BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 46, 3835—3839 (1973)

Synthetic Studies of Bacitracin. IX.[1] Synthesis of Peptide Fragments for Bacitracins of Cycloheptapeptide Formula[2]

Eisuke MUNEKATA,* Tetsuo SHIBA, and Takeo KANEKO**

Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka 560

(Received June 20, 1973)

In the synthetic study of bacitracins, peptide fragments corresponding to a branched part of bacitracin F, *i.e.*, (*S*)-2-(2-methylbutyryl)thiazole-4-carboxylic acid 1-succinimidyl ester (III) and L-leucyl-γ-*t*-butyl-D-glutamyl-L-isoleucine (IV), were prepared. As a macrocyclic part of bacitracin A and F of cycloheptapeptide formula, cyclo-(N^{α}-benzyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-β-benzyl-D-aspartyl-L-asparaginyl) (V) was synthesized through cyanomethyl ester method.

In the preceding paper,[1] the authors described the preparation of the intermediate heptapeptide derivative for the synthetic study of bacitracin A and F, the sequence of which was proposed by Stoffel and Craig.[3] Although Swallow and Abraham could not reduce four possibilities for the mode of linkage around L-lysine, L-aspartic acid and D-aspartic acid residues in the structure of bacitration A,[4] Stoffel and Craig proposed a structure I as the most probable one from rather inconceivable result of hydrazinolysis,[3] and this structure seemed to be accepted as the chemical structure of bacitracin A until 1966 when Ressler and Kashelikar revised the structure to formula II.[5] The latter group devised a new method for distinguishing asparagine and isoasparagine residues in peptide chain by dehydration of acid amide to nitrile followed by reduction and hydrolysis. Applying this technique to bacitration A, Ressler and Kashelikar recognized the presence of L-asparagine residue inside the peptide ring in lieu of D-isoasparagine residue outside the ring.[5] Recently, Craig and his collaborators also supported this structure II of a seven amino acids-membered ring

* Present address: Peptide Institute, Protein Research Foundation, Minoh, Osaka.

** Present address: Shiseido Laboratory, Nippa-cho, Kohoku-ku, Yokohama.

1) Part VIII: E. Munekata, Y. Masui, T. Shiba, and T. Kaneko, This Bulletin, **46**, 3187 (1973).

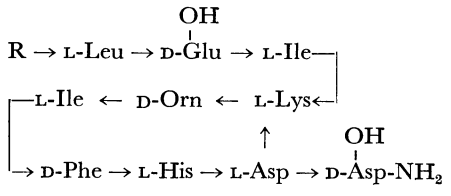
2) This work was presented at the 5th Symposium on Peptide Chemistry, Kyoto, November, 1967 and the 8th same Symposium, Osaka, November, 1970.

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

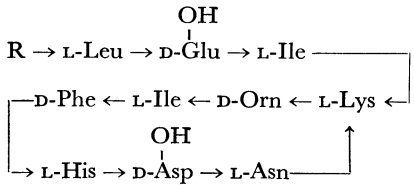
4) D. L. Swallow and E. P. Abraham, *Biochem. J.*, **72**, 326 (1959).

5) C. Ressler and D. V. Kashelikar, *J. Amer. Chem. Soc.*, **88**, 2025 (1966).

on a basis of their unpublished data.[6]

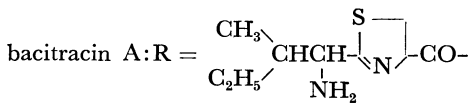


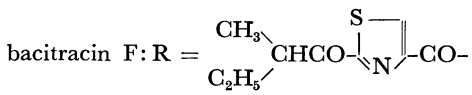
I



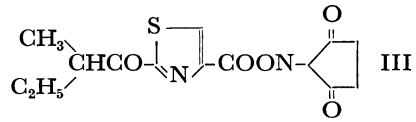
II

⟶ = CO→NH

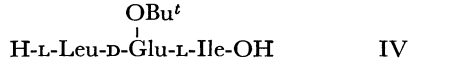




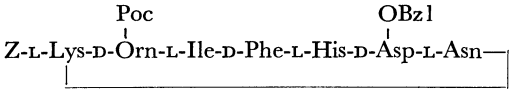
In this paper, we synthesized component fragments to build up the most reliable amino acid sequence II of bacitracin A and F, following the synthetic principle described in the previous paper[1] for an achievement of the total synthesis of the antibiotics. The principle is based on the coupling of thiazoline or thiazole moiety in the branched chain with macrocyclic peptide part. For the branched peptide part of bacitracin F, (*S*)-2-(2-methylbutyryl)-thiazole-4-carboxylic acid 1-succinimidyl ester (III) and L-leucyl-*γ*-*t*-butyl-D-glutamyl-L-isoleucine (IV) were prepared and for the macrocyclic part of the bacitracin A and F, cyclo-(N^{α}-benzyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-*β*-benzyl-D-aspartyl-L-asparaginyl) (V) was synthesized. These three peptide fragments cover the whole sequence of the antibiotic.



III



IV



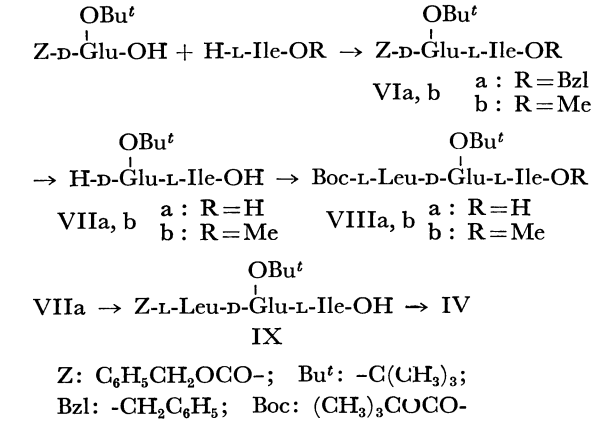
V

But: $-C(CH_3)_3$; Z: $C_6H_5CH_2OCO-$

Poc:  -OCO-; Bzl: $-CH_2C_6H_5$

For the synthesis of *N*-terminal part in bacitracin F, the 1-succinimidyl ester III was prepared by the usual manner for synthesis of the active ester using dicyclohexylcarbodiimide from (*S*)-2-(2-methylbutyryl)-thiazole-4-carboxylic acid, the preparation of which was already reported in our previous paper.[7] The synthetic route of the tripeptides derivative IV in the branched peptide chain of the antibiotic was shown in Scheme 1. Thus, benzyloxycarbonyl-D-glutamic acid *γ*-*t*-butyl *α*-1-succinimidyl ester was coupled with benzyl L-isoleucinate to give benzyloxycarbonyl-*γ*-*t*-butyl-D-glutamyl-L-isoleucine benzyl ester (VIa). Dipeptide derivative VIa thus obtained was hydrogenated using palladium charcoal as catalyst to give *γ*-*t*-butyl-D-glutamyl-L-isoleucine (VIIa). The resulting deblocked peptide derivative VIIa was acylated by *t*-butyloxycarbonyl-L-leucine 1-succinimidyl ester to obtain *t*-butyloxycarbonyl-L-leucyl-*γ*-*t*-butyl-D-glutamyl-L-isoleucine (VIIIa). In the alternative route for preparation of the tripeptide VIIIa, *t*-butyloxycarbonyl-L-leucyl-*γ*-*t*-butyl-D-glutamyl-L-isoleucine methyl ester (VIIIb) was synthesized as a precursor of VIIIa according to Scheme 1 through the methyl esters VIb and VIIb. However, the methyl ester of the resulting tripeptide derivative VIIIb was found to resist to alkaline hydrolysis and the wanted product VIIIa of a free carboxyl group could not be obtained. When *γ*-*t*-butyl-D-glutamyl-L-isoleucine (VIIa) was acylated with benzyloxycarbonyl-L-leucine 1-succinimidyl ester, other tripeptide derivative IX was obtained. This was hydrogenated in the presence of palladium charcoal to give L-leucyl-*γ*-*t*-butyl-D-glutamyl-L-isoleucine (IV).

The cycloheptapeptide derivative V corresponding to the macrocyclic part was synthesized starting from *C*-terminal L-asparagine residue as shown in Scheme 2. First, *t*-butyloxycarbonyl-*β*-benzyl-D-aspartyl-L-asparagine (X) was prepared by coupling of *β*-benzyl *t*-butyloxycarbonyl-D-aspartate with L-asparagine either by the mixed anhydride method or by the 1-succinimidyl ester method. Removal of *t*-butyloxycarbonyl group from X gave *β*-benzyl-D-aspartyl-L-asparagine (XI). When a principle of the stepwise elongation from *C*-terminal was applied to this dipeptide derivative XI, the resulting tripeptide derivatives, i.e., benzyloxycarbonyl or *t*-butyloxycarbonyl-L-histidyl-*β*-benzyl-D-aspartyl-L-asparagine, showed awfully hy-

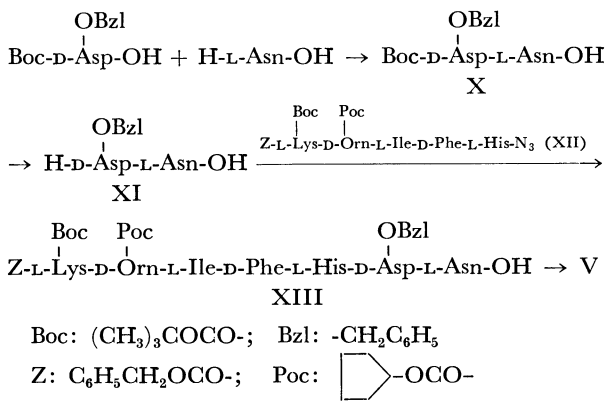


Z: $C_6H_5CH_2OCO-$; But: $-C(CH_3)_3$;
Bzl: $-CH_2C_6H_5$; Boc: $(CH_3)_3COCO-$

Scheme 1. Synthesis of the tripeptide derivative IV in a branched part of bacitracins.

6) R. E. Galardy, M. P. Printz, and L. C. Craig, *Biochemistry*, **10**, 2429 (1971).

7) Y. Ariyoshi, T. Shiba, and T. Kaneko, This Bulletin, **40**, 2654 (1967).



Scheme 2. Synthesis of the cycloheptapeptide derivative V corresponding to the macrocyclic part of bacitracin A and F of seven amino acids-membered formula.

groscopic and gelatinous properties which prevented isolation of a pure solid as the product. Furthermore, coupling of the deblocked product of the tripeptide mentioned above with *N*-acyl-D-phenylalanine could not give any pure tetrapeptide derivatives because of an extreme gelatinous character. Therefore, we turned to a route of coupling of XI with the acyl pentapeptide derivative XII, *i.e.*, N^{α}-benzyloxycarbonyl-N^{ε}-*t*-butyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidine azide, the preparation of which was reported in the preceding paper,[1] and obtained the expected heptapeptide derivative XIII as demonstrated in Scheme 2. In this plan of building up the heptapeptide, we used the special cyclopentyloxycarbonyl group for a protection of δ-amino group of ornithine residue by reason of selective stability as far as a final synthetic step and of cleavability with hydrogen fluoride.[1]

We are now ready to total synthesis of bacitracin F of Ressler's formula by securing all peptide fragments required. However, for a cyclization of the heptapeptide XIII obtained above, the choice of the condensation reagent is limited by possible dehydration of the acid amide group in asparagine residue or undesirable side reaction on imidazole ring in histidine residue. In fact, attempt to cyclize XIII between the carboxyl group of asparagine residue and ε-amino group of lysine residue after removal of *t*-butyloxycarbonyl protection has not been succeeded yet when *N*-ethyl-5-phenylisoxazolium-5'-sulfonate described as safe reagent particularly for asparagine peptide was used for cyclization. Only when the cyanomethyl ester method was applied to this cyclization, the expected cyclo (N^{α}-benzyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-β-benzyl-D-aspartyl-L-asparaginyl) was obtained in one experiment. However, a yield of this product was extremely low and reproducibility of this cyclization reaction was strangely poor. These results may indicate that the activation of the carboxyl group of asparagine residue for cyclization of this heptapeptide is not adequate presumably owing to undesirable side reactions. Therefore, it must be required on this stage of the study that either the more effective cyclization method should be applied or a linkage to be connected for the cyclization has to be searched at another place to accomplish the total synthesis of bacitracins.

Experimental

All melting points are uncorrected. For silica gel column chromatography, Kieselgel 0.05—0.2 mm (70—325 mesh ASTM) Merck, was used. The infrared spectrum was obtained in Nujol mull with a Nihon Bunko IR-S spectrophotometer.

(S)-2-(2-Methylbutyryl)-thiazole-4-carboxylic Acid 1-Succinimidyl Ester (III). To a solution of (*S*)-2-(2-methylbutyryl)-thiazole-4-carboxylic acid[7] (2.1 g, 10 mmol) and *N*-hydroxysuccinimide (1.2 g, 10 mmol) in tetrahydrofuran (50 ml), there was added *N,N'*-dicyclohexylcarbodiimide (2.1 g, 10 mmol) under ice-cooling. The reaction mixture was stirred at this temperature for 1 hr and then at room temperature for 4 hr. Dicyclohexylurea deposited was filtered off, and the filtrate was evaporated *in vacuo*. A solid residue thus obtained was recrystallized from ethanol. Yield, 2.5 g (81%); mp 101—102 °C; $[\alpha]_D^{20}$ +13.4° (*c* 1, methanol).

Found: C, 50.07; H, 4.62; N, 9.00; S, 10.24. Calcd for $C_{13}H_{14}O_5N_2S$: C, 50.31; H, 4.55; N, 9.03; S, 10.33%.

Benzyloxycarbonyl-γ-t-butyl-D-glutamyl-L-isoleucine Benzyl Ester (VIa). A mixture of benzyloxycarbonyl-D-glutamic acid γ-*t*-butyl-α-1-succinimidyl ester[8] (8.7 g, 20 mmol), benzyl L-isoleucinate *p*-toluenesulfonate[9] (8.0 g, 20 mmol) and triethylamine (3 ml) in methylene chloride (300 ml) was stirred at 0 °C for 1 hr and then at room temperature for 17 hr. The solution was washed successively with 5% aqueous sodium carbonate, water, 0.5 M hydrochloric acid and water. The organic layer was separated, dried over anhydrous sodium sulfate, and then evaporated *in vacuo*. The residual solid was recrystallized from ethyl acetate–petroleum ether. Yield, 8.3 g (77%); mp 79—81 °C; $[\alpha]_D^{17}$ +8.8° (*c* 1, methanol).

Found: C, 66.36; H, 7.54; N, 5.35%. Calcd for $C_{30}H_{40}O_7N_2$: C, 66.64; H, 7.46; N, 6.18%.

γ-t-Butyl-D-glutamyl-L-isoleucine (VIIa) Hydrate. The protected dipeptide ester VIa (4.0 g, 7.4 mmol) was dissolved in methanol (100 ml) containing acetic acid (10 ml). Hydrogen gas was bubbled through the solution in the presence of palladium charcoal at room temperature for 8 hr. The catalyst was filtered off with the aid of celite. The filtrate was concentrated and a residual precipitate was collected by filtration. The product was recrystallized from methanol–water. Yield, 2.4 g (97%); mp 165—168 °C; $[\alpha]_D^{17}$ −32.4° (*c* 1, methanol).

Found: C, 53.66; H, 9.18; N, 8.41%. Calcd for $C_{15}H_{28}O_5N_2 \cdot H_2O$: C, 53.87; H, 9.04; N, 8.38%.

t-Butyloxycarbonyl-L-leucyl-γ-t-butyl-D-glutamyl-L-isoleucine (VIIIa). *t*-Butyloxycarbonyl-L-leucine 1-succinimidyl ester[10] (2.3 g, 7 mmol) in ethyl acetate (50 ml) was added to a cold solution of VIIa (2.4 g, 7 mmol) in chloroform containing triethylamine (1 ml). The mixture was stirred under ice-cooling for 1 hr and at room temperature for 8 hr. The solvent was removed *in vacuo* and the residue was triturated with petroleum ether. The product was collected, washed with water and recrystallized from ethyl acetate–petroleum ether. Yield, 2.5 g (68%); mp 142—143 °C; $[\alpha]_D^{17}$ +10.4° (*c* 1, methanol).

Found: C, 58.81; H, 9.05; N, 7.85%. Calcd for $C_{26}H_{47}$-

8) R. Zabel and H. Zahn, *Z. Naturforsch.*, **20b**, 650 (1965).
9) L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).
10) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964).

O_8N_3: C, 58.95; H, 8.94; N, 7.93%.

Benzyloxycarbonyl-γ-t-butyl-D-glutamyl-L-isoleucine Methyl Ester (VIb). A mixture of benzyloxycarbonyl-D-glutamic acid γ-*t*-butyl α-1-succinimidyl ester[8] (2.2 g, 5 mmol) and methyl L-isoleucinate *p*-toluenesulfonate (1.6 g, 5 mmol) in chloroform (100 ml) containing triethylamine (0.8 ml) was stirred at 0 °C for 1 hr and then at room temperature for 10 hr. The solution was washed successively with 5% aqueous sodium hydrogen carbonate, water, 0.5 M hydrochloric acid and water, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and a resulting precipitate was recrystallized from diisopropyl ether–petroleum ether. Yield, 1.8 g (78%); mp 61—62 °C; $[\alpha]_D^{18}$ +5.9° (*c* 1, methanol).

Found: C, 61.96; H, 7.82; N, 6.17%. Calcd for $C_{24}H_{36}O_7N_2$: C, 62.05; H, 7.81; N, 6.03%.

γ-t-Butyl-D-glutamyl-L-isoleucine Methyl Ester (VIIb) p-Toluenesulfonate. The dipeptide methyl ester VIb (10 g, 2.2 mmol) was dissolved in methanol (20 ml) containing a few drops of acetic acid. Hydrogen gas was bubbled through the solution in the presence of palladium charcoal at room temperature for 6 hr. The catalyst was removed by filtration and a filtrate was concentrated to a half volume of the original solution. To a residual solution, *p*-toluenesulfonic acid hydrate (0.5 g) in ether was added. The deposit was collected by filtration and recrystallized from methanol–ethyl ether. Yield, 0.7 g (64%); mp 157—158 °C; $[\alpha]_D^{19}$ −21.0° (*c* 1, methanol).

Found: C, 54.88; H, 7.69; N, 5.54; S, 6.40%. Calcd for $C_{23}H_{38}O_8N_2S$: C, 54.96; H, 7.62; N, 5.57; S, 6.38%.

t-Butyloxycarbonyl-L-leucyl-γ-t-butyl-D-glutamyl-L-isoleucine Methyl Ester (VIIIb). *t*-Butyloxycarbonyl-L-leucine 1-succinimidyl ester[10] (1.3 g, 4.1 mmol) and VIIb *p*-toluenesulfonate (2.1 g, 4.1 mmol) were dissolved in chloroform (100 ml). To this solution, triethylamine (0.8 ml) was added on cooling and the mixture was stirred at room temperature for 20 hr. The chloroform solution was washed with 5% aqueous sodium carbonate, water, 0.5 M hydrochloric acid and water successively. The organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was kept under ethyl ether and a solid thus obtained was collected by filtration and recrystallized from diisopropyl ether. Yield, 1.4 g (63%); mp 131—132 °C; $[\alpha]_D^{20}$ −4.3° (*c* 1, methanol).

Found: C, 59.45; H, 9.00; N, 7.62%. Calcd for $C_{27}H_{49}O_8N_3$: C, 59.64; H, 9.08; N, 7.73%.

Benzyloxycarbonyl-L-leucyl-γ-t-butyl-D-glutamyl-L-isoleucine (IX). A solution of benzyloxycarbonyl-L-leucine 1-succinimidyl ester[10] (3.6 g, 10 mmol), VIIa (3.3 g, 10 mmol) and triethylamine (1.5 ml) in chloroform (80 ml) was stirred under ice-cooling for 1 hr, and then at room temperature for 12 hr. The solvent was evaporated *in vacuo* and a residue obtained was dissolved in ethyl acetate. The solution was washed with 5% aqueous citric acid, and then water. After drying over sodium sulfate, the solution was concentrated *in vacuo*. A residue obtained was purified by silica gel column chromatography using methanol–chloroform as an elution solvent to give oily product. Yield, 3.4 g (63%). This material was used to the following deblocking reaction directly.

L-Leucyl-γ-t-butyl-D-glutamyl-L-isoleucine (IV) Hydrate. The oily product IX (3.4 g, 6.0 mmol) obtained above was dissolved in tetrahydrofran (50 ml). Through the solution, hydrogen was bubbled at room temperature in the presence of palladium charcoal for 5 hr. After filtration of the catalyst, a filtrate was concentrated to yield crystals. They were filtered and reprecipitated from methanol–water. Yield, 2.2 g (85%); mp 181—184 °C; $[\alpha]_D^{20}$ +58.9° (*c* 1, methanol).

Found: C, 55.71; H, 9.39; N, 9.14%. Calcd for $C_{21}H_{39}O_6N_3 \cdot 1\frac{1}{2}H_2O$: C, 55.24; H, 9.27; N, 9.20%.

β-Benzyl t-Butyloxycarbonyl-D-aspartate. This compound was prepared from β-benzyl D-aspartate[11] (33.5 g, 0.15 mol) and *t*-butyl azidoformate (22.0 g, 0.15 mol) according to the procedure for L-antipode by Sandrin and Boissonnas.[12] Yield, 2.7 g (56%); mp 102—103 °C; $[\alpha]_D^{19}$ −8.3° (*c* 1, acetic acid). Lit,[12] L-antipode, mp 101 °C; $[\alpha]_D^{22}$ +9±1° (c 2, acetic acid).

Found: C, 59.39; H, 6.60; N, 4.35%. Calcd for $C_{16}H_{21}O_6N$: C, 59.43; H, 6.55; N, 4.33%.

t-Butyloxycarbonyl-D-aspartic β-Benzyl α-1-Succinimidyl Ester. Esterification of β-benzyl *t*-butyloxycarbonyl-D-aspartate (10 g, 31 mmol) with *N*-hydroxysuccinimide (3.5 g, 30 mmol) was carried out according to the procedure for L-antipode by Laufer and Blout.[13] Yield, 11.8 g (94%); mp 100—102 °C; $[\alpha]_D^{20}$ +20.5° (*c* 0.5, dioxane). Lit,[13] L-antipode, mp 102—103 °C; $[\alpha]_D^{26}$ −20.0° (c 0.55, dioxane).

Found: C, 57.30; H, 6.00; N, 6.63%. Calcd for $C_{20}H_{24}O_8N_2$: C, 57.13; H, 5.75; N, 6.66%.

t-Butyloxycarbonyl-β-benzyl-D-aspartyl-L-asparagine (X).

(i) By the Mixed Anhydride Procedure: A mixed anhydride was prepared from β-benzyl *t*-butyloxycarbonyl-D-aspartate (3.3 g, 10 mmol) with isobutyl chloroformate (1.3 g, 10 mmol) and triethylamine (1.3 ml) in tetrahydrofuran (20 ml) at −5—0 °C. To the mixed anhydride solution, L-asparagine hydrate (1.5 g, 10 mmol) and triethylamine (1.4 ml) in water (30 ml) were added. The mixture was stirred under ice-cooling for 2 hr and at room temperature for 8 hr. The mixture was extracted with two 20 ml portions of ether and aqueous layer was acidified with solid citric acid to pH 2—3. A resulting oily precipitate was extracted with two 80 ml portions of ethyl acetate. An organic layer was washed with water and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and a residual syrup was crystallized when kept under petroleum ether. The product was recrystallized from a large excess of ethyl acetate. Yield, 2.7 g (62%); mp 154—156 °C; $[\alpha]_D^{20}$ +37.1° (*c* 1, ethanol).

Found: C, 54.62; H, 6.43; N, 9.36%. Calcd for $C_{20}H_{27}O_8N_3$: C, 54.91; H, 6.22; N, 9.61%.

(ii) By the 1-Succinimidyl Ester Procedure: *t*-Butyloxycarbonyl-D-aspartic β-benzyl α-1-succinimidyl ester (14.4 g, 34 mmol) in tetrahydrofuran (160 ml) was added to an ice-cold solution of L-asparagine hydrate (5.2 g, 35 mmol) in water (200 ml) containing triethylamine (5 ml). The mixture was stirred at 0—3 °C for 1 hr and at room temperature for 8 hr. It was extracted with two 80 ml portions of ether and an aqueous layer was acidified with solid citric acid to pH 2—3. The resulting oily precipitate was taken up to ethyl acetate two times (500 ml, 200 ml). Combined ethyl acetate layer was washed with water and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and a residue was solidified by addition of petroleum ether. The product was recrystallized from excess of ethyl acetate. Yield, 11.8 g (79%); mp 154—156 °C; $[\alpha]_D^{20}$ +37.1° (*c* 1, ethanol).

Found: C, 54.83; H, 6.39; N, 9.51%.

β-Benzyl-D-aspartyl-L-asparagine (XI) Trifluoroacetate. The dipeptide derivative X(4.5 g, 10 mmol) was dissolved in anhydrous trifluoroacetic acid (50 ml) and the solution was stirred at room temperature for 30 min. Excess tri-

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

12) E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963).

13) D. A. Laufer and E. R. Blout, *J. Amer. Chem. Soc.*, **89**, 1246 (1967).

fluoroacetic acid was evaporated *in vacuo* and the residue was diluted with ether. The deposited solid was collected by filtration, washed with ether and dried over potassium hydroxide *in vacuo*. The product was recrystallized from methanol–ether. Yield, 4.4 g (98%); mp 131—134 °C; $[\alpha]_D^{18}$ +1.7° (*c* 1, methanol).

Found: C, 45.64; H, 4.50; H, 9.08%. Calcd for $C_{15}H_{19}O_6N_3$–CF_3COOH: C, 45.24; H, 4.47; N, 9.31%.

N^{α}-Benzyloxycarbonyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-β-benzyl-D-aspartyl-L-asparagine (XIII) Hydrate. A solution of sodium nitrite (0.5 g) in water (5 ml) was added to a solution of N^{α}-benzyloxycarbonyl-N^{ε}-*t*-butyloxycarbonyl-L-lysyl-N^{δ}-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidine hydrazide hydrate[1] (5.1 g, 4.9 mmol) in 70% acetic acid (300 ml) containing 20 ml of M hydrochloric acid at −1—−2 °C. The reaction mixture was stirred at this temperature for 20 min. A gelatinous mass started to deposit when excess cold water was added to the mixture. The resulting precipitate was collected by filtration and washed with water thoroughly. An azide XII thus obtained, which showed an absorption band at 2210 cm^{-1} in IR spectrum, was dissolved in ice-cold dimethylformamide (50 ml). This preparation was added to an ice-cold solution of XI trifluoroacetate (4.5 g, 10 mmol) in dimethylformamide (25 ml) containing triethylamine (1.3 ml) and the mixture was stirred at 2—5 °C for 24 hr and then at room temperature for 30 hr. The solution was concentrated *in vacuo* and a residue was triturated with water. The resulting amorphous material was collected by filtration and purified by silica gel column chromatography (elution solvent : chloroform–methanol, 8 : 2—7 : 3, v/v). Yield, 3.2 g (49%); mp 200—203 °C; $[\alpha]_D^{18}$ +12.6° (*c* 1, dimethylformamide).

Found: C, 59.30; H, 7.13; N, 12.37%. Calcd for $C_{66}H_{90}O_{17}N_{12}\cdot H_2O$: C, 59.09; H, 6.91; N, 12.53%.

Amino acid analysis: Lys+Orn, 1.93; Ile, 0.99; Phe, 1.00; His, 0.92; Asp, 2.00.

Cyclo-(N$^{\alpha}$-benzyloxycarbonyl-L-lysyl-N$^{\delta}$-cyclopentyloxycarbonyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-β-benzyl-D-aspartyl-L-asparaginyl) (V) dihydrate. To a solution of the protected heptapeptide XIII hydrate (7.0 g, 5.2 mmol) in dimethylformamide (70 ml), there were added chloroacetonitrile (4.5 ml) and triethylamine (6 ml). The reaction mixture was stirred at room temperature for 40 hr. After evaporation of the solvent under reduced pressure, water was added to the residue. Amorphous precipitate formed was filtered and dried *in vacuo* over phosphorus pentoxide. The product gave four spots on thin-layer chromatogram (developing solvent : methanol–chloroform, 2 : 8 v/v). One of them, showing positive Pauly and negative ninhydrin reaction, corresponds to the expected cyanomethyl ester while the other three spots seemed to an unreactive peptide and two cyanomethylated compounds on imidazole nuclei in histidine residues of the expected product and unreactive peptide from the coloring behaviors. The mixture (7.1 g) was dissolved in trifluoroacetic acid (80 ml) and stirred at room temperature for 45 min. Excess trifluoroacetic acid was removed by evaporation under reduced pressure, and ether was added to the residue. Precipitate thus formed was filtered and dried *in vacuo* over sodium hydroxide. A solution of this material in dimethylformamide (20 ml) and acetic acid (5 ml) was added to large excess of pyridine (800 ml) with stirring for 5 hr and then stirred at 45—50 °C for 20 hr. The solution was concentrated under reduced pressure. The reaction mixture showed five spots on thin-layer chromatogram (solvent system : methanol–chloroform 2 : 8, v/v). Among them, a spot of R_f 0.80 must be desired product, because it gave negative ninhydrin but positive Pauly reaction, while another spot of R_f 0.90 showing both negative ninhydrin and Pauly reactions seemed to be a cyclic peptide in which imidazole nucleus was cyanomethylated. All other three spots gave positive ninhydrin reactions. A product of R_f 0.80 was isolated by silica gel column chromatography (2.5×80 cm, elution solvent : methanol–chloroform, 1 : 19 (200 ml)—2 : 18 (300 ml) v/v). Fractions No. 21 to 42 (each fraction, 15 g) were collected and concentrated under reduced pressure to obtain a solid of the expected cyclic peptide (V) dihydrate, Yield, 90 mg (1.4%); mp 197—207 °C; $[\alpha]_D^{17}$ +11.4° (*c* 1, dimethylformamide).

Found: C, 58.96; H, 7.16; N, 13.63%. Calcd for $C_{61}H_{80}O_{14}N_{12}\cdot 2H_2O$: C, 59.02; H, 6.82; N, 13.54%.

Amino acid analysis: Lys+Orn, 2.11: Ile, 0.96; Phe, 0.94; His, 1.02; Asp, 2.00.

The authors are grateful to Peptide Center, Institute for Protein Research of Osaka University for carrying out the amino acid analyses and also indebted to Ajinomoto Co., Ltd. and Commercial Solvent Corporation, U.S.A. for their kind offers of amino acids.