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Chemoselective Peptide Ligation–Desulfurization Chemistry at Aspartate

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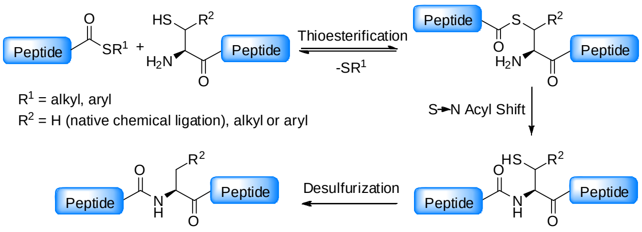


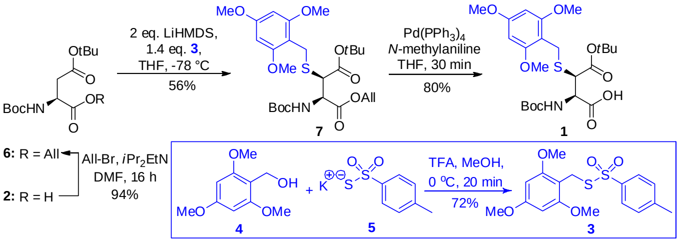
Scheme 1. Native chemical ligation and ligation at thiol-bearing amino acids followed by desulfurization.

Native chemical ligation is an extremely powerful method for the convergent assembly of proteins from smaller peptide fragments.[1] The methodology has been employed in the synthesis of numerous homogeneous proteins, including those possessing post-translational modifications, and has therefore contributed to our understanding of protein structure and function.[1d, 2] The process involves the reversible thioesterification reaction between a cysteine (Cys) residue, located at the N-terminus of a peptide fragment, with another peptide bearing a C-terminal thioester (Scheme 1). The resulting thioester intermediate subsequently rearranges through a rapid S→N acyl shift to provide the ligated peptide or protein product.

The Cys residue which remains at the ligation site following the reaction can be manipulated *via* hydrogenation[3] or radical-based[4] desulfurization chemistry, to convert Cys residues to alanine (Ala) [5] and has been widely adopted, featuring in the total chemical synthesis of several complex proteins and glycoproteins.[6] In addition, it has recently been demonstrated that desulfurization reactions can be carried out following native chemical ligation without purification of the intermediate Cys-containing peptide using solid-supported thiol scavengers, thus streamlining and improving the efficiency of the methodology.[7] Further expansion of the native chemical ligation–desulfurization concept has been made possible through synthetic amino acids bearing side-chain thiol groups, which can facilitate ligation reactions in a similar manner to a Cys residue when incorporated at the N-terminus of peptide fragments. These amino acids can be efficiently desulfurized to afford the native residue following the ligation event (Scheme 1).[8] Although these thiol-containing amino acids have greatly expanded the repertoire of peptide ligation chemistry, highlighted through their use in the assembly of large peptides and proteins,[8e, 8f, 8k, 9] the vast majority of building blocks have not found wide utility owing to the lengthy syntheses required to access them. Herein we describe our efforts toward the development of a short and scalable route to a suitably protected β-mercapto aspartate (Asp) residue and its implementation in ligation chemistry. Furthermore, we demonstrate that selective desulfurization of this residue within ligation products is possible in the presence of unprotected Cys residues.

We proposed access to the suitably protected β-mercapto Asp building block **1** *via* a robust three-step synthetic route from the affordable and commercially available amino acid Boc-Asp(O*t*Bu)-OH **2** (USD$10/g). The acid-labile 2,4,6-trimethoxybenzyl (Tmob) protected thiol moiety was installed at the β-position through the use of the novel sulfenylating reagent **3**, which was itself prepared in high yield through the condensation of 2,4,6-trimethoxybenzyl alcohol **4** and potassium toluene thiosulfonate **5** (Scheme 2, see Supporting Information for synthetic details). Allyl (All) ester protection of Boc-Asp(O*t*Bu)-OH **2** provided the fully protected Asp derivative **6**. Treatment of **6** with two equivalents of lithium hexamethyldisilazide (LiHMDS) at low temperature generated the corresponding dianion, which was treated with sulfenylating agent **3** to afford the Tmob-protected β-mercapto amino acid **7**, produced as a 9:1 diastereomeric mixture in favor of the *syn*-(*erythro*) isomer. These two diastereoisomers could be separated by column chromatography to provide *erythro*-**7** in 56% yield, the stereochemistry of which was confirmed by NMR coupling constant analysis (see Supporting Information).[10] Finally, palladium(0)-catalyzed All ester deprotection afforded the desired β-mercapto Asp building block **1** in 80% yield. Overall, **1** was prepared in three steps from **2** in 45% overall yield.

Having successfully prepared **1**, the building block was smoothly incorporated into model peptide **8** using standard Fmoc-strategy solid-phase peptide synthesis (SPPS) (see Supporting Information for synthetic details). Ligation reactions between peptide **8** and a number of peptide thioesters **9**–**13** bearing a representative selection of C-terminal residues were next carried out to determine the scope of the reaction (Table 1, see Supporting Information). Ligations were conducted in a denaturing buffer comprising 6 M guanidine hydrochloride (Gn•HCl), 200 mM HEPES and 50 mM tris-(2-carboxyethyl)phosphine (TCEP) at 37 °C and pH 7.3-7.5.



Scheme 2. Synthesis of β–mercapto Asp building block **1**

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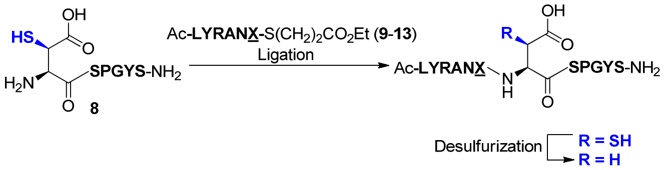
Gratifyingly, each peptide ligation proceeded to completion with rates comparable to those reported for native chemical ligation of peptides bearing N-terminal cysteine residues (see Supporting Information).[11] Interestingly, while it has been shown that the rate of ligation at β-mercapto leucine is faster with the *threo*-diasteroisomer,[8g] we determined that ligations at β-mercapto Asp were equally facile with either diastereoisomer (see Supporting Information for rate comparison studies). Following reverse-phase HPLC purification, the ligation products were isolated in excellent yields (71–82%, entries 1–5, Table 1). Following isolation, the ligation products were subsequently subjected to a radical-based desulfurization[4] reaction using VA-044 in the presence of TCEP and reduced glutathione,[8c] providing the native peptide products in 63–76% yields (entries 1–5, Table 1).

Table 1. Ligation–desulfurization reactions at Asp.



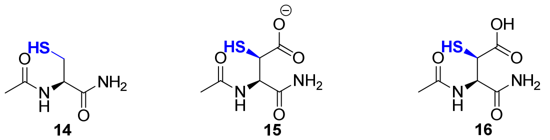
Entry	Peptide Thioester (**X** =)	Ligation yield[a] [%]	Desulfurization yield[a] [%]
1	G (**9**)	80	75
2	A (**10**)	82	71
3	M (**11**)	71	63
4	F (**12**)	78	76
5	V (**13**)	75	71

[a] Isolated yields after HPLC purification. **Ligation conditions:** 5 mM **8** in buffer (6 M Gn•HCl, 200 mM HEPES, 50 mM TCEP), PhSH (2 vol%), 37 °C, pH 7.3–7.5, 24 h. **Desulfurization conditions:** 5 mM in buffer (6 M Gn•HCl, 200 mM HEPES, 250 mM TCEP), reduced glutathione (40 mM), VA-044 (20 mM), pH 6.5–7.0, 37 °C, 16 h.

Although ligation–desulfurization reactions have greatly expanded the scope of ligation chemistry, a major limitation is the inability to chemoselectively desulfurize the installed thiol auxilliary in the presence of free sulfhydryl side chains of native Cys residues. This necessitates global protection of the Cys side chains in the sequence,[12] thus preventing the use of expressed protein ligation methodologies with recombinantly expressed fragments.[13]

Given these significant limitations, we were interested in exploring a chemoselective desulfurization reaction at β-mercapto Asp. We were inspired by the recent reports of radical deselenization of selenocysteine,[14] β-selenolphenylalanine[15] and γ-selenolproline,[8j] which could be effected in the presence of unprotected cysteine residues in the absence of a radical initiator by treating ligation products with TCEP and dithiothreitol (DTT). This selectivity may be attributed to the weaker selenium–carbon bond strength compared with the sulfur–carbon bond, together with the greater propensity of selenium to form radicals. It should be noted, however, that Cys residues have been reportedly desulfurized in the absence of a radical initiator when treated with excess phosphine, albeit upon heating.[16] We envisaged that a desulfurization reaction of thiol-containing amino acids may be correlated with C–S bond strengths i.e. the energy necessary to generate the β-carbon-centered radical, and that the propensity of radical formation would be governed by neighboring functional groups. For β-mercapto Asp, we hypothesized that the electronic properties of the carboxylate/carboxylic acid functionality at the β-position may weaken the C–S bond, thus affecting the rate of desulfurization. Through *in silico* investigations, carbon-centered radicals with an adjacent carboxylic acid group have been predicted to be stabilized relative to the unsubstituted counterparts and, as such, we anticipated that selective desulfurization of β-mercapto Asp over Cys may prove possible.[17]

To further probe this concept, we performed computational studies on model systems **14**–**16** to predict the bond dissociation energies (BDEs) corresponding to the cleavage of the C–S and S–H bonds in cysteine, β-mercapto aspartate and β-mercapto aspartic acid (Figure 1, see Supporting Information). The BDEs of the S–H bonds of **14**–**16**, calculated with the high-level G3X(MP2)-RAD procedure,[18] were found to be very similar (353.1–357.9 kJ mol^{-1}) and significantly larger than the energy required to break the C–S bonds in these molecules. There was a negligible difference between the C–S BDEs of **14** and **15** (308.5 and 308.0 kJ mol^{-1}, respectively) despite the presence of a carboxylate side chain in **15**. However, upon protonation of the carboxylate (as in **16**), the C–S bond was predicted to be significantly weaker (BDE = 298.3 kJ mol^{-1}). Notably, the ~10 kJ mol^{-1} lower BDE of **16** compared with **14** and **15** corresponds to a roughly two orders of magnitude increase in rate for the homolytic cleavage of the C–S bond at room temperature, suggesting that selective desulfurization of β-mercapto Asp may be possible in the presence of Cys.



14 (C–S BDE = 308.5 kJ mol^{-1}) **15** (C–S BDE = 308.0 kJ mol^{-1}) **16** (C–S BDE = 298.3 kJ mol^{-1})

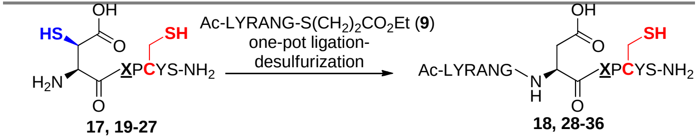
Figure 1. C–S BDEs of model peptides **14**–**16**.

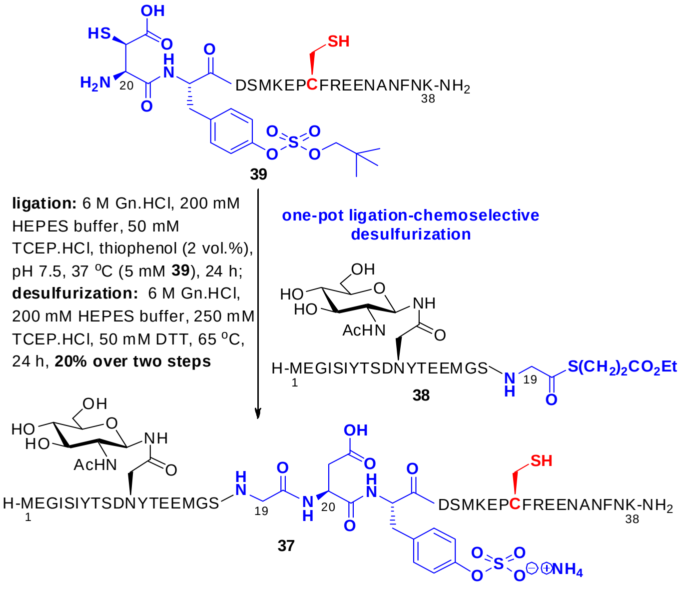
Based on our computational results, we moved to investigate the development of a one-pot chemoselective ligation–desulfurization reaction. To this end, we first synthesized peptide **17** bearing both a β-mercapto Asp residue on the N-terminus and a cysteine residue within the peptide sequence (Table 2). This peptide was reacted with peptide thioester **9** bearing a C-terminal Gly residue under identical conditions to those described previously (entry 1, Table 2). The reaction reached completion to provide the desired ligation product after 30 minutes, and after this time thiophenol was extracted from the ligation mixture to prevent this from hindering the desulfurization reaction rate.[19] Following removal of the aryl thiol, the mixture was immediately treated with 250 mM TCEP and 50 mM DTT and reactions at a variety of temperatures and pHs were assessed. The most rapid rate of desulfurization and complete selectivity was observed when the reaction was conducted at pH = 3 at 65 ºC. Increasing the pH of the desulfurization reaction led to a distinct decrease in the desulfurization rate of the β-mercapto Asp residue, thus leading to a loss in desulfurization selectivity over the side chain of Cys (see Supporting Information for HPLC data). This result is consistent with the computational results where the C–S bond is predicted to be significantly weaker when the side chain carboxylate is protonated (pKa of β-CO_2H of Asp = 3.86). After incubating for 20 h under the optimized conditions, HPLC-MS analysis revealed complete consumption of the ligation product and showed only the singly-desulfurized **18** as the major product, together with two minor by-products. Gratifyingly, purification by reverse-phase HPLC provided **18** in 48% isolated yield over the two steps, the identity of which was confirmed by spectroscopic comparison to an authentic sample of **18** (entry 1, Table 2).

Table 2. One-pot ligation–chemoselective desulfurization reactions.

[a] Isolated yields after HPLC purification over two steps. [b] Analytical yield from HPLC-MS analysis. **Conditions:** 5 mM **17**, **19**–**27** in buffer (6M Gn•HCl, 200 mM HEPES, 50 mM TCEP), PhSH (2 vol.%), 37 °C, pH 7.2–7.4, 2 h; then Et_2O washing and dilution to 2.5 mM in buffer (6M Gn•HCl, 200 mM HEPES, 250 mM TCEP), 50 mM DTT, 65 °C, pH 3.0, 20 h.

Entry	Peptide (**X** =)	Product (**X** =)	Yield[a] [%]
1	Ser (**17**)	Ser (**18**)	48
2	Gly (**19**)	Gly (**28**)	<5[b]
3	Pro (**20**)	Pro (**29**)	0[b]
4	Ala (**21**)	Ala (**30**)	45
5	His (**22**)	His (**31**)	59
6	Lys (**23**)	Lys (**32**)	47
7	Glu (**24**)	Glu (**33**)	57
8	Asn (**25**)	Asn (**34**)	50
9	Phe (**26**)	Phe (**35**)	63
10	Ile (**27**)	Ile (**36**)	58





Scheme 3. Synthesis of CXCR4(1-38) **37** *via* a one-pot Asp ligation–chemoselective desulfurization reaction.

Investigation into the identity of the minor by-products revealed that these arose from bond cleavage at the Asp-Ser junction to generate two peptides, Ac-LYRANGD-OH and H-SPCYS-OH. This reaction is a known degradation pathway of Asp-containing peptides and proteins at low pH, with the propensity of peptide bond cleavage dictated by the nature of the amino acid found on the C-terminal side of the Asp residue.[20] To further evaluate the nature of these effects, and thus the utility of the one-pot ligation–chemoselective desulfurization methodology, we synthesized a range of peptides **19**–**27** bearing a broad range of amino acids on the C-terminal side of the β-mercapto Asp residue (Table 2). These peptides were ligated to peptide thioester **9** and after 2 h the reactions were treated with TCEP (250 mM) and DTT (50 mM) at pH 3 to effect the selective desulfurization. After incubation for 20 h, the crude reaction mixtures were assessed by HPLC-MS. Pleasingly, in all but two cases the one-pot ligation–desulfurization reactions provided the native peptides as the major product, without any detectable Cys desulfurization and only minimal peptide cleavage by-products (see Supporting Information for all analytical HPLC data). An exception was the reaction of peptide **19** bearing glycine on the C-terminal side of the Asp residue which generated the corresponding aspartimide under the desulfurization conditions (entry 2, Table 2). Additionally, complete cleavage of the Asp–Pro bond of peptide **20** occurred under these conditions (entry 3, Table 2). These results were not unexpected and reflect the known lability of these bonds.[21] Gratifyingly, the one-pot ligation–chemoselective desulfurization reactions of all the remaining peptides possessing Ala (**21**), His (**22**), Lys (**23**), Glu (**24**), Asn (**25**), Phe (**26**) and Ile (**27**) residues on the C-terminal side of the β-mercapto Asp moiety provided excellent yields of the desired singly-desulfurized products (**30**–**36**) over the two steps (45–63%, entries 4–10, Table 2). These results suggest that the one-pot ligation–chemoselective desulfurization reaction represents a general methodology with broad substrate utility.

Having investigated the scope of the one-pot Asp ligation–selective desulfurization methodology, we next employed the methodology to assemble the extracellular N-terminal domain of the chemokine receptor CXCR4 bearing two homogeneous post-translational modifications (*N*-linked glycosylation and Tyr sulfation). Our laboratory has an interest in understanding the role of glycosylation and sulfation of receptor N-terminal domains on chemokine binding[22] and in this instance the presence of three Asp residues and one Cys residue within the 38 amino acid sequence provided a perfect target to test the synthetic utility of our method. We proposed to assemble doubly-modified CXCR4(1-38) **37** *via* ligation between glycopeptide **38** bearing a C-terminal Met thioester and neopentyl (nP) protected sulfopeptide **39** possessing an N-terminal β-mercapto Asp moiety (Scheme 3, see Supporting Information for synthesis of **38** and **39**). Ligation between **38** and **39** was carried out under the same conditions described for the model systems. After 24 h, LC-MS analysis indicated that the ligation reaction had proceeded to completion with concomitant nP ester deprotection (due to nucleophilic deprotection by TCEP in the ligation buffer). The thiophenol was next extracted before TCEP and DTT were added and the reaction heated at 65 °C at pH 3.0 for 24 h to effect chemoselective desulfurization. After 24 h of incubation, HPLC-MS analysis indicated successful single desulfurization of the ligation product as well as a minor by-product corresponding to imide formation between the backbone amide and the side chain of Asp20. We were pleased to find that the acidic desulfurization conditions did not lead to loss of the acid-labile sulfate ester moiety in **37**. Purification *via* reverse-phase HPLC then provided the full N-terminal domain of CXCR4(1-38) bearing an *N*-linked glycan and Tyr sulfation in 20% yield over the two steps.

In summary, we have successfully developed an expedient and scalable route to a suitably protected β-mercapto Asp building block that is capable of facilitating rapid ligation to peptide thioesters with rates similar to those observed for native chemical ligation at Cys. We have used computational studies to guide the development of an initiator-free desulfurization of β-mercapto Asp residues in the presence of free sulfhydryl side chains of Cys residues in peptides. A one-pot ligation–chemoselective desulfurization methodology at β-mercapto Asp proved to be efficient for a number of examples, and was successfully employed in the synthesis of the N-terminal domain of CXCR4 bearing two post-translational modifications. It should be noted that although the results of the selective desulfurization reactions are consistent with the calculated BDEs and a radical mechanism, we cannot rule out the potential for a pH dependent polar pathway operating. Further investigations into the mechanistic aspects of this reaction will be the subject of future work.

Given the straightforward synthesis of the β-mercapto Asp building block **1** and the operationally simple nature of the one-pot ligation–desulfurization protocol described, it is anticipated that this methodology will find widespread use in the chemical synthesis of peptides and proteins.

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