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Studies of Peptide Antibiotics. X. Syntheses of Cyclosemigramicidin S and Gramicidin S[1]

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The possibility of the occurrence of benzyloxycarbonyl-substituted cyclosemigramicidin S in the course of the cyclization reaction of a linear-pentapeptide-active ester with pyridine was investigated. It was indicated that the crude product of the cyclization of the linear-active ester was composed of two components. The protected gramicidin S, a less soluble material, was easily isolated by fractional crystallization, while the protected cyclosemigramicidin S, a more soluble material, was isolated by a column chromatography of Sephadex LH-20. The hydrogenolyses of these products in the presence of hydrogen chloride afforded the crystalline hydrochlorides of gramicidin S and cyclosemigramicidin S. The cyclic decapeptide was as active as the natural gramicidin S, however, cyclosemigramicidin S showed no activity in response to any of the microorganisms tested.

In 1957, Schwyzer and Sieber synthesized tosyl-substituted gramicidin S, *cyclo*-[L-Val-L-Orn(δ-Tos)-L-Leu-D-Phe-L-Pro]$_2$, by the drop-by-drop addition of a linear tosyl-substituted decapeptide active ester, H-[Val-Orn(δ-Tos)-Leu-D-Phe-Pro]$_2$-ONp, to a large amount of pyridine.[2,3] In a second approach, they synthesized Tos-substituted gramicidin S by the doubling reaction of a linear pentapeptide active ester with pyridine.[4] They also reported the synthesis of Tos- or Mz-substituted Lys$^{2,2'}$-gramicidin S by the doubling reaction of a H-Val-Lys(ε-Tos or Mz)-Leu-D-Phe-Pro-ONp.[4,5] They could not isolate the protected cyclosemigramicidin S or Lys^2-cyclosemigramicidin S from the reaction mixture of the cyclization of the linear pentapeptide active ester, and they surmised that the pentapeptide sequence might be present in a transition state favored in such a way that the cyclic decapeptide was formed exclusively.[6]

Recently, the present authors have demonstrated that the cyclization reaction of several linear-pentapeptide-active esters, in which their amino acid sequences have a strong resemblance to that of gramicidin S, affords a mixture of the protected cyclic penta- and decapeptide (see Table 1); for example, the cyclization of H-Val-Orn(δ-Z)-Leu-D-Phe-Sar-ONp yields a mixture of the Z-substituted cyclic penta- and decapeptide with a weight ratio of 85 : 15.[7] These results have prompted the present authors to investigate the possibility of obtaining the protected cyclosemigramicidin S in the course of the cyclization reaction of H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp with pyridine.

In this paper, we will describe the isolation of the protected cyclosemigramicidin S and gramicidin S from the reaction mixture of the cyclization of the pentapeptide active ester, together with the preparation and antibacterial properties of hydrochlorides of cyclosemigramicidin S and cyclic decapeptide.

As is shown in Fig. 1, the acylpentapeptide ethyl ester (II) was prepared in a yield of 81% by the condensation of the azide derived from the acyldipeptide

1) A part of this work has been briefly communicated: M. Waki and N. Izumiya, *J. Am. Chem. Soc.*, **89**, 1278 (1967). The nomenclature of cyclosemigramicidin S was introduced by Shröder and Lübke in their monograph ("The Peptide," Vol. 2, Academic Press, New York and London (1966), p. 429) for *cyclo*-(L-Val-L-Orn-L-Leu-D-Phe-L-Pro-).

2) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

3) The following abbreviations are from *Biochemistry*, **5**, 2485 (1966): Z-, benzyloxycarbonyl; Z(OMe)-, *p*-methoxybenzyloxycarbonyl; Mz-, *p*-methoxyphenylazobenzyloxycarbonyl; -ONp, *p*-nitrophenoxy.

4) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2186 (1958).

5) R. Schwyzer and P. Sieber, *ibid.*, **43**, 1910 (1960).

6) R. Schwyzer, CIBA Foundation, Symposium of Amino Acids and Peptides with Antimetabolic Activity, p. 171 (1958).

7) H. Aoyagi and N. Izumiya, This Bulletin, **39**, 1747 (1966).

hydrazide and tripeptide ethyl ester (I). The saponification of II afforded crystals of acylpentapeptide (III) in a yield of 84%. The treatment of III with an excess of di-*p*-nitrophenyl sulfite gave an amorphous acylpentapeptide *p*-nitrophenyl ester (IV), and the *p*-methoxybenzyloxycarbonyl group of IV was removed by the action of trifluoroacetic acid. A portion of the pentapeptide-active ester trifluoroacetate (V·CF_3COOH) obtained was treated with an appreciable amount of pyridine at 60°C

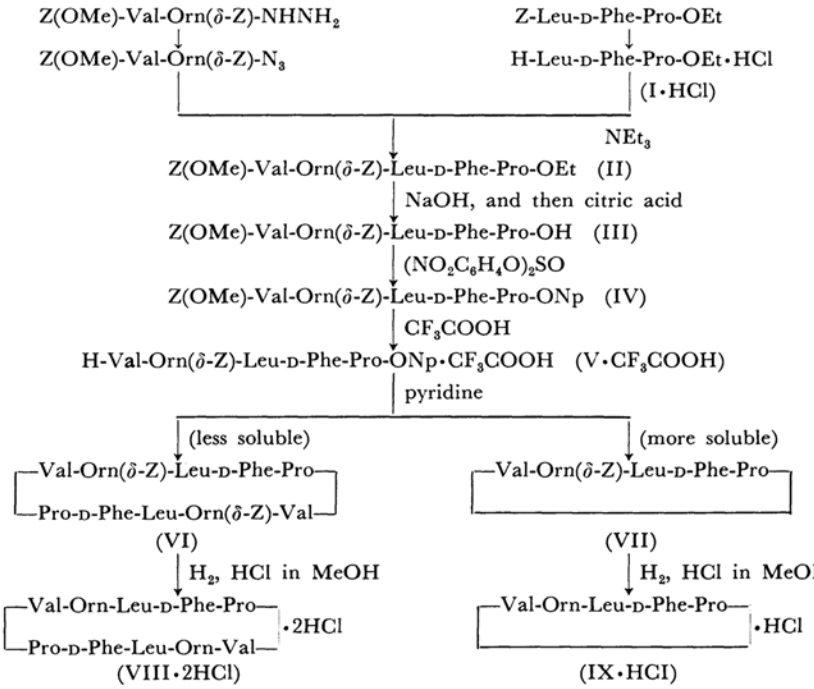

Fig. 1. Cyclization of linear pentapeptide active ester.

TABLE 1. RATIO OF PROTECTED CYCLIC PENTAPEPTIDE AND DECAPEPTIDE AFTER CYCLIZATION OF VARIOUS LINEAR PENTAPEPTIDE ACTIVE ESTERS

p-Nitrophenyl ester of	Ratio of two compounds in product after cyclization of *p*-nitrophenyl ester with pyridine[a]	
	Mono-Z-cyclic monomer[b]	Di-Z-Cyclic dimer[c]
H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH	32	68
H-Val-Lys(ε-Z)-Leu-D-Phe-Pro-OH[9]	29	71
H-Val-Orn(δ-Z)-Leu-D-Phe-Gly-OH[10,11]	79	21
H-Val-Orn(δ-Z)-Leu-D-Phe-Sar-OH[7]	85	15
H-Val-Orn(δ-Z)-Leu-Gly-Pro-OH[12]	0	100
H-Gly-Orn(δ-Z)-Leu-D-Phe-Pro-OH[13]	100	0
H-Ala-Orn(δ-Z)-Leu-D-Phe-Pro-OH[13]	91	9
H-Gly-Orn(δ-Z)-Leu-D-Phe-Gly-OH[14]	100	0
H-Orn(δ-Z)-Leu-D-Phe-Gly-Gly-OH[14]	100	0

a) The concentrations of linear pentapeptide *p*-nitrophenyl esters in pyridine were of approximately 3×10^{-3} M.
b) The figures were derived by calculation on the molar basis.
c) The figures were derived by calculation in which a half mole is used as an unit.

(concentration of V in pyridine: 3×10^{-3} M) for the cyclization reaction. After the evaporation of the reaction mixture, the residue dissolved in aqueous methanol was treated with columns of strongly basic and acidic ion-exchange resins to remove the linear peptides; the subsequent evaporation of the effluent yielded a semi-solid residue. This semi-solid material was found to be a mixture of two components by column chromatography and paper electrophoresis with a hydrogenated material of the residue. The less soluble component was easily isolated, by the fractional recrystallization of the semi-solid residue, as crystals with $[\alpha]_D$ −275° and in a yield of 23% from III; these crystals were identified as di-Z-substituted gramicidin S (VI). The hydrogenolysis of VI in the presence of two equivalents of hydrogen chloride yielded a cyclic decapeptide dihydrochloride octahydrate (VIII·2HCl·8H_2O), which is proved to be identical with natural gramicidin S hydrochloride.

Another protected cyclic peptide (VII), which is more soluble than VI, was isolated as follows. Since the proportion of VII to VI in the semi-solid residue increases with a decrease in the concentration of the active ester (V) in pyridine, as will be described later, a portion of V·CF_3COOH was treated with a large amount of pyridine at 60°C (concentration of V: 3×10^{-4} M) in order to elicit a cyclization reaction. An attempt to separate VII by the fractional crystallization of the semi-solid residue failed; however, the column chromatography of Sephadex LH-20, using methanol as the developing solvent, was successful in separating VII from VI; the product (VII) with $[\alpha]_D$ −49.7° was isolated in a pure state and was identified as Z-substituted cyclosemigramicidin S.[8] The hydrogenolysis of VII in the presence of an equivalent hydrogen chloride yielded the cyclosemigramicidin S hydrochloride as colorless crystals, with four moles of water of crystallization.

Table 1 shows the ratios of the protected cyclic monomer and dimer in the crude product after the cyclization of various linear pentapeptide active esters. As may be seen in Table 1, several pentapeptide active esters, wherein the partial sequence at the C-terminal part is D-phenylalanyl-L-prolyl, yield the cyclic monomer in some extent in addition to the cyclic dimer. Furthermore, it was indicated that the proportion of the Z-substituted cyclosemigramicidin S (VII) to the di-Z-substituted gramicidin S (VI) in the crude product after cyclization increases with a decrease in the concentration of the linear pentapeptide active ester (V) in pyridine. The weight ratios of the monomer (VII) and the dimer (VI) in the crude product were found to be 29 : 71 at 30×10^{-3} M, 32 : 68 at 3×10^{-3} M, and 45 : 55 at 0.3×10^{-3} M of V in pyridine. These results indicate that the sequence of the linear pentapeptide active ester in a very dilute solution is favorable for the intramolecular cyclization reaction, even though an active ester is still present in a transition state favored for the formation of the cyclic dimer.

The antibacterial activities of cyclosemigramicidin S and synthetic gramicidin S toward several microorganisms were also examined. It was found that the synthetic gramicidin S was as active as the natural gramicidin S against several microorganisms. On the other hand, cyclosemigramicidin S exhibited no antibacterial activity in relation to any of the microorganisms tested. Communications from this laboratory have described that Gly[5]- and Sar[5]-cyclosemigramicidin S exhibit no such activity either.[7,14] These results suggest that a certain ring size of a molecule, besides a specific amino acid sequence, is necessary for the exhibition of activity.

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 VIII·2HCl and IX·HCl compounds.

L-Leucyl-D-phenylalanyl-L-proline Ethyl Ester Hydrochloride (I). This compound was obtained by the hydrogenolysis of the benzyloxycarbonyl-L-leucyl-D-phenylalanyl-L-proline ethyl ester[15] in the presence of a 1.1 equivalent of hydrogen chloride in ethanol and palladium black. The filtrate from the catalyst was evaporated, and the oily residue was crystallized by letting it stand in a refrigerator for several days. It was then recrystallized from hot ethanol; yield, 93%; mp 225—228°C (decomp.); $[\alpha]_D^{18}$ −30.9° (*c* 0.5, acetic acid).

Found: C, 59.47; H, 7.83; N, 9.57%. Calcd for $C_{22}H_{33}O_4N_3\cdot HCl\cdot{}^1/_4H_2O$: C, 59.44; H, 7.82; N, 9.46%.

This compound has previously been reported to be an oily product.[15]

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline Ethyl Ester (II).** To a chilled solution of *p*-methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine hydrazide (13.6 g; 25 mmol)[10] in a

8) It is of interest to note that the solubility of *cyclo*-[Val-Orn(δ-Z)-Leu-D-Phe-Sar or Gly-] in any of the solvents tested is smaller than that of *cyclo*-[Val-Orn-(δ-Z)-Leu-D-Phe-Sar or Gly-]$_2$; therefore, the protected cyclic pentapeptide was easily separated from a mixture of the protected monomer and dimer by fractional crystallization.

9) M. Waki *et al.*, in preparation.

10) Isolation of cyclic decapeptide: see, H. Aoyagi *et al.*, *J. Am. Chem. Soc.*, **86**, 5700 (1964); This Bulletin, **38**, 2138 (1965).

11) Isolation of cyclic pentapeptide: see, H. Aoyagi, M. Kondo, T. Kato, S. Makisumi and N. Izumiya, This Bulletin, **40**, 1685 (1967).

12) R. Nagata, M. Waki, M. Kondo, H. Aoyagi, T. Kato, S. Makisumi and N. Izumiya, This Bulletin, **40**, 963 (1967).

13) M. Kondo and N. Izumiya, in preparation.

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

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

mixture of glacial acetic acid (300 m*l*) and dimethylformamide (150 m*l*), there were stirred N hydrochloric acid (55 m*l*) and sodium nitrite (1.9 g) in water (10 m*l*). After 6 min, cold water (1300 m*l*) was added. The azide which thereupon precipitated was collected by filtration, washed with water and 4% sodium bicarbonate, and then dried under a vacuum in a desiccator. The azide was added to a solution of I (11.6 g, 25 mmol) and triethylamine (3.5 m*l*) in dimethylformamide (170 m*l*). The mixture was then stirred for 3 days at 0°C and evaporated *in vacuo*. The precipitate which formed upon the addition of water (1000 m*l*) was collected, and then washed with 4% sodium bicarbonate, 10% citric acid, and water. It was recrystallized from dioxane-ether-petroleum ether; yield, 18.5 g (81%); mp 149—150°C; $[\alpha]_D^{24}$ −26.8° (*c* 2, dimethylformamide).

Found: C, 63.04; H, 7.18; N, 9.29%. Calcd for $C_{49}H_{66}O_{11}N_6 \cdot H_2O$: C, 63.07; H, 7.35; N, 9.01%.

p-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-lucyl-D-phenylalanyl-L-proline (III). To a solution of II (18.3 g, 20 mmol) in methanol (250 m*l*) and dioxane (120 m*l*) N sodium hydroxide (40 m*l*) was added, the solution was then allowed to stand for 5 hr at room temperature. After the addition of water (200 m*l*), the solution was acidified with 10% citric acid under cooling, the solution was concentrated *in vacuo* at a low temperature, and the residue was treated with water (2000 m*l*). After the residue had been stored in a refrigerator for several hours, the precipitate was collected by filtration. The product was recrystallized from methanol-ether; yield, 14.9 g (84%); mp 143—145°C; $[\alpha]_D^{20}$ −18.2° (*c* 1, dimethylformamide).

Found: C, 61.85; H, 6.87; N, 9.42%. Calcd for $C_{47}H_{62}O_{11}N_6 \cdot {}^3/_2H_2O$: C, 61.75; H, 7.17; N, 9.20%.

p-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline p-Nitrophenyl Ester (IV). To a solution of III (5.03 g, 5.7 mmol) in pyridine (30 m*l*), di-*p*-nitrophenyl sulfite[16] (9.2 g, 28.5 mmol) was added. After the mixture had been allowed to stand for 24 hr at room temperature, it was evaporated *in vacuo*. The oily residue was triturated with a mixture of ether and petroleum ether. The residual solid was collected and washed with a mixture of ether and petroleum ether until no yellow color could be discerned upon the addition of a sodium hydroxide solution to the filtrate. The yield was 5.92 g. The *p*-nitrophenyl-ester content of this product was spectrophotometrically estimated to be 106% by measuring the optical density of the compound at 412 mμ.[2]

L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline p-Nitrophenyl Ester Trifluoroacetate ($V \cdot CF_3COOH$). To the *p*-nitrophenyl ester (IV) (5.92 g), anisole (4 m*l*) and trifluoroacetic acid (27 m*l*) were added at 0°C. The solution was then evaporated *in vacuo*, and the residue was triturated with ether. The powder was collected by filtration in a cold room and washed with a mixture of ether and petroleum ether. The yield was 5.95 g. This material was then used in the next step.

***cyclo*-(L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl)$_2$ (VI).** The trifluoroacetate ($V \cdot CF_3COOH$) (1.19 g) was dissolved in dimethylformamide (12 m*l*) containing glacial acetic acid (0.4 m*l*). The solution was stirred, drop by drop over a period of 4 hr, into pyridine (360 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 (80 m*l*) and water (20 m*l*). The solution was passed successively through columns (1.5×10 cm, each) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (300 m*l*), and the combined effluent was evaporated to dryness *in vacuo*. The residual product was collected by filtration, and recrystallized several times from ethanol-ether-petroleum ether; yield, 0.189 g (23% from III); mp 250—252°C (decomp.); $[\alpha]_D^{20}$ −275° (*c* 0.3, acetic acid); R_f 0.96.[17]

Found: C, 62.98; H, 7.72; N, 11.46%. Calcd for $C_{76}H_{104}O_{14}N_{12} \cdot 2H_2O$: C, 63.13; H, 7.53; N, 11.62%.

The molecular weight of VI was determined by a Hitachi osmometer, type 115 (solvent; methanol). Found: 1420. Calcd for $C_{76}H_{104}O_{14}N_{12} \cdot 2H_2O$: 1446.

VI from Natural Gramicidin S. The natural product (gramicidin S dihydrochloride octahydrate) was kindly donated by the Meiji Seika Kaisha, Ltd. To a solution of the compound (120 mg, 0.08 mmol) in pyridine (10 m*l*), benzyloxycarbonyl chloride (660 mg) was added. The reaction mixture was stirred for 5 hr at 0°C and then evaporated *in vacuo*. The residual product was collected with the aid of water and washed with a mixture of ether and petroleum ether. Recrystallization from ethanol-water gave 104 mg (80%); mp 250—251°C (decomp.); $[\alpha]_D^{20}$ −273° (*c* 0.3, acetic acid); R_f 0.96.[17]

***cyclo*-(L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl) (VII).** The trifluoroacetate ($V \cdot CH_3COOH$) (1.19 g) in dimethylformamide (12 m*l*) containing acetic acid (0.4 m*l*) was added to pyridine (3600 m*l*) in the same manner as has been described for the preparation of VI. The residue obtained after the evaporation of the reaction mixture was dissolved in aqueous methanol, and the solution was passed through columns of Dowex 1 and 50. The effluent was evaporated, and the residual product was recrystallized several times from ethanol-ether-petroleum ether (the mother liquor was set aside for the isolation of the more soluble product (VII)). The yield of VI was 0.099 g (12% from III); mp 250—252°C; $[\alpha]_D^{20}$ −276°. The mother liquor was evaporated, and the residue was dissolved in methanol (1.5 m*l*). 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 a flow rate of 40 m*l* per hr; a 1.5 m*l* fraction was thus collected. The peptide content in the fractions was determined on a thin-layer plate by spraying on 47% hydrobromic acid and then a 0.2% ninhydrin acetone solution. When the optical density was plotted against the test tube number, the peak of VI appeared from tube numbers 26 to 32, and the peak of VII, from 34 to 41. When the fractions of 26—32 were combined and evaporated, crystals of VI were obtained; yield

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

17) The R_f value of the thin-layer chromatography with Merck silicagel refers to a solvent system of *n*-butanol - acetic acid - pyridine - water (4: 1: 1: 2, v/v).

TABLE 2. INHIBITORY ACTIVITY OF THREE COMPOUNDS ON MICROORGANISMS
Minimum inhibitory concentration, μg/ml

A. Bouillon agar medium[a)]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*
Natural GS	>100	>100	5	5	100
Synthetic GS	>100	>100	5	5	>100
Cyclosemi GS	>100	>100	>100	>100	>100

B. Synthetic medium[b)]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*
Natural GS	>100	>100	5	5	>100
Synthetic GS	>100	>100	5	5	>100
Cyclosemi GS	>100	>100	>100	>100	>100

a) Usual bouillon agar medium, pH 7.0.
b) Stephenson-Whetham's medium (modified); K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, Na-Glutamate 0.4%, casamino acid 0.2%, yeast-extract 0.05% and agar 2.0%, pH 7.0.

0.066 g (8%); mp 250—252°C. The total yield of VI was 20% from III. After the evaporation of the fractions of 34—41, there remained an oily product which crystallized out after standing several days in a refrigerator. The crystals were collected by filtration with the aid of a mixture of ether and petroleum ether; yield, 0.132 g (16% from III); decomposed over 240°C; $[\alpha]_D^{20}$ −49.7° (*c* 0.1, acetic acid).

Found: C, 62.83; H, 7.49; N, 11.23%. Calcd for $C_{38}H_{52}O_7N_6 \cdot H_2O$: C, 63.13; H, 7.53; N, 11.62%.

The molecular weight was determined as has been described above. Found: 710. Calcd for $C_{38}H_{52}O_7N_6 \cdot H_2O$: 723.

***cyclo*-(L-Valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl)$_2$Dihydrochloride Octahydrate (VIII·2HCl·8H$_2$O).** VI (72 mg, 0.05 mmol), dissolved in 0.05 N methanolic hydrogen chloride (2.2 ml), was subjected to hydrogenolysis in the presence of palladium black. The solution, after being filtered from the catalyst, was evaporated *in vacuo*. The residual product was recrystallized from methanol-ether; the yield of the air dried product was 65 mg (91%); mp 274—276°C (decomp.), reported value mp 277—278°C (decomp.);[2] $[\alpha]_D^{20}$ −271° (*c* 0.1 ethanol), reported value $[\alpha]_D^{21}$ −295° (70% ethanol);[18] R_f, 0.95[19] and 0.76;[17] amino acid ratios in acid hydrolysate, $Val_{1.0}Orn_{0.9}Leu_{1.0}Phe_{1.0}Pro_{0.9}$.

Found: C, 52.80; H, 8.03; N, 12.05%. Calcd for $C_{60}H_{92}O_{10}N_{12} \cdot 2HCl \cdot 8H_2O$: C, 53.04; H, 8.16; N, 12.37%.

Comparison of VIII and Natural Gramicidin S. The natural product, obtained from the Meiji Seika Kaisha, Ltd., showed a mp of 273—275°C and $[\alpha]_D^{20}$ −269° (*c* 0.1, ethanol). In addition to having the same R_f values in paper and thin-layer chromatographies and having indistinguishable paper electrophoretic patterns (Fig. 3), the natural and synthetic products had superimposable infrared spectra, and identical behavior in the carboxymethylcellulose column (Fig. 2). Furthermore, very close similarities were seen in the antibacterial activities of the two compounds with regard to microorganisms (Table 2).

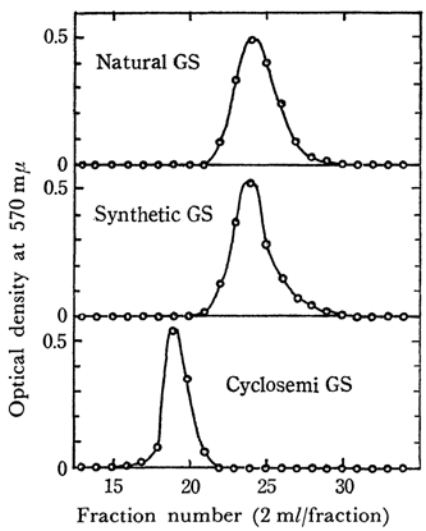

Fig. 2. Carboxymethylcellulose column chromatography of three compounds. GS, gramicidin S.

***cyclo*-(L-Valyl-L-ornithyl-L-leucyl-D-phenylalanyl L-prolyl) Monohydrochloride Tetrahydrate (IX·HCl·4H$_2$O).** VII (72 mg, 0.1 mmol) in 0.05 N methanolic hydrogen chloride (2.2 ml) was hydrogenated as has been described for the preparation of VIII·2HCl·8H$_2$O. The filtrate was evaporated, and the crystals which remained were collected with the aid of ether; yield of the air dried product, 63 mg (88%); mp 223—225°C (decomp.); $[\alpha]_D^{20}$ −76.1° (*c* 0.06, ethanol), R_f, 0.93[19] and 0.85;[17] amino acid ratios in acid hydrolysate, $Val_{1.0}Orn_{0.9}Leu_{1.0}Phe_{1.0}Pro_{1.0}$.

Found: C, 52.73; H, 7.90; N, 11.97%. Calcd for $C_{30}H_{46}O_5N_6 \cdot HCl \cdot 4H_2O$: C, 53.04; H, 8.16; N, 12.37%.

18) R. L. M. Synge, *Biochem. J.*, **39**, 363 (1945).
19) The R_f value of the paper chromatography refers to the same solvent system as has been previously described.[17]

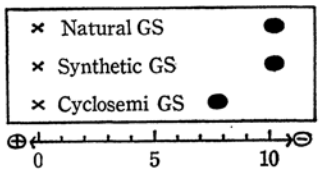

Fig. 3. Paper electrophoresis of three compounds.

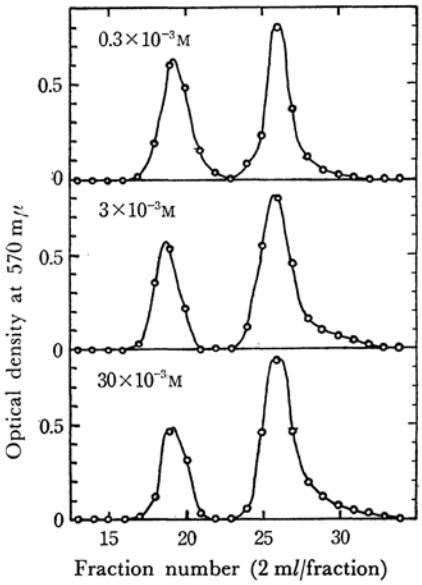

Fig. 4. Carboxymethylcellulose column chromatography of hydrogenated material after cyclization of V at different concentration.

Chromatography and Electrophoresis of Cyclosemigramicidin S (IX), and Synthetic and Natural Gramicidin S (VIII). A sample (0.5—1 mg) was dissolved in 0.2—0.3 m*l* of 0.2 M pyridinium acetate containing 30% methanol (pH 5.1), the solution was applied to a column (0.9×50 cm) with carboxymethylcellulose (Eastmen Organic Chem. 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 method described by Yemm and Cocking.[20] The results are shown in Fig. 2. In order to determine the sensibilities of IX and VIII to ninhydrin, a solution containing 1 μmol of IX and 0.5 μmol of VIII was also submitted to column chromatography; the ratio of intensities by ninhydrin between IX and VIII was found to be 100: 96 by calculating the areas in a chromatogram of optical density/fraction number.

Electrophoresis on Toyo Roshi No. 50 paper was carried out with a solvent system of formic acid - acetic acid - methanol - water (1: 3: 6: 10, v/v; pH 1.8) for 3 hr at 500 V/30 cm. Figure 3 shows that IX migrates faster toward the cathode than VIII, and that the mobility of synthetic gramicidin S is indistinguishable from that of the natural material.

Ratio of Protected Monomer (VII) and Protected Dimer (VI) after Cyclization of V. The pentapeptide-active ester trifluoroacetate (V·CF_3COOH) (119 mg) in dimethylformamide (1.2 m*l*) containing acetic acid (0.04 m*l*) was added to different volumes of pyridine at 60°C over a period of 4 hr. The volumes of pyridine used were 3.6 m*l* (30×10^{-3} M of V in pyridine), 36 m*l* (3×10^{-3} M), and 360 m*l* (0.3×10^{-3} M). Each of the reaction mixtures was evaporated, and the residue, dissolved in aqueous methanol, was passed through columns of Dowex 1 and 50. The effluent was evaporated, and the crude product (desingnated as X) was dissolved in methanolic hydrogen chloride (30 m*l*). The solution (1 m*l*) was subjected to hydrogenolysis, and the filtrate from the catalyst was evaporated to yield an oily residue. The residue was then subjected to carboxymethylcellulose column chromatography (Fig. 4). By the use of the value of 100: 96 obtained already as the ratio of the color intensities, it was calculated from Fig. 4 that the weight ratios of VII and VI in X are 29: 71 (30×10^{-3} M), 32: 68 (3×10^{-3} M), and 45: 55 (0.3×10^{-3} M) respectively.

Microbiological Assays. The microorganisms employed are listed in Table 2. 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 2, the cyclosemigramicidin S was found to be devoid of activity in reaction against any of the microorganisms utilized, whereas synthetic or natural gramicidin S exhibited considerable activity in reaction against some of the microorganisms.

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20) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).