SYNTHESIS OF BRADYKININ*

L. A. Shchukina, G. A. Ravdel, M. P. Filatova, E. P. Semkin,
and S. N. Krasnova

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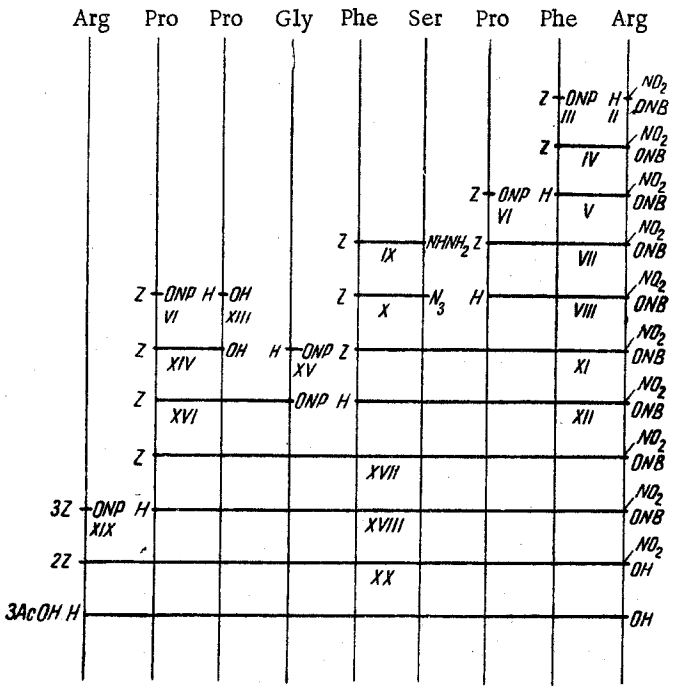
Investigations of the last few years have shown to a greater and greater extent the participation of substances of polypeptide nature in the mechanism of the regulation of vascular reactions in the organism. One of these substances, the polypeptide bradykinin (I), was first found in 1949 by Rocha-y-Silva et al., [1] by the action of trypsin or snake venom on the α_2-globulin fraction of the blood.

In 1960, Elliot et al., [2, 3] isolated bradykinin in the pure state and established its structure. Later, the structure of this peptide was refined by Boissonas et al., [4–6]:

*L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg***. (I)

The intensity and diversity of the biological activity of bradykinin*** has drawn the attention of investigators to it, their endeavors having been directed both to the synthesis of bradykinin itself and to an elucidation of the structural specificity of its molecules [11–14]. For this purpose, some groups of investigators have synthesized a considerable number of analogs of bradykinin and have studied their biological activity [15]. There is also a mass of literature data on the physiological activity of bradykinin [16] and its role in some pathological states (inflammation, shock, burns) [17, 18]. Recently, enzyme systems participating in the formation and degradation of bradykinin in the organism have been investigated [19, 20].

This paper describes a new synthesis of bradykinin ensuring high yields and optical purity both of the intermediate peptides and of the final bradykinin triacetate.



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** The symbols for the amino acids and their derivatives are in accordance with the resolution of the 5th European Peptide Symposium in Oxford (September 1962) [7]; in addition, TEA represents triethylamine and DMF dimethylformamide.

*** Bradykinin acts in doses $1 \times 10^{-7} - 1 \times 10^{-11}$ g/kg and is a powerful hypotensive agent, causing an increase in the permeability of the capillary vessels and a contraction of the smooth musculature; it takes part in the accumulation and migration of leucocytes [8–10].

As can be seen from the scheme, the starting material is p-nitrobenzyl ester of nitro-L-arginine (II), from which, by the p-nitrophenyl ester method, we obtained successively the dipeptide (IV) and the tripeptide (VII). The decarbobenzoxylation of the peptides (IV) and (VII) was effected by means of hydrogen bromide in glacial acetic acid.

Condensation of the tripeptide (VIII) with the azide of carbobenzoxy-L-phenylalanyl-L-serine (X) gave the pentapeptide (XI); by the action of hydrogen bromide in trifluoroacetic acid, the latter was converted into the pentapeptide (XII).

Biological Tests of Bradykinin

Pharmacological test	Threshold doses	
	Bradykinin synthesized	Data for a typical sample from Sandoz, Inc.
Contraction of isolated rat uterus, g/ml	$1\cdot10^{-10}$—$1\cdot10^{-11}$	$1\cdot10^{-9}$—$1\cdot10^{-11}$
Weakening action on an isolated section of rat duodenum, g/ml	$5\cdot10^{-9}$—$1\cdot10^{-10}$	$5\cdot10^{-9}$—$1\cdot10^{-10}$
Reduction of the blood pressure of the rat, g/kg weight	$2.5\cdot10^{-7}$	$4\cdot10^{-7}$
Increase in the permeability of the capillaries of rabbit skin, g/ml	$5\cdot10^{-9}$—$1\cdot10^{-10}$	$1\cdot10^{-9}$ (For guinea pig skin)

Note: The biological tests were carried out in the laboratory of biologically active peptides of the Institute of Biological and Medicinal Chemistry, AMS USSR by M. S. Surovikina and T. K. Egorova under the direction of Doctor of Biological Sciences T. S. Pashkina.

To lengthen the peptide chain further, we used the p-nitrophenyl ester of carbobenzoxy-L-prolyl-L-prolylglycine (XVI). This tripeptide, which contains an activated carboxy group, was synthesized with a high yield (86%) by condensing carbobenzoxy-L-prolyl-L-proline (XIV) with the p-nitrophenyl ester of glycine (XV) by means of sec-isobutyl chlorocarbonate.

The octapeptide obtained by the condensation of the tripeptide (XVI) with the pentapeptide (XII), after the elimination of the carbobenzoxy group with hydrogen bromide in trifluoroacetic acid, was condensed with the p-nitrophenyl ester of tricarbobenzoxy-L-arginine (XIX). Without special purification, the condensation product was subjected to alkaline hydrolysis, giving a nonapeptide in the form of the dicarbobenzoxy-substituted acid (XX). Hydrogenolysis of the acid (XX) over Pd black in a mixture of methanol and acetic acid gave the triacetate of bradykinin (I), the chemical and biological properties of which completely corresponded to those described in the literature (table).

Experimental

The purity of all the substances was checked by chromatography on Leningrad type "B" paper in the systems butan-1-ol—water—acetic acid (4:5:1) (R_{f_1}) and isoamyl alcohol-pyridine-water (35:35:30) (R_{f_2}). To reveal the spots, ninhydrin, potassium iodide-chlorine, and Sakaguchi's reagent were used.

The individuality of the substances was also checked by thin-layer chromatography on unsupported sliica gel; the methanol-ethyl acetate-benzene (2:3:5) system was used for the carbobenzoxy derivatives and methanol for compounds with a free amino group. Detection was carried out with iodine or ninhydrin.

The solutions, previously dried with sodium sulfate, were evaporated under vacuum. The substances for analysis were also dried under vacuum (0.1-0.5 mm) at a temperature of ~ 60° C.

p-Nitrophenyl ester of carbobenzoxy-L-phenylalanine (III). In drops at 0° C, 0.63 ml of $POCl_3$ was added over 10 min to a solution of 2 g of carbobenzoxy-L-phenylalanine [21] and 0.93 of p-nitrophenol in 10 ml of pyridine. The reaction mixture was kept for 15 min at 0° C and for 5 min at 20° C (on more prolonged standing it began to darken), after which it was poured into water containing ice. The precipitate which was deposited was washed with water, with 1 N hydrochloric acid, and again with water, and was dried in air and crystallized from alcohol. This gave 2.1 g (78%) of (III) with mp 126°-127° C, $[\alpha]_D^{20}$ −24.0° (c 2; DMF) (cf. [22]).

p-Nitrobenzyl ester of carbobenzoxy-L-phenylalanylnitro-L-arginine (IV). A solution of 1 g of the hydrobromide of the p-nitrobenzyl ester of nitro-L-arginine (II) [5] in 20 ml of DMF was treated at 0° C with 0.42 ml of TEA, and after careful stirring 0.96 g of the p-nitrophenyl ester of carbobenzoxy-L-phenylalanine (III) was added. The reaction mixture was kept for four days at 18°–20° C, the solvent was distilled off at 40°–45° C, and the dry residue was repeatedly washed with ether and was then dissolved in ethyl acetate. The solution was washed with water, with 1 N NH_4OH until the yellow color had disappeared, with 1 N hydrochloric acid, and again with water. The solution with the precipitate which had been deposited while it was being washed with hydrochloric acid was left at 5° C for 12 hr. The jelly-like precipitate that was deposited was filtered off. This gave 1.2 g (81%) of the dipeptide (IV), mp 169°–170° C (decomp.). After two crystallizations from ethyl acetate, the substance had mp 171°–173° C (decomp.); $[\alpha]_D^{20}$ −20° C (c 1; DMF) and −13° (c 1; methyl alcohol) (cf. [5]).

Found, %: C 56.36; H 5.49; N 14.85. Calculated for $C_{30}H_{33}O_9H_7$, %: C 56.69; H 5.21; N 15.43.

Hydrobromide of the p-nitrobenzyl ester of L-phenylalanylnitro-L-arginine (V). A suspension of 7 g of substance (IV) in 35 ml of glacial acetic acid was treated with 35 ml of a 36% solution of hydrogen bromide in glacial acetic acid [23]. The substance gradually dissolved, and after 20 min the solvent was distilled off. The residue was triturated with ether. A light yellow substance was obtained which was washed repeatedly with absolute ether and was dried in a vacuum desiccator over caustic potash. This yielded 6.8 g (94%) of the hydrobromide of the dipeptide (V) containing two moles of hydrogen bromide, mp 172°–174° C (decomp.); $[\alpha]_D^{21}$ −5° (c 1; DMF) (cf. [5]), R_{f_1} 0.83, R_{f_2} 0.86.

Found, %: Br 24.13. Calculated for $C_{22}H_{27}O_7N_7 \cdot 2HBr$, %: Br 24.44.

p-Nitrophenyl ester of carbobenzoxy-L-proline (VI). In drops at 0° C, 0.74 ml of $POCl_3$ was added to a solution of 2 g of carbobenzoxy-L-proline [21] and 1.2 g of p-nitrophenol in 15 ml of pyridine. The reaction mixture was left for 10 min at 0° C and for 1 hr at room temperature, after which it was poured into water containing ice. The precipitate which was deposited was filtered off, washed with water, with 1 N hydrochloric acid, and with water again, and was dried and crystallized from alcohol. This gave 2.45 g (82%) of substance (VI), mp 93°C; $[\alpha]_D^{23}$ −67.0° (c 2; DMF) (cf. [22]).

p-Nitrobenzyl ester of carbobenzoxy-L-prolyl-L-phenylalanylnitro-L-arginine (VII). A solution of 2 g of substance (V) in 15 ml of DMF was treated at 0° C with 0.87 ml of TEA and then at 20° C with 1.2 g of the p-nitrophenyl ester of carbobenzoxy-L-proline (VI). The reaction mixture was left for five days at 20° C. The solvent was distilled off, and the residue was dissolved in 100 ml of ethyl acetate and washed with water, with 1 N NH_4OH, with 1 N hydrochloric acid, and again with water. Crystals of the tripeptide (VII) deposited from the solution dried with sodium sulfate. Yield 1.9 g (86%); mp 95° C; $[\alpha]_D^{21}$ −47.5° (c 1.6; MeOH) (cf. [5]).

Found, %: C 57.37; H 5.69; N 15.25. Calculated for $C_{35}H_{40}O_{10}N_8$, %: C 57.37; H 5.53; N 15.29.

p-Nitrobenzyl ester of carbobenzoxy-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine (XI). A suspension of 2 g of substance (VII) in 10 ml of glacial acetic acid was treated with 10 ml of a 36% solution of hydrogen bromide in glacial acetic acid. After 40 min, the solution was evaporated to dryness and the residue was triturated with ether; the hydrobromide of the tripeptide (VIII) so obtained was washed with a large amount of ether. Then the substance was dissolved in a mixture of 30 ml of ethyl acetate and 16 ml of acetonitrile and, at 0° C, 14 ml of a saturated aqueous solution of potassium carbonate was added to this solution. The aqueous layer was separated off and washed twice with a mixture of ethyl acetate and acetonitrile. The combined organic fractions were dried for an hour, and evaporated to dryness to give 1.44 g (92%) of the tripeptide (VIII), R_{f_2} 0.87.

One gram of the hydrazide of carbobenzoxy-L-phenylalanyl-L-serine (IX) [5] was dissolved in a mixture containing 5 ml of glacial acetic acid, 5 ml of 2 N hydrochloric acid, and 16 ml of ethyl acetate; the solution was cooled to −5° C and, with vigorous stirring 0.22 g of $NaNO_2$ in 1 ml of water was added to it in drops. Stirring was continued for a further 10 min at −5° to 10° C, then 20 ml of water was added and the ethyl acetate layer was separated off. The aqueous layer was twice extracted with ethyl acetate and the extracts were combined with the main solution.

The ethyl acetate solution containing the azide (X) was washed with water, with a saturated solution of potassium carbonate, and with water again, and then it was dried (the washing and drying were carried out at 4°–5° C) and filtered into a solution of 1.4 g of substance (VIII) in 15 ml of DMF cooled to 0° C. The reaction mixture was left for 2 days at 5° C and then for 2 hr at room temperature, after which it was washed with water and with 1 N hydrochloric acid.

The monohydrate of the pentapeptide (XI)* deposited as a precipitate over 2 days (5° C). Yield 1.62 g (70%),

*This peptide was previously obtained by Boissonas et al., [5] under anhydrous conditions and with a different melting point.

mp 175°–177° C; $[\alpha]_D^{19}$ −35° (c 1; DMF); R_{f_1} 0.89; R_{f_2} 0.92.

Found, %: C 57.24; H 5.82; N 14.44; H_2O 2.29. Calculated for $C_{47}H_{54}O_{13}N_{10} \cdot H_2O$, %: C 57.30; H 5.72; N 14.21; H_2O 1.83.

Carbobenzoxy-L-prolyl-L-proline (XIV). With heating to 50°–55° C, 7.5 g of the p-nitrophenyl ester of carbobenzoxyl-L-proline (VI), 2.3 g of L-proline (XIII), and 2.9 ml of TEA were dissolved in 30 ml of methanol. The solution was kept at this temperature for 3 hr and at 18°–20° C for 4 days. The solvent was distilled off under vacuum and the residue was treated with 15 ml of water and was repeatedly extracted with ethyl acetate. The colored aqueous solution was decolorized by the addition of a few drops of 0.5 N hydrochloric acid and was extracted with ether and acidified with 5% hydrochloric acid to congo red. The precipitate which was deposited was filtered off, washed with cold water, and dried. This gave 5.46 g (79%) of the dipeptide (XIV), mp 185.5° −186.5° C; $[\alpha]_D^{20}$ −77° (c 1; DMF) (cf. [24]).

p-Nitrophenyl ester of carbobenzoxy-L-prolyl-L-prolylglycine (XVI). A solution of 3.46 g of substance (XIV) and 1.74 ml of TEA in 35 ml of dry $CHCl_3$ was cooled to −5° C, 1.7 g of sec-isobutyl chlorocarbonate [25] was added in drops, and the mixture was stirred for 20 min; then 2.9 g of the hydrobromide of the p-nitrophenyl ester of glycine (XV) [26] was added and, with vigorous stirring, in drops, 1.4 ml of TEA. Stirring was continued for another 3 hr at 5° C and for 1 hr at room temperature. The solution was washed with 2% hydrochloric acid and with water and was evaporated, the residue was dissolved in 50 ml of ethyl acetate, and the solution was left at 5° C for several days. This gave 4.47 g (86%) of the tripeptide (XVI), mp 143°–145° C (from ethyl acetate); $[\alpha]_D^{21}$ −87.5° (c 1; DMF).

Found, %: C 59.47; H 5.33; N 10.91. Calculated for $C_{26}H_{28}O_8N_4$, %: C 59.53; H 5.39; N 10.97.

p-Nitrobenzyl ester of carbobenzoxy-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginine (XVII). A solution of 1.8 g of substance (XI) in 15 ml of trifluoroacetic acid was saturated for an hour at 0° C with hydrogen bromide. The acid was distilled off and the residual oily substance was triturated with absolute ether until a deposit formed, which was repeatedly washed with ether. This gave 1.6 g (97%) of the hydrobromide of the pentapeptide (XII) containing 1.5 mole of hydrogen bromide. R_{f_1} 0.87; R_{f_2} 0.91.

Found, %: Br 12.91. Calculated for $C_{39}H_{48}O_{11}N_{10} \cdot 1.5\,HBr$, %: Br 12.58.

A solution in 15 ml of DMF of 1.5 g of the hydrobromide obtained was treated at 0° C with 0.33 ml of TEA and then with 0.87 g of the p-nitrophenyl ester of carbobenzoxy-L-prolyl-L-prolylglycine (XVI). The reaction mixture was left for 5 days at 20° C and then 100 ml of ethyl acetate was added and it was washed with water, with 1 N NH_4OH, and with 1 N hydrochloric acid, and dried, and the octapeptide (XVII) was precipitated with ether. Yield 1.6 g (83%); mp 120°–125° C; $[\alpha]_D^{17}$ −43° (c 1; DMF), R_{f_1} 0.83.

Found, %: C 57.12; H 5.83; N 14.72; H_2O 1.7. Calculated for $C_{59}H_{71}O_{16}N_{13} \cdot H_2O$, %: C 57.32; H 5.95; N 14.73; H_2O 1.62.

Hydrobromide of the p-nitrobenzyl ester of L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine (XVIII). A solution of 0.8 g of substance (XVII) in 10 ml of trifluoroacetic acid was cooled to 0° C and hydrogen bromide was passed for an hour through the solution maintained at this temperature. The solution was evaporated and the residual oily substance was triturated with ether. This gave 0.72 g (95%) of the hydrobromide of the octapeptide (XVII) in the form of an amorphous powder; R_{f_1} 0.82.

Found, %: Br 7.03. Calculated for $C_{51}H_{65}O_{14}N_{13} \cdot HBr$, %: Br 6.87.

Dicarbobenzoxy-L-arginyl-L-prolyl-L-phenylalanylnitro-L-arginine (XX). A solution of 0.74 g of the hydrobromide of compound (XVIII) in 5 ml of DMF was treated at 0° C with 0.095 ml of TEA, and then 0.45 g of the p-nitrophenyl ester of tricarbobenzoxy-L-arginine (XIX) [27] was added at room temperature. The solution was kept for 5 days at 20° C and then 30 ml of ethyl acetate was added and it was washed with water, with 1 N NH_4OH, with 1 N hydrochloric acid, and again with water. The ethyl acetate solution was dried and was evaporated to dryness. The dry residue was suspended in 3 ml of methanol, and 0.47 ml of 2 N NaOH was added. After 15 min, 0.5 ml of water was added to the solution, and after another 15 min 6 ml of water and 0.5 ml of 2 N hydrochloric acid were added. The oily substance liberated was separated off, dissolved in 0.6 ml of methanol, and treated with 10 ml of 4% $NaHCO_3$. The solution was extracted with ether and made acid to congo red with 5 N hydrochloric acid. The oil obtained solidified in the cold. After repeated trituration with water, the substance was dried in a vacuum desiccator over P_2O_5. The yield of the nonapeptide (XX) was 0.44 g (50%), mp 201°–203° C, $[\alpha]_D^{20}$ −52.5° (c 1; DMF) (cf. [11]), R_{f_1} 0.73, R_{f_2} 0.67.

Triacetate of L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-phenylalanyl-L-arginine (bradykinin) (I). 0.13 g of the nonapeptide (XX) was hydrogenated in a mixture of 10 ml of glacial acetic acid and 5 ml of methanol over Pd black at 25° C. The catalyst was filtered off, the filtrate was evaporated, and the residue was dissolved in 10 ml of water and was lyophilized. The yield of bradykinin triacetate (I) was 0.11 g (85%), mp 150°–160° C,

$[\alpha]_D^{20}$ −83.1° (c 1.1; water) (cf. [11]), R_{f_1} 0.31, R_{f_2} 0.46, R_f 0.35 (Whatman No. 1; butan-1-ol-acetic acid-water (63 : 10 : 27) system (cf. [28]).

Summary

A new method for the synthesis of bradykinin giving high yields in all stages has been developed. Biological tests of the compounds synthesized have shown that it is identical with the natural hormone.

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Institute of the Chemistry of Natural Compounds, AS USSR