Trifluoroethanethiol: An Additive for Efficient One-Pot Peptide Ligation-Desulfurization Chemistry

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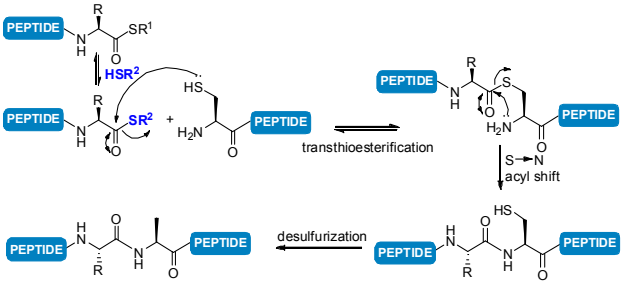
Supporting Information Placeholder

ABSTRACT: Native chemical ligation followed by desulfurization is a powerful strategy for the assembly of proteins. Herein, we describe the development of a high yielding, one-pot ligation-desulfurization protocol that employs trifluoroethanethiol (TFET) as a novel thiol additive. The synthetic utility of this TFET-enabled methodology is demonstrated by the efficient multi-step one-pot syntheses of two tick-derived proteins, chimadanin and madanin-1, without the need for any intermediary purification.

Chemical synthesis of proteins provides a means by which key structural and functional information of a given target can be elucidated.[1] Over the past two decades considerable advances in this area have been made possible owing to the development of the venerable native chemical ligation method.[2] This transformation involves the chemoselective reaction between a peptide containing a C-terminal thioester and a peptide bearing an N-terminal Cys residue to afford a native peptide bond in aqueous media at neutral pH (Scheme 1). Due to the ease of preparation and stability to long term storage, alkyl thioesters are often employed directly in ligation chemistry. However, this functionality is relatively inert, necessitating the inclusion of a thiol additive to generate a more reactive peptide thioester as the acyl donor in the ligation reaction. A trans-thioesterification, between the side chain of the Cys residue and the newly formed thioester moiety, then occurs followed by an irreversible intramolecular S→N acyl transfer to form a native peptide bond.



Scheme 1. Native chemical ligation-desulfurization.

Ligation technology has benefited greatly from the introduction of exogenous thiol additives to improve reaction rates *via* the *in situ* generation of reactive peptide thioesters. In a thorough study by Johnson and Kent, the relative reactivity of a range of commercially available thiols was investigated.[3] Aryl thiols with pKa values > 6 were shown to afford optimal ligation rates due to two key reactive properties: 1) the ability to rapidly exchange with alkyl thioesters to generate aryl thioesters and 2) excellent leaving group ability upon reaction with the N-terminal Cys residue. From this study the water soluble aryl thiol additive mercaptophenylacetic acid (MPAA, pKa = 6.6) was selected as an excellent additive that facilitated more rapid ligations compared with the two traditionally employed thiol additives, the water soluble alkyl thiol mercaptoethane thiolate sodium salt (MESNa, pKa 9.2) and the sparingly water soluble thiophenol (pKa = 6.6).

A significant advancement in ligation methodology has been the development of desulfurization chemistry which transforms Cys residues to Ala following the ligation event.[4] This methodology has sparked interest in the use of the native chemical ligation concept at a variety of unnatural mercapto- and seleno-amino acids that can subsequently be converted to native amino acids by desulfurization or deselenization.[5] Whilst desulfurization of Cys to Ala can be effected through the use of catalytic hydrogenation,[4a] radical desulfurization[4b] is the most widely employed method and has been used in the synthesis of a number of complex protein targets.[6] Given the high yielding nature of desulfurization chemistry, the union of this transformation with efficient ligation chemistry into a one-pot procedure would represent a powerful addition to the toolbox of methods available for use in chemical protein synthesis. Unfortunately the necessity of aryl thiol additives in the ligation reaction prohibits this capability due to the inherent radical quenching activity (even in trace amounts) of aryl thiols.[7] As such, products produced from ligation reactions require tedious purification and lyophilization before the submission of purified materials to desulfurization conditions. Solutions to this problem have therefore been sought, including the use of the alkyl thiol MESNa as the thiol additive which, despite the significantly slower ligation rates, does not interfere with the desulfurization chemistry.[6g] Alternatively, methods to remove aryl thiols from the reaction mixture have been employed, including extensive liquid-liquid extraction of aryl thiols such as thiophenol, or solid-phase extraction procedures.[5q] Recently, Brik and co-workers employed a synthetic bi-functional aryl thiol catalyst which could be captured with an aldehyde-derived solid-supported reagent prior to the desulfurization reactions.[8]

In an effort to streamline the two highly efficient reactions into a straightforward and operationally simple one-pot protocol, we sought to identify a novel thiol additive capable of facilitating rapid rates of ligation without disrupting the subsequent radical desulfurization. In this study we show that 2,2,2-trifluoroethanethiol (TFET) is an efficient thiol catalyst and, importantly, being an alkyl thiol permits *in situ* one-pot desulfurization reactions. To demonstrate the utility of TFET, we undertook the synthesis of two small tick-derived proteins, chimadanin and madanin-1, *via* the one-pot ligation-desulfurization of three peptide fragments either in the C- to N-terminal direction or through

kinetically-controlled ligation chemistry[9] in the N- to C-direction, respectively.

TFET has a pKa of 7.30 and has been shown to display a similar propensity to participate in thiol-thioester exchange compared with thiophenol.[10] Owing to the comparatively low pKa of TFET compared with other alkyl thiols, we envisaged that it would afford exchanged thioesters with similar acyl-donor capabilities to activated aryl thioesters. Furthermore, the fact that TFET is relatively volatile (b.p. 35-37 °C) permits facile removal following completion of the ligation if necessary (unlike MPAA which can co-elute with products during HPLC purification).

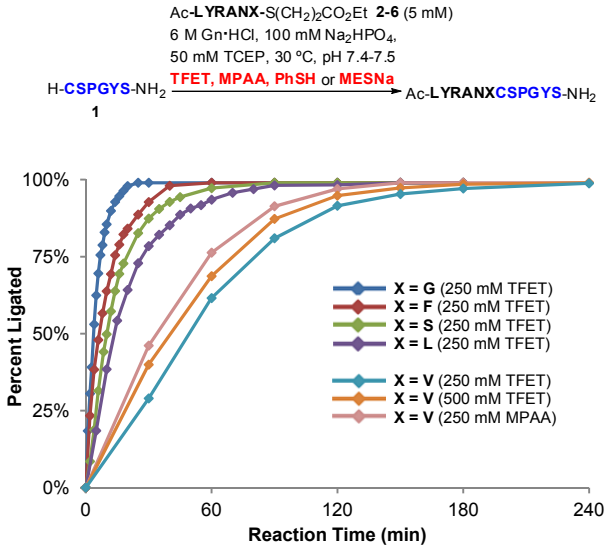
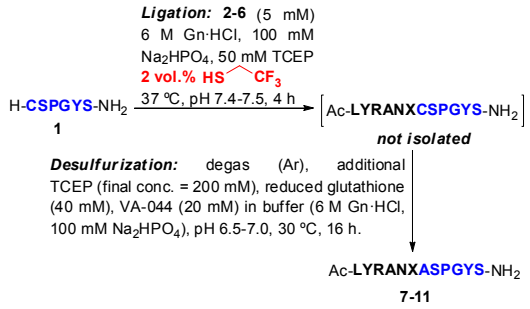


Figure 1. Kinetics for native chemical ligation reactions between peptide **1** and peptide thioesters Ac-**LYRANX**-S$(CH_2)_2CO_2$Et **2-6** (**2: X** = V, **3: X** = G, **4: X** = F, **5: X** = S, **6: X** = L). The percent ligated was calculated at each time point by integrating the areas under the peaks of analytical HPLC chromatograms at λ = 280 nm.

We began investigating the use of TFET as an additive in native chemical ligation by comparing with the commonly employed aryl thiol additives thiophenol and MPAA and the alkyl thiol additive MESNa, the most effective alkyl thiol catalyst currently known. To this end, we studied a challenging model ligation between peptide **1** and peptide thioester **2**, bearing a sterically hindered C-terminal Val residue, one of slowest sites for native chemical ligation.[11] Reactions were carried out in parallel in ligation buffer comprising 6 M Gn·HCl, 100 mM Na_2HPO_4 buffer at pH 7.4-7.5 in the presence of 250 mM TFET, PhSH, MPAA or MESNa as thiol additives. Gratifyingly, ligation reactions carried out in the presence of TFET were significantly faster than those employing PhSH and MESNa (see Supporting Information for data). The reaction reached completion within 4 h, comparable to the same transformation employing MPAA, the current gold standard additive (Figure 1). Unlike PhSH, TFET is freely soluble in aqueous solution up to a concentration of 250 mM and is supersaturated at 500 mM. The use of a saturating concentration of TFET (500 mM) led to only a slight enhancement in reaction rate (Figure 1) and, as such, a 250 mM (2 vol.%) concentration was used in all subsequent experiments.

Having demonstrated that TFET was an efficient thiol additive for ligation at a valyl-thioester, we next investigated the rate of reaction between **1** and peptide thioesters **3-6** bearing a range of C-terminal residues, representative of practical ligation junctions (Figure 1). Gratifyingly, reaction rates between **1** and **3-6** all proved to be rapid (Figure 1). Specifically, reaction of **1** with peptide thioester **3** containing a C-terminal Gly reached completion in 20 min, while reaction with thioester **6** bearing a sterically encumbered Leu residue reached completion in just 90 min.

Table 1. One-pot ligation-desulfurization reactions between peptide **1** and thioesters **2**–**6** using TFET as a thiol additive.



Entry	Thioester	Product	Yield (%)[a]
1	Ac-**LYRANV**-SR **2**	**7**	78
2	Ac-**LYRANG**-SR **3**	**8**	80
3	Ac-**LYRANF**-SR **4**	**9**	82
4	Ac-**LYRANS**-SR **5**	**10**	87
5	Ac-**LYRANL**-SR **6**	**11**	81

[a]Isolated yields over two steps following HPLC purification; R = $(CH_2)_2CO_2$Et.

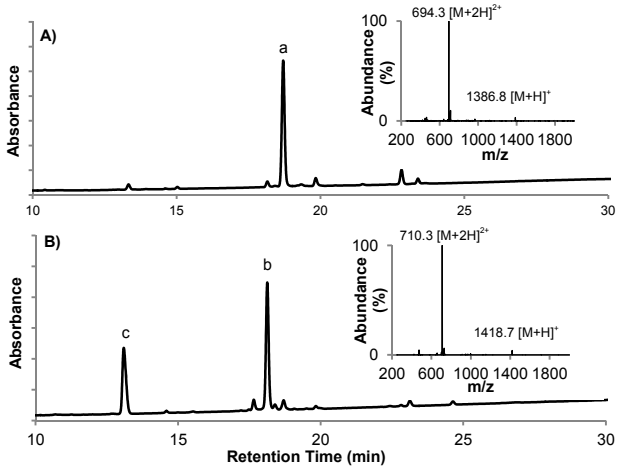
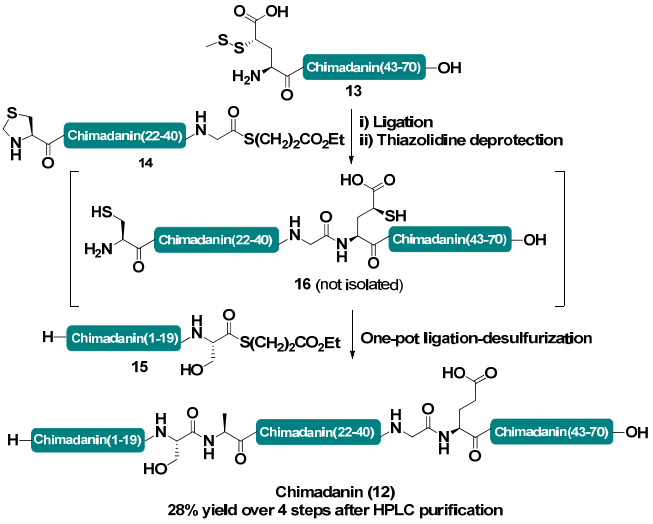


Figure 2. Crude analytical HPLC-MS for the one-pot ligation-desulfurization reaction between **1** and **4** (λ = 230 nm); **A**) Ligation reaction after 2 h: **a** = Ac-LYRANFCSPGYS-NH_2; **B**) Desulfurization after 16 h: **b** = **9**, **c** = S=P$(CH_2)_2CO_2$H.

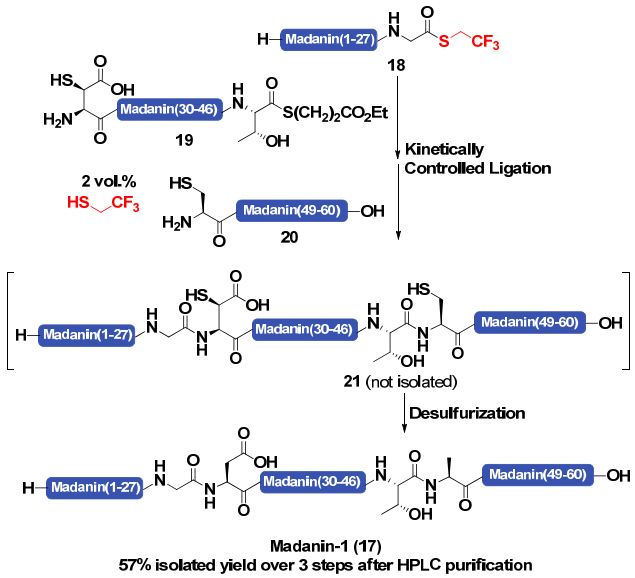
With the knowledge that TFET could promote extremely rapid and high yielding peptide ligation reactions, we next moved to investigate its ultimate utility in the context of one-pot ligation-desulfurization chemistry (Table 1). Ligation reactions were performed between peptide **1** and peptide thioesters **2-6** in the presence of 2 vol.% (250 mM) TFET and 50 mM TCEP (Table 1) and were left to proceed for 4 h, the time at which the slowest ligation (at Val thioester **2**) was complete (Figure 1). At this stage, the ligation product was not isolated, but rather the reaction mixture was thoroughly degassed by sparging with argon in preparation for the *in situ* radical desulfurization (see Supporting Information for full experimental details). This also led to the removal of the vast majority of the dissolved TFET owing to its volatility. At this point additional TCEP was added to the degassed solution to generate a final concentration of 200 mM, together with the radical initiator VA-044 (20 mM) and reduced glutathione[5b] (40 mM) as a hydrogen atom source. Reactions were incubated at 37 °C for

18 h to ensure complete desulfurization. Importantly, all one-pot ligation-desulfurization reactions proceeded smoothly under these conditions (see Figure 2 for crude analytical data for the ligation between peptide **1** and peptide thioester **4** and the Supporting Information for other raw data). Following HPLC purification, the native peptide products were isolated in excellent yields (78%-87%) over the two steps, highlighting the efficiency of the one-pot procedure (Table 1). It is important to note that whilst TFET was removed from the reaction mixture prior to desulfurization during the degassing step, we have also shown that TFET does not have a detrimental effect on the desulfurization reaction rate when present in solution (see Supporting Information for details).



Scheme 2. One-pot synthesis of Chimadanin (12). Conditions: *i) Ligation:* **14** (1.0 equiv.) and **13** (1.2 equiv.) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 25 mM TCEP), pH 6.8, conc. 2.5 mM with respect to **14**, 2 vol.% TFET, 30 °C, 2 h; ii) *Thiazolidine deprotection:* 120 µL of 0.2 M methoxyamine, final conc. 1.5 mM, 30 °C, 3 h; *One-pot ligation-desulfurization: Ligation:* pH readjusted to 6.8, addition of **15** (1.3 equiv. 3.0 mM) in buffer (6 M Gn. HCl, 100 mM Na_2HPO_4, 25 mM TCEP), pH 6.8, TFET (2 vol.%), conc. 1.0 mM with respect to **16**, 30 °C, 18 h. *Desulfurization:* readjust to 200 mM TCEP and 40 mM reduced glutathione in ligation buffer (500 µL), argon sparge, pH adjustment to 6.2, solid VA-044 (20 mM final conc.), 37 °C, 5 h.

Having demonstrated the utility of TFET as an additive for efficient and operationally simple one-pot ligation-desulfurization reactions, we were next interested in extending the scope of the methodology to the practical synthesis of some small protein targets. Our first target was the 70 amino acid thrombin inhibitory protein chimadanin (**12**) produced by the hard tick *Haemaphysalis longicornis* to facilitate the hematophagous activity of the organism.[12] We envisaged the synthesis of the protein *via* the assembly of three fragments in the C- to N-direction. Specifically, we proposed using a γ-thiol Glu ligation[5q] followed by a native chemical ligation-desulfurization at Cys that would proceed with concomitant desulfurization of the γ-thiol auxiliary on the Glu residue to generate the native protein. Importantly, this proposed one-pot strategy would abolish intermediary purification steps, thus limiting the exposure of the sensitive γ-thiol moiety to acidic HPLC buffers that can induce thiolactamization and peptide cleavage.[5q, 13] The synthesis began with preparation of the requisite fragments *via* Fmoc-strategy SPPS, including chimadanin (43-70) (**13**) possessing an N-terminal γ-thiol Glu[5q] residue, chimadanin (22-40) (**14**) bearing an N-terminal thiazolidine and a C-terminal thioester functionality, and chimadanin (1-19) thioester **15** (see Supporting Information). Peptide **13** (1.2 equiv.) bearing an N-terminal γ-thiol Glu residue was first ligated with peptide thioester **14** (1.0 equiv.) in the presence TFET. Following completion of the ligation reaction (as judged by HPLC-MS analysis) the reaction mixture was subsequently treated with methoxyamine at a pH of 4.2 to unmask an N-terminal Cys residue and afford intermediate **16**. Rather than purifying the intermediate, the pH of the reaction mixture was readjusted to 6.8 before the addition of the N-terminal chimadanin fragment, peptide thioester **15**. The ligation of **15** and **16** was again monitored by HPLC-MS and, upon completion, the reaction was degassed before treating with additional TCEP, reduced glutathione and VA-044 to effect global desulfurization affording the native protein. Gratifyingly, chimadanin was isolated in 28% yield over the one-pot four step sequence following a single HPLC purification step (> 72% average yield per step).



Scheme 3. Synthesis of Madanin-1 (17) *via* a one-pot kinetically-controlled ligation-desulfurization with TFET. Conditions: *Kinetically-controlled ligation:* **18** (1.2 equiv.), **19** (1.0 equiv., 5 mM) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP), pH 7.4–7.5, 37 °C, 1 h, then addition of **20** (1.8 equiv.), TFET (2 vol.%), 37 °C, 12 h; *Desulfurization:* Argon sparge, adjust to TCEP (200 mM), reduced glutathione (40 mM), VA-044 (20 mM) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4), 2.5 mM final conc. with respect to **21**, pH 6.5, 37 °C, 16 h.

To further probe the limits of the one-pot ligation-desulfurization reactions employing the TFET additive, we next investigated the potential of combining kinetically-controlled ligation[9] chemistry with our one-pot methodology to assemble the 60 amino acid protein madanin-1 (**17**, Scheme 3), a Cys-free thrombin inhibitor also produced by the hard tick *H. longicornis* that is also a substrate for thrombin cleavage.[14] It was envisaged that the use of a kinetically-controlled ligation sequence would enable the rapid assembly of multiple madanin-1 peptide segments in the N- to C-direction without intermediate purification steps through appropriate reactivity tuning of the requisite peptide thioesters.[9] With a view to future analogue generation, we were interested in assembling the protein *via* three short segments, namely madanin-1 (1-27) **18** as a preformed TFET-thioester, madanin-1 (30-46) **19** bearing an N-terminal β-thiol Asp residue and an unreactive C-terminal alkyl thioester and madanin-1 (49-60) **20** possessing an N-terminal Cys residue (Scheme 3 and Supporting Information). Peptide thioester **18** activated as the preformed TFET-thioester was first ligated with peptide alkyl thioester **19** bearing an N-terminal β-SH Asp[5r] moiety and a C-terminal Thr residue. Following completion of the ligation after 1 h (as judged by HPLC-MS analysis) peptide **20** was added in combination with 2 vol.% TFET to activate the alkyl thioester and facili-

tate a second ligation reaction. Following completion of the second ligation (12 h) the product **21** was not isolated but rather subjected to *in situ* desulfurization of both the Cys and β-thiol Asp residues to afford the native protein madanin-1 (**17**) in an excellent 57% yield over the 3 steps. To our knowledge, this represents the first report of a one-pot kinetically controlled ligation-desulfurization reaction and clearly underpins the utility of TFET in the context of chemical protein synthesis.

In summary, we have demonstrated that the alkyl thiol TFET can be successfully employed as an additive in native chemical ligation to facilitate ligations with rates comparable to the gold standard additive MPAA. More importantly, TFET can be used in ligation-desulfurization chemistry without the need for intermediate purification or capture from the reaction mixture. We have highlighted the utility of TFET as an additive for one-pot ligation-desulfurization reactions both on model peptide systems and in the assembly of multiple peptide fragments to access proteins. Specifically, we have used the additive for the efficient assembly of the tick-derived anti-thrombotic proteins chimadanin and madanin-1 through C- to N- assembly and kinetically controlled approaches, respectively. Given the efficiency and simplicity of ligations employing TFET (a commercially available and affordable reagent) it is anticipated that it will find widespread use in the ligation-based chemical synthesis of proteins and post-translationally modified proteins, greatly improving the efficiency of the processes and reducing handling and purification of intermediates. TFET should also serve as a useful replacement for MESNa in the generation of more reactive protein thioesters *via* intein technology.[15] These applications are currently under study in our laboratory and will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

Experimental protocols, crude analytical data of ligation-desulfurization reactions and characterization of peptides and proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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