Chapter 9
Genetics, Biosynthesis, Structure, and Mode of Action of Lantibiotics

Anneke Kuipers, Rick Rink, and Gert N. Moll

Abstract Lantibiotics are lanthionine-containing peptide antibiotics. They are characterized by having *meso*-lanthionine(s) and/or β-methyllanthionine(s) or both. These intramolecular monosulfide cross-links render the peptide resistant against breakdown by peptidases. Moreover, in several cases, the (methyl)lanthionines are essential for interaction with the so-called docking molecule lipid II. The best known lantibiotic, nisin, highly effectively inhibits growth of target cells via two mechanisms: (1) abduction of the cell wall precursor lipid II from the septum and (2) formation of pores composed of lipid II and nisin. (Methyl)lanthionines result from two enzyme-catalyzed posttranslational modifications: dehydration of serines/threonines and coupling of the resulting dehydro amino acids to cysteines. Besides the localization of the thioether bridges and dehydro amino acids in the lantibiotics, also the three-dimensional structure of some lantibiotics has been resolved by NMR. Genes encoding proteins involved in the biosynthesis of lantibiotics are present in clusters and may comprise combinations of the following genes in varying order: a structural gene that encodes a leader peptide and the lantibiotic propeptide, modification enzyme(s), a transporter responsible for the export of the lantibiotic and in some cases for cleavage of the leader peptide, a leader peptidase, a so-called immunity protein involved in self-protection of the host cell, components of a transporter also involved in self-protection, and two components of an autoinduction system.

Introduction

The name lantibiotics was introduced more than two decades ago (Schnell et al. 1988). Lantibiotics are ribosomally produced dehydroresidue- and (methyl)lanthionine-containing peptides (Fig. 9.1). Lanthionines are thioether-bridged amino acids

G.N. Moll (✉)
BiOMaDe Technology Foundation, Nijenborgh 4, 9747 Groningen, The Netherlands
e-mail: Moll@biomade.nl

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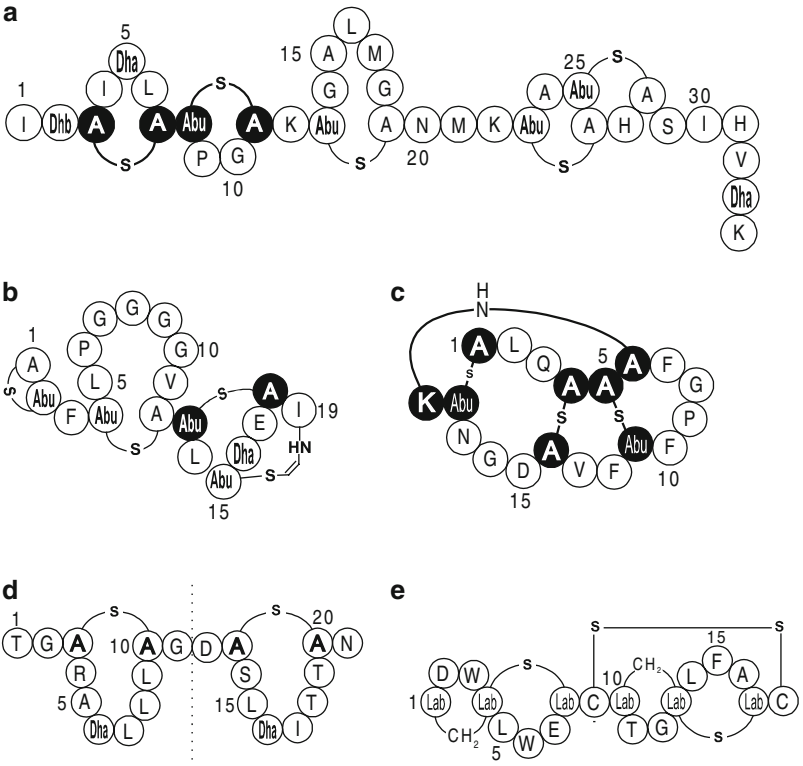

Fig. 9.1 Examples of lantibiotics. (**a**) nisin, (**b**) mersacidin, (**c**) duramycin, (**d**) sapB and (**e**) labyrinthopeptin A2. (Methyl)lanthionines and lysinoalanine in black are conserved within structural groups termed A, B, and C distinguished by Rink et al. (2005). The vertical dotted line indicates local structural symmetry in this morphogenetic lantibiotic (D). "Lab" in Figure E is labionine

(Fig. 9.2a). They are predominantly produced by Gram-positive bacteria, and those with antibiotic activity are principally effective against Gram-positive bacteria. Besides *meso*-lanthionine (Ala-S-Ala) and β-methyllanthionine, several other post-translational modifications may occur in lantibiotics (Table 9.1).

Nisin was the first lantibiotic described in literature (Rogers and Whittier 1928) and is the most studied lantibiotic. It is produced by different *Lactococcus lactis* strains. Already in 1969, nisin was approved for use as a food preservative (Delves-Broughton 2005). Nisin has a broad activity spectrum against Gram-positive bacteria, including strains of *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Lactobacillus*, *Bacillus*, *Listeria*, and *Clostridium*, (Thomas et al. 2000), and has antimicrobial activity in the nanomolar range (de Vos et al. 1993). Despite its world wide application as a food additive, virtually no resistance against nisin has occurred. Owing to their stability, high activity, and virtual absence of resistance development, lantibiotics are promising candidates for biomedical application. Their features make the

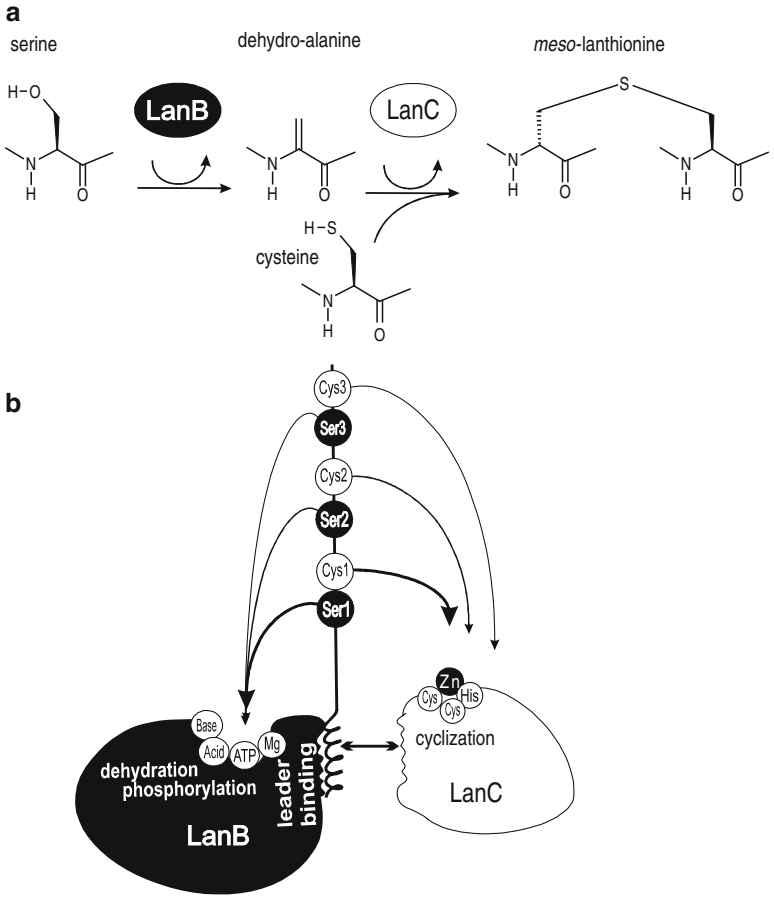

Fig. 9.2 (**a**) Enzyme-catalyzed dehydration and cyclization. (**b**) Hypothetical distributive mechanism of lantibiotic modification. A leader peptide binds to the modification enzyme, thus bringing the modifiable residues in the vicinity of the catalytic site. Due to the different distances to the catalytic site, residues 1, 2, and 3 will most frequently be modified in the order 1, 2, and 3, but all other orders may also occur. Dehydratase and cylase activities may alternate (Kuipers et al. 2008) due to vicinity of the leader-binding sites and or due to vicinity of the catalytic sites

search for new lantibiotic variants that are further improved by genetic engineering an exciting and relevant approach in the battle against multiple-antibiotic-resistant pathogens (Kuipers et al. 1992; Rink et al. 2007b; Field et al. 2008).

Nisin, subtilin, epidermin, Pep5, and some similar lantibiotics were first designated as type A lantibiotics, which are rod-shaped, flexible with an elongated structure, and mainly act by forming pores in the bacterial membrane (Jung 1991). Type B lantibiotics (e.g., cinnamycin, duramycin, and ancovenin) were discerned as having a higher degree of cyclization resulting in structures that are more globular and being devoid of pore-forming activity. To date, more than 60 different lantibiotics

Table 9.1 Residues occurring within lantibiotics

Residue	Lantibiotic	Reference
meso-Lanthionine	Most lantibiotics	Chatterjee et al. (2005b)
β-Methyllanthionine	Most lantibiotics	Chatterjee et al. (2005b)
2,3-Dehydroalanine	Most lantibiotics	Chatterjee et al. (2005b)
(Z)-2,3-Dehydrobutyrine	Most lantibiotics	Chatterjee et al. (2005b)
S-(Z)-Aminovinyl-D-cysteine	Epidermin, gallidermin, cypemycin	Allgaier et al. (1986), Kellner et al. (1988), Minami et al. (1994)
S-Aminovinyl-D-3-methylcysteine	Mersacidin	Chatterjee et al. (1992)
D-Alanine	Lactocin S, lactocin 3147	Skaugen et al. (1994); Martin et al. (2004)
Alloisoleucine	Cypemycin	Minami et al. (1994)
Dimethylalanine	Cypemycin	Minami et al. (1994)
Amide	Nisin(1-32)amide	Chan et al. (1989)
Erythro-3-hydroxy-L-aspartic acid	Cinnamycin, duramycin, duramycin B, duramycin C	Kessler et al. (1988); Naruse et al. (1989); Fredenhagen et al. (1991)
(2S, 8S)-Lysinoalanine	Cinnamycin, duramycin, duramycin B, duramycin C	Kessler et al. (1988); Naruse et al. (1989); Fredenhagen et al. (1991)
2-Oxobutyryl	Pep5	Kellner et al. (1989)
2-Oxopropionyl	Lactocin S, epilancin K7 (3-31)	Skaugen et al. (1994); van de Kamp et al. (1995a)
Hydroxypropionyl	Epilancin K7	van de Kamp et al. (1995b)
Acetyl	Paenibacillin	He et al. (2008)
Sulfoxide	Actagardine	Boakes et al. (2009)
disulfide	Thermophilin 1277	Kabuki et al. (2009)
5-Chloro-trypthopan	Microbisporicin	Castiglione et al. (2008)
Mono-/bis-hydroxylated proline	Microbisporicin	Castiglione et al. (2008)
Labionine	Labyrinthopeptins	Meindl et al. (2010)

have been discovered (Bierbaum and Sahl 2009), and several different posttranslational modifications have been described (Willey and van der Donk 2007; Table 1). After the finding of many new lantibiotics, the clarity of the old type A and type B classification diminished, and two (Oman and van der Donk 2010) or three (Pag and Sahl 2002; Willey and van der Donk 2007) groups were proposed on the basis of novel criteria. The three-group classification placed all lantibiotics in one of three classes initially on the basis of the biosynthesis machinery used for maturation of the peptide (class I and class II) or the absence of antibiotic activity (class III). Moreover, in class III lantibiotics, LanM maturation enzymes might act via a mechanism distinct from that of class II LanM enzymes.

Several detailed reviews on all lantibiotics exist (Chatterjee et al. 2005b; Bierbaum and Sahl 2009; Asaduzzaman and Sonomoto 2009) and also reviews on clinical applications of lantibiotics (Smith and Hillman 2008), on subgroups of lantibiotics (Lawton et al. 2007; Dufour et al. 2007; Willey et al. 2006), on a single

lantibiotic (Lubelski et al. 2008), and on the application of lantibiotic enzymes for modifying nonlantibiotic peptides (Moll et al. 2010). In this chapter, we focus on the genetics, synthesis, structure, and mode of action of lantibiotics including the most recent developments.

Genetics and Biosynthesis

The genes involved in lantibiotic synthesis are arranged in clusters. These gene clusters can be organized on a transposon (nisin), on the chromosome (subtilin), or on a plasmid (epidermin). Genes on these clusters have been designated the generic locus symbol *lan* (de Vos et al. 1991). In 1993, the gene cluster involved in the nisin biosynthesis was unraveled (van der Meer et al. 1993, Kuipers et al. 1993a). Besides the gene products required for the biosynthesis of the peptides, proteins that are needed for the processing (LanP), translocation (LanT), self-protection/immunity (LanI, LanEFG), and regulation (LanRK) are also encoded. Per type, many of these proteins encoded on the different gene clusters show amino acid homology, which indicates that indeed they have similar functions (Siezen et al. 1996; Kuipers et al. 1993a; Qiao et al. 1996).

The lantibiotics nisin, epidermin, and Pep5 belong to the class I lantibiotics. In class I lantibiotics, the prepeptide LanA is modified by two distinct enzymes, LanB and LanC. The lantibiotic prepeptide contains a leader sequence that is thought to be necessary for targeting the propeptide part to the separate modifying, processing, and translocating enzymes. LanB dehydrates the serines and threonines in the propeptide part of LanA, and LanC couples these dehydrated residues regio- and stereoselectively to cysteines to form respectively *meso*lanthionines and β-methyllanthionines. After translocation of the modified peptide via an ABC transporter LanT, the leader part is, in most class I lantibiotic systems, removed by a protease LanP, releasing the active lantibiotic (Willey and van der Donk 2007).

In class II lantibiotics, e.g., lacticin 481, mersacidin, and actagardin, only one enzyme is responsible for dehydration and cyclization of the propeptide LanA. These bifunctional LanM enzymes as well as LanB enzymes are both composed of about 1000 amino acids and share no sequence homology. The N-terminal parts of LanM enzymes are responsible for the dehydration reaction, but surprisingly, their sequences are about 400 amino acids shorter than those of LanB dehydratases, and they have no similarity to LanB enzymes. LanC enzymes are composed of around 400 amino acids. The C-terminal part of LanM enzymes has low sequence homology with the LanC enzymes (Siezen et al. 1996), including three zinc- coordinating amino acids (Patton and van der Donk 2005). Knockouts of one of these zinc ligands completely abolished the cyclase activity of NisC or LctM (Li et al. 2006, Paul et al. 2007). Another dissimilarity to class I lantibiotics is the dual functionality of LanT. Before translocation of the modified peptide, the peptide is intracellularly processed by the conserved N-terminal protease part of LanT (Pag and Sahl 2002, Willey and van der Donk 2007). Class II lantibiotics also comprises the two-component lantibiotics,

e.g., lacticin 3147 (Ryan et al. 1996) and haloduracin (McClerren et al. 2006). In the case of lacticin 3147, the two prepeptides LtnAα and LtnAβ are each separately modified by the two corresponding enzymes, respectively, LtnM1 and LtnM2. After modification, both peptides are processed and translocated by one LtnT enzyme. The gene cluster of lacticin 3147 contains a gene coding for an additional posttranslational modification enzyme, LtnJ. This enzyme converts some dehydroalanines in the prepeptides Ltnα and Ltnβ to D-alanines (Ryan et al. 1999).

The third class of lantibiotics was initially discerned as (methyl)lanthionine-containing peptides often devoid of antimicrobial activity. Instead, they would have other –for instance, morphogenetic– features that may be beneficial to the producing cells. The three lantibiotics that were first found for this group were SapB (Kodani et al. 2004; Fig. 9.1d), SapT (Kodani et al. 2005), and AmfS (Ueda et al. 2002). SapB and SapT are believed to be biosurfactants that may have a positive effect on the surface of aerial hyphae of the producer strains. In addition, SapT has antimicrobial activity against *Bacillus cereus* (Kodani et al. 2005). Interestingly, RamC, the presumed SapB modification enzyme has an N-terminal domain that resembles a Ser/Thr kinase and a central dimerization domain (Kodani et al. 2004). Furthermore, the enzymes involved in the biosynthesis of SapB and AmfS share homology with the C-terminal part of other LanM enzymes except for the zinc ligands, which are not conserved.

Three recent and exciting publications further characterized class III lantibiotics. Labyrinthopeptins were discovered by Meindl and coworkers by following the activity of labyrinthopeptin A2 against *Herpex simplex* virus. In addition, labyrinthopeptin A2 has an excellent efficacy against neuropathic pain in an in vivo mouse model. Labyrinthopeptins belong to class III lantibiotics and contain labionine, a carbacyclic triamino acid (Meindl et al. 2010; Fig. 9.1e). Subsequently, the in vitro reconstitution of the prelabyrinthopeptin A2 biosynthesis was demonstrated, which required guanosine triphosphate for the phosphorylation and dehydratation reaction of serines (Müller et al. 2010). A paper from the group of van der Donk demonstrated that a class III enzyme LanL comprises a kinase domain, which phosphorylates Ser/Thr, a phosphoSer/Thr lyase domain, and a cyclase domain comprising a zinc finger. It was proposed that LanL enzymes have evolved from stand-alone protein Ser/Thr kinases, phospho-Ser/Thr lyases, and enzymes catalyzing thiol alkylation. The name lantipeptides was suggested for compounds that by structure and biosynthesis are related to lantibiotics but that are not known to possess antimicrobial activity (Goto et al. 2010).

Engineering of Lantibiotics

With the elucidation of gene clusters involved in the biosynthetic pathways of lantibiotics, genetic engineering of lantibiotics became the next challenge. Much more studies have been performed on the engineering of the lantibiotics than on the mutagenesis of their leader peptides. The existence of natural variants among lantibiotics (e.g., nisin

A/nisin Z, nisin Q (Fukao et al. 2008), nisin U (Wirawan et al. 2006), epidermin/gallidermin) and the high homology between certain lantibiotics (i.e., nisin/subtilin/lantibiotic 97518 (Maffioli et al. 2009), mutacin II/lacticin 481) suggest that the identity of amino acids present at certain locations is flexible. Indeed, by site-directed mutagenesis of the structural genes and the development of expression systems, many lantibiotic variants were designed and produced in vivo (Cotter et al. 2005).

In 1992, the first nisin mutants were reported (Kuipers et al. 1992) followed by many other nisin mutants (reviewed by: Kuipers et al. 1996; Lubelski et al. 2008). Interestingly, a T2S mutant had increased activity (Kuipers et al. 1996) and some nisin hinge region mutants had antimicrobial activity against Gram-negative species (Yuan et al. 2004). By altering the charge of the nisin lantibiotic, solubility could be improved (Yuan et al. 2004). Randomization of the codons of the amino acids in nisin's ring A and ring B yielded a large number of mutants (Rink et al. 2007b). Nisin ring A mutants I4K/S5F/L6I and I4K/L6I showed enhanced activity against some target strains (Rink et al. 2007b), as did mutations M21V, N20P, and K22T in the hinge region (Field et al. 2008). Ring A mutants were obtained with enhanced activity against some strains, mutants that were not recognized by the self-protection systems, whereas opening of ring B caused loss of antimicrobial activity while the induction capacity remained intact (Rink et al. 2007b). Early successful mutagenesis studies also concerned subtilin (Liu and Hansen 1992) and gallidermin (Ottenwälder et al. 1995).

The first novel thioether bridge was introduced in Pep5. By the substitution A19C, a β- methyllanthionine was introduced in the peptide, which was formed between the Dhb on position 16 and the introduced cysteine at position 19. This mutant exhibited increased proteolytic stability against chymotrypsin and Lys-C. However, the novel thioether bridge had a negative effect on the antimicrobial activity of Pep 5 (Bierbaum et al. 1996). Also, in the class II lantibiotics, comprising mutacin II (Chen et al. 1998), mersacidin (Szekat et al. 2003), cinnamycin (Widdick et al. 2003), and actagardine (Boakes et al. 2009), new variants were made by site-directed mutagenesis. A systematic mutant analysis by alanine scanning of the two-peptide lantibiotic lacticin 3147 revealed the areas within the peptide that are amenable to changes and areas that are essential for the production. None of the mutants displayed an antimicrobial activity higher than that of the wild-type producer (Cotter et al. 2006). Succesful methods and strategies have been developed to engineer new lantibiotic variants and analyze libraries (Cortés et al. 2009).

By random mutagenesis and NNK scanning of nukacin ISK-1, a series of nukacin ISK-1 variants was generated to identify the positional importance of individual residues responsible for antimicrobial activity (Islam et al. 2009). Furthermore, by random mutagenesis of mersacidin, 80 mutants that produced mature mersacidin at good levels were made, and novel variants were obtained with improved overall bioactivity, such as F3W (Appleyard et al. 2009).

Novel lantibiotics with enhanced antimicrobial activity may be lethal for the producer itself. Many *pre*lantibiotics are inactive; apparently, the presence of the N-terminal leader peptide keeps these prelantibiotics inactive. To avoid lethality of engineered lantibiotics, a production system can be used without leader peptidase

(Rink et al. 2007b). After production, the leader can be removed. Another approach is using an in vitro modification system. The lantibiotics lacticin 481 and the two peptide lantibiotic haloduracin were both modified successfully by incubation of the precursor peptide with the LanM enzymes in vitro (Xie et al. 2004; McClerren et al. 2006). Furthermore, NisC could successfully cyclize dehydrated prenisin (Li et al. 2006).

Lantibiotic chimeras of nisin and subtilin have been made (Chakicherla and Hansen 1995). By replacing the propeptide-encoding sequence of one lantibiotic by the propeptide-encoding sequence of another lantibiotic, lantibiotics have been produced by the biosynthesis machinery of a system within the same class (Kuipers et al 1993b; Patton et al. 2008), and also of another class. Class II pneumococcins have been dehydrated, cyclized, and exported by Class I enzymes from the nisin system (Majchrzykiewicz et al. 2010). Overall, the biosynthetic systems used for the biosynthesis of lantibiotics seem to have a remarkable flexibility.

Mechanistic Aspects of the Biosynthesis of Lantibiotics

Lantibiotic genes code for prepeptides, which are composed of a leader peptide and a modifiable propeptide. The leader peptide appears to have a leading role in the posttranslational modification processes and in export (Patton et al. 2008). It induces the LanB/LanM-catalyzed dehydration of serines and threonines, the LanC/LanM-catalyzed cyclization, and the LanT-mediated export. However, it was demonstrated that presenting the leader in trans, not attached to the substrate, still leads to modification of the structural peptide (Levengood et al. 2007; Patton et al. 2008). Some, but only strongly reduced, LctM activity has also been demonstrated in the absence of the leader peptide. Since LanB, LanC, LanM, and LanT can be active in the absence of other lantibiotic enzymes, it is likely that each enzyme itself has a leader peptide-binding site. Possible roles of the leader could thus be stabilizing of an active conformation of the enzyme (Patton et al 2008) and bringing the modifiable substrate propeptide in the vicinity of the active center of the modification enzymes (Fig. 9.2b).

Alignments of leader peptides suggest at least two groups (Plat et al. 2010). One group containing an FNLD sequence, mainly occurring in Class I, LanB and LanC-modified prelantibiotics (Chatterjee et al. 2005b; Table 9.2). Mutagenesis of residues in this box affected nisin biosynthesis (van der Meer et al. 1994) and Pep5 production (Neis et al. 1997). Another group with an EVxxxEL sequence occur in Class II, LanM-modified prelantibiotics (Chatterjee et al. 2005b; Table 9.2). Some mutations of some leader peptide residues in mutacin II eliminated biosynthesis, whereas other mutations only affected the level of production (Chen et al. 2001). In the leader peptide of lacticin 481, not one of the conserved and not-conserved residues appears essential. Introduction of prolines, however, seems to interfere with the functionality of the leader peptide (Patton et al. 2008). For nukacin ISK-1, the importance of the α-helicity of the leader peptide was demonstrated (Nagao et al. 2009).

Table 9.2 Diversity of lantibiotic leader peptides. In bold: residues (partly) conserved within an alignment (sub)group

Lantibiotic	Leader peptide sequence	References for peptide and/or alignment
Nisin	MSTKD**FNLD**LVSVSKKDSGAS**P**R	Gross and Morell (1971); Chatterjee et al. (2005b)
Mutacin II	MNKLNSNAVVSLN**EV**SDS**EL**DTIL**GG**	Woodruff et al. (1998); Chatterjee et al. (2005b)
Cytolysin LS	MLNKENQENYYSNKLELVGPSFEELSLEE MEAIQGSGDVQAE	Gilmore et al. (1994)
Cinnamycin	MTASILQQSVVDADFRAALLENPAAFGASA AALPTPVEAQDQASLDFWTKDIAATEAFA	Widdick et al. (2003)
Michiganin	MNDILETETPVMVSPRWDMLLDAGEDTSP SVQTQIDAEFRRVVSPYM	Holtsmark et al. (2006)

The leader peptides from two component lantibiotics seem to differ from the aforementioned leader peptides, e.g., plantaricin Wα/Wß (Holo et al. 2001), staphylococcin C55α/ß (Navaratna et al. 1999). The leader peptides from the two-component lantibiotic cytolysin (Gilmore et al. 1994, Table 2) are processed at two cleavage sites in each peptide.

Most lantibiotic leader peptides are composed of about 20–35 amino acids. In contrast, the leader peptides from actagardine (Boakes et al. 2009), michiganin (Holtsmark et al. 2006, Table 9.2), and mersacidin (Bierbaum et al. 1995) are composed of respectively 45, 47, and 48 amino acids, whereas the leader peptide from the globular-shaped lantibiotic cinnamycin (Kessler et al. 1988) is even composed of 59 amino acids (Widdick et al. 2003; Table 9.2). Structures of some leader peptide-dependent lantibiotic modification enzymes may provide further insight into the roles of the leader peptide in the intriguing biosynthesis of lantibiotics. Indeed, the structure of NisC indicates a leader peptide binding site (Li et al. 2006).

Data on the substrate specificity of the lantibiotic modification enzymes have been obtained mainly for the nisin and lacticin 481 modification enzymes. The nisin biosynthesis machinery NisBTC proved to be highly versatile and can be used for the introduction of thioether rings in a broad spectrum of nonlantibiotic peptides (Rink et al. 2005; Kluskens et al. 2005; Rink et al. 2007c). Furthermore, successful dehydration of threonines/serines seems to be influenced by the flanking residues. Hydrophobic flanking residues on one or both sides may favor dehydration, whereas the simultaneous presence of hydrophilic flanking residues on both sides seems to disfavor dehydration (Rink et al. 2007a). Lantibiotic cyclases can catalyze the coupling of dehydroalanines and dehydrobutyrines to cysteines yielding lanthionines and β- methyllanthionines, respectively. However, dehydroalanines are reactive and can, in the absence of cyclase action, also spontaneously form a lanthionine when reacting with a cysteine under mild conditions (Rink et al. 2007c). Such nonenzymatic ring closure can result in a mixture of stereoisomers (Burrage et al. 2000).

Not only are small thioether-bridged peptides synthesized by utilization of NisBTC, but a more complicated substrate peptide with the sequence ITPGCKATVECKITGPCKATVECK can also be successfully modified to a peptide with four thioether linkages. Also, introduction of intertwined thioether rings is possible, thanks to the stereo- and regiospecificity of NisC (Rink et al. 2007c).

The lacticin 3147 enzymes LtnT and LtnM2 were also successfully used in vivo for the introduction of thioether bridges in nonnatural peptide substrates. Before translocation of the nonnatural substrate by LtnT, the peptide first has to be processed. The LtnA2 leader is intracellularly cleaved off by the same LtnT enzyme. Not all designed peptides could in vivo be produced via the Ltn enzymes. It is not clear whether the processing or the translocation itself limits the production of nonlantibiotic peptides in the case of cells containing LtnT (Kuipers et al. 2008).

Studies of lantibiotic biosynthesis systems for nisin, subtilin, and nukacin ISK1 revealed that the modification enzymes and transporters are arranged in multimeric membrane-associated enzyme complexes (Siegers et al. 1996; Kiesau et al. 1997; Nagao et al. 2005). Additionally, a study suggested the presence of a multimeric enzyme complex and the importance thereof for optimal production of prenisin (van den Berg van Saparoea et al. 2008). Alternatively, the presence of thioether rings might impose a structure, which is transported more efficiently by NisT (Kuipers et al. 2008) and is capable of autoinduction (Kuipers et al. 1995).

The composing enzymes of the nisin synthethase complex are separately functional in the absence of other lantibiotic enzymes (Kuipers et al. 2004, Kuipers et al. 2006). In the absence of other nisin enzymes, NisB-dehydrated peptides were exported via the Sec pathway when the nisin leader was preceded by a Sec signal sequence. Prenisin with or without preceding Sec or Tat signal was intracellularly fully modified by NisB and NisC in the absence of NisT, which precludes the necessity of either NisT or Sec action. In view of the above case, it is difficult to comprehend the proposed enzyme-complex-dependent NisT-driven-modification working model for nisin biosynthesis by Lubelski et al. (2009).

In vitro activity of the biosynthetic enzyme of lacticin 481 (Xie et al. 2004) of both haloduracin modification enzymes (McClerren et al. 2006), and of the cyclase of nisin (Li et al. 2006) has been attained (Li et al. 2009). Although LctM does not display an evident ATP-binding domain, ATP is necessary for functionality of LctM (Chatterjee et al. 2005a) and likely – in view of the kinase domain- ATP or GTP is necessary for RamC, the modification enzyme of SapB (Kodani et al. 2004). With the powerful in vitro thioether modification system, the substrate specificity and several mechanistic aspects of the LctM 481 enzyme were explored. Like NisB, LctM has high substrate promiscuity. LctM can dehydrate a range of nonlantibiotic peptides when attached to the N-terminal leader peptide (Chatterjee et al. 2006; Levengood and van der Donk 2008; Patton et al. 2008). Whereas hydrophilic amino acids, especially negatively charged amino acids, which flank dehydratable substrate residues, disfavor NisB-mediated dehydration, this does not appear to be the case for LctM (Chatterjee et al. 2006). Semisynthetic LctA derivates with nonproteinogenic amino acids like β-amino acids, D-amino acids, and N-alkyl amino acids, derived by expressed protein ligation, are successfully modified by LctM (Zhang and van der Donk 2007; Levengood et al. 2009a; Levengood et al. 2009b).

LctM phosphorylates the serines and threonines of its substrate prior to the dehydration of these residues (Chatterjee et al. 2005a; You and van der Donk 2007). An LctM T405A mutant was not affected in phosphorylation of serines and threonines of its substrate but was hampered in the phosphate-elimination step and thereby lost the ability to dehydrate its substrate. This mutant turned out to be a highly efficient kinase for a broad range of peptide substrates with serines, provided that the lacticin leader was N-terminally present (You et al. 2009). Recently, distributive and a tendency to an apparently directional behavior of the LctM enzyme were revealed (Lee et al. 2009). LctM has a high, though not strict, propensity for an apparent N- to C- directionality. When the leader peptide is present in trans, dehydrations were nondirectional. Also, intermediates were found, which were not completely dehydrated but already contained rings within their N-terminal region. This latter finding suggests that the dehydration and the cyclization activity of LctM can be alternating activities. This alternating feature was also observed for NisB- and NisC activity (Kuipers et al. 2008; Fig. 9.2b). The latter data were supported by data obtained by Lubelski et al., who also suggested that NisB and NisC are acting in an N- to C- direction (Lubelski et al. 2009). On the basis of the above observations, we propose a model depicted in Fig. 9.2b. When NisB and NisC have each a fixed leader peptide-binding site close to the active site, the distances of the residues that have to be modified in the prepeptide part of nisin to the active site are set. A residue that is located close to the leader and to the active site will have a much higher chance to be modified compared to a residue further away. The result will appear as directionality, but is actually the result of different binding constants of the residues determined by sequence and distance.

Lantibiotics and lantibiotic enzymes constitute a fascinatingly rich and diverse field of research. Important new mechanistic insights may follow from the crystallization of lantibiotic enzymes other than the cyclase NisC (Li et al. 2006) and the decarboxylase MrsD (Blaesse et al. 2003). In vitro reconstitution of LanB activity, which has not been attained despite many efforts in several laboratories, will hopefully be realized one day and facilitate further mechanistic studies.

Structure

The (methyl)lanthionines give lantibiotics their unique features such as thermostability and proteolytic resistance, and most (methyl)lanthionines are essential for high antimicrobial activity. The primary structure of lantibiotics is highly variable. Aligning the sequences of unmodified propeptides led to three structural groups with one or more conserved (methyl)lanthionine positions, represented by nisin (Fig. 9.1a), mersacidin (Fig. 9.1b), and duramycin (Fig. 9.1c), and a remaining number of apparently unrelated lantibiotics containing among others the morphogenetic sapB (Fig. 9.1d) (Rink et al. 2005). On the basis of primary lantibiotic structures also other groups have been discerned (Chatterjee et al. 2005b; Twomey et al. 2002).

Structural data have been reported on some lantibiotics that share the (methyl) lanthionine positions of rings A and B of nisin (Fig. 9.1a). The structure of nisin

was elucidated by chemical degradation (Gross and Morell 1971) and further studied by nuclear magnetic resonance (NMR) spectroscopy in the presence and absence of membrane-mimicking agents (van de Ven et al. 1991; Lian et al. 1992; van den Hooven et al. 1993, Van den Hooven et al. 1996). The data indicated 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 E that are joined by a flexible hinge region. Similarly, for gallidermin, in the presence of the structure-inducing solvent trifluoroethanol, an extended amphiphilic screw-shape structure was observed (Freund et al. 1991a, b). Also, the solution structure of mutacin 1140 measured in acetonitrile–water (80:20) indicated rigidity within the lanthionine rings as well as the flexibility of the C-terminal part (Smith et al. 2003).

NMR studies have also been performed on lantibiotics that share the position of the third ring of mersacidin (Fig. 9.1b). Mutacin II consists of an N-terminal helix formed by residues 1-8 (Novak et al. 1997). CD studies on SA-FF22 in lipid-mimicking conditions indicated a significant change compared to the structure in aqueous environment (Jack et al. 1994). The NMR solution structure of plantaricin C indicates a flexible, positively charged N-terminus and a rigid globular C-terminal domain (Turner et al. 1999). The mersacidin NMR structure in methanol solution is globular and has three domains formed by the thioether ring spanning residues (Prasch et al. 1997; Hsu et al. 2003). The X-ray crystallography structure of mersacidin (Schneider et al. 2000) largely resembled the solution structure. The NMR solution structure of actagardine was determined in a water–acetonitrile mixture. Actagardine has a compact globular structure composed of two domains. The N-terminal domain consists of a single lanthionine ring, while the C-terminal domain is composed of three intertwined methyllanthionine rings. Residues 7-8, 9-12, and 17-19 form a small, three-stranded β-sheet with one antiparallel and two parallel strands (Zimmermann and Jung 1997). Preliminary NMR solution structure data of LtnA1 (Martin et al. 2004) indicate a globular shape resembling mersacidin (Hsu et al. 2003).

Detailed structural studies are also known for highly globular lantibiotics which share the (methyl)lanthionine and lysinoalanine structure of duramycin (Fig. 9.1c). The NMR solution structure of cinnamycin has been determined in DMSO and a water–acetic acid mixture (Kessler et al. 1991). The conformation of cinnamycin changes in the presence of SDS micelles (Kessler et al. 1992). The lipophilic part of cinnamycin changed in the presence of SDS bilayers (Kessler et al. 1992) and in the presence of 1-dodecanoyl-*sn*-glycerophosphoethanolamine (C12-LPE) (Wakamatsu et al. 1990) due to interactions with hydrophobic segments of the lipids.

Taken together, detailed structural information for several lantibiotics has been obtained in solution and in the presence of lipophilic membrane-mimicking agents. Generally, lantibiotics contain a few rigid domains, and their conformation changes when interacting with lipophilic or membrane-mimetic surroundings. Furthermore, mechanistically highly important structures describing the interaction of lantibiotics with docking molecules have been obtained (Hsu et al. 2004).

Modes of Action

Pore Formation

Many studies have been performed on the capacity of several lantibiotics to permeabilize membranes. These studies involved liposomes, proteoliposomes, cell-membrane vesicles, black lipid membranes, lipid monolayers, and intact cells and provided much information on the biophysical aspects of the interaction of lantibiotics with these different membrane models. However, up to three orders of magnitude higher concentrations of nisin were needed for permeabilization of model membranes than for pore formation in intact cells. This difference was explained by an excellent study by Sahl and coworkers who were the first to demonstrate that nisin interacts with lipid II, a precursor in the cell wall synthesis. Nisin activity could be eliminated by adding ramoplanin, a compound interacting with lipid II (Brötz et al. 1998a). Subsequent studies demonstrated that hybrid pores composed of nisin and lipid II with a stoichiometry of 1:2 were formed in the target cell membrane. The presence of lipid II in liposome strongly reduced the concentration of nisin required for pore formation (Breukink et al. 1999). The presence of lipid II in black lipid membranes reduced the required threshold membrane potential from about 100 mV to 5–10 mV, allowed the induction of pores by not only trans-negative but also trans-positive membrane potential, and strongly prolonged the lifetime of the formed nisin pores from milliseconds to seconds. Nisin can also form pores in cells when there is no transmembrane electrical potential at all (Moll et al. 1997). Dissipation of a transmembrane electrical potential by itself is not leading to growth inhibition, but dissipation of the transmembrane pH gradient is sufficient for complete growth inhibition (Moll et al. 1999). The structure of nisin lipid II complexes reveals a pyrophosphate cage formed by ring A (Hsu et al. 2004). Interaction with lipid II, leading to pore formation, has also been suggested for a central part of the LtnA1 peptide from the two-component lantibiotic lacticin 3147 (Cotter et al. 2006). Interaction of LtnA1 with lipid II was experimentally demonstrated, and the role of LtnA2 in pore formation has been elucidated (Wiedemann et al. 2006b). Pep5 and epilancin K7 do not interact with lipid II or lipid I but still have high antibacterial activity. Likely, this high activity might be explained in the future by the interaction with (an) other docking molecule(s).

Antibacterial Activity by Interaction With Lipid II Without Pore Formation

Resistance against vancomycin is increasingly threatening. Hybrids of vancomycin and just an N-terminal nisin fragment, nisin(1-12), which does not span the membrane, could prevent the raise of such resistance. These hybrids simultaneously interact with two different sites of lipid II (Arnusch et al. 2008). Epidermin, which

shares the pattern of ring A and ring B with nisin, also interacts with lipid II. Likely, other lantibiotics, e.g., mutacin I, mutacin III, mutacin 1140, mutacin B-Ny266, gallidermin, ericin A, ericin S, subtilin, nisin Z, nisin Q, which all share this ring pattern of ring A and B interact with lipid II. Nisin can interact with lipid I and lipid II. Nisin and epidermin cause an accumulation of lipid I in in vitro peptidoglycan synthesis (Brötz et al. 1998b). Truncated nisin(1-23) variants have significant activity but are unable to dissipate the membrane potential (Rink et al. 2007b). Interaction of epidermin and mutacin 1140 with lipid II has been demonstrated. Epidermin whose activity can be even higher than that of nisin does not form pores, since it is too short to span the membrane; in thin model membranes, it does permeabilize the membrane. Breukink and coworkers demonstrated that nisin is able to dislocate lipid II from the septum, thus inhibiting growth of the cell wall (Hasper et al. 2006). It might be worthwhile to investigate whether this mechanism might be responsible for the activity of truncated nisin(1-23) and short lantibiotics that share the ring pattern of nisin's ring A and ring B. Also, other mechanisms have been described. Plantaricin C forms complexes with lipid I and lipid II and inhibits lipid II synthesis and the addition of the first glycine of the pentapeptide chain of lipid II (Wiedemann et al. 2006a, b). Mersacidin and actagardine, whose structures are entirely different from nisin, also bind to lipid II but do not form pores (Brötz et al. 1995; 1997; 1998b). Instead, they exert in vivo high antibacterial activity by blocking the transglycosylation step in the peptidoglycan synthesis.

Inhibition of the Outgrowth of Spores

Some lantibiotics such as nisin and subtilin inhibit the germination of spores of the species from the genera *Bacillus* and *Clostridium* (Thomas et al. 2002). It was suggested that the mechanism of inhibition resulted from the reaction of thiol groups in proteins of the spores with dehydroalanine in position 5 of subtilin and nisin (Morris et al. 1984). Indeed, replacement of the dehydroalanine in position 5 for an alanine caused loss of the capacity to inhibit the outgrowth of spores. However, nisin ringA mutants with other residues in position 5 even had enhanced activity in inhibiting the outgrowth of spores (Rink et al. 2007b).

Other Activities

Cinnamycin and its natural variant duramycin effectively inhibit phospholipase A2 by binding to its phosphatidylethanolamine substrate (Märki et al. 1991). The stoichiometry of the binding is 1:1, as measured by NMR, and binding appears to be specific for (lyso)phosphatidylethanolamine since no binding was observed with other phospholipids (Hosoda et al. 1996; Wakamatsu et al. 1990). Since in eukaryote cells amino phospholipids are usually localized in the inner layer of the plasma

membrane, either the lantibiotic has to translocate across the bilayer or it has to induce lipid flip-flop, or both. Lantibiotic-induced lipid flip-flop has been reported for nisin (Moll et al. 1998), but not yet for cinnamycin. Nisin and Pep5 induce autolysis of Staphylococcal strains. It is thought that these cationic peptides bind to lipoteichoic and teichoic acids and thereby displace and activate N-acetyl-alanine aminidase and N-acetylglucosaminidase, which normally interact with teichoic acids (Bierbaum and Sahl 1985). Ancovenin modulates the activity of angiotensin I-converting enzyme (Kido et al 1983). Antiviral activity and activity against neuropathic pain have been reported for some class III lantibiotics (Meindl et al 2010).

Development of Resistance of Gram-Positive Bacteria Against Lantibiotics

Despite its worldwide application, little resistance has been developed against nisin. Resistance against lantibiotics can be due to among others changes of the cell wall and or the cell membrane and has been reviewed in detail (Chatterjee et al. 2005b). Gravesen and coworkers have proposed that nisin resistance in a *Listeria monocytogenes* strain was due to shielding of lipid II from nisin through its binding to a penicillin-binding protein (Gravesen et al. 2001; Gravesen et al. 2004). Nisin resistance in *M. flavus* appeared to be independent of lipid II levels (Kramer et al. 2004). Studies on a resistant *Lactococcus lactis* strain, which was able to grow at a 75-fold higher nisin concentration than the parent strain, demonstrated that less nisin was able to bind to lipid II in the membranes of the resistant strain. The cell wall of the resistant strain displayed significantly increased thickness at the septum. Comparison of modifications in lipoteichoic acid revealed an increase in D-alanyl esters and galactose as substituents in the resistant strain, resulting in a less negatively charged cell wall. Shielding lipid II and thereby decreasing abduction of lipid II and pore formation appeared to be a major defense mechanism of *L. lactis* against nisin (Kramer et al. 2008).

Lantibiotic Activities Exploited for Clinical Developments

Animal studies demonstrated the in vivo efficacy of several lantibiotics. Human cells do not possess the target of several lantibiotics, i.e., lipid II. Duramycin (also called: Moli1901) is being developed for the treatment of reduced mucociliary clearance in cystic fibrosis. The molecular target of duramycin is the phospholipid phosphatidylethanolamine present in the cellular membrane. Duramycin binds to the polar head of phosphatidylethanolamine and induces changes in intracellular calcium levels, which in turn activate calcium-dependent chloride channels. These alternative calcium-activated chloride channels produce an output of chloride and

water. This may compensate for reduced or absent cystic-fibrosis-transmembrane-conductance-regulator/Cl^- channel function in cystic fibrosis patients. A phase II study in cystic fibrosis patients showed that the inhalation of duramycin over 5 days resulted in an improvement in pulmonary function parameters (Grasemann et al. 2007). Phase II clinical studies are also being carried out on duramycin for treating dry eyes (Grasemann et al. 2007). By opening the abovementioned alternate salt channel, duramycin promotes the hydration of epithelial tissue. Patients with dry eye disease may thereby experience increased hydration of the eyes. A product that contains *Streptococus salivarius* that produces salivaricin is marketed for oral care to counteract infections (Boakes and Wadman 2008).

Perspectives

Existing and novel effective lantibiotics are of great interest because of the increase in resistance to multiple antibiotics. The identification of docking molecules and the characterization of the detailed molecular interaction with docking molecules will enable contributions to the development of improved lantibiotics. Lantibiotics may also have therapeutic activities that are entirely different from antibiotic activities such as the anti-inflammatory inhibition of pospholipase A2 by duramycin via binding of the phospholipase substrate, phosphatidylethanolamine. Mechanistic studies on the lantibiotic modification enzymes will allow for their directed engineering and subsequent aimed for application. Thioether bridges may also stabilize a variety of peptides, which are drugs for diverse indications (Kluskens et al. 2005; Kluskens et al. 2009; Kuipers et al. 2009; Levengood and van der Donk 2008; Rink et al. 2010). By stabilization, these therapeutic peptides are less sensitive to proteolytic breakdown and accordingly need less frequent administration and/or in a lower dose. In addition, stabilization may allow oral and pulmonary delivery of short peptides (de Vries et al. 2010). These delivery ways are more patient-friendly than injection. Furthermore, the structural constraint resulting from the introduction of thioether bridges may enhance the receptor specificity and/or the efficacy of the receptor interaction, thus enhancing the therapeutic potential.

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