Chem. Pharm. Bull. 17(10)2135—2141(1969)

UDC 547.964.4.07 : 615.453.011.5

Synthesis of Peptides related to Corticotropin (ACTH). II.[1] Synthesis of the Protected Peptides Corresponding to the Sequence 1—3, 4—6, 7—10, 11—14, 15—19 and 20—23 of ACTH

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(Received June 13, 1969)

Syntheses of the six intermediary peptide fragments for the synthesis of α^{1-23NH_2}-ACTH, carbobenzoxyseryltyrosylserine hydrazide, *t*-butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine hydrazide, *t*-butyloxycarbonylphenylalanylnitroarginyltryptophanylglycine, *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysylprolylvalylglycine, *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysyl-N$^{\varepsilon}$-carbobenzoxylysylnitroarginylnitroarginylproline and valyl-N$^{\varepsilon}$-carbobenzoxylysylvalyltyrosine amide, are described.

In the previous paper,[1] we have reported briefly the synthesis of α^{1-23NH_2}-ACTH-tricosapeptide amide by the completely different route from those reported previously by several investigators.[4] For the synthesis of the protected tricosapeptide amide, the six intermediary peptide fragments, namely, carbobenzoxyseryltyrosylserine hydrazide (I), *t*-butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine hydrazide (II), *t*-butyloxycarbonylphenylalanylnitroarginyl tryptophanylglycine (III), *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysylprolylvalylglycine (IV), *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysyl-N$^{\varepsilon}$-carbobenzoxylysylnitroarginylnitroarginylproline (V) and valyl-N$^{\varepsilon}$-carbobenzoxylysylvalyltyrosine amide were employed.

In this paper, we wish to describe the syntheses of these six peptide fragments in detail. The protected N-terminal tripeptide hydrazide (I) was prepared as shown in Fig. 1. Carbobenzoxyseryltyrosine methyl ester (Ia) which was prepared by means of our oxime-ester method[5] was converted to the corresponding hydrazide (Ib), and coupled with serine methyl ester according to the method which has been reported by Hofmann, *et al.*[6] and Guttmann, *et al.*[7] The resulting methyl ester (Ic) was then converted to the corresponding hydrazide (I). For the preparation of the protected tripeptide hydrazide (II), *t*-butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine methyl ester was synthesized from γ-*t*-butylglutamylhistidine methyl ester[8] and *t*-butyloxycarbonylmethionine pentachlorophenyl

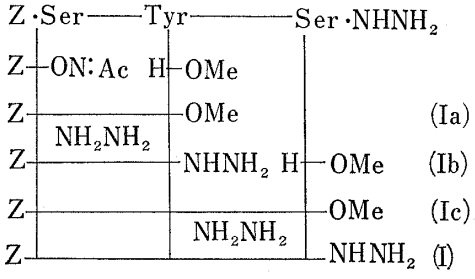

Fig. 1. Synthesis of Carbobenzoxyseryltyrosylserine Hydrazide (I)

Z: carbobenzoxy-
-OMe: methyl ester
-ON=Ac: acetoxime ester

1) Part I: M. Fujino, C. Hatanaka and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 2186 (1969).
2) All the amino acid residues of the L-configuration with the exception of glycine.
3) Location: *Juso, Higashiyodogawa-ku, Osaka.*
4) See E. Schröder and K. Lübke, "The Peptides," Vol. II, Academic Press, New York, 1966, p. 199.
5) M. Fujino and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1937 (1969).
6) K. Hofmann and A. Jöhl, *J. Am. Chem. Soc.*, **79**, 1636 (1957).
7) St. Guttmann and R.A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).
8) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).

ester which was prepared by the trichloroacetate method,[9] and then the resulting peptide ester was converted to the hydrazide (II) (Fig. 2).

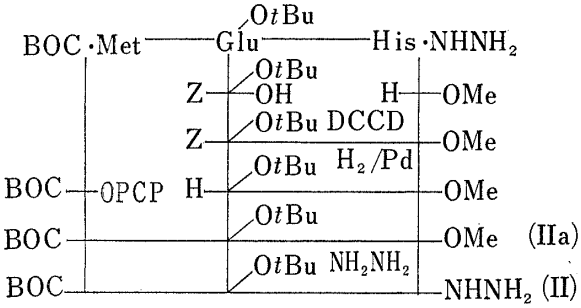

Fig. 2. Synthesis of *t*-Butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine Hydrazide (II)

BOC: *t*-butyloxycarbonyl
-O*t*Bu: *t*-butylester
-OPCP: pentachlorophenyl ester

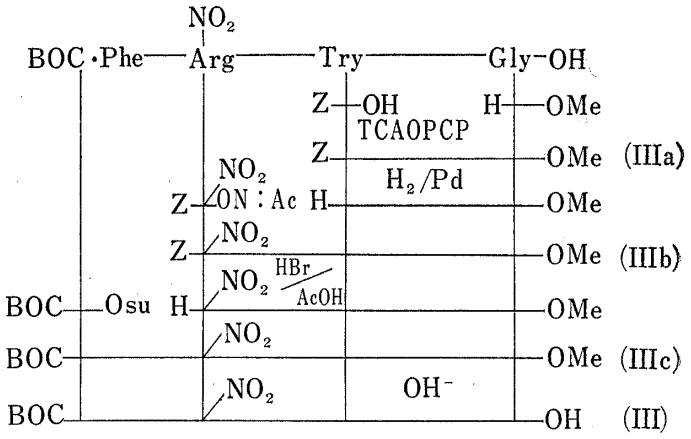

Fig. 3. Synthesis of *t*-Butyloxycarbonylphenylalanylnitroarginyltryptophanylglycine (III)

TCAOPCP: pentachlorophenyl trichloroacetate
-Osu: N-hydroxysuccinimide ester

The partially protected tetrapeptide (III) was synthesized as shown in Fig. 3. Carbobenzoxytryptophanylglycine methyl ester (IIIa) was prepared by means of the trichloroacetate method.[9] After the carbobenzoxy–group was removed from IIIa by catalytic hydrogenolysis, the resulting dipeptide ester was coupled with O-(carbobenzoxynitroarginyl)-acetoxime,[5] carbobenzoxynitroarginine 2,4-dinitrophenyl ester[10] or pentachlorophenyl ester[11] to yield carbobenzoxynitroarginyltryptophanylglycine methyl ester (IIIb) in 88.5, 84 or 53% yield, respectively. The carbobenzoxy–group was removed from the tripeptide derivative (IIIb) by a treatment with hydrogen bromide in glacial acetic acid at room temperature for 15 min. In this case, when the treatment with hydrogen bromide in glacial acetic acid was done for longer than 15 min, some undesired by–products were appeared on thin–layer chromatogram (solvent system II). The resulting tripeptide ester was then coupled with *t*-butyloxycarbonylphenylalanine N-hydroxysuccinimide ester[12] or with pentachlorophenyl ester to yield the fully protected tetrapeptide *t*-butyloxycarbonylphenylalanylnitroarginyltryptophanylglycine methyl ester (IIIc). The fully protected tetrapeptide (IIIc) thus obtained was then saponified by the standard procedure.

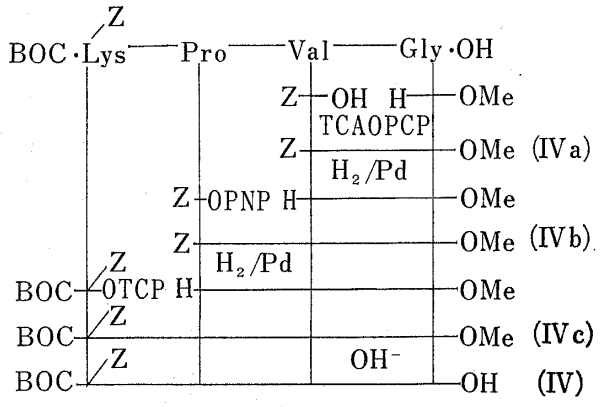

Fig. 4. Synthesis of *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysylprolylvalylglycine (IV)

-OPNP: *p*-nitrophenylester
-OTCP: 2,4,5-trichlorophenyl ester

The tetrapeptide derivative (IV) was obtained by a stepwise elongation, starting with glycine methyl ester (Fig. 4). During the stepwise process carbobenzoxyvaline pentachlorophenyl ester, carbobenzoxyproline *p*-nitrophenyl ester and *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysine 2,4,5-trichlorophenyl ester were used to introduce, respectively,

9) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
10) M. Bodanszky and M.A. Ondetti, *Chem. Ind.* (London), **1966**, 26.
11) J. Kovacs and M.Q. Ceprini, *Chem. Ind.* (London), **1965**, 2100.
12) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

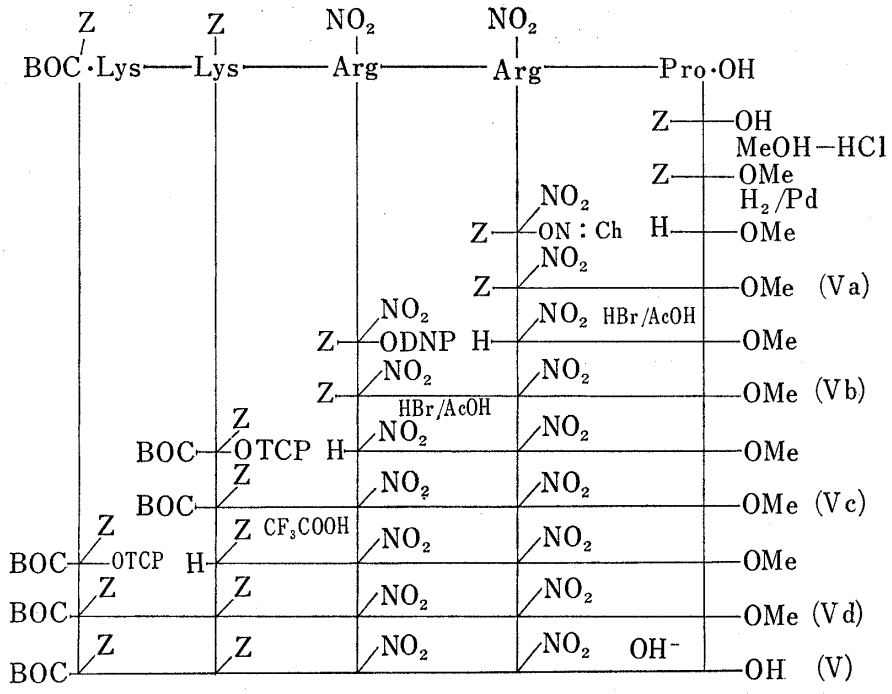

Fig. 5. Synthesis of *t*-Butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysyl-N$^{\varepsilon}$-carbobenzoxylysylnitroarginylnitroarginylproline

-ON:Ch: cyclohexanonoxime ester

those amino acids.

The pentapeptide derivative (V) was also prepared by a stepwise elongation using O-(carbobenzoxynitroarginyl)cyclohexanonoxime, carbobenzoxynitroarginine 2,4-dinitrophenyl ester and *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysine 2,4,5-trichlorophenyl ester (two steps) and a saponification of Vd with the standard manner (Fig. 5).

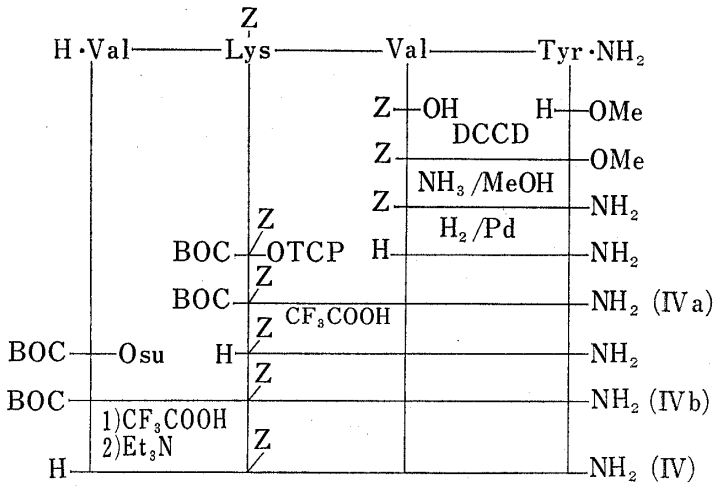

Fig. 6. Synthesis of Valyl-N$^{\varepsilon}$-carbobenzoxylysylvalyltyrosine Amide (VI)

DCCD: N,N'-dicyclohexylcarbodiimide

Finally, C-terminal tetrapeptide amide (VI) was synthesized from valyltyrosine amide[13] by a stepwise elongation with *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysine 2,4,5-trichlorophenyl ester, *t*-butyloxycarbonylvaline N-hydroxysuccinimide ester, and the acidolysis with trifluoroacetic acid at 10—15° for 20 min. The resulting tetrapeptide amide trifluoroacetate was then treated with triethylamine in methanol to yield the free form tetrapeptide amide (VI) as fine crystals.

Experimental

All melting points were uncorrected. Thin-layer chromatography was carried out on Merck's silica gel G with the solvent systems of $CHCl_3$–MeOH–AcOH (9:1:0.5, *Rf* I), AcOEt–pyridine–AcOH–H_2O (60:20:6:11, *Rf* II), *n*-BuOH–AcOH–H_2O (4:1:1, *Rf* III) and *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24, *Rf* IV).

13) K. Hofmann, T.T. Liu, H. Yajima, N. Yanaihara and S. Lande, *J. Am. Chem. Soc.*, **83**, 2294 (1961).

Carbobenzoxyseryltyrosylserine Hydrazide (I)——a) Carbobenzoxyseryltyrosine Hydrazide (Ib): Carbobenzoxyseryltyrosine methyl ester (IIa) was prepared by acetoxime method,[5] and the resulting dipeptide ester was hydrazinolyzed accodring to Guttmann, *et al.*[7] mp 213—214°, $[\alpha]_D^{23}$ −5.0° (c=2.0 in DMF) (lit. mp 213—214°,[6] $[\alpha]_D^{21}$ −0.0±0.5°[7] (c=2.0 in DMF)).

b) Carbobenzoxyseryltyrosylserine Hydrazide (I): The corresponding methyl ester (Ic) was prepared from Ib and serine methyl ester by the azide method, and Ic was hydrazinolyzed according to Hofmann, *et al.*[6] to give I: mp 225—226° (decomp.), $[\alpha]_D^{23}$ −4.4° (c=2.0 in DMF) (lit. mp 210—213°,[6] 234°,[7] $[\alpha]_D^{23}$ −4.2±0.5° (c=1.9 in DMF)).

***t*-Butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine Hydrazide (II)**——a) *t*-Butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine Methyl Ester (IIa): Carbobenzoxy-γ-*t*-butylglutamylhistidine methyl ester[8] (1.94 g, 3.97 mmole) was dissolved in 50 ml of MeOH containing AcOH (0.24 ml), and hydrogenated over Pd catalyst with the standard manner. After filtration, the filtrate was evaporated *in vacuo* and the residue was dissolved in 15 ml of DMF. To the solution, were added 0.55 ml of Et_3N and 1.95 g (3.97 mmole) of *t*-butyloxycarbonylmethionine pentachlorophenyl ester (mp 135.5—136.5°, $[\alpha]_D^{22}$ −31.9° (c=1.0 in DMF)) which was prepared by the trichloroacetate method,[9] and then this solution was stirred for 12 hr at room temperature. The reaction mixture was diluted with 300 ml of AcOEt and washed with 2N NH_4OH and H_2O. After dried over anhyd. Na_2SO_4, the AcOEt solution was evaporated to dryness. The residue was precipitated twice from AcOEt–ether to give IIa: Yield 1.42 g (61%), mp 142—144°, $[\alpha]_D^{22}$ −21.6° (c=2.0 in MeOH). *Rf* I; 0.42, *Rf* II; 0.84. *Anal.* Calcd. for $C_{26}H_{43}O_8N_5S$: C, 53.32; H, 7.40; N, 11.96; S, 5.47. Found: C, 52.72; H, 7.17; N, 11.75; S, 5.47.

b) *t*-Butyloxycarbonylmethionyl-γ-*t*-butylglutamylhistidine Hydrazide (II): To a solution of compound IIa (7.39 g, 12.6 mmole) in MeOH (80 ml) was added hydrazine hydrate (6.3 ml) and the reaction mixture was left stand for 40 hr at room temperature. The solvent was then evaporated *in vacuo*, and H_2O (50 ml) was added to the residue. The ensuing crystal was collected by filtration, washed with H_2O and then suspended in hot AcOEt (50 ml). After cooling, the crystal was collected by filtration: Yield, 6.45 g (87%), mp 175—176°, $[\alpha]_D^{22}$ −31.0° (c=1.0 in MeOH), *Rf* II; 0.50. *Anal.* Calcd. for $C_{25}H_{43}O_7N_7S$: C, 51.26; H, 7.40; N, 16.74; S, 5.47. Found: C, 51.17; H, 7.31; N, 16.59; S, 5.49.

***t*-Butyloxycarbonylphenylalanylnitroarginyltryptophanylglycine (III)**——a) Carbobenzoxytryptophanylglycine Methyl Ester (IIIa): To a cooled solution of carbobenzoxytryptophan (67.6 g, 0.2 mole) and Et_3N (28 ml, 0.2 mole) in 200 ml of DMF was added pentachlorophenyl trichloroacetate (82.3 g, 0.2 mole) and the whole was allowed to react for 10 min at 0° and then for additional 10 min at room temperature. To the reaction mixture was added a solution of glycine methyl ester hydrochloride (25.2 g, 0.2 mole) and Et_3N (28 ml, 0.2 mole) in $CHCl_3$ (100 mole). The mixture was stirred for 6 hr at room temperature, diluted with H_2O (2 liter), and extracted with $CHCl_3$ (800 ml). The extract was washed successively with 4% aqueous $NaHCO_3$, 1N HCl and H_2O. The $CHCl_3$ layer was dried over anhyd. $MgSO_4$ and evaporated *in vacuo* to yield crystalline residue, which was recrystallized from MeOH: Yield, 75.0 g (91.7%), mp 156—158°, $[\alpha]_D^{20}$ −11.7° (c=2.0 in AcOH) (lit.[14] mp 156—158°, $[\alpha]_D^{27}$ −11.0° (c=2.0 in AcOH). *Anal.* Calcd. for $C_{22}H_{23}O_5N_3$: C, 64.54; H, 5.68; N, 10.26. Found: C, 64.27; H, 5.56; N, 10.14.

b) Carbobenzoxynitroarginyltryptophanylglycnie Methyl Ester (IIIb): i) By Acetoxime Ester Method[5]——IIIa (40.9 g, 0.1 mole) was dissolved in 800 ml of MeOH containing AcOH (6 ml), and hydrogenated over a Pd catalyst for 4 hr. After filtration, the filtrate was evaporated *in vacuo* to yield an oily residue. The residue was dissolved in 300 ml of dioxane, and O-(carbobenzoxynitroarginyl)acetoxime (40.8 g, 0.1 mole) was added. The mixture was left stand for 16 hr at room temperature, and diluted with 600 ml of ether. The resulting precipitate was collected and crystallized from hot ethanol: Yield, 54.3 g (88.5%), mp 126—128° (decomp.), $[\alpha]_D^{22}$ −25.0° (c=1.0 in MeOH), (lit.[15] mp 126—128°, $[\alpha]_D^{22}$ −25.8° (c=1.3 in MeOH)). *Anal.* Calcd. for $C_{28}H_{34}O_8N_8$: C, 55.08; H, 5.62; N, 18.35. Found: C, 55.08; H, 5.71; N, 18.06.

ii) By 2,4-Dinitrophenyl Ester Method[10]——IIIa (40.9 g, 0.1 mole) was hydrogenated as described above. The hydrogenated IIIa in 200 ml of DMF was subjected to react for 6 hr with carbobenzoxynitroarginine 2,4-dinitrophenyl ester prepared from 42.2 g (0.12 mole) of carbobenzoxynitroarginine at room temperature. The reaction mixture was diluted with 2 liter of H_2O and extracted with AcOEt (1 liter). AcOEt layer was washed with 1N NH_4OH, 1N HCl and H_2O, and dried over anhyd. Na_2SO_4. The solvent was evaporated *in vacuo* and the resulting redisue was precipitated from hot ethanol: Yield, 51.0 g (83.6%), mp 114—117° (decomp.), $[\alpha]_D^{22}$ −26.0° (c=1.0 in MeOH). *Anal.* Found. C, 55.03; H, 5.60; N, 18.03.

iii) By Pentachlorophenyl Ester Method[11]——The reaction and subsequent purification procedure were essentially the same as described in (ii). Yield, 53%, mp 112—117° (decomp.). From the mother liquor of recrystallization, 1-nitroguanyl-3-carbobenzoxyamino-2-piperidone[16] was obtained. mp 146—147° (lit.[16] mp 146—147°). IR :5.78 μ (imide carbonyl), 5.91 μ (lactam), 6.25 μ (nitro). *Anal.* Calcd for $C_{14}H_{17}O_5N_5$: C, 50.14; H, 5.11; N, 20.89. Found: C, 50.22; H, 5.17; N, 20.86.

14) K. Hofmann, M.E. Woolner, G. Spühler and E. T. Schwarz, *J. Am. Chem. Soc.*, **80**, 1486 (1958).
15) R. Geiger, K. Sturm and W. Siedel, *Ber.*, **96**, 1080 (1963).
16) R. Paul, G.W. Anderson and F.M. Callahan, *J. Org. Chem.*, **26**, 3347 (1961).

c) *t*-Butyloxycarbonylphenylalanylnitroarginyltryptophanylglycine Methyl Ester (IIIc): IIIb (12.2 g, 20 mmole) was dissolved in glacial AcOH (20 ml), and 30% HBr in glacial AcOH (40 ml) was added to the solution. The mixture was then stirred for 15 min under nitrogen gas, and the product precipitated by adding dry ether was washed with dry ether by decantation, and dried over NaOH *in vacuo*. This dried powder was dissolved in DMF (90 ml), and Et_3N (5.6 ml) was added to the solution. The resulting Et_3N–HBr salt was removed off by filtration, and the filtrate was reacted with *t*-butyloxycarbonylphenylalanine pentachlorophenyl ester (10.2 g, 20 mmole) wihch was prepared by the trichloraocetate method[9] (mp 156—157.5°, $[\alpha]_D^{22}$ −52.7° (c=1.0 in DMF)) for 12 hr at room temperature. The reaction mixture was diluted with H_2O (1 liter) and extracted with AcOEt (300 ml×3). The AcOEt layer was then washed successively with 1N NH_4OH, 0.2N HCl and H_2O, dried over anhyd. Na_2SO_4 and evaporated *in vacuo* to dryness. The residue was dissolved in MeOH and then ether was added to give a precipitate as a fine powder, which was recrystallized twice from acetonitrile: Yield, 11.0 g (70%), mp 168—170.0° (decomp.), $[\alpha]_D^{21}$ −20.0° (c=1.0 in MeOH)(lit.[17] mp 172—173°, $[\alpha]_D^{25}$ −20.1±1° (c=2.045 in MeOH)). *Anal.* Calcd. for $C_{35}H_{45}O_9N_9$: C, 56.20; H, 6.42; N, 17.39. Found: C, 56.21; H, 6.26; N, 17.56.

IIIc was also prepared from *t*-butyloxycarbonylphenylalanine N-hydroxysuccinimide ester[12] and IIIb as described above. Yield, 93%, mp 168.0—170.0° (decomp.), $[\alpha]_D^{21}$ −19.8 (c=1.0 in MeOH).

d) *t*-Butyloxycarbonylphenylalanylnitroarginyltryptophanylglycine (III): A MeOH (80 ml) solution of IIIc (14.5 g, 20 mmole) was treated with 1N NaOH (20 ml) at room temperature for 1 hr, and neutralized with 1N HCl (22 ml). The mixture was then diluted with H_2O (500 ml) and extracted with AcOEt (300 ml×3). The AcOEt layer was washed with H_2O, dried over anhyd. Na_2SO_4, and then concentrated *in vacuo* to yield gelatinous precipitate. The precipitate was collected by filtration and recrystallized from MeOH–acetonitrile: Yield, 13.0 g (91.6%), mp 155—156° (decomp.), $[\alpha]_D^{26}$ −22.3° (c=1.0 in MeOH), *Rf* I; 0.25. *Anal.* Calcd. for $C_{33}H_{43}O_7N_9$: C, 55.84; H, 6.10; N, 17.75. Found: C, 55.55; H, 6.11; N, 17.88.

***t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysylprolylvalylglycine (IV)**——a) Carbobenzoxyvalylglycine Methyl Ester (IVa): To an ice cold solution of carbobenzoxyvaline (25.1 g, 0.1 mole) and Et_3N (14 ml) in DMF (100 ml) was added pentachlorophenyl trichloroacetate (41.2 g, 0.1 mole). The mixture was allowed to react for 10 min at 0° and then for additional 20 min at room temperature. A solution of glycine methyl ester hydrochloride (12.6 g, 0.1 mole) and Et_3N (14 ml) in $CHCl_3$ (50 ml) was added to the reaction mixture. The solution was stirred for 24 hr at room temperature, was diluted with H_2O (1.2 liter) and extracted twice with AcOEt (400 ml each). The AcOEt layer was washed with 1N NH_4OH and 1N HCl and dried over anhyd. Na_2SO_4. On a removal of the solvent, a crystalline residue was obtained, and recrystallized from MeOH; Yield, 20.8 g (64.6%), mp 159.5—161.5°, $[\alpha]_D^{26}$ −27.3° (c=1.0 in MeOH) (lit. mp 159—160°,[18] 160—161,[19] $[\alpha]_D^{25}$ −25.5° (c=1.0 in MeOH),[18] $[\alpha]_D^{24}$ −30.0° (c=1.85 in MeOH)[19]). *Anal.* Calcd. for $C_{16}H_{22}O_5N_2$: C, 59.60; H, 6.88; N, 8.69. Found: C, 59.47; H, 6.99; N, 8.83.

b) Carbobenzoxyprolylvalylglycine Methyl Ester (IVb): IVa (22.5 g, 70 mmole) was dissolved in 200 ml of MeOH containing 4.2 ml of AcOH, and hydrogenated over a Pd catalyst for 5 hr. After filtration, the filtrate was evaporated to dryness *in vacuo*. The residue was suspended in 300 ml of $CHCl_3$, and carbobenzoxy–proline *p*-nitrophenyl ester (25.9 g, 70 mmole) and Et_3N (9.8 ml) were added to this solution. The mixture was stirred for 30 hr at room temperature, and evaporated to dryness *in vacuo*. The residue redissolved in AcOEt was washed with 1N NH_4OH and 1N HCl, and was dried over anhyd. Na_2SO_4. The solution was evaporated *in vacuo* to yield a solid residue, which was reprecipitated from ether–petroleum. ether and then recrystallized from MeOH–H_2O: Yield, 27.7 g (94%), mp 127—129.5°, $[\alpha]_D^{23}$ −89.4° (c=1.0 in MeOH) (lit.[19] mp 125—127°, $[\alpha]_D^{25}$ −89.7° (c=1 in MeOH)). *Anal.* Calcd. for $C_{21}H_{29}O_6N_3$: C, 60.12; H, 6.97; N, 10.02. Found: C, 60.39; H, 7.24; N, 10.15.

c) *t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysylprolylvalylglycine Methyl Ester (IVc): IVb (29.4 g, 70 mmole) was dissolved in MeOH (200 ml) and hydrogenated over a Pd catalyst for 5 hr. The catalyst was filtered off, and the filtrate was evaporated *in vacuo*. The residue was dissolved in DMF (250 ml), and *t*-butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysine 2,4,5-trichlorophenyl ester (39.2g, 70 mmole) which was prepared from *t*-butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysine and 2,4,5-trichlorophenyl trichloroacetate by the standard procedure[20] (mp 100.5—102.0°, $[\alpha]_D^{22}$ −22.0° (c=1.0 in DMF)) was added. The mixture was stirred for 12 hr at room temperature, diluted with H_2O (2 liter) and extracted twice with AcOEt (800 ml each). The combined AcOEt solution was washed with 1N NH_4OH, 0.2N HCl and H_2O, dried over anhyd. Na_2SO_4, and evaporated out *in vacuo*. The residue was crystallized from MeOH–ether: Yield, 40.6 g (99%), mp 75—79°, $[\alpha]_D^{26}$ −81.0° (c=1.0 in MeOH). *Rf* I: 0.7. *Anal.* Calcd. for $C_{32}H_{49}$: O_9N_5: C, 59.33; H, 7.62; N, 10.81. Found: C, 59.41; H, 7.77; N, 10.79.

d) *t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysylprolylvalylglycine (IV): IVc (1.94 g, 3 mmole) was dissolved in MeOH (10 ml) and treated with 1N NaOH (3 ml) for 60 min at room temperature. The reaction

17) K. Inoue, *Bull. Chem. Soc. Japan*, **38**, 1148 (1965).
18) C.H. Li, J. Ramachandran, D. Chung and B. Gorup, *J. Am. Chem. Soc.*, **86**, 2703 (1964).
19) K. Hofmann, E. Sturz, G. Spuhler, H. Yajima and E.T. Schwarz, *J. Am. Chem. Soc.*, **82**, 3727 (1960).
20) M. Fujino and C. Hatanaka, Proc. of the 5th Symposium on Peptide Chemistry, Japan, 1967, p. 8.

mixture was acidified with 1N HCl (3.5 ml), and extracted with AcOEt (50 ml×3) after a dilution with H_2O (200 ml). The extract was washed with H_2O, dried over anhyd. Na_2SO_4, and then evaporated *in vacuo* to yield a waxy residue. The residue was crystallized from $CHCl_3$–ether and then recrystallized from MeOH–ether: Yield 1.70 g (89.5%), mp 174.0—175.0° (decomp.), $[\alpha]_D^{22}$ −77.0° (c=1.0 in MeOH). *Rf* I; 0.56. *Anal.* Calcd. for $C_{31}H_{47}O_9N_5$: C, 58.57; H, 7.48; N, 11.05. Found: C, 58.54; H, 7.64; N, 10.90.

t-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysyl-N$^\varepsilon$-carbobenzoxylysylnitroarginylnitroarginylproline (V)——a) Carbobenzoxynitroarginylproline Methyl Ester (Va): i) By Oxime–Ester Method——To an ice-cold solution of proline methyl ester acetate (oil, 5.04 g, 0.02 mole) in 50 ml of dioxane was added O-(carbobenzoxynitroarginyl)cyclohexanon oxime (9.6 g, 0.02 mole). The mixture was allowed to react for 18 hr at room temperature, and then evaporated *in vacuo* to yield an oily residue which was soon crystallized by a trituration with H_2O, and recrystallized from MeOH: Yield 8.4 g (90.3%), mp 159.0—160.5°, $[\alpha]_D^{22}$ −35.0° (c=1.0 in DMF) (lit.[21] mp 159—161°, $[\alpha]_D^{22}$ −36.04° (c=1.3 in DMF)). *Anal.* Calcd. for $C_{20}H_{28}O_7N_6$: C, 51.71; H, 6.08; N, 18.10. Found: C, 51.89; H, 5.87; N, 18.05.

ii) By 2,4-Dinitrophenyl Ester Method——To an ice-cold solution of proline methyl ester acetate (oil, 50.4 g, 0.2 mole) and Et_3N (28 ml) in 300 ml of DMF, was added carbobenzoxy-nitroarginine 2,4-dinitrophenyl ester prepared from 77.7 g (0.22 mole) of carbobenzoxynitroarginine. After allowed to react for 12 hr at room temperature, the reaction mixture was diluted with $CHCl_3$ (1 liter) and washed with 1N NH_4OH (400 ml×5) and H_2O (400 ml). On a removal of the solvent, a crystalline residue was obtained, and recrystallized from MeOH: Yield, 56.2 g (60.5%), mp 159—160°, $[\alpha]_D^{22}$ −35.0° (c=1.0 in DMF). *Anal.* Found. C, 52.00; H, 6.23; N, 18.22.

b) Carbobenzoxynitroarginylnitroarginylproline Methyl Ester (Vb): After Va (39 g, 83.8 mmole) was treated with 25% HBr in AcOH (80 ml) for 40 min, dry ether (400 ml) was added to the reaction mixture to produce a precipitate which was washed with dry ether by decantation, and dried over NaOH *in vacuo*. The dried powder was dissolved in DMF (100 ml), and Et_3N (23.6 ml) was added. The resulting Et_3N–HBr salt was removed off by filtration, and the filtrate was subjected to reaction with carbobenzoxynitroarginine 2,4-dinitrophenyl ester prepared from 35.3 g (0.1 mole) of carbobenzoxynitroarginine for 24 hr at room temperature. The reaction mixture was diluted with $CHCl_3$ (500 ml), and washed with 1N NH_4OH (300 ml×5), 0.5N HCl (300 ml) and H_2O (300 ml×2). The oily product separated from the $CHCl_3$ solution was collected and dissolved in MeOH (70 ml). Dry ether was added to the MeOH solution, and the separated oily product was titurated with ether to yield a fine powder, which was reprecipitated from acetone–ether: Yield, 43.0 g (77%), mp 87° (decomp., 83—85° sinter), $[\alpha]_D^{23}$ −46.0° (c=1.0 in MeOH). *Anal.* Calcd. for $C_{26}H_{39}O_{10}N_{11}$: C, 46.93; H, 5.90; N, 23.14. Found: C, 46.94; H, 5.97; N, 22.18.

c) *t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysylnitroarginylnitroarginylproline Methyl Ester (Vc): Vb (6.66 g, 10 mmole) was treated with 25% HBr in AcOH (35 ml) for 60 min. The product was precipitated by adding dry ether (150 ml) to the reaction mixture. The precipitate was washed with ether by decantation and dried over NaOH *in vacuo*. The dried powder was dissolved in an ice-cold DMF (50 ml), and Et_3N (4.5 ml) was added to the solution. The formed Et_3N–HBr salt was filtrated off, and the filtrate was then subjected to reaction with *t*-butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysine 2,4,5-trichlorophenyl ester (5.6 g, 10 mmole) for 16 hr at room temperature. To the reaction mixture was added 20% NH_4OAc–H_2O (300 ml) under cooling. The ensuing amorphous precipitate was collected by decantation and washed with ice-cold H_2O. The precipitate was then dissolved in MeOH (30 ml) and triturated with ether to give a fine powder, which was reprecipitated from acetone with ether: Yield, 6.50 g (72%), mp 99—101 °(decomp., 84° sinter), $[\alpha]_D^{23}$ −34.0° (c=1.0 in MeOH). *Rf* I; 0.60, *Rf* II; 0.75; *Rf* III; 0.87. *Anal.* Calcd. for $C_{37}H_{59}O_{13}N_{13}$: C, 49.71; H, 6.66; N, 20.37. Found: C, 49.41; N, 6.75; N, 20.17.

d) *t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysyl-N$^\varepsilon$-carbobenzoxylysylnitroarginylnitroagrinylproline Methyl Ester (Vd): Vc (6.0 g, 6.6 mmole) was treated with trifluoroacetic acid (30 ml) at 10° for 30 min. The product was precipitated by adding dry ether (200 ml) to the solution . The precipitate was washed with dry ether by decantation, and dried over NaOH *in vacuo* (*Rf* I, 0.0; *Rf* II, 0.40; *Rf* III, 0.55). The dried powder and Et_3N (0.98 ml) were dissolved in DMF (30 ml) and subjected to reaction with *t*-butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysine-2,4,5-trichlorophenyl ester (3.7 g, 6.6 mmole) for 6 hr at room temperature. The resulting product was purified as described above: Yield 6.6 g (86%), mp 89° (decomp.), $[\alpha]_D^{23}$ −30.2° (c=1.0 in DMF). *Rf* I, 0.54; *Rf* II, 0.66; *Rf* III, 0.87. *Anal.* Calcd. for $C_{51}H_{77}O_{16}N_{15}$: C, 52.16; H, 6.75; N, 17.93. Found: C, 52.33; H, 6.53; N, 17.88.

e) *t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxylysyl-N$^\varepsilon$-carbobenzoxylysyl-nitroarginylnitroarginylproline (V): A solution of Vd (3.47 g, 3 mmole) in 90% acetone (10 ml) was treated with 1N NaOH (4.5 ml) at room temperature for 3 hr. The solution was neutralized with 1N HCl (5 ml) and then diluted with ice-cold H_2O (40 ml) to give a precipitate which was collected by filtration and washed with H_2O. After dryness *in vacuo*, the resulting powder was reprecipitated from MeOH with ether: Yield 3.1 g (91%), mp 94—97° (decomp.), $[\alpha]_D^{21}$ −30.6° (c=1.0 in DMF). *Rf* I; 0.27. *Anal.* Calcd. for $C_{50}H_{75}O_{16}N_{15}\cdot H_2O$: C, 51.77; H, 16.68; N, 18.11. Found: C, 51.86; H, 6.94; N, 17.66.

21) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **46**, 1550 (1963).

Valyl-N$^{\varepsilon}$-carbobenzoxylysylvalyltyrosine Amide (VI)——a) *t*-Butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysyl-valyltyrosine Amide (VIa): Carbobenzoxy-valyl-tyrosine amide[13] (8.0 g, 20 mmole) was suspended in MeOH (400 ml) containing AcOH (1.5 ml), and then hydrogenated over a Pd catalyst. The catalyst was filtered off, and the filtrate was evaporated *in vacuo* to give a crystalline residue. The residue and Et_3N (2.8 ml) were dissolved in DMF (40 ml), and reacted with *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxylysine 2,4,5-trichlorophenyl ester (11.2 g, 20 mmole) for 10 hr at room temperature, and the full was diluted with ice-cold 1N NH_4OH (*ca.* 200 ml). The resulting precipitate was collected by filtration and washed with 1N NH_4OH and H_2O. The washed precipitate was crystallized from MeOH–H_2O: Yield, 11.0 g (82%), mp 187—188.5° (decomp.), $[\alpha]_D^{23}$ −16.5° (c=1.0 in DMF). *Anal.* Calcd. for $C_{33}H_{47}O_8N_5 \cdot 1.5H_2O$: C, 59.27; H, 7.38; N, 10.45. Found: C, 59.22; H, 6.98; N, 10.42.

b) *t*-Butyloxycarbonylvalyl-N$^{\varepsilon}$-carbobenzoxylysyl-valyltyrosine Amide (VIb): VIa (6.42 g, 10 mmole) was treated with trifluoroacetic acid (20 ml) at 10° for 30 min. The product was precipitated by adding dry ether (150 ml), collected by filtration, washed with dry ether and then dried over NaOH *in vacuo* (*Rf* I; 0.0, *Rf* II; 0.67). The dried powder and Et_3N (1.5 ml) were dissolved in DMF (70 ml) and subjected to react with *t*-butyloxycarbonyl-valine N-hydroxysuccinimide ester (prepared by the ester exchange reaction[20] from *t*-butyloxycarbonyl-valine and O-(dichloroacetyl)-N-hydroxysuccinimide in pyridine at 50° for 40 min for 10 hr at room temperature. The resulting gelatinous solid was triturated with 10% AcOH (250 ml) to yield a fine powder. The powder was collected by filtration, washed with H_2O, and crystallized from MeOH–H_2O: Yield, 6.20 g (82.5%), mp 215—217° (decomp.), $[\alpha]_D^{22}$ −18.3° (c=1.0 in DMF). *Anal.* Calcd. for $C_{38}H_{56}O_9N_6 \cdot H_2O$: C, 61.01; H, 7.82; N, 11.25. Found: C, 61.23; H, 7.65; N, 11.41.

c) Valyl-N$^{\varepsilon}$-carbobenzoxylysylvalyltyrosine Amide (VI): VIb (15.2 g, 20 mmole) was treated with trifluoroacetic acid (50 ml) at 10° for 20 min. The product was precipitated by adding dry ether (400 ml), collected by filtration, washed with dry ether, and then dried over NaOH *in vacuo*. The dried powder was dissolved in MeOH (200 ml), and Et_3N (10 ml) was added to the solution under stirring and cooling. The gelatinous precipitate was collected by centrifugation and washed with MeOH. The washed precipitate was crystallized from MeOH–H_2O to give the fine needle: Yield, 11.0 g (83.3%), mp 233—235° (decomp.), $[\alpha]_D^{22}$ −19.5° (c=1.0 in DMF), *Rf* I; 0.0, *Rf* II; 0.40, *Rf* III; 0.72. *Anal.* Calcd. for $C_{34}H_{48}O_7N_6$: C, 61.85; H, 7.55; N, 13.12. Found: C, 61.80; H, 7.49; N, 12.98.

Acknowledgement We wish to thank to Dr. S. Tatsuoka, Dr. Y. Abe and Dr. Y. Sanno, this Research and Development Division for their encouragement and useful discussion throughout this work. Thanks are also due to Mr. M. Kan and his staff in this laboratories for elemental analysis and Miss K. Mimuro for optical rotations.