Journal of Peptide Science
J. Peptide Sci. **11**: 512–515 (2005)
Published online 10 March 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.657

JOURNAL OF PEPTIDE SCIENCE

Amino acid deletion products resulting from incomplete deprotection of the Boc group from N^{π}-benzyloxymethylhistidine residues during solid-phase peptide synthesis

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Received 9 December 2004; *Accepted 29 December 2004*

Abstract: In peptide synthesis, the use of N^{α}-*tert*-butyloxycarbonyl-N^{π}-benzyloxymethylhistidine [Boc-His(π-Bom)] raises the problem of the Bom group generating formaldehyde during the hydrogen fluoride (HF) cleavage reaction. This can lead to modification of the functional groups on amino acids in the peptide chain. Besides this side reaction, the failure of N^{α}-Boc deprotection from the His(π-Bom) residue occurs during TFA treatment for the standard solid-phase peptide synthesis (SPPS) even in the case of a non 'difficult sequence'. This gives amino acid deletion products generated at the *N*-terminus of the His(π-Bom) residues. Reviewing the removability of the Boc group on amino acid derivatives showed that the group on the His(π-Bom) residue was much more resistant under the deprotecting conditions than expected. To circumvent this problem, special precautions, i.e. prolonged deprotection steps and/or increased concentrations of TFA, should be taken for a successful SPPS. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amino acid deletion; Boc-His(π-Bom); *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid

INTRODUCTION

The segment condensation method in solution employing a maximum protection strategy with Boc chemistry is ideal for the synthesis of large peptides or proteins [1]. A standard solid-phase peptide synthesis on an *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid (HMFS) linker developed by Rabanal *et al.* [2] was introduced recently for synthesizing a fully protected segment with a free α-carboxyl group for use in a subsequent coupling reaction in solution or on a solid support [3]. The HMFS linker belongs to the 9-hydroxymethylfluorene type protecting groups that are designed to be cleaved by nucleophiles such as piperidine and morpholine. Base-labile linkers are useful for practical peptide synthesis because they require no special equipment and no strict control of the reaction conditions during detachment of the protected segments from the resin. To apply the above strategy, however, high compatibility between the side-chain protecting groups of the segment and the anchoring groups is indispensable. The formyl (For) group for the Trp residue and the 2-bromobenzyloxycarbonyl (BrZ) group for the Tyr residue are particularly susceptible to the detachment procedure using 20% morpholine in DMF. This obstacle was overcome not only by developing base-resistant protecting groups, i.e. the cyclohexyloxycarbonyl (Hoc) [4] for Trp and the 3-pentyl (Pen) [5] for Tyr, compatible with Boc chemistry but also by configuring an alternative detachment procedure using a weaker nucleophile such as triethylamine (TEA) [6]. An alternative procedure using 20% TEA in DMF allowed us to employ the ordinary protecting group including the For and BrZ groups to prepare the protected segment since it causes no loss of these groups during the detachment reaction. However, closer examination of the segments by TLC, HPLC and ESI MS revealed that serious side reactions had occurred during elongation of peptide chains on the resin; i.e. dehydration of the Asn residue and amino acid deletion at the *N*-terminus of particular amino acid residues. The dehydration can be avoided by temporary protection using a 9-xanthydryl (Xan) group on the Asn residues [7], whereas amino acid deletion products generated at the *N*-terminus of the His(π-Bom) residues [8] resulted more or less from incomplete deprotection of the Boc group even with high concentrations of TFA. Although this disadvantage with Boc-His(π-Bom) had already been pointed out [9], its details had not been reported. The present study reviewed the removability of the Boc group on amino acid derivatives and found that the group on the His(π-Bom) residue was more resistant under the deprotecting conditions than expected. The resistivity to TFA treatment of the Boc group on the individual amino acid residue would be related to the extent of deletion of an amino acid located at the *N*-terminus of the corresponding residue.

Abbreviations: cHx, cyclohexyl; ClZ, 2-chlorobenzyloxycarbonyl; Doc, 2,4-dimethylpent-3-yloxycarbonyl; HMFS, *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid; Hoc, cyclohexyloxycarbonyl; IEX-HPLC, ion exchange high performance liquid chromatography; Pen, 3-pentyl; otherwise as defined in *J Peptide Sci* **9**: 1–8 (2003).

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MATERIALS AND METHODS

Boc-protected amino acids and other reagents for peptide synthesis were obtained from Peptide Institute Inc. (Osaka, Japan). All other chemicals and solvents of special grade were obtained from Nacalai Tesque (Kyoto, Japan) and were used without further purification. RP-HPLC analyses were performed on a Shimadzu liquid chromatograph Model LC-10AT (Kyoto, Japan), with a YMC-Pack A-302 C18 or A-202 C8 column (4.6 × 150 mm) using a flow rate of 1 ml/min and the following solvent systems: 0.1% TFA in H_2O (A), 0.1% TFA in MeCN (B). Molecular weights were measured with an ESI MS (HP 1100 LC/MSD, Palo Alto, CA, USA).

Synthesis of Protected GFP (197–201): Boc-Asp(OcHx)-Asn-His(Bom)-Tyr(Pen)-Leu-OH

Solid-phase peptide synthesis was performed with an ABI 433A peptide synthesizer using Boc strategy on an HMFS resin loading Boc-Leu (0.85 g, 0.50 mmol) [3]. The following side-chain-protected amino acids were employed: Asp(OcHx), His(π-Bom) and Tyr(Pen). The peptide chain was elongated using *in situ* neutralization protocols of coupling with Boc-amino acid/HBTU/HOBt/DIEA (4/4/4/6 eq) in NMP (single coupling, acetylation after each coupling step). The Boc group was removed by treatment with 50% TFA/DCM or neat TFA for 3 + 17 min.

A few milligrams of the resulting peptide resin was treated with 20% morpholine in DMF (100 µl/mg of the peptide resin) for 1 h with occasional shaking. The supernatant solution was directly applied to RP-HPLC (YMC-Pack C8 column 4.6 × 150 mm; flow rate 1 ml/min; detection at 220 nm) with a gradient of 45%–95% MeCN in 0.1% TFA to assess the homogeneity of the protected segment as shown in Figure 1. Peak 1 [Boc-His(π-Bom)-Tyr(Pen)-Leu-OH]: ESI MS, 721.84 (theoretical value: 721.90); AAA, Leu 1.00 (1) Tyr 0.98 (1) His 1.00 (1) NH_3 0.09 (0). Peak 2 [Boc-Asp(OcHx)-Asn-His(π-Bom)-Tyr(Pen)-Leu-OH]: ESI MS, 1033.23 (theoretical value: 1033.23); AAA, Asp 1.96 (2) Leu 1.00 (1) Tyr 0.96 (1) His 0.99 (1) NH_3 0.96 (1). Peak 3 [Boc-Asp(OcHx)-His(π-Bom)-Tyr(Pen)-Leu-OH]: ESI MS, 919.16 (theoretical value: 919.13); AAA, Asp 1.00 (1) Leu 1.00 (1) Tyr 1.00 (1) His 0.97 (1) NH_3 0.14 (0).

Removability of the Boc Group from Individual Amino Acid Residues and a Model Peptide

To 100 µl of a solution of individual Boc-amino acid (30 µmol) in DCM (1.0 ml) at room temperature was added TFA (100 µl). After 1, 3, 5, 10 and 15 min, an aliquot of each solution was applied to RP-HPLC (YMC-Pack A-302 C18 column 4.6 × 150 mm; flow rate 1 ml/min; detection at 220 nm) with a gradient of 10%–60% or 20%–70% MeCN in 0.1% TFA to measure the extent of recovery of the Boc group on the corresponding amino acid by comparison with the internal standard (Fmoc-Ala-OH or Fmoc-Gly-OH). To examine the removability of the Boc group from a model peptide having a Boc-His(π-Bom) residue at the *N*-terminus, Boc-His(π-Bom)-Asp(OcHx)-Ser(Bzl)-Gly-OPac (10 mg, 11 µmol) was added to various concentrations of TFA in DCM (300 µl) and a portion of each solution was subjected to RP-HPLC (YMC-Pack A-302 C18 column 4.6 × 150 mm; flow rate 1 ml/min; detection at 220 nm) with a gradient of 45%–95% MeCN in 0.1% TFA to measure the extent of recovery of the Boc group at regular intervals.

RESULTS AND DISCUSSION

In the synthetic procedure, highly homologous protected segments must be prepared for use in the subsequent segment coupling reactions either in solution or on a solid support. Therefore, the risk of side product formation must be excluded during the detachment of the protected segments from the HMFS resin by treatment with piperidine or morpholine, which is standard procedure for cleaving the Fmoc group. Under these conditions, the formation of aspartimide peptides is suspected. However, this side reaction was found to be negligible if 20% morpholine or 20% TEA in DMF was employed within 1 h for the detachment conditions, even if the protected segment contained sequences susceptible to base-catalysed conditions (e.g. Asp-Gly, Asp-Asn or Asp-Phe containing peptide) [3,6].

The HMFS resin can facilitate the release of high-purity protected segments. Therefore, the side reactions occurring during elongation of the peptide chains were directly examined by evaluating the purity of the protected segments detached from the HMFS resin on TLC, HPLC and ESI MS. The data revealed a serious side reaction during step-wise peptide elongation on the resin; amino acid deletion products generated at the *N*-terminus of the His(π-Bom) residues resulted from incomplete deprotection of the Boc group even with higher concentrations of

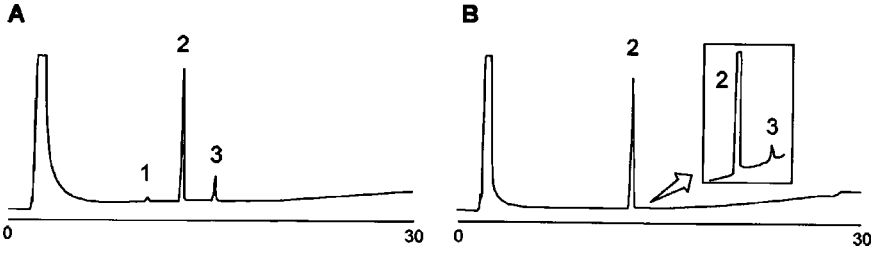

Figure 1 HPLC profiles of protected segments detached from the HMFS resin. Deprotection of the Boc group was performed with 50% TFA/DCM (3 + 17 min) (A) and 100% TFA (3 + 17 min) (B). Peak 1, Boc-His(π-Bom)-Tyr(Pen)-Leu-OH; Peak 2, Boc-Asp(OcHx)-Asn-His(π-Bom)-Tyr(Pen)-Leu-OH; Peak 3, Boc-Asp(OcHx)-His(π-Bom)-Tyr(Pen)-Leu-OH. Column: YMC-Pack A-202 C8 (4.6 × 150 mm). Eluent: 45%–95% CH_3CN in 0.1% TFA. Running conditions: 25 min gradient, 5 min holding; flow rate: 1 ml/min; temperature: 40 °C. Detection: 220 nm.

TFA. In general, it is presumed that a deletion product missing a neutral and hydrophilic amino acid would be extremely difficult to separate from the desired one in the form of a free peptide, even after being subjected to purification using RP- and IEX-HPLC. It is preferable to circumvent such a problem in the stage of elongation of the peptide chain on a solid support. To assess the extent of deletion arising from the incomplete deprotection of the Boc group on the His(π-Bom) residue, the protected peptide segment, green fluorescent protein (GFP) (197–201) [Boc-Asp(OcHx)-Asn-His(π-Bom)-Tyr(Pen)-Leu], was synthesized as a model peptide on the HMFS resin with an automatic synthesizer (ABI 433A) using standard Boc chemistry, in which 50% TFA in DCM was employed for Boc-deprotection (3 min $\times$1, 17 min $\times$1) [10,11]. In this protocol, quantitative removal of the Boc group by treatment with TFA for a short period is required for successful performance. However, ESI MS analysis of the product detached from the HMFS resin revealed that the desired peptide was contaminated by a significant amount of des[Asn^{198}]-GFP (197–201) (Figure 1A). Furthermore, the Boc group on the His(π-Bom) residue remained even after undergoing the Boc-deprotection procedure twice, which caused the peptide to lose two amino acid residues (peak 1). This incomplete N^{α} deprotection could be suppressed to less than 0.5% by employing neat TFA in place of 50% TFA/DCM (Figure 1B).

These findings prompted us to review the removability of the Boc group on individual amino acid residues. The Boc groups on almost all of the amino acid derivatives were removed within 5 min by treatment with 50% TFA/DCM except for the Boc group on the His(π-Bom) residue, which required much longer than 15 min for completion of deprotection (Figure 2). In addition to the Boc group on the His(π-Bom) residue, that on the Arg(Tos) residue was found to be somewhat resistant to TFA treatment although its resistivity of the Boc group and the extent of the resulting deletion with an amino acid located at its *N*-terminus were immaterial.

The His derivatives are known to be very prone to epimerization on activation and coupling steps in which the π-nitrogen is involved [12]. Therefore, regiospecific protection of the π-nitrogen is favourable for suppressing the epimerization, although the protecting groups conventionally used on the His residue with Boc chemistry are introduced at the τ-nitrogen, except for the Bom group. In the comparison of His(π-Bom) with several τ-protected His derivatives including His(τ-Bzl) and His(τ-Bom), both of which can not prevent epimerization on activation and coupling [13], the duration required to accomplish Boc-deprotection on the His(π-Bom) residue was much longer than those on others (Figure 3).

On the basis of the behaviour of Boc-His-OMe in TFA, increasing the electron-withdrawing characteristics of τ-protecting groups (e.g. Dnp and Tos) to attenuate the basicity of the imidazole ring tended to improve removability of the Boc group. In contrast, the τ-alkyl group (*i.e.* τ-Bom) which does not diminish the basicity was observed to impair Boc-deprotection. Although electron-withdrawing groups were introduced at the τ-position to attenuate the basicity, the Boc group on His(τ-2-Adoc) [14] and His(τ-Doc) [15] possessed comparable removability to that on His-OMe. This might be related to the steric bulk of these groups. A factor other than the basicity of the π and τ nitrogen atoms appeared to be responsible for the resistance to TFA with Boc-deprotection from the His(π-Bom)

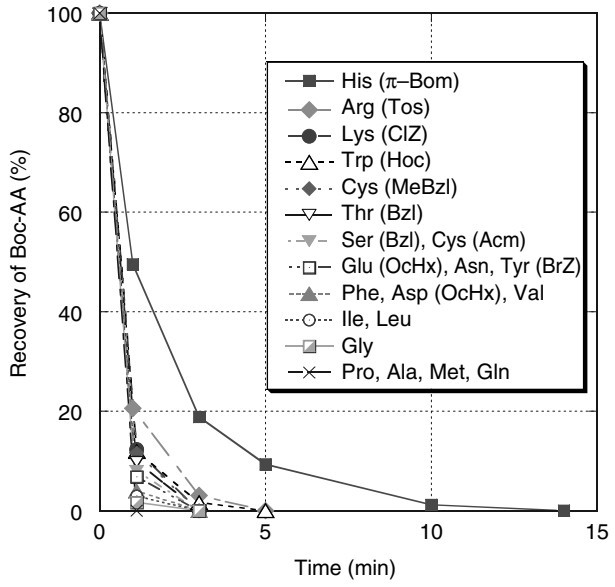

Figure 2 Removability of the Boc group from Boc-amino acids by 50% TFA/DCM.

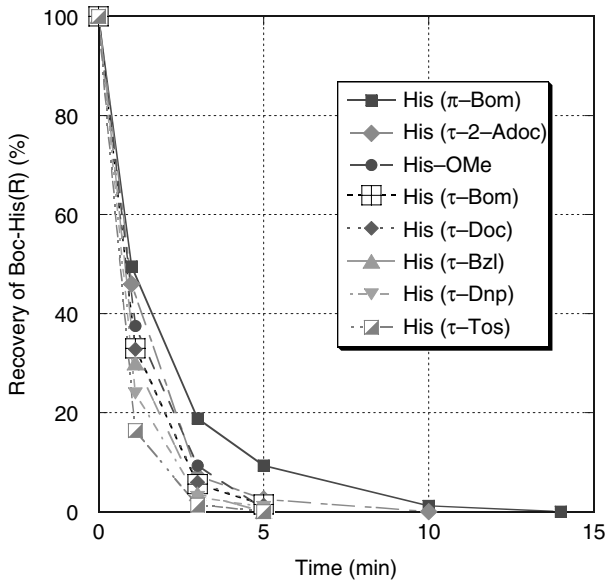

Figure 3 Removability of the Boc group from Boc-His derivatives by 50% TFA/DCM.

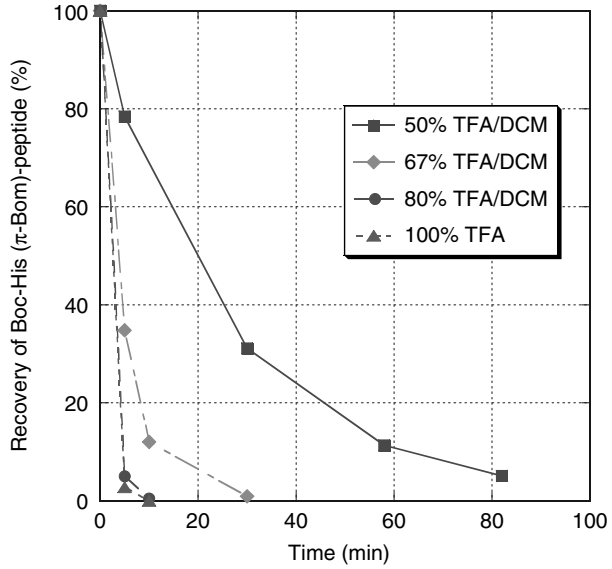

Figure 4 Removability of the Boc group from Boc-His(π-Bom)-Asp(OcHx)-Ser(Bzl)-Gly-OPac by various TFA concentrations.

residue. Furthermore, in the model experiment of the peptide having the Boc-His(π-Bom) residue located at the *N*-terminus, the Boc group was more resistant to TFA treatment than that in the case of the amino acid derivative (Figure 4).

The duration of TFA treatment decreased as the concentrations of TFA increased. However, regarding the concentration of TFA, there was no differentiation observed with reducing effect on Boc-deprotection time beyond 80% TFA. Because of the disadvantages of 100% TFA leading to poor swelling of resin especially in the initial deprotection cycle [16] and causing partial cleavage of side-chain protecting groups of the peptide chain and anchoring groups in the repetitive deprotection cycle, treatment with 80% TFA seemed to be suited for removing the Boc group from the peptide resin with the His(π-Bom) residue located at the *N*-terminus.

The His(π-Bom) derivative has been frequently used in Boc chemistry because it is susceptible in HF [8]. However, HF treatment generates 1 mole of formaldehyde from the Bom group, which leads to problems with other functional groups in the same peptide. In particular, when a Cys residue is located at the *N*-terminus of the peptide, it is cyclized to form a thiazolidine ring by the formaldehyde. This side reaction can be suppressed by the addition of excess free Cys as a scavenger to the HF reaction mixture [17]. Another reason for the failure of N^{α}-Boc deprotection associated with the use of His(π-Bom) could occur under TFA treatment for the standard SPPS even in the case of a non 'difficult sequence'. The results in the present study suggested that more attention should be paid to the complete deprotection of the Boc group on His(π-Bom) residue by prolonging the duration of TFA treatment and/or increasing the TFA concentrations.

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