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4-Methoxybenzyloxymethyl Group, a Racemization-Resistant Protecting Group for Cysteine in Fmoc Solid Phase Peptide Synthesis

Hajime Hibino[†] and Yuji Nishiuchi[*,†,‡]

SAITO Research Center, Peptide Institute, Inc., Ibaraki, Osaka 567-0085, Japan, and Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

yuji@peptide.co.jp

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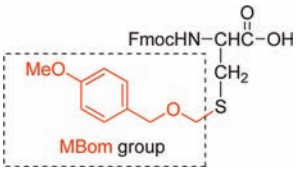
ABSTRACT



The 4-methoxybenzyloxymethyl (MBom) group was introduced for sulfhydryl protection of Cys in combination with Fmoc chemistry. The MBom group proved to substantially suppress racemization of Cys during its incorporation mediated by phosphonium or uronium reagents. Furthermore, this group was found to significantly reduce racemization of the C-terminal Cys linked to a hydroxyl resin during repetitive base treatment, in comparison with the usually used trityl (Trt) and acetamidomethyl (Acm) groups.

Disulfide linkages play an important role in the formation and stabilization of distinct three-dimensional structures in naturally occurring peptides and proteins. For the synthesis of such Cys-rich molecules, native chemical ligation (NCL)[1] involving the coupling of a peptide thio ester and a cysteinyl-peptide is an advantageous approach. In the course of peptide assembly, protection of the sulfhydryl group on Cys is indispensable for avoiding side reactions related to its high nucleophilicity, such as acylation, alkylation, or oxidation. For this purpose, many side-chain protecting groups for Cys have been developed.[2] Among them, the trityl (Trt) and acetamidomethyl (Acm) groups are widely accepted as a protecting group for Cys in the Fmoc strategy. During incorporation of these Cys derivatives onto the growing peptide chain, however, considerable base-catalyzed racemization of the Cys residue is known to always occur at the activating and coupling steps with phosphonium or uronium reagents such as PyBOP or HBTU, respectively.[3] This may hamper purification of the products including those obtained after the NCL and disulfide formation reactions. Therefore, the carbodiimide-mediated coupling method has been recommended to reduce the racemization rate during Cys incorporation to acceptable levels ($< 1.0\%$) although this method has no advantage in terms of coupling efficiency over that using phosphonium or uronium reagents.[4] The synthetic procedure would be complicated if the protocols for coupling reagents had to be changed every time incorporation of Cys is performed, especially in the case of

[†] Peptide Institute, Inc.
[‡] Osaka University.

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(4) Incorporation of Cys was performed by a protocol of coupling with Fmoc-amino acid/*N*,*N*′-diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt) (4:4:4 equiv with respect to the peptide; 5-min preactivation) in DMF. See: (a) Angell, Y. M.; Alsina, J.; Albericio, F.; Barany, G. *J. Pept. Res.* **2002**, *60*, 292–299. (b) Han, Y.; Albercio, F.; Barany, G. *J. Org. Chem.* **1997**, *62*, 4307.

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machine-assisted solid phase peptide synthesis (SPPS). In addition to this, racemization of the *C*-terminal Cys esterified to resins also occurs during the repetitive N^{α}-Fmoc deprotection using piperidine.[5] Even when employing a Trt-type resin with the aid of its steric hindrance, racemization of the *C*-terminal Cys associated with the base treatment is likely to occur.[6] In order to find an S-protected Cys derivative applicable for Fmoc chemistry that can efficiently suppress racemization of Cys both when its incorporation is conducted with phosphonium or uronium reagents and when it is linked to a resin via an ester linkage, we introduced the 4-methoxybenzyloxymethyl (MBom) group into the sulfhydryl function of Cys (Figure 1).

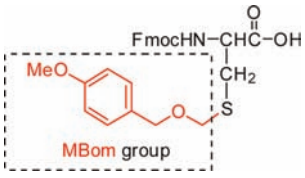
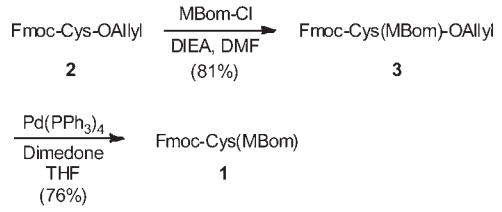


Figure 1. Structure of Fmoc-Cys(MBom) (**1**).

The synthesis of Fmoc-Cys(MBom) (**1**) is illustrated in Scheme 1. Fmoc-Cys-OAllyl (**2**)[7] was alkylated using 4-methoxybenzyloxymethyl chloride (MBom-Cl) to provide Fmoc-Cys(MBom)-OAllyl (**3**), which was subsequently deallylated by treatment with $Pd(PPh_3)_4$ and dimedone to give **1**.

Scheme 1. Synthesis of Fmoc-Cys(MBom) (**1**)



The MBom group was found to be completely stable during the repetitive Fmoc deprotection reaction using 20% piperidine/DMF but readily removable by TFA in the same manner as the Trt group, although the MBom group generates formaldehyde and an electrophilic alkylating species, methoxybenzyl cation, upon TFA cleavage.[8] Formaldehyde can lead to hydroxymethylation of the functional groups on peptides. A particularly serious one is that in which formaldehyde reacts almost quantitatively with a Cys residue located at the *N*-terminus to produce a thiazolidyl (Thz)-peptide during isolation from an acidolysis mixture.[9] This may hamper the preparation of peptides having an *N*-terminal Cys residue used for the subsequent NCL. To circumvent this conversion, methoxyamine hydrochloride ($MeONH_2 \cdot HCl$) has been recommended as a formaldehyde scavenger in the acidolytic cleavage reaction.[10] As for the methoxybenzyl cation, it was reported that alkylation of susceptible residues such as Cys can be effectively prevented by performing TFA cleavage in the presence of thiol compounds.[11] Thus, we applied Fmoc-Cys(MBom) to the synthesis of cysteinyl-angiotensin II (Cys-Ang II: CDRVYIHPF) to examine the byproduct formation associated with the use of this protecting group. As expected, performing TFA cleavage using the reagent K in the presence of $MeONH_2 \cdot HCl$ (5 equiv) was found to be accompanied by virtually no side products arising from formaldehyde and the methoxybenzyl cation (Table 1).

Table 1. Effect of Additives on Cys Modification during TFA Deprotection Using Reagent K[a]

	ratio of Cys-Ang II to Cys(X)-Ang II		
additive	Cys-Ang II	Thz-Ang II	Cys(MeOBzl)-Ang II
none	82	24	<1
$MeONH_2 \cdot HCl$ (5 equiv)	98	<1	<1

[a] Reagent K: TFA/H_2O/phenol/thioanisole/1,2-ethanedithiol (v/v, 82.5/5/5/5/2.5).

Next, the suppressive effect of S-MBom on racemization during incorporation of Cys was evaluated by synthesizing a model peptide, Gly-Cys-Phe-NH_2.[4] The peptide chain was elongated onto a Rink amide resin using the 1-min preactivation procedure of coupling with Fmoc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv) in DMF. The results are summarized in Table 2 in comparison with those of the Acm and Trt groups. Fmoc-Cys(MBom) was found to be accompanied by an acceptable level of racemization (0.4%) on the activating and coupling steps in the conventional SPPS compared with Fmoc-Cys(Trt) and Fmoc-Cys(Acm) (8.0% and 4.8%, respectively). Even in the case of the microwave-assisted SPPS performed at

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(11) Trp is also susceptible to the alkylation with a methoxybenzyl cation. In practical peptide synthesis, however, Trp alkylation is negligible as long as its indole ring is protected by the Boc group, which can produce the N^{in}-carbamic acid compound to prevent such electrophilic attacks during TFA treatment. See: Fields, C. G.; Fields, G. B. *Tetrahedron Lett.* **1993**, *34*, 6661.

50 °C/80 °C,[12] Fmoc-Cys(MBom) caused a significant reduction in the level of racemization (0.8/1.3%) while Fmoc-Cys(Trt) and Fmoc-Cys(Acm) led to a considerable level of racemization (10.9/26.6% and 8.8/15.3%, respectively) (Figure 2).

Table 2. Racemization during Synthesis of the Model Peptide, Gly-Cys-Phe-NH_2, as a Function of the Cys Protecting Group[a]

	racemization (%)[b]		
X	conventional	microwave 50 °C	microwave 80 °C
Trt	8.0	10.9	26.6
Acm	4.8	8.8	15.3
MBom	0.4	0.8	1.3

[a] The coupling reactions were performed using a 1-min preactivation procedure of coupling with Fmoc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv) in DMF. [b] Defined as (Gly-D-Cys-Phe-NH_2)/(Gly-L-Cys-Phe-NH_2) × 100. More details can be found in the Supporting Information.

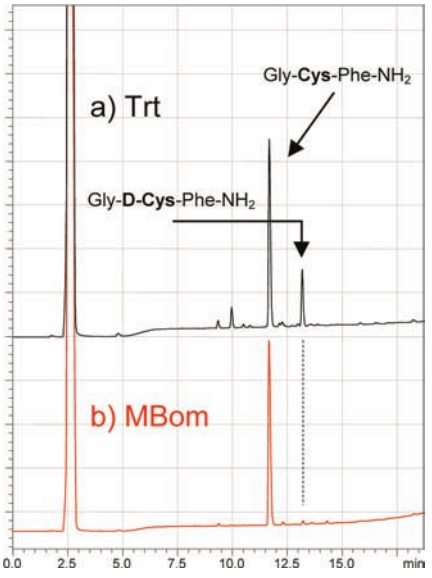

Figure 2. HPLC profiles of the products, Gly-Cys-Phe-NH_2, obtained by performing MW-assisted SPPS at 80 °C. Incorporation of Cys was performed by coupling Fmoc-Cys(Trt) (a) and Fmoc-Cys(MBom) (b). HPLC conditions are described in the Supporting Information.

As for racemization of the *C*-terminal Cys esterified to a Trt-type resin, the Bz-Ser(*t*Bu)-Cys(X)-NovaSynTGT resin, where X is MBom, Acm, or Trt, was exposed to 20% piperidine/DMF for a given period of time to estimate the racemization rate arising during the repetitive Fmoc deprotection reactions.[6] The products prepared using

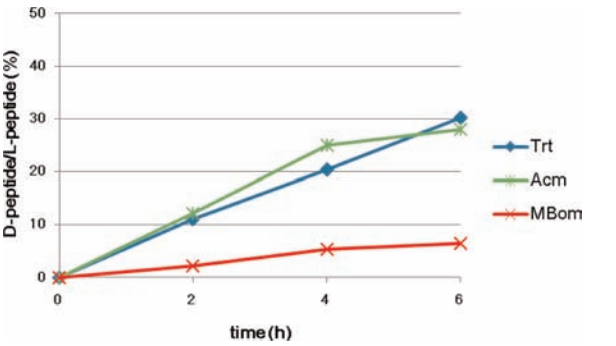

Figure 3. Racemization of the C-terminal Cys linked to Trt resin during exposure to 20% piperidine/DMF. Defined as (Bz-Ser-D-Cys)/(Bz-Ser-L-Cys) × 100. More details are described in the Supporting Information.

Cys(Trt) and Cys(Acm) contained significant amounts of isomers in that order. However, a much lower rate of racemization (6.4%) was detected even after a 6-h treatment with 20% piperidine/DMF when Cys(MBom) was used (Figure 3). This indicated that more attention should be paid to the suppression of racemization with the *C*-terminal Cys linked to a hydroxyl resin, especially when synthesizing a long peptide. These results suggested that use of the MBom group effectively suppresses racemization at the *C*-terminal Cys caused by the base treatment, probably due to an electron-donating effect. Racemization occurs through oxazolone and direct enolization. In particular, Cys tends to be racemized via enolization due to stabilization of the carbanion formed on α-proton abstraction.[13] To destabilize this enol form, therefore, an S-protecting group that possesses an electron-donating effect would be essential to prevent racemization of Cys.[14]

In order to demonstrate the usefulness of the MBom group on Cys, especially in the NCL chemistry, we synthesized human glucagon-like peptide 1 (GLP-1, 7−36 amide)[15] by Ala ligation involving a ligation−desulfurization approach[16] that consisted of NCL between the thioester peptide (7−24) and Cys^{25}-(26−36)-NH_2 followed by desulfurization to convert Cys^{25} to Ala^{25}. When synthesizing Cys^{25}-(26−36)-NH_2 by using Fmoc-Cys(Trt) with the aid of HCTU/6-Cl-HOBt/DIEA, the product was found to be contaminated by 7% of D-Cys^{25}-(26−36)-NH_2 at a moderate estimate. The diastereoisomer was eluted at the same retention time as the desired peptide on RP-HPLC using various gradient systems although the isocratic system made it difficult to separate because it was a shoulder peak

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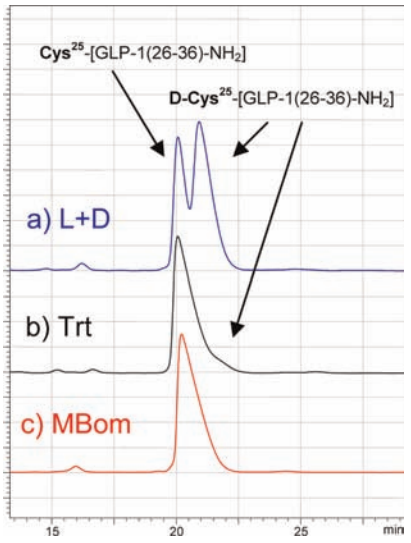

Figure 4. HPLC profiles of Cys^{25}-[GLP-1(26−36)-NH_2]. (a) A mixture of L-Cys^{25}- and D-Cys^{25}-[GLP-1(26−36)-NH_2]. The product obtained by using Fmoc-Cys(Trt) (b) or Fmoc-Cys-(MBom) (c). HPLC conditions are described in the Supporting Information.

(Figure 4). Even after NCL and desulfurization, the racemized peptides arising from D-Cys^{25} and D-Ala^{25}, respectively, could not be separated from the respective desired products by RP- and IEX-HPLC. Thus, there were no practical purification procedures available for removing these side products. In general, a diastereoisomer with racemization somewhere in its sequence tends to be difficult to separate from the intact peptide as its chain length increases. Therefore, it is important to exclude the risk of racemization with incorporation of Cys by using Fmoc-Cys(MBom) during the course of the chain assembly. In fact, racemization with the *N*-terminal Cys in Cys^{25}-(26−36)-NH_2 was found to be negligible when using Fmoc-Cys(MBom) as shown in Figure 4. This measure using Fmoc-Cys(MBom) can facilitate the avoidance of ambiguities in the quality of the synthesized peptides.

In summary, the MBom group on Cys was demonstrated to possess all of the chemical properties required for the Fmoc strategy and to effectively prevent racemization of Cys during its incorporation mediated even by phosphonium or uronium reagents as well as that of the *C*-terminal Cys esterified to the solid support during repetitive base treatment.

Supporting Information Available. Experimental procedures, NMR charts for key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.