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Synthesis of a Cyclic Peptide Containing Norlanthionine: Effect of the Thioether Bridge on Peptide Conformation

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Two diastereomeric analogues of ring C of nisin incorporating a novel norlanthionine residue have been synthesized via a triply orthogonal protecting group strategy. A full structural study was carried out by NMR, which elucidated the conformational properties of the two peptides and enabled the identity of each diastereoisomer to be proposed.

Introduction

The synthesis of cyclic peptides incorporating unnatural side-chain to side-chain linkages has recently received much attention.[1] This is due, in part, to the synthetic challenges and biological properties of naturally occurring, side-chain-bridged peptides[2] and, in part, to the metabolic stability and/or unusual conformational properties that may be conferred on a naturally occurring linear peptide by cyclization in this manner.[3] Cyclic peptides incorporating thioether bridges are of special interest. They occur naturally in a family of peptides known as the lantibiotics,[4] incorporating multiple lanthionine and methyllanthionine residues. On the basis of their structural properties, the lantibiotics have been subdivided into two classes, both possessing potent biological activity. Type A lantibiotics, such as nisin, have elongated structures and potent antimicrobial properties. Their biological activity is believed to stem from their ability to form pores in the cytoplasmic membrane of susceptible bacteria.[4d] Nisin itself may act in conjunction with lipid II,[4e] which is also believed to be the target of vancomycin action.[4g] Type B lantibiotics form compact, globular structures: some, for instance actagardine, are highly active against Gram-positive bacteria, including multi-antibiotic-resistant streptococci; others, such as cinnamycin, inhibit enzymes such as phospholipase A_2. As thioether-bridged peptides may be regarded as non-reducible analogues of cystine bridges, lanthionine[5] and analogues[6,7] have also been incorporated into medicinally relevant peptides as conformational constraints.

Only a single total synthesis of a complete lantibiotic has so far been reported. Shiba et al. synthesized nisin using solution-phase methods,[8] forming the lanthionine

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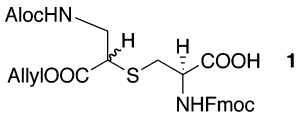
bridges by desulfurization of individual cystine-bridged cyclic peptides, and joining these by segment condensation. This method has recently been revisited;[9] however, it has not been optimized for solid-phase synthesis. Two solid-phase approaches have recently been reported for the synthesis of shorter lanthionine-containing peptides. The PCOR (peptide cyclization on an oxime resin) method involves the synthesis of a linear peptide containing an orthogonally protected lanthionine residue: head-to-tail cyclization, with concomitant cleavage of the cyclic peptide from the resin, affords the lanthionine-bridged peptide.[5] However, it would be difficult to generalize this approach for the total synthesis of polycyclic lanthionine-containing peptides on the solid phase. In the second, biomimetic, approach, a linear peptide containing a cysteine and a dehydroalanine residue is synthesized and the lanthionine bridge formed by intramolecular 1,4-addition of the cysteine thiol group to the dehydroalanine.[10−12] Complete diastereoselectivity was generally, although not always, observed in the 1,4-addition step. However, it would be difficult to predict the diastereoselectivity of this reaction for any particular peptide; furthermore, many lantibiotics contain multiple dehydroalanine and dehydrobutyrine residues, and controlling the regioselectivity of cyclization might also be problematic.

The relatively few methods available for the synthesis of lanthionine-containing peptides has meant that, despite their potential importance as lead structures for new classes of antibacterial drugs, the mode of action is poorly understood, and they have not been exploited for drug discovery. Structural information about the conformational preferences of lanthionine-bridged cyclic peptides is likewise scarce, although solution structures of some naturally occurring lantibiotics have been determined.[13] Finally, there have been no studies of the effect that incorporating other classes of thioether bridges may have on the conformation of the resulting cyclic peptides.

In the preceding paper, we reported the synthesis of protected nor-lanthionine **1**, via an unexpected rearrangement which afforded **1** as an inseparable mixture of two diastereoisomers.[14] In this paper, we report the incorporation of this material into peptides as a further proof of our methodology for synthesising cyclic peptides with novel, noncleavable side-chain linkages.[15,16] We also describe a full structural and conformational analysis of the resulting peptides. This was undertaken for two reasons: to verify and identify the norlanthionine diastereoisomers and to determine the effect that this previously unknown thioether bridge has on the conformation of small cyclic peptides.



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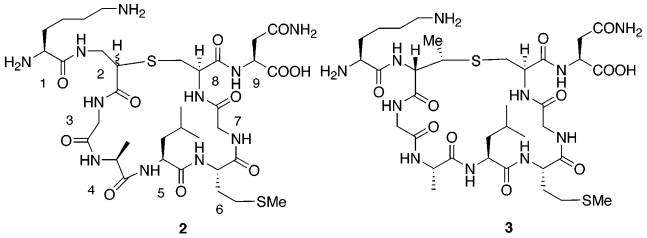
Results and Discussion

Synthesis of a Norlanthionine-Bridged Analogue of Ring C of Nisin. As a test of our approach for synthesizing cyclic peptides containing thioether bridges, we chose to synthesize the norlanthionine analogue **2** of ring C of nisin (**3**). We envisaged that this peptide would not present any problems, as the projected ring size was small enough for cyclization to occur readily yet large enough that ring constraints would not pose any problems.[2] Moreover, additional difficulties associated with the synthesis of lantibiotics, such as dehydroalanine or dehydrobutyrine residues, are not present within ring C of nisin, thus simplifying the problem.



2 **3**

After some preliminary investigations of possible solid supports and linkers, we elected to use the highly acid labile NovaSyn TGT alcohol resin, preloaded with Asn(Trt), for the synthesis. The peptide synthesis was carried out in a Merrifield bubbler.[17] Standard Fmoc deprotection, amino acid coupling, and capping steps (Scheme 1) were first used to synthesize the linear peptide **4**, which includes the differentially protected norlanthionine **1**. Deprotection of the orthogonal allyl/Aloc groups[18] in the presence of the other side-chain protecting groups, and without cleavage from the resin, was followed by removal of the N-terminal Fmoc group. On-resin cyclization, using PyAOP, then afforded the cyclic peptide **5**. The final Lys residue was then attached, the peptide cleaved from the resin, and the side-chain protecting groups removed to

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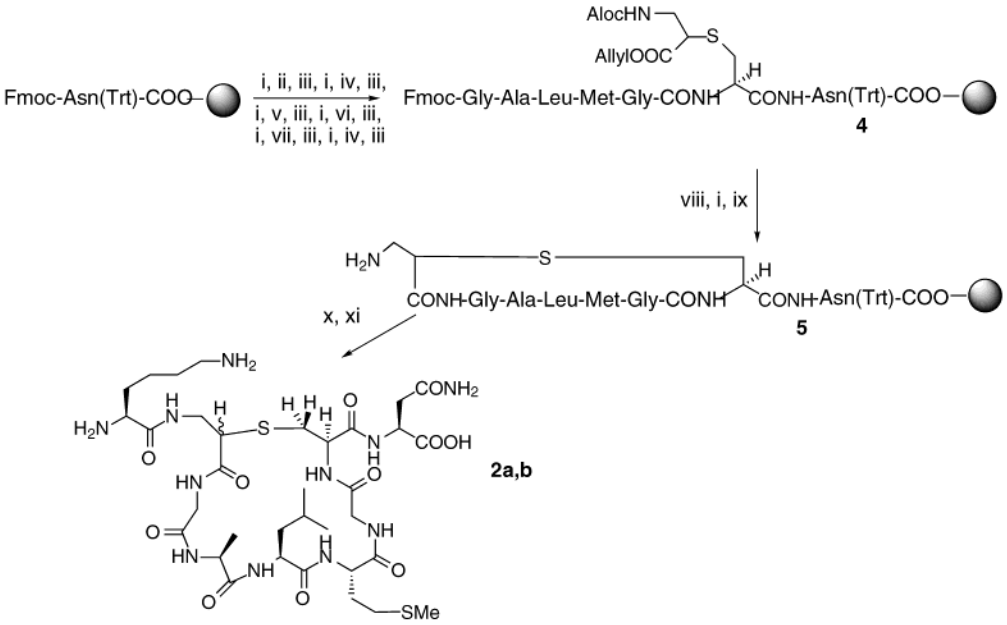
SCHEME 1[a]



[a] Key: (i) 20% piperidine; (ii) **1**, DIC, HOBt; (iii) 1-acetylimidazole, *i*-Pr_2EtN, HOBt; (iv) Fmoc-Gly, DIC, HOBt; (v) Fmoc-Met, DIC, HOBt; (vi) Fmoc-Leu, DIC, HOBt; (vii) Fmoc-Ala, DIC, HOBt; (viii) $Pd(PPh_3)_4$, $CHCl_3$/AcOH/NMM; (ix) PyAOP, HOAt, *i*-Pr_2EtN; (x) Fmoc-Lys, DIC, HOBt; (xi) TFA, H_2O, Et_3SiH.

give the crude peptide. The synthesis appeared to proceed well, as monitored by Kaiser test.[19] Cleavage, precipitation, and lyophilization under standard conditions yielded the crude material as an off-white powder. Analytical LCMS showed that there were two peaks on the HPLC trace, which gave identical mass spectra—the parent ion of the peptide and $[M + 2]^{2+}$. Purification of the peptide via preparative HPLC confirmed the presence of two peptides with the same mass in an approximately 3:1 ratio, giving a total yield of 55%. This suggested an average coupling efficiency of 94% over 10 steps.

Structure and Solution Conformation of the Norlanthionine-Bridged Analogue of Ring C of Nisin. With the two peptides in hand, we were keen to undertake a complete structural analysis by NMR. Clearly, it was important to confirm the structure of the two peptides, particularly given the uncertainty surrounding previously published routes to lanthionine.[14] Analysis of the peptides by NMR also promised to resolve the issue of the synthesis of two diastereoisomers of protected norlanthionine **1** and held out the possibility of determining which diastereoisomer was which.[15] Finally, as the norlanthionine bridge was previously unknown, it was clearly important to elucidate the effect that such a bridge might have on the conformation of the resulting cyclic peptide.

The individual ^1H NMR spectra for the isolated products of the peptide synthesis (**2a** and **2b**) show a single set of resonances indicating that each peptide adopts either a single conformation in solution or multiple conformations in fast exchange on the NMR time scale. Complete ^1H and ^{13}C NMR resonance assignments for the two peptides were determined by application of standard homonuclear and heteronuclear two-dimensional NMR techniques. Briefly, 2D [^1H,^1H] TOCSY (40 ms and 80 ms mixing time) was used to identify each amino acid spin system. 2D [^1H,^{13}C] HSQC and HMBC spectra were used to fully assign the ^{13}C resonances and resolve any overlap in the ^1H dimension. The sequence specific assignment of the two residues containing the norlanthionine moiety was based upon the chemical shift differences for the Cα and Cβ chemical shifts.[15] Confirmation that the nor-lanthionine bridge had been formed in each peptide was obtained from the [^1H,^{13}C] HMBC spectra, with $^3J_{C2\alpha,H8\beta}$ and $^3J_{H2\alpha,C8\beta}$ correlations observed as indicated (Figure 1). Sequence-specific resonance assignments were confirmed using 2D [^1H,^1H] NOESY spectra through identification of $d_{NN}(i, i + 1)$ or $d_{\alpha N}(i, i + 1)$ NOEs, and the pattern of which also allowed the resolution of the assignment of the two glycine residues.

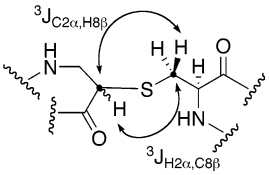


FIGURE 1.

Both peptides were found to have the same amino acid composition and norlanthionine bridge, and therefore, they differ only in the stereochemistry of the α-carbon position in the β-amino acid (residue 2). However, this conclusion leaves unresolved the identification of which peptide possesses the *R* configuration and which possesses *S* at norLan 2. Large ^{13}C chemical shift differences (>1 ppm) between the two peptides were observed for the Cα resonance of residue 2, and Cα and Cβ resonances of residue 8, as expected for a change in the stereochemistry about Cα in residue 2. Intriguingly, the Cα chemical shift of Met 5 also exhibits a large difference that

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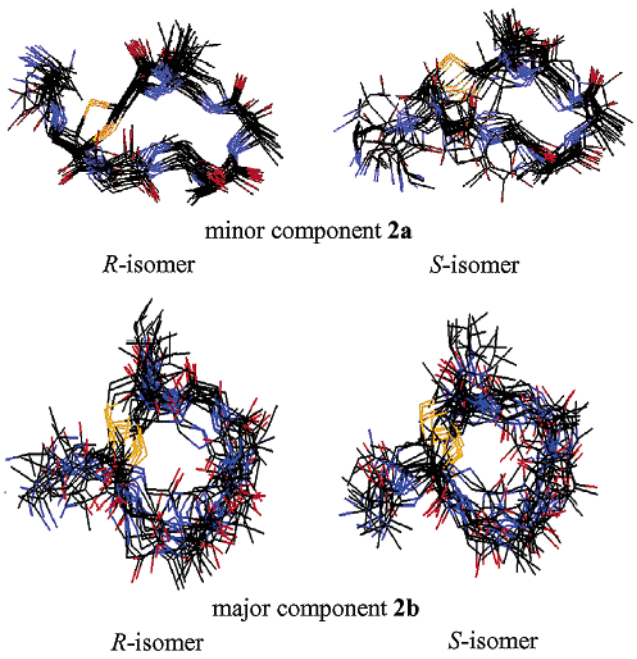

FIGURE 2. Superposition of 20 lowest energy conformers in each set.

TABLE 1. Pairwise RMSD Values for Main Chain and All Atoms Relative to the Lowest Energy Conformer

		R-isomer	*S*-isomer
minor component	main chain (Å)	0.41 ± 0.07	0.54 ± 0.22
	all atoms (Å)	0.86 ± 0.18	0.99 ± 0.18
major component	main chain (Å)	1.11 ± 0.22	1.16 ± 0.16
	all atoms (Å)	2.07 ± 0.14	1.96 ± 0.24

suggests the peptides adopt different conformations away from the center of stereochemical inversion.

To determine whether the stereochemistry for each peptide could be determined, the solution structure of the two peptides was investigated by 2D [^{1}H, ^{1}H] NOESY and [^{1}H, ^{1}H] ROESY measurements in the conventional manner. A total of 63 and 175 unique and unambiguous inter-proton cross-peaks were assigned for the major and minor peptide components, respectively. The cross-peaks were grouped into three categories according to their relative peak intensities, strong, medium, and weak, and were designated with the corresponding restraint limit of 1.8−2.8 Å, 1.8−3.5 Å, 1.8−5.0 Å, respectively. An additional 0.5 Å was added for distances that involved methyl groups to account for the three protons intensity and narrow line widths.

These restraints were applied in restrained dynamical simulated annealing calculations using as input 100 pseudo-randomly generated starting structures. For each set of distance restraints two separate calculations were performed assuming either *R* or *S* configuration about the Cα position of the *β*-amino acid in the nor-lanthionine bridge. The superposition of the 20 lowest energy conformers in each set is shown (Figure 2) and the RMSD values given in Table 1. In each set of conformers no restraint violation > 0.5 Å was observed.

For the minor peptide component from the synthesis the large set of distance restraints obtained (175) was sufficient to obtain a well-defined conformation with either *R*- or *S*-configuration. Comparing the lowest energy structures for the *R*- and *S*-isomers, residues 4−6 adopt a similar conformation, while the orientation of residues 2, 3, 7, and 8 is altered to accommodate the different stereochemistries. Each set of conformers equally satisfies the NMR data and is, therefore, a reasonable representation of the structure of the minor component. However, the conformers for the *R*-isomer have a significantly higher backbone and side-chain precision (lower RMSD) (Table 1). Additionally, the lowest energy conformer for the *R*-isomer has a lower overall energy (58.6 vs 66.1 kcal mol^{-1}) and lower Lennard-Jones potential (−44.8 vs −38.4 kcal mol^{-1}) as compared to the lowest energy *S*-isomer conformer. These data argue that the minor component from the synthesis is the *R*-isomer and, therefore, the major component must be the *S*-isomer. Interestingly, while the *R*-isomer model for the minor component adopts essentially a single conformation in the structure calculations, the *S*-isomer model, in marked contrast, adopts multiple conformations (as reflected in the higher RMSD), suggesting that it is free to sample a more expanded region of conformational space.

In contrast, because of the lower number of distance restraints obtained (63) from the ROESY spectra for the major component from the peptide synthesis, structure calculations for neither the *R*- nor the *S*-isomer resulted in a well-defined conformation, thereby yielding relatively high RMSD values (Table 1). Moreover, although the prochiral H2*β*s are at different chemical shifts, we were not able to identify which was which. The relatively small number of ROEs suggests that the major component, which is expected to be the *S*-isomer, adopts multiple conformations that are in fast exchange on the NMR time scale. This lends support to the idea that the *S*-isomer is able to sample a larger area of conformational space. However, this smaller set of observed ROE data for the major isomer could not unambiguously confirm that this diastereoisomer was in the *S* configuration, as both the *R* and *S* configurations equally satisfied the NOE-derived experimental restraints.

Conclusions

We have demonstrated that a triply orthogonal protecting group strategy can be effective for the solid-phase synthesis of thioether-bridged peptides from differentially protected bis-amino acids. The synthesis, and subsequent characterization, of the nor-lanthionine analogue **2** of ring C of nisin has confirmed the structure of the previously unknown nor-lanthionine **1** and allowed the identity of the two diastereoisomers to be assigned. This methodology has the potential for preparation of analogues of other subunits of nisin, and related lantibiotics.

Interestingly, the resulting cyclic peptides do not appear to adopt any previously known turn conformation, although **2a** adopts a single, well-defined conformation. Importantly, this also suggests that cyclic peptides bridged with nor-lanthionine residues could sample a region of conformational space not accessible with the 20 proteinogenic amino acids, with the potential for them to be used as lead structures in drug design.

Experimental

Unless otherwise noted, solvents and reagents were reagent grade and used without purification. Amino acids, coupling reagents and resins were obtained from Novabiochem and Perseptive Biosystems. Analytical grade DMF was obtained from BDH.

Synthesis of Peptide 2. All glassware was dried in the oven for 1 day prior to synthesis. Fmoc-Asn(Trt)-NovaSyn TGT resin (0.5 g, 0.1 mmol) was suspended in DMF (5 mL) for 20 min in a vial and then transferred to a Merrifield bubbler. The terminal Fmoc group was removed with piperidine-DMF (20% piperidine in DMF, 2 mL) with bubbling for 20 min, followed by thorough washing with DMF (6 × 10 mL). A qualitative Kaiser test showed a positive result.

3-[(*R*/*S*)-1-Allyloxycarbonyl-2-(allyloxycarbonylamino)ethylsulfanyl]-(*R*)-2-(fluoren-9-ylmethoxycarbonylamino)propionic acid (norlanthionine **1**) (0.13 g, 0.25 mmol, 2.5 equiv) was then activated under inert conditions, in a separate vial, with a HOBt solution in DMF (0.033 g, 0.25 mmol, 2.5 equiv, 1 mL of DMF (0.25 M)) and a DIC solution in DMF (0.039 mL of DIC, 0.25 mmol, 2.5 equiv, 1 mL of DMF (0.25 M)) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. After 10 min of the first coupling, another batch of **1** (0.13 g, 0.25 mmol, 2.5 equiv) was activated in the same manner. Removal of the first coupling solution was followed by addition of the second and bubbling for a further 30 min. The second coupling solution was then removed and the resin washed with DMF (6 × 10 mL). A qualitative Kaiser test showed a negative result. The resin was then capped with a solution of 1-acetylimidazole in DMF (0.16 g, 1.5 mmol, 4 mL of DMF (0.3 M)), and the terminal Fmoc group removed with piperidine−DMF (20% piperidine in DMF, 2 mL) with bubbling for 20 min, followed by thorough washing with DMF (6 × 10 mL).

Fmoc-Gly-OH (74.3 mg, 0.25 mmol, 2.5 equiv) was then activated under inert conditions, in a separate vial, with a HOBt solution in DMF (0.033 g, 0.25 mmol, 2.5 equiv,1 mL of DMF (0.25 M)) and a DIC solution in DMF (0.039 mL of DIC, 0.25 mmol, 2.5 equiv,1 mL of DMF (0.25 M)) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. After 10 min of the first coupling, another batch of Fmoc-Gly-OH (74.3 mg, 0.25 mmol, 2.5 equiv) was activated in the same manner. Removal of the first coupling solution was followed by addition of the second and bubbling for a further 30 min. The second coupling solution was then removed and the resin washed with DMF (6 × 10 mL). A qualitative Kaiser test showed a negative result. The resin was then capped with a solution of 1-acetylimidazole in DMF (0.16 g, 1.5 mmol, 4 mL of DMF (0.3 M)) and the terminal Fmoc group removed with piperidine-DMF (20% piperidine in DMF, 2 mL) with bubbling for 20 min, followed by thorough washing with DMF (6 × 10 mL). The activation, coupling, capping, and Fmoc removal stages were then repeated sequentially for Fmoc-Met-OH (92.9 mg), Fmoc-Leu-OH (88.4 mg), Fmoc-Ala-OH (77.8 mg), and Fmoc-Gly-OH to afford the linear peptidyl-resin **4**.

Simultaneous allyl ester and allyl chloroformate deprotection was then carried out. $Pd(PPh_3)_4$ (0.23 g, 0.20 mmol, 2 equiv) was dissolved by gentle heating in a solution of DMF/$CHCl_3$/AcOH/NMM (18.5:18.5:2:1 volume, 1.85/1.85/0.2/0.1 mL, where the concentration of palladium(0) catalyst in the cocktail solution = 0.050 M) and added to the peptidyl-resin **4**. The mixture was gently bubbled with Ar for 2 h in the dark. The resin was then washed sequentially with the following: 0.5% *i*-Pr_2EtN in DMF (v/v) (4 × 5 mL); DMF (6 × 10 mL); 0.5% sodium diethyldithiocarbamate trihydrate in DMF (w/v) (4 × 5 mL); followed by a final wash with DMF (6 × 10 mL). The qualitative Kaiser test was blue. The peptidyl-resin was then deprotected by bubbling with piperidine−DMF (1:4 v/v) (20 mL) for 20 min and thoroughly washed with DMF (6 × 10 mL). This was followed by a wash with 0.4% HOBt in DMF (w/v) (5 mL) to prevent the possible side reaction of the undesired conversion of the free carboxy group to a piperidyl amide.[20] This was followed by further DMF washes (2 × 10 mL). The qualitative Kaiser test was purple.

The cyclization reagents were prepared during the Fmoc deprotection. PyAOP (0.26 g, 0.5 mmol, 5 equiv) was dissolved in DMF (1 mL) and mixed with a solution of HOAt (0.068 g, 0.5 mmol, 5 equiv) in DMF (1 mL). The mixture was transferred to the bubbler, and a solution of *i*-Pr_2EtN (0.18 mL, 1.0 mmol, 10 equiv.) in DMF (0.5 mL) was added after 1 min. The mixture was bubbled with Ar for 1 h, after which time the qualitative Kaiser test was blue. The resin was again washed with DMF (6 × 10 mL).

Fmoc-Lys(Boc)-OH (117 mg, 0.25 mmol, 2.5 equiv) was then activated under inert conditions, in a separate vial, with a HOBt solution in DMF (0.033 g, 0.25 mmol, 2.5 equiv, 1 mL of DMF (0.25 M)) and a DIC solution in DMF (0.039 mL of DIC, 0.25 mmol, 2.5 equiv, 1 mL of DMF (0.25 M)) for 20 min. The activated amino acid solution was then added to the resin with Ar bubbling for 30 min. After 10 min of the first coupling, another batch of Fmoc-Lys(Boc)-OH (117 mg, 0.25 mmol, 2.5 equiv) was activated in the same manner. Removal of the first coupling solution was followed by addition of the second and bubbling for a further 30 min. The second coupling solution was then removed and the resin washed with DMF (6 × 10 mL). A qualitative Kaiser test showed a negative result. The resin was then capped with a solution of 1-acetylimidazole in DMF (0.16 g, 1.5 mmol, 4 mL of DMF (0.3 M)).

Removal of the terminal Fmoc group with 20% piperidine in DMF (2 mL) with bubbling for 20 min was followed by washing with DMF (6 × 10 mL) and then with CH_2Cl_2 (6 × 10 mL). Cleavage of the peptide from the resin was carried out by successively bubbling the resin with a 5% TFA, 2.5% H_2O, 2.5% Et_3SiH solution in DCM (5 × 10 mL; 1 min each portion). The cleavage cocktails were collected in a flask containing TFA (50 mL) and stirred vigorously under Ar for 3 h. The mixture was then concentrated in vacuo to approximately 1 mL, after which time ice-cold Et_2O was added (100 mL). The product was precipitated in the refrigerator overnight. Filtration followed by lyophilization of the precipitate from 20% AcOH/H_2O yielded the crude peptide (87.1 mg). This was purified by preparative reversed-phase HPLC. HPLC: Preliminary preparative HPLC of the crude material (10−20% MeCN in 30 min) afforded a crude separation of the two peptides: diastereoisomer **2a** t_R 17.0, **2b** t_R 20.1 min. Further purification of each each peak afforded the two peptides, **2a** and **2b**, as white solids. Diastereoisomer **2a**: t_R 8.9 min (15−18% MeCN in 30 min); 14.3 mg, 16.6 μmol, 16.6%. Diastereoisomer **2b**: t_R 11.7 min (15−18% MeCN in 30 min); **2b** 33.1 mg, 38.4 μmol, 38.4% (47.4 mg total, 55% yield overall). Mass spectrum: *m*/*z* (ES+) **2a** 863 ([M + H]$^+$, 63); **2b** 863 ([M + H]$^+$, 100).

NMR Spectroscopy. NMR spectra were acquired at either 283 or 298 K on Varian UNITYplus spectrometers (operating at nominal ^{1}H frequencies of 500 MHz and 600 MHz) equipped with triple resonance probe including *Z*-axis pulse field gradients. Assignments were obtained from 2D [^{1}H,^{1}H] TOCSY[21] employing 40 or 80 ms mixing times, [^{1}H,^{13}C] HSQC[22] and [^{1}H,^{13}C] HMBC[23] spectra. Suppression of the residual CD_3OH solvent signal was achieved using the WATERGATE[24] sequence. All spectra were processed using NMRpipe/NMRDraw[25] and analyzed using ANSIG[26] for OpenGL v1.0.3. Chemical shifts were referenced to the residual methanol methyl group signal at 3.5 ppm (^{1}H) and 49.5 ppm (^{13}C) at

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298 K. Interproton distance restraints were derived from the ANSIG cross-peaks file of 250 ms mixing time 2D [^{1}H,^{1}H] NOESY[27] on a sample dissolved in CD_3OH and 250 ms mixing time 2D [^{1}H,^{1}H] ROESY spectra[28] on a sample dissolved in CD_3OD. The cross-peaks were grouped into three categories according to their relative peak intensities, strong, medium and weak, and were designated in the restraint list with the corresponding distance limits of 1.8−2.8, 1.8−3.5, 1.8−5.0 Å, respectively. An additional 0.5 Å was added for distances that involved methyl groups.

Structure Calculations. The structure calculations were carried out within the CNS program[29] using the PARALLHDGv5.1 parameter, with nonbonded energy function of PROLSQ,[30] and modified to include parameters for the norlanthionine bridge. Sixty-three and 175 interproton distance restraints were applied in restrained molecular dynamics simulated-annealing calculations for major and minor components, respectively. Each calculation was performed separately assuming either the *R*- or *S*-configuration of the Cα position of residue 2.

The resulting conformers were clustered and best fit to the lowest energy conformation. The 20 lowest energy conformers of each set were selected for the computation of coordinate precision (RMSD) and presentation in Figure 2 using Rasmol.

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Supporting Information Available: ES+ spectra for purified **2a** and **2b** and the complete set of NMR spectra for each compound. This material is available free of charge via the Internet at http://pubs.acs.org.

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