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Bradykinin: Configurations of the Arginine Moieties and Biological Activity*

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ABSTRACT: Three new analogs of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) were prepared which contained arginine in the D configuration: 1-D-arginine, 1,9-bis-D-arginine, and 9-D-arginine bradykinin. The last analog was found to be contaminated with about 5% of bradykinin, but treatment with carboxypeptidase B eliminated this impurity. Bioassay of the three analogs in the dog hindquarters has shown them to have negligible vasodilatory activity. These results indicate that the two terminal arginines of bradykinin must be of the L configuration in order for the molecule to exhibit its characteristic biological effects. The analogs did not show any inhibitory effects to bradykinin.

The importance of the bradykinin C-terminal arginine (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) for biological activity has previously been demonstrated since its removal results in significant loss of activity (Elliott *et al.*, 1960; Nicolaides *et al.*, 1963a). It has also been shown that synthetic bradykinin is destroyed in blood by a carboxypeptidase-type enzyme that cleaves the C-terminal arginine (Erdös, 1961). This enzymatic inactivation process is very rapid and probably contributes heavily to the extremely brief biological half-life of bradykinin which has been found to be less than 0.5 minute in man (Saameli and Eskes, 1962) and 0.3 minute in the dog (D. A. McCarthy, D. E. Potter, and E. D. Nicolaides, submitted for publication).

In an effort to circumvent this rapid carboxypeptidase inactivation it appeared reasonable that introduction of an unnatural peptide bond at the point of attack would increase the biological half-life of the kinin. A somewhat similar approach has recently been described (Stewart and Woolley, 1964) for bradykinin in which the 8-phenylalanine was replaced by leucine. Although one would expect this type of peptide bond to be resistant to a chymotrypsinlike attack it would not be expected to provide protection from a carboxypeptidase cleavage.

Experimental

A half-life study of the 9-D-arginine analog would indicate the extent to which an aminopeptidase or other enzymatic attack was contributing to the inactiva-

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TABLE I: Synthesis of 9-D-Arginine and 1,9-Bis-D-arginine Bradykinin.[a]

Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg
								NO_2
							Z– –ONP	– –OCH_3
								NO_2
						Z– –ONP	–	– –OCH_3
								NO_2
				Z–	–	– N_3	–	– –OCH_3
								NO_2
			Z– –ONP	–	–	–	–	– –OCH_3
					OAc			NO_2
		Z– –ONP	–	–	–	–	–	– –OCH_3
					OAc			NO_2
	Z– –ONP	–	–	–	–	–	–	– –OCH_3
					OAc			NO_2
Triz– –ONP	–	–	–	–	–	–	–	– –OCH_3
								NO_2
Diz–	–	–	–	–	–	–	–	– ––OH
H–	–	–	–	–	–	–	–	– ––OH

[a] Z = carbobenzoxy; NP = *p*-nitrophenyl; Ac = acetyl.

tion of the kinin. The 1,9-bis-D-arginine analog was prepared to overcome a combined enzyme attack at both ends of the molecule.

The participation of the N-terminal amino acid in the biological activity of bradykinin has been well explored (Ondetti, 1963; Schröder, 1964; Nicolaides *et al.*, 1963b). Such major changes as substitution with other amino acids or even complete removal of the arginine from the molecule have resulted only in a reduction in kinin potency. The extent to which these changes reflect a change in receptor affinity or to intrinsic activity upon the receptors involved in producing the biological effect has been difficult to assess largely owing to the great variation in analog activities.

The possibility that the D-arginine analogs might be inactive was considered but previous work in the bradykinin area with D-amino acids (De Wald *et al.*, 1963; Nicolaides *et al.*, 1963c) showed that activity could be retained albeit to a lesser degree than the parent substance.

Chemistry. The 9-D-arginine and the 1,9-bis-D-arginine bradykinins were synthesized by the procedure used for bradykinin (Nicolaides and De Wald, 1961) which is shown in Table I. The 1-D-arginine analog was prepared by condensing the *p*-nitrophenyl ester of carbobenzoxynitro-D-arginyl-L-proline with the heptapeptide L-prolylglycyl-L-phenylalanyl-*O*-acetyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine methyl ester after the method of Boissonnas *et al.* (1960). The required N-terminal dipeptide was obtained in poor yield by a carbodiimide coupling between carbobenzoxynitro-D-arginine and L-proline *p*-nitrophenyl ester hydrobromide.

Peptide mapping of the carboxypeptidase B–treated 9-D-arginine and 1,9-bis-D-arginine analogs was done using paper electrophoresis and staining with ninhydrin and Sakaguchi's reagent. A few mg of the enzyme-treated analogs was obtained for a more detailed bioassay by peparative paper electrophoresis.

Bioassay. The potency of synthetic bradykinin was compared with that of the analogs containing D-arginine using vasodilation in the dog autoperfused hindquarters preparation. The same preparation was used to estimate the biological half-life of each of these polypeptides. The method has been described in detail elsewhere (D. A. McCarthy, D. E. Potter, and E. D. Nicolaides, submitted for publication). In brief, the test depends upon a vasodilatation produced in the hindquarters of the dog following the intraarterial injection of small quantities of the test substances dissolved in 0.1 ml H_2O. The hindquarters of each animal was perfused with its own blood by means of constant-output pump and the degree of vasodilatation was ascertained by measuring the fall in perfusion pressure

induced by the injected kinin. Delay coils interposed in the extracorporeal circuit maintained at body temperature permitted the kinins to be incubated for various periods of time with the animal's own blood before return to the hindquarters and eliciting a response proportional to the concentration of inactivated kinin remaining in the blood.

TABLE II: Biological Activity of the D-Arginine Analogs.

Material	No. of Dogs	Mean Potency (relative to bradykinin)	No. of Dogs	Mean $t^{1/2}$ (min) in Dog Blood
9-D-Arginine bradykinin (raw)	2	4×10^{-2}	5	0.28
9-D-Arginine bradykinin (treated with carboxypeptidase B)	1	3×10^{-4}	1	0.30
1,9-Bis-D-arginine bradykinin (raw)	3	10^{-4}	4	0.28
1,9-Bis-D-arginine bradykinin (treated with carboxypeptidase B)	1	2×10^{-5}	1	0.27
1-D-Arginine bradykinin (raw)	1	6×10^{-4}	1	0.24
Pure synthetic bradykinin	8	1	7	0.27

Results and Discussion

The results of the biological assays for potency and half-lives of the three D-arginine bradykinins are given in Table II. The 9-D-arginine analog in the dog gave an activity equal to 0.04 of bradykinin with no apparent change in half-life from that of bradykinin. This anomalous result indicated either that the kinins could be rapidly destroyed in blood by processes other than those dependent upon carboxypeptidase or that the peptide was contaminated with a small amount of bradykinin and was, in fact, inactive. To eliminate the latter possibility a few mg of the analog was treated with carboxypeptidase B–DFP. Samples were withdrawn every 5 minutes for 30 minutes and also at the end of 1 hour. A portion of each sample was immediately injected intraarterially into the dog preparation and/or also spotted on paper. Although paper electrophoresis showed no change in the character or mobility of the 9-D-arginine analog at the end of the first 5 minutes, the vasodilating activity became negligible. However, it was of interest to note that at the end of 10 minutes other ninhydrin-detected spots appeared, and at the end of 20 minutes these spots became very prominent. Although no effort was made to determine the identity of these spots, they are believed to be caused by cleavage of the peptide at the Pro—Phe bond or, less likely, to self-digestion of the enzyme. No arginine could be detected but a faint yellow spot corresponding to proline was evident. A control sample containing only enzyme gave one spot with ninhydrin after 20 minutes.

A control experiment using bradykinin as a substrate for carboxypeptidase B was also done; and paper electrophoresis demonstrated that destruction of the peptide was complete in less than 5 minutes, arginine, proline and phenylalanine being present, and also the octapeptide 9-desarginine bradykinin.

A study of the biological activities of the 1,9-bis-D-arginine and 1-D-arginine analogs showed them to be essentially inactive. The 1,9-D analog was found to have 1×10^{-4} the activity of bradykinin and the 1-D analog was of the order of 1.7×10^{-3} of bradykinin. Since the synthetic schemes for the three peptides were similar it is believed that elimination of the contaminating L isomer in the 1-D and 1,9-bis-D analogs was achieved during purification of the intermediates. The presence of 5% of L isomer is probably owing to racemization during synthesis. It is important that great care be used in the synthesis of D analogs of extremely potent peptides such as bradykinin or angiotensin since optical impurities will give erroneous biological results and will prevent the correct interpretation of receptor-site mechanisms.

An attempt was made to determine half-lives of all the analogs even though their activities were of such low orders of magnitude that the results have little meaning. The injection of large doses of the D analogs directly into the dog hindquarters does produce a vasodilatation, but to interpret these responses as kininlike is of little merit.

The negligible activity of the three D-arginine analogs demonstrates the stereospecificity of the N- and C-terminal amino acids of the bradykinin molecule. Scherrer (1964) has recently proposed a receptor-site mechanism for bradykinin which involves the C-terminal sequence Phe-Arg. This model has been used to explain the antibradykinin activities of certain antiinflammatory anthranilic acids. The results from the present study indicate that at least two receptor sites are required for vasodilator activity and that the correct spatial configuration of the terminal ends of the peptide chain is an indispensable feature of the molecule.

Experimental

Melting points were taken using a Thomas-Hoover capillary melting point apparatus and are corrected.

Carbobenzoxy-L-phenylalanylnitro-D-arginine Methyl Ester (I). To a cold (5°) solution of 20 g (0.074 mole) of nitro-D-arginine methyl ester hydrochloride, mp 157–158°, $[\alpha]_D^{24}$ −15.3° (*c*, 2; methanol); reported (Gibian and Schröder, 1961), mp 154–156°, $[\alpha]_D^{24}$ −14.7° (*c*, 2; methanol) in 100 ml of dimethylformamide was added 8 g (0.08 mole) of triethylamine. The precipitate was removed and to the filtrate was added 32 g (0.076 mole) of carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester. The solution was stirred for 3 days at room temperature and was evaporated to a thick oil which was taken up in 300 ml of ethyl acetate. The ethyl acetate solution was washed several times with 5% sodium carbonate solution and once with dilute hydrochloric acid, and dried over magnesium sulfate. The ethyl acetate was evaporated and ether was added giving a gummy solid which was crystallized from ethyl acetate–ether, yield of white solid, 37 g (97.5%), mp 85–90°, $[\alpha]_D^{24}$ +5° (*c*, 2; dimethylformamide).

Anal. Calcd for $C_{24}H_{30}N_6O_7$: C, 56.03; H, 5.87; N, 16.33. Found: C, 56.40; H, 6.00; N, 16.18.

Carbobenzoxy-L-prolyl-L-phenylalanylnitro-D-arginine Methyl Ester (II). Carbobenzoxy-L-phenylalanylnitro-L-arginine methyl ester (32 g, 0.062 mole) was dissolved in 250 ml of glacial acetic acid containing 30 g of dry hydrogen bromide. The solution was kept 2 hours at 25° and poured into 1 liter of vigorously stirred dry ether. The precipitate was collected by filtration and dried *in vacuo*. The crude product, 40 g, was dissolved in 150 ml of dimethylformamide and cooled to 0°, and 20 g of triethylamine was added. The precipitate was removed and 25 g (0.067 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester was added to the filtrate. The solution was stirred for 2 days at 25° and evaporated to an oil; the oil was taken up in ethyl acetate, and the ethyl acetate solution was washed with 5% sodium carbonate solution, water, and dilute hydrochloric acid, and dried. The solvent was removed and ether was added, yielding a cream-colored solid which was reprecipitated from ethyl acetate with ether; yield, 31 g (83%), mp 90–100°, $[\alpha]_D^{24}$ −42° (*c*, 2; dimethylformamide).

Anal. Calcd for $C_{29}H_{27}N_7O_8$: C, 56.94; H, 6.10; N, 16.03. Found: C, 56.78; H, 6.06; N, 15.85.

Carbobenzoxy-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-D-arginine Methyl Ester (III). The carbobenzoxy group was removed from 21 g (0.035 mole) of tripeptide II with HOAc-HBr, giving 24 g of crude product which was dissolved in 75 ml of cold (5°) dimethylformamide. To this solution was added 10 g (0.1 mole) of triethylamine, the precipitate was removed, and the filtrate was added to a cold ethyl acetate solution containing 0.035 mole of carbobenzoxy-L-phenylalanyl-L-seryl azide. The solution was stirred 2 days at 4° and evaporated to a small volume, and the residue was taken up in fresh ethyl acetate. The solution was washed with water, dilute sodium bicarbonate solution, water, and dilute hydrochloric acid, dried, and evaporated to a small volume, and ether was added. The precipitate was reprecipitated from ethyl acetate–methanol–ether as a cream-colored solid, mp 115–120°, $[\alpha]_D^{24}$ −33° (*c*, 1; dimethylformamide); yield, 24 g (83%).

Anal. Calcd for $C_{41}H_{51}N_9O_{11}$: C, 58.21; H, 6.08; N, 14.90. Found: C, 58.39; H, 5.88; N, 14.90.

Carbobenzoxyglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanylnitro-D-arginine Methyl Ester (IV). Removal of the carbobenzozy group from the pentapeptide III was effected with HOAc-HBr. From 39 g (0.047 mole) of pentapeptide was obtained 56 g of crude hydrobromide which was dissolved in 150 ml of dimethylformamide. The solution was cooled to 0° and 20 g of triethylamine was added. After 5 minutes the mixture was filtered and 18 g (0.0485 mole) of carbobenzoxyglycine *p*-nitrophenyl ester was added to the filtrate. The solution was stirred for 5 days at 30–35° and evaporated to a thick yellow oil, and 200 ml of ethyl acetate was added. A crystallized solid was removed, washed with ethyl acetate, ethanol, and ether; yield of white solid, 24 g (53%), mp 204–205°, $[\alpha]_D^{24}$ −63° (*c*, 1; dimethylformamide).

Anal. Calcd for $C_{45}H_{56}N_{10}O_{13}$: C, 57.20; H, 5.97; N, 14.83. Found: C, 56.85; H, 5.99; N, 14.87.

Carbobenzoxy-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanylnitro-D-arginine Methyl Ester (V). From 10 g (0.0106 mole) of carbobenzoxy-hexapeptide IV there was obtained 13 g of crude hexapeptide hydrobromide after treatment with HOAc-HBr (100 ml/10 g). The white solid was dissolved in 75 ml of dimethylformamide and cooled to 0°, and 5 g of triethylamine was added. The mixture was filtered after 5 minutes and 4 g (0.0108 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester was added to the filtrate. The solution was stirred for 3 days at 25° and evaporated to an oil, and addition of 200 ml of ethyl acetate produced a solid which was removed and recrystallized from ethanol–ethyl acetate as a white solid, mp 170–172°, $[\alpha]_D^{24}$ −68.3° (*c*, 1; dimethylformamide); yield, 10 g (91%).

Anal. Calcd for $C_{50}H_{63}N_{11}O_{14}$: C, 57.62; H, 6.09; N, 14.78. Found: C, 57.40; H, 6.35; N, 14.89.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanylnitro-D-arginine Methyl Ester (VI). The carbobenzoxyheptapeptide V (10 g, 0.0096 mole) was dissolved in 100 ml of glacial acetic acid containing 10 g of dry hydrogen bromide. After 2 hours the clear solution was poured into 1 liter of dry ether; the precipitate was removed, dried *in vacuo*, and dissolved in 50 ml of cold dimethylformamide. Four g of triethylamine was added to the solution, the mixture was filtered after 5 minutes, and 4 g (0.0108 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester was added to the filtrate. The solution was stirred for 5 days at 30° and evaporated to a thick oil, and ether was added giving a solid which was washed several times with ether and twice with warm ethyl acetate. The product was dissolved in 30 ml of ethanol and precipitated as a cream-colored solid by the addi-

tion of ether; yield, 9 g (82.5%), mp 115–125°, $[\alpha]_D^{24}$ −63° (*c*, 1.9; dimethylformamide).

Anal. Calcd for $C_{55}H_{70}N_{12}O_{15} \cdot 2\ H_2O$: C, 56.09; H, 6.35; N, 14.31. Found: C, 56.54; H, 6.41; N, 14.44.

*Tricarbobenzoxy-*L*-arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-O-acetyl-*L*-seryl-*L*-prolyl-*L*-phenylalanyl-nitro-*D*-arginine Methyl Ester (VII).* From 4.5 g (0.00395 mole) of the carbobenzoxyoctapeptide VI there was obtained 5 g of the crude decarbobenzoxylated octapeptide. This product was dissolved in 20 ml of cold dimethylformamide, 2 g of triethylamine was added, the mixture was filtered, and 2.9 g (0.00415 mole) of tricarbobenzoxy-L-arginine *p*-nitrophenyl ester was added to the filtrate. After 3 days the solution was evaporated to an oil, ether was added, and the oil solidified. The solid was washed well with ether and ethyl acetate and dried; yield of cream-colored solid, 6 g (97.5%), mp 130–140°, $[\alpha]_D^{24}$ −56° (*c*, 1; dimethylformamide).

Anal. Calcd for $C_{77}H_{94}N_{16}O_{20} \cdot 2\ H_2O$: C, 57.82; H, 6.17; N, 14.02. Found: C, 57.70; H, 6.54; N, 13.96.

*Dicarbobenzoxy-*L*-arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-*L*-seryl-*L*-prolyl-*L*-phenylalanylnitro-*D*-arginine (VIII).* To a solution of 6 g. (0.0038 mole) of the nonapeptide VII dissolved in 50 ml of methanol was added 3.5 ml of 2 N sodium hydroxide. The solution was kept 1 hour at 25° and diluted with 100 ml of water, and 4 ml of 2 N hydrochloric acid was added. The solid which precipitated was removed, washed with water and warm ethyl acetate, and reprecipitated from acetonitrile with ethyl acetate as a cream-colored solid; mp 165–175°, $[\alpha]_D^{24}$ −48° (*c*, 1; dimethylformamide), yield, 4 g (76%).

Anal. Calcd for $C_{66}H_{84}N_{16}O_{17} \cdot 2\ H_2O$: C, 56.24; H, 6.29; N, 15.90; H_2O, 2.56. Found: C, 55.56; H, 6.40; N, 15.77; H_2O, 2.91.

*L-Arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-*L*-seryl-*L*-prolyl-*L*-phenylalanyl-*D*-arginine Triacetate Salt (9-*D*-Arginine Bradykinin) (IX).* A solution of 500 mg of the dicarbobenzoxynonapeptide VIII in 20 ml of glacial acetic acid, 30 ml of methanol, and 200 mg of palladium black catalyst was hydrogenated for 24 hours at room temperature and atmospheric pressure. The catalyst was removed, and the filtrate was evaporated to an oil which was taken up in 50 ml of water and freeze-dried. A cream-colored solid, 432 mg (91%), was obtained, $[\alpha]_D^{24}$ −78° (*c*, 1; water). The nonapeptide behaved as a single component on paper chromatography (isopropyl alcohol–concd ammonium hydroxide–water, 70:5:25, R_F 0.52 and 2,6-lutidine–2,4,6-collidine–water, 1:1:1, R_F 0.51) and sepraphore electrophoresis (0.05 M acetate buffer, 300 volts, 2 hours) developed with bromphenol blue and Sakaguchi reagents.

Anal. Calcd for $C_{56}H_{85}N_{15}O_{17} \cdot 3\ H_2O$: C, 51.96; H, 7.09; N, 16.24. Found: C, 52.06; H, 6.85; N, 16.40.

*Tricarbobenzoxy-*D*-arginine.* This compound was prepared in the same manner as described by Zervas *et al.* (1957) for the L isomer. From 10 g of D-arginine hydrochloride there was obtained 4.5 g (17%) of the free acid, mp 132–134°.

Anal. Calcd for $C_{30}H_{32}N_4O_8$: C, 62.48; H, 5.60; N, 9.72. Found: C, 62.58; H, 5.47; N, 9.69.

*Tricarbobenzoxy-*D*-arginine p-Nitrophenyl Ester.* To a cold (0°) solution of 4 g (0.00695 mole) of tricarbobenzoxy-D-arginine in 20 ml of dimethylformamide was added 1 g (0.0072 mole) of *p*-nitrophenol and 1.5 g (0.0072 mole) of dicyclohexylcarbodiimide. The solution was kept 2 hours at 25°, filtered, and evaporated to an oil. The oil was washed with ether and crystallized from boiling ethanol as a white solid, mp 124–126°, $[\alpha]_D^{24}$ +14° (*c*, 1; dimethylformamide), yield, 2.5 g (52%).

Anal. Calcd for $C_{36}H_{35}N_5O_{10}$: C, 61.97; H, 5.06; N, 10.04. Found: C, 62.11; H, 4.98; N, 10.19.

*Tricarbobenzoxy-*D*-arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-O-acetyl-*L*-seryl-*L*-prolyl-*L*-phenylalanyl-nitro-*D*-arginine Methyl Ester (X).* To a solution of 2 g (0.00175 mole) of L-prolyl-L-prolylglycyl-L-phenylalanyl-*O*-acetyl-L-seryl-L-prolyl-L-phenylalanylnitro-D-arginine methyl ester hydrobromide in 20 ml of dimethylformamide at 5° was added 1 g of triethylamine. The solution was filtered after 5 minutes and 1.5 g (0.00214 mole) of tricarbobenzoxy-D-arginine *p*-nitrophenyl ester was added to the filtrate. The solution was kept 3 days at 25° and evaporated to a thick yellow oil, and the oil was washed with ether until a solid was obtained. The solid was washed with water, then with ether, and dried; yield, 2.5 g (91%), mp 130–135°, $[\alpha]_D^{24}$ −47° (*c*, 1; dimethylformamide).

Anal. Calcd for $C_{77}H_{94}N_{16}O_{20} \cdot 2\ H_2O$: C, 57.82; H, 6.17; N, 14.02. Found: C, 58.23; H, 6.01; N, 14.26.

*Dicarbobenzoxy-*D*-arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-*L*-seryl-*L*-prolyl-*L*-phenylalanylnitro-*D*-arginine (XI).* Hydrolysis of 2 g (0.00128 mole) of the tricarbobenzoxynonapeptide methyl ester X with 2 N sodium hydroxide in methanol gave 1.5 g (85%) of a cream-colored solid, mp 155–160°, $[\alpha]_D^{24}$ −52° (*c* 1; dimethylformamide).

Anal. Calcd for $C_{66}H_{84}N_{16}O_{17} \cdot 2\ H_2O$: C, 56.24; H, 6.29; N, 15.90. Found: C, 56.18; H, 6.13; N, 15.93.

*D-Arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-*L*-seryl-*L*-prolyl-*L*-phenylalanyl-*D*-arginine Triacetate Salt (1,9-Bis-*D*-arginine Bradykinin (XII).* Catalytic hydrogenation of 400 mg (2.9×10^{-4} mole) of the dicarbobenzoxynonapeptide XI in the manner previously described yielded, after lyophilization, 341 mg (90%) of cream-colored solid. Paper chromatography (isopropyl alcohol–concd ammonium hydroxide–water, 70:5:25, R_F 0.65) and paper electrophoresis (0.05 M Tris buffer, *p*H 8.0, 2 hours at 300 volts) developed with bromphenol blue and Sakaguchi reagents indicated the peptide to be homogenous.

Anal. Calcd for $C_{56}H_{85}N_{15}O_{17}$: C, 54.22; H, 6.91; N, 16.94. Found: C, 53.11; H, 6.89; N, 17.56.

L-Proline p-Nitrophenyl Ester Hydrobromide. Into a solution of 7 g (0.019 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester in 50 ml of glacial acetic acid was bubbled 5 g of hydrogen bromide gas. Carbon dioxide was vigorously evolved and a crystalline solid formed in *ca.* 15 minutes. After 30 minutes the mixture was poured into ether; the needles were removed, washed with ether, and dried; yield, 6 g (100%), mp 194–195°.

Anal. Calcd for $C_{11}H_{13}BrN_2O_4$: C, 41.66; H, 4.13; N, 8.83. Found: C, 41.77; H, 4.27; N, 8.74.

*Carbobenzoxynitro-*D*-arginyl-*L*-proline p-Nitrophenyl Ester (XIII).* To a solution of 5.8 g (0.0183 mole) of L-proline *p*-nitrophenyl ester–HBr in 40 ml of dimethylformamide at 0° was added 1.9 g of triethylamine. The precipitate was removed and 6 g (0.017 mole) of carbobenzoxynitro-D-arginine and 3.6 g (0.0174 mole) of dicyclohexylcarbodiimide were added to the filtrate. The solution was stirred at 30° for 18 hours and evaporated to 25 ml, and ether was added. The yellow oil which formed was taken up in ethyl acetate, washed with 5% sodium bicarbonate solution, water, and dilute hydrochloric acid, and dried, and ether was added giving 2 g (16%) of a yellow solid, mp 70–80°, $[\alpha]_D^{24}$ −59° (*c*, 1; methanol).

Anal. Calcd for $C_{25}H_{29}N_7O_9$: C, 52.50; H, 5.10; N, 17.20. Found: C, 53.28; H, 5.24; N, 17.13.

*Carbobenzoxynitro-*D*-arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-O-acetyl-*L*-seryl-*L*-prolyl-*L*-phenylalanyl-nitro-*L*-arginine Methyl Ester (XIV).* The reaction between the dipeptide XIII (2.5 g, 0.0035 mole) and the heptapeptide prolylglycylphenylalanyl-*O*-acetylserylprolylphenylalanylnitroarginine methyl ester (3.3 g, 0.0035 mole) was carried out in dimethylformamide for 4 days at 30°. The product was isolated by adding ether and recrystallizing from hot ethanol; yield, 2 g (43%), mp 160–175° (slow decomp), $[\alpha]_D^{24}$ −62.5° (*c*, 1; dimethylformamide).

Anal. Calcd for $C_{61}H_{81}N_{17}O_{18} \cdot 2\ H_2O$: C, 53.23; H, 6.23; N, 17.30. Found: C, 53.56; H, 6.22; N, 16.79.

D-*Arginyl-*L*-prolyl-*L*-prolylglycyl-*L*-phenylalanyl-*L*-seryl-*L*-prolyl-*L*-phenylalanyl-*L*-arginine Triacetate Salt (XV) (1-*D*-Arginine Bradykinin).* The methyl ester was removed from the nonapeptide XIV (1.7 g) with 1 N sodium hydroxide in methanol. The methanol was evaporated, and acidification with 1 N hydrochloric acid gave a solid which was recrystallized from ethanol, 700 mg. One-half of this product was hydrogenated for 24 hours in methanol–acetic acid (30 ml/10 ml) using palladium-black catalyst and atmospheric pressure. The product was isolated by evaporation of the methanol, addition of water, and freeze-drying, 270 mg of cream-colored solid.

Paper chromatography (isopropyl alcohol–ammonium hydroxide–water, 70:5:25, R_F 0.48; *t*-butyl alcohol–acetic acid–water, 2:1:1, R_F 0.8; 2,6-lutidine–2,4,6-collidine–water, 1:1:1, R_F 0.24) and paper electrophoresis (*p*H 5.6, 250 volts, 0.05 M ammonium acetate buffer) developed with Sakaguchi's reagent and bromphenol blue both gave single spots.

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, 51.08; H, 7.19; N, 15.85; H_2O, 4.07.

*Carboxypeptidase Treatment of Bradykinin, 9-*D*-Arginine, and 1,9-Bis-*D*-arginine Bradykinin.* In a typical experiment, 3 mg of the peptide was dissolved in 0.2 ml of 0.05 M Tris buffer at 37°, *p*H 7.8, and 0.05 ml of a solution of carboxypeptidase B, DFP treated (6.1 mg/ml) (Worthington), was added. At 5-minute intervals 0.015-ml samples were withdrawn and either spotted on paper strips or used for bioassay. The paper strips were then subjected to electrophoresis in 0.05 M ammonium acetate buffer at *p*H 5.6, 250 volts, for 2.5 hours. Control strips of arginine, phenylalanine, proline, and 9-desarginine bradykinin were run concurrently. The strips were dried and the spots were developed with ninhydrin and Sakaguchi's reagent. For preparative purposes, 20-mg samples of the two D analogs were treated with 0.18 mg of the enzyme for 10 minutes. The solution was heated to boiling and then spotted on 40 paper strips. After electrophoresis the strips were cut at the appropriate place and extracted with water. The product was isolated by freeze-drying, 12.7 mg. Paper chromatography of the product and the starting material in two different solvent systems gave identical R_F values.

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