Biomimetic Synthesis of Lantibiotics

Sarah Burrage,[a] Tony Raynham,[b] Glyn Williams,[b] Jonathan W. Essex,[a] Carl Allen,[a] Marianne Cardno,[a] Vinay Swali,[a] and Mark Bradley*[a]

Abstract: The lantibiotics are a class of highly posttranslationally modified small peptide antibiotics containing numerous lanthionine and dehydroamino acid residues. We have prepared peptides containing multiple dehydroamino acids and cysteine residues in order to probe the biomimetic synthesis of the lantibiotics from their precursor peptides. A novel synthetic methodology was developed to allow the synthesis of multiple dehydroamino acid containing peptides. Cyclisations were rapid, quantitative and regiospecific. Remarkably the peptide sequences alone appear to contain sufficient information to direct a series of stereo- and regiospecific ring closures. Thus both the two linear peptides for the B and E-rings closed stereoselectively. In the case of the A-ring precursor peptide which contained two dehydroamino acids, cyclisation was again totally regioselective, although not totally stereoselective.

Keywords: biomimetic synthesis · lantibiotics · peptides · solid-phase synthesis

Introduction

The lantibiotics are a family of polycyclic peptides produced by a broad range of bacteria, including *Bacillus*, *Lactococcus*, *Streptomyces* and *Staphylococcus*.[1] The first lantibiotic to be discovered was nisin in 1928,[2] with its structure being determined some 40 years later.[3] This was followed by the structural assignment of subtilin, a related lantibiotic.[4] Since this time many more lantibiotics have been discovered, isolated and characterised (Figure 1). These bacteriocins typically contain unusually high proportions of nonproteinogenic amino acids often accounting for one third of the total residues. Thus nisin and subtilin[5, 6] each contain eight unnatural amino acids, including two dehydroalanines, one dehydroaminobutyrate and five lanthionine residues. Detailed NMR studies on some of the lantibiotics have been reported,[7] while elegant total or partial synthesis of some members of this family has been achieved.[8]

Biosynthetically, the lantibiotics are ribosomally derived peptides which have been subjected to extensive posttranslational modifications.[9] Their direct ribosomal origin was first indicated by the inhibition of lantibiotic production by inhibitors of both RNA and protein synthesis. Ingram, inspired by the structures of the lantibiotics, proposed that certain serine and threonine residues were initially dehydrated to dehydroalanines and dehydroaminobutyrates, respectively. He further postulated that a series of subsequent stereospecific Michael additions yielded *meso*-(2*S*,6*R*)-lanthionines and (2*S*,3S,6*R*)-3-methyllanthionines (Figure 2),[10] a prediction that was confirmed some 20 years later when the primary transcripts of the structural genes were determined.[9] The dehydration of serine/threonine residues is thought to occur immediately after the primary transcript is released from the ribosome; indeed the existence of a multiply dehydrated precursor has been confirmed by ion-spray mass spectrometry[11] and amino acid analysis in the case of Pep5. It is proposed that nucleophilic addition of the cysteine thiol onto the α,β-unsaturated amino acid produces an "enol(ate)" intermediate which is selectively protonated affording the observed *(2S,6R)* stereochemistry.

Lanthionine formation—regio- and stereospecificity: Since the mature forms of the lantibiotics contain dehydroamino acids and multiple lanthionines, the rationality of both regio- and stereospecificities of cyclisation is raised. In addition, the relative stereochemical orientation of the *meso*-lanthionine varies within the lantibiotic family. The regio- and stereoselectivities observed may be the product of enzyme catalysis, or could simply result from the natural conformation of the peptide. The work reported here was aimed at unravelling this process and involved the synthesis of peptides containing

[a] Prof. M. Bradley, Dr. S. Burrage, Dr. J. W. Essex, C. Allen, Dr. M. Cardno, V. Swali
Department of Chemistry, Southampton University
Highfield, Southampton, SO17 1BJ (UK)
E-mail: mb14@soton.ac.uk

[b] Dr. T. Raynham,[+] Dr. G. Williams
Roche Discovery Welwyn
Broadwater Road, Welwyn Garden City, Herts, AL7 3AY (UK)

[+] Current address:
Cambridge Discovery Chemistry Ltd
(Formerly Cambridge Combinatorial Ltd)
The Merrifield Centre, 12 Rosemary Lane, Cambridge CB1 3LQ (UK)

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000 0947-6539/00/0608-1455 $ 17.50+.50/0

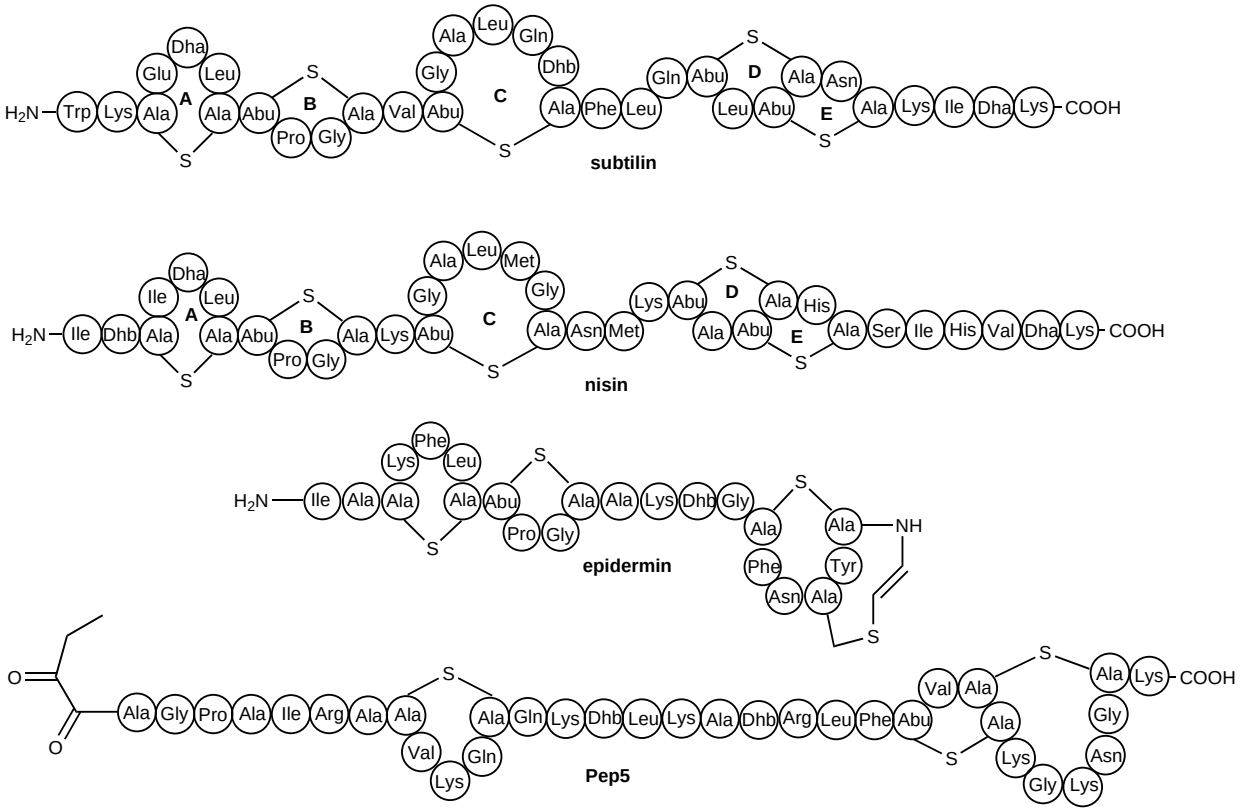


Figure 1. Some typical lantibiotics.

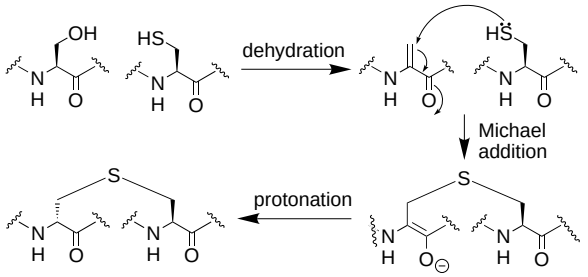


Figure 2. Ingram's proposal for the formation of the unusual amino acids.

single and multiple dehydroalanine and cysteine residues for biomimetic cyclisation studies. Evaluation of the cyclic products would give valuable information concerning the actual biosynthetic route to the lanthionine residues.

The B- and E-rings of subtilin and nisin (Figure 3) were selected as the first targets for cyclisation studies for a number of reasons. Firstly, regiochemistry was not an issue because the precursor peptides contain only one dehydroamino acid. Secondly, the central residues of the B-ring (Pro – Gly) are known to promote β-turns possibly favouring cyclisation. The B-ring has also been previously studied[12] and would allow us to determine if solid-phase based cyclisations would mimic those in solution, an important future issue with regards to the synthesis of these complex precursor peptides. The E-ring peptide would allow us to determine if the turn element was essential for cyclisation, as well as giving a competing lysine residue of interest in a number of lantibiotics which contain the lysinoalanine motif.

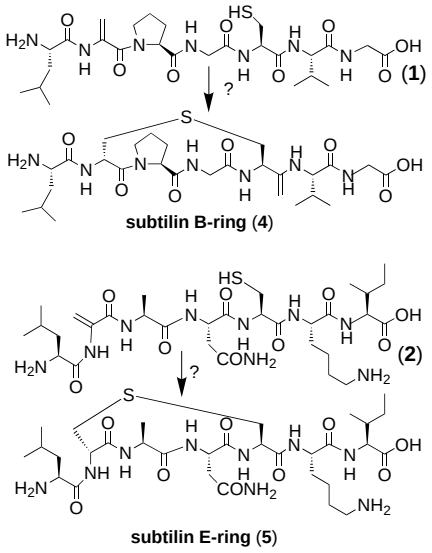


Figure 3. Proposed biomimetic B- and E-ring closures.

Having developed the synthetic methodology to make these relatively simple mono-dehydroamino acid containing peptides, the methodology could then be extended to prepare the A-ring precursor (Figure 4), containing two dehydroamino acids and a single thiol. This would allow monitoring of both the regio- and stereoselectivity of cyclisation, and could result in the formation of up to four possible cyclic products (two regioisomers each containing either D,L or L,L-lanthionines).

© WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000
0947-6539/00/0608-1456 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 8

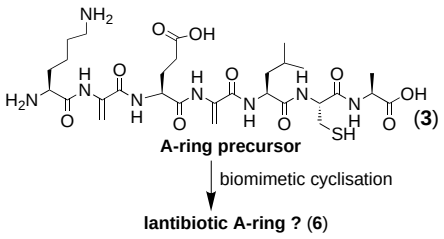


Figure 4. Proposed biomimetic A-ring cyclisation.

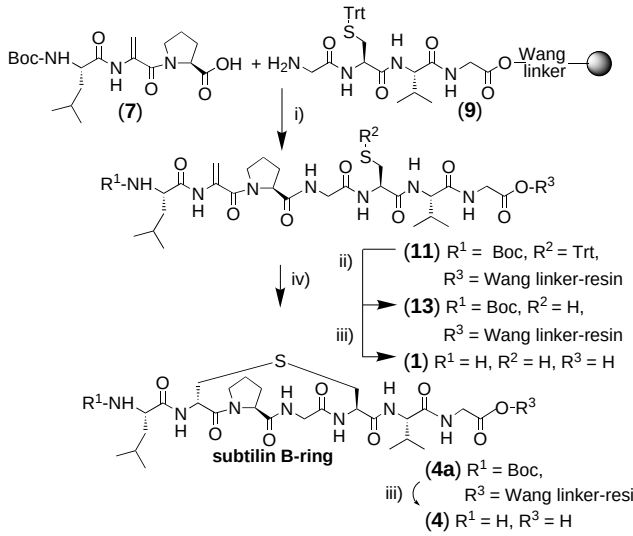
Synthetic routes to dehydroamino acid containing peptides: The chemistry of dehydroamino acids is well documented and has been extensively reviewed.[13] Methods of synthesis often rely on the activation of serine or threonine residues for example with dichloroacetyl chloride or tosyl chloride followed by elimination with triethylamine or DABCO, respectively.[14, 15] They also include phosphonium activation of serine and elimination (Ph_3P/DEAD)[16] or EDC activation followed by CuCl-mediated elimination.[17] On the solid phase fewer methods have been reported. Of principal importance are the coupling of preformed dehydroamino acid *N*-carboxyanhydrides (NCA's),[18] the solid-phase Hofmann elimination of quaternary amines[19] and the attachment of peptides by a cysteine side chain followed by oxidation to the sulfone and elimination.[20] The stepwise incorporation of dehydroamino acids residues into peptides by either a solid-phase or solution route is also often difficult. Dehydroamino acids couple very poorly[13, 21] and are inherently reactive towards nucleophiles (including amines). Protected peptides containing dehydroamino acid residues are always prone to decomposition, although they appear much more robust when unprotected, while *N*-terminal dehydroamino acids decompose to give the keto amide and ammonia.[13] Clearly, if such residues are to be incorporated into peptides they should be introduced as masked residues which can be converted to the α,β-unsaturated amino acids as late as possible in the synthetic scheme, or inserted within small peptide fragments such that the exposed termini couple efficiently.

Results and Discussion

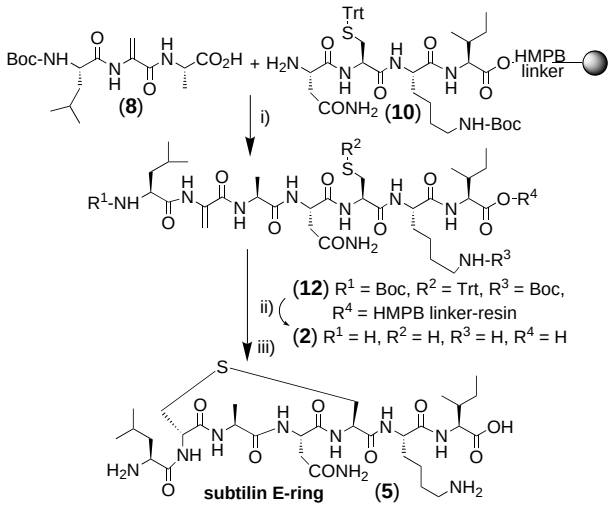
Synthesis of dehydroamino acid containing peptides: The dehydroamino acid and thiol containing peptides synthesised for this study were:

H-Leu-**Dha**-Pro-Gly-**Cys**-Val-Gly-OR (**1**)
H-Leu-**Dha**-Ala-Asn-**Cys**-Lys-Ile-OH (**2**)
H-Lys-**Dha-**Glu-**Dha**-Leu-**Cys**-Ala-OH (**3**)
(R = H or linker-resin) (**1**) = **B-ring** precursor
(**2**) = **E-ring** precursor
(**3**) = **A-ring** precursor

Peptides **1** and **2** were prepared by a fragment coupling procedure as outlined in Schemes 1 and 2. The crucial dehydroamino acid containing peptide Boc-Leu-Dha-Pro-OH (**7**) was synthesised in 23% yield from the tripeptide Boc-Leu-Ser(Bzl)-Pro-OMe,[22] by hydrogenation, followed by dehydration (CuCl/EDC)[15] and ester hydrolysis.[22] Using a similar approach the tripeptide Boc-Leu-Dha-Ala-OH (**8**) was prepared in 14% yield from Boc-Leu-Ser-Ala-OMe. In this case the serine residue was used without side chain protection throughout the synthesis without mishap. The dehydroamino acid containing peptides **7** and **8** were then available for coupling to the solid-phase linked peptides **9** and **10**, which were synthesised with standard solid-phase Fmoc chemistry,[23, 24] to give the full length linear protected peptides **11** and **12**. Following deprotection, peptides **1**, **2** and **13** were ready for solution and solid-phase cyclisation studies.



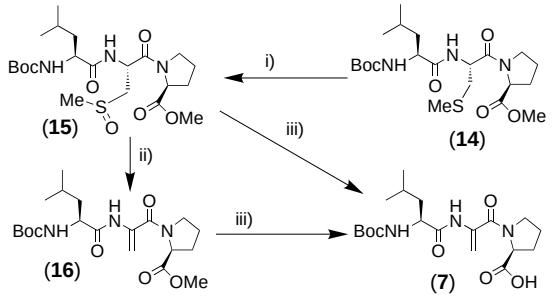
Scheme 1. Subtilin B-ring biomimetic synthesis. i) PyBrop, DMAP, DIPEA, CH_2Cl_2; ii) 2% TFA, 2% TIS, CH_2Cl_2; iii) 95% TFA, CH_2Cl_2; iv) pH 8.0, 50 mM $NEt_3 \cdot AcOH$ or 5% NMM, DMF for (**13**).



Scheme 2. Subtilin E-ring biomimetic synthesis. i) DIC, HOBt, CH_2Cl_2/DMF (9:1); ii) 50% TFA, 5% TIS, CH_2Cl_2; iii) pH 8.0, 50 mM $NEt_3 \cdot AcOH$.

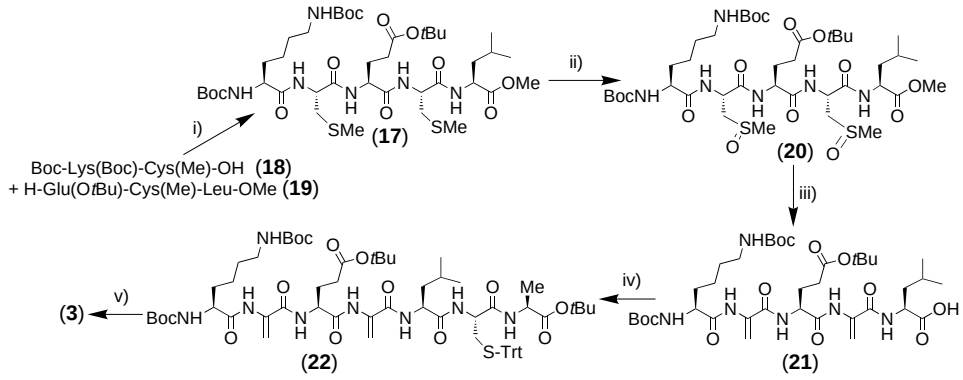
Peptide **3** (see Figure 4) containing two dehydroamino acids and one cysteine residue was prepared by a novel method, developed for the incorporation of multiple dehydroamino acids into peptides.[25] This was achieved by the synthesis of peptides containing *S*-methylcysteine and the pyrolytic elimination of the *S*-methylcysteine sulfoxides.[25] The use of *S*-methylcysteine obviated the need for side chain

protection, and if desired, Fmoc/*t*Bu orthogonality could be maintained throughout the synthesis. It was envisaged that oxidation and elimination would be performed immediately prior to global deprotection. As an initial trial study the tripeptide Boc-Leu-Cys(Me)-Pro-OMe (**14**) was assembled in solution with standard Boc chemistry in 34% overall yield. Addition of peptide **14** to a solution of cold sodium metaperiodate in dioxane (1.1 equiv) gave the diastereomeric sulfoxides **15** in excellent yield and purity. Pyrolytic elimination was found to be solvent dependent; the best results were obtained by refluxing the sulfoxides **15** overnight in dioxane. Purification by column chromatography led to isolation of the protected tripeptide **16** in 88% yield (Scheme 3). Elimination also occurred on treatment of the Boc protected sulfoxide tripeptide **15** with DBU in MeOH, whilst simultaneous elimination and saponification was accomplished in high yield by 1M NaOH.



Scheme 3. Thioether approach to dehydroamino acids. i) $NaIO_4$ (aq), dioxane (99%); ii) dioxane, reflux (88%) or DBU, MeOH (96%); iii) 1M NaOH, MeOH (95%).

This methodology was extended to the synthesis of peptide **3** (Scheme 4) which contains two dehydroamino acids. Thus the desired precursor peptide Boc-Lys(Boc)-Cys(Me)-Glu(O*t*Bu)-Cys(Me)-Leu-OMe (**17**) was obtained by fragment condensation of Boc-Lys(Boc)-Cys(Me)-OH (**18**) with the tripeptide H-Glu(O*t*Bu)-Cys(Me)-Leu-OMe (**19**) mediated by EDC and HOBt in an overall yield of 36% from the starting amino acids. Peptide **17** was oxidised to give **20** which was treated with 1M NaOH in MeOH to facilitate two eliminations and ester hydrolysis in under 2 h to give **21**. Subsequent coupling of **21** to H-Cys(Trt)-Ala-O*t*Bu gave **22** which was deprotected to give the desired peptide **3**. It was important that oxidation, elimination, deprotection and coupling was accomplished in a single day due to the observed lability of the protected dehydoamino acid containing peptides.



Scheme 4. Synthesis of a pentapeptide containing two dehydroalanine residues. i) EDC, HOBt, NEM, CH_2Cl_2, 75%; ii) $NaIO_4$ (aq), dioxane (99%); iii) a) DBU, MeOH (86%), 1M NaOH, MeOH (92%) or b) 1M NaOH, MeOH (93%); iv) H-Cys(Trt)-Ala-O*t*Bu (**21**), EDC, HOBt (72%); v) 50% TFA, 2% TIS, CH_2Cl_2.

Biomimetic cyclisations

Synthesis of the B-ring (Scheme 1): The linear peptide **1** was analysed by HPLC following cleavage from the solid phase and was shown to be essentially pure (>95%) by HPLC thereby giving confidence for the subsequent solid-phase and solution based cyclisations. Thus the trityl group was removed from **11** by repeated washes with 2% TFA/2% TIS/96% CH_2Cl_2 to give **13**. Quantification of the thiol in peptide **13** was attempted by the Ellman test.[26] However, this assay gave a much lower substitution than expected (0.05 mmol per g cf. 0.25 mmol per g), and lead to the development of a new test which was designed to monitor the consumption of the dehydroamino acid during cyclisation. This was based on the very well known addition of thiols onto dehydroamino acids. Thus the peptidyl resin **13** was treated with a large excess of β-mercaptoethylamine allowing the rapid addition of the thiol to the α,β-unsaturated amino acid. (A small portion of the resin was treated with TFA and the addition confirmed by ES-MS and HPLC). A quantitative ninhydrin assay was then carried out allowing solid-phase monitoring of the cyclisation.[27] Cyclisation was carried out with 5% NMM in DMF and monitored as described above. The reaction was complete within 1.5 h furnishing the cyclic peptide **4a**. This was cleaved and purified by RP-HPLC to give **4** which was fully characterised by NMR (see below).

The cyclisation of the B-ring in solution was undertaken to confirm the results above. The synthesis was identical to that above and peptide **9** was cleaved and deprotected from the resin prior to cyclisation to give **1**. Cyclisation to give **4** was carried out in 50mM triethylammonium acetate (pH 8) and was monitored by RP-HPLC by using a C_8 column (C_{18} columns did not separate the product and starting peptide). The cyclisation was found to proceed cleanly with complete consumption of starting material in under 10 min as determined by UV/Vis analysis of the cyclisation reaction and HPLC analysis, following removal of aliquots and quenching with acid. A single product, identical to that observed in the solid-phase cyclisation was observed by RP-HPLC. The cyclisation was also performed in deuterated buffer and incorporation of a single nonexchangeable deuteron was observed by ES-MS confirming that cyclisation had occurred with deuterium inclusion at the newly generated alpha centre.

Synthesis of the E-ring (Scheme 2): The E-ring cyclisation was similarly investigated.

© WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000
0947-6539/00/0608-1458 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 8

The linear peptide **2** was prepared in an analogous manner to the B-ring precursor. Cyclisation gave a single product **5** which was fully characterised by NMR studies (see below) and was determined to be the desired lanthionine cyclic peptide, clearly demonstrating that the turn element in the B-ring is unnecessary for cyclisation to take place. Again cyclisation was rapid and experiments in deuterated buffer showed the expected incorporation of a single deuterium atom into the cyclic product.

Synthesis of the A-ring (Figure 4): The linear A-ring precursor peptide **3** was produced in good yield and purity (86 % by RP-HPLC) (Scheme 4). Considering the problems of stability, the peptide was cyclised crude. The reaction was again found to be complete within 10 min but this time produced two products in a 3:1 ratio (RP-HPLC). These were separated by RP-HPLC and their structures determined by NMR, as detailed below, to be the stereo- rather than regioisomers of the natural A-ring.

NMR and modelling studies

The B-ring: The samples of cyclic peptide produced both on the solid phase and in solution were identical by RP-HPLC and NMR. The presence of the Lan-H_β at $\delta = 2.96$, 2.86 and 2.60 could be clearly detected (Figure 5). The appearances of resonances corresponding to the amide NHs and the loss of the olefinic resonances as well as a full spectral assignment confirmed that cyclisation had occurred. Two ring conformers were observed which interconverted on the NMR timescale, but which coalesced when recorded at 310 K. NOE studies were performed on the B-ring sample. Most importantly an NOE was observed between the Pro-H_α and the Lan^1-H_α indicating that the proline amide bond was *cis* configured as expected. Modelling indicated that the distance between these protons was about 2.4 Å. The lanthionine stereochemistry was determined as *meso* from these studies in accordance with the literature.[12]

The E-ring: 1-D NMR analysis verified the presence of only one compound and further 2-D NMR analysis enabled a full assignment of the cyclic product to be obtained. The loss of the olefinic protons, the splitting of the NH resonance (dehydroamino acid NH's are singlets) and the gain of a CH_α proton were all observed as expected. In contrast to the B-ring, only a single conformer was observed. A detailed examination of the ring was undertaken by COSY, TOCSY and ROESY NMR spectroscopy, allowing the sequential assignment of the amino acids (also confirmed by Edman sequencing). The stereochemistry of the lanthionine residue was examined by using the modified distance geometry program DIANA[28] and the NOE constraints obtained above measured from the spectrum. The best structure gave the expected *meso*-lanthionine, although there was not a substantial difference between the two target functions (4.9 versus 3.8). The pattern of coupling constants ($^3J_{NH-CH\alpha}$) was also analysed but again no concrete conclusions could be made with regard to the lanthionine stereochemistry. Modelling studies (MacroModel 5.0[29]) on the enolate suggested that protonation from the surrounding solvent would be most likely to give the D-amino acid with protonation taking place on the less hindered face opposite to that of the attacking sulfur.

The A-rings: A series of 1-D and 2-D NMR experiments showed that both of the new compounds were cyclic. Assignment was facilitated by TOCSY experiments and confirmed that each was a single diastereoisomer. The existence of a cyclic peptide in both cases was confirmed by a number of features in the 1-D NMR spectra, notably one set of resonances corresponding to Dha-H_β protons and Dha-NH were missing and there was the expected resonance for the Lan-NH. There were also signals corresponding to the

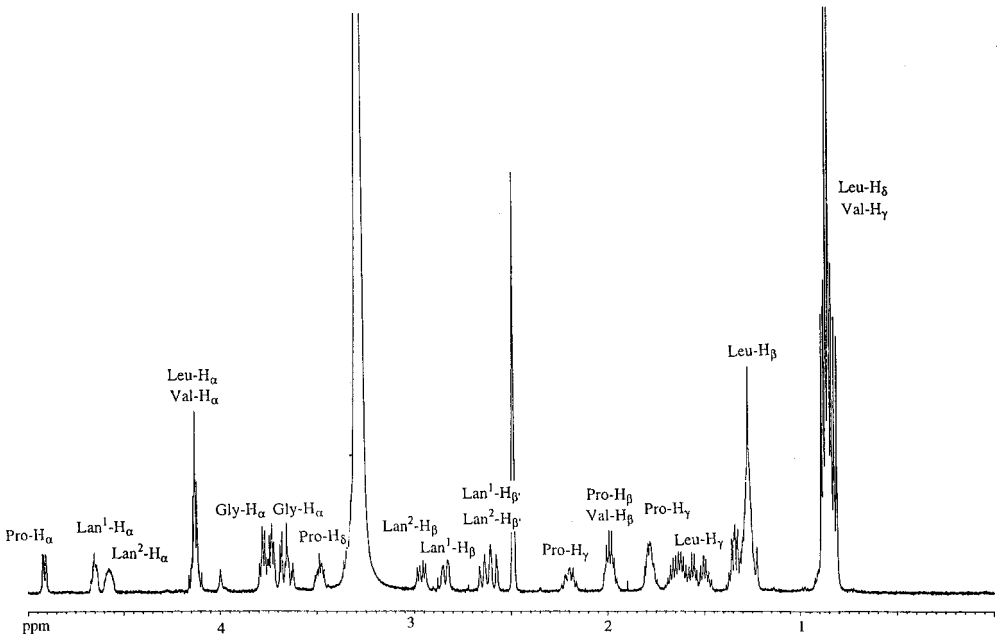


Figure 5. 500 MHz NMR spectrum ([D_6]DMSO, 310 K) of the B-ring **4** of subtilin.

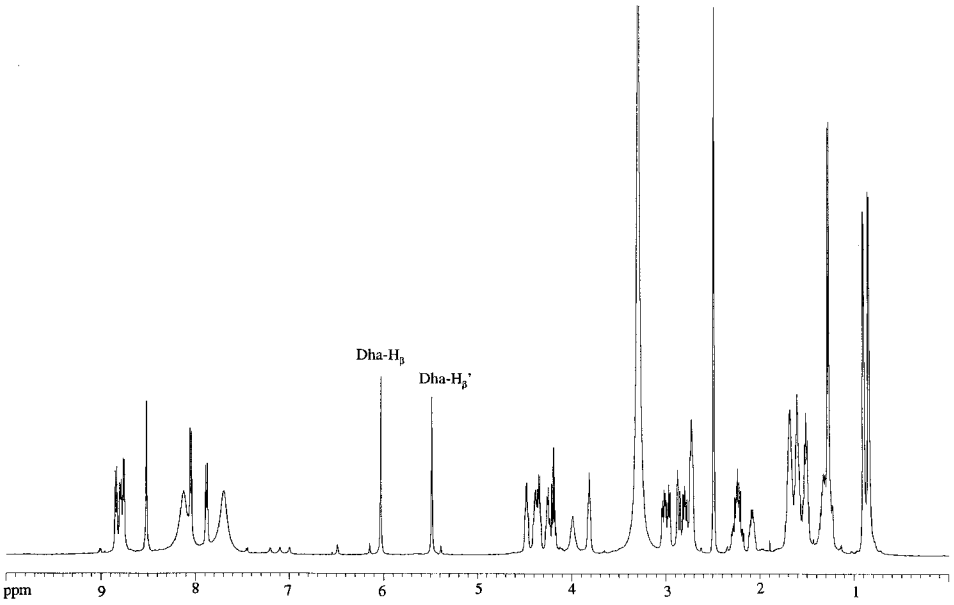


Figure 6. 500 MHz NMR spectrum ([D$_6$]DMSO, 303 K) of the major product formed by cyclisation of peptide H-Lys^1-Lan^2-Glu^3-Dha^4-Leu^5-Lan^6-Ala^7-OH (**3**)—A-ring of subtilin.

presence of only a single dehydroalanine residue and the characteristic ABX resonances observed for the Lan-H$_\beta$ were found just below $\delta = 3.0$, clearly displaying the presence of four protons (Figure 6).

Regiochemical assignment of the major product

Having confirmed cyclisation had occurred the regio- and stereoselectivity of the process was then established. The observation of an NOE between Lan^6-NH and Leu^5-H$_\alpha$ allowed the identification of the resonances associated with Lan^6 (Figure 7). In conjunction with the TOCSY experiments it was therefore possible to distinguish between the two lanthionine halves and fully assign the resonances.

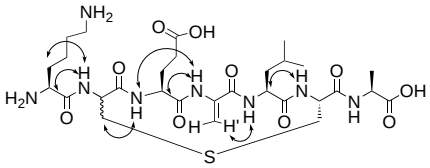


Figure 7. The major product H-Lys^1-Lan^2-Glu^3-Dha^4-Leu^5-Lan^6-Ala^7-OH formed as a result of cyclisation of peptide **3** showing the regiochemistry of the isomer formed as deduced from the depicted NOEs.

Cyclisation could have resulted in the formation of lanthionines at either residue 2 or 4. A series of NOEs were recorded which indicated that Michael addition had occurred at position 2. Considering first the NOEs associated with Lan^2, there was a strong correlation between Lan^2-NH and Lys^1-H$_\alpha$ and a medium NOE observed between Lan^2-NH and Lys-H$_\beta$. A medium NOE was observed between Lan^2-H$_\beta$ and the Glu^3-NH supporting the hypothesis that cyclisation had occurred at position 2. The NOEs related to the dehydroalanine residue further validated the regiochemical assignment. Strong correlations were found between Dha-NH and both Glu-H$_\alpha$ and Glu-NH. These NOEs again can only arise from a Dha at position 4. A strong NOE was observed between Dha-H$_\beta$ and Leu-NH, confirming that dehydroalanine was at position 4 in the peptide. In summary, the presence of several NOEs including those between Lan^2-NH and Lys^1-H$_\alpha$ and Dha^4-H$_{\beta'}$ and Leu^5-NH allowed the regiochemical assignment of the major product of cyclisation.

Regiochemistry of the minor product

The minor product was also analysed by a NOESY experiment. The NOE observed between the Lan-NH and Leu-H$_\alpha$ again facilitated the full assignment of the lanthionine protons. A larger number of NOEs were observed for this peptide than for the major isomer, including one between the Lan^2-H$_\beta$ and Lan^6-NH which indicated that a cyclic peptide had been formed. Investigation of the NOEs associated with Lan^2-NH showed a strong correlation with Lys-H$_\alpha$ and a medium correlation with Lys-H$_\beta$ as a result of cyclisation at position 2. The regiochemical assignment was confirmed by the NOEs which arose from the dehydroalanine residue. Again strong NOEs were observed between Dha^4-NH and both Glu-H$_\alpha$ and Glu-NH, indicating that cyclisation had occurred at position 2. Strong correlations were found between the Leu^5-NH and both the Dha^4-H$_\beta$ verifying that Dha was at position 4 in the peptide. The evidence described above supports the conclusion that cyclisation had occurred at position 2. The A-ring cyclisation was found to occur regiospecifically in the absence of enzymes. The results imply that the peptide conformation is sufficient to induce Michael addition only at position 2 since no cyclisation onto position 4 was detected. Interestingly, whilst the cyclisation appears to be regioselective in the absence of catalysis both possible stereoisomers at position 2 were obtained. However, there was some degree of stereospecificity since the isomers were produced in a 3:1 ratio.

Molecular modelling—E-ring

Unfortunately it was not possible to assign the stereochemistry of the newly generated alpha centre using the recorded NOEs directly. The correlations observed were very similar for both peptides and hence an unambiguous assignment could not be made.

In order to determine the absolute stereochemistry at residue 2, molecular modelling studies were undertaken. Both D,L-lanthionine and L,L-lanthionine were modelled and it was hoped that assignment would be possible by comparison of the experimentally observed NOEs with the calculated interproton distances. The A-ring was constructed with both

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000 0947-6539/00/0608-1460 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 8

a D and an L centre at residue 2. The lowest energy conformation of the rings was obtained by a two step procedure involving potential energy minimisation followed by molecular dynamics simulated annealing. The latter process constituted heating the molecules to a very high temperature (600 K) and then slow cooling over 1000 ps, thus furnishing them with sufficient energy to overcome local maxima and resulting in a conformation situated at an energy minimum. This was carried out using MacroModel 5.0[29] and was repeated three times to ensure that a true low energy conformer had been found. The OPLS force field was used throughout this work together with the GB/SA solvent model for water. For the L isomer, each anneal resulted in the generation of the same structure indicating a definite energy minimum that presumably is the global energy minimum. However, from the three anneals for the D isomer three different structures were obtained. The conformations resulting from the second (II) and third (III) anneal were both utilised in the subsequent simulations since both may represent the lowest energy conformation. A molecular dynamics simulation was then performed at 300 K so as to reproduce the molecular environment present in the NMR experiments. The simulation was run for 5000 ps with distance measurements recorded every 1 ps. From this data set the useful interatomic distances were selected, that is those where the proton–proton separation differed by >0.5 Å between the two isomers. These values were averaged to produce an effective interproton distance in Angstroms using Equation (1).

$$r_{av} = \langle r_{ij}^{-6} \rangle^{-1/6} \quad (1)$$

r_{av} is the final average simulation distance for atom pairs i, j, r_{ij} is the instantaneous value of this distance and indicates that an average is calculated over the course of the molecular dynamics trajectory (Table 1).[30]

Table 1. Average interproton distances from molecular modelling studies. Only the results which gave a predicted difference of over 0.5 Å between the D and L forms and where a difference in NOEs is observed are listed.[a]

		L Isomer	D Isomer (II)	D Isomer (III)	Major product	Minor product
Lan-H_β	Glu-NH	4.1	3.5	3.4	m	s
Glu-NH	Glu-H_α	2.2	2.9	2.9	w	s
Glu-NH	Dha-NH	2.4	1.8	1.9	–	m
Glu-H_α	Glu-H_γ	2.9	3.9	3.9	m	–
Glu-H_γ	Dha-NH	5.2	2.7	2.6	w	–
Dha-NH	Leu-NH	2.4	1.7	1.7	–	m
Leu-H_α	Lan-NH	3.6	3.0	2.8	s	m

[a] NOEs are given as strong (s), medium (m) or weak (w).

The intensity of an NOE is affected not only by proton separation but also by a number of other factors, including the timescale of the relative motions of the atoms involved. Therefore the assignment of strong, medium or weak to an observed NOE contact cannot simply be made by comparison with the above distance criteria. For example, the molecular modelling indicated that the separation between Glu-H_γ and Dha-NH is 5.2 Å in the L isomer and 2.7/2.6 Å in the D isomer. A weak NOE was observed for the major product and no NOE for the minor compound. However, assignment of the weak NOE to the L isomer, since it falls around the upper distance range, cannot be made from this information. It follows that conclusions regarding which isomer is the natural one (D isomer) can only be drawn from examples where an NOE was observed for each compound. In the case of the Lan[2]-H_β and Glu-NH there was a significant difference in the interatomic distance (4.1 Å in the L isomer cf. 3.5/3.34 Å in the D isomer) and a difference in the NOEs observed (medium for the major product and strong for the minor one). However, an assignment of the major product as the L isomer would be incorrect since neither interproton distance falls within the boundary for a strong NOE. The NOEs also observed between the Glu-H_α and Glu-NH are weak in the major product and strong in the minor compound. The proton–proton separation was found by modelling to be 2.2 Å for the L isomer and 2.9/2.9 Å for the D isomer. This implies that the major product has D stereochemistry. Further evidence to support this can be gained from the Leu-H_α and Lan-NH example. Here the interproton distance is 3.6 Å for the L isomer and 3.0/2.8 Å for the D isomer and a strong and a medium NOE observed in the major and minor products, respectively, again suggesting that the major product is the D isomer. Fewer NOEs were also observed for the major product further supporting the tentative assignment of the major product of cyclisation as the D isomer. The simulated annealing studies of the D isomer failed to produce a single lowest energy conformer. If the molecule does not have a single preferred lowest energy conformer and is constantly changing conformation then the number of NOEs observed would diminish. However, this cannot be used as proof of the stereochemistry at residue 2. Therefore, whilst the data obtained suggests that a D centre is present at residue 2 in the major product of cyclisation, it is not conclusive and cannot be used to generate a definite assignment of absolute stereochemistry.

Conclusion

Three biomimetic cyclisations have been studied based on the biosynthesis of the A-, B- and E-rings of subtilin. The required dehydroamino acid and cysteine containing peptides were synthesised by using both solid-phase and solution methodology. All cyclisations were found to be rapid and quantitative. In the case of the B- and E-rings single stereoisomers were produced. Although the stereochemistry was not determined beyond doubt the lanthionines in both of these cases are probably *meso* in accord with natures precedent. Numerous desulfurisation trials with Raney nickel were unsuccessful with the small amounts of compound available to us, nor was Edman sequencing in comparison with modified nisin conclusive. In the case of the A-ring two products were formed, in a 3:1 ratio. These were found to be cyclic with Michael addition having occurred regioselectively but not stereoselectively onto Dha[2], resulting in the isolation of the natural regioisomer of the subtilin A-ring. No addition onto the other dehydroamino acid could be detected. Unfortunately the assignment of absolute stereochemistry at this point

was not possible either by NOE studies, by desulfurisation or comparison with a D,L-lanthionine residue obtained from nisin. Molecular modelling however in conjunction with the NOE work indicated that the stereochemistry of the major product corresponded to the natural D,L-lanthionine. In peptides containing both cysteine and lysine residues the cysteine residue was not unexpectedly the only residue to cyclise. It is fascinating to begin to understand how these linear peptides contain sufficient information to direct both regio- and stereospecific ring closures. To further these aims linear peptides containing the precursors to the combined ABC rings are now being prepared.

Experimental Section

General information: NMR spectra were recorded on Bruker AC300 or Bruker DRX 500 spectrometers. ESI mass spectra were recorded by using a VG platform quadrupole electrospray ionisation mass spectrometer. High resolution accurate mass measurements were carried out at 10000 resolution by using mixtures of polyethylene glycols and/or polyethylene glycol methyl ethers as mass calibrants for FAB. Infrared spectra were recorded on a BioRad Golden Gate FTS 135. All samples were run as neat solids or oils. UV/Vis spectra were measured on a Hewlett Packard 8452A diode array spectrophotometer. RP-HPLC was carried out with a Hewlett Packard HP1100 Chemstation. 1) column: C_{18} ODS, i.d. 150 mm × 3 mm (0.5 mL min^{-1}), gradient: 0.1 % TFA/H_2O to 0.04 % TFA/MeCN over 20 min; 2) column: C_8 Techsphere, i.d. 250 mm × 4.6 mm (1 mL min^{-1}), gradient: 0.1 % TFA/H_2O to 0.04 % TFA/MeCN over 20 min; 3) column: C_{18} ODS, i.d. 250 mm × 10 mm (2.5 mL min^{-1}), gradient : 0.1 % TFA/H_2O to 0.04 % TFA/MeCN over 45 min.

Abbreviations: DABCO: 1,4-diazabicyclo[2.2.2]octane, DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, DCC: dicyclohexyl carbodiimide, DEAD: diethyl azodicarboxylate, DIC: diisopropyl carbodiimide, DMAP: 4-dimethylaminopyridine, DMF: *N*,*N*-dimethyl formamide, EDC: dimethylaminopropylethyl carbodiimide hydrochloride, HOBt: 1-hydroxybenzotriazole, NEM: *N*-ethyl morpholine, NMM: *N*-methyl morpholine, TIS: triisopropyl silane; resin: HMPB: 4-hydroxymethyl-3-methoxyphenoxybutyric acid.

General resin handling procedures (all reactions were performed manually): Manipulations using resin were carried out with 10 mL of solvent per gram of resin.

Solid-phase peptide synthesis:[23, 24]

i) Couplings: The resin was swollen in a minimum amount of CH_2Cl_2 for 30 min. *N*-Fmoc amino acid (2 equiv) and HOBt (2 equiv) were dissolved in CH_2Cl_2 with a few drops of DMF and stirred at room temperature for 10 min. DIC (2.2 equiv) was added and the mixture stirred for a further 10 min before addition to the resin. The resin was agitated at room temperature for 2 h to effect coupling. The resin was washed with DMF (3 ×), CH_2Cl_2 (3 ×), MeOH (3 ×) and Et_2O (2 ×). The resin was dried under vacuum for 30 min. For ester formation DMAP (0.3 equiv) was included in the coupling step.

ii) Fmoc removal: The resin was treated with 20 % piperidine in DMF with sequential treatments of 5 and 15 min. The resin was then filtered and washed with DMF (3 ×), CH_2Cl_2 (3 ×), MeOH (3 ×) and Et_2O (2 ×) and dried under vacuum.

iii) Cleavage: The resin (100 mg) was swollen in a minimum amount of CH_2Cl_2 (0.3 mL). TFA (9.5 mL)/TIS (0.2 mL) was added and the resin agitated at room temperature for 90 min. The resin was removed by filtration through a glass wool plug, washed with TFA, the filtrate concentrated to ca. 1 mL and Et_2O (25 mL) added. The resulting precipitate was collected by centrifugation and washed with Et_2O (4 × 25 mL).

Boc-Leu-Cys(Me)-Pro-OMe (14): TFA · H-Cys(Me)-Pro-OMe (2.0 g, 6 mmol, 1.0 equiv), Boc-Leu-OH (1.4 g, 1.0 equiv), HOBt (0.92 g, 1.0 equiv) and NMM (3.0 g, 3.3 mL, 5 equiv) were dissolved in CH_2Cl_2 and the mixture cooled on an ice bath whilst DCC (1.36 g, 1.1 equiv) was added portionwise. Stirring was continued on ice for 1 h and at room temperature for 16 h. The reaction mixture was then heated to 40 °C for 2.5 h. The reaction mixture was cooled, filtered and concentrated in vacuo. The resulting oil was dissolved in EtOAc (100 mL) and washed with sat. $NaHCO_3$ (100 mL), 10 % citric acid (100 mL), sat. $NaHCO_3$ (100 mL), water (100 mL) and brine (100 mL). The organic phases were dried ($MgSO_4$) and concentrated in vacuo to give an orange oil. Purification by column chromatography on silica gel (eluting with 1:1 EtOAc/hexane) yielded the title compound as a colourless foam (2.1 g, 76 % yield). R_f = 0.27 (EtOAc/hexane 1:1); IR: $\tilde{\nu}_{max}$ = 3286 (m), 2957 (m), 2369 (w), 1745 (m, ester, C=O), 1712 (s, urethane, C=O), 1638 (s, amide, C=O), 1514 (m), 1438 (m), 1366 (w), 1248 (w), 1212 (s), 1171 (s), 1041 (m), 1016 cm^{-1} (m); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.92 (2 d, 6 H, J = 6, 6 Hz; Leu-6H_δ), 1.45 (s, 9 H; C(CH_3)$_3$), 1.65 – 1.69 (m, 2 H; Leu-2H_β), 1.96 – 2.02 (m, 4 H; Pro-2H_β+Pro-2H_γ), 2.15 (s, 3 H; Cys-CH_3), 2.15 – 2.18 (m, 1 H; Leu-H_γ), 2.72 (*AB*X, 1 H, J = 14, 7 Hz; Cys-H_β), 2.94 (*AB*X, 1 H, J = 14, 6 Hz; Cys-H_β), 3.73 – 3.79 (s+m, 4 H; CO_2CH_3+Pro-H_δ), 4.10 – 4.14 (m, 1 H; Pro-H_δ), 4.49 (dd, 1 H, J = 7, 6 Hz; Cys-H_α), 4.93 – 4.99 (m+dd, 2 H, J = 6, 8 Hz; Leu-H_α+Pro-H_α), 6.82 (d, 1 H, J = 9 Hz; CON*H*), 6.99 (d, 1 H, J = 8 Hz; CON*H*); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.5 (Cys-CH_3), 23.1 (Leu-C_δ), 24.8 (Leu-C_γ), 25.0 (Pro-C_γ), 28.4 (C(CH_3)$_3$), 29.2 (Pro-C_β), 36.7 (Cys-C_β), 41.6 (Leu-C_β), 47.3 (Pro-C_δ), 49.8 (C_α), 52.4 (C_α), 52.3 (C_α), 59.1 (CO_2CH_3), 80.2 (*C*(CH$_3$)$_3$), 155.7 (O*C*ONH), 169.5 (*C*ONH), 172.3 (*C*ONH), 172.6 (CO_2CH_3); ES-MS: m/z: 460.3 [M+H]$^+$; HR-MS: [M+H]$^+$: $C_{21}H_{38}N_3O_6S$ calcd 460.2481, found 460.2492; HPLC 1) (λ_{220}): 18.0 min.

Boc-Leu-Cys((O)Me)-Pro-OMe (15): Sodium metaperiodate (1.05 g, 1.1 equiv) was dissolved in water (20 mL) and cooled to 0 °C. Boc-Leu-Cys(Me)-Pro-OMe (2.1 g, 4.6 mmol, 1.0 equiv) was dissolved in dioxane (40 mL) and added dropwise to the oxidant. Stirring was continued for 1 h. The reaction mixture was concentrated to remove organic solvent and the product extracted with CH_2Cl_2 (2 × 100 mL). The combined organic phases were washed with water (2 × 150 mL), brine (150 mL) and dried ($MgSO_4$). Concentration of the organic phases in vacuo yielded both diastereoisomers of the title compound as a colourless glass (2.15 g, 99 % yield). R_f = 0.38 (MeOH/EtOAc 1:9); IR: $\tilde{\nu}_{max}$ = 3281 (w), 2958 (w), 1743 (m, ester, C=O), 1705 (s, urethane, C=O), 1642 (s, amide, C=O), 1515 (m), 1437 (m), 1365 (w), 1247 (w), 1165 (s), 1042 (m), 1017 cm^{-1} (m); HPLC (λ_{220}): 12.3+12.5 min; (gradient 1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.89 – 0.94 (m, 6 H; Leu-6H_δ), 1.45 (s, 11 H; C(CH_3)$_3$+Leu-2H_β), 1.64 – 1.67 (m, 1 H; Leu-H_γ), 1.96 – 2.03 (m, 3 H; Pro-H_β+Pro-2H_γ), 2.20 – 2.24 (m, 1 H; Pro-H_β), 2.65+2.75 (2 s, 3 H; Cys-(O)CH_3), 2.96 – 3.11 (m, 2 H; Cys-H_β), 3.75 – 3.82 (s+m, 5 H; CO_2CH_3+Pro-2H_δ), 4.08 – 4.10 (m, 1 H; H_α), 4.48 – 4.52 (m, 1 H; H_α), 5.01 – 5.08 (m, 2 H; H_α+CON*H*), 7.36 (bd, 1 H, J = 8 Hz; CON*H*); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 21.8 (Leu-C_γ), 23.2+24.7 (Leu-C_δ), 24.9 (Pro-C_γ), 28.3 (C(CH_3)$_3$), 29.1 (Pro-C_β), 39.3+39.4 (Cys-(O)CH_3), 41.3 (Leu-C_β), 46.3 (Pro-C_δ), 52.5 (C_α), 53.1 (C_α) 53.4 (C_α), 57.9 (CO_2CH_3), 59.1+59.2 (Cys-C_β), 80.0 (*C*(CH$_3$)$_3$), 155.8 (O*C*ONH), 168.2 (*C*ONH), 172.3 (*C*ONH), 172.8 (CO_2CH_3); ES-MS: m/z: 498.4 [M+Na]$^+$; HR-MS [M+H]$^+$: $C_{21}H_{38}N_3O_7S$ calcd 476.2430, found 476.2445; HPLC 1) (λ_{220}): 12.2 and 12.4 min.

Boc-Leu-Dha-Pro-OMe (16): Boc-Leu-Cys((O)Me)-Pro-OMe (1.45 g, 3.1 mmol, 1.0 equiv) was dissolved in MeOH (15 mL), DBU (1.11 g, 1.10 mL, 2 equiv) added and the reaction mixture stirred at room temperature for 1.5 h. The reaction mixture was evaporated to dryness. The resulting glass was purified by column chromatography on silica gel (eluting with EtOAc/hexane 1:1) yielding the title compound as a colourless glass (1.22 g, 96 % yield). R_f = 0.58 (EtOAc); IR: $\tilde{\nu}_{max}$ = 3285 (w), 1957 (w), 2873 (w), 1744 (m, ester, C=O), 1679 (m, urethane, C=O), 1625 (s, amide, C=O), 1507 (m, alkene, C=C), 1365 (w), 1281 (w), 1163 (s), 1046 (w), 1021 cm^{-1} (w); ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 0.86 (2 d, 6 H, J = 6, 6 Hz; Leu-6H_δ), 1.45 – 1.52 (s+m, 11 H; C(CH_3)$_3$+Leu-2H_β), 1.60 – 1.64 (m, 1 H; Leu-H_γ), 1.95 – 2.01(m, 3 H; Pro-H_β+Pro-2H_γ), 2.20 – 2.23 (m, 1 H; Pro-H_β), 3.35 (s, 3 H; CO_2CH_3), 3.52 – 3.65 (m, 2 H; Pro-2H_δ), 4.02 (d, 1 H, J = 9 Hz; H_α), 4.29 (dd, 1 H, J = 5, 3 Hz; H_α), 4.80 (s, 1 H; Dha-H_β), 5.47 (s, 1 H; Dha-H_β), 7.04 (d, 1 H, J = 8 Hz; Leu-CON*H*), 9.77 (s, 1 H; Dha-CON*H*); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): δ = 21.3+22.9 (Leu-C_δ), 24.2 (Leu-C_γ), 24.7 (Pro-C_γ), 28.1 (C(CH_3)$_3$), 28.8 (Pro-C_β), 40.2 (Leu-C_β), 48.9 (Pro-C_δ), 51.8 (C_α), 52.7 (C_α), 58.4 (CO_2CH_3), 78.2 (*C*(CH$_3$)$_3$), 102.6 (Dha-C_β), 137.7 (Dha-C_α), 155.5 (O*C*ONH), 164.9 (*C*ONH), 171.9 (*C*ONH), 172.3 (CO_2CH_3); ES-MS: m/z: 412.4 [M+H]$^+$, 434.4 [M+Na]$^+$, 450.3 [M+K]$^+$; HR-MS [M+H]$^+$: $C_{20}H_{34}N_3O_6$ calcd 412.2448, found 412.2463; HPLC 1) (λ_{220}): 14.6 min.

© WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000
0947-6539/00/0608-1462 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 8

Boc-Leu-Dha-Pro-OMe (16): Boc-Leu-Cys((O)Me)-Pro-OMe (250 mg, 0.52 mmol) was dissolved in dioxane (5 mL) and the reaction mixture was refluxed. After the solution was heated under reflux for 18 hours, concentration of the reaction mixture yielded an orange oil which was purified by column chromatography on silica gel (eluting with EtOAc/hexane 1:1) giving the title compound as a colourless glass (210 mg, 88% yield).

Boc-Leu-Dha-Pro-OH (7):[12] Boc-Leu-Dha-Pro-OMe (750 mg, 1.8 mmol) was dissolved in MeOH (15 mL) and cooled on an ice bath whilst 1M NaOH (10 mL) was added dropwise. The reaction was allowed to warm to room temperature over 1.5 h. The reaction mixture was concentrated to ca. 15 mL, acidified to pH 4 with 2M $KHSO_4$ and the product extracted into CH_2Cl_2 (2×30 mL). The combined organics were washed with water (50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated in vacuo yielding the desired material as a colourless glass (680 mg, 95% yield). $R_f=0.26$ (EtOAc); IR: $\tilde{\nu}_{max}=3283$ (w), 2959 (w), 2933 (w), 1674 (s, urethane, C=O), 1643 (s, amide, C=O), 1619 (s, acid, C=O), 1515 (m), 1448 (m), 1367 (w), 1283 (w), 1162 (s), 1047 cm^{-1} (w); ^1H NMR (300 MHz, [D$_6$]DMSO): $\delta=$ 0.87 (2d, 6H, $J=6,6$ Hz; Leu-6H_δ), 1.45–1.51 (s+m, 11H; C(CH_3)$_3$)+Leu-2H_β), 1.78–1.89 (m, 4H; Pro-2H_γ+Pro-H_β+Leu-CH_γ), 2.20 (m, 1H; Pro-H_β), 3.32–3.45 (m, 2H; Pro-2H_δ), 4.04 (bdd, 1H, $J=9,5$ Hz; H_α), 4.23 (dd, 1H, $J=5,6$ Hz; H_α), 4.80 (s, 1H; Dha-H_β), 5.48 (s, 1H; Dha-H_β), 7.03 (d, 1H, $J=8$ Hz; Leu-CONH), 9.65 (s, 1H; Dha-CONH); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): $\delta=$21.4+23.1 (Leu-C_δ), 24.5 (Leu-C_γ), 28.2 (C(CH_3)$_3$), 29.0 (Pro-C_γ), 33.4 (Pro-C_β), 40.4 (Leu-C_β), 49.0 (Pro-C_δ), 52.8 (C_α), 58.6 (C_α), 102.4 (Dha-C_β), 138.0 (Dha-C_α), 155.5 (O*C*ONH), 164.8 (*C*ONH), 171.9 (*C*ONH), 173.2 (*C*O$_2$H); ES-MS: m/z: 342.2 [($M-t$Bu)+H]$^+$, 398.3 [M+H]$^+$.

Boc-Leu-Dha-Pro-Gly-Cys(Trt)-Val-Gly-Wang linker-resin (11): H-Gly-Cys(Trt)-Val-Gly-Wang-resin (**9**) (100 mg, 0.024 mmol, 1.0 equiv) was swollen in CH_2Cl_2 for 30 min. Boc-Leu-Dha-Pro-OH (**7**) (40 mg, 0.096 mmol, 4 equiv), PyBroP (50 mg, 4.4 equiv) and DMAP (7 mg, 2.4 equiv) were dissolved in a minimum amount of CH_2Cl_2 (ca. 1 mL) and DIPEA (12 mg, 17 μL, 4 equiv) added. This solution was added to the resin and the reaction mixture shaken at room temperature for 1.5 h. A ninhydrin test showed that the coupling was incomplete, therefore the coupling was repeated using Boc-Leu-Dha-Pro-OH (20 mg, 2 equiv), PyBroP (25 mg, 2.2 equiv), DMAP (3.5 mg, 1.2 equiv) and DIPEA (6 mg, 8 μL, 2 equiv). After the mixture had been shaken for 30 min at room temperature, a negative ninhydrin test was obtained. The resin was washed with DMF (3×5 mL), CH_2Cl_2 (3×5 mL), MeOH (3×5 mL), Et_2O (2×5 mL) and dried under high vacuum for 2 h.

TFA·H-Leu-Dha-Pro-Gly-Cys-Val-Gly-OH (1): Boc-Leu-Dha-Pro-Gly-Cys(Trt)-Val-Gly-HMPB-resin (**11**) (100 mg) was treated with 95% TFA/CH_2Cl_2 as described above. The resulting peptide was obtained as a white powder (18 mg, 75% yield). ^1H NMR (360 MHz, D_2O): $\delta=0.99$ (2d, 12H, $J=8,7$ Hz; Leu-6H_δ+Val-6H_γ), 1.75–1.83 (m+d, 3H, $J=7$ Hz; Pro-2H_γ+Leu-H_γ), 2.00–2.12 (m, 3H, Leu-2H_β+Pro-H_β), 2.18 (dq, 1H, $J=7,7$ Hz; Pro-H_β), 2.44 (dd, 1H, $J=8,7$ Hz; Val-H_β), 2.96 (t, 2H, $J=6$ Hz; Cys-2H_β), 3.70–3.74 (m, 1H, Pro-H_δ), 3.85–3.88 (m, 1H, Pro-H_δ), 4.02+4.04 (2s, 4H, 2Gly-2H_α), 4.13 (t, 1H, $J=8$ Hz; Leu-H_α), 4.24 (d, 1H, $J=7$ Hz; Val-H_α), 4.52 (t, 1H, $J=7$ Hz; Pro-H_α), 4.60 (dd, 1H, $J=6,5$ Hz; Cys-H_α), 5.38 (d, 1H, $J=2$ Hz; Dha-H_β), 5.52 (d, 1H, $J=2$ Hz; Dha-H_β); ^{13}C NMR (90 MHz, D_2O): $\delta=$18.5+19.3 (Val-C_γ), 21.8+22.7 (Leu-C_δ), 24.8 (Pro-C_γ), 25.7 (Leu-C_γ), 26.2 (Val-C_β), 30.5 (Pro-C_β), 31.1 (Cys-C_β), 40.8 (Leu-C_β), 42.1+43.5 (2Gly-C_α), 51.5 (Pro-C_δ), 52.7 (C_α), 56.8 (C_α), 60.5 (C_α), 62.0 (C_α), 110.1 (Dha-C_β), 136.1 (Dha-C_α), 167.8 (*C*ONH), 169.8 (*C*ONH), 172.4 (*C*ONH), 172.8 (*C*ONH), 174.0 (*C*ONH), 174.5 (*C*ONH), 175.8 (*C*O$_2$H); ES-MS: m/z: 614.3 [M+H]$^+$, 636.4 [M+Na]$^+$; HR-MS [M+H]$^+$: $C_{26}H_{44}N_7O_8S$ calcd 614.2972, found 614.2992; HPLC 1) (λ_{220}): 7.6 min.

Boc-Leu-Lan-Pro-Gly-Lan-Val-Gly-Wang linker-resin (4a): Boc-Leu-Dha-Pro-Gly-Cys(Trt)-Val-Gly-Wang-resin (95 mg, 0.023 mmol, 1.0 equiv) was shaken in 2%TFA/2%TIS/96% CH_2Cl_2 (3 mL) for 15 min at room temperature, filtered and the process repeated four more times. After five 15 min exposures the resin was washed with CH_2Cl_2 (2×5 mL), DMF (2×5 mL), CH_2Cl_2 (2×5 mL), MeOH (2×5 mL), Et_2O (2×5 mL) and dried. The resin (95 mg, 0.023 mmol, 1.0 equiv) was swollen in DMF (1 mL). 5%NMM in DMF (5 mL) was added and the mixture shaken at room temperature for 3 h. The resin was washed with DMF (3×5 mL), CH_2Cl_2 (3×5 mL), MeOH (3×5 mL), Et_2O (2×5 mL) and dried. Boc-Leu-Lan-Pro-Gly-Lan-Val-Gly-Wang-resin (50 mg, 0.25 mmol per g resin) was treated with 95% TFA as desribed above. The title compound was precipitated with Et_2O and isolated as a white powder (6 mg, 75% yield); RP-HPLC (3): 9.4 min; data as below.

H-Leu-Lan-Pro-Gly-Lan-Val-Gly-OH (4) B-ring: H-Leu-Dha-Pro-Gly-Cys-Val-Gly-OH (10 mg, 0.016 mmol) was dissolved in water (9 mL) and triethylammonium acetate buffer (5mM, pH 8, 1 mL) added. Aliquots were removed from the reaction every 30 s for 10 min and then every minute for a further 5 min. Analysis by RP-HPLC 2) (C_8 Techsphere column) showed the reaction to be complete after 10 min. The reaction mixture was lyophilised yielding the pure material (10 mg, 100% yield); ^1H NMR (500 MHz, [D$_6$]DMSO): $\delta=0.68-0.85$ (m, 12H, Leu-6H_δ+Val-6H_γ), 1.25–1.35 (m, 2H, Leu-2H_β), 1.65–1.90 (m, 1H, Leu-H_γ), 1.71–1.76 (m, 1H, Pro-H_γ), 1.95–2.04 (m, 3H, Pro-2H_β+Val-H_β), 2.23–2.27 (m, 1H, Pro-H_γ), 2.60 (ddd, 2H, $J=16,6,5$ Hz; Lan-2H_β), 2.86 (dd, 1H, $J=16,9$ Hz; Lan-H_β), 2.98 (dd, 1H, $J=16,9$ Hz; Lan-H_β), 3.33–3.48 (m, 2H; Pro-2H_δ), 3.65–3.75 (m, 4H; 2Gly-2H_α), 4.10 (2d, 2H, $J=8,7$ Hz; Leu-H_α+Val-H_α), 4.55–4.58 (m, 1H; Lan-H_α), 4.64–4.68 (m, 1H; Lan-H_α), 4.90 (dd, 1H, $J=$ 8, 4 Hz; Pro-H_α), 7.58 (s, 1H, $J=7$ Hz, Lan-NH), 7.86 (d, 1H, $J=8$ Hz, Val-NH), 8.23 (t, 1H, $J=6$ Hz; Gly-NH), 8.77 (t, 1H, $J=7$ Hz; Gly-NH), 8.98 (d, 1H, $J=8$ Hz; Lan-NH); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta=$ 18.5+19.3 (Leu-C_δ), 22.3+22.5 (Val-C_γ), 23.2 (Pro-C_γ), 24.9 (Pro-C_β), 31.4 (Leu-C_γ), 32.7 (Val-C_β), 35.8 (Leu-C_β), 40.9 (2Gly-C_α), 42.1 (Lan-C_β), 45.0 (Lan-C_β), 52.4 (Pro-C_δ), 52.8 (C_α), 53.8 (C_α), 60.3 (C_α), 61.4 (C_α), 64.4 (C_α), 171.2 (*C*ONH), 171.4 (*C*ONH), 172.0 (*C*ONH), 172.8 (*C*ONH), 174.0 (*C*ONH), 174.3 (*C*ONH), 175.8 (*C*O$_2$H); ES-MS: m/z: 614.2 [M+H]$^+$, 636.2 [M+Na]$^+$; HR-MS [M+H]$^+$: $C_{26}H_{44}N_7O_8S$ calcd 614.2972, found 614.3126; HPLC 1) (λ_{220}): 9.5 min.

H-Leu-[2-2H]Lan-Pro-Gly-Lan-Val-Gly-OH (4) B-ring: The above reaction was repeated with a solution of H-Leu-Dha-Pro-Gly-Cys-Val-Gly-OH (0.1 mg) in D_2O (900 μL). Deuterated phosphate buffer (100 μL, 5 mM, pD 8) was added and the reaction progress assessed (by UV) every 30 s for 10 min. The sample was then lyophilised, redissolved in water and lyophilisation repeated twice and the sample analysed by mass spectrometry. ES-MS: m/z: 615.3 [M+H]$^+$.

Boc-Leu-Dha-Ala-OH (8): Boc-Leu-Ser-OH (3 g, 9.4 mmol) was dissolved in freshly distilled THF (20 mL) together with Et_3N (0.95 g, 9.4 mmol) and cooled to $-15\,^oC$ for 15 min, under N_2. Isobutylchloroformate (1.28 g, 9.4 mmol) was added and the mixture stirred at $-15\,^oC$ for 30 min under N_2. To this mixture, HCl·H-Ala-OMe (1.45 g, 14.1 mmol) in water (18 mL) and Et_3N (1.42 g, 14.1 mmol) were added rapidly in one portion. The mixture was vigorously stirred at room temperature for 3 h. The reaction mixture was concentrated to approx. 20 mL and the product extracted with Et_2O (3×20 mL). The combined organic extracts were washed with water (50 mL), brine (50 mL), dried ($MgSO_4$), filtered and the solvent removed in vacuo to give the crude tripeptide. Purification by column chromatography (silica gel, hexane/EtOAc 80:20) gave the title compound as a white crystalline solid (1.65 g, 44% yield). $R_t=0.1$ (EtOAc/hexane 20:80); m.p.: 123–125 °C; IR: $\tilde{\nu}_{max}=3425$ (s, broad, urethane, amide, NH), 1744 (s, ester, C=O), 1654, 1508 cm^{-1} (s, urethane, amide, C=O); ^1H NMR (300 MHz, [D$_6$]DMSO): $\delta=0.97+1.00$ (2d, 6H, $J=6.5,6.5$ Hz; CH(CH_3)$_2$), 1.44 (d, 3H, $J=7$ Hz; CHCH_3), 1.49 (s, 9H; C(CH_3)$_3$), 1.55–1.62 (br m, 2H; CH_2CH(CH$_3$)$_2$), 1.69–1.83 (br m, 1H; CH$_2$CH(CH$_3$)$_2$), 3.76 (s, 3H; CO$_2$CH_3), 3.83 (dd, 2H, $J=6$ Hz; CH_2OH), 4.08–4.18 (m, 1H; CH_α), 4.43–4.53 (m, 2H; 2CH_α); ^{13}C NMR (75 MHz, DEPT analysis, [D$_6$]DMSO): $\delta=$17.5 (CHCH_3), 21.7+23.4 (CH(CH_3)$_2$), 25.8 (CH(CH$_3$)$_2$), 28.6 (C(CH_3)$_3$), 41.8 (CH_2(CH$_3$)$_2$), 49.5, 52.8, 54.7, 56.3 (CO$_2$CH$_3$)+(3CH_α), 63.0 (CH_2OH), 80.7 (*C*(CH$_3$)$_3$), 158.1 (O*C*ONH), 171.8, 174.4, 175.7 (2*C*ONH)+(*C*O$_2$CH$_3$); ES-MS: m/z: 829 [2M+Na]$^+$, 426 [M+Na]$^+$; HR-MS [M+H]$^+$ $C_{18}H_{34}O_7N_3$ calcd 404.2397, found 404.2412.

CuCl (0.08 g, 0.77 mmol) and EDC (0.48 g, 2.5 mmol) was added at room temperature to a stirred solution of Boc-Leu-Ser-Ala-OMe (1 g, 2.5 mmol) in dry MeCN (50 mL). The reaction was stirred for 3 h following which an equal volume of EtOAc was added and the precipitated urea removed by filtration. The organic product was washed with water (50 mL), brine (50 mL), dried over $MgSO_4$ and the solvent removed in vacuo. The title compound was obtained as a pale yellow oil after column chromatography (silica gel, hexane/EtOAc 7:3; 0.54 g, 57% yield). $R_f=0.18$ (hexane/EtOAc 7:3); IR: $\tilde{\nu}_{max}=3449$ (s, urethane, amide, NH), 1736 (s, ester, C=O), 1695 (s, urethane, C=O), 1663 cm^{-1} (urethane, amide, C=O); ^1H NMR (300 MHz, $CDCl_3$): $\delta=0.93+0.95$ (2d, 6H, $J=6.5,6.5$ Hz; CH(CH_3)$_2$), 1.44 (s, 9H; C(CH_3)$_3$), 1.37–1.56 (m, 4H; CHCH$_2$CH(CH$_3$)$_2$+CHCH_3), 1.57–1.69

(br m, 2H; CHCH_2CH(CH_3)$_2$), 3.78 (s, 3H; CO_2CH_3), 4.06 – 4.23 (br m, 1H; CH_α), 4.61 (dq, 1H, J = 7.5, 7.5 Hz, CH_α), 4.98 (d, 1H, J = 7.5 Hz, NH), 5.35+6.47 (2s, 2H; C=CH_2), 6.97 (br d, 1H, J = 9 Hz, NH), 8.61 (br s, 1H; NH); ^{13}C NMR (75 MHz, DEPT analysis, $CDCl_3$): δ = 18.23, 21.85, 23.14, 24.93 (CHCH_3)+(CH(CH_3)$_2$)+(CH(CH_3)$_2$), 28.40 (C(CH_3)$_3$), 41.59 (CH_2(CH_3)$_2$), 48.76, 52.82, 54.12 (CO_2CH_3)+(2 CH_α), 80.38 (C(CH_3)$_3$), 102.77 (C=CH_2), 133.80 (C=CH$_2$), 155.78 (OCONH), 163.42, 171.96, 173.37 (2 CONH)+(CO_2CH_3); ES-MS: m/z: 1178 [3M+Na]$^+$, 793 [2M+Na]$^+$, 408 [M+Na]$^+$; HR-MS [M+H]$^+$ $C_{18}H_{32}O_6N_3$ calcd 386.2291, found 386.2322.

Boc-Leu-Dha-L-Ala-OMe (0.39 g, 1.02 mmol) was dissolved in THF (2 mL), 2.5 M NaOH (50 mL) was added and the mixture stirred at room temperature for 2 h. The aqueous mixture was washed with EtOAc (100 mL) and subsequently acidified to pH 1 – 2 (2 M HCl). The precipitate was extracted with EtOAc (2 × 100 mL) and the combined organic extracts washed with water (100 mL), brine (100 mL), dried ($MgSO_4$) and the solvent removed in vacuo. The title compound was obtained as a colourless oil after purification by column chromatography (silica gel, hexane/EtOAc/AcOH 50:50:2; 0.31 g, 82 % yield). R_f = 0.26 (hexane/EtOAc/AcOH 50:50:2); IR: $\tilde{\nu}_{max}$ = 3329 (m, urethane, amide, NH), 1719 (s, acid, C=O), 1656 (s, urethane, C=O), 1630 (s, amide, C=O), 1508 cm^{-1} (s, urethane, amide, C=O); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.89+0.92 (2d, 6H, J = 6.5, 6.5 Hz, CH(CH_3)$_2$), 1.40 (s, 9H; C(CH_3)$_3$), 1.41 (d, 3H, J = 7 Hz; CHCH_3), 1.50 – 1.70 (2m, 3H; CH_2CH(CH_3)$_2$), 3.96 – 4.12 (br m, 1H; CH_α), 4.41 (d q, 1H, J = 7, 7.5 Hz; CH_α), 5.46+6.29 (2s, 2H; C=CH_2), 5.76 (br d, 1H, J = 9 Hz; NH), 7.29 (d, 1H, J = 7 Hz; NH), 8.69 (s, 1H; NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.8 (CHCH_3), 21.7+23.2 (CH(CH_3)$_2$), 24.9 (CH_2CH(CH_3)$_2$), 28.4 (C(CH_3)$_3$), 41.8 (CH_2CH(CH_3)$_2$), 48.9+53.9 (2 CH_α), 80.3 (C(CH_3)$_3$), 104.2 (C=CH_2), 133.8 (C=CH$_2$), 156.3 (OCONH), 163.9, 172.6, 175.8 (2 CONH)+(CO_2H); ES-MS: m/z: 765 [2M+Na]$^+$, 394 [M+Na]$^+$, 372 [M+H]$^+$; HR-MS [M+H]$^+$ $C_{17}H_{30}O_6N_3$ calcd 372.2135, found 372.2110.

Boc-Leu-Dha-Ala-Asn-Cys(Trt)-Lys(Boc)-Ile-HMPB-linker-resin (12): Boc-Leu-Dha-Ala-OH (**8**) was coupled to the H-Asn-Cys(Trt)-Lys(Boc)-Ile-HMPB-resin (**10**) (0.2 g, 0.038 mmol) as described above and the reaction followed by the ninhydrin test until negative. The peptide resin was then filtered, washed with DMF (1 × 15 mL), CH_2Cl_2 (4 × 5 mL) and MeOH (2 × 5 mL) and dried under vacuum. MALDI-TOF (DHB matrix, TFA cleavage in situ):m/z: 731.8 [M+H]$^+$ (H-Leu-Dha-Ala-Asn-Cys-Lys-Ile-OH).

H-Leu-Dha-Ala-Asn-Cys-Lys-Ile-OH (2): Boc-Leu-Dha-Ala-Asn-Cys(Trt)-Lys(Boc)-Ile-HMPB-resin (**12**) (0.21 g, 0.038 mmol) was shaken in 50 % TFA/CH_2Cl_2 containing 5 % TIS v/v for 10 min. The resin was filtered through a glass wool plug, the solvent was removed in vacuo and the residue redissolved in the minimum amount of methanol (0.5 mL). The peptide was precipitated into cold ether (10 mL) and isolated by centrifugation. The supernatant was discarded and the pellet resuspended in ether (10 mL), again pelleted by centrifugation and dried (N_2) (20.5 mg, 73 % yield). ES-MS: m/z: 730.7 [M+H]$^+$, 366.1 [M + 2H]$^+$; HR-MS [M+H]$^+$ $C_{31}H_{56}O_9N_9S$ calcd 730.3922, found 730.3888.

H-Leu-Lan-Ala-Asn-Lan-Lys-Ile-OH (E-ring) (5): H-Leu-Dha-Ala-Asn-Cys-Lys-Ile-OH (1 mg, 0.0014 mol) was cyclised as previously described. RP-HPLC 1): One major product observed, cyclic 7-mer (E-ring); ES-MS: m/z: 730.4 [M+H]$^+$, 366.1 [M + 2H]$^{2+}$ (cyclic 7-mer); HR-MS [M+H]$^+$ $C_{31}H_{56}O_9N_9S$ calcd 730.3922, found 730.3932; ^{1}H NMR (500 MHz, H_2O+one drop D_2O): δ = (residue, NH, αH, βH, others, $J_{NH,CH\alpha}$), Leu[1] 4.13, 1.73, 1.73 γH: 1.66 δCH$_3$: 0.95, 0.95; Lan[2] 9.49, 5.15, 3.00, 3.00, J = 8.5 Hz; Ala[3] 8.60, 4.25, 1.46, J < 2 Hz; Asn[4] 8.68, 5.06, 2.98, 2.62 γNH$_2$: 7.61, 6.93, J = 9.3 Hz; Lan[5] 7.50, 4.11, 3.62, 2.83, J = 5.3 Hz; Lys[6] 8.49, 4.42 1.88, 1.88 γCH$_2$: 1.45, 1.45 δCH$_2$: 1.82, 1.82, εCH$_2$: 3.04, 3.04, J = 7.5 Hz; Ile[7] 8.13, 4.27 1.94 γCH$_2$: 1.45, 1.25, γCH$_3$: 0.90, δCH$_3$: 0.87, J = 8.1 Hz.

H-Leu-Lan-Ala-Asn-Lan-Lys-Ile-OH—cyclisation in deuterated buffer: ES-MS: m/z: 731.4 [M+H]$^+$ (deuterated cyclic 7-mer).

Boc-Lys(Boc)-Cys(Me)-Glu-(O*t*Bu)-Cys(Me)-Leu-OMe (17): Boc-Lys-(Boc)-Cys(Me)-OH (2.3 g, 1.1 equiv), HOBt (882 mg, 1.2 equiv), EDC (1.03 g, 1.2 equiv) and NEM (1.04 g, 1.1 mL, 2 equiv) were dissolved in CH_2Cl_2 (150 mL). H-Glu(O*t*Bu)-Cys(Me)-Leu-OMe (2.0 g, 4.5 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (20 mL) and added to the reaction mixture. The reaction was stirred at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with sat. $NaHCO_3$ (250 mL), 5 % citric acid (250 mL), sat. $NaHCO_3$ (250 mL), water (250 mL) and brine (250 mL). The organics were dried ($MgSO_4$), filtered and the filtrate concentrated in vacuo to give a pale yellow foam. Purification by column chromatography on silica gel (eluting with EtOAc/hexane 1:1) produced the desired compound as a white solid (3.0 g, 75 % yield). R_f = 0.16 (EtOAc/hexane 1:1); IR: $\tilde{\nu}_{max}$ = 3271 (m), 2973 (w), 2932 (w), 1714 (m, ester, C=O), 1689 (m, urethane, C=O), 1634 (s, amide, C=O), 1515 (m), 1391 (w), 1365 (w), 1245 (m), 1154 (s); m.p.: 132 – 135 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.85 (2d, 6H, J = 6, 6 Hz; Leu-6H_δ), 1.25 – 1.30 (m, 2H; Lys-2H_β), 1.40 (s, 27H; 3C(CH_3)$_3$), 1.45 – 1.55 (m, 7H; Leu-H_γ+Leu-2H_β+Lys-2H_γ+Lys-2H_δ), 1.70 – 1.73 (m, 1H; Glu-H_β), 1.80 – 1.85 (m, 1H; Glu-H_β), 2.05 (2s, 6H; 2 Cys-CH_3), 2.15 (t, 2H, J = 8 Hz; Glu-2H_γ), 2.60 (*AB*X, 2H, J = 14, 7 Hz; 2 Cys-H_β), 2.75 (*AB*X, 2H, J = 14, 6 Hz; 2 Cys-H_β), 2.90 – 2.98 (m, 2H; Lys-2H_ε), 3.60 (s, 3H; CO_2CH_3), 3.90 – 3.96 (m, 1H; Lys-H_α), 4.25 – 4.30 (m, 2H; Leu-H_α+Glu-H_α), 4.50 (AB*X*, 2H, J = 7, 6 Hz; 2 Cys-H_α), 6.70 (br t, 1H, J = 7 Hz; OCONH), 6.90 (d, 1H, J = 7 Hz; Lys-CONH), 7.90 (d, 1H, J = 6 Hz; Cys-CONH), 8.05 (d, 1H, J = 7 Hz; Cys-CONH), 8.20 (d, 1H, J = 8 Hz; Glu-CONH), 8.40 (d, 1H, J = 7 Hz; Leu-CONH); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): δ = 15.8 (Cys-CH_3), 15.9 (Cys-CH_3), 21.6+21.8 (Leu-C$_\delta$), 23.1 (Lys-C$_\gamma$), 24.8 (Leu-C$_\gamma$), 28.2+28.6+28.7 (3C(CH_3)$_3$), 29.1 (Lys-C$_\beta$), 29.8 (Glu-C$_\beta$), 30.5 (Lys-C$_\delta$), 31.4 (Glu-C$_\gamma$), 38.8 (Cys-C$_\beta$), 40.9 (Cys-C$_\beta$), 48.0 (Lys-C$_\varepsilon$), 50.6 (C$_\alpha$), 51.8 (C$_\alpha$), 52.3 (C$_\alpha$), 52.6 (C$_\alpha$), 68.3 (CO_2CH$_3$), 77.7+78.3+80.8 (3C(CH$_3$)$_3$), 155.7 (2 OCONH), 169.4 (CONH), 170.1 (CONH), 170.7 (CONH), 171.4 (CONH), 171.7 (CO_2CH_3), 173.0 (CO_2*t*Bu); ES-MS: m/z: 893.4 [M+H]$^+$, 915.4 [M+Na]$^+$.

Boc-Lys(Boc)-Cys((O)Me)-Glu-(O*t*Bu)-Cys((O)Me)-Leu-OMe (20): Sodium metaperiodate (265 mg, 2.2 equiv) was dissolved in water (10 mL) and cooled on an ice bath. Boc-Lys(Boc)-Cys(Me)-Glu-(O*t*Bu)-Cys(Me)-Leu-OMe (500 mg, 0.56 mmol, 1.0 equiv) was dissolved in dioxane (20 mL) and added dropwise to the oxidant. The reaction was stirred on ice for 1 h and at 40 °C for 4 h. The reaction mixture was concentrated to ca. 10 mL, water (20 mL) added and the product extracted into CH_2Cl_2 (2 × 30 mL). The combined organic phases were washed with water (50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated in vacuo to give a colourless glass (510 mg, 99 % yield). R_f = 0.53 (10 % MeOH/CH_2Cl_2); IR: $\tilde{\nu}_{max}$ = 3286 (m), 2962 (w), 2933 (w), 1733 (m, ester, C=O), 1684 (m, urethane, C=O), 1638 (s, amide, C=O), 1514 (s), 1453 (w): 1366 (w), 1248 (w), 1158 (s), 1018 cm^{-1} (w); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.90 (d+m, 6H, J = 7 Hz; Leu-6H_δ), 1.17 – 1.28 (m, 2H, Lys-2H_γ), 1.42 (3s, 27H; 3C(CH_3)$_3$), 1.52 – 1.64 (m, 7H; Leu-H_γ+Leu-2H_β+Lys-2H_β+Lys-2H_δ), 1.84 – 1.93 (m, 2H; Glu-2H_β), 2.22 – 2.27 (m, 2H; Glu-2H_γ), 2.61 (2s, 6H; 2 Cys-(O)CH$_3$), 2.94 (dd, 2H, J = 8, 7 Hz; Lys-2H_ε), 3.14 – 3.35 (m, 4H; 2 Cys-2H_β), 3.62+3.67 (2s, 3H; CO_2CH_3), 3.98 – 4.03 (m, 1H; Lys-H_α), 4.33 – 4.46 (m, 2H; Leu-H_α+Glu-H_α), 4.71 – 4.78 (m, 2H; 2 Cys-H_α), 6.75 (br s, 1H; OCONH), 6.90 – 6.96(m, 1H; Lys-CONH), 8.21 – 8.27 (m, 3H; 3 CONH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 21.3+21.4 (Leu-C$_\delta$, 22.8 (Lys-C$_\gamma$), 24.2 (Leu-C$_\gamma$), 26.9 (Lys-C$_\beta$), 27.8+28.3+28.4 (3C(CH$_3$)$_3$), 29.3 (Glu-C$_\beta$), 30.9 (Lys-C$_\delta$), 31.4 (Glu-C$_\gamma$), 46.1+47.6 (2 Cys-(O)CH$_3$), 48.0 (Lys-C$_\varepsilon$), 50.6 (C$_\alpha$), 52.2 (C$_\alpha$), 52.4 (C$_\alpha$), 54.5 (C$_\alpha$), 55.0 (CO_2CH$_3$), 55.4+55.5 (2 Cys-C$_\beta$), 55.9 (C$_\alpha$), 77.4+78.3+79.7 (3C(CH$_3$)$_3$), 155.7 (2 OCONH), 169.4 (CONH), 169.9 (CONH), 170.0 (CONH), 170.9 (CONH), 171.7 (CO_2CH_3), 172.6 (CO_2-*t*Bu); ES-MS: m/z: 925.4 [M+H]$^+$, 947.4 [M+Na]$^+$; HR-MS: [M+H]$^+$ $C_{40}H_{73}N_6O_{14}S_2$ calcd 925.4626 found 925.4709.

Boc-Lys(Boc)-Dha-Glu(O*t*Bu)-Dha-Leu-OH (21): Boc-Lys(Boc)-Cys-((O)Me)-Glu(O*t*Bu)-Cys((O)Me)-Leu-OMe (475 mg, 0.51 mmol, 1.0 equiv) was dissolved in MeOH (22.5 mL) and cooled on an ice-salt bath. 1 M NaOH (103 mg, 2.5 mL, 5 equiv) was added dropwise and the reaction allowed to warm to room temperature over 1.5 h. The reaction mixture was concentrated in vacuo to ca. 3 mL and acidified to pH 4 with 2 M $KHSO_4$. The product was extracted into CH_2Cl_2 (3 × 40 mL) and washed with water (100 mL), brine (100 mL), dried ($MgSO_4$) and concentrated in vacuo to give a colourless foam (370 mg, 93 % yield). R_f = 0.29 (MeOH/EtOAc 1:9); IR: $\tilde{\nu}_{max}$ = 3302 (m), 2962 (w), 2934 (w), 1681 (s, urethane, C=O), 1652 (s, amide, C=O), 1630 (s, acid, C=O), 1504 (s), 1392 (w), 1367 (w), 1249 (w), 1161 (s), 1120 (w), 1020 cm^{-1} (w); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.91 (2d, 6H, J = 6, 6 Hz; Leu-6H_δ), 1.17 – 1.26 (m, 2H; Lys-2H_γ), 1.42 (br s, 27H; 3C(CH_3)$_3$), 1.52 – 1.64 (m, 7H; Leu-H_γ+Leu-2H_β+Lys-2H_β+Lys-2H_δ), 1.92 – 2.03 (2m, 2H; Glu-2H_β), 2.21 – 2.28 (m, 2H; Glu-2H_γ), 2.88 – 2.93 (m, 2H; Lys-2H_ε), 3.98 – 4.02 (m, 1H; Lys-H_α), 4.34 – 4.37 (m, 1H; Leu-H_α), 4.42 – 4.45 (m, 1H; Glu-H_α), 5.61 (2s, 2H; Dha-H_β), 6.20 (br s, 2H; Dha-H_β), 6.83 (br t, 1H, J = 6 Hz;

© WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000
0947-6539/00/0608-1464 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 8

OCONH), 7.31 (br d, 1H, $J=7$ Hz; Lys-CONH), 8.45 (br d, 1H, $J=7$ Hz; Glu-CONH), 8.62 (br d, 1H, $J=8$ Hz; Leu-CONH), 9.05+9.10 (2s, 2H; 2Dha-CONH); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): $\delta=21.3$ (Leu-C_δ), 23.0 (Lys-C_γ), 24.6 (Leu-C_γ), 26.5 (Lys-C_β), 27.9+28.3+28.4 (3C(CH_3)$_3$), 29.3 (Glu-C_β), 30.9 (Lys-C_δ), 31.7 (Glu-C_γ), 40.6 (Leu-C_β), 48.7 (Lys-C_ε), 51.1 (C_α), 53.8 (C_α), 55.5 (C_α), 77.5+78.6+79.9 (3C(CH_3)$_3$), 103.5 (2Dha-C_β), 134.6 (2Dha-C_α), 155.7 (OCONH), 163.8 (CONH), 164.3 (CONH), 170.5 (CONH), 171.7 (CONH), 171.9 (CO_2tBu), 173.8 (CO_2H); ES-MS: m/z: 683.3 [$(M-$Boc)+H]$^+$, 783.3 [M+H]$^+$; HR-MS: [M+H]$^+$ $C_{37}H_{63}N_6O_{12}$ calcd 783.4504, found 783.4559.

Boc-Lys(Boc)-Dha-Glu(O*t*Bu)-Dha-Leu-Cys(Trt)-Ala-O*t*Bu (22): Boc-Lys(Boc)-Dha-Glu(O*t*Bu)-Dha-Leu-OH (**21**) (370 mg, 0.47 mmol, 1.0 equiv) and H-Cys(Trt)-Ala-O*t*Bu (230 mg, 1.0 equiv) were dissolved in CH_2Cl_2 (15 mL) and cooled on an ice-salt bath under an inert atmosphere. HOBt (80 mg, 1.1 equiv) was added followed by EDC (110 mg, 1.2 equiv). The reaction was allowed to warm to room temperature over 3 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with sat. $NaHCO_3$ (50 mL), 10% citric acid (50 mL), sat. $NaHCO_3$ (50 mL), water (50 mL), brine (50 mL), dried ($MgSO_4$), and concentrated in vacuo yielding a yellow foam. Purification by column chromatography on silica gel (MeOH/CH_2Cl_2 3:97) followed by crystallisation with EtOAc/hexane gave the title compound as a colourless foam (423 mg, 72% yield). $R_f=0.22$ (EtOAc/hexane 1:2); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta=0.95$ (2d, 6H, $J=7$, 7 Hz; Leu-6H_δ), 1.30 (d, 3H, $J=7$ Hz; Ala-3H_β), 1.34–1.45 (m, 38H; 4C(CH_3)$_3$+Lys-2H_β), 1.60–1.75 (m, 7H; Leu-H_γ+Leu-2H_β+Lys-2H_γ+Lys-2H_δ), 1.98–2.10 (m, 2H; Glu-2H_β), 2.30–2.35 (m, 2H; Glu-2H_γ), 2.42 (*AB*X, 1H, $J=14$, 8 Hz; Cys-H_β), 2.53 (*AB*X, 1H, $J=14$, 7 Hz; Cys-H_β), 3.02–3.09 (m, 2H; Lys-2H_ε), 4.00–4.04 (m, 1H; Lys-H_α), 4.14 (dq, 1H, $J=7$, 7 Hz; Ala-H_α), 4.45–4.49 (dd+m, 3H, $J=8$, 7 Hz; Cys-H_α+Leu-H_α+Glu-H_α), 5.68 (s, 1H; Dha-H_β), 5.73 (s, 1H; Dha-H_β), 6.20 (s, 1H; Dha-H_β), 6.30 (s, 1H; Dha-H_β), 6.80 (br t, 1H, $J=7$ Hz; Lys$_\varepsilon$-OCONH), 7.32–7.45 (m, 15H; Trt-H), 8.15 (d, 1H, $J=9$ Hz; CONH), 8.17 (d, 1H, $J=7$ Hz; CONH), 8.53 (d, 1H, $J=8$ Hz; CONH), 8.68 (d, 1H, $J=7$ Hz; CONH), 9.70 (d, 1H, $J=7$ Hz; CONH), 9.18 (s, 1H; Dha-CONH), 9.25 (s, 1H; Dha-CONH); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta=18.1$ (Ala-C_β), 21.9+22.5 (Leu-C_δ), 23.0 (Lys-C_β), 24.8 (Leu-C_γ), 26.5 (Lys-C_γ), 27.3 (Glu-C_β), 27.9 (C(CH_3)$_3$), 28.1 (C(CH_3)$_3$), 28.3 (C(CH_3)$_3$), 28.5 (C(CH_3)$_3$), 29.7 (Lys-C_δ), 31.8 (Glu-C_γ), 33.3 (Cys-C_β), 41.1 (Leu-C_β), 49.0 (Lys-C_ε), 52.5 (C_α), 54.8 (C_α), 55.2 (C_α), 7.6 (C_α), 57.7 (C_α), 67.1 (C-Ph$_3$), 79.2 (C(CH_3)$_3$), 80.4 (C(CH_3)$_3$), 81.4 (C(CH_3)$_3$), 81.8 (C(CH_3)$_3$), 106.3+107.3 (Dha-C_β), 126.8 (*para*-C(Trt)), 128.0 (*ortho*-C(Trt)), 129.6 (*meta* C-(Trt)), 134.0 (Dha-C_α), 134.7 (Dha-C_α), 144.5 (*ipso*-C (Trt)), 156.2 (OCONH), 156.3 (OCONH), 163.8 (CONH), 164.4 (CONH), 169.1 (CONH), 170.4 (CONH), 171.5 (CONH), 171.7 (CONH), 173.1 (2CO_2tBu); ES-MS: m/z: 1255.6 [M+H]$^+$, 1277.6 [M+Na]$^+$.

H-Lys-Dha-Glu-Dha-Leu-Cys-Ala-OH (3): Boc-Lys(Boc)-Dha-Glu-(O*t*Bu)-Dha-Leu-Cys(Trt)-Ala-O*t*Bu (**22**) (250 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (9.6 mL) and TFA (10 mL) added to give a bright yellow solution. TIS (0.4 mL) was added dropwise, removing the yellow colour. The reaction was stirred at room temperature for 1 h. The reaction mixture was concentrated to ca. 1 mL and Et_2O (40 mL) added. The resulting precipitate was collected by centrifugation, washed with Et_2O (3 × 40 mL) and dried (135 mg, 96% yield). $R_f=0.25$ (CH_2Cl_2/MeOH/AcOH/H_2O 40:18:3:2); ^{1}H NMR (360 MHz, [D$_6$]DMSO): $\delta=0.94$ (2d, 6H, $J=6$, 6 Hz; Leu-6H_δ), 1.22 (d, 3H, $J=8$ Hz; Ala-3H_β), 1.33–1.75 (m, 9H; Leu-H_γ+Leu-2H_β+Lys-2H_β+Lys-2H_γ+Lys-2H_δ), 1.98–2.05 (m, 2H; Glu-2H_β), 2.40–2.65 (m, 2H; Glu-2H_γ), 2.83 (m, 3H; Lys-2H_ε+Cys-H_β), 2.90 (*AB*X, 1H, $J=14$, 6 Hz; Cys-H_β), 3.55–3.59 (m, 1H; H_α), 3.96 (s, 1H; H_α), 4.28 (t, 1H, $J=6$ Hz; H_α), 4.34 (dd, 1H, $J=8$, 4 Hz; H_α), 4.47 (dd, 1H, $J=6$, 4 Hz; H_α), 5.67 (s, 1H; Dha-H_β), 5.83 (s, 1H; Dha-H_β), 6.02 (s, 1H; Dha-H_β), 6.28 (s, 1H; Dha-H_β); ES-MS: m/z: 701.2 [M+H]$^+$; HR-MS [M+H]$^+$: $C_{29}H_{48}N_8O_{10}S$ calcd 701.3214, found 701.3480; HPLC 1) (λ_{220}): 8.6 min.

A-ring cyclisation of (3): H-Lys-Dha-Glu-Dha-Leu-Cys-Ala-OH (25 mg, 0.036 mmol) was dissolved in H_2O (22.5 mL) and pH 8.0 triethylammonium acetate buffer (50 mM, 2.5 mL) added. Small aliquots (100 μL) were removed every 30 s for 10 min then at 20 and 30 min, quenched with 2% TFA in water (20 μL). The reaction was complete within 10 min and was lyophilised. The peptides were purified by HPLC 3) (13 mg, 52% (major), 4 mg, 16% (minor)).

Major isomer H-Lys-Lan-Glu-Dha-Leu-Lan-Ala-OH (A-ring) (6a): ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta=0.84+0.88$ (2d, $J=6$, 6 Hz; Leu-6H_δ), 1.23 (d, $J=8$ Hz; Ala-3H_β), 1.30–1.39 (m, Lys-2H_γ), 1.48–1.53 (m, Lys-2H_δ), 1.60–1.64 (m, Leu-2H_β+Leu-H_γ), 1.67–1.70 (m, Lys-2H_β+Glu-H_β), 2.07–2.11 (m, Glu-H_β), 2.18–2.29 (m, Glu-2H_γ), 2.71–2.74 (m, Lys-2H_ε), 2.80 (dd, $J=15$, 6 Hz; Lan-H_β), 2.87 (d, $J=10$ Hz; Lan-H_β), 2.98 (dd, $J=10$, 6 Hz; Lan-H_β), 3.03 (dd, $J=15$, 5 Hz; Lan-H_β), 3.79–3.83 (m, Lys-H_α), 4.20 (dq; $J=8$, 7 Hz; Ala-H_α), 4.22–4.28 (m, Leu-H_α), 4.30–4.41 (m, Lan-H_α+Glu-H_α), 4.48–4.50 (m, Lan-H_α), 5.50 (s, Dha-H_β), 6.03 (s, Dha-H_β), 7.58–7.80 (br s, Lys-NH_3^+), 7.88 (d, $J=7$ Hz; Lan^2-NH), 8.05 (d, $J=8$ Hz; Ala-NH), 8.05–8.20 (br s, Lys-NH_3^+), 8.51 (s, Dha-NH), 8.74 (d, $J=6$ Hz; Lan-NH), 8.77 (d, $J=7$ Hz; Leu-NH), 8.50 (d, $J=8$ Hz; Glu-NH); ES-MS: m/z: 700.8 [M+H]$^+$, 723.3 [M+Na]$^+$; HPLC 1) (λ_{220}): 11.0 min.

Minor product H-Lys-Lan-Glu-Dha-Leu-Lan-Ala-OH (A-ring isomer) (6b): ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta=0.85+0.89$ (2d, $J=6$, 6 Hz; Leu-6H_δ), 1.23 (d, $J=8$ Hz; Ala-3H_β), 1.33–1.38 (m, Lys-2H_γ), 1.50–1.65

Table 2. NOEs found for the major product of cyclisation.[a]

		Lys		Lan^1			Glu				Dha			Leu			Lan^2			Ala		
		α	β	N	α	β	N	α	β	γ	N	β	β'	N	α	β	N	α	β	N	α	β
Lys	α		s	s																		
	β	s		m																		
Lan^1	N	s	m		m	s																
	α			m		s	s															
	β			s	s		m				w											
Glu	N				s	m		w	m	m	m											
	α						w		s	m	m											
	β						m	s														
	γ						m	m			w											
Dha	N					w	m	m		w		w	w	w					w			
	β										w		s	m	w	w						
	β'										w	s		s	w	w						
Leu	N										w	m	s		m	s	m					
	α											w	w	m		s	s					
	β											w	w	s	s		w					
	N													m	s	w		m	m	m		
Lan^2	α																m		s	w		
	β										w						m	s		w		
Ala	N																m	w	w		w	m
	α																			w		s
	β																			m	s	

[a] NOEs are given as strong (s), medium (m) or weak (w).

Table 3. NOEs observed for the minor product of cyclisation.[a]

		Lys			Lan			Glu				Dha			Leu			Lan			Ala		
		α	β	γ	N	α	β	N	α	β	γ	N	β	β'	N	α	β	N	α	β	N	α	β
Lys	α		s	m	s																		
	β	s			m																		
	γ	m			w																		
Lan	N	s	m	w		m	m	m															
	α				m		s	s				w											
	β				m	s		s				w			w			w					
Glu	N				m	s	s		s	m	w	m											
	α							s		s	m	m											
	β							m	s			w											
	γ							w	m														
Dha	N					w	w	m	m	w			w	w	m			w					
	β											w		s	m	w	w						
	β'											w	s		s	w	w						
Leu	N						w					m	m	s		m	s	m					
	α												w	w	m			m					
	β												w	w	s			w					
Lan	N						w					w			m	m	w		m	m	m		
	α																	m		s	s		
	β																	m	s		m		
Ala	N																	m	s	m		w	s
	α																				w		s
	β																				s	s	

[a] NOEs are given as strong (s), medium (m) or weak (w).

(m, Lys-2H_δ+Leu-H_γ+Leu-2H_β+Lys-2H_β), 1.84–1.89 (m, Glu-H_β), 2.15–2.33 (m, Glu-H_β+Glu-2H_γ), 2.68–2.80 (m, Lys-2H_ε+Lan^1-H_β+Lan-H_β), 2.98 (dd, J = 14, 6 Hz; Lan-H_β), 3.08 (dd, J = 15, 5 Hz; Lan-H_β), 3.81–3.84 (m, Lys-H_α), 4.12–4.19 (m, Glu-H_α+Ala-H_α), 4.39–4.44 (m, Leu-H_α), 4.49–4.54 (m, Lan-H_α+Lan-H_α), 5.52 (s, Dha-H_β), 6.00 (s, Dha-H_β), 7.58–7.80 (brs, Lys-NH_3^+), 7.98 (d, J = 7 Hz; Lan-NH), 8.12–8.20 (brs+d, J = 8 Hz; Lys-NH_3^++Ala-NH), 8.44 (d, J = 7 Hz; Leu-NH), 8.51 (d, J = 8 Hz; Glu-NH), 8.68 (s, Dha-NH), 8.65 (d, J = 6 Hz; Lan-NH); ES-MS: m/z: 700.8 $[M+H]^+$, 723.3 $[M+Na]^+$; HPLC 1) (λ_{max}): 11.3 min.

Acknowledgments

M.B. and J.W.E. would like to thank the Royal Society for their generous support the EPSRC for studentships to S.B., M.C. and C.A., Aplin and Barrett for supplying nisin as a reference compound, the referees for very helpful suggestions and David Turner for the NMR studies.

[1] a) J. N. Hansen, *Annu. Rev. Microbiol.* **1993**, *47*, 535–564; b) G. Jung, *Angew. Chem.* **1991**, *103*, 1067–1084; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 1051–1068; c) H.-G. Sahl, R. W. Jack, G. Bierbaum, *Eur. J. Biochem.* **1995**, *230*, 827–853; d) R. N. H. Konings, C. W. Hilbers (Eds.), *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiology*, **1996**, *69*, 85–202.
[2] L. A. Rogers, *J. Bacteriol.* **1928**, 321–325.
[3] G. W. Buchman, S. Banerjee, J. N. Hansen, *J. Biol. Chem.* **1988**, *263*, 16260–16266.
[4] S. Banerjee, J. N. Hansen, *J. Biol. Chem.* **1988**, *263*, 9508–9514.
[5] H. Allgaier, G. Jung, R. G. Werner, U. Schneider, H. Zähner, *Angew. Chem.* **1985**, *97*, 1052–1054; *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 1051–1053.
[6] H.-G. Sahl, M. Grossgarten, W. R. Widger, W. A. Cramer, H. Brandis, *Antimicrob. Agents Chemother.* **1985**, *27*, 836–840.
[7] a) D. E. Palmer, D. F. Mierke, C. Pattaroni, M. Goodman, *Biopolymers* **1989**, *28*, 397–408; b) L.-Y. Lian, W. C. Chan, S. D. Morley, G. C. K. Roberts, B. W. Bycroft, D. Jackson, *Biochem. J.* **1992**, *283*, 413–420; c) H. W. van den Hooven, H. S. Rollema, R. J. Siezen, C. W. Hilbers, O. P. Kuipers, *Biochemistry* **1997**, *36*, 14137–14145; d) F. J. M. van de Ven, G. Jung, *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **1996**, *69*, 99–107.
[8] a) A. Polinsky, M. G. Cooney, A. Toy-Palmer, G. Osapay, M. Goodman, *J. Med. Chem.* **1992**, *35*, 4185–4194; b) T. Wakamiya, K. Shimbo, A. Sano, K. Fukase, T. Shiba, *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2044–2049; c) K. Fukase, T. Wakamiya, T. Shiba, *Bull. Chem. Soc. Jpn.* **1986**, *59*, 2505–2508.
[9] N. Schnell, K.-D Entian, U. Schneider, F. Gotz, H. Zähner, R. Kellner, G. Jung, *Nature* **1988**, *333*, 276–278.
[10] L. Ingram, *Biochim. Biophys. Acta.* **1970**, *224*, 263–265.
[11] H.-P. Weil, A. G. Beck-Sickinger, J. Metzger, S. Stevanovic, G. Jung, M. Josten, H.-G Sahl, *Eur. J. Biochem.* **1990**, *194*, 217–233.
[12] P. L. Toogood, *Tetrahedron Lett.* **1993**, *34*, 7833–7836.
[13] U. Schmidt, A. Lieberknecht, J. Wild, *Synthesis* **1988**, *3*, 159–172.
[14] K. W. Li, J. Wu, W. N. Xing, J. A. Simon, *J. Am. Chem. Soc.* **1996**, *118*, 7237–7238.
[15] K. Goodall, A. Parsons, *Tetrahedron Lett.* **1995**, *36*, 3259–3260.
[16] R. J. Cherney, L. Wang, *J. Org. Chem.* **1996**, *61*, 2544–2546.
[17] M. J. Miller, *J. Org. Chem.* **1980**, *45*, 3131–3132.
[18] C. G. Shin, S. Honda, K. Morooka, Y. Yonezawa, *Bull. Chem. Soc. Jpn.* **1993**, *66*, 1844–1846.
[19] C. Blettner, M. Bradley, *Tetrahedron Lett.* **1994**, *35*, 467–470.
[20] M. Yamada, T. Miyajima, H. Horikawa, *Tetrahedron Lett.* **1998**, *39*, 289–292.
[21] C.-G. Shin, Y. Yonezawa, T. Yamada, J. Yoshimura, *Bull. Chem. Soc. Jpn.* **1982**, *55*, 2147–2152.
[22] M. Bodanszky, A. Bodanszky, *The Practice of Peptide Synthesis*, 2nd ed., Springer, Berlin, **1994**.
[23] G. B. Fields, R. L. Noble, *Int. J. Peptide Protein Res.* **1990**, *35*, 161–214.
[24] E. Atherton, R. C. Sheppard, *Solid Phase Synthesis—A Practical Approach*, IRL Press, **1989**.
[25] S. Burrage, T. Raynham, M. Bradley, *Tetrahedron Lett.* **1998**, *39*, 2831–2834.
[26] G. L. Ellman, *Arch. Biochem. Biophys.* **1959**, *70*, 82–89.
[27] V. K. Sarin, S. B. H. Kent, J. P. Tam, R. B. Merrifield, *Anal. Biochem.* **1981**, *117*, 147–157.
[28] P. Güntert, W. Braun, K. Wüthrich, *J. Mol. Biol.* **1991**, *217*, 517–530.
[29] F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, W. C. Still, *J. Comput. Chem.* **1990**, *11*, 440–467.
[30] G. J. Pernia, J. D. Kilburn, J. W. Essex, R. J. Mortishire-Smith, M. Rowley, *J. Am. Chem. Soc.* **1996**, *118*, 10220–10227.

Received: June 22, 1999
Revised version: October 8, 1999 [F1868]

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000 0947-6539/00/0608-1466 $ 17.50+.50/0