Fmoc-Compatible and External-Thiol-Free Peptide C-Terminus Alkyl Thioester Formation Using Cysteinylprolyl Imide

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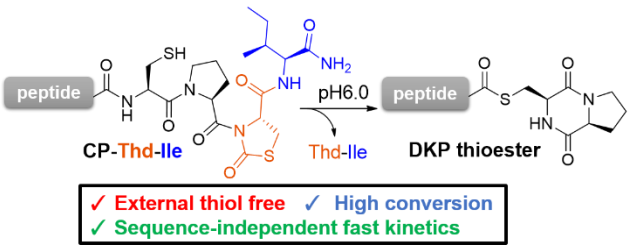
Supporting Information Placeholder



ABSTRACT: We report an Fmoc-compatible and external-thiol-free method of peptide C-terminus thioesterification with cysteinylprolyl imide. The newly synthesized structure, i.e., cysteinylprolyl-thiazolidinone, provided high conversion and sequence-independent fast kinetics (90 min) in the diketopiperazine thioester formation under relatively mild conditions: pH 6.0, 37 °C. Employing this thioesterification method, we synthesized histone H3.2 bearing K56 acetylation.

In chemical protein synthesis,[1–7] native chemical ligation (NCL) is a promising strategy to ligate two unprotected peptide fragments between an N-terminal cysteine peptide and a C-terminal peptide thioester.[8] To prepare peptide thioesters through Fmoc SPPS,[9] most previous procedures utilized an excess amount of external thiol against the various thioester surrogates[10-21], to accelerate the thioesterification and suppress competing hydrolysis. However, this procedure is sometime problematic because the particular amino-acid (aa) next to the thioester such as Thr, Val, Ile, or Pro, partially block the nucleophilic attack of external thiols due to its bulky side chain or stereoelectronic effects.[22,23]

To address this issue, a few external-thiol-free thioester preparation methods have been developed so far (Figure 1a). Melnyk and coworkers reported combinatorial use of SEA peptide and glyoxylic acid to trap thiazolidine thioester.[24] As another method, an enamide-containing aa that converts into a thioester through an irreversible intramolecular N-S acyl shift in the TFA cleavage step was developed.[25,26] However, these methods are still affected by neighboring aa residues probably due to the intermolecular reaction to trap stable thioester derivatives. Recently, we developed a N-S acyl-shift-type thioester surrogate, cysteinylprolyl imide (CPI),[27] in which the equilibrium of the N-S acyl shift can be trapped by intramolecular diketopiperazine (DKP) formation (Scheme S1). Kawakami and Aimoto originally applied this mechanism to NCL as a cysteinylprolyl ester peptide.[28] We assumed that the intramolecular thioester formation of CPI could be another Fmoc-compatible and external-thiol-free method for alkyl thioester preparation. It is notable that a CPI peptide can be synthesized from commercially available reagents and aa monomers, and it has a high thioesterification rate over a broad pH range.

In this study, we developed a new CPI peptide tethering thiazolidinone (Thd) as the imide moiety and compared it to the previously developed oxazolidinone (Oxd) and methyloxazolidinone (MeOxd) peptides. The Thd structure, formed by on-resin cyclization, was more robust than oxazolidinone derivatives and the obtained cysteinylproly thiazolidinone (CP-Thd) peptides showed efficient and sequence-independent DKP thioester conversion. Finally, we applied the CP-Thd-mediated DKP thioesters to the synthesis of histone H3 protein with K56 acetylation.

We commenced our study with the synthesis of a model peptide SYRKGCP-Thd-Tle(**1G**), in which the C-terminal *tert*-leucine (Tle) was introduced to improve the hydrolytic stability and the DKP formation kinetics.[29] For on-resin Thd

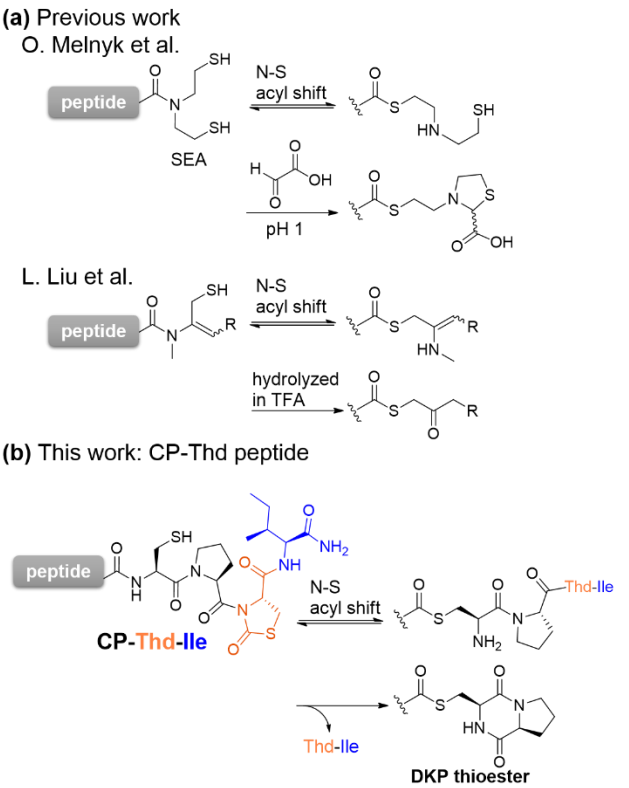


Figure 1. External-thiol-free peptide C-terminal thioesterification methods. (a) Previous works using SEA peptide (above) or enamide-containing peptide (below), and (b) this work used CP-Thd peptide to produce DKP thioesters.

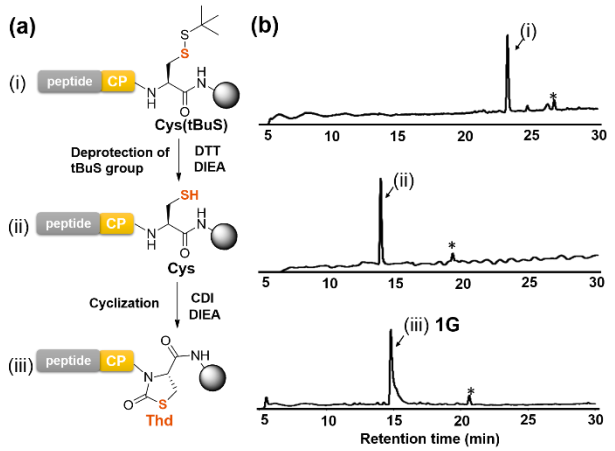


Figure 2. Synthesis of CP-Thd peptide. (a) Synthetic scheme of on-resin Thd formation. (b) HPLC traces of each crude peptide after small-scale TFA cleavage. HPLC gradient: acetonitrile 10–40% for 30 min. The peptide sequence is SYRKGCP-C(tBuS)-Tle-NH_2. *Byproduct derived from Rink amide resin.

formation, Fmoc-Cys(*t*BuS)-OH was coupled in SPPS and, after the elongation of the peptide chain, the *tert*-butylsulfanyl group (*t*BuS) was selectively removed by dithiothreitol (DTT) treatment, after optimization of the reaction conditions (Figures 2 and S2). Then, 1,1-carbonyldiimidazole (CDI) was added to form a 5-membered Thd ring (Figures 2 and S3). When using *N,N'*-disuccinimidyl carbonate in place of CDI, the reaction rate decreased. Although 4-dimethylaminopyridine was also added to the reaction mixture as a nucleophilic catalyst, it did not exhibit any significant effects on Thd formation (Figure S3). Notably, less CDI (10~20 equiv) was sufficient to form the imide structure in a quantitative fashion compared to in the case of a previously reported CP-MeOxd peptide[27] (50 equiv) (Figure S4). Ile was also tested as a C-terminal aa because it is less costly than Tle. Other model peptides (SYRKACP-Thd-Tle(**1A**); SYRKXCP-Thd-I: X=A(**2A**), V(**2V**), G(**2G**), S(**2S**), K(**2K**), P(**2P**), *D*-Ala(**2a**); SYRKACP-Oxd-I(**3**); and SYRKACP-MeOxd-I(**4**)) were also synthesized following the optimized protocol (Table S1, Figure S1). The isolated yield of each peptide calculated from the loading of resin was around 30~40%.

We examined the DKP thioester formation reaction using CP-Thd, CP-MeOxd, and CP-Oxd model peptides **2A**, **3**, and **4**, respectively (Figure 3a). These peptides were incubated under the following conditions: 1 mM peptide, 6 M Gn·HCl, 0.2 M sodium phosphate (pH 6.0), 20 mM TCEP·HCl, 37 °C (these are defined as 'condition A'). The time-course analysis of peptide **2A** indicated that DKP thioester formation was completed within 90 min with >98% conversion and the epimerization rate was negligible (HPLC monitoring) (Figure S9). On the other hand, peptides **3** and **4** showed slower kinetics (5 h for completion in both cases) and lower conversion yield (81% and 93%, respectively) than **2A** (Figures 3b, S5b–d). We then evaluated the pH dependency of the DKP thioester formation using peptides **2A** and **4**. At pH 5.0, the conversion took longer than 4 h and >12 h, respectively (Figures 3c, S6b, c and S7a). At pH 7.0, the reaction became faster than at pH 6.0 in the cases of both **2A** and **4**. At pH 7.0, the reaction was completed within 60 min; however, a thioester-linked dimer was observed as a byproduct, leading to a lower final conversion yield (Figures S6d, e and S7b). To compare Tle and Ile as a C-terminal aa of CP-Thd, we also conducted the DKP thioester formation reaction using peptide ACP-Thd-Tle(**1A**) at pH 6.0. The time required for completion of this reaction was 90 min and the conversion yield was 97%. This rate was almost the same as for **3A** (Figure S5e).

The results obtained for DKP thioester formation indicate that the CP-Thd moiety was the most efficient precursor for DKP thioester preparation among the different imide structures that we tested. Considering the conversion yield and time required for completion of the reaction, we concluded that 'condition A' was most preferable for the DKP thioester formation. (Additionally, at room temperature, the reaction proceeded at slower rate as shown at Figure S5f)

In an effort to determine the effect of an aa next to the CP-Thd moiety, the DKP thioesterification of **2A**, **2V**, **2G**, **2S**, **2K**, and **2P** was tested at 'condition A'. All the peptides showed similar conversion rates of DKP formation (Table 1 and Figure S8). Even in the case of **2P**, where a proline residue next to the CP-Thd was assumed to decrease the reactivity due to the stereoelectric effect,[22,23,30] DKP thioester formation was completed within 90 min (Figure S8f). This sequence independency can be explained as follows: the rate-limiting step of the reaction could the nucleophilic attack of the cysteinyl amine to the prolyl carbonyl group (6-membered-ring formation) after N-to-S acyl transfer, which would be so rapid that the effect of the aa residues next to the CP-Thd became negligible. The rate of formation of the 6-membered

ring is too low to be affected by the neighboring aa residues because there is sufficient distance between the prolyl carbonyl group and the side chain of the aa residue next to Cys.

Table 1. Time for Completion of the DKP Thioester Formation at Different pHs[a], and the Final Conversion Yields

C-terminus sequence[b]	pH 7.0	pH 6.0	pH 5.0
ACP-Thd-I (**2A**)	60 min (90%)	90 min (98%)	>4 h
GCP-Thd-I (**2G**)	-	60 min (93%)	-
VCP-Thd-I (**2V**)	-	90 min (98%)	-
KCP-Thd-I (**2K**)	-	90 min (98%)	-
SCP-Thd-I (**2S**)	-	90 min (96%)	-
PCP-Thd-I (**2P**)	-	90 min (99%)	-
ACP-Oxd-I (**3**)	-	5 h (81%)	-
ACP-MeOxd-I (**4**)	60 min (87%)	5 h (93%)	>12 h

[a]Conditions were based on 'condition A'. [b]Sequence of the model peptide is SYRKXCP-imide-X'.

To simplify the isolation process of DKP thioester, we then conducted the conversion of CP-Thd to DKP thioester with crude peptides. Crude ACP-Thd-I(**2A**) and VCP-Thd-I(**2V**) were incubated under the optimized conditions given above and the reaction was monitored by HPLC. The DKP thioester formation reaction was completed cleanly within 90 min and the isolated yields calculated from resin loading were 43% and 40%, respectively (Figure S10), hence suggesting that this direct thioesterification procedure is practical for the preparation of peptide alkyl thioesters.

Previously, cyclic-imide-containing peptides were used as thioester precursors and converted to thioesters by the addition of external thiols.[18] Therefore, we attempted to compare the procedures and efficiency of alkyl thioester preparation. To this end, model peptides (SYRKA-Thd-I(**5**) and SYRKA-MeOxd-I(**6**)) were synthesized through on-resin imide formation. In the case of **5**, the thioester-linked dimer and hydrolyzed byproduct were detected at a substantial rate (Figure S11b), suggesting that the thioester-linked dimer could be formed during the Thd formation of selectively deprotected cysteine residues and that the alanyl Thd seems to be hydrolytically labile. Although the isolated yield of **6** was higher than that of **5**, a hydrolyzed byproduct derived from **6** was still observed and the yield was lower than that of CP-Thd peptide **2A** (Table S1). These results suggest that the proline residue in CPI stabilizes the imide structure through the proline specific n–π* interaction,[22,23,30] leading to the increased isolated yields of peptide thioester. (As additional information, the data of thioesterification of cyclic-imide-containing peptides are shown at Figure S12.) These results suggest that the CP-Thd peptide has some advantages over the cyclic-imide-containing peptides without CP sequences in the preparation of peptide alkyl thioesters.

We applied the CPI-mediated DKP thioester preparation to the chemical synthesis of histone H3.2[31–39] bearing K56Ac modification. H3K56Ac is incorporated to replicating DNA and enhances the ability of two histone chaperones CAF-1

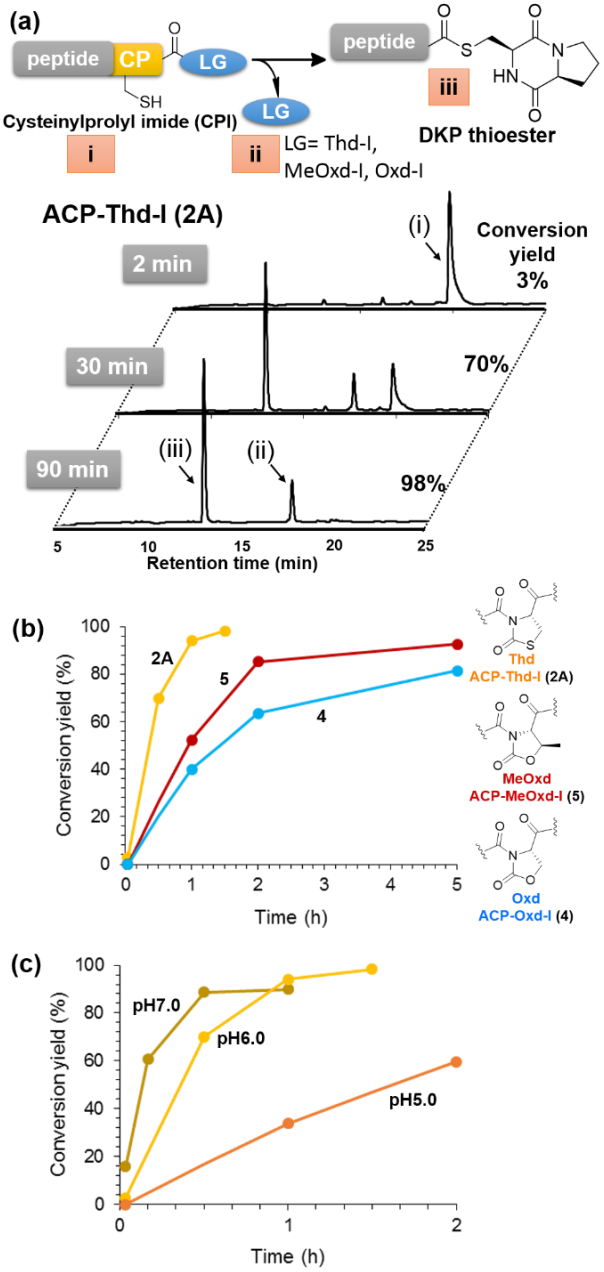


Figure 3. DKP thioester formation using CPI. (a) Scheme of the reaction using CPI peptide and time-course HPLC monitoring of DKP formation of peptide **2A**(Thd). Reaction mixtures were incubated at 'condition A'. HPLC gradient: acetonitrile 5–30% for 25 min. The conversion yields were calculated from HPLC peak areas. (b) Time-course analysis of the conversion yield with different imide structures using peptides **2A**, **3**(Oxd), and **4**(MeOxd). (c) Time-course analysis of the conversion yield at different pHs, using **2A**.

and Rtt106 to assemble DNA into nucleosomes by increasing the binding affinity of these chaperones.[40] We divided this protein into three fragments: F1(**7**), F2(**8**), and F3(**9**) (Figure 4a). CP-Thd-I was introduced to the C-terminus of F1 and F2. Thiazolidine, which can be readily introduced, was chosen as a protecting group of the N-terminus cysteine of F2 (Figure 4b). After the cleavage from the resin, the crude peptides of F1(**7'**) and F2(**8'**) were directly converted

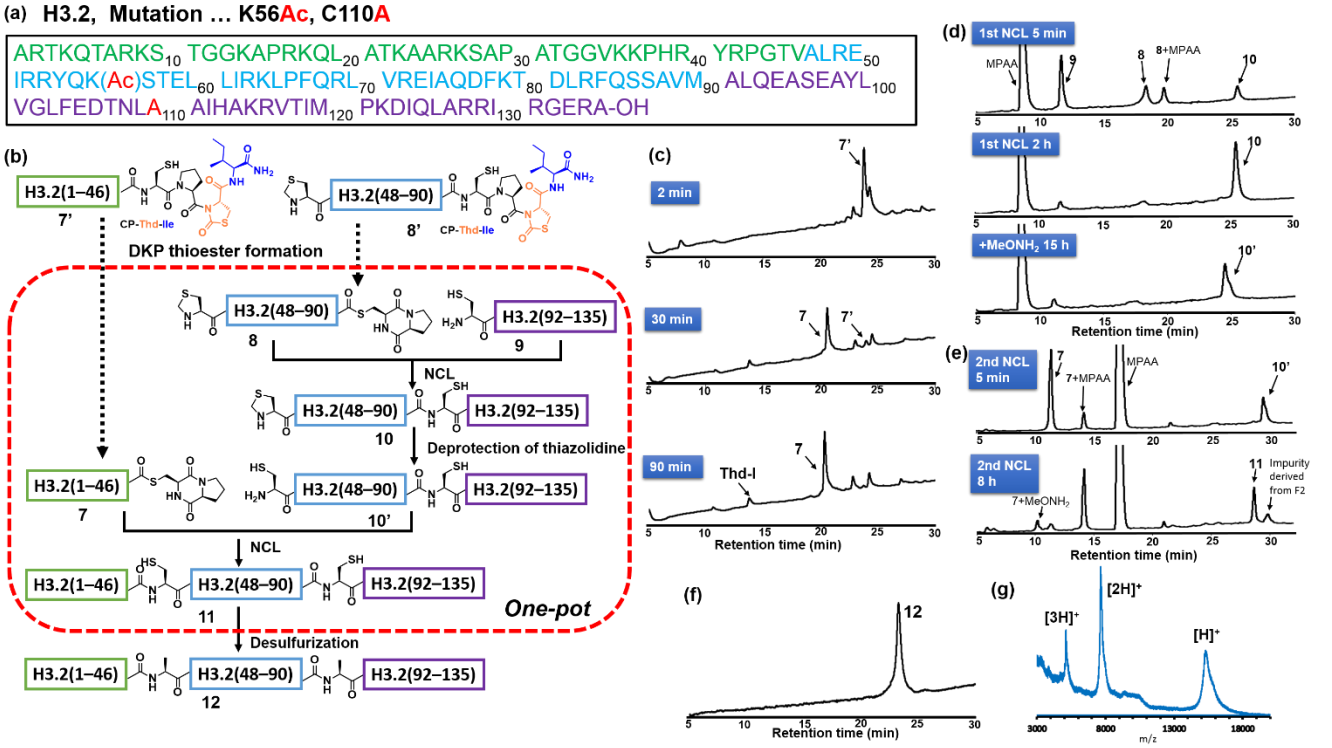


Figure 4. Chemical synthesis of H3K56Ac. (a) Sequence of H3.2 divided into three peptide fragments, shown in green, blue, and purple. (b) Synthetic scheme of H3K56Ac including C-to-N one-pot ligation using DKP thioester. (c) Time-course HPLC traces of DKP thioester formation of crude F1(**7**). HPLC gradient: acetonitrile 5–35% for 30 min. Conditions: 2.5 mM peptide (other conditions were the same as 'conditionA'). (d) and(e) HPLC monitoring of one-pot three-fragment ligation. HPLC gradient: acetonitrile 30–60% (d) or 10–60% (e), for 30 min. (f) HPLC profile of full length H3K56Ac(**12**) after desulfurization. HPLC gradient: acetonitrile 30–60% for 30 min. (g) MALDI-TOF/MS spectrum of purified full length H3K56Ac(**12**). The observed mass was 15269.9 Da (calcd 15267.8 Da, $[M+H]^+$).

to DKP thioester in 6 M Gn·HCl buffer at pH 6.0, 37 °C. The reaction was complete within 90 min (Figure 4c). These peptides were purified by RP-HPLC. The isolated yields calculated from resin loading of F1(**7**), F2(**8**), and F3(**9**) were 12%, 3.5%, and 11%, respectively (Figures S13–16).

These three peptide fragments were assembled in one-pot manner, as reported by Kent group[41] (Figure 4d, e). First, F2(**8**) and F3(**9**) were dissolved in NCL buffer (6 M Gn·HCl, 0.2 M sodium phosphate, 50 mM 4-mercaptophenylacetic acid (MPAA), 50 mM TCEP·HCl, pH 7.0) and the reaction mixture was stirred at 37 °C. F2-3(**10**) was cleanly produced within 2 h. To the solution, a buffer containing 0.4 M methoxyamine·HCl was added to convert the N-terminus thiazolidine to unprotected cysteine. The reaction was completed within 15 h. The concentration of MPAA was adjusted to 100 mM by adding solid MPAA and the pH was readjusted to 7.0. Hereafter, 2.0 equiv of solid F1(**7**) was added to the reaction mixture. The second NCL was completed within 8 h and, after purification by RP-HPLC, F1-2-3(**11**) was obtained in 23% isolated yield, calculated from the amount of F3(**9**). Subsequently, desulfurization at two ligation sites was conducted and the full length of H3K56Ac(**12**) was obtained in 72% isolated yield (Figure 4f,g). The total yield after three-fragment ligation and desulfurization was 16%.

In summary, we have developed a robust preparation strategy for Fmoc-compatible alkyl thioesters using a CPI peptide. A newly developed CP-Thd structure, as a thioester precursor, can be quantitatively synthesized from a Cys-Pro-Cys sequence via on-resin deprotection and subsequent Thd formation. The conversion of CP-Thd to alkyl thioester proceeded almost quantitatively in a neighboring-sequence-independent manner via intramolecular DKP formation, leading to an external thiol being unnecessary. Finally, this thioester preparation method was applied to the chemical synthesis of H3K56Ac.

In conclusion, CP-Thd peptides have several advantages, for example: good isolated yields, rapid thioester formation, and the requirements for only mild reaction conditions and no specialized reagents. We believe that the alkyl thioester preparation method developed here will offer a promising option for chemical protein synthesis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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