Synthesis of Peptides related to Bradykinin

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The synthesis of Arg-Gly$_n$-Arg † where n = 1—7 and of Arg-Gly$_3$-Phe-Gly$_3$-Arg, Arg-Gly$_3$-Phe-Gly$_2$-Phe-Arg, and Arg-Gly$_6$-Phe-Arg, peptide analogues of bradykinin, is described.

THE structural basis for the biological action of a polypeptide hormone is impossible to define because the mode of interaction at the molecular level with the biological receptor is completely unknown. It is possible only to make surmises on the basis of the presence or absence of various groupings in the side chains, the possibilities for hydrogen bonding, and so forth. Nevertheless, the smaller polypeptide hormones such as oxytocin, vasopressin, and bradykinin have received much attention by way of the synthesis of structural analogues in the hope that some light would be shed on this complex problem.

Studies on bradykinin (I), summarised recently by Schröder and Lübke,[1] indicate that both terminal arginine residues are essential for full biological activity; they cannot be replaced by other amino-acid residues, basic or otherwise. On the other hand, changes in amino-acid sequence in the rest of the molecule have not demonstrated such clear-cut structural requirements. It is possible that when bradykinin interacts with its receptor the two guanidine groups are not so far apart as they would be in the molecule in its fully extended form, and with this idea in mind a series of peptides was prepared with the general structure (II). It was hoped that the low steric effect of the glycine residues would enable the two structurally important guanidine groups to fit on their respective sites on the receptor with resulting agonistic or antagonistic effects. As this hope was not realised the work was extended to include the three phenylalanine-containing peptides (III), (IV), and (V). None of these showed significant agonistic or antagonistic activity. Bodanszky, Sheehan, Ondetti, and Lande [2] prepared peptide (II; $n = 7$) for reasons similar to those given here, but the work was not extended.

In a statistical study of correlations between amino-acids and their bearing on biological activity in a series of hormone analogues, it was shown that glycine, proline, and alanine formed a group within which the individual relationship was quite high.[3] It is also known that proline and glycine, when they occur at the ends of helical sections of a protein, both allow bending of the chain. It was found however that peptide (V), in which

† The abbreviations used in this paper follow those given in (*a*) R. Schwyzer, J. Rudinger, E. Wünsch, and G. T. Young, ' Peptides,' ed. G. T. Young, Pergamon, Oxford, 1963, p. 261; (*b*) E. Schröder and K. Lübke, ' The Peptides,' vol. 1, Academic Press, New York and London, 1966, p. xiii; (*c*) I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature, *Biochemistry*, 1967, **6**, 362.

[1] E. Schröder and K. Lübke, ' The Peptides,' vol. 2, Academic Press, New York and London, 1966, p. 4.

[2] M. Bodanszky, J. T. Sheehan, M. A. Ondetti, and S. Lande, *J. Amer. Chem. Soc.*, 1963, **85**, 994.

[3] P. H. A. Sneath, *J. Theor. Biol.*, 1966, **12**, 157.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
(I)

H-Arg-Gly$_n$-Arg-OH
(II) $n = 0$—7

H-Arg-Gly$_n$-Phe-Gly$_{n'}$Arg-OH
(III) $n = 3$, $n' = 3$

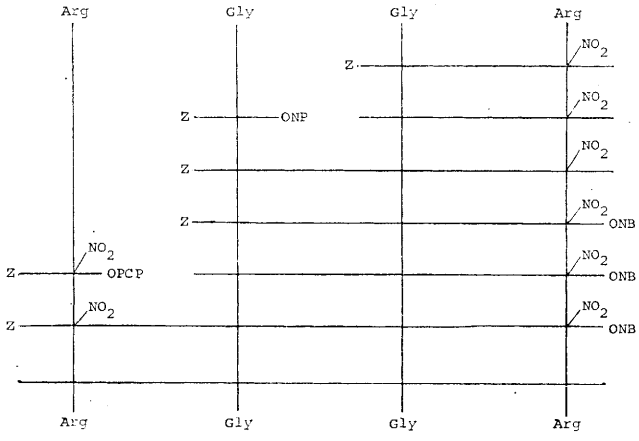
(IV) $n = 6$, $n' = 0$

H-Arg-Gly$_3$-Phe-Gly$_2$-Phe-Arg-OH
(V)

the three proline residues present in bradykinin had been replaced by glycine, had no biological activity even at a high level of dosage. In this analogue the 6-serine residue present in bradykinin had also been replaced by glycine, but it is known that when this is the only change made in bradykinin the analogue is almost equi-active with the hormone itself. In the study by Sneath [3] each of the attributes of the amino-acids was given equal weight, but in the case of bradykinin it appears that the unique properties of proline, for example its effect on tertiary structure, may far outweigh those which it has in common with glycine. This appears to be borne out by recent work on the synthesis of the 2-, 3-, and 7-mono-, 2,3-di-, and 2,3,7-tri-sarcosine analogues of bradykinin.[4] In this series, all compounds possessed activity and although this was progressively reduced as the degree of substitution was increased the 2,3,7-trisarcosine analogue was nevertheless appreciably active.

The routes used to prepare the various analogues are depicted in Schemes 1—10, with the exception of L-arginyl-L-arginine, which was prepared by the method used by Zervas and his co-workers.[5] Active ester coupling was used throughout, and the benzyloxycarbonyl residue was used for *N*-protection. As may be seen from the example of Scheme 1, the *p*-nitrobenzyl ester was chosen for the protection of the *C*-terminal arginine in the synthesis of the two smaller peptides. Initially nitro-L-arginine was coupled with benzyloxycarbonylglycine *p*-nitrophenyl ester [6] in aqueous pyridine containing sufficient sodium hydroxide to dissolve the amino-acid and maintain the pH at $\geqslant 8{\cdot}5$. After extraction of the excess of active ester the protected dipeptide was separated by acidification to give a 74% yield of product identical in m.p. and optical rotation with that described by Hofmann and his co-workers.[7] This acid was converted into the *p*-nitrobenzyl ester,* the *N*-terminal protecting group was removed, and the dipeptide ester was coupled with benzyloxycarbonyl-nitro-L-arginine pentachlorophenyl ester [9] (see Experimental section). Physical data for the resulting protected tripeptide are given in Table 1. Hydrogenolysis removed all the protecting groups simultaneously and the product was purified by chromatography on carboxymethylcellulose.

SCHEME 1



The preparation of arginyldiglycylarginine followed an analogous route (see Scheme 1). Protection of the *C*-terminal carboxy-group was delayed until the tripeptide stage in order to reduce the possibility of dioxopiperazine formation during the coupling of benzyloxycarbonylglycine *p*-nitrophenyl ester with the dipeptide. Again hydrogenolysis removed all protecting groups simultanesouly from the fully blocked tetrapeptide, and the resulting peptide was purified by chromatography on carboxymethylcellulose.

In one experiment the dipeptide derivative, *viz.* glycylnitro-L-arginine *p*-nitrobenzyl ester, was liberated as the free base in the absence of active ester. It transpired that *p*-nitrobenzyl, like methyl, gave no protection against dioxopiperazine formation and a good yield of the expected dioxopiperazine side product was obtained.

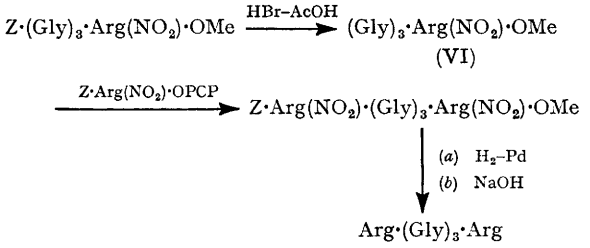
In the case of the larger peptides containing glycine and arginine it was possible to design syntheses that did not involve coupling on to dipeptide units and the danger of dioxopiperazine formation did not arise. The use of *p*-nitrobenzyl for carboxy-protection had been found to lead to intermediates of relatively low solubility. As this problem was expected to become more acute with larger molecules, nitro-L-arginine methyl ester was used as the protected intermediate for the *C*-terminal amino-acid. This change improved solubilities and obviated an esterification reaction in mid-synthesis. Benzyloxycarbonyltriglycylnitro-L-arginine methyl ester (Scheme 2) was prepared essentially as described by Bodanszky,[2] with triethylamine replacing

* The conditions used for esterification were not expected to lead to racemisation in view of the work of Theodoropoulos and Tsangaris.[8] This assumption is supported by our finding that the optical rotations of the final peptides made in this way and those of the same compounds made by the solid phase method (see later) were identical within the limits of experimental error.

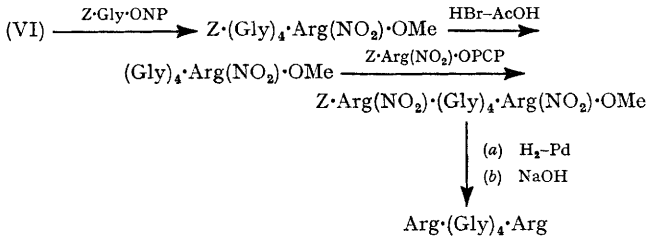
[4] N. Yanaihara, M. Sekiya, K. Takagi, H. Kato, M. Ichimura, and T. Nagao, *Chem. and Pharm. Bull.* (*Japan*), 1967, **15**, 110.
[5] L. Zervas, T. Otani, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, 1957, **22**, 1515.
[6] J. A. Farrington, P. J. Hextall, G. W. Kenner, and J. M. Turner, *J. Chem. Soc.*, 1957, 1407.
[7] K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Amer. Chem. Soc.*, 1956, **78**, 238.
[8] D. Theodoropoulos and J. Tsangaris, *J. Org. Chem.*, 1964, **29**, 2272.
[9] J. Kovacs and M. Q. Ceprini, *Chem. and Ind.*, 1965, 2100.

tributylamine, and an identical yield of product was obtained. Further stages were carried out as described above and depicted in Scheme 2. By conducting the

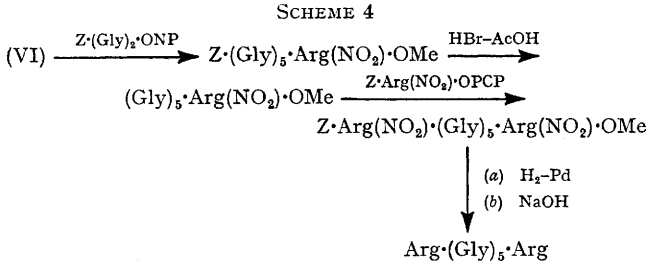
SCHEME 2



SCHEME 3



SCHEME 4



saponification after the urethane bond of the *N*-terminal protecting group had been eliminated from the molecule it was hoped to avoid the formation of side products involving hydantoin intermediates. These have been observed previously by, for example, MacLaren,[10] and also in the closely related case of benzyloxycarbonylnitro-L-arginyltriglycylglycine by Bodanszky and his co-workers.[2]

The synthesis of arginyltetraglycylarginine followed a similar course (Scheme 3), with the additional insertion of a coupling with benzyloxycarbonylglycine *p*-nitrophenyl ester into the method outlined for the lower homologue.

Arginylpentaglycylarginine was prepared analogously by the incorporation of two glycine units in one step (Scheme 4). The fully protected hexa- and heptapeptides represented the limiting case of a trend which had been observed through the series. A tendency towards decreased solubility in organic solvents with increasing molecular weight, which is often observed, was accompanied by an increase in water solubility, and an increased tendency to form gels in dimethylformamide solution. Thus benzyloxycarbonylpentaglycylnitro-L-arginine methyl ester could be recrystallised from hot water and benzyloxycarbonylnitro-L-arginylpentaglycylnitro-L-arginine methyl ester was insoluble in all the common organic solvents including methanol (but excluding dimethylformamide).

The succeeding member of the series, containing six glycine residues, was prepared analogously (Scheme 5); the same method of preparing the hexaglycylarginine derivative was used previously,[2] in which gel formation was also recorded during the coupling of benzyloxycarbonyldiglycylglycine *p*-nitrophenyl ester with triglycylnitro-L-arginine methyl ester in dimethylformamide. In addition Bodanszky and his co-workers [2] recrystallised the product from water. In our series the protected octapeptide containing *N*- and *C*-terminal arginine was insoluble in all the common organic solvents with the exception of dimethylformamide.

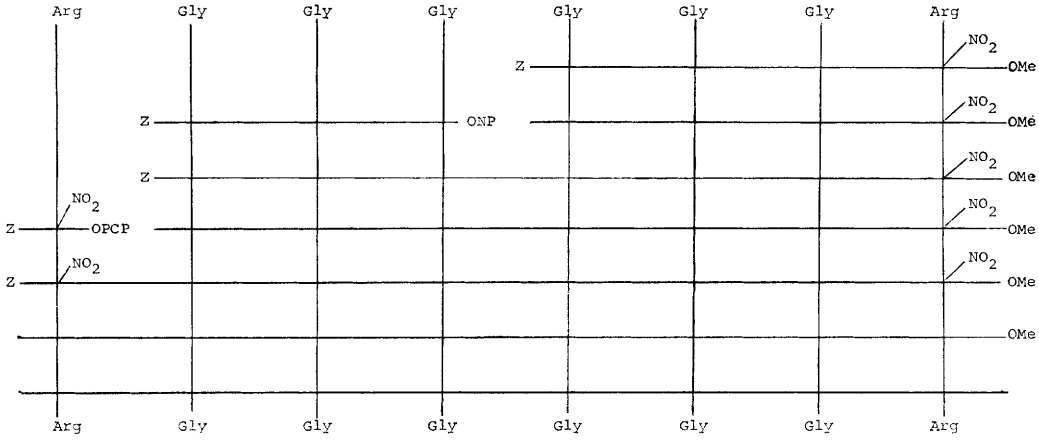
Two routes were used to prepare benzyloxycarbonylnitro-L-arginylheptaglycylnitro-L-arginine methyl ester. One (Scheme 6) involved coupling the *N*-terminal dipeptide unit in the form of a pentachlorophenyl ester to hexaglycylnitro-L-arginine methyl ester by a similar procedure to that of Bodanszky and his co-workers,[2] who used the *p*-nitrophenyl ester for this purpose. The yield of product was comparable. The dipeptide unit was prepared as the n-butyl ester, which presented no difficulties in saponification. The use of glycine n-butyl ester for the coupling with the active ester may have been more advantageous than the use of the corresponding methyl ester, as no dioxopiperazine formation was observed. The other route (Scheme 7) involved coupling the hitherto unknown benzyloxycarbonyltriglycylglycine *p*-nitrophenyl ester with triglycylnitro-L-arginine methyl ester to give an octapeptide derivative, which was again recrystallised from water. This was converted into the required nonapeptide derivative by methods similar to those used in Schemes 1—5, but dimethyl sulphoxide was employed as solvent. The yield was significantly lower than in the method outlined in Scheme 6. The protecting groups were removed and the free nonapeptide was isolated as before.

During the preparation of the three analogues containing L-phenylalanine in positions 5 and 8 [(III) (IV), and (V)] the tendency towards gel formation mentioned previously was not observed. Intermediates were significantly more soluble in the common organic solvents, although the protected nonapeptides did not differ much from the glycine analogue (II; $n = 7$) in solubility but could be recrystallised from aqueous alcohols. L-Arginylhexaglycyl-L-phenylalanyl-L-arginine was prepared from benzyloxycarbonyl-L-phenylalanylnitro-L-arginine methyl ester [7] by three successive additions of two glycine units followed by the *N*-terminal arginine as depicted in Scheme 8. Benzyloxycarbonyldiglycyl-L-phenylalanylnitro-L-arginine methyl ester, a tetrapeptide intermediate in this synthesis, was also used in the preparation of the Phe[5],Phe[8]-analogue (Scheme 9). After removal of the benzyloxycarbonyl protecting group a second phenylalanine residue was introduced, by use of benzyl-

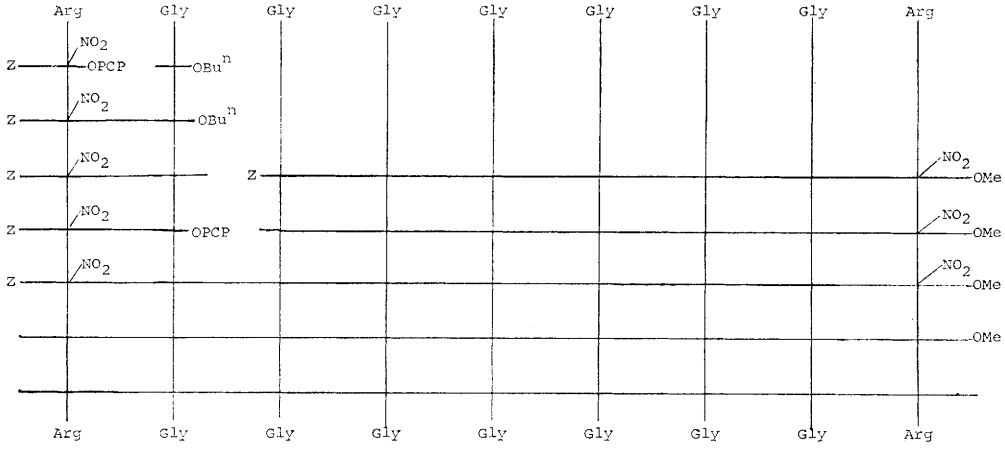
[10] J. A. MacLaren, *Austral. J. Chem.*, 1958, **11**, 360.

oxycarbonyl-L-phenylalanine pentachlorophenyl ester.[11] The succeeding three glycine residues were introduced in one stage and a final coupling with benzyloxycarbonyl-nitro-L-arginine pentachlorophenyl ester gave the required protected nonapeptide.

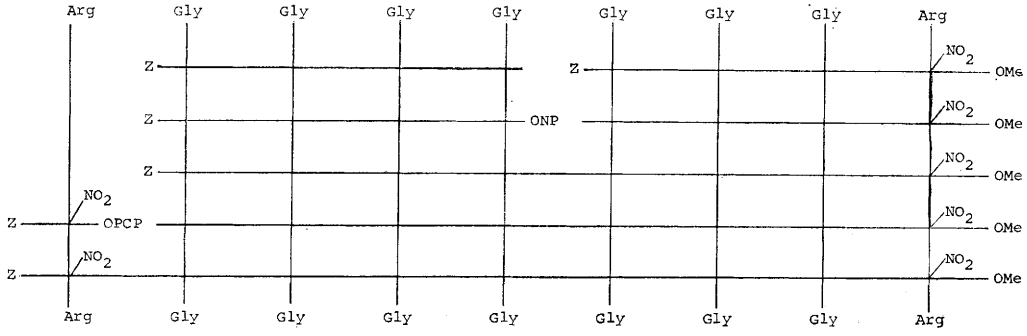
SCHEME 5



SCHEME 6



SCHEME 7



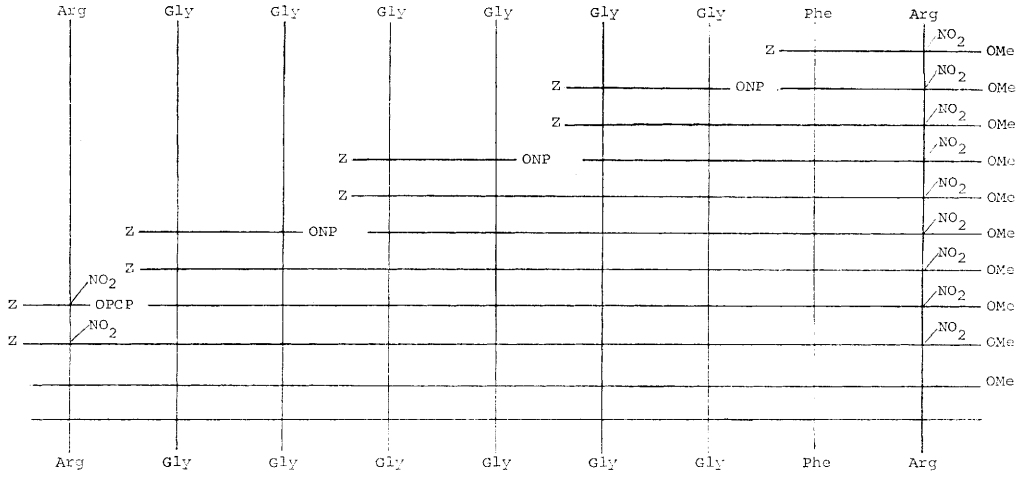
The Phe[5]-analogue was prepared from benzyloxycarbonyltriglycylnitro-L-arginine methyl ester by similar methods following the route shown in Scheme 10. The protected octapeptide in this synthesis was soluble in hot water but gel formation did not occur during its preparation.

In addition to the preparations already described attempts were made to prepare the same compounds concurrently by use of the solid phase peptide synthesis technique developed by Merrifield.[12] The products after cleavage from the resin support showed a multiplicity of spots on electrophoresis. From these mixtures it was

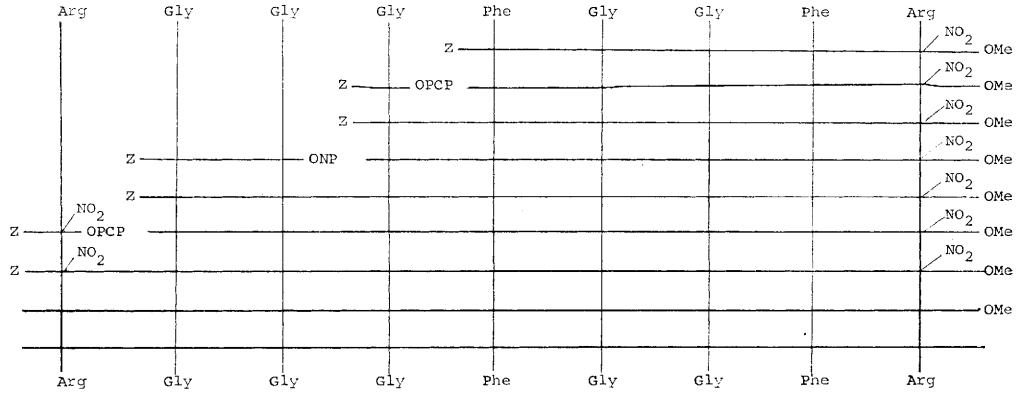
[11] J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1609.

[12] R. B. Merrifield, *Biochemistry*, 1964, **3**, 1385.

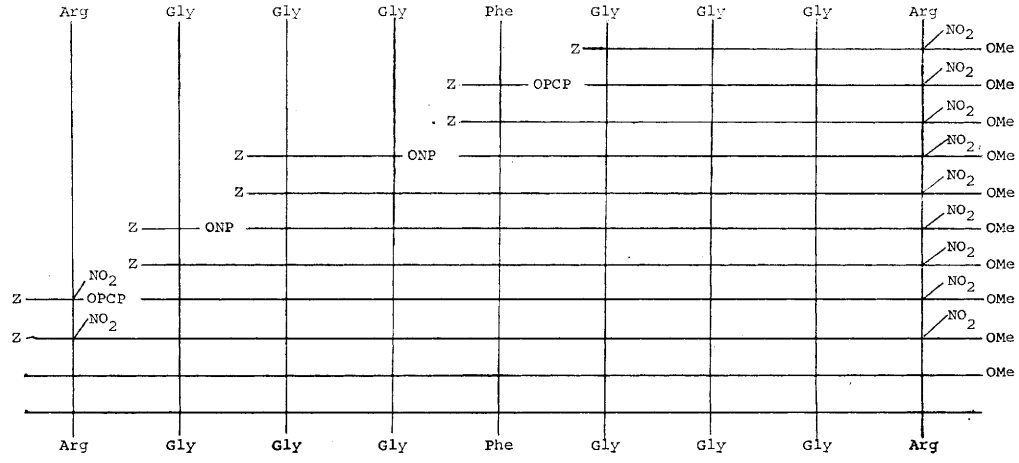
SCHEME 8



SCHEME 9



SCHEME 10



found possible to isolate the required products, as described for (II; $n = 1$ and 2) in the Experimental section, but the difficulties of purification caused us to abandon this mode of preparation for the other members of the series.

All the peptides prepared were tested for bradykinin and antibradykinin activity on isolated guinea pig ileum preparations.[13] No activity was detectable up to 10^5 times the dose of bradykinin giving 50% of the maximum response.

EXPERIMENTAL

Compounds were checked for purity by t.l.c. on silica gel. The most useful solvent systems were found to be (*a*)

[13] J. J. Lewis, 'Introduction to Pharmacology,' 3rd edn., Livingstone, Edinburgh and London, 1964, p. 481.

$MeOH–CHCl_3$ (5 : 95), (*b*) $MeOH–CHCl_3$ (20 : 80), (*c*) EtOAc–MeOH–cyclohexane (30 : 30 : 30), (*d*) $Bu^nOH–AcOH–H_2O$ (66 : 24 : 12), and (*e*) $Bu^nOH–AcOH–H_2O$ (100 : 10 : 30). Compounds were located with the aid of chlorine–starch–potassium iodide, ninhydrin, or Sakaguchi reagent as appropriate.[14] Electrophoresis was carried out on Whatman 3MM paper in a Locarte high voltage apparatus with a voltage gradient of 80 v/cm. Mobilities are expressed relative to arginine (R_{Arg}). Buffers used were pyridine (100 ml.)–glacial acetic acid(10 ml.)–water(2390 ml.) of pH 6·2, and 90% formic acid(78 ml.)–glacial acetic acid(148 ml.)–water(2274 ml.) of pH 1·85. Samples for amino-acid analysis were hydrolysed for 16 hr. at 115° in an evacuated tube and analysed on the Beckman 120C analyser.

The following general procedures were used where appropriate.

Debenzyloxycarbonylation followed by Active Ester Coupling.—The protected peptide derivative was dissolved in 40% hydrogen bromide in glacial acetic acid solution (from 8 ml./g. for small molecules up to 20 ml./g. for octapeptide derivatives). After 1 hr. at room temperature the solution was diluted with anhydrous ether (20 vol.). The precipitated peptide hydrobromide was washed rapidly on the filter three times with anhydrous ether then dissolved in dimethylformamide (15 ml./g. starting material). To this solution was added active ester (1 equiv. based on starting material) and the peptide amino-group was liberated by the addition of triethylamine (to pH 8—9; wet indicator paper). The reaction was judged complete when ninhydrin-positive material was no longer detectable by t.l.c. (1—3 days).

The solvent was evaporated off under reduced pressure, and the residue was triturated successively with ether, water, and in some cases ethanol or methanol and recrystallised from the solvent given in Table 1. Compounds 10, 11, and 19 (Table 1), and methyl benzyloxycarbonylnitroarginylheptaglycylnitroargininate were purified by repeated extraction with boiling methanol, in which they were insoluble. Physical characteristics of the products are given in Table 1.

TABLE 1

Protected intermediates

No.	Compound	M.p.	$[\alpha]_D$	*c* (Solvent)	Yield (%) ‡	Found (%) C	Found (%) H	Found (%) N	Formula	Required (%) C	Required (%) H	Required (%) N
1	Z-Gly-Arg(NO_2)-ONB	139—142° [a]	−14·4°	1·4(DMF) *	77	50·4	5·0	17·8	$C_{23}H_{27}N_7O_9$	50·6	5·0	18·0
2	Z-Arg(NO_2)-Gly-Arg(NO_2)-ONB	134—137 [b]	−12·2	2·1(DMF)	52	46·5	5·0	22·6	$C_{29}H_{38}N_{12}O_{12}$	46·6	5·1	22·5
3	Z-Gly_2-Arg(NO_2)-OH	83—85 [c]	−3·1	1·4(DMF)	87	45·8	5·6	21·2	$C_{18}H_{25}N_7O_8$	46·3	5·4	21·0
4	Z-Gly_2-Arg(NO_2)-ONB	107—110 [d]	−16·6	1·0(DMF)	60	49·7	5·1	18·8	$C_{25}H_{30}N_8O_{10}$	49·8	5·0	18·6
5	Z-Arg(NO_2)-Gly_2-Arg(NO_2)-ONB	108—111 [e]	−10·9	1·0(DMF)	48	46·6	5·4	22·4	$C_{31}H_{41}N_{13}O_{13}$	46·3	5·1	22·6
6	Z-Arg(NO_2)-Gly_3-Arg(NO_2)-OMe	115—119 [e]	−8·0	2·0(DMF)	58	43·7	5·7	24·8	$C_{27}H_{41}N_{13}O_{12}$	43·8	5·6	24·6
7	Z-Gly_4-Arg(NO_2)-OMe	129—134 [e]	−3·0	2·0(DMF)	87	46·2	5·7	21·1	$C_{23}H_{33}N_9O_{10}$	46·4	5·6	21·2
8	Z-Arg(NO_2)-Gly_4-Arg(NO_2)-OMe	110—115 [e]	−8·0	2·0(DMF)	64	43·6	5·6	23·6	$C_{29}H_{44}N_{14}O_{13}$	43·7	5·5	24·6
9	Z-Gly_5-Arg(NO_2)-OMe	169—174 [f]	−5·9	2·0(DMSO) †	94	45·8	5·5	21·7	$C_{25}H_{36}N_{10}O_{11}$	46·0	5·6	21·5
10	Z-Arg(NO_2)-Gly_5-Arg(NO_2)-OMe	152—158 [f]	−3·5	2·0(DMSO)	50	43·5	5·8	24·8	$C_{31}H_{47}N_{15}O_{14}$	43·6	5·6	24·6
11	Z-Arg(NO_2)-Gly_6-Arg(NO_2)-OMe	171—175 [f]	−1·8	2·0(DMSO)	43	40·8	5·4	23·2	$C_{33}H_{50}N_{16}O_{15},3H_2O$	41·1	5·8	23·2
12	Z-Arg(NO_2)-Gly-OBu^n	141—142 [g]	−3·0	5·0(DMF)	73	51·5	6·5	18·0	$C_{20}H_{30}N_6O_7$	51·3	6·4	18·4
13	Z-Arg(NO_2)-Gly-OPCP	198—202 [h]	−10·5	2·0(DMF)	64	40·3	3·5	12·5	$C_{22}H_{21}Cl_5N_6O_7$	40·1	3·2	12·8
14	Z-Gly_4-ONP	208—212 [i]			24	52·4	4·9	14·3	$C_{22}H_{23}N_5O_9$	52·7	4·6	14·0
15	Z-Gly_7-Arg(NO_2)-OMe	274—276 [f]	−1·9	1·0(DMSO)	49	44·9	5·8	21·9	$C_{29}H_{42}N_{12}O_{13}$	45·4	5·5	21·9
16	Z-Gly_2-Phe-Arg(NO_2)-OMe	126—129 [e]	−10·0	1·0(DMF)	80	52·0	6·0	17·6	$C_{28}H_{36}N_8O_9,H_2O$	52·0	5·9	17·3
17	Z-Gly_4-Phe-Arg(NO_2)-OMe	166—168 [f]	−12·0	1·0(DMF)	68	50·4	5·5	18·7	$C_{32}H_{42}N_{10}O_{11},H_2O$	50·5	5·5	18·4
18	Z-Gly_6-Phe-Arg(NO_2)-OMe	218—220 [j]	−11·0	1·0(DMF)	69	49·7	5·5	19·6	$C_{36}H_{48}N_{12}O_{13},H_2O$	49·4	5·7	19·2
19	Z-Arg(NO_2)-Gly_6-Phe-Arg(NO_2)-OMe	200—202 [f]	−4·0	2·0(DMF)	74	46·4	5·9	22·8	$C_{41}H_{59}N_{17}O_{15},2H_2O$	46·2	5·9	22·4
20	Z-Phe-Gly_2-Phe-Arg(NO_2)-OMe	194—195 [j]	−20·0	1·0(DMF)	80	56·5	5·8	16·3	$C_{37}H_{45}N_9O_{10}$	56·8	5·8	16·3
21	Z-Gly_3-Phe-Gly_2-Phe-Arg(NO_2)-OMe	176—180 [j]	−6·0	1·0(DMF)	64	53·9	6·1	16·8	$C_{43}H_{54}N_{12}O_{13},H_2O$	53·5	5·9	17·4
22	Z-Arg(NO_2)-Gly_3-Phe-Gly_2-Phe-Arg(NO_2)-OMe	135—140 [j]	−11·0	2·0(DMF)	65	48·9	5·7	19·8	$C_{49}H_{65}N_{17}O_{16},3H_2O$	48·9	5·9	19·8
23	Z-Phe-Gly_3-Arg(NO_2)-OMe	174—176 [k]	−16·0	2·0(DMF)	76	52·4	6·1	18·2	$C_{30}H_{39}N_9O_{10}$	52·6	5·7	18·4
24	Z-Gly_2-Phe-Gly_3-Arg(NO_2)-OMe	181—182 [e]	−16·5	2·0(DMF)	70	50·9	6·0	18·9	$C_{34}H_{45}N_{11}O_{12}$	51·1	5·6	19·3
25	Z-Gly_3-Phe-Gly_3-Arg(NO_2)-OMe	189—191 [f]	−3·0	1·0(DMF)	61	49·4	5·9	19·1	$C_{36}H_{48}N_{12}O_{13},H_2O$	49·4	5·7	19·2
26	Z-Arg(NO_2)-Gly_3-Phe-Gly_3-Arg(NO_2)-OMe	176—179 [k]	−1·0	2·0(DMF)	45	47·2	6·1	23·1	$C_{41}H_{59}N_{17}O_{15},H_2O$	47·0	5·8	22·7

* DMF = *NN*-Dimethylformamide. † DMSO = Dimethyl sulphoxide. ‡ Of final stage.
Cryst. from: [a] EtOAc; [b] MeOH–Et_2O; [c] AcOH–EtOAc; [d] Me_2CO–light petroleum; [e] MeOH; [f] H_2O; [g] EtOH; [h] DMF–Me_2CO; [i] DMF–Et_2O; [j] aq. EtOH; [k] aq. MeOH.

In the formation of a dioxopiperazine the benzyloxycarbonyl group was removed from *p*-nitrobenzyl benzyloxycarbonylglycyl-L-nitroargininate (19·8 g., 0·036 moles) as already described. The peptide hydrobromide was washed well with anhydrous ether then dissolved in dimethylformamide (50 ml.); triethylamine (25 ml.) was added and the solution was kept for 1 hr. Triethylammonium bromide was filtered off and the excess of triethylamine was removed on a rotary evaporator. The mixture was set aside overnight, and the solid which had separated was filtered off, washed with tetrahydrofuran, and dried. 3-[3-(*Nitroguanidino*)*propyl*]-2,5-*dioxopiperazine* (7·5 g., 80%) had m.p. 235—238° (Found: C, 37·0; H, 5·7; N, 30·6. $C_8H_{14}N_6O_4$ requires C, 37·2; H, 5·5; N, 32·5%).

Removal of Protecting Groups and Purification of Final Products.—The fully protected peptide (0·5 g.) was dissolved in a mixture of glacial acetic acid (10 ml.), methanol

[14] I. Smith 'Chromatographic and Electrophoretic Techniques,' vol. I, Heinemann, London, 1960, p. 97.

(200 ml.), and N-hydrochloric acid (2 ml.), and hydrogenated, at atmospheric pressure or slightly above, over palladium–charcoal (5%; 300 mg.). Samples were withdrawn from time to time for chromatography and the hydrogenation was continued while ninhydrin-negative, chlorine–starch–iodide-positive spots were apparent. When the reaction was complete (16—30 hr.) the catalyst was filtered off and the solvent evaporated off. Water (20 ml.) was distilled from the residue which was then dissolved in water (10 ml.), brought to pH 10·5 and maintained there for 1 hr. with a pH Stat by the addition of N-sodium hydroxide. Electrophoresis (pH 6·1) revealed that the spot corresponding to peptide ester had disappeared and a spot of lower mobility had appeared, indicating that the ester group had been completely saponified by this treatment.

TABLE 2

Peptides

No.	Compound	$[\alpha]_D^{22}$ *	Yield † (%)	R_{Arg} pH 6·1 ‖	R_{Arg} pH 1·9 ¶	R_F ‡ (A)	R_F ‡ (B)	Amino-acid ratio § Arg	Gly	Phe
1	Arg-Gly-Arg	+50·3	30	1·0	1·36	0·28	0·53	2·0	0·94	
2	Arg-Gly_2-Arg	+9·0	48	0·95	0·95	0·22	0·76	2·0	2·0	
3	Arg-Gly_3-Arg	+4·5	30	0·92	0·92	0·33	0·73	2·0	3·14	
4	Arg-Gly_4-Arg	+7·0	38	0·83	0·88	0·23	0·73	2·0	3·92	
5	Arg-Gly_5-Arg		28	0·82	0·92	0·09	0·74	2·0	5·04	
6	Arg-Gly_6-Arg	+6·0	34	0·76	0·88	0·16	0·74	2·0	6·4	
7	Arg-Gly_7-Arg	+1·5 **	20	0·77	0·89	0·10	0·76	2·0	6·93	
8	Arg-Gly_6-Phe-Arg	+10·0	32	0·70	0·76	0·37	0·90	2·0	6·24	1·04
9	Arg-Gly_3-Phe-Gly_3-Arg	+8·0	28	0·68	0·74	0·37	0·90	2·0	6·00	0·99
10	Arg-Gly_3-Phe-Gly_2-Phe-Arg	+9·5	32	0·60	0·74	0·25	0·91	2·0	5·12	2·04

* *c* 1 in water. † Overall (deprotection and purification). ‡ Descending on Whatman no. 4 paper in (A) Bu^nOH–pyridine–acetic acid–water (30 : 20 : 6 : 24) and (B) Pr^nOH–ammonia (*d* 0·88)–water (3 : 6 : 1). § Expressed in terms of Arg = 2·0. ‖ Pyridine acetate buffer. ¶ Formic acid–acetic acid. ** Lit. $[\alpha]_D^{22}$ −11·8° (*c* 1·1 in N-AcOH).

The pH was adjusted to 5·0 with glacial acetic acid and the solution was put on a column (25 × 2 cm.) of carboxymethylcellulose (Whatman CM 32) which had been precycled and equilibrated with 0·01M-ammonium acetate buffer (pH 5·0). Elution was carried out with a gradient of 0·01—0·5M-ammonium acetate (pH 5·0). Fractions were examined for Sakaguchi-positive material and positive fractions were characterised by electrophoresis. Fractions comprising each positive peak were combined and concentrated. Ammonium acetate was removed by evaporating off glacial acetic acid and water (several times each), and the *products* were isolated by lyophilisation from water. The fluffy powders were characterised by paper chromatography and electrophoresis and their specific rotations and amino-acid contents were determined. Data are presented in Table 2.

p-*Nitrobenzyl Esters of Benzyloxycarbonyl-glycyl- and -diglycyl-nitro-*L*-arginine.*—The appropriate acid (0·1 mole) was dissolved in dimethylformamide (150 ml.), *p*-nitrobenzyl chloride (25·8 g., 0·15 mole) and triethylamine (21 ml., 0·15 mole) were added, and the mixture was heated at 80° for 12 hr. The solvent was evaporated off under reduced pressure and the residue was dissolved in ethyl acetate (1 l.). The solution was washed successively with N-hydrochloric acid, water, N-ammonium hydroxide, and water again, dried, and concentrated. The solid which separated was filtered off and sucked dry. Solvents for crystallisation and physical data on the *esters* are given in Table 1 (nos. 1 and 4).

Synthesis of Arginylglycyl- and Arginyldiglycyl-arginine by the 'Solid Phase' Method.—(*a*) t-Butoxycarbonylnitro-L-arginine was esterified with chloromethylated copolystyrene–divinylbenzene (Bio–Rad); protecting groups were removed and the product was coupled essentially as described by Merrifield.[12] The resulting product was cleaved from the resin by the same author's procedure and hydrogenated by suspending the protected peptide (2 g.) in methanol (100 ml.) containing N-hydrochloric acid (2 ml.) in the presence of palladium–charcoal (10%; 1 g.). Hydrogenation was continued for 48 hr. at 3 atmos. The catalyst was then filtered off, and the filtrate was concentrated and then lyophilised from glacial acetic acid solution.

(*b*) The crude product (1 g.) was dissolved in 0·6M-pyridine acetate buffer pH 7·0 (5 ml.) and introduced on to a column (100 × 1·8 cm.) of Amberlite CG-50 resin. Elution was effected with a gradient from 0·6M (pH 7·0) to 0·5M (pH 5·8); fractions (15 ml.) collected were tested for ninhydrin and Sakaguchi-positive reaction.

(i) From fractions 41—46 of the column purification of crude arginylglycylarginine, a chromatographically homogeneous peptide (240 mg., 30%) was obtained, R_F(A) (see Table 2) 0·28, R_F(B) 0·51, R_{Arg} (pH 6·1) 1·0, R_{Arg} (pH 1·9) 1·30, $[\alpha]_D^{21}$ +48·2° (*c* 0·1 in H_2O); amino-acid ratio found: arg : gly = 1·94 : 1·00.

(ii) Similarly, from fractions 58—68 of the column purification of crude arginyldiglycylarginine a chromatographically homogeneous peptide (186 mg., 23%) was obtained, R_F(A) 0·30, R_F(B) 0·52, R_{Arg} (pH 6·1) 1·00, R_{Arg} (pH 1·9) 1·28, $[\alpha]^{20}$ +10·0 (*c* 1·0 in H_2O); amino-acid ratio found: arg : gly = 1·00 : 1·13.

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