# Biosynthesis and Mode of Action of Lantibiotics

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## 1. Introduction

Lantibiotics are peptide-derived antimicrobial agents that are ribosomally synthesized and posttranslationally modified to their biologically active forms. The name lantibiotics was introduced in 1988 as an abbreviation for lanthionine-containing antibiotic peptides.[1] The unusual amino acid lanthionine consists of two alanine residues cross-linked via a thioether linkage that connects their $\beta$-carbons (*S*-(alaninyl-3-yl)-cysteine) (Figure 1). These residues are the unifying structural motif present in all lantibiotics. Horn and co-workers reported the first isolation of this thioether-cross-linked amino acid from the treatment of wool with sodium carbonate and introduced the name lanthionine (Latin, *lana* = wool).[2] In all natural lantibiotics, the lanthionines are believed to have the *meso*-stereochemistry (Lan, Figure 1),[3] although this has only been rigorously established for a subset of known lantibiotics including nisin,[4] subtilin,[5] epidermin,[6] Pep5,[7,8] cinnamycin,[9–11] ancovenin,[12,13] actagardine,[14,15] and mersacidin,[16] and the *meso* stereochemistry is generally assumed for other family members.

Lantibiotics are produced by a large number of Gram-positive bacteria and have their lanthionines imbedded within cyclic peptides. They usually also contain a methyl-substituted lanthionine derivative, (2*S*,3*S*,6*R*)-3-methyllanthionine[17] (MeLan, Figure 1) and typically (but not always)[18] contain the unsaturated amino acids 2,3-didehydroalanine (Dha) and (*Z*)-2,3-didehydrobutyrine (Dhb).[19] Less frequently encountered posttranslationally introduced structures are lysinoalanine, $\beta$-hydroxy-aspartate, D-alanine, 2-oxobutyrate, 2-oxopropionate (pyruvate), 2-hydroxypropionate (lactate), *S*-aminovinyl-D-cysteine, and *S*-aminovinyl D-methylcysteine (Figure 1), and it is possible that other modifications remain to be discovered.

The widespread application of the prototype lantibiotic nisin (Figure 2) as a safe alternative for chemical reagents in food preservation (>80 countries for over 40 years)[20–22] spurred a rapid expansion of research activities directed at understanding lantibiotic biogenesis. Early investigations showed that their production by Gram-positive bacteria was sus-

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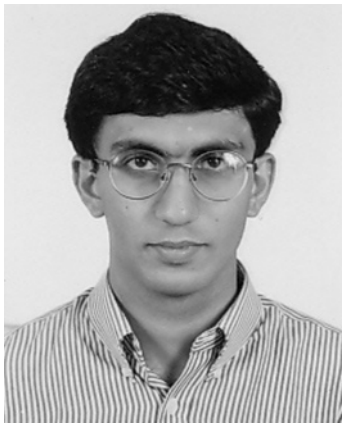
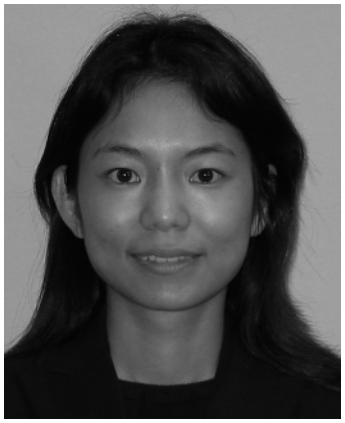


Champak Chatterjee was born in Calcutta, India. He received a B.S. in Chemistry in 1997 from the University of Bombay. After receiving an M.S. from I. I. T. Bombay in 1999 working with Professor Sambasivarao Kotha, he moved to the University of Illinois at Urbana-Champaign and joined the laboratories of Professor Wilfred A. van der Donk. He has been engaged in studying the mechanism and substrate specificity of the lacticin 481 synthetase LctM, and the protease involved in lacticin 481 maturation.



Lili Xie was born in China in 1976. She graduated from the University of Science and Technology of China (USTC) with a B.S. degree in Chemical Physics in 1998. She received her Ph.D. degree from the University of Illinois at Urbana-Champaign in 2003 under the supervision of Professor Wilfred van der Donk, where she successfully developed the first in vitro reconstitution of lantibiotic biosynthesis. Currently, she is carrying out her postdoctoral research with Professor Jon Clardy at Harvard Medical School.

Moushumi Paul was born in Mississippi and raised in Philadelphia, PA. She earned a B.A. in Chemistry from Barnard College in 1999 and is currently in the final stages of completing her Ph.D. in chemistry under the guidance of Prof. Wilfred van der Donk at the University of Illinois, Urbana-Champaign. Her doctoral work has focused on understanding the enzymes involved in lantibiotic biosynthesis.

Wilfred van der Donk received his B.S. and M.S. from Leiden University, The Netherlands, under the guidance of Prof. Jan Reedijk and his Ph.D. from Rice University in the laboratories of Prof. Kevin Burgess. After postdoctoral work with Prof. JoAnne Stubbe at MIT, he joined the faculty at the University of Illinois at Urbana-Champaign in 1997. The research in his laboratory focuses on understanding of the molecular mechanisms of enzyme catalysis and the use of enzymes in organic chemistry.

ceptible to inhibition by compounds that disrupt protein biosynthesis,[22] suggesting that lantibiotics are ribosomally synthesized as precursor peptides (prepeptides), which subsequently undergo post-translational modifications.[23,24] As such, they are considered members of the bacteriocins.[25] To distinguish lantibiotics from other bacteriocins that are not posttranslationally modified, they have been designated as a separate subgroup, the class I bacteriocins (Table 1). The biosynthesis of lantibiotics also distinguishes them from "classical" nongene encoded peptide antibiotics such as gramicidin, which are produced by modular nonribosomal peptide synthetases (NRPS).[26−30]

Nisin, the most studied lantibiotic, is produced by *Lactococcus lactis* and has been used extensively as a food preservative without substantial development of bacterial resistance.[20] Discovered in 1928,[31,32] one year prior to penicillin,[33] the compound is one of the oldest known antibacterial agents but its structure was not determined until elegant landmark studies by Gross and Morell in 1971 (Figure 2).[4] Around the same time as the structure elucidation studies, Ingram proposed that the dehydro amino acids in lantibiotics are the result of dehydration of serine and threonine residues to produce Dha and Dhb structures, respectively, and that the lanthionine and methyllanthionine rings are generated by intramolecular conjugate additions of cysteines to these $\alpha,\beta$-unsaturated amino acids.[35,36] This hypothesis was confirmed when the first gene clusters responsible for the biosynthesis of a number of lantibiotics were sequenced in the late 1980s.[1,37−39] In these studies, the genes encoding the precursor peptides for epidermin,[1] subtilin,[37,38] and nisin[39] were shown to contain codons for Ser, Thr, and Cys residues at the sites of posttranslational modifications. Only very recently has the biosynthesis of a lantibiotic (lacticin 481) been reconstituted in vitro,[40] which has provided further support for Ingram's hypothesis.

Nisin is active at low concentrations (MICs low nanomolar) against many strains of Gram-positive bacteria,[20] including drug resistant strains[41] and the

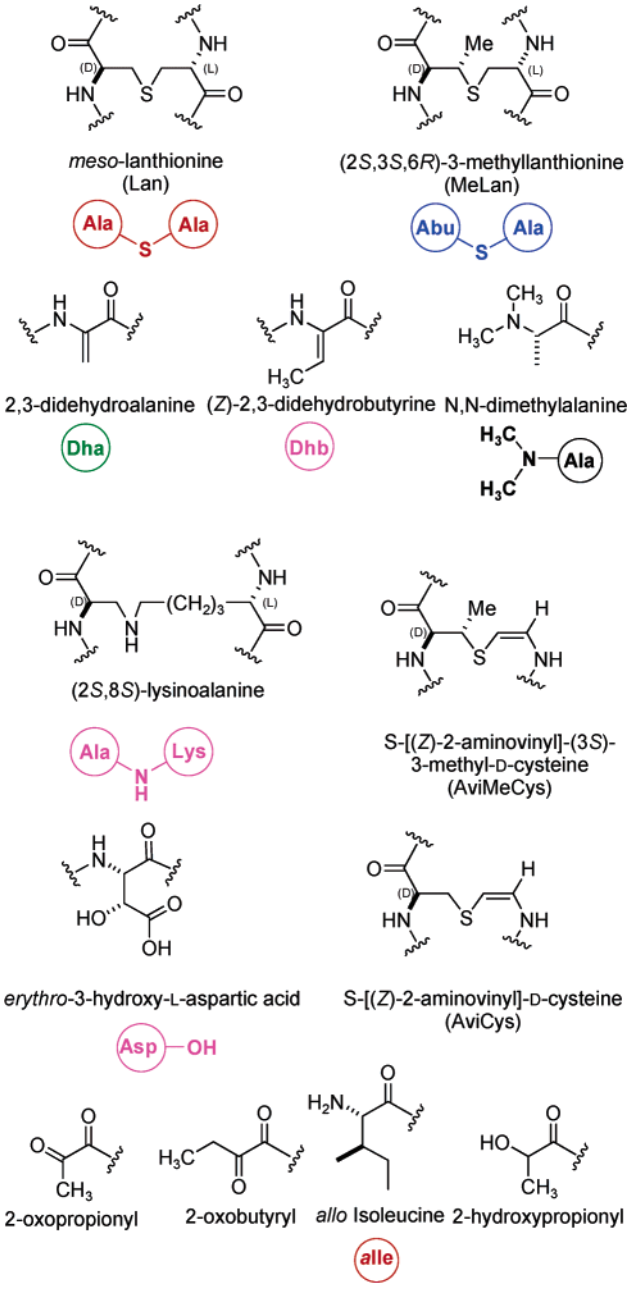



**Figure 1.** Structural motifs that are introduced into lantibiotics by posttranslational modifications. A shorthand notation for these modification that will be used elsewhere in this review is presented underneath several structures.

food-borne pathogens *Clostridium botulinum* and *Listeria monocytogenes*.[42−45] With an estimated 76 million cases of food-related illness in the United States each year,[46] translating into a cost of between $6.5 and 34.9 billion in 1997,[47] research into the modes of action and biosynthesis of nisin has increased dramatically in the past decade culminating in the demonstration that the cell wall biosynthetic intermediate lipid II constitutes its specific target.[41,48−54] Other lantibiotics show different interesting biological activities. These include high potency of epidermin against *Propionibacterium acnes*,[55] which may be exploited for topical treatment of acne, inhibition of phospholipase $A_2$ by cinnamycin and duramycin,[56,57] and inhibition of angiotensin converting

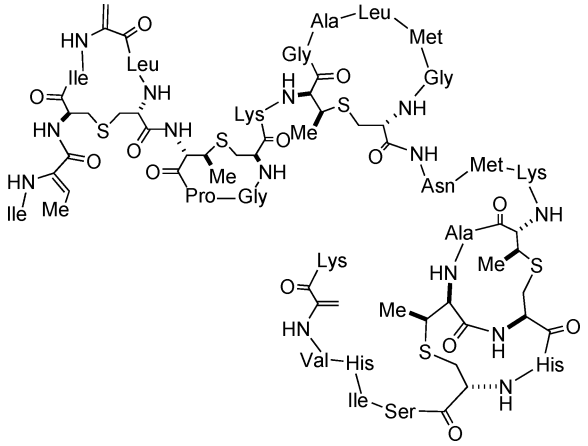


**Figure 2.** The structure of nisin A.

enzyme by ancovenin.[58] Moreover, mersacidin exhibits comparable antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) as vancomycin without showing any cross-resistance,[59−61] and mutacin B-Ny266 displays activities comparable to vancomycin and oxacillin against many strains and remains active against vancomycin-resistant strains.[62] We will not cover in this review the extensive studies dealing with practical applications of lantibiotics and refer the reader to various excellent recent reviews.[20,55,63,64] Instead, this work will focus on the available information regarding the mechanisms of biosynthesis and mode of action of this intriguing class of compounds.

## 2. Gene Organization

Similar to most biosynthetic pathways in bacteria, the genes for lantibiotic biosynthesis are clustered and have been designated the generic locus symbol *lan*, with a more specific genotypic designation for each lantibiotic member (e.g., *nis* for nisin, *gdm* for gallidermin, *cin* for cinnamycin). They may be found on conjugative transposable elements (e.g., nisin), on the chromosome of the host (e.g., subtilin), or on plasmids (e.g., epidermin, lacticin 481). Many of the *lan* genes have been sequenced in the past 15 years.[23,65,66] These studies have demonstrated a high level of similarity in the gene organization for production of the various compounds. The gene clusters for the biosynthesis of a select number of lantibiotics are depicted in Figure 3.[67] Although the gene order, complexity, and transcriptional organization of the various clusters differ, three genes have been identified that are involved in the biosynthesis of all type AII and type B lantibiotics (*lanAMT*), and four genes are present in all type AI lantibiotics gene clusters (*lanABCT*) (blue-colored genes, Figure 3. For a description of the type A and B classification, see section 3). These essential genes obviously include the structural genes encoding the precursor peptides for posttranslational maturation (prepeptides), which have been designated *lanA*, except for subtilin whose structural gene historically is named *spaS*. The *lanA* genes produce prepeptides that have an extension of 23−59 amino acids at their N-terminus compared to the mature lantibiotic product, which provided the first indication that an N-terminal leader peptide is

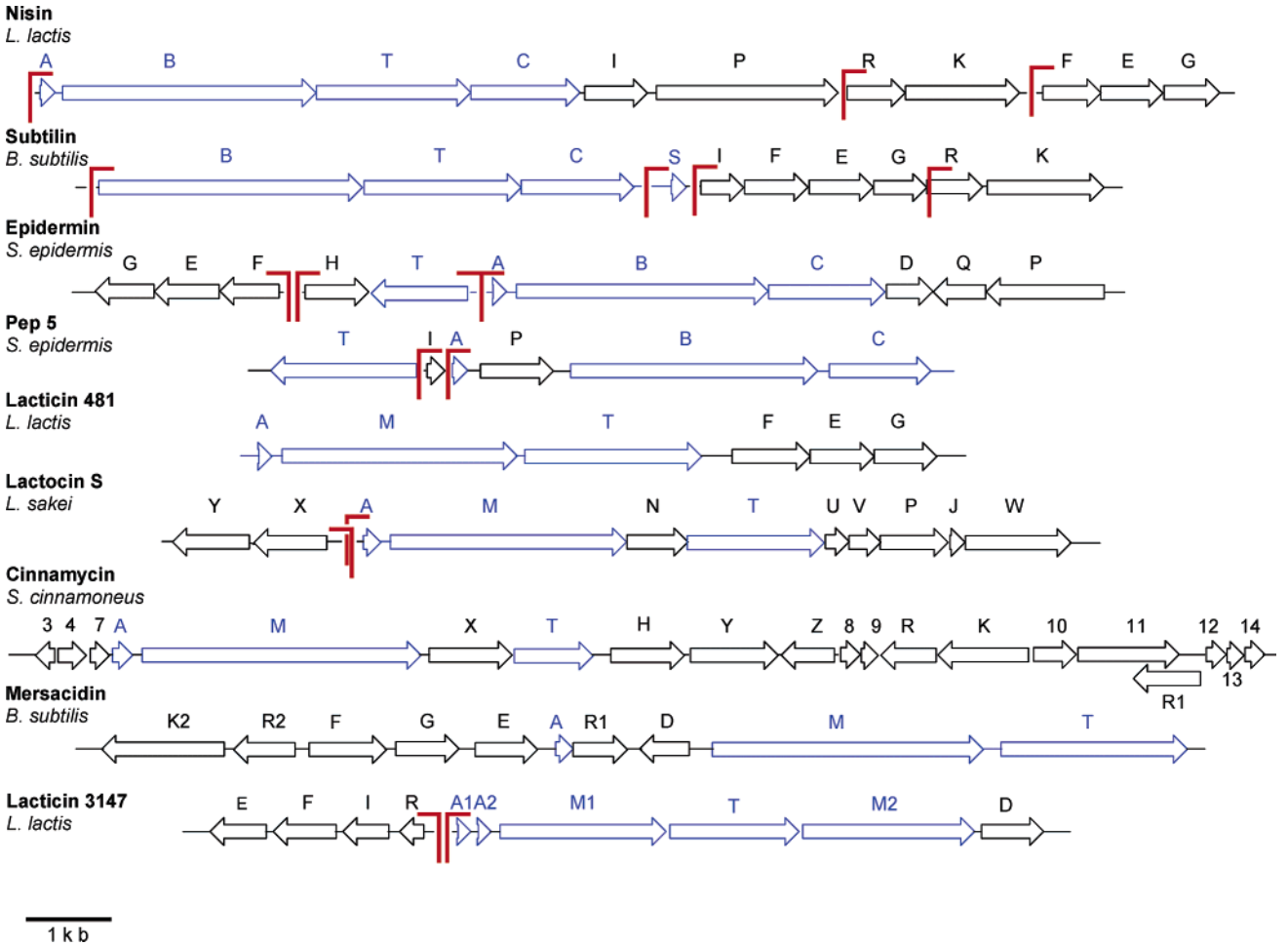



**Figure 3.** Representative biosynthetic gene clusters of the lantibiotics nisin,[214,259,305] subtilin,[68,73,211] epidermin,[76] Pep5,[75] lacticin 481,[148] lactocin S,[155] cinnamycin,[78] mersacidin,[262] and lacticin 3147.[227] In blue are those genes that are present in all known members with the LanB and LanC genes substituted by one LanM gene in some cases. Promoters for the transcriptional units in these clusters (where known) are indicated by red wedges.[23,75,76,155,211,260,268,272,289,294,295,329,467,468]

**Table 1. Classification of Bacterial Antimicrobial Peptides (Bacteriocins)[25,34]**

| class | characteristics | size | subclasses | example |
|---|---|---|---|---|
| Class I | posttranslationally modified peptides containing (methyl)lanthionines (lantibiotics) | < 5 kDa | Type A: elongated shape | nisin |
| | | | Type B: globular shape | mersacidin |
| Class II | heat-stable peptides of 37−58 amino acids; leader peptide removed during maturation | <10 kDa | Type IIa: N-terminal consensus YGNGVXC, *Listeria*-active, contain 1−2 disulfides | leucocin A |
| | | | Type IIb: two-peptide systems | lactococcin G |
| Class III | heat labile, large proteins | >30 kDa | | helveticin J |

important in lantibiotic biosynthesis. As mentioned in the introduction, sequencing of the *lanA* genes also indicated that Ser and Thr residues are the precursors to Dha and Dhb structures found in the final products, and that Ser+Cys and Thr+Cys residues are the precursors to the formation of the characteristic Lan and MeLan structures, respectively.

For the type AI lantibiotics, two genes, *lanB* and *lanC* code for proteins that have no similarity with any other entries in the databases.[68−70] They are believed to be required for the dehydration of Ser and Thr to Dha and Dhb, respectively (section 4.2), and the conjugate addition of Cys residues to these dehydro amino acids (section 4.3) to form Lan and MeLan (e.g., Figure 4 for nisin). In the type AII and type B lantibiotics, the *lanBC* genes are not present and instead a single gene (*lanM*) producing a protein with sequence homology at its C-terminus with the LanC proteins is found. No homology can be detected between the LanM and LanB proteins, indicating the *lanM* genes do not originate from a gene fusion event.[66] It was initially postulated, and recently confirmed both in vivo and in vitro,[40,71] that the *lanM* products carry out both the dehydration and cyclization reactions (section 4.4). It is very interesting that the operons for type AII lantibiotics as well as the sequences of their leader peptides have very strong similarity to those involved in the biosynthesis of

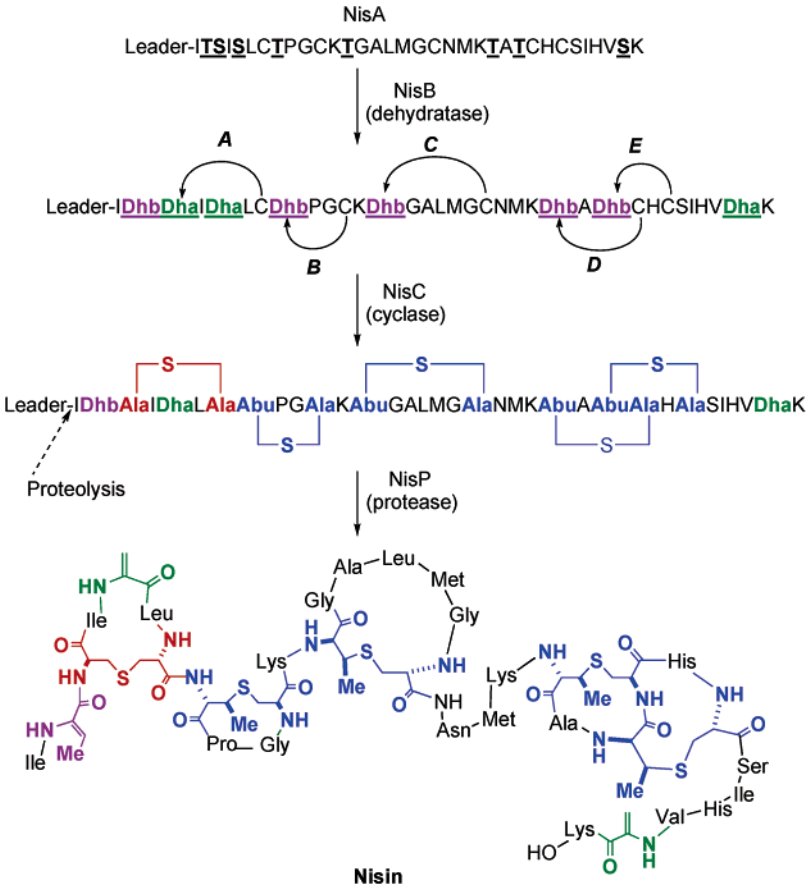



**Figure 4.** Representative example of the posttranslational maturation process of lantibiotics. The prepeptide NisA is ribosomally synthesized, followed by NisB catalyzed dehydration of underlined Ser and Thr residues in the propeptide region of NisA. NisC catalyzes the conjugate addition of Cys residues in a regio- and stereospecific manner to five of the Dha (green) and Dhb (purple) residues to generate five cyclic thioethers: one lanthionine (red) and four methyllanthionines (blue). It should be emphasized that although it is generally assumed that LanB proteins complete their dehydration of the targeted Ser and Thr residues before LanC proteins catalyze the cyclizations, at present it cannot be ruled out that the two proteins pass the substrate between them such that LanB dehydrates one Ser/Thr followed by LanC catalyzed ring formation before LanB dehydrates the next Ser/Thr. After dehydration/cyclization is complete, the leader peptide is proteolytically removed by the protease NisP. Sequence of the leader peptide: MSTKDFNLDLVSVSKKDSGASPR.

nonlanthionine-containing bacteriocins (class II, Table 1) except for the addition of the *lanM* gene. This may indicate that the recruitment of this single gene resulted in the transformation of class II bacteriocin producing bacteria to lantibiotic producing organisms.

An ATP-binding cassette (ABC) transport system (*lanT*) is found in all lantibiotic gene clusters except for epicidin 280.[72] The LanT proteins are responsible for secretion of either the final mature product or the posttranslationally modified product still attached to its leader sequence (section 4.7). Gene disruption and heterologous expression studies have shown that these transport systems are absolutely required in some cases (e.g., subtilin[73] and nisin[74]), but that alternative transport systems can substitute during biosynthesis of other lantibiotics (e.g., Pep5[75] and epidermin[69,76]).

Although all lantibiotics require proteolytic removal of the leader peptide, genes encoding the proteases are not always located in the biosynthetic gene clusters, suggesting that other cellular proteases can fulfill this role (e.g., subtilin[77] and cinnamycin).[78] When genes for dedicated proteases are found in the biosynthetic operons, they have been designated *lanP* and code for subtilisin-type serine proteases (section 4.7). Some clusters lacking a *lanP* gene have *lanT* genes with an N-terminal protease domain fused to the ABC-type transporter, similar to the transport systems of class II bacteriocins.[79] These protease domains appear to be cysteine proteases by sequence comparison with LagD, a transport-proteolysis system involved in the biosynthesis of the nonlantibiotic class IIa bacteriocin lactococcin G.[79] In addition to the transport system that excretes the lantibiotic, several gene clusters contain a second transport system comprised of three genes (*lanEFG*) that has been implicated in self-immunity in lantibiotic-producing strains that contain them (section 6). In addition to these transport proteins, a protein encoded by *lanI* is also believed to be involved in self-protection for some family members. Finally, two often found regulatory genes (*lanKR*) are important for regulation of lantibiotic production and comprise a two-component sensory system (section 5). A schematic representation of the overall process of lantibiotic biosynthesis is depicted for subtilin as a representative example in Figure 5.

As depicted in Figure 3, other genes are found in certain gene clusters, and they are in many cases

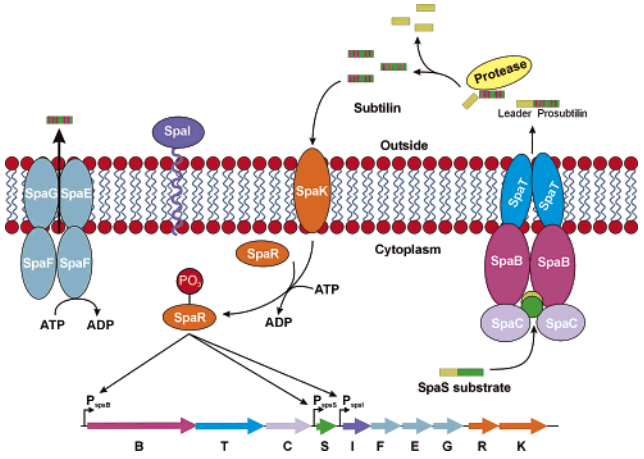



**Figure 5.** Schematic representation of the process of subtilin biosynthesis. Subtilin serves as the ligand for the receptor kinase SpaK,[272] which upon sensing subtilin first autophosphorylates a His residue and subsequently transfers the phosphate to an Asp residue on SpaR.[261] After phosphorylation, this transcription factor regulates the expression of three transcriptional units (*spaS*, *spaBTC*, and *spaIFEG*) involved in biosynthesis of and self-immunity against subtilin.[272] The SpaS substrate is acted upon by a membrane-associated multi-enzyme complex.[216] The biosynthetic enzymes SpaB and SpaC introduce the dehydro amino acids and (Me)Lan residues, respectively, and the modified peptide is secreted in an ATP-dependent manner by SpaT. Outside of the cell proteases remove the leader peptide generating mature subtilin.[77] SpaEFG constitute another ABC-type transporter believed to be important for immunity and SpaI is also involved in self-protection.[319]

involved in additional, less frequently encountered posttranslational modifications discussed in sections 4.5 and 4.6. A dramatic example was recently reported for cinnamycin with no less than 21 likely open reading frames (*orfs*) in the vicinity of the *cinA* structural gene (Figure 3).[78] This lantibiotic contains the highly unusual lysinoalanine residue as well as the unique $\beta$-hydroxy aspartate (Figure 6), and some of these unassigned *orfs* may be involved in their biogenesis as they have no similarities to known proteins.

## 3. Structures of Lantibiotics

At present about 40 different lantibiotics are known with varying structure, size, and mode of action. A representative collection is depicted in Figure 6 illustrating the high level of posttranslational modifications that typically amount to structural changes to about one-third of all amino acids in the peptide. The lantibiotics were classified by Jung as type A or B, based on the topology of their ring structures and their biological activities (Table 2).[80] The type A lantibiotics, with nisin as the prototype, exist as elongated amphipathic screw-shaped structures in solution, varying in length from 20 to 34 residues and bearing a net positive charge. Initially, their bactericidal action was believed to predominantly involve the formation of short-lived pores in cell membranes. More recently, a growing number of lantibiotics have been shown to interfere with peptidoglycan biosynthesis by binding to lipid II, but this activity is not confined to the type A lantibiotics (section 8). Type B lantibiotics such as cinnamycin and mersacidin are more globular and compact in structure (Figure 6), and they generally have no net charge or a negative charge at pH 7. A further subdivision within the type A lantibiotics is based upon the modification enzymes involved in their biosynthesis. The type A lantibiotics in which the Lan and MeLan residues are formed by the action of two distinct enzymes (LanB and LanC) are classified as type AI, whereas those that are formed by a single enzyme (LanM) are termed type AII (Table 2).[81] The structures and unique modifications present in representative examples from seven subgroups, each of which is likely derived from a common ancestor, will be discussed in this section (for an alternative classification scheme based on genetic data, see section 4.1).

### 3.1. Type AI Lantibiotics: Nisin Group

#### 3.1.1. Primary Structure

Nisin is produced by *L. lactis*,[38] and as mentioned previously, has been used as a preservative in the food industry for over 40 years without the appearance of significant bacterial resistance. The efforts to understand the molecular basis of its action have rendered it the most extensively studied lantibiotic.[82] The two common forms of nisin are nisin A and Z, which differ by a single amino acid at position 27, which is His in nisin A and Asn in nisin Z.[83,84] Recently, another natural variant, nisin Q, has been isolated from *L. lactis 61−14* that differs at four positions (Val15, Leu21, Asn27, and Val30) from nisin A (Ala15, Met21, His27, and Ile30).[85] The name nisin is derived from Lancefield Group N inhibitory substance, the initial classification of the compound.[86] The structure of nisin A was worked out by Gross in 1971[4] and later confirmed by genetic analysis of the prepeptide[38] and the landmark total synthesis by Shiba and co-workers.[87] Nisin contains three dehydrated amino acids and five thioether rings, which are not amenable to amino acid analysis by Edman sequencing. Gross was able to overcome this impediment by reductive desulfurization of the rings with Raney nickel to yield D- and L-Ala in the case of *meso*-lanthionine, and D-α-aminobutyric acid and L-Ala in the case of MeLan, which were identified by sequencing before or after proteolysis of the peptide. The positions of Lan and MeLan were identified by performic acid oxidation of the thioether linkages to the corresponding sulfones, which upon prolonged heating in sodium bisulfite underwent $\beta$-elimination to generate the α,$\beta$-didehydro amino acids. Subsequent addition of bisulfite generated sulfonic acid derivatives that could be identified by sequencing.[121] Dha and Dhb residues also interfere with Edman sequencing (and amino acid analysis) by the formation of a pyruvyl group under hydrolytic conditions.[122] Hence, these groups were hydrogenated or treated with thiol reagents prior to sequencing. The *S*-configuration at the $\beta$-position of MeLan was established by comparison of retention times during cation exchange chromatography with authentic samples.[17] Interestingly, in the 30 odd years since the initial determination of their stereochemistry, no reports

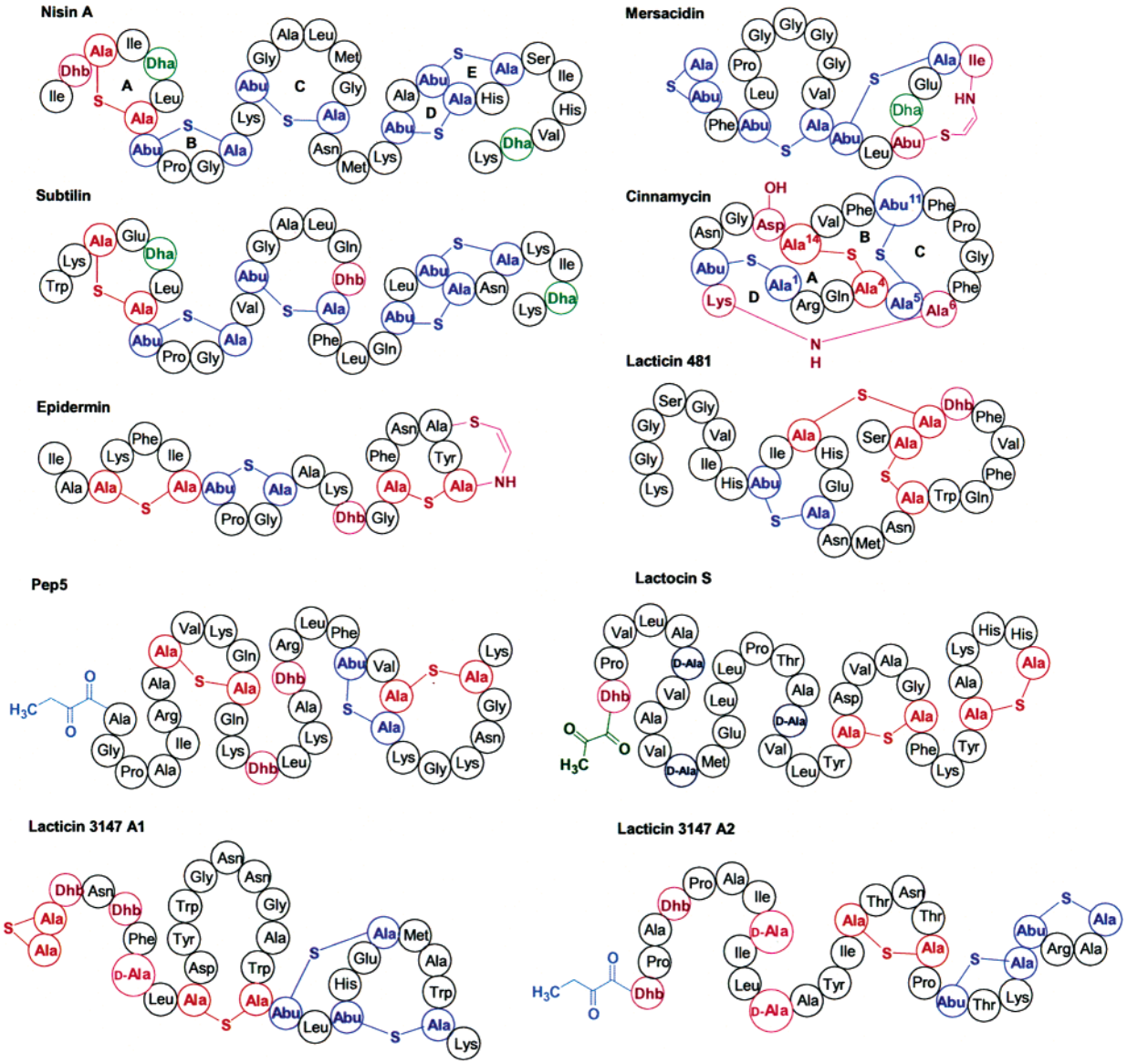



**Figure 6.** Representative structures of some lantibiotics using the shorthand notation and color coding defined in Figure 1. The ring numbering is shown for nisin and cinnamycin and is typically alphabetical from the N- to C-terminus.

have suggested the occurrence of other diastereomers of Lan/MeLan in lantibiotic isolates. Although this may be pending a more thorough characterization of various more recent isolates, a strong intrinsic preference for the natural diastereomers has been shown in the nonenzymatic biomimetic cyclization of the B-rings of subtilin and nisin (section 4.3).[123−126]

Subtilin is produced by *Bacillus subtilis* ATCC 6633[37] and is structurally closely related to nisin, showing 63% sequence identity including one Lan and four MeLan rings of identical size and position along the peptide chain (Figure 6). In addition, both contain Dha residues at position 5 and as their penultimate amino acid. The structure of subtilin was determined by Gross[5] and confirmed by two-dimensional NMR methods by Roberts and co-workers.[127] A natural variant of subtilin that is succinylated on the N-terminus, [N$^{\alpha}$-succinyl-Trp$^{1}$]-subtilin, has been identified in the culture broth of *B. subtilis* ATCC 6633 and found to have reduced antibacterial activity compared to subtilin (MICs against *Micrococcus luteus* are 0.05 and 0.34 $\mu$g/mL, respectively).[128]

Ericin A and S[88] whose precursor peptides have high identity with the subtilin precursor (75 and 92%, respectively) have been classified as type AI lantibiotics. A putative thioether bridging pattern has been proposed based on the results of Edman sequencing, digestion with peptidases, mass spectral analysis, and the similarity in the placement of Ser, Thr, and Cys residues with those of subtilin. Streptin isolated from *Streptococcus pyogenes*[129] is another type AI lantibiotic, the structure of which has been proposed based on its similarity to nisin.[89]

### 3.1.2. Three-Dimensional Structure

The solution structure of nisin has been determined using high-resolution NMR spectroscopy both in aqueous solution and in the presence of dodecylphosphocholine (DPC) and sodium dodecyl sulfate (SDS) micelles that mimic the cellular membrane.[130−132] These studies revealed an overall extended conformation and the presence of two amphipathic screw-shaped domains consisting of the N-terminal A-, B-, and C-rings, and the C-terminal fused rings D and

**Table 2. Lantibiotics Isolated as of June 2004**

| lantibiotic | producer strain | ref | Lan | MeLan | Dha | Dhb | amino acids |
|---|---|---|---|---|---|---|---|
| | | **Type A (I)** | | | | | |
| Nisin A[a] | *Lactococcus lactis* ATCC 11454 | 4 | 1 | 4 | 2 | 1 | 34 |
| Nisin Z[a] | *Lactococcus lactis* N8, NIZO22186 | 83 | 1 | 4 | 2 | 1 | 34 |
| Subtilin[a] | *Bacillus subtilis* ATCC6633 | 5 | 1 | 4 | 2 | 1 | 32 |
| Ericin S[b] | *Bacillus subtilis* A1/3 | 88 | 1 | 4 | 2 | 1 | 32 |
| Ericin A[b] | *Bacillus subtilis* A1/3 | 88 | 1 | 4 | 1 | 0 | 29 |
| Streptin[b] | *Streptococcus pyogenes* BL-T, M25 | 89 | 2 | 1 | 0 | 3 | 23 |
| Epidermin[a] | *Staphylococcus epidermis* Tü3298 | 6 | 2 | 1 | 0 | 1 | 22 |
| [Val1-Leu6]-epidermin[b] | *Staphylococcus epidermis* BN−V1, BN−V301 | 90 | 2 | 1 | 0 | 1 | 22 |
| Gallidermin[a] | *Staphylococcus gallinarium* Tü3928 | 91 | 2 | 1 | 0 | 1 | 22 |
| Mutacin 1140[a] | *Streptococcus mutans* JH1140 | 92 | 2 | 1 | 1 | 1 | 22 |
| Mutacin B−Ny266[b] | *Streptococcus mutans* Ny266 | 93 | 2 | 1 | 1 | 1 | 22 |
| Mutacin III[b] | *Streptococcus mutans* UA787 | 94 | 2 | 1 | 1 | 1 | 22 |
| Mutacin I[b] | *Streptococcus mutans* CH43 | 95 | 3 | 0 | 2 | 0 | 24 |
| Pep5[a] | *Staphylococcus epidermis* 5 | 7 | 2 | 1 | 0 | 2 | 34 |
| Epilancin K7[a] | *Staphylococcus epidermis* K7 | 96 | 2 | 1 | 2 | 2 | 31 |
| Epicidin 280[b] | *Staphylococcus epidermis* BN280 | 72 | 1 | 2 | 0 | 1 | 30 |
| | | **Type A(II)** | | | | | |
| Lacticin 481[a] | *Lactococcus lactis* CNRZ 481 | 97 | 2 | 1 | 0 | 1 | 27 |
| Variacin[b] | *Micrococcus varians* MCV8 | 98 | 2 | 1 | 0 | 1 | 25 |
| Mutacin II[a] | *Streptococcus mutans* T8 | 99 | 2 | 1 | 0 | 1 | 27 |
| StreptococcinA-FF22[a] | *Streptococcus pyogenes* FF22 | 100 | 1 | 2 | 0 | 1 | 26 |
| Salivaricin A[b] | *Streptococcus salivarius* 20P3 | 18 | 1 | 2 | 0 | 0 | 22 |
| [Lys2,Phe7]-salivaricin A[b] | *Streptococcus pyogenes* T11 (M type 4) | 101 | 1 | 2 | 0 | 0 | 22 |
| Lactocin S[b] | *Lactobacillus sakei* L45 | 102 | 2 | 0 | 0 | 1 | 37 |
| Cypemycin[a] | *Streptomyces OH-4156* | 103 | 0 | 0 | 0 | 4 | 22 |
| Plantaricin C[a] | *Lactobacillus plantarum* LL441 | 104 | 1 | 3 | 1 | 0 | 27 |
| Sublancin 168[b] | *Bacillus subtilis 168* | 105 | 0 | 1 | 1 | 0 | 37 |
| Butyrivibriocin OR79A[b] | *Butyrivibrio fibriosolvens* | 106 | 1 | 2 | 0 | 1 | 25 |
| | | **Type B** | | | | | |
| Cinnamycin[a] | *Streptomyces cinnamoneus* | 10,107 | 1 | 2 | 0 | 0 | 19 |
| Duramycin[a] | *Streptoverticillium hachijoense* DSM 40114 | 108 | 1 | 2 | 0 | 0 | 19 |
| Duramycin B[a] | *Streptoverticillium* R 2075 | 109,110 | 1 | 2 | 0 | 0 | 19 |
| Duramycin C[a] | *Streptomyces griseoluteus* R 2107 | 109,110 | 1 | 2 | 0 | 0 | 19 |
| Ancovenin[a] | *Streptomyces sp.* A647P-2 | 13 | 1 | 2 | 1 | 0 | 19 |
| Mersacidin[a] | *Bacillus sp. strain* HIL Y-85,54728 | 16 | 0 | 3 | 1 | 0 | 20 |
| Actagardine[a] | *Actinoplanes linguriae* ATCC 31048 | 14 | 1 | 2 | 0 | 0 | 19 |
| Ala(0)-actagardine[b] | *Actinoplanes linguriae* ATCC 31048 | 111 | 1 | 2 | 0 | 0 | 20 |
| | | **Two-Component Lantibiotics** | | | | | |
| Lacticin 3147A1[a] | *Lactococcus lactis* DPC3147 | 112 | 2 | 2 | 0 | 2 | 30 |
| Lacticin 3147A2[a] | *Lactococcus lactis* DPC3147 | | 1 | 2 | 0 | 2 | 29 |
| Staphylococcin C55α[b] | *Staphylococcus aureus* C55 | 113 | Lan/MeLan | 4 | Dha/Dhb | 3 | 30 |
| Staphylococcin C55β[b] | *Staphylococcus aureus* C55 | | Lan/MeLan | 3 | Dha/Dhb | 2 | 28 |
| Plantaricin Wα[b] | *Lactobacillus plantarum* LMG 2379 | 114 | 2 | 1 | 0 | 0 | 29 |
| Plantaricin Wβ[b] | *Lactobacillus plantarum* LMG 2379 | | 1 | 2 | 1 | 1 | 32 |
| Cytolysin $L_L$[b] | *Enterococcus faecalis* | 115 | Lan/MeLan | 2 | Dha/Dhb | 4 | 38 |
| Cytolysin $L_S$[b] | *Enterococcus faecalis* | | Lan/MeLan | 1 | Dha/Dhb | 4 | 21 |
| | | **Structures Not Yet Determined** | | | | | |
| Ruminococcin A | *Ruminococcus gnavus* E1 | 116 | | | | | |
| Carnocin UI 49 | *Carnobacterium piscicola* UI49 | 117 | | | | | |
| Macedocin | *Streptococcus macedonicus* ACA-DC 198 | 118 | | | | | |
| Bovicin HJ50 | *Streptococcus bovis* HJ50 | 119 | | | | | |
| Nukacin ISK-1 | *Staphylococcus warneri* ISK-1 | 120 | | | | | |
| SapB morphogen | *Streptomyces coelicolor* | 474 | | | | | |

[a] Structure established independently. [b] Proposed structure.

E that are joined by a flexible hinge region (residues 20−22, Asn-Met-Lys) as depicted in Figure 7. The secondary structure of subtilin was found to be similar to nisin.[127] The presence of a Dhb residue at position 18 in subtilin compared to Gly in nisin A did not significantly affect the conformational flexibility of the C-ring. In both compounds, the four-amino acid containing thioether rings are enforced to adopt a β-turn[131,133] (type I in rings B and C and type II and type II′ in rings D and E).[54] Although some regions of rigidity are present within the individual lanthionine rings, both nisin and subtilin were found to be overall flexible molecules.[127,130]

## 3.2. Type AI Lantibiotics: Epidermin Group

### 3.2.1. Primary Structure

The structure of the 22-residue lantibiotic epidermin was elucidated by Jung and co-workers in 1985.[6] Epidermin contains one Dhb, one MeLan, and two Lan residues besides the unusual ring structure containing *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) (Figures 1 and 6). Epidermin's structural elucidation was carried out by chemical and enzymatic fragmentation coupled with Raney-Ni catalyzed desulfurization, Edman sequencing, and FAB-MS analysis. The AviCys residue was characterized by its conversion

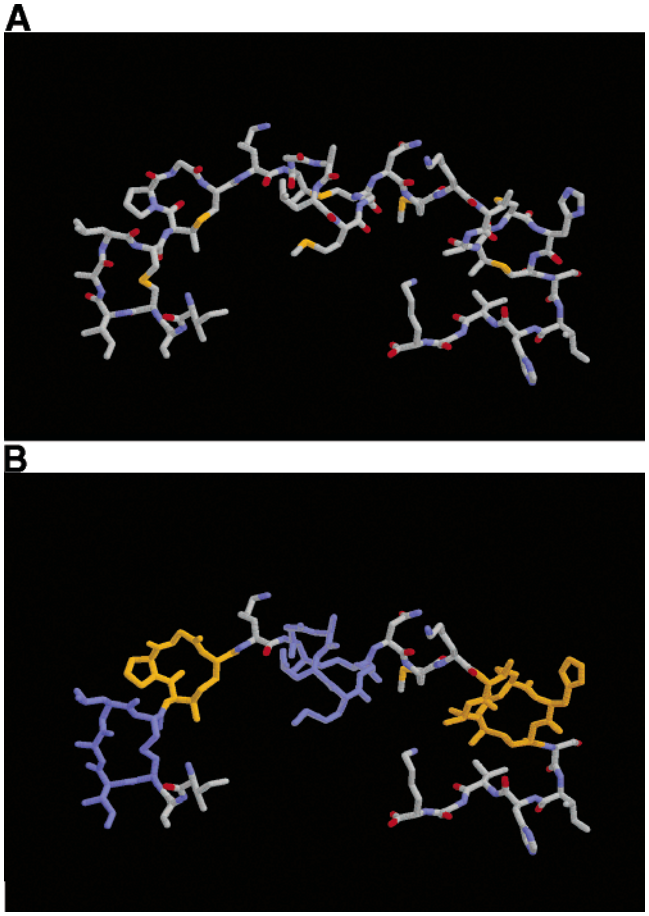



**Figure 7.** (A) One of the NMR structures of nisin in the presence of DPC micelles.[131] The molecule adopts a more or less extended conformation with the N- and C-termini somewhat curling back toward each other. The four amino acid rings B, D, and E all have enforced $\beta$-turn conformations, which is also adopted in a noncovalent fashion by residues 21−24. (B) Molecule shown from the same viewpoints as in panel A with the A-ring in blue, the B-ring in yellow, the C-ring in cyan, and the fused D- and E-rings in orange. Figures were generated using the program RASMOL.[469]

to *S*-[2-aminoethyl]-D-cysteine (thialysine) by reduction of epidermin with Pd/C and subsequent hydrolysis, and also by the formation of L-alanine-*N*-ethylamide upon Raney-Ni desulfurization of the C-terminal tryptic fragment. The absolute configuration of the Lan and MeLan residues was confirmed to be identical to that found in nisin by gas chromatography with a chiral stationary phase.

The natural epidermin variant gallidermin, produced by *Staphylococcus gallinarum*, differs from epidermin by a single amino acid, Leu6, which is Ile in epidermin.[91] The structural elucidation of the polypeptide revealed the presence of four thioether bridges identical to epidermin. Another natural variant of epidermin, initially named staphylococcin T, has been isolated from *Staphylococcus cohnii T*.[134] Amino acid analysis and DNA sequencing indicated that it is identical to gallidermin.

Mutacin 1140,[92,135] mutacin B-Ny266,[93] mutacin I,[95] and mutacin III[94] are other lantibiotics in the epidermin group that are all isolated from various strains of *Streptococcus mutans*. They bear close homology to each other and to epidermin. Mutacin III and mutacin 1140 were initially isolated, characterized, and named by different research groups but have identical structures that differ by two amino acids from mutacin B-Ny266, and share 77% identity with epidermin and 62.5% identity with mutacin I. All members of the epidermin group have the characteristic Lan ring between positions 3 and 7; however, only the mutacins have a Dha at position 5, which is also found in the nisin subgroup and has been implicated in its biological activity (section 8.3).[136−138]

#### *3.2.2. Three-Dimensional Structure*

In the presence of the structure inducing solvent trifluoroethanol (TFE) gallidermin adopts an extended amphiphilic screw-shape with a lipophilic C-terminus and positively charged hydrophilic N-terminus.[139,140] Flexibility in the fairly rigid peptide backbone is due to a hinge region from residues 12 to 15. The calculated average length of 30 Å and average diameter of 8−10 Å would allow the molecule to span the cell membrane once, which may be relevant for its pore forming activity. Obviously, multiple molecules would have to come together to generate the pore. The recent solution structure of mutacin 1140 as determined by Edison and co-workers in acetonitrile−water (80:20) retains the rigidity within the lanthionine rings that are seen in nisin and gallidermin as well as the flexibility of the hinge region.[141] The structure differs from that of gallidermin in being bent in the hinge region, giving it a horseshoe-like appearance.

### 3.3. Type AI Lantibiotics: Pep5 Group

#### *3.3.1. Primary Structure*

The representative lantibiotic Pep5 isolated from *S. epidermis 5*[142] is large (34 residues, 3488 Da) and basic in nature (pI > 10.5) due to the presence of eight positively charged amino acids. The structure of Pep5 was determined by Jung and co-workers[7] and contains two Dhb residues, two Lan, and one MeLan. The stereochemistry of the Lan and MeLan residues was identical to that in nisin and subtilin. Fragmentation of Pep5 by enzymatic cleavage with chymotrypsin and endoproteinase Arg-C gave rise to four fragments that were partially identified by Edman sequencing and FAB-MS. The N-terminus of Pep5 is blocked to sequencing, and $^{13}C$ NMR spectroscopy suggested the presence of a 2-oxobutyryl residue at this position. This was confirmed with the synthesis of an N-terminally 2-oxobutyrylated heptapeptide corresponding to the N-terminal proteolytic fragment of Pep5. The deduced structure was also consistent with the subsequently determined pre-Pep5 gene sequence that revealed a codon for Thr at position 1.[143] Presumably, this Thr at the N-terminus is converted to a Dhb during the maturation process, which spontaneously hydrolyzes to the observed 2-oxobutyryl structure. To eliminate the difficulty in Edman sequencing due to the presence of this residue and the other unusual amino acids in Pep5, Meyer and co-workers have outlined a noteworthy method of chemical derivatization.[144] They achieved the

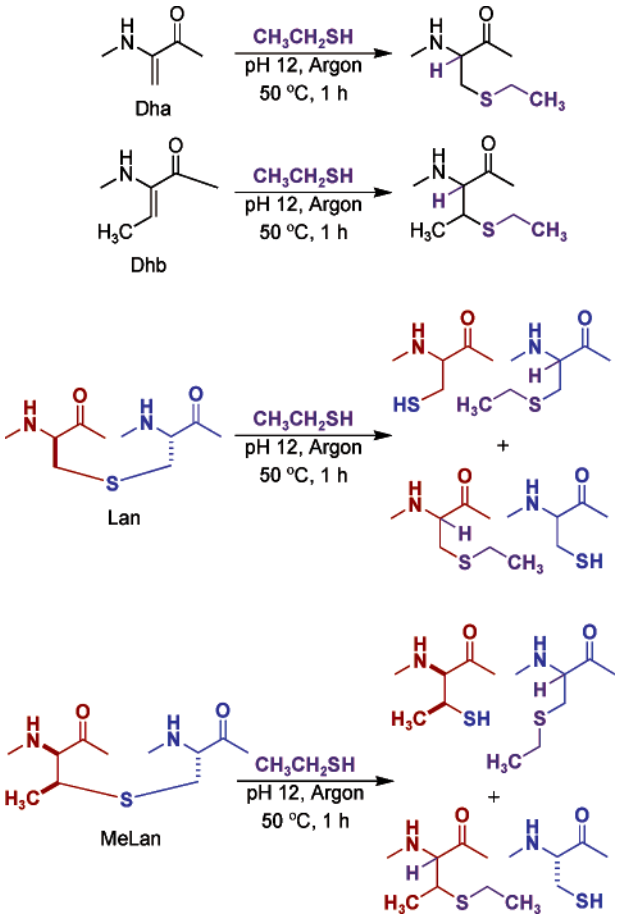



**Figure 8.** Modification of Dha, Dhb, Lan, and MeLan with ethanethiol to generate products that are amenable to Edman degradation.[144]

almost complete sequencing of Pep5 by the initial treatment with an alkaline mixture of ethanethiol that led to the opening of thioether rings and formation of cysteine and *S*-ethylcysteine from Lan, and $\beta$-methyl-*S*-ethylcysteine together with cysteine or $\beta$-methylcysteine and *S*-ethylcysteine from MeLan (Figure 8). This procedure was followed by an oxidative step with trifluoroperacetic acid that released propionic acid, $CO_2$, and freed the N-terminal amine of Pep5. This method was also shown to derivatize the AviCys residue in epidermin and gallidermin and allowed for the complete sequencing of the latter.

Epilancin K7, isolated from *S. epidermis* K7, is three residues shorter than Pep5 and contains two Dha residues that are absent in Pep5.[96] The structure of epilancin K7 was determined by extensive two-dimensional $^1$H-homonuclear and $^1$H,$^{13}$C-heteronuclear NMR spectroscopy as well as three-dimensional $^1$H-homonuclear NMR spectroscopy. An unprecedented 2-hydroxypropionyl (lactate) group was identified at the N-terminus and corresponds to a Ser residue in the ElkA prepeptide as determined by sequencing of the structural gene.[145] Like the oxobutyryl moiety in Pep5, this structure is probably the result of dehydration of the Ser residue followed by spontaneous hydrolysis of the N-terminal Dha residue. Unlike Pep5, the resulting ketone is subsequently reduced to the alcohol in a process for which currently the stereochemistry is not known. Epicidin 280 contains one Dhb, one Lan, and two MeLan residues.[72] It bears 75% identity in amino acid sequence to Pep5, which has led to the suggestion of a similar pattern of thioether bridging. Like epilancin K7, its N-terminus is blocked by the 2-hydroxypropionyl group.

### 3.3.2. Three-Dimensional Structure

The solution structure of Pep5 has been investigated in water and water−TFE mixtures by circular dichroism (CD) and NMR spectroscopy.[146] In CD studies, Pep5 presented a disordered, random coil type structure in water alone, while the addition of TFE was found to induce helicity. The helical nature of Pep5 was also seen in the presence of sodium dodecyl sulfate micelles that mimic the membrane environment. Thus, it was concluded that Pep5 remains in a disordered state in aqueous solution and adopts a (pore forming) helical shape in a lipophilic environment. NMR experiments in water echoed the mostly disordered state observed by CD. Similar to gallidermin, some rigidity was observed in the backbone of the thioether rings and residues immediately next to the Dhb. In the presence of TFE, the inflexibility observed in water was further extended as reflected in a higher number of backbone NH−NH cross-peaks. On the basis of these findings, some degree of helicity was inferred in the segment spanning residues 14−23, suggesting a partly rod-shaped structure in a membrane-like dielectric.

## 3.4. Type AII Lantibiotics: Lacticin 481 Group

### 3.4.1. Primary Structure

Lacticin 481[147] (also isolated as lactococcin DR)[148] was purified and partially sequenced by Piard and co-workers from *L. lactis CNRZ481*.[149] Two probable structures of lacticin 481 were proposed based on Edman degradation, amino acid analysis, NMR spectroscopy, and comparison with the predicted translational product of the *lctA* gene.[150] Lacticin 481 (ESI-MS 2901 Da) is 27 residues long with a high Gly content (11%), a high proportion of hydrophobic residues (75%), and no net charge at pH 7. It contains two Lan, one MeLan, and one Dhb residue. The precise location of the thioether bridges was determined by van de Hooven et al.[97] after cyanogen bromide digestion of lacticin 481, and analysis of the fragments by FAB-MS and NMR spectroscopy (Figure 9). The compact C-terminus, arising from overlapping rings, and the low homology of the prelacticin leader peptide with the prenisin and presubtilin leader sequences (section 4.1), led to a distinct classification of lacticin 481 and its analogues as type AII lantibiotics (Table 2). Mutacin II (3245 Da), a close relative of lacticin 481 (Figure 9), was isolated from *Streptococcus mutans T8* by Caufield and coworkers.[151] It contains 27 amino acids, including one MeLan, two Lan, and one Dhb residue.[151,152] The complete structure, including the bridging topology, was established by a combination of cyanogen bromide digestion, mass spectrometry, site-directed mutagenesis, and NMR spectroscopy (Figure 9).[99] Its primary structure agrees with the product of the *mutA* gene sequence.[153] The compound bears high

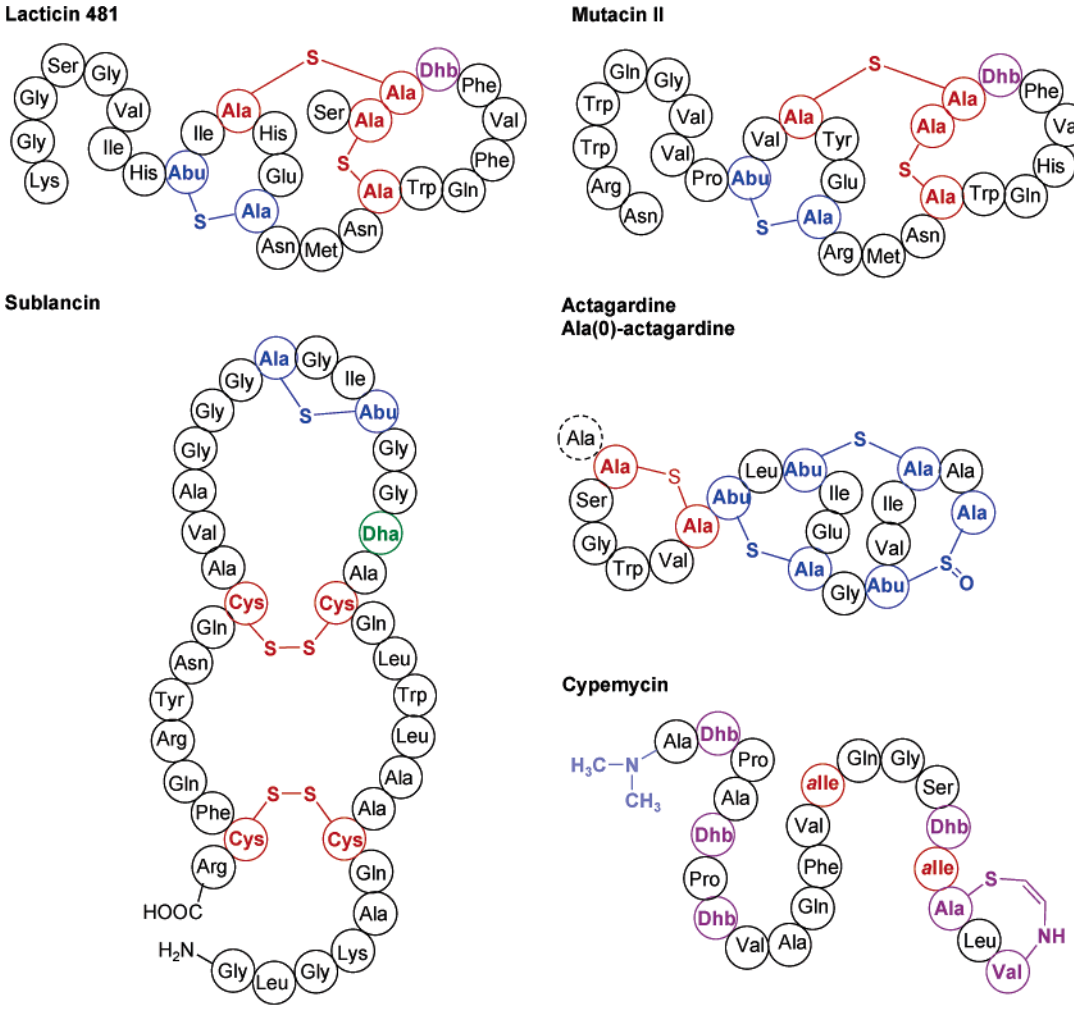



**Figure 9.** Structures of lacticin 481,[97] mutacin II,[99] sublancin 168,[105] actagardine,[14] and cypemycin.[103] The natural derivative Ala(0)-actagardine is shown as a dotted circle.

identity with lacticin 481 (59%), including an identically clustered ring structure and invariant position of a Dhb.

Lactocin S produced by *Lactobacillus sakei L45*[154] is a unique lantibiotic in that its structure contains three Ala of D-configuration.[102,155] The compound (3764 Da) consists of 37 residues, including two Lan and one Dhb residue. The N-terminus of lactocin S is blocked to Edman sequencing due to the presence of a 2-oxopropionyl group.[102] Its structure has been proposed but as yet has not been firmly established. StreptococcinA-FF22 (SA-FF22),[156] salivaricin A,[18] [Lys2,Phe7]-salivaricin A (salivaricin A1),[101,157] variacin,[98] plantaricin C,[158] and butyrivibriocin OR79A[106] are the other members of this family that share high homology, and a similar pattern of ring formation to that found in lacticin 481. At present, the ring structure has only been determined unequivocally for SA-FF22[100] and plantaricin C.[104] Salivaricin is an interesting case in that salivaricin A is active against strains of *S. pyogenes*, but use of *salA* from *Streptococcus salivarius* 20P3 as a DNA hybridization probe showed that no less than 63 out of 65 *S. pyogenes* strains tested contained a *salA* homologue.[101] Some of these strains were further investigated revealing that those strains in which the production of a salivaricin analogue was disrupted by deletions in the biosynthetic genes were sensitive to salivaricin A, whereas those that actually produced an analogue were not. One of the latter strains (*S. pyogenes* T11) was shown to produce the lantibiotic [Lys2,Phe7]-salivaricin A (salivaricin A1).[157]

Sublancin 168, produced by *B. subtilis 168*,[105] differs significantly from the other lantibiotics of this group due to the presence of two disulfide linkages, besides a MeLan and a Dha residue. The pattern of rings is also different from that of lacticin 481 (Figure 9), and it contains an as yet unknown modification of +164 Da.[105] Sublancin's inclusion with the lacticin subgroup results from the similarity of its leader peptide with other members of this group (section 4.1). Cypemycin[159] is an extraordinary lantibiotic containing four Dhb residues, one *allo*-Ile, one Avi-Cys, and an N-terminal *N*,*N*-dimethylalanine residue (Figure 9).[103] This molecule illustrates the range of posttranslational modifications that may take place during lantibiotic maturation.

### 3.4.2. Three-Dimensional Structure

The type AII lantibiotics are made up of a characteristic linear N-terminus and globular C-terminus. On the basis of NMR studies, the structure of mutacin II has been proposed to consist of an N-terminal amphipathic α-helix from residues 1−8,

with a hinge region around Pro9 separating it from the C-terminal Lan/MeLan rings.[160] The importance of this hinge region has been demonstrated by the mutation P9A, which leads to loss of antimicrobial activity.[161] CD studies conducted with SA-FF22 in water−TFE mixtures, 1% aqueous SDS, and in the presence of vesicles showed a change in secondary structure and suggested an ordered conformation different from that of the type AI lantibiotics Pep5 and gallidermin.[100] The solution structure of plantaricin C has been determined by NMR spectroscopy. It shows two distinct regions comprised of a positively charged, flexible N-terminus (residues 1−6) and a C-terminal rigid globular domain.[104]

## 3.5. Type B Lantibiotics: Mersacidin Group

### 3.5.1. Primary Structure

The representative lantibiotic from this group, mersacidin, was isolated from *Bacillus HIL Y-85,-54728*.[16,60] Its primary sequence was investigated by two-dimensional NMR spectroscopy, GC-MS analysis of the acid hydrolysate, and MS/MS studies of its desulfurized analogue.[162] Mersacidin is one of the smaller lantibiotics, 20 residues long (1825 Da), and contains three MeLan rings, one Dha, and the residue *S*-[(*Z*)-2-aminovinyl]-(3*S*)-3-methyl-D-cysteine, AviMeCys (Figure 1). The AviMeCys residue is presumably formed by the same oxidative decarboxylation mechanism as seen for the formation of AviCys in epidermin (section 4.5). The primary amino acid sequence of mersacidin was confirmed by cloning of the *mrsA* gene from the producing strain by Bierbaum et al.[61] In contrast to the type AI lantibiotics, mersacidin has no net charge.

Actagardine is a tetracyclic lantibiotic, 19 residues in length (1890 Da), and bears a single negative charge in neutral solution (Figure 9).[23,163] It has also been isolated under the name gardimycin.[164−167] The primary structure of this lantibiotic was first published by Kettenring and co-workers in 1990,[168] but Zimmermann and Jung proposed a revised structure in 1995 that is currently accepted.[14] Edman degradation and amino acid sequencing, before and after modification of actagardine with $\beta$-mercaptoethanol as per the protocol of Meyer et al. (i.e., Figure 8),[144] along with ESI-MS and multidimensional NMR experiments, established the presence of one Lan and three MeLan residues. Interestingly, the authors observed a major portion of actagardine to possess an unprecedented oxidized C-terminal MeLan residue (sulfoxide), while only a small amount corresponded to the non-oxidized actagardine.[14] A natural variant of actagardine, Ala(0)-actagardine, was isolated from *Actinoplanes linguriae* ATCC 31048 (Figure 9).[111] NMR spectroscopy, ESI-MS, Edman sequencing, and amino acid analysis indicated a primary sequence identical to actagardine with an additional N-terminal Ala residue. A synthetic Ala(0)-actagardine, prepared by coupling previously purified actagardine with Boc-protected *O*-*N*-hydroxysuccinimidyl alanine and subsequent deprotection of the N-terminus, had identical retention times during HPLC purification as the isolated wild-type compound.[111]

### 3.5.2. Three-Dimensional Structure

The activity of mersacidin[59] against the virulent strain *S. aureus* (see also Note Added in Proof) has produced efforts to uncover its key structural features in solution[169] and crystalline state.[170] The solution NMR structure in methanol showed three distinct structural domains formed by the thioether rings spanning residues 1−3, 4−12, and 13−20 (Figure 10).[169,171] The overall structure is globular with mostly neutral side-chains and only a few charged groups (Glu17 and the N-terminus) exposed to solvent. The glycine-rich sequence in domain II (residues 4−12) confers some conformational flexibility. Intramolecular hydrogen bonds were observed between domains I and III and within domain III that promote backfolding and rigidify the structure. The structure of mersacidin has been solved by X-ray crystallography using crystals obtained from a saturated solution of benzene and methanol.[170] This was the first and only example of an X-ray structure of a lantibiotic and showed good correspondence with the solution structure proposed by Griesinger and coworkers (vide supra) except for the glycine rich region in domain II.

Zimmerman and Jung determined the solution structure of actagardine in 1997 by two- and three-dimensional NMR spectroscopy in a water−acetonitrile mixture (3:7).[15] Actagardine has a compact globular structure comprised of two domains joined between residues 6 and 7 (Figure 11). The N-terminal domain consists of a single Lan ring, while the C-terminal domain is composed of three intertwisted MeLan rings. Residues 7−8, 9−12, and 17−19 form a small, three-stranded $\beta$-sheet with one antiparallel and two parallel strands that is not common to lantibiotic structures, and provides rigidity along with the thioether bridges. The fixed torsional angle between residues 6 and 7 and van der Waals interactions lead to an L-shaped planar arrangement of the two domains. Two putative binding pockets are present in actagardine. A hydrophilic pocket is made by Glu11 and Ser2 and the thioether bridges between residues 1−6 and 7−12. This resembles the sequence between residues 9 and 18 in mersacidin, and the similar position of the Glu residue suggests a role in the mode of action in both mersacidin and actagardine. The second pocket is formed by the backbone amide loop of residues 12−17 and the thioether bridge from residue 14−19 and may be involved in binding a hydrogen bond acceptor.

## 3.6. Type B Lantibiotics: Cinnamycin Group

### 3.6.1. Primary Structure

This group consists of the lantibiotics cinnamycin, duramycin, duramycin B and C, and ancovenin, all of which are very similar in their ring structure and amino acid composition (Figure 12). Cinnamycin[56,78,107,172] isolated from *Streptomyces cinnamoneus* was also purified as Ro 09-0918[173−175] from *Streptoverticillium griseoverticillatum* and as lanthiopep-

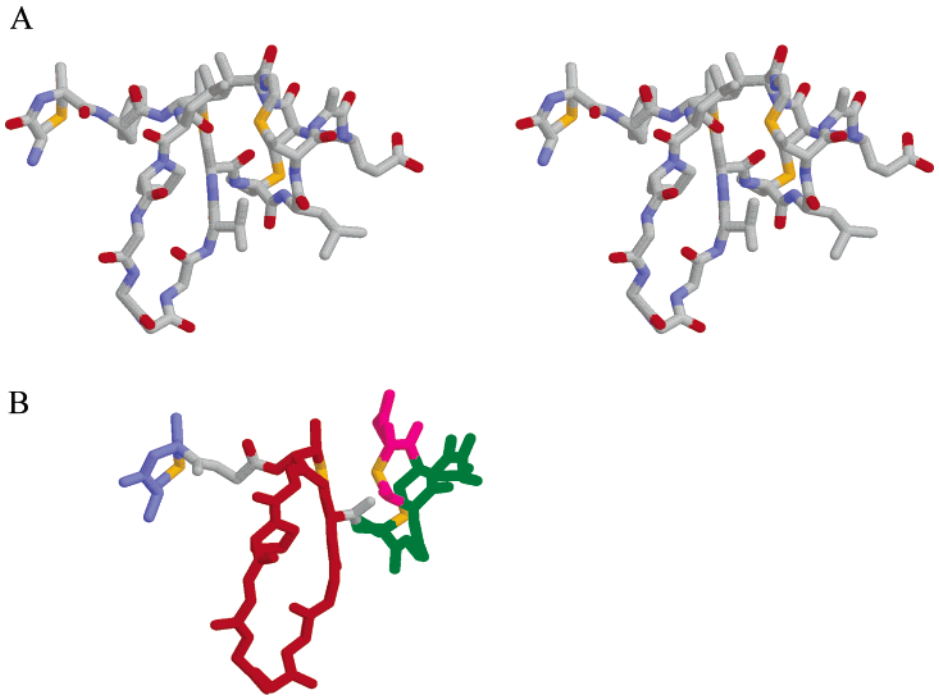



**Figure 10.** (A) Stereoview of one of the NMR structures of mersacidin in water-methanol.[171] (PDB accession number 1MQX). (B) View of mersacidin in the same orientation as in panel A in which the rings are highlighted and the side-chains are omitted. Shown in blue is the A-ring, in red the B-ring, in green the C-ring, and in magenta the D-ring. Figures were generated using the program RASMOL.[469]

tin by Shiba and co-workers from *Streptoverticillium* L337-2.[176] Structural determinations by various groups have shown that these compounds are identical.[56,174,176] The structure of cinnamycin was first suggested by Gross,[177] with later corrections to the amino acid sequence by Kessler[174] and Fredenhagen et al.[56,109] The revised structure is in agreement with the prepeptide gene sequences from *S. griseoverticillatum*[172] and *S. cinnamoneus*.[78] Cinnamycin, the duramycins, and ancovenin are all 19 residues long and contain one Lan and two MeLan rings in conserved positions. Except for ancovenin they all possess the unusual lysinoalanine ring that joins Lys19 to Dha6.[172] In ancovenin, Ser6 undergoes dehydration but no cyclization takes place onto Dha6.[13] Ancovenin is also the only family member in which Asp15 is not $\beta$-hydroxylated (section 4.6). To further distinguish them from the type A lantibiotics, the cinnamycin group contains two MeLan residues where the nucleophilic Cys is positioned N-terminally to the Dhb (Figure 12). This *reversed* direction of ring formation is also seen in the type-B lantibiotics mersacidin, the A1 peptide of the two-component lantibiotic lacticin 3147, and actagardine, but is not found in type A lantibiotics.

The current structure of duramycin was proposed by Hayashi[108] and Fredenhagen.[56] Duramycin (also isolated as leucopeptin by Kondo et al.[178]) differs from cinnamycin at a single residue at position 2, while the duramycins B and C differ by one and six amino acids, respectively (Figure 12). Ancovenin isolated from *Streptomyces sp. A647P-2* contains two MeLan rings, one Lan ring, and one Dha residue. Wakamiya et al.[12,13] confirmed the presence of Dha at position 6 by reduction of ancovenin with $Pd/H_2$, which resulted in Ala at residue 6, as well as by nucleophilic addition of the methyl ester of mercaptoacetic acid ($HSCH_2COOCH_3$). The position and stereochemistry of the sulfide bridges were assigned by comparison with synthetic samples and chiral GC analysis.[12,13]

### 3.6.2. Three-Dimensional Structure

The solution structure of cinnamycin has been determined in DMSO and a water−acetic acid mixture (9:1) by NMR spectroscopic techniques.[179] Its ring structure can be viewed as four structural domains. Residues 1−4 and 14−18 along with the thioether bridges spanning Ala1-Abu18 and Ala4-Ala14 together constitute the A-ring (Figure 6). This ring exists in an antiparallel $\beta$-sheet structure supported by backbone H-bonds between the Arg2 carbonyl and amide of Abu18, the carbonyl of Ala4 and amide of Gly16, and the carbonyl of the side-chain of Asn17 and the amide of Ala4. The C-ring formed by residues 5−11 showed a high degree of flexibility. The D-ring comprising Lys19 to Ala6 and the lysinoalanine is placed above the plane occupied by the other rings resulting in an amphipathic structure. The conformation of cinnamycin undergoes a strong change in the presence of SDS micelles.[180] NOE measurements conducted in water and SDS micelles indicated that the change takes place predominantly in a hinge region that connects the lipophilic and lipophobic portions of the molecule. Moreover, studies with cinnamycin in SDS bilayers[180] and in the presence of 1-dodecanoyl-*sn*-glycerophosphoethanolamine (C12-LPE)[181] indicated a conformational change in the lipophilic portion of the molecule due to interactions with hydrophobic segments of the lipids. Zimmerman et al.[110] have determined the solution structures of duramycins B and C and also observed a hinged amphiphilic structure. The authors noted that

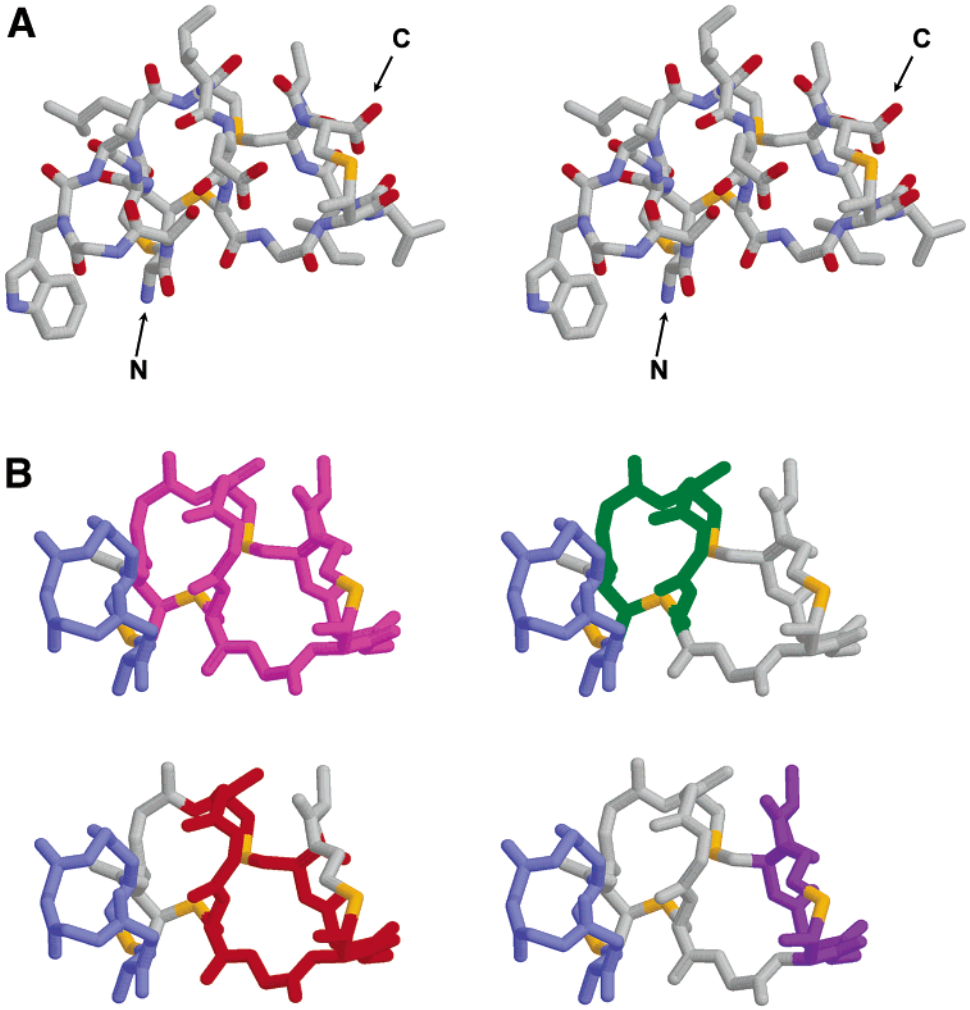



**Figure 11.** (A) Stereoview of one of the NMR structures of actagardine reported by Jung and Zimmermann.[15] (PDB accession number 1AJ1) (B) View of actagardine in the same orientation as in panel A in which the rings are highlighted and the side-chains are omitted. Shown in blue is the A-ring that makes up domain A and in magenta domain B formed by ring B (green), ring C (red), and ring D (purple). Figures were generated using the program RASMOL.[469]

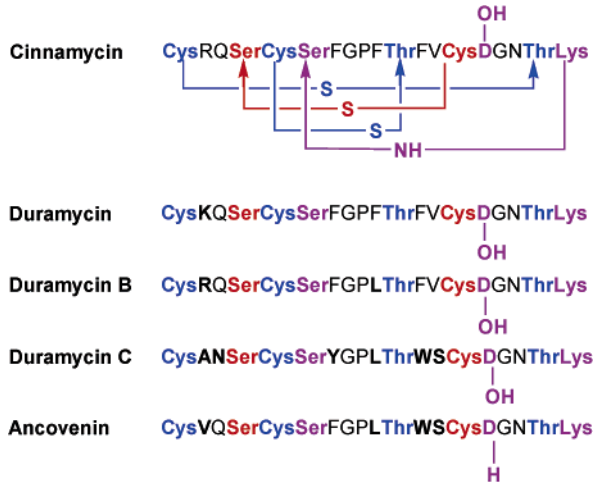



**Figure 12.** Sequence comparison of compounds from the cinnamycin group. The residues involved in ring formation are depicted in three letter code, whereas one letter code is used for the other residues. Arrows represent the directionality of cyclization. Ancovenin posseses neither the head-to-tail lysinoalanine bridge nor the hydroxyaspartic acid residue present in all other members of this group.[13]

the changes in the amino acid sequence compared to that of cinnamycin (Figure 12) did not disturb the overall structure but led to a decreased amphiphilicity in these compounds.

## 3.7. Two-Component Lantibiotics: Lacticin 3147

A separate subgroup is formed by the two-component lantibiotics that consist of two posttranslationally modified peptides that individually have weak activity but synergistically display strong antibacterial action. Two-component systems had been previously characterized in the class II bacteriocins, and only a few such systems have been found so far in lantibiotics.[182] At present, only the two polypeptides of lacticin 3147 have been structurally characterized.

### *3.7.1. Primary Structure*

The two components of lacticin 3147, LtnA1 and LtnA2, were initially purified and characterized from the producer strain *L. lactis DPC3147* by Hill and co-workers.[183] Amino acid analysis of both LtnA1 and A2 revealed the presence of Lan/MeLan residues as well as D-Ala by chiral-phase GC. Furthermore, the total number of Ala residues present in the two components was found to be greater than that predicted from the genetic sequence of the prepeptides. Edman sequencing of LtnA1 after derivatization with 1-pro-

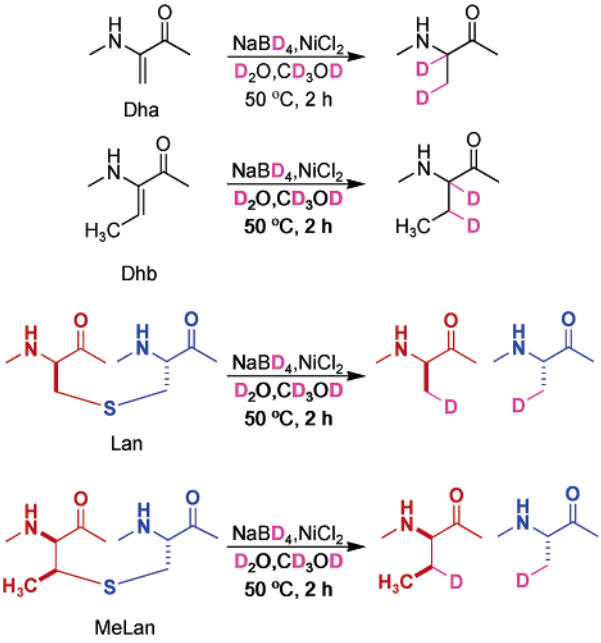



**Figure 13.** Modification of Dha, Dhb, Lan, and MeLan by reduction with $NaBD_4$ and nickel boride to generate a product that is amenable for Edman degradation.[112] This method allows distinction between dehydro amino acids and (methyl)lanthionines. Another method that achieves this was reported by Smith et al.[92]

panethiol indicated the presence of Lan/MeLan and/or Dha/Dhb residues. A genetically predicted Ser residue at position 7 was found to correspond to a D-Ala residue in the mature product suggesting posttranslational conversion of Ser to D-Ala. The Edman sequencing of LtnA2 was not possible even after chemical modification and was attributed to the presence of a 2-oxobutyryl residue at the N-terminus, formed by the hydration−deamination reaction of a Dhb residue at this position. Vederas and co-workers recently completed the characterization of the structure of LtnA1 and A2 by a novel method involving nickel boride ($Ni_2B$), an in situ generated hydrogenation and desulfurization catalyst.[112] Treatment of the lantibiotics with $Ni_2B$ in the presence of $NaBD_4$ in $CD_3OD/D_2O$ led to the desulfurization of Lan/MeLan and incorporation of a deuterium atom at the $\beta$-carbon of each of the constituent residues (Figure 13). Simultaneous reduction of the Dha/Dhb residues resulted in addition of two deuterium atoms across the double bonds. The LtnA2 peptide was deblocked prior to $Ni_2B$ treatment by the removal of two N-terminal residues upon treatment with 1,2-diaminobenzene in an acetic acid/sodium acetate buffer.

Automated Edman sequencing of the deblocked and reduced peptides along with NMR spectroscopy were used to assign the structures of both peptides. LtnA1 contains two Lan and two MeLan residues, two dehydrobutyrines, and one D-Ala at position 6. The overall structure resembles that of mersacidin (Figure 6). LtnA2 was found to contain one Lan and two MeLan residues besides two Dhb and two D-Ala residues. The A2 peptide may have similarity with lactocin S, the only other lantibiotic to contain D-alanine but whose proposed structure has not yet been unequivocally confirmed.[184] Since the structural gene sequences of both LtnA1/2 and lactocin S encode Ser residues at the positions of the observed D-alanines, it is believed that they arise from dehydration followed by stereospecific hydrogenation.[184,185] Although Lan/MeLan amino acids have been identified in the two-component lantibiotic systems plantaricin W,[114] staphylococcin C55,[113] and cytolysin L,[115] their structural determination is not complete and the position and nature of modified residues is only speculative based on limited sequence homology to known lantibiotics.

#### *3.7.2. Three-Dimensional Structure*

Preliminary NMR solution structures of LtnA1 and A2 show that they exist in different conformations.[112] LtnA1 exhibits a globular shape resembling mersacidin, while LtnA2 is similar in structure to the type AI lantibiotics, being elongated and screw-shaped. The similarity in lanthionine bridging patterns of LtnA1 and mersacidin, especially in the two N-terminal rings, have been invoked to suggest similar conformational changes for LtnA1 that are observed for mersacidin in the presence of the cell membrane and lipid II (section 8.2).[171]

## *4. Biosynthesis of Lantibiotics*

### 4.1. Lantibiotic Precursor Peptides

All lantibiotic precursor peptides (LanA) contain a C-terminal structural region that undergoes posttranslational modification (propeptide) and a relatively long N-terminal leader sequence containing between 23 and 59 amino acid residues, which remains unaffected during biosynthesis. Whereas both the leader sequence and propeptide region contain serine and threonine residues, cysteines have only been found in the propeptide segment. Comparisons of the leader sequences of a large number of lantibiotics have revealed two different conserved motifs (Figure 14),[186] which has been proposed as the basis for an alternative classification of lantibiotics[81] than that presented above (i.e., type AI, type AII, and type B). In this organization by genetics rather than activity profiles or three-dimensional structure, the class I lantibiotics all have a common "FNLD" motif between positions −20 and −15 and usually a Pro at position −2. The biosynthetic machinery that carries out the posttranslational modifications in this class consists of LanB and LanC. In contrast, class II peptides contain a characteristic "GG" or "GA" cleavage site (historically termed the "double Gly motif"),[186−188] contain multiple Asp and Glu residues, and are processed by one modification enzyme (LanM). An exception to this general rule are the salivaricins, which contain the typical leader peptide signature for the class II lantibiotics but which are actually modified by SalB and SalC proteins.[157] A few outlying sequences are found in cinnamycin and mersacidin that both have very long leader peptides, and in lactocin S (Figure 14). The cleavage site of the cinnamycin leader peptide has the AXA motif found for recognition by type I signal peptidases of the general secretory (*sec*) pathway.[78]

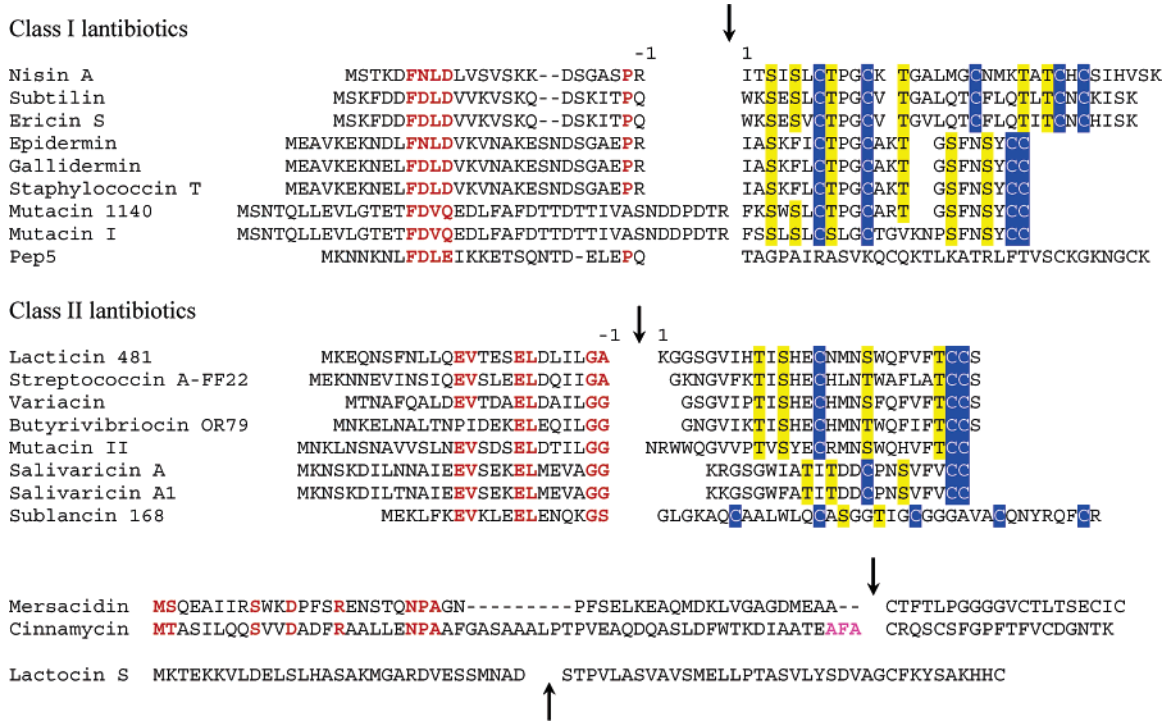



**Figure 14.** Sequence alignments of several prepeptides of lantibiotics showing conserved motifs discussed in the text in red. The classification is based on consensus sequences present in the leader peptides as well as the modification process that is catalyzed by two enzymes (LanB and LanC) for class I and by a single enzyme (LanM) in class II. It is clear that within each class subclasses can be identified of compounds that are essentially natural variants. Within class I there is high homology in the positions of Ser, Thr, and Cys residues that form the A- and B-rings with the exception of Pep5. In class II, the ring structures of compounds whose leader sequences end in the so-called double glycine motif[187,188] are all very similar with the exception of salivaricin A and A1 and sublancin. Arrows indicate proteolytic processing sites. Accession numbers: nisin A (P13068), subtilin (P10946), ericin S (AAL15569), epidermin (P08136), gallidermin (P21838), mutacin 1140 (O68586), mutacin I (AAG48564), Pep5 (CAA90023), lacticin 481 (P36499), SA-FF22 (AAB92600), variacin (A58711), butyrivibriocin (AAC19355), mutacin II (AAC38144), salivaricin A (P36500), sublancin 168 (O34781), mersacidin (I40461), cinnamycin (CAD60520), lactocin S (A55457).

The role of the leader sequences is at present unclear. With the exception of the leader peptide for cinnamycin,[78] they lack the typical characteristics of the *sec*-dependent transport signal sequences. Possible functions that have been suggested include signaling for export, protection of the producing strain by keeping the peptides inactive, and providing scaffolds for the posttranslational modification machinery.[19,189] Precedent for all three functions can be found in the literature on export proteins,[190] hormones,[191,192] class II bacteriocins,[193] and microcin biosynthesis,[194] and as discussed below all three functions appear important in lantibiotic biosynthesis.

In a recent study, a series of nonlantibiotic peptides attached to the C-terminus of the NisA leader sequence were transported by NisT, suggesting secretion is directed by the leader peptide.[195] Similarly, alkaline phosphatase fused to the subtilin leader peptide was exported in *B. subtilis*,[196] a process that was enhanced when the SpaT transporter was also present. Analogous experiments in *Escherichia coli* resulted in translocation of the fusion protein into the periplasmic space.[197] These studies support a role of the leader peptide in recognition by the secretion machinery and are also consistent with the extracellular membrane location of the NisP protease that removes the leader peptide and the observation that the leader of posttranslationally modified presubtilin is removed by extracellular proteases.[77] However, it is unclear how general this statement is across the lantibiotics family as several members contain cytoplasmic proteases that appear to remove the leader peptide prior to transport (section 4.7).

A protective role of the leader sequence is consistent with studies on nisin,[189,198,199] subtilin,[77,200] lacticin 481,[40] and mutacin II,[201] showing that the posttranslationally modified peptides with the leader sequence still attached exhibit little to no biological activity. NMR studies comparing posttranslationally modified prenisin with its leader sequence still attached and mature nisin suggested that a different interaction between the membrane and the N-terminal region of the modified propeptide in both compounds accounts for the loss of antimicrobial activity.[202] In light of the subsequent discovery that nisin's activity is mediated by binding to lipid II,[41] it would be interesting to revisit this issue in membranes containing lipid II. Indeed, in the recent NMR structure of nisin bound to lipid II, its N-terminal Ile is located at the interface of the two molecules, suggesting extension of the N-terminus may disrupt complementarity.[54]

A number of intriguing in vivo studies have been conducted with chimeras from the nisin and subtilin leader and structural regions. While expression of the nisin gene in a subtilin producing *Bacillus* strain did not lead to nisin-related modified peptides, a chimera consisting of the subtilin leader and nisin structural gene sequences produced a fully processed product.[203] This result suggested that the posttranslational

```
Class I bacteriocins (lantibiotics)
    LctA          MKEQNS--FNLLQEVTESELDLILGA
    MutA          MNKLNSNAVVSLNEVSDSELDTILGG
    SalA          MKNSKDILNNAIEEVSEKELMEVAGG
    ScnA          MEKNNE-VINSIQEVSLEELDQIIGA
Class II bacteriocins
    PedA          --------MKKIEKLTEKEMANIIGG
    LcnGα         ----------MKELSEKELRECVGG
    LcnGβ         MKNNNN-FFKGMEIIEDQELVSITGG
    LcnA          MKNQLN-FN----IVSDEELSEANGG
```

**Figure 15.** Sequence alignment of the leader sequences of type AII lantibiotic and class II bacteriocin prepeptides. LctA (lacticin 481), MutA (mutacin II), SalA (salivaricin A), ScnA (streptococcin A-FF22), PedA (pediocin PA-1), LcnGα (lactoccocin Gα), LcnGβ (lactococcin Gβ), LcnA (lactococcin A). Completely conserved residues are highlighted in red while strongly conserved residues are in blue. Alignment created with CLUSTAL W (v1.82). Entrez accession numbers: LctA, P36499; MutA, JC6526; SalA, P36500; ScnA, AAB92600; PedA, P29430; LcnA, A39443.

modification machinery of the host specifically recognized the leader sequence. However, when a similar chimera containing a subtilin leader and nisin structural region was expressed in a nisin-producing *Lactococcus* strain the structural region was processed.[204] Furthermore, the leader sequence of several type AII lantibiotics have similarity to the leader peptides of class II bacteriocins that are not posttranslationally modified (Figure 15).[25,150] These observations appear to argue against a role of providing a recognition motif for binding of the modifying enzymes. At least one modification enzyme has been demonstrated not to require the leader sequence. Isolated EpiD, an oxidative decarboxylase involved in the formation of AviCys (section 4.5), was able to process peptides without the leader sequence.[205]

The importance of the conserved residues in the leader peptides for proper posttranslational modifications and proteolytic processing has been probed by site-directed mutagenesis for several lantibiotics. These studies demonstrated a rather weak dependence of the maturation process on point mutations, as the single mutants Pro(−2)Gly, Pro(−2)Val, Asp(−7)Ala, and Lys(−9)Leu (Figure 16A) as well as the double mutants Ser(−10)Ala/Ser(−12)Ala and Val(−11)Asp/Val(−13)Glu still produced and secreted nisin Z. Mutation of Arg(−1) to Gln in the *nisA* gene for nisin Z resulted in production and excretion of both the fully modified product and the posttranslationally modified product that still contained the leader peptide.[189] Apparently proteolytic processing of the mutant peptide, which now actually has the same residues in the −1 and −2 positions as subtilin and Pep5, still occurs albeit with much reduced efficiency. A similar result was obtained for Ala(−4)Asp. Not all positions can tolerate substitutions, however, because strains containing mutant genes coding for Ser(−6)Leu, Asp(−15)Ala, Leu(−16)Lys, and Phe(−18)Leu NisA did not produce any detectable products. Hence, the conserved F-(N/D)-L-(N/D/E) motif in the class I leader peptides appeared important for the biosynthetic machinery. However, an analogous study on the leader sequence of Pep5 showed that the nonconservative mutations Phe(−19)Ser and Glu(−16)Lys within this motif as well as Asp(−6)Lys still resulted in respectable levels of Pep5 production (Figure 16B).[206] These findings indicate

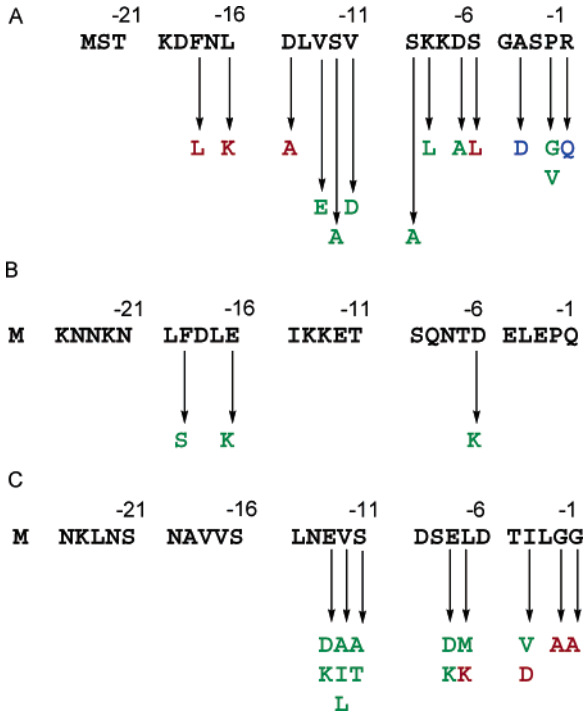



**Figure 16.** Sequence requirements of the leader peptides of (A) nisin, (B) Pep 5, and (C) mutacin II as determined by site-directed mutagenesis. In green are those mutants that still result in full processing of the prepeptides, in blue those that result in both mature lantibiotics and lantibiotics with their leader peptides still attached, and in red the mutants that do not lead to lantibiotic production.

that the recognition of the leader peptide by the processing enzymes is likely a complex process possibly involving recognition of tertiary structural elements rather than conserved residues. Early investigations using synthetic prepeptides and leader peptides did show that in structure-inducing hydrophobic solvents, the peptides adopted amphiphilic helical structures.[207,208] In addition to the low substrate specificity in the leader peptide, several studies have indicated substrate promiscuity in the propeptide region. These efforts have resulted in engineering of the structure of lantibiotics and are discussed in section 7.

Two studies have investigated the leader sequence requirement for the class II lantibiotics mutacin II and lacticin 481. Similar to the studies of nisin and Pep5, the investigation of mutacin II employed expression of a mutated structural gene in a modified producing strain.[201] Replacement of the double Gly motif in positions −1 and −2 with two Ala residues resulted in complete abolishment of production of bioactive peptides. Analysis of the intracellularly accumulated peptides showed the presence of premutacin. Whether these peptides had been posttranslationally modified in the propeptide region is not clear. Substitution of other conserved residues with similar amino acids (Figure 16C) did not affect mutacin production, and even replacement with oppositely charged residues at positions Glu−8 and Glu−13 only resulted in a decreased production of mature mutacin II. Two mutants close to the propeptide (Ile(−4)Asp and Leu(−7)Lys), however, did fail to produce mutacin and the prepeptides could not be detected.

The recent in vitro reconstitution of lacticin 481 biosynthesis allowed the first examination of the importance of the leader sequence and the structural region for correct modification with isolated peptides.[40] These studies demonstrate that the length of the prepeptide is not critical for the modification enzyme. For example, analogues of the prepeptide LctA with attachments of extra amino acid residues at either the N-terminus or the C-terminus did not affect the activity of the bifunctional dehydratase/cyclase LctM. In addition, LctA peptides truncated at the C-terminus still provided the expected products upon LctM catalysis. Very interestingly, although removal of the entire leader sequence resulted in loss of detectable modification by LctM, LctA mutants that lacked the first three (LctA5−51), eight amino acids (LctA10−51), or 11 amino acids (LctA13−51) were fully processed by LctM (Figure 26). On the other hand, a mutant that lacked the first 14 amino acids (LctA16−51) was not a substrate (Chatterjee and van der Donk, unpublished results). This observation suggests that the conserved residues in the segment spanning residues 17−24 (Figure 14) are essential for enzyme recognition. The highly conserved GG or GA sequence at the end of the leader sequence was not critical for LctM activity. The single point mutants $His_6$-LctA(G23V), $His_6$-LctA(A24D), and $His_6$-LctA(L20Q) were all dehydrated by LctM (Chatterjee and van der Donk, unpublished results). LctM also displayed high substrate promiscuity in the propeptide region as a variety of LctA mutants were processed (section 7.2).

## 4.2. The LanB Dehydratases

The selective dehydration of Ser and Thr residues in the LanA structural region leading to Dha and Dhb, respectively, is the key first reaction involved in the biosynthesis of lantibiotics. For the members of the type AI group, this modification is believed to be carried out by the putative LanB dehydratases based on in vivo genetic disruption studies. BLAST searches do not reveal homology of the LanB enzymes (~120 kDa) with any other known proteins. Among the LanB family, the overall sequence identity is only around 30%. The low similarity might be due to the significantly different prepeptide substrates and the formation of products of vastly different three-dimensional structures. In cases in which the products are structurally close, the dehydratase proteins also show higher homology such as in subtilin and ericin S with EriB sharing 83% identity with SpaB. The two products have identical ring locations with only four amino acid differences, and they contain identical leader peptides in the respective precursors.[88]

The isolation of a dehydrated Pep5 peptide after inactivation of *pepC* in a Pep5-producing strain provided the first indirect evidence that the LanB proteins are involved in the dehydration.[75] More recently, Dodd and co-workers generated a nisin A variant (H27K, H31K) in which Ser33 is partially dehydrated in about half of the processed product. Overexpression of NisB in various *L. lactis* hosts containing this mutated structural gene resulted in elevated cellular levels of NisB as well as increased efficiency of dehydration of Ser33, consistent with dehydratase activity for NisB.[209] Koponen et al. reported the isolation of unmodified nisin precursor NisA from strains in which NisB activity was impaired, whereas the dehydrated prepeptide was recovered from strains lacking NisC activity, indicating the importance of NisB for dehydration in the biosynthesis of nisin.[210] The same result was obtained by Kuipers et al. in studies expressing *nisABT* in a nonproducing *L. lactis* strain that yielded dehydrated prenisin without thioether rings.[195] In accord with the essential role of LanB proteins in carrying out the first step in posttranslational modification, deletion of *spaB* prevented subtilin production,[68,211] stable *epiB* mutants abolished epidermin formation,[69] disruption of *eriB* in the ericin gene cluster eliminated the production of both ericin A and ericin S,[88] and insertional inactivation of *salB* resulted in abrogation of salivaricin A production.[157]

In vitro reconstitution of the enzymatic dehydration, either in cell-free extracts or with (partially) purified proteins, has remained enigmatic to date.[75,209,210,212,213] Although a hydrophobicity plot indicates that LanB proteins are rather hydrophilic and contain no clear transmembrane domains, NisB and SpaB were found to co-sediment with membrane vesicles, suggesting a membrane-associated nature.[214] Furthermore, for nisin and subtilin biosynthesis, yeast two-hybrid[215,216] and immunoprecipitation[215−217] experiments suggest that LanB forms a membrane-associated multimeric complex with LanC and LanT, the transport protein.

EpiB involved in epidermin biosynthesis in *Staphylococcus epidermis* was detected in both the cytoplasmic and the membrane fraction, indicating a loose association with the cytoplasmic membrane. Initial attempts to express EpiB in *E. coli* resulted in low protein production possibly due to differences in codon usage. EpiB was then purified from the closely related strain *Staphylococcus carnosus* using an improved staphylococcal expression system; however, in vitro dehydration activity could not be detected.[212] Recently, Xie et al. succeeded in expressing SpaB in *E. coli* as a cytoplasmic protein with the aid of the GroEL/ES molecular chaperones.[217] Efforts to detect dehydratase activity of purified SpaB in the presence of the subtilin prepeptide (SpaS), SpaC, and a range of potential cofactors or metal ions were, however, unsuccessful. This lack of activity could be because other components of the multimeric lantibiotic synthetase were absent. Consequently, detailed studies on how the enzyme recognizes its substrate and carries out the multiple dehydrations are still lacking. Recently, in vitro dehydration activity has been reported for a LanM enzyme as described in section 4.4.

## 4.3. The LanC Cyclases

Even though dehydro amino acids are less electrophilic than other Michael-type acceptors by virtue of the built-in deactivating enamine moiety, nucleophilic additions to dehydro amino acids are well-known and relatively fast. Hence, compared with the dehydration reaction, the cyclization of a nucleophilic thiolate onto an electrophilic dehydro amino acid is

relatively easy. The challenge for the cyclase enzymes lies therefore not so much in chemical activation as in the control of regio- and stereochemistry. When biomimetic studies indicated that cyclization in short peptide analogues of the ring systems encountered in various lantibiotics occurred spontaneously to generate thioether rings of the correct stereochemistry,[123−125] the question of the need for a dedicated enzyme was raised. Early evidence of the requirement of the LanC proteins for in vivo cyclization was reported in 1995 using the Pep5 biosynthetic system.[75] In this work, a Pep5-producing strain of *S. epidermis* was depleted of its ability to generate the lantibiotic, but after supplementation with a plasmid encoding the *pepTIAPBC* gene cluster the production of fully modified Pep5 was restored. Subsequent disruption of the *pepC* gene led to the formation of the fully dehydrated prepeptide as well as fragments thereof, but none contained the correctly cyclized thioether bridges characteristic of the Pep5 structure. It was proposed that PepC needs to bind the fully dehydrated substrate in a specific conformation to facilitate the correct addition of cysteines to their dehydrated partners. These results were the first experimental evidence implicating a LanC protein as the cyclase involved in lantibiotic biosynthesis and offered counterevidence to the idea of spontaneous nonenzymatic cyclization of thioethers.

EpiC, involved in epidermin biosynthesis, has been overexpressed and purified from *S. epidermidis* by Kupke and Götz.[213] The enzyme showed no activity with unmodified EpiA, as expected since it would not be the substrate for the enzyme. Because dehydrated EpiA was not available, in vitro cyclization activity could not be tested. In these studies, it was noted that plasmids encoding *epiC* mutants where a conserved glycine residue was changed to a glutamate did not restore epidermin production in strains lacking *epiC*. The cause of inactivation of these mutants is still unknown.

In a series of experiments similar to the work probing the function of PepC, Koponen et al. engineered mutant strains of *L. lactis* lacking either the *nisB* or *nisC* genes.[210] A His-tagged nisin precursor peptide was coexpressed simplifying the purification of the products formed. Use of this tagged prepeptide in a strain lacking the NisB gene showed that no dehydrations or cyclizations took place. On the other hand, the NisC deficient strain yielded a dehydrated propeptide, but no cyclization products were observed. This was the first direct evidence in the nisin system that NisC was responsible for the cyclization of the thioether rings, either directly or by inducing cyclization activity in NisB. NisC has been shown to be localized at or in the cellular membrane by co-immunoprecipitation studies.[215] Furthermore, yeast two-hybrid studies indicated a specific interaction of NisC with NisT, NisB, and itself.[215] These findings led to a putative model of nisin biosynthesis involving a complex consisting of two NisT proteins, two NisC polypeptides, and one NisB protein, all associated with the cellular membrane (Figure 17). Similar studies were conducted with the subtilin system, yielding evidence for a multimeric biosynthetic complex for the modification of the SpaS precursor peptide (Figure 5).[216] Small differences involved the observation of an interaction between SpaB and SpaT that was not observed for nisin as well as an interaction of SpaB with itself suggesting an oligomeric structure.

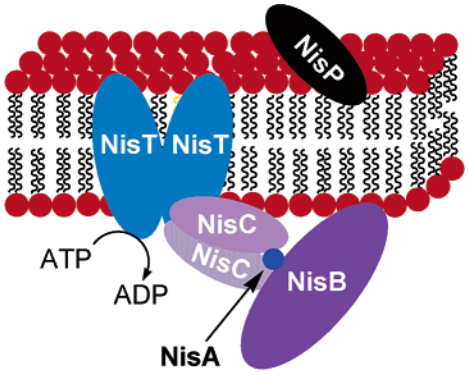



**Figure 17.** Proposed multi-enzyme complex involved in nisin production on the basis of co-immunoprecipitation and yeast two-hybrid studies.[215]

Recently, the LanC enzymes involved in nisin and subtilin production (NisC and SpaC) were cloned, overexpressed in *E. coli*, and purified to homogeneity. As isolated, the proteins are monomers and metal analysis showed that each contains a stoichiometric amount of zinc.[218] The difference in oligomerization state predicted by the two-hybrid studies and these in vitro experiments with heterologously expressed protein may be due to incorrectly folded protein in either *E. coli* or yeast or both. It has been postulated that the zinc may serve to activate the thiol substrates of the LanC enzymes,[218] a role similar to that in a number of other enzymes that catalyze thiol alkylation.[219] The zinc in these metalloenzymes is believed to activate the thiol of their substrates by lowering the p$K_a$ and enhancing the reactivity at neutral pH. For example, the pH dependence of the binding of a peptide substrate to farnesyltransferase indicates that the p$K_a$ of the cysteine is lowered from 8.3 for the free peptide (GCVLS) to approximately 6.4 upon binding to the enzyme.[220] Indeed, to sustain a reasonable rate of lanthionine formation at neutral pH, activation of the cysteine thiols of the substrate by deprotonation is required. For instance, the rate constant for the addition of free thiols to α,β-unsaturated centers is $10^{10}$-fold decreased compared to the corresponding thiolates.[221] One noticeable feature that is common among this family of zinc enzymes that catalyze thiol alkylations is the presence of two or more cysteine residues in the zinc coordination sphere and an overall net negative charge. In accord with this postulated role of the metal, extended X-ray absorbance fine structure (EXAFS) analysis of SpaC showed that the ligand environment surrounding the zinc is comprised of two cysteines and possibly two histidine residues, or one histidine and one water molecule.[218]

The LanC proteins share low sequence similarity (~20−30%, Figure 18). Only very few amino acids are strictly conserved, but these include two cysteine residues (Cys284 and Cys330, NisC numbering) and two histidines (His212, His331). These residues are

```
MrsM      864 ----- ----G FAHGA SG--- -IIWA LYEIY AITKQ TVFKE VAEKA LEFER 900
CinM      883 ----- ----G FSHGS GG--- -IGWA LIQLG RHTGR SDYIE AGRKA FAYED 919
LctM      723 ----- ----- YAHGN SG--- -IATA FVHGY KVTKN EKYLK IFHEL WNLEN 758
SpaC      219 AYPYG NFNMG LAHGI PGPIC VLSSA LIQGI KVKGQ ERAIE KMAN- FLLEF 267
EpiC      241 ----- NINLG LAHGI LGPLS LTALS KMNGI EIEGH EEFLQ DFTSF LLKPE 285
NisC      205 ----- CLNMG LAHGL AGVGC ILAYA HIKGY SNEAS LSALQ KIIFI YEKFE 249
PepC      200 ----- ---LG YAHGI PGIIN TLCNS YKRGY GIIKT KKILE QSIFT LLQNL 241
                              **   *

MrsM      901 TLFIP EKNNW ADIK- ---LE NGQFR NDN-- -FVAW CNGAA GIGLS RIL-- 941
CinM      920 RHVDE QEKDW YDLR- ---IN NGSAV KGARH FSNAW CNGAA GIGLA RISSW 965
LctM      759 SSK-- LRRGW TDSR- ---KV DSSY- ----- -SSQW CHGAS GQAIA RMEWI 795
SpaC      268 SEKEQ DSLFW KGIIS FEEYQ YGSPP NAVNF SRDAW CYGRP GVCLA LVKAG 317
EpiC      286 FKNNN EWFDR YDILE NYIPN YSVR- ----- --NGW CYGDT GIMNT LLLSG 327
NisC      250 LER-K KQFLW KDGLV ADELK KEKVI REASF IRDAW CYGGP GISLL YLYGG 298
PepC      242 KLENG TIYIP NDIE- ---SP NDYR- ----- --DAW CYGLP SVAYT IFNVS 279
                                                  * * *

MrsM      942 ----- --ILP HNQNE LIKDE AHVAI NTTLK --YGF EHD-Q SLCHG DLGN- 980
CinM      966 ----- -AALD RSDEQ LLRDA QQALS ATLRN --FPR LKN-H TLCHG TSGN- 1005
LctM      796 TVNKT ARFLS NSELI KVKKE LGELI DILKK --EGM YTDNF CLCHG ILGN- 842
SpaC      318 ----- ---KA LQNTE LINIG VQNLR YTISD --IRG IFS-P TICHG YSGIG 356
EpiC      328 ----- ---KA LNNEG LIKMS KNILI NIIDK --NND DLISP TFCHG LASHL 367
NisC      299 ----- ---LA LDNDY FVDKA EKILE SAMQR --KLG IDS-Y MICHG YSGLI 337
PepC      280 ----- ---ST LKNKS LIELS ESLLH QVFLR SDNAT KLISP TLCHG FSGVV 321
                                                        ***
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**Figure 18.** Partial sequence alignment of several LanC proteins and the C-terminal domains of a number of LanM proteins. The putative metal ligands[218] are in red font. Acccession numbers: SpaC-AAA22777, NisC-Q03202, EpiC-CAA44254, PepC-CAA90026, LctM-AAC72258, MrsM-CAB60261, CinM-CAD60521.

also conserved in the C-terminal part of the LanM proteins (section 4.4).[66,155] Mutants of SpaC with Cys303 and Cys349 changed to alanine residues were analyzed by EXAFS or inductively coupled plasma mass spectrometry (ICP-MS), confirming that they indeed bind significantly less zinc than the wild type protein and have only a single sulfur ligand.[218] As described above for the EpiC protein, activity determinations for the purified NisC and SpaC proteins have been hampered by the inability to produce the dehydrated prepeptides that are their putative substrates. Hence the effect of mutation of the Zn ligands on activity has not been determined, and at present the role for zinc is purely speculative and it cannot be ruled out that the zinc in SpaC and NisC provides structural integrity or acts as a Lewis acid for the electrophilic activation of a carbonyl group.[218]

Interestingly, while the LanC enzymes are responsible for the formation of cyclic lanthionines and methyllanthionines, the directionality of ring formation is not always uniform. The LanC enzymes involved in the biosynthesis of type AI lantibiotics such as nisin, subtilin, gallidermin, Pep5, and epidermin incorporate lanthionine rings solely in the N-to-C terminal direction, i.e., the cysteine involved in cyclization is always located on the C-terminal side of its dehydroalanine or dehydrobutyrine reaction partner.[172] Conversely, in the case of some type B lantibiotics such as cinnamycin (Figure 19),[172] mersacidin,[169] and the duramycins,[108,110] some of the (methyl)lanthionine rings in the final lantibiotic structures result from cyclization of cysteines onto dehydrobutyrines that are located downstream (i.e., toward the C-terminus).

The question of the origin of the stereoselectivity of the cyclization step has been investigated in a number of model studies after the initial work by Toogood showed that biomimetic reactions featuring the B-ring of epidermin provided the same stereochemical outcome as found in the natural product.[123] Similar findings have been reported in two subsequent studies on the biomimetic formation of lanthionine analogues of the subtilin and nisin B- and E-rings, which like the epidermin B-ring contain four amino acids.[124,125] More recently, the actual methyl-

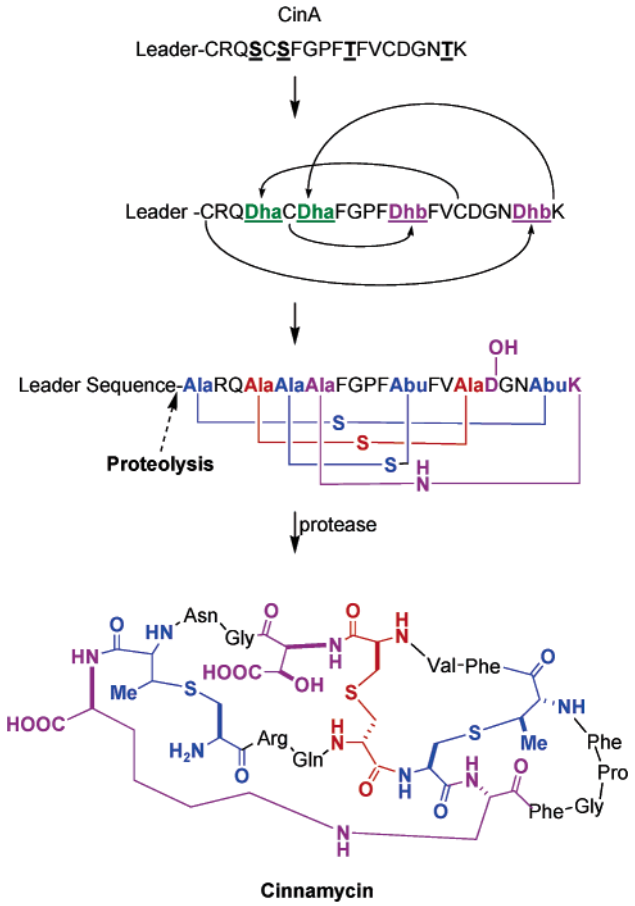
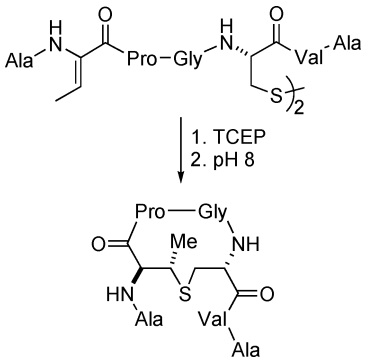
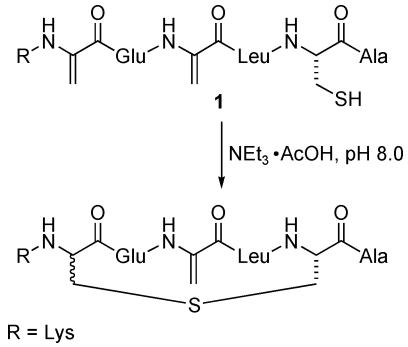



**Figure 19.** Schematic representation of the maturation process of cinnamycin illustrating the different directions of cyclization. The order of the cyclizations to form (methyl)-lanthionines and lysinoalanine is not known nor are the enzymes responsible for the formation of lysinoalanine and $\beta$-hydroxy aspartate.

**Scheme 1**




**Scheme 2**




lanthionine-containing B-ring of subtilin was prepared by nonenzymatic cyclization of a Cys onto a Dhb residue,[126] representing the first test of the stereochemistry of biomimetic formation of methyllanthionines (Scheme 1).[126] Through independent synthesis of the natural stereoisomer, it was shown that this Michael-type addition also occurred with very high selectivity in favor of the naturally occurring diastereomer. Interestingly, when the cyclization was carried out in the opposite direction (i.e., Cys located N-terminal to the Dha or Dhb), the reaction was not stereoselective.[126,222] This finding suggests that whereas the peptide substrates for type A lantibiotics involving cyclization in the C-to-N terminus direction have a propensity to provide the same stereochemistry observed in the natural products, the substrates of type B lantibiotics that undergo cyclization in the opposite direction do not have such an intrinsic stereoselectivity. The major difference between these two different modes of cyclization is whether an endocyclic (type A) or exocyclic enolate (type B) is generated.[126]

Two studies have examined the regioselectivity of biomimetic ring formation. Bradley and co-workers investigated the biomimetic formation of the A-ring of nisin (Scheme 2).[124] After deprotection of the cysteine of peptide **1**, a Michael-type addition led to two products in a 3:1 ratio. NMR analysis revealed that these compounds both contained the connectivity of the natural subtilin A ring, but with different stereochemistry at the newly formed stereocenter. Hence, whereas the correct regiochemistry was observed, the protonation of the enolate intermediate is less selective in this example than that observed for rings containing four amino acids (vide supra).

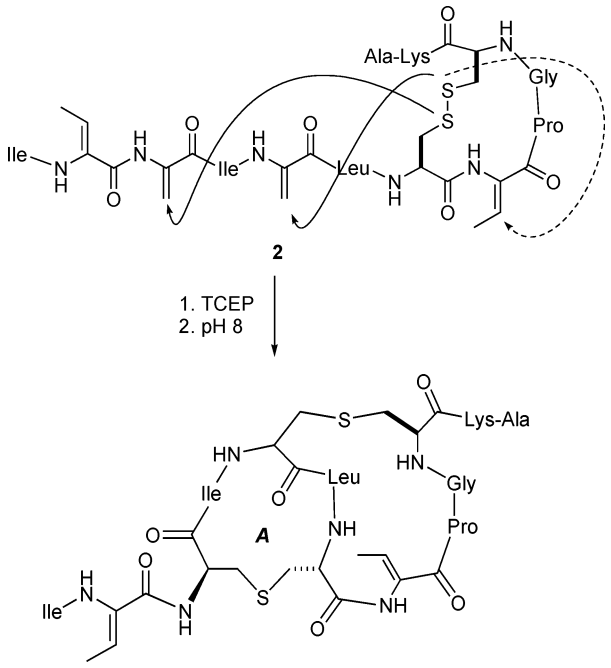



**Figure 20.** Attempted biomimetic synthesis of the A- and B-rings of nisin. On the basis of the result in Scheme 2, upon reduction of the disulfide bond in peptide **2**, the Cys at position 7 does form the A-ring, but the Cys at position 11 does not attack Dhb8 to generate the B-ring (dashed arrow) but rather adds to Dha5.

Zhu et al. recently addressed the chemo- and regioselectivity of the intramolecular Michael addition of the precursor peptide to the A- and B-rings of nisin (Figure 20).[222] $^1$H NMR analysis of the cyclization products revealed that the product did not consist of the A- and B-rings of nisin. Instead, the resonances of the vinyl protons of the two dehydrobutyrines were still present in the product, whereas the signals for the vinyl protons of the two Dha residues were absent. Hence, the much faster cyclization rate for lanthionines compared to methyllanthionines[126] prevents the biomimetic cyclization in which one Lan (A-ring) and one MeLan (B-ring) would have been formed (see nisin structure, Figure 2). The products that were obtained were assigned the structures depicted in Figure 20. The important conclusion from these studies is that lantibiotic biosynthesis clearly requires enzymatic control over the chemoselectivity and/or processivity of the cyclization reactions, in accordance with the observed genetic studies involving various LanC proteins. The much higher reactivity of the Dha residues compared to Dhb residues also explains why nonenzymatic cyclization of dehydrated prenisin and pre-Pep5 at elevated pH provided products in which free cysteines were no longer present but that did not have any biological activity.[75,195]

Intriguingly, a number of proteins with significant sequence similarity to the LanC family of enzymes has recently been discovered in mammalian erythrocytes. One of these, p40/GPR69A originally assigned as a member of the G-protein-coupled receptor superfamily, has been postulated to be a peptide-modifying protein based on its sequence homology to

the LanC enzymes.[223] Upon further characterization, p40/GPR69A was renamed to LANCL1 for LanC-like protein 1. Rat LANCL1 is highly expressed in the testis and brain, and is possibly involved in the immune surveillance of these particular organs, although no exact function of this protein has been determined.[224] If the hypothesis that the zinc serves an activating role to promote thiol alkylation proves correct, the LANCL1 proteins likely carry out alkylation of a currently unknown thiol substrate because the metal ligands are conserved. Since no proteins with sequence similarity with the lantibiotic dehydratases have been reported in mammals, the electrophile would probably not be Dha or Dhb.

## 4.4. The LanM Bifunctional Enzymes

A novel gene designated *lanM* encoding a 900−1000 amino acid protein is present in the gene clusters of the class II lantibiotics (nomenclature as per Figure 14).[148] The C-termini of LanM proteins have about 20−27% sequence identity with LanC proteins including the conserved motifs with the possible metal ligands (Figure 18). They show no sequence homology to LanB proteins, and hence their origin is unlikely to be from the fusion of *lanB* and *lanC* genes.[66] The unique sequences of LanM proteins and the fact that no other candidates for catalyzing the posttranslational modifications are present in the gene clusters of class II lantibiotics led to the proposal that they might be responsible for catalysis of both dehydration and cyclization reactions.[66,148] In support of this hypothesis, disruption of *lctM* in the lacticin 481 biosynthetic gene cluster prevented the production of the mature lantibiotic.[148,225] Similarly, mutacin II production was not observed in the absence of MutM, a frameshift in the gene for CylM eliminated cytolysin formation,[153,226] and LasM inactivation abolished the production of lactocin S.[155] A molecular interaction between the lacticin 481 prepeptide LctA and LctM was observed using the yeast two-hybrid system[225] similar to the observations for the LanB and LanC proteins with their LanA substrates discussed in sections 4.2 and 4.3. Interestingly, in the gene cluster of the two-component lantibiotic lacticin 3147 two independent genes encoding LtnM1 and LtnM2 are present (Figure 3). Disruption studies of the *ltnM* genes showed that each prepeptide (LtnA1 and LtnA2) requires a dedicated LtnM for modification.[71,227]

Direct evidence for the role of LanM proteins was recently provided by Xie et al. during the first reconstitution of an active lantibiotic synthetase (LctM) involved in the biosynthesis of lacticin 481.[40] In this report, LctM was shown to convert the prepeptide LctA into a 4-fold dehydrated species. The product was characterized by MALDI-MS and high-resolution FT-MS/MS demonstrating that the correct cyclization reactions had also taken place (Figure 21) thereby verifying that LctM is a bifunctional enzyme. Removal of the leader sequence from the product with the commercial protease Lys-C, which results in lacticin 481 lacking its N-terminal Lys residue, generated a bioactive peptide, whereas prior to proteolysis the product was devoid of any antimicrobial

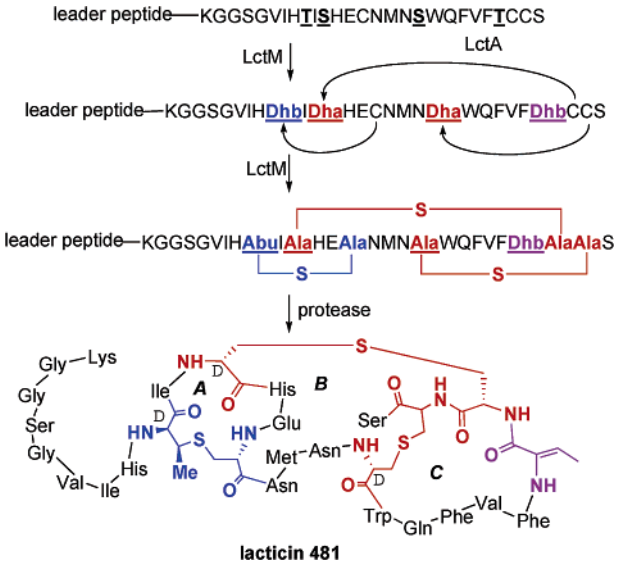



**Figure 21.** The posttranslational maturation process of lacticin 481. LctM catalyzes the dehydration of the underlined Ser and Thr residues in the propeptide region of LctA. The sequence of the leader peptide is MKEQNSFNLLQEVTESELDLILGA, and in the substrate used for in vitro reconstitution of the maturation process[40] an N-terminal $His_6$-tag linker was attached with the sequence GSSHHHHHHSSGLVPRGSH. LctM also catalyzes the conjugate addition of three Cys residues in a regiospecific manner to three of the Dha and Dhb residues to generate three cyclic thioethers, one methyllanthionine and two lanthionines. The leader peptide is proteolytically removed by the N-terminal protease domain of the LctT ABC-type transporter that excretes the final product (section 4.7).

activity at the concentrations tested. It should be emphasized that although it is generally assumed that the dehydration of all targeted Ser and Thr residues in the prepeptide is completed before cyclization commences (e.g., Figures 4 and 21), at present this has not been unambiguously established. In an alternative model, the two active sites on LanM (or the LanB and LanC proteins for type AI lantibiotics) could pass the substrate between them such that dehydration of one particular Ser/Thr is immediately followed by ring formation before dehydration of the next Ser/Thr.

ATP and $Mg^{2+}$ were required for LctM to carry out the posttranslational modifications, although at present the exact role of the cofactor is unknown. It may activate the serines and threonines for elimination by phosphorylation of their hydroxyl groups, or it may provide the energy for peptide translocation during the series of dehydration and cyclization reactions. ATP is converted into ADP in the process and use of non-hydrolyzable ATP analogues did not support catalysis. Although no dehydration is seen in the absence of ATP, ATP hydrolysis is observed in the absence of the peptide substrate (Xie and van der Donk, unpublished results). Hence, either LctM has ATPase activity or a contaminating protein is present that catalyzes this background reaction. Uncoupled ATP hydrolysis has also been reported for the biosynthesis of microcin B17, a nonlantibiotic posttranslationally modified antibiotic produced by *E. coli*.[228,229] The LctM-catalyzed process shown in Figure 21 is a remarkable example of molecular

recognition as only four of the 14 serine and threonine residues are dehydrated without the presence of a clear consensus sequence. Given this exquisite control it is surprising, although not unexpected given the results of in vivo mutagenesis studies on other lantibiotics (section 7.1), that the purified LctM demonstrated permissive substrate specificity processing a series of LctA mutants as well as C-terminally truncated LctA. The study of LctM not only solved the long-standing question regarding the exact function of the novel modification enzymes but also potentially opened the door for future in vitro lantibiotic engineering (section 7.2).[40]

## 4.5. The LanD Enzymes

The lantibiotics epidermin, gallidermin, cypemycin, and mutacin 1140 possess the unusual amino acid *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) (Figures 1 and 6) at their C-terminus. The enzymes responsible for the formation of AviCys are encoded by the *lanD* genes. EpiD, involved in the formation of the AviCys group in epidermin, was isolated by Kupke and coworkers in 1992[230] from the native producer *S. epidermis* Tü3298. The protein contains a stoichiometric noncovalently bound flavin mononucleotide (FMN). In vivo experiments with $His_6$-tagged EpiA[231] as well as in vitro experiments with heterologously expressed and purified EpiD and EpiA[232] revealed the loss of 46 Da from the substrate corresponding to the loss of $CO_2$ and two H atoms. This study represented the first in vitro activity of an enzyme involved in one of the posttranslational modifications of lantibiotics. The entire epidermin prepeptide EpiA, as well as a fragment containing only the structural region underwent decarboxylation, suggesting that the leader sequence of EpiA was not necessary for EpiD action. The substrate specificity of EpiD was probed by constructing a library of heptapeptides containing single amino acid mutations in the C-terminal sequence of EpiA (SFNSYCC).[205,233] EpiD showed low substrate specificity with only the C-terminal cysteine an absolute requirement. This Cys must be present as a free thiol and possess a free carboxylate for activity, suggesting that decarboxylation occurs prior to ring formation. A general consensus sequence for the final three amino acids at the C-terminal was reported to be [V/I/L/F/W/Y/(M)]-[A/S/V/T/C/(I/L)]-C. The mechanism of decarboxylation was investigated by means of heteronuclear correlation NMR using a model peptide KKSFNSYTC that was $^{13}C$-labeled at the $\beta$-carbon of the terminal cysteine.[234] This experiment showed the formation of an unusual enethiol in the product upon the action of EpiD. Addition of this enethiol ($pK_a$ 6.0)[235] to the Dha at position 19 of epidermin, presumably catalyzed by EpiC, would then yield AviCys (Figure 22).

Analysis of the effects of point mutations in an MBP-EpiD fusion protein on the decarboxylation of the substrate peptide SFNSYTC was used to identify the active site residues of EpiD.[236] On the basis of these experiments and its complete conservation in all other homologues, His67 was proposed as an active site base in EpiD. An X-ray structure of EpiD

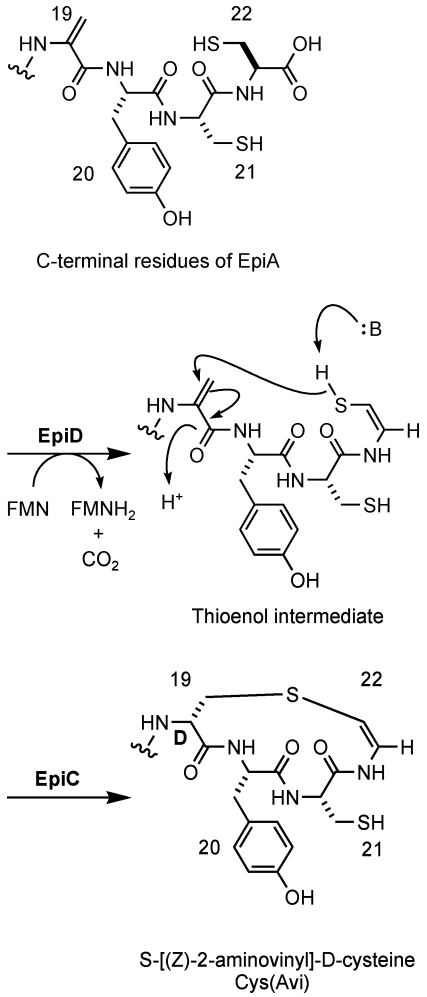



**Figure 22.** Proposed mechanism of EpiD-catalyzed formation of the enethiol intermediate and the putative EpiC catalyzed formation of *S*-[(*Z*)-2-aminovinyl]-D-cysteine.[237]

and H67N-EpiD complexed to a pentapeptide substrate DSYTC showed that it exists as a dodecamer consisting of tetrahedrally placed trimers.[237] Each monomer unit consists of a central parallel $\beta$-sheet domain of six strands flanked by nine helices in a Rossmann-type fold (Figure 23a). The pentapeptide substrate forms a parallel $\beta$-sheet with a $\beta$-strand stretching from Phe149 to Ile151 in EpiD and forms additional backbone hydrogen bonds with Asn117 and Asn14. On the basis of the proximity of the sulfur of the terminal Cys of the substrate to N5 of FMN, the authors proposed a mechanism featuring oxidation of the Cys to the thioaldehyde followed by spontaneous decarboxylation to form a thioenolate, as opposed to direct hydrogen removal from the $C_\alpha$ and $C_\beta$ positions of the Cys (Figure 23b).

The enzyme MrsD involved in biosynthesis of the *S*-[(*Z*)-2-aminovinyl]-(3*S*)-3-methyl-D-cysteine (AviMeCys) residue in mersacidin has also been purified and characterized.[238] Unlike EpiD, MrsD is a flavin adenine dinucleotide (FAD)-containing enzyme with a more stringent substrate requirement. Whereas MrsA proved to be a substrate, neither the EpiA-R30Q mutant nor a short peptide corresponding to the C-terminal eight residues of MrsA were processed by MrsD. However, similar to EpiD, mutation of the conserved His75 to Asn was found to abolish MrsD

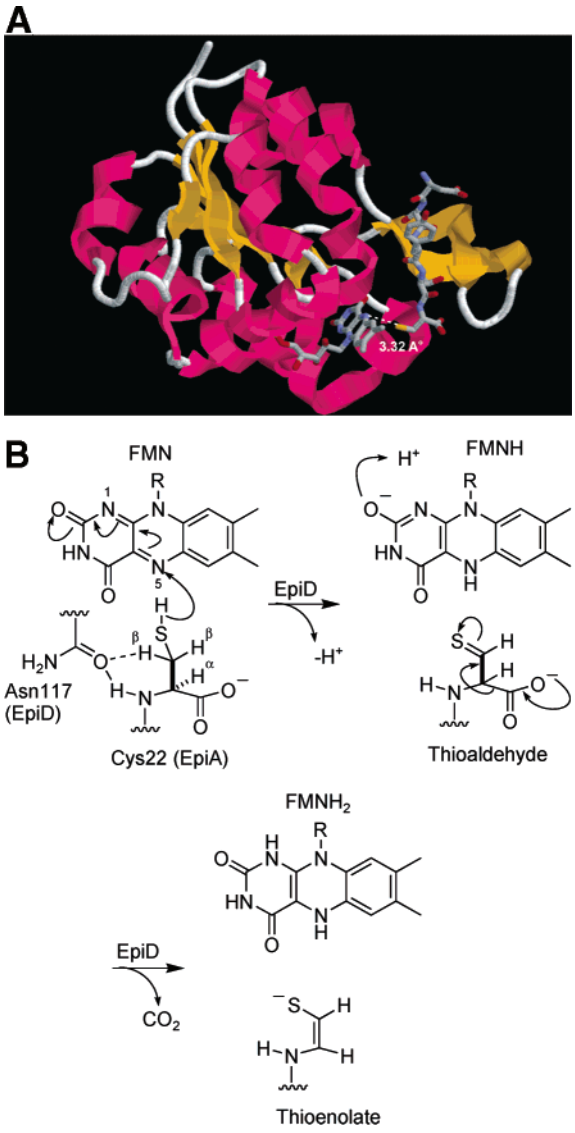



**Figure 23.** (A) X-ray structure of the EpiD His67Asn mutant complexed with a pentapeptide substrate, DSYTC.[237] A single monomer with the Rossmann-like fold is shown as well as the distance between N5 of the cofactor FMN and Cys-$S_\gamma$. Figure generated using the program RASMOL.[469] (B) On the basis of the proximity of $S_\gamma$ and N5 a direct oxidation of the cysteine thiol has been proposed.[237]

activity, suggesting the role of an active site base in decarboxylation or dehydrogenation. Like EpiD, MrsD has a dodecameric structure with a Rossmann-type fold and cofactor binding at the subunit interface.[239] On the basis of their similarity with bacterial[240] and plant[241] decarboxylases, EpiD and MrsD are members of the homooligomeric flavin-containing Cys decarboxylase (HFCD) superfamily.

## 4.6. Other Posttranslational Modifications

Many lantibiotics undergo further posttranslational modifications in addition to the characteristic Lan and MeLan ring formation and the aforementioned formation of AviCys and AviMeCys.[24,163,242,243] Presumably, after removal of the leader peptides nonenzymatic hydrolysis of Dhb at position 1 of Pep5,[7] and Dha at position 1 of lactocin S[102,154] and epilancin K7[96,145] leads to the formation of 2-oxobutyryl and 2-oxopropionyl groups, respectively. Reduction of the 2-oxopropionyl functionality to the 2-hydroxypropionyl group has been reported in the case of epilancin K7[96] and has been proposed for epicidin 280.[72] A putative oxidoreductase EciO was hypothesized to be involved for the latter compound. The stereochemistry of the reduction step is currently unknown. Structural elucidation of cinnamycin and the duramycins[109,174,176] has shown the presence of an *erythro*-3-hydroxy-L-aspartic acid resulting from the hydroxylation of a genetically encoded L-Asp at position 15 (Figures 6 and 12).[172,56] This unusual modification is also found in mammalian proteins, such as the vitamin K-dependent glycoprotein, Protein C,[244] and the epidermal growth factor (EGF)-like domain in human plasma factor IX.[245] The mammalian enzyme responsible for $\beta$-hydroxylation of Asp has been purified from native sources[246]and expressed in *E. coli*.[247−249] The Asp $\beta$-hydroxylase also hydroxylates Asn residues to produce *erythro*-3-hydroxy-L-asparagine. The enzyme is $O_2$/Fe(II)/$\alpha$-ketoglutarate-dependent, and a stoichiometric amount of $CO_2$ is released per Asp hydroxylated.[246] The role of $\beta$-hydroxylation in cinnamycin is currently still uncertain, although this residue is essential for recognition of its target phosphatidyl ethanolamine (section 8.4).

The cinnamycin group also exhibits a head-to-tail lysinoalanine bridge probably formed by addition of the $\epsilon$-amine of Lys19 to Dha6. Lysinoalanine is commonly found in processed and unprocessed food products such as eggs, meats, tortillas, and Chinese noodles, as well as in body organs, where it is possibly formed during the aging process.[250] The formation of lysinoalanine in these cases is due to chemical dehydration of Ser and conjugate addition of Lys to the resulting Dha and produces both diastereomers.[250] It is unclear at present whether the lysinoalanine bridge in cinnamycin is formed by CinM or by one of the genes of unknown function in its gene cluster (section 2).

A number of additional modifications for which the responsible enzymes are currently unknown have been reported. The presence of D-Ala in place of genetically encoded L-Ser is observed in lactocin S[102] and both components of the two-component lantibiotic lacticin 3147 (Figure 6).[112] The mechanism of D-Ala formation may involve stereospecific hydrogenation of the dehydrated serine (Dha) by a hitherto unknown enzyme. Subtilin has been shown to undergo $N^\alpha$-succinylation at late stages of cell growth that leads to a reduction in its biological activity.[128] Cypemycin isolated from *Streptomyces* contains a number of unique modifications including bis-methylation at Ala1 ($Me_2$N-Ala), the presence of *allo*-isoleucine at positions 13 and 18, and a AviCys involving residues 19 and 22 (Figure 9).[103] Finally, the lantibiotic sublancin 168 contains two unprecedented disulfide linkages and contains an additional modification of currently unknown structure (Figure 9).[105] The presence of a single disulfide bridge has also recently been reported for the lantibiotic bovicin HJ50, which awaits complete structural characterization.[119]

### 4.7. Proteases and Transporters

All lantibiotics known to date are ribosomally produced as two-segment precursor peptides consisting of a leader region and a propeptide that undergoes modification by the LanB/C or LanM enzymes. While still attached to the modified propeptide, the leader region has been shown to negate biological activity.[40,77,198,200,201] The *lanP* or *lanT* genes in the lantibiotic operon generally encode the protease responsible for removal of the leader region.[66,79] LanP proteases vary in size depending upon the presence or absence of an N-terminal *sec*-signal sequence and a C-terminal cell wall anchor sequence. They all share homology with the serine protease subtilisin, especially in the sequence near the residues involved in the catalytic triad (Asp, His, and Ser) and the Asn involved in oxyanion hole formation. In the case of the nisin protease (NisP), these residues were predicted to be Asp259, His306, Ser512, and Asn407, respectively.[198] Although the gene translation product of *nisP* corresponds to 682 amino acids and has a predicted mass of about 75 kDa, purification of NisP yielded a protein of only 54 kDa.[198] This loss of mass was attributed to peptidase cleavage of an N-terminal prosequence of 220 residues that contains the *sec*-dependent secretion signal and directs extracellular transport of NisP. The C-terminal 30 residues of NisP contain the sequence LPXTG, which is a consensus sequence involved in anchoring surface proteins in Gram-positive bacteria[251] (Figure 17). The observed in vitro proteolysis of a modified nisin precursor by intact *L. lactis NZ9800* producer cells or *E. coli* cells expressing NisP, but not by cellular supernatants or membrane-free extracts, supports this model.[198] On the other hand, disruption of the *lanT* gene in *L. lactis* N8 led to a mutant strain that accumulated fully processed nisin in the cytoplasm,[74] suggesting at first glance either that NisP is located at the cytoplasmic side of the membrane or that other cytoplasmic proteases in *L. lactis* N8 can also process the posttranslationally modified NisA peptide. Since the full-length NisP protein contains a *sec*-dependent secretion signal, the first possibility is unlikely and to date no intracellular proteases capable of removing the leader have been identified. The cytoplasmic fraction of this mutant strain was obtained by sonication and subsequent centrifugation, and hence the posttranslationally modified prepeptide was probably exposed to NisP during sample preparation leading to proteolytic processing.

The substrate requirement of NisP has been tested with a chimeric substrate consisting of the subtilin leader sequence and nisin Z structural region.[204] Although there is significant sequence similarity between the nisin and subtilin leader peptides (Figure 14), the subtilin leader ends in a Gln instead of the Arg found in nisin. The chimeric substrate was fully processed in *L. lactis* to a subtilin leader−nisin Z product, which did not undergo proteolytic removal of the leader peptide. The importance of an Arg at the cleavage site was also demonstrated in point mutants of the NisA substrate in which Arg(−1) (P1) and Asp(−4) (P4) were replaced, resulting in incomplete cleavage of the leader peptide.[189] In a very recent study, Kuipers and co-workers have shown that in vivo NisP catalyzed removal of the leader peptide occurs only upon formation of the thioether rings in prenisin.[195] Neither the unprocessed prepeptide nor the uncyclized dehydrated peptide were substrates for NisP.

EpiP, the protease responsible for maturation of epidermin, bears an overall 44% sequence identity with NisP. Its gene sequence predicts a 99-residue pre-prosequence that is absent from the enzyme purified from the culture supernatant of *S. carnosus*.[252] The C-terminal anchor sequence found in NisP is absent in EpiP, suggesting the protein is not covalently bound to the cell surface. Incubation of culture supernatants with the epidermin precursor EpiA resulted in cleavage between the Arg(−1) (P1) and Ile1 (P1′) residues, suggesting the protease is excreted as a soluble extracellular protein. Tests of substrate specificity revealed that EpiP was intolerant of mutation at Arg(−1) as it failed to cleave the leader from an EpiA-R(−1)Q mutant. Subsequent homology modeling based on the known crystal structures of subtilisin and other serine proteases predicted that the binding of NisP and EpiP to their respective substrates is dominated by electrostatic interactions at the P1 position.[253] The proteases PepP and ElkP involved in the maturation of Pep 5[75] and epilancin K7,[145] respectively, bear 44% sequence identity to each other and only about 20% sequence identity to NisP. The lack of an N-terminal *sec*-signal sequence as well as a cell wall anchor sequence suggests they are intracellularly localized. LasP, the homologous protein from the lactocin S biosynthetic cluster,[155] also lacks the pre-pro sequence and is probably localized within the cytoplasm.

The subtilin biosynthetic gene cluster does not contain a dedicated protease. Experiments conducted with subtilin−nisin Z prepeptide chimeras in *B. subtilis* resulted in removal of the leader region,[203] indicating that the substrate specificity of the protease(s) involved in subtilin production is more relaxed than that of NisP, which did not remove the leader peptide from this chimera as mentioned before. The observation that subtilin could also be obtained by cleavage of the leader peptide upon incubation with culture supernatants from a nonproducing strain[200] suggested the action of nonspecific proteases in subtilin maturation. Entian and coworkers have recently shown that at least three extracellular serine proteases, subtilisin (AprE), WprA, and Vpr may activate subtilin to its mature form.[77]

The two-component lantibiotic cytolysin is comprised of the peptides $CylL_L$ and $CylL_s$. They undergo stepwise proteolysis starting in the cytoplasm of the cell.[115] The leader sequence of each peptide was trimmed by an unknown protease to leave an identical six-residue tail attached to the structural region of each component. Those peptides were then secreted to the extracellular medium where the serine protease CylA (the cytolysin nomenclature differs from the general classification of lantibiotic genes) removed the last vestiges of the leader sequence and generated the active lantibiotic. Mixtures of the partly processed CylL peptides were found to have


**Figure 24.** Sequence alignment of the N-terminal domains of ABC-transporters involved in lantibiotic or class II bacteriocin proteolytic processing. Lantibiotic transporters: LctT (lacticin 481), MutT (mutacin II), SalT (salivaricin A), ScnT (streptococcin SA-FF22). Class II bacteriocin transporters: LcnC (lactococcin A), PedD (pediocin PA-1), LagD (lactococcin G). Completely conserved residues are highlighted in blue with the proposed catalytically active residues in red.[79] Alignment created with CLUSTAL W (v1.82) and numbering based on LctT sequence. Accession numbers: LctT, AAC72259; MutT, AAD01806; SalT, AAG32538; ScnT, AAB92603; LcnC, AAK04177; PedD, AAA25561; LagD, P59852.

biological activity only in the presence of CylA establishing its role in the final proteolytic step. CylA is a serine protease similar to EpiP in that it is exported outside the cell but does not anchor to the cell surface. It undergoes proteolytic activation by the loss of a prosequence comprising of 95 amino acids. Homology modeling of the interaction of CylA with the posttranslationally modified CylL substrates indicated a dominant electrostatic interaction between the Glu-P1 side-chain and His180 of the S1-binding pocket of CylA.[115]

Lantibiotics are generally transported to the extracellular medium by the LanT family of ATP-dependent transporters. There is some uncertainty as to the absolute necessity of a LanT protein for all lantibiotic systems, as evidenced by the nonrequirement of PepT in extracellular transport of Pep5 in *S. epidermis*[75] and the absence of a *lanT* gene for epicidin 280.[72] Conversely, in the case of nisin, it has been shown that NisT is an absolute requirement for extracellular transport. Deletion of the *nisT* gene in *L. lactis N8* led to a build-up of processed nisin in the cytoplasm and no extracellular nisin was detected.[74] NisT has relaxed substrate specificity as demonstrated by its ability to transport not only fusions of the leader region with processed or unprocessed forms of pronisin but also with peptide fragments from enkephalin and angiotensin.[195] Extracellular export of a protein consisting of the subtilin leader sequence fused to alkaline phosphatase in the subtilin nonproducer strain *B. subtilis 168* demonstrated recognition and transport in the absence of SpaT, albeit with lower efficiency.[196]

The type AI group of ATP binding cassette (ABC) transporters represented by NisT and SpaT are about 600 amino acids in length and bear significant homology to hemolysin B-like ATP-dependent transport proteins present in a range of organisms.[73,254] These proteins contain a hydrophobic N-terminal domain, a six-helix membrane spanning domain, and a C-terminal ATP-binding domain. In comparison, most of the type AII LanT enzymes such as LctT,[148] MutT,[255] and SunT[105] are about 700 residues long and contain an extra N-terminal peptidase domain besides the membrane spanning and C-terminal ATP-binding domains. The absence of a *lanP* equivalent in the gene clusters that encode these proteins suggests a role for the N-terminal peptidase domain in processing of the leader peptide concomitant with export. Indirect evidence for this model is found in the gene cluster of the type AII lantibiotic lactocin S, which encodes an abbreviated 535 amino acid LasT protein but also encodes a subtilisin-like LasP protein.[155] Although the protease function of a LanT protein has not been demonstrated, experiments with homologous transport proteins (Figure 24) responsible for class II bacteriocin production have shown peptidase activity in vitro and in vivo.[79,256,257] Håvarstein and co-workers reported in vitro cleavage of both precursors of the two-component bacteriocin lactococcin G by incubation with the N-terminal domain of 150 amino acids of the transporter LagD.[79] Investigation of the membrane topology of the lactococcin A transport protein (LcnC from *L. lactis*) revealed that both the N-terminal protease domain and the C-terminal ATP binding region were located at the cytoplasmic side of the cell membrane.[256] In accord with this finding, overexpression of the N-terminal 164 amino acids of LcnC in the wild-type lactococcin A producer along with the LcnA prepeptide led to the formation of mature LcnA in the cytoplasm. The leader sequences of the lantibiotics lacticin 481, mutacin II, streptococcin A-FF22, salivaricin A, and variacin exhibit significant similarity to these class II bacteriocin leader sequences including the double-glycine type cleavage site (Figure 15) where processing takes place. However, despite a 47% sequence identity with LctT and the presence of conserved Cys and His residues that suggests they are both cysteine proteases, the lactococcin A trans-

porter LcnC was not able to secrete lacticin 481 in an *L. lactis* strain bearing the LctA and LctM genes.[258] This suggests that the LanT proteins recognize regions of the substrate other than the double Gly motif alone.

### 5. Regulation of Lantibiotic Production

Lantibiotic-producing bacteria must maintain an inherent balance between bacteriocin production and immunity to their product. The production of these compounds is regulated such that it typically takes place late in the exponential growth phase. For instance, Engelke et al. monitored the growth-dependent expression of prenisin and NisB by using antibodies directed against the prepeptide and dehydratase.[259] Prenisin expression was highest when cells were in their mid-logarithmic growth phase; at earlier times during growth nisin is produced and excreted but remains adsorbed to the membrane and is released when the pH drops below 5.5.[260] NisB expression was not observed in the first 4 h of growth and increased in the late logarithmic state.[259] For many lantibiotics (e.g., nisin,[259] subtilin,[261] mersacidin,[262] streptococcin A-FF22,[263] and salivaricin A)[157] production is controlled by a typical two-component regulation system comprised of a receptor-histidine kinase (LanK) and a transcriptional response regulator (LanR, e.g., Figure 5).[264] In bacteria, these systems are often involved in quorum sensing, the intraspecies communication process that allows cells to sense other organisms in their surroundings in a cell-density-dependent manner.[265−267] The receptor-histidine kinases are present on the cellular surface and are involved in detecting extracellular changes, leading to a signal cascade initiated by autophosphorylation of a histidine residue. A typical case is exemplified by the nisin regulatory system[259,268−270] in which the phosphoryl group from NisK is transferred to an aspartate on NisR that initiates its binding to the *nisA* and *nisF* operators (see Figure 3 for transcriptional units). This in turn activates transcription of the *nisABTCIP* operon involved in nisin biosynthesis as well as *nisFEG* involved in self-immunity (section 6).[260,268] The *nisRK* genes themselves are under control of a separate *nisR* promoter.[268] Similar to nisin, most lantibiotic producers contain operons that include both the structural gene for the prepeptide as well as the biosynthetic enzymes (e.g., see Figure 3). Typically, the DNA region downstream of the structural gene allows limited readthrough to the biosynthetic genes (leaky termination of transcription) to ensure the desired stoichiometry between the substrate for posttranslational modification and the modification machinery (e.g., refs 155 and 271).

Nisin,[269] subtilin,[272] and salivaricin A[157] have been shown to serve as the sensing molecules that trigger the transcription of their prepeptides in an extracellular autoregulatory mechanism. In the case of nisin, this system is extraordinarily efficient in that as few as five nisin molecules are sufficient to activate transcription.[269] Strains of *L. lactis* with a four-base pair deletion in the middle of *nisA* (Δ*nisA*) such that they could not produce nisin were used by Kuipers et al. to monitor the effect of nisin A as an initiator of *nisA* transcription.[269] The authors showed that in the absence of any external inducers, Δ*nisA* was not transcribed. Upon addition of nisin A, transcription of Δ*nisA* was observed in a concentration-dependent manner. On the other hand, unmodified synthetic nisin A precursor peptide did not induce transcription, providing evidence that the posttranslational modifications within the nisin peptide are required for induction of *nisA* expression. Subtilin, lacticin 481, and Pep5 did not stimulate transcription. The structural requirements for induction were probed by utilizing synthetic nisin A fragments.[269] Truncated peptides lacking the first two N-terminal residues or composed of just the B- and C-, or the D- and E-rings did not retain induction capacity, whereas a synthetic fragment containing the first 11 residues including the A- and B-rings was sufficient for transcriptional activity. Nisin Z and several nisin Z mutants were also shown to be transcriptional activators of Δ*nisA*, but not all mutants retained efficient signaling capability (e.g., S3T in which ring A has a MeLan, Val32Glu/Dha33Ser, and Val32Trp/Dha33Ser).[269,273] In some cases, these nisin mutants stimulated transcriptional activity only at very high concentrations.[273] Disrupted or strongly reduced signal transduction potency is important for lantibiotic engineering (section 7) as mutants with weaker inductive properties may not be produced to the levels required to trigger the autoregulatory mechanism to support continued lantibiotic production.

Interruption of NisK expression yielded a strain of *L. lactis* that did not produce nisin upon addition of varying amounts of nisin A or Z, indicating its involvement in the signal transduction cascade.[269] To evaluate the minimal requirements for efficient induction of *nisA* transcription, the genes for *nisR* and *nisK* were incorporated into the chromosome of a nonlantibiotic producing *L. lactis* strain transformed with a plasmid carrying the *gusA* reporter gene under control of the *nisA* promoter. Addition of nisin to this strain resulted in *gusA* expression, illustrating that NisK and NisR are the only requirements for nisin activation of gene expression. The two-component regulatory system not only controls expression from the *nisA* promoter but also from the *nisF* promoter that regulates the *nisFEG* immunity genes (section 6),[260,268] whereas transcription from the *nisR* promoter that controls expression of NisR and NisK is nisin independent.[268] Two sets of TCT direct repeats located 39 and 107 bp upstream of the *nisA* transcription start site have been proposed as the putative binding site for NisR,[274] and as described below such binding has been established for the analogous system involved in subtilin production. The high efficiency of the nisin regulatory system has found utility as a heterologous-controlled protein expression system in food-grade lactic acid bacteria (NICE = nisin-controlled expression).[275−287]

Nisin-induced stimulation of its own biosynthesis is not the only mechanism of enhancing *nisA* transcription. It has been reported that transcription from the *nisA* promoter can also be induced in the absence of externally supplied nisin in a carbon-source-

dependent fashion, with galactose and lactose enhancing transcription significantly.[288] The *nisRK* signal transduction system was not involved, and the nisin and galactose/lactose induction regulators were shown to compete for the same recognition site. Galactose and lactose do not induce transcription from the *nisF* promoter. A possible rationale for this differential expression may be found in the presence of two TCT-$N_8$-TCT repeats upstream of the *nisA* start site and only a single such repeat upstream of the *nisF* start site.[274]

To understand the regulation of subtilin biosynthesis, experiments analogous to those performed with nisin have been carried out.[216] Deletions within the *spaR* and *spaK* genes resulted in failure to express SpaB and SpaC and eliminated subtilin production. Complementation with a plasmid encoding the *spaR* gene sequence restored the ability to produce the lantibiotic in the *spaR* mutant. Two molecules of the SpaR protein have been shown to bind to the *spaS*, *spaB*, and *spaI* promoter regions (Figure 5), which contain a pentanucleotide repeat separated by six nucleotides as the recognition motif (*spa*-box)[289] that is similar to that found in the *nisA* and *nisF* promoters. This *spa*-box is located upstream of the transcription initiation sites for all three promoters (*spaS*, *spaB,* and *spaI*).[271] While the subtilin and nisin systems are very similar in that both possess a LanRK signal transduction pathway that is autoinduced by the respective lantibiotic, an additional regulatory system governs subtilin biosynthesis. Stein et al. recently demonstrated a positive regulatory system for *spaR* expression utilizing sigma factor H (*sigH* gene), an endogenous regulator within subtilin producing *B. subtilis* strains that is also under cell-density-dependent control.[272] An additional *B. subtilis* regulator, AbrB, appears to negatively regulate lantibiotic production as strains lacking *abrB* exhibit a significant increase in the production of subtilin.[272]

The similarity between the nisin and subtilin regulatory systems has been illustrated via cross-talk experiments involving the incorporation of SpaK into the nisin induction system.[55] A plasmid encoding a reporter gene under control of the *nisA* promoter was introduced into a bacterial strain that contained the *nisR* gene on its chromosome. Upon introduction of a plasmid containing the *nisK* gene sequence, a completely functional nisin induction system was established, which used nisin as a transcriptional activator. Gene expression was also accomplished by introduction of a plasmid encoding *spaK* and using subtilin as inducer, illustrating that both SpaK and NisK can phosphorylate NisR upon activation by their respective lantibiotic. Furthermore, chimeric NisK−SpaK proteins, in which the N-terminus corresponded to that of SpaK while the C-terminal domain originated from NisK, can modulate the specificity of the inducer.[290] When this protein was expressed in place of NisK in a *L. lactis* strain equipped with the nisin signal transduction machinery, it resulted in a functional hybrid sensor kinase that activated transcription of the *nisA* promoter in the presence of subtilin. Not only do these results provide evidence that the N-terminal portion of the NisK protein is responsible for molecular interactions with the inducer, but they also suggest the numerous possibilities for protein engineering that are available within the lantibiotic family.

Epidermin production in *S. epidermis* Tü3298 is regulated by an accessory gene regulator (*agr*) quorum sensing system[267] that is also responsible for the transcriptional activation of many surface proteins in various strains of staphylococci.[291] The *agr* locus contains the response regulator AgrA, a sensor kinase AgrC, a pheromone precursor (AgrD), and AgrB, which is responsible for pheromone maturation. A transcription profiling study identified a lantibiotic locus that was under control of *agr*,[292] but the organism under investigation has not been shown to produce a lantibiotic and the mechanism of epidermin regulation by *agr* was not clear. Kies and co-workers recently demonstrated that unlike the nisin and subtilin systems in which interference with LanK/R directly affects lantibiotic production by blocking transcription of the biosynthetic genes, disruption of the *agr* regulatory system does not interfere with the epidermin biosynthetic proteins that introduce the dehydro amino acids and thioether bridges.[293] Instead, *agr* in *S. epidermis* controls removal of the leader peptide from posttranslationally modified preepidermin. It was suggested that the cleavage of the prosequence from EpiP, the protease responsible for removing the leader sequence (section 4.7), may be regulated by *agr* resulting in control over the activity of EpiP.[293]

The epidermin gene cluster also contains the *epiQ* gene that is essential for epidermin production.[294−296] EpiQ belongs to the family of response regulators, but the epidermin gene cluster does not contain a gene for a corresponding receptor histidine kinase. EpiQ is responsible for activating transcription from the *epiA*, *epiF*, *epiH*, and *epiT* promoters that leads to the expression of the proteins necessary for epidermin synthesis (*epiABCD*) and immunity (*epiFEG*, *epiT*, *epiH*, see section 6). Interference with the transcription of *epiQ* leads to complete loss of lantibiotic production. Peschel et al. showed that EpiQ binds directly to an inverted repeat that is the putative operator site for expression from the *epiA* promoter.[294]

Regulation of production of the type B lantibiotic mersacidin involves two LanR type proteins (MrsR1 and MrsR2) and one LanK sensor protein (MrsK2).[297] The adjacent *mrsR2* and *mrsK2* genes (Figure 3) encode proteins with significant similarity to the two-component regulatory systems present in nisin and subtilin biosynthetic operons, whereas the MrsR1 protein is an additional response regulator. Mersacidin-producing *Bacillus* strains lacking *mrsR1* were unable to produce mersacidin but retained self-immunity properties. On the other hand, inactivation of *mrsR2*/*K2* led to increased susceptibility to the lantibiotic with biosynthesis remaining intact. In the *mrsR2*/*K2* knockout mutant, the *mrsFGE* genes encoding an ABC-type transporter were not transcribed, suggesting they are involved in self-protection (section 6). These results provide evidence that

the mersacidin producer utilizes the MrsR2/K2 tandem to activate transcription of immunity genes and the MrsR1 protein is responsible for promoting biosynthesis of the lantibiotic.[297] At present, the stimulus for MrsR1 activation is unknown, nor is it clear whether MrsR1 requires phosphorylation for activity or which kinase would be involved. The results described above do rule out MrsK2 as the kinase. Other single regulatory proteins engaged in lantibiotic biosynthesis that lack a corresponding histidine kinase are the aforementioned EpiQ as well as MutR, LasX, and LtnR (see below).

Despite the high homology in the structures of the type AII lantibiotics, the regulation systems for these compounds are quite diverse. The gene cluster of lacticin 481 lacks regulator genes corresponding to the LanKR proteins. It was recently shown that lacticin 481 is regulated at the transcriptional level by pH control of P1 and P3 promoters located upstream of *lctA* in the lantibiotic operon.[298] During growth *L. lactis* produces lactic acid, which in turn leads to a decrease of the pH of the growth medium from 7.0 to 5.8. This natural acidification correlates to the amount of lacticin 481 that is produced. Medium that was acidified to pH 5.8 using acetic acid prior to lacticin production, although resulting in a slower growth rate, actually led to a higher production level of lacticin 481, indicating that control of lantibiotic expression is pH controlled, not lactic acid induced. Since the lacticin 481 operon does not contain a dedicated regulation system, transcription from the P1 and P3 promoters is probably governed by a general regulator.

Mutacin II production requires the MutR regulatory protein, but its biosynthetic gene cluster lacks a sensor histidine kinase analogous to NisK or SpaK. Although mutacin II is structurally very similar to lacticin 481 (Figure 9), their biosynthetic regulation systems are quite different. Mutacin II transcriptional regulation is controlled by the *mutA* and *mutR* promoters, and the *mutR* gene[299] encoding a protein homologous to the family of Rgg (regulator gene of glucosyltransferase) transcription regulators.[300] Activation of the *mutA* promoter responsible for transcription of the *mutAMTFEG* operon is dependent on MutR as well as currently unknown components in the growth medium.[301] Inactivation of the gene encoding MutR eliminates transcription of all genes in the *mutA* operon, including *mutA*, *mutM*, and the immunity genes *mutEFG*.[301] Although direct binding of MutR to the *mutA* promoter site was not determined and the protein sequence does not show obvious DNA binding motifs, the homology to Rgg is consistent with its direct interaction with DNA.

Unlike mutacin II and lacticin 481, regulation of the type AII compound salivaricin A in *S. salivarius* UB1309 is much more similar to the type AI lantibiotics nisin and subtilin. It also autoregulates its own production through a *salKR* two-component response system.[157] As mentioned in section 3.4, *S. pyogenes* strains produce very close relatives such as [Lys2,Phe7]-salivaricin A (salivaricin A1). Addition of salivaricin A1 to the growth medium of *S. salivarius* UB1309 induced transcription of the *salA* gene, showing that signaling not only occurs intraspecies but also interspecies.[157]

Dedicated repressors of lantibiotic gene expression have only been described for the two-component lantibiotics lacticin 3147 and cytolysin, and for lactocin S. McAuliffe et al. reported characterization of LtnR, the first example of a repressor encoded in a lantibiotic biosynthetic operon (see also section 6).[227] Biosynthesis of lacticin 3147 in *L. lactis* ssp. *lactis* DPC3147 is under control of the constitutive $P_{bac}$ promoter that governs the transcription of the *ltnA*$_1$*A*$_2$*M*$_1$*TM*$_2$*D* operon. A second divergently transcribed transcriptional unit *ltnRIFE* (Figure 3) responsible for immunity is negatively regulated by LtnR, which was shown to bind to the $P_{imm}$ promoter for this operon. A second interesting case of repression of lantibiotic production has been reported for cytolysin.[302] Mutational inactivation of either or both *cylR1* and *cylR2* led to the constitutive expression of high levels of *lacZ* placed under the control of the cytolysin promoter pL in an engineered strain, whereas in the presence of both genes no expression was observed unless the fully modified $CylL_S$ peptide was added to the growth medium. Adding $CylL_L$ or incompletely processed $CylL_S$ or $CylL_L$ did not induce transcription. These results indicate that CylR1 and CylR2 together are needed for repression of the expression of the biosynthetic machinery. This block is alleviated when $CylL_S$ reaches a certain threshold concentration, which corresponded to about $10^7$ colony-forming units per mL of the producer *Enteroccus faecalis*.[302] Hence, this is another example of quorum sensing that leads to autoinduction of lantibiotic production and differs from that described above for nisin, subtilin, and salivaricin A. CylR1 lacks homology to other known proteins and the CylR2 protein is a member of the helix−turn−helix family of DNA-binding proteins. Therefore, this system is distinct from other two-component signal transduction systems. A preliminary report of expression and crystallization of the transcriptional repressor CylR2 has recently appeared.[303] A third example of a repression system is found for lactocin S, the production of which is modulated by LasX, a protein that like MutR has significant homology to Rgg-type proteins. Interestingly, LasX serves as an activator of the promoter for transcription of the *lasAMNTUVPJW* operon and as repressor of the overlapping promoter for the divergently transcribed bicistronic *lasXY* operon (Figure 3).[304] It was proposed that this dual action may aid in maintaining a steady rate of lactocin S production.

### 6. Self-Immunity of the Producing Strains

Any bacterial strain producing antimicrobial compounds that are active against closely related strains must protect itself against its product. Although the picture of these mechanisms is still emerging for the lantibiotics, a number of recent studies have provided the first insights for some family members. Early work on the characterization of the nisin biosynthetic gene cluster revealed the presence of the *nisI* gene.[259,305] The NisI protein (245 amino acids) shows no homology with other proteins and has a hydro-

phobic N-terminus containing a consensus lipoprotein sequence.[305] It is membrane anchored by posttranslational removal of the first 19 amino acids and lipid modification of the Cys at the new N-terminus. Palmitoylation of NisI was confirmed by Qiao and coworkers[199] in both *E. coli* and *L. lactis* strains using [$^3$H]-palmitic acid. SDS−PAGE and Western blotting using anti-NisI antibodies showed that the protein is localized extracellularly at the cytoplasmic membrane. More recently, Koponen et al. showed that a significant percentage of NisI is secreted in a lipid-free form into the cytoplasm.[306] The presence of extracellular NisI may have a biological function, complexing external nisin before it can aggregate at the cell surface, thereby acting as an additional self-protection mechanism. Expression of *nisI* in nisin-sensitive *L. lactis* strains results in modestly increased levels of resistance,[199,214,259,305] indicating that the production of NisI is correlated to sensitivity to exogenous nisin but that NisI by itself does not impart full immunity. The extracellular membrane association may facilitate direct interaction with nisin at the exterior of the cell thereby possibly inhibiting pore formation. Circular dichroism spectroscopy and biomolecular interaction analysis have been used to demonstrate that NisI and nisin do interact with each other,[307] and interactions of purified $His_6$−NisI with nisin but not subtilin were detected by SDS−PAGE.[308] The lack of strong sequence similarity among the LanI proteins has been attributed to the specific nature of the recognition between the LanI protein and the lantibiotic against which it protects.[305]

Near wild-type levels of immunity could be conferred on nisin-sensitive strains via complementation with a plasmid encoding NisI as well as the prepeptide (NisA) and the posttranslational modification machinery.[305] At the time, the need of nisin production for immunity was not understood, but with the discovery that nisin is a quorum sensing molecule that regulates its own production as well as transcription of immunity genes (section 5),[269] the role of nisin production in immunity was revealed. In support of this model, strains containing disruptions or in-frame deletions of the *nisB*, *nisC*, and *nisT* genes were highly sensitive to externally added nisin, but upon preincubation with nisin, they regained about 20% of wild-type level immunity.[307] These gene disruptions led to the abolishment of *nisFEG* transcription in the absence of exogenous nisin, whereas prior addition of nisin resulted in increased production of NisFEG, thereby implicating these proteins in immunity. Since *nisFEG* transcription is under control of the NisR/K regulation system (section 5), these findings also explained why they are necessary for inducing immunity and why strains of nisin-producing *L. lactis* with NisK mutations are sensitive to nisin.[309] A similar role of the final lantibiotic product in self-protection was established for the salivaricins.[157] Collectively, these results indicate that full nisin immunity requires three components: nisin production and expression of the *nisI* and *nisFEG* genes. Interruption of either *nisI* or *nisFEG* results in strongly decreased immunity levels, indicating that these proteins actually operate in a cooperative manner.

The *nisFEG* genes were first identified by Siegers and Entian in 1995.[310] The NisE and NisF proteins are homologous to members of the type B ABC transporters of the HisP family.[311−313] NisF contains two potential ATP-binding sites, whereas NisE with NisG is thought to form the integral membrane segment of the transporter. NisG is a hydrophobic protein with sequence similarity to the immunity proteins found for several channel-forming colicins.[314,315] The latter proteins are believed to interact directly with the pore-forming domains of the corresponding peptides. Investigations into the HisP and MalK secretion systems showed that a complete complex contains two hydrophobic membrane spanning subunits and two ATPase subunits.[316] The similarity with the $MalFGK_2$ and $HisMQP_2$ transport systems involved in maltose[317] and histidine[318] transport, respectively, in *E. coli* suggests that the complex for nisin (and other lantibiotics, see below and Figure 5) consists of $NisF_2EG$. Individual disruptions of the *nisE*, *nisF*, and *nisG* genes did not affect the ability to produce nisin, indicating that their gene products are not directly involved in lantibiotic biosynthesis, but did lead to increased sensitivity to the lantibiotic.

Stein et al. reported that the genes involved in nisin immunity in *L. lactis* could produce the same phenotype in *B. subtilis* strains via coordinated expression.[308] *B. subtilis* cells expressing NisI showed significant and comparable immunity to nisin as cells containing the NisFEG transport proteins while cells transformed with both *nisI* and *nisFEG* displayed full nisin tolerance. Interestingly, nisin was associated in much decreased levels with cells expressing NisFEG than with control cells, suggesting the NisFEG proteins act by transporting nisin from the membrane into the extracellular space.

The proteins involved in self-protection of *B. subtilis* against subtilin were first characterized by Klein and Entian in 1994.[319] Like most other LanI proteins, SpaI is a mostly hydrophilic lipoprotein with an N-terminal hydrophobic domain postulated to anchor the protein in the cell membrane. The SpaE, SpaF, and SpaG proteins have a great deal of sequence similarity to ABC-transporters located in the biosynthetic gene clusters of other lantibiotics including NisE, NisF, and NisG (note: originally *spaE* and *SpaF* were reported to be one *orf*, *spaF*,[319] but they were later shown to consist of two genes).[272] Klein and co-workers demonstrated that these proteins are required for immunity and that their disruption does not lead to the abolition of subtilin production by the cell.[319] In a further similarity to the nisin immunity system, disruption of *spaS* led to subtilin-sensitive producer strains, which is well explained by the same type of autoregulation of expression of the immunity genes as reported for nisin.[272]

Epidermin-producing *S. epidermidis* strains also contain the *epiFEG* genes for self-immunity.[295] Their inactivation led to complete loss of immunity against epidermin, and expression of *epiFEG* in an epidermin-sensitive strain conferred a significant level of immunity. As mentioned above, it has been proposed

that the LanFEG proteins may scavenge lantibiotics that have penetrated the cytoplasmic membrane and secrete them into the extracellular medium.[295,320] Experimental support for this model has been reported by comparing epidermin concentrations in the supernatant of cells that do or do not express the *epiFEG* genes.[320] The efficacy of releasing epidermin from the cell surface was much higher than that with nisin, showing specificity of the transport system. A variation on this hypothesis proposed by Bierbaum and co-workers[321] has the LanEFG proteins detach the lantibiotic from its cellular target lipid II (see section 8.2). This proposal is supported by the observation that *lanEFG* genes are found in the clusters for nisin, epidermin, and mersacidin, which all interact with lipid II but are absent from the cluster of Pep5, which does not bind to lipid II.[322] A corollary of this hypothesis is that lantibiotics such as lacticin 481, mutacin II, streptococcin A FF-22, and lacticin 3147, which currently have not been reported to bind lipid II but do contain *lanEFG*, also have a cellular target.

More recently, the accessory factors *epiH* (formerly *epiT″*)[69,76] and *gdmH* that are unique to the epidermin and gallidermin gene clusters, respectively, were shown to mediate immunity by controlling lantibiotic secretion.[323] Since the EpiT transport system in the producer of epidermin is defective because of a 20-bp deletion, studies on the role of EpiH and GdmH have focused on gallidermin producing strains; gallidermin differs in only a single amino acid from epidermin (section 3.2). The EpiH and GmdH proteins were first shown to aid secretion of epidermin and gallidermin by GmdT, the one-component transport system responsible for lantibiotic export after posttranslational modifications.[296] The need for ancillary proteins for the assembly of active ABC-type transporters is not uncommon and has been described for other systems.[324] More recently, it was shown that a plasmid containing only the *gdmH* gene conferred a 2-fold decrease in sensitivity to gallidermin in the heterologous expression host *S. carnosus*, illustrating that *gdmH* also has a role in immunity.[323] Expression of the EFGHT proteins involved in gallidermin and/or epidermin production resulted in a much higher level of immunity than expression of only EpiFEG, and expression of the EpiEFGH proteins led to an immunity level equal to the additive effects of the EpiFEG and GdmH proteins. These studies demonstrated that while the GdmT protein is not necessary to confer protection, it is required for full cooperativity between the EFG and H proteins. These proteins did not confer any immunity to nisin, providing further evidence for the high specificity of the LanEFG proteins.[323]

The immunity mechanisms for bacterial strains responsible for producing epicidin 280,[72] Pep5,[325] and lactocin S[155] are quite similar on a genetic level. The operons for the biogenesis of all three compounds contain *lanI* genes but lack genes that encode LanEFG-type transporter proteins. The EciI and PepI proteins share 74.2% sequence identity, and EciI has significant similarity (42.1%) to the gene product of ORF57 in the lactocin S biosynthetic gene cluster.[72] All of these proteins have a similar size and charge distribution.[326] PepI (69 amino acids) is characterized by an apolar N-terminus and a hydrophilic C-terminal domain with a net positive charge. Early studies implicated a role of PepA production in immunity in the producing strain of Pep5,[325] suggesting possibly a similar regulation of self-protection by the mature product as described for nisin. Pag and co-workers[327] have subsequently shown, however, that *pepA* transcription plays a different essential role. They showed by complementation studies with plasmids encoding either *pepI* or *pepA* that *in trans* complementation is not sufficient to confer immunity against Pep5, but when both genes are encoded on the same plasmid immunity similar to that of the wild-type strain was observed. This unusual observation was explained by a stabilizing effect on the PepI mRNA by an inverted repeat located downstream of the *pepA* gene sequence, which also functions as a weak terminator that allows partial read-through to the *lanPBC* genes.[327] This inverted repeat is proposed to form a hairpin that protects the PepI mRNA from ribonucleases, possibly via direct binding. Hence, *pepA* transcription is necessary for efficient expression of PepI. The position of this stabilizing inverted repeat is not important, however, because placing the terminator sequence upstream of *pepI* resulted in a strain that was hyperimmune to Pep5. A dual role of the leaky transcriptional terminator downstream of a *lanA* has also been proposed for lactocin S, although in this case it protects the *lasA* transcript.[155] PepI confers cross immunity against epicidin 280 with which its shares 75% identity, the only such case in lantibiotics producing strains that has been reported.[242]

Recently, the localization of PepI has been studied using protein fusions with green fluorescent protein (GFP).[326] These PepI−GFP constructs revealed that PepI is found at the cell wall−membrane interface. Truncated proteins and site-directed mutants were generated to determine the functional role of the two domains of PepI. Introduction of charged amino acids into the apolar N-terminus blocked export of PepI, while shortening the C-terminal portion did not affect the localization of PepI but reduced immunity. These experiments illustrated that the two domains probably have distinct functions: the N-terminus serves a role in localization of the protein, while the C-terminal end is involved in conferring immunity against Pep5.

While investigating regulation systems utilized in mersacidin producers, Guder and co-workers were able to indirectly ascertain modes of self-protection as well.[321] In a mersacidin-producing strain, the *mrsR2/K2* genes were knocked out to investigate the role of these gene products in regulation (section 5). The resulting bacterial strain maintained its ability to produce mersacidin, but its sensitivity to its product increased significantly, although not as dramatically as with NisI inactivation in *L. lactis*. Analysis by reverse transcription-PCR revealed that in this mutant the *mrsEFG* genes had not been transcribed, therefore showing that lack of MrsEFG protein expression leads to loss of immunity.[321] These proteins have significant similarity to the LanEFG

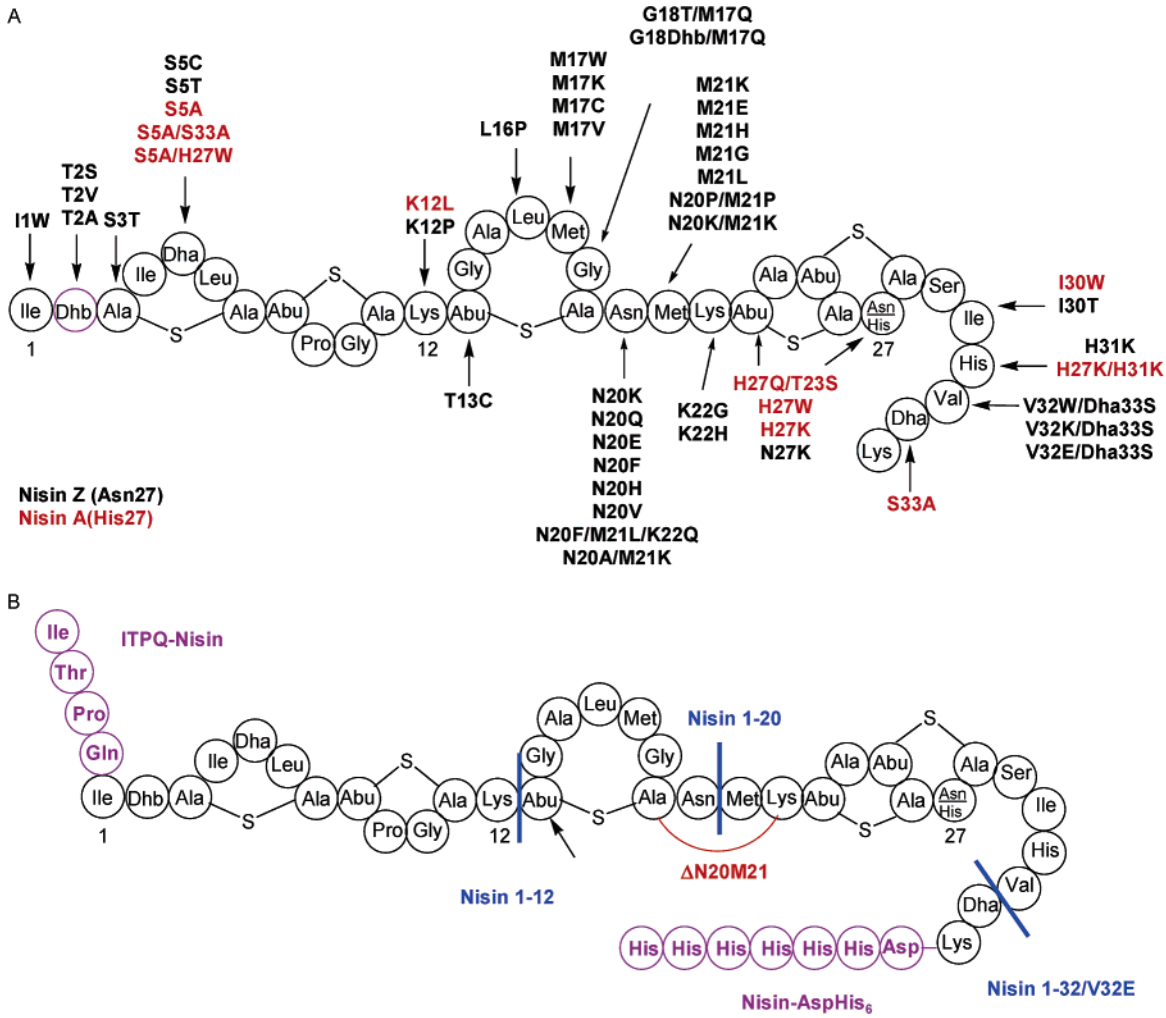



**Figure 25.** Representation of variants of nisin that have been reported. (A) Mutants that have been generated by site-directed mutagenesis.[48,136,273,331,335,337−339,378,389,470] Shown in black are mutants in the nisin Z background and in red mutants in the nisin A background. (B) Truncation,[273,392,416,471] contraction,[48] or extension[383,385] mutants were obtained either through molecular biology or by chemical[471] or proteolytic techniques.[392]

transporter systems described above, and it is believed that they actively extrude the lantibiotic.

In the gene cluster encoding the biosynthetic proteins involved in lacticin 481 production three genes (*lctEFG*) were identified with significant similarity to other *lanEFG* genes. Rincé and co-workers demonstrated that strains containing all three genes were immune to lacticin 481, while the absence of any one protein resulted in loss of immunity.[328] Homologues of the *lanFE* genes were also identified in the biosynthetic cluster of lacticin 3147 (*ltnFE*),[329] but they do not appear to play an important role in immunity. Instead, expression of *ltnI* is sufficient to confer levels of immunity to sensitive strains that are comparable to that of producing strains. LtnI is predicted to be a protein of 116 amino acids and bears no homology to any of the other LanI proteins.[329]

It is interesting to compare the various immunity mechanisms in the lantibiotics. Nisin, subtilin, epidermin, lacticin 3147, streptococcin A FF-22,[263] mutacin II,[255] and lacticin 481 all have the *lanFEG* genes (Figure 3), whereas Pep5, cytolysin,[330] epicidin,[72] lactocin S, and epidermin only require *lanI* for immunity. Interestingly, the compounds that both form pores and utilize lipid II as a docking molecule to increase the efficiency of pore formation and also interfere with peptidoglycan formation (nisin and epidermin, section 8) all contain both *lanI*/*lanH* and *lanEFG* genes.[321] Mersacidin, which also inhibits cell wall biosynthesis by binding to lipid II but does not form pores, only contains the *lanFEG* genes. Further investigation into the modes of action and self-protection will shed more light onto the specific mechanism of action of these proteins. For resistance mechanisms of nonlantibiotic producing bacterial strains against nisin, see section 9.

## 7. Lantibiotic Engineering

### 7.1. In Vivo Protein Engineering

The cloning of the gene clusters involved in the biosynthesis of many lantibiotics laid the foundation for genetic protein engineering aimed at in vivo production of novel compounds with potentially interesting properties. Many studies have indicated the feasibility of changing the molecular structures of lantibiotics by mutagenesis of the pre-lantibiotic genes.[331] So far, engineering of the nisin structure has been most extensively investigated (Figure 25), but engineered (heterologous) expression systems have also

been established for subtilin,[137] Pep5,[332] epidermin and gallidermin,[333] mutacin II,[161] and mersacidin.[334] In these studies, not only the biosynthetic machinery but also the immunity factors had to be considered for generating successful expression systems.

Replacement of Ser at position 5 by Thr in the structural region of the *nisZ* gene led to the production of Dhb instead of Dha in mature nisin Z.[335] The variant exhibited an increased resistance to chemical degradation albeit accompanied by a 2−10-fold reduction in bioactivity toward various indicator strains. In contrast, replacement of Dha at position 2 in nisin Z with Dhb (S2T mutation in NisA) resulted in a mutant that was twice as active as native nisin Z toward certain sensitive strains.[331] Replacement of Dha by Dhb and vice versa has also been reported for other lantibiotics. The gallidermin variant Dhb14Dha did not exhibit any noticeable decrease in activity,[331] and the Dhb10Dha mutant of mutacin II also showed similar activity to wild-type.[161] Very interesting is the production of a nisin mutant with a Dhb residue at position 18 in place of Gly after introduction of a Thr codon in *nisA*,[335] and the similar introduction of a novel Dha in place of a Lys at position 18 in Pep 5.[336] These studies clearly indicate low substrate specificity for the dehydratases involved. This low substrate requirement has been further explored in a nonproducing *L. lactis* strain by overexpressing the nisin dehydratase and transport proteins along with a chimera consisting of the nisin leader peptide fused at its C-terminus to the angiotensin heptapeptide NRSYICP. A single dehydration was observed in the exported chimera, suggesting that dehydratase function is independent of the nature of the structural region as long as the leader is present.[195]

Generally, removal of dehydro amino acids from lantibiotics reduces their biological activity. For instance, removal of the Dha at position 5 by replacement with Ala eliminated nisin and subtilin's activity against outgrowing spores (section 8.3).[136−138] Strains expressing nisin in which either Dha33 was replaced by Ala or both Dha5 and Dha33 were substituted with Ala resulted in greatly reduced activity (about 1% of wild-type nisin producing strains). Unfortunately, it was not established whether this reduction of activity was due to a less active antimicrobial peptide or to poor processing and secretion of the mutants and/or reduced expression in the engineered system.[337] Substitution of Dhb at positions 16 and 20 of Pep5 to Ala were also found to reduce its activity against four test strains,[336] and changing Dha16 in mersacidin to Ile greatly reduced its activity toward indicator strains such as *M. luteus* and *S. pyogenes*.[334] Not all amino acid substitutions are tolerated by the biosynthetic machinery, however. For example, attempts to generate the mutacin II mutant Dhb10Ala did not lead to detectable mutacin production,[161] and replacement of Ser3, Ser19, or Cys22 that are involved in lanthionines in gallidermin also resulted in loss of production.[333] For those lantibiotics that autoregulate their biosynthesis (section 5), the mutant structures may not be able to activate the two-component response system resulting in lack of production.[273]

Alterations in the Lan and MeLan structures have also been accomplished. Substitution of Ser at position 3 in nisin Z with Thr gave rise to MeLan instead of Lan with a dramatic reduction in activity.[331] Replacement of Thr13 with Cys produced a disulfide in place of MeLan in nisin Z resulting in reduced activity.[338] Intriguingly, a fourth thioether bridge (MeLan) was introduced between positions 16 and 19 in Pep5 by the mutation Ala19Cys.[336] This methyllanthionine increased proteolytic stability against the proteases chymotrypsin and Lys-C but also resulted in a significant decrease in antimicrobial activity. This decrease in activity may be due to rigidification of the flexible central region that otherwise aids pore formation by Pep5. Other Pep5 analogues in which ring structures had been deleted displayed a pronounced susceptibility toward proteolysis. Caufield and co-workers have mutated all three Cys residues involved in ring formation in mutacin II to Ala. While the Cys15Ala and Cys26Ala mutants exhibited no antimicrobial activity, Cys27Ala had low level activity (less than 10% of wild-type mutacin II).[161] Collectively, these studies on changing the thioether bridges reiterate the importance of the Lan/MeLan rings for antibiotic activity.

In addition to substitutions of the residues that are posttranslationally modified, a large number of mutants have been reported in which other amino acids in the polypeptide have been replaced (e.g., Figure 25 for nisin). Two nisin variants with higher solubility than the parent compound have been produced by substitution of Asn27 or His31 with Lys.[339] The introduction of an extra Cys in nisin Z by van Kraaij et al. (Ser5Cys and Met17Cys) resulted in a compound that required the presence of a reducing agent for bioactivity.[338] Natural selection may therefore explain the absence of any lantibiotic structures with free thiol groups, whereas the occurrence of unreacted Dha/Dhb residues is fairly common (Table 2). Several nisin mutants have been reported in which the residues in the so-called hinge region (Asn20, Met21, Lys22) were changed,[48,470] which resulted in very interesting changes in the bactericidal activities (section 8.2). Changing Glu4 of subtilin to Ile increased the biological activity 3−4-fold compared to wild type and significantly slowed chemical modification of Dha5, a process that leads to loss of certain biological activities of the compound (sections 8.2 and 8.3).[136−138] The Leu6Val gallidermin mutant was found to be twice as active as the wild-type against *M. luteus*, while the mutants Dhb14Pro and Ala12Leu showed increased resistance to proteolytic degradation.[333] Two mutants of MrsA were expressed in an engineered host,[341] and the corresponding mersacidin analogue Glu17Ala-mersacidin had strongly reduced activity whereas Phe3Leu-mersacidin displayed activity closer to the wild-type lantibiotic. Heterologous expression in *Streptomyces lividans* of the *cin* cluster containing mutated *cinA* genes has resulted in the production of Arg2Lys- and Phe10Leu-cinnamycin, which correspond to duramycin and duramycin B (section 3.6).[78] Attempts to produce the sextuple mutant R2A/Q3N/F7Y/F10L/F12W/V13S-cinnamycin (duramycin C) were not successful.


**Figure 26.** Sequences of $His_6$-LctA and its mutants used to investigate the minimal sequence requirements of the leader peptide and the substrate specificity for posttranslational modifications in the propeptide. Truncations in the leader sequence (black) and structural region (red) are depicted by dashed lines. Mutated residues are in yellow.

The important advancements in in vivo protein engineering of the lantibiotics have greatly contributed to a better understanding of lantibiotic biosynthesis and antimicrobial activity. However, very few mutant lantibiotics have been generated with improved antimicrobial activities, and none display enhanced activity for all test strains. A number of potential explanations could account for this. First, it might not come as a surprise that Nature has already optimized the biological activity of these compounds using the same tools, i.e., mutagenesis with 20 amino acids. Furthermore, the structural and functional space that can be sampled using genetic engineering of ribosomally synthesized proteins will likely always be limited although great strides have been made to overcome this impediment.[342−344] Another contributing factor to the absence of more potent compounds produced by genetically engineered lantibiotic producers may lie in the breakdown of self-immunity in cases in which more active compounds are actually generated. This might lead to either degradation of the intermediates or shutdown of antibiotic production. Indeed, degradation products or incompletely modified peptides are often observed even in cases in which fully processed novel materials are isolated.[137,336] Finally, it has been shown for several lantibiotics that the prepeptide and/or the final product fulfills a regulatory role in its production (section 5).[189,269,325,332] Structural variants, however, may lack the ability to induce in vivo synthesis resulting in reduced or abolished production.[273]

## 7.2. In Vitro Protein Engineering

In vitro engineering of the lantibiotic biosynthetic processes has several conceptual advantages over genetic protein engineering of lantibiotics. The structures of the prepeptides are not limited by the physiological amino acids, only by the ability to design and synthesize the amino acids and incorporate them into peptides using well-developed solid-phase synthesis and peptide ligation techniques. In addition, peptide synthesis is particularly amenable to combinatorial techniques, thereby dramatically increasing the number of rapidly accessible substrate candidates. Because of the in vitro nature of the approach, degradation of products is not a problem, nor will cytotoxic or regulatory properties of the products be a concern. This will permit exploration of the structural and functional tolerance of the biosynthetic enzymes in much greater detail because nonproteinogenic amino acids can be utilized in addition to the natural amino acids. Finally, although speculative, it may prove possible to use nonpeptide structures in part of the substrates to produce even more stable molecules.

While in vitro engineering of the posttranslationally produced oxazole and thiazole rings in the bacteriocin microcin B17 by a microcin synthetase complex has been demonstrated,[345] similar attempts to reconstitute an active lantibiotic synthetase in vitro proved challenging until recently. In 2004, an in vitro system for generation of the type AII lantibiotic lacticin 481 was the first example of its kind (section 4.4).[40] The prelacticin modifying enzyme LctM was cloned from *L. lactis CNRZ 481* and heterologously expressed in *E. coli*. The prelacticin peptide LctA was also expressed and purified with an N-terminal $His_6$-tag. The resulting functional in vitro system was then exploited to test the substrate specificity of LctM with $His_6$-LctA derived substrates (Figure 26). Engineering of LctA to obtain novel substrates was achieved at the genetic level by mutation and/or truncation of the *lctA* gene and also posttranslationally by expressed protein ligation (EPL)[346,347] with an LctA(1−37)-intein-chitin binding domain (CBD) fusion protein.

As expected, replacement of Thr48 with Ala resulted in only three dehydrations instead of the usual four found in $His_6$-LctA. An unexpected fifth dehydration was seen in the LctA-Cys49Ser mutant due to the introduction of an extra Ser. Obviously, the replacement of the Cys at this position also precluded formation of the B-ring, but it was not anticipated that the formation of an extra Dha at position 49 also interfered with formation of the C-ring. Interestingly, the LctA-Cys49Ala mutant underwent only two (Thr33 and Ser35) of the possible four dehydrations (Thr33, Ser35, Ser42 and Thr48), when a disulfide bond was present between Cys38 and Cys50. Reduction of this incompletely dehydrated product with DTT and reincubation with LctM led to the formation of products with up to four dehydrations, demonstrating the ability of LctM to further process partially dehydrated products. A C-terminally truncated His-LctA(1−37) peptide that contained two of the residues that undergo dehydration in the full

length substrate was found to be a substrate for LctM and dehydration was localized by tandem MS to Thr33 and Ser35. Furthermore, the His-LctA(1−38) and His-LctA(1−38)Cys38Sec mutant peptides that include the residues necessary for formation of the A-ring were accepted by LctM resulting in dehydration and cyclization to form thioether and selenoether rings, respectively. This example demonstrates the potential of combining semi-synthetic substrates generated by EPL with the substrate promiscuity of LctM.

It should be noted that some advantages of genetic engineering are lost in the in vitro strategy. One of the biggest assets of molecular biology is that it produces readily and rapidly renewable sources of manipulated genes and organisms, which is not true for chemically synthesized molecules and purified enzymes. A potential solution for the problem of rapidly generating mutant substrates with unnatural amino acids at desired positions would be the utilization of the in vivo amber codon suppression methodology developed by Schultz and co-workers.[348] It may prove, however, that such in vivo use of unnatural peptide substrates for lantibiotic engineering suffers to an even higher degree from the drawbacks described in section 7.1 with respect to discovering lantibiotics with new or improved biological activities.

The great promise of in vitro use of lantibiotic synthases is not limited to the production of lantibiotic analogues. These enzymes may also find application in installing dehydro amino acids or lanthionine rings into other synthetic targets. Cyclic lanthionine containing peptides have found use as mimics of natural products that contain disulfide bridges or as structures that limit the conformational flexibility of bioactive compounds.[349] The lanthionine moiety also provides higher chemical and proteolytic stability for such analogues. There are reasons to be optimistic that these type of compounds may be prepared using lantibiotic synthases, although to date they have only been produced by chemical synthesis. For instance, Goodman and co-workers have demonstrated the advantage of the structural constraints imposed by Lan in a somatostatin analogue.[350] Peptide cyclization on oxime resin (PCOR)[351,352] was employed to generate a conformationally rigid mimic of sandostatin, a somatostatin analogue, by replacing a disulfide linkage with a thioether bridge. The lanthionine-sandostatin analogue possessed enhanced receptor selectivity and an increased half-life toward enzymatic degradation.[350] Cyclic lanthionine containing enkephalin analogues have also been synthesized and the $\beta,\beta$-dimethyl substituted compounds were selective for the $\delta$-opiod receptor.[353]

In addition to using lanthionines themselves, several synthetic studies have incorporated lanthionine analogues into biologically relevant compounds.[350,353,354] Tabor and co-workers prepared the norlanthionine analogue of the C-ring of nisin (Figure 27).[355,356] The analogue was synthesized by solid-phase peptide synthesis in a linear fashion employing Fmoc-based chemistry and a triply protected norlanthionine monomer. Deprotection of the allyl and Fmoc protecting groups was followed by intramolecular amide bond

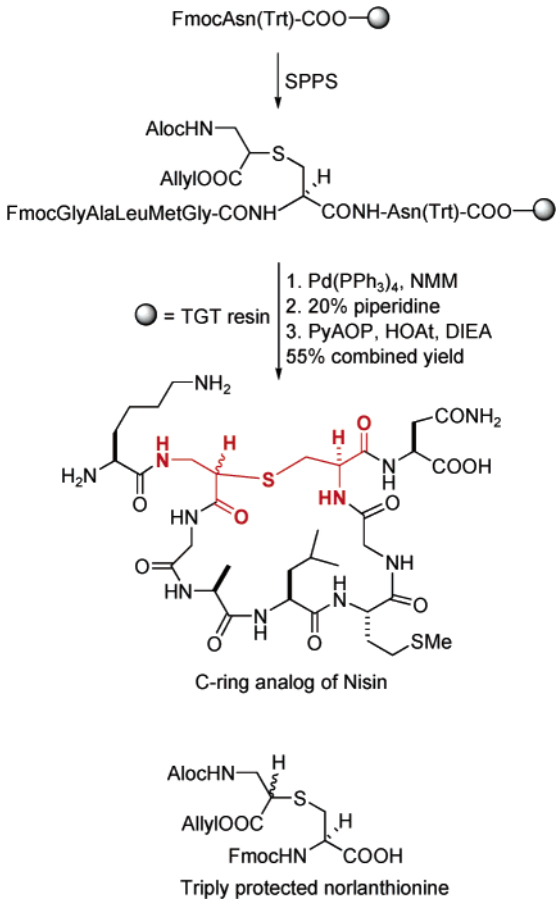



**Figure 27.** Solid-phase synthesis of the norlanthionine analogue of the nisin C-ring.[356]

formation on the resin. Grieco and co-workers[357] recently reported a variation on this concept. On-resin cyclization of homologated lanthionines with varying ring sizes was achieved by intramolecular amide bond formation. A method for the synthesis of thioether-bridged peptides that yields diastereomerically pure products was also developed by Yu and co-workers.[358] The *tert*-butyldimethylsilyl (TBDMS) ether of homoserine and *tert*-butyl disulfide protected Cys were included in a 14-mer peptide by linear solid-phase synthesis (Figure 28). Reaction with triphenylphosphine dichloride led to the conversion of the homoserine to the corresponding chloride. Cleavage from the resin and removal of the protecting groups was followed by base-induced cyclization to afford a homolanthionine-containing peptide. In addition to the synthetic use of Lan and its analogues, dehydro amino acids are also valuable synthons for further manipulation when incorporated into peptides as they constitute an electrophilic site for site-selective ligation with external nucleophiles.[359−361]

Aside from preparation of lanthionine containing structures by chemical synthesis, chemical modification strategies have been applied to natural lantibiotics. The single glutamic acid in actagardine (Figure 9) was converted selectively into a series of monocarboxamides in addition to variants that contained amide functionalities at both Glu11 and the C-terminal carboxylate.[362] Some of these semisynthetic analogues displayed improved solubility and antibacterial activity.

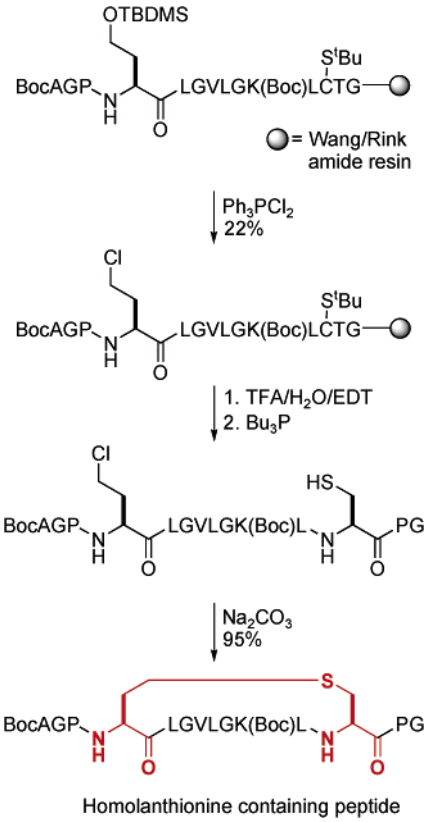



**Figure 28.** Solid-phase synthesis of cyclic homolanthionine containing peptides.

## 8. Mode of Action

It has become clear in recent years that the mechanisms by which lantibiotics exert their antimicrobial activities are more complex than initially thought. For several type AI compounds, antibiotic activity stems from more than one mechanism and may include disruption of cell wall biosynthesis, inhibition of spore outgrowth, and pore formation that may or may not be aided by prior docking on cellular targets. Nisin, for instance, and presumably also its close structural analogues, uses all of the above modes of action with the individual contributions depending on the target organism. The currently known details for these processes are discussed below.

### 8.1. Pore Formation in Model Membranes

Much of the work prior to the late 1990s focused on the permeabilization of bacterial cell membranes as the primary mode of action of nisin and other type AI lantibiotics. Efflux of cellular components from *Clostridium butyricum* upon treatment with nisin was observed as early as 1960.[363] Pore formation is a widespread property of antibacterial peptides,[364] and generally they are not expected to interact with a specific chiral receptor in the target organism. Indeed, the observation that peptides consisting of all D-amino acids provide the same activity as their L-amino acid counterparts for the nonlantibiotics magainin, cecropin, and melittin provided strong evidence for this supposition.[365,366] As a result, numerous studies focused on using cytoplasmic and artificial membrane vesicles to study the mode of action of nisin[82] and other cationic type AI lantibiotics. Sahl and Ruhr reported that the cytoplasmic membrane is the primary target of nisin and that membrane disruption results in efflux of metabolites and dissipation of vital ion gradients.[367,368] Subsequent studies showed that membrane depolarization occurs in a voltage dependent manner upon treatment with nisin,[367−372] subtilin,[373] Pep5,[374,375] epidermin,[376] gallidermin,[376] and streptococcin A-FF22.[377] The threshold potential for depolarization of black lipid membranes (planar lipid bilayers) was in the 50−100 mV range and required a trans-negative orientation (inside negative) with respect to the addition of nisin. The higher the content of anionic phospholipids the lower the threshold potential for dissipation of $\Delta\Psi$.[376] It has been proposed that the transmembrane potential aids in nisin's pore formation by effectively pulling the charged amino acids at the N-terminus into the membrane,[82] a hypothesis that is supported by the pore-forming activity of the Lys12Leu mutant that is independent of a membrane potential.[378] However, several studies have shown that a membrane potential, while increasing membrane permeabilization, is not absolutely necessary for nisin-induced leakage of certain solutes from negatively charged vesicles.[371,379−381] Moreover, nisin has been shown to dissipate a transmembrane pH gradient in the absence of a transmembrane electrical potential in sensitive *Lactococcus* cells and proteoliposomes.[382]

The pores that are assembled in the presence of a membrane potential have been studied in detail and are transiently formed with lifetimes of a few to several hundred milliseconds and with diameters ranging from 0.2 to 1−2 nm.[368] Nisin and Pep5 form pores that work only in one direction (rectifying)[369,374] with the nisin pores somewhat anion selective,[381] whereas gallidermin and epidermin form nonrectifying channels that are also more stable.[376] The lipid composition has a strong influence on the efficiency of pore formation in model membrane systems such as planar lipid bilayers and liposomes,[370] indicating a preferential interaction of nisin with anionic lipids.[372,375,378,379,381,383,384] These were important findings since Gram-positive bacteria have a high content of anionic lipids in the membrane. Indeed in a monolayer study, it was shown that antimicrobial activity correlated well with the nisin−anionic lipid interaction.[383] In addition to the charge state of the membrane, several studies have reported an optimal charge state for the nisin molecule itself.[380,385] Efflux of metabolites and depletion of the proton motive force have also been demonstrated in other studies focusing on the interactions of lantibiotics with artificial or bacteria-derived membranes.[44,367,368,373,382,386−388]

Two models have been proposed for the mechanism of pore formation, the barrel-stave and the wedge model. In the former, the cationic lantibiotic monomer (the stave) binds to the membrane surface through electrostatic attraction, and after assembly into a preaggregate, pores (barrel) are formed at a certain membrane potential in which the lantibiotic assumes a position perpendicular to the membrane.[368] In the wedge model, surface bound nisin molecules bind parallel to the membrane and produce

local strain leading to bending of the membrane such that the lipid molecules together with nisin make up the pore.[372] In both models, the hydrophobic regions of the amphiphilic nisin molecules presumably face the membrane, whereas the hydrophilic face forms the lumen of the pore. Although both models expected a more or less perpendicular orientation of the molecule with respect to the membrane surface, fluorescence studies on antimicrobially active variants in which tryptophans had been introduced at various positions by mutagenesis[379,389] suggested that nisin adopts an overall stable parallel orientation.[389] At least three regions of the peptide were shown to insert into membranes made up of anionic lipids with the N-terminus inserted more deeply than the C-terminus. These results are similar to an assembly of NMR structures obtained in DPC micelles (Figure 7).[131,390] Combining the results of the fluorescence and NMR studies, a model was proposed for nisin's orientation in negatively charged membranes.[389] In this picture, nisin is relatively elongated and lies parallel to the membrane surface with the positively charged side-chains of lysines 12, 22, and 34 pointing out of the lipid bilayer. Whereas this model represents the most stable orientation, formation of the transient pores most likely requires a conformational change that allows the molecule to traverse the membrane.

During the pore forming process, the highly positively charged C-terminus of nisin (Lys22, Lys34, His31) interacts initially with the anionic membrane surface as demonstrated by binding studies, which showed strongly reduced interaction of anionic lipids with a Val32Glu mutant of nisin Z.[381] Presumably, the negative charge introduced in this mutant results in electrostatic repulsion with the anionic headgroups of the lipids. The weak binding also resulted in a greatly reduced ability to release solutes from vesicles. The importance of the C-terminus for binding to the membrane was also shown by the strongly reduced affinity of a nisin[1−12] fragment for anionic phospholipids.[382] The binding isotherms with wt-nisin Z and negatively charged membranes show biphasic character, consistent with aggregation in the membrane.[381] Although the initial binding to the membrane surface seems to involve the C-terminus of nisin, studies with a variant of nisin Z in which a short peptide ($AspHis_6$) was fused to its C-terminus showed that the C-terminus translocates across the membrane, where it could be cleaved off behind Lys34 by trypsin encapsulated in the lumen of unilammellar vesicles.[385] This translocation of the C-terminus was correlated with pore-forming activity and both activities were anionic lipid dependent. Thus, after electrostatic binding of the C-terminal region to the membrane surface, the peptide adopts a membrane spanning orientation in which at least part of the molecules that form the pore have their C-terminus in the lumen of the vesicle. Alternatively, some of the nisin molecules may translocate completely across the membrane as has been shown for other nonlantibiotic pore-forming peptides such as magainin 2.[391] Several studies have been performed to analyze the pore-forming capabilities of lantibiotic mutants prepared by protein engineering (section 7.1). In these studies the antibacterial activity of nisin is mainly affected by changes in the first three rings of nisin,[136,331,335,339] which is now believed to originate from disrupted interactions with lipid II (section 8.2).

## 8.2. Highjacking of Lipid II for Pore Formation

The in vitro studies using model membrane systems have provided much information regarding their interaction with nisin and other lantibiotics, but many observations could not be explained. For instance, nisin's efficacy against intact cells (nM MIC) was 3 orders of magnitude higher than pore formation in model membranes ($\mu$M). Furthermore, although nisin[1−12] did not bind to anionic membranes, this fragment does antagonize nisin's action against intact cells.[392] Other unexplained issues involved nisin's spectrum of biological activity, for instance, *L. monocytogenes* has a high content of anionic lipids (50−88%), and yet it is relatively insensitive to nisin.[393] Moreover, it was unclear why mammalian membranes are only affected at millimolar concentrations of nisin.[367,394] These apparent discrepancies were explained when it was reported by Breukink and Sahl and co-workers that nisin interacts in a highly specific manner with lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc),[41,322] the essential membrane bound precursor for cell wall formation (Figure 29) that is present in very different amounts in various microorganisms (e.g., *E. coli* 2000 molecules per cell,[395] *Micrococcus lysodeikticus* $10^5$ molecules per cell).[396] In retrospect, several indications of nisin's interference with peptidoglycan biosynthesis had been reported prior to the unequivocal demonstration that it binds to lipid II. Linnet and Strominger showed that nisin interferes with peptidoglycan biosynthesis in an in vitro system made up of isolated bacterial membranes.[397] A subsequent study provided evidence that this inhibition is caused by binding to the lipid-associated peptidoglycan precursors lipid I (lacking the GlcNAc unit) and lipid II.[398] For many years, these early findings were not followed up on until a series of recent studies that have firmly established the interaction of nisin with lipid II.[41,48−52,322] Mersacidin,[399−401] epidermin,[322] actagardine,[167,322] and probably lacticin 3147[112] also inhibit peptidoglycan biosynthesis. For mersacidin, epidermin, mutacin 1140,[54] and actagardine, complex formation with lipid II has been demonstrated, although unlike nisin, mersacidin does not form pores. An NMR study on the interaction of mersacidin with lipid II in SDS micelles showed that it adopts a different structure than that observed in the absence of lipid II.[171] Interestingly, epidermin and nisin, but not mersacidin, have both been shown to cause an accumulation of lipid I during in vitro peptidoglycan biosynthesis assays, suggesting they may also interfere with the conversion of lipid I into lipid II.[322]

Binding of antimicrobial substances to lipid II interferes with peptidoglycan biosynthesis by physically sequestering the compound preventing utilization by transpeptidase and transglycosylase enzymes that install the cross-linked network of the bacterial

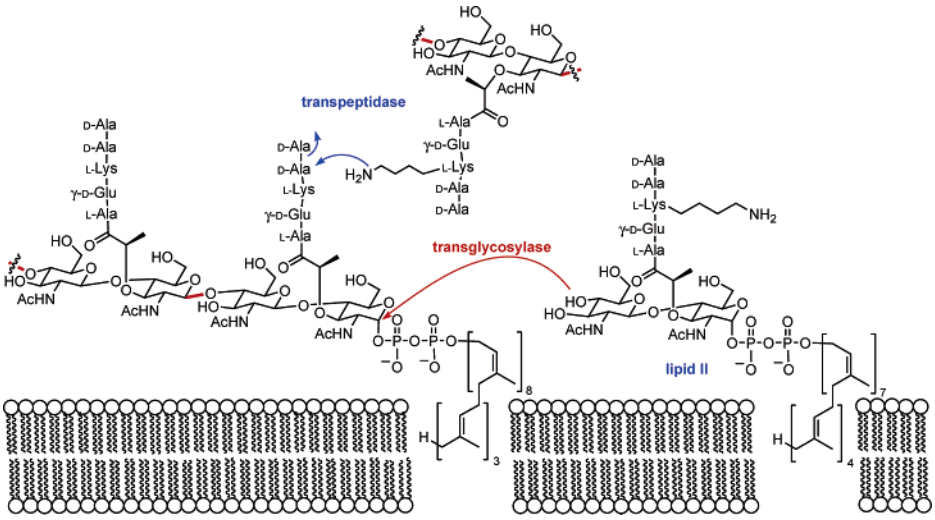



**Figure 29.** The structure of lipid II and its incorporation into the peptidoglycan by transpeptidase and transglycosylase enzymes. Lipid II is made up of an *N*-acetylglucosamine-$\beta$-1,4-*N*-acetylmuramic acid disaccharide connected to a $C_{55}$-lipid carrier undecaprenylpyrophosphate made up of eight *Z*-prenyl and three *E*-prenyl units.[413] The muramic acid bears a pentapeptide at O3 that contains a Lys for later cross-linking (or a *meso*-diaminopimelic acid in Gram-negative bacteria). Bonds made by the transglycosylase are shown in red.

cell wall (Figure 29).[402] Binding to lipid II is the primary mode of action of the well-studied glycopeptide antibiotics vancomycin and teicoplanin,[403,404] as well as ramoplanin[405−408] (see also the review by Walker et al in this issue).[409] As would be expected for a common target, treatment of *M. luteus* with ramoplanin prevented pore formation by nisin and epidermin,[322] and strongly decreased mersacidin binding to the cell surface.[401] Similarly, vancomycin protected *M. flavus* cells against membrane leakage induced by nisin but not by magainin.[41] The lantibiotics must bind to a different part of lipid II than vancomycin, which interacts with the L-Lys-D-Ala-D-Ala segment of the pentapeptide,[403,404] since nisin and mersacidin are active against vancomycin resistant enterococci.[41,399] Comparing existing NMR structural data,[169,410] McCafferty and co-workers suggested that mersacidin and ramoplanin exhibit very similar backbone conformations, which may be important for their mode of binding lipid II.[411] Binding of an antibiotic to a complex biosynthetic intermediate like lipid II has certain advantages over binding to a single enzyme involved in peptidoglycan assembly because changing the structure of lipid II is much more demanding on a microbe than changing the structure of the active site of one enzyme, thereby decreasing the odds of bacterial resistance. For instance, eight successive enzymes are required for the biosynthesis of lipid II from UDP-GlcNAc.[412,413] That resistance can nevertheless develop has been demonstrated in vancomycin-resistance when bacteria change the D-Ala-D-Ala unit of lipid II to D-Ala-lactate.[414,415] For resistance mechanisms to nisin, see section 9.

Although nisin, vancomycin, teicoplanin, and ramoplanin interact with the same target, nisin is unique in that it subsequently forms pores that include lipid II as an essential constituent.[51] For instance, when lipid II is present in membranes, nisin's pore-forming efficiency is increased 1000-fold, an increase that is not seen with other pore forming peptides such as magainin.[41] Interestingly, the aforementioned increased efficiency of pore formation in membranes made up of negatively charged lipids observed in the absence of lipid II was completely undone in its presence and the anion selectivity seen in model systems disappeared upon addition of lipid II.[48] These observations suggest that in the presence of lipid II the architecture of the pores is changed. Lipid II-mediated pore formation is very effective as only seven molecules of lipid II per vesicle (or 2 lipid II per $10^5$ phospholipid molecules) result in a dramatic decrease in the nisin concentrations needed to release dyes from vesicles.[41,322] The stoichiometry of complex formation between nisin and lipid II is 1:1 in solution and in SDS micelles.[48,50,54]

The ability to produce lantibiotic mutants by site-directed mutagenesis or chemical and enzymatic truncation as described in section 7.1 (Figure 25) has proven an extremely powerful asset in the study of their modes of action. Deletion of the two C-terminal amino acids (Dha33, Lys34) of nisin does not affect antimicrobial activity.[416] Mutation of Val32 to Lys or Glu, thereby introducing an additional positive or negative charge and also preventing dehydration of Ser33,[273] also had relatively little effect for the Val32Lys mutation whereas the Val32Glu mutant had about 4-fold decreased activity against certain test strains.[48] Hence, the C-terminus is relatively unimportant for biological activity. This is in keeping with the observation that epilancin K, which shares a very similar C-terminal double-ring system with nisin, does not appear to interact with lipid II.[322] On the other hand, several findings strongly suggested that the N-terminus of nisin is essential for binding. An inactive nisin fragment (nisin 1−12) was shown to antagonize nisin's bacteriocidal activity, suggesting it competes for the same binding site.[392] Furthermore, epidermin and nisin both bind to lipid II,[322] and they have identical topologies of their A- and B-rings (Figure 6). Moreover, chemical disruption of Dha5, thereby opening the A-ring, results in more than 500-fold reduction of biological activity,[416] whereas complete removal of the D- and E-rings by proteolysis

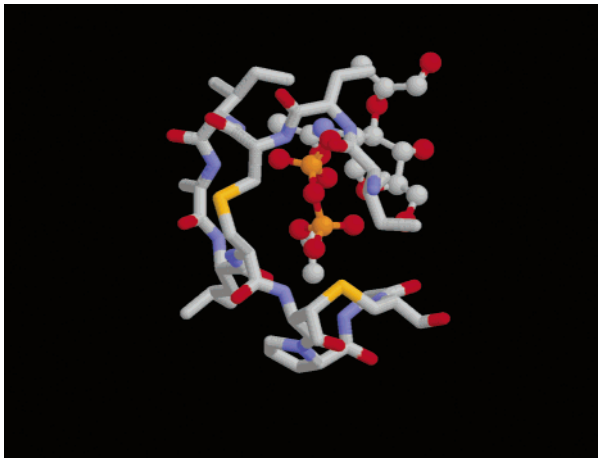


**Figure 30.** Depiction of the cage-like structure of the A- and B-rings of nisin around the pyrophosphate group of lipid II. The lipid II fragment containing the muramic acid and pyrophosphate is shown in ball-and-stick format. The A-ring is shown to the left of the pyrophosphate and the B-ring is below the pyrophosphate.

results in only a 100-fold decrease in potency.[392] Recent NMR studies on nisin in the presence of lipid II provide the most direct support for the importance of the N-terminus for binding. In SDS micelles containing lipid II, nisin residues located in the N-terminal region showed the largest perturbation in chemical shift.[50] A very recent exciting development is the determination of the solution structure of a complex between nisin and a lipid II analogue. The lipid II variant contained a shortened prenyl tail that consisted of just 3 rather than 11 isoprene units. The length of this isoprene tail does not affect pore-forming activity.[417] The structure of the 1:1 complex was solved in DMSO revealing a cage-like binding motif in which the N-terminus of nisin folds back onto the A- and B-rings.[54] The pyrophosphate moiety of lipid II is bound within this cavity with intermolecular hydrogen bonds between the amides of Dhb2, Ala3, Ile4, Dha5, and Abu8 on nisin and the oxygens of the pyrophosphate group of lipid II (Figure 30). In addition to the pyrophosphate group, MurNAc and the first isoprene unit make up the binding site for nisin recognition (Figure 31). This structure provides explanations of various previous observations including studies with nisin mutants. Extension of its N-terminus by the tetrapeptide IleThrProGln (Figure 25) significantly reduces the antimicrobial activity.[204] Furthermore, it is in full agreement with the observations that chemical opening of the A-ring essentially eliminates all activity,[416] and that changing the Lan in the A-ring to a MeLan decreases the affinity of the mutant nisin for lipid II 50-fold.[48] The NMR structure also reveals why nisin (and epidermin) binds to both lipid I and lipid II,[322] which share the recognition motif, and why nisin (and epidermin) is active toward vancomycin resistant strains[41,399] as its binding site (the L-Lys-D-Ala-D-Ala segment of the pentapeptide) on lipid II does not make contacts with nisin. The structure also provides an important lesson that it is not the side-chains on nisin that impart its biological activity but rather the backbone.

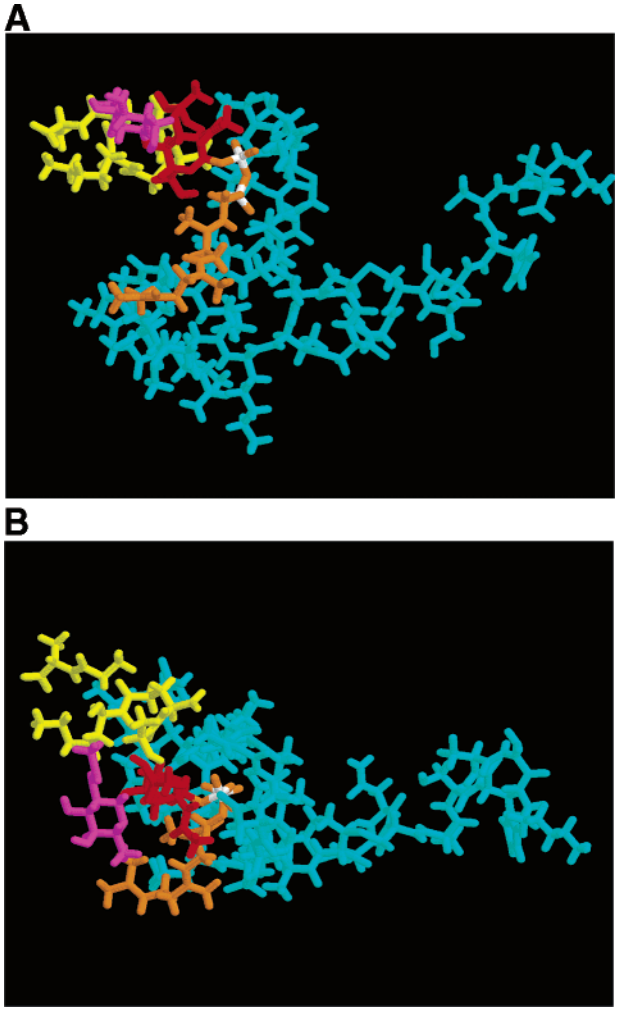



**Figure 31.** Two views of the NMR structure in DMSO of the 1:1 complex of nisin and a lipid II analogue with a truncated prenyl tail.[54] Figure generated using the program RASMOL[469] with PDB file 1UZT. Nisin is shown in cyan, the prenyl chain of lipid II is shown in orange, the two phosphate atoms of the pyrophosphate group are depicted in white, and the muramic acid is presented in red. The pentapeptide chain is shown in yellow and the GlcNAc in magenta.

Hence, it rationalizes why to date no significantly improved analogues have been reported in the bioengineering efforts (section 7). Whether this will also turn out to be true for other lantibiotics remains to be established.

The most interesting nisin mutants have changes in the flexible segment between the C- and D-rings ("hinge region"). Deletion of Asn20 and Met 21, thereby decreasing the length of this linker to a single residue, resulted in an 80% decrease of dye release from lipid II-supplemented vesicles even at high nisin concentrations ($\mu$M).[48] Replacement of these two amino acids with prolines decreased the lipid II promoted pore formation even further. The antimicrobial activities of these mutants against *Streptococcus thermophilus* were also strongly affected with MIC values that increased 40- and 25-fold, respectively. However, their activity against *Micrococcus flavus* was much less affected (3−9-fold), and a third mutant, Met21Gly, showed comparable activity as wt nisin Z against both strains despite having no pore

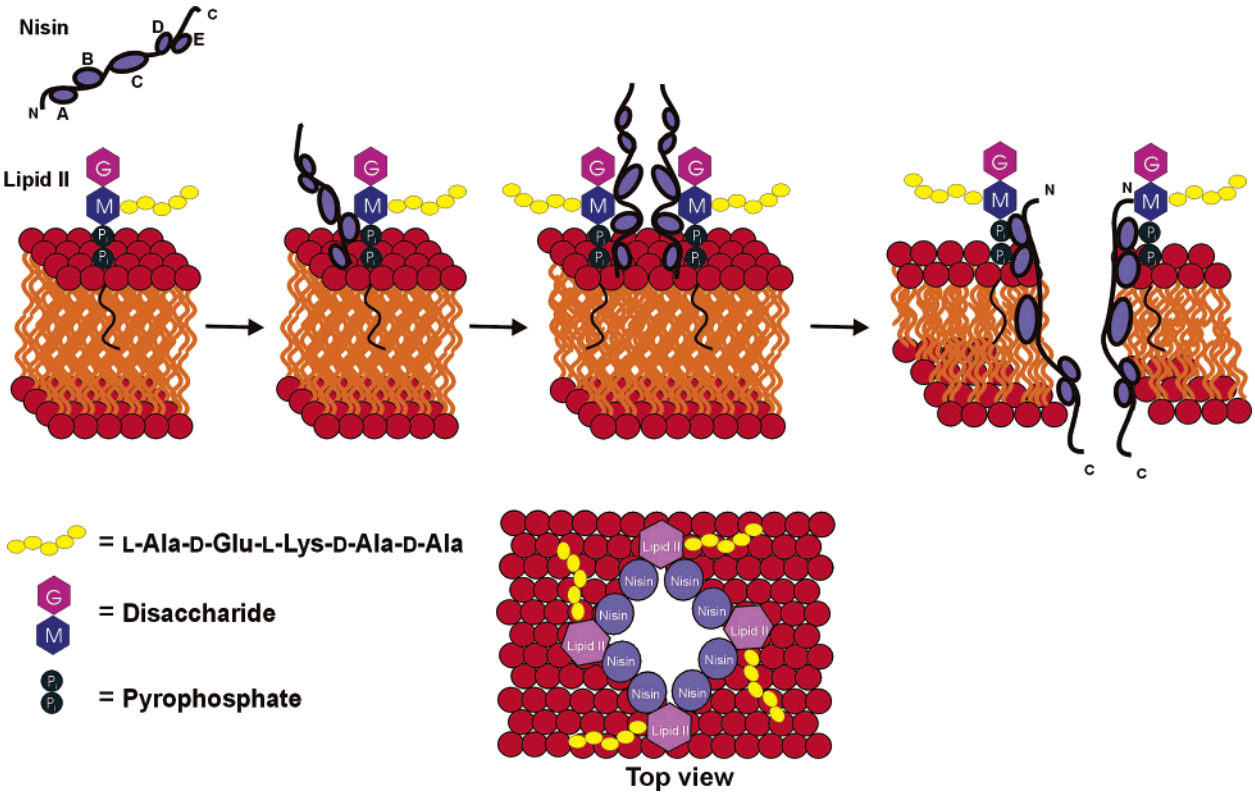



**Figure 32.** Proposed model for lipid-II mediated pore formation. The C-terminus of nisin is shown as residing in solution upon initial binding of the N-terminus to lipid II, but it may also interact with the negatively charged headgroups of the membrane.[54] The pore structure has been shown to be made up of four lipid II and eight nisin molecules, but their arrangement is not known and the shown architecture is therefore speculative.

forming activity. Hence, the binding to lipid II alone constitutes a very potent antimicrobial activity in vivo. Since pore formation by itself is also bactericidal, nisin truly has two distinct modes of action.

Using tryptophan fluorescence, it was demonstrated that lipid II changes the orientation of nisin from parallel to perpendicular with respect to the membrane surface.[49] Furthermore, the use of pyrene-labeled lipid II showed that it is recruited into a stable pore structure.[51,53] The distance between two labeled lipid II molecules was estimated to be about 18 Å[51] with a pore diameter of 2 nm.[52] A model has been proposed to explain all of the experimental data as shown in Figure 32.[48,49,53] The N-terminal rings of nisin bind to the disaccharide-pyrophosphate region of lipid II, whereas the positively charged C-terminus initially interacts with the headgroups of the lipids in the membrane bilayer (not shown). Multiple molecules of the lipid II−nisin complex[51] subsequently aggregate and form a pore of defined uniform structure. Whereas the stoichiometry of lipid II to nisin in solution is 1:1,[48,54] the stoichiometry in the pore is 1:2 as it is made up of four lipid II and eight nisin molecules.[53] How the different stoichiometry in the pore affects the structure of the lipid II−nisin complex that was determined by NMR spectroscopy (Figures 30 and 31) remains to be established. However, a promising observation with respect to establishing the structure of the pore at atomic resolution involves the solubilization of the pore complex using detergents such as *n*-octyl polyoxyethylene, *n*-octyl-*β*-D-glucopyranoside, and Tween 20 without any changes in the CD spectrum.[53] The stability of the pore complex has also been observed in electrophysiology experiments. The lifetime of the pore in the presence of lipid II is greatly enhanced from milliseconds in its absence to seconds in its presence.[52] Furthermore, the threshold potential for permeabilization was decreased from ~ 100 mV in the absence of lipid II to as low as 5−10 mV in its presence, and unlike the studies discussed in section 8.1, when lipid II was present pores were formed by applying either *trans*-negative or *trans*-positive membrane potentials.[52] The high stability of the pore complex is quite unique as other cationic pore forming antimicrobial peptides typically form transient pores of low stability and without uniform structures.[391,418]

Mersacidin and actagardine also bind to lipid II, but have no structural similarity with nisin and epidermin (Figures 6 and 9). In doing so, these compounds block the transglycosylation step of peptidoglycan biosynthesis (Figure 29).[400] As mentioned previously, mersacidin does not form pores once bound to lipid II, which may explain the moderate MIC values. However, the compound is very effective in vivo against systemic staphylococcal infections,[16,60] including methicillin resistant *S. aureus* (MRSA),[473] as well as against vancomycin resistant enterococci.[400] Hence, mersacidin and its close relative actagardine may have potential chemotherapeutic applications.

Except for nisin, epidermin, mutacin 1140,[54] and mersacidin, no other lantibiotics have been demonstrated to bind to lipid II, and some such as Pep5 and epilancin K7 have been specifically shown not

to interact with lipid I or lipid II.[322] However, these compounds still have activities against certain bacteria that are far greater than those of other pore formers. For instance, Pep5 is active in low nanomolar concentrations against *Staphylococcus simulans* and *S. carnosus*, which may indicate that it uses a different high-affinity receptor/docking molecule for its biological activity.[322,327]

### 8.3. Inhibition of Spore Outgrowth

In addition to their bactericidal activity against vegetative cells, nisin, subtilin and sublancin also inhibit the germination of spores from *Bacillus* and *Clostridium* species.[22,105] For nisin and subtilin, this activity has been proposed to result from covalent modification of a target on the spore coat by nucleophilic attack on Dha5.[138] Indeed, reactive thiol groups on the exterior of spores from *Bacillus cereus* react with reagents such as *S*-nitrosothiols or iodoactetate,[419] and nisin interferes with the modification of these sulfhydryl groups. This suggests that nisin's target during its inhibition of spore outgrowth may be these reactive thiol groups,[420] but to date a covalent mechanism has not been established. In both subtilin[137,138] and nisin,[136] Dha5 has been implicated as the putative site of attack as replacement by Ala via site-directed mutagenesis abolishes the inhibition of spore outgrowth. Interestingly, these mutations did not affect the growth inhibition activity of subtilin and nisin against vegetative cells of *Bacillus cereus*, and *L. lactis* and *M. luteus*, respectively. These studies clearly indicate that the inhibition of spore outgrowth is yet a different, third distinct biological activity of these compounds with a different structure−activity relationship. Given the recent use of spores of *Bacillus anthracis* in bioterrorism and the similarities of the structure of the spore coat in *Bacillus* species, these activities of subtilin and nisin may have interesting future applications.

### 8.4. Other Biological Activities

In addition to having bactericidal and hemolytic activity,[175] cinnamycin and the duramycins are potent inhibitors of phospholipase A2 by sequestering its phosphatidylethanolamine (PE) substrate.[57,109,181,421,422] When phosphatidylcholine or other lipids are the substrates of phospholipase, no inhibition and substrate binding is seen,[57,175,421,423] indicating specific recognition of PE. The binding stoichiometry deduced from NMR investigations is 1:1[181] and the binding constant as determined by isothermal titration calorimetry is dependent on the lipid matrix, being $10^7$−$10^8$ $M^{-1}$ in 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) and $10^6$ $M^{-1}$ in octyl glucoside micelles.[422,424] Since phospholipids such as PE reside in the inner leaflet of plasma membranes, it was unclear how cinnamycin accesses its target. A recent study addressed this issue and demonstrated that the compound induces transbilayer lipid movement, apparently in a PE-dependent fashion.[425]

Investigations of the conformation of cinnamycin in SDS bilayers indicate the insertion of the lipophilic portion of the molecule into the bilayer due to its interaction with the methylene (-$CH_2$-) groups of the lipid.[179,180] Conformational changes in the presence of 1-dodecanoyl-*sn*-glycerophosphoethanolamine (C12-LPE)[180,340] in DMSO were induced primarily by ionic interactions between the $\beta$-hydroxy-Asp15 of cinnamycin and the ammonium ion of glycerophosphoethanolamine, as well as via hydrophobic interactions between the lipophilic portion of cinnamycin (Gly8, Pro9, Val13) and the methine/methylene backbone of the headgroup. A three-dimensional $^1$H NMR structure of cinnamycin bound to 1-acetyl-*sn*-glycerophosphoethanolamine (C2-LPE) shows the formation of a rigid cylindrical complex, 11 Å in diameter, and 26 Å in length.[340] The glycerophosphoethanolamine headgroup resided in the hydrophobic pocket formed by residues Phe7 through Ala(S)14 of cinnamycin. The high specificity of cinnamycin was attributed to the limited space in the hydrophobic pocket that prevents binding of larger molecules.[340] In this study, C2-LPE and C12-LPE were thought to bind cinnamycin in a fashion independent of their acyl groups. However, more recent isothermal titration calorimetry (ITC) studies of cinnamycin binding to diacyl-phosphoethanolamine of varying chain lengths in both micellar and membrane environments indicate an optimal acyl chain length of eight methylene units for binding.[422] At least one acyl group is necessary for binding and diacyl-PE binds more strongly than lyso-PE.[422] This appears to argue in favor of an interaction between the lipophilic portion of cinnamycin and the hydrocarbon chains of diacyl-PE, which was not observed by $^1$H NMR spectroscopy.

In addition to their other activities, nisin and Pep5 induce autolysis of certain staphylococcal strains leading to cell wall break-down at the septa between dividing cells. It is thought that the interaction of the cationic lantibiotics with the negatively charged teichoic and lipoteichoic acids displace and activate *N*-acetyl-L-alanine amidase and *N*-acetylglucosaminidase enzymes that are usually associated with the teichoic acids.[426,427]

## *9. Resistance Against Nisin*

Microbial resistance to antibiotics is brought about by the evolutionary pressure exerted by exposure of bacteria to these compounds and is eventually unavoidable.[26,428] The advantage conferred by the dual mechanism of action of nisin (section 8) has attenuated the emergence of bacterial resistance to nisin, but resistance has been induced in laboratory settings. In this section, the various mechanisms of resistance to nisin will be discussed. For a discussion of self-immunity of the producing strains see section 6.

### 9.1. Gram-Negative Bacteria

In 1973, Linnet and Strominger observed that nisin and subtilin do not exert a significant bactericidal effect on intact Gram-negative organisms, such as *E. coli*, even though they inhibit peptidoglycan biosynthesis in cell-free assays.[397] Nisin also displays no

effects on the growth of the Gram-negative organisms *Serratia marcescens*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*.[429] The nonsusceptibility of Gram-negative bacteria to nisin (and subtilin) has been attributed to the outer membrane that prevents access of hydrophobic substances to the peptidoglycan layer. Therefore, agents that disrupt the outer lipopolysaccharide (LPS) rich membrane and allow nisin to access the inner membrane where lipid II is present increase the susceptibility to nisin. For instance, sensitization to nisin has been achieved by treatment with chemical agents such as EDTA[430,431] and trisodium phosphate[432] as well as by temperature shock.[433] In each of these cases, susceptibility was transient and was lost upon restoration of normal growth conditions. Incubation of nisin with various mutant strains of *E. coli* and *Salmonella enterica* containing truncated LPS demonstrated that resistance was generally reduced in the absence of the O-chain,[434] which has been reported to inhibit antibiotic action.[435] Interestingly, *Pectinatus frisingensis*, an anaerobic microorganism responsible for spoilage of beer, is nisin-sensitive.[436] Resistance to nisin (Nis-5000 strain) was induced in the laboratory by stepwise exposure to increasing nisin concentrations. Sensitivity to nisin could not be induced by treatment of Nis-5000 with EDTA in contrast to nisin-insensitive Gram-negative organisms, suggesting that the LPS layer is not responsible for resistance of Nis-5000. Chihib and co-workers have suggested an alternate mechanism of nisin-resistance in this strain that involves rigidification of the cellular membrane by changes in the fatty acid composition of the cytoplasmic membrane. This proposal is based on the observation of a 2-fold decrease in unsaturated fatty acids and an increase in saturated fatty acids in the cell membrane of Nis-5000.[436]

## 9.2. Gram-Positive Bacteria

Nisin resistance in Gram-positive bacteria is often developed in sensitive strains by repeated exposure to increasing amounts of nisin. Such "induced" resistance (in a laboratory setting) is typically a complex phenotype arising from changes in the bacterial cell wall and/or cell membrane, and sometimes requiring divalent cations.[437] Plasmid-mediated resistance[438,439] as well as resistance due to changes in expression levels of proteins such as a putative penicillin binding protein,[440,441] and a two-component signal transduction system[442] have also been observed as discussed below.

Resistance to nisin in several *Bacillus* strains was reported by Jarvis in 1967 and ascribed to the presence of nonproteolytic nisin inactivating enzymes.[443] This followed in the wake of an earlier proposal of an enzyme, nisinase, present in the cell extracts of *Streptococcus thermophilus*, that was capable of inactivating nisin but not subtilin.[444] Jarvis noted that the treatment of nisin or subtilin with cell-free extracts of *Bacillus cereus* and *Bacillus polymyxa* led to loss of their antibiotic activity, whereas antibiotics such as gramicidin S, polymyxin B, and bacitracin were not affected, suggesting the presence of lantibiotic-specific agents.[443] The inactivating activity of the *B. cereus* cell-free extracts was dependent on $Ca^{2+}$, $Co^{2+}$, and $Mg^{2+}$ ions and was inhibited by EDTA. Partial purification of the nisin-inactivating fraction was achieved, but an actual protein was never isolated.[445] Acid hydrolysis of inactivated and wild-type nisin before and after the addition of methylmercaptoacetate and $^{14}C$-labeled cysteine demonstrated a reduction in the number of dehydrated residues in inactivated nisin. Furthermore, partial hydrolysis of inactivated nisin showed the presence of Ala-Lys as well as pyruvate-Lys (presumably formed from Dha33-Lys34). On the basis of these results a putative dehydroalanine reductase role was suggested for the enzyme involved.[445]

Several instances of resistance to nisin have been attributed due to changes in the composition of the cell membrane.[393,446−449] The dissipative action of nisin on both components of the proton motive force ($\Delta$pH and $\Delta\psi$) was significantly reduced in a resistance-induced strain ($NIS^r$) of *L. monocytogenes* Scott A (*L. monocytogenes* ATCC 700302).[393] An evaluation of the content of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) in the $NIS^r$ strain revealed an increase in the PG/DPG ratio to 7, from a ratio of 5 in the wild type (wt) strain. On the basis of the persistence of this ratio even in the absence of exposure to nisin, the increased ratio was suggested to be caused by a decreased activity of the enzyme DPG synthetase, although this has not been experimentally verified. The higher negative charge density of DPG possibly contributes to stronger binding of the cationic nisin, and a greater ratio of PG/DPG may therefore increase resistance by reducing binding affinity. Other reported changes in the membrane composition of $NIS^r$ *L. monocytogenes* Scott A are a lower ratio of C15/C17 long-chain fatty acids, a significantly greater amount of PE, and lesser quantities of the anionic phospholipid cardiolipin compared to the wt strain.[437] These changes probably also result in reduced binding of the cationic nisin to the less negative cell membrane, and may also inhibit pore formation by increasing membrane rigidity.

Several studies have also reported constitutive compositional changes in the cell wall of $NIS^r$ cells that persist in the absence of nisin from the growth medium. For instance, $NIS^r$ *L. monocytogenes* cells exhibit increased resistance to lysozyme action and the antibiotics gramicidin S and gentamycin while displaying increased sensitivity to the cell wall targeting antibiotics benzylpenicillin and ampicillin.[437] The degree of resistance to nisin is dependent on the presence of divalent cations ($Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Ba^{2+}$) and their sequestering by EDTA leads to increased nisin sensitivity. The molecular identity of the changes to the cell wall remain to be determined, but no thickening of the cell wall was observed by transmission electron microscopy (TEM) of $NIS^r$ *L. monocytogenes* 700302 cells.[437] A proposed model for nisin resistance in *L. monocytogenes* ATCC 700302 is shown in Figure 33.

Nisin resistance in *L. monocytogenes*[446,447] and *C. botulinum* 169 B[448] has also been related to increased membrane rigidity arising from the presence of more straight-chained, monounsaturated, and saturated

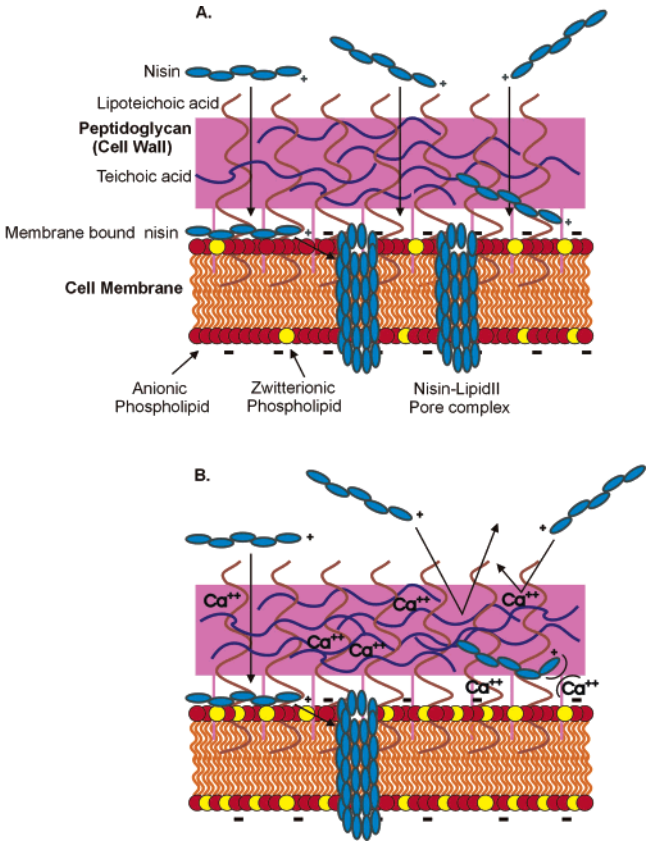



**Figure 33.** Proposed model of nisin resistance[437] in *L. monocytogenes* that includes changes in the cell wall, cytoplasmic membrane, and the requirement for divalent cations. (A) In wt strains nisin passes through the cell wall, binds to the cytoplasmic membrane via electrostatic interactions with anionic phospholipids, and initiates pore formation. (B) In NIS$^r$ cells, the permeability of the cell wall toward nisin is reduced; possibly due to increased amounts of teichoic acid as well as the presence of increased quantities of $Ca^{2+}$ ions. Furthermore, a reduced negative charge density of the cytoplasmic membrane in NIS$^r$ cells leads to weaker binding of nisin molecules. Finally, membrane bound $Ca^{2+}$ ions may repel the positively charged nisin molecules.

fatty acids and less branched, polyunsaturated fatty acids. Similarly, nisin-resistant variants of the pediocin producer, *Pediococcus acidilacti* UL5 exhibited a significant increase in the amounts of monounsaturated C16:1 and C18:1 fatty acids.[450] Interestingly, nisin-sensitive *L. monocytogenes* Scott A cells grown in the presence of 0.1% of the surfactant Tween 20 underwent changes in the cell membrane that did not affect membrane fluidity yet doubled the nisin binding capacity and increased their sensitivity to nisin.[449] While this result holds promise in reducing the growth of nisin-sensitive *L. monocytogenes*, a parallel study of the effect of Tween 20 on NIS$^r$ *L. monocytogenes* Scott A cells has not been reported to date.

Transmission electron microscopy of a NIS$^r$ strain of *Listeria innocua* did show a thickened cell wall that was less hydrophobic than that of wt, and showed an increased resistance to hydrolysis by lysozyme and mutanolysin.[451] The effect of antibiotics such as carbenicillin, vancomycin, and D-cycloserine on the growth of log-phase cultures of NIS$^r$ *L. innocua* was also attenuated in comparison to the wt.[451] It was suggested that cell wall changes in the NIS$^r$ variants may be due to the displacement/inhibition of autolysin by nisin and the activation of murein synthesis. A similar thickening of the cell wall was also observed in TEM experiments on NIS$^r$ strains of *Streptococcus thermophilus* INIA 463 after incubation at 37 °C with nisin for only 2 h.[452] However, contrary to most other cases in which resistance persists even after nisin is removed from the growth medium,[447,448,450,453] this NIS$^r$ strain lost resistance after a single transfer to nisin-free medium.[452] The involvement of the cell wall in acquisition of resistance to nisin has also been reported in *L. monocytogenes* F6861.[454] NIS$^r$ and wt cells became equally sensitive to nisin upon removal of the cell wall by treatment with lysozyme. Similar to the observations with *L. innocua*[451] these NIS$^r$ *L. monocytogenes* cells were less hydrophobic than wt when measured by their affinity for *n*-hexadecane and retention during hydrophobic interaction chromatography.[454] This reduced hydrophobicity of the cell surface may lead to reduced binding of the hydrophobic nisin and thus increased resistance.

Laboratory induced NIS$^r$ cultures of *Streptococcus bovis*, an opportunistic bacterium that resides in the rumen and causes ruminal acidosis in cattle[455] and is associated with colon cancer in humans,[456,457] contained more ampicillin-resistant cells, were unaffected by lysozyme, were less hydrophobic, and bound less (cationic) cytochrome *c*, than wt *S. bovis*.[453] After incubation with nisin, the cell-free supernatant from the NIS$^r$ culture depleted potassium from a second batch of nisin-sensitive cells, suggesting the supernatant did not inactivate the antibiotic nor was it sequestered by the NIS$^r$ cells. In contrast, cell-free supernatants from nisin sensitive cells grown in the presence of nisin, did not exhibit any $K^+$-depletion activity, suggesting that nisin is adsorbed to a much larger degree on the sensitive strain.[453]

A reduction of the net negative charge of the cell envelope has also been suggested to confer increased resistance to nisin in *B. subtilis*.[458] Expression of the enzymes involved in biosynthesis of PE as well as the D-alanylation of lipoteichoic (LTA) and wall teichoic acids (WTA) is controlled in part by the extracytoplasmic-function (ECF) $\sigma^X$ factor. Esterification of the glycerol moieties of teichoic acids with D-alanine introduces free amine (-$NH_2$) groups into the cell wall and leads to a reduction in negative charge. Similarly, the increased content of the zwitterionic PE molecule in place of anionic phospholipids lowers the overall negative charge. Both the *dlt* operon and *pssAybfMpsd* operon, which encode the enzymes responsible for D-alanylation and PE biosynthesis, respectively, are preceded by promoters recognized by the ECF $\sigma^X$ factor. Analysis of *sigX*, *dltA*, *pssA*, and *psd* mutants showed an increased sensitivity to nisin for all except the *pssA* mutant.[458] The authors suggested that extracellular conditions leading to activation of the $\sigma^X$ regulating factor could enhance expression of the *dlt* operon. The direct involvement of the *dlt* operon in resistance of *Staphylococcus xylosus* C2a and *S. aureus* Sa113 to the

lantibiotics nisin and gallidermin was demonstrated by Peschel et al.[459] Mutations of the *dltA* gene in *S. aureus* by homologous recombination, and of *dltA*, *dltB*, and *dltD* genes in *S. xylosus* by transposon insertion resulted in no detectable incorporation of D-alanine in either LTA or WTA of the mutant strains. These *dlt* mutants displayed an 8−50-fold increased sensitivity toward the cationic antimicrobials nisin and gallidermin, while no significant changes in sensitivity toward the neutral peptide gramicidin D was observed.[459] The effect of preventing D-alanylation on the overall charge of the cell envelope has been determined by the affinity toward the cationic molecules cytochrome *c* and gallidermin and the anionic green fluorescent protein (GFP). The *dlt* mutants bound smaller quantities of GFP and greater amounts of cytochrome *c* and gallidermin than the wt strains, suggesting an increased overall negative charge on the cell surface. D-Alanylation of LTA and WTA was regained in the *dlt* mutants by expression of the DltABCD proteins from the plasmid pRBdlt1. The *dlt* mutant strains bearing the pRBdlt1 plasmid regained wt-like resistance to nisin and gallidermin, and wt *S. aureus* and *S. xylosus* transformed with pRBdlt1 displayed a 1.4−1.6-fold increased resistance to the two antibacterials. That this increased resistance was indeed due to increased D-alanylation of LTA and WTA was confirmed by measuring the molar ratio of D-alanine to phosphorus in the mutant and wt strains, before and after the introduction of pRBdlt1. Thus, reduction of the negative charge of the cell envelope by esterification of LTA and WTA with D-alanine in Gram-positive *Staphylococcus* and *Bacillus* strains is one of the better understood mechanisms of resistance to nisin.

Nisin binds to lipid II prior to formation of a nisin-lipid II pore complex in the Gram-positive cell membrane (section 8). Kramer et al. recently investigated the relation between resistance to nisin and the amount of membrane associated lipid II in *M. flavus*, *L. monocytogenes* and their isogenic $NIS^r$ variants.[460] No significant differences were observed in the maximal amount of lipid II in the membranes of $NIS^r$ variants of *M. flavus* and *L. monocytogenes* and the wt strains,[460] indicating that resistance to nisin in these strains is not related to lipid II levels. The authors observed that spheroplasts of $NIS^r$ *M. flavus* that lack the cell wall showed greatly enhanced dissipation of the membrane potential in the presence of even 10 nM nisin, which had no detectable effect on the intact $NIS^r$ cells. This indicates that nisin resistance, at least in *M. flavus*, is determined by changes in the cell wall and is independent of lipid II levels.

Gravesen and co-workers have studied nisin resistance in *L. monocytogenes* 412 by analyzing changes in gene expression in a spontaneous mutant using restriction fragment differential display (RFDD).[440] A 2−4-fold increase in MIC of nisin toward *L. monocytogenes* 412N was associated with the increased expression of genes encoding a protein homologous to the glycosyltransferase domains of high-molecular-weight penicillin binding proteins (PBPs) (*pbp2229*), a histidine protein kinase (*hpk1021*), and an unknown protein (*lmo2487*).[440] Gene disruption studies demonstrated that PBP2229 and HPK1021 are directly involved in imparting nisin resistance while LMO2487 levels did not affect nisin sensitivity.[441] Moreover, the expression of PBP2229 was dependent on the expression of HPK1021, and may be controlled by a two-component signal transduction system that includes HPK1021.[441] Gravesen et al. also observed a 1.8-fold increase in DltA expression in *L. monocytogenes* 412N that might confer resistance by the same mechanism as reported for *S. aureus* by Peschel and co-workers discussed previously.[441,459] The authors proposed that nisin resistance in 412N is probably due to shielding of lipid II from nisin through its binding to PBP2229.

The two-component signal transduction system LisRK has been implicated in sensitivity of *L. monocytogenes* LO28 toward nisin and cephalosporins.[442] Inactivation of the histidine-kinase LisK (not identical to HPK-1021 in *L. monocytogenes* 412N) led to an increased resistance toward nisin. The direct role of the regulatory protein LisR in sensitivity to nisin was demonstrated by its overexpression in the LO28Δ*lisK* strain either constitutively or via nisin controlled expression (NICE[461]). High level induction of *lisR* could overcome the Δ*lisK* mutation and impart sensitivity to nisin.[442] The precise nature of the gene products controlled by the LisRK system is not known, however, similar to the observations of Gravesen et al., they include proteins that bear homology to a histidine kinase, a PBP, and a protein of unknown function. Interestingly in the LO28 strain, deletion of the *lisK* gene led to a greatly reduced transcription of these genes as well as increased nisin resistance,[442] which is in contrast to the observations of Gravesen et al for the 412N strain.[440] The difference between the two studies may be due to different growth phases of the cells that were studied.

Nisin resistance in the nisin nonproducer *L. lactis subspecies diacetylactis* DRC3 is associated with a 60 kbp plasmid, pNP40[462] containing a nisin resistance (*nsr*) gene. Homologous sequences to the nisin resistance protein (predicted mass 35 kDa) were not detected in the nisin producer *L. lactis* 11454, nor were homologous sequences found in the NBRF/PIR and SWISS−PROT data banks at the time of publication of this article (1991). Interestingly, a current BLAST alignment (November 2004) reveals the presence of a C-terminal conserved tail-specific protease domain (residues 107−303). Tail-specific proteases are endopeptidases that bind their polypeptide substrates at a nonpolar C-terminus prior to proteolysis.[463,464] Whether this resistance protein actually causes proteolysis of nisin itself remains to be established since its C-terminal six amino acids include Lys, His, and Ser.

## 10. Summary and Outlook

The posttranslational modifications involved in lantibiotics are unique in Nature and are essential for their biological activities. After the pioneering structural studies, the past decade has seen the accumulation of a wealth of information about their biogenesis from genetic studies. The biochemical

characterization of the proteins involved is only just starting to be explored and with the successful reconstitution of the dehydration, cyclization, and oxidative decarboxylation reactions the near future is likely to unveil many new exciting aspects of enzymatic catalysis. In addition, the relaxed substrate specificity of the biosynthetic machinery will see continuing exploration in vivo and in vitro to engineer lantibiotic variants. These will provide powerful tools to investigate the molecular mode(s) of action of lantibiotics that may result in more effective antimicrobials. The ubiquitous use of the cyclic lanthionine structural motif by Gram-positive organisms to build highly active compounds of very diverse three-dimensional structure argues that it is a natural privileged structure for constraining bioactive peptides. A related motif was recently determined in subtilosin A, a cyclic thioether containing nonlantibiotic posttranslationally modified antimicrobial peptide in which a Cys sulfur is cross-linked to the α-carbon of Phe and Thr.[465,466] This example further illustrates Nature's use of the thioether cross-link to achieve a stable constrained conformer of a bioactive peptide. Certainly, the recent revelations of the recognition of molecular targets such as lipid II and phosphatidyl ethanolamine with very high affinities and specificities suggest that these structures are excellent starting points for design of compounds with biological activities. Whether other lantibiotics may have other specific molecular targets is an open question that will likely be actively investigated in years to come.

The low substrate specificity of the lantibiotic biosynthetic machinery also bodes well for its use in engineering of molecular architectures that are unrelated to lantibiotics. The recent in vivo studies by Kuipers et al.[195] as well as unpublished in vitro results from our laboratory shows that nonlantibiotic prepeptides fused to the leader peptide are substrates for dehydration. Such installation of dehydroamino acids provides orthogonal electrophilic handles for the chemoselective and site-specific introduction of a wide array of functionalities including biophysical probes, thiosaccharides, and prenylthiols.[359−361] Furthermore, it may prove possible to prepare lanthionine analogues of natural cyclic peptides with impoved biological activity and/or stability using the biosynthetic enzymes rather than traditional synthetic chemistry. Clearly, the future of lantibiotic research and application of their biosynthetic machinery holds great promise.

## 11. Abbreviations

| | |
|---|---|
| ABC | ATP-binding cassette |
| Abu | l-α-aminobutyric acid |
| Agr | accessory gene regulator |
| Allo-Ile | *allo*-isoleucine |
| Aloc | allyloxycarbonyl |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| AviCys | *S*-[(*Z*)-2-aminovinyl]-D-cysteine |
| AviMeCys | *S*-[(*Z*)-2-aminovinyl]-(3*S*)-3-methyl-D-cysteine |
| BLAST | Basic Local Alignment Search Tool |
| Boc | *t*-butyl carbamate |
| C12-LPE | dodecanoyl-*sn*-glycerophosphoethanolamine |
| CBD | chitin binding domain |
| CD | circular dichroism |
| Dhb | dehydrobutyrine |
| Dha | dehydroalanine |
| DMSO | dimethyl sulfoxide |
| DPC | dodecylphosphocholine |
| DPG | diphosphatidylglycerol |
| DTT | dithiothreitol |
| ECF | extracytoplasmic-function |
| EGF | epidermal growth factor |
| EPL | expressed protein ligation |
| ESI-MS | electrospray ionization mass spectrometry |
| EXAFS | extended X-ray absorbance fine structure |
| FAB-MS | fast atom bombardment mass spectrometry |
| FAD | flavin adenine dinucleotide |
| FMN | flavine mononucleotide |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| FT-MS/MS | Fourier transform tandem mass spectrometry |
| GC | gas chromatography |
| GFP | green fluorescent protein |
| HFCD | homooligomeric flavin-containing Cys decarboxylase |
| HIF | hypoxia-inducible factor |
| HPLC | high performance liquid chromatography |
| ICP-MS | inductively coupled plasma-mass spectrometry |
| Lan | lanthionine |
| LanA | generic designation for precursor peptides for lantibiotic biosynthesis |
| LanB | generic designation for dehydratases |
| LanC | generic designation for cyclases |
| LANCL | LanC-like protein |
| LanE | generic designation for component of ABC transport protein involved in self-immunity |
| LanF | generic designation for component of ABC transport protein involved in self-immunity |
| LanG | generic designation for component of ABC transport protein involved in self-immunity |
| LanI | generic designation for lantibiotic immunity proteins |
| LanK | generic designation for lantibiotic receptor histidines kinase |
| LanM | generic designation of bifunctional enzymes catalyzing both dehydration and cyclization reactions |
| LanP | generic designation of proteases that remove the leader peptides |
| LanR | generic designation for lantibiotic response regulator protein |
| LanT | generic designation of ABC transporters that excrete lantibiotics after biosynthesis |
| LPS | lipopolysaccharide |
| LTA | lipoteichoic acid |
| MALDI-MS | matrix-assisted laser desorption/ionization |
| MBP | maltose binding protein |
| MeLan | methyllanthionine |
| MIC | minimal inhibitory concentration |
| MRSA | methicillin resistant *Staphylococcus aureus* |
| MS/MS | tandem mass spectrometry |
| NICE | nisin controlled expression |
| NMR | nuclear magnetic resonance |
| NRPS | nonribosomal peptide synthetase |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PCOR | peptide cyclization on oxime resin |
| PCR | polymerase chain reaction |
| PE | phosphatidylethanolamine |

| | |
|---|---|
| PG | phosphatidylglycerol |
| POPC | 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine |
| Rgg | regulator gene of glucosyltransferase |
| SDS | sodium dodecyl sulfate |
| Sec | secratory pathway |
| TBDMS | *tert*-butyldimethylsilyl |
| TEM | transmission electron microscopy |
| TFE | trifluoroethanol |
| wt | wild type |
| WTA | wall teichoic acid |

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## 13. Note Added In Proof

Several very recent studies have appeared in the area of lantibiotics research. The gene cluster of nukacin ISK-1 has been sequenced,[472] and the activity of mersacidin against MRSA has been further demonstrated.[473] The morphogenic peptide SapB from *Streptomyces coelicolor* is likely a lantibiotic,[474] which further illustrates the breadth of nature's choice of the lanthionine as a stable residue to contrain a peptide conformation. Interestingly, the putative SapB biosynthetic machinery contains a protein that has a kinase domain and a domain with sequence homology to the LanC-proteins. The kinase domain may be linked to phosphorylation of Ser/Thr residues to be dehydrated. Of note, the LanC-like domain lacks the putative metal binding residues. In other recent work, the LctT protein has been shown to be required for biosynthesis of lacticin 481,[475] and the substrate specificity of the NisB dehydratase and the NisT transporter has been further defined.[476]

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