Synthesis of the Pentapeptide Sequence 105—109 and Smaller Fragments of Human Encephalitogenic Protein Containing the Arginine Residue 107

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Peptides corresponding to part or all of sequence 105—109 of human encephalitogenic protein, and containing the arginine residue 107, have been synthesized. The peptides were inactive as substrates for the enzyme which methylates arginine-107 in the protein, suggesting a high structural specificity for this enzyme.

A BASIC protein isolated from myelin found in the central nervous system induces autoimmune encephalomyelitis when injected, with Freunds complete adjuvant, into experimental animals. This experimental autoimmune encephalomyelitis has been proposed as a possible model of the disease multiple sclerosis. The structure of the human encephalitogenic protein has been elucidated [1,2] and a synthetic undecapeptide with the sequence 111—121 of the 170-amino-acid protein is highly encephalitogenic,[1,3] as is the closely related bovine peptide.[3] The sequence 103—121 †

† The numbering of the sequence given in ref. 2 is used throughout the present work.

[1] P. R. Carnegie, *Nature*, 1971, **229**, 25.
[2] P. R. Carnegie, *Biochem. J.*, 1971, **123**, 57.
[3] F. C. Westall, A. B. Robinson, J. Caccam, J. Jackson, and E. H. Eylar, *Nature*, 1971, **229**, 22.

of the protein has been suggested as a receptor site for serotonin in the central nervous system.[4]

The human protein, as isolated, is methylated specifically on one of the nineteen arginine residues (Arg-107),[1,5] which occurs close to the antigenic determinant and within the proposed serotonin receptor site.

This paper describes the synthesis of seven di- to penta-peptides corresponding to parts of the sequence containing arginine-107, as potential substrates for or inhibitors of the arginine-107 methylase enzyme.[5] The peptides [(I)—(VII); Scheme 1] were synthesized as shown in Scheme 2.

· · · -Lys-Gly-Arg-Gly-Leu- · · ·
105 107 109

Gly-Arg	(I)
Arg-Gly	(II)
Arg-Gly-Leu	(III)
Gly-Arg-Gly	(IV)
Lys-Gly-Arg-Gly	(V)
Gly-Arg-Gly-Leu	(VI)
Lys-Gly-Arg-Gly-Leu	(VII)

SCHEME 1

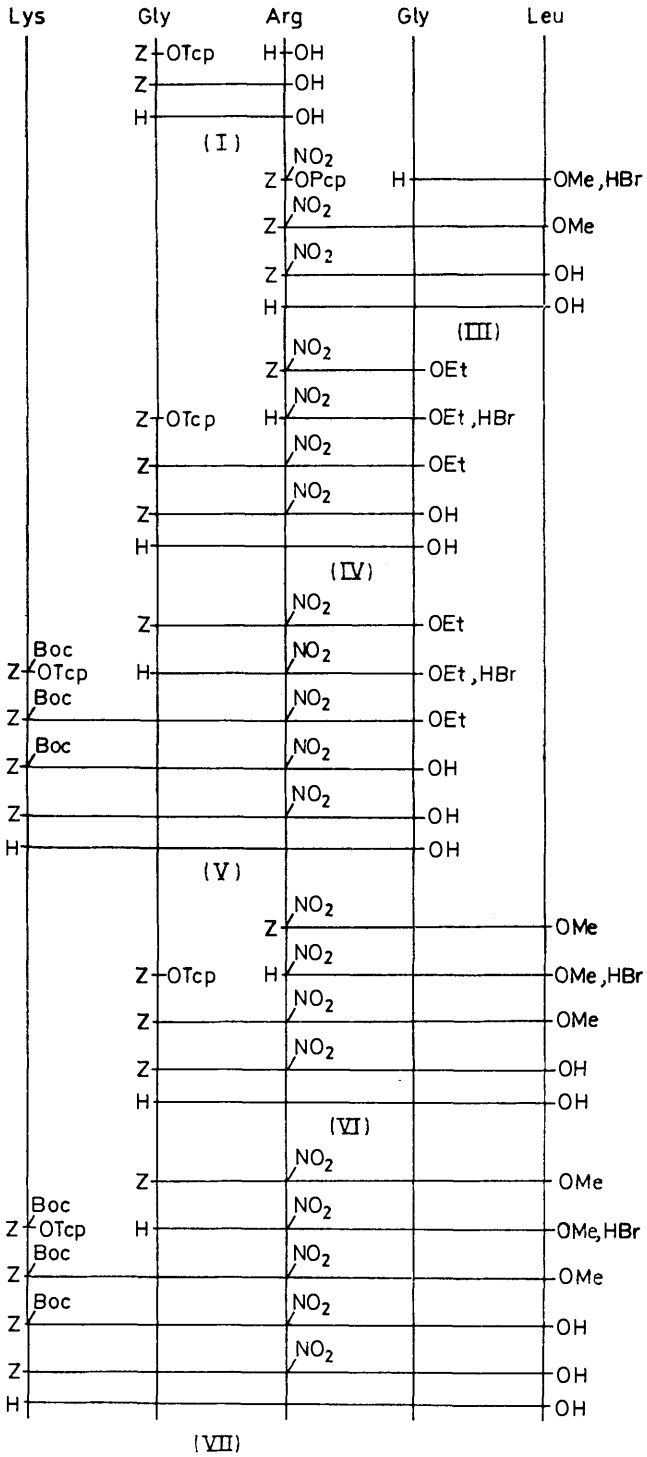
Glycyl-L-arginine was prepared by hydrogenation of the N^{α}-benzyloxycarbonyldipeptide formed by coupling benzyloxycarbonylglycine 2,4,5-trichlorophenyl ester with L-arginine, as the free base, in dimethylformamide. The crude hydrogenation product was purified by ion-exchange chromatography on carboxymethylcellulose using a single pyridine acetate buffer of pH 5·3 for elution, and this approach was also applied to the purification of the peptides (III)—(VII).

Protected intermediates corresponding to the peptides (II)—(VII) were synthesized by a stepwise approach from glycine ethyl ester hydrochloride or leucine methyl ester hydrochloride. Benzyloxycarbonylglycine 2,4,5-trichlorophenyl ester, benzyloxycarbonyl-N^{ω}-nitro-L-arginine pentachlorophenyl ester and N^{α}-benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-L-lysine 2,4,5-trichlorophenyl ester were used for coupling. Co-products could be removed simply by trituration with an organic solvent. Constants for protected derivatives are given in Table 1. Benzyloxycarbonyl groups were removed prior to coupling by hydrogen bromide in acetic acid.

For removal of the protecting groups to give the free peptides the following general procedures were used: saponification with sodium hydroxide in aqueous tetrahydrofuran, removal of t-butoxycarbonyl protection (when present) with hydrogen chloride in dioxan, and finally removal of benzyloxycarbonyl and N^{ω}-nitro-groups by hydrogenation in 80% acetic acid. T.l.c. of the crude hydrogenation products showed the presence of a single major component, and trace impurities were removed by chromatography on carboxymethylcellulose. Amino-acid ratios and electrophoretic mobilities are given in Table 2.

[4] J. R. Smythies, F. Bevington, and R. D. Morin, *Experientia*, 1972, **28**, 23.
[5] G. S. Baldwin and P. R. Carnegie, *Science*, 1971, **171**, 579.
[6] P. R. Carnegie, personal communication.

The peptides were inactive as substrates for the arginine methylase enzyme.[6] If the amino- and carboxy-groups are not interfering, this indicates a remarkably high structural specificity for this enzyme.



SCHEME 2 Abbreviations follow the recommendations of the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature (*Biochem. J.*, 1972, **126**, 773); also Tcp = 2,4,5-trichlorophenyl; Pcp = pentachlorophenyl

None of the peptides significantly inhibited the methylation of the protein by the enzyme.

EXPERIMENTAL

Silica gel GF_{254} (Merck) was used for t.l.c.; R_F values refer to the following systems: methanol–chloroform mixtures: (A1) 1 : 19, (A2) 1 : 9; n-butanol–acetic acid–water mixtures: (B1) 10 : 1 : 3; (B2) 4 : 1 : 1; (B3) 3 : 1 : 1; (B4) 1 : 1 : 1; (B5) 2 : 1 : 1; (C) n-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6); (D) cyclohexane–ethyl acetate–methanol (1 : 1 : 1); (E) chloroform–methanol–acetic acid (10 : 2 : 1); (F) t-butyl alcohol–methyl ethyl ketone–ammonia (*d* 0·880)–water (5 : 3 : 1 : 1); (G) propan-2-ol–pyridine–acetic acid–water (10 : 5 : 4 : 4); (H) chloroform–methanol–acetic acid–water (30 : 20 : 4 : 6); (J) methanol. Spots were detected by use of ninhydrin, chlorine and starch–iodide, and u.v. illumination.

Optical rotations were measured with a Perkin-Elmer 141 automatic polarimeter (1 dm cell). A Locarte high-voltage apparatus was used for electrophoresis at *ca.* 120 V cm^{-1} (Whatman 3MM paper). M.p.s were determined with a Kofler hot-stage apparatus. Organic solutions were dried over sodium sulphate. Samples for amino-acid analysis were hydrolysed at 110° for 16—18 h under N_2 and were analysed with a JEOL JLC 6AH machine; n.m.r. spectra were measured on a Varian T60 spectrometer with tetramethylsilane as internal standard; u.v. spectra were measured on a Unicam SP 800 spectrometer.

General Procedures.—Deprotection of benzyloxycarbonyl peptides. Hydrogen bromide in acetic acid (50% w/v; 6—12 ml per g of peptide) was added to a stirred solution of the peptide in the minimum volume of acetic acid. The solution was stirred for 1 h, then diluted with a large volume of ether, and the crystalline or amorphous solid hydrobromide was collected, washed with ether, and dried.

Isolation of protected peptide esters. The reaction mixture was filtered to remove triethylamine hydrobromide. Then the solution was either evaporated and the residue dissolved in ethyl acetate, or diluted with ethyl acetate. The resulting solution was washed with M-hydrochloric acid and saturated aqueous sodium hydrogen carbonate (both saturated with sodium chloride) and brine, dried, and evaporated. The residue was triturated with the appropriate solvent and the resulting solid washed with the solvent and dried.

Saponification of protected peptide esters. Sodium hydroxide (M; 10—30% excess) was added to a stirred solution of the ester (0·75—2 mmol) in tetrahydrofuran (3 ml). After 1 h, the solution was diluted with water (10—30 ml) and ether (10—20 ml). The aqueous layer was extracted with a further portion of ether, acidified with 2M-hydrochloric acid (0·67M-citric acid when a t-butoxycarbonyl group was present), and extracted with ethyl acetate (3—4 vol), and the extracts were washed (brine), dried, and evaporated.

Removal of the t-butoxycarbonyl group. Hydrogen chloride in dioxan (4M; 10—12 ml) was added to a solution of the t-butoxycarbonyl derivative (0·5—0·6 g) in dioxan (3—4 ml) and after 75 min an excess of ether was added. The precipitated hydrochloride was washed with ether.

Hydrogenation. The protected peptide in 80% acetic acid was hydrogenated over 10% palladium–charcoal until the reaction was complete (t.l.c.); the mixture was filtered through Celite and the filtrate was evaporated.

Column chromatography of the peptides (I) *and* (III)—(VII). The crude peptide was dissolved in buffer [pyridine (25 ml), acetic acid (10 ml), and water (to 2·5 l); pH 5·3] and put on a column (2 × 35 cm) of carboxymethylcellulose (Whatman CM32) equilibrated with and subsequently eluted with the same buffer. Fractions containing product (t.l.c.) were combined and evaporated, and the residue was dissolved in water; the solution was filtered (if necessary) and lyophilized.

Glycyl-L-arginine (I).—Benzyloxycarbonylglycine 2,4,5-trichlorophenyl ester (4·7 g, 12 mmol) was added to a stirred suspension of L-arginine (1·7 g, 10 mmol) in dimethylformamide (15 ml) and the mixture was stirred overnight. Ether was added to the resulting solution and the gum which precipitated was triturated with ether and reprecipitated from ethanol with ether, giving white, solid *benzyloxycarbonylglycyl-L-arginine* (2·7 g, 74%), R_F 0·14 (B1) and 0·52 (B3) (see Table 1). A solution of the protected dipeptide (1·1 g, 3 mmol) in 80% acetic acid (10 ml) was hydrogenated (100 mg catalyst; 3½ h) giving crude dipeptide (1·1 g). Carboxymethylcellulose chromatography of the crude product (0·68 g) gave the dipeptide (I) acetate (0·49 g), R_F 0·27 (B4), 0·05 (C), 0·02 (F), 0·18 (G), and 0·05 (H) (see Table 2).

L-*Arginylglycine* (II).—This was prepared as described [7] by hydrogenation of benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine (0·41 g, 1 mmol), giving the dipeptide (II) acetate (0·25 g), R_F 0·26 (B4) and 0·18 (G) (see Table 2).

L-*Arginylglycyl-L-leucine* (III).—Benzyloxycarbonyl-N^{ω}-nitro-L-arginine pentachlorophenyl ester [8] (6·0 g, 10 mmol) and triethylamine (in slight excess; 1·35 ml) were added to a stirred solution of glycyl-L-leucine methyl ester hydrobromide [9] (3 g, 11 mmol) in dimethylformamide (15 ml). The mixture was stirred at room temperature, kept at 0° overnight, then filtered, diluted with ethyl acetate (200 ml) and methanol (40 ml), washed, and dried. The solution deposited crystalline *benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycyl-L-leucine methyl ester* (3·8 g, 67%), R_F 0·28 (A2) and 0·65 (J), m.p. 170—172°; τ [$(CD_3)_2SO$] (tentative assignments) 1·7—2·8 (NH and Ph at τ 2·66), 4·96 ($PhCH_2$), 5·4—7·2 ($NH{\cdot}CH$ × 2, $NH{\cdot}CH_2{\cdot}CO$, and CO_2Me at τ 6·31), 6·6—7·0 ($NH{\cdot}CH_2{\cdot}CH_2$), 8·1—8·6 ($CH{\cdot}CH_2{\cdot}CHMe_2$ and $CH{\cdot}[CH_2]_2{\cdot}CH_2{\cdot}NH$), and 9·0—9·25 [$CH(CH_3)_2$]; $\lambda_{max.}$ (Me_2SO) 274 nm (Found: *M*, by comparison with benzyloxycarbonyl-N^{ω}-nitro-L-arginine, assuming equal extinction of the nitro-group in each compound, 533. $C_{23}H_{35}N_7O_8$ requires *M*, 537·6); other constants are given in Table 1. Saponification of the methyl ester (1·1 g, 2 mmol) gave benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycyl-L-leucine as a gum (0·72 g), R_F 0·0 (A2), 0·51 (B1), and 0·64 (J), with trace impurities. A solution of the crude acid (0·71 g) in 80% acetic acid (15 ml) was hydrogenated (100 mg catalyst; 3·5 h) and the crude product chromatographed, giving the *tripeptide* (III) *acetate* (0·36 g), R_F 0·36 (B5), 0·47 (B4), 0·30 (C), 0·08 (F), 0·54 (G), and 0·24 (H) (see Table 2).

Glycyl-L-arginylglycine (IV).—Removal of the benzyloxycarbonyl group from benzyloxycarbonyl-N^{ω}-nitro-L-

[7] M. E. Cox, H. G. Garg, J. Hollowood, J. M. Hugo, P. M. Scopes, and G. T. Young, *J. Chem. Soc.*, 1965, 6806.

[8] J. Kovacs, M. Q. Ceprini, C. A. Dupraz, and G. N. Schmit, *J. Org. Chem.*, 1967, **32**, 3696.

[9] E. Klieger and H. Gibian, *Annalen*, 1961, **649**, 183.

arginylglycine ethyl ester [10] (1·65 g, 3·8 mmol) gave white, solid *Nω-nitro-L-arginylglycine ethyl ester hydrobromide*, R_F 0·14 (B1). Triethylamine (in slight excess; 1·1 ml) was added to a stirred mixture of this hydrobromide and dimethylformamide (7·5 ml), followed by benzyloxycarbonylglycine 2,4,5-trichlorophenyl ester (1·8 g, 4·6 mmol), and the mixture was stirred overnight. The mixture was filtered and evaporated, and the residue (dissolved in ethyl acetate with the addition of a little ethanol) washed, dried, and evaporated. Trituration with ether gave *benzyloxycarbonylglycyl-Nω-nitro-L-arginylglycine ethyl ester* (1·4 g, 70% from the benzyloxycarbonyldipeptide), R_F 0·56 (B1) and 0·65 (B4) (see Table 1). Saponification of the ester (0·50 g, 1 mmol) gave benzyloxycarbonylglycyl-N^{ω}-nitro-L-arginylglycine as a gum (0·34 g), R_F 0·07 (A1) and 0·42 (B2), with trace impurities. Hydrogenation of this acid (0·34 g) in 80% acetic acid (5 ml; 50 mg catalyst; 4 h) gave the tripeptide (IV) acetate after chromatography (0·19 g), R_F 0·26 (B4), 0·04 (C), 0·02 (F), 0·12 (G), and 0·05 (H) (see Table 2).

TABLE 1

Protected intermediates

Compound	$[\alpha]_D^{25}$ (°) [a]	$[\alpha]_{546}^{25}$ (°) [a]	Found (%) C	H	N	Formula	Required (%) C	H	N
Z-Gly-Arg-OH	−3·1 [b]	−3·4 [b]	52·3	6·1	18·7	$C_{16}H_{23}N_5O_5$	52·6	6·35	19·2
Z-Arg(NO_2)-Gly-Leu-OMe	−12·3	−13·7	50·9	6·5	18·0	$C_{23}H_{35}N_7O_8$	51·4	6·6	18·2
Z-Gly-Arg(NO_2)-Gly-OEt	−7·7	−8·8	48·3	5·8	18·6	$C_{20}H_{29}N_7O_8$,0·25AcOEt	48·7	6·0	18·95
Z-Lys(Boc)-Gly-Arg(NO_2)-Gly-EOt	−10·0	−11·8	50·8	6·7	17·1	$C_{31}H_{49}N_9O_{11}$,0·5H_2O	50·8	6·9	17·2
Z-Gly-Arg(NO_2)-Gly-Leu-OMe	−13·4	−14·1	49·6	6·2	18·3	$C_{25}H_{38}N_8O_9$,0·5H_2O	49·7	6·5	18·6
Z-Gly-Arg(NO_2)-Gly-Leu-OH	−10·9	−11·0	48·7	6·2	18·4	$C_{24}H_{36}N_8O_9$,H_2O	48·2	6·4	18·7
Z-Lys(Boc)-Gly-Arg(NO_2)-Gly-Leu-OMe	−10·9	−13·0	53·0	7·0	16·8	$C_{36}H_{58}N_{10}O_{12}$	52·5	7·1	17·0

[a] *c* 1 in Me_2N·CHO unless stated otherwise. [b] $[\alpha]_{\lambda}^{20}$ at *c* 1 in 10% AcOH.

TABLE 2

Peptides

No.	$[\alpha]_D^{20}$ (°) [a]	$[\alpha]_{546}^{20}$ (°) [a]	R_{Arg} [c]	Amino-acid ratios [b] Arg	Gly	Leu	Lys
(I)	−0·8 [d]	−1·0	0·91	1·01	0·99		
(II)	+42·3 [e]	+45·8		0·98	1·02		
(III)	−0·6	+0·4	0·69	0·98	1·03	0·99	
(IV)	−18·7 [f]	−22·3	0·82	1·06	1·94		
(V)	−31·9	−37·8	0·65	1·00	2·03		0·97
(VI)	−30·2	−35·4		0·98	2·02	1·00	
(VII)	−14·5	−16·6	0·91	1·00	2·04	0·99	0·97

[a] *c* 1 in 10% AcOH unless otherwise indicated. [b] Average figure per amino-acid taken as 1·00. [c] Electrophoresis in pyridine (10%)–acetic acid (0·4%); pH 6·5. [d] 1·25 in H_2O; lit.,[10] $[\alpha]_D^{25}$ +1·6° (*c* 4·3 in H_2O). [e] *c* 1 in H_2O; lit.,[7] $[\alpha]_D^{23}$ +39·1° (*c* 1 in H_2O). [f] *c* 2·3 in H_2O; lit.,[11] $[\alpha]_D^{25}$ −17·6° (*c* 2·5 in H_2O).

L-Lysylglycyl-L-arginylglycine (V).—Deprotection of benzyloxycarbonylglycyl-N^{ω}-nitro-L-arginylglycine ethyl ester (0·8 g, 1·6 mmol) with hydrogen bromide in acetic acid gave glycyl-N^{ω}-nitro-L-arginylglycine ethyl ester hydrobromide (1 g), R_F 0·25 (B2). The hydrobromide (1 g) was stirred in dimethylformamide (5 ml) with the addition of triethylamine (in excess; 0·25 ml) and N^{α}-benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-L-lysine 2,4,5-trichlorophenyl ester (1·1 g, 2 mmol), and kept at 0—5° for 66 h. The mixture was filtered, diluted with ethyl acetate, washed, dried, and evaporated. Trituration of the residue with ethyl acetate gave N^{α}-*benzyloxycarbonyl-*N^{ϵ}-*t-butoxycarbonyl-L-lysylglycyl-*N^{ω}-*nitro-L-arginylglycine ethyl ester* as a white powder (0·68 g, 56% from the benzyloxycarbonyl tripeptide), R_F 0·30 (A2), 0·75 (B2), and 0·57 (D) (see Table 1). Saponification of the ester (0·54 g, 0·75 mmol) gave N^{α}-benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-L-lysylglycyl-N^{ω}-nitro-L-arginylglycine (0·46 g) as a gum, R_F 0·0 (A2), 0·62 (B5), and 0·69 (B4). Removal of the t-butoxycarbonyl group gave N^{α}-benzyloxycarbonyl-L-lysylglycyl-N^{ω}-nitro-L-arginylglycine hydrochloride (0·55 g), R_F 0·60 (B4) and 0·41 (B5) with impurities. The crude hydrochloride (0·55 g) was hydrogenated in 80% acetic acid (10 ml; 50 mg catalyst; 7 h) and chromatographed on carboxymethylcellulose; lyophilization gave a sticky product, which was precipitated from water with acetone, washed with acetone and ether, and dried, giving the *tetrapeptide* (V) *acetate* (0·17 g), R_F 0·19 (B4), 0·05 (C), 0·02 (F), 0·18 (G), and 0·05 (H) (see Table 2).

Glycyl-L-arginylglycyl-L-leucine (VI).—The benzyloxycarbonyl group was removed from benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycyl-L-leucine methyl ester (2·2 g, 4 mmol) and the resulting hydrobromide was dissolved with stirring in dimethylformamide (15 ml) with the addition of triethylamine (in excess; 1·2 ml) and benzyloxycarbonylglycine 2,4,5-trichlorophenyl ester (2·0 g, 5 mmol). The mixture was kept at 0—5° for 66 h, filtered, and evaporated, and the residue was dissolved in ethyl acetate (with the addition of methanol), washed, dried, and evaporated. The residue was triturated with ether giving *benzyloxycarbonylglycyl-Nω-nitro-L-arginylglycyl-L-leucine methyl ester* as a white powder (1·8 g, 75%), R_F 0·22 (A2), 0·61 (B1), and 0·53 (E) (see Table 1). Saponification of the ester (0·60 g, 1 mmol) gave *benzyloxycarbonylglycyl-Nω-nitro-L-arginylglycyl-L-leucine* as a powder (0·53 g, 90%) after trituration with ether; R_F 0·49 (B1) and 0·16 (E) (see Table 1). The acid (0·49 g) in 80% acetic acid (8 ml) was hydrogenated (50 mg catalyst; 4 h) and the crude product purified by chromatography on carboxymethylcellulose, giving the *tetrapeptide* (VI) *acetate* (0·37 g), R_F 0·38 (B4), 0·24 (B5), 0·24 (C), 0·06 (F), 0·47 (G), and 0·13 (H) (see Table 2).

L-Lysylglycyl-L-arginylglycyl-L-leucine (VII).—Deprotection of benzyloxycarbonylglycyl-N^{ω}-nitro-L-arginylglycyl-L-leucine methyl ester (0·89 g, 1·5 mmol) with hydrogen bromide in acetic acid gave glycyl-N^{ω}-nitro-L-arginylglycyl-L-leucine methyl ester hydrobromide, R_F 0·23 (B1). The hydrobromide was dissolved in dimethylformamide (7·5 ml) and triethylamine (in excess; 0·4 ml)

[10] P. M. Scopes, K. B. Walshaw, M. Welford, and G. T. Young, *J. Chem. Soc.*, 1965, 782.

[11] K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Amer. Chem. Soc.*, 1956, **78**, 238.

and N^{α}-benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-L-lysine 2,4,5-trichlorophenyl ester (1·0 g, 1·8 mmol) were added with stirring. After 16 h at 0°, the mixture was diluted with ethyl acetate (150 ml) and water, and the organic layer washed (0·67M-citric acid ×2, water, saturated aqueous sodium hydrogen carbonate ×2, and brine), dried, and evaporated. Trituration of the residue with methanol–ether and ether gave N$^{\alpha}$-*benzyloxycarbonyl*-N$^{\epsilon}$-*t-butoxycarbonyl*-L-*lysylglycyl*-N$^{\omega}$-*nitro*-L-*arginylglycyl*-L-*leucine methyl ester* as a white solid (0·91 g, 75% from the benzyloxycarbonyl tetrapeptide ester), R_F 0·20 (A2) and 0·55 (D); amino-acid analysis: Leu 1·00, Gly 2·00, Lys, Orn (not separated), and Arg present (see Table 1). Saponification of the ester (0·62 g, 0·75 mmol) and removal of the t-butoxycarbonyl group gave N^{α}-benzyloxycarbonyl-L-lysylglycyl-N^{ω}-nitro-L-arginylglycyl-L-leucine hydrochloride (0·7 g), R_F 0·75 (B4), with impurities. The crude hydrochloride (0·7 g) dissolved in 80% acetic acid (15 ml) was hydrogenated (100 mg catalyst; 6 h) and the crude product chromatographed, giving, after lyophilization, the *pentapeptide* (VII) *acetate* (0·22 g), R_F 0·23 (B4) (see Table 2).

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