# Unexpected Chain-Terminating Side Reaction Caused by Histidine and Acetic Anhydride in Solid-Phase Peptide Synthesis[1)]

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**Capping is a useful technique to facilitate purification of a crude deprotected peptide in solid-phase peptide synthesis. However, we observed a serious side reaction caused by the $Ac_2O$ capping procedure, when it was applied to a synthesis of a peptide containing His. The mechanism of the side reaction was studied.**

**Keywords** side reaction; termination; capping; acetic anhydride; 1-hydroxybenzotriazole; π-tosylhistidine; peptide; solid-phase synthesis

An automated solid-phase peptide synthesizer in combination with high-performance liquid chromatography (HPLC) is a powerful tool for preparing biologically active peptides with minimum expenditure of time and labor. However, the shortcoming of the solid-phase method is that purification of a synthetic intermediate is not possible, and purification of a crude deprotected peptide often becomes a difficult and time-consuming step. Capping procedures have been developed to facilitate the purification step and are widely used. In the course of solid-phase syntheses of long-chain peptides, however, we observed that syntheses with the capping procedure gave worse results than syntheses without the capping procedure when the peptides contained His residue(s). Here we describe a side reaction of His(Tos) and $Ac_2O$.

In order to simplify the analysis of the side reaction, model peptides were synthesized. Peptide syntheses with or without capping were carried out using the Boc chemistry as shown in Fig. 1. Protected amino acids were activated to preformed symmetric anhydrides except for Asn, Gln, and Arg. They were activated to HOBt active esters. Peptides were deprotected and cleaved from resins with HF. The crude products were analyzed and purified by HPLC on a C18 reversed-phase column.

A synthesis without the capping procedure gave a model peptide (**1**) with a purity of 70%. However, a synthesis with the $Ac_2O$ capping procedure gave a complex crude mixture, and the purity of **1** was only 27% (Fig. 2). Side products were purified by preparative HPLC and they were determined to be terminated peptides by mass spectral analyses (Table I). The purpose of the capping procedure is to terminate unreacted free amino terminals, but quantitative ninhydrin analyses[2)] indicated that such amino terminals were less than 0.3% in each step in the case of the synthesis with capping. So side products were derived from an unexpected side reaction. The Tos group is the most widely used protecting group for His. It was, however, removed by

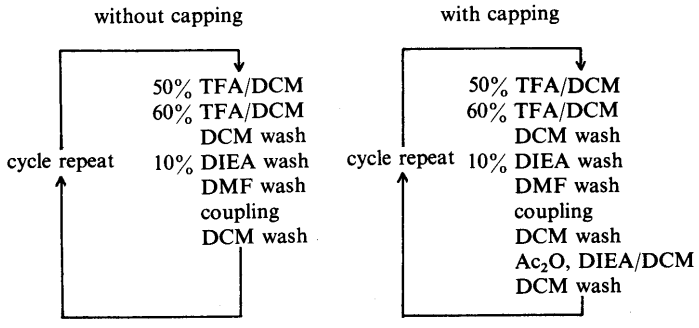


Fig. 1. Scheme of Peptide Synthesis Cycle

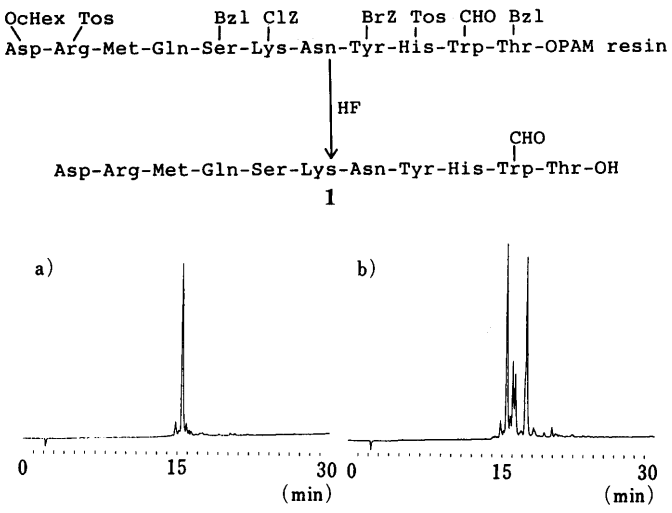


Fig. 2. HPLC of the Crude Model Peptide

a) Prepared without capping. b) Prepared with capping.

TABLE I. Structures of Side Products

| | $t_R$[a)] (min) | $m/z$[b)] | Structure |
|---|---|---|---|
| **2** | 16.0 | 1005 (M+1) | Ac–Ser–Lys–Asn–Tyr–His–Trp(CHO)–Thr–OH |
| **3** | 16.3 | 1536 (M+1) | Ac–Asp–Arg–Met–Gln–Ser–Lys–Asn–Tyr–His–Trp(CHO)–Thr–OH |
| **4** | 17.2 | 1264 (M+1) | Ac–Met–Gln–Ser–Lys–Asn–Tyr–His–Trp(CHO)–Thr–OH |
| **5** | 17.4 | 790 (M+1) | Ac–Asn–Tyr–His–Trp(CHO)–Thr–OH |

*a*) HPLC analysis data. YMC AM302 (C18, 5 μ, 4.0 mm × 150 mm), 1 ml/min, 0.1% aqueous TFA–0.1% TFA in $CH_3CN$ gradient elution (0%—50%, 30 min), ultraviolet (UV) 214 nm. *b*) Fast atom bombardment-MS.

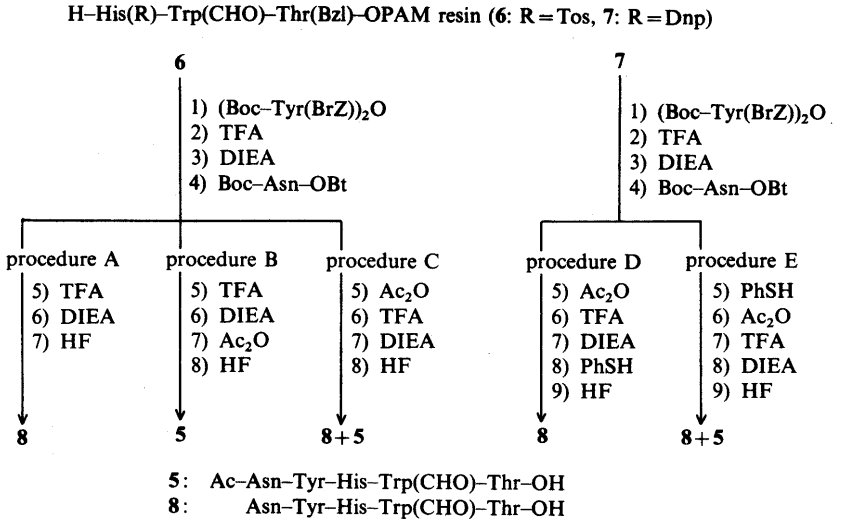


Fig. 3. $Ac_2O$ Treatments of Protected Peptide-Resins

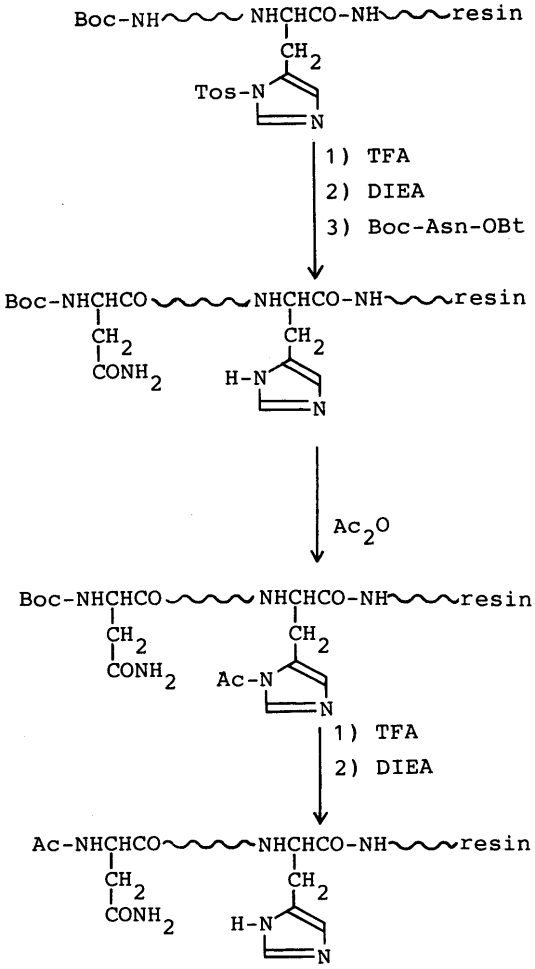


Fig. 4. Mechanism of Side Reaction

HOBt,[3] a common reagent in peptide synthesis, and side reactions involving the free imidazolyl group of His were reported.[4] In the case of the synthesis of **1**, HOBt active ester of Boc–Asn was used after incorporation of His(Tos).

The following model studies were performed to clarify the mechanism of the side reaction (Fig. 3). Peptide-resins (**6** and **7**) were prepared without the capping procedure. Authentic samples, Asn–Tyr–His–Trp(CHO)–Thr–OH (**8**) and its acetyl derivative (**5**), were prepared by procedure A and by procedure B, respectively. As shown in Fig. 1, where the $Ac_2O$ capping procedure was employed in the synthesis of **1**, the last step of a cycle was $Ac_2O$ treatment, and the first step of the next cycle was TFA treatment followed by DIEA treatment. So steps from 5 to 7 of procedure C were part of the actual synthesis of **1** from **6**, but procedure C gave a mixture of **8** and **5** in the ratio of 82 : 18. When His was protected with a Dnp group, procedure D, essentially the same procedure as procedure C (an extra PhSH treatment was added to remove Dnp), gave only **8**. When the Dnp group was removed before $Ac_2O$ treatment, a mixture of **8** and **5** in the ratio of 72 : 28 was obtained (procedure E). The results of procedures D and E indicated that the free imidazole ring of His at the acetylation step was necessary for the side reaction. When His(Tos) was used as the protected His residue, the Tos group was partially or fully deprotected with HOBt at the HOBt active ester coupling step, and $\pi$-acetylation of the non-protected His residue followed by acetyl transfer to the N-terminal caused the unexpected chain termination (Fig. 4). Barany and Merrifield pointed out that $\pi$-acylation with an activated amino acid was not an important problem in most synthetic schemes.[4] However, we revealed that $\pi$-acetylation gave rise to a serious problem and that His(Tos), use of HOBt, and the $Ac_2O$ capping procedure are not compatible.

In conclusion, we revealed that the combination of the common reagents, His(Tos) and HOBt, and the widely used $Ac_2O$ capping procedure causes a serious chain-terminating side reaction.

**Experimental**

Peptide syntheses were performed on an Applied Biosystems model 430A peptide synthesizer. HPLC was conducted with a Waters model M-600 and a Japan Spectroscopic model Twincle with a solvent programmer. Preparative HPLC was conducted on a C18 column (YMC, 20 mm × 300 mm, 5 $\mu$). Amino acid compositions of acid hydrolysates (110 °C, 24 h, 6 N HCl) were determined with a Hitachi 835 amino acid analyzer. Mass spectra (MS) were recorded on a JEOL HX-110 spectrometer.

**Asp–Arg–Met–Gln–Ser–Lys–Asn–Tyr–His–Trp(CHO)–Thr–OH (1)** Without Capping: Boc–Thr(Bzl)–OPAM resin (0.5 mmol) was used as a

solid support. Protected amino acids (2 mmol) were activated and coupled to the resin sequentially according to the procedures shown in Fig. 1. Boc–His(Tos) was used as a protected His residue. A portion of the resin was taken in each step for ninhydrin monitoring. Removal of the N-terminal Boc group afforded the protected peptide-resin (1.43 g, 86%). The resin (400 mg) was stirred with HF (9 ml) and anisole (1 ml) at −2 °C for 1 h. The HF and anisole were evaporated off *in vacuo* at the same temperature. The residue was washed with ether (2 × 20 ml), and extracted with 2 N AcOH (2 × 20 ml) and with 0.1 N AcOH (100 ml). After filtration, the combined extract was lyophilized to afford a colorless fluffy powder (151 mg). The crude product was purified by preparative HPLC using 0.1% aqueous TFA–$CH_3CN$ (injection 3 times, 13%—18% gradient, 80 min, 10 ml/min) as an eluent. The main peak was collected and lyophilized, followed by ion exchange with Dowex 1 × 2 ($AcO^-$ form) and lyophilization to afford **1** as a colorless fluffy powder, yield 77.1 mg, $t_R$ 15.5 min (YMC AM302, 4.6 mm × 150 mm, 5 $\mu$, 0.1% aq. TFA–0.1% TFA in $CH_3CN$ (0%—50% gradient, 30 min), 1 ml/min, UV 214 nm). MS *m/z*: 1493 ($M^+ + 1$). Amino acid ratio in acid hydrolysate: Arg, 0.89; Asp, 1.84; Glu, 1.00; His, 0.88; Lys, 0.94; Met, 0.97; Ser, 0.95; Thr, 0.94; Tyr, 1.00; $NH_3$, 1.86.

With Capping: Crude deprotected peptide (115 mg) was prepared by using essentially the same procedure as mentioned above except for capping reaction performed at each step of the chain elongation (Fig. 1). Five components of the crude peptide were purified by preparative HPLC (injection 6 times, 15%—18% gradient, 80 min, 10 ml/min). Each peak was collected and lyophilized to afford a fluffy powder, **1** (29.7 mg), **2** (0.4 mg), **3** (2.2 mg), **4** (4.6 mg), and **5** (13.2 mg).

**General Procedures of the Studies on $Ac_2O$ Treatment of Protected Peptide-Resins** Removal of Boc Group and Neutralization: Protected peptide-resin was vortexed with 50% TFA in DCM for 60 s and 60% TFA in DCM for 14 min successively. The resin was washed with DCM three times. The resin was treated twice with 10% DIEA in DCM for 45 s, and then washed with DCM.

Treatment with $Ac_2O$: The resin was vortexed with 25% $Ac_2O$ in DCM for 10 min and washed with DCM. The resin was then treated twice with 10% DIEA in DCM for 45 s, and washed with DCM.

Removal of Dnp: The resin was stirred twice with 5% PhSH in DMF for 30 min, then washed with DMF and DCM three times each.

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**References and Notes**

1) Abbreviations: Boc = *tert*-butoxycarbonyl, BrZ = 2-bromobenzyloxycarbonyl, Bzl = benzyl, ClZ = 2-chlorobenzyloxycarbonyl, cHex = cyclohexyl, DCM = dichloromethane, DIEA = diisopropylethylamine, DMF = dimethylformamide, Dnp = 2,4-dinitrophenyl, HOBt = 1-hydroxybenzotriazole, PAM = *p*-hydroxymethylphenylacetamidomethyl, PhSH = thiophenol, TFA = trifluoroacetic acid, Tos = *p*-toluenesulfonyl.
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