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Improving the Performance of an Acid-labile 4-Hydroxymethyl phenoxyacetic acid (HMP) Linker on Resin and SynPhase™ Grafted Solid-Supports

CHINH T. BUI*, FRANCESCA ERCOLE, YEN PHAM, RHONDA CAMPBELL, FIRAS A. RASOUL[1], N. JOE MAEJI[1] and NICHOLAS J. EDE

Mimotopes Pty. Ltd., Clayton, Victoria, Australia

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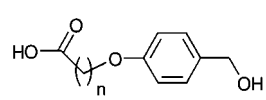
Abstract: A replacement of the acetic acid moiety by valeric acid within the 4-hydroxymethylphenoxyacetic acid (HMP) linker (Sheppard RC, Williams BJ. Acid-labile resin linkage agents for use in solid phase peptide synthesis. *Int. J. Peptide Protein Res*. 1982; **20**: 451–454) significantly improved its performance in terms of loading capacity, yield and purity of the final products. The results indicated the spacer–linker combination and type of solid supports are important factors for solid-phase synthesis. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: 4-hydroxymethylphenoxyacetic acid; 5-(4-hydroxymethylphenoxy)pentanoic acid; multipin solid support; solid phase organic chemistry

INTRODUCTION

High-throughput parallel solid phase synthesis of drug-like small organic molecules is an important methodology for accelerating drug discovery research [2]. Successful solid-phase organic synthesis depends on the choice of linker, spacer and solid support [3]. The 4-hydroxymethylphenoxyacetic acid linker **1** (HMP, Figure 1) has been used for many years as the acid-labile linker for solid-phase syntheses of peptide acids [4] and small organic molecules [5]. The linker **1** is also considered as an alternative version of the 4-hydroxymethylphenoxy-polystyrene (PS) (the Wang linker) [6] except that it has a terminal carboxylic acid group on the side chain for attachment on to an aminomethyl-PS support via an amide bond. Substrates including carboxylic acids or alcohols can be loaded on to the hydroxymethyl moiety within the linker via ester and ether linkages, respectively [7]. The final cleavage can be achieved using 50% trifluoroacetic acid/dichloromethane (TFA/DCM) [1,4]. However, we have recently found that the theoretical loading of carboxylic acids on to the linker **1** could not be achieved under standard loading conditions, and the final product was usually obtained in low yield and purity. In order to overcome this problem, we now describe the analogue **2**

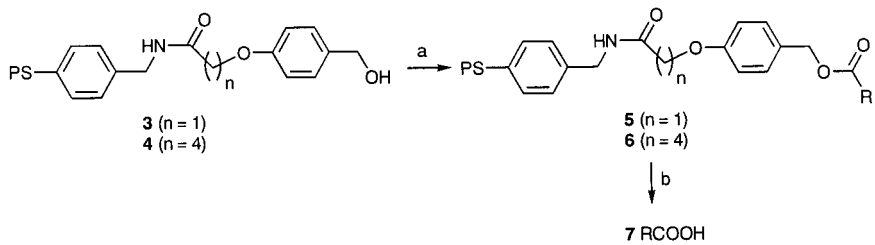


1 (n = 1)
2 (n = 4)

Figure 1 The HMP linker (1) and its analogue (2).

Abbreviations: oromethane; DIC, diisopropylcarbodiimide; DMA, *N*,*N*-dimethylacetamide; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; Dnp: dinitrophenyl; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenyl-methoxycarbonyl; HOBt, 1-hydroxybenzotriazole; PIP, piperidine; PS, polystyrene; RP-HPLC, reverse-phase high performance liquid chromatography; RT, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid.

* Correspondence to: Mimotopes Pty. Ltd., 11 Duerdin Street, Clayton, Victoria 3168, Australia
[1] Present address: Polymerat Pty. Ltd., 14 Rheanva Street, Berwick, Victoria 3806, Australia



Scheme 1 Esterification reaction on Lanterns **3** and **4**. (a) RCOOH/DIC/DMAP/20% DMF in DCM/25 °C (b) 50% TFA in DCM.

[5-(4-hydroxymethylphenoxy)pentanoic acid, Figure 1] which contains a longer carboxylic acid side-chain within the linker structure. This modification significantly improved the performance of the linker for solid phase synthesis in terms of yield and purity. A comparative study between two linkers **1** and **2** using the commercially available PS grafted Lantern [8] and resin solid supports is the main focus of this study.

MATERIALS AND METHODS

Solid phase synthesis was performed using SynPhase™ aminomethyl-PS grafted Lanterns with an average loading of 15 μmol (Mimotopes, Clayton, Australia), and aminomethylated PS resins (38–75 μm beads, 1.0 mmol/g and 130–212 μm, 1.1 mmol/g, NovaBiochem, PO Box 466, Croydon, Victoria, Australia). Lanterns are a lantern-shaped molded polypropylene co-polymer object (5 × 5 mm) with a mobile surface graft of linear PS. Chemicals and solvents were purchased from Aldrich Chemical Company (Castle Hill, Australia). Analytical reverse-phase high performance liquid chromatography (RP-HPLC), electrospray mass spectrometry (ES-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) analyses were performed as reported elsewhere [9].

Synthesis of 5-(4-hydroxymethylphenoxy)pentanoic acid 2

The methyl ester of 5-bromopentanoic acid (38.4 g, 0.19 mol) was added in portions to a magnetically stirred solution of 4-hydroxybenzaldehyde (20 g, 0.16 mol) and K^+t-BuO^- (18.4 g, 0.16 mol) in 100 ml dimethylformamide (DMF) at 25 °C. The reaction mixture was heated to 120 °C for 5 h under N_2 atmosphere. The reaction mixture was poured on to ice and the product was extracted with ether (3 × 30 ml). The combined extract was concentrated under reduced pressure and the resulting crude oil was immediately mixed with 100 ml of 2 M NaOH in 50% aqueous methanol at 25 °C for 4 h. The reaction mixture was finally poured into 100 ml of water and the aqueous phase was acidified with concentrated HCl until pH = 3.0. The solid was filtered and dried under reduced pressure to afford the product, 5-(4-formylphenoxy)pentanoic acid, as a white powder {R_t = 7.8 min, 97% purity, 87% overall yield, ^{1}H NMR (400 MHz, $CDCl_3$): 9.87 (s, 1H), 7.82 (d, J = 0.8 Hz, 2H), 6.98 (d, J = 0.8 Hz, 2H), 4.07 (t, J = 0.6 Hz, 2H), 2.43 (t, J = 0.7 Hz, 2H), 1.92–1.82 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): 190.9, 179.2, 164.0, 132.0, 129.8, 114.7, 67.7, 33.5, 28.3, 21.3}. The resulting product (10.2 g, 48.6 mmol) was then reduced with $NaBH_4$ (1.82 g, 48.6 mmol) in methanol (100 ml) at 25°C for 4 h. The solvent was then removed under reduced pressure and the residue was poured into water and the product was extracted with ether (3 × 50 ml). The combined extracts were dried, filtered and concentrated under reduced pressure to afford the title compound **2** as a white powder (9.5 g, 92% yield), {R_t = 6.91 (98% purity) ES-MS m/z [(M-H_2O) + H]$^+$, 207.1. ^{1}H NMR (400 MHz, $CDCl_3$): 7.28 (d, J = 1.0 Hz, 2H), 6.86 (d, J = 1.0 Hz, 2H), 4.57 (s, 2H) 3.97 (t, J = 0.6 Hz, 2H), 2.36 (t, J = 0.7 Hz, 2H), 1.86–1.76 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): 175.1, 157.9, 133.5, 128.0, 114.0, 67.1, 63.8, 33.4, 28.3, 21.2}.

Preparation of Lanterns 3 and 4 (Scheme 1)

The TFA salt of aminomethylated-PS Lanterns (100 Lanterns with loading = 15 μmol/Lantern) was neutralized by standing the Lanterns in 5% triethylamine (TEA) in DMF/DCM (1:1) for 15 min. After draining, the neutralization step was repeated and the Lanterns were washed with DMF/DCM (1:1) for 2 × 5 min and DCM for 2 × 5 min. The Lanterns were then derivatized with linker **1** or **2** under

standard conditions (0.12 M linker **1** or **2**/0.12 M diisopropylcarbodiimide (DIC)/0.15 M 1-hydroxybenzotriazole (HOBt)/20% DMF in DCM) to give **3** and **4**, respectively [4]. For the comparative study, resins were swollen in 5 ml of DCM:DMF (1:1) for 20 min and then treated with linkers **1** or **2** under similar reaction conditions.

Determination of loading capacity (Scheme 1)

Typical esterification reaction conditions with 9-fluorenyl-methoxycarbonyl (Fmoc)-Ala-OH used for resin and Lantern solid supports are as follows: Lanterns **3** or **4** (four Lanterns, 15 μmol/Lantern) were incubated with 1.2 ml of DMF:DCM (1:4) solution containing Fmoc-Ala-OH (44.8 mg, 144 μmol), DIC (22.5 μl, 144 μmol), 4-(dimethylamino)pyridine (DMAP) (5.8 μmol) at 25 °C for 16 h. Lanterns were removed, washed with DCM, DMF and DCM (3 ×) and dried in air for 15 min. Cleavage was effected by 50% TFA/DCM (2 h) and the resulting Fmoc-amino acid was dried under a stream of N_2 gas. The residue was dissolved in 10 ml acetonitrile and a 5 μl sample was injected for HPLC analysis. Similar reaction conditions were applied to resins for this study.

RESULTS AND DISCUSSION

Synthetic strategy of **2** was essentially as reported in our previous publication [10]. In brief, the linker **2** was prepared by coupling the methyl ester of 5-bromovaleric acid with 4-hydroxybenzaldehyde. The resulting product was saponified (NaOH/methanol) and followed by reduction with $NaBH_4$ to afford **2**. For comparative study of their performances, linkers **1** and **2** were attached to aminomethyl-PS solid supports (Lanterns or resins) under standard conditions [4]. The resulting products **3** and **4** were allowed to react with a series of Fmoc-amino acids using standard coupling conditions (DIC/DMAP/20% DMF in DCM) (Scheme 1) [4]. The optimized reaction conditions (2.5 molar excess of Fmoc-amino acid, 16 h incubation at 25 °C) were applied in this comparative study. After being washed with DCM, DMF and DCM (3 ×), the Lanterns **5** and **6** were subjected to 50% TFA/DCM (2 h) to afford the cleavage product **7**. Loading capacity was quantitated using HPLC analysis (peak area of the carboxylic acid product at 214 nm was used as a measure of the yield and thus of the original loading).

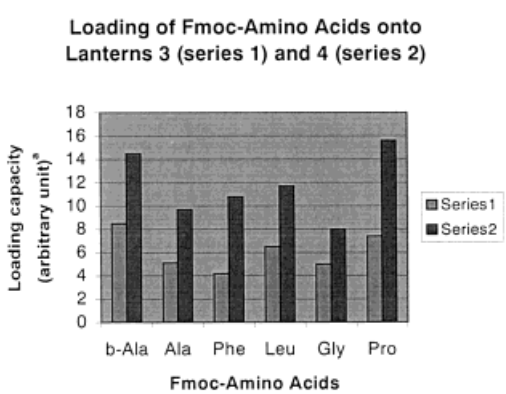


Figure 2 Comparative study of loading capacity between Lanterns **3** (Series 1) and **4** (Series 2). [a] Loading determination was based on HPLC peak areas of the cleavage products at 214 nm.

Figure 2 depicts the performance of the Lanterns **3** & **4**. The Lanterns **4** gave consistently higher loading values (up to 50%) over a wide range of tested Fmoc-amino acids. In addition, there were large variations in product purities. In one typical example, the RP-HPLC chromatograms (Figure 3) displayed the product **7** obtained from **4** as a single peak. However, the cleavage product derived from **3** gave the target **7** (Fmoc-amino acid) along with a significant amount of the impurity **10** which has an increase in molecular weight of 165 compared to the Fmoc-amino acid **7** (Table 1).

The by-product **10** was unstable and decomposed when the cleavage mixture was passed through a SiO_2 column. However, ^1H and ^{13}C NMR of the mixture clearly indicated the presence of linker **1**. In the control experiment, subjecting the Lantern **3** itself to 50% TFA in DCM released a compound which was chromatographically and spectroscopically identical to the authentic compound, 4-hydroxymethylphenoxyacetic acid **1**. In fact, the experimental data suggested the by-product **8** was generated during the assembly process of linker on aminomethyl-PS Lanterns (Scheme 2). After a successful esterification with Fmoc-amino acid, cleavage of the Lanterns gave the product **7** in *ca.* 80% yield as well as the by-product **10** in *ca.* 10–20% yield. Attempts to optimize the coupling step (a) of Scheme 2 by varying linker **1** concentrations failed to avoid a concomitant formation of the by-product **8** on the solid supports.

A comparative assessment of linkers **1** and **2** was repeated on resins and up to 40% of the by-product **10** was obtained (Table 2). Although similar reaction conditions were applied to both types of solid

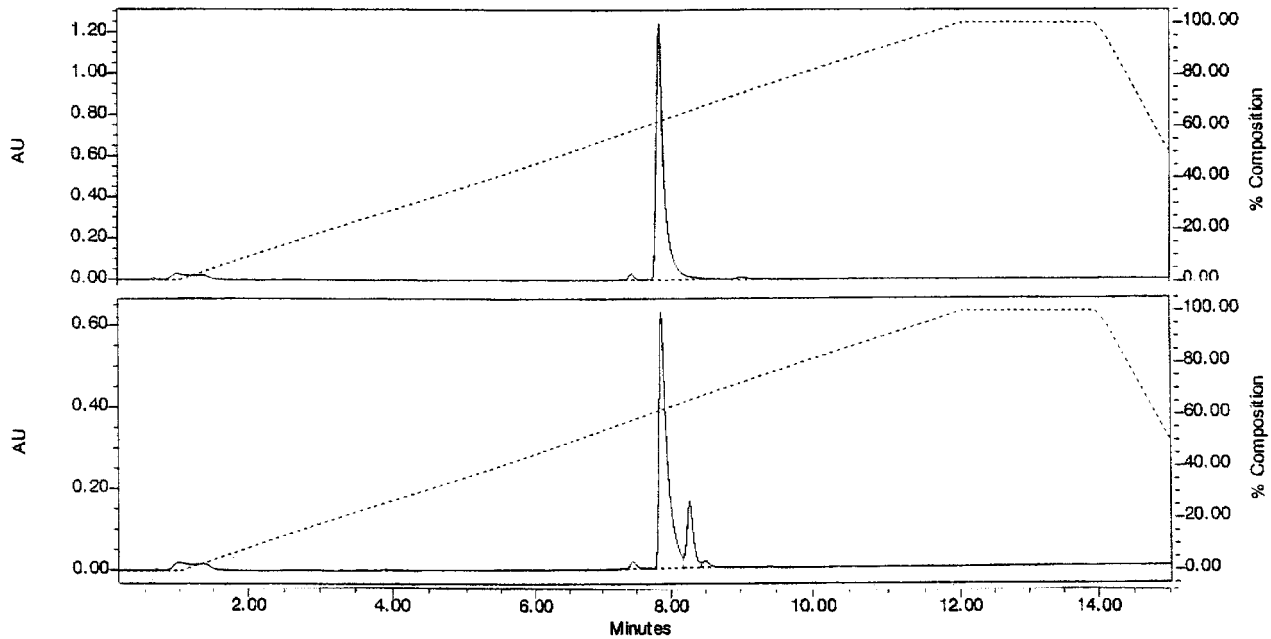


Figure 3 Typical HPLC chromatograms of the product **7** (Fmoc-Ala-OH) prepared from the Lanterns **4** (top) and **3** (bottom).

Table 1 Cleavage Products Derived from Lanterns **3**

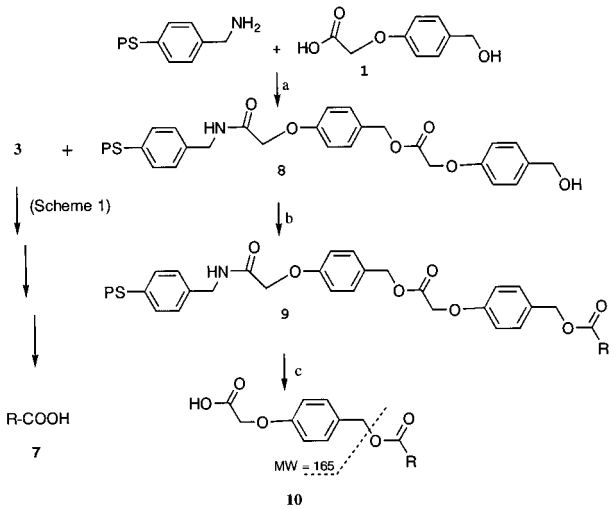
Loading materials	Product **7** RP-HPLC (R_t, min)/peak% [a]/ LC-MS observed $[RCOOH+H]^+$	Major by-product **10** RP-HPLC (R_t, min)/peak% [a]/ LC-MS observed $[RCOO+165+H]^+$
Fmoc-β-Ala-OH	7.6/84/312.0	8.1/13/475.9 (311+165)
Fmoc-Ala-OH	7.8/86/312.0	8.3/11/476.0 (311+165)
Fmoc-Phe-OH	9.2/81/388.2[b]	9.2/19/552.2 (387+165)[b]
Fmoc-Leu-OH	9.0/88/354.0[b]	9.0/12/518.2 (353+165)[b]
Fmoc-Gly-OH	7.5/60/297.9	8.0/10/462.1 (297+165)
Fmoc-Pro-OH	8.1/74/338.1	8.5/23/502.2 (337+165)

[a] % product and the major by-product were based on HPLC peak area (214 nm).
[b] % Product and by-product were based on their ES-MS peak heights as the resolution could not be achieved on HPLC.

support, different percentages of the by-product **10** were observed on resins and Lanterns, indicating the formation of **8** was strongly influenced by the type of solid support. Extension of the spacer between the linker and the polymeric supports by replacing acetic acid with the valeric acid completely prevented the hydroxymethylphenoxy moiety on the solid support from repeatedly coupling with **1**. The results were confirmed by the cleavage products obtained from **2** in excellent yields and purities (>95% purity) for all cases. Whether this problem is caused by the proximity of an amide bond to the hydroxymethylphenoxy moiety when linker **1** is on an aminomethyl PS remains unknown.

CONCLUSIONS

A comparative study of two acid-labile linkers, 4-hydroxyphenoxy acetic acid (**1**) and 5-(4-hydroxymethylphenoxy)pentanoic acid (**2**) has been carried out. The following results were obtained: (i) Solid supports derivatized with linker **1** gave poorer loading values. (ii) Attachment of the linker **1** to aminomethyl-PS solid supports is problematic as it further reacted with **1** to form 'double linkers' on solid supports. (iii) The percentage of by-product is higher on resins than on Lanterns. (iv) The problem can be avoided by extension of the spacer between the linker and the polymer support. As the general performance of **2** is superior to **1** the former linker is highly recommended for solid phase synthesis.



Scheme 2 Formation of the by-product **10** on Lantern derivatized with **1**. (a) HOBt/DIC/20% DMF in DCM/25 °C (b) RCOOH/DIC/DMAP/20% DMF in DCM/25°C (c) 50% TFA in DCM.

Table 2 Effect of Linkers and Solid Supports on the Formation of the By-product **10**

Solid support	Linker used	% By-product **10**[a]
Lantern	**1**	10–20
Resin (38–75 μm bead)	**1**	39
Resin (130–212 μm bead)	**1**	41
Lantern	**2**	0
Resin (38–75 μm bead)	**2**	0
Resin (130–212 μm bead)	**2**	0

[a] Study was carried out with Fmoc-Gly-OH.

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