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Synthesis and purification of aggregation-prone hydrophobic peptides by the incorporation of an Fmoc dipeptide with the peptide bond protected with a modified 2-hydroxy-4-methoxybenzyl (Hmb) group

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Abstract

The dipeptide Fmoc-Val-(2-hydroxy-4-methoxybenzyl)Gly-OBzl was synthesized and the 2-hydroxyl group carbamoylated to give a Boc-N(CH_3)CH_2CH_2N(CH_3)CO–, (Boc-Nmec-) modification of the 2-hydroxy-4-methoxybenzyl (Hmb) group. After catalytic hydrogenation and purification, the resulting dipeptide Fmoc-Val-(Boc-Nmec-Hmb)Gly-OH was used in solid phase peptide synthesis. During treatment with TFA, the peptide was released from the resin and the Boc group cleaved. The peptide could then be purified with an alkylated peptide bond carrying a cationic charge that both increased the solubility of the peptide during the purification steps and facilitated analysis by MALDI-TOF mass spectrometry. The Nmec group was cleaved by intramolecular cyclization under slightly alkaline conditions, followed by cleavage of the Hmb group by TFA to give the fully deprotected peptide.

Aggregation of the peptide chain during peptide synthesis is a major problem in both Boc and Fmoc chemistries. It results in incomplete acylation of the growing peptide chain and is the major source of deletion peptides. Incomplete acylation is usually caused by β-sheet formation, which has been studied extensively. Several methods to reduce the extent of the problem have been put forward.[1–6] Although much research has been devoted to solving problems associated with peptide aggregation during the synthesis of the peptide, relatively few studies have concentrated on the difficulties encountered after synthesis. RP-HPLC of poorly soluble peptides is a major practical problem resulting in low loading of the peptide onto the column, poor resolution and low yields. In addition, hydrophobic and aggregation-prone peptides are often very difficult to characterize by mass spectrometry because of poor ionization. These difficulties are especially severe during the purification of transmembrane sequences of integral membrane proteins, which contain long stretches of hydrophobic amino acid residues. In this case, the introduction of a C-terminal thioester building block containing several arginine residues has been reported to result in a large improvement in solubility.[7,8] This strategy is, however, dependent on the use of Boc chemistry to synthesize the C-terminal thioesters and the in situ neutralization method to counteract aggregation during the coupling steps; thus, this method is not directly relevant to Fmoc chemistry. The same concept has already been exploited by Choma et al. using a 'solubilising tail' to be used in both Boc and Fmoc chemistries.[9–11] Addition of cationic charges to the terminals of the peptide chain is, however, unlikely to have a major effect on β-sheet formation.

An efficient general strategy to counteract β-sheet formation during the synthesis of a peptide is reversible modification of selected peptide bonds.[12,13] Reversible protection of the peptide backbone using the

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N-(2-hydroxy-4-methoxybenzyl) (Hmb) group was introduced by Johnson et al.[14] With this method, selected residues are introduced as *N*,*O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl) amino acids, the Fmoc groups are cleaved with piperidine, and the coupling of the next residue to the relatively unreactive secondary amino group is facilitated by an intramolecular *N*⇒*O* acyl transfer. Hmb-protected peptides attached to a solid phase are less prone to β-sheet formation and coupling of the remaining residues usually proceeds with significantly enhanced efficiency.[15,16] This method has an important additional advantage and can, through the use of a modified protocol, be used to facilitate the purification of synthetic peptides. If the phenolic hydroxyl groups are acetylated prior to cleavage of the peptide with TFA, the peptide can be isolated and purified with acetylated Hmb groups still attached to the peptide backbone.[17] This increases the solubility of peptides that are prone to β-sheet formation and thereby improves the purification yield. This has been demonstrated by the synthesis and purification of the notoriously difficult β-sheet-prone amyloid β (1–43) peptide.[18] However, there are some potential difficulties associated with this strategy. During solid phase peptide synthesis (SPPS) the phenolic hydroxyl group of Hmb is acylated, thereby consuming up to 1 equiv of activated amino acid in each coupling step. Furthermore, the extent of *O*-acetylation of the Hmb group at the end of the synthesis cannot be monitored and in the synthesis of amyloid β (1–43) this acetylation step was therefore performed by an extended overnight treatment with anhydride/diisopropylethylamine. This prolonged treatment can be expected to lead to acetylation of the π-nitrogen of protected histidine side chains, and possibly even other, less reactive groups such as amide nitrogens. Acetylation of Hmb groups will also increase the hydrophobicity of the Hmb-protected peptide, which generally tends to reduce the solubility of the peptide and thereby render the purification more difficult.

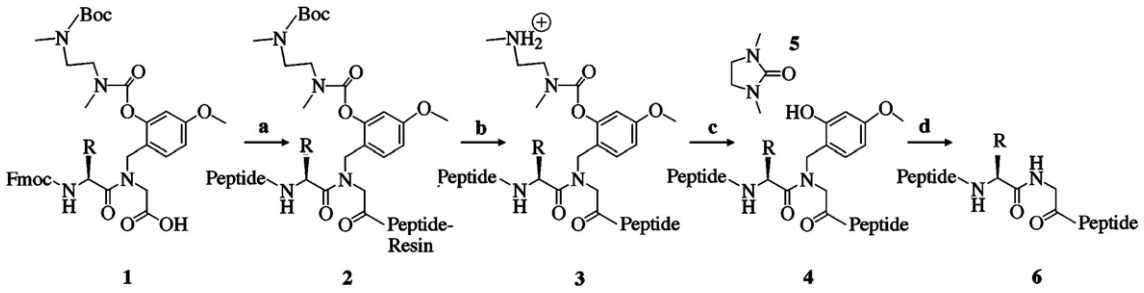
Despite these potential difficulties, synthesis of peptides with modified Hmb groups that are stable during the TFA cleavage step is a very attractive concept, as it can greatly facilitate *both* the synthesis and the purification of many hydrophobic and β-sheet-prone peptides.

One method that can be used to avoid some of these problems is to introduce modified Hmb groups into a peptide chain as dipeptides. This strategy is limited to dipeptides with C-terminal glycine residues, as activation of chiral residues in peptides is prone to racemization. In this context, however, glycine is an important amino acid as it is one of the six most frequently occurring residues in hydrophobic transmembrane sequences, often occurring in pairs with valine and isoleucine residues.[19]

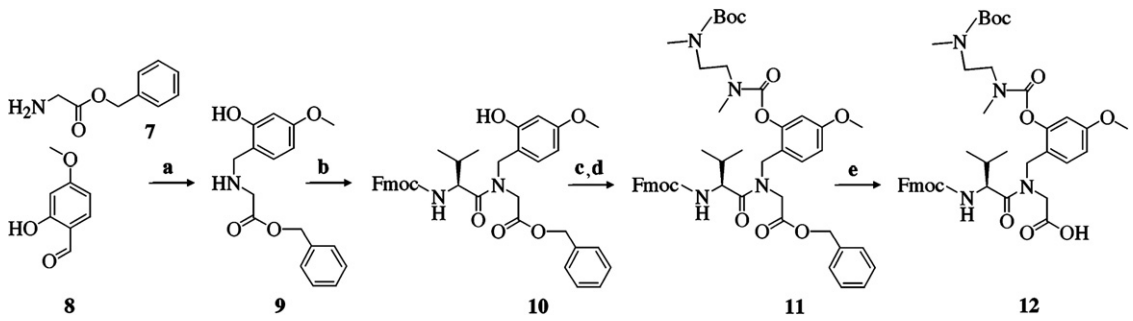
One advantage of using dipeptides with modified Hmb groups would be avoidance of the modification of the Hmb groups at the end of the synthesis, however, this requires that the modified Hmb groups are stable to both piperidine and TFA yet can be rapidly removed under mild reaction conditions.

Phenolic carbamates are known to be very stable to both nucleophiles, bases, and acids.[20–22] We therefore investigated the possibility of using Hmb-protected dipeptides where the phenolic oxygen in Hmb is protected with a Boc-*N*-methyl-*N*-[2-(methylamino)ethyl]carbamoyl group (Boc-Nmec, compound **1**, Scheme 1). This protecting group had previously been investigated as an intermediate in the synthesis of cyclization-activated prodrugs of phenolic compounds, [23] and we have recently used Boc-Nmec as a protective group for tyrosine residues in SPPS (unpublished results). During cleavage of the peptide from the resin with TFA, the Boc group was removed but the protonated methyl-(2-methylamino-ethyl) carbamoyl group remained attached to the Hmb group **3**. In this form, peptide will generally have a greatly increased solubility in aqueous solutions as a result of both the β-sheet-disrupting effect of a benzylated peptide bond and the solubilizing effect of the cationic charge. After purification, the peptide was exposed to slightly alkaline reaction conditions during which an intramolecular cyclization reaction produced the Hmb-protected peptide **4** and *N*,*N*′-dimethylimidazoldione (**5**). The Hmb group can be removed with TFA. In the fully deprotected form, the peptide **6** could be isolated either by an additional chromatographic step or, for aggregation-prone peptides, simply by precipitation.

We therefore synthesized Fmoc-Val-(Boc-Nmec-Hmb)Gly-OH (**12**) as outlined in Scheme 2. The Hmb derivative of the benzyl ester of glycine **9** was obtained by the reductive alkylation of H-Gly-OBzl (**7**) with hydroxymethoxybenzyl aldehyde (**8**) in ethanol, and the free base was isolated as a crystalline compound. Acylation



Scheme 1. General scheme for the use of Fmoc dipeptides with peptide bonds protected with modified 2-hydroxy-4-methoxybenzyl groups, R=$CH(CH_3)_2$. (a) Peptide synthesis; (b) TFA, RP-HPLC purification; (c) DMF/water, *N*-methylmorpholine; (d) TFA, diisopropylsilane.



Scheme 2. Synthesis of Fmoc-Val-(Boc-Nmec-Hmb)Gly-OH. (a) Sodium borohydride in ethanol; (b) Fmoc-L-Val-OH/*N*,*N*′-diisopropyl carbodiimide in DCM; (c) *p*-nitrophenylchloroformate/diisopropylethylamine in DCM; (d) Mono-Boc-*N*,*N*′-dimethylethylenamine/diisopropylethylamine in DCM; (e) H_2/10% Pd on charcoal in ethanol.

of **9** with Fmoc-Val-OH/*N*,*N*′-diisopropylcarbodiimide gave the dipeptide Fmoc-Val-(Hmb)Gly-OBzl (**10**) as a solid foam after purification by dry flash chromatography. The phenolic hydroxyl group was activated with *p*-nitrophenylchloroformate followed by treatment with mono-Boc-*N*,*N*′-dimethylethylenamine to give carbamate **11**. Introduction of the Boc-Nmec group resulted in a large increase in retention time of product **11** in TLC systems, and facilitated purification by dry flash chromatography. The benzyl ester was removed by catalytic hydrogenation, and the extent of the reaction was followed by TLC. The reaction was terminated when traces of 9-methylfluorene were detected by TLC. After purification by dry flash chromatography, the protected dipeptide **12** was obtained as a solid foam in an overall yield of 45%. The ^{1}H NMR and ^{13}C NMR spectra were in accordance with the expected structure (see Supplementary data).

Incorporation of dipeptide **12** into peptides during solid phase peptide synthesis generally proceeded more slowly than for Fmoc amino acids, but acylation of a β-branched residue such as valine was complete after 4 h when coupled as HOAt esters in NMP.

In order to estimate the effect of dipeptide **12** on the solubility of a very aggregation-prone peptide, amyloid β (35–42) (MVGGVVIA) was synthesized (Fig. 1). After cleavage from the resin, the peptide MV(NmecHmb)GGVVIA-OH had a solubility of more than 50 mg/ml in 50% acetonitrile/water while the unprotected peptide was almost insoluble (solubility <1 mg/ml).

The rate of the intramolecular cyclization reaction had been investigated previously for a hydroxyanisole derivative in aqueous solutions by Saari and co-workers, and the half-life at pH 7.4 was found to be 36.3 min.[23] Water is, however, generally a very poor solvent for hydrophobic, β-sheet-prone peptides and we therefore required a more general solvent system. The cyclization reaction was slow in the aprotic solvent DMF, but we found that DMF/water was a better solvent system. The reaction was studied using the model peptide KG(HmbNmec)VAKKKA-OH, which was designed to have high solubility even without a protonated Nmec group. The cyclization proceeded smoothly in 4 h in 30% DMF/H_2O using *N*-methylmorphiline as base. DMF was removed by evaporation under reduced pressure

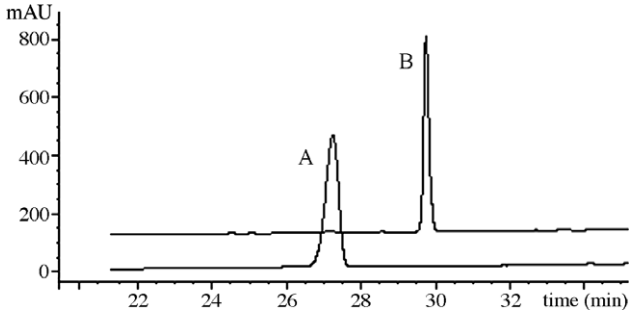


Fig. 1. (A) RP-HPLC elution profile of the Nmec-Hmb backbone-protected amyloid β (35–42) peptide; MV(NmecHmb)GGVVIA-OH B. Amyloid β (35–42) (MV(Hmb)GGVVIA-OH) after the treatment of the purified peptide for 8 h with 30% DMF/water and 10 equiv of *N*-methylmorpholine.

and the product was precipitated five times with diethyl ether. The resulting Hmb-protected peptide was redissolved in acetonitrile/water, lyophilized, and purified by RP-HPLC.

The Nmec-Hmb group also facilitated analysis of the peptides by MALDI-TOF mass spectrometry. For the unmodified amyloid β (35–42) only weak signals with poor signal-to-noise ratios were obtained. This is probably the result of rapid aggregation of the peptide on the matrix, as we observed precipitation of the peptide within 15–30 min during the preparation of the sample. To a lesser extent, the same problems were encountered for amyloid β (35–42) where Gly^{37} was modified with the Hmb group. In contrast, we always recorded robust signals and excellent signal-to-noise ratios for the same peptide protected with Nmec-Hmb. However, peptides modified with the Nmec-Hmb group were not completely stable in MALDI-TOF mass spectrometry. Even for purified peptides that appeared as homogenous peaks in HPLC, there was always partial cleavage of the carbamate group, as peptides with molecular masses of 114 less than expected were evident.

In conclusion, the problems associated with post-synthetic handling of peptides are frequently underestimated. These problems are inherent properties of the particular peptide sequence and are usually very difficult to counteract without reversible covalent modifications of the

peptide. Taking into account the fact that glycine residues frequently occur in large stretches of hydrophobic peptides, it is therefore likely that the use of the protected dipeptides discussed in this study will result in a major improvement in both the yield and purity of a large number of synthetic peptides.

Supplementary data

Experimental details for the synthesis of Fmoc-Val-(Boc-Nmec-Hmb)Gly-OH. ^{13}C NMR for Fmoc-Val-(Boc-Nmec-Hmb)Gly-OH. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.04.055.

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