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Tandem Thiol Switch Synthesis of Peptide Thioesters via N–S Acyl Shift on Thiazolidine

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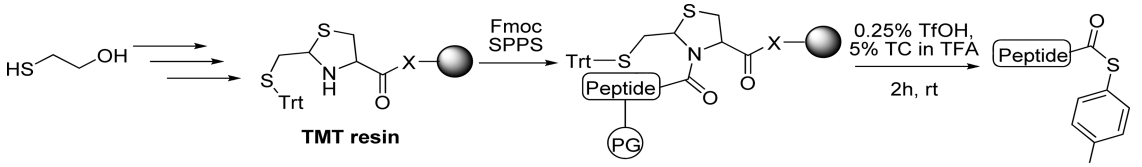
ABSTRACT



An efficient "thiol switch" approach for the synthesis of peptide thioesters via an acid-catalyzed N–S acyl shift and a thioester exchange reaction in tandem with concurrent removal of protecting groups is described. This method employs novel 2-(thiomethyl)thiazolidine (TMT)-anchored resins and is fully compatible with Fmoc chemistry.

Peptide thioesters are useful building blocks for protein modification and immobilization strategies in peptide and protein arrays.[1−3] Moreover, they are required for segment coupling reactions such as chemical ligation,[3] Staudinger ligation,[4] and Ag^{+}-mediated thioester ligation.[5] While peptide thioesters are compatible with the Boc/Bzl chemistry during solid-phase peptide synthesis (SPPS),[2] they are incompatible with the Fmoc-based methods because of their susceptibility to piperidine deprotection[1] steps. This has led to early attempts at developing Fmoc-compatible methods for preparing peptide thioesters based on optimized Fmoc deprotection cocktails,[6] activation of protected peptides in solution,[7] and thiol-labile safety-catch linkers.[8]

Recently, there has been increasing interest in methods involving acyl-transfer reactions for the Fmoc-compatible synthesis of peptide thioesters.[9−17] In principle, preparing an ester or a thioester from an amide is the reverse process of a ligation scheme. In practice, such a reversal is generally thermodynamically disfavored and would require activation,

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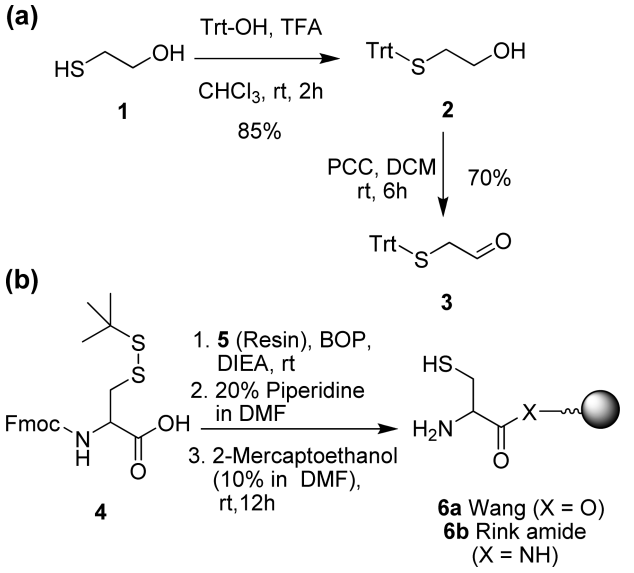
conformational assistance, or both to enable an internal hydroxyl or thiol nucleophile to undergo an intramolecular N−O or N−S acyl shift reaction to form an ester or thioester from an amide.[9−14]

The N−O or N−S acyl shift is known to occur in the early stage of protein splicing to afford an ester or thioester.[1b] The acyl shift is proximity driven and conformationally assisted with the scissile peptide bond in a cis conformation. Among the naturally occurring peptide bonds, only the tertiary Xaa-Pro bonds can favorably exist in a cis conformation without enzymatic assistance. To mimic the N−S acyl shift in protein splicing, many sought to develop tertiary amide or proline-like surrogates with a thiol handle to enable a ciscoid N−S acyl shift of a tertiary amide bond to form a thioester. Tertiary amide surrogates include structures such as N-alkylated Cys and its mimetics,[9] thiol-substituted proline,[10] oxazolidinone,[11] anilide,[12] or modified benzyl[13] groups. However, the reported conformationally assisted N−S acyl methods are often complicated by synthetic complexity in their preparation, by side reactions at either the activation or the conversion step, and in some cases, by the need of an additional step for their removal.

Based on our previous ligation chemistry using thiazolidoine and oxazolidoine as a proline surrogate,[18] we envisioned that a thiazolidine with a 2-thiomethyl group could undergo a proximity-driven N−S acyl shift reaction in a cis conformation, mimicking the N−S acyl shift of protein splicing. Herein, we describe the use of 2-thiomethylthiazolidine as an Fmoc-compatible proline surrogate to enable a tandem acid-catalyzed thiol switch to afford a thioester, first through an N−S acyl-transfer reaction and then a S−S (thiol thioester) exchange. To obtain the desired 2-thiomethylthiazolidine, our scheme began with 2-mercaptoethanol **1**, which was protected with a trityl group upon treatment with triphenylmethyl alcohol in the presence of trifluoroacetic acid (TFA) in $CHCl_3$ (Scheme 1a)[16] to afford tritylthioethyl alcohol **2** in 85% yield within 2 h. The purified alcohol **2** was then subjected to oxidation by reacting it with pyridinium chlorochromate[2] (PCC) under anhydrous conditions for 6 h to obtain the corresponding tritylthioethyl aldehyde **3** in 70% yield.

The desired thiazolidine can be synthesized from aldehyde **3** by allowing it to undergo intermolecular cyclization with a moiety containing a 1,2-aminothiol functionality as

Scheme 1. Preparation of starting Materials for Synthesis of Peptide Thioesters



previously reported by our group.[18] The unprotected cysteine anchored on an Fmoc-compatible resin support would provide such a functionality. Keeping this in mind, the synthesis of unprotected cysteine-anchored resins was carried out, first by coupling Fmoc-Cys(S-*t*-Bu)-OH **4** to the Wang or Rink-amide resin **5** using benzotriazole-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and *N*,*N*-diisopropylethylamine (DIEA), followed by the removal of Fmoc group (Scheme 1b). Treatment with 2-mercaptoethanol (10%, v/v) in DMF for 12 h resulted in removal of *tert*-butylsulfenyl protecting group, thereby rendering the unprotected cysteine **6** with desired 1,2-aminothiol functionality. The presence of free thiol was confirmed by treating **6** with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)], leading to the appearance of a red solution.

To synthesize the thiazolidine ring with a highly acid-sensitive trityl moiety both on the thiol side chain and resin supports (e.g., Rink resin), the tritylthioethyl aldehyde **3** was reacted with resin-coupled unprotected cysteine **6** under neutral conditions for 24 h in DCM (Scheme 2). Treatment of resin beads with Ellman's reagent did not produce red color, confirming the absence of free thiol group. To further characterize the cyclized product, the reaction was reproduced in the solution phase, confirming the quantitative formation of thiazolidine ring (Supporting Information Figure 3). The intermolecular cyclization was efficient and proceeded via an imine capture between the free amino group of **6** and the aldehyde carbonyl of **3**, followed by ring−chain tautomerization by the free thiol group leading to the formation of tritylthiomethylthiazolidine−Wang (TMT−W) or −Rink amide (TMT−R) resin **7**. Collectively termed as TMT resins, their main advantage is that the suitably placed trityl protected thiol

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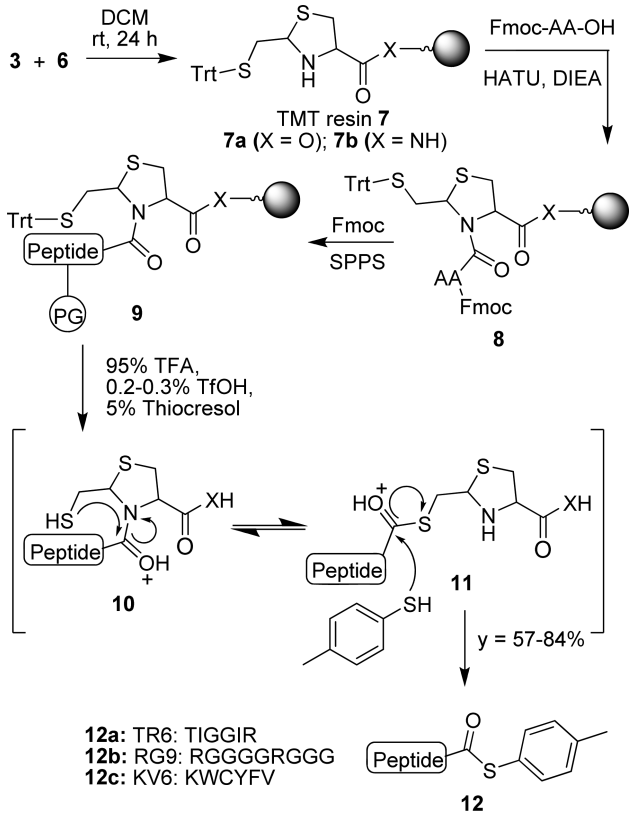
moiety can be released at the completion of peptide synthesis as a latent thiolate for the N−S acyl transfer reaction.

Scheme 2. Preparation of Peptide Thioesters via Acid-Catalyzed N−S and then S−S Acyl Shifts



The secondary amine of the synthesized thiazolidine ring offers a suitable site for carrying out the solid-phase peptide synthesis. Fmoc-protected amino acids were sequentially coupled to the TMT-resins in the presence of *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and DIEA, resulting in the formation of TMT-resin-coupled product **8** (Scheme 2). Subsequently, the Fmoc group was removed and other amino acids were further coupled via regular Fmoc-SPPS steps to afford an orthogonally protected peptide-TMT **9**.

Recently, our group has reported an efficient Fmoc-compatible method for preparing peptide thioesters via an acid-catalyzed tandem "thiol switch" of esters by an intramolecular O−S acyl shift followed by trapping the rearranged thioester with a large excess of thiocresol (TC) to form a stable thioester.[17] We envisioned that this approach might also work for the N−S acyl transfer reaction via the 2-thiomethylthiazolidine surrogate. In this context, the protected peptide-TMT **9** was treated with varying concentrations of TFA, trifluoromethanesulfonic acid (TfOH) and TC. Since the N−S acyl shift is dependent on the acidity function of the TfOH/TFA deprotecting solution, we tested a suitable acidic condition by varying the concentration of TfOH in three increments, 0.1, 0.25, and 1%, while keeping the concentrations of TFA and TC constant. Initially, TR6-TMT-W resin **9a** was treated with 0.1% TfOH and 5% TC (v/v) in TFA for 2 h. It led to the formation of the desired thioester TR6-TC **12a** (*m*/*z* 722) as the major product in MS profile with an isolated yield of 55% (Figure 1a), which was confirmed by MS/MS sequencing (Supporting Information Figure 8). Apart from the desired thioester product, the MS profile also revealed minor peaks at *m*/*z* 616 (< 5%) and *m*/*z* 781 (< 8%). The former peak corresponded to TR6-OH suggesting the incomplete attachment of the Fmoc-Cys(S-*t*-Bu)-OH to the resin, and the latter indicated an impurity peak which seems to be generated during peptide synthesis. A side reaction in preventing the N−S acyl shift is the trifluoroacetylation of the thiol group, with an observed peak at *m*/*z* 838.

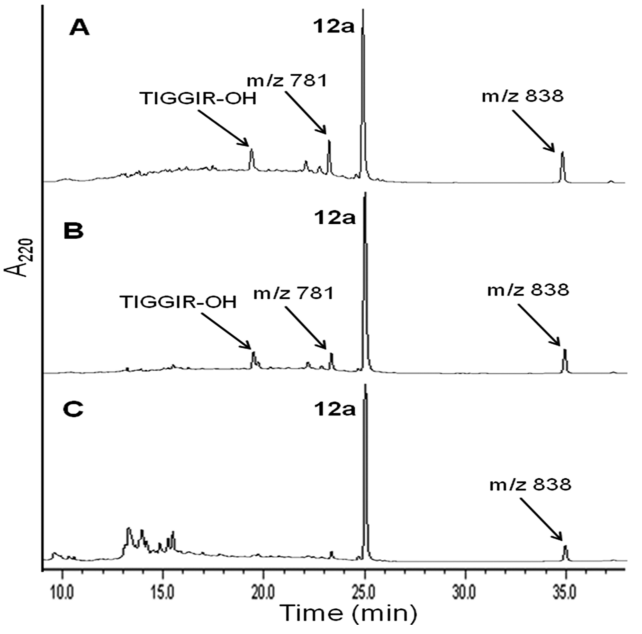


Figure 1. RP-HPLC profiles of the preparation of TR6 **12a** from TMTW-TR6 **9** using of 5% TC, TFA, and (A) 0.1% TfOH; (B) 0.25% TfOH; (C) 1% TfOH.

The yield of **12a** increased to 69% as the concentration of TfOH was increased to 0.25% (v/v) in TFA (Figure 1b). However, when the TfOH concentration was increased to 1% (v/v) of TFA, there was also an increase in competing reactions leading to a slight decrease in the thioester yield to 65% (Figure 1c). Considering this almost unchanged

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yield of thioester for a 4-fold increase in the TfOH concentration, the use of 0.25% TfOH (v/v) of TFA appeared to be a practical compromise so as to permit sufficient acid strength to protonate the amide carbonyl to effect the N−S acyl shift but not too strong to increase competing side reactions. To investigate suitable conditions for the second thiol switch of the thioester intermediates **10** and **11** by a thioester exchange, the concentration of TC was varied from 2.5% to 5% to 10%. These chagnes effectively resulted in the formation of TR6-TC in respective yields of 45%, 69%, and 73%. As the thioester yield did not increase much after 5% TC, we postulated that the suitable concentration of TC and TfOH for the acid-catalyzed tandem N−S-acyl transfer reaction and subsequent thioester exchange using TMT handle appears to be 5% TC (or lower %) and 0.25% TfOH (v/v) of TFA, respectively. Previously, we showed the L- and D-form of TIGGIR-TC and TIGGIr-TC, respectively, are well separated by HPLC.[17] We did not detect TIGGIr-TC by HPLC, suggesting racemization is minimal under the proposed conditions.

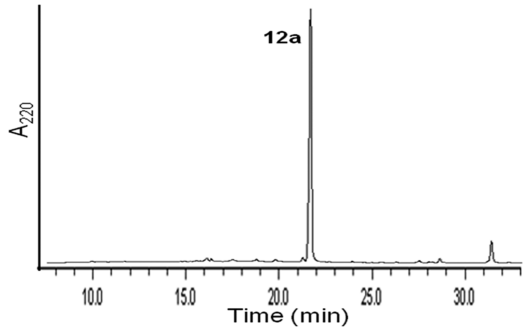

Figure 2. RP-HPLC profile of the preparation of TR6 **12a** from TMTR-TR6 **9** using 0.25% TfOH and 5% TC (v/v) of TFA.

The versatility of the acid-catalyzed N−S acyl shift reaction using the TMT handle was further verified with two additional peptide thioesters, RG9-TC **12b** and KV6-TC **12c**, which were synthesized using TMT-Wang resin **9** under similar conditions in 65% and 57% yields, respectively. Mechanistically, the acid-catalyzed thioester formation from peptide-TMT **9** resulted from a combination of two "thiol switch" reactions. The first thiol switch takes place after the release of the TMT handle **9** during the acid deprotection step to permit an acid-catalyzed intramolecular N−S acyl shift of **10** to **11** followed by the second thiol switch via a thioester exchange from **11** to **12** by thiocresol under a timely activation by TfOH/TFA protonating the amide carbonyl **10** at the C-terminus (Scheme 2). It should noted that TC also serves as a scavenger under the proposed conditions.

To determine the applicability of the current method for the Fmoc-compatible Rink amide resins, the Rink amide resin-coupled TR6-TMT was subjected to deprotection, N−S acyl transfer, and thioester exchange using the identified conditions. It resulted in the formation of thioester TR6-TC **12a** in 84% yield (Figure 2), suggesting that Rink amide resin is even more appropriate for thioester preparation than the Wang resin based on the proposed thiazolidine surrogate. The increase in yield is likely attributed to the ease of attachment of the TMT-handle to the Rink amide resins. This result also suggests that the proposed TMT-handle can be used for any Fmoc-compatible resin to afford a peptide thioester. Another important advantage of the tandem thiol switch reaction is that it provides a solution to circumvent the issues for a separate step of activation and auxiliary removal by allowing the deprotection of side chains under a one-pot reaction. Compared to O−S acyl shift, the thiol switch reaction seems to be cleaner for the N−S acyl shift under similar acidic conditions because of the absence of the reversible alkylation of the guandino side chain of Arg by hydroxyalkylthiol handle.[17]

In summary, the synthesis of peptide thioester via acid-catalyzed N−S-acyl shift on a 2-thiomethylthiazolidine[3] framework is a simple, novel, and practical Fmoc-based method for solid-phase peptide synthesis. It employs simple starting materials and is compatible with the Wang or Rink-amide resins. The proposed TMT resin **7** is relatively easy to synthesize and provides with a thioester surrogate where routine Fmoc-base peptide synthesis can be pursued to obtain the desired peptide thioester in relatively good yield after an acid-catalyzed tandem thiol switch reaction. The observed N−S acyl shift was dependent on acidity, and the protonation state of the amide carbonyl as the formation of thioester **12** was accelerated by the addition of a catalytic amount of TfOH. The proposed N−S acyl shift scheme bears similarity to the "low TFMSA" and "low HF" conditions with an acidity function of −5.2 and could be potentially used for ligation, convergent synthesis, cysteine-free ligation,[19] and tandem ligation[20] as well as for the synthesis of glyco- and phosphopeptides.[5c,21]

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