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Synthetic Study on Peptide Antibiotic Nisin. I. The Synthesis of Ring A[1]

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Synthesis of the ring A, a cyclic sulfide peptide part containing dehydroalanine residue, in peptide antibiotic nisin was successfully achieved by applications of two novel procedures. First, a sulfide ring was derived from a cyclic disulfide peptide by desulfurization with $P(Et_2N)_3$. Secondly, for the preparation of dehydroalanyl residue, a simple and convenient method through Hofmann degradation of 2,3-diaminopropionyl residue was newly exploited in the presence of sulfide linkage.

Nisin, a peptide antibiotic, was isolated from culture broth of *Streptococcus lactis* by Mattick *et al.* in 1947,[2] and Berridge *et al.* in 1952.[3] However, in spite of the early isolation, the structure of nisin was first proposed by Gross *et al.* in 1970.[4] As shown in Fig. 1, nisin has a quite unique structure composed of thirty four amino acids including three dehydroamino acid residues and five sulfide rings named A, B, C, D, and E from the *N*-terminal in the molecule.

An antimalarial activity as well as an inhibition of growth of *Staphylococcus aureus* were known as typical biological activities of this peptide.[5] However, few works are reported about the structure-biological activity relationships except a role of dehydroalanine residue for an exhibition of the antibacterial activity.[5] Thus, we started the synthetic study of nisin from the standpoint of not only a synthetic interest in such a complicated structure but also a confirmation of its unique structure and detailed elucidation on structure-activity relationships of this antibiotic.

For accomplishment of the synthesis of nisin, we are required to resolve two problems in the synthetic procedures, *i.e.*, i) introduction of the dehydroamino acid residue; ii) formation of the sulfide ring. Especially, both points are very important to carry out the synthesis of the ring A (Fig. 2) as the first target of the total synthesis of nisin. We attempted to solve the first problem by application of our novel method for the dehydroalanine synthesis through Hofmann degradation of 2,3-diaminopropionic acid (A_2pr).[6]

The second problem concerns to how to prepare lanthionine moiety in a cyclic peptide. For synthetic strategies of cyclic lanthionine peptide, possibly the following three procedures may be considered (Fig. 3). First of all, a cyclization can be performed by amide bond formation between carboxyl and amino groups at suitable positions in a lanthionyl peptide. Indeed, Photaki and her coworkers applied this concept to prepare ring A of nisin based on their extensive knowledge of lanthionine chemistry.[7] Although, a route a) seems to be quite reasonable, much efforts are required for preparation of the *meso*-lanthionyl residue. For instance, four functional groups in lanthionine moicty must be differentially protected and also made free step by step. This is not so easy task, and furthermore, a yield of cyclization by amide bond formation is sometimes not so high.

Therefore, we attempted to find more convenient method for sulfide ring formation. One of the promising ways may be an addition of mercapto group to the dehydroalanyl residue as described in route b). Although highly expensive D-cysteine is not required in this case, crucial disadvantage of this route is no assurance of stereoselective addition to obtain a desired steric form of lanthionine. Therefore, a final route c) seems to be most attractive procedure, if desulfurization of a cyclic disulfide peptide will successfully proceed, because a formation of –S–S– bond is rather easier than a cyclization by amide bond formation.

Based on the above considerations, the linear peptide **8** containing two cysteine residues of D- and L-configurations and 2,3-diaminopropionic acid residue as precursors of *meso*-lanthionine and dehydroalanine, respectively, was prepared as shown in Fig. 4. Protection of the mercapto groups in Cys residues and S–S bond formation were made by reference to Kamber's elucidation, which suggested an oxidative disulfide formation effected with I_2 from ~~Cys(Acm)~~Cys(Acm)~~ in DMF or ~~Cys(Trt)~~Cys(Acm)~~ in MeOH.[8] For reasons of easiness of *S*-tritylation compared to *S*-acetamidomethylation, convenience of experimental procedure in MeOH than DMF, and possible prevention of the intermolecular S–S linkage formation, the latter combination of protecting groups seemed to be preferable. On the other hand, N^{β}-amino group of A_2pr residue was protected with benzyl-

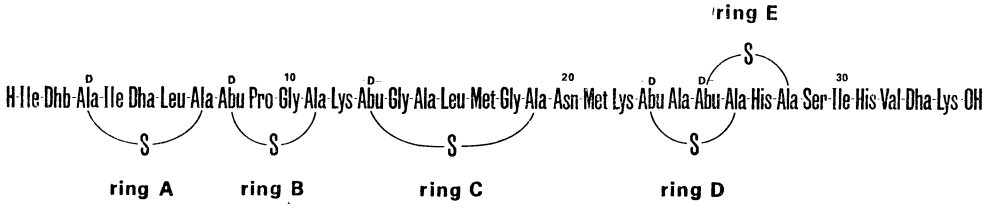

Fig. 1. The proposed structure of nisin. Abu: 2-aminobutyric acid; Dha: dehydroalanine; Dhb: dehydrobutyrine (=3-methyldehydroalanine); Ala Ala: lanthionine; Abu Ala: β-methyllanthionine.

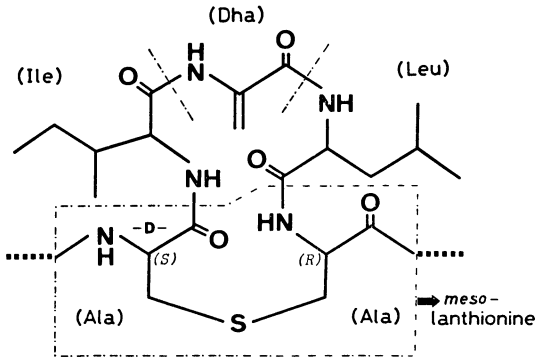

Fig. 2. The chemical structure of the ring A part in nisin.

oxycarbonyl (Z) group which is removable by the catalytic hydrogenation even though in the presence of sulfide moiety.[9] The peptide elongation was performed starting from the *C*-terminal by use of an active ester method.

The fully protected linear pentapeptide **8** thus obtained was then subjected to the preparation of the cyclic disulfide **9**. The oxidation was carried out smoothly to give the monomeric disulfide, whose molecular weight was confirmed by both mass spectrometry (FD, *m/z* 782; calcd 782) and osmometry (750± 37; calcd 783), in a good yield as expected.

The desulfurization reaction for the cystine peptide **9**, one of the important targets in this study, was achieved using tris(diethylamino)phosphine ($P(Et_2N)_3$) reported by Harpp for the preparation of lanthionine itself.[10] When the reaction was carried out in $CHCl_3$, the yield of the sulfide compound **10** was not reproducible and always poor. In order to check the reaction conditions, we then examined an effect of solvent on the desulfurization using a model compound, $(Z\text{-}Cys\text{-}OCH_3)_2$. The desirable lanthionine derivative was obtained only in DMF presumably *via* path (a) based on the concerted mechanism as shown in Fig. 5, while in solvents such as $CHCl_3$, THF, dioxane, or benzene, the dehydroalanine derivative was found to be a major product as a result of intramolecular β-elimination as shown in path (b). Thus, the cyclic disulfide compound **9** was successfully desulfurized in DMF to produce cyclic *meso*-lanthionyl peptide **10** without a formation of dehydroalanyl peptide. In this case, care must be taken to dry DMF absolutely, otherwise another side reaction as shown in Fig. 6 occurs.[11] The formation of *meso*-lanthionyl residue in this reaction was clearly ascertained by amino acid analysis of the hydrolyzate of **10**. (Fig. 8b)

A conversion of the A_2pr residue into the dehydroalanine residue was originally performed by a permethylation with CH_3I in the presence of $KHCO_3$.[6] However, according to this procedure, a methylation onto sulfur atom also occurred resulting in the undesirable formation of another dehydroalanyl residue. For example, *S*-methylcysteinyl peptide **14** as a model peptide gave a mixture of monodehydroalanyl peptide **15** and didehydroalanyl peptide **16**. (Fig. 7) Even under the most preferable conditions, the yield of **15** was less than 70% and the by-product **16** was still obtained in a yield of 10%.[6a] In order to prevent this undesirable *S*-methylation, we attempted to increase a reactivity of β-amino group of A_2pr compared to that of sulfide part. For this purpose, the β-amino group was once methylated to increase its basicity by an application of reductive *N*-alkylation with $NaBH_3CN$ and HCHO.[12] From model experiments using two simple peptides **14** and **18**, it was revealed that the former compound **14** gave dimethyl derivative **17**, while the latter **18** gave monomethyl one **19**. (Fig. 7) Probably, N^{α}-Boc group might sterically hinder the introduction of the second methyl group in the case of the methylation of **18**. These compounds were then subjected to Hofmann degradation. Interestingly, both **17** and **19** produced solely monodehydroalanyl peptides in good yields by use of a limited amount of CH_3I in the presence of an excess $KHCO_3$, respectively. The fact clearly indicated that the first *N*-methylation would be a rate-determining step during permethylation procedure in Hofmann reaction. By this modification, it has become feasible that our method for dehydroalanine synthesis is applicable to the A_2pr peptide involving the sulfide amino acid.

Along the above consideration, a final step of our synthesis of ring A was performed. Cyclic pentapeptide **10** was first debenzyloxycarbonylated by hydrogenation in the presence of Pd black catalyst which was required in more amount than that in usual case. When the hydrogenation did not proceed smoothly, the first catalyst was removed and then fresh catalyst

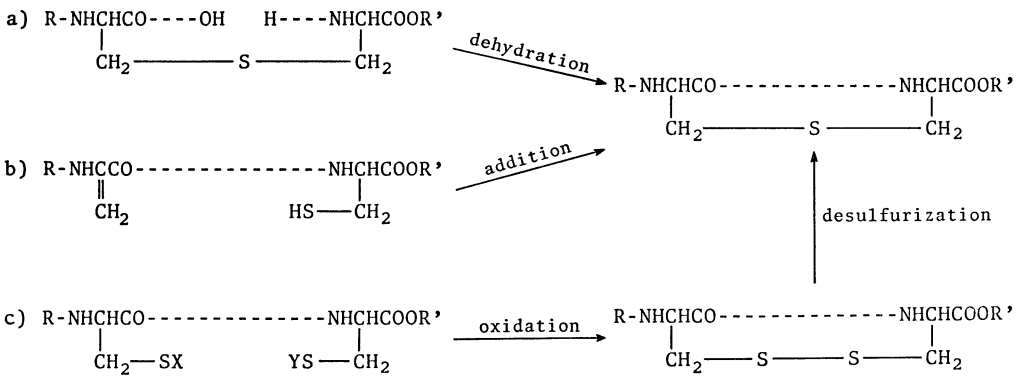

Fig. 3. Synthetic strategies of the cyclic lanthionyl peptide.

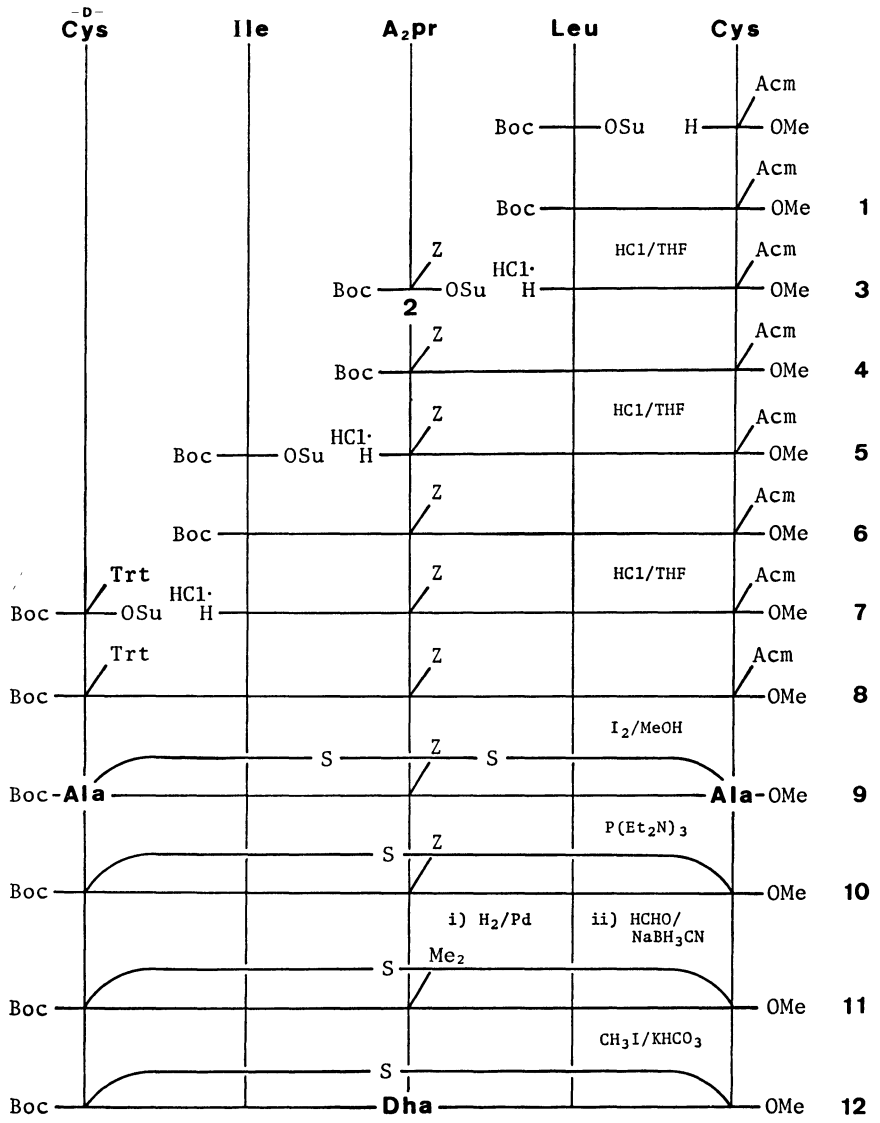

Fig. 4. The synthetic scheme of the protected ring A.

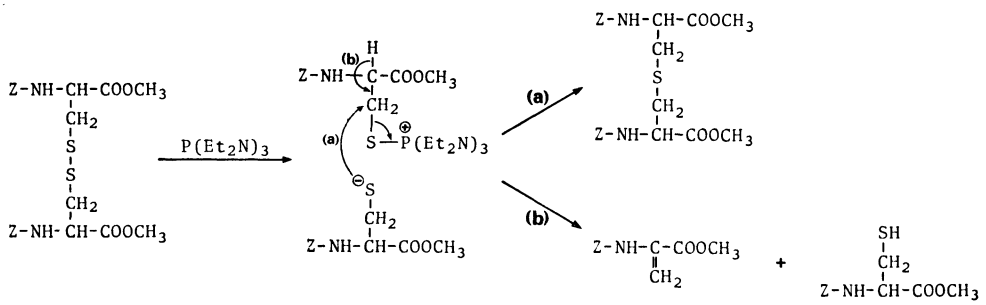

Fig. 5. The plausible reaction mechanism of the desulfurization.

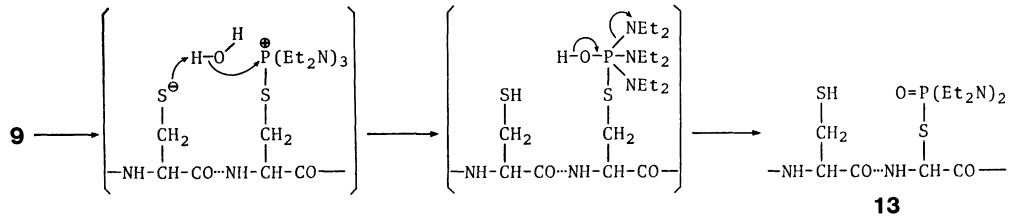

Fig. 6. The plausible by-product formation in the presence of water molecule during the desulfurization reaction.

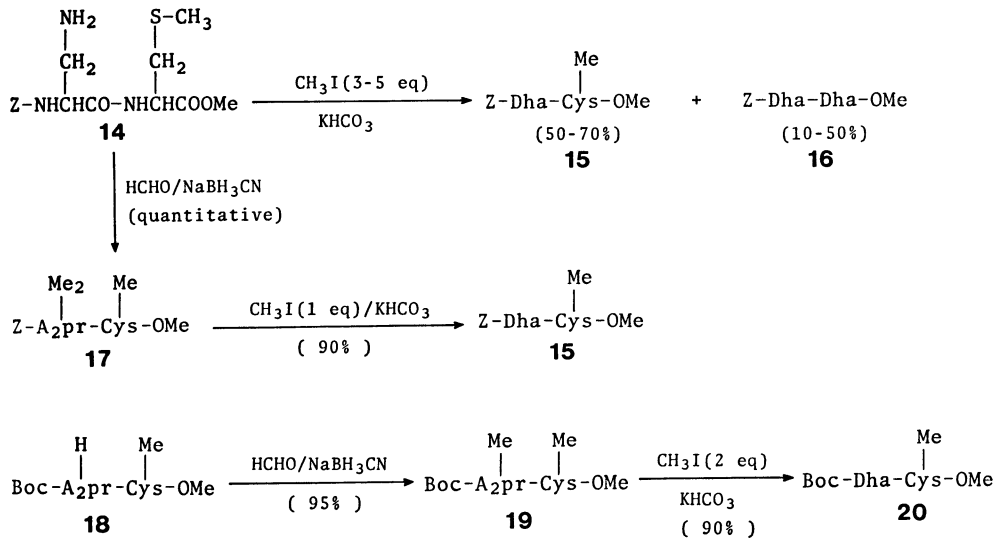

Fig. 7. An improved method of the dehydroalanine synthesis through Hofmann degradation *via* the N^{β}-methyl derivative of A_2pr residue.

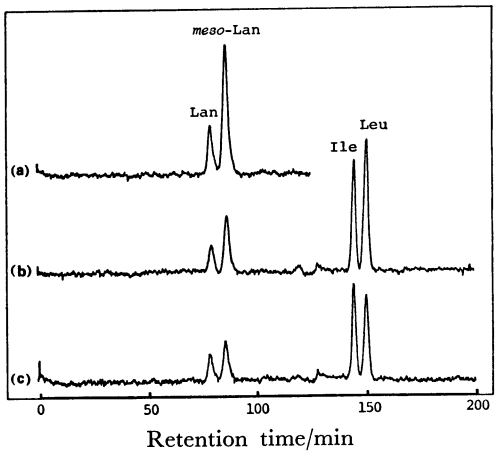

Fig. 8. Amino acid analyses of compounds **11** and **12**.
(a) When *meso*-lanthionine (*meso*-Lan) was treated under the hydrolytic conditions (6 M HCl, 110 °C, 20 h), a part of the sample was racemized to produce DL-lanthionine (Lan). (b) The hydrolyzate of desulfurization product **11** with 6 M HCl at 110 °C for 20 h. A chart of neutral component was shown. (c) The hydrolyzate of protected ring A **12**. A longer hydrolysis (6 M HCl, 110 °C, 40 h) caused more racemization of the *meso*-lanthionine residue.

must have been added. The hydrogenation product was immediately subjected to *N*-methylation with $NaBH_3CN$ and HCHO. Actually in the case of the cyclic peptide **10**, we could obtain dimethyl derivative **11** whose structure was confirmed by the molecular weight determination with FD-MS. Finally, the pentapeptide **11** could be converted through addition of 1.0—1.5 equivalent of CH_3I into desired dehydroalanyl peptide **12**, *i.e.*, protected ring A. The structure of the final product was definitely ascertained both chemically and physically. For example, the molecular weight was confirmed by FD-MS and the existence of the dehydroalanyl residue was confirmed from NMR spectrum. Furthermore, amino acid analysis of a hydrolyzate of the final product proved the presence of ll amino acids constituting ring A. (Fig. 8c)

As mentioned above, we could satisfactorily exploit a novel method for preparation of the dehydroalanine through Hofmann degradation in the synthesis of ring A in peptide antibiotic nisin. Thereby, it proved that this method is very useful and applicable even for syntheses of other complex natural products containing dehydroalanine residue such as AM-toxins. Furthermore, the successful application of desulfurization reaction from cystinyl peptide made the preparation of lanthionyl peptide possible and opened a promising way for the convenient syntheses of other rings in nisin. Indeed, we recently performed the synthesis of ring B in this extention and the result will be reported elsewhere.

Experimental

All melting points are uncorrected. NMR spectra were obtained with a Varian XL-100-15 and JEOL FX-90Q spectrometers using TMS as an internal standard. FD-MS spectra were obtained with a JEOL 01SG spectrometer. Molecular weights were measured with a Knauer vapor pressure osmometer. The specific rotations were obtained with a Perkin-Elmer 141 polarimeter. UV spectra were recorded on a Hitachi 124 spectrophotometer. Amino acid analyses were carried out with a Hitachi KLA-5 analyzer using the following conditions: neutral and acidic amino acids were run on 0.9×55 cm column warmed at 55 °C with citrate buffer of pH 3.25 to 4.25 and basic components were analyzed on 0.9×7 cm column with citrate buffer of pH 5.28. Samples for the analysis were hydrolyzed with constant boiling 6 M (1 M=1 mol dm^{-3}) HCl in a sealed tube at 110 °C for 20 h.

*Boc-Leu-Cys(Acm)-OMe (**1**).* To a solution of Boc-Leu-OSu[13] (20 g, 60.9 mmol) and HCl·H-Cys(Acm)-OMe[14] (17.7 g, 72.8 mmol) in 200 ml of DMF was added triethylamine (14.8 g, 0.146 mol) under ice cooling. The reaction mixture was stirred at room temperature overnight. Triethylamine hydrochloride was filtered off and the filtrate

was concentrated *in vacuo*. Ethyl acetate solution of the residue was washed with aqueous 10% citric acid, saturated $NaHCO_3$, and saturated NaCl solutions. The organic layer was dried over $MgSO_4$ and then concentrated *in vacuo*. The oily residue was crystallized from hexane to obtain colorless needles in a yield of 24.5 g (96.1%). Analytical sample was prepared by recrystallization from ethyl acetate and hexane: mp 79—81 °C; $[\alpha]_D^{17}$ −16.4° (*c* 1.42, AcOEt).

Found: C, 51.32; H, 7.85; N, 9.82; S, 7.71%. Calcd for $C_{18}H_{33}N_3O_6S$: C, 51.53; H, 7.93; N, 10.02; S, 7.64%.

HCl·H-Leu-Cys(Acm)-OMe (**3**). Compound **1** (1.00 g, 2.38 mmol) was dissolved in 5.4 ml of 4.4 M HCl/THF. To a reaction mixture allowed to stand at room temperature for 1 h was added 300 ml of ether under cooling in an ice bath. The precipitate filtered was reprecipitated from isopropyl alcohol and ether: yield 0.69 g (82%); mp 156—158 °C; $[\alpha]_D^{15}$ −14.5° (*c* 1.45, MeOH).

Found: C, 43.33; H, 7.33; N, 11.24; S, 8.83; Cl, 9.71%. Calcd for $C_{13}H_{26}N_3O_4SCl\cdot 0.4H_2O$: C, 43.00; H, 7.44; N, 11.57; S, 8.83; Cl, 9.76%.

Boc-A_2pr(Z)-Leu-Cys(Acm)-OMe (**4**). To a solution of **3** (4.05 g, 11.4 mmol) in 80 ml of DMF were added Boc-A_2pr(Z)-OSu[15] (4.50 g, 10.3 mmol) and triethylamine (2.08 g, 20.6 mmol) under cooling in an ice bath. After stirring at room temperature overnight, triethylamine hydrochloride precipitated was filtered off. The residue obtained by concentration of the filtrate was dissolved in ethyl acetate. The solution was washed with aqueous 10% citric acid, saturated $NaHCO_3$, and saturated NaCl solutions. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Crystalline residue obtained was recrystallized from AcOEt: yield 5.60 g (84.7%); mp 119—122 °C; $[\alpha]_D^{13}$ −53.6° (*c* 1.45, MeOH).

Found: C, 54.29; H, 7.03; N, 10.71; S, 4.79%. Calcd for $C_{29}H_{45}N_5O_9S$: C, 54.44; H, 7.09; N, 10.95; S, 5.01%.

HCl·H-A_2pr(Z)-Leu-Cys(Acm)-OMe (**5**). Protected tripeptide **4** (1.00 g, 1.56 mmol) was dissolved in 5.7 ml of 4.1 M HCl/THF. After the solution was allowed to stand at room temperature for 40 min, ether was added to precipitate **5** under cooling in an ice bath. White powder obtained was filtered and reprecipitated from isopropyl alcohol and hexane: yield 0.91 g (quantitative); mp 103—105 °C; $[\alpha]_D^{13}$ −46.4° (*c* 1.01, MeOH).

Found; C, 48.56; H, 6.60; N, 11.73; S, 5.75; Cl, 6.23%. Calcd for $C_{24}H_{38}N_5O_7SCl$: C, 48.81; H, 6.76; N, 11.86; S, 5.43; Cl, 6.00%.

Boc-Ile-A_2pr(Z)-Leu-Cys(Acm)-OMe (**6**). To a solution of tripeptide hydrochloride **5** (7.46 g, 13.0 mmol) in 50 ml of DMF were added Boc-Ile-OSu [3] (4.26 g, 13.0 mmol) and triethylamine (2.63 g, 26.0 mmol) under ice cooling. The reaction mixture was stirred at room temperature overnight. Triethylamine hydrochloride was filtered off and the filtrate was concentrated *in vacuo*. The residue was once dissolved in a small amount of hot AcOEt and the solution was cooled in an ice bath. The gelatinous precipitate filtered was reprecipitated from methanol and water: yield 8.65 g (88.6%); mp 169—170 °C (decomp); $[\alpha]_D^{15}$ −56.5° (*c* 1.00, MeOH).

Found: C, 55.46; H, 7.42; N, 11.15; S, 4.30%. Calcd for $C_{35}H_{56}N_6O_{10}S\cdot 1/4\,H_2O$: C, 55.50; H, 7.52; N, 11.10; S, 4.23%.

HCl·H-Ile-A_2pr(Z)-Leu-Cys(Acm)-OMe (**7**). To a solution of protected tetrapeptide **6** (5.60 g, 7.44 mmol) in 22 ml of MeOH was added 16 ml of 4.7 M HCl/THF. The mixture was allowed to stand at room temperature for 1 h and then ether was added to precipitate hydrochloride under cooling in an ice bath. Crude product was reprecipitated from methanol and ether: yield 4.80 g (93.6%); mp 197—198 °C; $[\alpha]_D^{17}$ −36.0° (*c* 1.00, MeOH).

Found: C, 51.59; H, 7.15; N, 12.03; S, 4.87; Cl, 5.11%. Calcd for $C_{30}H_{49}N_6O_8SCl$: C, 51.60; H, 7.22; N, 12.04; S, 4.59; Cl, 5.08%.

Boc-D-Cys(Trt)-Ile-A_2pr(Z)-Leu-Cys(Acm)-OMe (**8**). Boc-D-Cys(Trt)-OH·DCHA[16] (1.40 g, 2.18 mmol) was treated with ethyl acetate and 10% aqueous citric acid. Ethyl acetate layer taken was washed with water three times and dried over $MgSO_4$. Oily residue obtained by vacuum concentration was dissolved in 10 ml of absolute THF. To the solution was added *N*-hydroxysuccinimide (250 mg, 2.18 mmol) and then DCC (448 mg, 2.18 mmol) under ice cooling. The mixture was stirred at 0 °C for 1 h and at room temperature for 2 h. *N,N'*-Dicyclohexylurea precipitated was filtered off and the filtrate was concentrated *in vacuo*. Oily residue thus obtained and **7** (1.50 g, 2.18 mmol) were dissolved in 20 ml of DMF. To the solution was added triethylamine (0.44 g, 4.4 mmol) under cooling in an ice bath. The reaction mixture was stirred at 0 °C for 30 min and at room temperature overnight. Triethylamine hydrochloride was filtered off and the filtrate was concentrated *in vacuo*. Oily residue was triturated with cold ether to make powder which was reprecipitated from methanol and 2% aqueous acetic acid. The precipitate filtered was washed with water and ether: yield 1.85 g (77.4%); mp 169—172 °C; $[\alpha]_D^{26}$ −45.4° (*c* 1.00, MeOH).

Found: C, 61.09; H, 6.84; N, 8.93; S, 5.62%. Calcd for $C_{57}H_{75}N_7O_{11}S_2\cdot H_2O$: C, 61.30; H, 6.95; N, 8.82; S, 5.74%.

Boc-D-Cys-Ile-A_2pr(Z)-Leu-Cys-OMe (**9**). To a solution of linear peptide **8** (4.60 g, 4.19 mmol) in 840 ml of MeOH was added I_2 (3.19 g, 12.6 mmol) in 125 ml of MeOH under vigorous stirring in a minute. The oxidation was accomplished for 1 h at room temperature and stopped by an addition of 0.1 M $Na_2S_2O_3$ solution until the color of I_2 disappeared. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in water. Extraction with $CHCl_3$ was performed three times and the extract was concentrated *in vacuo* after drying over $MgSO_4$. The oily residue thus obtained was crystallized by an addition of ether to obtain pure material as a powder: yield 2.63 g (80.2%); mp 178—180 °C; $[\alpha]_D^{26}$ +2.3° (*c* 1.0, DMF); MW 750±37 (Calcd 783); FD-MS, *m/z* 782 (M^+).

Found: C, 53.12; H, 6.93; N, 10.42; S, 8.33%. Calcd for $C_{35}H_{54}N_6O_{10}S_2\cdot 1/2\,H_2O$: C, 53.08; H, 7.00; N, 10.61; S, 8.10%.

Boc-D-Ala-Ile-A_2pr(Z)-Leu-Ala-OMe (**10**). To a solution of the cyclic disulfide **9** (200 mg, 0.255 mmol) in 80 ml of anhydrous DMF was added tris(diethylamino)-phosphine (1.26 g, 5.11 mmol) in 20 ml of anhydrous DMF. The reaction mixture was stirred at 50 °C for 2 d and then concentrated *in vacuo*. The residue was once dissolved in AcOEt and washed with 10% citric acid and saturated NaCl solution. Organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The oily residue was easily crystallyzed by trituration with hexane in a yield of 164 mg (85.4%). Although the product was pure enough to be supplied for the next reaction, a part of the sample was recrystallized from methanol to obtain an analytical sample: mp 226.5—227 °C; $[\alpha]_D^{26}$ −47.6°C (*c* 1.00, DMF); FD-MS, *m/z* 750 (M^+). Amino acid analysis: Lan[17] (0.33), *meso*-Lan (0.72), Ile (0.76), Leu (1.00), A_2pr (1.15).

Found: C, 55.56; H, 7.18; N, 11.04; S, 4.31%. Calcd for $C_{35}H_{54}N_6O_{10}S$: C, 55.98; H, 7.25; N, 11.19; S, 4.27%.

Boc-D-Ala-Ile-$A_2pr(Me_2)$-Leu-Ala-OMe (**11**). The sulfide compound **10** (279 mg, 0.356 mmol) in 60 ml of methanol was hydrogenated in the presence of acetic acid (2.0 ml) by use of Pd black catalyst (*ca.* 2 g) under a gentle bubbling of hydrogen. A progress of the reaction was followed on TLC. After the hydrogenolysis was finished, the catalyst was filtered off. The filtrate was concentrated *in vacuo* and the residue was immediately dissolved in 25 ml of methanol for the following permethylation. To the solution were added 35% formaldehyde (153 mg, 1.78 mmol) and $NaBH_3CN$ (45 mg, 0.72 mmol). The reaction mixture was stirred at room temperature for 2 h and then concentrated *in vacuo*. The residue was treated with ethyl acetate and a saturated aqueous $NaHCO_3$. The organic layer was washed once with a saturated $NaHCO_3$ solution and three times with a saturated NaCl solution. The ethyl acetate extract was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was reprecipitated from ethyl acetate and hexane: yield 179 mg (78.3%); mp 192—193 °C; $[\alpha]_D^{24}$ −44.0° (*c* 1.00, MeOH); FD-MS, *m/z* 644 (M^+).

Found: C, 54.24; H, 8.09; N, 13.02; S, 4.61%. Calcd for $C_{29}H_{52}N_6O_8S$: C, 54.02; H, 8.13; N, 13.03; S, 4.97%.

Boc-D-Ala-Ile-Dha-Leu-Ala-OMe (Boc-Ring A-OMe) (**12**). To a solution of dimethyl derivative **11** (140 mg, 0.217 mmol) in 5 ml of methanol were added $KHCO_3$ (326 mg, 3.26 mmol) and CH_3I (30.8 mg, 0.217 mmol). Since a part of CH_3I vaporized during the reaction at room temperature, a small amount of CH_3I (3.1 mg) was added four times every 10—12 h. The reaction mixture was finally cooled in an ice bath to precipitate a desired product. The precipitates filtered were washed with a 10% critic acid solution and water to remove inorganic salts: yield 40.6 mg (31.2%); mp 228—229 °C; $[\alpha]_D^{17}$ −68.2° (*c* 1.00, DMF); FD-MS, *m/z* 599 (M^+); UV(MeOH)λ_{max}, 250 nm (ε, 1500). Amino acid analysis: Lan[17] (0.40), *meso*-Lan (0.70), Ile (0.94), Leu (1.00).

Found: C, 53.91; H, 7.56; N, 11.55; S, 5.27%. Calcd for $C_{27}H_{45}N_5O_8S$: C, 54.07; H, 7.56; N, 11.68; S, 5.35%.

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17) See the note in the caption of Fig. 8, (a).