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Studies of Peptide Antibiotics. VII. The Synthesis of Cyclo-D-phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-prolyl

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A cyclic hexapeptide, cyclo-D-phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-prolyl, was once reported to be an antibiotic gramicidin J_2, and the synthesis of the compound was described also. However, the natural occurrence of gramicidin J_2 was denied recently. For the purpose of comparing its antibacterial activity, if any, with gramicidin S, the synthesis of this compound was carried out. *p*-Methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-*δ*-benzyloxycarbonyl-L-ornithyl-L-valyl-*δ*-benzyloxycarbonyl-D-ornithyl-L-proline *p*-nitrophenyl ester was prepared, and, after the selective cleavage of *p*-methoxybenzyloxycarbonyl group, the hexapeptide ester was transformed to the cyclic benzyloxycarbonyl hexapeptide, which was then hydrogenated to afford the desired cyclic hexapeptide as dihydrochloride. The effects of the synthesized cyclic peptide on bacterial growth have been examined. No antibacterial activities were observed in reaction to the microorganisms utilized here.

A number of polypeptide antibiotics, such as gramicidin S, have three characteristic features in common; namely, the occurrence of D-amino acids, basic amino acids, and a cyclic structure in the molecule. For a study of the relationship between the chemical structure and the biological activities of peptide antibiotics, some dipeptide anhydrides, which are the simplest compounds possessing the characteristics mentioned above, were synthesized; however, these were all devoid

of any antibacterial activity.[1] A cyclic hexapeptide, cyclo-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycyl, which contains the partial amino acid sequence found in gramicidin S and tyrocidines, was then prepared, and its biological activity examined. However, in spite of its apparent resemblance to those natural antibiotics, this compound also was found to be inactive.[2] Thus, it was concluded that even the occurrence of a hexapeptide ring structure may not be a sufficient condition for a molecule to exhibit antibacterial activity. These results prompted the present study of another cyclic hexapeptide, cyclo-D-phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-prolyl (XXIV), which has been demonstrated to be an antibiotic named gramicidin J_2.[3]

D-Phe-D-Leu-L-Orn-L-Val-D-Orn-L-Pro

XXIV

Previously Noda[3] isolated an antibiotic peptide from a culture of *Bacillus brevis Nagano* and proposed its chemical structure to be that shown as XXIV. He furthermore reported a method of the chemical synthesis of the compound corresponding to XXIV, and described that the product synthesized was identical with natural gramicidin J_2.[4] Nevertheless, Kurahashi[5] and Otani and Saito[6] reported that natural gramicidin J_2 is the same compound as gramicidin S, a cyclic decapeptide.

To elucidate the ambiguity described above, the present authors attempted to synthesize the cyclic hexapeptide with the structure of XXIV and to test its antibacterial activity.

The sequence of reaction employed for the synthesis of the acylhexapeptide ester (XVII) is indicated in Figs. 1 and 2. The proline residue was selected as the *C*-terminal amino acid in a linear hexapeptide in order to avoid otherwise possible racemization. *p*-Methoxybenzyloxycarbonyl and benzyloxycarbonyl groups were employed for the selective blocking of the *N*-terminal amino group and the δ-amino group of ornithine residue respectively.

As is shown in Fig. 1, the *C*-terminal dipeptide derivative, the *t*-butyloxycarbonyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline ethyl ester (IV), was prepared via three different methods: (a) the mixed anhydride method,[7] (b) the active ester method using the diacyl D-ornithine *p*-nitrophenyl ester (III),[8] and (c) the dicyclohexylcarbodiimide method.[9] The last method gave the highest yield. The protected tripeptide ester (IX) was secured via two ways; (a) stepwise elongation from *C*-terminal amino acid, and (b) fragment condensation via the azide method. The former method gave a slightly better yield, but the latter method furnished two well-defined crystalline intermediate compounds, VII and VIII.

As is shown in Fig. 2, the *N*-terminal tripeptide derivative, the *p*-methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithine ethyl ester (XV), was obtained via stepwise elongation and converted to the corresponding hydrazide (XVI). However, the protected hexapeptide ester (XVII), derived from the azide of XVI and X, showed resistance to saponification. Hence, the preparation of the protected hexapeptide (XX) was carried out as is shown in Fig. 3.

Figure 3 indicates the route for the synthesis of the cyclic hexapeptides (XXIII, XXIV). XX was prepared by the coupling reaction of tripeptide azide (from XVI) with neutral free tripeptide (from XIX), and then XX was converted to the protected hexapeptide *p*-nitrophenyl ester (XXII). The removal of the *p*-methoxybenzyloxycarbonyl group from XXII by treatment with trifluoroacetic acid yielded the hexapeptide *p*-nitrophenyl ester trifluoroacetate. The cyclization reaction of the trifluoroacetate in pyridine gave the benzyloxycarbonyl-substituted cyclic peptide (XXIII), which was then purified by passing it through columns of Dowex 50 (H^+ form) and Dowex 1 (OH^- form). The molecular-weight determination using a vapor pressure osmometer demonstrated that the molecular size of XXIII corresponds to that of cyclic hexapeptide. The final product, XXIV, was obtained, upon the hydrogenolysis of XXIII in the presence of two equivalents of hydrogen chloride, as crystalline dihydrochloride, with five moles of water. The treatment of XXIII with hydrogen bromide in acetic acid furnished the corresponding cyclohexapeptide hydrobromide (the hydrobromide of XXIV). The homogeneity and identity of the two products ($XXIV \cdot 2HCl \cdot 5H_2O$ and hydrobromide of XXIV) was established by paper, thin-layer, and carboxymethyl cellulose column chromatography and by paper electrophoresis.

The chromatographic behavior and several other characteristics of the synthesized product, $XXIV \cdot 2HCl \cdot 5H_2O$, were compared with those

1) N. Izumiya, T. Kato, Y. Fujita, M. Ohno and M. Kondo, This Bulletin, **37**, 1809 (1964).
2) T. Kato, M. Kondo, M. Ohno and N. Izumiya, ibid., **38**, 1202 (1965).
3) Y. Noda, *J. Chem. Soc. Japan, Pure Chem. Sect. (Nippon Kagaku Zasshi)*, **79**, 662 (1958).
4) Y. Noda, ibid., **80**, 411 (1959).
5) K. Kurahashi, *J. Biochem.*, **56**, 101 (1964).
6) S. Otani and Y. Saito, ibid., **56**, 103 (1964).
7) J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951); J. R. Vaughan, Jr., and J. A. Eichler, ibid., **75**, 5556 (1953); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland, W. Kern and R. Sehring, *Ann.*, **569**, 117, 122 (1950).
8) M. Bodansky, *Nature*, **175**, 685 (1955); M. Bodansky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
9) J. C. Sheehan and G. P. Hess, ibid., **77**, 1067 (1955).

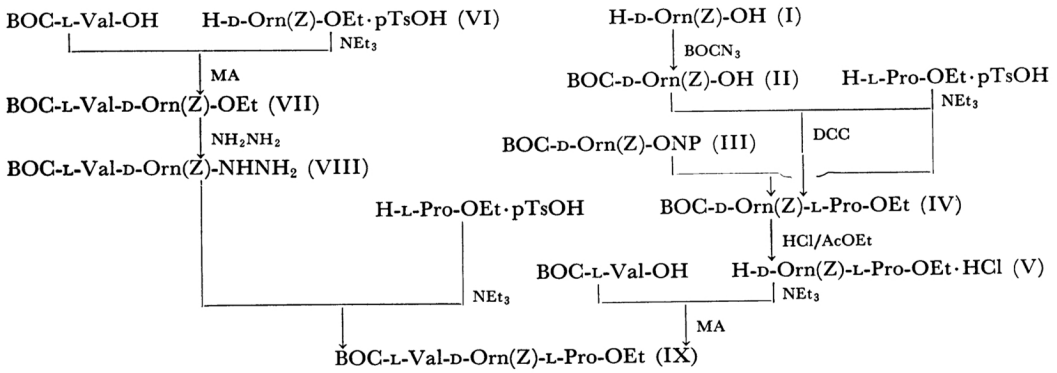

Fig. 1. The abbreviations used are: BOC, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; pMZ, *p*-methoxybenzyloxycarbonyl; NEt_3, triethylamine; DCC, dicyclohexylcarbodiimide; MA, mixed anhydride; OEt, ethyl ester; ONP, *p*-nitrophenyl ester; pTsOH, *p*-toluenesulfonic acid.

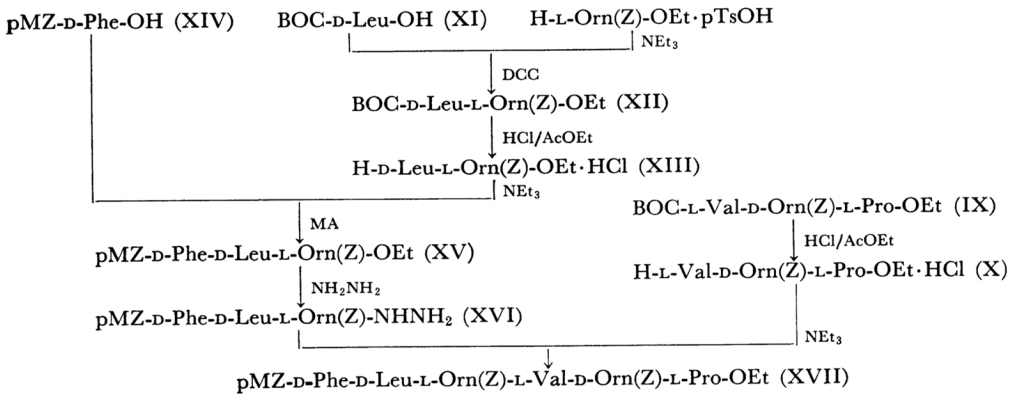

Fig. 2.

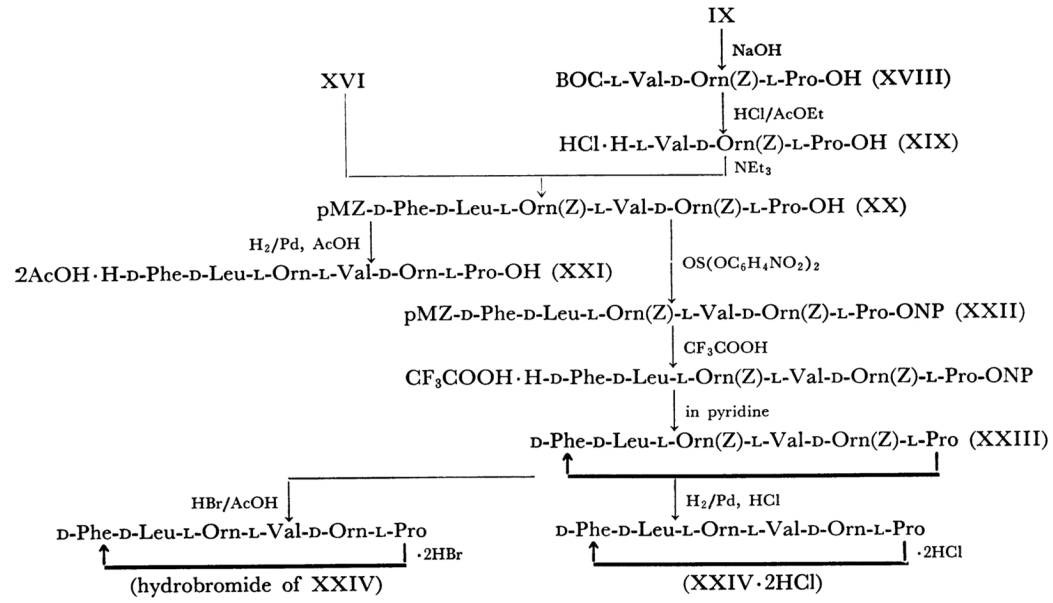

Fig. 3.

Table I. Comparison of XXIV with gramicidin S

	XXIV·2HCl·5H_2O	XXIV·2HCl by Noda[4]	Gramicidin S
Melting point	242—244°C (decomp.)	279—280°C (decomp.)	277—278°C (decomp.)[a,10]
Specific rotation	$+15.3°\left(\begin{smallmatrix}c\ 0.3,\ \mathrm{EtOH}\\ t=18°\mathrm{C}\end{smallmatrix}\right)$	$-313.1°\left(\begin{smallmatrix}c\ 0.4,\ \mathrm{EtOH}\\ t=14°\mathrm{C}\end{smallmatrix}\right)$	$-295°\left(\begin{smallmatrix}70\%\ \mathrm{EtOH}\\ t=21°\mathrm{C}\end{smallmatrix}\right)$[a,11]
R_f on silica gel	0.69		0.80[b]
on paper[c]	0.64	0.90	0.94[b]
Electrophoretic mobility (related to lysine) in solvent (i) pH 1.8	0.64		0.60[b]
(ii) pH 7.0	0.95		0[b]

a) Reported value for gramicidin S dihydrochloride.
b) Observed value for gramicidin S sulfate octahydrate, Astra Co., U.S.A.
c) The solvent used in this case was *n*-butanol - acetic acid - water (4: 1: 1, v/v).

of gramicidin S. These two cyclic peptides were quite different from each other in chromatographic behavior. Moreover, such constants as the specific rotation of XXIV·2HCl·5H_2O observed were quite different from those of XXIV·2HCl reported by Noda.[4] Table I compares the physical constants and R_f values of XXIV·2HCl·5H_2O synthesized in this paper and described by Noda,[4] and those of gramicidin S.

The antibacterial activity of XXIV·2HCl·5H_2O and gramicidin S in reaction to several microorganisms were examined. XXIV·2HCl·5H_2O had no retarding effect on the growth of the microorganisms, even at so high a concentration as 100γ per ml. of the assay medium. Gramicidin S, however, showed substantial activity under these conditions. It should be noted that the antibacterial activity of the product, XXIV·2HCl, synthesized by Noda[4] was reported to be the same as that of gramicidin S.

The findings described in this paper are in marked disagreement with the observations of Noda.[4] The authors have no explanation for the discrepancy between their results and those of Noda.[4,12]

Experimental

All the melting points are uncorrected. The paper chromatography was carried out on Toyo Roshi No. 52 paper. The thin-layer chromatography was carried out on Merck silica gel G. The developing solvent commonly used was *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2 v/v). Spots of materials possessing a free amino group on a thin-layer plate were detected by spraying ninhydrin, and those of the amino-group-blocked materials, by spraying 47% hydrobromic acid and then ninhydrin. Prior to analysis, the compounds were dried over phosphorus pentoxide to a constant weight at 80°C and 2 mmHg.

Amino Acids.—The amino acids used were as follows: D-phenylalanine, $[\alpha]_D^{24}$ +33.5° (*c* 2, water) (lit. for the L-isomer, −34.5°[13]), was obtained from acetyl-DL-phenylalanine through the asymmetric hydrolytic action of hog renal acylase.[14] D-Leucine, $[\alpha]_D^{24}$ +15.5° (*c* 2, 5 N HCl) (lit. for L-isomer −16.0°[13]), L-ornithine hydrochloride, $[\alpha]_D^{24}$ +21.8° (*c* 2, 5 N HCl) (lit. +22.2°[13]), L-valine, $[\alpha]_D^{24}$ +27.7° (*c* 2, 5 N HCl) (lit. +28.3°[13]), and L-proline, $[\alpha]_D^{24}$ −59.7° (*c* 2, 5 N HCl) (lit. −60.4°[13]), were commercial products. D-Ornithine hydrochloride, $[\alpha]_D^{24}$ −22.6° (*c* 2, 5 N HCl) (lit. for L-isomer +22.2°[13]), was obtained from DL-ornithine hydrochloride through optical resolution, using D-glutamic acid as the resolving reagent.[15]

δ-Benzyloxycarbonyl-D-ornithine (I).—This compound was obtained according to the procedure for the L-isomer.[16] Yield, 87%; m. p. 255°C; $[\alpha]_D^{24}$ −21.1° (*c* 1, 6 N HCl), −21.3° (*c* 1, 50% acetone + 2 eq. HCl) (lit. for L-isomer +17.0°[16]).

Found: C, 58.47; H, 6.85; N, 10.53. Calcd. for $C_{13}H_{18}O_4N_2$: C, 58.63; H, 6.81; N, 10.52%.

α-*t*-Butyloxycarbonyl - δ - benzyloxycarbonyl - D - ornithine (II).—To a stirred solution of I (2.295 g., 8.6 mmol.) and sodium bicarbonate (2.94 g., 35 mmol.) in water (50 ml.) at 50°C, a solution of *t*-butyloxycarbonyl azide[17] (2.419 g., 17 mmol.) in dioxane (50 ml.) was added. After 2 days' stirring at 45°C, the reaction mixture was evaporated to a half volume and extracted with ether. The aqueous layer was then acidified and extracted with ethyl acetate, and the organic layer was dried and evaporated. The residual oil weighed 2.574 g. (82%).

α-*t*-Butyloxycarbonyl - δ - benzyloxycarbonyl - D - ornithine *p*-Nitrophenyl Ester (III).—A solution of II (733 mg., 2 mmol.), dicyclohexylcarbodiimide[9] (412 mg., 2 mmol.) and *p*-nitrophenol (333 mg., 2.4 mmol.) in tetrahydrofuran was stirred for 3 hr. at 0°C, and then for 2.5 hr. at room temperature. After filtration,

10) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).
11) R. L. M. Synge, *Biochem. J.*, **39**, 363 (1945).
12) It may be pointed out that in his paper Noda reported that he had used di-*p*-nitrophenyl sulfide instead of di-*p*-nitrophenyl sulfite in the preparation of the *p*-nitrophenyl ester of trityl-D-phenylalanyl-D-leucyl-δ-tosyl-L-ornithyl-L - valyl - δ - tosyl - D - ornithyl-L-proline, and cited the literature (Org. Syntheses, Vol. 28, p. 82) on the synthesis of di-*p*-nitrophenyl sulfide.
13) J. P. Greenstein and M. Winitz, "The Chemistry of Amino Acid," John Wiley & Sons, New York (1961).
14) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).
15) M. Kondo and N. Izumiya, unpublished.
16) J. I. Harris and T. S. Work, *Biochem. J.*, **46**, 582 (1950).
17) R. Schwyzer, P. Sieber and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

the solution was evaporated to an oil. After the addition of a mixture of ether and petroleum ether (1 : 1, 4 ml.), the solution was stored in a refrigerator overnight. The crystals were filtered off, washed with a mixture of ether and petroleum ether (1 : 1), and dried (683 mg.). Recrystallization from ethyl acetate - ether - petroleum ether gave 611 mg. (63%); m. p. 108°C.

Found: C, 59.13; H, 6.00; N, 8.62. Calcd. for $C_{24}H_{29}O_8N_3$: C, 59.23; H, 5.99; N, 8.69%.

α-*t*-Butyloxycarbonyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline Ethyl Ester (IV).—*a) The Dicyclohexylcarbodiimide Method.*[9]—To a solution of II (1.042 g., 2.85 mmol.), L-proline ethyl ester *p*-toluenesulfonate[18] (967 mg., 3 mmol.), and triethylamine (0.46 ml.) in tetrahydrofuran (6 ml.), dicyclohexylcarbodiimide (618 mg.) was added at 0°C. After the stirring had been continued for 2 hr. at 0°C, and for more 2 hr. at room temperature, the mixture was left to stand overnight. The mixture was then evaporated in vacuo, and the residue was diluted with ethyl acetate (20 ml.). The solution which was separated from the dicyclohexylurea by filtration was washed successively with water, a 4% sodium bicarbonate solution, water, 10% citric acid, and water, and then dried over anhydrous sodium sulfate. The filtrate separated from the salt was evaporated in vacuo. The residual oil weighed 1.316 g. (89%).

b) The p-Nitrophenyl Ester Method.—A solution of III (488 mg., 1 mmol.), L-proline ethyl ester *p*-toluenesulfonate[18] (378 mg., 1.2 mmol.), and triethylamine (0.15 ml.) in chloroform (4 ml.) was prepared and treated as usual.[8] Yield, 266 mg. (53%).

δ-Benzyloxycarbonyl-D-ornithyl-L-proline Ethyl Ester Hydrochloride (V).—To a solution of IV (488 mg., 1 mmol.) in ethyl acetate (5 ml.), 4.38 N hydrogen chloride in ethyl acetate (5 ml.) was added; the reaction mixture was then allowed to stand for 2 hr. at room temperature. After it had been evaporated to dryness, the residual oil weighed 377 mg. (88%).

δ-Benzyloxycarbonyl-D-ornithine Ethyl Ester *p*-Toluenesulfonate (VI).—This compound was prepared according to the procedure of Kato et al.[1] Yield, 80%; $[\alpha]_D^{21}$ −8.9° (*c* 2, dimethylformamide) (lit. for L-isomer +8.9°[1]).

Found: C, 56.47; H, 6.67; N, 5.95. Calcd. for $C_{22}H_{30}O_7N_2S$: C, 56.63; H, 6.48; N, 6.01%.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-D-ornithine Ethyl Ester (VII).**—To a freshly-prepared mixed anhydride of *t*-butyloxycarbonyl-L-valine[17] (2.004 g., 9.22 mmol.), triethylamine (1.29 ml., 9.22 mmol.) and isobutyl chloroformate (1.20 ml., 9.22 mmol.)[7] in tetrahydrofuran (20 ml.), a solution of VI (4.301 g., 9.22 mmol.) and triethylamine (1.29 ml.) in chloroform (20 ml.) was added. The mixture was then washed and dried as has been described for IV. To the dried solution, a mixture of ether and petroleum ether was added, and the solution was stored in a refrigerator. The crystals which precipitated were collected and dried (3.97 g.). Recrystallization from ethyl acetate - ether - petroleum ether gave 3.505 g. (77%); m. p. 123—125°C; $[\alpha]_D^{20}$ −8.2° (*c* 1, acetic acid).

Found: C, 60.70; H, 7.73; N, 8.30. Calcd. for $C_{25}H_{39}O_7N_3$: C, 60.83; H, 7.96; N, 8.51%.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-D-ornithine Hydrazide (VIII).**—A solution of VII (987 mg., 2 mmol.) and hydrazine hydrate (2 ml., 40 mmol.) in dimethylformamide (4 ml.) was allowed to stand for 2 days at room temperature. Upon the addition of water (40 ml.), colorless crystals of VIII were precipitated; these were filtered off, washed with water, and dried (912 mg., 95%); m. p. 174°C; $[\alpha]_D^{20}$ −9.6° (*c* 1, ethanol).

Found: C, 57.60; H, 7.61; N, 14.33. Calcd. for $C_{23}H_{37}O_6N_5$: C, 57.60; H, 7.78; N, 14.60%.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline Ethyl Ester (IX).**—*a) From V and t-Butyloxycarbonyl-L-valine.*—To a freshly-prepared mixed anhydride gel of *t*-butyloxycarbonyl-L-valine[17] (100 mg., 0.46 mmol.), triethylamine (0.064 ml.), and isobutyl chloroformate (0.060 ml.) in tetrahydrofuran (2 ml.), a solution of V (205 mg., 0.46 mmol.) and triethylamine (0.064 ml.) in chloroform (3 ml.) was added. The mixture was treated as has been described for VII. The oily residue obtained weighed 215 mg. (79%).

b) From VIII and L-Proline Ethyl Ester.—The following operations were carried out in a cold room. Into a chilled solution of VIII (735 mg., 1.53 mmol.) in a mixture of glacial acetic acid (2 ml.) and dimethylformamide (4 ml.), N hydrochloric acid (1.7 ml.), 116 mg. of sodium nitrite, and additional N hydrochloric acid (1.7 ml.) were stirred. After 7 min., cold water (40 ml.) was added. The azide which was precipitated as a white mass was extracted thrice with ethyl acetate; the combined extract was washed successively with cold water, a saturated sodium bicarbonate solution, and water, and then dried over anhydrous sodium sulfate. The filtered azide solution was added to a solution of L-proline ethyl ester *p*-toluenesulfonate[18] (483 mg., 1.53 mmol.) and triethylamine (0.21 ml.) in dimethylformamide (10 ml.), and the mixture was stirred for 3 days at 0°C. The solution was evaporated and washed successively with water, a 4% sodium bicarbonate solution, water, 10% citric acid, and water, and then dried over anhydrous sodium sulfate. The filtrate was evaporated in vacuo; the oily residue weighed 728 mg. (81%).

L-Valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline Ethyl Ester Hydrochloride (X).—A solution of IX (215 mg., 0.36 mmol.) in 2.6 N hydrogen chloride in ethyl acetate (2.8 ml., 7.2 mmol.) was allowed to stand for 2 hr. at room temperature, and then evaporated to dryness in vacuo; the residue weighed 194 mg. Recrystallization from acetone-ether gave 148 mg. (77%) as hygroscopic crystals. R_f 0.93 on paper.

***t*-Butyloxycarbonyl-D-leucine (XI).**—This compound was prepared according to the general procedure of Schwyzer et al.[17] As a slight alteration, sodium bicarbonate was used instead of magnesium oxide, and a 1.2 equivalent of *t*-butyloxycarbonyl azide was utilized per equivalent of amino acid; yield, 76% (lit. for L-isomer 73%,[17] 72%,[19]); m. p. 84°C (lit. for L-isomer 78—81°C[17]) 74—80°C[19]); $[\alpha]_D^{21}$ +25.2° (*c* 2, acetic acid) (lit. for L-isomer −24.0°C[19]).

Found: C, 53.04; H, 9.39; N, 5.73. Calcd. for

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

19) F. C. Mckay and N. F. Albertson, *J. Am. Chem. Soc.*, **72**, 4648 (1957).

$C_{11}H_{21}O_4N \cdot H_2O$: C, 52.99; H, 9.30; N, 5.62%.

***t*-Butyloxycarbonyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithine Ethyl Ester (XII).**—To a solution of XI (2.493 g., 10 mmol.) and dicyclohexylcarbodiimide[9] (2.06 g., 10 mmol.) in absolute tetrahydrofuran (20 ml.) at 0°C, a solution of δ-benzyloxycarbonyl-L-ornithine ethyl ester *p*-toluenesulfonate[1] (4.665 g., 10 mmol.) and triethylamine (1.4 ml., 10 mmol.) in chloroform (20 ml.) was added. The mixture was stirred for 3 hr. at 0°C and then allowed to stand overnight at room temperature. After the solvent had been removed by evaporation in vacuo, the resultant oily residue was dissolved in ethyl acetate (50 ml.) and the mixture was stored in a refrigerator for several hours. After it had been filtered from the formed dicyclohexylurea, the solution was washed and dried as has been described for IV(a). The filtrate was evaporated in vacuo, and the residual oil was solidified after pertoleum ether (20 ml.) had been added and the mixture had been stored in a refrigerator for several days. Recrystallization from ethyl acetate - petroleum ether gave 3.913 g. (77%); m. p. 88°C; $[\alpha]_D^{21}$ +11.0° (*c* 2, acetic acid).

Found: C, 61.77; H, 8.05; N, 8.46. Calcd. for $C_{26}H_{41}O_7N_3$: C, 61.52; H, 8.14; N, 8.28%.

D-Leucyl-δ-benzyloxycarbonyl-L-ornithine Ethyl Ester Hydrochloride (XIII).—To a solution of XII (254 mg., 0.5 mmol.) in ethyl acetate (2 ml.), 4.38 N hydrogen chloride in ethyl acetate (2.3 ml., 10 mmol.) was added. When, after 3 hr. at room temperature, the solution was evaporated to dryness in vacuo, hygroscopic crystals were obtained (211 mg., 96%). R_f 0.93 on paper.

***p*-Methoxybenzyloxycarbonyl-D-phenylalanine (XIV).**—To a solution of D-phenylalanine (1.652 g., 10 mmol.) and sodium bicarbonate (2.52 g., 30 mmol.) in water (30 ml.), a solution of *p*-methoxybenzyloxycarbonyl azide[20] (2.693 g., 13 mmol.) in dioxane (30 ml.) was added; the reaction mixture was stirred for 48 hr. at room temperature, and then evaporated in vacuo. The residual aqueous solution was extracted twice with ether (20 ml. each) and acidified with 10% citric acid (40 ml.). The acidified mixture was extracted thrice with ethyl acetate (30 ml. each), and the combined extract was washed with water, dried over sodium sulfate, and evaporated. The residual oil solidified upon the addition of petroleum ether. Recrystallization from ethyl acetate - petroleum ether gave 2.555 g. (77%); m. p. 81—88°C; $[\alpha]_D^{24}$ −5.7° (*c* 2, acetic acid) (lit. for L-isomer +5.7°[20]).

Found: C, 65.61; H, 6.05; N, 4.53. Calcd. for $C_{18}H_{19}O_5N$: C, 65.64; H, 5.82; N, 4.25%.

***p*-Methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithine Ethyl Ester (XV).**—To a solution of XIV (988 mg., 3 mmol.) and triethylamine (0.42 ml.) in tetrahydrofuran (10 ml.), isobutyl chloroformate (0.4 ml., 3 mmol.) was added at 0°C. After 20 min., a solution of XIII (1.40 g., 3 mmol.) and triethylamine (0.42 ml.) in chloroform (10 ml.) was added thereto, and the reaction mixture was allowed to stand overnight at room temperature. The mixture was then concentrated in vacuo, and water (100 ml.) and petroleum ether were added. The crystals were collected by filtration, washed with 10% citric acid, water, a 4% sodium bicarbonate solution, and water, and then dried; 1.598 g. (74%). Recrystallization from ethanol - ether - petroleum ether gave 1.394 g. (65%); m. p. 159°C; $[\alpha]_D^{21}$ +8.4° (*c* 2.01, acetic acid).

Found: C, 64.84; H, 6.95; N, 7.86. Calcd. for $C_{39}H_{50}O_9N_4$: C, 65.16; H, 7.01; N, 7.82%.

***p*-Methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithine Hydrazide (XVI).**—XV was converted to the hydrazide as in the case of VIII. Yield, 97%; m. p. 205°C; $[\alpha]_D^{24}$ +9.7° (*c* 1.0, dimethylformamide).

Found: C, 63.10; H, 6.94; N, 11.95. Calcd. for $C_{37}H_{48}O_8N_6$: C, 63.05; H, 6.86; N, 11.93%.

***p*-Methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithyl-L-valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline Ethyl Ester (XVII).**—Into a chilled solution of XVI (310 mg., 0.44 mmol.) in a mixture of glacial acetic acid (2.5 ml.) and dimethylformamide (2 ml.), 1.09 N hydrochloric acid (0.5 ml.), 33 mg. of sodium nitrite, and additional 1.09 N hydrochloric acid were stirred. After 6 min., cold water (20 ml.) was added. The azide which precipitated was filtered off, washed successively with cold water, a sodium bicarbonate solution, and water, and then dried under a vacuum in a desiccator. The azide was added to a solution of X (236 mg., 0.44 mmol.) and triethylamine (0.061 ml.) in dimethylformamide (4 ml.), and the mixture was stirred for 3 days at 0°c. The insoluble substance was removed by filtration. The precipitate which formed upon the addition of water (40 ml.) to the filtrate was collected, washed with 0.5 M citric acid, a 4% sodium bicarbonate solution and water, and dried. Recrystallization from dioxane-ether gave 366 mg. (71%); m. p. 168—170°C; $[\alpha]_D^{22}$ −14.4° (*c* 2, acetic acid).

Found: C, 63.02; H, 7.06; N, 9.51. Calcd. for $C_{62}H_{82}O_{14}N_8 \cdot H_2O$: C, 63.03; H, 6.99; N, 9.49%.

XVII was only partially saponified, even if it was treated with ten equivalents of alkali for 48 hr. at 30°C.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline (XVIII).**—To a solution of IX (728 mg., 1.23 mmol.) in methanol (6 ml.), N sodium hydroxide (1.61 ml.) was added; the solution was then allowed to stand for 3 hr. at room temperature. After the addition of water (6 ml.), the solution was evaporated in vacuo. After filtration, the solution was acidified with 0.5 M citric acid (6 ml.) and extracted with ethyl acetate three times (20 ml. each); then the combined extract was dried over anhydrous sodium sulfate. The filtrate from the salt was evaporated in vacuo to yield an oily product; 654 mg. (94%).

L-Valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline Hydrochloride (XIX).—To a solution of XVIII (654 mg., 1.16 mmol.) in dioxane (2 ml.), 4.2 N hydrogen chloride in dioxane (5.5 ml.) was added. After 2 hr. at room temperature, the solution was evaporated in vacuo to afford hygroscopic powder. Yield, 522 mg. (90%); R_f 0.61 on paper, 0.54 on silica gel.

***p*-Methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithyl-L-valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline (XX).**—The powder of azide drived from XVI (895 mg., 1.27 mmol.), N hydrochloric acid (2.8 ml.), and sodium nitrite

20) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

(97 mg.) in a mixture of acetic acid (8 ml.) and dimethylformamide (2 ml.) were collected, washed, and dried as in the case of XVII. Then the azide was added to a solution of XIX (619 mg., 1.21 mmol.) and triethylamine (0.169 ml.) in dimethylformamide (10 ml.), and the mixture was stirred for 3 days at 0°C. Then the filtered solution was evaporated in vacuo, and the residual oil solidified upon the addition of ethyl acetate (4 ml.) and ether (20 ml.). The collected precipitate was washed with 10% citric acid and water. Recrystallization from dioxane - methanol - ether gave 893 mg. (65%); m. p. 163°C; $[\alpha]_D^{21}$ −15.7° (*c* 2, acetic acid).

Found: C, 63.26; H, 7.01; N, 9.77. Calcd. for $C_{60}H_{78}O_{14}N_8$: C, 63.47; H, 6.93; N, 9.87%.

D-Phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-proline Diacetate (XXI). — XX (9.0 mg., 0.007 mmol.), dissolved in a mixture of methanol (0.5 ml.), acetic acid (0.2 ml.) and water (0.3 ml.), was subjected to hydrogenolysis in the presence of palladium black. After being filtered from the catalyst, the solution was evaporated to dryness in vacuo. The hygroscopic powder which remained was collected with the aid of ether; yield, 6.3 mg.; R_f 0.43 on paper, and 0.47 on silica gel. The amino acid analysis gave the molar ratio of 1.0 : 1.0 : 2.2 : 0.8 : 1.1 for phe : leu : orn : val : pro.

***p*-Methoxybenzyloxycarbonyl-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithyl-L-valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proline *p*-Nitrophenyl Ester (XXII).**—To a solution of XX (795 mg., 0.7 mmol.) in pyridine (14 ml.), di-*p*-nitrophenyl sulfite[21] (1.814 g., 5.6 mmol.) was added. The reaction mixture was allowed to stand for 24 hr. at room temperature. After the solvent had been evaporated, the oily product was triturated in petroleum ether and washed repeatedly with a mixture of ether and petroleum ether (1 : 1) by decantation. The residual solid was filtered off and washed with a mixture of ether-petroleum ether until no yellow could be discerned upon the addition of N sodium hydroxide to the filtrate. The yield was 826 mg. The *p*-nitrophenyl-ester content in this product was spectrophotometrically estimated to be 80% by measuring the optical density of the compound at 412 mμ.[10,22]

Cyclo-D-phenylalanyl-D-leucyl-δ-benzyloxycarbonyl-L-ornithyl-L-valyl-δ-benzyloxycarbonyl-D-ornithyl-L-proyly (XXIII).—Anisole (0.5 ml.) and trifluoroacetic acid (4 ml.) were added to XXII (826 mg.) below 0°C. The solution was then evaporated in vacuo at 0°C, and the residue was triturated with ether. The hexapeptide *p*-nitrophenyl ester trifluoroacetate was collected in a cold room, washed with a mixture of ether and petroleum ether and dried in vacuo. The dried trifluoroacetate was then dissolved in dimethylformamide (10 ml.) containing 0.2 ml. of acetic acid, and the solution was stirred, drop by drop, into pyridine (200 ml.) which had been kept at 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 removed by filtration, and the filtrate was passed successively through the columns (2×10 cm. each) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (140 ml.), and the collected effluent was evaporated to dryness in vacuo. The residual product was dissolved in methanol, and the solution was evaporated. The residue was triturated and pulverized in a mixture of ether and petroleum ether (1 : 1). Recrystallization from ethyl acetate - ether - petroleum ether gave 322 mg. (48% form XX); m. p. 184°C; $[\alpha]_D^{24}$ +7.3° (*c* 1.01, acetic acid).

Found: C, 64.22; H, 7.59; N, 11.26. Calcd. for $C_{51}H_{68}O_{10}N_8$: C, 64.26; H, 7.19; N, 11.75%.

The molecular weight of XXIII was determined by a model 301 A Osmometer, Mechrolab Inc. (solvent methanol).[23]

Found: 955. Calcd. 953.

Cyclo-D-phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-prolyl Dihydrochloride (XXIV·2HCl).—XXIII (48.6 mg., 0.05 mmol.), dissolved in methanol (0.5 ml.) containing 0.48 N methanolic hydrogen chloride (0.23 ml.), was subjected to hydrogenolysis in the presence of palladium black. After being filtered from the catalyst, the solution was evaporated to dryness in vacuo. The residual crystals were then collected with the aid of a mixture of acetone and ether, and dried in air. Yield, 37.4 mg. (81%); m. p. 242—244°C (decomp.); $[\alpha]_D^{23}$ +6.6° (*c* 1.02, acetic acid); R_f 0.73 on paper, 0.69 on silica gel. The amino acid analysis gave the molar ratio of 1.0 : 1.1 : 2.3 : 1.0 : 1.2 for phe, leu, orn, val, and pro.

Found: C, 49.79; H, 7.90; N, 12.88. Calcd. for $C_{35}H_{58}O_6N_8Cl_2 \cdot 5H_2O$; C, 49.58; H, 8.08; N, 13.22%.

The air-dried product lost 8.8% of its weight after being dried over phosphorus pentoxide at 80°C (2 mmHg) to a constant weight. Calcd. for $4H_2O$: 8.5%.

The molecular weight of XXIV·2HCl·$5H_2O$ was determined by the procedure described above (solvent methanol).[23]

Found: 340. Calcd. for $1/3 \times (C_{35}H_{56}O_6N_8 \cdot 5H_2O \cdot 2HCl)$: 283.[24]

Cyclo-D-phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-prolyl Hydrobromide (Hydrobromide of XXIV).—Ten mg. of XXIII was dissolved in 2 N hydrogen bromide in acetic acid (0.2 ml.). After 45 min., the reaction mixture was evaporated in vacuo, and acetic acid (0.2 ml.) and ether (0.5 ml.) were added. The precipitate was filtered off, repeatedly washed with ether, and dried in vacuo. The hygroscopic powder obtained weighed 6.6 mg. Its homogeneity was established by paper (R_f 0.73), thin-layer (R_f 0.68), and carboxymethyl cellulose column chromatography (Fig. 4), and by paper electrophoresis (Fig. 5).

The Chromatography and Electrophoresis of XXIV and Related Compounds.—A sample (0.5 mg. —1mg.) was dissolved in 0.2—0.3ml. of 0.2M pyridinium acetate contining 30% methanol (pH 5.0); the solution was then applied to a column (0.9×50 cm.) with carboxymethyl cellulose (Eastman Organic Chem. 7796),

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

22) M. Ohno and N. Izumiya, This Bulletin **38**, 1839 (1965).

23) The authors are indebted to Mr. Michinori Waki of this laboratory for the molecular-weight determination.

24) This compound appears to dissociate into three molecules under the conditions under which the determination of the molecular weight was carried out.

TABLE II. INHIBITORY ACTIVITY OF XXIV·2HCl·5H_2O AND GRAMICIDIN S ON MICROORGANISMS

Minimum inhibitory concentrations, μg./ml.

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*
XXIV·2HCl·5H_2O	>100	>100	>100	>100	>100
Gramicidin S·H_2SO_4·8H_2O	>100	>100	5	5	>100

Medium used was Stephenson-Whetham's medium (modified); K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4$·7H_2O 0.05%, Na-glutamate 0.4%, casamino acid 0.2%, yeast-extract 0.05% and agar 2.0%, pH 7.0. The same results were obtained on bouillon agar medium.

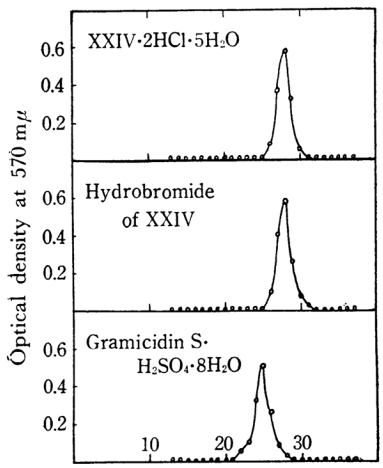

Fig. 4. Carboxymethyl cellulose column chromatography of three compounds.

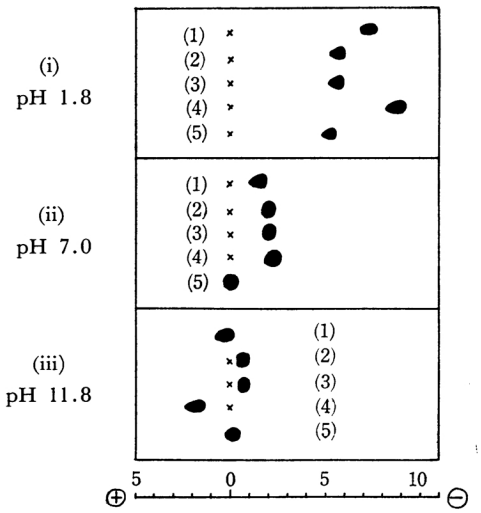

Fig. 5. Paper electrophoresis of (1) XXI, (2) XXIV·2HCl·5H_2O, (3) hydrobromide of XXIV, (4) L-lysine hydrochloride, and (5) gramicidin S·H_2SO_4·8H_2O.

and development was continued with the same solvent. Two milliliter fractions were collected at a flow rate of 20 ml. per hour. The peptide content of the fractions was determined by the method described by Yemm and Cocking.[25] Gramicidin S was used as the control. As is shown in Fig. 4, the same chromatographic pattern was obtained for XXIV·2HCl·5H_2O and for the hydrobromide of XXIV.

Paper electrophoresis was carried out under these conditions: paper, Toyo Roshi No. 52; voltage gradient, 500 V./30 cm.; charged period, 2 hr.; solvent system, (i) formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10, v/v) (pH 1.8), (ii) 0.02 M sodium citrate buffer - methanol (1 : 1, v/v) (pH 7.0), and (iii) triethylamine - concentrated ammonium hydroxide - methanol - water (1 : 1 : 20 : 40, v/v) (pH 11.8). L-Lysine hydrochloride and gramicidin S sulfate were used as the reference compounds. Figure 5 demonstrates the electrophoretic patterns of XXI, XXIV·2HCl·5H_2O, the hydrobromide of XXIV, lysine and gramicidin S. The mobility of XXIV·2HCl·5H_2O with relation to lysine was 0.64 in solvent i, and 0.95 in solvent ii; it was indistinguishable from one of the hydrobromides in each solvent.

Microbiological Assays. — The microorganisms employed are shown in Table II. The minimum amount of the compounds needed for the complete inhibition of growth was determined by a dilution method with a bouillon agar medium and with a synthetic medium. As Table II shows, the cyclic hexapeptide XXIV·2HCl·5H_2O was found to be devoid of activity in reaction against any of the microorganisms utilized, even at high concentrations of the test compound, whereas gramicidin S exhibited considerable activity in reaction against some of the microorganisms at substantial concentrations.

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25) B. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955); *Biochem. J.*, **58**, xii (1954).