Next generation bacitracin: reimagining a classic antibiotic

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1

Targeting membrane-bound bacterial cell wall precursors: A tried and true antibiotic strategy in nature and the clinic

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Abstract

Since Fleming's discovery of penicillin nearly a century ago, a bounty of natural product antibiotics have been discovered, many of which continue to be of clinical importance today. The structural diversity encountered among nature's repertoire of antibiotics is mirrored by the varying mechanisms of action by which they selectively target and kill bacterial cells. The ability for bacteria to construct and maintain a strong cell wall is essential for their robust growth and survival under a range of conditions. However, the need to maintain the cell wall also presents a vulnerability that is exploited by many natural antibiotics. Bacterial cell wall biosynthesis involves both the construction of complex membrane-bound precursor molecules and their subsequent crosslinking by dedicated enzymes. Interestingly, many naturally occurring antibiotics function not by directly inhibiting the enzymes associated with cell wall biosynthesis, but rather by binding tightly to their membrane-bound substrates. Such substrate sequestration mechanisms are comparatively rare outside of the antibiotics space with most small-molecule drug discovery programs instead aimed at developing inhibitors of target enzymes. In this chapter we provide the reader with an overview of the unique and ever increasing family of natural product antibiotics known to specifically function by binding to membrane-anchored bacterial cell wall precursors. In doing so, we highlight both our own contributions to the field as well as those made by other researchers engaged in exploring the potential offered by antibiotics that target bacterial cell wall precursors.

Introduction

The bacterial cell wall has historically been, and continues to be, one of the most important targets for antibacterial drugs. Despite the range of developments and advancements made in the antibiotics field since penicillin was first brought to the clinic, natural compounds that interfere with bacterial cell wall biosynthesis remain a cornerstone of the world's antibiotic arsenal.[1,2] Given the well-documented increase in antibiotic resistance, there is also a renewed interest in natural product antibiotics discovered in decades past, which in some cases were assumed to be unsuitable for use as systemic drugs due to innate toxicity or unfavourable pharmacokinetics and -dynamics. Recently, modern techniques have allowed for the re-examination of such compounds and in some cases revealed novel mechanisms and targets involving bacterial cell wall biosynthesis.[3] In parallel, modern semisynthetic approaches also offer the potential to overcome pharmacological or toxicological issues associated with some natural product antibiotics while maintaining, or even improving, their antibacterial efficacy.

The majority of approved drugs, including antibiotics, function by inhibiting enzymes that contribute to a disease of interest. However, within the world of antibiotics that interfere with bacterial cell wall biosynthesis, there are some that function by binding to and sequestering the biomolecular substrates of the cell wall synthesis enzymes, rather than the enzymes themselves. These membrane-bound, polyprenylated intermediates of peptidoglycan (PG) synthesis are produced and consumed as part of the tightly regulated cell wall biosynthesis cycle. Antibiotics that bind to these PG precursors, and in doing so remove them from the available cellular pool, can elicit potent antibacterial effects. Furthermore, given that PG precursors are non-proteinogenic, they are not easily mutated or changed. Also of note, these lipid-linked PG intermediates are synthesized on the inner leaflet of the cytoplasmic membrane, before being "flipped" to the outer leaflet, allowing large, often peptide-based, antibiotics to bind to them without needing to permeate the cell membrane.

This chapter will focus on natural product antibiotics (and compounds informed by natural products) of MW <5000 Da that specifically function by sequestering the key membrane-bound intermediates that are encountered on the outer bacterial cell surface during cell wall biosynthesis. These include: undecaprenyl phosphate ($C_{55}P$), undecaprenyl pyrophosphate ($C_{55}PP$), and lipid II. These targets are all fundamental to cell wall biosynthesis, unique to bacteria, and highly conserved throughout the bacterial world.[2] Several antibiotics that function by sequestering these polyprenylated biomolecules are amongst the most important, clinically relevant last-resort antibiotics, most notably vancomycin and ramoplanin.[3,4]

Bacterial cell wall biosynthesis

The cell wall (**Fig. 1**) of all bacteria is comprised of a glycopeptide polymer, peptidoglycan, whose basic subunit comprises a repeating disaccharide of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc).[5] The D-lactoyl group of each MurNAc residue is appended to a peptide whose composition is most often L-Ala-γ-D-Glu-L-Lys (or *meso*-2,6-diaminopimelic acid (*m*-DAP)) -D-Ala-D-Ala.[6,7] Polymerisation occurs through two types of covalent bonds. First, linear glycan strands are formed via the β-1,4-glycosidic bond between alternating MurNAc and GlcNAc sugars.[7] Secondly, the pentapeptide strands attached to each MurNAc unit are cross-linked via amide bond formation. Most commonly, peptide cross-linking occurs between the amino group of the ε-amino acid at position 3 of one peptide strand, and the carboxyl group of D-Ala at position 4 of another peptide strand (with concomitant removal of the D-Ala at position 5).[6,7] This cross-linking creates the web-like structure of PG, and is responsible for the mechanical strength that confers cell rigidity.[3,7] This is critical, as the main function of this giant, cell-sized macromolecule is to resist the effect of the otherwise lethal internal osmotic pressure.[5,8,9] Although Gram-negative and -positive bacteria exhibit significant diversity in their cell wall thickness (90% dry weight in Gram-positive vs. 10% in Gram-negative), the central lipid II cycle is highly conserved throughout the bacterial world.[2]

Bacterial cell wall biosynthesis is a complex cycle of interdependent enzymatic reactions (**Fig. 1**). The process begins in the cytoplasm with UDP-GlcNAc, which, following six sequential steps mediated by the MurA-F synthetases, generates UDP-MurNAc-pentapeptide, the last soluble cell wall precursor.[10] The MurNAc-pentapeptide portion is then transferred by the membrane protein MraY translocase to the membrane bound phospholipid undecaprenyl phosphate ($C_{55}P$) generating lipid I.[8,11]

From lipid I, a GlcNAc unit is ligated via a 1,4-glycosidic bond, mediated by the peripheral membrane glycosyltransferase, MurG.[3,12] This generates lipid II, which contains the completed peptidoglycan monomer (GlcNAc-MurNAc-pentapeptide), still attached to the undecaprenyl lipid carrier via a pyrophosphate moiety.[3,8,11] Lipid II is then translocated through the cytoplasmic membrane, before initiation of polymerisation by glycosyltransferases and subsequently transpeptidases, mediated by penicillin-binding proteins (PBPs), to produce the finished, cross-linked, peptidoglycan structure.[10,11,13,14] The resulting C_{55}-pyrophosphate is then dephosphorylated by UPP phosphotases (UppP/BacA) to reform C_{55}-phosphate, allowing the substrate to be recycled for another round through the biosynthetic cycle.[2,5,15–17]

Undecaprenyl phosphate ($C_{55}P$) is a unique biomolecule consisting of 11-isoprene subunits that is commonly referred to, together with its pyrophosphate form ($C_{55}PP$), as the universal lipid carrier.[14] $C_{55}P$ is utilised in numerous essential bacterial biosynthetic pathways. Peptidoglycan[18] provides structural integrity to the cell, teichoic acids[19] are important in the pathogenesis of Gram-positive bacteria, lipopolysaccharide[20] protects Gram-negatives from hydrophobic antibiotics, capsular polysaccharides[21] are virulence factors, colanic acid[22] promotes biofilm formation, and protein glycosylation[23] is involved in cell adhesion, protein stabilisation, and evasion of the host immune response. $C_{55}P$ appears under a variety of similar names in the literature, including C_{55}-isoprenyl phosphate, undecaprenyl phosphate, bactoprenyl phosphate, and will be used interchangeably in this chapter. Undecaprenyl phosphate functions as a transmembrane carrier of the precursors of peptidoglycan biosynthesis and is essential to the lipid II cycle. It serves to anchor peptidoglycan intermediates to the membrane and facilitates their crossing of the cytoplasmic membrane into the extracytoplasmic space. Here, additional processing and polymerisation into growing peptidoglycan occurs, after which $C_{55}P$ is recycled for additional cycles.[11,24]

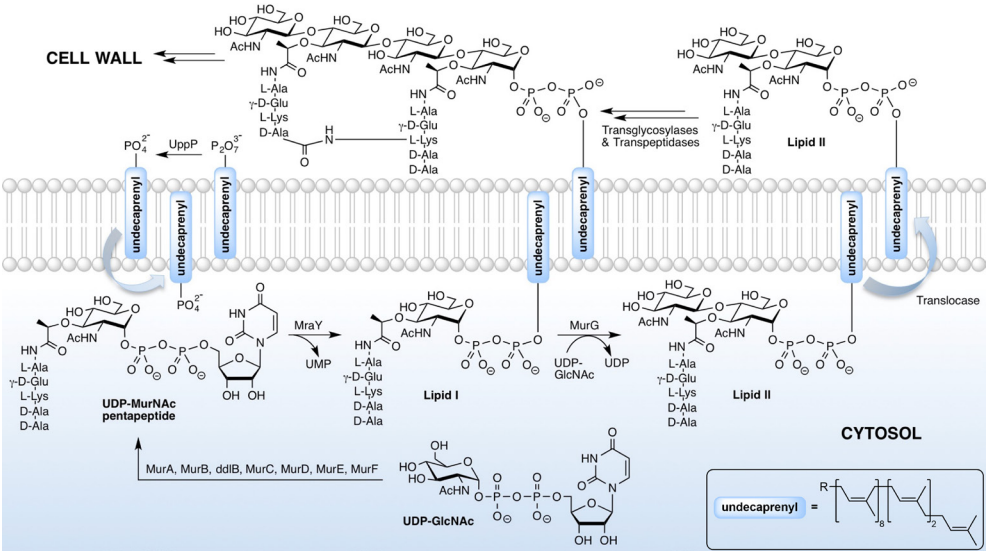


Figure 1. Schematic representation of cell wall biosynthesis. Synthesis commences in the cytoplasm with the conversion of UDP-GlcNAc to UDP-MurNAc-pentapeptide by sequential action of MurA to MurF enzymes. The first membrane bound intermediate, lipid I, is generated by MraY, before a GlcNAc moiety is ligated via a 1,4-glycosidic bond by MurG, generating lipid II. Next, lipid II is translocated across the membrane before being incorporated into the growing peptidoglycan by transglycosylation and transpeptidation, mediated by PBPs. The released $C_{55}PP$ is then dephosphorylated to $C_{55}P$, transported back into the cytoplasm and reused. The stempeptide shown contains L-Lys at position 3, which is representative of most Gram-positive bacteria. Gram-negative bacteria frequently bear *m*-DAP at this position. Other structural variations and modifications are known and have been reviewed elsewhere.[35]

The cell wall in Gram-positive bacteria is thought to be around 20 layers thick (Gram-negatives have ≈1.5 layers), with each individual layer compromising an enormous amount of peptidoglycan.[3] In stark contrast to this, only around 2×10^5 molecules of C_{55}-isoprenyl phosphate are present in any individual cell.[25–27] Therefore the lipid II cycle is a highly dynamic process, with each undecaprenyl lipid carrier involved in 1–3 transits through the cycle per second.[28] Consequently, this cycle also presents itself as a clear site of vulnerability and an ideal target for antibiotics.[3] In particular, the fact that C_{55}P, C_{55}PP and lipid II are all at some point localised in the extracytoplasmic space, makes them especially sensitive targets. Both these isoprenyl lipid-bound intermediates and the lipids themselves form a unique class of antibacterial targets. Indeed a great number of naturally occurring antibiotics target and tightly bind these polyprenylated biomolecules including vancomycin[29], nisin[30,31] and ramoplanin[32], which all bind lipid II, while bacitracin[33] sequesters C_{55}PP and friulimicin[34] does the same for C_{55}P. In this chapter, we will begin by discussing natural product antibiotics that specifically target C_{55}P and C_{55}PP followed by those that engage with the structurally more complex lipid II.

Undecaprenyl phosphate (C_{55}P)-targeting antibiotics

There are a number of lipopeptides known to target C_{55}P, two of which have been evaluated as human therapeutics, namely **amphomycin** and **friulimicin** (**Fig. 2**). This entire class of C_{55}P binders are part of the broader family of calcium dependent antibiotics (CDAs), with amphomycin the first reported in the literature.[36] In the subsequent decades, a series of structurally related compounds were described which can be divided into three classes. Namely, the amphomycins, the friulimicins, and the **glycinocins** (also called **laspartomcyins**). Structurally, these lipopeptide antibiotics all share a decapeptide macrocyclic core, bear the hallmark Asp-X-Asp-Gly motif that facilitates Ca^{2+} binding, contain a number of non-proteinogenic amino acids, and are lipid acylated at their N-termini.[37–40] This class of antibiotics is specifically active against Gram-positive bacteria.[38]

There is a rational naming scheme for the various members of the friulimicin family depending on the length of the lipid tail. We also recently proposed applying this nomenclature to clarify the structural similarities in the amphomycins (as shown in **Fig. 2**).[39] Indeed the friulimicins and amphomycins are structurally identical with the exception of the exocyclic Asp residue in amphomycin being replaced with an Asn in the equivalent friulimicin derivative. Early studies on amphomycin showed that its antibacterial activity involved inhibition of cell wall biosynthesis leading to a proposed mechanism based on the inhibition of MraY, a

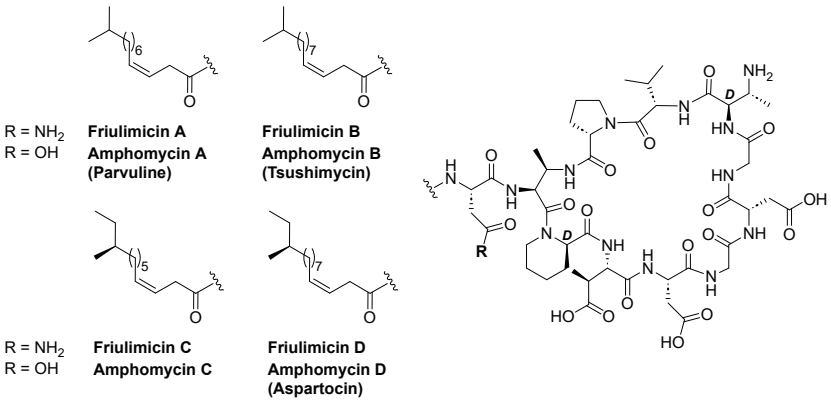


Figure 2. Structures of the friulimicin and aphomycin class of lipopeptide antibiotics.

UDP-MurNAc-pentapeptide phosphotransferase responsible for lipid I synthesis.[34] However, more recent publications from our group and others have convincingly demonstrated that members of this class of antibiotics inhibit cell wall biosynthesis by forming a defined complex with $C_{55}P$ and Ca^{2+}, thereby sequestering it and interrupting the lipid II cycle.[41–43]

The **glycinocins** (also called the **laspartomycins**) are the final subclass of the calcium dependent $C_{55}P$ binding lipopeptides. The first member of this class to be reported was isolated from *Streptomyces viridochromogenes* in 1968.[44] However, it was not until 2007 when the major component of this family, laspartomycin C, had its structure elucidated.[45] Separately, investigations into antimicrobial agents produced by a previously unidentified *Actinomycetes* species led to the discovery of the glycinocin family of CDAs. Subsequent research revealed that glycinocin A was structurally identical to laspartomycin C.[46] In a similar fashion to the amphomycins and friulimicins, other glycinocins, specifically B and C, have an identical peptide core and differ only in the length of their branched lipid acylated N-terminus (**Fig. 3A**). Glycinocin D bears an identical lipid tail to laspartomycin C but in place of Ile at position 10, it contains Val (analogous with the amphomycins and friulimicins).[46]

The first total synthesis of laspartomycin C was reported by our group in 2016, and we further demonstrated it to be a tight binder of $C_{55}P$ in the presence of Ca^{2+}.[43] In a follow-up study, the crystal structure of laspartomycin C bound to the water soluble prenyl phosphate ($C_{10}P$) was obtained (**Fig. 3B**).[47] This revealed that laspartomycin C forms a 1:1:2 complex with $C_{10}P$ and Ca^{2+}, providing a rationale for the previously observed tight binding. This work was notable in being both the first where a crystal structure of an antibiotic bound to a prenyl phosphate was obtained, and also the first of a CDA bound to its bacterial target. Payne and co-workers

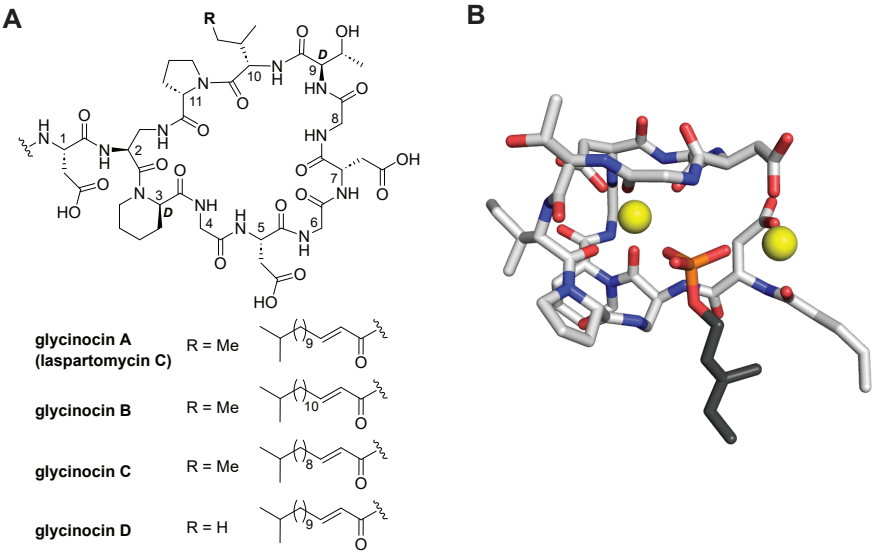


Figure 3. (A) Structures of the glycinocin (laspartomycin) family of lipopeptide antibiotics. **(B)** The crystal structure of glycinocin A (laspartomycin C) bound to $C_{10}P$, a water soluble prenyl phosphate, mediated by the coordination of 2 Ca^{2+} ions (yellow spheres). The $C_{10}P$ phosphate head group is coloured red and orange with the lipid tail in black (full chain of $C_{10}P$ not shown). Produced using PyMol from PDB file 5O0Z.[47]

subsequently also developed an effective total synthesis of the glycinocins.[48] Utilising this approach they prepared a small library of glycinocin derivatives bearing a variety of different fatty acyl substituents at the N-terminus.[38] These compounds were found to exhibit similar calcium dependence and antimicrobial activity as the parent compound. Chain length was determined to be the most important contributor to activity, with a sharp decrease in activity observed for acyl chains of less than 13 carbons.[38]

Recently, our group also prepared a series of hybrid CDAs to probe the impact of structural variations at residues 4, 9, and 10, which differ between the friulimicin/amphomycin and laspartomycin classes.[49] A surprising result from this investigation was that among lipopeptides resembling friulimicin/amphomycin, the introduction of Val at position 10 was strongly favoured compared to Ile at the same position, leading to an 8-fold lower MIC against methicillin-resistant *Staphylococcus aureus* (MRSA). The profound impact on activity associated with this remarkably subtle structural change was rationalised via analysis of the crystal structures of these analogues bound to $C_{10}P$. The slightly less bulky Val10 side chain (found naturally in the amphomycin/friulimicin class) allows for optimal packing of the peptide in the crystal structure allowing for enhanced interaction with the polyprenyl phosphate bacterial target, and is hypothesised to underscore the increased antibacterial activity observed.[49]

MX-2401 is a semisynthetic analogue of amphomycin that was taken into preclinical development for the treatment of serious Gram-positive infections in 2011.[50] MX-2401 is structurally different from amphomycin in two ways. Firstly, its lipid tail is 4-(dodecanamido) benzoic acid, rather than the Δ^3-*iso*-dodecenoic acid of the natural product. Secondly, the side chain amino group of Dab9 is acylated with a 3-aminopropinoyl group. Hancock and co-workers demonstrated that MX-2401's mechanism of action derives, like amphomycin, from its ability to bind $C_{55}P$ in a calcium dependent manner.[42] More recent NMR studies showed that when *S. aureus* cells are treated with MX-2401, a thinning of the cell wall, a decrease in D-alanine linked teichoic acids, and a reduction in peptidoglycan cross-linking are all observed.[51]

Undecaprenyl pyrophosphate ($C_{55}PP$)-targeting antibiotics

Undecaprenyl pyrophosphate ($C_{55}PP$) is released upon the transglycosylation of lipid II and is the direct precursor of $C_{55}P$. The only clinically used antibiotic that specifically binds $C_{55}PP$ is **bacitracin**.[52] The bacitracins are a family of closely related compounds, produced by strains of *Bacillus lichenformis* and *Bacillus subtilis*, the most abundant of which is bacitracin A (**Fig. 4A**).[53,54] Bacitracin was first described in the literature in 1945 and since then has been used extensively as both a topical human therapeutic and in livestock (as a growth promotor).[54–56] Bacitracin is a nonribosomal peptide antibiotic, with several D- and nonproteinogenic amino acids, and contains a heptapeptide ring formed by an amide bond linking the ε-amino group of Lys6 and the C-terminus of the peptide (Asn12).[57] Bacitracin has an exocyclic pentapeptide sidechain terminating in an aminothiazoline dipeptide moiety that is formed by the enzymatic condensation of Ile1 and Cys2.[58]

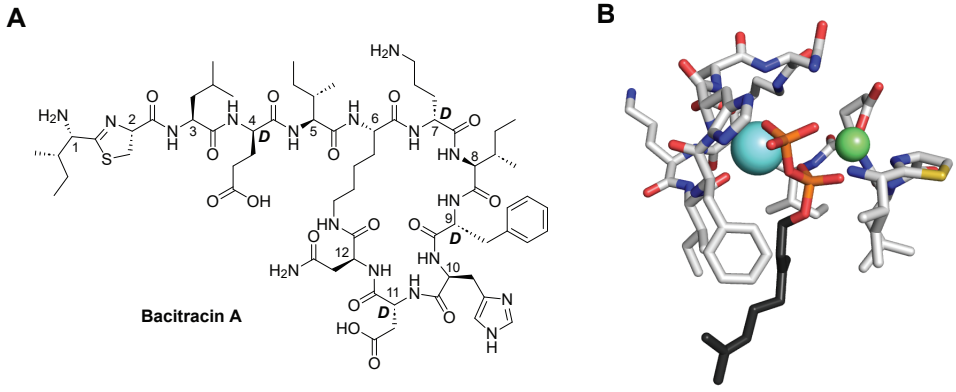


Figure 4. (A) The chemical structure of bacitracin A. **(B)** Crystal structure of bacitracin A bound to $C_{10}PP$ mediated by Zn^{2+} (green) and Na^+ (blue). The $C_{10}PP$ pyrophosphate head group is coloured red and orange with the lipid tail in black. Produced using PyMol from PDB file 4K7T.[59]

Bacitracin requires divalent metal ions for activity, with Zn^{2+} offering the highest potency. Bacitracin's affinity for, and activity with, various metal ions has been extensively studied.[33,52,60] Bacitracin's efficacy derives from its ability to form a well-defined ternary complex with C_{55}PP and a divalent metal ion, thereby sequestering C_{55}PP and preventing its subsequent dephosphorylation and recycling back into the lipid II cycle.[33,61–64] A recent crystal structure of bacitracin and Zn^{2+} bound to C_{10}PP revealed that bacitracin completely envelops the pyrophosphate head group of the lipid (**Fig. 4B**).[59] The Zn^{2+} ion bridges the peptide and pyrophosphate, with the aminothiazoline playing an important role in Zn^{2+} coordination. The crystal structure also clearly shows that when complexed to Zn^{2+} and C_{10}PP, bacitracin adopts an amphipathic conformation wherein all hydrophilic side chains are aligned on one face of the complex and all hydrophobic side chains on the other, presumably oriented toward the bacterial membrane, thus providing clues to its antibiotic mechanism.[59]

Resistance to bacitracin is known and, in fact, bacitracin resistance genes have even been identified in cave bacteria isolated from the outside world for 4 million years.[65] However, despite the clearly long history of bacitracin, and its more recent extensive use in the livestock industry, widespread and problematic resistance has not occurred.[52] Bacitracin's uses as a human therapeutic has been limited primarily to topical uses due to the nephrotoxicity of its degradation product, bacitracin F, which forms due to the oxidative deamination of the aminothiazoline moiety to a ketothiazole.[66–69]

Various syntheses of bacitracin have been attempted over the years, but have been hampered by the stereochemical fragility of the aminothiazoline moiety, which is prone to epimerisation, generating less potent isomers of bacitracin.[70–73] A total synthesis of bacitracin A was reported in 1996 by Griffin and co-workers where, using HPLC co-injections and NMR, they were able to show they could separate the correct, natural-occurring diastereomer.[74] By using this approach, they also went on to synthesise the enantiomer of bacitracin A, which was found to be equipotent to the natural product.[75] This further supports the proposed mechanism of bacitracin, considering the achiral nature of C_{55}PP. In 2006, Marahiel and co-workers employed a chemoenzymatic route to a range of derivatives of bacitracin featuring alternative N-terminal heterocycles.[76] While these analogues did display some antibacterial activity, in all cases they were less active than the natural product, demonstrating the importance of the aminothiazoline moiety. Recent work from our group led to an improved total synthesis of bacitracin that suppresses the problematic diastereomer formation (discussed in more detail in **Chapter 2**).[77] A series of novel bacitracin analogues were also prepared to investigate the possibility of replacing the N-terminal aminothiazoline moiety with alternate zinc-binding motifs and acyclic dipeptides. These new analogues were found to be inactive as antibacterials,

again illustrating the essentiality of the aminothiazoline moiety (see **Chapter 3)**.

Aside from bacitracin, little is known about other natural products that specifically target C_{55}PP. An additional class with this ability may be the **tripropeptins**, a group of cyclic calcium-dependent lipopeptide antibiotics with activity against Gram-positive bacteria (**Fig. 5**).[78] Using mass spectrometry and thin-layer chromatography-based assays, Hashizume and co-workers showed that tripropeptin C inhibits the lipid II cycle by binding C_{55}PP.[79] Unlike the calcium-dependent amphomycins and laspartomycins discussed above, tripropeptin C does not show affinity for C_{55}P. Also of note, mechanistic studies with the structurally similar empedopeptins indicate that they bind both C_{55}PP and lipid II (see discussion below). Whether the same is true for the tripropeptins, or if they are exclusive C_{55}PP binders, remains to be fully established.

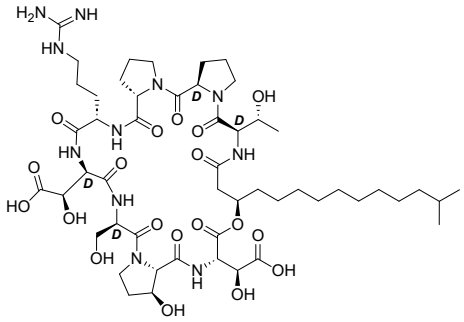


Figure 5. Chemical structure of tripropeptin C.

Lipid II-targeting antibiotics

Lipid II is by far the most important and widely exploited antibiotic target of those discussed in this chapter. Its structure is much more complex than $C_{55}P$ and $C_{55}PP$, as it contains both the disaccharide and peptide moieties that make up the cell wall monomer, attached to its lipid anchor via the pyrophosphate linkage (**Fig. 6**). This structural diversity provides a variety of binding epitopes that are exploited by a number of naturally occurring antibiotics. In fact, due to the large variety of antibiotic classes that target lipid II, it is often referred to as the bacterial "Achilles' heel".[3]

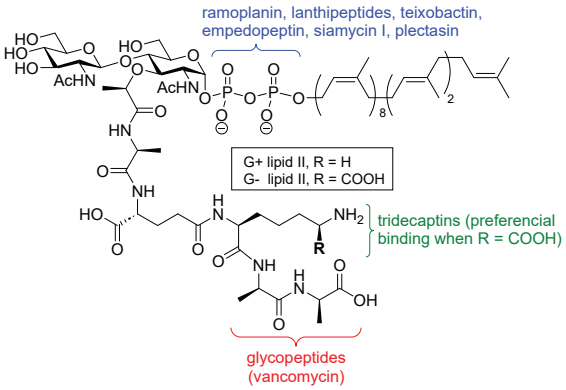


Figure 6. Lipid II including antibacterials with known specific lipid II binding motifs.

Vancomycin and the glycopeptide antibiotics

Vancomycin (**Fig. 7**) was the first glycopeptide antibiotic discovered and has been in clinical circulation since 1958. It is on the World Health Organization's list of essential medicines, highlighting its importance to human medicine. It is administered intravenously as a 'last resort' antibiotic for the treatment of severe Gram-positive infections.[80] It was first isolated in 1955 from *Streptomyces orientalis*, a fungus found in Bornean soil samples.[81] Despite extensive studies, it was not until 1983 that the structure of vancomycin was determined conclusively, at which point it had already been used in the clinic for 20 years.[82] Vancomycin is a heptapeptide, where five of the seven residues contain an aromatic side chain, each with a degree of oxidative cross-linking that forms the tricyclic ring structure and the associated distinctive three-dimensional conformation. The central residue is further modified with a glucose-vancosamine disaccharide moiety. The *N*-methylated N-terminal leucine residue is also essential for antibiotic activity.[83]

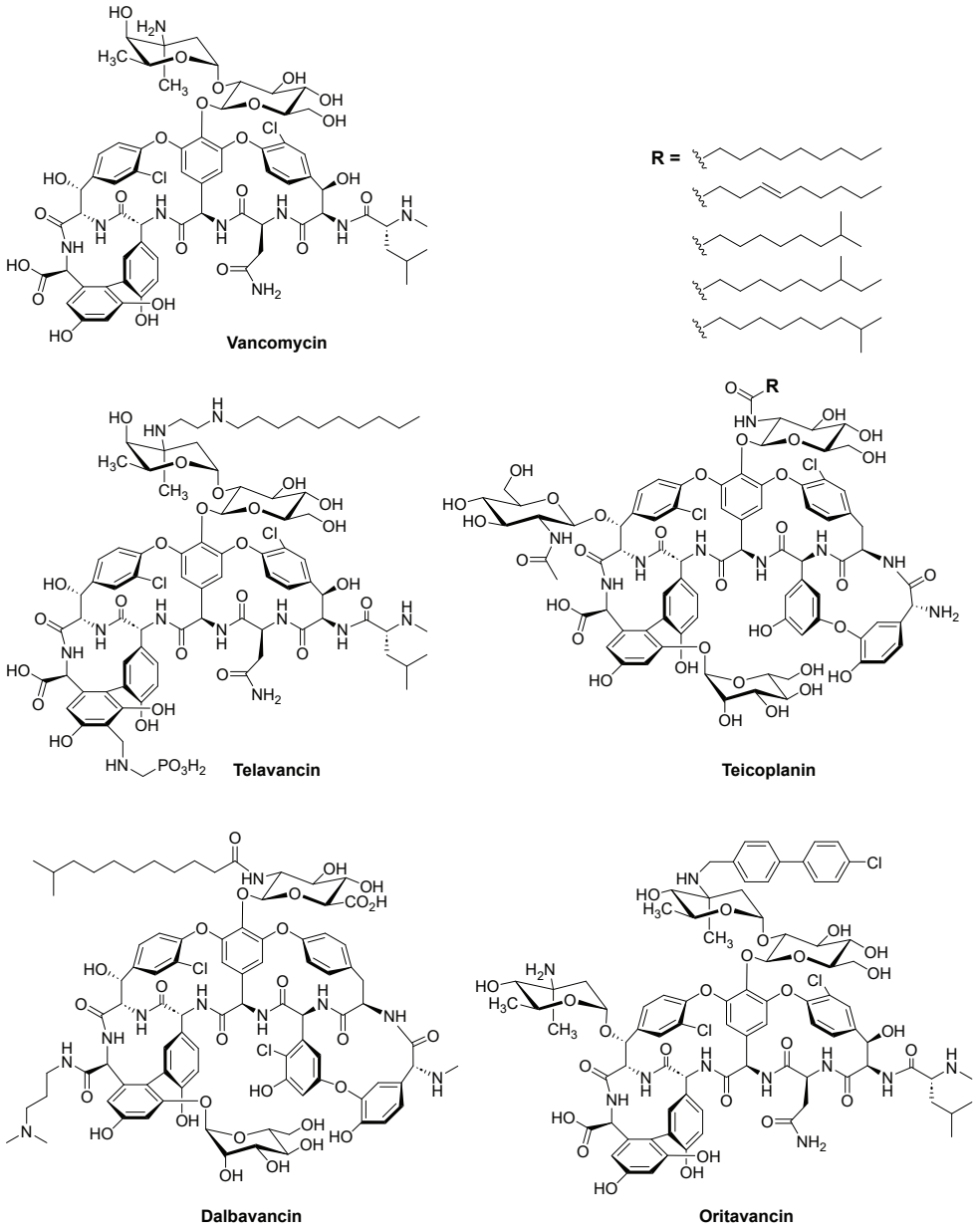


Figure 7. Structures of the glycopeptides vancomycin and teicoplanin, and the semisynthetic analogues telavancin, dalbavancin and oritavancin.

Studies into the mode of action of vancomycin observed an accumulation of UDP-MurNAc-pentapeptide in bacterial cells treated with vancomycin, indicating that it was targeting one or more of the late stages in cell wall biosynthesis.[84] In pursuit of vancomycin's target, Perkins already noted in 1969 that the simplest PG precursor fragment that vancomycin showed affinity for was acetyl-D-alanyl-D-alanine.[85] Further studies by Butcher, Hammond, and Williams confirmed that vancomycin does indeed have high affinity for binding to D-Ala-D-Ala, facilitated by hydrogen bonding.[86–88] They correctly concluded that vancomycin binding to the peptide portion of lipid I and lipid II inhibited cell wall synthesis, resulting in cell lysis and death. The hydrogen bonding interactions that govern the interaction were subsequently visualised by Aoki and co-workers who solved the X-ray crystal structure of vancomycin bound to the relevant lipid II peptide fragment (**Fig. 8A**).[89]

Resistance to vancomycin began to gain headlines in the late 1980s when the first vancomycin-resistant *Enterococci* (VRE) strains were reported.[90,91] Vancomycin resistance has now also been identified in *Staphylococcus aureus*, *Streptococcus bovis*, and *Clostridium difficile*. Resistance primarily arises through acquisition of genes that enable bacteria to produce a lipid II variant that incorporates a terminal D-Ala-D-Lac or D-Ala-D-Ser moiety in place of D-Ala-D-Ala. This in turn leads to loss of a key hydrogen bonding interaction that greatly diminishes the binding affinity of vancomycin for the modified lipid II target and results in the loss of antibacterial activity (**Fig. 8B**).[92]

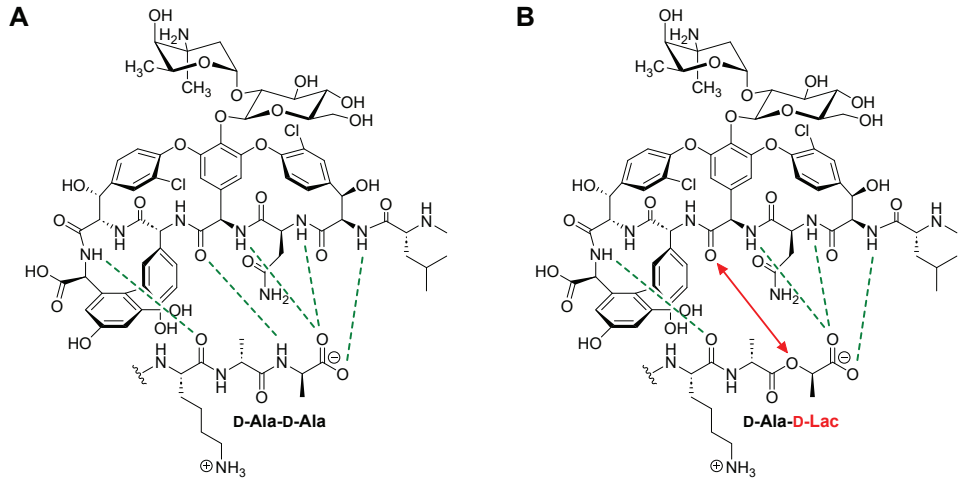


Figure 8. (A) Vancomycin's recognition of the D-Ala-D-Ala terminus of lipid II is governed by five hydrogen bonds (shown in green). **(B)** Vancomycin resistance genes result in a mutation in the pentapeptide sequence of lipid II to terminate with D-Ala-D-Lac, eliminating one of the hydrogen bonds and disfavouring the binding of vancomycin to lipid II (repulsive interaction indicated with red double headed arrow).

Teicoplanin (**Fig. 7**) was first isolated in 1978 from *Actinoplanes teichomyceticus* and has been used clinically since 1988.[93] It is structurally similar to vancomycin as it also contains a heptapeptide backbone but in this case all seven amino acids have aromatic side chains that are linked through oxidative cross couplings to give a tetracyclic structure. In place of a disaccharide, the phenolic side chain of the central amino acid is substituted with a single glucosamine bearing an *N*-linked fatty acid, whose length and degree of branching is analogue dependent. Teicoplanin also contains additional monosaccharide units consisting of glucosamine attached to the chain of residue 6 and glucose on the side chain of residue 7.[94] Teicoplanin exerts its bactericidal activity through the same mechanism as vancomycin: by binding to the D-Ala-D-Ala subunit of lipid II and inhibiting cell wall synthesis.[95]

In response to vancomycin and teicoplanin resistance, semisynthetic glycopeptides have also been developed and brought to the clinic (**Fig. 7**). Inspired by the increased activity of teicoplanin relative to vancomycin, these semisynthetic next-generation glycopeptides contain hydrophobic substituents that impart enhanced antibacterial activity, presumably due to productive membrane anchoring effects.[96–98] The first semisynthetic glycopeptide to receive clinical approval was **telavancin**. Starting from vancomycin, telavancin is synthesised by the addition of a hydrophobic side chain at the vancosamine moiety along with the introduction of a hydrophilic phosphonate group on the aromatic side chain of the C-terminal residue.[99] These features were found to provide enhanced antibacterial activity and improved drug bioavailability and distribution. **Dalbavancin** is a derivative of the teicoplanin-like natural product termed A40926, isolated from *Nonomuraea sp.* ATCC 39727.[100] The number and identity of monosaccharide units differs compared to teicoplanin as well as the methylated N-terminus. The semisynthetic preparation of dalbavancin from A40926 centres around the addition of the (3-dimethylamino)-1-propylamine moiety via amide bond formation at the C-terminal carboxyl group. Dalbavancin has improved activity compared to vancomycin against a majority of Gram-positive pathogens and an extremely long *in vivo* $t_{½}$ of >300 hours allowing for once-weekly dosing.[101] The third clinically used semisynthetic glycopeptide is **oritavancin**, a semisynthetic derivative of the natural product chloroeremomycin that is isolated from fermentation of *Amycolatopsis orientalis*.[102] The preparation of oritavancin from chloroeremomycin involves addition of the highly hydrophobic *p*-chlorophenylbenzyl substituent to the 4-epi-vancosamine moiety of the disaccharide attached to the phenolic side chain of the central amino acid. As in the case of dalbavancin, oritavcin also exhibits unusual pharmacokinetics with an *in vivo* $t_{½}$ of 200–300 hours.[103] The glycopeptides are a prime example in demonstrating the power and potential of combining natural product discovery with chemical synthesis to optimise drug properties and bring new therapeutic agents to the clinic.

Ramoplanin

The **ramoplanins**, consisting of ramoplanin A_1, A_2, and A_3 (**Fig. 9**) were first isolated from a species of *Actinomycetes* in 1984.[104,105] Technically also a class of glycopeptides, the ramoplanins are non-ribosomal cyclic depsipeptides with a relatively large 17-mer ring that is further decorated with a dimannose moiety. The peptide macrocycle contains seven residues in the D-configuration and several non-proteogenic amino acids: two ornithine, five hydroxyphenylglycine, one chlorohydroxyphenylglycine, one β-hydroxyasparagine and three *allo*-threonine residues.[104] The N-terminus bears an α,β,γ,δ-unsaturated lipid tail, the exact structure of which varies between the three (A_1, A_2, and A_3) factors. All three ramoplanins contain a dimannose unit, linked through the side chain of hydroxyphenylglycine at position 11. Ramoplanin displays potent activity against a broad spectrum of Gram-positive pathogens. One study found it to be four- to eight-fold more active than vancomycin against over 500 tested strains.[106] Crucially, it is active against MRSA, VRE, and metronidazole-resistant *C. difficile* but is not effective against the Gram-negative pathogens tested.[107–111] Ramoplanin is currently being evaluated in clinical trials for the treatment of vancomycin-resistant *enterococci* (VRE) and *Clostridium difficile* infections. Early studies into the mechanism of action of ramoplanin demonstrated that, in bacterial cells treated with the antibiotic, there was an accumulation of UDP-MurNAc-pentapeptide, indicating that it inhibits peptidoglycan biosynthesis. It was accepted for many years that ramoplanin was binding solely to lipid I, blocking MurG conversion of lipid I to lipid II.[112] However, Sahl and co-workers were the first to show that ramoplanin has affinity for both lipid I and lipid II through TLC binding assays.[113] In 2000, Walker and co-workers provided further evidence that lipid I was not ramoplanin's only target. Using radiolabelled lipid II, they could monitor the conversion of lipid II to peptidoglycan. In the presence of ramoplanin, lipid II was sequestered, proving that the antibiotic blocks the transglycosylation step of peptidoglycan synthesis. Subsequent NMR investigations established that ramoplanin binds lipid II with a 1:1 stoichiometry.[114] McCafferty and co-workers determined that the minimal structural feature of lipid II required for complexation with ramoplanin consists of the pyrophosphate-linked MurNAc-Ala-γ-D-Glu moiety.[115] This revealed a binding mode distinct from that of the vancomycin-like glycopeptides. **Enduracidin** (**Fig. 9**) is a sister compound of ramoplanin. Residues 1, 2, 9, 10, 12, 13, 15 and 17 deviate from those in ramoplanin and it also lacks the dimannosylation of residue 11.[116] While it has not garnered the same level of clinical interest as ramoplanin, experimental investigations show that enduracidin acts through the same lipid II mediated mechanism.[117]

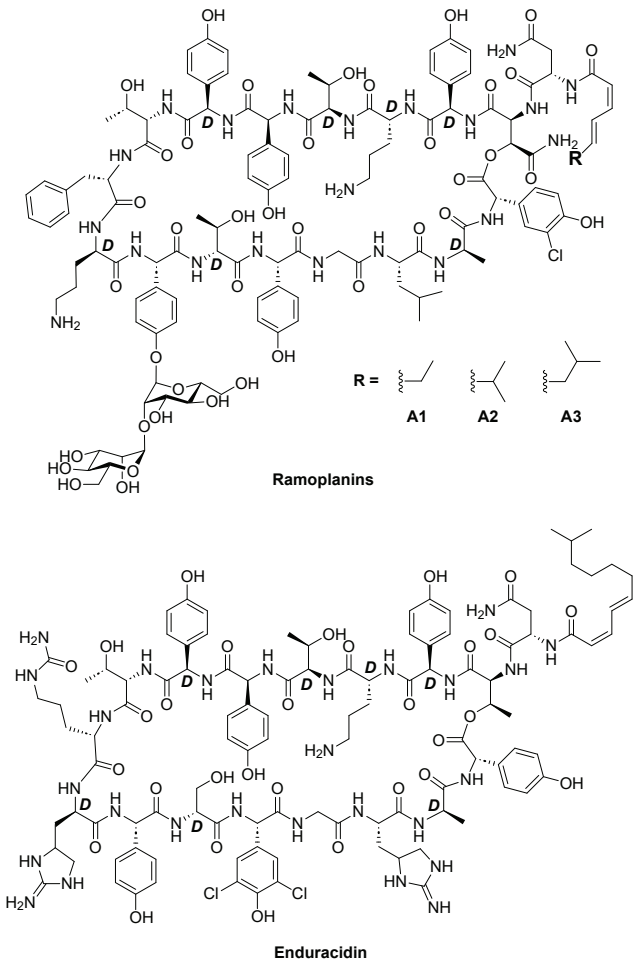


Figure 9. Chemical structures of the ramoplanins and enduracidin.

Lanthipeptides

The **lanthipeptides** are part of the broader family of ribosomally synthesised and post-translationally-modified peptide (RiPP) natural products. While lanthipeptides can contain a variety of posttranslational modifications, they all share distinctive β-thioether cross-linkages, commonly known as lanthionine bridges, formed by the enzyme-mediated Michael addition of a cysteine sulfhydryl group to a neighbouring dehydroalanine or dehydrobutyrine residue (formed by dehydration of serine and threonine respectively).[118–121] Notably, a significant number of lanthipeptides exhibit potent antibiotic activity. Many of these so-called lantibiotics (*lan*thionine-containing an*tibiotic*) target and kill bacteria by specifically binding to lipid II.

More than 60 lantibiotics have been described and they are commonly sorted into one of two distinct categories, namely the type A (nisin-like) lantibiotics which are elongated and positively charged, and type B (mersacidin-like) lantibiotics that are globular with a slight negative charge or neutral under physiological conditions.[122,123] The prototypical example of a type A lantibiotic is **nisin** (**Fig. 10A**), which was reported the same year in which penicillin was discovered.[124] However, it wasn't until 1971 that nisin's structure was elucidated.[125] Nisin, a widely used food preservative, is a potent antibiotic that displays a broad spectrum of activity against Gram-positive bacteria and is produced by *Lactococcus lactis*.[120,126] Nisin has a unique and well characterised dual mode of action involving lipid II targeted disruption of cell wall biosynthesis and pore formation in the cytoplasmic membrane.[9,113,127–129] Hsu *et al.* showed that the first ten N-terminal amino acids comprising the nisin A/B ring system form a cage that envelops the pyrophosphate moiety of lipid II with a unique mechanism not observed in other classes of antibiotics.[9,130] This sequestration of lipid II effectively prevents its further processing, halting peptidoglycan synthesis.[35,126] More recently, lipid II binding studies conducted in our group using isothermal titration calorimetry (ITC) further confirmed that the pyrophosphate moiety of lipid II is the primary binding site of nisin, and also showed that the complete MurNAc unit is required for high-affinity interaction.[30] This explains why nisin has a similar association constant for lipid I and lipid II. Although lipid I lacks the GlcNAc moiety found in lipid II, this is not involved in binding. Upon binding lipid II on the outer leaflet of the cell membrane, nisin's C-terminal region, containing the C-E rings, can insert into the lipid bilayer facilitating the assembly of a nisin-lipid II complex that stabilises pore formation and ultimately results in bacterial cell death.[31,131–134] This dual mechanism makes nisin a potent antibiotic and may go some way to explaining why, despite extensive use in the food industry, only limited reports of resistance to nisin have emerged.[26] However, despite these clear advantages of nisin, poor pharmacokinetics due to rapid proteolysis *in vivo*, limited solubility above pH 6, and immunogenicity issues have curtailed its use as a human therapeutic.[123,135,136] To address this issue, our group recently reported a chemoenzymatic approach generating semisynthetic nisin-derived antibiotics with improved stability.[137] While the C-terminal region of nisin (responsible for membrane insertion) is rapidly proteolyzed, the N-terminal A-B ring motif that drives nisin's binding to lipid II is comparatively stable.[138] With this in mind, we used trypsin to digest nisin to provide access to the A-B ring fragment after which a variety of lipids where coupled to mimic the membrane active C-terminal region.[137] This approach yielded semisynthetic derivatives of nisin with similar antibacterial activities and greatly improved proteolytic stability.

Mersacidin (**Fig. 10B**), and related type B lantibiotics, have a globular structure and also function by sequestration of lipid II, but lack the secondary pore-forming mechanism

common to nisin and other nisin-like class of lantibiotics.[1] Also unlike nisin, mersacidin carries no net charge and requires calcium ions for full activity.[139] Mersacidin binds lipid II primarily at the pyrophosphate moiety, though the presence of additional structural features such as the MurNAc sugar and polyprenyl lipid enhance binding, an effect also observed for nisin.[31,126]

Also of interest are the two-component lantibiotics as typified by **lacticin 3147** (**Fig. 10C**) in which two distinct lanthipeptides, each with negligible inherent antibacterial activity, elicit

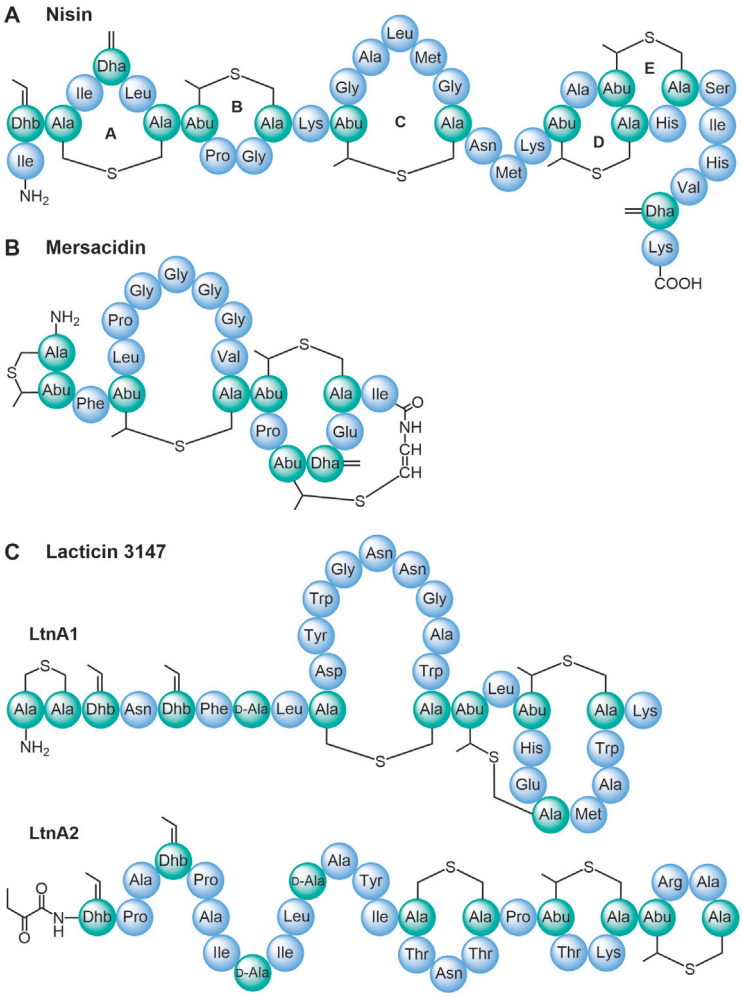


Figure 10. (A) The structure of the lantibiotic nisin with the rings A-E labelled. **(B)** The structure of mersacidin. **(C)** The structures of the two components of lacticin 3147, namely LtnA1 and LtnA2. Blue spheres represent proteogenic amino acids, whilst green spheres represent post-translationally modified amino acids. Dhb = dehydrobutyrine, Dha = dehydroalanine, Abu = aminobutyric acid.

a potent antibiotic effect when combined. Using a variety of biophysical and biochemical approaches it was shown that while only the LtnA1 peptide binds to lipid II, the LtnA2 peptide is also required for potent killing via lysis of the bacterial cell.[140,141] Since the discovery and structure elucidation of lacticin 3147, a growing number of two-component lantibiotics have been reported, further revealing the diversity of this unique class.[142–148]

Teixobactin

Reported by Lewis and co-workers in 2015, **teixobactin** (**Fig. 11**) was isolated from *Eleftheria terrae*, a β-proteobacteria, cultured using isolation chip (iChip) technology.[149] The iChip enables the lab growth of otherwise 'unculturable' bacteria.[150] Teixobactin was shown to be an 11-mer depsipeptide containing four D-amino acids, a methyl phenylalanine, and the unusual L-*allo*-enduracididine residue. Enduracididine contains a cyclic guanidinium moiety and has only ever been isolated from antimicrobial peptides.[151] Teixobactin showed potent activity against Gram-positive bacteria exclusively, with the exception of a strain of *Escherichia coli* asmB1 which does not possess an effective outer membrane barrier. Resistance studies did not identify measurable resistance which, coupled with positive outcomes in animal disease models, has led to great enthusiasm within the field and enabled the progression of teixobactin into early clinical trials. The treatment of bacterial cells with teixobactin was found to cause accumulation of UDP-MurNAc-pentapeptide indicating that teixobactin inhibits one or more steps in the peptidoglycan biosynthetic pathway. Furthermore, addition of exogenous lipid II inhibited teixobactin activity against *S. aureus*. Subsequent ITC-based binding studies by our group clearly demonstrated that teixobactin binds to lipid II in model membranes with a measured K_D of 100 nM.[152] Notably, teixobactin exerts activity against VRE strains, demonstrating that its binding mode differs from that of vancomycin.[149] Recent solid-state NMR studies by Weingarth and co-workers have provided new insights in this regard, showing that teixobactin forms an overall 1:1 complex with lipid II. In doing so, the enduracididine moiety of teixobactin binds to the pyrophosphate sugar moiety of one lipid II molecule while its linear N-terminal peptide tail interacts with the pyrophosphate sugar moiety of a teixobactin:lipid II-complex, resulting in a β-sheet arrangement of bound teixobactin molecules.[153,154] Perhaps unsurprisingly, considering its mode of target recognition, teixobactin has been shown to also bind undecaprenyl pyrophosphate and the wall teichoic acid (WTA) precursor lipid III.[155] Recent studies have suggested that the bactericidal nature of teixobactin comes not only from the sequestration of lipid II, and thus from inhibiting the biosynthesis of peptidoglycan, but also from the resulting teixobactin fibril that displaces phospholipids, compromising the membrane integrity.[153,156,157]

Since the discovery of teixobactin, the structurally similar **hypeptin** and **clovibactin** have also been characterized (**Fig 11**). **Hypeptin** was originally isolated from *Pseudomonas* sp. PB-6269 in 1989 and was recently produced in larger quantities by application of the same iChip technology that facilitated the discovery of teixobactin.[158] Containing three fewer amino acids that teixobactin, hypeptin is an octadepsipeptide containing a C-terminal macrocyclic tetrapeptide with four of its eight residues being β-hydroxylated. Given its similarities to teixobactin in both structure and activity, Schneider and co-workers speculated that hypeptin exerts its bactericidal activity through a similar mechanism.[159] Indeed, the accumulation of UDP-MurNAc-pentapeptide in *S. aureus* treated with hypeptin indicated that this antibacterial acts by interfering with the late stages of peptidoglycan biosynthesis. Binding assays determined that pyrophosphate containing peptidoglycan precursors (C_{55}PP, lipid I, lipid II and lipid III_{WTA}) are all bound by hypeptin. The binding was considerably stronger

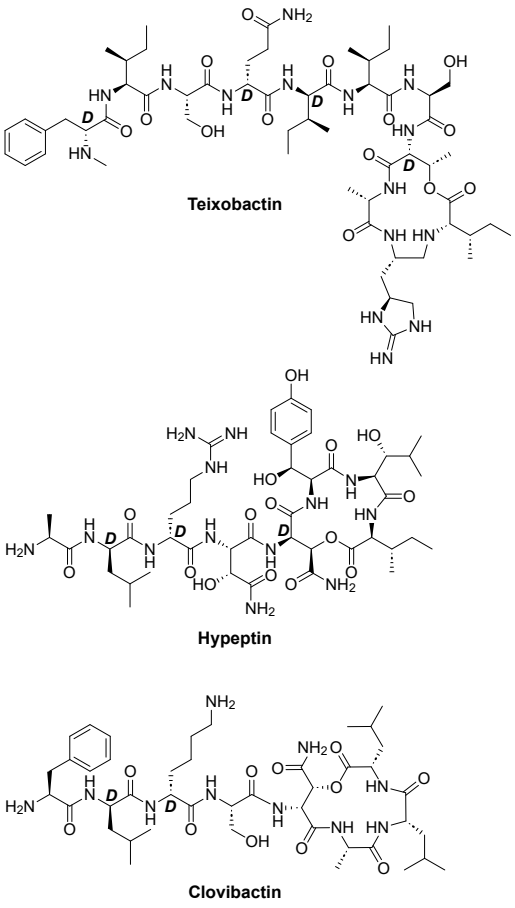


Figure 11. Chemical structures of teixobactin, hypeptin, and clovibactin.

(four-fold) to lipid I and lipid II than C_{55}PP and lipid III_{WTA}.[158] **Clovibactin**, also known as Novo29, is another teixobactin-like octadepsipeptide that was recently disclosed in the patent literature.[160] While clovibactin is reported to have potent anti-Gram-positive activity, as of yet no mechanistic studies have been reported. The Nowick group recently published the total synthesis of clovibactin that conclusively establishes its structure and provides reliable access to milligram quantities of the compound.[161]

Assorted macrocyclic peptides

In addition to the glycopeptides, lantibiotics, and teixobactins described above, a number of other lipid II-targeting macrocyclic peptide antibiotics are known. The following section highlights representative examples of these including the mannopeptimycin, lysobactin, empedopeptin, malacidin, and siamycin classes (**Fig. 12**).

In screens conducted in the 1950s, a series of antibacterial compounds were isolated from *Streptomyces hygroscopicus* but it was not until some 40 years later that their structures were elucidated to reveal the **mannopeptimycins** (**Fig. 12**).[162] These unique non-ribosomal peptide antibiotics are cyclic 6-mers wherein the residues sequentially alternate between L- and D- configurations. Three of the six amino acids are non-canonical including two β-hydroxyenduracididine residues and a β-methyl phenylalanine. These antibiotics get their name from the mannose disaccharide unit appended to the D-tyrosine portion and further mannosylation of the guanidine group at the D-β-hydroxyenduracididine residue. Semisynthetic modification of the mannose groups have yielded derivatives with excellent potency.[163–165] The mannopeptimycins act exclusively on Gram-positive bacteria, including VRE and MRSA.[166] Notably, they were found to lack activity against cell wall deficient *S. aureus*, indicating that their mechanism of action involves targeting some aspect of the bacterial cell wall. Investigations with a radioactive mannopeptimycin derivative demonstrated binding to both isolated and membrane-bound lipid II.[167] Competition assays with the pentapeptide portion of lipid II indicated that this is not the binding point of mannopeptimycins. Another competition assay with mersacidin, a disaccharide pyrophosphate-binding lantibiotic, showed that it did not disrupt mannopeptimycin binding to lipid II. These studies suggest that the mannopeptimycins may exploit a binding mode distinct from other lipid II-targeting antibiotics.[167] Further investigations are required to fully determine the precise binding configuration of mannopeptimycin to lipid II.

Lysobactin (katanosin B) (**Fig. 12**) was first isolated from *Lysobacter* sp. in 1988 and was shown to have potent activity (two- to four-fold more active than vancomycin) against a wide

range of Gram-positive bacteria.[168,169] Animal studies in mice also showed lysobactin to have good *in vivo* efficacy.[168] Early mechanistic studies showed that bacterial cells treated with lysobactin exhbited UDP-MurNAc-pentapeptide accumulation, indicating that the antibiotic interferes with cell wall biosynthesis. In the same studies it was also shown that addition of Ac-Lys-D-Ala-D-Ala did not antagonize lysobactin's bactericidal abilities.[170] Aside from work

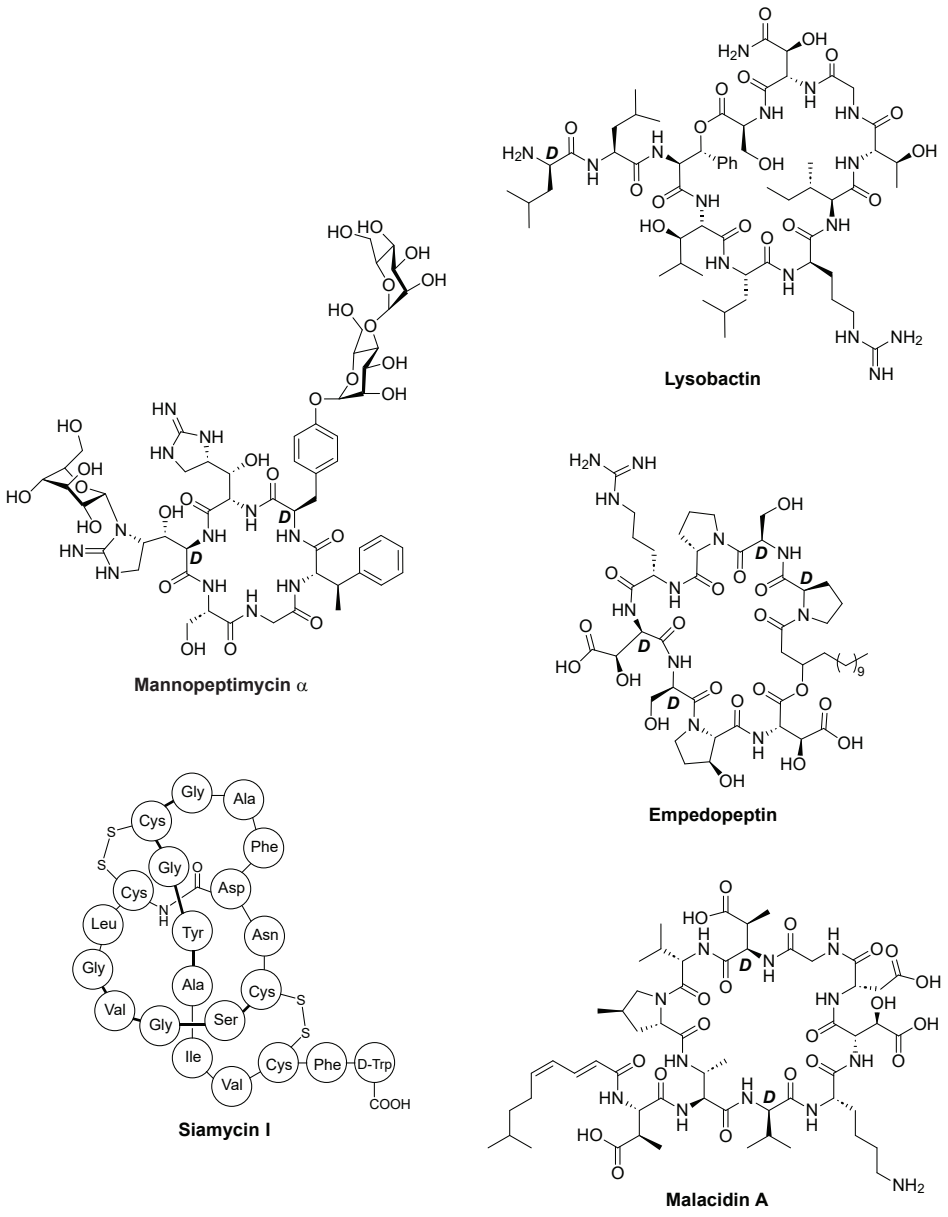


Figure 12. Chemical structures of mannopeptimycin α, lysobactin, empedopeptin, malacidin A, and siamycin I.

on its total synthesis,[171,172] there were few other studies into lysobactin until 2016 when Walker and co-workers published an in-depth investigation into its mode of action.[171] Through the use of enzyme inhibition assays for the key steps in peptidoglycan synthesis, it was shown that lysobactin forms 1:1 complexes with Lipid I, Lipid II, and Lipid II_A^{WTA}. This has led to the hypothesis that, like ramoplanin and teixobactin, lysobactin binds to the saccharide-pyrophosphate-polyprenyl portion of each intermediate.

Empedopeptin (**Fig. 12**) is a cyclic lipodepsipeptide, produced by Gram-negative soil bacteria, that was first identified in 1984.[173] Specifically, empedopeptin is a octapeptide lactone bearing a 3-hydroxymyristic acid lipid tail and is closely related to the previously discussed $C_{55}PP$ binder tripropeptin C.[174] A 2012 study revealed that empedopeptin sequesters lipid II in a calcium-dependent mechanism, blocking the incorporation of lipid II into peptidoglycan.[175] Empedopeptin appears to bind lipid II in a region that involves the pyrophosphate group, MurNAc, and elements of the stem peptide and undecaprenyl tail. In the same study, empedopeptin was shown to also bind $C_{55}PP$ and wall teichoic acid precursors, albeit with attenuated affinity.[175]

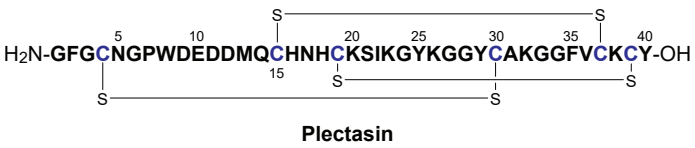
The **malacidins** (**Fig. 12**) are another class of calcium-dependent lipopeptides, first isolated from *Streptomyces albus* in 2018.[176] The two main variants, A and B, differ only in the structure of their N-terminal lipid tail. Although they lack the typical Asp-X-Asp-Gly calcium binding motif found in other calcium dependent lipopeptide antibiotics (e.g. laspartomycin C), strong affinity for calcium, which is necessary for antibiotic activity, was demonstrated. Malacidin has a wide spectrum of activity against Gram-positive pathogens, including MRSA, and was found to exhibit a low propensity to induce resistance. Fluorescent dye leakage studies indicate that malacidin is not likely to exert its activity through pore formation in the bacterial membrane. Rather, the accumulation of UDP-MurNAc-pentapeptide detected in bacterial cells treated with malacidin suggested that it targets the peptidoglycan synthesis pathway. TLC binding assays further demonstrated strong binding to lipid II, suggesting this cell wall precursor as the target of malacidin.[176]

Siamycin I (**Fig. 12**) was isolated from *Streptomyces sp*. in 1996 and found to be a 21-mer peptide that contains a distinctive tricyclic lasso structure stabilised through two disulfide bonds and an N-terminal macrolactam.[177] It shows potent activity against Gram-positive pathogens, including MRSA and VRE. Given its antibiotic properties, Nodwell and co-workers sought to identify its mode of action.[178179] Fluorescent dye assays indicated that siamycin I does not cause membrane depolarisation. Further studies revealed that the capacity for siamycin I to induce bacterial stress responses was antagonised by $C_{55}PP$, lipid I, and lipid II

but not by $C_{55}P$. These findings indicate that siamycin I binds the pyrophosphate moiety in the peptidoglycan precursors. Cellular uptake assays showed that siamycin I cannot pass through the cell membrane, pointing to lipid II as its likely target given that it is accessible from the cell surface. Siamycin I was further shown to inhibit transglycosylation in a dose dependent manner, confirming that its mode of action involves binding to lipid II and thus inhibiting peptidoglycan synthesis. Notably, Siamycin I is presently the only known lasso peptide that binds to lipid II, again highlighting the structural diversity of lipid II binding antibiotics.

Miscellaneous lipid II-binding natural products

Plectasin (**Fig. 13**) belongs to the family of defensins, a term used to describe a broad assortment of host defense peptides produced by a range of eukaryotic organisms to ward off invading pathogens.[180] Plectasin was isolated from the fungus *Pseudoplectania nigrella* in 2005 and is a 40-mer peptide containing an α-helix and two antiparallel β-strands that are stabilised through four disulfide bonds.[181] It was the first identified fungal defensin and bears remarkable parallels to the structure and amino acid sequences of invertebrate defensins, suggesting that such host defense compounds evolved in or before the last common ancestor shared by fungi and invertebrates, over a billion years ago.[182] Plectasin exhibits antibacterial activity against Gram-positive bacteria including clinical isolates and strains resistant to clinically used antibiotics.[181] Structure-activity studies have also led to the identification of novel plectasin analogues with enhanced *in vitro* and *in vivo* activity.[183,184] Mechanistic studies revealed that plectasin does not cause pore formation in bacterial cell membranes but does lead to accumulation of UDP-MurNAc-pentapeptide, indicating that it inhibits cell wall synthesis.[185] Using TLC binding assays and enzyme substrate inhibition assays, it was subsequently determined that plectasin binds to lipid I and lipid II with a 1:1 stoichiometry. NMR studies revealed key hydrogen bonding interactions between the amide protons of residues 2, 3, 4 and 37 in plectasin and the pyrophosphate subunit on lipid II. Following on these findings it was shown that two other fungal defensins, **oryzeasin** and **eurocin** as well as two invertebrate defensins, **lucifensin** and **gallicin**, also bind to lipid II. Shortly thereafter it was also found that two human defensins **hNP1** and **hBD3**, both produced by neutrophils, likely also exert their antimicrobial activity by binding to lipid II.[186,187]



Plectasin

Figure 13. Primary structure of plectasin with disulphide bridges connectivity shown.

The **tridecaptins** (**Fig. 14**) are a family of linear nonribosomal lipopeptides, isolated from strains of *Bacillus* and *Paenibacillus*, of which tridecatpin A_1 ($TriA_1$) is the most extensively characterised.[188–191] Structurally, they all contain 13 amino acids, bear a net positive charge, and are N-terminally acylated with branched lipids. Unlike all the other lipid II-targeting natural products covered above, the tridecaptins hold the unique distinction of exhibiting activity exclusively against Gram-negative bacteria. This makes them promising antibiotic agents and mechanistically intriguing. Given their promising anti-Gram-negative activity, a number of synthetic efforts and structure activity relationship studies on the tridecaptins have been reported in recent years.[189,192–198] These investigations revealed that the N-terminal tail is amenable to alteration without loss of activity and led to the identification of octanoyl tridecaptin A_1 (Oct-$TriA_1$) as a promising lead compound.[194] The enantiomer of Oct-tridecaptin A_1 (*Ent*-Oct-$TriA_1$) was shown to be four-fold less active than Oct-tridecaptin A_1, indicating that the tridecaptins specifically recognise a chiral biomolecular target.[199] The identity of this target was revealed when it was found that co-incubation of $TriA_1$ with one equivalent of Gram-negative lipid II abolished its activity against *E. coli*. Notably, the same was not observed for Gram-positive lipid II, which required up to seven equivalents before activity was impacted.[198] ITC and *in vitro* assays confirmed a strong affinity of $TriA_1$ to Gram-negative lipid II, and a much weaker affinity to the Gram-positive analogue. Solution-phase NMR studies with $TriA_1$ and Gram-negative lipid II showed that there was no change in the ^{31}P NMR of lipid II upon introduction of the antibiotic, suggesting it was not binding to the pyrophosphate unit. A significant change in ^{1}H shifts corresponding to the amide protons of lipid II revealed that $TriA_1$ was likely binding to the pentapeptide portion. This supports the observation that $TriA_1$ has a stronger affinity for Gram-negative lipid II than for Gram-positive lipid II, which differ only in their peptide region, with Gram-negative lipid II having *m*-DAP at the 3 position, rather than Lys as in Gram-positive lipid II (see **Fig. 6**). Docking studies

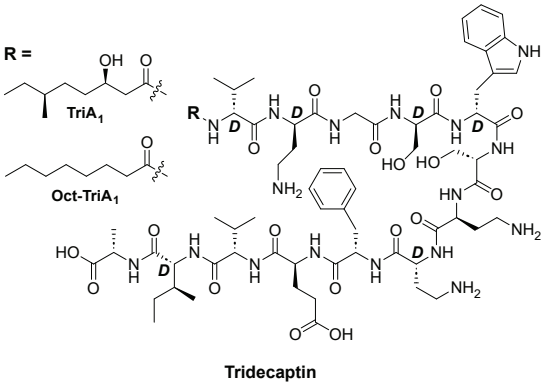


Figure 14. The general structure of the tridecaptin family of antimicrobials, with lipids corresponding to $TriA_1$ and Oct-$TriA_1$ shown.

suggest that a key hydrogen bond interaction between the *m*-DAP3 residue in lipid II and the D-Dab8 sidechain of $TriA_1$ drives this recognition.[199] The observation that the majority of lipid II targeting antibiotics are active against Gram-positive bacteria can be explained by the presence of the outer membrane found in Gram-negative species. While the outer membrane generally prevents access of peptide-based antibiotics to the periplasm where the cell wall is assembled in Gram-negatives, the tridecaptins are clearly able to circumvent this barrier. It will be interesting to see if additional classes of natural products capable of targeting PG precursors in Gram-negative bacterial will emerge in the years to come.

Conclusions and outlook

The bacterial cell wall biosynthesis pathway is a proven and effective target for antibiotics. Not only is this process crucial for bacterial cell viability, it is also unique to the bacterial world, and save a few structural variations, conserved among a broad range of different bacterial species. The membrane-bound polyprenyl intermediates of peptidoglycan synthesis are attractive targets, particularly $C_{55}P$, $C_{55}PP$, and lipid II. During peptidoglycan biosynthesis, all three present themselves on the outer leaflet of the cytoplasmic membrane, allowing access to the frequently bulky, peptide-based, antibiotics that target these biomolecules. In particular, the sheer quantity and variety of natural product antibiotics that bind lipid II demonstrate it to be an exploitable vulnerability in bacteria. Another major advantage is that as non-proteinaceous targets, $C_{55}P$, $C_{55}PP$ and lipid II are not readily mutable through DNA replication errors, a mechanism commonly attributed to the resistance encountered for clinical antibiotics that bind to proteins. This makes antibacterials that bind to these targets much less prone to resistance development. There are, however, limitations to the utility of natural product antibiotics that target these membrane-bound PG precursors. In general, their large size (relative to small molecules) prevents many of these compounds from passing the outer membrane of Gram-negative bacteria, resulting in innate resistance for a large portion of prokaryotic life. Many of these antibacterials also face major challenges in their clinical development as a result of unfavourable physicochemical properties related to their peptidic nature.

A large portion of the compounds discussed in this chapter have been known to possess antibiotic properties for decades. Yet, in many cases, their specific modes of action were only recently elucidated. This has provided invaluable insights into the binding mechanisms that underpin some of nature's best bactericidal strategies. These insights, paired with the progress made in the semisynthesis of natural products, today provide the opportunity to tune the properties of these natural products in order to both overcome their limitations and fully harness their potential. Also of particular note are developments in the technologies that allow researchers to investigate and identify antibiotics that target lipid II and other PG precursors. In recent years, the ability to access greater quantities of lipid II, paired with improvements in assay technology, has facilitated the characterisation of the mode of action of lipid II binding antibiotics. This is exemplified by the rapidly expanding volume of literature on natural product antibiotics that target PG precursors within the last two decades. The continued discovery of natural products that target bacterial cell wall biosynthesis, and our capacity to understand the underlying mechanisms by which they do so, will likely play a key role in addressing the critically depleted antibiotics pipeline.

Scope and outline of this thesis

The theme of this thesis is addressing antimicrobial resistance through the development of novel peptide antibiotics, prepared *via* rational design approaches, that are inspired by the mechanisms of natural product antibiotics. To this end, this thesis describes the design, synthesis and biological evaluation of novel antibacterials that function through underexploited mechanisms.

Chapter 2 focusses on the development of an optimised synthesis of the natural product antibiotic bacitracin A. Bacitracin is a cyclic peptide antibiotic used topically to treat Gram-positive bacterial infections and operates *via* a unique mechanism of action not seen in any other clinically used antibiotics. In order to leverage this mechanism for the purpose of preparing novel antibacterials, we developed an improved synthesis of bacitracin A. This allows for robust access to the bacitracin scaffold by resolving the previously unaddressed formation of epimers associated with the stereochemically fragile N-terminal aminothiazoline moiety.

In **Chapter 3** we apply our optimised synthesis of bacitracin to prepare a number of analogues wherein the N-terminal thiazoline motif is replaced with other known zinc-binding moieties. An alanine scan of the peptide was also performed in order to determine the contribution of individual amino acid residues towards bacitracin's antibacterial activity.

Chapter 4 builds upon this work by conducting a structure-activity relationship (SAR) investigation into the hydrophobic amino acid residues in bacitracin. This led to the design, synthesis, and evaluation of a series of novel analogues that possess enhanced hydrophobicity. A number of which were found to exhibit significantly enhanced antibacterial activity against clinically relevant, drug-resistant pathogens. We further demonstrate the mechanism of action of these potent new analogues to be driven by increased target binding affinity. As a result, we are able to report the first examples of analogues of bacitracin that are able to exploit its unique mechanism of action with higher potency than bacitracin itself. This opens the door for the development of a new class of antibiotics capable of more effectively targeting $C_{55}PP$, a key and underexploited bacterial target.

Chapter 5 describes the design, synthesis, and biological evaluation of inhibitors of lipoprotein signal peptidase II (LspA), a bacterial aspartyl protease with an essential role in Gram-negative bacteria. Inspired by the success of aspartyl protease inhibitors in the treatment of other diseases, we prepared peptidomimetic inhibitors of LspA, with the goal of developing the first

example of an aspartyl protease inhibitor used as an antibiotic. We applied a rational design approach, informed by a crystal structure of LspA bound to the natural product inhibitor globomycin, focussing on the inclusion of four distinct classes of non-cleavable motif into short peptides resembling the consensus sequence of LspA's natural substrate. The activity of these compounds was then evaluated *in vitro* against LspA and against a panel of Gram-negative bacteria.

Finally, the key findings presented in this work are summarised in **Chapter 6** and an outlook on its future prospects is provided.

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