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Silyl-protected propargyl glycine for multiple labeling of peptides by chemoselective silyl-deprotection

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ABSTRACT

We synthesized Fmoc-propargyl glycine derivatives bearing different silyl protecting groups that can be readily introduced by using a standard solid-phase peptide coupling procedures. Taking advantage of the orthogonality between the different silyl protecting groups, chemoselective incorporation of functional molecules into a 19-mer peptide through click reactions was demonstrated.

Site-specific chemical modification of peptides or proteins is an essential technique for biological research and drug discovery [1]. Total chemical protein synthesis, which consists of solid-phase peptide synthesis (SPPS) and peptide ligation, has become a promising technology to obtain homogeneously modified proteins [2,3]. Native chemical ligation [4], which utilizes the chemoselectivity between a C-terminal thioester and an *N*-terminal Cys residue, is the most widely employed peptide ligation technique to condense divided peptide segments and form native amide bonds. To synthesize proteins that do not contain Cys residues in the sequences, free-radical desulfurization to convert Cys residues into Ala residues has been exploited [5]. However, the radicals generated in the desulfurization often cause side reactions of functional molecules such as alkyne moieties and fluorescent dyes that contain π-extended conjugations [6,7], limiting the introduction of functional molecules into chemically synthesized proteins.

To address this issue, we previously developed a method to incorporate functional molecules into peptides or proteins site-selectively by using alkynes protected with different silyl groups. Silyl-protected alkynes tolerate the free-radical desulfurization conditions and can be removed orthogonally based on differing steric hindrance, enabling to site-selective labeling of peptides by copper-catalyzed azide–alkyne cycloaddition (CuAAC) [8].

However, these silyl-protected alkynes are generally incorporated at Lys residues or *N*-terminal amino groups, and the replacement of amino groups with hydrophobic silyl groups leads to significant reduction in the water solubility of the peptides. Moreover, we could not fine-tune the length of the linker when functional molecules were introduced at side chains of Lys residues, which were not suitable for application in distance-dependent analyses such as Förester resonance energy transfer (FRET) assays.

Herein, we developed two Fmoc-propargylglycine derivatives bearing dimethylethylsilyl (DMES)-protected or *t*-butyldimethylsilyl (TBS)-protected alkynes [Fmoc-Pra(DMES)-OH or Fmoc-Pra (TBS)-OH] that can be readily introduced by using standard coupling SPPS. We prepared 19-mer peptide with these Fmoc-amino acids, and site-selective CuAAC was preformed through the orthogonal deprotection of DMES and TBS groups (Scheme 1).

To synthesize Fmoc-Pra(DMES)-OH and Fmoc-Pra(TBS)-OH, 2-tetrahydropyranyl (THP)-protected propargylalcohol (**1**) was used as starting material (Scheme 2). The hydrogen at the terminal alkyne was abstracted by *n*-BuLi in tetrahydrofuran (THF) at −78 °C and then DMES or TBS chloride was added to introduce each silyl group onto the terminal alkyne. Upon completion, the THP group was removed under acidic conditions to afford compounds **2a** and **2b**. Diphenylphosphate, a bulky leaving group, was introduced at the hydroxy group of **2a** and **2b** in the presence of 4-dimethylaminopyridine (4-DMAP) and pyridine to obtain

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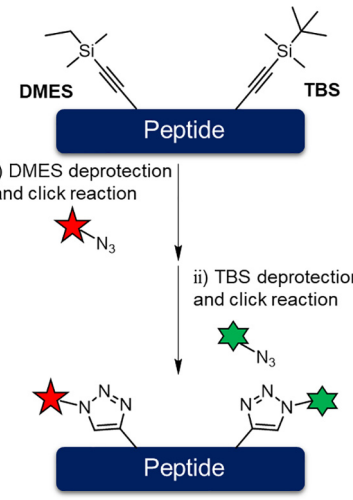
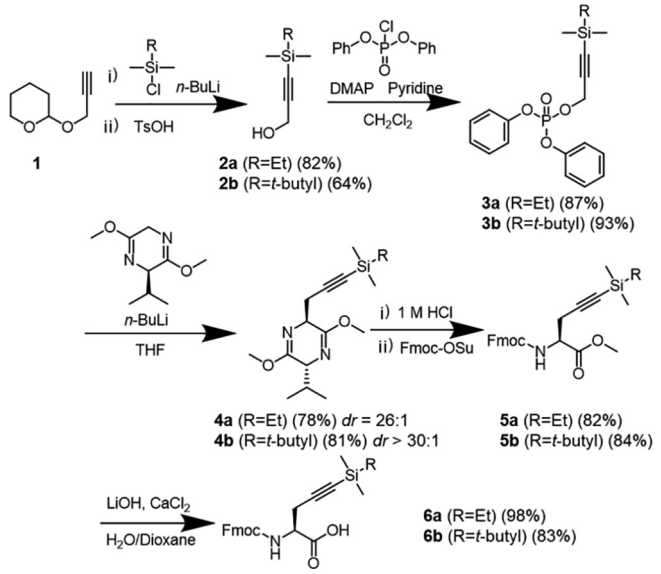


Scheme 1. General strategy of this study.

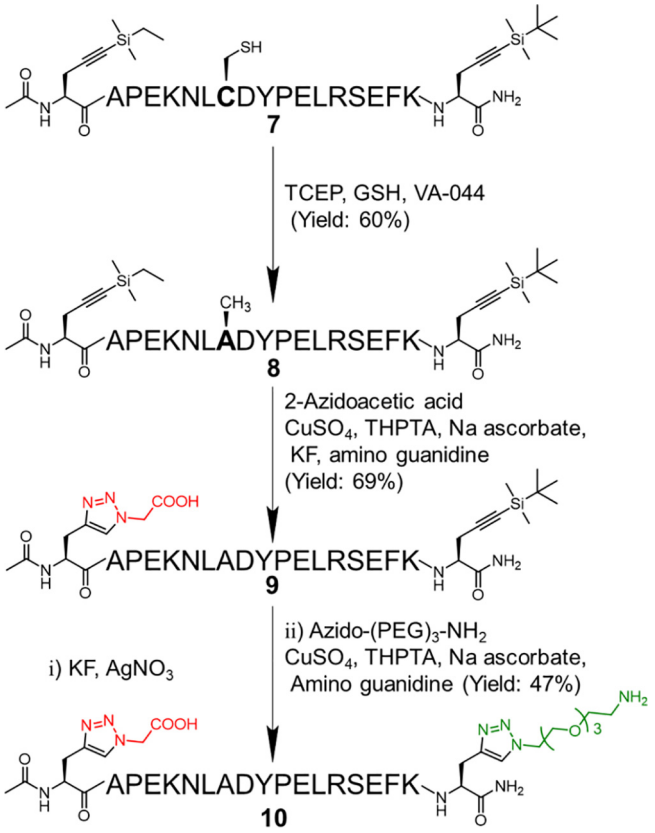


Scheme 2. Synthetic strategy of Fmoc-Pra(DMES)-OH (**6a**) and Fmoc-Pra(TBS)-OH (**6b**).

compounds **3a** and **3b**. To achieve enantioselective synthesis, we employed the Schollkopf reagent [9]. First, the proton with the higher acidity of the Schollkopf reagent was abstracted with *n*-BuLi in THF at −78 °C, and the mixture was added to the solution containing compound **3a** or **3b** bearing diphenylphosphate. The steric hindrance between the isopropyl group of the Schollkopf reagent and the diphenylphosphate led to the diastereoselective reaction (Scheme S1). While compound **4b** with TBS-protected alkyne was obtained without any byproducts, we observed a small amount of the diastereomer of compound **4a** with DMES-protected alkyne (Fig. S1), suggesting that the steric repulsion between the Schollkopf reagent and silyl groups might affect the diastereoselectivity. Compounds **4a** and **4b** were hydrolyzed under acidic conditions and each compound was mixed with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) in the presence of $NaHCO_3$ to protect the amino group with the Fmoc group to afford compounds **5a** and **5b**. Finally, the methyl ester moiety was hydrolyzed under basic conditions in the presence of $CaCl_2$, which prevented degradation of the Fmoc groups by reducing the concentration of the hydroxyl ions [10] to afford final products Fmoc-Pra(DMES)-OH (**6a**) and Fmoc-Pra(TBS)-OH (**6b**). Compounds **6a** and **6b** were synthesized in 45% and 34%, respectively, over five reaction steps, and the gram-scale synthesis of each compound was accomplished.

Next, peptide **7** was also prepared to perform chemoselective labeling on a peptide utilizing **6a** and **6b** (Scheme 3). After the incorporation of compound **6b** on rink amide resin using 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HBTU) as a condensation reagent, the peptide chain was extended by using an automated peptide synthesizer. Compound **6a** was then coupled, and the peptide was cleaved with trifluoroacetic acid (TFA) cocktail (92.5% TFA, 5% triisopropylsilane, 2.5% 1,3-dimethoxybenzene). The peptide was recovered by ether precipitation and the crude peptide was purified by high-performance liquid chromatography (HPLC); however, we observed the removal of DMES or TBS group during cleavage from the resin (Fig. S2), indicating that optimization of the cleavage conditions was required.

To check the stability of silyl-protected alkynes under desulfurization conditions, the Cys residue in peptide **7** was converted into an Ala residue through free-radical desulfurization (Scheme 3). Quantitative conversion into the desired Ala residue was observed after 2 h reaction without side reactions on silyl-protected alkynes, and peptide **8** was isolated by HPLC purification in 60% yield (Fig. S3).



Scheme 3. Strategy of free-radical desulfurization and dual incorporation of small molecules into a peptide. Desulfurization condition: peptide **7** (1.5 mM), TCEP (250 mM), GSH (50 mM), VA-044 (5 mM) in denaturing solution (6 M Gn-HCl, 0.2 M NaH_2PO_4 pH 6–7), 37 °C. Simultaneous DMES removal and click reaction condition: peptide **8** (1 mM), 2-azidoacetic acid (2 mM), $CuSO_4$ (1.5 mM), THPTA (5 mM), sodium ascorbic acid (20 mM), KF (4 mM), amino guanidine (20 mM) in H_2O/*t*-BuOH (7:3), 37 °C. TBS removal condition: peptide 9 (1 mM), KF (15 mM) and $AgNO_3$ (15 mM) in H_2O/*t*-BuOH (7:3), 37 °C. Click reaction condition: peptide (0.5 mM), azido-$(PEG)_3$-NH_2 (2 mM), $CuSO_4$ (2 mM), THPTA (5 mM), sodium ascorbic acid (20 mM) and amino guanidine (20 mM) in H_2O/*t*-BuOH (7:3), 37 °C.

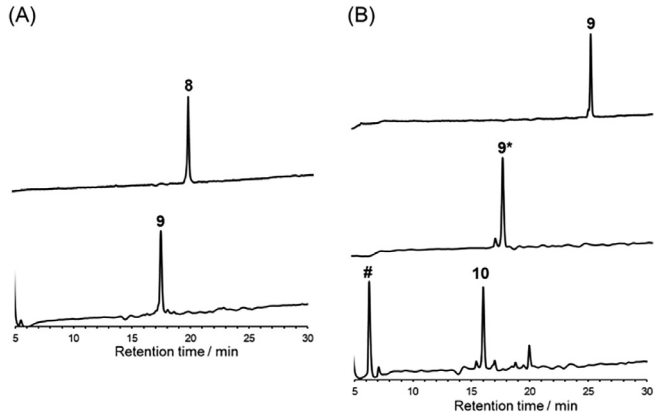


Fig. 1. Reaction tracking of dual labeling on the model peptide using the orthogonality between the DMES and TBS groups. HPLC peaks were monitored at 220 nm in a linear gradient of water/acetonitrile containing 0.1% TFA with $5C_{18}$-AR-II. (A) Simultaneous DMES deprotection and click reaction with 2-azidoacetic acid. Gradient: 20–50% for 30 min. (B) TBS deprotection, followed by click reaction with azido-$(PEG)_3$-NH_2. 9*= TBS-deprotected 9. # = Click reagents. Gradient: 15–45% for 30 min.

Site-selective modification that leveraged the orthogonality between the DMES and TBS groups was then performed. DMES removal with potassium fluoride (KF) and CuAAC with 2-azidoacetic acid were conducted in a one-pot manner. Notably, the deprotection of the DMES group was accelerated in the presence of Cu(I) cation, without affecting the TBS group [8,11]. HPLC analysis showed the quantitative conversion of peptide **8** into peptide **9** after 2 h reaction at 37 °C, and the desired product was isolated in 69% yield (Fig. 1A, S4). Peptide **9** was then treated with 15 equiv. of KF and 15 equiv. of $AgNO_3$ to remove the TBS group (Fig. 1B). After completion of the deprotection, sodium chloride was added to the reaction mixture to trap the silver ion via precipitation of AgCl salt, and the supernatant and precipitates were separated. To the supernatant were added azido-$(PEG)_3$-NH_2 and other reagents for CuAAC. After 1 h of reaction at 37 °C, the HPLC data showed a new peak derived from peptide **10** (Fig. 1B), which was identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and isolated in 47% yield (Fig. S5). The chemoselective incorporation of small molecules into a peptide was demonstrated using newly synthesized amino acids bearing different silyl-protected alkynes.

In conclusion, we synthesized Fmoc-Pra(DMES)-OH and Fmoc-Pra(TBS)-OH, which are compatible with Fmoc-SPPS. The chemoselective labeling of a peptide was accomplished by using the orthogonality based on the different steric bulk of the silyl-protecting groups. We envision that these Fmoc-amino acids bearing silyl-protected alkynes can be applied to produce a high diversity of peptides and proteins that will facilitate drug discovery.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2021.153093.

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