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Acid-Catalyzed Tandem Thiol Switch for Preparing Peptide Thioesters from Mercaptoethyl Esters

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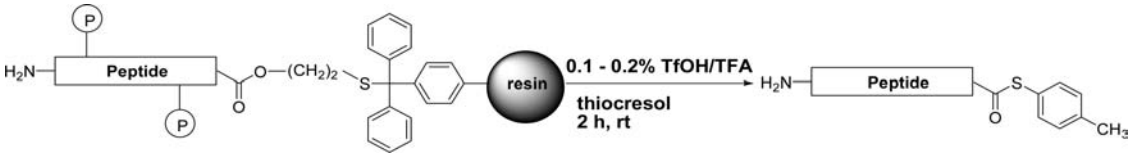
ABSTRACT



An efficient method compatible with Fmoc synthesis for preparing peptide thioesters via an acid-catalyzed tandem "thiol switch" of esters is described first by an intramolecular O−S acyl shift and then by an intermolecular S−S exchange, with concurrent deblocking of side chain protection groups.

Thioesters are versatile building blocks for various synthetic schemes to prepare lactone and peptides.[1] Thioesters are susceptible to base and intolerant to piperidine in Fmoc (fluorenylmethoxycarbonyl) chemistry and are currently the method of choice for many solid-phase syntheses of peptides, particularly those bearing glyco- and phospho-modifications on the peptidyl side chains.[2−4] These needs have prompted the development of new methods for preparing thioesters,[5−9] and many employ a "thiol switch" approach using a piperidine-resistant surrogate group during the Fmoc peptide synthesis steps.[2,4−9] Surrogate groups such as sulfonamide, hydrazine, ester, and amide with or without a thiol auxiliary[2−7,9−15] are then activated at the completion of peptide synthesis to undergo a thiol switch reaction via an O−S or N−S acyl shift effected either intra- or intermolecularly.

While each method has its merits, the use of a surrogate is often complicated by synthetic complexity, by side reactions at either the activation or the conversion step, and in some cases, by the need of an additional step for its removal. Consequently, a simple and practical method would be valuable for preparing peptide thioesters under an Fmoc-compatible scheme.

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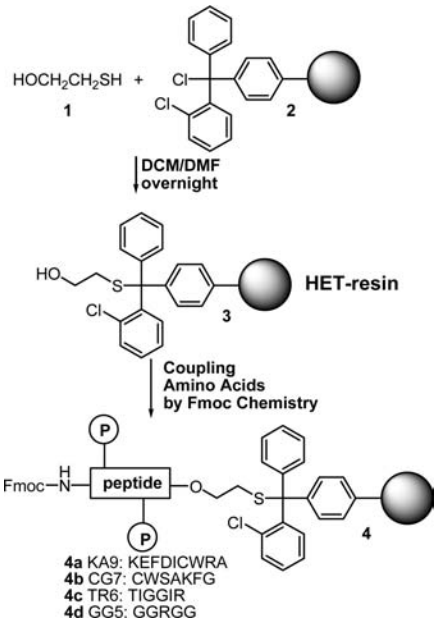
The N−S and O−S acyl shifts occur in the early stages of protein splicing, and their biomimetic versions to afford a thioester have been exploited.[10−15] Recently, several groups reported highly promising methods of thioester preparations using the conformation-assisted N−S acyl shift of tertiary amides.[12−15] In contrast, methods using O−S acyl shift of esters to prepare thioesters directly on resin supports are less developed.

Scheme 1. Preparation of Peptide-Thioesters on Hydroxyethylthiol (HET)-Resin



Esters (**4** in Scheme 1) are generally stable during the repetitive deprotection steps of Fmoc-chemistry and could serve as a thioester surrogate during the Fmoc synthesis of peptides. Although O−S acyl shift has been exploited for thioester preparation at pH 6.5 after the global deprotection of side chains,[10] hydrolysis is a worrisome side reaction. Hilvert's group has also explored the use of Lewis acid-thiolate on benzylic ester resin for preparing thioesters under rather severe conditions.[7] Here, we describe an efficient method for preparing peptide thioester by a tandem "thiol switch" of a peptide ester containing β-mercaptoethanol handle (OEtSH) which enables an intramolecular O−S acyl shift followed by an intermolecular S−S exchange reaction to afford a thioester.

Our approach makes use of the C-terminal alkoxythiol linker that we have previously developed for cysteine-free ligation.[16] It has the advantage of being readily accessible by reacting hydroxyethylthiol (HET) **1** or hydroxypropyl thiol (HPT) with a trityl chloride resin **2** to form HET- or HPT-resin, respectively (Scheme 1). The reactive thiol linked to the resin as thiol ether **3** is protected during the peptide synthesis stage and released as a latent thiolate for the first "thiol switch" reaction during the acid

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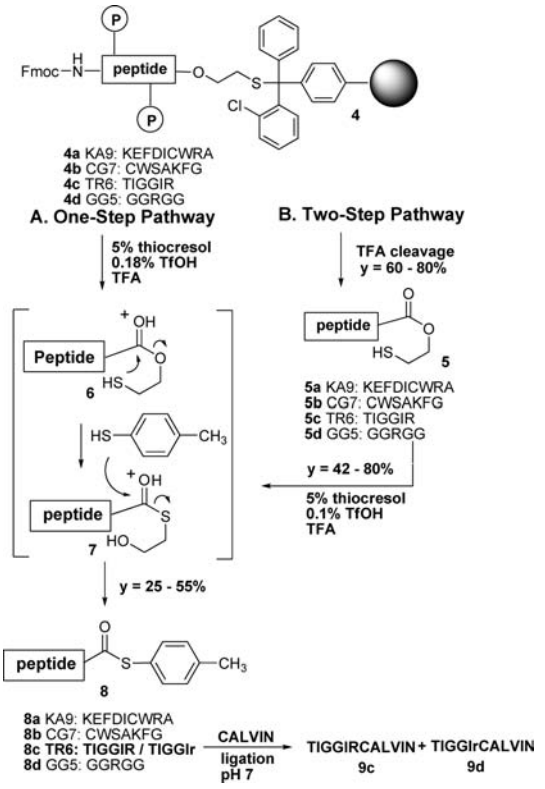
Scheme 2. Thioester Preparation under Acidic Conditions for Tandem Thiol Switch via O−S Acyl Shift and S−S Acyl Shifts[a]



[a] Isolated yield (Supporting Information Figure 3-1).

deprotection step at the completion of peptide assemblage. We reasoned that an ethoxythiol handle released during the acid deprotection step as a C-terminal peptide ester **5** would permit an acid-catalyzed intramolecular O−S acyl shift **6**→**7** followed by an intermolecular S−S exchange **7**→**8** with another thiol during the TFA (trifluoroacetic acid) deprotection step under a timely activation by protonating the ester carbonyl **6** at the C-terminus (Scheme 2). The proposed scheme would provide a solution to circumvent the issues for a separate step of activation and auxiliary removal, and concurrently permit deprotection of side chains under a one-pot reaction.

To determine the acidity requirement of the first O−S acyl step of our approach, we prepared a 9-residue peptide ester KA9-OEt-SH **5a** which was readily obtained from the standard TFA cleavage of KA9-O-Et-S-Trt-resin **4a**. TFA treatment of the purified **5a** without an external thiol for 2 h produced little rearranged product KA9-SEt−OH ($< 2\%$) and the starting material KA-OEt-SH **5a** remained as the major product ($> 80\%$). Prolonged TFA treatment for 18 h did not significantly increase the desired thioester. These results were in contrast of acid-catalyzed thiol switch reactions for preparing thioesters by N−S acyl shifts through tertiary amide surrogates which gave a mixture of both amide and thioester products after TFA treatment.[11−15] However, our results are consistent with the known basicity of the tertiary amide and ester moieties,

which have pK_a of 0 and −3, respectively. Since the acidity function of TFA is around −3, our results suggest that the O−S acyl shift will require an increase in acidity to protonate the ester carbonyl for a smooth thioester conversion.

Table 1. Effect of TfOH (v/v) in TFA on the Disappearance of KA9-OEtSH **5a** and the Formation of KA9-OH via 1,2-Elimination to Form Ethylene Sulfide

	KA9-OEt-SH (mol %)		KA9-OH (mol %)	
TfOH (vol %)	1 h	8 h	1 h	8 h
0.01	92	72		1
0.06	74	21	19	52
0.1	57	2	30	62
0.2	53	1	31	62
1	48	0	34	44

To increase acidity of the TFA for the O−S acyl shift of **5a**, we added incrementally TfOH (trifluoromethane sulfonic acid) from 0.01 to 1% by volume to TFA (Table 1). Since we were unable to observe a significant accumulation of the desired thioester KA9-SEt−OH **6a**, the rates of S−O acyl shift in the conversion of KA9-SEt−OH **6a** from KA9-OEt-SH **5a** were deduced on the basis of the starting material and the formation of the byproduct KA9-OH which lost the OEtSH handle by a competitive acid-catalyzed elimination reaction to form ethylene sulfide. The use of 0.1−0.2% (v/v) of TfOH in TFA appeared to be a suitable compromise so as to permit sufficient acid strength to protonate the ester carbonyl to effect the O−S acyl shift but not too strong to increase competing side reactions. We also concluded that the O−S and S−O acyl shifts were likely reversible in stronger acidic solutions than TFA alone. These results are consistent with our previous findings that the OEtSH handle of peptide ester **5** is fairly stable in TFA but susceptible to degradation in strong acids.[16,17]

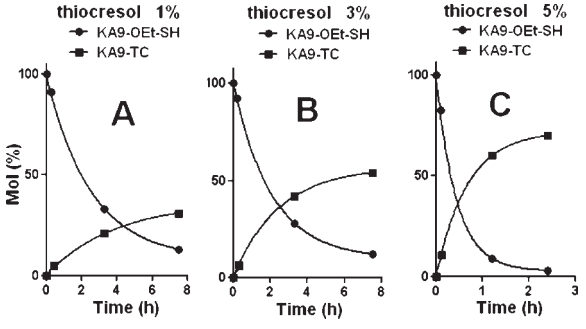


Figure 1. Thioester conversion under 0.1% TfOH/TFA in (A) 1%, (B) 3%, (C) 5% of thiocresol from KA9-OEt-SH to KA9-TC.

To investigate suitable conditions for the S−S exchange of transthioesterification in the second step of a tandem thiol switch scheme to afford a stable thioester and to terminate the equilibrations of O−S and S−O acyl shifts of the thioester intermediate **5b**, we used an increasing concentration of an external thiol, thiocresol (TC), in 0.1% TfOH in TFA. With 5% TC in 2 h, the desired thioester **8a** was obtained in 70% yield (Figure 1).

For the acid-catalyzed S−S exchange reactions, we found that, under our proposed conditions, simple thiols such as *p*-thiocresol and benzyl thiol were more suitable than the bifunctional thiols, such as mecaptoethanol or mecaptopropionic acid which gave a complicated product profile because of side reactions of their O-moiety participating in the reverse O−S acyl shift reactions.

In combining both steps, a direct one-step thioester conversion from the peptide-HET resin of **4a**, **4b**, and **4c** was successfully achieved in 2 h affording 25−55% yield with a deprotection mixture (TFA 9.3 mL, TC 0.5 mL, and TfOH 18 μL within 0.18 mL TFA, 1:5%:0.18%, v/v/v) from 60 mg of peptide resins. The conversion became sluggish when a Lewis acid such as $B(TFA)_3$ replaced TfOH at the same concentration of 0.1 to 0.2% in TFA (v/v).

The first of the two-thiol switch reactions is likely the rate-determining step for the tandem thiol switch scheme because it is an entropic-favored, intramolecular O−S acyl shift through a five-member ring intermediate. Control experiments, requiring an intermolecular O−S acyl shift using methylbenzoate as a model compound, did not afford thioester products under the proposed conditions of 0.1−0.3% TfOH (v/v) in TFA and 5% TC for 2 h as determined by HPLC. Similarly, the desired thiolactone from the first tandem thiol switch reaction was not observed with CG7-OEt-SH **5b** containing an N-terminal cysteine. Treatment of **5b** with 0.15% TfOH in TFA without TC for 8 h afforded CG7-OH as the main product suggesting the intramolecular O−S acyl shift by the N-terminal thiol to form a thiolactone of **5a** was unfavorable. Addition of 5% TC to the 0.15% TfOH/TFA solution for 4 h provided 42% CG7-TC **8b**, which was smoothly converted to the end-to-end cyclized lactam in 80% yield within 1 h at pH 7.5 or 8.5 (Supporting Information Figure 1) with $< 6\%$ the hydrolyzed product CG7-OH.

Two side reactions associated with the current scheme were also investigated. The first was the long-standing problem of racemization during the esterification of the first amino acid to a hydroxy resin such as the HET resin. To determine the extent of racemization, we subjected the esterification of Fmoc-Arg(Pbf)−OH or Fmoc-Ala-OH to a high loading HET resin (1.4 mmol/g) to completion by BOP-DIEA repetitively (three times) in the synthesis of TIGGIR-TC **8c** or KA9-TC **8a**, which produced 85 and 11% racemization, respectively. Interestingly, the D and L forms of both peptides **8c** could be clearly identified in HPLC in their thiocresol ester forms. For example, hexapeptide TIGGIR-TC **8c** afforded two peaks (Supporting Information Figure 3-1) in HPLC with the expected molecular weight. Native ligation with a hexapeptide

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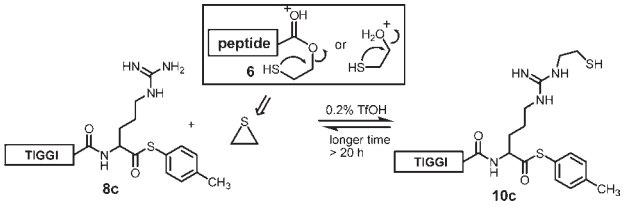
CALVIN-NH_2 gave two ligated products TIGGIRCALVIN **9c** and TIGGIrCALVIN **9d** with a different retention time in HPLC. Direct synthesis of TIGGIRCALVIN **9c** and TIGGIrCALVIN **9d** by solid phase synthesis confirmed D- and L-isomers of the C-terminal Arg position (Supporting Information Figure 2). Several esterification methods have been developed to address this side reaction,[7,18,19] and we recommended that prolonged esterification under basic conditions should be avoided to minimize racemization of the C-terminal amino acid during the attachment step.

Scheme 3. Reversible Alkylation Reaction of C-Terminal Arg to Form C-Terminal Arg(EtSH)



The second side reaction is a reversible alkylation of the guanidino side chain of Arg of **8c** by ethylene sulfide to form TIGGI-Arg(EtSH) **10c**, which was confirmed by MS/MS sequencing (Supporting Information Figure 3-2 and 3-3). Ethylene sulfide is the elimination byproduct of mercaptoethanol released from the S−S exchange of the HET handle or hydrolysis of peptide **6** (Scheme 3). It is an electrophile and can act as an alkylating agent to form adduct with nucleophilic side chains. Under our proposed deprotection conditions containing 0.1−0.2% TfOH in TFA with 5% TC, the alkylation appeared to be specific for the guanidino side chain when tested against Fmoc-Met, Fmoc-Tyr and Fmoc-Trp, and the large excess of TC likely served as a scavenger for these nucleophilic side chains. We found that the alkylation of TIGGIR-TC **8c** was 30% in 2 h, probably due to the proximal effect of the C-terminal Arg to the OEtSH handle (Scheme 3), but decreased to 1% in the model peptide GGRGG **5d** with an internally placed Arg. Similar to the acid-catalyzed modification and removal of the guanidino side chain of Arg by an electrophile such as NO to form Arg(NO), Arg(EtSH) was reversible to Arg in a longer time reaction > 20 h (30%). It did not escape our attention that the facile formation and the reversibility of Arg(EtSH) could provide a novel entry to peptide ligation of N-terminal Arg peptides

In a preliminary study to support our results, we found that the alkylation side reaction can be minimized in the synthesis of TIGGIR-TC **8c** by substituting 2-mercaptoethanol with 3-mercaptopropanol to form HPT-trityl resin. The acid-catalyzed conversion under our proposed thiol-switch scheme to form the four-member ring propylene sulfide was substantially slower than the three-member ring ethylene sulfide to effect the alkylation reaction to form Arg(PrSH).

In summary, the O−S acyl shift of ester **5** was dependent on acidity and the protonation state of the ester carbonyl. Addition of a catalytic amount of TfOH accelerated the O−S acyl shift to afford a thioester **7**. The addition of an external thiol into the reaction mixture permits the second "thiol switch" reaction and serves to favor the formation of stable thioester **8** by terminating the equilibration of the acyl migrations prone to the reverse reaction and degradation between O and S moieties. Our proposed scheme bridges an "acid gap" in preparing thioesters under acidic conditions. It bears similarity to the "low TFMSA" and "low HF" conditions which have an acidity function of −5.2 and could be used for the synthesis of glycol- and phospho-peptides.[2b,20]

Our method based on convenient starting materials provides a simple and practical solution to prepare thioester peptides compatible with Fmoc chemistry. It also provides a choice of an ester **7** with a thiol handle or thioesters for ligation, suitable for the cysteine-free,[3,16] convergent synthesis,[3] and the tandem ligation schemes.[21]

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Supporting Information Available. General procedures, additional HPLC and MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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