The main limitation in the application of therapeutic peptides is their rapid degradation by proteolytic enzymes. Thioether-bridges in peptides confer strong resistance against proteolytic degradation, can modulate receptor interaction and extend delivery possibilities. Their enzymatic introduction is chemo-, region- and stereo-specific and allows the stabilization of medically and economically highly important therapeutic peptides. This emerging technology has huge potential for the development of a large number of novel highly effective peptide drugs.

**Introduction**

Therapeutic peptides form a tremendously large market. Several single therapeutic peptides have a market size of over a billion dollar. The application of many therapeutic peptides faces limitations such as rapid breakdown by proteolytic enzymes, lack of specificity and lack of delivery options. Many (poly)peptide-modifying technologies involve the genetic or chemical coupling of large molecules to the therapeutic (poly)peptides. Well known are the coupling of polyethylene glycol (PEG), glycosylation or polysialylation, and the coupling of peptides or proteins such as albumin to the therapeutic (poly)peptide which all reduce renal clearance and prolong the half-life of the peptide drugs in the blood.

Cyclization of therapeutically important peptides has been a successful method to create more stable peptide analogs with improved pharmacodynamic properties [1]. Intramolecular thioether-bridge formation is an effective way to protect peptides against proteolytic degradation. Thioether-bridges are more stable than peptide bonds and disulfide bridges [2]. Here we describe the biological technology of using *Lactococcus lactis* containing the nisin-modifying enzymes for producing thioether-stabilized therapeutic peptides.

**Thioether-bridges introduced in therapeutic peptides by lantibiotic enzymes**

Chemical methods for peptide cyclization face several drawbacks, such as lack of stereo- and regio-specificity, multimerization instead of cyclization and inefficient multistep synthesis involving the use of protecting groups, in particular for the synthesis of larger peptides with multiple ring structures. Enzymatic cyclization overcomes several of the challenges for chemical synthesis and occurs during the synthesis of some cyclic antibiotics for instance: tyrocidin, gramicidin S, circularin A and of the lipopeptapeptide surfactin A. Peptide backbone modifications, comprising not only head to tail cyclization but also thiazole and oxazole cyclizations, occur in post-translationally modified microcins, a group of antimicrobial peptides from Gram-negative bacteria.

Thioether-bridges are more stable than peptide bonds and disulfide bridges. Lantibiotic enzymes can stereo- and regio-specifically introduce several thioether-bridges into a single
peptide. These enzymes dehydrate serines and threonines and subsequently couple the formed dehydroresidues to cysteines, thus forming thioether-bridges. By site-directed mutagenesis the positions of Ser/Thr/Cys can be chosen thus controlling the position, size and number of the lantibiotic-cyclase-generated thioether-rings. Lantibiotic enzymes form thioether-rings in peptides by sulfur-bridging a D-amino acid and an L-amino acid. Hence there is double protection against proteolytic degradation: first by the thioether-ring structure and second by the D-amino acid. Export of thioether-bridged peptides by bacteria containing lantibiotic enzymes offers a convenient production system with the opportunity to generate libraries.

Thioether-bridged peptides occur in nature among others as a class of antimicrobial peptides, lantibiotics, produced by some Gram-positive bacteria [3]. Lantibiotics are lanthionine-containing antibiotics. Lanthionines (Ala-S-Ala) and methyl-lanthionines (Abu-S-Ala) are thioether-bridged amino acids, which are formed by two enzymatic steps. Abu is amino butyric acid. First, the enzyme-catalyzed dehydration of serines and threonines takes place. Second, the resulting dehydroalanines and dehydrobutyrines are coupled to cysteines. This coupling process is cyclase-catalysed and thereby chemo-, regio- and stereo-specific process is enzyme-catalyzed (Fig. 1), as far as has been investigated the enzymatically formed thioether-bridged amino acids are DL amino acids. Dehydratase and cyclase activities are directed by a leader peptide which is located at the N-terminus of the substrate peptide. This leader peptide is cleaved off either intracellularly by a peptidase-part present in some of the dedicated transporters or extracellularly by a specific leader peptidase. Already two decades ago a Nature paper suggested exploiting the thioether-bridge synthesizing enzymes for stabilizing therapeutic peptides [4]. In the past three years several breakthroughs demonstrated the applicability of this idea.

In the biosynthesis pathway of some lantibiotics including nisin, four enzymes are involved, LanB dehydratases, LanC cyclases [5], LanT transporters and LanP leader peptidases. In the biosynthesis of other lantibiotics only two enzymes are involved in performing these four functions. For instance, in the biosynthesis pathway of the well-studied lactacin 481 one bifunctional enzyme LctM performs both the dehydration and the cyclization reaction [6]. In this system another enzyme, LctT, performs both the transport and the cleavage of the leader peptide [7]. LctM was the first lantibiotic enzyme whose activity has been reconstituted in vitro. Its cyclization reaction is region-specific [8]. This enzyme has also been engineered such that the cyclase activity is lost but the dehydratase activity is maintained [9]. Also the leader-cleaving peptidase activity of LctT, which appears to have relatively broad substrate specificity, has been reconstituted in vitro [7]. Cleavage of the leader peptide also could be achieved by engineering a Factor X cleavage site in the C-terminus of the leader peptide. LctM has been successfully applied for the synthesis of thioether-bridged bioactive peptides [10].

The pentacyclic lantibiotic nisin is produced as a prepeptide which is composed of a 23-amino-acid leader peptide followed by a 34-amino-acid propeptide. The dehydratase NisB dehydrates serines and threonines in the propeptide yielding dehydroalanine (or dehydrobutyrylne), a planar residue. The cyclase, NisC, couples dehydroalanine (or dehydrobutyrylne) to cysteine, yielding lanthionine (or methyl-lanthionine). This (methyl)lanthionine is composed of a D-amino acid (D-alanine or D-aminobutyric acid), a thioether-bridge and an L-alanine.

**Figure 1.** Lanthionine synthesis by the nisin modification enzymes. The dehydratase, NisB, dehydrates serine (or threonine) to yield dehydroalanine (or dehydrobutyrylne), a planar residue. The cyclase, NisC, couples dehydroalanine (or dehydrobutyrylne) to cysteine, yielding (methyl)lanthionine. This (methyl)lanthionine is composed of a D-amino acid (D-alanine or D-aminobutyric acid), a thioether-bridge and an L-alanine.

**Broad use of lantibiotic export systems**

We investigated the use of lantibiotic transporters for export of thioether-bridged therapeutic peptides. We dissected the presumed multimeric enzyme complex responsible for the synthesis of the pentacyclic thioether-bridged lantibiotic nisin. We found that NisT and LtnT are not specific for their corresponding prelantibiotic, fully modified prenisin and lacticin 3147 A1 or A2 [11,12]. *L. lactis* containing NisT,
irrespective of the presence or absence of the modification enzymes, is able to export (poly)peptides, that are unrelated to nisin, provided that the nisin leader peptide is present at the N-terminus of the substrate peptide. Especially, NisT-mediated export of short and cationic peptides seems to be very efficient. The leader peptidase, NisP, is however too specific to remove the leader peptide. Cleavage sites, for instance of factor X, can be engineered in the C-terminus of the leader peptide without interfering with the capacity of the leader peptide to induce modification by the nisin enzymes or export by NisT. A broad variety of peptides can thus be exported by lantibiotic transporters.

**Production of peptides with dehydroresidues by lantibiotic dehydratases**

The first step in the introduction of thioether-bridges in peptides is the dehydration of serines and threonines. This step can be separated from the subsequent cyclization. *L. lactis* containing NisT and NisB can produce peptides with dehydroresidues, and even polydehydroresidues, without thioether-rings [13]. Generally hydrophobic flanking residues of serines and threonines favor NisB-mediated dehydration [13,14]. One hydrophobic flanking amino acid can be sufficient for dehydration, whereas the simultaneous presence of hydrophilic amino acids on both sides, especially glycine and aspartate and glutamate, seems to preclude dehydration [13]. *L. lactis* containing NisC can also produce peptides with dehydroresidues via nonlantibiotic transporters, for instance the sec system. In the latter case, a construct is made as coding for a signal sequence for the sec system, followed by the nisin leader peptide, followed by the peptide of interest. The nisin leader peptide, although present internally in such a hybrid peptide is still able to induce modification by NisB and NisC. The advantage of the sec system is its well-established broad substrate specificity. Using the sec system for export, the extent of NisB-mediated dehydration can be enhanced by raising the intracellular NisB levels and or by reducing the export efficiency by using a twin arginine transport system (TAT) signal sequence. *L. lactis* does not have a TAT export system, but many TAT signal sequences are recognized by the sec system. Lower export efficiency in the case of a TAT signal sequence allows for enhanced extent of modification by lantibiotic enzymes [15]. Some thioether-bridged peptide could be successfully exported via the sec system [16]. The sec system therefore clearly provides an important alternative for peptides which are not efficiently exported via the lantibiotic transporter NisT.

Dehydroresidues can be installed in peptides at a distance from the leader peptide which is larger than the 32 amino acids preceding dehydroalanine33, the most remote dehydroresidue in nisin [13]. They can also be introduced at 21 residues distance from the leader peptide while none of these preceding 21 residues is dehydrated [14]. Dehydroalanine and dehydrobutyrine constitute two additional amino acids which broaden the possibilities of peptide libraries, that can be screened for desired properties. Dehydroresidues have a special planar shape and have high reactivity especially dehydroalanine. These residues are known to be essential for the activity of a variety of (poly)peptides. Dehydroresidues can also be introduced chemically via selenium [17]. The biological production of peptides containing dehydroresidues constitutes an easy and cheap way for specific peptides with (poly)dehydroresidues and for dehydroresidue-containing peptide libraries.

**Thioether-bridges in peptides: proteolytic resistance, modulated receptor interaction, extended delivery options**

As discovered and clearly demonstrated in the past three years, *L. lactis* containing NisB and also the cyclase NisC can introduce thioether-bridges in a variety of leader peptide fusion peptides [18,19]. The bacterial production of thioether-bridged peptides allows generating libraries by randomizing the codons for one or more positions in the peptide. Subsequent screening allows selecting peptides with desired properties. Multiple thioether-rings and also intertwined thioether-rings of different sizes can be introduced in single peptides which are unrelated to nisin. Dehydroalanines can spontaneously react with cysteines when these cysteines are at appropriate distance to the dehydroalanines, for example present in an n = 3 position. Therefore, NisC-catalyzed lanthionine (Ala-S-Ala) formation is not readily discriminated from spontaneous lanthionine formation. In the case of spontaneous thioether-bridge formation a mixture of stereo- and regio-isomers might be formed, whereas NisC-catalyzed thioether-bridge formation probably results in one specific isomer. By contrast dehydrobutyrines are less reactive than dehydroalanines. Therefore, under fermentation conditions, methyllanthionine (Abu-S-Ala)-formation results exclusively from NisC activity. To be sure that stereo- and regio-specific thioether-bridge formation takes place it is preferred to use peptides containing threonines, which yield less reactive dehydrobutyrines, rather than peptides containing serines, that yield the reactive dehydroalanines [19].

On the basis of all at that moment known lantibiotic structures, in silico analyses have provided some insight into the design of peptides that could be cyclized by lantibiotic cyclases. Generally hydrophilic amino acids seem to be preferred as flanking residues of cysteines when one aims at cyclase-mediated thioether-bridge formation. Glutamate often occurs at the N-terminal side of cysteines, whereas bulky residues like tryptophan are not common in that position. Some experimental support for the applicability of these guidelines was obtained by subjecting designed leader–hexapeptide fusions to NisB and NisC [14].

NisC specificity has also been studied by the randomization of the three amino acid residues in ring A and in different
experiments the two amino acids in ring B of nisin followed by screening for antimicrobial activity. Because screening was based on activity it is very well possible that the capacity of NisC to introduce thioether-bridges is underestimated, as a result of the occurrence of inactive fully cyclized mutants. Nevertheless, these studies already clearly demonstrated that NisC cyclizes a broad range of ring A mutants. Even the simultaneous presence of more than one aromatic residue within ring A is accepted in this n to n+4 cyclization. Ring B mutants, comprising n to n+3 cyclization, had predominantly small amino acids [20].

Thioether-bridges protect against proteolytic degradation [21–24]. This has the important advantage that higher local concentrations of a particular therapeutic peptide in the body can be reached as well as prolonged higher concentrations. Therefore it might be that such thioether-stabilization allows less frequent addition of a therapeutic peptide. Importantly, the proteolytic stability prevents the generation of breakdown products with toxic or opposite effects. Thioether angiotensin-(1–7) was fully resistant against degradation by ACE and significantly more resistant against a variety of protease-rich organ homogenates than the native linear angiotensin-(1–7). Simultaneous infusion of rats of natural angiotensin-(1–7) and thioether-bridged angiotensin-(1–7) led to up to several orders of magnitude higher concentration of the thioether-bridged one in the plasma. This indicates that thioether-bridged peptides may reach higher local concentrations and probably may reach sites within tissues which are not reached by the natural linear peptides. Labeled thioether-bridged peptides which bind a specific receptor may have an important potential in the field of diagnostics. Their proteolytic stability ensures that the detection of the label really reflects the presence of the receptor and not that of the free label liberated from the peptide by proteolytic cleavage. Although it has not been studied in detail to what extent thioether-bridges protect against peptide-degrading enzymes, it seems that generally peptide bonds that are present within the (methyl)lanthionine ring structure as well as two peptide bonds adjacent to the ring are protected against proteolysis.

The introduction of thioether-bridges can modulate the receptor interaction. The interaction may be lost, may become more selective or become enhanced. In silico studies may in principle guide the design of thioether-bridged peptides. However, in many cases screening of several variants to select the active one(s) may be faster. Thioether-bridged peptides may also be obtained via base-assisted sulfur extrusion from disulfide-bridged peptides. This method generally is, however, neither chemo-, nor regio-, nor stereo-specific. Therefore it may be of use for generating several variants that can be used for a first screening for activity of variants of a specific therapeutic peptide. Predictability of the optimal position of a thioether-bridge may also have severe limitations. Properties of single site mutants cannot be readily extended to the properties of peptides in which this particular single site is bridged to a second site. For instance d-Pro7 and d-Ala7 analogs of angiotensin-(1–7) act as antagonists which suggests that the modification of position 7 may cause loss of agonist activity. Surprisingly thioether-bridging of positions 4 and 7 led to an even more effective angiotensin-(1–7) agonist [24]. Stabilization of an agonist by introducing a thioether-bridge will be generally more difficult than stabilization of an antagonist because agonists not only have to maintain their receptor binding capacity but also have to maintain their role in signal transduction. In some cases, for instance when a novel receptor-binding thioether-bridged peptide is sought, libraries of thioether-bridged peptides will strongly increase the chances of success.

Introduction of a thioether-bridge in somatostatin led to a molecule with highly specific receptor interaction. Interaction with some of the receptors was completely lost whereas the interaction with one receptor remained unaltered [22]. Also the receptor interaction of enkephalin could be modulated by the introduction of a thioether-bridge [23]. Thioether angiotensin-(1–7) caused 10- to 100-fold more effectively vasodilation of rat aorta rings also in the presence of protease inhibitors indicating that this is the result of strongly enhanced receptor interaction [24].

Oral delivery of therapeutic peptides is frequently referred to as the Holy Grail in peptide research. Obviously oral delivery is much more patient-friendly than frequent injections in the blood. Thioether-bridged angiotensin-(1–7) could successfully be delivered both orally and pulmonarily (Biomade, unpublished data).

Conclusions

Thioether-bridges are more stable than peptide bonds and disulfide bridges. Lantibiotic enzymes can be broadly applied for the chemo-, regio- and stereo-specific introduction of one or multiple thioether-bridges in peptides. Thioether-bridges can be introduced chemically, but multistep region- and stereo-specific chemical synthesis of thioether-bridged peptides is costly and cumbersome, especially when multiple thioether-rings are introduced in a single peptide. Thioether-bridges confer proteolytic stability, may modulate receptor interaction and may allow pulmonary and oral delivery.

The strong proteolytic resistance causes longer in vivo lifetime of thioether-bridged peptides. Proteolytic degradation may also be reduced to some extent by multimerization, introduction of d-amino acids without rings and modification of the termini, for example, pGlu formation and/or amidation. Acylation may cause di-multimerization and albumin binding. Therefore it also exerts indirect effects to increase the molecular mass and concomitant decreased renal clearance. Main technologies aim at prolonged in vivo lifetime by coupling genetically or chemically large molecules to the therapeutic
peptide thus strongly reducing renal clearance (Table 1). An example of an interesting new development is recombinant protein chains with PEG-like properties by Amunix Inc. These methods may strongly reduce the secretion but in some cases necessary uptake into tissue will be reduced as well.

The introduction of thioether-bridges may cause loss of activity, may make the receptor interaction more specific or may enhance the receptor interaction. Amidation is for about 50% of the number of therapeutic peptides required for effective receptor interaction and furthermore counteracts carboxypeptidase-mediated breakdown. Amidation both enzymatically and chemically involving 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) is very well compatible with thioether-bridged peptides (R. Rink, unpublished data).

Oral delivery of thioether-bridged peptide has recently been measured within Biomade. A selection of some main peptide modification technologies to extend the delivery possibilities of therapeutic peptides is summarized in Table 2. They include the design of prodrugs that exploit transepithelial uptake systems, lipidization and coupling to amphiphilic polymers. Potential future opportunities are coupling to bile acid, biotinylation and coupling to cell penetrating peptides.

There are no thioether-bridged therapeutic peptide hormones on the market as far as we know. Owing to their great potential based on the conferred proteolytic resistance, capacity to modulate receptor interaction and extended delivery options this is likely to change.

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References
11 Kuipers, A. et al. (2004) NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated, and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. J. Biol. Chem. 279, 22176–22182
13 Rink, R. et al. (2005) Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes. Biochemistry 44, 8873–8882
15 Kuipers, A. et al. (2009) Translocation of a thioether-bridged azurin peptide fragment via the Sec pathway in L. lactis. Epub ahead of print
19 Rink, R. et al. (2007) NisC, the cyclase of the lantibiotic nisin, can catalyze cyclization of designed non-lantibiotic peptides. Biochemistry 46, 13179–13189
29 Russell-Jones, G.J. (2004) Use of targeting agents to increase uptake and localization of drugs to the intestinal epithelium. J. Drug Target. 12, 113–123