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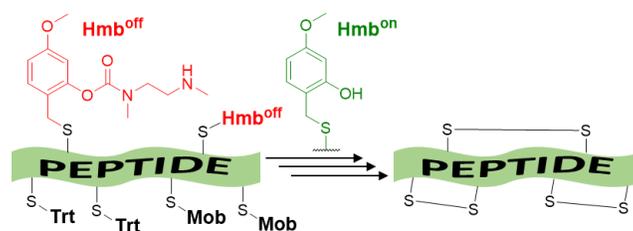
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2-hydroxy-4-methoxybenzyl as thiol-protecting group for directed-disulfide bond formation

Claude Zoukimian,^{†,‡} Rémy Bérout,[‡] and Didier Boturyn^{*,†}

[†] Univ. Grenoble Alpes, CNRS, Department of Molecular Chemistry, 570 rue de la chimie, CS 40700, Grenoble 38000, France

[‡] Smartox Biotechnology, 6 rue des platanes, Saint-Egrève 38120, France



ABSTRACT: The chemical synthesis of disulfide-rich peptides such as toxins can be accomplished by using numerous orthogonal cysteine-protecting groups. Herein we report the use of Hmb^{off/on} protecting group for directed disulfide bond formation. Its combination with classical Trt, Acn and Mob groups was studied for the synthesis of NMB-1 and Phlotoxin-1 toxins highlighting new orthogonal strategies for directed disulfide bond formation.

Disulfide bonds are key structural elements of numerous peptides and proteins as the correct three-dimensional structure is essential to function.¹ Although most peptides and proteins fold correctly spontaneously, many have limitations on their folding abilities leading to misfolded isomers. To this end, the use of orthogonal thiol-protecting groups may provide an access to disulfide-rich peptides (DRPs).² To date, several cysteine protecting groups that are compatible with the Fmoc-based solid-phase peptide synthesis (SPPS) were exploited.³ Most of them are acid-labile protecting groups such as trityl (Trt) and methoxybenzyl (Mob) derivatives that are removed under conditions that prevent scrambling of disulfide bonds in peptides. Besides, other orthogonal protecting groups like the acetamidomethyl (Acm) or the tert-butylsulfenyl (*S*-*t*Bu) moieties allow the regioselective formation of multiple disulfide bonds.⁴ Very recently, Ashraf Brik and coll. has described an ultrafast one-pot formation of three disulfide by using the disulfiram, UV light and PdCl₂.⁵ Undoubtedly, this strategy will pave the way for the access of DRPs that contains up to three disulfide bridges.

To expand the scope of those strategies, the development of new thiol-protecting groups is desirable. In this study, we present the development and study of 2-hydroxy-4-méthoxybenzyl (Hmb) derivative as cysteine-protecting group for the synthesis of DRPs. The Hmb group was initially designed as a backbone-protecting group for the synthesis of difficult peptide sequences preventing aggregation.⁶ This group can be removed by using TFA during Fmoc-based SPPS. Lei Liu and co-workers have later developed an acylated 2-hydroxyl-containing Hmb derivative, the *N*-methyl-*N*-[2-(methylamino)-ethyl]-carbamoyl group (Hmb^{off}), which is less electron-rich and stable to TFA.⁷ Restoring Hmb, namely Hmb^{on}, was then carried out by using neutral buffered conditions (Figure 1). It is important to note that Hmb^{off} is fully compatible with SPPS. Interestingly, this Hmb^{off/on} system was recently exploited as thiol-protecting group for the synthesis of protein *via* the native chemical ligation.⁷

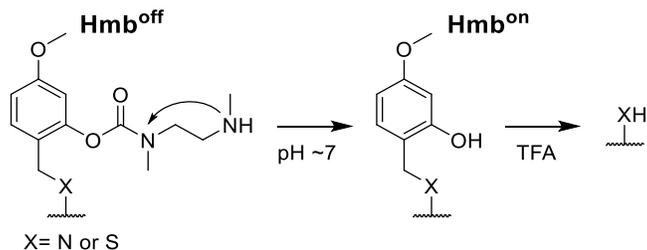
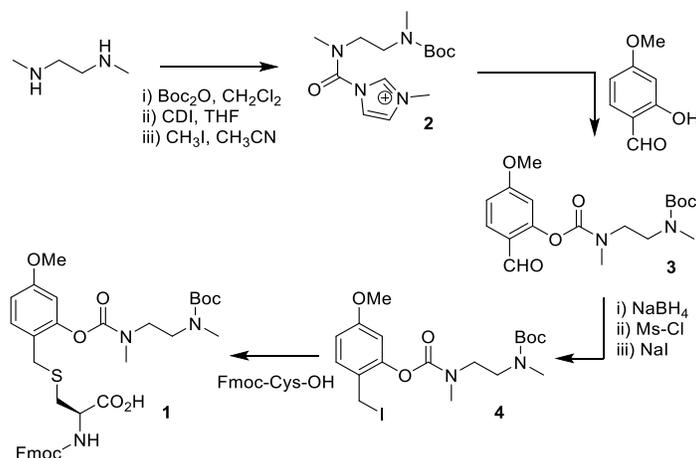


Figure 1. Chemical structures and deprotection scheme of the Hmb^{on/off} protecting group

Scheme 1. Synthetic scheme for Fmoc-Cys(Hmb^{off})-OH



Herein we report a novel strategy for the synthesis of DRPs combining the Hmb^{off/on} group with classical thiol-protecting groups. Prior to the DRP synthesis, the Fmoc-Cys(Hmb^{off}) **1** was prepared as described⁸ with some modifications (Scheme 1). Briefly, Boc protection of *N,N'*-Dimethylethylenediamine was followed by reaction with carbonyldiimidazole (CDI) affording the compound **2**. The intermediate **3** is then obtained by reaction of the carbamide-containing compound **2** with 4-methoxy-2-hydroxybenzaldehyde. Reduction of the aldehyde function to alcohol using sodium borohydride and treatment by mesyl chloride (MsCl) and NaI produced the compound **4**. Finally, the Fmoc-Cys(Hmb^{off})-OH **1** was obtained from the coupling of Fmoc-Cys-OH.

In order to evaluate the orthogonality of the Hmb^{off/on} group with Trt, Acn and Mob groups, we performed the synthesis of the NMB1 toxin (Figure 2).⁹ This peptide contains 19 amino acids with four cysteine residues forming the C⁵-C¹⁹ and C⁶-C¹⁰ bridges.

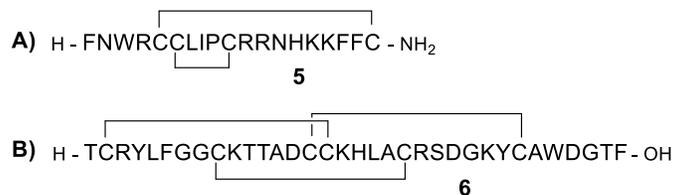
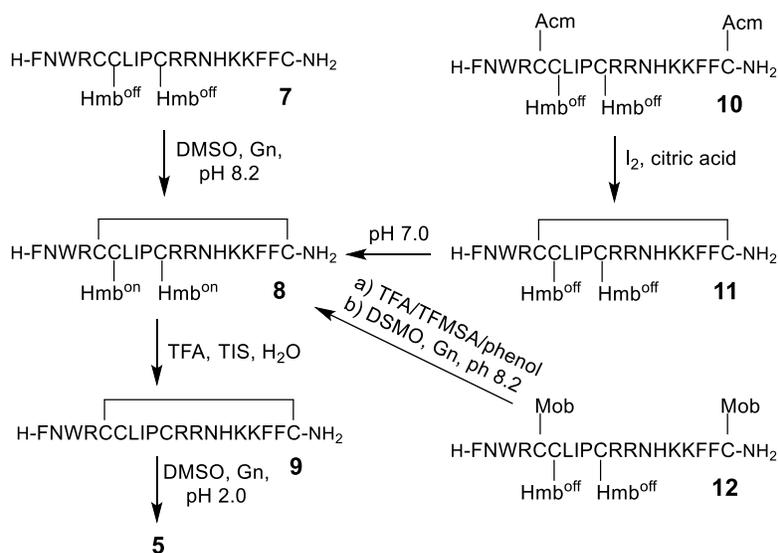


Figure 2. Structures of A) NMB1 and B) PhlTx1

We first sought to confirm the orthogonality of Hmb^{off} with standard Trt protective group (Scheme 2). For that purpose, we synthesized NMB1 containing Trt on Cys⁵ and Cys¹⁹ and Hmb^{off} on Cys⁶ and Cys¹⁰. TFA acidolysis provided the linear toxin derivative **7** displaying two free thiols and two Cys(Hmb^{off}). A first disulfide formation was carried out under alkaline conditions overnight using DMSO as oxidant and guanidine improving peptide folding, and was confirmed by HRMS analysis. It is important to note that under these conditions we observed the

conversion of Hmb^{off} into Hmb^{on} without undesired side-reaction providing compound **8** (Figure S-3). Then, Hmb^{on} groups were removed in a mixture of TFA/triisopropylsilane/water (95:2.5:2.5) as described.⁸ HPLC and MS analyses confirmed the formation of the desired fully unprotected peptide **9** with slight amount of Hmb^{on}-containing adducts (Figures S-7 and S-8). The latter were partially removed by adding 2,2'-(Ethylenedioxy)diethanethiol (DODT) in the reaction mixture. The formation of the last disulfide bond was carried out in a buffer (pH 2.0) containing DMSO and guanidine overnight. The acidic conditions are required in order to avoid scrambling.¹⁰

Scheme 2. Synthetic scheme for NMB1



We then evaluated Hmb^{off} orthogonality with Acm group starting with the NMB1 derivative that contains Acm on Cys⁵ and Cys¹⁹ (Scheme 2). The protected peptide **10** was applied to Acm cleavage cocktail (iodine in acidic buffer) during one hour.¹¹ The desired C⁵-C¹⁹ disulfide bond (peptide **11**) was obtained without affecting Hmb^{off} groups. The same experiment was conducted with peptide displaying Acm and Hmb^{on} obtained by incubating protected NMB1 in a neutral buffered solution prior to Acm cleavage cocktail. MS analysis revealed that applying this approach resulted in the random formation of both disulfide bonds (data not shown). This indicates that Hmb^{on} can be easily removed by iodine with concomitant disulfide bond formation, and that Acm and Hmb^{on} are not orthogonal. Conversely, Acm group is known to be resistant to conditions used to deprotect Hmb^{on}.¹²

Next, Hmb^{off/on} orthogonality with Mob group was studied by using NMB1 derivative **12** that contains Mob on Cys⁵ and Cys¹⁹. Mob groups were removed by using a solution containing TFA/TFMSA/phenol 8:1:1 at 0°C,¹³ and disulfide bridge was subsequently obtained under oxidative alkaline conditions. Nevertheless, major undesired products were observed corresponding to the loss of one or two Hmb^{off} group and indicating that this group is sensitive to Mob cleavage conditions. This results in a low 4% yield. It is important to note that Hmb^{on} can be removed before Mob groups since Mob groups are resistant to TFA.¹⁴

oxidation of Cys(Hmb^{on}) using iodine could not be carried out since the AcM group is not stable under these conditions. Hmb^{on} were then removed under acidic conditions as described for NMB-1. Attempt of the following disulfide formation of crude peptide **14** in an acidic oxidative buffer produced the desired product **15** and several side-products corresponding to the loss of one or two AcM group, not stable under a prolonged exposure to acidic oxidative conditions (Figure S-25). We then decided to carry out the reaction by using iodine in glacial acetic acid for a short time (5 min). These conditions are known to preserve the AcM group.¹¹ The Cys²-Cys¹⁷ disulfide bond was obtained and the product **15** isolated in 16% yield including the Hmb^{on} deprotection step. In these conditions, no major side products due to the use of iodine were observed during the reaction (Figures S-21 and S-22). It is important to note that Hmb^{on} is stable under these conditions preventing concomitant Cys(Hmb^{on}) deprotection and oxidation. Another attempt to produce this disulfide bond from **14** was done using supported Ellman's reagent (ClearOx resin) in 1 % acetic acid buffer.¹⁷ Unfortunately, the two disulfide bonds-containing peptide was obtained in lower yield, due to the formation of oxidized by-products. The last Cys¹⁶-Cys²⁹ disulfide bond was finally formed by using several equivalents of iodine in acidic buffer affording AcM deprotection and subsequent oxidation. Here again, no iodine adducts nor over-oxidized by-products were observed using these conditions (Figure S-26), and the PhITx1 **6** was isolated in 48 % yield (~1.5% overall yield including SPPS).

In parallel, the synthesis of toxin **6** was carried out by using the Trt/Hmb^{off/on}/Mob strategy (Scheme 3B). Linear PhITx1 **16** was synthesized as previously described with Mob moiety instead of AcM as protecting group for Cys¹⁶/Cys²⁹ pairs. As the Mob group is stable under iodine conditions unlike AcM, a sequential one-pot formation of Cys²-Cys¹⁷ and Cys⁹-Cys²² disulfide bonds was carried out as described for the Trt/AcM/Mob strategy.¹⁶ The crude peptide **16** was then incubated overnight in a citrate buffer (pH 7.4) that contains DMSO and guanidine. Formation of C⁹-C²² disulfide bond and the full conversion of Hmb^{off} into Hmb^{on} was observed by HPLC-HRMS. The pH was dropped to 1.5 and iodine was then added over 20 minutes. As expected, HPLC-HRMS analysis confirmed the formation of the second disulfide bond, without major side products formation (Figure S-33). The product **17** was isolated in 13 % overall yield including the SPPS. Mob groups were removed in a mixture of TFA/phenol/TFMSA 8:1:1 at 0°C and peptide **18** was isolated as a white powder after ether precipitation. Finally, the last disulfide bond was formed in an acidic oxidative buffer containing guanidine. After 48 hours, the native-like PhITx1 **6** was obtained as the major product (Figure S-28) and was isolated from RP-HPLC purification in 2 % overall yield including the SPPS. This result indicates that racemisation was minimized during this step. Moreover, the same folding pattern was obtained either using Trt/Hmb^{off/on}/AcM or Trt/Hmb^{off/on}/Mob strategy (See Figure 3), which was identical to the bioactive product previously obtained with the Trt/AcM/Mob strategy.¹⁶ It is important to note that the fully oxidized PhITx1 shows two peaks in analytical HPLC as previously observed, unless the column is heated.^{16,18} Recently, Gonçalves *et al.* have described the chemical synthesis of PhITx1 by using a random disulfide oxidation strategy but no information concerning synthetic yields was given.¹⁸

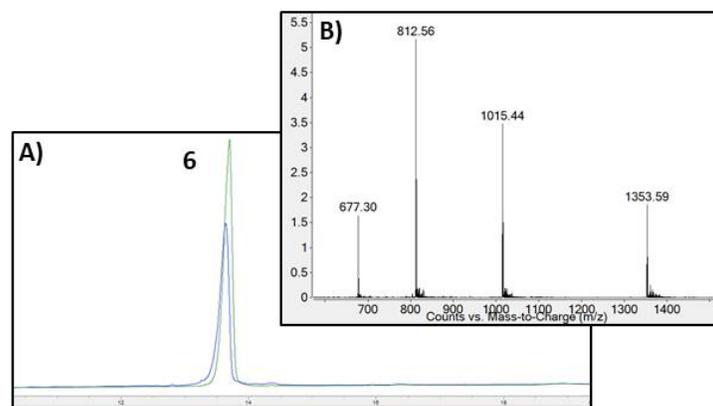


Figure 3. Characterization of Phlotoxin-1 **6**. A) HPLC chromatograms of **6** obtained by Trt/Hmb^{off/on}/AcM (blue) and Trt/Hmb^{off/on}/Mob (green) strategies; HPLC column was heated to 55°C. B) HRMS analysis of **6**: Exact mass calculated for C₁₈₃H₂₅₄N₄₆O₄₈S₆ 4055.7173; found 4055.7448.

In conclusion, this report expands the range of chemical strategies for the synthesis of DRPs with comparative yields to Trt/AcM/Mob strategy. Moreover, regarding the peptide sequence the Hmb^{off/on} thiol protection provides a very useful alternative to current protocols for directed disulfide bond formation avoiding iodine or TFMSA used

respectively for Acn or Mob removal. Finally, considering the orthogonality of Hmb^{off/on} the regioselective formation of four disulfide bonds could be envisioned.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General procedures and methods for SPPS, experimental details and characterization data for compounds **1**, **5-8** and **10-17**.

AUTHOR INFORMATION

Corresponding Author

* Give contact information for the author(s) to whom correspondence should be addressed.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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