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α-(PHENYLACETAMIDO)BENZYLPOLYSTYRENE (PAB-RESIN)

A NEW POLYMERIC SUPPORT FOR PEPTIDE SYNTHESIS WITH IMPROVED ACID STABILITY

E. GIRALT,* D. ANDREU, M. PONS and E. PEDROSO
Departament de Química Orgànica, Facultat de Química, Universitat de Barcelona, Diagonal 645, Barcelona-28, Spain

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Abstract—Solid phase peptide synthesis carried out on conventional chloromethyl polystyrene support has the disadvantage of the acidolysis of the peptide-resin linkage during the deprotection step. In addition, when trifluoroacetic acid is used as deprotecting agent, intrapolymeric trifluoroacetyl transfer may give rise to truncated trifluoroacetylated peptides. A new polymer support devised to minimize these problems, α - (4 - chloromethylphenylacetamido)benzylcopoly(styrene-1%-divinylbenzene) ($ClCH_2$-Pab-resin), has been synthesized. The acetamido bridge between the peptide and the resin increases the acid stability of the new support thus allowing the synthesis of purer peptides in higher yields. Boc-aminoacyl-OCH_2-Pab-resins can be readily prepared in a versatile way by reaction of Boc-amino acid caesium salts with chloromethyl-Pab-resin. This fact contrasts with the rather elaborate procedures required for previously described similar resins.

The synthetic viability of the chloromethyl-Pab-resin is shown by the synthesis of Ac–His–Arg–Tyr–Arg–Pro–OH (fragment 39-43 of histone H3). The synthesis and subsequent purification are exhaustively compared with the parallel synthesis carried out on a standard chloromethyl polystyrene support. The presence of the acetamido linkage provokes, as expected, a decrease in the HF cleavage yield. Nevertheless, this disadvantage is balanced by the increase in global purification yields even for the synthesis of a pentapeptide for which only five deprotection steps are required.

A serious inconvenience of the standard methodology of solid phase peptide synthesis is the cleavage of the benzyl ester bond between the peptide and the solid support under the acidic conditions necessary to remove the t-butyloxycarbonyl (Boc) protection of the α-amino group[1] (usually 30% trifluoroacetic acid (TFA)–CH_2Cl_2). This laboratory has recently reported[2] that treatment of Boc–Gly–OCH_2-resin,† Boc–Phe–OCH_2-resin and Boc–Gly–Gly–OCH_2-resin with 30% TFA–CH_2Cl_2 for 25 hr results in losses of 27, 29 and 31% of the initial load respectively. On the other hand, recent work by Kent *et al.*[3] has shown that trifluoroacetylation of hydroxymethyl sites resulting from acidolysis can give rise to truncated trifluoroacetylated peptide sequences. Therefore, an improvement of the acid stability of the peptide-resin bond is desirable not only in order to improve synthetic yields but also to increase the purity of the crude peptide material obtained at the end of the synthesis.

Several solid supports which minimize acidolytical losses have been described hitherto by Blake and Li,[4] Mitchell *et al.*[5] and Sparrow,[6] all of them being derived from aminomethyl-resins and having as a common feature the presence of a phenylacetamido group which exerts an electron-withdrawing effect upon the peptide ester bond. Our laboratory has recently developed α-(4-chloromethylphenylacetamido)benzylcopoly(styrene - 1% -divinylbenzene) (chloromethyl-Pab-resin) (3), which derives from the easily accessible benzhydrylamine support (1).[7] The new Pab-resin allows a more acid-resistent anchoring of the C-terminal amino acid onto the polymer. Thus, the acidolytical losses of a Boc–Gly–OCH_2–Pab-resin submitted to the usual deprotecting conditions (30% TFA-CH_2Cl_2) are practically negligible (*ca* 1%) after 24.5 hr, which is the global deprotection time required for the synthesis of a 49-residue peptide.

Chloromethyl-Pab-resin (3) is obtained (Fig. 1) from 1 by treatment with the symmetric anhydride **2a** of 4-chloromethylphenylacetic acid (2). Quantitative acylation is easily achieved by using excess **2a**. If a lower substitution degree is desired, the starting polymer 1 may be treated with a limited amount of **2a** and subsequently acetylated to block unreacted amino groups. If resin 3 is synthesized by N,N'-dicyclohexylcarbodiimide (DCCI)-mediated coupling of 1 and 2, basic titrable groups other than primary amines are detected at the end of the synthesis, probably due to N-alkylation of 1.

Attachment of different Boc-amino acids to resin 3 can be effected by direct esterification via the corresponding caesium salts, in the same way as with standard chloromethyl-resin. This procedure contrasts with more elaborate methods employed in the preparation of other Boc-aminoacyl-polymers of similar structure. For instance, the preferred route to Boc-aminoacyl-OCH_2-Pam-resin[5] implies for each amino acid the preparation through a three-step process of the corresponding 4 - (Boc-aminoacyloxymethyl)phenylacetic acid, which is subsequently coupled to an aminomethyl-resin.[8] Furthermore, as Mitchell *et al.*[9] have shown, the obtention of pure aminomethyl-resins is in itself a laborious process.

The synthetical viability of the new chloromethyl–Pab-resin (3) was tested by the synthesis of the pentapeptide Ac–His–Arg–Tyr–Arg–Pro–OH (fragment 39–43 of histone H3), which was compared with a completely parallel synthesis performed on a conventional chloromethyl-resin. Both Boc–L–Pro–OCH_2–Pab-resin (0.34 mmole/g) and Boc–L–Pro–OCH_2-resin (0.42 mmole/g) were prepared by the caesium salt procedure.[10] N^{α}–Boc–L–Arg(N^{G}-tosyl)–OH and N^{α}–Boc–L-His(N^{im}-tosyl)–OH

†In general, "resin" stands for copoly(styrene-1%-divinylbenzene).

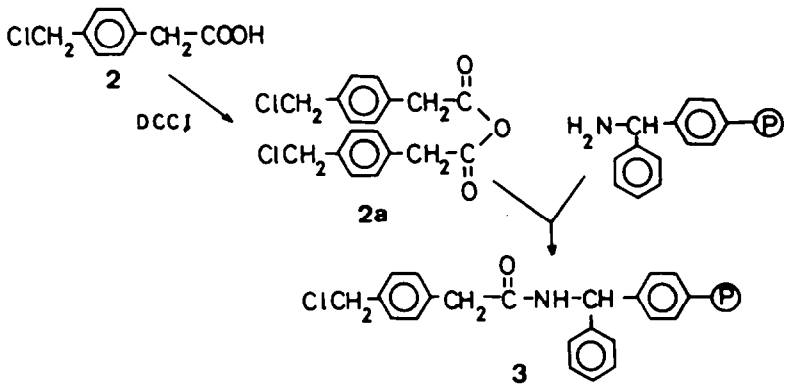

Fig. 1. Synthesis of chloromethyl-Pab-resin.

were assembled into the desired sequence by stepwise coupling as described in the experimental part. N^{α}-Boc-L-Tyr(O-2,6-dichlorobenzyl)-OH was coupled by the method of Suzuki *et al.*[11] to minimize diketopiperazine formation. The extent of each coupling was monitored by either fluorescamine or ninhydrin tests.[12]. Cleavage from the support was achieved in both cases by HF treatment.[13] As expected, the increased acid stability of the peptidyl-OCH_2-Pab-resin results in a somewhat lower HF cleaving yield. In the synthesis of larger peptides, however, this inconvenient may be expected to be overweighed by the minimization of undesired acidolytic cleavage during N^{α}-deprotection steps.

The purification procedures were identical for both syntheses. The crude peptide obtained by HF treatment of peptidyl-OCH_2-Pab-resin (207 mg) was filtered through Bio-Gel P-2 (Fig. 2a) giving a major peak (Ia), boxymethylcellulose (Fig. 2b) to give three peaks: a major component (Id, 135 mg, 80%) with the desired structure (Table 1), a non-peptide component (Ib) and a minor peptide fraction (Ic, 5%), whose amino acid analysis was consistent with the structure Ac-Arg-Tyr-Arg-Pro-OH and which arises from incomplete coupling of histidine (see experimental part). Finally, Id was desalted on Sephadex G-10 giving a single peak in a practically quantitative yield.

Purification of the crude peptide synthesized on the conventional chloromethyl-resin is shown in Fig. 3. Gel filtration of the crude led to peak IIa (61%) which was again resolved into three fractions by cation-exchange chromatography. Fraction IIb was non-peptide material Fraction IIc (8%) corresponds to the tetrapeptide mentioned above though slightly impurified by other peptide material. The desired product IId (77%) was finally desalted on Sephadex G-10 as above.

Both purification processes were further controlled by reverse-phase hplc and high-voltage paper electrophoresis, showing the crude product synthesized on the new support to be slightly purer than that from the conventional resin. This was also reflected in the fact that global purification yields were again favourable (64% vs 47%) to the new support. The final purified peptides were homogeneous to both hplc and electrophoresis.

EXPERIMENTAL

Abbreviations not previously defined are as follows: DMF-Dimethylformamide, Tos-tosyl, Dcbzl-2,6-dichlorobenzyl, AcOH-acetic acid, MeOH-methanol, DIEA-diisopropylethylamine, EtOH-ethanol, Et_2O-diethyl ether. CH_2Cl_2

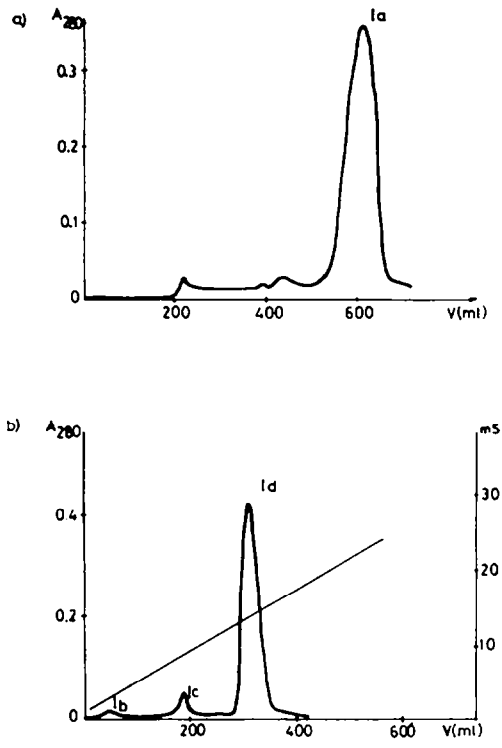

Fig. 2. Chromatography of Ac-His-Arg-Tyr-Arg-Pro-OH synthesized on a Pab-resin.

Table 1. Amino acid analyses of Ac-His-Arg-Tyr-Arg-Pro-OH along the purification process

Fraction	Pro	Tyr	His	Arg
crude from Pab resin	1.28	0.81	0.81	2.09
crude from standard resin	0.93	1.15	1.00	1.92
Ia	0.96	1.02	1.03	1.99
IIa	1.02	0.93	0.95	2.10
Id	0.98	0.98	1.02	2.02
IId	0.96	1.11	0.99	1.94
Ic	1.04	0.93	-	2.03
IIc	1.14	0.90	-	1.97

Table 2. Electrophoretic properties of Ac-His-Arg-Tyr-Arg-Pro-OH along the purification process. A, B and C refer respectively to ninhydrin, Pauly and phenanthraquinone reagents

fraction		Ia	IIa		Id	IId	Ic	IIc	
mobility relative to arginine		0.61;0.81	0.53;0.61;0.81		0.81	0.81	0.61	0.53;0.61	
visualization	A	- -	- - -		-	-	-	-	-
	B	+ +	- + +		+	+	+	-	+
	C	+ +	+ + +		+	+	+	+	+

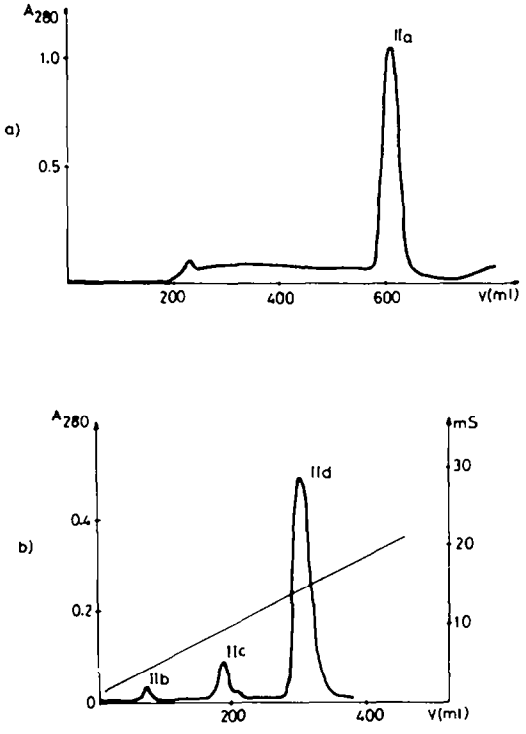

Fig. 3. Chromatography of Ac-His-Arg-His-Arg-Pro-OH synthesized on a standard resin.

was dried over anhydrous K_2CO_3 and distilled over it immediately before use. DMF was dried over 4 Å molecular sieves and freed of amines by nitrogen bubbling until negative 1-fluoro-2,4-dinitrobenzene test.[14] All other solvents and chemicals were reagent grade. Boc-L-Pro-OH was from Fluka. Boc-L-Arg(Tos)-OH and Boc-L-His(Tos)-OH were from Serva. Boc-L-Tyr(Dcbzl)-OH was synthesized in our laboratory as described by Yamashiro and Li.[15] Copoly (styrene-1%-divinylbenzene) beads (200–400 mesh) (Bio-Beads SX1) and its chloromethylated derivative (0.89 mmole Cl/g)were from Bio-Rad Laboratories. Hydrolysates for amino acid analyses from peptide or pepidyl-resin samples were prepared by treatment with 6N HCl for 24 hr or 12N HCl/AcOH (1:1) for 48 hr respectively in vacuum degassed sealed tubes at 110°, adding norleucine as internal standard. Amino acid analyses were run in a Beckman 119 C autoanalyzer. Hplc was performed in a Waters Associates ALC/GPC 205 U apparatus equipped with a μ Bondapack C_{18} column eluted isocratically with 0.01 M ammonium acetate pH 4.0/CH_3CN (91:9). High-voltage paper electrophoreses were performed on 90×45 cm Whatman 3M sheets in a pyridine-AcOH-water (1:10:455) pH 6.4 buffer for 2 hr at 3000V, using a Savant Instruments HV-5000A power supply. Spots were visualized with Pauly and phenanthraquinone reagents.[14]

4-Chloromethylphenylacetic acid (2). The synthetic procedure of Bodganov[16] was followed with minor modifications. Recently, Mitchell *et al.*[17] have described a revised procedure for the synthesis of this compound.

Boc-aminoacyl-Pab-resins

Method (i). Complete coupling of **2a** *and* **1**. In a typical experiment 3 g of **1** (substitution degree 0.67 mmole NH_2/g) were exhaustively washed with CH_2Cl_2, DMF, MeOH, CH_2Cl_2, 30% TFA-CH_2Cl_2, CH_2Cl_2, 5% DIEA-CH_2Cl_2 and CH_2Cl_2 and treated with 1.5 equivalent of **2a** (prepared *in situ* from 1.22 g of the acid **2**[18]) and 1.5 equiv (0.68 g) DCCI† in 25 ml CH_2Cl_2 for 2 hr at room temp. After filtration and washings with CH_2Cl_2, DMF, CH_2Cl_2, 5% DIEA-CH_2Cl_2, CH_2Cl_2 and DMF coupling of **2a** was repeated (14 hr, 1.5 equiv **2a** and 1.5 equiv DCCI) and the polymer was finally washed with DMF and CH_2Cl_2 to give 3.3 g of **3** (negative ninhydrin, fluorescamine and picric acid[19] tests). Reaction of **3** (3.2 g) with caesium Boc-valinate (0.88 g) in 30 ml DMF for 18 hr at 50°C afforded 3.34 g of Boc-valyl-OCH_2-Pab-resin. Deprotection of an aliquot followed by picric acid titration gave 0.57 mmole NH_2/g (99% overall yield from **1**).

Method (ii). Limited coupling of **2a** *and* **1**. **1** (6 g; 1.04 mmole NH_2/g) was treated once with 0.4 equiv of **2a** (prepared from 0.92 g of **2**) and 0.4 equiv. (2.1 g) of DCCI following a procedure parallel to that described in *Method (i)*. 0.31 mmole unreacted NH_2/g was detected by picric acid titration after this coupling. The polymer was then acetylated with AcOH (5 equiv) and DCCI (5 equiv) and extensively washed with 30% TFA-CH_2Cl_2, CH_2Cl_2, DMF, CH_2Cl_2 and MeOH to give **3** (negative ninhydrin and picric acid tests). 3 g of **3** were treated with 0.51 g of caesium Boc-prolinate (see *Method (i)*) to afford Boc-Pro-OCH_2-Pab-resin (0.34 mmole NH_2/g determined by deprotection and picric acid titration). This polymer was subsequently used for the peptide synthesis described below.

Method (iii). Direct coupling of **2** *and* **1**. The treatment of (1.04 mmole NH_2/g) with 0.8 equiv of **2** and 0.8 equiv of DCCI led to a polymer with 0.32 mmole NH_2/g. After acetylation (negative ninhydrin test), caesium Boc-glycinate was attached in the usual way but the resulting polymer showed an amine content prior to deprotection of 0.15 mmole NH_2/g, which rose to 0.38 mmole NH_2/g when the picric acid titration was performed on a previously deprotected sample.

Ac-His-Arg-Tyr-Arg-Pro-OH

residue except Tyr: (1) 40 ml CH_2Cl_2 (4×2 min); (2) 40 ml 30% 3 g) was placed in the reaction-vessel of a custom-made synthesizer[20] and treated as follows for the incorporation of each residue except Tyr: (1) 40 ml CH_2Cl_2 (4×2 min); (2) 40 ml 30% TFA-CH_2Cl_2 for 2 min; (3) 40 ml 30% TFA-CH_2Cl_2 for 30 min; (4) 40 ml CH_2Cl_2 (4×2 min); (5) 40 ml 5% DIEA-CH_2Cl_2 for 2 min; (6) 40 ml 5% DIEA-CH_2Cl_2 for 30 min; (7) 40 ml CH_2Cl_2 (4×2 min); (8) 40 ml 5% DMF-CH_2Cl_2 (only for Boc-L-Arg(Tos)-OH incorporation); (9) shaken with 2.0 equiv Boc-amino acid in 40 ml CH_2Cl_2 (5% DMF-CH_2Cl_2 for Arg); (10) without

†This additional amount of DCCI is used in order to recycle excess **2** formed during the coupling reaction.

filtration, 2.2 equiv DCCI in 2 ml CH_2Cl_2 for 150 min; (11) 40 ml CH_2Cl_2 (4 × 2 min); (12) 40 ml DMF (4 × 2 min); (13) 40 ml CH_2Cl_2 (4 × 2 min); (14) repeat from (5). The same program was employed for final acetylation using AcOH (5 equiv) in step (9). Boc-L-Tyr(Dcbzl)-OH was incorporated by the method of Suzuki *et al.*[11] The final Ac-His(Tos)-Arg(Tos)-Tyr(Dcbzl)-Arg(Tos)-Pro-OCH_2-Pab-resin was washed with 30% TFA-CH_2Cl_2, CH_2Cl_2 and EtOH to give 4.27 g of dry resin which was subsequently treated with 60 ml anhydrous HF and 9 ml anisole for 1 hr at 0°. Hydrolysis and amino acid analysis of peptidyl-resin aliquots before and after HF gave 94% cleaving yield. These seemingly too high value may be due to incomplete acid hydrolysis of peptidyl chains,[21] which would precisely leave unaffected those peptide chains less accessible to HF. The cleaved material was washed with Et_2O (3 × 50 ml) and dissolved in AcOH-water (1:1) (100 ml). The resulting soln was quantitated by hydrolysis and amino acid analysis and referred to the amine content of the peptidyl-resin at the end of the synthesis as determined by picric acid titration. By this method, a 68% HF cleaving yield was found. Bio-Gel P-2 chromatography was performed on a 100 × 2 cm column loaded with *ca* 200 mg crude peptide eluted with 0.1 M ammonium acetate pH 8.5 (37 ml/hr) and monitored by UV absorption at 280 nm. Two parallel cation-exchange chromatographies were performed on 20 × 2 cm carboxymethylcellulose CM-52 Whatman columns loaded each with *ca* 85 mg peptide and eluted with a linear gradient of ammonium acetate pH 5.0 from 1 mS to 39 mS. Desalting was carried out on Sephadex G-10 (70 × 2 cm) or G-15 (100 × 2 cm) columns using 0.1 M AcOH (30 ml/hr).

From standard resin. Boc-L-Pro-OCH_2-resin (0.42 mmole/g) was prepared by reaction of 3 g of chloromethyl-resin (0.89 mmole Cl/g) with 0.58 g of caesium Boc-prolinate for 16 hr at 50° in DMF. Exactly the same protocol as above was followed for the incorporation of each amino acid. Fluorescamine was used in addition to ninhydrin to monitor couplings. After His incorporation a negative ninhydrin but a positive fluorescamine test were found. A third coupling was not considered, though, for the sake of strict comparability between both syntheses. HF cleavage yields calculated as above were 96% and 79% respectively. A purification procedure parallel to the above one was followed.

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