# Facile Synthesis of Protected *C*-terminal Peptide Segments by Fmoc/Bu$^t$ Solid-phase Procedures on *N*-Fmoc-9-amino-xanthen-3-yloxymethyl Polystyrene Resin

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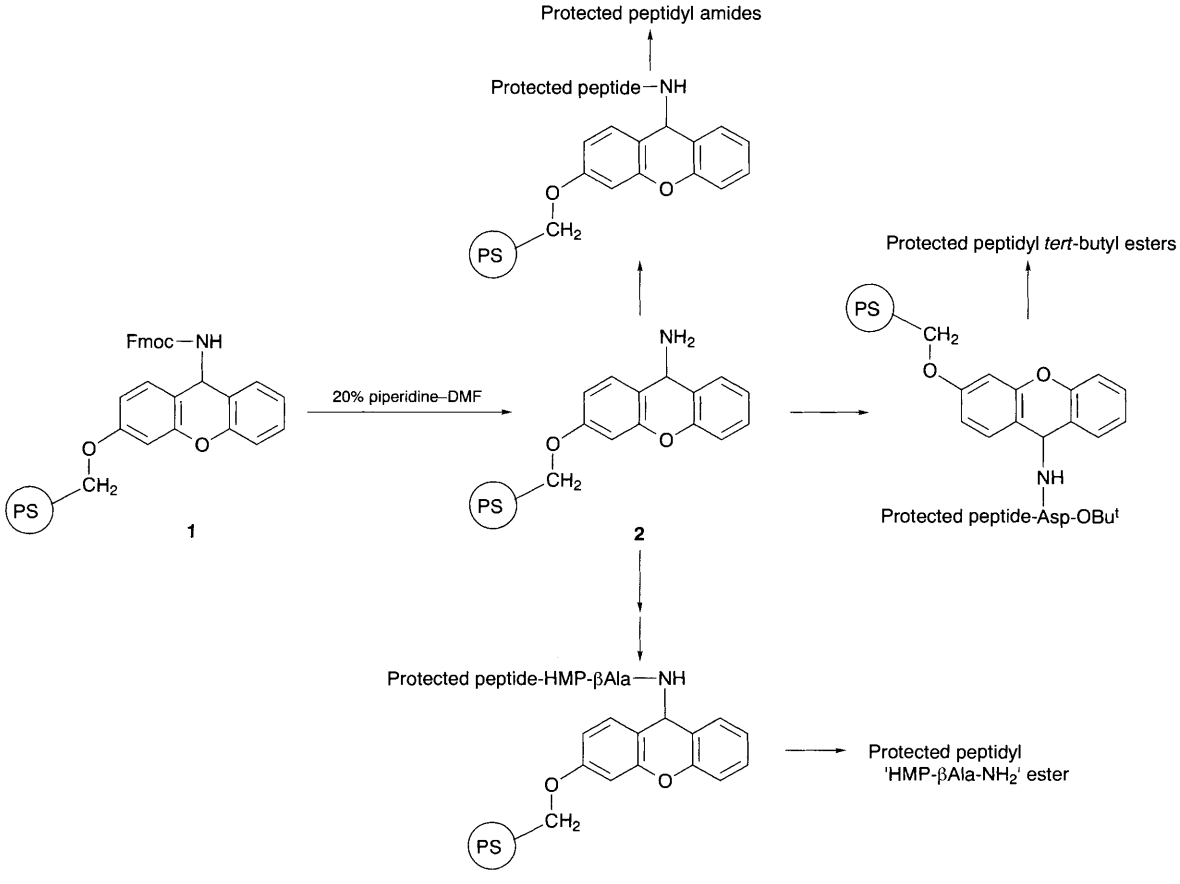
Side-chain protected peptidyl amides, peptidyl *tert*-butyl esters, and the novel peptidyl 4-[*N*-(oxyacetyl)-β-alaninamide]-benzyl ester (HMP-β-Ala-$NH_2$ ester) have been synthesised in excellent yields on *N*-Fmoc-9-amino-xanthen-3-yloxymethyl polystyrene resin and selectively cleaved from the solid support using 1% trifluoroacetic acid in dichloromethane within 7–8 min.

Total synthesis of peptides by convergent methods is increasingly being used as an alternative to the established step-wise solid-phase procedures.[1] This methodology overcomes some of the sequence-dependent difficulties, such as peptide aggregation, encountered during step-wise assembly. Convergent peptide synthesis is achieved by condensation of the appropriately protected *N*-terminal segment with the *C*-terminal segment which is either in solution or bound to a solid support *via* a linker moiety.

Currently, the method of choice for synthetic assembly of peptides is that based on efficient Fmoc/Bu$^t$ procedures.[2] Thus, the synthesis of protected peptide segments required for convergent synthesis is best achieved utilising this methodology. For the synthesis of the protected $N^{\alpha}$-Fmoc-*N*-terminal segments, a number of highly acid-labile linker/anchors, *e.g.* 4-carboxy-trityl[3] and 2-chlorotrityl-polystyrene resin (-PS)[4] are available which allow selective cleavage of the segments from the solid support using 30–40% *v/v* acetic acid in dichloromethane.

In contrast, similar linker or anchors for the synthesis of *C*-terminal protected segments (with the carboxyl-terminus protected) by Fmoc/Bu$^t$ methods have not been reported. To date, *C*-terminal segments are either utilised whilst attached to a solid support (for convergent solid-phase approach) or obtained by laborious post-synthetic modifications of protected $N^{\alpha}$-Fmoc peptide acids to peptidyl *tert*-butyl esters *via* carboxyl *O*-alkylation with *tert*-butyl 2,2,2-trichloroacetamidate followed by *N*-Fmoc deprotection.[1] We now report an efficient route to *C*-terminal protected segments (Scheme 1) using *N*-Fmoc-9-amino-xanthen-3-yloxymethyl-PS **1**[5]† and Fmoc/Bu$^t$ chemistry. The assembled peptides have been selectively cleaved from the anchor-resin with 1% *v/v* TFA in $CH_2Cl_2$ to afford the protected segments in excellent yields and purities. This methodology has been successfully applied for the synthesis of




**Scheme 1** Fmoc/Bu$^t$ strategies for the synthesis of protected *C*-terminal peptide segments using *N*-Fmoc-9-amino-xanthen-3-yloxymethyl polystyrene resin

a novel protected peptidyl benzyl ester which has general application for the convergent solution-synthesis of pharmaceutical peptides.

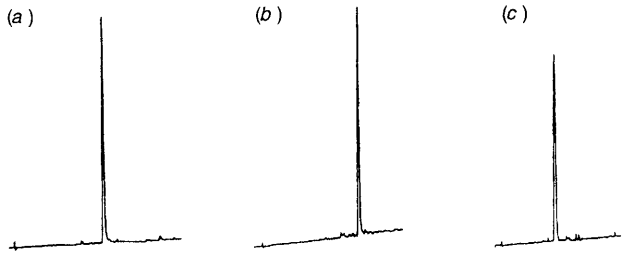
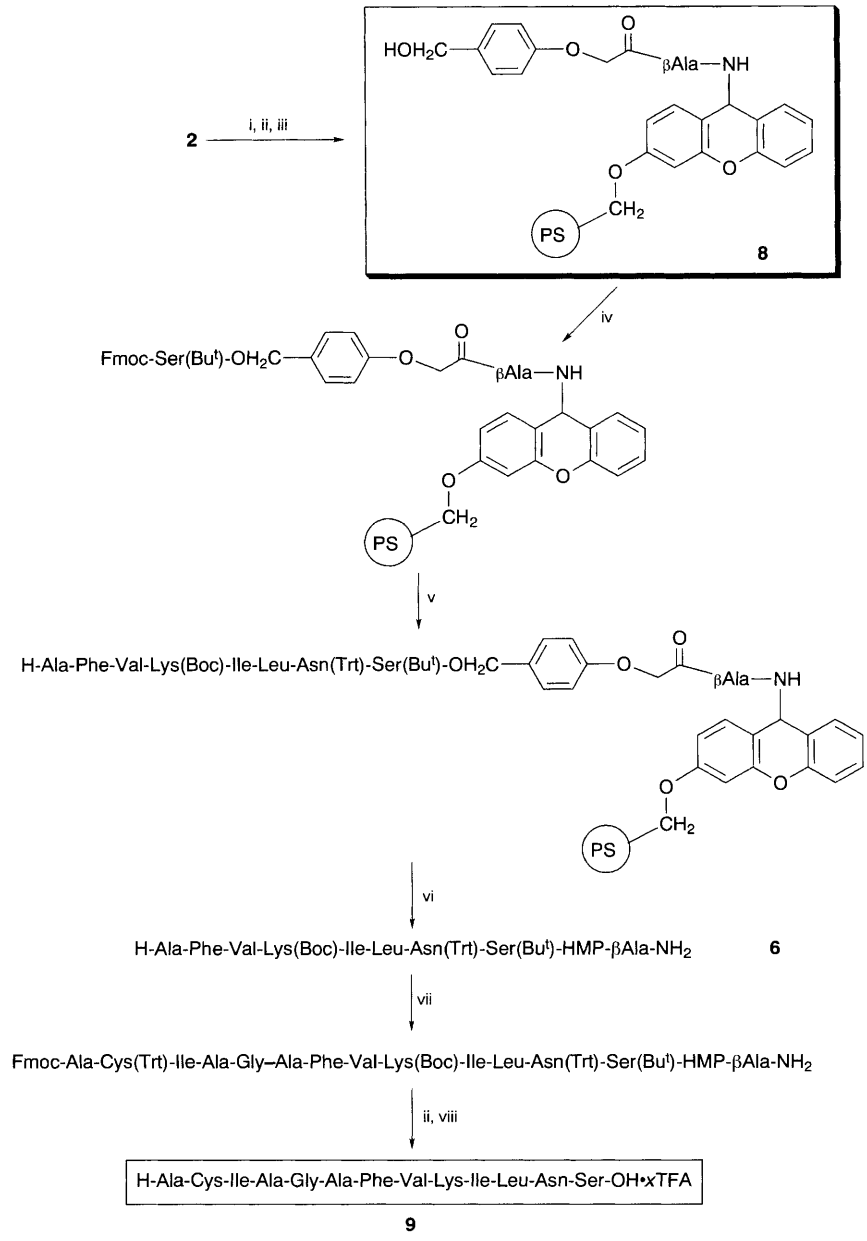



**Fig. 1** RP-HPLC profile of crude synthetic protected peptide segments (*a*) **3**, (*b*) **4** and (*c*) **5** analysed on Hypersil Pep $C_{18}$ column.§

Using previously reported procedures,[5] the Fmoc-anchor resin **1**† was obtained *via* an optimised reduction of the intermediate 9-keto-xanthen-3-yloxymethyl-PS to 9-hydroxy-xanthen-3-yloxymethyl-PS with $LiBH_4$ in refluxing THF followed by coupling with Fmoc-$NH_2$ by acidic catalysis. Fmoc-deprotection yielded 9-amino-xanthen-3-yloxymethyl-PS **2**. The *C*-terminal sequences **3** and **4** of porcine pancreastatin[6] and salmon calcitonin,[7] respectively, were readily assembled by step-wise Fmoc/Bu$^t$ solid-phase procedures (Scheme 1).[2]‡ In both cases, the peptidyl-**2** resins were obtained in *ca.* 98% yields, thus establishing the stability of the peptidyl anchor linkage to standard Fmoc/Bu$^t$ synthetic conditions.

We next investigated procedures for selective cleavage from the resin by very mild acidolysis to afford protected peptide amides. Sieber has previously shown that peptide amides are obtained in good yields by treating peptide-**2** with 2% TFA in 1,2-dichloroethane for extended periods;[5a] under such conditions, significant side-chain deprotection (*e.g.* of Trt, Bu$^t$) is




**Scheme 2** Synthetic procedure for the construction of the chimeric peptide **9** by application of solid-phase and convergent solution-phase peptide synthesis methodologies.

*Reagents and conditions*: i, Fmoc-β-Ala-OH-HBTU-HOBt-DIPEA (1 : 1 : 1 : 2); ii, 20% piperidine in DMF; iii, HMP-OH-DIPCDI-HOBt (1 : 1 : 1); iv, Fmoc-Ser(Bu$^t$)-OH-DIPCDI (2 : 1), DMAP (0.1 equiv.); v, standard Fmoc/Bu$^t$ solid-phase procedures‡; vi, 1% TFA in $CH_2Cl_2$, 7–8 min; vii, Fmoc-Ala-Cys(Trt)-Ile-Ala-Gly-OH **7**-HATU-DIPEA (1 : 1 : 2), 18 h, room temp.; viii, TFA–$Pr^i_3SiH$–$H_2O$ (95 : 2.5 : 2.5), 2 h, room temp.

**Table 1** Peptides synthesised utilising solid-phase methodologies

| | |
|---|---|
| H-Ala-Pro-Gln(Trt)-Gly-Leu-Phe-Arg(Pmc)-Gly-$NH_2$[6] | **3** |
| H-Arg(Pmc)-Thr(Bu$^t$)-Asn(Trt)-Thr(Bu$^t$)-Gly-Ser(Bu$^t$)-Gly-Thr(Bu$^t$)-Pro-$NH_2$[7] | **4** |
| H-Ile-Pro-Lys(Boc)-Glu(OBu$^t$)-Tyr(Bu$^t$)-Leu-Asn-OBu$^t$ | **5** |
| H-Ala-Phe-Val-Lys(Boc)-Ile-Leu-Asn(Trt)-Ser(Bu$^t$)-HMP-β-Ala-$NH_2$[9] | **6** |
| Fmoc-Ala-Cys(Trt)-Ile-Ala-Gly-OH | **7** |
| H-Ala-Cys-Ile-Ala-Gly-Ala-Phe-Val-Lys-Ile-Leu-Asn-Ser-OH | **9** |

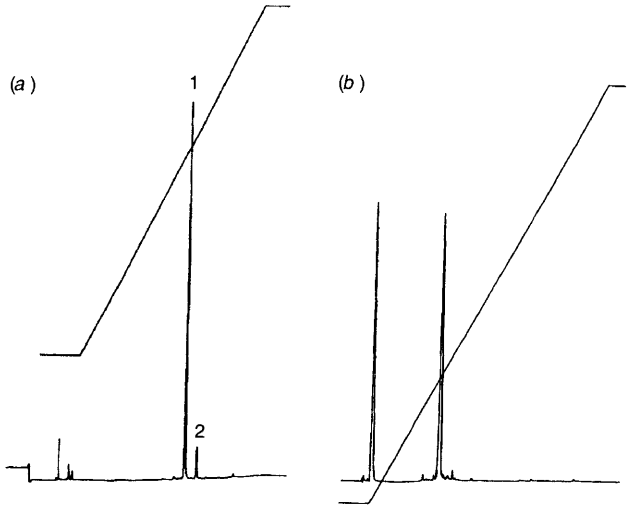



**Fig. 2** RP-HPLC profile of crude synthetic protected peptide segment (*a*) 1 = **6** and 2 = H-Ala-Phe-Val-Lys(Boc)-Ile-Leu-Asn(Trt)-Ser(Bu$^t$)-HMP-HMP-β-Ala-$NH_2$ (30–100% B in 35 min), and the corresponding fully deprotected peptide (*b*) H-Ala-Phe-Val-Lys-Ile-Leu-Asn-Ser-OH **6a** [5–100% B in 50 min] analysed on TSK-120 ODS column.§

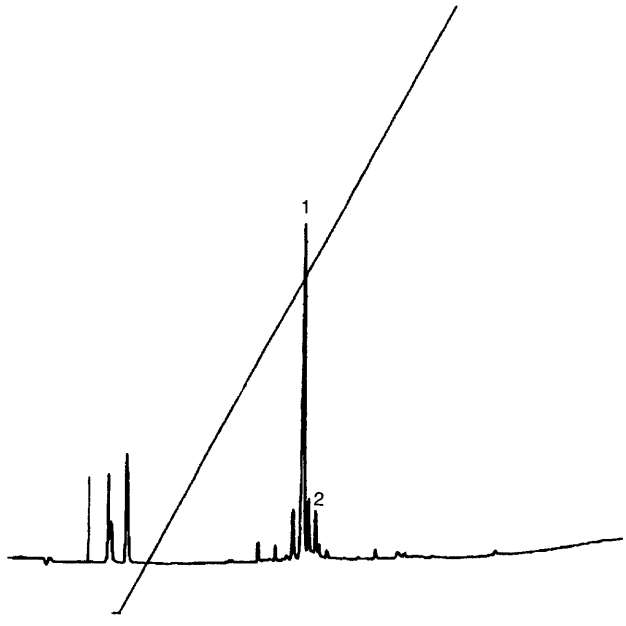



**Fig. 3** Analytic profile on TSK-120 ODS column§ [5–100% B in 50 min] of crude synthetic chimeric peptide **9** prepared by convergent method outlined in Scheme 2; 1 = **9** and 2 = Disulphide-bridged dimer of **9**

expected. We have now found that the peptidyl amino xanthene bond can be selectively cleaved by continuous-flow treatment with 1% TFA in $CH_2Cl_2$ within 7–8 min to yield the desired protected peptides, as well as the resin-bound mesomeric stabilised xanthen-3-yloxymethyl carbocation. Using this procedure our model protected peptidyl amides **3** and **4** were obtained in good yields and purities§ in excess of 95% when analysed by reversed-phase (RP)-HPLC (Fig. 1).

In addition, the protected peptide *tert*-butyl ester **5** was synthesised using resin **1**. This was achieved by first acylation of **2** with β-carboxyl activated Fmoc-Asp-OBu$^t$, and then followed by standard solid-phase synthetic procedures. Selective cleavage of the assembled peptide-resin with 1% TFA in $CH_2Cl_2$ afforded **5** in *ca.* 90% yield.§ This strategy for producing protected *C*-terminal segments is applicable in cases where the residue Asn or Gln occurs at the *C*-terminus of the final target peptide.

The versatility of the Fmoc-anchor resin **1** is further illustrated by the efficient synthesis of side-chain protected peptides blocked at the *C*-terminus carboxyl by the novel 4-alkoxy benzyl ester protecting group, 4-[*N*-(oxyacetyl)-β-alaninamide]-benzyl ester (HMP-β-Ala-$NH_2$). The chemical stability of the peptidyl-HMP ester bond is comparable to those of the amino acid side-chain protecting groups, and is therefore stable to 1% TFA but is readily cleaved with 90% TFA.[8] To validate the approach, a protected *C*-terminal domain of the antibacterial peptide magainin[9] **6** was synthesised (Scheme 2). In this case, solid-phase assembly was first achieved by acylation of **2** with activated Fmoc-β-Ala-OH, deprotected, and acylated with activated 4-hydroxymethyl-phenoxyacetic acid (HMP-OH) for 1–2 h¶ to yield the key component **8**. This was followed by esterification of the resin-bound benzyl alcohol moiety with Fmoc-Ser anhydride, and peptide synthesis was then continued using standard procedures‡. Selective cleavage of the assembled peptide with 1% TFA afforded **6** as a white solid. A sample of **6** was readily fully deprotected using the acidolysis cocktail TFA–triisopropylsilane–$H_2O$ (95 : 2.5 : 2.5% *v*/*v*) to give the peptide product **6a** with the RP-HPLC elution profile shown in Fig. 2. This unique strategy for synthesis of protected *C*-terminal peptide segments with built-in *C*-terminus carboxyl protecting group has general application for the total chemical construction of pharmaceutical peptide acids by convergent solution-phase methodologies.

As an example, the utility of 'HMP-βAla-$NH_2$' carboxy protection is demonstrated by the convergent synthesis of the chimeric peptide **9**, prepared firstly, in solution by the condensation of **6** and **7** using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)[10]–DIPEA (1 : 2 molar equiv.) carboxyl activation in DMF. Following *N*-Fmoc deprotection and acidolysis with the TFA cocktail, the target peptide **9** was obtained in excess of 80% purity (see Fig. 3).§

In conclusion, we have outlined several facile routes for the Fmoc/Bu$^t$ solid-phase synthesis of protected *C*-terminal segments using the anchor-resin **1**. These segments, *C*-terminus carboxyl protected as amides, *tert*-butyl esters or 'HMP-β-Ala-$NH_2$' esters have been obtained in excellent purities and hence exploitable for convergent large-scale (solution-phase) manufacture of biologically and pharmaceutically important peptides and peptide amides.

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## Footnotes

† The anchor-PS **1** was found to have a final loading (based on Fmoc) of 0.38 mmol $g^{-1}$.

‡ Solid-phase assembly of synthetic peptide segments were accomplished by Fmoc continuous-flow procedures using a Millipore PepSynthesizer 9050. Carboxyl activation was achieved by the mixture *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)-HOBᵗ-*N*,*N*-diisopropyl-ethylamine (DIPEA) (1 : 1 : 2 molar ratios), and $N^{\alpha}$-Fmoc deprotection with 20% *v*/*v* piperidine in DMF.

The dried peptidyl resin (0.1 mmol) was placed in a glass column, allowed to swell in $CH_2Cl_2$ for 20 min, and then cleaved by eluting with 1% *v*/*v* TFA in $CH_2Cl_2$ (flow rate 6 ml $min^{-1}$, 7–8 min elution) into a flask containing 5% *v*/*v* pyridine in methanol (15 ml). The mixture was evaporated *in vacuo* to dryness and following trituration with ice-cold water afforded the desired protected *C*-terminal peptidyl amides, *tert*-butyl or HMP-β-Ala-$NH_2$ esters as white solids in typically 65–90% yields.

Acid-labile side-chain protecting groups used in Fmoc/Buᵗ peptide synthesis have previously been shown to be stable to the cleavage reagent 1% *v*/*v* TFA in $CH_2Cl_2$.[1,3,4]

The protected *N*-terminal peptide **7** was prepared by solid-phase peptide assembly on 2-chlorotrityl-PS resin followed by selective cleavage from the resin using 0.5% TFA in $CH_2Cl_2$.

§ Crude peptides **3–5** were analysed by RP-HPLC on Hypersil Pep $C_{18}$ column (4.6 × 150 mm). The elution gradient was 40–100% B in 30 min at 1.20 ml $min^{-1}$ (A = 0.06% aq. TFA, B = 0.06% TFA in 90% aq. MeCN) and the eluate was monitored at 220 nm. Peptides **6**, **6a** and **9** have been analysed on TSK-120 ODS column (4.6 × 250 mm).

All purified synthetic peptides gave expected plasma desorption (PD)-MS or laser desorption (LD)-MS data: **3** requires 1353.7, found 1352.4; **4** requires 1623.1, found 1626.3; **5** requires 1145.5, found 1148.1; **6** requires 1524.9, found 1525.8; **6a** requires 891.1, found 891.4; **9** requires 1306.6, found 1307.0.

¶ β-Alanine is used as a spacer residue between HMP and **2**-PS, since we observed that the peptidyl HMP-**2**-PS amino xanthene bond is comparatively less labile to 1% *v*/*v* TFA in $CH_2Cl_2$. The coupling reaction of HMP activated with 1,3-diisopropylcarbodiimide (DIPCDI)-HOBᵗ should be treated with caution; with extended reaction time (18 h) we have observed rather significant self-esterification to afford the oligomers HMP-HMP-β-Ala-**2**-PS and HMP-HMP-HMP-β-Ala-**2**-PS.

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