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140. Kenji Suzuki and Takashi Abiko: Synthesis of β-Alanine-containing Analogs of Bradykinin.*1

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The amino acid sequence of bradykinin was elucidated by Elliot, *et al.*[1] and established by synthesis by Boissonnas, *et al.*[2] A large number of analogs of bradykinin has since been prepared and investigated biologically.[3] While these studies have been concerned with the substitution of one amino acid within bradykinin for another α-amino acid, the substitution for β-alanine which elongates peptide chain of bradykinin by one methylene group, and the resultant effects on biological activity have not been explored. In the present paper, the first example of bradykinin analogs, 6-β-alanine-, 4-β-alanine-, and 4,6-di-β-alanine-bradykinin which have β-alanine unit in the internal position of bradykinin were reported.

The synthetic route for 6-β-alanine-bradykinin (Ⅵ) is illustrated in Chart 1. Esterification of N-benzyloxycarbonyl-β-alanine[4] with *p*-nitrophenol by the N,N′-dicyclohexylcarbodiimide method[5] gave N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (Ⅰ) with N(N$^{\alpha}$-benzyloxycarbonyl-β-alanyl)-N,N′-dicyclohexylurea. N-Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester[6] was debenzyloxycarbonylated with hydrogen bromide-acetic acid solution and L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester thereby formed was condensed with N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (Ⅰ) to yield N-benzyloxycarbonyl-β-alanyl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (Ⅱ). After the removal of the benzyloxycarbonyl group from Ⅱ, the resulting tetrapeptide ester was condensed with N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester[7] to yield N-benzyloxycarbonyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (Ⅲ). After the removal of the benzyloxycarbonyl group from Ⅲ, the resulting pentapeptide ester was condensed with N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester[8] to yield N-benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (Ⅳ). After the removal of the benzyloxycarbonyl group from Ⅳ, the resulting heptapeptide ester was condensed with N$^{\alpha}$-benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginyl-L-proline *p*-nitrophenyl ester[8] to yield N$^{\alpha}$-benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (Ⅴ). The fully protected nonapeptide (Ⅴ) was submitted to catalytic hydrogenation over 10% palladium-carbon in aqueous acetic acid solution for 40 hours and the reduction product was purified through carboxymethyl (CM) cellulose column to obtain

*1 Nomenclature of bradykinin homologs and abbreviation of amino acids followed those given in Proc. 2nd Intl. Pharmcol. Meeting, Vol. 10. Oxytocin, Vasopressin, and their Structural Analogues. Ed. J. Rudinger. xi (1964). Czechoslovak Medical Press, Praha.

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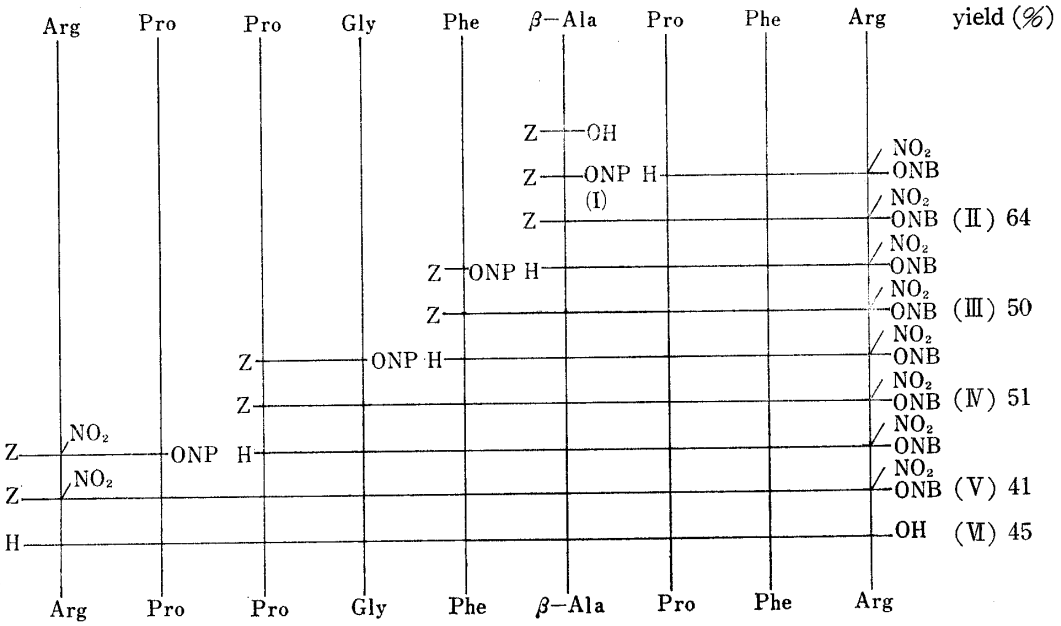
1) D. F. Elliot, G. P. Lewis, E. W. Horton: Bioch. Biophys. Res. Commun., **3**, 87 (1960).
2) R. A. Boissonnas, St. Guttmann. P. A. Jaquaenoud: Helv. Chim. Acta, **43**, 1349 (1960).
3) H. D. Law: "Progress in Medicinal Chemistry," Ⅳ, 86 (1965). Ed. G. P. Ellis, G. B. West. Butterworths Scientific Publication, London.
4) R. H. Sifferd, V. Du Vigneaud: J. Biol. Chem., **108**, 753 (1935).
5) J. C. Sheehan, C. P. Hess: J. Am. Chem. Soc., **77**, 1067 (1955).
6) K. Suzuki, T. Abiko, M. Asaka: This Bulletin, **14**, 217 (1966).
7) M. Bodanszky, V. Du Vigneaud: J. Am. Chem. Soc., **81**, 6072 (1959).
8) M. A. Ondetti: J. Med. Chem., **6**, 10 (1963).

L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-L-arginine triacetate salt (Ⅵ). The nonapeptide (Ⅵ) so obtained was found to be unity from the result of paper chromatography using two different solvent systems. Ratio of amino acids in the acid hydrolysate agreed well with the theoretical value.



Z=benzyloxycarbonyl, ONB=*p*-nitrobenzyl, ONP=*p*-nitrophenyl

Fig. 1. Synthesis of 6-β-Alanine-bradykinin

After the removal of the benzyloxycarbonyl group from N-benzyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester,[2,6] the resulting L-phenylalanyl-O-acethyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester was condensed with N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (I) to yield N-benzyloxycarbonyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (Ⅶ). After the removal of the benzyloxycarbonyl group from Ⅶ, the resulting hexapeptide ester was condensed with N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester[9] to yield N-benzyloxycarbonyl-L-propyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (Ⅷ). After the removal of the benzyloxycarbonyl group from Ⅷ, the resulting heptapeptide ester was condensed with N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester to yield N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester dihydrate (Ⅸ). The fully protected nonapeptide (Ⅸ) was submitted to catalytic hydrogenation over 10% palladium-carbon in aqueous acetic acid solution for 40 hours. The reduction product was purified through CM-cellulose column to obtain L-arginyl-L-prolyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine triacetate (X). The nonapeptide (X) so obtained was found to be unity from the result of paper chromatography using two different solvent systems. Ratio of amino acids in the acid hydrolysate agreed well with the theoretical value. Determination of the acteyl-ester group by the hydroxamic acid method[10] was 73.0% of the theoretical value. Saponification of the nonapeptide (X) with 1*N* sodium hydroxide solution afforded 6-β-alanine-bradykinin (Ⅺ). The nonapeptide (Ⅺ) here obtained was found to be unity from the result of

9) M. Bodanszky, V. Du Vigneaud: J. Am. Chem. Soc., **81**, 5688 (1959).
10) S. Hestrin: J. Biol. Chem., **180**, 249 (1949).

paper chromatography using two different solvent systems and the ratio of amino acids in the acid hydrolysate agreed well with the theoretical value.

After the removal of the benzyloxycarbonyl group from N-benzyloxycarbnoyl-L-phenylalanyl-β-alanyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (Ⅲ), the resulting pentapeptide ester was condensed with N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (Ⅰ) to yield N-benzyloxycabonyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (Ⅻ). After the removal of the benzyloxycarbonyl group from Ⅻ, the resulting hexapeptide ester was condensed with N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester to yield N-benzyloxycarbonyl-L-prolyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-niprobenzyl ester (XIII). After the removal of the benzyloxycarbonyl group from XIII, the resulting heptapeptide ester was condensed with N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester to yield N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester trihydrate (XIV). The fully protected nonapoptide (XIV) was submitted to catalytic hydrogenation over 10% palladium-carbon in aqueous acetic acid solution. The reduction product was purified through CM-cellulose column to obtain L-arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-L-arginine triacetate (XV). The nonapeptide (XV) so obtained was found to be unity from the result of paper chromatography using two different solvent systems. Ratio of amino acids in acid hydrolysate agreed well with the theoretical value.

Quantitative examinations were made on the bradykinin-like activity, antibradykinin action, and potentiation of bradykinin activity of the nonapeptides synthesized

Table I. Biological Activities of Bradykinin Analogs[a]

	Bradykinin-like activity	Bradykinin potentiating activity	Antibradykinin activity[e]
Bradykinin	1		
4-β-Alanine-bradykinin (Ⅺ)	1/5	−	−
4-β-Alanine-6-O-acetyl-L-serine	1/100	−	−
6-β-Alanine-brakykinin (Ⅵ)	1/20[c]	−	+[d]
4,6-Di-β-alanyl-bradykinin (XV)	−[b]	−	−

a) Assayed by Magnus method on a mouse ileum (male). +: active; −: inactive.
b) Inactive by 9×10^{-8} g./ml.
c) Active in a mouse out of three.
d) Antagonized ca. 20% by 1×10^{-6} g./ml. (in bath).
e) Assayed on the inhibition to contract an ileum induced bradykinin 3×10^{-7} g./ml. (in bath).

in the present work.*3 Result of these biological examination is given in Table I. The bradykinin-like activity of 6-β-alanine-bradykinin (Ⅵ), 4-β-alanine-6-O-acetyl-L-serine-bradykinin (Ⅹ), and 4-β-alanine-bradykinin (Ⅺ) is lower than that of bradykinin but activity is fairly apparent. The fact that the activity of O-acetyl derivative (Ⅹ) is lower than that of 4-β-alanin-bradykinin (Ⅺ) with free hydroxyl is similar to the other bradykinin analogs reported in the previous papers.[6,11] 6-β-Alanine-bradykinin (Ⅵ) in the concentration of 1×10^{-6} g./ml. in bath inhibited 20% to contract a mouse ileum induced bradykinin in concentration of 3×10^{-7} g./ml.

*3 The details of the biological assays will be reported in separate paper by Dr. Tsutomu Kameyama of this College.

11) K. Suzuki, M. Asaka, T. Abiko: This Bulletin, **14**, 211 (1966).

Experimental

Melting points are uncorrected. For paper chromatography, the protected peptides were debrocked with HBr in AcOH unless otherwise mentioned and the resulting hydrobromides were chromatographed on fiter paper, Toyo Roshi No. 51, at room temperature. Rf^1 value refer to the Partridge system,[12] and Rf^2 value refer to the system of BuOH–pyridine–AcOH–H_2O (30:20:6:24).[13] The amino acid composition of the acid hydrolysates was determined according to the directions given by Moore, *et al.*[14]

N-Benzyloxycarbonyl-β-alanine *p*-Nirophenyl Ester (I)——To a cold solution of N-benzyloxycarbonyl-β-alanine (1.00 g.) and *p*-nitrophenol (0.68 g.) in tetrahydrofurane (10 ml.) N,N′-dicyclohexylcarbodiimide (1.00g.) was added. The reaction mixture was stirred for 30 min. at 0° and 3 hr. at room temperature. To the reaction mixture a few drops of AcOH was added and the solution was stirred for 20 min. The precipitated N,N′-dicyclohexylurea was filtered off and washed with tetrahydrofurane. The combined filtrates were evaporated to dryness in vacuum and the residue was recrystallized from EtOH–petroleum ether (1:10) 3 times. The crystalline product was dissolved in acetone (10 ml.) and added petroleum ether (40 ml.). The fine needles which appeared in 10 to 15 min. were collected by filtration; yield 0.20 g. of by-product, m.p. 131°. After rabbed the wall of the glass wear, the filtrate was kept in refregirator. The precipitate thereby formed was recrystallized from the same solvent; yield 0.60 g. (39%), m.p. 89°. *Anal.* Calcd. for $C_{17}H_{16}O_6N_2$: C, 60.00; H, 4.74; N, 8.23. Found : C, 59.50; H, 4.22; N, 7.87.

N(N^{α}-Benzyloxycarbonyl-β-alanyl)-N,N′-dicyclohexylurea——The by-product described above is assumed to be N(N^{α}-benzyloxycarbonyl-β-alanyl)-N,N′-dicyclohexylurea from the result of elemental analysis. *Anal.* Calcd. for $C_{24}H_{35}O_4N_3$: N, 9.78. Found : N, 9.97.

N-Benzyloxycarbonyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (II)——N-Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (1.00 g.) was dissolved in AcOH (0.7 ml.) and 5.7*N* HBr in AcOH (0.7 ml.). After 40 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 (5 ml.) N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (0.50 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 (3 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1 *N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and concentrated to small volume. Petroleum ether was added and the precipitate thereby formed was reprecipitated from AcOH, H_2O, and 50% NH_4OAc 2 times; yield 0.70 g. (64%), m.p. 78°, $[\alpha]_D^{15}$ −10.3° (c=1.1, AcOH). *Anal.* Calcd. for $C_{38}H_{45}O_{11}N_9$: C, 56.78; H, 5.64; N, 15.68. Found : C, 57.12; H, 5.22; N, 15.44. Deblocked peptide ester: Rf^1 0.55, Rf^2 0.84, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N′-nitro-L-arginine *p*-Nitrobenzyl Ester Dihydrate (III)——The protected tetrapeptide ester (II) (110 mg.) was dissolved in AcOH (0.3 ml.) and 5.7*N* HBr in AcOH (0.3 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 (5 ml.) N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (58 mg.) 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 (3 ml.), stirred for 1 hr., and diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH (3 ml.), stirred for 1 hr., and diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and concentrated to small volume in vacuum. Petroleum ether was added to the residue and the precipitate was reprecipitated from AcOH, H_2O, and 50% NH_4OAc 3 times; yield 75 mg. (58%). m.p. 94~100°, $[\alpha]_D^{15}$ −17.5° (c=0.6, AcOH). *Anal.* Calcd. for $C_{47}H_{52}O_{12}N_{10}\cdot 2H_2O$: C, 57.31; H, 5.73; N, 14.22. Found : C, 57.81; H, 5.31; N, 14.06. Deblocked peptide ester : Rf^1 0.67, Rf^2 0.91, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester Monohydrate (IV)——The protected pentapeptide ester (III) (564 mg.) was dissolved in AcOH (2.5 ml.) and 4.3*N* HBr in AcOH (2.5 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (6 ml.) N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester (294 mg.) 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 (0.6 ml.), stirred for 1 hr., and then diluted with 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 concentrated to small volume. Petroleum ether was added to the

12) S. M. Partridge : Biochem. J., **42**, 238 (1948).
13) S. G. Waley, G. Watson : *Ibid.*, **55**, 328 (1953).
14) S. Moore, D. H. Spackman, W. H. Stein : Anal. Chem., **30**, 1185 (1958).

residue and the precipitate was reprecipitated from AcOH, H_2O, and 50% NH_4OAc; yield 322 mg. (51%), m.p. 108~114°, $[\alpha]_D^{28}$ −31.3°(c=1.2, AcOH), *Anal.* Calcd. for $C_{54}H_{64}O_{14}N_{12}\cdot H_2O$: C, 57.74; H, 5.92; N, 14.97. Found: C, 57.98; H, 5.67; N, 14.94. Debloked peptide ester: Rf^1 0.69, Rf^2 0.88, single ninhydrin positive spot.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine-*p*-Nitrobenzyl Ester (V)——The protected heptapeptide ester (Ⅳ) (250 mg.) was dissolved in AcOH (1.5 ml.) and 4.3*N* HBr in AcOH (1.5 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (4 ml.) N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (129 mg.) was added, followed by Et_3N to keep the solution slightly alkaline. After 2 days at room temperature, the reaction mixture was diluted with 1*N* NH_4OH (2 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH and H_2O. A few percent of AcOH toward the EtOAc solution was added to prevent precipitation and EtOAc solution was washed with 1*N* HCl and H_2O. The EtOAc solution was dried over $MgSO_4$ and evaporated to dryness. The residue was reprecipitated from AcOH, H_2O, and 50% NH_4OAc; yield 130 mg. (41%), m.p. 123~129°, $[\alpha]_D^{28}$ −46.0° (c=0.8, AcOH), *Anal.* Calcd. for $C_{65}H_{82}O_{18}N_{18}$: C, 55.62; H, 5.89; N, 17.97. Found: C, 55.98; H, 6.21; N, 17.36. Deblocked peptide ester: Rf^1 0.65, Rf^2 0.89, single ninhydrin positive spot.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-L-arginine Triacetate Salt (VI)——The fully protected nonapeptide (V) (90 mg.) was hydrogenated in 10:5 mixture of AcOH and H_2O (15 ml.) for 40 hr. in the presence of 10% Pd-C. The catalyst was removed by filtration with the aid of Cellite. The solution was evaporated to dryness in vacuum and the residue was dried over KOH pellets in vacuum. The solution of the hydrogenated product in H_2O (10 ml.) was added to a (2.0×6.0 cm.) CM-cellulose column which was eluted with a linear gradient elution from H_2O (300 ml.) in a mixing chamber to 0.1*M* NH_4OAc buffer solution (pH 6.50) (300 ml.) in a reservoir. Fractions of 13 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collector. Arginine-containing peptide was located in the eluate by Sakaguchi reaction. The eluates in tubes No. 25 to 33 containing the nonapeptide were pooled, evaporated to dryness in vacuum, and lyophilized. The solution of the residue (45 mg.) in BuOH-pyridine-AcOH-H_2O (30:20:6:24) (5 ml.) was added to a (2.0×30 cm.) cellulose powder (200 to 300 mesh) column which was eluted with the same solvent. Fractions 13 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collector. Arginine-containing peptide was located in the eluate by Sakaguchi reaction. The eluates No. 5 to 9 containing the nonapeptide were pooled, evaporated to dryness, and lyophillized; yield 35 mg. (45%), $[\alpha]_D^{28}$ −50.1° (c=0.7, H_2O), Rf^1 0.35, Rf^2 0.52, single ninhydrin and Sakaguchi positive spot, amino acid ratios in acid hydrolysate: Arg 1.9, Pro 3.1, Phe 2.1, Gly 1.0, β-Ala 1.0.

N-Benzyloxycarbonyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-Nitrobenzyl Ester (VII)——N-Benzyloxycarbony-L-phenylalanyl-L-sery-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (451 mg.) was dissolved in AcOH (2.2 ml.) and 4.5*N* in AcOH (2.2 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (5 ml.) N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (176 mg.) 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 (0.5 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and concentrated to small volume. Petroleum ether was added to the residue. The precipitate was reprecipitated from dimethylformamide and 1*N* NH_4OH; yield 274 mg. (57%), m.p. 130~144°, $[\alpha]_D^{22}$ −32.8° (c=0.8, AcOH), *Anal.* Calcd. for $C_{52}H_{61}O_{15}N_{11}$: C, 57.82; H, 5.69; N, 14.27. Found: C, 57.39; H, 5.99; N, 14.15. Deblocked peptide ester: Rf^1 0.72, Rf^2 0.93, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (VIII)——The protected hexapeptide ester (Ⅶ) (230 mg.) was dissolved in AcOH (2 ml.) and 4.5*N* HBr in AcOH (2 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (4 ml.) N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (97 mg.) 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 (1 ml.), stirred for 1 hr., and then poured into 1*N* NH_4OH (40 ml.) with stirring. The precipitate thereby formed was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The dried precipitate was reprecipitated from acetone and ether; yield 86 mg. (66%), m.p. 159~164°, $[\alpha]_D^{22}$ −40.70° (c=0.3, AcOH), *Anal.* Calcd. for $C_{57}H_{68}O_{16}N_{12}$: C, 58.15; H, 5.82; N, 14.28. Found: C, 58.14; H, 6.11; N, 14.43. Deblocked peptide ester: Rf^1 0.72, Rf^2 0.90, single ninhydrin positive spot.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester Dihydrate (IX)——The protected heptapeptide ester (Ⅷ) (140 mg.) was dissolved in AcOH (1.5 ml.) and 4.5*N* HBr in AcOH (1.5 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate

thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (3 ml.) N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (83 mg.) was added, followed by Et_3N to keep the solution slightly alkaline. After 2 days at room temperature, the reaction mixture was diluted with 1*N* NH_4OH (0.5 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and concentrated to small volume. Petroleum ether was added to the residue and the precipitate was reprecipitated from AcOH, H_2O, and 50% NH_4OAc; yield 73 mg. (38%), m.p. 130~137°, $[\alpha]_D^{20}$ −75.5° (c=0.8, AcOH), *Anal.* Calcd. for $C_{68}H_{86}O_{20}N_{18}\cdot 2H_2O$: C, 54.03; H, 6.00; N, 16.68. Found: C, 54.20; H, 5.83; N, 16.60. Deblocked peptide ester: Rf^1 0.64, Rf^2 0.86, single ninhydrin positive spot.

L-Arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Triacetate (X)——The fully protected nonapeptide (IX) (102 mg.) was hydrogenated in 10:5 mixture of AcOH and H_2O (15 ml.) for 40 hr. in the presence of 10% Pd-C. The catalyst was removed by filtration with the aid of Cellite. The filtrate was evaporated to dryness in vacuum and the residue was dried over KOH pellets in vacuum. The solution of the hydrogenated product in H_2O (10 ml.) was added to a (2.0×6.0 cm.) CM-cellulose column which was eluted with a linear gradient elution from H_2O (300 ml.) in a mixing chamber to 0.20*M* pyidinium acetate buffer solution (pH 5.20) (300 ml.) in a reservoir. Fractions of 13 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collecter. Arginine-containing peptide was located in the eluate by Sakaguchi reaction. The eluates in tubes No. 21 to 29 containing the nonapeptide were pooled, evaporated to dryness in vacuum, and lyopillized. The solution of residue in the upper layer of Partridge system (5 ml.) was added to a (2.0×30 cm.) cellulose powder (200 to 300 mesh) column which was eluted with the same solvent. Fractions of 13 ml. each were colleted at a flow rate of 3 to 4 ml./min. with an automatic fraction collector. Arginine-containing peptide was located in the eluate by Sakaguchi reaction. The eluates in tubes No. 5 to 9 containing the nonapeptide were pooled, evaporated to dryness, and lyophillized; yield 49.8 mg. (57%), $[\alpha]_D^{17}$ −59.8 (c=0.52, H_2O) Rf^1 0.31, Rf^2 0.51, single ninhydrin and Sakaguchi positive spot, amino acid ratios in acid hydrolysate: Arg 1.9, Pro 3.0, β-Ala 1.0, Phe 2.1, Ser 0.8. Acetyl ester group was 73.0% of theory.

L-Arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Triacetate (XI)——The nonapeptide (X) (49.8 mg.) was dissolved in H_2O (1 ml.) and saponified with 1*N* NaOH (0.6 ml.) for 1 hr. at room temperature. The solution was neutralized with 1*N* AcOH and added to a (2.0×6.0 cm.) CM-cellulose column which was eluted as described above. The eluates in tubes No. 33 to 43 were pooled, evaporated to dryness in vacuum, and lyophillized to constant weight; yield 29.8 mg. (62%), $[\alpha]_D^{19}$ −80.1° (c=0.9, H_2O), Rf^1 0.32, Rf^2 0.62, single ninhydrin and Sakaguchi positive spot, amino acid ratios in acid hydrolysate: Arg 2.0, Pro 2.0, β-Ala 1.0, Phe 2.1, Ser 0.9.

N-Benzyloxycarbonyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (XII)——The protected pentapeptide ester (III) (398 mg.) was dissolved in AcOH (0.8 ml.) and 4.3*N* HBr in AcOH (0.8 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was centrifuged, and reprecipitated from MeOH and dry ether, and then dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (5 ml.) N-benzyloxycarbonyl-β-alanine *p*-nitrophenyl ester (155 mg.) 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 (1 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4CH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and concentrated to small volume. Petroleum ether was added to the residue and the precipitate thereby formed was reprecipitated from acetone and Et_2O; yield 256 mg. (62%), m.p. 95~110°, $[\alpha]_D^{20}$ −12.9° (c=0.4, AcOH), *Anal.* Calcd. for $C_{50}H_{55}O_{13}N_{11}$: C, 58.75; H, 5.82; N, 15.08. Found. C, 57.74; H, 5.64; N, 14.96. Deblocked peptide ester: Rf^1 0.76, Rf^2 0.93, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (XIII)——The protected hexapeptide ester (XII) (256 mg.) was dissolved in AcOH (0.8 ml.) and 4.3*N* HBr in AcOH (0.8 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide (5 ml.) N-benzyloxycarbonyl-L-pronine *p*-nitrophenyl ester (94 mg.) 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 (1 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed succesively with 1*N* NH_4OH, H_2O 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and concentrated to small volume. Petroleum ether was added to the residue and the precipitate was reprecipitated from acetone and ether; yield 256 mg. (67%), m.p. 106~124°, $[\alpha]_D^{22}$ −87.7° (c=0.5, AcOH), *Anal.* Calcd. for $C_{55}H_{66}O_{14}N_{12}$: C, 59.02; H, 5.94; N, 15.02. Found: C, 59.10; H, 6.28; N, 14.50. Deblocked peptide ester: Rf^1 0.76, Rf^2 0.90, single ninhydrin positive spot.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester Trihydrate (XIV)——The protected heptapeptide ester (XIII) (158 mg.) was dissolved in AcOH (0.8 ml.) and 4.5*N* HBr in AcOH (0.8 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH pellets in vacuum. To a solution of this product in dimethylformamide

(5 ml.) N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (98 mg.) was added, followed by Et_3N to keep the solution slightly alkaline and after 1 day, added more the N-protected dipeptide *p*-nitrophenyl ester (30 mg.). After 2 days at room temperature, the reaction mixture was diluted with 1*N* NH_4OH (2 ml.), stirred for 1 hr., and then diluted with EtOAc containing MeOH. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc containing MeOH solution was dried over $MgSO_4$ and concentrated to small volume. Petroleum ether was added to the residue, the precipitate thereby formed was reprecipitated from hot MeOH and ether; yield 129 mg. (65%), m.p. 135~138°, $[\alpha]_D^{22}$ −64.2° (c=0.7, AcOH), *Anal.* Calcd. for $C_{66}H_{84}O_{18}N_{18}\cdot 3\,H_2O$: C, 53.87; H, 6.17; N, 17.13. Found : C, 54.00; H, 6.40; N, 17.15. Deblocked peptide ester : Rf^1 0.77, Rf^2 0.92, single ninhydrin positive spot.

L-Arginyl-L-prolyl-L-prolyl-β-alanyl-L-phenylalanyl-β-alanyl-L-prolyl-L-phenylalanyl-L-arginine Triacetate (XV)——The fully protected nonapeptide (XIV) (94 mg.) was hydrogenated in 10:5 mixture of AcOH and H_2O (15 ml.) in the presence of 10% Pd-C for 40 hr. The catalyst was removed by filtration with the aid of Cellite. The solution was evaporated to dryness in vacuum and the residue was dried over KOH pellets in vacuum. The solution of the hydrogenated product in H_2O (10 ml.) was added to a (2.0×6.0 cm.) CM-cellulose column which was eluted with a linear gragient elution from H_2O (300 ml.) in a mixing chamber to 0.15*M* pyrdinium acetate buffer solution (pH 5.10) (300 ml.) in a reservoir. Fractions 13 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collector. Arginine-containing peptide was located in the eluate by Sakaguchi reaction. The eluates in tubes No. 26 to 38 containing the nonapeptide were pooled, evaporated to dryness, and lyophilized to constant weight; yield 61 mg. (72%), $[\alpha]_D^{22}$ −81.6 (c=0.6, H_2O), Rf^1 0.45, Rf^2 0.48, single ninhydrin and Sakaguchi positive spot, amino acid ratios in acid hydrolysate : Arg 2.0, Pro 3.0, β-Ala 2.0, Phe 2.1.

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Summary

The synthesis of 6-β-alanine, 4-β-alanine, and 4,6-di-β-alanine-bradykinin is described. The biological activity of three analogs were compared with that of bradykinin.

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141. Shoji Shibata, Isao Kitagawa,*1 and Haruhiro Fujimoto : The Chemical Studies on Oriental Plant Drugs. XV. On the Constituents of *Bupleurum* Spp. (2).*2 The Structure of Saikogenin A, a Sapogenin of *Bupleurum falcatum* L.*3

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As reported in the previous paper,[1] the root of *Bupleurum falcatum* L. (Mishima-saiko) (Umbelliferae) which is currently used as an important drug (Saiko : Chai-hu) in Chinese medicine contains a considerable amount of saponins,*5 whose thin-layer

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*2 Part XIV (1) : S. Shibata, I. Kitagawa, R. Takahashi, H. Fujimoto : Yakugaku Zasshi, in press.
*3 Preliminary report of this paper appeared in Tetrahedron Letters, No. 42, 3783 (1965).
*4 Hongo, Tokyo (柴田承二, 北川 勲, 藤本治宏).
*5 S. Ezawa reported[2] the existence of saponin in the *Bupleurum* root.
1) S. Shibata, I. Kitagawa, R. Takahashi, H. Fujimoto : Yakugaku Zassai, in press.