The Modification of Tryptophyl Residues during the Acidolytic Cleavage of Boc-groups. I. Studies with Boc-Tryptophan[1]

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The formation of a component, X, was observed when a reaction mixture of *t*-butyloxycarbonyl-tryptophan (Boc-Trp), trifluoroacetic acid (TFA), and 1,2-ethanedithiol was analyzed by cellulose TLC. The side product was isolated and further separated into two components by column chromatography on silica gel. The subsequent analysis of the NMR spectra of these components confirmed that they were N^{in}-Bu^t-Trp and C^{in}-Bu^t-Trp.** The reaction of Boc-Trp with TFA and with anhydrous hydrogen fluoride (HF) was examined as model systems in order to determine conditions which would minimize the formation of the X component during the synthesis of peptides containing Trp. The addition of 10 molar equivalents of 1,2-ethanedithiol completely suppressed the formation of the X component in HF, but additives were less efficient in TFA. A combination of dimethyl sulfide and 1,2-ethanedithiol was the most effective method for suppressing the formation of the X component in TFA. The behavior of N^{in}- and C^{in}-Bu^t-Trp in acidic media was also investigated, and a possible pathway for the *t*-butylation of the tryptophyl residue will be proposed.

The acidolytic cleavage of protective groups from Trp-containing peptides is known to be accompanied by several side reactions. The first group of side reactions is the so-called oxidative destruction of the tryptophyl residue. This is a series of complicated reactions of the highly reactive indole ring characterized by coloration[2] or by an increase in the fluorescence[3] of the reaction mixture. These reactions can be effectively avoided by the addition of such antioxidants as 2-mercaptoethanol[4] or 1,2-ethanedithiol[2] to the acidic reagents. The second group of side reactions is the alkylation of the tryptophyl residue by fragments of the protective groups. The *t*-butylation of the indole ring during the deprotection of the Boc groups was first observed in 1970 by Alakhov *et al.*[5] using mass spectrometry. Boc-Trp-Gly-OMe was treated with TFA to remove the Boc group. The newly formed amino group was acetylated, and the product was subjected to mass spectrometry. The spectrum indicated the presence of an ion at *m/e*(M+56) in addition to the original molecular ion. Alakhov *et al.* also observed that 95% TFA gave less butylated by-products, as did 2 M HCl in acetic acid or ethyl acetate. The formation of side products was still noticeable in all cases. In 1972, Wünsch *et al.*[6] reported that a significant amount of side products was formed when a protected analog of human gastrin was treated with TFA to remove the Boc and Bu^t groups. They later isolated a side product and found that the tryptophyl residue was *t*-butylated at the NH group of the indole ring.[7,8] Kisfaludy *et al.*[7] observed a similar phenomenon during the synthesis of ACTH. They then examined the reaction of Trp with isobutylene under relatively drastic conditions and isolated a crystalline product, 2,5,7-tri-Bu^t-Trp. From these observations, Wünsch and Kisfaludy concluded that the tryptophyl residue is readily converted to mono-or multi-butylated Trp by isobutylene in acid or during the deprotection of Boc or Bu^t groups with TFA, and that N^{in}-Bu^t-Trp was a major product. They emphasized that HF caused more alkylation of the tryptophyl residue than did TFA and that the yield of alkylated products was as high as 50%.[7]

We also observed that side products, detectable by TLC, were produced when Trp-containing peptides were treated with TFA to remove the Boc group, even though 1,2-ethanedithiol was added to the system. We examined the reaction of Boc-Trp with TFA and with HF as model systems. TFA and HF were chosen as acids since they are commonly used active reagents for the removal of protective groups and also since they are excellent solvents for almost all peptide derivatives.

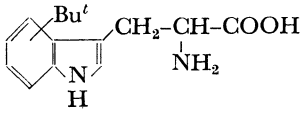
This paper will deal with the isolation of *t*-butylated products from the reaction mixture and with the evaluation of additives in the deprotection reaction, with the goal of minimizing the formation of side products. We will also discuss the different characteristics of HF and TFA as deprotecting reagents.

Results

Isolation and Structure Determination of Side Products. The cellulose TLC of reaction mixtures of Boc-Trp with TFA containing 1,2-ethanedithiol always indicated the presence of one major side component (X component) and some minor ones, as is shown in Fig. 1(A). The isolation of the X component from the reaction mixture was achieved by column chromatography on Dia-ion HP-20. The reaction mixture was placed in the top of the column, and Trp was washed out with 20% aqueous methanol. The minor components were eluted by increasing the concentration of methanol to 30%, and then to 40%. X component was eluted with 50% aqueous methanol. The yield of the X component was about 10% of the starting material. Silica gel TLC [Fig. 1(B)] revealed that the X component consisted primarily of almost equal amounts of two sub-components, Xa and Xb, which were both ninhydrin- and Ehrlich-positive. Therefore, these two components were separated by column chromatography on silica gel.

Elemental analysis showed that both components, Xa

** C^{in}-Bu^t-Trp:



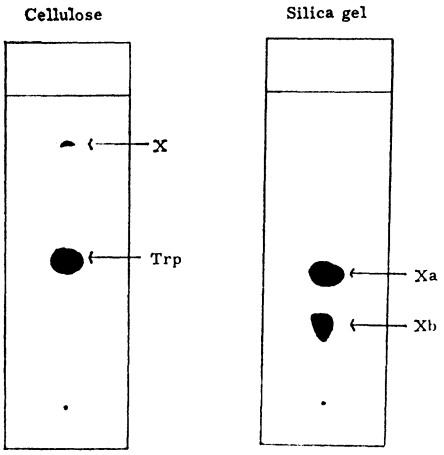


Fig. 1. TLC of products of Boc-Trp in TFA containing 1,2-ethanedithiol.

and Xb, had the same composition, mono-*t*-butylated Trp. The ^{1}H-NMR spectra of the Xa component in a mixture of C_5D_5N–D_2O (1 : 1) and in a mixture of DMSO-d_6–D_2O (1 : 1) (Fig. 2) clearly indicated that one Bu^t group was attached at the indole nitrogen of Trp. This conclusion was also confirmed by the analysis of the ^{13}C-NMR spectrum. However, the ^{1}H-NMR spectrum of the Xb component indicated 4H on the indole carbons and one Bu^t group at a carbon somewhere in the indole ring, but the location of the Bu^t group could not be determined. It was considered that the Xb component was a mixture of mono-*t*-butylated tryptophans substituted at various positions on the indole ring.

Determination of the X Component and Trp; Effect of Additives as Scavengers. The elution pattern of

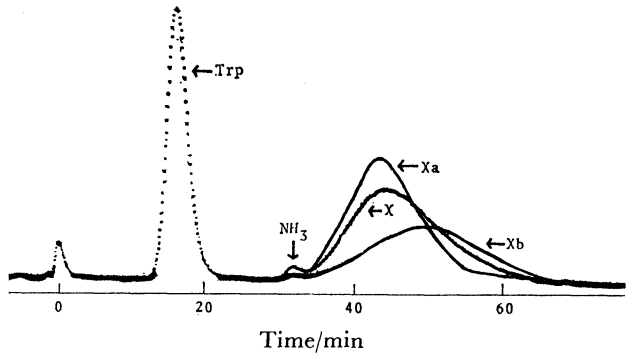


Fig. 3. Ion-exchange chromatogram of Trp, X, Xa, and Xb by amino-acid analyzer.

Xa, Xb, and Trp on an amino-acid analyzer is shown in Fig. 3. No efficient condition was found for determining Xa and Xb separately on the same chromatogram. Therefore, an isolated X component was used as a standard for measuring the total amount of mono-*t*-butylated Trp, Xa and Xb, on an amino-acid analyzer.

By using this procedure, we measured the *t*-butylated Trp and the intact Trp in reaction mixtures of Boc-Trp in TFA and in HF under various conditions (Tables 1 and 2). If no scavenger was added with the TFA, the reaction mixture turned brown, a large quantity of the X component was formed (14%), and the recovery of Trp was low (67%). The addition of 1,2-ethanedithiol effectively prevented coloration, but the addition of even 50% of this material to the TFA failed to reduce the formation of the X component to less than 4.2%. A combination of a large excess of dimethyl sulfide with 1,2-ethanedithiol was the best scavenger in TFA. This mixture gave the maximum recovery of intact Trp after deprotection, yet the formation of the X component was significant (see Table 1). The addition of anisole, which is frequently used as a scavenger during acidolysis, was ineffective in suppressing *t*-butylation.[9]

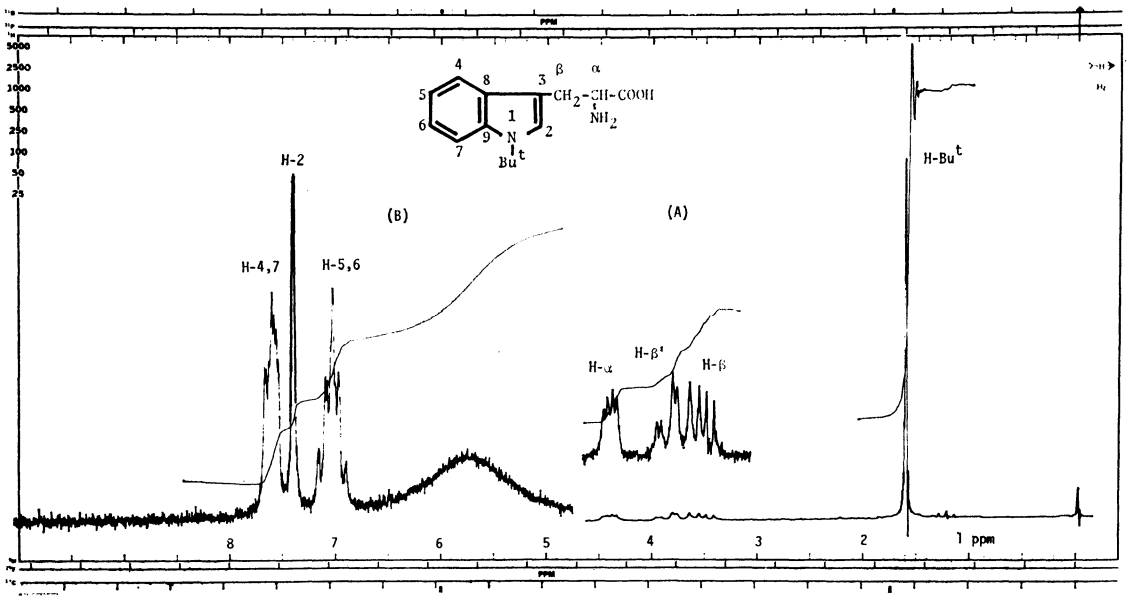


Fig. 2. NMR spectra of Xa in C_5D_5N–D_2O (1 : 1)(A) and in DMSO-d_6–D_2O (1 : 1)(B) at 100 MHz.

Table 1. Effect of additives on the formation of X component in TFA[a)]

Additives		Recovery/%	
1,2-Ethanedithiol	Others	X	Trp
None		14.2	67
1.0 ml	None	4.2	93
0.3 ml		5.1	91
	Me-S-Me 1.0 ml[b)]	1.1	99
0.1 ml	0.5 ml	2.6	97
	0.3 ml	4.3	96
	Me-S-Et 1.0 ml	2.6	97
	Me-S-Ph 1.0 ml	3.2	96
0.1 ml	Et-S-Pr^i 1.0 ml	4.9	94
	Pr^i-S-Pr^i 1.0 ml	6.4	92
	Anisole 1.0 ml	6.3	91

a) For the reaction conditions, see the Experimental section. b) 137 equiv against Boc-Trp.

Table 2. Effect of additives on the formation of X component in HF[a)]

Additives	Amount (equiv)[b)]	Recovery/%	
		X	Trp
None		15.6	57
1,2-Ethanedithiol	10	ND[c)]	99
Anisole+E[d)]	10+10	ND[c)]	99
Me-S-Me+E[d)]	10+10	ND[c)]	99
Anisole	10	2.4	94

a) For the reaction conditions, see the Experimental section. b) Equiv against Boc-Trp. c) Not detectable. d) 1,2-Ethanedithiol.

On the other hand, the formation of the X component in HF was negligible when 10 equivalents of 1,2-ethanedithiol were added to the reaction mixture, even without the addition of anisole or dimethyl sulfide (Table 2). This result differs from that reported in the absence of scavengers by Wünsch *et al.*[7,10] Scavengers seem to be effective in HF, but not in TFA.

Behavior of Xa and Xb in Acidic Reagents. The chemical stability of Bu^t groups of Xa and Xb was examined in acidic reagents. The Xa and Xb were dissolved separately in TFA and in HF in the presence of additives, and the reaction mixtures were examined with an amino-acid analyzer and by TLC. As is shown in Table 3, over 90% of the Xa was converted to Trp in HF, which indicates that the N^{in}-Bu^t-group is quite unstable in HF. In TFA, however, only 50% of the N^{in}-Bu^t-group was removed, and about 10% of the starting Xa was converted to Xb during the treatment. No formation of N^{in},C^{in}-di-*t*-butylated Trp was observed. Although a small amount of Trp was detectable in a reaction mixture of Xb with HF, Xb seemed to be fairly stable in both reagents, as was to be expected. Interestingly, the C^{in}-Bu^t-group was removable under the conditions of the acid hydrolysis of peptides.

Table 3. Stability of Bu^t groups in N^{in}-Bu^t-Trp (Xa) and C^{in}-Bu^t-Trp (Xb) in acidic media

Conditions	Xa Recovery/%		Xb Recovery/%	
	Trp	Xa+Xb	Trp	Xb
HF/HS-CH_2-CH_2-SH/Anisole 0 °C, 1 h	92	Trace	5	90
TFA/HS-CH_2-CH_2-SH 0 °C, 10 min and rt, 50 min	50	40+10[a)]	None	100
6 M HCl/Mercaptoacetic Acid[11)] 108 °C, 22 h	99	None	84	Trace

a) The ratio of Xa and Xb was estimated from the silica gel TLC.

Discussion

In discussing the modification of tryptophyl residues during deprotection procedures, the contribution of scavengers cannot be eliminated. Wünsch and others have shown that the modification of the tryptophyl residues is higher in HF than in TFA when no scavenger is added to the reaction mixture. Similar results were obtained in the reactions of Boc-Trp in this study; the recovery of intact Trp was 67% in TFA and 57% in HF under the conditions used for the removal of Boc groups (see Tables 1 and 2). In the presence of scavengers, however, the situation changed dramatically. The formation of *t*-butylated Trp was negligible in HF when 10 molar equivalents of 1,2-ethanedithiol were added, but a considerable amount of modified Trp was detectable in reaction mixtures with TFA, even when an excess of promising scavengers was added. This result may be explained by the reactivity with acidic reagents of Bu^t groups attached to the N or C atom of the indole group. As can be seen in Table 3, the N^{in}-Bu^t-group is reasonably stable in TFA, but quite unstable in HF. Therefore, in HF the scavengers must compete with the C^{in}-*t*-butylation reaction. The effectiveness of scavengers in HF can be clearly explained if the *S*-*t*-butylation is much faster at 0 °C than the *C*-*t*-butylation of the indole moiety. In TFA at room temperature, however, the scavenger must compete with the N^{in}-*t*-butylation reaction and the formation of N^{in}-Bu^t-Trp may be much faster than the formation of the *S*-Bu^t-derivatives of scavengers.

In the presence of 10% 1,2-ethanedithiol in TFA, the formation of C^{in}-Bu^t-Trp from N^{in}-Bu^t-Trp was 10%, and 50% of the N^{in}-Bu^t-Trp was converted to Trp. Under these conditions, the formation of C^{in}-Bu^t-Trp from Boc-Trp was only 5%. No *t*-butylation at the indole nucleus was observed when Boc-Trp(For)*** was treated with TFA (see Experimental section). The above facts suggest that N^{in}-Bu^t-Trp is more reactive with Bu^t cations than is an unsubstituted tryptophyl residue. We therefore propose the following scheme for the formation of these side products in TFA: (1) N^{in}-*t*-butylation is the first step in the side reaction, (2) once a Bu^t group is attached to the indole nitrogen, the carbon atoms of the indole moiety become more susceptible to attack by Bu^t cations, and (3) the N^{in}, C^{in}-

*** For: N^{in}-formyl.

di-Bu^t-Trp thus formed degrades to C^{in}-Bu^t-Trp.

It must be noted that the formation of *t*-butylated Trp cannot be detected after the acid hydrolysis of the peptide (see Table 3). Thus, *t*-butylation must be detected in the intact peptide or after the enzymatic digestion of the peptide.

Since the present study used Boc-Trp as a model compound, the above observations must be confirmed with peptides which are protected with Boc or Bu^t groups. These studies are now being carried out in our laboratory.

Experimental

All melting points are uncorrected. Amino-acid analysis was performed on a JEOL JLC-6AH Amino-Acid Analyzer. The UV spectra were obtained on a Hitachi 124 Spectrophotometer, and the IR spectra, on a Hitachi EPI-G2 Grating Infrared Spectrophotometer. The optical rotations were measured with a Perkin-Elmer 141 Polarimeter. The ^{1}H-NMR and ^{13}C-NMR measurements were performed with a Varian XL-100 Spectrometer, using tetramethylsilane as the internal reference. The solvent systems used for TLC on silica gel G (Merck Art 7731) or cellulose (Merck Art 5552) were (A) 1-butanol–acetic acid–water (4: 1: 5, upper phase) and (B) chloroform–methanol–acetic acid (85: 15: 2). For silica gel-column chromatography, silica gel 60 (Merck Art 7734) was used.

Isolation of By-products, Xa and Xb. A solution of Boc-Trp (10 g, 33 mmol) in TFA (30 ml) containing 1,2-ethanedithiol (3 ml) was allowed to react at 0 °C for 10 min and then at room temperature for 50 min. The TLC of this reaction mixture is shown in Fig. 1(A). The solution was concentrated under reduced pressure, and the residue was triturated with ether and hexane to obtain a powder. The product was collected by filtration and dried in a vacuum desiccator over sodium hydroxide pellets at room temperature for 24 h. The dried material was dissolved in a mixture of water (400 ml) and methanol (20 ml), and the solution was neutralized with 5% aq sodium hydrogencarbonate. This solution was then poured into the top of a column (4×20 cm) of Dia-ion HP-20, Mitsubishi Kasei Co., Ltd., which was then eluted with 2 l of water, 1-l portions each of 20%, 30%, and 40% aq methanol, and 2 l of 50% aq methanol successively. Each fraction was monitored by TLC, the X component was found to be eluted by 50% aq methanol. This fraction was then collected and condensed. The residue was purified by reprecipitation from methanol with ether and dried over P_2O_5 at 35 °C for 24 h; yield, 1.0 g (11%); R_f 0.85 on cellulose TLC using Solvent A. Found: C, 67.79; H, 8.09; N, 10.22%. Calcd for $C_{11}H_{11}O_2N_2 \cdot C(CH_3)_3 \cdot 1/3\ H_2O$: C, 67.64; H, 7.82; N, 10.52%.

This product, X component, gave two spots on silica gel TLC, as is shown in Fig. 1(B). It was applied to the top of a column (3×20 cm) of silica gel and was eluted with a mixture of chloroform–methanol–acetic acid (85: 15: 2).

The fraction containing Xa was collected and concentrated to a residue, which was then triturated with ether to obtain a powder. The product was purified by reprecipitation from methanol with ether and dried over P_2O_5 *in vacuo* at 45 °C for 120 h; yield, 421 mg (4.9%); mp 179 °C (dec). $[\alpha]_D^{29.5}$ −29.6° (c 0.5, 80% aq ethanol). UV (H_2O) max: 286 nm (ε 6000), 223 nm (ε 37000). ^{1}H-NMR [100 MHz, C_5D_5N–D_2O (1: 1)]: H-Bu^t: δ 1.61 (9H, s), H-β: 3.55 (1H, dd, J=9.0, 16.0 Hz), H-β': 3.91 (1H, dd, J=4.0, 16.0), H-α: 4.44 (1H, dd, J=4.0, 9.0), [100 MHz, DMSO-d_6–D_2O (1:1)]: H-5, 6: 7.03 (2H, m), H-2: 7.36 (1H, s), H-4, 7: 7.62 (2H, d, J=9.0), [100 MHz, DMSO-d_6]: Absence of indole NH signal. ^{13}C-NMR [25.2 MHz, DMSO-d_6]: C-β: δ 27.1, *C*-methyl (Bu^t): 29.2, C-α: 55.0, *C*-methine (Bu^t): 55.6, C-3: 107.9, C-7: 111.5, C-4: 118.2, C-6: 119.2, C-5: 120.7, C-2: 125.6, C-8: 129.6, C-9: 135.3, C-(C=O): 171.4. IR(KBr): 2955 cm^{-1} (ν_{C-H}, Bu^t), 1215 cm^{-1} (ν_{C-C}, Bu^t). Found: C, 69.50; H, 7.63; N, 10.78%. Calcd for $C_{11}H_{11}O_2N_2 \cdot C(CH_3)_3$: C, 69.20; H, 7.74; N, 10.76%. From the above data, the Xa component was identified as N^{in}-Bu^t-Trp.

The fraction containing Xb was collected and treated in the same manner as Xa; yield, 455 mg (5.3%). ^{1}H-NMR [100 MHz, DMSO-d_6]: H-Bu^t: δ 1.32, 1.34, 1.44 (9H, three singlet signals), H-indole ring: 6.90—7.54 (4H, unresolved signals). Found: C, 69.31; H, 7.69; N, 10.73%. Calcd for $C_{11}H_{11}O_2N_2 \cdot C(CH_3)_3$: C, 69.20; H, 7.74; N, 10.76%. From the above data, the Xb component was considered to be mono-*t*-butylated Trp, substituted at various positions on C atoms of the indole ring.

Determination of X Component and Trp on the Amino-acid Analyzer. A mixture of X component (0.35 μmol) and Trp (0.20 μmol) was analyzed on the amino-acid analyzer under the following conditions: length of column with LC-R-2 resin, ϕ 0.8×15 cm; solvent, 0.5 M sodium citrate buffer at pH 5.24 containing 1% benzyl alcohol; flow rate, 1.0 ml/min; jacket temperature, 52 °C. The pattern obtained is shown in Fig. 3.

Reaction of Boc-Trp with Cleavage Reagents for the Evaluation of Additives. *(A) Reaction with TFA*: Boc-Trp (30.4 mg, 0.1 mmol) was dissolved in TFA (1 ml) containing various additives, and the solution was stirred at 0 °C for 10 min and at room temperature for 50 min. The reaction mixture was then concentrated *in vacuo*, and the residue was diluted with 0.5 M sodium citrate buffer (pH 5.20) to 10 μmol/ml and to 0.2 μmol/ml. These solutions were then analyzed under the conditions described above for determining the recovery of X component and Trp respectively. The analytical data are shown in Table 1.

(B) Reaction with HF: Boc-Trp (30.4 mg, 0.1 mmol) was treated with HF (1 ml) in the presence of various additives at 0 °C for 1 h in the usual manner.[12] The reaction mixture was concentrated at reduced pressure, and the residue was redissolved in acetic acid (10 ml). The solvent was evaporated *in vacuo*, and the residue was diluted with 0.5 M sodium citrate buffer (pH 5.20). The recovery of X component and Trp was determined in the same manner as has been described previously in the case of TFA treatment. X component was not detected on the chromatogram of amino-acid analysis using only 10 equiv of 1,2-ethanedithiol as an additive. The analytical data are shown in Table 2.

Reaction of Xa or Xb with Acids. *(A) Reaction of Xa or Xb with TFA*: Xa (26.0 mg, 0.1 mmol) was treated with TFA (1 ml) in the presence of 1,2-ethanedithiol (0.1 ml) at 0 °C for 10 min, the solution was then maintained at room temperature for 50 min and worked up as has been described for the reaction of Boc-Trp with TFA. The recovery of Trp and X was determined on the amino-acid analyzer. A part of the reaction mixture was placed in the top of the column of Dia-ion HP-20 and eluted with 20% aq methanol, and then methanol. When each fraction was monitored by TLC, only Trp was detected in the 20% aq methanol fraction. X component was found in the methanol fraction. This fraction was collected, concentrated, and analyzed by TLC on a silica gel G plate using the B solvent system. The ratio of Xa and Xb was estimated to be 4: 1, and N^{in},C^{in}-di-Bu^t-Trp was not detected. The analytical data are shown in Table 3. Xb (26.0 mg, 0.1 mmol) was treated in the same manner as Xa.

(B) Reaction of Xa or Xb with HF: Xa (26.0 mg, 0.1 mmol) was treated with HF (1 ml) in the presence of 1,2-ethanedithiol (0.1 ml) and anisole (0.1 ml) at 0 °C for 1 h and then worked up as has been described for the reaction of Boc-Trp with HF. The recovery of Trp and X component was determined on the amino-acid analyzer. The treatment of Xb (26.0 mg, 0.1 mmol) was carried out in the same manner as Xa.

(C) Reaction of Xa or Xb with 6 M HCl: Xa (2.6 mg, 10 μmol) was treated with 6 M HCl in the presence of 2% mercaptoacetic acid[11] in an evacuated, sealed ampule at 108 °C for 22 h and then analyzed on the amino-acid analyzer. Xb (2.6 mg, 10 μmol) was treated in the same manner as Xa.

Reaction of Boc-Trp(For)[13] with TFA. Boc-Trp(For) (33.2 mg, 0.1 mmol) was treated with TFA (1 ml) containing 1,2-ethanedithiol (0.1 ml) at 0 °C for 10 min, and then the mixture was maintained at room temperature for 50 min. The reaction mixture was concentrated and the residue was treated with 0.1 M piperizine (1 ml) for 1 h at room temperature to remove the For group. The solution was then applied into the top of a column of Dia-ion HP-20 and eluted with water, 20% aq methanol, and methanol. Each fraction was collected and analyzed by TLC. Only Trp was eluted by 20% aq methanol. Although other by-products were present in the methanol fractions, Xa and Xb were not detected. Thus, no *t*-butylation occurred during this reaction.

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