Biological, chemical, and biochemical strategies for modifying glycopeptide antibiotics

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Abstract

Since the discovery of vancomycin in the 1950s, the glycopeptide antibiotics (GPAs) have been of great interest to the scientific community. These non-ribosomally biosynthesized peptides are highly crosslinked, often glycosylated, and inhibit bacterial cell wall assembly by interfering with peptidoglycan synthesis. Interest in glycopeptide antibiotics covers many scientific disciplines, due to their challenging total syntheses, complex biosynthesis pathways, mechanism of action, and high potency. After intense efforts, early enthusiasm has given way to a recognition of the challenges in chemically synthesizing GPAs and of the effort needed to study and modify GPA-producing strains to prepare new GPAs in order to address the increasing threat of microbial antibiotic resistance. Although preparation of GPAs, either by modifying the pendant groups such as saccharides or by functionalizing the N- or C-terminal moieties are readily achievable, the peptide core of these molecules – the GPA aglycone – remains highly challenging to modify. This review aims to present a comprehensive analysis of the results of GPA modification obtained with the three major approaches developed to date: in vivo strain manipulation, total chemical synthesis, and chemoenzymatic synthesis methods.
Antibiotics are essential compounds that underpin modern medicine through their ability to treat infections and limiting the spread of bacteria. However, bacteria have developed many mechanisms of resistance through selective pressure, since they are literally fighting for their survival.(1) The overuse or misuse of antibiotics dramatically accelerates the development of resistance, which is a process that also occurs naturally due to competition between different bacteria.(2) To date, microbial diversity remains the major source of important discoveries of both new antibiotics as well as their mechanism of action from the point of view of resistance mechanisms.(3) However, conversion of natural compounds into clinical antibiotics is highly time consuming and has a limited chance of success, due in no small part to the differences in the natural usage of antibiotics as opposed to a clinical application of these molecules. Currently, it is common for antibiotics to undergo 10 or more years of studies prior to their use in the clinic.(4) Consequently, the modification of existing, current FDA-approved antibiotics is an important strategy for antibiotic development since significant volumes of data have already been collected in terms of their mechanism of action, production, toxicity and structure/activity relationships. In such a strategy, the main challenge often faced for antimicrobial development lies in the complex structures of natural antibiotics, as these need to be modified to address newly developed resistance mechanisms.

The glycopeptide antibiotics (GPAs) – which include vancomycin (1) and teicoplanin (2) – are clinically utilized agents of last resort to treat resistant bacterial infections. All GPAs are non-ribosomally-biosynthesized heptapeptides produced by soil dwelling microbes, which consist of a high proportion of aromatic amino acid residues that are extensively crosslinked through the side chains of these residues. Beyond the peptide core of these molecules, GPAs are often highly decorated with other functional groups including sugars (as indicated in their naming), acyl chains, sulfate groups and chlorine atoms, all of which can contribute to the activity and specificity of these different antibiotics.

Despite their effectiveness, this compound class is also becoming inactive against certain bacterial strains due to emerging antimicrobial resistance.(5-7) This is part of a growing trend, where various “superbugs”, both gram-positive and negative, are emerging as the first stones in a potential avalanche of resistant strains that could herald a future antimicrobial resistance crisis. Amongst the current gram-positive “superbugs”, all FDA approved antibiotics are inactive against Enterococcus faecalis/faecium (VRE strains - Figure 1B) or are reaching toxicity limitations against Staphylococcus aureus (VISA and MRSA strains).

The mechanism of action of GPAs toward gram-positive bacteria functions through GPA binding to the D-Ala-D-Ala dipeptide terminus of the lipid II precursor to peptidoglycan. GPA binding then blocks transglycosidase (TG) and to some extend the transpeptidase (TP) enzymes that construct the bacterial cell wall (Figure 1A), leading to eventual cell lysis. This interaction, which centers on hydrogen bonding interactions between the dipeptide D-Ala-D-Ala terminus of lipid II and the peptide backbone of the GPA, is...
the primary mode through which GPAs bind and thus inhibit bacterial cell wall biosynthesis. The binding of GPAs to the central precursor in cell wall biosynthesis means that this a “target rich” environment for antibiotic activity, and further peripheral modifications to the peptide core of GPAs can lead to further mechanisms of binding and inhibition, and even improve their activity to the extent that the central interaction is no longer required for their antibacterial activity. In the most “hard-to-kill” VRE strains (Figure 1B), a central hydrogen bonding interaction in the GPA/lipid II complex is disrupted, resulting in a 1000-fold loss of binding due the repulsion between a carbonyl group of the GPA (4-hydroxyphenylglycine – Hpg-4) and an oxygen atom in the lipid II D-Lac ester moiety. The effect that such small changes can have on the activity of GPAs has become the focus of intense research, for in the case of VRE resistance is rising rapidly and has the potential to be a major problem in the clinic,(5) particularly with only one new antibiotic in preclinical trials to treat such infections.(4) Furthermore, the transfer of GPA resistance from VRE strains to MRSA mediated by enterococcal plasmids is further leading to the emergence of VRSA strains and the need of new compounds to also target these strains.(8)

To date, the structural complexity of GPAs has rendered their diversification through synthesis mostly restricted to total chemical synthesis. Nevertheless, a focus on understanding how bacterial producer strains produce such GPAs has the highest chances to provide GPAs with the lowest manufacturing cost,(9) which is often the drawback associated with total chemical synthesis. To this end, a comprehensive understanding of the GPA biosynthesis processes mediated by non-ribosomal peptide synthetases (NRPS) and cytochrome P450 enzymes (P450s) has led to the recent development of a chemoenzymatic strategy combining the strengths of both chemical and biochemical methods.(10-12) This review aims to summarize the current state-of-the-art with regards to how and where GPAs can be modified by in vivo strain manipulation, total chemical synthesis or chemoenzymatic approaches.

1. An overview of GPA synthesis approaches

The structure of GPAs comprises a heptapeptide backbone that is rigidified by extensive side chain crosslinks between aromatic amino acids. GPAs are classified in five classes depending on the extent of side chain crosslinking and either the structure of the peptide core or the nature of the modifications appended to the peptide itself (Figure 2). Type I GPAs, exemplified by vancomycin, contain three side chain crosslinks, which are known as the A-B (linking residues 5 and 7), C-O-D (linking residues 4 and 6) and D-O-E (linking residues 2 and 4) rings. The remaining two positions of the peptide (residues 1 and 3) contain residues bearing aliphatic side chains, and the peptide is also glycosylated. Type II GPAs (exemplified by avoparcin) share the crosslinked structure of type I GPAs, however the aromatic amino acid residues are now found at positions 1 and 3 of the peptide. Type III and IV GPAs contain an additional crosslink linking aromatic residues at positions 1 and 3 of the peptide when compared to type I/II GPAs (F-O-G ring in addition to type I/II crosslinks), and differ in terms of whether the peptide is modified with
an acyl chain (type IV GPAs) or is lacking such a modification (type III GPAs). Type V GPAs include compounds such as complestatin and kistamycin, which do not exhibit the same antimicrobial activity as the other GPA classes, and thus are of less interest as antibiotics. The minimum number of crosslinks within the peptide core of GPAs (known as the aglycone) that is required for antibacterial antibiotics is three – the A-B, C-O-D and D-O-E rings – as this this confers the constrained structure needed for GPA antibacterial activity (Figure 1). Following the biosynthesis of the GPA aglycone, the peptide core of these antibiotics is subsequently modified by enzymatic processes, including N-terminal amino group methylation and glycosylation/sulfation of diverse hydroxyl groups, to produce the final structures of natural GPAs.(13,14) As noted above, a major difference between type I-III and type IV GPAs is the presence of an N-lipidated glucosamine on the position R4 of type IV GPAs (Figure 2B). Inspired by such particular feature, the N-alkylation of epi-vancosamine (chloroeremomycin - G2) and vancosamine (vancomycin - G3) have been explored, leading to the development of second generation, semi-synthetic GPAs including the compounds oritavancin and telavancin (Figure 2A. CM1 and 2). In these semi-synthetic GPAs, the hydrophobicity introduced at R4 is balanced by the presence of hydrophilic group such epi-vancosamine (G6) for oritavancin (6), (phosphonomethyl)aminomethyl group (CM3) for telavancin (7) and dimethylaminopropyl (CM4) for dalbavancin (9). The ability to modify existing GPA aglycones and to isolate GPAs with improved properties is also highly promising for the future development of this class of antibiotics.

When the structure of vancomycin was determined, total chemical syntheses by Evans (1-aglycone in 1998), Nicolaou (1 in 1999) and Boger (1-aglycone in 1999 and 2-aglycone in 2000) were achieved at a time where GPA resistance was already on the rise.(15-18) Later, the group of Boger had notable successes in developing modified GPAs that were active against by VRE strains.(19-22) The common routes adopted in these total syntheses are the formation of the bicyclic C-terminal tetrapeptide moiety including both C-O-D and A-B rings installed, with the N-terminal linear tripeptide assembled separately (Figure 4A). The peptide coupling of both moieties and the formation of the D-O-E ring then enable the formation of the vancomycin aglycone.(23) Despite this impressive synthetic work, commercial GPA production remains tied to biosynthesis in vivo due to GPA structural complexity and the limits of scale up of this challenging chemical synthesis.(9)

In contrast to chemical synthesis, the heptapeptide core of GPAs is biosynthesized by non-ribosomal peptide synthetases (NRPS), which generate a linear heptapeptide precursor bound to the final module of the NRPS (module 7) through the pantetheine linker of the carrier protein (PCP) domain (Figure 3).(24-26) Next, side chain/side chain crosslinking (i, i + 2) is mediated by cytochrome P450 (Oxy) enzymes,(27,28) which are recruited to the NRPS bound linear heptapeptide intermediate by the unique GPA P450 recruitment domain, the X-domain.(29-32) The GPA crosslinking cascade occurs in a distinct order, which involves initial C-O-D ring formation by OxyB, followed by
OxyA-mediated insertion of the D-O-E ring and finally A-B ring insertion performed by OxyC.\(^{33-35}\) The cleavage of the complete GPA peptide aglycone from the final NRPS module is mediated by the actions of a selective thioesterase (TE) domain,\(^{36}\) which releases the GPA aglycone to then be further modified. Indeed, it is through these various modification processes that the majority of the natural diversity found in GPAs is introduced.\(^{24,36}\) The current inability to redesign the NRPS-machinery, which is highly specific for the biosynthesis of one peptide sequence, is the current major limitation in the \textit{in vivo} production of new GPAs. Such specificity is maintained through the combination of specificities enforced by the amino acid selection (adenylation, A), peptide bond forming (condensation, C) and epimerization (E) domains present in each NRPS module and that are together responsible for the step by step incorporation of monomers into the growing peptide chain.\(^{26,37}\) Modifying this complex NRPS machinery is essential if the \textit{in vivo} GPA production machinery is to be utilized to generate new GPAs: this is clearly a major challenge for the field, with step-wise approaches needing to be developed to assist in the identification of valid GPA targets to first make such biosynthetic redesign efforts worthwhile.

Studies of the final crosslinking steps of GPA aglycone biosynthesis have led to the development of a hybrid synthesis route known as chemoenzymatic synthesis, in which the final NRPS module is simplified to a PCP-X didomain and is used as a platform to present synthetic peptides for cyclisation using the Oxy enzymes \textit{in vitro}. The peptide substrates utilized in this method are first prepared by solid phase peptide synthesis (SPPS), before being further modified to generate their coenzyme A thioesters that then allows their enzymatic loading onto the PCP-X didomain using a promiscuous phosphopantetheinyl transferase.\(^{38}\) Next, Oxy enzymes are added to reproduce the GPA enzymatic cascade, leading to formation of the GPA aglycone that can be released either by addition of a chemical agent (such as an amino group) or by hydrolysis, be it enzymatic or chemical (Figure 4B).\(^{39}\) Importantly, such a chemoenzymatic cascade has been developed to use the Oxy enzymes as biocatalysts, which differ to \textit{in vivo} biosynthesis where the entire machinery is stoichiometric. Furthermore, the activity of the Oxy enzymes is maintained by a ferredoxin reductase/ferredoxin coupled enzyme pair that transfers the electrons needed for the crosslinking reaction from NADH to each Oxy enzyme. Each of these methods described in figure 2 for the synthesis of the vancomycin aglycone has led to various modified GPA architectures, which will now be described in terms of their location(s)/nature within these modified GPAs.

\section{Peptide backbone modifications}

\subsection{Total synthesis}

After the successful synthesis of both vancomycin and teicoplanin aglycones,\(^{15,16}\) the Boger group made a concerted and highly impressive effort to modify the peptide backbone of GPAs, initially to avoid the repulsion created by the D-Ala substitution into D-Lac in VanA resistance, and secondly to recreate the missing hydrogen bonding with D-
Lac (Figure 5. Compound 10-11). One way to avoid the problem of lone pair repulsion from GPAs to the lipid II target of resistant strains was to replace the original carbonyl group by a methylene group, forming a “reduced” bond. (19) To this end, special amino acid building blocks were used to generate the modified dipeptide between the Hpg residues 4 and 5. After protecting the secondary amine group as a methyl carbamate, amino acid 6 (tyrosine) was introduced followed by the formation of the C-O-D ring by SNAr cyclization of the fluoro atom (tyrosine 6) by one of the phenol group from the Hpg-5 (10a). From there, a Suzuki cross coupling reaction allows the addition of the amino acid 7 precursor (Dpg-7), forming the biaryl bond. After several intermediate steps, macrolactamization between the Tyr-6 and the Dpg-7 closes the A-B ring, leading to the bicyclic C-terminal tetrapeptide moiety containing both C-O-D and A-B rings (10b). The remaining synthesis follows a previously described strategy, with the peptide coupling between 10b and the N-terminal tripeptide and formation of the D-O-E ring by SNAr cyclization. Final global deprotection was performed with AlBr$_3$-EtSH, which promotes the removal of all protecting groups including the methyl carbamate to produce the desired $[\Psi$-CH$_2$NH]Tpg$^4$ vancomycin aglycone (10). This compound was found to be 40-fold more potent than 1 (MIC: 650µg/ml) against VanA resistant Enterococcus faecalis (MIC: 31µg/ml), whilst being 30-fold less potent than 1 against sensitive strains of Enterococcus faecalis. Despite these encouraging results, this strategy to avoid lone pair repulsion was insufficient to fully recover the potency of GPAs against VRE strains. Consequently, Boger and coworkers next targeted the insertion of an amidine group (11), which can act as both a hydrogen donor and acceptor and would thus be able to bind lipid II molecules ending with either D-Ala or D-Lac (Figure 5. Compound 11). (21,22) The key intermediary targeted here was the vancomycin aglycone incorporating a thioamide bond between the Hpg-4 and 5 (11c). The synthesis followed the successful Boger strategy until the formation of the tripeptide 5-4-6 incorporating the C-O-D ring. Thionation with Lawesson’s reagent selectively generated thioamide (11a), after which the synthesis was modified to account for the reactivity of the thioamide bond. Thus, the thioamide was first converted into the methyl thioimidate before the Suzuki coupling reaction was performed and removed immediately afterwards, leading to the bicyclic C-terminal tetrapeptide moiety with both C-O-D and A-B rings after the macrocyclization (11b). The $[\Psi$[C (C=SNH]Tpg$^4$] vancomycin aglycone (11c) was obtained following further standard synthetic steps – peptide coupling between 11b and the N-terminal tripeptide, formation of the D-O-E ring by SNAr cyclization and final global deprotection with AlBr$_3$-EtSH. Finally, the direct conversion of the thioamide (11c) into the desired amide moiety (11) was achieved with an optimized protocol using AgOAc–NH$_3$ in methanol. (20) This compound exhibited an impressive 0.5µg/ml MIC against VanA resistant Enterococcus faecalis (BM4166 strain). Both modified vancomycin aglycones 10 and 11 could be further improved by adding the saccharide units and by modifying the C-terminal moiety using either chemical or enzymatic methods.
2. 2. Chemoenzymatic strategy

Inspired by in vivo biosynthesis, the GPA chemoenzymatic strategy is a powerful strategy that has been developed to assess peptide substrate/Oxy enzyme compatibility. The philosophy behind such strategy is to use this as a platform to identify GPA modifications that will be compatible with the in vivo biosynthesis machinery. To do this, NRPS and P450s assays are utilized to understand the feasibility of biosynthesis of a particular peptide sequence or aglycone prior to the eventual translation of this modified sequence into biosynthesis in vivo, which is crucial as the redesign of the multi-modular NRPS machinery to incorporate new amino acids is highly challenging.(40,41) The substitution of the NRPS with SPPS provides access to wide range of amino acid building blocks, which allows the question of the substrate acceptance by the crucial P450 enzymes to be addressed in a relatively facile manner. Recently, the group of Seyedsayamdost confirmed the feasibility of an analog \([\psi[C=C(S)NH]Tpg4]\) vancomycin aglycone (11c without both β-hydroxyl groups)) through such a chemoenzymatic strategy (Figure 6A).(42) To this end, a 3-step organic synthesis was performed from Fmoc-D-Hpg to afford the corresponding benzotriazolide activated compound, which was used immediately for SPPS. The conversion of the linear peptide substrate into an analog of 11c is low, however, which highlights the initial major drawback of this strategy.

Another example of the use of a chemoenzymatic synthesis route was the testing of incorporation of β-Dpg residues in positions 3 and 7 of the GPA precursor peptide in order to understand their potential acceptance by the P450-catalyzed cyclization cascade (Figure 6B).(43) Despite not being involved in the C-O-D and D-O-E rings, the introduction of β-Dpg-7 had a dramatic effect on ring formation on D-O-E ring formation by the OxyA enzyme, essentially preventing the insertion of this ring. The introduction of β-Dpg-3 is, however, led to a peptide substrate that was well accepted by both OxyB and OxyA enzymes and led to the formation of a 17-membered D-O-E ring in a bicyclic intermediate for either teicoplanin or actinoidin-type GPA sequences. At the time of this study, an effective tricyclization cascade was not available and the incorporation of the final A-B ring was not tested. Drawbacks with this chemoenzymatic approach has recently been addressed by the alteration of the assay to prevent oxidative damage to the Oxy enzymes, which increases the activity of the final enzyme OxyC and, as a consequence the formation of the A-B ring, from at most 20% to more than 50% in the majority of cases.(44) In such an optimized assay, the feasibility of peptide cyclization by the Oxy enzyme cascade can be tested on any desired sequence in a facile manner, which will be of great value to avoid unforeseen outcomes whilst attempting to translate GPA synthesis in vitro to production in vivo.

3. Modifications to GPA halogenation

Vancomycin (1) and teicoplanin (2) have been the focus of various studies concerning their chloro- substituent(s) and more generally concerning the halogen atoms present on the aglycone moiety, given that these moieties bear interesting physical properties. Chlorine atoms
present in GPAs are involved in stabilizing the overall three-dimensional structure of vancomycin, and their removal has direct effects on the antibiotic activity of modified GPAs.(45-47) In one example, Suzuki-Miyaura cross-coupling was directly performed on the vancomycin chlorine atoms to obtain mono- or di-substituted vancomycin derivatives bearing either aryl or alkenyl groups (Figure 7A).(48) The majority of the di-alkenyl GPAs were inactive against susceptible strains, while some of the compounds monosubstituted on the D-O-E ring broadened the antibacterial spectra of activity to include activity against strains typically resistant to GPAs. Here, the compound with the highest contains a bis-phenyl moiety, similar to that found in oritavancin, which clearly shows the importance of such a modification for GPA activity, whilst monosubstitution of the D-O-E ring with arylboronic acids led to slight loss of activity against sensitive strains. Moreover, the selective palladium(0)-catalyzed borylation of vancomycin aglycone corroborated the monosubstitution on the D-O-E ring and a small loss of antimicrobial properties after various substitution of the boronic acid. (45,46) This can be explained by the disruption of GPA dimerization, as the chloride present on the D-O-E ring is known to be involved in this process.(49) Subsequently, a two-step dechlorination/ cross-coupling protocol allowed the selective substitution of the chlorine atom found on the GPA C-O-D ring.(47) Taken together, these results strengthen the importance of the chlorine atoms found in GPAs, particularly on the C-O-D ring, where the chloride (and hence subsequent modifications) face towards the lipid II binding site, potentially disrupt lipid II binding to these modified GPAs. Introducing other halogens such as Br or I in place of the Cl atoms is of great interest, since aryl chlorides are relatively unreactive, and Br/I containing GPA scaffolds would facilitate further exploration of modifications to these positions. To this end, bromination of A40926 aglycone or iodination of vancomycin and ristocetin have been successfully performed to introduce a bromo- or iodo- substituent between the 2 phenol groups in A-B ring or on the F-O-G ring for ristocetin.(50,51) More recently, Miller and coworkers studied the modification of GPAs using reagents such as N-bromophtalimide in combination with catalytic quantity of peptides related to the lipid II terminus, the natural GPA target.(52) Such peptide catalysts are designed to bind to the GPA and then to catalyse site-specific modifications of the GPA. Their findings provide a useful approach in the design of a selective reactions using the interaction of vancomycin with a ligand, and also have had success in introducing other modifications beyond bromine.(53,54) The chemical yield of such GPA modifications, however, often remains a major drawback to the application of such routes in functionalizing GPA scaffolds for further development.

Mutasynthesis of GPAs in vivo has been used to provide access to several alternative of balhimycin (3), where both chloride atoms have been substituted by fluoride or bromide (Figure 7B). In the first step, a gene responsible for the production of the essential β-hydroxytyrosine amino acid precursor of balhimycin was deleted.(55) Next, chemically prepared analogues of tyrosine were added to the culture of this mutated deletion strain, leading the
identification of fluorobalhimycin. The selection of 3-fluoro-β-hydroxytyrosine by the A domain of module 2 and 6 in the NRPS machinery is most likely due to the small radius of fluorine in this case. After the completion of these and related experiments, the timing of GPA halogenation was confirmed to occur once the tyrosine residue is attached to the peptidyl carrier protein (PCP) domain of the NRPS. For this reason, the strategy utilized to produce fluorobalhimycin is unlikely to work for other halogenated tyrosine residues, as was demonstrated by the failure to prepare balhimycin derivatives while supplementing the culture media with 3-chloro-β-hydroxytyrosine. To overcome this, the Süssmuth group investigated the supplementation of fluoride, bromide and iodide instead of amino acids in the culture medium of Amycolatopsis balhimycina. While fluoride and iodide salts were toxic, bromide supplementation led to the isolation of bromobalhimycin, where both chlorine atoms were replaced by bromine. Most of these modifications led to bioactive compounds against sensitive bacterial strains, but provided no noticeable improvement in activity against GPA resistant strains. None the less, as a mechanism for introducing reactive chemical handles for further modification of GPAs through chemical means these techniques hold significant future interest.

4. N-and C-terminal modifications of GPAs

The structural rigidity and highly crosslinked nature of GPAs renders modification of the aglycone core complex, as 5 of 7 amino acids are crosslinked in type I and II GPAs whilst all 7 amino acids in type III and IV are crosslinked due to the extra F-O-G ring. For this reason, GPA modifications have often targeted the peptide termini (Figure 8). C-terminal modifications are readily achieved by coupling various amino functions with a pre-activated carboxylic acid moiety of the GPA to generate an amide bond, and this has been extensively utilized due the simplicity of this process (9, 14-15). A common theme in the C-terminal modification of GPAs is the introduction of positively charged amino acids (such as lysine and arginine) in order to increase the affinity for the membrane. As an example, the modification of vancomycin with a poly-(D)arginine peptide led to the discovery of a compound capable of biofilm disruption, which is a mechanism commonly exhibited by virulent bacterial strains. Interestingly, the addition of a single arginine residue also led to activity towards gram-negative bacteria. Importantly, the killing mechanism of GPAs by osmotic insult can often be complemented by membrane lysis through the addition of an aliphatic chain to GPAs. One of the most successful outcomes of such an approach led to the synthesis of dalbavancin (9), which is the product of the modification of the natural type-IV GPA A40926 (Figure 2B, compound 8) with 3-(dimethylamino)-1-propylamine. In this case, C-terminal modification of the GPA with short chain amino- or quaternary ammonium salt groups added cell permeabilization to the existing mechanism of transglycosidase and transpeptidase inhibition. Beyond direct conjugation to the GPA C-terminus, the selective chemical
modification of the Dpg-7 position of GPAs has recently been demonstrated by Pentelute and co-workers, who could exploit the reactivity of this residue in a modification with selenocysteine-containing peptides and proteins.(65) Through this approach, the conjugation of dermaseptin (antimicrobial peptide-AMP) to vancomycin (12) was affected, which improved the overall MIC against VanB resistant Enterococcus faecalis but had no effect against VanA resistant such as dalbavancin (9). Further exploration of this approach allowed the identification of a vancomycin-kinocidin conjugate (13) that displays an excellent MIC against the bacteria Acinetobacter baumannii, a strain that is traditionally unaffected by GPA administration. Additionally, mutasynthesis has also been used to generate further derivatives of balhimycin via the supplementation of chemically synthesized amino acids into a strain in which Dpg biosynthesis has been disrupted. This showed that several modifications/alterations of the phenol groups present on this residue were tolerated by both the NRPS and the Oxy enzyme cyclization cascade.(66) Related work demonstrated that the GPA A-B ring can also form when a Hpg residue is present at position 7 of the precursor peptide, but that this expansion of the A-B ring by a single atom is sufficient to eliminate the antibiotic activity of such GPA derivatives.(67) This serves as further evidence indicating how sensitive the essential crosslinked ring systems are in GPAs and how modifications to these compounds must be carefully assessed to ensure that these do not result in unwanted loss of antibiotic activity from modified compounds produced through these pathways.

Concerning the N-terminus of GPAs, one major area of focus has been the removal of the N-methyl-leucine residue in order to replace it with other amino acids and complete SAR at this position (Figure 8). It was known from the structure of vancomycin that the aliphatic chain of the leucine residue helps to stabilize the tertiary structure of the GPA. However, the impact of such amino acids on the antibiotic activity of GPAs was not known until several groups focused on removal of this residue, accomplished either by chemical or enzymatic means. In the latter approach, vancomycin was added to a bioconversion fermentation broth containing Actinomadura citrea (NRRI 18382), which was shown to be responsible for the enzymatic removal of the N-methylleucine residue, generating des-leucyl vancomycin.(68) Actinomadura citrea was selected for this work since it was one of the rare soil microbes able to inactivate vancomycin by degradation, forming des-leucyl vancomycin. This compound and its aglycone equivalent were also obtained chemically after Edmann degradation via treatment with phenyl isothiocyanate.(69,70) Boger and coworkers obtained further insightful results by acylating the N-terminal of the vancomycin aglycone with various phenylalanine incorporating amino-, hydrazino- or guanidine substituent. All the compounds tested lost significant antimicrobial activity against a vancomycin-sensitive strain of S. aureus, but testing against vancomycin-resistant E. faecalis (VanA type of resistance) demonstrated that significant improvement in activity could be obtained using such modifications (a 4-fold improvement compared to vancomycin aglycone for compound 16).(71) N-terminal modification
in a chemoenzymatic approach has also been tested using the natural methyltransferase enzymes from pekiskomycin biosynthesis, a type I GPA,(72) which demonstrated that these enzymes can effectively mono- or dimethylate the N-termini of various GPA derivatives and precursors.(73) Furthermore, the cofactor S-
adenosylmethionine – the source of the methyl groups – can be replaced with other synthetic cofactors that can be used to expand the structures appended to the GPA N-terminus.(73) This clearly has interest for the future redesign potential of the in vivo biosynthesis of GPAs.

5. Modification of residue 3 of the GPA peptide

The pool of amino acids used in the biosynthesize GPAs is limited due to their high conserved sequences and structures. Despite a greater diversity of amino acids found in position 3 of type I and II GPAs (L-Asn, L-Glu, L-Phe), little is known about the effects of these different residues on GPA binding to lipid II or their antimicrobial properties. To date, only two reports describe the modification of this position, the first being hydrolysis of the primary amide of the Asn residue in ereromycin, leading to the conversion of this residue into a carboxylic acid. The second report of modification of this Asn residue, this time in vancomycin, was by dehydration leading to a nitrile group.(74,75) The hydrolysis approach generated a carboxylic acid functionality that was subsequently used for further modifications of ereromycin, whilst the same approach failed when being applied to vancomycin.(75) The results of testing the derivatives produced by these approaches showed that there was no particular effect on the binding and activity of such compounds compared to the unmodified GPA, although the scope of these studies is clearly limited and should not be taken as proof that no benefits can be made through modification of this residue in future. Further diversity at residue 3 is found for type III and IV GPAs, such as teicoplanin, which include phenylglycine residues, but these structures also include an additional F-O-G ring linking positions 1 and 3, making modification even more challenging in these cases. Thus, there is clear gap in our current understanding of the role of the amino acid residue in position 3 of GPAs. This lack of knowledge can potentially be overcome in future by the application of chemoenzymatic GPA synthesis to understand the possible impact of modifying this position in terms of GPA binding, dimerization, antibacterial activity or even evasion of resistance mechanisms.

6. Peripheral modifications of the GPA aglycone

The complexity of the chemical synthesis of GPA aglycones has in general led researchers to prioritize peripheral modifications of GPAs as a diversification strategy, which includes the adoption of chemo-enzymatic and chemical approaches.(76-78) Overall, the best outcomes with such derivatization strategies has been the identification of second-generation GPAs, represented by oritavancin (6) and telavancin (7).(72,73) Grouped with the type I GPAs, oritavancin is generated via the alkylation of chloroeremomycin with a para-chlorophenylbenzyl group,(79) while telavancin is prepared from vancomycin by alkylation and...
aminomethylation following a Mannich reaction (Figure 9A). (80) In both compounds, the vancomycin aglycone core is conserved and the common point is the introduction of a hydrophobic tail on the vancosamine moiety. The outcome of such modifications in both cases is increased in vivo stability and hydrophobicity, which favors an interaction of the GPA with lipid II as it is located in the bacterial membrane. These improvements in activity are significant and second generation of GPAs are potent against VISA, MRSA strains in the case oritavancin and even some VRE strains despite the mutation of the lipid II D-Ala-D-Ala dipeptide terminus into D-Ala-D-Lac (VanA – Figure 1B). (81,82) The multiplicity of lytic mechanisms was determined to be crucial for the impressive activity displayed by oritavancin.

Studying peripheral modifications in the case of GPAs is a process facilitated by facile access to the GPA aglycone. Indeed, the disaccharide moiety found in vancomycin can be easily removed in acidic conditions, leading the perfect scaffold to study the effect of various sugars (plus other modifications) on the antibiotic activity of these compounds. Thorson, Walsh and coworkers have exploited various glycosyltransferases such as GftE, (83) which is responsible of the incorporation of the D-glucose unit on residue 4 of the GPA aglycone. (84) Other than its native substrate, GftE has been shown to be promiscuous in its choice of substrate, accepting more than 20 other nucleotide diphosphosugars that have in turn led to the preparation of new mono-glycosylated vancomycin derivatives, including three with azidosugars and one with a thiosugar. (85) Such glycosyltransferases have also been shown to be capable of modifying GPAs by exploiting the natural reversibility of these enzymes (Figure 9B). (86) The ability to incorporate sugars containing easily diversifiable handles such as 6-azido-α-D-glucose has been further exploited by the addition of various alkynes via Huisgen 1,3-dipolar cycloaddition (click chemistry). This combinatorial biochemistry-chemistry approach has been highly successful, and allowed the rapid assessment of the effect of sugar modifications on the central Hpg-4 of GPAs, a process that is highly challenging by chemical syntheses alone. (86,87) Recently, the amino group found in the vancosamine moiety of vancomycin was shown to even be able to be directly and selectively converted into an azido group using fluorosulfuryl azide. (88) Miller and co-workers have further exploited their GPA binding dipeptide catalysts to allow the specific phosphorylation of three of the sugars in teicoplanin, and lipidation of two sugar sites in vancomycin, highlighting yet another viable approach to the diversification of the glycosyl groups found in GPAs. (89,90) Recent work has also made substantial process towards overcoming a major drawback with type IV GPA, which is the in vivo incorporation of various lipids leading to non-homogeneous compounds (Figure 2. Compounds 2, 8 and 9). Here, in vivo mutasynthesis was used to generate a de-acylated A90226 (8) that was chemically modified for preparing a homogeneous dalbavancin analog containing a C10 aliphatic chain and the dimethylaminopropyl modification. (91) Importantly, the deletion of Dbv8 inhibited the action of Dbv29, which oxidizes the primary alcohol group into a
carboxylic acid group (Figure 10A). Homologous enzymes to Dbv8/Dbv29 have also studied from teicoplanin biosynthesis (2) and the insights gained into the mechanism Dbv29 led to the development of a methodology in which the intermediary aldehyde formed during the oxidation could trapped either as aminated or amidated compounds (Figure 10B).(92) As mentioned earlier in relation to studies on A40926, this modification via the trapping of an activated intermediate has been combined with Dbv21 de-acylation and subsequent acylation to generate altogether libraries of compounds containing doubly modified sugars.

**Future directions**

Within the field of GPA research, interdisciplinary efforts have arguably made the great contributions to progress. Oritavancin serves as one of the best achievements in terms of practical therapeutic improvements to GPA activity and use in the clinic, which was made possible by sourcing the GPA from its producer, introducing chemical modifications that led to identification of new lytic mechanism and then the ability to produce this compound at large scale by sourcing the most complex structural features in the final antibiotic from *in vivo* biosynthesis. The inclusion of peptide backbone modifications by Boger and co-workers is arguably the most impressive example of GPA redesign to combat resistance, although the application of these types of compounds remain hindered by the challenges of GPA total synthesis and the inability to access to new GPA aglycones at scale. Behind this issue lies the complexity of both total synthesis and the biosynthetic NRPS/P450 machinery used for the production of GPA aglycones *in vivo*. Thus, it seems fair to argue that the main challenge for the field lies in overcoming our current inability to produce modified GPA aglycones at appreciable scale. Here, Liskamp’s pioneering work on mimicking the C-O-D and D-O-E rings found in GPAs by synthetic triazolo moieties is a great example of a novel strategy to provide alternative synthetic routes to such GPA aglycones.(93,94) This strategy uses a linear precursor that is then crosslinked using copper/ruthenium catalysts, and as such is a powerful example of the utility of biomimetic synthesis for GPAs. In this way, the next major breakthroughs in GPA research will be only accessible by re-thinking the chemical or biochemical synthesis of these compounds, and using the biosynthetic logic of the natural NRPS/P450 machinery as an inspiration in order to alter these highly conserved peptide sequences to maximize the chances of translational impact. The recent optimization of a chemoenzymatic strategy that combines chemical peptide synthesis with P450-biocatalysis to form tricyclic GPA aglycones now offers a glimpse into a future that would allow detailed structure/activity relationships to be deduced for novel GPA aglycones.(44) The use of peptide synthesis and the availability of thousands of amino acids provide an boundless source of potential GPA modifications at any position of the peptide, which augers well for future GPA reengineering and production if the key NRPS machinery can be successfully reengineered – this is clearly a priority for the field.

**Conclusions**
Over the past 70 years, GPAs have been effectively used as a last resort therapeutic option for the treatment of serious gram-positive infections. The increase in prevalence of “superbugs” such as VRE has led to major efforts from the entire scientific community to identify and generate new GPAs to counter such growing threats to modern medicine. In this regard, “mixing and matching” methodologies from chemistry, biochemistry, enzymology and microbiology have provided huge potential benefits in the diversification of GPAs and have broadening their antibacterial spectra. However, such research must now continue if we are to innovate our way out of an antibiotic crisis that may only be just beginning, with the evolution of bacterial resistance likely to remain a constant problem even if we gain access to improved antibacterial agents. Furthermore, diversification of GPAs offers a potential pathway to the generation of selective antibacterial agents, which can be tuned to target pathogenic strains of interest: this remains one of our best chances to slow the evolution of new resistance mechanisms in the future, and should be a priority for antibiotic development. Beyond innovating our way out of the current antimicrobial resistance crisis that we find ourselves in, we also as a community must address the fact that we need to carefully manage all aspects of our use of antibiotics. We must more carefully guard against the inappropriate future exploitation of this precious resource for questionable benefits, such as for weight gain in agriculture. Evolution has provided us with a wealth of antibiotic resources to use and thus improve our lives through modern medicine, but also can just as quickly lead to rapid and widespread resistance if we do not learn our lesson from current events.

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Figures

Figure 1. A comparison of the interactions between GPAs and their lipid II target shown for vancomycin against sensitive (A) and resistant (B) bacteria. In the case of sensitive bacteria, the interaction between the GPA and lipid II is centered on five hydrogen bonds between the peptide backbones of both compounds (green arrows), which result in complex formation that then inhibits the actions of cell wall synthesis enzymes (TP – transpeptidase, TG – transglycosylase) and leads to eventual cell lysis. Resistance mediated via the exchange of the final D-Ala moiety for a D-Lac leads to the loss of one hydrogen bond and replaces this with lone pair/lone pair repulsion (red arrow), leading to a loss of GPA binding affinity of three orders of magnitude and rendering GPAs ineffective against such bacterial strains.
Figure 2. Diversity and differences between type I (A) and IV (B) GPAs.

(*) second generation; G: Glycosyl-; CM: Chemically modified.
Figure 3. Balhimycin biosynthesis. Peptide extension is achieved by the NRPS multi-modular machinery, which comprises seven modules spread across three polypeptide chains. Chlorine atoms (blue) are added when the tyrosine is linked to the PCP domain in the NRPS by a halogenase, whilst the and β-hydroxyl groups (orange) are added prior to activation of β-hydroxytyrosine by the main NRPS assembly line (A – adenylation domain, C – condensation domain, PCP – peptidyl carrier protein domain, E – epimerization domain, X – Oxy recruitment domain, TE – thioesterase domain; Oxy enzymes – cytochrome P450s). Bht: β-hydroxytyrosine, Hpg: 4-hydrophenylglycine; Dpg: 3,5-dihydroxyphenylglycine.
Figure 4. Vancomycin aglycone: total chemical retrosynthesis, in vivo biosynthesis and chemoenzymatic synthesis. (A) The 5 key steps in the vancomycin aglycone total synthesis by Evans, Nicolaou and Boger. (B) Comparison of in vivo biosynthesis and chemoenzymatic synthesis. S_NAr: nucleophilic aromatic substitution, CoA: coenzyme A, C: condensation domain, A: adenylation domain, PCP: peptidyl carrier protein domain, X: Oxy-recruitment domain TE: thioesterase domain, Oxy enzymes – cytochrome P450s.
Figure 5. The intermediaries in the backbone modified vancomycin aglycone \([\Psi[\text{CH}_2\text{NH}]\text{Tpg}_4]\) (10) and \([\Psi[\text{C}=\text{NH}\text{NH}]\text{Tpg}_4]\) (11) syntheses. 10a/11a: Fully protected tripeptide 4-to-6 with C-O-D ring incorporating the backbone modification; 10b/11b: tetrapeptide 4-to-7 with both A-B and C-O-D rings; 11c: vancomycin aglycone \([\Psi[\text{C} (=\text{S})\text{NH}]\text{Tpg}_4]\) bearing a thioamide function between Hpg 4 and 5. Building blocks indicated in dashed square are commonly used in all synthetic routes from Boger and co-workers. Atoms shown in orange are labeled to allow these to be traced from their origins to their positions in the final modified backbone. The green arrows show the effect of 3 and 4 on their interaction with the D-Ala-D-Lac target sequence.
Figure 6. Peptide backbone modifications and their Oxy enzymes acceptance. (A) Chemoenzymatic synthesis of an analog 11c (Ψ[C(=S)NH]Tpg)]. (B) The incorporation of β-Dpg on teicoplanin and actinoidin sequences and its effect on the formation of the C-O-D and D-O-E rings. Building blocks indicated in the dashed squares represent the modified starting amino acids. Atoms shown in red are labeled to allow these to be traced from their origins to their positions in the final modified backbone.
Figure 7. Modifying the halogen pattern of GPAs. (A) Chemical approaches explored with vancomycin. (B) In vivo approaches tested with the balhimycin system. NBP: \textit{N}-bromophtlimide
Figure 8. Routes developed to modify the N- and C-termini of vancomycin. All modifications can be carried out either with or without the disaccharides present.
Figure 9. Chemical and chemoenzymatic methodologies for GPA diversifications. (A) Chemical synthesis of oritavancin (6) and telavancin (7) from naturally biosynthesized GPAs chloroeremomycin and vancomycin. (B) Combinatorial syntheses from the vancomycin aglycone: glycorandomization at the D-glucose moiety through GftE-mediated incorporation of modified sugars and click chemistry on a 6-azido-α-D-glucose moiety introduced by the actions of GftE.
Figure 10. Further modifications of type IV GPAs. (A) In vivo modification of A40926 producer strain to allow the preparation of homogenous dalbavancin analogs. (B) Chemoenzymatic de-acylation/ re-acylation of teicoplanin (2) and aldehyde trapping leading to the isolation of various amidated or aminated products.
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