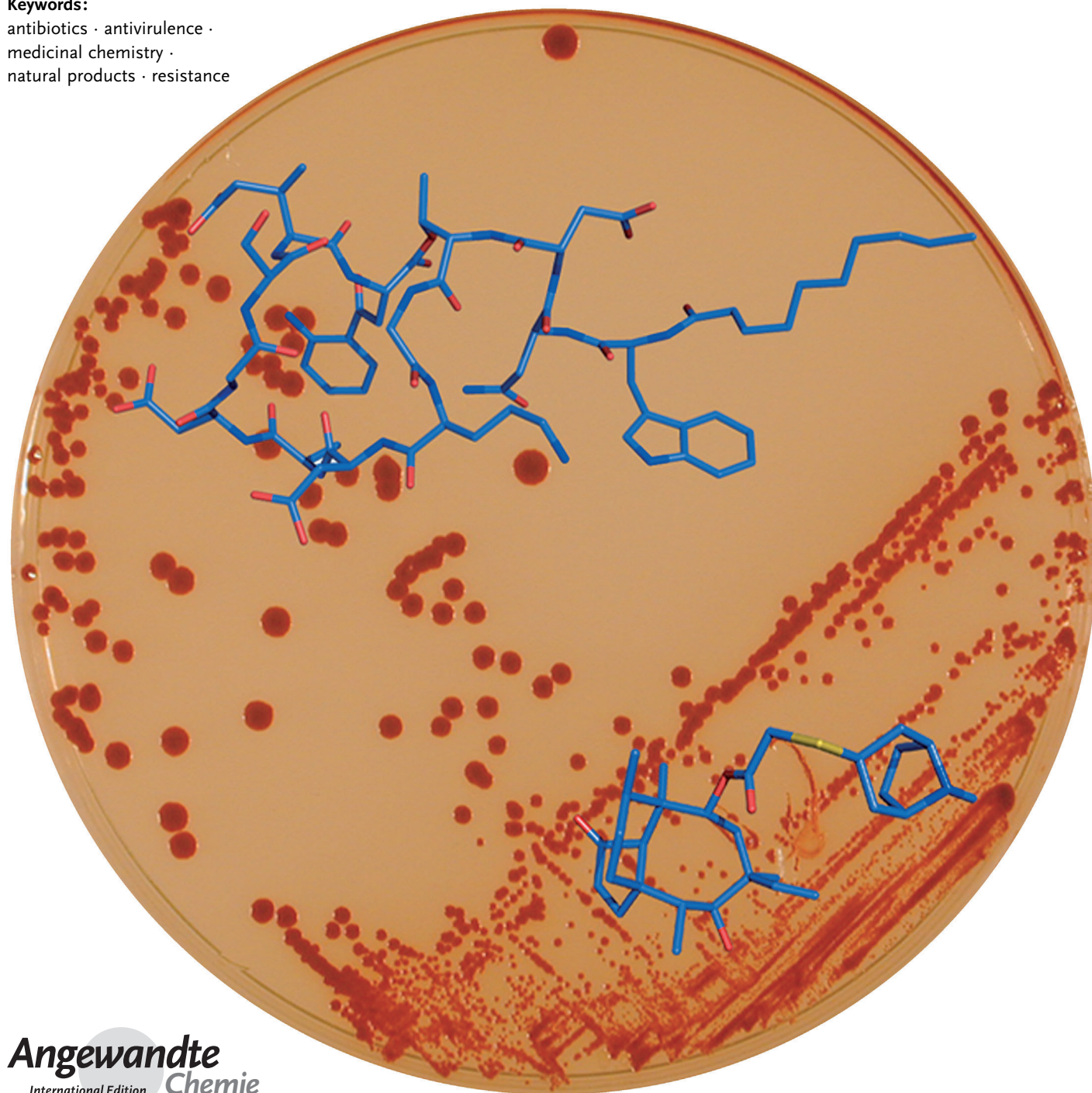# Combating Multidrug-Resistant Bacteria: Current Strategies for the Discovery of Novel Antibacterials

Kieron M. G. O'Connell, James T. Hodgkinson, Hannah F. Sore, Martin Welch, George P. C. Salmond, and David R. Spring

*T*he introduction of effective antibacterial therapies for infectious diseases in the mid-20th century completely revolutionized clinical practices and helped to facilitate the development of modern medicine. Many potentially life-threatening conditions became easily curable, greatly reducing the incidence of death or disability resulting from bacterial infections. This overwhelming historical success makes it very difficult to imagine life without effective antibacterials; however, the inexorable rise of antibiotic resistance has made this a very real and disturbing possibility for some infections. The ruthless selection for resistant bacteria, coupled with insufficient investment in antibacterial research, has led to a steady decline in the efficacy of existing therapies and a paucity of novel structural classes with which to replace them, or complement their use. This situation has resulted in a very pressing need for the discovery of novel antibiotics and treatment strategies, the development of which is likely to be a key challenge to 21st century medicinal chemistry.

From the Contents

1. Introduction and Overview

Lead compounds for antibacterial chemotherapy, as for all chemotherapy, are obtained from two sources: de novo chemical synthesis and natural products. For antibacterials, natural products have historically been by far the more important, with only three clinically used classes having purely synthetic heritage. The remaining classes of antibacterials all have their roots in natural products obtained from microbial sources. One view is that the production of these compounds may have evolved over millennia to enable competitive fitness of their microbial producers at the expense of less competitive organisms; their efficacy in antagonizing bacteria is, therefore, clear. It is, therefore, unsurprising that the isolation and evaluation of these bioactive compounds has proved a very fruitful line of investigation for medicinal chemistry.

The development of antibacterial chemotherapy has its roots in the late 19th century, with the observations of contemporary microbiologists, including eminent scientists such as Robert Koch and Louis Pasteur, about the antagonistic activity between microbial populations, and in Paul Ehrlich's search for a "magic bullet" capable of selective toxicity towards bacterial cells.[1] However, it was not until the mid-20th century and the introduction of the sulfonamides (as chemical therapies) in 1935 and β-lactams (as natural product therapies) in 1940 that antibacterials found widespread use and the modern age of antibacterial treatment began.[1] The introduction of these compounds also heralded the start of a period of intense activity, sometimes termed the "golden age" of antibacterial discovery,[2] and during this period, from the 1940s to the 1960s, the majority of antibacterial drugs in current use were discovered.

There then followed a significant "innovation gap" of almost 40 years, with no new antibacterial scaffolds introduced into clinical practice between the quinolones in 1962 and the oxazolidinone linozolid (Zyvox) in 2000.[3] Most of the antibacterials introduced during the golden age were discovered as a result of screening natural products obtained from microbial fermentations. Therefore, a significant contributory factor to the innovation gap would appear to be that most of the "low-hanging fruit" available from this approach had quickly been picked, thereby leading to subsequent programs rediscovering existing treatments.[4] In addition, the remarkable improvement in antibacterial chemotherapy from the 1930s to the 1960s led to a degree of complacency regarding bacterial diseases, and the availability of many effective therapeutics made the area progressively less appealing from an industrial investment perspective.[5]

Antibacterial research remained an active field in the decades between the golden age and the introduction of linozolid, but the compounds brought into clinical use during this period were modified versions of existing classes rather than novel chemical entities in their own right.

Today the development of novel antibacterials continues to represent an unattractive investment for big pharma.[6] The principal reason for this is the "auto-obsolescence" of antibacterials: if an antibacterial is effective, it rapidly cures the malady that necessitated its use, thus obviating the need for the patient to continue treatment. In contrast, for chronic disorders such as hypertension, therapeutics can be used continuously for decades. The mature nature of antibacterial research, the uncertain lifecycles for new drugs, and the general regulatory environment affecting the pace of translational exploitation, all diminish the commercial impetus for

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investing in antibacterials in a very difficult market place.[7] For these reasons, it may be that the fastest advances in antibacterial research will now come from the academic and small and medium-sized biotechnology laboratories.[8]

In this Review, we discuss briefly the various bacterial processes targeted by antibacterial agents as well the development of resistance mechanisms to these agents. The main focus of this Review is to present an overview of the more recent and significant areas in contemporary antibacterial research. The choice of topics included is intended to cover a broad range of the science being carried out in the antibacterial arena. However, the Review is necessarily not exhaustive, as to comprehensively review such a dauntingly huge field would be an all but impossible task.

Recent developments in "classical" discovery methods are discussed, including the application of modern genetic techniques in the screening of bacterial-derived natural products and examples of the medicinal chemistry campaigns that continue to breathe new life into existing classes of antibacterials. We then go on to present more exploratory approaches including the development of hybrid antibacterials and the potential harnessing of host-defence peptides for therapeutic intervention. Finally, we review the potential of quorum sensing inhibitors as antivirulence agents and as an alternative approach for developing novel antibacterials. Particularly, we focus on the inhibition of quinolone signaling in *Pseudomonas aeruginosa* and autoinducer peptide signaling in *Staphylococcus aureus*, two clinically relevant pathogens commonly found in hospital-acquired infections.

2. Bacterial Targets and Resistance Mechanisms

Antibacterial agents act upon bacteria by targeting essential processes such as inhibiting their cell-wall construction, disrupting the structure and function of their cell membrane, preventing the synthesis of vital proteins or by interfering with the synthesis of RNA or DNA (Table 1).[9] Some of these agents, such as those that inhibit cell-wall construction actively bring about bacterial cell death, and so are termed bactericidal. Other agents, such as the tetracyclines, which inhibit protein synthesis, are referred to as bacteriostatic, as they simply prevent the growth of the bacteria. Some antibacterial agents are only effective against a narrow spectrum of bacteria, for example, the glycopeptides only display activity against Gram-positive organisms, whereas other antibacterials, such as β-lactams, target processes that are common across species and are classified as broad-spectrum antibacterial agents.[9]

Bacteria have evolved a range of protective mechanisms to deactivate, remove, or otherwise circumvent the toxicity of antibacterial compounds, thereby leading to today's multi-drug-resistant organisms.[9b] This is known as acquired resistance, in other words the bacteria have developed resistance mechanisms to an antibacterial agent it had previously been sensitive to. Bacterial resistance can essentially be defined as the continued growth of bacteria in the presence of cytotoxic concentrations of antibacterials.[10] In clinical practice, an organism is resistant to a therapeutic agent if treatment with that agent results in clinical failure at the in vivo concen-

Kieron O'Connell obtained his MChem in Chemistry with a year in Industry from the University of Reading in 2007. He then completed his PhD in 2011 at the University of Cambridge under the supervision of Dr. David Spring. His research focused on the synthesis of diversity-oriented libraries for antibacterial screening along with method development to generate new scaffolds. After a short postdoctoral stay in the Spring group, he joined Peakdale Molecular as a synthetic organic chemist.

Hannah Sore worked as a medicinal chemist at Millennium Pharmaceuticals and Astex Therapeutics, where she developed small-molecule inhibitors against inflammation and oncology biological targets. In 2006 she began a PhD at the University of Cambridge under the supervision of Dr. David Spring. Her studies focused on the development of new silicon-based cross-coupling methods. Upon finishing her PhD she took up a position as a healthcare consultant at Frost & Sullivan. Currently she is a postdoctoral research assistant for Dr. Spring and is working on the development of antibacterials.
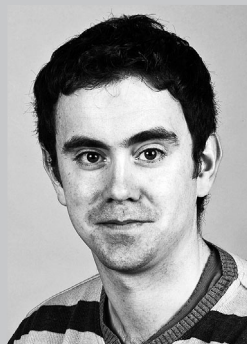
James Hodgkinson obtained his BSc in Chemistry from Queens University Belfast in 2007. He moved to the University of Cambridge for his PhD studies, where he worked at the chemical/biological interface in the area of Quorum Sensing under the supervision of Dr. David Spring and Dr. Martin Welch. Upon completing his PhD in 2011 he became a Junior Research Fellow at Trinity College Cambridge.

Martin Welch studied Biochemistry at Oxford University. In 1989 he started a PhD at the Weizmann Institute of Science. In 1994 he took up a long-term EMBO fellowship and moved to Toulouse, where he solved the X-ray crystal structure of a bacterial chemotaxis protein. In 1997 he returned to the UK, where he carried out studies on bacterial virulence and quorum sensing. After working as a Royal Society University Research Fellow, he took up a University lectureship at Cambridge. His goal is to understand how bacterial lifestyle and interaction with the environment influences microbial physiology.

Table 1: Antibacterial classes and their modes of action.

Antibacterial classes	Mode of action
penicillins, cephalosporins, carbapenems, monobactems, glycopeptides, polypeptides	**cell-wall construction** inhibitors of peptidoglycan synthesis or cross-linking functions resulting in osmotic lysis
lipopeptides, polypeptides	**cell-membrane disruption** altering the structure and function of the cell membrane, thus causing cellular leakage
aminoglycosides, tetracyclines	**protein synthesis inhibitors** binding to the 30S ribosomal subunit, thus preventing translation initiation and tRNA binding
macrolides, oxazolidinones, streptogramins, phenicols	**protein synthesis inhibitors** binding to the 50S ribosomal subunit, thus disrupting translocation and peptidyl transferase activity
rifampin	**RNA synthesis inhibitors** preventing the synthesis of mRNA by binding to DNA-directed RNA polymerase
quinolones	**DNA synthesis inhibitors** prevent DNA replication by binding to topoisomerase IV or DNA gyrase
trimethoprim	**folic acid metabolism inhibitors** preventing the synthesis of nucleotide bases by blocking the synthesis of tetrahydrafolate
sulfonamides	**folic acid metabolism inhibitors** inhibiting nucleic acid synthesis by preventing the synthesis of folate
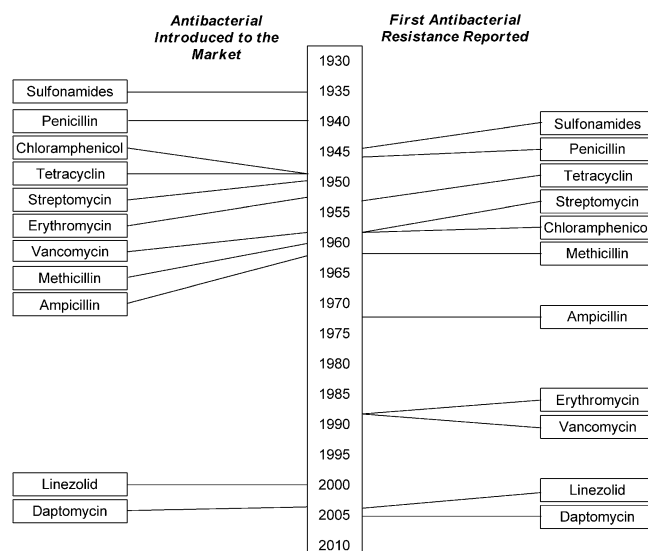
Figure 1. Timeline showing the time between the introduction of an antibacterial and the development of clinically significant resistance.

George Salmond studied microbiology at the universities of Strathclyde and Warwick. His PhD was on bacteriophage–bacterium interactions. He is currently Professor of Molecular Microbiology and Deputy Head of the Department of Biochemistry, University of Cambridge and a Fellow of Wolfson College, Cambridge. Before moving to Cambridge in 1996, he was Professor of Microbiology at the University of Warwick. His research interests cover various areas of molecular microbiology, including quorum sensing, pathogenesis, protein secretion, antibiotics, and other bioactive secondary metabolites.

David Spring is currently an EPSRC Established Career Fellow at the University of Cambridge Chemistry Department. Previous to this, he spent two and a half years as a Wellcome Trust Postdoctoral Fellow and Fulbright Scholar at Harvard University with Prof. Stuart L. Schreiber. He gained his DPhil for work on the proposed biosynthesis of the manzamine alkaloids at Oxford University under the supervision of Prof. Sir Jack E. Baldwin FRS. His research program is focused on diversity-oriented synthesis and the use of small molecules to probe biological processes.

tration achieved.[10] As such, the limiting factor can often be the bioavailability of the therapeutic agent. Figure 1 shows an approximate timeline for the introduction of some major antibacterials and the subsequent emergence of clinically significant resistance.
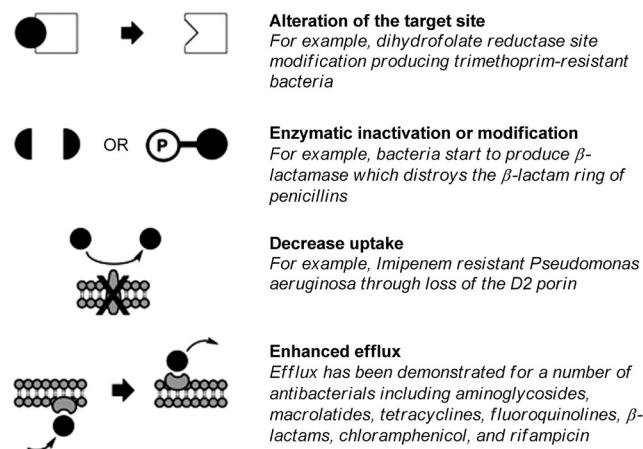
Genetic resistance arises in one of two ways, either as a result of a chromosomal mutation or, more commonly, through the acquisition of an antibiotic resistance gene from another bacteria via mobile plasmids or transposons.[9b,11] This horizontal gene transfer between bacterial populations is the primary reason for the spread of antibacterial resistance. Genetic resistance mechanisms include (Figure 2): the modification or over-expression of drug targets;[12] controlling cellular antibacterial concentrations (by either the expression of efflux pumps or by mechanisms that reduce influx);[13] and the expression of enzymes that can deactivate antibacterials.[14] This impressive array of protective mechanisms, coupled with inadequate investment in antibacterial research,

Alteration of the target site
For example, dihydrofolate reductase site modification producing trimethoprim-resistant bacteria

Enzymatic inactivation or modification
For example, bacteria start to produce β-lactamase which distroys the β-lactam ring of penicillins

Decrease uptake
For example, Imipenem resistant Pseudomonas aeruginosa through loss of the D2 porin

Enhanced efflux
Efflux has been demonstrated for a number of antibacterials including aminoglycosides, macrolatides, tetracyclines, fluoroquinolines, β-lactams, chloramphenicol, and rifampicin

Figure 2. Genetic resistance mechanisms that bacteria develop.

has been termed the "perfect storm" of antibacterial resistance.[15]

We tend to think of resistance as a relatively modern development, brought about by the selective pressure applied to bacteria by the clinical use of antibacterials over the last 70 years. However, this is a misconception, because there is evidence that resistance mechanisms existed in bacteria many years prior to our exploitation of antimicrobials as therapeutic agents in the clinic.[10]

A recent study involving the metagenomic analysis of 30 000 year-old DNA found in Beringian permafrost sediments discovered genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibacterials.[16] This study has shown beyond doubt that these resistance mechanisms are at least 30 000 years old, but, in all likelihood, they are considerably older still. Bacteria are thought to have originated over 3.8 billion years ago and, based on the genetic divergence of antibacterial gene clusters, antibacterial-producing bacteria at least hundreds of millions of years ago.[17] As antibacterial-producing bacteria must coevolve resistance mechanisms for self-preservation if they also possess the target for the drug produced, it is reasonable to assume that resistance mechanisms have existed for just as long. The ability of bacteria to transfer these resistance genes horizontally through populations, coupled with the selective pressure applied by the clinical use of antibacterials, has led, in recent years, to the development of multidrug-resistant pathogens.

Therefore, an unfortunate drawback to using natural products as clinical antibacterials is that, while production of these bioactive compounds has evolved over millennia, continuously optimizing activity, the bacteria have had an equally long time to develop resistance to them. At first sight, this might suggest that future antibacterial discovery should be refocused towards non-natural compounds. However, bacteria have proved equally adept at developing resistance to purely synthetic agents, such as the sulfonamides and linezolid. Indeed, clinically significant resistance has developed to every antibacterial ever used, sometimes only a few years after their introduction.[18]

The generation of new antibacterials alone is not sufficient to combat the issue of multidrug resistance. More needs to be done to alter the behavior and use of antibacterials to control or even reduce the extent of resistance to existing agents. The level of resistance to Gram-negative pathogens increased across Europe in 2011 according to the European Centre for Disease Prevention and Control annual surveillance report.[19] However, resistance to Gram-positive pathogens was found to be stable, if not improving, in some European countries. For the pathogens monitored, a north-south divide was typically seen, with lower levels of resistance observed for northern European countries compared to the south.[19] The differences reflect the policies that have been developed and adopted in the north, such as the prudent use of antibacterials in the clinic (for example, not always prescribing for viral infections) and throughout agriculture, as well as extensive measures for infection control.[19]

It is clear that there can be no single answer to the problem of countering resistance to antibacterials and that no antibacterial will remain efficacious forever. However, any research that leads to the discovery of novel antibacterials or aids our understanding of resistance mechanisms (and how to overcome them) will prove critically important in a future with the looming specter of widespread multidrug-resistant bacterial infections that are effectively untreatable. Therefore, the urgent need for new antibacterial research is both critical and undeniable.

3. New Antibacterials from Natural Products

Historically, the most successful means of antibacterial discovery has been the screening of natural products obtained from microbial sources. Although many of the most easily accessible compounds have been discovered, there is still value in this approach, as indicated by the on-going research efforts in this area.[20] In fact, according to current thinking, only a tiny fraction of the potentially relevant natural product chemical space has been explored.

3.1. Delving to New Depths To Bring the Next Generation of Natural Products

The majority of clinically used antibacterials from natural products have been discovered from soil actinomycetes, thus reflecting a historical bias towards this class of easily obtainable and culturable bacteria. A result of this bias is that a considerable amount of microbial flora, such as that found in "unconventional" ecological niches, has not yet been evaluated systematically for the ability to produce potentially useful antibacterial compounds. One subgroup reported to have particular promise for novel bioactive production is marine bacteria. In 2004, Süssmuth and co-workers identified the abyssomicins (Figure 3), an interesting new class of
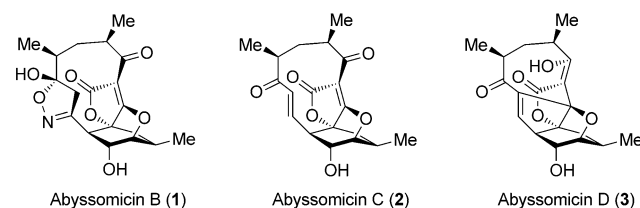
Figure 3. Abyssomicins; a new class of natural product antibacterials from deep-sea sediment. Scientists are now exploring less accessible areas of the globe in the search for the next generation of natural product antibacterials.

antibacterials, from an actinomycete found in deep-sea sediment samples.[21] Abyssomicin C (**2**) shows promising activity against Gram-positive bacteria such as methicillin- and vancomycin-resistant strains of *Staphylococcus aureus* with MIC$_{50}$ values (MIC = minimal inhibitory concentration) of 4 µg mL^{-1} and 13 µg mL^{-1}, respectively.[21] It also displays an unprecedented mode of action. Abyssomicin C acts by preventing the conversion of chorismate into *p*-aminobenzoic acid (*p*ABA), a precursor in tetrahydrofolate biosynthesis.

Inhibiting *p*ABA biosynthesis is an attractive target for antibacterials, as it is found in many microorganisms but not in humans.[22]

3.2. Culturing the "Uncultured"

Even if efforts are limited to easily accessible soil bacteria, it is estimated that the number of species successfully cultured and evaluated from soil represent less than one percent of the total population.[23] This is because many microorganisms are unculturable under standard laboratory conditions and, therefore, were historically unavailable for investigation.[24] The existence of these "uncultured" bacteria has been acknowledged in the microbiology community for over a century; the large discrepancy between the total microscopically determined cell count and the viable plate count is known as the Great Plate Count Anomaly.[25] Clearly, this anomaly has greatly limited the evaluation of bacterial populations for their ability to produce antibacterials. However, modern biological and genetic techniques can go some way to circumvent this difficulty and could ultimately make it possible to evaluate the entire microbiological genome.

There are several possible ways to approach this problem. The first is to address the methods of growing uncultured bacteria by developing nontraditional technologies for cultivation. At its simplest, this research can involve varying incubation times of samples, temperatures of cultivation, or the nutrient composition of media, but more sophisticated techniques that are intended to mimic the natural environment of the bacteria are also being explored.[26] One such method, the use of diffusion chambers for incubation, has been used by Novobiotic Pharmaceuticals to discover several antibacterial agents.[27] These diffusion chambers are effective because the growth of many strains of bacteria is dependent on the presence of growth factors produced by other microorganisms within their environment. By allowing the diffusion of these growth factors into the chambers, they are able to mimic the molecular environment of a multispecies community, while allowing a single strain to grow in isolation within the chamber.

Another approach with huge potential in this area is the heterologous expression of antibacterial biosynthetic genes in a readily culturable host.[28] The success of this approach can require that the antibacterial biosynthesis genes and the intrinsic resistance genes are located on a contiguous section of DNA. Fortunately, this clustering of biosynthetic and resistance genes is often the case for antibacterial natural products. Additional difficulties may be encountered if there is a difference in codon usage between the host and donor organisms (leading to problems with gene expression),[29] or where biosynthetic proteins may require posttranslational modification for activation, as for some polyketide and nonribosomal peptide biosyntheses.[30]

Despite these difficulties, successful examples of the expression of drug candidates from a genetically engineered host do exist, such as the successful expression and subsequent identification of the pantocin antibacterials (Figure 4). Pantocin A and pantocin B act by inhibiting histidine and
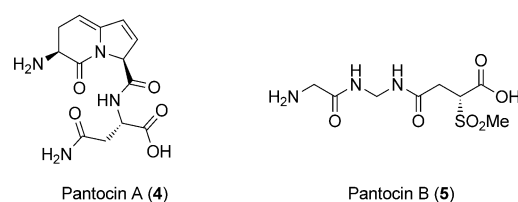

Figure 4. Pantocin A and B; a new class of natural product antibacterial generated from a genetically engineered host.

arginine biosynthesis, respectively. They are naturally produced by *Pantoea agglomerans*. It has been known since the 1980s that this species can produce antibiotics; however, it was not until their biosynthetic genes were expressed by recombinant DNA methods in *Escherichia coli* that the compounds could be positively identified.[31]

3.3. Metagenomics

Heterologous techniques are not limited to the expression of the DNA from single colonies of specific bacteria; they can be used also to evaluate environmental DNA (sometimes known as eDNA) samples for the production of natural products.[32] The direct extraction and analysis of DNA from the entire microbial population within an environmental sample is called metagenomics.[33] The main potential benefit of metagenomic approaches is that they circumvent the necessity for the laboratory cultivation of bacterial strains to evaluate their ability to produce antibacterials.

Metagenomics involves the extraction of DNA from every microorganism within an environmental sample, subsequent purification, and insertion into vectors such as plasmids, cosmids, or bacterial artificial chromosomes (BACS), then propagation of the DNA in hosts such as *E. coli*.[33,34] The resulting complex DNA library can then be screened functionally to test for the production of novel antibacterials or other biologically interesting secondary metabolites.[33,34] Another advantage of this approach is that the discovery of novel antibacterials is intrinsically coupled to the discovery of their biosynthetic genes.

Several examples of the successful discovery and identification of novel antibacterials by using metagenomics have been reported, and it is very likely that more will follow as the field matures and techniques become more sophisticated. The first reported incidence of antibacterial discovery from the heterologous expression of environmental DNA came in 2000 when Brady and Clardy reported the discovery of long-chain saturated and unsaturated *N*-acyl-L-tyrosines CSL12-A to -M (**6**, Figure 5a).[35] These 13 compounds were discovered as a result of screening around 700 000 clones obtained from a soil sample, and were shown to have activity against a drug-resistant strain of the Gram-positive bacterium *Bacillus subtilis*. The extent of antibacterial activity was dependent on the chain length, with C_{13}–C_{16} saturated and unsaturated acetyl derivatives being the most active. Five years later, in 2005, the same authors discovered the isocyanide-indole compound **7**, which also exhibited activity against *B. subtilis*
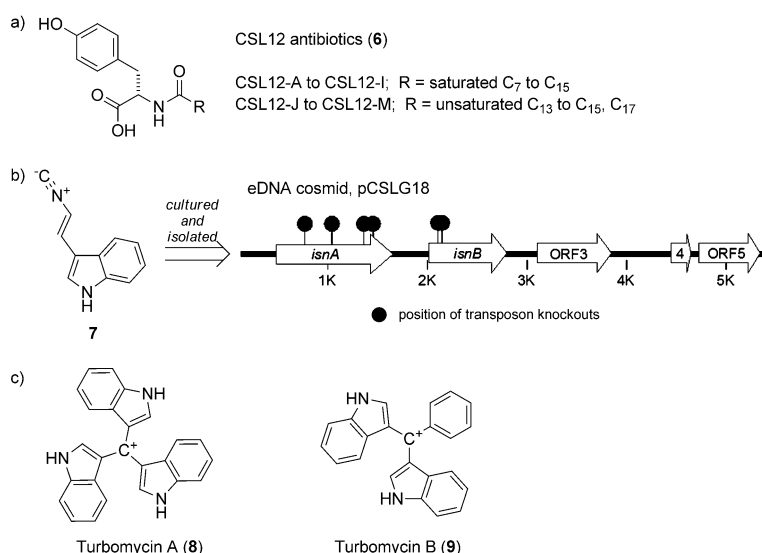
Figure 5. Antibacterials discovered by using metagenomic screening techniques. A) CSL12-G to CSL12-I and CSL12-J to CSL12-L displayed potent antibacterial activity against *B. subtilis*. B) **7** exhibited antibacterial activity against *B. subtilis*. C) The MIC_{50} value was 6.2 $\mu g\,mL^{-1}$ for turbomycin A against *E. herbicola*, *B. subtilis*, *S. aureus*, and *S. pyogenes*, and 12.5 $\mu g\,mL^{-1}$ against *S. enerica* ssp 1 serovar Typhimurium.

(Figure 5b).[36] Compound **7** was derived from the eDNA cosmid pCSLG18, which contains the biosynthetic genes *isn*A/B.[36] Another example is the discovery of the antibacterials turbomycin A and B (**8** and **9**; Figure 5c) by Handelsman and co-workers.[37] These cationic natural products have activity against a range of Gram-positive and Gram-negative bacteria including: *Erwinia herbicola*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. Interestingly, turbomycin A had previously been obtained from the yeast *Saccharomyces cerevisiae*, but never before as a bacterial metabolite; turbomycin B is a novel natural product.[38] These early indications suggested that metagenomics may be a very promising field for antibacterial discovery.

It is likely that considerable progress can still to be made in both culture-dependent and culture-independent methods for the discovery of antibacterials, and that many biologically interesting metabolites remain to be uncovered. In addition to the discovery of new antibacterials, metagenomics is also being used to provide insights into mechanisms of bacterial resistance.[39] For these reasons, antibacterial discovery from natural products will continue to be an important field for the foreseeable future.

3.4. The Progression of Lantibiotics

Lantibiotics, a subclass of bacteriocins, are peptidic toxins produced by bacteria to inhibit the growth of closely related bacteria.[40] They are ribosomally synthesized antibacterials that have a high degree of posttranslational modification that leads to the formation of multiple macrocyclic rings within their 3D structure. The macrocycles are generated from a linear peptide sequence by formation of lanthionine,

methyllanthionine, and/or labionin bridges through a process of dehydration and cyclization typically involving cysteine and serine/theronine residues (Figure 6a).[40c, 41] Jung and co-workers were the first to report on the genetic source for the biosynthsis of epidermin.[42] They demonstrated that the structural gene *epiA* coded for the antibiotic epidermin, a ribosomally synthesized lantibiotic with four sulfide-fused macrocyclic rings. Since this report, a significant number of novel lantibiotic structures have been discovered, isolated, and reported including more recently; actagardine,[43] deoxyactagardine B (DAB),[44] entianin,[45] curvopeptin,[46] prochlorosins,[47] catenulipeptin,[41] erythreapeptin,[48] avermipeptin,[48] griseopeptin,[48] and labyrinthopeptins.[49] Lantibiotics have the potential to offer a novel source of antibacterial agents in the future. Actagardine (**10**; Figure 6b), for example, demonstrated potent antibacterial properties against Gram-positive bacteria such as *Clostridium difficile* by binding to lipid II, thereby disrupting cell biosynthesis.[43b] The structurally similar lantibiotic
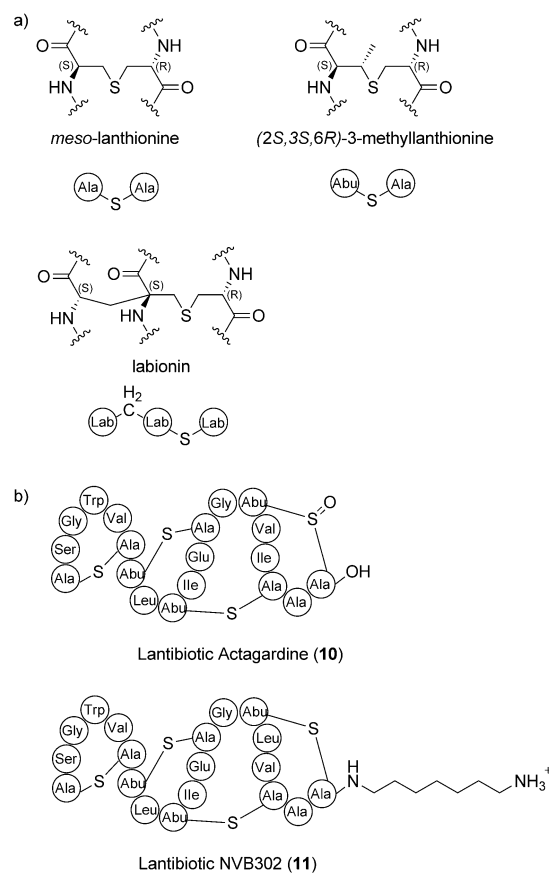

Figure 6. Lantibiotics structures. A) Common bridges found in lantibiotics established during posttranslational modification to generate the globular 3D structure. B) Structurally similar lantibiotics actagardine (**10**) and NVB302 (**11**) both show significant antibacterial activity, and NVB302 is currently in clinical trials for the first-line treatment of *C. difficile*.

NVB302 (**11**, Figure 6b) discovered by Novacta is currently progressing through Phase 1 clinical trials for the treatment of *Clostridium difficile* infections.[43b, 50]

4. Modification of Existing Classes

A significant proportion of the anti-infective research being carried out today is still focused on medicinal chemistry efforts to improve the activity or profile of existing classes.[51] These efforts are showing that, despite many of the classes being in use for over 50 years, with the correct alterations the next generation can still prove efficacious. Making logical and systematic changes to these compounds can increase potency and activity spectrum and counter bacterial resistance mechanisms.

4.1. Developing the Next Generation of Cephalosporins

Figure 7. Different generations of cephalosporins.

One class of antibacterials that has been subjected to extensive medicinal chemistry exploration is the cephalosporin class of β-lactam antibacterials. β-Lactams inhibit the transpeptidation of neighboring pentapeptides, thereby preventing the formation of cross-links between the peptide–glycan units which are essential for cell-wall biosynthesis.[52] The β-lactams bind to the enzymes known as penicillin-binding proteins (PBPs) through acylation of the β-lactam amide bond, with the nucleophilic serine residue in the active site. This acyl enzyme is stable, thereby preventing binding of the natural D-ala-D-ala section of the pentapeptide.[52] The broad activity spectrum, proven efficacy, and favorable safety profile of this class has made it one of the most widely prescribed clinical classes. There are now at least four recognized generations of the cephalosporins (Figure 7), which are differentiated by their efficacy and activity spectrums rather than by structural similarity.[53] The existence of several generations might imply that the earliest compounds to be approved are now obsolete; however, this is not the case and examples of each of the classes are still in clinical use today.

First-generation cephalosporins have activity against Gram-positive cocci; second-generation compounds maintain this activity and also display activity against Gram-negative organisms; third-generation compounds have decreased activity against Gram-positive organisms but an increased Gram-negative activity profile. Fourth-generation cephalosporins show an increased activity profile against both Gram-postive and Gram-negative organisms and also show activity against strains that produce some β-lactamase enzymes.

Recently, cephalosporins displaying useful activity against methicillin-resistant *Staphylococcus aureus* (MRSA) have been identified and these compounds display an improved profile against Gram-positive bacteria, but, as they display no great improvemen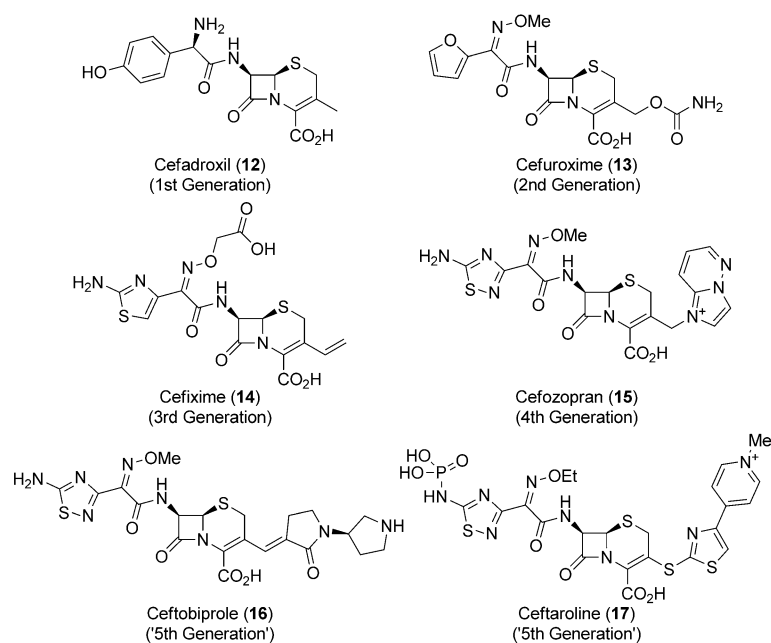t against Gram-negative organisms, there is some debate as to whether they represent a new generation.[54] Despite this, the anti-MRSA compounds ceftobiprole (**16**)[55] and ceftaroline (**17**)[54,56] are often referred to as fifth-generation cephalosporins.

More important than the presence of β-lactamase enzymes, the insensitivity of MRSA to many β-lactam antibacterials is attributed to the presence of penicillin-binding protein 2a (PBP2a), which is not found in methicillin-susceptible strains (Figure 8).[57] There are several PBPs found in *Staphylococcus aureus,* the majority of which are susceptible to β-lactams; however, PBP2a has very low affinity for β-lactams and so is unaffected by most antibacterials in the class. Consequently, even when the other PBPs are effectively inhibited, PBP2a can continue to mediate cell-wall biosynthesis, thus leading to high-level β-lactam resistance.

Ceftobiprole is a broad-spectrum antibacterial and is effective for the treatment of skin and soft tissue infections arising from MRSA (and shows activity against *Streptococcus pneumoniae*). The compound shows high potency against MRSA, with an MIC_{50} value of 2 µg mL^{-1} compared to the ineffective third-generation cephalosporin cefotaxime, which has an MIC_{50} value of > 64 µg mL^{-1}.[55b] Ceftobiprole monotherapy has been shown to display equally effective activity against MRSA skin infections as vancomycin and ceftazidime (a third-generation cephalosporin) combination therapy.[58] Strynadka and co-workers succeeded in obtaining a crystal structure of ceftobiprole bound to the PBP2a active site, thus providing insight into the inhibition mechanism of ceftobiprole.[59] The elongation, the planarity, and hydrophobic nature of R^2 were all essential for effective binding to the narrow cleft of the active site (Figure 9). The modification of the active site is compensated by additional interactions such as van der Waals contacts between the enzyme and R^2, thereby leading to the formation of a stabilized complex

Methicillin Susceptible Strains
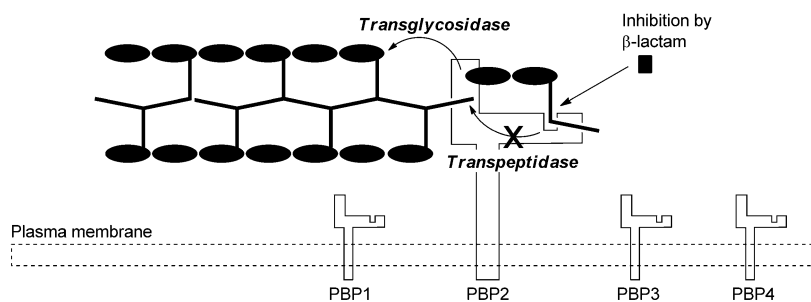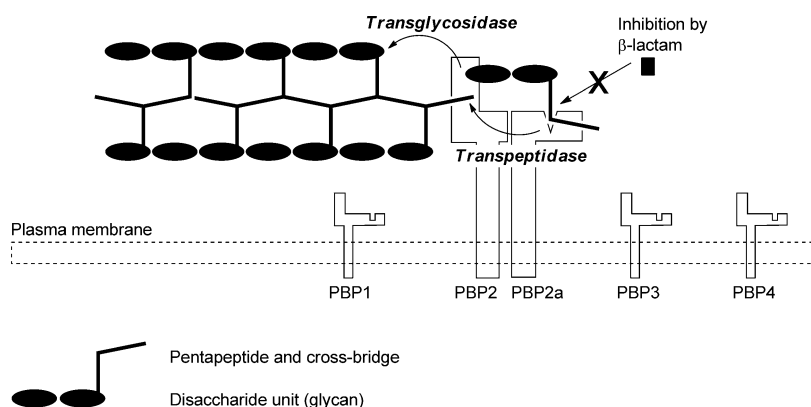

Methicillin Resistant Strains

Figure 8. Synthesis of the bacterial cell wall of methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA). In MSSA, β-lactam antibacterials bind to PBP2, thereby preventing the transpeptidation of the pentapeptide to the peptide–glycan complex. In MRSA, the PBP2a site has a different active site that is less susceptible to β-lactams, thus leading to resistance to the majority of β-lactams.
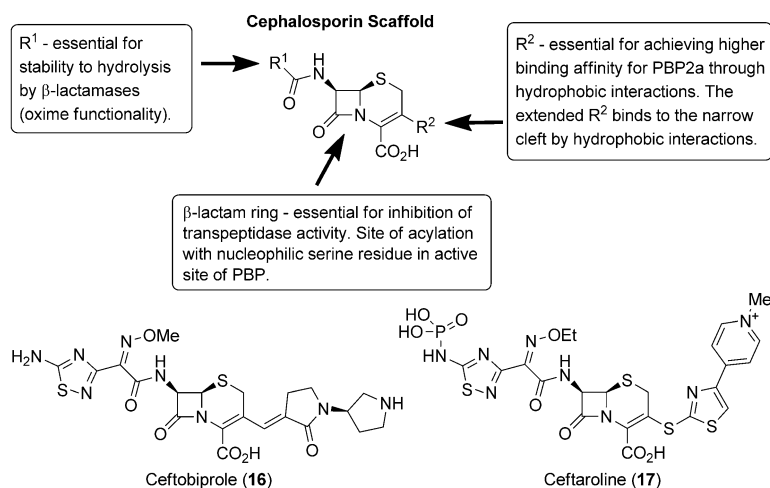

Figure 9. Key structural aspects of cephalosporins for achieving activity against bacteria with β-lactamases and PBP2a binding sites.

followed by acylation. The anti-MRSA activity of ceftobiprole and ceftaroline is due to their increased binding affinity, increased rate of acylation, and lower deacylation rate to PBP2a.[59,60]

Ceftaroline displays a similar activity profile as ceftobiprole and was approved in 2010 for the treatment of acute bacterial skin infections and community-acquired bacterial pneumonia. Ceftaroline displays potent antibacterial activity against a number of strains of *S. aureus*, including *S. aureus* 510 (VRS2), which is methicillin-resistant, vancomycin-resistant, and has β-lactamase activity.[57b] Ceftaroline had a vastly superior activity against *S. aureus* 510 (VRS2), with an MIC value of 1 μg mL^{-1} when compared to the other β-lactams penicillin G, ceftriaxone, and cefotaxime, which had activities of 32, > 64, and > 64 μg mL^{-1}, respectively. The mechanism by which ceftaroline is able to bind to PBP2a was investigated by Mobashery and co-workers.[61] Using X-ray crystallography, they discovered that the binding site of free PBP2a is closed and only becomes accessible once the enzyme has been allosterically activated. Further experiments indicated that ceftaroline can achieve this allosteric activation and, therefore, gain access to the binding site through its elongated R^2 substituent.[61]

4.2. Overcoming Vancomycin Resistance

Vancomycin (**18**, Figure 10) provides another interesting illustration of the ability that medicinal chemistry can have to potentiate activity and counter resistance. Scientists at Eli Lilly discovered vancomycin from microbes in soil samples taken from the jungle in Borneo in the 1950s, and it quickly became the first glycopeptide antibacterial to be introduced to the clinic in 1959.[62] Since then, glycopeptides have proved highly effective for the parenteral treatment of Gram-positive infections. They lack activity against Gram-negative bacteria because their size prevents them from penetrating their outer membrane.

As a consequence of their toxicity and the absence of cross-resistance with other antibacterials, glycopeptides have been used as antibacterials of last resort for multidrug-resistant infections. For some time, resistance to vancomycin was slow to develop. However, as infections such as MRSA have become more prevalent, vancomycin use has increased, and has led to an increase in resistant organisms. High-level resistance to vancomycin was first reported in 1988, around 30 years after its first clinical use. The first microorganisms to display resistance were enterococci and these vancomycin-resistant enterococci (VRE) were first reported in European hospitals, but they have since spread and are now encountered worldwide.[63] The resistance
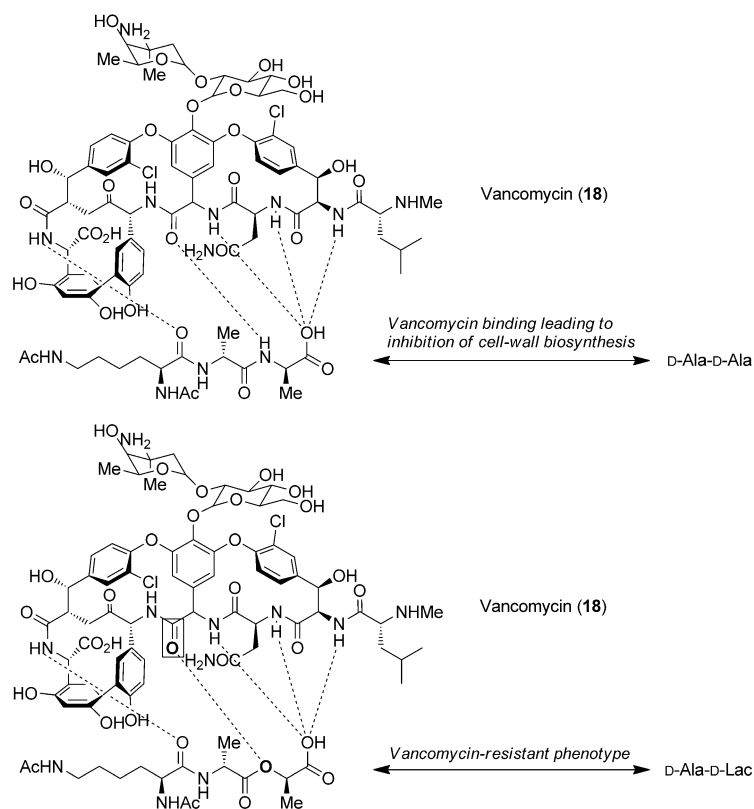
Figure 10. Vancomycin resistance resulting from the substitution of D-Ala-D-Ala for D-Ala-D-Lac.

displayed by VRE may be transferred horizontally, and other vancomycin-resistant pathogens have now emerged, including some staphylococci.

Glycopeptides inhibit cell-wall biosynthesis, but they do so with an unusual mode of action, in that they do not bind to an enzymatic target to modulate its function. Instead, they bind tightly to the L-Lys-D-Ala-D-Ala residues at the termini of peptidoglycan strands and so act to sequester these vital precursor units. Once bound to the peptidoglycan, the antibacterial physically prevents transpeptidation and transglycosylation, thereby impeding the maturation of the cell wall and eventually causing cell lysis. This glycopeptide mode of action is at the cell surface and avoids some common cellular resistance mechanisms such as efflux pumps and bacterial modification. Vancomycin has been shown to bind to the L-Lys-D-Ala-D-Ala residues by five specific hydrogen-bonding interactions, shown in Figure 10.[64]

The most common resistance phenotypes encountered (VanA and VanB) result from the substitution of the terminal alanine residue of the peptidoglycan chains for a lactic acid moiety (D-Ala-D-Ala to D-Ala-D-Lac).[65] This single substitution (NH to O) removes one hydrogen-bond interaction and replaces it with a destabilizing interaction between lone pairs of electrons, thereby resulting in a 1000-fold reduction in the binding affinity between the antibacterial and ligand.[66]

Boger and co-workers have carried out a large body of work aimed at exploring these interactions and restoring antibacterial activity.[67] By using an impressive strategy based on the de novo synthesis of a number of vancomycin aglycon

analogues, they were able to probe the effects of making small structural changes to the binding pocket of vancomycin. Their efforts focused on altering the carbonyl group at the C14-position, in the hope of creating a compound with comparable activity against both D-Ala-D-Ala and D-Ala-D-Lac peptidoglycan strands (Figure 11).

The first change that they explored was the removal of the carbonyl oxygen atom to leave a methylene group in its place (**19**), so removing one potential hydrogen bond from the D-Ala-D-Ala interaction and the repulsive interaction between the lone pairs of electrons in the case of the D-Ala-D-Lac interaction.[68] This deoxygenated analogue displayed a 40-fold increase in binding affinity for a model D-Ala-D-Lac ligand and a 5-fold reduction in affinity for the corresponding D-Ala-D-Ala ligand, thus resulting in relatively balanced binding characteristics for both variants. It also displayed a 40-fold increase in potency against a VanA-resistant strain of *Enterococcus faecalis* (VanA VRE BM4166) compared to vancomycin, with an MIC_{50} value of 31 $\mu g\,mL^{-1}$.

Replacing the amide functionality with an amidine group (**20**) resulted in significantly improved binding characteristics for both ligands.[69] The binding affinity of **20** for D-Ala-D-Ala was 15-fold greater than for methylene compound **19** and
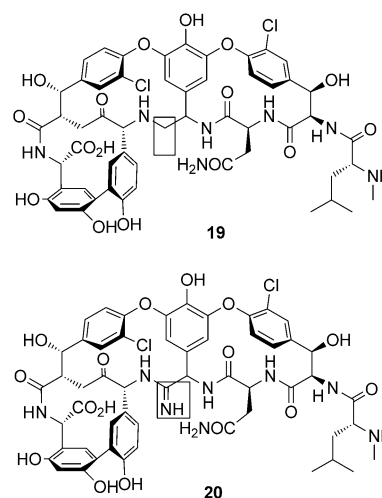
Figure 11. Vancomycin aglycon analogues synthesized by Boger and co-workers.

only around 2-fold less than vancomycin aglycon itself, which suggests that the amidine group can function as a hydrogen-bond acceptor for the amide group in the ligand and so act as an isostere for the amide group of vancomycin. The results for D-Ala-D-Lac were even more impressive, with **20** displaying comparable binding affinity to that of the D-Ala-D-Ala ligand, which corresponds to a 600-fold increase compared to vancomycin aglycon and an over 10-fold improvement

compared to **19**. The amidine compound **20** also displayed very potent antimicrobial activity against VRE BM4166 with an MIC_{50} value of 0.31 μg mL^{-1}, which is comparable to the activity displayed by vancomycin and vancomycin aglycon against susceptible strains ($MIC_{50} = 0.3$–2 μg mL^{-1}).

Rather than relying on time-consuming and laborious total synthesis to produce analogues, the majority of medicinal chemistry campaigns around antibacterial scaffolds use fermentation techniques to obtain the natural products before modifying them synthetically. The compounds produced by these methods are more likely to vary in their peripheral features than the core scaffold, as is the case for Boger's studies.

Semisynthetic medicinal chemistry efforts on the glycopeptides have focused on introducing structural elements that either promote dimerization of the antibacterial or improve affinity for the bacterial cell membrane (Figure 12). The dimerization of vancomycin improves ligand binding by rigidifying the peptide backbone, which leads to improved hydrogen-bonding interactions.[70] Hydrophobic side chains, such as those found in the naturally occurring glycopeptide teicoplanin (**21**), serve to anchor the antibacterial within the phospholipid bilayer of the cell membrane, thereby bringing it into proximity with its peptidoglycan target and so favoring binding interactions. Both dimerization and cell-membrane anchoring greatly enhance their activity.[71]

Three semisynthetic glycopeptides have progressed to clinical trials: oritavancin (**22**), dalbavancin (**23**), and telavancin (**24**; Figure 12). Oritavancin is the 4-chlorobiphenylmethyl analogue of chloroeremomycin, a glycopeptide produced by *Amycolatopsis orientalis*. Chloroeremomycin is 4 to 8 times more active than vancomycin against susceptible strains, but displays insignificant activity against vancomycin-resistant strains.[72] However, the addition of the hydrophobic 4-chlorobiphenylmethyl substituent of oritavancin confers a clinically significant increase in activity against vancomycin-resistant enterococci.[73] The addition of this substituent has a profound effect on the ability of oritavancin to form dimers. The dimerization constant of oritavancin is 100 times greater than that of chloroeremomycin and four orders of magnitude higher than vancomycin.[74] The biphenyl substituent also serves to improve the membrane-anchoring ability of oritavancin. In combination, these two effects (which both lead to intramolecular ligand binding) appear to override the loss of binding efficacy encountered with the substitution of D-Ala-D-Ala for D-Ala-D-Lac, and consequently bestow useful activity against VanA-resistant organisms.

Dalbavancin (**23**) is a close analogue of teicoplanin, and displays a similar activity profile. It shows activity against many Gram-positive bacteria and is generally slightly more
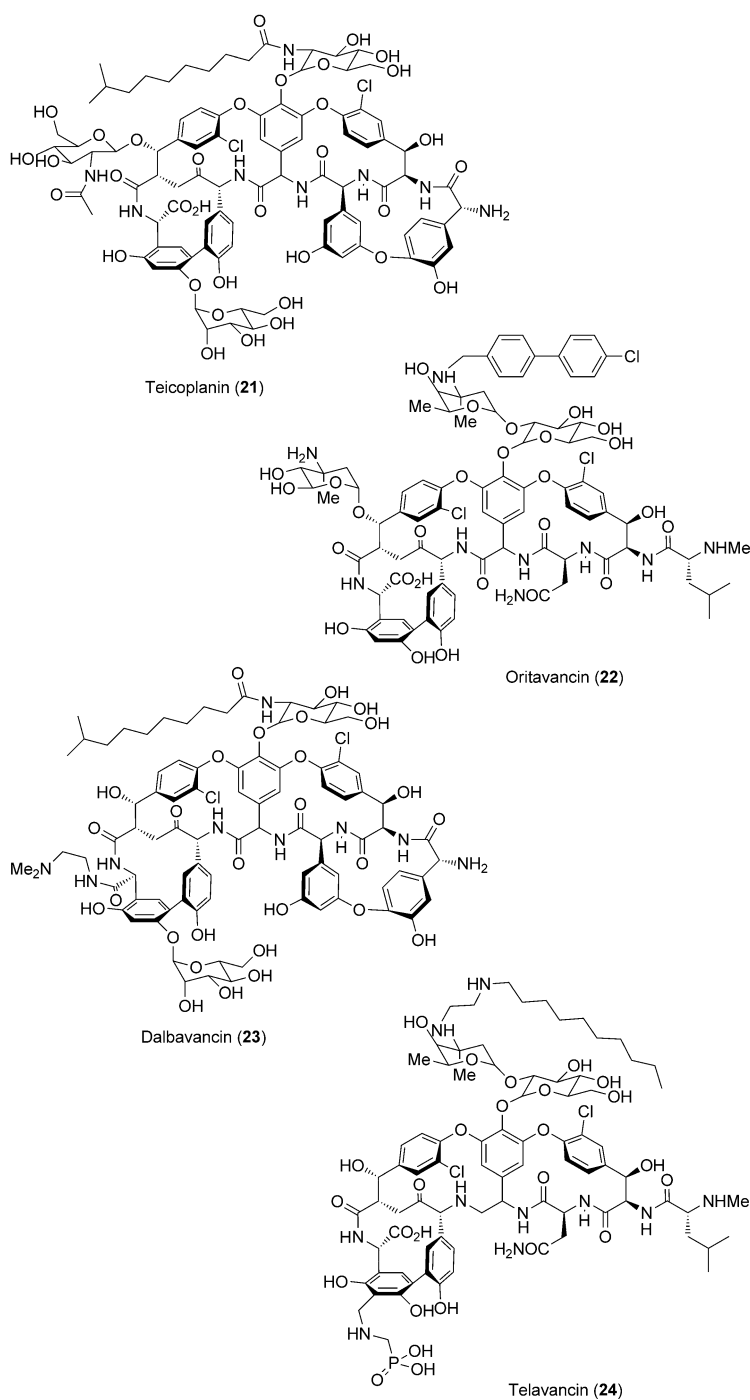
Figure 12. Semisynthetic glycopeptides.

potent than vancomycin; however, it lacks activity against VanA-resistant strains.[75] The other useful characteristic of dalbavancin is that it exhibits a uniquely long half-life of 170–210 h, thus making once weekly dosing a possibility.[76]

Telavancin (**24**) is the youngest of the semisynthetic variants and the first to be approved for clinical use. It was approved by the FDA for the treatment of complicated skin and skin-structure infections (cSSSIs) in 2009. Telavancin was one of several semisynthetic vancomycin analogues explored by chemists at Theravance, and provides a nice illustration of

the application of principles of medicinal chemistry to glycopeptide antibacterials.[77] It was found that, as expected, the hydrophobic *N*-decylaminoethyl group conveyed increased potency and favorable in vitro activity against vancomycin-resistant strains. However, it negatively influenced pharmacokinetic factors, producing poor absorption, distribution, and excretion properties in animal models. It was then necessary to append polar functionality in the form of a phosphonate group to counterbalance the hydrophobic substituent; this had the desired effect of improving the pharmacokinetics while maintaining potency.

Telavancin has been described as a multifunctional lipoglycopeptide and its potency has been attributed to a dual mode of action.[78] It maintains the common glycopeptide mechanism of inhibiting cell-wall biosynthesis, but additionally appears to be able to disrupt the integrity of the cell wall, thus leading to depolarization, leakage, and cell death. It is hoped that this dual mode of action may have the effect of slowing the development of bacterial resistance to telavancin.

5. Hybrid Antimicrobials

The fusing of two antimicrobial agents with distinct modes of action to produce a single bifunctional entity is a concept that has been around for several decades.[8a] The rationale behind the production of such compounds is that they may display an extended spectrum of activity over their constituent agents, including against resistant organisms, and, with their dual mode of action, be slower at driving the emergence of bacterial resistance. The major difficulty associated with this strategy is that, for it to be deemed successful, the hybrid agents must prove to be more effective than the sum of their parts.

Historically, this approach has not been overwhelmingly successful, usually because the hybrid agent offered no significant advantage over the two individual agents. This was the case for the quinalactams (fluoroquinolone–cephalosporin hybrids),[79] and rifamycin–fluoroquinolone analogues.[80] Both of these research programmes have now been abandoned.[8a] Despite this, research in this area continues, and one of the more promising lines of enquiry is the investigation of aminouracil-fluoroquinolone hybrids.

Derivatives of 6-(3-ethyl-4-methylanilino)uracil (EMAU) display moderate activity against a range of Gram-positive organisms, as a result of their ability to disrupt DNA synthesis by inhibiting DNA polymerase IIIC (Pol IIIC).[81] The fluoroquinolone (FQ) antibacterials are effective against both Gram-positive and Gram-negative species, and act by inhibiting bacterial topoisomerases and gyrases, and so affect the "winding up" of DNA. Butler et al. from Microbiotix under-
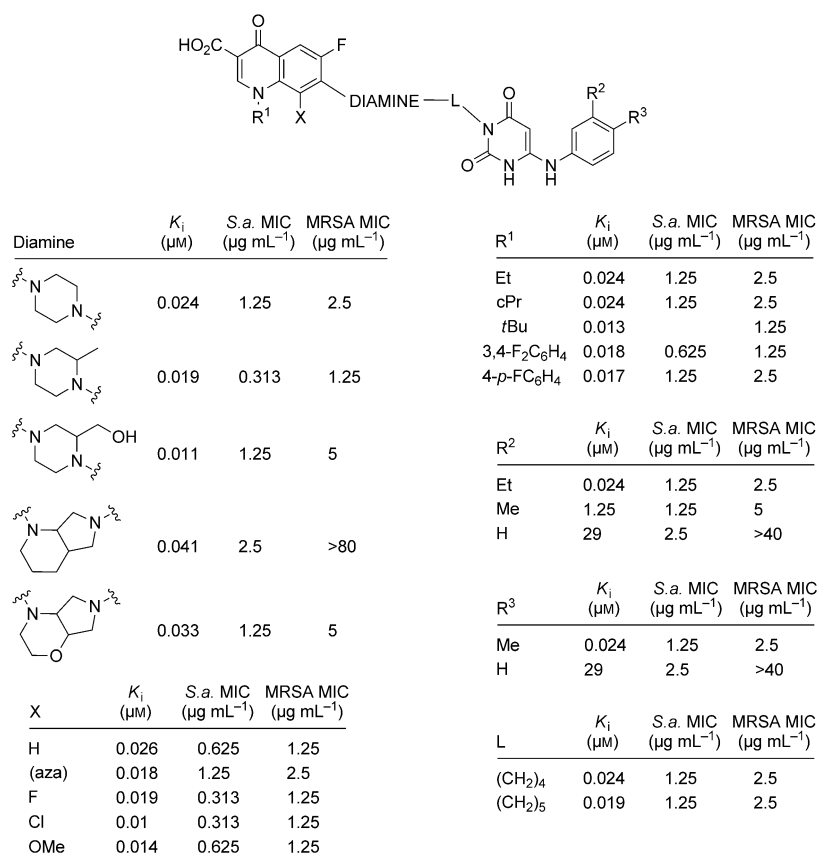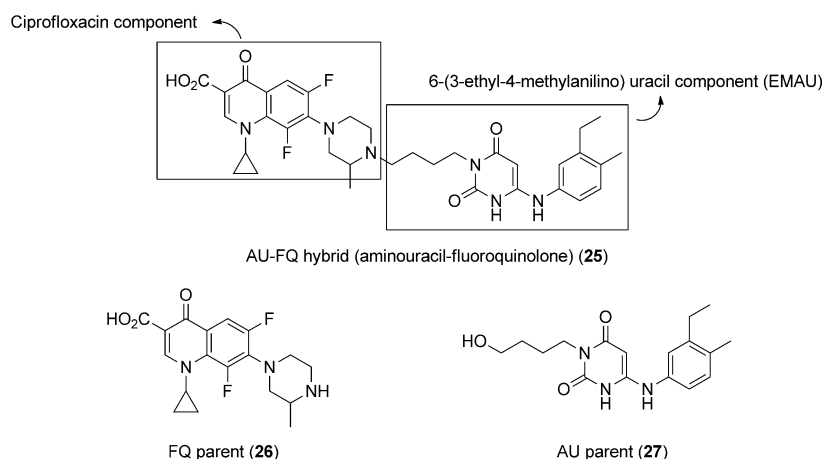

Figure 13. Inhibition of Pol IIIC and antibacterial activity of anilinouracil–fluoroquinoline (AU-FQ) hybrids against the Gram-positive bacteria *S. aureus* 25923 and methicillin-resistant *S. aureus* (MRSA) 1094. cPr = cyclopropyl.

Diamine	K_i (µM)	S.a. MIC (µg mL^{-1})	MRSA MIC (µg mL^{-1})
piperazine	0.024	1.25	2.5
methylpiperazine	0.019	0.313	1.25
hydroxymethylpiperazine	0.011	1.25	5
pipyridyl bicycle	0.041	2.5	>80
morpholine bicycle	0.033	1.25	5

X	K_i (µM)	S.a. MIC (µg mL^{-1})	MRSA MIC (µg mL^{-1})
H	0.026	0.625	1.25
(aza)	0.018	1.25	2.5
F	0.019	0.313	1.25
Cl	0.01	0.313	1.25
OMe	0.014	0.625	1.25

R^1	K_i (µM)	S.a. MIC (µg mL^{-1})	MRSA MIC (µg mL^{-1})
Et	0.024	1.25	2.5
cPr	0.024	1.25	2.5
tBu	0.013		1.25
3,4-F$_2$C$_6$H$_4$	0.018	0.625	1.25
4-p-FC$_6$H$_4$	0.017	1.25	2.5

R^2	K_i (µM)	S.a. MIC (µg mL^{-1})	MRSA MIC (µg mL^{-1})
Et	0.024	1.25	2.5
Me	1.25	1.25	5
H	29	2.5	>40

R^3	K_i (µM)	S.a. MIC (µg mL^{-1})	MRSA MIC (µg mL^{-1})
Me	0.024	1.25	2.5
H	29	2.5	>40

L	K_i (µM)	S.a. MIC (µg mL^{-1})	MRSA MIC (µg mL^{-1})
(CH$_2$)$_4$	0.024	1.25	2.5
(CH$_2$)$_5$	0.019	1.25	2.5

took the synthesis of a range of anilinouracil–fluoroquinolone (AU-FQ) hybrids (Figure 13) in an attempt to maximize the potency and spectrum of anilinouracil-based compounds.[82] Only marginal effects were observed on the potency against Pol IIIC and the antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* when R^1, X, and the linker length were varied.[82b] However, more dramatic and detrimental effects were seen with variations in the diamine as well as the R^2 and R^3 substituents.[82b] The removal of substituents at the R^2 and R^3 positions of the anilinouracil phenyl ring significantly reduced the antibacterial activity of the hybrid. Variation of the diamine from a piperazine ring to a pipyridyl bicycle or morpholine bicyclic ring reduced the activity; however, methyl substitution of the piperazine improved the antibacterial activity.

The optimal hybrid compound (**25**) maintained in vitro activity against both DNA polymerase IIIC, the AU target, as well as against topoisomerases and gyrases, the FQ targets, when compared to the parent compounds.[82a] The activity of hybrid **25** against DNA polymerase IIIC in enzymatic assays was significantly more potent than the AU parent compound. The activity of the hybrids against the FQ targets was equivalent to norflaxin, but was 10-fold lower than newer fluoroquinolones such as ciprofloxacin.[82a] Hybrid **25** displayed excellent in vitro antibacterial activity against a broad panel of Gram-positive and Gram-negative strains, with

MIC values up to 64-fold more potent than the parent AUs and an improved activity spectrum when compared to either the AU or FQ parents. Significantly, the hybrid compound displayed antibacterial activity against both AU- and FQ-resistant strains, such as, VREF F118 (linezolid-resistant) and MRSA B42876, respectively.[82a] To confirm the activity was not the result of simply administering the two AU and FQ parents together, Butler et al. assessed this additive combination against the activity of the hybrid and found that the covalently bonded hybrid version of the FQ and AU parent compounds was significantly more potent (Figure 14).[82a] The

Figure 14. A comparison of the antibacterial activity of the AU-FQ hybrid compound versus the FQ and AU parent compounds and the FQ and AU administered together.[82a]

Bacterial Strain	MIC of **25** (μM)	MIC of **26** (μM)	MIC of **27** (μM)	MIC of **26+27** (μM)
S. aureus Smith ATCC 13709	0.88	0.26	28	0.88
MRSA clinical isolate 1094	2.6	30	28	30
E. faecium ATCC 19434	3.75	30	30	30
VRE clinical isolate B42762	2.1	>60	30	45

hybrid compound clearly displays a dual-target activity and, therefore, two distinct mechanisms for disrupting DNA replication.

Another hybrid combination that has recently been investigated is that of fluoroquinolone–aminoglycoside antibacterials, specifically hybrids of ciprofloxacin and neomycin B.[83] Aminoglycosides act by selectively disrupting bacterial protein synthesis and exhibit activity against both Gram-positive and Gram-negative organisms. They have been widely used for over 50 years, and have resulted in the development of many resistance mechanisms, which have severely limited their usefulness.[84] Baasov and co-workers used a copper-catalyzed azide–alkyne cycloaddition (CuAAC) to form a series of hybrid compounds, with the ciprofloxacin component connected to the neomycin B component through a 1,2,3-triazole linkage.[83] The compounds were created to potentiate the activity of neomycin B, particularly against aminoglycoside-resistant strains.

A number of hybrid compounds were synthesized, which differed only in the nature and length of the linker units between the antibacterial groups (Figure 15).[83] The nature of

the linker groups had a significant effect on the antibacterial activity of the hybrid. The majority of the hybrids proved significantly more potent than the parent neomycin B, particularly against Gram-negative bacteria and MRSA. The hybrid compounds also performed well against strains of *E. coli* that were engineered to express aminoglycoside-modifying enzymes (for example, *E. coli* XL1 blue and the resistant variant *E. coli* XL1 blue pSF815), with activities up to 240-fold higher than neomycin B and maintains a relatively consistent performance against resistant and nonresistant strains (Figure 15).[83] The hybrids were, however, considerably less potent than ciprofloxacin in the antibacterial assays, despite, surprisingly, proving superior in enzymatic assays against DNA gyrase and topoisomerase IV. The disparity in the antibacterial screening results was, therefore, attributed to the reduced cell-penetrating ability of the hybrids compared to ciprofloxacin.[83] In terms of just antibacterial activity, therefore, it cannot be claimed that these hybrids offer significant advantages over ciprofloxacin monotherapy, but they do appear to be valuable in restoring the antibacterial activity of neomycin B against resistant strains.

Perhaps more interestingly, the hybrids were then evaluated in assays to test for their ability to induce resistance compared to the individual antibacterials.[83] This was achieved by subjecting strains of *E. coli* and *B. subtilis* to sub-inhibitory (0.5 MIC) concentrations of antibacterials over 15 successive subcultures. A hybrid compound was tested against neomycin B and ciprofloxacin individually and as a combination therapy.[83] The relative MIC_{50} values of ciprofloxacin, neomycin B, and the mixture increased by 75-, 4-, and 20-fold, respectively against *E. coli* and 37.5-, 8-, and 7.6-fold against *B. subtilis*, whereas the values for the hybrid compound remained essentially unchanged, thus providing compelling evidence for their reduced propensity to induce resistance under the test conditions.

These two combinations provide some evidence to suggest that hybrid antibacterials may have something to offer in the ongoing fight against resistant bacteria. However, there is no reason that hybrid agents must be limited to this "dual warhead" approach. An alternative approach is the pairing of an antibacterial with an agent that either enhances its activity or enables it to reach its site of action more efficiently. Such agents could include molecules that counter resistance or allow transport into the bacterial cell.

An interesting example of the latter approach is the combination of an antibacterial with an iron(III)-chelating siderophore group. These siderophore–drug conjugates may find utility in the treatment of Gram-negative pathogens such as *P. aeruginosa*, which are resistant to many classes of antibacterials. A significant factor in Gram-negative antibacterial resistance is the cell envelope, which is impervious to
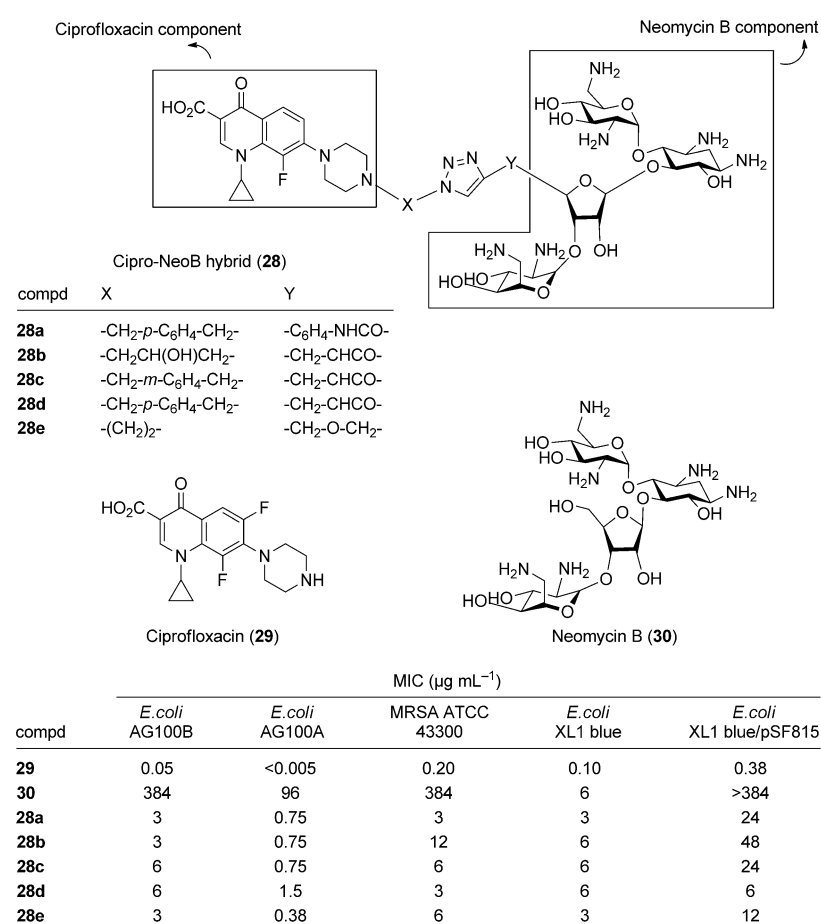
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Ciprofloxacin component

Neomycin B component

Cipro-NeoB hybrid (28)

compd	X	Y
28a	-CH$_2$-p-C$_6$H$_4$-CH$_2$-	-C$_6$H$_4$-NHCO-
28b	-CH$_2$CH(OH)CH$_2$-	-CH$_2$-CHCO-
28c	-CH$_2$-m-C$_6$H$_4$-CH$_2$-	-CH$_2$-CHCO-
28d	-CH$_2$-p-C$_6$H$_4$-CH$_2$-	-CH$_2$-CHCO-
28e	-(CH$_2$)$_2$-	-CH$_2$-O-CH$_2$-

Ciprofloxacin (29)

Neomycin B (30)

MIC (μg mL^{-1})

compd	*E.coli* AG100B	*E.coli* AG100A	MRSA ATCC 43300	*E.coli* XL1 blue	*E.coli* XL1 blue/pSF815
29	0.05	<0.005	0.20	0.10	0.38
30	384	96	384	6	>384
28a	3	0.75	3	3	24
28b	3	0.75	12	6	48
28c	6	0.75	6	6	24
28d	6	1.5	3	6	6
28e	3	0.38	6	3	12

Figure 15. The antibacterial activity of a series of ciprofloxacin–neomycin B hybrid compounds against Gram-negative *E. coli* strains, a corresponding engineered variant of *E. coli* that encodes for a resistance enzyme, and a Gram-positive MRSA stain.

many classes of antibacterials. However, Gram-negative bacteria actively transport Fe^{III}–siderophore complexes into the cell via specific receptors.[85] By attaching an antibacterial to a siderophore, it is hoped that a "Trojan horse" compound will be created that is capable of delivering the drug to its site of action.[86]

Miller and co-workers reported a recent example of this approach when they produced a drug conjugate of a penicillin antibacterial attached to an artificial tris(catecholate) siderophore (Figure 16).[87] The tris(catecholate) siderophore represents an easily accessible substitute for the naturally occurring enterobactin siderophore. Conjugates of ampicillin and amoxicillin were produced and screened, along with their parent drugs, for their effects against several strains of *P. aeruginosa*. As expected, the parent compounds were essentially inactive against the majority of the strains, with MIC$_{90}$ values generally > 200 μM; by contrast, the conjugates frequently attained values of < 0.5 μM, thus indicating potent activity. The conjugates performed particularly well in iron-deficient media, where uptake of the complexes is most likely to be induced. This marked enhancement of antibacterial activity as a result of conjugation to a siderophore has very positive implications for this approach, although further investigation is required to determine its utility.
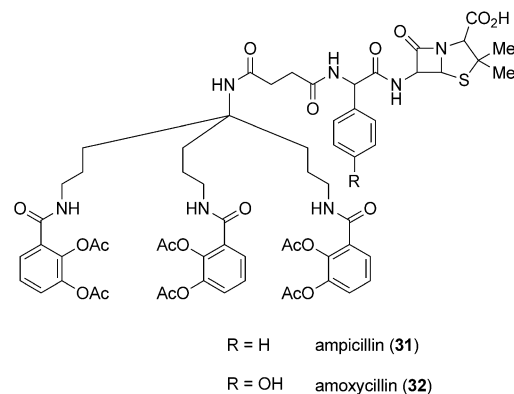
6. Antimicrobial Peptides

Antimicrobial peptides (AMPs) are released by virtually every eukaryotic life form, from insects and plants to human beings, as part of the innate immune response to infections.[88] Over 600 of these "host-defence" peptides have been reported to kill pathogenic microorganisms, including Gram-positive and Gram-negative bacteria, viruses, protozoa, and fungi, and also to be involved in promoting and regulating the immune response.[89] Although their structures are extremely diverse, these peptides share a number of important characteristics. They are relatively small, generally containing less than 50 amino acid residues, bear an overall positive charge, imparted by the presence of multiple arginine and lysine residues, and they also contain a substantial number of hydrophobic residues (typically accounting for over 50% of the amino acid residues).[90] As a result of this distribution of hydrophilic (cationic) and hydrophobic amino acids, the peptides are able to adopt amphipathic structures, often as a result of interacting with their microbial targets, which are fundamental to their mechanism of action.

6.1. Mechanism of Action

Antimicrobial peptides principally target the phospholipid membranes of bacterial cells. The selectivity that they display for bacterial cells is due to their cationic nature. In contrast to eukaryotic cells, the outer surface of both Gram-positive and Gram-negative bacterial cell membranes is heavily populated with anionic phosphate head groups. These negatively charged groups provide an electrostatic attraction to the cationic peptides, thereby causing them to congregate at the membrane surface, and thus allowing them to exert their antimicrobial effects. In

R = H   ampicillin (**31**)

R = OH   amoxycillin (**32**)

Figure 16. Penicillin–siderophore conjugate by Miller et al.

the case of Gram-negative bacteria, the peptides can achieve transit across the outer membrane by self-promoted uptake.[91] The cationic peptides outcompete the native Mg^{2+} and Ca^{2+} ions bound to the lipopolysaccharides and displace them, thereby resulting in destabilized regions through which the peptides can pass. The outer membrane surface of eukaryotic cells is generally neutral, which results in a reduced affinity for the cationic peptides. In these membranes, the negatively charged groups are instead oriented inwards towards the cell cytoplasm. The presence of collagen in eukaryotic membranes also increases their stability and has a protective effect against host-defence-type peptides.

Once the peptides are aggregated at the membrane surface, they are able to insert into the membrane and consequently exert their antibacterial effects. The peptides form micellar aggregates, which either disrupt the physical integrity of the membrane bilayer or facilitate the transport of the peptides into the bacterial cytoplasm, where they are able to act on cellular targets. A number of mechanisms have been suggested by which antimicrobial peptides disrupt membrane function.[92] The peptides can orient themselves perpendicular to the membrane to form either "barrel-stave" or "toroidal" transmembrane pores. Alternatively, the peptides can orient themselves parallel to the membrane as in the "carpet model", where they are attracted to many sites at the membrane surface, eventually forming a continuous carpet-like layer. Once a critical concentration is reached, the peptides then disrupt the membrane in a detergent-like manner, forming cracks, pores, and holes in the membrane. This leads to complete disintegration of the cell membrane into micelles, and the death of the cell. The exact mechanism that operates for a given case can depend on the structure of the AMP or cell membrane and the concentration of the AMP.

It is not always clear whether this disruption of the cell membrane is the primary killing mechanism, and AMPs have been found to attenuate a wide range of cytoplasmic processes, including nucleic acid synthesis, protein synthesis, cell-wall synthesis, and protein folding.[93] Examples of AMPs that have been shown to exert intracellular effects include PR 39,[94] CP 10A,[95] pleurocidin,[96] indolicidin,[97] and buforin II.[98] Interestingly, AMPs often appear to modulate several targets with similar, moderate potency, thus providing a sharp contrast to most clinically developed antibacterials, where the preference has been for high-affinity binding to a single target. The evolution of AMPs, therefore, seems to have followed an alternative path to that of microbe-derived antibacterials, with the "fittest" AMPs appearing to be promiscuous binders.[99] For this reason, it is thought that the activity of AMPs may be the result of their ability to act at multiple sites simultaneously, including the cell membrane as well as intra- and extracellular targets, thereby exposing the cell to many stresses that cumulatively cause the cell to die.

6.2. Antimicrobial Peptides as Potential Antibacterial Agents

The exploration of AMPs as antibacterial agents is an intriguing prospect that is currently attracting a good deal of attention as it is thought that such therapies may offer a number of advantages over more traditional antibacterials. Clearly a consideration of paramount importance in the development of novel antibacterials is their propensity to induce resistance. While it is inevitable that AMPs will (and do) induce resistance, there is a considerable amount of evidence to suggest that the onset of such resistance is slower than for conventional treatments. For example, one study involving 30 generational transfers of *Pseudomonas aeruginosa* with sub-inhibitory concentrations of synthetic AMPs resulted in only a 2- to 4-fold increase in resistance.[100] A similar study involving the antibacterial gentamicin found that it induced a 190-fold increase after only 11 transfers.[101] The AMP protegrin-1 was also tested in the second study and was not found to induce resistance to any appreciable degree.

This slow development of resistance has been attributed to a combination of the interaction of the AMPs with the cell membrane and their ability to act at multiple targets. It has been postulated that a mutation capable of furnishing resistance by the reorganization of the cell membrane may often prove to be too metabolically expensive for survival.[93] Additionally, if multiple cellular targets can be affected with similar potency, then resistance to a single mode of action will not necessarily confer complete resistance to the AMP.

Although the development of resistance to AMPs appears to be slow, high-level resistance could have grave consequences if the therapeutic use of AMPs were to lead to cross-resistance with innate immune response peptides.[102] However, laboratory studies have suggested that the occurrence of cross-resistance to other peptides as a result of repeated exposure to a single AMP is relatively low-level and not universal.[103] Also, the immunomodulatory and anti-endotoxin effects of host-defence peptides would not be affected by the development of resistance to their bactericidal actions. It is worth mentioning that resistance mechanisms against native host-defence peptides have been observed, and another possible strategy for chemotherapy would be to target these mechanisms, thereby removing the pathogens' defence against endogenous AMPs.[104]

Aside from their apparent reduced propensity to engender resistance, the immunomodulatory, anti-inflammatory, and anti-endotoxin activities of AMPs are also clearly appealing from a therapeutic point of view. These activities have promising implications for the use of AMPs synergistically with other antibacterials, as well as antibacterials in their own right. The fact that they are frequently bactericidal rather than bacteriostatic is also viewed positively.

Despite their many favorable qualities, few AMPs have been approved for clinical use, the most apparent exception being the polymyxins, cationic lipopeptides obtained from the Gram-positive bacterium *Bacillus polymyxa*. Polymyxin E (colistin, **33**; Figure 17) is currently used as a drug of last resort for some multidrug-resistant *P. aeruginosa* infections.[105] Colistin is generally administered topically to treat maladies such as wound infections; however, its prodrug
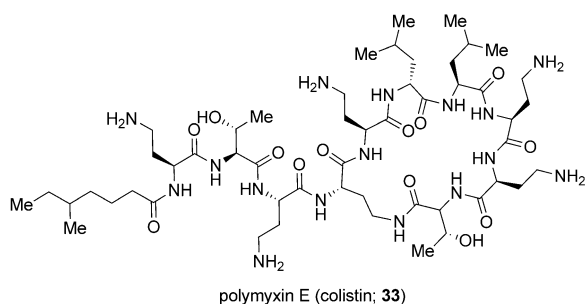
Figure 17. Polymyxin E, also known as colistin, is one of the few antimicrobial peptides approved for clinical use.

peptidomimetic sequences, the most potent compounds discovered were co-oligomers of lysine and 8-aminooctanoic acid.[111] The activity of these compounds was then improved further by capping the N terminus with a dodecanoyl group to impart greater hydrophobicity. Best among the lysine–aminooctanoic acid oligomers was the octameric compound denoted $C_{12}K-7_{\alpha 8}$ (Figure 18 a).[111]

$C_{12}K-7_{\alpha 8}$ was found to be rapidly bactericidal, displaying potent activity (MIC range: 1.6–12.5 μM) against a range of Gram-negative bacteria, including strains of *Acinetobacter*, *Klebsiella*, and *Pseudomonas*, and was also shown not to induce resistance in *E. coli* after 15 subcultures. Additionally,
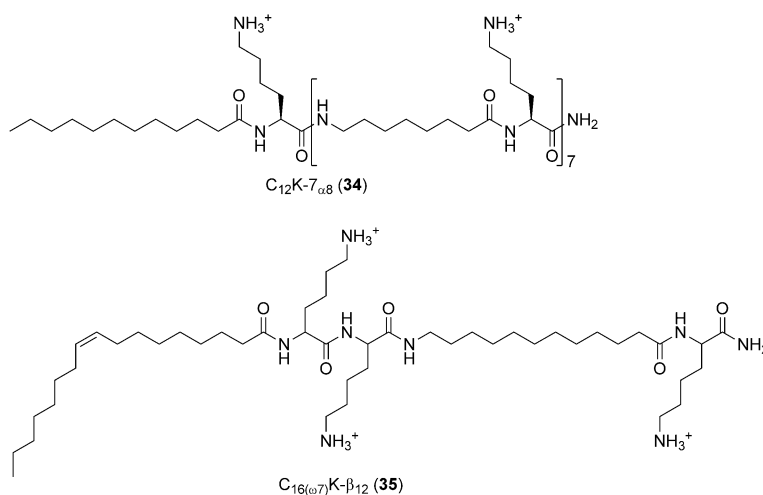
colomycin, in which the acidic groups are neutralized by methane sulfonation, is used systemically for the treatment of pulmonary infections in cystic fibrosis patients.[106] Various AMPs have also reached clinical trials for the treatment of a range of conditions, including impetigo, gingivitis, and catheter infections, but most have not been approved because of failure to demonstrate increased efficacy over existing treatments.[107]

In addition to a lack of efficacy, the inauspicious history of AMPs as therapeutics has been attributed to several factors.[108] Among the most important of these is the high cost of peptide synthesis, which is unappealing from a manufacturing point of view. Additionally, they display short half-lives in vivo as a result of their susceptibility to proteolytic enzymes, and can present issues with toxicity. For these reasons there is a great deal of interest in the development of peptidomimetics so as to mitigate some of these difficulties.[109]

6.3. Recent Advances in the Development of Antimicrobial Peptides

An exciting area of antibacterial research is the exploration and development of chemical mimics of host-defence peptides. It is hoped that the novel AMPs would overcome some of the challenges currently faced with natural AMPs, such as ease of synthesis, increased stability, and reduced toxicity. One strategy that shows some promise is the use of acyllysine oligomers to mimic the effects of naturally occurring peptides.[110] Mor and co-workers deliberately designed these peptidomimetics to have an alternating acyl chain (A) and cationic amino acid (K) structure, to preclude the formation of stable secondary structure in the resulting oligo-AKs (OAKs). The intention of this approach is that it would allow the ampipathic structures necessary for activity to be adopted upon interaction with the bacterial cell membrane.[111] Initial studies on the OAKs involved variation of the number and identity of the A and K units to balance the overall charge and hydrophobicity, and so achieve optimal activity. After the synthesis and testing of a library of linear

Figure 18. Molecular structures of two highly potent antibacterial OAKs, $C_{12}K-7_{\alpha 8}$ and $C_{16(\omega 7)}K-\beta_{12}$.

it proved capable of treating systemic *E. coli* infections in mice with similar efficacy to the conventional antibacterials ciprofloxacin and imipenem, and markedly superior efficacy to the naturally occurring AMPs MSI-78 and S4 (1–16), which were completely ineffective.[111] Studies on the mode of action of $C_{12}K-7_{\alpha 8}$ suggested that it was able to disrupt the bacterial cell membranes in a similar fashion to that of the naturally occurring AMPs tested.[111] Cumulatively, these results suggest that OAKs can potentially go a long way towards circumventing the problems encountered with standard AMPs.

A recent study by Mor and co-workers has revealed that the cationic peptide-based antimicrobial OAK $C_{16(\omega 7)}K-\beta_{12}$ is highly potent against a broad range of bacterial species, including 50 different bacterial strains, with MIC values ranging from 1.2 to 10 μg mL^{-1} (Figure 18 b).[112] The potency and mechanism could be influenced by environmental factors such as the pH, salt concentration, and temperature.[112,113] Under the optimal incubation conditions of pH 8.5, 85 mM NaCl, and 48 °C, $C_{16(\omega 7)}K-\beta_{12}$ had an MIC value of 0.08 μg mL^{-1} against *E. coli* and rapid bactericidal kinetics (0.25 h). Significantly, it was the rate of bactericidal activity against a particular strain that gave the most insight into the mechanism of action, with fast killing rates indicative of

disruption of the cell membrane or cell wall and slower rates associated with intracellular processes such as inhibiting macromolecule synthesis or DNA replication.[112] Interestingly, Mor and co-workers proposed that $C_{16(\omega7)}K\text{-}\beta_{12}$ acted by a distinct mechanism for a particular strain and that the mechanism of action adopted by the OAK would vary between stains. In a similar study by Gellman and co-workers, the concentration of AMP also had an impact on the mechanism adopted, with lower concentrations resulting in slower killing rates and, therefore, intracellular activity, whilst higher concentrations of AMP led to rapid killing rates, thereby indicating there was sufficient amounts of the peptide to inhibit cell-wall or membrane synthesis.[114]

An alternative approach for the development of novel AMPs is based on the concept of disrupting the bacterial cell membrane with tubular structures (Figure 19).[115] Potent antibacterial activity was achieved with a series of six- and eight-residue cyclic peptides, which self-assemble into hollow tubular open-ended structures within bacterial cell membranes and lead to an increased permeability and bacterial death.[115b] The cyclic peptides were constructed of alternating D- and L-α-amino acids which generate flat ring conforma-
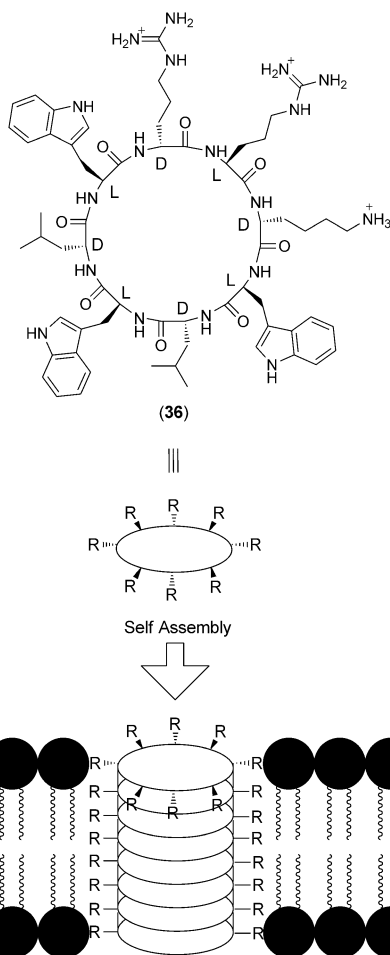
tions, with the R groups oriented on the outside of the ring and the amide backbone at right angles to the plane of the ring. In the bacterium cell membrane, the cyclic peptides stack together with the amide backbone through the formation of hydrogen bonds between the rings and create the tubular structure. The nature of the R group was essential for achieving potency, as these interact with the bacterial membrane; for example, increasing the number of basic groups from two to three residues in the cyclic peptide yielded higher activities against MRSA, ranging from 6 to 12 $\mu g\,mL^{-1}$, whereas the presence of acid groups was detrimental to potency due to unfavorable electrostatic interactions with the cell membrane.[115b] The rapid bactericidal properties of the self-assembled peptides and the rate of membrane depolarization was consistent with the proposed mode of action.[115b,116]

Another approached currently being explored is the use of β-peptides to disrupt the bacterial cell membrane.[117] Amphiphilic β-peptides typically adopt a variety of helical structures, and in biological systems behave in a similar manner to related natural α-peptides, such as magainin and cecropin.[118] β-Peptide mimics were found to be more conformationally stable, resilient to proteases, and have a better activity profile when compared to the natural α-amino acid antimicrobial peptides.[119] Early β-peptide analogues had a significant drawback in terms of selectivity, as despite their antibacterial profile they also often exhibited haemolytic activity.[118a,120] Extensive research by the research groups of DeGrado,[118a] Gellman,[118b,121] and Seebach[120] identified β-peptides that maintained antimicrobial activity but importantly were more selective for bacterial cells than human red blood cells.

7. Antivirulence Strategies by Quorum Sensing Inhibition

Quorum sensing (QS) is a cell–cell signaling phenomenon utilized by bacteria cells within a given population to collectively coordinate gene expression. The inhibition of QS has attracted considerable attention for the development of novel antibacterial agents.[18a,122] The first phenotype recognized to be under QS regulation was bioluminescence in the marine bacterium *Vibrio fischeri* during the 1970s.[123] However, the term "quorum sensing" was not used until 1994 by Fuqua et al.[124] QS is achieved by the free diffusion (or active transport) of chemical signaling molecules between cells in a given population.[125] These signaling molecules are detected by specific cognate receptor proteins, some of which can be associated with the cell membrane.[126] The signaling molecule (autoinducer) is produced proportionally to the cell density in the population and when the concentration of the signal reaches a critical threshold value (reflecting the necessary bacterial quorum) the autoinducer binds its specific receptor protein.[127] The bound receptor acts as a transcriptional regulator and activates (or represses) the expression of genes under QS control.[128] The signaling molecules are often referred to as autoinducers because, in some cases, once bound to their specific receptors they may up-regulate their

(36)

Self Assembly

Figure 19. Potent antibacterial cyclic D,L-α-peptide (36) with an MIC value of 6 $\mu g\,mL^{-1}$ against *S. aureus* (MRSA). The cyclic peptides self-assemble to form tubular structures within the bacterial cell membrane, ultimately leading to increased permeability and cell death.

own biosynthesis, thereby creating a positive auto-feedback loop.[129]

Many Gram-negative and Gram-positive bacteria utilize QS to regulate a range of phenotypes. Phenotypes regulated by QS include: virulence factor production, biofilm formation, bioluminescence, swarming motility, and sporulation.[130] Clinically relevant pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* utilize a form of QS to regulate the production of the virulence factor.[131] QS allows such pathogenic bacteria to make a coordinated attack on the host when the bacterial population is high, thus overwhelming the host's defence system and enhancing the survival chances of the bacteria.[130a,132] The most studied signaling molecules are acylhomoserine lactones (AHLs), used by Gram-negative bacteria. LuxI-type synthases are required for AHL biosynthesis and the AHLs produced are detected by cognate LuxR-type receptor proteins.[130b,133] The chemical structure of the AHLs varies depending on the strain of bacteria, and variations include: modifications to the acyl chain length and the presence/absence of a hydroxy or ketone group on the third position of the acyl chain[134] (37–39, Figure 20). This has led to the hypothesis that multiple bacterial species in a given

population can be involved in interspecies signaling or "bacterial cross talk" via LuxR homologues and AHL signaling molecules.[135] Indeed, it has been well-documented that gene regulation in specific bacterial species can be affected by non-endogenous QS signals.[136] Aside from AHLs, there are other classes of quorum sensing molecules which differ in chemical structure from the AHL framework produced by bacteria.[130a] Some of these chemical structures include quinolones (40 and 41), cyclic peptides (43), and borate diesters (42; Figure 20).

One exciting prospect for a potential novel antibacterial strategy is the attenuation of virulence by inhibition of QS (quorum quenching).[137] Attenuating virulence in this fashion could allow the host's immune system to overcome the bacterial infection independently of, or in combination with, antibacterial treatment. As such, many competitive antagonists of QS signaling molecules and their receptor proteins have been reported.[137] Indeed, in vivo studies in animals with QS inhibitors have shown in many cases reduced virulence production and attenuation of bacterial infection.[138] An appealing hypothesis is that, as the QS inhibitors do not affect bacterial growth, the evolutionary selection for the survival of resistant bacteria will be removed or reduced compared to cytotoxic antibacterials.[122] However, this may be a naïve view arising from laboratory-based studies, because the real-life fitness implications associated with loss of QS in bacteria are not clear. This view that the evolution of resistance could be circumvented, or massively reduced by through quorum quenching strategies has been challenged.[139] Nevertheless, inhibiting QS as a novel antibacterial strategy is in its infancy and remains a tractable prospect. Of all the QS molecules currently being investigated, the AHL and AI-2 signaling molecules have been more extensively studied, and comprehensive reviews have been written on the disruption of virulence factor production by the inhibition of AHL and AI-2 quorum sensing pathways.[140] PQS and quinolone signaling has recently attracted considerable interest within our research group, and in others. For these reasons, the subsequent section will focus more on the advances in the inhibition of the quinolone and cyclic peptide QS systems.
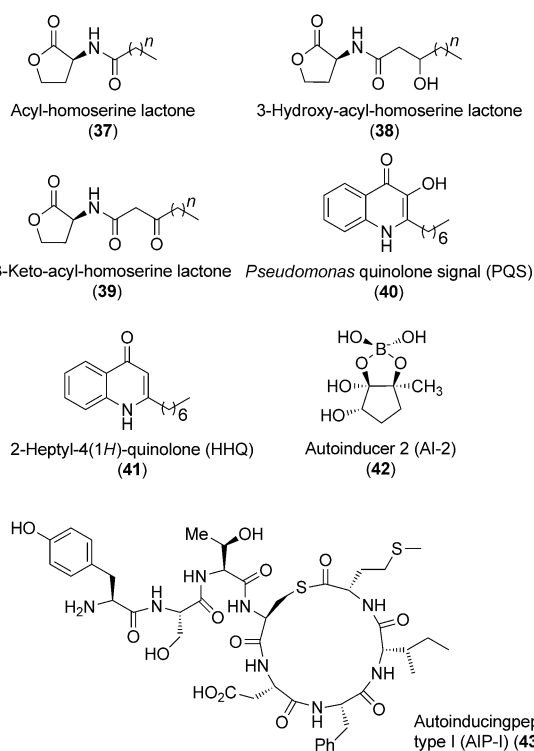

Figure 20. QS signaling molecules produced by bacteria. Acylhomoserine lactones (AHLs) are produced by many Gram-negative bacteria, variations across bacterial species include the presence/absence of an oxygen atom at the 3-position of the AHL and varying alkyl chain length (*n*). PQS and HHQ are the only quinolone QS molecules reported to date from the opportunistic pathogen *P. aeruginosa*. Borate diester signaling, including AI-2, is utilized by both Gram-negative and Gram-positive bacteria. Peptide QS signaling is found in Gram-positive bacteria including the opportunistic pathogen *S. aureus* which has evolved to use one of four AIP signaling molecules including AIP-I.

7.1. Quinolone Quorum Sensing

2-Heptyl-3-hydroxy-4(1*H*)-quinolone (40), also known as the *Pseudomonas* quinolone signal (PQS), is a quorum sensing signaling molecule in *P. aeruginosa*, which was first described by Pesci et al. in 1999 (Figure 21).[141] Although bacterial species of the genera *Burkholderia* and *Alteromonas* also produce quinolones, no quinolone quorum sensing signaling system (receptor proteins) has been identified.[142] The quinolone signaling system in *P. aeruginosa* is involved in the regulation of a number of virulence phenotypes including elastase production, pyocyanin production, biofilm formation, pyoverdine production, and membrane vesicle formation.[141,143] In *P. aeruginosa*, the *las* and *rhl* QS systems, which utilize AHLs, are also interlinked with the PQS signaling system. The enzymes PqsA, PqsB, PqsC, and PqsD encoded by the *pqsABCDE* operon convert anthranilic acid (44) into
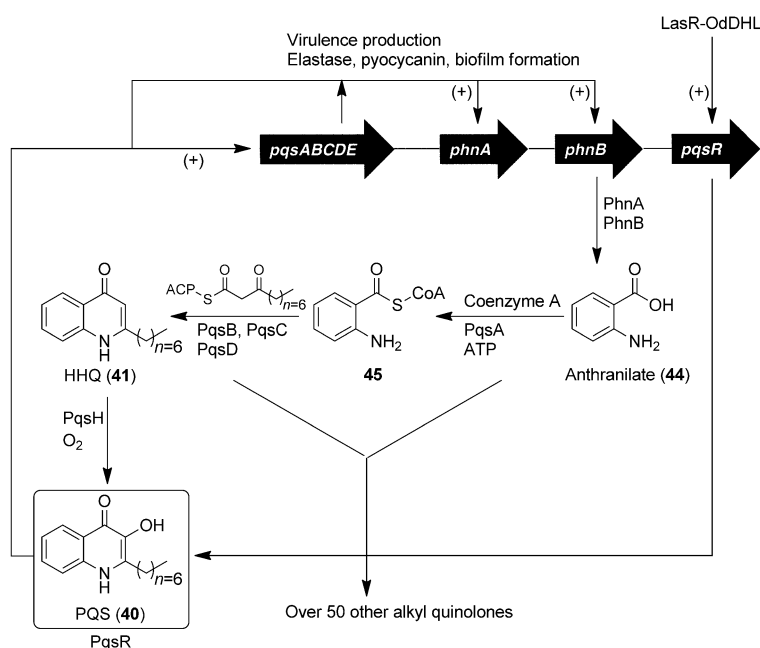
Figure 21. PQS biosynthetic pathway and positive auto-feedback loop; PhnA and PhnB are responsible for anthranilic acid production. PqsA is an acyl-CoA ligase protein involved in activating anthranilic acid. PqsA, PqsB, PqsC, and PqsD are required for HHQ biosynthesis and 50 other alkyl quinolones produced by *P. aeruginosa*. PqsH is required for the oxidation of HHQ to PQS. PQS binds PqsR and further up-regulates transcription of the pqsABCDE operon, phnA, and phnB, thereby resulting in the further biosynthesis of PQS and other alkyl quinolones. It is important to note that HHQ is capable of binding PqsR and up-regulating quinolone biosynthesis and virulence. The PQS signaling system is also interlinked with the las and rhl AHL signaling systems (not shown in diagram).

over 50 alkyl quinolones including 2-heptyl-4(1*H*)-quinolone (HHQ, **41**), the biosynthetic precursor to PQS (**40**; Figure 21).[144]

HHQ is oxidized to PQS by the monoxygenase enzyme PqsH.[145] PQS bound to PqsR interacts with the *pqsA* promoter region, thereby resulting in the transcription of the *pqsABCDE* operon and further biosynthesis of PQS and alkyl quinolones, thus creating a positive auto-feedback loop.[146] HHQ is also considered a signaling molecule, as it can also bind PqsR and up-regulate alkyl quinolone biosynthesis via the *pqsA* promoter region.[146] Although the exact function of PqsE is unknown, it is essential for full virulence production.[147] It has been speculated that one of the major purposes of the PQS-PqsR complex is to upregulate PqsE for virulence production.[148]

7.2. Quinolone Quorum Quenching by Modulation of PqsR

Despite the discovery of PQS as a signaling molecule over ten years ago, the targeted inhibition and modulation of the Pqs receptor protein (PqsR) with synthetic QS modulating compounds is a relatively new field. We recently reported the development of an operationally simple two-step procedure to generate PQS analogues by using microwave irradiation.[149] The PQS analogues generated were assayed in a number of phenotypic assays including the ability to

activate PqsR, thereby providing structure–activity data on the PQS–PqsR interaction.[150] This was achieved by testing the ability of the analogues to stimulate PqsR-dependent transcription from the *pqsA* promoter in the *E. coli* strain DH5α containing the plasmid pEAL08-2 (generated by Hogan et al.).[151] Such a heterologous reporter assay removes other potential complications that may arise in *P. aeruginosa* and allows a more direct assay for measuring PqsR stimulation. Results revealed that small modifications in chain length (reduction/addition) did not diminish agonist activity greatly. However, major modifications such as the introduction of phenyl substituents to the heptyl chain or the replacement of the heptyl chain with a methyl group resulted in a dramatic decrease or complete removal of the agonist activity. The introduction of substituents on the quinolone ring also affected agonist activity, with strongly electron-donating groups dramatically reducing activity. However, no significant competitive antagonist activity of PqsR was observed with any of the PQS analogues.

Lu et al. reported the first competitive PqsR antagonists based on HHQ analogues.[152] In this study 30 HHQ analogues were synthesized and screened using the same *E. coli* strain. The analogues generated included modifications to the heptyl chain and the introduction of substituents around the quinolone ring. Three of the most potent antagonists **46–48** (Figure 22a) contained strongly electron-withdrawing groups at the 6-position of the quinolone and were found to have IC_{50} values of approximately 259, 54, and 51 nM, respectively, in competition with 50 nM PQS. Interestingly, analogues with the same substituents at the 7- or 8-position of the quinolone ring
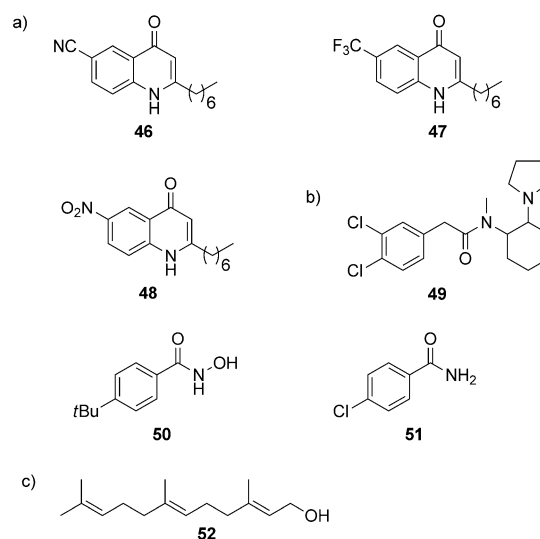

Figure 22. PqsR antagonists that have been reported: a) HHQ quinolone analogues; b) K-opioid receptor agonists and fragment-based analogues; c) natural sesquiterpene farnesol.

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resulted in a dramatic loss in antagonist activity. This high-lights the importance of the electronics of the quinolone ring and the position of the substituent for agonist/antagonist activity. In phenotypic virulence assays with *P. aeruginosa*, analogues **46** and **47** (Figure 22 a) were capable of inhibiting pyocyanin production in the low micromolar range and importantly growth of *P. aeruginosa* was not affected by the antagonists. This study has provided the first synthetic PqsR antagonists based on quinolone signaling molecule analogues. Considering the abundance of AHL and AI-2 analogue based antagonists, this study should pave the way for future quinolone-based quorum quenching compounds.

Klein et al. recently reported the synthesis and identification of a number of benzamide derivatives which act as antagonists of PqsR.[153] The K-opioid receptor agonist (**49**; Figure 22 b) affects *pqsABCDE* transcription in *P. aeruginosa*. The authors hypothesized this effect may be due to the inhibition of PqsR and, indeed in the *E. coli* reporter strain used in previous studies, **49** acted as a moderate antagonist. Klien et al. then applied a rational design strategy that involved the simplification of **49** into smaller fragments and related analogues. Combining the *E. coli* reporter strain and biophysical techniques, including surface plasmon resonance (SPR) and isothermal calorimetry (ITC), the authors identified a small number of PqsR antagonists including **50** and **51** (Figure 22 b). Site-directed mutagenesis and ITC revealed that the Gln194 and Phe221 amino acid residues of PqsR are integral for antagonist binding. Antagonist **50** had an IC$_{50}$ value of 12.5 μM in the *E. coli* reporter strain and an IC$_{50}$ value of 23.6 μM in pyocyanin inhibition in *P. aeruginosa*. Although the IC$_{50}$ values are higher than those reported for the antagonists of Lu et al., the fact that these antagonists bear little resemblance to HHQ or PQS and given that no crystal structure was available to guide the design, makes the study an impressive feat.

Hogan and co-workers reported that the addition of the natural sesquiterpene farnesol **52** (Figure 22 c) to *P. aeruginosa* results in reduced production of pyocyanin and alkyl quinolone.[151] DNA motility shift assays revealed that, similar to PQS, farnesol interacts with PqsR, and affects the transcription of the *pqsA* promoter region. A dose-dependent inhibition of PqsR binding to the *pqsA* promoter region was observed in the presence of farnesol. The long-chain alcohol dodecanol was inactive, while the farnesol derivatives farnesyl acetate and geranyl linalool had moderate activity, but were not as potent as farnesol. Importantly, this study highlights how natural products could be a potential source for future PqsR antagonists.

7.3. Other Quinolone Quorum Quenching Strategies

Aside from targeting the inhibition of PqsR, other approaches for inhibiting quinolone signaling have attracted considerable interest. An approach explored by a number of research groups has involved inhibiting the PQS biosynthesis pathway. Pesci and co-workers discovered that anthranilic acid is a precursor to the biosynthesis of PQS and other alkyl quinolones produced by *P. aeruginosa*.[154] During this study it

was found that the addition of methylanthranilic acid to *P. aeruginosa* cultures inhibited the production of alkyl quinolone and the virulence factor elastase.[155] The authors hypothesized that this was a result of methylanthranilic acid inhibiting the PQS biosynthetic pathway, which requires anthranilic acid. Further studies by Lecis et al. also revealed a number of halogen-substituted anthranilic acids **53**, **54**, and **55** (Figure 23 a), which inhibited PQS production and viru-
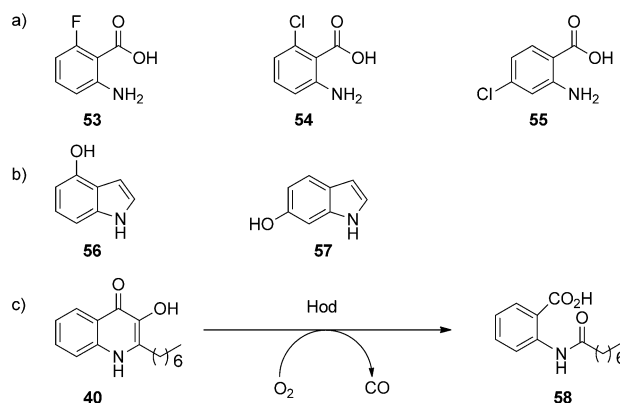

Figure 23. Alternative strategies to quinolone quorum quenching: a) The substituted anthranilic acids compete for the PqsA binding site, thereby abolishing quinolone biosynthesis and reducing virulence; b) the indoles are believed to compete with tryptophan degradation and anthranilic acid production, hence reducing alkylquinolone biosynthesis; c) the enzyme Hod degrades PQS, thus providing an alternative enzymatic approach to quinolone quorum quenching.

lence in *P. aeruginosa*. Not all the halogen-substituted anthranilic acids were active; the position of the halogen substituent was found to be important for inhibitory activity.[156] The authors provided evidence that the substituted anthranilic acids target the binding site of PqsA, competing with anthranilic acid for binding. Interestingly, the substituted anthranilic acids reduced virulence in mice infected with *P. aeruginosa* and increased survival rates, further confirming the targeting of quinolone signaling as a potential novel antibacterial strategy.

Indole and hydroxyindoles **56** and **57** (Figure 23 b) have also been shown to inhibit PQS, pyocyanin, pyoverdine, and rhamnolipid production in *P. aeruginosa*.[157] Interestingly, the compounds also increased the antibacterial susceptibility of *P. aeruginosa*. A similar study by Tasherio et al. with indole and substituted indoles revealed that substituted indoles can also repress production of membrane vesicles.[158] Although the exact mechanism by which indole inhibits virulence in *P. aeruginosa* is unknown, it is hypothesized that the production of anthranilic acid is affected, as indole may interfere with tryptophan degradation to generate anthranilic acid.

Another exciting alternative strategy to inhibit quinolone quorum sensing is the use of quinolone-degrading enzymes; similar strategies have been applied to AHL signaling molecules. Pultensy et al. hypothesized that the enzyme Hod (1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase) from the soil bacterium *Arthrobacter nitroguajacolicus* could be

used to degrade PQS (Figure 23 c).[159] In the natural environment, Hod catalyzes the degradation of 3-hydroxy-2-methyl-4(1*H*)-quinolone to *N*-acetylanthranilic acid and carbon monoxide. Hod was capable of cleaving PQS to *N*-octanoyl-anthranilic acid and resulted in the reduction of PQS production, pyocyanin, and rhamnolipid production in *P. aeruginosa*. However, the cleavage of Hod by extracellular proteases produced by *P. aeruginosa*, and the inhibition by HHQ, reduced the efficiency of Hod. Despite this, the enzyme degradation of quinolone signaling molecules is an exciting prospect.

7.4. Antibacterial Activity of Quinolone Quorum Sensing Compounds

Although PQS and HHQ are thought primarily to be signaling molecules, recent studies have highlighted the antibacterial activity of both molecules against certain bacterial species. The quinolones produced by *P. aeruginosa* have been known to have antibacterial activity since the 1950s.[160] However, the antibacterial activities of PQS and HHQ have only recently been discovered. This dual property is not uncommon to QS quorum sensing signaling molecules as the degradation products of AHLs (tetramic acids) exhibit potent antibacterial activity.[161] Toyofuku et al. have shown that PQS represses the growth of several Gram-positive and Gram-negative bacteria.[162] In this study, bacterial species exposed to PQS during growth exhibited an extended lag phase and decreased growth rates. The amount of repression varied depending on the bacterial species. The authors noted that the effect on growth was not like that of traditional bacteriostatic or bacterolytic antibacterials; instead, the PQS caused the bacteria to grow at a slower rate. Oxygen consumption was affected in some bacterial species, and this may have an underlying effect on the growth repression effects observed. The addition of iron also abolished growth repression; however, this is most likely due to the formation of the PQS-Fe chelate complex, which renders the PQS inactive.

Reen et al. have reported on the antibacterial effects of HHQ which exhibited potent bacteriostatic activity against several Gram-negative bacterial species, including the human pathogens *Vibrio cholerae* and *Vibrio vulnificus* at 10 μM.[163] Both HHQ and PQS also repressed bacterial motility, which is associated with virulence in *E. coli* and *S. aureus*. *Burkholderia* and *Alteromonas* spp. also produce over 50 quinolones, such as *P. aeruginosa*. These studies highlight how these signaling molecules should not be overlooked for their antibacterial activity or their potential for signaling modulation.

7.5. Autoinducer Peptide Signaling in Gram-Positive Bacteria

A number of Gram-positive bacteria utilize oligopeptides as QS signals and are commonly referred to as autoinducer peptides (AIPs). As a consequence of its clinical relevance, the most widely studied AIP signaling system is in *S. aureus*.
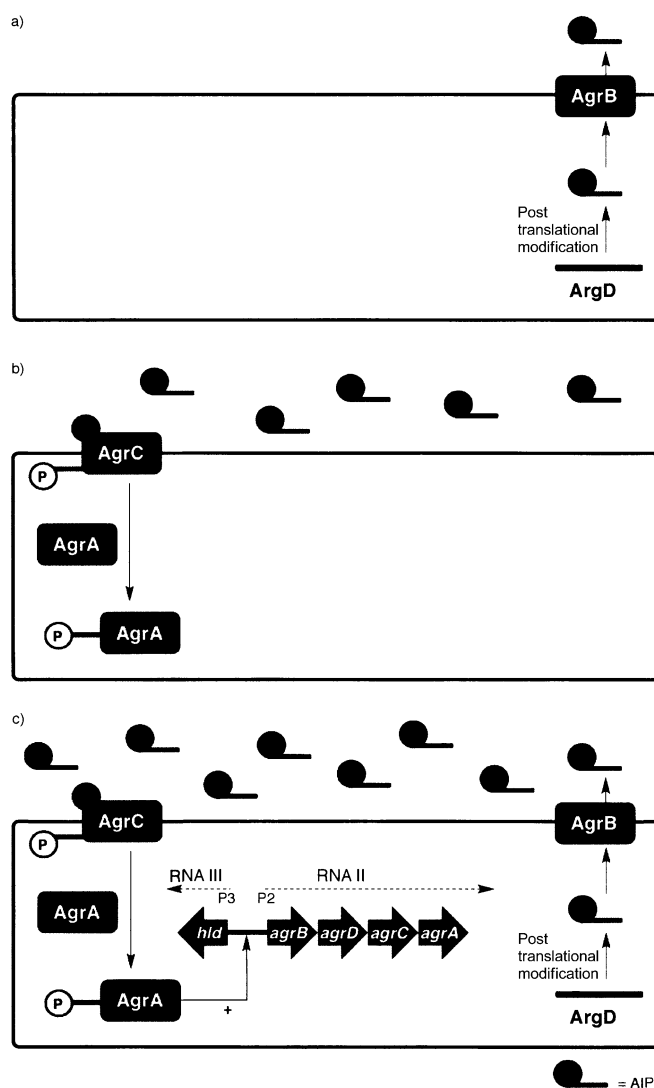

Figure 24. AIP signaling pathway in *S. aureus*. a) The pro-peptide (AgrD) undergoes posttranslational modification and is exported outside the cell by AgrB. b) When the critical quorum level is reached, the AIP binds the receptor AgrC which then phosphorylates AgrA. c) AgrA in turn acts as a transcriptional activator, activating genes responsible for further AIP biosynthesis (via RNA II) and virulence production (via RNA III).

The AIPs are ribosomally synthesized as pro-peptides (AgrD); after posttranslational modification, the AIP is exported in its active form to the extracellular environment via a membrane-associated transporter protein (AgrB; Figure 24).[164]

The detection of the AIP autoinducers by bacterial cells consists of a two-component receptor/response system including a membrane-associated receptor histidine kinase protein (AgrC) and an intracellular response regulator protein (AgrA).[165] At a critical threshold concentration of AIP, the AIP binds AgrC, thereby resulting in autophosphorylation and subsequent phosphorylation of AgrA.[165] AgrA acts as a transcriptional regulator activating transcription of genes responsible for AIP biosynthesis and virulence production.[166] Some of the virulence phenotypes regulated by the AIP

signaling system include: extracellular toxins, cell-surface adhesion factors, tissue degrading enzymes, and exoproteins.[167] Unlike AHL signaling systems in Gram-negative bacteria, which tend to have species-specific signaling systems, *S. aureus* AIP signaling has evolved so there are four distinct autoinducer peptides (I–IV, **59–62**; Figure 25).[168]
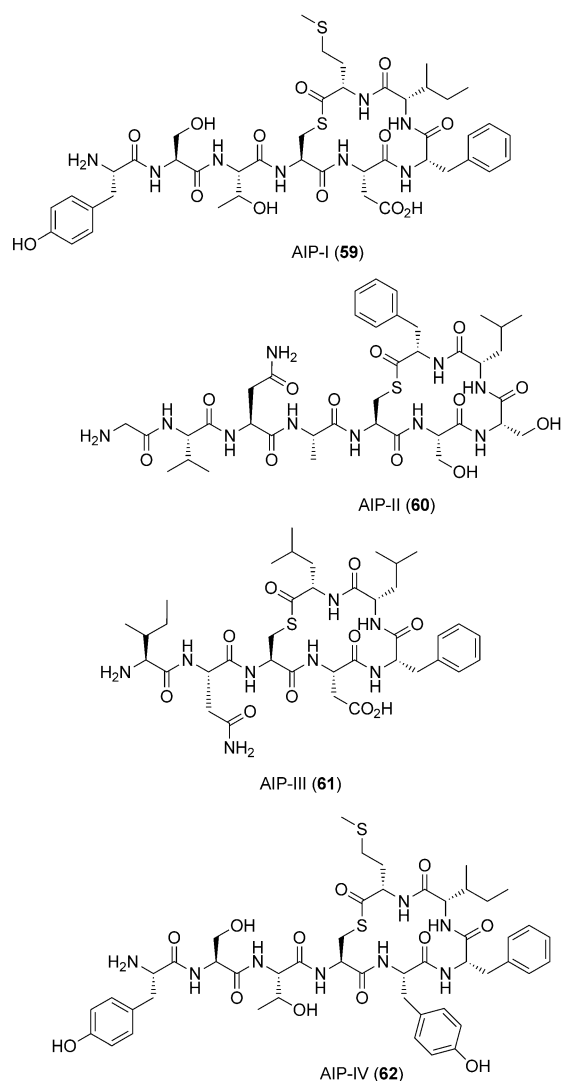

AIP-I (**59**)

AIP-II (**60**)

AIP-III (**61**)

AIP-IV (**62**)

Figure 25. Chemical structures of the four AIP signals utilized by *S. aureus*. AIP-I and AIP-IV only differ by one amino acid, yet have differing cognate receptors, AgrC-I and AgrC-IV. The amino acid sequences are: type I YSTCDFIM, type II GVNACSSLF, type III INCDFLL, type IV YSTCYFIM.

Differing bacterial strains of *S. aureus* utilize one of four distinct AIP signaling systems, for example, AIP-II (**60**) would activate the distinct AgrC receptor within a specific *S. aureus* strain, in this case AgrC-II, to induce the signaling cascade. The four AIP signaling molecules utilized by *S. aureus* are 7–9 amino acids long and consist of an N-terminal tail region and a thiolactone macrocycle moiety (Figure 25).

7.6. Quorum Quenching by Modulation of AgrC

The inhibition of AgrC would prevent the further biosynthesis of AIPs as well as the initiation of virulence-factor synthesis, thus making it a potential target for novel antibacterial therapies. Natural AIPs can exhibit potent inhibitory activity with other noncognate AgrC receptors, sometimes termed cross-group inhibitors.[168,169] For example, AIP-I is capable of inhibiting AgrC-II and AgrC-III, while AIP-II has inhibitory activity against AgrC-I and AgrC-III. Studies in mice infected with *S. aureus* (type I) revealed that treatment with AIP-II resulted in attenuation of the infection, thus highlighting the potential of AIP signaling inhibition as a novel antibacterial strategy.[169] Many studies on structure–activity relationships of AIP analogues on cognate and noncognate AgrC receptor proteins have been carried out, and most of the AgrC inhibitors reported in the literature are based on AIP analogues.[169,170] Although differing structure–activity relationships are observed against each AIP and AgrC receptor, the following general trends have been reported:[171]

1) linear noncyclic AIP peptide derivatives are inactive;
2) replacement of the thiolactone bond with a lactone or lactam bond dramatically reduces agonist activity for the cognate receptor, but, such analogues can still retain cross-group antagonist activity;
3) modification of amino acids in the tail residue dramatically reduces agonist activity, but such analogues can still maintain antagonist activity; and
4) modification of residues within the macrocycle effect both agonist and antagonist activity.

Perhaps one of the most exciting recent advances was the identification of a potent AgrC antagonist by Novick and co-workers.[172] The authors synthesized a truncated analogue of AIP-II **63** (Figure 26), which is a potent inhibitor of all four AgrC receptors. Toxin production was inhibited in *S. aureus*, with an IC_{50} value of 10 nM reported. Follow-up studies involved the parallel synthesis of 10 analogues by utilizing a novel linker strategy for fluorenylmethoxycarbonyl (Fmoc) based thioester synthesis.[170a] The cysteine, leucine, and phenylalanine residues of **63** proved to be essential for inhibitory activity.

AIP-I and AIP-IV vary in structure by one amino acid situated within the macrocyclic ring: aspartate in AIP-I is replaced by tyrosine in AIP-IV. Lyon et al. synthesized three AIP-I and AIP-IV analogues, where the key amino acid was varied with the aim of understanding the structure–activity relationship responsible for the differential activity of AIP-1 and AIP-IV towards AgrC-I and AgrC-IV.[170e] Analogue **64**, which contains one amino acid replacement, aspartic acid in AIP-I for alanine, had impressive IC_{50} values of 5, 8, 0.3, and 3 nM against AgrC I–IV, respectively (Figure 26).

Williams and co-workers identified the minimum scaffold of AIP-I necessary for antagonistic activity against all *S. aureus* AgrCs.[173] Analogue **65** had IC_{50} values of 5, 5, 0.1, and 5 nM against AgrC I–IV, respectively (Figure 26).[173] Blackwell and co-workers used the peptide scaffold **65** as a basis to synthesize a number of peptide–peptoid hybrids
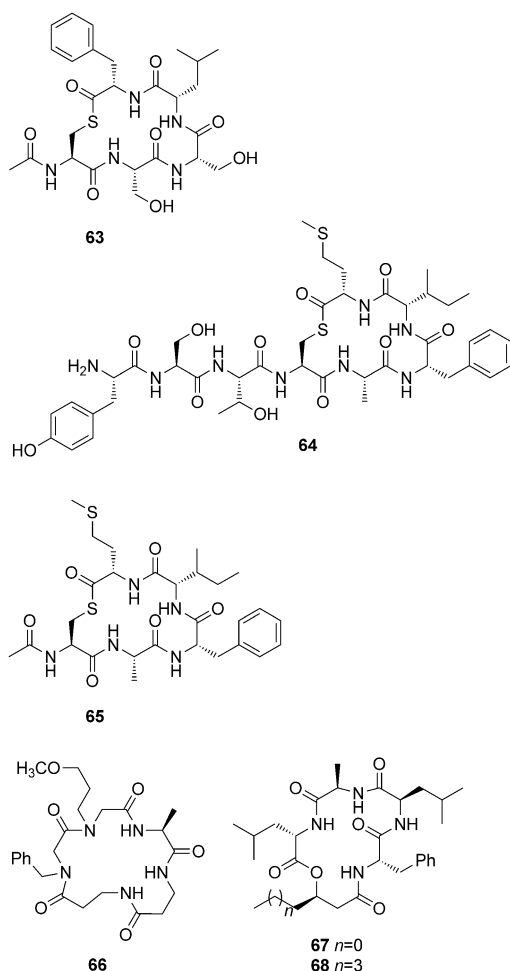
Figure 26. Recently reported Agr-modulating compounds. **63–65** are potent antagonists of AgrC I–IV. **66** affects biofilm formation in *S. aureus* which is hypothesized to be due to Agr modulation. Solonamides A and B (**67** and **68**) inhibit virulence in *S. aureus* without affecting growth; as a result of their similar structure to AIPs, they are hypothesized to inhibit AgrC.

(peptomers) aimed at modulating AgrC-AIP signaling.[174] Analogue **66** discovered in the study was found to be capable of modulating biofilm formation in *S. aureus*. Although no antagonists were identified, the ease of synthesis of such peptomers is an attractive alternative to lengthy synthetic routes towards AIP analogues. Natural products have also provided a source for the AIP signaling inhibition. Recently, Larsen and co-workers isolated two natural products, solonamide A and B (**67** and **68**), from marine photobacteria which inhibited *agr* regulation in gene reporter assays.[175] Although the exact target of the natural products is unknown, their structural resemblance to AIP signaling molecules means it is likely the solonamides may be AgrC antagonists.

7.7. Other AIP Quorum Quenching Strategies

Balaban and co-workers have shown that the natural product hamamelitannin (**69**) inhibits MRSA infections
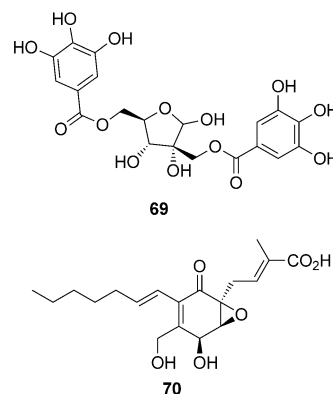

Figure 27. The natural products hamamelitannin (**69**) and ambuic acid (**70**). Hamamelitannin reduces the production of RNA III and virulence. Ambuic acid inhibits AIP biosynthesis.

in vivo without affecting growth, which indicates that hamamelitannin may be inhibiting QS (Figure 27).[176] Hamamelitannin, which is isolated from the bark of *Hamamelis virginia*, also inhibits RNA III and reduces virulence production. Although the exact mode of action is unknown, the authors hypothesize that hamamelitannin does not inhibit AgrC, but rather affects cellular process upstream of *agr* signaling. Further mode of action studies are necessary, as they could lead to novel strategies for inhibiting QS in *S. aureus*. Ambuic acid **70** has been shown to reduce *agr* gene expression in *S. aureus* (Figure 27).[177] The authors suggest the mode of action is through inhibition of the AIP biosynthesis pathway, as AIP production is abolished; however, the exact target is unknown. Virulence was also affected, but the authors highlighted the need for more studies to generate more potent inhibitors.

Janda and co-workers have generated an alternative approach to AIP quorum quenching, which involves the generation of AIP antibodies.[178] The authors synthesized a rationally designed hapten based on AIP-IV to elicit an antibody immune response in mice. The hapten contained a lactone linkage rather than the natural thiolactone linkage in AIPs, as the authors speculated the thiolcatone may be predisposed to aminolysis and the formation of undesired degradation products in the immunization process. The antibody generated (AP4-24H11) was capable of reducing virulence production in *S. aureus* and inhibited the formation of abscesses in mice from *S. aureus*. AP4-24h11 also had a high binding affinity for AgrC-IV, which was specific for the peptide. Such immunopharmacotherapeutic strategies could provide an exciting alternative, or supplement, to receptor-based antagonists.

8. Summary and Outlook

Resistance to antibacterials has become a major issue over the past few decades, especially as the generation of new antibacterial agents seemingly dried up. As this Review has shown, all is not lost; there are many active avenues of research on-going to develop the next generation of anti-

bacterial drugs. The search for novel antibacterial drugs that are more effective and able to overcome bacterial resistance mechanisms is continuing in earnest, both in academic and small and medium-sized biotech settings, but also with renewed interest from large pharmaceutical companies. Recently identified natural products and those products originating from modified versions of existing antibacterial classes have shown significant promise in delivering more effective antibacterial agents. Initial research involving the combination of two active antibacterial agents into one hybrid compound has the potential to partially combat resistance mechanisms. Another area of enormous potential is the development of antimicrobial peptides and peptide mimics. These are believed to act on multiple targets, thereby possibly lowering the rate of evolution of resistance mechanisms. In addition to the methods discussed in this Review, many alternative approaches are also being explored as ways to potentially combat antibacterial resistance; these include modulating or stimulating immunity,[179] combination therapies, and the re-development of off-patent drugs or discarded drug candidates.

The majority of existing antibacterial drugs on the market or in clinical development today only focus on a small number of biological targets. With an increasing incidence of resistance to the drugs that act upon those few targets, there is an urgent need to search for novel targets. The identification of new biological targets and approaches that could lead to antibacterials with a novel mode of action against processes such as bacterial biosynthesis, essential metabolic pathways, disrupting bacterial membrane function,[180] or cell-wall biosynthesis are critical and active areas of research. One interesting example is the discovery of the novel target FabF 1B, which is involved in lipid biosynthesis.[181] It is hoped that resistance would develop slower for drugs acting with a novel mechanism, but, from an evolutionary perspective, that might prove to be a naïve leap of faith. Quorum sensing (QS) may be one physiological process that offers an interesting array of novel targets to explore as antibacterial agents.

Compared to the large array of AHL QS inhibiting strategies, the AIP and quinolone QS quenching strategies still remain relatively untapped sources. PqsR antagonists are sparse and need to be further expanded, including quinolone inhibitors and non-quinolone inhibitors. Quinolone analogues should provide compounds with extremely interesting biological activity considering their dual properties, including: PqsR activation, antibacterial activity, membrane vesicle formation, and their involvement in interspecies signaling. Another advantage of quinolone analogues is that quinolone antibacterials (such as ciprofloxacin) are currently used as drugs. Therefore, it could be that quinolone quorum quenching compounds that are cytotoxic to bacterial cells but have desirable pharmacokinetics could be developed. Other targets for quinolone quorum quenching also need to be investigated. Such targets could include PqsB, PqsC, PqsD, and possibly PqsE. The synthesis of AIP analogues and their modulation of AgrC receptors are well-represented in the literature. However, other methods of AIP quorum quenching are relatively few and the specific target is often unknown.

Inhibitors for other targets of the AIP QS pathway also need to be explored further and could include kinase inhibitors for AgrC, inhibition of the export of AIPs via AgrB, and the inhibition of enzymes involved in the posttranslational modification of AgrD. Such strategies should lead to the development of novel QS quenching compounds and are an exciting prospect for the future.

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