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A New Approach to Hmb-backbone Protection of Peptides: Synthesis and Reactivity of N^{α}-Fmoc-N^{α}-(Hmb)amino Acids[1]

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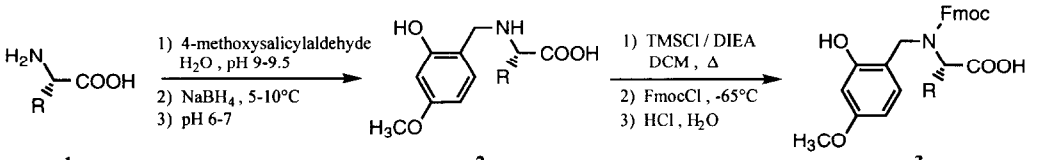
Abstract: *The use of* N-*Fmoc*-N-*(Hmb)amino acids for the introduction of the Hmb backbone protection into peptides during solid-phase synthesis is described. An efficient two step synthesis of these derivatives is reported and their coupling to the peptide chain is studied.*

During the last few years, the solid-phase methodology for peptide synthesis has evolved very favourably.[2] One of the main drawbacks of the method is the production of deletion peptides[3] resulting from incomplete acylations during the coupling of hindered amino acids, especially those having β-branched side-chains. These incomplete couplings can be improved by recoupling. In such cases, the use of a different coupling method and/or solvent is advisable. More intractable coupling difficulties are due to intrachain (i.e. reverse turns) or interchain interactions (i.e. β-sheets).[3a,4] To overcome this problem, Sheppard and co-workers[5] have recently described the protection of the amide bond in peptides with the Hmb group, which is compatible with the Fmoc/tBu strategy. This protecting group has also been used in the orthogonal solid-phase synthesis of *N*-glycopeptides,[6] to avoid aspartimide formation.[7] and to improve the solubility of protected peptides,[8] one of the main drawbacks of the convergent approach.[9]

Classically, the Hmb group is incorporated into the peptide chain using *N,O*-bis-Fmoc-*N*-(Hmb)amino acids under conditions similar to those used for amide bond formation in solid phase peptide synthesis.[5-7,10] These derivatives are obtained by alkylation of the amino acid through a reductive amination followed by reaction with Fmoc-Cl to protect both amino and phenol functions.[5a] Although the synthesis of these products looks straightforward, impure products have usually been obtained in our laboratory, even after careful purification by column chromatography.[11] We thought that these synthetic problems might arise from an overactivation of the carboxyl function by the Fmoc-Cl,[12] similar to that described during the preparation of Fmoc-amino acids with Fmoc-Cl. Other reagents such as Fmoc-N_3[12] or Fmoc-OSu[13] were tried, but these proved to be less reactive and were not suitable for protection of the secondary amino function. This moved us to focus our attention on the *N*-Fmoc-*N*-(Hmb)amino acids because they can be easily obtained from the corresponding *N*-(Hmb)amino acids after *in situ* protection of the amino, carboxylic and phenol functions using the method described by Bolin.[14] The fact that there is no protection for the phenol function should not be a problem, because, independently of the strategy chosen (the use of either *bis*-Fmoc or Fmoc-derivatives), the phenol group will remain unprotected during the growth of the peptide chain once the corresponding amino acid derivatives were coupled and the Fmoc group(s) removed.[15]

Alanine (a difunctional amino acid), aspartic acid (a trifunctional amino acid) and threonine (a trifunctional β-branched amino acid) were chosen as representative amino acids to carry out this study. The corresponding *N*-Fmoc-*N*-(Hmb)amino acids (**3a**, R = CH_3; **3b**, R = $CH_2COOtBu$; and **3c**, R = (*R*)-$CH(OtBu)CH_3$; Scheme 1) were synthesized from the *N*-Hmb-amino acids (**2a**, **2b** and **2c**), which were obtained using similar conditions to those reported in the literature[5a] but with some modifications. Thus, it was found that strict control of the temperature and pH is crucial for the purity of the final product. The reaction of the amino acid with the aldehyde was carried out at room temperature, keeping the pH around 9-9.5. The reduction with $NaBH_4$ was performed at 5-10°C instead of heating at 60°C, as reported by Sheppard et al.[5a] The obtention of **3** was achieved after protection with TMSCl, using Fmoc-Cl at -65°C to room temperature. The desired products were obtained without any detectable racemization as determined using Marfey's method.[16]

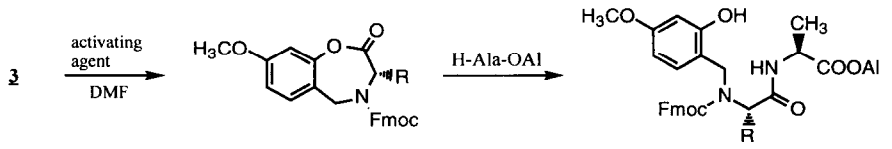


1 a, R = CH_3
b, $CH_2COOtBu$
c, (*R*)-$CH(OtBu)CH_3$

2 **3**

Scheme 1

To demonstrate the validity of *N*-Fmoc-*N*-Hmb-amino acids (**3**) for the preparation of Hmb containing peptides, the coupling of these derivatives to alanine allyl ester was studied in solution.[17] The reactions were carried out at room temperature in DMF (75 μM solutions) and were followed by HPLC. Equimolar quantities of both **3** and the coupling reagent (DCC, DIPCDI, PyBOP, PyOAP, HBTU or HATU) were used.[18] Activation of **3** afforded the same intermediate in all cases, which could be isolated as oils and characterised as the 4,5-dihydro-8-methoxy-1,4-benzoxazepin-2(3H)-one **4** (Scheme 2).[19] The formation of this product took place extremely quickly (the reaction is quantitative after 5 min in the case of the onium salts), due to the attack of the phenol function to the carboxylic group that has been activated by the coupling agent.[20] The active especies **4** afforded the desired dipeptides **5** with yields that depended on the nature of the *N*-Fmoc-*N*-Hmb-amino acid. Thus, **5a** and **5b** were obtained in 80% yield after 1.5 h at room temperature, while **4c** proved to be less reactive with a 29% yield after the same time and 80% after 72 h.[21] No other products were detected during the process in all cases. The different results that were achieved can be explained in terms of the steric hindrance that provokes the β-branched side chain of threonine, which impedes the nucleophilic attack on the seven-membered ring of the **4**.



3 **4** **5**

Scheme 2

Amino acid derivatives **5a**, **5b** and **5c** were used in the solid phase synthesis of the model peptide H-Asp-Asp-Thr-Nle-Lys-Asp-Ala-Gly-OH. These amino acids were introduced at positions 2 (Asp), 3 (Thr) and 7 (Ala) in order to suppress aspartimide formation. A chlorotrityl-resin was used[22] and couplings were carried out at room temperature[23] using 6 eq of reagents (amino acid and DIPCDI). **5a** was assembled with a single coupling, and triple couplings were used to incorporate **5b** and **5c** respectively (2 h per coupling). The cleavage of the peptide from the polymeric support afforded the right sequence (77%, as determined by HPLC-MS), but one of the Hmb groups proved to be especially resistant to acidolytic conditions.[24] Moreover, a 23% of the peptide with a deletion of the two *N*-terminal Asp residues was detected after careful analysis of the HPLC profile.[25]

In summary, *N*-Fmoc-*N*-(Hmb)amino acids can be an excellent alternative to *N,O*-bis-Fmoc-*N*-(Hmb)amino acids for the introduction of the Hmb backbone protection into peptides. Couplings can be achieved with quantitative yields using standard solid phase methodologies. Amide bond formation takes place through the obtention of a benzoxazepine which acts as the activated species in this particular case. However, special attention has to be paid in the case of difficult sequences such in the case of the coupling of an *N*-Hmb protected amino acid to another *N*-Hmb protected residue. Alternative conditions for amino acid coupling and for the final deprotection of the amide bond are under investigation.

Experimental Procedures

Synthesis of* N-*(Hmb)amino acids. To a three-necked 100 mL round-bottom flask provided with an addition funnel, a thermometer and a pH electrode, were added the amino acid (11.4 mmols) and H_2O (20 mL). The pH was raised to 9 by dropwise addition of a 2 M aqueous solution of NaOH. Finely powdered 2-hydroxy-4-methoxybenzaldehyde (1 .7 g, 1 eq) was added and the resulting suspension was left for 2 h with magnetic stirring at room temperature. The pH was kept at 9-9.5 by ocasional addition of small quantities of 2 M aqueous solution of NaOH. The temperature of the final solution was lowered to 5°C when a 0.76 M aqueous solution of $NaBH_4$ (1 eq) plus three drops of 2 M aqueous solution of NaOH were added dropwise during 20 min. The mixture was left for 1 h at 5-10°C with magnetic stirring and was transferred to a 250 mL flask. The pale yellow solution was taken to pH 6-7 adding dropwise a 2 M aqueous HCl solution with vigorous magnetic stirring and keeping the temperature below 20°C. A white solid was formed and the suspension was left in the refrigerator overnight. The solid was filtered, washed with water and dried under vacuum (60-80% yields). The products were characterized by MS, IR, ^1H NMR and ^{13}C NMR.

Synthesis of* N-*Fmoc* -N*(Hmb)amino acids. To a three-neck 100 mL round-bottom flask provided with an addition funnel and a thermometer were added the *N*-Hmb-amino acid (8.9 mmols) and anhydrous CH_2Cl_2 (30 mL) under argon. Then DIEA and TMSCl (9.3 mL and 6.7 mL respectively, 6 eq) were added through a septum. The solution was left with magnetic stirring for 3 h and at room temperature when it was cooled down to -65°C. A 0.89 M solution of Fmoc-Cl in anhydrous CH_2Cl_2 was added dropwise during 10 min and the mixture was left to rise to room temperature. The reaction was quenched with 40 mL of 2 M aqueous HCl and the organic solution was washed with 5x30 mL of 2 M aqueous HCl. The organic solution was dried over $MgSO_4$ and volatiles were removed under vacuum. The resulting orange oily crude was chromatographed on a silica gel column using $CHCl_3$ and $CHCl_3$/MeOH (9:1) as eluents.

The final products were obtained as pale yellow solids in 75-85% yields. The products were characterized by MS, IR, ^{1}H NMR and ^{13}C NMR.

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References and Notes

1. Abbreviations used for amino acids and peptides follow the rules of the IUPAC-IUB Comission of Biochemical Nomenclature in *European J. Biochem.*, **1984**, *138*, 9-37 and *J. Biol. Chem.*, **1989**, *264*, 633-673. The following additional abbreviations are used: CH_2Cl_2, dichloromethane; DCC, diciclohexylcarbodiimide; DIEA, diisopropylethylamine; DIPCDI, diisopropylcarbodiimide; DMF, dimethylformamide; ES-MS, electrospray mass espectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-Cl, 9-fluorenylmethoxycarbonyl chloride; Fmoc-N_3, 9-fluorenylmethoxycarbonyl azide; Fmoc-OSu, 9-fluorenylmethoxy-carbonyl succinimide; FmOH, 9-fluorenylmethanol; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *O*-benzotriazolyl-*N*-*N*-*N*'-*N*'-tetramethyluronium hexafluorophosphate; Hmb, 2-hydroxy-4-methoxybenzyl; HPLC, high performance liquid chromatography; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; PyOAP, 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; t-Bu, *tert*-butyl; TFA, trifluoroacetic acid; TMSCl, trimethylsilylchloride.
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11. As an example, the final product that was obtained in the synthesis of *N,O*-bis-Fmoc-*N*-(Hmb)-Ala-OH was analysed by HPLC-MS. FmOH, *N*-Fmoc-*N*-(Hmb)-Ala-OH and by-products that probably resulted from intermolecular reactions in which the latter was involved, were detected in the purified material. The commercial product afforded a similar result.
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18. One extra eq of DIEA was added when phosphonium salts or uronium salts were used.
19. ES-MS *m/z* : **5a**, 430.4 $[M + 1]^+$ ($C_{26}H_{23}NO_5$ requires 429.5); **5b**, 530.3 $[M + 1]^+$ ($C_{31}H_{31}NO_7$ requires 529.6); **5c**, 516.4 $[M + 1]^+$ ($C_{31}H_{33}NO_6$ requires 515.6).
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23. The assembly of the three amino acid derivatives was followed by HPLC of crude materials that were obtained after acidolytic treatment of peptide-resin aliquotes. No starting materials were observed after the couplings.
24. The cleavage of the peptide from the resin using reagent R for 2 h (Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R.I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730-3743) yielded the desired peptide (29%) and the octapeptide with an Hmb group (71%).
25. A similar result was obtained when *N*-Fmoc-Asp(OtBu)-OH was used for the incorporation of Asp^2.

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