Peptide Cyclization

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Cyclization of Peptides by using Selenolanthionine Bridges**

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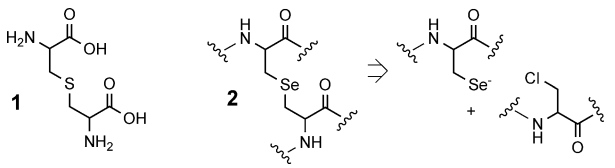
The unusual bis(amino acid)lanthionine **1** is the key component of many natural products, including the so-called lantibiotics, a family of multiple thioether-bridged antimicrobial peptides produced by bacteria.[1] Lantibiotics have found broad application as food preservatives (for example, nisin) and are considered to be a promising class of antibiotic agents.[2] In nature, the monosulfide linkage of lanthionines is formed post-translationally and derived from the cyclase-mediated Michael addition of a cysteine (Cys) to a dehydroalanine (Dha) residue within the same peptide sequence.[1]

The cyclization of peptides by using lanthionine bridges is also particularly interesting in peptide chemistry, as these moieties can be effective surrogates of disulfide bonds with the advantage of being more stable to reducing environments. This stabilizing modification has been applied to bioactive peptides, such as sandostatin,[3] angiotensin,[4] and a cell-adhesion modulator.[5] Moreover, the short monosulfide bridge can be engineered into linear peptides to introduce conformational rigidity to increase their biological activity and stability against proteolytic degradation, as demonstrated for an analgesic cyclic version of enkephalin.[6]

Current synthetic strategies for lanthionine formation include the Michael addition of Cys to a Dha residue that is generated by either chemical or biological transformation.[7,8] Other related methods include desulfurization of disulfides and substitution of a leaving group by cysteine.[7,9] A complementary approach to peptide cyclization is through amide bond closure using preformed orthogonally protected lanthionine building blocks,[7] as recently demonstrated by Vederas et al. for the challenging total synthesis of lactocin S and lacticin 3147.[10] Although each of these methods has its specific advantages, limitations over the stereocontrol of thioether formation and laborious synthetic routes restrict their applicability and practicability. A fast and simple synthetic approach to make lanthionine macrocycles that can be applied to a wide range of peptides, including non-lantibiotic sequences, is therefore highly desirable.

Herein we report a new approach for the synthesis of lanthionine-like cyclic peptides. Whereas most of the reported chemical strategies for the lanthionine construction are based on the selection of a proper reactive partner for Cys, we have modified the reactivity of the cysteine residue itself by replacing it with an isosteric selenocysteine (Sec). Sec is a rare but naturally occurring amino acid that shares many physicochemical properties with Cys, including similar C_β–X_γ bond length (X = S 1.82 Å; X = Se 1.95–1.99 Å).[11] Recently, several reports have highlighted the advantages of substituting disulfide (S–S) by diselenide (Se–Se) bonds in peptides bearing disulfide bridges, which include an improvement in folding properties, stability, and selectivity for target receptors.[11,12] In those cases, the Se replacements did not disturb the structure of the peptides, and their biological activity was maintained and in some cases improved. Interestingly, no discrimination between Cys and Sec motifs were observed for a lantibiotic enzyme, where LctM was able to catalyze conjugate addition of a Sec residue to form a selenolanthionine ring in lacticin 481.[13]

Herein we evaluate the chemical synthetic advantage of substituting thioethers by selenoethers. Despite their similarity, Sec exhibits higher nucleophilicity than Cys. The pK_a value for the selenol in Sec is about 5.4, while the value for the thiol in Cys is 8.2.[14] As recently reported, the selenol pK_a value can be even lower (pK_a 3.3–4.3) when the Sec residue is found within a peptide.[15] Based on this fact, we envisioned that a cyclic selenolanthionine scaffold such as **2** could be readily prepared by Se-alkylation of Sec by an internal β-halogenated alanine residue (Scheme 1). When carried out with the Cys counterpart, the same reaction is expected to be problematic owing to the strong basic conditions required for thiolation.[7,9] We also anticipated that such intramolecular alkylation with the more reactive Sec residue could be carried out under milder conditions at lower pH, thus circumventing the lability issues of the halogenated amino acid (for example, elimination to Dha).[9]



Scheme 1. Lanthionine **1** and selenolanthionine **2**.

To demonstrate the scope of the new cyclization method, we chose first to build a monoselenide analogue of ring C of the lantibiotic nisin. The synthesis involved the assembly on solid support of the linear peptide equipped with a β-chloroalanine (with either a D- or L-configuration) and a selenocysteine residue at the respective sites for cyclization employing either Boc or Fmoc-based peptide chemistry (Scheme 2). The linear precursor **3**, the α-carbon of which features a D-configuration at the 2-position, was assembled via Boc-based solid-phase peptide chemistry (SPPS)on a 4-

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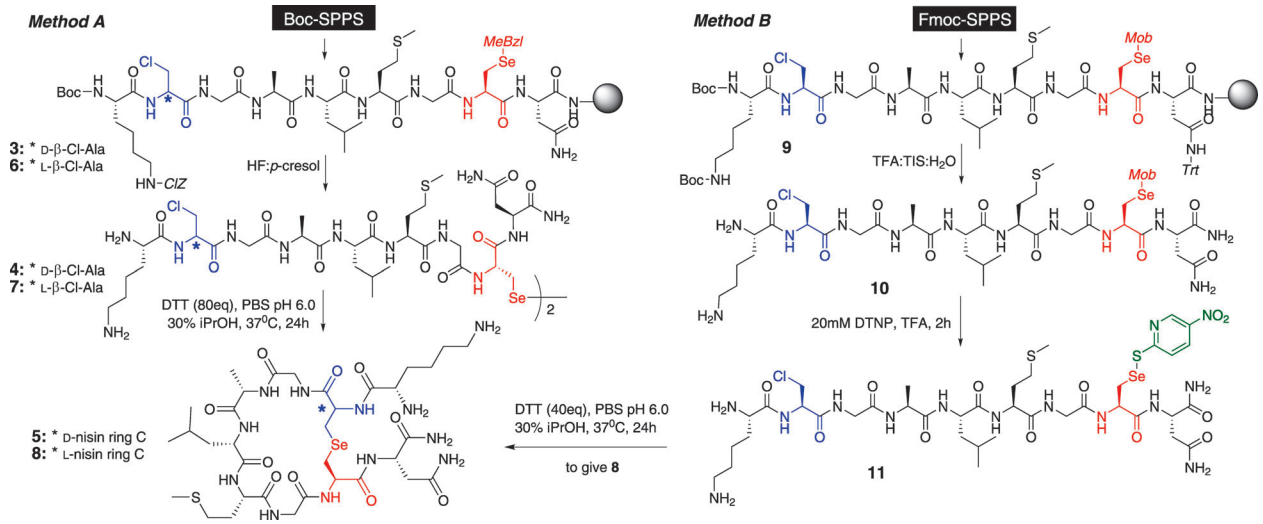


Scheme 2. Synthesis of the selenolanthionine analogues **5** and **8** of nisin ring C by using Boc (*tert*-butoxycarbonyl) and Fmoc (9-fluorenylmethoxycarbonyl) protection strategies.

methylbenzylhydrylamine (MBHA) resin utilizing Boc-β-D-chloroalanine and the *p*-methylbenzyl (MeBzl) group for the side-chain Sec protection (Scheme 2, Method A).[12f] The Boc-β-D-chloroalanine amino acid was coupled to the resin using diisopropylcarbodiimide (DIC) as the activating agent, whereas all of the other residues were coupled using in situ neutralization with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation.[16] The peptide was finally cleaved from the solid support by acidolysis with HF, which removed the MeBzl protecting group from the Sec residue and led to formation of diselenide **4**.

The key feature of the selenolanthionine cyclization is the diselenide reduction of the linear peptide with concomitant intramolecular Se-alkylation of the generated selenolate by the β-chloro-alanine residue. Dithiothreitol (DTT), a widely used reducing reagent, was shown to be a suitable reductant to promote the cyclization. When used in excess, DTT is capable of cleaving diselenide bonds in situ while leaving the chloride moiety intact. DTT adducts were only observed when the reaction was carried out at high pH after prolonged time periods (pH ≥ 11 over two days). Cyclization of the linear precursor **4** to selenoether **5** was investigated under various conditions and carried out in phosphate buffer over 24 h using a 40- to 80-fold excess of DTT. Incubation at 37 °C and addition of isopropanol to the reaction buffer improved the cyclization rate. The extent of selenide crosslinking in different pH media was also examined and monitored using analytical HPLC (Figure 1 A). A high rate of formation of cyclic product **5** was obtained from pH 5.0 to 9.0 with optimal conditions for the cyclization found to be at slightly acidic pH (pH 5–6) where ring closure was 95 %. We then used these conditions to prepare compound **5** as depicted in Scheme 2, followed by purification by RP-HPLC. Using the same synthetic route, an analogue of nisin ring C with the L-configuration at residue 2 was prepared employing Boc-β-L-chloroalanine for the chain assembly of **6** that was cleaved from the resin to give precursor **7** and cyclized to L-nisin ring C **8** (Scheme 2). The selenoether ring closure was again very

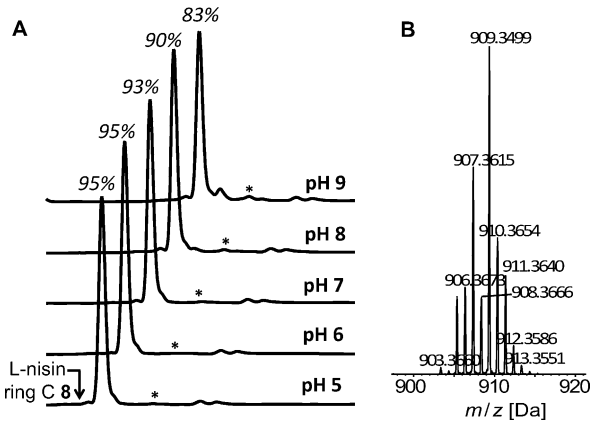


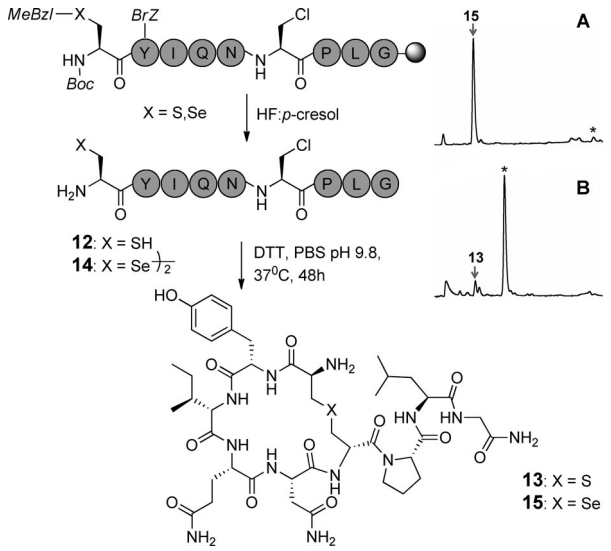
Figure 1. A) HPLC traces showing cyclization of peptide **4** at different pH values. The major peak corresponds to the selenoether **5** and the percent macrocyclization after 24 h is indicated in italics. The asterisk denotes the retention time for the precursor **4**. The arrow points to the expected retention time for the diasterisomer **8**. B) TOF-mass spectrum of selenoether **8** showing the characteristic Se isotopic distribution (expected mass for the major peak: 909.3514).

efficient, with 90 % of macrocycle **8** being produced. The two selenolanthionine compounds were characterized by mass spectrometry (Figure 1 B) and NMR spectroscopy (Supporting Information, Figure S1). The fact that the two diastereoisomers **5** and **8** are clearly distinguishable by RP-HPLC analysis (Supporting Information, Figure S2) allowed us to evaluate the level of epimerization at the α-carbon in the 2-position during the course of the selenoether cyclization. As depicted in Figure 1 A, there is no significant indication of epimerization of selenolanthionine **5** to **8** (or production of **5** during cyclization of **8**, data not shown), thus confirming the high level of stereoselectivity of the selenide cyclization.

The chlorinated Sec intermediate can also be synthesized by employing Fmoc-SPPS (Scheme 2, Method B) where the side-chain of the Sec residue was protected with the standard *p*-methoxybenzyl (Mob) group and Fmoc-β-L-chloro-alanine

was employed during the assembly of peptide **9**. Again, DIC was used to couple the chloride-containing residue and HBTU for the other amino acids. To minimize the exposure of the Mob-protected Sec and chloride moieties to piperidine, a short Fmoc-deprotection step was applied throughout the synthesis (2 × 1 min with 20% piperidine in DMF). Next, peptide **9** was cleaved from the resin by standard trifluoroacetic acid (TFA) treatment that led to the Mob-protected precursor **10**. Based on the procedure reported by Hondal et al,[17] the Mob group was removed by treatment of the crude peptide with 2,2′-dithiobis(5-nitropyridine) (DTNP) in TFA for 2 h. The deprotection step gave largely the TNP-selenosulfide **11** plus traces of diselenide **7** and other complex species that were eventually converted into the selenolate upon reduction.[17] Following isolation of the crude peptide by Et_2O trituration, the peptide was submitted to intramolecular cyclization in the presence of DTT in phosphate buffer pH 6.0 as described for precursor **4** to give selenolanthionine **8** in good overall yield (39%). The MeBzl group may also be employed for Sec protection during Fmoc assembly, as it may also be removed by the DTNP/TFA treatment to give the cyclic selenoether **8** upon DTT reduction (Supporting Information, Figure S3).

During the course of our studies on neuropeptide derivatives, we became interested in the synthesis of a lanthionine mimetic of the hormone oxytocin with a thioether linkage as a surrogate for the native disulfide bond. Although this analogue can be produced by means of the lanthionine building block approach after several synthetic steps,[18] we sought simpler access to this compound. Intramolecular *S*-alkylation of the β-L-chloroalanine-containing oxytocin precursor **12** failed to produce thioether analogue **13** even under strong basic conditions (Scheme 3, Supporting Information, Figure S4). The thiolation reaction was not sufficiently efficient to promote the unfavorable oxytocin cyclization to a 19-membered ring formation. However, when the diselenide precursor **14** was submitted to the same cyclization under DTT reducing conditions, the selenolanthionine oxytocin **15** could be efficiently synthesized (Scheme 3). Unlike compound **4** and **7**, cyclization of precursor **14** to **15** required higher pH conditions and was best carried out at pH 8 to 10 over the course of two days (Supporting Information, Figure S4).



Scheme 3. Synthesis of the oxytocin lanthionine **13** and selenolanthionine **15**. Upper right: HPLC traces for the cyclization of **14** (A) and **12** (B) at pH 9.8. The retention time for the cyclic product and linear precursor is indicated by an arrow or asterisk, respectively.

Analysis of the novel selenolanthionine peptides **8** and **15** by two-dimensional ^{1}H NMR confirmed the structure of the selenoether linkage (Supporting Information). The presence of the Se atom at the cyclization site leads to an additional analytical method to assess selenoether formation, as the coupling of Se and its neighboring protons can be visualized by 2D heteronuclear ^{1}H–^{77}Se HMQC spectroscopy.[19] As shown in Figure 2, there are clear correlations between the Se atom and β protons on either side of the selenium bridge for selenoethers **8** and **15**, thus unambiguously confirming its position within the new cycles.

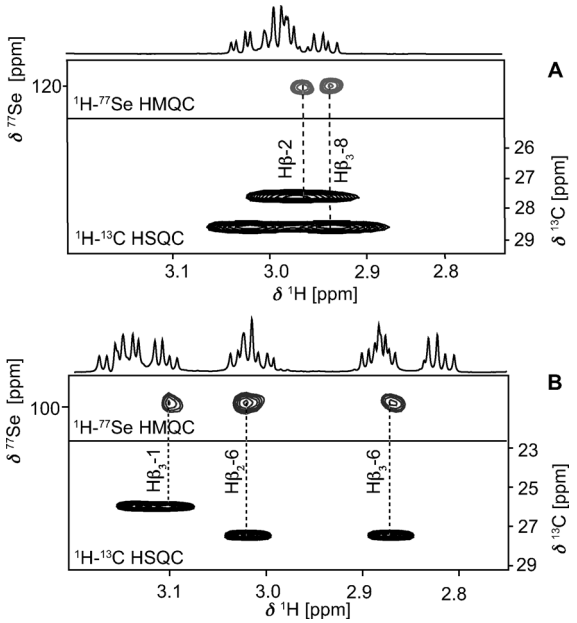
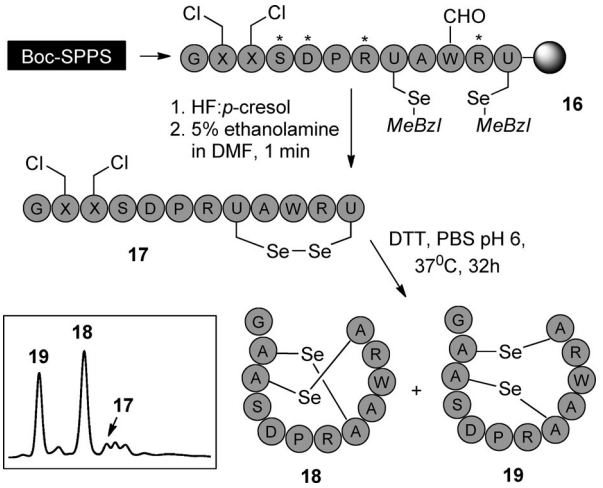


Figure 2. Portion of ^{1}H–^{77}Se HMQC (upper rectangle) and ^{1}H–^{13}C HSQC (lower rectangle) spectrum of selenolanthionine **8** (A) and **15** (B). The ^{1}H–^{13}C HSQC spectrum shows correlations between the β-carbon atoms and their attached protons (measured at a ^{1}H frequency of 900 MHz). The ^{1}H–^{77}Se HMQC shows correlations between the Se atom and protons two bonds away (measured at ^{1}H frequency of 500 MHz). The 1D ^{1}H spectrum of the same region at 900 MHz is shown at the top in each panel.

The high levels of efficacy and stereoselectivity observed in the synthesis of nisin ring C and oxytocin selenoether derivatives prompted us to apply the selenolanthionine macrocyclization strategy to a more complex system featuring double intercalating selenoether bridges. The α-conotoxin ImI, an antagonist of the α7 nicotinic acetylcholine receptor,[20] was employed as a model peptide. Native α-ImI has the sequence of GCCSDPRCAWRC-NH_2 and it contains two disulfide bonds with the connectivity Cys2–Cys8 and Cys3–

Cys12. We thus prepared a double selenoether α-ImI derivative where both native disulfide bonds were replaced by selenolanthionine bridges. The synthesis began with the assembly of the doubly chlorinated intermediate **16** by Boc-SPPS (Scheme 4). HF cleavage of the peptide from the resin



Scheme 4. Synthesis of the double selenolanthionine bridged α-conotoxin ImI **18**. Residues with asterisks were assembled using standard protecting groups during SPPS. Bottom left: HPLC trace for the cyclization of **17** at pH 6.0 after 32 h under DTT reducing conditions. X = β-L-chloroalanine.

followed by removal of the formyl protecting group of the Trp residue by treatment with 5% ethanolamine in DMF for 1 min gave the double chlorinated diselenide precursor **17**. This compound was then purified by RP-HPLC and cyclized in a sodium phosphate buffer pH at 6.0 containing 30% isopropanol and excess DTT. After reaction at 37 °C for 32 h, approximately 81% of the precursor peptide was doubly cyclized into two products **18** and **19** in a 3:2 ratio (Scheme 4). The two cyclic products corresponded to the two possible regioisomers: **18** is the globular form, with selenolanthionine bridges between residues 2–8 and 3–12, whereas **19** is the ribbon version of the molecule with selenolanthionine bonds between residues 2–12 and 3–8 (Supporting Information, Figure S5). The regioselectivity of the selenoether closure here seems to follow the same folding pattern of native α-ImI under similar folding conditions.[21] After purification by RP-HPLC, the correctly folded double selenolanthionine α-ImI analogue **18** was obtained in 13% overall yield and characterized by mass spectrometry and NMR (Supporting Information). The synthesis of this overlapping double selenoether cyclized peptide described herein is much simpler than if the same kind of peptide framework was to be prepared by the conventional building block approach, where two different fully orthogonally protected lanthionine bisamino acids would have to be constructed in solution to build the doubly cyclized thioether peptide on solid support, although in this case full regiocontrol over the bridging can be achieved.[10,22]

In conclusion, we have demonstrated for the first time the use of selenocysteine in the synthesis of lanthionine-like cyclic peptides. The synthetic difficulties attributed to the intramolecular thioether formation for the manufacture of lantipeptides can be overcome by substitution with the more reactive selenocysteine residue. The approach shown herein is simple and versatile: the solid-phase synthesis of the linear precursors can be performed by either Boc or Fmoc protection strategies and the cyclization can be carried out using a wide range of pH conditions. It does not require the synthesis of building blocks; all of the reacting partners are commercially available amino acids. More importantly, unfavorable macrocyclizations, as illustrated for oxytocin analogue **15**, can be driven by the power of the Se-alkylation reaction.

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