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Synthetic Studies of Bacitracin. VIII.[1] Synthesis of Cyclohexapeptide Moiety[2]

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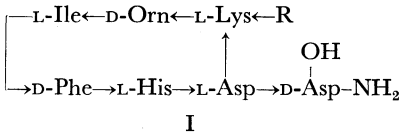
For the purpose of synthesis of antibiotic bacitracin of six amino acids-membered ring formula, the following intermediate peptides, *i.e.*, cyclo-(N^{α}-benzyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-α-methyl-L-aspartyl) (III) and N^{α}-benzyloxycarbonyl-N^{ε}-*t*-butyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-N^{im}-benzyl-L-histidyl-L-aspartyl-D-isoasparagine benzyl ester (IV) were prepared. In this synthesis, cyclopentyloxycarbonyl group was introduced to protect δ-amino group of ornithine residue and cleavability of this protecting group for hydrogen fluoride was investigated.

Bacitracin A is an antibiotic peptide produced by *Bacillus licheniformis*.[3] The investigations on the chemical structure of bacitracins were performed by several groups since twenty five years ago.[4,5] Craig and his collaborators had suggested a chemical structure I of six amino acids-membered ring for bacitracin A as the most probable one in 1961.[5] However, Ressler *et al.* later proposed another formula II of seven amino acids-membered ring for the antibiotic.[6] A decisive judgment for a correct structure must be awaited after completion of the total synthesis of this antibiotic peptide unless it is conclusively determined by unobjectionable method like X-ray analysis. In connection with the situation, we attempted to synthesize both of the proposed structures in order to identify with the natural antibiotic.

In our previous papers,[7,8] we prepared a peptide



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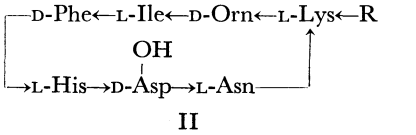

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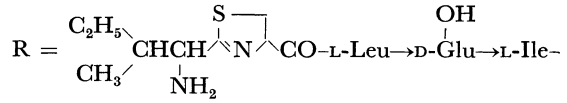

analogue possessing a cysteinyl residue in the place of a thiazoline ring in bacitracin A of Craig's formula I, and demonstrated that it was unable to lead to the compound I through thiazoline ring cyclization from the cysteinyl peptide by the treatment with strong acid such as concentrated hydrochloric acid.[9] Therefore, the ring closure of the cysteine peptide in the final step of a synthetic program must be avoided. On the contrary, it seems most promising to accomplish the synthesis by the condensation of thiazoline moiety corresponding to *N*-terminal peptide with a remaining peptide fragment containing a macrocyclic structure at an appropriate synthetic stage followed by an exhaustive deprotection. We already developed the synthetic method of thiazoline peptides through the coupling of iminoether derivative of *N*-protected amino acids with cysteine ester or cysteinyl peptide.[1,10] This synthetic

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1) Part VII: Y. Hirotsu, T. Shiba, and T. Kaneko, This Bulletin, **43**, 1870 (1970).

2) This work was presented at the 5th Symposium on Peptide Chemistry, Kyoto, November, 1967 and at the 8th same Symposium, Osaka, November, 1970.

3) B. A. Johnson, H. Anker, and F. L. Meleney, *Science*, **102**, 376 (1945).

4) D. W. Swallow and E. P. Abraham, *Biochem. J.*, **72**, 326 (1959).

5) W. Stoffel and L. C. Craig, *J. Amer. Chem. Soc.*, **83**, 145 (1961).

6) C. Ressler and D. V. Kashelikar, *ibid.*, **88**, 2025 (1966).

7) Y. Ariyoshi, T. Shiba, and T. Kaneko, This Bulletin, **40**, 1709 (1967).

8) Y. Ariyoshi, T. Shiba, and T. Kaneko, *ibid.*, **40**, 2648 (1967).

9) Y. Hirotsu, T. Shiba, and T. Kaneko, *ibid.*, **40**, 2950 (1967).

10) Y. Hirotsu, T. Shiba, and T. Kaneko, *ibid.*, **40**, 2945 (1967).

strategy may also be applied to a synthesis of bacitracin F which is known to be a peptide transformed from bacitracin A *via* oxidative deamination under mild alkaline or neutral condition and possess a keto-thiazole moiety instead of amino-thiazoline in *N*-terminal part of the molecule of bacitracin A.

In this paper, we synthesized hexa- and heptapeptide derivatives corresponding to the ring peptide moiety of the structure I proposed by Craig *et al.*[5] in order to investigate a possibility to elongate these intermediate peptides to the whole structure of bacitracins. However, prior to this synthesis, we must overcome the difficult problem concerned with the selection of protective groups in building up these peptide chains. Particularly, the protection of δ-amino group of D-ornithine residue is in problem, since this protective group must remain throughout the whole synthetic pathway including cyclization of macro-ring as far as the final condensation step on the one hand, and, for the removal of this protection, reaction conditions such as in catalytic hydrogenation, reduction with sodium in liquid ammonia, and saponification, cannot be applied in view of instability of thiazoline or thiazole ring on the other hand. Furthermore, this protective group is also required to be resistant enough for catalytic hydrogenation, hydrazinolysis as well as mild acidolysis, those conditions being needed on a way of making up the cyclic peptide containing ornithine residue. The fact that both thiazoline and thiazole ring are stable under acidic condition, suggests a possible use of anhydrous hydrogen fluoride as reagent for removal of the all protective groups at the final step of the synthesis. These considerations lead us to utilize *s*-alkyloxycarbonyl group for protection of δ-amino group of ornithine residue, since this special protection may resist to the reaction condition mentioned above with the exception of facile cleavability with hydrogen fluoride.

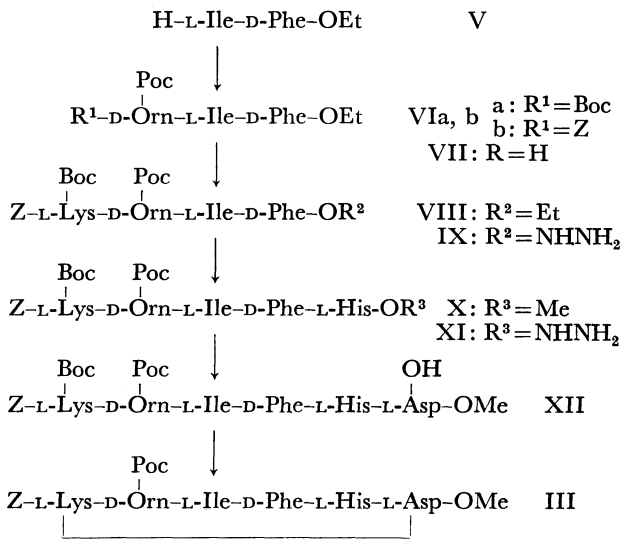
In preliminary tests, *s*-alkyloxycarbonyl groups such as isopropyloxycarbonyl, *s*-butyloxycarbonyl, 1-ethylpropyloxycarbonyl, cyclopentyloxycarbonyl, and cyclohexyloxycarbonyl were introduced into δ-amino group of L-ornithine. The derivatives thus prepared were treated with anhydrous hydrogen fluoride respectively. From the results obtained in the experiments as shown in Table 1, it was demonstrated that cyclopentyloxycarbonyl group was the most suitable for our purpose. Although this protective group had been introduced by McKay and Albertson who used hydrogen bromide in glacial acetic acid as removing agent,[11] its behavior for hydrogen fluoride has not been reported yet.

Table 1. CLEAVABILITY OF N^{δ}-*s*-ALKYLOXYCARBONYL GROUP OF L-ORNITHINE IN TREATMENT WITH HYDROGEN FLUORIDE

	0 °C 30 min	0 °C 60 min	20 °C 60 min
Isopropyloxycarbonyl		−	−
s-Butyloxycarbonyl		−	−
1-Ethylpropyloxycarbonyl	−	±	+
Cyclopentyloxycarbonyl	±	+	
Cyclohexyloxycarbonyl	−	±	+

+: cleavable −: stable

Using such a special protective group, the hexa- and heptapeptides, *i.e.*, cyclo-(N^{α}-Z-L-lysyl-L-N^{δ}-Poc-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-α-methyl-L-aspartyl) (III)[12] and N^{α}-Z-N^{ε}-Boc-L-lysyl-N^{δ}-Poc-D-ornithyl-L-isoleucyl-D-phenylalanyl-N^{im}-benzyl-L-histidyl-L-aspartyl-D-isoasparagine benzyl ester (IV)[12] were prepared.



Poc: cyclopentyloxycarbonyl, Boc: *t*-butyloxycarbonyl, Z: benzyloxycarbonyl

Scheme 1.

Thus the synthesis of these peptides was started with the coupling reaction of Z-L-isoleucine with ethyl D-phenylalaninate through the active ester method as shown in Scheme 1. The resulting acyl dipeptide ester was treated with hydrogen bromide in glacial acetic acid to afford V hydrobromide.[7] This compound was coupled with N^{α}-Boc-N^{δ}-Poc-D-ornithine or N^{α}-Z-N^{δ}-Poc-D-ornithine either by the active ester method or by the mixed anhydride method to give two corresponding acyl tripeptide derivatives VIa and VIb respectively. The Boc group was removed from VIa by exposing to hydrogen chloride in ethyl acetate and the Z group of VIb was removed by catalytic hydrogenation in the presence of hydrogen chloride.

The same tripeptide ester VII hydrochloride thus obtained was coupled with N^{α}-Z-N^{ε}-Boc-L-lysine *via* the active ester method to give VIII. This acyl tetrapeptide ester VIII was converted to a corresponding hydrazide IX. The protected tetrapeptide azide prepared from IX was coupled with methyl L-histidinate dihydrochloride to afford acyl pentapeptide ester X. The ester X was converted to the hydrazide XI which was then used for azide coupling reaction with aspartic acid moiety. Thus, removal of Z group from α-methyl Z-L-aspartate was carried out by heating with trifluoroacetic acid applying Weygand's technique[13]

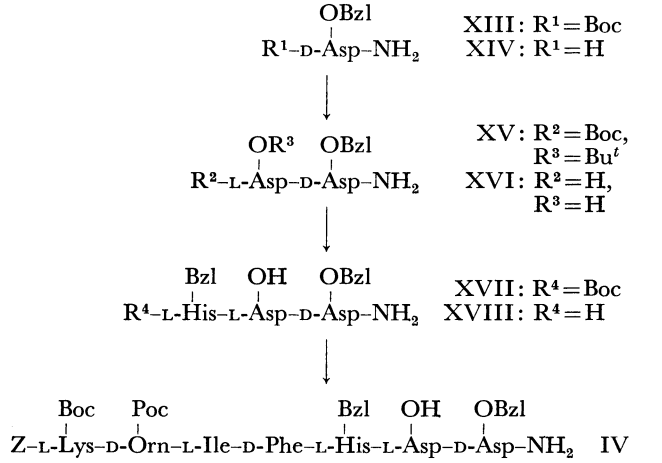
11) F. C. McKay and N. F. Albertson, *J. Amer. Chem. Soc.*, **79**, 4686 (1957).

12) Abbreviations: Z, benzyloxycarbonyl; Poc, cyclopentyloxycarbonyl; Boc, *t*-butyloxycarbonyl.

13) F. Weygand and W. Steglich, *Z. Naturforsch.*, **14b**, 472 (1959).

to give α-methyl L-aspartate trifluoroacetate. This compound was condensed with the azide prepared from the protected pentapeptide hydrazide XI mentioned above in the presence of triethylamine to afford a linear hexapeptide derivative XII. Attempts to obtain the same peptide by stepwise elongation method starting from *C*-terminal side failed, because of difficulty in purification of intermediate tri- or tetrapeptides due to their hygroscopic natures or troublesome tendencies to form gelatinous solid.

After removal of N^{ε}-Boc group of lysine residue in hexapeptide derivative XII obtained above, cyclization between ε-amino group of lysine residue and β-carboxyl group of aspartic acid residue was attempted in high dilution by *p*-nitrophenyl ester method, 1-succinimidyl ester method or carbodiimide method. However no satisfactory results were obtained in all cases. Only when cyanomethyl ester of the hexapeptide XII was tried to be cyclized, expected cyclohexapeptide III was obtained but in an extremely low yield. This cyclic peptide corresponds to macrocyclic part of Craig's formula. However, this cyclohexapeptide methyl ester III was resistant to conversion into a hydrazide which is a necessary intermediate to be coupled with *C*-terminal isoasparagine residue by the azide method. Therefore, we could not extend this synthetic route to reach to the heptapeptide (6—12) of the Craig's formula, and turned our efforts to an alternative way through coupling of the tetrapeptide fragment IX with *C*-terminal tripeptide derivative as shown in Scheme 2.



Bzl: benzyl, Z: benzyloxycarbonyl, Boc: *t*-butyloxycarbonyl, Poc: cyclopentyloxycarbonyl, Bu^t: *t*-butyl

Scheme 2.

Thus, β-benzyl Boc-D-aspartate was converted to benzyl Boc-D-isoasparaginate (XIII) by the active ester method. Boc group in XIII was removed by trifluoroacetic acid and the resulting deblocked product XIV was coupled with β-*t*-butyl Boc-L-aspartate by the 1-succinimidyl ester method to give acyl dipeptide ester XV. This compound XV thus obtained was treated with trifluoroacetic acid to afford L-aspartyl-D-isoasparagine benzyl ester (XVI) which was then acylated with N^{α}-Boc-N^{im}-benzyl-L-histidine-2,4,5-trichlorophenyl ester. In this case, we used benzyl group for protection of imidazole nitrogen in histidine residue in order to prevent the side reaction at cyclization reaction by the cyanomethyl ester method and to facilitate a purification of the product by silica gel column chromatography. The resulting tripeptide derivative XVII was exposed to trifluoroacetic acid to give deblocked ester XVIII. This compound XVIII was coupled with the tetrapeptide azide derived from the hydrazide IX prepared before to afford a linear heptapeptide derivative IV. All attempts to cyclize this compound, after removal of N^{ε}-Boc group on lysine residue, either by dicyclohexylcarbodiimide method, 1-succinimidyl ester method or cyanomethyl ester method did not give satisfactory results at the stage of this investigation.

Therefore, the presented results indicate that synthetic approaches from the intermediate hexa- and heptapeptides mentioned above to the whole structure of bacitracins of six amino acids-membered ring formula must be reinvestigated.

Experimental

All melting points are uncorrected. Silica gel G according to Stahl, Merck was used for thin-layer chromatography. Paper electrophoresis was carried out using Toyo filter paper No. 51 in a Toyo paper electrophoresis C apparatus at a potential gradient of 13 V/cm for 1—2 hr. The buffer used was pyridine–acetic acid–water (30: 4: 966, v/v/v, pH 6.4) unless otherwise stated. For silica gel column chromatography, Kieselgel 0.05—0.2 mm, Merck 70—325 mesh ASTM, was used.

N^{δ}-*s*-*Alkyloxycarbonylornithines.* These compounds were prepared by the reaction of copper complex of ornithine with corresponding *s*-alkyloxycarbonyl chlorides through usual Schotten-Baumann type acylation. The complexes of the N^{δ}-acylornithines thus obtained were operated with hydrogen sulfide and the products were recrystallized from water. Reaction yields, melting points, elemental analyses, and optical properties of those compounds are shown in Table 2.

Treatment of N^{δ}-*Alkyloxycarbonyl-*L-*ornithine with Hydrogen Fluoride.* N^{δ}-*s*-Alkyloxycarbonyl-L-ornithines were treated with hydrogen fluoride under the following three reaction conditions, *i.e.*, i) at 0 °C, for 30 min, ii) at 0 °C, for 60 min, iii) at 20 °C, for 60 min, respectively. After the given reaction period, excess hydrogen fluoride was removed *in vacuo* and the reaction vessel was dried over sodium hydroxide under reduced pressure. The residue was dissolved in water and the solution was extracted with ether. An aqueous layer was concentrated by lyophilization. The degree of deprotection was checked by thin-layer chromatography and paper electrophoresis. The quantitative results are shown in Table 1.

N^{δ}-*Cyclopentyloxycarbonyl-*D-*ornithine.* This compound was prepared using cyclopentyloxycarbonyl chloride[11] by the same procedure described above for N^{δ}-*s*-alkyloxycarbonylornithines; yield, 64%; mp 251—253 °C; $[\alpha]_D^{20}$ −17.6° (*c* 1, 3 M HCl).

Found: C, 54.05; H, 8.29; N, 11.51%. Calcd for $C_{11}H_{20}O_4N_2$: C, 54.08; H, 8.25; N, 11.47%.

N^{α}-t-*Butyloxycarbonyl-*N^{δ}-*cyclopentyloxycarbonyl-*D-*ornithine.* This compound was prepared by acylation of N^{δ}-Poc-D-ornithine (22.3 g, 0.09 mol) with *t*-butyl azidoformate (15 g, 0.1 mol) and sodium carbonate (12 g) in dioxane–water (1: 1, v/v 800 ml). The reaction mixture was operated in the usual manner and the product was recrystallized from

TABLE 2. RESULTS OF PREPARATIONS OF N^{δ}-*s*-ALKYLOXYCARBONYL-L-ORNITHINE

s-Alkyloxycarbonyl group	Formula of derivatives	Yield (%)	Mp (°C) (Decomp.)	Elemental analysis Found (Calcd) C	H	N	$[\alpha]_D$ (Temperature, Concentration)
Isopropyloxycarbonyl	$C_9H_{18}O_4N_2$	52.0	237—240	49.43 (49.53)	8.34 (8.31)	12.82 (12.84)	+18.6° (18°, *c* 1, M HCl)
s-Butyloxycarbonyl	$C_{10}H_{20}O_4N_2$	44.0	227—230	51.38 (51.70)	8.60 (8.68)	11.92 (12.06)	+20.6° (19°, *c* 1, M HCl)
1-Ethylpropyloxycarbonyl	$C_{11}H_{22}O_4N_2$	59.2	225—228	53.43 (53.64)	9.06 (9.00)	11.31 (11.37)	+22.1° (19°, *c* 1, M HCl)
Cyclopentyloxycarbonyl	$C_{11}H_{20}O_4N_2$	64.0	250—253	54.13 (54.08)	8.39 (8.25)	11.25 (11.47)	+17.7° (20°, *c* 1, 3M HCl)
Cyclohexyloxycarbonyl	$C_{12}H_{22}O_4N_2$	56.0	229—232	54.99 (54.84)	8.70 (8.63)	10.48 (10.66) [a]	+16.3° (19°, *c* 1, M HCl)

a) Calculated values are based on formula of $C_{12}H_{22}O_4N_2 \cdot 1/4\ H_2O$.

ethyl acetate–petroleum ether; yield, 28.5 g (91%); mp 144—146 °C; $[\alpha]_D^{18}$ +7.9° (*c* 1, dimethylformamide).

Found: C, 55.63; H, 8.11; N, 7.83%. Calcd for $C_{16}H_{28}O_6N_2$: C, 55.80; H, 8.20; N, 8.13%.

Dicyclohexylammonium salt: (recrystallized from ethanol); mp 170—172 °C; $[\alpha]_D^{19}$ −9.3° (*c* 1, ethanol).

Found: C, 64.08; H, 9.83; N, 8.06%. Calcd for $C_{16}H_{28}O_6N_2 \cdot C_{12}H_{23}N$: C, 63.97; H, 9.78; N, 7.99%.

N^{α}-t-*Butyloxycarbonyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithine* p-*Nitrophenyl Ester*. *N,N'*-Dicyclohexylcarbodiimide (6.6 g, 32 mmol) was added to an ice-cold solution of N^{α}-Boc-N^{δ}-Poc-D-ornithine (10.3 g, 30 mmol) and *p*-nitrophenol (4.2 g, 30 mmol) in ethyl acetate (200 ml) with stirring. The mixture was stirred at 0—3 °C for 1 hr and then at room temperature for 4 hr. Dicyclohexylurea formed was filtered off, washed with ethyl acetate and the combined filtrate was concentrated under reduced pressure. The residue was recrystallized from ethanol; yield, 8.0 g (57%); mp 146—147 °C; $[\alpha]_D^{19}$ +27.9° (*c* 1, dimethylformamide).

Found: C, 56.77; H, 6.77; N, 8.93%. Calcd for $C_{22}H_{31}O_8N_3$: C, 56.76; H, 6.71; N, 9.03%.

N^{α}-t-*Butyloxycarbonyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithine 1-Succinimidyl Ester*. *N,N'*-Dicyclohexylcarbodiimide (3.1 g, 15 mmol) was added to an ice-cold solution of N^{α}-Boc-N^{δ}-Poc-D-ornithine (5.2 g, 15 mmol) and *N*-hydroxysuccinimide (1.8 g, 16 mmol) in tetrahydrofuran (150 ml) with stirring. The stirring was continued at 0—3 °C for 30 min and then at room temperature for 5 hr. Dicyclohexylurea formed was filtered off and washed with tetrahydrofuran. The combined filtrate was concentrated *in vacuo*. The residual syrup was crystallized from 2-propanol–isopropyl ether; yield, 5.2 g, (79%); mp 152—155 °C; $[\alpha]_D^{18}$ +21.1° (*c* 1, dimethylformamide).

Found: C, 54.48; H, 7.04; N, 9.42%. Calcd for $C_{20}H_{31}O_8N_3$: C, 54.41; H, 7.08; N, 9.52%.

N^{α}-t-*Butyloxycarbonyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanine Ethyl Ester (VIa)*. *(i) By 1-Succinimidyl Ester Procedure*: A solution of N^{α}-Boc-N^{δ}-Poc-D-ornithine 1-succinimidyl ester (5.7 g, 13 mmol) in dimethylformamide (60 ml) was added to an ice-cold solution of V hydrobromide[7] (5.0 g, 13 mmol) in dimethylformamide (150 ml) containing triethylamine (1.9 ml). The mixture was stirred in an ice bath for 1 hr and then at room temperature for 30 hr. The solution was concentrated under reduced pressure. Precipitate formed upon addition of water to the residue was filtered, washed with water and recrystallized from methanol; yield, 6.6 g, (80%); mp 188—190 °C; $[\alpha]_D^{19}$ +18.7° (*c* 1, dimethylformamide).

Found: C, 62.59; H, 8.30; N, 8.91%. Calcd for $C_{33}H_{52}O_8N_4$: C, 62.63; H, 8.28; N, 8.85%.

(ii) By p-*Nitrophenyl Ester Procedure*: The same compound VIa was prepared by coupling of N^{α}-*t*-Boc-N^{δ}-Poc-D-ornithine *p*-nitrophenyl ester (5.1 g, 11 mmol) and V hydrobromide[7] (4.3 g, 11 mmol) according to the usual procedure; yield, 5.3 g, (76%); mp 188—190 °C; $[\alpha]_D^{20}$ +18.9° (*c* 1, dimethylformamide).

Found: C, 62.60; H, 8.25; N, 8.86%.

(iii) By Mixed Anhydride Procedure: The same compound VIa was also prepared from a mixed anhydride of N^{α}-Boc-N^{δ}-D-ornithine (26.5 g, 77 mmol) with ethyl chloroformate (8.4 g, 77 mmol), and V hydrobromide[7] (29.8 g, 77 mmol) by the usual procedure; yield, 42 g, (86%); mp 187—190 °C; $[\alpha]_D^{20}$ +18.9° (*c* 1, dimethylformamide).

Found: C, 62.43; H, 8.41; N, 8.69%.

N^{α}-*Benzyloxycarbonyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithine*. This compound was prepared by acylation of N^{δ}-Poc-D-ornithine (10 g, 40 mmol) with benzyloxycarbonyl chloride (8.5 g, 50 mmol) and aqueous 1M sodium hydroxide by the usual procedure. The oily product (14.3 g, 95%) thus obtained was used directly to the following esterification.

N^{α}-*Benzyloxycarbonyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithine* p-*Nitrophenyl Ester*. *N,N'*-Dicyclohexylcarbodiimide (6.8 g, 33 mmol) was added to the chilled solution of N^{α}-Z-N^{δ}-Poc-D-ornithine (12.5 g, 33 mmol) and *p*-nitrophenol (4.6 g, 33 mmol) in ethyl acetate (50 ml). The mixture was stirred in an ice-bath for 1 hr and at room temperature for 3 hr. Dicyclohexylurea precipitated was filtered off and the residue was washed with ethyl acetate. The combined filtrate was concentrated and residual oil was kept under petroleum ether. The solidified material was collected by filtration and recrystallized from ethanol; yield, 12.9 g (78%); mp 118—120 °C; $[\alpha]_D^{21}$ +22.4° (*c* 1, dimethylformamide).

Found: C, 60.28; H, 5.85; N, 8.42%. Calcd for $C_{25}H_{29}O_8N_3$: C, 60.11; H, 5.85; N, 8.41%.

N^{α}-*Benzyloxycarbonyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanine Ethyl Ester (VIb)*. N^{α}-Z-N^{δ}-Poc-D-ornithine *p*-nitrophenyl ester (5.0 g, 10 mmol) in dimethylformamide (30 ml) was added to a solution of V hydrobromide[7] (4.0 g, 10 mmol) in dimethylformamide (100 ml) containing triethylamine (1.4 ml) with stirring in an ice-bath. The stirring was continued on ice cooling for 30 min and at room temperature for 20 hr. The solvent was removed *in vacuo* and residue was triturated with water. The precipitate was collected, washed with 5% sodium carbonate, water, 1M hydrochloric acid and water successively. The product was reprecipitated from ethanol; yield, 5.7 g (85%); mp 231—233 °C, $[\alpha]_D^{20}$ +9.6° (*c* 1, dimethylformamide).

Found: C, 64.94; H, 7.57; N, 8.40%. Calcd for $C_{36}H_{50}$-

O_8N_4: C, 64.84; H, 7.56; N, 8.40%.

N^{δ}-*Cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanine Ethyl Ester (VII) Hydrochloride.* *(i) From VIa*: The tripeptide derivative VIa (6.6 g, 10.4 mmol) was suspended in ethyl acetate (300 ml) and treated with hydrogen chloride in ethyl acetate (about 6.4 M, 150 ml). As vigorous stirring was continued, a clear solution was obtained and then the deposition of crystals was observed. The reaction mixture was kept at room temperature for 40 min. Upon addition of petroleum ether (800 ml), crystalline hydrochloride was deposited. It was filtered, washed with petroleum ether and dried over potassium hydroxide *in vacuo*. The crude product was combined with a second crop secured from the mother liquor, and recrystallized from methanol–ethyl ether; yield, 5.4 g, (92%); mp 197—200 °C (decomp.); $[\alpha]_D^{19}$ +1.4° (*c* 1.5, dimethylformamide).

Found: C, 58.93; H, 8.05; N, 9.78; Cl, 6.32%. Calcd for $C_{28}H_{44}O_6N_4 \cdot HCl$: C, 59.09; H, 7.97; N, 9.85; Cl, 6.23%.

(ii) From VIb: The tripeptide derivative VIb (10 g, 15 mmol) was dissolved in ethanol (200 ml) containing concentrated hydrochloric acid (2 ml). Palladium black was added to the solution and hydrogen gas was bubbled through the solution at room temperature for 10 hr. The catalyst was filtered off with the aid of celite and the filtrate was concentrated *in vacuo*. The precipitated solid was collected and recrystallized from methanol–ether; yield, 7.7 g, (91%); mp 198—199 °C (decomp.); $[\alpha]_D^{20}$ +1.2° (*c* 1.5, dimethylformamide).

Found: C, 59.17; H, 8.25; N, 9.61; Cl, 6.27%.

N^{α}-*Benzyloxycarbonyl*-N^{ε}-t-*butyloxycarbonyl*-L-*lysyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanine Ethyl Ester (VIII).* *(i) By 1-Succinimidyl Ester Procedure*: N^{α}-Z-N^{ε}-Boc-L-lysine 1-succinimidyl ester[14] (13.5 g, 28 mmol) in dimethylformamide (100 ml) was added to an ice-cold solution of the tripeptide ester VII hydrochloride (16 g, 28 mmol) and triethylamine (4 ml) in dimethylformamide (400 ml). The mixture was stirred at 0—3 °C for 30 min and then at room temperature for 30 hr. After most of the solvent had been removed under reduced pressure, the residue was diluted with water to yield a precipitate. It was collected by filtration, washed with 5% aqueous sodium carbonate, water, 5% aqueous citric acid and water successively. The product was reprecipitated from dimethylformamide–water and then dried *in vacuo* at 30 °C over phosphorus pentoxide; yield, 23.7 g, (94%); mp 218—221 °C (decomp.); $[\alpha]_D^{19}$ −10.8° (*c* 1, dimethylformamide).

Found: C, 63.09; H, 8.01; N, 9.35%. Calcd for $C_{47}H_{70}O_{11}N_6$: C, 63.06; H, 7.88; N, 9.39%.

Amino acid analysis: Lys+Orn, 2.10; Ile, 1.00; Phe, 0.97.

(ii) By p-*Nitrophenyl Ester Procedure*: The same tetrapeptide derivative VIII was obtained from N^{α}-Z-N^{ε}-Boc-L-lysine *p*-nitrophenyl ester[15] (3.2 g, 6.4 mmol) and the tripeptide ester VII hydrochloride (3.6 g, 6.3 mmol) by the usual method; yield, 4.4 g, (79%); mp 216—219 °C (decomp.); $[\alpha]_D^{19}$ −11.1° (*c* 1, dimethylformamide).

Found: C, 62.75; H, 7.96; N, 9.33%.

N^{α}-*Benzyloxycarbonyl*-N^{ε}-t-*butyloxycarbonyl*-L-*lysyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanine Hydrazide (IX) Hemihydrate.* Hydrazine hydrate (15 ml) was added to a solution of the tetrapeptide derivative VIII (7.3 g, 8.2 mmol) in dimethylformamide (150 ml) and the mixture was kept at room temperature for 48 hr. The solvent was removed *in vacuo* and the residue was solidified by addition of water. The resulting gelatinous mass was collected, washed with water, and dried *in vacuo*; yield, 6.8 g (93%); mp 187—190 °C (decomp.); $[\alpha]_D^{20}$ +8.9° (*c* 1, dimethylformamide).

Found: C, 60.56; H, 7.80; N, 12.46%. Calcd for $C_{45}H_{68}O_{10}N_8 \cdot 1/2\ H_2O$: C, 60.72; H, 7.81; N, 12.59%.

N^{α}-*Benzyloxycarbonyl*-N^{ε}-t-*butyloxycarbonyl*-L-*lysyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanyl*-L-*histidine Methyl Ester (X) Dihydrate.* A solution of sodium nitrite (1 g) in water (5 ml) was added to an ice-cold solution of the tetrapeptide hydrazide IX hemihydrate (8.9 g, 10 mmol) in a mixture of 60% acetic acid (200 ml) and 1M hydrochloric acid (20 ml) at −3—0 °C with vigorous stirring. After 10 min, excess cold water was added to the mixture. The resulting precipitate was filtered and washed thoroughly with water. The acyl tetrapeptide azide thus obtained was dissolved in dimethylformamide (200 ml) and cooled to 2—3 °C. This solution was added to an ice-cold solution of methyl L-histidinate dihydrochloride (5 g, 21 mmol) in dimethylformamide (100 ml) containing triethylamine (4 ml) with stirring. The mixture was stirred at 0—2 °C for 18 hr and then at room temperature for 24 hr. The mixture was concentrated *in vacuo* and the residue was triturated with water. The amorphous solid precipitated was collected and washed thoroughly with water. The product was reprecipitated from dimethylformamide–water, and dried over phosphorus pentoxide at 30—35 °C; yield, 8.4 g, (80%); mp 186—190 °C; $[\alpha]_D^{19}$ +6.0° (*c* 1, dimethylformamide).

Found: C, 59.53; H, 7.58; N, 11.90%. Calcd for $C_{52}H_{75}O_{12}N_9 \cdot 2H_2O$: C, 59.24; H, 7.55; N, 11.96%.

Amino acid analysis: Lys+Orn, 1.95; Ile, 1.00; Phe, 1.05; His, 0.96.

N^{α}-*Benzyloxycarbonyl*-N^{ε}-t-*butyloxycarbonyl*-L-*lysyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanyl*-L-*histidine Hydrazide (XI) Hydrate.* Hydrazine hydrate (10 ml) was added to a solution of the pentapeptide ester X dihydrate (5.5 g, 5.2 mmol) in dimethylformamide (150 ml). After the mixture had been kept at room temperature for 48 hr, the solvent was evaporated *in vacuo* and the residue obtained was diluted with water. The amorphous solid precipitated was filtered and washed with water. The product was reprecipitated from dimethylformamide-water and dried *in vacuo* over phosphorus pentoxide at 35 °C; yield, 5.1 g, (94%); mp 206—208 °C (decomp.); $[\alpha]_D^{17}$ +6.3° (*c* 1, dimethylformamide).

Found: C, 59.07; H, 7.46; N, 14.74%. Calcd for $C_{51}H_{75}O_{11}N_{11} \cdot H_2O$: C, 59.11; H, 7.49; N, 14.87%.

α-Methyl L-*Aspartate Trifluoroacetate.* α-Methyl Z-L-aspartate[16] (11.3 g, 40 mmol) prepared from the corresponding dicyclohexylammonium salt was dissolved in trifluoroacetic acid (40 ml) and the solution was heated under refluxing for 30 min. Excess acid was removed *in vacuo* and the residue was triturated with ethyl ether. The precipitate formed was collected by filtration and recrystallized from methanol–ethyl ether; yield, 9.4 g, (90%); mp 117—119 °C; $[\alpha]_D^{15}$ +13.0° (*c* 1, methanol).

Found: C, 32.31; H, 3.95; N, 5.36%. Calcd for $C_5H_9O_4N \cdot CF_3COOH$: C, 32.19; H, 3.86; N, 5.36%.

N^{α}-*Benzyloxycarbonyl*-N^{ε}-t-*butyloxycarbonyl*-L-*lysyl*-N^{δ}-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanyl*-L-*histidyl*-L-*aspartic Acid α-Methyl Ester (XII).* The pentapeptide hydrazide XI hydrate (4.0 g, 3.9 mmol) was dissolved in aqueous acetic acid (CH_3COOH 40 ml, H_2O 16 ml). Sodium nitrite (400 mg) in water (1 ml) was added to the soltuion at 0 °C and the reaction mixture was vigorously stirred at the

14) H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, This Bulletin, **39**, 882 (1966).

15) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).

16) E. Wünsch and A. Zwick, *Z. Physiol. Chem.*, **333**, 108 (1963).

same temperature for 10 min. Saturated sodium chloride solution (250 ml) was added to the solution on ice cooling and the precipitated material was collected and washed thoroughly with ice-cold water. The solid thus obtained was dissolved in cold dimethylformamide (200 ml). This preparation was added to the cooled solution of α-methyl L-aspartate trifluoroacetate (1.6 g, 6.1 mmol) in dimethylformamide (100 ml) containing triethylamine (2 ml). The mixture was stirred at 0 °C for 12 hr and at room temperature for 24 hr. The solvent was removed *in vacuo* and excess water was added to the residue. The precipitate formed was collected, washed with water and reprecipitated from dimethylformamide–water; yield, 4.1 g, (93%); mp 167—175 °C; $[\alpha]_D^{19}$ +9.1° (*c* 1, dimethylformamide).

Found: C, 59.77; H, 7.60; N, 11.49%. Calcd for $C_{56}H_{80}O_{15}N_{10}$: C, 59.35; H, 7.12; N, 12.36%.

Cyclo-(N$^{\alpha}$-benzyloxycarbonyl-L-lysyl-N$^{\delta}$-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-α-methyl-L-aspartyl) (III) Hydrate. To a solution of the hexapeptide derivative XII (6.0 g, 5.3 mmol) in dimethylformamide (50 ml), there were added chloroacetonitrile (3 ml) and triethylamine (6 ml). The reaction mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and water was added to the residue. Solid formed was filtered and dried over phosphorus pentoxide *in vacuo*. The product (5.8 g) was treated with trifluoroacetic acid (60 ml) at room temperature for 30 min with stirring. Excess trifluoroacetic acid was removed *in vacuo*, and the residue was triturated with ethyl ether. The deblocked product thus obtained was dissolved in a mixture of dimethylformamide (20 ml) and acetic acid (5 ml). The solution was dropped into large excess pyridine (5 l) for 6 hr and the reaction mixture was stirred at 40—45 °C for 48 hr. The reaction mixture gave a spot showing positive Pauly and negative ninhydrin reaction at R_f 0.71, besides the original spot of deblocked linear peptide at R_f 0.1—0.2, and a minor spot of positive iodine test but negative both to Pauly[17] and ninhydrin reaction at R_f 0.91 on thin-layer chromatography using methanol–chloroform (2: 8, v/v) as developing solvent. The solvent was evaporated *in vacuo* and the residue was purified by silica gel column chromatography (elution solvent: chloroform–methanol, 1:19—1:9 v/v) to obtain the cyclization product of R_f 0.71 on thin-layer chromatogram; yield, 53 mg (0.97%); mp 160—165 °C (decomp.); $[\alpha]_D^{18}$ +11.4° (*c* 1, dimethylformamide).

Found: C, 59.04; H, 7.03; N, 13.78%. Calcd for $C_{51}H_{70}O_{12}N_{10}\cdot H_2O$: C, 59.29; H, 7.03; N, 13.56%.

Amino acid analysis: Lys+Orn, 1.98; Ile, 1.11; Phe, 1.00; His, 0.95; Asp, 0.89.

Benzyl t-Butyloxycarbonyl-D-isoasparaginate (XIII). Concentrated aqueous ammonia (5 ml) in tetrahydrofuran (50 ml) was added to a cooled solution of Boc-D-aspartic acid β-benzyl α-1-succinimidyl ester (21.0 g, 50 mmol) in tetrahydrofuran (200 ml). The mixture was stirred at room temperature for 3 hr. The solution was concentrated and the residual solid was recrystallized from methanol–ethyl ether; yield, 12.3 g, (76%); mp 159—160.5 °C (decomp.); $[\alpha]_D^{15}$ −1.3° (*c* 1, methanol).

Found: C, 59.54; H, 6.89; N, 8.69%. Calcd for $C_{16}H_{22}O_5N_2$: C, 59.61; H, 6.83; N, 8.69%.

Benzyl D-Isoasparaginate (XIV) Trifluoroacetate. The compound XIII (4.8 g, 15 mmol) was dissolved in trifluoroacetic acid (30 ml). The solution was stirred at room temperature for 20 min. Excess trifluoroacetic acid was evaporated and the residue obtained was triturated with anhydrous ethyl ether. The precipitate was recrystallized from methanol–ethyl ether; yield, 4.5 g, (90%); mp 185—186.5 °C (decomp.); $[\alpha]_D^{15}$ −1.1° (*c* 1, methanol).

Found: C, 46.32; H, 4.52; N, 8.33%. Calcd for $C_{11}H_{14}O_3N_2\cdot CF_3COOH$: C, 46.43; H, 4.50; N, 8.33%.

t-Butyloxycarbonyl-L-aspartic Acid β-t-Butyl α-1-Succinimidyl Ester. *N*-Hydroxysuccinimide (11.5 g, 0.1 mol) and β-*t*-butyl Boc-L-aspartate obtained from its dicyclohexylammonium salt (47.1 g, 0.1 mol) by desalting with citric acid were dissolved in tetrahydrofuran (300 ml) and cooled on an ice-bath. *N,N'*-Dicyclohexylcarbodiimide (20.6 g, 0.1 mol) was added to the solution and the reaction mixture was stirred on ice cooling for 2 hr and then at room temperature for 3 hr. The precipitate was filtered off and the combined filtrate was concentrated *in vacuo*. The residual solid was collected by filtration with the aid of petroleum ether and recrystallized from ethyl acetate–petroleum ether; yield, 27.5 g, (71%); mp 105—105.5 °C; $[\alpha]_D^{15}$ −15.2° (*c* 1, ethyl acetate).

Found: C, 52.97; H, 6.82; N, 7.21%. Calcd for $C_{17}H_{26}O_8N_2$: C, 52.84; H, 6.78; N, 7.25%.

t-Butyloxycarbonyl-β-t-butyl-L-aspartyl-D-isoasparagine Benzyl Ester (XV). A solution of Boc-L-aspartic acid β-*t*-butyl α-1-succinimidyl ester (9.7 g, 25 mmol) in chloroform (100 ml) was added to an ice-cold solution of benzyl D-isoasparaginate (XIV) trifluoroacetate (8.4 g, 25 mmol) in chloroform (200 ml) containing triethylamine (4 ml). The mixture was stirred on an ice-bath for 1 hr and at room temperature for 12 hr. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (200 ml). The ethyl acetate solution was washed with water and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the residue was kept under petroleum ether. The precipitate was collected and recrystallized from methanol–petroleum ether; yield, 11.0 g (89%); mp 120—121 °C; $[\alpha]_D^{14}$ −3.2° (*c* 1, methanol).

Found: C, 58.35; H, 7.12; N, 8.52%. Calcd for $C_{24}H_{35}O_8N_3$: C, 58.40; H, 7.15; N, 8.51%.

L-Aspartyl-D-isoasparagine Benzyl Ester (XVI) Hemihydrate. The dipeptide diester XV (4.0 g, 8.1 mmol) was dissolved in trifluoroacetic acid (15 ml). The mixture was stirred at room temperature for 30 min. Excess trifluoroacetic acid was evaporated *in vacuo*. The residue was kept under ethyl ether and the resulting solid was recrystallized from methanol–ethyl ether. During recrystallization, trifluoroacetic acid in salt form of the product was eliminated from the deblocked peptide derivative; yield, 2.6 g, (93%); mp 160—165 °C; $[\alpha]_D^{15}$ +6.7° (*c* 1, acetic acid).

Found: C, 52.26; H, 5.53; N, 12.05%. Calcd for $C_{15}H_{19}O_6N_3\cdot 1/2H_2O$: C, 52.02; H, 5.82; N, 12.13%.

t-Butyloxycarbonyl-N^{im}-benzyl-L-histidyl-L-aspartyl-D-isoasparagine Benzyl Ester (XVII) Hydrate. Boc-*N*^{im}-benzyl-L-histidine 2,4,5-trichlorophenyl ester[18] (7.9 g, 15 mmol) in dimethylformamide (30 ml) was added to a chilled solution of the compound XVI hemihydrate (5.2 g, 15 mmol) in dimethylformamide (70 ml) containing triethylamine (4.2 ml). The mixture was stirred at 0 °C for 1 hr and at room temperature for 12 hr. The solvent was removed *in vacuo* and the residual oil was diluted with water. The precipitate formed was collected by filtration. The product was purified by silica gel column chromatography (elution solvent: chloroform–methanol 9: 1—7: 3 v/v); yield, 3.2 g, (31%); mp 125—128 °C; $[\alpha]_D^{15}$ +6.5° (*c* 1, methanol).

Found: C, 58.48; H, 6.22; N, 12.20%. Calcd for $C_{33}H_{40}$-

17) Faint yellow: This spot is assumed to be *N*^{im}-cyanomethylated cyclohexapeptide.

18) B. O. Handford, T. A. Hylton, K.-T. Wang, and B. Weinstein, *J. Org. Chem.*, **33**, 4251 (1968).

$O_9N_6 \cdot H_2O$: C, 58.05; H, 6.20; N, 12.31%.

Amino acid analysis: N^{im}-Bzl-His, 1.08; Asp, 2.00; N^{im}-benzylhistidine was eluted with citrate buffer of pH 6.80 from the short column of amino acid analyzer.

N^{im}-*Benzyl*-L-*histidyl*-L-*aspartyl*-D-*isoasparagine Benzyl Ester (XVIII) Bistrifluoroacetate.* The compound XVII hydrate obtained above (683 mg, 1 mmol) was dissolved in trifluoroacetic acid (5 ml). The solution was stirred at room temperature for 30 min and excess acid was evaporated *in vacuo*. The residue was triturated with ethyl ether. The precipitate formed was collected by filtration. This product was homogeneous on paper electrophoresis at pH 4.8; yield, 713 mg (90%); mp 104—118 °C (decomp.); $[\alpha]_D^{15}$ +4.4° (*c* 1, methanol).

Found: C, 48.51; H, 4.42; N, 10.50%. Calcd for $C_{28}H_{32}O_7N_6 \cdot 2CF_3COOH$: C, 48.49; H, 4.32; N, 10.60%.

N$^{\alpha}$-*Benzyloxycarbonyl*-N$^{\varepsilon}$-t-*butyloxycarbonyl*-L-*lysyl*-N$^{\delta}$-*cyclopentyloxycarbonyl*-D-*ornithyl*-L-*isoleucyl*-D-*phenylalanyl*-N^{im}-*benzyl*-L-*histidyl*-L-*aspartyl*-D-*isoasparagine Benzyl Ester (IV) Hydrate.* The tetrapeptide hydrazide IX hemihydrate (291 mg, 0.33 mmol) was dissolved in a mixture of acetic acid (4 ml) and 1M hydrochloric acid (1.7 ml). A solution of sodium nitrite (40 mg) in water (0.5 ml) was added to the cooled solution mentioned above and the mixture was stirred at 0 °C for 30 min. Ice-cold saturated aqueous sodium chloride (50 ml) was added to the above solution. The deposit was collected by filtration and washed with cold water thoroughly. The product was dissolved in dimethylformamide (30 ml) and added to a solution of the tripeptide derivative XVIII bistrifluoroacetate (270 mg, 0.34 mmol) in dimethylformamide (20 ml) containing triethylamine (0.1 ml) at 0 °C. The solution was stirred at 5 °C for 48 hr and then at room temperature for 24 hr. The solvent was removed *in vacuo* and the residual oil was triturated with water. The precipitate was collected by filtration. The crude product was purified by silica gel column chromatography (elution solvent: chloroform–methanol 9:1—8:2; v/v); yield, 330 mg (70%); mp 185—195 °C; $[\alpha]_D^{15}$ +15.0° (*c* 1, dimethylformamide).

Found: C, 60.80; H, 7.04; N, 11.74%. Calcd for $C_{73}H_{96}O_{17}N_{12} \cdot H_2O$: C, 61.24; H, 6.90; N, 11.74%.

Amino acid analysis: Lys+Orn, 1.92; Ile, 1.00; Phe, 0.95; N^{im}-Bzl-His, 1.05; Asp, 1.85.

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