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Synthesis and application of N^{α}-Fmoc-N^{π}-4-methoxybenzyloxymethylhistidine in solid phase peptide synthesis

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The 4-methoxybenzyloxymethyl (MBom) group was introduced at the N^{π}-position of the histidine (His) residue by using a regioselective procedure, and its utility was examined under standard conditions used for the conventional and the microwave (MW)-assisted solid phase peptide synthesis (SPPS) with 9-fluorenylmethyoxycarbonyl (Fmoc) chemistry. The N^{π}-MBom group fulfilling the requirements for the Fmoc strategy was found to prevent side-chain-induced racemization during incorporation of the His residue even in the case of MW-assisted SPPS performed at a high temperature. In particular, the MBom group proved to be a suitable protecting group for the convergent synthesis because it remains attached to the imidazole ring during detachment of the protected His-containing peptide segments from acid-sensitive linkers by treatment with a weak acid such as 1% trifluoroacetic acid in dichloromethane. We also demonstrated the facile synthesis of Fmoc-His (π-MBom)-OH with the aid of purification procedure by crystallization to effectively remove the undesired τ-isomer without resorting to silica gel column chromatography. This means that the present synthetic procedure can be used for large-scale production without any obstacles. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: histidine; 4-methoxybenzyloxymethyl (MBom) group; microwave (MW)-assisted solid phase peptide synthesis (SPPS); protecting group; racemization

Introduction

In the synthesis of histidine (His)-containing peptides, appropriate protection of the imidazole ring of the His residue is indispensable for avoiding side reactions associated with the nucleophilicity of the imidazole ring, such as N^{im}-acylation followed by acyl transfer reactions, during the course of chain assembly. In particular, the His residue is known to be extremely prone to racemization in activating and coupling processes involving the π-nitrogen of the imidazole ring [1]. Therefore, regioselective protection of the π-nitrogen should enable avoidance of racemization during incorporation of His derivatives onto the growing peptide chains [2]. In Boc chemistry, the N^{π}-benzyloxymethyl (Bom) group is widely accepted as a protecting group for His because it can effectively suppress the risk of racemization and can be readily removed by HF or trifluoromethanesulfonic acid [3]. On the other hand, the N^{τ}-Trt group is often used in combination with N^{α}-Fmoc protection [4]. Although the steric hindrance and electron withdrawing effect of the Trt group on the τ-nitrogen may help reduce racemization to a certain extent, the N^{τ}-protecting group cannot inherently prevent racemization. In particular cases such as the formation of ester bonds or when the amino component is sterically hindered, the N^{τ}-Trt group on His raises the risk of racemization. In addition, MW-assisted SPPS performed using Fmoc-His(τ-Trt)-OH results in a serious level of racemization [5]. To offer the N^{π}-protection of His compatible with Fmoc chemistry, the N^{π}-*t*-butoxymethyl and N^{π}-1-adamantyloxymethyl groups have been developed [6,7]. However, the preparation of Fmoc-His-OH derivatives substituted with these protecting groups at the π-nitrogen requires a purification procedure using column chromatography on silica gel to separate the undesired τ-isomer. This would hamper large-scale production, thus preventing such derivatives from gaining widespread application in the practical peptide synthesis. To obtain an N^{π}-protected His derivative applicable to Fmoc chemistry and easily purified by crystallization, we introduced the MBom [8], 2,4-DMBom, and 3,4-DMBom groups at the π-nitrogen of the imidazole ring. Among them, the MBom group was found to be a suitable protecting group of the His residue applicable for Fmoc chemistry to eliminate the side-chain-induced racemization during incorporation of His performed even by MW-assisted SPPS at a high temperature.

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Abbreviations used: *Ang II, angiotensin II; Boc, t*-butoxycarbonyl; *t*Bu, *t*-butyl; 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; DCC, *N,N′*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; 2,4-DMBom, 2,4-dimethoxybenzyloxymethyl; 3,4-DMBom, 3,4-dimethoxybenzyloxymethyl; ESI MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyoxycarbonyl; HCTU, 1-[*bis*(dimethylamino) methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate; HPLC, high performance liquid chromatography; MBom, 4-methoxybenzyloxymethyl; MW, microwave; NPW30, neuropeptide W-30; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RP-HPLC, reversed phase HPLC; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; THCar, tetrahydroxy-β-carboline; Thz, thiazolidine; TMSOTf, trimethylsilyl triflate; Trt, trityl; Trt(2-Cl), 2-chlorotrityl; Z, benzyloxycarbonyl.

Materials and Methods

All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), Wako Chemical (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan), and Watanabe Chemical Industries (Hiroshima, Japan). Analytical HPLC was performed on a Shimadzu liquid chromatograph Model LC-10AT (Kyoto, Japan) with a DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm) using a flow rate of 1 ml/min and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in MeCN (B). Purities are based upon area percent of the peaks detected at 220 nm. High resolution mass spectra were measured with a Bruker Esquire 200T (Billerica, MA, USA). Molecular weights were measured with a MALDI-TOF MS (Voyager-DESTR, Applied Biosystems) and an ESI MS (HP1100 LC/MSD). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL-ECX400 spectrometer (Tokyo, Japan) in dimethyl sulfoxide-d_6 (d_6-DMSO) with the solvent residual peak (d_6-DMSO: ^{1}H = 2.49 ppm, ^{13}C = 39.52 ppm) as an internal reference unless otherwise stated.

Synthesis of Fmoc-His(π-MBom)-OH (1a) and its Related Compounds (1b, 1c)

Boc-His(π-MBom)-OH (4a)

A solution of Boc-His(τ-Ac)-OMe (**3a**) (6.10 g, 19.6 mmol) and 4-methoxybenzyloxymethylchloride (MBom-Cl: 4.51 g, 24.5 mmol) in CH_2Cl_2 (60 ml) was stirred at room temperature for 4 h. After removal of the solvent, the residue was triturated with diethyl ether to give Boc-His(π-MBom)-OMe (HCl form, 10.6 g). A solution of Boc-His(MBom)-OMe obtained previously in MeOH (40 ml) was treated with 1 M NaOH aq. (40 ml) and stirred at room temperature for 4 h. The reaction mixture was adjusted with 1 M HCl to pH 4. After removal of the solvent, the residue was extracted with $CHCl_3$. The extract was washed with water and dried over Na_2SO_4. After removal of the solvent, the residue was triturated with AcOEt to give Boc-His(π-MBom)-OH (4.92 g, 62% from Boc-His(τ-Ac)-OMe). ^{1}H NMR (DMSO-d_6) 1.34 (s, 9H), 2.82–3.12 (m, 2H), 3.72 (s, 3H), 4.20 (br s, 1H), 4.33 (s, 2H), 5.37 (br s, 2H), 6.75 (s, 1H), 6.88 (d, J = 8.70 Hz, 2H), 7.15–7.25 (m, 3H), 7.73 (s, 1H).; ^{13}C NMR (DMSO-d_6) 25.3, 28.2, 52.8, 55.1, 69.0, 73.2, 78.1, 113.7, 127.6, 127.9, 129.0, 129.5, 138.3, 155.4, 158.9, 173.3; ESI MS Calcd for $[C_{20}H_{27}N_3O_6 + H]^+$ 406.198, found 406.1.

Fmoc-His(π-MBom)-OH (1a)

To a vigorously stirred solution of Boc-His(π-MBom)-OH (10.0 g, 23.8 mmol) and 2,6-lutidine (33.3 ml, 191 mmol) in CH_2Cl_2 (75 ml) at 0 °C was added TMSOTf (34.6 ml, 191 mmol). After 10 min, the ice bath was removed, and the mixture was stirred at room temperature for 18 h. The reaction mixture was placed again in an ice bath, and ice-cold water (50 ml) was added. Subsequently, the mixture was washed with $CHCl_3$ (2 × 10 ml). The aqueous layer was added to DMF (50 ml) and treated with Fmoc-OSu (8.03 g, 23.8 mmol) and stirred at room temperature for 4 h. The solution was adjusted with Na_2CO_3 aq. to pH 7–8. After removal of the solvent, the residue was washed with H_2O. The residue was triturated with $CHCl_3$/MeOH and Et_2O to give Fmoc-His(π-MBom)-OH (11.8 g, 84% yield): ^{1}H NMR (DMSO-d_6) 2.90–3.17 (m, 2H), 3.71 (s, 3H), 4.15–4.38 (m, 6H), 5.38 (q, 2H, J = 10.0 Hz) 6.74–6.87 (m, 3H), 7.15–7.42 (m, 6H) 7.60–7.94 (m, 6H); ^{13}C NMR (DMSO-d_6) 25.2, 46.6, 50.1, 53.2, 55.0, 63.8, 65.7, 69.0, 73.2, 113.7, 119.8, 120.1, 125.2, 126.8, 127.0, 127.1, 127.6, 127.8, 128.9, 129.5, 138.3, 140.6, 143.7, 145.2, 155.9, 158.8, 172.9.; ESI MS Calcd for $[C_{30}H_{29}N_3O_6 + H]^+$ 528.21, found 528.2; $[\alpha]_D$ −20.0 (c 1.12, DMF).

Fmoc-(2,4-DMBom)-OH (**1b**) and Fmoc-(3,4-DMBom)-OH (**1c**) were prepared as described earlier for Fmoc-His(π-MBom)-OH (**1a**) by using 3,4-DMBom-Cl and 2,4-DMBom-Cl, respectively.

Fmoc-His(2,4-DMBom)-OH (**1b**); ^{1}H NMR (d_6-DMSO) δ = 7.89 (2H, d, J = 6.87 Hz), 7.75 (1H, d, J = 8.70 Hz), 7.66 (2H, d, J = 7.79 Hz), 7.48 (2H, t, J = 7.20 Hz), 7.30 (2H, t, J = 7.20 Hz), 7.26 (2H, d, J = 8.80 Hz), 6.65 (1H, d, J = 2.70 Hz), 6.50 (1H, d, J = 8.80, 2.70 Hz), 4.73 (2H, s), 4.44 (2H, dd, J = 16.03, 10.99 Hz), 4.35–4.10 (4H, m), 3.71 (3H, s), 3.10–2.78 (2H, m).; ^{13}C NMR (d_6-DMSO) δ = 32.0, 46.6, 54.3, 55.0, 55.8, 65.8, 66.7, 72.7, 100.3, 106.2, 118.2, 120.1, 125.2, 125.3, 127.1, 127.6, 128.9, 137.3, 140.7, 143.8, 156.0, 158.8, 159.8, 172.3; HRMS Calcd for $[C_{31}H_{31}N_3O_7H]^+$, m/z 558.2235, found 558.2238.

Fmoc-His(3,4-DMBom)-OH (**1c**); ^{1}H NMR (d_6-DMSO) δ = 7.88 (2H, d, J = 6.87 Hz), 7.75 (1H, d, J = 8.70 Hz), 7.70 (2H, d, J = 7.79 Hz), 7.40 (2H, t, J = 7.20 Hz), 7.30 (2H, t, J = 7.20 Hz), 7.10 (2H, d, J = 2.70 Hz), 6.87 (1H, d, J = 8.80, 2.70 Hz), 6.58 (1H, d, J = 8.80 Hz), 4.73 (2H, s), 4.44 (2H, dd, J = 16.03, 10.99 Hz), 4.35–4.10 (4H, m), 3.71 (3H, s), 3.10–2.78 (2H, m); ^{13}C NMR (d_6-DMSO) δ = 32.0, 46.6, 54.3, 55.0, 55.2, 65.8, 68.7, 72.7, 112.7, 114.6, 120.1, 121.9, 125.2, 125.3, 127.1, 127.6, 128.2, 128.9, 137.3, 140.7, 143.8, 148.8, 156.0, 158.8, 172.3; HRMS: Calcd for $[C_{31}H_{31}N_3O_7H]^+$: m/z 558.2235, found 558.2237.

Examination of His Racemization during Synthesis of the Model Peptide, Z-Ala-His-Pro-OH

Starting with H-Pro-Trt(2-Cl) resin (0.29 g, 0.69 mmol/g), manual peptide chain assembly was carried out using the protocol of 30-min coupling with Fmoc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv with respect to the peptide resin, 0.2 M, 0–5 min preactivation) in DMF. MW heating was performed in a 25-ml polypropylene open vessel placed into the MW cavity of a 300 W single-mode manual MW peptide synthesizer (CEM, Discover SPS) set at 50 or 80 °C; power pulsing sequences of 30 W were used for His coupling steps (5 min). Fmoc deprotection was carried out with 20% piperidine in DMF, followed by washing with DMF (5 × 2 min). The final release of peptides was achieved with TFA/triisopropylsilane/H_2O (95/5/5) containing $MeONH_2$.HCl (5 equiv with respect to the peptide resin) at room temperature for 1 h. The crude peptides were directly analyzed by HPLC and ESI MS analyses.

Loading of the Fmoc-His-OH Derivatives onto Wang Resin

Wang resin (4-methoxybenzylalcohol resin) (185 mg, 1.00 mmol) was left to swell in THF/DMF (3:1, 10 ml) for 1 h before adding the Fmoc-His-OH derivative [Fmoc-His(π-MBom)-OH (**1a**) or Fmoc-His (τ-Trt)-OH (1.50 mmol)] and DCC (1.50 mmol). The suspensions were cooled to −15 °C, and then, 0.06 eq of DMAP was added. The reaction mixture was kept at −15 °C with slight shaking for 4 h and left in an ice bath overnight. Fmoc deprotection was carried out with 20% piperidine in DMF, followed by washing with DMF (5 × 2 min). The resin was then filtered off and washed with DMF and DCM. After an aliquot (ca. 50 mg) of the resin had been treated with 6 ml of TFA/water (v/v, 95:5) for 1 h, the filtrate was evaporated to give the residue, which was checked for optical purity by using Merfey's method [9].

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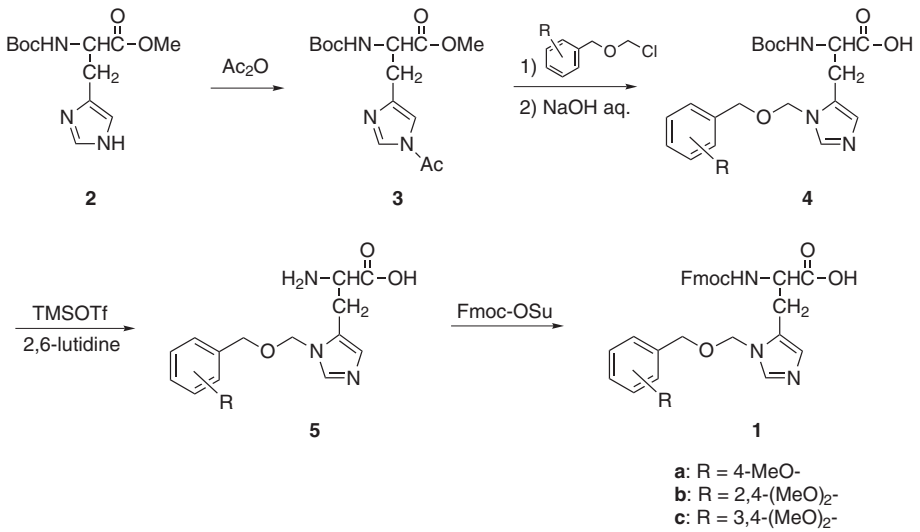

Scheme 1. Synthetic route of Fmoc-His(MBom)-OH (**1a**) and its related compounds (**1b**, **1c**).

Synthesis of Cys-Ang II, Trp-Ang II, and NPW30 Related Peptides

The protected Cys-Ang II, Trp-Ang II, NPW30, and its related peptides were assembled with an ABI 433A (Forester, CA, USA) using Fmoc strategy on Wang-PEG resin (0.37 g, 0.27 mmol/g). The peptide chain was elongated using the FastMoc® protocol of coupling with Fmoc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv) in 1-methyl-2-pyrrolidinone. The following side-chain-protected amino acids were used: Trp(Boc), Tyr(*t*Bu), Asp(O*t*Bu), Lys(Boc), His(τ-Trt)/His(π-MBom), Ser(*t*Bu), Arg(Pbf), Cys(Trt), and Thr(*t*Bu). Final cleavage and deprotection of peptides were achieved with TFA/triisopropylsilane/H_2O (95/5/5) in the presence of $MeONH_2$.HCl (5 equiv) at room temperature for 1 h. The crude peptides were directly analyzed by HPLC and ESI MS analysis.

NPW30 (10–15): H-Tyr-His-Thr-Val-Gly-Arg-OH

Analytical HPLC condition: linear gradient of solvent B in solvent A, 10% to 60% over 25 min, retention time = 16.1 min, LRMS (ESI) *m/z* Calcd for $C_{32}H_{49}N_{11}O_9$ ($[M+H]^+$) 732.4, found 732.4.

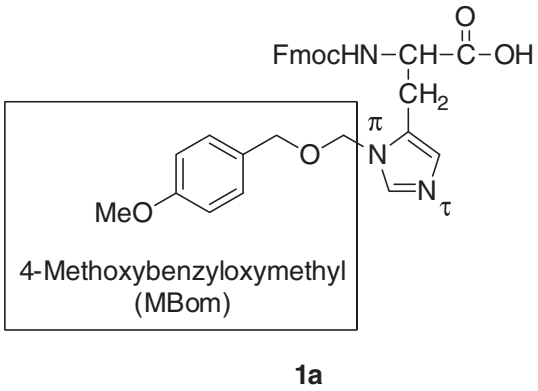

Figure 1. Structure of Fmoc-His(MBom)-OH (**1a**).

Results and Discussion

Preparation of the His Derivatives

Each N^{π}-protecting group was introduced by regioselective alkylation of Boc-His(τ-Ac)-OMe using the chloride of the respective mono-methoxy and di-methoxy substituted benzyloxymethanols. Removal of the methyl ester by saponification with NaOH aq gave intermediates in the synthesis of the corresponding Fmoc derivatives (Scheme 1). The combination of the protecting groups on these intermediates, that is, N^{α}-Boc and respective N^{π}-groups, facilitated purification by crystallization to separate the undesired τ-isomer from the product without resorting to column chromatography, thus making it possible to use the present synthetic procedure for large-scale production (Figures 1 and 2). Furthermore, recrystallization of these intermediates could be effectively performed to remove the respective D-isomers arising from saponification [10]. To convert **4a–c** into the corresponding Fmoc derivatives **1a–c**, the N^{α}-Boc group on these intermediates had to be selectively cleaved without affecting the N^{π}-protecting groups, which are acid-sensitive in the same way as the Boc group. Thus, selective removal of the N^{α}-Boc group avoiding the use of acidic conditions was achieved by treatment with TMSOTf/2,6-lutidine to give **5a–c** in quantitative yield [11], with the α-amino functions being protected by the Fmoc group. It is known that transformation of an *N*-Boc group to a TMS carbamate using TMSOTf/2,6-lutidine is compatible with the presence of other acid sensitive groups, that is, *t*Bu esters and ethers [12]. The optical purities of **1a–c** synthesized in this manner were >99.9% as determined by Marfey's method after removing the protecting groups [9].

The stability and removability of these N^{π}-protecting groups under the standard conditions used for Fmoc-SPPS were examined by RP-HPLC and summarized in Table 1 in comparison with those of the Trt group. The MBom, 2,4-DMBom, and 3,4-DMBom groups were found to be completely stable during the repetitive Fmoc deprotection reaction using 20% piperidine in DMF but readily removable by TFA in the same manner as the Trt group. Among them, the MBom group was the only protecting group that remained attached to the imidazole ring during detachment of the protected peptide segments with a free carboxyl group from the Trt(2-Cl) resin by treatment with 1% TFA/DCM or DCM/trifluoroethanol/AcOH (v/v, 3/1/1) [13]: The Trt group is known to be susceptible to this condition. When applying the protected His-containing peptide segments prepared on acid-sensitive linkers for the convergent synthesis, partial loss of the N^{im}-protecting group on the His residue may not only

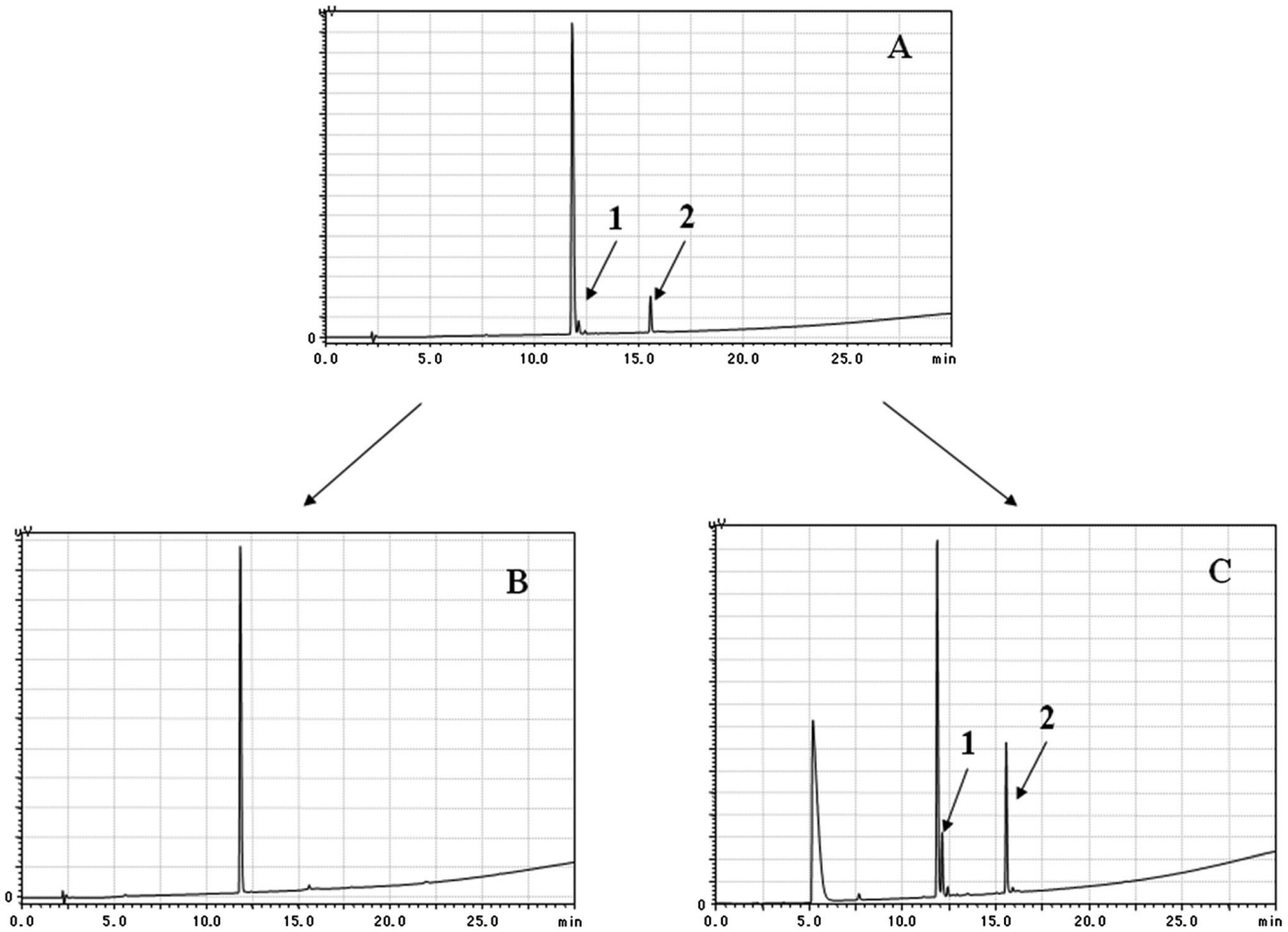

Figure 2. HPLC profiles of the products, Boc-His(π-MBom)-OH (**4a**). (A) Crude product without purification; (B) product obtained after crystallization from $CHCl_3$/AcOEt; and (C) mother solution. Eluent: 10–60% MeCN in 0.1% TFA. Peaks 1 and 2 indicate Boc-His(τ-MBom)-OH and Boc-His(π,τ-diMBom)-OH, respectively.

hamper their purification procedure but also cause side reactions involving the nucleophilicity of the imidazole ring in the subsequent segment condensation. These results clearly indicated that the MBom group for the His residue can offer permanent N^{im}-protection throughout the synthesis of the protected peptides.

Application of Fmoc-His(π-MBom)-OH in SPPS

Suppressive effect of N^{π}-MBom on racemization

The suppressive effect of the N^{π}-MBom group on racemization during the incorporation of His was evaluated by synthesizing a model peptide, Z-Ala-His-Pro-OH [10], via the conventional and the MW-assisted SPPS. The peptide chain was elongated onto an H-Pro-Trt(2-Cl) resin by the widely accepted protocol of coupling with Fmoc (or Z)-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv) in DMF. Upon activation of amino acid derivatives as a carboxyl component, this protocol is known to be more or less accompanied by their racemization in correlation with the length of preactivation [14]. The rate of racemization of Fmoc-His(τ-Trt)-OH during the activating procedure significantly increased in a time-dependent fashion because of its unprotected π-nitrogen, which is involved in promoting racemization pathways, whereas that of Fmoc-His(π-MBom)-OH increased very slightly, and it was comparable with those observed with other ordinary Fmoc-amino acids (Table 2) [15]. As long as the duration of preactivation was limited to no more than 1 min, which is the standard condition, Fmoc-His(π-MBom)-OH was found to be accompanied by virtually no racemization on activating and coupling steps of the conventional SPPS compared with that of Fmoc-His(τ-Trt)-OH (2.9%). This indicates that the N^{π}-MBom group would effectively prevent racemization even in the case of a slow coupling process where the amino component is sterically hindered [16]. Furthermore, Fmoc-His(π-MBom)-OH

Table 1. Stability of Fmoc-His(X)-OH under detachment conditions using a weak acid

		Imidazole protection			
Conditions	X =	π-MBom	2,4-DMBom	3,4-DMBom	τ-Trt
1% TFA/CH_2Cl_2	rt 3 h	100	87	92	72
DCM/trifluoroethanol/AcOH(v/v, 3/1/1)	rt 3 h	100	94	100	88

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Table 2. Racemization of His during the synthesis of the model peptide, Z-Ala-His-Pro-OH, as a function of the length of preactivation with HCTU

Preactivation (min)	Racemization (%)[a]	
X =	π-MBom	τ-Trt
0	<0.1[b]	1.0
1	<0.1[b]	2.9
5	0.3	7.8

[a]Defined as (Z-Ala-D-His-Pro)/(Z-Ala-L-His-Pro) × 100.
[b]Below detection limit.

provided significant reduction in the level of racemization even when performing the MW-assisted SPPS at 50 and 80 °C (0.2% and 0.5%, respectively), whereas Fmoc-His(τ-Trt)-OH led to a considerable extent (3.7% and 16.6%, respectively) as shown in Table 3. MW-assisted SPPS is accepted as a valid support of enhancement of the coupling efficiency in a short time. The coupling reactions are usually carried out under MW irradiation at a high temperature of around 80 °C [17]. However, recent publications have drawn attention to the susceptibility to racemization of Fmoc-His(τ-Trt)-OH under these conditions [5,18]. Thus, it is recommended that incorporation of Fmoc-His(τ-Trt)-OH onto a peptide chain be carried out at 50 °C or less, although there is no advantage in terms of coupling efficiency over the procedure performed at 80 °C. In contrast, Fmoc-His (π-MBom)-OH proved to reduce the rate of racemization to an acceptable level even when performing MW-assisted SPPS at 80 °C (Figure 3). This measure using Fmoc-His(π-MBom)-OH can facilitate the exclusion of racemization with His during the course of chain assembly by MW-assisted SPPS.

The efficient acylation of hydroxyl-functionalized solid supports such as Wang resin with Fmoc-protected amino acids without racemization is the essential first step for SPPS. Esterification of the His derivatives with the aid of the DCC/DMAP method to a hydroxyl group on the resin is known to be accompanied by severe racemization [19], although several methods involving activation of the hydroxyl group for linking to Fmoc amino acids, for example, the use of trichloroacetimidate Wang resin and the Mitsunobu reaction, are available to avoid the side reaction with racemization [20,21]. Thus, we tried to apply the DCC/DMAP method for loading Fmoc-His(π-MBom)-OH to Wang resin in order to evaluate the optical stability during this process. Fmoc-His(π-MBom)-OH caused a dramatic reduction in the level of racemization during its loading, whereas Fmoc-His(τ-Trt)-OH led to a serious level of racemization (Table 4).

Final deprotection of His(π-MBom)-containing peptides

After the final TFA deprotection and cleavage of the peptide resin, the generation of formaldehyde and an electrophilic-alkylating species, a methoxybenzyl cation, from the MBom group occurs in side reactions. Formaldehyde can lead to hydroxymethylated modification of α-amino and ε-amino groups, although the extent is inconsequential. On the other hand, when a Cys or a Trp residue is located at the *N*-terminus, formaldehyde can react with it to produce a Thz-peptide or THCar-peptide, respectively, during isolation from an acidolytic mixture [22,23]. The methoxybenzyl cation can cause alkylation of susceptible residues such as Cys and Trp [24]. Thus, we applied Fmoc-His(π-MBom)-OH to the synthesis of Cys-Ang II (CDRVYIHPF) and Trp-Ang II to examine their by-product formation associated with the use of this protecting group as summarized in Tables 5 and 6, respectively. As has been reported, the formation of Thz-peptide or THCar-peptide arising from the generation of formaldehyde during TFA treatment was almost completely suppressed by the addition of methoxyamine hydrochloride ($MeONH_2$·HCl, 5 equiv) to the reaction mixture [10,25]. The alkylation of Trp with a carbocation evolved from the MBom group was negligible in amount as long as its indole ring was protected by the Boc group, which is known to produce the

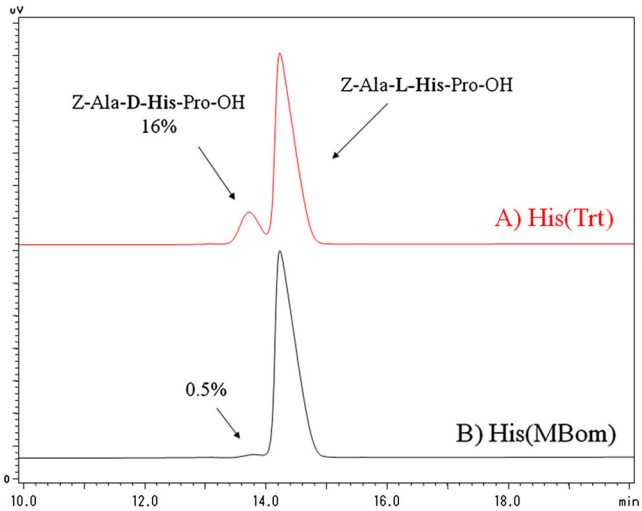

Figure 3. HPLC profiles of the products, Z-Ala-His-Pro-OH, obtained by performing MW-assisted SPPS at 80 °C. Incorporation of His was performed by coupling Fmoc-His(τ-Trt)-OH (A) and Fmoc-His(π-MBom)-OH (B). Eluent: 15–40% MeCN in 0.1% TFA.

Table 3. Racemization of His during MW-assisted SPPS of the model peptide, Z-Ala-His-Pro-OH, as a function of the coupling temperature

Temperature (°C)	Racemization (%)	
X =	π-MBom	τ-Trt
50	0.2	3.7
80	0.5	16.6

Table 4. Racemization during the loading of Fmoc-His(X)-OH onto Wang resin

X	Racemization (%)
τ-Trt	31.4
π-MBom	0.3

Table 5. Effect of additives on Cys modification during TFA deprotection

Additives	Ratio of Cys-Ang II to Cys(X)-Ang II		
	Cys-Ang II	Thz-Ang II	Cys(MeOBzl)-Ang II
None	88	5.3	7.1
$MeONH_2$·HCl	91	<1.0	7.8
$MeONH_2$·HCl/PhSH	99	<1.0	<1.0

Table 6. Effect of additives on Trp modification during TFA deprotection

Additives	Ratio of Trp-Ang II to Trp(X)-Ang II		
	Trp-Ang II	THCar-Ang II	Trp(MeOBzl)-Ang II
None	90	7.8	1.2
$MeONH_2$·HCl	98	<1.0	1.2
$MeONH_2$·HCl/PhSH	99	<1.0	<1.0

N^{in}-carbamic acid to prevent such electrophilic additions during TFA treatment (Table 6) [26]. In contrast, performing TFA cleavage in the presence of thiol compounds was necessary to avoid the alkylation of Cys (Table 5). This measure using $MeONH_2$·HCl and thiols could effectively prevent the side reactions associated with the use of the MBom group.

Synthesis of rat neuropeptide W-30

To demonstrate the usefulness of the N^{π}-MBom group on His, we synthesized rat NPW30 (WYKHVASPRY^{10}HTVGRASGLL^{20}MGLRR-SPYLW^{30}), a food intake-regulating peptide [27], by Fmoc SPPS using a 1-min preactivation procedure of coupling with Fmoc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv) in DMF/1-methyl-2-pyrrolidinone. When synthesizing NPW30 by using Fmoc-His(τ-Trt)-OH, the product was found to be contaminated by 4% of D-His^4-diastereoisomer at a moderate estimate; this could be only detected as a shoulder peak by RP-HPLC when an isocratic system was used. The other isomer, D-His^{11}-diastreoisomer, was eluted at the same retention time as the desired peptide on RP-, ion exchanged HPLC, and capillary zone electrophoresis, although RP-HPLC analysis of the segment (10–15) obtained by digesting the synthetic NPW30 with trypsin revealed that racemization had occurred at His^{11} (3.2%) as shown in Figure 4. Thus, there were no practical purification procedures available for removing the racemized peptides arising from D-His^4 or D-His^{11}. Because it is known that a diastereoisomer of biologically active peptides with racemization somewhere in its sequence may pose the risk of developing biological efficacy and/or pharmacological targets that differ from those of the natural product, extreme care is required in their chemical synthesis, especially of biologically active peptides, to avoid ambiguity in the quality of the synthesized peptides. Therefore, it is important to exclude the risk of racemization with incorporation of His by using Fmoc-His(π-MBom)-OH during the course of the chain assembly. No racemization with His^{11} was found in the segment (10–15) on synthesis using Fmoc-His (π-MBom)-OH.

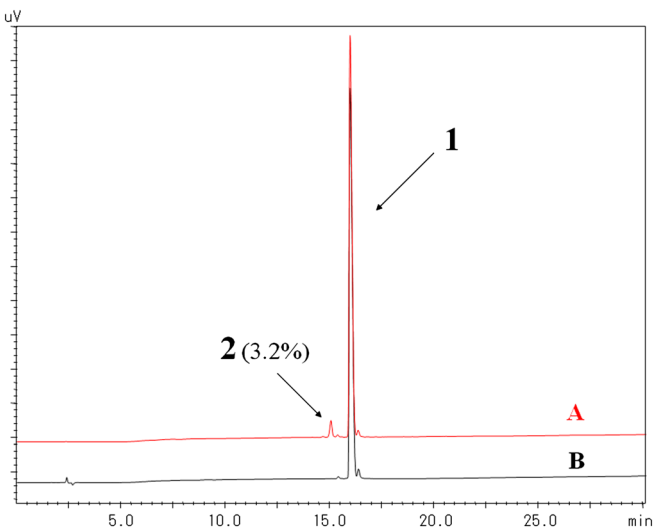

Figure 4. HPLC profiles of the tryptic segments, NPW30 (10–15), synthesized by using Fmoc-His(τ-Trt)-OH (A) or Fmoc-His(π-MBom)-OH (B). Peaks 1 and 2 indicate the desired product and [D-His^{11}]-isomer, respectively. Eluent: 10–60% MeCN in 0.1% TFA.

Conclusions

The MBom group proved to be an ideal protecting group for the His residue in terms of offering N^{π}-protection and Fmoc compatibility to eliminate side-chain-induced racemization during incorporation of His. The suppressive effect of N^{π}-MBom on racemization occurred even in the case of MW-assisted SPPS performed at 50 and 80 °C. In particular, the MBom group was shown to be a suitable protecting group for the convergent synthesis because it remains attached to the imidazole ring during detachment of the protected peptide segments from acid-sensitive linkers. Furthermore, we were able to confirm the applicability of the present synthetic procedure of Fmoc-His(π-MBom)-OH for large-scale production.

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