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N-Alkyl cysteine-assisted thioesterification of peptides

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Abstract—A new method for the preparation of peptide thioester by the post-solid phase peptide synthesis (SPPS) approach was developed. A series of *N*-alkyl cysteine derivatives were prepared and used as the C-terminus residue of the peptides prepared by the Fmoc SPPS. The synthetic peptides released from resin by TFA were readily converted to the peptide thioester in aqueous 3-mercaptopropionic acid (MPA) without significant side reactions.

A peptide thioester was first introduced in 1991 as a key intermediate for the thioester method.[1] Since then, its usefulness was demonstrated by the chemical synthesis of various proteins by the thioester method[2] as well as native chemical ligation.[3,4] The preparation of peptide thioesters has been mainly accomplished by the Boc method. Recently, the Fmoc method has become more prevalent than the Boc method, as the Fmoc method does not use HF, which requires special precaution and apparatus for its handling. In addition, the Fmoc method is preferable for the preparation of peptides carrying an acid-sensitive moiety, such as carbohydrates. However, application of the Fmoc method for peptide thioester synthesis is difficult in that the thioester linkage is labile to piperidine used for the Fmoc removal. Recently, various Fmoc methods have been developed to avoid this issue.[5–21] Among them, post-SPPS thioesterification using a sulfonamide linker is widely used.[7,9] In this method, peptide thioester can be obtained by activation of the sulfonamide group by alkylation after the peptide chain assembly, followed by thiolysis. However, in one case the thioesterification reaction did not proceed well.[22] Unverzagt and co-workers reported that acetyl capping unexpectedly acetylates the nitrogen atom in the sulfonamide linker, which results in cleavage of the peptide chain during the solid-phase synthesis.[23] Other Fmoc methods also have potential drawbacks. Thus, further efforts are required to accomplish the Fmoc compatibile peptide thioester preparation.

Recently, we reported that 5-mercaptomethyl proline linked at the C-terminal of a peptide effectively promotes thioesterification by 3-mercaptopropionic acid (MPA) under microwave irradiation conditions.[21] The reaction seems to proceed via intramolecular *N*- to *S*-acyl migration, followed by the intermolecular thioester exchange reaction. This post-SPPS thioesterification is fully compatible with the conventional Fmoc strategy and gives peptide thioester in good yield. However, the preparation of 5-mercaptomethyl proline is rather tedious. In addition, without microwave irradiation, the thioesterification is a slow reaction, which requires about a week for completion. It seems that the driving force of the first intramolecular *N*- to *S*-acyl shift reaction is the lability of the amide bond with an imino acid, proline.

Thus, we speculated that the same migration reaction might proceed with other imino acids. Here, we examined a series of *N*-alkyl cysteine derivatives for use as an N- to S-migratory device in the synthesis of peptide thioesters modeled after the partial structure of the emmprin intermediate[24,25] as shown in Scheme 1.

The synthesis of Fmoc–N-alkylated cysteine derivatives was carried out as shown in Scheme 2. Commercially available Cys(Trt) was condensed with aldehydes to form Schiff bases, which was reduced by $NaBH_4$ and purified by silica gel column chromatography. The purification of *N*-methyl–Cys(Trt) was achieved by reversed-phase HPLC, since the separation from contaminated

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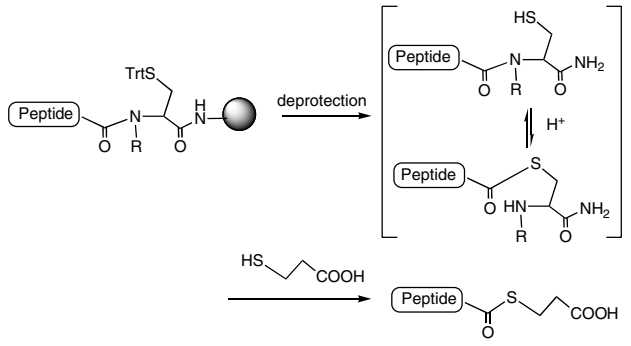
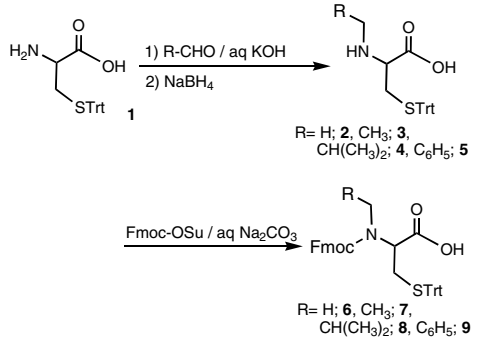
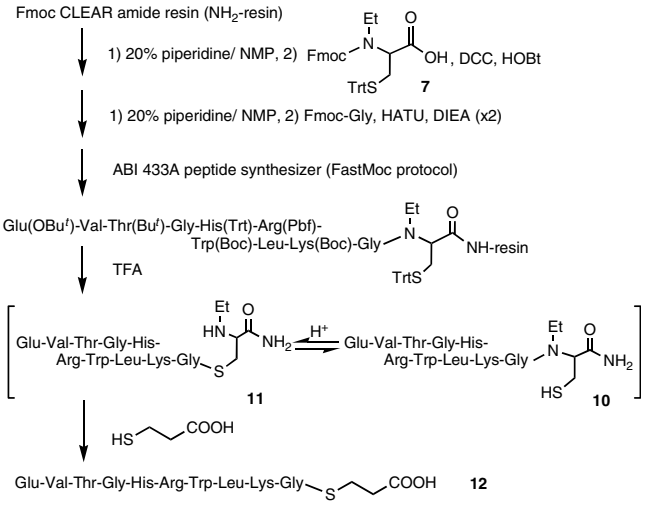


Scheme 1. Post-SPPS thioesterification using *N*-alkyl cysteine as *N*- to *S*-acyl transfer device.



Scheme 2. Synthetic route for *N*-alkyl cysteine derivatives.

Cys(Trt) was incomplete using silica gel column chromatography. The obtained *N*-alkyl cysteine derivatives were protected by the Fmoc group using Fmoc–OSu to obtain key devices **6**–**9**.[26] Then, these units were used for the synthesis of emmprin (49–58), Glu-Val-Thr-Gly-His-Arg-Trp-Leu-Lys-Gly by the Fmoc method. The synthetic route for the *N*-ethyl derivative is exemplified in Scheme 3. Fmoc–CLEAR amide resin was treated with 20% piperidine NMP for 5 and 15 min. After washing with NMP, Fmoc–*N*-ethyl cysteine derivative **7** (2 equiv to the amino groups in the resin) was introduced by the DCC–HOBt method. Then, Fmoc–Gly (10 equiv to the amino group) was reacted using HATU (9.5 equiv) in the presence of DIEA (20 equiv) at 50 °C for 1 h. The reaction was repeated with the same amount of Fmoc–Gly and reagents. Then the resin was subjected to the automated synthesis using a peptide synthesizer (ABI 433A) by the Fmoc method. After completion of the chain assembly, the resin was treated with Reagent K[27] for 2 h at room temperature. Then the crude peptide was analyzed by HPLC. The results are shown in Figure 1 (0 h). The major peak (Peak A) had the desired mass number of peptides **10** and **11**.[28] In addition, Peak B also showed the desired mass number. When Peak A collected by HPLC was redissolved in aq $NaHCO_3$ (pH 8) and immediately analyzed by HPLC, it disappeared and was converted to Peak B. In contrast, Peak B was slowly converted to Peak A under acidic conditions (3% aq TFA, ~12 h for completion). Peak A was positive to Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)), whereas peak B was negative. These results correspond well to the previous result that this peptide is in an equilibrium between the thioester (Peak B, **11**) and the amide form (Peak A, **10**).[21] Then the crude peptide was dissolved in 5% aq MPA (pH ~ 1) and was made to stand at room temperature overnight. As shown in Figure 1 (24 h), the peptide was cleanly converted to the thioester of MPA **12**[28] without serious side reactions. The reaction rate is almost comparable in all *N*-alkyl cysteine, but becomes slightly higher according to the increase of the alkyl chain from methyl to isobutyl. These results are in marked contrast to the pre-



Scheme 3. Synthetic route for model peptide thioester **12** using C-terminal *N*-ethyl cysteine as N- to S-migratory device.

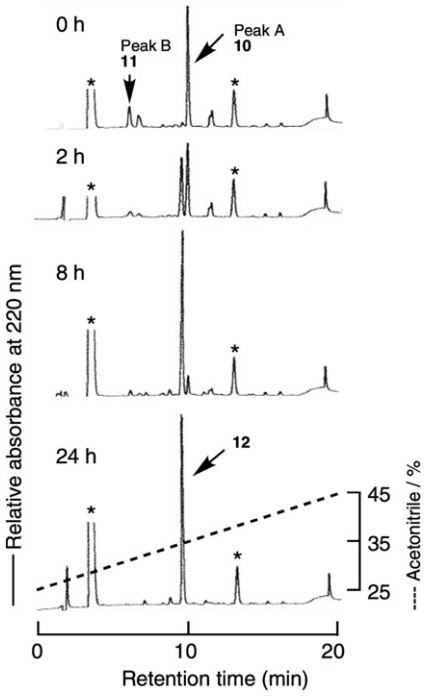


Figure 1. HPLC profile for thiol exchange reaction to obtain peptide thioester **12** using MPA. HPLC elution conditions: Eluent; aqueous acetonitrile containing 0.1% TFA, Column: Mightysil RP-18 GP (4.6 × 150 mm) at a flow rate of 1 ml/min. * denote non-peptidic compounds derived from MPA.

vious result using mercaptomethylated Pro, which required one week for the reaction to complete even in 40% aq MPA.[21] The yields of peptide thioesters are summarized in Table 1. The decreased yield in the *i*Bu and Bn derivatives compared to the Et derivative was derived from the incomplete introduction of the C-terminal Gly residues due to steric hindrance. It is known that the introduction of amino acid after N-alkylated amino acid is difficult.[29,30] Thus, in point of the ease of the preparation and the high yield of the peptide thioester, the *N*-ethyl derivative seems to be the most practical device for peptide thioester preparation. By the semi-preparative scale synthesis using the *N*-ethyl derivative, preparation of product **12** in 10 mg scale was easily accomplished.

We next examined the synthesis of peptide thioesters having chiral amino acids at their C-terminus to examine the general applicability of this method. Based on the results in Table 1, the *N*-ethyl Cys derivative was used in this synthesis. To the *N*-ethyl–Cys(Trt)–CLEAR amide resin, Fmoc–Leu, Lys(Boc), or Glu(OBu^t) was introduced by HATU-DIEA. The introduction was repeated using the same amount of amino acid and reagents. However, the coupling yield of these chiral amino acids was less than 50%, which decreased the total yield of the peptide thioester. Further optimization of the coupling conditions of chiral amino acids is required. Chain elongation on this resin was then achieved using the peptide synthesizer. After completion of the chain assembly, the resin was treated with Reagent K. Then the crude peptide was dissolved in 5% MPA and the thioesterification reaction was carried out. To analyze the epimerization ratio during thioesterification, a peptide thioester carrying D-amino acids was also prepared in the same manner. As in the case of Gly series in Table 1, the conversion proceeded without significant side reactions, but at slower rates. The complete conversion required 2–3 days. The result of the synthesis is shown in Table 2. The epimerization ratio of C-terminal Glu was not determined, since the peptide thioesters having C-terminal L-Glu and D-Glu were not separated on HPLC. These data show that the novel method is applicable to the preparation of peptide thioesters carrying chiral amino acids at their C-termini in acceptable yields and epimerization ratio.

In conclusion, we have developed an efficient post-SPPS thioesterification reaction using C-terminal *N*-alkyl cysteine as N- to S-migratory device, which can be easily prepared from a commercially available cysteine derivative. The thioesterification after peptide chain assembly proceeds without serious side reactions in good yields. This method is fully compatible with the conventional Fmoc strategy. Thus, the novel method eliminates problems inherent in peptide thioester preparation by the Fmoc strategy. In addition, due to the mild reaction conditions for the thioesterification, this method would be easily extended to the preparation of peptide thioesters carrying acid-sensitive moieties, such as carbohydrates. Further optimization of the introduction of the amino acid next to C-terminal *N*-alkyl cysteine as well as suppression of the epimerization during thioesterification are now under study. The preparation of glycoprotein using the novel strategy is also in progress.

Table 1. Result of the *N*-alkyl cysteine assisted thioesterification

R	Yield[a] (%)
Methyl	33
Ethyl	34
*i*Butyl	28
Bn	17

[a] Isolated yields based on the amino groups in the initial resin.

Table 2. Preparation of peptide thioesters having chiral amino acids at the C-terminus

Sequence	Yield[b] (%)	D/L[c]
ATEVTGHRWL-SR[a]	7.0	<0.068
TEVTGHRWLK-SR[a]	4.4	<0.025
WLKGGVVLKE-SR[a]	5.3	N.D.

[a] SR denotes SCH_2CH_2COOH.

[b] Isolated yields based on the amino groups in the initial resin.

[c] Calculated by the comparison of peak areas of the peptide thioester composed of all L-amino acids with C-terminally epimerized peptide thioester.

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