannot be counteracted so easily by administering only a lozenge. Although more studies are necessary to analyse the biological properties of EOs and elucidate their mechanism of action, EO use in the treatment of viral diseases has been confirmed by many studies throughout the most recent decades.

In a preliminary attempt, antiviral activity was investigated in different fractions of rosemary extracts. An ethanol extract of steam-distilled rosemary EO was further fractionated by dichloromethane and hexane extraction and dried extracts were tested for activity against herpes simplex virus (HSV) propagated in human embryo lung fibroblast (HELF) cell cultures. The hexane extract was shown to have activity against HSV-2 and was not cytotoxic to HELF cells.

In later research, the antimicrobial activity of *Salvia fruticosa* EO exhibited cytotoxic activity against African green monkey kidney (Vero) cells and high levels of virucidal activity against HSV-1, a ubiquitous human virus (Sivropoulou et al. 1997).

The EO and extracts obtained from *Origanum acutidens* and methanol extracts from callus cultures were evaluated for their antiviral activities. Inhibitory effects of the methanol extracts from herbal parts on the reproduction of HSV-1 and a slight antiherpetic effect of callus cultures were observed. In contrast, none of the extracts inhibited the reproduction of influenza A/Aichi virus (Sökmen et al. 2004).

EOs from *Artemisia arborescens*, *Eugenia caryophyllus*, *Cedrus libani* and *Melissa officinalis* were tested against HSV-1 and HSV-2 and showed the ability to inhibit both viruses and cell-to-cell virus diffusion (Saddi et al. 2007; Loizzo et al. 2008; Schnitzler et al. 2008). The most promising oil, which had the highest activity against HSV-1, was *Juniperus oxycedrus* oil with an IC50 value of 200 μg/ml. The major constituents of this oil were found to be α-pinene and β-myrcene.

In a comparative study, the antifungal effects of various EOs (anise, hyssop, thyme, ginger, chamomile and sandalwood) against HSV-2 were tested. The screen identified chamomile as the most promising EO due to its higher selectivity index (Koch et al. 2008). In another study, the EO of star anise (*Illicium verum*) was also shown to be a promising antiviral candidate against HSV-1. Later on, the same authors reported antiviral activities of the EOs from eucalyptus, tea tree and thyme and of their major monoterpene compounds against HSV-1 (Astani et al. 2010). The highest selectivity index was shown by α-pinene and α-terpineol. Moreover, the mixtures of different monoterpenes, which are present in natural tea tree EO, revealed a 10-fold higher selectivity index and a lower toxicity than their isolated single monoterpenes.

Tea tree EO has also been tested against polio type 1, ECHO 9, Coxsackie B1, adeno type 2, HSV-1 and HSV-2 (Garozzo et al. 2009). As a result, tea tree EO showed no virucidal activity against polio 1, adeno 2, ECHO 9, Coxsackie B1, HSV-1 and HSV-2 but exhibited a slight virucidal effect against HSV-1 and HSV-2. The results of this study showed that tea tree oil can be considered a promising drug in the treatment of influenza virus infection.

The inhibitory effect of EOs of *Lippia alba*, *Lippia origanoides*, *Origanum vulgare* and *Artemisia vulgaris* on yellow fever virus (YFV) replication was investigated, showing antiviral activity against YFV through direct virus inactivation (Meneses et al. 2009). A subsequent study investigated the antiviral activity of Mexican oregano (*Lippia graveolens*) EO and its major component, carvacrol, against different human and animal viruses. An antiviral effect was detected against acyclovir-resistant herpes simplex virus type 1 (ACVR-HSV-1), acyclovir-sensitive HSV-1, human respiratory syncytial virus (HRSV), bovine herpesvirus type 2 (BoHV-2) and bovine viral diarrhoea virus (BVDV). The human rotavirus (RV) and BoHV-1 and 5 were not inhibited by the EO. Carvacrol alone exhibited high antiviral activity against RV, but it was less efficient than its effect on the other viruses.

The application of hyssop (*Hyssopus officinalis*) and marjoram EOs was evaluated for inactivation of non-enveloped viruses using murine norovirus and human adenovirus as models. A significant reduction of virus titres (TCID 50) was observed when EOs were used at different temperatures and times (Kovač et al. 2012).

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# Chapter 4
# New Antimicrobial Agents of Plant Origin

**Javier Sampedro and Elene R. Valdivia**

**Abstract** Plants are constantly under attack by microbial pathogens. As part of their defensive arsenal, they use antimicrobial peptides such as thionins, defensins, lipid transfer proteins, hevein-like peptides, knottins, cyclotides, $\beta$-barrelins, and others. In addition, they produce a diversity of antimicrobial metabolites. Those where the evidence for a role in plant defense is stronger include benzoxazinoids, camalexin, and glucosinolates among the alkaloids; flavonoids and stilbenes among the phenylpropanoids; and also terpenoids such as saponins. Our understanding of these plant antimicrobial agents has increased significantly in recent years with new information on their distribution, synthesis, regulation, in vivo function, and mechanism of action. Plant antimicrobial agents have a large potential for biotechnological applications. Engineered plants with increased disease resistance have been achieved using almost every family of antimicrobial peptides. There have been also been successes in using metabolic engineering to increase the production of antimicrobial compounds, as in the case of stilbenes and glucosinolates. Commercial applications using both approaches are likely to appear soon. The use of plant antimicrobials in human medicine is probably further in the future, although there are promising antifungal agents like defensin peptides, and saponins. With more than a quarter million species and a particularly diverse specialized metabolism, the richness of plant antimicrobials has barely been explored.

## 4.1 Plant Antimicrobial Defenses

Wild plants are quite successful at keeping bacterial and fungal pathogens at bay. In addition to physical barriers, they have an immune system capable of detecting and responding to the presence of pathogens. The first layer of defense is based on

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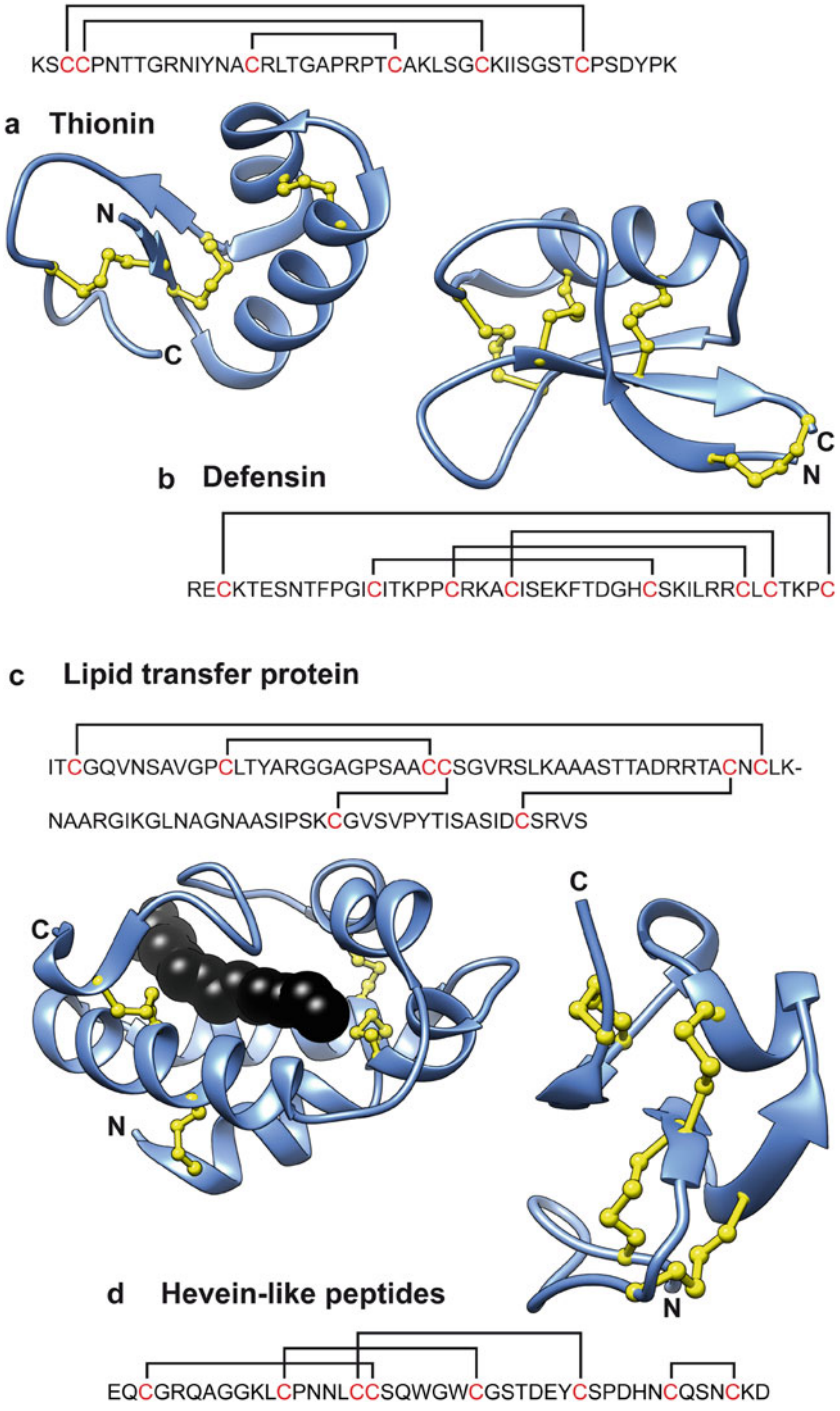
a series of receptors capable of recognizing conserved microbial products, like bacterial flagellin or fungal chitin, as well as signs of compromised cell wall integrity, such as lytic polysaccharide fragments (Schwessinger and Ronald 2012). Activation of these receptors triggers a series of responses called pattern-triggered immunity which includes the production of broad-spectrum antimicrobial compounds. Pathogens can overcome this barrier by producing a range of specific effectors, proteins, and other molecules that suppress pattern-triggered immunity or otherwise enhance their virulence. A second layer of defense is activated when the plant detects directly or indirectly the presence of these effectors through specific receptors called R proteins (Spoel and Dong 2012). The effector-triggered immune response is stronger than the pattern-triggered response and can activate programmed cell death in the affected area. Plants can also produce multiple mobile immune signals that are transported through the vasculature, usually after an effector-triggered response. These signals preventively activate defenses in unaffected organs and can also induce chromatin modifications that establish immune memory and lead to stronger responses in subsequent attacks.

Plant antimicrobial peptides and secondary or specialized metabolites are a critical part of the immune response, even if some of them are also produced constitutively. While some groups are ubiquitous, many are restricted to specific families. Secondary metabolites involved in defense responses are called phytoalexins when are synthesized in response to an attack and phytoanticipins if they are produced as inactive precursors and are activated upon attack (Morant et al. 2008; Ahuja et al. 2012). According to their biosynthetic pathway they can be separated into alkaloids, phenylpropanoids, and terpenoids, which is the classification that will be used in this chapter.

Increasing the production of antimicrobial peptides or specialized metabolites through genetic engineering has lead to enhanced resistance in many cases and will probably result soon in improved commercial crops (Collinge et al. 2010). While introducing a new antimicrobial peptide is relatively easy, engineering the synthesis of specialized metabolites is much more complex, but has now become a real possibility (Großkinsky et al. 2012). Regarding potential uses in human medicine, large-scale screens have failed to find potent and safe plant antibiotics (Lewis and Ausubel 2006). However, there is evidence that some plant antimicrobials act synergistically and may need to be combined to produce effective treatments.

## 4.2 Ribosomal Peptides

Antimicrobial peptides belong to a number of unrelated families, but in many cases they share some common characteristics. Despite their small size, below 10 kD, most of them have several disulfide bridges that increase their stability. The pattern of cysteines is characteristic of each family and is sometimes the easier way to identify new peptides, due to poor sequence conservation (Fig. 4.1).


KSCCPNTTGRNIYNACRLTGAPRPTCAKLSGCKIISGSTCPSDYPK
a Thionin
N
C
b Defensin
C
N
RECKTESNTFPGICITKPPCRKACISEKFTDGHCSKILRRCLCTKPC
c Lipid transfer protein
ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLKAAASTTADRRTACNCLK-
NAARGIKGLNAGNAASIPSKCGVSVPYTISASIDCSRVS
C
N
C
N
d Hevein-like peptides
EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKD

◀ **Fig. 4.1** Sequence and structure of plant antimicrobial peptides from different families with disulfide bridges indicated. **a** Viscotoxin A3 from *Viscum album* (PDB identifier 1ED0), **b** NaD1 from *Nicotiana alata* (1MR4), **c** nsLTP1 from *Oryza sativa* complexed with myristic acid (1UVA), **d** Hevein from *Hevea brasiliensis* (1HEV), **e** PAFP-S from *Phytolacca americana* (1DKC), **f** Kalata B1 from *Oldenlandia affinis* (1NB1), **g** MiAMP1 from *Macadamia integrifolia* (1C01)

Peptides from several different families are basic and amphiphilic, with both hydrophobic and positively charged hydrophilic areas. Many of these seem to interact with lipids as part of their mechanism of action and in some cases, they seem to penetrate and disrupt lipid membranes. An online database of plant antimicrobial peptides called PhytAMP is available (Hammami et al. 2009).

### *4.2.1 Thionins*

Thionins, whose name comes from a high proportion of sulfur-containing cysteines, are also known as family 13 of pathogenesis-related proteins (PR-13). Wheat thionins were the first plant antimicrobial peptide to be isolated, when they were identified as the component of wheat flour toxic to yeast cells (Balls et al. 1942).

Thionins have been characterized in a limited range of angiosperm species (Stec 2006; Hammami et al. 2009). Most of those with proven antimicrobial activity were obtained from monocot species of the grass (*Poaceae*) family (Oard et al. 2004; Fujimura et al. 2005) or dicot plants of the mistletoe (*Viscaceae*) family (Giudici et al. 2004). However, related sequences are present in many other plants suggesting that many more thionins with antimicrobial properties remain to be discovered (Silverstein et al. 2007).

In vitro thionins are toxic at micromolar concentrations to a wide range of organisms from bacteria and fungi to protozoa and human cells (Fujimura et al. 2005; Loeza-Ángeles et al. 2008; Berrocal-Lobo et al. 2009). Thionins have been found in both seeds and vegetative organs, such as leaves and stems (Stec 2006). While some are expressed constitutively, others are induced by fungal infections, supporting an in vivo defensive function for these peptides. It has also been proposed that they could have a role in redox regulation and that seed thionins could function as storage proteins (Castro and Fontes 2005).

Mature thionin peptides have 45–47 amino acids and are processed from longer pre-pro-proteins with a signal peptide and an acidic C-terminal domain of unknown function (Abbas et al. 2013). The degree of sequence similarity in mature thionins is quite high and those with proven antimicrobial activity are basic and amphiphilic (Stec 2006). The three-dimensional structure of more than 10 thionins from different groups has been resolved (Stec 2006). They have the shape of an L with two $\alpha$-helixes on one arm and a two-stranded antiparallel $\beta$-sheet on the other (Fig. 4.1a). This structure is stabilized by three conserved disulfide bridges, which are supplemented by a fourth one in some groups.

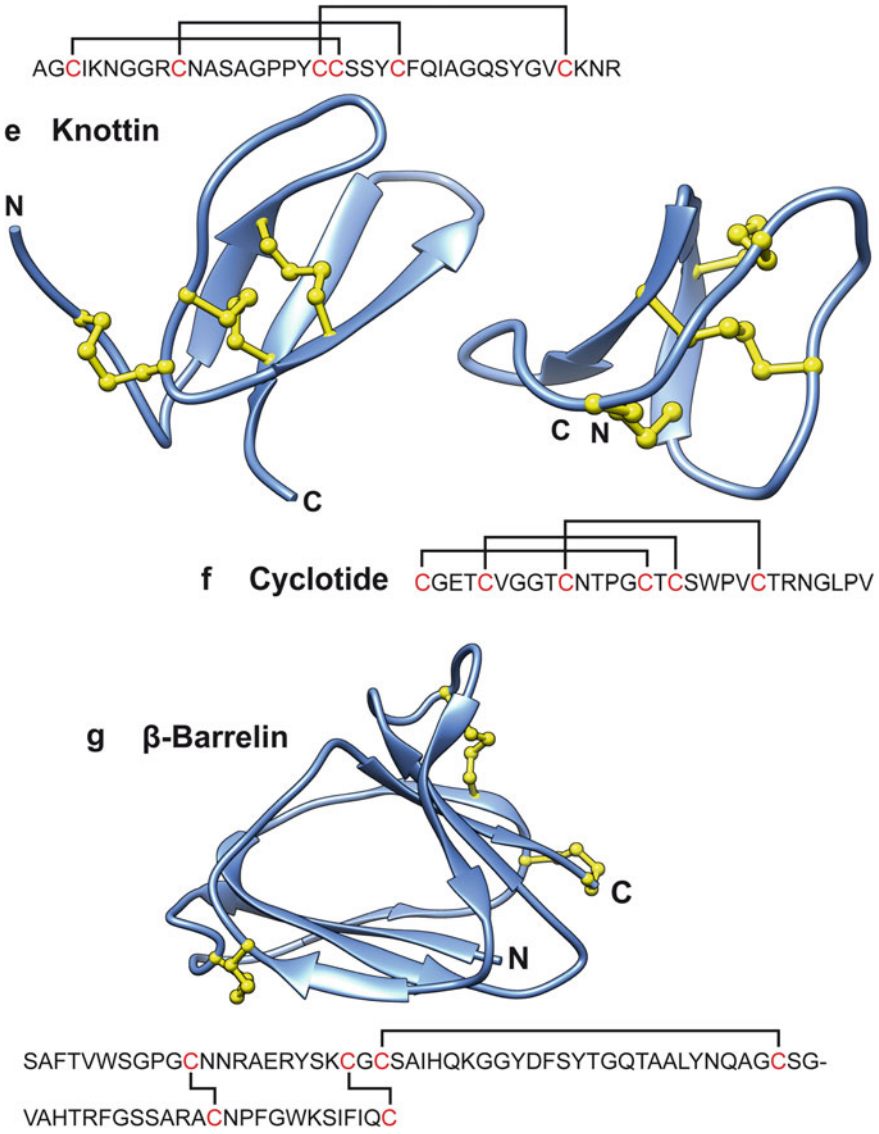



**Fig. 4.1** continued

The toxicity of thionins is associated with an increase in membrane permeability that causes depolarization and cell death (Giudici et al. 2004; Berrocal-Lobo et al. 2009). Their positive charges allow them to bind negatively charged phospholipid heads while a hydrophobic region could interact with the lipid tails. Several mechanisms have been proposed to explain the interaction of thionins with membranes (Stec 2006). In the first hypothesis, a complex of thionin molecules

forms transmembrane channels that allow the selective passage of ions (Hughes et al. 2000). Another proposed mechanism is that binding of thionins to the outer membrane surface creates rigid patches that allow the formation of transient pores at their margins (Coulon et al. 2002). It has also been suggested that thionins could bind and solubilize individual lipid molecules contributing in the process to membrane disruption (Stec et al. 2004). A recent proposal suggests that thionins become inserted in the outer leaflet of the membrane where they form half-channels that allow water to reach the interior of the membrane, disrupting its organization and creating transient pores (Oard 2011). Truncation of a thionin sequence showed that the α-helixes are sufficient for antimicrobial activity and membrane permeabilization (Vila-Perelló et al. 2005). In addition to this primary effect, additional mechanisms of toxicity have been suggested, such as DNA or RNA binding, although the evidence is limited (Stec 2006).

There have been some successes in engineering transgenic crops with improved resistance against pathogens by overexpressing thionins. Expression of a barley thionin in sweet potato, for instance, increased resistance against a fungal disease (Muramoto et al. 2012) and an oat thionin protected rice seedlings against bacterial infections (Iwai et al. 2002). However, the broad-spectrum toxicity of these peptides has to be taken into account, as is the case too with the possibility of using thionins to treat infectious human diseases. An Arabidopsis thionin was found to be active against human pathogens, both bacterial and fungal, but it also reduced the viability of mammalian cells (Loeza-Ángeles et al. 2008). The same is likely to be the case for a wheat thionin that has shown strong activity against the protozoan parasite responsible for human leishmaniasis (Berrocal-Lobo et al. 2009).

### *4.2.2 Defensins*

Defensins were originally called γ-thionins (Colilla et al. 1990), but were later separated in their own family because of the structural differences between the two groups of antimicrobial peptides (Terras et al. 1995). The new name was based on their similarity to animal defensins, which are also antimicrobial peptides. Plant defensins are also known as family 12 of pathogenesis-related proteins (PR-12). There is an online database for defensins which includes both plant and animal sequences (defensins.bii.a-star.edu.sg/).

Defensins are ubiquitous in plants, and a single species can have hundreds of defensin-like sequences in its genome (Silverstein et al. 2005). Sequence conservation is generally poor, but these peptides share a common pattern of disulfide bridges. A recent phylogenetic analysis of 139 defensins has divided the family in 18 groups (van der Weerden and Anderson 2013). Many defensins from different groups have been shown to have in vitro antifungal activity at micromolar concentrations (Carvalho and Gomes 2011). Antibacterial activity against both gram-positive and gram-negative species has been proven for a smaller number of

defensins, many of which belong to divergent groups (van der Weerden and Anderson 2013).

There is abundant evidence that plants use defensins in vivo to protect themselves from the attack of fungal pathogens (De Coninck et al. 2013). Some of them are produced constitutively during seed germination and others accumulate in vegetative tissues in response to pathogen inoculation. The best understood activation pathway is that of Arabidopsis PDF1.2a and closely related defensins which are induced by necrotrophic fungi. This pathway involves the production of the hormones ethylene and methyl jasmonate and a number of transcription factors have been identified (Çevik et al. 2012). Some defensins seem to have additional functions not related to antimicrobial defense such as a role in pollen recognition (Carvalho and Gomes 2011; van der Weerden and Anderson 2013).

Mature defensins have approximately 50 aminoacids. Their precursors have a signal peptide and in some cases additional domains of unknown function (De Coninck et al. 2013). Defensins share a common structure which has been determined for many different peptides (van der Weerden and Anderson 2013). Three disulfide bridges connect an $\alpha$-helix with an antiparallel $\beta$-sheet formed by three strands. A fourth bridge further stabilizes the structure by joining the N terminus with the C terminus (Fig. 4.1b). The second and third strands of the $\beta$-sheet together with the intermediate loop form the $\gamma$-motif or $\gamma$-core which is critical for antimicrobial activity and can indeed maintain this activity on its own (Sagaram et al. 2011).

The mechanism of action of antifungal defensins has been studied extensively (Kaur et al. 2011; De Coninck et al. 2013). NaD1, a defensin from *Nicotiana alata*, appears to bind a receptor in the outer cell wall layer (van der Weerden et al. 2010). This leads to the formation of membrane pores that allow it to enter the cytoplasm, where it could reach intracellular targets (van der Weerden et al. 2008). RsAFP2, a radish defensin, also interacts with a cell wall receptor, but it does not appear to be internalized (Thevissen et al. 2012). In this case, the receptor was identified as a sphingolipid that is present in both membrane and cell wall. Other defensins have also been shown to interact specifically with different fungal sphingolipids (De Coninck et al. 2013).

Membrane permeabilization has been demonstrated for a number of defensins from different species, including RsAFP2 and NaD1 (Sagaram et al. 2011; De Coninck et al. 2013). An interesting result is that NaD1 cannot induce permeabilization in liposomes, suggesting that it does not act directly on the membrane (van der Weerden et al. 2010). In addition to membrane permeabilization, the antifungal activity of several defensins appears to involve formation of reactive oxygen species and activation of apoptosis (De Coninck et al. 2013). One of these is RsAFP2 which appears to act extracellularly by compromising cell wall integrity (De Coninck et al. 2013). On the other hand, a pea defensin that, like NaD1, enters the cytoplasm, has been shown to localize to the nucleus where it interferes with the cell cycle (Lobo et al. 2007). These results clearly indicate that defensins can have different modes of action.

There is a large potential for biotechnological applications of defensins (Carvalho and Gomes 2011). Numerous groups have reported the use of constitutively expressed defensins to obtain crops with improved resistance to diverse fungal pathogens and commercial applications are under development. Among the plants where this approach has been successful are potato, tomato, rice, wheat, and banana (Kaur et al. 2011; Ghag et al. 2012; De Coninck et al. 2013). While most experiments have been carried out in growth chambers or greenhouses, two different transgenic potatoes have shown effective resistance in field tests to the agronomically important pathogens *Verticillium dahliae* and *Phytophthora infestans* (Gao et al. 2000; Portieles et al. 2010). Using NaD1, the Australian biotechnology company Hexima developed and field-tested cotton plants with increased resistance to *Fusarium* and *Verticillium* wilt (Kaur et al. 2011).

Regarding medical applications, several plant defensins have been considered promising candidates for the treatment of *Candida* infections in human (Thevissen et al. 2007). A radish defensins has already been shown to be effective in vivo as a treatment against systemic candidiasis in mice (Tavares et al. 2008). Unlike thionins, defensins do not appear to be toxic to human cells. While commercial-scale production of large defensin peptides would require heterologous systems, the small $\gamma$-motif can be chemically synthesized. It has been reported recently that a tomato defensin $\gamma$-motif has strong activity against bacterial human pathogens, both gram-positive and gram-negative, with no cytotoxic effect on human cells (Rigano et al. 2012).

### *4.2.3 Lipid Transfer Proteins*

Antimicrobial lipid transfer proteins (LTPs) are also called nonspecific lipid transfer proteins (nsLTPs), because they can associate with a broad range of phospholipids and transfer them from one membrane to another. LTPs have also been classified as family 14 of pathogenesis-related proteins (PR-14).

Dozens of different LTPs are present in a single plant genome and many more if we also include LTP-like sequences (Silverstein et al. 2007; Boutrot et al. 2008). Phylogenetic analyses of LTPs recognize up to nine different types, some specific to particular plant groups (Boutrot et al. 2008). Type I genes are widespread in seed plants and seem to be the most numerous in plant genomes. LTPs with proven antimicrobial activity belong to this group. They were discovered in the 1990s and have been purified from a number of species including radish, barley, spinach, onion, or coffee (Yeats and Rose 2008; Ng et al. 2012). Type I LTPs have shown activity against diverse fungi, as well as gram-positive and gram-negative bacteria, although the specificity varies among them (Carvalho and Gomes 2007). Onion Ace-AMPl, for instance, has activity against fungi and gram-positive bacteria at nanomolar concentrations, but shows no activity against gram-negative bacteria or

human cells (Cammue et al. 1995). In the same work, maize and wheat type I LTPs showed no antimicrobial activity.

Lipid transfer proteins were originally thought to be involved in the transport of lipids among different cellular compartments, but they are generally located extracellularly. A role for type I LTPs in plant defense is suggested by their in vitro activity against plant pathogens, but also by the induction of a number of LTPs upon pathogen attack (Carvalho and Gomes 2007; Yeats and Rose 2008). However, other possible functions have been suggested for type I LTPs, such as transportation of waxes to the cuticle or regulatory functions that involve interaction with hydrophobic molecules (Carvalho and Gomes 2007; Yeats and Rose 2008). In this regard, an Arabidopsis LTP-like gene has been shown to be necessary for the correct formation of the stem cuticle (DeBono et al. 2009). Even if lipid transport is the primary function of LTPs, their antimicrobial activity could also be beneficial to the plant.

Mature type I LTPs are basic peptides of 90–95 amino acids, preceded by a signal peptide in the pre-protein (Carvalho and Gomes AdO and Gomes 2007; Yeats and Rose 2008). Sequence conservation is not very high, but all share eight strictly conserved cysteines. The three-dimensional structure reveals four α-helixes that surround a hydrophobic tunnel and are connected by the four disulfide bridges (Fig. 4.1c). The tunnel can bind different types of lipids, as can be seen in several of the structures that have been resolved (Yeats and Rose 2008). Interestingly, in onion Ace-AMPl, the hydrophobic tunnel is blocked by the presence of voluminous side-chains, but the antimicrobial activity is not affected (Tassin et al. 1998).

Not much is known about the antimicrobial mechanism of action of LTPs. Several groups have observed membrane permeabilization, although the amount of leakage was small in some cases (Tassin et al. 1998; Regente et al. 2005; Zottich et al. 2011). Onion Ace-AMPl is one of the LTPs that causes membrane leakage, despite its inability to bind isolated lipids or transport them between membranes (Cammue et al. 1995; Tassin et al. 1998).

A small number of studies have found increased pathogen resistance in plants overexpressing antimicrobial LTPs. A barley LTP reduced the symptoms caused by the bacterial pathogen *Pseudomonas syringae* in tobacco and Arabidopsis (Molina and García-Olmedo 1997). Similarly, overexpression of Ace-AMPl from onion resulted in increased resistance to a fungal pathogen in wheat, as had previously been found also in geranium and rose (Roy-Barman et al. 2006). An LTP from motherwort also improved resistance against fungal pathogens in tobacco and poplar (Jia et al. 2010). It is possible that some of these results are caused by indirect effects such as alterations in defense signaling or cuticle properties, as suggested by an Arabidopsis mutant in an LTP-like gene with a defective cuticle and increased susceptibility to fungal attack (Lee et al. 2009). Biotechnological applications of LTPs also need to consider the fact that these peptides are potent allergens (Egger et al. 2010).

### 4.2.4 Hevein-Like Peptides

Hevein is an antifungal peptide that was isolated from rubber-tree latex using chitin-affinity chromatography (Parijs et al. 1991). Sequences homologous to hevein and capable of binding chitin have been found as peptides in other species, but also as domains that form part of larger proteins, such as chitinases (Porto et al. 2012).

Database searches show that hevein-like peptides are present in dicots and monocots and even in spikemosses, suggesting that they are widespread in vascular plants (Porto et al. 2012). Hevein-like peptides with antifungical activities at micromolar or lower concentrations have been purified from *Eucommia ulmoides* bark, ginkgo leafs, elderberry fruits and seeds of amaranth, morning glory, or wheat (Koo et al. 1998; Van Damme et al. 1999; Huang et al. 2000; Huang et al. 2002; Lipkin et al. 2005; Odintsova et al. 2009). These peptides appear to be expressed constitutively, suggesting a possible preventive role against fungal attacks. A hevein-like peptide from spindle tree bark, in addition to inhibiting the growth of several fungi, also showed activity against gram-positive bacteria, but not gram-negative species or yeasts (Van den Bergh et al. 2002).

There are two types of hevein-like peptides. The larger ones, like hevein itself, have 40–45 amino acids, while the shorter ones are only 30 amino acids long with a truncated C-terminal region (Egorov and Odintsova 2012). The three-dimensional structure of the longer peptides is anchored by a central three-stranded antiparallel $\beta$-sheet (Fig. 4.1d). Four conserved disulfide bridges connect the central $\beta$-sheet with the rest of the peptide, resulting in high thermal and pH stability. Some sequences have an additional fifth bridge, which can adopt several configurations (Van den Bergh et al. 2002; Odintsova et al. 2009). A cluster of hydrophobic residues forms the chitin-binding site, while the opposite face of the peptide is enriched in charged residues (Xiang et al. 2004). Short hevein-like peptides are missing the last strand of the $\beta$-sheet and have only three disulfide bridges (Martins et al. 1996). Precursor proteins include a signal peptide and a C-terminal pro-peptide of variable length, and they can sometimes contain more than one hevein-like peptide (Van Damme et al. 1999; Egorov and Odintsova 2012).

Although hevein-like peptides bind to chitin with high affinity, it is not clear what role this plays in their mechanism of action. Several peptides have shown activity against fungi with no chitin in their walls or against gram-positive bacteria (Koo et al. 1998; Van den Bergh et al. 2002; Huang et al. 2002; Odintsova et al. 2009). A hevein-like peptide from morning glory was shown to cause actin depolimerization resulting in burst hyphae (Koo et al. 2004). Analysis of resistant mutants suggests that the target of this peptide might be a membrane glycoprotein and that it activates the cell wall integrity pathway.

Constitutive expression of a hevein-like peptide from morning glory enhanced resistance of tobacco and tomato against different fungal species (Choon Koo et al. 2002; Lee et al. 2003). Similarly expression of hevein-like peptides from *Stellaria media* increased resistance against phytopathogenic fungi in Arabidopsis, tomato and tobacco (Khaliluev et al. 2011; Shukurov et al. 2012). A possible obstacle to

the biotechnological application of these peptides is that hevein is a major latex allergen (Chen et al. 1997).

### 4.2.5 Knottins and Cyclotides

Knottin is a generic term for small proteins with a particular type of cysteine knot motif known as inhibitor cysteine knot (Isaacs 1995). Cysteine knots require two disulfide bridges that form a circular structure with the protein backbone. The knot is formed when a third disulfide bridge crosses this circular structure. In the case of inhibitor cysteine knots, this third bridge is the one formed by cysteines III and VI (Fig. 4.1e,f). The knottin structural family encompasses a number of distinct protein families with apparently unrelated sequences. Knottins can be found in plants, animal, and fungi and they have toxic, inhibitory, and regulatory functions. The KNOTTIN database is available online and has knottin sequences and structures from different families (http://knottin.cbs.cnrs.fr/).

Two families of plant peptides with an inhibitor cysteine knot have been found to possess antimicrobial activity. The first family is simply referred to as antimicrobial knottins and was discovered when two closely related peptides were purified from *Mirabilis jalapa* seeds (Cammue et al. 1992). These peptides showed in vitro activity against a diverse range of phytopathogenic fungi and gram-positive bacteria, but not against gram-negative bacteria or human cells. Another antimicrobial peptide with a related sequence was purified by two different groups from seeds of *Phytolacca americana*, a species from the same order as *M. jalapa* (Shao et al. 1999; Liu et al. 2000). This peptide also showed activity against fungi and gram-positive bacteria, but not against gram-negative bacteria. The KNOTTIN database indicates that similar sequences exist in other species such as poplar, but it is currently unknown if they also have antimicrobial activity.

The characterized antimicrobial plant knottins are 36–38 amino acids long and have high temperature and pH stability as a result of the cysteine knot. The three-dimensional structure of the *P. americana* peptide was determined by NMR (Gao et al. 2001). It has an antiparallel $\beta$-sheet formed by three strands (Fig. 4.1e). This peptide is an amphiphile with a hydrophobic surface next to a patch of hydrophilic residues that includes positive charges. The precursor proteins of knottins include a signal peptide and are expressed specifically in seeds (Bolle et al. 1995; Liu et al. 2000).

There is little information on the mechanism of action of these peptides beyond the fact that the *P. americana* knottin binds to the sphingolipids of fungal membranes and its antifungal activity is critically dependent on the presence of the hydrophobic patch (Peng et al. 2005). As for biotechnological applications, one of the *M. jalapa* peptides increased resistance to the fungus *Alternaria solani* when introduced in tomato (Schaefer et al. 2005).

The cyclotides form the second family of antimicrobial peptides with an inhibitor cysteine knot. They are approximately 30 amino acids long and most are

cyclic peptides, with the N-terminal and C-terminal amino acids connected by a peptide bond (Fig. 4.1f). This increases their stability and makes them resistant to exoproteases (Craik 2009; Pinto et al. 2012). Cyclotides seem to be widespread in the violet (*Violaceae*) family and are common in the coffee (*Rubiaceae*) and dogbane (*Apocynaceae*) families, with individual species producing 10–100 different peptides (Gruber et al. 2008). They have also been found in isolated species of the potato (*Solanaceae*) and pea (*Fabaceae*) families, although genomic analyses suggest that they are not common (Poth et al. 2012). Sequences related to cyclotides are present in the grass (*Poaceae*) family but the peptides appear to be exclusively linear (Nguyen et al. 2012). Linear cyclotides, also called acyclotides or uncyclotides, are rare in species from other families, where they are always found together with the more abundant circular ones.

Cyclotides were discovered in a medicinal herb used in DR Congo to accelerate delivery and were later found to be toxic to a wide range of organisms (Craik 2009). Four cyclotides from different species showed moderate antifungal activity, but strong antibacterial activity against a range of gram-positive and gram-negative human pathogens (Tam et al. 1999). In another study, a cyclotide from violet was found to have strong activity against gram-negative human pathogens, but not gram-positive *Staphylococcus* species (Pränting et al. 2010). Finally, a linear grass cyclotide showed activity against *Escherichia coli* (Nguyen et al. 2012). All these cyclotides were also found to be cytotoxic to human cells. The function of cyclotides in plants is unclear. Since they accumulate at high concentration, a defensive function is possible and insects have been suggested as their main target (Craik 2009). There are no reports of antimicrobial activity against plant pathogens, but their antibacterial activity does not appear to be highly specific.

Cyclotides are synthesized in ribosomes as part of larger pre-pro-proteins (Pinto et al. 2012). In addition to a signal peptide, they have N and C-terminal domains that are removed during maturation. Precursor proteins can contain up to three cyclotides separated by intermediate sequences. Cyclization appears to be catalyzed by an asparaginyl endopeptidase that cuts the C terminus of the mature peptide (always Asn or Asp) and transfers it to the N-terminal amino acid in a transpeptidation reaction (Craik 2009). This process occurs on the vacuole where cyclotides are stored (Conlan et al. 2011). The main mechanism of action of cyclotides seems to involve the disruption of lipid membranes (Henriques et al. 2011). These peptides appear to bind specifically to phosphoethanolamine creating pores when they become inserted into membranes (Wang et al. 2012).

### *4.2.6 β-Barrelins*

The $\beta$-barrelin or MiAMP1 family is present in gymnosperms, angiosperms, and spikemosses, although they seem to be missing in several species with sequenced genomes (Manners 2009). It was discovered when a peptide purified from macadamia nuts, called MiAMP1, showed antimicrobial activity against several fungi

and a gram-positive bacteria, but not gram-negative bacteria or mammalian cells (Marcus et al. 1997). In vitro antifungal activity against several phytopathogens, as well as yeast, has also been demonstrated for homologous peptides from three different pine species (Sooriyaarachchi et al. 2011; Canales et al. 2011; Zamany et al. 2011). The $\beta$-barrelin precursor proteins have a signal peptide and in gymnosperms expression is induced by fungal attack, supporting an in vivo defensive role (Manners 2009; Sooriyaarachchi et al. 2011).

The family has been named $\beta$-barrelins due to the three-dimensional structure of MiAMP1 (Fig. 4.1g). This protein, which is 76 amino acids long, forms a barrel of eight $\beta$-strands that includes three disulfide bonds (McManus et al. 1999). Sequence conservation is high in the family and basic amino acids are abundant.

The mechanism of action of these peptides against fungi appears to involve binding specifically to $\beta$-(1,3)-glucans in fungal cell walls through conserved polar and aromatic residues (Sooriyaarachchi et al. 2011). The three-dimensional structure of $\beta$-barrelins is very similar to that of the fungal killer toxin HM-1, which interacts with $\beta$-(1,3)-glucans and blocks their synthesis (Kasahara et al. 1994; McManus et al. 1999). Regarding biotechnological applications, expression in canola of $\beta$-barrelins from macadamia and pine resulted in increased resistance to fungal pathogens (Kazan et al. 2002; Verma et al. 2012).

### *4.2.7 Other Peptides*

An antibacterial peptide from maize kernels, called MBP-1, showed activity against several fungi and both gram-positive and gram-negative bacteria (Duvick et al. 1992). Closely related peptides were purified from seeds of two other grass species and antifungical activity was demonstrated for one of them (Egorov et al. 2005; Nolde et al. 2011). These peptides have approximately 35 amino acids and their three-dimensional structure consists of two $\alpha$-helixes that form a hairpin stabilized by two disulfide bridges (Nolde et al. 2011). A similar cysteine pattern, but with low sequence similarity, was found in several macadamia nut peptides with activity against diverse fungi and a gram-positive bacterial phytopathogen (Marcus et al. 1999). The macadamia peptides are all produced from a large pre-pro-protein precursor that belongs to the vicilin family of seed storage proteins. Vicilins from other species include similar sequences which could be released as antimicrobial peptides. The names $\alpha$-helical hairpin and 4-cys peptides have been proposed for this family, which also includes peptides that inhibit trypsin or ribosomes (Egorov and Odintsova 2012). The peptide from barnyard grass is internalized in fungal spores and hyphae suggesting an intracellular target, but it does not seem to disrupt the cell membrane (Nolde et al. 2011).

Other antimicrobial peptides have been identified in a single species. The seeds of *Impatiens balsamina*, for example, accumulate four antimicrobial peptides that are produced from the same pre-pro-protein (Tailor et al. 1997). They were shown to be highly effective against a range of phytopathogenic fungi and gram-positive

bacteria. The mature peptides have only 20 amino acids, are highly basic and form a loop stabilized by two disulfide bridges that contains a hydrophobic patch (Patel et al. 1998). The cysteine pattern is clearly different from that of the 4-cys peptides discussed above and no homologs have been identified in other species. A recent study that evaluated the potential use of one of these peptides to control foodborne pathogens found it promising, although toxicity against some types of human cells was significant (Wu et al. 2013).

Two peptides of about 7 kD were purified from potato tubers on the basis of their ability to inhibit bacterial growth (Segura et al. 1999; Berrocal-Lobo et al. 2002). They belong to the snakin/GASA family of small proteins, which is widely distributed in angiosperms and appears to be involved in the regulation of plant development and stress responses (Nahirñak et al. 2012). The potato sequences are highly divergent, suggesting a possible change of function. The mature peptides are derived from the C-terminal region of the precursor protein, have a positive charge and include 12 conserved cysteines. They showed antifungal and antibacterial activity in vitro and overexpression of the corresponding genes resulted in transgenic plants with enhanced resistance to fungal and bacterial pathogens (Almasia et al. 2008; Balaji and Smart 2012).

## 4.3 Alkaloids

Alkaloid can be used either as a generic term for all nitrogen containing specialized metabolites or just for those derived from amino acids. In both definitions, alkaloids are a highly diverse group of compounds that that do not share a common biosynthetic pathway. The best understood classes of plant antimicrobial alkaloids are benzoxazinoids, mostly found in grasses, and two groups of sulfur-containing alkaloids that are found in many species of the mustard (*Brassicaceae*) family, also known as crucifers (Niemeyer 2009; Bednarek 2012). The two groups of sulfur-containing alkaloids are glucosinolates and indole-type compounds, of which camalexin is the best known.

Both benzoxazinoids and camalexin-type compounds are derived from tryptophan or its precursors. Glucosinolates can be produced from a number of different amino acids. Benzoxazinoids and glucosinolates are produced in inactive form, conjugated with glucose, and only become toxic when this sugar is removed. Camalexin and glucosinolates have been extensively studied because they are both produced by *Arabidopsis thaliana*, the most popular model species for plant molecular biology. Both types of compounds most likely acquire their sulfur from the tripeptide glutathione and also share some other biosynthetic steps (Bednarek 2012). They act synergistically against fungal attacks with constitutively produced glucosinolates offering a first line of defense, while induction of camalexin biosynthesis at later stages creates a second barrier against infection (Schlaeppi et al. 2010).

### 4.3.1 Benzoxazinoids

Benzoxazinoids are a small group of glucosylated derivatives of indole-3-glycerol phosphate, a precursor of tryptophan (Fig. 4.2). They are found in the grass (*Poaceae*) family and in isolated dicotyledonous species. This distribution seems to be the result of convergent evolution (Frey et al. 2009; Dick et al. 2012).

The entire synthesis pathway of benzoxazinoids has been established in maize, where nine genes code for the enzymes that participate in the process, including two partially redundant glucosyltransferases (Frey et al. 2009). The synthesis pathway starts in the plastid, continues in the microsomes, and ends in the cytosol, where active benzoxazinoids are glucosylated to avoid autotoxicity before they are stored in the vacuole.

In vitro antifungal and antibacterial activity against plant and human pathogens has been reported for benzoxazinoids and their decomposition products, such as benzoxazolinones (Bravo et al. 1997; Glenn et al. 2001; Maresh et al. 2006; Rostás 2007). Other plant fungal pathogens can tolerate these compounds only because they have developed the ability to metabolize them (Glenn and Bacon 2009). The best evidence for an in vivo function in plant antimicrobial defense is a maize mutant unable to produce benzoxazinoids that showed increased susceptibility to a fungal pathogen (Ahmad et al. 2011). However, this mutant also showed altered defense responses suggesting that benzoxazinoids could have an additional regulatory function. Further support for an antifungal function comes from several studies that have found significant correlations between benzoxazinoid content and resistance to fungal pathogens (Niemeyer 2009). In addition benzoxazinoids seem to have an important role in defense against insects and neighboring plants (Rostás 2007; Niemeyer 2009; Ahmad et al. 2011).

Benzoxazinoids stored in the vacuole can be activated by plastid glucosidases upon tissue disruption, but in root exudates or in case of fungal attack benzoxazinoids appear to be exported directly to the apoplast, where they are activated by unknown glucosidases (Niemeyer 2009; Ahmad et al. 2011). Removal of the glucose generates an unstable compound with a highly electrophilic carbon that can easily react with nucleophiles, such as thiol groups of proteins, forming stable conjugates (Niemeyer 2009; Dixon et al. 2012). Benzoxazinoids can also decompose to toxic benzoxazolinones, whose mechanism of action is less clear. In the case of the plant pathogen *Agrobacterium tumefaciens* benzoxazinoids do not affect viability but appear to target instead the host recognition system (Maresh et al. 2006).

### 4.3.2 Camalexin and Related Indole-Type Metabolites

Camalexin is a sulfur-containing derivative of tryptophan produced by *Arabidopsis thaliana* (Fig. 4.2). It forms part of a group of related phytochemicals produced by species in the *Brassicaceae* family. More than 44 different compounds are known,

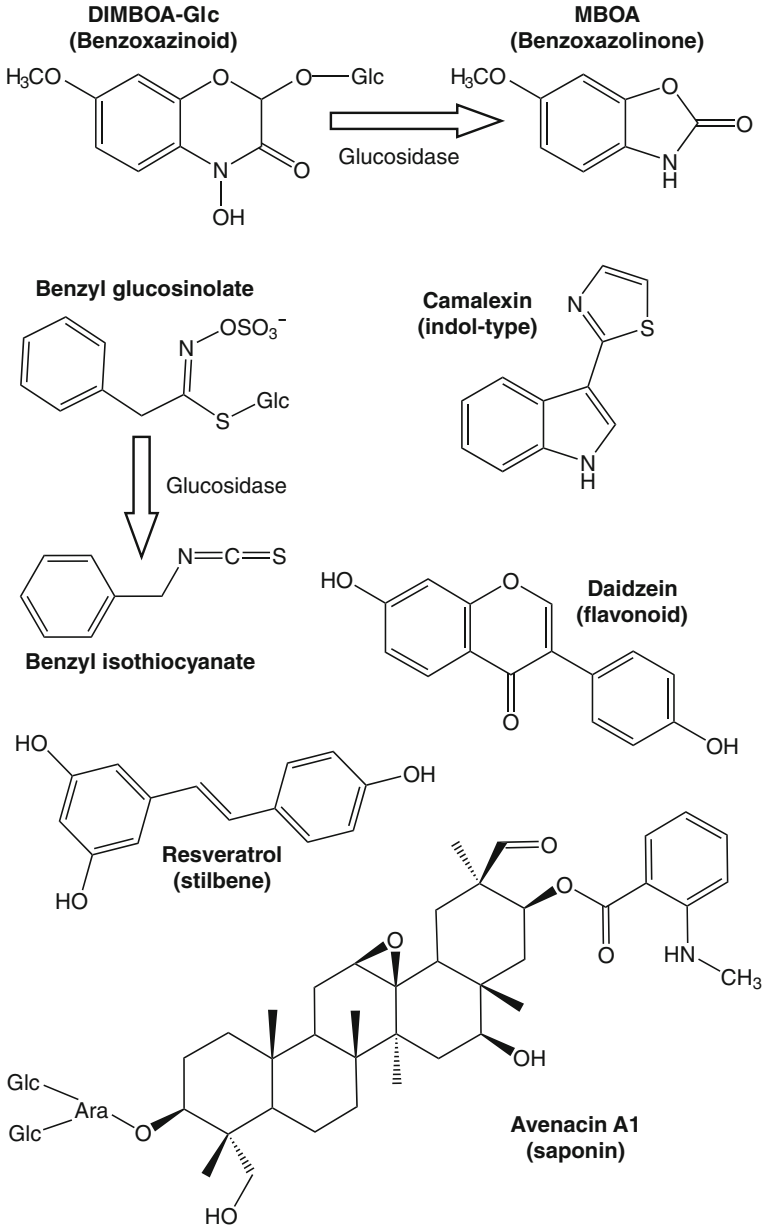



**Fig. 4.2** Structure of representative plant antimicrobial metabolites from the main groups discussed in the text

although many others are likely to exist, since only a minority of species in the family have been analyzed (Pedras et al. 2011).

The synthesis of camalexin and related compounds is quite well understood (Pedras et al. 2011; Bednarek 2012; Ahuja et al. 2012). Thanks to the availability of Arabidopsis mutants, most of the genes involved in the seven-step camalexin pathway have been identified, as well as some of the transcription factors that regulate the process. A glutathione S-transferase is most likely responsible for the incorporation of sulfur and most of the remaining steps are catalyzed by cytochrome P450 enzymes.

There is evidence of in vitro antimicrobial activity for 44 of these indole-type metabolites, mainly against fungi but in some cases against gram-positive and gram-negative bacteria too (Pedras et al. 2011). Production of these compounds, which are also toxic to plant cells, is only activated in case of stress, including bacterial and fungal infections (Pedras et al. 2011; Ahuja et al. 2012). In vivo antimicrobial activity has only been demonstrated for camalexin in Arabidopsis, with enhanced susceptibility to fungal pathogens in mutants unable to produce this compound (Schlaeppi et al. 2010; Stotz et al. 2011). Camalexin is a lipophilic molecule and its mechanism of action, while not well understood, appears to involve membrane disruption in both fungi and bacteria (Rogers et al. 1996; Joubert et al. 2011; Ahuja et al. 2012). In fungi camalexin treatment activates cell wall integrity and high osmolarity pathways that contribute to pathogen virulence.

### *4.3.3 Glucosinolates*

Glucosinolates can derived from six to nine different amino acids (Ala, Val/Leu, Ile, Met, Phe/Tyr, Trp, and possibly Glu) and include a sulfur atom linked to the anomeric carbon of a $\beta$-glucose residue (Fig. 4.2). More than 100 different glucosinolates have been identified in the *Brassicaceae* and closely related families (Agerbirk and Olsen 2012).

The synthesis of glucosinolates shares some steps with the synthesis of camalexin and related compounds (Sønderby et al. 2010; Bednarek 2012). Glutathione is again the most likely donor of the first sulfur. The rest of the tripeptide is removed and the remaining thiol group is then glucosylated. A sulfate group is later added from 3′-phosphoadenosine 5′-phosphosulfate.

The glucose residue can be removed by thioglucosidases called myrosinases (Halkier and Gershenzon 2006; Bednarek 2012). Some myrosinases are stored in specialized cells and become mixed with glucosinolates when the tissue is damaged by herbivores, but others are produced in cells under pathogen attack. The resulting aglycone is unstable and it rapidly decomposes. The main direct products are isothiocyanates (Fig. 4.2), which are responsible for the bitter and pungent flavors of crucifers like cabbage, mustard or wasabi. Isothiocyanates are toxic in vitro to a wide variety of organisms, including fungi and bacteria (Aires et al. 2009; Schlaeppi et al. 2010; Shin et al. 2010; Wu et al. 2011; Stotz et al. 2011).

An in vivo role for glucosinolates as antimicrobial defense compounds is now well established in Arabidopsis. Mutants deficient in glucosinolate synthesis show

enhanced susceptibility to various fungal pathogens (Schlaeppi et al. 2010; Stotz et al. 2011). In addition to producing toxic compounds, glucosinolate hydrolysis results in molecules that appear to have a regulatory role in plant defense responses (Bednarek 2012). The mechanism of action of isothiocyanates involves the central carbon of the functional group, which is a reactive electrophile that can readily form stable covalent bonds with nucleophilic atoms in thiol and amine groups of proteins and peptides (Brown and Hampton 2011).

There has been interest in the possibility of using isothiocyanates as natural preservatives, to prevent bacterial and fungal growth on diverse food products, from chicken meat to apples (Shin et al. 2010; Wu et al. 2011; Wilson et al. 2013). While they are generally considered safe for human consumption, the strong odor and flavor of these chemical can be unpleasant. The most promising approach is to apply them as a vapor, as they are volatile and can be effective at low concentrations.

The biosynthetic pathway of glucosinolates is quite well understood and dozens of genes have been identified in Arabidopsis, opening the way for plant genetic engineering (Sønderby et al. 2010). The introduction in Arabidopsis of different cytochrome P450 genes, responsible for the first committed step, resulted in the production of two different types of glucosinolates and led to increased resistance to specific bacterial pathogens (Brader et al. 2006). While this approach would work in crucifer crops, engineering glucosinolate production in species from other families is a more complex task. The simultaneous expression of six heterologous genes was sufficient to obtain stable tobacco transformants capable of synthesizing benzylglucosinolate from phenylalanine (Møldrup et al. 2012). Production of a the glucosinolate glucoraphanin, on the other hand, was achieved by transient expression of 13 different genes in tobacco leafs (Mikkelsen et al. 2010). Recently the synthesis of glucosinolates in yeast was achieved by introducing a seven-step pathway (Mikkelsen et al. 2012).

## 4.4 Phenylpropanoids

Phenylpropanoids, also referred to as phenolics or polyphenols, are compounds with aromatic rings derived from phenylalanine, and to a lesser extent tyrosine, by the loss of their amine group (Vogt 2010). This pathway is responsible for the formation of lignin monomers as well as a wide variety of other specialized metabolites, some of which have antimicrobial activities (Daglia 2012). Flavonoids and stilbenes are the two groups of phenylpropanoids where evidence for an in vivo role in plant defense is clearest. It is also likely that a tobacco coumarin with in vitro antimicrobial activity plays an important role in resistance to bacterial and fungal pathogens (El Oirdi et al. 2010; Großkinsky et al. 2011). Flavonoid, stilbenes and coumarins are all derived by different routes from p-coumaroyl-CoA, a key branching point in the phenylpropanoid pathway.

### 4.4.1 Flavonoids

The flavonoids are generally tricyclic compounds with two aromatic rings (Fig. 4.2). They are a very large group of specialized metabolites with more than 6,000 known molecules (Falcone Ferreyra et al. 2012). They are present in all land plants and have a diversity of functions, including providing color to flowers, fruits and seeds.

The synthesis of flavonoids starts with one molecule of p-coumaroyl-CoA and three of malonyl-CoA. The enzyme chalcone synthase catalyzes a series of decarboxylation, condensation, and cyclization reactions that produce naringenin chalcone, a C6-C3-C6 compound with two aromatic rings connected by a chain of three carbons (Winkel-Shirley 2001). With the collaboration of chalcone reductase, chalcone synthase can produce isoliquiritigenin, also known as trihydroxychalcone, which has one less OH group. Chalcone isomerase can then act on both products to form the tricyclic molecules naringenin and liquiritigenin, precursors to most flavonoids. Glycosylation is common in flavonoids, usually with one or two sugar residues.

A large number of flavonoid compounds have shown in vitro antifungal and antibacterial activity, sometimes at very low concentrations, and against a wide range of species that includes plant and human pathogens (Rivera-Vargas et al. 1993; Cushnie and Lamb 2011; Pistelli and Giorgi 2012). The clearest evidence for an in vivo role in antimicrobial plant defense comes from studies of isoflavonoids or its pterocarpan derivatives in the pea (*Fabaceae*) family. The synthesis of these compounds starts with isoflavone synthase acting on naringenin or liquiritigenin to change the position of the aryl group (Du et al. 2010). Isoflavonoid synthesis is induced upon fungal infection in many *Fabaceae* species (Ahuja et al. 2012). Overexpression of an isoflavone biosynthetic enzyme in alfalfa resulted in increased accumulation of these compounds and enhanced resistance to a fungal pathogen (He and Dixon 2000). On the other hand silencing of another biosynthetic enzyme in pea led to increased susceptibility to a different fungal pathogen (Wu and VanEtten 2004). The same result was obtained when isoflavone synthase or chalcone reductase were silenced in soybean (Subramanian et al. 2005; Graham et al. 2007). Silencing of another enzyme required for isoflavone synthesis resulted in increased susceptibility of soybean to the bacterial pathogen *Pseudomonas syringae*, (Zhou et al. 2011). *P. syringae*, unlike fungal pathogens, was not inhibited by isoflavones in vitro, suggesting that isoflavones could also have a role in defense signaling (Rivera-Vargas et al. 1993; Zhou et al. 2011). In particular soy isoflavonoids appear to activate programmed cell death around the infected area (Graham et al. 2007).

The antimicrobial mechanism of action of flavonoids is currently unclear. Various hypotheses have been proposed for their antibacterial activity, including membrane alterations, inhibition of DNA modifying enzymes and interference with energy metabolism. However, a recent review concluded that many published studies, in addition to presenting methodological problems, cannot discriminate

between causes and consequences (Cushnie and Lamb 2011). A proposed mechanism that could that could explain toxicity of flavonoids to both eukaryotes and prokaryotes is direct inhibition of ATP synthase, as flavonoids can bind the $\gamma$-subunit and block its rotation (Gledhill et al. 2007; Dadi et al. 2009). Several studies also indicate that flavonoids could reduce the pathogenicity of bacteria by interfering with quorum-sensing mechanisms (Vandeputte et al. 2011; Cushnie and Lamb 2011).

There has been several successful attempts to manipulate flavonoid biosynthesis in plants, introducing for example isoflavone synthase in species that do not normally produce isoflavonoids (Du et al. 2010). However these studies have been centered on the possible health benefits of dietary isoflavonoids as phytoestrogens, instead of their antimicrobial properties. There have also being significant advances in the production of flavonoids, including isoflavonoids, in bacterial and yeast heterologous systems (Du et al. 2010; Falcone Ferreyra et al. 2012). Finally a number of flavonoids have been considered for clinical use against human pathogens such as antibiotic-resistant bacteria (Liu et al. 2009; Cushnie and Lamb 2011; Pistelli and Giorgi 2012).

### *4.4.2 Stilbenes*

Stilbenes have two aromatic rings connected by a two-carbon bridge (Fig. 4.2). More than 400 stilbenes have been described and they can appear as monomeric or oligomeric structures (Shen et al. 2009). They have been found in several unrelated families of land plants, including mosses, ferns, gymnosperms and angiosperms, in what appears to be a case of convergent evolution (Tropf et al. 1994; Riviere et al. 2012). Among economically important plants stilbenes have been found in grapevine, pines, peanut and sorghum (Chong et al. 2009).

Stilbene synthases are the enzymes responsible for the first committed synthesis step. These enzymes have independently evolved several times from chalcone synthases. They produce a C6-C2-C6 skeleton instead of the C6-C3-C6 skeleton of flavonoids (Chong et al. 2009). Like chalcone synthases, stilbene synthases can use three molecules of malonyl-CoA and one of p-coumaroyl-CoA as substrates, resulting in the production of resveratrol. Some stilbene synthases can also utilize cinnamoyl-CoA instead of p-coumaroyl-CoA to produce pinosylvin. Similarly to flavonoids, stilbenes are frequently glycosylated.

There are numerous reports of significant in vitro antifungal activity for different stilbenes (Jeandet et al. 2002; Chong et al. 2009). Antibacterial activity against gram-positive and gram-negative species has also been reported (Jeandet et al. 2012; Plumed-Ferrer et al. 2013). Some stilbenes are cytotoxic to human cells and have antitumoral activities (Jane Lunt et al. 2011; Jeandet et al. 2012). Regarding their physiological role in vivo, resveratrol and its derivatives are produced at higher levels during fungal infections in grapevine, while in conifers pinosylvin derivatives accumulate in the heartwood constitutively, probably

protecting it from decay (Jeandet et al. 2002; Chong et al. 2009). The best evidence for the effectiveness of stilbenes in vivo is the enhanced resistance of different species transformed with stilbene synthase and the linkage between fungal pathogenicity and the capacity to metabolize stilbenes (Chong et al. 2009; Jeandet et al. 2012). As for the mechanism of action of stilbenes, one possibility, as in the case of flavonoids, is inhibition of ATP synthase by direct binding (Gledhill et al. 2007; Dadi et al. 2009).

The first demonstration of enhanced disease resistance in a transgenic plant producing a new antimicrobial compound was achieved by introducing stilbene synthase in tobacco (Hain et al. 1993). The transgenic tobacco produced the stilbene resveratrol and showed increase resistance to a fungal pathogen. The same experiment has been repeated in numerous other species including rice, barley, wheat, apple, papaya, banana, tomato, lettuce and pea (Jeandet et al. 2012). In many cases increased resistance against fungal pathogens was observed. Tobacco plants producing pterostilbene, a derivative of resveratrol with higher antifungal activity, were obtained by introducing an *O*-methyltransferase in addition to stilbene synthase (Rimando et al. 2012). Synthesis of resveratrol from amino acids phenylalanine and tyrosine has been achieved in yeast (Jeandet et al. 2012). It is also possible to produce resveratrol in *E. coli*, but the culture needs to be fed p-coumaric acid (Lim et al. 2011).

## 4.5 Terpenoids

Terpenoids, also called isoprenoids, are the most diverse class of specialized metabolites, but they also include core metabolites, such as sterol lipids. They are derived from the five carbon isomers isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAP). These compounds are produced in the cytosol and chloroplast by different pathways. Addition of one to three IPP subunits to DMAP produces skeletons of 10 (geranyl diphosphate), 15 (farnesyl diphosphate) or 20 carbons (geranylgeranyl diphosphate) that are further modified to produce the huge diversity of plant terpenoids (Vranová et al. 2012).

Terpenoids are usually classified according to the number of IPP or DMAP units used in their synthesis. Sesquiterpenes (C15 or three subunits) appear to have a role as antimicrobial metabolites in species from the potato (*Solanaceae*) family. In addition to antimicrobial activity in vitro, transgenic tobacco and cotton that produced higher amounts of sesquiterpenes, due to altered pathogen response pathways, show enhanced resistance against fungal pathogens (Parkhi et al. 2010; Großkinsky et al. 2011). A novel family of sesquiterpenes induced by fungal infection and with antifungal activity in vitro has also being discovered in maize (Huffaker et al. 2011). There is also evidence for a role of diterpenes (C20) as antifungal compounds in plants from the grass (*Poaceae*) family. Mutants or transgenic rice plants with altered defense pathways that have increased or

decreased levels of diterpenes shows correlated changes in fungal disease resistance (Mori et al. 2007; Kim et al. 2009). Direct proof of an in vivo role, however, is only available for a group of triterpenes (C30) called saponins.

### *4.5.1 Saponins*

Saponins are glycosylated triterpenoids whose name derives from their ability to form soap-like foams in solution due to the combination of hydrophilic and hydrophobic moieties (Fig. 4.2). Saponins appear to be widespread in angiosperms and are found in both monocots and dicots (Vincken et al. 2007).

The synthesis of saponins starts with two farnesyl diphosphate molecules that are linked and oxidized to produce oxidosqualene. The cyclization of this linear compound can be carried out by a number of oxidosqualene cyclases that generate different saponin precursors, which are further modified and glycosylated, usually with 2–5 monosaccharide units (Vincken et al. 2007; Sawai and Saito 2011; Augustin et al. 2011; Osbourn et al. 2011). The two main types of saponin are C27 steroidal saponins and C30 triterpenoid saponins, but the latter can be subdivided in 10 different groups according to their skeleton (Vincken et al. 2007). Although saponins accumulate constitutively in many species, saponin levels can also increase in response to fungal attacks (Augustin et al. 2011).

There are numerous reports of saponins with in vitro toxicity against diverse fungi including plant and human pathogens (Coleman et al. 2010; Saha et al. 2010). Antibacterial activity against gram-positive and gram-negative species has also being reported (Sung and Lee 2008). Many saponins are also hemolytic, but this property does not seem to correlate with antifungal activity (Augustin et al. 2011).

The best evidence for an in vivo defensive function for saponins comes from oat. This plant constitutively accumulates in roots fluorescent saponins called avenacins. This has made it possible to identify a number of saponin-deficient mutants that showed reduced resistance against fungal pathogens (Papadopoulou et al. 1999). Five of the genes directly involved in the synthesis of avenacins have already been identified, with mutants in four of them resulting in enhanced disease susceptibility (Mugford et al. 2013). In addition to active avenacins in the root, oat accumulates inactive forms in the leaves called avenacosides (Morant et al. 2008). The inactive saponins have an additional $\beta$-glucose residue at C26 that can be removed by a specific $\beta$-glucosidase.

The main mechanism of action of saponins seems to involve membrane disruption leading to an increase in permeability (Coleman et al. 2010; Augustin et al. 2011). It appears that the triterpenoid moiety of saponins binds sterol lipids in the membrane. The attached sugars can force the membrane to curve when the local saponin concentration is high. This could lead to disruption of lipid rafts or even pore formation. It is unclear how plants avoid these effects when they accumulate

active saponins, possibly in vacuoles, but differences is sterol concentration or composition might be responsible (Augustin et al. 2011).

Triterpenoid saponins have not been found in cereals other than oat. Engineering other species to synthesize these compounds could enhance their resistance against fungal pathogens. A first step has been taken with the introduction in rice of a $\beta$-amyrin synthase from oat, an oxidosqualene cyclase that catalyzes the first committed step in avenacin biosynthesis (Inagaki et al. 2011). There have also being some successes at engineering yeast for the production of saponin precursors (Sawai and Saito 2011). The achievement of this objective would first require the identification of all the steps in the pathway. Regarding clinical applications, saponins have been found to be effective against *Candida albicans* using a nematode model, showing no hemolytic activity at inhibitory concentrations (Coleman et al. 2010).

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# Chapter 5
# Advances in Beta-Lactam Antibiotics

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**Abstract** The $\beta$-lactam antibiotics continue providing health to the world population by virtue of industrial production and discoveries of new molecules with useful activities. Sales of these remarkable compounds, including penicillins, cephalosporins, cefoxitin, monobactams, clavulanic acid, and carbapenems, have reached over $20 billion dollars per year. Strain improvement of the penicillin-producing strains of *Penicillium chrysogenum* has been truly remarkable, with present strains producing about 100,000 times more penicillin than the original *Penicillium notatum* of Sir Alexander Fleming. The traditional strain improvement programs based on random mutation and screening in combination with recombinant DNA techniques allowed an impressive $\beta$-lactam yield enhancement at industrial scale. A remarkable amount of information has been gathered on the biosynthetic enzymes involved, the pathways of biosynthesis of $\beta$-lactams as well as their regulation, and the genomics and proteomics of the producing organisms. The rational metabolic engineering of $\beta$-lactam-producing microorganisms has resulted in productivity increments and the design of biosynthetic pathways giving rise to new antibiotics. A legal framework has been developed for the confined manipulation of genetically modified organisms (GMOs). Modern aspects of the processes are discussed in the present review including genetics, molecular biology, metabolic engineering, genomics, and proteomics.

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## 5.1 Introduction

The β-lactam antibiotics industrially produced (Demain and Elander 1999) includes: (1) penicillins, such as penicillin G and penicillin V, and its semisynthetic derivatives amoxicillin, ampicillin, cloxacillin, nafcillin, and piperacillin; (2) cephalosporin C and its semisynthetic products such as cephalothin, cephaloridine, cephalexin, and cefaclor; and (3) cephamycins such as cefoxitin. The semisynthetic penicillins are all made from 6-aminopenicillanic acid (6-APA) which is produced by removing the side chain of penicillin G or V with penicillin acylase. In addition, β-lactams include nonclassical structures such as (1) monobactams, including aztreonam; (2) clavulanic acid, which is combined with amoxicillin to produce the drug Augmentin®; and (3) thienamycin, a carbapenem which is chemically transformed into imipenem, a component of the combination drug primaxin.

Penicillin was the first β-lactam antibiotic discovered (Fleming 1929), and the importance of the β-lactams can be seen by the continuous increase in their commercial production. According to the data from Michael Barber and Associates (Elander 2003; Ozcengiz and Demain 2013), 26,400 tons of penicillin G and 9,980 tons of penicillin V were manufactured in 1995, representing a turnover of $1.06 billion. By 2000, production of penicillin had risen to more than 60,000 tons, of which 25,000 tons were bulk products (Thykaer and Nielsen 2003). Production of the intermediates (6-APA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA) amounted to 9,680 and 2,150 tons, respectively, and other intermediates amounted to 2,340 tons. Bulk production of cephalosporin C in 1999 was 4,700 tons and for the intermediate 7-aminocephalosporanic acid (7-ACA), 2,350 tons. Two thirds of semisynthetic cephalosporins are made from 7-ACA, production of which in 2002 amounted to 3,000 tons.

By 2003, the market of β-lactam antibiotics reached over $15 billion, which represented 65 % of the world antibiotic market (Elander 2003). Cephalosporins (there are more than 50 marketed) were at $10 billion, penicillins at $5 billion, and clavulanic acid over $1 billion. Clavulanic acid combined with amoxicillin (Augmentin®) reached $2 billion.

Industrial penicillin production has been carried out with *Penicillium chrysogenum* for over 70 years. Production was rather poor with *Penicillium notatum* (Fleming's strain), i.e., only 1.2 mg/L. *P. chrysogenum* NRRL-1951 was isolated in 1943 from a moldy cantaloupe showing improved penicillin production, i.e., 150 mg/L. Further improved strains of *P. chrysogenum*, include the Panlabs (Taiwan) strain P2, the DSM (The Netherlands) strain D504825, and the Antibióticos S.A. (Spain) strains AS-P-78 and E1 (Rodríguez-Sáiz et al. 2001; Rodríguez-Sáiz et al. 2005). Penicillin titers in industry in 1993 were as high as 100,000 units/ml = 60 g/L (Elander 2003). It has been estimated that recent industrial strains produce 100,000 times more penicillin than Fleming's original strain of *P. notatum* (Rokem et al. 2007).

Commercial cephalosporin C production by *Acremonium chrysogenum* (formerly *Cephalosporium acremonium*) is at least 30 g/L (Seidel et al. 2002). Cephalosporin C has only weak antibiotic activity. It has a minimal inhibitory concentration (MIC) of 25–100 μg/mL against Gram-positive bacteria and 12–25 μg/mL versus Gram-negative bacteria. The first generation of semisynthetic cephalosporins (cephalothin, cephaloridine, cephaloglycine, cephazoline, and cephaprin) are relatively resistant to penicillinase and have improved activity versus *Escherichia coli* and *Klebsiella pneumoniae*, but are inactive against *Pseudomonas* (Sonawane 2006). The second generation (including cefamandole, cefoxitin, cefuroxime, cefaclor, and cefadroxil) are more active against Gram-negative bacteria including *Proteus* spp. and *Enterobacter* spp. The third-generation compounds (including cefotaxime, cefoperazone, ceftazidime, ceftizoxime, cefsulodin, and ceftiaxon) have increased activity against a broad range of microbes.

The hydrophobic $\beta$-lactams, e.g., benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V), contain the nonpolar side chains called phenylacetate and phenoxyacetate, respectively, and are made only by filamentous fungi (e.g., *P. chrysogenum* and *Aspergillus nidulans*). The antibacterial spectrum of the hydrophobic penicillins is essentially Gram positive. The hydrophilic $\beta$-lactams include penicillin N, cephalosporins, and cephamycins (7-$\alpha$-methoxycephalosporins), which are made by filamentous fungi, actinomycetes, and unicellular bacteria. They all contain the polar side chain D-$\alpha$-aminoadipate (D-AAA).

The biosynthetic pathways for penicillins, cephalosporins, and cephamycins are shown in Fig. 5.1. All $\beta$-lactam pathways possess the first two steps in common, catalyzed by the enzymes $\delta$-(L-$\alpha$-aminoadipyl)-L-cysteinyl-D-valine (ACVS) and IPN synthase (IPNS), respectively, and all cephalosporin pathways go through deacetylcephalosporin C. A great number of modified cephalosporins including cephamycins are produced by unicellular and filamentous bacteria. They are usually altered by attachment of additional groups to the C3 acetoxy side chain; these include di-, tri-, and tetrapeptides and some have formylamino (-NH-CHO) rather than H or methoxy at C7. They include "cephabacins" produced by *Xanthomonas lactamgena* and *Lysobacter lactamgenus* (Sohn et al. 2001), "chitinovorins" by species of *Flavobacterium*, and 7-formamido-cephalosporins and "oganomycins" by *Streptomyces oganonensis*. Some are resistant to and/or inhibit $\beta$-lactamases. Other $\beta$-lactams, such as clavams, carbapenems, nocardicins, and monobactams, are biosynthesized by different pathways and are exclusively the products of prokaryotic unicellular bacteria and actinomycetes.

## 5.2 The Biosynthetic Pathway of Penicillin G

The biosynthetic pathways of the main $\beta$-lactam families (penicillins, cephalosporins, and cephamycins) are common in their first steps (Fig. 5.1), i.e., those catalyzed by the enzymes ACVS and IPNS, encoded by the *pcbAB* and *pcbC* genes,

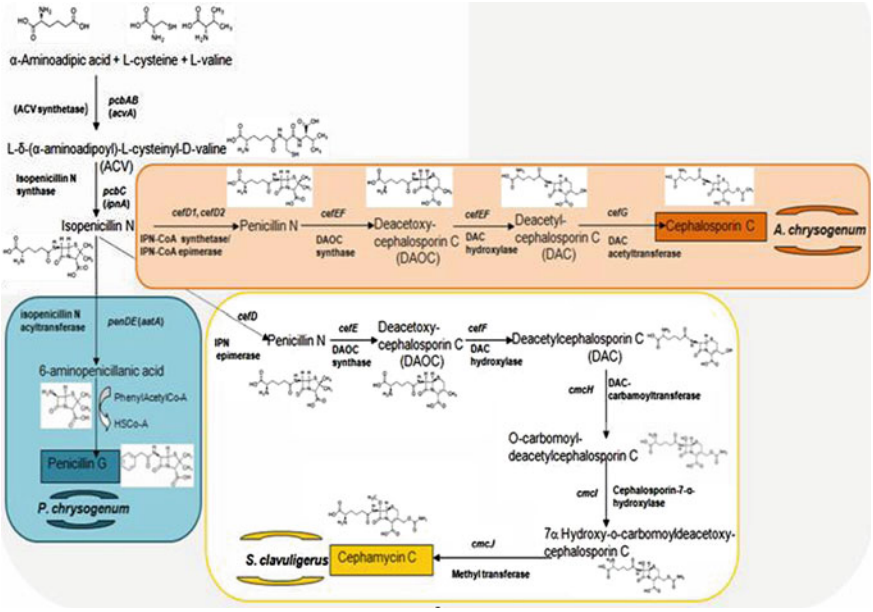


**Fig. 5.1** Biosynthetic pathways of penicillin G, cephalosporin C, and cephamycin C by *Penicillium chrysogenum*, *Acremonium chrysogenum,* and *Streptomyces clavuligerus*, respectively (from Ozcengiz and Demain 2013 with permission)

respectively. ACVS catalyzes the condensation of L-cysteine (L-Cys) and L-α-aminoadipic acid (L-Aaa) to form the intermediate L-α-aminoadipyl-L-cysteine (AC). Then, L-valine (L-Val) is epimerized to the D-form during activation and addition to form the LLD-tripeptide, i.e., δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). ACV is subsequently converted to the hydrophilic isopenicillin N (IPN) by IPNS. *P. chrysogenum* and other penicillin-producing fungi exchange the α-aminoadipyl side chain of IPN with aromatic acids such as phenylacetic or phenoxyacetic acids as catalyzed by IPN acyltransferase activity (IAT), encoded by the *penDE* gene (Barredo et al. 1989a), resulting in the biosynthesis of penicillin G or V, respectively (Fig. 5.1). This IAT activity is absent in *A. chrysogenum* and other cephalosporin producers because they lack a gene homologous to *penDE*.

### 5.2.1 *The δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine Synthetase (ACVS) Encoded by the* pcbAB *Gene*

The start of the common pathway in fungi is the condensation of L-Cys and L-Aaa to form the intermediate AC. In bacterial producers of β-lactam antibiotics such as cephamycins, L-Aaa is usually produced from lysine. The conversion occurs by

the action of lysine-6-aminotransferase (LAT) and piperideine-6-carboxylate dehydrogenase (PCD). In *Streptomyces clavuligerus*, both genes *lat* and *pcd* are within the cephamycin gene cluster. Unexpectedly, elimination of *pcd* did not eliminate production of cephamycin (Alexander et al. 2007). 30–70 % of wild-type levels of cephamycin were produced by such mutants whereas *lat* mutants completely lost production ability. Addition of L-Aaa to the *pcd* mutants restored production to wild-type levels. The same occurred when an intact copy of *pcd* was added to the mutant by complementation. L-Val is epimerized to the D-form during activation and addition to form ACV. ACVS is the enzyme that catalyzes this step and it is encoded by the *pcbAB* gene. ACV is the key intermediate in the formation of all penicillins and cephalosporins by eukaryotic and prokaryotic microorganisms. The motifs in the C-terminal region of ACVS, which are essential for valine epimerization and processivity of ACV formation, have been recently described (Wu et al. 2012).

### 5.2.2 *The IPN synthase (IPNS, "Cyclase") Encoded by the* pcbC *Gene*

ACV is converted to the hydrophilic IPN by the next enzyme in the pathway, i.e., IPNS. The conversion of the reduced form of ACV to IPN by this cyclase has been demonstrated in virtually all producers of penicillins and cephalosporins. In media lacking the side-chain precursors, *P. chrysogenum* produces IPN, which contains L-Aaa as its side chain. Although IPN is found in both mycelia and broth filtrates in precursor-free fermentations, its usual location is intracellular where it predominates over hydrophobic penicillins. IPNS is soluble and is stimulated by $Fe^{2+}$ and reducing agents such as ascorbate and dithiothreitol (DTT). It is a unique enzyme reaction with no precedent in biochemistry. Its mechanism appears to involve the formation of the $\beta$-lactam ring followed by the closure of the thiazolidine ring. No free intermediates are apparent. The ACV sulfur atom binds to the active site iron of the enzyme. The enzyme catalyzes the transfer of four hydrogen atoms from ACV to dioxygen. Via subcellular fractionation and immunoelectron microscopy, IPNS was shown to be localized in the cytosol (van der Lende et al. 2002).

### 5.2.3 *The Isopenicillin Acyltransferase (Acylcoenzyme A IPN Acyltransferase; IAT) Encoded by the* penDE *Gene*

*P. chrysogenum* produces penicillin G (=benzylpenicillin) when phenylacetate is fed to the fermentation medium. On the other hand, penicillin V (=phenoxymethylpenicillin) is only produced when phenoxyacetate is added. Many different

penicillins can be made by feeding diverse side-chain acids to the fermentation medium, but some others cannot be made at all by fermentation, presumably because their side-chain acids cannot be taken up by the mycelia or cannot be activated once inside the mycelia. These penicillins are manufactured by chemical reactions which attach the side chain to the 6-APA nucleus in processes grouped under the term "semisynthesis." 6-APA is an intermediate in the conversion of IPN to penicillin G. Some of these penicillins can also be made by enzymatic synthesis.

Industrial strains show complete conversion of phenylacetate into benzylpenicillin whereas a historic strain such as *P. chrysogenum* Wisconsin 54-1255 incorporates only 17 % and metabolizes the remainder. Uptake of phenoxyacetate into cells of *P. chrysogenum* occurs by active carrier-mediated transport at low phenylacetate concentrations but mainly by simple diffusion when high concentrations of phenylacetic acid are used as in commercial penicillin production. Phenylacetyl-CoA and phenoxyacetyl-CoA arise from phenylacetate and phenoxyacetate, respectively, and coenzyme A by the action of an idiophasic side-chain activating enzyme. It does not appear to be a specific acyl-CoA ligase but rather the acetyl-CoA synthetase of *P. chrysogenum* and *A. nidulans*. Genetic analysis of penicillin production in old and newer strains of *P. chrysogenum* indicates that a limiting factor for further improvement is the level IAT (Nijland et al. 2010).

Phenylacetate can be undesirably degraded in *A. nidulans* via homogentisate which is broken down to fumarate and acetoacetate. The first step is a hydroxylation at C-2 followed by another hydroxylation at C-5 of the aromatic ring. Gene *phacA* encodes the first enzyme, a cytochrome P450 monoxygenase catalyzing formation of 2-hydroxyphenylacetate. Disruption of this gene eliminated the ability to use phenylacetate and increased penicillin production by three- to five-fold. One of the early mutations in the Wisconsin family of penicillin producers was the mutation of *P. chrysogenum* WIS48-701 to WIS49-133, giving a 100 % increase in penicillin titer and more efficient usage of phenylacetic acid. The mutation decreased destruction of phenylacetate by phenylacetate 2-hydroxylase encoded by *pahA* (Rodríguez-Sáiz et al. 2001). Expression of the gene was found to be inversely correlated with penicillin productivity in a series of strains.

The short hydrophobic branch which occurs in *Penicillium* and *Aspergillus* but not in *Acremonium* or prokaryotes is a simple side-chain exchange reaction catalyzed by IAT. The hydrophilic L-Aaa side chain in IPN is exchanged for a hydrophobic side-chain acid, e.g., phenylacetic acid present as its coenzyme A ester. IAT is intracellular, idiophasic, found in fungi producing hydrophobic penicillins but not in nonproducing species, present at higher levels in superior producers, and accepts those side chains which are present in the natural penicillins normally made in fermentations. The absence of IAT in producers of hydrophilic penicillins, such as *A. chrysogenum*, *Emericellopsis glabra,* and actinomycetes, is responsible for their inability to make penicillin G or any other penicillin with a hydrophobic side chain.

In the absence of a CoA ester of a functional hydrophobic acid, IPN is hydrolyzed to 6-APA by IAT. Since 6-APA accumulates in fermentation media

lacking an added side-chain precursor, controversy has existed as to whether free 6-APA is an intermediate between IPN and benzylpenicillin or a shunt product which would be converted to benzylpenicillin by IAT when phenylacetyl-CoA became available. Since *P. chrysogenum* extracts can convert IPN (but not penicillin N) to benzylpenicillin, this is consistent with 6-APA serving as a shunt product but it still had to be established whether the conversion is direct or involves a prior hydrolysis of IPN to 6-APA, especially since the extracts used were also capable of phenylacetylating free 6-APA.

Purified IAT accepts, as a substrate, coenzyme A esters of phenylacetate, phenoxyacetate, octanoate, hexanoate, and heptanoate; it then can transfer the acid to the intermediate 6-APA. Whether or not the purified enzyme transfers side-chain acids to the 6-APA moiety of IPN or only to free 6-APA is important in deciding whether free 6-APA is an intermediate or a shunt product. A pure preparation of the enzyme was capable of accepting IPN although the monomeric enzyme did not use penicillin G, 7-aminocephalosporanic acid (7-ACA), cephalosporin C, or isocephalosporin C as donors of the 6-APA or 7-ACA moiety, nor did it remove the side chain of penicillin G. Four nonproducing mutants of *P. chrysogenum* lacked the enzyme. Transformation of the penicillin acyltransferase gene into *penDE* mutants of *P. chrysogenum* restored penicillin production. The cloned *P. chrysogenum* enzyme isolated from *E. coli* accepts both 6-APA and IPN. Thus, a single enzyme is responsible for phenylacetylating free 6-APA and the 6-APA moiety of IPN.

Many activities have been shown in purified IAT, including (1) isopenicillin acyltransferase activity converting IPN to penicillin G; (2) IPN amidohydrolase activity converting IPN to 6-APA; (3) 6-APA acyltransferase activity converting 6-APA to penicillin G; (4) penicillin transacylase activity which interconverts hydrophobic penicillins and also interconverts 6-APA and penicillins; and (5) penicillin amidase activity converting penicillin G to 6-APA. Since the IPN amidohydrolase specific activity is much lower than that of the acyltransferase, it appears that free 6-APA is not a true intermediate in penicillin G formation. Thus, the conversion of IPN to hydrophobic penicillins is a two-step reaction catalyzed by a single enzyme, and 6-APA is a bound intermediate which is released in the absence of an activated side-chain acid.

The IATs from *A. nidulans* and *P. chrysogenum* have a 40-kDa proacyltransferase $\alpha\beta$-heterodimer (precursor). This heterodimer is maintained at that size in *A. nidulans*, whereas the *P. chrysogenum* proenzyme is processed into the 29-kDa $\beta$-subunit and the 11-kDa $\alpha$-subunit (Fernández et al. 2003). The $\beta$-subunit of *P. chrysogenum* is active, but the $\alpha$-subunit is not. However, the activity is highest when the two subunits associate autocatalytically (i.e., without another enzyme).

In *P. chrysogenum*, the three enzymes of penicillin biosynthesis (ACVS, IPNS, and IAT) appear to be located in three different cellular compartments. ACVS is associated with membranes or small organelles, perhaps vacuoles or Golgi vesicles. IPNS is in the cytosol and IAT is located in the peroxisomes (van de Kamp et al. 1999). The latter localization is essential for completion of penicillin biosynthesis. There is a correlation between peroxisome numbers and penicillin

production rates in *P. chrysogenum* and the production strictly requires the function of intact peroxisomes, as it is significantly reduced in peroxisome-deficient mutants (Meijer et al. 2010). The authors proposed that peroxisomes most likely create a unique microenvironment, suitable for the performance of the side-chain precursor-activation enzyme and IAT, catalyzing the two final steps of penicillin production. On the contrary, in *A. nidulans*, blocking peroxisomal localization of IAT reduces penicillin biosynthesis but does not shut it down completely, indicating that cytosolic IAT is also functional (Sprote et al. 2009).

Enzymes involved in the biosynthesis of secondary metabolites, such as non-ribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs), require post-translational phosphopantetheinylation for activity. This is true for penicillin biosynthesis (e.g., ACVS) and lysine formation in *P. chrysogenum* (García-Estrada et al. 2008). 4′-phosphopantetheine is produced from coenzyme A and is added to the inactive enzyme by PPTase (4′-phosphopantetheinyl transferase). The encoding gene *ppt* is present as a single copy in wild-type *P. chrysogenum* and in industrial strains. Its amplification increases production of IPN and benzylpenicillin.

## 5.3 The Biosynthetic Pathway of Cephalosporin C

The cephalosporin C biosynthetic pathway has been investigated in depth in *A. chrysogenum*. The route begins with three enzymatic reactions common to penicillins and cephalosporins (Fig. 5.1). The first one is the condensation of L-Cys and L-Aaa to form the dipeptide AC. Then, L-Val is epimerized to the D-form, activated, and condensed with AC to form the tripeptide ACV (Banko et al. 1986, 1987). The first two reactions are catalyzed by the activity of ACVS, encoded by the *pcbAB* gene (Gutiérrez et al. 1991; Rodríguez-Sáiz et al. 2004a, b). The purified ACVS has a molecular weight of 360 kDa and was suggested to be a dimeric structure. Its activity is dependent on L-Aaa, L-Cys, ATP, and $Mg^{+2}$, or $Mn^{+2}$. The third reaction is the cyclization of ACV to IPN catalyzed by the IPN synthase (IPNS or "cyclase"), encoded by the *pcbC* gene (Zhang and Demain 1990). The IPNS has a molecular weight of 41 kDa (Hollander et al. 1984) and is stimulated by $Fe^{+2}$, reducing agents, and oxygen (Sawada et al. 1980). In *A. chrysogenum*, the IPN epimerase activity (IPNE), responsible for the epimerization of the L-AAA side chain of IPN to the D-configuration of penicillin N, has been shown to be a two-component protein system catalyzed by the enzymes isopenicillinyl-CoA ligase and isopenicillinyl-CoA epimerase, encoded, respectively, by the *cefD1* and *cefD2* genes (Ullán et al. 2002a; Martín et al. 2004), whereas the single *cefD* gene encodes IPNE in cephamycin or cephalosporin-producing bacteria such as *S. clavuligerus*, *Nocardia lactamdurans,* and *L. lactamgenus* (Kovacevic et al. 1990; Coque et al. 1993; Kimura et al. 1996).

Although formerly, penicillin N and cephalosporin C were thought to be products of different biosynthetic pathways, the discovery of ring expansion of penicillin N to DAOC revealed the key step for a common pathway (Kohsaka and

Demain 1976). The ring expansion of penicillin N to form DAOC is catalyzed by an α-ketoglutarate-dependent dioxygenase named DAOC synthase (DAOCS or "expandase") (Kohsaka and Demain 1976) requiring $Fe^{+2}$, ascorbate (Sawada et al. 1980), oxygen, and α-ketoglutarate (Felix et al. 1981). DAOCS and the following enzyme of the pathway, DACS, are encoded by separate genes in bacteria (*cefE* and *cefF*, respectively) (Kovacevic et al. 1989; Kovacevic and Miller 1991), but both activities are present as a single protein encoded by the *cefEF* gene located on chromosome II in *A. chrsogenum* (Skatrud and Queener 1989; Samson et al. 1987; Scheidegger et al. 1984; Wu et al. 2005). The DAOCS of *S. clavuligerus* is a monomer of 34.6 kDa (Dotzlaf and Yeh 1989) whose catalytic activity is stimulated by DTT and ascorbate but not by ATP. On the other hand, the fungal bifunctional DAOCS-DACS enzyme is a monomer of 41 kDa with an isoelectric point of 6.3 (Dotzlaf and Yeh 1987). It requires $Fe^{+2}$, α-ketoglutarate, and oxygen and is stimulated by ascorbate, DTT, and ATP. Finally, the methoxy group of DAC, located at C3, is acetylated by a DAC acetyltransferase (DAC-AT) encoded by the *cefG* gene (Gutiérrez et al. 1992; Martín et al. 1994) to biosynthesize cephalosporin C.

The genes of *A. chrysogenum* encoding the first two enzymes of the cephalosporin biosynthetic pathway (*pcbAB* and *pcbC*) are clustered in chromosome VII, whereas the genes coding for the enzymes that catalyze the last two steps of the pathway (*cefEF* and *cefG*) are clustered in chromosome I (Gutiérrez et al. 1999a, b). Both clusters are present as a single copy per genome in the wild-type and in the high cephalosporin-producing strains of *A. chrysogenum*. Altered electrophoretic karyotypes as a result of chromosomal rearrangements have been described in industrial strains of *A. chrysogenum* obtained by classical mutagenesis (Walz and Kück 1991). After functional analysis of promoter sequences of cephalosporin C biosynthesis genes from *A. chrysogenum* to define specific DNA–protein interactions, the winged helix transcription factor CPCR1, involved in the regulation of β-lactam biosynthesis, was characterized (Schmitt et al. 2001, 2004a, 2004b).

### *5.3.1 The IPNE*

The conversion of the L-Aaa side chain of IPN to the D-configuration of penicillin N is catalyzed by the extremely labile epimerase enzyme called IPNE. This is the first reaction of the longer hydrophilic branch in cephalosporin- and cephamycin-producers. IPNE has been shown to be a two-component enzyme composed of IPN-CoA synthetase and IPN-CoA epimerase, encoded, respectively, by the *cefD1* and *cefD2* genes, in *A. chrysogenum* (Ullán et al. 2002a; Martín et al. 2004). A single *cefD* gene encodes IPNE in cephamycin or cephalosporin-producing bacteria (Kovacevic et al. 1990; Coque et al. 1993; Kimura et al. 1996). Most probably *P. chrysogenum* and other producers of hydrophobic β-lactams lack IPNE and thus cannot produce penicillin N and cephalosporins.

### 5.3.2 The Deacetoxycephalosporin C Synthase (DAOCS; "Expandase") and Deacetoxycephalosporin C 3′-Hydroxylase (DACOH; "3′-Hydroxylase")

The expandase catalyzes the conversion of penicillin N into deacetoxycephalosporin C (DAOC) (Valegård et al. 1998; Lee et al. 2001a). It requires $Fe^{2+}$, ascorbate, oxygen, and α-ketoglutarate. $CO_2$ is liberated from the α-ketoglutarate during the reaction. Expandase is an α-ketoglutarate-linked dioxygenase, although it does not technically fit the definition since the two atoms of oxygen do not end up in the products. One atom of oxygen is incorporated into succinate during the oxidative decarboxylation of the cosubstrate, α-ketoglutarate. The other oxygen atom is presumably incorporated into an intermediate which is converted to DAOC and water, the second oxygen atom ending up in $H_2O$. Expandase was the first enzyme discovered that requires $Fe^{2+}$, α-ketoglutarate, and oxygen that performs an oxidative cyclization/desaturation instead of a hydroxylation. A second one is the clavaminate synthase of the clavulanic acid pathway in *S. clavuligerus*. The *A. chrysogenum* expandase is inactivated by α-ketoglutarate if this cosubstrate is added before $Fe^{2+}$ and ascorbate. The α-ketoglutarate apparently prevents $Fe^{2+}$ from binding to the enzyme and a dead-end complex is formed, resulting in enzyme inactivation. DTT is capable of reactivating the *S. clavuligerus* expandase after inactivation.

Epimerase (*cefD*) and expandase (*cefE*) genes in *S. clavuligerus* form an operon. In contrast to the unienzymatic epimerization of IPN to penicillin N in bacteria, the epimerization in the fungus *A. chrysogenum* occurs by a two-enzyme system (Ullán et al. 2002a). Genes *cefD1* and *cefD2* encode proteins resembling long-chain acyl-CoA synthetases and acyl-CoA racemases from higher forms of life, respectively. The function of CefD1 and CefD2 two-component system has been shown to constitute rate-limiting steps of cephalosporin biosynthesis in *A. chrysogenum* (Ullán et al. 2004). In *A. chrysogenum*, early cephalosporin biosynthetic enzymes are cytosolic (van de Kamp et al. 1999) while the CefD1–CefD2 epimerization system was shown to be compartmentalized in microbodies, implying the intracellular transport of IPN and penicillin N (Martín et al. 2010). Although expandase and 3′-hydroxylase are separate dioxygenases in *S. clavuligerus* and *N. lactamdurans*, the two-enzyme activities are present on a single protein in *A. chrysogenum*. The *S. clavuligerus* expandase is a monomer which requires α-ketoglutarate, $Fe^{2+}$, and oxygen and is specifically stimulated by ascorbate and DTT.

The possibility to modify the expandase to act on penicillin G has been a topic of interest in the pharmaceutical industry. Availability of the crystal structure of *S. clavuligerus* expandase (Lee et al. 2001a; Lloyd et al. 1999; Valegård et al. 1998) and proposed roles for certain amino acid residues in catalysis (Chin and Sim 2002; Lee et al. 2000, 2001b; Lipscomb et al. 2002; Sim and Sim 2000) have led to rational approaches to engineer the enzyme.

The 3′-hydroxylation of DAOC to deacetylcephalosporin C (DAC) is carried out by an α-ketoglutarate-linked dioxygenase. It is stimulated by α-ketoglutarate, ascorbate, DTT, and $Fe^{2+}$ and incorporates oxygen from molecular oxygen. Expandase and 3′-hydroxylase appear to be the rate-limiting enzymes in *A. chrysogenum*.

### 5.3.3 *The DAC Acetyltransferase (Acetyl-Coenzyme A:Deacetylcephalosporin C O-Acetyltransferase; "Acetyltransferase") Encoded by the* cefG *Gene*

DAC is acetylated in *A. chrysogenum* to biosynthesize cephalosporin C, whereas in actinomycetes, DAC is carbamoylated. Mutants lacking the acetyltransferase accumulate DAC as their sole extracellular antibiotic. The reaction in the wild type, catalyzed by acetyltransferase and producing cephalosporin C, is the final reaction in cephalosporin-producing fungi. Acetyltransferase appears to be a rate-limiting enzyme in *A. chrysogenum*, possibly due to a weak promoter. The limitation is probably the major reason for the accumulation of high concentrations of DAC in cephalosporin C fermentation broths.

The *cefG* gene encoding acetyltransferase was cloned from *A. chrysogenum*. The gene contains two short introns of 79 and 65 bp and is closely linked to the expandase *cefEF* gene. The separation is via a 1,114-bp segment from which the genes are divergently transcribed. Disruption of *cefG* leads to lack of cephalosporin C production and a greater level of DAC in the broth. The structural gene is 1.2 kb in length, encoding a protein with a deduced molecular weight of 49,269, compared to a native molecular weight of 52,000. Cloning of *cefG* revealed clustering to *cefEF*, two introns, and transcription in the opposite direction from the same promoter region. This cluster is on chromosome II whereas the early genes of the pathway are on chromosome VI.

## 5.4 The Biosynthetic Pathway of Cephamycin C

Some bacteria can convert DAC into cephamycin C but fungi are unable to do it. First of all, DAC is converted into O-carbamoyldeacetylcephalosporin C by a carbamoyl transferase encoded by the *cmcH* gene. Then, the carbamoyldeacetylcephalosporin C is hydroxylated by an α-ketoglutarate-linked dioxygenase, encoded by the *cmcI* gene, to 7α-hydroxycarbamoyldeacetylcephalosporin C, which is finally methylated to cephamycin C by a methyltransferase encoded by the *cmcJ* gene.

### 5.4.1 The Carbamoyl Phosphate-3-Hydroxymethyl-Cephem-O-Carbamoyl Transferase ("Carbamoyl Transferase") Encoded by the **cmcH** Gene

DAC is converted in *S. clavuligerus* and *N. lactamdurans* to O-carbamoyldeacetylcephalosporin C by an ATP-dependent O-carbamoyltransferase, encoded by the *cmcH* gene, using carbamyl phosphate as the carbamoyl donor. The carbamoyltransferase gene of *N. lactamdurans* is part of the cephamycin C gene cluster. It has been cloned in *Streptomyces lividans* and found to contain a 1,563-bp ORF encoding a protein of 520 residues with a deduced Mr of 57,149 and a pI of 5.2. It requires $Mg^{2+}$ in addition to ATP and is rather unstable. The *cmcH* gene is closely liked to *cefF*, and a similar gene was found in *Streptomyces cattleya*, but not in *Streptomyces griseus* or *Streptomyces lipmannii*, which cannot make carbamoylated cephems.

### 5.4.2 The Carbamoyldeacetylcephalosporin C 7-Hydroxylase ("7 α-Hydroxylase") Encoded by the **cmcI** Gene

Carbamoyldeacetylcephalosporin C is hydroxylated to 7α-hydroxycarbamoyl-deacetylcephalosporin C by another α-ketoglutarate-linked dioxygenase. The purified hydroxylase has a Mr of 32,000 (by SDS-PAGE), an optimum pH of 7.3–7.7, an optimum temperature of 20–30 °C, and a Km for cephalosporin C of 0.72 mM. The enzyme requires α-ketoglutarate, $Fe^{2+}$, and a reducing agent (ascorbic acid) which confirms its identity as the third α-ketoglutarate-linked dioxygenase in the cephamycin C pathway.

The hydroxylase shows no ability to act on penicillin N or deacetoxycephalosporin C, but it also acts on cephalosporin C, a substrate more readily available than O-carbamoyldeacetylcephalosporin C. Antibacterial activity of 7α-hydroxycarbamoyl-deacetylcephalosporin C is similar to that of cephalosporin C but weaker against *Bacillus subtilis, Staphylococcus aureus,* and *Proteus mirabilis*. It is degraded by cephalosporinase.

### 5.4.3 The 7α-Hydroxycarbamoyldeacetylcephalosporin C Methyl Transferase ("Methyl Transferase") Encoded by the **cmcJ** Gene

The term "cephamycin" is used to designate 7α-methoxycephalosporins. 7α-hydroxycarbamoyldeacetylcephalosporin C is methylated to cephamycin C by a methyltransferase using S-adenosylmethionine as methyl donor. Methoxylation is

very important for activity against β-lactamase-producing bacteria. Semisynthetic cephamycins, e.g., cefoxitin, are thus very useful in the clinic against β-lactam-resistant bacteria.

Cell-free extracts of *S. clavuligerus* carry out the two-step methoxylation of carbamoyldeacetylcephalosporin C in the presence of S-adenosylmethionine, α-ketoglutarate, $Fe^{2+}$, and a reducing agent such as ascorbate. The methoxylation reaction is catalyzed by a two-protein ($P_7$ and $P_8$) system encoded by genes *cmcI* and *cmcJ* of the cephamycin C biosynthetic cluster. The C-7 of the cephem ring is first hydroxylated by an α-ketoglutarate- and NADH-dependent hydroxylase, followed by methylation of the hydroxyl group by a methyltransferase using S-adenosylmethionine as donor of the methyl group. Component $P_7$ is encoded by *cmcI* and is thought to have catalytic centers for the two enzymatic activities of the methoxylation system. The $P_8$ component is encoded by *cmcJ* and may be a helper protein. Methylation of hydroxycephalosporin C is optimal at pH 7.5. Methoxylation works only feebly on DAOC and not at all on DAC.

The cephalosporin C acetylhydrolase of *N. lactamdurans* attacks cephalosporin C and 7-ACA but not cephamycin C (Cardoza et al. 2000).

## 5.5 Cloning and Characterization of Genes Involved in β-Lactam Biosynthesis

Biosynthetic gene clusters for fungal and bacterial cephems are shown in Fig. 5.2. The penicillin biosynthetic pathway is found on chromosome II (9.6 Mb) in *P. notatum* and on chromosome I (10.4 Mb) in *P. chrysogenum*. Each of the species has four chromosomes. The total genome size is 32.1 Mb for *P. notatum* and 34.1 Mb for *P. chrysogenum.* The penicillin cluster in *A. nidulans* is on chromosome VI in strain ATCC 28901. A noteworthy result in the concurrent cloning programs of the late 1980s was that a 35-kb DNA fragment carrying two penicillin biosynthetic genes was amplified to six to nine copies (Barredo et al. 1989b), as was the entire biosynthetic cluster of ca. 57.4 kb containing *pcbAB*, *pcbC,* and *penDE* which was present in 8–16 copies (Smith et al. 1989) in high-producing industrial strains of *P. chrysogenum* obtained by classical mutagenesis. It was demonstrated by Fierro et al. (1995, 1996) and Newbert et al. (1997) that the amplifications in the high titer strains are tandem, linked by a conserved TTTACA hexanucleotide, implying crossing-over, resulting in amplification or deletion occurring within this hot-spot for site-specific recombination after random mutation. On the other hand, the lack of a linear relationship between the copy numbers of clusters and penicillin titers (Newbert et al. 1997), as well as the presence of only a single copy in a producer strain lineage with varying penicillin titers (Elander 2002), led the investigators to consider the impact of pathway-specific regulation. Metabolic characterization of high- and low-yielding strains of *P. chrysogenum* (Christensen et al. 2000), application of metabolic flux analysis

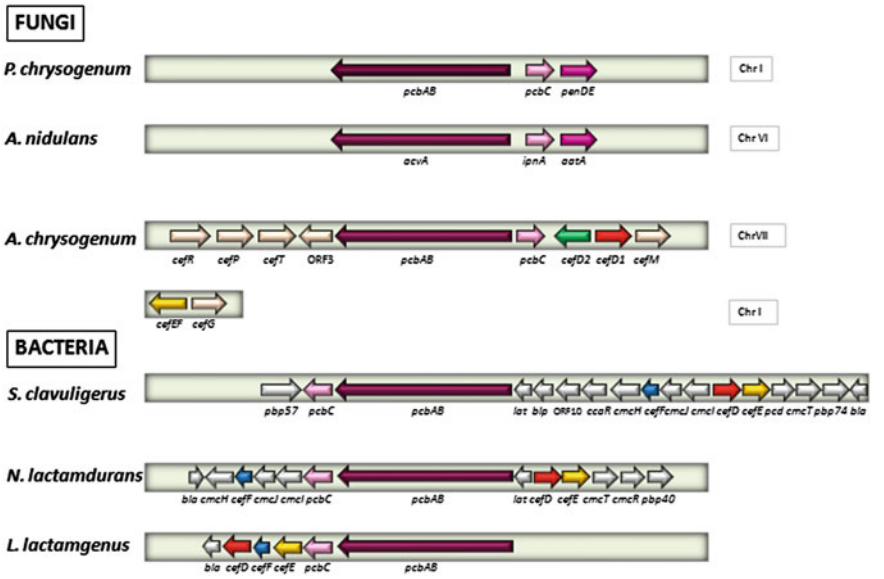


**Fig. 5.2** Clusters of biosynthetic genes of $\beta$-lactams produced by fungi (*Penicillium chrysogenum*, *Aspergillus nidulans,* and *Acremonium chrysogenum*) and bacteria (*Streptomyces clavuligerus*, *Nocardia lactamdurans,* and *Lysobacter lactamgenus*). The direction of transcription of the genes is shown by arrows and the bacterial, and fungal homologous genes are colored the same. ORFs specify functionally unknown open reading frames. The rest of the abbreviations are mentioned in the text (from Ozcengiz and Demain 2013 with permission)

for the identification of metabolic bottlenecks in the biosynthesis of penicillin G (van Gulik et al. 2000), and the relationship between growth and penicillin production in a high-producing strain of *P. chrysogenum* (van Gulik et al. 2001) have been studied. Still, in spite of some conflicting reports on transcriptional activities and a putative binding site for *pcbAB* expression (Kosalkova et al. 2000) or candidate ORFs (Fierro et al. 2006) within the penicillin biosynthetic gene cluster, a master control switch could not be identified. Detailed bioinformatic analyses revealed a detectable transcript for most of the 12 predicted ORFs in the cluster. However, the genes encoding the three penicillin biosynthetic enzymes alone were sufficient to restore full $\beta$-lactam synthesis in a mutant lacking the complete region (van der Berg et al. 2007). On the other hand, in addition to the inactivation of homogentisate pathway of phenylacetic acid degradation, the industrial strains of *P. chrysogenum* harbored some other genetic alterations in specific ORFs that reduced unwanted $\beta$-lactam degradation and transport capabilities as well as the synthesis of competing homologous secondary metabolites (Wang et al. 2008, van den Berg 2010).

The genomic region containing genes *pcbAB, pcbC,* and *penDE*, which encode the three penicillin biosynthetic enzymes, is amplified in tandem repeats in strain AS-P-78 (Barredo et al. 1989a; Fierro et al. 1995, 1996; Newert et al. 1997). This

region appears only as a single copy in strains NRRL-1951 and Wisconsin 54-1255. Another genetic modification in the improved strains is a greater number of microbodies (peroxisomes) which are involved in the final steps of penicillin production, i.e., they contain IAT and phenylacetyl-CoA ligase.

The availability of the 32.19 Mb genome sequence (van den Berg et al. 2008) of *P. chrysogenum* Wis 54-1255 (an improved strain but still a low producer) opened new insights by making transcriptome, proteome, and metabolome (Nasution et al. 2008) analyses possible (van den Berg 2011). DNA microarrays were used to compare Wis 54-1255 to the high-producing industrial strain DS17690 (van den Berg et al. 2008). Upregulated genes in the high producer were those involved in the formation of precursor amino acids (valine, cysteine, α-aminoadipic acid) and of microbodies. Conditions stimulating penicillin production favored transcription of genes encoding transporters, i.e., proteins involved in moving penicillin out of the cell. Penicillin is usually found externally at ten times the concentration of internal penicillin. The high-producing strain *P. chrysogenum* DS17690 and its cluster-free derivative DS50661 were compared with respect to their genome-wide gene expression profile in the presence and absence of phenylacetic acid, revealing for the first time, clear-cut target genes for metabolic engineering (Harris et al. 2009a).

Proteomic analysis of *P. chrysogenum* NRRL 1951 (the wild-type), Wis 54-1255, and AS-P-78 (the high producer) revealed changes such as increases in biosynthesis of the penicillin precursor cysteine, pentose phosphate pathway enzymes, and stress response proteins, and a reduction in the production of pigments and isoflavanoids (Jami et al. 2010a). The increase in cysteine biosynthesis was due to higher levels of two enzymes, i.e., cysteine synthase, forming cysteine from serine, and cystathionine β-synthase, forming cysteine from methionine by transsulfuration. Also, there were reductions in virulence proteins and enzymes of other secondary metabolite biosynthetic pathways. In addition, enzymes utilizing carbon sources such as cellulose and sorbitol were deleted. The comparison of the extracellular proteins of these three strains of *P. chrysogenum* provided a further step in their characterization, showing that enzymes related to plant pathogenesis, tissue invasion, and infectivity were all diminished during the improvement of the wild-type strain (Jami et al. 2010b; Barreiro et al. 2012).

The *pcb*AB and *pcb*C genes of *A. chrysogenum* are linked together in chromosome VII of 4.6 Mb, forming the so-called early cephalosporin gene cluster (Gutiérrez et al. 1999a, b). Genes *cefD1* and *cefD2* are located in the region downstream of *pcbC* of this early gene cluster (Ullán et al. 2002a). For the final steps of cephalosporin biosynthesis, genes *cefEF*, coding for the bifunctional expandase-hydroxylase, and *cefG*, coding for the DAC acetyltransferase (Velasco et al. 1999), are linked together in the so-called late cephalosporin cluster on chromosome I of 2.2 Mb. The systems involved in the secretion of secondary metabolites are essential to avoid suicide of the producer organisms. The genes for resistance to such toxic metabolites are frequently harbored by the biosynthetic clusters. The ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) predominate in fungi and bacteria. The *cefT* gene was

identified in the early cephalosporin cluster coding for a transmembrane protein belonging to MSF (Ullán et al. 2002b). It was later shown to be a hydrophilic transporter involved in the secretion of hydrophilic β-lactams containing the α-aminoadipic acid side chain, i.e., IPN, penicillin N, and DAC (Ullán et al. 2008b). In an attempt to characterize the genes in the region located downstream of the *cefD1* gene (distal to *cefT*), the *cefM* gene, coding for another protein of the MFS with 12 transmembrane domains, was found to be located in the opposite end of the "early" cephalosporin gene cluster of *A. chrysogenum* (Teijeira et al. 2009). Its targeted disruption resulted in a drastic reduction in cephalosporin production. Instead, the disrupted mutant accumulated about 7-fold higher intracellular penicillin N than the parental strain. When a fused *cefM-gfp* gene complemented the *cefM*-disrupted mutant, the fusion was targeted to intracellular microbodies. It therefore appeared that the IPN to penicillin N epimerization takes place in the peroxisome matrix, the CefM transporter being involved in the translocation of penicillin N from the lumen of the peroxisome (or peroxisome-like microbodies) to the cytosol, to be converted into cephalosporin C. Also found in the "early" CPC cluster is another gene named *cefP* encoding a putative transmembrane protein containing an 11 transmembrane spanner (Ullán et al. 2010). Like CefM, CefP was essential for cephalosporin biosynthesis in that the disrupted mutant was unable to synthesize cephalosporins and secreted a significant amount of IPN, indicating that the mutant is blocked in the conversion of IPN into penicillin N. Interestingly, cephalosporin production in the cefP-disrupted mutant was restored by transformation with both *cefP* and *cefR* (a regulatory gene located upstream of *cefP*), but not with *cefP* alone. As shown by fluorescence microscopy studies with an EGFP–SKL (Ser-Lys-Leu) protein (a peroxisomal targeted marker) as a control, the red fluorescence-labeled CefP protein co-localized in the peroxisomes with the control peroxisomal protein. Targeted inactivation of *cefR*-delayed expression of the *cefEF* gene, increased penicillin N secretion and decreased cephalosporin production, while its overexpression decreased penicillin N secretion and increased cephalosporin C production by preserving precursors (Teijeira et al. 2011). Expression analyses showed that *cefR* acts as a repressor of the exporter *cefT*.

*Kallichroma tethys*, a wood-inhabiting marine fungus phylogenetically related to *A. chrysogenum*, has a *pcbAB-pcbC-cefD2* cluster organization similar to that in *A. chrysogenum* (Kim et al. 2003). Complementation analyses suggested that at least *pcbAB* is functional, although active antibiotic could not be isolated from the culture. Cephamycin- and cephabacin-producing prokaryotes, on the other hand, possess larger β-lactam clusters (Liras and Martín 2006, Liras et al. 2008). In *S. clavuligerus*, the cephamycin C gene cluster and the adjacent clavulanic acid gene cluster together form the entire supercluster of cephamycin C-clavulanic acid genes which extends for ca. 50 kb along the chromosome. In the cephamycin biosynthetic gene cluster, besides the early genes *pcbAB* and *pcbC* which are expressed from a polycistronic transcript (Alexander et al. 2000), the genes of intermediate steps are *cefD* encoding a pyridoxal phosphate-dependent enzyme catalyzing a single-step conversion of IPN to penicillin N and two different genes,

*cefE* and *cefF*, encoding enzymes for ring expansion in two sequential steps. Interestingly, CefE and CefF proteins have 70 % identity in amino acids, and they are 60 % identical to the protein encoded by *cefEF* in fungi. Thus, they are thought to represent an example of gene duplication followed by "specialization" (Liras and Martín 2006). Genes *cmcH* and *cmcI-CmcJ* required for carbamoylation, C-7 hydroxylation and subsequent methylation, respectively, represent the late genes of cephamycin biosynthesis. In cephamycin-producing actinomycetes, two other genes, *lat* and *pcd*, coding for the respective enzymes lysine-6-aminotransferase and piperideine-6-carboxylate dehydrogenase for the provision of the precursor α-aminoadipic acid from lysine in two steps, are also located in the cephamycin gene cluster. The genes (1) for β-lactam resistance such as *bla* and *blp* coding for β-lactamase and a protein similar to the extracellular β-lactamase-inhibitory protein BLIP, respectively; (2) an ORF; (3) the *cmcT* gene encoding a putative cephamycin transport protein; and (4) the regulatory gene *ccaR*, encoding a *streptomyces*-activator regulatory protein (SARP) regulatory protein, are among the other components of the cluster. The *S. clavuligerus bla* is 5.1 kb downstream of *cefE*; its β-lactamase is weak, acts against penicillin G and cefoxitin, but not against natural cephalosporins.

The early genes *lat, pcbAB,* and *pcbC* in the cephamycin cluster in *N. lactamdurans* are tightly clustered. Four late genes, i.e., *cefF* (encoding DAOC hydroxylase), *cmcI* and *cmcH* (encoding the two-protein component cephem-7-methoxylase), and *cmcH* (encoding the 31-hydroxymethylcephem canbamoyltransferase), are immediately downstream of *pcbC*. Genes *cefD* and *cefE* are immediately upstream of *lat* and expressed in the opposite direction. There is only a 0.6kb space between *lat* and *cefD*. The gene cluster is expressed as three distinct mRNAs from a bidirectional promoter region in this intergenic space and from a promoter located inside the *lat* gene. In *N. lactamdurans*, promoter activity for *pcbAB* is 60–70 nt upstream of the translation start codon. A very long polycistronic RNA (at least 16 kb) starts at the *pcbAB* promoter covering *pcbAB-pcbC-cmcI-cmcJ-cefF-cmcH*. The *pcbAB* promoter is more active than the *lat* promoter. The cluster of cephamycin biosynthetic genes in *N. lactamdurans* also contains genes encoding a penicillinase, a penicillin-binding protein (PBP) and a transmembrane protein. The PBP does not bind to cephamycin C. The transmembrane protein is thought to be involved in antibiotic secretion. The cephamycin gene cluster contains gene *cmcT* that appears to encode a transport protein in *N. lactamdurans* and also in *S. clavuligerus*, as mentioned earlier in this section. It contains transmembrane domains (11 and 14, respectively) and sequences similar to proteins in other organisms involved in transport of antibiotics, sugars, and phosphate via a protein gradient. In *N. lactamdurans*, *cmcT* is downstream of *cefE* where its GTG initiation codon overlaps the TGE stop codon of *cfe*. These proteins may offer resistance to cephamycin. β-Lactamase-encoding genes (*bla*s) are present in *N. lactamdurans*, *S. clavuligerus,* and *L. lactamgenus.* The *L. lactamgenus* β-lactamase does not act on cephalosporins. Overexpression of *bla* in *N. lactamdurans* increases resistance to penicillin G and its disruption increases sensitivity. The β-lactamases of *N. lactamdurans* and *S. clavuligerus* appear to be

in the periplasmic space. Gene *blp*, which occurs in *S. clavuligerus* and encodes the β-lactamase-inhibitory protein BLIP, is not present in the cephamycin cluster of *N. lactamdurans*. Genes encoding PBPs are present in the cephamycin clusters of *N. lactamdurans* and *S. clavuligerus*. Gene *pbp40* is 1 kb downstream of *cmcR* in *N. lactamdurans* and its encoded protein is in the plasma membrane. *S. clavuligerus* has *pbp57*, immediately downstream of *pcbC*, which encodes a protein anchored in the membrane, and the other is *pbp74*, immediately downstream of *bla.* Thus, the two genes that appear to be involved in resistance to β-lactams are at opposite ends of the cephamycin C cluster.

*P. chrysogenum* Wis 54-1255 was transformed with individual genes, pairs of genes, and the entire three genes of the penicillin pathway. Major increases occurred when all three genes were overexpressed (Theilgaard et al. 2001). Transformation with the *pcbc-penDE* fragment actually decreased production. The transformant containing three extra copies of *pcbcAB*, one extra copy of *pcbC,* and two extra copies of *penDE* produced 299 % of control shake flask production and 276 % of control productivity in continuous culture.

Cyclase genes have also been cloned and sequenced from *P. chrysogenum*, *A. nidulans*, *S. clavuligerus*, *S. lipmanii,* and *Streptomyces jumonjinensis*. It appears that the cyclase genes from prokaryotes and eukaryotes are related and probably evolved from a common ancestral gene. The three fungal genes show 74–80 % relatedness and their proteins 73–81 % similarity in sequence. The corresponding figures for the actinomycetes are 70–81 % and over 70 %. The relatedness between fungal and actinomycete genes is 56–62 % and that between the enzymes 54–56 %. There may have been divergent evolution of the bifunctional *cefEF* gene of *A. chrysogenum*, the *cefE* gene, and the *cefF* gene of *S. clavuligerus*, i.e., they may have all evolved from a common ancestral gene. The active site appears to be the same in all three enzymes and the sizes of the three enzymes are similar.

## 5.6 Rational Metabolic Engineering

In certain strains, the β-lactam biosynthetic pathways have some bottlenecks caused by rate-limiting enzymes. Cloning and overexpression of their biosynthetic genes, even entire gene clusters, is one way to increase antibiotic production. A number of regulatory genes have recently been detected in organisms producing β-lactam antibiotics. Thus, other useful manipulations include increasing dosage of positively acting regulatory genes (transcription factors or other pleiotropic regulators), while completely disrupting or decreasing dosage of negatively acting genes. Replacing a weak promoter with a strong promoter is another means of raising antibiotic titer. Downregulation or deletion of certain genes in the branched pathways to eliminate nonproductive/undesirable reactions at the level of both primary and secondary metabolism has also been among the goals. Directed evolution of biosynthetic genes, inactivation of nonhomologous end joining

(NHEJ) to improve knock-in and knock-out constructions, and RNA-silencing for knock-downs appear as new and promising molecular tools to improve penicillin and cephalosporin-producing strains.

### 5.6.1 Penicillin Metabolic Engineering

The improvement in penicillin production by mutagenized strains of *P. chrysogenum* is reflected in their production of individual enzymes. Strain AS-P-78, an old Antibioticos strain, has 6.7 times more cyclase than ancestral strain Wis 54-125. The Beecham strain BW 1890 has 170–1,340 times more cyclase activity than NRRL 1951. A Novo Nordisk production strain produces 273 times more ACVS, 100 times more cyclase, and over 10 times more penicillin acyl transferase than NRRL 1951.

Metabolic engineering of penicillin-producing *P. chrysogenum* strains corroborated the positive role of penicillin biosynthetic gene amplification detected in high-producing industrial strains, showing that increases can be obtained by introducing extra copies of biosynthetic genes and by increasing copy number and high transcription levels of the whole cluster. Flux control is exerted only by ACVS at the beginning of the fed-batch culture, but it later shifts to cyclase. Penicillin production is also increased by overexpressing the gene encoding phenylacetic acid activating CoA ligase from *Pseudomonas putida.*

ACVS is the rate-controlling step in the production of penicillin V by the Novo strain of *P. chrysogenum* up to 50 h and, after that, IPNS is the most limiting enzyme. In *A. nidulans* strain WG355, ACVS limits the production of penicillin more so than the other two enzymes of the pathway, i.e., IPNS and IAT. Expression of the *pcbAB* gene is only one-third as great as *penDE*, which in turn is expressed to a lower degree than the *pcbC*. In *A. nidulans*, an intergenic region of 872 bp separates *pcbAB* from *pcbC* and the *pcbAB* promoter is in this region; it is the weakest of three promoters. Use of a strong promoter for *pcbAB* expression (e.g., the *alcA* promoter of alcohol dehydrogenase) increased penicillin production 30-fold in *A. nidulans* 191.

Penicillin production attains high levels in *P. chrysogenum* not only by enhanced gene expression but also by gene amplification. Increased levels of mRNA corresponding to *pcbAB*, *pcbC,* and *penDE* are found in high penicillin-producing strains of *P. chrysogenum* as compared to wild-type strains. High-producing strains contain an amplified region which is at least 35 kb. A 6- to 16-fold increase in copies of the biosynthetic genes per genome is observed. A 106kb region amplified five to six times as tandem repeats is present in a high-producing strain. Only a single copy has been found in wild-type *P. chrysogenum* and Fleming's original strain of *P. notatum.*

Penicillin productivity depends on the resistance of the precursor phenylacetic acid to oxidative destruction. Breakdown of phenylacetic acid was studied in *A. nidulans* and it was found that it is initiated by 2-hydroxylation by the microsomal cytochrome P450 monooxygenase encoded by the *phacA* gene. When

the gene was inactivated, penicillin production by *A. nidulans* increased by 5-fold (Mingot et al. 1999). Rodríguez-Sáiz et al. (2001) compared the sequence of *phaA* gene from *P. chrysogenum* wild-type strain NRRL 1951, Wis 54-1255, and the industrial strain E1, showing a base change at position 598 of the ORF in that the T present in strains Wis 54-1255 and E1 was formerly a C in wild-type strain NRRL 1951. This mutation causes a single amino acid substitution at position 181 of the protein: a leucine residue in the wild-type strain was substituted with phenylalanine in the improved strains and was responsible for the reduced function in industrial strains. Therefore, a C to T substitution (L181F mutation) in this gene of *P. chrysogenum* accounted for the historic choice of this species over the Fleming strain of *P. notatum* (Rodríguez-Sáiz et al. 2005).

A way to control pathway activity by clustering enzymes with their substrates inside specific membrane-bound structures sequestered from the cytosol was the compartmentalization of specific parts of the *β*-lactam biosynthesis pathways (Evers et al. 2004). Overexpression of *P. chrysogenum* cDNA encoding Pc-Pex11p, a peroxin that is involved in microbody abundance, resulted in massive proliferation of microbodies and up to a 2.5-fold increase in penicillin level in the culture medium (Kiel et al. 2005). This was not due to an effect on the levels of the penicillin biosynthetic pathway enzymes, but rather as a result of an increase in the fluxes of penicillin and/or its precursors across microbody membranes. More recently, *P. chrysogenum* microbody matrix enzymes were identified by in silico and proteomic approaches (Kiel et al. 2009).

In submerged penicillin fermentations, oxalate is an undesirable by-product, not only because its formation diverts carbon flow and decreases product yield, but it also results in the need for additional downstream processing steps. *Pc22g24830/PcoahA* is the sole oxaloacetase gene in *P. chrysogenum* and its deletion led to complete elimination of oxalate production, while improving yields of the cephalosporin precursor ad-6-APA (Gombert et al. 2011). Deletion of the *P. chrysogenum* ortholog of *Saccharomyces cerevisiae* serine-threonine kinase *atg1* brought about increased levels of the penicillin biosynthetic pathway enzymes and enhanced production of penicillin (Bartoszewska et al. 2011). This provided a rescue from significant amounts of cytosolic and peroxisomal protein degradation via autophagy under penicillin-producing conditions.

Gene replacements in penicillin- and cephalosporin-producing fungi were not very efficient, and the split-marker technique was developed to ensure three crossing-over events for substituting the target gene by homologous recombination, thus improving efficiency (Casqueiro et al. 1999; Liu et al. 2001b). Later, the NHEJ system that is responsible for random integration in fungi was inactivated by eliminating its main component, the DNA-PK complex, *hdfA* gene (Hoff et al. 2010), and both *hdfA* and *hdfB* genes (Snoek et al. 2009) of *P. chrysogenum*, respectively.

RNA-silencing has been considered as another new tool for exploring gene function in the genomes of filamentous fungi (Kück and Hoff 2010; Li et al. 2010; Salame et al. 2011). The dsRNA expression cassette included in plasmid pJL43-RNAi was promising to facilitate post-transcriptional gene attenuation by

targeting the *pcbC* gene in *P. chrysogenum* and the *cefEF* gene in *A. chrysogenum* (Ullán et al. 2008a) with an efficiency of 15–20 % for selected transformants. By using a hairpin-expressing vector, the *DsRed* gene coding for an autofluorescent reporter, either alone or together with *pcbC* in *A. chrysogenum*, was successfully silenced (Janus et al. 2007). All transformants having a colorless phenotype showed simultaneous downregulation of the *pcbC* gene. In another study, under the control of the constitutive *trpC* promoter or the inducible *xylP* promoter, gene *DsRed* and morphogene *PcbrlA*, which controls fungal conidiophore development in *P. chrysogenum*, were silenced, leading to a dramatic reduction in the formation of conidiospores (Janus et al. 2009).

### 5.6.2 Cephalosporin C Metabolic Engineering

The first engineering of an antibiotic-producing industrial strain was described by Eli Lilly researchers in the cephalosporin C producer *C. acremonium* 394-4 (Skatrud and Queener 1989). This strain accumulates in the broth a substantial quantity of penicillin N during industrial scale fermentation. Developing a new transformation system based on hygromycin B resistance (Queener et al. 1985), extra copies of the *cefEF* gene in a 7kb *Bam*HI genomic DNA fragment were introduced. Fermentation testing of a selected transformant showed an increase in the specific activity DAOCS, a substantial decrease in the amount of penicillin N excreted, and an important increase in the cephalosporin C produced with respect to the untransformed recipient strain. Southern analysis of this recombinant strain showed the presence of a single heterologous integration event. Nevertheless, later cloning of the *cefG* gene (Gutiérrez et al. 1992) revealed that it was linked to the *cefEF* gene and was included in the 7kb *Bam*HI fragment used in the above mentioned transformations. This fact raised the possibility that the effects observed in the transformants were more attributable to the enhanced expression of the *cefG* gene, reported to be limiting in *C. acremonium* (Mathison et al. 1993).

An industrial strain improvement program based on genetic transformation showed that the best genes to increase cephalosporin C production in *A. chrysogenum* are *cefEF*, encoding expandase-hydroxylase, and *cefG*, encoding acetyltransferase (Rodríguez-Sáiz et al. 2004a, b). The increased gene dosage increased cephalosporin C and decreased production of intermediates such as DAC and DAOC.

The two main strategies to improve the expandase function have been directed evolution and the rational approach (Goo et al. 2009). Since a more hydrophobic substrate-binding pocket may favor its interaction with hydrophobic penicillins, candidate residues in *S. clavuligerus* expandase were systematically substituted with a noncharged hydrophobic leucine residue for the production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) from penicillin G (Chin et al. 2001). The mutant N304L was found to catalyze the conversion of penicillin G with an activity increased by about 2-fold with respect to the wild-type enzyme. A

complete library of amino acid alterations at N304 was next prepared and the specific activities of the wild and mutant enzymes for penicillin G, ampicillin, amoxicillin, phenethicillin, carbenicillin, penicillin V, and metampicillin conversion were determined (Chin et al. 2004). Replacing N304 with amino acids harboring a strictly aliphatic or basic side chain, particularly R and K, exhibited outstanding enhancement of enzyme activities (up to 730 %), possibly by incorporating favorable hydrophobic or charge interaction between these mutant enzymes and their prime substrates. The role of the C-terminal R306 residue of *S. clavuligerus* expandase in the catalysis of penicillin substrates was investigated by replacing it with the other 19 proteinogenic amino acids (Goo et al. 2008a). The results emphasized the importance of hydrophobic packing around this site as substitutions to nonpolar residues, leucine, isoleucine, and methionine were able to improve the ampicillin, penicillin G, phenethicillin, and carbenicillin conversion activity of the enzyme. The same authors reported the effect of pairing V275, C281, N304, I305, R306, and R307 mutations on enzyme catalysis (Goo et al. 2008b). C-terminal mutations (N304X [where X is alanine, leucine, methionine, lysine, or arginine], I305 M, R306L, and R307L) in combination with C281Y substantially increased the conversion of ampicillin, carbenicillin, and phenethicillin up to 491, 1,347, and 1,109 % of the wild-type activity, respectively.

Random mutants of the *S. clavuligerus cefE* gene were hydroxylamine-generated resulting in three point mutations: G79E, V275I, and C281Y with improved activity (Wei et al. 2003). Each of the six sites M73, L158, R160, V303, N304, and I305 surrounding the aminoadipoyl moiety of ACV was then changed first to an Ala residue and then to a positively charged residue (Lys), a negatively charged residue (Asp), a hydrophobic residue (Leu), and a sulfur-containing residue (Met) by site-directed mutagenesis. Three selected mutants, N304 K, I305L, and I305 M showed improvement, the best being I305L, with a 14-fold increase in kcat/Km, and I305 M with an 11-fold increase. This rationally based approach was then used to create all possible combinations of the six substitutions in order to see whether these could have an additive effect on activity. The best three were the V275I, I305 M double mutant which showed a 32-fold increase in Kcat/Km and a 5-fold increase in activity on penicillin G, the triple mutants V275I, C281Y, I305 M and G79E, V275I, I305 M showing a 13- and 11-fold increase in activity on penicillin G, respectively.

Eight *cefE* expandase-homologous genes were directly evolved by using the DNA shuffling technique to improve the substrate specificity for penicillin G: *cefE* genes from *S. clavuligerus, N. lactamdurans* [reclassified as *Amycolatopsis lactamdurans* (Barreiro et al. 2000)], *S. jumonjunensis*, the newly isolated *Streptomyces ambofaciens,* and *Streptomyces chartreusis*, the *cefF* gene from *S. clavuligerus,* the *cefEF* gene of *A. chrysogenum*, and the *cefF* gene from the soil actinomycete isolate 65PH1 (Hsu et al. 2004). The evolved enzyme from a two-round-shuffled clone had the highest known *k*cat/*Km* value, 2,121 ($M^{-1}$ $s^{-1}$), for penicillin G, which is 118-fold higher than that for the *S. clavuligerus* expandase.

The *cefE* gene from *S. clavuligerus* was directly modified, via error-prone PCR-based random mutagenesis and subsequent DNA shuffling, to obtain mutants

which have the highest relative activities for penicillin G expansion (Wei et al. 2005). Subsequently, DNA shuffling was carried out to screen possible combinations of substitutions. One quaternary mutant (C155Y/Y184H/V275I/C281Y) with a 41-fold higher *k*cat/*Km* ratio was found. The study also provided insight into structure–function relationship of the protein which will pave the way to rational engineering, for solving substrate inhibition and increasing substrate specificity. *In vivo* recombination between the *cefE* genes of *S. clavuligerus* and *N. lactamdurans* was made to construct *S. lividans* strain W25 containing a hybrid expandase (Adrio et al. 2002; Gao et al. 2003). This strain carried out four to five times more effective-level bioconversion of penicillin G to deacetoxycephalosporin G than the previously used strain, *S. clavuligerus* NP1.

The R308 residue located in close proximity to the C-terminus of *A. chrysogenum cefEF* was mutated to the other 19 amino acids (Wu et al. 2011). Substitution with L, I, T, and V, all possessing short aliphatic side chains, brought about significant improvement in the ability of the engineered enzyme to convert penicillin analogs and confirmed the role of R308 in controlling substrate selectivity.

Gene *acveA* of *A. chrysogenum* regulates production of cephalosporin C and hyphal fragmentation (Dreyer et al. 2007). The protein AcVEA, present in the nucleus, controls transcription of six cephalosporin C biosynthetic genes. Disrupted strains show a decrease in cephalosporin C production of 80 %. In the disrupted strains, hyphal fragmentation occurs early (48 h) whereas in the parent strain, it occurs later (after 96 h). *A. chrysogenum* produces cephalosporin C but also excretes the intermediate DAOC at 1–2 % of the cephalosporin C level. This undesirable situation can be modified by engineering the strain with two extra copies of the *cefEF* gene. The new strain excretes only half as much of this intermediate with no effect on cephalosporin C production.

The disruption and one-step replacement of the *cefEF* gene of *A. chrysogenum* with the *cefE* gene from *S. clavuligerus* yielded recombinants producing high titers of DAOC. The subsequent two-step enzymatic DAOC deacylation, yielding the important nucleus 7-ADCA free of other cephalosporin intermediates, can be used for the preparation of medically useful semisynthetic cephalosporins (Velasco et al. 2000).

The expression of the *A. chrysogenum* genes *cefD1*, *cefD2*, *cefEF,* and *cefG* in a *P. chrysogenum* strain lacking the IPN acyltransferase led to significant amounts of intracellularly produced and accumulated DAC (Ullán et al. 2007). Intracellular accumulation was as expected since *P. chrysogenum* is not a natural cephalosporin producer in which the *cefT* gene associated with cephalosporin secretion in *A. chrysogenum* has never been demonstrated (Martín et al. 2005; Ullán et al. 2002b). Introduction of *cefT* into an ad7-ACCCA-producing *P. chrysogenum* strain led to a 2-fold increase in cephalosporin production while decreasing penicillin by-product formation (Nijland et al. 2008). Koetsier et al. (2010) reported the first successful identification and characterization of a broad substrate specificity acyl-CoA ligase (encoded by *aclA*) activity from *P. chrysogenum* that may activate the side-chain precursor adipic acid during production of

cephalosporin precursors with recombinant *P. chrysogenum* strains, thus providing a potential target for molecular engineering of the cephalosporin pathway in *P. chrysogenum*.

DAC is known to be far more resistant to nonenzymatic breakdown in fermentation broth than cephalosporin C. An approach based on this matter involved the construction of a recombinant *A. chrysogenum* expressing an extracellular cephalosporin C esterase protein of *Rhodosporidium toruloides*, resulting in the conversion of excreted cephalosporin C to DAC in culture fluids (Basch et al. 2004).

Integration of the *A. chrysogenum* genes *cefEF* and *cefG,* along with the bacterial hemoglobin gene *vgb*, into the chromosome of an industrial strain of *A. chrysogenum* resulted in a significant increase in cephalosporin C production, whereas recombinant incorporation of *cefT* into this combination showed only little effect (Liu et al. 2010). In another successful approach, *A. chrysogenum cefEF* and *S. clavuligerus cmcH* genes were expressed in a penicillin G-overproducing strain of *P. chrysogenum* to produce adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (ad7-ACCCA) when grown in the presence of adipic acid (Harris et al. 2009b).

### *5.6.3 Cephamycin Metabolic Engineering*

In *S. clavuligerus,* there is a branched pathway to biosynthesize L-lysine from aspartate and then L-Aaa from L-lysine. L-Aaa supply is essential for cephamycin C formation. Elimination of the competitive branch yielding threonine, methionine, and isoleucine, by disruption of homoserine dehydrogenase, increased intracellular lysine production and cephamycin C formation (Yilmaz et al. 2008). Aspartokinase is the initial enzyme of the aspartate pathway in *S. clavuligerus*, the producer of cephamycin C. This pathway leads to the production of L-Aaa as well as methionine, isoleucine, and lysine. Inserting multiple copies of the *ask* gene, encoding aspartokinase, tripled cephamycin C production, presumably due to increased production of L-Aaa (Ozcengiz et al. 2010).

An extra copy of the *lat* gene encoding lysine aminotransferase was inserted in *S. clavuligerus* NRRL 3585 genome. Lat activity increased 8-fold and cephamycin production from 1- to 4-fold. The level of ACVS did not change, nor did the ratio between cephamycin C and O-carbamoyl-DAC. The increase in antibiotic production normally observed upon lysine addition did not occur in the recombinant culture which apparently produced enough lysine to synthesize both cellular protein and L-Aaa for cephamycin. The recombinant culture accumulated 80 % more L-Aaa in the culture supernatant than the wild-type strain. The work demonstrates that Lat is the rate-limiting enzyme in *S. clavuligerus* for synthesis of cephamycins.

Wild-type *N. lactamdurans* contains very low levels of Lat, ACVS, IPNS, epimerase, DAOCS, DACS, carbamoyl transferase, 7-cephem hydroxylase, and methyltransferase. Expression of *lat* from promoters of *S. griseus* increased Lat from 8- to 15-fold, increasing cephamycin C productivity by 50–200 % (Chary et al. 2000).

A genome-minimized *Streptomyces avermitilis* was constructed as a heterologous host with a 1.4-Mb deleted genome. The intact gene cluster for cephamycin C biosynthesis was efficiently expressed at levels even higher than that in the original producer *S. clavuligerus* ATCC 27064, and this engineered organism was also useful in that it no longer produced the major endogenous secondary metabolites of its parental strain (Komatsua et al. 2010).

## 5.7 Concluding Remarks

The global market of $\beta$-lactams, the most widely used antibiotics in human medicine, is still increasing. Since the introduction of penicillin into clinical practice, studies have been undertaken to improve the penicillin titer of industrial *P. chrysogenum* strains by at least 3 orders of magnitude and to optimize the fermentation processes. A major expansion of the $\beta$-lactam field occurred in the early 1960s with the development of the semisynthetic $\beta$-lactam antibiotics. Starting from the early 1970s, the development of cell-free systems from $\beta$-lactam-producing organisms led to the elucidation of the biosynthetic steps and biochemical properties of the enzymes involved. Rational metabolic engineering programs conducted from the late 1980s were quickly followed by biosynthetic cluster manipulation with the aim of overproducing $\beta$-lactams, altering their pathway activities and/or the nature of final metabolites. Availability of the genome sequences of *P. chrysogenum* and *S. clavuligerus* opened new insights by making genome-wide gene expression analyses possible. These are being used to solve the complex puzzle related to high productivities of industrial strains or rationally engineered ones. These attempts will also help to uncover "cryptic" metabolites. Molecular regulation of the biosynthetic clusters and the impacts from central metabolism will be more easily clarified, leading to rational strain improvement programs. These will involve the application of gene- or RNA-targeting tools to manipulate fungal recipients especially when combined with metabolomics and fluxomics. Fascinating advances in structural, synthetic, and systems biologies and their integration will drive the creation of *de novo* designed $\beta$-lactam factories of superior microbes. Knowledge and experience accumulated with genetically manipulated organisms (GMOs) has not revealed any particular safety and/or environmental problem. These encouraging results will simplify the use of GMOs at industrial scale. The power and potential of metabolic engineering are great and must be exploited wisely and safely for the benefit of humans and the environment.

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# Chapter 6
# The Cornerstone of Nucleic Acid-Affecting Antibiotics in Bacteria

**M. Gacto, M. Madrid, A. Franco, T. Soto, J. Cansado and J. Vicente-Soler**

**Abstract** Relatively few new antibiotics targeted against nucleic acids have been developed in the last 50 years. Rifamycins and a wide group of related microbial compounds block RNA synthesis by specific inhibition of bacterial RNA polymerase without interacting with mammalian analog enzymes. Others, such as actinomycins or doxorubicin, interfere with transcription in both bacterial and mammalian systems and may be used as antitumor drugs because the fast growth favors a higher percentage of death in malignant cells respect to normal counterparts. Another group of antibiotics interferes with DNA synthesis by acting on the bacterial DNA gyrase involved in the mechanism of replication of closed-circular DNA. Most members of this group, including aminocoumarin antibiotics, work by binding to the ATPase active site located on one subunit of the gyrase enzyme. Moreover, antibiotics effective against mammalian topoisomerases or able to prevent normal DNA distribution into daughter cells are being also used as antineoplastic drugs. In spite of the wide variety of mechanisms classically invoked to explain the primary action of antibacterial drugs, a new view is emerging whereby the killing damage behind all major classes of antibiotics appears to stem from the generation of destructive molecules that fatally damage nucleic acids through a long chain of cellular events.

## 6.1 Introduction

All cells, either prokaryotic or eukaryotic, have DNA as depository material of their genetic traits and both cell types also contain RNA as template for protein synthesis. Since the basic structure of DNA and RNA is universal at the molecular

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level, one might anticipate that the selective toxicity of antibiotics affecting nucleic acids should be rather scarce. In fact, the use of this type of compounds for clinical purposes is comparatively limited in comparison to the wide usage of antibiotics pertaining to other antibiotic families, like beta-lactams, aminoglycosides, or macrolides, whose targets rely on specific bacterial structures that are absent in eukaryotic host cells. However, there is still enough room to control bacterial cell growth by using compounds acting on nucleic acids that are able to show some selective toxicity. Such therapeutic specificity is often based on particular features of the enzymes involved in bacterial RNA synthesis or on aspects related to the circular nature of the chromosomal DNA and its supercoiling disposition. In the case of antibiotics against fungi, which are also briefly considered here, the selective interaction with components of the mitotic apparatus has been similarly exploited. Despite their occasional relative toxicity, some of these antibiotics may be useful not only as antimicrobial agents but also in cell biology studies as well as in anticancer treatments.

The comparatively short list that follows on nucleic acid-affecting antibiotics is based in the notion that true antibiotics, according to the definition by Waksman, are antimicrobial chemical substances of microbial origin (Waksman 1947). Hence, synthetic type compounds which are not produced by microorganisms in nature, such as quinolones or nalidixic acid, will not be analyzed in depth in this chapter although they are able to efficiently affect nucleic acid synthesis in bacterial systems. On the other hand, the field of antibiotics is in continuous flux and only a selected core list of this kind of antibiotics is presented attending to the current level of knowledge on their molecular mechanisms of action. The arsenal of nucleic acid-affecting antibiotics includes compounds inhibiting transcription or replication in bacteria, and some of them exhibit antifungal or antitumor properties. Because transcription and translation are directly linked and these two processes can occur nearly simultaneously in bacterial cells, chemotherapy agents targeting transcription may also affect protein synthesis in vivo. Likewise, agents affecting transcription also have effects on DNA replication and vice versa. Therefore, conventional consideration of the critical points of antibiotic action often refers solely to the primary interaction that triggers subsequent alterations in the normal cell machinery. It is worth to mention, however, that some observations support a novel interpretation of the main antibiotic action that can modify our classic mechanistic view. As suggested in the final paragraph of the present chapter, antibiotics structurally quite distant, such as penicillin or streptomycin, might share a common effect on nucleic acid metabolism irrespective of their chemical nature.

## 6.2 Inhibition of Bacterial Transcription by Antibiotics

As mentioned above, bacterial transcription may be aborted by limited translational events or by blockages in the mechanism that impairs unwinding of the DNA strand serving as template for messenger RNA synthesis. In general, two main initial

episodes can be set in motion by antibiotics that greatly influence the transcription process: (i) either direct interaction with DNA to impede the spatial formation of the transcription complex or (ii) inhibition of the enzyme function involved in the generation of messenger RNA. As described below, both types of interference can be found among the antibiotics affecting RNA synthesis.

### 6.2.1 The Family of Actinomycins

Actinomycins constitute a family of chromopeptide antibiotics containing lactone groups whose members differ in the bicyclic peptide portions of the molecules (Fig. 6.1). The first members of this family were identified in cultures of *Actinomyces antibioticus* as highly active against Gram-positive bacteria. However, early studies also revealed the limited therapeutic value of these compounds and their analogues due to their high toxicity. Despite this restriction, actinomycins have become an important research tool in molecular and cellular biology to inhibit transcription. Some actinomycin derivatives are used for staining fluorescent microscopy and flow cytometry, and their affinity for regions of GC-rich DNA convert them in good DNA markers.

Many species of actinomycetes (mostly including strains from the genera *Streptomyces* and *Micromonospora*) produce some kind of actinomycins and about thirty compounds of this set of antibiotics are currently known (Kurosawa et al. 2006; Praveen and Tripathi 2009). The mechanism of action of Actinomycin D has been studied in more detail. The direct binding of actinomycins to DNA in the complex of transcription initiation and elongation prevents the normal function of the DNA-dependent RNA polymerase, so that the final outcome of the initial interaction with DNA results in both inhibition of the polymerase enzyme and decreased protein synthesis. The exact DNA-binding mechanism has been a matter of controversy. Some studies suggested that the primary binding mode is intercalation into the double-stranded DNA whereas others pointed that single-stranded DNA binding is most relevant. Recent studies, based on quantification of

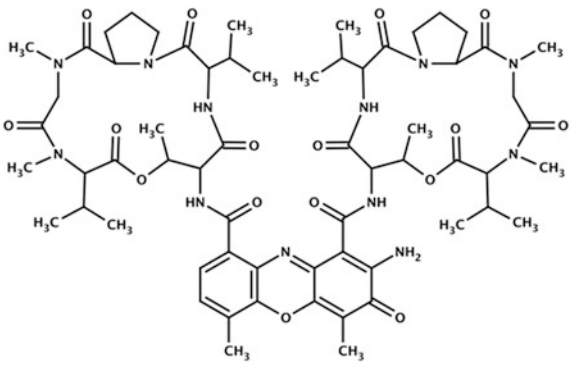

**Fig. 6.1** Structural composition of actinomycin D or dactinomycin

actinomycin D equilibrium and kinetic DNA-binding properties, support a model in which the active mode of actinomycin D binding is to pre-melted double-stranded DNA, as found in transcription bubbles (Paramanathan et al. 2012). Destabilization of double stranded DNA greatly facilitates the slow on and off rates of actinomycin D-DNA interaction, with a much stronger effect on association, resulting in overall enhancement of equilibrium actinomycin D binding. In short, the preferred binding mode appears to be to two DNA strands, but major duplex deformations are a pre-requisite for effective binding (Paramanathan et al. 2012). This mechanism can explain why DNA binds efficiently and rapidly low actinomycin D concentrations in transcriptionally hyperactive cancer cells: locking of the antibiotic within double-strand DNA favors blockage of RNA synthesis and leads to cell death due to a slow dissociation. As actinomycins can bind to a DNA duplex by promoting interstrand crosslinking they also interfere with DNA replication. Actinomycins thus exert their mechanism in the same way as the antibiotic mitomycin (see Sect. 6.3.4) although in this case the agent is a rather symmetrical molecule (Fig. 6.1). Other chemical agents, such as hydroxyurea, operate in a somewhat similar way.

A great deal of research has focused during the last years on the structural modification of pre-existing actinomycins and their resulting biofunctions. However, more classical approaches are still currently underway. For example, the characterization of actinomycins of the X series from novel sources of marine actinomycete strains is providing new effective agents, especially for antituberculosis therapies (Chen et al. 2012). It has been reported that, unrelated to the effect of inhibition of mRNA synthesis by anchoring into purine-pyrimidine DNA base pairs, actinomycins show strong antineoplastic activity. There are results suggesting that they have an antagonistic role in the interaction of components of the RAS signaling pathway to inhibit cell proliferation (Kim et al. 1999).

## *6.2.2 Antibiotics that Inhibit Bacterial DNA-Dependent RNA Polymerase*

### 6.2.2.1 Structural Features of RNA Polymerase

The multisubunit DNA-dependent RNA polymerase, the central enzyme of bacterial gene expression, is a complex molecular machine whose architecture at the structural level is well known following high-resolution studies. The structure and function of this bacterial enzyme has been the subject of detailed reviews (Darst 2001; Lee et al. 2012; Sekine et al. 2012). The basic composition of the catalytically competent core of bacterial RNA polymerase typically consist of five polypeptides named $\beta$, $\beta'$, $2\alpha$, and $\omega$, with a total molecular weight close to 400 kDa. This form of polymerase is capable of DNA-dependent RNA synthesis but unable to locate promoters and initiate direct specific transcription. An

additional key factor is the $\sigma$ subunit, which carries determinants for promoter recognition. The $\beta\beta'2\alpha\omega\sigma$ form of polymerase is known as the holoenzyme that is competent for transcription initiation in vivo. In the functional condition of the enzyme the subunits $\beta$ and $\beta'$ form a channel that contains the active site where RNA is being synthesized, as well as a small segment of RNA:DNA hybrid, duplex DNA, and single-stranded RNA newly formed. Another secondary channel connects the active site to the passageway for the entry of nucleoside triphosphates and exit of the resulting pyrophosphate molecules. The overall structure of the main channel within bacterial and eukaryotic RNA polymerases is quite similar (Cramer 2002). However, the primary sequences of the bacterial polymerase subunits are not conserved in the various nuclear RNA polymerases of eukaryotic cells. This fact determines differences in the affinity for various compounds and makes the eukaryotic enzymes insensitive to most inhibitors of bacterial RNA polymerases, thus providing bases for specific toxicity and therapeutic selectivity.

Because of their key role in gene expression, bacterial RNA polymerases are essential for viability and, hence, a good target for antibacterial therapy. Surprisingly however, among a broad range of known antibiotics, few of them have been described as specific inhibitors of this enzyme, despite their potential as antibacterial drugs and as tools to investigate the transcription mechanism. In fact, only one inhibitor, rifampicin (and its derivatives), has reached significant medical use. Also, some structural variations among bacterial RNA polymerases cause different sensitivity to this type of inhibitors (Lane and Darst 2010).

The best studied examples of antibiotics directed against the function of bacterial RNA polymerase include the antituberculosis agent rifampicin (Floss and Yu 2005), streptolydigin (Schobert and Schlenk 2008), the cyclic peptide microcin J25 (Adelman et al. 2004; Mukhopadhyay et al. 2004), and sorangicins (Campbell et al. 2005). To avoid the development of bacterial resistance, synthetic rifamycin-quinolone hybrids have been developed as compounds representative of the dual-action/targeting concept that employs complementary mechanisms of action (Barbachyn 2008). More recently, a new class of natural drugs has been identified targeting the so-called "switch region" within the bacterial RNA polymerase (Sousa 2008; Srivastava et al. 2011). The switch region is a structural element subject to conformational changes required for loading and retaining DNA into the cleft of the RNA polymerase that contains the active-center during transcription initiation. Several segments of the switch region undergo changes in local conformation upon opening and closing of the functional enzyme complex. This particular region is highly conserved across the taxonomic domain *Bacteria* and, consequently, inhibitors that function through this region will typically inhibit RNA polymerase in both Gram-positive and Gram-negative bacterial species. Moreover, since the switch region does not overlap the rifamycins binding site, inhibitors that function through the switch region do not typically share cross-resistance with rifamycins (Mukhopadhyay et al. 2008). In this context, four main natural products that bind to the switch region have been so far identified, namely myxopyronin, corallopyronin, ripostatin, and fidaxomicin. All of them inhibit bacterial RNA polymerase function and exhibit broad-spectrum antibacterial activity.

### 6.2.2.2 Rifamycins and Streptovaricin

Rifamycins, a subclass of compounds within the group of ansamycins, are currently used to control some bacterial diseases produced by Gram-positive or Gram-negative infectious agents. A rare actinomycete, *Amycolatopsis mediterranei* (originally known as *Streptomyces mediterranei* and later as *Nocardia mediterranei*) is known to produce the commercially important antibiotic rifamycins -notably rifampicin, rifabutin and rifapentine- whose basic structure is shown in Fig. 6.2. These antibiotics function by binding to and inhibiting bacterial RNA polymerase (Campbell et al. 2001; Floss and Yu 2005; Villain-Guillot et al. 2007; Ho et al. 2009; Mariani and Maffioli 2009) and they are among the few anti-tuberculosis agents able to kill non-replicating tuberculosis bacteria.

In particular, rifampicin is one of the most potent and broad spectrum representative antibiotics against bacterial pathogens and a key component of therapy treatments for mycobacterial diseases, including tuberculosis and leprosy. This antibiotic diffuses easily into living cells and bacteria, thus resulting quite effective against intracellular pathogens (Floss and Yu 2005). Nevertheless, its use is very limited and nearly reserved to tuberculosis because indiscriminate use for minor infections can promote extensive selection of resistant bacteria and prevent greater usefulness of this drug. Resistance to rifampicin involves mutational substitutions of residues that directly decrease binding of the antibiotic to the RNA polymerase (Ho et al. 2009; Mariani and Maffioli 2009). The relatively high occurrence of these changes implies that rifampicin should not be used as a single drug and therefore combined therapy is recommended. The use of rifamycins in combination with quinolones (ciprofloxacin) and translation-inhibiting lincosamides (clindamycin) avoids resistance development (Srivastava et al. 2011).

The bactericidal activity of all rifamycins is a consequence of their high affinity binding to the bacterial RNA polymerase that promotes transcriptional inhibition. Transcription initiation involves: (i) a first step comprising the binding of the RNA polymerase to the promoter region of DNA to yield a closed complex, in which DNA is outside the enzyme active-center cleft and fully double-stranded; (ii), an

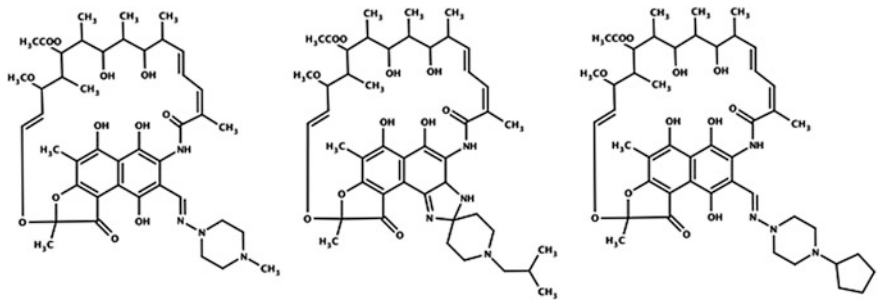

**Fig. 6.2** Basic structure of rifamycins as exemplified by rifampicin (*left*), rifabutin (*centre*) and rifapentine (*right*)

isomerization step of the closed complex occurs to yield an open complex in which DNA is inside the polymerase active-center cleft and some base pairs of the duplex DNA are unwound around the transcription start site; (iii) thereafter, synthesis of the first nucleotides of the RNA product takes place as an initial transcribing complex provided that appropriate nucleoside triphosphates are present; (iv) finally, the breakage of the interactions between the promoter and the RNA polymerase yields a RNA polymerase-DNA elongation complex (Saecker et al. 2011). The exact point where rifamycins inhibit transcription initiation appears to occur at the level of the third stage, i.e. during initial synthesis of RNA when the polymerase is still bound to the promoter (Floss and Yu 2005; Villain-Guillot et al. 2007). The antibiotic binds in a pocket of the $\beta$ enzyme subunit, deep within the DNA/RNA channel, but away from the active site, blocking both the path of the elongating RNA and the RNA exit pathway. The final result is that, in the presence of rifamycins, the polymerase enzyme initiates RNA chain synthesis but is unable to elongate the RNA product beyond a reduced length of nucleotides (Campbell et al. 2001). Fortunately, the enzymology of the transcription process is substantially different in prokaryotes and eukaryotes and rifamycins suppress the formation of chains in the synthesis of RNA from bacteria while does not affect human RNA polymerases. Although this inhibition might occur in the mitochondria of mammalian cells, much higher concentrations are required to inhibit the bacterial-type enzyme located within this organelle.

Other ansamycins, such as streptovaricin, are chemically similar to rifamycins and share the same RNA polymerase binding site and an analogous mechanism of transcription inhibition.

#### 6.2.2.3 Streptolydigin and Tirandamycin

The spectrum of biological activity displayed by natural products containing the structural tetramic acid ring is remarkable and includes potent antibiotic properties like those of streptolydigin and tirandamycin (Schobert and Schlenk 2008). Streptolydigin was first obtained as secondary metabolite from cultures of *Streptomyces lydicus* and shows the structural features of a typical member of the tetramic acids family (Fig. 6.3). Related compounds which lack the complex dioxabicyclononane moiety and the diene chromophore present in these antibiotics exhibit no antimicrobial activity and are without effect on bacterial RNA polymerase, indicating that one or both of these structural features may be critical for antibacterial activity.

Streptolydigin and tirandamycin inhibit initiation, elongation, and pyrophosphorolysis by bacterial RNA polymerases. However, these two antibiotics do not act on eukaryotic RNA polymerases, which share high three-dimensional structural similarity with their bacterial counterpart but low sequence identity (Cramer 2002). Streptolydigin and similar antibiotics show only limited cross-resistance with rifamycins, or with other characterized inhibitors of bacterial RNA polymerases, including microcin J25 and sorangicin, suggesting a closely related but

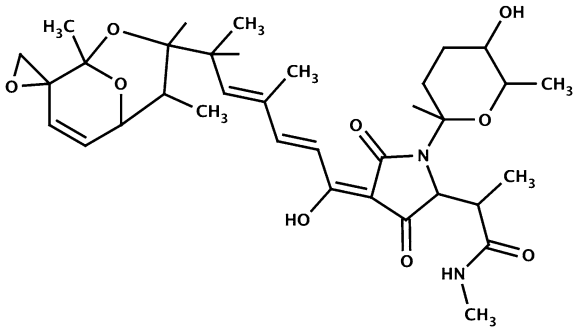


**Fig. 6.3** The tetramic acid antibiotic streptolydigin inhibits transcription by stabilization of a straight-bridge-helix active-center conformation of the bacterial RNA polymerase

different point of primary action. Chemical synthesis of both streptolydigin and tirandamycin has been accomplished by assembly of commercially available precursors in a convergent and stereocontrolled fashion (Pronin and Kozmin 2010; Yadav et al. 2012).

Several substitutions in the $\beta$ and $\beta'$ subunits of bacterial RNA polymerase that confer resistance to streptolydigin have been isolated. Initial biochemical analyses of these functional changes concluded that the antibiotic affects substrate binding by preventing the nucleotide triphosphate insertion step with subsequent inhibition of enzyme catalysis and DNA translocation. However, later studies have shown that the inhibitor binding site is located somehow away from the RNA polymerase active site and includes the bridge helix and the trigger loop, two elements that are considered crucial for the function of the catalytic center (Temiakov et al. 2005). Hence, concluding evidence supports that streptolydigin does not primarily affect the binding of the nucleotide triphosphate substrates, DNA translocation, or phosphodiester bond formation. Rather, the complex structure originated by the interaction between streptolydigin and the bacterial RNA polymerase points to an inhibitory mechanism by which the antibiotic stabilizes catalytically inactive substrate bound transcription intermediates, thereby the blocking structural isomerization of the polymerase to an active configuration (Temiakov et al. 2005). Using a combination of genetic and biochemical approaches, other studies have further defined the target and the structural basis of the mechanism of this inhibition. Streptolydigin binds to a site adjacent to, but not overlapping, the active center of the enzyme which is located in close proximity to the binding site for the inhibitor microcin J25 (Yuzenkova et al. 2002; Adelman et al. 2004), and the inhibition appears associated with effects on the translocational state of the polymerase. In essence, the antibiotic interacts with three structural elements within the enzyme: part of the inhibitory molecule interacts with a receiving pocket and also with the bridge helix, while the tetramic-acid moiety interacts with the trigger-loop region of the enzyme (Tuske et al. 2005). The proposed model highlights that the antibiotic stabilizes a conformational state of the RNA polymerase active center to a straight bridge helix configuration. However, cycling

between alternative straight bridge-helix and bent-bridge-helix conformations of the polymerase active-center is required for enzyme function. Thus, transcription results inhibited by blocking conformational changes of the RNA polymerase during the nucleotide addition cycle, by trapping the trigger loop in its unfolded conformation and the bridge helix in its straight conformation (Tuske et al. 2005).

#### 6.2.2.4 Microcin J25

The finding that the peptide microcin J25 was synthesized by strains of *Escherichia coli* and showed antibacterial properties through inhibition of the bacterial RNA polymerase generated wide interest in the last decade (Bayro et al. 2003; Rosengren et al. 2003). This antibiotic is produced only by *E. coli* strains that harbor a plasmid-borne antibiotic synthesis and an antibiotic export cassette, consisting of a gene for the precursor form, two genes for factors that process the precursor into the final microcin J25, and one gene for export from the cell (Solbiati et al. 1999). The peptide, whose lineal primary structure is shown in Fig. 6.4, adopts an unusual three-dimensional structure with particular folds forming what has been called a lassoed tail (Wilson et al. 2003). Microcin J25 exhibits bactericidal activity against a wide range of Gram-negative bacterial species, including *E. coli.*

The proposed mechanism of action for microcin J25 is that inhibits transcription by, firstly, binding within the secondary channel on the RNA polymerase molecule (known as the nucleotide triphosphate uptake channel or pore), and, second, obstructing this channel in the way a cork acts in a bottle. Blockage of this secondary channel represents a novel mechanism for inhibition of a nucleotide polymerase and an attractive target for discoveries of new drugs showing affinity for this determinant (Mukhopadhyay et al. 2004).

Another related compound, microcin B17, is also a post-translationally modified bactericidal compound that contains thiazole and oxazole heterocycles in a peptide backbone (Fig. 6.4). However, the mode of action of this antibiotic is significantly different from that of microcin J25, and it is considered here because of his genetic

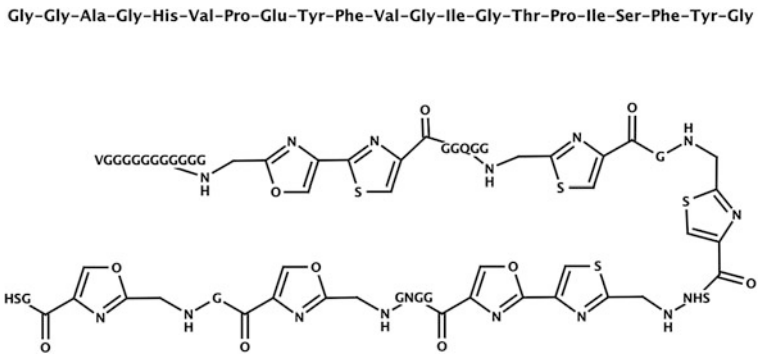


**Fig. 6.4** Primary structures of microcin J25 (*upper formula*) and microcin B17 (*lower formula*)

relatedness. This antibiotic is also coded in nature by *E. coli* strains bearing a plasmid with an operon of similar organization to that of microcin J25, i.e. with genes encoding the precursor polypeptide, the transforming enzymes and the mechanism required for the export of the mature antibiotic. The total synthesis of the 43 amino acid composing this antibacterial peptide has been described (Thompson et al. 2011). Unlike the mode of action of microcin J25, this microcin appears to function as a poison for DNA gyrase. As will be discussed below (see Sect. 6.3), DNA gyrase is a prokaryotic type II topoisomerase whose role is to perform negative supercoiling of closed-circular DNA using the free energy of ATP hydrolysis. This enzyme introduces supercoils by wrapping a segment of DNA around itself, cleaving the DNA in both strands, passing another segment through the break and then resealing the DNA. One of its components, the gyrase A subunit is responsible for the DNA wrapping and DNA breaking step whereas another, the gyrase B, subunit contains the site of ATP hydrolysis as well as the clamp which captures the piece of DNA to be passed through the break. In essence, microcin B17 exerts its effects by binding to the A subunit of DNA gyrase through a mechanism that has similarities to that of the quinolone antibacterial agents, such as the widely used ciprofloxacin, rather to that of microcin J25 (Heddle et al. 2001).

#### 6.2.2.5 Sorangicins

Sorangicins are a family of architecturally complex macrolides polyether antibiotics isolated from the myxobacteria *S. cellulosum*. The family includes potent inhibitors of bacterial, but not eukaryotic DNA-dependent RNA polymerases. Sorangicin A, the most potent and prevalent member of this family, has remarkable antibiotic activity against a broad spectrum of both Gram-positive and Gram-negative bacteria. Despite the lack of apparent chemical and structural similarity between this type of antibiotics and rifamycins, sorangicins inhibit transcription initiation, but not elongation, similar to rifamycins (Irschik et al. 1985). The intricate architecture of soragicins (Fig. 6.5), in conjunction with their important biological properties, has stimulated considerable interest to initiate synthetic approaches. An effective total synthesis of sorangicin A by chemical procedures has been achieved (Smith et al. 2011).

Studies with isolated mutants of RNA polymerase from Gram-negative bacteria showing resistance to sorangicin revealed that they also share partial resistance to rifamycins. This observation led to the initial conclusion that the binding sites for each type of antibiotic largely overlap. In contrast, however, not all mutants isolated for rifamycins resistance show sorangicin resistance, suggesting that there are determinants of the polymerase enzyme that are necessary for interacting with rifamycin but not with sorangicin. A similar study on antibiotic resistance due to mutational changes in the RNA polymerase in Gram-positive bacteria came to the same conclusion (O'Neill et al. 2000). Other congruent result that supports the existence of subtle differences in the way the two antibiotics interact with the

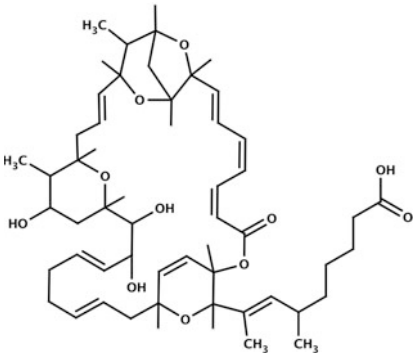


**Fig. 6.5** The molecular structure of sorangicin A

bacterial RNA polymerase is that sorangicin has the potential to work as an anti-tuberculosis drug in rifamycin-resistant strains.

Structural analyses confirm that sorangicin binds in the same $\beta$ subunit pocket of the bacterial RNA polymerase as rifamycins, with almost complete overlap of binding determinants. On the other hand, parallel functional analyses have indicated that both antibiotics inhibit transcription initiation by directly blocking the path of the elongating transcript. Moreover, genetic studies support that the binding of rifamycins is extremely sensitive to mutations expected to change the shape of the antibiotic binding pocket, while this is not the case for sorangicin. Although both antibiotics share the same $\beta$-subunit pocket on the RNA-polymerase, sorangicin displays an advantageous profile against rifamycins-resistant microbial mutants, likely due to the increased conformational flexibility of sorangicin. In contrast to the rigid conformation of rifamycins, sorangicin would show a better adaption to mutational changes in the binding pocket (Campbell et al. 2005).

#### 6.2.2.6 Myxopyronins

Myxopyronins (Fig. 6.6) are α-pyrone antibiotics produced by the myxobacteria *Myxococcus fulvus* that potently inhibit bacterial RNA polymerases without inhibiting the orthologue eukaryotic enzymes (Tupin et al. 2009). The antibacterial activity of these antibiotics, particularly that of myxopyronins A and B, can be directed against a broad spectrum of Gram-positive bacteria and some Gram-negative bacteria. The absence of antibacterial activity against many Gram-negative species appears due to cellular-uptake barriers and the presence of efficient efflux mechanisms. Their bactericidal properties do not exhibit cross-resistance with rifamycins nor with other small-molecule inhibitors of bacterial RNA polymerase described above which function through sites other than the switch region. Also, when co-administered with rifampicin, the myxopyronins show synergistic antibacterial activity (O'Neill et al. 2000; Srivastava et al. 2011).

During the last years, efforts to optimize myxopyronins as attractive antibacterial therapeutic agents have been in progress and more than a hundred analogs

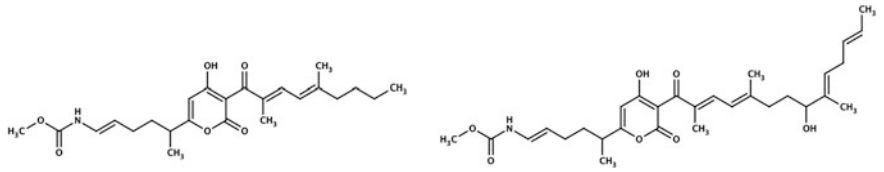

**Fig. 6.6** Myxopyronin B (*left*) and corallopyronin A (*right*)

are today available (Doundoulakis et al. 2004; Lira et al. 2007). Total synthesis of myxopyronins and also of the secondary metabolite corallopyronin (see below) has been recently achieved (Rentsch and Kalesse 2012).

The specific target is located in the RNA polymerase switch region. This antibiotic binds within a nearly completely enclosed, predominantly hydrophobic pocket formed by the so called switch 1, switch 2, and adjacent segments of $\beta$ and $\beta'$. The target does not overlap the rifampicin target, and it is conserved in bacterial RNA polymerases, although three residues of this region on the $\beta$ subunit are radically different in eukaryotic RNA polymerases. These observations explain that myxopyronins do not display cross-resistance with rifampicin and exhibits broad-spectrum activity against bacterial RNA polymerase, although not against its eukaryotic counterparts (Mukhopadhyay et al. 2008). Unlike rifampicin (see above), myxopyronins inhibit transcription initiation by inhibiting the isomerization stage (Mukhopadhyay et al. 2008; Belogurov et al. 2009). In more detail, biochemical experiments with promoter subfragments indicate that they interfere with interactions between the polymerase enzyme and a promoter DNA segment that must be loaded into the RNA polymerase active-center cleft and unwound during the isomerization (Mukhopadhyay et al. 2008). Other experiments show that myxopyronins interfere with opening of the RNA polymerase clamp, which is predominantly open in the absence of the antibiotics, and closed in their presence (Srivastava et al. 2011). Altogether, two mutually non-exclusive models have been proposed for the mechanism inhibiting the isomerization stage. Myxopyronins might interfere with either switch-region conformational changes required for opening of the enzyme clamp to load DNA into the polymerase active-center cleft (Mukhopadhyay et al. 2008) or with contacts between the switch region and the unwound DNA template strand required for DNA unwinding (Belogurov et al. 2009).

### 6.2.2.7 Corallopyronin

Corallopyronin is another α-pyrone antibiotic structurally related to the myxopyronins described above, but possesing an additional side-chain extension (Fig. 6.6). This compound is produced by the myxobacteria *Corallococcus coralloides*, and it shows inhibitory and antibacterial activities quite similar to those of myxopyronins, although with a lower potency (Mukhopadhyay et al. 2008). Other properties, as the

cross-resistance and the frequency the spontaneous resistance, are also essentially identical to those of myxopyronins (O'Neill et al. 2000; Mariner et al. 2011).

The primary target of corallopyronin within the bacterial RNA polymerase was identified by elegant analyses of cross-resistance properties of previously identified myxopyronin-resistant mutants. Other approaches based on experiments involving direct isolation and sequencing of mutants resistant to corallopyronin after mutagenesis of genes coding for subunits of the RNA polymerase have led to similar conclusions. Taken together, the results of these studies strongly support the interpretation that its specific target overlaps the myxopyronin target and includes all residues of the later area plus one additional residue on the $\beta$ subunit (Mukhopadhyay et al. 2008). The target in *S. aureus* is the same as in *E. coli* (Mariner et al. 2011). Like myxopyronins, corallopyronin inhibits the isomerization stage of the polymerase, interferes also the interaction of the enzyme with the promoter, and prevents the opening of the polymerase clamp (Mukhopadhyay et al. 2008; Srivastava et al. 2011). Hence, the mechanism of action of this antibiotic appears to mirror that of myxopyronins.

#### 6.2.2.8 Ripostatins

Ripostatins are macrocyclic-lactone antibiotics structurally unrelated to other switch-region-target inhibitors that are produced by the myxobacteria *S. cellulosum* (Fig. 6.7). Total synthesis of bacterial RNA-polymerase inhibitors ripostatins A and B has been recently reported (Winter et al. 2012). Again, as indicated before for corallopyronins, the properties of ripostatins with respect to the cross-resistance and the resistant mutant frequency are essentially identical to those shown by myxopyronins (O'Neill et al. 2000; Mukhopadhyay et al. 2008). The antibacterial activities steaming from their inhibitory effect on the bacterial RNA polymerase also parallel those of myxopyronins, although they show a narrow antimicrobial spectrum and lower potency.

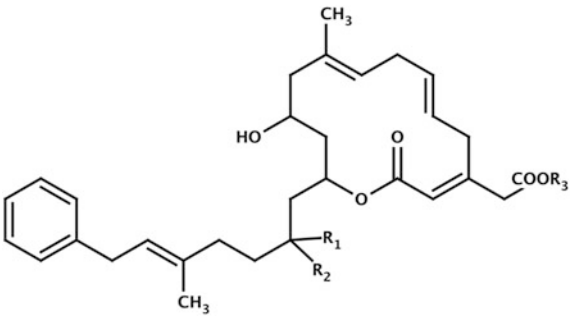


**Fig. 6.7** Basic structural features of ripostatin-type antibiotics. The different nature of the substitutes *R1*, *R2* and *R3* gives rise to the various ripostatins. For instance, in the case of ripostatin B, $R1 = H$, $R2 = OH$, $R3 = H$

A stricking feature of ripostatins is that, in spite of their lack of structural similarity with myxopyronins and corallopyronins, they share common overlapping targets. All these antibiotics inhibit the isomerization stage of the bacterial RNA polymerase, interfere in its interaction with promoter positions and block the opening of the enzyme clamp (Mukhopadhyay et al. 2008; Srivastava et al. 2011).

#### 6.2.2.9 Fidaxomicin

Fidaxomicin (Fig. 6.8), which is also known as clostomicin, tiacumicin, diffimicin, lipiarmycin, dificid or OPT-80, is a macrocyclic-lactone antibiotic produced as fermentation product by various species of actinomycetes, namely *Dactylosporangium aurantiacum, Actinoplanes deccanensis* and *Micromonospora echinospora* (Gerber and Ackermann 2008; Miller 2010). No chemical synthesis of this antibiotic has been described, although several analogs have been prepared by fermentation of the producer strains in the presence of unnatural precursors or by the use of engineered gene-disruption mutants (Srivastava et al. 2011). Fidaxomicin shows potent antibacterial activity against most Gram-positive bacteria and some Gram-negative bacteria. However, the most characteristic feature of this antibiotic is that it exhibits especially potent antibacterial activity against the infectious agent causing pseudomembranous colitis, *Clostridium difficile*, which is often resistant to the treatment by other antibiotics (O'Neill et al. 2000; Ackermann et al. 2004; Artsimovitch et al. 2012; Venugopal and Johnson 2012). Because of its good behavior upon oral administration, fidaxomicin antibiotic was recently approved for the treatment of this kind of infection and associated diarrheas (Traynor 2011).

Fidaxomicin exhibits no cross-resistance with rifampicin (Miller 2010) or with other RNA polymerase inhibitors that function through sites other than the switch region (Srivastava et al. 2011). The frequency of spontaneous resistance to this antibiotic is comparable to, or lower than, the frequency of spontaneous resistance to rifampicin.

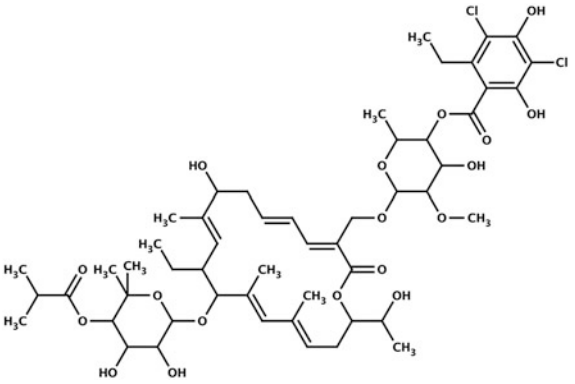


**Fig. 6.8** The macrocyclic antibiotic fidaxomicin approved for selective eradication of pathogenic *C. difficile* strains

The fidaxomicin target appears to be adjacent to the target for myxopyronin, corallopyronin and ripostatin, but without significant overlap (Srivastava et al. 2011). Likely, this is the reason by which fidaxomicin resistant mutants do not show cross-resistance to the above antibiotics or rifampicin (Kurabachew et al. 2008; Gualtieri et al. 2009). Initial biochemical results supported that fidaxomicin inhibits the isomerization step of the bacterial RNA polymerase in a way at least in part similar to the action of myxopyronin, corallopyronin and ripostatin, interfering also with its interactions with promoter regions and with the opening of the enzyme clamp (Srivastava et al. 2011). However, more recent experiments have been conducted to identify the precise blocked step of transcription by bacterial RNA polymerase in which fidaxomicin was added at different stages of transcriptional initiation. DNA footprinting techniques were also performed to further elucidate the stage inhibited in this multistep process. Notably, fidaxomicin was shown to block initiation only if added before the formation of the "open promoter complex," in which the template DNA strands have separated but RNA synthesis has not yet begun. Binding of fidaxomicin precludes the initial separation of DNA strands that is a prerequisite to RNA synthesis. Hence, these studies show that fixadomicin has a mechanism distinct from that of elongation inhibitors, such as streptolydigin, and from the transcription initiation inhibitors myxopyronin and the rifamycins (Artsimovitch et al. 2012).

## 6.3 Inhibition of DNA Replication by Antibiotics

DNA replication is another vital function for living cells since DNA must be copied as part of the normal process of cell division. In the course of an infectious disease, pathogens must be able to replicate its DNA as a prerequisite for spreading. The unzipping and binding with complementary nucleotides on leading and lagging strands requires a complex enzyme machinery. Also, because bacterial DNA is much longer than cell size, it needs to be packed in a narrow cellular space by supercoiling.

### *6.3.1 Selective Toxicity and DNA Replication*

Many inhibitors of DNA replication bind to DNA in both prokaryotic and eukaryotic systems and thus are too toxic for clinical use. However, selective toxicity can be established on the bases of peculiar properties of some of the bacterial enzymes involved in DNA replication.

In particular, bacterial DNA gyrase is a type II topoisomerase which directs the negative supercoiling of closed-circular DNA at the expense of ATP hydrolysis (see also Sect. 6.2.2, microcin B17). Active gyrase is a heterotetramer that consists of two gyrase A catalytic subunits, responsible for DNA breakage and reunion, and

two gyrase B subunits which contain the ATPase active site (Maxwell and Lawson 2003). The key functions of this prokaryotic enzyme, which controls the topological state of DNA molecules during transcription, replication, and cell division, makes gyrase a good target for antibiotics. Although also present in some eukaryotes, the ortholog enzymes are not entirely similar in structure or sequence and show different affinities for different compounds. Moreover, gyrase is not present in humans.

Several natural products inhibit DNA gyrase by blocking the binding of ATP to the domain containing the ATPase activity on the gyrase B subunit. The mechanism of action of these compounds, mainly aminocoumarins and cyclothialidine antibiotics, has been characterized by biochemical methods and crystallographic studies (Oblak et al. 2007). Another subset of representative compounds is also shown whose mode of action is different and well supported by actual data.

### *6.3.2 Novobiocin and Related Aminocoumarins*

Aminocoumarin antibiotics are produced by several species of *Streptomyces*, including *S. niveus, S. caeruleus* or *S. spheroids* (novobiocin; also known as albamycin, streptonivicin or cathomycin), *S. roseochromogenes* (clorobiocin) and *S. rishiriensis* (coumermycin A1) (Wang et al. 2000). These antibiotics act by binding to an N-terminal nucleotide-binding domain of the gyrase B subunit and promoting the inhibition of the enzyme-catalyzed hydrolysis of ATP (Maxwell and Lawson 2003). Aminocoumarins also inhibit DNA topoisomerase IV, which is a type II topoisomerase similar to gyrase and which is involved both in the control of DNA supercoiling and in the decatenation of daughter chromosomes after DNA replication (Anderle et al. 2008). They show poor solubility in water and poor oral absorption. Moreover, in contrast to quinolones (synthetic DNA gyrase inhibitors) which are broad spectrum antimicrobial agents, coumarins show low levels of activity against Gram-negative bacteria and display some toxicity in eukaryotes (Downes et al. 1985).

The structure of novobiocin and that of the closely related aminocoumarin clorobiocin (Fig. 6.9) contains a hydroxybenzoyl moiety, a dihydroxycoumarin moiety with a methyl group and a chlorine atom, respectively, and a substituted deoxysugar. The coumarin moiety and the substituted deoxysugar moiety are essential for their binding to the gyrase B subunit of the bacterial enzyme. Clorobiocin also differs from novobiocin in the substitution of a noviose carbamoyl (novobiocin) for a 5-methyl-2-pyrrolyl-carboxyl group (clorobiocin) able to occupy an additional hydrophobic pocket in the gyrase B subunit and to displace two water molecules (Lewis et al. 1996), that results in an enhancement of the inhibition of gyrase in vitro. Thereby, clorobiocin binds more effectively to the gyrase B subunit than novobiocin.

The ring moiety that represents the 3-dimethylallyl-4-hydroxybenzoic acid component shared by novobiocin and clorobiocin (at the right of their respective

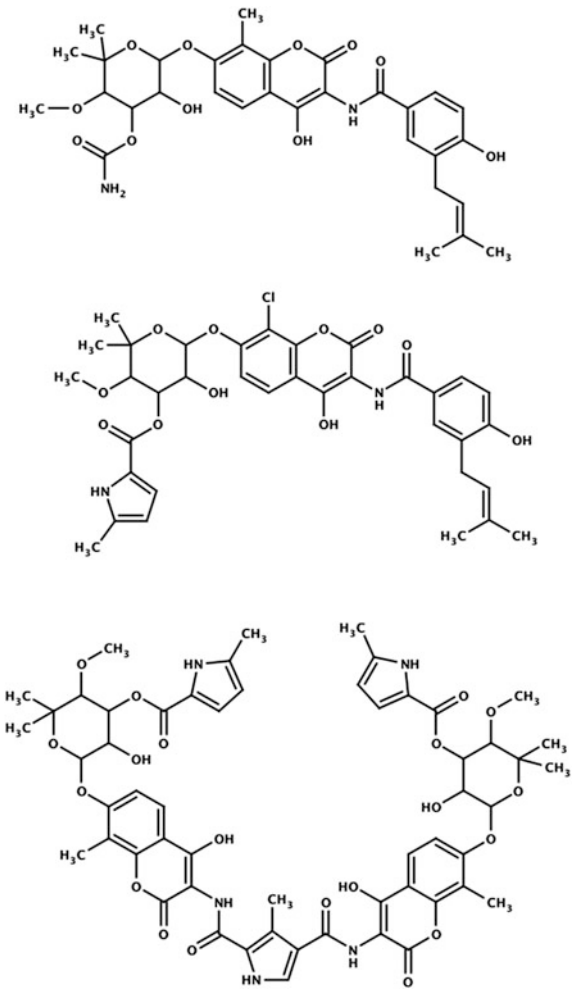

**Fig. 6.9** The aminocoumarin antibiotics novobiocin (*up*), clorobiocin (*centre*) and coumermycin A1 (*down*)

formula in Fig. 6.9), interacts barely via hydrophobic bonds with the B subunit of gyrase and contributes only weakly to the antibacterial activity (Lafitte et al. 2002). However, this ring appears to influence the uptake of the compound into the bacterial cell. Furthermore, a comparison of the ability of clorobiocin and novobiocin to inhibit the DNA supercoiling reaction and bacterial growth reveals substantial differences in the relative coumarin concentrations required in vitro and in vivo.

The coumermycin A1 molecule (Fig. 6.9) contains two active aminocoumarin-deoxysugar moieties and has been shown to stabilize a dimer form of a 43-kDa fragment of gyrase B. Therefore, coumermycin A likely cross-links the two gyrase B subunits of the intact gyrase heterotetramer. Consequently, its affinity for intact gyrase is extremely high as compared to novobiocin, or the synthetic quinolones norfloxacin and nalidixic acid. Likewise, coumermycin A1 has been found to

exhibit much higher antibacterial activity than novobiocin. In coumermycin A1, a methylpyrrole-2-carboxylic acid unit is attached to the 3-OH of each deoxysugar moiety. The same pyrrole unit is also contained in clorobiocin and has been shown to result in a higher affinity for gyrase than that of the carbamoyl group found in the corresponding position of novobiocin (Tsai et al. 1997). In contrast, the ring shared by novobiocin and clorobiocin indicated above is absent in coumermycin A1 confirming the observation that such part of the molecule may not be quite essential for biological activity. Altogether, these features make coumermycin A1 a most interesting starting compound for the development of new aminocoumarin antibiotics, which may serve as anti-infective agents against multiresistant bacteria. The chemical synthesis of a series of new aminocoumarin antibiotics has been accomplished (Brvar et al. 2012) and combinatorial biosynthesis with the biosynthetic gene clusters for the aminocoumarin antibiotics could provide additional possibilities for the discovery of novel anti-infective agents. The genetic manipulation of the biosynthesis of antibiotics has already succeeded in the production of new and even clinically useful "hybrid" antibiotics (Wang et al. 2000).

Nevertheless, the real state of the art reveals that novobiocin is the only aminocoumarin which has been so far licensed for human therapy. It is mainly used as an orally active antibiotic for the treatment of infections with multiresistant Gram-positive bacteria, such as *Staphylococcus epidermidis* and *Staphylococcus aureus* (Anderle et al. 2008; Schröder et al. 2012). Except in these cases it is rarely employed because of the high incidence of adverse reactions and the frequent emergence of resistant strains. For this reason, it is usually given in combination with another antibacterial compounds. Novobiocin derivatives have garnered the attention of numerous researchers as attractive agents for various treatments (Tambo-ong et al. 2011). It has been reported that this antibiotic binds to the Hsp90 C-terminal nucleotide-binding site and induces degradation of Hsp90 client proteins (Allan et al. 2006). Also, structural modifications of novobiocin have led to analogues with 1000-fold greater efficacy in anti-proliferative assays against various cancer cell lines (Donnelly and Blagg 2008).

### *6.3.3 Cyclothialidine*

Cyclothialidine contains a lactone ring partly integrated into a pentapeptide chain (Fig. 6.10) and it is produced by *Streptomyces filipinensis* (Watanabe et al. 1994). Although structurally distinct from coumarin compounds, it can exert similar inhibitory effects on nucleotide binding by interfering with the ATPase activity of the B subunit of DNA gyrase and also inhibits DNA topoisomerase IV. This antibiotic had been shown to be a valuable lead structure for the discovery of new antibacterial classes able to overcome bacterial resistance to clinically used drugs (Angehrn et al. 2011; Saíz-Urra et al. 2011).

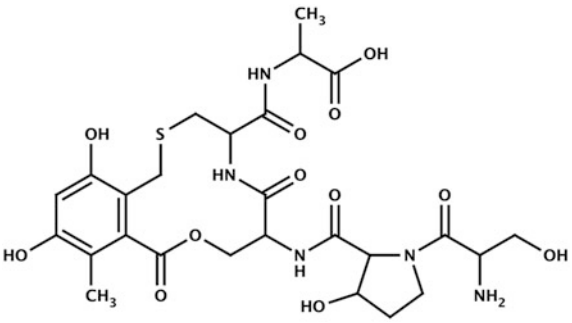

**Fig. 6.10** The DNA gyrase inhibitor cyclothialidine isolated from *S. filipinensis*

## *6.3.4 Antibiotics Affecting DNA with Antitumor Properties*

### 6.3.4.1 Mitomycins

The mitomycins are a family of potent antibacterial antibiotics isolated from several *Streptomyces* strains, like *S. lavendulae*. They show clinical interest because of their activity as anti-cancer compounds in a broad range of tumors. The more common mitomycins are named from A to K. They have an asymmetric structure and exhibit a tetracyclic pyrrolo-indole skeleton an aziridine ring, a carbamoyl moiety and a bridged carbinolamine packed in a constrained architecture (Fig. 6.11). The presence of such a concentration of functional groups renders this type of molecule particularly reactive. Mitomycins were rapidly identified to act as pro-drugs and their unique activity was thought to originate from their ability to transform in vivo to generate the active metabolite. This was followed by decades of investigations to understand in detail their singular mode of action. It was found that the aziridine played a crucial role, allowing an irreversible bis-alkylation of DNA (Yudin 2006). Mitomycins attach to the DNA helix and inhibit or block the functional expression of the DNA polymerase enzyme and thus DNA replication.

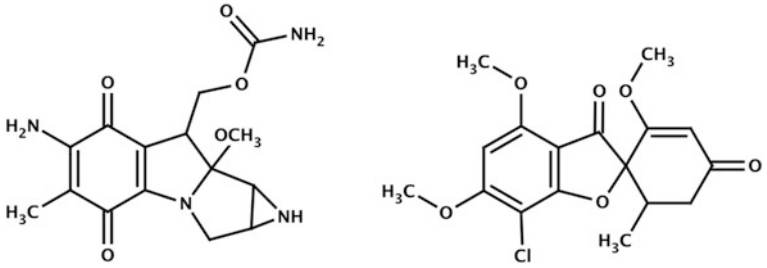

**Fig. 6.11** The DNA crosslinker mitomycin C (*left*) and the antifungal drug griseofulvin (*right*), originally developed as antimicrobial agents, show properties for potential cancer treatments

One of these compounds, mitomycin C, has been characterized as a potent DNA crosslinker. Interstrand cross-links are among the most cytotoxic DNA lesions to cells because they prevent the two DNA strands from separating, thereby precluding strand opening during replication and transcription. This agent reacts principally at the N2 position of guanine to form a G–G interstrand cross-link at CpG sites, or a G–G intrastrand cross-link at GpG sites (Warren and Hamilton 1996). A single crosslink per genome has shown to be effective in killing bacteria. This is accomplished by reductive activation followed by two N-alkylations that are sequence specific for a guanine nucleoside in the sequence described above, 5′-CpG-3′ or 5′-GpG-3′ (Tomasz 1995). Considering this mode of action, potential bis-alkylating heterocylic quinones have been synthesized in order to explore their antitumoral activities by bioreductive alkylation.

Mitomycin C is useful as a chemotherapeutic agent due to its dual ability as antitumor and antibacterial activity. It has been used against a variety of tumors and become one of the most effective drugs against lung carcinoma, as well as other soft and solid tumors (Bradner 2001; Wolkenberg and Boger 2002). This antibiotic has been used topically and intravenously in several areas but causes delayed bone marrow toxicity so that prolonged use may result in permanent damage. Besides the well-known antibiotic and antitumor properties of these compounds, other semi-synthetic derivatives have been investigated in clinical trials (Casely-Hayford et al. 2005). The natural products themselves are so sensitive that only minor modifications have been possible in connection with medicinal chemistry studies. Even fifty years after their discovery, the mitomycins are still regarded as challenging synthetic targets, and a practical synthetic route has yet to be discovered (Andrez 2009).

#### 6.3.4.2 Griseofulvin

Griseofulvin (Fig. 6.11) is not an antibacterial antibiotic but an antifungal compound produced by various species of *Penicillium*, like *P. griseofulvum*. Although not directly involved in inhibition of DNA synthesis, this compound has been considered as related to nucleic acid-affecting antibiotics due to its effects on DNA distribution in dividing fungal cells. Griseofulvin also inhibits plant and mammalian cells by inducing abnormal mitosis and promoting blockage at G2/M phase of the cell cycle (Panda et al. 2005; Rebacz et al. 2007). This antibiotic interacts with tubulin as well as microtubule associated proteins. However, its selective toxicity relies in the fact that different organisms exhibit different degrees of sensitivity due to a differential affinity of the antibiotic toward tubulins of various origins. The concentration required to inhibit the growth of fungal cells is much lower than that required to inhibit mammalian cells (Czymmek et al. 2005). On this basis, griseofulvin has been extensively used both in humans and in animals to treat epidermal fungal infections by oral administration. Griseofulvin has tissue-affinity for the skin, accumulates finally in the keratin layers of the epidermis and inhibits there fungal growth. However, it is ineffective topically, and it is

considered relatively of low toxicity to human beings (Chan and Friedlander 2004). Once absorbed by the blood is transported to the basal cells and establishes stable complexes in keratin precursor cells. The drug reaches its final site of action when the skin is replaced by keratinized cells that retain sufficient amount of drug to inhibit the growth of the dermatophyte fungi. The antibiotic enters the fungal cells through energy dependent transport mechanisms and binds to fungal microtubules altering the processing for mitosis.

In contrast to normal cells, the presence of supernumerary centrosomes and aneuploidy are characteristics associated with the malignant phenotype of tumor cells. To solve the threatening problem of multipolar spindles, cancer cells use a singular mechanism of centrosomal clustering during division to ensure that the correct genetic material is present within each of the tumor daughter cells. Essentially, supernumerary centrosomes are clustered into two functional spindle poles to allow for bipolar mitotic division. Interestingly, griseofulvin may inhibit proliferation of different types of tumor cells and induce apoptosis by interfering the microtubule assembly dynamics, blocking centrosome coalescence, promoting mitotic arrest, and leading to subsequent cell death (Rebacz et al. 2007; Bramann et al. 2012) Hence, as part of future strategies against cancer, this antibiotic may further improve its effectiveness combined with other anticancer drugs in adjuvant therapy and lead to the development of more effective drug treatments with low toxic side effects (Rathinasamy et al. 2010). The characterization of novel derivatives of griseofulvin as potent inhibitors of centrosomal clustering in malignant cells is being currently investigated (Raab et al. 2012).

Although tubulin was identified as the primary target of griseofulvin in early studies, the exact binding site in tubulin is still a matter of controversy. It has been suggested that it binds at a site distinct from the colchicine binding site in tubulin (Chaudhuri and Luduena 1996). In any case, even at low concentrations, griseofulvin exerts a strong suppressive effect on the dynamics of the individual microtubules that has an inhibitory effect on cell proliferation (Panda et al. 2005). More recently, two potential binding sites in mammalian tubulin have been identified and a mechanistic explanation of how the antibiotic stabilizes microtubule dynamics has been provided (Rathinasamy et al. 2010).

#### 6.3.4.3 Other Antibiotics Affecting DNA with Antitumor Properties

In line with the above mentioned antibiotics it is worth to mention the existence of other types of natural compounds of microbial origin that can be exemplified by doxorubicin and clerocidin. DNA topoisomerases are the common targets of these antibacterial and anticancer drugs. While bacterial type II topoisomerase (gyrase) is the target of aminocoumarin antibiotics and synthetic quinolones (see above), the function of human type II topoisomerases can be inhibited by a series of novel antibiotics able to affect tumor cells through DNA inhibition. In this context, doxorubicin (adriamycin or daunoribucin) can be considered quite different from griseofulvin in structure and mode of action but functionally related to mitomycins

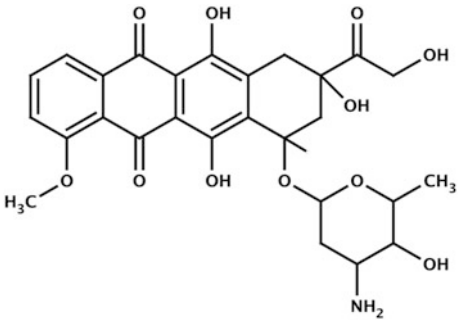


**Fig. 6.12** The anthracycline antibiotic doxorubicin is useful for treatment of a wide range of cancers

because of its direct interaction with DNA. Doxorubicin is a photosensitive anthracycline (Fig. 6.12) produced by *Streptomyces peuceticus* as a red pigment. The mechanism of inhibition relies on its intercalation in the double helix, thus inhibiting the progression of the human topoisomerase II which relaxes supercoils in DNA during transcription and replication. The blockage of the topoisomerase II complex after breakage of the DNA chain for replication prevents the double helix from being resealed thus producing an arrest in the cell division (Pommier et al. 2010). The analog mitoxantrone, which is an anthracenedione derivative of doxorubicin, also engages in intercalation, disruption of DNA synthesis and DNA repair. Another structurally unrelated antibiotic with potent antibacterial and antitumor activity is clerocidin, a microbial terpenoid capable of poisoning both the prokaryotic DNA gyrase and the eukaryotic DNA topoisomerase II (Richter et al. 2004). The continued development of this kind of novel antibiotics with attenuated or low levels of toxicity may open new therapeutic pathways to control malignant cells.

## 6.4 Unified Theory of Antibiotic Action on Nucleic Acids

Some unanswered questions remain on the mechanism of killing by many antibiotics. For example, we do not know for certain how beta-lactams induce autolysis nor how exactly mistranslation caused by aminoglycosides leads to cell death. In this line, the traditional view that bactericidal antibiotics kill by modulating their respective targets has been challenged by studies that propose an alternative, unified mechanism of killing. Although recent works have questioned the relevance of such interpretation, this is still a matter of current debate.

The classification of antibiotics by their mode of action is based upon drug-target interaction and whether the resultant inhibition of cellular function is lethal to bacteria. However, the complete understanding of many of the bacterial responses occurring as a consequence of the primary drug-target interaction remains incomplete and there have been suggestions that a common mechanism of cellular death might be underlying the action of all classes of bactericidal antibiotics

(Kohanski et al. 2007). Some work reveals that the real killing mechanism of antibiotics might be predominantly due to the common production of highly destructive reactive oxygen species (ROS) which promote hydroxyl-induced damage to the nucleotide base guanine, one of the DNA constituents (Foti et al. 2012). The evidence obtained supports that the oxidized guanine in the nucleotide pool appears to play an important role in the cell death mediated by the presence of diverse and structurally unrelated antibiotics, ranging from beta-lactams to aminoglycosides, or the synthetic nucleic acid-affecting drugs quinolones. The damaged guanine is included into DNA by the DNA-copying enzyme DinB (DNA polymerase IV), which inserts the oxidized guanine opposite to its correct base partner cytosine on the complementary strand during DNA replication. However, the enzyme also does the same opposite to its incorrect partner adenine. Attempting to replace the inaccurately paired base, cells try then to correct the complementation damage by setting in motion the repair DNA system through the action of specific enzymes which make cuts in one of the strands. Hence, when two such repairs occur in close proximity and in opposite DNA strands, the DNA molecule suffers a double-strand break and the loss of its physical continuity, making the process of replication impossible and leading to cell death. Even penicillin might act this way. The bactericidal action of aminoglycosides could additionally result from mistranslation due to the incorporation of oxidized guanine into newly synthesized mRNAs. When too many oxidized guanines incorporate into new mRNA strands the genetic messages are highly altered and the resulting lesions entail loss of viability. Under this view, because most existing antibiotics should ultimately to affect nucleic acids, a vast array of structurally distant antibiotic compounds might likely be included under the general title of this chapter.

Evidence against the hypothesis that classic antibiotics kill bacteria by stimulating the formation of ROS rests on the fact that antibiotic treatment does not necessarily accelerate the formation of hydrogen peroxide nor elevate in all cases the intracellular essential reactants for the production of lethal damage (Liu and Imlay 2013; Keren et al. 2013). Also, the survival of bacteria treated with various antibiotics under aerobic or anaerobic conditions was found similar and some DNA repair mutants were not hypersensitive, thus undermining the idea that toxicity arises from oxidative DNA lesions. Ampicillin was used in such studies to block cell-wall synthesis, kanamycin to target translation, and norfloxacin to disrupt DNA replication. Consequently, these controversial studies conclude that lethality more likely results from the direct inhibition of cell-wall assembly, protein synthesis, and DNA replication, respectively. In other words, they support that the major classes of antibiotics exert their lethal actions through the classical mechanisms rather than through common oxidative stress pathways.

In the last few decades only a handful of new classes of antibiotics have been introduced. The possible existence of a common mechanism of cell death for bactericidal antibiotics offers several outcomes and has broad implications for their effectiveness. Since a new model suggests that DNA polymerases and DNA repair enzymes play an important role in the intrinsic susceptibility of bacteria to antibiotics such possibility should be further explored. If correct, bactericidal antibiotics

could be potentiated by targeting proteins involved in repairing double-stranded DNA breaks, or by influencing the incorporation of oxidized guanine into the DNA and RNA or the consequences of this incorporation (Foti et al. 2012).

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# Chapter 7
# Genetic Analysis and Manipulation of Polyene Antibiotic Gene Clusters as a Way to Produce More Effective Antifungal Compounds

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**Abstract** The present clinical scenario is one of the growing numbers of immunocompromised patients infected with a variety of fungal pathogens. AIDS, tuberculosis, immunosuppressive therapy, cancer chemotherapy, or the use of broad-spectrum antibiotics contribute to the boost of such patient category. However, progress in the treatment of fungal infections has been slow. Different polyenes and/or azoles are available for the treatment of systemic fungal infections, but it is becoming clear that new and more effective antifungal agents are urgently required. Fortunately, biosynthetic gene clusters for several antifungal polyene macrolides have been characterized, and this opened the way to generate improved antifungal compounds via genetic engineering as well as to understand the molecular mechanisms that regulate polyene production. Here, we will review the history of the discovery of antifungal polyenes, their environmental role, and the strategies currently used for their identification and improvement.

## 7.1 Introduction

The polyene antibiotics form a subgroup of the macrolide antibiotics containing hydroxylated macrocyclic lactone rings and usually one or more sugars. From the biosynthetic point of view, the macrolides are a homogenous group, being synthesized from acetate, propionate, and other short-chain fatty acids via the polyketide pathway (Hopwood and Sherman 1990). The macrolide antibiotics are divided into two subgroups: (a) polyene macrolides (mostly antifungal) and (b)

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nonpolyene macrolides (antibacterial) antibiotics. The polyene macrolides have lactone rings of 26–38 carbon atoms, which are much larger than those of the nonpolyene macrolides.

The polyene subgroup has a chromophore formed by a system of three to seven conjugated double bonds in the macrolactone ring. Polyene macrolides are amphipathic molecules containing both a rigid planar lipophilic portion and a flexible hydrophilic polyhydroxylated region. The chromophore accounts for some of the characteristic physical and chemical properties of the polyenes (strong light absorption, photolability, and poor solubility in water) and appears to be responsible for the differences in the mode of action of the polyene and nonpolyene macrolide subgroups.

Polyene macrolide compounds have a characteristic three-peak UV absorbance spectrum, and the number of conjugated double bonds determines the wavelengths of the peak maxima. The UV absorbance spectrum can therefore be used to identify potential polyene macrolide-producing isolates. The chromophore gives a typical multipeak ultraviolet-visible light absorption spectrum. Polyene macrolides are subdivided into trienes, tetraenes, pentaenes, hexaenes, and heptaenes according to the number of conjugated double bonds in the chromophore (Fig. 7.1 and Table 7.1).

A large number of polyene macrolide antibiotics have an aminosugar (or neutral sugar) moiety that is linked to the macrolide ring by a glycoside bond. In all cases, the aminosugar is attached to a carbon bearing a hydroxyl group adjacent to the chromophore. Two different aminosugars have been described as components of polyene macrolide antibiotics, namely mycosamine (3-amino-3, 6'dideoxy-D-mannose) and its isomer perosamine (4-amino-4, 6-dideoxy-D-mannose) (Table 7.2).

Mycosamine is present in all the aminosugar-containing polyene macrolides described so far, with the exception of the heptaene perimycin (fungimycin) (Borowski et al. 1961) and the tetraene JBIR-13 (Komaki et al. 2009), which contain perosamine.

Until 1979, it was believed that neutral sugar did not occur in polyene macrolides, but Zielinski et al. (1979) isolated the first neutral sugar from nystatin A3, candidinin, and polyfungin B and identified it as 2,6-dideoxy-L-ribohexopyranose (L-digitoxose); the pentaene lienomycin contains the neutral sugar rhamnose (Pawlak et al. 1980), and there are also polyene antibiotics with two sugar moieties as antibiotic NPP (Lee et al. 2006), nystatin P1 (Barke et al. 2010), or antibiotic 67-121C (Stephens et al. 2013).

Several polyenes belonging to the heptaene subgroup (ascosin, aureofungin, ayfactin, candicidin, heptamycin, levorin, trichomycin, DJ 400 B2, vacidin, etc.)

**Table 7.1** Absorption spectrum (UV-visible, nm) of the different polyene macrolide antibiotics

| | | | |
|---|---|---|---|
| Trienes | 262 | 272 | 283 |
| Tetraenes | 291 | 304 | 318 |
| Pentaenes | 317 | 331 | 350 |
| Methylpentaenes | 323 | 340 | 357 |
| Hexaenes | 340 | 358 | 380 |
| Heptaenes | 360 | 382 | 405 |

**Table 7.2** Chronology of the polyene antibiotic discovery

| Name | Producer strain | Chromophore subgroup | Aminosugar moiety | Aromatic moiety[a] | Reference |
|---|---|---|---|---|---|
| Nystatin (fungicidin) | *Streptomyces noursei* | Tetraene | Mycosamine | | Hazen and Brown (1950) |
| Rimocidin | *Streptomyces rimosus* | Tetraene | | | Davisson et al. (1951) |
| Ascosin | *Streptomyces canescus* | Aromatic heptaene | Mycosamine | PAAP | Hickey et al. (1952) |
| Candicidin | *Streptomyces griseus* | Aromatic heptaene | Mycosamine | PAAP | Lechevalier (1953) |
| Flavacid | *Streptomyces flavus* | Hexaene | | | Takahashi (1953) |
| Trichomycin A | *Streptomyces hachijoensis* | Aromatic heptaene | Mycosamine | PAAP | Hosoya et al. (1953) |
| Candidin | *Streptomyces viridoflavus* | Heptaene | Mycosamine | | Taber et al. (1954) |
| Amphotericin B | *Streptomyces nodosus* | Heptaene | Mycosamine | | Oura et al. (1955) |
| Eurocidin | *Streptomyces eurocidicus* | Pentaene | Mycosamine | | Osato et al. (1955) |
| Filipin (Duramycin) | *Streptomyces filipensis* | Methylpentaene | | | Ammann et al. (1955) |
| Fungichromin | *Streptomyces cellulosae* | Pentaene | | | Tytell et al. (1955) |
| Aureofacin (ayfactin) | *Streptomyces aureofaciens* | Aromatic heptaene | Mycosamine | PAAP | Igarashi et al. (1956) |
| Etruscomycin (lucensomycin) | *Streptomyces lucensis* | Tetraene | Mycosamine | | Arcamone et al. (1957) |
| Flavofungin | *Streptomyces flavofungini* | Carbonyl pentaene | | | Uri and Bekesi (1958) |
| Tennecetin (pimaricin) | *Streptomyces chattanoogensis* | Tetraene | Mycosamine | | Burns and Holtman (1959) |
| Tetrin A and B | *Streptomyces* sp. | Tetraene | Mycosamine | | Gottlieb and Pote (1960) |
| Perimycin | *Streptomyces coelicolor* | Aromatic heptaene | Perosamine | MPAAP | Borowski et al. (1961) |
| Dermostatin | *Streptomyces viridogriseus* | Hexaene | | | Thirumalachar and Menon (1962) |
| Hamycin | *Streptomyces primprina* | Aromatic heptaene | Mycosamine | PAAP | Gokhale (1963) |
| Levorin | *Streptomyces levoris* | Aromatic heptaene | Mycosamine | PAAP | Mylyshkina et al. (1963) |
| Aureofungin | *Streptomyces cinnamomeus* | Aromatic heptaene | Mycosamine | PAAP | Thirumalachar et al. (1964) |
| Monicamycin | *Streptoverticillium cinnamomeus* var. *monicae* | Heptaene | | | Gupta (1964) |
| Mycoheptin | *Streptoverticillium mycoheptinicum* | Heptaene | Mycosamine | | Borovskii et al. (1965) |
| Mycoticin A and B | *Streptomyces ruber* | Hexaene | | | Wasserman et al. (1967) |

(continued)

**Table 7.2** (continued)

| Name | Producer strain | Chromophore subgroup | Aminosugar moiety | Aromatic moiety[a] | Reference |
|---|---|---|---|---|---|
| Roflamycoin (flavomycoin) | *Streptomyces roseoflavus* | Pentaene | | | Schlegel and Thrum (1968) |
| DJ-400-B2 | *Streptomyces surinam* | Aromatic heptaene | Mycosamine | PAAP | Bohlmann et al. (1970) |
| DJ-400-B1 | *Streptomyces surinam* | Aromatic heptaene | Mycosamine | MPAAP | Bohlmann et al. (1970) |
| Tetramycin | *Streptomyces noursei* var. *jenensis* | Tetraene | Mycosamine | | Dornberger et al. (1971) |
| Chainin | *Chaina* sp. | Methylpentaene | | | Pandey et al. (1972) |
| Heptafungin | *Streptomyces longisporolavandulae* | Aromatic heptaene | Mycosamine | PAAP | Kalasz et al. (1972) |
| Sch 16656. | *Actinoplanes* NRRL5325 | Heptaene | | | Wagman et al. (1975) |
| Candihexin A | *Streptomyces viridoflavus* | Hexaene | Mycosamine | | McDaniel (1976) |
| Aurantinin | *Bacillus aurantinus* | Triene | | | Nishikiori et al. (1978) |
| Hexafungin | *Streptomyces hygroscopicus* | Hexaene | | | Thrum et al. (1978) |
| Hydroheptin | *Streptomyces* IMRU3962 | Heptaene | Mycosamine | | Tunac et al. (1979) |
| Lienomycin | *Actinomyces diastatochromogenes lienomycini* | Pentaene | Rhamnose | | Pawlak et al. (1980) |
| Elizabethin | *Streptomyces elizabethii* | Pentaene | | | Pirt et al. (1981) |
| Acmycin | *Streptomyces* AC2 | Heptaene | | | Chakrabarti and Chandra (1982) |
| Tetrafungin | *Streptomyces albulus* subsp. *tetrafungini* | Tetraene | | | Veiga and Fabregas (1983) |
| PA-5 | *Streptoverticillium* sp 43/16 | Pentaene | | | Soliveri et al. (1987) |
| PA-7 | *Streptoverticillium* sp 43/16 | Heptaene | | | Soliveri et al. (1987) |
| Vacidin A | *Streptomyces griseus* | Aromatic heptaene | Mycosamine | PAAP | Sowinski et al. (1989) |
| Faerifungin | *Streptomyces griseus autotrophicus* | Pentaene | | | Nair et al. (1989) |
| Trichomycin B | *Streptomyces hachijoensis* | Aromatic heptaene | Mycosamine | PAAP | Komori (1990) |
| YS-822A | *Streptoverticillium eurocidicum* var. *asterocidicus* | Tetraene | Mycosamine | | Itoh et al. (1990) |
| AB023 A y B | *Streptomyces* | Pentaene | | | Bortolo et al. (1993) |
| HM17 | *Spirillospora* | Methylpentaene | | | Hacene et al. (1994) |

(continued)

**Table 7.2** (continued)

| Name | Producer strain | Chromophore subgroup | Aminosugar moiety | Aromatic moiety[a] | Reference |
|---|---|---|---|---|---|
| Ab021 | *Streptomyces* sp. | Not reported | | | Scacchi et al. (1995) |
| Linearmicin A | *Streptomyces* n° 30 | Linear polyene | | | Sakuda et al. (1995) |
| 3874 H1 | *Streptomyces* sp. HAG003874 | Aromatic heptaene | | PAAP | Vertesy et al. (1998) |
| 3874 H3 | *Streptomyces* sp. HAG003874 | Heptaene | | | Vertesy et al. (1998) |
| HA-94 | *Streptoverticillium cinnamomeus* var. *scleroticum* | Pentaene | | | Paradkar et al. (1998) |
| Tetrin C | *Streptomyces* sp. GK9244. | Tetraene | | | Ryu et al. (1999) |
| AB-400 | *Streptomyces costae* | Tetraene | Mycosamine | | Canedo et al. (2000) |
| HA-2-91 | *Streptomyces arenae* var. *ukrainiana* | Tetraene | Mycosamine | | Gupte and Kulkarni (2002) |
| Takanawaenes | *Streptomyces* sp. K99-5287 | Pentaene | | | Fukuda et al. (2003) |
| CE-108 | *Streptomyces diastaticus* 108 | Tetraene | | | Perez-Zuniga et al. (2004) |
| ECO-02301 | *Streptomyces aizunensis* NRRLB-11277 | Linear polyene | Mycosamine | | McAlpine et al. (2005) |
| NPP | *Pseudonocardia autotrophica* KCTC9441 | Tetraene | Two sugars | | Lee et al. (2006) |
| Mediomicins | *Streptomyces mediocidicus* | Linear polyene | | | Cai et al. (2007) |
| SJA-95 | *Streptomyces* sp. S24 | Heptaene | | | Naik et al. (2007) |
| RKG-A2215A | *Streptomyces* A2215 | Pentaene | | | Osada et al. (2009) |
| JBIR-13 | *Streptomyces bicolor* NBRC12746 | Tetraene | Perosamine | | Komaki et al. (2009) |
| Marinisporolides | *Marinispora* | Pentaene | | | Kwon et al. (2009) |
| Nystatin P1 | *Pseudonocardia* P1 | Tetraene | Two sugars | | Barke et al. (2010) |
| 32,33-didehydroroflamycoin | *Streptomyces durmitorensis* | Pentaene | | | Stodulkova et al. (2011) |
| Urticifoleno | *Vernonia urticifolia* (a plant) | Linear polyene | | | Kiplimo et al. (2011) |
| Antifulgamicin | *Streptomyces padanus* | Tetraene | | | Xiong et al. (2012) |
| Bahamaolides A and B | *Streptomyces* sp. | Hexaene | | | Kim et al. (2012) |
| 67-121C | *Actinoplanes caeruleus* | Aromatic heptaene | Two sugars | PAAP | Stephens et al. (2013) |

[a] *PAAP* *p*-aminoacetophenone, *MPAAP* N-methyl-*p*-acetophenone

were described as aromatic polyenes, and they have an aromatic *p*-aminoacetophenone moiety. Other aromatic heptaenes, including DJ 400 B1, and perimycin, have an aromatic N-methyl-*p*-aminoacetophenone moiety (N-methyl-PAAP) (Table 7.2).

The polyene antibiotics were essentially described as antifungal compounds that directly target the plasma membrane via a specific interaction with the main fungal sterol, ergosterol, often resulting in membrane permeabilization (Hamilton-Miller 1973; Zotchev 2003). This was accepted by the scientific community, but there was an earlier work where it was described that pimaricin formed complexes with cholesterol which resulted in membrane pores, but they were of insufficient length to traverse the cell membrane (de Kruijff and Demel 1974). Recently, it was demonstrated that natamycin/pimaricin inhibits growth of yeasts and fungi via the immediate inhibition of amino acid and glucose transport across the plasma membrane (Welscher et al. 2012). It was proposed that inhibition of membrane proteins ergosterol-dependent is a general mode of action of all the polyene antibiotics, being permeabilization of plasma membrane a collateral effect (Welscher et al. 2012).

Polyene antibiotics, as other secondary metabolites, showed a variety of unexpected functions and applications (Vaishnav and Demain 2011), and they include hypocholesterolemic activity (Schaffner and Gordon 1968), antibacterial activity (Nair et al. 1989), immunomodulation (Henry-Toulme et al. 1989), inhibition of HIV replication (Pontani et al. 1989), anticancer (Feigin 1999), antiprion (Soler et al. 2008), and antiprotozoal activity (Tewary et al. 2006).

Polyene antibiotics are synthesized by the common pathway for all polyketides, in which basic units derived from acetate, propionate, or butyrate are condensed onto the growing chain by a polyketide synthase (PKS), in similar way to the biosynthesis of the long-chain fatty acids, except that the beta-keto function introduced at each elongation step may undergo all, part or none of a reductive cycle comprising beta-keto reduction, dehydration, and enoyl reduction (Hopwood and Sherman 1990)

Polyene antibiotics are produced by the activity of type I modular PKSs that consist of several extremely large polypeptides in which different modules (sets) of enzymatic activities catalyze each successive round of elongation (Aparicio et al. 2003). The biosynthesis requires one starter unit (typically activated as acetyl-CoA, propionyl-CoA, or *p*-aminobenzoyl-CoA), and a highly variable number of malonate or methylmalonate (as malonyl-CoA and methylmalonyl-CoA) units are similarly attached together. In the aromatic polyene biosynthesis, as candicidin, the starter unit is PABA, activated as *p*-aminobenzoyl-CoA (Campelo and Gil 2002).

The structural variation between naturally occurring polyenes arises, as in other polyketide macrolides, largely from the way in which the PKS controls the number and type of starter and extender units used, and from the extent and stereochemistry of reduction at each cycle. The polyketide is further modified by tailoring enzymes to imbue them with various biological activities and include the action of glycosylases, methyltransferases, and oxidative enzymes (Martin and Aparicio 2009).

## 7.2 Discovery of Polyene Producer Microorganisms

Polyene macrolide antibiotics include more than 200 members produced by actinomycetes, mainly of the genus *Streptomyces*, although the production of polyenes by species of *Streptoverticillium*, *Actinosporangium*, *Actinoplanes*, *Chainia, Pseudonocardia, Spirollospora,* and *Marinispora* among others has been reported (see Table 7.2).

Since 1950, many different polyene antibiotics were isolated and some of them are described in Table 7.2. It should be noticed that the rate of polyene discovery was not very high, as pharmaceutical companies did not spend enough money for the discovery of new antifungal compounds due to the fact that fungal infections were not clinically important. However, in the last 20 years, an increased number of immunocompromised people (VIH and transplanted) were infected by fungi and no efficient treatments were available. The antifungal agents currently available to treat invasive fungal infections in those patients were limited in both number and usefulness. The approaches to overcome this situation involve the discovery and development of new antifungal agents, the chemical modification of the old ones, or formulations with advantages over and/or complementary to existing drugs.

Treatment with the polyene amphotericin B (Fig. 7.1) is the most useful antifungal chemotherapy. Amphotericin B is a heptaene isolated from *Streptomyces nodosus* in 1955 (Oura et al. 1955) that contained the aminosugar mycosamine (Dutcher et al. 1956). Its name comes from its chemical's amphoteric properties. Since 1956, amphotericin was used for the treatment of histoplasmosis, cryptococcosis (Louria et al. 1956), and cutaneous candidiasis (Kozinn et al. 1956). Today, treatment with amphotericin B, and with several azoles, is still the most important antifungal chemotherapy.

### *7.2.1 Isolation of Polyene-Producing Organisms from Terrestrial Environments*

The first useful polyene antibiotic was isolated by Hazen and Brown in 1950, and it was firstly named "fungicidin" and renamed "nystatin" (Fig. 7.1) for the New York State Department of Health, where Elizabeth L. Hazen was working (Brown et al. 1953). It was effective against *Candida albicans*, *Aspergillus*, *Penicillium*, and common phytopathogenic fungi (Verona and Gambogi 1957). The producer organism was a strain of *Streptomyces* named *noursei* after William Nourse, the farm's owner where Hazen isolated this microorganism.

*S. nodosus* (the amphotericin B producer) was found from the soil collected in the Orinoco River region of Venezuela in 1955. *Streptomyces natalensis* (natamycin/pimaricin producer) was isolated from a sample of soil collected in Natal (today KwaZulu-Natal), a province of South Africa (Fig. 7.1). The producer of filipin (Fig. 7.1) was isolated from a soil of the Phillipines Islands (Ammann et al. 1955). The tetraene antibiotic AB-400 producer *Streptomyces costae* was isolated

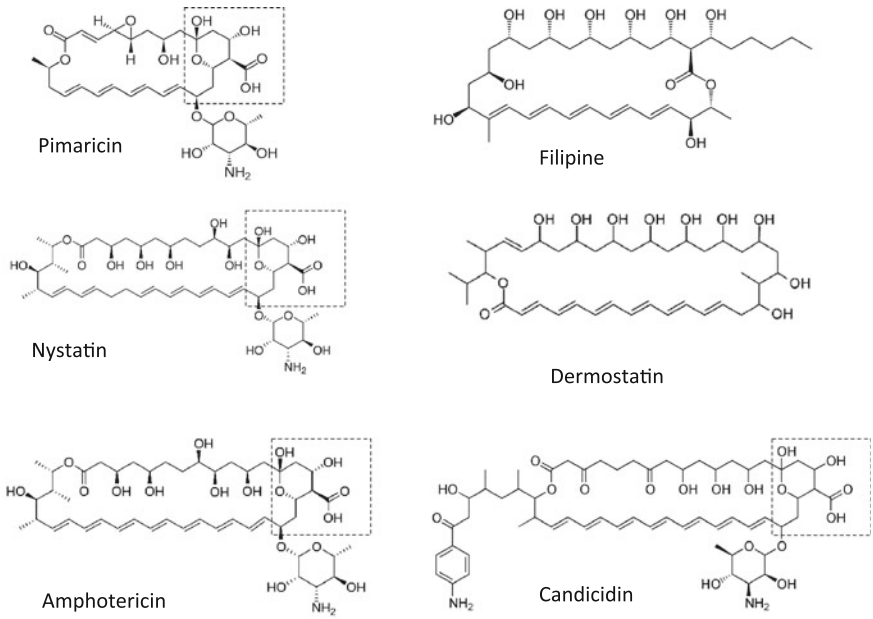


**Fig. 7.1** Chemical structures of different polyene macrolide antibiotics: tetraene (pimaricin), pentaene (filipin), hexaene (dermostatin), degenerated heptaene (nystatin $A_1$), heptaene (amphotericin B), and aromatic heptaene (candicidin D). The conserved regions in the aglycones of pimaricin, nystatin, amphotericin, and candicidin are indicated with dashed rectangles (Lei et al. 2013)

from a soil sample collected in the surrounding of Madrid, Spain (Canedo et al. 2000). These are a few examples to show that *Streptomyces* polyene producer species are normal inhabitants in the soils and can be isolated from soils of different continents.

The polyenes are usually formed as mixtures of polyene and nonpolyene products. This situation, common in secondary metabolism, has given rise to great difficulties in separating the different polyene entities and in determining their precise molecular chemical structures. Although separation of polyene macrolide antibiotics is difficult, considerable progress has been achieved in their purification by HPLC (Hansen and Thomsen 1976) and in the determination of their complex chemical structure by sensitive analytical methods, such as proton magnetic resonance (Pandey and Rinehart 1976) X-ray, and mass spectrometry (MS) (Raatikainen et al. 1991).

### *7.2.2 Isolation of Polyene-Producing Organisms from Marine Environments*

The marine environment represents a treasure of useful products awaiting discovery for the treatment of infectious diseases. A small number of marine plants,

animals, and microbes have already yielded more than 12,000 novel chemicals, with hundreds of new compounds still being discovered every year (Donia and Hamann 2003).

Perhaps the first report of antimicrobial compounds from marine organisms was carried out in the 1960s by Dr. Ross F. Nigrelli, a marine biologist who gained a worldwide reputation for his work on fish diseases (Nigrelli 1952) as well as by the poison secreted by the sea cucumber (Nigrelli and Jakowska 1960). Nigrelli's group showed that extracts of the red sponge *Microciona porifera* inhibited the growth of *C. albicans* (Jakowska and Nigrelli 1960).

However, the first systematic screening of 1,140 species of marine invertebrates to find antifungal compounds against *C. albicans* was performed by Burkholder and Ruetzler in 1969. Burkholder was one of the scientists who discovered the antibiotic chloromycetin (chloramphenicol) (Ehrlich et al. 1947).

In 1993, a collection of 116 marine sponges, ascidians, and cnidarians was assayed for the presence of antifungal compounds (Antonio and Molinski 1993). Samples with significant activity against *C. albicans* were found in that study. The activity of a potent antifungal from the sponge, *Jaspis* sp., was reduced by ergosterol concentrations as low as 10 μg/liter. This ergosterol antagonism is similar to the well-known ergosterol dependence of polyene antifungals, such as amphotericin B, and suggests a common mode of action.

The ability of these marine invertebrates to produce antifungal metabolites was in fact due to the microflora associated with these organisms, as reported in other cases with anticancer agents. The symbiont hypothesis, however, remained unproven until very recently because of a general inability to cultivate the suspected producers. However, Piel et al. (2004) using metagenomic analysis of the marine sponge *Theonella swinhoei*, which is the source of the antitumor polyketides onnamides and theopederins, showed that the responsible gene clusters belongs to a prokaryotic genome responsible for the biosynthesis of almost the entire portion of the polyketide structure (Piel et al. 2004).

There are a lot of publications describing the isolation of marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities (Glaser and Mayer 2009; Mayer et al. 2011), but after an extensive revision of the described antifungal compounds, and to our knowledge, none of them were polyene antibiotics. Most of the isolated compounds were cytotoxic, and some had anticancer activity as trabectedin (Yondelis; PharmaMar) being the first marine anticancer drug to be approved in the European Union (Molinski et al. 2009).

As we indicated previously, the majority of polyene-producing streptomycetes have been isolated from terrestrial environments, while antibiotic-producing streptomycetes from the marine sources remain largely unexplored. Therefore, streptomycetes from marine sediments have been studied in different laboratories for unravelling their potential for polyene production.

Pisano et al. (1987) found a high number of actinomycetes from estuarine sediments in the Hudson River, between New York and New Jersey. A total of 22

strains out of the 165 isolated produced antifungal compounds, being the heptaene group the most common (9 out of 22).

In 2007, 55 actinomycete strains isolated from the Mediterranean Sea were assayed for their antifungal activity. The more active strain (*S. griseus* AFM 105) produced the aromatic heptaene candicidin (Mahmoud et al. 2007).

A more systematic and selective screening of actinomycetes in marine sediments and neuston layer in the Trondheim fjord (Norway) were performed by Sergey Zotchet's group in 2009. They were able to isolate 3,708 actinomycetes strains and 1,044 of them showed antifungal activity against *C. albicans* and/or *C. glabrata* (Jorgensen et al. 2009). The UV/visible scans indicated that 655 of the 1,044 isolates produced polyene antibiotics, and a high proportion of the isolated *Streptomyces* strains produce the antifungal polyene macrolide candicidin; one of the *Streptomyces* strains contained a linear plasmid that could be responsible of the spreading of the candicidin cluster (Jorgensen et al. 2009).

In 2012, two hexaene antibiotics called bahamaolides A and B were extracted from a marine *Streptomyces* sp. isolated from a sediment sample collected at North Cat Cay in the Bahamas. Bahamaolide A, but not bahamaolide B, showed important fungicidal activity against *C. albicans* and various pathogenic fungi such as *Aspergillus fumigatus, Trichophyton rubrum*, and *T. mentagrophytes* (Kim et al. 2012). Interestingly, bahamaolide A inhibited isocitrate lyase activity in *C. albicans*; this enzyme cleaves isocitrate to glyoxylate and succinate and is one of the enzymes of the glyoxylate cycle which serves to bypass the $CO_2$-generating steps of the TCA cycle and permits the net assimilation of carbon from $C_2$ compounds, allowing microorganisms to replenish the pool of TCA cycle intermediates necessary for gluconeogenesis and other biosynthetic processes. A clear role for isocitrate lyase in the pathogenesis of *C. albicans* was reviewed (Dunn et al. 2009). Mutants of *C. albicans* in the isocitrate lyase gene (ICL1) are less virulent in mice than the wild type (Lorenz and Fink 2001). Isocitrate lyase is also a target to develop antimycobacterial drugs as this enzyme plays a key role for the survival of *Mycobacterium tuberculosis* during the chronic stage of infection (Kratky and Vinsova 2012).

### 7.2.3 Discovery of Polyene-Producing Organisms by DNA Hybridization

One of the main methods to find new producer strains or to clone gene clusters involved in antibiotic production is by DNA hybridization using previously cloned biosynthetic genes or antibiotic resistance genes. This was possible due to the clustering of antibiotic production and resistance genes on the streptomycete chromosome as it have been clearly demonstrated in many different species (Chater and Bruton 1985). In the case of polyene producer microorganisms, as the producer strain does not have polyene-resistant genes, only biosynthetic genes can be used for this purpose.

The first cloned gene involved in polyene production from *Streptomyces* was *pab* (Gil and Hopwood 1983). The *pab* gene of the candicidin producer *S. griseus* IMRU3570 codes for a protein with two domains: a PabA domain at the amino-end, and PabB domain at the carboxy-end; thus, the gene has been renamed *pabAB*. The gene contains an open reading frame for a protein of 723 amino acids (Criado et al. 1993). The *pab* genes of *S. lividans* are organized like those of the primary PABA pathway in *B. subtilis*, with *pabB* upstream of *pabA* (Arhin and Vining 1993).

Gil and Hopwood (1983) showed that the *pabAB* gene from *S. griseus* hybridized with chromosomal DNAs from *S. griseus* IMRU3570, *S. griseus* 38A and *S. fradiae* ATCC10745 but not with *S. lividans* 1326, *S. coelicolor* A3 (2), *S. clavuligerus* ATCC27064, *S. parvulus* ATCC12534 or *S. griseus* 52.2 (Gil and Hopwood 1983). Therefore, it was concluded that in streptomycetes, there are two sets of genes directing PABA biosynthesis, one for primary (*pabA-pabB*) and the other one for secondary metabolism (*pabAB*).

*pabAB* from *S. griseus* IMRU3570 has been used as a probe to find new aromatic polyene-producing *Streptomyces* strains. The *pabAB* gene hybridizes with 5 out of 16 *Streptomyces* strains (*S. acrimycini* JI2236, *S. coelicolor* JI2159, *S. coelicolor* JI1157, *S. griseus* JI2212, and *Streptomyces albus* G). The antibiotic produced by these strains contained an aromatic moiety of *p*-aminoacetophenone, the aminosugar mycosamine and they were active against *C. utilis* (Gil et al. 1990).

The *pabAB* gene was also used as probe by Sergey Zotchet's group in 2009 to identify candicidin producer actinomycetes isolated from marine sediments (Jorgensen et al. 2009). They also used internal (400–500 bp) fragments of the genes *fscTI/canRA* (ABC transporter) and *fscC/canP3* (PKS) (Fig. 7.2), but the best results were obtained with *pabAB* and *canRA*.

Another strategy used to discover new polyene producer strains was by DNA hybridization using a cytochrome P450 hydroxylase gene as probe. The probe was designed taking into account the amino acid sequences from polyene cytochrome P450 hydroxylases encoded by *amphN* (amphotericin-producing *S. nodosus*), *nysN* (nystatin-producing *S. noursei*), *pimG* (pimaricin-producing *S. natalensis*), and *canC* (candicidin-producing *S. griseus*) (Fig. 7.2)

Using this polyene cytochrome P450 hydroxylase-specific probe, a genomic library of *Pseudonocardia autotrophica* was screened and a cosmid clone, which contained a DNA fragment of 34.5 kb, was isolated. The complete sequencing of this DNA fragment revealed a total of seven complete and two incomplete ORFs that were highly similar to the previously described polyene biosynthetic genes (Lee et al. 2006) (Fig. 7.2). The whole cluster was really involved in the biosynthesis of a pentaene antibiotic named Nystatin-like Pseudonocardia Polyene (NPP) (Kim et al. 2009). As we indicated previously, this pentaene has two sugar moieties: mycosamine and N-acetyl-glucosamine connected by an α (1–4) bond (Lee et al. 2012)

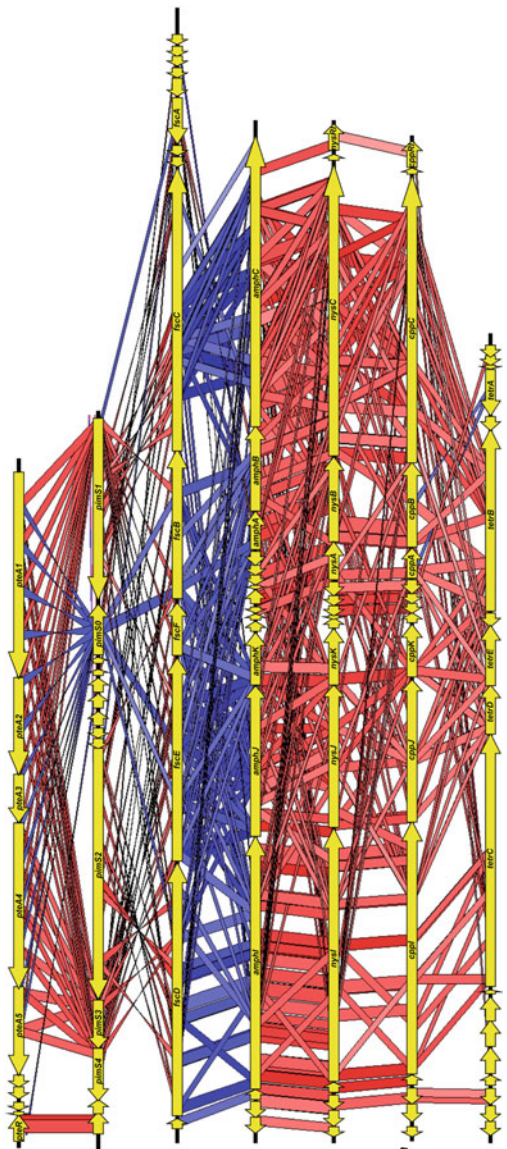


**Fig. 7.2** Artemis Comparison Tool (ACT) synteny (Carver et al. 2005) and homology pairwise comparison of the polyene biosynthetic clusters sequenced from different actinomycetes. Genes are drawn as *arrows* indicating transcriptional direction. Significant similarity calculated by tBLASTx is represented by *colored lines* (McGinnis and Madden 2004). *Red lines* reflect homology between sequences located in the same orientation. *Blue lines* connect similar sequences located in reverse orientation. The color intensity reflects the homology strength between two similar sequences

### 7.2.4 Isolation of Polyene-Producing Organisms from Symbiosis

There is increasing evidence that streptomycetes are not just free-living soil bacteria, but also form symbioses with other organisms, especially with invertebrates and plants. A recent review described the different types of symbioses established between *Streptomyces* spp. and fungi, plants and animals (Seipke et al. 2012). In several cases, *Streptomyces* spp. form protective mutualistic symbioses in which the host feeds and protects *Streptomyces* and the bacteria provide antibiotics to protect the host from pathogens. Therefore, the screening of symbiontic antibiotic producer strains has been considered as a new method to discover new polyene antibiotics. We will focus our attention to polyene antibiotics produced by *Streptomyces* living in symbioses with invertebrate and plants.

Bacterial symbionts of eukaryotes constitute promising candidates for the production of antibiotics with potential applicability in human medicine, because the involved chemical compounds should not harm the eukaryotic host and are therefore less likely to have harmful side effects on humans.

#### 7.2.4.1 Symbiosis with Insects

One of the first descriptions of production of antifungal antibiotics from *Streptomyces* living in a symbiontic consortium was made by Currie et al. (1999). They were studying the ancient and highly evolved mutualism between the leaf-cutting ants *Acromyrmex octospinosus* that live in obligate symbiosis with fungi of the genus *Leucoagaricus* growing with harvested leaf material. The symbiotic fungi, in turn, serve as a major food source for the ants (Currie et al. 1999).

Cultivation of fungus for food originated about 45–65 million years ago in the ancestor of fungus-growing ants (Formicidae, tribe Attini), representing an evolutionary transition from the belligerent life of hunters and gatherers to the peaceful life of a farmer subsisting on cultivated fungi (Mueller et al. 2001).

The obligate symbiosis between the ant *A. octospinosus* and *Leucoagaricus* sp. is disturbed by the specialized pathogenic fungus *Escovopsis* sp., which can overcome *Leucoagaricus* sp. and thus destroy the ant colony. The third mutualist in this consortium is a filamentous bacterium of the genus *Streptomyces* that produces uncharacterized antibiotics specifically targeted to suppress the growth of the specialized garden-parasite *Escovopsis*. As this *Streptomyces* strain was present in all species of fungus-growing ants studied, they conclude that is transmitted vertically (Currie et al. 1999). In a corrigendum appeared in 2003, they announced that the specialized symbiotic bacterium associated with *Acromyrmex* was not a species of *Streptomyces*, but a member of the actinomycetous family *Pseudonocardiaceae* (Currie et al. 2003).

In a recent study, by analyzing three different *Acromyrmex* species (*A. octospinosus, A. echinatior*, and *A. volcanus*), 19 leaf-cutting ant-associated

microorganisms were isolated, being *Pseudonocardia* and *Streptomyces* the most abundant. One *Streptomyces* strain produced the aromatic polyene candicidin and was very active against the pathogen *Escovopsis* but do not significantly affect the growth of *Leucoagaricus*. They also showed that each of the *Acromyrmex* species contained a *Streptomyces* strain that produced candicidin and concluded that candicidin plays an important role in protecting the fungus gardens of leaf-cutting ants against pathogenic fungi (Haeder et al. 2009).

Recent evidence obtained by 454-sequencing data suggests that attine ants are associated with a more complex community of actinobacteria, including members of the genera *Pseudonocardia*, *Streptomyces*, and *Amycolatopsis*, and that antibiotic-producing actinomycetes can be horizontally acquired through ant male dispersal and sampling of actinomycetes from the soil (Sen et al. 2009).

As candicidin-producing *Streptomyces* are abundant in the soil and sediments (Jorgensen et al. 2009; Mahmoud et al. 2007; Pisano et al. 1987), they were probably acquired from the environment, and thus, attine ants took actinomycete bacteria from the soil, selecting and maintaining those species that make useful antibiotics to fight against the pathogen *Escovopsis* (Barke et al. 2010).

A different situation might happen with *Pseudonocardia*, which produce unspecific antifungal compounds that inhibit a great diversity of fungi, but most strongly suppress or even kill the ant-cultivated fungi *Leucoagaricus* sp. The antibiotic produced by *Pseudonocardia* was nystatin P1. Nystatin P1 has a similar aglycone as the classical nystatin A1, and the main difference is the presence of a disaccharide moiety (possibly mycosamine-glucose) in nystatin P1 and only mycosamine in nystatin A1 (Barke et al. 2010).

The candicidin producer *Streptomyces* S4 strain present in leaf-cutting ants was sequenced and, as many *Streptomyces* strains already sequenced, contains multiple biosynthetic gene clusters coding for known and predicted bioactive secondary metabolites. As expected, *Streptomyces* S4 contains the biosynthetic gene cluster that directs the biosynthesis of candicidin, but also the gene cluster of the antifungal antimycin, the gene cluster of the antibacterial gramicidin, the gene cluster of anticancer agents (fredericamycin and kendomycin) as well as four cryptic biosynthetic gene clusters whose products are unknown (Seipke et al. 2011a). Genetic disruption of genes involved in candicidin and antimycin abolished completely antifungal production in vivo, but the double mutant retained antifungal activity against *Escovopsis*. It was concluded that additional antifungal agent(s) may be encoded by any of the cryptic biosynthetic gene clusters identified in the *Streptomyces* S4 genome (Seipke et al. 2011b).

One of the pioneering researchers postulated that the leaf-cutting ants should be considered "walking pharmaceutical factories" that could be a source of novel antimicrobials and from which we can learn how to make better antibiotics by studying how the bacteria have adapted to fight the parasite in an ancient evolutionary arms race (Hayden 2009). However, most of the antibiotics discovered to date in this system are either well known or belong to known classes.

#### 7.2.4.2 Symbiosis with Plants and Lichens

There are also examples of antibiotics influencing symbioses with plants. Thus, antibiotics of some plant-associated streptomycetes appear to provide protection to the plant against pathogens, while the streptomycetes presumably benefit from the plant exudates (Castillo et al. 2002; Tokala et al. 2002); on the other hand, undefined substances secreted by mycorrhizal helper streptomycetes appear to promote the plant–mycorrhizal symbiosis by affecting the branching pattern of the fungal hyphae (Schrey et al. 2007).

The relationships between *Streptomyces* and plants ranged from benign saprophytes to beneficial plant endosymbionts to plant pathogens. *Streptomyces* and fungi have a competitive advantage over many other microorganisms in soil ecosystems, because of their filamentous and sporulating lifestyle, which allows them to persist during strict environmental conditions. The filamentous lifestyle of *Streptomyces* allows colonizing nearby plants roots and subsequently entry into the host, leading to endophytic and pathogenic phenotypes (Seipke et al. 2012). We will discuss here only about endophytic *Streptomyces* that appear to provide protection to the plant against fungal or bacterial pathogens.

Endophytic *Streptomyces* have been isolated from different plants and forest. Thus, an ambitious study screened for antitumour and antimicrobial activities of endophytic actinomycetes isolated from pharmaceutical plants in a rainforest in China. They isolated 41 *Streptomyces* strains, and most of them were cytotoxic or antibacterial, but 12 % of them were active against *C. albicans.* They investigated for the presence of polyketide synthases (PKS-I, PKS-II) and nonribosomal peptide synthetases (NRPS) sequences by PCR amplification, and as expected, high frequencies of positive amplification were obtained in all cases (Li et al. 2008) suggesting the richness in these biosynthetic pathways, and its potential to isolate new antimicrobial or antifungal compounds.

In a recent revision (Joseph et al. 2012), authors described the different antibiotics produced by endophytic *Streptomyces* such as broad-spectrum antibiotics (p-aminoacetophenonic acids, munumbicins A-D, and antimycin $A_{18}$), antifungal (arylcoumarins, kakadumycin A, and fistupyrone), and anticancer compounds (arylcoumarins, pterocidin, and salaceyins A and B). It is interesting to know that the *p*-aminoacetophenonic acids produced by the endophytic *Streptomyces* sp. HK10552 of the mangrove plant *Aegiceras corniculatum* show structural similarity to the polyene antibiotic, candicidin. As the *p*-aminoacetophenone moiety of candicidin is synthesized from chorismic acid via the aromatic amino acid pathway and *p*-aminobenzoic acid (PABA) (Criado et al. 1993), it was suggested the presence of this biosynthetic pathway in *Streptomyces* sp. (strain HK10552) (Wang et al. 2010)

Actinomycetes also live in association with lichens and represent an extremely rich reservoir for the isolation of a wide diversity of actinomycetes, mainly *Micromonospora* (40 %) and *Streptomyces* (36 %). By PCR amplification with specific probes, it was possible to detect genes for polyketide synthases (PKS-I, PKS-II) and NRPS, aminoglycosides (APH) and isoprenoid compounds (Gonzalez et al. 2005). All strains were assayed for antibacterial or antifungal activity, and

10 % of the strains were active against *C. albicans*, and perhaps they represent a new source of antifungal compounds.

As far as we know, there is only a description of a polyene antifungal antibiotic produced by an endophytic *Streptomyces* isolated from the medically important plant *Catharanthus roseus*, commonly known as the Madagascar periwinkle (Rakotoniriana et al. 2012). The polyene produced is fungichromin, an already known methylpentaene macrolide antibiotic discovered in 1955 (Tytell et al. 1955).

Further research on protective symbioses will undoubtedly continue to yield novel antibiotics that might prove valuable in the arms race against increasingly resistant human pathogens.

### 7.2.5 *Polyene Antibiotics Isolated from Plants and Linear Polyenes from* Streptomyces

Traditional medicine uses different species of plants to treat diseases. One of these plants is *Vernonia urticifolia* which possesses antimicrobial activity. Kiplimo et al. (2011) isolated a new linear polyene, urticifolene, that exhibited inhibitory activity against all the bacteria investigated, and it was very active against *Enterococcus faecium* and *Pseudomonas aeruginosa* but its potential antifungal activity was not described or tested (Kiplimo et al. 2011). The antibacterial activity of a linear polyene is not an exception, as there are a few descriptions of polyene antibiotics with antibacterial activity, and one of them is the pentaene faerifungin (Nair et al. 1989). Faerifungin was described as a unique polyene with activity against fungi, nematodes, mosquitoes, and bacteria, and its mode of action against bacteria is still unknown (Mulks et al. 1990).

There is another description of linear polyene isolated from *Streptomyces* sp. named linearmycin A (Sakuda et al. 1995). This antibiotic has a long 60 units carbon chain and resembles typical polyene macrolide antibiotic except for its linearity. Linearmycin A is synthesized from one $\gamma$-aminobutyric acid, 24 acetate, and 4 propionate molecules, by a polyketide pathway (Sakuda et al. 1996).

A linear polyene molecule is also the starting point of the biosynthesis of enediyne, one of the most potent anticancer drugs ever discovered (Zhang et al. 2008) produced by *Streptomyces globisporus*. This polyene molecule, 1, 3, 5, 7, 9, 11, 13-pentadecaheptaene, has been identified as the first intermediate produced by the enediyne polyketide synthase (PKSE) before the formation of the nine-membered enediyne core (Zhang et al. 2008).

## 7.3 Cloning of Polyene Clusters

A number of excellent articles described the analysis of gene clusters and the biosynthetic mechanisms of several polyene antibiotics, such as pimaricin (Aparicio et al. 1999, 2000), nystatin (Brautaset et al. 2000), rimocidin (Seco et al.

2004), candicidin/FR-008 (Campelo and Gil 2002; Hu et al. 1994), and amphotericin (Caffrey et al. 2001). The biosynthesis of a macrolide aglycone often requires not only large PKS subunits for the formation of the macrolactone ring, but also some enzymes for post-PKS tailoring steps. The length of these clusters ranged from 84.9 kb (pimaricin) up to 137.2 kb (candicidin/FR-008).

All of these PKSs are type I modular enzymes. The loading modules of nystatin, amphotericin, and pimaricin, unlike those of other modular type I PKS, such as that for erythromycin biosynthesis are located on a separate protein, while the extension modules are distributed over several PKS components organized as bi-, tri-, tetra-, or hexa-modular proteins. The candicidin cluster seems to be structurally intermediate, with the loading domain fused to the first elongation module.

The cloning and characterization of these clusters (Fig. 7.2) were performed using different strategies:

1. *DNA Hybridization with the pabAB Gene*. The genes involved in the biosynthesis of candicidin and the antibiotic FR-008 were cloned separately in two laboratories using a similar strategy (Campelo and Gil 2002; Hu et al. 1994). In both cases, a *pabAB* gene from the candicidin producer *S. griseus* was used as probe to clone the set of genes involved in the biosynthesis of those aromatic heptaenes.
2. *DNA Hybridization with Type I PKS Domains*. The clusters of genes involved in pimaricin biosynthesis (Aparicio et al. 1999) were identified by hybridization using as probes DNA from both the PKS genes from the rapamycin (Schwecke et al. 1995) and candicidin (Criado et al. 1993) producer strains. The biosynthetic genes involved in amphotericin (Caffrey et al. 2001) and rimocidin (Seco et al. 2004) production were cloned using the *eryAII* gene from the erythromycin producer (Cortes et al. 1990)
3. *PCR Screening with Polyene Specific Cytochrome P450 Hydroxylase*. The genes for the production of the nystatin-like polyene (NPP) from *P. autotrophica* were identified by PCR screening using several actinomycete genomic DNAs. A pair of degenerated primers was designed from conserved regions present in cytochrome P450s from the producers of candicidin, pimaricin, amphotericin, and nystatin (Lee et al. 2006).

There are several review articles comparing different clusters of polyene antibiotic already known (Aparicio et al. 2003, 2004; Caffrey et al. 2008; Martin and Aparicio 2009; Zotchev and Caffrey 2009), and by this reason, we are not concentrating in these aspects.

In Fig. 7.2, an Artemis Comparison Tool (ACT) pairwise comparison (Carver et al. 2005) of the sequence homology of different polyene biosynthetic clusters already known revealed that the evolution of the genetic units involved in secondary metabolite production is driven by the duplication of highly conserved modules present in enormous genes coding for molecular mega-factories. The common feature of all these comparisons is the repetition of a single region from a specific gene in multiple regions of different genes. This homology crosslinking reflects that the duplication of specific genetic modules is quickly providing the adjustments required

to produce new secondary metabolites with entirely novel functions. This modular structure resembles the industrial pipelines of production used in modern days, where the factory may adapt quickly to synthesize new products by only changing specific parts of the machinery without altering the entire production system.

## 7.4 Genome Mining as a New Approach to Discover Polyene Producer Strains

Up to date, more than 2,000 microbial genomes are available in the data bank (http://www.ncbi.nlm.nih.gov/genome/browse/), and many of these genomes have been deeply analyzed in order to identify cryptic biosynthetic pathways for new antimicrobial agents. Genome mining involves looking at the sequenced genome of bacteria and fungi to determine whether gene clusters involved in the production of new antibiotics can be found in these organisms (Scheffler et al. 2013). As many useful antibiotics are produced by actinomycetes, genome mining is focused on *Streptomyces* and, more specifically, on cryptic gene clusters possibly involved in the formation of new antibiotics.

Once a bacterial strain has been selected to be genome-mined, there are a number of methods (Scheffler et al. 2013) that can be utilized to determine the possible product of a cryptic gene cluster and include:

(a) *Bioinformatics*: After defining the gene sequence, the data are used to predict the structure of the enzyme and the identity of the product as closely as possible. The predicted product should be isolated from the fermentation broth and analyzed its biological activity.
(b) *Genome Isotopic Approach*: Based on the predicted structure by the bioinformatic method, labelled intermediates are added to the bacterial culture to obtain the labelled predicted product.
(c) *Gene Inactivation Method*: A gene possibly involved in the biosynthesis of the unknown product can be deleted or inactivated followed by the confirmation that the unknown product is absent in the fermentation broth.
(d) *Heterologous Expression*: It consists of the transfer of genes possibly involved in the biosynthesis of the unknown product into a different host and looking for its production.
(e) *Transcriptional Activators*: In the case, the genes cannot be transferred into a different host, and its production can be increased by manipulation of activator genes from the cluster.
(f) *Removal or Inactivation of Inhibitors to Express a Silent Gene*: Some of the gene clusters are not expressed due to the presence of inhibitor gene(s). Removal of this gene will allow the silent gene to be expressed, and therefore, the product isolated and identified.

In all cases, the technique used for identification of products in the fermentation broth is liquid chromatography/mass spectrometry (LC/MS) as well as matrix-

assisted laser desorption/ionization-time of flight (MALDI-TOF) imaging mass spectrometry (IMS) (see later).

Genome mining was initiated in 2000 by Challis and Ravel who predicted the structure of a new tripeptide siderophore (coelichelin) produced by a nonribosomal peptide synthetase present in the genome of *S. coelicolor* A3(2) (Challis and Ravel 2000). Due to the increased need for new antibiotics (Hopwood 2007), genome mining has been considered an adequate alternative method for finding novel antibiotics.

We will describe here only genome mining papers related with the discovery of antifungal antibiotics.

One of the first polyene antibiotics discovered by genome mining was a pentaene cluster located in the left 2 Mb subtelomeric region of *Streptomyces avermitilis* (Omura et al. 2001). *S. avermitilis* has the highest proportion of predicted secondary metabolite gene clusters and 25 clusters involving the biosynthesis of melanin, carotenoid, siderophore, polyketide, and peptide compounds among others. The total lengths of these gene clusters were estimated to be about 560 kb, suggesting that 6.43 % of the *S. avermitilis* genome is devoted to the biosynthesis of secondary metabolites. One of these clusters is the *pte* cluster having five genes encoding PKSs. These PKSs consist of 13 modules carrying 57 catalytic domains yielding a 26-membered pentaene compound (Ikeda et al. 2003) that was identified as filipin (Xu et al. 2009) (Fig. 7.1).

In 2005, a compound of novel and predictable structure was discovered by analyzing the genome of *Streptomyces aizunensis* NRRL B-11277. The biosynthetic locus for the production of the novel compound spans ca.176 kb of DNA and encodes for 38 proteins. The compound structure was predicted with satisfactory precision to allow direct detection and purification of the compound from the culture supernatants of *S. aizunensis* (McAlpine et al. 2005). The structure of the purified compound coincided with the predicted one and turned out to be a linear polyene containing mycosamine; the compound was named ECO-02301 (Fig. 7.3) and showed a potent antifungal activity against *C. albicans* and a broad anticancer activity against several types of human cancer.

Another example of genome mining was the discovery of the tetraene JBIR-13 by analyzing an unreported type I polyketide synthase (PKS-I) gene present in the genome of *Streptomyces bicolor* NBRC 12746. This PKS-I showed 79 and 78 % sequence similarity to the KS domains in PKS genes for pimaricin and candicidin biosynthesis, respectively. *S. bicolor* produces the compound JBIR-13 that is a pimaricin analog containing perosamine instead of mycosamine (Fig. 7.3), and the side chain length of the aglycone is different from those of pimaricin (Komaki et al. 2009).

A recent work by Cao et al. 2012 identified the presence of the polyene macrolide antibiotic tetramycin biosynthetic gene cluster in the genome of *Streptomyces hygrospinosus* var. *beijingensis* (Cao et al. 2012). Tetramycin (Fig. 7.3) was previously described as a tetraene antibiotic produced by *S. noursei* var *jenensis* JA 3789 (Dornberger et al. 1971). Up to date, and in relation with polyene antibiotics, no new polyene antibiotics were discovered by genome mining, except for the linear polyketide polyene ECO-02301.

Perhaps, scientists should look for new antibiotics producer strains in soil, as soil microorganisms have been the most valuable source of natural products. One gram of soil is estimated to contain millions of bacteria, archaea, viruses, and eukaryotic microorganisms of which only a small percentage has been cultivated in the laboratory (Kakirde et al. 2010). Microorganisms in natural environments may contain genes that encode and express biosynthetic pathways of interest that have never been identified using culture-dependent methods; therefore, the development of molecular cultivation-independent approaches should be used.

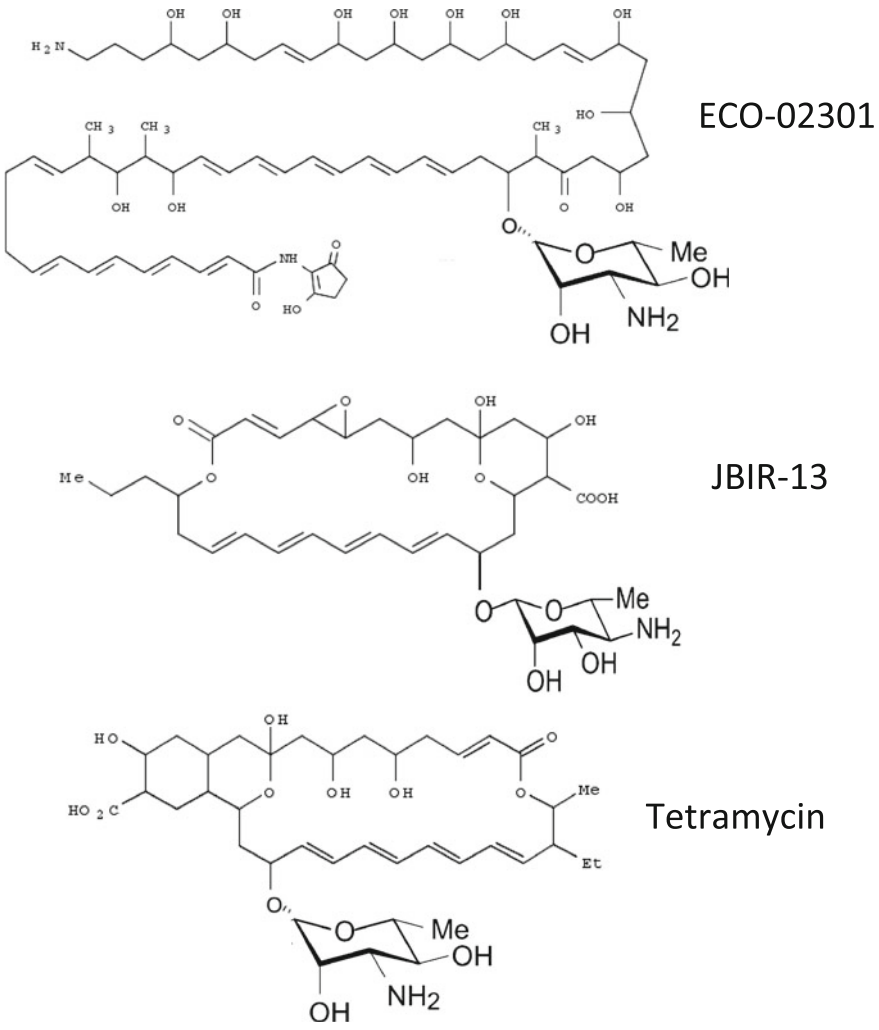


**Fig. 7.3** Structures of some polyene antibiotics discovered (ECO-02301 and JBIR-13) or rediscovered (tetramycin) by genome mining. ECO-02301 is a linear polyene containing mycosamine (McAlpine et al. 2005), and JBIR-13 is a pimaricin analog containing the aminosugar perosamine instead of mycosamine (Komaki et al. 2009)

As polyene antibiotics are produced by modular enzymatic pathways that can be identified using specific DNA probes, soil metagenomics could be used to discover new gene clusters and clone them into heterologous hosts that are easier to manipulate in vitro, such as *E. coli* or *S. lividans.* The construction and screening of complex libraries derived from the soil or marine metagenome provide opportunities to fully explore and exploit the enormous genetic and metabolic diversity of soil or marine microorganisms (Daniel 2004). As far as we know, only two descriptions of antifungal compounds were discovered by metagenomic analysis: a 36 amino acids peptide that inhibited the growth of *C. albicans* and *Aspergillus niger* (Pushpanathan et al. 2012) and a type II polyketide compound of unknown structure (Chung et al. 2008). In this particular case, two fosmid libraries from a forest soil were screened against *Saccharomyces cerevisiae,* and an *E. coli* clone expressing antifungal compounds was identified; however, they were unable to find an extraction procedure that can separate the active antifungal component. These results suggested the metagenomic approach is an alternative to search for novel antifungal antibiotics from unculturable soil bacteria (Pushpanathan et al. 2012)

In the last couple of years, a more powerful technique was developed to decipher the production of new compounds at the level of a single colony in agar plate. This technique is MALDI-TOF-IMS settled by the Dorrestein's group (Gonzalez et al. 2012). This method gives a broad view of the metabolites produced by the entire bacterial colony, including the surrounding agar medium, and increases the possibility for detecting new molecules produced by a bacterial colony alone or by interaction with other bacterial species (Watrous et al. 2013). The combination of genome mining with MALDI-TOF-IMS permitted the identification of arylomycins and their biosynthetic gene cluster from the daptomycin-producing *S. roseosporus* (Liu et al. 2011) and opened the way to discover polyene antibiotics by IMS and genome mining.

## 7.5 Engineered Biosynthesis of New Polyene Antibiotics

The methodology for genetic manipulation of the amphotericin and nystatin producers, *S. nodosus* and *S. noursei*, used to engineer the biosynthesis of several analogs of both polyenes, as well as the methods for production, identification, purification, and characterization of new analogs, was published in a special issue of Methods in Enzymology (Zotchev and Caffrey 2009) and we are not going to describe here.

The starting point to get new polyenes by engineered biosynthesis was taken from the manipulation of the PKS genes for erythromycin, producing a library of new erythromycins by combinatorial alterations of catalytic activities in the biosynthetic pathway (McDaniel et al. 1999; Rodriguez and McDaniel 2001).

The PKSs involved in polyene antibiotic biosynthesis, because of their size and number of modules, represent a more exciting system to create chemical diversity via genetic engineering and combinatorial biosynthesis. The post-PKS modification

enzymes responsible for the biosynthesis and attachment of mycosamine/perosamine, and formation of additional hydroxyl, epoxide, and carboxyl groups on the polyene macrolactone ring must be considered as well, since these moieties seem to be important for activity and toxicity of the polyenes (Cybulska et al. 1995).

The first example of manipulation of a polyene biosynthetic gene was the phage-mediated gene disruption of the *pimD* gene (encoding a P450 monooxygenase) in the pimaricin cluster of *S. natalensis.* The *pimD* mutant accumulates 4,5-deepoxypimaricin at high yield, thus establishing a role for PimD as 4,5-deepoxypimaricin epoxidase (Mendes et al. 2001). This enzyme, which converts 4,5-deepoxypimaricin into pimaricin by epoxidation of the C-4–C-5 double bond on the macrolactone ring, was overproduced in *E. coli* and showed the expected in vitro activity (Mendes et al. 2005) Unfortunately, the biological activity of deepoxypimaricin was found to be substantially lower than that of pimaricin, showing a minimal inhibitory concentration 10 times higher than the one for pimaricin, which clearly demonstrated the importance of the epoxy group for the pimaricin antifungal activity (Mendes et al. 2001).

Another pioneering experiment of manipulation of the nystatin PKS was performed in *S. noursei*. It was known that the tetraene nystatin is a degenerated heptaene (See Fig. 7.1), and the experiment tried to convert the single bond present in the hydrophobic region of the macrolide ring (C-28–C-29) into a double bond by removing the ER domain in the module 5 of the NysC PKS protein (Brautaset et al. 2002) (Figs. 7.4 and 7.5). Although the desired mutant (ERD44) was obtained, it produced very little of the expected heptaene derivative, which was named S44HP, and its identity as a nystatin with seven double bonds was confirmed by NMR (Bruheim et al. 2004).

As most of the engineered biosynthesis experiments to produce new polyene antibiotics were performed using the nystatin producer *S. noursei*, we will describe here the proposed model for the biosynthesis of nystatin (Fig. 7.4). The synthesis of nystatin starts with loading the acetyl-CoA onto the NysA protein and proceeds through condensation of three methylmalonyl-CoA and 15 malonyl-CoA extender units by NysB, NysC, NysI, NysJ, and NysK PKS. After cleavage of the mature polyketide chain from the PKS complex by the thioesterase (TE) domain of NysK, the chain is cyclized to form the nystatin aglycone. The next two steps in the nystatin biosynthesis are probably accomplished by the NysL and NysN monooxygenases, which perform hydroxylation and oxidation of the macrolactone ring at C-10 and C-16, respectively (Brautaset et al. 2000).

Biosynthesis of the aminosugar mycosamine presumably starts with the L-fructose-6-phosphate, which is converted to GDP-D-mannose through the action of a phosphomannoisomerase (PMI), phosphomannomutase (PMM), and a GDP-mannose pyrophosphorylase (GMPP) (Fig. 7.4). Phosphomannose isomerase (ManA) and phosphomannomutase (ManB) were studied in *S. nodosus*, and they were involved in amphotericin biosynthesis as nonmycosaminated amphotericin was produced by a *S. nodosus* strain lacking *manA* and *manB* (Lochlainn and Caffrey 2009).

GDP-D-mannose probably serves as a substrate for the NysDIII protein, which converts GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose. A GDP-3-keto-6-

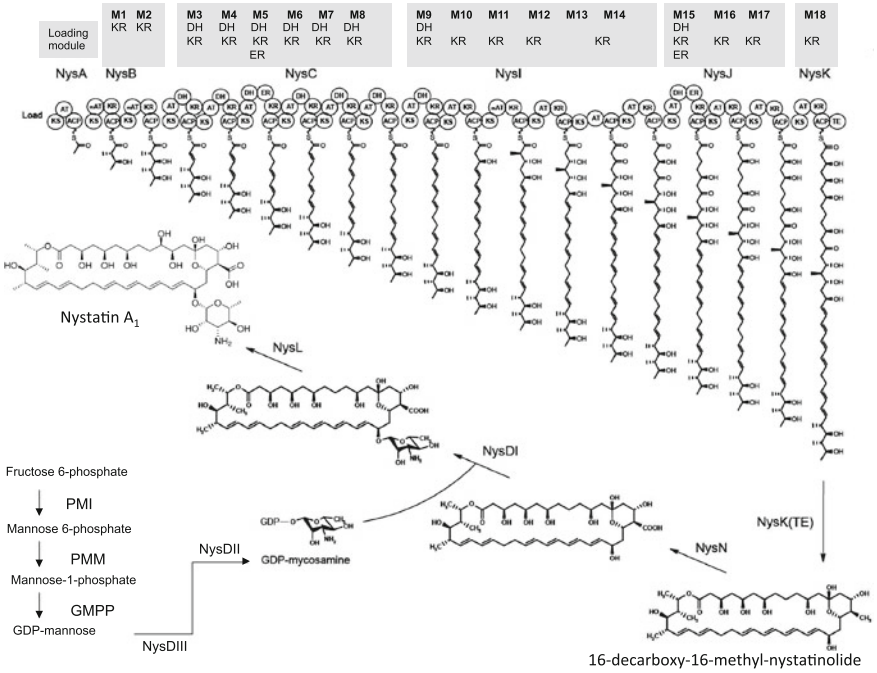


**Fig. 7.4** Proposed nystatin $A_1$ biosynthesis in *Streptomyces noursei*. Only reductive domains in the nystatin PKS modules (*upper part* of the figure) are shown. Redraw from (Brautaset et al. 2000; Brautaset et al. 2008; Lochlainn and Caffrey 2009). For details, see text

deoxy-D-mannose isomerase must perform the next step in mycosamine biosynthesis, probably followed by the NysDII-mediated amidation leading to synthesis of GDP-mycosamine. The glycosyltransferase NysDI completes the pathway by attaching the mycosamine moiety to the modified nystatin aglycone (Brautaset et al. 2000).

### *7.5.1 Modifications in the Hydrophobic Region*

As indicated above, removing the ER domain in the module 5 of the NysC PKS originated *S. noursei* ERD44 strain that produced the heptaene derivative S44HP (Fig. 7.5) and also produced an octaene nystatin analog with an expanded macrolactone ring due to the "stuttering" of the modified PKS NysC (Bruheim et al. 2004). This mutant also produced a tetraene (NYST1070) and a heptaene (NYST1068) with two sugars (Fig. 7.5). It is interesting to note that one component of nystatin (nystatin A3) contained two sugars: L-mycosamine attached to C-19 and 2,6-dideoxy-L-ribo-hexopyranose (L-digitoxose) at C-35 (Zielinski et al. 1988). The tetraene produced by the mutant ERD44 contained mycosamine and

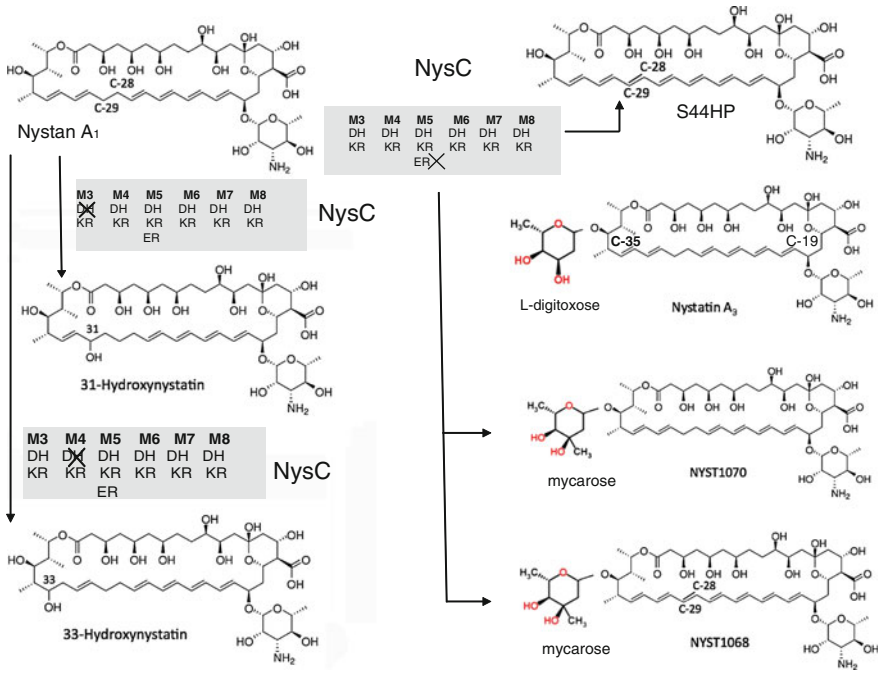


**Fig. 7.5** Molecular structures of nystatin $A_1$ and nystatin $A_3$ and its analogs produced by genetic manipulation of the genes involved in the biosynthesis of the hydrophobic region of nystatin. Note that the degenerated heptaene nystatin $A_1$ is converted into the heptaene S44HP by manipulation of the ER domain (module 5) NysC. Redraw from (Brautaset et al. 2008)

2,6-dideoxy-3-C-methyl-L-ribo-hexopyranose (mycarose), and the heptaene NYST1068 contained mycosamine and mycarose. The presence of a mycarose moiety has been found in other polyketide antibiotic molecules with antibacterial activities, such as mithramycin A, tylosin, and erythromycin A intermediates, but never before in polyene antibiotics (Bruheim et al. 2004).

The biological activity of the different nystatin derivatives obtained was assayed for antifungal activity against *C. albicans*, and the main conclusions were that the most active compound was S44HP (28,29-didehydro nystatin) followed by the octaene. The presence of the second sugar reduces the activity of the heptaene NYST1068 (28,29-didehydro 35-mycarosyl nystatin). In all cases, the heptaene derivatives are better antifungal compounds that the corresponding tetraene ones (Bruheim et al. 2004).

However, another mutant selected in the same screening was shown to produce significant amounts of hexaene nystatin derivatives. Genetic analysis of this mutant revealed an in-frame deletion in the *nysC* gene resulting in elimination of one complete module from the protein. Purification and analysis of the hexaene derivative suggest that it present a contracted macrolactone ring which is substantially less active that nystatin (Brautaset et al. 2002).

As we indicated previously, polyene antibiotics are amphipathic molecules with low solubility in water and therefore with a reduced use in medical practice. In order to increase its solubility in water, several derivatives were made by chemical methods (Falk et al. 1999); with all the genetic information about the biosynthesis of a given polyene antibiotic, and with efficient genetic tools, it is now possible to rationally engineer the polyene biosynthetic pathways in order to produce more active and less toxic polyene antibiotics by introducing changes in the polyene molecule (i.e., introducing extra hydroxy groups) that might increase its solubility.

This was made by Borgos et al. (2006) who introduced hydroxyl groups at positions either C31 or C33 by inactivating the DH domains in modules 3 and 4, respectively, on the nystatin PKS NysC (Borgos et al. 2006). The structures of the new polyenes (31- and 33-hydroxynystatin, Fig. 7.5) produced by these mutants were confirmed by RMN, and their solubility was found to be 2,000 times higher than that of nystatin, but their antifungal activity was almost lost (no antifungal activity up to 200 μg/ml). These results indicate that introduction of either C33 or C31 hydroxyls groups with subsequent elimination of C30–C31 or C32–C33 double bonds is detrimental for its antifungal activity (Borgos et al. 2006).

### 7.5.2 *Modifications in the Hydrophilic (Polyol) Region and Exocyclic Carboxy Group*

It was described that the exocyclic carboxy group of amphotericin B was important for selective toxicity and activity (Mazerski et al. 1995), and therefore, several changes were introduced by genetic engineering to modify exocyclic carboxy group of amphotericin B in order to modify its pharmacological properties (Carmody et al. 2005; Power et al. 2008). Similar experiments were performed with nystatin (Carmody et al. 2005; Power et al. 2008)

The exocyclic carboxy group of nystatin and of the nystatin heptaene S44HP was removed by deleting the corresponding *nysN* gene encoding a C-16 methyl oxidase/P450 monooxygenase. The production of the resulting 16-decarboxy-16-methyl-nystatin did not reach the 2 % of the nystatin production by wild-type strain, but the new antibiotic has a reduced hemolytic activity without affecting its antifungal action (Brautaset et al. 2008). These data agreed with the obtained previously with 16-decarboxy-16-methyl-amphotericin (Carmody et al. 2005). The 16-decarboxy-16-methyl-S44HP derivative was named BSG005 (Fig. 7.6) and improved the pharmacological properties of S44HP as the new compound is less hemolytic and 20-fold more active than nystatin (Brautaset et al. 2008).

Different experiments were performed to change the polyol region of amphotericin (Power et al. 2008) and nystatin (Brautaset et al. 2008). As an example, we will describe here a S44HP derivative with a modified polyol region. As we can see in Fig. 7.6, the polyol region of nystatin A1 or S44HP has three hydroxyl groups in C-3, C-5, and C-7, and because the number of hydroxyl groups might modify the hydrophilic properties of the polyene, the C-5 and C-7 hydroxyl groups

were replaced separately by keto groups. In the biosynthesis of nystatin, the C-5 keto group is converted in C-5 hydroxyl group by the action of the ketoreductase (KR17) of NysJ (Brautaset et al. 2000). When a mutation was introduced in the gene encoding KR17 NysJ and reintroduced in *S. noursei,* the recombinant strain produced the desired antibiotic (BSG013) (Fig. 7.6). Its production rate was 56 % of the S44HP production, and its antifungal activity was reduced twofold (Brautaset et al. 2008). The new antibiotic seems to be identical to the previously described polyene mycoheptin (Borowski et al. 1978).

The deletion of the *nysN* gene in the BSG013 producer strain originated a new antibiotic with a modified polyol region and lacking the carboxy group at C-16 that was named BSG020 (Fig. 7.6). This genetic engineering modified nystatin has a further reduced hemolytic activity, a reduced toxicity in a mouse model, and an improved antifungal activity (Brautaset et al. 2008). These data suggest that the combination of the oxo substitutions in the polyol region and the presence of a C-16 methyl group improved the therapeutic value of S44HP and are at least as efficient as amphotericin B, the unique polyene currently used for the treatment of systemic fungal infections.

Both in the nystatin molecule and in S44HP the C-1–C-15 polyol region is interrupted by a saturated C-9–C-10 saturated bond formed as a result of the activity ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains in the module 15 of NysJ (Fig. 7.6). The DH15 domain of NysJ was inactivated in *S. noursei* and the resulting strain produced the polyene BSG002 that has acquired a C-9 hydroxyl group but lacks the C-10 hydroxyl group. This new polyene is less hemolytic than nystatin but its antifungal activity was reduced fourfold (Brautaset et al. 2008). Using a similar strategy, a S44HP derivative (BSG003) (Fig. 7.6) was obtained and, as above, the antifungal activity was severely reduced as well as the hemolytic properties (Brautaset et al. 2011). When the antifungal properties of BSG003 were analyzed in detail, it was shown that S44HP derivatives with a C-10 to C-9 repositioned hydroxyl group display fungistatic activity. It was concluded that the perfect string of hydroxyl groups in the polyol region increases the inelasticity of the macrolactone ring, and its penetration in the cell membrane to form channels is altered (Brautaset et al. 2011).

Another approach to get new derivatives was the chemical modification of S44HP, but this process is complicated by the high molecular weight of the polyene as well as their solubility properties and photolability. For this purpose, several amidation reactions of the exocyclic C-16 carboxy group were performed. One of the resulting compounds, when the carboxy group was replaced by $CONH(CH_2)_3N(CH_3)_2$, exhibited higher antifungal activity, lower hemolytic activity, and lower toxicity than either S44HP or amphotericin B (Preobrazhenskaya et al. 2009).

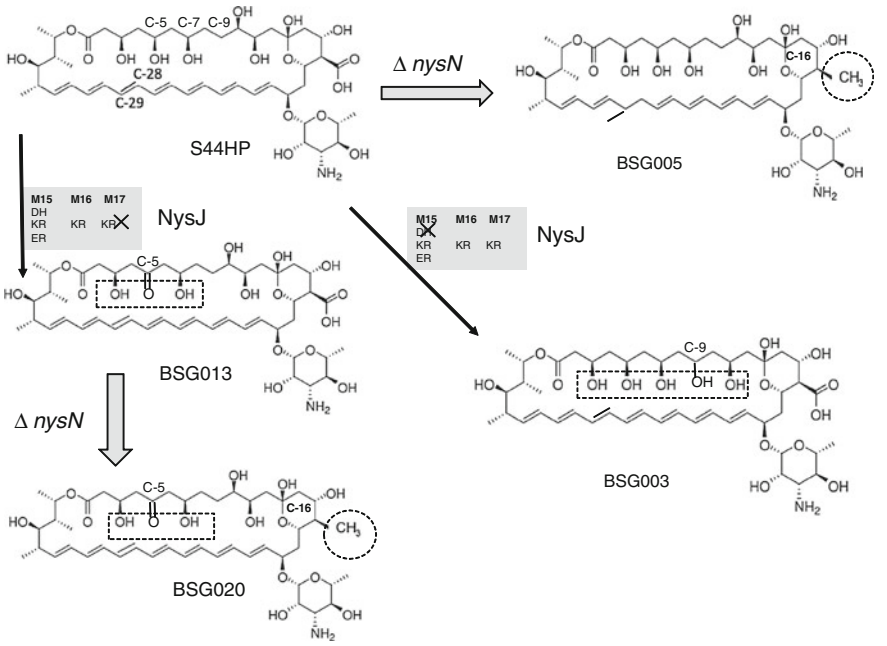


**Fig. 7.6** Molecular structures of the heptaene S44HP and its analogs produced by genetic manipulation of the genes involved in the biosynthesis of the hydrophilic region and replacement of the C-16 carboxyl with a methyl group. Dashed rectangles and circles indicate chemical modifications related to the heptaene nystatin derivative S44HP. Redraw from (Brautaset et al. 2008; Brautaset et al. 2011)

## 7.6 Modification in the Aminosugar Moiety

The set of genes involved in the last biosynthetic steps of the aminosugar mycosamine and its attachment to the macrolide aglycone were found in the clusters of candicidin (*canG, canA,* and *canM*), FR-008 (*fscM1*, *fscM2*, and *fscM3*), amphotericin B (*amphDI, amphDII,* and *amphDIII*), nystatin (*nysDI, nysDII*, and *nysDIII*), and pimaricin (*pimK*, *pimC*, and *pimJ*). These three set of genes encoded for glycosyltransferase, GDP-ketosugar aminotransferase, and GDP-mannose-4, 6-dehydratase activities (Fig. 7.4).

Some of these genes were inactivated in *S. griseus* (Chen et al. 2003), *S. nodosus* (Byrne et al. 2003), and *S. noursei* (Nedal et al. 2007). Disruption of the putative glycosyltransferase and GDP-ketosugar aminotransferase functionalities resulted in the productions of a set of nonmycosaminated aglycones (Chen et al. 2003; Nedal et al. 2007). The antifungal and hemolytic activities of the purified nystatin aglycone (nystatinolide) were shown to be strongly reduced compared to those of nystatin (Nedal et al. 2007). In the case of *S. nodosus*, the antifungal activity of the amphoteronolides showed a dramatically reduced activity compared to amphotericins extracted from wild-type *S. nodosus* (Byrne et al. 2003). These

results confirm the importance of the mycosamine moiety for the biological activity of nystatin/amphotericin, and it is consistent with previous findings that a positive charge on the amino group of mycosamine is essential for the amphotericin antifungal activity (Cheron et al. 1988).

Engineering of post-synthesis glycosylation pathways is another important route to generate new polyketide structures, and they are several examples of novel structures being generated by the transfer of glycosylation genes from one polyketide antibiotic producing organism to another (Han et al. 2012; Jung et al. 2007; Olano et al. 1999).

In order to get new polyene derivatives, a *S. nodosus* strain (lacking the AmphN cytochrome P450) was constructed by inactivation of the *amphDI* gene (encoding mycosamine glycosyltransferase). The new mutant produced 8-deoxy-16-methyl-16-descarboxyl amphoteronolides in high yield and can be used for in vivo and in vitro glycosylation engineering (Stephens et al. 2012). As mycosamine is produced from GDP-mannose, nonmycosaminated amphotericin was also produced by a *S. nodosus* strain lacking phosphomannose isomerase (ManA) and phosphomannomutase (ManB) (Lochlainn and Caffrey 2009).

The production of new polyene derivatives can be also carried out transforming a mutant that produce a nonmycosaminated antibiotic with plasmids carrying genes involved in the biosynthesis of a different aminosugar. This was achieved by introducing in *S. nodosus* genes involved in the biosynthesis of perosamine, the aminosugar present in the polyene perimycin produced by *S. aminophilus* (Borowski et al. 1961). The *perDII* perosamine synthase alone or linked to *perDI* perosaminyltransferase genes from *S. aminophilus* were used to replace the *S. nodosus* chromosomal *amphDII* or *amphDI–DII* genes, respectively. Only a small amount of the desired perosaminyl-amphoteronolide B was obtained, suggesting that AmphDI can perosaminylate amphoteronolides, but very inefficiently (Hutchinson et al. 2010). To increase the yield of the putative perosaminylated analog, hybrid glucosyltransferases were constructed in which the putative 8-deoxyamphoteronolide-binding *N*-terminal domain of AmphDI was fused to the putative GDP-perosamine-binding *C*-terminal domain of PerDI (Hutchinson et al. 2010). One of these hybrid glycosyltransferases was introduced into *S. nodosus* Δ*amphDII* and 40 mg/L of perosaminyl-amphoteronolide B were obtained. The new polyene is slightly more soluble in water than amphotericin B but showed similar minimal inhibitory concentration and approximately the same hemolytic activity (Hutchinson et al. 2010). The biosynthesis of 19-*O*-perosaminyl-amphoteronolide B is the first rational redesign of polyene glycosylation by genetic engineering of a producer microorganism and demonstrates the potential of combinatorial biosynthesis for the generation of new polyenes carrying diverse sugars.

Recently, it was found that homologous glycosyltransferase genes including *amphDI*, *nysDI*, and *pimK* can catalyze the conversion of candicidin aglycone into candicidin/FR-008-III in a *fscMI* mutant, being the FscM activity slightly more efficient that the other three homologous glycosyltransferases (Lei et al. 2013). This result suggests the presence of a conserved region of the polyene structure that could be essential for glycosyltransferase recognition, and effectively the four

polyenes have a conserved region around the exocyclic carbon where mycosamine is attached (see Fig. 7.1). Attempts to modify this region in the FR-008/candicidin molecule, together with the complementation analysis, confirm that polyene glycosyltransferases have relaxed specificity and that it is possible to create new polyene analogs with altered aglycones with improved bioactivities.

Another approach to get new polyenes containing different sugar derivatives is by using *E. coli* as heterologous host as it was demonstrated for the biosynthesis of erythromycins. Bio-conversion experiments carried out by feeding 6-deoxyerythronolide B (6-dEB) or 3-α-mycarosylerythronolide B (MEB) demonstrated that the genetically modified *E. coli* B strain was able to produce 60- and 25-fold more erythromycin D (EryD) than the original strain K207-3, respectively (Peiru et al. 2008). To our knowledge, no attempts have been made to express polyene clusters in *E. coli.*

## 7.7 Concluding Remarks

After many years of looking for new antifungal compounds, polyene antibiotics are the more effective drugs. Its use is hampered by the toxicity and low solubility of the already available polyenes. Amphotericin B is the best option to treat fungal infections, but using genetic engineering, it has been possible to "create" nystatin derivatives with similar antifungal activity than amphotericin B, lower toxicity and higher solubility.

These technologies will lead scientists to produce new polyene derivatives that can be used in the future to combat emerging resistant strains and to increase their efficiency and application spectrum, or to minimize unwanted secondary effects. These will be certainly needed due to the constant increase in chronic and immunocompromised hospital patients, which are exposed to multiple infection agents including fungi. These novel approaches to identify new antibiotherapies are becoming essential since new multidrug resistant strains are being isolated at a rate inversely proportional to the discovery of novel antimicrobial drugs by traditional means. The effort invested in this research area during the next decade will be decisive for preventing the return of our clinical care facilities to a pre-antibiotic era.

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# Chapter 8
# Enzybiotics: The Rush Toward Prevention and Control of Multiresistant Bacteria (MRB)

**Patricia Veiga-Crespo, Angeles Sanchez-Perez and Tomás G. Villa**

**Abstract** The phenomenon of antibiotic resistance of pathogenic microorganisms represents a worldwide hot-spot in the healthcare of all countries, since new antibiotic-resistant strains are emerging at a constant rate and traditional treatments, based on antibiotic therapy, are now failing. The search for new antimicrobial alternatives is thus necessary and mandatory and the solution to this problem of antibiotic resistance may not be as far away as initially thought. Bacteriophages are viruses that attack and lyse bacteria. They produce two types of enzymes with therapeutic use: holins and lysins. These enzymes are able to degrade the bacterial cell wall, thus causing cell lysis and death. The search for phage-based products to use in pharmaceutical formulations opens a new window of hope for medicine in the struggle against illness currently resistant to antibiotic treatment.

## 8.1 Prevalence of Multiresistant Bacteria (MRB)

The discovery of penicillin by Fleming and its subsequent industrial exploitation marked the beginning of the antibiotic era. The historical perspective in antibiotic development was to maximize the treatment efficiency of the drug while minimizing its toxicity, regardless of the evolution of antibiotic resistance. Antibiotics

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have irreversibly changed the practice of medicine in the last century, significantly reducing the occurrence of diseases such as diphtheria, syphilis, and whooping cough (Lauter 1989).

Several major programs have been deployed to monitor the antimicrobial resistance phenomena; these include the National Nosocomial Infection Surveillance System, Intensive Care Antimicrobial Resistance Epidemiology (ICARE), and SENTRY Antimicrobial Surveillance Program (Diekema et al. 1999; Fridkin et al. 1999; Gales et al. 2001; Sahm et al. 1999). The European Commission, as well as the Food and Drug Administration (FDA-USA) establish strict rules for antibiotic usage, in an attempt to control the antibiotic misuse that was common in the past.

Resistance phenomena represent not only an important healthcare issue but also a huge economic problem, with an estimated cost of between 17 and 26 billion dollars per year in the USA alone. According to the US Centers for Disease Control and Prevention, 1,7 million people per year, in the USA alone, suffer from hospital-acquired infections, resulting in 6 % of deaths (Control 2002). In order to control this problem, both the European Union and the US Government have established directives aimed at coordinating the fight against antibiotic resistance and toward the rational use of antibiotics (Council 1999; U.S. Centers for Disease Control 2000).

However, these initiatives do not seem to be sufficient. Methicillin-resistant *S. aureus* (MRSA) caused nearly 60 % of nosocomial infections in 2001 and was responsible for more than 58 % of the new cases isolated in Europe (ECDC 2009). Currently, 30 % of *Streptococcus pneumoniae* strains are already penicillin-resistant and the number of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* strains are constantly increasing. Another important group of antibiotic-resistant strains are the opportunistic pathogens that infect immunocompromised patients, such as multidrug-resistant *Stenotrophomonas maltophilia* and *Acinetobacter* species (Gales et al. 2001). A WHO report in 2010 established that multiresistant *Mycobacterium tuberculosis* was responsible for around 1.7 million deaths, per year, worldwide (WHO 2010).

Antibiotic resistance is also a major problem in cattle care and veterinary medicine in general. It has been reported that mastitis, an infection caused mainly by *Streptococcus agalactiae, S. aureus, Streptococcus uberis,* and *Streptococcus dysgalactiae*, generated losses estimated at between 1700 and 2000 million dollars per year (Donovan et al. 2006).

One of the major concerns with the antibiotic resistance problem is the high cost associated with treatment failure, both in hospitals and industry, and the short period of time in which a new antibiotic remains active and useful.

Bacterial resistance to antibiotics has been progressively growing until it became a threat to modern medicine (Nordberg 2005). Different strategies have been proposed in this fight, they range from reduction in the amount of antibiotics given to using a combination of several drugs, and include optimizing the dosage and duration of the antibiotic treatments (Geli et al. 2012). However, the studies were not extensive enough to conclude which were the right strategies

(Metlay et al. 2006). Major studies carried out on the dynamics of infection and immunity must be taken into account in order to determine the right treatment regimen.

In the fight against antibiotic resistance, two major problems have to be considered: A) multidrug-resistant (MDR) bacteria, able to survive in the presence of different types of antibiotics; and B) the localization of the infection, since the pharmacodynamic properties of antibiotics vary depending on which organ in the patient is affected. Some studies have highlighted the importance of knowing the optimal drug dosage and suggest this as a worthwhile strategy (Geli et al. 2012).

The abuse and ill-use of antibiotics in the past has been suggested as one of the causes for the increase and generation of resistant strains of bacteria. Additionally, the relationship between different factors such as drug dosage, pharmacokinetics, and efficiency is now starting to be factored in (Olofsson and Cars 2007). The route of drug administration could also be a contributing factor in the increase in vancomycin-resistant strains of *Clostridium difficile* in the United States (Rice 2001). Currently, there have been attempts to unify all of the above criteria and to determine the influence of all of these factors in order to produce a more efficient treatment without the drawback of creating a positive pressure to create resistant strains (Geli et al. 2012).

The antibiotic market generates sales of 42 billion dollars per year around the world. In Europe, the best selling antibiotics, from 1997 to 2003, were penicillins, although there were relevant differences within the European countries (Cars et al. 2001; Ferrara 2008; Turnidge and Christiansen 2005).

### *8.1.1 Discovery and Development of New Antibiotics*

Ever since the discovery of penicillin and streptomycin, there have constantly been worldwide programs, financed by different countries, to attempt the isolation and identification of novel and more potent antibiotics. Mainly due to the spectacular initial success of these two quintessential antibiotics and their significance to the human race, in terms of millions lives they have saved. For the first time, human beings were on the edge of fully controlling disease and consequently were able to extend life expectancy by a factor of 20–30 %. Undoubtedly, the industrial exploitation of penicillin first, and streptomycin later, marked the beginning of the antibiotic era and antibiotics appeared to be the embodiment of Erlich's magic-bullet concept (Sulakvelidze 2005a). Since then, the development of novel natural, synthetic, or semi-synthetic antibiotics has been a continuous labor, with hundreds of antibiotics isolated, although only a handful of them have been used in medicine.

Despite the fact that antibiotic resistance has considerably increased in the last few years, antibiotic resistance is not a new phenomenon. As early as 1943, a few years after the discovery of penicillin, some penicillin-resistant *S. aureus* strains had already been described, and a few years later more than 80 % of the strains of

this bacterium isolated from hospitals were penicillin-resistant (Chambers 2001; Levy and Marshall 2004). Fleming, after the discovery of penicillin, pointed out in the New York Times, in 1945: "… the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and from them to others until they reach someone who gets a septicemia or a pneumonia which penicillin cannot save…" (Levy 2002). Since then, the emergence of MDR bacterial strains has contributed to the chronic problem of hospital-acquired infections.

Despite the golden era of antibiotics, since Florey and Chain were able to purify penicillin and produce it in quantities sufficient for clinical assays, one of the major drawbacks in the discovery and development of new antibiotics was the enormous failure rate experienced by sulfa-based drugs during the 1930s. This happened after a US company developed a raspberry flavored liquid to mask the natural bad taste of sulfa-based drugs. The use of diethylene glycol to solubilize the sulfa powder caused originally the death of 107 people, mostly children (Kiefer 2001), with more deaths to follow. At first, the strategy to combat MDR bacteria was to develop new antibiotics. However, recently the finding and industrial development of new antibiotics has been dramatically reduced, although a significant proportion of the worldwide pharmaceutical production is dedicated to antibiotics. A cost reduction policy became the norm in the different healthcare systems around the world, therefore increasing the importance of generic drugs. Traditionally, pharmaceutical industries have capitalized on the discovery of new antibacterial second metabolites, but the current policy has contributed to the failure in developing new antibiotics, with industry concentrating on more profitable drugs, such as those treating heart failure and chronic diseases. Another contributory factor is the reduction in antibiotic prescription by physicians. In 2000, amoxicillin-clavulanate was the only antibiotic in the top 20 prescription drugs (Kregling 2001). So, the major pharmaceutical companies have practically stopped their research and development of new antibiotics (Talbot et al. 2006).

The FDA new antibiotic approvals suffered a near 56 % decline during the last 20 years (Hancock 2005) (Table 8.1). The need for antibiotics and antibacterials in general will increase in the near future, due to factors such as population aging, the plight of immunocompromised patients, bacterial resistance, and organ transplantation.

The methods used for new drug discovery were based on direct observation, such as in the case of cephalosporin C-producing organisms (Powers 2004). Antibiotics have traditionally been recovered from nature and nearly all of today's antibiotics are variations on the natural ones. Antibiotic development was based on chemical modifications of previously existing drugs. Some of these new generation antibiotics were more effective against diseases than the original drugs. The chemical synthesis of entirely new antibiotics is limited to the fluoroquinolones, a group of broad-spectrum antimicrobials based on the chemistry of nalidixic acid, the subject of another chapter in this book. Today, the strategy for obtaining new antibiotics is very laborious, although it can be approached in different ways. The traditional perspective involves the screening of large libraries of compounds and a massive

**Table 8.1** New antimicrobial agents approved by the U.S. Food and drug administration during the last few years

| Generic name | Manufacturer | Indications for use |
|---|---|---|
| Moxifloxacin hydrochloride | Bayer Corporation | Modified guidelines for treatment of community-acquired pneumonia to combat penicillin-resistant strains of *Streptococcus pneumoniae* |
| Gatifloxacin | Allegan | Treatment of bacterial conjunctivitis |
| Moxifloxacin | Alcon | Treatment of bacterial conjunctivitis |
| Gemifloxacin mesylate | LG Life Sciences | Infections caused by susceptible strains of designated microorganisms in acute bacterial exacerbations of chronic bronchitis and community-acquired pneumonia |
| Levofloxacin | Ortho-McNeil | Treatment of chronic bacterial prostatitis due to *Escherichia coli, Enterococcus faecalis* or *Staphylococcus epidermidis* |
| Ciprofloxacin and dexamethasone | Alcon | Treatment of acute otitis media, in pediatric patients, due to *Staphylococcus aureus, S. pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* or *Pseudomona aeruginosa* |
| Linezolid | Pharmacia & Upjohn | Treatment of diabetic foot infections |
| Gemifloxacin mesylate | GeneSoft Pharmaceuticals | Treatment of infections, caused by susceptible strains of designated microorganisms, in acute bacterial exacerbations of chronic bronchitis and community-acquired pneumonia, including those caused by multidrug-resistant strains of *S. pneumoniae* |
| Ciprofloxacin | Bayer corporation | Treatment of complicated urinary tract infections and acute uncomplicated pyelonephritis |
| Daptomycin | Cubist Pharmaceuticals | Treatment of complicated skin and skin structure infections caused by susceptible strains *of S. aureus* (including methicillin-resistant), *Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus dysgalactidae* subsp *equisimilis or E. faecalis* (vancomycin-susceptible only) |
| Doripenem | Ortho-McNeil-Janssen | Treatment of complicated intra-abdominal and urinary tract infections |
| Tigecycline | Wyeth Pharmaceuticals Ltd | Complicated skin and skin structure infections and complicated intra-abdominal infections |
| Biskalcitrate, metronidazole, and tetracycline hydrochloride | Axcan candipharm | Treatment of patients with *Helicobacter pylori* infection and duodenal ulcer disease (to eradicate *H. pylori*) |
| Tobramycin/dexamethasone | Alcon | Superficial bacterial ocular infections or when there is a risk of bacterial ocular infection |

number of producer organisms. Currently, new approaches, such as reverse engineering and genotyping the resistant strains, are being carried out in order to better understand the antibiotic resistance phenomena (Struble and Gill 2006). Additionally, old-fashioned drugs are now been revisited and assessed for future usage; one of the most important avenues currently resides in the development of new uses for "old drugs". This means finding a new application for antibiotics, that were originally designed for a particular disease, in the treatment of other diseases. One such example is Comtan, a drug used in the treatment of Parkinson but that also has potential in the treatment of diseases caused by *M. tuberculosis* (Chong and Sullivan 2007; Kinnings et al. 2009; Wozniak et al. 2012).

### *8.1.2 Mechanisms of Drug Resistance*

Better knowledge about antibiotic resistance mechanisms will undoubtedly translate into the design of better novel drugs.

Resistance to antibiotics can be active, such as that resulting from a specific evolutionary pressure, or passive, where resistance originates from a general adaptation process (Wright 2005).

The mechanism of resistance depends not only on the particular microorganism, but also on the antimicrobial agent. The different possibilities thus far proposed, can be summarized into several categories:

1. Drug target modification, for example single mutations in target genes such as *gyrA* (very common in fluoroquinolone resistant strains) that can also accumulate mutations in the same gene thus increasing the resistance level (Piddock 1999). Target modification can also include the region-selective modification catalyzed by enzymes, such as the Erm (Wright 2011).
2. Drug molecule modification by specialized enzymes, such as the $\beta$-lactamases in the case of penicillin, known since 1940 (Abraham and Adler 1940).
3. Reduced accumulation of the drug inside the bacteria, by modification of the cell wall permeability or by pumping out the drugs. A good example of this are resistant Gram-negative bacteria: AcrAB-TolC in *Escherichia coli* (*E. coli*) and MexAB-OprM in *Pseudomonas aeruginosa* (*Ps. aeruginosa*) (Kumar and Schweizer 2005),
4. Alternative metabolic pathways (Wright 2011).

The current explosion of "omics" is opening an array of new possibilities, such as the relationship between gene mutations and antibiotic resistance. The information about putative target genes is mainly stored in Databases such as Drugbank.ca or ARDB (Antibiotic Resistance Genes Database) (Knox et al. 2011; Liu and Pop 2009).

The knowledge of both the way of action of antibiotics and the mechanisms of resistance is essential to identify the resistant microbial strains. Only with this in mind, it was possible to develop gentamicin, an antibiotic active against

aminoglycoside-resistant bacteria (Wright et al. 1998). Semi-synthetic β-lactams, such as penems and carbapenems, were designed to overcome the problem of β-lactamase-resistance (Edwards and Betts 2000). Consequently, the knowledge about resistance mechanisms has led us to the design of new strategies for fighting them. Hence, to overcome penicillin-resistance, an inhibitor of lactamases, such as clavulanic acid, is administered together with the antibiotic (Drawz and Bonomo 2010). Another possibility could be to combine the antibiotic with bacterial cell wall proteins, to facilitate its penetration into the bacterial cell, thus lowering the minimal inhibitory concentration (MIC) threshold (Veiga-Crespo et al. 2011). Antibiotics such as β-lactams, chloramphenicol, and fluoroquinolones permeate across the Gram-negative outer membrane via porins (Nikaido 2003; Yoshimura and Nikaido 1985). *Serratia marcescens* exhibits resistance to aminoglycosides and β-lactams due to loss of porins (Goldstein et al. 1983). Porin loss is also responsible for *E. cloacae* resistance to meroprem (Cornaglia et al. 1996).

Different types of antibiotics have different mode of action. For example, tetracycline acts by binding to the bacterial 30S ribosomal subunit, thus inhibiting protein synthesis, while erythromycin binds to the 23S rRNA molecule and gentamicin binds to the 30S subunit of the bacterial ribosome complex. β-lactam antibiotics (penicillin, oxacillin, and methicillin) bind to transpeptidase proteins, inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall. As mentioned above, fluoroquinones, such as ciprofloxacin, bind to gyrase A, and the most frequent mechanism of resistance to these antibiotics results from a mutation in the gyrase gene. On the other hand, the most common mechanism that results in tetracycline-resistance involves the ribosome protection proteins (RPPs), such as *tet* and *tetM* in *S. aureus* (Connell et al. 2003). Resistance against β-lactams can involve either the presence of β-lactamase enzymes, able to degrade penicillin but not methicillin and oxacillin (Methodology 2010; Sabath 1982), or a mechanism mediated by proteins capable of functionally substituting for PBPs that renders them effective against penicillin, methicillin and oxacillin. There are three known mechanisms involved in erythromycin resistance. The most common is the enzyme-mediated methylation of domain V of the 23S rRNA molecule; this resistance can be mediated by either macrolide efflux pumps or by inactivation of the drug by specialized enzymes, such as *EreA* or *EreB* (Schmitz et al. 2000). The use of drug-modifying enzymes appears to be the most frequent mechanism in gentamycin-resistant microorganisms.

## 8.2 Enzybiotics

### *8.2.1 Definition*

The term enzybiotic is a hybrid created from the words “enzyme” and “antibiotic”. Its usage refers to phages, viruses that attack and lyse bacteria, which can potentially help us to fight bacterial diseases. The most precise definition of the term refers to a

group of phage-associated enzymes. These enzymes are actively produced during the lytic phase of the phage's cycle and are released from the bacterial cell through specific lytic processes working on the peptidoglycan layer, this is holins and lysins. Accordingly, the term "enzybiotic" has now acquired a broader meaning than originally designed for and it should include all enzymes, regardless of their biological origin, that are able to act as antibiotics. All of them have an enormous potential in the struggle against microorganism-caused diseases.

### *8.2.2 Phage-Based Therapies*

Phages are the most abundant and diverse biological agents in the planet. They have been identified in all kind of environments, although they are more abundant in water, both in freshwater and marine habitats.

Phages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to their morphology and nucleic acid content. Bacteriophage genomes consist of either single- or double-stranded DNA or RNA, with a highly variable size and can be circular or lineal. Bacteriophages have been classified into 12 virus families (Table 8.2).

Phages may undergo two life cycles: a lysogenic cycle, in which the phage DNA is inserted into the bacterial chromosome, and a lytic cycle, in which the phage uses the cellular machinery to replicate its own DNA and assemble its viral components. After assembly, the phage progeny lyse the bacterial membrane and cell wall, thus killing the bacterial host. In this last step of lysis and liberation of progeny is where holins and lysins play a crucial role: holins insert themselves into the bacterial plasma membrane to form pores; lysins then travel across these pores and degrade the peptidoglycan layer of the bacterial cell wall.

The discovery of phages is linked to the Ganges and Juma rivers in India. In 1896, Hankin reported the "curative" properties of these waters against bacterial infection, although he was not able to identify the responsible substance (Hankin 1896). A couple of decades later, Twort and D'Herelle introduced the term "bacteriophage" and started their application in Medicine (D'Herelle 1917; Twort 1915). Progressively these antibacterial applications included more infections, not only in humans but also in cattle and in the control of plant pathogens; with even commercially available preparations such as Bacté-coli-phage and Bacté-rhino-phage (Bruynoghe 1921; Summer 2005). D'Herelle described the first intravenous application of a solution containing bacteriophages for cholera treatment (d'Herelle 1931). Both in Europe and in the United States, phage-based therapies were applied for the treatment of different infections such as furunculosis, meningitis, and staphylococcal septicemia (Larkum 1929; Schless 1932; Schultz 1929).

However, the discovery of antibiotics, together with the early controversial results due to lack of knowledge about phages, limited their therapeutic uses. Since 1940, studies of phages were mainly the field of the nascent discipline of Molecular Biology, except for the work carried out in a few Institutions, such as

**Table 8.2** Phage classification

| Virus family | Nucleic acid | Main characteristics and representative members |
|---|---|---|
| *Myoviridae* | dsDNA | T4, T2 or Mu bacteriophages |
| *Siphoviridae* | dsDNA | $\lambda$ bacteriophage |
| *Podoviridae* | dsDNA | P22 or T7 phages |
| *Tectiviridae* | dsDNA | Bam35 or PRD1 phages, Thermus phage 37–61 |
| *Plasmaviridae* | dsDNA | Infect *Mycoplasma* |
| *Corticoviridae* | dsDNA | Infect *Pseudomonas* |
| *Microviridae* | ssDNA | G4 and ΦX174 bacteriophages |
| *Inoviridae* | ssDNA | M13 bacteriophage |
| *Cysaviridae* | ssRNA | Infect *Pseudomonas* |
| *Leviviridae* | ssRNA | MS2 and Q$\beta$ coliphages |
| *Lipothrixviridae* | dsDNA | Infect members of Archaea |
| *Fuselloviridae* | dsDNA | Infect members of Archaea |

the Hirszfeld Institute, in Poland, and the Elieva Institute in the Republic of Georgia (Sulakvelidze et al. 2001).

In Eastern Europe, studies on the use of bacteriophages for therapy were centered on the prophylaxis and treatment of bacterial infections in humans, and targeted a variety of pathogens and illnesses, such as *Staphylococcus, Pseudomonas, E. coli, Klebsiella,* and *Salmonella,* as well as infections located mainly in the gastrointestinal tract, skin, head, and neck (Litvinova et al. 1978; Slopek et al. 1984; Slopek et al. 1983a, b; Slopek et al. 1985a, b, c; Slopek et al. 1983c), but also lung infections and allergoses (Meladze et al. 1982; Sakandelidze 1991). It was in the Hirszfield Institute that the concept of 'a therapy must be designed for each particular patient' was introduced. When an infection was detected, the physicians applied specific mixtures of phages specially designed by the Institute researchers and they reported high success rates in patient recovery (Slopek et al. 1987).

Clinical use of phages in the West involved either typification of clinical strains of bacterial species such as *S. aureus, E. coli* 0157, *Listeria,* and *Campylobacter* (Khakhria et al. 1990; Lossner et al. 1990; Owen et al. 1990; Parker 1978) or diagnosis confirmation for *V. cholerae* 01 infections.

Since the late 1990 s, the lack of new antibiotics and the emergence of resistant bacterial strains made it mandatory to review the old therapies and approaches used thus far to control infectious diseases. Unfortunately, the firsts few years concentrated mainly on nonhuman therapeutical uses focusing on aquaculture, plague control, and veterinary matters (Barrow et al. 1998; Donovan et al. 2006; Goodridge 2004; Heuer et al. 2002; Higgins et al. 2005; Huff et al. 2005; Leverentz et al. 2004). In 1979, Lang et al. (Lang et al. 1979) described the use of bacteriophages to eliminate chronic infections in orthopedics. Phage therapy is based on the specificity of phages against bacteria. A bacteriophage can only infect and kill a particular type of bacteria. The largest users of phage preparations so far have been the Soviets. The Soviet Army, during the Georgian civil war in the early 1990s, provided its soldiers with sprays containing phage preparations against *S. aureus, E. coli, P. aeruginosa, S. pyogenes,* and *P. vulgaris* (Stone 2002).

The last few years saw a fast increase on the usage of phage-based therapies; for instance, the Southwest Regional Wound Care Center (USA) used phages to treat antibiotic-resistant infections (Clark and March 2006).

Some pharmaceutical companies have been created to develop phage products, such as "PhagoBioDerm" (sold by "Phage International"), used in the treatment of infected venous stasis ulcers and other poorly healing wounds, where antibiotics are unable to penetrate due to poor wound vascularization. PhagoBioDerm is a biodegradable polymer impregnated with phages (PyoPhage, BioPharm-L, Georgia), antibiotics (ciprofloxacin, benzocaine) and proteolytic enzymes (α-chymotrypsin), which can be used for both prophylactic and therapeutical purposes (www.phageinternational.com). "PhageDent" is similar to "PhagoBioDerm", but it is used in oral applications only (Shishniashvili 1999). The most extensive phage safety trials in humans were undertaken by Delmont Laboratories (USA) for Staphage Lysate. This compound was administered intra-nasally, topically, orally, subcutaneously, and intravenously. After 12 years, only minor side effects were observed (Sulakvelidze 2005b). A preparation containing a mixture of phages active against *E. coli, P. aeruginosa,* and *S. aureus* (Rhoads et al. 2009) were tested in a FDA-approved phase I trial, to use in skin ulcerations and other wounds; the trial was completed in 2008 at the Wound Care Center. This trial also constituted an example of different Institutions (Wound Care Center and Eliava Institute) and a company (Intralytix) working together. Other clinical trials are currently being carried out, but thus far only preliminary results are available (Kutter et al. 2010; Wright et al. 2009).

The use of cocktails of phages against cattle pathogens has also been evaluated and their use in biocontrol has been validated (O'Flynn et al. 2004).

The FDA has approved the use of bacteriophages in cheese, to control *Listeria monocytogenes*, thus recognizing phages as GRAS (generally recognized as safe) in 2006 (http://www.cfsan.fda.gov/~rdb/opa-g198.html). In 2007, the GRAS status of these phages was updated to include their use on all food products (http://www.cfsan.fda.gov/~rdb/opa-g218.html).

### 8.2.3 Structure of Phage Enzymes

As mentioned above, there are two main types of phage enzymes that have therapeutical utility: holins and lysins, both essential during the lytic phase of the viruses.

Lysins are classified according to their cleavage site in the peptidoglycan layer: N-acetylmuramidase or lysozymes, endo-β-N-acetylglucosaminidases, transglycosylases, endopeptidases, and N-acetylmuramoyl-L-alanine amidases (Borysowski et al. 2006).

The lysins contain two domains: the catalytic domain, a well conserved area that is responsible for the enzymatic cleavage of peptidoglycan, and a variable binding domain, that changes with different species and strains, and able to

distinguish discrete epitopes, mainly carbohydrates or teichoic acids (Lopez et al. 1997; Nelson et al. 2006).

Despite the fact that all lysins share this basic organization, some lysins exhibit some particular characteristics. For example, the lysin PlyGBS has more than one catalytic domain, while the amidase domain present in amidase Pal is totally different from the equivalent domain in other amidases with lytic activity against pneumococci; additionally, the lysin isolated from T7 phage does not have a binding domain but only contains the catalytic domain (Cheng et al. 2005; Cheng et al. 1994; Navarre et al. 1999; Pritchard et al. 2004; Varea et al. 2004).

Lysins are usually monomeric enzymes, but that is not always the case. PlyC from streptococcal $C_1$ bacteriophage is multimeric (Nelson et al. 2006) and this is also the case for phages that infect *M. tuberculosis*.

Holins are responsible for the formation of holes in the bacterial cellular membrane, they open the way for lysins to reach the peptidoglycan layer. Holins are classified according to the number of transmembrane domains (TMD) they span. This results in two major classes: class I includes enzymes with two TMD, while class II members span three TMD (Young 2002).

### *8.2.4 Therapies Based on Phage Enzymes*

Advances in molecular biology techniques have allowed researchers to focus on two main phage-based therapies: the administration of whole phages and the administration of phage lytic enzymes. Lysins are capable of degrading peptidoglycan when applied topically, as purified recombinant proteins, and lysozymes play a relevant role in the control of bacterial populations in a huge variety of environments and applications.

Lysozymes can be used as food additives and food preservatives in a variety of processes, from the production of cheese and wine, to medical uses, such as in eye drops and even in toothpaste (Burman et al. 1991; Ibrahim et al. 2002; Shearman et al. 1994; Touch et al. 2003).

One of the first phage enzymes tested was the enzyme PlyG, from phage $\gamma$, that can kill *Bacillus anthracis* strains; when administered in vivo the enzyme was shown to protected mice from *B. anthracis* infections (Schuch et al. 2002). Lytic enzymes have been successfully used to combat infections caused by vancomycin-resistant *E. faecium* and also by vancomycin-resistant *E. faecalis* (Biswas et al. 2002; Yoong et al. 2004). The development of both genetic engineering and cloning techniques led to the possibility of creating hybrid proteins containing domains originating from different phages. This opened a new opportunity, the creation of recombinant lysins with different binding and catalytic domains, but still retaining the same bacterial specificity (Fischetti 2005).

One of the major advantages of phages over antibiotics is their specificity, but at the same time this is also their drawback. But the advent of recombinant purified enzymes allows researchers to break this barrier. The lysine plyGBS, isolated from

the GBS bacteriophage NCTC 11261, displays different lytic activity within the different groups of streptococci and additionally exhibits enzymatic activity against other bacterial species, such as *S. salivarius, S. gordonii,* and *S. mutans* (Cheng et al. 2005). This enzyme can be used in the treatment of infections caused by *S. agalactiae*, whether located in the vagina or in the oropharynx.

One of the current major health threats is posed by the methicillin-resistant *S. aureus* (MRSA) strains. Some of the cloned lysins, such as p17 lysin or LysK from a variety of phages, have been shown to be efficient against MRSA (O'Flaherty et al. 2005; Takac and Blasi 2005).as $\varphi$13 and $\varphi$6, are effective against *Pseudomonas syringae* (Daugelavicius et al. 2005).

An even more promising field is the use of purified phage enzymes in combination with antibiotics. For example, the use of lysin together with antibiotics against *S. pneumoniae* (Djurkovic et al. 2005); or even, protein cocktails containing enzymes with different lytic activities (Jado et al. 2003; Loeffler and Fischetti 2003).

### *8.2.5 Pros and Cons of Phage Therapies*

The reported failures in the use of whole phage-based therapies were mainly due to the scarcity of knowledge about phage biology, with mistakes in the selection of the therapeutic phages to be used, problems with the titer of phage preparations, bad methodology in the preparation of phage solutions and their administration, incorrect identification of causal agents, and an overall lack of knowledge about the toxins released after cell lysis (Kutter 2001). But all these pitfalls can now be solved.

The current limitations of phage therapy that hamper its applications are: the limited host range, the requirement for a prior identification of the pathogenic bacteria, and finding a way to sterilize the phage solutions without damaging the viruses (Hermoso et al. 2007).

Not all phages are suitable for phage therapy. There are two major classes of phages: lytic and lysogenic phages, with only the former being good candidates for their use as therapeutic agents. Lysogenic phages integrate their genome inside the host genome and this can lead to the transfer of virulence genes from one host to another, via the process known as transduction.

Of course, the naturally occurring evolution phenomena can result in bacteria developing resistance against phages but, even then, it would be easier and cheaper to develop new phage alternatives than to develop new antibiotic compounds.

The side effects of phage-based therapies are lower than those of the antibiotic-based therapies, because of the specificity of interaction between the virus and its bacterial host, hence, even if the target pathogen is not present, the phage would not have any effect on the patient's normal microflora (Bruttin and Brussow 2005; Sulakvelidze et al. 2001). The broad spectrum of antibiotics is, in this case, a clear disadvantage.

It is even possible to use cocktails of bacteriophages without the need for specific identification of the pathogenic agent. Recently, the FDA has approved a cocktail of bacteriophages for use against *L. monocytogenes* in ready-to-eat meat and poultry products (Fischetti et al. 2006).

A deep knowledge about the pharmacokinetics of the therapeutic phage preparations used is a must. The whole particles are live organisms, so storage conditions are critical if they are to remain alive. Additionally, phages self-replicate inside the host, so the initial phage dosage and the administration cycles must be carefully calculated. Another challenge would be to exactly replicate the composition of the phage preparations from batch to batch (Gill and Hyman 2010). This requires phage purification in the laboratory, and we must adhere to strict protocols to avoid bacterial contaminations in the purified phage preparations. It is also possible that we may need to develop new mathematical models to project phage growth inside the human body (Payne and Jansen 2003; Weld et al. 2004).

Experiments in mice have shown that phages are capable of crossing the blood–brain barrier and are more effective in areas of the body with a poor blood circulation (Barrow et al. 1999; Brussow 2005; Kutter 2001). It is also noteworthy the ability of phages to reach far away locations in the body, this was described as early as 1943, when experiments in mice demonstrated that peritoneal injections of phages were able to combat infections located in the brain, hence confirming that phages are able to reach the bloodstream and cross the blood–brain barrier to reach their bacterial host (Dubos et al. 1943). This ability is a huge advantage because, for the phage therapy to succeed, and result in the bacteria being cleared from the body, it is imperative that sufficient amounts of phage reach the area of the body colonized by their target bacteria. Nevertheless, in cases like this, when exogenous lytic enzymes are used in therapy, a new problem must be dealt with. Most lysins lack a secretory signal to enable them to cross the bacterial cellular membrane. In the case of Gram-positive bacteria, exogenous lysins would have no difficulty reaching the peptidoglycan layer. However, in Gram-negative bacteria the cell wall is more complicated, mainly due to the presence of an outer membrane, hindering access of the lysin to the peptidoglycan layer. Further research is needed in order to solve this problem.

Intracellular pathogens, such as *M. tuberculosis* or *Mycobacterium avium*, pose one of the major challenges in the medical treatment of illnesses. But, even in these cases, phage-based therapies show promise, since Broxmeyer et al. (2002) reported finding phages inside macrophages.

The immunology of phages has also been the subject of scrutiny (Sulakvelidze 2005b). While overall immunity was not negatively affected by whole phage particles, the case was different for purified enzymes, which could display immunotoxicity. On the other hand, treatment of hyperimmunized rabbits with lysins did not result in the production of lysin-neutralizing antibodies (Fischetti 2006).

An important ethical question has to be addressed that particularly concerns the Pharmaceutical industry when cash investments are required. Can a patent on a phage be justified? And more importantly, would the pharmaceutical industry be

willing to invest money in research and development into phages and accept a limited patent protection?

The use of phages has several advantages: i) phages are self-replicating; ii) they are more specific than antibiotics and hence do not cause damage to the normal microbiota; iii) they have low side effects; iv) they represent a real alternative for patients allergic to antibiotics; v) production costs are low; vi) they could be used for prophylaxis in hospital settings; vii) phages can be administered in several ways; viii) they may exhibit synergistic effects with antibiotics or even other medicines; ix) whole phage particles are able to replicate at the site of infection and so particle concentration only occurs at the sites where they are needed; and x) the search for and development of new alternatives to treat antibiotic-resistant bacteria is faster and cheaper than the development of new antibiotics.

Evidently, antibiotics have certain advantages over bacteriophages, the main being that they can have a broad spectrum of action, this means that they can be used without knowing the exact nature of the disease-causing bacteria. However, this type of wasteful use of antibiotics has been the main reason for the increase in the number of antibiotic-resistant bacterial strains.

## 8.3 Additional Enzymatic Therapies

There are many enzymes that can be considered as enzybiotics, these include not only the phage lytic enzymes, but also antifungal enzymes, such as glucanases and chitinases (Veiga-Crespo et al. 2007). However, this list must be extended to include all medically important enzyme preparations, regardless of their origin (Biziulevicius et al. 2008).

Additionally, to provide a complete revision of this topic we must include other enzyme groups, such as those that can act as helpers for enzybiotics. One important group of helper-enzymes includes the enzymes than can degrade the exopolysaccharides required in biofilm formation, such as Dispersin B (Itoh et al. 2005; Lu and Collins 2007). It has been shown that phage T4 can infect and replicate within *E. coli* biofilms, hence disrupting biofilm morphology by killing the bacterial cells (Corbin et al. 2001).

Alginate lyase is produced by bacterial species such as *Azotobacter chroococcum, Bacillus circulans,* and *P. aeruginosa* (Hansen and Nakamura 1985; Pecina et al. 1999; Schiller et al. 1993). In patients with cystic fibrosis, secretion of alginate by *P. aeruginosa* is a major problem, but the enzyme alginate liase can degrade it, thus facilitating the entry of other antibacterial drugs, including bacteriophages.

Lysostaphin, an endopeptidase, is naturally produced by *Staphylococcus simulans* strains, and it was approved in the Former Soviet Union, not only for the treatment of gastrointestinal and gynecological diseases for which its efficacy was tested (Biziulevicius and Zukaite 1999), but also for biofilm disruption on artificial surfaces (Kusuma et al. 2007).

Zoocin A is a peptidoglycan hydrolase, produced by *Streptococcus equi spp. zooepidemicus* 4881, that targets a number of pathogenic streptococci, such as *S. equi, S. pyogenes,* and *S. mutans*. Zoocin's ability to bind to and hydrolyze the peptidoglycan layer of several strains of *Streptococcus* has been tested in vitro, confirming its potential to be used as an enzybiotic (Akesson et al. 2007).

Finally, the group of enzymes that blocks the synthesis of the bacterial peptidoglycan layer also deserves a place here. Additionally, the RNA-bearing Q$\beta$ bacteriophage must also be considered, in this context, as a potential therapeutic alternative (Bernhardt et al. 2001).

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# Chapter 9
# New Cell Wall-Affecting Antifungal Antibiotics

**Juan Carlos Ribas, Ángel Durán and Juan Carlos G. Cortés**

**Abstract** Fungi have emerged worldwide as increasingly frequent causes of healthcare-associated infections. Invasive fungal infections can be life-threatening. However, the number of antifungal agents available and their use in therapy is very limited. Recently, a new family of specific fungal cell wall synthesis inhibitors has emerged as an alternative antifungal therapy and is gaining increasing relevance yearly. The cell wall is a multilayer dynamic structure, essential to the integrity and shape of the fungal cell, whose function is to counteract the osmotic forces that could otherwise produce fungal cell lysis. The cell wall is absent in nonfungal cells, therefore representing a useful target in discovering selective drugs for the treatment of fungal infections without causing toxicity in the host. Although fungi exhibit a considerable diversity in their cell wall structure, all present $\beta$(1,3)-, $\beta$(1,6)- and $\alpha$(1,3)-glucans, chitin, and mannoproteins as their major cell wall components. Three different cell wall synthesis inhibitors of the lipopeptide family of echinocandins, named caspofungin, micafungin, and anidulafungin, are commercially available and new classes of cell wall synthesis inhibitors are emerging. This review provides an overview of what is so far known about the different classes of cell wall-affecting antifungal agents and their mechanism of action, offering new alternatives with clinical potential.

## 9.1 Introduction

Invasive fungal infections are devastating. Despite the progressive development of new antifungal therapies, the mortality rates for invasive mycoses often reach the 50% (Lai et al. 2008; Park et al. 2009). Although fungal infections affect to

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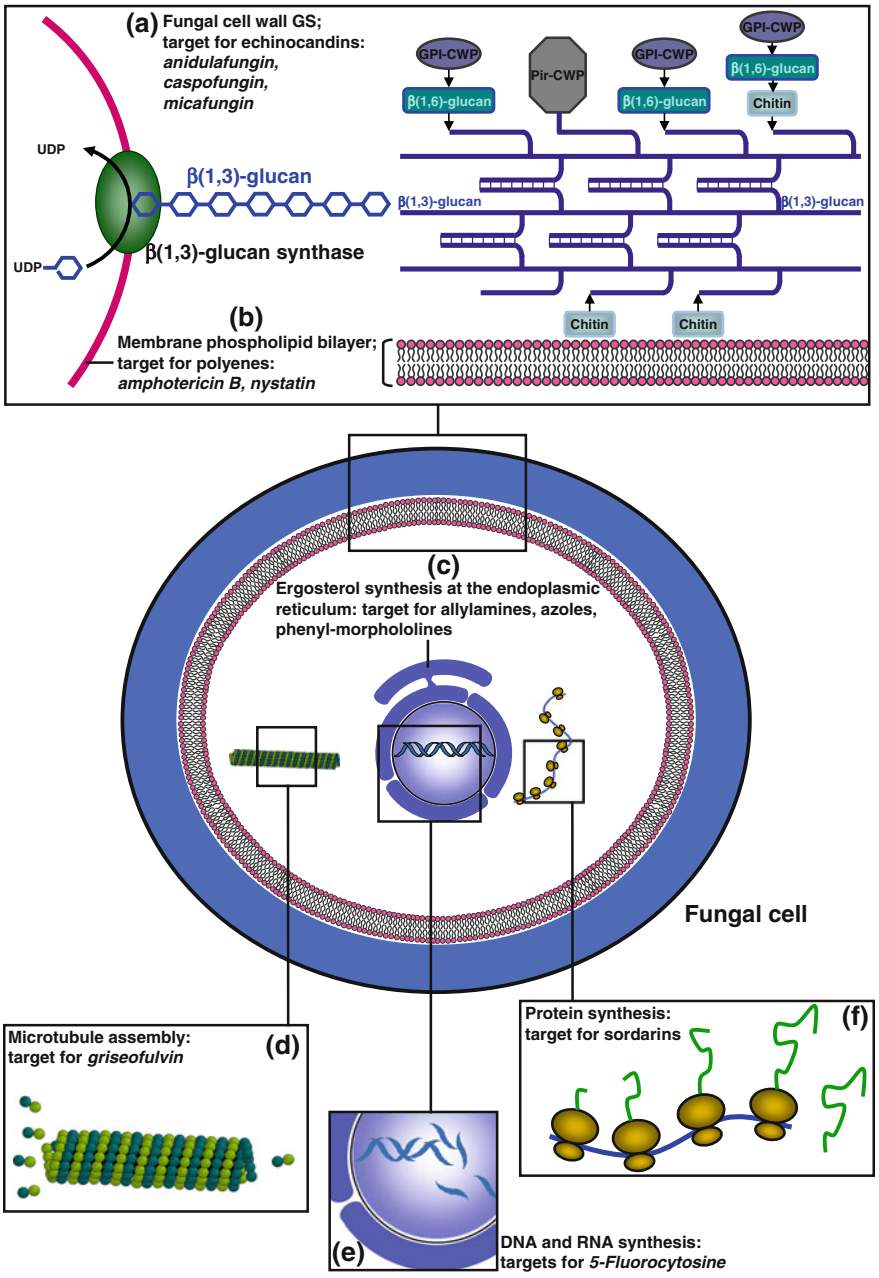
individuals with intact immune systems, invasive aspergillosis and mucormycosis continue increasing in parallel with the growth of immunocompromised patients (Brown et al. 2012b). Improved diagnostics, new epidemiological analysis, and new antifungals available have changed the field of medical mycology in the past few decades (Ostrosky-Zeichner et al. 2010).

The high phylogenetic similarity between fungi and humans makes that relatively few differential targets can be used for antifungal drug development. Fungi originate a huge collection of extracellular enzymes and secondary metabolites to counteract and digest other fungi and microorganisms from their environment; thus many antimicrobial agents have been isolated from fungi themselves. The best example is penicillin, which was isolated from *Penicillium notatum* (now *Penicillium chrysogenum*). Similarly, the echinocandins, a class of antifungal compounds now in extensive clinical use, are semisynthetic derivatives of fungal-produced cyclic hexapeptides (Denning and Hope 2010; Chain et al. 1993).

Presently, five classes of antifungal drugs are used for the treatment of fungal infections in humans: allylamines, azoles, echinocandins, polyenes, and pyrimidine analogs. These drugs show some limitations, such as the nephrotoxicity of amphotericin B and the emerging resistance to the azoles (Cartledge et al. 1997), despite several improvements, such as new lipid formulations of polyenes with lower toxicity and new triazoles with a wider spectrum of action (Granier 2000). The development of new antifungal agents, preferably naturally occurring with novel mechanisms of action, is an urgent medical need (Vicente et al. 2003). In the last years, new synthetic and semisynthetic inhibitors of the cell wall synthesis have emerged (Hector and Bierer 2011), being effective against the main infectious agents of life-threatening mycoses. In addition to the more traditional classes of cell wall synthesis inhibitors, this chapter focuses and reviews some of the new and more promising classes of such compounds, and their mechanism of action, concentrating on the most promising candidates that are nearing or currently in clinical development.

## 9.2 Sites of Action of the Antifungal Agents

The sites of action of all the antifungal agents used in clinic therapy are summarized in Fig. 9.1, showing the extensive range of antifungal targets that already exist. Nevertheless, the classes of agents that can be used against life-threatening mycoses are heavily focused, directly or indirectly, on the pathogen cell envelope (cell wall and plasma membrane), and particularly on the membrane complex $\beta$(1,3)-glucan synthase (GS), in charge of the biosynthesis of the cell wall $\beta$(1,3)-glucan; and on the membrane ergosterol and its biosynthesis (Chap. 8 of this book). Targets elsewhere in the cell would therefore be a welcome advance for systemically bioavailable antifungal agents. The introduction of the echinocandins (anidulafungin, caspofungin, and micafungin are the only new drugs licensed within the last 10 years) and the third-generation triazoles (voriconazole and


(a) Fungal cell wall GS; target for echinocandins: anidulafungin, caspofungin, micafungin
GPI-CWP
Pir-CWP
GPI-CWP
GPI-CWP
β(1,6)-glucan
β(1,6)-glucan
β(1,6)-glucan
Chitin
UDP
β(1,3)-glucan
β(1,3)-glucan
β(1,3)-glucan
UDP
β(1,3)-glucan synthase
(b)
Membrane phospholipid bilayer; target for polyenes: amphotericin B, nystatin
Chitin
Chitin
(c)
Ergosterol synthesis at the endoplasmic reticulum: target for allylamines, azoles, phenyl-morpholines
Fungal cell
Microtubule assembly: target for griseofulvin
(d)
(e)
DNA and RNA synthesis: targets for 5-Fluorocytosine
Protein synthesis: target for sordarins
(f)

◀ **Fig. 9.1** Sites of action of antifungal agents. Illustration of the sites of action of currently available antifungals. **a** Echinocandins inhibit the $\beta$(1,3)-glucan synthase (GS) and the formation of the $\beta$(1,3)-glucan of the fungal cell wall. It is shown a schematic representation of the synthesis and organization of the $\beta$-glucans. The synthesis of linear $\beta$(1,3)-glucan chains starts in the plasma membrane by the GS. Then cell wall transglycosidases form branched $\beta$(1,3)$\beta$(1,6)-glucan bound to chitin via $\beta$(1,4) linkages. Proteins are covalently attached to $\beta$(1,3)$\beta$(1,6)-glucans through the GPI remnant or through a glutamine residue following a transglutaminase reaction. **b** Polyene antifungals bind to fungal ergosterol, disturbing the plasma membrane function. **c** Azole agents target the biosynthesis of ergosterol at the endoplasmic reticulum, leading to an altered plasma membrane depleted of ergosterol. **d** Griseofulvin presumably binds to tubulin, thus interfering with the assembly of microtubules and inhibiting the nuclear mitosis. **e** 5-Fluorocytosine or Flucytosine interferes with DNA and RNA synthesis. **f** Sordarins block the fungal translation thus interfering with protein synthesis

posaconazole), in particular, have enhanced therapeutic options for many fungal infections (Brown et al. 2012a; Ostrosky-Zeichner et al. 2010).

## 9.3 The Fungal Cell Wall

The extracellular fungal wall is responsible for the cell shape, provides mechanical protection, and supports the internal osmotic pressure of fungal cells. In addition, its rigid structure is useful for penetration into and colonization of insoluble substrates. This structure is not simply a rigid exoskeleton, but has the flexibility required for allowing cell growth, cell division, and the formation of numerous cell types during the fungal life cycle. The functional equivalent of the fungal cell wall in animal cells is denominated extracellular matrix, but its composition and osmotic and structural properties are totally different from those of the cell wall. Therefore, although both structures are functionally equivalent, their differences permit that the cell wall-affecting antifungals specifically inhibit the fungal cell wall synthesis without affecting the extracellular matrix of animal cells (Durán and Pérez 2004; Free 2013; Latge 2007; Lesage and Bussey 2006; Levin 2011; Lu et al. 2012; White and Bednarek 2003; Oh et al. 2012).

The cell wall is also critical for biofilm formation, a process that many fungi participate in, and which is an important ecological niche for a variety of fungi. For fungal pathogenicity, the cell wall is the surface of interaction between pathogen and host, being critical for its virulence and pathogenicity, providing both adhesive properties critical for invasion of host tissue and protection against the host defense mechanisms. Alterations of cell wall structure have a deep impact on the growth, morphology, and integrity of the fungal cell, often leading to lysis by plasma membrane breaking and release of cytoplasm content. Given the essential role of the cell wall in fungal survival, this structure has long been considered an excellent target for antifungal agents (Free 2013).

To build their walls, the fungal cells must synthesize the wall components in or export them across the plasma membrane, and assemble them outside the cell

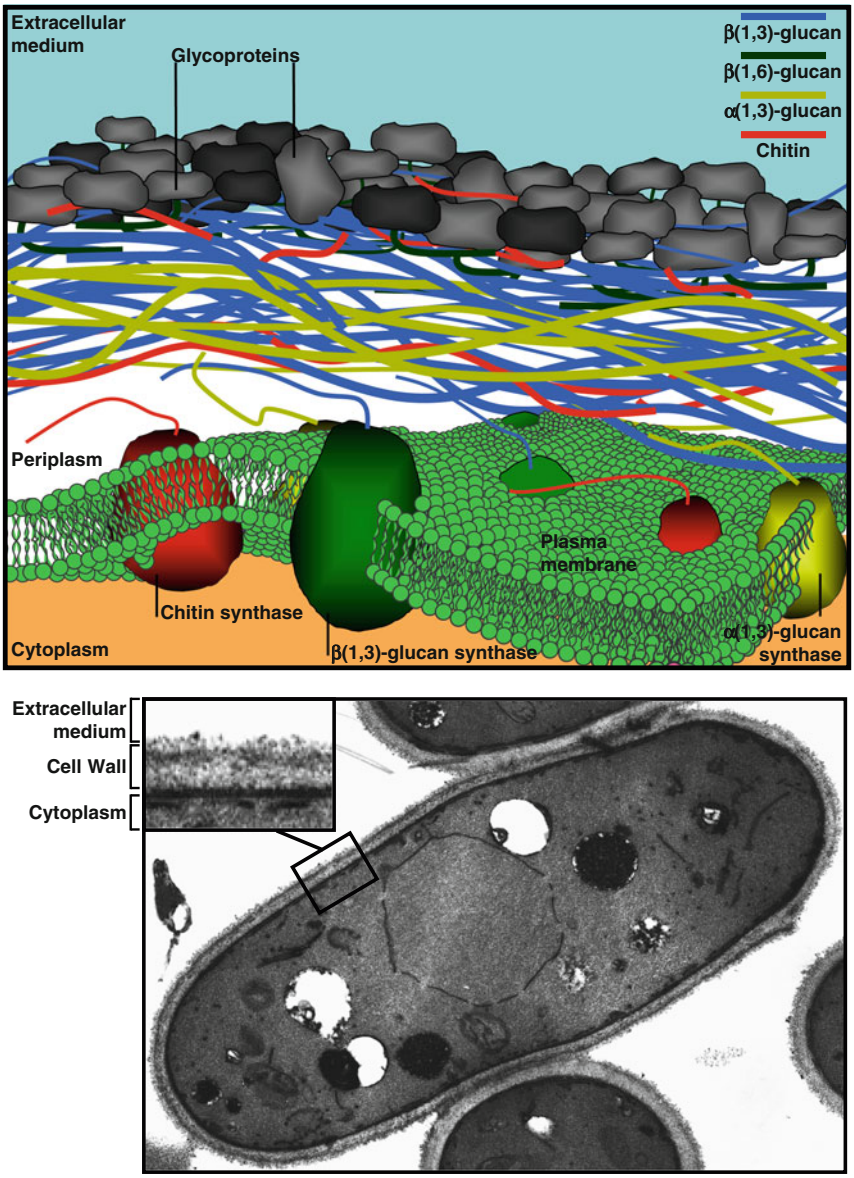



**Fig. 9.2** Structure and composition of fungal cell wall. The *upper panel* shows a cartoon of the organization and composition of the main fungal cell wall layers with the cell wall synthases embedded in the plasma membrane. Transmission electron micrographs of a fission yeast cell and a detail of the cell wall are presented in the *lower panel*

(Fig. 9.2). The wall is composed basically of polysaccharides (70–90 %) and glycoproteins (10–30 %). Although composition varies among fungal species, most walls have a common structure (Latge 2007). When observed by

transmission electron microscopy, the cell walls show two electron dense external layers rich in glycoproteins and an internal layer more transparent to the electrons, which mainly contains fibrillar polysaccharides (Fig. 9.2).

The main fungal wall fibrillar components are: glucose homopolymers, β(1,3)-glucan with different percentages of β(1,6) branches depending on the fungal species; chitin, a β(1,4)-N-acetylglucosamine polymer; and α(1,3)-glucan. Chitin accounts for only 1–2 % of the yeasts wall (Cabib et al. 1988; Lesage and Bussey 2006), whereas filamentous fungi, such as *Neurospora* or *Aspergillus*, contain 10–20 % of chitin in their walls (Latge 2007). In both yeasts and filamentous fungi, chitin forms microfibrils by interchain hydrogen bondings, which have enormous tensile strength and significantly contribute to the overall integrity of the cell wall (Cabib and Kang 1987). Similarly, α(1,3)-glucan is essential for the adhesion strength of the cell wall components (Cortés et al. 2012).

The wall polysaccharides are formed at the plasma membrane by synthase enzymes and extruded to the periplasmic space (Fig. 9.2), where they bind to each other. The linkages among the different components, which result in a tightly linked network, are generated by transglycosylation (Cabib et al. 2007, 2008) and are responsible for the mechanical strength of the cell wall (Cabib and Kang 1987; Kapteyn et al. 1997; Kollar et al. 1995, 1997).

## 9.4 Cell Wall Components

### *9.4.1 β(1,3)-Glucan*

β(1,3)-glucan is the main structural polysaccharide of the wall and represents 50–60 % of the total cell wall polysaccharides. Most of glucan polymers are composed of glucose units linked with β(1,3) bonds (65–90 %), although there are also some β(1,6), β(1,4), α(1,3), and α(1,4) glucans. The β(1,3)-glucan chains display a coiled spring-like structure that confers elasticity and tensile strength to the cell wall. Usually, the β(1,3)-glucan is formed by a main backbone of β(1,3)-glucan and by β(1,6)-linked side chains (Fig. 9.1, upper panel). In these linear chains of β(1,3)-glucan, new glucose units bind forming β(1,6) branches in variable proportion depending on the organism, from almost linear to highly branched. Then, the β(1,6)-branched β(1,3)-glucan can bind to other glucans, to chitin or to glycoproteins, providing a great mechanical resistance to the wall, which is essential to maintain the fungal cell integrity (Klis et al. 2002; Kollar et al. 1995, 1997; Lesage and Bussey 2006).

#### 9.4.1.1 β(1,3)-Glucan Synthase (GS) Complex

β(1,3)-glucan chains are synthesized by the enzyme complex β(1,3)-glucan synthase (GS), which is located in the plasma membrane. The echinocandins interfere with the production of β(1,3)-glucan and target the GS directly (Douglas 2001). This family of enzymes use uridine-diphospho-glucose (UDP-Glc) as substrate and catalyze the reaction 2n UDP-Glc → [Glc-β-1,3-Glc]n in the presence of GTP, resulting in the formation of linear glucan chains composed of approximately, 1,500 β(1,3)-bound glucose residues (Fig. 9.1, upper panel) (Cabib and Kang 1987). The GS complex is composed by at least two subunits, which were identified by detergent and high ionic strength extraction from the plasma membrane, followed by an in vitro assay for GS activity. Under these conditions, the GS complex was separated into a membrane-bound and a cytosolic fraction, containing the catalytic and regulatory GTP-binding subunits, respectively (Kang and Cabib 1986):

**Catalytic subunit**

The genes coding for the putative GS catalytic subunit were initially identified in *S. cerevisiae* and named *FKS1* and *GSC2*/*FKS2* (Douglas et al. 1994b; Mazur et al. 1995). *FKS1* (for ***FK***506 ***s***ensitive) was initially cloned by complementation of a mutant hypersensitive to the calcineurin inhibitor FK506 (Parent et al. 1993). The discovery that *ETG1* and *PBR1* (whose mutants are resistant to GS inhibitors), *CND1* (the *cnd1* mutant requires a functional calcineurin pathway), and *CWH53* (required for resistance to calcofluor white) are all identical to *FKS1*, established the link between Fks1 and the cell wall (Castro et al. 1995; Douglas et al. 1994a; El-Sherbeini and Clemas 1995; Garrett-Engele et al. 1995; Ram et al. 1995). *FKS1* mutants show decreased GS activity, and Fks1 is enriched in membrane fractions enriched in GS activity, suggesting that Fks1 could be a GS catalytic subunit (Douglas et al. 1994a; Inoue et al. 1995).

Fks2/Gsc2 is 88 % identical to Fks1 and presents similar topology and domain organization to that of Fks1. Fks1 and Fks2 are thought to be alternative subunits of the GS complex, with a double *fks1*Δ *fks2*Δ mutant being nonviable. This synthetic lethality and the finding that membrane extracts from cells expressing only *FKS2*, show a GS activity that can be depleted after treatment with anti-Fks2 antibodies, suggested that Fks2 is involved in β(1,3)-glucan synthesis (Inoue et al. 1999; Mazur et al. 1995).

*FKS1* and *FKS2* show differential expression patterns. *FKS1* transcription is cell-cycle regulated, and linked to cell wall remodeling. *FKS2* transcription is calcineurin dependent (Kurtz and Rex 2001). *FKS1* is expressed during mitotic growth, in agreement with its proposed function as the major GS subunit. *fks1*Δ cells show reduced GS activity and altered cell wall composition, with decreased β(1,3)- and β(1,6)-glucan levels, and increased chitin and mannan levels (Dijkgraaf et al. 2002; Douglas et al. 1994a; Lesage and Bussey 2006). In contrast, during vegetative growth, the *fks2*Δ mutant does not display cell wall or cell

growth defects. However, during sexual differentiation, the *fks2*Δ cells are impaired in spore wall assembly. The strength of the phenotypes observed in *fks1*Δ compared to those observed in *fks2*Δ cells, suggest that Fks1 is the main responsible for GS activity and β(1,3)-glucan synthesis during vegetative growth, while Fks2 would function under more stressful conditions (Lesage and Bussey 2006). The *S. cerevisiae FKS* family has a third member, *FKS3*, whose product is required for sporulation (Deutschbauer et al. 2002; Ishihara et al. 2007).

The Fks protein family of GS is very well conserved in fungi and plants, and are large multispan membrane proteins with a hydrophilic central region (Johnson and Edlind 2012). Orthologs of these genes have been described in the main fungal genera, encoding proteins with a high degree of identity ranging from 56 % (*Cryptococcus neoformans*) to 83 % (*Candida glabrata*) (Latge 2007, Lesage and Bussey 2006; Pérez and Ribas 2004; Cortés et al. 2002).

The fission yeast *Schizosaccharomyces pombe* has provided an appealing model for studies addressing cell wall synthesis and morphogenesis. The cell wall of *S. pombe* has no detectable chitin (Durán and Pérez 2004), but it contains three different essential β(1,3)-glucans as follows: a β(1,6)-branched β(1,3)-glucan, which is the major contributor to the cell wall structure; a minor linear β(1,3)-glucan, concentrated in the primary septum, with residual amounts in the cell wall; and a minor branched β(1,6)-glucan (Cortés et al. 2007; Humbel et al. 2001). *S. pombe* contains four genes, *bgs1*$^+$ to *bgs4*$^+$, which encode four different GS catalytic subunits of the Fks protein family, and three of them (Bgs1, 3, and 4) are essential during vegetative growth. Differently to what has been described for *S. cerevisiae* and some other fungi, where Fks1 and Fks2 have been shown to have redundant functions (see above), *S. pombe* GS catalytic subunits display differential essential nonoverlapping functions in the assembly and the transport of different β(1,3)-glucans during polarized cell wall growth, septum synthesis, and ascospore wall assembly. The different essential functions of Bgs proteins in cell wall synthesis and morphogenesis make them good targets for the search and study of new improved antifungal drugs that specifically inhibit β(1,3)-glucan synthesis. *bgs1*$^+$ was initially cloned by phenotype complementation of a mutant that displays hypersensitivity to Cyclosporin A as well as Papulacandin B, and the encoded protein Bgs1 is responsible for the synthesis of the linear β(1,3)-glucan and primary septum (Cortés et al. 2002, 2007; Ishiguro et al. 1997; Liu et al. 2002). *bgs2*$^+$ encodes a protein essential for spore wall maturation, and *bgs3*$^+$ was identified as a suppressor of a mutant that shows hypersensitivity to echinocandin. Bgs3 is essential, although its function remains unknown (Carnero et al. 2000; Liu et al. 2000; Martín et al. 2000). *bgs4*$^+$ encodes the only subunit that has been shown to form part of the GS enzyme. It is responsible for most of the cell wall β(1,3)-glucan synthesis and in vitro GS activity, and it is essential for the maintenance of cell integrity during cell growth and mainly septum synthesis. To date, the only identified mutants of *S. pombe* that display reduced levels of β-glucan and GS activity, or resistance to specific GS inhibitors, are due to point mutations in the Bgs4 sequence (Castro et al. 1995; Cortés et al. 2005; Martins et al. 2011; Ribas et al. 1991a, b).

The presence of multiple *FKS*/*bgs* genes in some fungi might be the result of the ancient and selective retention of duplicated genes through increased fitness conferred by their specialized application of GS functions (Lesage and Bussey 2006). This complexity is also found in plants, where the Fks/Bgs protein family of GS is also present. Plant GS synthesize a $\beta$(1,3)-glucan polymer denominated callose. Although $\beta$(1,3)-glucan is less abundant in plants than in fungi, the number of GS genes is much higher in plants than in fungi. Thus, *Arabidopsis thaliana* or *Oryza sativa* contain 12–18 callose synthase genes, whereas the highest number of genes in fungi are the 4 *bgs* genes identified in *S. pombe*. Plant callose is involved in pollen development (primary and secondary cell wall of the pollen, germination pore and pollen tube) as well as wound repair after injury or disease. Similarly to *S. pombe* $\beta$(1,3)-glucans, all steps in which callose participates should involve different callose synthase genes, which would explain the existence of multiple callose synthase genes in plants. Despite that plant and fungi belong to different kingdoms, plant and fungal GS display high sequence identity (Cortés et al. 2007; Latge 2007; Verma 2001; Verma and Hong 2001).

The GS catalytic subunit is thought to extrude $\beta$(1,3)-glucan chains produced on the periplasmic face of the plasma membrane for incorporation into the wall. Although the GS enzyme has never been purified to homogeneity, the central hydrophilic domain of partially purified *Neurospora crassa* Fks protein was shown to crosslink to azido-UDP-glucose, supporting the conclusion that this protein is the catalytic subunit (Schimoler-O'Rourke et al. 2003). Purification of the complete and active GS catalytic subunits will require new advances in our knowledge of enzymatically active integral membrane protein complexes (Lesage and Bussey 2006; Levin 2011).

**The regulatory subunit Rho1**

Besides the catalytic subunit, fungal GS require GTP-bound Rho1 GTPase for their activity. Essential Rho1 is the prototype of small G proteins, which in their active GTP-bound state bind and activate their effectors. After synthesis in the endoplasmic reticulum, Rho1 is geranylgeranylated, allowing its anchoring to the membrane through a C-terminal prenylated tail, which is required for Rho1-membrane association and activation of GS activity (Arellano et al. 1998; Díaz et al. 1993; Inoue et al. 1999). Different biochemical and genetic approaches have pointed out to Rho1 GTPase as a regulator of GS: (i) Rho1 colocalizes and copurifies with Fks1 and cofractionates with GS activity, (ii) thermosensitive *rho1* mutations cause thermolabile GS activity, (iii) a hyperactive *rho1* allele shows a GTP-independent GS, and (iv) overproduction of Rho1 is able to partly suppress the GS deficiency of a geranylgeranyltransferase type I mutant (Arellano et al. 1996; Drgonova et al. 1996; Mazur and Baginsky 1996; Qadota et al. 1996; Kondoh et al. 1997). Geranylgeranylated Rho1p is transported to the plasma membrane, where is thought to be activated by its GDP/GTP exchange factors.

This localized activation on the plasma membrane is required for proper cell wall β(1,3)-glucan synthesis (Abe et al. 2003; Díaz et al. 1993; Inoue et al. 1999; Perez and Rincon 2010). Although the Fks family members are well established as Rho1 effectors, the interaction domains between the GS catalytic and regulatory subunits and the basis for Rho1 activation on the GS catalytic subunits remain unknown (Lesage and Bussey 2006).

### *9.4.2 β(1,6)-Glucan*

In addition to β(1,3)-glucan, most of fungal walls contain a second β-linked glucan, the β(1,6)-glucan. This polymer is shorter than β(1,3)-glucan, it does not form a fibrillar structure, and acts as a flexible glue by forming covalent cross-links to β(1,3)-glucan, chitin, and glycoproteins (Kollar et al. 1997). Comparative studies show the variability of β(1,6)-glucan structures among fungi, with the most highly branched *S. pombe* diglucan, likely a variant of the β(1,6)-glucan found in *S. cerevisiae* and *C. albicans* (Lesage and Bussey 2006). To date, most of the genes implicated in the synthesis of β(1,6)-glucan have been identified in *S. cerevisiae*. The site of β(1,6)-glucan synthesis has been controversial for many years. Defects in β(1,6)-glucan synthesis are observed in mutations of genes as *CWH41*, *ROT2, KRE5,* and *CNE1*, encoding glucosidases I and II and UDP-glucose: glycoprotein glucosyltransferase- [UGGT]- and calnexin-related proteins, respectively. These proteins are involved in different steps of the secretory pathway (Page et al. 2003; Lesage and Bussey 2006; Shahinian and Bussey 2000), suggesting that biosynthesis of this polymer could start in the endoplasmic reticulum. In *S. pombe*, an immunoelectron microscopy analysis showed particles of β(1,6)-glucan associated to the Golgi apparatus (Humbel et al. 2001), suggesting that biosynthesis of this polymer progresses in the Golgi, and is completed at the cell surface. Indeed, *S. cerevisiae* glucosyl hydrolases (or transglucosylases) Kre6 and Skn1 are critical for β(1,6)-glucan synthesis and reside in the Golgi (Roemer and Bussey 1991; Roemer et al. 1994). However, a late secretory pathway mutant displayed β(1,6)-glucan exclusively accumulated in the cell surface, indicating that a block of the secretion does not cause an accumulation of intracellular β(1,6)-glucan (Montijn et al. 1999), suggesting that β(1,6)-glucan, like β(1,3)-glucan, may be synthesized at the plasma membrane. An in vitro assay for β(1,6)-glucan synthesis using specific antibodies against β(1,6)-glucan has been developed (Vink et al. 2004). This assay requires membrane extracts, UDP-glucose, and GTP and shows enhanced activity in cells overexpressing Rho1, suggesting that the β(1,6)-glucan could be synthesized at the sites of polarized cell growth where Rho1 is detected. Besides, the use of membrane extracts from defective β(1,6)-glucan mutants correlated the decreased in vitro β(1,6)-glucan synthase activity with the low levels of in vivo cell wall β(1,6)-glucan (Levin 2011; Vink et al. 2004).

### 9.4.3 Chitin

Chitin is a $\beta$(1,4)-linked homopolymer of *N*-acetylglucosamine that forms microfibrils stabilized by hydrogen bonds. This polymer is present in the walls of all fungi studied to date with the exception of *S. pombe*. Chitin represents 1–2 % of the yeast cell wall whereas in filamentous fungi, it can reach up to 10–20 % (Latge 2007). Chitin is synthesized from N-acetylglucosamine units by the enzyme chitin synthase (CS) that deposits microfibrils of chitin outside of the plasma membrane. This family of enzymes use uridine-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) as substrate and catalyze the reaction 2n UDP-GlcNAc → [GlcNAc-$\beta$-1,4-GlcNAc]n. Chitin biosynthesis is best understood in *S. cerevisiae*, where it has been broadly studied. Three CS activities (CSI, CSII, and CSIII) have been identified in membrane extracts, and the corresponding catalytic subunits have been identified as Chs1, Chs2 and Chs3, respectively. The three Chs proteins are integral membrane proteins, each one responsible for the synthesis of a chitin (Cabib et al. 2001) at different times and places during cell growth. Chs1 acts as a repair enzyme during cell separation; Chs2 is responsible for synthesis of the primary septum chitin; and Chs3 synthesizes most of the cell wall chitin and is responsible for the increase in chitin synthesis observed when the cell wall is stress-affected (Roncero and Sanchez 2010; Schmidt et al. 2002). The number of CS genes varies from 1 to 20 according to the fungal species. The large family of CS enzymes falls into seven classes according on the evolution of their amino acid sequences (Roncero 2002). The multiplicity of enzymes suggests that they have redundant roles in chitin synthesis and makes it difficult to find functional significance to the different classes (Lenardon et al. 2010).

### 9.4.4 α(1,3)-Glucan

Many fungi contain α(1,3)(1,4)-glucan in their cell wall. However, the corresponding in vitro α(1,3)-glucan synthase activity has not been detected yet. A putative catalytic subunit was first described in *S. pombe* (Cortés et al. 2012; Hochstenbach et al. 1998; Katayama et al. 1999). Ags1/Mok1 is a multidomain integral membrane protein with a predicted cytoplasmic synthase domain, multiple transmembrane domains and an extracellular transglycosylase domain. The cytoplasmic synthase domain would add glucose residues to the non reducing end of an α(1,3)-glucan glucan chain. Interestingly, the large extracellular N-terminal region presents homology to transglucanases, which could function in cross-linking newly synthesized α(1,3)-glucan to other cell wall components (Grun et al. 2005; Vos et al. 2007). The α(1,3)-glucan synthesized by Ags1/Mok1 is vital for cell integrity during polar growth and mainly cell separation, and to maintain the adhesion between cell wall components, primarily in the septum during cell separation (Cortés et al. 2012). *S. pombe* contains five genes coding for Ags/Mok proteins, and genomes of other fungi, including several pathogens in which the cell

wall α-glucan accounts for around 35 % of the total wall polysaccharides, present sequences of predicted proteins homologous to the Ags/Mok family (Edwards et al. 2011; García et al. 2006; Henry et al. 2011).

### *9.4.5 Glycoproteins*

Glycoproteins represent 30–50 % of the dry weight of the walls of *S. cerevisiae* or *Candida*, and around 15–20 % of the dry weight of *S. pombe* and filamentous fungi walls. The glycoproteins present in the cell wall are expansively modified with both N- and O-linked carbohydrates, predominantly or exclusively formed by mannose residues known as mannan. In some cases, the mannan backbone presents single residues or side chains of different sugars, galactomannan, rhamnomannan, glucogalactomannan, rhamnogalactomannan, etc. (Bowman and Free 2006; Leal et al. 2010). Most of the wall glycoproteins are attached through a glycosylphophatidyl inositol (GPI) remnant to β(1,3)-D-glucan or chitin, via a β(1,6)-glucan linker. Other wall glycoproteins are directly covalently attached to β(1,3)-D-glucan (Klis et al. 2006). The wall glycoproteins contribute to the preservation of the cellular shape, participating in adhesion processes, transmitting signals to cytoplasm, and remodeling the components of the wall (Latge 2007).

## 9.5 Inhibitors of Cell Wall Biosynthesis

As described above, the cell wall structure and rigidity depend on the layering and interlinking of β(1,3)-glucan, β(1,6)-glucan, chitin, α(1,3)-glucan, and mannoproteins. Because the cell wall is absent in mammalian cells, its polysaccharides are attractive targets for the discovery and development of antifungal drugs. However, the types of polysaccharides, their extent, and the linkages between them are quite variable across the fungal kingdom, and can change during the different growth phases. Therefore, the use of inhibitors of the synthesis of a specific wall component might result in different effects depending on the fungus or its stage of growth.

### *9.5.1 Inhibitors of the β(1,3)-Glucan Synthase (GS)*

To date, the GS is the only component of the cell wall synthesis machinery that has successfully led to the development and commercialization of new drugs on the market. In 2001, the Merck inhibitor of the GS caspofungin (Cancidas®, Merck, Sharp and Dhome, MSD) was approved for clinical use. Since then, antifungal drugs that inhibit the GS have extensively been used in clinic. The collection of

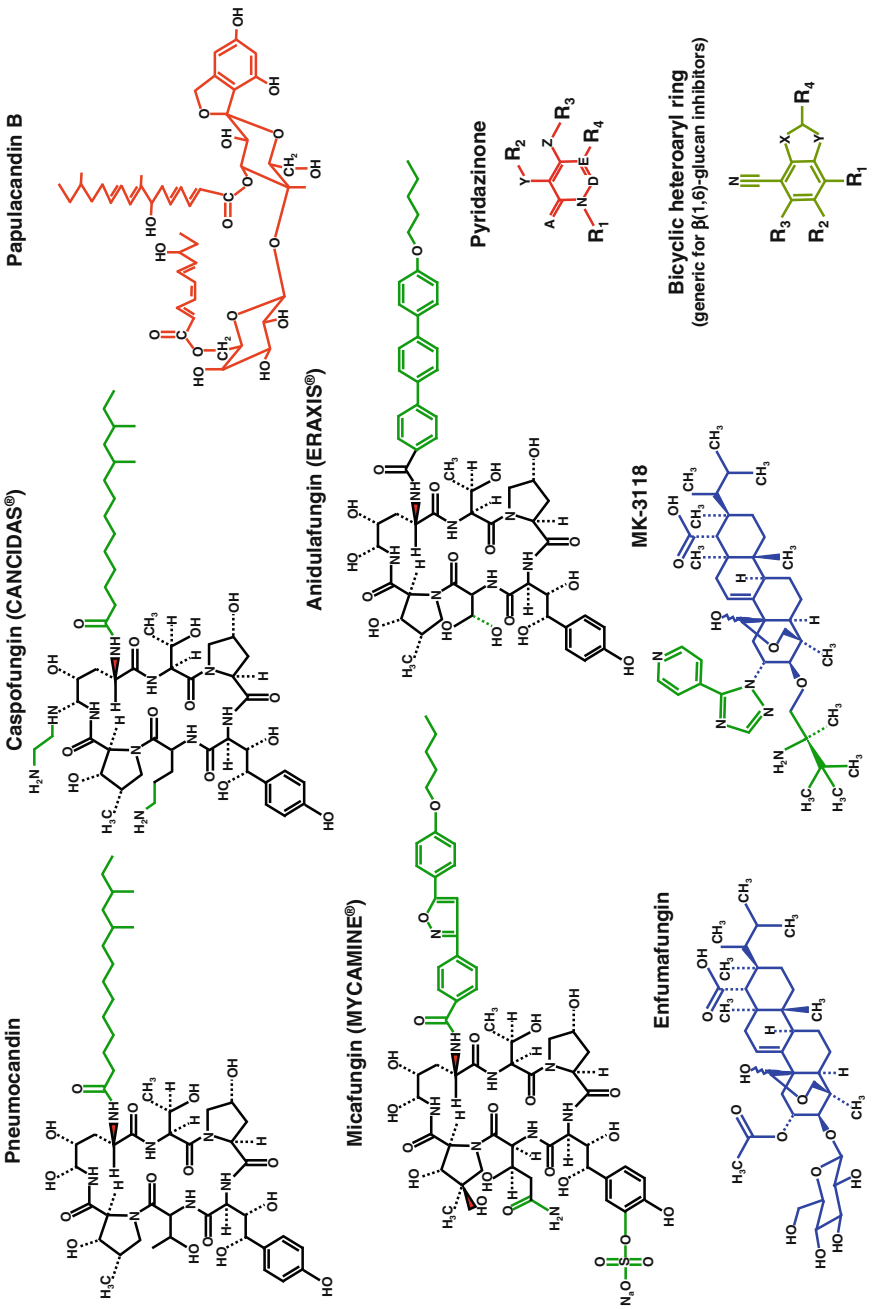



**Fig. 9.3** Chemical structures of the main inhibitors of cell wall $\beta$-glucan biosynthesis. Echinocandins (pneumocandin, caspofungin, micafungin, and anidulafungin), papulacandins (papulacandin B), acidic terpenoids (enfumafungin and MK-3118), and pyridazinone derivatives inhibit the GS activity and thus the synthesis of $\beta(1,3)$-glucan. Derivatives of bicyclic heteroaryl ring inhibit the synthesis of $\beta(1,6)$-glucan. For simplicity, the generic forms of pyridazinone and the bicyclic heteroaryl ring are shown. See the text for more details

marketed echinocandins also includes micafungin (Mycamine®, Astellas Pharma) and anidulafungin (Eraxis® or Ecalta®, Pfizer), which were approved for the treatment of invasive candidiasis and aspergillosis in 2005 and 2006–2007, respectively (Sable et al. 2008; Walsh et al. 2008; Chapman et al. 2008). Their structures are shown in Fig. 9.3. Additional members of this general class of GS inhibitors have emerged as preclinical candidates over the years, including papulacandins (Ciba-Geygy, Novartis), aerothricin analogs (Chugai Pharmaceutical and Basilea Pharmaceutica), cryptocandins (HMV Corporation), and other analogs (MSD, Astellas Pharma and Eli Lilly) (Hector and Bierer 2011). However, this class of antifungals displays limitations as the lack of oral formulations, restricting them to parenteral formulations, and their semisynthesis based on fermentation products. In addition, these compounds have a limited spectrum of activity showing no activity against, for example, *C. neoformans*. New $\beta$-glucan inhibitors with a broad spectrum activity against the main fungal pathogens, some of them with oral bioavailability, have started to emerge (Hector and Bierer 2011).

#### 9.5.1.1 Mode of Action

The primary mode of action of this class of antifungals is the obstruction of the biosynthesis of the fungal cell wall by inhibiting the GS enzyme (Douglas et al. 1994b; Pérez et al. 1981; Yamaguchi et al. 1985), but little is known about their mechanisms of action. Inhibitors of $\beta$(1,3)-glucan synthesis also have secondary effects on other components of the cell, including a decrease in the ergosterol and lanosterol content and an increase in the chitin content of the cell wall (Pfaller et al. 1989). Echinocandins bind non-competitively to the catalytic subunit of GS. The inhibition of $\beta$(1,3)-glucan synthesis requires the uptake of echinocandins by sensitive cells. At low concentrations (<1 mg/ml), a high-affinity facilitated-diffusion transporter mediates the uptake of caspofungin, a semisynthetic pneumocandin $B_0$ derivative (see below) in *Candida albicans*. At higher concentrations, nonspecific uptake can also progress (Paderu et al. 2004). The specificity of this class of antifungals for $\beta$(1,3)-glucan synthesis was demonstrated by the observation that echinocandins were ineffective in inhibiting chitin or mannan synthesis (Pérez et al. 1981). Substantial work has gone into the mechanistic understanding where echinocandins bind to the GS, but this question still remains obscure, largely because a membrane-associated protein is involved (Denning 2003).

In yeasts, inhibitors of the GS increase the osmotic sensitivity of the cells and cause cell lysis; however, resistant mutants with different behaviors against distinct antifungals compounds have been described, suggesting specific modes of action against the GS within this antifungal class (Martins et al. 2011). Only some *S. cerevisiae* and *S. pombe* mutants have been identified as resistant to the glycolipid papulacandin (Castro et al. 1995; Ribas et al. 1991b), in each case defining a single gene called *pbr1*, later found to be allelic to *FKS1* and *bgs4*$^+$, respectively. In *S. pombe*, wild type and the resistant mutant strains *pbr1-8* and *pbr1-6*, display differences both in vivo and in vitro between papulacandin, enfumafungin, and

echinocandins (Martins et al. 2011). In *S. pombe* wild-type cells, papulacandin and enfumafungin produce generalized cell lysis, while echinocandins are different; the lysis of wild-type cells is incomplete, and the surviving cells become rounded and maintain a residual cell growth. Besides, whereas *pbr1-8* and *pbr1-6* are highly resistant to papulacandin and enfumafungin, they exhibit opposite behaviors with regard to aculeacin; *pbr1-8* is highly resistant and *pbr1-6* is sensitive (Martins et al. 2011). No GS activation by GS inhibitors has been reported, except for papulacandin in *S. cerevisiae* wild-type GS and for enfumafungin, pneumocandin, and caspofungin in *S. pombe pbr1-8* GS (Kang et al. 1986, Martins et al. 2011). In the case of *S. cerevisiae*, the drug activation affects the wild-type GS and is dependent on low substrate concentrations. It is possible that some mutations, such as that *S. pombe pbr1-8* GS, in the presence of an antifungal could mimic the proposed preferential binding of substrate to the active form of the enzyme. Caspofungin displays special properties as regard the in vitro GS activity, with an inhibitory concentration lower than that of other drugs, except papulacandin, and with two previously unreported inhibitory effects of high and low affinity (Fig. 9.4), suggesting the presence of two GS interaction sites with caspofungin (Martins et al. 2011).

It is clear that the target to which these antifungals bind is the catalytic subunit of the GS, but their inhibitory effects on $\beta$(1,3)-glucan synthesis do not necessarily

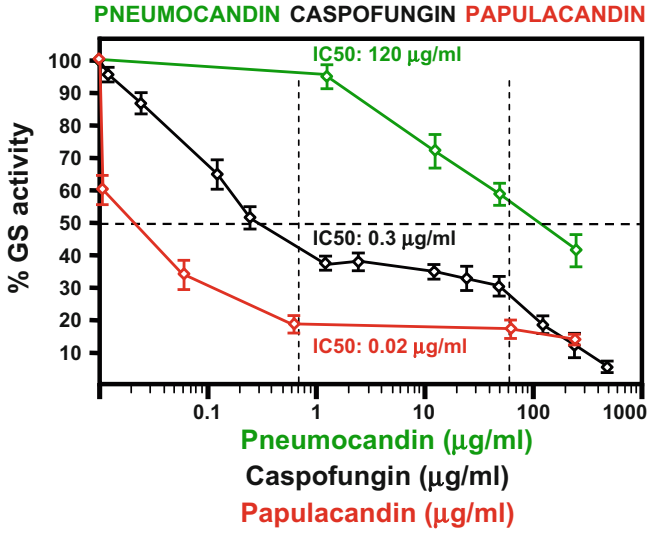



**Fig. 9.4** Differential inhibitory effect of pneumocandin B0, caspofungin, and papulacandin B on the in vitro GS activity of *Schizosaccharomyces pombe* wild-type cell extracts. Caspofungin exhibits a higher inhibitory capacity than pneumocandin and lower than papulacandin B. Besides caspofungin displays a dual inhibitory effect, at low and high concentrations (1st and 3rd sections marked by *dotted lines*), separated by a plateau of a 100-fold drug increase with no increase in inhibition (2nd section marked by *dotted lines*). Adapted from Martins et al. 2011

would involve the catalytic subunit itself, nor is it clear whether their binding site on GS catalytic subunit is external or internal to the plasma membrane (Odds et al. 2003). For example, it has recently been suggested that the acyl side chain of the echinocandins may interact with the plasma membrane (Chen et al. 2011).

#### 9.5.1.2 Echinocandins

The commonly known as echinocandins (variously called lipopeptides, cyclic hexapeptides, pneumocandins, etc.) is a class of antifungals that specifically target the GS enzyme. Echinocandins (Fig. 9.3) are large lipopeptide molecules, produced by fungi as secondary metabolites. The first echinocandin-type antimycotics, aculeacin A, and echinocandin B, were isolated independently by random screenings in the 1970s. A modified form of echinocandin B, cilofungin, was developed to clinical use, but was abandoned when its formulation showed toxicity to patients in the trials. All the echinocandin derivatives in clinical use or development are amphiphilic cyclic hexapeptides with an N-linked acyl lipid side chain (Denning 2003; Kurtz and Rex 2001; Odds et al. 2003). The three echinocandins approved for clinic use (caspofungin, micafungin, and anidulafungin) exhibit linear pharmacokinetics, are highly protein bound (97–99 %), and are not dialyzable. The echinocandins present some advantages which make them useful when used as additional antifungal treatment. Among common fungal pathogens, only *C. neoformans* is excluded from the echinocandins spectrum; but they also lack activity against emerging pathogens, such as *Fusarium* spp. and *Scedosporium* spp. However, they are active against *Pneumocystis jiroveci* (Odds et al. 2003). Besides, echinocandins display an improved hepatic and renal safety profile compared with those of the azoles and polyenes, and decreased cytochrome-mediated drug interactions compared with those of the azoles (Walker et al. 2011). However, the main disadvantage of available echinocandins is that all of them have limited oral bioavailability, and therefore must be administered by intravenous infusion (Emri et al. 2013; Sable et al. 2008).

##### Caspofungin

In 2001 Caspofungin (Cancidas®) was the first cell wall antifungal commercialized by MSD and approved for clinical use in both the United States and the European Union. Caspofungin is used as salvage therapy for invasive aspergillosis. Caspofungin is a semisynthetic echinocandin derived from pneumocandin $B_0$ via chemical modification of the hexapeptide scaffold (Fig. 9.3). Pneumocandins are natural products derived from the fermentation of the fungus *Glarea lozoyensis*. The introduction of additional amino groups in the peptide ring of pneumocandin $B_0$ increased the solubility of the molecule and the potency against fungal pathogens by two orders of magnitude. The caspofungin group has lower rates of nephrotoxicity, infusion-related events, and drug-related adverse events (Denning 2003; Emri et al. 2013; Sable et al. 2008; Vicente et al. 2003).

### Micafungin

Micafungin (Mycamine®) was commercialized by Astellas Pharma and approved for clinical use in the United States and the European Union in 2005. It is a semisynthetic echinocandin produced from the echinocandin B FR901379 via substitution of the fatty acid side chain (Fig. 9.3). Micafungin was approved for the treatment of esophageal candidiasis and for the prevention of *Candida* infections in patients undergoing hematopoietic stem cells transplantation. Micafungin is shown to be superior to fluconazole when given as prophylaxis in stem cell transplantation, and show less drug interactions than caspofungin (Denning 2003; Odds et al. 2003; Sable et al. 2008).

### Anidulafungin

Anidulafungin was commercialized by Pfizer and approved for clinical use in the United States in 2006 (Eraxis®) and in the European Union in 2007 (Ecalta®). It is a semisynthetic echinocandin produced from echinocandin B, via substitution of the fatty acid side chain (Fig. 9.3). It is used against esophageal candidiasis, candidemia, and other *Candida* infections (intra-abdominal abscess and peritonitis). It is highly active in vitro against a wide range of *Candida* spp, including those that are resistant to azoles (*C. krusei*), amphotericin B (*C. lusitaniae*) or other echinocandins (*C. parapsilosis*), and also against species of *Aspergillus* spp. (Denning 2003; Emri et al. 2013; Kathiravan et al. 2012; Sable et al. 2008).

### Echinocandin B derivatives

A series of cyclohexapeptide echinocandin semisynthetic derivatives and formulations are the subject of several patents. In one of these patents it is claimed that the natural products echinocandin B, aculeacin, pneumocandin $A_0$, pneumocandin $B_0$, pneumocandin $C_0$, and cilofungin may also be used as synthetic starting points. Activity against several *Candida*, *Aspergillus,* and *Cryptococcus* strains is mentioned for the described compounds, but biological data are not provided. Although not shown, it is stated that some of the echinocandin B derivatives present good activity in vitro against the GS of *C. albicans* and *A. fumigatus* (Hector and Bierer 2011).

## 9.5.1.3 Enfumafungin and MK-3118

Enfumafungin is a hemiacetal triterpene glycoside (Fig. 9.3) that was originally isolated by the fermentation of *Hormonema* sp. It exists as a mixture of two interconverting forms at the hemiacetal state, and it was determined that the natural product specifically inhibits the GS (Pelaez et al. 2000, Onishi et al. 2000; Martins et al. 2011). MK-3118 (Fig. 9.3) is an orally active, semisynthetic derivative of enfumafungin with in vitro and in vivo activity against *Candida* spp

and *Aspergillus* spp. MK-3118 and other derivatives of enfumafungin are potent inhibitors of fungal GS, yet these compounds are structurally distinct from the echinocandins. The sites of mutations in Fks GS that are associated with resistance to the echinocandins are in some cases distinctly different from those causing decreased susceptibility to the enfumafungin derivatives. MK-3118 displays in vitro an excellent activity against wild-type *Candida* spp, and wild-type and itraconazole-resistant strains of *Aspergillus* (Hector and Bierer 2011; Pfaller et al. 2013a, b; Walker et al. 2011). In vivo, the preclinical results demonstrate a comparable level of activity for MK-3118 against *Candida* spp. compared with caspofungin, while mouse efficacy results for aspergillosis suggest a somewhat inferior response compared with caspofungin. Importantly, the demonstration of oral bioavailability with MK-3118 suggests that formulations of this drug may be dosed either orally or parenterally, providing a much needed flexibility to the class of GS inhibitors as antifungal agents (Hector and Bierer 2011).

#### 9.5.1.4 Papulacandins

The glycolipids papulacandins (Fig. 9.3) are a series of naturally occurring antifungal agents containing a benzannulated spiroketal unit, which has been the signature of a wide series of bioactive natural products and has inspired ample synthetic activity. The papulacandins A–E were isolated from the fermentation broths of *Papularia sphaerosperma* (Traxler et al. 1977; van der Kaaden et al. 2012). They block the synthesis of $\beta$(1,3)-glucan by inhibition of GS (Baguley et al. 1979; Pérez et al. 1981; Varona et al. 1983). Interestingly, it has been observed a general decline in the inhibition of GS as the concentration of the substrate UDP-glucose is decreased. Surprisingly, at very low concentrations of UDP-glucose, papulacandin B even acted as a stimulator, suggesting that UDP-glucose might act as an allosteric ligand, shifting the enzyme from one conformation to another once its concentration increases. Within the range of substrate concentrations in which papulacandin B is inhibitory, the inhibition appears to be of the mixed type, although very close to noncompetitive (Kang et al. 1986; Pérez et al. 1981). Papulacandins display a very high specific activity against several yeasts, but they are largely inactive against filamentous fungi, bacteria and protozoa (Traxler et al. 1977). Direct comparison (Fig. 9.4 and not shown) between glycolipids (papulacandin B), echinocandins (pneumocandin, caspofungin and aculeacin) and acidic terpenoids (enfumafungin) has shown that papulacandin B inhibitory effect of GS activity is superior in several orders of magnitude to that of the other antifungals (Martins et al. 2011). Several new compounds structurally related to papulacandins have been isolated (van der Kaaden et al. 2012). Their structures diverge with respect to the two partially unsaturated acyl chains on the sugars. Drastic changes in these tails or the lack of one of the tails severely reduce their activity compared with the most active papulacandin B (van der Kaaden et al. 2012). Due to their limited potency in animal models, neither papulacandin B nor any of its derivatives have been developed as GS inhibitory drugs for clinical use (Vicente et al. 2003).

#### 9.5.1.5 Pyridazinone Derivatives

Since the echinocandins used in clinic are delivered only parenterally, there is a significant interest in identifying new and unrelated GS inhibitors. A search for antifungal bioactivities combined with mechanism-of-action studies identified a new class of piperazinyl-pyridazinones that target the GS. The generic form of pyridazinone compounds that inhibit the GS is shown in Fig. 9.3 (Hector and Bierer 2011). These compounds exhibit in vitro activity comparable, and in some cases superior, to that of echinocandins (Walker et al. 2011, Hector and Bierer 2011, Butts and Krysan 2012). The pyridazinone compounds inhibit GS in vitro, with a strong correspondence between enzyme inhibition and in vitro antifungal activity. Moreover, the compounds cause cell lysis and release of cytoplasmic contents as other GS inhibitors. Importantly, this novel class of small-molecule GS inhibitors present oral efficacy in a murine model of disseminated *C. glabrata* infection. The oral availability of these pyridazinone derivatives distinguishes them from the echinocandins (Walker et al. 2011).

#### 9.5.1.6 Other Inhibitors of the GS

Besides echinocandins, other cyclic peptides have been described as inhibitors of $\beta$(1,3)-glucan synthesis (Vicente et al. 2003, Hector and Bierer 2011). The echinocandin-related Cryptocandin has an inhibitory activity against *C. albicans* and *Trychophyton* spp (Strobel et al. 1999). Arborcandins are other antifungal agents described as GS inhibitors, containing a 10-aminoacid ring and two lipophilic tails (Ohyama et al. 2000). Similarly, Aerothricin3/FR901469 is a macrocyclic lipopeptidolactone composed of 12 amino acids and a 3-hydroxypalmitoyl moiety with GS inhibitory activity (Fujie et al. 2001; Kondoh et al. 2002). The piperazine propanol derivative GSI578 [(2,6-Difluoro-phenyl)-carbamic acid 3-(4-benzothiazol-2-yl-piperazine-1-yl)-propyl ester] is a synthetic antifungal drug described as GS inhibitor (Kondoh et al. 2005). Clavariopsins, cyclic depsipeptides lacking a long lipophilic radical, have also been described as inhibitors of glucan synthesis (Kaida et al. 2001). New chlorogenic, quinic, and caffeic acid derivatives that were coupled with an H2 N-orn-4-(octyloxy) aniline group have been described to display antifungal activities by partial inhibition of GS (Ma et al. 2010). Additionally, several new synthetized 4-aryl-4-*N*-arylamine-1-butene compounds display antifungal properties and specific inhibitory activity on the GS (Urbina et al. 2000).

#### 9.5.1.7 Resistance to GS Inhibitors: The importance of *FKS* Hot spots

Nowadays, a significant problem of the public health is the rising prevalence of resistance to antimicrobial agents among important human pathogens, which is severely restricting the availability of treatments for common infections (Ben-Ami

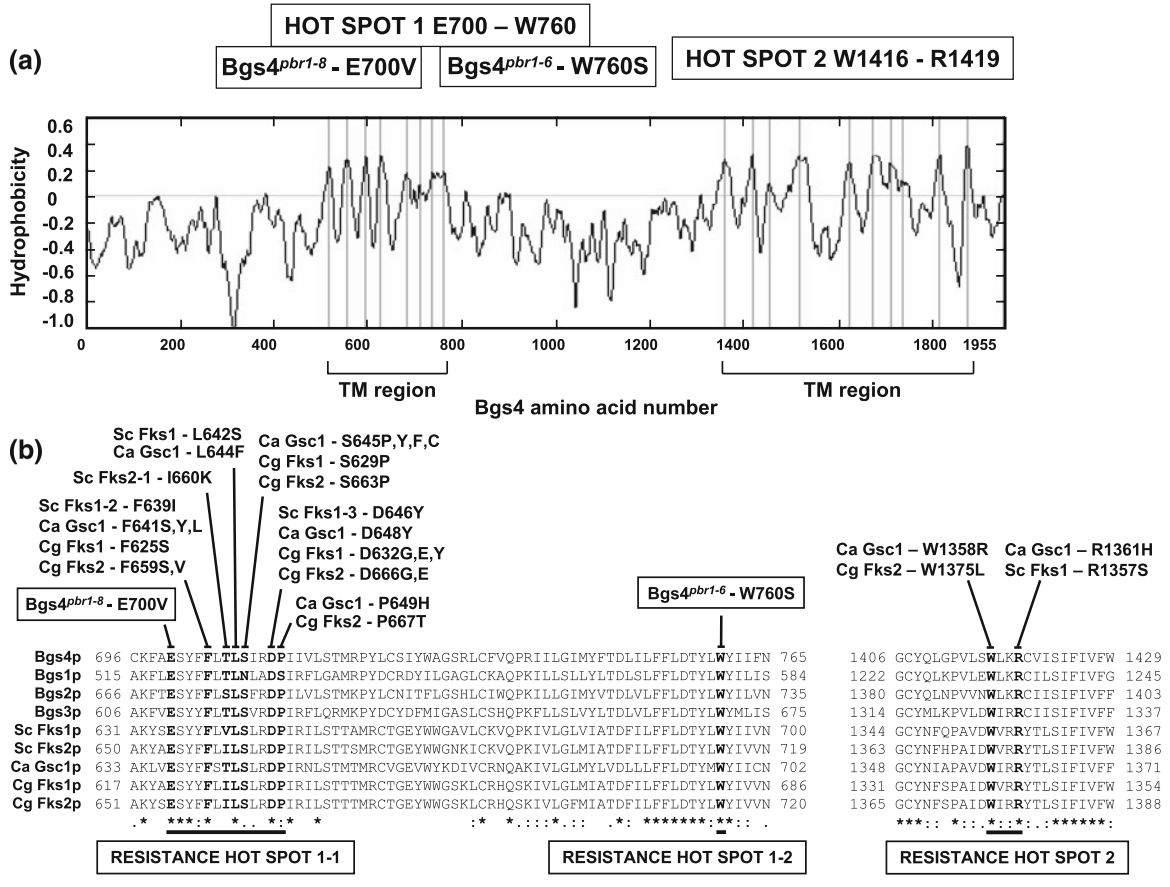
and Kontoyiannis 2012). Although resistance to echinocandins and other GS inhibitors is still relatively uncommon, it is increasingly encountered; moreover, the clinical susceptibility breakpoints for echinocandins result in the inclusion of a greater proportion of clinical isolates in the resistant category (Pfaller et al. 2011a; Ben-Ami and Kontoyiannis 2012).

Fungal resistance to GS inhibitors is clearly associated with mutations grouped in conserved short regions (hot spots) of the Fks proteins (Fig. 9.5b), indicating that this resistance mechanism is well conserved in fungi (Perlin 2007; Rocha et al. 2007; Walker et al. 2010). In addition, some fungi are naturally resistant to echinocandins, as they contain natural substitutions in the conserved Fks hot spot regions that are determinants of their resistance (Katiyar and Edlind 2009; Perlin 2007; Walker et al. 2010). *FKS* hot spot mutations which confer resistance to echinocandin are frequently associated with changes in cell wall thickness, attributed to increased cell wall chitin content due to upregulation of chitin synthesis as a result of activation of cell wall salvage pathways (Walker et al. 2008; Ben-Ami and Kontoyiannis 2012). In these cases, the combination of echinocandins with chitin synthase inhibitors (see below) avoids the increase of cell wall chitin, preventing the growth of echinocandin-resistant strains that contain *FKS1* hot spot mutations (Walker et al. 2008; Munro 2013).

Another resistance effect associated to just activation of compensatory mechanisms from the cell integrity and calcineurin pathways is the termed paradoxical growth effect, or Eagle effect, described for *Candida* spp. This paradoxical effect consists of an in vivo attenuation of growth inhibition at drug concentrations above the inhibitory concentration. The result is growth inhibition followed by a resumption of growth at higher antifungal concentrations and a new inhibitory effect when the drug concentration increases. In this case, the resistance is not due to Fks hot spot mutations and therefore, it is only observed on the in vivo cell growth but not on the in vitro GS activity (Fleischhacker et al. 2008; Wiederhold 2007).

Clearly, a weak point in all the $\beta$(1,3)-glucan synthesis inhibitors discovered or developed up to date is their lack of activity against *C. neoformans*. This is something intriguing since the *FKS1* homologue gene of this fungus has been shown to be essential, leading to the proposal that its GS enzyme could be relatively resistant to the action of echinocandins and the rest of the $\beta$(1,3)-glucan synthesis inhibitors. However, the in vitro GS assays have demonstrated that the GS from *C. neoformans* is in fact very sensitive to caspofungin and cilofungin (Maligie and Selitrennikoff 2005), indicating that *C. neoformans* is resistant to echinocandins through other mechanisms.

*S. pombe* is a good model for in vivo and in vitro studies of the resistance mechanisms to GS inhibitors (see above). It is interesting that exclusively some *S. cerevisiae* and *S. pombe* mutants display resistance or have been found resistant to papulacandin (see above and Fig. 9.5a). Although *S. pombe* vegetative cells contain three essential Bgs subunits which all contain in their hot spots the conserved aminoacid sequences associated to natural antifungal sensitivity, the antifungal resistance is only associated with Bgs4 hot spot mutations, suggesting that


(a)
HOT SPOT 1 E700 – W760
Bgs4pbr1-8 - E700V
Bgs4pbr1-6 - W760S
HOT SPOT 2 W1416 - R1419
Hydrophobicity
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
0
200
400
600
800
1000
1200
1400
1600
1800
1955
TM region
TM region
Bgs4 amino acid number
(b)
Sc Fks1 - L642S
Ca Gsc1 - L644F
Ca Gsc1 - S645P,Y,F,C
Cg Fks1 - S629P
Cg Fks2 - S663P
Sc Fks2-1 - I660K
Sc Fks1-2 - F639I
Ca Gsc1 - F641S,Y,L
Cg Fks1 - F625S
Cg Fks2 - F659S,V
Sc Fks1-3 - D646Y
Ca Gsc1 - D648Y
Cg Fks1 - D632G,E,Y
Cg Fks2 - D666G,E
Bgs4pbr1-8 - E700V
Ca Gsc1 - P649H
Cg Fks2 - P667T
Bgs4pbr1-6 - W760S
Ca Gsc1 – W1358R
Cg Fks2 – W1375L
Ca Gsc1 – R1361H
Sc Fks1 – R1357S
Bgs4p 696 CKFAESYFFLTLSIRDPIIVLSTMRPYLCSIYWAGSRLCFVQPRIILGIMYFTDLILFFLDTYLWYIIFN 765
Bgs1p 515 AKFLESYFFLTLNLADSIRFLGAMRPYDCRDYILGAGLCKAQPKILLSLLYLTDLSLFFLDTYLWYILIS 584
Bgs2p 666 AKFTESYFFLSLSFRDPILVLSTMKPYLCNITFLGSHLCIWQPKILLGIMYVTDLVLFFLDTYLWYILVN 735
Bgs3p 606 AKFVESYYFLTLSVRDPIRFLQRMKPYDCYDFMIGASLCSHQPKFLLSLVYLTDLVLFFLDTYLWYMLIS 675
Sc Fks1p 631 AKYSESYYFLVLSLRDPIRILSTTAMRCTGEYWWGAVLCKVQPKIVLGLVIATDFILFFLDTYLWYIIVN 700
Sc Fks2p 650 AKYAESYFFLILSLRDPIRILSTTSMRCTGEYWWGNKICKVQPKIVLGLMIATDFILFFLDTYLWYIVVN 719
Ca Gsc1p 633 AKLVESYFFSTLSLRDPIRNLSTMTMRCVGEVWYKDIVCRNQAKIVLGLMYLVDLLLFFLDTYMWYIICN 702
Cg Fks1p 617 AKYAESYYFLILSLRDPIRILSTTTMRCTGEYWWGSKLCRHQSKIVLGLMIATDFILFFLDTYLWYIVVN 686
Cg Fks2p 651 AKYSESYFFLILSLRDPIRILSTTTMRCTGEYWWGSKLCRHQSKIVLGFMIATDFILFFLDTYLWYIVVN 720
1406 GCYQLGPVLSWLKRCVISIFIVFW 1429
1222 GCYQLKPVLEWLKRCILSIFIVFG 1245
1380 GCYQLNPVVNWLKRCIISIFIVFF 1403
1314 GCYMLKPVLDWIRRCIISIFIVFF 1337
1344 GCYNFQPAVDWVRRYTLSIFIVFW 1367
1363 GCYNFHPAIDWVRRYTLSIFIVFW 1386
1348 GCYNIAPAVDWIRRYTLSIFIVFF 1371
1331 GCYNFSPAIDWVRRYTLSIFIVFW 1354
1365 GCYNFSPAIDWIRRYTLSIFIVFW 1388
RESISTANCE HOT SPOT 1-1
RESISTANCE HOT SPOT 1-2
RESISTANCE HOT SPOT 2

◀ **Fig. 9.5** Mapping of the Fks hot spots that confer fungal resistance to GS inhibitors. **a** Hydropathy profile of Bgs4. The two predicted transmembrane (TM) regions and the sites where Bgs4$^{pbr1-8}$-E700 V and Bgs4$^{pbr1-6}$-W760S are located in the hot spot 1 in the first transmembrane region and the sites where the hot spot 2 is located in the second transmembrane region are shown. **b** Sequence alignment of two conserved regions of 70 and 24 amino acids of Bgs1, Bgs2, Bgs3, and Bgs4 from *S. pombe*, Fks1 and Fks2 from *Saccharomyces cerevisiae* (Sc), Gsc1 (Fks1) from *Candida albicans* (Ca), and Fks1 and Fks2 from *Candida glabrata* (Cg). The amino acid mutations described to confer resistance to echinocandins in *S. cerevisiae*, *C. albicans,* and *C. glabrata*, defining two resistance hot spot 1 and hot spot 2 of 9 and 4 aminoacids, respectively, are shown. The Bgs4$^{pbr1-8}$ mutation is located 4 amino acids N-terminal from hot spot 1, increasing the cluster to a 13-amino acid hot spot 1-1 of resistance to papulacandin, enfumafungin, and echinocandins. The Bgs4$^{pbr1-6}$ change is located 48 amino acids C-terminal from hot spot 1-1, defining a novel hot spot 1-2 of resistance to the three antifungal families. Adapted from Martins et al. 2011

Bgs1 and Bgs3 are natural intrinsic resistant subunits (Fig. 9.5b). The analysis of mutants resistant to papulacandin expanded the resistance hot spot 1 to 13 aminoacids and defined a new resistance hot spot 1-2 (Martins et al. 2011). These new sites, which are important for resistance and interaction with antifungals, should help to understand the mechanism of action of antifungals, and the resistance mechanism to GS inhibitors of the Fks proteins.

### *9.5.2 New Inhibitors of β(1,6)-Glucan Synthesis*

*C. neoformans* is an encapsulated pathogenic yeast that is responsible for pulmonary infections and fatal meningoencephalitis in humans. Disseminated cryptococcosis is one of the main causes of death among immunocompromised patients, while cryptococcal pneumonia is one of most common manifestation of cryptococcosis in AIDS patients. As stated above, to date all the described or developed $\beta$(1,3)-glucan synthesis inhibitors are ineffective against *C. neoformans*. Since its GS is very sensitive to echinocandins (Maligie and Selitrennikoff 2005), one hypothesis for the resistance of *C. neoformans* is that the pathogen capsule could inhibit the access of the drug to the transmembrane GS. However, limitation of accessibility is an unlikely explanation since the minimal inhibitory concentrations of echinocandins for acapsular strains are similar to those for encapsulated strains (Feldmesser et al. 2000). Other theoretical explanation may be that echinocandins do not inhibit $\beta$(1,6)-glucan synthesis, which seems to be the main glucan in *C. neoformans* wall (Free 2013; Feldmesser et al. 2000). Besides *C. neoformans*, in other fungal species (*Candida parapsilosis*, *Candida guilliermondii*, *Neurospora crassa*, *Fusarium graminearum*, *Fusarium solani*, *Fusarium verticillioides* and *Magnaporthe grisea*) it has been found that their wild type Fks1 sequence contains natural substitutions in their hot spot region that are determinants of their resistance (Walker et al. 2010). Therefore, the search, design and development of novel drugs that specifically target the synthesis of $\beta$(1,6)-glucan might be essential to bypass the resistance of *C. neoformans* and other fungi, which are intrinsic resistant to GS inhibitors. Besides

the importance of the $\beta$(1,6)-glucan as new target in those resistant species, this cell wall polymer is essential for virulence in *C. albicans* (Herrero et al. 2004; Umeyama et al. 2006), representing an additional target for antifungals.

Recently several works and a patent have described new bicyclic heteroaryl ring derivatives as inhibitors of $\beta$(1,6)-glucan synthesis (Fig. 9.3 shows the generic bicyclic heteroaryl ring), which have been reported to have in vitro and in vivo activity against a range of *Candida* spp (Kitamura et al. 2009a, b, 2010; Takeshita et al. 2010; Hector and Bierer 2011). Unfortunately, like the $\beta$(1,3)-glucan synthesis inhibitors reported to date, the $\beta$(1,6)-glucan synthesis inhibitors have little or no in vitro activity against *C. neoformans*. As stated above, most of the wall glycoproteins are bound to the cell wall through a glycosylphophatidyl inositol (GPI) residue via a branched $\beta$(1,6)-glucan linker (Fig. 9.1). Recently, the novel compound E1210, orally active isoxazole-based inhibitor of glycosylphosphatidylinositol (GPI)-linked protein biosynthesis, has been shown to have a good activity against *C. neoformans*, as well as a wide range of medically relevant yeasts and molds (Pfaller et al. 2011b, c; Miyazaki et al. 2011).

### 9.5.3 Chitin Synthase (CS) Inhibitors as a Target for Antifungal Therapy

Chitin is one of the main wall polysaccharides, which is vital for the maintenance of cell wall structure and integrity. Therefore, inhibition of chitin synthesis has been proposed as an attractive target for antifungal treatments. Differently from GS, no CS inhibitor has ever been developed into drug for clinical use (Munro 2013). Existing CS inhibitors such as peptidyl nucleoside antibiotics polyoxins (or nikkomycins) are more potent and specific against class I enzymes, and less effective against the other classes. Their structures imitate the structure of the Chs substrate and thus, they act as competitive inhibitors (Gaughran et al. 1994; Munro 2013; Lenardon et al. 2010). The finding that *CHS1* is essential for the viability of *C. albicans* (Munro et al. 2001) pointed to the search of novel inhibitors of the class II CS. The compound RO-09-3143 inhibits specifically to Chs1, and it is only fungicide to *C. albicans* in the absence of Chs2 (Sudoh et al. 2000), suggesting the existence of compensatory mechanisms between the different CS enzymes. Supporting this observation, *C. albicans* yeast cells harboring *chs1*$\Delta$ deletion (absence of Chs1) were able to grow when treated with $Ca^{2+}$ and the chitin-interfering fluorochrome Calcofluor white, due to an increase of the chitin content and the synthesis of a new remedial septum (Walker et al. 2008). In conditions of stress or when the integrity of the cell wall is affected, this structure is reinforced by increasing the synthesis of chitin, either in *S. cerevisiae* or in *C. albicans* (Lenardon et al. 2010). In addition, *C. albicans fks1* mutants display increased cell wall thickness, which is attributed to higher cell wall chitin content. Something similar is observed when fungi are grown in the presence of sub-lethal concentrations of echinocandins (Ben-Ami and Kontoyiannis 2012).

Synthesis of chitin-rich cell walls results from activation of cell wall salvage pathways, including the high osmolarity mitogen-activated protein kinase, the protein kinase C, and the $Ca^{2+}$/calcineurin pathways. These compensatory mechanisms are thought to reduce the sensitivity of *C. albicans* to echinocandins. Therefore, the combined treatment of echinocandins with CS inhibitors is more effective than individual drug treatments (Walker et al. 2008; Munro 2013; Lenardon et al. 2010). In addition, the treatment with antagonists of the calcineurin pathway, which regulates chitin synthesis in *C. albicans* and *A. fumigatus*, as well as the response to echinocandin drugs, have shown a synergistic effect when combined with echinocandins and CS inhibitors (Fortwendel et al. 2009; Hill et al. 2013; Lamoth et al. 2012; Lenardon et al. 2010; Munro 2013; Walker et al. 2008; Wiederhold et al. 2005).

## 9.6 Perspective

In the past decade, there have been important advances in antifungal therapy. Several new antifungal agents have received authorization for clinical use in the United States and European Union. Sadly, with some exceptions against certain fungi, available antifungal compounds are fungistatic rather than fungicidal; and therefore, treatments for life-threatening mycoses require prolonged periods of time, conducing to the emergence of drugs resistance in fungal pathogens, which is compromising the efficiency of the available antifungal drugs. The combined treatment of inhibitors of the main cell wall components, together with azole antifungals and antagonists of pathways involved in mechanisms of antifungal resistance, is a powerful strategy to abrogate fungal resistance and combat invasive fungal infections. In addition to orally available azole compounds, the new formulations with oral bioavailability, the broad array of natural, semisynthetic, and synthetic compounds that have shown an specific activity against $\beta$(1,3)-glucan synthesis, together with the new inhibitors of $\beta$(1,6)-glucan synthesis, will supply more powerful increased activity and spectrum of activity against most of the pathogenic fungi. Unfortunately, with the exception of the three closely related echinocandins now on the market, no new antifungals have been developed to advance clinical testing. However, the diversity of antifungal classes with activity against $\beta$-glucans reviewed in the present chapter, plus the prospect of oral bioavailability, will open new strategies to develop novel, safe, and more effective drugs as additional treatments and management of life-threatening mycoses.

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# Chapter 10
# Perspectives in the Research on Antimicrobial Peptides

**Miguel Viñas, Francesc Rabanal, Roland Benz, Teresa Vinuesa and Ester Fuste**

**Abstract** Peptide molecules exhibiting antimicrobial properties are found in nature as a product from animals, plants, or microbes. Additionally, peptide molecules can be synthesized in chemical laboratories covering in principle an unlimited extension of chemical structures, sequences, and spectra of action. The use of peptides as antimicrobials has been relatively extended in industrial microbiology. Lantibiotics and bacteriocins from lactic bacteria have been used as food preservatives worldwide. In the past few years, the eventual application of such molecules to fight against infectious diseases is growing due, in part to the narrow investment in searching for new antibiotics and the increasing number of infectious diseases caused by multi-drug resistant bacteria. The mechanism of action of antimicrobial peptides involves in general membrane damage but they act also on internal targets, such as protein synthesis, folding and translation, cell division and DNA/RNA synthesis. Thus, such agents can have not only antibacterial but also antiviral and antifungal activities. The study of damage caused by antimicrobials into the membranes is a powerful approach to explore chemical modifications and strategies of delivery that can enhance the antimicrobial activity of such compounds. In this chapter, different kinds of antimicrobial peptides, their chemical structure, antimicrobial action and mechanisms are reviewed. Single channel conductance measurements are used to explore the properties and nature of peptide/membranes interaction.

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## 10.1 Introduction

One of the drivers of the vertebrate evolution has been to build systems able to defense species against infection. Infection is normally produced in all cases when a microorganism reaches habitats in which nutrient availability and environmental conditions allow the colonization of biological surfaces. Besides the removal of pathogens, toxins, and cell debris by phagocytes, the production of peptides having direct microbicidal activity seems to be an ancient mechanism of this protective system. These molecules being chemically peptides and having antimicrobial activity are normally called Antimicrobial Peptides or AMP. Their role has been recently recognized in many traditional "antimicrobial agents" such as honey (bee peptides) (Israili 2013), and have been shown to play a crucial role in skin innate immunity. Defects in the production of AMPs seem to play a relevant role in periodontitis in obese individuals.

A renewed interest in the research antimicrobial peptides derives directly from the critical situation to defeat bacterial infections. A large variety of antibiotics and other antimicrobial agents have been used not only to fight against infections in humans but also for other applications, for example to promote growth in livestock. This has resulted in the emergence of antibacterial drug resistance, which has led to major problems in the control of infectious diseases. Different strategies have been used to find new antimicrobial agents, including research on short peptides produced by bacteria and other living organisms (Yeung et al. 2011).

## 10.2 Natural AMPs

In principle, when looking at their origin two completely different classes of AMP exist. The first one comprises AMP synthesized at ribosomal level, being gene-encoded. It seems that almost all organisms (if not all) have the ability to produce these peptides. A second class comprises non-ribosomally synthesized peptide antibiotics; these are typically produced by prokaryotic microorganisms (bacteria) and also by fungi. These AMP are normally synthesized by ribosomally independent biochemical processes. They contain natural as well as non-natural amino acids and frequently formed cyclic structures. Some of these peptides are used in clinics from many years ago, and in some cases are almost unique therapeutic options. For instance, Colistin (polymyxin E) is in some cases the unique antibiotic that can be used to treat respiratory tract infections in Cystic fibrosis patients when they became infected by multiresistant *Pseudomonas aeruginosa* strains. Some more AMP are well established in human clinics such as bacitracin, gramicidin S, polymyxin B, streptogramins, or glycopeptides such as teicoplanin and vancomycin.

Ribosomally synthesized AMPs can be divided into two major classes depending on their origin: (i) AMPs of bacterial origin and (ii) AMPs of eukaryotic origin. The first group is currently referred as bacteriocins while the term AMP is gradually restricted to the ones produced by eukaryotes.

Bacteriocins are well known from many years ago. They represent an extremely heterogneous group of molecules including some very small highly modified peptides but also large proteins with complex structures. In general bacteriocins have a narrow spectrum of activity. Normally they act on bacteria closely related with the producer. In this sense, they seem to represent a competition mechanism preventing that related microbes can compete for nutrients or in the colonization process in the environment with the producer. On the other hand, they have highly specific mechanisms of recognition. In fact, in former times, they were extensively used to type bacteria at subspecies level in many species like *Serratia marcescens* or *Shigella* (Merino et al. 2000; Traub et al. 1971).

Small changes in surface structures completely block activity. Colicins (the bacteriocins produced by *Escherichia coli*) can be used to type related bacteria such as other enterobacteria and even to be used in research to recognize small differences in protein structure in the bacterial surface. As example Colicin E1, one of the best known colicins from *E. coli* uses the vitamin B12 receptor BtuB to bind to bacteria followed by transport though the channel-tunnel TolC. Strain C-600 is susceptible to colicin E1, whereas clones *E. coli* C600*tolC*, Tn*5* having TolC interrupted are, of course, resistant. A construct of C600 carrying TolC from another enterobacteria (*Klebsiella oxytoca*) is also resistant as is *K. oxytoca*. Differences between TolC of *E. coli* and *K. oxytoca* involves only six amino acids forming part of a loop exposed to the external side of the outer membrane. It is supposed that this region would play some role in the attachment of colicins and bacteriophages (Fenosa et al. 2009).

For instance bacteriocins produced by lactic acid bacteria (called LAB-bacteriocins) are of interest because of their effect on microorganisms responsible for both infectious diseases and food spoilage; and have been deeply investigated, received progressively more attention and occasionally used as food preservatives (Hoover and Steenson 1993). For instance, the use of the LAB-bacteriocin nisin is permitted in most industrialized countries; however, its interest as a preservative is limited due to both its narrow spectrum and the high frequency of mutations leading to nisin-resistance. In this sense there is a parallel situation with antibiotics. Many years ago we have explored the antibacterial action of LAB-bacteriocin pairs (Mulet-Powell et al. 1998). Several combinations were described as having a synergistic or additive effect, whereas, in some cases, indifference and even antagonism were detected. Nisin and lactacin F have additive bactericidal activity. Nisin is a well-known LAB-bacteriocin whose mechanism of action has been extensively investigated. Lactacin F is produced by *Lactobacillus johnsonii* and has antibiotic activity against other species of *Lactobacillus* and against *Enterococcus faecalis*. It is composed of two polypeptide subunits, LafA and LafX, which are encoded together with a promoter and the putative immunity protein ORFZ on a 1-kb polycistronic operon, similar to the genetic organization

of lactococcin M. LafA and LafX are produced with N-terminal extensions characterized by a special cleavage site. Both subunits are necessary for biological activity, i.e., the two have to be expressed together. The action of lactacin F on other species of *Lactobacillus* has been investigated in detail. Addition of lactacin F to the susceptible cells results in a sudden loss of internal potassium and rapid depolarization of the cytoplasmic membrane. The response to uncouplers of oxidative phosphorylation suggests that the proton-motive force is not essential for lactacin F action on target cells. This is in contrast to the effect of other bacteriocidins, such as lantibiotics, in which membrane potential plays an essential role. Eukaryotic AMPs frequently contain less than 50 amino acid residues and do not have post-transcriptional modifications (with exception of some frog AMPs). On the other hand when looking at their spectrum of activity, eukaryotic AMPs exhibit a wide broad-spectrum activity.

## 10.3 Measuring Antibacterial Activity of Antimicrobial Peptides

The first step in the evaluation of the *in vitro* efficacy of antimicrobial peptides begins with a type of antimicrobial assay. Antimicrobial peptide activity can be assessed in solution or in solid substrates where AMP can be immobilized. The traditional methods for measuring the growth inhibition include the microdilution broth assay and viable plate count method (Otvos and Cudic 2007).

The broth microdilution protocol is used to establish the minimal inhibitory concentration (MIC). It is a convenient method for susceptibility testing of several antimicrobials on a large number of bacterial isolates, in 96-well microtiter plates, in a short time. Overnight cultures of bacterial cells are diluted in both full-strength and ¼ Mueller–Hinton broths (MHB) and grown to mid-logarithmic phase. Decreasing concentrations (expressed in μM) of the peptides are incubated with the microorganisms. Growing bacteria produce turbidity in the wells that is cleared if the peptides inhibit bacterial growth. The lowest concentration preventing growth is considered the MIC.

Results can be recorded by visual inspection or read in a spectrophotometer at 600 nm. The assay is a 3-day procedure, with growing bacteria at day 1, peptide addition at day 2, and reading plates at day 3. As often peptides are sensitive to serum degradation is useful to assay the antibacterial activity also in the presence of 25 % serum. All the assays must be repeated three separate times to ensure reproducibility. While the method is appropriate for testing multiple peptide concentrations it does not distinguish between bacteriostatic or bactericidal action.

The half inhibitory concentration figure $IC_{50}$ is more appropriate for expressing peptide activity than MIC and is defined as the concentration where the activity curve crosses the 50 % between growth control and blank of medium. To determine the minimal bactericidal concentration the viable plate count method is employed to determine viability, starting with a peptide concentration lower that

the MIC. The method can also be used to determine bactericidal kinetics although it is unsuitable for the study of a large range of peptide concentrations.

Due to the potential applications of immobilized peptides (medical devices and textiles), new interest has arisen to study their proprieties when attached to solid surfaces. In order to study the antibacterial activity of immobilized AMP, indirect qualitative activity has been determined by means of optical density and respiration measurements, fluorescence microscopy and luminescence. The traditional methods such as microdilution broth and viable plate growth assays have also been employed in this setting but do not accurately depict the relationship between the dose and the time-dependent activity of immobilized AMP.

A new kinetic microplate method has been described for determining antimicrobial activity of peptides anchored to solid surfaces. The method gives information taking into consideration concentrations and time of exposition, revealing the MBC in a single, microplate-based platform Arcidiacono et al. (2011).

Antimicrobial peptides are candidates to be used in synergic combinations with currently available antibiotics or with other peptides, however, there is a need in the field of standardization and critical evaluation of testing and quantification methods, characterization of the molecular mechanism of action, and study of indirect antibacterial activity.

To test synergies MICs can be determined for each antimicrobial agent and checkerboard assays can be used to determine fractional inhibitory concentration index (FICI) values for dual combinations of AMPs. Viability assays must be performed for the same combinations in order to investigate microbiocidal interactions.

## 10.4 Drug Delivery Systems

The development of technologies that enable the selective delivery of micro- and nano-particulate (NP) medicines is gaining attention among antimicrobial peptide delivery systems as strategy to improve their pharmacokinetics, to increase its efficacy and reduce its toxicity.

Antimicrobial peptides can be successfully loaded onto NPs and when necessary their surface can be functionalized with specific biomolecules to reach the target sites. Due to the small size of NPs (from 10 to 1000 nm of diameter) interiorized peptides can cross barriers through small capillaries into individual cells, lowering the side effects and toxicity and enhancing efficacy. Some key aspects when dealing with nanodevices are the chemical composition as well as the manufacturing process which affect the peptide loading capacity, the release profile, the stability, and the toxicity of the final product.

Nanotechnologies formulate therapeutic agents in biocompatible nanocarriers such as nanoparticles, nanocapsules, micellar systems, and dendrimers. Polymeric nanoparticles (NPs) can present the therapeutic agent dispersed in the matrix (nanosphere) or encapsulated (nanocapsule). Solid Lipid Nanoparticles (SLNs) are made from lipids that are solid at room and body temperature, and are stabilized by

surfactants. Polymeric micelles are polymers assembled in aqueous solutions as outer hydrophilic layer and inner hydrophobic core (Salvador et al. 2011).

The most commonly used polymeric NP are polymer (D,L-lactic-co-glycolic) acid (PLGA) and its derivates. Most of them are biodegradable and biocompatible allowing sustained release of the drug along weeks. Other polymers have also been used: polylactide (PLA), chitosan, poly($\gamma$-glutamic acid). The peptides can be located inside or adsorbed on the surface of the particles. The delivery can be continuous, pulsated, or stimulated by external factors as changes in pH, temperature, ionic strength, or electric or magnetic fields.

The particle size is a determinative factor in the release rate and should be optimized for the particular case. The route of administration can be systemic (oral or parenteral) or local (cutaneous or mucosal). SLN are formulated with highly purified triglycerides or waxes. Their main advantage is a good tolerability and biodegradability. These particles are administered via parenteral, pulmonary, and dermal routes.

Polymeric micelles are composed of a core of hydrophobic blocks stabilized by a corona of hydrophilic chains (generally PEG blocks). They can be classified depending on their structure and size as unilamellar or multilamellar vesicles Elizondo et al. (2012). This system offers a set of advantages over the other methods like an increase of water solubility, improvement of bioavailability by enhancing permeability across physiological barriers, minimal toxicity, high drug-loading capacity, and controlled release profile for the incorporated peptide.

## 10.5 Mechanisms of Action

A large amount of literature has suggested that death of cells affected by AMPs seems to be the result of membrane potential disruption and subsequent exhaustion of the cell (Jack et al. 1994). Interaction between antimicrobial peptides and bacterial membranes can be explored by using similar tools than those used to investigate transmembrane proteins. The so-called "black lipid bilayer conductance measurements" can be applied to such purpose. Black lipid bilayer membranes can be formed by laboratory lipids (i.e., 1 % solution of a variety of lipids (a good supplier is Avanti Polar Lipids, Alabaster, Ala., USA) in *n*-decane or several other organic solvents as is known from many years ago (Benz et al. 1978). The instrumentation consists of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of about 0.5 $mm^2$, across which the membranes can be easily formed. The aqueous salt solutions could be used either unbuffered or buffered. Peptides should be added from concentrated stock solutions to the aqueous phase on one side, bathing a membrane in the black state. The temperature is generally maintained at 25 °C throughout.

The membrane current is measured with a pair of Ag/AgCl electrodes with salt bridges switched in series with a voltage source and a current amplifier (Keithley 427). The output signal of the amplifier is monitored on a strip-chart recorder and

can be fed via an A/D converter into a personal computer. The digital data can be analyzed with a home-made computer program. Analog data can be manually analyzed. Zero-current membrane potential can be measured by establishing a salt gradient across membranes containing a given number of channels (100–1,000) as described elsewhere (Benz et al. 1979).

When *E. faecalis* is treated with lactacin F a rapid decrease of the membrane potential is observed accompanied by a loss of intracellular K (Abee et al. 1994). It was suggested that these effects were the result of channel formation in the cytoplasmic membrane by the two components of lactacin F, the peptides LafA and LafX that form the lactacin F complex (Klaenhammer 1993). Lipid bilayer experiments were carried out with Lactacin F added in small concentrations (20 μg/mL) to the aqueous phase of one or both sides of a black lipid membrane made of diphytanoyl phosphatidylcholine (DPhPC)/*n*-decane. After a lag time of about 2 min, the membrane conductance started to increase and reached a conductance maximum after about 20–30 min (Fig. 10.1). Furthermore, a considerable increase of the current noise of the membrane indicating rapid fluctuations of the conductive units was observed. The effect of lactacin F on the conductance of lipid bilayer membranes was strongly dependent on its concentration in the aqueous phase. When the lactacin F concentration was decreased by a factor of two (addition of 10 μg/mL to one or both sides of the membrane), the conductance increase was considerably smaller (Fig. 10.1). A possible explanation for this is that several lactacin F molecules are needed to form a conductive unit. In these experiments, several different lipids were tested to check whether the effects described above were lipid-specific. Lactacin F was added to black lipid membranes in smaller amounts and the sensitivity of the current measuring device was greatly increased, but channels in single channel recordings were only occasionally observed, indicating that the lactacin F concentration was below the critical limit needed for the formation of conductive units. This is consistent with the idea that some peptides can open channels as the result of cooperation of several molecular units in a complex.

Non-ribosomally encoded antimicrobial peptides have also drastic effects on membranes. It has been shown that polymyxin B acts on the outer membrane of most Gram-negative bacteria by disrupting the general structure and originating blebs; in fact, the outer membranes of treated bacteria are completely disorganized as it has been shown in many publications (Vaara 2013). This can be easily seen in ultrathin sections when observed by electron microscopy. Physiologically, this is also detected as it results in a completely different scenario concerning ability of solutes to penetrate bacteria (Lauferska et al. 1983). The occurrence of colistin-resistant clinical isolates is increasing in the past few years. It has been shown that such a resistance tends to disappear when bacteria are cultured in laboratory conditions. We have investigated a pair of isolates of *P. aeruginosa* called 328S and 328R isolated from the same patient at the same time. 328S had a MIC of 0.5 μg/mL colistin whereas 328R gave a value of 16 μg/mL. Cultures at different concentrations of colistin were visualized by atomic force microscopy (AFM). AFM is a non-destructive powerful tool to visualize and characterize biological

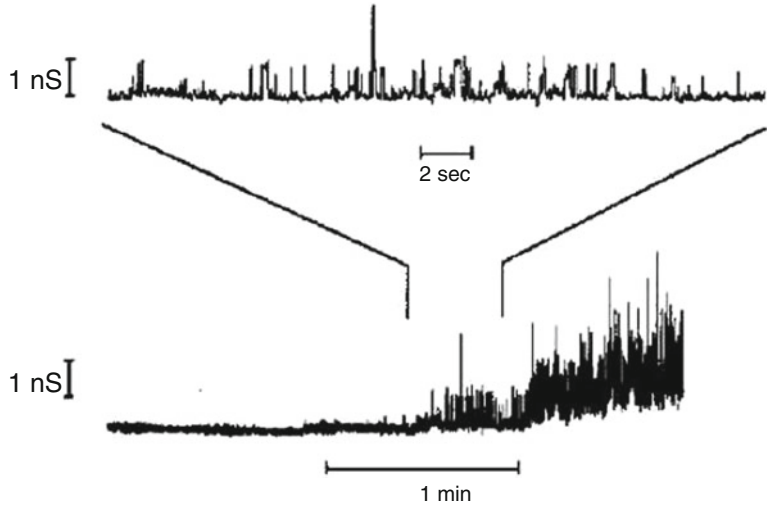


**Fig. 10.1** Single channel recording of a DPhPC/*n*-decane membrane in the presence of 1 μg lactacin F/mL added to the cis-side of the membrane. The aqueous phase contained 1 M KCl. The membrane potential applied at the cis-side was 10 mV; $T = 20$ °C. The time scale of part of the single channel conductance recording was spread by a factor of five to allow resolution of the single channel (from Dalmau et al. 2002) (©Springer-Verlag and SEM 2001)

samples at the molecular level obtaining high-resolution images, without any special preparation of the sample that can produce alterations on the structure. Imaging of both colistin-susceptible and colistin-resistant *P. aeruginosa* after incubation for 36 h in Muller-Hinton Agar (MHA) and MHA supplemented with different concentrations of colistin (0, 4 or 500 μg/mL) showed an increase in outer membrane damage as the concentration of this antimicrobial agent was higher. Furthermore, substantial topographical changes such as marked bulges and surface deformations were observed in bacteria upon treatment with 500 μg/mL of colistin. These alterations were consistent with a remarkable increase in surface roughness of bacteria growth with 500 μg/mL of colistin (24.93 ± 2.1 for strain 328S and 28.58 ± 2.3 for strain 328R) when compared with untreated bacteria or bacteria growth at low concentrations of colistin (9.22 ± 3.9 for strain 328S growth without colistin; 11.21 ± 1.8 for strain 328S growth with 4 μg/mL of colistin; and 16.9 ± 2.3 for strain 328R growth with 4 μg/mL of colistin). Mortensen et al. (2009) evaluated the changes in *P. aeruginosa* morphology and nanomechanical properties due to exposure to colistin in a liquid environment, and found that treatment with this cationic peptide caused an increase in the rigidity of the bacterial cell wall and their surface changed morphologically from smooth to wrinkle. These morphological changes were suggested to be due to the loss of surface proteins or LPS since lipopolysaccharide is necessary for the initial interaction needed for colistin uptake. Soon et al. (2011) observed that surface of colistin-resistant *A. baumanii* strains exposed to high concentration of colistin (32 μg/mL) were smoother than colistin-susceptible ones when exposed to the

same concentration of colistin. In our case, surface roughness of both 328R and 328S *P. aeruginosa* were able to growth at 500 μg/mL (when adapted) of colistin and do not exhibit remarkable differences.

These authors already pointed out that surface of colistin-resistant *A. baumanii* when treated with colistin exhibit similar alterations as susceptible strains. In other words, the ability of colistin to interact with the OM seems to be independent from the final output of antimicrobial/bacterial interaction. This is in agreement with the results observed in intrinsically resistant species. For instance, *S. marcescens*, which is naturally resistant to polymyxins can modify its behavior in the presence of colistin and related antimicrobials. This strongly suggests that injuries in outer membrane are needed for antimicrobial effect of polymyxins on Gram-negative bacteria, but are not enough to limit the viability of treated bacteria. In summary, although it seems that interaction between colistin and LPS is the first step of colistin antimicrobial action, it becomes apparent that bactericidal effect is due to the injuries induced in the cytoplasmic membrane. Thus, the role of LPS in resistance is probably a consequence of its capacity to prevent the entry of colistin and the subsequent interaction between the antimicrobial and the membrane.

Our AFM results obtained with *P. aeruginosa* confirmed this idea, since AFM images of colistin-susceptible and colistin-resistant strains revealed similar surface disruptive effects Fig. 10.2.

On the other hand, another approach to enlarge knowledge of the intimate mechanism of colistin action is to explore membrane damaging by electrophysiology. Colistin action on the artificial membranes by using the single channel conductance measurements technique revealed that it is feasible to obtain recordings of the channel-forming behavior of colistin in 1 % DiphPC/*n*-decane membranes.

Similar to other studies with antimicrobial peptides, we observed that these peptides required high concentrations (approximately 1 μg/mL or more) to allow the observation of a reasonable number of channels. All the studies were performed by using concentrations of colistin between 1 and 32 μg/mL. Certain generalizations could be made regarding the observed activities.

First, it is required a high voltage across the membrane. In general, voltages above ±180 mV initiated conductance events. When the applied voltage was subsequently decreased to ±130 mV, conductance events were still observed but often at a lower frequency.

Second, in contrast with other studies (Wu et al. 1999), conductance events in DiphPC planar bilayer membranes were initiated by both positive and negative applied voltages (e.g. ±180 mV). Other studies obtained similar results in the cases of cationic antimicrobial peptides such as tritrpticin peptide (a cationic peptide with 13 amino acids), exhibiting ionic channel-forming activity in both negative and positive voltages. At identical concentrations of colistin, conductance events in DiphPC/*n*-decane planar lipid bilayers were observed less frequently and were less relevant than in planar lipid bilayers containing negatively charged lipid PS. This result seems reasonable given the positive charge of the peptide. Most of the events observed involved very rapid conductance alterations which were widely variable in magnitude. There were some situations where substantial

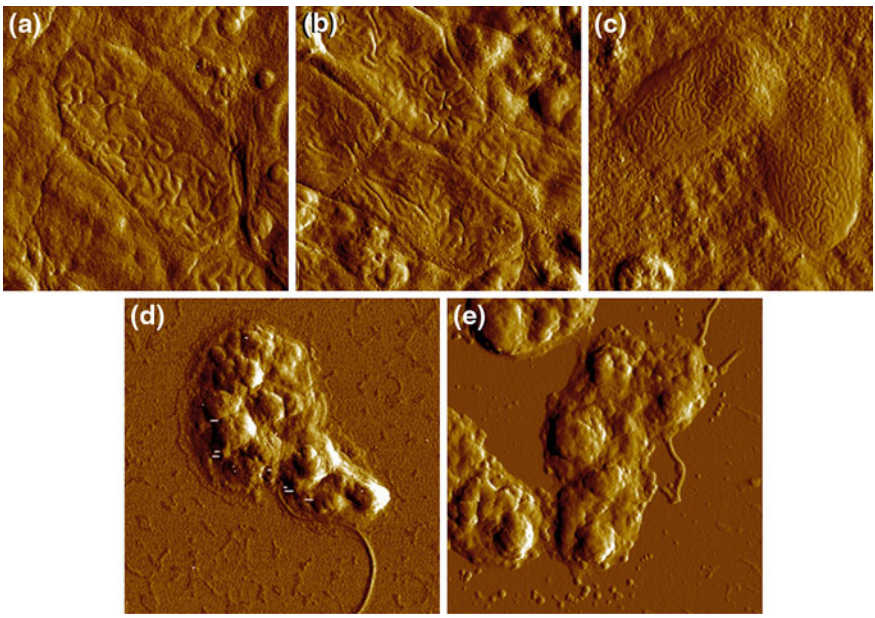


**Fig. 10.2** AFM amplitude images of colistin-susceptible, colistin-resistant, and ATCC 27853 *P. aeruginosa.* Bacteria were visualized in air by using the Atomic Force Microscope XE-70 (Park Systems) previously air dried at RT in a dust-free environment and images were collected in non-contact mode using pyramidal-shaped silicon cantilevers. **a** *P. aeruginosa* 328S growth without colistin; **b** *P. aeruginosa* 328S growth with 4 mg/L of colistin; **c** *P. aeruginosa* 328R, growth with 4 mg/L of colistin; **d** *P. aeruginosa* 328S, growth with 500 mg/L of colistin; and **e** *P. aeruginosa* 328R, growth with 500 mg/L of colistin

increases in transmembrane conductance were observed for a short period of time, followed by a fast return to the conductance base line as can be seen in Figs. 10.3 and 10.4. Some of the experiments performed clearly showed that channel-like conductance events increase with higher concentrations of colistin, although the low reproducibility of these experiments led to a few contradictory results. This would indicate the need to refine the methods to obtain reliable data.

In general, increments in conductance for colistin and other antimicrobial peptides are irregular, unstable, and represent fast openings and closings of pores. This is in contrast to the conductance events observed for the majority of channel-forming proteins, which tend to be regular, stable, and in the form of steps (stepwise manner). Cationic peptides are generally able to interact electrostatically with the negatively charged headgroups of bacterial phospholipids and then insert into the cytoplasmic membrane, forming conductance events which are proposed to lead to the leakage of cell contents and cell death. In accordance with the general model proposed by Matsuzaki et al. (1998), antimicrobial peptides bind to the outer leaflet of model membranes and flip inward, carrying lipids with them and creating brief disruptions in permeability. Besides, Wu et al. (1999) suggested that these compounds contain irregular aggregates of peptide molecules within the

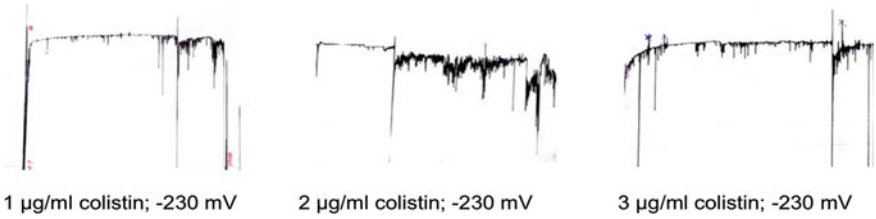


**Fig. 10.3** Chart recorder tracings of conductance events that occurred upon the addition of different concentrations of colistin to the solution (1 M KCl) bathing a planar lipid bilayer. The indicated voltages were applied. 1 % DiphPC/*n*-decane membranes. Approximately 3 min chart recordings are shown

membrane that will form in a concentration- and voltage-dependent manner creating informal aqueous channels. These informal aqueous channels probably allow the passage of at least ions and possibly larger molecules. As long as these supramolecular complexes are of variable size and stability, this would explain the observed variations in both the magnitude and duration of conductance events observed in our and other planar bilayer studies (Fig. 10.4).

## 10.6 Penetration of Antimicrobial Peptides Through the Gram-Negative Outer Membrane

A crucial question when focusing the fight against infections caused by Gram-negative bacteria is how these molecules can pass through the outer membrane. It seems that some peptides can induce the formation of transient channels in lipid bilayers and subsequently opening themselves "doors" to penetrate bacteria. In any case it is not known if this possibility exists in strongly asymmetric membranes such as the outer membrane of Gram-negative bacteria. Recently, it has been shown that extracellular DNA induces peptide resistance in Gram-negative bacteria in *Pseudomonas* and *Salmonella* (Johnson et al. 2013; Lewenza (2013)). These authors showed evidences that extracellular DNA is a component of the *Salmonella* Typhimurium extracellular matrix when grown in biofilms. Moreover, the addition of DNA to planktonic cultures resulted in cations chelation (i.e., $Mg^{2+}$ limited environment) and increased expression of some operons more highly expressed in biofilms, than in planktonic cultures. The addition of cDNA to planktonic cultures also led to increased antimicrobial peptide resistance. It seems that extracellular DNA could have a general role as a cation chelator inducing AMP resistance in biofilms. This can ensure long-term survival of bacteria in biofilms.

Other antimicrobial peptides use porins to penetrate outer membrane. Although permeation through outer membrane of Gram-negative bacteria by antimicrobial peptides is unknown, a few studies have shown that interaction at a single-molecule level occurs. Two different peptides (magainin 2 and HPA3P) interact with

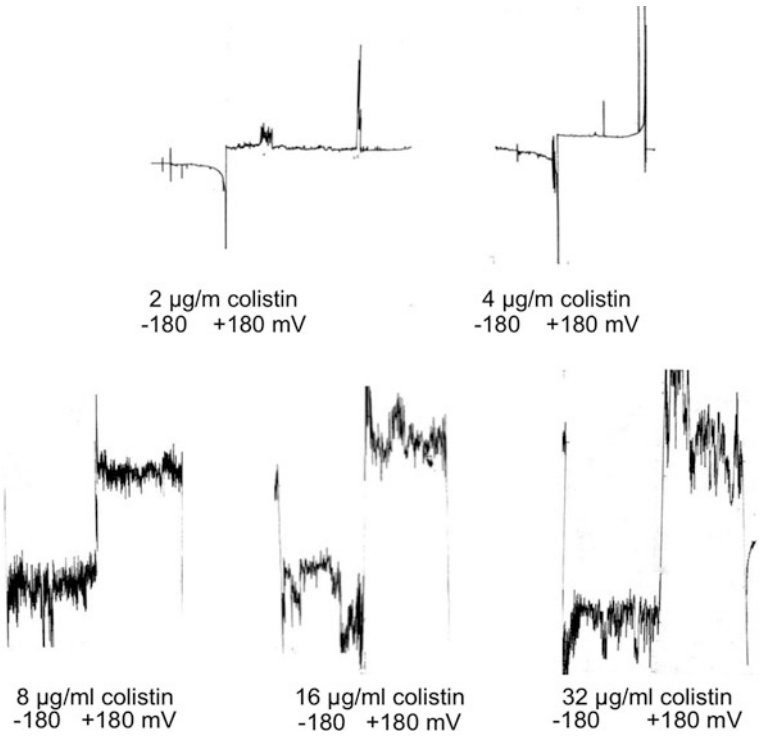


**Fig. 10.4** Chart recorder tracings of conductance events that occurred upon the addition of different concentrations of colistin to the solution (1 M KCl) bathing a planar lipid bilayer (0.8 % DiphPC in *n*-decane). The indicated voltages were applied. Approximately 3 min recordings are shown

OmpF from *E. coli*. HPA3P is an analog of the antimicrobial peptide HP(2–20) isolated from the N-terminal region of the *Helicobacter pylori* ribosomal protein. It was demonstrated that HPA3P peptide is more accessible to the inner volume of the OmpF than magainin 2. The capacity of HPA3P peptides to interact with OmpF in a voltage- and concentration-dependent manner was demonstrated. Unexpectedly, they found that increasing the applied voltage led to an increase of the residence time of HPA3P peptide inside the pore, possibly reflecting electric field-induced changes in pore and peptide geometry (Apetrei et al. 2010). Recently, the specificity of porin channels has been revisited by Kojima and Nikaido (2013). At the beginning of the "porin era" channels were originally thought as non-specific that discriminate solutes only on the basis of their gross chemical and physical properties such as size, electric charge, hydrophobicity, etc. Further work emphasized on the receptors detected in many porins and a point of view more much "specific" was adopted. It was generally accepted that specific interactions play a key role in the permeation of most drugs. Kojima and Nikaido (2013) showed after a careful examination that the original non-specific model is valid and that the emphasis for the role of binding sites is misleading.

## 10.7 Interaction Between AMPs

The narrow spectrum of several AMPs and the knowledge that many antimicrobials can be combined to originate cooperative antimicrobial action have lead to the exploration of combination of AMPs or combination of AMPs with other kind of antimicrobials. Mulet-Powell et al. (1998) observed antagonisms (lactacin 481 and nisin), lack of interaction (lactacin B and lactacin F), and synergism (nisin + Pediocin; Nisin + Lactacin B; nisin + Lactacin F, Pediocin + Lactacin F, etc.) when they studied bacteriocins of lactic acid bacteria. The interest in exploring the use of combinations of several antibiotics and also in the rescue of old antimicrobials whose use had ceased several decades ago because of its potential toxicity is recently a field of great interest. In some cases; some combinations have been used and assayed in several antibacterial combinations to achieve synergy. Recently, combination of old antimicrobials is being explored to treat multiresistant bacterial infections (Segura et al. 2013). Theoretically, such an approach should include alternative therapeutic agents such as antimicrobial peptides and photosensitizers and light. It has been pointed out that such an approach is feasible *in vitro* and eventually is going to be also useful *in vivo* (Mataraci and Dosler 2012).

## 10.8 Synthetic AMPs

The development of AMPs as therapeutic agents requires that these compounds show certain pharmaceutical properties to become market drugs. To start with, they need to be active against the target pathogen, have low toxicity to the host at the therapeutic dose (high therapeutic index), and show stability *in vivo*. Natural AMPs showing a standard polypeptide structure will hardly meet these conditions. Hence, AMP-based pharmaceuticals aim at the development of modified peptides (peptide-based therapeutics) or mimetics to overcome the pharmacological drawbacks of natural peptide compounds, namely, stability, toxicity, immunological recognition, and in general, pharmacokinetic properties. Synthetic peptide-based antimicrobial analogs are directly based on the natural structure of the peptide compound and include modifications to optimize those pharmacological shortcomings (peptide-based therapeutics). Such modifications may consist of minimizing the length of the sequence and/or systematically substituting each residue with other coded or non-coded amino acids. Structure activity relationships (SAR) are thus generated and allow for the rationale design of therapeutic candidates (Fjell et al. 2012).

AMP mimetics use partially or totally non-peptide-based chemical structures with the idea of maintaining the amphipathic character of the naturally occurring compound while sparing the inherent drawbacks of the peptide structure. Examples of peptide mimetics include arylamide foldamers, peptoids, oligoureas, ceragenins, and phenylene-ethynylene oligomers.

Needless to say, progress toward clinical development of peptide antibiotics has been hampered by numerous difficulties. However, some synthetic AMPs are currently in preclinical and clinical trials for anti-infective and/or anti-inflammatory indications, mostly by topical administration. On the one hand, synthetic peptide-based analogs of AMPs such as protegrin (iseganan and analogs IB-367 and POL7080), magainin (pexiganan), histatin (PAC-113), plectasin (NZ2114) indolicin (Omiganan and Omigard), lactoferricin (hLF1-11), BPI (XOMA 629), or designed LTX-109 and PTX peptides have reached preclinical or clinical phase at different stages. Computationally, designed analogs such as lipohexapeptides HB1345, HB 1275, or oligo-acyl-lysine BL-2060 are also in development. On the other hand, mimetics of AMPs such as amphiphilic arylamides (PMX-30063 and PMX 10070; analogs of defensins), ceragenins (sterol-based cationic amphiphiles), or foldamers such as aromatic and aliphatic *N*,*N*′-linked oligourea foldamers, are similarly in different stages of development (Devocelle 2012; Pasupuleti et al. 2012; Yount and Yeaman 2012).

It is worth mentioning that a few among the already mentioned AMPs are already in the market, such as cyclic lipopeptides polymyxin (polymyxin B and colistin) and daptomicin (Cubicin). The latter was launched in 2003 and it is indicated for skin (and skin structure) infections, *S. aureus* bacteraemia, and right-sided endocarditis. Polymyxins were discovered in 1947 but their use was discontinued due to their nephrotoxicity and neurotoxicity problems. However, polymyxins are recently finding some use as last resort drugs due to the scarcity of antibiotics, but their problems of toxicity remain and careful monitoring of the patient is needed. Fortunately, research of promising new polymyxin analogs is carried out to obtain new chemical entities that reduce the drawbacks posed by such molecules and improve their therapeutic index (Rabanal et al. 2013; Vaara 2013).

In summary, despite initial difficulties that greatly delayed their general pharmaceutical development, recent technological breakthroughs indicate that synthetic AMP may become an entirely novel class of anti-infective agents able to fight even the most resistant microorganisms. In the next few years, we shall see whether this statement is hopefully right.

## 10.9 Perspectives in Human Treatment

Several pathologies and the difficulties in finding effective treatments warrant the research in this field. Several examples can be mentioned. Cystic fibrosis, which is a severe genetic disease leading to a dysfunction of secretion processes is characterized by recurrent respiratory tract infections. *P. aeruginosa* is the etiological agents most frequently encountered in these infections. As a consequence there is a gradual destruction of the respiratory epithelium being the main cause of death in these patients. *P. aeruginosa* infecting cystic fibrosis patients has become more and more resistant to antibiotics and nowadays most of the isolates are fully resistant to most of routinely used antibiotics. Thus, in some cases colistin is the

last hope drug. The exploration of novel peptides combined with new delivery strategies constitutes a main objective of research on antimicrobials. Another example is *Clostridium difficile* associated diarrhea which has dramatically increased in the last few years and is a major cause of morbidity and mortality among hospitalized patients. Moreover, hypervirulent strains have emerged making antibiotic usage a major risk factor. The use of bacteriocins, probiotics, and phages to act as antimicrobials agents against *C. difficile* infection in the gut has been envisaged and proposed (Rea et al. 2013). At present, there are different clinical trials in progress for the treatment of a myriad of microbial produced diseases with AMP. Because of the uncertainties of the mechanisms of action and their potential toxicities, most clinical trials have focused on topical rather than systemic treatments. The clinical infectious conditions in which they have been assayed include: the prevention of catheter-associated infections, the prevention of diabetic foot ulcers and promotion of wound healing, the prevention of oral mucositis in radiation therapy patients, rosacea, chronic bacterial middle-ear otitis, and fungal infections of the toenail among others.

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# Chapter 11
# Glycopeptides and Bacterial Cell Walls

**Fernando Santos-Beneit, Juan F. Martín and Carlos Barreiro**

**Abstract** The discovery of antibiotics prompted a new era in the treatment of microbial infections. However, from the very beginning of antibiotic utilization, bacterial resistance to these compounds also emerged. Thus, the resistance to penicillin was reported only 1 year after its adoption in clinic and the same process has been reported later with other important drugs. In contrast, glycopeptide antibiotics have been an intriguing exception during a long period of time, which led to their adoption as drugs of last resort treatments. Enterococci strains presented resistance to vancomycin, which is the most important member of this class of antibiotics, in 1987, many years after its introduction in clinic in 1958. Later, this resistance was also spread to important pathogens like 'methicillin-resistant *Staphylococcus aureus*' (MRSA). In this chapter, we will focus on the origin of glycopeptides in the context of the antibiotic discovery, the structure, biochemistry, regulation, and action mechanism of these compounds, as well as the resistance appearing especially in the producer and nonproducer *Streptomyces* spp. Besides, a special attention is paid to the cell wall modifications, which leads to the glycopeptide resistance. New trends in semisynthetic glycopeptides production are also reviewed.

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## 11.1 Introduction

The reference to the work of Sir Alexander Fleming is obligated in every document related with antibiotics. This history started accidentally in 1928 at the St. Mary's Hospital (London) with the discovery of the antimicrobial activity of a fungus contaminating a Petri dish cultured with *Staphylococcus* sp. The mold identified as responsible of the antibacterial effect was *Penicillium rubrum* (Fleming 1929). Later in 1940, a group of scientists from the Sir William Dunn School of Pathology at Oxford University were able to commence complete studies on penicillin (Florey et al. 1949). However, massive production was the result of a huge cooperative effort among industrial laboratories and universities during the World War II led to multiple large-scale clinical trials to treat those wounded in battle in England and in the United States during 1942 and 1943 (Kong et al. 2010). In 1945, Ernst B. Chain, Howard W. Florey, and Sir Alexander Fleming were awarded with the Nobel Prize for Physiology and Medicine. A year later, penicillin was finally available in the open market (Barreiro et al. 2012; Demain and Sanchez 2009). Nowadays, antibiotics represent 5 % of the global drug market and provided $42 billion in annual sales in 2009 (Hamad 2010).

The discovery of antibiotics more than 70 years ago was supported by a geneticists' mistaken idea. It was suggested that, taking into account the low frequency of spontaneous mutants appearance observed in nature, the emergence of resistance during therapy was unlikely (Méndez-Alvarez et al. 2000). But the reality is that the antibiotics discoveries have been tempered by the emergence of resistant microbes (Livermore 2009; Wright 2007). The well-known bacterial gene transference promiscuity, coupled with the high pressure owing to the indiscriminate use of antibiotics, have burst different resistance mechanisms (Méndez-Alvarez et al. 2000). This fact led to the idea that antibiotic resistance in pathogenic bacteria is a modern phenomenon due to the antibiotics use and abuse. In contrast, D'Costa and coworkers (2011) have recently demonstrated, by metagenomic analyses of ancient DNA from permafrost sediments, that a highly diverse collection of genes encoding resistances ($\beta$-lactam, tetracycline, or glycopeptide antibiotics) exist in 30,000 year-old samples. This fact is congruent with the bacterial need of resistance mechanisms to survive against antibiotic-related chemical weapons.

The old phenomenon of antimicrobial resistance is a critical health issue today as the World Health Organization currently recognizes. Thus, the antimicrobial resistance has evolved to become a worldwide health threat that shows the limited effective lifespan of antibiotics and turn many common and life-threatening infections into difficult or even impossible to treat, which entails an ever increasing health and economic burden (World Health Organization 2012). As an example, the presence of *Escherichia coli* in bacteraemias due to the extended-spectrum $\beta$–lactamases (resistants to fluoroquinolones, oxyimino-cephalosporins or both) in England, Wales and Northern Ireland has been documented (Livermore 2009) as a prominent increase of resistants after 2000.

One of the main reasons of the antimicrobial resistance increase is the wide and in vast quantity use of antibiotics to promote the growth and ensure the health of livestock, poultry, and fish reared for food production. To decrease this dangerous situation, some countries have banned the use of antibiotics as growth promoting compounds. This is the case of The Netherlands, which prohibited this application in 2006, and the next target is to decrease up to 50 % the whole antibiotic application in livestock not later than 2013 (World Health Organization 2012). This stringent regulation has raised the challenge of how to substitute the antibiotics traditionally used in animal husbandry. The question is tackled from different approaches as the use of: (i) probiotics; (ii) prebiotics; (iii) enzymes; (iv) immune modulators; (v) organic acids (acidifers); (vi) feed supplements (e.g.: minerals, vitamins, conjugated linoleic acid, carnitine, amino acids, etc.); or (vii) vaccines (Doyle 2001; Falcão-e-Cunha et al. 2007). But, does it mean the end of the antibiotics use? Or, has the era of untreatable infections arrived? (Livermore 2009). Fortunately, not or at least not yet. At present, most infections can be still treated including the antibiotics in the drug arsenal. On one hand, indiscriminate antibiotic use is being legally regulated. On the other hand, new antibiotics or modifications of the existing ones are carried out (although at a slower rate than recommendable) (Jovetic et al. 2010). And finally, the microbial resistance to some antibiotics takes longer times than other. Thus, vancomycin resistance onset happened three decades after its use began (James et al. 2012). This last question about how the microorganisms resist this latter antibiotic barrier, as well as the severe public health problem generated (Table 11.1), are the core of this chapter.

**Table 11.1** Estimated annual human burden of infections due to antibiotic-resistant bacteria in EU member states, Iceland and Norway (2007)

| Antibiotic-resistant bacteria | Infections | Deaths | Days |
|---|---|---|---|
| Antibiotic-resistant Gram-negative bacteria | | | |
| *E. coli*: 3rd-generation cephalosporin-resistant | 32,500 | 5,100 | 358,000 |
| *Klebsiella pneumoniae*: 3rd-generation cephalosporin-resistant | 18,900 | 2,900 | 208,000 |
| *Pseudomonas aeruginosa*: carbapenem-resistant | 14,1900 | 10,200 | 809,000 |
| Antibiotic-resistant Gram-positive bacteria | | | |
| *S. aureus*: methicillin-resistant (MRSA) | 17,1200 | 5,400 | 10,500,00 |
| *E. faecium*: vancomycin-resistant | 18,100 | 1,500 | 111,000 |
| *S. pneumoniae*: penicillin-resistant | 3,500 | – | – |

*Note* infected patients incurred three times higher hospital costs and remain in hospital 2.5 times longer than those of uninfected patients (Plowman et al. 2001), which result in the presented data. The data present the number of infections, extra deaths, and extra hospital days. Based on data published by: European Centre for Disease Prevention and Control (ECDC), European Medicines Agency (EMEA), and World Health Organization (World Health Organization 2012; ECDC/EMEA 2009)

## 11.2 Discovery of Glycopeptide Antibiotics

From the 1940s to the 1960s, many new antibiotics were provided by the pharmaceutical industry, including some with new mechanisms of action, such as glycopeptides, that evaded the problems caused by multidrug-resistant bacteria (Fig. 11.1).

Vancomycin is the most widely recognized member of the large family of glycopeptide antibiotics. This antibiotic came from a productive soil screening program of the pharmaceutical company Eli Lilly. Thus, McCormick and coworkers (1956) isolated a Gram-positive active component from the fermentation broth of a novel actinomycete that was named *Streptomyces orientalis* (now renamed as *Amycolatopsis orientalis*). The initial isolate came from a soil sample collected by a missionary in Borneo, although the same antibiotic was found later in samples from Indian soil (Levine 2006). Early studies on animals indicated a low toxicity of the antibiotic. However, due to significant impurities from the fermentation process, vancomycin was described to cause serious side effects in patients, especially related to nephrotoxicity. Later improvements in the purification process led to a much purer and harmless product. Therefore, it was rapidly approved for the treatment of penicillin-resistant staphylococcal infections in 1958. Nevertheless, because of the introduction of methicillin in 1960, the use of vancomycin in clinic was overshadowed. It was not until the 1970s, with the

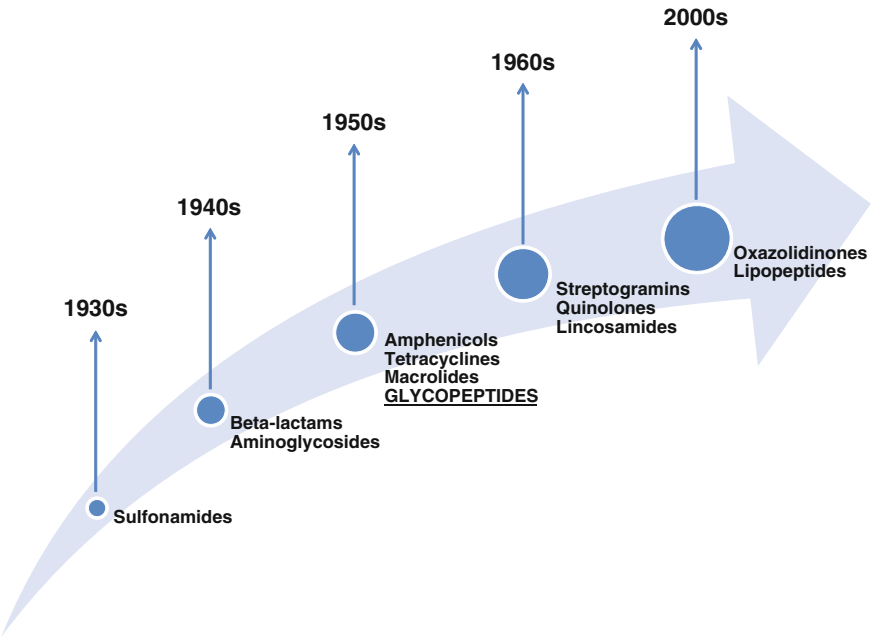


**Fig. 11.1** Evolution of the new classes of antibiotics discovery

spread of methicillin-resistant *Staphylococcus aureus*, when vancomycin was resurrected as an important antibiotic. In the 1980s, another glycopeptide antibiotic used in clinic today, teicoplanin, was also introduced, first in France and Italy, and then in other countries (Reynolds 1989). It was not until 1983, almost 30 years after the introduction in clinic of vancomycin, when the structure of the glycopeptide was brought to light (Harris et al. 1985; Barna and Williams 1984) (Fig. 11.2).

The glycopeptides are complex structures that consist of a multiring peptide core (the aglycone component) and sugar molecules that are attached at various sites. Actually, the term "glycopeptide antibiotics" is generally applied to designate the dalbaheptides, including both vancomycin and teicoplanin (Nicolaou et al. 1999). These compounds have a central heptapeptidic core with at least five amino acid residues bearing aromatic side chains. The heptapeptide backbone of vancomycin and vancomycin-type glycopeptides consists of two proteinogenic amino acids (leucine and asparagine) and five non-proteinogenic amino acids including $\beta$-hydroxytyrosine, 4-hydroxyphenylglycine, and 3,5-dihydroxyphenylglycine. In the teicoplanin family, tyrosine is the only proteinogenic amino acid of the scaffold. In all cases, these amino acids are cross-linked to form a unique trimacrocyclic or tetramacrocyclic structure which is further glycosylated. Despite being vancomycin and teicoplanin structurally similar, they belong to different glycopeptide families (Fig. 11.2). Nowadays, glycopeptide antibiotics are classified into five structural subtypes, I–V (Nicolaou et al. 1999). The main difference between type I glycopeptides, such as vancomycin, and the rest of structures is related with the presence of aliphatic or aromatic side chains, respectively. In addition, types III and IV contain an extra ring system, and in the case of type IV, such as teicoplanin, they also contain an additional long fatty acid chain. It has been shown that modification of glycopeptides with additional hydrophobic side chains increases the capacity of the resultant semisynthetic lipoglycopeptides to impair cell wall synthesis likely by stabilizing the interaction of the antibiotic with its target (Beauregard et al. 1995; Arhin et al. 2012).

Glycopeptide biosynthesis can be subdivided in three steps. In the first step, there is a supply of the basic building blocks by the specific biosynthesis pathways.

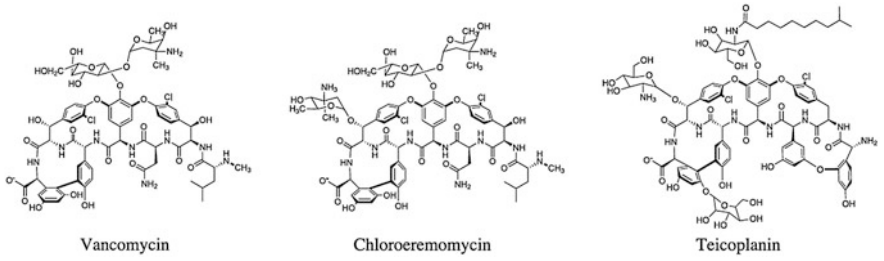


**Fig. 11.2** Comparison of vancomycin and the vancomycin-like glycopeptide chloroeremomycin (type I) with teicoplanin (type IV)

In the second step, the building blocks are linked by multienzyme complexes involving different non-ribosomal peptide synthetases resulting in a closely packed structure. Finally, there are a number of selective chemical modifications of the heptapeptide by glycosyltransferases, methyltransferases, acyltransferases, and sulfotransferases (reviewed by Kahne et al. 2005; Li et al. 2012; Sosio and Donadio 2006; Stegmann et al. 2010)

Although the screening of bacterial culture broths for the presence of glycopeptide antibiotics initially yielded a large number of novel congeners, up to date only six gene clusters involved in the biosynthesis of glycopeptide antibiotics have been reported [balhimycin (*bal*), chloroeremomycin (*cep*), A47934 (*sta*), A40926 (*dbv*), teicoplanin (*tcp*) and complestatin] (Chiu et al. 2001; Li et al. 2004; Pelzer et al. 1999; Pootoolal et al. 2002; Sosio et al. 2004; Sosio et al. 2003; van Wageningen et al. 1998). These glycopeptide clusters have been derived from two *Streptomyces* spp: *Streptomyces toyocaensis* (*sta*) and *Streptomyces lavendulae* (complestatin), two *Amycolatopsis* spp: *A. orientalis* (*cep*) and *Amycolatopsis balhimycina* (*bal*), an *Actinoplanes* sp: *Actinoplanes teichomyceticus* (*tcp*), and a *Nonomuraea* sp: *Nonomuraea* sp. ATCC 39727 (*dbv*). Among the glycopeptides referred to here, complestatin, balhimycin, and chloroeremomycin belong to the vancomycin family, while A40926 and A47934 are related to teicoplanin. Actually, vancomycin is different from chloroeremomycin only by lacking a sugar moiety at the penultimate amino acid (Fig. 11.2). In addition to these clusters, the biosynthetic gene cluster *teg*, which encodes the biosynthesis of a polysulfated teicoplanin congener (sulfoteicoplanin) has been found in an environmental DNA megalibrary (Banik and Brady 2008).

Vancomycin and teicoplanin are the only two glycopeptides currently used in the treatment of Gram-positive infections. These antibiotics are also used for the treatment of patients on dialysis, undergoing cancer chemotherapy, or allergic to $\beta$-lactam antibiotics. Nowadays, three semisynthetic glycopeptides (oritavancin, dalbavancin, and telavancin) are being introduced into the clinic to combat Gram-positive pathogens. Telavancin has been approved in 2009 for complicated skin infections treatments (reviewed by Arhin et al. 2012). Whereas, dalbavancin and oritavancin are under investigation in clinical trials (see below).

## 11.3 Semisynthetic Glycopeptides

Natural products are rich in structural diversity and possess a wealth of biological activities (e.g.: antimicrobial, antitumor, and immunosuppression). Nevertheless, many efforts have been made to expand the diversity of bioactivities by selective chemical modifications of the natural occurring products. The combination of synthetic chemical tools and recombinant enzymes, particularly through engineering of genes for secondary metabolites, has an advantage over other strategies. General approaches have been explored for modifying glycopeptide antibiotics, typically through the derivatization of the natural products themselves or in few

cases through chemical total synthesis. Because of the structural complexity of glycopeptides, nearly all new structures have been derived by semisynthetic modification of the natural products (Li et al. 2012). Many of the most significant modifications have introduced hydrophobic moieties into the glycopeptide structure (Beauregard et al. 1995). Actually, strategically placed hydrophobic groups have been shown to rise both antibiotic dimerization in solution and membrane anchoring ability, leading to increased binding to the peptidoglycan terminus and consequently to an augmented activity (see Mechanism of action). Additionally, the direct inhibition of transglycosylase enzymes, mediated by a glycopeptide-modified carbohydrate has been proposed as a second mechanism by which the resulting new glycopeptides increase their antimicrobial properties. A huge number of selective chemical modifications have been described, mainly glycosylation, alkylation, acylation, deglycosylation, realkylation, cyclization, aminomethylation, esterification, and sulfation (Arhin et al. 2012). Moreover, important changes in the structure of the glycopeptides such as removal of amino acids of the dalbaheptide scaffold and the subsequent rebuilding of the molecule have been also reported (Allen et al. 2002; Malabarba et al. 1997). These selective chemical modifications have led not only to a better understanding of the impact of key structural elements in the pharmacology of glycopeptides, but also to the development of new congeners (Van Bambeke 2004). Among the novel glycopeptides telavancin, oritavancin, and dalbavancin constitute an interesting advance.

Telavancin is obtained in three synthetic steps from vancomycin (Leadbetter et al. 2004). It bears a hydrophobic N-decylamino group increasing the activity against resistant organisms and a hydrophilic phosphonic acid side chain that provides improved pharmacokinetic properties. Telavancin shows to function both through the bacterial membrane integrity disruption and also through the traditional glycopeptide mechanism of cell wall synthesis inhibition (see below), which combines a uncommon mechanism for other glycopeptides. Telavancin was approved for complicated skin and skin–structure infections on September of 2009. Recently, a new drug application for telavancin has been submitted for the treatment of nosocomial pneumonia (Arhin et al. 2012; James et al 2012).

Oritavancin is derived by the reductive alkylation of chloroeremomycin, a vancomycin-type glycopeptide (Nicas et al. 1996). It was originally developed by Eli Lilly in a program aiming to maintain the activity of chloroeremomycin against *S. aureus*. Oritavancin possesses the vancomycin core but different sugar residues and inhibits late stages of peptidoglycan biosynthesis, as vancomycin does. Studies on the mechanism of action for oritavancin and related compounds have shown that the chlorobiphenyl side chain of the molecule promotes antibiotic dimerization and membrane anchoring; favoring antimicrobial activity against resistant organisms (Allen and Nicas 2003). Oritavancin is currently in Phase 3 of clinical development for the complicated skin and skin–structure indication (Arhin et al. 2012).

Dalbavancin is a three step synthetic derivative from the naturally occurring teicoplanin-like glycopeptide parvocidin (A 40926) by a 3,3-dimethylaminopropyl amide substitution on the peptide carboxyl group (Malabarba and Goldstein 2005). The antimicrobial spectrum of dalbavancin most closely resembles that of

teicoplanin; however, it has greater potency against many Gram-positive organism groups, including anaerobes (Biedenbach et al. 2009; Raad et al. 2005). In skin and soft tissue infections, a 92–94 % of microbiological and clinical response, respectively, was found in an open label Phase 2 comparative dosing trial (Seltzer et al. 2003). Dalbavancin is currently in Phase 3 clinical development for its improved activity, pharmacokinetics, and pharmacodynamics (Van Bambeke 2004).

In addition to these three novel glycopeptides, the formation of hybrid antibiotics by modifications of the glycopeptide scaffold is especially interesting. Notably are the glycopeptide–cephalosporin hybrid TD-1792 and the glycopeptide–aminoquinoline hybrid PA1409 or also called vancomyquine. These two hybrid antibiotics are extremely potent against both staphylococci and enterococci (Chau et al. 2011; Long et al. 2008).

## 11.4 Spread of Vancomycin Resistance: A Serious Public Health Problem

Hospital-acquired infections (HAI) are a major source of morbidity and mortality in hospitals and other health care facilities (Plowman et al. 2001). Most of the bacterial species causing HAI can be classified as Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci, and Gram-negative bacilli. Among them, Gram-positive cocci have emerged as an increasingly problematic cause of HAI, and also a reason of spreading into the community. *S. aureus* is one of the major hospital-acquired pathogen (Parker and Jevons 1964). It produces a variety of toxins and antigens which predispose to invasive skin and soft tissue infections, and also necrotizing pneumonias and other similar infectious localizations, like heart, central nervous system, and bones (Lowy 1998; Becker et al. 2003).

After the introduction of penicillin over 70 years ago, infections by *S. aureus* and other important pathogens, like *Streptococcus pyogenes* and *Streptococcus pneumoniae*, finally became treatable. However, within a short period of time, *S. aureus* developed resistance to penicillin and later also to methicillin, the next generation drug for the treatment of penicillin-resistant strains (giving the so-called 'methicillin-resistant *S. aureus*' MRSA; Wenzel 1982). The major spread of antibiotic resistance started in the early 1980s with the diffusion of virulent clones of methicillin-resistant *S. aureus* worldwide (Chambers and Deleo 2009). Today, MRSA is an incipient community pathogen in many geographical regions and endemic in most hospitals of the world (Lowy 1998).

The onset of vancomycin resistance was long-delayed in comparison to all other antibiotics. Vancomycin-resistant phenotypes were first reported in *Enterococcus faecium* and *Enterococcus faecalis* (Leclercq et al. 1988) that were named 'vancomycin-resistant enterococci' (VRE) becoming a major hospital-acquired pathogen. Some years later, vancomycin resistance was also reported in

staphylococci; first in coagulase-negative staphylococci, like *S. epidermidis* (Schwalbe et al. 1987) and then in coagulase-positive staphylococci, like *S. aureus* (Hiramatsu et al. 1997). Staphylococci strains with reduced susceptibility to teicoplanin were also identified (Tenover et al. 2001; Bertrand et al. 2003). Depending on the mechanism of resistance, these *S. aureus* strains are variably referred to as vancomycin-resistant *S. aureus* (VRSA), glycopeptide-resistant *S. aureus* (GRSA), vancomycin intermediate-resistance *S. aureus* (VISA), and 'glycopeptide intermediate-resistance *S. aureus*' (GISA). The first cases of fully 'vancomycin-resistant MRSA' strains (VRSA) were reported in 2002 and, worryingly, there have been an increasing number of cases in the subsequent years (Chang et al. 2003; Alzolibani et al. 2012). Actually, the potential of the spread of vancomycin resistance has prompted researchers to use the term 'crisis' when analyzing the VRSA spread (Larkin 2003).

A majority of VRSA has been found in patients co-infected with VRE, implicating horizontal gene transfer as the current method for acquiring vancomycin resistance (Zhu et al. 2010). Indeed, the Tn*1546* transposon harbored within a multiresistant conjugative plasmid from *E. faecalis* was found in VRSA clinical isolates in 2002 (Weigel et al. 2003). Consequently, the spread of VRE in hospitals creates a risk of new resistant strains emerging. Therefore, a rational vancomycin prophylaxis, with a not prolonged duration of treatment, should be taken into account in determining policies for antibiotic use. Actually, several national healthcare systems recommend that glycopeptide treatments should be limited to those patients with known MRSA infections in order to limit appearance of new glycopeptide-resistant strains (Gemmell et al. 2006). Alternatively, as the prevalence of VRE and VRSA increases, and as both horizontal and vertical gene transfer of resistance are established, new antibiotics with the efficacy of vancomycin will be required to contain their impact.

## 11.5 Why to Focus on Cell Wall Biosynthesis?

The publication of the emergence of antibiotic-resistant strains from previously sensitive bacteria is regularly observed in the media becoming one of the major themes of the current research. Thus, even when the resistance to antiparasitic, antiviral, or antifungal drugs is increasing, the attention is mainly focused on the resistance to antibacterial agents (Wright 2003). The occurrence of 'new' infectious agents, multidrug resistance, or resurgent pathogens is closely related with the bacterial cell wall structure. This external wrapping conditions the bacterial morphology (e.g.: rods, cocci), the number of units that can result in a single, clusters of cells as pairs (e.g.: *Neisseria* sp.), tetrads, or embedded masses within a capsule. Similarly, the fungi can be found as a mycelia (e.g.: *Penicillium chrysogenum*) or as single yeasts (e.g.: *Saccharomyces cerevisiae*) based on the cell wall. The simplest bacterial classification, but nonetheless effective, is based on the differential staining method developed by the Danish bacteriologist Hans

Christian Joachim Gram (1853–1938). This staining system is focused on the peptidoglycan amount present in the cell wall (Fig. 11.3). Besides, cell wall composition is directly related with the bacterial resistance to antibiotics.

Today, glycopeptide antibiotics, which inhibit cell wall synthesis (Fig. 11.3), are the last resort drugs for hospital-acquired Gram-positive infections, including 'methicillin-resistant *S. aureus*' (MRSA) (Jovetic et al. 2010). The target of glycopeptides, such as vancomycin, is the peptidoglycan or murein (Fig. 11.3). This is a polymer composed of repeating disaccharide-pentapeptide units synthesized in the cytoplasm. It shapes a layer outside the plasma membrane of bacteria, but not in Archaea. The sugar components are residues of $\beta$-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid. The N-acetylmuramic acid presents a peptide chain of 3–5 amino acids, which are: (i) in Gram-positive bacteria: L–alanine, D-glutamine, L-lysine, and D-alanine; (ii) and in Gram-negative bacteria: L–alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanine. The peptidoglycan layer in Gram-positive bacteria constitutes around 90 % of the dry weight (thickness 20–80 nm), whereas in Gram-negative bacteria it is only 10 % (7–8 nm) (Madigan et al. 2010). Due to this large different of peptidoglycan composition the glycopeptides impair cell wall synthesis only in Gram positive bacteria.

## 11.6 Mechanism of Action

The peptidoglycan is an impressive macromolecule, which surrounds the bacterial cell providing the scaffold for other cell wall components as well as the bacterial morphology. The peptidoglycan synthesis is the result of several protein interactions never reproduced in vitro with purified proteins from Gram-positive bacteria (Zapun et al. 2012). In short, a lipid carrier (undecaprenylpyrophosphate), commonly known as lipid II, is the vehicle that transports disaccharide-pentapeptide units through the cell membrane (Fig. 11.3, detail). The C-terminal residues of this muramyl pentapeptide are the substrates of transglycosylases and transpeptidases activities (peptidoglycan backbone extension and growing chain cross-linking to the cell wall, respectively) involved in the nascent peptidoglycan arrangement (Périchon and Courvalin 2012; Arhin et al. 2012).

$\beta$-lactam and glycopeptide antibiotics, which are the first and last line of antibacterial defence, work in different ways for their antibiotic activities (Jovetic et al. 2010). On one hand, $\beta$-lactams directly inhibit the transpeptidase enzyme, whereas, on the other hand, vancomycin binds the enzyme substrate [acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala) (Fig. 11.3, detail)] and prevents the enzyme access (James et al. 2012). Thus, the transpeptidase inhibition due to the formation of a glycopeptide–peptidoglycan complex maintains the wall integrity and stops the cell wall biosynthesis blocking the cell division and growth (Barna and Williams 1984). In contrast, the lipopolysacchararide outer membrane of Gram-negative bacteria transforms these microorganisms in natural resistant cells to

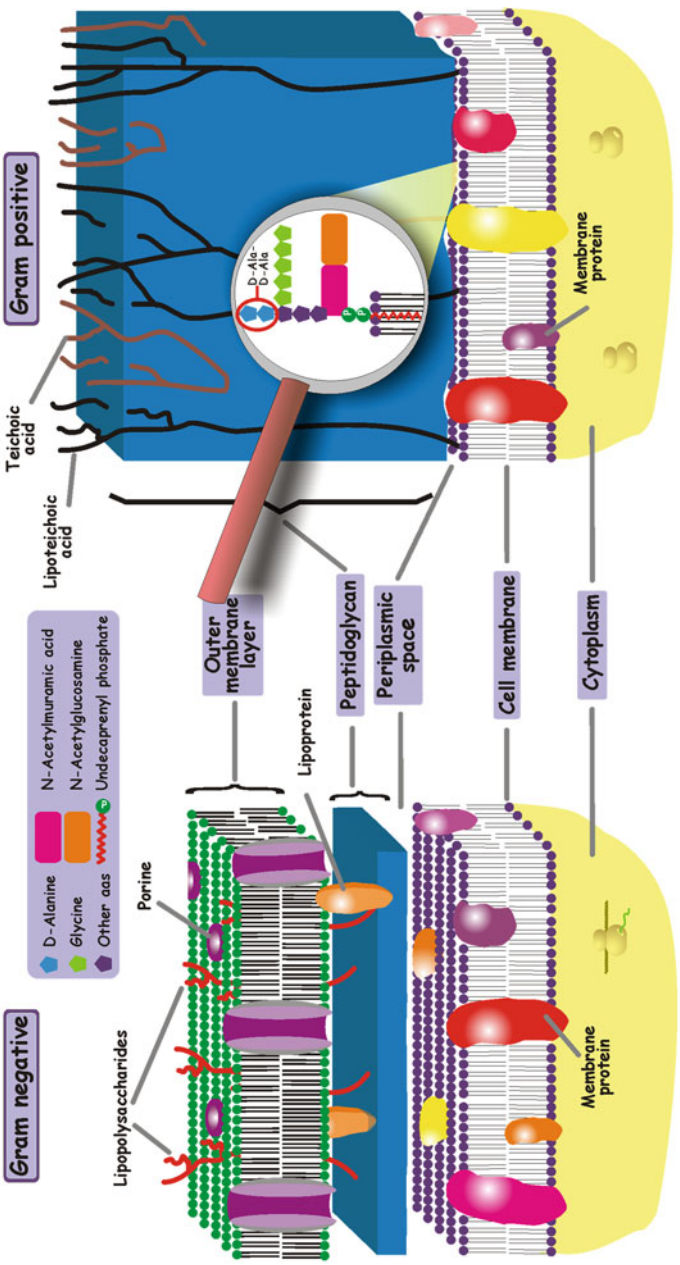


**Fig. 11.3** Gram-negative and Gram-positive cell wall differences. The main components of the peptidoglycan layer, which is the target of the glycopeptide antibiotics, are shown (magnifying glass) and detailed (*gray box*). D-Ala-D-Ala target is highlighted

glycopeptide antibiotics since their peptidoglycan layer is inaccessible to these drugs (Wright 2003). The vancomycin presents a well-established activity pattern binding to the carboxyl-terminal of the acyl-D-Ala-D-Ala residues of the pentapeptide moiety of lipid II. This fact sterically obstructs the transglycosylases activity (Reynolds 1989). Vancomycin binds the acyl-D-Ala-D-Ala residues through five hydrogen bonds and hydrophobic van der Waals contacts between the Ala motif and the central pocket of the glycopeptide (Williams and Bardsley 1999). This network depends on the precise alignment of the antibiotic and the acyl-D-Ala-D-Ala of peptidoglycan (Wright 2003). This mechanism of action reduced the acquisition of resistance to the glycopeptide antibiotics more than the majority of the other antibiotic groups (Reynolds 1989).

## 11.7 Resistance to Glycopeptide Antibiotics: Cell Wall Remodelling

The emergence and dissemination of antibiotic resistance are the two key steps of the antimicrobial drug resistance. This is an unstoppable part of the bacterial evolution, which presents a difficult prediction of its future progression. In order to understand the bacterial resistances, on one hand, the nature of the antimicrobial components must be distinguished. Thus, these can be: (i) naturally produced by the microorganisms from the environment (e.g., kanamycin); and (ii) semisynthetic (e.g., amikacin); (iii) or fully synthetic (e.g., quinolones), which are produced under human intervention (Courvalin 2005). On the other hand, the way to face up the antibacterial attack should also be considered. Thus, the antibiotic resistance can be: (i) natural type I (intrinsic) due to the lack of target sites or low permeability as is the case of *Corynebacterium jeikeium*, which accumulates genetic events resulting in nonspecific mechanisms that increased the antibiotic efflux or modified the permeability of the corynebacterial cell wall (Tauch et al. 2005); (ii) natural type II (producer bacteria), since the natural antibiotic producers tend to ensure their own survival against their own products (Courvalin 2005; Cundliffe and Demain 2010) or (iii) acquired (e.g.: enzymatic inhibitors of antibiotic agent, target mutation/modification, active efflux pumps, target overproduction, etc.) (Byarugaba 2010). Then, under this panoramic view, the antibiotic resistance points to the cell wall and the cell membrane since it is the first line of contact where the antibiotic target (e.g.: peptidoglycan) or efflux pumps are located.

In particular, the resistance against glycopeptides is originated when the hydrogen bonds between the glycopeptide and the acyl-D-Ala-D-Ala residues are interfered by an atom substitution in the ligand that serves to remove the central H–bond and to introduce repulsive lone pair/lone pair interactions. These effects decrease up to 1,000-fold the affinity (James et al. 2012). Thus, the most common resistance mechanism to glycopeptides involves the transformation of D-Ala-D-

Ala to the depsipeptide D-Ala-D-Lac (D-lactate) or to D-Ala-D-Ser (D-Serine). Additionally, peptidase-mediated elimination of D-Ala-D-Ala moieties from the cell is a frequent method of resistance (Périchon and Courvalin 2012; Kalan et al. 2013).

Vancomycin-resistance gene clusters can be detected in three bacterial groups: (i) human pathogens (e.g.: *E. faecalis*), (ii) glycopeptide-producing actinomycetes (e.g.: *A. orientalis),* and (iii) non-glycopeptide-producing actinomycetes (e.g.: *Streptomyces coelicolor*) (Hong et al. 2008). The groups of actinobacteria-resistant strains will be reviewed below. Regarding the human pathogen-resistant strains, Depardieu et al. (2007) have established eight types of glycopeptide resistance in enterococci against the most common glycopeptides vancomycin and teicoplanin (Table 11.2). These eight types have been enlarged later with two new types [VanN (Lebreton et al. 2011); VanM (Xu et al. 2010)]. It is the so-called 'van alphabet' (based on the primary sequence of the structural gene for the resistance ligase), which is encoded by specific operons (*vanA*, *B*, *C*, *D*, *E*, *F*, *G* and *L*) (Périchon and Courvalin 2012). These operons are responsible for: (i) the synthesis of new target such as D-Lac (VanA, B, D, and F-types) or D-Ser (VanC, E, G, and L-types) and (ii) the elimination of the normal precursors ending in D-Ala (Depardieu et al. 2007; Périchon and Courvalin 2012). Other mechanisms like thickened cell walls, reduced peptidoglycan cross-linking, and increased numbers of D-Ala-
D-Ala residues have been also described like mechanisms leading to reduced susceptibility to glycopeptides. Some staphylococci strains (e.g.: the so-called 'vancomycin-intermediate *S. aureus*'; VISA), show reduced susceptibility to glycopeptides due to a thickened and a poorly cross-linked cell wall that sequestered glycopeptides at the periphery and precludes the drugs from reaching their target (Walsh and Howe 2002).

Interestingly, a three-gene cluster similar to the basic resistance operon *vanH–vanA–vanX* (*vanHAX*) has been recently detected in 30,000-year-old Beringian permafrost sediments; therefore glycopeptide resistance is an ancient event (D'Costa et al. 2011). In parallel to the resistance development and the different Van types discovery (Woodford 1998), the need of a careful screening to ensure the detection of resistant organisms is required (Woodford and Johnson 1994). Accordingly, the development of detection methods was observed as crucial for clinical purposes (Dutka-Malen et al. 1995). Nowadays, different multiplex PCR detection systems has been described for the recognition of these Van types (Patel et al. 2001), as well as real-time PCR methodologies (Eisner et al. 2005).

**Table 11.2** Van types currently described in *Enterococcus*, *Ruminococcus,* or *Clostridium* species. Abreviations: Van: Vancomycin; Tei: Teicoplanin; R: resistent; S: sensitive; H: high; M: moderate; L: low. Based on: (Périchon and Courvalin 2012; Sujatha and Praharaj 2012)

| Type | Resistance | Genes | Genetic location | Action | References |
|---|---|---|---|---|---|
| VanA | $Van^{HR}/Tei^{HR}$ | *vanA* operon:-element movement (transposase, resolvase)-glycopeptide resistance (*vanR, vanS, vanH, vanA, vanX, vanY,* and *vanZ)* | Self-transferable plasmids (transposon Tn*1546*); bacterial chromosome (large conjugative elements) | Synthesis D-Ala-D-Lac | (Handwerger et al. 1990; Handwerger and Skoble 1995; Leclercq et al. 1989; Leclercq et al. 1988) |
| VanB (subtypes: *vanB1, vanB2, vanB3*) | $Van^{HR}/Tei^{S}$ | *vanB* operon: resistance, accessory, regulatory genes ($vanH_B$, *vanB*, $vanX_B$, $vanY_B$, $vanR_B$, $vanS_B$, *vanW*) | Plasmids; conjugative transposon (Tn*5382*;Tn*1549*); large conjugative elements (up to 250 kb) transferable from chromosome based on Tn*1547* or Tn*5382* | Synthesis D-Ala-D-Lac | (Dahl et al. 1999; Evers and Courvalin 1996; Quintiliani and Courvalin 1994; Rice et al. 1998) |
| VanC | $Van^{LR}/Tei^{S}$ | *vanC* operon: *vanC*, $vanXY_C$, *vanT*, $vanR_C$, $vanS_C$ | Chromosome | Synthesis D-Ala-D-Ser | (Billot-Klein et al. 1994a; Handwerger et al. 1994) |
| VanD (subtypes: *vanD-1*, *vanD-4*) | $Van^{MR}/Tei^{LR}$ | *vanD* operon: $vanR_D$, $vanS_D$, $vanY_D$, $vanH_D$, *vanD*, $vanX_D$ | Chromosome | Synthesis D-Ala-D-Lac | (Depardieu et al. 2009; Depardieu et al. 2003b) |
| VanE | $Van^{LR}/Tei^{S}$ | *vanE* operon: *vanE*, $vanXY_E$, $vanT_E$, $vanR_E$, $vanS_E$ | Chromosome | Synthesis D-Ala-D-Ser | (Abadía-Patiño et al. 2004; Fines et al. 1999) |
| VanF | $Van^{R}/Tei^{S}$ | *vanF* operon: $vanR_F$, $vanS_F$, $vanY_F$, $vanZ_F$, $vanH_F$, *vanF*, $vanX_F$ | Chromosome | Synthesis D-Ala-D-Lac | (Fraimow et al. 2005; Patel et al. 2000) |

(continued)

**Table 11.2** (continued)

| Type | Resistance | Genes | Genetic location | Action | References |
|---|---|---|---|---|---|
| VanG | $Van^{LR}/Tei^{S}$ | *vanG1* operon: $vanU_G$, $vanR_G$, $vanS_G$, $VanY_G$, $vanW_G$, *vanG*, $vanXY_G$, $vanT_G$<br>*vanG2* operon: $vanU_G$, $vanR_G$, $vanS_G$, $vanW_G$, *vanG*, $vanXY_G$, $vanT_G$<br>*vanG-like C. difficile* operon: $vanR_G$-*like*, $vanS_G$-*like*, *vanG-like*, $vanXY_G$-*like*, $vanT_G$-*like*<br>*vanG-like Ruminococcus* sp. operon: partial $vanS_G$-*like*, *vanZ*, *vanG-like*, $vanT_G$-*like* | Chromosome | Synthesis D-Ala-D-Ser | (Boyd et al. 2006; Depardieu et al. 2003a; Domingo et al. 2007; McKessar et al. 2000; Sebaihia et al. 2006) |
| VanL | $Van^{LR}/Tei^{S}$ | *vanL* operon: *vanL*, $vanXY_L$, $vanTm_L$, $vanTr_L$, $vanR_L$, $vanS_L$ | Chromosome | Synthesis D-Ala-D-Ser | (Boyd et al. 2008) |

## 11.8 Gene Organization and Regulation of Highly Inducible Vancomycin Resistance

As described above, resistance to vancomycin can be acquired by several different mechanisms. The most common involves conversion of D-Ala-D-Ala precursors to the depsipeptide D-Ala-D-Lac or D-Ala-D-Ser. The affinity of vancomycin for precursors terminating in D-Ala-D-Lac and D-Ala-D-Ser is approximately 1,000-fold and sixfold lower than for precursors terminating in D–Ala-D-Ala, respectively (Billot-Klein et al. 1994b). Some of the enzymes implicated in the D-Ala-D-Ser resistance pathway are different from those involved in the D-Ala-D-Lac ending precursors, suggesting the existence of, at least, two routes of evolution causing resistance (Reynolds and Courvalin 2005). A third mechanism by which resistance can be acquired (reported in staphylococci) is through mutation of endogenous two-component signal transduction systems that control normal cell wall metabolism (like the WalRK system) or systems that mediate the cellular response to cell wall damage, like VraRS and GraRS (Jansen et al. 2007; Howden et al. 2008; Kato et al. 2010). The highest resistance is achieved with the conversion of D-Ala-D-Ala to D-Ala-D-Lac, coupled with a peptidase-mediated elimination of D-Ala-D-Ala dipeptides from the cell wall (found in strains expressing *vanA*, *vanB,* or *vanD* clusters) (Table 11.2). We will focus on the comparison of these clusters together with that found in streptomycetes (see below).

The *vanA* gene cluster, characterized by an inducible high level of resistance to both vancomycin and teicoplanin, is mainly carried by the transposon Tn*1546* (Table 11.2). This element is composed by two regulatory genes (*vanR* and *vanS*), three glycopeptide-resistance genes (*vanH*, *vanA*, and *vanX*), and two accessory

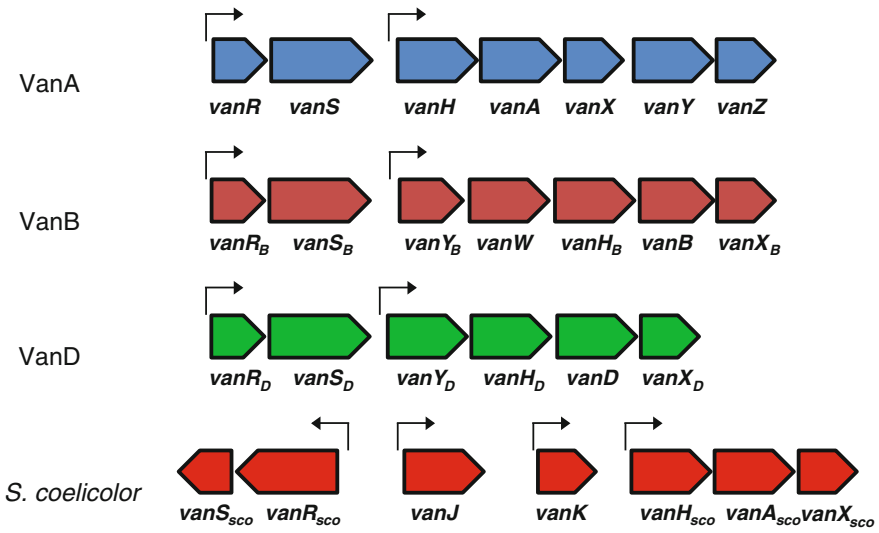


**Fig. 11.4** Comparison of the *vanA*, *vanB,* and *vanD* operons with that of *S. coelicolor*

genes (*vanY* and *vanX*), in addition to the two genes responsible for transposition (ORF1 and ORF2) (Fig. 11.4 and Table 11.2). The *vanH*, *vanA,* and *vanX* genes code for proteins that are necessary for expression of resistance. *vanH* codes for a dehydrogenase which converts pyruvate to D-Lac (Arthur et al. 1992), *vanA* codes for a D-Ala-D-Lac ligase (Bugg et al. 1991), and *vanX* codes for a D,D-dipeptidase that cleaves D-Ala-D-Ala dipeptides to ensure that only altered peptidoglycan precursors terminated in D-Ala-D-Lac are built up (Reynolds et al. 1994). Transcriptional activation of *vanHAX* genes is regulated by the two-component system VanS-VanR in response to extracellular glycopeptide antibiotics (Handwerger et al. 1992). The number of genes present in the resistance cluster can vary, but the "core" cluster usually consists of these five genes (*vanSRHAX*). The accessory protein VanY is a membrane-bound penicillin-insensitive D,D-carboxypeptidase that cleaves the D-Ala C-terminal residue of the pentapeptide precursors synthesized from the D-Ala-D-Ala dipeptide that has escaped VanX hydrolysis (Arthur et al. 1996). The *vanZ* gene confers low-level resistance to teicoplanin by an unknown mechanism (Arthur et al. 1995).

VanB, the other major type of inducible vancomycin resistance involving precursors ending in D-Ala-D-Lac, confers resistance to vancomycin but not to teicoplanin (Table 11.2). The organization of the *vanB* cluster is similar to that of the *vanA* cluster; only differing in the location of the glycopeptide resistance and accessory genes (Fig. 11.4). The function of the accessory gene *vanW* (different to *vanZ*) is unknown. The *vanB* gene cluster is generally carried by large conjugative elements that are transferable from chromosome to chromosome, like the 250 kb genetic element which contains the Tn*1547* transposon (Table 11.2). Tn*1547* has two insertion sequences allowing its dissemination by both vertical and horizontal transfer (Quintiliani and Courvalin 1994). The *vanB* element can also be spread by plasmids (Rice et al. 1998) or disseminated directly by conjugative transposons, like Tn*5382* (Carias et al. 1998) or Tn*1549* (Garnier et al. 2000).

The *vanD* cluster confers moderate levels of resistance to vancomycin and teicoplanin and involves also the synthesis of precursors ending in D-Ala-D-Lac (Table 11.2). This cluster, mainly found in enterococci, is exclusively chromosomally located and therefore is not transferable by conjugation to other bacteria. Although the organization of the *vanD* cluster is similar to that of *vanA* or *vanB* clusters, this cluster lacks *vanZ* or *vanW* genes (Fig. 11.4). Moreover, the *vanS* or *vanR* genes carry mutations responsible for the synthesis of inactive VanS or VanR proteins, which make the cluster to be constitutively expressed (Depardieu et al. 2009). It is important to note that the VanS and VanR proteins from enterococci strains are distantly related in amino acid sequence. In VanA and VanB types, exposure of cells to vancomycin triggers autophosphorylation of VanS on a histidine residue. Although the kinases are presumed to detect glycopeptides, a direct interaction has been demonstrated only for VanS from *S. coelicolor* (Koteva et al. 2010). The transference of the phosphoryl group from VanS to an aspartate residue of VanR results in transcriptional activation of *vanH*, *vanA,* and *vanX* genes, as well as of the accessory genes and the own *vanRS* genes. VanS is not strictly necessary for full activation of the promoters, since VanR can be phosphorylated

independently by acetylphosphate or kinases encoded by the host chromosome (Arthur et al. 1999). To date, VanR is the only transcriptional activator that has been shown to activate the transcription of the vancomycin-resistance genes. Nevertheless, other proteins different to VanR have been shown to modulate *van* transcription, mainly in *S. coelicolor* (see next section). It is not clear why in VanB strains teicoplanin does not induce *van* expression while in VanA strains it does. It has been proposed that the phosphatase activity of VanS could account for this teicoplanin susceptibility. Thus, VanB-type teicoplanin-resistant strains harboring mutations that impair VanS phosphatase activity have been obtained in vitro and in vivo (Hayden et al. 1993).

A *van* gene cluster, similar to that in VanB-type strains, has been also described in the non-pathogenic and non-glycopeptide-producing actinomycete *S. coelicolor*. This bacterium is highly resistant to vancomycin but susceptible to teicoplanin. The resistance cluster of *S. coelicolor* consists of seven genes divided into four transcription units: *vanRS*, *vanJ*, *vanK,* and *vanHAX* (Hong et al. 2004). At least four VanR-dependent promoters have been identified in this cluster, in contraposition to the only two found in *vanA*, *B,* or *D* cluster (Fig. 11.4). VanRSHAX proteins have the same function to those previously described in VanA, B, and D types. By contrast, *vanJ* and *vanK* have no orthologues in the vancomycin-resistance gene clusters of pathogenic enterococci. VanK is essential for vancomycin resistance in *S. coelicolor* while VanJ is not critical (Hong et al. 2005; Novotna et al. 2012). VanK is a member of the Fem family of peptidyltransferases which add the cross-bridge amino acids to the stem pentapeptide of cell wall precursors. The absence of *vanK* orthologues in the vancomycin-resistance gene clusters of pathogenic enterococci implies that enterococcal FemX can recognize precursors terminating in either D-Ala-D-Lac or D-Ala-D-Ala. Interestingly, *S. coelicolor femX* null mutants are viable only under presence of glycopeptide induction (Hong et al. 2005). The other specific Van protein of *S. coelicolor* in relation to pathogenic strains, VanJ, is a membrane located protein oriented with its C-terminal active site exposed to the extracytoplasmic space. VanJ is not involved in vancomycin resistance and it seems to be involved in an uncharacterized teicoplanin-resistance mechanism (Novotna et al. 2012). In addition, this protein seems to confer resistance to other teicoplanin-like antibiotics (ristocetin and A47934) as well as to a broad range of semisynthetic teicoplanin derivatives; but not generally to antibiotics or semisynthetic derivatives with vancomycin-like structures. *vanJ* homologues are found ubiquitously in streptomycetes and include the *staP* gene from the *S. toyocaensis* A47934 biosynthetic gene cluster (Pootoolal et al. 2002).

## 11.9 Glycopeptide Resistance in *Streptomyces*

As stated above, vancomycin-resistance gene clusters (*van*) are found in human pathogens such as *E. faecalis*, *E. faecium,* or *S. aureus*, glycopeptide-producing actinomycetes such as *A. orientalis*, *A. teichomyceticus,* or *S. toyocaensis* and

non-glycopeptide-producing actinomycetes like *S. coelicolor*. Hong and co-workers (2004) reported in *S. coelicolor* the first example of vancomycin resistance in a non-pathogenic and non-glycopeptide-producing bacterium. Recently, new cases have been also reported, like those of *Streptomyces lividans* and *Bacillus subtilis* BP341A (Bisicchia et al. 2011; Santos-Beneit and Martín 2013). This last bacterium carries a *vanB* cluster (introduced in vitro) and a mutation in the endogenous *ddl* gene that impairs D-Ala-D-Ala ligase activity (Bisicchia et al. 2011). Naturally occurring vancomycin-resistant *B. subtilis* strains have not been reported, although a VanA-type phenotype has been found in a clinical isolate of *Bacillus circulans* VR0709 (Fontana et al. 1997). In relation to *Streptomyces*, at least 23 vancomycin-resistant environmental strains have been identified in a recent work from a collection of VanB-type strains; although it is not reported whether these strains are glycopeptide producers or not (Kalan et al. 2013). Due to the recent explosion in microbial genome sequencing, many *vanHAX* orthologous genes have been found among *Streptomyces* species (Novotna et al. 2012). On the contrary, none of the *Streptomyces* complete genomes available in the Strep database (*Streptomyces avermitilis*, *Streptomyces griseus*, *Streptomyces clavuligerus*, *Streptomyces scabies* and *Streptomyces venezuelae*), except *S. coelicolor*, have *vanHAX* orthologs. In addition, only two (*S. coelicolor* and *S. lividans*) of ten different streptomycetes (*S. coelicolor* M145, *S. lividans* 1326, *Streptomyces avidinii* NRRL 3077, *S. clavuligerus* NRRL 3585, *Streptomyces hygroscopicus* NRRL 3602, *S. griseus* NRRL 3851, *Streptomyces tsukubaensis* NRRL 18488, *S. avermitilis* NRRL 8165, *Streptomyces natalensis* NRRL 2651 and *S. toyocaensis* NRRL 15009) were shown to be vancomycin-resistant in a recent work of Santos-Beneit and Martín (2013). All these findings may indicate that although vancomycin resistance in streptomycetes is not rare, the mechanism is not ubiquitously found in this genus.

Despite the fact that *S. coelicolor* carries a complete set of glycopeptide-resistance genes, it is sensitive to teicoplanin, because teicoplanin fails to induce expression of the *van* gene cluster (Hong et al. 2004; Koteva et al. 2010). However, both vancomycin and DS-A47934 (the A47934 desulfo derivative), even at concentrations as low as 0.1 μg $ml^{-1}$, have been shown to induce teicoplanin resistance in this bacterium (Kalan et al. 2013; Novotna et al. 2012). In addition to vancomycin and DS-A47934, ristocetin, chloroeremomycin, and A47934 have been also shown to act as inducers of the *S. coelicolor van* genes (Hutchings et al. 2006). In contrast, glycopeptide resistance in *S. toyocaensis* is induced by A47934, but not by vancomycin or teicoplanin (Neu and Wright 2001). Therefore, understanding the molecular basis for induction of glycopeptide resistance is essential for the proper utilization of antibiotics in the clinic and for the development of new semisynthetic derivatives.

Another interesting finding in *Streptomyces* is that the induction of glycopeptide resistance is very influenced by the nutritional conditions of the culture media. Actually, different minimal inhibitory concentration values (MIC) of vancomycin, ranging from 20 to 512 μg $ml^{-1}$, have been determined for *S. coelicolor* grown in different media (Hesketh et al. 2011; Hong et al. 2004; Hutchings et al. 2006;

Kalan et al. 2013; Koteva et al. 2010; Novotna et al. 2012; Santos-Beneit and Martín 2013; Tan et al. 2002). In particular, phosphate concentration has been proved to be a key step for vancomycin resistance. Thus, addition of phosphate to the medium decreased more than four times the MIC of vancomycin in *S. coelicolor* (Santos-Beneit and Martín 2013).

A last interesting finding reported in different works with *Streptomyces*, as well as in other bacteria like *Staphylococcus*, *Mycobacterium,* and *Bacillus*, is the relation between glycopeptide resistance and cell wall stress (Bisicchia et al. 2011; Hesketh et al. 2011; Hong et al. 2002; McCallum et al. 2006; Provvedi et al. 2009). In *S. coelicolor*, cell wall hydrolytic enzymes and cell wall acting antibiotics, like penicillins and glycopeptides, are able to induce the *sigE* signal transduction system. This system senses and responds to certain changes in the integrity of the cell envelope by activating different genes, some of them involved in the biosynthesis of cell wall glycans (Hong et al. 2002). Interestingly, vancomycin resistance is significantly decreased in a *S. coelicolor sigE* null mutant; pointing to a role of this sigma factor in glycopeptide resistance (Hesketh et al. 2011). Other genes that could be implicated in *S. coelicolor* vancomycin resistance are *sigB* (codes for an osmotic stress sigma factor), *relA* (encodes for a ppGpp synthetase), SCO3397 (codes for a lysyl-tRNA synthetase), and SCO1875 (encodes a penicillin binding protein). Disruption of these genes highly increases vancomycin susceptibility in *S. coelicolor* (Hesketh et al. 2011). Finally, according to recent results of our group (Santos-Beneit, F. unpublished), it is very probable that *whiB*, coding for a transcriptional regulator, may be also implicated in *S. coelicolor* vancomycin resistance. This fact is not strange since the WhiB homologue in *Mycobacterium tuberculosis* (WhiB2) has been involved in the resistance to cell wall acting antibiotics (Geiman et al. 2006). Moreover, transcription of *whiB* is regulated by SigE, which, as mentioned before, has been shown to be involved in vancomycin resistance (Hesketh et al. 2011).

## 11.10 Conclusions and Future Perspectives

The launching of medical application of every antibiotic has been and will be followed by resistance in the targeted bacteria. Therefore, there is a constant urgency to develop new agents to compete with the increase of bacterial resistances. However, since the 1970s only three systemically administered antibiotics (quinupristindalfopristin, linezolid, and daptomycin) have been marketed in the EU to treat infections caused by multidrug-resistant Gram-positive bacteria (http://www.ecdc.europa.eu/). The growing gap between the increasing frequency of multidrug-resistant bacteria emerging and the decline in development of new antibiotics alerts us to suffer a return to the preantibiotic era. Strategies to diminish the spread of multidrug-resistant bacteria have met with limited success. Therefore, the continued improvement of effective antibiotics must be considered as a 'common good'.

The emergence of multidrug-resistant strains has raised the importance of searching for alternative targets to develop new drugs. Due to the importance of its physiological role and its difference from the eukaryotic cell surface, the bacterial cell wall is one of the best candidates in the search for new drug targets. A large number of antibacterial drugs currently in use, like $\beta$-lactams, cephalosporins, glycopeptides, phosphomycin, bacitracin, and cycloserine, are directed against cell surface components or metabolic pathways that are involved in their synthesis. However, in spite of a good knowledge of the chemical composition of the bacterial surface, not much is known about its organization and physiology.

In this perspective, recent efforts to understand cell wall physiology in conjunction with glycopeptide biosynthesis and resistance have been reported. These findings in the future may lead to redesign glycopeptide antibiotics for the treatment of resistant microbial infections, including VRE and VRSA, and to provide a more powerful class of antibiotics much less prone to bacterial resistance.

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# Index


**A**
*Acinetobacter*, 216
AcrAB-TolC, 220
Actinomycetes, 2, 3, 5–7, 19
Actinomycin, 151–152
Acyclotides, 94
Alginate lyase, 228
Alkaloids, 84, 96–100
Amino-glycoside-resistant, 221
Amoxicillin-clavulanate, 218
Antibacterial, 52–54, 60–68, 71
Antibiotic resistance, 215, 217, 220, 286, 292, 296
Antibiotics, 177, 178, 182–186, 189–199, 201, 206, 215–218, 220–228, 285–288, 290, 293–296, 302, 304, 305
Antifungal, 52–54, 68–73, 178, 182, 183, 185, 186, 189–192, 195, 197, 198, 201–204, 206
Antifungal compounds, 182, 183, 185, 186, 190–192, 197, 201, 206
Antimicrobial peptides, 269, 270, 272–275, 277, 279, 280
Antiviral, 52–54, 72–74
APS, 31
Atomic force microscopy (AFM), 275–278
*Azotobacter chroococcum*, 228

**B**
*Bacillus anthracis*, 225
*Bacillus circulans*, 228
Bacté-coli-phage, 222
Bacté-rhino-phage, 222
Bacterial resistance, 216, 218
Bacteriophage, 222–229
$\beta$-barrelins, 94–95
Benzocaine, 224
Benzoxazinoids, 96, 97
Bernal, J. D., 28
Biofilm, 228
Biosynthesis, 118, 121–124, 127–131, 134, 139
Biosynthesis gene clusters, 3, 6, 13, 14, 18, 19, 190, 191
Biskalcitrate metronidazole, 219
Blood–brain barrier, 227
Born approximation, 39
Bragg equation, 36
Bragg, W. H., 28, 36, 43
Bragg, W. L., 28, 36, 43

**C**
$C_1$ bacteriophage, 225
Camalexin, 96–99
*Campylobacter*, 223
Carbapenems, 221
Cell membranes, 29, 44, 46
Cell wall, 285, 289, 291, 293–297, 300, 304, 305
Cell wall antifungals, 252
Cephalosporin, 119, 121–127, 129–131, 133–135, 137, 138
Cephalosporin C, 218
Cephamycins, 116–118, 127, 138
$\alpha$-chymotrypsin, 224
Ciprofloxacin, 219, 221, 224
*Clostridium difficile*, 217
Cocktail of bacteriophages, 227
Coherent scattering, 40, 43
Combinatorial biosynthesis, 5, 6, 8, 11, 12, 19
Constructive interference, 36
Corallopyronin, 153, 160–163
Coumarins, 100
Cross section, 36
Crystallography, 27, 28
Cyclothialidine, 164, 166–167
Cyclotides, 93, 94
4-cys peptides, 95, 96



T. G. Villa and P. Veiga-Crespo (eds.), *Antimicrobial Compounds*,
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**D**
Daptomycin, 219
Defensins, 88–90
Dexamethasone, 219
D'Herelle, F., 222
Diamond light source, 31
Diffraction, 28, 32, 34–36, 41–43, 45
Dirac delta, 39
Dispersin B, 228
Doripenem, 219

**E**
Echinocandins, 238, 240, 243, 249–252, 254–256, 258–260
Elastic scattering, 32, 34, 40, 43
Electron density, 28, 41
Elieva Institute, 223
Endopeptidases, 224
Endo-$\beta$-N-acetylglucosaminidases, 224
Engineering, 129, 132, 133, 135, 137–139
*Enterococcus cloacae*, 221
*Enterococcus faecalis*, 216, 219
*Enterococcus faecium*, 216
Environmental role, 186
Enzybiotics, 221–228
Enzymes, 29
Erlich's, 217
Ernest Rutherford, 28
*Escherichia coli* (*E. coli*), 46, 219, 220, 223, 224, 228
ESRF, 31
ESS, 31, 32
Essential oils (EOs), 52–74

**F**
Fermi pseudo-potential, 39
Fidaxomicin, 153, 162–163
*FKS* hot spots, 255, 258
Flavonoids, 101–103
Fleming, 215, 218
Fluoroquinolones, 218
Flux, 29, 31, 41, 43
Food preservation, 52, 57, 63, 70, 71
Fourier transform, 38, 40
Fraunhofer diffraction, 34
Fungi, 238, 240, 242, 244–248, 252, 254, 256, 258–260

**G**
Gatifloxacin, 219
GBS bacteriophage NCTC 11261, 226
Gemifloxacin mesylate, 219
Genetic engineering, 198, 202, 205, 206
Genome mining, 5, 6, 15, 17, 19
Glucanases, 228
$\beta$(1,3)-glucan synthase inhibitors, 248–258
Glucosinolates, 96, 99–100
Glycopeptide, 285, 288–297, 301–305
Gram-positive bacteria, 227
GRAS, 224
Griseofulvin, 167–169

**H**
Haemoglobin, 28
Havighurst, R. J., 28
Health, 52, 54–57, 60, 69, 70, 72
Helpers for enzybiotics, 228
Hevein-like peptides, 92–93
Hirszfeld Institute, 223
Holins, 222, 224, 225

**I**
ICARE, 216
Incoherent scattering, 40
Indole-Type Metabolites, 97–99
Inelastic scattering, 32, 38, 40
Instruments, 29, 31, 41, 42, 47
Integer, 35
Intensity, 31, 33–38, 42–44
Interatomic forces, 29
Interference, 27, 28, 32, 35, 36
Interference function, 35
Invasive mycoses, 237
IPS, ANL, 32
ISIS, RAL, 32
Isoflavonoids, 101, 102
Isoprenoids, 103

**J**
James Chadwick, 28

**K**
*Klebsiella*, 223
Knottins, 93–94

**L**
$\beta$-lactam antibiotics, 116, 118, 132, 139
$\beta$-lactamase, 220, 221
Lactamases, 221
LANSCE, LANL, 32

Lattice, 27, 29, 34–36
Levofloxacin, 219
Linezolid, 219
Lipid bilayer, 44–46
Lipid Transfer Proteins (LTPs), 90–91
*Listeria monocytogenes*, 223, 224
Lysin PlyGBS, 225
Lysins, 222, 224–227
Lysogenic cycle, 222
Lysogenic phages, 226
Lysostaphin, 228
Lysozymes, 224, 225
Lytic cycle, 222
Lytic enzymes, 225, 227, 228

**M**
MAX IV, 31
Max von Laue, 27
MDR bacteria, 217, 218
Mechanism, 52, 61, 69, 73
Membrane channels, 276, 277
Methicillin-resistant *S. aureus* (MRSA), 216, 219, 226
MexAB-OprM, 220
MiAMP1 family, 94
Microcin J25, 153, 155–158
Minimal inhibitory concentration (MIC), 221
Mitomycin, 152, 167–169
Momentum transfer, 36, 37
Moxifloxacin, 219
Moxifloxacin hydrochloride, 219
Multidrug-resistant (MDR) bacteria, 217
*Mycobacterium avium*, 220, 225, 227
*Mycobacterium tuberculosis*, 220, 225, 227
Myoglobin, 28
Myxopyronin, 153, 159–163

**N**
N-acetylglucosaminidases, 224
N-acetylmuramidase, 224
N-acetylmuramoyl-L-alanine amidases, 224
Natural products, 1–3, 7, 9, 11–14, 18, 19
Neutron beams, 29, 37, 41
Neutrons, 28, 29, 31, 32, 36–38, 40, 43
Neutron sources, 31, 41
Normal microflora, 226
Novobiocin, 164–166
Nucleic acids, 29

**O**
Oliphant, 30
Organs, 29

**P**
*Pseudomonas aeruginosa*, 223, 224, 228
*Proteus vulgaris*, 223
Penicillin, 116, 117, 119, 215, 217, 218, 220, 221
Penicillin-resistance, 221
Penicillin-resistant, 216, 217
Pepsin, 28
Peptidoglycan, 224, 225, 227, 229
Phage therapy, 223, 226, 227
PhageDent, 224
Phages, 221–228
PhagoBioDerm, 224
Phase Stability Principle, 30
Phenolics, 100
Phenylpropanoids, 100–103
Phytoalexins
Phytoanticipins, 84
Planck's constant, 36
Plant antimicrobial peptides, 84, 86
PlyG, 225
Polyene antibiotic, 186, 189, 192, 193, 195–204
Polyphenols, 100
PR-12, 88
PR-13, 86
PR-14, 90
*Pseudomonas*, 223
*Pseudomonas aeruginosa*, 219, 220, 223, 224, 228

**Q**
Q$\beta$ bacteriophage, 229

**R**
Radius of gyration, 42
Resistance, 45, 46, 215–218, 220, 221, 226
Resistance to antifungals, 256, 260
Resveratrol, 98, 102, 103
Rifamycin, 153–155, 158, 159, 163
Ripostatin, 153, 161–163

**S**
*Salmonella*, 223
Saponins, 104, 105
Scattering, 27–29, 31–47
Scattering angle, 38, 41, 43
Scattering length, 37, 39, 40, 43, 44

Scattering length density, 43, 44
Scattering plane, 34
Scattering vector, 34, 35, 38, 43
SENTRY, 216
Single channel conductance, 276
SINQ, PSI, 32
Small-angle neutron scattering (SANS), 43, 44, 46
Small-angle X-ray scattering (SAXS), 41, 42, 46
SNS, ORNL, 32
Sorangicin, 153, 155, 158–159
Spallation sources, 29, 31
*Staphylococcus*, 223
*Staphylococcus aureus*, 46, 216, 217, 219, 221, 223, 224
*Staphylococcus simulans*, 228
*Stenotrophomonas maltophilia*, 216
Stilbenes, 100, 102–103
*Streptococcus agalactiae*, 216, 219
*Streptococcus dysgalactiae*, 216
*Streptococcus equi spp. zooepidemicus* 4881, 229
*Streptococcus pneumoniae*, 216, 219, 226
*Streptococcus pyogenes*, 223, 229
*Streptococcus uberis*, 216
Streptolydigin, 153, 155–157, 163
Streptomyces, 2, 6, 11, 12, 14, 16
Streptomycin, 217
Streptovaricin, 154–155
Synchrotron, 29–31, 42

**T**
Terpenoids, 84, 103–104
Tetracycline hydrochloride, 219
Thionins, 86–88, 90
Thomson scattering, 32
Tigecycline, 219
Tirandamycin, 155–157
Tissues, 29
Transglycosylases, 224
Twort, F. W., 222

**V**
*Vibrio cholerae*, 223
Vacuum permittivity, 33
Vancomycin, 285, 287–294, 296–298, 300–304
Vancomycin-resistant, 216, 217, 225

**W**
Wavelength, 27, 28, 32, 33, 35, 36, 40, 41, 43
Wave-particle duality, 36
Wave vectors, 33, 34, 36
X-rays, 27–30, 32, 35, 36, 40, 41, 43

**Z**
Zoocin A, 229