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Studies of Peptide Antibiotics. XIII. Synthesis of Retrogramicidin S[1]

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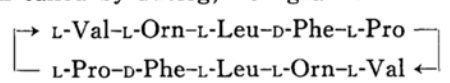
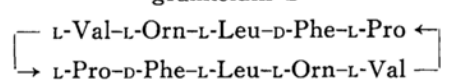
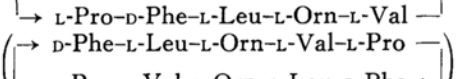
In order to investigate a contribution for an antibacterial activity of a direction of the peptide bonds in gramicidin S molecule, retrogramicidin S with the reverse peptide sequence was prepared through a cyclization reaction of linear decapeptide active ester in pyridine. The cyclization of linear pentapeptide active ester yielded a mixture of retrocyclosemigramicidin S and retrogramicidin S. Retrogramicidin S thus obtained exhibited a weak activity against *Bacillus subtilis* and *Staphylococcus aureus*, whereas retrocyclosemigramicidin S showed no activity against any of microorganisms tested.

In studies of the relationship between chemical structure and biological activity of gramicidin S, various analogs wherein several amino acid residues are replaced by other amino acids have been synthesized in this laboratory.[2] For example, it was found that [5,5′-glycine]-gramicidin S[3,4] possessed a stronger activity in several-fold than natural gramicidin S. As a different type of synthetic analogs of gramicidin S, we have designed the synthesis of retrogramicidin S in order to determine what degree the direction of the peptide bonds contributes to a biological activity. The retrogramicidin S can be regarded as a cyclodiastereomer, which has been called by Prelog,[5] of gramicidin S.

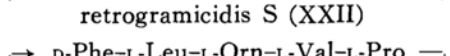


gramicidin S





retrogramicidis S (XXII)



retrocyclosemigramicidin S (XXIII)

In the studies of synthetic analogs of linear biologically active peptides, a retro-analog of bradykinin has been synthesized by two groups,[6] they observed that the retrobradykinin was completely inactive in the rat uterus assay. As an analog of cyclic active peptides, Shemyakin *et al.* demonstrated that the retroenantio-[5,5′-glycine]-gramicidin S was highly active as the parent compound, [5,5′-glycine]-gramicidin S.[7]

For the conformation of gramicidin S in the solid- and solution-state, several models have been proposed.[8] A possible model (Fig. 1) is the intramolecular antiparallel β form with four hydrogen bond which has been suggested by Hodgkin and Oughton with the results of X-ray crystallographic investigation.[9] Schwyzer also suggested the same model with the occurrence of dimerization reaction on the

1) A part of this work was presented at the 5 th Symposium of Peptide Chemistry, Kyoto, November, 1967, and communicated briefly in *Tetrahedron Letters*, **1968**, 3083.

2) See for recent example, M. Waki, O. Abe, R. Okawa, T.Kato, S. Makisumi and N. Izumiya, This Bulletin, **40**, 2904 (1967).

3) We followed the rules naming synthetic modifications of natural peptides in *Biochemistry*, **6**, 362 (1967).

4) H. Aoyagi, T. Kato, M. Ohno, M. Kondo and N. Izumiya, *J. Am. Chem. Soc.*, **86**, 5700 (1964); This Bulletin, **38**, 2139 (1965).

5) V. Prelog and H. Gerlach, *Helv. Chim. Acta*, **47**, 2288 (1964).

6) S. Lande, *J. Org. Chem.*, **27**, 4558 (1962); K. Vogler, P. Lanz and W. Lergier, *Helv. Chim. Acta*, **45**, 561 (1962).

7) M.M. Shemyakin, Yu. A. Ovchinnikov, V.T. Ivanov and I.D.Ryabova, *Experientia*, **23**, 326 (1967). These authors have mentioned that retro-[5,5′-glycine]-gramicidin S is highly active as [5,5′-glycine]-gramicidin S in foot note.

8) See for recent example, D. Balasubramanian, *J. Am. Chem. Soc.*, **89**, 5445 (1967).

9) D. C. Hodgkin and B. M. Oughton, *Biochem. J.*, **65**, 752 (1957).

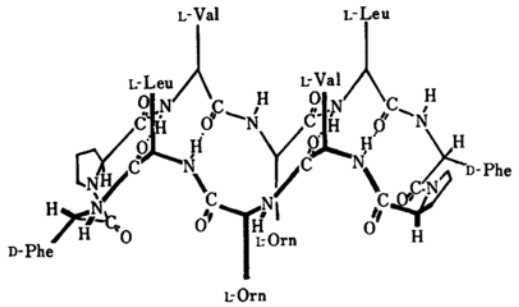


Fig. 1. A possible structure of gramicidin S.

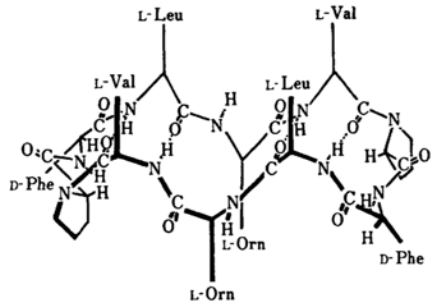


Fig. 2. A possible structure of retrogramicidin S.

occasion of gramicidin S synthesis.[10] In this model, the hydrophilic ornithyl side chains are on one side of the pleated sheet, thus the model appears to be a cationic detergent. Rothen's surface studies on gramicidin S, quoted in Craig's article,[11] also seem to support this model. We attempted to construct the retrogramicidin S model (Fig. 2) with the antiparallel β form, which has been suggested by Hodgkin and Oughton, as a principal frame by molecular model study. It is observed that the hydrophobic proline residues are directed to the side where the ornithyl side chains are located on. Since the character as a specific cationic detergent for gramicidin S is an important factor for the antibacterial activ-

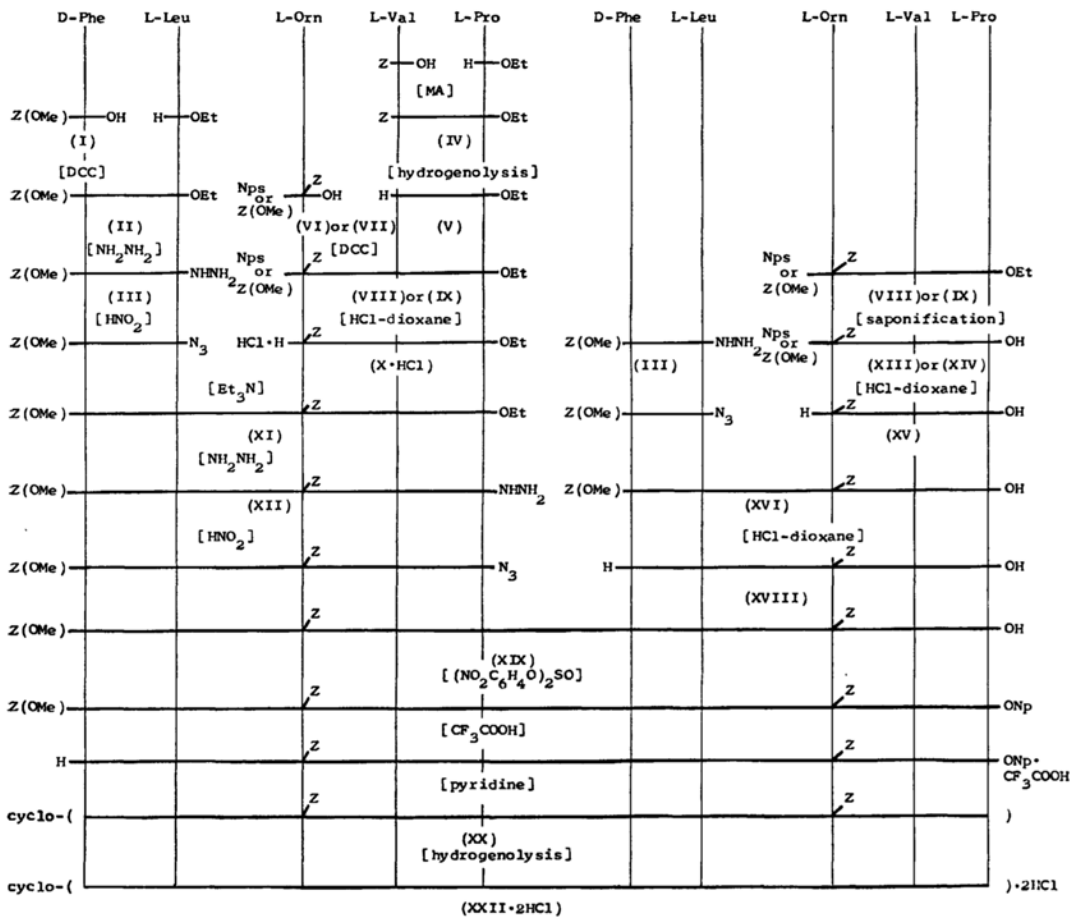


Fig. 3. Synthesis of retrogramicidin S.

10) R. Schwyzer, *Chimia*, **12**, 53 (1958).

11) L.C. Craig, *Science*, **144**, 1093 (1964).

ity,[12] two proline residues in retrogramicidin S may disturb an interaction of the compound and a microorganism. Thus, we tended to surmise that retrogramicidin S would possess weaker or no activity compared with gramicidin S.

The sequence of reaction employed for the synthesis of retrogramicidin S (XXII) from a linear decapeptide nitrophenyl ester is shown in Fig. 3. The sequence of steps was chosen specifically to preclude racemization. The tripeptide ester (X) was obtained by treatment of hydrogen chloride on either VIII or IX. In this step, the use of *o*-nitrophenylsulfenyl group seemed more advantageous than that of *p*-methoxybenzyloxycarbonyl; *o*-nitrophenylsulfenyl chloride,[13] which was used for VI synthesis, was prepared more easily than *p*-methoxybenzyloxycarbonyl azide,[14] and the selective removal of nitrophenylsulfenyl group from VIII or XIII was easier than that of methoxybenzyloxycarbonyl group from IX or XIV. The condensation of the azide derived from acyldipeptide hydrazide (III) with X gave acylpentapeptide ester (XI).

To obtain an acylpentapeptide acid (XVI), the acylpentapeptide ester (XI) was subjected to saponification. When XI was treated with dilute alkali (Fig. 4), there was formed a crude product which was composed of two compounds. The mixture was separated into two pure components by use of a Sephadex LH-20 column; the desired acylpeptide acid (XVI) was obtained from the faster eluting fraction, and a compound with mp 121—123 ℃ was obtained from the slower fraction. The elemental analysis of the latter compound and the neutral equivalent by quantitative titration procedure favored the dibasic acid of structure

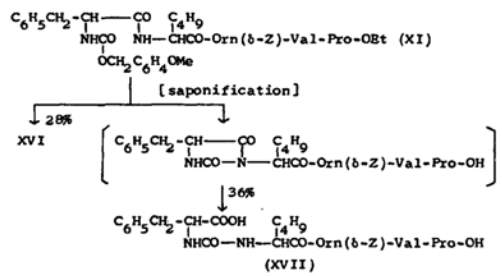


Fig. 4. Saponification of acylpentapeptide ethyl ester (XI).

12) E.P.Abraham, "Comprehensive Biochemistry," Vol. 11, Elsevier, Amsterdam (1963), p. 181.

13) M. H. Hubacher, "Organic Syntheses," Coll. Vol. II, p. 455 (1943); L. Zervas, D. Borvas and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).

14) F.Weygand and K.Hunger, *Chem. Ber.*, **95**, 1 (1962).

XVII. It appeared that the cyclization with loss of *p*-methoxybenzyl alcohol, followed by hydrolytic opening of the hydantoin intermediate, had occurred in addition to the desired hydrolysis reaction. Such rearrangements have been observed already by Maclaren[15] and Bodanszky *et al.*,[15] the latter group demonstrated that alkaline treatment of *α*-benzyloxycarbonyl-*ω*-nitroarginyl-tetraglycine ethyl ester yielded a dibasic acid, 5-[1-carboxy-4(3-nitroguanidino)butyl]-hydantoyl-triglycine.

The acylpentapeptide acid (XVI) was prepared more efficiently as follows. Saponification of acyltripeptide ester (VIII or IX) gave the acyltripeptide acid (XIII or XIV) without appreciable side reactions, then either XIII or XIV was transformed to the free tripeptide (XV). The condensation of the azide from III with XV afforded the acylpentapeptide acid (XVI) in a good yield. Then, the condensation of the azide from XII with free pentapeptide (XVIII), which had been obtained from XVI, gave the acyldecapeptide acid(XIX). The successive treatments of XIX with di-*p*-nitrophenyl sulfite, trifluoroacetic acid and excess pyridine afforded the benzyloxycarbonyl-substituted cyclic decapeptide (XX) in a good yield (49% from XIX). The hydrogenation of XX in the presence of two equivalents of hydrogen chloride yielded the desired retrogramicidin S dihydrochloride as colorless crystals containing water of crystallization.

The synthesis of the protected cyclic decapeptide (XX) was also attempted by possibe dimerization reaction of the pentapeptide active ester (XXIV) (Fig. 5). The compound XXIV was treated with excess pyridine, and after evaporation the reaction mixture was passed through Dowex 1 and 50 columns; the subsequent evaporation of the effluent yielded a semisolid residue. This

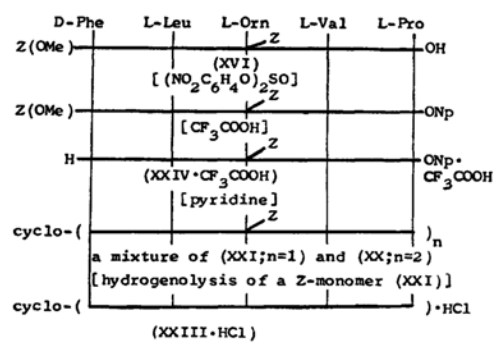


Fig. 5. Synthesis of retrocyclosemigramicidin S.

15) J.A.Maclaren, *Austral. J.Chem.*, **11**, 360(1958); M. Bodanszky, J. T. Sheehan, M. A. Ondetti and S Lande, *J. Am. Chem. Soc.*, **85**, 991 (1963).

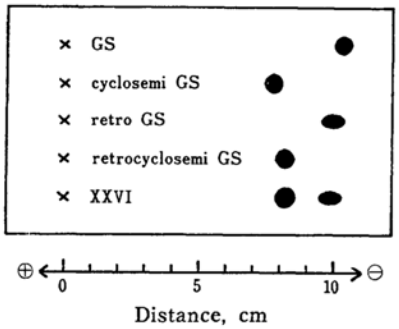


Fig. 6. Paper electrophoresis of the compounds. GS, gramicidin S; XXVI, hydrogenated material after cyclization of pentapeptide ester (XXIV).

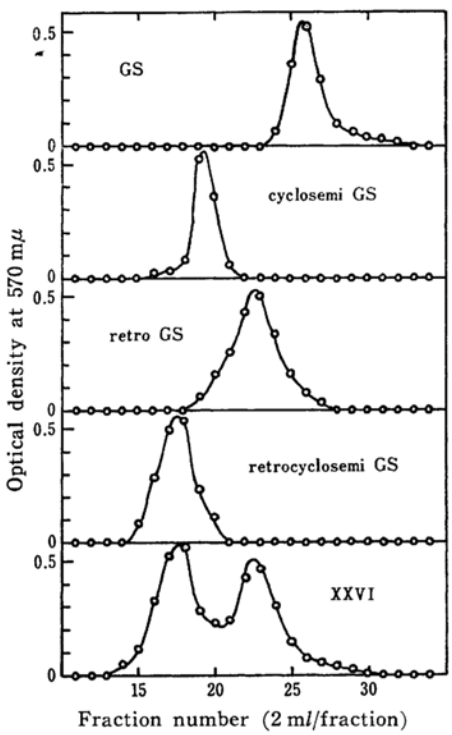


Fig. 7. Carboxymethylcellulose column chromatography of the compounds.

residue was found to be a mixture of two components by analyses with a hydrogenated material of a small part of the residue (see, Figs. 6 and 7). The residue was applied with a Sephadex LH-20 column to be separated into the components; dimer (XX) was obtained in a yield of 22.6% and the monomer (XXI) obtained in 25.3%. Thus, the weight ratio of XXI and XX is calculated as 53 : 47; the result indicates that an appreciable portion of the active ester (XXIV) is present in a transition state favored for the formation of the cyclic dimer even though some active esters are directed to the intramolecular cyclization reaction. We reported already that the cyclization reaction of H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp (XXVII) had afforded a mixture of the protected cyclosemigramicidin S and the protected gramicidin S with a weight ratio of 32 : 68.[16] In spite of the profound difference of the amino acid sequences between XXIV and XXVII, the formation of the protected cyclic decapeptides in considerable amount by the dimerization reaction in both cases favors for the stable comformation of a XX and protected gramicidin S molecule.

The antibacterial activity of gramicidin S, retrogramicidin S (XXII) and retrocyclosemigramicidin S (XXIII) toward several microorganisms was examined. The monomer XXIII showed no activity for any of the microorganisms even at 100 μg/ml of the assay medium.[17] Gramicidin S and XXII showed also no activity for the Gram negative microorganisms (*E. coli* and other), whereas minimum concentrations of growth-inhibition for the Gram positive microorganisms (*B. Subtilis* and other) were found to be 5 μg/ml with gramicidin S and 50 μg/ml with XXII. Since this results agree in some extent with the surmise described already, the proposed structure (Fig. 2) for a retrogramicidin S molecular as a possible conformation will be useful for understanding the mode of antibacterial activities of gramicidin S and its analogs.

Experimental

All the melting points are uncorrected. Prior to analysis, the compounds were dried over phosphorus pentoxide to a constant weight at 80 °C and 2 mmHg, except for the cyclic peptide hydrochlorides.

Z(OMe)-D-Phe-OH·DCHA(I·DCHA).[18] A solution of dicyclohexylamine (6 ml) in ether (10 ml) was added to *p*-methoxybenzyloxycarbonyl-D-phenylalanine which was prepared from D-phenylalanine (0.03 mol).[19] Ether was evaporated *in vacuo*, and the residual oil was crystallized by the addition of petroleum ether. It was recrystallized from ethyl acetate-petroleum ether; yield, 9.85 g (64% from

16) M. Waki and N. Izumiya, This Bulletin, **40**, 1687 (1967); *J. Am. Chem. Soc.*, **89**, 1278 (1967).

17) We have observed that the cyclic pentapeptides synthesized in this laboratory, cyclosemigramicidin S[15] and its analogs, exhibit no activity.

18) The following abbreviations are from *Biochemistry*, **5**, 2485 (1966); Z-, benzyloxycarbonyl; Z(OMe)-, *p*-methoxybenzyloxycarbonyl; Nps-, *o*-nitrophenylsulfenyl; -ONp, *p*-nitrophenoxy; ·DCHA; dicyclohexalammonium salt; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide. Amino acid symbols except D-Phe denote the L-configuration.

19) T. Kato and N. Izumiya, This Bulletin, **39**, 2242 (1966).

D-phenylalanine); mp 154—155°C; $[\alpha]_D^{20}$ −23.6° (*c* 1, methanol); R_f 0.77.[20]

Found: C, 70.36; H, 8.27; N, 5.22%. Calcd for $C_{30}H_{42}O_5N_2$: C, 70.56; H, 8.29; N, 5.49%.

Z(OMe)-D-Phe-Leu-OEt (II). To a solution of I·DCHA (10.21 g, 20 mmol), L-leucine ethyl ester *p*-toluenesulfonate[21] (6.63 g, 20 mmol) in chloroform (120 m*l*) was added dicyclohexylcarbodiimide[22] (4.13 g, 20 mmol) at 0 °C. The reaction mixture was stirred for 1 hr at 0 °C, and then kept overnight in a refrigerator. The mixture was evaporated *in vacuo*, and ethyl acetate (150 m*l*) was added to the residue. After the dicyclohexylurea was filtered off, the filtrate was washed successively with 0.5 M citric acid, 4% sodium bicarbonate and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo*, and the residue was crystallized by the addition of ether and petroleum ether. It was recrystallized from methanol-ether-petroleum ether; yield, 7.25 g (77%); mp 108—109°C; $[\alpha]_D^{25}$ −0.6° (*c* 1, DMF); R_f 0.97.[20]

Found: C, 66.31; H, 7.35; N, 6.01%. Calcd for $C_{26}H_{34}O_6N_2$: C, 66.36; H, 7.28; N, 5.95%.

Z(OMe)-D-Phe-Leu-NHNH$_2$ (III). A solution of II (7.06 g, 15 mmol) and hydrazine hydrate (14.6 m*l*, 300 mmol) in dimethylformamide (20 m*l*) was allowed to stand at room temperature for 2 days. The hydrazine was evaporated *in vacuo*, and then water (250 m*l*) was added to the residue. The resulting crystals were collected by filtration ; yield, 6.51 g (95%); mp 176—177°C; $[\alpha]_D^{20}$ −7.5° (*c* 1, DMF); R_f 0.87.[20]

Found: C, 63.23; H, 7.05; N, 12.18%. Calcd for $C_{24}H_{32}O_5N_4$: C, 63.14; H, 7.07; N, 12.27%.

Z-Val-Pro-OEt (IV). To a chilled solution of benzyloxycarbonyl-L-valine (10.05 g, 50 mmol) and triethylamime (5.6 m*l*, 40 mmol) in tetrahydrofuran (60 m*l*), isobutylchloroformate (5.24 m*l*, 40 mmol) was added. After 15 min, a mixture of L-proline ethyl ester *p*-toluenesulfonate[21] (12.61 g, 40 mmol), triethylamine (5.6 m*l*, 40 mmol) and chloroform (60 m*l*) was added to the solution. The reaction mixture was allowed to stand overnight and then evaporated to dryness *in vacuo*. After the residual oil was dissolved in ethyl acetate, the solution was washed successively with 3% hydrochloric acid, 4% sodium bicarbonate and water, dried over sodium sulfate, and then the filtrate was evaporated. The product was obtained as an oil; yield, 10.36 g (69%); R_f 0.94.[20]

HCl·H-Val-Pro-OEt (V·HCl). A solution of IV (7.53 g, 20 mmol) dissolved in a mixture of 3.97 N ethanolic hydrogen chloride (5.04 m*l*) and ethanol (100 m*l*) was hydrogenated in the presence of palladium black. After 3 hr, the filtrate from the catalyst was evaporated to dryness *in vacuo*. The oil weighed 5.35 g (96%); R_f 0.79.[20]

Nps-Orn(δ-Z)-OH·DCHA(VI·DCHA). δ-Benzyloxycarbonyl-L-ornithine (10.65 g, 40 mmol) was dissolved in a mixture of 2 N sodium hydroxide (40 m*l*, 80 mmol) and dioxane (160 m*l*). To the solution, *o*-nitrophenysulfenyl chloride[13] (7.59 g, 40 mmol) and 2 N sodium hydroxide (15 m*l*, 30 mmol) was added 5 portions over a period of 30 min under vigorous stirring. The reaction mixture was diluted with water (600 m*l*), a small amount of the insoluble material was filtered off and the filtrate was acidified with 0.5 M citric acid at 0 °C. The solution was extracted with ethyl acetate, and the extract was washed with water and dried over sodium sulfate. The filtrate from the salt was evaporated *in vacuo*. To the residue the solution of dicyclohexylamine (8 m*l*) in ether (10 m*l*) was added. This solution was treated following the same procedure described for the preparation of I·DCHA. The crude product was recrystallized from methanol-ether-petroleum ether; yield, 17.06 g (71%); mp 167—169 °C; $[\alpha]_D^{25}$ −25.0° (*c* 1, DMF); R_f 0.85.[20]

Found: C, 61.81; H, 7.38; N, 9.06%. Calcd for $C_{31}H_{44}O_6N_4S$: C, 61.97; H, 7.38; N, 9.33%.

Z(OMe)-Orn(δ-Z)-OH·DCHA (VII·DCHA). *p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithine[23] was prepared from δ-benzyloxycarbonyl-ornithine (0.05 mol) and then changed to VII·DCHA as described for the preparation of I·DCHA; yield, 20.56 g (67%), mp 121—122 °C; $[\alpha]_D^{20}$ −4.0° (*c* 1, methanol); R_f 0.81.[20]

Found: C, 66.80; H, 8.15; N, 6.77%. Calcd for $C_{34}H_{49}O_7N_3$: C, 66.75; H, 8.09; N, 6.87%.

Nps-Orn(δ-Z)-Val-Pro-OEt (VIII). To a suspension of VI·DCHA (6.01 g, 10 mmol) in ethyl acetate (100 ml) at 0 °C, 0.5 M citric acid (40 ml) was added under stirring. After 1 hr, the organic layer was separated and washed with water. It was then dried over sodium sulfate and evaporated to dryness to give an oily product (4.20 g) of VI. To a solution of the oily VI in chloroform (60 m*l*) was added V·HCl (3.07 g, 11 mmol) and triethylamine (1.54 m*l*, 11 mmol). Dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added to the solution at 0 °C, and then the reaction mixture was treated in the same manner as that used for the preparation of II. The residual oil weighed 6.37 g (99%); R_f 0.98.[20]

Z(OMe)-Orn(δ-Z)-Val-Pro-OEt (IX). A crystalline product VII (4.35 g) from VII·DCHA (6.12 g, 10 mmol) was coupled with V·HCl (3.07 g, 11 mmol) by dicyclohexylcarbodiimide (2.06 g, 10 mmol) following essentially the same procedure described above. The oily residue obtained weighed 6.29 g (96%); R_f 0.94.[20]

HCl·H-Orn(δ-Z)-Val-Pro-OEt (X·HCl). From VIII. To a solution of VIII (5.15 g, 8 mmol) in

20) The R_f value of the thin-layer chromatography with Merck silica gel refers to a solvent system of *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, v/v). Spots of materials possessing a free amino group on a thin-layer plate were detected by spraying ninhydrin, and those of the amino group-blocked materials, by spraying 47% hydrobromic acid and then ninhydrin.

21) T.Kato, S.Makisumi, M.Ohno and N.Izumiya, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **83**, 1151 (1962).

22) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

23) M. Kondo, H.Aoyagi, T.Kato and N.Izumiya, This Bulletin, **39**, 2234 (1966).

dioxane (16 ml), 2 N hydrogen chloride in dioxane (16 ml) was added. After the solution was allowed to stand at room temperature for 30 min, the solution was evaporated *in vacuo*. The residual oil was washed several times with ether by the way of decantation ; yield of the oily X·HCl, 5.11 g (97 %); R_f 0.82.[20]

From IX. A solution of IX (5.24 g, 8 mmol) and anisole (2 ml) in dioxane (20 ml) was treated with 3 N hydrogen chloride in dioxane (53 ml) for 3 hr; yield of the oily X·HCl, 5.27 g (100%); R_f 0.82.[20]

Z(OMe)-D-Phe-Leu-Orn(δ-Z)-Val-Pro-OEt (XI). To a chilled solution of III (4.57 g, 10 mmol) in glacial acetic acid (140 ml) and dimethylformamide (70 ml), there were stirred 1 N hydrochloric acid (22 ml), sodium nitrite (0.76 g) in water (2 ml). After 10 min, cold water (500 ml) was added. The azide which thereupon precipitated was collected by filtration, washed with water, a saturated sodium bicarbonate solution and water, and then dried in a desiccator. The azide was added to a solution of X·HCl (5.27 g, 10 mmol) and triethylamine (14 ml) in dimethylformamide (50 ml). The mixture was then stirred for 3 days at 0 ℃ and evaporated *in vacuo*. The precipitate which was formed upon the addition of water (400 ml) was collected, and then washed with 4 % sodium bicarbonate, 0.5 M citric acid and water. It was recrystallized from dioxane-ether-petroleum ether. Yield, 6.26 g (68%); mp 158—159 ℃; $[\alpha]_D^{20}$ −31.0° (*c* 1, DMF); R_f 0.98[20]

Found: C, 64.38; H, 7.31; N, 9.00 %. Calcd for $C_{49}H_{66}O_{11}N_6$: C, 64.31; H, 7.27; N, 9.19 %.

Z(OMe)-D-Phe-Leu-Orn(δ-Z)-Val-Pro-NHNH$_2$ (XII). To a solution of XI (0.92 g, 1 mmol) in dimethylformamide (8 ml), hydrazine hydrate (1.21 ml, 25 mmol) was added and the solution was allowed to stand for 7 days at room temperature. The reaction mixture was then evaporated *in vacuo*. The hydrazide which precipitated upon the addition of water was collected by filtration and dried. The product was recrystallized from methanol-ether; yield, 0.75 g (83 %); mp 160—161 ℃; $[\alpha]_D^{20}$ −29.4° (*c* 0.5, DMF); R_f 0.88[20].

Found: C, 62.14; H, 7.27; N, 12.12 %. Calcd for $C_{47}H_{64}O_{10}N_8 \cdot 1/2H_2O$: C, 62.03; H, 7.20; N, 12.31 %.

Nps-Orn(δ-Z)-Val-Pro-OH (XIII). To a solution of VIII (5.31 g 8 mmol) in methanol (40 ml) and dioxane (40 ml), N sodium hydroxide (32 ml) was added; the solution then was allowed to stand for 24 hr at room temperature. After the addition of water (40 ml), the solution was evaporated *in vacuo* to remove organic solvents. After the solution was extracted with ether, it was acidified with 0.5 M citric acid at 0 ℃. The solution was extracted with ethyl acetate and the organic layer was dried over sodium sulfate. The filtrate was evaporated to dryness *in vacuo* to afford an oily product; yield, 3.05 g (62%); R_f 0.86.[20]

Z(OMe)-Orn(δ-Z)-Val-Pro-OH (XIV). To a solution of IX (3.27 g, 5 mmol) in methanol (50 ml), 1 N sodium hydroxide (10 ml) was added. The solution was treated following the same procedure described above. The residual oil weighed 1.89 g (60 %); R_f 0.84.[20]

HCl·H-Orn(δ-Z)-Val-Pro-OH (XV·HCl). From XIII. To a solution of XIII (3.08 g, 5 mmol) in dioxane (20 ml), 2 N hydrogen chloride in dioxane (15 ml) was added at room temperature. After 30 min the solution was evaporated to dryness, and the residue was washed repeatedly with ether by decantation. The remaining hygroscopic crystals were collected by filtration with the aid of ether; yield, 1.90 g (76 %); mp 135—140 ℃ (decomp.); $[\alpha]_D^{20}$ −18.0° (*c* 0.5, DMF); R_f 0.71.[20]

Found: C, 53.60; H, 7.19; N, 10.57 %. Calcd for $C_{23}H_{34}O_6N_4 \cdot HCl \cdot H_2O$: C, 53.43; H, 7.21; N, 10.84%.

From XIV. XV·HCl was also prepared from XIV (1.88 g, 3 mmol), anisole (0.9 ml) and 2.53 N hydrogen chloride in dioxane (23.7 ml) for 2 hr following the same procedure described above; yield, 1.03 g (69 %); $[\alpha]_D^{25}$ −17.9° (*c* 0.4, DMF); R_f 0.71.[20]

H-Orn(δ-Z)-Val-Pro-OH (XV). XV·HCl (998 mg, 2 mmol) was dissolved in small volume of a mixture of water and methanol. After triethylamine (0.28 ml) had been added, the solution was evaporated to dryness *in vacuo*. The crystalline product was collected by filtration with the aid of water ; yield, 898 mg (97 %); mp 174—176 ℃ (decomp.); $[\alpha]_D^{25}$ −34.0° (*c* 0.4, acetic acid); R_f 0.71.[20]

Found: C, 53.34; H, 7.68; N, 10.91 %. Calcd for $C_{23}H_{34}O_6N_4 \cdot 2H_2O$: C, 55.41; H, 7.68; N, 11.24 %.

Z(OMe)-D-Phe-Leu-Orn(δ-Z)-Val-Pro-OH (XVI). From III and XV. The azide derived from III (2.15 g, 4.7 mmol) was added to a solution of XV·HCl (2.50 g, 5 mmol) and triethylamine (1.4 ml, 10 mmol) in dimethylformamide (25 ml), and the mixture was stirred for 3 days at 0 ℃. The mixture was evaporated *in vacuo*, and the residue was added to ethyl acetate (300 ml). The mixture was washed successively with water, 0.5 M citric acid, and water, and then dried over sodium sulfate. After the filtrate was evaporated, the residual crystals were collected with the aid of a mixture of ether and petroleum ether. It was recrystallized from methanol-ether-petroleum ether; yield, 2.92 g (70 %); mp 137—138℃; $[\alpha]_D^{25}$ −29.5° (*c* 0.5, DMF); R_f 0.82,[20] 0.68.[24]

Found: C, 63.53; H, 6.75; N, 9.66 %. Calcd for $C_{47}H_{62}O_{11}N_6$: C, 63.64; H, 7.65; N, 9.48 %.

From XI. To a solution of XI (275 mg, 0.3 mmol) in ethanol (4 ml) and dioxane (2 ml), 1 N sodium hydroxide (0.6 ml) was added at room temperature. The hydrolysis was almost complete after 4 hr. The solution was concentrated *in vacuo* to small volume, and acidified with 0.5 M citric acid under cooling. The separated crystalline solid was taken up with ethyl acetate, and the ethyl acetate solution was washed with water, dried over sodium sulfate. The filtrate was evaporated, and the residual crystals were collected by filtration with the aid of a mixture of ether and petroleum ether; yield of the crude product, 208 mg. The crude product was separated into the two pure components as follows. A solution of the crude product (200 mg) in dioxane (0.6 ml)

24) The R_f value of the thin-layer chromatography refers to a solvent system of chloroform-methanol (5 : 1, v/v).

was applied to a column (0.9×48 cm) with Sephadex LH-20, and the development continued with dioxane. Elution was carried out at room temperature, the flow rate 7 m*l* per hr, and 1.5 m*l* fraction collected. When the peptide content in the fractions was determined on a thin-layer plate, two compounds appeared separately in tube numbers 10 to 13 and 14 to 20. The fractions of 10—13 were combined, evaporated and the crystals of XVI were obtained. Yield, 73 mg (28 % from XI); mp 136—137 °C; $[\alpha]_D^{25}$ −30.6° (*c* 0.2, DMF); R_f 0.82[20], 0.68.[24]

2-Isobutyl-5-[1-carboxy-2-(phenyl)-ethyl]-hydantoyl-Orn(∂-Z)-Val-Pro-OH (XVII). The fractions of 14—20 obtained in above was evaporated *in vacuo*, and the residual product was recrystallized from methanol-water; yield, 80 mg (36 % from XI); mp 121—123 °C; $[\alpha]_D^{25}$ −23.3° (*c* 0.3, DMF); R_f 0.65.[24]

Found: C, 61.21; H, 7.49; N, 10.55 %; neut equiv, 784. Calcd for $C_{39}H_{54}O_{10}N_6$: C, 61.08; H, 7.10; N, 10.96 %; neut equiv, 767.

HCl·H-D-Phe-Leu-Orn(∂-Z)-Val-Pro-OH (XVIII·HCl). To a solution of XVI (1.06 g, 1.2 mmol) and anisole (0.3 m*l*) in dioxane (6 m*l*), 2.57 N hydrogen chloride in dioxane (9.3 m*l*) was added at room temperature. After 4 hr, the solution was treated following the same procedure for the preparation of XV·HCl. Yield, 0.82 g (90 %); mp 201—203 °C (decomp.); $[\alpha]_D^{25}$ −53.6° (*c* 0.3, DMF); R_f 0.77.[20]

Found: C, 58.72; H, 7.25; N, 10.75 %. Calcd for $C_{38}H_{54}O_8N_6 \cdot HCl \cdot H_2O$: C, 58.71; H, 7.39; N, 10.81 %.

H-D-Phe-Leu-Orn(∂-Z)-Val-Pro-OH (XVIII). XVIII·HCl (152 mg, 0.2 mmol) was dissolved in a mixture of water and methanol. After triethylamine (0.06 m*l*) had been addded, the solution was evaporated to dryness *in vacuo*. The crystalline product was collected by filtration with the aid of methanol. It was recrystallized from hot methanol-ether, and washed with a small amount of water; yield, 105 mg (72 %); mp 199—201°C (decomp.); $[\alpha]_D^{20}$ −84.3° (*c* 0.3, acetic acid); R_f 0.77.[20]

Found: C, 60.35; H, 7.50; N, 11.04 %. Calcd for $C_{38}H_{54}O_8N_6 \cdot 2H_2O$: C, 60.14; H, 7.70; N, 11.08 %.

Z(OMe)-D-Phe-Leu-Orn(∂-Z)-Val-Pro-D-Phe-Leu-Orn(∂-Z)-Val-Pro-OH (XIX). The azide was prepared from XII (451 mg, 0.5 mmol), 1N hydrochloric acid (1.1 m*l*), and sodium nitrite (38 mg) in a mixture of acetic acid (6 m*l*) and dimethylformamide (3 m*l*) as in the case of the preparation of XI. Then the azide dried was added to a solution of XVIII·HCl (380 mg, 0.5 mmol) and triethylamine (0.14 m*l*, 1 mmol) in dimethylformamide (10 m*l*). After the reaction mixture was stirred for 3 days at 0°C, it was treated following the same procedure that employed for the preparation of XVI. The crude product was recrystallized from hot dioxane-methanol-ether; yield, 600 mg (75 %); mp 174—175 °C; $[\alpha]_D^{25}$ −31.4° (*c* 0.5, DMF); R_f 0.94,[20] 0.45.[24]

Found: C, 62.78; H, 7.24; N, 10.58 %. Calcd for $C_{85}H_{114}O_{18}N_{12} \cdot 2H_2O$: C, 62.71; H, 7.31; N, 10.33 %.

***cyclo*-(D-Phe-Leu-Orn(∂-Z)-Val-Pro-)$_2$ (XX). From XIX.** To a solution of XIX (557 mg, 0.35 mmol) in pyridine (10 m*l*), di-*p*-nitrophenyl sulfite[25] (2.27 g, 7 mmol) was added. After the mixture had been allowed to stand for 24 hr at room temperature, it was evaporated *in vacuo*. The residual solid was collected by filtration with the aid of a mixture of ether-petroleumether (1 : 1, v/v); 589 mg. The *p*-nitrophenyl ester content for this product was estimated to be 85 % by measuring the optical density of the compound at 412 mμ.[26] To the acyldecapeptide *p*-nitrophenyl ester (585 mg) thus obtained, anisole (0.5 m*l*) and trifluoroacetic acid (3.5 m*l*) were added at 0 °C. After the solution was evaporated *in vacuo*, the residual powder was collected by filtration with the aid of ether. The decapeptide *p*-nitrophenyl ester trifluoroacetate thus obtained was dissolved in dimethylformamide (10 m*l*) and acetic acid (0.1 m*l*). The solution was then stirred, drop by drop and over a period of 4 hr, into pyridine (170 m*l*) which had been kept at 60 °C; the stirring was then continued for an additional 2 hr at the same temperature. After the solvent had been removed, the residue was dissolved in a mixture of methanol (40 m*l*) and water (10 m*l*). After the insoluble substance was removed, the filtrate was passed successively through columns (1.8×10 cm, each) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (200 m*l*), and the combined effluent was evaporated *in vacuo* to yield a crystalline product. The product was collected by filtration with the aid of water, and recrystallized from methanol-ether-petroleum ether; yield, 242 mg (49 % from XIX); mp 250—251°C (decomp.); $[\alpha]_D^{20}$ −41.3° (*c* 0.3, acetic acid).

Found: C, 64.44; H, 7.46; N, 11.73 %; mol wt, 1413.[27] Calcd for $C_{76}H_{104}O_{14}N_{12} \cdot 1/2H_2O$: C, 64.34; H, 7.46; N, 11.85 %; mol wt, 1419.

From XVI. The acylpentapeptide XVI (2.22 g, 2.5 mmol) was converted to the pentapeptide *p*-nitrophenyl ester trifluoroacetate (XXIV·CF_3COOH) following the same procedure as described above. XXIV·CF_3COOH (2.39 g) thus obtained was added to pyridine (800 m*l*). After the filtrate was passed through columns of Dowex 1 and Dowex 50 as described above, the effluent was evaporated to dryness, the residual product was collected by filtration with the aid of water; yield of the crude product (XXV), 830 mg. The solution of XXV (50 mg) in methanol (1 m*l*) was applied to a column (2.7×50 cm) with Sephadex LH-20, and the development continued with methanol. Elution was carried out at room temperature, the flow rate 30 m*l* per hr, and 3 m*l* fraction collected.

When the peptide content in the fractions was determined on a thin-layer plate, the peak of XX appeared from the test tube numbers 45 to 52 and the peak of XXI from 54 to 65. The same chromatography was repeated further nine times; thus, 500 mg of XXV was separated into two component. The ten fractions of 45—52 were combined, evapo-

25) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1760 (1960).

26) R. Schwyzer and P. Sieber, *ibid.*, **40**, 624 (1957).

27) Molecular weight was determined by a Hitachi Osmometer, type 115 (solvent, methanol).

TABLE 1. INHIBITORY ACTIVITY OF FOUR COMPOUNDS ON MICROORGANISMS
Minimum inhibitory concentration, μg/ml

A. Bouillon agar medium[a)]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*
GS	>100	>100	5	5	>100
retro GS	>100	>100	50	50	>100
retrocyclosemi GS	>100	>100	>100	>100	>100

B. Synthetic medium[b)]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*
GS	>100	>100	5	5	>100
retro GS	>100	>100	50	50	>100
retrocyclosemi GS	>100	>100	>100	>100	>100

a) Usual bouillon agar medium, pH 7.0.
b) Stephenson-Whetham's medium (modified).

rated *in vacuo* and the crystals (240 mg, 22.6% from XVI) of XX were obtained; yield after recrystallization, 234 mg (22%); mp 251—252 °C (decomp.); $[\alpha]_D^{20}$ −40.8° (*c* 0.3, acetic acid).

***cyclo*-D-Phe-Leu-Orn(δ-Z)-Val-Pro (XXI).** The ten fractions of the test tube numbers 54—65 obtained in above were evaporated *in vacuo* to afford an oily residue. It was crystallized after standing of several days in a refrigerator; 271 mg (25.5% from XVI). It was recrystallized from methanol-ether-petroleum ether (235 mg, 22%); mp 230—232 °C (decomp.); $[\alpha]_D^{20}$ −50.9° (*c* 0.2, acetic acid).

Found: C, 63.19; H, 7.20; N, 11.33%; mol wt, 697.[27)] Calcd for $C_{38}H_{52}O_7N_6 \cdot H_2O$: C, 63.13; H, 7.53; N, 11.62%; mol wt, 723.

Retrogramicidin S, *cyclo*-(D-Phe-Leu-Orn-Val-Pro-)$_2$ (XXII). A solution of XX (85 mg, 0.06 mmol) in methanol (3 ml) and 0.4 N methanolic hydrogen chloride (0.32 ml), was subjected to hydrogenolysis in the presence of palladium black for 3 hr. The solution, after being filtered from the catalyst, was evaporated *in vacuo*. The residual product was recrystallized from methanol-ether-petroleum ether; yield of the air-dried product, 69 mg (94%); mp 272—276°C (decomp.); $[\alpha]_D^{20}$ −36.3° (*c* 0.1, etnanol); R_f 0.96,[28)] 0.75[20)]; amino acid ratios in acid hydrolysate, $Phe_{0.8}Leu_{1.0}Orn_{1.1}Val_{1.0}Pro_{1.0}$.

Found; C, 54.64; H, 7.89; N, 12.51%. Calcd for $C_{60}H_{92}O_{10}N_{12} \cdot 2HCl \cdot 6H_2O$: C, 54,49; H, 8.08; N, 12.71%. The air-dried product lost 8.6% of its weight after being dried for 3 hr at 80°C and 2 mmHg. Calcd for $6H_2O$; 8.2%.

Retrocyclosemigramicidin S, *cyclo*-D-Phe-Leu-Orn-Val-Pro (XXIII). XXI (42 mg, 0.06 mmol) was treated as described for the preparation of XXII·2HCl; yield, 31 mg (84%); mp 252—253 °C (decomp.); $[\alpha]_D^{20}$ −60.0° (*c* 0.5, ethanol); R_f 0.92,[28)] 0.61; [20)] amino acid ratios in acid hydrolysate, $Phe_{0.9}Leu_{1.0}Orn_{1.0}Val_{0.9}Pro_{1.1}$.

Found: C, 54.27; H, 7.77; N, 12.85%. Calcd for $C_{30}H_{46}O_5N_6 \cdot HCl \cdot 3H_2O$: C, 54.49; H, 8.08; N, 12.71%. The air-dried product lost 8.7% of its weight after drided for 3 hr at 80 °C and 2 mmHg. Calcd for $3H_2O$: 8.2%.

Electrophoresis and Carboxymethylcellulose (CMC) Chromatography. These experiments were carried out as has been described before.[16)] A part of the crude product (XXV), which was obtained after the cyclization reaction of the pentapeptide *p*-nitrophenyl ester (XXIV), was hydrogenated, and the hydrogenated product thus obtained was designated as XXVI. As shown in Figs. 6 and 7, retro GS and retrocyclosemi GS was clearly separated in the electrophoresis, whereas these compounds could be only partially separated with a column (0.9×50 cm) of CMC.

Microbiological Assays.[29)] The microorganisms employed are listed in Table 1. The minimum amount of the compound necessary for the complete inhibition of growth was determined by a dilution method using a bouillon agar medium and a synthetic medium. As is shown in Table 1, retrogramicidin S (XXII) was found to exhibit weak antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. Whereas retrocyclosemigramicidin S (XXIII) exhibited no antibacterial activity against the microorganisms tested.

28) The R_f value of the paper chromatography refers to the same solvent system described.[20)]

29) We are indebted to Dr. M. Shibata of Takeda Chemical Industries, Ltd. for the assay.