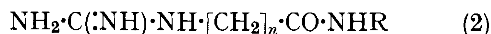
Synthesis of Analogues of Bradykinin with Replacement of the Arginine Residues by 4-Guanidinophenyl-L-alanine

By **Stanley Moore** and **Harry D. Law,** Department of Chemistry, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF
Derek E. Brundish, Donald F. Elliott, and **Roy Wade,*** CIBA Laboratories, Horsham, Sussex RH12 4AB

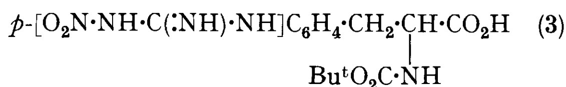
The synthesis is described of three analogues of bradykinin in which the terminal arginine residues were separately or together replaced by 4-guanidinophenyl-L-alanine. The analogues were considerably less potent than the parent peptide in their action on smooth muscle *in vitro*.

ANALOGUE studies of the local tissue hormone bradykinin have indicated that a guanidino-function in the side chain of each terminal amino-acid is essential for high biological activity. Replacement of guanidino by a less strongly basic amino-group leads to compounds possessing significant activity but of a lower potency.[1] Bradykinin itself can be generated by tryptic fission of a precursor molecule and presumably is less well bound to the trypsin receptor than is its precursor. Possibly bradykinin possesses only a portion of the structural requirements of the trypsin receptor. However the common requirement of the trypsin receptor and the receptor responding to bradykinin for basic side chains led us to consider whether other characteristics were possessed in common by the two receptor types. It is well known that compounds such as 4-aminobenzamidine dihydrochloride [2] and some guanidine derivatives [3] (1) are competitive inhibitors of trypsin. Moreover the latter group of compounds have similarities to other compounds (2) which exhibit anti-bradykinin activity.[4] The present study was therefore directed to the preparation of molecules of the bradykinin type possessing some feature of the known competitive inhibitors of trypsin. In addition their biological activities were measured to ascertain whether such structural modification would generate bradykinin antagonistic activity. We now describe the synthesis of three analogues of bradykinin in which the terminal arginine residues are separately or together replaced by *p*-guanidinophenyl-L-alanine.

p-[NH_2·C(:NH)·NH]C_6H_4·CO_2·[CH_2]$_n$R (1)



NH_2·C(:NH)·NH·[CH_2]$_n$·CO·NHR (2)



p-[O_2N·NH·C(:NH)·NH]C_6H_4·CH_2·CH($NHCO_2Bu^t$)·CO_2H (3)

p-Guanidinophenylalanine (4) has been prepared in racemic form previously by one of us [5] using a malonate synthesis, but an alternative route was developed starting from N^{α}-butoxycarbonyl-*p*-nitrophenyl-L-alanine in order to avoid a resolution step and to introduce functional groups suitably protected for subsequent peptide synthesis. Catalytic reduction of the nitro-group was followed by conversion into nitroguanidine with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [6] or the modified Habeeb reagent,[7] the former being preferred for practical reasons. The intermediate (3) containing protected α-amino and side-chain guanidino-groups was tested in two model peptide couplings and was then used for the introduction of the required amino-acid residue at the *N*-termini of the nonapeptides. The same intermediate (3) was converted into the benzyl ester,[8] retaining side-chain protection, for use in the *C*-terminal position (see Scheme 1).

Couplings were carried out stepwise from the *C*-terminus, most frequently using butoxycarbonylamino-acid hydroxysuccinimide esters [9] or hydroxysuccinimide-assisted dicyclohexylcarbodi-imide. Serine, with the side chain unprotected, was coupled by the azide method. Physical characteristics of the oligopeptide intermediates are given in Table 1. Peptides were deprotected with trifluoroacetic acid and the resulting peptide esters were precipitated as hydrochlorides by addition of ether to a solution in benzyl alcohol containing hydrogen chloride (Table 2). Final nonapeptides were deprotected first by treatment with trifluoroacetic acid and then by hydrogenation over palladium–charcoal. Purification was effected by ion-exchange chromatography on carboxymethylcellulose.

An outline of the synthesis of [9-(guanidinophenylalanine)]-bradykinin is given in Scheme 2. The analogue containing two amino-acid replacements was obtained

[1] E. D. Nicolaides, H. A. De Wald, and M. K. Craft, *J. Medicin. Chem.*, 1963, **6**, 739; E. Schröder, *Annalen*, 1964, **673**, 220; E. Schröder, H. S. Petras, and E. Klieger, *ibid.*, 1964, **679**, 221; E. Wunsch, H. G. Heidrich, and W. Grassmann, *Chem. Ber.*, 1964, **97**, 1818.

[2] M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, 1965, **240**, 1579.

[3] H. Mix and H. J. Trettin, Ger. Offen. 1905813 (*Chem. Abs.*, 1970, **72**, 1,003,364).

[4] S. Hashimoto, K. Okada, R. Sakakibara, and S. Fuji, Ger. Offen. 2015650 (*Chem. Abs.*, 1970, **73**, 130,649m).

[5] D. F. Elliott and C. R. Harington, *J. Chem. Soc.*, 1949, 1374.

[6] A. F. McKay, *J. Amer. Chem. Soc.*, 1949, **71**, 1968.

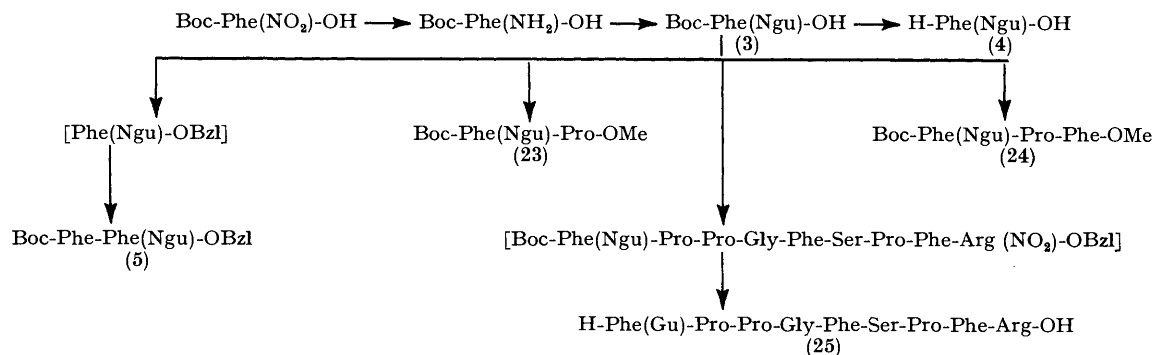
[7] A. F. S. A. Habeeb, *Biochim. Biophys. Acta*, 1964, **93**, 533.

[8] J. D. Cipera and R. V. V. Nicholls, *Chem. and Ind.*, 1955, 16.

[9] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, 1964, **86**, 1839.

by coupling the octapeptide ester (18) with butoxycarbonyl(nitroguanidinophenyl)-L-alanine (3) and proceeding in the usual way. [1-(Guanidinophenylalanine)]-bradykinin was prepared from the normal sequence bradykinin octapeptide by coupling with (3) followed by deprotection as before.

sample of (3) was hydrolysed with 6N-acid and the products were separated and characterised as described in the Experimental section. Tyrosine, chlorotyrosine, aminophenylalanine, and guanidinophenylalanine were the major hydrolysis products identified, which are clearly derived from p-nitroguanidinophenylalanine.



SCHEME 1

TABLE 1
Protected peptides †

No.	Compound	M.p. (°C)	[α]D (°)	c [a]	Yield (%)	Found (%) C	H	N	Formula	Required (%) C	H	N
(7)	Boc-Pro-Phe-Phe(Ngu)-OBzl	85—90 [b]	−15.4	0.13	85	61.8	6.0	13.8	$C_{36}H_{43}N_7O_8$	61.6	6.2	14.0
(9)	Boc-Ser-Pro-Phe-Phe(Ngu)-OBzl	90—100 [b]	−26.8	0.1	58	59.3	6.0	14.0	$C_{39}H_{48}N_8O_{10}$	59.4	6.1	14.2
(11)	Boc-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	136—138 [b]	−28.2	0.12	64	61.4	6.5	13.5	$C_{48}H_{57}N_9O_{11}$	61.6	6.1	13.5
(13)	Boc-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	134—136 [b]	−8.0	0.1	83	60.4	6.0	14.0	$C_{50}H_{60}N_{10}O_{12}$	60.5	6.1	14.1
(15)	Boc-Pro-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	145—150 [b]	−34.8	0.6	79	60.8	6.1	14.0	$C_{55}H_{67}N_{11}O_{13}$	60.6	6.2	14.1
(17)	Boc-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	140—145 [c]	−39.5	0.7	57	60.6	6.5	14.0	$C_{60}H_{74}N_{12}O_{14}$	60.7	6.3	14.2
(19)	Z-Arg(NO$_2$)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	149—152 [d]	−46.3	0.2	46	58.3	5.8	16.6	$C_{69}H_{83}N_{17}O_{17}$	58.3	5.9	16.7
(21)	Boc-Phe(Ngu)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	180—182 [e]	−80.0	0.2	23	58.2	5.8	16.6	$C_{70}H_{85}N_{17}O_{17}$	58.5	6.0	16.6
(23)	Boc-Phe(Ngu)-Pro-OMe	138—140 [b]	−14.0	0.3	69	52.9	6.4	17.4	$C_{21}H_{30}N_6O_7$	52.7	6.3	17.6
(24)	Boc-Phe(Ngu)-Pro-Phe-OMe	110—112 [b]	−11.0	0.8	61	57.4	6.1	15.5	$C_{30}H_{39}N_9O_8$	57.6	6.3	15.7

[a] In DMF. [b] From EtOAc–light petroleum. [c] From $CHCl_3$–Et_2O. [d] From $CHCl_3$–light petroleum. [e] From MeOH–Et_2O.

† Abbreviations follow those given by the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature reprinted in Chem. Soc. Specialist Periodical Reports, vol. 4, ed. G. T. Young, p. 441, and vol. 5, ed. R. C. Sheppard, p. 476; also Phe(Gu) = 4-guanidinophenyl-L-alanine; Phe(Ngu) = 4-nitroguanidinophenyl-L-alanine.

TABLE 2
Peptide hydrochlorides

No.	Compound	Yield (%)	M.p. (°C)	[α]D (°)	c in DMF
(6)	Phe-Phe(Ngu)-OBzl	80	103—105	−4.1	1.1
(8)	Pro-Phe-Phe(Ngu)-OBzl	95	130—135	+6.4	0.11
(10)	Ser-Pro-Phe-Phe(Ngu)-OBzl	98	148—150	−24.8	0.8
(12)	Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	94	144—146	−12.2	1.0
(14)	Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	86	158—160	−17.0	0.2
(16)	Pro-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	100	155—158	−23.6	0.6
(18)	Pro-Pro-Gly-Phe-Ser-Pro-Phe-Phe(Ngu)-OBzl	94	173—175	−37.6	0.17

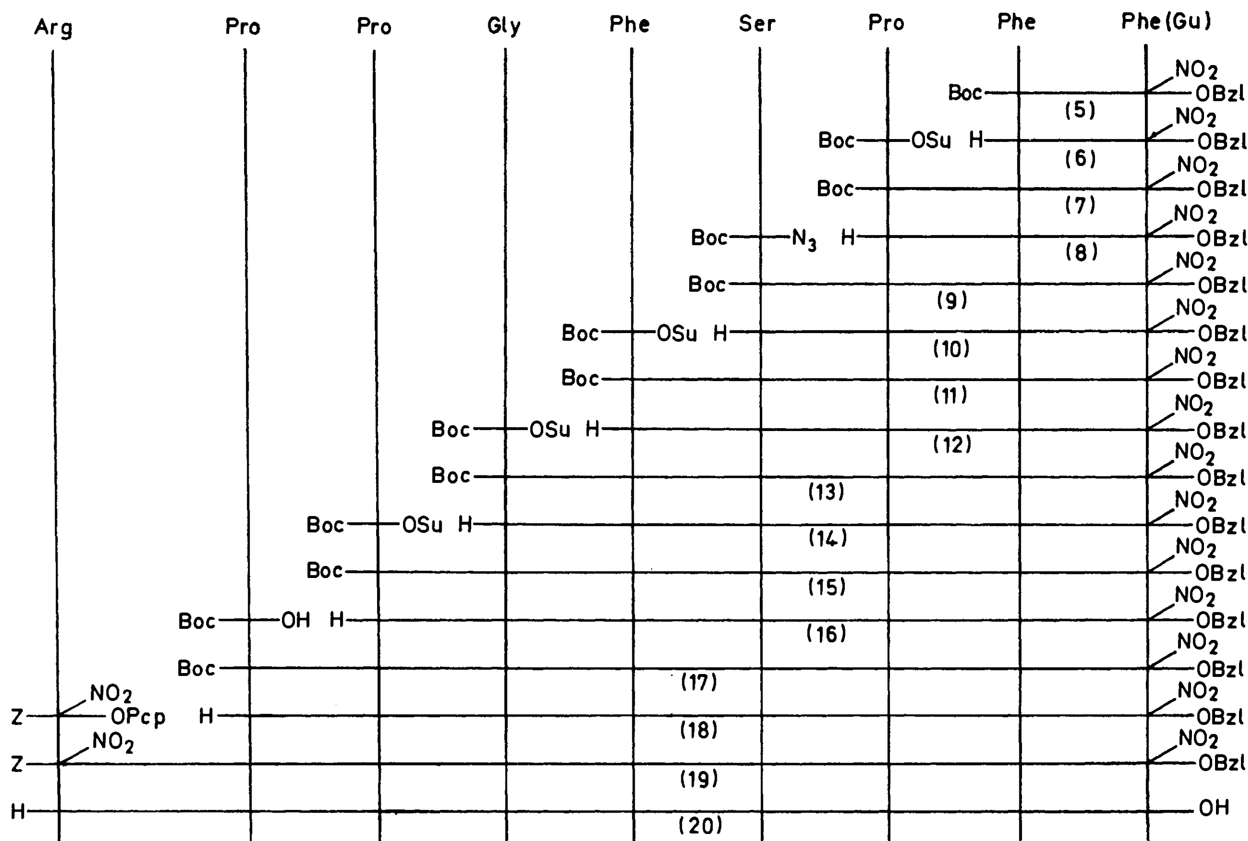
Amino-acid analysis was used as an additional means of characterising protected peptides during the later stages of the synthesis. When guanidinophenylalanine was present protected as the nitro-derivative, acidic hydrolysis prior to analysis caused several new peaks to appear on the analyser trace. In order to elucidate the nature of these ninhydrin-positive materials a

The three nonapeptides were obtained in solid form only by drying from the frozen state. Such materials contain variable amounts of water, a portion of which may be bound quite firmly. It is not always possible to interpret the results of elemental microanalysis in these cases in a straightforward manner. The preferred criteria are the amino-acid analysis data presented, in

which the values for the individual amino-acid contents are close to the theoretical values. The amino-acid content of an aliquot portion of a solution of a freeze-dried sample was used as the basis for calculating biological activity; this avoids the pitfalls of attempting to weigh a freeze-dried product of unknown and variable moisture content.

The three nonapeptides were tested *in vitro* for bradykinin-like and antibradykinin activity on smooth muscle. On guinea-pig ileum all three showed similar potencies of 1—3% of that of bradykinin. However on rat uterus the 1-, 9-, and 1,9-bis-substituted analogues revealed potencies of 2, 0.1, and 0.001% of that of bradykinin. No analogue showed any anti-bradykinin activity.

A possible reason for the sharp reduction in potency in tests on smooth muscle may lie in the rigidity introduced into the side chain by the incorporation of a benzene ring. This ring is presumably conjugated with the guanidino-group to make a large coplanar system which prevents the latter group from taking up many conformations relative to the peptide chain. In actual distance from the chain a closer aliphatic analogue would be a bradykinin containing homoarginine rather then arginine. The three analogues (1-, 9-, and 1,9-) containing homoarginine have been prepared by Arold and his collaborators [10] and were found [11] to possess activity much closer to that of bradykinin than the analogues in the present study. We ourselves have examined one of these analogues ([1-homoarginine]-bradykinin) and have confirmed this result. Arold and his collaborators also examined the three norarginine analogues and found these to possess potency reduced to values similar to those possessed by our guanidinophenylalanine analogues. The conclusion may therefore be drawn that for high activity the guanidino-group must be located beyond a certain minimum distance from the peptide chain. If the distance is significantly greater than this minimum, as in the case of homoarginine, the flexibility of the aliphatic chain can accommodate the extra distance by folding. The introduction of rigidity (as by incorporation of a benzene ring) seemingly prevents folding, and a lower potency results. This hypothesis could be tested by examining the potency of analogues containing for example arginine replaced by dehydrohomoarginine or by 4-(guanidinomethyl)phenylglycine. Because of their intermediate flexibility it would be expected that bradykinin analogues containing these amino-acids would be intermediate in potency. In a similar manner replacement of guanidine by amidine, as described in Pinker and his collaborators,[12]



Scheme 2

[10] H. Arold and D. Stibenz, *J. prakt. Chem.*, 1970, **312**, 1161.

[11] S. Reissmann, I. Paegelow, and H. Arold, *Acta Biol. Med. Germ.*, 1974, **33**, 77.

[12] T. G. Pinker, G. T. Young, D. F. Elliott, and R. Wade, *J.C.S. Perkin I*, 1976, 220.

may alter fundamentally the receptor-binding direction of the side-chain basic function and this altered presentation may force a conformation on the side chain which is difficult to accommodate. This is tantamount to reducing flexibility and would be expected to give rise to a marked reduction of potency as was found to be the case.

EXPERIMENTAL

T.l.c. was carried out on silica gel G (Merck) plates, the most useful solvents being (A) Bu^nOH–AcOH–H_2O (66 : 12 : 26), (B) Bu^nOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24), (C) $CHCl_3$–MeOH (98 : 2), (D) EtOAc–MeOH (85 : 15), and (E) Bu^sOH–3% NH_3 aq. (60 : 22). After development compounds were located with chlorine and starch–KI, ninhydrin, or Sakaguchi reagent as appropriate. Electrophoresis was carried out on Whatman 3 MM paper in a Locarte high voltage apparatus with a gradient of 80 V cm^{-1}. Mobilities are expressed relative to arginine (R_{Arg}). Samples for amino-acid analysis (Beckman 120C analyser) were hydrolysed with 6N-hydrochloric acid for 16 h at 115 °C in evacuated tubes.

N-t-Butoxycarbonyl-4-nitrophenyl-L-alanine [13] was prepared from the amino-acid by the action of t-butyl azidoformate using either the procedure of Schwyzer, Sieber, and Kappeler [14] or that of Schnabel [15] (at pH 9.8). Yields were similar (65—70%) in each case.

N$^{\alpha}$*-t-Butoxycarbonyl-4-aminophenylalanine*.—The nitro-compound (8.0 g) was hydrogenated in ethyl acetate (80 ml) over 5% palladium–charcoal. When t.l.c. [system (A)] showed that starting material had disappeared the catalyst was filtered off and the solution was evaporated to dryness. The residue was recrystallised from ethyl acetate–light petroleum to give *N*$^{\alpha}$*-t-butoxycarbonyl-4-aminophenyl-L-alanine* (6.0 g, 90%), m.p. 126—128°, $[\alpha]_D$ +33.2° (*c* 1 in MeOH) (Found: C, 59.9; H, 7.15; N, 9.85. $C_{14}H_{20}N_2O_4$ requires C, 60.0; H, 6.5; N, 10.0%).

N$^{\alpha}$*-t-Butoxycarbonyl-4-(nitroguanidino)phenyl-L-alanine* (3).—A solution of the amino-compound (22.0 g) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [6] (13.2 g) in 50% aqueous ethanol (60 ml) was boiled under reflux for 20 min. The solvent was removed under reduced pressure and the residue was partitioned between aqueous N-sodium hydrogen carbonate and ethyl acetate. The layers were separated and the aqueous phase was washed again with ethyl acetate, then acidified with cold (5 °C) saturated citric acid solution. The product was extracted into ethyl acetate (3 × 100 ml) and the combined organic layers were washed 3 times with 10% brine, dried (Na_2SO_4), and concentrated. The residue was recrystallised from ethyl acetate–petroleum 1 : 1) containing a trace of methanol to give the *nitroguanidine derivative* (17.3 g, 60%), m.p. 179—180° (sinters), $[\alpha]_D$ +12.3° (*c* 1 in methanol) (Found: C, 49.0; H, 5.8; N, 19.1. $C_{15}H_{21}N_5O_6$ requires C, 49.2; H, 5.8; N, 19.1%).

An alternative procedure involved boiling under reflux a mixture of the protected amino-acid (6.0 g) with 3,5-dimethyl-1-nitroguanidinopyrazole [7] (21 g) in dioxan–water (2 : 1; 110 ml) for 6 h. Evaporation to dryness and treatment of the residue as before gave a similar yield of the required product.

N$^{\alpha}$*-t-Butoxycarbonyl-L-phenylalanyl-(4-nitroguanidinophenyl)-L-alanine Benzyl Ester* (4).—(a) *Esterification of nitroguanidinophenylalanine*. The butoxycarbonyl derivative (3) (10 g) was boiled under reflux with benzyl alcohol (15 ml) in benzene (50 ml) containing toluene-*p*-sulphonic acid (5.24 g) using a Dean–Stark trap to collect evolved water. After 5 h the solution was cooled and treated with anhydrous ether. The precipitate of impure ester toluenesulphonate was washed several times with ether by decantation and then dried; yield 8.5 g (59%).

(b) *Ester free base*. The ester salt (6.0 g) was dissolved in water–butan-1-ol–ethyl acetate (1 : 1 : 4; 50 ml) and treated with ice-cold aqueous 50% potassium carbonate (20 ml). After shaking, the phases were separated and the aqueous layer was extracted again with butanol–ethyl acetate (1 : 4; 40 ml). The combined organic layer was washed with N-sodium hydrogen carbonate solution (5 times) and with water, dried, and evaporated. Attempts to crystallise the residual oil (3.2 g) were unsuccessful, although it appeared essentially homogeneous by t.l.c.

(c) *Coupling with butyloxycarbonylphenyl-L-alanine N-hydroxysuccinimide ester*.[9] The free base (2.5 g) described above was stirred with active ester (1.5 g) in dimethylformamide (DMF) (10 ml) at 0 °C for 1 h, then overnight at room temperature. The mixture was poured into water (250 ml) and the resulting suspension was extracted with ethyl acetate (3 × 50 ml). *NN*-Dimethyltrimethylenediamine (0.1 ml) was added to the combined organic layer, which after 5 min was washed successively with cold citric acid solution, water, aqueous sodium hydrogen carbonate, and 10% brine, dried (Na_2SO_4), and evaporated to an oil (2.8 g). Chromatography of this oil (8 g) on a column of silica gel (chloroform as eluant) gave *N*$^{\alpha}$*-t-butoxycarbonyl-L-phenylalanyl-(4-nitroguanidinophenyl)-L-alanine benzyl ester* (4) (4.0 g), m.p. 80—85° [from ethyl acetate–light petroleum (b.p. 60—80 °C)], $[\alpha]_D$ −21.9° (*c* 0.28 in DMF) (Found: C, 61.7; H, 6.1; N, 13.7. $C_{31}H_{36}N_6O_7$ requires C, 61.6; H, 6.0; N, 13.9%).

4-(Nitroguanidino)phenyl-L-alanine (22).—The butoxycarbonyl derivative (3) (1.0 g) was treated with anhydrous trifluoroacetic acid (10 ml) for 1 h at room temperature. The solution was evaporated and the residue crystallised by trituration with ether. After precipitation from methanolic solution with ether the salt was dissolved in hot water, and the solution was neutralised with ammonia and allowed to cool. The crystalline amino-acid was filtered off and recrystallised (charcoal) to give *4-(nitroguanidino)phenyl-L-alanine* (0.45 g, 62%), m.p. 250° (decomp.), $[\alpha]_D$ −43.75° (*c* 1.6 in M-HCl) (Found: C, 44.8; H, 5.0; N, 26.1. $C_{10}H_{13}N_5O_4$ requires C, 44.9; H, 4.9; N, 26.2%).

Deprotection of Peptides and Isolation of Hydrochlorides.—The protected peptide was dissolved in anhydrous trifluoroacetic acid (4 ml g^{-1}) and the solution stored for 1 h at room temperature. The solvent was evaporated off, the residue triturated with dry ether, and the solid redissolved in the minimum volume of benzyl alcohol. A 4M-solution of hydrogen chloride in benzyl alcohol (4 equiv.) was added, and the mixture was poured into vigorously stirred dry ether (10—15 vols.). The hydrochloride salt was filtered off and dried (NaOH). Physical constants of the salts obtained in this way are given in Table 2.

Peptide Couplings.—The peptide hydrochloride (Table 2) was dissolved in DMF (5 ml g^{-1}) and the solution was cooled

[13] R. Schwyzer and M. Caviezel, *Helv. Chim. Acta*, 1971, **54**, 1395.

[14] R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, 1959, **42**, 2622.

[15] E. Schnabel, *Annalen*, 1967, **702**, 188.

to −5 to 0 °C. Triethylamine (1 equiv.) and the appropriate butoxycarbonylamino-acid active ester (see Scheme 2) (1 equiv.) were added, and the mixture was stirred at the same temperature for 1 h then at room temperature overnight. In other couplings the peptide salt was treated with triethylamine (1 equiv.) at −10 °C and triethylamine hydrochloride was filtered off. The filtrate was treated with a solution of the acid azide (1 equiv.) or the appropriate acid (1 equiv.), dicyclohexylcarbodi-imide (DCCI) (1.4 equiv.), and *N*-hydroxysuccinimide (2 equiv.), and stored at 4 °C overnight.

The products were isolated by pouring the mixture into a large (20-fold) volume of water and extracting 3 times with ethyl acetate or chloroform [for (17)]. The organic layer was washed successively with aqueous 5% citric acid, water, aqueous 5% sodium hydrogen carbonate, and water, then dried (Na_2SO_4), and evaporated. When present dicyclohexylurea (DCU) was removed by trituration with acetone. Products were purified by loading them in chloroform [ethyl acetate for (18)] solution on to a column of silica gel and eluting with pure solvent or solvent containing 2, 4, or 10% methanol as necessary. Fractions containing the required peptide were combined and evaporated, and the residue was recrystallised from the solvent indicated in Table 1.

L-*Arginyl*-L-*prolyl*-L-*prolylglycyl*-L-*phenylalanyl*-L-*seryl*-L-*prolyl*-L-*phenylalanyl*-(4-*guanidinophenyl*)-L-*alanine* {[9-(4-*Guanidinophenyl*)*alanine*]-*bradykinin*} (20).—The protected nonapeptide (19) (280 mg, 200 μmol) was dissolved in 80% acetic acid (10 ml) containing concentrated hydrochloric acid (0.2 ml). Palladium–charcoal (5%; 30 mg) was added and the mixture stirred for 20 min. The catalyst was filtered off and replaced by a fresh sample, and the mixture hydrogenated until hydrogenolysis was complete (2 days). After filtering off the catalyst the solution was evaporated, water (10 ml) was added and evaporated off, and the residue was stored overnight in an evacuated desiccator (NaOH). The crude product was dissolved in deionised water and loaded on to a column (40 × 2 cm) of carboxymethylcellulose which had been equilibrated with 0.05M-triethylammonium acetate (pH 5.0). The column was eluted by using a convex gradient to 0.6M-triethylammonium acetate (pH 7.0). Fractions containing pure product were combined, evaporated, and freeze dried twice from water to give [9-(4-*guanidinophenyl*)-L-*alanine*]-*bradykinin* (88 μmol, 44%), R_F (A) 0.09, R_F (B) 0.49, R_{Arg} (pH 6.1) 0.56, R_{Arg} (pH 1.8) 0.72, $[M]_D$ +870° (*c* 0.1 in H_2O); amino-acid analysis: Ser, 0.90; Pro, 3.03; Gly, 1.00; Phe, 2.03; Arg, 1.01; Phe(Gu), 1.00 (Found: C, 54.3; H, 7.1; N, 15.6. $C_{54}H_{73}N_{15}O_{11}$,3AcOH,$2H_2O$ requires C, 54.4; H, 6.8; H, 15.9%).

(4-*Guanidinophenyl*)-L-*alanyl*-L-*prolyl*-L-*prolylglycyl*-L-*phenylalanyl*-L-*seryl*-L-*prolyl*-L-*phenylalanyl*-(4-*guanidinophenyl*)-L-*alanine* {[1,9-*Bis*-(4-*guanidinophenyl*)*alanine*]-*bradykinin*} (22).—The protected nonapeptide (21) (180 mg) was dissolved in anhydrous trifluoroacetic acid and stirred for 1 h at room temperature, then the solution was evaporated to dryness. The residue solidified on trituration with ether and the solid was dissolved in 80% acetic acid (10 ml) containing conc. hydrochloric acid (0.3 ml). Palladium–carbon (5%; 25 mg) was added, the suspension stirred for 20 min then filtered, and a similar quantity of fresh catalyst added to the filtrate. The mixture was then hydrogenated, and the product was worked up and purified by chromatography on carboxymethylcellulose as described for compound (20). The 1,9-*bis-substituted bradykinin analogue* was isolated as a freeze-dried powder (45.8 μmol, 36%), R_F (A) 0.08, R_F (B) 0.55, R_{Arg} (pH 6.1) 0.51, R_{Arg} (pH 1.8) 0.64, $[M]_D$ +990° (*c* 0.1 in H_2O); amino-acid analysis: Ser, 0.86 Pro, 2.98; Gly, 1.00; Phe, 1.96; Phe(Gu) 1.93 (Found C, 57.5; H, 6.6; N, 15.6. $C_{58}H_{73}N_{15}O_{11}$,3AcOH requires C, 57.5; H, 6.4; N, 15.7%).

(4-*Guanidinophenyl*)-L-*alanyl*-L-*prolyl*-L-*prolylglycyl*-L-*phenylalanyl*-L-*seryl*-L-*prolyl*-L-*phenylalanyl*-L-*arginine* {[1-(4-*Guanidinophenyl*)*alanine*]-*bradykinin*} (25).—(a) *Coupling*. A solution of L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginine benzyl ester[16] (0.74 g), *N*-hydroxysuccinimide (0.163 g), and compound (3) (0.26 g) in DMF (4 ml) was stirred at −20°. DCCI (0.204 g) was added, and the mixture was stirred at −20° for 1 h then at room temperature for 48 h. Evaporation was followed by dissolution of the residue in chloroform; the chloroform was washed with acid and base in the usual way, dried, and evaporated. Trituration with acetone was followed by filtration to remove DCU and addition of ether to precipitate the protected nonapeptide (600 mg). The crude derivative was purified by chromatography on silica gel using increasing concentrations of methanol in chloroform; yield 0.20 g (20%).

(b) *Deprotection*. The protected nonapeptide (0.18 g) was dissolved in trifluoroacetic acid and left for 1 h. Evaporation was followed by trituration of the residue with ether. The solid was washed with ether then dissolved in 80% acetic acid (10 ml) to which conc. hydrochloric acid (0.3 ml) had been added. Hydrogenation and purification of the free peptide were as described for compound (22). [1-(4-*Guanidinophenyl*)*alanine*]-*bradykinin* was obtained as a freeze-dried powder (42 μmol, 25%) R_F (A) 0.075, R_F (B) 0.5, R_{Arg} (pH 6.1) 0.51, R_{Arg} (pH 1.8) 0.68, $[M]_D$ +887° (*c* 0.1 in H_2O); amino-acid analysis: Ser, 0.88; Pro, 2.88; Gly, 1.00; Phe, 2.02; Arg, 0.98; Phe(Gu), 0.97 (Found: C, 52.55; H, 6.7; N, 15.55. $C_{54}H_{73}N_{15}O_{11}$,3AcOH,$5H_2O$ requires C, 52.5; H, 6.95; N, 15.25%).

N^{α}-*t-Butoxycarbonyl*-(4-*nitroguanidinophenyl*)-L-*alanyl*-L-*proline Methyl Ester* (23).—A solution of L-proline methyl ester hydrochloride (0.18 g) was dissolved in the minimum volume of DMF; the solution was cooled to −5 to 0 °C and triethylamine (0.19 ml), N^{α}-t-butyloxycarbonyl-(4-nitroguanidinophenyl)-L-alanine (0.5 g), and DCCI (0.31 g) were added. After stirring overnight at 4 °C the precipitate was filtered off, the filtrate was poured into water, and the product was extracted with ethyl acetate (2 × 10 ml). The combined organic phase was washed to neutrality as described above, dried, and evaporated. The residue was dissolved in chloroform and passed through a column (10 × 1 cm) of silica gel, eluting with the same solvent. The purified product was recrystallised from ethyl acetate–light petroleum to give the dipeptide. Physical data are given in Table 1.

N^{α}-*t-Butoxycarbonyl*-(4-*nitroguanidinophenyl*)-L-*alanyl*-L-*prolyl*-L-*phenylalanine Methyl Ester* (24).—L-Prolyl-L-phenylalanine methyl ester hydrobromide (0.357 g) was dissolved in DMF (5 ml); the solution was cooled to −20 °C and triethylamine (0.14 ml) was added. Triethylamine hydrobromide was filtered off and t-butoxycarbonyl-(4-nitroguanidinophenyl)-L-alanine (0.37 g), *N*-hydroxysuccinimide (0.23 g, 2 equiv.), and DCCI (0.286) were added.

[16] W. F. Johnson, H. D. Law, and R. O. Studer, *J. Chem. Soc.* (*C*), 1971, 748.

The mixture was stirred for 1 h at −20 °C and overnight at room temperature. Removal of the solvent was followed by dissolution of the residue in chloroform. Subsequent treatment was as described in the general coupling procedure above and the *tripeptide* was recrystallised from ethyl acetate–light petroleum. Physical data are given in Table 1.

Decomposition of (4-*Nitroguanidinophenyl*)-L-*alanine by Acid*.—A solution of N^{α}-t-butoxycarbonyl-(4-nitroguanidinophenyl)-L-alanine (250 mg) in 6N-hydrochloric acid (60 ml) was boiled under reflux for 18 h then evaporated to dryness. A sample (150 mg) of the residue was passed through a short column of Dowex 1 (AcO^- form) and the eluate concentrated (to 1 ml) before applying to a column (20 × 0.9 cm) of Amberlite CG-50 (200—400 mesh; H^+ form). The column was developed by gradient elution (0—1M-AcOH). Samples of eluate were tested with ninhydrin and positive fractions were examined on the amino-acid analyser. Fractions containing individual products were pooled and concentrated. In addition to these main products two minor ninhydrin-positive substances were detected, appearing on the analyser trace between *p*-aminophenylalanine and *p*-guanidinophenylalanine, but were not characterised further.

The compounds contained in pools 1 (fractions 29—32), 3 (fractions 57—64), and 4 (fractions 81—93) were identified as tyrosine, *p*-aminophenylalanine, and *p*-guanidinophenylalanine, respectively, by comparison with standard samples run on the amino-acid analyser. For this purpose column 2 of the analyser was packed (6.7 cm) with fractionated ZeoKarb 225 sulphonated polystyrene (8% cross-linked). Elution was carried out with pH 5.28 buffer (Beckman) for pools 1 and 3 or the same molarity buffer adjusted to pH 10.1 with 4N-sodium hydroxide for pool 4. Elution times (min) and peak widths (dots) were: pool 1, 15.9, 11.1; pool 3, 19.0, 11.0; pool 4, 37.0, 21.0.

The content of pool 2 was identified by mass spectrometry of the derivative prepared by conversion into the methyl ester followed by trifluoroacetylation. Details of the relative abundances of the observed fragments were as follows [fragment, *m/e*, relative abundance (%)]: M^+, 325, 16; $(M - CO_2CH_3)^+$, 226, 6.5; $(M - CF_3CONH_2)^+$, 212, 73; $(M - CF_3CONH_2 - CH_3O)^+$, 181, 8.1; $(M - CO_2CH_3 - COCF_3)^+$, 169, 4.9; $(ArCH \cdots CH)^+$, 153, 4.9; $(ArCH_2)^+$, 141, 100. An accurate mass measurement (*m/e* 325.0316) on the molecular ion corresponded to the expected empirical formula $C_{12}H_{11}ClF_3NO_4$ for trifluoroacetylchlorotyrosine methyl ester (calc. *M* 325.0329). An ion of *m/e* 212.0243 ($C_{10}H_9ClO_3$; *M* 212.0241) was assigned to $M - CF_3CONH_2$. In confirmation, the compound contained in pool 2 exhibited the same peak elution time (19.7 min) and peak width (13.7 dots) on the amino-acid analyser (pH 5.28 buffer) as an authentic sample of 3-chlorotyrosine.

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