The expanding role of lipid II as a target for lantibiotics

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Lipid II is an essential cell-wall precursor required for the growth and replication of both Gram-positive and Gram-negative bacteria. Compounds that use lipid II to selectively target bacterial cells for destruction represent an important class of antibiotics. Clinically, vancomycin is the most important example of an antibiotic that operates in this manner. Despite being considered the 'antibiotic drug of last resort', significant bacterial resistance to vancomycin now manifests itself worldwide. In this paper we review recent progress made in understanding the lipid II-associated antibacterial characteristics of various naturally occurring compounds, with particular focus on the lantibiotic peptides.

Antibiotics revolutionized medicine in the early 20th Century and continue to be a cornerstone in the battle against infectious disease. The accelerated appearance of drug-resistant bacteria over the past two decades, however, presents a serious threat to human health and fuels the demand for the development of new antibiotics. The sequencing of the first complete bacterial genome in 1995 heralded a new era of hope for antibacterial drug discovery. Despite the promise for genomic approaches, useful antibiotics have not emerged using these strategies [1]. This has led to a renewed interest in natural products as a source for antimicrobials. This review examines antibiotics derived from natural sources, specifically compounds belonging to the lantibiotic family that utilize lipid II in their antibacterial modes of action.

Bacteria synthesize peptidoglycan, starting from the common building block, lipid II. Peptidoglycan is the continuous, covalent, macromolecular structure that provides the strength and rigidity to the cell walls of both Gram-positive and Gram-negative bacteria (**Figure 1**). This polymeric network is comprised of alternating amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) [2]. These glycan polymer chains are cross-linked by a pentapeptide, typically with the sequence L-alanyl-γ-D-glutamyl-diaminopimelyl (or L-lysyl)-D-alanyl-D-alanine, which is attached to the MurNAc sugar. The glycosidic linkages in peptidoglycan are generated by bifunctional high molecular weight penicillin binding proteins (PBPs) or monofunctional transglycosylases. Two of these high molecular weight PBPs have recently been characterized at the structural level [3,4] and represent an, as of yet, unexploited target for

antibiotic development. Alternatively, the enzyme activity responsible for cross-linking the pentapeptide units in peptidoglycan has historically been among the most important antibiotic targets. The penicillins, cephalosporins, carbapenems and monobactams all operate by inhibition of the peptidoglycan-cross-linking transpeptidases [5].

As mentioned above, lipid II is the fundamental building block from which bacteria synthesize peptidoglycan. Each lipid II monomer contains the GlcNAc–MurNAc disaccharide and pentapeptide as well as a C55 carbon chain attached to the disaccharide via a pyrophosphate linkage (**Figure 2**).

The pathway by which lipid II is biosynthesized has been the subject of previous reviews [6,7] and is here described in brief (**Figure 3**). Lipid II assembly occurs on the cytoplasmic side of the bacterial cell membrane, where the UDP-MurNAc-pentapeptide is coupled to the membrane associated C55 lipid-phosphate by the action of the membrane protein MraY, to yield the intermediate lipid I. Addition of a GlcNAc sugar to lipid I is catalyzed by the membrane-associated enzyme MurG, providing lipid II. At this stage the intact lipid II monomer is translocated across the plasma membrane and is delivered to the periplasmic (exterior) side of the bacterial cell for incorporation into the growing peptidoglycan network. While the mechanism by which this translocation occurs is unknown, recent evidence has shown that it is not a spontaneous process and may be coupled to transglycosylation on the periplasmic cell surface [8].

The cell wall of Gram-positive bacteria contains multiple layers of peptidoglycan (~20) while Gram-negative bacteria require a thinner

Figure 1. Peptidoglycan layer in the bacterial cell wall.

N-acetylmuramic acid (MurNAc)

N-acetylglucosamine (GlcNAc)
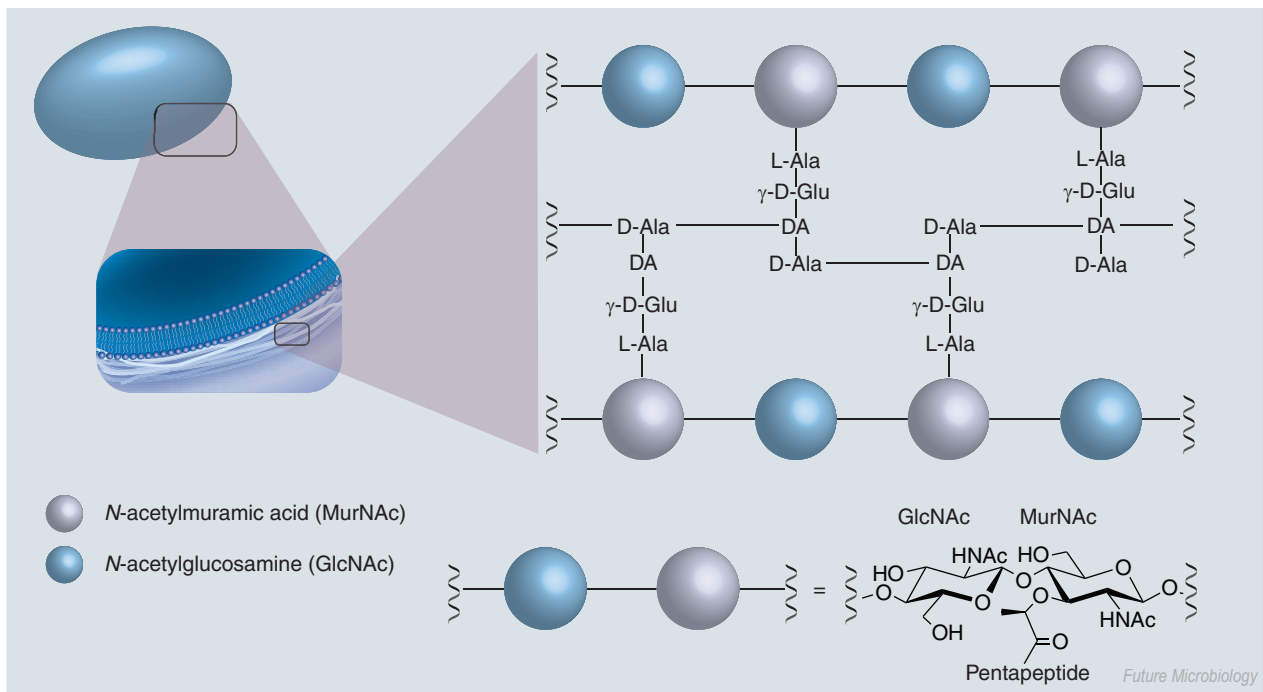
DA: Diamino acid (diaminopimelate or lysine).

cell wall, estimated to be 1.5 layers thick [9]. Despite the need for a continuous supply of lipid II, there exist approximately only 2000 intact molecules of this essential building block in a Gram-negative cell at any given time. This is likely due to the limited amount of C55 phospholipid (estimated to be 2×10^5 molecules per cell [10,11]) available for lipid II synthesis inside the cell and transport across the bacterial membrane. This suggests that the process by which lipid II is synthesized and incorporated into peptidoglycan is dynamic with respect to the C55 phopholipid (turnover rate for lipid II incorporation is estimated to be 1–3 transfers/sec/C55 phospholipid [12]). Given that bacteria rely on a relatively small amount of available lipid II, the targeted exploitation of this cycle presents a promising avenue for antibiotic discovery and development. In the subsequent sections we describe those antibiotics known to act by interfering with the lipid II cycle, as well as the adaptive resistance mechanisms that bacteria have developed to compensate.

Figure 2. Structure of lipid II with the specific recognition elements for vancomycin and nisin indicated.
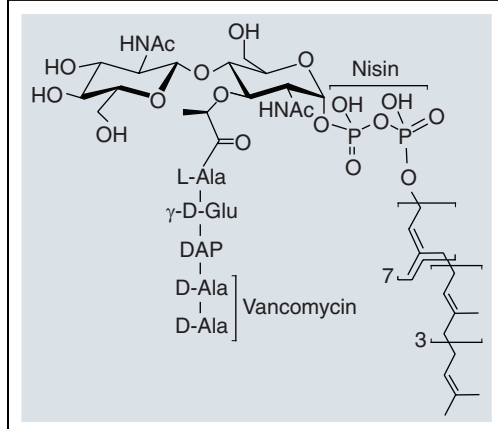
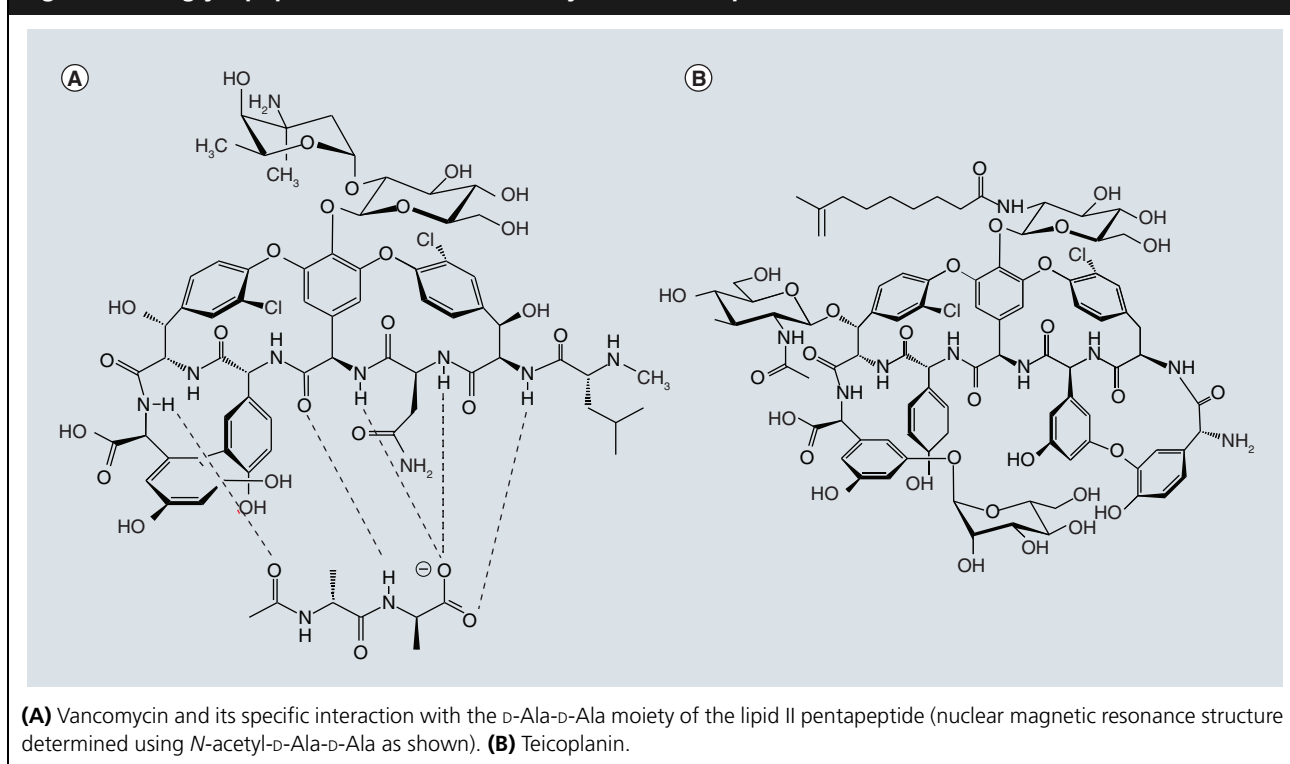## Vancomycin: the pre-eminent lipid II-binding antibiotic

The first identified and most studied example of an antibiotic with a lipid II-specific mode of action is vancomycin (**Figure 4a**). Discovered in 1956, vancomycin was isolated from the soil bacterium *Amycolatpsis orientalis* and has been shown to have a broad spectrum of activity [13]. Vancomycin, and the structurally similar teicoplanin (**Figure 4b**), belong to the glycopeptide family of antibiotics and have been approved for human clinical use. Both contain a nonribosomally synthesized peptide aglycone as well as a number

Figure 3. Lipid II biosynthesis and incorporation into peptidoglycan.
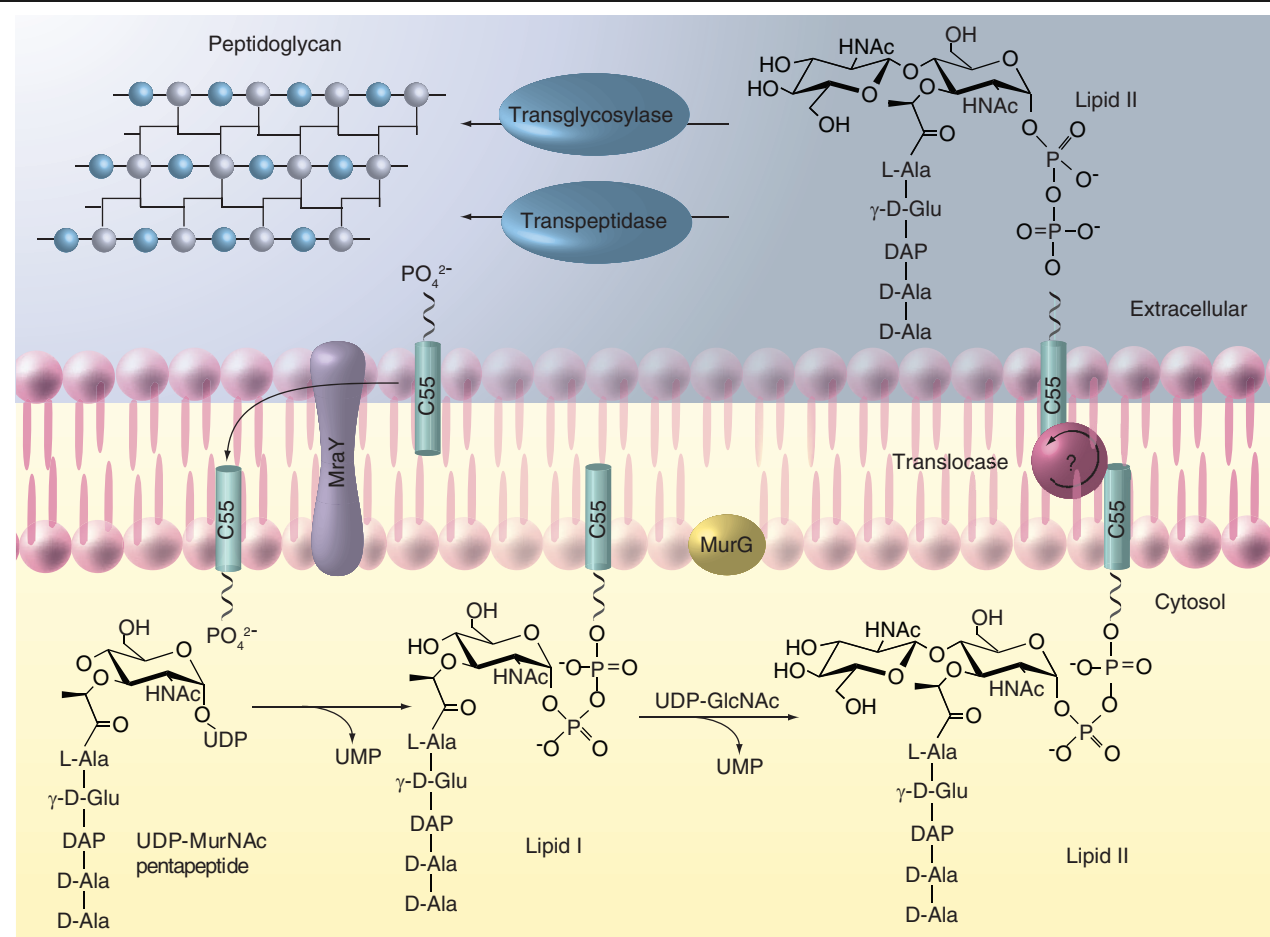
GlcNAc: *N*-acetylglucosamine; MurNAc: *N*-acetylmuramic acid.

of associated carbohydrate groups (and a C10 lipid in the case of teicoplanin). The aglycone of each contains the unnatural amino acids hydroxyphenylglycine and dihydroxyphenylglycine, the biosynthetic details of which have recently been described [14]. Cyclization of the peptide backbone was also recently shown to be the result of three unique enzymatic oxidative couplings [15,16]. The resulting polycyclic scaffold provides vancomycin and teicoplanin with a structural rigidity that contributes to their lipid II-binding ability [17–19]. Interaction of vancomycin with the D-Ala–D-Ala moiety of the lipid II pentapeptide (as illustrated in **Figures 2 & 4a**) was first observed using chromatographic, electrophoretic and differential ultraviolet spectral approaches [20,21], and this was later confirmed by nuclear magnetic resonance spectroscopy [18,22]. These experiments employed small fragments of the lipid II pentapeptide and recent investigations using a more representative lipid II species have suggested that there may be additional complexity to the interaction [23].

As illustrated in **Figure 4a**, vancomycin binds to the sequence D-Ala-D-Ala through a specific network of five hydrogen bonds. By doing so, vancomycin effectively sequesters lipid II and prevents its normal incorporation into the growing peptidoglycan network, which ultimately leads to cell death. In response, certain bacteria have adapted to this mechanism of action and are capable of mutating the terminal D-Ala residue to D-lactate in the lipid II pentapeptide [24]. This mutation greatly reduces the affinity of vancomycin for lipid II and leads to a resistant strain [25–27]. In response, drug makers are now developing new semisynthetic glycopeptides including dalbavancin [28], telavancin [29] and oritavancin [30]. While retaining the vancomycin aglycone, these second generation glycopeptides are elaborated with various hydrophobic groups on the disaccharide moiety, rendering the compounds active against otherwise resistant organisms. In addition, a small number of novel, nonribosomal peptide

Figure 4. The glycopeptide antibiotics vancomycin and teicoplanin.

(A) Vancomycin and its specific interaction with the D-Ala-D-Ala moiety of the lipid II pentapeptide (nuclear magnetic resonance structure determined using *N*-acetyl-D-Ala-D-Ala as shown). **(B)** Teicoplanin.

antibiotics have recently been described, including the ramoplanins [31–33], mannopeptimycins [34,35], plusbacins [36,37] and katanosins [38]. While these compounds have all been described as lipid II-binding antibiotics [39], they will not be discussed in further detail here with the remaining focus of this review being the lantibiotic class of antibiotics.

Lantibiotics

In addition to conventional small molecule antibiotics, another family of lipid II-targeting antimicrobial agents, the lantibiotics, is rapidly gaining recognition. Lantibiotics are small (2–6 kDa) ribosomally synthesized bacterial defense peptides that contain extensive post-translational modifications [40,41]. The discovery of the first lantibiotic, nisin (**Figure 5**), in 1928 [42], predates Flemming's discovery of penicillin and, to date, approximately 50 different lantibiotics have been identified from approximately 30 different bacteria. Over the past 20 years there has been an increasing interest in such compounds as possible preservative agents for food and as potential supplements or replacements for currently used antibiotics. Many lantibiotics show promising activity towards a variety of pathogenic bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and

vancomycin-resistant *Enterococcus* (VRE) [43]. Detailed descriptions pertaining to the activity spectra and therapeutic potential of lantibiotics have been previously described [39,44–48].

The lantibiotic peptides can be classified based upon a number of features including size, shape, charge and mode of action [40,49] and have traditionally been subdivided into two major groups – the type A and B lantibiotics – that comprise peptides with straight-chain and globular structures, respectively [50]. Additionally, a third class, the two-component lantibiotics, is now gaining recognition wherein two structurally different peptides act synergistically to kill target bacteria. In the following sections we describe the progress made in understanding the biosynthesis and modes of action of the major subgroups of lantibiotics.

Lantibiotic biosynthesis

Nisin is the most thoroughly studied lantibiotic and contains a number of unique post-translational modifications common to all lantibiotics (**Figure 5**) [51,52]. These include the dehydration of Ser and Thr residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, with subsequent cyclization by conjugate addition of Cys residues to Dha and Dhb, generating the thioether cross-links

lanthionine (Lan) and methyllanthionine (MeLan), respectively. These latter structures have given lantibiotics their family name [53]. Lantibiotics are ribosomally synthesized as pre-peptides and then enzymatically modified followed by proteolytic removal of a leader peptide to generate the active species with concomitant export from the cell [41]. Recently, the biosynthetic details of a number of lantibiotics including nisin have been investigated [54,55]. This work has shown that in the biosynthesis of nisin, two separate enzymes carry out the dehydration (NisB) and cyclization (NisC) reactions, prior to export and leader peptide removal by translocase (NisT) and protease (NisP) enzymes. Analogous enzyme systems have also been reported for a number of other lantibiotics [56–59].

Figure 5. Biosynthesis of nisin.
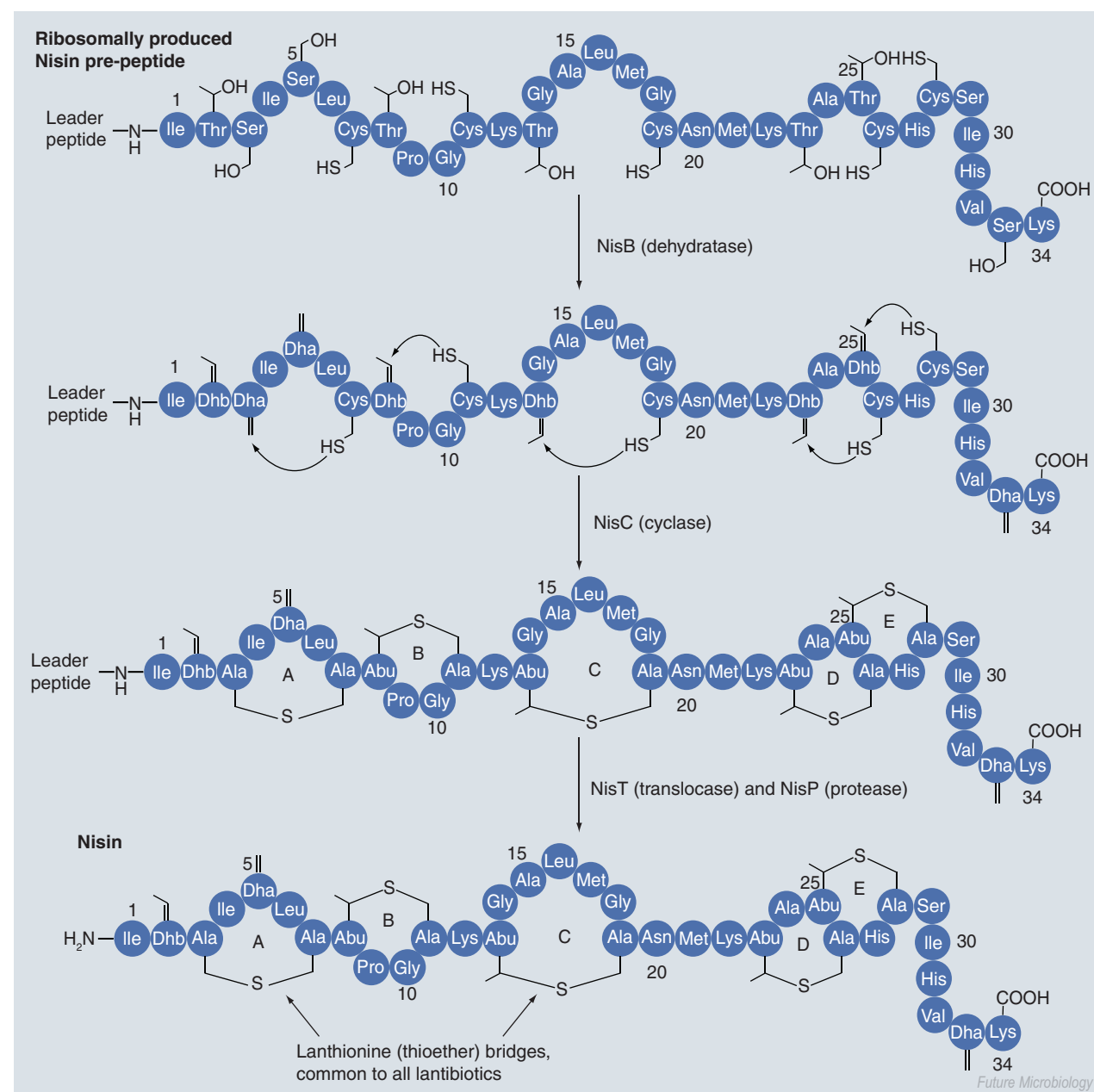
The ribosomally produced nisin prepeptide is enzymatically modified to yield the mature lantibiotic containing the unnatural amino acids Lan, MeLan, Dha and Dhb.

Lantibiotic mode of action
Nisin & the type A lantibiotics

Nisin is comprised of 34 amino acids and at physiological pH bears a net positive charge (+4). Of the five lanthionine-based rings in the peptide core of nisin, the A, B and C rings are separated from rings D and E by a flexible 'hinge' region (residues 20–22). Despite being the oldest known antibiotic, the precise mode of action by which nisin operates has only recently been brought to light. It was initially suggested that nisin killed bacteria via binding to lipid II in a manner similar to that described for vancomycin [60,61]. However, unlike vancomycin, nisin was later shown to cause the rapid outflow of small cytoplasmic compounds such as amino acids, ATP or preaccumulated rubidium, as well as the collapse of vital ion gradients, when administered to Gram-positive bacteria [50,62]. These results supported a mode of action involving perturbation of the plasma membrane. Further experiments, focusing on the interaction of the cationic nisin with model membranes containing anionic lipids found in the plasma membrane of Gram-positive bacteria [63–66], led to a perplexing observation: while the concentration of nisin required for membrane perturbation in model experiments was in the micromolar range, the concentration at which nisin was able to effectively kill bacteria was in the nanomolar range. These results were ultimately reconciled by experiments that showed nisin has a twofold mode of action whereby it uses lipid II as a 'docking molecule' to form pores in bacterial membranes in a targeted manner and with high efficiency [67,68]. It has also since been shown that the pores formed by nisin in membranes containing lipid II are much more stable than pores formed in the absence of the receptor [69–72]. Furthermore, two independent approaches have demonstrated that lipid II is a constituent of the pore complex [73,74], the stoichiometry of which is four lipid II molecules and eight nisin molecules (Figure 6) [75].

Recently, NMR investigations have provided molecular-level insight into the interaction of nisin with lipid II [76]. This work showed that nisin has a unique mode of binding to lipid II, entirely different from glycopeptides (as earlier indicated in Figure 2). A defined network of five intermolecular hydrogen bonds between nisin's peptide backbone and the pyrophosphate moiety


Figure 6. Model for the target-directed pore-formation mechanism of nisin.
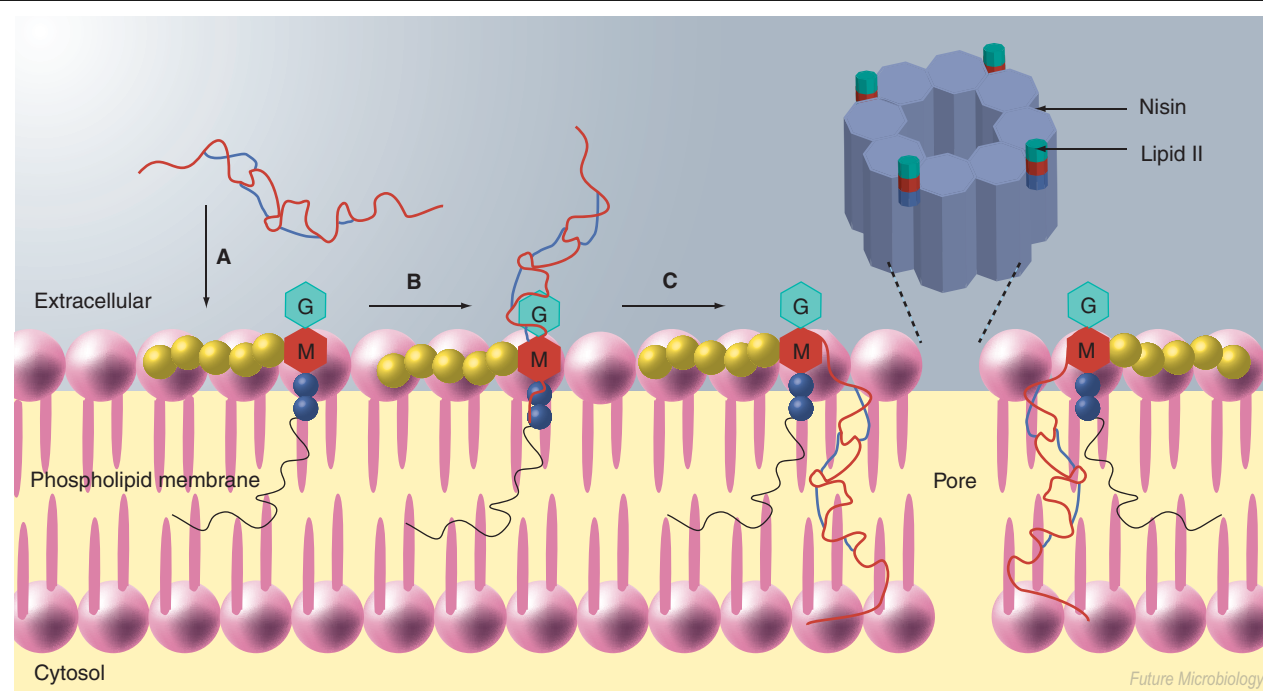
(A) Nisin reaches the bacterial plasma membrane and (B) selectively binds to lipid II. This is then followed by pore formation (C), which involves a stable transmembrane orientation of nisin. During or after assembly of four 1:1 (nisin:lipid II) complexes, four additional nisin molecules are recruited to form the pore complex.
Figure reproduced with permission from [39] © (2006) Macmillan Publishers Limited.

**Figure 7. Hydrogen-bonding network between nisin N-terminal residues and lipid II pyrophosphate moiety.**
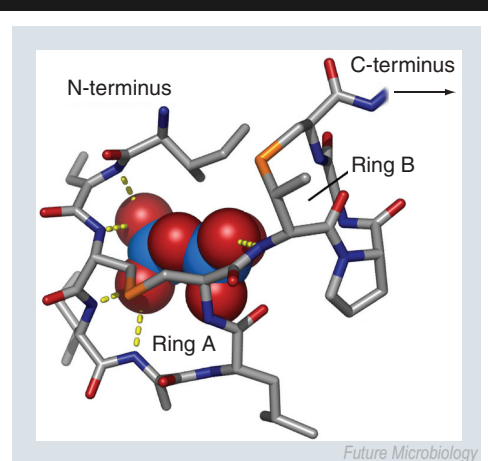
For clarity, only the pyrophosphate moiety of lipid II is shown, as a red and blue ball representation. Yellow dashes indicate intermolecular hydrogen bonds.
Adapted from published coordinates [76].

of lipid II are responsible for the association (**Figure 7**). Interestingly, only the first ten N-terminal amino acids, containing lanthionine rings A and B, contribute to the 'pyrophosphate cage' with which nisin binds lipid II. Nisin's lanthionine rings are critical to its biological activity and likely provide a pre-ordered structure with a lower entropic cost upon binding to lipid II [77].

The N-terminal A/B lanthionine ring system of nisin is also maintained in a number of other lantibiotics, which suggests that these peptides also interact with lipid II in a similar manner (**Figure 8**). The closest relative of nisin, subtilin, differs at three places in the first eleven residues of the sequence. However, these three places
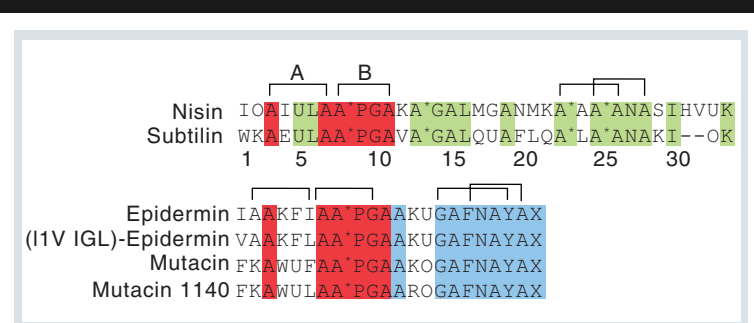
are not key positions, and thus the cage structure remains intact with only the side chains differing.

Members of the epidermin family of lantibiotics also share the nisin A/B ring system, suggesting a lipid II-mediated mode of action. The epidermins are highly potent antibiotics with MIC values comparable to nisin [78]. However, these peptides are of insufficient length to span a bacterial membrane and cannot induce leakage from model membrane systems in the presence of lipid II. Recent fluorescence microscopy experiments have suggested that the epidermins functions to sequester lipid II in a unique manner, effectively removing it from the cell division site (septum) and blocking peptidogylan synthesis [79].

Mersacidin & the type B lantibiotics

Mersacidin is the most studied of the type B lantibiotics (**Figure 9**). While smaller and more compact in structure, this class of lantibiotic also targets lipid II and inhibits bacterial cell-wall synthesis (without pore-formation as for nisin) [80].
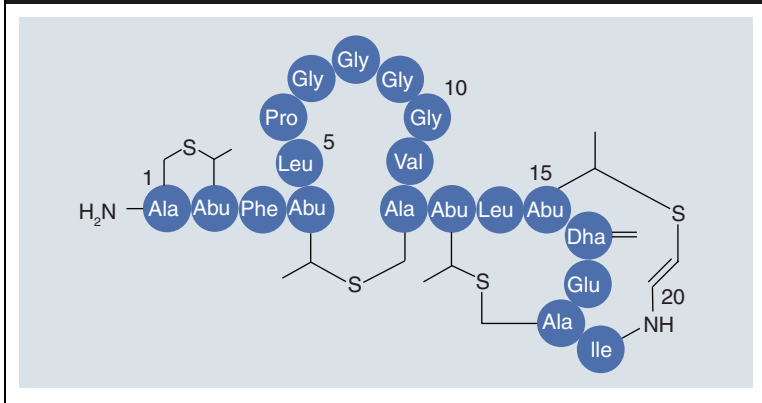
The lipid II-binding interaction of mersacidin is different to that of nisin and includes the terminal GlcNAc sugar [80]. Comparison of mersacidin with similar lantibiotics reveals a conserved sequence that comprises residues 12–18, suggesting that these residues may form the core lipid II-binding site. While the measured affinity of mersacidin for purified lipid II is much lower than that of nisin [80,81], it remains a potent antibiotic indicating that additional factors likely contribute to its overall antibacterial mode of action [67,81].

Lacticin 3147 & the two-component lantibiotics

The two-component lantibiotics are comprised of two structurally unique peptides that act in synergy to provide potent antibacterial activity [82,83]. Lacticin 3147-A1/A2 (A1 and A2 referring to the two peptides) is the best-studied member of this class and was the first two-component lantibiotic system to be fully characterized at the structural level (**Figure 10**) [84,85].

In the case of lacticin 3147, both the A1 and A2 peptides show modest antibacterial activity when administered in isolation. When co-administered however, a synergistic effect is observed with the resulting antibiotic activity many-fold greater than the sum of the individual activities (**Figure 11**).

Figure 8. A/B ring system homology between nisin and related lantibiotics.

Red denotes homology between the nisin and epidermin families, green within the nisin family and blue within the epidermin family.

Figure 9. Mersacidin, a type B lantibiotic.

The apparent rarity of two-component lantibiotics is most likely due to the fact that investigators have traditionally been biased towards looking for single active compounds. With the identification of four new two-component lantibiotics in the past 2 years, seven such systems have now been documented [87–93], suggesting that two-component lantibiotics may indeed be more common than previously realized.

Lantibiotic-resistance mechanisms

Investigations into the acquired bacterial resistance to lantibiotics have primarily focused on nisin. It has been shown that many nisin-sensitive Gram-positive bacteria, including clinically relevant strains, can acquire nisin resistance upon repeated exposure to increasing nisin concentrations [10,44,94,95]. This type of resistance is often lost once nisin pressure is removed [10] and is more accurately described as a physiological adaptation, although the nisin resistance of *Streptococcus bovis* was claimed to be stable, resistant cells were rapidly overgrown by sensitive ones [94]. Unlike vancomycin resistance, this adaptive mechanism does not involve an alteration of either the structure or

Recent investigations have provided insight into the lipid II-mediated mode of action for lacticin 3147 and show that when administered together, the two peptides cause pore formation in bacterial membranes in a manner reminiscent of nisin [86]. The model proposed involves a sequence of events in which the A1 peptide of lacticin binds first to lipid II to generate a binding site for the A2 peptide. The subsequent binding of the A2 peptide drives the system towards pore formation.

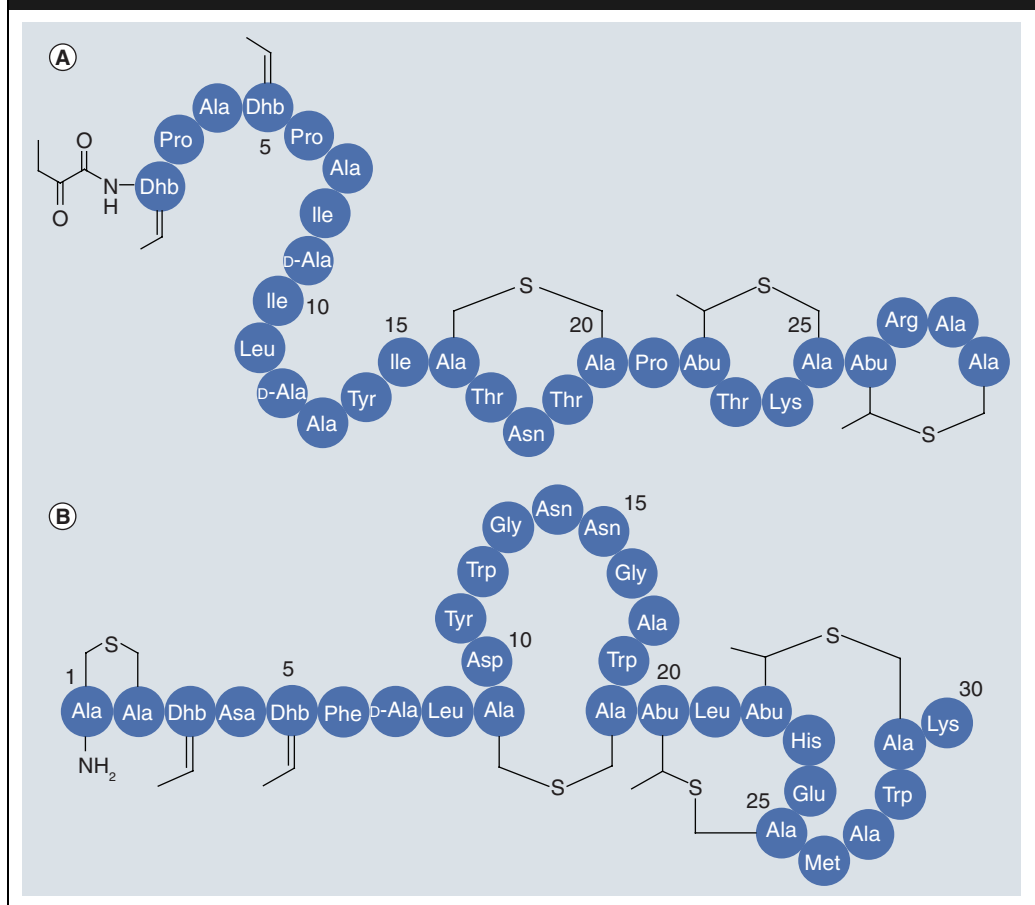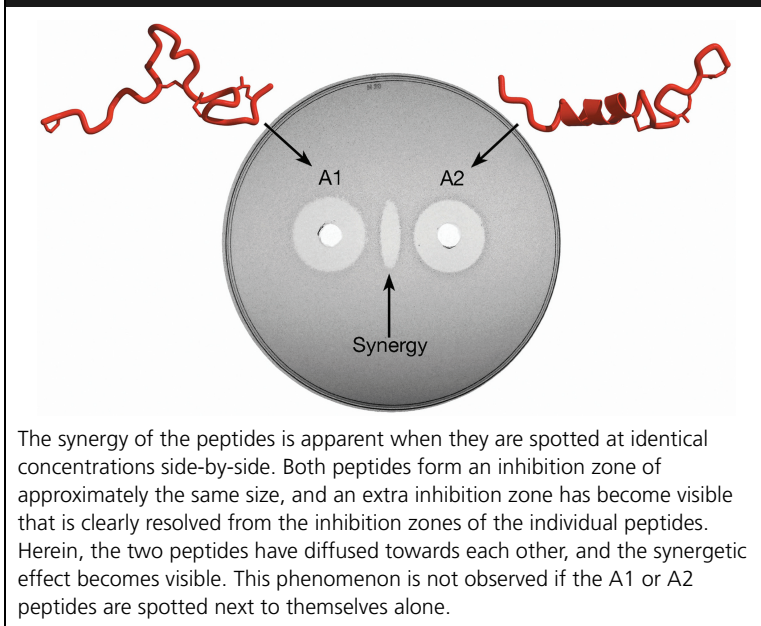## Figure 10. Structures of the lacticin 3147 (A) A1 and (B) A2 lantibiotic peptides.

Figure 11. Complementary activity of lacticin 3147-A1 and -A2 against *Lactococcus lactis* subsp. *cremoris* HP.

The synergy of the peptides is apparent when they are spotted at identical concentrations side-by-side. Both peptides form an inhibition zone of approximately the same size, and an extra inhibition zone has become visible that is clearly resolved from the inhibition zones of the individual peptides. Herein, the two peptides have diffused towards each other, and the synergetic effect becomes visible. This phenomenon is not observed if the A1 or A2 peptides are spotted next to themselves alone.

the net negative charge of the cell wall and has recently been shown to be a major cause of nisin resistance in strains of *S. bovis*, *Lactococcus lactis* and *Bacillus subtilis* [94,100,101]. Strains displaying elevated levels of D-Ala containing teichoic acids also exhibited an upregulation of the dlt operon which regulates D-Ala incorporation [101,102]. These results indicate that the main bacterial adaptation leading to nisin resistance involves an electrostatic shielding mechanism; incorporation of positive charges in the cell wall serve to repel nisin, preventing it from reaching lipid II.

Conclusion

Lantibiotics target lipid II in a variety of ways and operate with several modes of action. To date, all lantibiotics tested show promising *in vitro* activity against multiple-drug-resistant and vancomycin-resistant strains. Lipid II is a uniquely prokaryotic biomolecule with no known metabolic role in humans, and as such represents a promising target for the development of selective antibiotics with low toxicity. While certain pharmacokinetic obstacles remain to be fully addressed, the clinical development of lantibiotics may benefit from the information gleaned from the use of nisin in the food industry as a preservative, on issues relating to formulation, conditions of use and stability. Also, with the rapidly increasing body of knowledge pertaining to the structures and of the modes of action of these peptides, it should be possible to modify lantibiotics to circumvent these barriers. The clinical development of the lipid-II-targeting lantibiotics is likely to play an important role in future approaches to dealing with infectious disease.

quantity of lipid II. Rather, lipid II is effectively 'shielded' from nisin by a change in the cell-wall composition [10,94,96–99]. Normally, the cell wall of a Gram-positive bacterium is highly negatively charged due to the phosphate groups of teichoic acid, another major component of the cell wall (**Figure 12**).

Teichoic acids are either covalently linked to the peptidoglycan layer (as in **Figure 12**), or membrane linked via a lipid anchor. A variety of substituents can be attached to the negatively charged phosphoglycerol moiety, one of which is D-ala. Incorporation of D-ala into teichoic acid reduces

Figure 12. (A) Cell wall-associated teichoic acid structure in nisin-sensitive organisms and (B) D-Ala modified teichoic acid with reduced net negative charge as found in nisin-resistant bacteria.
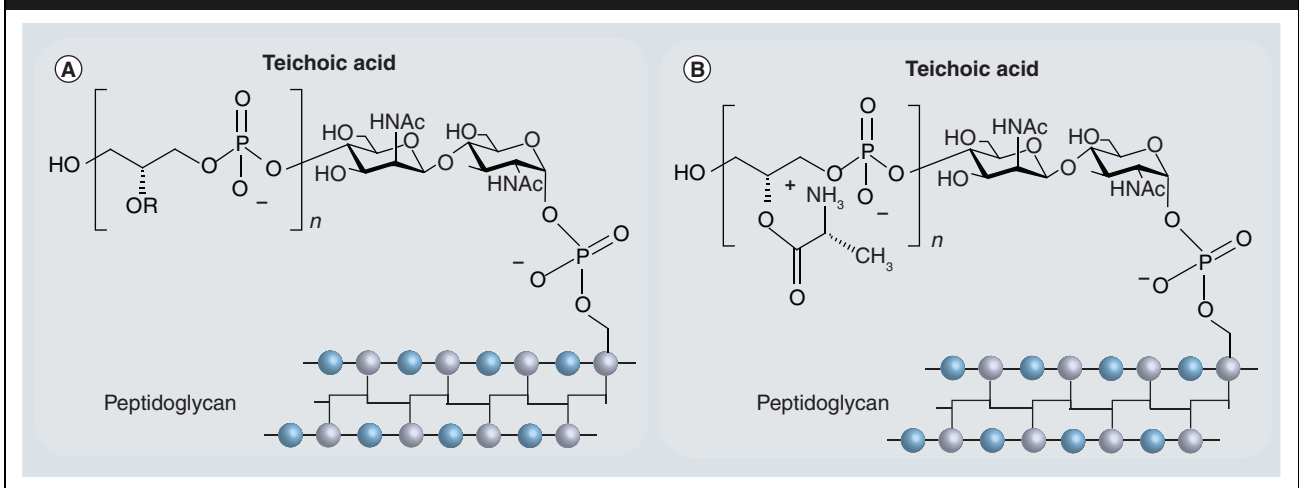
Executive summary

The expanding role of lipid II as a target for lantibiotics

- This review describes recent progress made in understanding the lipid II-associated antibacterial characteristics of a variety of naturally occurring compounds, with particular focus on the lantibiotic peptides.

Lantibiotic mode of action

- There is growing evidence to support a lipid II-associated mode of action by which lantibiotics specifically target bacteria.
- The interaction of nisin with lipid II (recently characterized at the molecular level by nuclear magnetic resonance spectroscopy) provides insight into approaches for targeting bacteria that are unique from those employed by conventional antibiotics.

Future perspective

- While pharmacokinetic obstacles remain to be fully addressed, the clinical development of lipid II-targeting lantibiotics is likely to play an important role in future approaches to dealing with infectious disease.

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