Citrulline-Containing Analogs of Bradykinin

BY MIGUEL A. ONDETTI

The Squibb Institute for Medical Research, New Brunswick, N. J.

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Three new analogs of bradykinin have been prepared, in which each one or both of the arginine moieties have been replaced by citrulline. They are: L-citrullyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine (1-citrulline bradykinin), L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrulline (9-citrulline bradykinin), and L-citrullyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrulline (1,9-bis-citrulline bradykinin). The nitrophenyl ester method has been employed in practically all of the coupling steps. The biological activity of the free nonapeptides is reported.

The enhancement and increased duration of melanocyte stimulating activity achieved by treatment of α-MSH (*alpha*-melanocyte stimulating hormone) with dilute sodium hydroxide has been ascribed to the transformation of the arginine moiety of that peptide hormone into an ornithine residue.[1] The possibility, however, that the guanidino group might have been only partially degraded to a ureido group rather than to the amine as is the case when streptidine is treated with mild alkali had not previously been considered. Such degradation could have led to a citrulline-containing peptide and since peptides containing this amino acid have not been isolated from natural sources nor prepared synthetically it was of interest to synthesize some analogs of naturally-occurring peptides with L-citrulline in place of L-arginine.[2,3]

(1) C. H. Li, E. Schnabel and D. Chung, *J. Am. Chem. Soc.*, **82**, 2062 (1960); E. Schnabel and C. H. Li, *ibid.*, **82**, 4376 (1960).

(2) M. Bodanszky, M. A. Ondetti, J. J. Piala, B. Rubin, J. Friedl, J. T. Sheehan and C. A. Birkhimer, *Nature*, **194**, 482 (1962).

(3) M. Bodanszky, M. A. Ondetti, J. T. Sheehan, and S. Lande, *Proc. New York Acad. Sci.*, in press.

TABLE I

SYNTHESIS OF 9-CITRULLINE BRADYKININ AND 1,9-BIS-CITRULLINE BRADYKININ

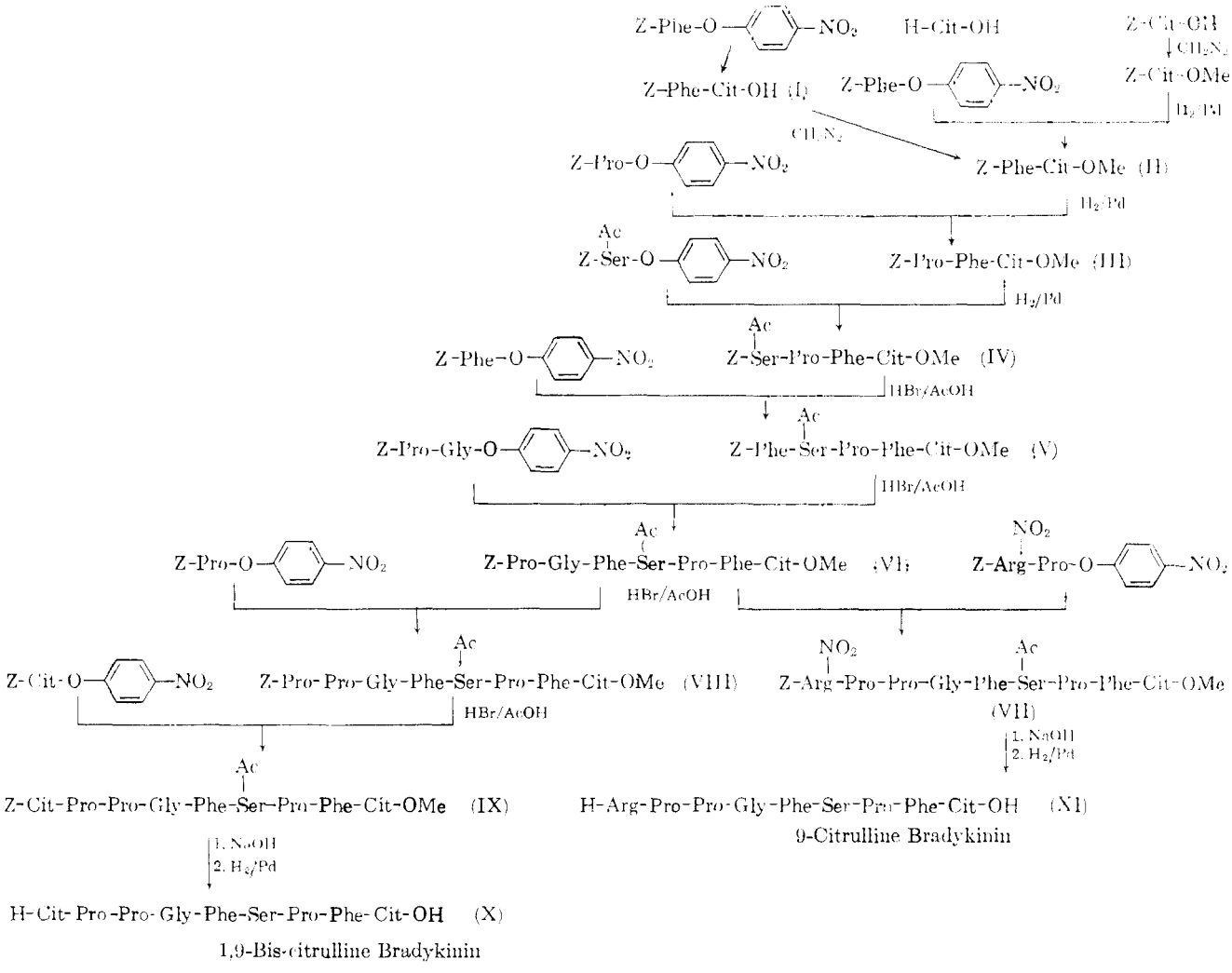
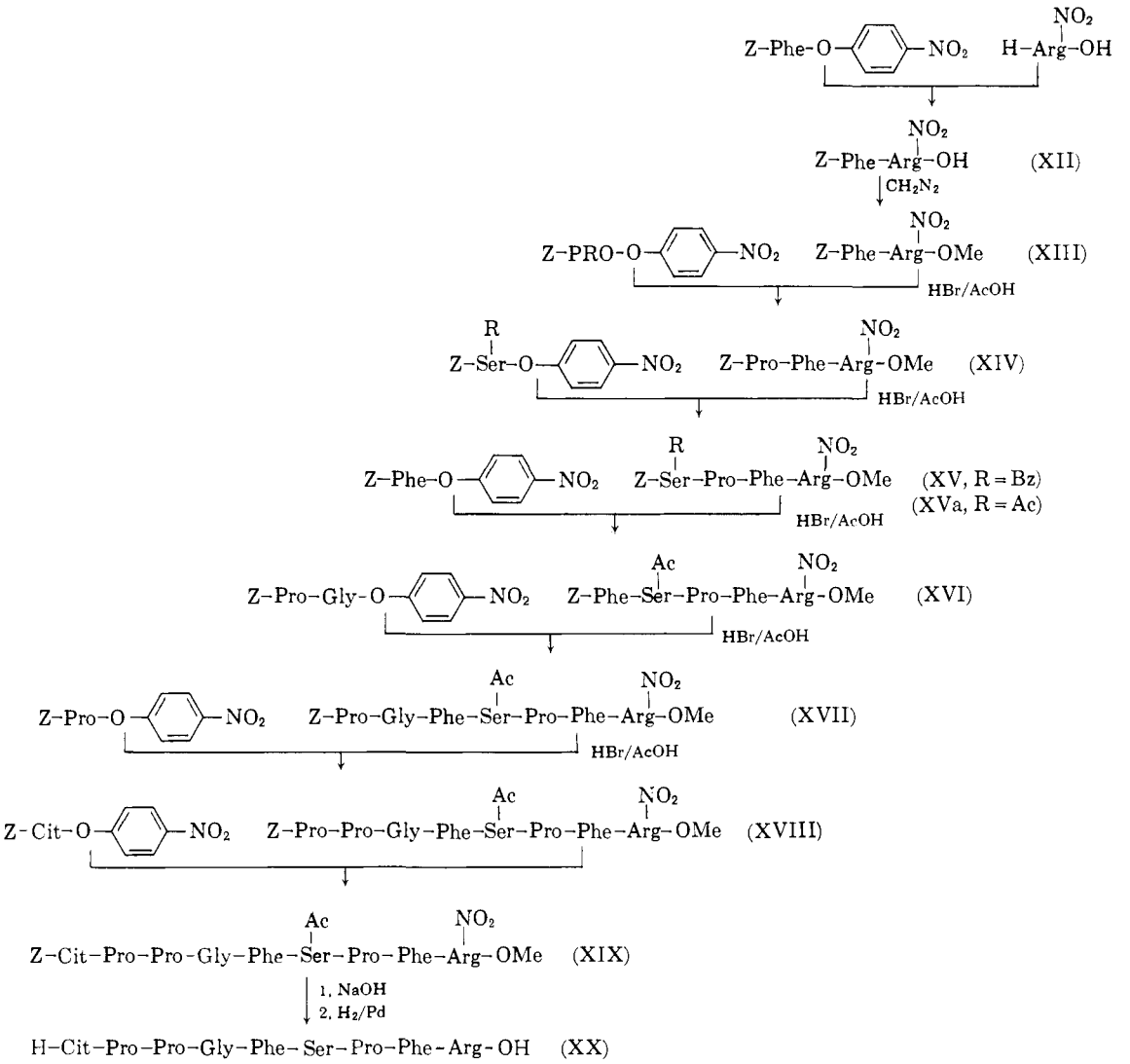


TABLE II

SYNTHESIS OF 1-CITRULLINE BRADYKININ



Because of the prominence of arginine in the biologically highly active nonapeptide bradykinin, in which it serves both as the C- and N-terminal amino acid this peptide appeared admirably suited for the stated purpose. Replacing each of the arginine moieties individually and then both together we have prepared 1-citrulline bradykinin, 9-citrulline bradykinin and 1,9-bis-citrulline bradykinin.

The synthetic schemes (Tables I and II) employed in this work followed essentially the stepwise approach starting from the carboxyl end, using the amino acid *p*-nitrophenyl esters for coupling. This mode of chain lengthening has proved to be a useful route in the synthesis of oxytocin,[4a] lysine vasopressin[4b] and bradykinin.[4c] Minor deviations from the stepwise scheme consisted of the use of the nitrophenyl esters of two dipeptides: *viz.*, nitrophenyl N-benzyloxycarbonyl-nitro-L-arginyl-L-prolinate and nitrophenyl benzyloxycarbonyl-L-prolylglycinate. These intermediates can be used without danger of racemization, either in the preparation of the active esters or in the coupling reaction. The first of these compounds already reported by Boissonas and his co-workers[5] was prepared in a different way and the second, benzyloxycarbonyl-L-prolylglycine, was obtained by acylation of glycine with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester at a constant pH in a pyridine-water mixture, a method found very useful also in many other instances.

The presence of serine in the molecule presented the problem of preparing an active ester of this amino acid. Failure experienced in the preparation of the *p*-nitrophenyl ester of N-benzyloxycarbonyl-L-serine with the aid of dicyclohexylcarbodiimide, led to the study of hydroxyl protected derivatives. Crystalline active esters were obtained from O-benzyl-N-benzyloxycarbonyl-L-serine and O-acetyl-N-benzyloxycarbonyl-L-serine. Both have been found useful from the synthetic point of view; however, since the route leading to the O-benzyl derivative is long and cumbersome and since the latter was shown to be converted into the O-acetyl derivative during the hydrobromic acid–acetic acid treatment, the O-acetyl compound became the intermediate of choice.

The preparation of 9-citrulline bradykinin presented some difficulty due to side reactions not observed with

(4) (a) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959), *J. Am. Chem. Soc.*, **81**, 5688 (1959); (b) M. Bodanszky, J. Meienhofer and V. du Vigneaud, *J. Am. Chem. Soc.*, **82**, 3195 (1960); (c) E. D. Nicolaides and H. A. DeWald, *J. Org. Chem.*, **26**, 3872 (1961).

(5) R. A. Boissonnas, St. Guttmann and P. A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1481 (1960).

the corresponding arginine peptides. Acylation of free citrulline with benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester proceeded at a much slower rate than the acylation of nitro-L-arginine. As a result, hydrolysis of the nitrophenyl ester became sufficiently competitive as to lead to contamination of the final product with benzyloxycarbonyl-L-phenylalanine. For this reason, an alternate route, consisting of coupling the above mentioned *p*-nitrophenyl ester with citrulline methyl ester in an anhydrous medium was devised for the synthesis of methyl benzyloxycarbonyl-L-phenylalanyl-L-citrullinate.

During the preparation of the protected tripeptide ester intermediate methyl benzyloxycarbonyl-L-prolyl-L-phenylalanyl-L-citrullinate, the formation of the diketopiperazine of L-phenylalanine and L-citrulline was observed. Fortunately, the proper choice of solvents permitted a simple and clear-cut separation of the two components.

The free nonapeptides were purified by ion exchange chromatography on carboxymethylcellulose or by counter-current distribution until they were homogeneous on paper chromatography and paper electrophoresis. Their quantitative aminoacid analyses gave the expected molar ratios.

The two analogs in which only one arginine moiety of bradykinin was replaced by citrulline showed weak but definite activity in a variety of test systems in which bradykinin is highly active. In contrast the analog with both arginines similarly replaced showed activity only in one of the tests. Results of the biological assays[6] are summarized in Table III.

TABLE III

BIOLOGICAL ACTIVITIES OF SYNTHETIC NONAPEPTIDES

Nonapeptide	Isolated rat uterus[a]	*In vitro* cat heart coronary vasodilation[b]	Vasodepression in rat[c]	Capillary permeability[d]
1-Citrulline bradykinin	1/400	$\leqslant$ 1/100[e]	$\leqslant$ 1/100	1/1000
9-Citrulline bradykinin	1/400	1/10	$\leqslant$ 1/100	1/10–1/100
1,9-Bis-citrulline bradykinin	<1/5000	...	<1/100	$\leqslant$ 1/100
Bradykinin	1	1	1	1

[a] Uteri from rats pretreated with estradiol benzoate for 3 days (2×10^{-3} mg./day i.m.) were mounted in a 10 ml. bath of modified Tyrode's solution containing 0.22 g./l. of $MgCl_2 \cdot 6H_2O$. Each peptide was allowed to act for 5 min. before washing. [b] F. F. Anderson and B. N. Craver, *J. Pharmacol. Exptl. Therap.*, **93**, 135 (1948). [c] J. Dekanski, *Brit. J. Pharmacol.*, **7**, 567 (1962). [d] Modification of procedure used for histamine by S. F. Reinhard and J. V. Scudi, *Proc. Soc. Exp. Biol. Med.*, 66, 512 (1948). Each analog was injected intradermally at concentrations ranging from 5×10^{-6} to 0.5 mg./ml. [e] The use of the symbol $\leqslant$ indicates that the response obtained at the dosage ratio shown in the Table was insufficient to ascertain an accurate estimate or range of potency.

It has been shown[2] that even minor changes in the structure of bradykinin may lead to complete or at least very significant loss of biological activity. The octapeptide L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-phenylalanyl-L-arginine is inactive on the isolated rat uterus even at concentrations of 10 γ/ml., which corresponds to less than 10^{-5} of the activity of bradykinin.

The replacement of any one of the strongly basic guanido groups by the neutral ureido group has yielded compounds that show the biological properties of bradykinin in concentrations that are still significantly low. This would suggest that the strong basicity of the arginine moiety is not an indispensable feature of the molecule even though its contribution cannot be overlooked. The insignificant degree of activity of the 1,9-bis-citrulline analog clearly emphasizes this latter point.

Experimental

***p*-Nitrophenyl O-Benzyl-N-benzyloxycarbonyl-L-serinate.**[7]—O-Benzyl-N-carbobenzyloxy-L-serine (6.6 g.) prepared from ethyl 2,3-dibromopropionate and resolved *via* its N-acetyl derivative[8] with acylase (Nutritional Biochemicals Corp.), was esterified with *p*-nitrophenol (3.0 g.) in 75 ml. of ethyl acetate containing dicyclohexylcarbodiimide (4.2 g.). The mixture was stirred first for 0.5 hr. at 0° and then for 1.5 hr. at room temperature. Glacial acetic acid (0.5 ml.) then was added to the mixture and the insoluble material filtered off and washed with ethyl acetate. The filtrate and washings were combined and the solvent was evaporated under vacuum at room temperature. The residue which did not crystallize was dissolved in a small amount of ethyl acetate to remove additional small amounts of the urea and the solvent again evaporated as above. The final residue was an oil and weighed 9 g. This was extracted with 18 liters of hot hexane, from which the ester separated slowly, first as an oil, which on further standing at −5° turned to fine crystals. Filtered off and dried the product weighed 6 g. (66%) and melted at 45–47°; $[\alpha]^{22}D$ −12.2° (*c*, 2, dimethylformamide containing 1% acetic acid).

Anal. Calcd. for $C_{24}H_{22}N_2O_7$: C, 63.99; H, 4.92; N, 6.22. Found: C, 64.27; H, 5.15; N, 6.37.

O-Acetyl-N-benzyloxycarbonyl-L-serine *p*-Nitrophenyl Ester.—To a solution of O-acetylserine[9] (29.4 g.) in 1 *M* bicarbonate (800 ml.), benzyloxyearbonyl chloride (48 ml.) was added in 5 equal portions during a period of 30 min., while the mixture was stirred vigorously and the temperature kept at 18–20°. After the final addition stirring was continued for another 3.5 hr. and the mixture was then extracted with ether, acidified and extracted with ethyl acetate. The organic phase was dried and the solvent removed *in vacuo* leaving an oily residue that was dried overnight *in vacuo* over sodium hydroxide. This residue (48 g.) was dissolved in ethyl acetate (400 ml.), the solution was cooled in an ice-water bath and *p*-nitrophenol (25 g.) and dicyclohexylcarbodiimide (35 g.) were added. The mixture was stirred for 0.5 hr. in the cooling bath and 3 hr. at room temperature; acetic acid (17 ml.) was added, the stirring was continued for another 15 min. and then the urea was filtered and washed with fresh ethyl acetate. The filtrate was concentrated to dryness and the residue was taken up with ether from which it crystallized readily. The product was filtered, washed with ether, dried and recrystallized from absolute ethanol (containing 1% Ac(OH); yield, 40 g. (50%), m.p. 94–96°; $[\alpha]^{23}D$ −42.5° (*c*, 2, dimethylformamide).

Anal. Calcd. for $C_{19}H_{18}N_2O_8$: C, 56.71; H, 4.47; N, 6.96; OAc, 10.6. Found: C, 56.89; H, 4.62; N, 7.15; OAc, 10.7.

Benzyloxycarbonyl-L-prolylglycine.—To a solution of glycine (1.7 g.) in water (50 ml.) benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (7.4 g.) in pyridine (55 ml.) was added. The suspension was stirred and the pH kept at 8.3–8.5 by the addition of *N* sodium hydroxide. After about 6 hr. and a total consumption of 37.2 ml. of *N* sodium hydroxide the pH remained constant and a clear solution was obtained. The solution was diluted with water (50 ml.), neutralized and saturated with solid sodium bicarbonate. The mixture was extracted several times with ethyl acetate and then acidified to congo red with concd. hydrochloric acid. An oil separated and turned readily into a crystalline

(6) The pharmacological assays have been performed by Dr. B. Rubin to whom we express our sincere appreciation.

(7) This compound was prepared by Dr. John T. Sheehan.

(8) K. Okawa, *Bull. Chem. Soc. Japan*, **29**, 488 (1956).

(9) J. C. Sheehan, M. Goodman and G. P. Hess, *J. Am. Chem. Soc.*, **78**, 1367 (1956).

solid. This was filtered and washed with water. The dry product weighed 5.02 g. (82%), m.p. 124–125° (lit.[10] m.p. 122–123°).

p-**Nitrophenyl Benzyloxycarbonyl-L-prolylglycinate.**—To a solution of benzyloxycarbonyl-L-prolylglycine (VII) (918 mg.) in ethyl acetate (19 ml.) and dimethylformamide (1.5 ml.), *p*-nitrophenol (0.5 g.) was added and the solution was cooled in an ice-water bath. Dicyclohexylcarbodiimide (0.62 g.) was added and the mixture was stirred for 0.5 hr. in an ice-water bath and then for 2 hr. at room temperature. The urea derivative was filtered off and the solution was evaporated to dryness. The oily residue when treated with boiling ether readily crystallized giving 1.1 g. (86%) of product, m.p. 140–142°. After recrystallization from ethanol containing 1% acetic acid the product melted at 143.5–145°; $[\alpha]^{22}D$ −63° (*c*, 1, dimethylformamide).

Anal. Calcd. for $C_{21}H_{21}N_3O_7$: C, 59.01; H, 4.92; N, 9.84. Found: C, 59.35; H, 5.12; N, 9.83.

Benzyl Benzyloxycarbonyl-nitro-L-arginyl-L-prolinate.—A solution of benzyloxycarbonyl nitro-L-arginine (3.53 g.) and triethylamine (1.4 ml.) in a mixture of dioxane (100 ml.) and dimethylformamide (5 ml.) was stirred and cooled to 11–12° and isobutyl chloroformate (1.4 ml.) was added. The stirring and cooling was continued for 30 min. after which the suspension was added to a mixture of proline benzyl ester hydrochloride[11] (2.41 g.), dioxane (30 ml.) and triethylamine (1.4 ml.). The stirring was continued for 1.5 hr. at room temperature and the solution was concentrated *in vacuo* to a few ml. The residue was diluted with ethyl acetate, washed twice with *N* hydrochloric acid, twice with *N* sodium carbonate and several times with water, dried over magnesium sulfate and evaporated to dryness. The oily residue was taken up with methanol and soon began to crystallize. Upon cooling, 2.9 g. (56%) of material, m.p. 145–147° was obtained. Recrystallization from methanol raised the m.p. to 147–148.5°; $[\alpha]^{20}D$ −42° (*c*, 1, dimethylformamide).

Anal. Calcd. for $C_{16}H_{32}N_6O_7$: C, 57.77; H, 5.92; N, 15.55. Found: C, 57.69; H, 5.90; N, 15.67.

Benzyloxycarbonyl-nitro-L-arginyl-L-proline.—To a solution of benzyl benzyloxycarbonyl-nitro-L-arginyl-L-prolinate (770 mg.) in dioxane (10 ml.) and dimethylformamide (1.5 ml.), *N* sodium hydroxide was added, at first 1.5 ml. and after 1.5 hr. another 0.5 ml. Half an hour later the solution was diluted with 60 ml. of water and extracted twice with ethyl acetate. The aqueous solution was acidified and extracted 4 times with ethyl acetate. This extract was dried over magnesium sulfate and concentrated to dryness. The residue was crystallized from methanol, 0.550 g. (82%), m.p. 108–110° was obtained; $[\alpha]^{20}D$ −30° (*c*, 2, dimethylformamide), lit.[5] m.p. 119°; $[\alpha]^{22}D$ −26.5°.

Anal. Calcd. for $C_{19}H_{26}N_6O_7$: N, 18.70. Found: N, 18.00.

The ethyl acetate extract from the still alkaline reaction mixture gave 70 mg. of the unhydrolyzed ester, m.p. 143–146°.

p-**Nitrophenyl Benzyloxycarbonyl-nitro-L-arginyl-L-prolinate.**—A solution of benzyloxycarbonyl-nitro-L-arginyl-L-proline (1.35 g.) and *p*-nitrophenol (0.51 g.) in dimethylformamide (8 ml.) and acetonitrile (2.5 ml.) was stirred and cooled in an ice bath and after a few min. dicyclohexylcarbodiimide (0.61 g.) was added. The cooling bath was removed after 0.5 hr. and the stirring continued for 18 hr. at room temperature. The solvents were evaporated *in vacuo*, the residue was taken up in ethyl acetate, the urea derivative was filtered and the ethyl acetate solution was washed 3 times with *N* sodium bicarbonate and 3 times with water, dried and evaporated to dryness. The residue was dissolved in hot ethanol, cooled to 0° for 2 hr., the ethanol was decanted and the gummy residue treated with boiling ether, decanted and treated again with fresh ether until a solid (1 g., 66%) (m.p. 58–66°) was obtained. On repetition of the treatment with ethanol and ether 0.85 g., m.p. 55–70° was obtained; $[\alpha]^{20}D$ −64° (*c*, 1.07, methanol), lit.[5] m.p. 60°; $[\alpha]^{23}D$ −62°.

Anal. Calcd. for $C_{25}H_{29}N_7O_9$: C, 52.50; H, 5.10; N, 17.20. Found: C, 52.33; H, 5.40; N, 16.50.

Methyl Benzyloxycarbonyl-L-citrullinate.—An ethereal solution of diazomethane was added to a solution of benzyloxycarbonyl-L-citrulline (23.8 g.)[12] in methanol (210 ml.) until a persistent yellow color was obtained. After 15 min. the excess diazomethane was destroyed with acetic acid and the solvents were removed *in vacuo*. The crystalline residue was taken up in ethyl acetate, filtered and washed with the same solvent; yield, 20.15 g. (80%), m.p. 155–157°. This product was used without any further purification in the preparation of II.

Benzyloxycarbonyl-L-phenylalanyl-L-citrulline (I).—A solution of benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (54.6 g.) in tetrahydrofuran (20 ml.) was added to a solution of L-citrulline (23 g.) in water (200 ml.). The two phase mixture was stirred and kept at pH 9.2 by controlled addition of 5 *N* sodium hydroxide. After about 7 hr. a clear solution resulted, which was diluted with water (100 ml.) acidified to pH 8, saturated with sodium bicarbonate and extracted several times with ethyl acetate. Upon acidification of the aqueous layer an oily precipitate formed which solidified readily. The dried solid weighed 58 g. and melted at 150–152° (sintering 148°). The crude product (30 g.) was dissolved in hot methanol and precipitated with water. The amorphous product thus obtained was dissolved in a mixture of hot ethyl acetate–ethanol (9:1), a small amount of impurity was removed by filtration, and the solution was allowed to cool slowly to room temperature. Crystalline I (17 g., 55%) was obtained, m.p. 155–157°; $[\alpha]^{20}D$ −7° (*c*, 0.9, dimethylformamide).

Anal. Calcd. for $C_{23}H_{28}N_4O_6$: C, 60.50; H, 6.21; N, 12.28. Found: C, 60.54; H, 6.43; N, 11.79.

Methyl Benzyloxycarbonyl-L-phenylalanyl-L-citrullinate (II).—(A) To a solution of benzyloxycarbonyl-L-phenylalanyl-L-citrulline (I) (17 g.) in methanol (170 ml.) an ethereal solution of diazomethane was added until a persistent yellow color was obtained. After 15 min. the excess diazomethane was destroyed with a few drops of acetic acid and the solvents were removed *in vacuo*. The amorphous residue readily became crystalline on treatment with boiling ethyl acetate. This crude product (15.8 g., m.p. 169–172°) was recrystallized from absolute ethanol and gave 15 g. (85%) of II, m.p. 172–173° (sint. at 160°); $[\alpha]^{20}D$ −10.9° (*c*, 2, dimethylformamide).

Anal. Calcd. for $C_{24}H_{30}N_4O_6$: C, 61.27; H, 6.42; N, 11.91. Found: C, 61.23; H, 6.57; N, 12.15.

(B) A solution of benzyloxycarbonyl-L-citrulline methyl ester (20.1 g.) in methanol (400 ml.) containing acetic acid (4 ml.) was hydrogenated at atmospheric pressure for 2 hr. in the presence of 10% palladium on charcoal (2 g.). After the removal of the catalyst, the solvent was evaporated *in vacuo* leaving an oily residue of L-citrulline methyl ester which was dissolved in pyridine (60 ml.) together with benzyloxycarbonyl-L-phenylalanyl *p*-nitrophenyl ester (29.4 g.). On standing at room temperature the product (II) started to crystallized from the reaction mixture. After 5 days the solid mass was diluted with ethyl acetate, concentrated to dryness, diluted with fresh ethyl acetate, filtered and dried (19.7 g., m.p. 169–172° sintering at 150°). The product was recrystallized from absolute ethanol and gave 17.6 g. (59%), m.p. 170–172° sintering at 162°; $[\alpha]^{20}D$ −12.4° (*c*, 2, dimethylformamide).

Methyl Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-L-citrullinate (III).—A solution of methyl benzyloxycarbonyl-L-phenylalanyl-L-citrullinate (II) (18 g.) in methanol (300 ml.) acetic acid (20 ml.) was hydrogenated at ordinary pressure for 2.5 hr. in the presence of 10% palladium on charcoal (1 g.). After filtration of the catalyst the solvent was removed *in vacuo* and the residue was dissolved in pyridine (60 ml.) together with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (14.8 g.). After 3 days at room temperature the semisolid mass was triturated with ethyl acetate, the solvent removed, fresh ethyl acetate was added and the process repeated once more. After filtration and drying the product weighed 18.5 g., m.p. 175–180°, sintering 140°. Recrystallization from a mixture of ethyl acetate–ethanol gave 14.5 g., m.p. 187–189°, sintering 155°.

Paper chromatography of this product in butanol–acetic acid–water (4:1:5) showed in addition to the main product a second component, which likewise gave a positive test with *p*-dimethylaminobenzaldehyde. The two components were separated easily by boiling the mixture with acetonitrile, filtering the less soluble material (1.5 g., m.p. 235–236°; $[\alpha]^{20}D$ −40.7° (*c*, 1.0, dimethylformamide)) and concentrating the filtrate to dryness. Crystallization of the residue from ethyl acetate–ethanol gave 10.5 g. (46%) of the desired protected tripeptide (III): m.p. 196–197° (sint. 158°); $[\alpha[^{20}D$ −43.0° (*c*, 1.0, dimethylformamide).

Anal. Calcd. for $C_{29}H_{37}N_5O_7$: C, 61.37; H, 6.57; N, 12.34. Found: C, 61.21; H, 6.72; N, 12.39.

The less soluble component (m.p. 235–236°) showed, on hydrolysis with 6 *N* HCl and paper chromatography of the hydrolysate, the spots corresponding to phenylalanine and citrulline.

(10) W. Grassmann and E. Winisch, *Chem. Ber.*, **91**, 449 (1958).
(11) R. E. Neuman and E. L. Smith, *J. Biol. Chem.*, **193**, 97 (1951).
(12) M. Bodanszky and C. A. Birkhimer, *J. Am. Chem. Soc.*, in press.

The analytical figures agree with the values calculated for the diketopiperazine containing these two amino acids.

Anal. Calcd. for $C_{15}H_{20}N_4O_3$: C, 59.19; H, 6.62; N, 18.41. Found: C, 59.11; H, 6.75; N, 18.31.

Methyl N-Benzyloxycarbonyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrullinate (IV).—A solution of the benzyloxycarbonyl tripeptide methyl ester (III) (16.8 g.) in methanol (200 ml.) was hydrogenated as described for III. After filtration of the catalyst the solvent was removed *in vacuo* and the residue was dissolved in dimethylformamide (30 ml.) together with N-benzyloxycarbonyl-O-acetyl-L-serine *p*-nitrophenyl ester (13.4 g.). After 4 days at room temperature the semisolid mass was triturated under ethyl acetate, the solvent was removed, and the same process repeated twice more. The residue when taken up with fresh ethyl acetate, filtered, washed with ethyl acetate and absolute ethanol furnished IV (11.6 g.), m.p. 158–162°. The crude product was recrystallized from acetonitrile–water and gave 11.1 g. (52%), m.p. 170–172°; $[\alpha]^{20}D$ −53.6 (*c*, 1.02, dimethylformamide).

Anal. Calcd. for $C_{34}H_{44}N_6O_{10}$: C, 58.60; H, 6.36; N, 12.06. Found: C, 58.70; H, 6.56; N, 12.01.

Methyl Benzyloxycarbonyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrullinate (V).—A solution of *ca.* 40% hydrobromic acid in acetic acid (40 ml.) was added to a suspension of the benzyloxycarbonyl tetrapeptide methyl ester (IV) (9.7 g.) in acetic acid (40 ml.). The clear solution was kept for 1 hr. at room temperature, diluted with acetic acid (*ca.* 40 ml.) and freeze dried. The residue was dissolved in dimethylformamide (20 ml.), the solution was made alkaline with tributylamine (8.5 ml.) and benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (6.5 g.) was added. After 3 days at room temperature the solid mass was triturated with ethyl acetate, filtered, washed with ethyl acetate and absolute ethanol. Recrystallization from acetonitrile-water gave 6.9 g. (58%) of V; m.p. 206–208° sintering 185°; $[\alpha]^{20}D$ −57.4° (*c*, 1.0, dimethylformamide).

Anal. Calcd. for $C_{43}H_{53}N_7O_{11}$: C, 61.19; H, 6.33; N, 11.62. Found: C, 61.10; H, 6.49; N, 11.40.

Methyl Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrullinate (VI).—The benzyloxycarbonyl group of the protected pentapeptide methyl ester (V) (5.7 g.) was removed by hydrobromic acid-acetic acid treatment and the resulting hydrobromide coupled (4 days at room temperature) with benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester (3.2 g.) in the way described above for the preparation of V. The dried solid obtained by trituration of the reaction product with ethyl acetate was extracted with hot 95% ethanol, and dried. Yield of purified product (VI), was 5.8 g. (86%); m.p. 214–216°; $[\alpha]^{20}D$ −47.7° (*c*, 1.09, dimethylsulfoxide).

Anal. Calcd. for $C_{50}H_{63}N_9O_{13}$: C, 60.17; H, 6.36; N, 12.63. Found: C, 60.21; H, 6.26; N, 12.38.

Methyl α-Benzyloxycarbonyl-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrullinate (VII).—The benzyloxycarbonyl group of the protected heptapeptide methyl ester (VI) (1 g.) was removed and benzyloxycarbonyl-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (0.62 g.) was coupled (5 days) to the resulting hydrobromide, as described above for V. The resulting product was dissolved in hot absolute ethanol and cooled. The resulting oil was separated by decantation and triturated with ethyl acetate until it solidified. Yield was 0.63 g. (48%); m.p. 155–160° (sintering 115°); $[\alpha]^{20}D$ −61.3° (*c*, 0.99, dimethylformamide).

Anal. Calcd. for $C_{61}H_{81}N_{15}O_{17}$: C, 56.50; H, 6.30; N, 16.21. Found: C, 56.07; H, 6.43; N, 15.73.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrulline (9-Citrulline Bradykinin) (XI).—To a solution of the protected nonapeptide methyl ester (VII) (135 mg.) in methanol (2 ml.) 2 *N* sodium hydroxide (0.2 ml.) was added.[13] After 1 hr. at room temperature the solution was diluted with water (3.5 ml.) and acidified with *N* hydrochloric acid. The cloudy mixture was kept overnight in the refrigerator, centrifuged, the supernatant was decanted and the residue was dried *in vacuo* over sodium hydroxide. This residue was triturated with acetonitrile, filtered and dried (110 mg.). The amorphous solid thus obtained was hydrogenated at atmospheric pressure, dissolved in a mixture of acetic acid-water (1:1) in the presence of 5% palladium on barium sulfate[14] (200 mg.). After 30 hr. the catalyst was removed by filtration and the filtrate was freeze dried leaving a residue of 100 mg. This crude residue (90 mg.) was applied to a column of carboxymethylcellulose (*ca.* 4 g., 0.9 meq./g.) and eluted with a linear gradient of ammonium acetate. This gradient was obtained with a seven chamber Autograd (Technicon, Inc., Chauncey, New York) using solutions of ammonium acetate (250 ml.) of the concentrations: 0.007, 0.009, 0.01, 0.025, 0.03, 0.04, and 0.05 *M*. After elution the absorption of the 10 ml. fractions was measured at 230 mμ. The tubes containing the main peak were pooled and lyophilized 3 times. A white powder (62 mg., $[\alpha]^{25}D$ −88.7° (*c*, 0.99, *N* acetic acid]) was obtained which was shown to be homogeneous by paper chromatography (butanol-acetic acid–water, 4:1:5; R_f 0.41 and butanol-ethanol-0.1% aqueous acetic acid, 4:1:5; R_f 0.47) using ninhydrin, *p*-dimethylaminobenzaldehyde, and Sakaguchi reagent for detection. It was also shown to be homogeneous by paper electrophoresis (pyridine acetate buffer (pH 4.0 and ammonium citrate buffer pH 5.3) with the aid of the same reagents.

The quantitative amino acid analysis gave the ratio of amino acids: serine:proline:glycine:phenylalanine:arginine:citrulline-ornithine; 0.9:3.1:1.0:2.0:1.0:1.0. Hydrolysis of citrulline under similar conditions and quantitative analysis led to the recovery of 60–70% of citrulline with the simultaneous formation of about 30–40% ornithine.

Methyl Benzyloxycarbonyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrullinate (VIII).—The benzyloxycarbonyl group was removed from the protected heptapeptide methyl ester (VI) (3 g.) and the hydrobromide thus obtained was allowed to react with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (1.23 g.) for 5 days in the way described for the preparation of V; yield, 2.5 g. (76%); m.p. 182–184° (sintering at 175°); $[\alpha]^{26}D$ −64° (*c*, 1.02, dimethylformamide).

Anal. Calcd. for $C_{55}H_{70}N_{10}O_{14}$: C, 60.32; H, 6.44; N, 12.79. Found: C, 59.90; H, 6.43; N, 13.28.

Methyl Benzyloxycarbonyl-L-citrullyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrullinate (IX).—The benzyloxycarbonyl group of the protected octapeptide methyl ester (VIII) (2 g.) was removed and benzyloxycarbonyl-L-citrulline *p*-nitrophenyl ester[12] (0.79 g.) was coupled (6 days) to the resulting hydrobromide in the way described for the preparation of V; yield, 1.15 g.; m.p. 128–130° (50%); $[\alpha]^{20}D$ −61.9° (*c*, 1.0, dimethylformamide). A small sample was dried *in vacuo* at 78° for analysis; m.p. was raised to 136–138°.

Anal. Calcd. for $C_{61}H_{81}N_{13}O_{16}$: C, 58.51; H, 6.52; N, 14.54. Found: C, 58.44; H, 6.39; N, 14.23.

L-Citrullyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-citrulline (1,9-Bis-citrulline Bradykinin) (X).—To a solution of the benzyloxycarbonyl nonapeptide methyl ester (262 mg.) in methanol (2 ml.), was added *N* sodium hydroxide (0.6 ml.). After 30 min. at room temperature water (2 ml.) was added and after another 15 min. the solution was diluted with more water (1 ml.). On acidification with *N* hydrochloric acid an oily precipitate was formed. After overnight standing in the refrigerator, the supernatant was decanted and the residue was dried *in vacuo* over sodium hydroxide (193 mg.). This crude protected nonapeptide acid crystallized from methanol upon standing for 3 days in the refrigerator. The crystals were filtered, washed with cold methanol and dried (90 mg., m.p. 175–185° sintering at 135°). The crystalline intermediate was dissolved in a 1:1 mixture of acetic acid and water (12 ml.) and hydrogenated for 16 hr. in the presence of 10% palladium on charcoal (90 mg.). The catalyst was filtered off and the filtrate was freeze-dried (65 mg.). The free nonapeptide thus obtained behaved as a single component on paper chromatography (butanol-ethanol-0.1% aqueous acetic acid, 4:1:5, R_f 0.59 and butanol-acetic acid–water, 4:1:5, R_f 0.28) and paper electrophoresis (pyridine acetate buffer, pH 4.0 and ammonium citrate buffer, pH 5.3) developed with ninhydrin and Ehrlich (*p*-dimethylaminobenzaldehyde) reagents. Quantitative amino acid analysis gave the ratio: serine:proline:glycine:phenylalanine:ammonia:citrulline-ornithine: 0.8:3.06:0.95:2.13:0.77:2.1. The mother liquors from the crystallization of the protected nonapeptide acid were concentrated to dryness and the residue was hydrogenated as described above. The crude product was puri-

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(14) R. Kuhn and J. Haas, *Angew. Chem.*, **67**, 785 (1955).

fied by counter current distribution in the system butanol-ethanol–0.1% aqueous acetic acid (4:1:5). From the main peak (K = 0.43) the free nonapeptide (35 mg.) was isolated with the properties described above; $[\alpha]^{21}D$ −96° (*c*, 1.0, *N* AcOH).

Benzyloxycarbonyl-L-phenylalanyl-nitro-L-arginine (XII).—To a solution of nitroarginine (9.68 g.) in a mixture of *N* sodium hydroxide (45 ml.) and water (80 ml.) a solution of benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (16.8 g.) in pyridine (90 ml.) was added. Precipitation occurred and the resulting suspension was stirred at room temperature. When the pH dropped to 9–9.3 it was kept at this level by addition of 2 *N* sodium hydroxide. After 5–6 hr. and a total consumption of 18.35 ml. of 2 *N* sodium hydroxide, the pH remained constant and the clear reaction mixture gave no turbidity upon dilution with water. The solution was treated with concd. hydrochloric acid to pH 8 and saturated with solid sodium bicarbonate, extracted 7 times with ethyl acetate to remove *p*-nitrophenol and pyridine and acidified to congo red with concd. hydrochloric acid. An oil separated, which readily crystallized and could be filtered, washed with water and dried. There was obtained 16.7 g. of XII, m.p. 170–173° (sintering 168°). On trituration with ether a product weighing 15.7 g. (71%), m.p. 173–176° was obtained; $[\alpha]^{20}D$ +2.1° (*c*, 2, pyridine); lit.[13a] m.p. 185–186°; $[\alpha]^{27}D$ +1.5; lit.[13b] 171.5–173°.

Methyl Benzyloxycarbonyl-L-phenylalanyl-nitro-L-argininate (XIII).—Benzyloxycarbonyl - L - phenylalanyl - nitro - L - arginine (XII) (7.7 g.) was methylated with diazomethane in the way described for the preparation of II. The residue was crystallized from methanol; 6.6 g. of product, m.p. 148–151° (softens at 145°) was obtained. Another recrystallization from methanol gave 6 g. (76%) of XIII, m.p. 150–152°; $[\alpha]^{20}D$ −13.5° (*c*, 1, MeOH), lit.[13a] m.p. 160–161°; $[\alpha]^{28}D$ −16.2°.

Methyl Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-nitro-L-arginiinate (XIV).—The protecting group was removed from methyl benzyloxycarbonyl-L-phenylalanyl-nitro-L-argininate (XIII) (6.7 g.) and the resulting hydrobromide was allowed to react with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (4.8 g.) by the procedure described in the preparation of V. After 2 days at room temperature the mixture was diluted with ethyl acetate, washed with *N* hydrochloric acid, *N* ammonium hydroxide and water. The ethyl acetate solution was dried over magnesium sulfate and evaporated to dryness. The resulting residue was crystallized from methanol–water. The protected tripeptide ester XIV (4.8 g., 60%) melted at 137–139° (soft. 135°); $[\alpha]^{20}D$ −43° (*c*, 2.0, dimethylformamide), lit.[4c] m.p. 137–139°; $[\alpha]^{23}D$ −43°.

Anal. Calcd. for $C_{29}H_{32}N_2O_8$: C, 57.00; H, 6.05; N, 16.00; OMe, 5.60. Found: C, 57.65; H, 6.23; N, 16.18; OMe, 5.46.

Methyl O-Benzyl-N-benzyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-argininate (XV).—The benzyloxycarbonyl group was removed from the protected tripeptide methyl ester (XIV) (3.05 g.) as described for V. The hydrobromide of the tripeptide ester was dissolved in methanol, the solvent was removed *in vacuo* and the residue again dissolved in methanol (20 ml.). To this solution Amberlite IRA-400 (acetate cycle) was added with stirring until the reaction for bromide became negative. The solution was filtered through a layer of resin and concentrated to dryness. The residue was dissolved in dimethylformamide (12.5 ml.) and *p*-nitrophenyl O-benzyl-N-carbobenzyloxy-L-serinate (IV) (2.25 g.) was added. After 2.5 days at room temperature the product was isolated as described above for the preparation of XIV. The crude protected tetrapeptide ester XV was dissolved in ethyl acetate–methanol and precipitated with ether, yielding 2.6 g. (66%), m.p. 100–102°; $[\alpha]^{20}D$ −40° (*c*, 1.0, dimethylformamide).

Anal. Calcd. for $C_{39}H_{48}N_8O_{10}$: C, 59.50; H, 6.10; N, 14.20. Found: C, 59.10; H, 6.20; N, 14.20.

Methyl O-Acetyl-N-benzyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-argininate (XVa).—The benzyloxycarbonyl tripeptide methyl ester (XIV) (19 g.) was treated with hydrobromic acid in acetic acid as described for V. The resulting hydrobromide was dissolved in a mixture of saturated potassium carbonate solution (65 ml.), ethyl acetate (195 ml.) and acetonitrile (97 ml.). The organic layer was separated off and the aqueous phase was extracted 3 times with ethyl acetate–acetonitrile (2:1). The organic phases from two such batches were pooled, dried and concentrated to dryness. The oily residue, methyl L-prolyl-L-phenylalanyl-nitro-L-argininate, and O-acetyl-*N*-benzyloxycarbonyl-L-serine *p*-nitrophenyl ester (25 g.) were dissolved in pyridine (70 ml.). After 3 days at room temperature the mixture was diluted with ethyl acetate, washed 3 times with *N* hydrochloric acid, 7 times with *N* ammonium hydroxide, once with *N* hydrochloric acid and finally with water. The solution was dried over $MgSO_4$, the solvent was removed *in vacuo* and the oily residue was boiled with ethyl acetate. This resulted in a crystalline precipitate very insoluble in ethyl acetate. The crystals were filtered, washed with ethyl acetate and dried; weight 10 g., m.p. 170–172°; $[\alpha]^{20}D$ −56.6° (*c*, 1.06, dimethylformamide). The mother liquors on concentration deposited a second crop (16 g., total yield 56%) of the same m.p. and rotation.

Anal. Calcd. for $C_{34}H_{44}N_8O_{11}$: C, 55.13; H, 5.99; N, 15.12. Found: C, 54.92; H, 6.14; N, 15.09.

Methyl Benzyloxycarbonyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-argininate (XVI).—(A) The benzyloxycarbonyl group was removed from the protected tetrapeptide methyl ester XV (1.94 g.) and the hydrobromide treated as described for XV. The tetrapeptide ester acetate was dissolved in a 1:1 mixture of pyridine and dimethylformamide (7.5 ml.) and benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester was added. After 2.5 days at 37° the mixture was diluted with ethyl acetate, washed once with *N* hydrochloric acid and once with water. The solution was dried over magnesium sulfate and concentrated to about 5 ml.; the product began to crystallize. The crystals were filtered, washed with ethyl acetate and dried, yielding 910 mg. (45%) of XVI, m.p. 208–211°; $[\alpha]^{20}D$ −54° (*c*, 1.0, dimethylformamide).

Anal. Calcd. for $C_{43}H_{53}N_9O_{12}$: C, 58.18; H, 5.98; N, 14.20; O-Ac, 4.8. Found: C, 58.29; H, 6.21; N, 14.38; O-Ac, 4.5.

(B) The benzyloxycarbonyl group of the protected tetrapeptide methyl ester XVa (25 g.) was removed with hydrobromic acid–acetic acid and the hydrobromide thus obtained was allowed to react with benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (15.2 g.) in the way described for V. After 3 days at room temperature the reaction mixture was diluted with ethyl acetate, concentrated almost to dryness and the crystalline residue was taken up with ethyl acetate, filtered and washed with ethyl acetate and methanol. After drying 15 g. (50%) of XVI was obtained, m.p. 209–212°. A small portion boiled with methanol gave m.p. 214–216°; $[\alpha]^{23}D$ −57° (*c*, 1, dimethylformamide).

Methyl Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-argininate (XVII).—The benzyloxycarbonyl group was removed from the protected pentapeptide methyl ester (XVI) (0.6 g.) and the resulting hydrobromide was coupled with benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester (0.33 g.) by the procedure described for V. After 2.5 days the mixture was processed as in the preparation of XVa. XVII was obtained as crystals weighing 0.61 g. (83%), m.p. 188–190° (sintering 185°). It can be recrystallized from 95% ethanol (m.p. 193–196°); $[\alpha]^{20}D$ −59° (*c*, 1.3, dimethylformamide).

Anal. Calcd. for $C_{50}H_{63}N_{11}O_{14}$: C, 57.63; H, 6.05; N, 14.79; O-Ac, 4.1. Found: C, 57.54; H, 6.13; N, 14.73; O-Ac, 5.3.

Methyl Benzyloxycarbonyl-L-prolyl-L-prolylglycyl-L-phenylalanyl - O - acetyl - L - seryl - L - prolyl - L - phenylalanyl-nitro-L-argininate (XVIII).—The benzyloxycarbonyl heptapeptide methyl ester (XVII) (3.85 g.) was treated with hydrobromic acid-acetic acid in the usual way and the hydrobromide obtained was coupled with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (1.5 g.) by the procedure employed for V. After 3 days at room temperature the reaction mixture was diluted with ethyl acetate–acetonitrile and washed once with *N* hydrochloric acid and water. After drying the solvents were removed *in vacuo* and the oily residue was triturated with ethyl acetate, which caused it to solidify; yield, 2.5 g. (59%), m.p. 131–135°; $[\alpha]^{20}D$ −60° (*c*, 1.0, dimethylformamide).

Anal. Calcd. for $C_{55}H_{70}N_{12}O_5$: C, 57.99; H, 6.15; N, 14.76. Found: C, 58.51; H, 6.51; N, 14.38.

Methyl Benzyloxycarbonyl-L-citrullyl-L-prolyl-L-prolylglycyl-L - phenylalanyl - O - acetyl - L - seryl - L - prolyl - L - phenylalanyl-nitro-L-argininate (XIX).—The benzyloxycarbonyl octapeptide methyl ester (XVIII) (2.4 g.) was treated with hydrobromic acid-acetic acid and benzyloxycarbonyl-L-citrulline *p*-nitrophenyl ester (0.9 g.) was coupled (4 days) to the resulting hydrobromide by the procedure described for the preparation of V. The reaction mixture was processed as in the preparation of XVIII; yield, 1.5 g. (55%), m.p. 132–140° sintering at 110°; $[\alpha]^{20}D$ −58° (*c*, 1, dimethylformamide).

Anal. Calcd. for $C_{61}H_{81}N_{15}O_{17}$: C, 56.50; H, 6.30; N, 16.21. Found: C, 56.89; H, 6.40; N, 16.24.

L-Citrullyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine (1-Citrulline Bradykinin) (XX).—To a solution of the benzyloxycarbonyl nonapeptide methyl ester (XIX) (130 mg.) in methanol (1 ml.) 2 *N* sodium hydroxide (0.1 ml.) was added. After 1 hr. at room temperature water (2 ml.) was added and after another 15 min. the solution was acidified with *N* hydrochloric acid and kept in a refrigerator overnight. The supernatant was decanted and the semi-solid precipitate was dried *in vacuo* over sodium hydroxide. The solid residue thus obtained (88 mg.) was dissolved in a mixture of acetic acid-water (2:1) (12 ml.) and hydrogenated at atmospheric pressure for 48 hr. in the presence of 5% palladium on barium sulfate.[14] After removal of the catalyst the solution was freeze-dried. The residue was chromatographed on carboxymethylcellulose, using a gradually increasing concentration of ammonium acetate for the elution, as described for the purification of XI. The product was freeze-dried several times to remove residual ammonium acetate. The final product (30 mg., $[\alpha]^{20}D$ −91.2° (*c*, 1.0, *N* acetic acid)) was homogeneous on paper chromatograms (butanol–acetic acid–water 4:1:5, R_f 0.30) and by paper electrophoresis (pyridine acetate buffer pH 4.0 and ammonium acetate buffer pH 5.3) when developed with ninhydrin, Ehrlich (*p*-dimethylaminobenzaldehyde) and Sakaguchi reagents.

Amino Acid Analysis.—Gly:Ser:Pro:Phe:Arg:Cit-Orn-1:0.8:3:2:1.1:1.0.

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Metal Complexation of the Tetracycline Hydrochlorides

JAMES T. DOLUISIO

Philadelphia College of Pharmacy and Science, Philadelphia 4, Pennsylvania

AND ALFRED N. MARTIN

Purdue University School of Pharmacy, Lafayette, Indiana

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Therapeutically active tetracycline analogs, tetracycline·HCl, chlorotetracycline·HCl, oxytetracycline·HCl, 7-chloro-6-demethyltetracycline·HCl, and anhydrochlorotetracycline·HCl, were found to complex with cupric, nickel and zinc ions to form 2:1 complexes with the same avidity. Inactive tetracycline analogs, anhydro-4-epitetracycline·HCl, 7-chloro-4-epitetracycline·HCl and isochlorotetracycline·HCl, were found to form only 1:1 complexes with cupric, nickel and zinc ions. By analysis of potentiometric data and examination of Stuart and Briegleb models, it is postulated that tetracycline chelation with these metal ions occurs through coördination with the C.4 dimethylanino group and either the C.3 or C.12a hydroxyl group.

Many possible modes of tetracycline action have been suggested, but one is of particular interest. Hunter and Lowry[1] have suggested that tetracyclines uncouple aerobic phosphorylation (that is, they inhibit the formation of ATP, which is a primary source of energy for cellular functions, without affecting oxygen consumption) by interaction with magnesium bound to an enzyme without actually removing it. In this investigation experiments were undertaken to determine whether there existed a correlation between the metal binding properties of the tetracyclines and effectiveness of antibacterial activity.

Albert[2,3] has calculated stability constants for the interaction of many metal ions with tetracycline·HCl, oxytetracycline·HCl and chlorotetracycline·HCl and has stated that chelation is likely to play a part in the mode of action "because substances with constants of such magnitude could not fail to compete with metals in the tissues."[4] He also points out that the action of the tetracyclines on bacteria is much slower than that of oxine and they are active in iron-depleted media.

Miura, *et al.*,[5] have shown that oxytetracycline and chlorotetracycline exhibit their activity by uncoupling aerobic phosphorylation. Recent work has indicated that manganese is essential for phosphorylation.[6] Saz and Slie[7,8] have presented evidence that manganese is essential for reduction of DPN^+ by malate in certain *Escherichia coli* extracts and suggest that the inhibition of nitroreductase by chlorotetracycline in such preparations is due to complexing with manganese and preventing the formation of DPNH which is essential for reduction. Brody, *et al.*,[9] have shown that the uncoupling of phosphorylation is prevented if excess magnesium is included in the medium; however, Burstall[10] suggests that small amounts of metal enhance, and may in some cases be necessary for, inhibition. Pancreatic lipase is inhibited by chlorotetracycline only in the presence of divalent ions.[11] Hamner[12] recently has reported the potentiation of demethylchlorotetracycline·HCl in the presence of zinc cations. This result was in contrast to effects obtained with nickel, magnesium, iron, copper and aluminum ions.

Goldman[13] has studied the inhibition of alanine dehydrogenase by oxytetracycline. He noted that crude

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