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Comparative study of reductive amination reaction on 5-(4-formyl-3,5-dimethoxyphenoxy)valeric acid and its monomethoxy analog using the Multipin^{TM} approach

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Summary

The 5-(4-formyl-3,5-dimethoxyphenoxy)valeric acid (Barany) linker and its monomethoxy analog were applied to the Multipin^{TM} method of solid phase synthesis. A comparative assessment of reductive amination and cleavage of these linkers under conditions of multiple synthesis indicated that both were applicable to a broad range of primary amines including aniline and 4-nitroaniline. Apart from the greater lability of the dimethoxy version under TFA cleavage, there was no observable advantage of one linker over the other within the described experiment.

Abbreviations: HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane; DIC, N,N-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MS, mass spectrometry; TFA, trifluoroacetic acid; MeCN, acetonitrile; Dnp, dinitrophenyl.

Introduction

Choice of linkers is an important factor in achieving good success in solid phase synthesis. The minimum requirements for a suitable linker are stability under conditions of synthesis and quantitative cleavage to release the target product without any competing side reactions. With the advent of combinatorial strategies and multiple/parallel organic synthesis [1,2], the need for a diversity of end groups (other than carboxylic acid and carboxamides) and efficient incorporation of monomers of varying reactivity to the linker also became important issues. Research into linkers that give different end groups on cleavage is a major activity in solid phase synthesis [3] but, as yet, there are limited studies on the relative efficiency of linkers in a combinatorial synthesis situation. For example, N-alkylamides can be readily obtained via reductive amination reactions using the linker 5-(4-formyl-3,5-dimethoxyphenoxy)valeric acid derivatised on solid supports **1** [4,5]. Some elegant examples have been demonstrated on the solid phase synthesis of C-terminal peptide amides [6], aliphatic peptide secondary amides [7], benzodiazepines [8] and sulfonamides [9]. More recently, it has been reported that the formyl group of this linker is sterically hindered due to two flanking methoxy groups, and that such an electron-rich system was not required to facilitate cleavage when the leaving group was a secondary amide or a sulfonamide [10]. As an alternative, the monomethoxy analog **2** (Acid sensitive MEthoxy BenzAldehyde, AMEBA) has also been introduced for the solid phase synthesis of secondary amides [9].

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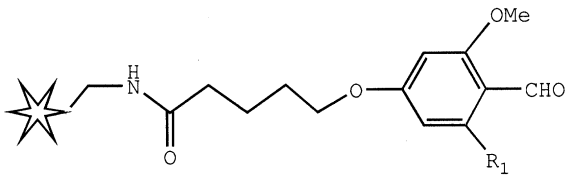


1 R_1=OMe
2 R_1=H

The relative advantages of these two linkers that give the same end groups on cleavage have not been fully explored [11]. Here, we report on the comparative study of these linkers, **1** and **2**, in two key steps of *N*-alkylamide formation, i.e., the reductive amination conditions suitable for a broad range of amines and the relative efficiency of cleavage of these linkers. This study was performed on SynPhase crowns used with the Multipin™ approach to solid phase synthesis.

Materials and methods

Preparation of linker, 5-(4-formyl-3-methoxyphenoxy)valeric acid, for synthesis of **2**

To a suspension of potassium *t*-butoxide (1.36 g, 12.1 mmol) in DMF (20 ml) was added dropwise 4-hydroxy-2-methoxybenzaldehyde (1.7 g, 11 mmol) in 2 ml DMF at room temperature under a nitrogen atmosphere. The reaction mixture was allowed to stir at room temperature for 15 min and methyl-5-bromovalerate (2.14 g, 11 mmol) was then injected into the mixture via a syringe. The reaction mixture was finally heated to 136 °C for 4 h before being poured onto ice and then extracted with ether (3 × 20 ml) and ethyl acetate (20 ml). The combined organic phase was concentrated under reduced pressure and the resulting yellowish oil was added to a solution of NaOH (8 ml of 4M aqueous NaOH + 12 ml of methanol) at room temperature for 12 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 to a pH of ∼ 3.0. The product was extracted with EtOAc (3 × 30 ml) and the combined organic phase was dried over $MgSO_4$, and finally concentrated under reduced pressure to afford a pale yellow powder. Recrystallisation of this material (5% ethyl acetate in petroleum spirit) afforded 5-(4-formyl-3-methoxyphenoxy)valeric acid as pale yellow needles (2.1 g, 75% overall yield). R_f = 0.25 (in 50% EtOAc in petroleum spirit 40–60 °C), Rt = 6.22 min [17]. Melting point = 126.7 °C. ES-MS m/z 253.1 $[M+H]^+$, 505.0 (55%) $[2M+H]^+$, 522.1 $[2M+NH_4]^+$. ^{1}H NMR (400 MHz, 5% DMSO-d_6 in $CDCl_3$) δ: 10.2 (s, 1H), 7.68 (d, 1 Hz, 1H), 6.58–6.52 (m, 2H), 4.08 (t, J = 0.6 Hz, 2H), 3.92 (s, 3H), 2.26 (t, J = 0.4 Hz, 2H), 1.84–1.66 (m, 4H). ^{13}C NMR (100 MHz, 5% DMSO-d_6 in $CDCl_3$) δ: 186.1, 173.2, 164.2, 162.2, 128.5, 117.1, 105.3, 96.8, 66.5, 54.3, 32.1, 26.9, 19.9.

Preparation of linker, 5-(4-formyl-3,5-dimethoxyphenoxy)valeric acid, for synthesis of **1**

Similar reaction conditions were applied to 4-hydroxy-2,6-dimethoxy benzaldehyde for synthesis of 5-(4-formyl-3,5-dimethoxyphenoxy)valeric acid. The product was obtained in 73% overall yield. R_f = 0.43 (in EtOAc), melting point = 129 °C. ES-MS m/z 283.0 (100%) $[M+H]^+$, 565.2 (12%) $[2M+H]^+$. ^{1}H NMR (200 MHz, 5% DMSO-d_6 in $CDCl_3$) δ: 10.32 (s, 1H); 6.09 (s, 2H, Ar); 4.06 (t, J = 6.1 Hz, 2H); 3.88 (s, 6H, OCH_3); 2.38 (t, J = 6.8 Hz), 2H; 1.95–1.75 (m, 4H). ^{13}C NMR (50 MHz, 5% DMSO-d_6 in $CDCl_3$) δ: 187.0, 174.9, 165.3, 163.5, 108.0, 90.2, 67.3, 55.5, 33.1, 29.9, 21.0.

Attachment of 5-(4-formyl-3-methoxyphenoxy)valeric acid to the crown solid support

The TFA salt of aminomethylated-PS crowns (100 crowns with loading = 30 μmol/crown) was neutralised by standing the crowns in 5% TEA in DMF/DCM (1:1) for 10 min. After draining, the neutralisation step was repeated and the crowns were washed with DMF/DCM (1:1) for 5 min, DCM for 5 min, DMF/DCM (1:1) for 5 min, and DCM for 5 min. The crowns were then incubated with a coupling solution containing 5(4-formyl-3-methoxyphenoxy)valeric acid (1.69 g, 6.7 mmol), HOBt (1.02 g, 6.7 mmol) and *N,N′*-diisopropylcarbodiimide (1.1 ml, 6.7 mmol) in 50 ml dry DMF for 24 h.

The coupling solvent was decanted and the crowns were washed with DMF (5 min) and DCM (2 × 5 min) and then allowed to dry under reduced pressure for 4 h to give linker **2**. Linker **1**, 5-(4-formyl-3,5-dimethoxyphenoxy)valeric acid, derivatised on aminomethylated polystyrene crowns is available from Chiron Technologies Pty Ltd. For comparative studies, exactly the same batch of crowns used to produce linker **2** was used to produce linker **1** (loading of 30 μmol/ crown).

General conditions for reductive amination

(a) Two-step reductive amination reaction. For example, an I-series crown (linker **1** or **2)** was incubated

with 1.5 ml of 2.0 M 2,4-dimethoxyaniline in DMF at 60 °C for 2 h (in a closed 5 ml vial). The crown was then removed, washed with DMF (5 min) and DCM (2 × 5 min) and placed in a closed 5 ml vial containing 1.5 ml of 0.05M $NaBH_3CN$ in THF at 60 °C for 2 h. After incubation, the crown was removed and washed with DCM (3 × 5 min) to afford **3** or **4** (where R_2-NH_2 = 2,6-dimethoxyaniline), see Scheme 1.

(b) One-step reductive amination reaction. For example, two I-series crowns (linker **1** or **2)** were incubated with 5 ml of 1M 2,4-dimethoxyaniline in DCM:DMF (1:1) (pre-adjusted at pH = 5 with acetic acid) and 0.15 ml of 0.5M $NaBH_3CN$ in DCM:DMF (1:1) at 60 °C for 4 h. The crowns were then removed and washed with DCM, DMF and DCM (3 × 5 ml) and dried in air for 30 min to afford **3** or **4** (where R_2-NH_2 = 2,6-dimethoxyaniline), see Scheme 1.

General acylation procedures and cleavage

After reductive amination (**3** or **4**), the crowns were transferred to a vial containing 1.5 ml of 0.1M Fmoc-β-Ala-OH, 0.1M DIC and 0.01M DMAP in DMF/DCM (1:1). After incubation at room temperature for 12 h, the crowns were removed and washed with DMF (5 min), DCM (2 × 5 min) and dried under reduced pressure. The crowns from linkers **1** and **2** were cleaved in 50% TFA in DCM for 2 h to afford the target compound **7p** (melting point = 150 °C). ES-MS m/z 447.1 $[M+H]^+$; 464.3 $[M+NH_4]^+$; 910 $[2M+NH_4]^+$. For **7r**, the crowns (**3** or **4**) were allowed to react with acetyl chloride (50 μl in 5 ml of DMF/DCM, 1:1) at room temperature for 4 h. The crowns were then removed, washed and cleaved as in the general procedure to give product **7r** (melting point = 216 °C). ES-MS m/z 180.9 $[M+H]^+$; 197.9 $[M+NH_4]^+$; 378.4 $[2M+NH_4]^+$.

Determination of yield

Compound yields of **7a** to **7r** were quantitated by using a standard curve established by HPLC peak area at 401 nm under standard running conditions, versus the concentration of the pure amides **7a**–**r** which had been prepared using solution phase synthesis. Typical solution-phase synthesis of compound **7p** was carried out with the following standard procedure: the 2,4-dimethoxyaniline (0.1 mol) was treated with Fmoc-β-alanine (0.1 mol) in the presence of HOBt (0.1 mol) and DIC (0.1 mol) as coupling reagents at room temperature for 2 h. The product was extracted and recrystallised in EtOAc and petroleum spirit (40–60 °C) to afford **7p** as a white powder. A series of standard solutions of **7p** were prepared for HPLC analysis and the peak areas at 401 nm against quantity (0 to 10 nmol) were established to create the standard curve.

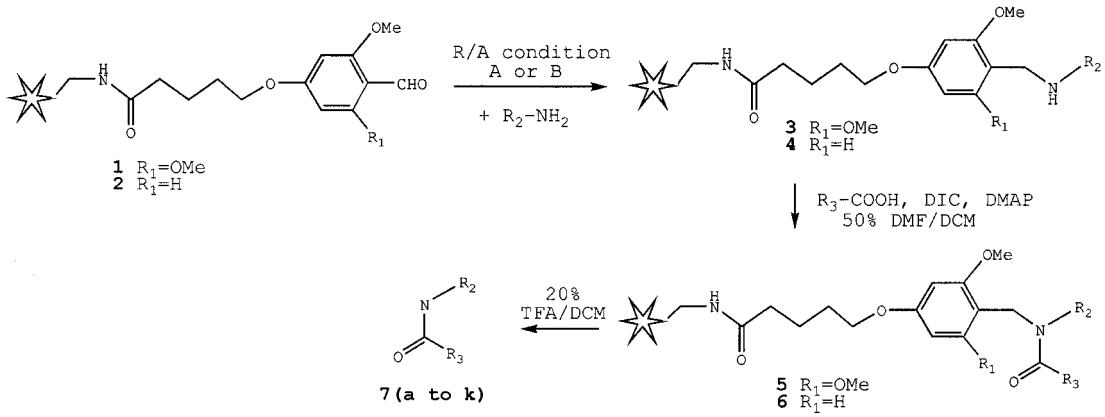
Cleavage study

To assess the cleavage efficiency of the two linker types, 40 crowns of **5q** or **6q** (R_2 = benzyl; R_3 = Dnp-β-Ala) were incubated in duplicate with five different concentrations of TFA (5%, 10%, 20%, 50% and 95%) in DCM. At 15, 30, 60, 90 and 120 min, crowns were removed and transferred to 95% TFA in DCM for completion of cleavage (2 h incubation). The cleavage solutions were aerated with nitrogen gas to dryness and the resulting solid (chromophoric **7q**) 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 by Biotex 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.).

Results

The two linkers, 5-(4-formyl-3,5-dimethoxyphenoxy)-valeric acid (Barany) [5] and 5-(4-formyl-3-methoxyphenoxy)valeric acid (monomethoxy analog) [9] can be readily derived in good yield from the commercially available starting materials, 2,6-dimethoxy-4-hydroxybenzaldehyde, 4-hydroxy-2-methoxybenzaldehyde and methyl 5-bromovalerate. The preformed linkers were coupled overnight to aminomethylated-PS crowns [12] using a twofold excess of linkers with DIC/HOBt activation in DMF/DCM. Completion of the coupling reaction was confirmed by amine stain tests using TNBSA. As shown in Scheme 1, the linkers **1** and **2** were subjected to reductive amination with benzylamine (R_2-NH_2) to give **3** and **4**, followed by acylation with Fmoc-β-Ala-OH (R_3-COOH) to afford the corresponding **5** and **6**. Cleavage of the amide bound linkers with TFA/DCM (1:4) afforded the target amide **7**.

Under the conditions of two-step reductive amination (Table 1), the reaction with benzylamine gave the product (R_2-NH_2 = benzylamine, R_3-COOH = Fmoc-β-Ala-OH) in above 90% purity on both linkers **1** and **2** [13]. However, with more sterically hindered amines such as 2,4-dimethoxy aniline, these



Scheme 1. Solid phase synthesis of the model molecule **7**. Reductive amination (R/A) condition A: two-step process; condition B: one-step process.

same procedures gave the target product (R_2-NH_2 = 2,4-dimethoxyaniline, R_3-COOH = Fmoc-β-Ala-OH) in only 59% purity with linker **1**. The remaining 41% impurity by HPLC was mainly Fmoc-β-Ala-OH which was obtained due to incomplete imine formation resulting in **10** and **11** (Scheme 2). The less sterically hindered linker **2** improved conversion to product of 77% purity (with 23% Fmoc-β-ala-OH). Further attempts to improve reaction conversion of these more bulky amines by more forcing conditions and other reducing agents (including $NaBH(OAc)_3$ and BH_3.pyridine) [14,15] were not successful. The HPLC data (Figure 1) clearly indicates the presence of the product **7p** (Rt = 8.8 min) and the by-product Fmoc-β-Ala-OH (Rt = 7.6 min) in the reaction mixtures when linkers **1** and **2** were employed in a two-step reductive amination reaction.

For the one-step process [16], the more effective general procedure for loading primary amines was determined to be 1M amine, 0.05M $NaBH_3CN$, pH ~6.0 with acetic acid, in DCM:DMF (1:1) at 60 °C for 4 h. With R_2-NH_2 = benzylamine, R_3-COOH = Fmoc-β-Ala-OH (Table 2), the one-step reaction gave the product **(7f)** in > 95% purity on both linkers. Even with the more sterically hindered amines, e.g., R_2-NH_2 = 2,4-dimethoxyaniline, R_3-COOH = Fmoc-β-Ala-OH (Table 2) the product (**7p)** was obtained essentially as a single peak (Figure 1). The optimal condition for the one-step process was successfully applied to a broad range of primary amines using both linkers **1** and **2**. In all cases, the reactions cleanly gave the desired product with minimal formation of the R_3-COOH by-product (Table 2).

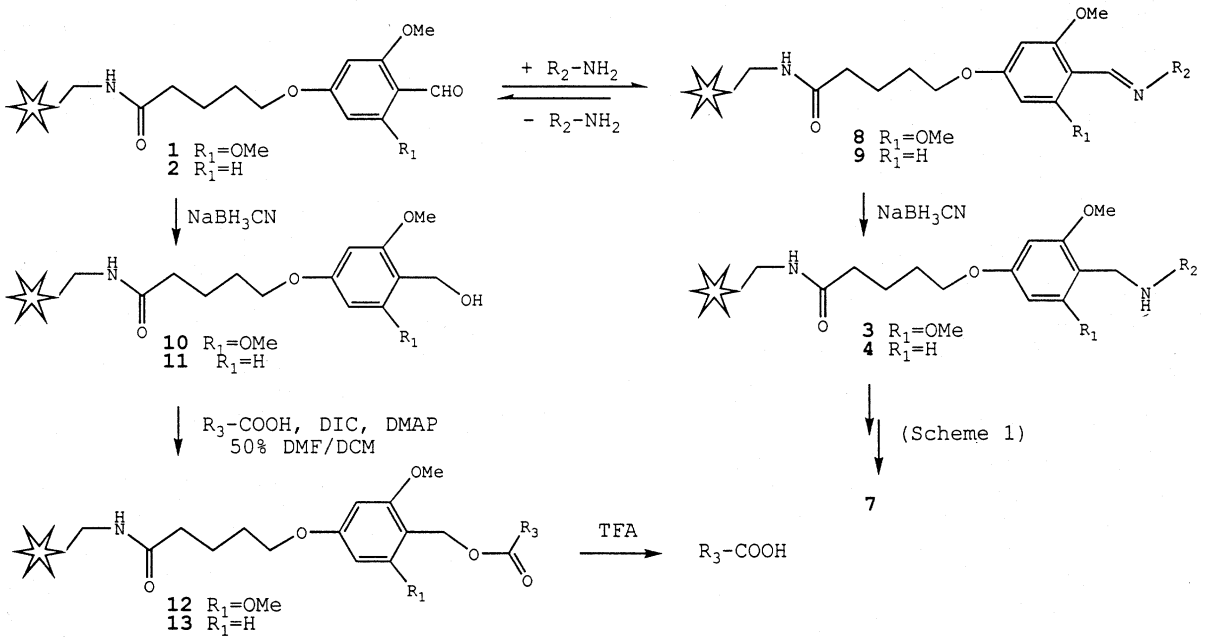
To confirm that HPLC data was consistent with the expected product, all target products were independently synthesized by solution phase methods to confirm the identity of the results from solid phase synthesis. Compounds obtained from the two procedures were identical in melting point, HPLC and MS spectral data. HPLC [17] and electron-spray MS [18] and other data is presented in Table 3. A series of standard solutions of the various products made by solution phase methods was prepared for HPLC analysis and the peak areas at 401 nm against quantity (0 to 10 nmol) were established for the standard curve.

Cleavage in trifluoroacetic acid

Efficiency of cleavage from linkers **1** and **2** was based on the release of the chromophoric compound **7q** (Table 2) from the corresponding solid support. Briefly, SynPhase crowns derivatised with linkers **1** and **2**, and reacted with benzylamine by the one-step process were acylated with Dnp-β-Ala-OH (compounds **5q** and **6q**, Scheme 1). These crowns were incubated with 5 different concentrations of TFA in DCM (5%, 10%, 20%, 50% and 95%). For each experiment, 40 crowns were used and 2 crowns were removed at different times over a period of 2 h to assess the relative efficiency of cleavage with time. The cleavage solutions containing chromophoric compound **7q** were directly quantitated by measuring the absorbance at 405 nm [19] and the results are described in Figure 2.

Discussion

Comparative assessment of linkers **1** and **2** was initiated to find the most suitable linker for *N*-alkylamides



Scheme 2. Two-step reductive amination reaction on linkers **1** and **2**.

Table 1. Synthesis of **7** via two-step reductive amination on linkers **1** and **2**

Product **7**	Linker **1**, % Target (% by-product)	Linker **2**, % Target (% by-product)
7f: R_2-NH_2 = benzylamine; R_3-COOH = Fmoc-β-alanine	90 (10)	95 (5)
7p: R_2-NH_2 = 2,4-dimethoxyaniline; R_3-COOH = Fmoc-β-alanine	59 (41)	77 (23)
7r: R_2-NH_2 = 4-nitroaniline; R_3-COCl = acetylchloride	NR	NR

% Target was based on the HPLC peak area of the product at 214 nm. NR: No reaction.

in library synthesis. The primary goal was achieving efficient reactivity with a broad range of amines including a number of poor nucleophiles such as aniline and nitroaniline. The basic conclusion of this study indicates that the procedure used for reductive amination was the primary factor in synthesis success. Studies on the two-step reductive amination method with 2,4-dimethoxy aniline, gave the target product in 59% and 77% purity for linkers **1** and **2**, respectively. A difference of 18% is indicative of linker **2** being more accessible to bulky amines; however, there were large variations in product purities by the two-step approach and certain amines such as 4-nitroaniline failed to react at all. Equilibrium considerations (Scheme 2, **1** to **8** and **2** to **9**) became an important factor with concentration, steric effects or nucleophilicity of the incoming amines, and competing aldehyde reduction giving poor percentages of target product (Scheme 2) [20]. It is expected that this problem is more severe with linker **1** as the steric effect of the extra methoxy group would slow down initial nucleophilic attack on the carbonyl carbon and drive the equilibrium in the wrong direction.

The one-step reductive amination procedure favoured the formation of amine bound solid supports **3** and **4** and the competing aldehyde reduction was significantly reduced. This was achieved with a lower concentration of amine (1 M versus 2 M) and the reaction worked well with hindered amines like 2,4-dimethoxyaniline, and poor nucleophiles, like aniline and 4-nitroaniline. Except for **7m**, the products were obtained as single peaks by HPLC with small amounts of by-products (R_3-COOH, $< 5\%$), implying that the key step reductive amination had gone to completion. The one-step process effectively suppressed any steric effects due to the extra methoxy

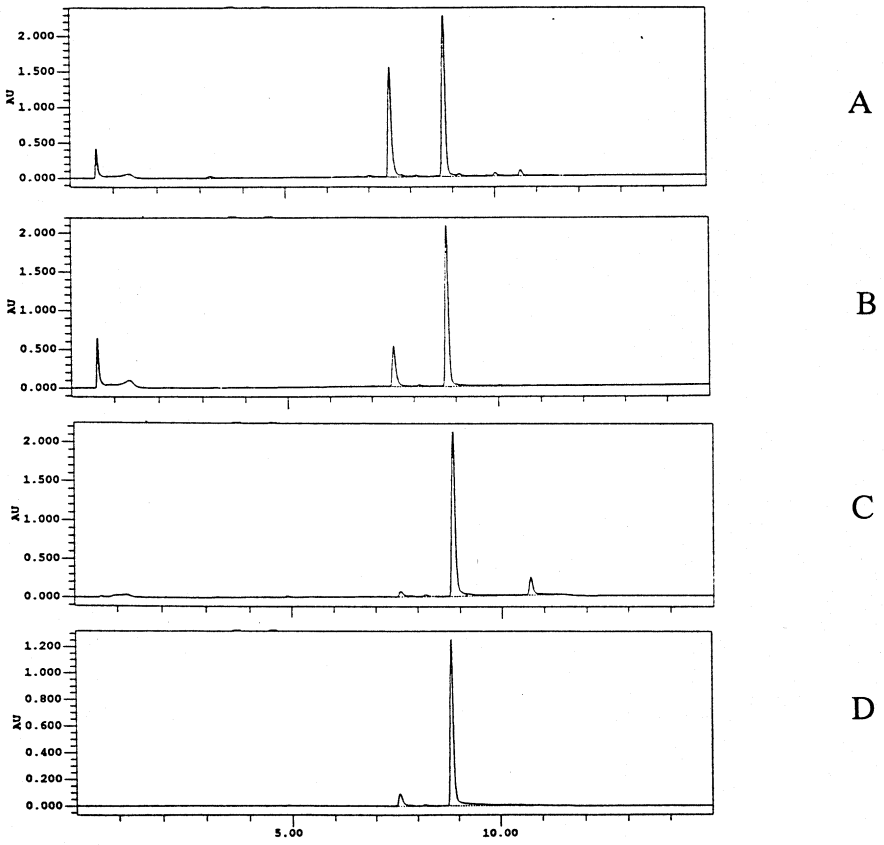


Figure 1. RP-HPLC of products from solid phase synthesis of compound **7p**. From top to bottom: (A) Linker **1**, two-step process, (B) Linker **2**, two-step process, (C) Linker **1**, one-step process and (D) Linker **2**, one-step process. Product **7p** (Rt = 8.8 min), by-product (Fmoc-β-Ala-OH, Rt = 7.6 min).

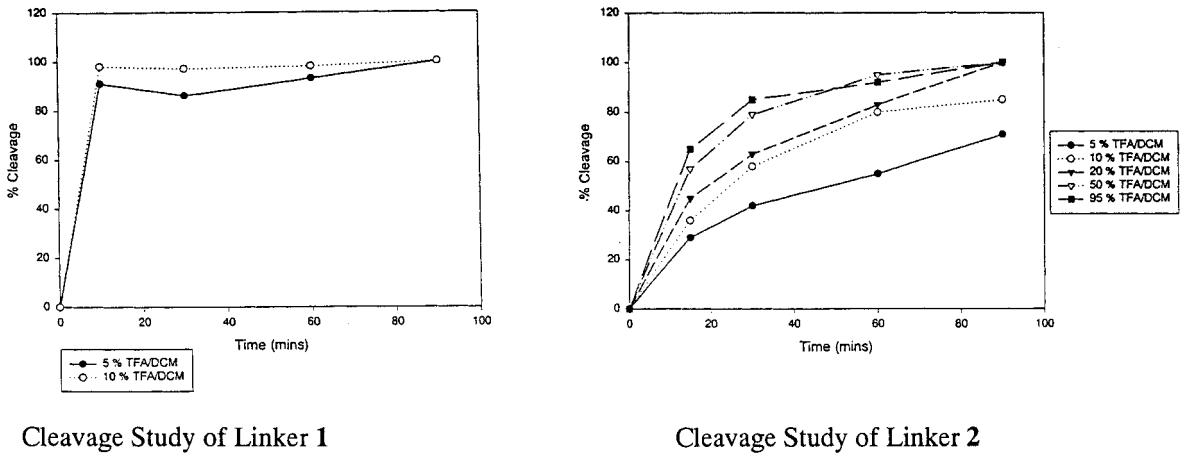


Figure 2. Comparative cleavage study on two linkers **1** and **2**. {% Cleavage = initial O.D./ (initial O.D. + final O.D.)}.

Table 2. Synthesis of **7** (**a**–**r**) by one-step reductive amination on linker bound solid supports **1** and **2**

Entry	R_2-NH_2	R_3-COOH	Linker **1**, % Yield	Linker **2**, % Yield	Linker **1**, % Target[a]	Linker **2**, % Target[a]
7a	Ethylamine	Fmoc-β-Ala-OH	29	33	78	98
7b	Butylamine	Fmoc-β-Ala-OH	42	46	86	77
7c	*iso*-Propylamine	Fmoc-Phe-OH	80	80	89	94
7d	Cyclopropylamine	Fmoc-Phe-OH	45	36	84	95
7e	Cyclopentylamine	Fmoc-Phe-OH	21	20	82	80
7f	Benzylamine	Fmoc-β-Ala-OH	70	75	99	98
7g	4-Methoxybenzylamine	Fmoc-β-Ala-OH	34	35	91	95
7h	4-Nitrobenzylamine	Fmoc-β-Ala-OH	30	21	83	94
7i	4-Amino-biphenyl	Fmoc-β-Ala-OH	54	34	80	82
7k	2,2-Diphenylethylamine	Fmoc-β-Ala-OH	57	40	80	80
7l	2-Methoxyphenethylamine	Fmoc-β-Ala-OH	64	67	91	90
7m	5-Amino-2-methoxypyridine	Fmoc-β-Ala-OH	76	11	97	50
7n	Aniline	Fmoc-β-Ala-OH	60	52	94	85
7o	4-phenoxyaniline	Fmoc-β-Ala-OH	66	56	91	83
7p	2,4-dimethoxyaniline	Fmoc-β-Ala-OH	46	52	88	98
7q	Benzylamine	Dnp-β-Ala-OH	63	38	94	72
7r	4-Nitroaniline	Acetyl chloride	nd	nd	100	96

[a] % Target is based on the target peak area in an analytical HPLC trace monitored at 214 nm. In all cases, less than 5% by-product (R_3-COOH) was observed. Nd: not determined.

Table 3. Characterization data for compounds **7a** to **7r**

Entry	HPLC,[17] Rt (min)	Melting point (°C)	ES-MS Calculated $[M+H]^+$	ES-MS[18] Observed $[M+H]^+$
7a	9.38	160–162	339.2	339.3
7b	10.21	139–140	367.2	367.4
7c	9.33	182	429.2	429.2
7d	8.98	186–187	427.2	427.3
7e	9.76	166	455.2	455.3
7f	10.91	178	401.2	401.4
7g	8.45	183–185	431.2	431.1
7h	8.54	181–183	446.2	446.1
7i	9.98	162	463.2	463.2
7k	8.82	154–155	491.2	491.2
7l	8.97	129	445.2	445.2
7m	9.86	193–195	418.2	418.2
7n	10.58	180–181	387.2	387.4
7o	11.63	187–188	479.2	479.2
7p	8.80	150	447.3	447.3
7q	7.46	175–178	345.2	345.2
7r	5.38	216	181.0	180.9

group in linker **1** as there is no observable advantage in product yield or purity of linker **2** over linker **1** (Table 3). Analysis of the 16 amines shown in Table 1 shows that both linkers gave comparable purities (<5% difference) with 8 amines. Of the remaining 9 amines, approximately half gave better results on one linker compared to the other. There were no observable trends with both linkers giving high purities with nitroaniline and high but different purities with aniline and 2,4-dimethoxyaniline. However, in the case of 4-nitroaniline, the standard acylation reaction failed and only coupling with acetyl chloride was attempted. With the exception of compound **7m**, yields of the products obtained from both linkers were very similar and ranged from 30 to 80% in overall yield. For **7m** (from 5-amino-2-methoxypyridine), linker **1** gave 97% purity and 76% yield while linker **2** gave 50% purity and a poor yield of only 11%. With the latter, there was large by-product formation (Fmoc-β-Ala-OH) and attempts to improve purity and yield by using more forcing conditions (100 times excess of amine) failed. The reason for these differences is as yet unclear.

In our model study with **7q**, linker **2** was sufficiently acid-sensitive that the extra methoxy group was not necessary for cleavage. However, a higher concentration of TFA (at least 20%) and an increased time (minimum of 2 h incubation) were required for maximum cleavage. In contrast, linker **2** achieved quantitative cleavage in 5% TFA/DCM within 10 min. Our general cleavage procedure used 50% TFA/DCM for 2 h so any differences in yield in our studies are unlikely to be due to poor cleavage.

Conclusions

Both linkers **1** and **2** are useful tools for the preparation of a wide range of *N*-alkyl or aryl amides. As previously mentioned, linker **1** is less accessible than **2**, with the former giving poorer purities in the two-step process. This was not the case with the one-step process which has been found to be suitable for the attachment of a wide range of primary amines onto both linkers. The cleavage study showed that linker **1** is highly susceptible to TFA (5%) while linker **2** requires at least 20% TFA/DCM for completion of cleavage within 2 h. Assuming that appropriate synthesis and cleavage conditions are used, both linker systems provide target products in good yield and purity.

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13. The optimization (two-step reductive amination reaction) has been studied with three variables: solvents (DMF, DCM, THF), temperature (25 °C and 60 °C), duration (2 h and 16 h), and the optimal condition was described in the Experimental section.
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17. Reverse phase high performance liquid chromatography (RP-HPLC) was conducted with Rainin, Microsorb-MV Cat.# 86-200-F3, 50×4.6 mm column using gradient mobile-phase 0–100% B over 11.5 min. Flow rate: 1.5 ml/min (solvent A: 0.1% ortho-phosphoric acid in water; solvent B: 0.1% ortho-phosphoric acid in 90% acetonitrile). Detection: 214 nm.
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