

# Bacterial Lantibiotics: Strategies to Improve Therapeutic Potential

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**Abstract:** Lantibiotics are ribosomally-synthesised antimicrobial peptides produced by Gram-positive bacteria that are characterised by the presence of lanthionine and/or methyllanthionine residues. Other unusual post-translationally modified amino acids, most frequently dehydroalanine and dehydrobutyrine, can also be present. While it has been frequently suggested that these peptides have the potential to be utilised in a wide range of medical applications, to date no actual therapeutic applications have been convincingly described. More recently, however, they have been the focus of much attention as a consequence of improved biotechnological capabilities, an improved understanding of lantibiotic biosynthesis and mode of action, and their high specific activity against multi-drug resistant bacteria. This review concerns the fundamental analyses that have revealed the importance of individual amino acids in these peptides and has permitted the implementation of rational mutagenesis strategies ('intelligenetics') to alter individual residues with a view to ultimately widening the active pH range, improve stability, and enhance binding to cell wall targets with the ultimate aim of optimising their antimicrobial activity. It is hoped that as a consequence of this improved knowledge the most suitable application of individual lantibiotics will become apparent. It should also prove possible, in the near future, to generate tailor-made lantibiotics and utilise biosynthetic enzymes to incorporate modified amino acids into non-lantibiotic peptides. In the shorter term, the extensive characterisation of lantibiotics will be instrumental in reassuring drug industry regulators of their safety and facilitate the widespread application of these novel antimicrobial agents in medicine.

**Keywords:** Lantibiotic, medical applications, genetic engineering, modified amino acids.

## INTRODUCTION

The lantibiotics are a group of ribosomally-synthesised antimicrobial peptides that show enormous chemotherapeutic potential. This is especially significant as it has now been 20 years since a genuinely new class of antibacterial compounds has reached the market place and bacteria now exist that are resistant to all clinically important antibiotics. Thus it is increasingly important that these under-utilised peptides deliver on their undoubted potential. Lantibiotics display a number of remarkable characteristics. While a number of cationic peptides, such as those produced by mammals, insects, fish and amphibians have antimicrobial properties, the active concentrations of such peptides are in the micromolar ( $\mu\text{M}$ ) range, whereas some lantibiotics have been shown to be active in nanomolar (nM) amounts [9]. In addition to possessing a number of beneficial physico-chemical properties such as a resistance to high temperatures and good stability, relatively recent advances have demonstrated that at least some lantibiotics are among a rare group of antibacterial compounds that target the lipid II component of the bacterial cell wall (others include the lipoglycopeptide antibiotic ramoplanin and the glycopeptide vancomycin). In some instances such binding facilitates a dual mode of action, i.e. the prevention of peptidoglycan transglycosylation and the formation of pores [9].

Many of these features are associated with the modification of serine, threonine and cysteine residues in the peptide. The typical reaction associated with the biosynthesis of mature peptides involves the dehydration of serine and threonine residues, resulting in the formation of dehydroalanine or dehydrobutyrine, respectively. When one of these modified amino acids reacts with an intrapeptide cysteine, a thioether bond is formed generating lanthionine (in the case of dehydroalanine) or -methyl-lanthionine (in the case of dehydrobutyrine). Traditionally these peptides have been classified, on the basis of structural and functional aspects, as being either type A or type B lantibiotics [53]. Type A lantibiotics, such as nisin, pep5, and epidermin, are elongated amphiphilic peptides that act by forming pores in the cell membranes of susceptible cells. In contrast, type B lantibiotics such as mersacidin and actagardine have a globular structure and act by inhibiting various enzyme functions by binding to membrane lipids. However, the distinction between type A and type B lantibiotics became blurred when it was observed that nisin, in addition to its pore-forming capabilities, and mersacidin both inhibit peptidoglycan synthesis in sensitive Gram positive cells by binding to the peptidoglycan precursor lipid II. Binding to lipid II facilitates nisin pore-formation by acting as a docking molecule [9].

The recent renewed interest in lantibiotics originates from the emergence of clinical isolates resistant to vancomycin, the drug of last resort against infections from Gram-positive pathogens for almost thirty years. Vancomycin functions by binding the N-acyl-D-Ala-D-Ala

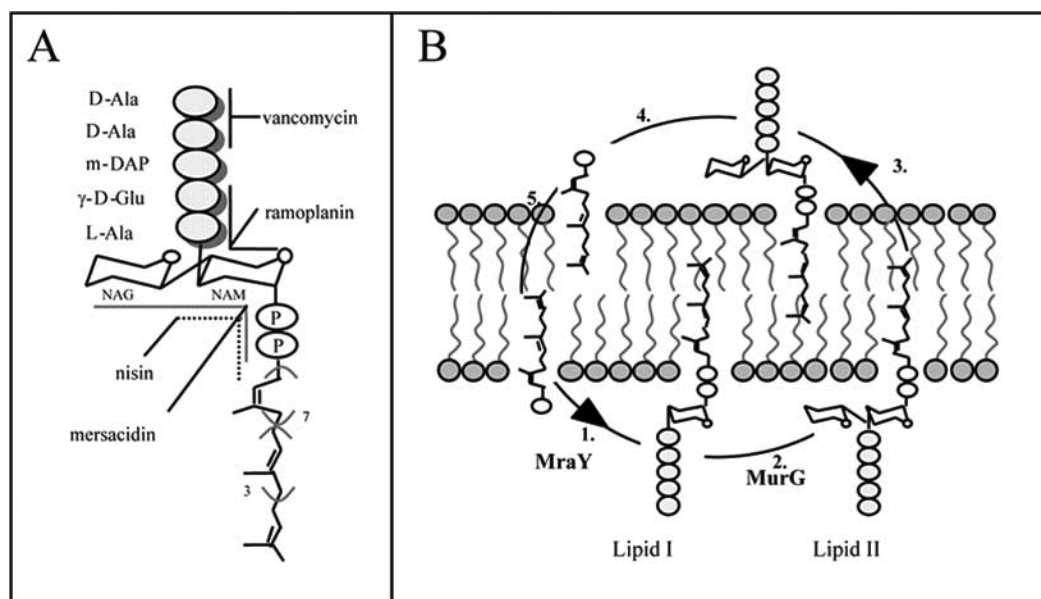
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of lipid II, (Fig. 1). While the addition of vancomycin inhibits the ability of nisin to bind to lipid II, the binding motif of nisin for lipid II is distinctly different, given that vancomycin resistant strains remain sensitive to nisin [9]. Nisin and epidermin can bind both lipid I and lipid II (suggesting GlcNAc is not a target) [15], and nisin can bind lipid II derivatives with altered prenyl chains [10] (suggesting this is also not the prime target) leaving N-Ac-Muramyl-pentapeptide as the likely target. Mersacidin and actagardine bind only lipid II [14, 15] and share a conserved backbone fold with ramoplanin, which targets the MurNAc-Ala- $\gamma$ -D-Glu pyrophosphate, suggesting that these have overlapping lipid II binding sites [27] (Fig. 1). The dual mechanism of action of nisin may also give us an insight as to how a two-component lantibiotic such as lactacin 3147 may function. This lantibiotic functions optimally through the combined activity of two lanthionine-containing peptides, one of type A and one of type B.

In addition to having developed an improved understanding of the mode of action of some lantibiotic peptides the mechanisms involved in their biosynthesis are also being unravelled. In particular LanB and LanC proteins, which catalyse dehydration and lanthionine bridge formation respectively in type A lantibiotics [99], and LanM proteins, which carry out an equivalent role in the formation of type B lantibiotics [106], have been the subjects of much attention. This has been demonstrated most successfully in the case of LcnM. This lactacin 481 modification enzyme has demonstrated a capacity to modify various mutants of lactacin 481 as well as to be capable of *in vitro* activity [128]. This study represents a major advance in lantibiotic research and makes the possibility of generating novel, synthetic lantibiotics more realistic.

## OVERVIEW OF MEDICAL APPLICATIONS

Even before their use in the treatment of drug resistant bacteria was suggested it has been apparent that lantibiotics could have potential medical applications. Lantibiotics have been investigated since 1928 when the prototypical lantibiotic nisin was first reported as having activity against tubercle bacilli [93]. More recently the use of nisin has become widespread and it is currently sold in more than 40 countries as a food preservative. It was added to the positive list of food additives by the EU as additive number E234 [34] and has been approved by the FDA [28]. Not only is it recognised as safe but it is also active against a very wide range of bacteria, making it suitable for a number of different applications. Despite the relative success of nisin in the food arena the lantibiotics can be considered in general to be an under-utilised resource. This trend may change in the near future, however, as a consequence of advances in our biotechnological capabilities and improved understanding of the synthesis and novel mode of action of lantibiotics. Fundamental analysis has revealed the importance of individual amino acids in these peptides and has permitted the implementation of rational mutagenesis strategies to alter these residues with a view to ultimately widening the active pH range, improve stability, and enhance binding to cell wall targets with the ultimate aim of optimising the antimicrobial activity of lantibiotics. A better understanding of these antimicrobial peptides increases the likelihood of practical applications and also improves the possibility that tailor-made or hybrid lantibiotics can be generated to target particular bacteria. An improved understanding of the biosynthetic enzymes involved in lantibiotic production may also facilitate the introduction of modified amino acids into non-lantibiotic peptides. In the shorter term the extensive characterisation of lantibiotics will be instrumental in



**Fig. (1).** A. Sites on lipid II (undecaprenylpyrophosphorylMurNAc(pentapeptide)-GlcNAc) to which vancomycin, ramoplanin, nisin and mersacidin attach. B. Biosynthesis of lipid II. (1) MrdY catalyses the transfer of a UDP N-acetylmuramyl(NAM)pentapeptide moiety to the membrane lipid carrier undecaprenyl (UDP) phosphate to generate lipid I. (2) MurG catalyzes the coupling of lipid I to N-acetylglucosamine (NAG). (3) Lipid II is translocated and is (4) transglycosylated to form linear glycan chains and UDP phosphate is released to be (5) transferred to the inside of the cell membrane to begin the cycle again.

**Table 1. Lantibiotics with Potential Applications (Adapted from [98])**

Lantibiotic	Producing strain	Inhibitory activity of commercial interest	Potential Biomedical Applications
Nisin A	<i>Lactococcus lactis</i>	Gram-positive bacteria, Gram negative bacteria including <i>Helicobacter pylori</i>	Bacterial mastitis, Oral hygiene, Treatment of methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) and enterococcal infections, Cosmetic deodorants and topical formulations, peptic ulcer treatment, treatment of enterocolitis, lung mucus clearing
Lactacin 3147	<i>Lactococcus lactis</i>	Gram-positive bacteria	Bacterial mastitis, Treatment of MRSA and enterococcal infections, Oral hygiene, Acne
Gallidermin/ Epidermin	<i>Staphylococcus gallinarum</i> <i>Staphylococcus epidermidis</i>	<i>Propionibacterium acnes</i> , staphylococci, and streptococci	Acne, eczema, folliculitis, impetigo
Mutacin 1140	<i>Streptococcus mutans</i>	<i>Streptococcus mutans</i>	Prevention of dental caries
Mersacidin/ Actagardine	<i>Bacillus subsp.</i> <i>/Actinoplanes subsp.</i>	Staphylococci including methicillin-resistant strains, streptococci	Treatment of MRSA and streptococcal infections
Duramycin	<i>Streptomyces subsp.</i> and <i>Streptoverticillium subsp.</i>	Weak bacterial inhibition, inhibitor of phospholipase A2	Inflammation
Cinnamycin	<i>Streptomyces cinnamoneus</i>	Inhibitor of phospholipase A2, angiotension converting enzyme (ACE), and herpes simplex virus	Inflammation, Blood pressure regulation, viral infection treatment
Ancovenin	<i>Streptomyces subsp.</i>	Inhibitor of ACE	Blood pressure regulation

reassuring drug industry regulators of their safety and facilitate the widespread application of these novel antimicrobial agents in medicine.

Potentially the most significant application of lantibiotics may be in the treatment of antibiotic resistant pathogens. Lantibiotics such as mersacidin and actagardine have been shown to display considerable activity against *S. aureus* [72], including methicillin resistant strains (MRSA). While the glycopeptide antibiotic vancomycin can still be used to treat MRSA and enterococcal infections, vancomycin insensitive and resistant strains have emerged. Significantly it has been shown that vancomycin-resistant strains remain sensitive to mersacidin [12,13,14], indicating that cross-resistance should not pose an immediate problem. Experiments carried out with mice show that concentrations of mersacidin similar to those of vancomycin are effective in treating infections with MRSA, thus indicating that this lantibiotic may have considerable therapeutic application. Additionally, mersacidin is not readily susceptible to proteolytic digestion, and it has proven very difficult to raise antibodies to this lantibiotic [72]. Nisin may also have a role in the treatment of systemic disease caused by antibiotic-resistant streptococci. It exhibits excellent activity against clinical isolates of *Strep. pneumoniae*, including penicillin-resistant strains [40]. When this study was extended to include a mouse infection model, it was observed that nisin was 8 to 16 times more active than vancomycin against one clinical isolate. Additionally, low

blood and tissue levels of nisin appear to be sufficient to treat mice infected with *Strep. pneumoniae* [40]. Nisin has also been shown to be active against a number of other multidrug-resistant Gram-positive pathogens [105]. When tested against 56 multidrug-resistant *Strep. pneumoniae*, 33 *S. aureus*, and 29 vancomycin-resistant *Enterococcus faecium* and *E. faecalis* isolates it brought about a 3 to 4 log reduction in titre in the vast majority of cases, although a concentration of up to 10 to 20 mg/l was required for the MRSA and enterococci. Notably, however, nisin-resistant mutants appear rapidly following repeated exposure, indicating that as with existing antibiotics, bacteria are also capable of adapting to the inhibitory effect of nisin [105]. In contrast to the high levels of nisin required to kill drug resistant bacteria significantly less is required to inhibit their growth. Levels of nisin between 1 and 32 µg/ml inhibit all of 40 nosocomial isolates of MRSA tested and at the higher levels stable mutants did not emerge [39]. It has also been shown that lactacin 3147 is effective against methicillin resistant *St. aureus*, vancomycin resistant *Ent. faecalis*, penicillin resistant *Pneumococcus*, *Propionibacterium acnes*, and *Strep. mutans* [38] suggesting it also may have a range of medical applications.

Lantibiotics have also demonstrated activity against bacteria including antibiotic-resistant variants responsible for bovine mastitis. At present, antibiotics are used in the treatment and prevention of mastitis but problems associated

with the potential presence of antibiotic residues in milk and the emergence of antibiotic-resistant strains means that an alternative treatment is desirable. It is thus significant that the first clinical testing of nisin involved its evaluation as an alternative to intramammary antibiotics for the treatment of bovine mastitis in the 1940s [113]. In that instance promising results were generated, however problems due to irritancy, which probably arose as a consequence of the presence of impurities, were encountered. More recently this area has received renewed attention, undoubtedly as a consequence of the ability of nisin to inhibit a wide range of mastitis-causing pathogens [11]. While its full potential in the treatment of mastitis may be restricted by its poor solubility at physiological pH, it has been used successfully in combination with lysostaphin [85] resulting in efficient cure rates of 66%, 95%, and 100% for animals infected with *S. aureus*, *Strep. agalactiae* and *Str. uberis*, respectively [102, 103, 104]. Lactacin 3147, which is active at physiological pH, has also produced promising results when incorporated into a teat seal product, an oil-based formulation that forms a physical barrier against infection in the area of the teat canal and sinus [96]. It was found that lactacin 3147 does not cause teat irritancy and retains activity eight days after its introduction into an adult cow teat [97]. *In vivo* tests with the mastitic pathogen *Streptococcus dysgalactiae* showed that only 6% of treated quarters either developed clinical mastitis or were shedding the challenge organism (relative to 61% of control quarters) [97]. A similar trend was observed when *Staphylococcus aureus* was introduced to simulate a natural mastitis infection [114]. It has also been proposed that lantibiotics could simply be used as germicidal sanitizers for teat skin surfaces [104]. Notably, nisin is the active ingredient in two commercial products that are used in the prevention of mastitis, Consept (a teat dip) and Wipe-Out (a teat wipe).

Following the same principle, some research has focused on examining the potential of nisin for topical use to inhibit skin pathogens [118]. While research is required to determine the consequences should it, or any degradation product from it, penetrate the skin it has been shown that the cumulative amount of nisin that permeated the skin during 6 hours of iontophoretic transport corresponds to only 0.015% [3]. It has also been suggested that the activity of the lantibiotics gallidermin and lactacin 3147 against *Propionibacterium acne*, one of the causative agents in skin diseases such as acne, could be utilised in the treatment of such diseases [1]. This condition is very often treated with antibiotics such as erythromycin, but given the emergence of antibiotic-resistant strains alternative strategies such as the use of lantibiotics are extremely attractive.

In a related application lantibiotics could be used in the preservation of cosmetics and deodorants [51]. In particular gallidermin is stable at skin pH (5.4) and has quite a narrow spectrum of inhibition, thereby reducing possible side effects. In addition, the strains that produce gallidermin are isolated from human skin and are most probably normal flora of the skin, where they provide natural protection.

Nisin may also have a role in the prevention of periodontal disease, one of the major diseases responsible for tooth loss in the adult population. While such a role has been

mooted for quite some time [23, 26] it has only been in recent years that the application of nisin in this way has been examined more thoroughly. The effect of a mouth rinse containing nisin was investigated [46] for its effectiveness against the development of plaque and gingivitis in beagles. It was found that the nisin-treated groups showed an inhibition of gingivitis development. Such is the potential of nisin that a number of patents have been filed for products, including chewing gum, in which it is incorporated as the active ingredient against tooth decay [6,78,89]. A genetically modified *Streptococcus mutans* has demonstrated the feasibility of using a lantibiotic to prevent dental caries. The strain in question, BCS3-L1, lacks the lactate dehydrogenase gene and thus is not acidogenic. This strain has also been altered to produce elevated levels of the lantibiotic mutacin 1140 giving it a strong selective advantage over most other strains of *S. mutans*. This strain is significantly less cariogenic and it has been suggested that it is capable of permanent implantation and displacement of indigenous disease-causing *S. mutans* [44].

Nisin may also have gastroenterological applications. Significantly, nisin exhibits limited activity against Gram-negative bacteria, such as *H. pylori*, that may be enhanced by the presence of a chelator. The advantages to using nisin as a potential treatment for peptic ulcers include its stability at acid pH and its resistance to the stomach protease pepsin [7]. In addition it is broken down in the intestine reducing its effect on the intestinal microflora [29]. Nisin is also inhibitory to *Clostridium difficile*, which is associated with antibiotic-induced enterocolitis. The management of diarrhea caused by *C. difficile* infection is often complicated by relapses after apparently successful antibiotic treatment. Nisin may offer an alternative treatment for this condition, as it prevents the germination of clostridial spores and preliminary results suggest that the killing kinetics are similar to those of vancomycin [56]. Following the same principle lantibiotics may also have potential as animal feed additives. Feedstuffs are fermented in the rumen of animals, and methane, heat, and ammonia are produced as metabolic side-products. As a result of subsequent metabolic reactions involving methane, the ratio of acetate to propionate increases, resulting in decreased energy retention by the animal. At present, the ionophore monensin is used to inhibit methane production by dissipating the ion gradients of Gram-positive bacteria. However, adverse side effects in both animals and humans have resulted in a search for alternative inhibitors of Gram-positive ruminal bacteria. Due to its inhibitory spectrum and nontoxicity, nisin was evaluated for activity against ruminal bacteria in comparison to monensin. Results indicated that both monensin and nisin can inhibit ruminal methane formation, decrease the acetate to propionate ratio, and prevent amino acid deamination. Although more detailed investigation is required, these preliminary findings are encouraging [16].

Unlike the other lantibiotics, the two-component lantibiotic cytolysin, produced by *Enterococcus faecalis*, is medically significant in that it is lethal for a broad range of eukaryotic as well as prokaryotic cells. It is frequently produced by particularly virulent strains, and a number of models and epidemiological studies suggest that its

production can be associated with patient mortality [25] (see paper by Gilmore in this issue).

The cinnamycin-like lantibiotics, i.e., cinnamycin, ancovenin, and the duramycins may have medical applications but for reasons unlike those for other lantibiotics. These peptides share considerable sequence homology, varying in only six of nineteen amino acids and although their antimicrobial activity is restricted to quite a narrow spectrum (specifically some *Bacillus* sp.), their novel activities against the functions of specific medically-significant human enzymes, such as phospholipase A2 and angiotensin converting enzyme (ACE), have resulted in renewed interest. Phospholipase A2 is involved in the immune system, while ACE is involved in the maintenance of circulatory blood pressure. Ancovenin inhibits ACE [58], the duramycins inhibit phospholipase A2 [35], while cinnamycin is inhibitory to both ACE and phospholipase A2 [57]. Thus, while the cinnamycin-like bacteria are medically significant, it is not as a consequence of their antimicrobial activity.

While the potential applications of lantibiotics are apparent the development of protein engineering strategies that could improve their biological properties and thereby extend their range of uses could prove to be rewarding. A large number of mutants have been generated, but perhaps as might be expected, the majority of mutations have resulted in decreased antimicrobial activity, with only a few exceptions. However, our ever-improving understanding of the mode of action of these peptides will allow us to better predict the consequences of specific mutations in the future. The continued identification of variants of the established lantibiotics and the identification of conserved motifs within novel peptides will also allow us to identify significant structural features of lantibiotics. It is to be expected that an improved understanding of the role of lantibiotics and other features typical of lantibiotics will facilitate their optimisation with respect to antimicrobial activity, stability, solubility, or protease insensitivity and may ultimately lead to the creation of novel synthetic lantibiotics. The permissiveness of lantibiotics to change has also been demonstrated by the analysis of natural variants that differ by a small number of amino acids. These natural variants confirm that at least some amino acids can be modified. The comparison of more distantly related lantibiotics may reveal even more information in that such analysis allows us to identify conserved amino acids/motifs that are likely to play a major role in the structure or activity of a particular group of lantibiotics.

## BIOENGINEERING OF LANTIBIOTICS

### Natural Variants

Natural variants of a number of lantibiotics have been described. The existence of natural variants suggests that the identity of amino acids present at certain locations is flexible and it may thus be possible to generate mutants with changes that enhance a particular phenotypic trait of the peptide without reducing activity. These natural variants may highlight regions of lantibiotics that demonstrate a greater propensity and permissiveness to change while the comparison of more distantly related peptides permits the

identification of conserved regions that are likely to be essential for activity. In all cases described below the relevant amino acid changes in the predicted propeptides (i.e. unmodified structural peptide) are highlighted (see also Table 2).

Two natural variants of nisin A have been identified. Nisin Z differs from nisin A by having an asparagine rather than a histidine residue at position 27 [82]. The *nisZ* gene is widely distributed, having been found in 54% of nisin producing *L. lactis* strains analysed [31]. The activity spectra of the two peptides are 83% similar [80] and they also differ in that nisin Z exhibits a higher rate of diffusion [31] but is less soluble at low pH [94]. Nisin Q differs to an even greater extent from nisin A having a methionine to glutamine change at position 17, a methionine to leucine change at position 21, an isoleucine to valine change at position 30 as well as possessing, like nisin Z, the His27Asn change. The antibacterial spectrum of nisin Q is similar, but not identical to that of nisin A and Z. Interestingly subtilin, which demonstrates great structural similarity with nisin [42] has, like nisin Q, a leucine at position 21 and an asparagine at position 27. While subtilin demonstrates high homology with nisin the lantibiotic which it most closely resembles is a natural variant (N-succinyl-Trp1) subtilin, which is succinylated at its N-terminus and is 6-20 fold less active than subtilin [19]. Another two subtilin-like lantibiotics, ericin S and ericin A, are produced by *B. subtilis* A1/3 [110]. Ericin S differs from subtilin by only 4 amino acids changes (Val6, Val15, Ile24 and His28) and demonstrates similar antimicrobial activity. In contrast ericin A differs to an even greater extent (12 residues) and has a truncated C-terminus. Its lack of activity may be due to the presence of two C-terminal rings that differ from those of subtilin [110].

A number of other examples of variant lantibiotics exist. Gallidermin differs from epidermin as a consequence of having a leucine rather than an isoleucine at position 6 [55] while another epidermin variant, epidermin', demonstrates this change in addition to an Ile1Val variation [50]. Gallidermin is more active than epidermin against various Gram-positive pathogens such as *S. aureus* SG 511 and *Propionib. acnes* IF 31002 as well as *M. luteus* 15957 [55]. The mutacin III subgroup (mutacin B-Ny266, mutacin I and mutacin III/mutacin 1140) also shows very high homology with the epidermin-type lantibiotics with the exception of the N-terminal amino acids of the propeptides. Mutacin III/mutacin 1140 and mutacin B-Ny266 differ by only two amino acids, 1140 having leucine and arginine rather than phenylalanine and lysine at positions 6 and 13, respectively [43, 109]. Mutacin I is also positioned amongst this group of lantibiotics on the basis of homology at the C-terminus and the conserved Ser3, Cys7, Gly10 and Cys11 residues at the N-terminus.

Another lantibiotic, streptin, would seem to be distantly related to the epidermin/mutacin group on the basis of some similarities at the N-terminus [125]. Interestingly, the structural genes of both mutacin I and mutacin III (designated *mutA* in both cases) appear to have undergone a duplication event resulting in the presence of second genes (*mutA'* in both cases) which are ~90% identical to the respective *mutA* at the DNA level. Inactivation of the

mutacin I mutA' results in a slight decrease in antimicrobial activity of the strain [92] whereas mutagenesis of the equivalent gene in a mutacin III producer had no impact [91].

There are a number of lantibiotic peptides that show high similarity with the lantibiotic lactacin 481. These include lactacin J46, mutacin II, butyrivibriocin, ruminococcin, streptococcin SA-F22, nukacin, variacin and the salivarinicins [115]. Such is the number of peptides/variants within this group and the variety of residues that differ upon alignment that it becomes obvious which amino acids are highly conserved i.e. Gly5, Thr9, Ser/Thr11, Glu/Asp13, Cys14, Asn17, Ser/Thr18, Phe23, Cys25 and Cys26 (as located in lactacin 481) while the others differ with high frequency. The existence of two salivarinic variants, A and A1, which differ by only two amino acids is particularly noteworthy [107, 117], and there are also instances where two or more genes that are identical with respect to the region encoding mature structural peptides are present in a single strain. This is the case with streptococcin SA-F22 which possesses 2 such genes located adjacently [49] while ruminococcin producers have three such structural genes i.e. *rumA1*, *rumA2* and *rumA3* [41,75]. Finally bovicin HJ50 has been placed in the lactacin 481 group by virtue of the similarity of the N-terminus of the unmodified propeptide with those of other lactacin 481-type lantibiotics. It should be noted however that its C-terminus differs greatly [126].

Of the residues conserved among lactacin 481-type lantibiotics, a number are especially interesting in that they are also conserved in peptides of the mersacidin group, which comprises Rumb, mersacidin, actagardine, C55a, LtnA1, Plw, BhaA1 [114] and possibly plantaricin C. This

suggests that these amino acids are significant with respect to the structure and/or activity of these peptides. In all cases where the structure of peptides belonging to either the lactacin 481 or mersacidin groups has been elucidated, the residues corresponding to Ser/Thr11, Cys14 and Cys25 in lactacin 481 have been shown to be involved in bridge formation [61, 76, 90, 119, 131]. In addition it has been shown that in mersacidin the residue corresponding to Glu13 in lactacin 481 (Table 2) experiences a large chemical shift perturbation in the presence of DPC micelles and undergoes a further shift in response to lipid II [48]. This residue seems to be essential on the basis that site-directed mutagenesis (see below, [112]) or amidation of the carboxyl group of this glutamate results in the inactivation of mersacidin [48] supporting suggestions that the residue is crucial in the interaction of mersacidin with lipid II. Furthermore, the conservation of this residue suggests that all of the lactacin 481- and mersacidin-like peptides may bind lipid II (Table 2).

In addition to those amino acids that the mersacidin-like peptides have in common with lactacin 481, an additional glycine, corresponding to position nine in mersacidin, is conserved among all peptides bar Rumb of this group while the cysteine at position 11 is fully conserved (see Table 2). Within this group particular peptides show a high degree of homology. LtnA1 and C55a display the highest similarity differing by only 4 amino acids while Plw and the putative BhaA1 peptide are also quite similar.

It is noteworthy that while the beta peptides of a number of two-component lantibiotics also demonstrate homology it is to a much lesser degree than that observed among the corresponding alpha peptides. Of the two-component

Table 2. Lantibiotic subgroups

Group	Lantibiotic	Unmodified sequence of structural peptide
Nisin	Nisin A	I T S I S L C T P G C K I G A L M G C N M K T A I C H N C S I H V S K K
	Nisin Z	I T S I S L C T P G C K I G A L M G C N M K T A I C H N C S I H V S K K
	Nisin Q	I T S I S L C T P G C K I G A L M G C N M K T A I C H N C S I H V S K K
	Subtilin	W K S I S L C T P G C K I G A L M G C N M K T A I C H N C S I H V S K K
	Ericin A	V L S I S L C T P G C K I G A L M G C N M K T A I C H N C S I H V S K K
Epidermin	Epidermin	I A S K F L C T P G C A K T G S F N S Y C C C
	Epidermin'	I A S K F L C T P G C A K T G S F N S Y C C C
	Gallidermin	V A S K F L C T P G C A K T G S F N S Y C C C
	Staphylococcin T	V A S K F L C T P G C A K T G S F N S Y C C C
	NV256	F S S L S L C T P G C A K T G S F N S Y C C C
Streptin	Streptin	V G S I R Y L C T P G S C V V K L V C F T T T V K
	Pep5	I A G P A I R A S V K Q G Q K T L K A T R L F V S C K K K N D G C K
	Epidermin 280	S L G P A I K A T R I G V G C P K K A T R F V T V S C K K K N D G C K
	Epidermin 287	S A S V L K T S I K V S K K K Y C K G V T L T C G C N I T G G K
	Lactacin 481	K G G S G V I H T I S H E C N M N S W Q F V F T C C S
Lactacin 481	Lactacin J46	N R W W Q G V I H T I S H E C N M N S W Q F V F T C C S
	Butyrivibriocin	G N G V I K T I S H E C N M N S W Q F V F T C C S
	RumA	G N G V I K T I S H E C N M N S W Q F V F T C C S
	Streptococcin SA-F22	K K K S G V I P T T V S H E C H M N S F Q F V F T C C S
	Nukacin	K K K S G V I P T T V S H E C H M N S F Q F V F T C C S
Mersacidin	Variacin	K K K S G V I P T T V S H E C H M N S F Q F V F T C C S
	Salivarin A	K K K S G V I P T T V S H E C H M N S F Q F V F T C C S
	Salivarin A1	K K K S G V I P T T V S H E C H M N S F Q F V F T C C S
	Bovicin HJ50	A D R G W I A K T L T K D C F N V I S S I C A G T I I A C K N C A
	Rumb	G E P R V S D G S G F C I S Y K E C N W G I M F V C C
Mersacidin	Plantaricin C	C T F T L C G G V C T L T Y S E C D H L A T W V C X
	Mersacidin	C S T N T F S L S D Y W G N N F G W V C T L T I E C G
	Actagardine	C S T N T F S L S D Y W G N N F G W V C T L T I E C C
	C55a	C S T N T F S L S D Y W G N N F G W V C T L T I E C C
	LtnA1	K C K W W N I S C D L G N N G H V C C T L S H E C C
LtnA2	Plw	G T P L A L L G A A T I S I L S A Y I S N Q T C P T T A C T R A C
	C55b	G T P L A L L G A A T I S I L S A Y I S N Q T C P T T A C T R A C
	Plw	G D V H A Q T F G I G A V A A S I A V C P T T A C T R A C
	LtnA2	G D V H A Q T F G I G A V A A S I A V C P T T A C T R A C
	CylII	G D V A E T T F A C A C F T I G I G V G G L F S A V I V V S L K H C
Cytolysin	CylI	G D V A E T T F A C A C F T I G I G V G G L F S A V I V V S L K H C
	CylII	G D V A E T T F A C A C F T I G I G V G G L F S A V I V V S L K H C
	CylIII	G D V A E T T F A C A C F T I G I G V G G L F S A V I V V S L K H C
	CylIV	G D V A E T T F A C A C F T I G I G V G G L F S A V I V V S L K H C
	CylV	G D V A E T T F A C A C F T I G I G V G G L F S A V I V V S L K H C
Lactosin S	Lactosin S	T P V I A S V A V S M E L L P T A S V L Y S D V A G G F K Y S A K H H C
	Chromomycin	C K Q S C S F G P F T F V C D G N T K K
	Duramycin	C K Q S C S F G P F T F V C D G N T K K
	Duramycin B	C K Q S C S F G P F T F V C D G N T K K
	Duramycin C	C K Q S C S F G P F T F V C D G N T K K
Sublancin	Sublancin	L G K A Q C A A L W L Q C A S G G T I G C G G G A V A C Q N Y R Q F C R
	Sublancin 168	L G K A Q C A A L W L Q C A S G G T I G C G G G A V A C Q N Y R Q F C R
	Sublancin 169	L G K A Q C A A L W L Q C A S G G T I G C G G G A V A C Q N Y R Q F C R
	Sublancin 170	L G K A Q C A A L W L Q C A S G G T I G C G G G A V A C Q N Y R Q F C R
	Sublancin 171	L G K A Q C A A L W L Q C A S G G T I G C G G G A V A C Q N Y R Q F C R

Alignments are based on primary sequence comparisons of the mature peptides (both known and predicted). Amino acids conserved across more than one subgroup are indicated by boxed, bold font text, amino acids conserved among all members of a particular subgroup are indicated by white text on a black background, amino acids conserved among at least 50% of the members of a particular subgroup are indicated by light grey boxes while amino acids conserved among less than 50% of a particular subgroup are indicated by dark grey boxes.

lantibiotics cytolysin is unusual in that the two peptides are homologous to one another but not to any other lantibiotics. This would suggest that they originated from an ancient duplication event in the same way that mutacin I and III as well as streptococcin SA-F22 and ruminococcin have multiple homologues of the structural genes.

As outlined previously the cinnamycin-like lantibiotics have activities that differentiate them from other lantibiotics. This is also true of their amino acid sequences and structures [35, 130]. However, these lantibiotics, which are produced by GC-rich Gram positive bacteria, are very similar to one another and are in essence a collection of variants. Among these lantibiotics cinnamycin is regarded as the prototype and duramycin A (Arg2Lys alteration), duramycin B (Phe10Leu alteration) and duramycin C (Arg2Ala, Gln3Asn, Phe7Tyr, Phe10Leu, Phe12Trp, and Val13Ser) as variants.

Thus it is apparent that quite a number of natural variants exist and it is likely that there are a large number of other variants that have yet to be isolated. The examination of these natural variants assists in the identification of regions that are more amenable to change. While the creation of mutant peptides with enhanced capabilities is a rare occurrence, the potential rewards are great. In addition, the knowledge gained by identifying essential residues and structures will greatly enhance our understanding of the mechanism of action of these complex peptides and potentially assist in the construction of hybrid or synthetic lantibiotics in the future.

### Site-directed Mutagenesis

A number of different systems have been used to mutate specific amino acids in lantibiotics [87]. The consequences of these mutations are numerous and thus, for the purpose of clarity these mutations have been subdivided according to a number of different criteria. The various site-directed mutants characterised to date are summarised in (Fig. 2).

### Thiol bridge Formation

The generation of thiol bridges as a consequence of lanthionine and -methylanthionine formation is the key trait that gives lantibiotics their name. It is thus likely that any mutations that interfere with the formation of these rings are likely to interfere with the structure of the peptide and consequently its activity. In most cases, elimination of a thiol ring has a devastating effect on antimicrobial activity due to the peptide itself not being active or because production of the mutant peptide is diminished, possibly as a consequence of the biosynthetic machinery not being compatible with this peptide. In some cases, the nature of the ring at a particular location is not of great importance and thus lanthionines and -methylanthionines can be interchangeable.

Unsurprisingly, the replacement of hydroxyamino acids involved in thiol bridge formation by non-hydroxyamino acids (Ser3Asn and Ser19Ala) [86] eliminated epidermin production and activity. The consequences were the same when cysteine residues involved in thioether bridging were deleted (Cys21,22 and Cys22) [86]. Ser16<sub>gal</sub> and Ser19<sub>epi</sub> were mutated to determine the compensatory effects when serine residues involved in bridging were replaced with threonine residues in gallidermin and epidermin. These

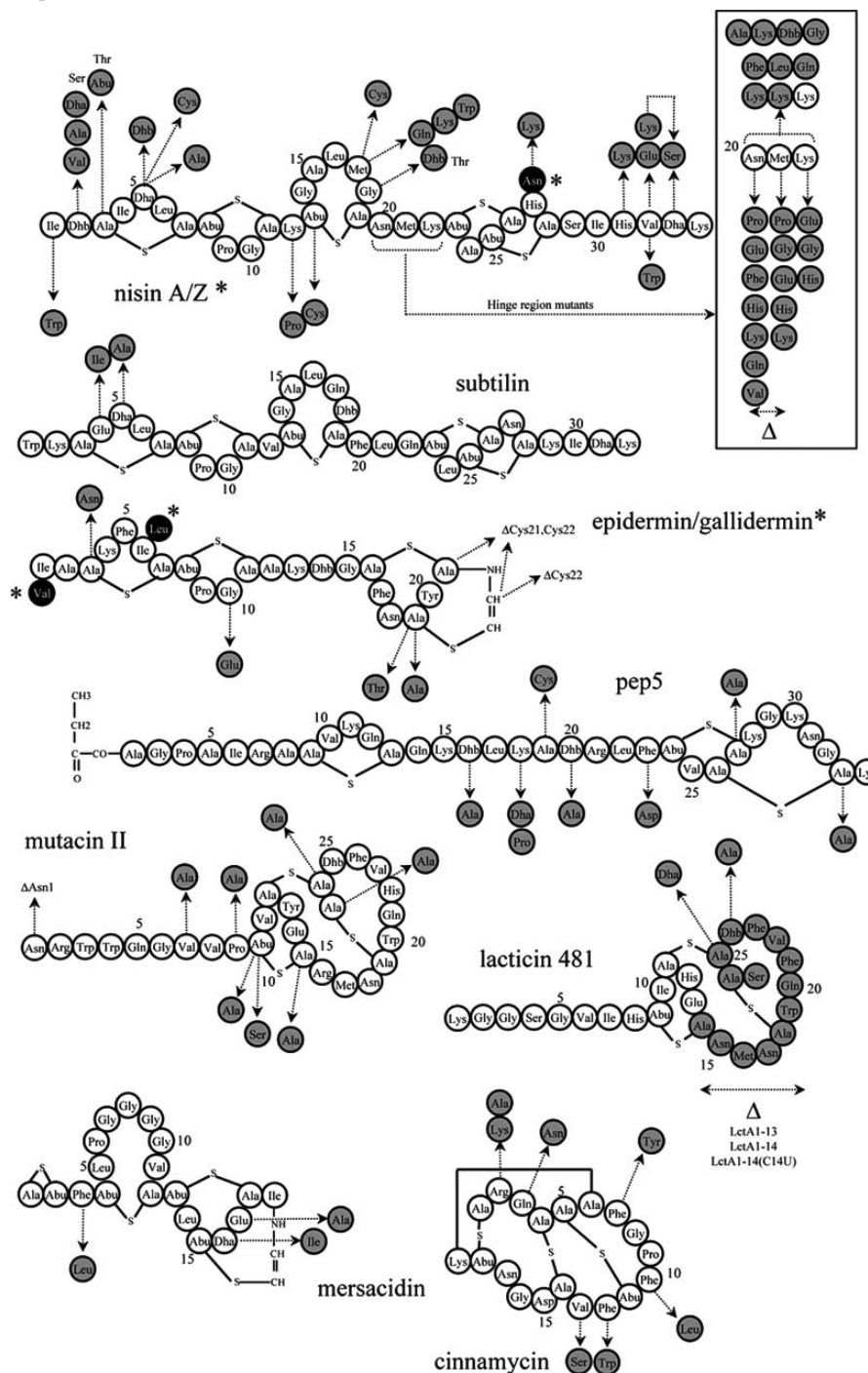
mutant peptides demonstrated antimicrobial activity but were produced in extremely low amounts and thus a strong decrease in activity was observed in both instances [86]. It may be that Thr16 and Thr19 are modified inefficiently and consequently only a small percentage of the peptide is exported. A Ser3Thr mutation of nisin Z also showed dramatically decreased antimicrobial activity [63].

Analysis of mutacin II mutants revealed similar results. Mutation of Thr10 [22], which was subsequently shown to be involved in thioether bond formation [61], to alanine resulted in non-secretion of the mutant peptide. However, when this threonine was converted to a serine residue the resultant peptide was correctly processed and, significantly, exhibited antimicrobial activity similar to that of wild-type mutacin [22]. Yet again the consequences of mutating cysteines involved in thioether bond formation were devastating in that Cys15Ala and Cys26Ala peptides, though successfully produced, lacked antimicrobial activity while Cys27Ala peptides displayed greatly reduced activity. These mutants were of further interest in that analysis of the Cys15Ala, Cys26Ala and wild-type peptides by mass spectrometry and nuclear resonance spectrometry permitted elucidation of the bridging pattern of mutacin II [61]. Mutagenesis of cysteine residues in Pep5 also affected antimicrobial activity. A Cys27Ala peptide had reduced activity as is the case with Cys33Ala, though not to the same extent [5].

Given the important nature of cysteine residues in lantibiotics the incorporation of additional cysteine residues is also likely to have a large impact on production and activity. When it was noted that the side-chain of Ala19 was on the same side of the Pep5 peptide as the side chain of Dhb16 [36] an Ala19Cys mutant was created in the hope that it would form a new bridge with Dhb16. Small quantities of this peptide were produced though its activity was diminished [5]. A more extensive analysis involved altering 11 different codons in nisin to encode cysteines in order to determine which mutations could be tolerated. The majority of these mutants, Ile1Cys, Ile4Cys, Ser5Cys, Leu6Cys, Thr13Cys, Met17Cys, Met21Cys, His31Cys, Val32Cys, Ser33Cys and Lys34Cys, however, are not produced [122]. An interesting observation was made with respect to the Thr13Cys mutant. Mutation of Thr13 was predicted to impact on the third lanthionine ring of the peptide (ring C) (see Fig. 2). It was found, however, that a new bridge is formed as a consequence of Cys13 and Cys19 interacting to generate a disulphide bridge [122] which, until the identification of disulphide bridges in sublancin 168 [88], was unique among lantibiotics. This mutation is still detrimental as antimicrobial activity drops to less than 1% of wild type peptide. Two other mutant peptides, Ser5Cys and Met17Cys, were also produced. In this instance, it was found that the incorporated cysteines were not modified making these the first lantibiotics known to contain an additional cysteine residue that is present as a free thiol. As a consequence the secretion and activity of these peptides was strongly dependent on a reducing environment [122].

### Dha/Dhb

The unusual amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) have also been the subject of a great



**Fig. (2).** Lantibiotic mutants that have been created. Black circles indicate amino acids that differ between natural variants in situations where different variants have been the subject of mutation. Grey circles indicate mutations made.

deal of research. Site directed mutagenesis has been used to determine their importance in a number of instances. Dha5 of subtilin is an interesting example (see Fig. 2). This residue exhibits a great tendency to undergo spontaneous modification resulting in diminished activity. Thus minimising the level of spontaneous modification was desirable. As the R carboxyl of Glu4 was thought to participate in the chemical modification of the adjacent Dha5, Glu4 was altered to an isoleucine, i.e., the residue

present at the corresponding location in nisin. It was found that the rate of loss of activity of the mutated subtilin was dramatically reduced and furthermore the peptide demonstrated an increased specific activity. It is thought that increased activity resulted as a consequence of the alteration enhancing the hydrophobicity of the amino terminus of the peptide which may allow it to enter the membrane more easily. This was the first example of a lantibiotic whose properties have been improved by mutagenesis. The critical



nature of the Dha5 residue was demonstrated when it was found that a double mutant, Glu4Ile-Ser5Ala, lacked the ability to prevent spore outgrowth [73]. The corresponding residue in nisin is also of great importance. A study of nisin degradation products shows that saturation of the double bond in Dha5 leads to a dramatic reduction in the bacteriocidal activity of nisin. Its importance was also demonstrated when it was found that a Ser5Ala mutation abolishes the sporicidal activity of nisin [20,32]. When attempts were made to complement the Dha residue with Dhb by creating a Ser5Thr prepeptide it was found that activity was reduced. The fact that Dhb could not replace Dha in this instance may be as a consequence of the additional methyl group on Dhb either affecting the three-dimensional structure of the peptide or altering the interaction of the peptide with target cell membranes [18, 71]. Interestingly, however, the Dha5Dhb mutant does show improved stability as demonstrated by its resistance to acid-catalysed chemical degradation [94].

The Thr2Ser mutant of nisin Z is particularly interesting. This peptide was modified correctly resulting in the generation of a Dha residue and displayed a two-fold increase in activity against *Micrococcus flavus* and *Streptococcus thermophilus* [63, 124]. However, this residue would seem to be quite flexible to change as Thr2Ala and Thr2Val mutants also displayed either wild-type or close to wild-type levels of activity [124]. In another nisin Z mutant a dehydro residue was introduced rather than altered. This was done to generate a Met17Gln/Gly18Thr double mutant such that ring C of the mutated nisin would be identical to that of subtilin. The result was the production of two mutant species with either a Thr (minority) or Dhb (majority) at position 18. The Dhb18 version demonstrates similar activities to nisin Z whereas the Thr 18 variant was four-fold less active against two indicators and twice as active against another [62] making it also an example of a mutant peptide with some enhanced antimicrobial activities. Additional studies have demonstrated that Dha33 is not essential for nisin activity as a Ser33Ala mutant yields similar activity as that from the wild-type strain [65].

Various mutants of gallidermin, in which threonine 14 was mutated, were generated in order to determine the importance of the dehydrated residue present in the modified peptide and to assay the sensitivity of the peptides to trypsin. The sensitivity of gallidermin and epidermin to proteases could potentially impact on their utilisation as therapeutic agents against acne and it is thought that trypsin acts at the flexible middle region (or hinge), i.e., amino acids 12 to 15 [37] (see Fig. 2). Thus the generation of mutants with increased resistance to protease action but with good antimicrobial activity was desirable. When a Thr14Ser mutant of gallidermin was generated two peptides were produced. The first Dhb14Dha exhibited antimicrobial activity similar to wild type gallidermin while Dhb14Ser was less active [86]. A Dhb14Ala mutant generally displayed even less activity while a Dhb14Pro mutant showed the least activity. Dhb14Pro was, however, the only one of the mutant peptides that did not demonstrate increased susceptibility to tryptic cleavage. In a related study, it was found that the incorporation of a bulky residue within the hinge (Ala12Leu) resulted in diminished activity but did bring about a dramatic

increase in resistance to trypsin [86]. It may be that the creation and analysis of other hinge region mutants would reveal alterations that would increase trypsin resistance without impacting on activity.

Dhb residues in Pep5 have been altered by generating Thr16Ala and Thr20Ala mutants resulting in a significant loss of activity [5]. It has also been demonstrated that it is possible to incorporate dehydroamino acids, as was the case when Lys18Ser was generated resulting in the incorporation of a Dha. The portion of fully modified peptide generated demonstrated lower antimicrobial activity than Pep5 [5].

A Dha residue was also mutated in a Ser16Ile derivative of mersacidin such that the third ring of the mutant mersacidin and the second of actagardine would be identical. However, this was produced only in small amounts and there was a marked reduction in activity [112].

The consequence of mutating Dha and Dhb residues remains largely unpredictable, and is probably dependent on the location of the residue within a particular peptide. The relatively small impact in the cases of the nisin and subtilin examples presented above probably reflects the fact that the residues in question are present in rigid parts of the peptide. In contrast the pep5 and epidermin residues discussed are in question are located at a flexible location.

### Hinge Region

As outlined above with respect to gallidermin, mutation of the hinge region of a lantibiotic can have devastating effects on the antimicrobial activity of the peptide. A hinge region has been described as any region, flexible or rigid, that connects the thioether ring domain to the other domains of a lantibiotic molecule [22]. A number of other examples exist. As the N terminus of mutacin II is thought to form an alpha helix [83] it has been speculated that proline 9 is part of a hinge region within this peptide. This suggestion is consistent with the observation that a Pro9Ala mutant displays dramatically reduced activity [22]. In addition, while production of a Lys18Pro mutant of Pep5 was not affected, it was found that this mutant displayed dramatically diminished activity. This phenotype, like that of the previously described Lys18Thr mutant, is probably as a result of this mutation interfering with the hinge region. The hinge region in nisin corresponds to the section between rings C and D (see Fig. 2). Its importance is immediately apparent by the drastic reduction in activity of Asn20/ Met21, Asn20Pro/Met21Pro and Met21Gly mutant peptides against *S. thermophilus*, suggesting an impact on pore formation, though the reduction in activity was not as apparent when assayed against *M. flavus*, indicating lipid II binding capability is still intact [124]. A more extensive mutagenesis of the hinge-region of nisin Z resulted in the generation of mutant peptides that were sub-grouped on the basis of their antimicrobial activity against *Strep. thermophilus* and *M. flavus*. The introduction of a negative charge into the hinge region resulted in non-production of the mutant peptide (Lys22Glu) or an almost complete elimination of activity (Asn20Glu, Met21Glu) while the introduction of a hydrophobic amino acid (Asn20Val) or the alteration of the hinge to more closely resemble that of epidermin/gallidermin (Asn20Ala-

Met21Lys-Dhb-Lys22Gly) dramatically reduced activity. A number of other mutants (Asn20His, Asn20Gln, Asn20Phe, Met21Gly, Met21His, Lys22Gly, Lys22His, Asn20Lys-Met21Lys, Asn20Phe-Met21Leu-Lys22Gln) all displayed activities that were slightly reduced relative to the wild-type peptide. Of this group the activity of the Asn20Phe-Met21Leu-Lys22Gln mutant (in which the nisin hinge region is replaced with the subtilin hinge region) is notable in that its activity remains greater than that of wild-type subtilin. It was also found that, though less active, the Asn20Gln and Met21Gly peptides were more stable at higher temperatures and at neutral/alkaline pHs than the wild-type peptide (129). However of the peptides generated in this study the Asn20Lys and Met21Lys mutants are by far the most significant. While they display activity similar to wild-type nisin Z against *Strep. thermophilus* and *M. flavus* they are much more soluble, especially at alkaline pHs. More importantly these peptides show activity against Gram-negative bacteria (*Shigella*, *Pseudomonas* and *Salmonella*) in the absence of other stress factors (129) presumably due to an enhanced interaction with the target membranes. These mutants are an excellent example of how site-directed mutagenesis can enhance the antibacterial activity of a lantibiotic.

### Altered Charge

A number of lantibiotics are charged. In fact, one of the primary characteristics associated with type A lantibiotics is their cationic nature. While the charge of these peptides influences their solubility, it is also likely to have a major role in their capacity as antimicrobials. While these charges are likely to contribute to the pore-forming capacity of certain lantibiotics this characteristic explains the high tolerance of organisms such as *Staphylococcus aureus* and *Staphylococcus xylosus* to these peptides. D-Ala esters, present in high amounts on the teichoic acid within the cell wall of these organism, carry a positive charge making the microbial surface less likely to bind cationic peptides. In the case of a nisin Z mutant with a reduction in its positive charge, Lys12Pro, it was found that the activity of this peptide was unaffected but it was thought that it may influence the solubility of the peptide [65]. However, as it is likely that it is the C-terminus of nisin that plays the major role in pore-formation three other nisin Z mutants, Val32Glu, Val32Lys and Val32Trp, were also generated to determine the consequences of altering the charge at this location. However 1H-NMR analyses showed that these modifications also affected the efficiency of peptide modification resulting in the presence of an unmodified serine residue at position 33, instead of the usual dehydroalanine. The activity of the Val32Lys/Ser33 peptide (with an additional positive charge) against *Micrococcus flavus* was similar to that of wild-type nisin, while the Val32Trp/Ser33 and Val32Glu/Ser33 (possessing an additional negative charge) mutants exhibited 3-5-fold reduced activity, indicating that negative charges in the C-terminal part of nisin Z are detrimental for activity [121, 124]. Similarly a Met17Lys change results in the corresponding peptide demonstrating a greatly reduced capacity to form pores suggesting that a charged residue in the central segment of the molecule was detrimental. Despite this, it retained quite a good activity, probably due to

increased accumulation counteracting the effects of reduced activity [124]. It has also been shown that it is possible to alter charge in a beneficial way to improve the solubility of nisin in water at neutral pH. This was done by introducing a Lys residue (Asn27Lys and His31Lys nisin Z). These variant peptides maintained a comparable antimicrobial activity and spectrum but displayed a four- and seven-fold increase in solubility at neutral pH, respectively [94].

### Mutagenesis Resulting in 'Intermediate' Lantibiotics

It has been outlined above that when glutamate 4 of subtilin was altered to an isoleucine, to mimic the situation in nisin which is less susceptible to modification of Dha5, activity increases. On the other hand, a Ser16Ile derivative of mersacidin, which more closely resembles actagardine, is produced at greatly reduced levels. A number of similar strategies have been implemented. Since gallidermin (Leu6) is more active than epidermin (Ile6) against various Gram-positive pathogens [55] gallidermin mutants were generated to determine the result of changing Leu6 to either glycine or valine. These peptides were produced and modified successfully. It was found that while the Leu6Gly mutant was slightly less active against all indicators tested the Leu6Val peptide was twice as active against *M. luteus* and *C. glutamicum*. These results represent an excellent example whereby a difference between natural variants has highlighted a location within these peptides that is amenable to change with potentially beneficial consequences [86].

As outlined previously it has been shown that members of the duramycin family of lantibiotics display high levels of similarity. In studies aimed at determining whether the biosynthetic machinery of a cinnamycin producer could recognise and modify cinnamycin mutants that are altered to create other duramycin-like lantibiotics, it was found that it was possible to generate duramycin (Arg2Lys change) and duramycin B (Phe10Leu change). However, the cinnamycin biosynthetic machinery does not permit the production of duramycin C, undoubtedly as a consequence of being unable to tolerate the six differences between these peptides, i.e. Arg2Ala, Gln3Asn, Phe7Tyr, Phe10Leu, Phe12Trp, and Val13Ser. Of the six changes the most detrimental are likely to be the nonconservative ones, Arg2Ala and Val13Ser [123].

### Mutagenesis to Elucidate Mode of Action

A number of mutants have also been generated which have been of benefit to nisin research, not necessarily as a consequence of an enhanced property of the mutant peptide, but due the role they have played in improving our understanding of the mode of action of the peptide.

Several mutants have demonstrated that binding of nisin to lipid II is mediated through the N terminus of the peptide. In fact, an N-terminal 1-12 fragment of nisin was capable of antagonising the binding of wild-type nisin [21]. However, when the N terminus was analysed in greater detail, it was found that mutants such as Thr2Ser, Thr2Ala and Thr2Val had only a slight effect on the minimum inhibitory concentrations against *Micrococcus flavus* and *Streptococcus thermophilus*. In contrast dye release from lipid II-containing liposomes by a Ser3Thr mutant was strongly affected and its activity against *M. flavus* was greatly reduced [124] (again

reflecting a deficiency in lipid II binding as *M. flavus* contains about  $10^5$  lipid II molecules in its membrane, [111]). Thus, there appears to be high structural selectivity at position 3 while position 2 is more tolerant of change. These observations were supported by studies in which the nisin Z/lipid II interaction in SDS micelles was determined. It was found that the N-terminal part of nisin first binds to lipid II, with rings A and B acting as a binding interface, and a subsequent structural rearrangement takes place with the C-terminal part of nisin playing a role in pore formation [47]. In the case of mersacidin, it is thought that glutamate17 is essential for binding to lipid II. The observation that a Glu17Ala has negligible levels of antimicrobial activity supports this theory [112].

A variety of mutants with tryptophan residues incorporated at different locations i.e. Ile1Trp, Met17Trp, Val32Trp [30, 64, 121] were instrumental in determining the orientation of nisin in lipid II plus and lipid II minus membranes by measuring tryptophan fluorescence emissions. While these mutants displayed slightly diminished activities against *M. flavus* [8, 120] they did demonstrate that the presence of lipid II in artificial membranes changes the overall orientation of nisin in the membranes from parallel to perpendicular with respect to the membrane surface [120].

The identification of specific target molecules within bacterial membranes will greatly facilitate the rational optimisation of the antibiotic activity. Even among lantibiotics known to bind lipid II, it is apparent that the binding site is not always the same, i.e., mersacidin and nisin are not antagonistic to each other. The accumulation of this information will not only lead us to determine which lantibiotic is best suited to target particular strains. It may ultimately be possible to mutate lantibiotics to improve their activity on the basis of an improved understanding of the role of residues at particular locations.

A number of other mutants do not fit into any of the categories described above. It has been shown that the conversion of certain amino acids in epidermin to amino acids of different character (e.g. Gly10Glu, Tyr20Gly) results in the elimination of activity [86] while a Phe23Asp mutant of Pep5 was not secreted and appeared to accumulate intracellularly [4]. In contrast a Phe3Leu mutant of mersacidin has only moderately reduced activity [112]. Other mutations which are likely to interfere with cleavage of the leader region result in the non-secretion of the mutant peptide as is the case with the Asn1 mutant of mutacin II [22].

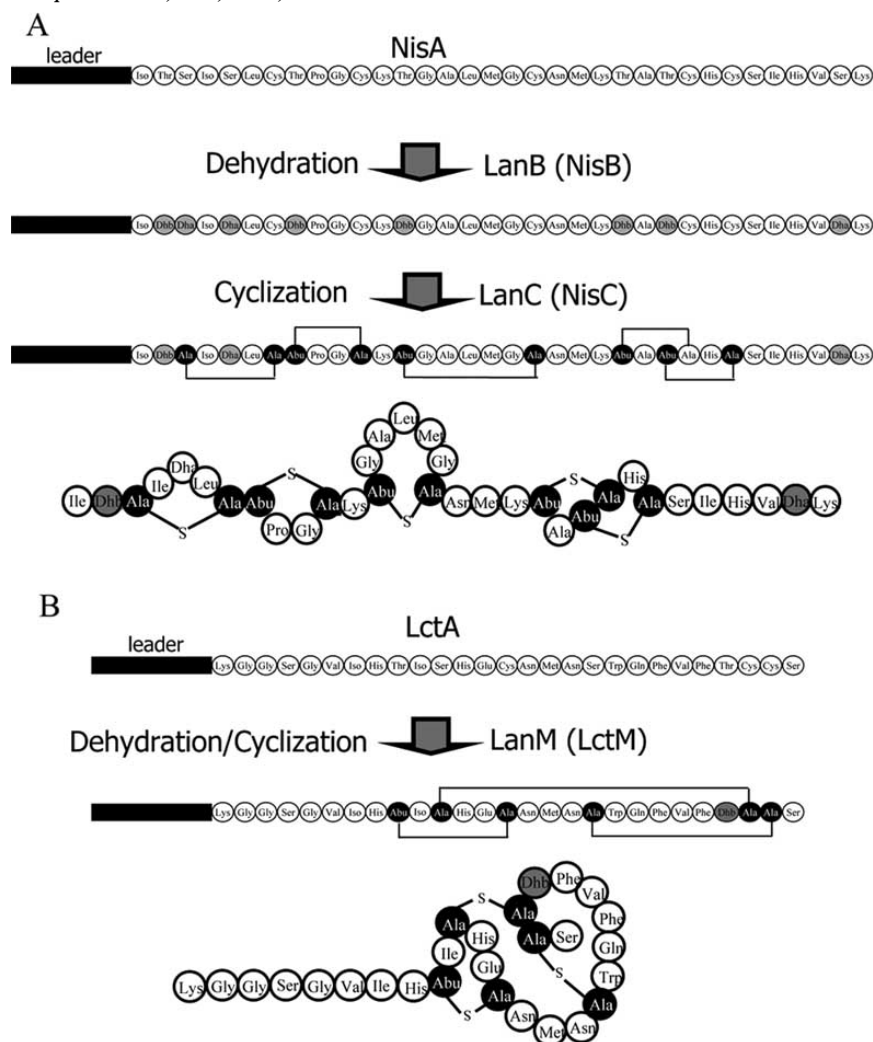
The continuing study of mutated peptides will be greatly aided by new developments such as the use of mass spectrometry to detect lantibiotics from whole bacteria grown on plates. This has already been used to detect Ser27 and Ser4Thr mutants of lactacin 481, while the mass of the peptides also suggests that the correct alterations have occurred. This technique will also quickly reveal whether mutations resulting in the elimination of antimicrobial activity do so as a result of the prevention of bacteriocin production or whether an inactive antimicrobial peptide is produced [45].

## Peptide-modifying Enzymes

It has been shown above that novel amino acids and novel bridging structures [5, 128] may be introduced into lantibiotics. The development of new peptide compounds, without time-consuming and costly chemical syntheses, will benefit from the isolation of modification enzymes involved in lantibiotic biosynthesis from producing strains and their use to transform peptides into novel compounds of biotechnological interest. *In vitro* synthesis eliminates problems associated with the construction of expression systems or inadequate immunity systems. The chemical reactions synonymous with lantibiotic peptides involve the dehydration of hydroxyl amino acids to generate Dha and Dhb and cyclization of cysteine residues onto these dehydroamino acids to assemble the thioether ring structures. The production of type A lantibiotics is dependent on these reactions being carried out by LanB (dehydration) and LanC (cyclization) [60, 106] while bacteria producing type B lantibiotics possess LanM enzymes that seem to catalyse both dehydration and cyclization [77, 106] (see Fig. 3). It is likely that the cyclization capacity of the LanM proteins lies with the C-terminal end where it displays homology to LanC enzymes [116].

Results based on studies of NisC and SpaC suggests that the LanC enzymes are zinc proteins and may function by activating cysteine residues for Michael addition to the dehydroamino acids [84]. Two conserved cysteines and two conserved histidines are suggested to act as ligands to the metal [84] and interestingly these are also conserved in LanM enzymes. In addition to the purification of NisC and SpaC, NisB [54] has been overexpressed while EpiC [69] and SpaB [127] have been purified. However the extent to which M proteins may be of biotechnological significance has been best demonstrated in the case of LctM. This enzyme, which modifies lactacin 481, has been expressed in, and isolated from, *E. coli* and displays activity in the presence of  $Mg^{2+}$  and adenosine triphosphate (ATP). As well as having the capacity to produce fully modified lactacin 481 fused to its leader peptide LctM also successfully dehydrated Thr9 and Ser11 (numbered on the basis of their location in mature lactacin 481) in an LctA(1-13) derivative. Incubation with LctM also resulted in the dehydration and cyclization of LctA(1-14) and LctA(1-14)C14U derivatives indicating that the enzyme demonstrates a degree of flexibility with respect to its substrate. It was also found that, as expected, the removal of a post-translationally modified residue (Thr24Ala) resulted in a reduction in the number of dehydrations occurring and that, more significantly, the converse is also true i.e. it is possible to incorporate an additional dehydration (Cys25Ser) [128].

Studies have also highlighted the role of the leader peptide of unmodified lantibiotics. Previously, it had been suggested that conserved amino acids in the leader peptides may act as a recognition sequence either for modifying enzymes or for transport. Significantly while nisin cannot be modified by the subtilin biosynthetic machinery under normal circumstances a nisin derivative in which the seven amino acids at the C-terminus of the nisin leader are replaced by the corresponding region from the subtilin leader is processed and is active [17]. It would thus seem that the C-



**Fig. (3).** Roles of LanB, LanC and LanM proteins. Nisin is used as an example to demonstrate the role of its LanB and LanC proteins in dehydration and cyclization, respectively, of the prepeptide. Using lactacin 481 as an example, the dual functionality of LanM proteins is demonstrated. Grey Residues – dehydrated amino acids, black residues – amino acids involved in lanthionine bridge formation.

terminus of the leader region is responsible for recognition of the prepeptide by modification proteins. This theory is supported by studies. LctM was found to modify LctA when the leader region (24 amino acids in length) is present [128] but not in its absence. Furthermore mutants lacking residues 2-4 or 2-9 of the leader were fully processed indicating that residues 10-24 play an important role in the interaction of LctA with the modification machinery [128].

These developments demonstrate that mutant peptides that cannot be exported could now be synthesised and suggest that the generation of systems that would permit the creation of novel peptides may be more feasible than previously thought. In addition to dehydrated amino acids and lanthionines, lantibiotics may contain any of a number of other modifications e.g. 3-methylanthionines, lysino-alanines, lanthionine sulfoxides, D-alanines, allo-isoleucines, erythro-3-hydroxyaspartates, 2-oxopyruvates, 2-oxobuty-rates, hydroxypyruvates, S-(2-aminovinyl)-D-cysteines and S-(2-aminovinyl)-3-methyl-D-cysteines to name but a few [52, 87, 99]. It is thus also desirable to identify, and purify,

enzymes that may catalyse such reactions in order to appropriately modify lantibiotics when synthesis becomes a reality or to incorporate additional modifications into other lantibiotic, or even non-lantibiotic, peptides where desirable.

In the last few years, several lantibiotics with unsaturated thioether bridges at the COOH terminus have been characterized. S-[(z)-2-aminovinyl]-D-cysteine is present in epidermin [2], gallidermin [55], cypemycin [79] and some of the mutacins [81, 91, 92, 108], whereas mersacidin contains a COOH-terminal S-[(Z)-2-aminovinyl]-3-methyl-D-cysteine residue [59, 101].

Extensive research has been carried out on the flavoprotein EpiD, which is required for the oxidative decarboxylation of the C-terminal Cys residue of preepidermin [66, 67, 68]. Unsurprisingly, mutations at the C-terminus of epicidin (Ser19Ala, Cys21, 22 and Cys22) prevent this reaction taking place [86], however in order to determine the sequence specificity of EpiD, it was tested in an system using synthetic peptide substrates [68]. Heptapeptide libraries with one variable amino acid residue

at positions 1-7 of the peptide substrate SFNSYCC were incubated with EpiD, and the reaction products were identified. When Cys6 was replaced with Ser, Thr, Ala, or Val decarboxylation was unaffected. It was also found that Tyr5 could be replaced with other hydrophobic amino acids [68] while SFNSCCC has also been shown to be a suitable substrate [100]. In each case the final Cys was modified from a peptidyl cysteine to a peptidyl aminoethiol [70]. This experimental approach may be a valuable tool for the investigation of the post-translational reactions involved in lantibiotic biosynthesis, as well as for applications of the modification enzymes.

Oxidative decarboxylation of the C terminus to an aminoethiol residue is carried out by the flavoprotein MrsD [74]. Like EpiD it belongs to a family of homooligomeric flavin-containing Cys-decarboxylases (HFCD family). In contrast to EpiD, MrsD is a FAD and not an FMN-dependent flavoprotein and the two enzymes have different substrate specificities i.e. MrsD doesn't act on epidermin. This group of enzymes are characterised by a conserved histidine residue that forms part of the active site. Exchange of this histidine residue for an asparagine led to inactivation of MrsD [74].

The lantibiotic sublancin 168 produced by *Bacillus subtilis* is unusual in that it contains two disulphide bonds. It has been shown that thiol-disulphide oxidoreductases are required for disulphide bond formation in proteins that are exported from the cytoplasm. *Bacillus subtilis* has four such enzymes, BdbA, BdbB, BdbC, and BdbD. The *bdbA* and *bdbB* genes are located in one operon that also contains the genes specifying the lantibiotic sublancin 168 and the ATP-binding cassette transporter SunT. It was found that SunT and BdbB, but not BdbA, are required for the production of active sublancin 168. Furthermore, the BdbB paralogue BdbC is at least partly able to replace BdbB in sublancin 168 production [33].

## CONCLUSION

The potential benefits of applying lantibiotics for medical use have already been demonstrated. In addition lantibiotics can also provide additional medical and industrial benefits through the inhibition of pathogens and food-spoilage organisms in food [24, 95, 115]. In this respect, these peptides can frequently be produced relatively inexpensively in industrial quantities. While nisin remains the only lantibiotic that is commercially produced, it is likely that the emergence of antibiotic-resistant pathogens will necessitate the use of alternate antimicrobials and given the activity of specific lantibiotics against such strains this group of peptides may provide at least part of the solution. The application of lantibiotics will benefit greatly from continuing research that is providing us with answers to the many questions associated with lantibiotics. For example, (i) a target has been identified in a number of instances giving us a better understanding of the mode of action of the associated peptides, (ii) the structures of an ever increasing number of lantibiotics has been elucidated and mutagenesis strategies have given us an insight into the importance of specific sites and residues while (iii) the enzymes involved in modification of lantibiotics are now receiving greater

attention to the extent that it may ultimately be possible to produce these lantibiotics on a large scale. Moreover, *in vivo* and *in vitro* based systems for bioengineering of novel lantibiotic structures offers tremendous potential for the development of novel more efficacious antimicrobials for biomedical applications in the future. It would thus seem to be potentially the beginning of a golden-era in lantibiotic research which may have multiple and wide-ranging benefits.

## NOTES ADDED IN PROOF

It has recently been established that the combined region of pyrophosphate, MurNAc and the first isoprene of lipid II represent the target site for nisin binding [Hsu, S.T., Breukink, E., Tischenko, E., Lutters, M. A., de Kruijff, B., Kaptein, R., Bonvin, A.M., van Nuland, N.A. (2004) *Nat. Struct. Mol. Biol.* 11, 963-967.].

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