The reduction of oxidized methionine residues in peptide thioesters with NH_4I–Me_2S†

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Oxidized methionine residues in peptide thioesters can be reduced rapidly with NH_4I to the corresponding sulfide by using Me_2S as coreductant. Comparative reduction studies employing a 28-amino acid peptide thioester with an *N*-terminal methionine oxide as model system revealed the importance of the Me_2S addition to avoid hydrolysis of the reactive thioester functionality. In addition, an NH_4I–Me_2S containing cleavage cocktail has been used for the global deprotection of various thioesters which revealed no hydrolysis or oxidative side products. These results demonstrate the general applicability of sulfoxides as protecting groups in advanced peptide synthesis techniques by facilitating the preparation and handling of methionine containing peptide thioesters for native chemical ligation (NCL).

Introduction

Peptide thioesters have become valuable compounds and useful building blocks in modern bioorganic chemistry. A common feature of thioesters is a rapid thioester equilibrium at neutral pH upon the addition of thiols.[1] This thioester–thiol exchange reaction has found widespread spectroscopic and synthetic applications. Backbone thioester exchange (BTE) has been used to determine the conformational stability and formation of peptidic secondary and tertiary structures.[2] Most importantly, thioester exchange can facilitate the connection between a polypeptide thioester and an *N*-terminal Cys protein or peptide through native chemical ligation (NCL).[3] Expressed protein ligation (EPL),[4] which enables the connection of expressed protein fragments with synthetic peptides *via* NCL, has proven to be particularly suitable for accessing semisynthetic proteins with various natural and unnatural protein modifications in the N- or C-terminal part of the protein.

Various routes for the access of thioesters have been developed. A common biochemical process is intein expression, which delivers large proteins with a *C*-terminal thioester.[5] Synthetic thioesters can be derived from Fmoc-based solid phase peptide synthesis (SPPS) using different resins and cleavage strategies.[6] These include the alkane-sulfonamide "safety-catch" resin,[7] which requires an activation step with diazomethane or iodoacetonitrile prior to peptide cleavage with thiols,[8] or the conversion of peptides on HMBA (4-hydroxymethylbenzoic acid) or PAM (4-hydroxymethyl-phenylacetamidomethyl polystyrene) resins to thioesters with alkylaluminium thiolates.[9] Alternatively, highly acid-labile resins (TGT[10] or 2-chlorotrityl resin[11]) have been used to allow orthogonal cleavage under mild acidic conditions without deprotection of peptide side chains. Subsequently, the *C*-terminal carboxylic acid can be transformed selectively to the thioester by HBTU–DIEA activation.

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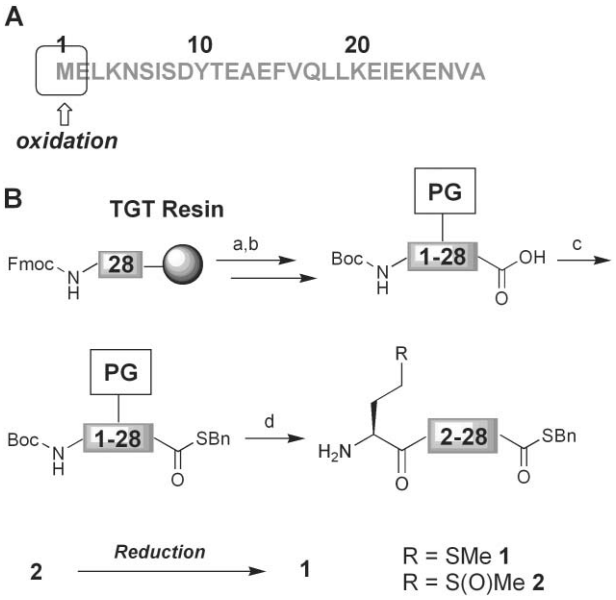
The synthetically attractive but sometimes problematic feature of thioesters is their high electrophilicity, which often leads to hydrolysis to the carboxylic acid. In consequence, the high thioester reactivity often requires the development of more sophisticated protocols for the access and handling of peptide thioesters, as exemplified in the development of Fmoc deprotection protocols with non-nucleophilic bases to replace commonly employed piperidine.[12]

In this paper a convenient protocol for the nucleophilic reduction of sulfoxides with NH_4I *in the presence* of a thioester functionality is presented. In the course of these investigations it was demonstrated that the use of Me_2S as a coreductant and a careful optimization of the reaction conditions could prevent thioester hydrolysis, which is initiated by treatment of thioesters with NH_4I alone. The motivation for the development of a protocol to reduce sulfoxides in peptide thioesters is twofold. First, it presents the first strategy to reduce Met sulfoxide containing peptide thioesters, which showed *undesired* sulfoxide formation during the synthesis or prolonged storage. Second, the method extends the use of methionine sulfoxides as *protected methionine analogues* in peptide thioester syntheses, because oxidized methionines have been proposed as methionine surrogates to avoid alkylations of the methionine thioethers during peptide cleavage after the SPPS. The latter application could provide important advantages in thioester syntheses *via* the sulfonamide catch strategy for the use in NCL. In previous investigations it was shown that the activation reagent iodoacetonitrile alkylates methionines and thus lowers the yield for the final thioester product.[13]

Results and discussion

Peptide synthesis

The 28-amino acid thioester **1** with an *N*-terminal Met residue was employed as a model system for the reduction studies (Scheme 1A). The thioester was accessed in reduced (**1**) and oxidized form (**2**) *via* the acid-labile linker (TGT) strategy by using a non-reductive cleavage cocktail for the final global deprotection (Scheme 1B).



Scheme 1 A) Im7 peptide sequence (1–28); B) SPPS synthesis of thioester **1** using the acid-labile TGT resin. *Reagents and conditions*: (a) i) 20% piperidine–DMF; ii) Fmoc-Xaa-OH or Boc-Met-OH, HBTU, HOBt, DIEA, NMP; (b) 0.5% TFA, CH_2Cl_2; (c) BnSH, HBTU, DIEA, THF; (d) modified cleavage cocktail K (TFA, phenol, PhSMe, TIS, H_2O). See Experimental section for details.

Thioester **1**, resembling the *N*-terminal part of the 87-amino acid immunity protein Im7, has recently been employed in the semisynthesis of Im7 variants *via* EPL for protein folding studies.[14]

All 27 amino acids were coupled to the commercially available Fmoc-Ala-TGT-Resin using standard Fmoc-based SPPS coupling conditions with HBTU–HOBt activation. The *N*-terminal Met was coupled as its Boc-protected analogue to deliver the unprotected *N*-terminus in the final deprotection step. Finally, the peptide was cleaved from the resin under mild acidic conditions (0.5% TFA in CH_2Cl_2) and treated with benzylmercaptan, HBTU and DIEA in THF to furnish the corresponding thioester, which was globally deprotected with reagent K[15] in which EDT was omitted. After HPLC analysis, considerable amounts of the Met oxidized analogue **2** were detected (Fig. 1A), which were isolated by HPLC and used for subsequent reduction studies (Fig. 1B).[16]

Reduction of thioester sulfoxides with NH_4I–Me_2S in TFA

Several groups have previously reported protocols for the reduction of methionine sulfoxides in peptide systems using NH_4I in TFA.[17,18] This reaction is based on a nucleophilic iodide attack on the protonated sulfoxide leading to the reduced methionine sulfide and the formation of iodine as the reduction byproduct (Scheme 2). In order to minimize the formation of iodine and iodosulfonium ions, which have been reported to induce side reactions like Trp dimerizations to 2,2′ indolylindoline derivatives,[19] Me_2S was previously found to accelerate the reaction and serve as an efficient coreductant leading to the formation of dimethyl sulfoxide and iodide. Although it is well documented that iodine promotes the cleavage of thioesters to acids,[20] we envisioned that a careful optimization of the reaction conditions and the use of Me_2S as coreductant could avoid the formation of iodine and thereby lead to a selective nucleophilic reduction of the methionine sulfoxide **2** to **1** while leaving the thioester intact.

Initial attempts to reduce the oxidized thioester **2** with 20 equivalents NH_4I in TFA at 0 °C for 60 min led to formation of iodine, as observed as a brown precipitate which dissolved upon the addition of ascorbic acid. Subsequently, complete decomposition of the starting material was observed, since only

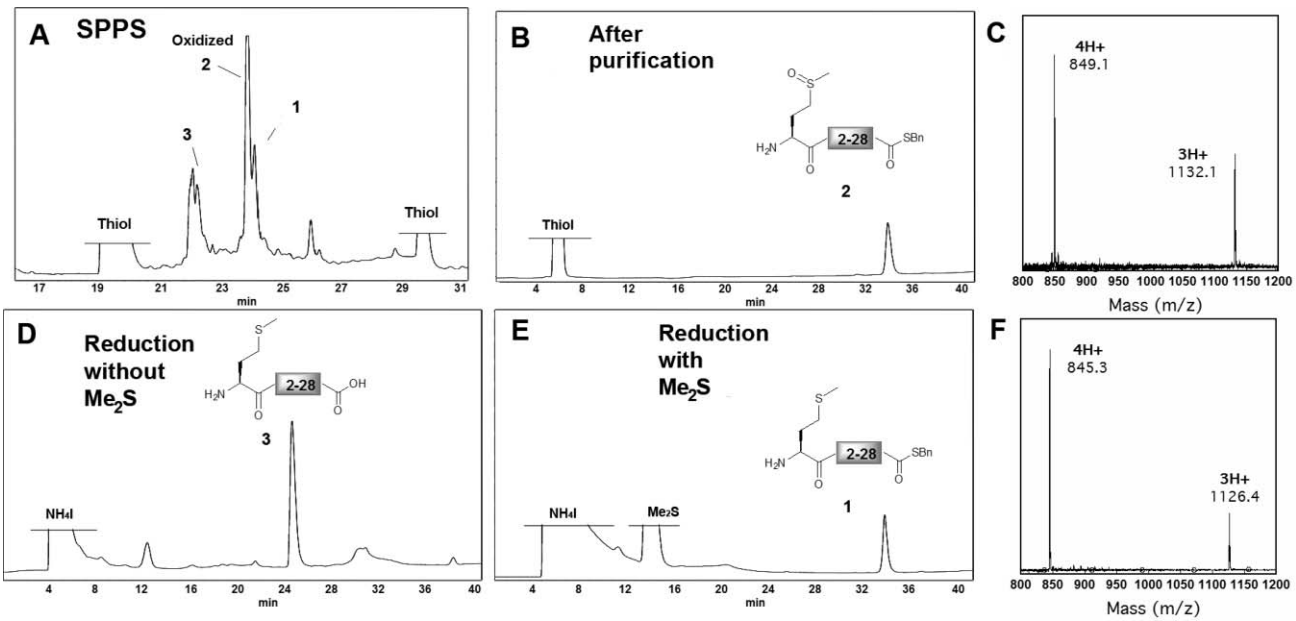
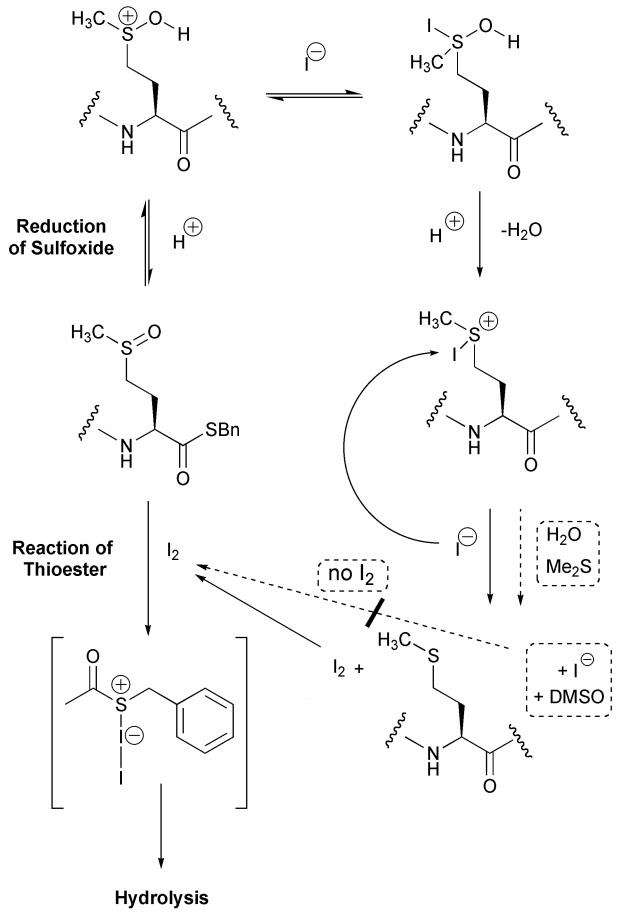


Fig. 1 HPLC spectra of Im7 peptides. A) Peptide thioester **1** with oxidized side product **2** and non converted peptide **3** after SPPS thioester synthesis and cleavage with cocktail K; B) oxidized thioester **2** after purification; C) ESI-MS of purified oxidized thioester **2**; D) hydrolyzed peptide **3** after 60 min reduction in TFA, 0 °C with 20 equiv. NH_4I; E) reduced thioester **1** after 30 min reduction in TFA, 0 °C with 20 equiv. Me_2S–NH_4I; F) ESI-MS of reduced thioester **1**. For cleavage cocktail contents and HPLC gradients see supporting Experimental part.



Scheme 2 Mechanism and potential hydrolysis pathway for the reduction of sulfoxides with NH_4I in TFA in peptidic systems with (solid line) and without (dashed line) the addition of Me_2S .

the hydrolyzed peptide **3** was detected by HPLC-MS (Fig. 1D). Fortunately, a reaction of the oxidized peptide thioester **2** with NH_4I–Me_2S in TFA at 0 °C showed full conversion to the reduced peptide thioester (Fig. 1E). In the course of further optimization studies, it became evident that the reaction was complete after 30 minutes using 20 equivalents of Me_2S. Prolonged treatment with NH_4I–Me_2S (>6 hours) at 0 °C led to the formation of iodine and the destruction of the thioester functionality. Trials to reduce thioester **2** with Me_2S without the addition of NH_4I showed no reduction capability.

The NH_4I–Me_2S containing cleavage cocktail H for the global deprotection of thioesters

The successful reduction and stability of sulfoxide thioesters prompted us to use NH_4I–Me_2S as additives for cleavage cocktails for global deprotections of thioesters (Step d in Scheme 1 and Experimental section for details). The use of these reagents in the cleavage cocktail H has previously been restricted to the synthesis of methionine containing peptides without a *C*-terminal thioester functionality.[21] We employed this cleavage cocktail in various thioester syntheses containing mutations in the Im7 sequence using the TGT resin and obtained the reduced thioester in very good overall conversions with no Met-sulfoxide or hydrolysis byproducts. (Scheme 3 and Experimental section). These included glycopeptides like **5** and **6** which carry an N-linked chitobiose modification at position 27 or 5. It is important to mention that the use of cleavage cocktail H in glycopeptide thioester deprotections had another beneficial effect: The use of cleavage cocktail H for the deprotection of glycopeptide thioesters prepared from building block **4**[22] did lead to the removal of all carbohydrate TBDMS protecting groups. Comparative cleavages with cocktail K resulted in the isolation of a side product still containing one TBDMS group.[23]

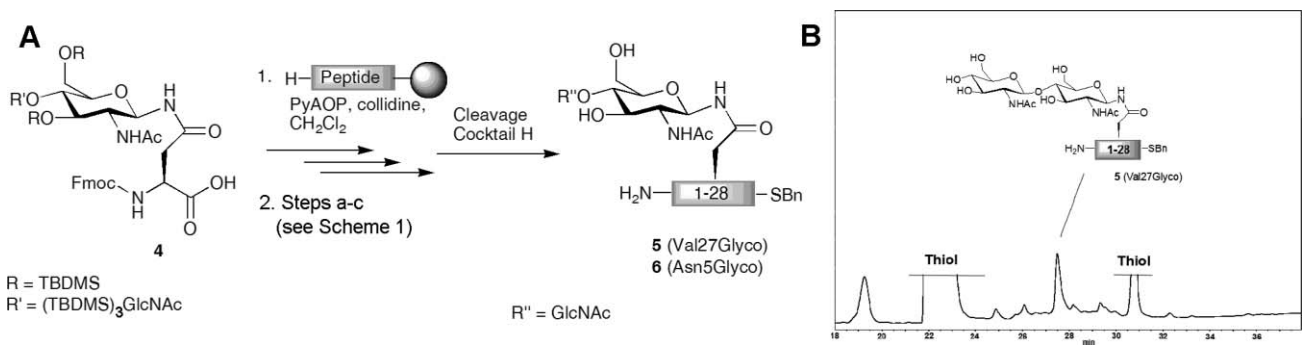
Conclusion

In summary, a protocol for a fast reduction of oxidized methionine thioesters with NH_4I has been developed. Undesired hydrolysis of the thioester can be avoided by adding an excess of Me_2S to the reaction mixture. These optimized reduction conditions led to the development of a modified cleavage cocktail H, which allows the reduction (or prevention) of oxidized methionine residues *after* the installation of the thioester functionality. These studies furthermore support the use of sulfoxides as protecting groups for methionine amino acids during peptide synthesis as their reduced analogues in highly functional and reactive thioester building blocks have become accessible by this strategy.

Experimental

General methods

All reagents, solvents, and amino acids, were purchased from commercial suppliers and used without further purification. All solvents were reagent grade and used as received. HPLC



Scheme 3 A) Use of a modified cleavage cocktail H for the deprotection of peptide thioesters after SPPS; B) HPLC spectra of glycopeptide thioester **5** (gradient: 7% to 95% CH_3CN with 0.1% TFA over 35 min) after cleavage with the modified cleavage cocktail H.

measurements were performed on a Waters HPLC system, specific conditions are given in the appropriate section. ESI-MS spectra were recorded on a PE Biosystems Mariner mass spectrometer. See Fig. 1C and 1F and the supplementary material for all recorded MS spectra.†

Peptide synthesis

Peptide thioesters were synthesized on an ABI 431A peptide synthesizer on a TGT-Resin (Novabiochem) to which the first amino acid (alanine) was already attached. Standard amide double coupling protocols (HBTU–HOBt in NMP) were used throughout the solid phase peptide synthesis.

Thioester synthesis

The thioester synthesis was pursued as described previously. Briefly, the desired peptide was cleaved from the TGT resin with 0.5% TFA in DCM for 2 h delivering the peptide selectively deprotected at the *C*-terminus. The resin was filtered off and washed with CH_2Cl_2. To the combined filtrates ice-cold hexanes (50 mL) were added which precipitated the protected peptides. After removal of the organic solvents *in vacuo*, the protected peptides were dissolved in THF (5 mL), and HBTU (4 equiv.), DIEA (8 equiv.) and benzylmercaptan (4 equiv.) were added for the conversion into the corresponding thioesters. THF was removed *in vacuo* and the peptide was globally deprotected with the following cleavage cocktails.

a) Modified cleavage cocktail K (without ethanedithiol (EDT)): 90% TFA; 5% thioanisole; 5% H_2O; with additional phenol (75 mg), and triisopropylsilane (TIS, 100 μL) added per mL;

b) Modified cleavage cocktail H: 82.5% TFA; 2.5% EDT; 5% thioanisole; 5% H_2O; 5% dimethyl sulfide (Me_2S); with additional phenol (75 mg), NH_4I (50 mg) and triisopropylsilane (TIS, 100 μL) added per mL.

Peptide thioesters were precipitated from ice-cold ether (40 mL), redissolved in H_2O–CH_3CN mixtures (1 : 1) and purified *via* preparative HPLC (C_{18}-column, flow: 15 ml min^{-1}, water–acetonitrile (with 0.1% TFA) gradient). Electrospray ionization mass spectrometry (ESI-MS) analysis was used to confirm the identities of the unglycosylated and glycosylated thioesters.

Synthesis of the reduced and oxidized thioesters 1 and 2

The peptide thioester, containing the 1–28 Im7 sequence (Scheme 1A) was globally deprotected with the modified cleavage cocktail K as described in the previous section. HPLC analysis (gradient of 7% to 95% CH_3CN over 30 min) revealed the presence of the reduced (~20%) and the oxidized (~50%) thioesters **1** and **2**, whereas **1** eluted at 24.1 min and **2** at 23.7 min (See Fig. 1A).

Thioesters **1** and **2** were purified with HPLC (gradient of 7% to 95% CH_3CN over 50 min), in which **1** eluted at 34.0 min and **2** at 33.5 min (Fig. 1B).

MS (ESI-MS) of 1. 1126.4 ($[M/3]^+$, observed), 1126.9 ($[M/3]^+$, calculated); 845.1 ($[M/4]^+$, observed), 845.4 ($[M/4]^+$, calculated) (see supplementary material†).

MS (ESI-MS) of 2. 1132.1 ($[M/3]^+$, observed), 1132.2 ($[M/3]^+$, calculated); 849.1 ($[M/4]^+$, observed), 849.4 ($[M/4]^+$, calculated) (Fig. 1C).

Reduction studies of thioester 2 with NH_4I and Me_2S

General procedure: the oxidized peptide thioester **2** was dissolved in an Eppendorf tube in TFA to obtain a 1 mM solution and cooled to 0 °C by inserting the tube in a 50 mL falcon tube with ice. NH_4I (20 equiv.) and/or Me_2S (20 equiv.) were added to the Eppendorf tube and the falcon tube was shaken for the time mentioned in the text (if necessary, the ice was refilled). The reaction was quenched with a saturated solution of ascorbic acid and the final mixture was directly analyzed by HPLC and ESI-MS.

Peptide thioester **2** was reduced according to the general procedure with different reducing agents.

During the reduction with NH_4I (20 equiv., 60 min at 0 °C) a brown iodine precipitate was observed which disappeared after quenching with ascorbic acid. HPLC and ESI-MS analysis revealed the hydrolysis of thioester **1**.

Reduction with NH_4I and Me_2S (20 equiv. each, 30 min at 0 °C) showed full reduction to **1**, which was confirmed by HPLC (Fig. 1E) and ESI-MS (Fig. 1F).

MS (ESI-MS) of 1. 1126.4 ($[M/3]^+$, observed), 1126.9 ($[M/3]^+$, calculated); 845.3 ($[M/4]^+$, observed), 845.4 ($[M/4]^+$, calculated) (Fig. 1F).

Treatment with Me_2S (20 equiv., 60 min at 0 °C) did not reduce **2**. Prolonged treatment with NH_4I and Me_2S (20 equiv. each, 6 hours at 0 °C) lead to the formation of a brown iodine precipitate.

Global deprotection of thioesters with the modified cleavage cocktail H

Peptide thioesters containing different mutations in the Im7 sequence were prepared as described in the thioester synthesis section and globally deprotected with the modified cleavage cocktail H, containing NH_4I–Me_2S. Glycosylated thioesters were synthesized similarly using building block **4** during the SPPS.[21] Thioesters were purified *via* HPLC at a gradient of 7–95% CH_3CN over 35 min and analyzed with ESI-MS. See supplementary material for all MS spectra.†

MS (ESI-MS) of 1. 1126.4 ($[M/3]^+$, observed), 1126.9 ($[M/3]^+$, calculated); 845.2 ($[M/4]^+$, observed), 845.4 ($[M/4]^+$, calculated).

MS (ESI-MS) of 1 with Val27Asn. 1132.0 ($[M/3]^+$, observed), 1131.9 ($[M/3]^+$, calculated); 849.2 ($[M/4]^+$, observed), 849.2 ($[M/4]^+$, calculated).

MS (ESI-MS) of glycopeptide thioester 5 (Val27Glyco). 1267.5 ($[M/3]^+$, observed), 1267.2 ($[M/3]^+$, calculated); 950.9 ($[M/4]^+$, observed), 950.7 ($[M/4]^+$, calculated).

MS (ESI-MS) of glycopeptide thioester 6 (Asn5Glyco). 1262.4 ($[M/3]^+$, observed), 1262.3 ($[M/3]^+$, calculated); 947.1 ($[M/4]^+$, observed), 946.9 ($[M/4]^+$, calculated).

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