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SOLID-PHASE SYNTHESIS USING A NEW POLYACRYLIC RESIN

SYNTHESIS OF THE FRAGMENT 14-21 OF THE AMINO ACID SEQUENCE OF HISTONE H4

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Abstract—The solid-phase synthesis of the octapeptide **1** AcGly–Ala–Lys–Arg–His–Arg–Lys–ValOMe, which represents the fragment 14-21 of the amino acid sequence of the chromosomal histone H4, as well as of the structurally related nonapeptide **2** AcGly–Ala–Lys–Leu–Arg–His–Arg–Lys–ValOMe, is described using a new polyacrylic resin containing a glycolamide ester linkage (resin–NHCO–CH_2–OCO–peptide) acting as a labile anchoring moiety between the resin and the peptide.

After elongation of the polypeptide chain using classical protecting groups, i.e. t-butyloxycarbonyl for the α-NH_2 function, benzyloxycarbonyl, nitro and 2,4-dinitrophenyl groups for the side-chains of Lys, Arg and His respectively, both peptides **1** and **2** were obtained in good yields and with a high purity as shown by high-pressure liquid chromatography, by amino-acid analysis and by high-field proton NMR spectroscopy.

This work demonstrates the ability of the newly introduced polyacrylic resin to act as a convenient support for solid-phase peptide synthesis.

A very promising improvement in the solid-phase peptide synthesis methodology is provided by the use of polyacrylic resins first introduced by Atherton *et al.*[1–5] Because of its structure a polyacrylic polymer displays remarkable swelling properties in a great variety of solvents commonly used in peptide synthesis (or polynucleotide synthesis) as well as in aqueous media. Long peptides (10 to 30 amino acids) have been thus obtained using a polyacrylic matrix with good yields and a well-controlled purity.[4,5]

We report the use of a new polyacrylic resin[6] as a solid support in the stepwise synthesis of the highly basic octapeptide AcGly–Ala–Lys–Arg–His–Arg–Lys–ValOMe **1**, which represents the fragment 14-21 of the amino acid sequence of the chromosomal histone H4. This peptide contains the major site of enzymatic acetylation *in vivo*, i.e. Lys 16, and it has been shown to be a substrate of the acetyltransferase which acetylates this site on the intact protein in chromatin.[7]

We also report the synthesis of the related nonapeptide AcGly–Ala–Lys–Leu–Arg–His–Arg–Lys–ValOMe **2**. The synthesis of the octapeptide **1** with its C-terminal position substituted by a primary amide group has been previously reported,[7,26] using a benzhydrylamide polystyrene resin.

As previously described,[6] the polyacrylic support used in this work has been prepared through copolymerisation of N-acryloylpyrrolidine **3** as the basic monomer, bis-(acryloyl) 1,2-diaminoethane **4** as the cross-linking agent and the methyl ester of N-acryloyl-β-alanine **5** as the functionalising agent. A reversible chemical linkage between the peptide chain and the polymeric backbone was provided by using a recently introduced,[8] glycolamide ester moiety. Such a chemical anchor can be readily cleaved by nucleophilic attack, thus leading to the production of a carboxylic acid or a methyl ester or a primary amide at the C-terminal position of the synthesised peptide.

The usefulness of the new polyacrylic resin has been assessed by the synthesis of the two above mentioned peptides **1** and **2** which were characterised by high pressure liquid chromatography (HPLC) and amino acid analysis. The use of spin–spin decoupling and two dimensional correlation in high field proton NMR (360 MHz) has also proved to be very valuable in establishing the purity of **1** and **2**.

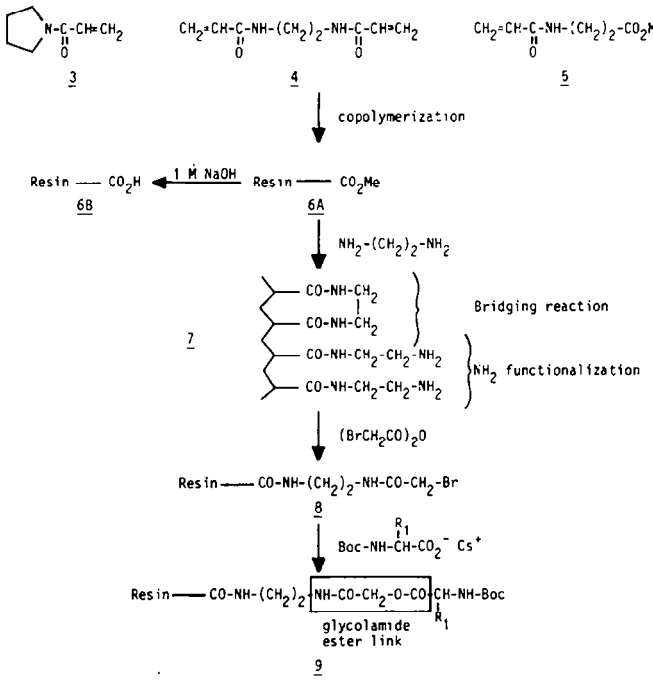
The preparation of histone fragments of high purity by chemical synthesis is of interest insofar as it provides a tool for the study of chromatin structure and function.

RESULTS

The polymeric support

A regularly beaded polyacrylic resin of high porosity (as judged by electron microscopy) was obtained as previously reported,[6] through copolymerisation of **3**, i.e. N-acryloylpyrrolidine,[11,12] of **4**, i.e. bis-(acryloyl)1,2-diaminoethane[12] and of **5**, i.e. methyl ester of N-acryloyl-β-alanine.[6]

The degree of functionalisation by the β-alanine moiety was determined by direct amino acid analysis of resin–CO_2–CH_3 **6A** (yielding 0.93 mmol β-Ala/g of dry resin, using Nor Leu as internal standard) and by titration of the CO_2H functions in resin **6B** (yielding 0.86 mmol of CO_2H/g of dry resin) obtained after saponification of **6A**. The two analytical methods



Scheme 1.

afford very similar results for the degree of functionalisation of the polyacrylic resin. No further characterisation of the polymeric support was carried out and it was then transformed into the basic polymer resin–CONH–$(CH_2)_2$–NH_2 **7** by allowing 1,2-diaminoethane to react with **6A** under conditions previously described by Arshady *et al.*[2] for a different polyacrylic support.

The content of primary amino groups in **7** was determined by the ninhydrin assay, first proposed by Sarin *et al.*[13] for a polystyrene resin and it corresponds to 0.55 mmol/g of dry resin. A loss of functionalisation is systematically observed after treatment of **6A** with 1,2-diaminoethane which is likely to be due to the bridging of the two functionalising groups by a given diamine molecule[12,14] (Scheme 1). In agreement with this assumption, it was shown that no methyl ester group remains on the resin after treatment with 1,2-diaminoethane. (An aliquot of the resin **7** was acetylated by acetic anhydride in DMF[5] in order to substitute all primary amino groups and it was then hydrolysed with 1 M NaOH for 4 hr at ambient temperature. The polymer was treated with 1 M HCl and washed with water until neutrality and successively with ethanol and ethyl ether. After drying, there was no evidence of any free carboxylic group on the polymeric support as indicated by titration with 0.1 M NaOH.)

In order to obtain the peptide free in soln, a labile anchoring group was introduced by allowing bromoacetic anhydride to react with the free NH_2 groups of resin **7**.[6,8] Substitution of **7** by the bromoacetamido group is apparently complete as judged by the absence of any primary amino group[15] and by the content of Br atoms (0.48 mmol Br/g of dry resin) which was determined by titration of the liberated bromide ions following a modified Charpentier–Volhard procedure after hydrolysis of **8** for 10 min in boiling 1 M NaOH.[16]

The bromine functionalised polyacrylic resin **8** was used without further treatment for synthesising the octapeptide **1** and the nonapeptide **2**.

Stepwise solid-phase synthesis

The cesium salt of Boc–Val–OH[17] was reacted with resin **8** suspended in DMF. The extent of coupling was monitored by the ninhydrin assay of Sarin *et al.*[13] on carefully weighed resin aliquots after acidolysis of the Boc protecting group and it was shown to be quantitative after about 40 hr. Amino acid analysis of the Boc–Val substituted resin gives a value of 0.404 mmol Val/g of dry resin.

The Boc protection was adopted for all amino acids used in this work, i.e. Boc–L–Val–OH, Boc–L–Lys(Z)–OH, Boc–L–Arg(NO_2)–OH, Boc–L–His(Dnp)–OH, Boc–L–Ala–OH and Boc–L–Leu–OH, except for Ac–Gly–OH. Symmetrical anhydrides of these amino acid derivatives were prepared according to a standard procedure.[4,5,18] The general protocol for the synthesis is summarised in Table 1.

The progression of the synthesis was monitored by a qualitative ninhydrin assay[15] at the end of each coupling step and by the above-mentioned quantitative ninhydrin assay[13] at the end of each Boc-deprotection step. Monitoring by amino acid analysis was also carried out at some steps of the peptide chain elongation as given in Table 2. Since acidic hydrolysis of a given amount of resin–peptide yields a constant quantity of β-alanine, this amino acid was therefore used as an internal standard for determining the content of peptide linked to the resin.

As is apparent from the results in Table 2, the amount of peptide linked to the resin markedly decreases during

Table 1. Procedure for the solid-phase synthesis

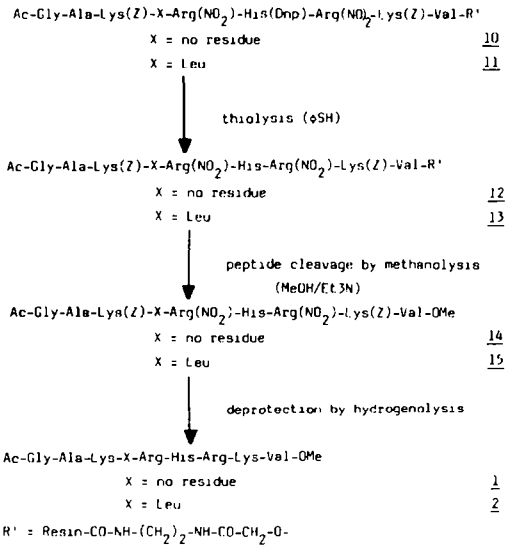
Step	Time	Solvent or reagent
Boc-deprotection	1 × 2 min	TFA (30% by vol.) in CH_2Cl_2
	1 × 30 min	TFA (30% by vol.) in CH_2Cl_2
Washing	4 × 2 min	CH_2Cl_2
Neutralisation	1 × 2 min	DIEA (5% by vol.) in CH_2Cl_2
	1 × 30 min	DIEA (5% by vol.) in CH_2Cl_2
Washing	4 × 2 min	CH_2Cl_2
Coupling	1 × 45 min	Symmetrical anhydride of Boc amino acid (3 equiv in CH_2Cl_2)[a] and 1 equiv of DIEA
Washing	4 × 2 min	DMF
	4 × 2 min	CH_2Cl_2

[a] Boc–Arg(NO_2)–OH and AcGly–OH were solubilised in the minimum amount of [2]DMF and the volume was completed with CH_2Cl_2.

synthesis since the final step only represents about 52% of the value expected on the basis of the initial valine content. The release of peptide from the support occurs primarily after the second coupling step (as suggested by the ninhydrin assay[13] after Boc-deprotection) and it could correspond to the formation of a diketopiperazine adduct during the prolonged neutralisation step (Table 1). Such a loss is likely to be due to the observed lability of the glycolamide group in the presence of a nucleophilic agent[8] (free NH_2 group of the deprotected lysyl residue). However, the loss of the peptide can be minimised to less than a few percent by reducing the time of the neutralisation step (Discussion).

Since both peptides **1** and **2** have a partial common sequence, the intermediate Boc – Arg(NO_2) – His(Dnp) – Arg(NO_2) – Lys(Z) – Val – O – CH_2 – CO – NH – $(CH_2)_2$ – NH – CO – resin was first synthesised and divided in two distinct parts (65% and 35% in weight) as precursors of **1** and **2** respectively.

After completion of the synthesis, the polyacrylic resin **10** substituted with the fully protected octapeptide was treated with thiophenol at room temperature by a procedure previously described.[19]



Scheme 2.

Table 2. Control of the quantity of peptide linked to the resin during the synthesis of octapeptide **1** (starting amount of resin 1.089 g) as inferred from amino acid analysis after direct hydrolysis of the peptide–resin

Peptidyl moiety attached to the resin	Amount of amino acid (mmol) attached to the resin							Amount of peptide linked to the resin (mean value, mmol)	Molar ratio of peptide to β-alanine
	Gly	Ala	His	Lys	Val *a*	Arg + Orn *b*	*a*+*b*		
Boc–Val–					0.438			0.438	0.470
Boc–His(Dnp)–Arg(NO_2)–Lys(Z)–Val–			—[a]	0.253 (1.0)[c]	0.268[b] (1.06)	0.238 (0.94)	0.506 (2.0)	0.253	0.272
Ac–Gly–Ala–Lys(Z)–Arg(NO_2)–His–Arg(NO_2)–Lys(Z)–Val	0.246 (1.08)	0.237 (1.04)	0.207[d] (0.91)	0.447 (1.96)	0.280[b] (1.23)	0.394 (1.73)	0.674 (2.96)	0.228	0.245

[a] No His detected, (His(Dnp) not eluted in the chosen gradient system).
[b] Since Val and Arg(NO_2) are eluted as a single peak, *a*+*b* represents the amount of Arg and Val in the peptide.
[c] Values in brackets correspond to ratios of the amount of a given amino acid to the amount of peptide.
[d] Resin after thiolysis.

The time course of the reaction was monitored by measuring the concentration of the liberated disulphide adduct by UV absorbance. As shown in Fig. 1, the thiolysis reaction is complete after 10 min. The release of 0.22 mmol of disulphide corresponds to the value expected on the basis of the total amount of peptide **1** (0.23 mmol) linked to the support as measured by amino acid analysis (Table 2).

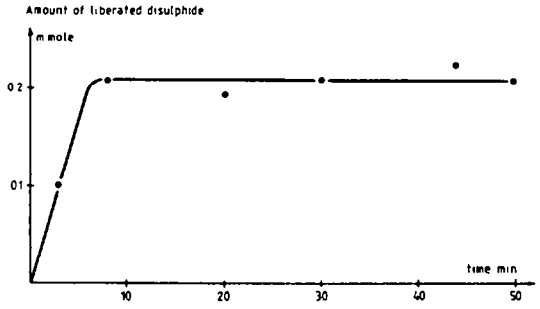


Fig. 1. Time course of the deprotection of the histidyl residue of Ac–Gly–Ala–Lys(Z)–Arg(NO_2)–His(Dnp)–Arg(NO_2) – Lys(Z) – Val – R' **10** by treatment with thiophenol (15 equivalents in DMF) at 20°. The concentration of $C_6H_3(NO_2)_2 - S - C_6H_5$ as measured by UV absorbance at 337 nm is given as function of time (ε = 10,250 in DMF[19]).

Since, as previously discussed,[7] the purpose of this work was to synthesise the histone fragment 14-21 with both N- and C-terminal positions blocked with non-charged chemical groups, the cleavage of the Dnp-deprotected octapeptide from the resin (**12** → **14**) was carried out by methanolysis[20] yielding a methyl ester group at the C-terminal position. The release of the soluble peptide **14** was monitored by UV absorbance at 270 nm (arginyl nitro group) and it was complete after 100 hr. Peptide **14** was finally deprotected by catalytic hydrogenation. The reaction was monitored by UV absorbance at 270 nm and it was complete after 24 hr. The crude peptide **1** thus obtained was analysed by

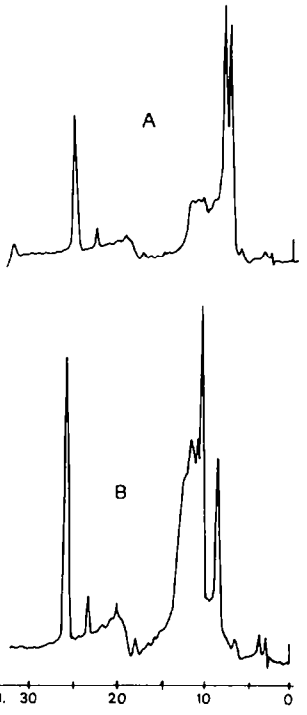


Fig. 2. Reverse phase HPLC analysis of crude peptides **1** curve A) and **2** (curve B) after deprotection step (see Scheme 2) on Lichrosorb RP-18 Merck (column size 250 × 4 mm, particle size 7 μm). Elution was carried out with the buffer A, 0.076 M triethylammonium phosphate pH 3.2 (H_3PO_4) and B, acetonitrile Lichrosolv, Merck, linear gradient from 0% to 90% B into A in 25 min, 1 ml/min. UV absorbance was monitored at 220 nm at a full scale sensitivity of 0.64 absorbance unit. Paper feed rate 5 mm/min.

analytical HPLC as shown in Fig. 2A. Peptide **2** was obtained from **15** by the same procedure (Scheme 2) and the pattern observed by analytical HPLC is displayed in Fig. 2B. Although the partially protected peptides **14** and **15**, which are released from the resin by

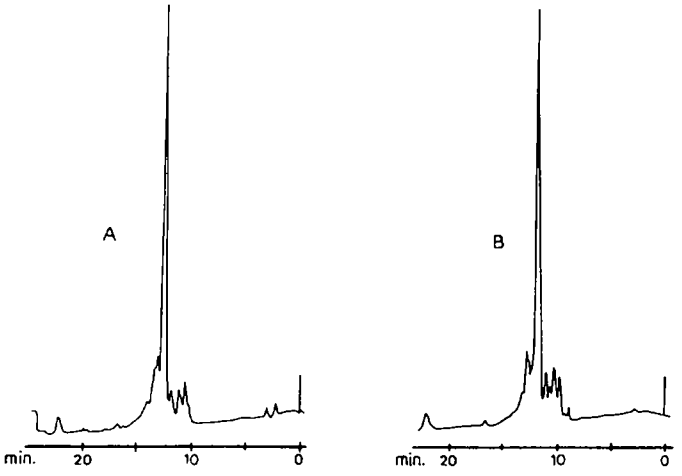


Fig. 3. Reverse phase HPLC analysis of peptides **14** (curve A) and **15** (curve B) on Lichrosorb RP-18 Merck (column size 250 × 4 mm, particle size 7 μm). Elution was carried out with the buffer A 0.076 M triethylammonium phosphate pH 3.2 (H_3PO_4) and B, acetonitrile Lichrosolv, Merck, linear gradient from 15% to 90% of B into A in 20 min, 1 ml/min. UV absorbance was monitored at 270 nm at a full scale sensitivity of 0.64 absorbance unit. Paper feed rate 5 mm/min.

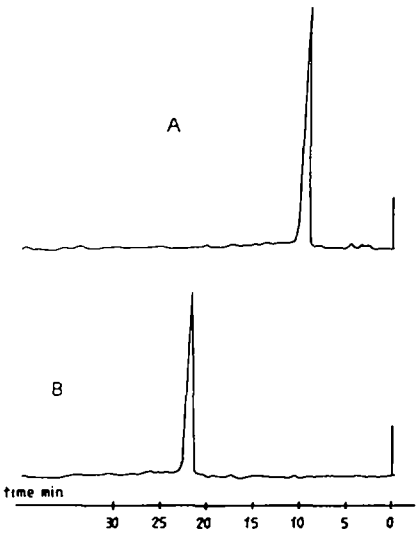


Fig. 4. Reverse phase HPLC analysis of peptides **1** (curve A) and **2** (curve B) on Lichrosorb RP-18 Merck (column size 250 × 4 mm, particle size 7 μm). Elution was carried out with the buffer A, 0.076 M triethylammonium phosphate pH 3.2 (H_3PO_4) and B, acetonitrile RS, Carlo Erba, linear gradient from 0 to 25% of B into A in 30 min. UV absorbance was monitored at 220 nm at a full scale sensitivity of 1.28 absorbance units. Paper feed rate 5 mm/min. Flow 1 ml/min.

methanolysis, are reasonably homogeneous as judged by HPLC (Fig. 3). The homogeneity of both peptide preparations after hydrogenolysis appears to be rather poor as judged by the complexity of their HPLC patterns (Figs 2A and 2B). As discussed below this is strong indication that the hydrogenation reaction gives rise to side-products, certainly in relation to the presence of the nitro-protecting groups on the arginyl residues.

Since peptides **1** and **2** are highly basic, the reaction mixture after hydrogenolysis was chromatographed through a carboxymethyl–cellulose ion-exchange column using a NaCl gradient as previously described for the octapeptide **1** substituted with a primary amide group[7] at the C-terminal position. In both cases, elution occurs at about 0.3 M NaCl as expected for peptides with 4 to 5 positive charges.[7]

The pooled fractions were desalted by Sephadex G10 gel filtration in 0.01 M HCl and the peptide solution was finally freeze-dried, resulting in a white powdered residue which was characterised by analytical HPLC (Fig. 4) and by amino acid analysis ($Gly_{0.95}Ala_{1.04}Lys_{2.0}Arg_{2.0}His_{0.97}Val_{1.0}$ for peptide **1** and $Gly_{1.0}Ala_{1.04}Lys_{1.94}Leu_{1.01}Arg_{2.02}His_{0.95}Val_{1.03}$ for peptide **2**). The total yields of purified peptides **1** and **2** were determined by amino acid analysis using Nor Leu as internal standard and they correspond to about 50% of the expected values on the basis of the initial valine content of the resin.

^{1}H-NMR at 360 MHz was used to characterise both peptides **1** and **2** in order to assess their purities. The NMR spectrum of octapeptide **1** dissolved in DMSO-d_6 is given in Fig. 5. Furthermore, the octapeptide **1** was extensively analysed by the classical decoupling technique as well as by the two-dimensional correlation (COSY) spectroscopy technique as shown in Fig. 6. Such an analysis, which enables the assignment of almost all of the signals in the NMR spectrum of **1**, is summarised in Table 3.

In the case of nonapeptide **2**, the ^{1}H-NMR spectrum (not shown) contains all the signals characteristic of peptide **1** with the addition of the signals corresponding to a leucyl residue.

It must be noted that all chemical shifts observed on the NMR spectrum of **1** are very close to those reported for the series of tetrapeptides Ac–Gly–Gly–*X*–Ala–OMe in DMSO-d_6 where *X* stands for any natural occuring amino acid.[21] A marked difference is, however, observed for the chemical shift of the histidyl C_2–H, which is found at 9.10 ppm in the octapeptide **1** whereas it is observed at 8.35 ppm in the histidyl model tetrapeptide[21] (no significant difference is observed for

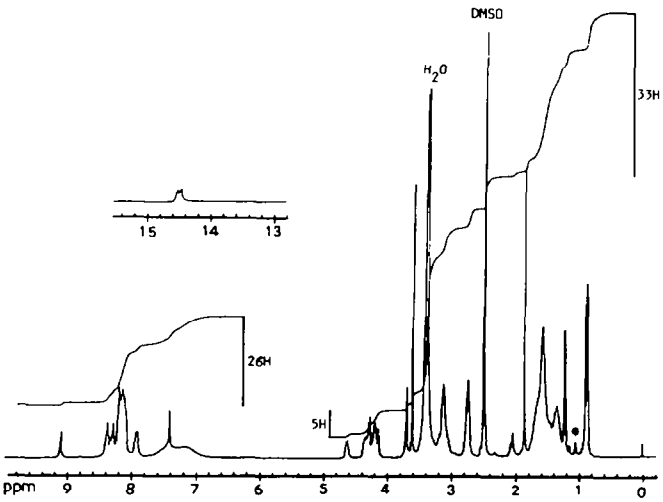


Fig. 5. ^{1}H-NMR spectra of peptide **1** at 360 MHz (5 mg of **1** in 0.5 ml of DMSO-d_6, 128 scans). * Impurity (solvent).

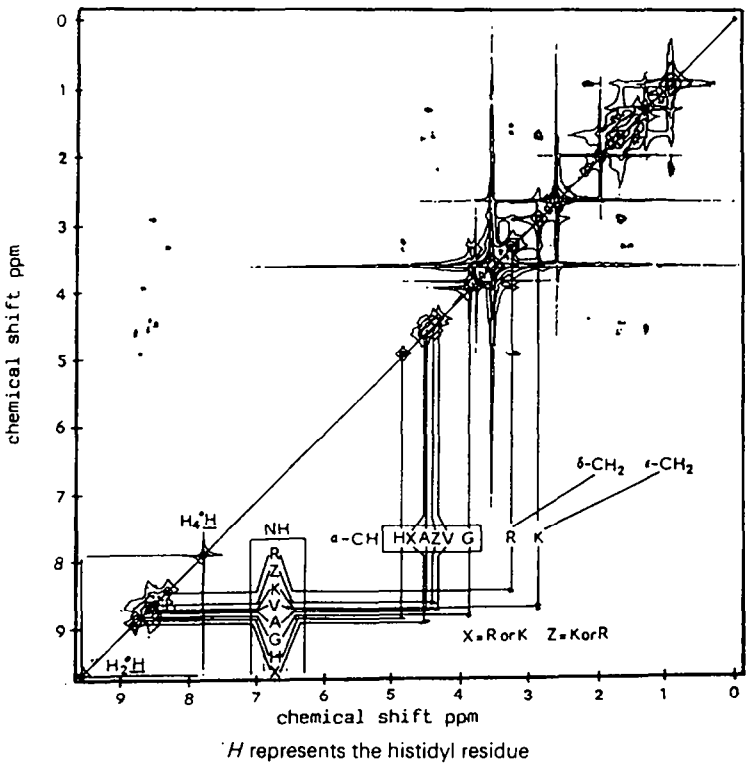


Fig. 6. 2D ^{1}H-NMR spectra of peptide **1** AcG–A–K–R–H–R–K–VOME. This interpretation is only given for the αNH–αCH connectivities.

C_4–H). It is likely that the low-field position of C_2–H in **1** reflects the highly polar environment of the histidyl residue (two adjacent arginyl residues) as well as the total protonation of the imidazole ring as shown by the appearance of two distinct one-proton signals at very low-field position (*ca* 14.5 ppm).

The high-field ^{1}H-NMR spectrum of **1** in DMSO-d_6 clearly indicates that the polar groups of the arginyl and lysyl side-chains are fully protonated. Indeed, integration of the 6.5–8.5 ppm region indicates about 25 protons. If one excludes the C_4–H proton at 7.417 ppm of the single histidyl residue such a value can be accounted for by: (i) 14 protons between 8.0 and 8.5 ppm which correspond to 8 amide NH protons and 6 protons (about 8.14 ppm) originating from two ε NH_3^+ groups of the lysyl residues; (ii) two protons at about 7.93 ppm for the two δ NH arginyl groups and (iii) 8 protons originating from two guanidinium groups of the arginyl residues (broad signal). The origin of the low intensity signal at 8.90 ppm is likely to correspond to the unprotonated form of histidine in octapeptide **1**, since this signal disappears upon addition of acid.

DISCUSSION

The purpose of this work was to demonstrate the ability of the new polyacrylic resin[6] substituted with the glycolamide ester linkage[8] to act as a convenient polymeric support for solid-phase peptide synthesis using classical protecting groups for the α NH_2 functions as well as for the side-chains of the trifunctional amino acids. The yield and the purity of both peptides **1** and **2** thus prepared demonstrate that the new polyacrylic resin is well adapted to solid-phase peptide synthesis. These results are to be compared to those reported by Sheppard's laboratory using a polyacrylic resin prepared by bead copolymerisation of N,N-dimethylacrylamide, bis-(acryloyl) 1,2-diaminoethane as a cross-linking agent and acryloylsarcosine methyl ester as a functionalising agent.[2,3]

At this time, a variety of peptide–resin anchorages has been proposed in the case of a polyacrylic support.[22–24] Such labile linkages involve the formation of a benzylic ester with the COOH function of the first attached amino acid. The lability of the ester bond to acidic or nucleophilic reagents can be modified by introducing electron-donating or withdrawing substituents on the aromatic ring as well as by altering the length and/or the chemical nature of the handle which links the benzyl ester moiety to the resin.[22–24] The use of varied peptide–resin linking agents thus increases the versatility of the solid-phase peptide synthesis. However, such linking agents are not readily available and this makes their use somewhat restricted. In contrast, the glycolamide ester link used in this work is readily introduced on the polyacrylic support (Scheme 1). The newly introduced linkage allows different chemical groups to be readily introduced at the C-terminal position of the synthetic peptide, either a carboxylic acid, or a primary amide, or a methyl ester.[8]

It must be noted that the yield of the purified octapeptide **1** (50% as determined by amino acid analysis) is very close to the expected value on the basis of the amount of peptide linked to the resin at the end of the synthesis (0.228 mmol thus corresponding to a yield of 52% on the basis of the initial valine content; Table 2). This must be due to the fact that the intermediate protected peptides **14** and **15**, which were obtained after cleavage from the resin by methanolysis (Scheme 2), are

Table 3. Characterisation of the protons (83) in the octapeptide AcGly–Ala–Lys–Arg–His–Arg–Lys–ValOMe (protonated $C_{42}H_{83}N_{18}O_9$, not protonated $C_{42}H_{78}N_{18}O_9$). The spectrum was recorded in DMSO-d_6 at 360 MHz (see Fig. 5)

Residue	Chemical group	Chemical shift (ppm)	Multiplicity and number of protons	Coupling constant(s) (Hz)
Gly 14	CH_3 (N-acetyl)	1.870	S (3H)	
	α-CH_2	3.710	D (2H)	5.7
	NH	8.282	T* (1H)	5.8
Ala 15	β-CH_3	1.235	D (3H)	7.2
	α-CH	4.288	Qd (1H)	7.2
	NH	8.209	D (1H)	6.8
Lys 16 and Lys 20	ε-CH_2	2.75	Multiplet (4H)	
	β,δ-CH_2	1.5 to 1.75	Multiplet (8H)	
	γ-CH_2	1.36	Multiplet (4H)	
	α-CH*			
	NH	8.386 and 8.377	D (2H)	7.5 and 6.8
	ε-NH_3	8.14	Dd (6H)	
Arg 17 and Arg 19	δ-CH_2	3.13	Multiplet (4H)	*ca* 5
	β,γ-CH_2	1.55 to 1.65	Multiplet (8H)	
	α-CH*			
	NH	8.102 and 8.081	D (2H)	4.5 and 4.15
	δ-NH	7.908 and 7.932	T* (2H)	5.3 and 5.6
	ω-NH_2	6.9 to 7.3	Broad signal (8H)	
His 18	β-CH^a_{2b}	3.050	Dd (1H)	15.2 and 8.3
		3.177	Dd (1H)	15.2 and 4.7
	α-CH	4.633	Ddd (1H)	8.3 and 4.9
	NH	8.318	D (1H)	
	C2-H	9.102	S (1H)	7.9
	C4-H	7.417	S (1H)	
	$NH(im)^a_b$	14.462	S (slightly broadened) (1H)	
		14.527	S (slightly broadened) (1H)	
Val 21	γ-CH^a_{3b}	0.874	D	6.8
		0.906	D 6H	6.8
	β-CH	2.054	Multiplet (1H) (6 apparent lines)	J_{mean} = 6.7
	α-CH	4.150	Dd (1H)	7.5 and 6.8
	NH	8.189	D (1H)	7.5
	OCH_3	3.623	S (3H)	

* No distinction could be obtained between the α-CH protons of arginyl and lysyl residues which appear as two groups of two-proton signals at about 4.20 ppm (signal Z in Fig. 6) and at about 4.35 ppm (signal X in Fig. 6).

rather homogeneous as judged by HPLC (Fig. 3) and ^{1}H-NMR (not shown). The complex HPLC elution pattern, which is observed after final deprotection (Fig. 2), is therefore due to catalytic hydrogenation. The observation that most of the contaminant products are common to both peptide preparations strongly suggests that they originate during this reaction.

It has been shown that the glycolamide ester anchoring group is sensitive to nucleophilic attack by primary and secondary amines.[8] It might therefore be that the observed peptide loss (about 48%; see Table 2) is due to an intramolecular reaction involving the free α-NH_2 group during the neutralisation step performed after deprotection of Boc–Lys(Z)–Val–resin. A re-investigation of the three first coupling steps has been carried out by using the Ddz protecting group.[25] We allowed the time of contact with DIEA during neutralisation (Table 1) to be substantially reduced (about 2 min in all). Under these conditions the loss of peptide from resin was negligible (to be published). It therefore appears that the new polyacrylic resin is able to provide peptides with high yields.

Peptide **1** with its C-terminal position blocked by a primary amide group was previously synthesised by using a benzhydrylamine polystyrene resin.[7,26] The purified peptide was characterised by amino acid analysis, paper electrophoresis, field desorption mass spectrometry and ^{1}H-NMR spectra. By using these criteria the purity appears to be identical to that reported for peptide **1** methyl ester. However, the overall yields of the synthesis performed with the benzhydrylamine polystyrene resine are substantially lower (total yield about 10%) than that obtained for peptides **1** and **2** (total yield about 50%) with the newly introduced polyacrylic resin.

The optical purity of the synthesised peptides **1** and **2** was not controlled during this work. However, a careful investigation of the extent of racemisation was previously carried out for octapeptide **1** (synthesised on polystyrene support) substituted by a primary amide instead of a methyl ester.[26] The extent of racemisation of each amino acid did not exceed 0.5% as shown by enzymatic hydrolysis using L-amino acid oxidase, the strict similarities of the ^{1}H-NMR at 360 MHz of both

peptides (1 amide and 1 methyl ester) clearly indicate that no detectable racemisation occurs during the synthesis of the latter on the polyacrylic support. It is known that ^{1}H-NMR is well adapted for detecting diastereoisomeric effects in peptides.[27]

As shown in this work, high-field proton NMR provides a convenient method for testing the purity of a synthetic peptide. Although a few extraneous low-intensity signals are present (due to slight contamination by solvent molecules; Fig. 5), it can thus be inferred that the octapeptide 1 is structurally homogeneous. It must be emphasised that the present state of purification of peptides 1 and 2 is only a practical one. No attempts have been made to purify them more extensively (e.g. by crystallisation). It is nevertheless remarkable to obtain these highly basic peptides in good yields and with a high purity in a relatively straightforward manner. This demonstrates the ability of the new polyacrylic resin substituted by the glycolamide ester group to act as a convenient support for solid-phase peptide synthesis.

EXPERIMENTAL

Materials and methods

Abbreviations are as follows: DDC, dicyclohexylcarbodiimide; DIEA, N-ethyldiisopropylamine; DMF, N,N-dimethylformamide; TFA, trifluoroacetic acid; Boc, t-butyloxycarbonyl; Z, benzyloxycarbonyl; Dnp, 2,4-dinitrophenyl; TMEDA, N,N,N',N'-tetramethylenediamine.

CH_2Cl_2 was dried overnight on anhyd K_2CO_3 and distilled on P_2O_5 before use. DMF (Fluka, puriss) was dried on molecular sieves (4 Å) and amino impurities eliminated by bubbling dry nitrogen (negative test with 1-fluoro 2,4-dinitrobenzene).[28]

Amino acid derivatives, Boc–L–Val–OH, Boc–L–Lys(Z)–OH, Boc–L–Arg(NO_2)–OH, Boc–L–His(Dnp)–OH, Boc–L–Ala–OH, Boc–L–Leu–OH and AcGly–OH were purchased from Fluka and used without any purification.

All operations of solid-phase peptide synthesis were carried out, as previously described,[26] under mechanical shaking using a glass reactor (about 150 ml total volume) equipped with two polyethylene sinters and a lateral outlet stopped with a screwed Teflon cap (Fig. 7). The amino acid derivatives (symmetrical anhydrides usually), as well as the reactants during the deprotection and neutralisation steps were introduced through the lateral outlet whereas all washing solvents were introduced through the polyethylene sinters, under a stream of dry nitrogen. The general procedure is given in Table 1.

Hydrolyses of peptides and peptide–resins were carried out under vacuum in the presence of 6 N HCl (Merck, Suprapur) for 24 or 48 hr using norleucine as an internal standard. Amino acid analyses were obtained with a Chromakon 400 apparatus (Kontron, Germany). Analytical and semi-preparative HPLC separations were performed on a Perkin–Elmer apparatus (3 B series). Ion-exchange chromatography on carboxymethylcellulose (Whatman) was performed as previously described.[7] NMR spectra were obtained on a Bruker NMR spectrometer WM 360.

Preparation of copolymer **6A**.[6] The reaction was conducted in a 4 l spherical vessel equipped with a half-circular Teflon stirrer (15 mm in diameter and 4 mm in thickness). 920 ml of thick paraffin oil (Paraffin liquid, high viscosity, Fluka), 180 ml of n-heptane and 0.3 ml of SPAN 85 (Fluka) were stirred at 150 rev/min. 50 g (400 mmol) of **1**,[11,12] 4.83 g (28.8 mmol) of **2**[12] and 8.61 g (54.9 mmol) of **3**[6] were dissolved in 200 ml of anhyd EtOH and 230 ml of distilled H_2O. This soln was introduced into the vessel and the mixture was flushed with N_2 at room temp. After 30 min a soln containing 0.90 g of ammonium persulfate in 2 ml of distilled H_2O was added to the mixture which was stirred during 10 min, finally TMEDA (1.6 ml) was added. A few min after TMEDA addition the temp rose to about 31° and the stirring was continued for 30 min.

The mixture was filtered on a Büchner equipped with a nylon cloth (100 μm) and the resin beads were thoroughly washed with 2 l of each of the following solvents: n-heptane, EtOH 95%, water, acetone and ethyl ether. The resin was dried *in vacuo* in the presence of P_2O_5 to constant weight, yield: 63.4 g of beaded resin.

Preparation of the amino functionalised polymer **7**.[2,6] 15 g of resin **6A** were placed in a 1 l Erlenmeyer flask to which 510 ml of 1,2-diaminoethane (distilled over KOH) were added. Shaking under N_2 was carried out overnight and the mixture was then filtered on a Büchner equipped with a nylon cloth (100 μm). The resin was washed successively with water (to neutrality), with acetone (3 × 500 ml), with EtOH 95% (3 × 500 ml), with acetone (3 × 500 ml) and with ethyl ether (5 × 500 ml) and dried *in vacuo* in the presence of P_2O_5 to constant weight, yield: 17 g.

Titration of the amino groups incorporated on the resin was performed on 2.5 mg aliquots following the technique of Sarin *et al.*[13] yielding a mean value of 0.55 ± 0.05 mmol of NH_2/g of dry resin. The functionalised resin displays good swelling properties in varied solvents protic or non-protic (CH_2Cl_2, $CHCl_3$, DMF, DMSO, pyridine, EtOH, MeOH, H_2O): 1 g of dry resin occupies 20 ml in DMF, while it does not swell in acetone, ethyl ether and hydrocarbon solvents.

Preparation of the bromine functionalised polymer **8**.[6] 1.377 g of bromoacetic acid (9.91 mmol, Fluka) were dissolved in 30 ml of CH_2Cl_2 to which DCC (1.02 g, 4.95 mmol) in 10 ml of CH_2Cl_2 was added at 0°. After stirring for 15 min, precipitated dicyclohexylurea was eliminated and the soln concentrated *in vacuo* without heating. A colourless oily residue was thus obtained and dissolved in 80 ml of DMF. The soln was then added to 3 g of **7** previously washed with DMF (4 × 80 ml). The mixture was stirred at 25° for 15 min. At this time DIEA (0.45 ml, 2.6 mmol) was added and the stirring was continued for 30 min.

The resin was washed with CH_2Cl_2 (4 × 2 min) with ethyl ether (4 × 2 min) and dried *in vacuo* 1 g of bromine functionalised resin **8** was treated in boiling 1 N NaOH in order to determine the amount of Br incorporated[16] (0.48 mmol Br^-/g).

Preparation of symmetrical anhydrides and anhydride coupling.[5,18] The protected amino acid (6 equiv) was dissolved in CH_2Cl_2 (3 ml/mmol). If necessary, the minimum amount of DMF was added to achieve a clear soln. A soln of DCC (3 equiv) in CH_2Cl_2 (5 ml/mmol) was added and the mixture stirred at 0° for 20 min. Precipitated dicyclohexylurea was

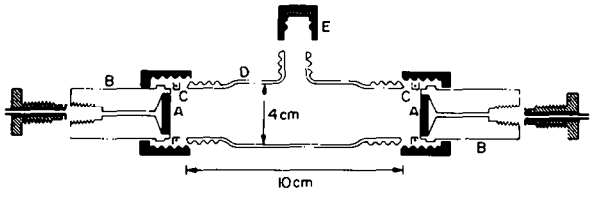


Fig. 7.

removed by filtration and the filtrate was added immediately to the amino-resin. 1 equiv of DIEA was added to the mixture.

Solid-phase synthesis of the octapeptide **1** *(acetylglycyl – alanyl – lysyl – arginyl – histidyl – arginyl – lysyl – valylmethyl ester) and of the nonapeptide* **2** *(acetylglycyl – alanyl – lysyl – leucyl – arginyl – histidyl – arginyl – lysyl – valylmethyl ester).* 3 g (1.5 mmol NH_2) of **7** were treated as described above by the anhydride of bromoacetic acid. The resin **8** also obtained was treated by the cesium salt of Boc–Val–OH[17] (in 30 ml of DMF) starting from 2.17 g (10 mmol) of Boc–Val–OH. After 40 hr of shaking, the resin was washed with DMF (4 × 2 min), H_2O (4 × 2 min), DMF (4 × 2 min) and ethyl ether (4 × 2 min) and carefully dried (weight 3.31 g). The degree of substitution by the C-terminal amino acid was determined by titration of the NH_2 groups liberated after removal of the Boc protecting group[13] (0.43 mmol of Val/g) and by amino acid analysis (0.404 mmol of Val/g).

Using 1.667 g (0.673 mmol of Val) of Boc–Val–resin the stepwise elongation (Table 1) of the peptide chain was carried out until step 5, thus yielding the immobilised pentapeptide Boc – Arg(NO_2) – His(Dnp) – Arg(NO_2) – Lys(Z) – Val – resin. The resin–peptide was carefully dried (weight 2.6 g) and divided in two parts: 1.7 g and 0.9 g respectively. The first part was used to synthesise **1**. After the completion of the synthesis, **10** was treated with 25 ml of DMF containing 0.541 g (4.925 mmol) of thiophenol for 40 min (Fig. 1) and then extensively washed with DMF (until a colourless filtrate is obtained) and finally washed with CH_2Cl_2 (4 × 2 min) and ethyl ether (4 × 2 min). The resin **12** was then dried *in vacuo* overnight and suspended in 25 ml of MeOH–Et_3N mixture (80:20, v/v) at ambient temp for 100 hr.[20] After extensive washing with MeOH, the pooled filtrates were concentrated *in vacuo* without heating. The oily residue **14** was dissolved in 50 ml of MeOH–AcOH–H_2O (80:10:10, v/v/v) and hydrogenated in the presence of 320 mg of Pd–$BaSO_4$ catalyst (Fluka) under normal pressure for 12 hr. After removal of the catalyst by filtration on celite and washing with water the peptide soln was concentrated *in vacuo*. The octapeptide **1** which was chromatographed on a CMC column (2 × 15 cm) using a linear NaCl gradient (0 to 0.5 M) at pH 5.0[7] was eluted at 0.31 M NaCl and finally desalted by gel filtration on a Sephadex G 10 column (2 × 90 cm, HCl 10^{-2} M). Lyophylisation yielded a white powdered product. The yield determined by amino acid analysis performed on an aliquot of the purified **1** was 49.6% based on the starting polyacrylic NH_2.

On the remaining 0.9 g of Boc – Arg(NO_2) – His(Dnp) – Arg(NO_2)–Lys(Z)–Val–resin, Boc–Leu–OH, Boc–Lys(Z) – OH, Boc – Ala – OH and AcGly – OH were successively coupled. After completion of the synthesis, **2** was cleaved and deprotected in the same manner as described for **1**. The yield determined by amino acid analysis was 51% based on the starting polyacrylic NH_2.

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