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Studies on the Synthesis of Polypeptides. Bradykinin

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The synthesis of the nonapeptide, L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine is described. The reactions leading to the nonapeptide consisted of either the *p*-nitrophenyl ester method of peptide synthesis or the classical azide reaction. The nonapeptide has been found to be identical with natural trypsin bradykinin.

Bradykinin was first isolated[1] from a plasma globulin by the action of snake venom or trypsin and was described as a hypotensive and smooth muscle stimulating factor. More recent work[2] on the isolation and purification of this substance resulted in a proposal[3] for the structure of bradykinin as a straight chain octapeptide, L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-phenylalanyl-L-arginine.

Following the synthesis of the octapeptide[4-6] and its lack of the expected biological activity, the structure was revised[7] to that of a nonapeptide, L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine.

In the present report, we wish to describe the synthesis of this nonapeptide.[8]

The method used for the synthesis of the nonapeptide closely parallels that used in our synthesis of the octapeptide[4] since this method has given in our hands crystalline intermediates of high purity and in good yields. The fact that the octapeptide and the nonapeptide were obtained chromatographically and analytically pure without the necessity of using prolonged and laborious countercurrent distribution methods is of prime importance in evaluating the synthetic method. Extensive use has been made of the *p*-nitrophenyl ester[9] procedure for peptide synthesis, and we have found it to be an excellent method for the synthesis of moderate size peptides in most cases. In a few instances, however, this method has been found to be lacking due to poor yields of the *p*-nitrophenyl esters and difficulties encountered in their purification. This necessitated in one case of resorting to another method of forming a peptide bond. The *p*-nitrophenyl esters of carbobenzoxy-L-serine and carbobenzoxynitro-L-arginine[10] were obtained in low yields and of dubious purity for the purpose for which they were envisioned. Better methods were found in the use of carbobenzoxy-L-serine azide and the *p*-nitrophenyl ester of tricarbobenzoxy-L-arginine,[11] the latter substance being obtained as an analytically pure crystalline compound. The remainder of the *p*-nitrophenyl esters were prepared as suggested by Elliott and Russell[12] and were isolated as pure, crystalline compounds before they were used.

The dipeptide, L-phenylalanylnitro-L-arginine methyl ester (I), when allowed to react with carbobenzoxy-L-proline *p*-nitrophenyl ester was converted in good yield to the crystalline carbobenzoxy-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (II). Removal of the carbobenzoxy group from the tripeptide was accomplished with anhydrous hydrogen bromide in glacial acetic acid and the resulting product was condensed with carbobenzoxy-L-serine azide at 5° yielding the tetrapeptide III. The tetrapeptide, after removal of its protecting carbobenzoxy group, was transformed to the pentapeptide IV on treatment with the *p*-nitrophenyl ester of carbobenzoxy-L-phenylalanine. A second and equally successful approach to the synthesis of the pentapeptide was found in the reaction of carbobenzoxy-L-phenylalanyl-L-serine azide with L-prolyl-L-phenylalanylnitro-L-arginine methyl ester. The pentapeptides obtained by the two different procedures at first did not appear to be identical. The melting points disagreed by some 60° and the rotations by 15°. Both compounds, however, gave the correct analysis. The proof that the two pentapeptides were the same substance was obtained when they were separately converted to the hexapeptide, carbobenzoxyglycyl-L-phenylalanyl-L-

(1) M. Rocha E. Silva, W. T. Beraldo, and G. Rosenfeld, *Am. J. Physiol.*, **156**, 261 (1949).

(2) D. F. Elliott, G. P. Lewis, and E. W. Horton, *Biochem. J.*, **74**, 15 p. (1960).

(3) D. F. Elliott, G. P. Lewis, and E. W. Horton, *Biochem. J.*, **76**, 16p (1960).

(4) E. D. Nicolaides, H. A. De Wald, P. G. Shorley, and H. O. J. Collier, *Nature*, **187**, 773 (1960).

(5) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud, H. Konzett, and E. Strümer, *Experientia*, **16**, 326 (1960).

(6) R. Schwyzer, W. Rittel, P. Sieber, H. Kappeler, and H. Zuber, *Helv. Chim. Acta*, **43**, 1130 (1960).

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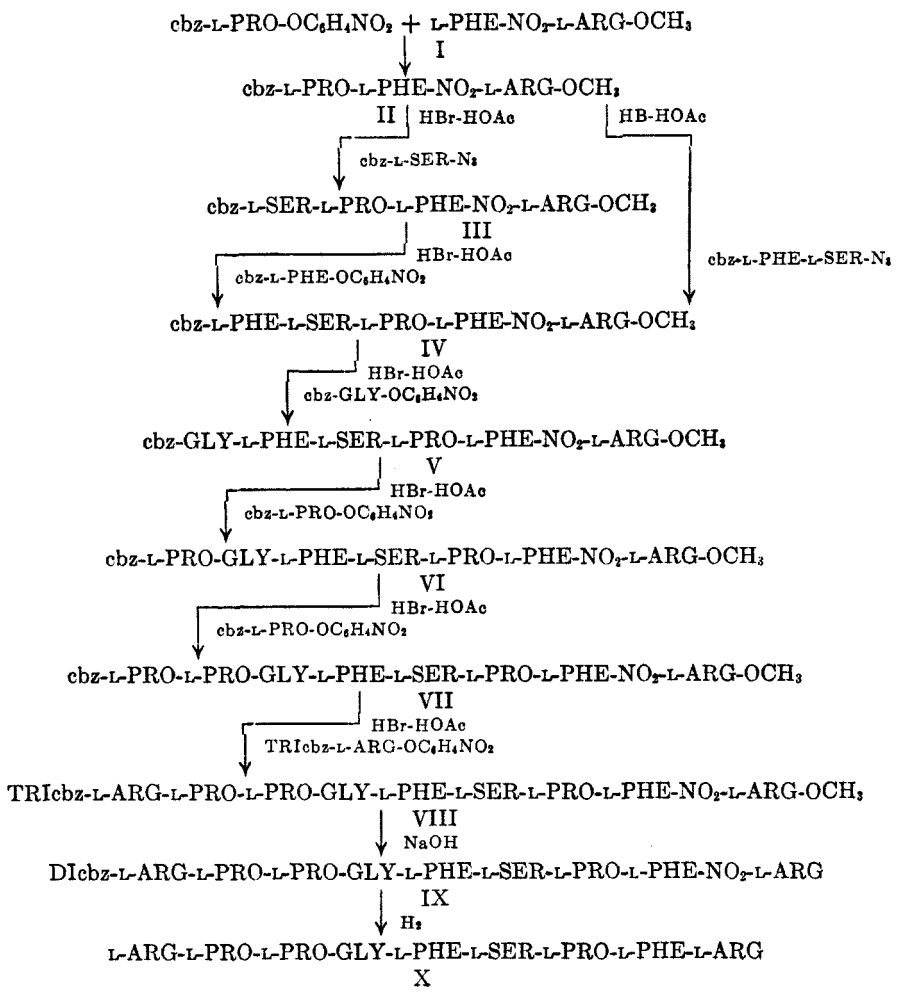
(8) A synthesis of the nonapeptide has been reported. R. A. Boissonnas, St. Guttmann, and P.-A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).

(9) M. Bodanszky, *Nature*, **175**, 685 (1955).

(10) In a recent report, M. Bodanszky and J. T. Sheehan, *Chemistry & Industry*, **41**, 1268 (1960), the authors found that the attempted preparation of the *p*-nitrophenyl ester of carbobenzoxynitro-L-arginine led to the formation of the lactam. A similar type of lactam formation has also been reported for N^{α},N^{ϵ}-dicarbobenzoxy-L-arginine; L. Zervas, T. T. Otani, M. Winitz, and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959).

(11) L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

(12) D. F. Elliott and D. W. Russell, *Biochem. J.*, **66**, 49 (1957).



seryl - L - prolyl - L - phenylalanylnitro - L - arginine methyl ester (V). Both hexapeptides were identical. The next two proline residues were added stepwise to the peptide chain by condensation of carbobenzoxy-L-proline *p*-nitrophenyl ester with the decarbobenzoxylated hexapeptide and then with the decarbobenzoxylated heptapeptide. A progressive lowering of the melting point was observed as these last two amino acids were attached to the chain, but the products were still readily recrystallized. The attachment of the last amino acid, arginine, to form the fully protected nonapeptide (VIII) appeared, at first, to be a problem of some magnitude since it was felt that the use of a carbodiimide or a mixed anhydride reaction on a large molecule such as the octapeptide would not be likely to give as pure a product as was desired. The use of the *p*-nitrophenyl ester of tricarbobenzoxy-L-arginine has been found by us to be a practical procedure for the introduction of this amino acid into the *N*-terminal position of a peptide chain even though the yield of tricarbobenzoxy-L-arginine is very low and its preparation somewhat difficult. The fully protected nonapeptide, tricarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl - L - prolyl - L - phenylalanylnitro - L - arginine methyl ester (VIII) was obtained in good yield, but possessed a somewhat low, broad melting point. Repeated recrystallization did not improve its melting range. On one occasion the loss of one of the carbobenzoxy groups was noted during the work-up of the reaction mixture and the dicarbobenzoxy nonapeptide methyl ester was obtained. Since the loss of this group occurred so readily it was intentionally removed from the tricarbobenzoxy nonapeptide along with the methyl ester by using two equivalents of base. The product obtained, dicarbobenzoxy - L - arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine (IX), crystallized as needles from methanol. Complete removal of the remaining protecting groups was accomplished by hydrogenation over palladium black catalyst in acetic acid–methanol solution. The final product, L-arginyl-L-prolyl-L-prolylglycyl-L- phenylalanyl - L - seryl - L - prolyl - L-phenylalanyl-L-arginine (X) was isolated as the triacetate salt by lyophilization from an aqueous solution. The nonapeptide on paper chromatography and paper electrophoresis showed a single spot. Direct comparison of the nonapeptide with natural trypsin bradykinin showed the two compounds to be identical.[18,19]

EXPERIMENTAL[13]

Carbobenzoxy-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (II). To a cold (5°) solution of 46 g. (0.1 mole) of L-phenylalanylnitro-L-arginine methyl ester hydrobromide[4] in 150 ml. of dimethylformamide was added 13 g. (0.13 mole) of triethylamine. The triethylamine hydrobromide which precipitated was removed by filtration and to the filtrate was added 37 g. (0.1 mole) of carbobenzoxy-L-prolyl *p*-nitrophenyl ester. The resulting yellow solution was allowed to stand for 4 days at room temperature and was then evaporated to 75 ml. Ether was added giving a yellow gum which was washed several times with ether, dissolved in 300 ml. of hot ethyl acetate, filtered, and the solution washed several times with dilute ammonium hydroxide solution to remove *p*-nitrophenol. The solution was washed with dilute hydrochloric acid, three times with water and dried over anhydrous magnesium sulfate. The solution was evaporated *in vacuo* to 150 ml. and ether added, giving a faint yellow solid which was twice recrystallized from ethyl acetate–ether, yield of white solid, 53 g. (87%) m.p. 137–139°, $[\alpha]_D^{23}$ − 43.3° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{29}H_{27}N_7O_8$: C, 56.94; H, 6.10; N, 16.03. Found: C, 56.58; H, 6.11; N, 16.27.

L-Prolyl-L-phenylalanylnitro-L-arginine hydrobromide. Into a cool (10°) solution of 60 g. (0.1 mole) of carbobenzoxy-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester dissolved in 200 ml. of glacial acetic acid was bubbled in 50 g. (0.62 mole) of hydrogen bromide gas. The temperature was kept below 20° during the addition of the hydrogen bromide. The solution was kept for 2 hr. at room temperature with occasional swirling. The solution was rapidly poured into 2 l. of vigorously stirred ether. The white precipitate which formed was allowed to settle, the supernate decanted and the solid washed several times with ether–ethyl acetate (4:1), collected on a glass-sintered funnel, washed copiously with ether and dried overnight in a vacuum desiccator. The yield of product, 64 g., was considerably more than theory (56 g.), and the excess weight was attributed to incomplete removal of hydrogen bromide. Only after prolonged drying, 1–2 weeks, did the yield approach that of the theoretical. The excess hydrogen bromide did not adversely affect the subsequent reaction and was normally taken care of by using an excess of triethylamine before reaction with a *p*-nitrophenyl ester or an acid azide.

Carbobenzoxy-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (III). A solution of 12 g. (0.05 mole) of carbobenzoxy-L-serine hydrazide[14] in 130 ml. of water, 15 ml. of glacial acetic acid, and 4 ml. of concd. hydrochloric acid was cooled to −1° and a solution of 3.5 g. (0.05 mole) of sodium nitrite in 10 ml. of water was added dropwise with stirring keeping the temperature at 0°. After the addition of the nitrite was completed, 400 ml. of cold (−5°) ethyl acetate was added, the organic layer was separated and quickly washed with cold, saturated sodium bicarbonate solution until the wash water was basic. The ethyl acetate solution was dried at 0°, filtered and to this solution was added a solution of L-prolyl-L-phenylalanylnitro-L-arginine methyl ester which had been prepared previously by dissolving 20.9 g. of the crude tripeptide hydrobromide in 100 ml. of dimethylformamide, cooling to 4°, treating with an excess of triethylamine and filtering to remove triethylamine hydrobromide. The reaction mixture was kept at 4° overnight, then at 25° for 2 hr. The solution was washed with water, dilute aqueous potassium carbonate solution, water, dilute hydrochloric acid, saturated sodium chloride solution, dried and evaporated to 200 ml. Colorless needles separated and the mixture was cooled, the needles removed and washed with cold ethyl acetate, yield, 13.2 g. (64%), m.p. 123–125°, $[\alpha]_D^{23}$ − 42.4° (*c* 1, dimethylformamide). An analytical sample from methanol melted at 128–130°.

Anal. Calcd. for $C_{32}H_{42}N_8O_{10}$: C, 55.00; H, 6.06; N, 16.04. Found: C, 55.12; H, 6.24; N, 16.20.

Carbobenzoxy-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (IV). Carbobenzoxy-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (9.1 g., 0.013 mole) was dissolved in 200 ml. of glacial acetic acid containing 15 g. of dry hydrogen bromide. The solution was kept 1 hr. at room temperature and poured into 700 ml. of cold, dry ether. The precipitate was collected by filtration, washed well with ether and dried *in vacuo* over potassium hydroxide pellets. The product weighed 10.4 g. The solid was dissolved in 60 ml. of dimethylformamide, cooled to 4° and 5.5 ml. of triethylamine was added. The precipitate was removed and to the filtrate was added 5.5 g. (0.013 mole) of carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester. The solution was allowed to remain at room temperature for 24 hr. and was then diluted with 250 ml. of ethyl acetate. The solution was washed with water, aqueous sodium carbonate and saturated sodium chloride solution. A finely divided solid separated which was removed, washed with water and dried, 8.6 g. (78%), m.p. 210–213°. The product was quite insoluble in most organic solvents except dimethylformamide. The solid was washed with boiling methanol yielding 8.4 g. of white solid, m.p. 216–218°, $[\alpha]_D^{23}$ − 57° (*c* 1, dimethylformamide). An analytical sample from dimethylformamide water and dimethylformamide-ethyl acetate melted at 218–220°.

Anal. Calcd. for $C_{41}H_{51}N_9O_{11}$: C, 58.21; H, 6.08; N, 14.90. Found: C, 58.05; H, 6.06; N, 14.83.

Carbobenzoxy-L-phenylalanyl-L-serine methyl ester. To a stirred, cold (−10°) solution of 30 g. (0.1 mole) of carbobenzoxy-L-phenylalanine in 300 ml. of methylene dichloride was added 10 g. (0.1 mole) of triethylamine followed by 10.8 g. (0.1 mole) of ethyl chloroformate. The solution was stirred at −10° for 25 min. and a cold (0°) mixture of 15.6 g. (0.1 mole) of L-serine methyl ester hydrochloride and 10 g. of triethylamine in 100 ml. of methylene chloride was added. The reaction mixture was stirred at 0° for 2 hr. and overnight at room temperature. The solution was washed with water, aqueous sodium bicarbonate, water, dilute hydrochloric acid, water, and dried. The solvent was removed leaving a colorless residue which was recrystallized from ethyl acetate–petroleum ether, yield, 38 g. (95%), m.p. 122–123°, $[\alpha]_D^{23}$ − 5.7° (*c* 2, dimethylformamide), (reported,[8] m.p. 125°, $[\alpha]_D^{21}$ −5.7° (*c* 1, dimethylformamide).

Carbobenzoxy-L-phenylalanyl-L-serine hydrazide. This compound was prepared by treating the above dipeptide methyl ester with a slight excess of anhydrous hydrazine in methanol solution. The product was obtained as white needles, 36 g. (97%), m.p. 187–188°, $[\alpha]_D^{23}$ −6.4° (*c* 2, dimethylformamide), (reported,[8] m.p. 193°, $[\alpha]_D^{21}$ −2.6° (*c* 1, dimethylformamide).

Synthesis of the carbobenzoxy pentapeptide via an acid azide. To a cold (5°) solution of 40 g. (0.1 mole) of carbobenzoxy-L-phenylalanyl-L-serine hydrazide in 200 ml. of glacial acetic acid and 60 ml. of 2*N* hydrochloric acid was added in portions a solution of 7.5 g. (0.11 mole) of sodium nitrite in 15 ml. of water. The solution was swirled for 5 min. and diluted with 700 ml. of ice water. The gum which precipitated was extracted with ice cold ethyl acetate (500 ml.) and the ethyl acetate solution was quickly washed with ice water three times, with cold, saturated aqueous sodium carbonate solution until the wash water was basic and dried over magnesium sulfate at 0°. To a previously prepared solution of 56 g. (0.1 mole) of L-prolyl-L-phenylalanylnitro-L-arginine methyl ester hydrobromide in 150 ml. of dimethylformamide at −5° was added 15 g. of triethylamine. The mixture was filtered and the filtrate was added to the ethyl acetate solution containing the dipeptide azide. The reaction was kept 2 days at 4° and 4 hr. at 25°. The solution was washed with water, dilute aqueous sodium bicarbonate solution, water, dilute hydrochloric acid, dried and evaporated to a small volume. Ether was added giving a gummy solid which was

(13) Melting points were taken using a Thomas-Hoover capillary melting point apparatus and are uncorrected.

(14) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

recrystallized from ethyl acetate–ether, methanol-ether as a white solid, yield, 48 g. (58%), m.p. 152–153°, $[\alpha]_D^{23}$ −42.3° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{41}H_{51}N_9O_{11}$: C, 58.21; H, 6.08; N, 14.90. Found: C, 58.08; H, 5.83; N, 14.97.

Carbobenzoxyglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (V). Into a suspension of 48 g. (0.058 mole) of the carbobenzoxy pentapeptide methyl ester (IV) in 250 ml. of glacial acetic acid at 10° was bubbled in 45 g. (0.55 mole) of hydrogen bromide gas. The solid dissolved and the solution was kept for 2.5 hr. at room temperature and then poured into 1.5 l. of vigorously stirred dry ether. The white precipitate was removed by filtration, washed thoroughly with dry ether and dried for 2 days *in vacuo* giving 63 g. (theory = 45 g.) of white solid. The crude product was dissolved in 200 ml. of dimethylformamide, cooled to 0°, and 26 g. of triethylamine added. The mixture was filtered and 20 g. (0.061 mole) of carbobenzoxy glycine *p*-nitrophenyl ester was added to the filtrate. The yellow solution was kept for 2 days at room temperature, evaporated to 100 ml., and diluted with 300 ml. of ethyl acetate. The ethyl acetate solution was washed with water and saturated sodium carbonate solution. During the carbonate washing, a precipitate began to form so the organic layer was separated, evaporated to half volume and an equal volume of ether added. The yellow precipitate was removed and washed with dilute ammonium hydroxide until colorless, then with water and ether and dried, weight, 45 g. The product was twice recrystallized from dimethylformamide-ether, yield, 40 g. (76%), m.p. 218–220°, $[\alpha]_D^{23}$ − 52.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{43}H_{54}N_{10}O_{12}$: C, 57.20; H, 6.03; N, 15.52. Found: C, 57.43; H, 6.11; N, 15.00.

Carbobenzoxy-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (VI). The carbobenzoxy hexapeptide methyl ester (V), 33 g. (0.037 mole), was dissolved in 200 ml. of glacial acetic acid containing 25 g. (0.31 mole) of dry hydrogen bromide gas. The solution was kept at room temperature for 2 hr. and poured into 1 l. of dry ether. The precipitate was removed, washed thoroughly with ether, and dried *in vacuo*. The yield of crude product was 39 g. The solid was dissolved in 150 ml. of dimethylformamide, cooled to 0°, and 15 g. of triethylamine was added. The precipitate was removed and to the filtrate was added 15 g. (0.040 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester. The ensuing solution was kept for 2 days at room temperature, evaporated to about 100 ml. and diluted with 500 ml. of ethyl acetate. The solution was washed with water twice and once with aqueous saturated sodium chloride. The ethyl acetate solution was concentrated to half volume. Ether was added and the white solid removed and recrystallized from methanol–ethyl acetate, yield, 30 g. (81%) m.p. 196–197°, $[\alpha]_D^{23}$ −65.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{48}H_{61}N_{11}O_{13}$: C, 57.65; H, 6.15; N, 15.41. Found: C, 57.34; H, 6.10; N, 15.24.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (VII). Into a cold (10°) solution of 29 g. (0.029 mole) of the carbobenzoxy heptapeptide methyl ester (VI) in 150 ml. of glacial acetic acid was bubbled in 29 g. (0.36 mole) of dry hydrogen bromide gas. The solution was kept at room temperature for 1.5 hr. with occasional swirling and was then poured into 1 l. of vigorously stirred ether. The precipitate was removed by filtration, quickly washed with a large amount of dry ether and dried *in vacuo*, crude yield, 36 g. (theory = 27 g.). The product was dissolved in 100 ml. of dimethylformamide, the solution cooled to 0°, and 15 g. of triethylamine added. The mixture was filtered after 5 min. and 13 g. (0.035 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester was added to the filtrate. The yellow solution was allowed to stand for 5 days at room temperature and was then evaporated to a small volume. Ether was added to the yellow solution precipitating an oil which was repeatedly washed with ether and was then twice recrystallized from methanol–ethyl acetate giving a white solid, 25 g. (75%), m.p. 150–152°, $[\alpha]_D^{23}$ −64.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{53}H_{68}N_{12}O_{14}$: C, 58.01; H, 6.25; N, 15.32. Found: C, 57.66; H, 6.13; N, 14.98.

Tricarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester (VIII). Twenty-five grams (0.0236 mole) of the carbobenzoxy octapeptide methyl ester (VII) was treated with 20 g. (0.025 mole) of dry hydrogen bromide gas in 150 ml. of glacial acetic acid for 2.5 hr. at 25°. The solution was poured into ether, the solid removed, washed well with ether and dried *in vacuo* for 2 days giving 30.5 g. of white solid. The crude hydrobromide salt was dissolved in 100 ml. of dimethylformamide, the solution cooled to 0° and 10 g. of triethylamine added. The mixture was filtered after 5 min. and to the clear filtrate was added 19 g. (0.027 mole) of tricarbobenzoxy-L-arginine *p*-nitrophenyl ester.[4] The solution was allowed to stand for 24 hr. at room temperature and was then evaporated to a small volume. Ether was added giving an oil which solidified after repeated washing with ether. The solid was digested for 10 min. with 150 ml. of hot ethyl acetate, the mixture cooled and the solid removed and dried, yield, 35 g. (97%), m.p. 125–145°, $[\alpha]_D^{23}$ −53.5° (*c* 1, dimethylformamide). An analytical sample from acetonitrile–ethyl acetate melted at 135–150°.

Anal. Calcd. for $C_{75}H_{92}N_{16}O_{19}$: C, 59.20; H, 6.10; N, 14.73. Found: C, 58.92; H, 6.14; N, 14.66.

In a similar reaction on a 0.0025-mole size the reaction mixture was concentrated, diluted with ethyl acetate, and the solution was washed with water containing triethylamine, aqueous potassium carbonate, and saturated sodium chloride. The solution was dried and evaporated giving a yellow solid which was recrystallized from methanol as a white solid, 0.6 g., m.p. 156–158°. The product analyzed for dicarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester with one mole of water.

Anal. Calcd. for $C_{67}H_{86}N_{16}O_{17}\cdot H_2O$: C, 57.25; H, 6.31; N, 15.95. Found: C, 57.30; H, 6.21; N, 16.18.

Dicarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine (IX). To a solution of 35 g. (0.023 mole) of the tricarbobenzoxy nonapeptide methyl ester (VIII) dissolved in 150 ml. of methanol at room temperature was added in one portion 25 ml. of 2*N* sodium hydroxide (0.050 mole). The solution was swirled occasionally and after 15 min. 25 ml. of water was added. The solution became cloudy and was allowed to stand for another 15 min. More water was added (50 ml.) and the solution remained clear. The solution was diluted with 300 ml. of water and 26 ml. of 2*N* hydrochloric acid was added. A white gum formed which solidified on cooling. The solid was removed, washed with water, air dried, and recrystallized twice from hot methanol. The product formed small needles in rosettes, yield, 20 g., m.p. 204–205°, $[\alpha]_D^{23}$ −52.6° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{66}H_{84}N_{16}O_{17}\cdot H_2O$: C, 56.96; H, 6.23; N, 16.10. Found: C, 56.90; H, 6.16; N, 16.27.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine triacetate salt (X). One gram (7.3×10^{-4} mole) of dicarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine (IX) was dissolved in 40 ml. of glacial acetic acid, 600 mg. of palladium black catalyst was added and 20 ml. of methanol. The resulting mixture was hydrogenated for 48 hr. at room temperature and 2–3 lb. pressure. The catalyst was removed by filtration and was washed with 10 ml. of glacial acetic acid. The filtrate was evaporated to dryness *in vacuo*, the residue was dissolved in 30 ml. of water, the solution was filtered, shell frozen, and lyophilized leaving 850 mg. of colorless solid. The product melted at 150–160° $[\alpha]_D^{23}$ −82.8° (*c* 1.1, water).

Anal. Calcd. for $C_{56}H_{85}N_{15}O_{17}\cdot 3\ H_2O$: C, 51.96; H, 7.09; N, 16.24; H_2O, 4.17. Found: C, 52.33; H, 6.94; N, 16.38; H_2O,

3.94. A sample of the product was dried at 110° *in vacuo* for 12 hr.

Anal. Calcd. for $C_{56}H_{85}N_{15}O_{17}$: C, 54.22; H, 6.91; N, 16.94. Found: C, 54.05; H, 7.16; N, 17.36.

For the paper chromatography of the synthetic bradykinin, five different solvent systems were employed: *t*-butyl alcohol:acetic acid:water (2:1:1); benzene: *n*-butyl alcohol: pyridine:water (1:5:3:3); *n*-butyl alcohol saturated with 3% ammonium hydroxide; *n*-butyl alcohol: 2.5% phenol; 2% piperidine-water saturated; isopropyl alcohol: concd. ammonium hydroxide:water (70:5:25). The corresponding R_f values obtained were 0.64; 0.1; 0.175; 0.108; 0.44. The spots were developed with ninhydrin, Bromphenol Blue, and Sakaguchi reagents and in all cases only single spots were observed. Paper electrophoresis of the final product was carried out in acetate buffer, pH = 5.6, using a constant current of 30 milliamp. for 3 hr. The product migrated as a single component toward the cathode a distance of 6.7 cm. from the origin.

High voltage electrophoresis[16] of bradykinin gave a well defined single spot with Sakaguchi reagent. The peptide migrated 11 to 12 cm. toward the cathode during 60 min. at a voltage of 43 v. per cm. using a pyridine acetate buffer, pH 3.5, with Varsol as a paper coolant.

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The synthetic nonapeptide was assayed on the guinea-pig ileum for its bradykinin activity and was found to possess the full potency of natural trypsin bradykinin.[17]

A previous sample of synthetic nonapeptide which appeared less pure by paper chromatography and paper electrophoresis possessed bradykinin activity,[18,19] but was found to be about 75% as active as the pure synthetic material described above.

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[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES, A DIVISION OF AMERICAN CYANAMID CO.]

Preparation of 6-Methoxycorticoids

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The synthesis of 6α- and 6β-methoxycortisone, -hydrocortisone, -prednisolone, and 6α-methoxy-9α-fluoroprednisolone is described.

The report[1] that a methoxy function has been found on metabolites of known active steroid hormones, prompts one to synthesize this type of compound in the corticoid field. Corticoids with a methoxyl group at positions C-9[2] and C-16[3] of the steroid molecule have been described. It is the purpose of this report to detail the synthesis of C-6 methoxylated corticoids.[4]

Treatment of a suspension of 21-acetoxy-5ξ,6ξ-epoxy-3-ethylenedioxy-17α-hydroxy-pregnene-11,-20-dione (I)[5] (mixture of α- and β-epoxides) in methanol with 70% perchloric acid for twenty-two hours yielded 6β-methoxycortisone acetate (IIa) after reacetylation. This structure was supported by an analysis for a methoxyl function and by the ultraviolet absorption spectrum, which showed a peak at 230 mμ. It is most probable that part of I which existed as the 5α,6α-epoxide was opened under the acidic conditions in methanol to an intermediate 5α-hydroxy-6β-methoxy-3-one, which was further dehydrated to the 6β-methoxy-Δ⁴-3-one (IIa). The 21-acetate IIa was then easily saponified to 6β-methoxycortisone (IIb). The hypsochromic effect (8 mμ) and the lowering of the molecular extinction coefficient by about 3000 in the ultraviolet spectra of IIa or IIb when compared to the spectra of cortisone acetate or cortisone strongly suggests the 6β-methoxyl configuration in IIa and IIb. It is, of course, well known that a 6β-hydroxy or acetoxy group exerts a hypsochromic effect of 3–5 mμ[5,6] on the ultraviolet spectrum of a Δ⁴-3-one. Further proof of this configuration is discussed later. The small shift in the conjugated carbonyl absorption of

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