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A carbamoyl-protective group for tyrosine that facilitates purification of hydrophobic synthetic peptides

Karolina Wahlström, Ove Planstedt, Anders Undén *

Department of Neurochemistry, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden

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Abstract

The Boc-*N*-methyl-*N*-[2-(methylamino)ethyl]carbamoyl group (Boc-Nmec) is reported as a new side chain-protective group for tyrosine in Fmoc solid-phase peptide synthesis. Tyrosine is incorporated into the peptide as Fmoc-Tyr(Boc-Nmec)-OH by standard coupling methods. During the cleavage of the peptide from the resin with TFA the Boc group is simultaneously cleaved while the cationic *N*-methyl-*N*-[2-(methylamino)ethyl]carbamoyl group remains attached to the tyrosine residue, thereby increasing the solubility of the peptide. After purification of the peptide, the Nmec protective group can be cleaved under neutral or mild alkaline conditions via an intramolecular cyclization reaction.

Keywords: Solid-phase peptide synthesis; Protective group; HPLC; Tyrosine; Fmoc amino acids

The solubility of synthetic peptides is often a major problem in solid-phase peptide synthesis (SPPS). Poor solubility is often a result of the formation of β-sheet structures that favor aggregation of the peptide, a process that occurs both during the assembly of the peptide chain and during the purification steps.[1,2]

Purification of the synthetic peptides is routinely carried out under aqueous acidic conditions using reversed phase HPLC, and good solubility of the peptide in water or in a water/acetonitrile mixture facilitates this step. Synthetic peptides with histidine, lysine, or arginine residues generally increase the solubility of peptides in aqueous solution as a result of the combined effect of increased hydration and electrostatic repulsion between the peptide chains. For synthetic peptides with few or no cationic charges, however, the purification is usually hampered by aggregation and low solubility.

In this Letter, we report a method in which these problems can be reduced to a significant extent for peptides with tyrosine residues, by employing a protective group that is cleaved in a two-step procedure.

It has been shown that phenyl carbamates can be hydrolyzed by hydroxyl ions by two different mechanisms. N-Unsubstituted and N-monosubstituted carbamates undergo an ElcB elimination reaction by proton abstraction and isocyanate formation while N-disubstituted carbamates are cleaved by the nucleophilic attack on the carbonyl carbon.[3] The latter mechanism proceeds much more slowly, and it could therefore be expected that N-disubstituted phenyl carbamates would be stable to the reaction conditions in Fmoc SPPS where the weaker nucleophile piperidine is used.[4] N-Disubstituted phenyl carbamates could even be expected to be too stable to be used in peptide synthesis, however, Saari et al. have shown that N-disubstituted phenyl carbamates can be cleaved under neutral or weakly basic conditions via an intramolecular cyclization reaction.[5] In that study, the use of *N*-methyl-*N*-[2-(methylamino)ethyl]carbamates as potential prodrugs for phenolic drugs was investigated, and it was found that cleavage occurred relatively quickly even in aqueous solution at pH 7.4, with a half-life of 36 min. The cleavage was shown to proceed via an intramolecular cyclization

* Corresponding author. Tel.: +46 816 4117; fax: +46 816 13 71.
E-mail address: andersu@neurochem.su.se (A. Undén).

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resulting in the generation of *N*,*N*′-dimethylimidazolidinone and the free phenol.

Taken together, these reports suggest that tyrosine residues with a Boc-*N*-methyl-*N*-[2-(methylamino)ethyl]carbamoyl group (Boc-Nmec) as a side chain-protective group for tyrosine residues could be expected to be stable under the reaction conditions in Fmoc SPPS. After treatment with TFA, the Boc group is cleaved but the protonated Nmec group would still be attached to the peptide, resulting in increased solubility. After purification, the unprotected peptide can be obtained by short exposure to a weak alkaline aqueous solution.

We therefore synthesized Fmoc-Tyr(Boc-*N*-methyl-*N*-[2-(methylamino)ethyl]carbamoyl)-OH, Fmoc-Tyr(Boc-Nmec)-OH (**3**), as shown in Figure 1. The phenacyl ester of Fmoc-Tyr-OH (**1**) was treated with a slight excess of *p*-nitrophenyl chloroformate and diisopropylethylamine (DIPEA) in methylene chloride for 30 min followed by a threefold excess of mono-Boc protected *N*,*N*′-dimethylethylenediamine and DIPEA. After aqueous work-up and dry flash chromatography, the phenacyl ester **2** was cleaved with zinc in acetic acid. Additional work-up and crystallization from ethyl acetate/petroleum ether gave the final product with an overall yield of 89%.[6]

The product was characterized by ^{1}H and ^{13}C NMR and the results were in agreement with the expected structure (see Supplementary data).

The stability of the Boc-Nmec group in 20% piperidine in DMF was studied by treating the peptide H-Ala-Tyr-(Boc-Nmec)-Phe-Lys(Boc)-Lys(Boc)-OH attached to the Wang resin for 24 h. After cleavage with TFA, the major product was the Nmec-protected peptide. No significant increase in the levels of by-products as compared to the peptide not exposed to prolonged piperidine treatment was observed by HPLC analysis and MALDI-TOF mass spectrometry. These results are in agreement with those obtained by Vontor et al. who determined the half-life of phenyl *N*,*N*-dimethylcarbamate in 1 M aqueous NaOH at 40 °C to be 20 min.[7] It can therefore be concluded that the Boc-Nmec group will be stable to piperidine treatment even during the synthesis of large peptides.

The rate of removal of the Nmec group will be dependent on the peptide sequence, the pH, the temperature, and the solvent. Saari et al. recorded a half-life of 36 minutes at pH 7.4 and 37 °C for Nmec-protected hydroxyanisole in aqueous solution.[5] For peptides that are prone to aggregation, especially very hydrophobic peptides with more than one Nmec protected tyrosine residue, it is important that the peptide should remain in solution during the cleavage as the solubility will be reduced when the cationic Nmec group is cyclized to *N*,*N*′-dimethylimidazolidinone. The selection of solvents for this reaction is therefore highly dependent on the sequence of the peptide, but in general we found that combinations of DMF and water gave the most reliable results.

To demonstrate the potential value of the Nmec group in the synthesis of hydrophobic peptides, the peptide H-Ala-Tyr-Val-Leu-Tyr-Tyr-Ala-OH, was synthesized using Boc-Nmec protection of all the tyrosine residues. The HPLC elution profile of the crude product is shown in Figure 2.

The expected Nmec-protected peptide was obtained as the major product. The purified Nmec- protected peptide was dissolved in 30% DMF/water (1 mg/ml) and 10 equiv of *N*-methyl morpholine was added. *N*-Methylmorpholine is a weak non-nucleophilic base (pK_a 7.41), and these mild reaction conditions will minimize base-catalyzed side-reactions. The cyclization reaction was monitored by removing small samples at different time points, quenching with acetic acid, and concentrating in vacuo. After being redissolved in 30% acetonitrile, the samples were analyzed by HPLC. The cyclization reaction was complete after 4 h. As expected, cleavage of the Nmec group resulted in signif-

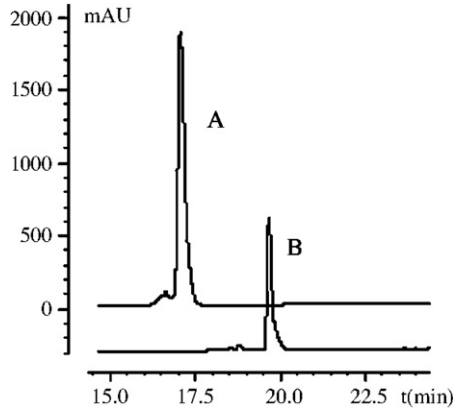

Fig. 2. HPLC elution profile of the model peptide H-Ala-Tyr-Val-Leu-Tyr-Tyr-Ala-OH synthesized by Boc-Nmec protection of the side chains of all the tyrosine residues. (A) H-Ala-Tyr(Nmec)-Val-Leu-Tyr(Nmec)-Tyr(Nmec)-Ala-OH after cleavage with TFA. (B) H-Ala-Tyr-Val-Leu-Tyr-Tyr-Ala-OH after treatment of the purified peptide for 4 h with 30% DMF/water and 10 equiv of *N*-methylmorpholine.

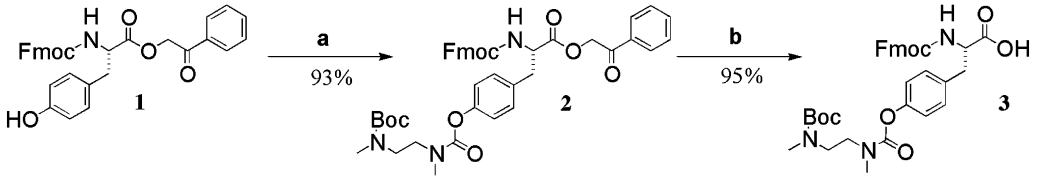

Fig. 1. Synthesis of Fmoc-Tyr(Boc-Nmec)-OH. Reagents: (a) (1) *p*-nitrophenyl chloroformate, DIPEA, DCM; (2) mono-Boc- *N*,*N*′-dimethylethylenediamine, DIPEA, DCM; (b) Zn/HOAc.

icantly longer retention times on HPLC as a result of loss of the cationic Nmec group.

In MALDI-TOF mass spectrometry the Nmec group is partly cleaved, and we always recorded molecular masses corresponding to the loss of one or more Nmec groups for peptides that eluted as a single peak on HPLC. MALDI-TOF analysis of the Nmec-protected peptide gave excellent signal-to-noise ratios, while removal of the Nmec group resulted in weaker signals; and when redissolved, the purified, lyophilized fully deprotected peptide gave a very poor signal-to-noise ratio. This poor signal-to-noise ratio in MALDI-TOF for the deprotected peptide is probably the result of aggregation and of the reduced cationic character of the peptide leading to poor ionization.

In conclusion, for hydrophobic peptides containing tyrosine residues, side chain protection using the Boc-Nmec group is likely to be a valuable tool. The synthesis of Fmoc-Tyr(Boc-Nmec)-OH can be carried out in a few simple synthetic steps, the yield is good, and the product is easy to crystallize. Once incorporated into a peptide, it is stable to the reaction conditions in Fmoc chemistry. After cleavage from the resin, the solubility of the peptide is increased and usually the peptide gives stronger signals in MALDI-TOF mass spectrometry. Finally, the fully deprotected and purified peptide can be generated under mild reaction conditions.

Supplementary data

Supplementary data (^{1}H NMR and ^{13}C NMR for Fmoc-Tyr(Boc-Nmec)-OH) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.04.014.

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