# Studies of Peptide Antibiotics. II. Cyclo-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycyl

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A number of polypeptide antibiotics1) such as gramicidin S, tyrocidine A and polymyxin B have several characteristic features in common. These features are, namely, the occurrence of D-amino acids, basic amino acids, and a cyclic structure in the molecule. For a study of the relationship between chemical structure and biological activities of antibacterial cyclic peptides, some dipeptide anhydrides, which are the simplest compounds possessing the characteristics mentioned above, were prepared and their biological activities were tested as described in a previous paper.2) However, they were found to be devoid of antibacterial activity. Although the reason for the inactivity of the dipeptide anhydrides is not yet clear, it appears that in addition to the characteristics mentioned above, other structural features such as a certain minimum ring size and/or a specific amino acid sequence, are necessary in this type of molecule for the exhibition of antibacterial activity. Therefore, it became of interest to determine whether or not a larger cyclic peptide with an appropriate amino acid sequence would exhibit the antibacterial properties.

It appears that difficulties have been encountered in the synthesis of cyclic tripeptides. It has been found, for example, that a linear tripeptide active ester or azide yields a cyclic hexapeptide through cyclization by the dimerization reaction.3,4) Furthermore, cyclization of a linear tetra or pentapeptide gave a cyclic tetrapeptide in very poor yield3) or was accompanied with the dimerization reaction to yield a cyclic decapeptide.5) On the other hand, it has been found that several cyclic hexapeptides can be synthesized without any difficulty.3,6) Therefore, we have decided to attempt the synthesis of a cyclic hexapeptide with the appropriate amino acid sequence.

This paper is concerned with the synthesis and an antibacterial assay of the cyclic hexapeptide, cyclo-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycyl, which contains the partial sequence -L-Val-L-Orn-L-Leu-D-Phe-L-Pro- found in gramicidin S and tyrocidines.1)

The sequence of reactions employed in the synthesis of the cyclic hexapeptide (XII) is indicated in the following scheme (p. 1203). The final product XII was obtained as colorless crystals containing five moles of water of crystallization; its homogeneity was demonstrated by paper and thin layer chromatographies.

In order to ascertain whether or not the cyclic peptide XII described herein possesses antibacterial activity, the effect of various levels of XII on the growth of *Escherichia coli*, *Bacillus subtilis*, and several other microorganisms was examined. It was found that there was no retardation of growth of any of these microorganisms.

Hence, it appears that even the presence of six amino acid residues in a cyclic peptide does not satisfy the requirements for antibacterial activity. Experiments on the preparation and studies on the properties of several other cyclic hexapetides are in progress in this laboratory in order to extend this study further.

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1) R. O. Studer and K. Vogler, *Helv. Chim. Acta*, **45**, 819 (1962).

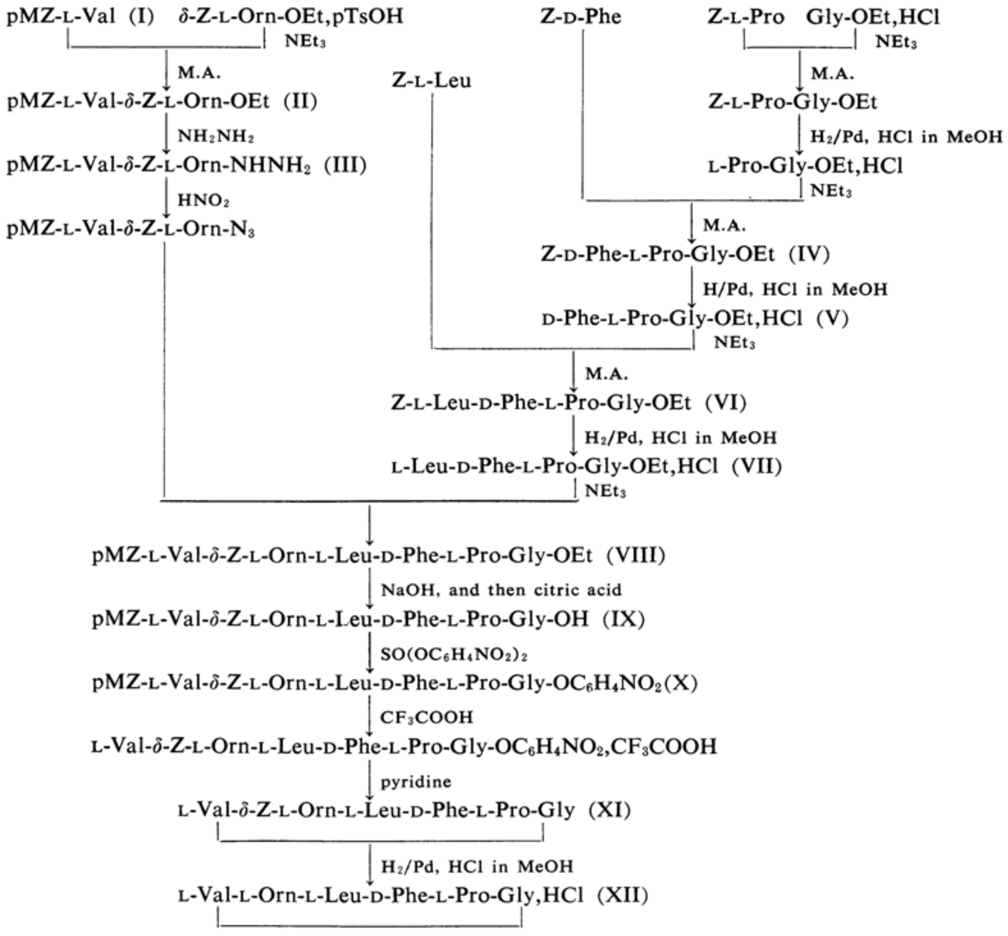
2) N. Izumiya, T. Kato, Y. Fujita, M. Ohno and M. Kondo, This Bulletin, **37**, 1809 (1964).

3) R. Schwyzer, B. Iselin, W. Rittel and P. Sieber, *Helv. Chim. Acta*, **39**, 872 (1956).

4) J. C. Sheehan and W. L. Richardson, *J. Am. Chem. Soc.*, **77**, 6391 (1955); G. W. Kenner, P. J. Thompson and J. M. Turner, *J. Chem. Soc.*, **1958**, 4148; R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2190 (1958); M. Rothe, K. D. Steffen and I. Rothe, *Angew. Chem.*, **75**, 1207 (1963).

5) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2186 (1958); German Patent, *Chem. Abstr.*, **57**, 949 (1962).

6) T. Wieland and K. W. Ohly, *Ann.*, **605**, 179 (1957); R. Schwyzer and B. Gorup, *Helv. Chim. Acta*, **41**, 2199 (1958); K. D. Kopple and D. E. Nitecki, *J. Am. Chem. Soc.*, **84**, 4457 (1962); J. C. Sheehan and D. N. McGregor, ibid., **84**, 3000 (1962).



pMZ: $CH_3OC_6H_4CH_2OCO$-, Z: $C_6H_5CH_2OCO$-, M.A.: mixed anhydride method

In this connection, it should be noted that it was recently reported that gramicidin $J_2$, previously described as a cyclic hexapeptide, is identical with gramicidin S,[7] a cyclic decapeptide.

## Experimental

All melting points are uncorrected.

***p*-Methoxybenzyloxycarbonyl-L-valine (I).**—To a solution of L-valine (1.172 g., 10 mmol.) and sodium bicarbonate (1.64 g., 20 mmol.) in water (20 ml.) was added a solution of *p*-methoxybenzyloxycarbonyl azide[8] (2.486 g., 12 mmol.) in dioxane (20 ml.), and the reaction mixture stirred for 48 hr. at room temperature. After removal of dioxane in vacuo, water (20 ml.) was added to the residue. The aqueous solution was extracted with ethyl acetate (20 ml.). The combined aqueous layer was acidified with 0.5 M citric acid to pH 5 in an ice bath, and then thrice extracted with ethyl acetate (30 ml. each). The extract was dried over anhydrous sodium sulfate, and after removal of the salt by filtration, evaporated in vacuo. The residual oil weighed 2.036 g. (72%).

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine Ethyl Ester (II).**—To a solution of I (2.036 g.) and triethylamine (1.0 ml.) in absolute tetrahydrofuran (20 ml.) at −5°C, was added isobutyl chloroformate (0.94 ml.). After 15 min., a cold mixture of δ-benzyloxycarbonyl-L ornithine ethyl ester *p*-toluenesulfonate (3.359 g.)[2] and triethylamine (1.0 ml.) in chloroform (20 ml.) was added, and the mixture was allowed to stand overnight at room temperature. After removal of the solvent by evaporation in vacuo, the resultant oily residue solidified. The solidified product was treated with water and the mixture was filtered; the residue was washed successively with 4% sodium bicarbonate, water, 10% citric acid, and water; yield, 3.379 g. This solid was recrystallized from methanol-ether-petroleum ether to give 3.126 g. (76%); m. p. 140—144°C; $[\alpha]_D^{23}$ −7.8° (*c* 1, acetic acid).

Found: C, 62.22; H, 6.96; N, 7.61. Calcd. for $C_{29}H_{39}O_8N_3$: C, 62.46; H, 7.05; N, 7.54%.

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine Hydrazide (III).**—To a solution of II (1.115 g., 2 mmol.) in dimethylformamide (4 ml.) was added hydrazine hydrate (1 ml.)

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[7] K. Kurahashi, *J. Biochem.*, **56**, 101 (1964).

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

and the solution was stored for 48 hr. at room temperature. The reaction mixture was evaporated in vacuo below 50°C. The hydrazide which precipitated upon the addition of water (20 ml.) was filtered off, washed with water and dried in a desiccator; 991 mg. (91%); m. p. 211—212°C (decomp.); $[\alpha]_{D}^{23}$ $-4.2°$ (*c* 1, dimethylformamide).

Found: C, 59.53; H, 6.87; N, 12.77. Calcd. for $C_{27}H_{37}O_7N_5$: C, 59.65; H, 6.86; N, 12.88%.

**Benzyloxycarbonyl-D-phenylalanyl-L-prolylglycine Ethyl Ester (IV).**—To a chilled solution of benzyloxycarbonyl-D-phenylalanine (2.155 g., 7.2 mmol.) and triethylamine (1.01 ml.) in tetrahydrofuran (15 ml.) was added isobutyl chloroformate (0.94 ml.). After 15 min. a mixture of L-prolylglycine ethyl ester hydrochloride (1.805 g.),[9] triethylamine (1.01 ml.) and chloroform (20 ml.) was added. The mixture was left overnight at room temperature and then evaporated in vacuo. The residual oil was dissolved in ethyl acetate (50 ml.), and the solution was washed successively with 2% hydrochloric acid, 4% sodium bicarbonate and water, and then dried over anhydrous sodium sulfate. The filtered solution was evaporated in vacuo. The residual oil solidified after the addition of ether and petroleum ether. The product was filtered off, dried in air and weighed; 2.588 g. (75%); m. p. 105—106°C; $[\alpha]_{D}^{23}$ $-52.6°$ (*c* 2, dimethylformamide).

Found: C, 63.85; H, 6.51; N, 8.25. Calcd. for $C_{26}H_{31}O_6N_3 \cdot 1/2H_2O$: C, 63.66; H, 6.57; N, 8.56%.

When dried at 100°C in vacuo, the crystals lost 1.5% of their weight. Calcd. for $1/2H_2O$: 1.8%.

**D-Phenylalanyl-L-prolylglycine Ethyl Ester Hydrochloride (V).**—To a solution of IV (2.407 g., 5 mmol.) in methanol (20 ml.) was added 0.494 N methanolic hydrogen chloride (10.7 ml.). The solution was then subjected to hydrogenolysis in the presence of palladium black. After being filtered from the catalyst, the solution was evaporated to dryness in vacuo; yield, 1.944 g. (100%).

**Benzyloxycarbonyl-L-leucyl-D-phenylalanyl-L-prolylglycine Ethyl Ester (VI).**—To a chilled solution of benzyloxycarbonyl-L-leucine (1.327 g., 5 mmol.) and triethylamine (0.70 ml.) in tetrahydrofuran (15 ml.) was added isobutyl chloroformate (0.66 ml.). After 15 min., a solution of V (1.944 g., 5 mmol.) and triethylamine (0.70 ml.) in chloroform (20 ml.) was added at −10°C. The mixture was left overnight at room temperature and evaporated in vacuo. The residual oil was treated in the manner described above. The oily residue weighed 2.910 g. (97%).

**L-Leucyl-D-phenylalanyl-L-prolylglycine Ethyl Ester Hydrochloride (VII).**—VI (1.189 g., 2 mmol.) was subjected to hydrogenolysis in the presence of palladium black and 0.48 N methanolic hydrogen chloride (4.5 ml.), and then treated as mentioned above. The resulting oil gradually solidified to form an amorphous powder; yield, 961 mg. (97%).

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycine Ethyl Ester (VIII).**—The following operations were carried out in a cold room. To a chilled solution of III (544 mg., 1 mmol.) in glacial acetic acid (12 ml.) were added successively with stirring 1.09 N hydrochloric acid (1 ml.), 76 mg. of sodium nitrite in 1 ml. of water, and additional 1.09 N hydrochloric acid (1 ml.). After 6 min., cold water (50 ml.) was added. The azide which precipitated as a white mass was filtered off and washed successively with cold water, saturated sodium bicarbonate solution, and water, and then dried under vaccum in a desiccator. The azide was added to a solution of VII (497 mg., 1 mmol.) and triethylamine (0.14 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 addition of water (50 ml.) to the filtrate was collected, washed with 0.5 M citric acid, 4% sodium bicarbonate solution and water, and dried. Recrystallization from dioxane and ether gave 696 mg. (72%) of VIII; m. p. 125—129°C; $[\alpha]_{D}^{23}$ $-33.5°$ (*c* 2, dimethylformamide).

Found: C, 62.15; H, 7.01; N, 9.93. Calcd. for $C_{51}H_{69}O_{12}N_7 \cdot 1/2H_2O$: C, 62.42; H, 7.19; N, 9.99%.

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycine (IX).**—To a solution of VIII (990 mg., 1 mmol.) in methanol (15 ml.) was added 0.52 N sodium hydroxide (3 ml.), and the solution was allowed to stand for 4 hr. at 40°C. After the addition of water (15 ml.), the solution was concentrated in vacuo, extracted with ether and acidified with 0.5 M citric acid in an ice-cold bath. After storing in a refrigerator, the crystals were collected by filtration, washed with water and dried. Recrystallization from acetone and ether did not raise the melting point. The yield was 736 mg. (79%); m. p. 154—158°C; $[\alpha]_{D}^{23}$ $-39.4°$ (*c* 2, dimethylformamide).

Found: C, 62.09; H, 6.83; N, 10.20. Calcd. for $C_{49}H_{65}O_{12}N_7$: C, 62.34, H, 6.94; N, 10.39%.

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycine *p*-Nitrophenyl Ester (X).**—To a solution of IX (472 mg., 0.5 mmol.) in pyridine (4 ml.) was added di-*p*-nitrophenyl sulfite[10] (648 mg., 2 mmol.), and the reaction mixture was allowed to stand for 24 hr. at room temperature. After evaporation of the solvent, the oily product was triturated in petroleum ether and washed repeatedly with a mixture of ether and petroleum ether (1 : 1) by decantation. When the reaction mixture had been washed completely, the washings did not give the yellow color of *p*-nitrophenol on addition of sodium hydroxide solution. The residual solid was filtered off, washed with a mixture of ether and petroleum ether (1 : 1) and dried. It weighed 509 mg. (95%). A *p*-nitrophenyl group estimation was carried out spectrophotometrically by measuring the optical density of the compound at 412 m$\mu$. The purity of the product was estimated to be 101%.

**Cyclo-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyglycyl (XI).**—To X (788 mg., 0.74 mmol.), were added successively

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[9] H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, 1956, 3642.

[10] B. Iselin and R. Schwyzer, *Helv. Chim. Acta* 3, 1760 (1960).

TABLE I. INHIBITORY ACTIVITY OF PEPTIDE XII AND GRAMICIDIN S ON MICROORGANISMS*
Minimum inhibitory concentrations, μg./ml.

| Compound | Synthetic medium[a] | | Bouillon agar medium[b] | |
|---|---|---|---|---|
| | *E. coli*[c] | *B. subtilis*[d] | *E. coli*[c] | *B. subtilis*[d] |
| XII | >100 | >100 | >100 | >100 |
| Gramicidin S[e] | >100 | 10 | >100 | 10 |

a) Stephenson-Whetham's medium (modified) ;
$KH_2PO_4$ lg., NaCl lg., $FeSO_4 \cdot 7H_2O$ 0.03 g., $MgSO_4 \cdot 7H_2O$ 0.7 g., Na-glutamate 4 g., glucose 5 g./1000 ml. and agar 20 g.
b) Usual bouillon agar medium, pH 7.0.
c) *Escherichia coli IRO 3044.*
d) *Bacillus subtilis PCI 219.*
e) A product of Astra Co, U.S.A.

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* In addition to *E. coli* and *B. subtilis*, microbiological assays were also performed against *Proteus vulgaris*, *Staphylococcus aureus*, *Bacillus cereus*, *Mycobacterium avium*, *Mycobacterium avium* (Streptomycin resistant St.), *Mycobacterium SP 607*, *Mycobacterium semegmatis*, *Candida albicans*, *Saccharomyces cerevisiae* and *Piricularia oryzae* in bouillon agar medium. Peptide XII was inactive at 50 μg./ml. against any of microorganisms tested here. Gramicidin S inhibits the growth of *Proteus vulgaris* in 10 μg./ml., *Staphylococcus aureus* in 5 μg./ml. and *Mycobacterium semegmatis* in 10 μg./ml. in bouillon agar medium.

anisole (0.4 ml.) and trifluoroacetic acid (3 ml.) at a temperature below 0°C. The solution was evaporated in vacuo at 0°C, and the residue was triturated with ether (10 ml.). The crude hexapeptide *p*-nitrophenyl ester trifluoroacetate was collected in the cold room, washed with ether - petroleum ether mixture and dried in vacuo over phosphorous pentoxide.

The dried trifluoroacetate was dissolved in dimethylformamide (5 ml.) containing 0.1 ml. of glacial acetic acid, and the solution was added dropwise to pyridine (200 ml.) at 62°C over a period of 3 hr. under continuous stirring; stirring was then continued for an additional 2 hr. at the same temperature. After removal of the solvent, the residual oil was dissolved in a mixture of methanol (40 ml.) and water (10 ml.), the insoluble substance was removed by filtration, and the filtrate was passed successively through columns of Dowex 1 ($OH^-$ form) and Dowex 50 ($H^+$ form). The columns were eluted with the same solvent (150 ml.), the collected effluent was evaporated to dryness in vacuo. The residual amorphous product (455 mg.) was dissolved in a mixture of ethyl acetate (20 ml.) and methanol (5 ml.). After removal of insoluble substances by filtration, the solution was evaporated, and the residue was triturated with ethyl acetate (4 ml.) and ether (1 ml.). The product which solidified as needles was filtered off, and recrystallized by the same procedure mentioned above. The yield was 139 mg. (32%); m. p. 224—226°C; $[\alpha]^{16}_{D}$ $-22.9°$ (*c* 1.95, dimethylformamide); $R_f = 0.66$ on thin layer chromatography.[11]

Found: C, 62.28; H, 7.18; N, 12.66. Calcd. for $C_{40}H_{55}O_8N_7 \cdot 1/3H_2O$: C, 62.56; H, 7.30; N, 12.77%. Mol. wt. Found: 652, 598.[12] Calcd. 762.

The air-dried compound lost 0.75% of its weight after drying over phosphorus pentoxide for 3 hr. at 120°C, 2 mmHg. Calcd. for $1/3H_2O$: 0.78%.

**Cyclo-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycyl Hydrochloride** (XII).—XI (38 mg., 0.05 mmol.), dissolved in methanol (2 ml.) containing 0.0455 N methanolic hydrogen chloride (1.22 ml.), was subjected to hydrogenolysis in the presence of palladium black. The solution after being filtered from the catalyst was evaporated to dryness in vacuo. The residue was dissolved in ethanol (0.2 ml.), then acetone (4 ml.) and ether (1 ml.) were added dropwise to the solution which was then stored overnight at 0°C. The crystals of XII which formed were filtered off, washed with acetone-ether (1 : 1) and dried in air. The yield was 24 mg. (72%); m. p. 283°C (decomp.); $[\alpha]^{34}_{D}$ $-125°$ (*c* 1.01, 50% methanol, v/v); $R_f = 0.88$ on paper,[11] 0.61 on silica gel.[11]

Found: C, 50.77; H, 7.70; N, 12.83. Calcd. for $C_{32}H_{50}N_7O_6Cl \cdot 5H_2O$: C, 50.95; H, 8.02; N, 12.89%.

The compound lost 10.4% of its weight after drying over phosphorus pentoxide for 3 hr. at 120°C, 2 mmHg. Calcd. for $5H_2O$: 11.9%.

XII, after hydrolysis with 6 N hydrochloric acid at 110°C for 16 hr., gave six distinctly separated spots when chromatographed on paper. $R_f$ values were 0.69, 0.65, 0.53, 0.38, 0.23 and 0.17. The $R_f$ values of authentic L-Leu, D-Phe, L-Val, L-Pro, Gly and L-Orn are 0.68, 0.63, 0.51, 0.36, 0.22 and 0.16, respectively.[11]

**Microbiological Assays.***—The microorganisms employed are shown in Table I. The minimum amount of compound necessary for complete inhibition of growth was determined by a dilution

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[11] Toyo Roshi No. 52 paper was used for paper chromatography, and Merck silica gel G for thin layer chromatography. Solvent system used in both cases was a mixture of *n*-butanol: acetic acid: pyridine: water, 4:1:1:2 in v/v.

[12] Micro Rast method. The solvent used was hexahydro-*p*-amino benzoic acid lactam (G. Wendt, *Chem. Ber.*, 75, 425 (1962)).

* The authors are indebted to Research Laboratories, Takeda Chemical Industries, Ltd., Osaka, for the microbiological assays.

method with bouillon agar medium and with synthetic medium. As shown in Table I, cyclohexapeptide XII exhibited no antibacterial activity against any of the microorganisms tested, even at high concentration of the test compound, whereas gramicidin S, a cyclic decapeptide, exhibited considerable activity against some of the microorganisms at comparable concentration.

## Summary

A cyclic hexapeptide, cyclo-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolylglycyl, was synthesized for the purpose of comparing its antibacterial action, if any, with the cyclopeptide antibiotics, such as gramicidin S, with which it shares several common structural features.

Synthesis was achieved by condensation of *p*-methoxybenzyloxycarbonyl-L-valyl-$\delta$-benzyloxycarbonyl-L-ornithine azide with L-leucyl-D-phenylalanyl-L-prolylglycine ethyl ester, followed by treatment of the resulting diacyl hexapeptide ester with alkali to remove the ethyl ester group. The diacyl hexapeptide so derived was converted to the *p*-nitrophenyl ester by the action of di-*p*-nitrophenyl sulfite, and after removal of the *p*-methoxybenzyloxycarbonyl group with trifluoroactetic acid, the hexapeptide *p*-nitrophenyl ester was transformed to the cyclic benzyloxycarbonyl-substituted hexapeptide by treatment with a large excess of pyridine. The cyclic benzyloxycarbonyl-substituted peptide was hydrogenated in the presence of palladium black to yield the desired cyclic hexapeptide hydrochloride.

The effects of the synthesized cyclic peptide on bacterial growth were examined. No antibacterial activities were observed against the microorganisms utilized here.

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