Journal of Peptide Science
*J. Peptide Sci.* **5**: 403–409 (1999)

# The Synthesis of 'Difficult' Peptides Using 2-Hydroxy-4-Methoxybenzyl or Pseudoproline Amino Acid Building Blocks: a Comparative Study

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*Received 20 April 1999*
*Accepted 5 May 1999*

Abstract: A comparative study has been undertaken between Hmb-protected amino acid and pseudoproline building block analogues for use in the solid phase synthesis of 'difficult' peptides. Both of these derivatives act by blocking inter- and intramolecular hydrogen bonding, which has been shown to be a major cause of poor synthesis/quality/efficiency. While the two were shown to result in substantial improvements in the purity of crude peptides, pseudoproline incoporation was found to be superior to Hmb backbone protection. This was due to slow and incomplete coupling of the amino acid immediately following the Hmb amino acid.


Keywords: 'difficult' peptide sequences; Hmb protected amino acids; pseudoproline dipeptides

## INTRODUCTION

Since Merrifield's landmark development of solid phase peptide synthesis, many refinements have been introduced to minimize side reactions that occur during synthesis and cleavage of the peptide sequence. Improvements in side-chain protecting groups, polymeric support systems, linkers, activation methods and automation have resulted in an overall procedure that can, on the whole, be performed by laboratories from a variety of disciplines [1]. These improvements are largely concerned with the underlying chemistry of peptide synthesis. However, problems associated with incomplete couplings and deprotections resulting in inferior yields and homogeneity of peptide products still occur regularly. Inter- and intramolecular hydrogen bonding resulting in aggregation of peptide chains to form secondary structures, such as $\beta$-sheet formation [2], is now thought to be a major cause of synthesis problems. Various strategies have been attempted in the past to circumvent difficult synthesis, including solvent composition [3], elevated temperatures [4], the use of chaotropic salts [5] or solubilizing protecting groups [6]. However, these strategies have not addressed the underlying cause of poor synthesis, which is intermolecular aggregation of the growing peptide chain caused by hydrogen bonding. Interestingly, peptides which have proline residues spaced fortuitously along the peptide chain often result in successful synthesis presumably by disrupting secondary structure formation by preventing

Abbreviations: AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; DMF, dimethylformamide; EDT, 1,2-ethanedithiol; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HEMA, hydroxyethylmethacrylic acid; Hmb, 2-hydroxy-4-methoxy-benzyl; HOBT, *N*-hydroxybenzotriazole; MeCN, acetonitrile; NMM, *N*-methyl morpholine; O*t*Bu, *tert*-butoxy; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl; Trt, trityl.

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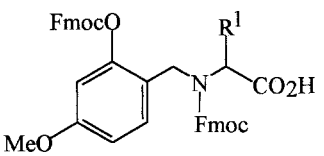
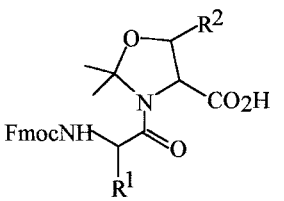
CCC 1075–2617/99/090403-07$17.50

interchain hydrogen bonding. This observation resulted in the development of peptide synthesis reagents that are designed to disrupt secondary structure formation during solid phase synthesis and has resulted in substantial improvements in the synthetic outcome of difficult peptides. These reagents include the 2-hydroxy-4-methoxybenzyl (Hmb) protected amino acids **1**, developed in Sheppard's laboratory [7,8], Bayer's Tmob backbone protecting group [9] and the pseudoproline protected dipeptide building blocks **2** developed in Mutter's laboratory [10–12]. All three backbone protecting group strategies act by preventing inter- and intramolecular hydrogen bonding, which are a source of solid-support bound peptide aggregation and subsequent failed synthesis. The pseudoprolines act further by causing the peptide chains to be 'kinked' during assembly. An obstacle when selecting sequences for pseudoproline substitution is the requirement for either a serine or threonine residue. While pseudoprolines can also be derived from cysteine, no dipeptide analogues are commercially available. The application of Hmb protection is much wider. However, the coupling of the following residue after Hmb incorporation, while being assisted by an O to N acyl shift, is difficult for hindered amino acids (particularly $\beta$-branched). The coupling of the following amino acid to a Tmob protected residue cannot be assisted by an O to N acyl shift mechanism, and must be incorporated as a Tmob protected dipeptide unit. This subsequently results in some racemization of the Tmob protected amino acid.




**1**




**2**

H-Met-Glu-Asp-Ser-Thr-Tyr-Tyr-Lys-Ala-Ser-Lys-Gly-Cys-$NH_2$

**3**

H-Met-Glu-Asp-Ser-Thr-PTyr-Tyr-Lys-Ala-Ser-Lys-Gly-Cys-$NH_2$

**6**

H-Pro-Lys-Tyr-Leu-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr-Gly-Met-Arg-Asn-Val-Pro-Glu-Lys-Gln-Thr-Thr-OH

**9**

**4,7,10**
Ala incorporated as Fmoc-(Fmoc-Hmb)Ala-OH (**1**, R1=Me)

**5, 8**
Ala-Ser incorporated as Fmoc-Ala-Ser($\psi^{Me,Me}$ pro)-OH (**2**, R1=Me, R2=H)

**11**
Ala-Thr incorporated as Fmoc-Ala-Thr($\psi^{Me,Me}$ pro)-OH (**2**, R1=Me, R2=Me)

We were interested in comparing the efficiency of each of these building blocks **1** and **2** during the synthesis of some particularly difficult sequences. Our initial efforts to synthesize the non-phosphorylated peptide **3**, the corresponding *O*-phosphotyrosine peptide **6** and a sequence **9** derived from the influenza virus hemagglutinin [13] (to $>95\%$ purity at 214 nm) yielded products of low purity. Mass spectral evidence showed that synthesis difficulty occurred at the *N*-terminal regions. Owing to the fact that these peptides had amino acid sequences that were suitable for accommodating Hmb or pseudoproline derivatives, they were deemed excellent models for evaluating their effectiveness in improving synthetic yields.

## MATERIALS AND METHODS

Peptides were synthesized on SynPhase™-HM-P Series crowns [(P-series, loading 25 μmol/crown), available from Chiron Technologies (Melbourne, Australia)] [14]. These supports had been radiation grafted with hydroxyethylmethacrylic acid (HEMA) and functionalized with the Fmoc-Rink amide (stock code SPHMPRAM) or hydroxymethylphenoxy acetic acid (stock code SPHMPHMP) linker. Fmoc protected amino acids were used throughout. Side-chain protection was afforded by: Trt for Cys, Boc for Lys, *t*Bu for Tyr, Thr, Ser, Asp and Glu and $PO_3$BzlH for phosphotyrosine. Fmoc protected Hmb-amino acids and pseudoprolines were supplied by NovaBiochem (Nottingham, UK).

Standard amino acid couplings, including Fmoc-(Fmoc-Hmb)Ala-OH (**1**, $R^1 = Me$), 'Fmoc-Ala-Ser($\psi^{Me,Me}$pro)-OH' (**2**, $R^1 = Me$, $R^2 = H$) and 'Fmoc-Ala-Thr($\psi^{Me,Me}$pro)-OH' (**2**, $R^1 = Me$, $R^2 = Me$) were carried out in distilled DMF at a concentration of 120 mM using HBTU/HOBT/NMM activation (1:1:1.5 equivalents), approximately threefold excess of reagents over loading. Phosphotyrosine couplings were carried out at a concentration of 120 mM using HBTU/HOBT/DIEA activation (1:1:2 equivalents). Fmoc-Lys(Boc)-OH or Fmoc-Leu-OH following Hmb-Ala incorporation was double coupled as its symmetric anhydride (in 20% DMF/DCM). Reagents were mixed prior to coupling and reaction carried out for 1.75 h or 16 h at 25°C in Beckman 2 ml deep well trays. A total volume of 1.3 ml was used per crown. Bromophenol Blue indicator (12 mM in DMF) was added to the reaction mixture (1:200 v/v) [15]. Fmoc deprotection was carried out between amino acid coupling cycles with 20% distilled piperidine in DMF for 20 min.

After synthesis, crowns were washed with DMF and methanol and air dried. Simultaneous side-chain deprotection and cleavage was carried out using 2.5 ml per crown of a solution of 82.5% TFA/5% thioanisole/5% anisole/5% water/2.5% EDT for 2 h. The TFA solution was reduced under vacuum and the cleaved peptide precipitated in 10 ml cold diethyl ether/petroleum ether (b.p. 40–60°C) (1:2 v/v), washed, and then air dried before sampling for analysis.

Analytical RP-HPLC was performed on a Waters chromatography system using a Rainin Microsorb-mv (# 86-200-F3) RP-18 column (100 A, 3 μm). The following conditions were used: Buffer A = water (0.1% $H_3PO_4$); Buffer B = 90% acetonitrile/water (0.1% $H_3PO_4$); linear gradient A to B from 1 to 11 min; flow rate 1.5 ml/min. Absorbances were recorded at 214 and 254 nm. HPLC purities were determined by peak area at 214 nm.

Mass spectral analysis was performed on a Perkin Elmer Sciex API III ion spray mass spectrometer. The data were processed by software developed at Chiron Technologies Pty., Ltd. [16].

## RESULTS

Synthesis of the peptides **3**, **6** and **9** was initially carried out on crowns under our standard laboratory conditions with resulting low crude purities (as measured by RP-HPLC at 214 nm) of 27, 25 and 24% obtained, respectively (Table 1, Figure 1A–C). Subsequent purification of these peptides to high purity ($>95\%$ at 214 nm) and with sufficient mass recovery proved difficult. Confirmation of the parent peak of **3**, **6** and **9** was obtained by LC-MS analysis. Deletions in the order of 3–5% for the *N*-terminal residues, as well as *N*-terminal truncated and Fmoc protected sequences, were evident in the ESMS analysis of peptides **3**, **6** and **9**.

Resynthesis of peptides **3**, **6** and **9** using commercially available Fmoc-(Fmoc-Hmb)Ala-OH to give **4**, **7** and **10**, the pseudoproline derivative Fmoc-Ala-Ser($\psi^{Me,Me}$pro)-OH to give **5** and **8** or Fmoc-Ala-Thr($\psi^{Me,Me}$pro)-OH to give **11** was then undertaken. The analytical data obtained with both analogues were significantly improved with respect to the original peptides. The *N*-terminal deletions and truncations were essentially eliminated in both the Hmb and pseudoproline peptides. However, an additional 5–10% impurity with an added *t*-butyl group

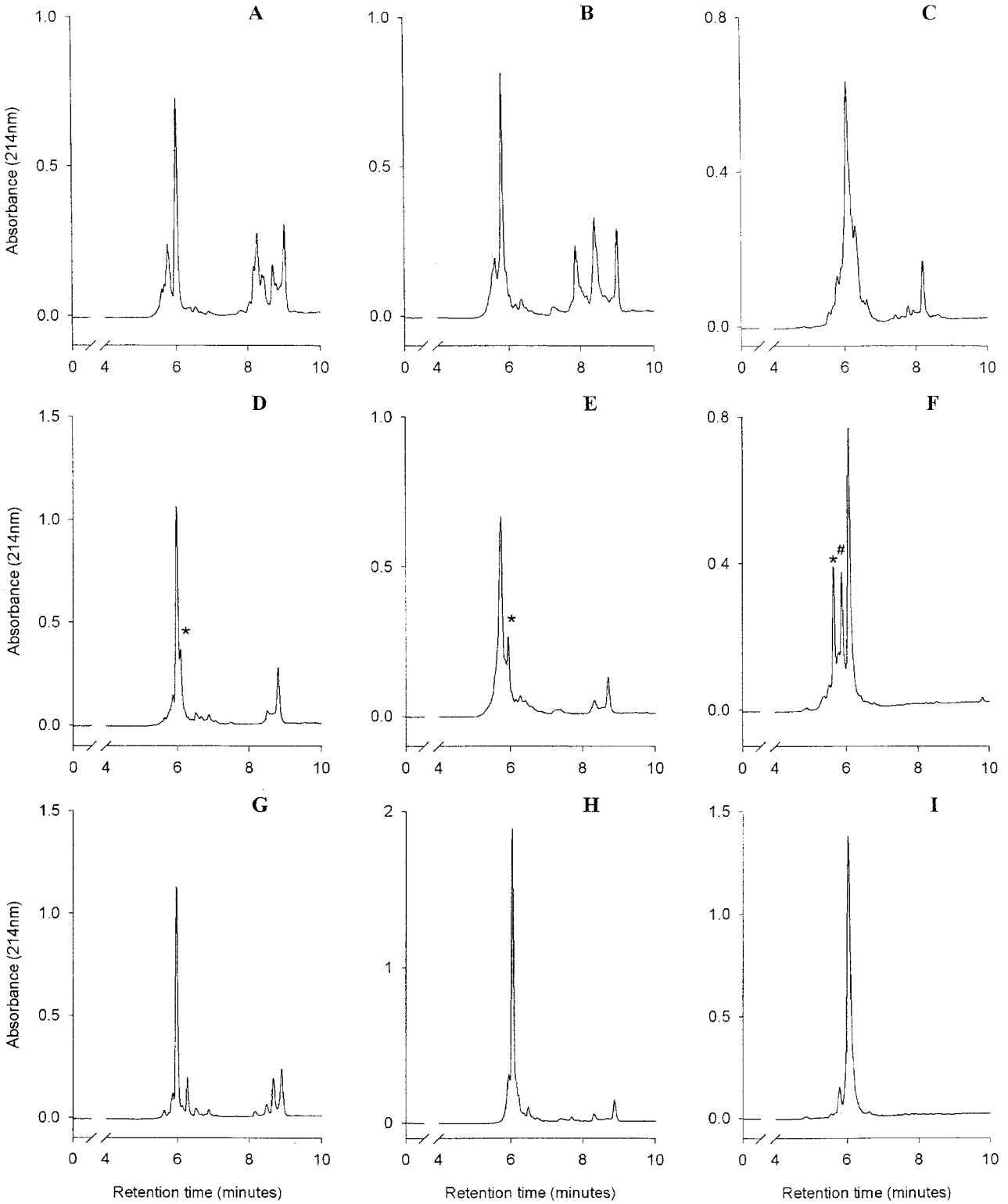



Figure 1 HPLC profiles of crude products after cleavage from the solid support. A: peptide **3**, B: peptide **6**, C: peptide **9**, D: peptide **4**, E: peptide **7**, F: peptide **10**, G: peptide **5**, H: peptide **8**, I: peptide **11**. * Indicates deletion of lysine; # indicates deletion of leucine.

(M + 56) was observed for peptides **4** and **7**. The pseudoproline produced slightly higher purities **5**, **8** and **11** at 45, 54 and 78%, respectively, while the Hmb **4**, **7** and **10** yielded purities of 39, 50 and 43% respectively (Table 1, Figure 1D–I). Subsequent purification yielded fractions of both high purity and mass from a lower crude starting mass.

## DISCUSSION

The beneficial aspects of incorporating Hmb protected amino acids and to a lesser extent pseudoproline analogues have been well documented. 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. 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 $\beta$-amyloid (1–43) [17], acyl carrier protein (65–74) [18], the 3-repeat region of human tau-2 [19] and a sequence derived from the HA chain of influenza [20] 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 [21–23]. In contrast, use of pseudoprolines for both improved synthesis and also as solubilizing building blocks has thus far been restricted to the laboratory of Professor Manfred Mutter. Elegant syntheses utilizing the pseudoproline building block for the synthesis of bis-amphiphilic secondary structure forming peptides, sarafotoxin-S6b and $\alpha$-helix transmembrane TASP molecules have been demonstrated [10–12].

In our studies here, analysis of the mass spectral data obtained from the initial purification fractions of peptides **3**, **6** and **9** indicated significant deletions within the *N*-terminal region of the peptide, as well as truncated Fmoc protected peptide sequences. This pattern of deletion is consistent with secondary structure formation by either inter- or intrachain hydrogen bonding during synthesis. The results of this comparative study have reconfirmed that the synthesis of 'difficult' peptide sequences can be improved by utilizing either Hmb or pseudoproline building blocks. It has been the view that improved synthesis with these building blocks is a result of drastically changed conformational and physicochemical properties of the peptide during synthesis, notably the disruption of secondary structures such as $\beta$-sheets. The comparable results presented here indicate that both types of building block are most likely working through the same mechanism. An additional benefit of these building blocks is their ability to improve mass recovery from HPLC purification. A correlation can be drawn between poor chemical synthesis resulting in highly structured, deleted and truncated sequences, and the subsequent poor dissolution of the crude peptide for purification. Inclusion of the Hmb or the pseudoproline building blocks in the synthesis of these peptides results in crude products that are both more soluble and result in superior recovery.

The use of pseudoproline building blocks is more convenient since synthesis can continue unhindered immediately following its incorporation. In comparison, our experience suggested that efficient coupling of the residue following the Hmb amino

Table 1 Analytical Data for Peptides Synthesized

| Peptide | Sequence | Molecular weight | Synthesis method | % Purity by HPLC[a] |
|---|---|---|---|---|
| **3** | H-MEDSTYYKASKGC-$NH_2$ | 1481.7 | Standard | 27 |
| **4** | H-MEDSTYYKASKGC-$NH_2$ | 1481.7 | Hmb | 39 |
| **5** | H-MEDSTYYKASKGC-$NH_2$ | 1481.7 | Pseudoproline | 45 |
| **6** | H-MEDST(PTyr)YKASKGC-$NH_2$ | 1561.1 | Standard | 25 |
| **7** | H-MEDST(PTyr)YKASKGC-$NH_2$ | 1561.1 | Hmb | 50 |
| **8** | H-MEDST(PTyr)YKASKGC-$NH_2$ | 1561.1 | Pseudoproline | 54 |
| **9** | H-PKYLQNTLKLATGMRNVPEKQTT-OH | 2646.4 | Standard | 24 |
| **10** | H-PKYLQNTLKLATGMRNVPEKQTT-OH | 2646.4 | Hmb | 43 |
| **11** | H-PKYLQNTLKLATGMRNVPEKQTT-OH | 2646.4 | Pseudoproline | 78 |

[a] Purity estimation obtained by peak integration at 214 nm.

acid is best achieved via the symmetric anhydride with double coupling. For many amino acids, particularly bulky, hydrophobic residues, this post-Hmb coupling may not always proceed to completion. For peptides **4** and **7**, an 8 and 5% deletion of lysine, which is the amino acid immediately following HmbAla, was observed. This deletion of the amino acid immediately following the Hmb protected residue was significantly worse for peptide **10** (15% deletion of leucine and 9% double deletion of leucine/lysine). However, the severe *N*-terminal deletions present in peptides **3**, **6** and **9** are eliminated using Hmb protection.

In conclusion, the use of Hmb and pseudoproline building blocks can substantially improve the purities and yields obtained with 'difficult' sequences. This is most probably due to the disruption of secondary structure formation during synthesis. Both types of building blocks are fully compatible with Fmoc synthesis since full deprotection of the Hmb and pseudoproline analogues can be effected in TFA during cleavage to restore the unprotected amino acid. Our results suggest that use of pseudoproline is superior to Hmb backbone protection for the synthesis of 'difficult' peptides, providing the sequence being assembled is compatible with incorporation of a pseudoproline dipeptide analogue. The use of pseudoproline derivatives warrants wider consideration by peptide chemists when preparing the synthesis of sequences predicted to be 'difficult'.

## Acknowledgements

The authors thank Dr Steve Hanks, Vanderbilt University, USA, for permission to present the peptide sequences **3** and **6** described in this study and Thao Nguyen, Chi Pham, Ben Gubbins, Elizabeth Huezo, Philip Rea and Janice Williams for their technical assistance.

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