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Recent advances in synthetic analogues of lantibiotics: What can we learn from these?

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ABSTRACT

The lantibiotics are a family of antibacterial cyclic peptides distinguished by one or more thioether linkages between amino acid side chains, and by unique modes of action. Recent developments in the chemical synthesis, mutagenesis and mutasynthesis of these peptides are providing insights into the structural requirements for antibacterial activity and into the mode of action, as well as having the potential to produce analogues with greater stability, potency and bioavailability. This Review provides a survey of these recent advances.

1. Introduction

The lantibiotics are a family of highly constrained cyclic peptides with unique structural and biological properties [1]. The defining structural feature of these peptides is that they contain one or more thioether linkage between amino acid side chains, arising from the incorporation of one of two bis-amino acids, either lanthionine (Lan) or threo-β-methyl lanthionine (MeLan) (Fig. 1). Other non-natural amino acids, such as dehydroalanine (Dha), dehydrobutyrine (Dhb), *S*-[(*Z*)-2-aminovinyl]-(3*S*)-3-methyl-D-cysteine (AviMeCys) and labionin (Lab) may also be present.

Lantibiotics are antibacterial peptides produced by Gram-positive bacteria to eliminate other competing bacteria. They are ribosomally synthesized with a leader peptide attached, and then undergo an extensive series of post-translational modifications, including dehydration of serine or threonine residues, thioether bridge formation by Michael addition of cysteine residues to the resulting Dha or Dhb, export and leader peptide cleavage to give the final lantibiotic structure. The biosynthesis of these peptides has been the focus of intensive research over the past 15 years and the roles of the various biosynthetic enzymes in the gene clusters are now well understood [2,3].

Many lantibiotics exert their antibacterial action through complexation with lipid II, a key precursor in bacterial cell wall biosynthesis, either by preventing the cell wall biosynthesis through sequestration of lipid II, or by the formation of lipid II-peptide pores in the cell membrane, causing cell lysis [4,5]. Cinnamycin and related lantibiotics, however, specifically bind to phosphatidylethanolamine, thus by removing the substrate of phospholipase A2 [6].

As these modes of action are unique to lantibiotics, and as they have not yet been used extensively in a clinical setting, pathogenic bacteria have not yet evolved resistance to lantibiotics. They therefore hold considerable potential as the next generation of antibacterial agents [7,8]. However, lantibiotics are not ideal as therapeutic entities, as they are unstable and/or insoluble at physiological pH, prone to oxidation and proteolysis, and cannot be orally administered. There is therefore a great need to find effective routes to synthesise variant lantibiotics, both in order to understand in more depth their mode of action, and also to prepare analogues with potentially superior pharmacokinetic properties. In this review I have surveyed firstly recent advances in strategies for the synthesis of modified lantibiotics. This is followed by a review of novel analogues of lantibiotics reported over the last five years, with the emphasis on what we have learned about both potency, mode of action and development of more stable analogues from these studies. Finally, the synthesis of modified lantibiotics as chemical biology tools or as therapeutic agents is discussed.

2. Synthetic analogues of lantibiotics: a testing-ground for new methodology

The unique structural properties of the lantibiotics, and in particular the multiple sequential and overlapping thioether

Abbreviations: Aha, azidohomoalanine; Aloc, allyloxycarbonyl; Dha, didehydroalanine; Dhb, didehydrobutyrine; Eth, Ethionine; Hpg, homopropargyglycine; Mob, *p*-methoxybenzyl; *p*-NB, *p*-nitrobenzyl; Nle, norleucine; *p*-NZ, *p*-nitrobenzyloxy carbonyl.

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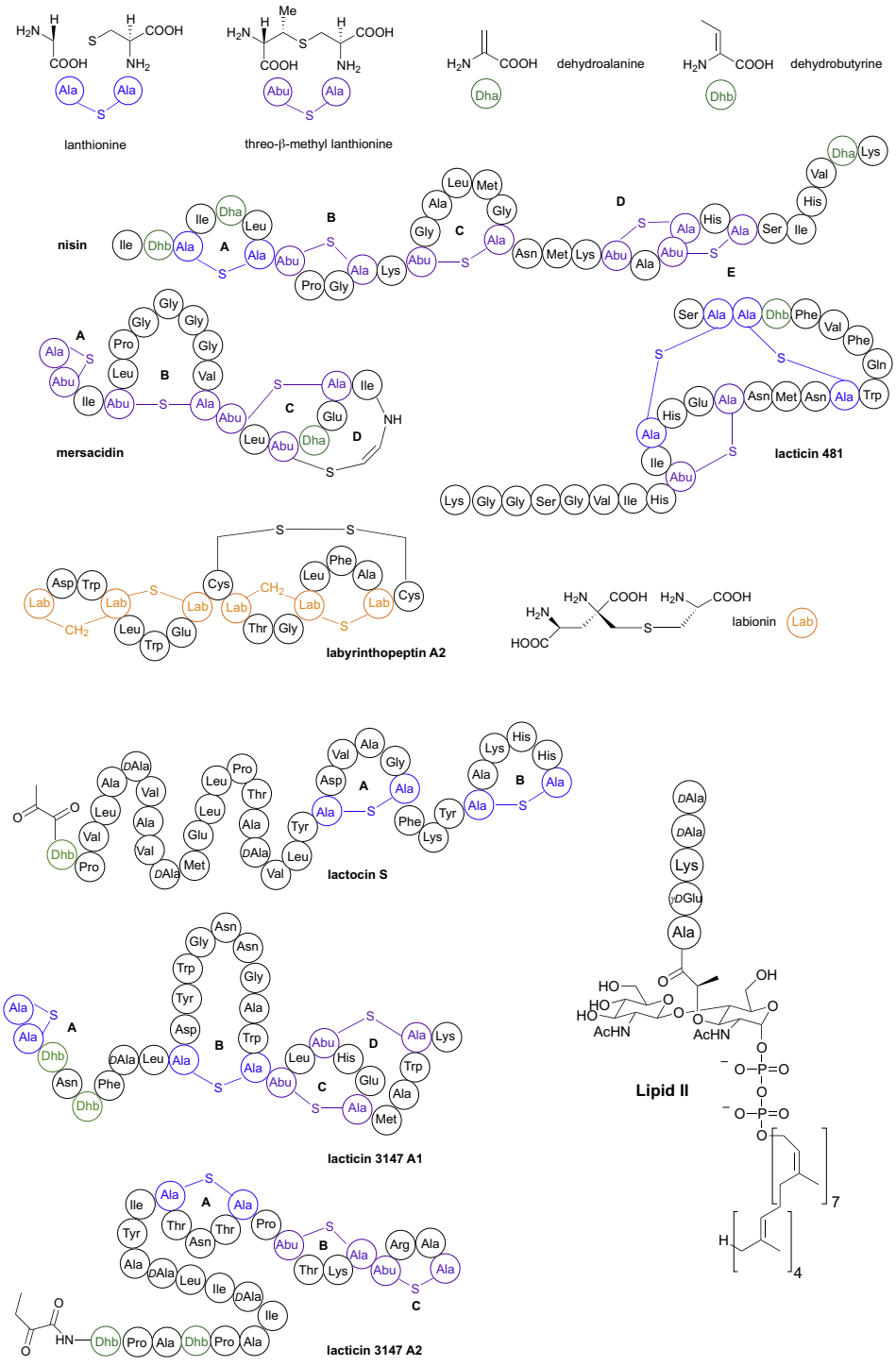


Fig. 1. Structures of representative lantibiotics, posttranslationally modified amino acids, and lipid II.

side-chain bridges, have posed a formidable synthetic challenge to peptide chemists over the last two decades. A number of different strategic approaches have been explored, and these were last comprehensively reviewed in 2011 [9]. For the preparation of synthetic analogues of lantibiotics incorporating thioether bridges, two approaches stand out as most successful: the solid-phase synthesis (SPPS) of these peptides using orthogonally protecting lanthionine or methyl lanthionine building blocks; and a biotransformation approach, utilizing the enzymes responsible for the biosynthesis of lantibiotics *in vivo* to manipulate synthetic peptide precursors. Since the previous review, several key advances have been made in both of these areas, and new synthetic routes to orthogonally protected building blocks have also been reported.

2.1. Recent developments in solid phase synthesis of lantibiotics

An important advance in the SPPS of lantibiotics in the past few years was the development of quadruply orthogonal protecting group strategies for synthesizing lantibiotics with overlapping thioether bridges. We recently reported [10] the synthesis of an ana-

logue of the D and E rings of nisin. Using orthogonally protected lanthionines **1** and **2**, linear peptide **3** was synthesized using standard solid-phase methodology (Scheme 1). The allyl ester and Aloc protecting groups from **1** were then selectively removed and the peptide cyclized on resin to give ring E analogue **4**, in which the lanthionine bridge had been formed regioselectively. This was followed by chain extension and selective removal of the orthogonal Teoc and TMSE groups (from **2**), allowing a second on-resin cyclisation to take place to form ring D. Further chain extension and resin cleavage afforded **5**.

Vederas and co-workers recently disclosed the solid-phase synthesis of both peptides, A1 and A2, that form the two-component lantibiotic lacticin 3147 [11]. The overlapping thioether bridges of rings C and D at the C-terminus of lacticin 3147 A1 presented the greatest challenge, and were again tackled *via* an orthogonal protecting group strategy. The orthogonally protected MeLan residues **6** and **7** were used to synthesise the resin-bound linear peptide **8** (Scheme 2). Selective deprotection of allyl ester and Aloc protecting groups followed by on-resin cyclisation gave peptide **9** with ring D in place. Chain extension and selective deprotection of the p-NZ and p-NB groups from **7**, followed by on-resin cyclisation, gave resin-bound peptide **10** with both rings C and D cyclised. In each case the selective removal of the mutually orthogonal protecting groups on **6** and **7** enabled each MeLan bridge to be formed regioselectively. Further rounds of SPPS, incorporating lanthionine **1** and forming ring B via a similar strategy, gave resin-bound peptide **11**. The N-terminal segment **12**, with its extensive posttranslational modifications, was synthesized separately in solution via a segment condensation approach, and then attached to the N-terminus of **11** to complete the peptide. Deprotection and cleavage from the resin afforded lacticin 3147 A1. Knerr and van der Donk subsequently used this approach to synthesise analogues of epilancin 15X [12], with the overlapping bridges of rings B and C again installed by using the orthogonally protected MeLan residues **6** and **7**.

2.2. Unusual amino acid building blocks

This approach to the solid-phase synthesis of lantibiotics relies on the availability of orthogonally protected lanthionine building blocks. Until recently, only synthetic routes to protected lanthionine derivatives were available, with the corresponding methyl lanthionine derivatives being hard to access efficiently and with the correct stereochemistry. In the past few years three new synthetic routes to methyl lanthionine have been published. Vederas and co-workers carried out a ring-opening of aziridine **13** (derived from Trt-Ser-OH) with Fmoc-Cys-OH to afford methyl lanthionine **7**, which was used directly in their synthesis of lacticin 3147 A1 (Scheme 3) [11].

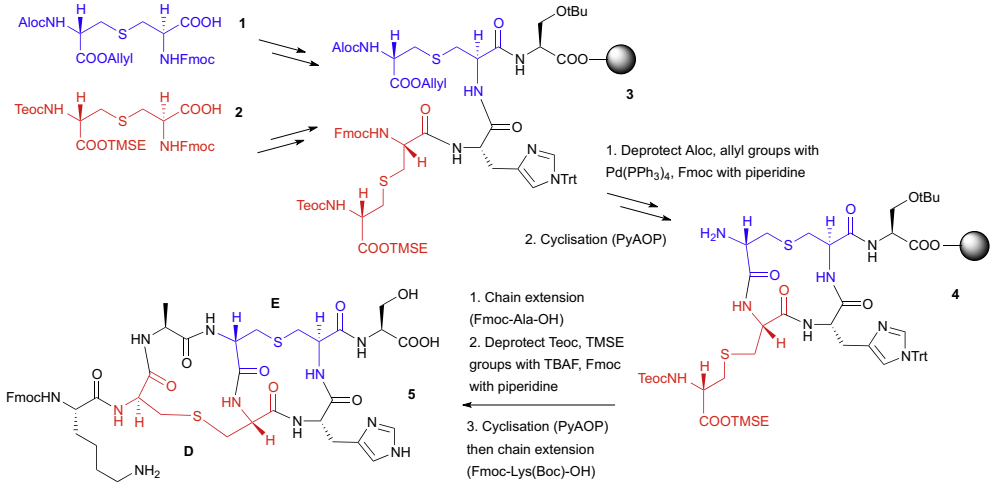
Knerr and van der Donk [12] used a different approach. β-Methyl-D-cysteine derivative **14** (derived from D-Thr) was deprotected and coupled to L-bromoalanine **15** under phase transfer conditions, giving **7** as a single diastereoisomer (Scheme 4).

Several groups have in the past attempted to synthesise lanthionine and methyl lanthionine via a biomimetic approach, imitating the Michael addition of cysteine to dehydroalanine or dehydrobutyrine. However until recently it has not been possible to control the stereoselectivity of the Michael addition. Avenoza and co-workers have recently published the first asymmetric sulfa-Michael addition routes to lanthionine and methyl lanthionine [13]. For example (Scheme 5) the chiral didehydrobutyrine derivative **16** was reacted with Boc-Cys-OH to give **17**, which was deprotected to give the naturally occurring methyl lanthionine as a single diastereoisomer.

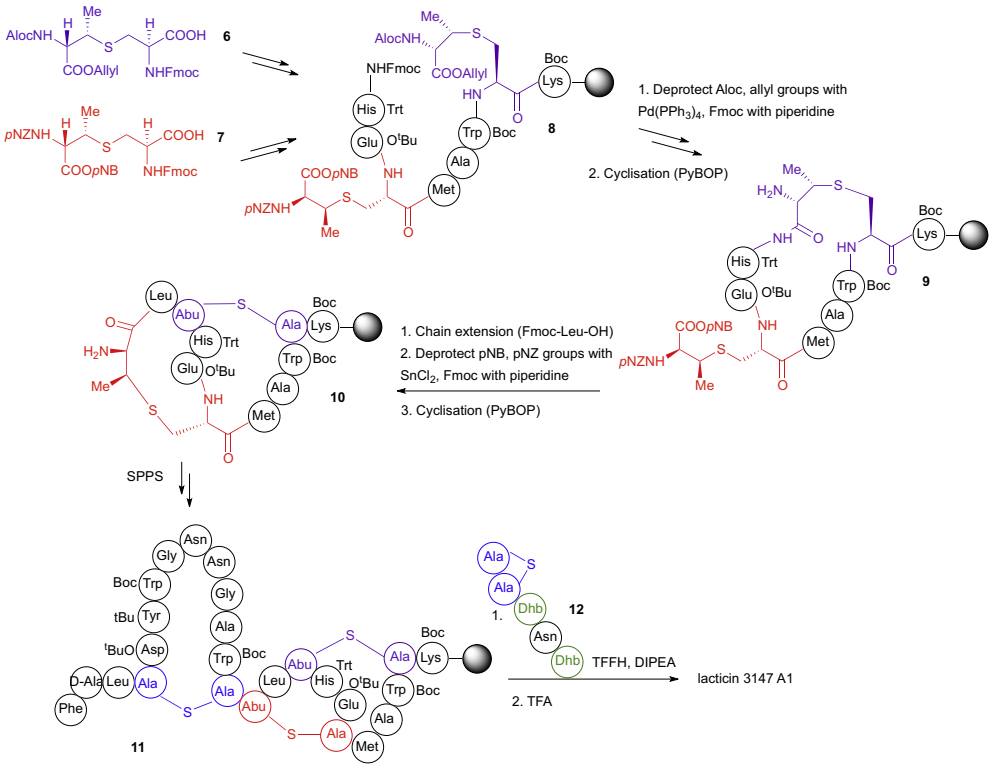
A new class of lantibiotics, the labyrinthopeptins, have recently been discovered and their structure established [14]. They have a previously unknown posttranslational modification, an α,α-disubstituted amino acid labionin. This effects a double bridge in this class of lantibiotics, one thioether bridge and one unusual carbocyclic bridge. The solid-phase synthesis of the labionins will require the development of a new amino acid moiety, with three sets of orthogonal protecting groups. Süssmuth and co-workers have published preliminary work towards this goal [15]. The α,α-disubstituted amino acid **18** [16] was prepared from D-serine and converted to **19** (Scheme 6). The orthogonal functionality in **19** should allow for eventual incorporation into labyrinthopeptin A2 via the strategy shown in Scheme 6, although the use of this amino acid in peptide synthesis has not yet been reported.

One post-translational modification that is found in lantibiotics such as epidermin, cypemicin and mersacidin is an extra amino vinyl thioether bridge formed via the inclusion of the unusual amino acid AviMeCys at the C-terminus. Recent biosynthetic studies (reviewed in [17]) have led to a proposed mechanism of AviCys formation via dehydration of a Ser residue to give a Dha, followed by oxidative decarboxylation of the C-terminal Cys residue of the precursor to form an enethiolate. It is believed that the enethiolate then undergoes 1,4-conjugate addition to the Dha residue to give the AviCys unit (Scheme 7), following the known precedent of lanthionine and methyllanthionine biosynthesis [1].

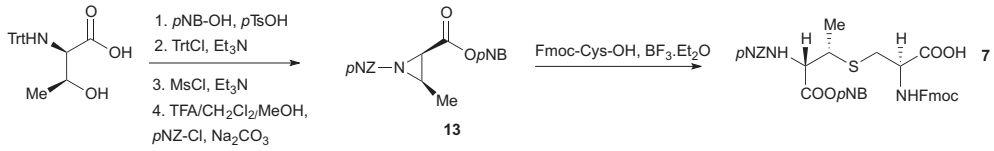
Inspired by this insight, VanNieuwenhze and co-workers have developed a stereoselective decarbonylative pathway to AviMeCys-containing peptides [18]. This has been applied this to



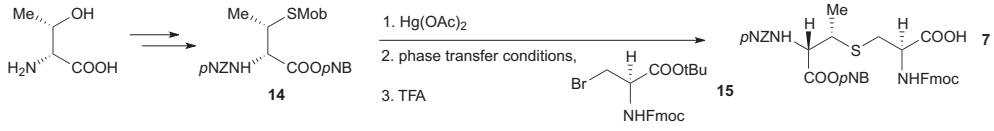
Scheme 1. SPPS of an analogue of the D and E rings of nisin.



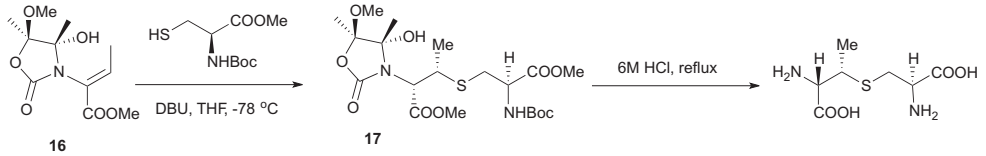
Scheme 2. Synthesis of lacticin 3147 A1 by solid phase peptide synthesis methods.



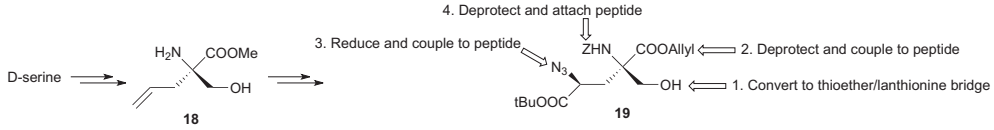
Scheme 3. Vederas' synthesis of protected methyllanthionine **7**.



Scheme 4. Van der Donk's synthesis of protected methyllanthionine **7**.



Scheme 5. Asymmetric sulfa-Michael addition to give methyllanthionine.



Scheme 6. Key amino acid precursor for the eventual synthesis of labyrinthopeptins.

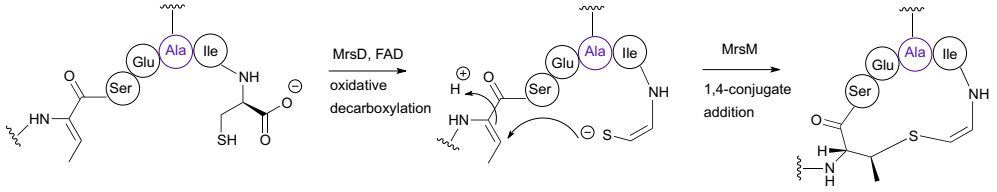
the synthesis of an analogue of the C-terminal D-ring of mersacidin [19] (Scheme 8) in which the precursor peptide **20** was oxidatively decarbonylated to give predominantly the desired (*Z*)-enamide **21**.

2.3. In vitro mutasynthesis

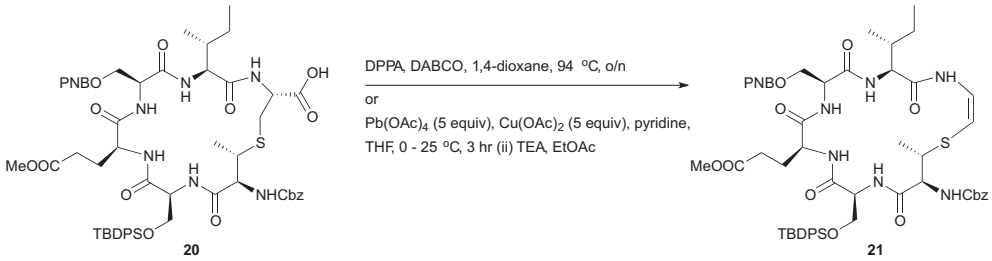
An equally powerful approach to the synthesis of lantibiotic analogues containing nonproteinogenic amino acids is to harness the enzymes involved in lantibiotic synthesis to process unnatural substrates. This "in vitro mutasynthesis" (IVM) approach was pioneered by Van der Donk and co-workers [20,21] and relies on the discovery that many of the lantibiotic synthetase enzymes will accept a wide range of possible peptide substrates for processing, provided that the peptide includes a leader sequence recognized by the enzyme.

This approach is illustrated by the IVM of lacticin 481 analogues containing novel amino acids (Scheme 9). Precursor peptides such as **22** were synthesized with the leader sequence recognized by lacticin 481 synthetase (LctM) attached to the mutant substrate by click chemistry. These peptides were successfully processed by the isolated enzyme LctM to give lanthionine-bridged peptides **23** which were then cleaved from the leader sequence by LysC protease. A range of different non-proteinogenic amino acids were included at the positions shown to give single mutant peptides, as well as a mutant with Gly2, Gly3 and Gly5 all replaced by β-Ala (Scheme 9).

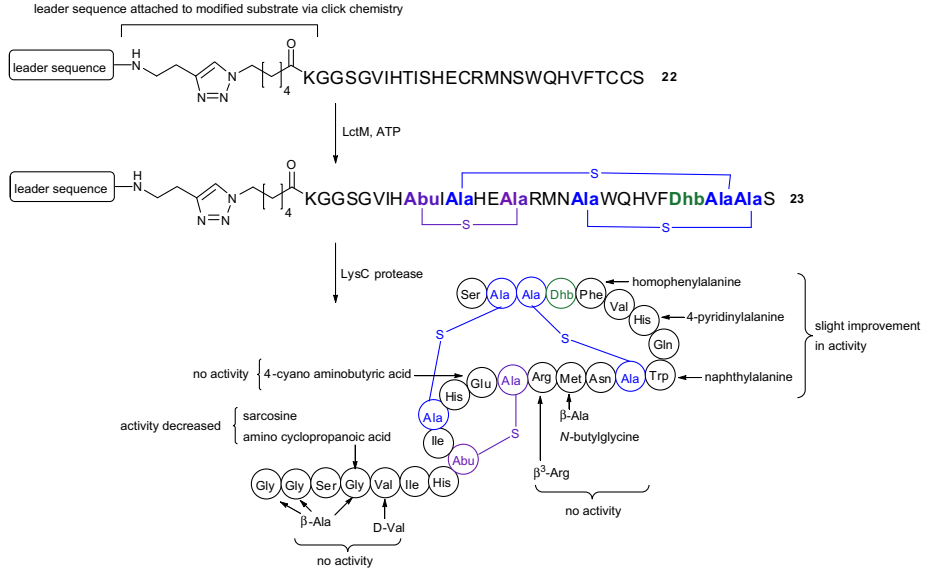
Süssmuth and co-workers have also developed an approach to the *in vivo* production of variant lantibiotics containing nonproteinogenic amino acids [22]. In order to generate mutants of the two-component lantibiotic lichenicidin, an auxotrophic *E. coli* strain B834 (Met $^-$) was transformed with both a plasmid encoding the sequence of the prepropeptide of one of the components (Bliα), and with a fosmid encoding the genes for the posttranslational modification, processing and export of this lantibiotic. As this auxotrophic *E. coli* strain cannot synthesise Met, growing the transformed cells in the presence of various non-coding amino acids such as Hpg (homopropargylglycine) and then induction of peptide synthesis with IPTG, resulted in the incorporation of these amino



Scheme 7. Proposed biosynthetic pathway for the C-terminal modification of mersacidin.



Scheme 8. VanNieuwenhze route to an analogue of the C-terminal D-ring of mersacidin.



Scheme 9. Unnatural mutants of lacticin 481 produced by IVM.

acids in place of the Met28 residue in the wild-type sequence (Scheme 10). Similarly, the auxotrophic *E. coli* strain JM83 (Pro $^-$) was used to replace the Pro residues in Bliα and Bliβ with (4*R*-OH)Pro, (4*S*-F)Pro, (4*R*-F)Pro or thioproline, and the auxotrophic *E. coli* strain ATCC 49980 (Trp $^-$) was used to replace the Trp residue in Bliβ with (4-F)Trp, (5-OH)Trp or (7-Aza)Trp.

3. Exploring structure–activity relationships through synthetic analogues

As lantibiotics represent a potentially novel strategy for combating the rise of antibiotic-resistant bacteria, there is currently intense interest in developing novel lantibiotics which do not suffer from the limitations of stability and pharmacokinetic properties of the naturally occurring peptides. Recent studies have focused on mutagenesis studies to incorporate either proteinogenic or non-proteinogenic amino acids, on replacement of the sulfur atoms in the lanthionine or methyl lanthinonine with carba-bridges or with oxygen atoms, and on studying the stereochemistry of the lanthionine and methyl lanthionine residues themselves.

3.1. Mutagenesis with proteinogenic and non-natural amino acids

Enhanced understanding of how these peptides are biosynthesized by the producing organisms has allowed many groups to study structure–activity relationships by traditional mutagenesis studies, utilizing the 20 proteinogenic amino acids. Nisin, lacticin 481, nukacin ISK-1, mersacidin, and the two-component lantibiotics lacticin 3147 A1/A2 and haloduracin αβ, have all been studied in this manner. Since the recent comprehensive review of this area [23] several further advances in bioengineering derivatives of nisin and lacticin 3147 with enhanced antimicrobial activity have been reported.

The two-component lantibiotic, lacticin 3147, consists of two peptides, lacticin 3147 A1 and lacticin 3147 A2, which act synergistically against a range of Gram-positive organisms. The mode of action involves the more compact lacticin 3147 A1 binding to lipid II; it has been proposed that this complex then interacts with lacticin 3147 A2 to inhibit cell wall biosynthesis and promote pore formation [24]. Cotter, Hill and co-workers have recently carried out saturation mutagenesis on selected residues within lacticin 3147 A1 [25]. In contrast to this group's earlier studies of lacticin 3147 A1 and A2 by alanine scanning [26], they demonstrated that the A1 peptide can tolerate a wider range of amino acid changes and still retain biological activity. Mutations which prevent lanthionine/methyl lanthionine bridge formation abolish antimicrobial activity altogether. However, conservative mutations in the region believed to mediate interaction with lacticin 3147 A2 (Phe6, D-Ala7, Trp12, Asn14) are tolerated, with the exception of Phe6 mutants, which were all inactive (Fig. 2). Conservative mutations in the putative lipid II binding region (Leu21 and Glu24) are also tolerated, whilst Trp18 mutants were also all inactive. Importantly, a His23Ser mutant was found to have improved antimicrobial activity when acting synergistically with the wild-type lacticin 3147 A2 peptide. This is the first time such an increase in antibacterial properties has been observed for bioengineered two-component lantibiotics, and may arise either from an improved interaction between the two peptides or from an enhanced interaction with lipid II.

The mode of action of nisin has been extensively studied [4,5]. The target for this lantibiotic is also lipid II, and it has been established that rings A and B of nisin form a "cage" structure which complexes with the pyrophosphate moiety of lipid II. This positions the C-terminus (rings D and E) in such a way as to form pores in the cell membrane, with the pore being made up of 4 lipid II and 8 nisin molecules. Although the exact details of the pore assembly, and the role of ring C, remain to be elucidated, nisin has been the subject of extensive SAR studies in recent years (Fig. 3). These have established that all five of the lanthionine/methyl lanthionine

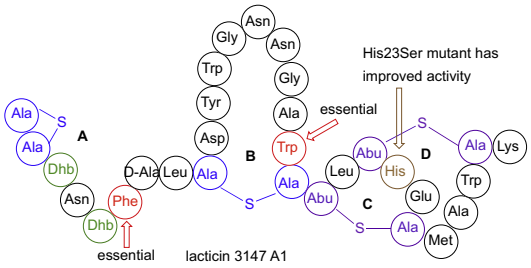
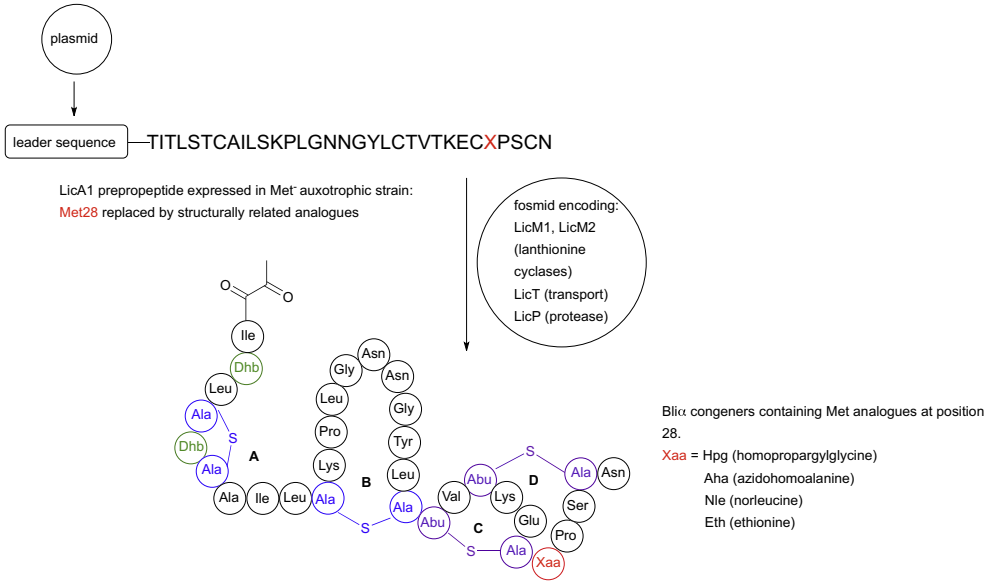


Fig. 2. Mutants of lacticin 3147 A1 which preserve or improve biological activity.



Scheme 10. Süssmuth's *in vivo* synthesis of mutants of the two-component lantibiotic lichenicidin.

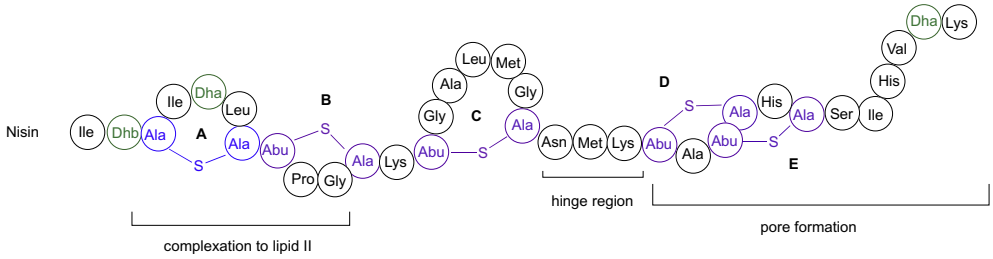


Fig. 3. Mode of action of nisin.

rings are important for biological activity. Truncated mutants (nisin(1–22)) lacking the C-terminal rings display some antibacterial action through binding to lipid II and thereby preventing cell wall biosynthesis, although these truncated mutants cannot form pores. Mutations in ring B are generally not tolerated, whereas in ring A several different substitutions are possible. Another region of nisin which is of crucial importance is the "hinge" region between rings C and D, although the exact role of the hinge in formation of the nisin:lipid II complex is not well understood. Building on the previous SAR studies, Cotter, Hill and co-workers have been able to rationally predict regions of the nisin sequence where saturation mutagenesis should lead to variants with enhanced antibiotic activity. Thus, saturation mutagenesis of position 29, which has been previously shown to be crucial for biological activity, led to the isolation of a number of mutations with enhanced activity against both Gram positive and Gram negative bacteria [27]. Similarly, investigation of mutants at position 12 (the link between ring B and ring C) identified other sequences with enhanced antimicrobial activity [28]. Finally, intensive mutagenesis of the three amino acids (positions 20–22) which form the hinge region led to the isolation of mutants with hinge sequences Ala-Ala-Ala and Ser-Ala-Ala with greater activity against several bacterial strains [29].

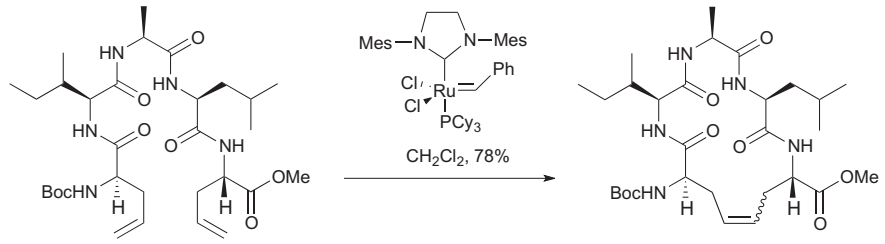
The recent advances in both solid-phase synthesis and mutasynthesis of lantibiotics containing non-natural amino acids (Sections 2.1 and 2.3) have also shed some light on SAR for the peptides studied. Synthetic studies on epilancin 15X [12] have shown that the N-terminal sequence (residues 1–8) is important, as an analogue with these removed displayed 100-fold less activity than the wild type sequence. Replacement of the N-terminal DLac group with a pyruvyl moiety had little effect on activity, suggesting that this particular post-translational modification mainly results in a peptide with increased stability. Surprisingly, replacement of the three Dha and Dhb residues by their saturated counterparts (Ala and Abu) also led to derivatives with a small decrease in activity. Some of the unnatural amino acid substitutions in lacticin 481 were also tolerated [20]. Conservative substitutions of aromatic amino acids in ring C gave mutants with similar or slightly improved activity (Scheme 9). However, substitutions at the N-terminus gave mutants with significantly reduced activity, and mutations in rings A/B abolished activity completely. This is in line with earlier mutagenesis studies with proteinogeneic amino acids (reviewed in [23]) which showed that N-terminal truncated lacticin 481 analogues had decreased activity, and that substitutions in rings A and B were generally not tolerated.

In contrast, few studies have been carried out on the two-component lantibiotic lichenicidin, and much less is known about the structure–activity relationships of this lantibiotic. Based on preliminary alanine scan studies [30], NMR structures of both Bliα and Bliβ [31], and comparison to lantibiotics with similar structures such as mersacidin [32] and the two-component lantibiotic haloduracin [33] it has been proposed [31] that Bliα binds to lipid II, possibly via rings C and D. The Glu17 residue in ring D is believed to be criticial for this binding, as mutation of this residue abolished biological activity. The resulting Bliα/lipid II complex is then believed to interact with Bliβ, which adopts a largely helical conformation, and may result in pore formation. In the Süssmuth group studies of lichenicidin variants with unnatural Met, Trp and Pro analogues incorporated, only the Bliα variants with unnatural Met analogues (Scheme 10) were tested for biological activity, in synergy with wild-type Bliβ [22]. Of these analogues, none showed enhanced activity compared to the wild-type Bliα, however the Aha analogue showed equal antibacterial activity.

3.2. Modified lantibiotics with carba- or oxy-bridges

One feature of the lantibiotics that currently limits their usefulness in the clinic are the thioether linkages, which are prone to aerial oxidation. It has been demonstrated that oxidation of the thioether bridges in nisin diminishes its ability to bind to lipid II and causes a complete loss of antibacterial activity [34] and this may well be the case for other families of lantibiotics. In recent years, research has therefore focused on using advances in synthetic methodology to prepare lantibiotic analogues with different, non-natural bridges, and assessing the biological properties of these novel analogues.

Liskamp and co-workers have explored replacing the thioether bridges in nisin with carbon bridges. Analogues of each of the individual rings A (Scheme 11) B and C were synthesized by ring-closing metathesis of linear precursors containing allyl glycine or β-methyl allyl glycine residues [35,36]. These ring fragments were then joined together by standard methods (EDC, HOBt) to give analogues of the N-terminal AB (**25**, **26**) and ABC rings **29** (Fig. 4) with C=C double bonds replacing the thioether bridges. All of these peptides were produced as a mixture of E and Z double bonds, which were not separated: these peptides were also hydrogenated to give the saturated analogues **27** and **28**. In order to simplify the synthesis, and to further increase the stability of the peptides, the Dha and Dhb residues at positions 2 and 5 were replaced by Ala, and the Met residue at position 17 by norleucine (Nle). The AB ring analogue **24** with the (*S*,*S*) stereochemistry (opposite to that seen in nisin) was also prepared. These analogues were then evaluated for binding to the target, lipid II, by measuring their ability to inhibit the release of carboxyfluorescein (CF) from large unilamellar vesicles (LUVs) containing lipid II [36]. The AB and ABC analogues **24**–**29** were not able to form pores in the LUVs in their own right, and were not as effective as wild type nisin (1–12) (the native AB rings) or nisin(1–20) (the native ABC rings) in competing with full-length nisin for lipid II binding and hence inhibiting the release of CF. The highest affinity for lipid II was shown with the saturated analogue **27**, which has the same stereochemistry as the wild type nisin(1–12); by contrast the saturated analogue **28**, with a methyl group mimicking the methyl group of the methyllanthionine residue normally found at this point, was completely inactive. Interestingly, molecular modeling studies of all six of these peptides, and comparison with modeling of wild



Scheme 11. Synthesis of a carba-analogue of nisin ring A by RCM.

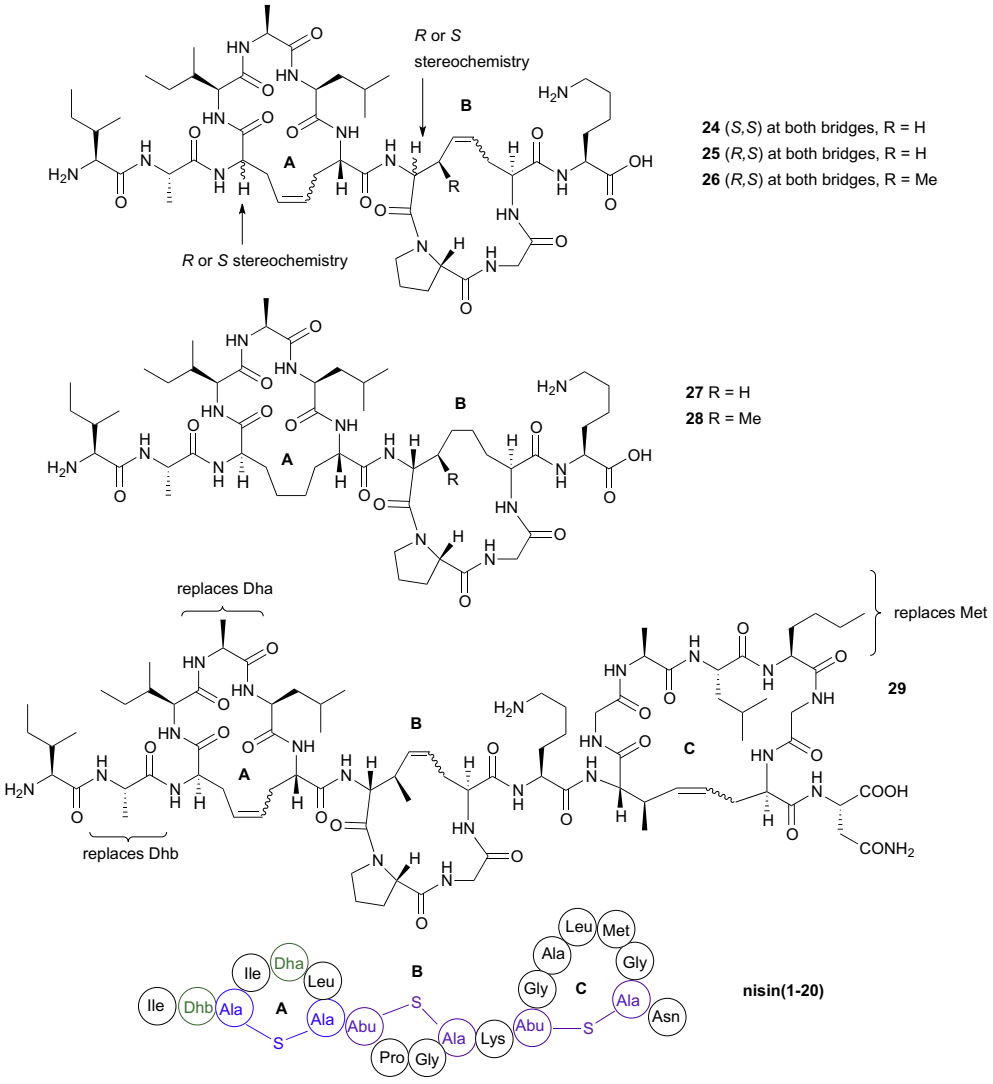


Fig. 4. Carba analogues of nisin AB and ABC rings.

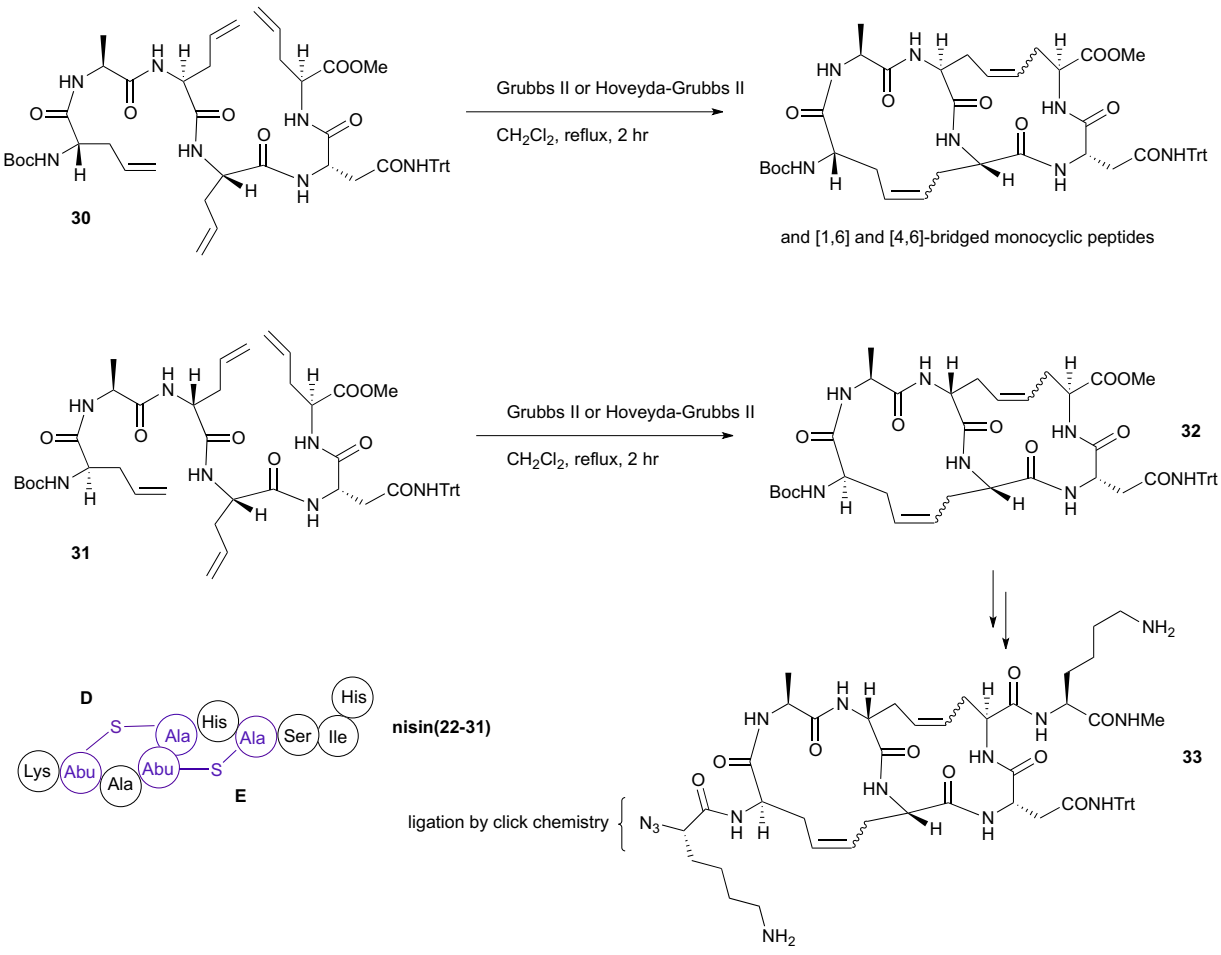
type nisin(1–12), indicated that the saturated analogue **24**, with the opposite stereochemistry at the α-carbons of residues 3 and 8 to that seen in the wild-type peptide, appeared to be closest in structure to the wild type nisin(1–12). Alkyne-bridged analogues of ring A, ring B and ring C of nisin have also been prepared by ring-closing alkyne metathesis [37], however these have not been joined together to form N-terminal analogues with the potential for binding lipid II, and their biological and structural properties have not yet been evaluated.

A RCM approach was also successfully used to prepare mimics of the overlapping D/E rings of nisin. Initially, an analogue of rings D and E with L-stereochemistry at all of the backbone amino acids was synthesized via a stepwise approach. The C=C bridged analogue of ring E was first prepared on resin by cross-metathesis between two allylglycine residues, followed by synthesis of the first ring, chain extension and RCM to form the second C=C bridge [38,39]. However, when the direct synthesis of the C=C mimic of rings D and E was attempted, with both C=C bridges formed at the same time from a linear peptide **30** with L-stereochemistry at all of the backbone amino acids, a complex mixture of products was formed (Scheme 12). In contrast, when a similar linear peptide **31** with D-stereochemistry at the first and third allylglycine residues was cyclized, using either Grubbs II or Hoveyda–Grubbs II catalysts, a bicyclic hexapeptide **32** with exclusively the same crossed ring pattern (1→ 4/3→6) as the nisin D/E ring system was produced (Scheme 12) [38–40]. This may suggest that the

linear peptide with the D-stereochemistry at residues corresponding to the D-amino acids in rings D and E of nisin is favourably pre-organised for this cyclisation. All four of the possible diastereoisomers, corresponding to the four possible double bond geometries (Z^{1-4}/Z^{3-6}, Z^{1-4}/E^{3-6}, E^{1-4}/Z^{3-6} and E^{1-4}/E^{3-6}) were produced and could be separated by HPLC and characterised [40] with the highest yield being the E^{1-4}/E^{3-6} isomer.

Molecular modeling of each diastereoisomer suggested that the Z^{1-4}/E^{3-6} had the lowest energy, and this superimposed well on the structure of rings D and E from nisin itself [39]. The biological properties of the mixture of diastereoisomers were evaluated [40] by growth inhibition assays and by interaction studies with CF-loaded LUVs and compared with the native nisin(22–31) fragment [41]. Unsurprisingly, as the C-terminal portion of nisin is known [1] to require the N-terminal ring AB system for docking to lipid II and subsequent pore formation, neither the native peptide nor the carba-bridged analogue showed any biological effect. However, Slootweg et al. were also able to prepare a derivative of the carba-bridged analogue **33** suitable for ligation to the N-terminus of nisin by click chemistry [40], and this may shed further light on whether the carba-bridged analogue **32** is a good mimic of the thioether-bridged native peptide. In contrast to the analogues of rings AB and ABC, it proved impossible to access the saturated analogues of rings D/E by hydrogenation [40]. Furthermore, in this more crowded structure with overlapping bridges, steric considerations also precluded the preparation of ring D/E analogues from alkyne [37] or β-methyl allyl glycine [35,36] residues.

The Vederas group have also used RCM to synthesise a C=C bridged analogue of lacticin 3147 A2 **34** [42] (Fig. 5). The three C-terminal rings of this lantibiotic were built up by sequential on-resin RCM, with each bridge being formed on-resin using Grubbs' 2nd generation catalyst before adding the next series of amino acids. Cis-trans mixtures at each double bond were obtained. As with this group's total synthesis of the wild-type peptide [11] the N-terminal pentapeptide sequence, which includes two Dhb residues and an N-terminal α-ketoamide, was synthesized separately in solution and then coupled to the remainder of the peptide on the solid support. This group has also successfully synthesized an oxa-analogue of lacticin 3147 A2 **35**, using an orthogonal protecting group strategy and incorporating the protected oxa-lanthionines **36** and **37**. Finally, the bis(desmethyl) analogue **38** of lacticin 3147 A2 has also been prepared by solid phase peptide synthesis, replacing all of the methyl lanthionine residues with lanthionine. The antibacterial properties of all three peptides was evaluated, individually and in synergy with lacticin 3147 A1. The ring-expanded, C=C analogue **34** showed no biological activity [42] either alone or with lacticin 3147 A1, suggesting that the correct ring size is crucial for this lantibiotic, in contrast to the nisin carba analogues reported by Liskamp. Oxa analogue **35** retained some intrinsic antibacterial activity [43], albeit 20-fold lower than the native lacticin 3147 A2, but displayed no synergy with wild type lacticin 3147 A1. The opposite is true for the bis(desmethyl) analogue **38**, which had no intrinsic antibacterial activity but retained a reduced level of synergistic activity when combined with wild type lacticin 3147 A1 [44].



Scheme 12. Synthesis of C=C analogues of nisin D/E rings.

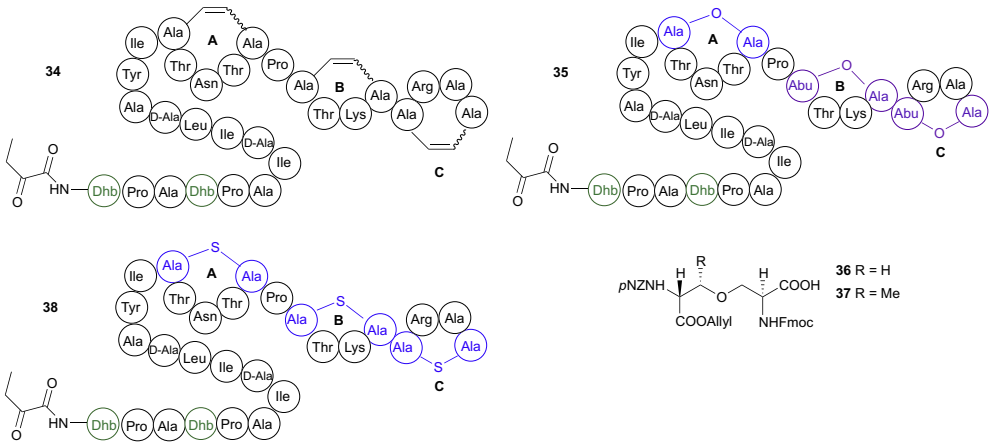


Fig. 5. Synthetic analogues of lacticin 3147 A2 with C=C and oxa bridges.

Whilst RCM approaches to carba bridged analogues of lantibiotics result in a larger ring size than the wild-type peptides, it is now also possible to synthesise carba bridged analogues with exactly the same ring size. The Vederas group has recently reported the solid-phase synthesis of carba bridged analogues **39**, **40** and **41** of lactocin S, in which the alkyl bridge is installed by incorporating orthogonally protected diaminopimelate **42** [45] (Fig. 6). In order to further stabilize the lactocin analogues against aerial oxidation, a further series of analogues in which the Met12 residue was replaced by either Nle or Leu (**43**–**45**) were also prepared [46]. Finally, an analogue in which the planar terminal ketoamide-Dhb unit was replaced by an oxazole, **46**, was also synthesized. Both the mutants **43** and **44** with lanthionine bridged and with Leu12 or Nle12 respectively retained full biological activity. Importantly, analogue **39**, in which the A ring has an alkyl bridge, also retained full biological activity. This is the first time a lantibiotic analogue with the thioether bridge replaced has been as active as the parent structure. Very little is known about the mode of action of lactocin S, however these results suggest that ring A is less crucial for interaction with the target of this peptide. The oxazole-substituted analogue **46** was completely inactive. Finally, aerial oxidation of these analogues, followed by re-testing for biological activity, indicated that mutant **45**, with one lanthionine and one methionine residue substituted with CH_2 groups, was the least affected by oxidation.

3.3. The role of lanthionine stereochemistry

A further benefit of solid-phase synthesis of lantibiotics is that it is comparatively easy to replace (*D,L*) lanthionine and methyl lanthionine with other diastereoisomers. In their first total synthesis of a naturally occurring lantibiotic [47], the Vederas group were able to confirm the stereochemistry of the lanthionine rings in lactocin S, which had previously been isolated from the producing strain in quantities too small for unambiguous assignment. Knerr and van der Donk investigated the effects of replacing each of the (*D,L*) lanthionine and methyl lanthionine residues in lacticin 481 with the (*L,L*) diastereoisomer [48]. All of the analogues incorporating (*L,L*) diastereoisomers were inactive, suggesting that they have modified three-dimensional structures when compared to the wild-type peptide.

4. Modified and hybrid lantibiotics as tools or therapeutics

4.1. Combining biological activity through conjugation to other bioactive molecules

Although the chemical sensitivity of many lantibiotics makes them tricky to derivatise, two research groups have recently succeeded in synthesizing bioconjugates of lantibiotics with other bioactive molecules in a bid to combine the best features of both components. Breukink and co-workers investigated the synthesis of hybrid antibiotics which target different features of lipid II [49]. The nisin(1–12) fragment, which binds to the pyrophosphate moiety of lipid II, was prepared by enzymatic cleavage of the wild type peptide. The C-terminus of the nisin(1–12) fragment was derivatised with 1-amino-3-azidopropane and conjugated to vancomycin, which targets the Lys-D-Ala-D-Ala section of lipid II, using click chemistry. Hybrids with the nisin(1–12) fragment attached to either the N-terminus or C-terminus of vancomycin were prepared, with different lengths of n-ethylene glycol (n-EG) spacers, and tested in an inhibition assay against various bacteria. The most

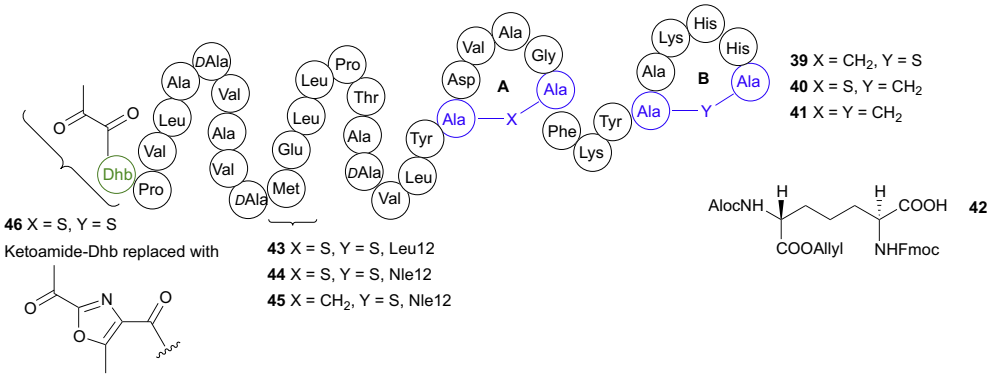


Fig. 6. Synthetic analogues of lactocin S.

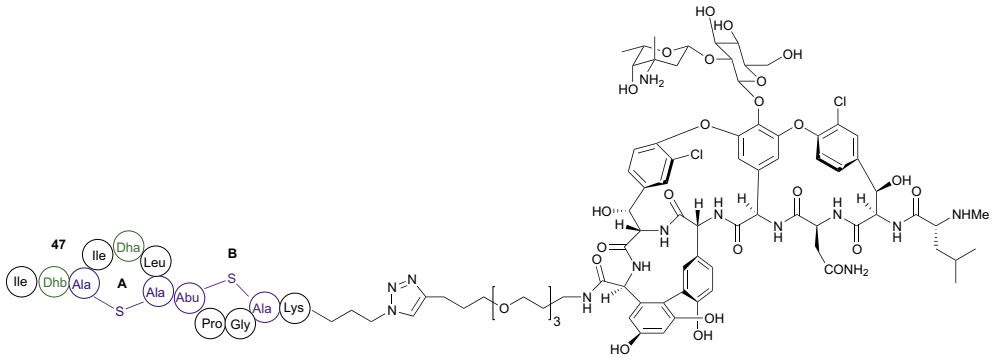


Fig. 7. Nisin(1-12)-vancomycin bioconjugate.

potent of these, **47**, showed a 40-fold increase in activity compared to each of the components separately (Fig. 7).

The Vederas group have also recently prepared gallidermin–siderophore conjugates [50] in which the wild-type gallidermin was modified at the Lys side chains by conjugating pyochelin, agrobactin or desferrioxamine B through squarate ester linkages. The aim of this study was to increase the efficacy of gallidermin against Gram-negative bacteria by providing a mechanism for it to penetrate the outer membrane via uptake by siderophore transporters. Regioselective bioconjugation of the siderophores was achieved, however it did not appear that the hybrid lantibiotics were taken up into Gram-negative bacteria in sufficient amounts to exert an antibacterial effect.

In preliminary studies of the feasibility of preparing antibacterial coatings from lantibiotics, McGuire and co-workers synthesized block copolymers with nisin reversibly conjugated [51]. In this work the N-terminal Ile was modified to introduce a thiol group, which was then coupled to PEO–PPO–PEO triblocks end-activated with pyridyl disulfide groups. In this work, these modified block copolymers also showed antibacterial activity, which was removed after reduction of the S-S linkage.

4.2. Modified lantibiotics as tools for chemical biology

N-terminal modification of nisin is generally problematic, as this may interfere with binding of the AB ring system to lipid II. In a previous study it was shown that PEGylation of nisin at the N-terminus completely abolished antibacterial activity [52]. C-terminal modification has more frequently been used to prepare labelled nisin for a variety of biological studies. Derivatives of nisin with fluorophores at the C-terminus, prepared by standard conjugation chemistry, have been used to demonstrate co-localisation of nisin in clusters with lipid II in giant unilamellar vesicles (GUVs) [53] and also to visualize how nisin interacts with the biosynthetic precursors of lipid II [54]. Fluorescein-labelled nisin has also been used to show localization at the division site of *Listeria* spp cells, where the concentration of lipid II is greatest [55]. Biotin has also been conjugated to nisin at the C-terminus, although this material has not so far been used for any biological studies [56].

The Liskamp group have recently published the C-terminal modification of nisin with propargylamine to give a derivative that can be used in click chemistry [57]. This has so far been used to prepare nisin with a C-terminal dansyl or carboxyfluorescein group, and also to construct nisin dimers joined together at the C-terminus by a short PEG linker. All of these derivatives retained broad antibacterial activity and were able to form pores with lipid II in membrane leakage assays from LUVs. Whilst the nisin dimer was able to permabilize the membrane more efficiently than the monomer, possibly because the nisin-lipid II pore complex forms more readily, this did not translate into enhanced antimicrobial activity. The CF-labelled material, on the other hand, showed both reduced pore-forming activity and reduced antimicrobial activity, possibly indicating that the fluorophore interferes with the ability of the peptide to complex lipid II.

Van der Donk and co-workers have made further advances in methodology which allow modified lantibiotics to be produced for chemical biology studies [58]. Genes encoding for both lantibiotic precursor peptides and the posttranslational modification enzymes have been cloned into plasmids and expressed in *E. coli*. This approach has been used to introduce a photo-cross-linking amino acid, p-benzoyl-L-Phe (pBpa) into prochlorosin 3.2, by introducing an amber stop codon at the required residue and co-expressing the precursor peptide and the synthase enzyme in the presence of the appropriate tRNA. Likewise, the two-component lantibiotic haloduracin α and β has also been successfully biosynthesized in *E. coli* in minimal medium suitable for isotopic labeling. The synthetic methods developed for the production of carba-bridged lantibiotic analogues have also allowed the synthesis of dye-modified analogues. Slootweg et al. [40] were able to attach CF to the N-terminus of the carba bridged nisin D/E analogues **33** using click chemistry.

Finally, the cinnamycin group of lantibiotics have found multiple applications as probes for phosphatidylethanolamine. Derivatives bearing biotin, ^{99m}Tc, and Gd^{3+}, and liposomes bearing duramycin, have all been used to track PE in vitro and *in vivo*, and this work has been recently reviewed [59].

5. Conclusions

In the last five to ten years a number of important and very promising advances in strategies to prepare modified lantibiotics have been reported. Although none of the peptides that have been produced so far show greatly enhanced antibacterial activity, the majority of the studies reported have been preliminary "proof of concept" work that showcase the power of each technique. However, the work reported so far with mutations of individual amino acids, whether with proteinogenic or non-proteinogenic amino acids, has already advanced understanding of the structure–activity relationships of many lantibiotics. In the case of nisin, this has certainly led to the production of mutants with increased stability to proteolysis and increased antibacterial activity. Recent synthetic studies replacing the thioether bridges with other linkages have generally produced peptides with enhanced resistance to aerial oxidation, as predicted. Further work will clearly be needed in order to understand what structural features are important when replacing the thioether bridges. In some lantibiotics, such as nisin, the ring size does not appear to be crucial, whereas it is clearly key in peptides such as lacticin 3147 A2. The conformational constraints imposed by each bridge replacement are so far not well understood, nor are the effects of replacing the thioether on pep-

tide-peptide or peptide-lipid recognition currently clear. Molecular modeling studies are limited in their predictive power in this field, however, once structural studies on ring-modified lantibiotics are available this will in turn enable more accurate predictions to be made. However, it is clear that the stereochemistry at the α-position of each lanthionine residue is very important to the biological activity of the peptide, and that the configuration found in any particular lantibiotic is not merely a constraint imposed by the specificity of the enzymes involved in their biosynthesis. With these new synthetic and mutasynthetic techniques in hand, and with the chemical biology tools now available for exploring structure–activity relationships and mode of action of the lantibiotics, the time is right to unlock the therapeutic potential of these challenging peptides.

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