BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 44, 143—148 (1971)

Studies of Peptide Antibiotics. XXIII. Syntheses of Linear Decapeptide Analogs with Gramicidin S Sequence

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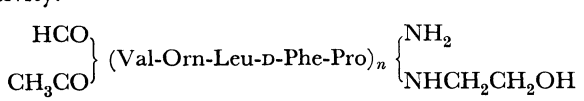
(Received June 13, 1970)

Six linear decapeptides having the same sequence of amino acids of gramicidin S, wherein the *N*-terminal amino acid is acylated with formyl or acetyl group, the *C*-terminal amino acid is in a form of its amide or ethanolamide and the *N*,*C*-terminal ones are free, were synthesized to obtain a more reliable picture of the cyclic structure to the antibacterial activity of gramicidin S. All these linear decapeptide analogs were found to be active to some extent against several microorganisms.

As a part of the program to synthesize linear analogs of gramicidin S, the syntheses of decapeptide analogs (II)[1] were undertaken. These compounds differ from gramicidin S in two ways: (a) they are non-cyclic and (b) both their terminal residues are blocked with an acyl and an amide group found in nature. Antibacterial assays of these analogs may clarify the influence of the cyclic structure of gramicidin S to its antibacterial activity.



I ($n=1$), II ($n=2$)

H-(Val-Orn-Leu-D-Phe-Pro)$_2$-OH

III

It has been reported[2] that pentapeptide analogs (I) of this type were found to possess no antibacterial activity, although Erlanger and Goode[3] reported that *N*,*C*-terminal free linear decapeptide (III) having the gramicidin S sequence was found to possess weaker activity than gramicidin S itself. In order to obtain a more reliable picture of the cyclic structure to the antibacterial activity of gramicidin S, the activities of various synthetic linear peptides were compared with that of natural peptide. The present paper describes the syntheses and antibacterial properties of several acyl decapeptide amide analogs and the *N*,*C*-terminal free decapeptide which is used as a reference compound.

The steps involved in the syntheses of the acyl decapeptide amide analogs are shown in Fig. 1.[4] The reaction schemes are similar to those of the pentapeptide analogs[2] in general. For removal of the BOC

1) Amino acid symbols except D-Phe denote L configuration.

2) S. Makisumi, M. Waki, and N. Izumiya, *Mem. Fac. Sci., Kyushu Univ., Ser. C.* in press.

3) B. F. Erlanger and L. Goode, *Nature*, **174**, 840 (1954).

4) Abbreviations used: BOC, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; DCC, *N*,*N'*-dicyclohexylcarbodiimide; HOSu, *N*-hydroxysuccinimide; CM, carboxymethyl.

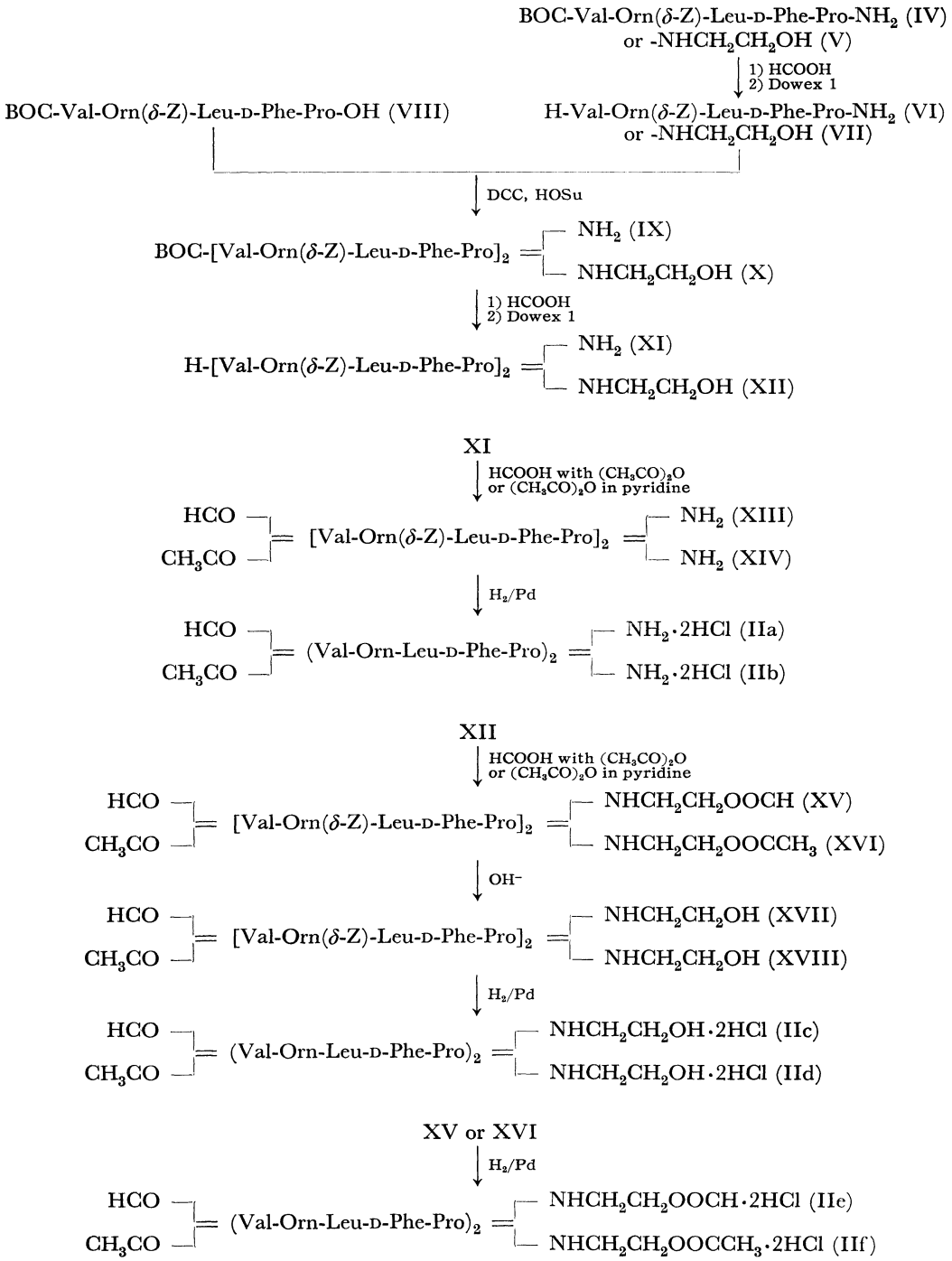


Fig. 1. Synthetic schemes of the acyl decapeptide amides.

group, 98% formic acid was used as reported by Halpern and Nitecki.[5] This method is good enough to prevent the partial deblocking of the Z groups in a peptide. Removal of the BOC group from BOC-pentapeptide amides (IV and V) by treatment with 98% formic acid and subsequent neutralization with Dowex 1 in methanol yielded free base of the pentapeptide amides (VI and VIII), respectively. Further purification of the products with column chromatography on CM-cellulose used in a previous work[2] was not needed in these cases, since the Z group remained intact.

Condensation of BOC-pentapeptide acid (VIII)[6] with VI or VII by the DCC-HOSu procedure[7] gave BOC-decapeptide amide derivative (IX or X) in a good yield. Purification of the crude product was performed by filtration over a column of Dowex 50 (H^+ form) and a Dowex 1 (OH^- form) in methanol. The method, reported by Sarges and Witkop,[8] was

5) B. Halpern and D. E. Nitecki, *Tetrahedron Lett.*, **1967**, 3031.

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

7) F. Weygand, D. Hoffmann, and E. Wünsch, *Z. Naturforsch.*, **21b**, 426 (1966); J. E. Zimmerman and G. W. Anderson, *J. Amer. Chem. Soc.*, **89**, 7151 (1967).

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

very useful for removing the contaminated amine and acid components in the fully protected peptide product obtained by coupling of the two components. Removal of the BOC group from IX or X by treatment with formic acid yielded a crystalline product of *N*-terminal free decapeptide amide derivative (XI or XII).

Acylation of the decapeptide amide (XI) with formic acid - acetic anhydride[9] or with acetic anhydride in pyridine[10] afforded formyl or acetyl decapeptide amide derivative (XIII or XIV). Removal of the Z groups from XIII or XIV by catalytic hydrogenation in the presence of hydrogen chloride in methanol provided formyl or acetyl decapeptide amide as dihydrochloride (IIa or IIb). On the other hand, acylation of decapeptide ethanolamide (XII) by the methods described above gave *N,O*-diacyl decapeptide derivative (XV or XVI) which was then converted to *N*-monoacyl derivative (XVII or XVIII) by saponification with alkali. Hydrogenolyses of XVII, XVIII, XV, and XVI in the presence of hydrogen chloride afforded *N*-acyl decapeptide ethanolamide analogs (IIc and IId) and *N,O*-diacyl decapeptide ethanolamide analogs (IIe and IIf) as hydrochloride, respectively. The final products IIa, IIb, IIc, IId, IIe, and IIf were shown to be practically homogeneous by thin-layer chromatography and paper electrophoresis. However, the formyl analogs (IIa, IIc, and IIe) were found to be contaminated with a minute amount of the deformylated peptides by paper electrophoresis as shown in Fig. 3. Attempts to remove these impurities have so far not been successful. It seems likely that the formyl group in these peptides is appreciably labile.

Erlanger *et al.* synthesized the *N,C*-terminal free decapeptide (III) by treatment of Z-[Val-Orn(δ-Tos)-Leu-D-Phe-Pro]$_2$-OH with sodium in liquid ammonia.[11] In this study, compound III was prepared *via* a different route shown in Fig. 2. Condensation of VIII with the formate (XIX) of neutral pentapeptide in the presence of two equivalents of triethylamine by the DCC-HOSu procedure gave BOC-decapeptide acid derivative (XX). Removal of BOC group from XX by an exposure to formic acid and subsequent crystallization in the presence of hydrogen chloride yielded *N,C*-terminal free decapeptide derivative as monohydrochloride (XXI). Catalytic hydrogenation of XXI gave III which was crystallized as trihydrochloride.

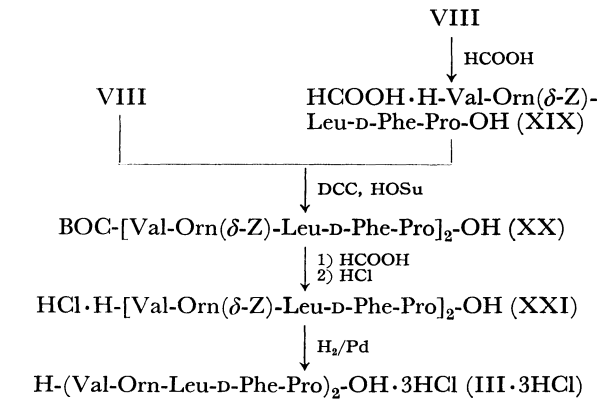


Fig. 2. Synthetic scheme of the *N,C*-terminal free decapeptide analog.

Comparison of the antibacterial activity of gramicidin S with that of the synthetic peptides showed that all of the linear decapeptide analogs have antibacterial activity against several microorganisms, though the activity is not so high compared with natural peptide (Table 1). It is of interest to note that the *N,C*-terminal free decapeptide (III) showed the same high activity as formyl-decapeptide amide (IIa) on Gram positive microorganisms, *S. aureus* and *B. subtilis*. The results we obtained have led us to conclude that even though the cyclic structure of gramicidin S is not essential for antibacterial activity, the specific activity of this compound is related to its unique cyclic structure.

Experimental

Melting points are uncorrected. The purity of the peptide intermediates after each synthetic step was ascertained by thin layer chromatography on silica gel plate. R_f^A refers to the solvent system chloroform-benzene-methanol (6 : 3 : 1, *v/v*) and R_f^B to the system chloroform-methanol (5 : 1, *v/v*). Homogeneity of the final product was confirmed by thin layer chromatography and by electrophoresis on paper. The solvent systems used are abbreviated as follows: C, ethyl acetate-pyridine-acetic acid-water (60 : 20 : 6 : 11, *v/v*); D, *n*-butanol-acetic acid-water (4 : 1 : 4, *v/v*); E, *sec*-buthanol-formic acid-

Table 1. Inhibitory activity of linear decapeptide analogs on microorganisms

Minimum inhibitory concentration, μg/ml

	Staphylococcus aureus FDA 209P	*Bacillus subtilis* ATCC 6633	*Escherichia coli* IAM 1253	*Proteus vulgalis* IAM 1052	*Shigella sonnei* 191—66	*Candida albicans* IAM 4888
GS·2HCl	1.56	0.79	25	>100	25	12.5
IIa	25	3.13	100	>100	100	50
IIb	50	6.25	100	>100	100	50
IIc	25	6.25	100	>100	>100	50
IId	50	12.5	100	>100	>100	100
IIe	50	6.25	100	>100	>100	50
IIf	25	12.5	100	>100	>100	100
III·3HCl	25	3.13	25	>100	50	50

9) J. C. Sheehan and D. D. H. Yang, *ibid.*, **80**, 1154 (1958).
10) H. Neumann, Y. Levin, A. Berger, and E. Katchalski, *Biochem. J.*, **73**, 33 (1959).
11) B. F. Erlanger, H. Sachs, and E. Brand, *J. Amer. Chem. Soc.*, **76**, 1806 (1954).

water (4 : 1 : 1, *v/v*). Gramicidin S was taken as the referene compound for the linear analogs and R_f^{GS} refers to the ratio of the distance which substances traveled to that which gramicidin S traveled from the origin on the same chromatogram. Paper electrophoresis was carried out on Toyo Roshi No. 50 with formic acid-acetic acid-methanol-water (1 : 3 : 6 : 10, *v/v*) buffer of pH 1.8. Electrophoretic mobilities are recorded as R^{His}, the ratio of the distance the compounds moved to that which a standard histidine spot moved on the same electrophoreogram. Compounds possessing free amino group were detected by spraying with ninhydrin and those with blocked amino groups by deblocking with 47% hydrobromic acid followed by staining with ninhydrin. Prior to microanalysis, desiccated samples were opened in air to a constant weight. Air dried samples were also subjected to measurement of specific rotation and $[\alpha]_D$ refers to 1% solution in methanol at 23°C unless otherwise noted.

The following compounds were prepared as described previously.[2] BOC-Val-Orn(δ-Z)-Leu-D-Phe-Pro-NH_2 (IV), BOC-Val-Orn(δ-Z)-Leu-D-Phe-Pro-$NHCH_2CH_2OH$ (V) and BOC-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH (VIII).

H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-NH_2 (VI). BOC-pentapeptide amide (IV, 823 mg, 1 mmol) was dissolved in 25 m*l* of 98% formic acid and the solution kept at room temperature for 3 hr. The reagent was then removed *in vacuo* (bath temperature <30°C)and the residue was triturated with ether. The crude solid (formate) was dissolved in 10 m*l* of methanol and the solution was filtered over a column (1.2×8 cm) of Dowex 1 (OH^- form preserved in methanol). The effluent and washings were combined and the solution was evaporated *in vacuo*. The resulting residue was crystallized by addition of ether. The product was collected by filtration, yield 671 mg (93%), mp 118—122°C, $[\alpha]_D$ −68.1°, R_f^A 0.27, R_f^B 0.62.

Found: C, 62.12; H, 7.84; N, 13.02%. Calcd for $C_{38}H_{55}N_7O_7 \cdot H_2O$: C, 61.69; H, 7.77; N, 13.25%.

H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-$NHCH_2CH_2OH$ (VII). Treatment of 866 mg (1 mmol) of V with 25 m*l* of 98% formic acid followed by crystallization and filtration over a column of Dowex 1 as described above gave 728 mg (95%) of peptide ethanolamide, mp 102—104°C, $[\alpha]_D$ −66.5°, R_f^A 0.29, R_f^B 0.63.

Found: C, 61.61; H, 7.99; N, 12.28%. Calcd for $C_{40}H_{59}N_7O_8 \cdot H_2O$: C, 61.28; H, 7.84; N, 12.51%.

BOC-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-NH_2 (IX). A suspension of 412 mg (0.5 mmol) of BOC-pentapeptide acid (VIII), 361 mg (0.5 mmol) of pentapeptide amide (VI) and 58 mg (0.5 mmol) of HOSu in a mixture of dioxane (5 m*l*) and dimethylformamide (4 m*l*) was cooled in an ice-salt bath. DCC (103 mg, 0.5 mmol) was added to the chilled solution and the mixture was stirred under cooling; meanwhile the suspension dissolved to form a clear solution. After it had been allowed to stand overnight at room temperature, the reaction mixture was stirred again with a few drops of water for 1 hr at 0°C. Crystals of dicyclohexylurea were removed by filtration and washed with cold dioxane. The filtrate and washings were evaporated to a small volume and the residual syrup was solidified by the addition of ice water. The solid obtained was collected by filtration, washed successively with water, 4% sodium bicarbonate, 10% citric acid and water, and dried. The product was dissolved in ethyl acetate and a small amount of insoluble dicyclohexylurea was removed. The filtrate was evaporated and the residue was crystallized by the addition of ether. Further purification was carried out as follow. The crystals were dissolved in 10 m*l* of methanol and the solution was filtered on a series of columns (1.2×8 cm, each) of Dowex 50 (H^+ form) and Dowex 1 (OH^- form) which were equilibrated with methanol. The effluent and the washings were evaporated and the residue was crystallized by the addition of ether and petroleum ether. The product was recrystallized from ethyl acetate-ether, yield 629 mg (82%), mp 203—205°C, $[\alpha]_D$ −123°, R_f^A 0.40.

Found: C, 63.19; H, 7.56; N, 11.64%. Calcd for $C_{81}H_{115}N_{13}O_{16} \cdot H_2O$: C, 62.97; H, 7.63; N, 11.79%.

BOC-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-$NHCH_2CH_2OH$ (X). By the DCC-HOSu method as described above, 823 mg (1 mmol) of VIII was allowed to couple with 766 mg (1 mmol) of pentapeptide ethanolamide (VII) in the presence of equimolar amount of DCC and HOSu. The product was purified as above to give 1399 mg (89%) of BOC-decapeptide ethanolamide, mp 127—130°C, $[\alpha]_D$ −110°, R_f^A 0.45.

Found: C, 62.45; H, 7.69; N, 11.28%. Calcd for $C_{83}H_{119}N_{13}O_{17} \cdot H_2O$: C, 62.74; H, 7.68; N, 11.46%.

H-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-NH_2 (XI). Treatment of 534 mg (0.35 mmol) of BOC-decapeptide amide (IX) with 10 m*l* of 98% formic acid followed by evaporation, crystallization and filtration on a column of Dowex 1 as described for the preparation of pentapeptide amide (VI) gave 481 mg (96%) of XI, mp 199—200°C, $[\alpha]_D$ −136°, R_f^A 0.30, R_f^B 0.66.

Found: C, 62.91; H, 7.56; N, 12.43%. Calcd for $C_{76}H_{107}N_{13}O_{14} \cdot 1.5H_2O$: C, 62.79; H, 7.63; N, 12.53%.

H-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-$NHCH_2CH_2OH$ (XII). Removal of the BOC group from 1304 mg (0.83 mmol) of BOC-decapeptide ethanolamide (X) by treatment with 98% formic acid followed by neutralization with Dowex 1 afforded 1185 mg (97%) of *N*-terminal free decapeptide ethanolamide, mp 114—116°C, $[\alpha]_D$ −123°, R_f^A 0.34, R_f^B 0.68.

Found: C, 62.52; H, 7.80; N, 11.93%. Calcd for $C_{78}H_{111}N_{13}O_{15} \cdot 1.5H_2O$: C, 62.55; H, 7.67; N, 12.16%.

HCO-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-NH_2 (XIII). To a solution of 200 mg (0.14 mmol) of decapeptide amide (XI) dissolved in 5 m*l* of 99.8% formic acid at 0°C was added 1.5 m*l* of acetic anhydride over a period of 15 min under stirring, and the mixture was kept at 0°C for 30 min at room temperature overnight. The solvent was removed *in vacuo* and the residue was triturated with ice water. The solid obtained was collected by filtration and dried. It was recrystallized from ethyl acetate-petroleum ether, yield 193 mg (95%), mp 215—216°C, $[\alpha]_D$ −145°, R_f^A 0.33.

Found: C, 62.03; H, 7.56; N, 12.05%. Calcd for $C_{77}H_{107}N_{13}O_{15} \cdot 2H_2O$: C, 62.04; H, 7.51; N, 12.21%.

CH_3CO-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-NH_2 (XIV). To a solution of 200 mg of XI in 5 m*l* of anhydrous pyridine, cooled in an ice-salt bath, 1.5 m*l* of acetic anhydride was added dropwise during a period of 10 min under stirring, and the mixture was kept in an ice-salt bath for 30 min at room temperature overnight. After the solvent had been evaporated, the oily product was solidified by the addition of ice water. The solid obtained was collected by filtration and dried. It was recrystallized from ethyl acetate-petroleum ether, yield 202 mg (98%), mp 230—232°C, $[\alpha]_D$ −133°, R_f^A 0.37.

Found: C, 62.48; H, 7.57; N, 12.00%. Calcd for $C_{78}H_{109}N_{13}O_{15} \cdot 2H_2O$: C, 62.26; H, 7.57; N, 12.10%.

HCO-(Val-Orn-Leu-D-Phe-Pro)$_2$-$NH_2 \cdot 2HCl$ (IIa). A solution of 102 mg (0.07 mmol) of formyl decapeptide amide (XIII) in 6 m*l* of methanol containing 0.7 m*l* of N/10 hydrochloric acid was hydrogenated in the presence of palladium black. After 3 hr, 0.7 m*l* of the hydrochloric acid was added and hydrogenation was continued. Completion of hydrogenolysis was confirmed by thin-layer chromatography. Palladium black was filtered off and the solution was evaporated *in vacuo*. When the residue was triturated with ether peptide was obtained as a crystalline hydrochloride. Recrystallization from methanol-ether gave 83 mg (94%), mp 247—

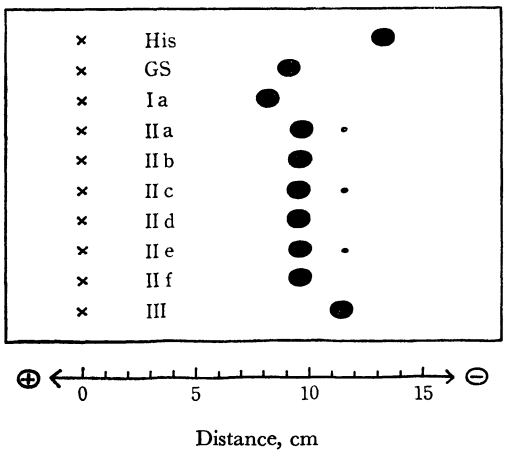


Fig. 3. Paper electrophoretic patterns of gramicidin S and its linear penta- and decapeptide analogs.
Buffer: formic acid-acetic acid-methanol-water (1 : 3 : 6 : 10, *v/v*) of pH 1.8. Duration: 600 V, 2 hr.

248°C, $[\alpha]_D$ −155°; R_f^{GS} 0.88(C), 0.90(D), 0.69(E); R^{His} 0.73.

Found: C, 53.42; H, 8.01; N, 13.32%. Calcd for $C_{61}H_{95}N_{13}O_{11}\cdot 2HCl\cdot 6H_2O$: C, 53.58; H, 8.03; N, 13.32%.

The product was contaminated with a minute amount of the deformylated derivative which was deduced from its higher electrophoretic mobility (R^{His} 0.87) (Fig. 3). Attempts to remove it have so far not been successful.

CH_3CO-(Val-Orn-Leu-D-Phe-Pro)$_2$-$NH_2\cdot 2HCl$ (IIb). When 103 mg (0.07 mmol) of XIV was hydrogenated as described above, after recrystallization from methanol - ether, 86 mg (97%) of the acetyl decapeptide amide dihydrochloride was obtained, mp 245—246°C, $[\alpha]_D$ −134°; R_f^{GS} 0.90(C), 0.80(D), 0.67(E); R^{His} 0.74.

Found: C, 53.59; H, 7.99; N, 13.16%. Calcd for $C_{62}H_{97}N_{13}O_{11}\cdot 2HCl\cdot 6H_2O$: C, 53.90; H, 8.10; N, 13.18%.

HCO-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-$NHCH_2CH_2OOCH$ (XV). Formylation of 441 mg (0.3 mmol) of the decapeptide ethanolamide (XII) was carried out in 10 m*l* of 99.8% formic acid with 3 m*l* of acetic anhydride in a similar manner to that employed for the preparation of XIII. Recrystallization from ethyl acetate-petroleum ether gave 441 mg (96%) of the diformyl decapeptide ethanolamide, mp 153—155°C, $[\alpha]_D$ −136°, R_f^A 0.38, R_f^B 0.60.

Found: C, 62.12; H, 7.44; N, 11.46%. Clacd for $C_{80}H_{111}N_{13}O_{17}\cdot H_2O$: C, 62.20; H, 7.37; N, 11.79%.

CH_3CO-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-$NHCH_2CH_2OOCCH_3$ (XVI). Acetylation of 441 mg of XII was carried out in 10 m*l* of pyridine with 3 m*l* of acetic anhydride in a similar manner to that employed for the preparation of XIV. Recrystallization from ethyl acetate-petroleum ether gave 444 mg (95%) of the diacetyl decapeptide ethanolamide, mp 179—181°C, $[\alpha]_D$ −123°, R_f^A 0.43. R_f^B 0.61.

Found: C, 62.54; H, 7.50; N, 11.45%. Calcd for $C_{82}H_{115}N_{13}O_{17}\cdot H_2O$: C, 62.62; H, 7.50; N, 11.58%.

HCO-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-$NHCH_2CH_2OH$ (XVII). A solution of 242 mg (0.16 mmol) of the diformyl derivative (XV) in 15 m*l* of methanol was saponified with 0.8 m*l* of N sodium hydroxide for 2 hr at room temperature. The reaction mixture was filtered over a column (1.8×8 cm) of Dowex 50 (H^+ form, equilibrated with methanol) and the effluent and washings were evaporated. The residue was dissolved in a mixture of methanol and ethyl acetate, and the solvent was removed. After this treatment had been repeated twice, the residue was crystallized by the addition of ether to yield 213 mg (89%), mp 148—150°C, $[\alpha]_D$ −132°, R_f^A 0.37.

Found: C, 62.47; H, 7.57; N, 11.74%. Calcd for $C_{79}H_{111}N_{13}O_{16}\cdot H_2O$: C, 62.56; H, 7.51; N, 12.00%.

CH_3CO-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-$NHCH_2CH_2OH$ (XVIII). Saponification of 233 mg (0.15 mmol) of XVI in 15 m*l* of methanol with 0.75 m*l* of N alkali as described above gave 219 mg (96%) of the monoacetyl derivative, mp 230—233°C, $[\alpha]_D$ −121°, R_f^A 0.40.

Found: C, 62.73; H, 7.57; N, 11.74%. Calcd for $C_{80}H_{113}N_{13}O_{16}\cdot H_2O$: C, 62.77; H, 7.57; N, 11.89%.

HCO-(Val-Orn-Leu-D-Phe-Pro)$_2$-$NHCH_2CH_2OH\cdot 2HCl$ (IIc). Hydrogenolysis of 105 mg (0.07 mmol) of XVII in 5 m*l* of methanol with 1.4 m*l* of N/10 hydrochloric acid as has been described for the preparation of IIa gave, after recrystallization from methanol - ether, 87 mg (96%) of the dihydrochloride of monoformyl decapeptide ethanolamide, mp 239—241°C, $[\alpha]_D$ −142°; R_f^{GS} 0.83(C), 0.82(D), 0.66(E); R^{His} 0.73.

Found: C, 53.99; H, 7.88; N, 13.02%. Calcd for $C_{63}H_{99}N_{13}O_{12}\cdot 2HCl\cdot 5H_2O$: C, 54.30; H, 8.03; N, 13.07%. This material was contaminated with a minute amount of the deformylated derivative (R^{His} 0.88).

CH_3CO-(Val-Orn-Leu-D-Phe-Pro)$_2$-$NHCH_2CH_2OH\cdot 2HCl$ (IId). By hydrogenolysis of 106 mg (0.07 mmol) of XVIII in methanol in the presence of hydrogen chloride, after recrystallization from methanol - ether, 88 mg (96%) of the dihydrochloride of monoacetyl decapeptide ethanolamide was obtained, mp 226—227°C, $[\alpha]_D$ −124°; R_f^{GS} 0.93(C), 0.76 (D), 0.58(E); R^{His} 0.72.

Found: C, 54.68; H, 8.09; N, 12.99%. Calcd for $C_{64}H_{101}N_{13}O_{12}\cdot 2HCl\cdot 5H_2O$: C, 54.61; H, 8.09; N, 12.94%.

HCO-(Val-Orn-Leu-D-Phe-Pro)$_2$-$NHCH_2CH_2OOCH\cdot 2HCl$ (IIe). Hydrogenolysis of 93 mg (0.06 mmol) of the diformyl derivative (XV) in 7 m*l* of methanol in the presence of hydrogen chloride for 5 hr gave, after recrystallization from methanolether, 76 mg (95%) of the diformyl decapeptide ethanolamide dihydrochloride, mp 234—236°C, $[\alpha]_D$ −138°; R_f^{GS} 0.91(C), 0.76(D), 0.65(E); R^{His} 0.71.

Found: C, 53.62; H, 7.83; N, 12.91%. Calcd for $C_{64}H_{99}N_{13}O_{13}\cdot 2HCl\cdot 6H_2O$: C, 53.39; H, 7.91; N, 12.65%.

The product was contaminated with a minute amount of the deformylated derivative (R^{His} 0.87).

CH_3CO-(Val-Orn-Leu-D-Phe-Pro)$_2$-$NHCH_2CH_2OOCCH_3\cdot 2HCl$ (IIf). By hydrogenolysis of 93 mg (0.06 mmol) of the diacetyl derivative (XVI) in 7 m*l* of methanol in the presence of hydrogen chloride 77 mg (94%) of the dihydrochloride of diacetyl decapeptide ethanolamide was obtained, mp 220—222°C, $[\alpha]_D$−119°; R_f^{GS} 0.84(C), 0.84(D), 0.71(E); R^{His} 0.73.

Found: C, 54.27; H, 7.83; N, 12.53%. Calcd for $C_{66}H_{103}N_{13}O_{13}\cdot 2HCl\cdot 6H_2O$: C, 54.01; H, 8.04; N, 12.41%.

H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH·HCOOH (XIX). BOC-pentapeptide acid (VIII; 101 mg, 0.123 mmol) was dissolved in 2 m*l* of 98% formic acid and the solution was kept at room temperature for 2 hr. The solvent was removed *in vacuo* and the residue was solidified by the addition of ethyl acetate and ether. The product was collected by filtration and washed with ether. The very hygroscopic material was dried in a vacuum; yield 96 mg (99%), R_f^A 0.66, R_f^B 0.34. The formate was used for the next reaction without further purification, since the free pentapeptide had not been crystallized.[6]

BOC-[Val-Orn(δ-Z)-Leu-D-Phe-Pro]$_2$-OH (XX). To a solution of 101 mg of VIII and 14 mg (0.123 mmol) of HOSu in 2 m*l* of dioxane, 25 mg (0.123 mmol) of DCC was added at 5°C and the reaction mixture was stirred for 2 hr. To this solution was added a solution of pentapeptide formate (XIX; 96 mg, 0.122 mmol) in dioxane (3 m*l*) and dimethylformamide (2m*l*) containing 0.034 m*l* (0.244 mmol) of triethylamine, and the mixture was left to stand overnight in

a refrigerator. Crystals of dicyclohexylurea were removed by filtration and washed with dioxane. The filtrate and washings were concentrated to a small volume and the urea deposited further was filtred off. The filtrate was evaporated and the resulting syrup was solidified by the addition of water. The solid was collected by filtration, washed successively with water, 10% citric acid and water, and dried. The dried material was dissolved in 10 m*l* of methanol and filtered over a column (1.2×4 cm) of Dowex 50 (H^+ form) and the filtrate was evaporated. The residue crystallized upon the addition of ether. Recrystallization from methanol-ether gave 160 mg (95%) of the pure peptide, mp 173—175°C, $[\alpha]_D^{25}$ −109°, R_f^A 0.08, R_f^B 0.55.

Found: C, 61.56; H, 7.53; N, 10.86%. Calcd for $C_{81}H_{114}N_{12}O_{17}\cdot 3H_2O$: C, 61.50; H, 7.65; N, 10.63%.

*H-[Val-Orn(δ-Z)-Leu-*D*-Phe-Pro]$_2$-OH·HCl (XXI).* Compound XX (76 mg, 0.05 mmol) was dissolved in 3 m*l* of 98% formic acid and kept at room temperature for 90 min. The solvent was removed and the residue was dissolved in methanol containing 0.55 m*l* of N/10 hydrochloric acid. The solution was evaporated and the residue was dried over sodium hydroxide in a vaccum. The material obtained was then dissolved in a minute volume of methanol and precipitated by the addition of 10 m*l* of petroleum ether. Recrystallization from methanol-petroleum ether gave 69 mg (95%), mp 155—157°C, $[\alpha]_D^{30}$ −132°, R_f^A 0.10, R_f^B 0.57.

Found: C, 59.66; H, 7.30; N, 10.96%. Calcd for $C_{76}H_{106}N_{12}O_{15}\cdot HCl\cdot 4H_2O$: C, 59.42; H, 7.54; N, 10.94%.

*H-(Val-Orn-Leu-*D*-Phe-Pro)$_2$-OH·3HCl (III·3HCl).* By hydrogenolysis of 59 mg (0.04 mmol) of XXI in methanol containing an excess of hydrogen chloride in a similar manner to that employed for the preparation of IIa 49 mg (96%) of the trihydrochloride of the peptide was obtained, mp 233—234°C; $[\alpha]_D$ −135°; R_f^{GS} 0.77(C), 0.72(D), 0.70(E); R^{His} 0.86. Reported values, mp 233°C, $[\alpha]_D^{28}$ −89° (0.5N HCl).[11]

Found: C, 51.83; H, 7.90; N, 12.07%. Calcd for $C_{60}H_{94}N_{12}O_{11}\cdot 3HCl\cdot 6.5H_2O$: C, 52.00; H, 8.00; N, 12.13%.

Microbiological Assay.[12] The minimum amount of compounds needed for the complete inhibition of growth was determined by a dilution method with 10^3—10^4 organisms per milliliter using a Sabouraud bouillon as an incubation medium (Heart Infusion Broth for pre-incubation). The antibacterial activities of the decapeptide analogs are listed in Table 1.

12) We are indebted to Meiji Seika Co., Ltd. for the microbiological assays.