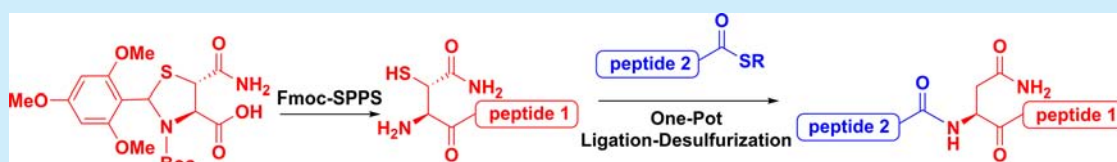# Thiazolidine-Protected *β*-Thiol Asparagine: Applications in One-Pot Ligation−Desulfurization Chemistry

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S *Supporting Information*

ABSTRACT: The synthesis of a *β*-thiol asparagine derivative bearing a novel (2,4,6-trimethoxyphenyl)thiazolidine protecting group is described. The efficient incorporation of the amino acid into the N-termini of peptides is demonstrated as well as the utility of the *β*-thiol asparagine moiety for rapid ligation reactions with peptide thioesters. The streamlined synthesis of native peptide products could be accomplished using a one-pot radical desulfurization of the *β*-thiol auxiliary following the ligation event. The utility of the amino acid is highlighted in the efficient one-pot assembly of the HIV entry inhibitor enfuvirtide.

The convergent assembly of unprotected peptide fragments by native chemical ligation remains the most robust and versatile method for accessing proteins by chemical synthesis.[1] The methodology involves the chemoselective reaction of a peptide bearing a thioester functionality on the C-terminus with a peptide containing an N-terminal cysteine (Cys) residue through an initial trans-thioesterification followed by a rapid S → N acyl rearrangement to afford a native amide bond (Scheme 1).

Scheme 1. Native Chemical Ligation−Desulfurization and Ligation−Desulfurization at Thiolated Amino Acids
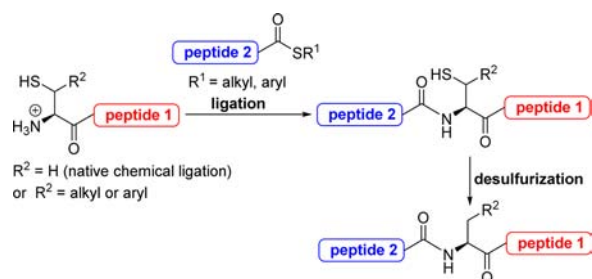
Over the past decade, researchers have focused on expanding the native chemical ligation methodology to enable reactions with amino acids other than Cys at the N-terminus of one of the peptide fragments, an area fueled by an initial report from Yan and Dawson[2] which outlined a platform for the post-ligation desulfurization of Cys to afford Ala (Scheme 1).[3] To date, 13 of the 20 proteinogenic amino acids have been prepared as suitably protected thiol-derived building blocks and have been shown to be competent in ligation chemistry via a native chemical ligation mechanism.[4] Following the ligation event, desulfurization of the thiol auxiliary, commonly through a radical process,[5] provides native peptides. The popularity of native chemical ligation and the associated ligation chemistry at thiol-derived amino acids arises from the ability to utilize unprotected fragments in aqueous media to facilitate rapid peptide bond formation in a chemoselective manner as well as the recent streamlining of ligation and desulfurization reactions via one-pot processes.[4u,6]

In order to further expand the toolbox of reagents available for the assembly of peptides and proteins by ligation−desulfurization chemistry, we were interested in accessing a suitably protected thiol-derived asparagine (Asn) residue. Our initial attempt at preparing such a building block was inspired by our prior syntheses of *β*-thiol aspartic acid[4d] and *γ*-thiol glutamic acid.[4f] Specifically, sulfenylation chemistry was employed as a key step to target the synthesis of **1** bearing Trt protection of the side-chain carboxamide of Asn and 2,4-dimethoxybenzyl (Dmb) protection of the *β*-thiol moiety (Figure 1; see the Supporting Information
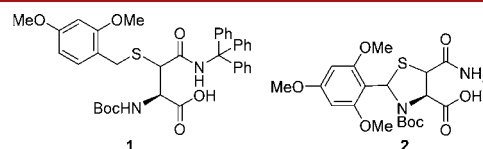

Figure 1. Target *β*-thiol Asn residues **1** and **2** for use in ligation−desulfurization chemistry.

for synthetic details). Unfortunately, while **1** was successfully synthesized, the practical use of the building block was plagued by two factors: (1) the steric bulk of the Dmb thioether in combination with the Trt amide protection led to inefficient coupling of the amino acid to solid-supported peptides and (2) removal of the side chain Trt from the N-terminal Asn of peptides was extremely sluggish,[7] requiring extended strong acid treatment

to effect deprotection of resin-cleaved peptides. It should be noted that while this work was in progress, Otaka and co-workers reported the successful use of an N-acetylglucosamine-derived β-thiol Asn residue bearing Tmb protection of the thiol moiety in ligation chemistry.[8] This suggests that groups less sterically demanding than a trityl may be tolerated for on-resin coupling when appended to the carboxamide side chain.

Herein, we report our efforts to solve the problems associated with building block **1** through the synthesis of a β-thiolated Asn building block (general structure **2**, Figure 1) bearing a novel protection scheme, namely a (2,4,6-trimethoxyphenyl)-thiazolidine (Tmp-thiazolidine) group. We also describe the efficient incorporation of this amino acid into peptides via Fmoc solid-phase peptide synthesis (SPPS) and the utility of the building block in one-pot peptide ligation–desulfurization chemistry to afford native Asn residues at the ligation junction. Synthesis of the target β-thiol Asn building block began from the commercially available and affordable amino acid Boc-Asp(OAll)-OH **3** (USD \$14/g) which was first treated with 2-phenylisopropyl (Ph*i*Pr) trichloroacetimidate[9] to yield the fully protected Asp derivative **4** (Scheme 2). Low-temperature double

Scheme 2. Synthesis of Tmp-thiazolidine-Protected β-Thiol Asn Building Blocks 2a and 2b
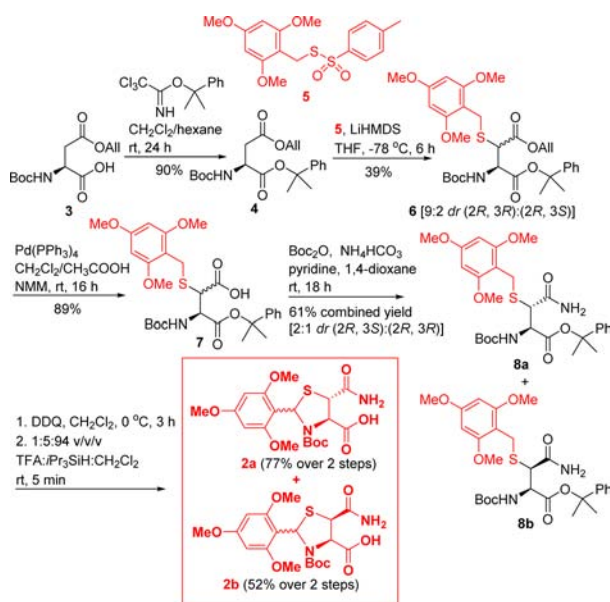
deprotonation of **4**, followed by treatment with sulfenylating reagent **5**,[4d] gave protected β-thiol Asp derivative **6** as an inseparable 9:2 mixture of the (2*R*,3*R*):(2*R*,3*S*) diastereoisomers as judged by NMR spectroscopy (coupling constant and NOESY analysis). Following Pd-catalyzed allyl ester deprotection of the diastereomeric mixture, acid **7** was generated in 89% yield and then treated with Boc anhydride, ammonium bicarbonate, and pyridine[10] to generate the corresponding diastereomeric carboxamides **8a** and **8b**. Conveniently (for the ultimate preparation of two diastereomers of **2**), these conditions led to epimerization of the β-center to afford a 2:1 ratio of the (2*R*,3*S*)-**8a**:(2*R*,3*R*)-**8b** diastereoisomers, which were easily separable by column chromatography and isolated in 61% yield (**8a** and **8b** combined).

At this stage we sought to test whether a building block possessing a Tmb thioether moiety but lacking side-chain carboxamide protection could be coupled to the N-terminus of

a resin-bound peptide, thus reducing the number of steps necessary to prepare a suitable β-thiol Asn residue. To this end, the Ph*i*Pr ester of diastereomer **8a** was selectively deprotected by treatment with a weakly acidic cocktail, and introduction of the resulting β-thiol Asn building block into a peptide was attempted (not shown). Unfortunately, the lack of protection on the side chain led to quantitative succinimide formation via side-chain carboxamide attack onto the carbonyl of the α-carboxylate when activated with a coupling reagent (see the Supporting Information).

As a means to hinder the participation of the carboxamide in deleterious side reactions during coupling, we sought to lock the conformational freedom of the side chain through cyclization with the α-nitrogen of the amino acid. While the use of oxidative cyclization chemistry is widespread as a means to functionalize the benzylic position of electron-rich benzylic oxo-ethers, there is, to our knowledge, no precedent for this chemistry at benzylic thioethers or Cys derivatives. We rationalized that the highly stabilizing trimethoxyphenyl moiety should, after treatment with DDQ in the absence of water, result in conversion to a benzylic carbocation. This reactive intermediate could then be trapped intramolecularly by the mildly nucleophilic α-nitrogen of the carbamate, affording a Tmp-thiazolidine ring. Gratifyingly, submission of both **8a** and **8b** to DDQ-mediated oxidative cyclization under anhydrous conditions smoothly provided the corresponding thiazolidines. As expected, two diastereomers were generated from the ring closure, epimeric at the benzylic center. However, these mixtures were inconsequential for the ultimate use of β-thiolated Asn in ligation chemistry as the Tmp-thiazolidine was designed to be removed under the acidic conditions employed for resin cleavage and side-chain deprotection via the Fmoc-SPPS protocol. The final step in the synthetic sequence involved selective deprotection of the Ph*i*Pr ester to afford target β-thiol Asn residues **2a** and **2b** in 77% and 52% yield, respectively, over the two steps. Overall, the amino acids were synthesized in six steps from **3**.

With the target Tmp-thiazolidine-protected β-thiol Asn amino acids **2a** and **2b** in hand, we next attempted to incorporate these residues into resin-bound peptides. To this end, model resin-bound pentapeptide **9** was first synthesized via standard Fmoc-SPPS (Scheme 3 and Supporting Information). Optimized

Scheme 3. Synthesis of Model Peptides Bearing β-Thiol Asn
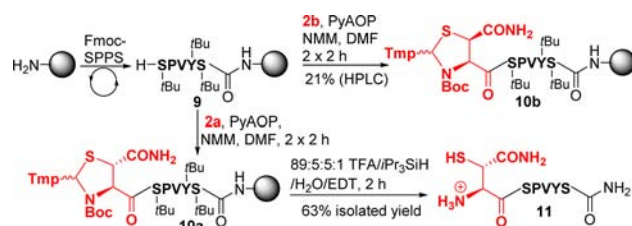
conditions for the coupling of **2a** and **2b** involved a 2 h double coupling of 1.2 equiv of the building blocks with the phosphonium coupling reagent PyAOP and NMM as the base. Under these conditions, the coupling of **2a** (with 2*R*, 3*S* stereochemistry) proceeded smoothly to afford resin-bound **10a**. Treatment with an acidic cocktail comprising 89:5:5:1 v/v/v/v TFA/triisopropylsilane/water/ethanedithiol (EDT) followed by purification by preparative HPLC then provided **11** in 63% yield based on the original resin loading. In contrast to **2a**, coupling of the β-thiol Asn building block **2b** (with 2*R*,3*R* stereochemistry) to **9** led to poor yields of the desired resin-bound peptide **10b** (21%, together with

79% unreacted **9** based on HPLC analysis). The poor coupling was hypothesized to result from rapid intramolecular succinimide formation upon activation of **2b** or the formation of another dehydration product, e.g., the corresponding nitrile.[4g]

Given the inefficient coupling of **2b**, we decided to proceed with the exploration of ligation chemistry with only diastereomer **2a**. To this end, peptide **11** was submitted to ligation reactions with peptide thioesters **12−16**, bearing a variety of C-terminal residues, at a final concentration of 5 mM. Reactions were performed in ligation buffer (6 M Gn·HCl, 100 mM Na$_2$HPO$_4$, 50 mM TCEP) at 30 °C and at a final pH of 7.0−7.4 (Scheme 4A). Trifluoroethanethiol (TFET) was used at saturating concentrations as a thiol additive (5 vol %) with a view to accelerating ligation reactions while enabling the subsequent desulfurization step to be carried out in one pot as we and others have described previously.[4u,6a,b] Reactions were initially carried out on an analytical scale with aliquots removed from the reaction at regular time intervals and quenched with 0.1% TFA in water before being submitted to HPLC−MS to gauge the relative rates of the reactions. It should be noted that quenching samples with this acidic solution was found to lead to gradual elimination of the β-thiol Asn residue. We hypothesize that this cleavage of the β-thiol Asn residue from the peptide is likely a result of acid-catalyzed succinimide formation.[11] Aliquots of ligation reactions were therefore diluted 20-fold with H$_2$O, flash-frozen, and thawed immediately before analysis by HPLC (see the Supporting Information). Pleasingly, all reactions proceeded cleanly and to completion to afford the desired ligation products (see Scheme 4C,D for crude HPLC traces of the ligation reaction between peptide **11** and peptide thioester **12**). More specifically, reactions at thioesters containing C-terminal alanine (Ala, **12**), glycine (Gly, **13**), methionine (Met, **14**), and phenylalanine (Phe, **15**) all proceeded to completion in 10−20 min, whereas the reaction with the more sterically encumbered valine thioester **16** required 4 h to reach completion, comparable to native chemical ligation at Cys using TFET,[6a] as well as ligation between **11** and **16** using the same concentration of MPAA in place of TFET (see Scheme 4B and the Supporting Information).

Having studied the rate of ligation reactions at a range of peptide thioesters, we next performed one-pot ligation−desulfurization on a preparative scale. The ligations were carried out under the same conditions described for the analytical studies above (6 M Gn·HCl, 100 mM Na$_2$HPO$_4$, 50 mM TCEP, 5 vol % TFET, 30 °C, pH of 7.0−7.4). Following completion of the ligation reactions, the crude products were not purified but rather sparged with argon and diluted with degassed buffer (6 M Gn·HCl, 100 mM Na$_2$HPO$_4$, 500 mM TCEP, pH adjusted to 7.0) before the addition of VA-044 and reduced glutathione[4j] to effect smooth in situ desulfurization of the β-thiol auxiliary in all cases between 6 and 16 h at 37 °C (see Scheme 4E for a crude HPLC trace of a desulfurization reaction). Gratifyingly, following purification of the ligation−desulfurization reactions by re-versed-phase HPLC, native peptide products **17−21** were isolated in excellent yield over the two steps (64−76%, Scheme 4A).

We next aimed to utilize building block **2a** for the ligation-based assembly of the medicinally relevant polypeptide drug enfuvirtide **22**, a combination drug for the treatment of HIV which inhibits viral fusion. The 36-residue peptide is produced commercially through two condensation reactions of three protected peptide fragments,[12] and as such, assembly through a single ligation event would serve as a more streamlined route to the therapeutic. Because of the centrally located Asn residue at position 19 within

Scheme 4. (A) One-Pot Ligation−Desulfurization between Peptide 11 and Peptide Thioesters 12−16. (B) Rates of Ligation Reactions between 11 and Peptide Thioesters 12-16. (C) Crude HPLC of Ligation Reaction between 11 and 12 at *t* = 0. (D) *t* = 10 min. (E) Crude HPLC−MS of One-Pot Ligation−Desulfurization Reaction between 11 and 12
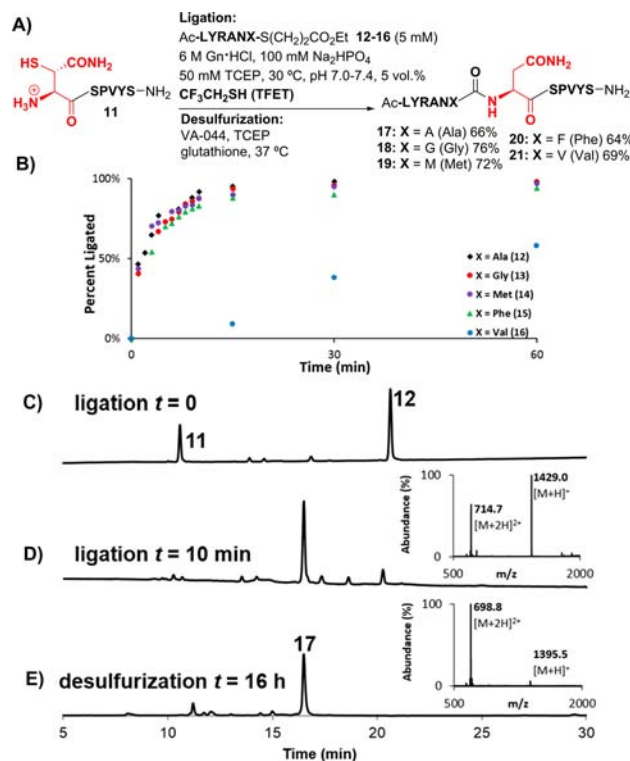
the drug, it was envisaged that the peptide could be accessed through a one-pot ligation−desulfurization reaction between enfuvirtide(19−36) **23** displaying an N-terminal β-thiol Asn residue and enfuvirtide(1−18) **24** bearing a C-terminal lysine (Lys) thioester (Scheme 5A). Both target fragments were synthesized using Fmoc-SPPS (see the Supporting Information for synthetic details) before being submitted to the TFET-mediated ligation conditions employed above, ensuring that the reaction was maintained at pH = 7.0 and 30 °C to prevent intramolecular lactamization of the ε-amine of Lys-18 onto the reactive thioester. The reaction was monitored by HPLC−MS, which indicated that the reaction had reached completion to afford **25** after 3 h without any lactam byproduct (see Scheme 5B). At this stage, the ligation product was not purified but subjected directly to radical desulfurization with VA-044, TCEP, and glutathione,[4j] which reached completion after 24 h (see Scheme 5B). Following purification by HPLC, enfuvirtide **22** was isolated in 61% yield.

In summary, we have developed a six-step synthesis of two β-thiol Asn diastereomers bearing a novel Tmp-thiazolidine protecting group. We have shown that the (2*R*,3*S*) diastereomer can be efficiently incorporated into resin-bound peptides and can facilitate rapid ligation reactions with peptide thioesters, including at sterically hindered junctions. Moreover, we show that the ligation reaction can be employed in concert with one-pot radical desulfurization through the use of TFET as a thiol additive. Finally, we demonstrate that the one-pot ligation−desulfurization methodology at β-thiol Asn is amenable to the preparation of larger polypeptides, highlighted in the high yielding preparation

Scheme 5. (A) Synthesis of Enfuvirtide (22) via a One-Pot Ligation−Desulfurization. (B) Crude HPLC Traces of the Ligation Reaction at *t* = 0 and 3 h and the *in situ* Desulfurization at *t* = 24 h. (C) ESI Mass Spectrum of Synthetic Enfuvirtide (22)
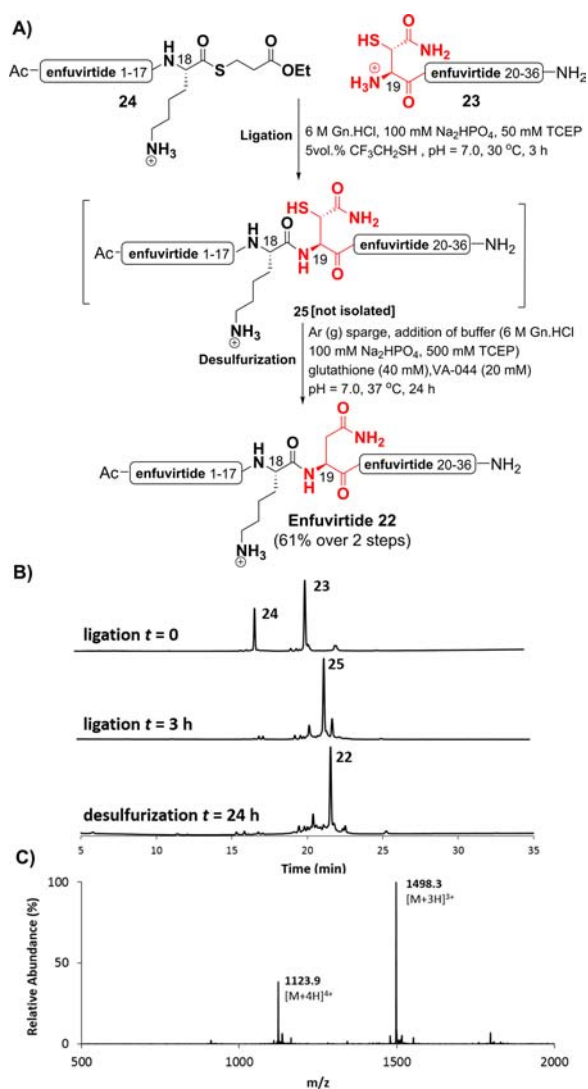
of the HIV fusion inhibitor enfuvirtide. The work described in this study lays the foundation for the use of β-thiol Asn ligation chemistry in the synthesis of other peptides and proteins which will be the focus of future research efforts in our laboratory.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02468.

Detailed experimental procedures, analytical HPLC traces, and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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