Synthetic Study on Peptide Antibiotic Nisin. V. Total Synthesis of Nisin[1)]

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Total synthesis of a lanthionine peptide nisin was achieved by the successive condensations of four segments including cyclic lanthionine peptide parts and the *C*-terminal linear segment including dehydroalanine, followed by the deprotection procedure with anhydrous HF. Modified reaction conditions for the Hofmann degradation were applied to prepare the dehydroalanine residue from the 2,3-diaminopropionic acid residue. The final deprotection with anhydrous HF proceeded without decomposition of the dehydroamino acid residues which are generally labile in acidic media. The synthetic nisin was completely identical with the natural one in all respects, resulting in a confirmation of the proposed structure.

The peptide antibiotic nisin is known to be the first lantibiotic (=biologically active lanthionine peptide)[2)] found in nature. Nisin has been used as an important food preservative widely in Europe,[3)] since it shows remarkable growth-prevention activity against Gram-positive microorganisms, particularly *Clostridium botulinum*.[4)] However, this peptide was not developed as a chemotherapeutic agent because of the poor solubility in water and the instability at physiological pH value. Although nisin had been found in a culture broth of *Lactococcus lactis* in 1928,[5)] the isolation of nisin was delayed up to 1947[6)] or 1952.[7)] The complex structure of nisin composed of 34 amino acid residues was first proposed by Gross et al. in 1971 as shown in Fig. 1.[8)] This unique peptide includes five residues of unusual sulfide amino acids, i.e., one *meso*-lanthionine (*meso*-Lan) and four (2*R*,2′*S*,3′*S*)-methyllanthionines (*threo*-Melan) which are involved in five cyclic parts termed rings A, B, C, D, and E in the molecule. In addition, it is also noteworthy that nisin was the first naturally occurring peptide containing the dehydroamino acid residues, i.e., dehydroalanine (Dha) and dehydrobutyrine (Dhb).

Nisin and subtilin[9)] had been the only lantibiotics whose structures were proposed before our structural determination of an enzyme inhibitor, ancovenin in 1984.[10)] Thereafter, many lantibiotics were found and their structures were determined in succession, i.e., lanthiopeptin[11)] (=cinnamycin, Ro 09-0198), duramycin, epidermin, gallidermin, mersacidin, nisin Z, and Pep5.[12)] Although many investigations of the lantibiotics were performed concerning with structural determination, biological activity, conformational analysis, or biosynthesis,[12)] there have been few synthetic studies of these peptides reported so far except for the synthesis of ring A in nisin by Photaki et al.[13)] Therefore, we aimed at the total synthesis of nisin not only for the development of the synthetic method of lantibiotics but also for the confirmation of the proposed structure and the investigation of the structure-activity relationships of this unique peptide as well.

Results and Discussion

We first exploited the novel method for a preparation of lanthionine peptide from disulfide peptide by desulfurization[14)] with hexaethylphosphorous triamide [$P(NEt_2)_3$]. Thus, all cyclic parts in nisin were successfully synthesized as reported in previous papers.[15–22)] In the synthesis of nisin, we also applied a novel and mild method based on the Hofmann degradation to the preparation of the Dha residue from the 2,3-diaminopropionic acid (A_2pr) residue in a peptide.[15,16,23)] On the other hand, the Dhb residue was prepared by dehydration of the threonine residue with carbodiimide and copper(I) chloride (CuCl).[24)]

For the total synthesis of nisin, a synthetic strategy based on the segment condensation method was adopted, since a successive construction of five cyclic parts from the *C*-terminus seemed to be not a reasonable procedure as far as we studied. Thus, nisin molecule was divided into five segments which were basically

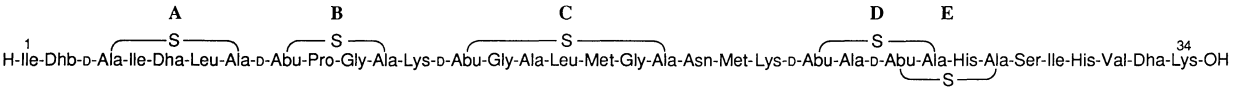

Fig. 1. Structure of nisin. Ala–S–Ala=*meso*-lanthionine; Abu–S–Ala=*threo*-methyllanthionine.

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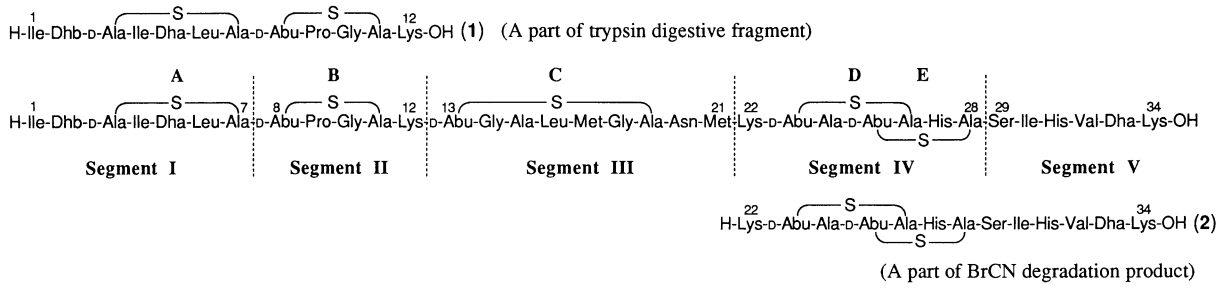


Fig. 2. Five fragments for the total synthesis of nisin.

designed as we can confirm the structures of two synthetic intermediates by comparison with the authentic samples, i.e., *N*-terminal dodecapeptide, nisin-(1-12) (**1**), and *C*-terminal tridecapeptide, nisin-(22-34) (**2**), obtained by enzymic and BrCN degradation of natural nisin,[17] respectively. (Fig. 2) Throughout this study, conventional *t*-butyl and benzyl type protective groups were applicable respectively for temporary and persistent protections of any functional groups, since we confirmed that the dehydroamino acid residues are sufficiently stable in TFA and HF under anhydrous conditions.

Along the synthetic strategy mentioned above, we started from the syntheses of five segments. Segment I [Z-Ile-Dhb-(ring A)-OH (**14**)] including two dehydroamino acid residues was prepared as shown in Fig. 3.[25] In the previous synthetic study of ring A (**5**), we realized that the first *N*-methylation of the β-amino group in the A_2pr residue is a rate-determining step in the Hofmann degradation, and that *S*-methylation of β-thioamino acid such as *S*-methylcysteine or lanthionine in the same molecule also occurs slowly to afford sulfonium ion which results in a β-elimination to produce another Dha residue.[15,16] According to these observations, the β-amino group in the A_2pr residue was first dimethylated and then the Hofmann degradation was carried out by control of the final permethylation. However, since the yield of this reaction was not quite satisfactory, we reinvestigated the reaction conditions for the Hofmann degradation in the present study. So far as we studied, *N*-permethylation step with CH_3I could be remarkably accelerated by use of DMF as the reaction solvent in place of MeOH resulting in a differentiation from the *S*-methylation. Thereafter, we used DMF as a single

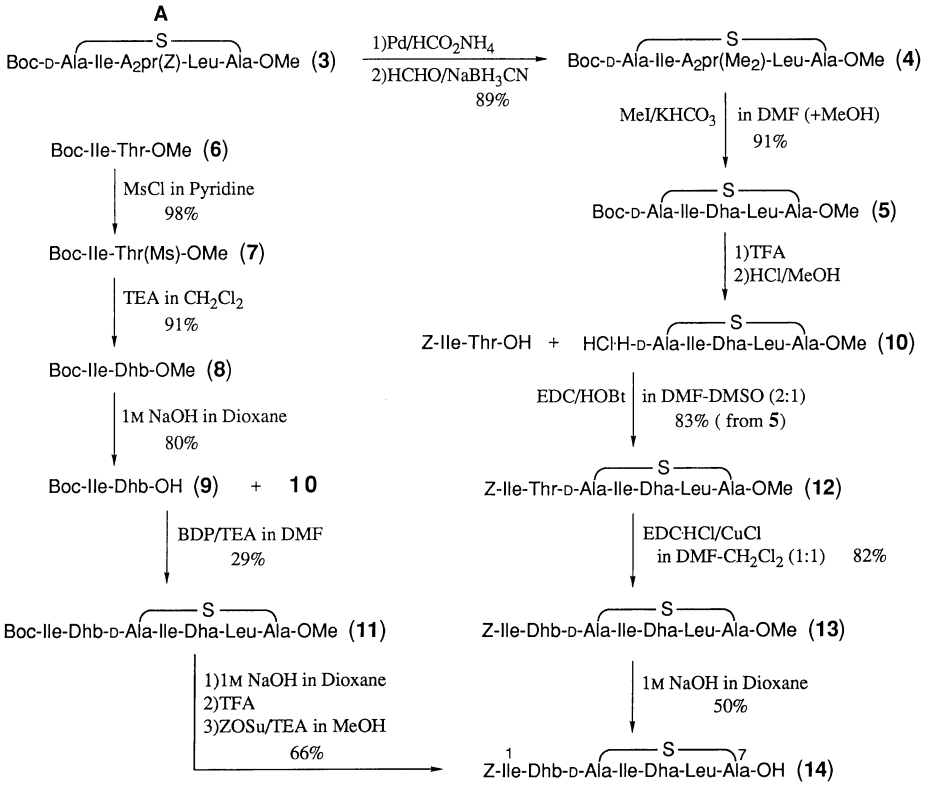


Fig. 3. Preparation of segment I.

solvent for methylation and then MeOH was newly added for the acceleration of β-elimination of the quaternary ammonium intermediate to give ring A part **5** containing the desirable Dha residue.[26] Thus, the yield of the Hofmann degradation was highly improved from 31% to 91%. Another improvement in the preparation of ring A was in the application of catalytic transfer hydrogenation using Pd-black and $HCOONH_4$[27] in place of general catalytic hydrogenation to the removal of the N^{β}-Z group in the A_2pr residue. As a result of this modification, the yield of peptide **4** was raised from 78% up to 89%.

The removal of the Boc group in peptide **5** was carried out with TFA, followed by the quick treatment with hydrogen chloride in MeOH to give hydrochloride **10** without decomposition of Dha group. The coupling of Boc-Ile-Dhb-OH (**9**) with **10** did not proceed so smoothly because of the low reactivity of the conjugated carboxyl group in the Dhb residue, and the product **11** was obtained in unsatisfactory yield (HOSu ester, pentachlorophenyl ester, EDC-HOBt, and BDP methods; 14%, 17%, 14%, and 29%, respectively).[28] Therefore, we chose an alternate route based on the dehydration of the Thr residue at the step of peptide **12** prepared by the coupling of Z-Ile-Thr-OH with **10**. Since *O*-mesylation of the Thr residue in **12** did not proceed at all, the dehydration with EDC·HCl-$CuCl_2$[24] was applied to the preparation of peptide **13** in good yield. The presence of doublet methyl signal of Dhb at 1.78 ppm[29] on ^{1}H NMR of **13** clearly indicated a sole formation of the desired *Z*-isomer of Dhb in this reaction.[30] Finally, *N*-terminal segment I (**14**) was obtained by the saponification of methyl ester in peptide **13**.

Segment II [HCl·H-(ring B)-Lys(Z)-OBu^t (**24**)] corresponding to the residue 8-12 in nisin was prepared as shown in Fig. 4. Since the methyl ester derivative of ring B prepared in the previous study[15,18] was easily decomposed under saponification conditions, the benzyl ester derivative of ring B (**20**) was newly prepared in the present work. In the synthetic studies of rings A to D-E, the most suitable solvent for desulfurization varied depending on the substrate, i.e., DMF for Boc-(ring A)-OMe,[15,16] benzene for Boc-(ring B)-OMe,[15,18] Boc-(ring D-E)-OMe,[21,22] and THF for Boc-(ring C)-OBzl.[19,20,31] In the case of the benzyl ester derivative **19**, DMF was known to be the appropriate solvent for this purpose. After the deprotection of both the Boc and the Bzl groups with HF, the liberated amino group was reprotected with Troc. The protected ring B (**22**) was coupled with the Lys derivative to give peptide **23**. Finally, the Troc group in **23** was selectively removed by reduction with Zn-AcOH and the segment II was prepared as its hydrochloride **24** (Fig. 4).

Preparation of segment III [HCl·H-(ring C)-Asn(Mbh)-Met-OBu^t (**30**)] was carried out in a manner similar to the preparation of segment II (**24**) (Fig. 5). The *N*-Troc derivative of ring C (**26**) was prepared from the benzyl ester derivative **25**,[19,20] and coupled with the dipeptide moiety **28**. The branched amide group of the Asn residue in the dipeptide was protected with Mbh to prevent dehydration or imide formation under the usual conditions for peptide synthesis. The *N*-Troc group in the coupling product **29** was removed and the segment III was prepared as its hydrochloride **30**.

Segment IV [Boc-Lys(Z)-(ring D-E)-OMe (**33**)] was prepared as shown in Fig. 6. The *N*-Boc group in the methyl ester derivative **31** of ring D-E part[21,22] was removed, and the resulting peptide **32** was coupled with Boc-Lys(Z)-OH. The segment IV derivative **33** thus obtained was then converted into the hydrazide derivative **34** to subject for the coupling with segment V by azide method.

Segment V corresponding to the *C*-terminal linear hexapeptide was prepared as shown in Fig. 7. The Dha

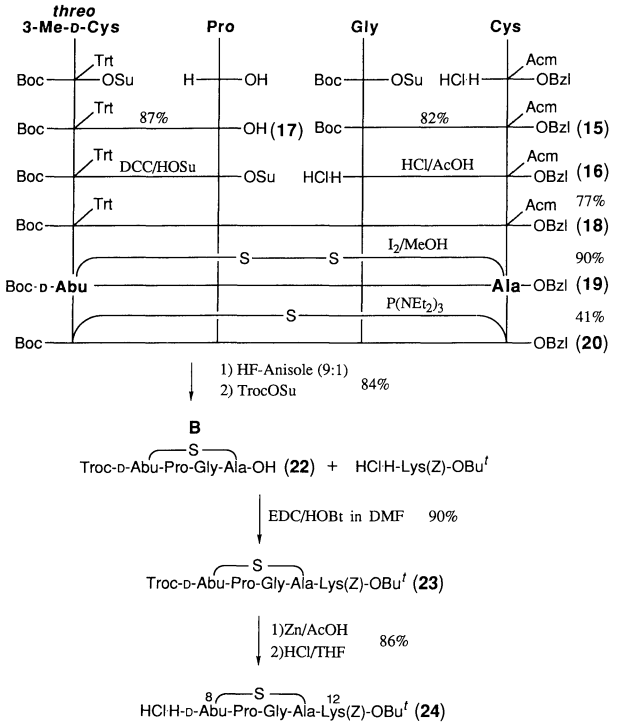


Fig. 4. Preparation of segment II.

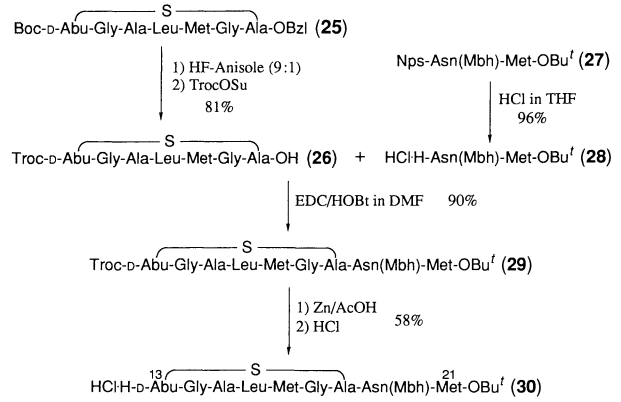


Fig. 5. Preparation of segment III.

residue in this segment was also prepared from the A_2pr residue by the Hofmann degradation. In this case, the N^{β}-amino group in the A_2pr residue was protected with the Troc group which can be removed selectively in the presence of the Z and the Bzl groups protecting the N^{ε}-amino and the carboxyl groups of the *C*-terminal Lys residue, respectively. The Hofmann degradation was carried out before introduction of the His residue, since the imidazole moiety in this residue may be methylated with CH_3I. Thus, tripeptide **37** containing the A_2pr residue was prepared, and then the Troc group in **37** was removed with Zn–AcOH. The free N^{β}-amino group of the A_2pr residue in the resulting peptide **38** was directly degraded by use of CH_3I and $KHCO_3$ in MeOH in this case. Tripeptide **39** containing the Dha residue was then elongated to hexapeptide **42** by DCC–HOBt method. The N^{im}-Ts group of the His residue removed during the coupling in the presence of HOBt was retosylated after each coupling step to facilitate purification of the product by silica-gel column chromatography, though finally removed with HOBt to obtain segment V (**43**).

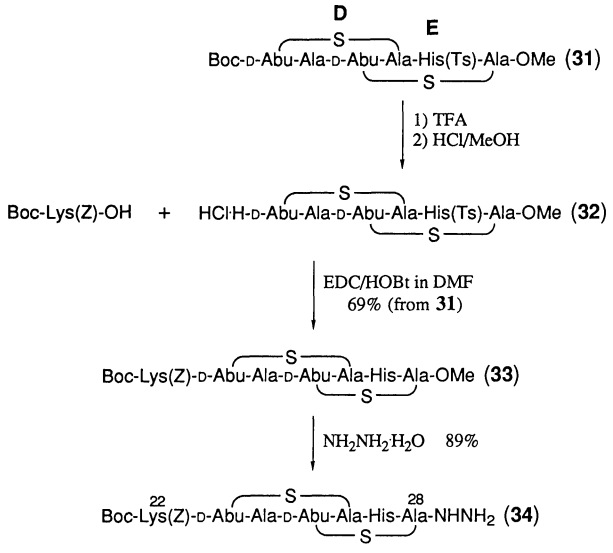


Fig. 6. Preparation of segment IV.

The coupling of five segments prepared as mentioned above was carried out as shown in Fig. 8. Segment I (**14**) and segment II (**24**) were first coupled by EDC–HOBt method to give dodecapeptide **44** in a 56% yield. The Bu^t ester of **44** was then removed with TFA, and the crude product was purified by reversed phase HPLC (Cosmosil $5C_{18}$ AR) to give dodecapeptide **45** in a 79% yield. The structure of **45** was confirmed by comparison with the authentic compound prepared by benzyl-

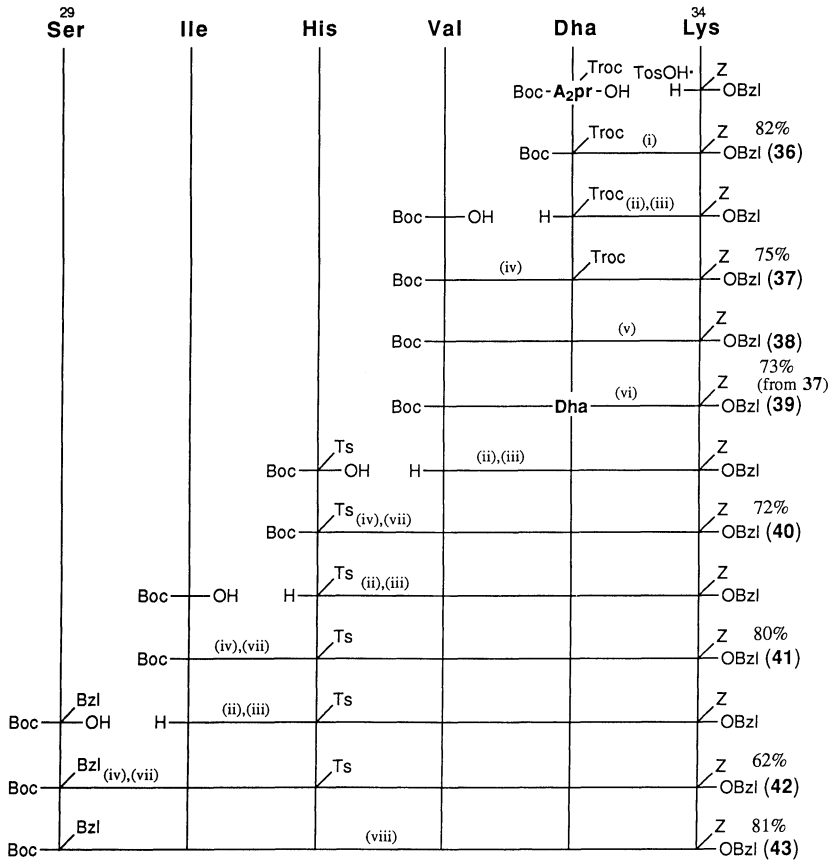


Fig. 7. Preparation of segment V. (i) DCC/HOBt/TEA; (ii) TFA; (iii) $NaHCO_3$; (iv) DCC/HOBt; (v) Zn/AcOH; (vi) $CH_3I/KHCO_3$ in MeOH; (vii) TsCl/TEA; (viii) HOBt in THF.

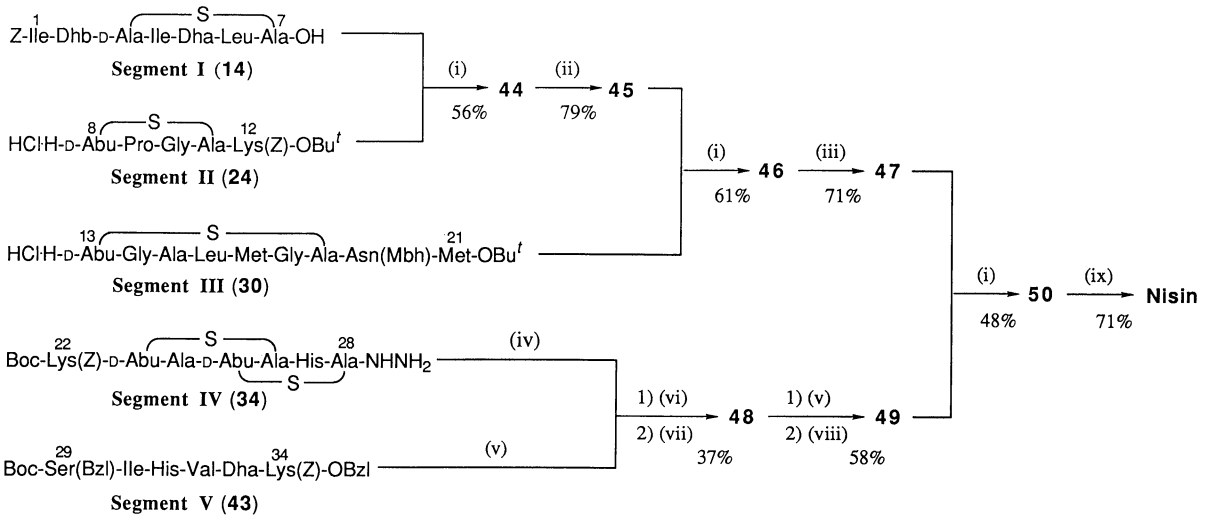


Fig. 8. Couplings of segments I to V for the total synthesis of nisin. (i) EDC/HOBt in DMF; (ii) TFA–CH_2Cl_2 (1 : 1); (iii) TFA–anisole (10 : 1); (iv) isopentyl nitrite/HCl in DMF; (v) TFA; (vi) TEA; (vii) $(Boc)_2O$; (viii) HCl; (ix) HF–anisole (10 : 1).

oxycarbonylation of nisin-(1-12) (**1**), i.e., one of the trypsin digestive fragments[17] of natural nisin. Both synthetic and authentic **45** were completely identical with each other in respects of TLC ($CHCl_3$–MeOH–AcOH=9 : 1 : 1, Merck silica gel 60 F_{254}, Art.5554, R_f 0.38), HPLC (Cosmosil 5C_{18}, 4×125 mm, gradient elution with 50—70% of CH_3CN–0.1% TFA (2% min^{-1}), flow rate: 1.0 ml min^{-1}, retention time: 8.2 min), and ^1H NMR. The protected nisin-(1-12) (**45**) was then coupled with segment III (**30**) to obtain **46** in a 61% yield, which was purified by reversed phase HPLC (Asahipak C4P-50). Both the Bu^t ester and the Mbh groups in **46** were then removed with TFA in the presence of anisole. Since the N^{ε}-Z groups of the Lys residues were partially removed during the reaction, the liberated amino groups were reprotected with ZOSu. The crude product was purified by HPLC (Asahipak C4P-50) to obtain pure carboxyl component **47** for final step in a 71% yield.

Segment IV (**34**) was treated with isopentyl nitrite in the presence of HCl and then coupled with segment V (**43**) removed the Boc group at *N*-terminus with TFA. After the coupling, the imidazole groups of both the His^{27} and the His^{31} residues were once protected with the Boc group to facilitate the column chromatographic purification of the product. The structure of **48** thus obtained was also confirmed by comparison with the authentic sample in HPLC. Namely, the retention time of the free peptide prepared from **48** by deprotection with anhydrous HF was definitely identical with that of nisin-(22-34) (**2**), one of the BrCN-degradation products of natural nisin, on reversed-phase support (Cosmosil 5C_{18}, 4×125 mm, gradient elution with 10—30% of CH_3CN–0.1% TFA (2% min^{-1}), flow rate: 1.0 ml min^{-1}, retention time: 10.4 min).

The Boc groups in the peptide **48** were removed with TFA and the amine component isolated as hydrochloride **49** was subjected to the final coupling with the carboxyl component **47**. The coupling was carried out by EDC–HOBt method and the product **50** was obtained in a 48% yield after HPLC purification. All protecting groups in **50** were removed with anhydrous HF in the usual way and the crude product was purified

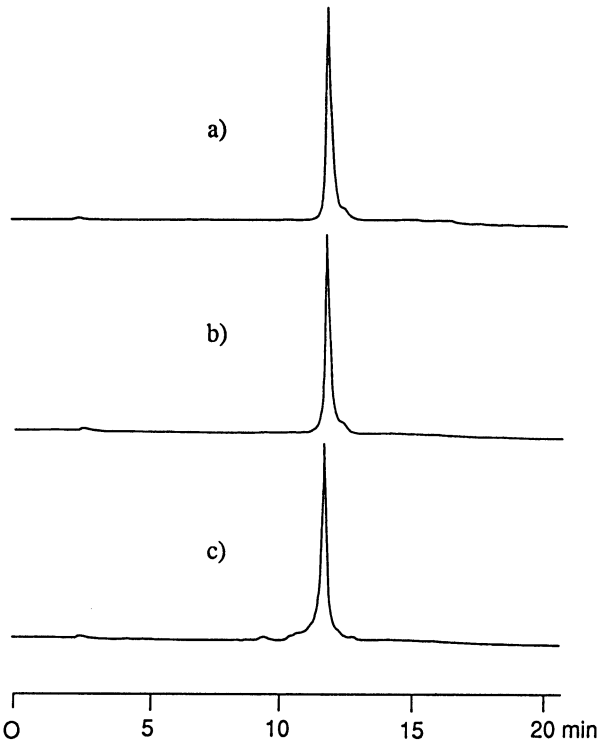


Fig. 9. HPLC profile of nisin. a) natural nisin; b) synthetic nisin after HPLC purification; c) synthetic nisin after HF cleavage. Cosmosil 5C_{18} AR column, 4.6×250 mm, gradient elution with 30—50% of CH_3CN–0.3 M guanidine hydrochloride (2% min^{-1}), flow rate: 1.0 ml min^{-1}, detection: UV at 220 nm.

Table 1. Antibacterial Activities of Nisin

Test organism	Natural	Synthetic
Staphylococcus aureus ATCC 6538p	12.5	12.5
Staphylococcus epidermidis sp-al-l	12.5	12.5
Staphylococcus epidermidis ATCC 12228	>100	>100
Streptococcus pyogenes A089	1.6	1.6
Streptococcus faecalis 030021	50	100
Micrococcus luteus ATCC 10240	<0.2	0.4
Micrococcus luteus IFO 3333	6.3	3.1
Corynebacterium diphteriae IID 527	12.5	12.5
Bacillus subtilis ATCC 6633	25	25
Escherichia coli NIHJ-JC2	>100	>100
Salmonella typhimurium ATCC 14028	>100	>100
Shigella flexneri IID 642	>100	>100
Proteus vulgaris OX-19	>100	>100

by HPLC to give pure nisin in a 71% yield. Synthetic nisin thus obtained was completely identical with natural compound in all respects such as ^{1}H NMR,[32] FAB-MS, retention time in HPLC [(a) Nucleosil 300-7C_{18}, 6×250 mm, gradient elution with 30—50% of CH_3CN–0.01 M HCl (2% min^{-1}), flow rate: 1.0 ml min^{-1}, retention time: 7.9 min; (b) Cosmosil 5C_{18} AR, 4.6×250 mm, gradient elution with 30—50% of CH_3CN–0.3 M guanidine hydrochloride (2% min^{-1}), flow rate: 1.0 ml min^{-1}, retention time: 11.6 min] (Fig. 9), and antibacterial activities (Table 1).

As mentioned above, we first succeeded in the total synthesis of lantibiotic nisin, and consequently, confirmed the structure of nisin synthetically. Thus, both novel synthetic methods for Dha peptide based on the Hofmann degradation and for lanthionine peptide from disulfide peptide using the desulfurization proved to be very practical for the general synthetic method for lantibiotics found in nature. Another valuable information obtained through the present study is that the dehydroamino acid residues, which are generally very labile under acidic conditions, are surprisingly stable in HF and TFA as well as for quick treatment with hydrogen chloride in organic solvent such as MeOH under anhydrous conditions. This fact was one of the important factors leading to the success of our nisin synthesis. In addition, the use of HPLC made possible the easy purification of the synthetic intermediates. Indeed, the fragments **45**, **46**, **47**, **49**, and protected nisin-(1-34) (**50**) were effectively purified by the reversed phase HPLC, and the combined yield of final two steps was improved from 10% in the preliminary work up to 34% by this experimental advantage.

According to our strategy for the synthesis of the lantibiotics, more than ten kinds of nisin fragments and analogs have already been prepared which could be used for construction of many analogs for the investigation of structure-activity relationship of nisin.[1c,33]

Experimental

All melting points are uncorrected. The ^{1}H NMR spectra were obtained with a Varian XL-100-15, JEOL FX-90Q, JEOL JNM-GSX 270, JEOL JNM-EX 270, or JEOL JNM-GSX 400 spectrometer. In general, the chemical shifts are given in δ values (ppm) from TMS used as an internal standard, while those of nisin are relatively given from the chemical shift of DMSO-d_6 adjusted at 2.49 ppm. FD-MS and FAB-MS spectra were obtained with a JEOL JMS-01SG-2, JMS HX-100 double-focusing, or JMS-DX 300 mass spectrometer. Specific rotations were obtained with a Perkin-Elmer 141 or 241 polarimeter. HPLC was carried out with a Shimadzu LC-6A or LC-6AD liquid chromatograph. Silica-gel column chromatography was carried out with Merck silica gel 60 (Art. 9385, 230—400 mesh) at medium pressure (2—10 kg cm^{-2}). Amino acid analysis was carried out with Hitachi 655A type liquid chromatograph system equipped with 655-3410 unit (column: Hitachi custom #2619F, 4×150 mm, 58 °C; buffer A: sodium citrate dihydrate 7.34 g, NaCl 5.02 g, citric acid monohydrate 20.56 g, EtOH 104 ml, 2,2′-thiobisethanol 5.22 ml, and octanoic acid 0.1 ml in 1000 ml of aqueous solution; buffer B: sodium citrate dihydrate 26.67 g, NaCl 54.35 g, citric acid monohydrate 6.10 g, EtOH 40 ml, and octanoic acid 0.1 ml in 1000 ml of aqueous solution; gradient: A 100% (0—12 min), A 85%/B 15% (12—37 min), A 19%/B 81% (37—47 min), B 100% (47—62 min); flow rate: 0.4 ml min^{-1}). Samples for amino acid analysis were hydrolyzed with 6 M HCl in sealed tubes at 110 °C. Deprotection of peptide derivatives with anhydrous hydrogen fluoride was carried out in HF-reaction apparatus.[34]

Boc-D-Ala-Ile-A$_2$pr(Me)$_2$-Leu-Ala-OMe (4). To a mixture of Boc-D-Ala-Ile-A$_2$pr(Z)-Leu-Ala-OMe (**3**)[15,16] (750 mg, 1.00 mmol), HCO_2NH_4 (2.25 g, 35.7 mmol), and TEA (470 mg, 4.6 mmol) in MeOH (125 ml) was added Pd-black (1.0 g),[27] and the mixture was stirred at room temperature for 6 h. The catalyst was filtered off and the filtrate was concentrated in vacuo. To a solution of the residue in MeOH (30 ml) was added 35% HCHO solution (0.43 ml, 5.0 mmol) and $NaBH_3CN$ (138 mg, 2.20 mmol). The solution was stirred at room temperature for 2 h, and concentrated in vacuo. The residue was treated with EtOAc and saturated aqueous $NaHCO_3$ solution. The organic layer was washed with brine, and worked up as usual, i.e., dried over anhydrous $MgSO_4$ and concentrated in vacuo. The residue was precipitated from EtOAc and hexane to give **4** as powder which was subjected to

the next reaction without further purification: Yield 575 mg (89%).

Boc-D-Ala-Ile-Dha-Leu-Ala-OMe (5). To a solution of compound **4** (800 mg, 1.24 mmol) in DMF (10 ml) were added K_2CO_3 (1.86 g, 18.6 mmol) and CH_3I (212 mg, 1.49 mmol). The mixture was stirred for 30 h at room temperature, and MeOH (5 ml) was added. The mixture was additionally stirred for 3 h,[26] and concentrated in vacuo. Powdery precipitate obtained by addition of water to the residue was collected by filtration and washed with 10% citric acid, water, and diethyl ether, successively: Yield 678 mg (91%); mp 235—237 °C; $[\alpha]_D^{21}$ −65.6° (*c* 0.247, DMF); FD-MS, *m/z* 600 [(M+H)$^+$], [lit,[16] mp 228—229 °C; $[\alpha]_D^{17}$ −68.2° (*c* 1.00, DMF)]. Anal. ($C_{27}H_{45}O_8N_5S \cdot 0.5H_2O$) C, H, N, S.

Boc-Ile-Thr-OMe (6). To a solution of Boc-Ile-OH (34.7 g, 84.0 mmol) and HCl·H-Thr-OMe (13.2 g, 84.0 mmol) in DMF (100 ml) were added TEA (8.50 g, 84.0 mmol), HOBt (11.3 g, 84.0 mmol), and DCC (17.3 g, 84.0 mmol) at 0 °C, successively. After the mixture was stirred at room temperature overnight, an insoluble material precipitated was filtered off. The filtrate was concentrated in vacuo, and to the residue were added water and EtOAc. The organic layer was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and then worked up as usual. The residue was triturated with hexane to give **6** as colorless prisms: Yield 23.4 g (81%); mp 125—128 °C; $[\alpha]_D^{13}$ −30.7° (*c* 1.02, MeOH). Anal. ($C_{16}H_{30}O_6N_2$) C, H, N.

Boc-Ile-Thr(Ms)-OMe (7). To a solution of **6** (3.46 g, 10.0 mmol) in pyridine (20 ml) was added methanesulfonyl chloride (4.9 g, 43 mmol) at −20 °C. The mixture stirred at −20 °C for 2 h was poured into ice, and extracted with AcOEt. The extract was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and worked up as usual. The crystalline residue was collected by filtration and washed with hexane: Yield 4.05 g (98%). The product **7** thus obtained was subjected to the next reaction without further purification.

Boc-Ile-Dhb-OMe (8). To a solution of **7** (1.00 g, 2.36 mmol) in CH_2Cl_2 (10 ml) was added TEA (954 mg, 9.43 mmol). The mixture was stirred at room temperature for 2 h, washed with water, and worked up as usual. The crystalline product was recrystallized from AcOEt and hexane to give colorless prisms: Yield 702 mg (91%); mp 125—126.5 °C; $[\alpha]_D^{13}$ −26.1° (*c* 1.06, MeOH); ^{1}H NMR (100 MHz, $CDCl_3$) δ=0.9—1.1 (6H, Ile; γ-CH_3×2), 1.3 (2H, m, Ile; γ-CH_2), 1.48 (9H, s, Boc), 1.78 (3H, d, *J*=7 Hz, Dhb; γ-CH_3), 2.0 (1H, m, Ile; β-CH), 3.87 (3H, s, OCH_3), 4.1 (1H, dd, Ile; α-CH), 5.1 (1H, bd, *J*=9 Hz, Ile; NH), 6.85 (1H, q, *J*=7 Hz, Dhb; β-CH), 7.4 (1H, bs, Dhb; NH). Anal. ($C_{16}H_{28}O_5N_2 \cdot 0.4H_2O$) C, H, N.

Boc-Ile-Dhb-OH (9). To a solution of **8** (1.76 g, 5.36 mmol) in dioxane (21 ml) was added 1 M NaOH (8.0 ml, 8.0 mmol) and the solution was stirred at room temperature for 2 h. To the solution were added AcOEt and 10% citric acid, and the organic layer was washed with brine and worked up as usual. The crystalline product was recrystallized from AcOEt and hexane to give colorless needles: Yield 1.34 g (80%); mp 150—152 °C; $[\alpha]_D^{13}$ −33.6° (*c* 1.04, MeOH). Anal. ($C_{15}H_{26}O_5N_2$) C, H, N.

HCl·H-D-Ala-Ile-Dha-Leu-Ala-OMe (10). Compound **5** (250 mg, 0.417 mmol) was dissolved in TFA (3.5 ml) and allowed to stand for 1 h at room temperature. The reaction mixture was concentrated in vacuo. To the residue in $CHCl_3$ (10 ml) and MeOH (10 ml) was added 10.2 M HCl in MeOH (81.7 μl, 0.834 mmol), and the solution was concentrated in vacuo. To the residue was added benzene and concentrated in vacuo again. The residue was suspended in benzene and lyophilized. Colorless powder thus obtained was subjected to the next coupling without further purification: Yield 223 mg (quant).

Boc-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-OMe (11). To a solution of the hydrochloride **10** (89.5 mg, 0.167 mmol), Boc-Ile-Dhb-OH (**9**) (62.8 mg, 0.200 mmol) in DMF (1 ml) was added BDP[35] (60 mg, 0.22 mmol) and TEA (65 mg, 0.64 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 2 d and then concentrated in vacuo. To the residue were added water and EtOAc, and the organic layer was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and worked up as usual. The residue was purified by silica-gel column chromatography (8 g, 1.1×15 cm, $CHCl_3$–acetone=2:1) to give **11** which was precipitated from $CHCl_3$, MeOH, and hexane: Yield 38 mg (29%); mp 230—232 °C; ^{1}H NMR (90 MHz, DMSO-d_6/$CDCl_3$=1/1) δ=1.41 (9H, s, Boc), 1.73 (3H, d, *J*=7 Hz, Dhb; γ-CH_3), 3.70 (3H, s, OCH_3), 5.5 (1H, s, Dha; β-CH), 6.1 (1H, s, Dha, β-CH). Anal. ($C_{37}H_{61}O_{10}N_7S \cdot 0.5H_2O$) C, H, N, S.

Z-Ile-Thr-D-Ala-Ile-Dha-Leu-Ala-OMe (12). To a solution of the hydrochloride **10** (502 mg, 0.937 mmol), Z-Ile-Thr-OH (374 mg, 1.02 mmol), and HOBt (138 mg, 1.02 mmol) in DMF (12 ml) was added EDC (189 mg, 1.22 mmol) under cooling at −70 °C. The reaction mixture was stirred at −20 °C overnight and then concentrated in vacuo. To the residue were added water and EtOAc. The organic layer was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and then worked up as usual. The residue was reprecipitaed from $CHCl_3$, MeOH, and diethyl ether to give colorless powder, which was subjected to the following reaction without further purification: Yield 660 mg (83%).

Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-OMe (13). To a solution of compound **12** (560 mg, 0.660 mmol) in CH_2Cl_2 (6 ml) and DMF (6 ml) were added CuCl (196 mg, 1.98 mmol) and EDC·HCl (1.27 g, 6.60 mmol). The mixture was stirred at room temperature overnight, and concentrated in vacuo. To the residue were added EtOAc and water, and the organic layer was worked up as usual after the removal of insoluble material by filtration. The residue was purified by silica-gel column chromatography (25 g, 1.5×30 cm, $CHCl_3$–MeOH, =19:1). The fraction containing **13** was concentrated in vacuo and the residue was precipitated from $CHCl_3$, MeOH and hexane to give colorless powder: Yield 450 mg (82%); mp 225—242 °C (decomp); $[\alpha]_D^{28}$ −47.3° (*c* 0.260, DMF); ^{1}H NMR (270 MHz, $CDCl_3$–CD_3OD=10:1) δ=0.8—1.0 (18H, Ile; γ-CH_3×2, δ-CH_3×2, Leu; δ-CH_3×2), 1.1—1.9 (8H, m, Ile; β-CH, γ-CH_2×2, Leu; β-CH_2, γ-CH), 1.78 (3H, d, *J*=7.2 Hz, Dhb; γ-CH_3), 2.16 (1H, m, Ile; β-CH), 2.76 (1H, dd, *J*=4.0, 12.1 Hz, Ala_L; β-CH), 2.9—3.1 (3H, m, Ala_L; β-CH, β-CH_2), 3.78 (3H, s, OCH_3), 4.06 (1H, m, α-CH), 4.26—4.33 (2H, m, α-CH), 4.72—4.78 (2H, m, α-CH), 4.97 (1H, d, *J*=12.5 Hz, $CH_2C_6H_5$), 5.14 (1H, d, *J*=12.5 Hz, $CH_2C_6H_5$), 5.26 (1H, s, Dha; β-CH), 6.19 (1H, s, Dha; β-CH), 6.34 (1H, q, *J*=7.2 Hz, Dhb; β-CH), 7.2—7.4 (5H, m, $CH_2C_6H_5$). Anal. ($C_{40}H_{59}$-

$O_{10}N_7S \cdot 2.5H_2O$) C, H, N, S.

Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-OH (14). To a solution of **13** (60.0 mg, 72.3 μmol) in dioxane (1.3 ml) was added 1 M NaOH (79.5 μl, 79.5 μmol) at 10 °C and the solution was stirred overnight at the same temperature. The reaction mixture was diluted with AcOEt and acidified with 10% citric acid. The organic layer separated was washed with brine and worked up as usual. The residue was purified by silica-gel column chromatography (20 g, 1.5×24 cm, $CHCl_3$–MeOH–AcOH=15 : 1 : 1). The fraction containing **14** was concentrated in vacuo and the residue was precipitated from $CHCl_3$, MeOH, and hexane to give colorless powder: Yield 29.5 mg (50%); mp 218—222 °C; $[\alpha]_D^{28}$ −47.0° (*c* 0.164, DMF); FAB-MS, *m/z* 854 [$(M+K)^+$]. Amino acid analysis (6 M HCl, 110 °C, 36 h): Lan(0.16), *meso*-Lan(0.78), Ile(1.90), Leu(1.00). Anal. ($C_{39}H_{57}O_{10}N_7S \cdot H_2O$) C, H, N, S.

Boc-Gly-Cys(Acm)-OBzl (15). To a solution of Boc-Gly-OSu[36] (4.05 g, 15.7 mmol) in DMF (20 ml) were added HCl · Cys(Acm)-OBzl[37] (5.01 g, 15.7 mmol) and TEA (1.59 g, 15.7 mmol). The mixture was stirred at room temperature overnight and concentrated in vacuo. The residue was dissolved in EtOAc and 10% citric acid. The organic layer separated was washed with saturated aqueous $NaHCO_3$ solution, brine, and then worked up as usual. The residue was triturated with hexane to give **15** as colorless prisms: Yield 5.65 g (82%); mp 59—61 °C; $[\alpha]_D^{15}$ −35.8° (*c* 1.01, MeOH). Anal. ($C_{20}H_{29}O_6N_3S$) C, H, N, S.

HCl · H-Gly-Cys(Acm)-OBzl (16). Compound **15** (1.01 g, 2.30 mmol) was dissolved in 1.4 M HCl in AcOH (20 ml) and allowed to stand for 20 min at room temperature. The reaction mixture was concentrated in vacuo, and the residue was triturated with diethyl ether. The powdery product thus obtained was subjected to the next coupling without further purification: Yield 843 mg (98%).

Boc-3-Me-D-Cys(Trt)-Pro-OH (17). To a solution of Boc-3-Me-D-Cys(Trt)-OSu[18,38,39] (6.90 g, 12.0 mmol) in DMF (20 ml) were added proline (4.14 g, 36.0 mmol) and TEA (3.64 g, 36.0 mmol), and the mixture was stirred at room temperature for 3 d. 1-(2-Aminoethyl)piperazine (155 mg, 1.20 mmol) was added to the mixture, stirred for 1 h, and concentrated in vacuo. The residue in EtOAc was washed with 10% citric acid and brine, and worked up as usual. The oily residue was triturated with hexane to obtain **17** as colorless powder: Yield 6.02 g (87%); mp 93—96 °C; $[\alpha]_D^{28}$ −85.8° (*c* 0.592, MeOH). Anal. ($C_{33}H_{38}O_5N_2S \cdot 0.25H_2O$) C, H, N, S.

Boc-3-Me-D-Cys(Trt)-Pro-Gly-Cys(Acm)-OBzl (18). To a solution of **17** (1.27 g, 2.21 mmol) and HOSu (254 mg, 2.21 mmol) in THF (10 ml) was added DCC (456 mg, 2.21 mmol) under ice cooling. After the mixture was stirred at room temperature for 2 h, *N,N'*-dicyclohexylurea precipitated was filtered off, and the filtrate was concentrated in vacuo. To a solution of the residue in DMF (3 ml) were added **16** (830 mg, 2.21 mmol) in DMF (3 ml) and TEA (224 mg, 2.21 mmol) under ice cooling. The mixture was stirred at room temperature overnight and concentrated in vacuo. The residue was dissolved in EtOAc and 10% citric acid. The organic layer separated was washed with saturated aqueous $NaHCO_3$ solution and brine, and worked up as usual. The oily residue was triturated with hexane to obtain **18** as colorless powder, which was reprecipitated from $CHCl_3$ and hexane: Yield 1.52 g (78%); mp 88—93 °C; $[\alpha]_D^{28}$ −70.5° (*c* 1.10, MeOH). Anal. ($C_{48}H_{57}O_8N_5S_2 \cdot 0.5H_2O$) C, H, N, S.

Boc-3-Me-D-Cys-Pro-Gly-Cys-OBzl (19). To a solution of **18** (1.42 g, 1.58 mmol) in MeOH (800 ml) was added I_2 (1.23 g, 4.83 mmol) in MeOH (50 ml) under vigorous stirring. The mixture was stirred at room temperature for 30 min, and 0.2 M $Na_2S_2O_3$ solution was then added until the color of I_2 disappeared. The mixture was concentrated in vacuo, and the residue was dissolved in EtOAc and saturated aqueous $NaHCO_3$ solution. The organic layer separated was washed with brine, and worked up as usual. The desired product purified by silica-gel column chromatography (70 g, 2.5×48 cm, $CHCl_3$–MeOH=20 : 1) was recrystallized from $CHCl_3$ and hexane to give colorless needles: Yield 826 mg (90%); mp 148—150 °C; $[\alpha]_D^{28}$ +28.7° (*c* 1.17, MeOH); FD-MS, *m/z* 580 (M^+). Anal. ($C_{26}H_{36}O_7N_4S_2 \cdot 0.5H_2O$) C, H, N, S.

Boc-D-Abu-Pro-Gly-Ala-OBzl (20). To a solution of **19** (485 mg, 0.835 mmol) in anhydrous DMF (290 ml) was added $P(NEt_2)_3$ (4.13 g, 16.7 mmol) under N_2 atmosphere.[31] The solution was stirred at room temperature for 7 d, and then concentrated in vacuo. The residue in EtOAc was washed with 10% citric acid and brine, and worked up as usual. The desired product purified by silica-gel column chromatography (70 g, 2.5×48 cm, $CHCl_3$–acetone=5 : 2) was recrystallized from $CHCl_3$, MeOH and hexane to give colorless needles: Yield 187 mg (41%); mp 185—188 °C; $[\alpha]_D^{20}$ +42.1° (*c* 0.368, MeOH); FAB-MS, *m/z* 549 [$(M+H)^+$]. Amino acid analysis (6 M HCl, 110 °C, 24 h): Pro(1.08), Melan(0.89), Gly(1.00). Anal. ($C_{26}H_{36}O_7N_4S \cdot 0.5H_2O$) C, H, N, S.

H-D-Abu-Pro-Gly-Ala-OH (21). The compound **20** (300 mg, 0.546 mmol) was dissolved in 2 ml of TFA and allowed to stand for 1 h at room temperature. The reaction mixture was diluted with benzene and then lyophilized. To the residue transferred into HF reaction apparatus was added anisole (1 ml), and anhydrous HF (9 ml) was introduced into the apparatus under cooling at −70 °C. The mixture was stirred at 0 °C for 1 h, and then HF was evaporated in vacuo at 0 °C. To the residue was added water and the aqueous solution was washed with diethyl ether several times. The aqueous layer was lyophilized to give **21** as colorless powder, which was subjected to the following reaction without purification.

Troc-D-Abu-Pro-Gly-Ala-OH (22). To a solution of **21** (532 mg, 1.49 mmol) in DMF (15 ml) were added TEA (452 mg, 4.47 mmol) and TrocOSu (476 mg, 1.64 mmol) at 0 °C. The mixture was stirred at room temperature for 1 h and concentrated in vacuo. The residue was dissolved in water and washed with diethyl ether. The aqueous layer was applied to a column of HP-20 (4.2×20 cm), and the column was washed with water and 7% aqueous acetic acid. Compound **22** was eluted with MeOH, and the eluate was concentrated in vacuo. The product was precipitated as powder from $CHCl_3$, MeOH, and hexane: Yield 670 mg (84%); mp 262—270 °C. The powdery compound thus obtained was subjected to the next coupling without further purification.

Troc-D-Abu-Pro-Gly-Ala-Lys(Z)-OBu^t (23). To a solution of compound **22** (104 mg, 0.195 mmol), HCl · H-Lys(Z)-OBu^t[40] (72.7 mg, 0.195 mmol), and HOBt (26.3 mg, 0.195 mmol) in DMF (1 ml) was added EDC (30.3 mg, 0.195 mmol) at −70 °C. The solution was stirred at −70 °C for 10 min, at −15 °C for 3 h and at room temperature overnight. The

reaction mixture was concentrated in vacuo and to the residue was added EtOAc. The organic layer washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine was worked up as usual. The crystalline residue was recrystallized from CH_2Cl_2 and hexane to give colorless prisms: Yield 150 mg (90%); mp 118—119 °C; $[\alpha]_D^{19}$ +36.1° (*c* 0.410, MeOH). Anal. ($C_{35}H_{49}N_6O_{10}SCl_3 \cdot 0.5H_2O$) C, H, N, S, Cl.

HCl · H-D-Abu-Pro-Gly-Ala-Lys(Z)-OBu^t (24). To a solution of compound **23** (85.2 mg, 0.100 mmol) in AcOH (3 ml) was added Zn powder (270 mg, 4.1 mmol), and the mixture was stirred at 40 °C for 1 h. The insoluble inorganic material was filtered off and the filtrate was concentrated in vacuo. To the residue were added EtOAc and saturated aqueous $NaHCO_3$ solution, and once again the insoluble material formed was filtered off. The organic layer was washed with brine, and worked up as usual. To the residue in THF (500 μl) was added 5.2 M HCl in THF (40 μl, 0.20 mmol), and the solution was concentrated in vacuo. Compound **24** was precipitated as powder from $CHCl_3$, diethyl ether, and hexane: Yield 61.0 mg (86%); mp 143—146 °C; $[\alpha]_D^{19}$ −14.2° (*c* 0.190, MeOH). Amino acid analysis (6 M HCl, 110 °C, 48 h): Pro(1.03), Melan(1.03), Gly(1.00), Lys(0.90). Anal. ($C_{32}H_{49}N_6O_8SCl \cdot 1.3H_2O$) C, H, N, S, Cl.

Troc-D-Abu-Gly-Ala-Leu-Met-Gly-Ala-OH (26). Deprotection of Boc-D-Abu-Gly-Ala-Leu-Met-Gly-Ala-OBzl (**25**)[20] (390 mg, 0.473 mmol) was carried out stepwise using TFA (3 ml) and then HF–anisole (9 ml/1 ml). The Troc group was introduced with TrocOSu (206 mg, 0.710 mmol) and TEA (144 mg, 1.42 mmol) in DMF (5 ml), and the product was purified as described for the preparation of **22**: Yield 310 mg (81%); mp 189—194 °C; $[\alpha]_D^{28}$ −17.0° (*c* 0.379, MeOH). Anal. ($C_{28}H_{44}N_7O_{10}S_2Cl_3 \cdot 1.5H_2O$) C, H, N, S, Cl.

Nps-Asn(Mbh)-Met-OBu^t (27). To a solution of Z-Met-OBu^t[41] (846 mg, 2.49 mmol) in MeOH (25 ml) were added $HCOONH_4$ (4.71 g, 74.7 mmol), Pd-black (635 mg), and TEA (1.04 ml, 7.47 mmol). After the mixture was stirred at 40 °C for 5 h, the catalyst was filtered off, and to the filtrate were added CH_2Cl_2 and brine. The organic layer separated was washed with water, brine, and dried over $MgSO_4$, and then concentrated in vacuo until the solution became 40 ml. To the solution were added Nps-Asn(Mbh)-OH[42] (1.27 g, 2.49 mmol), HOBt (336 mg, 2.49 mmol), and EDC · HCl (477 mg, 2.49 mmol) under ice cooling. The solution was stirred at room temperature overnight and concentrated in vacuo. The residue in EtOAc was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and worked up as usual. The product was purified by silica-gel column chromatography (85 g, 3.5×25 cm, $CHCl_3$–acetone=20 : 1). The oily residue obtained by vacuum concentration of fractions containing **27** was triturated with hexane to give yellow needles: Yield 748 mg (45%); mp 182—184 °C (decomp); $[\alpha]_D^{24}$ +22.4° (*c* 1.05, DMF). Anal. ($C_{34}H_{42}O_8N_4S_2$) C, H, N, S.

HCl · H-Asn(Mbh)-Met-OBu^t (28). To a solution of **27** (297 mg, 0.425 mmol) in THF (6 ml) was added 6 M HCl (425 μl, 2.55 mmol). The solution was stirred at room temperature for 2 h, and then concentrated in vacuo. The residue was triturated with diethyl ether to give powdery product which was reprecipitated from MeOH and diethyl ether. Colorless powder thus obtained was subjected to the next coupling without further purification: Yield 238 mg (96%).

Troc-D-Abu-Gly-Ala-Leu-Met-Gly-Ala-Asn(Mbh)-Met-OBu^t (29). To a solution of **26** (472 mg, 0.583 mmol), **28** (340 mg, 0.583 mmol), and HOBt (78.8 mg, 0.583 mmol) in DMF (5 ml) was added EDC (127 mg, 0.816 mmol) at −70 °C. The mixture was stirred at −20 °C overnight, and concentrated in vacuo. To the residue was added water, and the precipitate was collected by filtration and was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and water, successively: Yield 704 mg (90%). A part of the product was purified by HPLC (Cosmosil $5C_{18}$ AR, 20×250 mm, gradient elution with 65—85% CH_3CN in 0.1% aqueous TFA (2% min^{-1}), 10 ml min^{-1}) to obtain an analytical sample; mp 217—222 °C; $[\alpha]_D^{28}$ −24.0° (*c* 0.165, DMF). Anal. ($C_{56}H_{81}N_{10}O_{15}S_3Cl_3 \cdot H_2O$) C, H, N, S, Cl.

HCl · H-D-Abu-Gly-Ala-Leu-Met-Gly-Ala-Asn(Mbh)-Met-OBu^t (30). The Troc group of **29** (500 mg,0.374 mmol) was removed with Zn powder (980 mg, 15 mmol) in AcOH (12 ml) as described for the preparation of **24**. The product was purified by silica-gel column chromatography (25 g, 1.5×30 cm, $CHCl_3$–MeOH=9 : 1), and to a solution of the pure compound in $CHCl_3$–MeOH (1 : 1, 1.0 ml) was added 6 M HCl (80 μl, 0.48 mmol) and benzene (10 ml). The mixture was concentrated in vacuo, and the residue was suspended in benzene, followed by lyophilization to give colorless powder: Yield 260 mg (58%); mp 194—197 °C (decomp); $[\alpha]_D^{28}$ −17.0° (*c* 0.250, MeOH). Amino acid analysis (6 M HCl, 110 °C, 48 h): Asp (1.00), Melan(0.86), Gly(1.89), Ala(1.12), Met(1.88), Leu (1.17).

HCl · H-D-Abu-Ala-D-Abu-Ala-His(Ts)-Ala-OMe (32). The Boc group of Boc-D-Abu-Ala-D-Abu-Ala-His(Ts)-Ala-OMe[21,22] (**31**) (710 mg, 0.819 mmol) was removed with TFA (5 ml) as described for the preparation of **10**. To the oily TFA salt obtained was added 12.5 M HCl in MeOH (92 μl, 1.15 mmol), and the mixture was concentrated in vacuo. To the residue was added diethyl ether to give powdery hydrochloride **32**, which was subjected to the next coupling without further purification: Yield 660 mg (quant).

Boc-Lys(Z)-D-Abu-Ala-D-Abu-Ala-His-Ala-OMe (33). Condensation of Boc-Lys(Z)-OH (327 mg, 0.860 mmol) and compound **32** (0.819 mmol) was carried out in DMF (8 ml) using EDC (140 mg, 0.901 mmol) and HOBt (116 mg, 0.860 mmol) as described for the preparation of **12**. To the reaction mixture was added HOBt (443 mg, 3.28 mmol) to complete the removal of the N^{im}-Ts group. The solution was stirred at room temperature for 9 h, and then concentrated in vacuo. The residue in EtOAc was washed with saturated aqueous $NaHCO_3$ solution and brine, and worked up as usual. The coupling product was precipitated as powder from EtOAc, MeOH, and hexane. The precipitate collected by filtration was further washed with water and diethyl ether: Yield 547 mg (69%); mp 164—166 °C (decomp); $[\alpha]_D^{19}$ −10.0° (*c* 0.260, MeOH). Anal. ($C_{43}H_{62}O_{12}N_{10}S_2 \cdot 2H_2O$) C, H, N, S.

Boc-Lys(Z)-D-Abu-Ala-D-Abu-Ala-His-Ala-NHNH$_2$ (34).

To a solution of compound **33** (400 mg, 0.410 mmol) in DMF (4 ml) was added $NH_2NH_2 \cdot H_2O$ (410 μl, 8.2 mmol), and the solution was allowed to stand at room temperature for 7 h. To the solution was added water, and the mixture was lyophilized. The residue was suspended in water and lyophilized again. The powdery residue thus obtained was reprecipitated from MeOH and diethyl ether. The precipitate was collected by filtration and washed with water and diethyl ether, successively: Yield 355 mg (89%). Compound **34** thus obtained was subjected to the next coupling without further purification.

Boc-A_2pr(Troc)-OH·DCHA (35). To a solution of Boc-Asn-OH (4.00 g, 17.2 mmol) in DMF (30 ml) and water (30 ml) were added pyridine (2.78 ml, 34.4 mmol) and bis-*I*-(trifluoroacetoxy)iodobenzene (11.1 g, 25.8 mmol) at 0 °C.[43] The solution was stirred at 0 °C for 30 min and then at room temperature for 3 h. To the reaction mixture was added $NaHCO_3$ until the evolution of CO_2 ceased. The solution was washed with diethyl ether, and then saturated with $NaHCO_3$. To this basic solution was added TrocOSu (5.49 g, 18.9 mmol), and the mixture was stirred at room temperature overnight, followed by filtration. The filtrate was once washed with EtOAc, acidified with citric acid, and then extracted with EtOAc. The extract was washed with 10% citric acid and brine, and worked up as usual. To a solution of the residue in EtOAc was added dicyclohexylamine (3.37 ml, 17.2 mmol). The crystalline DCHA salt **35** precipitated by addition of hexane was collected by filtration to give colorless needles: Yield 6.60 g (68%); mp 196—197 °C; $[\alpha]_D^{18}$ +7.3° (*c* 1.1, MeOH). Anal. ($C_{23}H_{40}O_6N_3Cl_3$) C, H, N, Cl.

Boc-A_2pr(Troc)-Lys(Z)-OBzl (36). Compound **35** (2.60 g, 4.60 mmol) was treated with EtOAc and 10% citric acid. The organic layer was washed with water, and worked up as usual. To a solution of the residue, TsOH·H-Lys(Z)-OBzl[44] (2.50 g, 4.60 mmol), and HOBt (621 mg, 4.60 mmol) in THF (25 ml) was added DCC (948 mg, 4.60 mmol) at 0 °C. After the mixture was stirred at room temperature overnight, an insoluble material precipitated was filtered off, and the filtrate was concentrated in vacuo. The residue in EtOAc was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and worked up as usual. Compound **36** was obtained as powder by reprecipitation of the residue from EtOAc and hexane: Yield 2.76 g (82%); mp 105—107 °C; $[\alpha]_D^{21}$ −16.3° (*c* 1.04, MeOH). Anal. ($C_{23}H_{41}O_9N_4Cl_3$) C, H, N, Cl.

Boc-Val-A_2pr(Troc)-Lys(Z)-OBzl (37). Compound **36** (2.50 g, 3.41 mmol) in TFA (6 ml) was allowed to stand at room temperature for 30 min, and the solution was concentrated in vacuo. The residue dissolved in EtOAc was washed with saturated aqueous $NaHCO_3$ solution and brine, and worked up as usual. To a solution of the obtained H-A_2pr(Troc)-Lys(Z)-OBzl in THF (20 ml) were added Boc-Val-OH (778 mg, 3.58 mmol), DCC (737 mg, 3.58 mmol), and HOBt (484 mg, 3.58 mmol), and worked up as described for the preparation of **36**. Compound **37** was obtained by reprecipitation of the product from EtOAc, diethyl ether, and hexane: Yield 2.12 g (75%); mp 158—161 °C; $[\alpha]_D^{21}$ −26.6° (*c* 1.06, MeOH). Anal. ($C_{37}H_{50}O_{10}N_5Cl_3$) C, H, N, Cl.

Boc-Val-A_2pr-Lys(Z)-OBzl (38). The Troc group of **37** (420 mg, 0.505 mmol) was removed with Zn powder (1.00 g, 15.2 mmol) in AcOH (10 ml) as described for the preparation of **24** and the obtained oily product was subjected to the following reaction without purification.

Boc-Val-Dha-Lys(Z)-OBzl (39). To a solution of **38** (0.505 mmol) in MeOH (6 ml) were added $KHCO_3$ (900 mg, 9.00 mmol) and CH_3I (1.28 g, 9.00 mmol). To the mixture stirred at room temperature overnight were added EtOAc and water. The organic layer was washed with brine, and worked up as usual. The residue was purified by silica-gel column chromatography (20 g, 1.5×24 cm, $CHCl_3$–acetone=20 : 1) to give **39** as an oily material: Yield 236 mg (73%); $[\alpha]_D^{21}$ −19.3° (*c* 1.07, MeOH); ^{1}H NMR (100 MHz, $CDCl_3$) δ=0.93 (6H t, *J*=6.3 Hz, Val; γ-CH_3×2), 1.2—1.4 (2H, m, Lys; γ-CH_2), 1.44 (9H, s, Boc), 1.45—2.0 (4H, m, Lys; β, δ-CH_2), 2—2.2 (1H, m, Val; β-CH), 3.0—3.2 (2H, m, Lys; ε-CH_2), 4.60 (1H, m, α-CH), 4.84 (1H, m, α-CH), 5.08 (2H, s, C$\underline{H}_2C_6H_5$), 5.15—5.25 (2H, m, C$\underline{H}_2C_6H_5$), 5.42 (1H, bs, Dha; β-CH), 6.46 (1H, bs, Dha; β-CH), 7.0 (1H, bd, *J*=7.3 Hz, NH), 7.2 (1H, m, NH), 7.25—7.4 (10H, m, $CH_2C_6\underline{H}_5$), 7.5 (1H, m, NH), 8.47 (1H, bs, NH). Anal. ($C_{34}H_{46}O_8N_4 \cdot 0.3H_2O$) C, H, N.

Boc-His(Ts)-Val-Dha-Lys(Z)-OBzl (40). The Boc group of the compound **39** (4.20 g, 6.57 mmol) was removed with TFA (25 ml) as described for the preparation of **37**. H-Val-Dha-Lys(Z)-OBzl thus prepared was coupled with Boc-His(Ts)-OH[45] (2.69 g, 6.57 mmol) in CH_2Cl_2 (40 ml) using DCC (1.35 g, 6.57 mmol) and HOBt (888 mg, 6.57 mmol) at room temperature for 3 h, and then to the reaction mixture were added TsCl (1.71 g, 8.95 mmol) and TEA (906 mg, 8.95 mmol). The mixture was stirred at room temperature for 1 h, and concentrated in vacuo. The residue was dissolved in EtOAc, and the insoluble material was filtered off. The filtrate was concentrated in vacuo, and the residue was purified by silica-gel column chromatography (150 g, 3×45 cm, $CHCl_3$–MeOH=40 : 1). Compound **40** was obtained as powder from EtOAc and hexane after the chromatography: Yield 4.42 g (72%); mp 68—74 °C; $[\alpha]_D^{21}$ −18.7° (*c* 1.03, MeOH). Anal. ($C_{47}H_{59}O_{11}N_7S \cdot H_2O$) C, H, N, S.

Boc-Ile-His(Ts)-Val-Dha-Lys(Z)-OBzl (41). The Boc group of the compound **40** (5.17 g, 5.56 mmol) was removed with TFA (30 ml) as described for the preparation of **37**. To a solution of H-His(Ts)-Val-Dha-Lys(Z)-OBzl in CH_2Cl_2 (40 ml) were added Boc-Ile-OH (1.34 g, 5.56 mmol), DCC (1.15 g, 6.57 mmol), and HOBt (751 mg, 5.56 mmol). After stirring at room temperature overnight, reintroduction of the N^{im}-Ts group was carried out using TsCl (1.38 g, 7.23 mmol) and TEA (731 mg, 7.23 mmol) as described for the preparation of **40**. The crude product was purified by silica-gel column chromatography (75 g, 2.5×50 cm, $CHCl_3$–MeOH–acetone=40 : 1 : 3), and the desired product was precipitated as powder from EtOAc and hexane after the chromatography: Yield 4.64 g (80%); mp 109—115 °C; $[\alpha]_D^{13}$ −27.2° (*c* 1.05, MeOH). Anal. ($C_{53}H_{70}O_{12}N_8S \cdot H_2O$) C, H, N, S.

Boc-Ser(Bzl)-Ile-His(Ts)-Val-Dha-Lys(Z)-OBzl (42). The Boc group of compound **41** (4.64 g, 4.45 mmol) was removed with TFA (30 ml) as described for the preparation of **37**. To a solution of H-Ile-His(Ts)-Val-Dha-Lys(Z)-OBzl in CH_2Cl_2 (25 ml) were added Boc-Ser(Bzl)-OH (1.32 g, 4.45 mmol), DCC (917 mg, 4.45 mmol), and HOBt (601 mg, 4.45 mmol). To the mixture stirred at room temperature overnight were added TsCl (1.38 g, 7.23 mmol) and TEA (731 mg, 7.23 mmol) as described for the preparation of **40**. The crude product was purified by silica-gel column chromatography (75 g, 2.5×50 cm, $CHCl_3$–MeOH–acetone=35 : 1 : 2), and compound **42** was obtained as powder from EtOAc and hexane after the chromatography: Yield 3.39 g (62%); mp 138—143 °C; $[\alpha]_D^{13}$ −27.0° (*c* 1.03, MeOH). Anal. ($C_{63}H_{81}O_{14}N_9S$) C, H, N, S.

Boc-Ser(Bzl)-Ile-His-Val-Dha-Lys(Z)-OBzl (43). To a

solution of compound **42** (783 mg, 0.642 mmol) in THF (10 ml) was added HOBt (520 mg, 3.85 mmol), and the solution was allowed to stand at 30 °C for 3 d, followed by dilution with EtOAc. The solution was washed with saturated aqueous $NaHCO_3$ solution and brine, and worked up as usual. The residual compound **43** was precipitated as powder from $CHCl_3$ and hexane: Yield 544 mg (81%); mp 120—123 °C; $[\alpha]_D^{13}$ −36.9° (*c* 1.03, MeOH). Amino acid analysis (6 M HCl, 110 °C, 48 h): Ser(1.00), Val(1.20), Ile(1.07), His(0.87), Lys(0.97). Anal. ($C_{56}H_{75}O_{12}N_9 \cdot 0.75H_2O$) C, H, N.

Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-D-Abu-Pro-Gly-Ala-Lys(Z)-OBu^t (44). To a solution of **14** (208 mg, 0.255 mmol), **24** (182 mg, 0.255 mmol), and HOBt (34.5 mg) in DMF (3 ml) was added EDC (59.4 mg, 0.383 mmol) at −70 °C. The mixture was stirred at −20 °C overnight, and then concentrated in vacuo. The residue dissolved in EtOAc was washed with 10% citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and worked up as usual. The residue was purified by silica-gel column chromatography (25 g, 1.5×30 cm, $CHCl_3$–MeOH=30 : 1), and compound **44** was obtained as powder from diethyl ether and hexane after the chromatography: Yield 210 mg (56%); mp 200—210 °C; $[\alpha]_D^{28}$ −20.0° (*c* 0.183, MeOH). Anal. ($C_{71}H_{103}O_{17}N_{13}S_2 \cdot 2.3H_2O$) C, H, N, S.

Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-D-Abu-Pro-Gly-Ala-Lys(Z)-OH (45). To a mixture of **44** (46.0 mg, 0.0312 mmol) in CH_2Cl_2 (1 ml) was added TFA (1 ml) at 0 °C, and the solution stirred at 15 °C for 2 h was diluted with benzene. After evaporation of CH_2Cl_2 in vacuo, the reaction mixture was lyophilized and purified by HPLC (Cosmosil $5C_{18}$ AR, 20×250 mm, gradient elution with 60—90% CH_3CN in 0.1% aqueous TFA (2% min^{-1}), 10 ml min^{-1}). The fraction obtained was concentrated in vacuo, and the residue was precipitated from $CHCl_3$ and hexane to give **45** as powder: Yield 32.0 mg (72%) [recovery of **44** 4.0 mg (9%)]; mp 151—154 °C; $[\alpha]_D^{28}$ −10.0° (*c* 0.251, DMF); ^1H NMR (270 MHz, $CDCl_3$–CD_3OD=10 : 1) δ=0.8—1.05 (18H, Ile; γ-CH_3×2, δ-CH_3×2, Leu; δ-CH_3×2), 1.1—2.0 (16H, m), 1.28 (3H, d, *J*=7.4 Hz, Abu_L; γ-CH_3), 1.78 (3H, d, *J*=7.2 Hz, Dhb; γ-CH_3), 2.0—2.15 (2H, m), 2.2—2.4 (1H, m), 2.8—3.5 (11H, m), 3.6 (1H, m), 3.8—4.0 (2H, m), 4.09 (1H, d, *J*=7.2 Hz), 4.2—4.4 (3H, m), 4.45 (1H, m), 4.65 (1H, m), 4.78 (1H, m), 4.88 (1H, m), 5.02 (1H, d, *J*=12.4 Hz, $C\underline{H}_2C_6H_5$) 5.08 (2H, s, $C\underline{H}_2C_6H_5$), 5.15 (1H, d, *J*=12.4 Hz, $C\underline{H}_2C_6H_5$), 5.50 (1H, s, Dha; β-CH), 6.03 (1H, bs, Dha; β-CH), 6.34 (1H, q, *J*=7.2 Hz, Dhb; β-CH), 7.25—7.4 (10H, m, $CH_2C_6\underline{H}_5$×2). Amino acid analysis (6 M HCl, 110 °C, 48 h): Pro(1.00), Melan(0.98), *meso*-Lan(1.07), Gly(1.00), Ile(2.00), Leu(1.05), Lys(0.93). Anal. ($C_{67}H_{95}O_{17}N_{13}S_2 \cdot 2.5H_2O$) C, H, N, S.

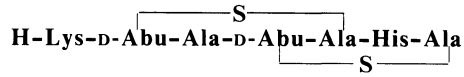
Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-D-Abu-Pro-Gly-Ala-Lys(Z)-OH (45) from Peptide 1. To a solution of H-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-D-Abu-Pro-Gly-Ala-Lys-OH[17] (**1**) (46 mg, 0.040 mmol) in MeOH (2.2 ml) were added ZOSu (30 mg, 0.12 mmol) and TEA (12 mg, 0.12 mmol). The solution was stirred at room temperature for 2 h, and then concentrated in vacuo. The residue was purified by silica-gel column chromatography (10 g, 1.5×12 cm, $CHCl_3$–MeOH–AcOH=9 : 1 : 1) to give **45** as powder: Yield 32 mg (56%); mp 150—155 °C; $[\alpha]_D^{19}$ −11° (*c* 0.26, DMF).

Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-D-Abu-Pro-Gly-Ala-Lys(Z)-D-Abu-Gly-Ala-Leu-Met-Gly-Ala-Asn(Mbh)-Met-OBu^t (46). A condensation of compound **45** (38.0 mg, 26.8 μmol) with **30** (35.3 mg, 29.5 μmol) was carried out in DMF (1 ml) using EDC (5.4 mg, 3.5 μmol) and HOBt (4.4 mg, 3.2 μmol) as described for the preparation of **44**. The crude product was purified by HPLC (Asahipak C4P-50, 21.5×300 mm, gradient elution with 60—80% CH_3CN in H_2O (2% min^{-1}), 5 ml min^{-1}) and the fraction was concentrated in vacuo to give **46** as powder: Yield 41.7 mg (61%); mp 174—178 °C; $[\alpha]_D^{27}$ −36.0° (*c* 0.176, MeOH). Amino acid analysis (6 M HCl, 110 °C, 60 h): Asp(1.09), Pro(0.88), Melan(2.23), *meso*-Lan(1.13), Gly(2.91), Ala(1.00), Met(1.85), Ile(1.68), Leu (1.93), Lys(0.82). Anal. ($C_{120}H_{173}O_{29}N_{23}S_5 \cdot 3H_2O$) C, H, N, S.

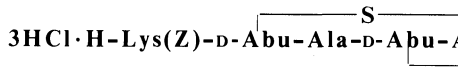
Z-Ile-Dhb-D-Ala-Ile-Dha-Leu-Ala-D-Abu-Pro-Gly-Ala-Lys(Z)-D-Abu-Gly-Ala-Leu-Met-Gly-Ala-Asn-Met-OH (47). The compound **46** (38.0 mg, 14.8 μmol) was dissolved in TFA (600 μl) and anisole (60 μl), and the solution was allowed to stand at 18 °C for 2 h. The reaction mixture was diluted with benzene, and lyophilized. To a solution of the residue in MeOH (3 ml) were added ZOSu (5 mg, 0.02 mmol) and TEA (10 mg, 0.1 mmol). The solution was stirred at room temperature for 2 h, and then applied to HPLC purification (Asahipak C4P-50, 21.5×300 mm, gradient elution with 60—90% CH_3CN in 0.1% aqueous TFA (2% min^{-1}), 5 ml min^{-1}). The eluate was concentrated in vacuo, and the product **47** was obtained as powder from $CHCl_3$ and hexane: Yield 24.0 mg (71%); mp 177—180 °C; $[\alpha]_D^{26}$ −43° (*c* 0.118, MeOH). Amino acid analysis (6 M HCl, 110 °C, 48 h): Asp(1.00), Pro(1.00), Melan(2.01), *meso*-Lan(1.00), Gly(3.10), Ala(1.19), Met(1.47), Ile(2.07), Leu(2.40), Lys(0.94). Anal. ($C_{101}H_{151}O_{27}N_{23}S_5 \cdot 10H_2O$) C, H, N.

Boc-Lys(Z)-D-Abu-Ala-D-Abu-Ala-His(Boc)-Ala-Ser(Bzl)-Ile-His(Boc)-Val-Dha-Lys(Z)-OBzl (48). Boc-Ser(Bzl)-Ile-His-Val-Dha-Lys(Z)-OBzl (**43**) (460 mg, 0.432 mmol) was dissolved in TFA (7 ml) and allowed to stand at 0 °C for 15 min. To the solution was added benzene and the mixture was lyophilized to give TFA · H-Ser(Bzl)-Ile-His-Val-Dha-Lys(Z)-OBzl as powder in a quantitative yield. To a solution of hydrazide **34** (418 mg, 0.428 mmol) in DMF (4 ml) were added 4.70 M HCl in THF (340 μl, 1.60 mmol) and isopentyl nitrite (75 mg, 0.64 mmol) at −70 °C, and the solution was stirred at −20 °C for 2 h. To the mixture were added TFA · H-Ser(Bzl)-Ile-His-Val-Dha-Lys(Z)-OBzl and TEA (216 mg, 2.14 mmol) at −70 °C, and the solution was stirred at −20 °C for 5 h. After addition of Boc_2O (748 mg, 3.42 mmol) to the mixture, the solution was further stirred overnight, and then concentrated in vacuo. The residue dissolved in EtOAc was washed with brine, and worked up as usual. The crude product was purified by silica-gel column chromatography (55 g, 2.5×38 cm, $CHCl_3$–MeOH=35 : 1). The pure compound **48** was obtained as powder from $CHCl_3$, MeOH, and hexane: Yield 334 mg (37%); mp 220—225 °C (decomp); $[\alpha]_D^{19}$ −31.8° (*c* 0.110, DMF). Amino acid analysis (6 M HCl, 110 °C, 48 h): Ser(0.86), Melan(2.20), Ala(1.00), Val(1.10), Ile(0.85), His(1.79), Lys(1.84). Anal. ($C_{103}H_{141}$-

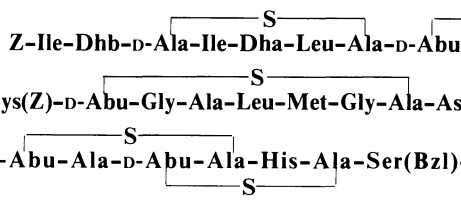
$O_{25}N_{19}S_2 \cdot 2.5H_2O$) C, H, N, S.



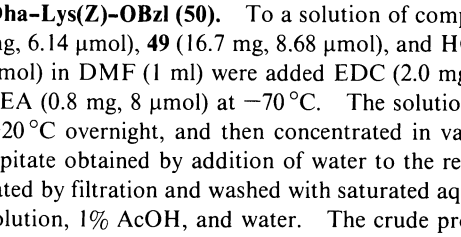
Val-Dha-Lys-OH (2). A solution of **48** (4.5 mg, 2.4 μmol) in TFA (700 μl) was allowed to stand at room temperature for 20 min. TFA was removed by blowing of nitrogen stream and the residue was dried over NaOH in vacuo. To the residue was added anisole (500 μl) and anhydrous HF (4.5 ml) was introduced into the mixture under cooling at −70 °C. The mixture was stirred at 0 °C for 1 h, and then HF was evaporated in vacuo at 0 °C. To the residue was added water and the mixture was washed several times with diethyl ether. The aqueous layer was lyophilized to give **2** as colorless powder: Yield 3.2 mg (quant.)



Ser(Bzl)-Ile-His-Val-Dha-Lys(Z)-OBzl (49). Compound **48** (80.6 mg, 38.2 μmol) was dissolved in TFA (2 ml), and allowed to stand at 0 °C for 1.5 h. To the solution was added benzene and the mixture was lyophilized. The product was purified by HPLC (Nucleosil $7C_{18}$, 8×250 mm, gradient elution with 35—50% CH_3CN in 0.01 M HCl (1% min^{-1}), 3.0 ml min^{-1}) and the eluate was lyophilized to obtain **49** as powder: Yield 41.5 mg (58%). Amino acid analysis (6 M HCl, 110 °C, 48 h): Ser(0.87), Melan(2.07), Ala(1.00), Val(1.06), Ile(0.83), His(1.76), Lys(1.84).



Dha-Lys(Z)-OBzl (50). To a solution of compounds **47** (14.0 mg, 6.14 μmol), **49** (16.7 mg, 8.68 μmol), and HOBt (1.4 mg, 10 μmol) in DMF (1 ml) were added EDC (2.0 mg, 12 μmol) and TEA (0.8 mg, 8 μmol) at −70 °C. The solution was stirred at −20 °C overnight, and then concentrated in vacuo. The precipitate obtained by addition of water to the residue was separated by filtration and washed with saturated aqueous $NaHCO_3$ solution, 1% AcOH, and water. The crude product was purified by HPLC (Cosmosil $5C_{18}$ AR, 20×250 mm, gradient elution with 55—80% CH_3CN in 0.1% aqueous TFA (2% min^{-1}), 10 ml min^{-1}) and the eluate was concentrated in vacuo to give **50** as powder: Yield 12.0 mg (48%), mp 180—185 °C, $[\alpha]_D^{27}$ −31.0° (*c* 0.103, MeOH). Anal. ($C_{189}H_{266}O_{45}N_{42}S_7 \cdot 4CF_3CO_2H \cdot 7H_2O$) C, H, N.



OH (Nisin). To a suspension of compound **50** (6.0 mg, 1.5 μmol) in anisole (200 μl) was introduced HF (2 ml) under cooling at −70 °C. The mixture was stirred at 0 °C for 1 h, and then HF was evaporated in vacuo at 0 °C. To the residue were added H_2O and diethyl ether, and the aqueous layer was lyophilized to give powder which was purified by HPLC (Cosmosil $5C_{18}$ AR, 20×250 mm, gradient elution with 30—50% CH_3CN in 0.3 M guanidine hydrochloride (2% min^{-1}), 10 ml min^{-1}). The eluate containing nisin was lyophilized and the residue was purified again by HPLC (Cosmosil $5C_{18}$ AR, 20×250 mm, gradient elution with 30—50% CH_3CN in 0.03 M HCl (2% min^{-1}), 10 ml min^{-1}) in order to remove guanidine hydrochloride. The eluate was lyophilized to give pure nisin: Yield 3.5 mg (71%).[46] FAB-MS, *m/z* 3352.7 [$(M+H)^+$]. Calcd for $C_{143}H_{231}O_{37}N_{42}S_7$: M+H, 3352.6. ^1H NMR (270 MHz, DMSO-d_6) δ=0.75—0.95 (33H), 0.97 (3H, Ile^1; γ-CH_3), 1.0—1.5 (33H), 1.5—1.9 (19H), 1.71 (3H, Dhb^2; γ-CH_3), 1.9—2.1 (7H), 2.00 (3H, Met; *S*-CH_3), 2.02 (3H, Met; *S*-CH_3), 2.1—2.6 (5H), 2.6—2.9 (13H), 2.9—3.8 (21H), 3.8—4.0 (5H), 4.0—4.9 (24H), 4.96 (1H, Abu_L^8; α-CH), 5.50 (1H, Dha^5; β(*E*)-CH), 5.58 (1H, Dha^{33}; β(*E*)-CH), 6.00 (1H, Dha^5; β(*Z*)-CH), 6.15 (1H, Dha^{33}; β(*Z*)-CH), 6.27 (1H, Dhb^2; β-CH), 6.9—9.1 (NH, $His^{27,31}$ aromatic), 9.17 (1H, Dha^{33}; NH), 9.73 (1H, Dhb^2; NH).[32] Amino acid analysis (6 M HCl, 110 °C, 40 h): Asp(0.97), Ser(1.06), Pro(1.12), Melan(3.88), *meso*-Lan(1.03), Gly(3.13), Ala(2.00), Val(0.90), Met(1.90), Ile(3.01), Leu(2.21), His(1.71), Lys(2.99).

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25) Abbreviations according to IUPAC-IUB commission, *Eur. J. Biochem.*, **138**, 9 (1984), are used. Abu: 2- (or α)-aminobutyric acid; A_2pr: 2,3- (or α,β)-diaminopropionic acid; Acm: acetamidomethyl; BDP: 1-benzotriazolyl diethyl phosphate; Boc: *t*-butoxycarbonyl; Boc_2O: di-*t*-butyl dicarbonate; Bu^t: *t*-butyl; Dha: dehydroalanine; Dhb: dehydrobutyrine; DCC: dicyclohexylcarbodiimide; DCHA: dicyclohexylamine; DMF: *N,N*-dimethylformamide; DMSO: dimethyl sulfoxide; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FAB-MS: fast atom bombardment mass spectrometry; HOBt: 1-hydroxybenzotriazole; HOSu: *N*-hydroxysuccinimide; 3-MeCys: 3-methylcysteine; Mbh: 4,4′-dimethoxybenzhydryl; Ms: methanesulfonyl (or mesyl); Nps: *o*-nitrophenylsulfenyl (or *o*-nitrophenylthio); TEA: triethylamine; TFA: trifluoroacetic acid; THF: tetrahydrofuran; Troc: 2,2,2-trichloroethoxycarbonyl; TrocOSu: *N*-(2,2,2-trichloroethoxycarbonyloxy)succinimide; Ts: *p*-toluenesulfonyl (or tosyl); Trt: triphenylmethyl (or trityl); Z: benzyloxycarbonyl; ZOSu: *N*-(benzyloxycarbonyloxy)-succinimide.
26) An addition of a little amount of water will accelerate the β-elimination.
27) An addition of TEA to the reaction mixture accelerated the hydrogenation.
28) The condensation of Z-Ile-Dhb-OH with ring A did not also proceed smoothly giving a complex mixture which was difficult to be purified by silica-gel column chromatography.
29) The methyl signal in peptide **13** appeared at 1.78 ppm in $CDCl_3$-CD_3OD (10 : 1) whereas at 1.70 ppm[1a,b] in DMSO-d_6.
30) In general, the doublet methyl signal of *Z*-isomer of the Dhb residue appeared nearly at 1.7 ppm whereas that of *E*-isomer nearly at 2.0 ppm: A. Srinivasan, R. W. Stephenson, and R. K. Olsen, *J. Org. Chem.*, **42**, 2256 (1977).
31) Since the contamination of a trace amount of water or bases in the reaction medium caused serious side reactions, the desulfurization reaction must be carried out under anhydrous conditions and N_2 atmosphere using freshly and carefully distilled solvents. The procedures of preparation of anhydrous solvents are as follows: DMF; commercially available solvent was dried over molecular sieves 3A for 3 d, distilled under reduced pressure, dried over anhydrous $CuSO_4$ for about 1 week, and then distilled under reduced pressure: THF; commercially available solvent was distilled, dried over Na, refluxed in the presence of Na and benzophenone until the color of the solvent turned purple, and then distilled: benzene; prepared in the same manner as THF.
32) The some proton signals of synthetic nisin were slightly shifted from that of natural ones perhaps due to the difference of composition of the salt: 1.71 from 1.70 (Dhb^2; γ-CH_3), 4.96 from 4.92 (Abu$_L^8$; α-CH), 5.50 from 5.54 (Dha^5; β(*E*)-CH), 5.58 from 5.63 (Dha^{33}; β(*E*)-CH), 6.00 from 6.07 (Dha^5; β(*Z*)-CH), 6.15 from 6.10 (Dha^{33}; β(*Z*)-CH), 6.27 from 6.50 (Dhb^2; β-CH), 9.17 from 9.18 (Dha^{33}; NH), 9.73 from 10.01 (1H, Dhb^2; NH). However, the ^{1}H NMR of the mixture of natural and synthetic nisin was completely identical with that of synthetic nisin. All proton signals of natural nisin in DMSO-d_6 solution were already assigned by Goodman et al. except the assignment of *S*-methyl resonances to a specific methionine.[47]
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