Synthetic Study on Peptide Antibiotic Nisin. II. The Synthesis of Ring B[1)]

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(Received March 5, 1986)

Synthesis of a cyclic sulfide moiety ring B in peptide antibiotic nisin was successfully achieved by desulfurization from a corresponding disulfide peptide with tris(diethylamino)phosphine. The configuration on β-carbon atom of *threo*-3-methyl-D-cysteine residue in the disulfide peptide was retained through the reaction to give a desired natural *threo* form of methyllanthionine.

Peptide antibiotic nisin was isolated from culture broth of *Streptococcus lactis* by Mattick and Hirsh in 1947,[2)] and Berridge et al. in 1952.[3)] Nisin shows several biological activities such as antibacterial activity against Gram-positive microorganisms, particularly *Clostridium botulinum*, as well as antimalarial activity, and so on.[4)] Nisin is now in practical use widely in European countries as an important food preservative but not as a chemotherapeutic agent because of low solubility at physiological pH value.

In 1971, quite unique and complicated structure of nisin was proposed by Gross and Morell as shown in Fig. 1.[5)] It is composed of thirty-four amino acid residues including three dehydroamino acids and five lanthionines which construct cyclic moieties via sulfide bond. These five ring parts were named A, B, C, D, and E respectively from *N*-terminal in the molecule.

From the standpoint of a synthetic interest in such a complicated peptide as well as an elucidation on structure-activity relationship of nisin, we started the synthetic study of this antibiotic, and have already accomplished the synthesis of ring A.[6)]

In the synthetic study of ring A, we could establish an advantageous preparative method of cyclic lanthionine peptide by an elimination of one sulfur atom from a corresponding cystine peptide using tris(diethylamino)phosphine [$P(NEt_2)_3$] as desulfurization reagent[7)] (Fig. 2). According to the application of this procedure, synthesis of ring B (Fig. 3) was now carried out as shown in Fig. 4.

threo-3-Methyl-D-cysteine required for this synthesis was prepared from D-threonine by use of our novel method via 2-aziridinecarboxylic acid derivative.[8)] Mercapto groups on L-cysteine and *threo*-3-methyl-D-cysteine residues were protected with acetamidomethyl (Acm) and trityl (Trt) groups, respectively as in ring A synthesis. These protecting groups can be readily removed with I_2 oxidation in methanol to form a cyclic disulfide compound directly.[9)] Since *S*-tritylation is better than *S*-acetamidomethylation in convenience on manipulation and in yield on reaction, the mercapto group of precious *threo*-3-methyl-D-cysteine residue was protected with trityl group. The protected linear tetrapeptide **7** was first prepared successively from the *C*-terminal by stepwise elongation with either a mixed anhydride method or an active ester method. The peptide **7** thus obtained was then subjected to I_2 oxidation. The monomeric disulfide **8** was satisfactorily obtained by high dilution method. In order to confirm the formation of monomer, the molecular weight of the compound **8** was measured by mass spectrometry (*m/z* 504; calcd 504).

In the synthetic study of ring A, the desulfurization reaction was successfully carried out in *N*,*N*-dimethylformamide (DMF). Thus the desulfurization reaction of disulfide **8** was first performed in DMF. Although we could obtain the desired monomeric sulfide peptide **9**, whose molecular weight was confirmed by mass spectrometry (*m/z* 472; calcd 472), the yield did not exceed 30% even though under the

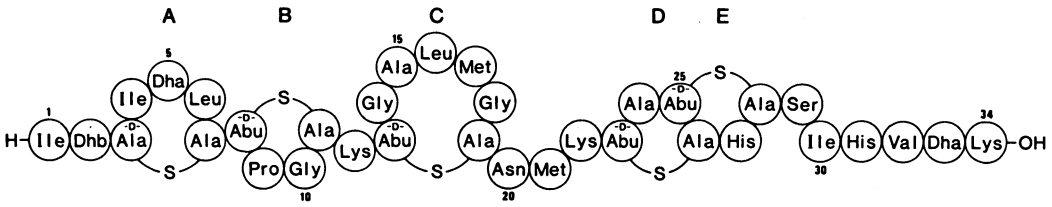

Fig. 1. The structure of nisin. Abu: 2-aminobutyric acid; Dha: dehydroalanine; Dhb: dehydrobutyrine (=3-methyldehydroalanine); Ala Ala: lanthionine; Abu Ala: methyllanthionine.

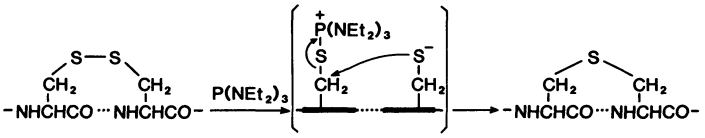

Fig. 2. The reaction mechanism of the desulfurization.

optimum conditions used in the case of ring A synthesis (Table 1). In order to improve the reaction conditions in the present case, we examined effects of the solvent, reaction temperature, amount of $P(NEt_2)_3$, and concentration of the substrate (Table 1). Among general organic solvents tested, benzene and DMF can be utilized while other solvents such as dioxane, THF, $CHCl_3$, ethyl acetate, CH_3CN, and so on were not suitable because of considerable occurrence of undesirable side reactions.

In view of reproducibility in yield, easiness in purification of the reaction product, and readiness of preparation of absolute solvent, benzene is better than DMF as reaction solvent in the present study. The yield was improved when desulfurization reaction was performed under higher dilution as far as it reached to the optimum at 0.62 mM (1 M=1 mol dm^{-3}) at room temperature as shown in Table 1. Thus, the cyclic sulfide peptide **9** was satisfactorily obtained in a 57% yield.

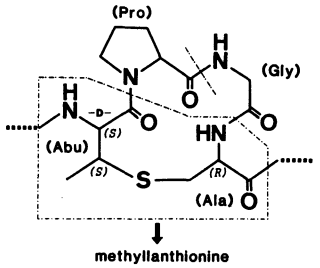

Fig. 3. The chemical structure of the ring B part in nisin.

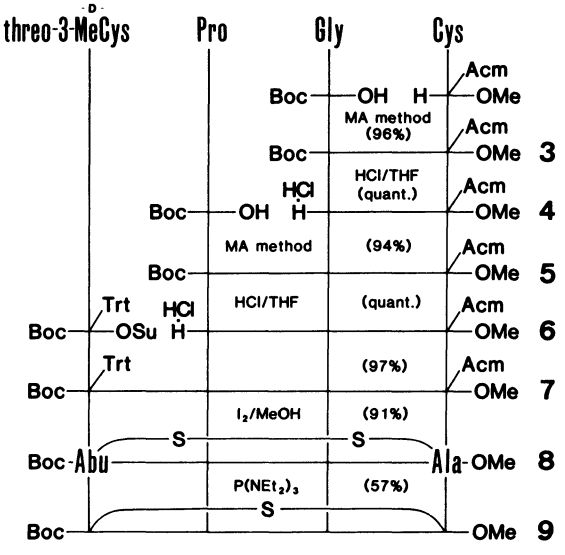

Fig. 4. The synthetic scheme of the protected ring B.

In the case of ring B preparation, it is very important to investigate about two possible desulfurization pathways (Fig. 5). When the reaction proceeds via path (a), the configuration on β-carbon atom of *threo*-3-methyl-D-cysteine is retained to give a *threo* form of methyllanthionine i.e., natural form in nisin. On the other hand, the reaction via path (b) will cause an inversion of the configuration on the β-carbon of 3-methylcysteine residue to form *erythro*-methyllanthionine.

Amino acid analysis of the hydrolyzate of the final product **9** clearly indicated the sole formation of desirable *threo*-methyllanthionine[10] residue via path (a) (Fig. 6). This result can be explained by following two plausible reasons; i) because of steric hindrance of 3-methyl group in *threo*-3-methyl-D-cysteine residue, the nucleophilic attack took place selectively at the sulfur atom of L-cysteine residue, ii) thiolate anion of 3-methylcysteine residue is more stable than that of cysteine residue.

Thus, ring B in nisin was successfully synthesized, verifying a possibility for preparation of *threo*-methyllanthionine peptide from a disulfide peptide composed of *threo*-3-methyl-D-cysteine and L-cysteine by the desulfurization with $P(NEt_2)_3$. Based on the synthetic studies of rings A and B, we could confirm that the desulfurization is very versatile method for the preparation of lanthionine peptide in general. In fact, we recently applied this method successfully to the synthesis of ring C as well as conjunctive ring D-E moiety in nisin, whose results will be reported soon elsewhere.

Experimental

All melting points are uncorrected. The FD-MS spectra were obtained with a JEOL JMS-01SG-2 spectrometer on silicone emitter. Specific rotations were obtained with a Perkin-Elmer 141 polarimeter. Amino acid analyses were carried out with a Hitachi KLA-5 analyzer. Sample for the amino acid analysis was hydrolyzed with constant boiling

Table 1. Yields of Compound **9** on Various Reaction Conditions

Solvent	Concn/mM	$P(NEt_2)_3$	Temp/°C	Reaction time/h	Yield/%
DMF	2.3	40 eq	50	96	28
DMF[a)]	2.4	20 eq	50	96	24
C_6H_6	2.5	40 eq	50	1	12
C_6H_6	2.5	40 eq	25	24	34
C_6H_6	2.5	10 eq	25	24	31
C_6H_6	1.3	40 eq	25	24	43
C_6H_6	0.62	40 eq	25	48	57
C_6H_6	0.31	40 eq	25	48	53

a) The optimum conditions in the case of ring A synthesis.

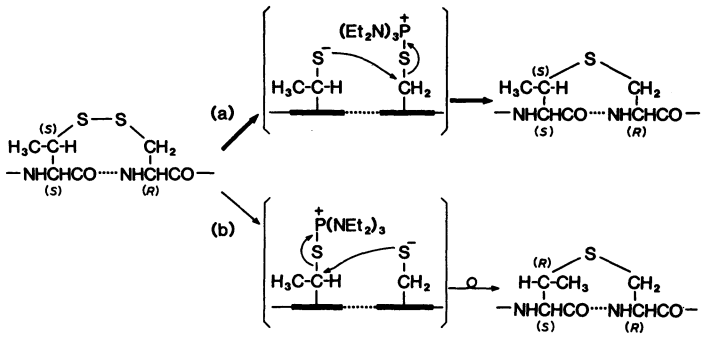

Fig. 5. Plausible desulfurization pathways in ring B preparation.

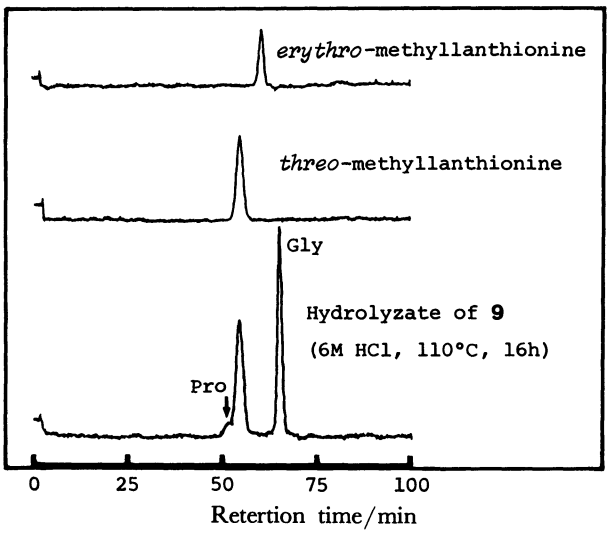

Fig. 6. Amino acid analysis of compound **9**. Column: Hitachi #2618 resin (0.9×25 cm, 55°C), buffer: 0.20 M sodium citrate (pH 3.0).

6 M HCl in a sealed tube at 110°C for 16 h.

H-3-Me-D-Cys(Trt)-OH (1). *threo*-3-Methyl-D-cysteine[8] (11.6 g, 85.9 mmol) and triphenylmethanol (22.3 g, 85.9 mmol) were dissolved in 150 ml of trifluoroacetic acid. The clear solution was allowed to stand at room temperature for 15 min and then trifluoroacetic acid was removed in vacuo. To an ether solution of the residue was added 10% aqueous sodium acetate until pH of the aqueous layer became 5 to precipitate H-3-Me-D-Cys(Trt)-OH (**1**). The precipitate collected was washed with water and ether: Yield 31.7 g (97.8%). A part of the product was recrystallized from ethanol and ether to obtain an analytical sample: Mp 163—164°C; $[\alpha]_D^{17}$ −28.9°(*c* 1.00, MeOH).

Found: C, 69.98; H, 6.23; N, 3.55; S, 8.16%. Calcd for $C_{23}H_{23}NO_2S \cdot H_2O$: C, 69.85; H, 6.37; N, 3.54; S, 8.11%.

Boc-3-Me-D-Cys(Trt)-OH·DCHA (2). To a suspension of the compound **1** (3.07 g, 8.14 mmol) in 60 ml of methanol were added di-*t*-butyl dicarbonate (2.10 g, 9.80 mmol) and triethylamine (0.906 g, 8.95 mmol). After stirring at room temperature overnight, the reaction mixture was concentrated in vacuo and the residue was dissolved in ethyl acetate. The solution was washed with 10% aqueous citric acid and saturated sodium chloride solutions. The organic layer was dried over $MgSO_4$ and then concentrated in vacuo. An oily residue thus obtained was dissolved in hexane to which dicyclohexylamine (1.77 g, 9.76 mmol) was added. After the reaction mixture was cooled in an ice bath, the precipitate was filtered: Yield 5.10 g (95.1%). A part of the product was recrystallized from ethyl acetate and hexane to obtain an analytical sample: Mp 186.5—187°C; $[\alpha]_D^{23}$ −83.0° (*c* 1.07, MeOH).

Found: C, 72.72; H, 8.29; N, 4.27; S, 4.81%. Calcd for $C_{40}H_{54}N_2O_4S$: C, 72.91; H, 8.26; N, 4.25; S, 4.87%.

Boc-Gly-Cys(Acm)-OMe (3). To a solution of Boc-Gly-OH (20.0 g, 114 mmol) in 80 ml of DMF were added ethoxycarbonyl chloride (12.4 g, 114 mmol) and triethylamine (11.5 g, 114 mmol) under cooling in an ice-salt bath. After the reaction mixture was stirred in the chilled bath for 50 min, a solution of HCl·H-Cys(Acm)-OMe[13] (23.1 g, 95.2 mmol) and triethylamine (9.63 g, 95.2 mmol) in 200 ml of DMF was added. The solution was stirred under cooling in an ice bath for additional 20 min and then kept at room temperature overnight. The reaction mixture was concentrated in vacuo to the residue, which was dissolved in chloroform, and washed with 10% aqueous citric acid, saturated sodium hydrogencarbonate, and saturated sodium chloride solutions. The organic layer was dried over $MgSO_4$ and then concentrated in vacuo to give an oily product: Yield 33.3 g (96.2%); $[\alpha]_D^{18}$ −9.50° (*c* 2.78, $CHCl_3$).

Found: C, 45.30; H, 6.94; N, 11.21; S, 8.72%. Calcd for $C_{14}H_{25}N_3O_6S \cdot 1/2H_2O$: C, 45.15; H, 7.04; N, 11.28; S, 8.61%.

HCl·H-Gly-Cys(Acm)-OMe (4). Protected dipeptide **3** (33.3 g, 91.6 mmol) was dissolved in 290 ml of 4.67 M hydrogen chloride in THF. The solution was allowed to stand at room temperature for 1 h, and then diluted with 2 l of ether. After cooling in an ice-salt bath for 1 h, the supernatant was decanted to separate dipeptide hydrochloride precipitated. The hydrochloride **4** thus obtained was dried over NaOH in vacuum desiccator and then subjected to the following reaction without purification.

Boc-Pro-Gly-Cys(Acm)-OMe (5). Ethoxycarbonyl chloride (11.0 g, 101 mmol) and triethylamine (10.2 g, 101 mmol) were added to a solution of Boc-Pro-OH (21.7 g, 101 mmol) in 70 ml of DMF under cooling in an ice-salt bath. To the mixture stirred in the chilled bath for 15 min was added a solution of dipeptide hydrochloride **4** (91.6 mmol) and triethylamine (9.27 g, 91.6 mmol) in 210 ml of DMF. The reaction mixture was stirred for 1 h and then concentrated in vacuo. The residue was dissolved in chloroform and the solution was washed with 10% aqueous citric acid, saturated sodium hydrogencarbonate, and saturated sodium chloride solutions. The organic layer was dried over $MgSO_4$ and then concentrated in vacuo to give an oily product: Yield

39.6 g (93.8%); $[\alpha]_D^{18}$ −55.1° (*c* 1.04, $CHCl_3$).

Found: C, 48.47; H, 7.26; N, 11.70; S, 6.75%. Calcd for $C_{19}H_{32}N_4O_7S \cdot 1/2H_2O$: C, 48.60; H, 7.08; N, 11.93; S, 6.83%.

HCl·H-Pro-Gly-Cys(Acm)-OMe (6). Protected tripeptide **5** (15.3 g, 33.2 mmol) was dissolved in 110 ml of 4.67 M hydrogen chloride in THF to give tripeptide hydrochloride which was obtained in a quantitative yield after the same treatment as mentioned in the preparation of **4**.

Boc-3-Me-D-Cys(Trt)-Pro-Gly-Cys(Acm)-OMe (7). Boc-3-Me-D-Cys(Trt)-OH·DCHA (**2**) (20.0 g, 30.4 mmol) was treated with ethyl acetate and 10% aqueous citric acid. Ethyl acetate layer separated from the aqueous layer was washed with water and dried over $MgSO_4$. The oily residue obtained by vacuum concentration was dissolved in 120 ml of THF. To the solution were added *N*-hydroxysuccinimide (3.50 g, 30.4 mmol) and dicyclohexylcarbodiimide (6.26 g, 30.4 mmol) under ice cooling. The mixture was stirred at 0 °C for 40 min and then at room temperature for 2 h. *N,N'*-Dicyclohexylurea precipitated was filtered off and the filtrate was concentrated in vacuo. The oily residue dissolved in 30 ml of chloroform was added to a solution of tripeptide hydrochloride **6** (33.2 mmol) and triethylamine (3.36 g, 33.2 mmol) in 60 ml of chloroform. After stirring at room temperature overnight, the mixture was concentrated in vacuo. Ethyl acetate was added to the residue and an insoluble material was filtered off. The filtrate was washed with 10% aqueous citric acid, saturated sodium hydrogencarbonate, and saturated sodium chloride solutions. The organic layer dried over $MgSO_4$ was concentrated in vacuo. The oily residue was washed by trituration with hexane several times to remove off unreacted Boc-3-Me-D-Cys(Trt)-OH and then precipitated from ethyl acetate and hexane: Yield 24.2 g (97.2%); mp 76—80 °C; $[\alpha]_D^{28}$ −39.5° (*c* 1.03, $CHCl_3$).

Found: C, 60.91; H, 6.52; N, 8.38; S, 7.61%. Calcd for $C_{42}H_{53}N_5O_8S_2 \cdot 1/2H_2O$: C, 60.85; H, 6.57; N, 8.45; S, 7.73%.

Boc-3-Me-D-Cys-Pro-Gly-Cys-OMe (8). To a solution of protected linear tetrapeptide **7** (620 mg, 0.757 mmol) in 150 ml of methanol was added I_2 (576 mg, 2.27 mmol) in 23 ml of methanol under vigorous stirring. The oxidation was carried out at room temperature for 1 h and stopped by an addition of 0.2 M sodium thiosulfate solution until the color of I_2 disappeared. The reaction mixture was concentrated in vacuo and the residue was suspended in water. Extraction with chloroform was repeated three times and the extract was concentrated in vacuo after drying over $MgSO_4$. The oily residue thus obtained was crystallized from ethyl acetate and hexane: Yield 345 mg (90.3%); mp 177—179 °C; $[\alpha]_D^{28}$ +38.1° (*c* 0.950, $CHCl_3$); FD-MS, *m/z* 504 (M^+).

Found: C, 46.49; H, 6.33; N, 10.71; S, 12.12%. Calcd for $C_{20}H_{32}N_4O_7S_2 \cdot 1/2H_2O$: C, 46.77; H, 6.48; N, 10.91; S, 12.48%.

Boc-D-Abu-Pro-Gly-Ala-OMe (9). To a solution of **8** (100 mg, 0.198 mmol) in 320 ml of anhydrous benzene was added tris(diethylamino)phosphine[7] (1.96 g, 7.94 mmol). The reaction mixture was stirred at room temperature for 2 d and then concentrated in vacuo. The residue was dissolved in ethyl acetate and washed with 10% aqueous citric acid and saturated sodium chloride solutions three times, respectively. Organic layer was dried over $MgSO_4$ and concentrated in vacuo. An oily residue obtained was applied to Sephadex LH-20 column (1.8×56 cm) and eluted with DMF. The column-chromatographic purification was repeated twice to give the product **9** as powder: Yield 53 mg (57%). A part of the product was recrystallized from ethyl acetate to obtain an analytical sample: Mp 206—207.5 °C (decomp); $[\alpha]_D^{16}$ +62.8° (*c* 0.931, DMF); FD-MS, *m/z* 472 (M^+). Amino acid analysis: Melan (0.92), Pro (1.01), Gly (1.00).

Found: C, 49.09; H, 7.05; N, 11.18; S, 6.39%. Calcd for $C_{20}H_{32}N_4O_7S \cdot H_2O$: C, 48.97; H, 6.99; N, 11.42; S, 6.54%.

The present work was partially supported by a Grant-in-Aid for Scientific Research No. 57540305 from the Ministry of Education, Science and Culture.

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