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Isobornyloxycarbonyl Function, a New Convenient Amino-Protecting Group in Peptide Synthesis. II.[1] Synthesis of Bradykinin[2]

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Bradykinin, a nonapeptide local tissue hormone, was synthesized by the use of the isobornyloxycarbonyl (IBOC-)-protecting group for the temporary protection of the all amino groups of the intermediary peptides. During the synthesis of bradykinin, no complication occurred in the processes involving the introduction and removal of the IBOC-group, and the final synthetic hormone showed up to possess the full biological activity and appeared to be pure by chemical criterions.

In the previous communication,[1] we have reported the preparation and properties of N-isobornyloxycarbonyl (IBOC-)-amino acids, and have suggested that the IBOC-group should be of great use in the synthesis of complicated peptides as an amino-masking group.

As an example, we now wish to report the synthesis of bradykinin by the use of the (*d*-) IBOC-protecting group for the temporary protection of the amino-group of the intermediary peptides.

The plan for the synthesis of bradykinin is outlined in Fig. 1.

To remove, at the final stage, the side-chain and *c*-terminal protecting groups by catalytic hydrogenolysis, the guanidino-group of arginine was protected by formation of the nitro-derivative[4] and the *c*-terminal carboxy group was converted to the *p*-nitrobenzyl ester. The IBOC-group which was employed for the temporary protection of all the N^{α}-amino group of the intermediates was removed at each stage by treating the blocked peptides with trifluoroacetic acid at room temperature for 30 min.

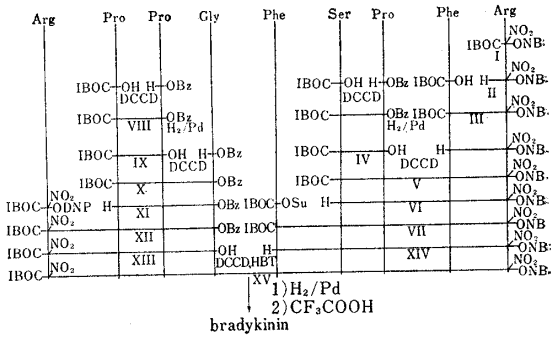

Fig. 1. Synthesis of Bradykinin

–ODNP=2,4-dinitrophenyl ester, –OBz=benzyl ester, –OSu=N-hydroxysuccinimide ester, –ONB=*p*-nitrobenzyl ester, NO_2=nitro, DCCD=N, N′-dicyclohexylcarbodi-imide, HBT=hydroxybenztriazole

IBOC-nitroarginine was treated with *p*-nitrobenzyl bromide in the presence of triethylamine to obtain the IBOC-nitroarginine *p*-nitrobenzyl ester (I) in 93% yield. The free base of this ester (II) was obtained by treatment of (I) with trifluoroacetic acid followed by neutralization with triethylamine and was then coupled with IBOC-phenylalanine *via* the corresponding N-hydroxysuccinimide ester[5] to yield IBOC-phenylalanylnitroarginine *p*-nitrobenzyl ester (III) in 76% yield. This dipeptide ester was treated with trifluoroacetic acid and the resulting free base was acylated with IBOC-seryl-proline (IV), which was prepared from IBOC-serine and proline benzyl ester

1) Part I: M. Fujino, S. Shinagawa, O. Nishimura and T. Fukuda, *Chem. Pharm. Bull.* (Tokyo), **20**, 1017(1972).
2) The amino acids, peptides and their derivatives (except glycine) mentioned in this paper are of the L-configuration.
3) Location: *Juso-Nishino-cho, Higashiyodogawa-ku, Osaka.*
4) M. Bergmann, L. Zervas and H. Rinke, *Z. Physiol. Chem.*, **224**, 40 (1934).
5) G.W. Anderson, F.M. Callahan and J.E. Zimmerman, *J. Am. Chem. Soc.*, **89**, 178 (1967).

by the N,N'-dicyclohexylcarbodi-imide (DCC) procedure[6] followed by catalytic hydrogenation, to yield IBOC-seryl-prolyl-phenylalanyl-nitroarginine *p*-nitrobenzyl ester (V) as an amorphous powder in 76% yield. The free base of this tetrapeptide ester (VI) was again obtained by treatment with trifluoroacetic acid followed by neutralization with triethylamine, and was coupled with IBOC-phenylalanine *via* the corresponding N-hydroxysuccinimide ester to give IBOC-phenylalanyl-seryl-prolyl-phenylalanyl-nitroarginine *p*-nitrobenzyl ester (VII) in 70% yield.

The synthesis of the other intermediary protected peptide, IBOC-nitroarginyl-prolyl-prolyl-glycine (XIII), involved first the reaction of IBOC-proline N-hydroxysuccinimide ester with proline benzyl ester followed by catalytic hydrogenation to produce IBOC-prolyl-proline (IX) in a crystalline form and the free acid of the dipeptide was coupled with glycine benzyl ester by the DCC method to form IBOC-prolyl-prolyl-glycine benzyl ester (X) in crystalline in 70% yield. The free base of the tripeptide ester (XI) was obtained by treating X with trifluoroacetic acid, which was then reacted with IBOC-nitroarginine, *via* the 2,4-dinitrophenyl ester,[7] to give IBOC-nitroarginyl-prolyl-prolyl-glycine benzyl ester (XII) in 97% yield.

This IBOC-tetrapeptide ester was saponified by the usual manner in dioxane–water to yield the free acid of IBOC-tetrapeptide (XIII).

The *c*-terminal pentapeptide ester (VII) was treated with trifluoroacetic acid and the resulting free base (XIV) was then treated with the N-terminal tetrapeptide derivative (XIII), *via* the N-hydroxybenztriazole method,[8] to form the fully protected bradykinin, IBOC-nitroarginyl-prolyl-prolyl-glycyl-phenylalanyl-seryl-prolyl-phenylalanyl-nitroarginine *p*-nitrobenzyl ester (XV), as a fine powder in 76% yield.

After removing the nitro group, as well as the *p*-nitrobenzyl group of the fully protected peptide (XV) by catalytic hydrogenation, the IBOC-group of N-terminal amino position was removed by treating with trifluoroacetic acid for 1 hr to give the trifluoroacetate of bradykinin.

The resulting trifluoroacetate was exchanged to the corresponding acetate by the treatment with Amberlite IRA-400 (acetate from). Column chromatography on carboxymethylcellulose using a gradient elution with ammonium acetate buffer was employed for the final purification of the hormone.

The peptide isolated from the column was homogeneous and identical with authentic bradykinin[9] in paper chromatography and paper electrophoresis. It was also pure as judged by amino acid and elemental analyses, and moreover the product showed the full biological activity in assay[10] on guinea pig ileum when compared with the authentic standard.[9]

As mentioned above, during the synthesis of bradykinin, no complication occurred and the final product had the full activity. Due to an easy accessibility of IBOC-amino acids and IBOC-chloride and the good solubility in organic solvents of IBOC-peptide derivatives, it is concluded that the IBOC-protecting method is useful for the synthesis of complicated peptidies.

Experimental

All melting points were taken by the capillary method and are uncorrected. Evaporations were all carried out with a rotary evaporator. The purity of products was tested by thin layer chromatography. Solvent systems used were: $CHCl_3$–MeOH–AcOH (9:1:0.5, Rf^1), AcOEt–pyridine–AcOH–H_2O (60:20:6:10, Rf^2), *n*-BuOH–AcOH–H_2O (4:1:1, Rf^3), *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24, Rf^4).

6) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
7) M. Bodanszky and M.A. Ondetti, *Chem. Ind.*, **1966**, 26.
8) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
9) Obtained from Peptide Center, Institute for Protein Research, Osaka University.
10) We thank Dr. M. Kanno and his staff of this Division for performing the assay.

***d*-Isobornyloxycarbonyl-N^G-nitro-arginine *p*-Nitrobenzyl Ester (I)**——To a solution of *d*-isobornyloxycarbonyl-N^G-nitroarginine (10.0 g, 25 mmole) and *p*-nitrobenzyl bromide (7.56 g, 35 mmole) in dimethylformamide (40 ml) was added triethylamine (4.9 ml). After standing the reaction mixture for 2 hr at 80°, water (200 ml) was added with cooling. The formed oil was extracted with AcOEt (100 ml×2), and the AcOEt was washed with water and dried over anhydr. Na_2SO_4. The dried solution was evaporated *in vacuo* and the residue was reprecipitated from AcOEt-pet. ether to give the pure ester; 12.5 g (93.3%), mp 115—116°. $[\alpha]_D^{25}$ −34.2° (c=1.0 in EtOH). Rf^1=0.67. *Anal.* Calcd. for $C_{24}H_{34}O_8N_6$: C, 53.92; H, 6.41; N, 15.72. Found: C, 54.00; H, 6.71; N, 14.93.

***d*-Isobornyloxycarbonyl-phenylalanyl-N^G-nitroarginine *p*-Nitrobenzyl Ester (II)**——*d*-Isobornyloxycarbonyl-N^G-nitro-arginine *p*-nitrobenzyl ester (10.6 g, 20 mmole) was treated fo 30 min with trifluoroacetic acid (50 ml) at room temperature. The excess of acid was evaporated off. The residue (Rf^2=0.62) was triturated with ether and dissolved in dioxane (50 ml). N-hydroxysuccinimide ester of *d*-isobornyloxycarbonylphenylalanine (8.84 g, 20 mmole: oil, prepared from acylamino acid and N-hydroxysuccinimide by the N,N′-dicyclohexylcarbodiimide method[5]) and triethylamine (2.8 ml) were added to the dioxane solution. After standing for 12 hr, the solution was evaporated, AcOEt (200 ml) was added. The AcOEt solution was washed with 0.2N HCl and 4% $NaHCO_3$, dried, and evaporated. The residue was solidified under pet. ether to give the pure product; 10.2 g (75.7%), mp 111—115°. $[\alpha]_D^{28}$ −21.0° (c=1.0 in EtOH). Rf^1=0.55. *Anal.* Calcd. for $C_{33}H_{53}O_9N_7$: C, 58.14; H, 6.36; N, 14.38. Found: C, 57.82; H, 6.34; N, 14.17.

***d*-Isobornyloxycarbonyl-seryl-proline (IV)**——Dicyclohexylammonium solt of *d*-isobornyloxycarbonyl-serine (5.6 g, 12 mmole) was dissolve in dether (200 ml), and the solution was washed with 0.2N H_2SO_4 (150-ml×3) and water, dried (anhydr. Na_2SO_4), and evaporated. The residue was dissolved in acetonitrile (200 ml), the solution was cooled to 0°, and triethylamine (1.68 ml) and N,N′-dicyclohexylcarbodiimide (27 g) was added. The mixture was stirred overnight at 0—5°, filtered, and evaporated to dryness.

The residue was dissolved in EtOAc (200 ml), which was washed (0.2N HCl and 4% $NaHCO_3$), dried, and evaporated. The residue (oil, Rf^1=0.69) was dissolved in MeOH (100 ml), and hydrogenated for 4 hr over palladium-black. The mixture was filtered and evaporated. The residue was crystallized from AcOEt-pet. ether to give the acid which was recrystallized from hot ether; 2.2 g (48%), mp 161—162°. $[\alpha]_D^{31}$ −91.1° (c=0.5 in MeOH). Rf^1=0.59. *Anal.* Calcd. for $C_{19}H_{30}O_6N_2$: C, 59.67; H, 7.91; N, 7.33. Found: C, 60.02; H, 8.26; N, 7.78.

***d*-Isobornyloxycarbonyl-seryl-prolyl-phenylalanyl-N^G-nitroarginine *p*-Nitrobenzyl Ester (V)**——*d*-Isobornyloxycarbonyl-phenylalanyl-N^G-nitro-arginine *p*-nitrobenzyl ester (3.0 g, 4.4 mmole) was treated with trifluoroacetic acid (20 ml) for 30 min at room temperature. To this was added a solution of HCl in AcOH (4N, 4 ml) and the mixture was poured into ether with vigorous stirring under cooling. The fine white solid which formed was collected by filtration, washed with ether and dried *in vacuo*, to give the dipeptide ester hydrochloride (yield, quantitative).

The hydrochloride (1.9 g, 3.54 mmole) and triethylamine (0.84 ml) were dissolved in acetonitrile (20 ml) with cooling, and after addition of *d*-isobornyloxycarbonyl-seryl-proline (1.45 g, 3.8 mmole) and N,N′-dicyclohexylcarbodi-imide (824 mg), the mixture was stirred overnight at 0°, filtered, and evaporated to dryness. The residue was dissolved in AcOEt (100 ml) and washed with 0.5N HCl and 4% $NaHCO_3$, dried (Na_2SO_4), and evaporated. The residue was triturated with pet. ether and the crude solid material dissolved in AcOEt was reprecipitated with pet. ether to give the pure product; 2.50 g (76.0%), mp 130—134°. $[\alpha]_D^{31}$ −64.4° (c=0.5 in MeOH). Rf^1=0.58. *Anal.* Calcd. for $C_{41}H_{55}O_{12}N_9 \cdot H_2O$: C, 55.71; H, 6.50; N, 14.29. Found: C, 55.70; H, 6.47; N, 14.50.

***d*-Isobornyloxycarbonyl-phenylalanyl-seryl-prolyl-phenylalanyl-N^G-nitro-arginine *p*-Nitrobenzyl Ester (VII)**——*d*-Isobornyloxycarbonyl-seryl-prolyl-phenylalanyl-N^G-nitro-arginine *p*-nitrobenzyl ester (2.0 g, 2.3 mmole) was treated with trifluoroacetic acid (20 ml) for 30 min at room temperature. The excess of acid was evapora ted off and the residue was triturated with ether to give a fine white solid which was collected by filtration, and dried *in vacuo* over NaOH-pellet. The dried powder (Rf^2=0.73) was dissolved in dimethylformamide (20 ml), and cooled to 0°. To this solution was added triethylamine (0.56 ml) and *d*-isobornyloxycarbonylphenylalanine N-hydroxysuccinimide ester (1.62 g), and the mixture was stirred for 15 hr at room temperature. The reaction mixture was diluted with AcOEt (100 ml), and washed with 0.2N HCl and 4% $NaHCO_3$, dried, and evaporated. The residue was triturated with pet. ether to give a solid which was collected by filtration, and purified by reprecipitation from AcOEt–ether to give the pure product; 1.80 g (78%), mp 133—139° (decomp.), $[\alpha]_D^{28}$ −54.8° (c=1.0 in MeOH). Rf^1=0.69. *Anal.* Calcd. for $C_{50}H_{64}O_{13}N_{10}$: C, 58.24; H, 6.45; N, 13.58. Found: C, 57.99; H, 6.39; N, 13.32.

***d*-Isobornyloxycarbonyl-prolyl-proline (IX)**——To a solution of proline benzyl ester hydrochloride (9.67 g, 40 mmole) and triethylamine (5.6 ml) in ice-cold acetonitrile (50 ml) was added *d*-isobornyloxycarbonyl-proline (11.8 g, 40 mmole) and N,N′-dicyclohexylcarbodiimide (9.06 g), and stirred at 0° for 14 hr. The formed dicyclohexylurea was filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in AcOEt (200 ml), washed (0.2N HCl and 4% $NaHCO_3$), dried (Na_2SO_4), and evaporated to give an oily substance (20 g, Rf^1=0.60). The oil was dissolved in MeOH (150 ml) and hydrogenated by the usual manner for 4 hr over palladium-black. The solution was evaporated to dryness *in vacuo*, and the residue was triturated with pet. ether to give crystals. The crystals were collected by filtration and recrystal-

lized from AcOEt–EtOH to give fine needles; 11.2 g (71.3%), mp 182—183°. $[\alpha]_D^{24}$ −154.3° (c=1.0 in MeOH). Rf^1=0.53. *Anal.* Calcd. for $C_{21}H_{32}O_5N_2$: C, 64.26; H, 8.22; N, 7.14. Found: C, 64.34; H, 8.32; N, 7.08.

***d*-Isobornyloxycarbonyl-prolyl-prolyl-glycine Benzyl Ester (X)**——To a solution of glycine benzyl ester *p*-toluenesulfonate (8.43 g, 25 mmole), triethylamine (3.5 ml) and *d*-isobornyloxycarbonyl-prolyl-proline (9.8 g, 25 mmole) in acetonitrile (40 mle) was added N,N′-dicyclohexylcarbodiimide (5.2 g), and the solution was stirred for 15 hr at 5°. The formed dicyclohexylurea was filtered off, and the filtrate was evaporated *in vacuo*, and the residue was dissolved in AcOEt (150 ml), washed with 0.2N HCl and 1N NH_4OH, dried (Na_2SO_4), and evaporated to dryness to give an oil. The oil was triturated with pet. ether to give crystals which were collected by filtration and recrystallized from AcOEt–pet. ether; 9.1 g (68%), mp 125—126°. $[\alpha]_D^{26}$ −152.3° (c=1.0 in EtOH). Rf^1=0.59. *Anal.* Calcd. for $C_{30}H_{41}O_6N_3$: C, 66.77; H, 7.66; N, 7.79. Found: C, 66.56; H, 7.57; N, 7.93.

***d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-prolyl-glycine Benzyl Ester (XII)**——*d*-Isobornyloxycarbonyl-prolyl-prolyl-glycine benzyl ester (3.8 g, 7 mmole) was treated with trifluoroacetic acid (30 ml) for 30 min at room temperature, and to this solution, 1N HCl in AcOH (8 ml) was added, and the mixture was poured into vigorously stirred ether with cooling. The resulting fine white solid was collected by filtration, and dried *in vacuo*, to give the tetrapeptide ester hydrochloride (yield, quantitative, Rf^2=0.62).

The hydrochloride and triethylamine (1.0 ml) were dissolved in dioxane (10 ml) with cooling, and to this *d*-isobornyloxycarbonyl-N^G-nitro-arginine 2,4-dinitrophenyl ester (acylamino acid 2.8 g was esterified by the N,N′-dicyclohexylcarbodiimide method[7]) were added, and the mixture was stirred for 5 hr at room temperature. The reaction mixture was filtered, and the filtrate was evaporated to dryness. The residue solidified by treatment with pet. ether, was reprecipitated from AcOEt–ether to give the pure product; 5.0 g (96.5%), mp 115—118°. $[\alpha]_D^{27}$ −109.9° (c=1.0 in MeOH). Rf^1=0.57. *Anal.* Calcd. for $C_{36}H_{52}O_9N_8\cdot H_2O$: C, 56.98; H, 7.17; N, 14.77. Found: C, 56.90; H, 7.19; N, 14.57.

***d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-prolyl-prolyl-glycine (XIII)**——*d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-prolyl-prolyl-glycine benzyl ester (2.1 g, 3 mmole) was dissolved in dioxane (10 ml), 1N NaOH (3.3 ml) was added in dropwise with cooling, and the solution was stirred for 1.5 hr at room temperature. After the solution was neutralized with 1N HCl (4 ml) with cooling, the bulk of the dioxane was removed *in vacuo*, and the formed oil was extracted with $CHCl_3$ (50 ml×2), washed with water, dried (Na_2SO_4), and evaporated *in vacuo*. The residue solidified after trituration with ether, and was collected by filtration. The solid was then purified by reprecipitation from AcOEt–pet. ether to give the pure acid; 1.50 g (76.7%), mp 160—164° (decomp.). $[\alpha]_D^{28}$ −116.5° (c=1.0 in MeOH). Rf^1=0.40. *Anal.* Calcd. for $C_{29}H_{46}O_9N_8\cdot H_2O$: C, 52.09; H, 7.23; N, 16.76. Found: C, 52.20; H, 7.21; N, 16.67.

***d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-prolyl-prolyl-glycyl-phenylalanyl-seryl-prolyl-phenylalanyl-N^G-nitro-arginine p-Nitrobenzyl Ester (XV)**——*d*-Isobornyloxycarbonyl-phenyalanyl-seryl-prolyl-prolyl-phenylalanyl-N^G-nitro-arginine *p*-nitrobenzyl ester (1.01 g, 1 mmole) was treated with trifluoroacetic acid (10 ml) for 30 min at room temperature. The solution was poured into vigorously stirred ether containing 0.5 ml of 4N HCl in AcOH. The fine solid was collected by filtration, washed with ether, and dried *in vacuo* to give the pentapeptide ester hydrochloride. The hydrochloride was dissolved in dimethylformamide (10 ml) and neutralized with triethylamine (0.15 ml).

To this solution were added *d*-isobornyloxycarbonyl-N^G-nitro-arginyl-prolyl-prolyl-glycine (626 mg, 1 mmole), hydroxybenztriazole (366 mg, 2 mmole) and N,N′-dicyclohexylcarbodiimide (247 mg, 1.2 mmole) at 0°. The mixture was kept at 0—5° for 25 hr and poured into AcOEt (100 ml), and the formed dicyclohexylurea was filtered off. The filtrate was washed with 0.2N-HCl and 4% $NaHCO_3$, dried (Na_2SO_4), and evaporated to a small volume. Addition of ether caused the precipitation of the product, which was collected by filtration and purified by chromatography on a silica gel column (50 g) ($CHCl_3$–MeOH, 9:1) to give the pure protected nonapeptide ester; 792 mg (55%), mp 125—131° (decomp.). $[\alpha]_D^{28}$ −77.0° (c=0.5 in MeOH). Rf^1=0.45. *Anal.* Calcd. for $C_{66}H_{92}O_{19}N_{18}\cdot 2H_2O$: C, 54.39; H, 6.44; N, 16.79. Found: C, 54.60; H, 6.18; N, 16.41.

Arginyl-prolyl-prolyl-glycyl-phenylalanyl-seryl-prolyl-phenylalanyl-arginine (Bradykinin)——A solution of the nonapeptide ester (432 mg, 0.3 mmole) in MeOH (50 ml) and AcOH (1.0 ml) was hydrogenated for 20 hr over palladium-black. The mixture was filtered and the solvent was evaporated. The residue was triturated with ether to give N^a-isobornyloxycarbonyl-bradykinin (hield, 368 mg, 90%: Rf^2=0.22, Rf^3=0.28, Rf^4=0.60). The product (334 mg, 0.245 mmole) was treated with trifluoroacetic acid (4 ml) for 1 hr at room temperature. The excess of acid was evaporated off *in vacuo* and the residue was dissolved in 2% AcOH (20 ml). The solution was passed through a column of Amberlite CG-400 (AcOH, 30 ml), and the column was washed well with 2% AcOH. The eluate and washings were combined and lyophilized to give 259 mg (84%) of bradykinin as acetate form. The product was then subjected to chromatography on a carboxymethylcellulose column (2×35 cm) and washed with 0.01M ammonium acetate (300 ml) and then eluted by a gradient elution method (0.01M/0.3M=700 ml/700 ml). The homogeneous bradykinin was eluted in 300—360 ml fractions. The fractions were combined and lyophilized: 161 mg, $[\alpha]_D^{22}$ −83.4° (c=0.46 in H_2O) (lit.[11] $[\alpha]_D^{13}$ −80.6° (c=0.28 in H_2O)). *Anal.* Calcd. for $C_{50}H_{73}O_{11}N_{15}\cdot 3CH_3COOH\cdot 3H_2O$:

11) S. Sakakibara, N. Nakamizo, Y. Kishida and S. Yoshimura, *Bull. Chem. Soc. Japan*, **41**, 1477 (1968).

C, 51.98; H, 7.09; N, 16.24. Found: C, 52.11; H, 6.88; N, 16.18. Amino acid analysis: Arg 1.93 (2), Ser 0.97 (1), Pro 3.00 (3), Gly 1.00 (1), Phe 2.00 (2) (average recovery 99.5% as triacetate-trihydrate).

The compound synthesized behaved exactly like authentic bradykinin[9] when compared by paper chromatography ($Rf^4=0.57$), paper electrophoresis ($R_{arg}=0.98$, pH 1.9, 500 V, 60 min), and the bioassay (guinea pig ileum contraction).

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