

# **One-Pot Peptide Ligation–Desulfurization at Glutamate**

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**Supporting Information** 

**ABSTRACT:** An efficient methodology for ligation at glutamate (Glu) is described. A  $\gamma$ -thiol-Glu building block was accessed in only three steps from protected glutamic acid and could be incorporated at the N-terminus of peptides. The application of these peptides in one-pot ligation-desulfuriza-



tion chemistry is demonstrated with a range of peptide thioesters, and the utility of this methodology is highlighted through the synthesis of the osteoporosis peptide drug teriparatide (Forteo).

N ative chemical ligation is the most widely used method for the construction of peptides and proteins from smaller peptide fragments.<sup>1–3</sup> This methodology has greatly expanded our understanding of protein structure and function by facilitating access to a diverse range of proteins, including post-translationally modified proteins that are difficult or impossible to obtain through expression technologies.<sup>4-6</sup> Mechanistically, the first step of the reaction involves a reversible trans-thioesterification between a cysteine (Cys) residue on the N-terminus of a peptide fragment with a thioester moiety on the C-terminus of another peptide reaction partner. A rapid S to N acyl shift, which proceeds through a five-membered ring transition state, then facilitates the formation of the native peptide bond in high yield and with complete chemoselectivity. In addition to the rapid and highyielding nature of this reaction, a further strength of the protocol is that ligations proceed in aqueous media and at neutral pH in the presence of unprotected amino acid side chains.

The low abundance of Cys residues within peptides and proteins has prompted the development of post-ligation desulfurization chemistry, whereby the Cys residue used to facilitate the ligation can be desulfurized to provide an alanine (Ala) residue,<sup>7</sup> either through hydrogenation or via a radicalmediated approach, for example, through the use of the popular water-soluble radical initiator 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044).<sup>8–10</sup> To expand the scope of ligation chemistry, extensive research efforts have recently focused on the synthesis of  $\beta$ -,  $\gamma$ -, and  $\delta$ -thiol-derived amino acids as well as thiol-derived aromatic amino acids (Scheme 1),<sup>11–25</sup> which can be implemented in ligation–desulfurization chemistry through a similar mechanism to native chemical ligation.

Herein we report the first synthesis of a suitably protected  $\gamma$ thiol-Glu building block through a short and scalable route, as well as its utility in peptide ligation—desulfurization chemistry. Although this reaction would proceed through a six-membered ring during the S to N acyl shift, owing to the  $\gamma$ -position of the thiol (Scheme 1B), we envisaged that the ligation reaction would still be facile based on prior ligation studies at Scheme 1. (A) Native Chemical Ligation–Desulfurization at Cys/ $\beta$ -Thiol Amino Acids and (B)  $\gamma$ -Thiol-Mediated Ligation–Desulfurization

A) Native Chemical Ligation–Desulfurization at Cys or  $\boldsymbol{\beta}\text{-thiol}$  amino acids



homocysteine<sup>26</sup> and other  $\gamma$ -thiol amino acids.<sup>8,27</sup> Furthermore, we report conditions for a one-pot ligation—desulfurization at Glu whereby desulfurization can be carried out on the crude ligation reaction, without the need for intermediate purification.

Synthesis of the initially proposed  $\gamma$ -thiol-Glu building block 1 proceeded from Boc-Glu(OtBu)-OAll (2) and began with installation of a 2,4-dimethoxybenzyl (DMB)-protected thiol at the  $\gamma$ -position (Scheme 2). This was facilitated by sulfenylating reagent 3 following double deprotonation of 2 (see Supporting Information for synthetic details).<sup>22,28–31</sup> Gratifyingly, the resulting DMB-protected  $\gamma$ -thiol-Glu 4 was isolated in good yield (83%, Scheme 2) and as a single diastereoisomer (>99% dr). Finally, 4 was subjected to Pd-catalyzed allyl ester deprotection conditions to afford the desired  $\gamma$ -thiol-Glu building block 1 in excellent yield. As it has recently been shown that both diastereomers of  $\beta$ -thiol-Asp facilitate ligation at comparable rates,<sup>22</sup> we decided to focus only on the isolated diastereomer for ligation studies.

With suitably protected  $\gamma$ -thiol-Glu 1 prepared, this building block was next incorporated at the N-terminus of model

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## Scheme 2. Synthesis of $\gamma$ -Thiol-Glu Building Blocks 1 and 10



Scheme 3. Synthesis of Model Peptide 12



peptide 5 following standard Fmoc strategy solid-phase peptide synthesis (SPPS), starting from Rink amide resin, to afford resin-bound peptide 6 (Scheme 3). Unfortunately, after concomitant resin cleavage and deprotection (90:5:5 v/v/v TFA/*i*Pr<sub>3</sub>SiH/H<sub>2</sub>O), pentapeptide 7 and thiolactone 8 were isolated, rather than the desired peptide displaying the  $\gamma$ -thiol-Glu on the N-terminus. In this case, the acidic cleavage conditions facilitated peptide splicing at the N-terminus, resulting from attack by the nucleophilic thiol moiety, revealed after deprotection of the acid-labile DMB protecting group, onto the amide backbone via a favorable five-membered ring transition state.<sup>26</sup>

To circumvent this unwanted pathway, we decided to exchange the acid-labile DMB-thiol protecting group for an acid-stable but reductively labile methyl disulfide protecting group (Scheme 2). This transformation was achieved by subjecting DMB-protected  $\gamma$ -thiol-Glu 1 to the reagent dimethyl(methylthio)sulfonium tetrafluoroborate (9),<sup>32</sup> which facilitated this protecting group exchange in moderate yield (S5%, Scheme 2).<sup>33</sup> Gratifyingly, incorporation of this modified  $\gamma$ -thiol-Glu building block 10 into resin-bound peptide 11 employing (benzotriazol-1-yloxy)tripyrrolidinophosphonium-hexafluorophosphate (PyBOP) and N-methylmorpholine (NMM) afforded the desired model peptide 12 in good yield after acidic deprotection, cleavage of the peptide from the resin and HPLC purification (68%, Scheme 3), without any trace of unwanted peptide splicing products.

At this stage, the stereochemical outcome of the sulfenylation reaction was confirmed by single-crystal X-ray analysis of cyclic derivative **13**, which was synthesized following reductive thiolactonization of **10**, employing tris(2-carboxyethyl)-phosphine (TCEP) followed by PyBOP and *N*,*N*-diisopropyl-ethylamine (DIPEA) (Scheme 2). The stereochemistry of thiolactone **13** was confirmed to be (2S,4S), indicating that the (2S,4S) diastereoisomer of **4** was obtained following sulfenylation.

With the desired model peptide **12** in hand, we next turned our attention to investigating the utility of the N-terminal  $\gamma$ -

thiol-Glu moiety in peptide 12 in ligation–desulfurization chemistry using a variety of C-terminal model peptide thioesters to probe the scope of these reactions (entries 1-5, Table 1) (see Supporting Information for synthetic details).

Table 1. Scope of  $\gamma$ -Thiol-Glu Ligation–Desulfurization Chemistry

HO H <sub>2</sub> N	0 S <sup>-S</sup> SPGYS-NH <sub>2</sub> 12	1. Ligation: Ac-LYRANX–S 2. Desulfurization (or one-pot ligation–desulfur	(CH <sub>2</sub> ) <sub>2</sub> CO <sub>2</sub> Et ization) ► Ac-LYRANX <sup>^</sup>	
entry	thioester (X =)	ligation yield <sup>a</sup> (%)	desulfurization yield <sup>a</sup> (%)	one-pot yield <sup>c</sup> (%)
1	Gly	72	89 (64) <sup>b</sup>	73
2	Ala	77	91 $(70)^b$	67
3	Met	83	98 $(81)^b$	72
4	Phe	80	84 $(67)^b$	74
5	Val	68	98 $(67)^b$	56

<sup>a</sup>Isolated yields after HPLC purification. Ligation: 5 mM **12** in buffer (6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM TCEP), PhSH (2 vol %), 37 °C, pH 7.2–7.4, 16 h. Desulfurization: 500 mM TCEP in buffer (6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>), reduced glutathione (40 mM), VA-044 (200 mM), pH 6.5–6.8, 65 °C, 16 h. <sup>b</sup>Yield over two steps. <sup>c</sup>Desulfurization reactions were carried out at 37 °C in the one-pot protocol.

Ligation reactions were carried out in ligation buffer (6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM TCEP, 5 mM with respect to **12**) at 37 °C and pH 7.2–7.4 with the addition of thiophenol as an aryl thiol catalyst. Pleasingly, each of the ligation reactions proceeded to completion within 16 h and in excellent yields after reverse-phase HPLC purification (68–83%, Table 1). It should be noted that HPLC fractions containing ligation product were immediately lyophilized to avoid acid-mediated peptide splicing caused by the acidic HPLC eluent.

Next we subjected the purified ligation products to radicalbased desulfurization<sup>10</sup> using VA-044 as the radical initiator, in the presence of TCEP and reduced glutathione.<sup>15</sup> In all cases, desulfurization reactions proceeded to completion within 16 h at 65 °C, affording the native peptide products in excellent yields after reverse-phase HPLC purification (Table 1, 84–98% yield).

On the basis of prior reports of one-pot ligationdesulfurization chemistry at Cys<sup>34</sup> and  $\beta$ -thiol-Asp,<sup>22</sup> we were next interested in developing this concept to effect the one-pot transformation at  $\gamma$ -thiol-Glu containing model peptides (Table 1, entries 1-5). We envisaged that this would not only streamline the methodology by preventing additional purification steps but also avoid peptide splicing facilitated by the  $\gamma$ thiol during purification. Specifically, each ligation reaction was first carried out with careful monitoring and shown to proceed to completion within 4 h, as determined by LC-MS analysis, with the exception of the ligation reaction with model thioester Ac-LYRANV-S(CH<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>Et (entry 5) which required a reaction time of 16 h to reach completion. After this time, thiophenol was extracted from the reaction mixture by washing with diethyl ether in order to prevent poisoning of the desulfurization reaction by thiophenol. $^{19,22}$  The ligation reaction mixture was immediately treated with TCEP (500 mM), reduced glutathione (40 mM), and VA-044 (200 mM), affording the desired peptide products in excellent yields over

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the two steps (Table 1, 56–74% yield). It should be noted that the one-pot reaction was also attempted in the absence of VA-044 to effect selective desulfurization in the presence of Cys residues, as we have recently described at  $\beta$ -thiol-Asp.<sup>22</sup> Unfortunately, due to peptide splicing which occurs at the acidic pH required for the selective desulfurization protocol (Scheme 3), a complex mixture of products resulted, and hence we were unable to achieve the analogous selective desulfurization reaction.

For one-pot ligation–desulfurization reactions with peptide thioesters bearing C-terminal Ala, Met, Phe, and Val residues, peptide byproduct 14 was also observed, which may result from reaction of the resulting VA-044 radical with the peptide radical formed following H-abstraction from the  $\gamma$ -thiol (Figure 1).<sup>10</sup>



**Figure 1.** (a) Analytical HPLC trace ( $\lambda = 280 \text{ nm}$ , 0–40% CH<sub>3</sub>CN over 30 min) of crude reaction mixture for one-pot ligation–desulfurization between 12 and Ac-LYRANG-S(CH<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>Et without the formation of byproduct 14. (b) Analytical HPLC trace ( $\lambda = 280 \text{ nm}$ , 0–40% CH<sub>3</sub>CN over 30 min) of crude reaction mixture for one-pot ligation–desulfurization between 12 and Ac-LYRANM-S-(CH<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>Et, indicating the formation of byproduct.

As this byproduct was not observed in the two-step ligation– desulfurization procedures, it is likely that this pathway results from trace amounts of aryl thiol remaining despite the extraction protocol.<sup>34</sup> Nonetheless, in all cases, this side reaction did not significantly impact the isolated yield or the overall efficiency of the reaction methodology (Figure 1).

Having demonstrated the scope and efficiency of the ligation-desulfurization reactions at  $\gamma$ -thiol-Glu, including the one-pot protocol, we next focused our attention on applying the methodology to the synthesis of the peptide drug teriparatide (15) (Forteo, Eli Lilly). Used in the treatment of glucocorticoid-induced osteoporosis, this recombinant 34 amino acid parathyroid hormone is the only osteoporosis drug on the market that stimulates bone growth.<sup>35</sup> We envisaged that this peptide could be easily accessed via a ligation reaction between two fragments, namely, peptide thioester 16, corresponding to teriparatide (1-18) and bearing a C-terminal Met residue, and peptide 17 possessing an Nterminal  $\gamma$ -thiol-Glu residue, representing teriparatide (19–38) (Scheme 4). To this end, peptide 18 corresponding to teriparatide (20-34) was first synthesized on trityl chloride resin using standard Fmoc-SPPS procedures (see Supporting Scheme 4. Synthesis of Teriparatide (15)



Information for synthetic details). Coupling of the  $\gamma$ -thiol-Glu building block 10 and acidic cleavage and deprotection from the resin then afforded the desired peptide fragment 17 in 18% yield after reverse-phase HPLC purification (based on the original resin loading) (Scheme 4). Ligation of peptide 17 with Met-thioester 16 (see Supporting Information for synthetic details) proceeded cleanly to completion over 16 h under the ligation condition described previously. After HPLC purification, the desired ligation product 19 was isolated in excellent yield (82%). Desulfurization of 19 under the radical conditions described above also proceeded smoothly to afford the native teriparatide peptide (15) in excellent yield (95%) after purification. Finally, we also synthesized teriparatide (15) by employing our one-pot ligation-desulfurization strategy, which gratifyingly afforded the desired peptide product in excellent vield (71%, Scheme 4).

In conclusion, we have developed a concise and scalable synthesis of a novel  $\gamma$ -thiol-Glu building block **10** which can be readily incorporated into a variety of peptides to faciliate ligation chemistry. The resulting  $\gamma$ -thiol-Glu peptides undergo facile ligation reactions with a range of thioesters and can be desulfurized to the native peptide products using radical-based conditions. Furthermore, we have extended this methodology to include a one-pot ligation-desulfurization cascade which proved to be efficient and high-yielding, while reducing the need for intermediate purification of the ligation products. Moreover, this methodology has been successfully employed in the high-yielding preparation of the FDA-approved osteoporosis drug teriparatide (15), which suggests that this methodology will have wide utility in the synthesis of peptides and proteins in the future. Further research in our laboratories will focus on the expansion of this methodology to include the synthesis of protein targets.

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ASSOCIATED CONTENT

#### **Supporting Information**

Detailed experimental procedures, analytical HPLC traces, and characterization data. 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 interest.

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