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Acetophenone-based Linker for Solid-phase Peptide Synthesis

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Abstract: A new and cost-effective linker for the generation of carboxylic acid end groups on Multipin supports (SynPhase™ crowns) has been developed. Synthesis of the linker was based on modification of grafted polystyrene (PS) crowns to generate a hydroxyethyl moiety which is acid labile in 10–20% trifluoroacetic acid (TFA) in dichloromethane (DCM). Solid-phase syntheses of model decapeptides using this linker are described. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hydroxyethyl polystyrene; acetophenone-based linker; solid-phase peptide synthesis; solid-phase organic chemistry

INTRODUCTION

Solid-phase synthesis is the method of choice for peptide and oligonucleotide synthesis, and with the advent of combinatorial chemistry, it is of increasing importance in general organic/medicinal chemistry [1,2]. With this method of synthesis, the initial attachment of starting material, stability under the following synthesis conditions but quantitative cleavage of end product from the solid support, are key requirements for a successful synthesis. Consequently, many linkers and methods for derivatization of the solid-phase polymer matrices have been developed for anchoring a wide range of functional groups [3]. However, if the key requirements are fulfilled, then linker selection is primarily a matter of cost, availability and flexibility of use. As part of a linker program for parallel synthesis with flexibility to scale up to larger quantities, we recently developed acetophenone-based linkers **1** and **4** (Scheme 1) for solid-phase synthesis of *N*-alkyl peptide amides and sulfonamides [4]. The two versions of the acetophenone linker are simple to assemble from relatively cheap starting materials and, on average, were found to be comparable with other sec-amide forming linkers such as the 3,5-dimethoxy-4-formylphenoxyvaleric acid linker (Barany) [5], and its monomethoxy analog (acid sensitive methoxy benzaldehyde, AMEBA) linker [6].

The latter AMEBA linker can be reduced to produce a hyperacid labile, 4-hydroxymethyl-3-methoxyphenoxy type linker for the synthesis of protected peptide fragments by the 9-fluorenylmethoxycarbonyl (Fmoc) method of peptide synthesis [7]. Similarly, the same acetophenone linkers, **1** and **4**, can be converted to the two potentially new and related hydroxyethyl polystyrene (PS) linkers, **2** and **5** (Scheme 1) for the generation of carboxylic acid end groups. Synthesis of **2** was based on direct functionalization of PS grafted crowns to give the acetophenone functionality which on reduction with $NaBH_4$ afforded hydroxyethyl-PS **2** [8]. For the

Abbreviations: DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMA, *N*,*N*-dimethylacetamide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; Dnp, dinitrophenyl; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenyl-methoxycarbonyl; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; PIP, piperidine; PS, polystyrene; R_t, retention time; TEA, triethylamine; TFA, trifluoroacetic acid.

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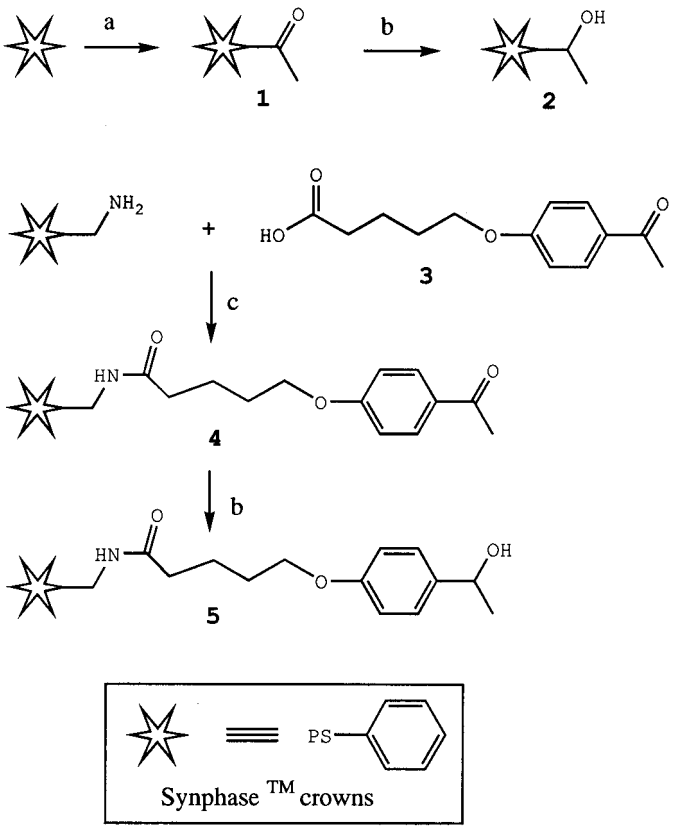

Scheme 1 Synthesis of linkers **2** and **5**. (a): $AlCl_3/CH_3COCl/CH_2Cl_2/25°C$ (b) $NaBH_4$/THF/RT (c) HOBt/DIC/DMF/25°C.

purpose of attachment to different solid supports, **5** was also prepared by coupling the preformed linker **3** with commercially available aminomethyl PS crowns. This was followed by reduction with $NaBH_4$ to afford **5**. Both **2** and **5** were used for attachment of carboxylic acids and their utility as linkers for peptide synthesis is the focus of this study.

MATERIALS AND METHODS

Solvents and fine chemicals were purchased from Aldrich Chemical Company (Castle Hill, Australia) and used as supplied. Amino acids were obtained from Senn Chemicals (Dielsdorf, Switzerland). Solid-phase synthesis was performed using SynPhase™ PS grafted crowns and aminomethyl PS crowns (loading = 20 μmol/I-series crown) available from Chiron Technologies (Clayton, Australia).

Reverse phase-high performance liquid chromatography (RP-HPLC): RP-HPLC was conducted with a Rainin, Microsorb-MV Cat.# 86-200-F3, 50 × 4.6 mm column using a gradient mobile-phase 0–100% B over 11.5 min. Solvent A: 0.1% *ortho*-phosphoric acid in water; Solvent B: 0.1% *ortho*-phosphoric acid in 90% acetonitrile. Detection: 214 nm. Flow rate increased from 0 to 1.5 ml/min during the first 0.5 min then remained constant for 15 min running time (system A) or flow rate was constant at 1.5 ml/min for 15 min running time (system B).

Mass spectrometer analysis: electrospray mass spectrometry (ES-MS) was conducted with a Perkin-Elmer Sciex API III using 0.1% acetic acid in 60% acetonitrile.

Fourier transformed-infra red spectroscopy (FT-IR): the PS surface (crown) was placed on the internal reflexion element (IRE) under a pressure of 1–2 lbs and the spectrum was obtained in the range 4000–400 cm^{-1} with 16 scans at a resolution of 4 cm^{-1} on a Perkin-Elmer System 2000 FT-IR spectrophotometer connected to the SPA (SplitPea accessory) [9].

Proton (^1H) and carbon (^{13}C) NMR spectra were recorded with a Varian^{UNITY} INOVA 400 spectrometer operating at 400 MHz for proton and 100 MHz for carbon. All spectra were recorded in $CDCl_3$ at 25°C.

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PREPARATION OF LINKERS 1 AND 2

Twenty I-series PS crowns (Chiron Technologies) were gently shaken in a glass bottle containing acetyl chloride (0.42 ml, 6 mmol) and $AlCl_3$ (0.7 g, 5 mmol) in dichloromethane (DCM) (10 ml) for 16 h at 25°C. The crowns were then removed and washed with DCM (3 ×), dimethylformamide (DMF) (3 ×) and DCM (3 ×) and finally dried under reduced pressure for 8 h to give **1**. FT-IR ν_{max}: 2917.3, 2849.5, 1678.3 (strong), 1604.4, 1416.0, 1357.9, 1268.3, 1182.5, 956.4, 829.8, 731.4, 718.7, 600.4 cm^{-1}. Crowns **1** were incubated with 20 ml of 0.8 M $NaBH_4$ in THF under N_2 gas at 25°C for 16 h. The crowns were removed, washed with DCM (2 ×), DMF (3 ×), DCM (3 ×) and then dried under reduced pressure for 4 h to afford the linker **2**. FT-IR ν_{max}: 3338.3 (broad), 2917.8, 2849.7, 1419.9, 942.5, 718.8 cm^{-1} (Figure 1).

To optimize the acylation reaction condition (step a, Scheme 1) the hydroxyethyl-PS grafted crowns **2** were prepared under similar conditions except that the PS grafted crowns (25 I-series crowns) were incubated with five different concentrations of acetyl chloride (0.012, 0.12, 0.3 0.6 and 1.2 M) and $AlCl_3$ (0.01, 0.1, 0.25, 0.5 and 1.0 M) in 10 ml of DCM.

To determine the loading of the hydroxyethyl-PS crown **2**, the crowns (ten I-series hydroxyethyl-PS crowns) were incubated with Fmoc-Ala-OH (0.311 g, 0.001 mol), diisopropylcarbodiimide (DIC) (0.126 g, 0.001 mol) and 4-dimethylamidopyridine (DMAP) (12.2 mg, 0.0001 mol) in 10 ml of 25% DMF in DCM for 12 h at 25°C. The crowns were washed carefully with DCM (3 ×), DMF (3 ×) and DCM (3 ×). The crowns were treated with 20% piperidine (PIP)/DMF (10 ml) for 30 min at room temperature. The loading of the crowns were determined by reading the absorbance of the deprotection solution at 301 nm after diluting the deprotection solution 1:10 in 20% PIP/DMF, and using the equation: Loading (nmol) = (Abs/0.0078) × 110 [10].

CLEAVAGE STUDY

To assess cleavage efficiency of the crown **2**, 24 crowns **2** derivatized with compound **7** (Dnp-β-Ala-Ala-OH) were incubated in duplicate with four different concentrations of trifluoroacetic acid (TFA) (5, 10, 20, and 50%) in DCM. At 15, 30 and 60 min ('initial' sampling points), crowns were removed and transferred to 95% TFA in DCM for completion of cleavage (2 h incubation, 'final' sampling point). The cleavage solutions were aerated with nitrogen gas to dryness and the resulting chromophoric compound **7** was dissolved in 5 ml of 50% ethanol in water. These solutions were pipetted into the wells of a microtitre plate (200 µl per well) and the absorbance at 405 nm was measured on a Biotek microplate reader (Model MA 310). The percentage cleavage at each time point was calculated from the ratio of 'initial' optical density/('initial' O.D. + 'final' O.D.) [11].

PREPARATION OF LINKER 3

To a suspension of potassium *t*-butoxide (5.6 g, 0.05 mol) in DMF (50 ml) was added dropwise 4-hydroxyacetophenone (6.8 g, 0.05 mol) in 2 ml DMF at 25°C under a nitrogen atmosphere. The reaction mixture was stirred at 25°C for 15 min and methyl-5-bromovalerate (9.75 g, 0.05 mol) was then injected into the mixture via syringe. The reaction mixture was heated up to 80°C for 6 h before being poured onto ice. The reaction mixture was extracted with ether (50 ml) and ethyl acetate (50 ml), and the combined organic phase concentrated under reduced pressure. The resulting colorless oil was added to an aqueous solution of NaOH (40 ml of 4N NaOH + 60 ml of methanol) at 25°C for 4 h. The reaction mixture was then poured onto ice and the aqueous phase was washed with EtOAc (30 ml) and then acidified with concentrated HCl until pH ~3.0. The product was filtered and washed with 5% EtOAc in petroleum spirit and dried under reduced pressure for 4 h to afford a pale yellow powder **3** (10.1 g, 85% overall yield). $R_f = 0.39$ (in 40% EtOAc in petroleum spirit 40–60°C), $R_t = 6.2$ min. Ion spray MS *m/z* 237.0 $[M + H]^+$, 254.1 $[M + NH_4]^+$, 472.8 $[2M + H]^+$, 490.2 $[2M + NH_4]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.93 (d, 9 Hz, 2H), 6.91 (d, 9 Hz, 2H), 4.05 (t, *J* 6 Hz, 2H), 2.56 (s, 3H), 2.46 (t, *J* 7 Hz, 2H), 1.83–1.89 (m, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): 197.0, 179.3, 162.8, 130.6, 130.2, 114.1, 67.5, 33.5, 28.5, 26.3, 21.3.

PREPARATION OF LINKER 5

The TFA salt of aminomethylated-PS crowns (50 crowns with loading = 20 µmol/crown) was neutralized by standing the crowns in 5% triethylamine (TEA) in 1:1 DMF:DCM for 10 min. After draining, the neutralization step was repeated and the

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crowns were washed with 1:1 DMF:DCM, DMF (2 ×) and DCM (2 ×). The crowns were then incubated with a coupling solution containing **3** (0.71 g, 3.0 mmol), 1-hydroxybenzotriazole (HOBt) (1.02 g, 6.7 mmol) and *N*,*N*′-diisopropylcarbodiimide (0.47 ml, 3.0 mmol) in 50 ml dry DMF for 24 h. The coupling solvent was decanted and the crowns were washed with DMF (2 ×) and DCM (2 ×) and then allowed to dry under reduced pressure for 4 h to give **4**. The acetophenone on **4** was reduced under similar conditions as described above to afford **5**.

PEPTIDE SYNTHESIS

Peptide synthesis was performed by Fmoc synthesis as previously described [12]. Briefly, the first Fmoc protected amino acid was directly attached to linker **2** (10 μmol/I-series crown) or **5** (20 μmol/I-series crown) via formation of an ester linkage. This initial coupling step was carried out by using our standard coupling conditions (0.1 M amino acid/0.1 M DIC/0.01 M DMAP/25% DMF in DCM) [12]. In the case of linker **2**, repeat esterification was required to get maximum loading, but this was not necessary for linker **5**, presumably due to greater flexibility imparted by the role of the spacer [13,14] [15]. After Fmoc deprotection (20% PIP/DMF) the resulting product was then coupled with the second amino acid via formation of an amide bond under the standard condition (0.1 M amino acid/0.1 M HOBt/0.1 M DIC/DMF) [12]. Completion of the amide coupling step was monitored by stain tests (bromophenol blue) [16]. This repeating cycle of Fmoc deprotection and coupling was then employed for the assembly of the amino acid sequence. Finally, the desired peptide was cleaved off the solid support using 20% TFA in DCM without any scavenger (for dipeptides) or a cocktail solution of 20% TFA, 2.5% anisole and 2.5% EDT (for decapeptides). After the usual TFA removal step (under a stream of N_2 gas) and work-up [12] crude products were analyzed by HPLC and MS spectroscopy (Table 3).

RESULTS AND DISCUSSION

In the initial phase, direct functionalization of PS, general conditions of esterification and cleavage efficiencies were studied. By adjusting acetyl chloride and $AlCl_3$ concentrations, different loading levels could be obtained on the PS surface. Loading determination was only possible after reduction of **1** to **2** and esterification with Fmoc-Ala-OH. Loading, as determined by a quantitative Fmoc test [10], with different acetylation conditions is shown in Table 1. The maximum loading achieved under these sets of conditions was 10.3 μmol/crown with more forcing conditions giving no great improvements in loading. Repeat experiments with different solvents (chloroform and carbon disulfide) gave very similar results.

The assigned structures of **1** and **2** were confirmed by FT-IR (Figure 1). The FT-IR spectra of **1** and **2** displayed very strong stretching bands associated with C=O and OH groups at 1678.3 and 3338.3 cm^{-1}, respectively.

The cleavage conditions were also optimized on linker **2**. Linker **2** derivatized with compound **7** was subjected to a series of TFA concentrations (5, 10, 20 and 50% in DCM). After cleavage, the chromophoric product **7** was quantitated by spectroscopy at 405 nm [11]. As shown in Table 2, linker **2** was almost quantitatively cleavable at 20% TFA/DCM within 1 h. The same cleavage condition was also suitable for linker **5**.

For the purpose of attachment to different solid supports, the preformed acetophenone linker **3** was coupled onto aminomethyl-PS crowns under the standard coupling conditions (HOBt/DIC/DMF) [12] to afford crowns carrying the sec-amide and sulfonamide forming linker **4** [4]. Under similar reduction conditions ($NaBH_4$/THF), the ketone functionality of **4** was converted to the hydroxyethyl moiety to give the linker **5**. Both **2** and **5** are functionally identical and were subjected to further assessment.

Syntheses of some model dipeptides (**6** and **7**) and decapeptides (**8** and **9**) on the new linkers have been demonstrated by using the standard Fmoc

Table 1 Optimization of the Friedel–Crafts Reaction on PS Crowns

[Acetyl chloride] (M)[a]	[$AlCl_3$] (M)[a]	Loading (μmol/crown)[b]
0.0	0.0	0.0
0.012	0.01	2.0
0.12	0.1	4.2
0.3	0.25	8.1
0.6	0.5	10.3
1.2	1.0	9.6

[a] The PS grafted crowns (25 I-series crowns) were incubated with five different concentrations of acetyl chloride and $AlCl_3$ in 10 ml of DCM.
[b] Average loading value was obtained from five crowns with variation less than 7% in all cases.

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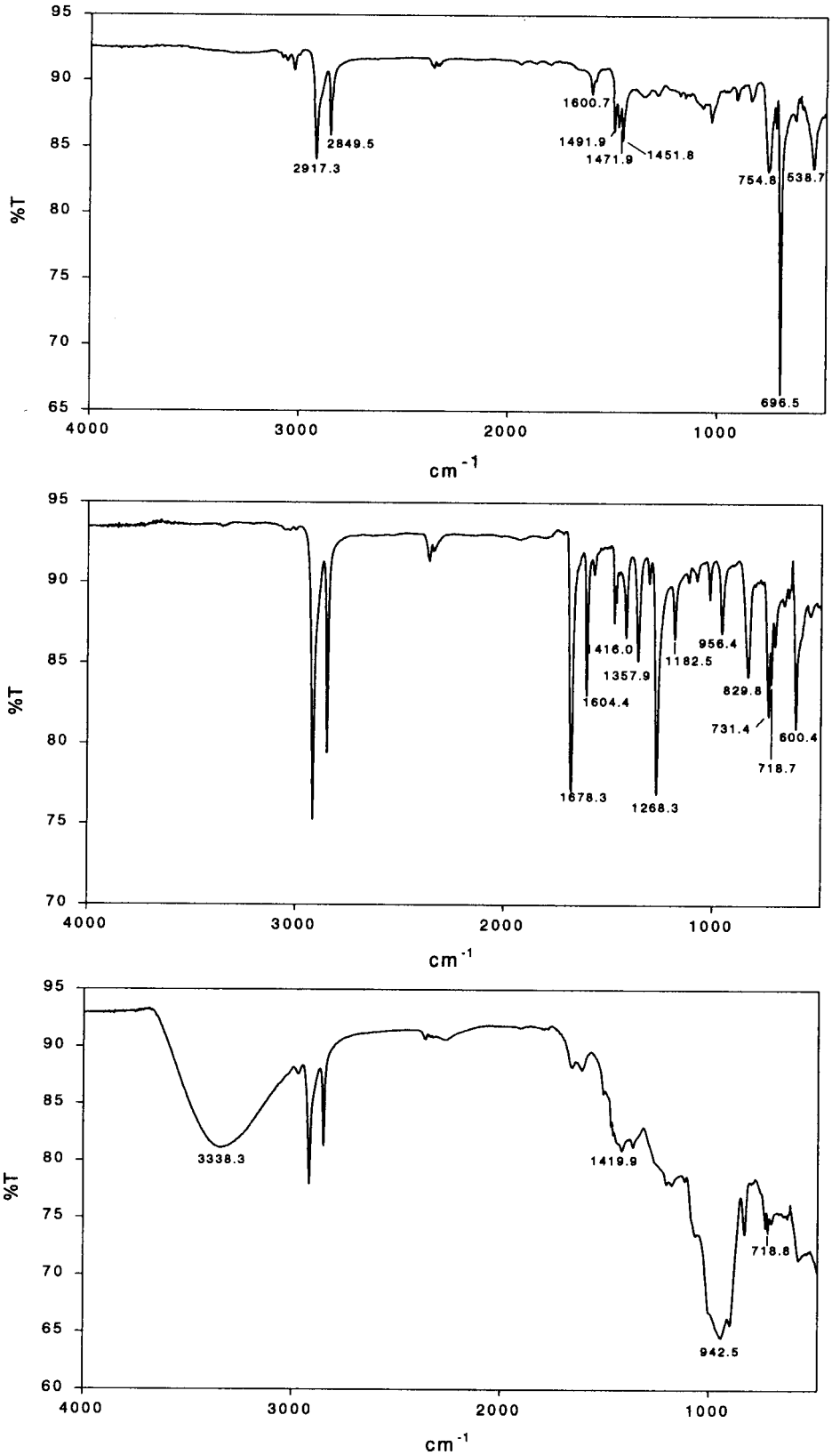

Figure 1 FTIR spectra of PS grafted crowns (top), linker **1** (middle) and linker **2** (bottom).

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Table 2 Cleavage Study of Linker **2**

Time	% Cleavage[a]			
(min)	5% TFA/DCM	10% TFA/DCM	20% TFA/DCM	50% TFA/DCM
0	0	0	0	0
15	64	86	93	97
30	78	91	96	98
60	88	93	97	97

[a] Cleavage study of linker **2** was based on the release of the chromophoric compound **7** in different concentrations of TFA. The experiment was carried out in duplicate.

peptide synthesis procedures [12]. Characterization of the model peptides (**8** and **9**) and all truncated versions was carried out by HPLC and MS (Figure 2, Tables 3–5). This is illustrated in Tables 4 and 5 in which peptides were cleaved off the solid support after each amino acid coupling and analyzed by reverse phase HPLC and MS spectroscopy. In all cases a single major peak with the correct molecular weight was obtained.

Previously, we reported the application of acetophenone-based linker **4** for the synthesis of sec-amides and sulfonamides [4]. Reduction of the ketone functionality on this same linker generates an acid labile linker **5** for synthesis of carboxylic acids. These linkers are of interest due to their simplicity, flexibility, cleavage properties and cost. All the starting materials, 4-hydroxyacetophenone, valeric acid and acetylchloride are relatively cheap,

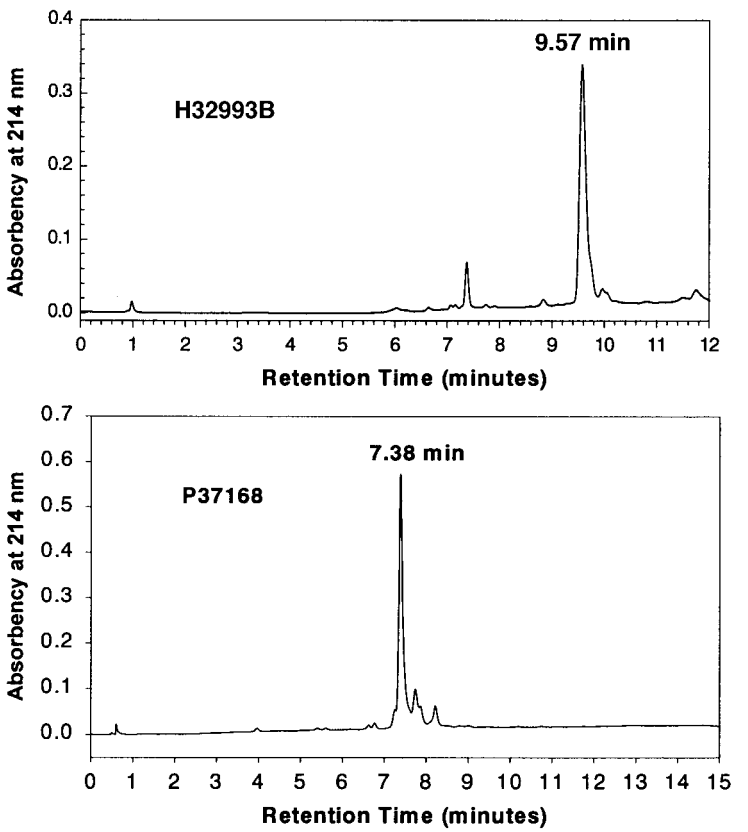

Figure 2 RP-HPLC chromatograms of compound **8** (upper, $R_t = 9.57$ min. Fmoc-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH) and compound **9** (lower, $R_t = 7.38$ min. Fmoc-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-OH).

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Table 3 Characterization Data for Peptides **6**, **7**, **8** and **9**

Peptides	Linker used	HPLC R_t (min)[a]	% Purity	% Yield[b]	ES-MS $[M+H]^+$ Observed	ES-MS $[M+H]^+$ Calculated
6 Fmoc-Phe-Ala-OH	**2**	8.32	90	81	459.3	459.3
6 Fmoc-Phe-Ala-OH	**5**	8.32	93	94	459.2	459.2
7 Dnp-β-Ala-Ala-OH	**2**	7.55	88	93	327.4	327.1
8 Decapeptide-1[c]	**2**	9.57	75	64	1423.7	1423.6
9 Decapeptide-2[d,e]	**5**	7.38	70	nd	1216.3	1216.4

[a] R_t of compounds **6**, **7** and **8** were obtained on HPLC (system A) and compound **9** on HPLC (system B).
[b] Overall crude yield (based on the initial loading of the crowns), nd: not determined.
[c] Compound **8** (Decapeptide-1): Fmoc-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH.
[d] Compound **9** (Decapeptide-2): Fmoc-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-OH.
[e] HPLC and MS spectral data of **9** was identical with the authentic compound which was prepared by using the commercially available HMP linker (4-hydroxymethylphenoxyacetamido handle, Chiron Technologies, Pty. Ltd.).

Table 4 Stepwise Analysis of Truncations of Peptide **8**

Peptides	ES-MS $[M+H]^+$ Observed	ES-MS $[M+H]^+$ Calculated
Fmoc-Pro-Gly-OH	395.1	395.1
Fmoc-Arg-Pro-Gly-OH	551.4	551.2
Fmoc-Leu-Arg-Pro-Gly-OH	664.4	664.3
Fmoc-Gly-Leu-Arg-Pro-Gly-OH	721.5	721.4
Fmoc-Tyr-Gly-Leu-Arg-Pro-Gly-OH	884.6	884.4
Fmoc-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH	971.6	971.4
Fmoc-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH	1157.8	1157.5
Fmoc-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH	1295.0	1294.6
Fmoc-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH	1423.7	1423.6

Table 5 Stepwise Analysis of Truncations of Peptide **9**

Peptides	ES-MS $[M+H]^+$ Observed	ES-MS $[M+H]^+$ Calculated
Fmoc-Leu-Pro-OH	451.6	451.5
Fmoc-Ile-Leu-Pro-OH	564.7	564.7
Fmoc-Pro-Ile-Leu-Pro-OH	661.8	661.8
Fmoc-Thr-Pro-Ile-Leu-Pro-OH	762.8	762.9
Fmoc-Asp-Thr-Pro-Ile-Leu-Pro-OH	878.0	878.0
Fmoc-Ser-Asp-Thr-Pro-Ile-Leu-Pro-OH	965.1	965.1
Fmoc-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-OH	1062.3	1062.2
Fmoc-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-OH	1119.6	1119.3
Fmoc-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-OH	1216.3	1216.4

commercially available starting materials. Both **2** and **5** are cleavable at low concentration of TFA (10% TFA/DCM for 2 h) indicating that both the phenyl and methyl groups act as electron-donating groups to facilitate the acid cleavage of the C–O bond. This property makes this new class of linker quite distinct from the current commercially available linkers such as hydroxymethyl-PS [17] (cleavage requires the strong acid, HF) and hydroxymethylphenoxy type linker (cleavage requires 50% TFA/DCM) [18]. In principle, direct assembly of linker **2** on a PS surface is a convenient process as it can avoid the presence of the potentially unstable benzyl ether linkage and also the

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extra work of solution phase synthesis of the preformed linker **3**. Both linkers **2** and **5** were found to be very stable throughout the reaction conditions used.

CONCLUSIONS

A new and cost-effective functionalized grafted polymer **2** for solid-phase synthesis of peptide acids is provided. The alternative method to generate the hydroxyethyl linker **5** is also described. The linkers cleave under mild acidic conditions (10–20% TFA) to release target products in high yield and purity, and are a simple alternative to more expensive commercially available linkers [17–19].

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