BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 1975—1980 (1967)

Studies of Peptide Antibiotics. XI. Analogs of Gramicidin S and Cyclosemigramicidin S Containing Glycine or Alanine in Place of Valine

Michio KONDO and Nobuo IZUMIYA

Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Fukuoka

(Received March 20, 1967)

To investigate the contribution for the antibacterial activity of the valine residues in a gramicidin S molecule, two analogs of gramicidin S, 1, 1'-glycine- and 1, 1'-L-alanine-gramicidin S, were prepared through the cyclization reaction of linear decapeptide active esters in pyridine. 1-Glycine- and 1-L-alanine-cyclosemigramicidin S were also prepared through the cyclization of linear pentapeptide active esters. The effects of the four cyclic peptides thus obtained on bacterial growth were tested; only 1, 1'-alanine-gramicidin S was as active as natural gramicidin S, while the other three analogs showed no activity in relation to any of the microorganisms tested. The results indicate that the side chains of the valines in gramicidin S can be replaced by smaller aliphatic side chains, the methyls, without the influence for the activity.

Regarding the relationship between the chemical structure and the biological activity of gramicidin S (Fig. 1), it has been reported in the papers from

$$\begin{bmatrix} \text{X-L-Orn-L-Leu-D-Phe-L-Pro} \\ \\ \text{L-Pro-D-Phe-L-Leu-L-Orn-X} \end{bmatrix}$$

Fig. 1. Structure of gramicidin S and its analogs. X represents an amino acid residue such as L-Val (gramicidin S), Gly (XIII-G) or L-Ala (XIII-A).

this laboratory that 5, 5'-glycine- and 5, 5'-sarcosine-gramicidin S were as active as gramicidin S against several microorganisms.[1-3] The results showed that the side chains of the proline residues in the 5- and 5'-positions are not indispensable to its full activity. On the other hand, the authors demonstrated previously that 1, 1', 5, 5'-glycine-gramicidin S exhibited no antibacterial activity even at level as 100 μg per m*l* of assay medium.[4] From this finding, it appeared that the isopropyl side chains of the valine residues in the sequence of gramicidin S are of importance for the exhibition of the activity. In order to investigate in what degree the valine side chains contribute to the biological activity, we attempted further to prepare two analogs of gramicidin S in which the valine residues are replaced by amino acids with smaller side chains than the isopropyl. The present paper will describe the syntheses and antibacterial properties of 1, 1'-glycine- and 1, 1'-alanine-gramicidin S (XIII-G and XIII-A) besides the preparations of the cyclic pentapeptides, 1-glycine- and 1-alanine-cyclosemigramicidin S (XIV-G and XIV-A).

The sequence of reactions employed for the syntheses of the cyclic decapeptides (XIII-G and XIII-A) is shown in Fig. 2. The condensation of the azide derived from an acylpentapeptide hydrazide (V-G or V-A) with corresponding pentapeptide (VII-G or VII-A) gave an acyldecapeptide (X-G or X-A). Alternatively, as indicated in Fig. 3, X-G was obtained by saponification of corresponding acyldecapeptide ethyl ester which had been prepared by the condensation of VI-G with pentapeptide ethyl ester (VIII-G) by means of the mixed anhydride method. The treatment of each of X-G and X-A with an excess of di-*p*-nitrophenyl sulfite gave an amorphous acyldecapeptide *p*-nitrophenyl ester. Its *p*-methoxybenzyloxycarbonyl group was removed by the action of trifluoroacetic acid, and the decapeptide *p*-nitrophenyl ester trifluoroacetate thus obtained was treated with a large amount of pyridine for the cyclization reaction. The benzyloxycarbonyl-substituted cyclic decapeptide (XI-G or XI-A) was then subjected to hydrogenolysis, and the final product (XIII-G·2HCl or XIIIA·2HCl) was obtained as colorless crystals containing some of water of crystallization.

The cyclization reaction of linear pentapeptide *p*-nitrophenyl esters was also carried out to investigate whether the substances produced are composed with benzyloxycarbonyl(Z)-substituted cyclic pentapeptide by a monomerization reaction and/or Z-substituted cyclic decapeptide by a dimerization. It was found that a linear pentapeptide active ester containing glycine residue as *N*-terminal amino acid produces exclusively Z-substituted monomer (XII-G) after the cyclization

1) The abreviation followed are from *J. Biol. Chem.*, **241**, 2491 (1966); Z(OMe), *p*-methoxybenzyloxycarbonyl; -ONp, *p*-nitrophenoxy.
2) H. Aoyagi *et al.*, *J. Am. Chem. Soc.*, **86**, 5700 (1964); This Bulletin, **38**, 2139 (1965).
3) H. Aoyagi and N. Izumiya, This Bulletin, **39**, 1747 (1966).
4) M. Kondo, H. Aoyagi, T. Kato and N. Izumiya, *ibid.*, **39**, 2234 (1966).

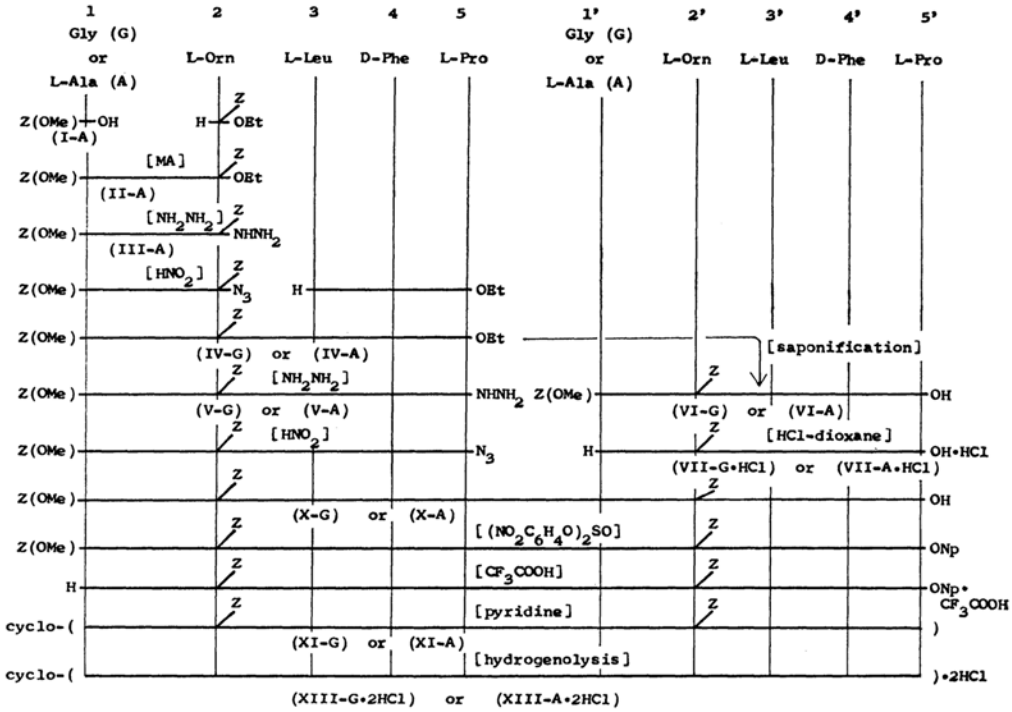

Fig. 2. Cyclization of linear decapeptide active esters.

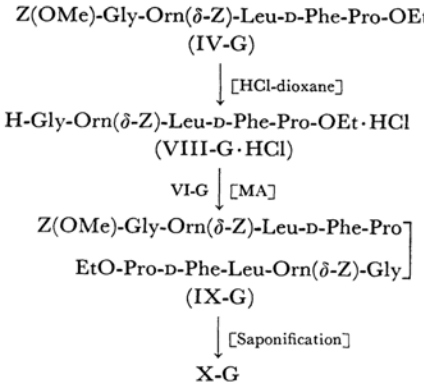

Fig. 3. Saponification of acyldecapeptide ester.

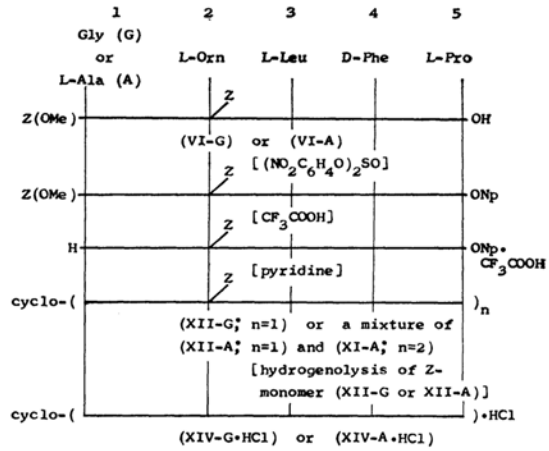

Fig. 4. Cyclization of linear pentapeptide active esters.

reaction in pyridine. Whereas a linear pentapeptide ester with *N*-terminal alanine residue yields a mixture of Z-substituted monomer (XII-A) and dimer (XI-A); the ratio in weight of XII-A and XI-A was found to be approximately 90 : 10 (see Fig. 6). In this connection, it would be of interest to note that the active ester of Gly-Orn(δ-Z)-Leu-D-Phe-Gly produced exclusively Z-substituted cyclic monomer after the cyclization reaction,[4] whereas the active ester of Val-Orn(δ-Z)-Leu-D-Phe-Pro produced a mixture of Z-substituted monomer and dimer.[5] These results may suggest that the presence of glycine as the *N*-terminal residue of a pentapeptide active ester in pyridine solvent is favorable for the intramolecular reaction to form a peptide bond with an amino group of glycine residue and a carboxyl group belonged to the same molecule.

The antibacterial activity of the cyclic deca- and pentapeptides toward several microorganisms were examined. It was observed that 1, 1'-glycine-gramicidin S (XIII-G) exhibited no antibacterial activity, whereas the alanine analog (XIII-A)

5) M. Waki and N. Izumiya, This Bulletin, **40**, 1687 (1967).

was active as natural gramicidin S in affecting several microorganisms. These observations indicate that the side chains, the isopropyls, of the valine residues in a gramicidin S molecule can be replaced by smaller aliphatic side chains, the methyls, without the influence for the antibacterial activity, while glycine residues which occupy the valines are too small to maintain the activity. It was also found that the two cyclic pentapeptides, 1-glycine and alanine-cyclosemigramicidin S (XIV-G and XIV-A), exhibited no antibacterial activity. This finding agrees with that found previously in this laboratory for a cyclosemigramicidin S[5] and its several analogs such as 5-sarcosine-cyclosemigramicidin S.[3]

Experimental

The 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 (XIII·2HCl and XIV·HCl).

Z(OMe)-Ala-OH (I-A). The synthetic procedure[6] was modified as follows. A mixture of L-alanine (4.46 g, 50 mmol), water (100 m*l*), dioxane (100 m*l*), sodium bicarbonate (10.9 g) and *p*-methoxybenzyloxycarbonyl azide (12.4 g, 60 mmol) was stirred at room temperature for 60 hr. The solution was evaporated *in vacuo* to small volume, extracted with ether, acidified with 0.5 M citric acid, and then extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated to dryness, and the residual oil was solidified by the addition of petroleum ether. This was recrystallized from ethyl acetate-petroleum ether; yield, 9.10 g (72%); mp 82—83°C; $[\alpha]_D^{18}$ −12.5° (*c* 2, acetic acid). Reported values;[6] yield, 59%, mp 74—75°C, $[\alpha]_D^{20}$ −11.9° (acetic acid).

Z(OMe)-Ala-Orn(δ-Z)-OEt (II-A). To a mixed anhydride prepared at −5°C from I-A (2.03 g) with isobutyl chloroformate (1.05 m*l*) and triethylamine (1.12 m*l*) in tetrahydrofuran (16 m*l*), a chilled solution of δ-benzyloxycarbonyl-L-ornithine ethyl ester *p*-toluenesulfonate (3.74 g) in triethylamine (1.12 m*l*) and chloroform (16 m*l*) was added. After the reaction mixture had been allowed to stand overnight, it was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (80 m*l*), and the solution was washed with 0.5 M citric acid, 3% sodium bicarbonate and water, dried over sodium sulfate, and then evaporated to dryness *in vacuo*. The oily residue was crystallized by the addition of ether. Recrystallization from ethyl acetate - ether gave 2.8 g (66%); mp 90—92°C; $[\alpha]_D^{18}$ −2.0° (*c* 1, dimethylformamide); R_f 0.87.[7]

Found: C, 61.12; H, 6.88; N, 8.09%. Calcd for $C_{27}H_{35}O_5N_3$: C, 61.23; H, 6.66; N, 7.94%.

Z(OMe)-Ala-Orn(δ-Z)-NHNH$_2$ (III-A). A solution of II-A (5.85 g) and hydrazine hydrate (11 m*l*) in dimethylformamide (40 m*l*) was allowed to stand for 2 days at 30°C. The solution was then concentrated *in vacuo* to a small volume. The hydrazide which precipitated upon the addition of water was collected and recrystallized from dioxane-ether; yield, 4.55 g (80%); mp 190—193°C; $[\alpha]_D^{18}$ −1.2° (*c* 1, dimethylformamide).

Found: C, 58.10; H, 6.59; N, 13.32%. Calcd for $C_{25}H_{33}O_7N_5$: C, 58.24; H, 6.45; N, 13.59%.

Z(OMe)-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OEt (IV-G). To a Z(OMe)-Gly-Orn(δ-Z)-NHNH$_2$[4] (2.36 g, 4.7 mmol) dissolved in acetic acid (30 m*l*) and 3 N hydrochloric acid (4.7 m*l*) was added M sodium nitrite (5.17 m*l*, 5.2 mmol) at −5°C. After 5 min, the solution was diluted with water (150 m*l*). The azide separated as a viscous mass was extracted with ethyl acetate, and the ethyl acetate solution was washed with 3% sodium bicarbonate and water. After 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 (2.06 g, 4.7 mmol)[8] dissolved in triethylamine (0.66 m*l*) and dimethylformamide (10 m*l*). After stirred for 3 days at 0°C, the solution was diluted with ethyl acetate (100 m*l*) and washed successively with 0.5 M citric acid, 3% sodium bicarbonate and water, dried over sodium sulfate and then evaporated *in vacuo*. The oily residue was solidified by the addition of petroleum ether, and the product was recrystallized from ethyl acetate - ether - petroleum ether; yield, 2.61 g (63%); mp 94—96°C; $[\alpha]_D^{26}$ −32.7° (*c* 1, dimethylformamide); R_f 0.86;[7] R_f of hydrogenolyzed product of IV-G, 0.73[7] and 0.84.[9]

Found: C, 62.90; H, 6.86; N, 9.57%. Calcd for $C_{46}H_{60}O_{11}N_6$: C, 63.28; H, 6.93; N, 9.63%.

Z(OMe)-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-OEt (IV-A). The azide derived from III-A (1.07 g) was condensed with H-Leu-D-Phe-Pro-OEt·HCl (0.98 g) and triethylamine as described above; yield, 1.37 g (75%); mp 184—185°C; $[\alpha]_D^{16}$ −28.0° (*c* 1, dimethylformamide); R_f 0.93;[7] R_f of hydrogenolyzed product of IV-A, 0.72[7] and 0.83.[9]

Found: C, 63.44; H, 7.41; N, 9.71%. Calcd for $C_{47}H_{62}O_{11}N_6$: C, 63.64; H, 7.05; N, 9.48%.

Z(OMe)-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-NHNH$_2$ (V-G). A solution of IV-G (1.05 g, 1.2 mmol) and hydrazine hydrate (2.4 m*l*, 48 mmol) in dimethylformamide (12 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, water (100 m*l*) was added to the residual solution, and the product was obtained as a hygroscopic powder; yield, 0.845 g (82%). The homogeneity was certified by paper chromatography with regard to the hydrogenolyzed product; R_f 0.70.[9]

Z(OMe)-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-NHNH$_2$ (V-A). This was obtained from IV-A (0.887 g, 1 mmol) and hydrazine hydrate (2 m*l*, 40 mmol) as described above; yield 0.816 g (93%); mp 88—89°C; $[\alpha]_D^{16}$ −36.0° (*c* 1, dimethylformamide); R_f of hydrogenolyzed product of V-A, 0.60.[9]

Found: C, 61.00; H, 7.26; N, 12.54%. Calcd for $C_{45}H_{61}O_{10}N_8 \cdot \frac{1}{2}H_2O$: C, 61.21; H, 7.08; N, 12.69%.

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

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

8) M. Ohno, T. Kato, S. Makisumi and N. Izumiya, This Bulletin, **39**, 1738 (1966).

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

Z(OMe)-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OH (VI-G). To a solution of IV-G (1.66 g, 1.9 mmol) in methanol (30 ml), N sodium hydroxide (5.7 ml) was added at 30°C. The hydrolysis was complete after 6 hr. After the addition of water (10 ml), the solution was concentrated *in vacuo* to small volume, and the residual solution was acidified with 1.22 N hydrochloric acid (4.7 ml) under cooling. The crystals resulted were collected by filtration, washed with water, and dried. Recrystallization from dioxane-ether-petroleum ether gave 1.42 g (89%); mp 97°C; $[\alpha]_D^{26}$ −37.8° (*c* 1, dimethylformamide); R_f 0.79.[7]

Found: C, 61.28; H, 6.61; N, 10.09%. Calcd for $C_{44}H_{56}O_{11}N_6 \cdot H_2O$: C, 61.24; H, 6.54; N, 9.74%.

Z(OMe)-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-OH (VI-A). To IV-A (0.887 g, 1 mmol) in methanol (15 ml) and dioxane (15 ml), 0.46 N sodium hydroxide (2.3 ml) was added. After 20 hr, the solution was concentrated *in vacuo* under cooling (10°C), and the residue was suspended in 10 ml of water and then extracted with ether. The aqueous layer was diluted with dioxane (10 ml) and acidified with N hydrochloric acid (1.07 ml). The product resulted was recrystallized from dioxane-ether-petroleum ether; yield, 0.672 g (78%); mp 105—108°C; $[\alpha]_D^{21}$ −33.0° (*c* 1, dimethylformamide); R_f 0.81.[7]

Found: C, 62.22; H, 7.18; N, 9.26%. Calcd for $C_{45}H_{58}O_{11}N_6 \cdot \frac{1}{2}H_2O$: C, 62.27; H, 6.85; N, 9.68%.

H-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OH·HCl (VII-G·HCl). To a mixture of VI-G (0.207 g, 0.24 mmol) and anisole (0.1 ml), 4 N hydrogen chloride in dioxane (1.2 ml) was added at room temperature. After 2 hr, the solution was evaporated to dryness, and the residue was triturated with ether and washed repeatedly with ether by decantation. The crystals remained were collected with the aid of ether; yield, 0.162 g (94%); mp 92—95°C; $[\alpha]_D^{18}$ −34.5° (*c* 1, dimethylformamide); R_f 0.90.[9]

Found: C, 56.69; H, 7.02; N, 11.50%. Calcd for $C_{35}H_{48}O_8N_6 \cdot HCl \cdot H_2O$: C, 57.17; H, 6.94; N, 11.43%.

H-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-OH·HCl (VII-A·HCl). This was obtained from VI-A (0.668 g) as described above; yield, 0.54 g (96%); mp 108—110°C; $[\alpha]_D^{18}$ −29.5° (*c* 1, dimethylformamide); R_f 0.70[7] and 0.88.[9]

Found: C, 57.06; H, 7.11; N, 11.00%. Calcd for $C_{36}H_{50}O_8N_6 \cdot HCl \cdot 3/2H_2O$: C, 57.02; H, 7.18; N, 11.08%.

H-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OEt·HCl (VIII-G·HCl). IV-G (0.775 g) in anisole (0.2 ml) was treated with 4 N hydrogen chloride in dioxane (4.5 ml) as described for the preparation of VII-G·HCl. Yield, 0.585 g (89%); mp 119—121°C; $[\alpha]_D^{18}$ −34.0° (*c* 0.5, dimethylformamide); R_f 0.76[7] and 0.88.[9]

Found: C, 56.45; H, 6.88; N, 10.88%. Calcd for $C_{37}H_{52}O_8N_6 \cdot HCl \cdot 2H_2O$: C, 56.87; H, 7.35; N, 10.76%.

Z(OMe)-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OEt (IX-G). To a mixed anhydride prepared from VI-G (0.635 g) in tetrahydrofuran was added a mixture of VIII-G·HCl (0.559 g) and triethylamine (0.11 ml) in chloroform as described for the preparation of II-A. The crude product obtained was recrystallized from ethyl acetate-ether-petroleum ether. Yield, 0.825 g (71%); mp 108—110°C; $[\alpha]_D^{18}$ −35.5° (*c* 1, dimethylformamide); R_f 0.86.[7]

Found: C, 62.17; H, 7.03; N, 10.77%. Calcd for $C_{81}H_{106}O_{18}N_{12} \cdot H_2O$: C, 62.61; H, 7.01; N, 10.82%.

Z(OMe)-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OH (X-G). *From IX-G.* A solution of IX-G (0.69 g, 0.45 mol) in methanol was treated with 2 N sodium hydroxide (2.25 ml) at 30°C for 6 hr. The crude product obtained as described for the preparation of VI-G was recrystallized from ethyl acetate-ether; yield, 0.571 g (84%); mp 105—109°C; $[\alpha]_D^{26}$ −37.0° (*c* 1, dimethylformamide); R_f 0.79;[7] R_f of hydrogenolyzed product of X-G, 0.45[7] and 0.68.[9]

Found: C, 61.20; H, 6.83; N, 10.95%. Calcd for $C_{79}H_{102}O_{18}N_{12} \cdot 2H_2O$: C, 61.46; H, 6.92; N, 10.89%.

From V-G and VII-G. To V-G (0.845 g) dissolved in dimethylformamide (10 ml) and 1.2 N hydrochloric acid (2.5 ml) was added sodium nitrite (0.083 g) in water. After 10 min, water (50 ml) was added, and the azide precipitated was extracted with ethyl acetate. After the organic layer was washed and dried, it was added to a mixture of VII-G·HCl (0.707 g) in dimethylformamide and triethylamine (0.28 ml). The mixture was stirred for 3 days at 0°C, and then evaporated *in vacuo*. The residual oil was dissolved in ethyl acetate and treated as described for the preparation of IV-G. The crude product was recrystallized from ethyl acetate-ether; yield, 0.716 g (48%); mp 104—109°C; $[\alpha]_D^{26}$ −37.5° (*c* 1, dimethylformamide); R_f 0.79.[7]

Found: C, 61.49; H, 7.11; N, 11.15%.

Z(OMe)-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-OH (X-A). The azide derived from V-A (0.518 g) was condensed with a mixture of VII-A·HCl (0.493 g) and triethylamine as described above. The crude product was recrystallized from dioxane-ether-petroleum ether; yield, 0.903 g (87%); mp 115—118°C; $[\alpha]_D^{18}$ −41.0° (*c* 1, dimethylformamide); R_f 0.87;[7] R_f of hydrogenolyzed product of X-A, 0.49.[9]

Found: C, 62.02; H, 7.12; N, 10.61%. Calcd for $C_{81}H_{106}O_{18}N_{12} \cdot 2H_2O$: C, 61.89; H, 7.05; N, 10.69%.

***cyclo*-(Gly-Orn(δ-Z)-Leu-D-Phe-Pro-)$_2$ (XI-G).** To X-G (0.528 g, 0.35 mmol) in pyridine (5 ml) was added di-*p*-nitrophenyl sulfite at room temperature. After 18 hr, the solution was evaporated and the residue was collected with the aid of a mixture of ether and petroleum ether (1 : 3). The yield of acyldecapeptide *p*-nitrophenyl ester was 0.559 g, and the *p*-nitrophenyl ester content was estimated to be 98% by measuring the optical density at 412 mμ.[10] This was treated with anisole (0.2 ml) and trifluoroacetic acid (4 ml) at −5°C. After 30 min, the solution was evaporated, and the residue was collected with the aid of a mixture of ether and petroleum ether (1 : 1). The decapeptide *p*-nitrophenyl ester trifluoroacetate so obtained was dissolved in dimethylformamide (6 ml) and acetic acid (0.1 ml). The solution was added dropwise into pyridine (120 ml) 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 and water (3 : 1). After the insoluble substance in small amount was filtered off, the filtrate was treated with columns (1.8×10 cm) of Dowex 1 and 50. The throughout effluent (*ca.* 400 ml) was evaporated,

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

and the crystals remained were collected with the aid of water; 179 mg. The crude product was purified with a Sephadex LH-20 column using methanol as a developing solvent. The main fractions were evaporated and recrystallized from methanol-ether. Yield, 129 mg (28% from X-G); mp 137—138°C; $[\alpha]_D^{15}$ −102° (*c* 0.5, acetic acid); R_f 0.93.[7]

Found: C, 60.13; H, 6.96; N, 12.46%; mol wt, 1330.[11] Calcd for $C_{70}H_{92}O_{14}N_{12}\cdot 4H_2O$: C, 60.16; H, 7.21; N, 12.03%; mol wt, 1398.

***cyclo*-(Ala-Orn(∂-Z)-Leu-D-Phe-Pro-)$_2$ (XI-A).** X-A (0.614 g, 0.4 mmol) was converted to the acyldecapeptide *p*-nitrophenyl ester (0.68 g) as described above in which the *p*-nitrophenyl ester content was estimated to be 104%. This substance was treated with trifluoroacetic acid, and the decapeptide ester trifluoroacetate obtained was added to pyridine (150 m*l*). The throughout effluent from the Dowex 1 and 50 columns was evaporated, and the crystals were collected with the aid of water. This was recrystallized from dioxane-methanol-ether; yield of the air-dried product, 0.18 g (34% from X-A); mp 255—258°C (decomp.); $[\alpha]_D^{18}$ −254° (*c* 0.5, acetic acid); R_f 0.92.[7]

Found: C, 62.13; H, 7.22; N, 12.55%; mol wt, 1320.[12] Calcd for $C_{72}H_{96}O_{14}N_{12}\cdot 2H_2O$: C, 62.23; H, 7.25; N, 12.10%; mol wt, 1355.

***cyclo*-Gly-Orn(∂-Z)-Leu-D-Phe-Pro (XII-G).** VI-G (0.634 g, 0.75 mmol) was treated with di-*p*-nitrophenyl sulfite (1.21 g, 3.7 mmol) in pyridine (3 m*l*), and the acylpentapeptide *p*-nitrophenyl ester (0.69 g) was obtained as an amorphous powder; its *p*-nitrophenyl ester content was estimated to be 100%. The substance was treated with trifluoroacetic acid, and the pentapeptide *p*-nitrophenyl ester trifluoroacetate obtained as a hygroscopic powder was added to pyridine (240 m*l*) as described for the preparation of XI-G. The throughout effluent from the ion-exchangers was evaporated to dryness, a few mg of the residue (XV-G) were subjected to hydrogenolysis, and the carboxymethylcellulose (CMC) column chromatography of the hydrogenated material showed one peak by a cyclic pentapeptide (XIV-G). The residue (XV-G) was recrystallized from methanol - ether - petroleum ether; yield, 0.223 g (45% from VI-G); mp 165—168°C; $[\alpha]_D^{24}$ −84.6° (*c* 0.5, acetic acid); R_f 0.75.[7]

Found: C, 62.55; H, 7.26; N, 12.58%; mol wt, 670.[11] Calcd for $C_{35}H_{46}O_7N_6\cdot \frac{1}{2}H_2O$: C, 62.57; H, 7.05; N, 12.51%; mol wt, 672.

***cyclo*-Ala-Orn(∂-Z)-Leu-D-Phe-Pro (XII-A).** VI-A (0.558 g, 0.65 mmol) was converted to the acylpentapeptide *p*-nitrophenyl ester (0.6 g) as described in the case of the preparation of XI-G. The *p*-nitrophenyl ester content was estimated to be 80%. This product was added to trifluoroacetic acid, and the pentapeptide ester trifluoroacetate obtained was treated with pyridine (100 m*l*). The throughout effluent was evaporated to dryness, a few mg of the residue (XV-A) were hydrogenated, and the CMC column chromatography of the hydrogenated material (XVI-A) showed two peaks (Fig. 6). It was determined that the faster peak contained the cyclic pentapeptide (XIV-A), the slower peak the cyclic decapeptide (XIII-A), and the ratio of the integrated areas of the two peaks was calculated to be 91 : 9. If the color intensities resulting from ninhydrin between XIV-A on the molar base and XIII-A on the base wherein a half mole is used as an unit are assumed to be same, the ratio in weight of XII-A and XI-A in the residue (XV-A) is calculated to be 91 : 9. When the residue (XV-A) was recrystallized from methanol - petroleum ether, XI-A admixed in the XV-A preparation was easily removed; yield of XII-A, 92 mg (21% from VI-A); mp 106—108°C; $[\alpha]_D^{15}$ −134° (*c* 0.5, acetic acid); R_f 0.84.[7]

Found: C, 62.32; H, 7.35; N, 11.65%; mol wt, 690.[11] Calcd for $C_{36}H_{48}O_7N_6\cdot H_2O$: C, 62.23; H, 7.25; N, 12.10%; mol wt, 695.

***cyclo*-(Gly-Orn-Leu-D-Phe-Pro-)$_2$•2HCl (XIII-G·2HCl).** A solution of XI-G (69.9 mg, 0.05 mmol) in 0.1 N methanolic hydrogen chloride (1.1 m*l*) was subjected to hydrogenolysis in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness, and the crystals remained were collected with the aid of a mixture of ether and petroleum ether (2 : 1); yield of the air-dried product, 55.5 mg (91%); mp 192—195°C (decomp.); $[\alpha]_D^{18}$ −108° (*c* 0.4, acetic acid); R_f 0.72[7] and 0.95;[9] amino acid ratios in acid hydrolysate, $Val_{1.0}Orn_{1.0}Leu_{1.1}Phe_{1.0}Pro_{0.9}$.

Found: C, 53.34; H, 7.35; N, 13.45%. Calcd for $C_{54}H_{80}O_{10}N_{12}\cdot 2HCl\cdot 5H_2O$: C, 53.15; H, 7.60; N, 13.77%.

The air-dried product lost 7.7% of its weight after it had been dried for 3 hr at 80°C and 2 mmHg. Calcd for $5H_2O$: 7.4%.

***cyclo*-(Ala-Orn-Leu-D-Phe-Pro-)$_2$•2HCl (XIII-A•2HCl).** A solution of XI-A (0.05 mmol) in acetic acid (2 m*l*) was hydrogenated, and the filtrate was evaporated to dryness. The residue was dissolved in 0.05 N methanolic hydrogen chloride (2.2 m*l*), and the solution was again evaporated to dryness; yield, 83%; mp 243—244°C (decomp.); $[\alpha]_D^{18}$ −252° (*c* 0.4, acetic acid); R_f 0.85[7] and 0.95;[9] amino acid ratios in acid hydrolysate, $Val_{1.0}Orn_{1.0}Leu_{1.0}Phe_{0.9}Pro_{1.0}$.

Found: C, 52.09; H, 7.74; N, 12.95%. Calcd for $C_{58}H_{84}O_{10}N_{12}\cdot 2HCl\cdot 8H_2O$: C, 51.64; H, 7.89; N, 12.91%. The air-dried product lost 10.8% of its weight after dried for 3 hr at 80°C and 2 mmHg. Calcd for $8H_2O$; 11.0%.

***cyclo*-Gly-Orn-Leu-D-Phe-Pro•HCl (XIV-G•HCl).** XII-G (0.1 mmol) was treated as described for the preparation of XIII-G·2HCl; yield, 83%; mp 190—192°C (decomp.); $[\alpha]_D^{18}$ −96.0° (*c* 0.4, acetic acid); R_f 0.62[7] and 0.81;[9] amino acid ratios in acid hydrolysate, $Val_{1.1}Orn_{1.0}Leu_{0.9}Phe_{1.0}Pro_{1.0}$.

Found: C, 51.01; H, 7.61; N, 12.96%. Calcd for $C_{27}H_{40}O_5N_6\cdot HCl\cdot 4H_2O$: C, 50.87; H, 7.75; N 13.19%. The air-dried product lost 10.9% of its weight after dried. Calcd for $4H_2O$: 11.3%.

***cyclo*-Ala-Orn-Leu-D-Phe-Pro·HCl (XIV-A·HCl).** XII-A was treated as described for the preparation of XIII-G·2HCl; yield, 86%; mp 194—196°C (decomp.); $[\alpha]_D^{18}$ −163° (*c* 0.4, acetic acid); R_f 0.80[7] and 0.84;[9] amino acid ratios in acid hydrolysate, $Ala_{1.0}Orn_{1.0}Leu_{1.1}Phe_{1.0}Pro_{1.0}$.

Found: C, 53.39; H, 7.70; N, 12.92%. Calcd for $C_{28}H_{42}O_5N_6\cdot HCl\cdot 3H_2O$: C, 53.08; H, 7.80; N, 13.27%. The air-dried product lost 8.8% of its weight after dried. Calcd for $3H_2O$: 8.6%.

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

12) Dimethylformamide was used as a solvent; see Ref. 11.

TABLE 1. AMOUNT OF COMPOUND NECESSARY FOR COMPLETE INHIBITION OF GROWTH (μg/ml)

	B. subtilis	*St. aureus*	*E. coli*	*Pr. vulgaris*	*M. avium*
		A. Bouillon agar medium			
GS	5	5	>100	>100	100
1,1'-Gly-GS (XIII-G)	100	100	>100	>100	>100
1,1'-Ala-GS (XIII-A)	5	5	>100	>100	50
1-Gly-semiGS (XIV-G)	>100	>100	>100	>100	>100
1-Ala-semiGS (XIV-A)	100	100	>100	>100	>100
		B. Synthetic medium			
GS	2	5	>100	>100	>100
1,1'-Gly-GS (XIII-G)	100	100	>100	>100	>100
1,1'-Ala-GS (XIII-A)	5	5	>100	>100	50
1-Gly-semiGS (XIV-G)	100	>100	>100	>100	>100
1-Ala-semiGS (XIV-A)	50	100	>100	>100	>100

Electrophoresis and Carboxymethylcellulose (CMC) Chromatography. Electrophoresis on Toyo Roshi No. 52 paper was carried out with a solvent system, formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10, v/v; pH 1.8) for 3 hr at 500V/30 cm. Figure 5 shows that XIII-G and XIII-A migrate toward the cathode faster than XIV-G and XIV-A, and that the mobilities of XIII-G and XIII-A were indistinguishable from that of gramicidin S. In CMC column chromatography, a sample (0.1—1 mg) was dissolved in 0.2—0.3 m*l*

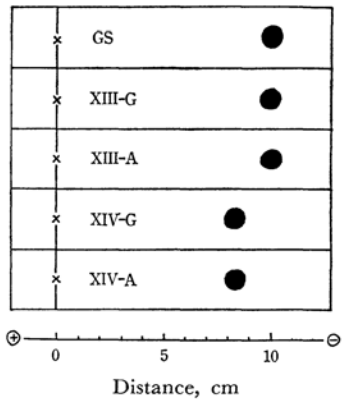

Fig. 5. Paper electrophoresis of gramicidin S (GS), XIII and XIV.

of 0.2 M pyridinium acetate containing 30% methanol (pH 5.1), 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 hour. The peptide content in the fractions was determined by the ninhydrin method, and the results are shown in Fig. 6.

Microbiological Assays.[13] The minimum amount of the compounds necessary for the complete inhibition of growth was determined by a dilution method with a

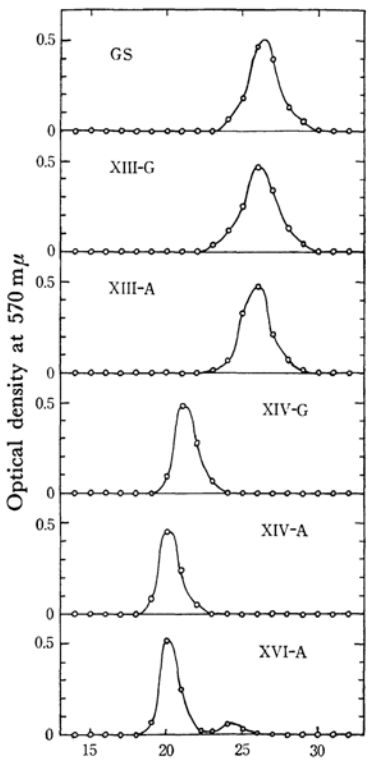

Fig. 6. Carboxymethylcellulose column chromatography of gramicidin S, XIII, XIV and XVI-A (hydrogenated material after cyclization of pentapeptide ester containing alanine).

bouillon agar medium and with a synthetic medium. As is shown in Table 1, 1,1'-alanine-gramicidin S (XIII-A) was found to be as active as natural gramicidin S against *B. subtilis* and *Staph. aureus*, whereas 1,1'-glycine-gramicidin S (XIII-G) and the cyclic pentapeptides exhibited no antibacterial activity against any of the microorganisms tested.

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