BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 46, 977—985 (1973)

Studies of Peptide Antibiotics. XXVIII.[1] Syntheses of Sesquigramicidin S and Digramicidin S[2,3]

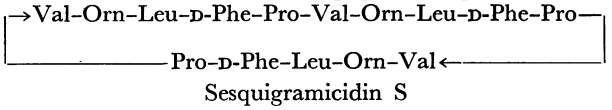
Shuji MATSUURA, Michinori WAKI, Tetsuo KATO, and Nobuo IZUMIYA

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

(Received September 6, 1972)

Two macro-ring analogs of gramicidin S, namely *cyclo*(–L-Val–L-Orn–L-Leu–D-Phe–L-Pro–)$_3$ (sesquigramicidin S) and *cyclo*(–L-Val–L-Orn–L-Leu–D-Phe–L-Pro–)$_4$ (digramicidin S), were prepared to investigate the influence of the ring size of gramicidin S for antibacterial activity. Several linear analogs such as H–(L-Val–L-Orn–L-Leu–D-Phe–L-Pro)$_n$–OH (n=2, 3 and 4) were also prepared as reference compounds. All these macro-ring and linear analogs showed weaker activity than gramicidin S and synergistic property when each of the analogs is mixed with gramicidin S. Measurements of optical rotatory dispersion were made with these analogs and gramicidin S in solvents of ethanol and 8 M aqueous urea. From these experiments, it was suggested that the mode of antibacterial action of the macro-ring analogs and their conformations are similar to those of the linear analogs.

In studies of the relationship between chemical structure and antibacterial activity of gramicidin S (GS), various analogs have been synthesized.[5] Particularly in regard to the ring size, several cyclic peptides with smaller ring size than that of GS were prepared, but none of them were active.[4,6] As only one analog with a larger ring size than GS, [β-Ala$^{5,5'}$]-GS was synthesized, but this compound was also inactive.[7] In order to determine further the influence of larger ring size on the activity, we have designed the syntheses of sesquigramicidin S[3] and digramicidin S[3] which contain decapeptide sequence of GS.



Sesquigramicidin S

1) Part XXVII: S. Matsuura, M. Waki, and N. Izumiya, This Bulletin, **45**, 863 (1972).

2) Presented at the 9th Symposium on Peptide Chemistry, Shizuoka, November, 1971. Part of this work has been briefly communicated: S. Matsuura and N. Izumiya, *Experientia*, **28**, 1402 (1972).

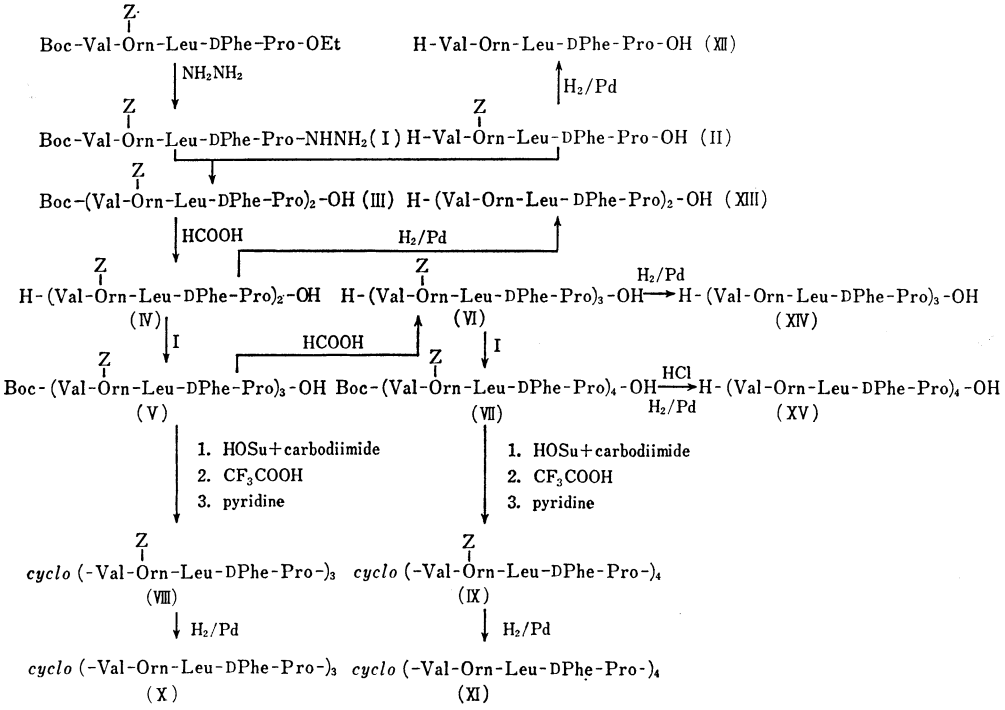
3) We introduced the naming of cyclosemigramicidin S for *cyclo*(-Val-Orn-Leu-D-Phe-Pro-) primarily,[4] but changed this naming as semigramicidin S in the recent review.[5] Therefore, we employ the namings of sesquigramicidin S and digramicidin S for *cyclo*(-Val-Orn-Leu-D-Phe-Pro-)$_3$ and *cyclo*(-Val-Orn-Leu-D-Phe-Pro-)$_4$, respectively.

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

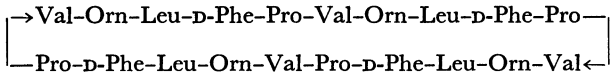
5) T. Kato and N. Izumiya, "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," Vol. 2, B. Weinstein, Ed., Marcel Dekker, New York (1972), in press.

6) N. Izumiya, T. Kato, Y. Fujita, M. Ohno, and M. Kondo, This Bulletin, **37**, 1809 (1964); T. Kato, M. Kondo, M. Ohno, and N. Izumiya, *ibid.*, **38**, 1202 (1965); O. Abe, K. Kuromizu, M. Kondo, and N. Izumiya, *ibid.*, **43**, 914 (1970).

7) S. Matsuura, M. Waki, S. Makisumi, and N. Izumiya, *ibid.*, **43**, 1197 (1970).



Scheme 1.



Digramicidin S

This paper describes the syntheses, antibacterial properties and ORD[8] measurements of two macro-ring analogs of GS besides those of several linear analogs.

Scheme 1 indicates the routes for syntheses of the macro-ring and linear analogs. The azide derived from Boc–pentapeptide hydrazide (I) was condensed with a neutral pentapeptide (II), and the resulting acyl-decapeptide acid (III) was converted to a neutral decapeptide (IV) by the action of formic acid. The azide derived from I was again condensed with VI, the resulting Boc–pentadecapeptide acid (V) was transformed to Boc–pentadecapeptide *N*-hydroxysuccinimide ester by the action of HOSu and water soluble carbodiimide, and its Boc group was removed with trifluoroacetic acid. Pentadecapeptide ester trifluoroacetate thus obtained was treated with pyridine for the cyclization reaction.[9] The reaction mixture yielded a pure Z-substituted cyclic pentadecapeptide (VIII) with a fairly good yield. The hydrogenolysis of VIII afforded the desired sesquigramicidin S (X) as a crystalline trihydrochloride. In a similar manner, the desired digramicidin S (XI) was prepared as a crystalline tetrahydrochloride as shown in Scheme 1.

The linear penta- (XII), deca- (XIII), and pentadecapeptide (XIV) were prepared from the corresponding Z-substituted peptides by hydrogenolysis.

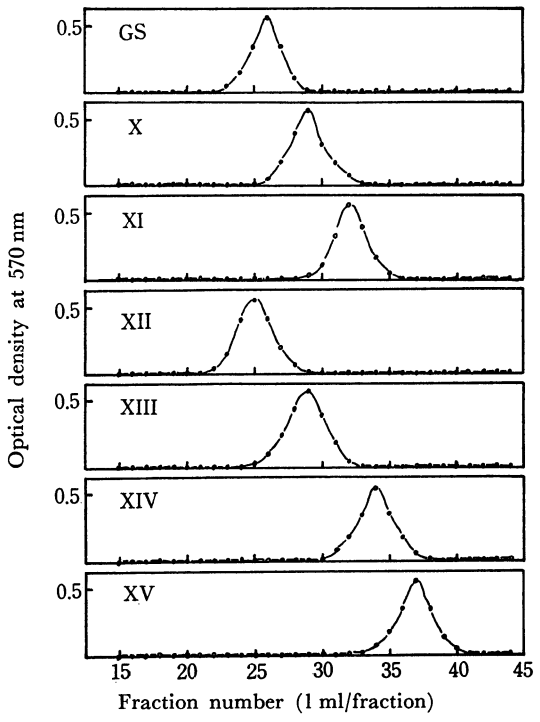


Fig. 1. CMC column chromatography of macro-ring and linear analogs of GS.

8) Abbreviations according to IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972), are used. Additional abbreviations are as follows: ORD, optical rotatory dispersion; CMC, carboxymethyl cellulose; HOSu, *N*-hydroxysuccinimide; TEA, triethylamine; DMF, dimethylformamide. Amino acid symbols except D-Phe denote the L-configuration.

9) For the cyclization reaction we have used very often linear peptide *p*-nitrophenyl esters. However, we recognized recently that the cyclization of linear peptide *N*-hydroxysuccinimide ester gives better yield than that of *p*-nitrophenyl ester in many cases. Some features using *N*-hydroxysuccinimide ester for the cyclization were presented at the 24th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1971.

Table 1. Antibacterial activity of the compounds
(Minimum inhibitory concentration, μg/ml)

Compound[a,b]	Medium for assay[c]	*Staphylococcus aureus*	*Bacillus subtilis*
semiGS	B, S	>100	>100
GS	B	5	2
	S	5	5
X (sesquiGS)	B	50	20
	S	50	50
XI (diGS)	B	20	10
	S	20	10
XII (penta)	B, S	>100	>100
XIII (deca)	B	50	50
	S	100	20
XIV (pentadeca)	B	50	50
	S	100	20
XV (eicosa)	B	20	10
	S	20	20
GS+X[d]	B	10	2
	S	5	5
GS+XI[d]	B	5	2
	S	5	2
GS+XIII[d]	B	5	5
	S	5	5
GS+XIV[d]	B	5	5
	S	5	5
GS+XV[d]	B	5	5
	S	5	5

a) The μg in the minimum inhibitory concentration refers net weight of a peptide without either HCl or H_2O in the compound. b) All compounds showed no activity for *Echerichia coli*. c) B, usual bouillon agar medium of pH 7.0. S, synthetic Stephensen-Whetham's medium of pH 7.0. d) A mixture was prepared with 1 : 1 weight ratio of GS and an analog.

The eicosapeptide (XV) was derived from α-Boc-δ-Z-substituted eicosapeptide (VII) by successive treatments of hydrogen chloride in ethyl acetate and hydrogenolysis. The purities of these macro-ring and linear analogs were ascertained by CMC column chromatography (Fig. 1) besides other experiments such as elemental analysis.

The antibacterial activities of the macro-ring and linear analogs toward microorganisms were tested (Table 1). As like that semigramicidin S was inactive,[4] the corresponding linear pentapeptide (XII) was also inactive. The linear decapeptide (XIII) showed weak activity as reported previously.[10,11] It is interesting to note that a level of the activity of sesquigramicidin S and digramicidin S was approximately same with that of the corresponding linear peptides, XIV, and XV, respectively. Furthermore, it seems that level of the specific activity increased with an increase of molecular size in both the macro-ring and linear analogs. Erlanger and Goode observed that some linear decapeptides such as XIII reveal synergistic

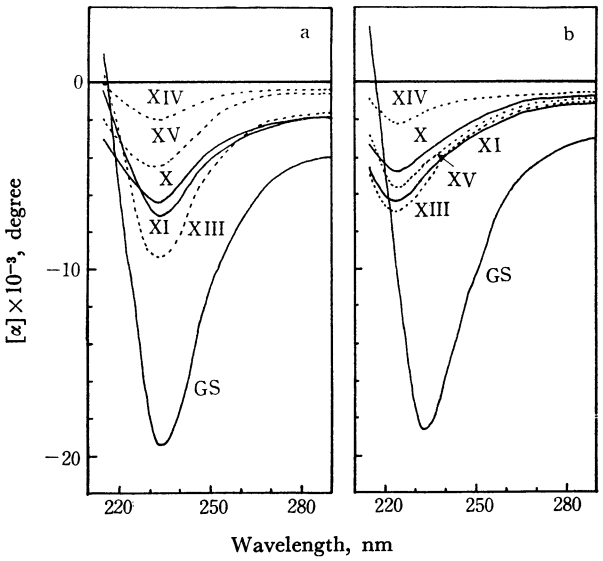


Fig. 2. ORD curves of macro-ring and linear analogs of GS. Solvent; a, ethanol; b, 8 M urea.

activity with GS against microorganism, and noted that the mode of antibacterial action by XIII may differ from that of GS.[12] As shown in Table 1, a mixture of GS and each of macro-ring and some linear analogs showed the same activity as GS itself; these analogs revealed synergistic activity with GS. We thus assumed that the mode of antibacterial action of the macro-ring analogs is similar to that of the linear analogs, and differ from that of GS.

The assumption mentioned is favored by the experiments of ORD. The ORD curves measured in a solvent of ethanol are shown in Fig. 2-a. The macro-ring and linear analogs have similar shaped curves with a negative trough at 232 nm as GS possesses the same. In a solvent of 8 M aqueous urea which causes denaturation of some polypeptides,[13] the troughs of the peptides except GS were moved to 225 nm whereas that of GS remained constant (Fig. 2-b). The results may indicate that a conformation of the macro-ring analogs is similar to that of the linear analogs, whereas that of GS is very stable even in 8 M aqueous urea. In this connection, it would be noteworthy that GS has the rigid β-pleated sheet structure having an antiparallel tripeptide sequence with four hydrogen bondings.[14] In conclusion, the presented data suggest that a cyclic character in a pentadeca- or eicosapeptide which contains a sequence of GS is not essential to exhibit the activity, whereas in a linear decapeptide sequence in GS, the cyclic character is important to form the rigid structure and consequently to exhibit a strong and characteristic antibacterial activity.

10) B. F. Erlanger and L. Goode, *Nature*, **174**, 840 (1954).
11) S. Makisumi, M. Waki, and N. Izumiya, This Bulletin, **44**, 143 (1971).
12) B. F. Erlanger and L. Goode, *Science*, **131**, 669 (1960).
13) B. Jirgensons, "Optical Rotatory Dispersion of Proteins and Other Macromolecules," Springer-Verlag, Berlin (1969).
14) D. C. Hodgkin and B. M. Oughton, *Biochem. J.*, **65**, 752 (1957); W. A. Gibbons, J. A. Sogn, A. Stern, and L. C. Craig, *Nature*, **227**, 840 (1970); F. Quadrifoglio and D. W. Urry, *Biochem. Biophys. Res. Commun.*, **29**, 785 (1967); S. L. Laiken, M. P. Printz, and L. C. Craig, *Biochemistry*, **8**, 519 (1969).

Experimental

Thin layer chromatography was carried out on Merck silica gel G with the following solvent systems: R_f^1, *n*-butanol–acetic acid–pyridine–water (4 : 1 : 1 : 2, v/v); R_f^2, chloroform–methanol (5 : 1, v/v); R_f^3, *sec*-butanol–formic acid–water (4 : 1 : 1, v/v).

Boc–Val–Orn(Z)–Leu–D-Phe–Pro–NHNH$_2$ (I). A solution of Boc–Val–Orn(Z)–Leu–D-Phe–Pho–OEt[15] (8.72 g, 10.3 mmol) and hydrazine hydrate (9.9 ml, 205 mmol) in DMF (35 ml) was allowed to stand at room temperature for 7 days. The solution was evaporated *in vacuo*, and then water (150 ml) was added to the residue. The resulting crystals were collected by filtration and recrystallized from methanol–ether; yield, 7.73 g (90%); mp 183—185°C; $[\alpha]_D^{15}$ −19.2° (*c* 0.5, DMF); R_f^1 0.67, R_f^2 0.60.

Found: C, 60.75; H, 7.88; N, 13.53%. Calcd for $C_{43}H_{64}O_9N_8 \cdot 1/2H_2O$: C, 61.04; H, 7.74; N, 13.25%.

Boc–(Val–Orn(Z)–Leu–D-Phe–Pro)$_2$–OH (III). I (2.23 g, 2.66 mmol) was dissolved in DMF (10 ml) and 2 N hydrogen chloride in dioxane (2.7 ml). To the solution at −30°C was added isoamyl nitrite (0.45 ml, 3.2 mmol). After some 10 min, disappearance of the hydrazide was ascertained by the detection method for hydrazide.[16] To the reaction mixture was added TEA (0.74 ml, 5.3 mmol). After 5 min at 0°C, a solution of H–Val–Orn(Z)–Leu–D-Phe–Pro–OH·HCOOH (II·HCOOH)[11] (2.46 g, 3.2 mmol) in DMF containing TEA (0.9 ml) was added to the mixture. After 3 days at 4°C, the reaction mixture was added to cold 0.2 M citric acid (1000 ml). The resulting solid was recrystallized from methanol–ether–petroleum ether; yield, 3.49 g (86%); mp 173—175°C; $[\alpha]_D^{25}$ −105° (*c* 1, MeOH). The same compound was prepared already from Boc–Val–Orn(Z)–Leu–D-Phe–Pro–OH and II by the method of HOSu plus DCC; $[\alpha]_D^{23}$ −109° (MeOH).[11] No depression of melting point was observed on a mixture of III with the previous product.[11]

H–(Val–Orn(Z)–Leu–D-Phe–Pro)$_2$–OH (IV). A solution of III (2.57 g, 1.68 mmol) in 99% formic acid (30 ml) was allowed to stand for 5 hr at room temperature. The solvent was removed by evaporation, and the residue was dissolved in a mixture of TEA (12 ml) and methanol (70 ml). The solution was evaporated, and the resulting crystals were collected by filtration with the aid of water;[17] yield, 2.19 g (87%); mp 155—157°C; $[\alpha]_D^{25}$ −21.8° (*c* 0.3, DMF); R_f^1 0.80, R_f^2 0.70. Hygroscopic monohydrochloride of IV was prepared previously.[11]

Found: C, 60.86; H, 7.51; N, 11.17%. Calcd for $C_{76}H_{106}O_{15}N_{12} \cdot 4H_2O$: C, 60.86; H, 7.66; N, 11.20%.

Boc–(Val–Orn(Z)–Leu–D-Phe–Pro)$_3$–OH (V). I (515 mg, 0.62 mmol) in DMF (5 ml) was treated with isoamyl nitrite (0.1 ml, 0.74 mmol) as in the case of III. To the azide solution was added a solution of IV (1.02 g, 0.68 mmol) in DMF (4 ml) containing TEA (0.095 ml). After being stirred for 5 days at 4°C, the reaction mixture was added to 0.2 M citric acid (300 ml). The resulting solid was collected and recrystallized from methanol–ether–petroleum ether; yield, 1.13 g (80%); mp 150—152°C; $[\alpha]_D^{25}$ −22.8° (*c* 0.5, DMF); R_f^1 0.98, R_f^2 0.64.

Found: C, 62.64; H, 7.54; N, 11.22%. Calcd for $C_{119}H_{166}O_{24}N_{18} \cdot 3H_2O$: C, 62.49; H, 7.58; N, 11.03%.

H–(Val–Orn(Z)–Leu–D-Phe–Pro)$_3$–OH (VI). This was prepared from V (2.26 g, 0.99 mmol) with 99% formic acid (60 ml) as in the case of IV: yield, 1.96 g (90%); mp 158—160°C; $[\alpha]_D^{25}$ −77.4° (*c* 0.5, DMF); R_f^1 0.90, R_f^2 0.65.

Found: C, 61.85; H, 7.52; N, 11.37%. Calcd for $C_{114}H_{158}O_{22}N_{18} \cdot 4H_2O$: C, 62.10; H, 7.50; N, 11.44%.

Boc–(Val–Orn(Z)–Leu–D-Phe–Pro)$_4$–OH (VII). The azide solution was prepared from I (1.04 g, 1.24 mmol) as in the case of III. To this was added a solution of VI (2.11 g, 0.96 mmol) in DMF (15 ml) containing TEA (0.13 ml). The solid was obtained as in the case of V and recrystallized from methanol–ether–petroleum ether; yield, 2.38 g (82%); mp 151—154°C; $[\alpha]_D^{25}$ −47.6° (*c* 1.0, DMF); R_f^1 0.90, R_f^2 0.70.

Found: C, 62.40; H, 7.67; N, 11.09%. Calcd for $C_{157}H_{218}O_{31}N_{24} \cdot 5H_2O$: C, 62.28; H, 7.59; N, 11.10%.

cyclo*(–Val–Orn(Z)–Leu–D-Phe–Pro–)$_3$ (VIII).* To a solution of V (320 mg, 0.14 mmol) in dichloromethane (2 ml) and DMF (1 ml) at 0°C, *N*-hydroxysuccinimide (32 mg, 0.28 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[18] (54 mg, 0.28 mmol) was added. After 12 hr at 4°C, the mixture was evaporated. The residual solid was collected by filtration with the aid of cold water; yield, 314 mg. To Boc–pentadecapeptide *N*-hydroxysuccinimide ester obtained, trifluoroacetic acid (3 ml) was added at 0°C. After 20 min, the solution was evaporated, and the residual powder was collected by filtration with the aid of ether. Pentadecapeptide *N*-hydroxysuccinimide ester trifluoroacetate obtained was dissolved in DMF (5 ml), and the solution was added into pyridine (50 ml) at room temperature. The stirring was continued for 2 hr. The solvent was removed, and the residue was dissolved in a mixture of methanol (50 ml) and water (10 ml). The solution was passed through columns (1.9×20 cm, each) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (200 ml), the combined effluent was evaporated, and the product was collected by filtration with the aid of water. The product was further purified by a column (2.4×115 cm) with Sephadex LH–20; the product dissolved in methanol (3 ml) was applied to the column, and developed with methanol. Main fractions were evaporated, and the residual solid was recrystallized from methanol–ether–petroleum ether; yield, 144 mg (47% from V); mp 128—130°C; $[\alpha]_D^{25}$ −15.6° (*c* 0.3, DMF); R_f^1 0.90, R_f^2 0.75.

Found: C, 62.39; H, 7.49; N, 11.34%; mol wt 2110.[19] Calcd for $C_{114}H_{156}O_{21}N_{18} \cdot 4H_2O$: C, 62.61; H, 7.56; N, 11.53%; mol wt 2186.

cyclo*(–Val–Orn(Z)–Leu–D-Phe–Pro–)$_4$ (IX).* VII (1.91 g, 0.63 mmol) was converted to IX as described for the preparation of VII; yield, 911 mg (50% from VII); mp 153—155°C; $[\alpha]_D^{25}$ −23.0° (*c* 0.5, DMF); R_f^1 0.98, R_f^2 0.75.

Found: C, 63.00; H, 7.32; N, 11.37%; mol wt 2900.[19] Calcd for $C_{152}H_{208}O_{28}N_{24} \cdot 4H_2O$: C, 63.13; H, 7.53; N, 11.63%; mol wt 2891.

cyclo*(–Val–Orn–Leu–D-Phe–Pro–)$_3$ (Sesquigramicidin S) (X).* A solution of VIII (39 mg, 0.018 mmol) in 0.01 N methanolic hydrogen chloride (6 ml) was subjected to hydrogenolysis in the presence of palladium black, and the filtrate was evaporated. The product was recrystallized from methanol–ether–petroleum ether; yield of air-dried product (X·3HCl·

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

16) H. Ertel and L. Horner, *J. Chromatog.*, **7**, 268 (1962).

17) In the case of H-Val-Orn(Z)-Leu-D-Phe-Pro-OH (II), the neutral peptide (II) was never solidified, whereas monohydrochloride of II was obtained as nice crystals.

18) J. C. Sheehan, P. A. Cruickshank, and G. L. Boshart, *J. Org. Chem.*, **26**, 2525 (1961).

19) Molecular weight was determined on a Hitachi Osmometer, type 115, using methanol as a solvent.

$8H_2O$), 27 mg (76%); mp 163—165°C (decomp.); $[\alpha]_D^{25}$ −33.0° (*c* 0.4, ethanol); R_f^1 0.82, R_f^2 0.10, R_f^3 0.80. Amino acid ratios in acid hydrolysate; Phe 0.9, Leu 1.0, Orn 1.1, Val 1.0, Pro 0.9.

Found: C, 55.31; H, 8.22; N, 13.04%. Calcd for $C_{90}H_{138}O_{15}N_{18}\cdot 3HCl\cdot 8H_2O$: C, 54.99; H, 8.05; N, 12.83%.

cyclo*(-Val-Orn-Leu-*D*-Phe-Pro-)*$_4$ *(Digramicidin S) (XI).* IX (153 mg, 0.053 mmol) was converted to XI·4HCl·$9H_2O$ as described for the preparation of X; yield of air-dried product, 117 mg (85%); mp 205—207°C (decomp.); $[\alpha]_D^{25}$ −51.6° (*c* 0.5, ethanol); R_f^1 0.80, R_f^2 0.10, R_f^3 0.70. Amino acid ratios in acid hydrolysate; Phe 0.8, Leu 1.0, Orn 1.0, Val 1.0, Pro 0.9.

Found: C, 55.93; H, 8.02; N, 13.40%. Calcd for $C_{120}H_{184}O_{20}N_{24}\cdot 4HCl\cdot 9H_2O$: C, 55.62; H, 8.01; N, 12.98%.

*H-Val-Orn-Leu-*D*-Phe-Pro-OH (XII).* A solution of II·HCOOH[11] (546 mg, 0.71 mmol) in 95% acetic acid (10 ml) was subjected to hydrogenolysis, and the filtrate was evaporated to dryness. The residue was dissolved in 0.2 N methanolic hydrogen chloride (7.5 ml) and evaporated. The residual solid was recrystallized from methanol–ethyl acetate–ether; yield of air-dried product, 406 mg (82%); mp 200—202°C (decomp.); $[\alpha]_D^{25}$ −62.5° (*c* 1.4, ethanol); R_f^1 0.85, R_f^2 0.01, R_f^3 0.70.

Found: C, 52.18; H, 7.90; N, 11.94%. Calcd for $C_{30}H_{48}O_6N_6\cdot 2HCl\cdot 2H_2O$: C, 51.64; H, 7.80; N, 12.05%.

*H-(Val-Orn-Leu-*D*-Phe-Pro)*$_3$*-OH (XIV).* VI (485 mg, 0.22 mmol) was converted to XIV·4HCl·$8H_2O$ as described for the preparation of XII; yield of air-dried product, 329 mg (74%); mp 245—247°C (decomp.); $[\alpha]_D^{25}$ −61.1° (*c* 0.5, ethanol); R_f^1 0.85, R_f^2 0.01, R_f^3 0.70.

Found: C, 53.83; H, 6.78; N, 12.86%. Calcd for $C_{90}H_{140}O_{16}N_{18}\cdot 4HCl\cdot 8H_2O$: C, 53.50; H, 6.99; N, 12.48%.

*H-(Val-Orn-Leu-*D*-Phe-Pro)*$_4$*-OH (XV).* XII (206 mg, 0.068 mmol) was dissolved in 2.5 N hydrogen chloride in ethyl acetate (5.4 ml). After 30 min, the solution was evaporated, and the residue was treated as described for the preparation of XII; yield of air-dried product, 127 mg (70%); mp 260—262°C (decomp.); $[\alpha]_D^{25}$ −69.5° (*c* 0.5, ethanol); R_f^1 0.85, R_f^2 0.01, R_f^3 0.70.

Found: C, 54.40; H, 7.31; N, 12.88%. Calcd for $C_{120}H_{186}O_{21}N_{24}\cdot 5HCl\cdot 10H_2O$: C, 54.11; H, 7.04; N, 12.62%.

Paper Electrophoresis and CMC Column Chromatography. To ascertain further the purities of the macro-ring and linear analogs of GS, each peptide was subjected to the analyses mentioned. The experiments were carried out as described before.[20] Semigramicidin S hydrochloride[4] and H-(Val-Orn-Leu-D-Phe-Pro)$_2$-OH·3HCl[11] were the products prepared before. The electrophoresis of each peptide revealed a single spot; R^{GS} for X, XI, XII, XIII, XIV, and XV were 1.04, 1.09, 1.05, 1.05, 1.07, and 1.07, respectively. The CMC chromatography revealed also a single peak as shown in Fig. 3.

Microbiological Assays.[21] The minimum amount of the compounds necessary for the complete inhibition of growth was determined by a dilution method using a bouillon agar and synthetic mediums, and the results are shown in Table 1.

ORD Measurements. The measurements were performed with JASCO spectropolarimeter model ORD–CD/UV-5 over a wavelength range of 220 to 300 nm. Cell of path length 0.1 cm was used and the runs were made at ambient temperature. Patterns in solvent of ethanol and 8 M aqueous urea are shown in Fig. 2-a and Fig. 2-b, respectively. When 2-chloroethanol and 1% aqueous sodium dodecyl sulfate were used instead of ethanol and 8 M aqueous urea, respectively, the similar patterns as Fig. 2-a and Fig. 2-b were observed.

20) H. Aoyagi, T. Kato, M. Waki, O. Abe, R. Okawa, S. Makisumi, and N. Izumiya, This Bulletin, **42**, 782 (1969).

21) We are indebted to the staff of Takeda Chemical Industries, Ltd. for the assay.