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Studies of Peptide Antibiotics. VI. Syntheses of Cyclic Penta and Decapeptides with a Glycyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl Sequence

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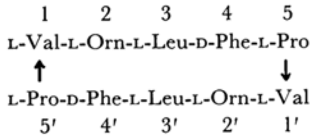
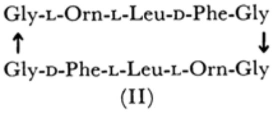
A cyclic decapeptide dihydrochloride, cyclo-(glycyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl)$_2$·2HCl, which is an analog of gramicidin S, and a cyclic pentapeptide monohydrochloride, cyclo-glycyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl·HCl, were synthesized in order to compare their antibacterial activities with that of gramicidin S. The cyclization reaction via two linear decapeptide active esters was found to yield the same cyclic bezyloxycarbonyl-substituted decapeptide; this was converted by hydrogenolysis to the cyclic decapeptide dihydrochloride mentioned above. The cyclization reaction of a linear pentapeptide active ester, in which the glycines are both terminal amino acid residues, yielded a cyclic benzyloxycarbonyl-substituted pentapeptide exclusively. When another linear pentapeptide active ester and a pentapeptide azide were also subjected to cyclization reaction, they afforded the same cyclic benzyloxycarbonyl-substituted pentapeptide; this was then hydrogenolyzed to the corresponding cyclic pentapeptide monohydrochloride. The effects of the two cyclic peptide hydrochlorides on the bacterial growth were tested; no retardation of growth was observed with any of the microorganisms tested.

For a study of the relationsihp between the chemical structure and the biological activity of such antibacterial cyclic peptides as gramicidin S (I) and tyrocidine A, various cyclic peptides which possess several characteristic features have been prepared and their biological activities have been tested, as has been described in previous papers from this laboratory. It was observed that some of them,[1] such as a cyclic hexapeptide related to gramicidin S, were devoid of antibacterial activity, but an analog of gramicidin S, 5, 5'-sarcosine-gramicidin S,[2] was as active as gramicidin S against several microorganisms. Furthermore, 5, 5'-glycine-gramicidin S was found to possess approximately 10 times as much activity as gramicidin S in a synthetic medium.[3] The result shows that the replacement of the proline residues by glycines or sarcosines in the sequence of gramicidin S does not reduce the activity, and that the side chain of proline is not indispensable to its full activity. In order to investigate whether or not the valine residues in the 1- and 1'-positions of gramicidn S might be replaced by an amino acid of a smaller molecule, glycine, without a decrease in the antibacterial activity, we attempted to prepare 1, 1', 5, 5'-glycine-gramicidin S (II). The present paper will describe the syntheses and antibacterial properties of this cyclic decapeptide (II) and of the cyclic pentapeptide, cyclo-glycyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl (III), which is related to II.

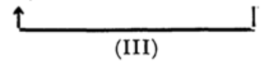


(I)

The structure of gramicidin S, with numbers indicating the positions of the individual amino acid residues.



(II)



(III)

The protected cyclic decapeptide (XXVI), which was converted to II by hydrogenolysis, was synthesized via two different routes, shown in Figs. 1 and 2.

Figure 1 indicates one route for the synthesis of XXVI via the linear decapeptide active ester (XXV), in which glycylglycine is the C-terminal

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[1] 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).

[2] H. Aoyagi and N. Izumiya, ibid., **39**, 1747 (1966).

[3] H. Aoyagi, T. Kato, M. Ohno, M. Kondo, M. Waki, S. Makisumi and N. Izumiya, ibid., **38**, 2139 (1965); H. Aoyagi, T. Kato, M. Ohno, M. Kondo and N. Izumiya, *J. Am. Chem. Soc.*, **86**, 5700 (1964).

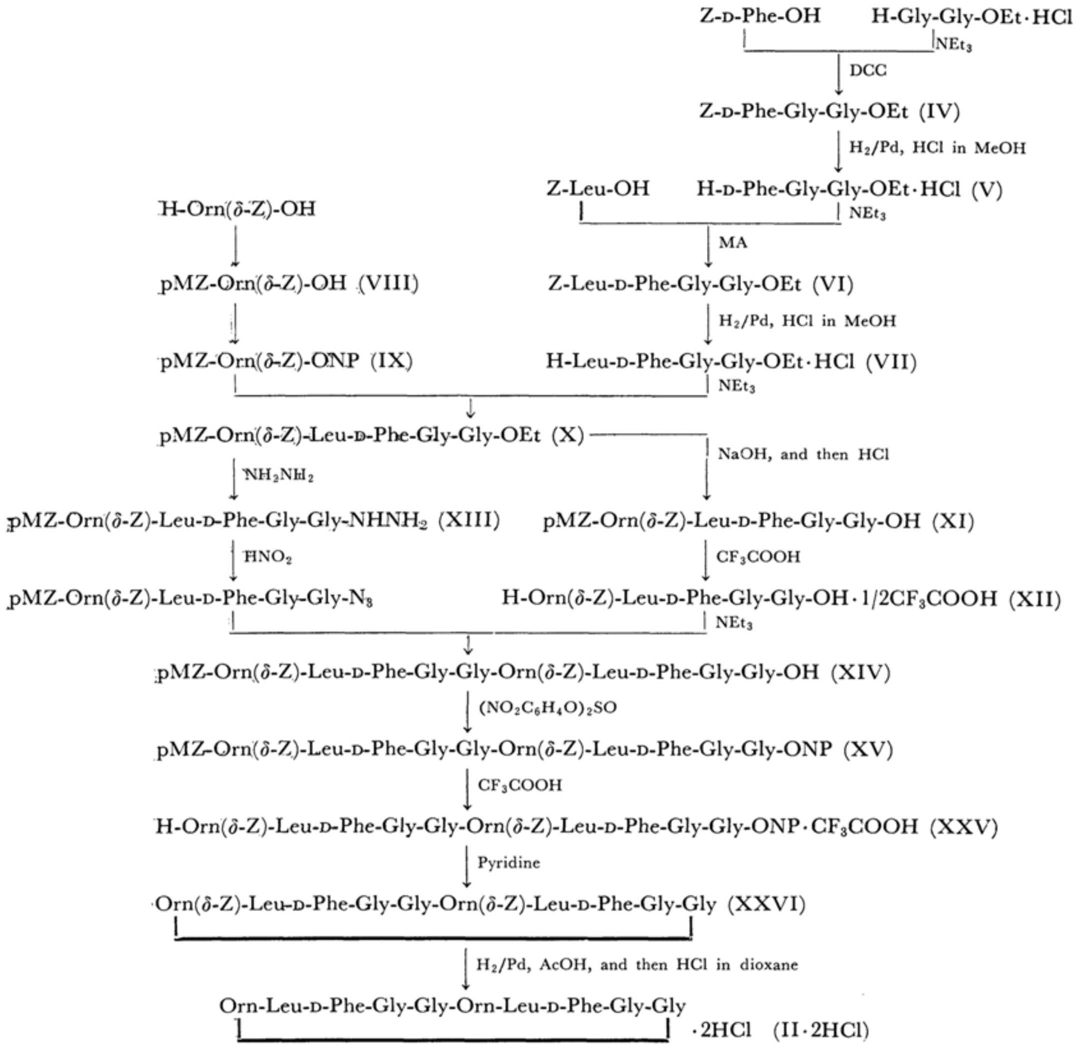

Fig. 1.

Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; pMZ, *p*-methoxybenzyloxycarbonyl; ONP, *p*-nitrophenoxy ester; MA, mixed anhydride method. An amino acid residue except Gly and D-Phe is of L-configuration.

sequence. The *C*-terminal tetrapeptide derivative (VI) was prepared by a step-by-step elongation by means of the dicyclohexylcarbodiimide method and the mixed anhydride method. Syntheses of *p*-methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithylglycine benzyl ester by the coupling of diacylornithine (VIII) with the glycine ester were attempted by several methods as a preliminary experiment for the preparation of diacylpentapeptide ester (X) by the coupling of VIII with the tetrapeptide ester (VII); it was found that the reaction of the diacylornithine active ester (IX) with the glycine ester gave the pure compound in the best yield. In fact, the reaction of IX with VII afforded the crystalline pentapeptide derivative (X) in a good yield. The azide, which was derived from XIII, was then made to react with XII to give the protected decapeptide (XIV) in a 68% yield. XIV was converted to the *p*-nitrophenyl ester (XV) by the action of di-*p*-nitrophenyl sulfite, and the removal of the *p*-methoxybenzyloxycarbonyl group from XV with trifluoroacetic acid gave the amorphous decapeptide *p*-nitrophenyl ester trifluoroacetate (XXV). The cyclization reaction of XXV in pyridine gave the crude benzyloxycarbonyl-substituted cyclic peptide (XXVI), which was then purified by passing it through columns of acidic and basic ion exchangers; the yield of the crystalline XXVI from XIV was found to be 21% after recrystallization. The molecular weight determination by the micro Rast method demonstrated that the molecular size of this compound corresponded to that of cyclic decapeptide (XXVI).

Fig. 2.

The final product (II·2HCl) was obtained as colorless crystals containing four moles of the water of crystallization. Its homogeneity was demonstrated by carboxymethyl(CM) cellulose column chromatography (Fig. 6) and by paper electrophoresis (Fig. 7).

The protected cyclic decapeptide (XXVI) was obtained again via another route, this time from the linear decapeptide active ester (XXVII), as is shown in Fig. 2, in which the glycines are the *N*- and *C*-terminal amino acid residues. It should be noted that the sequence of reactions presented in Fig. 2 is similar to that used for the preparation of 5, 5′-sarcosine-gramicidin S.[2] The cyclization reaction of XXVII gave the crystalline product (XXVI) in a yield of 36% from XXIII. From a comparison of melting points and specific rotations, this product is demonstrated to be identical with the protected cyclic decapeptide (XXVI) prepared from XXV.

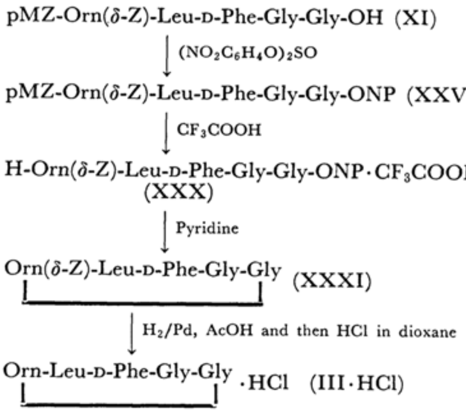

Fig. 3.

The synthesis of the protected cyclic decapeptide (XXVI) was further attempted by the dimerization reaction of a pentapeptide active ester (XXX). However, the benzyloxycarbonyl-substituted cyclic peptide obtained in a yield of 23% from XI was found to be a monomer, shown as XXXI, by the results of the molecular weight determination. The hydrogenolysis of XXXI in the presence of an equivalent amount of hydrogen chloride yielded a cyclic pentapeptide hydrochloride (III·HCl). The experiments of CM-cellulose column chromatography showed that the product (III·HCl) gives only one peak, which is at a position different from that of the cyclic decapeptide (II·2HCl).

The cyclizations of pentapeptides via different routes were also performed, as Figs. 4 and 5 show. Although it has been reported that cyclic hexaglycyl was obtained in a good yield from triglycine azide,[4] the linear pentapeptide azide, which was derived from XXXII, gave only a very small amount of the protected cyclic pentapeptide (XXXI) (Fig. 4). It should be noted, in this connection, that a paper from this laboratory has reported that the yield of the cyclization of pentaglycyl-ε-benzyloxycarbonyl-L-lysine azide attempting to obtain the corresponding cyclic hexapeptide was very poor.[5]

pMZ-Orn(δ-Z)-Leu-D-Phe-Gly-Gly-NHNH$_2$ (XIII)

$\downarrow$ CF_3COOH

H-Orn(δ-Z)-Leu-D-Phe-Gly-Gly-NHNH$_2$·2CF$_3$COOH (XXXII)

$\downarrow$ HNO_2

H-Orn(δ-Z)-Leu-D-Phe-Gly-Gly-N$_3$

$\downarrow$ $NaHCO_3$

XXXI

Fig. 4.

pMZ-Gly-Orn(δ-Z)-Leu-D-Phe-Gly-OH (XX)

$\downarrow$ $(NO_2C_6H_4O)_2SO$

pMZ-Gly-Orn(δ-Z)-Leu-D-Phe-Gly-ONP (XXIX)

$\downarrow$ CF_3COOH

H-Gly-Orn(δ-Z)-Leu-D-Phe-Gly-ONP·CF$_3$COOH (XXXIII)

$\downarrow$ pyridine

XXXI

Fig. 5.

Figure 5 shows the reaction sequence for the cyclization of the pentapeptide active ester, in which the glycines are both terminal amino acid residues. The cyclization reaction of the pentapeptide active ester (XXXIII) in a large amount of pyridine gave crude, protected cyclic peptide (XXXIV), which was found to contain only protected cyclic pentapeptide (XXXI), and not even a trace of the protected cyclic decapeptide (XXVI), from the criteria of CM-cellulose chromatography and paper electrophoresis of the hydrogenolyzed material of XXXIV. The protected cyclic pentapeptide (XXXI) in an analytically-pure state was obtained in a yield of 37% (from XX) after the recrystallization of XXXIV from methanol and ether. This result is of great interest, for it is in contrast with the fact that the cyclization reaction of L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-glycine (or -sarcosine) active ester[2,3] yielded an appreciable amount of the protected cyclic decapeptide by the dimerization reaction, besides a large amount of the monomeric cyclic pentapeptide.

The antibacterial activities of 1, 1′, 5, 5′-glycine-gramicidin S (II) and cyclic pentapeptide (III) toward several microorganisms were also examined. Neither compound had any retarding effect on the growth of the microorganisms, even at so high level as 100 γ per ml. of assay medium. From these findings, it appears that the valine residues in the gramicidin S sequence are of importance for the activity, for 5, 5′-glycine-gramicidin S showed a strong activity. In this connection, it is of interest to note that 1, 1′-glycine-2, 2′-lysine-gramicidin S, which has a structure similar to that of II, was found to possess some antibacterial activity.[6]

To clarify the effectiveness of the valine residues in gramicidn S further, the syntheses of several analogs of gramicidin S in which the valine residues are replaced by appropriate amino acids are in progress in this laboratory.

Experimental

All the melting points are uncorrected. The optical rotations were measured on a Yanagimoto Photometric Polarimeter, OR-20 type. Prior to analysis, the compounds were dried to a constant weight over phosphorus pentoxide at 80°C and 2 mmHg, except in the case of II·2HCl and III·HCl.

Benzyloxycarbonyl-D-phenylalanylglycylglycine Ethyl Ester (IV).—To a solution of benzyloxycarbonyl-D-phenylalanine (6.0 g.), glycylglycine ethyl ester hydrochloride (3.9 g.) and triethylamine (2.8 ml.) in chloroform (80 ml.), dicyclohexylcarbodiimide[7] (4.12 g.) was added at 0°C. The reaction mixture was stirred for 2 hr. at 0°C and then kept overnight in a refrigerator. The mixture was evaporated in vacuo, and the residue was extracted with ethyl acetate (120 ml.). After the dicyclohexylurea thus formed was filtered off, the filtrate was washed successively with a 4% sodium bicarbonate solution, 3% hydrochloric acid, and water, and then dried over anhydrous sodium sulfate. The filtered solution was evaporated in vacuo; the residual oil solidified after ether had been added. Recrystallization from ethyl acetate-ether gave 5.9 g. (67%); m. p. 95—96°C; $[\alpha]_D^{20}$ $+17.3°$ (c 2, dimethylformamide).

Found: C, 62.36; H, 6.19; N, 9.63. Calcd. for $C_{23}H_{27}O_6N_3$: C, 62.57; H, 6.16; N, 9.52%.

D-Phenylalanylglycylglycine Ethyl Ester Hydrochloride (V).—IV (8.83 g.) was subjected to hydrogenolysis in the presence of palladium black and 0.43 N methanolic hydrogen chloride (50.6 ml.). The filtrate from the catalyst was then evaporated to dryness in vacuo; yield of oil, 6.9 g. (100%); R_f 0.85.[8]

Benzyloxycarbonyl-L-leucyl-D-phenylalanylglycylglycine Ethyl Ester (VI).—To a chilled solution of benzyloxycarbonyl-L-leucine (5.17 g.) and triethylamine (2.72 ml.) in tetrahydrofuran (40 ml.), isobutyl

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[4] J. C. Sheehan and W. L. Richardson, *J. Am. Chem. Soc.*, **76**, 6329 (1954); **77**, 6391 (1955).

[5] M. Ohno and N. Izumiya, This Bulletin, **38**, 1839 (1965).

[6] German Patent, *Chem. Abstr.*, **57**, 949 (1962).

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

[8] 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.

chloroformate (2.52 ml.) was added. After 15 min., a mixture of V (6.9 g.), triethylamine (2.72 ml.), and chloroform (40 ml.) was added to the solution. The reaction mixture was allowed to stand overnight and then evaporated to dryness in vacuo. The residual oil was dissolved in ethyl acetate (150 ml.), and the solution was wahsed successively with 4% sodium bicarbonate, 3% hydrochloric acid and water, dried over sodium sulfate, and then evaporated to dryness in vacuo. The product was obtained as an oil; yield, 8.32 g. (75%).

L-Leucyl-D-phenylalanylglycylglycine Ethyl Ester Hydrochloride (VII).—VI (6.1 g.) was treated as has been described in the case of V. The product was obtained as a hygroscopic powder; yield, 4.7 g. (94%); R_f 0.87.[8]

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithine (VIII).**—A mixture of δ-benzyloxycarbonyl-L-ornithine (2.67 g.), sodium bicarbonate (2.52 g.), water (100 ml.), dioxane (150 ml.), and *p*-methoxybenzyloxycarbonyl azide[9] was stirred for 60 hr. at room temperature. The solution was then evaporated in vacuo in order to remove dioxane. After it had been extracted with ether, the solution was acidified with 0.5 M citric acid (40 ml.) and then extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated to dryness in vacuo. The residual oil was solidified by the addition of petroleum ether; yield, 3.42 g. (79%). For analysis, a part of the product was recrystallized from ethyl acetate-petroleum ether; m. p. 73—75°C; $[\alpha]_D^{20}$ $-2.3°$ (c 2, dimethylformamide).

Found: C, 60.96; H, 6.22; N, 6.77. Calcd. for $C_{22}H_{26}O_7N_2$: C, 61.38; H, 6.09; N, 6.51%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithine *p*-Nitrophenyl Ester (IX).**—To a stirred solution of VIII (3.34 g.) and *p*-nitrophenol (1.31 g.) in ethyl acetate (25 ml.), dicyclohexylcarbodiimide (1.60 g.) was added at 0°C. The stirring was continued for 2 hr. at 0°C, and then for 3 more hours at room temperature. The dicyclohexylurea which precipitated was filtered off and washed with ethyl acetate, and the combined filtrates were evaporated to dryness. The cyrstalline residue was recrystallized from hot ethanol; yield, 3.34 g. (78%); m. p. 123—124°C; $[\alpha]_D^{20}$ $-14.1°$ (c 2, dimethylformamide).

Found: C, 60.63; H, 5.25; N, 7.80. Calcd. for $C_{28}H_{29}O_9N_3$: C, 60.97; H, 5.30; N, 7.62%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithylglycine Benzyl Ester.**—*a) MA Method.*—The mixed anhydride prepared from VIII (1 mmol.) was coupled with the glycine benzyl ester (1 mmol.), following the procedure used in the preparation of VI. The crude product was recrystallized from ethyl acetate-ether; yield, 60 mg. (21%); m. p. 141—143°C. The melting point of this product was not depressed by admixture with the product (m. p. 143—144°C) obtained by the ONP-method.

b) DCC Method.—The same product was obtained by the carbodiimide method in the usual manner; yield, 144 mg. (50%); m. p. 141—143°C.

c) ONP Method.—The product was prepared with IX (1mmol.) and the glycine benzyl ester in the usual manner; yield, 188 mg. (65%); m. p. 143—144°C; $[\alpha]_D^{20}$ $-3.0°$ (c 2, dimethylformamide).

Found: C, 64.44; H, 6.47; N, 7.53. Calcd. for $C_{31}H_{35}O_8N_3$: C, 64.46; H, 6.11; N, 7.28%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine Ethyl Ester (X).**—To a solution of VII (4.7 g.) and triethylamine (1.87 ml.) in dimethylformamide (14 ml.), IX (5.68 g.) was added, and the solution was allowed to stand overnight at room temperature. Cold water (100 ml.) was then added slowly to it, and the mixture was kept in a refrigerator for several hours. The crystals were collected by filtration and washed with 4% sodium bicarbonate, M citric acid, and water. They were then recrystallized from dioxane-ether; yield, 6.12 g. (71%); m. p. 172—174°C; $[\alpha]_D^{16}$ $+1.5°$ (c 2, dimethylformamide); R_f 0.92.[10]

Found: C, 61.60; H, 6.58; N, 10.01. Calcd. for $C_{43}H_{56}O_{11}N_6$: C, 62.00; H, 6.78; N, 10.09%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine (XI).**—To a solution of X (510 mg.) in methanol (8 ml.), 1.03 N sodium hydroxide (0.71 ml.) was added. The solution was then allowed to stand overnight at room temperature. After the addition of water (60 ml.), the solution was concentrated in vacuo in order to remove methanol. It was extracted with ether and acidified with 1.22 N hydrochloric acid (0.61 ml.) under cooling. After they had been stored in a refrigerator for several hours, the crystals were collected by filtration, washed with water, and dried. Recrystallization from methanol-ether gave 420 mg. (84%); m. p. 190—192°C; $[\alpha]_D^{16}$ $-0.7°$ (c 2, dimethylformamide); R_f 0.72.[10]

Found: C, 60.10; H, 6.66; N, 10.49. Calcd. for $C_{41}H_{52}O_{11}N_6 \cdot H_2O$: C, 59.84; H, 6.61; N, 10.21%.

δ-Benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine Hemitrifluoroacetate (XII).—To a mixture of XI (916 mg.) and anisole (0.4 ml.), trifluoroacetic acid (4 ml.) was added at 0°C. After it had been permitted to stand for 40 min. at 0°C, the solution was evaporated in vacuo. The residue was triturated with ether (20 ml.) and washed repeatedly with ether by decantation. Crystals were filtered off with the aid of ether; yield, 783 mg. (96%); m. p. 183—184°C; $[\alpha]_D^{20}$ $+13.2°$ (c 2, dimethylformamide). R_f 0.85[8] and 0.64.[10]

Found: C, 54.28; H, 6.68; N, 11.71. Calcd. for $C_{32}H_{44}O_8N_6 \cdot 1/2CF_3COOH \cdot 2H_2O$: C, 54.10; H, 6.66; N, 11.45%.

An attempt to crystallize the CF_3COOH-free pentapeptide failed.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine Hydrazide (XIII).**—A solution of X (440 mg.) and hydrazine hydrate (0.53 ml.) in dimethylformamide (4 ml.) was allowed to stand for 2 days at 30°C. The solution was then concentrated in vacuo to a small volume. The hydrazide which precipitated upon the

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[9] F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

[10] The R_f of the thin-layer chromatography with Merck silica gel G refers 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 with ninhydrin, and those with blocked amino groups, by spraying with 47% hydrobromic acid and then with ninhydrin.

addition of water (20 ml.) was collected and recrystallized from methanol-ether; yield, 352 mg. (80%); m. p. 201—202°C; $[\alpha]^{16}_{D}$ +2.1° (*c* 2, dimethylformamide).

Found: C, 59.76; H, 6.60; N, 13.36. Calcd. for $C_{41}H_{54}O_{10}N_8$: C, 60.13; H, 6.65; N, 13.68%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine (XIV).**—The following operations were carried out in a cold room. Into a chilled solution of XIII (820 mg.) in dimethylformamide (12 ml.), 1.22 N hydrochloric acid (2.45 ml.) and sodium nitrite (83 mg.) in water (2 ml.) were stirred. After 5 min., cold water (80 ml.) was added to the solution. The azide, which precipitated as a white mass, was collected by filtration and washed with cold water, 4% sodium bicarbonate, and water, and then dried under a vacuum in a desiccator. The azide was added to a solution of XII (735 mg.) and triethylamine (0.28 ml.) in dimethylformamide (15 ml.). The mixture was stirred for 3 days at 0°C and then evaporated in vacuo. The precipitate which formed upon the addition of ethyl acetate (8 ml.) and ether (4 ml.) was collected, washed with 0.5 M citric acid, and water, and dried. Recrystallization from dioxane-ether did not raise the melting point. The yield was 971 mg. (66%); m. p. 235°C (decomp.); $[\alpha]^{16}_{D}$ +0.4° (*c* 2, dimethylformamide); R_f 0.87,[10] R_f of the hydrogenolyzed product of XIV, 0.52[10] and 0.69[8].

Found: C, 60.44; H, 6.50; N, 11.63. Calcd. for $C_{73}H_{94}O_{18}N_{12}\cdot 2H_2O$: C, 60.31; H, 6.59; N, 11.56%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine *p*-Nitrophenyl Ester (XV).**—To a solution of XIV (855 mg.) in a mixture of dimethylformamide (4.5 ml.) and pyridine (1.46 ml.), di-*p*-nitrophenyl sulfite[11] (1.94 g.) was added. After the mixture had then been allowed to stand for 20 hr. at room temperature, the solvent was removed in vacuo and the residual oil was solidified by the addition of a mixture of ether and petroleum ether (1 : 1). The product was collected by filtration and washed with a mixture of ether and petroleum ether (1 : 1) until no yellow color could be discerned upon the addition of N sodium hydroxide to the filtrate. The yield was 920 mg. The *p*-nitrophenyl ester content in this product was spectrophotometrically estimated to be 98% by measuring the optical density of the compound at 412 mμ.[5,12]

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithine Ethyl Ester (XVI).**—*p*-Methoxybenzyloxycarbonylglycine[5] (1.19 g.) was coupled with δ-benzyloxycarbonyl-L-ornithine ethyl ester *p*-toluenesulfonate[1] (2.32 g.) by dicyclohexylcarbodiimide (1.03 g.), following essentially the same procedure as that employed in the preparation of IV. As a minor alternation, however, 0.5 M citric acid was used instead of 2% hydrochloric acid to wash the reaction mixture. Recrystallization from ethyl acetate-ether gave 1.59 g. (62%); m. p. 102°C; $[\alpha]^{20}_{D}$ −6.0° (*c* 2, dimethylformamide).

Found: C, 60.44; H, 6.29; N, 7.89. Calcd. for $C_{26}H_{33}O_8N_3$: C, 60.57; H, 6.45; N, 8.15%.

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithine Hydrazide (XVII).**—XVI (1.38 g.) was converted to the hydrazide (XVII) as has been described in the case of XIII; yield, 1.21 g. (90%). For analysis, a part of the product was recrystallized from dioxane-ether; m. p. 170—171°C; $[\alpha]^{20}_{D}$ −1.3° (*c* 2, dimethylformamide).

Found: C, 57.42; H, 6.13; N, 13.96. Calcd. for $C_{24}H_{31}O_7N_5$: C, 57.47; H, 6.23; N, 13.97%.

L-Leucyl-D-phenylalanylglycine Ethyl Ester Hydrochloride (XVIII).—Benzyloxycarbonyl-L-leucyl-D-phenylalanylglycine ethyl ester[3] (3.98 g.) was subjected to hydrogenolysis as has been described in the case of V. The oily residue was crystallized by the addition of ether; yield, 3.14 g. (97%); m. p. 182—184°C; $[\alpha]^{20}_{D}$ +68.8° (*c* 0.5, dimethylformamide); R_f 0.90[8] and 0.75[10].

Found: C, 55.65; H, 7.74; N, 10.27. Calcd. for $C_{19}H_{30}O_4N_3Cl\cdot 1/2H_2O$: C, 55.80; H, 7.64; N, 10.28%.

XVIII had been obtained in this laboratory as an oily product earlier.[3]

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine Ethyl Ester (XIX).**—XVII (3.77 g.), in a mixture of glacial acetic acid (30 ml.) and 3.0 N hydrochloric acid (7.6 ml.), was treated with an aqueous solution of sodium nitrite (570 mg.) at −5°C. After 5 min., cold water (100 ml.) was added to the mixture. The azide was then extracted with ethyl acetate, and the organic layer was washed with 4% sodium bicarbonate and water, and dried over sodium sulfate. The filtered solution was added to a solution of XVIII (3.07 g.) and triethylamine (1.05 ml.) in dimethylformamide (15 ml.). The mixture was stirred for 3 days at 0°C and then evaporated in vacuo to a small volume. The precipitate which formed upon the addition of water was collected, washed with 0.5 M citric acid, 4% sodium bicarbonate, and water, and dried. Recrystallization from methanol-dixoane-ether gave 4.90 g. (78%) of XIX; m. p. 180—181°C; $]\alpha]^{20}_{D}$ +1.0° (*c* 2, dimethylformamide); R_f 0.90[10]; R_f of hydrogenolyzed product of XIX, 0.74[8] and 0.68[10].

Found: C, 61.67; H, 6.76; N, 10.14. Calcd. for $C_{43}H_{56}O_{11}N_6$: C, 62.00; H, 6.67; N, 10.09%.

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine (XX).**—To a solution of XIX (1.67 g.) in a mixture of methanol (10 ml.) and dioxane (5 ml.), 2 N sodium hydroxide (1.1 ml.) was added. After the solution had then been allowed to stand overnight at room temperature, the desired product was obtained by the procedure described in the case of XI. It was recrystallized from dioxane-ether; yield, 1.50 g. (93%); m. p. 157—159°C; $[\alpha]^{20}_{D}$ −1.5° (*c* 2, dimethylformamide); R_f 0.74.[10]

Found: C, 60.60; H, 6.45; N, 10.43. Calcd. for $C_{41}H_{52}O_{11}N_6\cdot 1/2H_2O$: C, 60.50; H, 6.56; N, 10.33%.

Glycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine Hydrochloride (XXI).—To a solution of XX (1.29 g.) and anisole (0.4 ml.) in dioxane (8 ml.), 3.9 N hydrogen chloride in dioxane (8 ml.) was added at room temperature. After 2 hr., the solution was evaporated to dryness in vacuo and the residue was collected with the aid of ether; yield,

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

[12] R. Schwyzer and P. Sieber, ibid., **40**, 624 (1957).

1.10 g. (98%); m. p. 188—191°C; $[\alpha]_D^{20}$ −7.6° (*c* 2, dimethylformamide); R_f 0.87[8] and 0.69[10].

Found: C, 55.48; H, 6.96; N, 12.12. Calcd. for $C_{32}H_{45}O_8N_6Cl\cdot H_2O$: C, 55.28; H, 6.82; N, 12.09%.

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine Hydrazide (XXII).**—XIX (1.67 g.) was converted to the hydrazide (XXII) as has been described in the case of XIII; yield, 1.60 g. (97%). For analysis, a part of the product was recrystallized from methanol-ether; m. p. 163—164°C; $[\alpha]_D^{20}$ −2.5° (*c* 2, dimethylformamide).

Found: C, 59.47; H, 6.65; N, 13.38. Calcd. for $C_{41}H_{54}O_{10}N_8\cdot 1/2H_2O$: C, 59.48; H, 6.67; N, 13.54%.

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine (XXIII).**—To XXII (828 mg.) in dimethylformamide (10 ml.), 1.22 N hydrochloric acid (2.45 ml.) and sodium nitrite (83 mg.) in water (1 ml.) water added at 0°C. After 7 min., cold water (70 ml.) was added to the solution. The azide which formed was extracted with ethyl acetate (100 ml.), and the extract was treated as has been described in the case of XIX. The solution of the azide was added to a solution of XXI (695 mg.) and triethylamine (0.28 ml.) in dimethylformamide (25 ml.). Then, the desired acyldecapeptide (XXIII) was obtained by the procedure used in the preparation of XIV. It was recrystallized from dioxane-ether; yield, 1.01 g. (70%); m. p. 239—242°C; $[\alpha]_D^{20}$ −0.6° (*c* 2, dimethylformamide); R_f 0.75[10]; R_f of hydrogenolyzed product of XXIII, 0.68[8] and 0.35[10].

Found: C, 60.92; H, 6.68; N, 11.78. Calcd. for $C_{73}H_{94}O_{18}N_{12}\cdot H_2O$: C, 60.65; H, 6.62; N, 11.63%.

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine *p*-Nitrophenyl Ester (XXIV).**—A solution of XXIII (723 mg.) and di-*p*-nitrophenyl sulfite (1.62 g.) dissolved in pyridine (7.5 ml.) was allowed to stand overnight at room temperature and then treated in the manner described in the case of XV. The powder obtained weighed 770 mg. The *p*-nitrophenyl-ester content of this product was estimated to be 81%. The product was used for the next reaction without further purification.

Cyclo-(glycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl)$_2$ (XXVI). — *a) From XV.*—To XV (890 mg.) and anisole (0.5 ml.), trifluoroacetic acid (4 ml.) was added at −5°C. After 20 min., the solution was evaporated in vacuo at 0°C, and the residue was triturated with ether. The decapeptide *p*-nitrophenyl ester trifluoroacetate (XXV) was collected in a cold room by filtration, washed with ether, and dissolved in dimethylformamide (18 ml.) containing glacial acetic acid (0.4 ml.). The solution was then stirred, drop by drop, into pyridine (180 ml.) which had been kept at 60—65°C over a period of 6 hr.; the stirring was then continued for an additional 2 hr. at the same temperature. After the solvent had been removed, the residue was dissolved in a mixture of methanol (200 ml.) and water (80 ml.). The insoluble substance was removed by filtration, and the filtrate was passed successively through columns (2.5×10 cm.) of Dowex 1 (OH$^-$ form) and Dowex 50 (H$^+$ form). The columns were then washed with the same solvent (300 ml.), the combined effluents were evaporated to dryness in vacuo, and the product was suspended in water (20 ml.), collected by filtration, and dried (174 mg.). This substance was then recrystallized from methanol-ethyl acetate-ether; yield of the air-dried product, 153 mg. (21% from XIV); m. p. 274—275°C (decomp.); $[\alpha]_D^{20}$ −5.0° (*c* 0.51, dimethylformamide); R_f 0.90.[10]

Found: C, 60.56; H, 6.83; N, 13.31. Calcd. for $C_{64}H_{84}O_{18}N_{12}\cdot H_2O$: C, 60.83; H, 6.86; N, 13.30%.

The air-dried product lost 1.40% of its weight after it had been dried for 2 hr. at 115°C, 2 mmHg. Calcd. for H_2O: 1.43%. The molecular weight[13] was determined by the micro Rast method using a solvent of hexahydro-*p*-aminobenzoic acid lactam.[14]

Found: 1100. Calcd. for $C_{64}H_{84}O_{18}N_{12}$: 1245.

b) From XXIV.—XXIV (765 mg.) was converted to the decapeptide active ester trifluoroacetate (XXVII) as has been described above. XXVII was allowed to react in pyridine (180 ml.), after with a treatment similar to that mentioned above was further carried out to give a crude cyclic product (284 mg.). This was recrystallized from methanol-ethyl acetate-ether; yield of the air-dried product, 233 mg. (36% from XXIII); m. p. 274—276°C (decomp.); $[\alpha]_D^{20}$ −4.9° (*c* 1, dimethylformamide); R_f 0.90.[10]

Found: C, 60.70; H, 6.82; N, 13.35. Calcd. for $C_{64}H_{84}O_{14}N_{12}\cdot H_2O$: C, 60.83; H, 6.86; N, 13.30%.

The molecular weight was determined by the micro Rast method described above. Found: 1030. Calcd. for $C_{64}H_{84}O_{14}N_{12}$: 1245.

Cyclo-(glycyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl)$_2$ Dihydrochloride (II·2HCl). — A solution of XXVI (84 mg.) in glacial acetic acid (2 ml.) was subjected to hydrogenolysis in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness in vacuo. The residue was dissolved in a mixture of methanol (1 ml.) and dioxane (1 ml.), and then 0.4 N hydrogen chloride in dioxane (0.5 ml.) was added to the solution. After the solution had then been evaporated in vacuo, the resulting crystals were filtered with the aid of a mixture of ether and petroleum ether (1 : 1); yield of the air-dried product, 59 mg. (84%); m. p. 239—242°C (decomp.); $[\alpha]_D^{20}$ −42.0° (*c* 0.5, acetic acid); R_f 0.88[8] and 0.65.[10] The amino acid analysis[3] gave the molar ratio of 1.9 : 1.1 : 1.0 : 1.1 for glycine, ornithine, leucine and phenylalanine.

Found: C, 51.52; H, 7.47; N, 14.91. Calcd. for $C_{48}H_{74}O_{10}N_{12}Cl_2\cdot 4H_2O$: C, 51.37; H, 7.37; N, 14.98%.

The air-dried product lost 6.6% of its weight after it had been dried over phosphorus pentoxide for 3 hr. at 80°C; 2 mmHg. Calcd. for $4H_2O$: 6.4%.

***p*-Methoxybenzyloxycarbonyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycylglycine *p*-Nitrophenyl Ester (XXVIII).**—To a solution of XI (393 mg.) in a mixture of dimethylformamide (1 ml.) and pyridine (1,2 ml.), di-*p*-nitrophenyl sulfite

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[13] The molecular weight determination by the use of an osmometer of Mechrolab, Inc., could not be applied to the protected cyclic decapeptide (XXVI) and pentapeptide (XXXI) because these products are insoluble in methanol or dioxane at room temperature.

[14] G. Went, *Ber.*, **75**, 425 (1942).

(640 mg.) was added. The mixture was then allowed to stand overnight at room temperature. After evaporation, the residual oil was triturated with petroleum ether and washed with a mixture of ether and petroleum ether (1 : 1) by decantation until no yellow color could be discerned on the addition of a sodium hydroxide solution to the washings. The product was collected by filtration and dried. It weighed 373 mg. The *p*-nitrophenyl ester content of this product was estimated to be 70%.

***p*-Methoxybenzyloxycarbonylglycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycine *p*-Nitrophenyl Ester (XXIX).**—A solution of XX (403 mg.) and di-*p*-nitrophenyl sulfite (975 mg.) dissolved in pyridine (3 ml.) was allowed to stand overnight at room temperature; then it was treated as above. The *p*-nitrophenyl ester content of this product was estimated to be 94%.

Cyclo-glycyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl (XXXI). — *a) From XXVIII.*—XXVIII (368 mg.) and anisole (0.2 ml.) were dissolved in trifluoroacetic acid (1 ml.) at −5°C. After 20 min., the solution was concentrated to a small volume in vacuo at 0°C, and then the residue was triturated with ether. The *p*-nitrophenyl ester trifluoroacetate (XXX) collected was dissolved in dimethylformamide (4 ml.) containing glacial acetic acid (0.1 ml.). The solution was stirred, drop by drop, into pyridine (80 ml.), which had been kept at 55—60°C over a period of 4 hr.; the stirring was then continued for an additional 2 hr. at the same temperature. After the solvent had been removed, the residue was dissolved in a mixture of methanol (50 ml.) and water (20 ml.). The insoluble substance was filtered off, and the filtrate was passed through columns of Dowex 1 and Dowex 50. The columns were eluted with the same solvent, the combined effluents were evaporated to dryness in vacuo, and the residue was collected by filtration with the aid of water (77 mg.). It was recrystallized from methanol-ether; yield, 71 mg. (32% form XI); m. p. 282—283°C (decomp.); $[\alpha]_D^{15}$ −49.7° (*c* 1.01, dimethylformamide); R_f 0.79.[10]

Found: C, 61.63; H, 6.67; N, 13.41. Calcd. for $C_{32}H_{42}O_7N_6$: C, 61.72; H, 6.80; N, 13.50%.

The molecular weight was determined by the micro Rast method. Found: 600. Calcd. for $C_{32}H_{42}O_7N_6$: 623.

b) From XIII.—To XIII (234 mg.) and anisole (0.1 ml.), trifluoroacetic acid (0.32 ml.) was added at −5°C. After 30 min., the solution was evaporated in vacuo at 0°C, and the residue was triturated with ether. The hydrazide ditrifluoroacetate (XXXII) was collected by filtration. To a solution of XXXII in water (2 ml.), 0.1 N hydrochloric acid (3.1 ml.) and sodium nitrite (22.3 mg.) in water (0.5 ml.) were added at −5°C. After 15 min., the reaction mixture was added to a solution of water (200 ml.) containing sodium bicarbonate (1.2 g.) and dioxane (100 ml.) at 0°C. The mixture was stirred for 2 days at 0°C; then it was brought to pH 5.0 with N hydrochloric acid. The mixture was evaporated in vacuo, and the residue was collected by filtration with the aid of 5 ml. of water (120 mg.). This was treated in a mixture of methanol (90 ml.) and water (30 ml.). The insoluble substance was filtered off, and then the filtrate was passed through columns, as has been mentioned above. The combined effluents were evaporated in vacuo to dryness; yield of crude product, about 8% from XIII. A solution, after the hydrogenolysis of the crude product in methanol containing hydrogen chloride, showed only one spot on paper and thin-layer chromatograms, a spot which was in a position identical with that of III·HCl obtained from XXX or XXXIII.

c) From XXIX.—XXIX (460 mg.) was converted to the pentapeptide active ester trifluoroacetate (XXXIII) by the method described for XXX. XXXIII was allowed to react in pyridine (160 ml.), and the reaction mixture was treated as has been described in the case of XXXI a). The combined effluents obtained after passing through the ion-exchangers were evaporated to dryness; the crude product which resulted weighed 136 mg. (XXXIV). A few mg. of XXXIV was subjected to hydrogenolysis, and the solution was submitted to CM-cellulose column chromatography and paper electrophoresis. The chromatography showed only one peak or spot resulting from presence of III. Therefore, it was concluded that the product (XXXIV) contains only protected cyclic pentapeptide (XXXI). XXXIV was recrystallized from methanol-ether; yield, 115 mg. (37% from XX); m. p. 281—282°C (decomp.); $[\alpha]_D^{20}$ −50.1° (*c* 1, dimethylformamide); R_f 0.80.[10]

Found: C, 61.70; H, 7.04; N, 13.35. Calcd. for $C_{32}H_{42}O_7N_6$: C, 61.72; H, 6.80; N, 13.50%.

The molecular weight was determined by the micro Rast method. Found: 610. Calcd. for $C_{32}H_{42}O_7N_6$: 623.

Cyclo-glycyl-L-ornithyl-L-leucyl-D-phenylalanylglycyl Hydrochloride (III·HCl).—A solution of XXXI (53.2 mg.) in glacial acetic acid (1 ml.) was subjected to hydrogenolysis in the presence of palladium black, and then treated with the method which has been described in the case of II·2HCl. Yield of the air-dried product, 38.2 mg. (85%); m. p. 270—272°C (decomp.); $[\alpha]_D^{20}$ −54.4° (*c* 0.5, acetic acid); R_f 0.81[8] and 0.61.[10] The amino acid analysis[3] gave the molar ratio of 1.9 : 1.1 : 1.0 : 1.0 for glycine, ornithine, leucine and phenylalanine.

Found: C, 53.47; H, 7.09; N, 15.17. Calcd. for $C_{24}H_{37}O_5N_6Cl\cdot H_2O$: C, 53.08; H, 7.23; N, 15.47%.

The air-dried product lost 3.19% of its weight after it had been dried over phosphorus pentoxide 3 hr. at 80°C; 2 mmHg. Calcd. for H_2O: 3.31%.

Chromatography and Electrophoresis of II, III and Gramicidin S (I).—A sample (0.5—1 mg.) was dissolved in 0.2—0.3 ml. of 0.2 M pyridinium acetate containing 30% methanol (pH 5.1); the solution was then applied to a column (0.9×50 cm.) with carboxymethyl cellulose (Eastmen Organic Chem. 7796), and development was continued with the same solvent. Two-ml. fractions were collected at a flow rate of 20 ml. per hour. The peptide content in the fractions was determined by the method described by Yemm and Cocking.[15] Gramicidin S was used as a control. The results are shown in Fig. 6. In order to determine the sensibilities for ninhydrin with the cyclic peptides, II and III, a solution containing 1 μ mole of III and 0.5 μ mole of II was also submitted to column chromatography; the ratio of intensities caused by ninhydrin between III and II was found to be 5 : 4 by calculating

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[15] E. W. Yemm and E. C. Clcking, *Analyst*, **80**, 209 (1955); *Biochem. J.*, **58**, xii (1954).

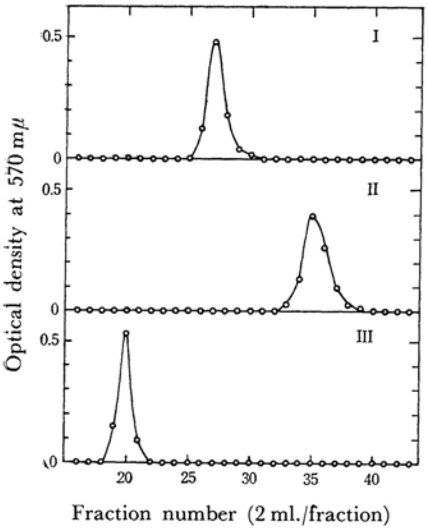

Fig. 6. Carboxymethylcellulose column chromatography of II, III and gramicidin S (I).

the areas in a chromatogram of the optical density-fraction number.

Electrophoresis on Toyo Roshi No. 52 paper was carried out with a formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10, v/v; pH 1.8) solvent system for 3 hr. at 500 V./30 cm. The peptides were detected with ninhydrin. Figure 7 shows that II migrates faster toward the cathode than III, and that the mobility of II is indistinguishable from that of gramicidin S (I).

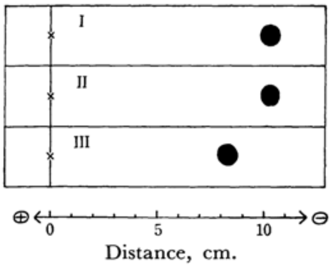

Fig. 7. Paper electrophoresis of II, III and gramicidin S (I).

Microbiological Assays. — The microorganisms employed are *E. coli, P. vulgaris, S. aureus, B. subtilis* and *M. avium*. A minimum amount of compound for complete inhibition of growth was determined by a dilution method with a bouillon agar medium and with a synthetic medium. Synthetic cyclic peptides II and III exhibited no antibacterial activity against any of the microorganisms tested even at levels of 100 γ per ml. of assays medium, whereas gramicidin S exhibited considerable activity against some of the microorganisms.

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