Introduction

The idea to exploit lantibiotic biosynthesis enzymes for the stabilization of peptide hormones by introducing thioether bridges was already brought up in a Nature paper from 1988. In that same article Schnell et al. introduced the name lantibiotics for antimicrobial peptides that contain lanthionines (183). The first description of a lanthionine was in a publication of Horn and co-workers, who isolated a thioether-cross-linked amino acid from sodium carbonate treated wool (63). The name lanthionine (Latin, lana = wool) was introduced and represents two alanine residues coupled via a thioether linkage. Thioether bridges are more stable than disulfide linkages and peptide bonds (205). Before 1988 several lantibiotics, among which nisin, subtilin, epidermin and Pep5 (1, 52, 53, 72), were discovered and in 1971 the structure of the lantibiotic nisin was elucidated by chemical degradation (Fig. 1). This study also revealed that all lanthionines and methyllanthionines are composed of one D-amino acid coupled by a mono-sulfide linkage to one L-amino acid. Hence their synthesis takes place stereospecifically. The thioether bridge pattern in the peptide nisin was subsequently confirmed by nuclear magnetic resonance (NMR) spectroscopy (109, 210, 214).

Figure 1. The lantibiotic nisin (Gross and Morrell 1971).

Nisin is a pentacyclic peptide. The first ring is a lanthionine (Ala-S-Ala) and the remaining four are methyllanthionines (Abu-S-Ala), of which the last two are intertwined. Furthermore, the peptide contains 3 dehydroresidues and 21 unmodified amino acids. These (methyl)lanthionines give lantibiotics their unique features like
thermostability, proteolytic resistance and most (methyl)lanthionines are essential for high antimicrobial activity.

The assumption that enzymes were involved in the stereospecific introduction of these thioether bridges in peptides, urged the search for the genes implicated in the biosynthesis of lantibiotics. In 1993 the gene cluster involved in the nisin biosynthesis was unraveled (90, 212). In 1999 a project was initiated for the starting Biomade Technology Foundation. This project aimed at the utilization of the nisin modification enzymes for stabilization of nonnatural substrate peptides. New thioether-bridged peptides may be novel antibiotics, which are of great interest because of the increase in resistance to multiple antibiotics, or may be stabilized peptide drugs. 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. 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.

Research at BiOMaDe demonstrated the feasibility of these concepts and resulted in several publications that partly contributed to this thesis. This thesis discusses the dissection and exploitation of the nisin synthetase and the lacticin 3147 synthetase complexes and their utilization for stabilization of nonnatural substrate peptides. Furthermore, this thesis deals with the examination of the different transport routes that can be used for translocation of the modified peptides across the membrane of the producing bacteria.

Lantibiotics

Lantibiotics are ribosomally produced peptides, containing posttranslationally modified amino acids, mainly (methyl)lanthionines and dehydroresidues. Lantibiotics are predominantly produced by Gram-positive bacteria and are principally effective against Gram-positive bacteria. The antimicrobial activity can be effected by disruption of the membrane of the target organism by pore formation (85). Lantibiotics may use lipid II as a docking molecule to efficiently generate hybrid pores (13, 17). They may also inhibit cell growth by binding to lipid II and displacing this precursor of cell wall synthesis (56). Besides, they may inhibit the germination of spores of the species *Bacillus* and *Clostridium* (200). All the above-mentioned modes of action are valid for the lantibiotic nisin.

Nisin was the first lantibiotic described in literature (166) and is the most studied lantibiotic. It is produced by different *Lactococcus lactis* strains which are designated as GRAS (generally recognized as save) organisms. Already in 1969, nisin was approved for use as a food preservative (36). Nisin has a broad activity spectrum against Gram-positive bacteria, amongst others against strains of *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Lactobacillus*, *Bacillus*, *Listeria* and *Clostridium* (199),
and has antimicrobial activity in the nanomolar range (38). These features make the search for novel nisin variants by genetic engineering an interesting approach in the battle against multiple resistant pathogens (49, 88, 159). By their stability, high activity and virtual absence of resistance development, lantibiotics are promising candidates for biomedical application (113). For example, like nisin, lacticin 3147 can be used for prevention and/or treatment of bacterial mastitis and MRSA (27).

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 which mainly act by forming pores in the bacterial membrane (70). Type B lantibiotics (e.g. cinnamycin, duramycin and ancovencin) were discerned as having a higher degree of cyclization resulting in structures that are more globular and being devoid of pore-forming activity. Nowadays, more than 60 different lantibiotics have been discovered (10) and no less than 15 different posttranslational modifications have been described (224). After the finding of many new lantibiotics the old type A and type B classification became blurred and it was suggested in a scheme by Pag and Sahl to use three groups for classification (143, 224). This new classification places all lantibiotics in one of the three classes 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 may act via a mechanism distinct from that of class II LanM enzymes.

The lantibiotics biosynthesis machineries

The genes involved in lantibiotic synthesis are genetically arranged in gene 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 \( lan \) (37). Besides gene products required for the biosynthesis of the peptides, also proteins are encoded which are needed for the processing (\( lanP \)), translocation (\( lanT \)), self-protection (\( lanI, lanEFG \)) and regulation (\( lanRK \)). Per type, many of these proteins encoded in the different gene clusters show amino acid homology, which indicates that indeed they have similar functions (90, 153, 190). The lantibiotics nisin, epidermin and Pep5 belong to the class I lantibiotics and their gene clusters were characterized as depicted in figure 2.
In class I lantibiotics, reviewed by Willey and van der Donk, the prepeptide LanA is modified by two distinct enzymes, LanB and LanC. The LanA prepeptide contains a leader sequence that is thought to be necessary for targeting the propeptide part to the 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 lanthionines and methyllanthionines (Fig. 3).

![Figure 2. Gene clusters of class I lantibiotics](image)

Figure 2. Gene clusters of class I lantibiotics. Genes involved in the modification (lanB and lanC) and transport (lanT) of the peptide (lanA) are illustrated with filled arrows. Promoters are indicated by wedges (21).

![Figure 3. Introduction of an intramolecular thioether bridge by lantibiotic enzymes](image)

Figure 3. Introduction of an intramolecular thioether bridge by lantibiotic enzymes. LanB dehydrates serine (1) (or threonine) and LanC couples the formed dehydroalanine (2) (or dehydrobutyryl) stereoselectively to a cysteine (3), thus forming one DL-lanthionine (4).
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 (224).

In class II lantibiotics, only one enzyme is responsible for dehydration and cyclization of the propeptide LanA. These bifunctional LanM enzymes bear no homology with the LanB enzymes. However, the C-terminal part of these LanM enzymes has low sequence homology with the LanC enzymes, including three zinc-coordinating amino acids (146, 190). Knockouts of one of these zinc ligands completely abolished the cyclase activity of NisC or LctM (106, 149). 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 (143, 224). Prototypes of class II lantibiotics are mersacidin and lacticin 481. The second class also comprises the two-component lantibiotics, like lacticin 3147 (168). The two prepeptides LanAα and LanAβ are each separately modified by the two enzymes, respectively LtnM1 and LtnM2. After modification, both peptides are processed and translocated by one LtnT enzyme. The gene clusters of the group II lantibiotics are depicted in Figure 4. The gene cluster of lacticin 3147 contains an additional post-translational modification enzyme, LtnJ. This enzyme converts some dehydroalanines in the prepeptides Ltnα and Ltnβ to D-alanines (169).

**Figure 4. Gene clusters of class II lantibiotics.** Genes involved in the modification (lanM) (lanJ) and transport (lanT) of the peptide (lanA) are illustrated with filled arrows. Promoters (known ones) are indicated by wedges (60, 21).

The third class of lantibiotics contains (methyl)lanthionine-containing peptides are mainly devoid of antimicrobial activity. Instead, they have other -for instance morphogenetic- features that may be beneficial to the producing cells. Three lantibiotics in this group are known by now: SapB (79), SapT (80) and AmfS (207). SapB and SapT are believed to be biosurfactants that may have a positive effect on the surface of aerial hyphae of the producer strains. Furthermore, the LanM enzymes involved in the biosynthesis of SapB and AmfS have homology with the C-terminal part of other LanM enzymes except for the zinc ligands, which are missing.
Engineering of lantibiotics

With the elucidation of gene clusters involved in the biosynthetic pathways of lantibiotics, the next challenge became genetic engineering of lantibiotics. The existence of natural variants among lantibiotics (i.e. nisin A/nnisin Z, epidermin/gallidermin) and the high homology between certain lantibiotics (i.e. nisin/subtilin, mutacin II/lacticin 481) suggests that the identity of amino acids present at certain locations is flexible. Generation of mutant lantibiotics with enhanced biological activity or improved physical properties therefore seems promising. In fact, by site directed mutagenesis of the structural genes and the development of expression systems many lantibiotic variants were designed and produced in vivo (Fig. 5AB).

Figure 5A. Some early mutants of some class I lantibiotics created by site directed mutagenesis. Black circles indicate amino acid differences between natural variants. Grey circles indicate mutations (adapted from Cotter 2005a).
The most engineered lantibiotic is nisin. In 1992 the first nisin mutants were reported (88) and these mutants were followed by many other nisin mutants, which have been reviewed (92, 113). The alteration of residues that take part in formation of the third ring of nisin by the substitution T13C resulted in reduced antimicrobial activity of the nisin mutant. Also the substitution S3T, changing ring A of nisin from a lanthionine in a methyllanthionine, led to a dramatic loss of bioactivity. The mutation T2S resulted in an interesting mutant that displayed a two-fold higher antimicrobial activity against two target organisms (92). Some hinge region mutants had antimicrobial activity against Gram-negative species and furthermore by altering the charge of the nisin lantibiotic, solubility could be improved (233).

**Figure 5B.** Some class II mutants obtained by site directed mutagenesis. Grey circles for the lantibiotics mutacin II, cinnamycin and mersacidin indicate mutations (adapted from Cotter 2005a). For lacticin 3147, comprising LtnA1 and LtnA2, grey circles represent essential residues. Continuous lines indicate essential domains/amino acids, dashed lines indicate domains that are for the most part variable (adapted from Cotter 2005a).
In addition, other lantibiotics were altered by site-directed mutagenesis. The subtilin E4I substitution displayed a 57-fold improvement in stability and had 3-4 fold the specific activity in suppression of bacterial spore outgrowth (110). Interesting gallidermin mutations were the substitutions L6V, A12L and Dhb14P in the mature peptide. The L6V gallidermin variant had an increased antimicrobial activity, whereas the A12L and Dhb14P variants resulted in a remarkable resistance against proteolytic breakdown (141). The first introduced novel thioether bridge in a lantibiotic reported was for Pep 5. By substitution of A19C, a 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 an increase in proteolytic stability against chymotrypsin and Lys-C. However, the novel thioether bridge had a negative effect on the antimicrobial activity of Pep 5 (8). Also in the class II lantibiotics, comprising mutacin II (24), mersacidin (196) and cinnamycin (221) new variants were made by site-directed mutagenesis. A systematic mutant analysis by alanine scanning of the two-peptide lantibiotic lactacin 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 (30).

More recently mutagenesis and screening were accelerated by genetic randomization of specific amino acid coding sites within lantibiotic genes. By random mutagenesis and NNK scanning of nukacin ISK-1, a bank of nukacin ISK-1 variants was generated to identify the positional importance of individual residues responsible for antimicrobial activity (63). Furthermore, by random mutagenesis of mersacidin, 80 mutants were made that produce mature mersacidin at good levels and novel variants were obtained with improved overall bioactivity, such as F3W (4). In addition, novel variants of nisin with improved bioactivity were found by random mutagenesis. Nisin ring A mutants I4K/S5F/L6I and I4K/L6I showed enhanced activity against some target strains (159) even as mutations M21V, N20P and K22T in the hinge region (49).

Novel lantibiotics with enhanced bioactivity may be lethal for the producer itself. For the nisin producer this was circumvented by using a production system without the presence of NisP. Without removal of the leader, there is no antimicrobial activity. After production, the leader can be removed by trypsin. Another approach is using an in vitro modification system. The lantibiotics lactacin 481 and the two peptide lantibiotic haloduracin were both modified successfully by incubation of the precursor peptide with the LanM enzymes in vitro (121, 226). In addition, the dehydrated precursor of nisin was successfully cyclized by incubation with NisC in vitro (106). Overall, the biosynthetic system used for the biosynthesis of lantibiotics seems to have a remarkable flexibility.
The application of these enzymes for the modification and production of modified peptides that are *entirely* different in size and sequence from their native substrates is subject of this thesis.

**Production and secretion**

In this thesis the modification and transport enzymes used in the biosynthesis of nisin (NisBTC) and lactacin 3147 (LtnTM2) were applied to investigate the feasibility to introduce thioether bridges in nonantibiotic peptides. Both systems, NisBTC and LanTM2 were derived from *Lactococcus lactis* strains. Accordingly, the first approach to produce therapeutic peptides with thioether bridges made use of *Lactococcus lactis* NZ9000. Lactis NZ9000 is a plasmid-free and prophage-cured *L. lactis* MG1363 strain with *nisRK* integrated on the chromosome (93). The two-component regulatory system NisRK, in which NisR is a response regulator and NisK is a histidine kinase, is involved in the autoregulation of nisin biosynthesis. The fully maturated nisin induces via NisRK activation of the *Pnis* promoter, which controls transcription of the *nisABTC* genes (40, 91). These components led to the development of the well known NICE, Nisin Controlled Gene Expression, system. Nowadays this system is widely and successfully used for gene expression in Gram-positive bacteria, including bacterial genera other than *Lactococcus* (44, 76).

In our lab we developed a two-plasmid system in which the two plasmids are compatible with each other for the expression and translocation across the membrane of modified peptides (77, 157)(Fig. 6).

![Figure 6. Nisin inducible two-plasmid system for the production of modified peptides by *L. lactis*](image-url)
Chapter 1

The genes encoding the enzymes NisBTC or LtnTM2 were cloned behind the nisin inducible Pnis promoter on a pIL-derived plasmid (192). This plasmid replicates bidirectionally and is appropriate for expression of larger proteins. The encoding sequence for the substrate that has to be modified was fused to the C-terminus of the leader-encoding sequences of NisA or LtnA2 under control of the nisin-inducible promoter. The expression plasmid used for this purpose is a high copy rolling-circle-replicating plasmid, a pNZ8048 derived plasmid (93). When possible, translocation occurred via NisT or via LtnT, respectively, and the modified peptide was harvested from the medium.

*L. lactis* is a suitable producer-strain for peptides *in vivo*. An advantage of *L. lactis* as a producer is the absence of production of lipopolysaccharides or proteases like occurring in *E. coli* and *B. subtilis*, respectively. Extracellular production of peptides simplifies purification methods, especially in the case of *L. lactis*, which secretes only a very low number of proteins in the culture media. Moreover, it has been shown that the production level of secreted proteins reached mostly a higher level than that of proteins that were produced intracellularly (101). *L. lactis* NZ9000 harbors a wide range of enzymes (peptidases, housekeeping proteases) committed to intracellular proteolysis. On the contrary, it possesses only one extracellular housekeeping protease, HtrA (152).

In *L. lactis*, the conserved Sec pathway is successfully used for translocation of homologous and heterologous proteins. These proteins are preceded by a Sec signal sequence that targets the proteins to the Sec pathway. During translocation across the membrane, this signal sequence is removed and the mature protein is integrated in the membrane, anchored to the cell wall or released into the medium. The Sec pathway translocates unfolded proteins across the membrane (45, 59). In this thesis, besides the dedicated lantibiotic transporters NisT and LtnT, also the Sec pathway is examined for transport of modified peptides. Another translocation pathway, which might be of interest, is the Tat (twin arginine translocation) pathway. The Tat pathway translocates folded proteins across the membrane and may consequently be an ideal route for transport of the more bulky (lanthionine-containing) peptides. *E. coli* and *B. subtilis* have both a well studied Tat export system (97). However, *L. lactis* lacks the Tat pathway, which might be a disadvantage for heterologues expression. When a Tat pathway can be introduced there will be no competition with homologues substrates.

**Outline of this thesis**

As already mentioned above, the introduction of thioether bridges in peptides can have a tremendous impact on the stability of the peptide. Moreover, thioether-bridge-imposed peptide structures can improve the pharmacodynamic properties of peptides. Examples of improved therapeutic peptide variants with thioether bridges are enkephalin (155) and somatostatin (138). Both had increased stability and improved pharmacodynamic properties. These improved peptides with thioether
bridges were chemically synthesized. Importantly, thioether peptides produced via lantibiotic enzymes contain only one isomer, which is a significant advantage. A biologically introduced thioether bond bridges a D-amino acid to an L amino acid, whereas chemically induced thioether formation can lead to several stereo isomers (i.e. DL, LL, LD and DD). In the case of engineering more than one thioether bridge in one peptide, regiospecificity of the lantibiotic enzymes can have an additional advantage. For more complex peptides with intertwined or multiple rings and for larger polypeptides the biological production may dramatically reduce the cost and time of synthesis compared to chemical synthesis.

Dehydroresidues can also have several valuable properties. For instance, they may play a role in inhibition of biological processes (131, 132) or they can function as attachment sites for further chemical modifications. Therefore, the introduction of lanthionines as well as the introduction of dehydroresidues in nonlantibiotic peptides, exploiting the lantibiotic enzymes NisBC and LtnM2, have a huge potential. The feasibility of engineering these residues in a broad range of peptides will be outlined in this thesis.

Chapter 2 focuses on the applicability of the NisT transporter for export of nonlantibiotic peptides and the dissection of the NisBTC enzyme complex. By mass spectrometry, this chapter shows that NisT transports dehydrated NisA prepeptides in the presence of NisB and the absence of NisC. In the absence of NisB and NisC, the unmodified prepeptide NisA is transported. These findings demonstrate that NisT can function independently and that NisB can function without the presence of NisC. Furthermore, it is proven for the first time that NisB can dehydrate and NisT can transport peptides unrelated to nisin when preceded by the nisin leader, like variants of angiotensin, vasopressin and enkephalin.

Progress in further exploiting the NisBTC enzymes for posttranslational modification of therapeutic peptides is presented in Chapter 3. The development of the two-plasmid system has a huge beneficial impact on the production level of modified peptides and makes analysis of these new peptide variants more straightforward. This chapter demonstrates that NisB has a wide substrate specificity. Furthermore, it demonstrates for the first time that NisC can cyclize nonlantibiotic peptides and that NisT can transport these novel thioether-bridged peptide variants. In conclusion, the NisBTC enzyme complex can successfully be used for the synthesis of stabilized potential therapeutic peptides.

Chapter 4 reports the dissection and utilization of the LtnTM2 part of the lacticin 3147 synthetase complex for modification and transport of nonnatural substrate peptides. Class II lantibiotic synthetase systems may be essential tools for the production of more globular peptides with a higher degree of cyclization. Although the LtnTM2 enzymes appear successful in modification and transport of nonnatural substrate peptides, it is not clear whether the substrate specificity of LtnT and LtnM were as broad as, respectively, NisT and NisBC. Analysis is hampered by lack of
secretion of a number of peptides. Whether this is caused by improper processing by LtnT or by blocked LtnT-dependent translocation is still unknown.

In Chapters 5 and 6, the well known Sec pathway is studied as a possible alternative secretion route for posttranslationally modified peptides. First, it is demonstrated that even though the nisin leader is preceded by a signal sequence up to 44 amino acids, the NisA peptide is still modified by NisB and NisC. Thioether bridged pronisin is too large for translocation via the Sec pathway, but the Sec pathway successfully translocates dehydrated peptides and the thioether bridged peptide fragment of azurin. These data reveal once more that the nisin synthetase complex can completely be dissected and that the enzymes NisB and NisT can function independently. Taken together the Sec pathway might be a successful alternative for the secretion of modified peptides.

The impact of this thesis is well illustrated in Chapter 7, which describes the development and therapeutic potential of thioether-bridged angiotensin-(1-7). Further perspectives and results are discussed and summarized in Chapter 8.