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Incorporation of 2-Hydroxy-4-methoxybenzyl Protection during Peptide Synthesis via Reductive Alkylation on the Solid Phase

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Abstract. *A method has been developed for the synthesis of Hmb protected amino acid residues via reductive alkylation of 2-hydroxy-4-methoxybenzaldehyde with resin bound amino acids/peptides. The methodology potentially allows incorporation of Hmb protected amino acids at any point in the synthesis of difficult peptides. Synthesis of ACP (65-74) illustrates the method.*

Protection of the main chain amide bond with the 2-hydroxy-4-methoxybenzyl (Hmb) group during solid phase peptide synthesis has been shown to greatly improve the synthesis of 'difficult sequences', presumably by suppression of secondary structure formation[1]. Synthesis of longer peptide sequences utilising standard peptide synthesis methodologies can now be attempted by virtually any peptide synthesis group. Incorporation of Hmb protected amino acids every fifth or sixth coupling during synthesis seems to be adequate in eliminating formation of secondary structures, and improved syntheses of β-amyloid (1-43)[2], acyl carrier protein (65-74)[3], the 3-repeat region of human tau-2[4], and HIV-1_{Bru} *tat*[1-72][5] have been achieved. In addition, the solid phase side reaction, aspartimide formation, is suppressed when Axx, in the sequence Asp-Axx, is protected by Hmb[6,7,8]. At present, reversible protection of the peptide amide bond is achieved through coupling of preformed N,O-bis-Fmoc 2-hydroxy-4-methoxy benzyl derivatives of amino acids. These are available commercially[9].

The solid phase reductive alkylation of amino acid aldehydes with support-bound peptides to yield Ψ-[CH_2NH]- pseudopeptides has been previously demonstrated[10]. Solution phase synthesis of Hmb analogues involves reductive alkylation of 2-hydroxy-4-methoxybenzaldehyde with the required amino acid followed by Fmoc protection. Like numerous others, our laboratory has become increasingly involved in solid phase organic chemistry[11,12,13], among which a relavent example is the reductive alkylation/amination of amines with

aldehydes and ketones[12,13]. In a useful extension to this work, synthesis of Hmb protected amino acids on the solid phase and in situ incorporation during peptide synthesis of ACP(65-74) peptide **(1)** forms the basis of this communication.

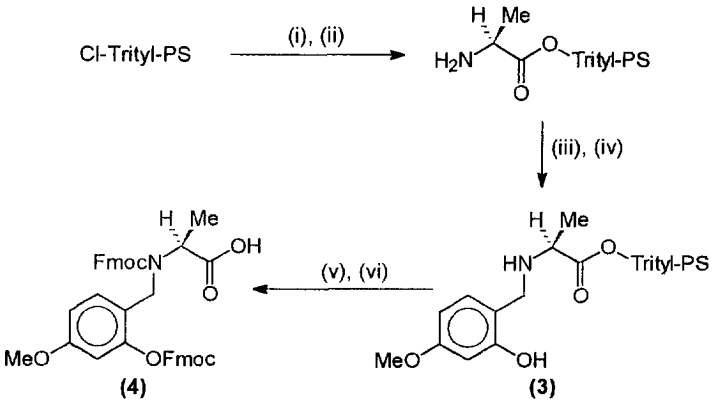
Val^{65}-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly^{74}

(1)

Ac-Val^{65}-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly^{74}
AcHmb

(2)

Scheme 1 outlines our strategy for the solid phase synthesis of N,O-bisFmoc-N-(Hmb)-L-alanine **(4)**. The advantage of solid phase over solution phase synthesis is that reactions can be driven to completion by using a large excess of reactants. An important consideration when selecting the linker was that the Hmb protecting group is TFA labile and thus very mild acid cleavage conditions were required for synthesis. The chlorotrityl [Cl-C(Ph)$_3$-PS (polystyrene)] handle serves this purpose (1% TFA/DCM cleavage (30 min.)).



(4) **(3)**

(i) Fmoc-Ala-OH, DIEA, DCM; (ii) 20% pip/DMF; (iii) Hmb, 1% AcOH/DMF
(iv)0.1M $NaCNBH_3$ 25% MeOH/DMF;(v) Fmoc-Cl, DCM; (vi)1%TFA/DCM

Scheme 1: Synthesis of Hmb analogue of Fmoc-Ala-OH

Thus Fmoc-Ala-OH was loaded onto high loading chlorotrityl polystyrene resin[14]. After deprotection of the Fmoc group (20% piperidine/DMF), reductive alkylation of 2-hydroxy-4-methoxybenzaldehyde (0.1M, solvent = 1% AcOH/DMF) with deprotected Ala was attempted in the presence of $NaBH_3CN$. Interestingly, **(3)** was not obtained even when the $NaBH_3CN$ was added 10 min. after the Hmb. Only the respective Schiff base (M+1, 224) was isolated in 100% yield. However, if the Schiff base was first washed with DMF to remove excess aldehyde, reduction with either 1M $NaBH_4$ or $NaBH_3CN$ in 25%MeOH/DMF, gave quantitative conversion to **(3)** (M+1, 226). Reaction of the HmbAla resin with 0.1M Fmoc-Cl in DCM for 1 hr yielded the

Bis Fmoc analogue **(4)** in >95% yield (yield calculated by HPLC integration). The cleaved and isolated product **(4)**, co-eluted on HPLC with authentic material from Novabiochem.

To demonstrate the usefulness of solid phase incorporation of the Hmb protecting group, we synthesised the ACP(65-74) peptide **(1)**[15]. Thus, ACP(65-74) peptide was synthesised up to Ala68 after which the resin was divided in order to perform five separate experiments concurrently (see Table 1). Control experiment **A**: synthesis of **(1)** was completed using standard HBTU coupling[15]; Experiment **B** and **C**: Hmb incorporated on the solid phase at Ala68 in situ[16] for 5 min. and 2 hrs respectively. In all Hmb examples, Ala67 was coupled as its symmetrical anhydride (0.1M, solvent = 25%DCM/DMF) and the final two residues coupled with HBTU as above. Coupling of Val65 is difficult due to secondary structure forming after deprotection of the penultimate Gln. This peptide is often synthesised by us as a control, and deletion of Val of up to 12 % has been observed. As presented in Table 1, the control ACP (65-74) yielded a deletion of 10 % Val (Exp. **A**) as expected. However, the deletion of Val was eliminated in experiments **B** and **C** indicating incorporation of Hmb had been successful. To quantitate the amount of in situ Hmb incorporation, acetylation of the Hmb peptides (experiments **D** and **E**) to yield **(2)** insured that the Hmb group was retained after cleavage[5]. The results show that reductive alkylation of 2-hydroxy-4-methoxybenzaldehyde with resin bound peptide may have been incomplete since a byproduct of acetylated **(1)** of 20% after 5 min., and 15% after 2 hrs, was evident by HPLC/MS. Nevertheless, the high purity of the final product **(1)** obtained in experiment **B** and **C** suggests that 100% incorporation of the Hmb analogue may not be necessary for disruption of secondary structure formation, and therefore improved synthesis conditions.

Table 1. Characterisation data for Hmb analogues of ACP(65-74)

EXPERIMENT	Reverse Phase HPLC Data*		Ion Spray Mass Spectral Data** MW (g/mol)			MAJOR BYPRODUCTS	
	Rt(min.)	% Target	% Target	Theoretical	Actual	% **	identity
A	6.72	74	77	1062.19	1062.29	10	_QAAIDYING
B	6.71	87	89	1062.19	1062.26		none identified
C	6.73	86	91	1062.19	1062.29		none identified
D	8.09	58	41	1282.42	1282.46	20	AcVQAAIDYING
E	8.08	73	37	1282.42	1282.45	15	AcVQAAIDYING

A. control synthesis of ACP(65-74); **B.** [Hmb] = 0.1M, solvent = 1%AcOH in DMF, time = 5min, [$NaBH_3CN$] = 0.1M, solvent = 25% MeOH/DMF, time = 1h; **C.** [Hmb] = 0.1M, solvent = 1%AcOH in DMF, time = 2hr, [$NaBH_3CN$] = 0.1M, solvent = 25% MeOH/DMF, time = 1h; **D.** acetylate **B** before cleavage to give **(2)**; **E.** acetylate **C** before cleavage to give **(2)**. *Analytical HPLC was performed on a Waters chromatography system using a Ranin microsorb-mv (#86-200-F3) RP-18 column (100A, 3 μm). The following conditions were used: buffer A = water (0.1% H_3PO_4); buffer B = 90% acetonitrile/10% water (0.1% H_3PO_4); linear gradient A to B from 1 to 11 min; flow rate = 1.5 mL min^{-1} . Absorbances were recorded at 214 and 254 nm. HPLC purities were determined by peak area at 214 nm. **MS analysis to identify products including impurities was performed on a Perkin Elmer Sciex API III ion spray mass spectrometer. The data were processed by software developed at Chiron Mimotopes Pty.Ltd[17].

In conclusion, synthesis of protected Hmb amino acid derivatives in high yield and purity with no solution phase chemical work-up is readily achievable, on the solid phase. Furthermore, incorporation of Hmb at any point during peptide synthesis via solid phase reductive alkylation has been shown to be a viable alternative to the use of pre-synthesised analogues.

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REFERENCES AND NOTES

Abbreviations: HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt: 1-hydroxybenzotriazole, DMF: dimethylformamide, DCM: dichloromethane, Fmoc: fluorenylmethoxycarbonyl, HPLC: high performance liquid chromatography, MS: mass spectrometry, NMM: N-methylmorpholine, TFA: trifluoroacetic acid, DIEA: diisopropylethylamine, AcOH: acetic acid, MeCN: acetonitrile.

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15. The control peptide is H-VQAA^{68}IDYING-NH_2. Peptides were synthesised on polyethylene glycol-polystyrene (PEG-PS) Rapp Polymere resin derivatised with Rink handle. A standard activation solution of Fmoc-Axx-OH/HBTU/HOBt/NMM (0.1M in DMF, 1.5equiv. NMM) was utilised. The Hmb protection was incorporated after coupling of Ala^{68}. Acetylation in experiments D and E results in acetylation of both the Hmb protecting group and the N-terminus.
16. The Fmoc deprotected resin was reacted with 0.1M 2-hydroxy-4-methoxybenzaldehyde (solvent = 0.1% acetic acid/DMF) for 5 min. or 2 hrs after which the resin was washed successively with DMF (3 x 10 mL). The resulting Schiff base was reduced with 0.1M $NaBH_3CN$ (25% MeOH/DMF) for 1 hr after which the resin was washed as before. The following residue (Ala) was coupled as its symmetrical anhydride (diisopropylcarbodiimde activation in 25% DCM/DMF) and then Gln and Val coupled using HBTU/HOBt/NMM activation. The peptide was cleaved with 95% TFA/H_2O and taken up in 75% MeCN/H_2O for HPLC and MS analysis.
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