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RESEARCH ARTICLE

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Unmasking latent thioesters under hydrophobic-compatible conditions

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Hydrophobic latent C-terminal thioesters were converted into thioesters, and were also coupled with cysteine in one-pot reactions, using conditions generally compatible with hydrophobic materials. The reaction conditions (ethanethiol and triethylamine in a mixture of DMF and THF) are compatible with acid-labile protecting groups (Boc/t-Bu) that are standard in Fmoc peptide synthesis.

KEYWORDS
chemoselective, hydrophobic, latent thioester, ligation, organic

1 | INTRODUCTION

Methods linking peptide fragments have received much attention in the years since the initial reports of native chemical ligation (NCL) and similar chemoselective reactions.[1–4] Many of these approaches employ C-terminal thioesters as reactants or intermediates.[5] Due to the inherent electrophilicity of thioesters, peptides containing this functionality are often accessed using Boc chemistry, which involves particularly hazardous HF cleavage. A number of groups have therefore developed methods for generating peptide thioesters via Fmoc/t-Bu solid-phase peptide synthesis (SPPS),[6,7] which is milder, or Fmoc/t-Bu-compatible latent thioesters or thioester surrogates that can be employed in place of thioesters.[8–20] Entering molecules with these functionalities into ligation reactions generally requires aqueous conditions, which either renders hydrophobic protected fragments inaccessible by these methods or prevents their further ligation due to the requirement for an aqueous environment. Dittman et al[21] reported conditions for performing NCL with hydrophobic C-terminal thioesters that struggle to react under aqueous conditions and further demonstrated that the reaction using their organic conditions proceeds racemization-free.[22] A useful addition to the repertoire of acyl donors for ligation would combine these features: compatibility with Fmoc SPPS and both aqueous and hydrophobic reaction conditions.

2 | MATERIALS AND METHODS

2.1 | General methods

All commercial materials were used as received unless otherwise noted. Diethyl ether (Et_2O), tetrahydrofuran (THF), and methylene chloride (DCM) were obtained from a PureSolv 400 dry solvent system and used without further drying. Water used in reactions was HPLC grade from Fischer Scientific. Dimethylformamide (DMF), also from Fischer, was Sequencing Grade. Reagents were purchased from Aldrich or Fischer Scientific, or as noted, except for amino acids, which were purchased from NovaBiochem (EMD). Older reagents (up to several years) were distilled prior to use, including triethylamine (TEA). Silica gel for flash column chromatography was purchased through Fisher Scientific from EM (Silica Gel 60, M9385-9); thin-layer chromatography (TLC) plates were purchased similarly from EMD Chemicals (Silica Gel 60, F254, M5715-7). Analytical TLC visualization was accomplished first by UV and then by heating after exposure to ceric ammonium molybdate [CAM; 0.5 g Ce $(NH_4)_2(NO_3)_6$, 24 g $(NH_4)_6Mo_7O_{24}\bullet 4H_2O$, 500 ml H_2O, 28 ml conc. H_2SO_4]. ^1H and ^{13}C NMR data were obtained on a Varian 400MR spectrometer at 400 and 100 MHz, respectively, and were internally referenced to chloroform (7.26 and 77.0 ppm, respectively). High-resolution mass spectral analysis was completed using positive-ion mode electrospray ionization with an Apollo II ion source on a Bruker 10 T APEX-Qe

FTICR-MS, a hybrid mass spectrometer. After ionization, the ions fly through a quadrupole and a hexapole before detection in an ion cyclotron resonance cell.

2.2 | General procedure A: Diester 1

Diol **7** (1 equiv), Boc-Xxx-OH (**8**) (3.7 equiv), EDC·HCl (4.9 equiv), and DMAP (10 mol%) were combined and placed under argon. THF (5 ml) and DCM (15 ml) were added, and the reaction was stirred for 1–6 h. The reaction mixture was transferred to a separatory funnel using EtOAc and DCM and the organics washed once each with H_2O, sat. $NaHCO_3$, and sat. brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by flash column chromatography (gradient from 10% to 40% EtOAc-hexane) afforded diester **1**.

2.3 | General procedure B: Thioester 2

To diester **1** (1 equiv) dissolved in a combination of DCM, DMF, and THF (total 100 μl) were added TEA (~5 equiv) and ethanethiol (~20 equiv), and the reaction was placed under argon, sealed tightly (to retain the ethanethiol), stirred vigorously, and allowed to run until completion as determined by TLC (about 24 h). The reaction mixture was concentrated in vacuo. Purification by flash column chromatography (EtOAc-hexane eluent) afforded thioester **2**.

2.4 | General procedure C: Cys-dipeptide 6

To diester **1** (1 equiv) and cysteine methyl ester hydrochloride (H-L-Cys-OMe·HCl) (~2.5 equiv) in DMF (190 μl) were added TEA (~5 equiv) and ethanethiol (~20 equiv), and the reaction was placed under argon, sealed tightly (to retain the ethanethiol), stirred vigorously, and allowed to run until completion as determined by TLC (about 24 h). Reduction of any disulfides was accomplished by addition of TCEP·HCl (1 equiv) and water (~50 equiv) and stirring for an additional 1–2 h. The reaction mixture was concentrated in vacuo, then transferred to a separatory funnel with EtOAc and H_2O. The organics were washed with 0.5 M $KHSO_4$ (three times), H_2O, sat. $NaHCO_3$, and sat. brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by flash column chromatography (gradient from 40% to 70% EtOAc-hexane) afforded dipeptide **6**.

3 | RESULTS AND DISCUSSION

The latent thioester in Figure 1 meets all of the desired criteria: compatibility with Fmoc SPPS and both aqueous and organic unmasking conditions. We have already demonstrated its suitability for Fmoc/t-Bu SPPS and subsequent aqueous ligation;[23,24] here, we establish that our latent thioester also functions under conditions compatible with hydrophobic materials, in addition to the previously

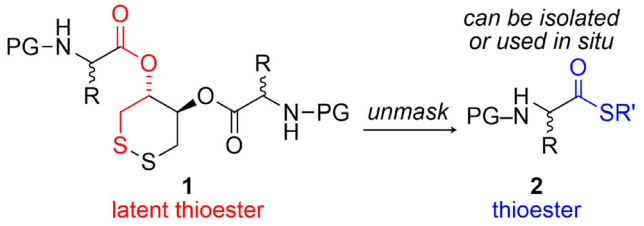


FIGURE 1 Our latent thioester substituted (twice) with a generic, protected amino acid; unmasking yields a thioester, which can be isolated or used in situ in ligation with cysteine

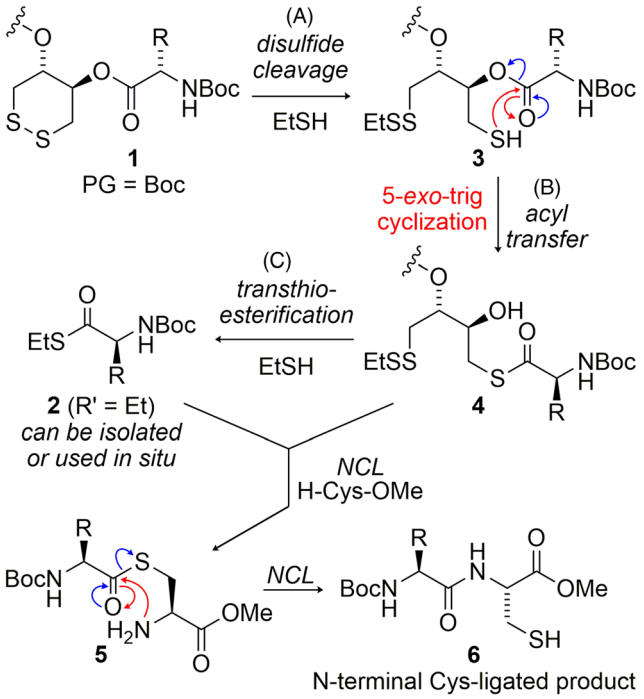


FIGURE 2 Unmasking a latent thioester (**1**) and further reactions. With an appropriate thiol (here, ethanethiol), thioester **2** can be isolated. In the presence of an N-terminal Cys residue, NCL ensues to give thioester **5**, then ligated **6**

reported aqueous conditions. As seen below, these organic reaction conditions can be employed to convert protected latent thioesters into thioesters or to enter directly into chemoselective ligations, if desired.

Latent thioester **1** comprises a disulfide-protected sulfur atom positioned two carbon atoms away from an ester oxygen atom, which enables an entropically favorable 5-*exo*-trig O → S acyl transfer when unmasked.[25] (In fact, **1** contains two such functional groups, symmetrically disposed. The solid-phase linker version of this material contains one latent thioester along with a carboxylic acid moiety.[24]) Though this intramolecular acyl transfer may favor the ester rather than the thioester, the steady-state concentration of thioester appears great enough to allow the reaction to proceed. For this type of latent thioester to work, as noted in Figure 2, the reaction conditions must (a) cleave the intramolecular disulfide

(shown in Figure 2 with ethanethiol); (b) facilitate the intramolecular acyl transfer (red and blue arrows); and (c) provide a partner to couple with the resulting thioester. When the coupling partner in step (c) is an appropriate thiol, the result is a thioester that can be isolated.

Ideally, a latent thioester could be employed not only to generate a thioester but also to proceed directly into ligation with an N-terminal cysteine residue unprotected at its α-amine and side-chain thiol, as illustrated in Figure 2. The only additional requirement would be the N-terminal Cys coupling partner with a thiol capable of intercepting thioester **4** and/or **2** to give a new thioester (**5**), thus entering into NCL; the result is the thermodynamically favored native peptide bond (**6**).

Using the latent thioester that we demonstrated to function in aqueous media,[23,24] we envisioned adopting the reported ligation conditions for hydrophobic thioesters[21,22] to effect both transformations outlined in Figure 2. Esterification of C_2-symmetrical diol **7**[26] with protected amino acids to give **1a–c** allowed us to test the functionality of our latent thioester. The amino acids in Table 1, including Boc-Phe-OH (**8a**), Boc-D-Phe-OH (**8b**), and Boc-Tyr(t-Bu)-OH (**8c**), were selected to demonstrate the system's ability to handle hydrophobic (**1a–b**) or t-Bu protected polar (**1c**) side chains in the latent thioester. Diastereomers **1a** and **1b** served to probe potential issues with epimerization at the α-position of the activated acyl donor under the reaction conditions.

In synthesizing **1a–c**, we obtained the most consistent results using EDC·HCl in a mixture of DCM and THF. Conditions generating diester **1** using DCC were complicated by difficulties separating the desired material from dicyclohexylurea (DCU). Enantiomerically pure diol **7**, with its C_2-symmetry, demonstrated no appreciable difference when employing L- or D-phenylalanine in this esterification.

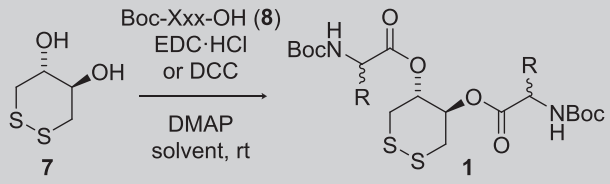
Diesters **1a–c** were subjected to organic reaction conditions designed to unmask the latent thioester and produce the corresponding ethyl thioesters **2a–c** (Table 2). Both amino acid substituents on **1** contain latent thioesters due to the C_2-symmetry of **1**; the isolated yields listed in Table 2 are therefore based on two equivalents of ethyl thioester. The reaction appears insensitive to the solvent used among DMF, DCM, and THF, producing good yields in all of the solvent combinations attempted. Additional excess TEA (Entry 6) did not lead to improved conversion to the ethyl thioester.

The reaction worked under polar, aprotic solvent conditions in the presence of a tertiary amine base to produce thioesters **2a–c**. Though the unmasking conditions are incompatible with the Fmoc protecting group itself, producing fluorenylmethylene, as a practical matter during SPPS this only precludes Fmoc as the protecting group for the final, N-terminal amino acid—the carbamate that remains after the peptide synthesis is complete. Using a Boc-protected amino acid rather than an Fmoc-amino acid for the N-terminal residue would not affect the endgame global deprotection scheme involving Fmoc/t-Bu SPPS because side chains are also protected using acid-labile *tert*-butyl groups. The reactions of diesters **1a–b** suggest that the stereochemistry of the amino acids relative to the C_2-symmetrical diol does not significantly affect the outcome of the process.

Diesters **1a–c** were subjected to organic reaction conditions designed to ligate protected amino acids **8a–c** with H-Cys-OMe·HCl (Table 3). The reaction conditions operate first to unmask the latent thioester yielding a thioester capable of undergoing NCL, and then to complete this NCL as expected. Initial experiments involved five equivalents of TEA and upwards of 10 equivalents of thiol. Preliminary attempts of the reaction with Boc-Phe diester **1a** yielded minimal to no desired product. Operating on the principle that the presence of the hydrochloride salt required additional base, we employed nine equivalents of TEA, which produced much better yields of the coupled products **6a–c** (again based on two equivalents of **6** because both amino acids on **1** are competent in this reaction).

Formation of protected dipeptides **6a–c** proceeded with no observed epimerization of the latent thioester partner, as expected

TABLE 1 Esterification generating latent thioesters



Entry #	Boc-Xxx-OH Amino Acid 8	Equiv. of EDC	Equiv. of DCC	Solvent(s)	1	Yield (%)[a]
1	**8a**, Phe	5	—	DMF	**1a**	34
2	**8a**, Phe	5	—	DCM, THF	**1a**	64
3	**8b**, D-Phe	5	—	DCM, THF	**1b**	62
4	**8c**, Tyr(t-Bu)	4.5	—	DCM, THF	**1c**	76
5	**8c**, Tyr(t-Bu)	—	4	DMF	**1c**	54
6	**8c**, Tyr(t-Bu)	—	3	DMF	**1c**	79

[a]Isolated yields.

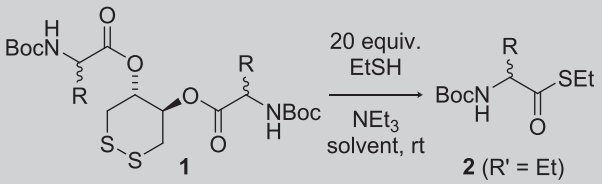


TABLE 2 Unmasking to generate ethyl thioesters

Entry #	Diester 1	Equiv. of NEt_3	Solvent(s)	2	Yield (%)[a]
1	**1a**, Phe	5	DMF	**2a**	73
2	**1a**, Phe	5	DCM, THF	**2a**	62
3	**1a**, Phe	5	DMF, THF	**2a**	61
4	**1b**, D-Phe	5	DMF, THF	**2b**	67
5	**1c**, Tyr(t-Bu)	5	DMF, THF	**2c**	51
6	**1c**, Tyr(t-Bu)	10	DMF	**2c**	45

[a]Isolated yields based on two equivalents of **2**.

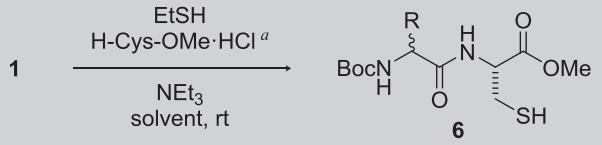


TABLE 3 Unmasking and in situ cysteine ligation

Entry #	Diester 1	Equiv. of NEt_3	Equiv. of EtSH	Solvent(s)	6	Yield (%)[b]
1	**1a**, Phe	5	10	DMF	**6a**	6
2	**1a**, Phe	5	15	DCM	**6a**	—
3	**1a**, Phe	5	16	DMF, THF	**6a**	—
4	**1a**, Phe	9	17	DMF	**6a**	74
5	**1b**, D-Phe	9	17	DMF	**6b**	66
6	**1c**, Tyr(t-Bu)	9	17	DMF	**6c**	64

[a]Either 2.5 or 5 equivalents; see Supporting Information.
[b]Isolated yields based on two equivalents of **6**.

based on reported precedent.[22] This result was straightforward to verify by comparing the results from the reactions with L- and D-Phe; in each case, a single Phe–Cys diastereomer was observed.

4 | CONCLUSIONS

In conclusion, we have demonstrated that C-terminal latent thioesters of hydrophobic and/or protected amino acids can be unmasked using organic, in addition to aqueous, reaction conditions. Further coupling with cysteine demonstrates that this process retains the stereochemical integrity of the activated amino acid. To our knowledge, this is the first system of its kind that is demonstrated, under organic reaction conditions, to generate a hydrophobic and/or protected peptide C-terminal thioester from a latent thioester, and which can be further ligated to cysteine in a one-pot reaction. We envision this method being useful for coupling larger protected peptide fragments; such experiments are underway, and the results will be reported in due course.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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