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Studies of Peptide Antibiotics. XIV. Analogs of Gramicidin S Containing D-Valine or D-Leucine in Place of D-Phenylalanine

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Two analogs of gramicidin S, 4,4'-D-valine- and 4,4'-D-leucine-gramicidin S (XVI-A and XVI-B), have been synthesized and tested for the antibacterial properties. The crude product obtained after the cyclization of a linear pentapeptide active ester was composed of the protected monomer and dimer. The pure protected dimer was obtained by a column chromatography of Sephadex LH-20, and the hydrogenolysis of this product in the presence of hydrogen chloride afforded the crystalline hydrochloride of XVI-A or XVI-B. These analogs were as active as natural gramicidin S; the results indicated that the aromatic side chains of the D-phenylalanines in the molecule of gramicidin S can be replaced by the aliphatic side chains without an influence for the activity.

In connection with a series of studies in this laboratory on a significance of the side chains of the amino acid residues of gramicidin S (Fig. 1)

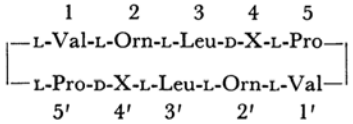

Fig. 1. Structure of gramicidin S (GS) and its analogs. X represents an amino acid residue such as Phe (GS), Val (XVI-A) or Leu (XVI-B).

to its antibacterial activity, it has been reported in the previous paper that an analog of the antibiotics was prepared in which the benzyl groups of D-phenylalanine residues in 4- and 4'-positions of the molecule were replaced by the hydrogens of glycines, and 4,4'-glycine-gramicidin S thus prepared was found to possess almost no activity toward several microorganisms.[3] From this finding, it appeared that the side chains of D-phenylalanines in the sequence of gramicidin S are of considerable importance for an exhibition of the activity. In order to investigate an effect of replacing the benzyl side chains of D-phenylalanines by the aliphatic side chains of D-valines or D-leucines, we attempted to prepare several analogs of gramicidin S. The present paper will describe the syntheses and antibacterial properties of 4,4'-D-valine- and 4,4'-D-leucine-gramicidin S besides the preparations of the cyclic pentapeptides, 4-D-valine- and 4-D-leucine-cyclosemigramicidin S.

It would be noteworthy that D-valine residue is a constituent in several natural antibiotics such as actinomycins and fungisporin, and D-leucine residue in etamycin, esperin and malformin.[4] Some peptide antibiotics such as sporidesmolide I[4] and gramicidin A[5] contain both D-valine and D-leucine as a constiuent. The closely related polymyxin B_1 or E_1 has been shown to be homodetic cyclic decapeptide wherein an amino group of the molecule is acylated with 6-methyl-octanoic acid. Of special interest in connection with the present paper was the fact that polymyxin E_1 differs from polymyxin B_1 in having an D-leucine-residue in place of the D-phenylalanine at 6-position of polymyxin B_1.[6]

Since the cyclization of H-Val-Orn(δ-Z)-Leu-Gly-Pro-ONp[7] afforded exclusively the benzyloxycarbonyl-substituted dimer through the doubling reaction,[3] the syntheses of the protected cyclic decapeptides (XIV-A and -B) were attempted by possible dimerization reaction of the pentapeptide

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

4) E. Schröder and K. Lübke. "The Peptides," Vol. 2, Academic Press, New York and London (1966), p. 396.

5) R. Sarges and B. Witkop, *J. Am. Chem. Soc.*, **86**, 1861, 1862 (1964).

6) T. Suzuki, H. Inouye, K. Fujikawa and S. Nagasawa, *J. Biochem.*, **54**, 173 (1963); S. Wilkinson and L. A. Lowe, *Nature*, **204**, 185, 993 (1964).

7) The following abbreviations are from *Biochmistry*, **5**, 2485 (1966); Z-, benzyloxycarbonyl; Z(OMe)-, *p*-methoxybenzyloxycarbonyl; ONp, *p*-nitrophenoxy. Amino acid symbols except D-Phe, D-Val or D-Leu denote the L configuration.

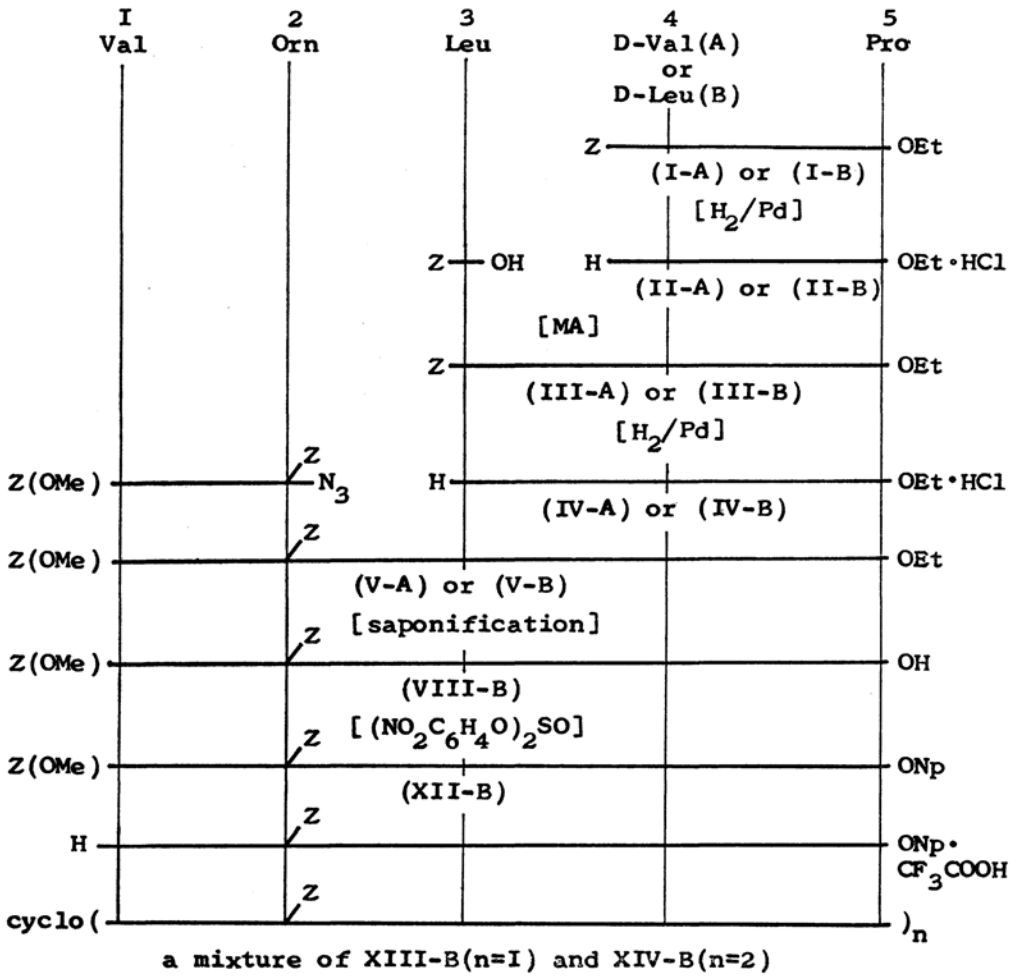

Fig. 2. Cyclization of linear pentapeptide active ester containing D-Leu.

active esters (XII-A and -B) as shown in Figs. 2 and 3. To obtain an acylpentapeptide acid (VIII-A or -B) which is a starting material for the active ester, the corresponding acylpentapeptide ethyl ester was subjected to the saponification; the reaction of the acylpeptide ester (V-B) containing D-leucine proceeded smoothly to afford the acylpeptide acid (VIII-B) in a good yield, whereas the ester (V-A) containing D-valine was found to resist for the saponification reaction. In this connection, it would be noteworthy that several investigators have reported the resistance of a peptide ester containing valine or isoleucine residue for the saponification.[8,9] Therefore, the acylpentapeptide acid (VIII-A) containing D-valine was prepared by the condensation of Z(OMe)-Val-Orn(δ-Z)-N_3 with neutral tripeptide (VII-A). Acyltripeptide acid (VI-A) which is a material for the neutral tripeptide was prepared by the saponification of acylpeptide ester (III-A) without any difficulty though the ester contained a valine residue.

The treatment of an acylpentapeptide acid (VIII-A or -B) with di-*p*-nitrophenyl sulfite gave an amorphous acylpeptide active ester, and its *p*-methoxybenzyloxycarbonyl group was removed by the action of trifluoroacetic acid. The pentapeptide active ester trifluoroacetate obtained was treated with a large amount of pyridine (concentration of the ester in pyridine, 3×10^{-3} M). After the evaporation of the reaction mixture, the residue dissolved in aqueous methanol was treated with columns of Dowex 1 and 50 to be free from the undesired products. The subsequent evaporation of the effluent yielded a semi-solid residue which was found to be a mixture of the protected monomer (XIII-A or -B) and dimer (XIV-A or -B). Attempts to separate the components in the residue by the fractional crystallization with methanol-ether failed because of no appreciable difference in solubility between

8) R. Sarges and B. Witkop, *J. Am. Chem. Soc.*, **87**, 2020 (1965).

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

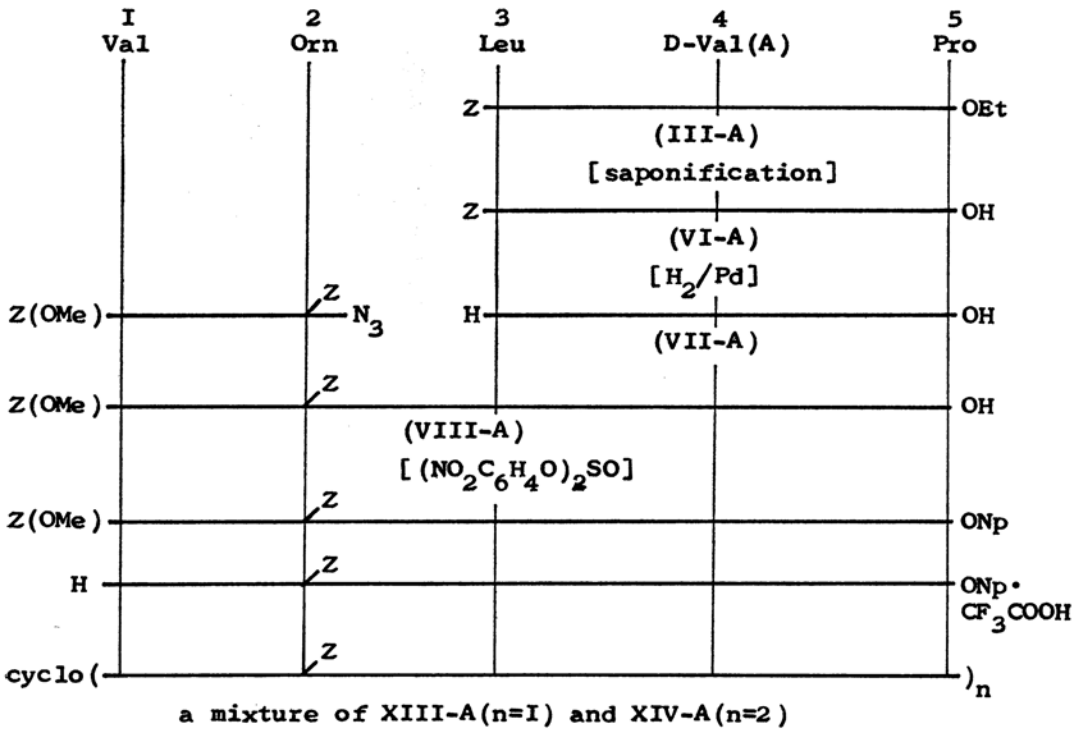

Fig. 3. Cyclization of linear pentapeptide active ester containing D-Val

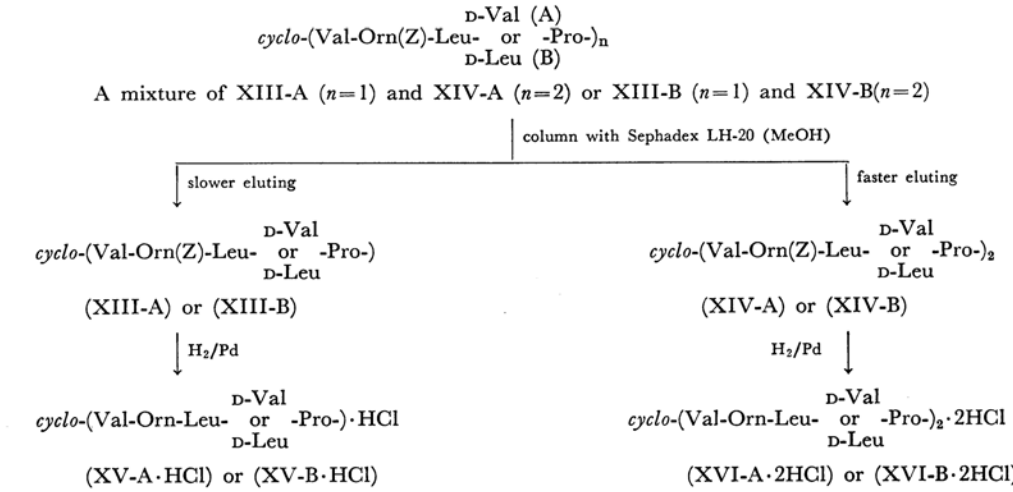

Fig. 4. Separation of the protected monomer and dimer, and preparation of the required GS and cyclosemi GS analogs.

the two components.[10] The separation of the components was achieved by the use of a Sephadex LH-20 column with methanol as an eluting solvent; the protected dimer (XIV-A or -B) was obtained from the faster eluting fraction, and the monomer (XIII-A or -B) from the slower fraction (Fig. 4). The weight ratio of both components calculated

10) In the previous papers, we reported that the pure protected GS,[11] diZ-substituted [Lys$^{2,2'}$]-GS or [Dba$^{2,2'}$]-GS[12] was obtained easily by the fractional crystallization from the mixture of the protected cyclic decapeptide and pentapeptide because of the great difference between their solubilities.

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

12) M. Waki, O. Abe, R. Okawa, T. Kato, S. Makisumi and N. Izumiya, *ibid.*, **40**, 2904 (1967).

Table 1. Ratio of protected monomer and dimer after cyclization of linear pentapeptide active esters

p-Nitrophenyl ester of[a]	Ratio of compounds in product[b] Z-cyclic monomer	diZ-cyclic dimer
H-Val-Orn(Z)-Leu-D-Phe-Pro-OH[11]	32	68
-Gly-[3]	0	100
-D-Val-	17	83
-D-Leu-	15	85

a) After the first compound listed, only variation of the residue will be shown.
b) The concentrations of linear pentapeptide *p*-nitrophenyl esters in pyridine were ~3×10^{-3} M.

are shown in Table 1 with the previous results related to the present paper.

To establish the structure of the protected dimer containing D-leucine derived from the linear pentapeptide active ester, a linear decapeptide active ester was cyclized to yield a benzyloxycarbonyl substituted cyclic decapeptide as shown in Fig. 5. Then the properties of the obtained crystals (XIV-B in Fig. 5) agreed well with that of the protected dimer (XIV-B in Fig. 4).

The hydrogenolysis of each of the protected peptides in the presence of an equivalent hydrogen chloride yielded a crystalline hydrochloride of the required cyclic peptide (XV-A, -B, XVI-A or -B). The antibacterial activities of these peptides toward several microorganisms were further examined (Table 2). It was found that the two cyclosemigramicidin S analogs (XV-A and -B) exhibited no antibacterial activities as same as cyclosemigramicidin S itself[11] and its analogs replaced by glycine[13] or sarcosine[14] at 5-position

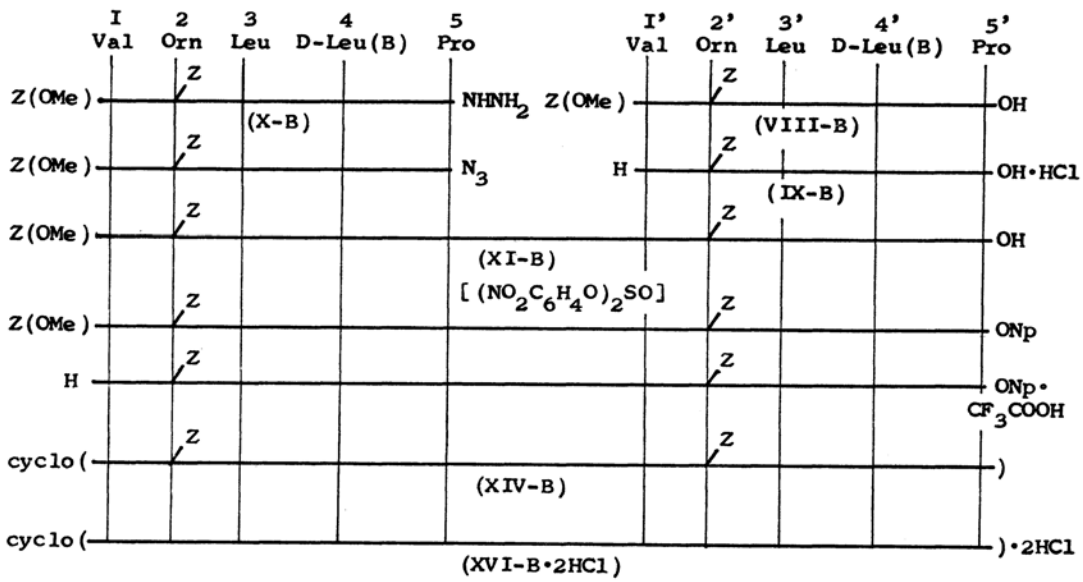

Fig. 5. Cyclization of linear decapeptide active ester.

Table 2. Inhibitory activity of the compounds on microorganisms
Minimum inhibitory concentration, μg/ml[a]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*
GS	>100	>100	5	5	>100
[D-Val^4]-semiGS (XV-A)	>100	>100	>100	>100	>100
[D-Leu^4]-semiGS (XV-B)	>100	>100	>100	>100	>100
[D-Val$^{4,4'}$]-GS (XVI-A)	>100	>100	10	10	>100
[D-Leu$^{4,4'}$]-GS (XVI-B)	>100	>100	10	5	>100

a) The same results were obtained with an usual bouillon agar medium (pH 7.0) and a Stephenson-Whetham's synthetic medium.

13) H. Aoyagi, M. Kondo, T. Kato, S. Makisumi and N. Izumiya, *ibid.*, **40**, 1685 (1967).
14) H. Aoyagi and N. Izumiya, *ibid.*, **39**, 1747 (1966).

of the molecule. On the other hand, 4,4'-D-valine- and D-leucine-gramicidin S were as active as the natural gramicidin S against several microorganisms. The results indicated that the aromatic side chains of D-phenylalanine residues of 4- and 4'-position can be replaced by the bulky aliphatic side chains such as isopropyl and isobutyl groups without any influence in the activity.

Experimental

All melting points are uncorrected.

Z-D-Val-Pro-OEt (I-A). To a solution of benzyloxycarbonyl-D-valine[8] (5.02 g; 20 mmol) and L-proline ethyl ester *p*-toluenesulfonate[15] (6.30 g; 20 mmol) in chloroform (40 m*l*), there were stirred triethylamine (2.80 m*l*) and dicyclohexylcarbodiimide[16] (4.12 g) at 0°C. After it had been allowed to stand overnight at 0°C, the mixture was evaporated *in vacuo*, and ethyl acetate was added to the residue. After the dicyclohexylurea was filtered off, the filtrate was washed successively with 4% sodium bicarbonate, 3% hydrochloric acid and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo*; yield of oil, 3.99 g (58%); R_f, 0.95.[17]

Z-D-Leu-Pro-OEt (I-B). (a) This was obtained from oily benzyloxycarbonyl-D-leucine (6.92 g; 26 mmol) and L-proline ethyl ester *p*-toluenesulfonate (8.20 g; 26 mmol) as described above; yield of oil, 7.48 g (74%); R_f, 0.97.[17] (b) To a solution of benzyloxycarbonyl-D-leucine dicyclohexylammonium salt[18] (4.46 g; 10 mmol) and L-proline ethyl ester *p*-toluenesulfonate (3.15 g; 10 mmol) in chloroform (20 m*l*), there were stirred dicyclohexylcarbodiimide (2.02 g) at 0°C. After it had been allowed to stand overnight at 0°C, the mixture was treated as described above; yield, 3.06 g (78%); R_f, 0.97.[17]

H-D-Val-Pro-OEt·HCl (II-A). A solution of I-A (3.00 g; 8 mmol) dissolved in 0.48N methanolic hydrogen chloride (17.5m*l*) was subjected to hydrogenolysis in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness *in vacuo;* yield of oil, 2.20g (99%); R_f, 0.75[17].

H-D-Leu-Pro-OEt·HCl (II-B). This was obtained from I-B (4.70g; 12mmol) as described above; yield of oil, 3.44g (98%); R_f, 0.65.[17]

Z-Leu-D-Val-Pro-OEt (III-A). Benzyloxycarbonyl-L-leucine dicyclohexylammonium salt[19] (3.57g; 8mmol) was coupled with II-A (2.23g; 8mmol) as described for the preparation of I-B (b). Yield of oil, 3.60g (92%); R_f, 0.92.[17]

Z-Leu-D-Leu-Pro-OEt (III-B). This compound was obtained from benzyloxycarbonyl-L-leucine dicyclohexylammonium salt (2.55g; 5.7mmol) and II-B (1.67g; 5.7mmol) as described above. The crude product was recrystallized from ethyl acetate - petroleum ether; yield, 2.02g (71%); mp 135°C; $[\alpha]_D^{22}$ −21.8° (*c* 1, DMF); R_f, 0.97.[17]

Found: C, 64.12; H, 8.30; N, 8.50%. Calcd for $C_{27}H_{41}O_6N_3$: C, 64.39; H, 8.21; N, 8.34%.

H-Leu-D-Val-Pro-OEt·HCl (IV-A). This was obtained from III-A (4.90g; 10mmol) as described for the preparation of II-A; yield of oil, 3.85g (98%); R_f, 0.70.[17]

H-Leu-D-Leu-Pro-OEt·HCl (IV-B). This was obtained from III-B (3.02g; 6 mmol); yield of oil, 2.44 g (100%); R_f, 0.67.[17]

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Val-Pro-OEt (V-A). To a Z(OMe)-Val-Orn(δ-Z)-$NHNH_2$[20] (1.09g; 2mmol) dissolved in a mixture of acetic acid (20m*l*), dimethylformamide (10 m*l*) and 1N hydrochloric acid (4.4 m*l*) was added sodium nitrite (152mg) in water (1m*l*). After 6 min cold water (100m*l*) was added to the solution. The azide which precipitated was collected by filtration and washed with 4% sodium bicarbonate and water, and then dried *in vacuo*. The azide was added to a solution of IV-A (0.77 g; 2mmol) and triethylamine (0.28m*l*) in dimethylformamide (20 m*l*). The mixture was stirred for 3 days at 0°C and evaporated *in vacuo*. The precipitate which formed upon the addition of water was collected, washed successively with 4% sodium bicarbonate, 10% citric acid and water. It was recrystallized from methanol-dioxane-ether; yield, 1.17 g (69%); mp 180—183°C; $[\alpha]_D^{22}$ −10.8° (*c* 1, DMF); R_f, 0.92.[17]

Found: C, 62.49; H, 7.64; N, 9.92%. Calcd for $C_{45}H_{66}O_{11}N_6$: C, 62.33; H, 7.69; N, 9.69%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Leu-Pro-OEt (V-B). The azide derived from Z(OMe)-Val-Orn(δ-Z)-$NHNH_2$ (3.26 g; 6 mmol) was condensed with IV-B (2.44 g; 6 mmol) and triethylamine (0.84 m*l*) as described above. The crude product was recrystallized from methanol - dioxane - ether - petroleum ether; yield, 4.04 g (77%); mp 142—144°C; $[\alpha]_D^{22}$ −11.0° (*c* 1, DMF); R_f, 0.88.[17]

Found: C, 62.46; H, 7.78; N, 9.76%. Calcd for $C_{46}H_{68}O_{11}N_6$: C, 62.70; H, 7.78; N, 9.53%.

Z-Leu-D-Val-Pro-OH (VI-A). To a solution of III-A (7.20 g; 14.7 mmol) in a mixture of methanol (40 m*l*) and dioxane (40 m*l*), 2N sodium hydroxide (11 m*l*) was added, and the solution was allowed to stand for 5 hr at 30°C. After the addition of 1N hydrochloric acid (23 m*l*), the solution was evaporated *in vacuo*. The oily product extracted with ethyl acetate (50 m*l*) and dried over sodium sulfate. The filtrate was evaporated *in vacuo*; yield of oil, 6.5 g (96%); R_f, 0.77.[17]

H-Leu-D-Val-Pro-OH (VII-A). A solution of VI-A (3.35 g; 7.3 mmol) in a mixture of acetic acid (15 m*l*), methanol (12 m*l*) and water (3 m*l*) was subjected to hydrogenolysis in the presence of palladium black. The

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

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

17) The R_f values refer to the thin-layer chromatography with Merck silica gel G and 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 them with ninhydrin, and those with blocked amino groups, by spraying them with 47% hydrobromic acid, and then with ninhydrin.

18) This compound was obtained according to the procedure for the L-isomer; mp 151—152°C, $[\alpha]_D^{22}$ +7.7° (*c* 2, MeOH). Values for the L-isomer;[19] mp 151.5—152°C; $[\alpha]_D^{20}$ −7.86° (MeOH).

19) E. Klieger, E. Schröder and H. Gibian, *Ann.*, **640**, 157 (1966).

20) T. Kato, M. Kondo, M. Ohno and N. Izumiya, This Bulletin, **38**, 1202 (1965).

filtrate was evaporated to dryness *in vacuo*; yield of oil, 2.26 g (95%); R_f, 0.72.[17]

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Val-Pro-OH (VIII-A). The azide derived from Z(OMe)-Val-Orn(δ-Z)-$NHNH_2$ (3.80 g; 7 mmol) was added to a solution of VII-A (2.35 g; 7 mmol) and triethylamine (0.98 m*l*) in dimethylformamide (50 m*l*). The mixture was stirred for 3 days at 0°C and evaporated *in vacuo*. The residue was triturated with 10% citric acid, and the precipitate was collected by filtration and washed with water. It was recrystallized from methanol - ether - petroleum ether; yield, 4.02 g (65%); mp 173—175°C; $[\alpha]_D^{22}$ −12.2° (*c* 1, DMF); R_f, 0.82.[17]

Found: C, 60.41; H, 7.30; N, 10.04%. Calcd for $C_{43}H_{62}O_{11}N_6 \cdot H_2O$: C, 60.18; H, 7.53; N, 9.84%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Leu-Pro-OH (VIII-B). To a solution of V-B (4.41 g; 5 mmol) in a mixture of methanol (20 m*l*) and dioxane (20 m*l*), 2 N sodium hydroxide (5 m*l*) was added, and the solution was allowed to stand for 4 hr at 30°C. After the addition of 10% citric acid (75 m*l*) under cooling, the solution was concentrated *in vacuo* at a low temperature, and the precipitate was collected by filtration. This was recrystallized from methanol - ether - petroleum ether; yield, 3.34 g (78%); mp 149—151°C; $[\alpha]_D^{22}$ −20.0° (*c* 1, DMF); R_f, 0.75.[17]

Found: C, 61.53; H, 7.70; N, 9.63%. Calcd for $C_{44}H_{64}O_{11}N_6 \cdot 1/2H_2O$: C, 61.30; H, 7.60; N, 9.75%.

H-Val-Orn(δ-Z)-Leu-D-Leu-Pro-OH·HCl (IX-B). To a solution of VIII-B (3.42 g; 4 mmol) in dioxane (40 m*l*), 4.0 N hydrogen chloride in dioxane (36 m*l*) was added at room temperature. After 2 hr, the solution was evaporated, and the residue was triturated with ether. The crystals were collected with the aid of ether; yield, 2.94 g (98%); mp 163—165°C; $[\alpha]_D^{18}$ −14.0° (*c* 0.5, DMF); R_f, 0.72.[17]

Found: C, 57.63; H, 7.84; N, 11.56%. Calcd for $C_{35}H_{57}O_8N_6Cl$: C, 57.95; H, 7.92; N, 11.59%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Leu-Pro-$NHNH_2$ (X-B). To a solution of V-B (3.52 g; 4 mmol) in dimethylformamide (15 m*l*), hydrazine hydrate (4 m*l*) was added and the solution was allowed to stand for 7 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, 3.01 g (87%); mp 147—149°C; $[\alpha]_D^{18}$ −20.4° (*c* 1, DMF); R_f, 0.78.[17]

Found: C, 60.50; H, 7.62; N, 12.50%. Calcd for $C_{44}H_{60}O_{10}N_8 \cdot 1/2H_2O$: C, 60.24; H, 7.71; N, 12.79%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Leu-Pro-Val-Orn-(δ-Z)-Leu-D-Leu-Pro-OH (XI-B). The azide derived from X-B (1.04 g; 1.2 mmol) was added to a solution of IX-B (0.87 g; 1.2 mmol) and triethylamine (0.33 m*l*) in dimethylformamide (20 m*l*). The crude product was obtained as described for the preparation of VIII-A and recrystallized from ethyl acetate - petroleum ether; yield, 1.54 g (67%); mp 148—151°C; $[\alpha]_D^{18}$ −34.0° (*c* 0.5, DMF); R_f, 0.80.[17]

Found: C, 61.24; H, 7.89; N, 10.82%. Calcd for $C_{79}H_{118}O_{18}N_{12} \cdot H_2O$: C, 61.54; H, 7.85; N, 10.90%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Val-Pro-ONp (XII-A). To a solution of VIII-A (2.04 g; 2.3 mmol) in pyridine (10 m*l*), di-*p*-nitrophenyl sulfite[21] (2.98 g; 9.2 mmol) was added. After the mixture had been allowed to stand overnight at room temperature, it was evaporated *in vacuo*. The residual solid was collected with the aid of a mixture of ether and petroleum ether; yield, 1.73 g. The *p*-nitrophenyl ester content was estimated to be 94% by measuring the optical density at 412 mμ.[22]

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Leu-Pro-ONp (XII-B). The compound VIII-B (0.853 g; 1 mmol) was converted to XII-B (0.890 g) as described above in which *p*-nitrophenyl ester content was estimated to be 111%.

Mixture of *cyclo*-(Val-Orn(δ-Z)-Leu-D-Val-Pro-) (XIII-A) and *cyclo*-(Val-Orn(δ-Z)-Leu-D-Val-Pro-)$_2$ (XIV-A). The compound XII-A (1.73 g) was treated with anisole (2 m*l*) and trifluoroacetic acid (12 m*l*) at 0°C. After 20 min, the solution was evaporated, and the solid was collected with the aid of a mixture of ether and petroleum ether. The pentapeptide *p*-nitrophenyl ester trifluoroacetate thus obtained was dissolved in dimethylformamide (20 m*l*) and acetic acid (1 m*l*). The solution was added dropwise into pyridine (600 m*l*) at 55—60°C during 5 hr and the stirring was continued for additional 2 hr. The solution was evaporated, and the residue was dissolved in a mixture of methanol (25 m*l*), dioxane (25 m*l*) and water (10 m*l*). The solution was treated with columns (1.8×15 cm) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (150 m*l*), and the combined effluent was evaporated to dryness. The residual product was collected by filtration with the aid of water and dissolved in 4 m*l* of methanol (solution A). A drop of solution A was subjected to hydrogenolysis, and the electophoresis of the hydrogenated material showed two spots.[23] Half of solution A (2 m*l*) was applied to a column (2.7×52 cm) with Sephadex LH-20, and the development continued with methanol. Elution was carried out at room temperature, at a flow rate of 30 m*l* per hr; a 3 m*l* fraction was collected. The peptide content in the fractions was determined as described previously.[11] The first peak appeared from tube numter 47 to 58, and the second, from 62 to 68. The fractions 59 to 61 contained both components. The other half of solution A was chromatographed; the first peak, 43 to 61 and the second, 64 to 72.

***cyclo*-(Val-Orn(δ-Z)-Leu-D-Val-Pro-) (XIII-A).** The fractions 62—68 and 64—72 were combined and evaporated *in vacuo*, and the product was collected by filtration with the aid of water. The crude product weighed 86 mg. It was recrystallized from methanol - ether - petroleum ether; yield, 68 mg (4.4% from VIII-A); mp 161—163°C; $[\alpha]_D^{22}$ −74.7° (*c* 0.3, DMF); R_f, 0.95.[17]

Found: C, 60.92; H, 7.97; N, 12.43%; mol wt, 704.[24] Calcd for $C_{34}H_{52}O_7N_6 \cdot H_2O$: C, 60.51; H, 8.08; N, 12.46%; mol wt, 675.

***cyclo*-(Val-Orn(δ-Z)-Leu-D-Val-Pro-)$_2$ (XIV-A).** The fractions 47—58 and 43—61 were treated as described above; yield of a crude product, 394 mg. Recrystallization from methanol - ether - petroleum ether gave 346 mg (22% from VIII-A); mp 212—214°C; $[\alpha]_D^{22}$ −137° (*c* 0.3, DMF); R_f, 0.93.[17]

Found: C, 60.38; H, 7.88; N, 12.36%; mol wt,

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

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

23) Each of the hydrogenated materials was designated as XVII-A in this case or XVII-B in the case containing D-leucine, and the results of carboxymethylcellulose column chromatography are shown in Fig. 7.

1372.[24] Calcd for $C_{68}H_{104}O_{14}N_{12}\cdot 2H_2O$: C, 60.51; H, 8.08; N, 12.46%; mol wt. 1350.

Mixture of *cyclo*-(Val-Orn(δ-Z)-Leu-D-Leu-Pro-) (XIII-B) and *cyclo*-(Val-Orn(δ-Z)-Leu-D-Leu-Pro-)$_2$ (XIV-B). Pentapeptide *p*-nitrophenyl ester trifluoroacetate obtained from XII-B (0.887 g) was added to pyridine (200 m*l*) as described for the preparation of a mixture of XIII-A and XIV-A. A crude product was dissolved in 2 m*l* of methanol (solution B). A quarter of solution B (0.5 m*l*) was applied to a column (2.7×52 cm) with Sephadex LH-20 as described. The first peak appeared from tube number 35 to 49, and the second, 53 to 60. The fractions from 50 to 52 contained both components. Other three quarters of solution B were treated similarly.

***cyclo*-(Val-Orn(δ-Z)-Leu-D-Leu-Pro-) (XIII-B).** The four fractions which were eluted slower such as from 53 to 60 were pooled and treated as described for the preparation of XIII-A; yield, 59 mg. It was recrystallized from ethyl acetate-petroleum ether; Yield, 53 mg (7.6% from VIII-B); mp 167—170°C; $[\alpha]_D^{18}$ −30.6° (*c* 0.3, DMF); R_f, 0.96.[17]

Found: C, 60.36; H, 7.97; N, 11.85%; mol wt, 680.[24] Calcd for $C_{35}H_{54}O_7N_6\cdot 3/2H_2O$: C, 60.23; H, 8.23; N, 12.03%; mol wt, 698.

***cyclo*-(Val-Orn(δ-Z)-Leu-D-Leu-Pro)$_2$ (XIV-B).** *(a) From VIII-B.* The four fractions which were eluted faster such as from 35 to 49 were treated as described above; yield, 312 mg. It was recrystallized from ethyl acetate-petroleum ether; yield, 296 mg (43% from VIII-B); mp 205—208°C; $[\alpha]_D^{18}$ −108° (*c* 0.5, DMF); R_f, 0.91.[17]

Found: C, 60.67; H, 8.32; N, 12.34%; mol wt, 1343.[24] Calcd for $C_{70}H_{108}O_{14}N_{12}\cdot 2H_2O$: C, 61.02; H, 8.19; N, 12.20%; mol wt, 1378.

(b) From XI-B. The compound XI-B (0.308 g; 0.2 mmol) was treated with di-*p*-nitrophenyl sulfite (0.65 g) in pyridine (2 m*l*), and the acyldecapeptide *p*-nitrophenyl ester (0.33 g) was obtained as amorphous powder; its *p*-nitrophenyl ester content, 107%. The product was treated with trifluoroacetic acid (2 m*l*), and the decapeptide *p*-nitrophenyl ester trifluoroacetate obtained was added to pyridine (100 m*l*). The effluent from the Dowex 1 and 50 columns was evaporated, and the crystals were collected with the aid of water; yield, 138 mg. This was recrystallized from ethyl acetate-petroleum ether; yield, 123 mg (45% from XI-B); mp 207-210°C; $[\alpha]_D^{18}$ −110° (*c* 0.5, DMF); R_f, 0.91.[17] The mixed mp with XIV-B from VIII-B was not depressed.

***cyclo*-(Val-Orn-Leu-D-Val-Pro-)·HCl (XV-A·HCl).** A solution of XIII-A (20 mg; 0.03 mmol) dissolved in 0.01 N methanolic hydrogen chloride (3.37 m*l*), was subjected to hydrogenolysis in the presence of palladium black. The solution, after being filtered from the catalyst, was evaporated to dryness *in vacuo*. The crystals were collected with the aid of ether; yield of the air-dried product, 16.2 mg (92%); mp 251—254°C (decomp.); R_f, 0.80[17] and 0.97;[25] amino acid ratios in acid hydrolysate, $Val_{2.1}Orn_{1.0}Leu_{1.0}Pro_{1.1}$.

Found: C, 51.72; H, 8.64; N, 13.47%. Calcd for $C_{26}H_{47}O_5N_6Cl\cdot 2.5H_2O$: C, 52.08; H, 8.49; N, 13.56%.

***cyclo*-(Val-Orn-Leu-D-Leu-Pro-)·HCl (XV-B·HCl).** The compound XIII-B (0.03 mmol) was converted to XV-B as described above; yield of the air-dried product, 90%; mp 240—243°C (decomp.); R_f, 0.75[17] and 0.97;[25] amino acid ratios in acid hydrolysate, $Val_{0.9}Orn_{0.9}Leu_{2.0}Pro_{0.9}$.

Found: C, 51.56; H, 8.93; N, 13.34%. Calcd for $C_{27}H_{49}O_5N_6Cl\cdot 3H_2O$; C, 51.70; H, 8.80; N, 13.40%.

***cyclo*-(Val-Orn-Leu-D-Val-Pro-)$_2$·2HCl (XVI-A·2HCl).** The compound XIV-A (0.05 mmol) was treated

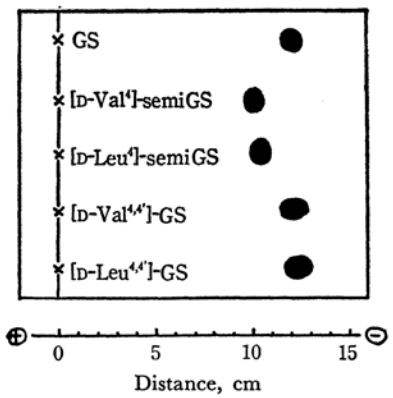

Fig. 6. Paper electrophoresis of the compounds.

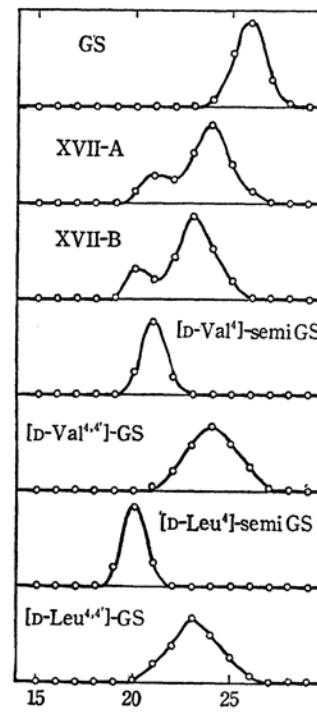

Fig. 7. Carboxymethylcellulose column chromatography of the compounds.
XVII-A or XVII-B, hydrogenated material after cyclization of pentapeptide active ester containing D-Val or D-Leu.

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

25) 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.

as described for the preparation of XV-A; yield of the air-dried product, 96%; mp 275—277°C (decomp.) $[\alpha]_D^{21}$ −133° (*c* 0.3, DMF; R_f, 0.75[17] and 0.97[25]); amino acid ratios in acid hydrolysate, $Val_{1.8}Orn_{0.9}Leu_{1.0}Pro_{0.9}$.

Found: C, 51.78; H, 8.68; N, 13.91%. Calcd for $C_{52}H_{94}O_{10}N_{12}Cl_2 \cdot 5H_2O$: C, 52.08; H, 8.49; N, 13.56%.

The air-dried product lost 8.9% of its weight after drying for 3 hr at 80°C and 2 mmHg. Calcd for $6H_2O$: 8.8%.

***cyclo*-(Val-Orn-Leu-D-Leu-Pro-)$_2$·2HCl (XVI-B·2HCl).** The compound XIV-B (0.04 mmol) was treated as described for the preparation of XV-A; yield of the air-dried product, 86%; mp 256—260°C (decomp.); $[\alpha]_D^{25}$ −100° (*c* 0.3, DMF); R_f, 0.73[17] and 0.95;[25] amino acid ratios in acid hydrolysate, $Val_{0.9}Orn_{1.0}Leu_{2.0}Pro_{0.9}$.

Found: C, 51.72; H, 8.95; N, 13.30%. Calcd for $C_{54}H_{98}O_{10}N_{12}Cl_2 \cdot 6H_2O$: C, 51.70; H, 8.80; N, 13.40%.

The air-dried product lost 6.9% of its weight after dried for 3 hr at 80°C and 2 mmHg. Calcd for $5H_2O$; 7.2%.

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 2.5 hr at 600 v/30 cm. Figure 6 shows that XVI-A and XVI-B migrate toward the cathode faster than XV-A and XV-B, and that mobilities of XVI-A and XVI-B were comparable with that of gramicidin S. In CMC column chromatography, 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 CMC (Eastman No. 7796), and development was continued with the same solvent. Two-m*l* fractions were collected at flow rate of 20 m*l* per hr. The peptide content in the fractions was determined by the ninhydrin method, and the results are shown in Fig. 7.

Microbiological Assays.[26] The minimum amount of the compounds necessary for the complete inhibition of growth was determined by a dilution method using a bouillon agar medium and a synthetic medium. As is shown in Table 1, [D-Val$^{4,4'}$]- and [D-Leu$^{4,4'}$]-gramicidin S were found to be as active as natural gramicidin S against *Staph. aureus* and *B. subtilis*. Whereas cyclo-semigramicidin analogs, XV-A and -B, exhibited no antibacterial activity against the microorganisms tested.

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