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Solid Phase Synthesis of *C*-terminal Peptide Amides: Development of a New Aminoethyl-polystyrene Linker on the Multipin™ Solid Support

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Abstract: A new aminoethyl-polystyrene linker, stable at low concentrations of TFA, has been developed for the solid phase synthesis of peptide amides. The described linker is stable under conditions which remove Bu^t protecting groups (30–50% TFA in DCM) and the desired product can be finally cleaved off the solid support in 95% TFA (5% H_2O). Model peptide amides and other *N*-alkylated peptide amides have been successfully synthesized in good yield and purity. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: aminoethyl-polystyrene; acetophenone-based linker; solid-phase synthesis of peptide amide; multipin solid support

INTRODUCTION

Peptide primary amides and their analogs represent a group of biologically active molecules that are the target of syntheses by solution [1] and solid phase [2] methodologies. On solid phase [3] the commercially available linkers, and therefore the ones most commonly used for the synthesis of carboxamides, are the benzhydrylamine-type linkers [4] such as 4-methylbenzhydrylamine (MBHA) and its derivatives substituted with electron-donating alkoxy groups such as in the Rink-amide linker [5]. The former requires strong acids (hydrogen fluoride or trifluoromethanesulfonic acid) for the final cleavage and is principally used in the Boc method of peptide synthesis. The latter is susceptible to mild acidic cleavage conditions (30% TFA) and is commonly used with the 9-fluorenyl-methoxycarbonyl (Fmoc) method where the α-amino group of the incoming amino acid is protected by a base-labile group [6,7]. In our continuing study on the acetophenone class of linkers **6** [8] (Scheme 1) we report a new and cost effective aminoethyl-polystyrene linker **5** (Scheme 1) for solid-phase synthesis of carboxamides. Unlike the above linkers, the aminoethyl-polystyrene linker has been found to be compatible with acid-labile side-chain protecting groups such as Boc and Bu^t groups as it is stable at low concentrations of TFA (50% TFA/dichloromethane (DCM)) but cleavable at 95% TFA (5% H_2O). This property allowed us to remove the acid-labile protecting groups which are commonly used in peptide synthesis in a simple and convenient manner. The final cleavage (95% TFA/5% H_2O) afforded the desired product in good yield and purity. The synthetic procedure for the preparation of this new linker and its application in the synthesis of peptide amides with side-chain modification are the main focus of this study.

Abbreviations: DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenyl-methoxycarbonyl; HF, hydrogen fluoride; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; PIP, piperidine; PS, polystyrene; R_f, retardation factor; R_t, retention time; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid.

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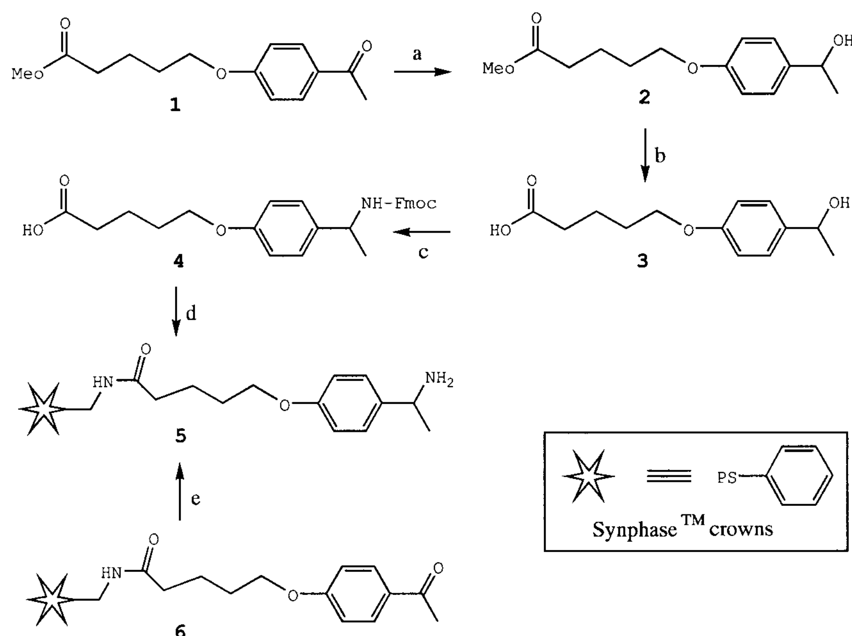

Scheme 1 Synthesis of linker **5**. (a) $NaBH_4$/MeOH/25°C. (b) NaOH/H_2O/MeOH/25°C. (c) Fmoc-NH_2/CH_3COOH/25°C. (d) Aminomethylated-PS crowns/HOBt/DIC/DMF/25°C then 20% PIP/DMF. (e) $NaBH_3CN$/NH_4OAc/THF:EtOH/60°C (5:1).

MATERIALS AND METHODS

All chemicals and amino acids were obtained from Aldrich Chemical Company (Castle Hill, Australia) and Senn Chemicals (Dielsdorf, Switzerland), respectively. Fmoc solid-phase peptide synthesis was performed on Synphase™ aminomethylated polystyrene grafted crowns (loading = 20 μmol/crown, Chiron Technologies Pty. Ltd, Clayton, Australia).

Analytical high performance liquid chromatography (HPLC), electrospray mass spectrometry (ES-MS) and NMR analyses were performed as reported previously [8].

Methyl 5-(4-Acetylphenoxy)valerate (1)

Methyl 5-bromovalerate (14.6 g, 0.075 mol) was added to a suspension of potassium *t*-butoxide (8.4 g, 0.075 mol) and 4-hydroxyacetophenone (10.2 g, 0.075 mol) in dimethylformamide (DMF) (100 ml) at 25°C under nitrogen atmosphere. The reaction mixture was heated to 80°C for 6 h and then poured on to ice. The mixture was extracted with ether (2 × 50 ml) and ethyl acetate (50 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give **1** (14.5 g, 77% yield). $R_t = 6.98$ min with a 94% purity by HPLC. ES-MS m/z 251.0 $[M+H]^+$, 501.3 $[2M+H]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.92 (d, $J = 0.8$ Hz, 2H), 6.92 (d, $J = 0.8$ Hz, 2H), 4.03 (t, $J = 6$ Hz, 2H), 3.67 (s, 3H), 2.54 (s, 3H), 2.41 (t, $J = 6.8$ Hz, 2H), 1.90–1.80 (m, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): 196.6, 173.6, 162.7, 130.4, 130.1, 113.9, 67.5, 51.4, 33.4, 28.4, 26.1, 21.4.

Methyl 5-(4-(1-Hydroxyethyl)phenoxy)valerate (2)

To a solution of ketone **1** (1.25 g, 0.005 mol) in 20 ml of methanol was added $NaBH_4$ (0.38 g, 0.01 mol). The reaction mixture was stirred at 25°C for 1 h and the solvent was removed under reduced pressure. The resulting oil was poured into water and the product was extracted with ether (3 × 50 ml). The combined extracts were concentrated under reduced pressure to give the crude product **2** (1.2 g, 95% yield) $R_t = 6.33$ min with a 92% purity by HPLC. ES-MS m/z 235.3 $[M-OH]^+$, 469.2 $[2(M-OH)]^+$, 522.5 $[2M+NH_4]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.21 (d, $J = 9$ Hz, 2H), 6.80 (d, $J = 9$ Hz, 2H), 4.75 (m, 1H), 3.98–3.92 (m, 2H), 3.60 (s, 3H), 2.37 (s, broad, 1H), 2.35–2.30 (m, 2H), 1.79–1.71 (m, 4H), 1.40 (d, $J = 7$ Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.8, 158.1, 138.0, 126.5, 114.3, 69.6, 67.3, 51.4, 38.5, 28.5, 24.9, 21.5.

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5-(4-(1-Hydroxyethyl)phenoxy)valeric Acid (3)

Methyl 5-[(4-(1-hydroxyethyl)phenoxy]valerate (**2**) (1.2 g, 0.005 mol) was stirred in 25 ml of a 4:6 mixture of 4 N NaOH and methanol at 25°C until the starting material had been consumed as determined by t.l.c. analysis. The reaction mixture was then poured into water and acidified with HCl until pH 2–3. The product was extracted with ethyl acetate (3 × 50 ml) and the combined extracts were dried over $MgSO_4$ and concentrated under reduced pressure to give the crude oil which was used for next step without further purification.

5-{4-(1-(9-Fluorenylmethoxycarbonylamino)ethyl)-phenoxy}valeric Acid (4)

To a solution of **3** (1.2 g, 0.005 mol) in 20 ml of acetic acid was added Fmoc-NH_2 (1.2 g, 0.005 mol) and a few drops of concentrated H_2SO_4. The reaction mixture was stirred at 25°C for 20 h and then poured into water. The product was filtered to give the crude light yellow powder (1.1 g, 48% yield). Chromatography of the crude (SiO_2, 40% EtOAc in petroleum spirit 40–60°C) afforded the product **4** as a white powder. R_f (retardation factor) = 0.3 (40% EtOAc in petroleum spirit 40–60°C), R_t (retention time) = 8.2 min with a 95% purity by HPLC. ES-MS *m/z* 460.2 $[M+H]^+$, 477.1 $[M+NH_4]^+$, 919.4 $[2M+H]^+$, 936.5 $[2M+NH_4]^+$. ^{1}H-NMR (400 MHz, DMSO-d_6): 7.76 (d, $J = 0.8$ Hz, 2H), 7.64 (d, $J = 0.6$ Hz, 2H), 7.38 (t, $J = 0.6$ Hz, 2H), 7.32–7.18 (m, 4H), 6.82 (d, $J = 0.8$ Hz, 2H), 4.7 (m, 1H), 4.32 (d, $J = 0.7$ Hz, 2H), 4.18 (m, 1H), 3.94 (t, $J = 0.6$ Hz), 3.2 (s, broad, 1H), 2.31 (t, $J = 0.7$ Hz, 2H), 1.78 (m, 4H), 1.4 (d, $J = 0.6$ Hz, 3H), 1.26 (s, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 174.3, 157.4, 155.3, 143.9, 143.8, 140.7, 136.9, 127.5, 127.0, 125.1, 120.0, 114.1, 67.1, 65.2, 49.4, 46.8, 33.3, 28.1, 22.6, 21.2.

Preparation of Linker (5)

Five I-series crowns (loading = 20 μmol/crown) were incubated with a mixture of **4** (0.069 g, 0.15 mmol), HOBt (0.023 g, 0.15 mmol), diisopropylcarbodiimide (DIC) (0.019 g, 0.15 mmol) in DMF (3 ml) at 25°C for 16 h. Crowns were carefully washed with DMF:DCM (50:50) and DCM and dried under reduced pressure for several hours. Before being used, the crowns were treated with 20% piperidine (PIP)/DMF for 1 h and then washed thoroughly with DMF, DMF:DCM (50:50) and DCM to give **5**.

Table 1 Analytical Data of the Model Molecules

Target molecules	HPLC[a] (min)	ES-MS[a] $[M+H]^+$ observed	ES-MS $[M+H]^+$ calculated	% Yield[b]	% Purity[c]
8a Fmoc-Gly-$CONH_2$	6.73	297.2	297.3	86	99
8b Fmoc-β-Ala-$CONH_2$	7.06	311.1	311.3	67	99
8c Fmoc-Val-$CONH_2$	9.45	339.0	339.4	81	99
8d Fmoc-Ile-$CONH_2$	9.83	353.0	353.4	85	96
8e Fmoc-Pro-$CONH_2$	8.98	337.2	337.4	86	97
8f Fmoc-Glu-$CONH_2$	6.26	369.1	369.5	85	99
8g Fmoc-Asp-$CONH_2$	6.77	354.9	354.4	77	96
8h 1-Naphthylacetamide	7.41	186.2	186.2	73	93
8i $C_6H_5CH_2CH_2CH_2CONH_2$	7.09	164.2	164.2	79	97
8j $(C_6H_5)_2CHCONH_2$	6.42	212.2	212.3	82	100
8k 3,4-$(CH_3O)_2C_6H_3CH_2CONH_2$	4.12	196.2	196.2	66	87
8l 2-$ClC_6H_4CH_2CH_2CONH_2$	5.96	184.0	184.1	73	86
Peptide **1**[d]	8.10	614.4	614.7	71	96
Peptide **2**[d]	7.77	801.5	801.8	78	62
11	7.94	458.1	458.3	86	72
13	8.85	605.2	605.4	78	86

[a] HPLC and ES-MS data of **8a** to **8l**, peptide **1** and peptide **2** were identical with the authentic compounds obtained by using Rink-amide linker.
[b] Crude yield was based on the initial loading of crown.
[c] % purity was based on the HPLC at 401 nm.
[d] Peptide **1**, Fmoc-Val-Gly-Phe-Ala-$CONH_2$; peptide **2**, Fmoc-Tyr-Pro-Phe-Pro-Gly-$CONH_2$.

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Synthesis of Compound 8

General procedure for **8b**: two derivatized crowns **5** were treated with Fmoc-β-Ala-OH (0.311 g, 0.001 mol)/ 1-hydroxybenzotriazole (HOBt) (0.156 g, 0.001 mol)/DIC (160 μl, 0.001 mol) in 10 ml of DMF at 25°C for 16 h. Crowns were washed with DCM:DMF (50:50) and DCM and 20% TFA in DCM for 10 min (pre-washing). For cleavage, the crowns were incubated with 95% TFA (5% H_2O) in DCM for 5 h and TFA was evaporated under a stream of N_2 gas to dryness to afford **8b** (Table 1). Compounds **8a**–**8l** are identical to the authentic samples prepared by using the Rink-amide linker (results not shown).

Synthesis of Compounds 11 and 13

Two crowns derivatized with linker **5** were treated with Fmoc-Glu(OBu^t)-OH (0.425 g, 0.001 mol), DIC (160 μl, 0.001 mol), HOBt (0.156 g, 0.001 mol) in DMF (10 ml) for 16 h. The crowns were then washed with DCM, DMF and DCM and dried in air for 1 h. The crowns were then subjected to 30% TFA in DCM for 30 min and carefully washed with DCM, DMF and DCM before being incubated in a DMF solution of benzylamine (0.107 g, 0.001 mol), DIC (160 μl, 0.001 mol), HOBt (0.156 g, 0.001 mol) for 16 h. After a normal washing step (DMF and DCM) the crowns were then transferred to a solution of 20% PIP/DMF for 1 h. The crowns were washed with DMF and DCM and finally treated with Fmoc-Phe-OH (0.387 g, 0.001 mol), DIC (160 μl, 0.001 mol), HOBt (0.156 g, 0.001 mol) in 10 ml of DMF for 16 h at 25°C. The crowns were washed with DMF, DCM and 20% TFA in DCM (10 min) and subjected to the cleavage condition (95% TFA and 5% H_2O) for 5 h (crowns turned deep purple). The TFA was evaporated under a stream of N_2 gas and the residue was dried under reduced pressure to afford the product **13** (Table 1). To obtain **11**, after the coupling with benzylamine, the resulting crown was treated with 95% TFA (5% H_2O) for 5 h and the cleavage solution was dried under a stream of N_2 gas to afford the desired product **11** (Table 1).

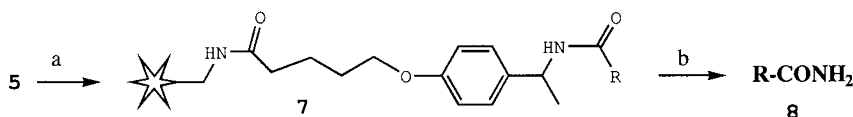
Synthesis of Peptide 1 (Fmoc-Val-Gly-Phe-Ala-CONH$_2$)

Two crowns **5** were incubated with Fmoc-Ala-OH (0.311 g, 0.001 mol), DIC (160 μl, 0.001 mol) and HOBt (0.156 g, 0.001 mol) in 10 ml of 25% DMF in DCM at 25°C for 16 h. The crowns were then removed, washed with DCM, DMF and DCM and finally dried in air for 30 min. For further elongation steps, the crowns were treated with 20% PIP/DMF for 1 h and then washed thoroughly with DCM, DMF and DCM. The crowns were incubated with 10 ml of subsequent Fmoc-amino acid solution (0.1 M), HOBt (0.1 M) and DIC (0.1 M) in DMF at 25°C for 8 h.

For cleavage, the crowns were incubated with 1 ml of 95% TFA, 5% H_2O at RT for 5 h. The crowns were removed and the solution was evaporated with N_2 gas for 20 min to afford the desired peptide **1**. A similar procedure was applied for the synthesis of peptide **2** (Fmoc-Tyr-Pro-Phe-Pro-Gly-CONH$_2$).

Cleavage Study

Two sets of 12 crowns were derivatized, one with linker **5** and one with Rink-amide. The crowns were then coupled with Fmoc-β-Ala-OH (0.622 g, 0.002 mol), HOBt (0.312 g, 0.002 mol) and DIC (320 μl, 0.002 mol) in 20 ml of DMF at 25°C for 16 h. The crowns were incubated in duplicate with two different concentrations of TFA (30% and 50% TFA in DCM). At 15, 30 and 60 min, crowns were removed and transferred to 95% TFA (5% H_2O) for completion of cleavage (16 h incubation). The cleavage solutions were dried with a stream of N_2 gas. The resulting products were dissolved in acetonitrile and analysed by HPLC. The percentage cleavage at each time point (Table 2) was calculated from the ratio of the peak area of initial cleavage/(total peak area of initial cleavage plus final cleavage).



Scheme 2 Synthesis of amides. (a) HOBt/DIC/DMF/25°C. (b) 95% TFA (5% H_2O).

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RESULTS AND DISCUSSION

Scheme 1 describes the detailed synthetic pathways to linker **5**. Our work was initially focussed on a direct reductive amination route to convert **6** to **5** using standard conditions ($NaBH_3CN$ and NH_4OAc in 5:1 THF:ethanol) [9]. However, this reaction did not go to completion due to a concomitant formation of the hydroxyethyl moiety as the byproduct on the solid supports. Attempts to optimize the reaction conditions failed to improve the yield and purity of the final product [10,11]. Alternatively, the linker **5** could be derived from the previously reported acetophenone-based linker **1** [8]. Thus, **1** was reduced under standard reduction conditions ($NaBH_4$ in THF) to afford the alcohol **2**. The compound **2** was then saponified (aq. NaOH/MeOH) and followed by treatment with Fmoc-NH_2 in acetic acid to afford the preformed linker **4** (48% overall yield, two steps from **2**) [6,10]. Attachment of the linker **4** to aminomethyl–polystyrene (PS) crowns under standard coupling conditions (HOBt/DIC/DMF), followed by Fmoc deprotection (20% PIP/DMF) afforded the linker **5** in quantitative yield [12].

Usefulness of linker **5** was established by coupling with a wide range of carboxylic acids (R-COOH/HOBt/DIC/DMF) in Scheme 2. After the usual washing step, the resulting derivatized crown **7** was subjected to the cleavage solution (TFA:H_2O, 95:5) for 5–10 h to give the target compound **8** in good yield (Table 1). Cleavage of most compounds was completed within 5 h. However, cleavage time can be shortened by using sonication. Under similar reaction conditions, two model peptide amides have been successfully prepared via the Fmoc strategy on this linker system [8]. In brief, linker **5** was coupled with the first Fmoc-amino acid (DIC/HOBt/DMF). After Fmoc deprotection the resulting product was allowed to react with the second amino acid (DIC/HOBt/DMF) and so on for a total of four cycles. At the end of the coupling cycles the desired peptide was cleaved off the solid support using 95% TFA (5% H_2O) for 5–10 h. The test compounds were analysed by HPLC and MS [13]. In all cases, the desired products were obtained in very high purity (Table 1 and Figure 1). Unlike the benzhydrylamine-type linker which requires strong acid conditions such as HF and trifluoromethanesulfonic acid (TFMSA), the aminoethyl-PS linker is TFA sensitive, suggesting that both phenyl and methyl groups act as electron donating groups to facilitate the cleavage process of the C–N bond.

Comparative TFA stability of aminoethyl-PS linker **5** and the commercially available Rink-amide linker was assessed. Both linkers were coupled with Fmoc-β-Ala-OH and the resulting products were subjected to 30% and 50% TFA in DCM at 25°C. The result (Table 2) showed that the linker **5** is stable under mild acidic conditions. At 50% TFA in DCM only 4% cleavage was observed within 30 min. The differential cleavage rates also allowed us to successfully remove the Bu^t protecting groups in the presence of linker **5**. In one example described in Scheme 3, linker **5** was derivatized with Fmoc-Glu(OBu^t)-OH under the previous conditions to afford compound **9**. The Bu^t protecting group of compound **9** was removed with 30% TFA/DCM (30 min). The resulting free carboxylic acid side-chain group was allowed to react with benzylamine under standard coupling conditions (DIC/HOBt/DMF) [12] to give compound **10**. Treatment of **10** with 95% TFA (5% H_2O) afforded the product **11**.

Further chemical transformation of **10** by sequential treatment with 20% PIP/DMF and Fmoc-Phe-OH/DIC/HOBt afforded the *N*-alkyl solid support bound peptide **12**. After cleavage in 95% TFA, the product **13** was obtained in good yield

Table 2 Comparative Cleavage Study on Linkers **5** and Rink-amide

Time	% Cleavage in 30% TFA/DCM		% Cleavage in 50% TFA/DCM	
(min)	Linker **5**	Rink-amide linker	Linker **5**	Rink-amide linker
15	3	80	2	92
30	3	89	4	93
60	9	92	12	94

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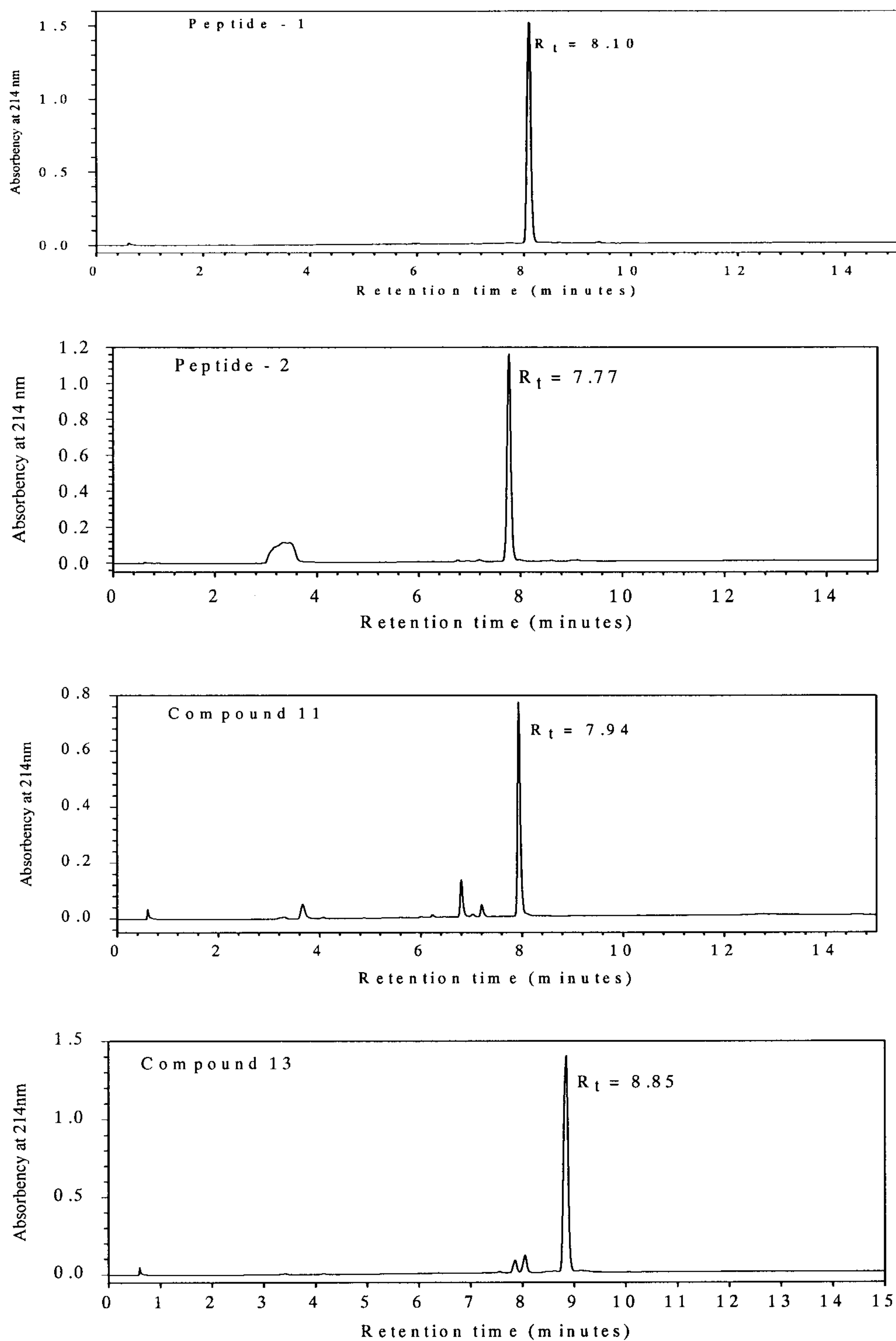

Figure 1 HPLC traces of the model molecules: peptide **1**, peptide **2**, **11** and **13**.

and purity. All products were characterized using HPLC and ES-MS spectroscopy and listed in Table 1 and Figure 1. HPLC of the crude product **13** indicated the quantitative cleavage of the Bu^t protecting group under the above described conditions.

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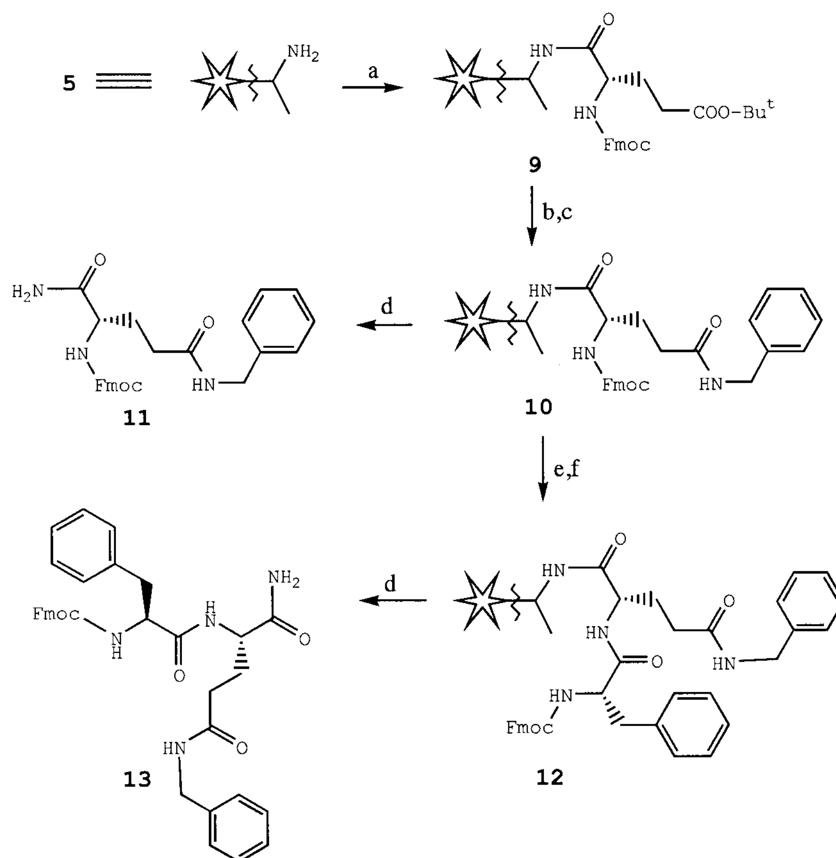

Scheme 3 Synthesis of the model compounds **11** and **13.** (a) Fmoc-Glu(OBu^t)-OH/DIC/HOBt/DMF/25°C. (b) 30% TFA/DCM. (c) Benzylamine/DIC/HOBt/DMF/25°C. (d) 95% TFA (5% H_2O). (e) 20% PIP/DMF. (f) Fmoc-Phe-OH/DIC/HOBt/DMF/25°C.

CONCLUSION

In our previous reports, we have shown that the universal acetophenone class of linkers can be used to give a number of end groups. Successful examples are syntheses of secondary amides, sulfonamides [8], peptide acids [14], phenol type molecules, hydroxamic acids [15] and now the synthesis of carboxamides. Compared to other commercially available linkers, the described linker is simple and readily prepared from cheap and commercially available starting materials, i.e. acetophenone and 5-bromovaleric acid. The linker is very versatile for the preparation of a broad range of peptide amides with or without modification of Bu^t protected amino acid side-chains during peptide assembly, a strategy which is not feasible using previous linkers designed for Fmoc peptide chemistry.

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