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Synthesis of Thiazolidine Thioester Peptides and Acceleration of Native Chemical Ligation

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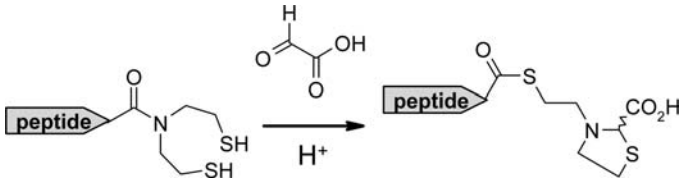
ABSTRACT



Thiazolidine thioester peptides were synthesized by reacting bis(2-sulfanylethyl)amido peptides with glyoxylic acid at pH 1. A significant increase in Native Chemical Ligation (NCL) rate was observed with thiazolidine thioesters compared to 3-mercaptopropionic acid-thioester analogues. The method is of particular interest for accelerating valine-cysteine peptide bond formation.

Ligation chemistries are powerful synthetic tools for the convergent and controlled assembly of complex molecular scaffolds. In particular, native peptide ligation methods allow the formation of native peptide bonds between unprotected peptides and thus the total or hemi synthesis of proteins.[1a−d] Native chemical ligation (NCL)[1a,2] and related extended methodologies[3a−c] are certainly the most frequently used techniques for protein total synthesis. NCL is based on the reaction of peptide thioesters with cysteinyl peptides. The reaction proceeds through a series of thiol-thioester exchanges first with an exogenous aromatic thiol used in excess and then with the side-chain thiols of cysteine residues. N-terminal cysteine leads to a key thioester-linked intermediate that rearranges spontaneously by acyl migration from sulfur to nitrogen, resulting in the formation of a native peptide bond.

One crucial aspect in ligation chemistry is the rate of bond formation in water. Fast reactions are needed for avoiding long reaction times, which can be harmful to sensitive biomolecules, and for compensating low reactant concentrations. The discovery of chemical systems allowing the acceleration of ligation chemistries is thus of utmost importance.[4a−f] In particular, NCL was shown to be accelerated by exogenous aromatic thiols which convert the starting thioester, usually an alkylthioester, into a more reactive aryl thioester.[4e,f] The other roles played by the external thiol are to maintain cysteine thiols in a reduced form and to permit the reversal of nonproductive thioester intermediate formation with internal cysteine residues. Acceleration of NCL by conformationally assisted positioning of reactive ends has also been described.[5]

In parallel, tremendous efforts have been focused on the synthesis of peptide thioesters using Boc[6] and later on Fmoc-solid phase peptide synthesis (SPPS), which is now

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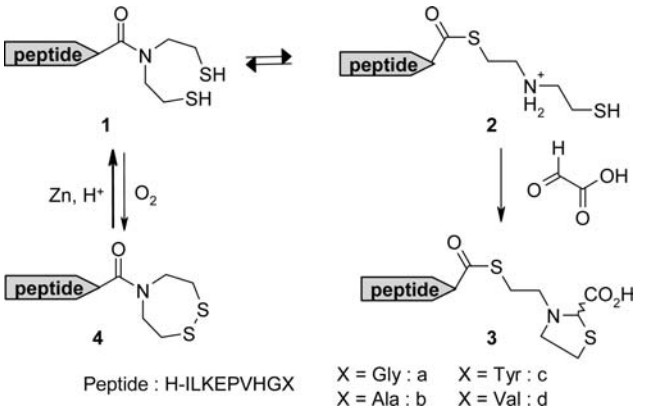
the most popular technique for peptide synthesis. To circumvent the instability of the thioester group in the presence of bases, additives such as hydroxybenzotriazole (HOBt) were used in combination with piperidine or other bases to remove the Fmoc group on thioester-linked peptidyl resin.[7a,b] Alternately, various methods introduce the thioester functionality after the peptide elongation step. This can be done either on the solid phase,[8a−e] during the cleavage from the solid support,[9] in solution or in situ during NCL.[9c,10] In particular, *N*,*S*-acyl shift-based methods have gained increasing importance recently for peptide thioester synthesis,[8c,9d,10b,10e,10j,10k] or for designing novel native ligation methods relying on the in situ generation of peptide thioesters.[10a,d,g−i,l,11]

Previous works have often targeted the synthesis of peptide thioesters derived from 3-mercaptopropionic acid or other simple alkylthiols.[2,6,11] A significant advance in the field would be to create a novel peptide thioester scaffold with enhanced reactivity in NCL compared to 3-mercaptopropionic acid-thioesters, to facilitate ligation at encumbered residues. The use of Fmoc-SPPS to access these peptide thioesters would be a plus.

We describe hereinafter a potential solution to these highly challenging goals. First we describe a simple method for the generation of thioesters **3** featuring a thiazolidine moiety on the thiol handle (Scheme 1). Bis(2-sulfanylethyl)-amido (SEA) peptides **1** at the basis of the method described here are easily synthesized by using Fmoc-SPPS.[10a,l] Second, we report that a significant increase in Native Chemical Ligation (NCL) rate was observed with thiazolidine thioesters compared to 3-mercaptopropionic acid-thioester analogues.

Scheme 1. Conversion of Bis(2-sulfanylethyl)amido Peptides **1a−d** into Thiazolidine Thioesters **3a−d** in the Presence of Glyoxylic Acid



Peptides **1** used in this study (Scheme 1) were synthesized on the solid phase by using a supported bis(2-sulfanylethyl)-amino reagent as described elsewhere.[10a] In brief, this solid support, which features a bis(2-sulfanylethyl)amino moiety linked to a trityl polystyrene resin through both sulfur atoms, is fully compatible with standard automated Fmoc-SPPS. Deprotection and cleavage of the peptide from the resin furnished peptide amides **1**. To avoid any interference of peptide amide **1**−peptide thioester **2** equilibrium during purification, **1** was converted into the cyclic disulfide **4** by air oxidation in basic medium.

The method described in Scheme 1 is based on the interconversion between amide form **1** and thioester form **2**. Formation of thioester **2**, which is detected only below pH 4 by RP-HPLC, is driven by the protonation of the amino group within the thiol handle. Thioester **2** is the major specie below pH ∼3 for Ala, Tyr, and Val analogues, and below pH ∼2 for Gly derivative.[10a]

To displace efficiently the equilibrium between **1** and **2** toward a stable thioester form, we envisaged blocking the β-aminothiol moiety within **2** by forming a thiazolidine ring in the presence of an aldehyde. Indeed, thiazolidine ligation between cysteinyl peptides and aldehyde derivatives is a robust ligation method that proceeds efficiently below pH 4,[12] i.e., at a pH where the fraction of thioester form **2** is significant. This chemistry has met a lot of success with glyoxylic acid derivatives as the aldehyde partners, due to high reactivity and compatibility with peptides and other biomolecules.[13]

Toward this goal, peptides **4a−d** were reduced back to peptide amides **1** with zinc at pH 1 and simply filtered to remove excess zinc.[14] At pH 1, thioester form **2** is the major species.[10a] Thus, thiazolidine formation was carried out at

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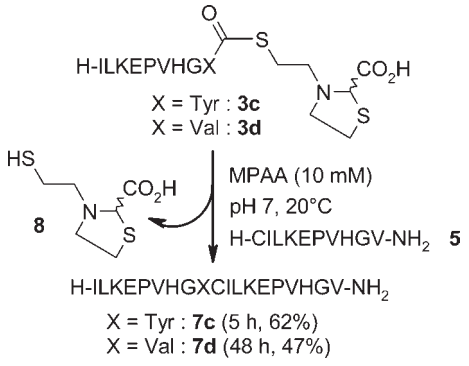
Table 1. Synthesis of Thiazolidine Peptides **3a**−**d**

X	peptide	reaction time (h)	yield[a] (%)	% D-enantiomer[b]
Gly	**3a**	16	52	
Ala	**3b**	16	62	0.14
Tyr	**3c**	24	56	0.32
Val	**3d**	72	45	<0.1

[a] Isolated yields. [b] Determined by chiral GC-MS after acid hydrolysis.[15]

this pH by just adding an excess of glyoxylic acid to the resulting solution. In these conditions, reoxidation of amide form **1** into cyclic form **4** by molecular oxygen was not observed probably due to the low aqueous solution pH and the use of an inert atmosphere. Gratifyingly, the capping of β-aminothiol moiety within peptide thioester **2** by glyoxylic acid appeared to be an efficient process. Thiazolidine thioester peptides **3a**−**d** were purified by using standard RP-HPLC techniques and isolated in good yield (Table 1). Moreover, chiral GC-MS analysis of peptides **3b**−**d** after acid hydrolysis showed that thiazolidine formation proceeded without significant racemization of C-terminal residue.[15]

Scheme 2. NCL between Peptide Thioesters **3c**,**d** and Cysteinyl Peptide **5**



We next examined NCL of thiazolidine peptide thioesters **3** with model cysteinyl peptide **5** (Scheme 2). We were particularly interested in comparing the ligation rates between thioesters **3** and peptide thioesters frequently used in NCL, i.e., 3-mercaptopropionic acid-derived thioesters **6** (Figure 1).[11] The comparison was done with thioester peptides presenting a Tyr (**3c**/**6c**) or Val (**3d**/**6d**) residue on the C-terminus. The reactions were performed by using typical experimental conditions for NCL, i.e., at pH 7 with each peptide thioester at 1 mM, in the presence of an excess of (4-carboxymethyl)thiophenol (MPAA) as the exogenous aromatic thiol and of TCEP to maintain all thiols in

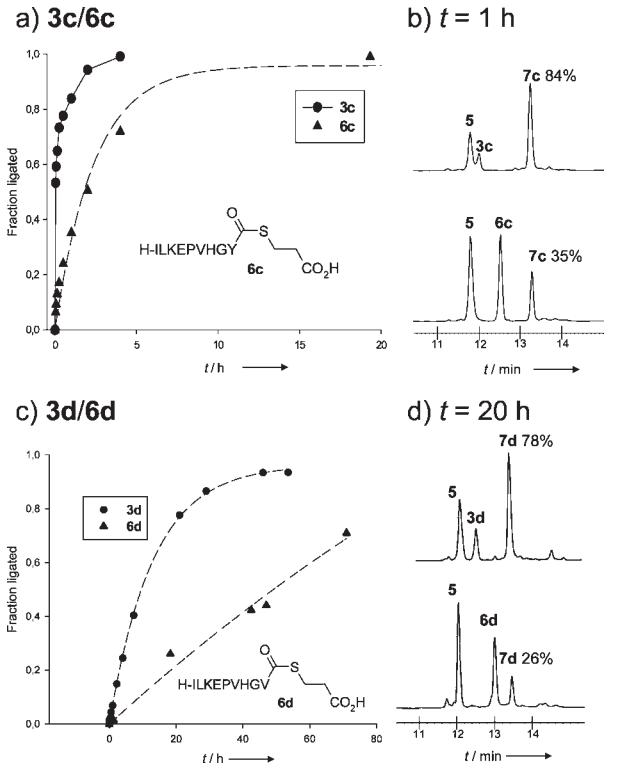


Figure 1. Time-course of the ligation reaction for peptide thioesters **3c**, **6c** (a) or **3d**, **6d** (c). The dotted line corresponds to a first-order rate fit. Peptide thioesters (1 mM) were reacted at room temperature with cysteinyl peptide **5** (1.5 mM) in the presence of MPAA (10 mM) and TCEP (80 mM) at pH 7.4. (b) **3c** or **6c** after 1 h. (d) **3d** or **6d** after 20 h.

reduced form.[4e] The reactions were followed by RP-HPLC (215 nm). The resulting data for peptide thioesters **3d**, **6c**, and **6d** could be fitted to the first-order rate equation (dotted curves in Figure 1a,c), by considering peptide thioesters **3**/**6** as limiting reactants. The data for thiazolidine peptide thioester **3c** could not be fitted to a simple first- or second-order rate equation.

For thioester **6c**, the half-time ($t_{1/2}$) of the reaction, that is, the time required to reach 50% conversion, was 120 min ($k_{obs} = 6.5 \pm 0.16 \times 10^{-5}$ s^{-1}). The $t_{1/2}$ for thiazolidine thioester **3c** was, however, dramatically reduced to 2 min, showing a ~60-fold increase in the reaction rate compared to thioester **6c**. The fraction ligated after 1 h for thiazolidine thioester peptide **3c** was 84%, which must be compared to 35% for thioester **6c** (Figure 1b). Chiral GC-MS analysis of peptide **7c** after acid hydrolysis showed, however, 7% of D-Tyr, and thus a partial racemization of C-terminal Tyr residue during the ligation step.

Similarly, the $t_{1/2}$ for thioester **6d** was 44 h ($k_{obs} = 4.4 \pm 0.5 \times 10^{-6}$ s^{-1}), whereas the $t_{1/2}$ for thiazolidine thioester **3d** was 10 h only ($k_{obs} = 1.9 \pm 0.01 \times 10^{-5}$ s^{-1}), showing a ~4.4-fold acceleration of the ligation reaction for Val C-terminal residue. The fraction ligated after 20 h for thiazolidine thioester peptide **3d** was as high as 78%, but only 26% for thioester **6d** (Figure 1d). Gratifyingly, chiral

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GC-MS analysis of peptide **7d** after acid hydrolysis showed 0.2% of D-Val only, and thus the absence of racemization of C-terminal Val residue during the ligation step. NCL with thiazolidine thioester **3d** furnished peptide **7d** with an isolated yield of 47% after 48 h (Scheme 2). For comparison, NCL of thioester **6d** with peptide **5** required up to seven days to go to completion (55% conversion after 72 h) and furnished **7d** with only 33% isolated yield. NCLs with peptide thioesters featuring a C-terminal valine residue require unusually long reaction times (> 48 h) and are often challenging. Indeed, the bulkiness of valine side chain induces a shielding of valine carbonyl group toward nucleophilic attack by exogenous thiol or cysteine peptide. The work presented here shows that the use of thiazolidine thioester peptides is a potential solution to sluggish NCLs involving sterically demanding amino acids.

It is widely accepted that the rate limiting step in Cys NCL is the thiol-thioester exchange between peptide alkylthioesters and the aromatic thiol such as MPAA.[4e] In accord with this hypothesis, MPAA-thioesters were never shown to accumulate during NCL involving **3** or **6**. Once formed, they are rapidly consumed in the reaction mixture. Thus, the acceleration of NCL with thiazolidine thioester peptide **3** relative to thioester **6** might be due to an acceleration of the MPAA-thioester forming step.

The reaction of a thiol anion RS^- with a thioester R″COSR′ proceeds through formation of a tetrahedral intermediate. When the attacking thiol RSH is less basic, i.e., a poorer nucleophile and a better leaving group than the thiol R′SH of the thiol ester, expulsion of the thioester thiolate $R'S^-$ is rate determining.[16] This is probably the case in this study since pK_a of thiol MPAA is 6.6,[4e] whereas pK_a of 3-mercaptopropionic acid thiol's group is 10.2[17] and pK_a of thiol **8** (Scheme 2) was estimated to be 9.4 using SPARC[18] algorithm. On this basis, thiol **8** is expected to be a slightly better leaving group than 3-mercaptopropionic acid. However, the question whether a ΔpK_a value of 0.8 between the two thiols can account for the 60-fold increase in ligation rate observed for Tyr derivatives **3c**/**6c** remains to be explored. Other factors may contribute to the acceleration of MPAA-thioester formation. Indeed, previous work from Dawson's group has shown that peptide thioesters featuring a histidine or cysteine residue on the C-terminus react faster in Cys NCL than alanine analogue.[2] Internal participation of histidine or aspartic acid residues in sugar-assisted ligation was also noted.[19] These residues were shown to accelerate the rate limiting *S*, *N*-acyl transfer step through probably an intramolecular general base catalysis mechanism. Internal participation of thiazolidine and/or carboxy groups present within thiol handle **8** might increase the rate of MPAA-thioester formation and thus of Cys NCL observed with thiazolidine thioester peptides **3c**/**d**. Studies aimed at deciphering the factors involved in this acceleration effect are underway.

In conclusion, we show that thiazolidine thioester peptides are easily prepared by combining Fmoc-SPPS of SEA peptides and a simple chemical step in solution with glyoxylic acid. The rate of NCL with thiazolidine thioester peptides is significantly higher than that with 3-mercaptopropionic acid-thioester analogues. The acceleration is significant even for a sterically demanding amino acid such as valine. In this case, no racemization of valine residue was observed. We believe that the ease of preparation of thiazolidine thioester peptides as well as their high reactivity will extend the frontier attainable to NCL, and in particular constitute a potential solution to "difficult" NCLs.

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Supporting Information Available. Experimental procedures and characterization data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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