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Fmoc Synthesis of Peptide Thioesters without Post-Chain-Assembly Manipulation

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Supporting Information

ABSTRACT: An operationally simple method for the synthesis of peptide thioesters is developed using standard Fmoc solid-phase peptide synthesis procedures. The method relies on the use of a premade enamide-containing amino acid which, in the final TFA cleavage step, renders the desired thioester functionality through an irreversible intramolecular N-to-S acyl transfer.

C-terminal peptide thioesters are key intermediates for the chemical synthesis of proteins through native chemical ligation.[1] The Boc solid-phase peptide synthesis (SPPS) on a thioester-linked resin has served as an effective protocol for the preparation of peptide thioesters.[2] In contrast, synthesis of a peptide thioester through Fmoc SPPS is problematic because of the requirement for repeated Fmoc removal under basic conditions. However, many laboratories use Fmoc SPPS exclusively due to safety and regulation concerns. Besides, Fmoc protocols are favored for the synthesis of phosphorylated[3] and glycosylated peptides.[4] In this context, many methods have been developed to synthesize peptide thioesters through Fmoc SPPS.[5] Some famous examples include the safety-catch linker system,[6] the C-terminal peptide *N*-acylurea method,[7] and the thiolysis of a backbone pyroglutamyl imide.[8] A common feature of these methods is *the requirement for a post-chain-assembly treatment* (e.g., C-terminal activation and thiolysis) that demands the expertise of a skilled professional.

We reasoned that a more user-friendly Fmoc SPPS method for peptides thioester synthesis may only use the standard Fmoc procedures without any post-chain-assembly manipulation. The earlier methods of optimizing Fmoc deprotection cocktails for peptide thioester synthesis partially satisfy this criterion, but they suffer from having low yields and racemization at the C-terminal amino acid.[9] Another related approach is the *in situ* peptide thioester generation via an intramolecular O-to-S or N-to-S acyl transfer.[10,11] However, these acyl transfer methods do not directly produce an isolable peptide thioester unless an alkylation activation[12] or thiolytic release[13] step is added. For instance, the recently developed N-alkylated Cys method[14] needs to use excess amounts of thiol to promote the thioester formation. Such a thiolytic release step may induce fragmentation at normal Cys junctions. On the other hand, direct ligation of the acyl transfer precursors with an N-Cys peptide through *in situ* thioester generation often suffers from significant hydrolysis leading to relatively low ligation yields.[11,15] Thus, straightforward synthesis of peptide thioesters by using standard Fmoc-SPPS procedures without post-chain-assembly manipulation remains an unsolved challenge.

Herein, we describe a practical method for direct synthesis of peptide thioesters using just standard Fmoc SPPS procedures (Scheme 1). A special amino acid is needed to achieve the goal, but it can be readily available once made in large quantities. This special amino acid carries an enamide moiety that is stable to both amino acid coupling and Fmoc deprotection steps. The target thioester is produced through an *in situ* N-to-S acyl transfer in the TFA cleavage media, which generates an enamine intermediate that is irreversibly hydrolyzed by water in the same cleavage cocktail. The advantage of the new thioester synthesis method is twofold: (1) the method is operationally simple because it uses standard Fmoc SPPS protocols; (2) the crude peptide thioester cleaved from the resin is fairly clean due to the irreversible nature of the acyl transfer process.

Our study began with examining the enamides of amino acids carrying mercaptoalkyl side chains. Compound **1** was tested first (Scheme 2), which is completely converted by the TFA/TIPS/H_2O treatment. However, the desired thioester product is obtained in only 17% yield, whereas detailed analysis of the reaction mixture reveals the formation of a thiazole byproduct in 83% yield. We hypothesized that the thiazole was produced through a favorable aromatization process of the five-membered ring intermediate. To prevent the formation of an aromatic ring, we next designed compounds **2**−**4** that should undergo the acyl transfer through a six-membered ring intermediate. Unfortunately, these compounds fail to produce any thioester after many tests.

We then changed our strategy to put a methyl group on the amide nitrogen, hoping that this would disfavor the formation of a thiazole. To our delight, the treatment of compound **5** with TFA/TIPS/H_2O at room temperature for 12 h indeed generates the desired thioesters in high yields (Scheme 3). For Gly the yield is as high as 93%, whereas for amino acids with modest steric hindrance (Phe, Ser, Leu) the yields are 70−86%. Even a sterically demanding amino acid (i.e., Val) can be used in this method, which gives the corresponding thioester in 55% yield. These results verify the principle of the new thioester formation strategy. Moreover, our test of compound **5** with the conditions for Fmoc deprotection shows that **5** remains intact after treatment with 20% piperidine in DMF for 48 h.

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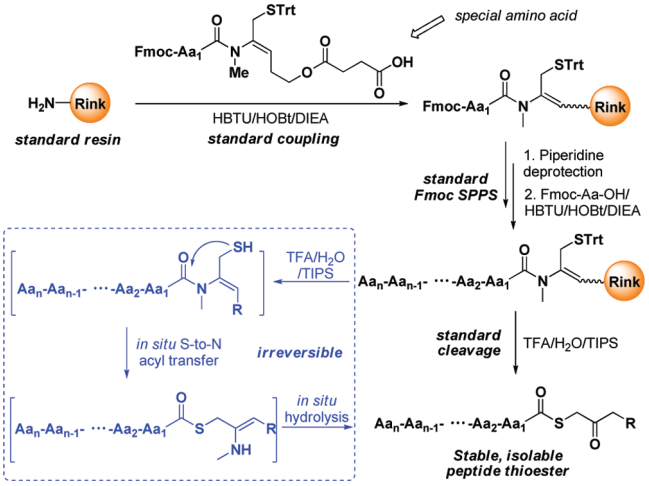
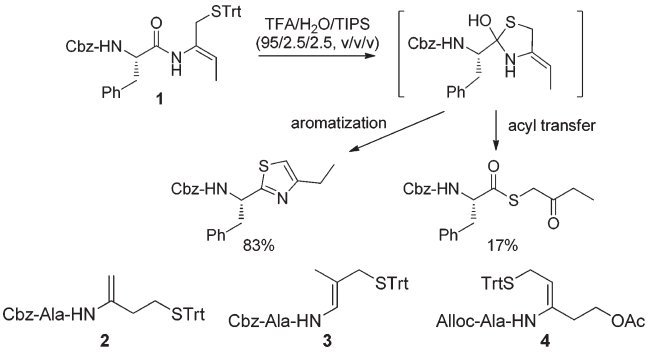
Scheme 1. Peptide Thioester Synthesis through Standard Fmoc SPPS Procedures[a]

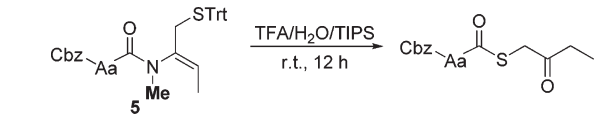


[a] Aa = amino acid.

Scheme 2. Thiazole Formation versus Acyl Transfer



Scheme 3. Thioester Formation from Compound 5[a]

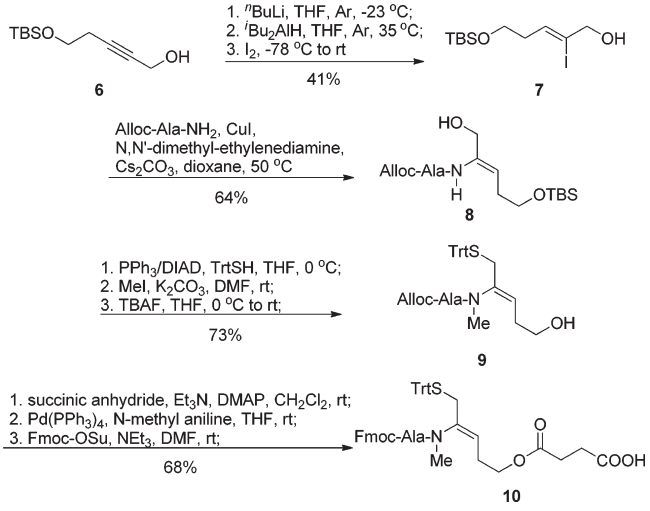


Amino acid	Gly	Phe	Ser	Leu	Val
Yield%	93%	86%	79%	70%	55%

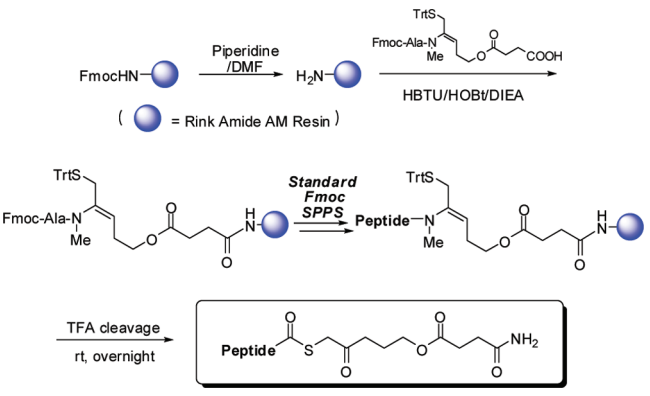
[a] Note that for Ser the starting material has a *t*-Bu protection group at Ser's OH, which is removed during the transformation.

To apply the above thioester formation method to SPPS, we need to incorporate the enamide moiety into the C-terminal amino acid. As shown in Scheme 4, this task can be accomplished through several synthetic steps from a known compound **6**.[16] Hydroiodination of **6** by using $^{i}Bu_2AlH/I_2$ affords a vinyl iodide **7**. The enamide **8** with Alloc protection is then obtained through a key step of Cu-catalyzed C−N cross-coupling.[17] Next, trityl-mercapto and N-methyl groups are installed to afford **9**, which upon reaction with succinic anhydride generates an acid linker

Scheme 4. Synthesis of Enamide-Containing Amino Acids



Scheme 5. Preparation of Peptide Thioester through a Standard Fmoc Procedure without Any Post-Chain-Assembly Manipulation



group. In the final step, Alloc is converted to Fmoc to satisfy the need of Fmoc SPPS, generating the desired special amino acid **10**.

The resulting enamide-containing amino acid **10** can be coupled onto a standard resin (e.g., Rink amide AM resin) using customary peptide coupling conditions (Scheme 5). Through routine Fmoc SPPS protocols, the remaining amino acids are assembled onto the resin in a stepwise manner according to the predesigned sequence. At the end of peptide chain assembly, standard TFA cleavage (12 h, rt) is conducted to release the peptide and remove the side-chain protection groups. During this step, the *in situ* N-to-S acyl transfer takes place, which is followed by an enamine hydrolysis to generate the desired peptide thioester. Note that several previously established TFA cleavage conditions have been examined and they do not show much difference in the thioester conversion. As to the model 7-mer peptide thioester **11**, the isolated yields under the four cleavage conditions are 81%, 76%, 79%, and 84%, respectively (Supporting Information).

To examine the racemization at the C-terminal amino acid during the thioester formation, we made the enantiomer of compound

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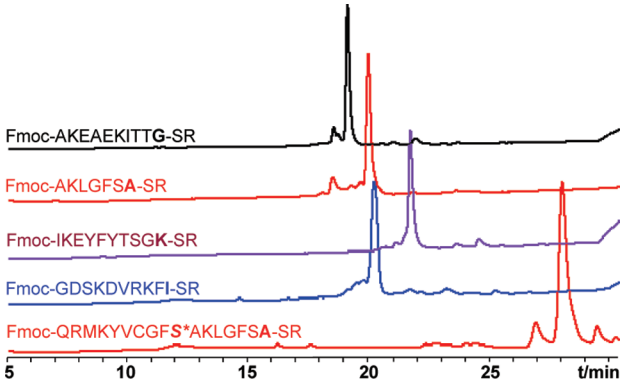
Table 1. Fmoc SPPS Synthesis of Peptide Thioesters

Entry	target peptides	purity %[a]	yield %[b]
1	Fmoc-AKEAEKITTG-SR[c]	91	70
2	Fmoc-AKLGFSA-SR	88	81
3	Fmoc-IKEYFYTSGK-SR	85	64
4	H-AVRTTGIF-SR	82	68
5	Fmoc-GGAGSAQAMPL-SR	91	78
6	Fmoc-MFVFAVRTTGIF-SR	75	60
7	Fmoc-GDSKDVRKFI-SR[d]	85	70
8	Fmoc-AELVDALQFV-SR[d]	56	32

[a] Purity of crude peptides based on HPLC detection trace at 214 nm. [b] Isolated yield based on the loading of the enamide-containing amino acid. Please see the Supporting Information for more details. [c] R = $CH_2COC_3H_6COC_2H_4-CONH_2$. [d] Cleavage for 24 h at 30 °C.

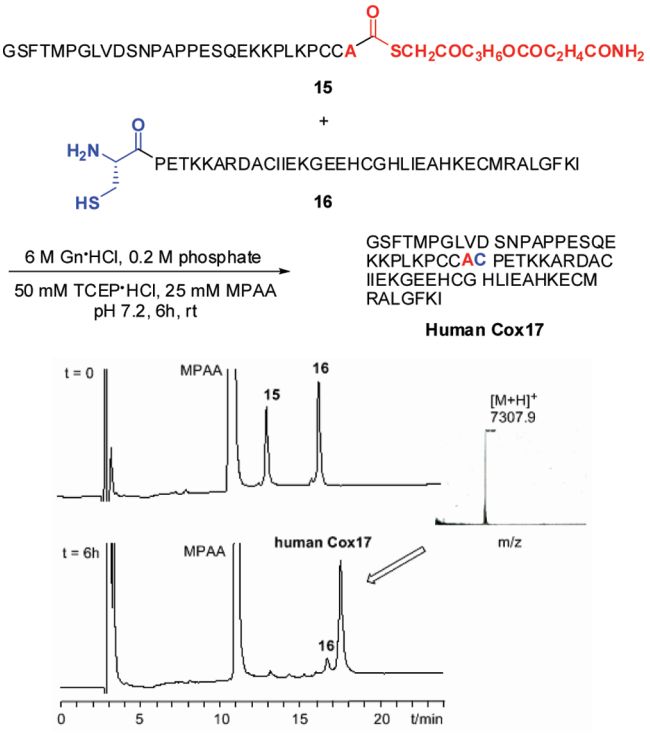
Scheme 6. HPLC Chromatogram of Crude Peptide Thioesters with Different C-Terminal Amino Acid Residues after the TFA Cleavage (12 h, rt)[a]



[a] Detailed yields can be found in Table 1. S* denotes Ser[β-D-Glc(OAc)$_4$]. R = $CH_2COC_3H_6COC_2H_4-CONH_2$.

10 corresponding to a D-alanine. With this compound, we then used the above Fmoc SPPS method to prepare another 7-mer peptide thioester **12** (i.e., Fmoc-Ala-Lys-Leu-Gly-Phe-Ser-[D]Ala-COSR, where R = $CH_2COC_3H_6COC_2H_4-CONH_2$). HPLC analysis of peptides **11** and **12** (Supporting Information) reveals that the epimerization at the C-terminal chiral center is less than 1%. To test the utility of the new method for the synthesis of peptide thioesters with post-translational modifications, we applied it to the preparation of two glycopeptide thioesters, i.e. Fmoc-Gly-Phe-Ser[β-D-Glc(OAc)$_4$]-Ala-Lys-Leu-Gly-Phe-Ser-Ala-COSR (**13**, a 10-mer) and Fmoc-Glu-Arg-Met-Lys-Tyr-Val-Cys-Gly-Phe-Ser[β-D-Glc(OAc)$_4$]-Ala-Lys-Leu-Gly-Phe-Ser-Ala-COSR (**14**, a 17-mer). The two model glycopeptides were obtained in 56% and 49% isolated yields. Moreover, we have made the enamide-containing amino acids for Gly, Lys, Phe, Leu, Val, and Ile (Supporting Information). With these special amino acids in hand we can readily prepare the corresponding peptide thioesters

Scheme 7. Chemical Synthesis of Human Cox17 and the Corresponding HPLC and MALDI-TOF Mass Data[a]



[a] MPAA = 4-mercaptophenylacetic acid, TCEP = tris(carboxyethyl)phosphine, Gn = guanidine.

in good yields and purities (Table 1 and Scheme 6). Note that the thioester formation step requires more time (24 h, 30 °C) for Ile and Val.

The method was further applied to the synthesis of a 67-mer protein, namely, human cytochrome oxidase 17 (human Cox17),[18] as shown in Scheme 7. This protein recruits copper ions to mitochondria for incorporation into the Cox apoenzyme. The 29-mer peptide thioester fragment **15** was prepared using the Fmoc method described in Scheme 7, which was successfully isolated in 42% yield. The other fragment **16** which is a 38-mer peptide was also synthesized using the standard Fmoc SPPS method. The ligation was performed under the standard conditions. Within 6 h at room temperature, the desired full-length peptide was obtained in about 95% ligation yield according to the HPLC and MALDI-TOF analysis.

In summary, we have introduced a new method for the synthesis of peptide thioesters using just routine Fmoc SPPS procedures. The method relies on the use of a premade enamide-containing amino acid which, in the final TFA cleavage step, renders the desired thioester functionality through an irreversible intramolecular N-to-S acyl transfer. An important feature of the new method is its operational simplicity and potential to be automatized with conventional procedures. Straightforward access to peptide thioesters may widen the application of peptide ligation methods for the synthesis of complex post-translationally modified proteins. The current limitation of the method is that the synthesis of enamide-containing amino acids remains laborious. We expect that more efficient amino acid loading may ultimately be achievable using analogous linker principles.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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