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Next generation bacitracin: reimagining a classic antibiotic

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Summary and outlook

Given the accelerating appearance of antimicrobial resistance and the broken economics associated with industrial antibacterial drug discovery and development, there is an urgent need for more fundamental research into novel antibiotic strategies. In this light, natural product antibiotics remain a rich source of structural and mechanistic diversity and will likely play a key role in addressing the critically depleted antibiotics pipeline in the future. The work in this thesis aims to assist in addressing this global problem by generating novel insights through the preparation of antibacterial agents that operate *via* underexploited mechanisms.

In **Chapter 1** an introduction and overview is provided on natural product antibiotics that inhibit bacterial cell wall biosynthesis by targeting and sequestering the membrane-bound intermediates of peptidoglycan synthesis. Specifically, the antibiotics that are known to function by binding to the membrane-bound substrates $C_{55}P$, $C_{55}PP$ and lipid II, and highlight both the importance and potential of these uniquely bacterial biomolecules as antibacterial targets (**Fig. 1**). This overview serves to emphasise the continued significance of natural compounds in the antibiotic arsenal. In addition, we highlight the growing interest in re-evaluating previously disregarded natural products using modern techniques that have been successful in revealing new insights in the antibacterial mechanisms that underpin these compounds. These insights, paired with advances in synthetic chemistry, provide the opportunity to tune

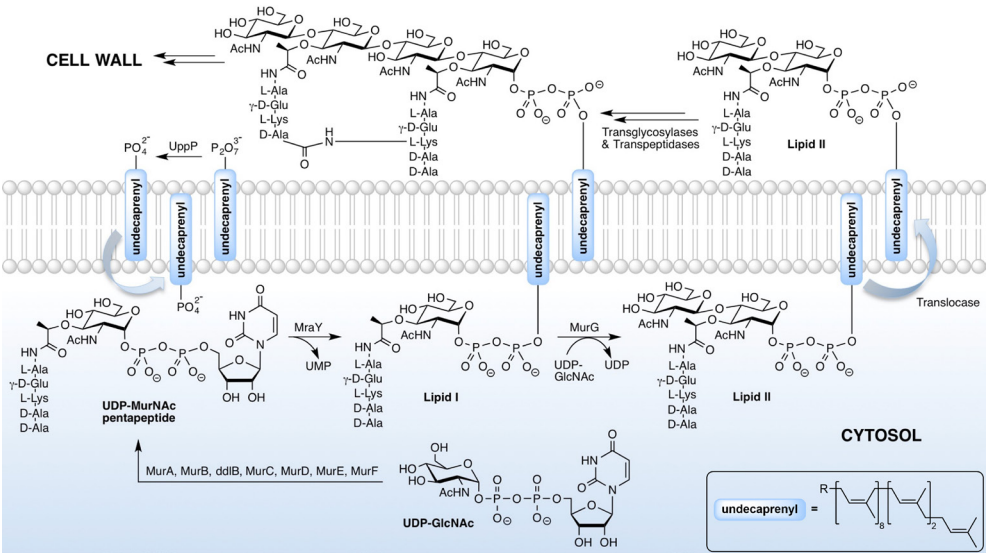

Figure 1. Schematic representation of cell wall biosynthesis. Synthesis commences in the cytoplasm with the conversion of UDP-GlcNAc to UDP-MurNAc-pentapeptide by sequential action of MurA to MurF enzymes. The first membrane-bound intermediate, lipid I, is generated by MraY, before a GlcNAc moiety is 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 properties of antibacterial natural products in order to both overcome their limitations and fully harness their distinctive mechanisms of action.

An example of one such antibiotic is bacitracin (**Fig. 2A**), and in **Chapter 2** we describe the development of an optimised chemical synthesis of bacitracin A. Previous syntheses were found to lead to a high degree of epimerisation of bacitracin's N-terminal aminothiazoline moiety, making the preparation of analogues unfeasible. Our improved synthesis resolves these previous unaddressed limitations by supressing the formation of lower potency isomeric forms of bacitracin, while maximising the yield of the desired product. This robust route in turn opens the door for the preparation of novel bacitracin analogues in order to better elucidate its specific mechanism of action and develop improved variants with enhanced properties and antibacterial activity.

The most serious limitation of bacitracin is the fragility of its N-terminal aminothiazoline moiety, which plays an important role in bacitracin's antibacterial activity by mediating Zn^{2+} chelation. However, *in vivo* reactivity of the aminothiazoline leads to the formation of bacitracin F (**Fig. 2B**) which is both inactive and nephrotoxic, limiting bacitracin's systemic use. In **Chapter 3** we attempt to address these issues by preparing a number of bacitracin analogues where the aminothiazoline moiety is replaced with alternate zinc-binding motifs. Unfortunately the replacement of the aminothiazoline moiety led to a precipitous loss in antibacterial activity, highlighting the important role the aminothiazoline plays in bacitracin's potency. The bacitracin scaffold was then further explored through the use of an alanine scan in order to reveal the contribution of individual amino acid residues to bacitracin's antibacterial activity. This revealed the key role that bacitracin's hydrophobic residues play in its antibacterial activity. Conversely, the majority of bacitracin's polar residues were more amenable to substitution than their nonpolar counterparts. In particular, Glu4, Orn7, and

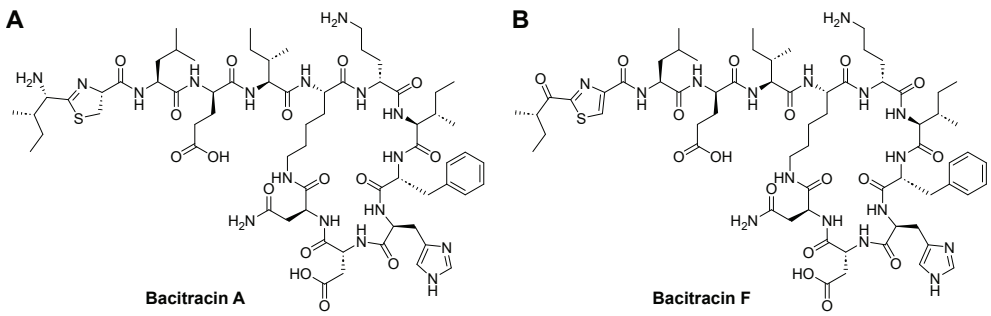

Figure 2. Chemical structures of **(A)** Bacitracin A and, **(B)** Bacitracin F which bears an N-terminal ketothiazole moiety in place of bacitracin A's aminothiazoline.

His10 were identified as sites amenable to further structural elaboration, for example in conjugating to fluorophores to generate tools for visualising bacterial cells or for linking to other active compounds in the preparation of novel hybrids.

In **Chapter 4** we report the preparation of analogues of bacitracin A designed to probe the specific contributions of the constituent nonpolar residues to bacitracin's antibacterial activity. This approach was informed by a recently reported crystal structure which revealed that bacitracin adopts a highly ordered amphipathic conformation upon binding to its target. Among the bacitracin variants prepared, substitution of the branched 4-carbon side chains of Leu3 or Ile8 with an 8-carbon linear aliphatic side chain was found to result in a dramatic enhancement of antibacterial activity (**Fig. 3**). We show that this enhancement is driven by increased target engagement rather than non-specific membrane disruption. Of note is the finding that the most potent analogues are particularly active against antibiotic-resistant bacteria including those bearing the clinically challenging *vanA*-type resistance. Numerous mechanistic studies demonstrated that our lead compounds maintain the specific binding of C_{55}PP characteristic of bacitracin and do so with a higher affinity than observed for the natural product. We here show that the bacitracin scaffold is indeed amenable to structural modification in designing next-generation variants of this classic antibiotic with improved activity. These insights point to the potential for developing novel antibiotics that selectively target C_{55}PP, a promising and currently underexploited strategy in antibacterial drug discovery.

Finally, **Chapter 5** focuses on the development of peptidomimetic inhibitors of lipoprotein signal peptidase II (LspA), an aspartyl protease that plays a vital role in the lipoprotein processing pathway of Gram-negative bacteria (**Fig. 4**). The rational design approach we pursued explored the use of four classes of non-cleavable motifs, commonly incorporated into peptidomimetic aspartyl protease inhibitors, that can mimic the tetrahedral transition state during proteolysis. The peptidomimetics are based on the conserved sequence of amino acid residues found in the lipobox of the signal peptide, and lipid substitutions were explored. All peptidomimetics were synthesised using convenient SPPS approaches and subsequently evaluated for both their inhibitory activity against LspA and their antibacterial activity against a panel of Gram-negative bacteria. These findings contribute to a better understanding of the lipidated peptidomimetics recognised by LspA and will help to inform the development of high-affinity LspA inhibitors as a means of pursuing novel antibiotics for Gram-negative bacteria. Future work will be aimed at exploring additional non-cleavable motifs or lipid substitutions in order to increase binding affinity and by employing structural studies to generate new insights into the interaction between the inhibitors and LspA. This will aid the rational design of new compounds with enhanced potency and selectivity.

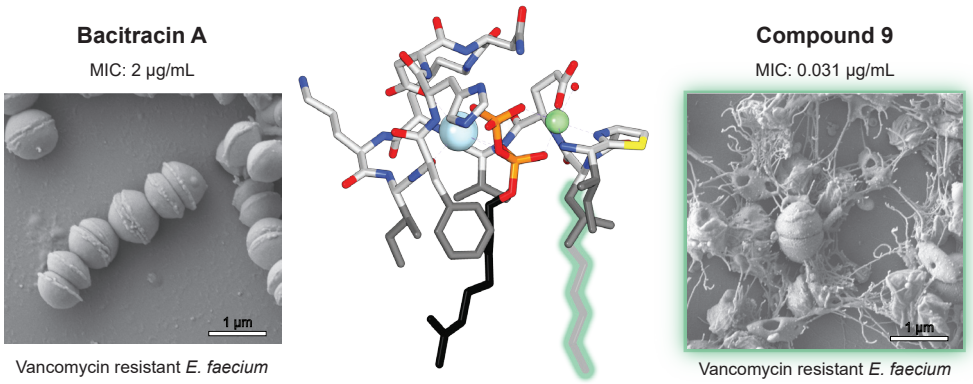

Figure 3. Comparison of the scanning electron micrographs at 20,000× magnification of vancomycin-resistant *E. faecium* E155 cells upon treatment with 4 μg mL^{-1} of bacitracin A and compound **9** (**Chapter 4**). The proposed active conformation of compound **9** is overlaid (green halo) on the crystal structure of bacitracin A complexed with geranyl-pyrophosphate ($C_{10}PP$) mediated by Zn^{2+} (light green) and Na^{+} (light blue). Adapted from PDB: 4K7T.

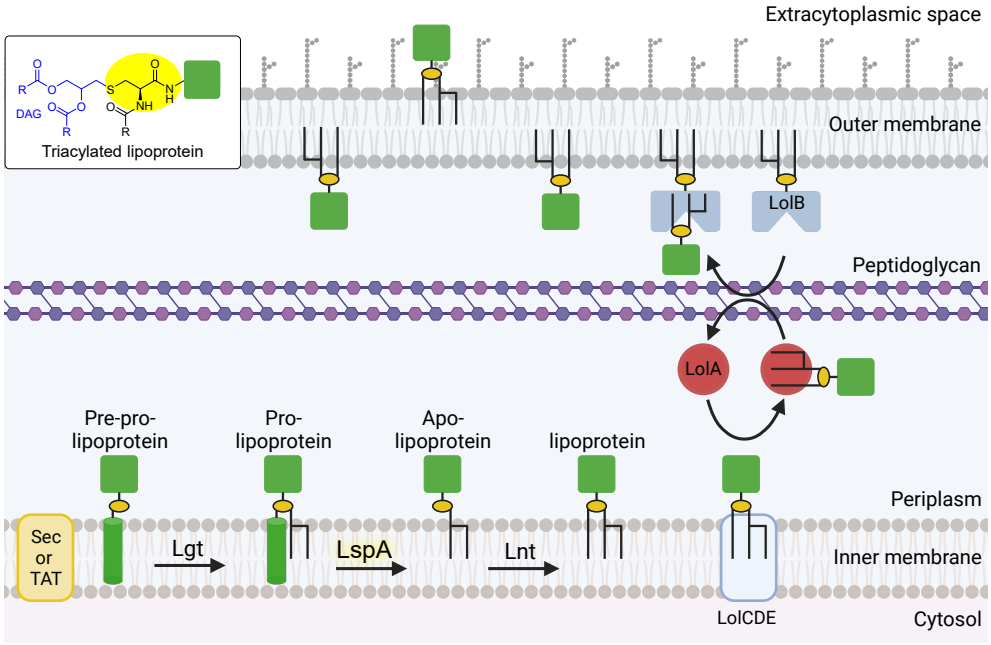

Figure 4. Lipoprotein posttranslational processing pathway in Gram-negative bacteria. Pre-prolipoproteins enter the cytoplasmic membrane via the Sec or Tat pathways, with an N-terminal signal peptide (green cylinder) anchoring the lipoprotein domain (green square) to the membrane. The action of prolipoprotein diacylglyceryl transferase (Lgt) dagylates the pre-prolipoprotein through the side chain of a cysteine residue (yellow oval). LspA then recognises a consensus sequence in the prolipoprotein leading to cleavage of the signal peptide. Some lipoproteins undergo further modification at the newly freed N-terminus by *N*-acyl transferase (Lnt), leading to further lipidation of the lipoprotein. The now mature lipoprotein is then trafficked to the outer membrane by the lipoprotein outer membrane localisation (Lol) pathway. This chapter focussed on developing peptidomimetic inhibitors of LspA in order to disrupt this essential pathway for Gram-negative bacteria. Made with Biorender.

Future Directions

Our future work will focus on optimising the lead compounds identified in **Chapter 4** (analogues **9** and **11**). This will be achieved by conducting a lipid screen at positions 3 and 8 of bacitracin. We will incorporate a range of lipids with different lengths, as well as branched and cyclic lipids, to further refine our structure/activity insights with the aim of further enhancing antibacterial potency. Additionally, we will investigate the intriguing discovery that our hydrophobic bacitracin analogues exhibit heightened activity without the need for exogenous Zn^{2+}. This is potentially due to the structural changes in these analogues affecting the target binding pocket and conceivably predisposing it to the active conformation resulting in stronger binding affinity without the need for zinc and/or possibly allowing for activation by other more common divalent metal ions. To test this hypothesis, ITC and MIC experiments will be conducted with varying divalent cations, as well as in the presence and absence of chelating agents.

While our attempts to replace the aminothiazoline moiety with more chemically stable motifs led to a marked loss in the compound's antibacterial activity (**Chapter 3**), the hope remains that in future, other commensurate structural alterations could potentially recover this lost activity, resulting in analogues with reduced toxicity, enhanced stability and potent activity. A study from Marahiel and co-workers indicated that the replacement of the N-terminal aminothiazoline moiety with an aminothiazole or aminooxazole leads to only a moderate increase in MIC. In **Chapter 4** we demonstrate the feasibility of enhancing bacitracin's antibacterial activity through the selective incorporation of lipidated amino acids at the 3 and 8 positions. We hypothesise that if these changes were paired with a commensurate inclusion of an N-terminal aminothiazole or -oxazole, novel analogues could be prepared that benefit from both the heightened potency conferred by the lipid component along with elimination of the chemically fragile aminothiazoline moiety that is also responsible for bacitracin's toxicity. Such strategies could be the key to unlock the true potential of this unique antibiotic. Our future work will focus on applying these structural insights through a targeted strategy to simultaneously enhance efficacy and chemical stability, facilitating the preparation of analogues with enhanced properties that continue to exploit bacitracin's unique mechanism of action.