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Studies of Peptide Antibiotics. XXV. Synthesis of an Immediate Precursor of Gramicidin S[1]

Satoru MAKISUMI, Shuji MATSUURA, Michinori WAKI, and Nobuo IZUMIYA
Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Fukuoka

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A linear decapeptide, HCO-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-$NHCH_2CH_2OH$, which has been identified as an immediate precursor of gramicidin S was synthesized in order to define the physical and biological properties of this peptide. The synthetic product possessed practically no antibacterial activity against any of the microorganisms tested, though the optical rotatory dispersion feature of this peptide was quite similar to that of gramicidin S. Stability of the terminal blocking groups of this peptide to the action of hydrogen chloride in methanol was also discussed.

For clarifying the influence of the cyclic structure of gramicidin S (GS) (I) on its antibacterial activity, several linear decapeptide analogs (II) having an amino acid sequence of the gramicidin S in which the α-amino group is acylated with formyl or acetyl and *C*-terminal amino acid in the form of its amide or ethanolamide have been synthesized in this laboratory.[2] Recently, Pollard *et al.*[3] observed the formation of another linear decapeptide (III), with formate linked to the *N*-terminal amino acid and ethanolamine to the *C*-terminus of the chain, in the cell-free biosynthesis systems as a possible intermediate of GS. However, they did not describe any of the physical and biological properties of this immediate precursor of GS. It can easily be seen that the natural analog (III) and the synthetic analogs (II) are very similar structurally, differing only in the sequence of amino acids. In connection with our original program it appeared of interest to synthesize the naturally occuring linear decapeptide and test its biological activity. This paper describes the details of the synthesis and properties of the product.

┌—Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro—┐
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GS (I)

1) Part of this work has been briefly communicated: S. Makisumi and N. Izumiya, *Tetrahedron Letters*, **1970**, 227.

2) S. Makisumi, M. Waki, and N. Izumiya, This Bulletin, **44**, 143 (1971).

3) L. W. Pollard, N. V. Bhagovan, and J. B. Hall, *Biochemistry*, **7**, 1153 (1968).

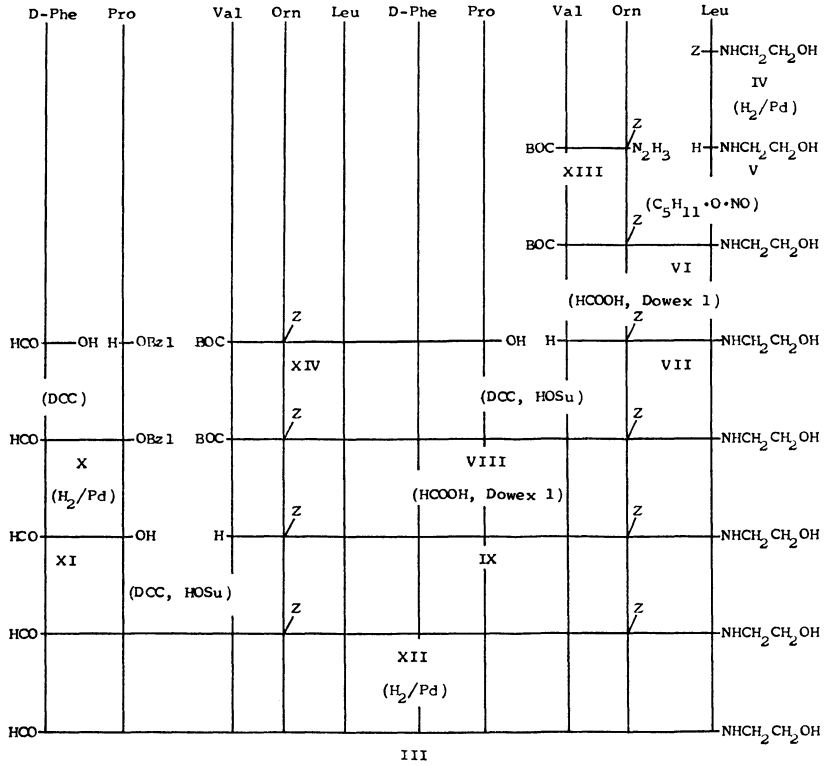

Fig. 1. Synthetic schemes of the formyl decapeptide ethanolamide.

HCO- / CH_3CO- } Val-Orn-Leu-D-Phe-Pro-

Val-Orn-Leu-D-Phe-Pro { -NH_2 / -$NHCH_2CH_2OH$

Linear decapeptide analogs (II)

HCO-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-
Val-Orn-Leu-$NHCH_2CH_2OH$

Immediate precursor of GS (III)

The steps involved in the synthesis of the formyl decapeptide ethanolamide are shown in Fig. 1.[4] Z-leucine ethanolamide (IV) was prepared by the reaction of Z-leucine with ethanolamine by means of the mixed anhydride method.[5] Compound (IV) was hydrogenolyzed to remove the Z-group and the product was isolated as the crystalline hydrochloride (V·HCl). BOC-tripeptide ethanolamide (VI) was synthesized from BOC-dipeptide hydrazide and V with the use of isoamylnitrite. Syntheses of BOC-dipeptide hydrazide (XIII) and BOC-pentapeptide acid (XIV) have already been described.[6]

Removal of the BOC-group from VI by treatment with 98% formic acid[7] and subsequent neutralization with Dowex 1 in methanol afforded free base of the tripeptide ethanolamide (VII). Condensation of BOC-pentapeptide acid with VII by the DCC–HOSu procedure[8] gave BOC-octapeptide ethanolamide (VIII) in a good yield. The BOC-group was removed from VIII by the method described above giving the free base of

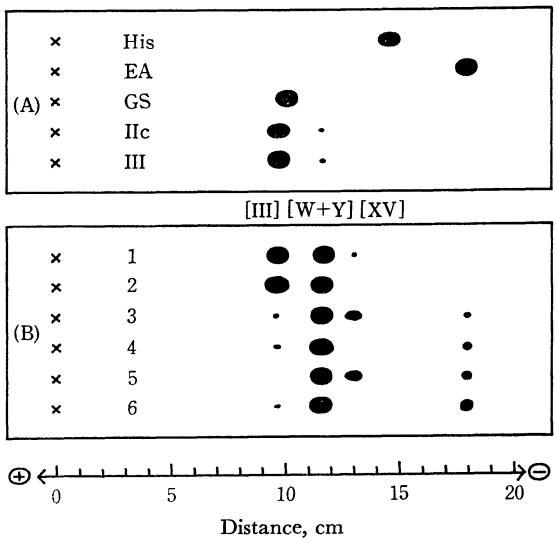

Fig. 2. Paper electrophoresis of the compounds. A: Synthetic peptide and reference compounds. EA, ethanolamine. B: Methanolysis products of the synthetic peptide; the odd numbers indicate the hydrolysates obtained before and the even after treatment with ammonia. 1,2: 0.8N HCl/MeOH, 50 min at room temperature; 3,4: 0.8N HCl/MeOH, 3 hr at room temperature; 5,6: 0.9N HCl/MeOH, 3 hr reflux.

4) Abbreviations used: Z, benzyloxycarbonyl; BOC, *t*-butyloxycarbonyl; OBzl, benzyl ester; DCC, dicyclohexylcarbodiimide; HOSu, *N*-hydroxysuccinimide. Amino acid symbol denotes L configuration otherwise noted.

5) J. R. Vaughan, Jr. and R. L. Osato, *J. Amer. Chem. Soc.*, **74**, 676 (1952).

6) M. Ohno and N. Izumiya, *ibid.*, **88**, 376 (1966); M. Ohno, T. Kato, S. Makisumi, and N. Izumiya, This Bulletin, **39**, 1738 (1966).

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

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

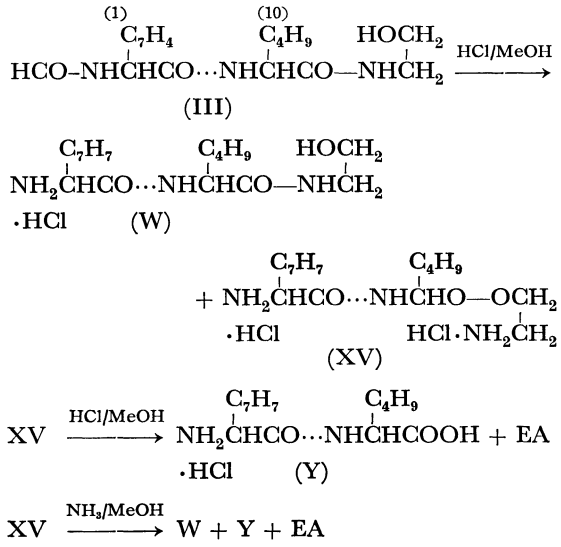

Fig. 3. Supposed reaction sequences of III by hydrogen chloride and subsequent treatment with ammonia.
EA, ethanolamine.

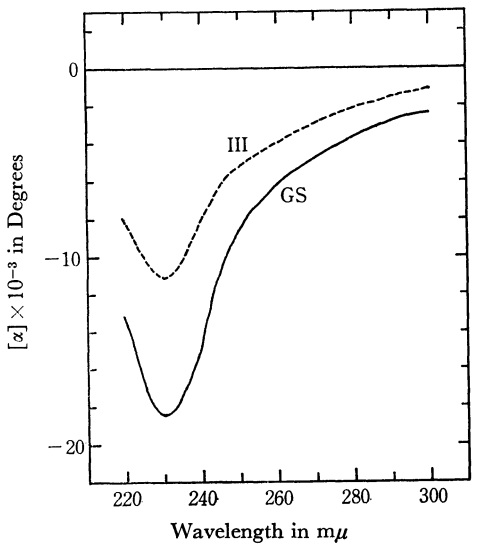

Fig. 4. Optical rotatory dispersion curves of GS (—) and linear decapeptide ethanolamide (···) in ethanol.

TABLE 1. INHIBITORY ACTIVITY OF SYNTHETIC DECAPEPTIDE AND RELATED COMPOUNDS ON MICROORGANISMS
Minimum inhibitory concentration, μg/ml

	Staphylococcus aureus FDA 209P	*Bacillus subtilis* ATCC 6633	*Escherichia coli* IMA 1253	*Proteus vulgaris* IAM 1025	*Shigella sonnei* 191— 66	*Candida albicans* IAM 4888
GS·2HCl	1.56	0.75	25	>100	25	25
IIc·2HCl	25	6.25	100	>100	>100	50
III·2HCl	100	50	>100	>100	>100	>100

octapeptide ethanolamide (IX).

Formyl dipeptide benzyl ester (X) was synthesized from formyl-D-phenylalanine and proline benzyl ester with the use of DCC.[9] Dipeptide (X) was obtained as an oil and could not be crystallized. X was hydrogenolyzed to remove the benzyl ester group and the product was isolated as crystalline dicyclohexylammonium salt. Pure formyl dipeptide (XI) obtained from the salt by treatment with Dowex 50 in methanol was coupled with IX by the DCC-HOSu procedure. This gave the protected decapeptide ethanoalmide (XII) in a good yield. Hydrogenolysis of XII in the presence of two equivalent amounts of hydrogen chloride in methanol provided the desired formyl decapeptide ethanolamide (III) as dihydrochloride.

The final product (III·2HCl) was found to be practically pure by means of elemental analysis and amino acid analysis. However, it was found to be contaminated with a minute amount of deformylated peptide by paper electrophoresis as shown in Fig. 2. It seems probable that the *N*-terminal formyl group of the peptide (III) is easily decomposed. This is comparable to the finding obtained for the formyl analogs of compound II.[2] On the contrary, Pollard *et al.*[3] reported that the formyl group in the natural peptide could not be removed from the peptide by treatment with 1.5N hydrogen chloride in methanol for 1 hr at room temperature. However, it was found that the formyl group in the synthetic peptide was relatively labile and removed easily under acidic conditions. It can be seen from Fig. 2 that the *C*-terminal ethanolamide group is apt to be rearranged by N→O acyl migration[10] with hydrogen chloride in methanol affording a deformylated decapeptide 2-aminoethyl ester (XV) to some extent. Peptide XV was easily converted to the parent peptide (W or Y) by treatment with ammonia. A supposed reaction sequence is summarized in Fig. 3.

Optical rotatory dispersion measurement at the region of 220—300 mμ revealed that the linear decapeptide exhibited apparent negative Cotton effect (see Fig. 4). This suggests that the conformational feature of the linear peptide is similar to that of GS, though the solution-state conformation of the latter compound has so far not been characterized completely.[11] In view of this experimental fact, it was expected that the linear peptide would have antibacterial activity. However, it was found that peptide (III) possessed practically no activity for any of microorganisms tested, whereas another formyl decapeptide ethanolamide (IIc) showed appreciable activity toward several strains of the Gram-positive microorganisms (Table 1). The result indicates that some linear decapeptide derivatives such as IIc possess substantial antibacterial activity though weaker than GS. Therefore, an amino acid sequence of a linear derivative affords important influence on the activity.

Experimental

Melting points are uncorrected. In thin-layer chromatography on a silica gel plate, the solvent systems used are abbreviated as follows: A, chloroform - benzene - methanol (6 : 3 : 1, *v/v*); B, chloroform - methanol (5 : 1, *v/v*); C, *s*-butanol - formic acid - water (4 : 1 : 1, *v/v*); D, ethyl acetate - pyridine - acetic acid - water (60 : 20 : 6 : 11, *v/v*); E, *n*-butanol - acetic acid - water (4 : 1 : 4, *v/v*) and F, *n*-butanol - acetic acid -

9) J. C. Sheehan and D.-D.H. Yang, *J. Amer. Chem. Soc.*, **80**, 1154 (1958).
10) A. P. Phillips and R. Baltzly, *ibid.*, **69**, 200 (1947).
11) D. Balasubramanian, *ibid.*, **89**, 5445 (1967).

pyridine - water (4 : 1 : 1 : 2, *v/v*). 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 at 600 V/30 cm for 2 hr. Prior to analysis, desiccated samples were left in air to a constant weight. $[\alpha]_D$ of air dried samples refers to 1% solution in methanol at 23°C.

Z-Leu-NHCH$_2$CH$_2$OH (IV). To a mixed anhydride prepared at −20°C from 2.65 g (10 mmol) of Z-Leu-OH, 1.32 m*l* (10 mmol) of isobutyl chloroformate and 1.4 m*l* (10 mmol) of triethyamine in 40 m*l* of tetrahydrofuran, a chilled solution of 0.61 g (10 mmol) of ethanolamine dissolved in 5 m*l* of tetrahydrofuran was added. The mixture was then allowed to stand in a refrigerator overnight. The solvent was removed *in vacuo* and the residue was dissolved in 50 m*l* of ethyl acetate. The solution was washed successively with 3% sodium bicarbonate, 10% citric acid and water, dried over sodium sulfate, and then evaporated. The residual syrup was solidified by the addition of petroleum ether. The product was recrystallized from ethyl acetate - ether; yield 2.20 g (71%); mp 125—126°C; $(\alpha)_D$ −17.9°; R_f 0.32 (A).

Found: C, 62.23; H, 7.86; N, 8.99%. Calcd for $C_{16}H_{24}N_2O_4$: C, 62.32; H, 7.84; N, 9.08%.

H-Leu-NHCH$_2$CH$_2$OH·HCl (V·HCl). A solution of 1.23 g (4 mmol) of IV dissolved in 45 m*l* of methanol containing 4.2 m*l* of N hydrochloric acid was subjected to hydrogenolysis in the presence of palladium black. After 3 hr, the filtrate from the catalyst was evaporated to dryness and left in a desiccator over sodium hydroxide. The residue was crystallized from methanol-ethyl acetate-ether; yield 0.78 g (93%); mp 153—154°C; $[\alpha]_D$ +22.7°; R_f 0.09 (A), 0.43 (C).

Found: C, 45.42; H, 9.04; N, 13.11%. Calcd for $C_8H_{18}N_2O_2\cdot HCl$: C,45.60; H, 9.09; N, 13.30%.

BOC-Val-Orn (δ-Z)-Leu-NHCH$_2$CH$_2$OH (VI). To a chilled solution of 959 mg (2 mmol) of BOC-Val-Orn(δ-Z)-N_2H_3 (XIII)[6] in 10 m*l* of dimethylformamide were added successively with stirring 1.45 m*l* (4 mmol) of 2.76N hydrogen chloride in dioxane and 0.272 m*l* (2 mmol) of isoamylnitrite at −5°C. After 10 min, 0.56 m*l* (4 mmol) of triethylamine and a solution of 421 mg (2 mmol) of V·HCl in 6 m*l* of dimethylformamide containing 0.28 m*l* (2 mmol) of triethylamine were added successively to the solution, and the mixture was stirred for 3 days at 0°C. After removal of the solvent, the residue was triturated with a mixture of ethyl acetate (5 m*l*) and water (50 m*l*). The product solidified was collected by filtation, washed successively with 4% sodium bicarbonate, 10% citric acid and water, and dried. It was recrystallized from methanol - ethyl acetate - ether; yield 949 mg (76%); mp 197—198°C; $[\alpha]_D$ −35.2°, R_f 0.41 (A), 0.95 (C).

Found: C, 60.09; H, 8.27; N, 11.12%. Calcd for $C_{31}H_{51}N_5O_8$: C, 59.88; H, 8.27; N, 11.26%.

H-Val-Orn(δ-Z)-Leu-NHCH$_2$CH$_2$OH (VII). BOC-tripeptide ethanolamide (VI, 622 mg, 1 mmol) was dissolved in 20 m*l* of 98% formic acid and the solution was kept at room temperature for 3 hr. The reagent was removed 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, equilibrated with methanol). The effluents and the washings were evaporated. The resulting residue was crystallized by the addition of ether. The product was collected by filtration; yield 491 mg (94%); mp 180—182°C; $[\alpha]_D$ −19.8°; R_f 0.20 (A), 0.51 (B).

Found: C, 59.39; H, 8.24; N, 13.21%. Calcd for $C_{26}H_{43}N_5O_6$: C, 59.86; H, 8,31. N, 13.43%.

BOC-Val-Orn(δ-Z)-Leu-D-Phe-Pro-Val-Orn(δ-Z)-Leu-NHCH$_2$CH$_2$OH (VIII). A suspension of 412 mg (0.5 mmol) of BOC-pentapeptide acid (XIV),[6] 261 mg (0.5 mmol) of VII and 58 mg (0.5 mmol) of HOSu in a mixture of dioxane (5 m*l*) and dimethylformamide (5 m*l*) was cooled in an ice-salt bath. DCC (103 mg, 0.5 mmol) was added to the 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 one drop of acetic acid for 3 hr at 0°C. Crystals of dicyclohexylurea were removed by filtration and washed with cold dioxane. The filtrate and the washings were evaporated to a minute 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 an isoluble material was removed. The filtrate was evaporated and the residue was crystallized by the addition of ether. The crystals obtained were dissolved in 10 m*l* of methanol and the solution was filtered over 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 petroleum ether; yield 622 mg (94%); mp 156—158°C; $[\alpha]_D$ −91.0°; R_f 0.42 (A), 0.82 (B).

Found: C, 61.18; H, 7.79; N, 11.36%. Calcd for $C_{69}H_{103}N_{11}O_{15}\cdot 2H_2O$: C, 60.82; H, 7.91; N, 11.31%.

H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-Val-Orn(δ-Z)-Leu-NHCH$_2$CH$_2$OH (IX). Treatment of 398 mg (0.3 mmol) of VIII with 6 m*l* of 98% formic acid followed by crystallization and filtration over a column of Dowex 1 as described above gave 335 mg (91%) of the octapeptide ethanolamide, mp 113—117°C; $[\alpha]_D$ −107°; R_f 0.22 (A), 0.56 (B).

Found: C, 60.99; H, 7.90; N, 11.84%. Calcd for $C_{64}H_{95}N_{11}O_{13}\cdot 2H_2O$: C, 60.88; H, 7.90; N, 12.20%.

HCO-D-Phe-Pro-OBzl (X). A solution of 0.97 g (5 mmol) of HCO-D-Phe-OH and 1.2 g (5 mmol) of H-Pro-OBzl·HCl in a mixture of 15 m*l* of chloroform and 15 m*l* of tetrahydrofuran containing 0.70 m*l* (5 mmol) of triethylamine was cooled in an ice-bath. With vigorous stirring 1.03 g (5 mmol) of DCC was introduced. After it had been allowed to stand overnight at 0°C, the mixture was evaporated, and ethyl acetate was added to the residue. After the insoluble dicyclohexylurea was filtered off, the filtrate was washed with 4% sodium bicarbonate, 10% citric acid and water, and dried over sodium sulfate. The filtrate was evaporated; yield of oil, 1.66 g (87%); R_f 0.44 (A).

HCO-D-Phe-Pro-OH·DCHA (XI·DCHA). A solution of 1.14 g (3 mmol) of X in 15 m*l* of methanol containing 3 drops of acetic acid was subjected to hydrogenolysis in the presence of palladium black. The filtrate was evaporated to dryness and the residue was dissolved in 10 m*l* of ethyl acetate. To the solution 0.6 m*l* (3 mmol) of dicyclohexylamine was added, and the mixture was allowed to stand in a refrigerator for 3 hr. The DCHA salt precipitated was collected, washed with ethyl acetate and ether, and dried; yield 1.02 g (72%); mp 177—178°C; $[\alpha]_D$ −51.1°.

Found: C, 66.19; H, 8.80; N, 8.58%. Calcd for $C_{27}H_{41}N_3O_4\cdot H_2O$: C, 66.23; H, 8.85; N, 8.58%.

HCO-D-Phe-Pro-Val-Orn(δ-Z)-Leu-D-Phe-Pro-Val-Orn(δ-Z)-Leu-NHCH$_2$CH$_2$OH (XII). The DCHA salt (94 mg, 0.2 mmol) of XI was dissolved in 5 m*l* of methanol and the solution was filtered through a column (1.2×4 cm) of Dowex 50 (H^+ form) in methanol. The effluent and the washings were evaporated. The formyl dipeptide acid (XI) thus obtained was reacted with 245 mg (0.2 mmol) of IX by the procedure using equimolar amount of DCC and HOSu as

has been described for the preparation of VIII. The product was purified by filtration over a series of columns of Dowex 50 and Dowex 1; yield 257 mg (86%); mp 132—135°C; $[\alpha]_D$ −81.0°; R_f 0.39 (A).

Found: C, 62.00; H, 7.50; N, 11.97%. Calcd for $C_{79}H_{111}N_{13}O_{16}\cdot 2H_2O$: C, 61.82; H, 7.55; N, 11.86%.

The molecular weight of a dried sample of XII was determined with a Hitachi Type 115 apparatus using methanol as a solvent. Found: 1477. Calcd: 1499.

HCO-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-NHCH$_2$-CH$_2$OH·2HCl (III·2HCl). A solution of 150 mg (0.1 mmol) of XII in 8 m*l* of methanol containing 0.4 m*l* of 0.25N methanolic hydrogen chloride was hydrogenolyzed over palladium black catalyst. After 3 hr, another portion of 0.4 m*l* of the hydrogen chloride was added and hydrogenolysis was continued. The progress of the reaction was checked by thin-layer chromatography. After additional 3 hr the filtrate was evaporated. When the residue was triturated with ether the peptide was obtained as a crystalline hydrochloride. Recrystallization from methanol - ether gave 107 mg (82%); mp 204—205°C; $[\alpha]_D$ −113°.

Found: C, 55.11; H, 8.06; N, 13.23; H_2O, 5.1%. Calcd for $C_{63}H_{99}N_{13}O_{12}\cdot 2HCl\cdot 4H_2O$: C, 55.01; H, 7.99; N, 13.24; H_2O, 5.2%.

The product was practically pure. However it was found to be contaminated with a minute amount of a ninhydrin positive compound which was deduced as a deformylated derivative (W) of III on the basis of electrophoretic mobility as shown in Fig. 2. The R_f values of III·2HCl and GS in several solvent systems are summarized in Table 2. Amino acid analysis of the hydrolysate of this peptide gave the molar ratios of the components as follows: Phe 2.2, Pro 1.9, Val 2.2, Orn 1.8, Leu 2.3 and ethanolamine 1.0.

TABLE 2. R_f VALUES OF SYNTHETIC LINEAR DECAPEPTIDE AND GS IN THIN LAYER CHROMATOGRAPHY

Solvent	III·2HCl	GS·2HCl
C	0.48	0.56
D[a)]	0.47	0.60
E	0.33	0.56
F	0.66	0.66

a) Pollard *et al.*[3] reported that the naturally occurring linear decapeptide (III) migrated in this solvent system with an R_f value of 0.82, whereas GS had an R_f of 0.45.

Methanolysis of III. A few mg of III·2HCl was dissolved in about 10 m*l* of 0.8N hydrogen chloride in methanol and the solution was allowed to stand at room temperature. After various periods of time two aliquots of the reaction mixture were withdrawn and evaporated to dryness *in vacuo*. One of them was dissolved in methanol and the solution was subjected to paper electrophoresis. The other was dissolved in ammonia in methanol and evaporated immediately after mixing prior to electrophoretic analysis. Another few mg of III·HCl was dissolved in about 10 m*l* of 0.9N hydrochloric acid and the mixture allowed to reflux for 3 hr. The reaction products obtained before and after treatment with ammonia were subjected to electrophoresis on paper. A typical electrophoreogram is shown in Fig. 2 (B). Two unidentified peptides spots were detected. Peptide spot migrated a little more rapidly than peptide III was not characterized completely but deduced to be a mixture of a deformylated decapeptide (Y) and its ethanolamide (W) (Fig. 3). The second peptide (XV) in small amounts was not also characterized but was deduced as a 2-aminoethyl ester (Fig. 3) of Y. Occurrence of the spot corresponding to ethanolamine suggests the formation of deformylated decapeptide (Y) after the hydrolysis of 2-aminoethyl ester bond of XV. However, Y could not be distinguishable from W on this electrophoreogram because of their similar electric character under the conditions applied as for *N*, *C*-terminal free decapeptide and deformylated decapeptide ethanolamide.[2] XV peptide was not found in the reaction mixture after ammonia treatment, suggesting the loss of one basic group resulting from the reverse rearrangement of XV to W or the hydrolysis of XV to Y under alkaline condition. From the results it seems likely that the *N*-terminal formyl group is labile to be cleaved and that the *C*-terminal ethanolamide is apt to be rearranged by $N\rightarrow O$ acyl migration by mild methanolysis in presence of hydrogen chloride.

Optical Rotatory Dispersion. ORD measurements were performed with a JASCO Model ORD/UV-5 spectropolarimeter. Cell of path length 1.0 cm was used and the runs were made at ambient temperature. In Fig. 4 are reported the ORD curves (range 220—300 mμ) of the synthetic peptide and GS in ethanol solution (7.8 mg/50 m*l*).

Microbiological Assays.[12] Minimum amounts of the compounds for the complete inhibition of growth were determined by a dilution method with 10^3—10^4 organisms per milliliter using a Sabouraud bouillon as an incubation medium.

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