BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 1850—1854 (1970)

Studies of Peptide Antibiotics. XIX. Syntheses of 1,1′-Leucine-gramicidin S and 1-Leucine-cyclosemigramicidin S

Michio KONDO* and Nobuo IZUMIYA**

*Department of Chemistry, Faculty of Science and Engineering, Saga University, Saga
**Laboratory of Biochemistry, Faculty of Science, Kyushu University, Fukuoka

(Received December 25, 1969)

Analogs of gramicidin S, 1,1′-L-leucine-gramicidin S (XI) and 1-L-leucine-cyclosemigramicidin S (XII), have been synthesized and tested for antibacterial properties. The cyclization reaction of a linear decapeptide active ester, in which δ-amino functions of ornithine residues were protected with benzyloxycarbonyl groups, yielded exclusively a protected cyclic decapeptide which was hydrogenated to afford the dihydrochloride of XI. A linear pentapeptide active ester afforded a mixture of protected cyclic penta and decapeptide. The pure protected cyclic pentapeptide was obtained with the use of a Sephadex LH-20 column, and the hydrogenolysis of the product afforded the hydrochloride of XII. The decapeptide analog (XI) was as active as natural gramicidin S, whereas the pentapeptide (XII) showed no activity toward any of the microorganisms tested.

We reported that 1,1′-L-alanine gramicidin S was as active as natural gramicidin S (GS) (Fig. 1) toward several microorganisms.[1] The result indicated that the side chains of valine residues, the isopropyls, in a GS molecule can be replaced by smaller aliphatic side chains, the methyls, without a decrease in the antibacterial activity. From this finding, it seemed of interest to investigate the antibacterial properties of an analog of GS with larger side chains than the isopropyls of the valines.

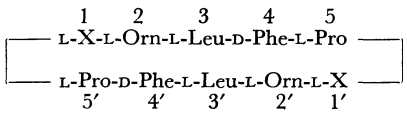

Fig. 1. Structure of GS and its analog.
X represents amino acid residue such Val (GS) or Leu (XI).

This paper will describe the synthesis and antibacterial properties of 1,1′-L-leucine-GS besides the preparation of the cyclic pentapeptide, 1-L-leucine-cyclosemiGS.

Figure 2 indicates the route for the synthesis of the desired cyclic decapeptide (XI).[2] Condensation of the azide derived from *p*-methoxybenzyloxycarbonyl pentapeptide hydrazide (V) with the corresponding neutral pentapeptide (VII) gave an acyldecapeptide acid (VIII). Acid VIII was transformed to an acyldecapeptide nitrophenyl ester, and its *p*-methoxybenzyloxycarbonyl group was removed by means of trifluoroacetic acid.

TABLE 1. RATIO OF PROTECTED CYCLIC MONOMER AND DIMER AFTER CYCLIZATION OF LINEAR PENTAPEPTIDE ACTIVE ESTERS

p-Nitrophenyl ester of[a]	Ratio of compounds in product[b]	
	Z-cyclic monomer	di-Z-cyclic dimer
H-Gly-Orn(Z)-Leu-D-Phe-Pro-OH[1]	100	0
-Ala-[1]	91	9
-Leu-	78	22
-Val-[3]	32	68
-Gly-Lys(Z)-[c]	100	0
-Val-Lys(Z)-[d]	29	71

a) The first compound is listed and only variations of the residue are shown.
b) The concentration of pentapeptide nitrophenyl esters in pyridine was 3×10^{-3} M.
c) M. Kondo and N. Izumiya, to be published.
d) M. Waki, O. Abe, R. Okawa, T. Kato, S. Makisumi and N. Izumiya, This Bulletin, **40**, 2904 (1967).

1) M. Kondo and N. Izumiya, This Bulletin, **40**, 1975 (1967).

2) Abreviations: GS, gramicidin S; Z-, benzyloxycarbonyl; Z(OMe)-, *p*-methoxybenzyloxycarbonyl; -ONp, *p*-nitrophenoxy; CMC, carboxymethyl cellulose; DMF, dimethylformamide; MA, mixed anhydride; TLC, thin-layer chromatography. Amino acid symbols except D-Phe denote the L-configuration.

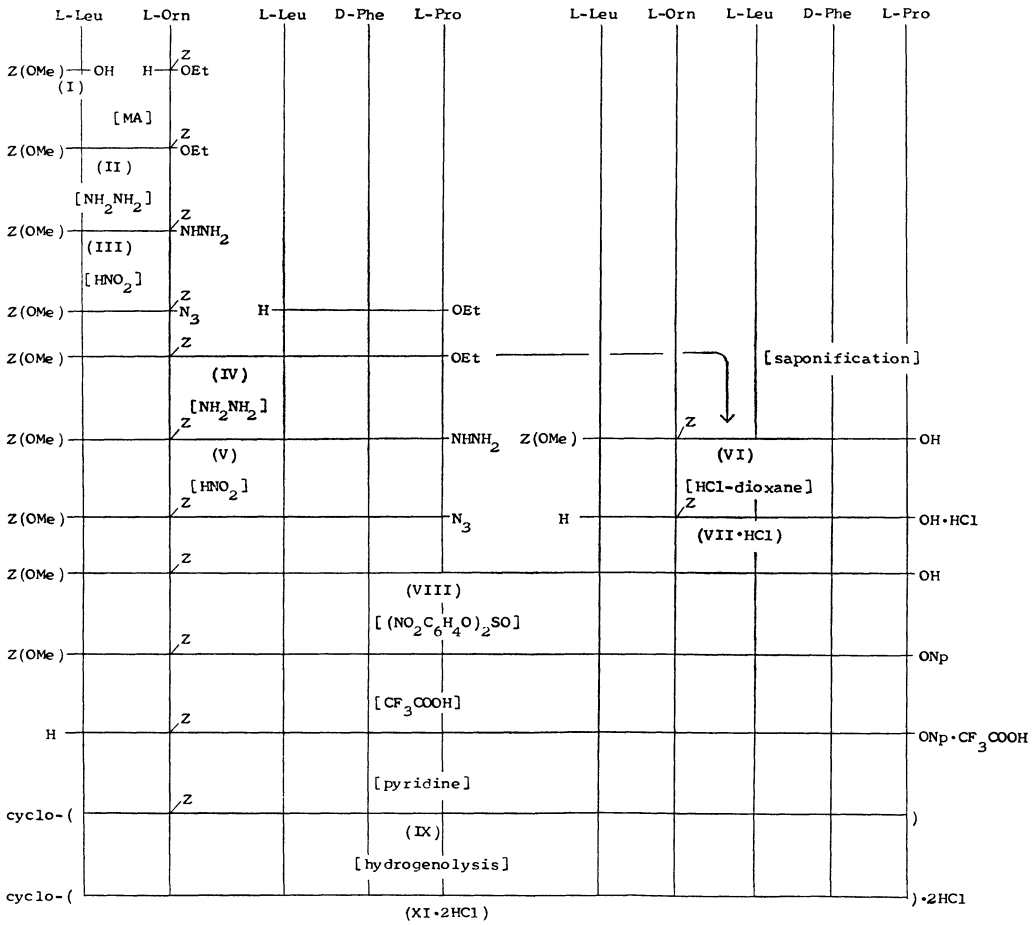

Fig. 2. Cyclization of linear decapeptide active ester.

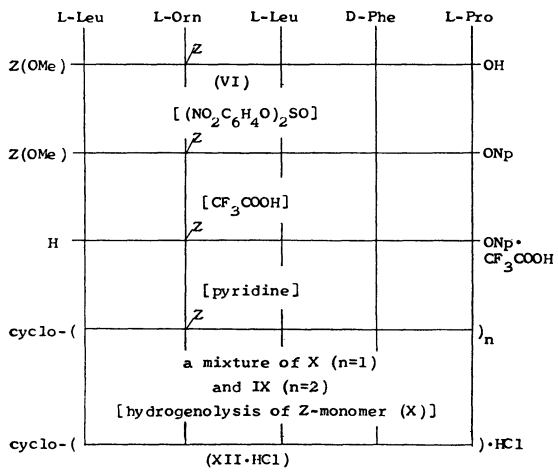

Fig. 3. Cyclization of linear pentapeptide active ester.

Decapeptide ester trifluoroacetate thus obtained was treated with pyridine for the cyclization reaction. The reaction mixture yielded a pure benzyloxycarbonyl-substituted cyclic decapeptide (IX) which was hydrogenated to afford the desired cyclic decapeptide (XI) as a crystalline dihydrochloride.

Synthesis of the protected cyclic decapeptide (IX) was attempted by possible dimerization reaction of the linear pentapeptide active ester which was derived from *p*-methoxybenzyloxycarbonyl pentapeptide acid (VI) (Fig. 3). Treatment of the active ester with pyridine afforded a mixture of the protected monomer (X) and dimer (IX); the ratio in weight of X and IX in the mixture was found to be approximately 78 : 22. Separation of the components was achieved with the use of a Sephadex LH-20 column with methanol as an eluting solvent, the protected monomer (X) being obtained as a pure crystalline material. Hydrogenolysis of X in the presence of hydrogen chloride yielded a crystalline hydrochloride of the cyclic pentapeptide (XII).

The weight ratios of the protected cyclic monomer and dimer in the crude product after the cyclization reaction of the linear pentapeptide active ester are shown in Table 1 with the previous results related to the present paper. It is recognized that the

Table 2. Amount of compound necessary for complete inhibition of growth, $\mu g/ml$

	A. Bouillon agar medium, pH 7.0				
	B. subtilis	*St. aureus*	*E. coli*	*Pr. vulgaris*	*M. avium*
GS	5	5	>100	>100	>100
Leu-GS (XI)	10	5	>100	>100	>100
Leu-semiGS (XII)	>100	>100	>100	>100	>100
	B. Synthetic medium, pH 7.0				
	B. subtilis	*St. aureus*	*E. coli*	*Pr. vulgaris*	*M. avium*
GS	2	5	>100	>100	>100
Leu-GS (XI)	5	10	>100	>100	>100
Leu-semiGS (XII)	>100	>100	>100	>100	>100

pentapeptide sequence with the alanine or leucine residue as N-terminus is more favorable for the intramolecular cyclization reaction compared with that with the valine residue.[3] This means that the degree of the monomer formation is influenced more profoundly by the shape of the side chain of the N-terminus in the pentapeptide active ester than its size; the side chain of valine is indicated as R–CH(R)– (R, methyl), whereas that of alanine or leucine as R–CH_2– (R, H or isopropyl). In this connection, the syntheses of 1,1′-L-isoleucine-GS and several related compounds are in progress.

The antibacterial activities of the synthetic cyclic peptides toward several microorganisms are listed in Table 2. It was observed that 1-leucine-cyclo-semiGS (XII) exhibited no antibacterial activity similar to those of 1-glycine and 1-alanine-cyclosemi-GS,[1] whereas 1,1′-leucine-GS was as active as natural GS against several microorganisms. The results indicate that the side chains of the valine residues in a GS molecule can be replaced with larger aliphatic side chains, the isobutyls, without influencing antibacterial activity.

Experimental

Melting points were not corrected. Prior to analysis, the compounds were dried to a constant weight over phosphorus pentoxide at 60°C and 2 mmHg, except in the case of the cyclic peptide hydrochlorides (XI·2HCl and XII·HCl).

Z(OMe)-Leu-OH (I). The general procedure[4] to prepare Z(OMe)-amino acid was modified as follows. A mixture of L-leucine (3.93 g, 30 mmol), water (90 ml), dioxane (90 ml), sodium bicarbonate (6.05 g) and *p*-methoxybenzyloxycarbonyl azide (7.46 g, 36 mmol) was stirred at room temperature for 60 hr, the reaction being followed by TLC. The solution was evaporated *in vacuo* to a small volume, extracted with ether, acidified with 0.5M citric acid, and then extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated to dryness. The product was obtained as an oil; yield, 6.55 g (74%). Reported yield for the oil, 28%.[4] A part of the oily product was converted to crystalline dicyclohexylamine salt; yield 85%; mp 163°C; $[\alpha]_D^{15}$ −6.8° (*c* 2, methanol). Reported value for the salt; mp 162°C; $[\alpha]_D^{24}$ −6.67° (methanol).[4]

Z(OMe)-Leu-Orn(δ-Z)-OEt (II). To a chilled solution of I (5.02 g) and triethylamine (1.99 ml) in tetrahydrofuran (28 ml), isobutylchloroformate (1.86 ml) was added. After 15 min, a mixture of H-Orn(δ-Z)-OEt·TsOH,[5] triethylamine (1.99 ml) and chloroform (38 ml) was added to the chilled solution. The reaction mixture was allowed to stand overnight, evaporated *in vacuo*, and the residue was dissolved in ethyl acetate (100 ml). The solution was washed with 0.5M citric acid, 4% sodium bicarbonate and water, dried over sodium sulfate and then evaporated *in vacuo*. The oily residue was crystallized by the addition of a mixture of ether and petroleum ether. It was recrystallized from ethyl acetate-ether-petroleum ether; yield, 5.97 g (81%); mp 109—110°C; $[\alpha]_D^{15}$ −6.3° (*c* 2, DMF).

Found: C, 62.74; H, 7.12; N, 7.41%. Calcd for $C_{30}H_{41}O_8N_3$: C, 63.03; H, 7.23; N, 7.35%.

Z(OMe)-Leu-Orn-(δ-Z)-$NHNH_2$ (III). A solution of II (5.15 g) and hydrazine hydrate (9 ml) in DMF (35 ml) was allowed to stand for 2 days at 30°C. The solution was concentrated *in vacuo* to a small volume. The hydrazide which precipitated upon the addition of water was collected; yield, 4.70 g (94%); mp 147—148°C; $[\alpha]_D^{15}$ −7.4° (*c* 1, DMF).

Found: C, 58.41; H, 7.17; N, 12.19%. Calcd for $C_{28}H_{39}O_7N_5 \cdot H_2O$: C, 58.42; H, 7.18; N, 12.17%.

Z(OMe)-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-OEt (IV). To III (4.47 g, 8 mmol) dissolved in acetic acid (35 ml) and N hydrochloric acid (16 ml) was added sodium nitrite (0.66 g) in water (5 ml) at −5°C. After 10 min, the solution was diluted with water (120 ml). The azide precipitated was extracted with ethyl acetate (150 ml), and the organic layer was washed with 4% sodium bicarbonate and water. After being dried over sodium sulfate for 15 min at 0°C, the filtrate was added to a solution of H-Leu-D-Phe-Pro-OEt·HCl (3.87 g, 8.8 mmol)[6] dissolved in triethylamine (1.24 ml) and DMF (50 ml). The mixture was stirred for 3 days at

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

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

5) N. Izumiya, T. Kato, Y. Fujita, M. Ohno and M. Kondo, This Bulletin, **37**, 1807 (1964).

6) M. Ohno, T. Kato, S. Makisumi and N. Izumiya, *ibid.*, **39**, 1738 (1966).

0°C, and then evaporated *in vacuo*. The precipitate formed upon the addition of water was collected, and washed with 4% sodium bicarbonate, 0.5M citric acid and water. The product was recrystallized from methanol-ether-petroleum ether; yield, 5.81 g (78%); mp 147—148°C; $[\alpha]_D^{15}$ −31.0° (*c* 1, DMF); R_f 0.98;[7] R_f of hydrogenated product of IV, 0.77[7] and 0.91.[8]

Found: C, 64.44; H, 7.62; N, 8.96%. Calcd for $C_{50}H_{68}O_{11}N_6$: C, 64.63; H, 7.38; N, 9.05%.

Z(OMe)-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-NHNH$_2$ (V). A solution of IV (0.929 g, 1 mmol) and hydrazine hydrate (2 m*l*) in DMF (10 m*l*) was allowed to stand for 3 days at 30°C. The solution was then evaporated *in vacuo* in order to remove the excess hydrazine. The product which precipitated upon the addition of water was collected by filtration; yield, 0.851 g (93%); mp 144—147°C; $[\alpha]_D^{15}$ −38.2° (*c* 1, DMF); R_f of hydrogenated product of V, 0.77.[8]

Found: C, 62.24; H, 7.36; N, 11.89%. Calcd for $C_{48}H_{67}O_{10}N_8\cdot\frac{1}{2}H_2O$: C, 62.32; H, 7.41; N, 12.11%.

Z(OMe)-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-OH (VI). To a solution of IV (0.929 g, 1 mmol) in methanol (15 m*l*) and dioxane (15 m*l*), 0.46N sodium hydroxide (2.3 m*l*) was added at 0°C. The solution was allowed to stand overnight at room temperature and then concentrated *in vacuo* at 10—15°C. To the residue was added water (20 m*l*), and the mixture was extracted with ether. The aqueous layer was diluted with dioxane (10 m*l*) and acidified with N hydrochloric acid (1.07 m*l*) at 0°C. The mixture was evaporated to remove dioxane *in vacuo* at 0°C, and the crystals produced were collected by filtration. It was recrystallized from dioxane-ether-petroleum ether; yield, 0.692 g (77%); mp 109—112°C; $[\alpha]_D^{15}$ −33.6° (*c* 0.5, DMF); R_f 0.82.[7]

Found: C, 63.21; H, 7.40; N, 9.20%. Calcd for $C_{48}H_{64}O_{11}N_6\cdot\frac{1}{2}H_2O$: C, 63.35; H, 7.20; N, 9.24%.

H-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-OH·HCl (VII·HCl). To a mixture of VI (0.70 g, 0.77 mmol) and anisole (0.1 m*l*), 3.4N hydrogen chloride in dioxane (4.5 m*l*) was added at room temperature. After 2 hr, the solution was evaporated to dryness, and the residue was triturated with ether. The crystals were collected with the aid of ether; yield, 0.584 g (97%); mp 142—144°C; $[\alpha]_D^{15}$ −28.0° (*c* 1, DMF); R_f 0.79[7] and 0.81.[8]

Found: C, 60.05; H, 7.44; N, 10.55%. Calcd for $C_{39}H_{56}O_8N_6\cdot HCl\cdot\frac{1}{2}H_2O$: C, 59.87; H, 7.47; N, 10.74%.

Z(OMe)-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-OH (VIII). To V (0.601 g) dissolved in DMF (8 m*l*) and N hydrochloric acid (2 m*l*) was added sodium nitrite (0.052 g) in water at −5°C. After 15 min, cold water was added to the solution. The azide precipitated was collected by filtration and washed with 4% sodium bicarbonate and water, and then dried in a vacuum in a desiccator. The azide was added to a solution of VII (0.507 g) and triethylamine (0.18 m*l*) in DMF (12 m*l*). The mixture was stirred for 3 days at 0°C and then evaporated *in vacuo*. The precipitate formed upon the addition of water was collected, washed with 0.5M citric acid and water, and dried. The product was recrystallized from dioxane-ether-petroleum ether; yield, 0.968 g (89%); mp 119—121°C; $[\alpha]_D^{15}$ −50.0° (*c* 1, DMF); R_f 0.90;[7] R_f of hydrogenated product of VIII, 0.60.[7]

Found: C, 63.08; H, 7.56; N, 10.13%. Calcd for $C_{87}H_{118}O_{18}N_{12}\cdot 2H_2O$: C, 63.10; H, 7.43; N, 10.15%.

***cyclo*-(Leu-Orn(δ-Z)-Leu-D-Phe-Pro-)$_2$ (IX).** To a solution of VIII (0.662 g, 0.4 mmol) in pyridine (5 m*l*) was added di-*p*-nitrophenyl sulfite at room temperature. After 20 hr, the solution was evaporated and the residue was collected with the aid of a mixture of ether and petroleum ether. The yield of acyldecapeptide *p*-nitrophenyl ester was 0.655 g, and the *p*-nitrophenyl ester content was estimated to be 99% by measuring the optical density at 412 mμ.[9] This was treated with anisole (0.2 m*l*) and trifluoroacetic acid (4 m*l*) at −5°C. After 30 min, the solution was evaporated *in vacuo* at 0°C, and the residue was collected with ether. The decapeptide *p*-nitrophenyl ester trifluoroacetate so obtained was dissolved in DMF (8 m*l*) and acetic acid (0.1 m*l*). The solution was added dropwise into pyridine (150 m*l*) at 60°C for 4 hr and the stirring was continued for additional 2 hr. The solution was evaporated, and the residue was dissolved in a mixture of methanol (40 m*l*) and water (15 m*l*). After the insoluble substance in small amount was filtered off, the filtrate was passed through columns (1.8×10 cm) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were then washed with the same solvent, the combined effluents (400 m*l*) were evaporated, and the product was collected by filtration with the aid of water (yield, 0.235 g). It was recrystallized from methanol-ether-petroleum ether; yield, 0.222 g (39% from VIII); mp 138—140°C; $[\alpha]_D^{15}$ −188° (*c* 0.25, acetic acid); R_f 0.93.[7]

Found: C, 64.34; H, 8.04; N, 11.89%; mol wt, 1482.[10] Calcd for $C_{78}H_{108}O_{14}N_{12}\cdot H_2O$: C, 64.35; H, 7.62; N, 11.55%; mol wt, 1456.

***cyclo*-(Leu-Orn(δ-Z)-Leu-D-Phe-Pro-) (X).** Compound VI (0.586 g, 0.65 mmol) was treated with di-*p*-nitrophenyl sulfite (1.68 g) in pyridine (4.5 m*l*) as described for the preparation of acyldecapeptide ester, and acylpentapeptide *p*-nitrophenyl ester (0.66 g) was obtained as an amorphous powder; its *p*-nitrophenyl ester content was estimated to be 96%. The substance was treated with trifluoroacetic acid, and the pentapeptide *p*-nitrophenyl ester trifluoroacetate was added to pyridine (180 m*l*) as described above. The effluent from the ion-exchangers was evaporated to yield a powdery residue (0.186 g); the residue was designated as XIII. Two mg of XIII was hydrogenated, and the CMC column chromatography of the hydrogenated material (XIV) showed two peaks (Fig. 5-XIV). It was determined that the faster peak contained the cyclic pentapeptide (XII), the slower peak the cyclic decapeptide (XI), and the ratio of the integrated areas of the

7) The R_f of TLC with Merck Silica Gel G refers to the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, v/v) system. Compounds possessing a free amino group were detected by spraying with ninhydrin, and those with blocked amino groups, by spraying with 47% hydrobromic acid and then ninhydrin.

8) The R_f of the paper chromatography with Toyo Roshi No. 52 refers to the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, v/v) system.

9) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **43**, 1760 (1960).

10) The molecular weight was determined on a Hitachi Osmometer, type 115, using methanol as the solvent.

two peaks was calculated to be 78 : 22. If the color intensities resulting from ninhydrin between XII (molar base as an unit) and XI (a half molar base as an unit)[1] are assumed to be same, the ratio in weight of X and IX in the residue (XIII) is calculated to be 78 : 22. A half (0.092 g) of the residue (XIII) was dissolved in methanol (1 m*l*), and the solution was applied to a column (1.7×40 cm) with Sephadex LH-20, and the development continued with methanol. Elution was carried out at room temperature, at flow rate of 50 m*l* per 1 hr; a 1.5 m*l* fraction was collected.[3] The main peak appeared from tube number 32 to 42. The other half of the residue (XIII) was chromatographed similarly. The combined fractions were evaporated, and the product was recrystallized from methanol-ether-petroleum ether; yield, 0.121 g (26% from VI); mp 101—104°C; $[\alpha]_D^{15}$ −112° (*c* 0.25, acetic acid); R_f 0.93.[7]

Found: C, 63.40; H, 7.91; N, 11.20%; mol wt, 757.[10] Calcd for $C_{39}H_{54}O_7N_6 \cdot H_2O$: C, 63.56; H, 7.66; N, 11.41%; mol wt, 737.

***cyclo*-(Leu-Orn-Leu-D-Phe-Pro-)$_2$·2HCl(XI·2HCl).** A solution of IX (102 mg, 0.07 mmol) in 0.04N methanolic hydrogen chloride (2 m*l*) was subjected to hydrogenolysis in the presence of palladium black. The filtrate was evaporated, and the crystals were collected with the aid of ether; yield of the air dried product, 78 mg (81%); mp 242—244°C (decomp.); $[\alpha]_D^{15}$ −197° (*c* 0.3, acetic acid); R_f 0.97;[8] amino acid ratios in acid hydrolysate; Leu 2.0, Orn 1.0, Phe 0.9, Pro 1.0.

Found: C, 55.26; H, 7.96; N, 12.42%. Calcd for $C_{62}H_{96}O_{10}N_{12} \cdot 2HCl \cdot 6H_2O$: C, 55.14; H, 8.21; N, 12.45%. The air-dried product lost 6.14% of its weight after it had been dried over phosphorus pentoxide at 80°C (2 mmHg) to a constant weight. Calcd for $5H_2O$: 6.74%.

***cyclo*-(Leu-Orn-Leu-D-Phe-Pro-)·HCl (XII·HCl).** Compound X (74 mg, 0.1 mmol) was treated as described above; yield of the air-dried product, 58 mg (84%); mp 200—201°C (decomp.); $[\alpha]_D^{15}$ −143° (*c* 0.3, acetic acid); R_f 0.92;[8] amino acid ratios in acid hydrolysate; Leu 2.0, Orn 1.0, Phe 1.0, Pro 0.9.

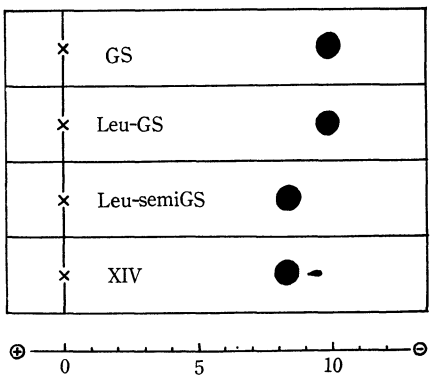

Fig. 4. Paper electrophoresis of GS, Leu-GS (XI), Leu-semiGS (XII) and the hydrogenated material (XIV) after cyclization of pentapeptide ester.

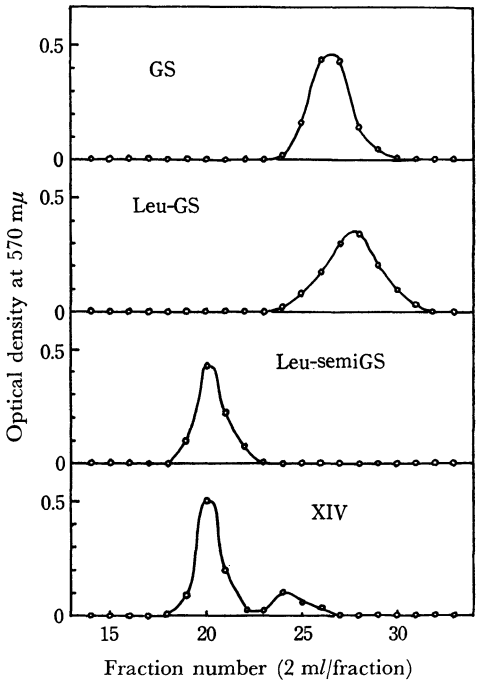

Fig. 5. CMC column chromatography of the compounds.

Found: C, 55.29; H, 8.01; N, 12.50%. Calcd for $C_{31}H_{48}O_5N_6 \cdot HCl \cdot 3H_3O$: C, 55.13; H, 8.21; N, 12.45%. The air-dried product lost 5.84% of its weight after being dried. Calcd for $2H_2O$: 5.49%.

Electrophoresis and CMC Chromatography. Electrophoresis on Toyo Roshi No. 52 paper was carried out at pH 1.8 (formic acid-acetic acid-methanol-water, 1 : 3 : 6 : 10, v/v) at 500 V/30 cm for 3 hr. Figure 4 shows that XI migrates toward the cathode faster than XII and that the mobility of XI was similar to that of GS. In CMC column chromatography, a sample (0.5—1 mg) was dissolved in 0.2 m*l* of 0.2M pyridinium acetate containing 30% methanol (pH 5.0), the solution was applied to a column (0.9×50 cm) with CMC (Eastman No. 7796), and development was continued with the same solvent. Two-m*l* fractions were collected at a flow rate of 20 m*l* per 1 hr. The peptide content in the fractions was determined by the ninhydrin method, and the results are shown in Fig. 5.

Microbiological Assays.[11] The minimum amount of compounds necessary for the complete inhibition of growth was determined by a dilution method with a bouillon agar medium and with a synthetic medium. As is shown in Table 2, 1,1'-Leu-GS (XI) was found to be as active as natural GS against *B. subtilis* and *St. aureus*, whereas the cyclic pentapeptide (XII) exhibited no antibacterial activity against the microorganisms tested.

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