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# Synthesis and Application of Fmoc-His(3-Bum)-OH†

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Abstract: This paper presents a reevaluation of the synthesis and properties of Fmoc-His(3-Bum)-OH regarding its application in SPPS with minimal racemization of histidine residues during coupling and esterification reactions. By-product formation during the deprotection of the test peptides could be significantly reduced by scavenging the concomitantly formed HCHO, e.g. with methoxyamine. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: histidine; *im*-Bum protection; racemization; SPPS

## INTRODUCTION

Carboxy-activated derivatives of $N^{\alpha}$-protected histidine are notoriously difficult to prevent from racemization which is promoted by the $\pi$-nitrogen of the imidazole ring. Only blocking of the $N^{\pi}$-position is believed to reliably suppress this undesired side reaction. However, mixtures of regioisomers result when introducing a $N^{im}$-protecting group, and usually the formation of the undesired $\tau$-isomer is favoured. An unambiguous $N^{\pi}$-substitution is usually achieved by alkylating a $\tau$-protected His derivative [1]. Based on these considerations, we aimed at the investigation of Fmoc-His(3-Bum)-OH to prevent racemization during esterification and peptide coupling reactions.

Fmoc-His(3(or $\pi$)-Bum)-OH (Figure 1) has been reported to present a His derivative suitable for Fmoc/*t*Bu-based SPPS [2], but only few syntheses employing the derivative Fmoc-His(3-Bum)-OH have been published up to now. Nowadays, the derivative Fmoc-His(1(or $\tau$)-Trt)-OH is generally applied due to the limited availability of $\pi$-protected derivatives. Recent studies have shown that the corresponding 3-Trt derivative cannot be obtained [3]. This contribution led us to reconsider the synthesis and application of His(3-Bum) derivatives in SPPS.

## MATERIALS AND METHODS

$^1$H-NMR measurements were performed on a Bruker Avance DRX 500 spectrometer, 500 MHz, employing tetramethylsilane as an internal standard. ESMS and LC-MS spectra were recorded in the positive mode using a Finnigan Mat LCQ mass spectrometer coupled to a Waters Alliance HPLC system. The optical purity of the His derivatives was determined by CAT GmbH, Tübingen (Germany). Analytical RP-HPLC-chromatograms were obtained on a Merck-Hitachi HPLC system consisting of: pump L-6200, UV-detector L-4000, integrator D-2500, column thermostat L-5025. TLC were run on Merck silica gel plates (Kieselgel 60 $F_{254}$). The following solvent systems were applied: chloroform/MeOH/AcOH/water (90:10:0.5:1) (A), chloroform/32% AcOH/MeOH (15:4:1) (B), chloroform/32%

Abbreviations are as set out in *J. Peptide Sci.* **5**: 465–471 and as follows: ACTH, adrenocorticotropic hormone; EDT, 1,2-ethanedithiol; ESMS, electrospray ionization mass spectrometry; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulphonyl; TGF, transforming growth factor.

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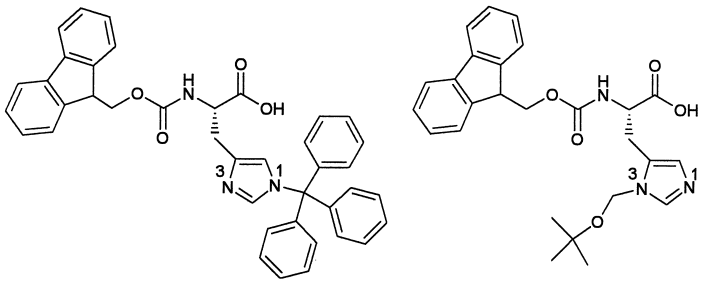


Figure 1 Structures of the derivatives Fmoc-His(1-Trt)-OH and Fmoc-His(3-Bum)-OH.

AcOH/MeOH (5:1:3) (C). Detection was performed by UV, by KI/2-tolidine after chlorination as a general method, by ninhydrin to monitor the presence or absence of free amino groups or by 4-diazobenzenesulphonic acid to identify deblocked imidazole.

### Boc-His(3-Bum)-OMe

*t*-Butyloxymethyl chloride (Bum-Cl) was obtained as described by Jones *et al.* [4] (1.45 g, max 11.8 mmol) and reacted with Boc-His($\tau$-Boc)-OMe (3.66 g, 10 mmol) in DCM (20 mL) for 6 h at room temperature. The solvent was removed *in vacuo* and the resulting foam was taken up in 150 mL of EtOAc and extracted with 100 mL of 5% aqueous $NaHCO_3$. The organic phase was washed with 100 mL of water and 50 mL of brine and dried over $Na_2SO_4$. After evaporation of the solvent, the residue was applied to a silicagel column (212 g). Elution with 2 L of EtOAc followed by EtOAc/MeOH (19:1, 1 L, then 9:1, 2 L) yielded Boc-His(3-Bum)-OMe (1.93 g, 55%), an oil containing only traces of the $\tau$-isomer. TLC: $r_f$ 0.43 (system A); ESMS: 356.1 ($MH^+$); $^1$H-NMR ($CDCl_3$): $\delta$ 1.29 (9H, s, *t*BuOCH$_2$); 1.41 (9H, s, *t*BuOCO); 3.05–3.20 (2H, m, $\beta$-CH$_2$); 3.75 (3H, s, OCH$_3$); 4.53–4.57 (1H, m, $\alpha$-CH); 5.26 (2H, s, $N^{\pi}$-CH$_2$O); 5.45 (1H, d, $J = 7.3$ Hz, brd, NH); 6.83 (1H, s, *im*-H4); 7.52 (1H, s, *im*-H2).

### Boc-His(1-Bum)-OMe

Crude Bum-Cl (1.39 g, max 11.3 mmol) was stirred with Boc-His-OMe (2.69 g, 10 mmol) in DCM (20 mL) for 5 h at room temperature. Removal of the solvent yielded a foam which was redissolved in EtOAc and extracted as described above. TLC monitoring showed the formation of a considerable amount of Boc-His-OMe, which could not be removed by aqueous extraction, due to premature cleavage of Bum. The solvent was removed and chromatography on silica gel (245 g) was performed. The column was washed with EtOAc (4 L) to remove apolar impurities. Elution with EtOAc/MeOH (19:1, 1 L and 9:1, 2 L) yielded the $\tau$-isomer followed by only a few fractions of a mixture of the regioisomers. Evaporation yielded only 390 mg (10%) of the desired $\tau$-isomer still contaminated with 5% of the regioisomer. TLC: $r_f$ 0.55 (system A); ESMS: 356.3 ($MH^+$); $^1$H-NMR ($CDCl_3$): $\delta$ 1.22 (9H, s, *t*BuOCH$_2$); 1.44 (9H, s, *t*BuOCO); 2.99–3.11 (2H, m, $\beta$-CH$_2$); 3.69 (3H, s, OCH$_3$); 4.55 (1H, m, $\alpha$-CH); 5.24 (2H, s, $N^{\tau}$-CH$_2$O); 5.88 (1H, d, $J = 7.8$ Hz, NH); 6.81 (1H, s, *im*-H4); 7.50 (1H, s, *im*-H2).

### *Z*-His(3-Bum)-OMe and *Z*-His(1-Bum)-OMe

Bum-Cl (2.57 g, max 21.0 mmol) was reacted with the crude mixture of the regioisomers *Z*-His(1-Boc)-OMe and *Z*-His(3-Boc)-OMe (4.83 g, 12.0 mmol; ratio of isomers ca. 5:1) in EtOAc (35 mL) for 4 h. The resulting solution containing *Z*-His(3-Bum)-OMe (main product) and contaminating *Z*-His(1-Bum)-OMe was stirred with 5% aqueous $NaHCO_3$ (50 mL) for 25 min, extracted with brine (50 mL) and dried with $Na_2SO_4$. Separation of the isomers was achieved by chromatography on silica gel (118 g) in the solvent system EtOAc/MeOH (39:1, 2 L) yielding 2.34 g (50%) of *Z*-His(3-Bum)-OMe and 300 mg (6%) of *Z*-His(1-Bum)-OMe. TLC: $r_f$ 0.44 (*Z*-His(3-Bum)-OMe), 0.53 (*Z*-His(1-Bum)-OMe), in system A; ESMS: 390.3 ($MH^+$ *Z*-His(3-Bum)-OMe); 390.1 ($MH^+$ *Z*-His(1-Bum)-OMe).

### *Z*-His(3-Bum)-OH

*Z*-His(3-Bum)-OMe (3.04 g, 7.81 mmol) was dissolved in THF (25 mL) at room temperature. Water (10 mL) was added followed by 10 mL of 1 M NaOH. The resulting turbidity disappeared after 5 min. The

 

saponification was stopped after 15 min by adding 1 M $KHSO_4$ (10 mL). Isolation of the desired product was achieved by adjusting to pH 4.5 and removal of THF. The resulting precipitate was filtered off and washed with cold water. Careful drying yielded 2.62 g (87%) of *Z*-His(3-Bum)-OH. The pH of the filtrate was readjusted to yield another 200 mg of the product. TLC: $r_f$ 0.45 (system B); ESMS: 376.3 ($MH^+$); D-enantiomer: $< 0.2\%$.

### *Z*-His(1-Bum)-OH

By analogy, *Z*-His(1-Bum)-OH (1.16 g, 79%) was obtained starting from *Z*-His(1-Bum)-OMe (1.53 g, 3.93 mmol). TLC: $r_f$ 0.45 (system B); D-enantiomer: $< 0.2\%$.

### Fmoc-His(3-Bum)-OH

*Z*-His(3-Bum)-OH (2.60 g, 6.92 mmol) was dissolved in 150 mL of EtOH/$H_2O$ (1:1). Hydrogenation at atmospheric pressure was carried out in the presence of 5% Pd/C (240 mg). The reaction proceeded rapidly and, after an hour, the catalyst was filtered off and washed with EtOH/water (1:1). The resulting product (TLC: $r_f$ 0.27 (system C)) was not isolated, merely the alcohol was removed and the resulting solution was adjusted to pH 7 with 5% aqueous $Na_2CO_3$. A solution of 2.49 g (7.38 mmol) of Fmoc-OSu in THF (20 mL) was slowly added while pH 7 was maintained by adding aqueous $Na_2CO_3$. Stirring was continued overnight, THF was removed and water (80 mL) was added. The resulting suspension was extracted with *t*-butyl methyl ether (50 mL) to remove apolar by-products. The insoluble material which proved to be pure product was filtered off. A second crop was produced by adjusting the pH of filtrate to pH 4.5 and collecting the resulting precipitate. After washing with water and drying, 2.91 g of Fmoc-His(3-Bum)-OH (79%) were obtained, m.p. 178–180°C (175–176°C [2]). TLC: $r_f$ 0.49 (system B); Purity according to HPLC: 99.3% (Conditions: 4.6 × 250 mm column $C_{18}$, 300 Å (Vydac), buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in acetonitrile, linear gradient 25–100% B in 25 min, flow: 1 mL/min, detection: 260 nm); ESMS: 464.2 ($MH^+$); D-enantiomer: $< 0.2\%$.

### Fmoc-His(1-Bum)-OH

By analogy to Fmoc-His(3-Bum)-OH, 1.27 g (89%) of the $\tau$-isomer were obtained from 1.16 g (3.09 mmol) of *Z*-His(1-Bum)-OH. TLC: $r_f$ 0.48 (system B); ESMS: 464.1 ($MH^+$); D-enantiomer: $< 0.2\%$.

### Esterification of Sasrin™

Three portions of Sasrin™ (2-methoxy-4-alkoxybenzyl alcohol resin) (each 1.45 g, 1.48 mmol) were left to swell in THF/DMF (3:1, 20 mL) for 1 h before adding the Fmoc-His derivative (Fmoc-His(3-Bum)-OH, Fmoc-His(1-Bum)-OH or Fmoc-His(1-Trt)-OH) of choice and DCC, 1.5 equivalent each. The Fmoc-His derivatives contained 0.1–0.2% of the D-enantiomer. The suspensions were cooled to −20°C, then 0.06 eq of DMAP were added. The reaction mixtures were kept at −20°C with slight shaking for 4 h and left in an ice-bath overnight. Then the resin was filtered off and washed with isopropanol to remove the dicyclohexyl urea. Determination of the loading was carried out by photometric analysis of the piperidine-dibenzofulvene adduct in piperidine/DMF (1:4) [5]. Samples (ca. 450 mg) of the loaded resins were treated with 6 mL of TFA/water (95:5) for 1 h, followed by filtration and evaporation of the solvent. The crude product, Fmoc-His-OH in each case, was checked for optical purity (Table 1). Further on, resin samples were cleaved in test tubes containing DCM/TFA (99:1) for 5 min and the resulting solutions were applied for TLC analysis. The plates were developed in system B and inspected under UV light.

### Peptide Synthesis

Couplings were performed with a threefold excess of the derivatives and reagents in DMF (8–10 mL/g

Table 1 Esterification of Fmoc-His(R)-OH to Sasrin™ in THF/DMF (3:1) at −20°C

| *R* | Molar excess Fmoc-His(R)-OH | Amount of DMAP (%)[a] | Load (meq/g) | Yield (%) | Racemization (% D) |
|---|---|---|---|---|---|
| 3-Bum | 1.50 | 4.0 | 0.64 | 91 | 0.5 |
| 1-Bum | 1.50 | 4.0 | 0.66 | 94 | 45.0 |
| 1-Trt | 1.50 | 4.0 | 0.68 | 100 | 43.2 |

[a] Molar percentage with respect to His derivative

 

resin). Monitoring was performed using the acetaldehyde/chloranil test (coupling to Pro) [6], the Kaiser test [7], and the 2,4,6-trinitrobenzenesulphonic acid test [8], respectively. Fmoc was split off by repetitive treatment with piperidine/DMF (1:4, 5 and 10 min). Side-chain functionalities were protected as follows: Arg(Pbf), Cys(Trt), His(3-Bum or 1-Trt), Glu(O*t*Bu), Lys(Boc), Ser(*t*Bu) and Tyr(*t*Bu).

### *Z*-Ala-His-Pro-OH and *Z*-Ala-D-His-Pro-OH

The model peptide *Z*-Ala-His-Pro-OH, and for control purposes the peptide *Z*-Ala-D-His-Pro-OH, were obtained by SPPS on H-Pro-chlorotrityl resin (1.11 mmol/g). The derivative Fmoc-His(Trt)-OH, Fmoc-D-His(Trt)-OH, or Fmoc-His(3-Bum)-OH was coupled after activation with TBTU and DIPEA. Completion of the reaction was observed after 1–1.5 h. *Z*-Ala-OH was coupled using activation by TBTU/DIPEA for 1 h. The cleavage was performed in TFA/water (95:5) (10 mL/g resin). After 1 h the resin was filtered off, following evaporation of the filtrate and analysis by HPLC. The optical purity was checked by gradient RP-HPLC using a $4.6 \times 250$ mm Nucleosil-column, $C_{18}$, 50 Å (Macherey-Nagel, Oensingen, Switzerland) at 40°C in a buffer system containing 0.095 M $H_3PO_4$ and 0.09 M triethylamine in water (pH 2.3), buffer A: 10% acetonitrile, buffer B: 60% acetonitrile; linear gradient: 10–40% B in 45 min; flow: 0.6 mL/min; detection: 220 nm) (see Table 2).

### H-Met-Glu-His-Lys-Phe-Phe-Gly-Lys-OH

The peptide was obtained following standard Fmoc/tBu-SPPS on Wang resin. The *N*-terminal amino acid was introduced as its $N^{\alpha}$-Boc derivative. Derivatives were activated with TBTU/collidine. Samples of peptide resins were subjected to the following cleavage conditions: A, TFA/water (95:5), 2 h; B, 5% EDT in TFA/water (95:5), 2 h; C, 5% methoxyamine × HCl (w/v) in TFA/water (95:5), 1 h. All cleavages were performed using 1 mL of cleavage cocktail/100 mg of peptide resin at room temperature, followed by precipitation in cold *t*-butyl methyl ether. The Met sulphonium salt, a by-product identified by LC-MS (see below), was cleaved by dissolving the crude peptide in 1% aqueous AcOH (2 mg/mL). This solution was kept at room temperature for 2 days. HPLC-chromatograms of the peptides were recorded, and mass spectra showed the expected masses: 1023.4 ($MH^+$), 512.2 ($MH_2^{2+}$, major peak). LC-MS was performed on the crude product of the His(Bum)-synthesis (cleavage conditions A, 1 h) to characterize by-products: H-Met-($t$Bu)$^+$-Glu-His-Lys-Phe-Phe-Gly-Lys-OH 1079.2 ($M^+$), 540.3. ($MH^{2+}$, major peak); H-Met-Glu-His-(Bum)-Lys-Phe-Phe-Gly-Lys-OH (incomplete deprotection) 1109.5 ($M^+$), 555.4 ($MH^{2+}$, major peak).

Table 2 HPLC-determination of the Amount of Z-Ala-D-His-Pro-OH (Crude Product)

| Imidazole protection | Coupling reagent | Base | Racemization (% D) |
|---|---|---|---|
| 3-Bum | TBTU | DIPEA | $<0.3$[a] |
| 1-Trt | TBTU | DIPEA | 4.5 |

[a] Below detection limit.

HPLC-chromatograms of the peptides were recorded on a $4.6 \times 250$ mm Bakerbond-column, $C_{18}$, 300 Å, at room temperature; buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in a water-acetonitrile mixture (2:3); gradient 20% B to 50% B in 30 min; flow: 1 mL/min; detection: 220 nm.

### H-Cys-His-Ser-Gly-Tyr-Val-Gly-Val-Arg-Cys-OH (TFG$^{\alpha}$(34–43))

The peptide was obtained by standard Fmoc/*t*Bu-SPPS on Wang resin or Sasrin™. The *N*-terminal amino acid was introduced as its $N^{\alpha}$-Boc derivative. SPPS was performed as described for H-Met-Glu-His-Lys-Phe-Phe-Gly-Lys-OH. Samples of peptide resin were subjected to the following cleavage conditions: B, 5% EDT in TFA/water (95:5), 2 h; D, 5% EDT, 5% methoxyamine × HCl (v/w/v) in TFA/water (95:5), 2 h; E, 5% EDT in TFA/water (95:5) containing 30 eq of Cys × HCl, 2 h. All cleavages were performed using 1 mL of cleavage cocktail/100 mg of peptide resin at room temperature, followed by precipitation in cold *t*-butyl methyl ether. The fully protected TGF-fragments were obtained by repetitive short (2–3 min) treatments of the peptide Sasrin resin with 1% TFA/DCM. Filtrates containing the desired product were neutralized immediately with pyridine and precipitated with *t*-butyl methyl ether. Trituration with water removed the pyridinium trifluoroacetate. The dried fragments were deblocked as described above (conditions: B, D, E, 30 mg of fragment/mL of cleavage cocktail). MS showed the expected masses, 1080.5 ($MH^+$), 540.9 ($MH_2^{2+}$, major peak). LC-MS was performed on the crude cleavage product obtained from the fully protected fragment of the His(Bum)-synthesis (cleavage conditions B, 1 h) to characterize the most abundant apolar by-product (formaldehyde adduct): 1092.4 ($MH^+$), 546.9 ($MH_2^{2+}$, major peak).

 

HPLC-chromatograms of the peptides were recorded on a 4.6 × 250 mm Bakerbond-column, $C_{18}$, 300 Å, at 30°C in a buffer system containing 0.095 M $H_3PO_4$ and 0.09 M triethylamine in water (pH 2.3), buffer A: 5% acetonitrile, buffer B: 60% acetonitrile; gradient: 0% B to 30% B in 45 min; flow: 1 mL/min; detection: 220 nm.

## RESULTS AND DISCUSSION

### Synthesis of the His Derivatives

Boc-His(1-Boc)-OMe was obtained by regioselective acylation of H-His-OMe with $Boc_2O$ following the published protocol [1]. Treatment with Bum-Cl resulted in conversion to Boc-His(3-Bum)-OMe. The corresponding regioisomer Boc-His(1-Bum)-OMe was obtained by $N^{im}$-alkylation of Boc-His-OMe using Bum-Cl. The resulting mixture of regioisomers could be separated by chromatography on silica. Yields may be considerably reduced due to premature cleavage of the Bum group and $N^{\alpha}$-alkylation yielding the Boc(Bum) derivative in the presence of bases. Since labile imidazole hydrochlorides and chlorides were produced upon alkylation, a neutralization step had to be included in the protocol. $^1$H-NMR-spectra of Boc-His(1-Bum)-OMe and Boc-His(3-Bum)-OMe were recorded to characterize the regioisomers. Interestingly, the NMR-spectra differed considerably. The regions of the imidazole and methylester protons are displayed in Figure 2. Unexpectedly, chemical shifts of nearly all the protons in the molecule are influenced by the position of the Bum group. Merely the signal of the $CH^{\alpha}$ is only slightly affected. This behaviour and also the different $r_f$ values obtained from TLC analysis of the regioisomers may be explained by an intramolecular hydrogen bond NH $\cdots N^{im}$, which can only be formed by the 1-isomer. Our findings, especially the reduced polarity of the 3-isomer, are in agreement with this proposal. *Z*-His(1-Bum)-OMe and *Z*-His(3-Bum)-OMe, which represent the intermediates in the synthesis of the corresponding Fmoc derivatives, showed similar differences with respect to polarity according to TLC analysis.

For the evaluation of His(Bum) derivatives in SPPS, Fmoc-His(3-Bum)-OH and, for reasons of comparison, Fmoc-His(1-Bum)-OH, were synthesized starting from *Z*-His-OMe. Acylation of *Z*-His-OMe with $Boc_2O$ yielded a mixture of *Z*-His(1-Boc)-OMe and *Z*-His(3-Boc)-OMe [9], which was treated with Bum-Cl. The resulting mixture of the regioisomers *Z*-His(3-Bum)-OMe (major component) and *Z*-His(1-Bum)-OMe could be smoothly separated by chromatography on silica. Hence, both regioisomers could be obtained simultaneously.

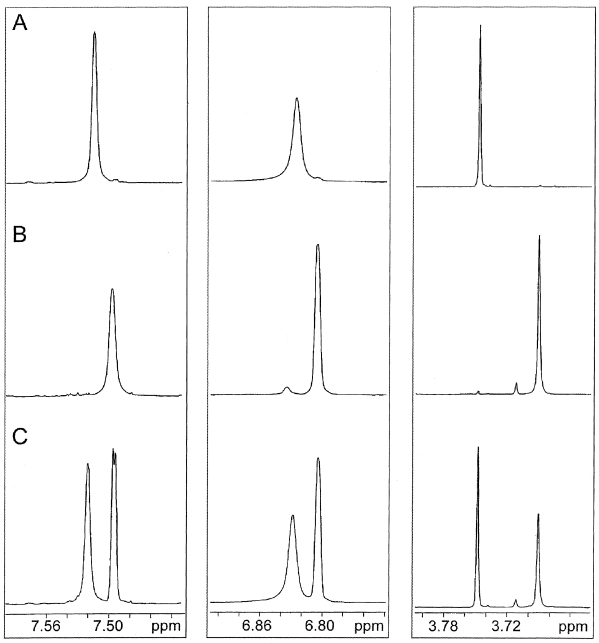


Figure 2 $^1$H-NMR spectra of Boc-His(1-Bum)-OMe (A), Boc-His(3-Bum)-OMe (B) and a 1:1 mixture of the two compounds (C) recorded in $CDCl_3$. The regions of the imidazole protons (7.48–7.54 ppm and 6.80–6.84 ppm) and the methylester protons (3.69–3.75 ppm) are displayed.

Saponification of the methyl ester had to be optimized to achieve minimal racemization. A slight excess of NaOH in aqueous THF presented the optimal conditions for hydrolysis, which only took 15 min for completion. The subsequent hydrogenolysis step and the acylation with Fmoc-OSu had no effect on the optical purity of the final derivatives.

### Application of Fmoc-His(3-Bum)-OH in SPPS

Esterification of His derivatives, e.g. by employing activation with DCC/DMAP, to resins carrying a linker functionality is usually accompanied by considerable racemization [10,11]. Therefore, these conditions represent an unambiguous test for the optical stability during this process. Thus, Fmoc-His(3-Bum)-OH, Fmoc-His(1-Bum)-OH and Fmoc-His(1-Trt)-OH were coupled to Sasrin™ (2-methoxy-4-alkoxybenzyl alcohol resin) using DCC activation in the presence of catalytic amounts of DMAP [10] (see Table 1). Furthermore, the His derivatives were

 

cleaved from the resin and the resulting Fmoc-His-OH was checked for racemization. These results are included in Table 1. As expected, severe racemization occurred when activating the $\tau$-protected derivatives, whereas the coupling reaction involving Fmoc-His(3-Bum)-OH shows an acceptable low degree of racemization. Treatment of the loaded resins with 95% aqueous TFA yielded Fmoc-His-OH and only traces of by-products were detected by TLC. Cleavage of the resins with 1% TFA/DCM yielded the corresponding $N^{im}$-protected His derivatives as verified by TLC.

Based on this stringent testing for Fmoc-His(3-Bum)-OH, we decided to evaluate its properties for peptide coupling reactions. Racemization of activated His derivatives during amide bond formation depends not only on the type and position of imidazole protection but also on the choice of coupling reagent. Recently, an astonishingly high degree of racemization was detected by NMR measurements for the sequence H-Ala-His-Gly-OH [12,13]. The authors used the derivative Fmoc-His(1-Trt)-OH to assess the effects of a new coupling reagent. Unfortunately, the diastereomers of this sequence could only be distinguished by NMR-spectroscopy. Therefore, we identified the simple model peptide, *Z*-Ala-His-Pro-OH, which is amenable to HPLC analysis of the corresponding L- and D-His epimers. In addition, the coupling reaction of any His derivative to Pro is considered to represent a good model for a rather difficult coupling step. As a positive control, the D-epimer was synthesized and applied for identification of this diastereoisomer in the chromatogram. Optimized conditions permitted the detection of 0.3% *Z*-Ala-D-His-Pro-OH. The results of our coupling experiments are summarized in Table 2. Figure 3 shows the corresponding HPLC-chromatograms. In principle, no racemization could be detected when coupling Fmoc-His(3-Bum)-OH with the standard reagent TBTU [14] in the presence of one equivalent of DIPEA. In our study, the percentage of racemization for TBTU-assisted coupling reactions was confirmed by applying the derivative Fmoc-D-His(1-Trt)-OH. In this case, we determined 3.9% of the epimer Z-Ala-L-His-Pro-OH. In contrast to the studies on the sequence H-Ala-His-Gly-OH, we would conclude that in most cases the proportion of D-His epimer remains below 5%. Our model peptide and the optimized HPLC procedure may find further application as a simple, rapid and sensitive test for assessing the efficacy of coupling reagents and/or protecting groups designed to overcome the problem of racemization with respect to His.

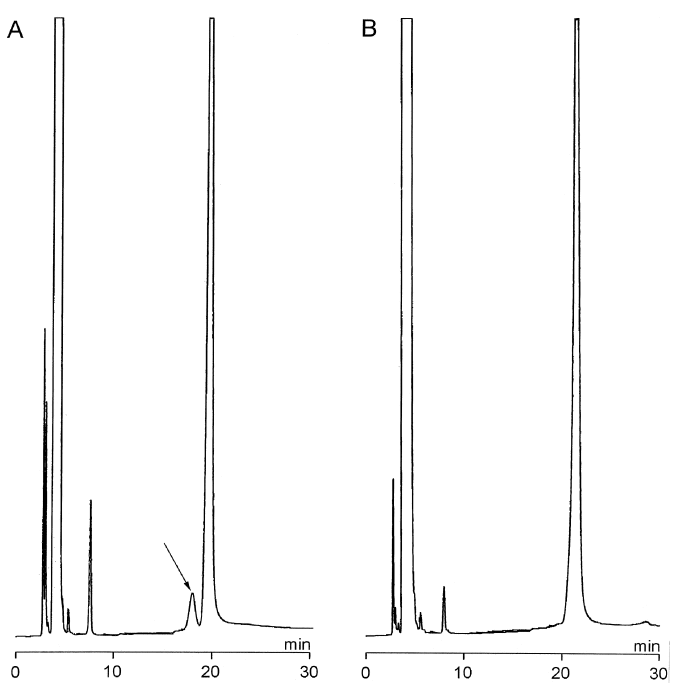


Figure 3 HPLC chromatograms of the crude peptide *Z*-Ala-His-Pro-OH synthesized using the derivatives Fmoc-His(1-Trt)-OH (A) or Fmoc-His(3-Bum)-OH (B). An arrow indicates the presence of the peptide Z-Ala-D-His-Pro-OH in chromatogram A. Additional experimental details are given in Materials and Methods.

 

Since the results obtained on esterification reaction and on the model peptide were all in favour of the derivative Fmoc-His(3-Bum)-OH, we decided to apply this derivative for challenging peptide sequences to check for by-product formation. When intending to employ Bum for $N^{im}$-protection, another problem inherent in the structure of the Bum group had to be considered: the inevitable formation of formaldehyde during its removal. This highly reactive species may result in modification of the desired peptide. By-product formation due to the liberation of formaldehyde during acidolytic cleavage has been investigated more thoroughly in Boc-based SPPS employing $N^{im}$-Bom protection [15]. The amount of by-products could be reduced by adding scavengers with high reactivity towards HCHO, but, usually, the unwanted reaction could not be completely prevented. Peptides containing an *N*-terminal Cys are especially prone to reaction with formaldehyde yielding an *N*-terminal thiazolidine (thiaproline). Considerable amounts of this by-product are formed if HCHO is not properly scavenged [15]. The side-chain functionalities of Lys, Trp and the *N*-terminus are further potential targets for modification. To overcome this problem, Cys derivatives have been recommended as formaldehyde scavengers when deblocking His(Bom)-containing peptides with HF [15]. For our Fmoc/tBu-based SPPS, we also examined the properties of methoxyamine hydrochloride as an additive to the final cleavage cocktail. EDT may scavenge HCHO, but not very efficiently, which has already been noted during investigations on the cleavage of Bom.

The first example, H-Met-Glu-His-Lys-Phe-Phe-Gly-Lys-OH, a modification of a partial sequence of ACTH, was synthesized on Wang resin employing either the derivative Fmoc-His(3-Bum)-OH or Fmoc-His(1-Trt)-OH. During final cleavage, Bum was deprotected rather sluggishly unless methoxyamine·HCl was added. Otherwise, the cleavage time had to be prolonged, e.g. complete removal of Bum took 2 h when treating the peptide resin with 95% aqueous TFA. By contrast, in the presence of methoxyamine (cleavage conditions C) 1 h was sufficient for complete removal of Bum. Fmoc-His(3-Bum)-OH and Fmoc-His(1-Trt)-OH supported syntheses did not differ with respect to purity of the crude product (Figure 4(A) and (B)). Mass spectra of the crude peptides obtained via Bum protection showed only traces of formaldehyde adducts that could not be related to peaks in the chromatograms. On the other hand, varying amounts of a slightly

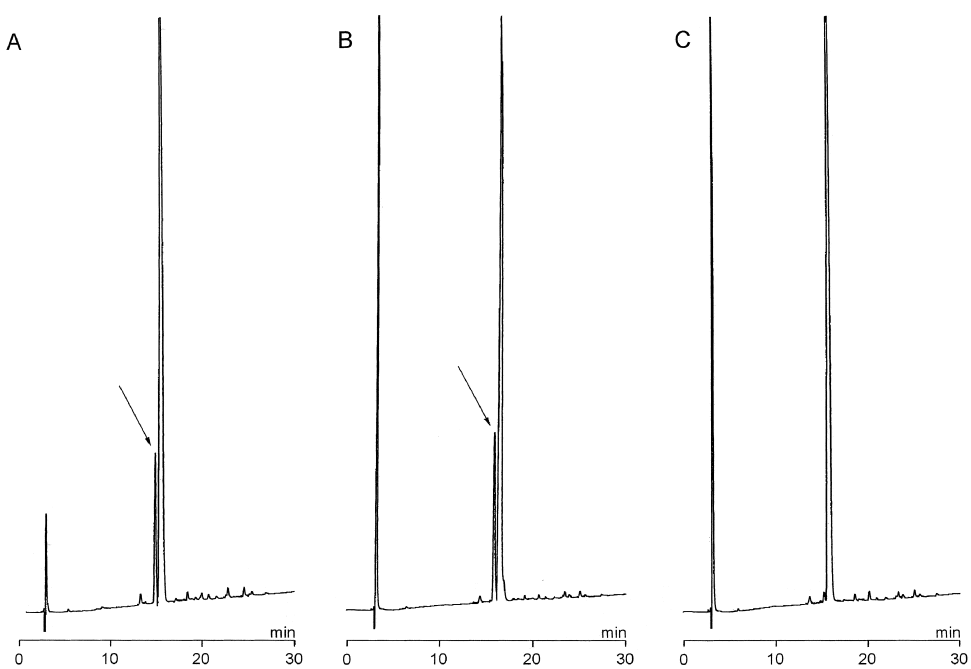


Figure 4 HPLC chromatograms of the crude peptide H-MEHKFFGK-OH synthesized using the derivatives Fmoc-His(1-Trt)-OH (A) or Fmoc-His(3-Bum)-OH (B). The sulphonium salt formed during cleavage form the support is indicated by arrows in chromatograms A and B. The crude product obtained with the derivative Fmoc-His(3-Bum)-OH was treated 2 days in dilute acetic acid and submitted for HPLC analysis (C). Additional experimental details are given in Materials and Methods.

 

more polar by-product could be detected. The quantities observed (HPLC: 6–15% of the main peak) showed no correlation to the type of imidazole protection or to the cleavage conditions. The structure of this impurity was determined by LC-MS to represent a Met sulphonium salt, obviously generated by *t*-butylation. By-products resulting from methionine alkylation have rarely been described in Fmoc/*t*Bu-SPPS, thus we thought to report this observation in our contribution. This side-reaction, normally suppressed when EDT is present in the cleavage medium, was surprising. However, the sulphonium salt slowly decomposes if the peptide is left standing in dilute aqueous AcOH (Figure 4(C)).

The peptide TGF$^{\alpha}$(34–43), H-Cys-His-Ser-Gly-Tyr-Val-Gly-Val-Arg-Cys-OH, containing an *N*-terminal Cys residue adjacent to His, represented the most challenging example. This peptide was synthesized employing the derivatives Fmoc-His(3-Bum)-OH or Fmoc-His(1-Trt)-OH on Wang resin and on the more acid-labile Sasrin™. In the latter case, the fully protected peptide could be obtained by cleavage with 1% TFA/DCM leaving $N^{im}$-Bum as well as $N^{im}$-Trt protection intact (data not shown). Thus, Bum and the remaining protecting groups were removed in solution, permitting a more thorough fine-tuning of cleavage conditions. HPLC chromatograms obtained after final TFA cleavage of the His(3-Bum)- and the His(1-Trt)-protected peptides are shown in Figure 5. The chromatogram shown in Figure 5(C), nicely demonstrates that thiazolidine formation was completely suppressed by adding methoxyamine·HCl to the cleavage cocktail (conditions D, 2 h). However, cysteine as a scavenger was less effective (condition E, data not shown).

To our surprise, the removal of the Bum group was somewhat sluggish when compared to Boc or O*t*Bu cleavage. Especially longer peptides containing His(Bum) definitely require a longer acid treatment than their His(Trt)-containing analogues. On the other hand, the presence of methoxyamine accelerated the cleavage of Bum, perhaps via a transacetalization process. In the case of cysteine and methoxyamine × HCl, both, reaction products and excess of scavenger, precipitated with the peptide, thus rapid processing of the crude product is required to avoid post-cleavage side-reactions. Fortunately, except for the case of thiazolidine formation, only very small amounts of formaldehyde-

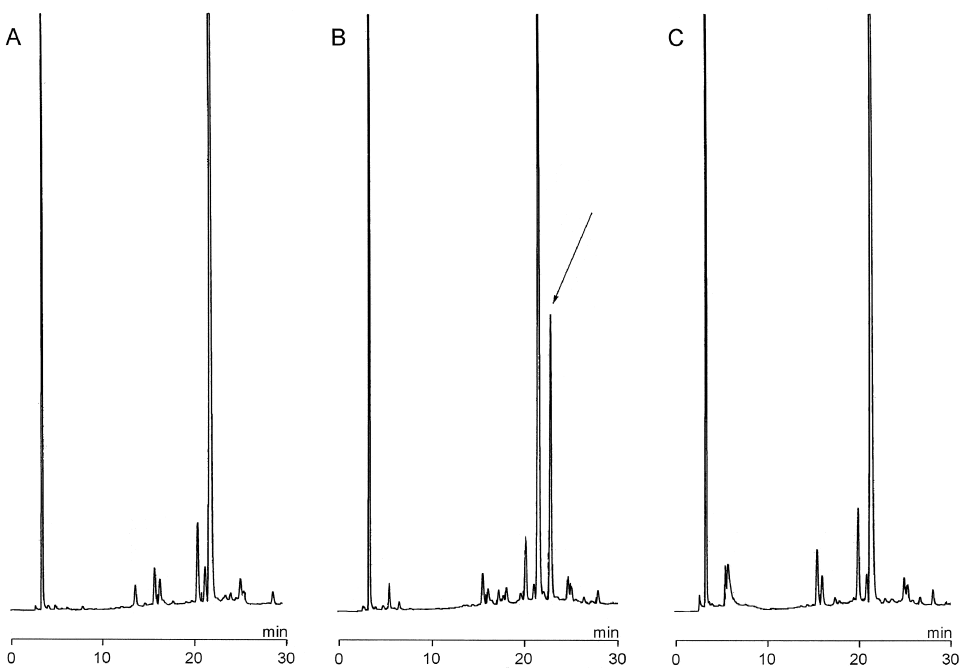


Figure 5 HPLC chromatograms of the crude peptide TGF$^{\alpha}$(34–43). Synthesis was performed using the derivatives Fmoc-His(1-Trt)-OH (A) or Fmoc-His(3-Bum)-OH (B) and final cleavage was carried out with cocktail B. The thiazolidine-derivative generated during cleavage of the Bum group is indicated by an arrow in chromatogram B. Crude TGF$^{\alpha}$(34–43) was also obtained by synthesis with the derivative Fmoc-His(3-Bum)-OH and final cleavage with cocktail D containing methoxyamine (C). Additional experimental details are given in Materials and Methods.

 

derived by-products could be detected by MS and HPLC, even when refraining from the addition of a particular HCHO scavenger. In general, an evaluation of cleavage conditions (composition of cocktail, duration of cleavage) is strongly recommended to achieve an optimal quality of the crude product.

## CONCLUSIONS

In the first part of our contribution, we were able to prove that indeed $\pi$-Bum protection of His minimizes racemization during esterification and peptide coupling reactions. The simple peptide, *Z*-Ala-His-Pro-OH, permitted easy separation and quantification of the L- and D-His diastereoisomer by HPLC and, therefore, this model peptide may find applications for future studies related to His-activation and/or -protection. In addition, we provided evidence for the efficient suppression of formaldehyde related by-products, which are expected to be formed during the final TFA cleavage. In our hands, methoxyamine represented a very potent scavenger, even for the very unfavourable situation of an *N*-terminal Cys adjacent to the Bum protected His, present in the peptide TGF$^{\alpha}$(34–43). In summary, the stringent testing confirmed that the derivative Fmoc-His(3-Bum)-OH could be routinely applied for peptide synthesis. Unfortunately, its synthesis could not be markedly improved. Consequently, the limited availability and/or the high price may prevent a more widespread application. However, in case of difficult coupling reactions, which result in slow conversion of the activated His to form the amide bond, we would recommend to consider the replacement of the readily available Fmoc-His(1-Trt)-OH by Fmoc-His(3-Bum)-OH. In addition, a replacement of His(1-Trt) has to be taken into account for the synthesis of peptides with $>30$ amino acids, especially, if a number of His-residues are present in the sequence. In this situation, even a racemization rate below 5% for a single incorporation of His may spoil the product, since, most likely, the corresponding diastereoisomers will not easily be separated by conventional purification methods due to negligible differences with respect to the overall properties of a long peptide.

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