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Regioselective Formation of Multiple Disulfide Bonds with the Aid of Postsynthetic S-Tritylation

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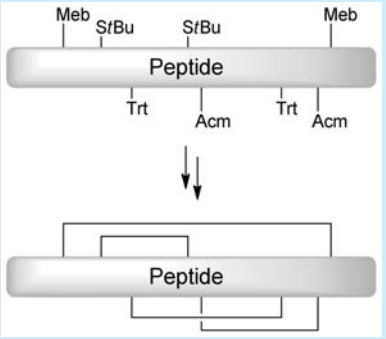
Supporting Information

ABSTRACT: Disulfide bond formation performed in solution with the *tert*-butylthio (S*t*Bu) group was accomplished using a free peptide having only the sulfhydryl groups of Cys protected with the aid of postsynthetic S-tritylation. This facilitated removal of the S*t*Bu group with subsequent disulfide formation without any difficulty. This strategy using the S*t*Bu group in combination with the widely used acetamidomethyl (Acm), 4-methylbenzyl/4-methoxybenzyl (Meb/Mob), and trityl (Trt) groups enables reliable and regioselective synthesis of multicystine peptides.



Disulfide bond formation plays an important role in stabilizing the native conformation of peptides and proteins.[1] Thus, a particular combination with disulfide bonding in Cys-rich peptides/proteins is critical for expressing their intrinsic biological activities.[2] To assess the biological activities of disulfide-coupled peptides, extreme care is required in the chemical synthesis to avoid ambiguity in the quality of the synthesized peptides, especially in their disulfide connectivity. In practical peptide synthesis, disulfide formation of Cys-rich peptides is often performed by subjecting their reduced precursor peptides to the freely oxidative folding reaction. This procedure is believed to predominantly provide the native disulfide pairings under thermodynamic control using redox reagents such as gluthatione (GSH) and oxidized gluthatione (GSSG).[3] Even in this situation, the obtained disulfide-coupled products need to be verified as to whether they possess the correct disulfide pairings on the basis of chemical and/or spectroscopic analyses.[4] A promising alternative option is to use a regioselective strategy involving stepwise formation of disulfide bonds to synthesize disulfide-coupled peptides having the native conformation.[5] This can be accomplished by using various orthogonal protection schemes of the side-chain protecting groups for Cys. In Fmoc chemistry, the most commonly used combinations consist of the trityl (Trt), acetamidomethyl (Acm), 4-methybenzyl (Meb)/4-methoxybenzyl (Mob), *tert*-butyl (*t*Bu), and *tert*-butylthio (S*t*Bu) groups. Among them, the reliability of the combination of the Trt, Acm, and Mob/Meb groups has been established by synthesizing various tricystine peptides via regioselective formation of disulfide bonds.[6] To extend the applicability with this combination into the synthesis of a tetracystine peptide, the *t*Bu group[7] or a safety-catch protecting group, such as 4,4′-dimethylsulfinylbenzhydryl (Msbh),[8] was suggested as a fourth thiol protecting group. However, removal of the *t*Bu and Msbh groups and their concomitant disulfide formation conducted by dimethylsulfoxide (DMSO)/trifluoroacetic acid (TFA)[9] or silyl chloride sulfoxide[10] and NH_4I/DMS/TFA,[8] respectively, led to considerable modification of the unprotected Trp residue(s) in target peptides, although this was not accompanied by a disulfide exchange reaction, except in the silyl chloride sulfoxide method.[11] In view of this obstacle with Trp, we decided to try the S*t*Bu group with the fourth thiol protecting group possessing complete orthogonality to the Trt, Acm, and Meb/Mob groups.

The S*t*Bu group is removable by treatment with reducing agents such as thiols and trialkylphosphines. Therefore, this protecting group must be involved with the formation of the first disulfide bond in the course of the regioselective steps in order to avoid the cleaving and/or scrambling of pre-existing disulfide bond(s) in the molecule. For this reason, removal of the S*t*Bu group and subsequent disulfide formation has almost always been performed on resin prior to TFA acidolysis. However, on-resin folding with the S*t*Bu group often results in the formation of side products and low yields,[12] presumably due to the lack of uniformity in reaction processes as well as steric hindrance caused by side-chain protecting groups. Thus, the S*t*Bu group has not been widely used in practical peptide synthesis. In order to avoid the problem in on-resin manipulations with the S*t*Bu group and to facilitate its removal and the subsequent disulfide formation, we carried out these procedures in solution by using a peptide in which all the side-chain protecting groups had been removed except those for the

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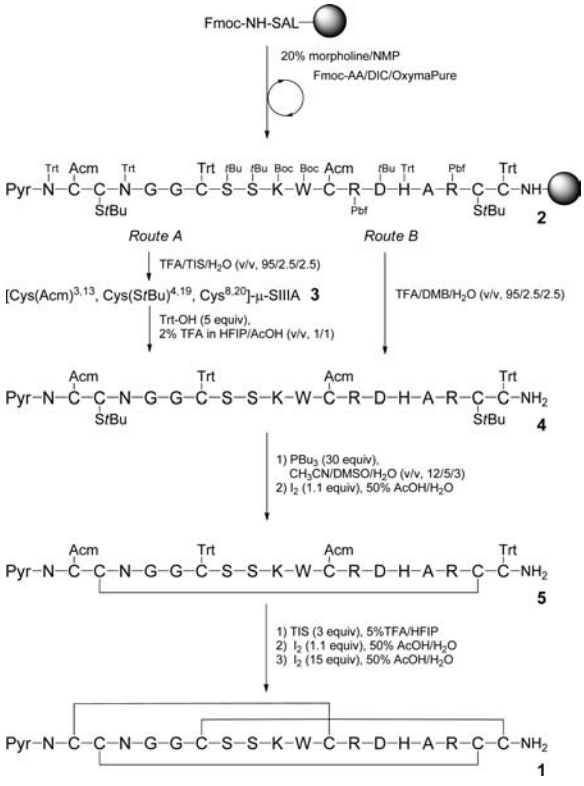
Cys residues. The standard TFA treatment, that is, TFA/triisopropylsilane (TIS)/H_2O (v/v, 95/2.5/2.5) in the absence/presence of thiols, provides peptide cleavage from a resin and global deprotection of the side-chain protecting groups including the Trt group on Cys, while the Acm, Meb/Mob, and *S*t*Bu* groups on Cys remain intact. Thus, there are two approaches to obtaining such a free peptide having only the sulfhydryl groups of Cys protected: (A) reintroduction of the Trt group into Cys sulfhydryl groups after TFA cleavage or (B) retrapping of the Trt cation by Cys sulfhydryl groups during TFA cleavage. The former is achieved by postsynthetic modification using Trt−OH in hexafluoro-2-propanol (HFIP) to selectively promote S-tritylation in the presence of peptide functionalities.[13] In the latter case, the Trt cation generated during TFA cleavage is trapped again in Cys by changing the carbocation scavengers in TFA acidolysis cocktails from TIS/thiols to 1,3-dimethoxybenzene (DMB) since DMB is a less effective Trt cation scavenger than the Cys sulfhydryl group due to steric hindrance.[14]

Applying this approach, we regioselectively synthesized a tricystine peptide having the Trp residue, μ-conotoxin SIIIA (μ-SIIIA), with the aid of orthogonal Cys protection using the Acm, S*t*Bu, and Trt groups. μ-SIIIA is a 20 residue peptide isolated from *Conus striatus* and is considered to be a selective blocker of tetrodotoxin-resistant neuronal sodium channels.[15] Chain assembly was performed on a Rink-amide resin by using Fmoc chemistry with diisopropylcarbodiimide/ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure)[16] activation for coupling to prevent racemization of the Cys residues.[17] Furthermore, to avoid aspartimide (Asi) formation with the susceptive sequence Asp[15]−His[16] in the μ-SIIIA molecule, repetitive Fmoc deprotection was conducted by substituting 20% morpholine/*N*-methyl-2-pyrrolidone (NMP) (5 min × 4) for 20% piperidine/NMP (2.5 min × 4), although prolonged deprotection steps were required for complete removal of the Fmoc group.[18]

This measure led to a significant reduction in Asi formation (<1%). After completion of the chain assembly, the peptide resin **2** was treated by TFA/TIS/H_2O (v/v, 95/2.5/2.5) or TFA/DMB/H_2O (v/v, 95/2.5/2.5) at room temperature for 1 h (route A or B in Scheme 1, respectively). The latter directly yielded Cys(Acm)^{3,13}/Cys(S*t*Bu)^{4,19}/Cys(Trt)^{8,20} μ-SIIIA (**4**), while the former gave Cys(Acm)^{3,13}/Cys(S*t*Bu)^{4,19} μ-SIIIA with free thiols at Cys^{8,20} (**3**), which was subsequently subjected to postsynthetic tritylation to yield **4**. We previously reported the tritylation method using Trt−OH in HFIP, which can offer S-protection on Cys located in fully unprotected peptides, but it was accompanied by a side product (2−5%) in which the Trt group was attached to the τ-nitrogen of His.[13] This obstacle was overcome by configuring an alternative tritylation procedure using Trt−OH in HFIP/AcOH (v/v, 1/1) containing 2% TFA (Supporting Information Figure S1). Product **4** obtained via route A was comparable to that via route B in terms of the high-performance liquid chromatography (HPLC) profile of crude products and the isolated yield after purification (Figure 1), although the latter route is not applicable when a thiol compound has to be included as a carbocation scavenger in TFA cocktails (e.g., the reagent K). To construct the first disulfide bond, the S*t*Bu groups on Cys^{4,19} were readily removed by tributylphosphine (PBu_3) in DMSO/CH_3CN/H_2O, and the liberated thiols were subsequently oxidized with I_2 (1.1 equiv) in AcOH/H_2O to give **5**. After removal of the Trt groups on Cys^{8,20} by 5% TFA/HFIP in the presence of TIS (5 equiv) followed by dilution of the reaction mixture with AcOH/H_2O, consecutive disulfide formation with Cys^8−Cys^{20} and Cys(Acm)^3−Cys(Acm)^{13} by the tandem addition of 0.1 M I_2/MeOH (1.1 and 15 equiv, respectively) gave single major peaks at the respective steps (Figure 2).[19] Synthetic μ-SIIIA (**1**) was identical with the product that had been oxidatively folded from the hexathiol precursor in the presence of GSH/GSSG, by an analytical procedure using reverse-phase (RP)-HPLC (Supporting Information Figure S6). This freely oxidized product had been confirmed to possess the native disulfide connectivity by a chemical procedure.[20]

Scheme 1. Regioselective Synthesis of μ-SIIIA



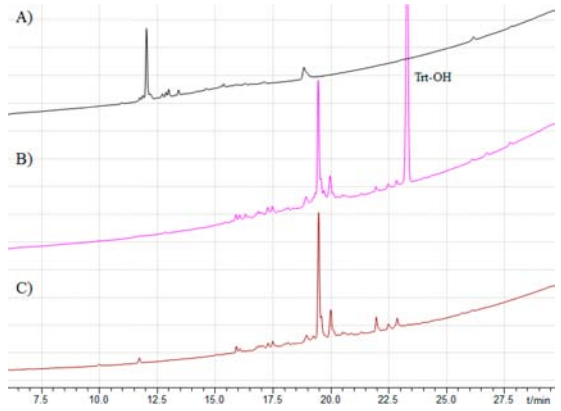


Figure 1. HPLC profiles of the synthetic intermediates of μ-SIIIA. Crude reaction products are shown. (A) [Cys(Acm)^{3,13}, Cys(S*t*Bu)^{4,19}, Cys^{8,20}]-μ-SIIIA (**3**). (B) [Cys(Acm)^{3,13}, Cys(S*t*Bu)^{4,19}, Cys(Trt)^{8,20}]-μ-SIIIA (**4**) obtained by route A. (C) **4** obtained by route B. HPLC conditions: column, YMC-Pak ODS (4.6 × 150 mm); elution, 10−80% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

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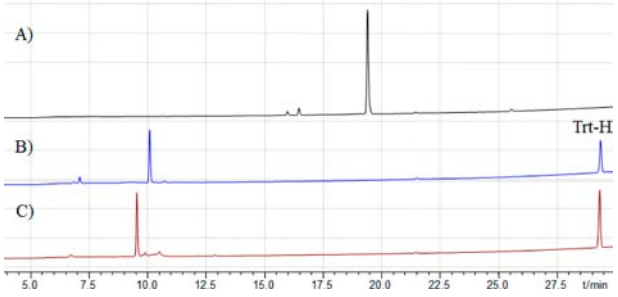
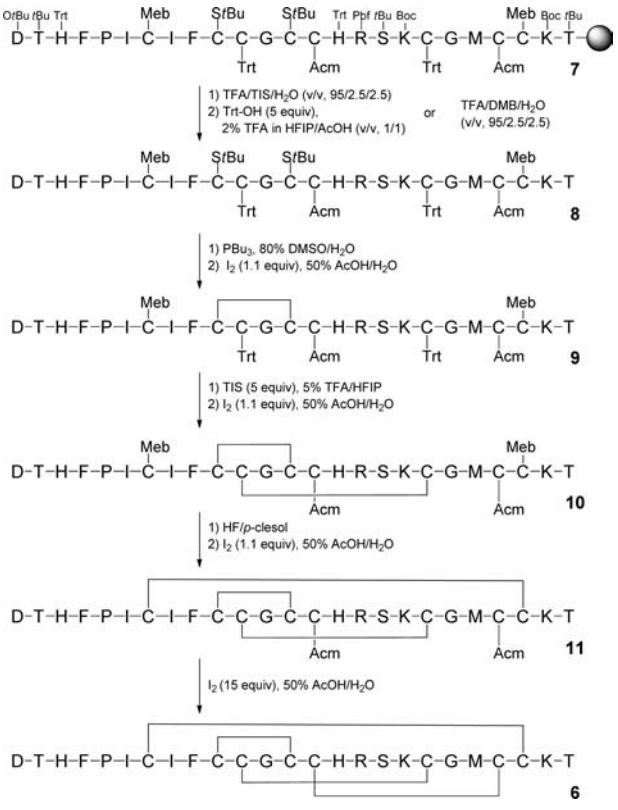


Figure 2. RP-HPLC profiles showing stepwise formation of disulfide bonds in the μ-SIIIA molecule. (A) 1SS, [Cys(Acm)^{3,13}, Cys^{4}–Cys^{19}, Cys(Trt)^{8,20}]-μ-SIIIA (**5**). (B) 2SS, [Cys(Acm)^{3,13}, Cys^{4}–Cys^{19}, Cys^{8}–Cys^{20}]-μS-IIIA. (C) 3SS, [Cys^{3}–Cys^{13}, Cys^{4}–Cys^{19}, Cys^{8}–Cys^{20}]-μ-SIIIA (**1**). HPLC conditions: column, YMC-Pak ODS (4.6 × 150 mm); elution, 1–60–98% CH_3CN in 0.1% TFA (20–10 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

Next, the strategy using the S*t*Bu group was applied in combination with the Trt, Acm, and Meb groups to the regioselective synthesis of a tetracystine peptide, human hepcidin. Chain assembly was performed using an ABI433A on an Fmoc–Thr(*t*Bu)–Wang resin by the same procedure as that for μ-SIIIA except for the use of Fmoc deprotection, which was done using 20% piperidine/NMP (2.5 min × 4). Peptide resin **7** was treated by TFA/TIS/H_2O to give Cys(Meb)^{7,23}/Cys(S*t*Bu)^{10,13}/Cys(Acm)^{14,22} hepcidin with free thiols at Cys^{11,19}, which was subjected to S-tritylation using Trt–OH in HFIP/AcOH containing 2% TFA to produce the peptide **8**, with all the sulfhydryl groups protected (Scheme 2).

Alternatively, peptide **8** could be directly obtained by treating peptide resin **7** with TFA/DMB/H_2O (Supporting Information Figure S8). Cleavage and oxidation of the Cys derivatives in the order corresponding to the S*t*Bu and Trt groups furnished the first and second disulfide bond formation, respectively. The Meb groups on Cys^{7,23} were removed with HF/*p*-cresol, and the liberated thiols were subsequently oxidized with I_2 (1.1 equiv) in 50% AcOH/H_2O to give **11**. In the last step, treatment of **11** with I_2 (15 equiv) resulted in removal of the Acm groups with simultaneous disulfide bond formation between Cys^{14} and Cys^{22} to give the product **6**, which was identical to a commercial sample of human hepcidin (PI 4392s),[8] having the native fold according to RP-HPLC analysis (Figure 3).

Scheme 2. Regioselective Synthesis of Human Hepcidin



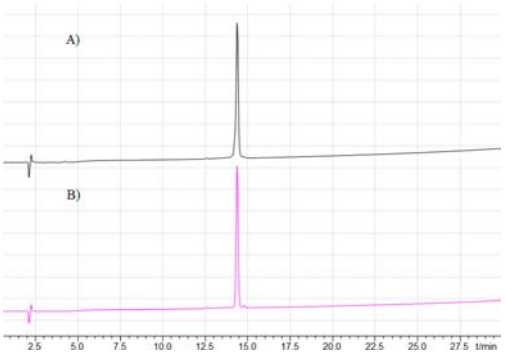


Figure 3. RP-HPLC profiles of the synthetic human hepcidine. (A) Regioselectively synthesized human hepcidin **6**. (B) Commercial human hepcidin (Peptide International, PI4392s). HPLC conditions: column, YMC-Pak ODS (4.6 × 150 mm); elution, 10–60% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

Applying well-established procedures for removal of Cys protection and disulfide formation with the Acm, Meb/Mob, S*t*Bu, and Trt groups, we found no significant side reactions including disulfide rearrangement and Trp modification upon construction of the individual disulfide bonds of μ-SIIIA and human hepcidin.

In summary, we successfully synthesized μ-SIIIA and human hepcidin by regioselective formation of disulfide bonds using commercially available protecting groups for Cys, such as Acm, Meb, S*t*Bu, and Trt, which are completely orthogonal to one another. In particular, the in-solution folding with the S*t*Bu group was accomplished by using peptides with postsynthetic *S*-Trt protection. This facilitated removal of the S*t*Bu group and the subsequent disulfide formation that was performed in solution to afford a high yield without any side reaction. The strategy using the S*t*Bu group in combination with the Acm, Meb/Mob, and Trt groups enables reliable and regioselective synthesis of multiple cystine peptides.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, characterization, and chromatographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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