[Chem. Pharm. Bull. 16(10)1997—2004(1968)]

UDC 615.225.2.011.5 : 547.964.4.07

Synthesis of Bradykinyl-peptide and Peptidyl-bradykinin[1]

Kenji Suzuki and Takashi Abiko

Tohoku College of Pharmacy[2]

(Received February 16, 1968)

[6-O-Acetyl-L-serine]-bradykinyl-glycyl-L-valyl-L-glutamine, glycylglycyl-L-lysyl-bradykinin, and bradykinylglycine were synthesized for the elucidation of structure activity relationship of bradykinin and their properties as substrates for bradykinin-releasing enzymes. The *p*-nitrophenyl ester method and stepwise elongation procedure were extensively used for the peptide synthesis.

The amino acid sequence being adjacent to the C-terminus arginine residue of L-methionyl-L-lysyl-bradykinin moiety in bovine serum kininogen has been elucidated by Habermann[3] as follows: L-methionyl-L-lysylbradykinyl-L-seryl-L-valyl-L-glutaminyl-L-valyl-L-methionine. By the same authors[3] it was reported that kinins were released on incubation with kinin-releasing enzymes, for example trypsin, kallikreins,and so on, from L-methionyl-L-lysyl-bradykinyl-L-seryl-L-valyl-glutamine synthesized by Schröder, by whom the experimental details of the synthesis have not been reported yet. In addition to the above investigations, it was suggested that the kinin moiety was located at the carboxyl terminal portion in the polypeptide chain of bovine serum kininogen-I[4] and in the internal of bovine serum kinonogen-II.[5] In view of these investigations it was of interest to examine the biological properties of bradykinyl-peptide and peptidyl-bradykinin which was modified the C- or N-terminal vicinity being adjacent to bradykinin moiety in kininogens. In a previous communication[6] one of the authors have reported that the synthesized bradykinin derivatives, namely glycylglycyl-L-lysyl-[6-O-acetyl-L-serine]-bradykinin, [6-O-acetyl-L-serine]-bradykinyl-glycyl-L-valyl-L-glutamine, and bradykinyl-glycine, were practically inactive in the assay on an isolated guinea pig ileum and bradykinin was released in large quantities from the two bradykinin derivatives with the exception of bradykinyl-glycine on incubation with trypsin, but not with hog pancreatic kallikrein from the three. In this paper the details of the synthesis of the three bradykinin derivatives are described. Bradykinyl-glycine has been synthesized by Woolley, *et al.*[7] using solid phase method.[8] The same peptide was synthesized by so-called classical method by the authors for the purpose described above. The method of peptide synthesis used here was virtually similar with a previous paper on the synthesis of bradykinin and its analogs.[9]

1) Abbreviations of amino acid derivatives and peptides, and naming synthetic modification of the natural peptide used herein are those recommended by IUPAC-IUB. commission on Biochemical Nomenclature in July 1965; *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967).
2) Location: *Nankozawa, Sendai.*
3) E. Habermann, "Hypotensive Peptide," Proceeding of the International Symposium, Oct. 25—29, 1965, Florence, Italy, Ed. E.G. Erdös, N. Bach, F. Sicuteri and A.F. Wilde, Springer-Verlag, New York Inc., 1966, p. 116.
4) M. Yano, S. Nagasawa, K. Horiuchi and T. Suzuki, *J. Biochem.* (Tokyo), **62**, 504 (1967).
5) S. Nagasawa, T. Mizushima, T. Sato, S. Iwanaga and T. Suzuki, *J. Biochem.* (Tokyo), **60**, 643 (1966).
6) K. Suzuki, K. Sasaki and T. Kameyama, *Japan. J. Pharmacol.*, **16**, 486 (1966). The authors of this communication wish to correct an error in calculation as follows: For '3' read '30' in the second line of the Table I from the bottom.
7) J.M. Stewart and D.W. Woolley, *Nature*, **207**, 1160 (1965).
8) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
9) K. Suzuki, T. Abiko and M. Asaka, *Chem. Pharm. Bull.* (Tokyo), **14**, 217 (1966).

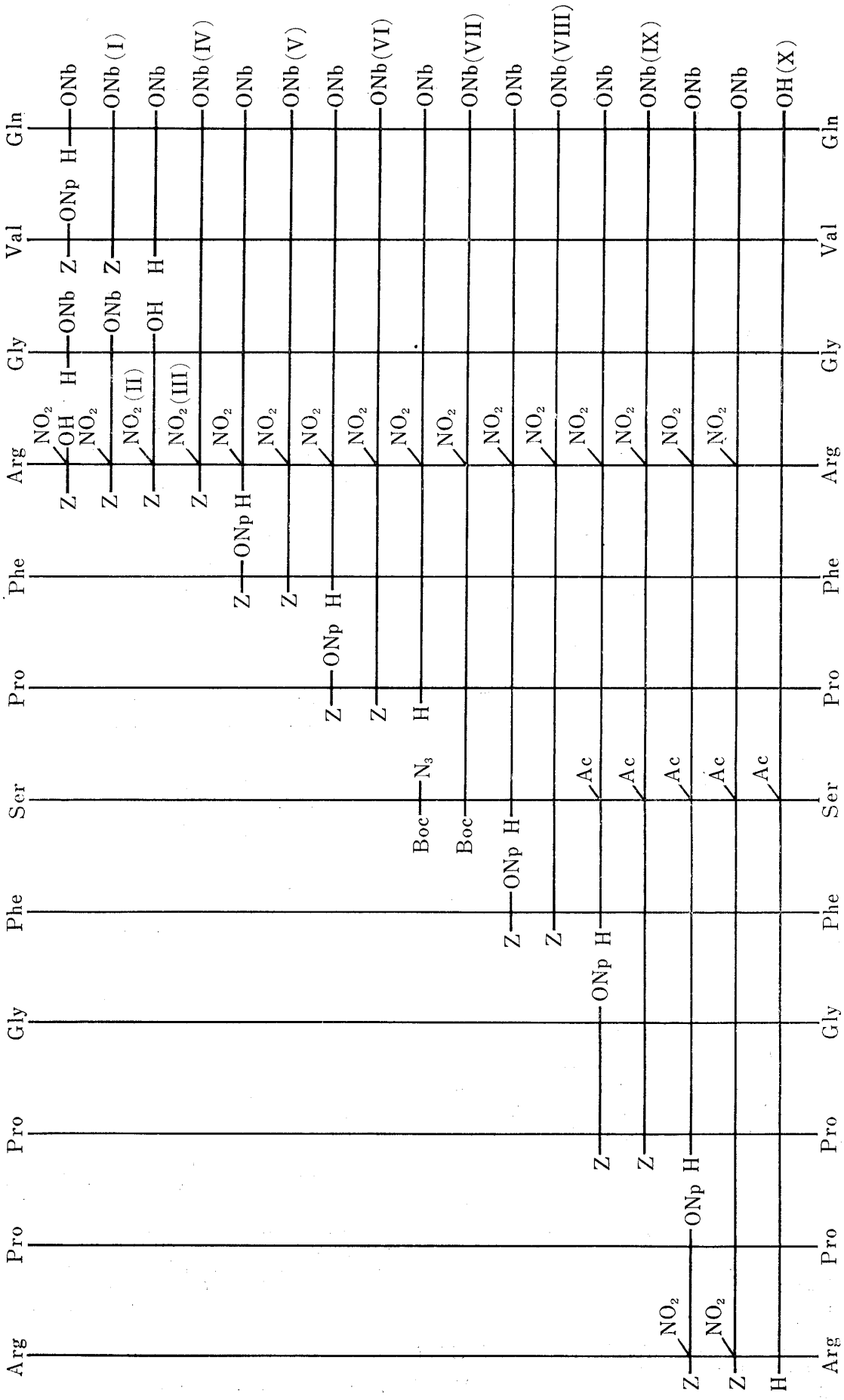

Fig. 1. Synthesis of [6-O-Acetyl-L-serine]-bradykinyl-glycyl-L-valyl-L-glutamine (X)

The synthetic route for the undecapeptide, [6-O-acetyl-L-serine]-bradykinyl-glycyl-L-valyl-L-glutamine (X), is illustrated in Fig. 1 as an example.

N-Benzyloxycarbonyl-L-glutamine *p*-nitrobenzyl ester[10] was de-benzyloxycarbonylated with a hydrogen bromide-acetic acid solution in the presence of anisole and the resulting amino acid ester was condensed with N-benzyloxycarbonyl-L-valine *p*-nitrophenyl ester[11] to yield N-benzyloxycarbonyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (I). N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginine[12] was condensed with glycine *p*-nitrobenzyl ester[13] by the N,N'-dicyclohexylcarbodiimide procedure[14] to yield N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine *p*-nitrobenzyl ester (II), which formed fine needles by recrystallization from ethanol. Saponification of II yielded N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine (III).[15] The N-protected dipeptide (III) was identical with the compound prepared by the mixed anhydride procedure.[15b] After the removal of the benzyloxycarbonyl group of I, the resulting dipeptide ester was condensed with III by the N,N'-dicyclohexylcarbodiimide procedure to yield N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (IV). After the removal of the benzyloxycarbonyl group of IV, the resulting tetrapeptide ester was condensed with N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester[16] to yield N-benzyloxycarbonyl-L-phenylalanyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (V). After the removal of the benzyloxycarbonyl group of V, the resulting pentapeptide ester was condensed with N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester[17] to yield N-benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (VI). After the removal of the benzyloxycarbonyl group of VI, the resulting hexapeptide ester was condensed with N-*tert*-butyloxycarbonyl-L-serine azide[18] to yield N-*tert*-butyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (VII). As described in a previous paper[9] the introduction of N-*tert*-butyloxycarbonylseryl group is preferable to avoid O→N acetyl shift due to O-acetylseryl group, resulted from the de-benzyloxycarbonylation of N-benzyloxycarbonylseryl-peptide with hydrogen bromide-acetic acid solution. After the removal of the *tert*-butyloxycarbonyl group of VII with trifluoroacetic acid,[19] the resulting heptapeptide ester was condensed with N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester to yield N-benzyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (VIII). After the removal of the benzyloxycarbonyl group of VIII, the resulting octapeptide ester was condensed with N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester[20] to yield N-benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester (IX). After the removal of the benzyloxycarbonyl group of IX, the resulting decapeptide ester was condensed with N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester[20] to yield N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-phenylalanyl-N^{ω}-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-nitrobenzyl ester

10) D. Theodoropoulos and I. Souchleris, *J. Org. Chem.*, **31**, 4009 (1966).
11) B. Iselin, W. Rittel, P. Sieber and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).
12) M. Bergmann, L. Zervas and H. Rinke, *Z. Physiol. Chem.*, **224**, 40 (1934).
13) H. Schwarz and K. Arakawa, *J. Am. Chem. Soc.*, **81**, 5691 (1959).
14) J.T. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
15) *a*) H. Van Orden and E.L. Smith, *J. Biol. Chem.*, **208**, 751 (1954); *b*) K. Hofmann, W.D. Peckham and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).
16) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 6072 (1959).
17) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
18) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **44**, 169 (1961).
19) L.A. Carpino, *J. Am. Chem. Soc.*, **79**, 98 (1957); R. Schwyzer, W. Rittel, H. Kappeler and B. Iselin, *Angew. Chem.*, **70**, 915 (1960).
20) M.A. Ondetti, *J. Med. Chem.*, **6**, 10 (1963).

whose peptide ester de-benzyloxycarbonylated formed a single spot on paper in two solvent systems, but it was not analized. The protected undecapeptide was hydrogenated over 10% palladium on carbon in aqueous acetic acid for 2 days. Analysis of the hydrogenated product by paper chromatography using the Partridge system revealed the presence of one major ninhydrin and Sakaguchi positive spot with *Rf* 0.28, and one minor with *Rf* 0.43. The hydrogenated product was purified through carboxymethyl (CM-) cellulose column to obtain [6-O-acetyl-L-serine]-bradykinylglycyl-L-valyl-L-glutamine (X). The undecapeptide (X) so obtained was found to be homogeneous from the results of paper chromatography using two solvent systems. The ratio of amino acids in the acid hydrolysate agreed well with the theoretical value.

The other two peptides, glycylglycyl-L-lysyl-bradykinin and bradykinylglycine, were prepared essentially in a similar approach that had let to the formation of X. Glycylglycyl-L-lysine and glycyl-L-valyl-L-glutamine also was synthesized for the standard peptides in comparing with that released from the bradykinin derivatives which were incubated with kinin-releasing enzymes.[6]

Experimental

Melting points are uncorrected. For paper chromatography, the protected peptides were deblocked with HBr in AcOH unless otherwise mentioned and the resulting hydrobromides were chromatographed on a filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 value refer to the Partridge system,[21] and Rf^2 value refer to the system of BuOH—pyridine—AcOH—H_2O (30:20:6:24).[22] The amino acid composition of the acid hydrolysates was determined according to the directions given by Moore, *et al.*[23] N^{ω}-Nitro-L-arginine[12] was prepared from L-arginine hydrochloride in conc. H_2SO_4 adding NH_4NO_3 portionwise at room temperature according to the method described by Noguchi, *et al.*[24]

N-Benzyloxycarbonyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester (I)——N-Benzyloxycarbonyl-L-glutamine *p*-nitrobenzyl ester (2.0 g) was dissolved in AcOH (7 ml) and 7 N HBr in AcOH (7 ml). After 50 min at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was washed with dry ether and dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (DMF) (7 ml), N-benzyloxycarbonyl-L-valine *p*-nitrophenyl ester (2.0 g) was added, followed by Et_3N to keep the solution slightly alkaline. After 24 hr at room temperature, the reaction mixture was diluted with 1 N NH_4OH (50 ml) with stirring. The resulting precipitate was collected by filtration, washed successively with 1 N NH_4OH, H_2O, 1 N HCl, and H_2O. The product was recrystallized from EtOH; 1.4 g (57%) of fine needles, mp 198°, $[\alpha]_D^{13}$ +11.0° (c=0.7, DMF). *Anal.* Calcd. for $C_{25}H_{30}O_8N_4$: C, 58.36; H, 5.88; N, 10.89. Found: C, 58.91; H, 5.95; N, 10.86. Deblocked peptide ester: Rf^1 0.53, Rf^2 0.79, single ninhydrin positive spot.

N-Benzyloxycarbonylglycyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester——This compound was prepared from I (1.0 g) and N-benzyloxycarbonylglycine *p*-nitrophenyl ester (0.66 g) as described above. The product was washed with hot acetone. Yield 0.8 g (73%), mp 206°, $[\alpha]_D^{13}$ +65.4° (c=0.2, DMF). *Anal.* Calcd. for $C_{27}H_{33}O_9N_5$: C, 56.73; H, 5.82; N, 12.25. Found: C, 56.41; H, 6.05; N, 11.82. Deblocked peptide ester: Rf^1 0.57, Rf^2 0.66.

Glycyl-L-valyl-L-glutamine Hemihydrate——The above protected tripeptide (260 mg) was hydrogenated in 1:1 AcOH and H_2O (14 ml) in the presence of 10% Pd-C for 6 hr. The product was recrystallized from H_2O and EtOH. Yield 130 mg (92%), mp 216—218° (decomp.), $[\alpha]_D^{13}$ −45.7° (c=0.7, H_2O), Rf^1 0.20, Rf^2 0.29, single ninhydrin positive spot. *Anal.* Calcd. for $C_{12}H_{22}O_5N_4 \cdot \frac{1}{2}H_2O$: C, 46.29; H, 7.45; N, 18.00. Found: C, 46.87; H, 7.60; N, 17.56.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine *p*-Nitrobenzyl Ester (II)——To a solution of glycine *p*-nitrobenzyl ester hydrobromide in 1:1 mixture of acetonitrile and methylene chloride (50 ml), Et_3N (2.8 ml) was added, stirred for 30 min, and then cooled in an ice bath. To the cold solution, N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginine (7.1 g) and N,N'-dicyclohexylcarbodiimide (4.4 g) was added and stirred overnight at 5°. After adding AcOH (2 ml) to the reaction mixture, the precipitate was filtered and washed with DMF. The combined filtrate was diluted with EtOAc containing MeOH. The EtOAc solution was

21) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).
22) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).
23) S. Moore, D.H. Spackmann and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
24) J. Noguchi and T. Hayakawa, Abstracts of Papers, the 4th Symposium on Peptide Chemistry, Osaka, Nov. 1965, p. 21.

washed successively with 1 N HCl, H_2O, 1 N NH_4OH, and H_2O. The EtOAc solution containing MeOH was dried over $MgSO_4$ and evaporated to dryness. The residue was recrystallized from EtOH (400 ml); yield 6.4 g (59%) of needles, mp 132°, $[\alpha]_D^{20}$ +47.2° (c=0.6, DMF). *Anal.* Calcd. for $C_{23}H_{27}O_9N_7$: C, 50.64; H, 4.99; N, 17.98. Found: C, 50.89; H, 4.61; N, 17.74. Deblocked peptide ester: Rf^1 0.36, Rf^2 0.75, single ninhydrin positive spot.

N$^{\alpha}$-Benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginylglycine (III)——The protected dipeptide (II) (5.0 g) was suspended in MeOH (30 ml) and added 1 N NaOH (11.0 ml). The mixture was stirred for 1.5 hr at room temperature and the solution was concentrated to small volume in vacuum. The residue was diluted with H_2O and the aqueous solution was washed EtOAc. The aqueous layer was acidified to Congo red with 5 N HCl and kept in a refregerator overnight. The resulting crystalline product was collected and recrystallized from aqueous EtOH. Yield 3.9 g (95%) of needles, mp 112° (lit.[15b] mp 111—113°), $[\alpha]_D^{14}$ −13.7° (c=1.02 MeOH) (lit.[15b] $[\alpha]_D^{28}$ −16.8° (c=1.05, MeOH)).

N$^{\alpha}$-Benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester (IV)——The protected dipeptide ester (I) (0.51 g) was de-benzyloxycarbonylated as described above. To a solution of the de-benzyloxycarbonylated compound in a mixture of tetrahydrofuran (10 ml) and DMF (3 ml), Et_3N (0.18 ml) was added and after 30 min the mixture was cooled in an ice bath. To the cold mixture, N$^{\alpha}$-benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginylglycine (0.42 g) in DMF (2 ml) was added, followed by N,N′-dicyclohexylcarbodiimide (0.21 g), the mixture was stirred in an ice bath for 1 hr, and at room temperature overnight. The precipitate thereby formed was filtered and washed with DMF. The combined filtrate was evaporated to small volume in vacuum and the residue was taken up in EtOAc. The EtOAc solution was washed successively with 1 N NH_4OH, H_2O, 1 N HCl, and H_2O. The solution was dried over $MgSO_4$ and evaporated to dryness. The residue was recrystallized from EtOH. Yield 0.45 g (58%), mp 112—115°, $[\alpha]_D^{14}$ −14.6° (c=0.7, DMF). *Anal.* Calcd. for $C_{33}H_{44}O_{12}N_{10}$: C, 51.29; H, 5.74; N, 18.13. Found: C, 51.44; H, 5.97; N, 18.28. Deblocked peptide ester: Rf^1 0.26, Rf^2 0.69, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester (V)——This compound was prepared from IV (850 mg) and N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (500 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from AcOH with H_2O. Yield 650 mg (65%), mp 160—170°, $[\alpha]_D^{12}$ −13.9° (c=0.4, DMF). *Anal.* Calcd. for $C_{42}H_{53}O_{13}N_{11}$: C, 54.83; H, 5.81; N, 16.75. Found: C, 54.47; H, 6.15; N, 16.49. Deblocked peptide ester: Rf^1 0.54, Rf^2 0.87, single ninhydrin postive spot.

N-Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester Monohydrate (VI)——This compound was prepared from V (300 mg) and N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (135 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from acetone with ether. Yield 250 mg (75%), mp 145—167°, $[\alpha]_D^{14}$ −25.6° (c=0.7, DMF). *Anal.* Calcd. for $C_{47}H_{60}O_{14}N_{12}\cdot H_2O$: C, 54.54; H, 6.04; N, 16.24. Found: C, 54.66; H, 6.28; N, 15.34. Deblocked peptide ester: Rf^1 0.43, Rf^2 0.81, single ninhydrin positive spot.

N-*tert*-Butyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester Dihydrate (VII)——The protected hexapeptide (VI) (365 mg) was de-benzyloxycarbonylated as described above. To a solution of the de-benzyloxycarbonylated compound in DMF (10 ml) containing Et_3N to keep the solution slightly alkaline, was added an EtOAc solution (5 ml) of N-*tert*-butyloxycarbonyl-L-serine azide prepared from 279 mg of the hydrazide. The reaction mixture was stirred at 5° for 24 hr and at room temperature for 1 hr. The reaction mixture was diluted with EtOAc and washed successively with H_2O, 1 N citric acid, H_2O, 1 N $NaHCO_3$, and H_2O. The EtOAc solution was dried over $MgSO_4$ and evaporated in vacuum to dryness. The residue was reprecipitated from acetone with ether. Yield 220mg (56%), mp 118—124°, $[\alpha]_D^{14}$ −16.0° (c=0.4, DMF). *Anal.* Calcd. for $C_{47}H_{67}O_{16}N_{13}\cdot 2H_2O$: C, 51.03; H, 6.47;N, 16.46. Found: C, 51.36; H, 6.13; N, 16.36. For paper chromatography a sample was removed its *tert*-butyloxycarbonyl group with trifluoroacetic acid: Rf^1 0.64, Rf^2 0.86, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginylglycly-L-valyl-L-glutamine *p*-Nitrobenzyl Ester One and A Half Hydrate (VIII)——The protected heptapeptide ester (VII) (204 mg) was dissolved in anhydrous trifluoroacetic acid (1 ml) and the solution was kept at room temperature for 20 min, when dry ether was added. The resulting precipitate was condensed with N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (91 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from DMF with 1 N NH_4OH; yield 135 mg (58%), mp 123—128°, $[\alpha]_D^{14}$ −27.6° (c=0.4, DMF). *Anal.* Calcd. for $C_{59}H_{74}O_{17}N_{14}\cdot 1\frac{1}{2}\ H_2O$: C, 55.43; H, 6.07; N, 15.34. Found: C, 55.38; H, 5.99; N, 15.03. Deblocked peptide ester: Rf^1 0.71, Rf^2 0.90, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginylglycyl-L-valyl-L-glutamine *p*-Nitrobenzyl Ester Monohydrate (IX)——This compound was prepared from the protected octapeptide (X) (135 mg) and N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester (55 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from DMF with 1 N NH_4OH. Yield 75 mg(49%), mp 130—140° $[\alpha]_D^{12}$ −22.2° (c=0.3, DMF). *Anal.* Calcd. for $C_{68}H_{86}O_{20}N_{16}\cdot H_2O$: C, 55.73; H, 6.05; N, 15.29. Found: C, 55.62; H, 6.41; N, 15.00. Deblocked peptide ester: Rf^1 0.67, Rf^2 0.89, single ninhydrin positive spot.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginylglycyl-L-valyl-L-glutamine Triacetate (X)——The protected decapeptide (IX) (90 mg) and N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (42 mg) was condensed essentially in the same manner as described in the prepartion of I. The time for coupling reaction was for 2 days. The product was reprecipitated from DMF with 1 N NH_4OH. Yield 65 mg, mp 127—134°. The deblocked peptide ester: Rf^1 0.54, Rf^2 0.83, single ninhydrin positive spot. The product was hydrogenated for 2 days as described above. The aqueous solution (5 ml) of the hydrogenated product was added to a CM-cellulose column (2.0×6.0 cm) which was eluted with a linear gradient eluation from 0.01 M NH_4OAc buffer (pH 6.50) (300 ml) in a mixing chamber to 0.10 M NH_4OAc buffer (pH 6.50) (300 ml) in a reservoir. Fractions of 9.5 ml each were collected at a flow rate of 1.5 to 2.0 ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 230 mμ. The eluates in tubes No. 32 to 37 containing the undecapeptide were pooled, evaporated in vacuum to dryness, and lyophilized to constant weight, colorless fluffy material. Yield 26 mg (26%), mp 167—180°, $[\alpha]_D^{12}$ −57.1° (c=0.4, H_2O), Rf^1 0.28, Rf^2 0.38, single ninhydrin and Sakaguchi positive spot, amino acid ratios in the acid hydrolysate; Arg 2.14, Pro 2.74, Gly 1.65, Phe 1.93, Ser 0.91, Val 1.00, Glu 0.80, NH_3 1.10 (average recovery 101%). Acetyl ester group was 64.0% of theory.

N^{ε}-*tert*-Butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester Monohydrate (XI)——The compound was prepared from N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester[9] (300 mg) and N^{α}-*tert*-butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester[25] (120 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from acetone with ether. Yield 235 mg (60%), mp 117—123°, $[\alpha]_D^{14}$ −35.1° (c=0.1, DMF). *Anal.* Calcd. for $C_{78}H_{104}O_{23}N_{20}\cdot H_2O$: C, 54.85; H, 6.26; N, 16.41. Found: C, 55.02; H, 6.11; N, 15.99. For paper chromatography the *tert*-butyloxycarbonyl group of the fully protected peptide was deblocked with trifluoroacetic acid: Rf^1 0.73, Rf^2 0.92, single ninhydrin positive spot.

N-Benzyloxycarbonylglycylglycyl-N^{ε}-benzyloxycarbonyl-L-lysyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester Dihydrate (XII)——The compound was prepared from the XI (78 mg) de-*tert*-butyloxycarbonylated and N-benzyloxycarbonylglycylglycine *p*-nitrophenyl ester[26] (20 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from MeOH with ether. Yield 70 mg (82%), mp 120—127°, $[\alpha]_D^{14}$ −42.8° (c=0.5, DMF). *Anal.* Calcd. for $C_{86}H_{108}O_{25}N_{22}\cdot 2H_2O$: C, 54.77; H, 5.99; N, 16.34. Found: C, 55.01; H, 6.30; N, 16.14. Deblocked peptide ester: Rf^1 0.15, Rf^2 0.49, single ninhydrin positive spot.

Glycylglycyl-L-lysyl-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Tetraacetate Octahydrate (XIII)——The fully protected undecapeptide (XII) (70 mg) was hydrogenated for 2 days as described above. The aqueous solution (10 ml) of the hydrogenated product was added to a CM-cellulose column (2.0×6.0 cm), which was eluted with a linear gradient method from H_2O (300 ml) in a mixing chamber to a 0.33 M pyridinium acetate buffer (pH 5.1) (300 ml) in a reservoir. Fractions of 12 ml each were collected at a flow rate 4 ml/min with an automatic fraction collector. The arginine-containing peptide was located in the eluate by Sakaguchi reaction. The eluates in tubes No.30 to 39 containing the undecapeptide were pooled, evaporated to dryness in vacuum, and lyophilized. Yield 50 mg (78%) of colorless fluffy material, mp 170—180° (decomp.), $[\alpha]_D^{19}$ −88.9° (c=0.2, H_2O), Rf^1 0.14, Rf^2 0.41, single ninhydrin and Sakaguchi positive spot. *Anal.* Calcd. for $C_{62}H_{93}O_{15}N_{19}\cdot 4CH_3COOH\cdot 8H_2O$: C, 48.63; H, 7.29; N, 15.40. Found: C, 48.33; H, 7.66; N, 15.71.

Glycylglycyl-L-lysyl-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Tetraacetate——Glycylglycyl-L-lysyl-[6-O-acetyl-L-serine]-bradykinin (XIII) (30 mg) in H_2O (0.2 ml) was saponified with 1 N NaOH (0.3 ml) for 1 hr at room temperature. The solution neutralized with 1 N AcOH was added to a (2.0×6.0 cm) CM-cellulose column, which was eluted as described above. The eluates containing the peptide were evaporated to dryness in vacuum and lyophilized. Yield 25 mg (94%), mp 163—176°, $[\alpha]_D^{12}$ −76.3° (c=0.4, H_2O), Rf^1 0.08, Rf^2 0.35, single ninhydrin and Sakaguchi positive spot; amino acid ratios in the acid hydrolysate; Arg 1.60, Lys 1.00, Pro 3.12, Gly 2.80, Phe 1.96, Ser 0.73(averatge recovery 83%).

Glycylglycyl-L-lysine Diacetate Monohydrate——The coupling reaction of N-benzyloxycarbonylglycylglycine *p*-nitrophenyl ester and N^{ε}-benzyloxycarbonyl-L-lysine[27] was carried out essentially in the same manner described by Ondetti.[20] To a suspension of N^{ε}-benzyloxycarbonyl-L-lysine (0.84 g) in H_2O (30 ml) N-benzyloxycarbonylglycylglycine *p*-nitrophenyl ester (1.29 g) in pyridine (30 ml) was added. The suspension was stirred and the pH kept at 8.3—8.5 by the addition of 1 N NaOH until the pH remained constant, when a clear solution was obtained. The solution was diluted with H_2O (30 ml) and saturated with $NaHCO_3$. The mixture was washed 8 times with EtOAc and then acidified to Congo red with 5 N HCl. An oil separated

25) K. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **14**, 909 (1966).
26) M. Bodanszky, J.T. Sheehan, M.A. Ondetti and S. Lande, *J. Am. Chem. Soc.*, **85**, 991 (1963).
27) M. Bergmann, L. Zervas and W.F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

was extracted with EtOAc and the solution was treated as usual manner, when the solvent was evaporated to give solid, wt. 300 mg. The crude product (100 mg) was hydrogenated as usual manner and the hydrogenated product was submitted to a linear gradient CM-cellulose column chromatography (300 ml of H_2O in a mixing chamber, 300 ml of 0.06 M pyridinium acetate buffer (pH 5.1) in a reservoir) as described above. The tripeptide was located in the eluates by ninhydrin test. The eluates in tubes No. 26 to 34 containing the tripeptide were pooled, evaporated to dryness in vacuum, and lyophilized. Yield 38 mg, mp 98—108°, $[\alpha]_D^{12}$ −23.8° (c=0.4, H_2O), Rf^1 0.09, Rf^2 0.16, single ninhydrin positive spot. *Anal.* Calcd. for $C_{10}H_{20}O_4N_4 \cdot 2CH_3COOH \cdot H_2O$: C, 42.20; H, 7.59; N, 14.06. Found:C, 42.54; H, 7.85; N, 14.28.

N-Benzyloxycarbonyl-L-phenylalanyl-N$^\omega$-nitro-L-arginylglycine *p*-Nitrobenzyl Ester (XIV)——This compound was prepared from II (5.4 g) and N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (4.6 g) essentially in the same manner as described in the preparation of I. The product was reprecipitated from EtOAc with petroleum ether. Yield 6.9 g (100%), mp 94—99°, $[\alpha]_D^{20}$ −0.0° (c=0.8, DMF). *Anal.* Calcd. for $C_{32}H_{36}O_{10}N_8$: C, 55.48; H, 5.24; N, 16.18. Found: C, 56.49; H, 5.89; N, 15.78. Deblocked peptide ester: Rf^1 0.54, Rf^2 0.58, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginylglycine *p*-Nitrobenzyl Ester (XV)——This compound was prepared from XIV (5.0 g) and N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (3.0 g) essentially in the same manner as described in the preparation of I. The product was washed with hot EtOH. Yield 4.6 g (77%), mp 216°, $[\alpha]_D^{20}$ −76.0° (c=0.6, DMF). *Anal.* Calcd. for $C_{38}H_{43}O_{12}N_9$: C, 55.81; H, 5.30; N, 15.42. Found: C, 56.12; H, 5.05; N, 15.97. Deblocked peptide ester: Rf^1 0.49, Rf^2 0.82, single ninhydrin positive spot.

N-*tert*-Butyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginylglycine *p*-Nitrobenzyl Ester (XVI)——This compound was prepared from XV (400 mg) and N-*tert*-butyloxycarbonyl-L-serine azide prepared from the hydrazide (140 mg), essentially in the same manner as described in the prepartion of VII. The product was reprecipitated from acetone with ether. Yield 200 mg (49%), mp 110—112°, $[\alpha]_D^{20}$ −43.8° (c=0.6, DMF). *Anal.* Calcd. for $C_{38}H_{50}O_{14}N_{10}$: C, 52.41; H, 5.79; N, 16.09. Found: C, 52.73; H, 6.17; N, 15.92. For paper chromatography the *tert*-butyloxycarbonyl group was removed with trifluoroacetic acid: Rf^1 0.69, Rf^2 0.83, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginylglycine *p*-Nitrobenzyl Ester Monohydrate (XVII)——The protected pentapeptide (VI) (532 mg) and N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (292 mg) was coupled essentially in the same manner as described in the preparation of VIII. The reaction mixture was diluted with EtOAc. The EtOAc solution was washed successively with 1 N NH_4OH, H_2O, 1 N HCl, and H_2O, when dried over $MgSO_4$. The EtOAc solution was concentrated in vacuum and addded petroleum ether to give solid. The product was reprecipitated from EtOH with ether. Yield 493 mg (76%), mp 106—111°, $[\alpha]_D^{28}$ −12.1° (c=0.5, DMF), *Anal.* Calcd. for $C_{49}H_{57}O_{14}N_{11} \cdot H_2O$: C, 56.47; H, 5.71; N, 14.79. Found: C, 56.49; H, 5.64; N, 14.79. Deblocked peptide ester: Rf^1 0.64, Rf^2 0.89, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginylglycine *p*-Nitrobenzyl Ester Monohydrate (XVIII)——This compound was prepared from XVII (300 mg) and N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester (134 mg) essentially in the same manner as described in the preparation of I. The product was reprecipitated from hot EtOH with ether. Yield 280 mg (79%), mp 110—119°, $[\alpha]_D^{28}$ −8.6° (c=0.6, DMF), *Anal.* Calcd. for $C_{58}H_{69}O_{17}N_{13} \cdot H_2O$: C, 56.26; H, 5.78; N, 14.71. Found: C, 56.13; H, 5.48; N, 14.62. Deblocked peptide ester: Rf^1 0.66, Rf^2 0.94, single ninhydrin positive spot.

N$^\alpha$-Benzyloxycarbonyl-N$^\omega$-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginylglycine *p*-Nitrobenzyl Ester Dihydrate (XIX)——This compound was prepared from XVIII (140 mg) and N$^\alpha$-benzyloxycarbonyl-N$^\omega$-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (80 mg) essentially in the same manner as described in the preparation of I. The time for coupling reaction was for 2 days. The product was reprecipitated from MeOH with ether. Yield 82 mg (46%), mp 126—134°, $[\alpha]_D^{28}$ −50.7° (c=0.5, DMF), *Anal.* Calcd. for $C_{69}H_{87}O_{21}N_{19} \cdot 2H_2O$: C, 53.29; H, 5.90; N, 17.12. Found: C,53.39; H, 5.83; N, 16.68. Deblocked peptide ester: Rf^1 0.56, Rf^2 0.89, single ninhydrin positve spot.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginylglycine Triacetate Trihydrate (XX)——The fully protected decapeptide (XIX) (80 mg) was hydrogenated in the manner as described in the preparation of XIII. The hydrogenated product was submitted to CM-cellulose chromatography (0.15 M pyridinium acetate buffer in a mixing chamber) as described above. The eluates in tubes No. 30 to 45 containing the decapeptide were pooled, evaporated to dryness in vacuum, and lyophilized;yield 40 mg (56%), mp 170—182°, $[\alpha]_D^{15}$ −75.0° (c=0.4, H_2O). *Anal.* Calcd. for $C_{54}H_{84}O_{16}N_{16} \cdot 3CH_3COOH \cdot 3H_2O$: C, 51.71; H, 6.94; N, 16.08. Found: C, 51.54; H, 7.19; N, 15.63. Rf^1 0.31, Rf^2 0.37; single ninhydrin and Sakaguchi postive spot, Acetyl ester group was 90.0% of theory.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginylglycine Triacetate Dihydrate——This decapeptide was prepared from XX (26 mg) as described in the preparation of glycylglycly-L-lysyl-bradykinin. Yield 22 mg (90%), mp 170—181°, $[\alpha]_D^{20}$ −74.0° (c=0.7, H_2O), *Anal.* Calcd. for

$C_{52}H_{76}O_{14}N_{16}\cdot 3CH_3COOH\cdot 2H_2O$: C, 51.02; H, 6.79; N, 16.41. Found: C, 51.11; H, 6.81; N, 16.02. Rf^1 0.23, Rf^2 0.46, single ninhydrin and Sakaguchi positive spot.

Acknowledgement The authors wish to thank Mrs. Y. Abiko for the preparation of a part of the starting materials and the staff of Central Analysis Laboratory, Department of Chemistry, Faculty of Sciences, University of Tohoku for elemental analysis.