Peptide Ligation

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Chemoselective Peptide Ligation–Desulfurization at Aspartate**

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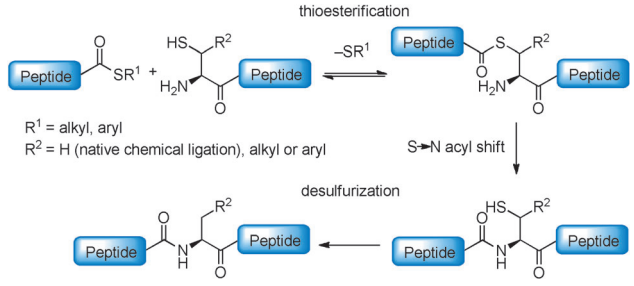
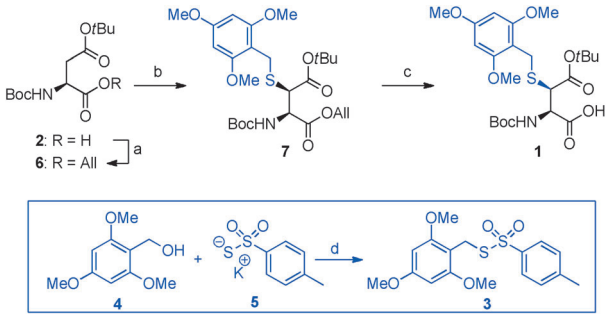
Native chemical ligation is an extremely powerful method for the convergent assembly of proteins from smaller peptide fragments.[1] The methodology has been employed in the synthesis of numerous homogeneous proteins, including those possessing post-translational modifications, and has therefore contributed to our understanding of protein structure and function.[1d,2] The process involves the reversible thioesterification between a cysteine (Cys) residue, located at the N-terminus of a peptide fragment, with another peptide that bears 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.



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

The Cys residue, which remains at the ligation site following the reaction, can be manipulated by hydrogenation[3] or radical-based[4] desulfurization to convert Cys residues to alanine (Ala).[5] This method has been widely adopted, for example in the total chemical synthesis of several complex proteins and glycoproteins.[6] Desulfurization can also 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 that bear 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,f,k,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 utility 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 envisaged access to the suitably protected β-mercapto Asp building block **1** through 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 thiol moiety, which is protected by a 2,4,6-trimethoxybenzyl (Tmob) group, was installed at the β-position using 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 the 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*)



Scheme 2. Synthesis of β-mercapto Asp building block **1**. Reaction conditions: a) All-Br, iPr_2EtN, DMF, 16 h, 94%; b) LiHMDS (2 equiv), **3** (1.4 equiv), THF, −78 °C, 56%; c) $Pd(PPh_3)_4$, *N*-methylaniline, THF, 30 min, 80%; d) TFA, MeOH, 0 °C, 20 min, 72%.

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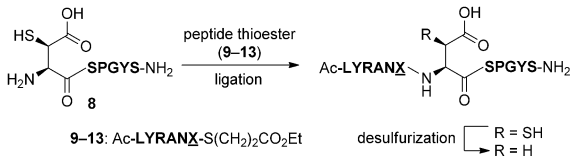
isomer. These two diastereoisomers could be separated by column chromatography to provide *erythro*-**7** in 56% yield; the stereochemistry of **7** was confirmed by analysis of NMR coupling constants (see the Supporting Information).[10] Finally, palladium(0)-catalyzed deprotection of the All ester afforded the desired β-mercapto Asp building block **1** in 80% yield. Overall, **1** was prepared in three steps from **2** in 45% yield.

Table 1: Ligation–desulfurization reactions at Asp.



Entry	X	Peptide thioester	Yield[a] [%] Ligation	Desulfurization
1	Gly	**9**	80	75
2	Ala	**10**	82	71
3	Met	**11**	71	63
4	Phe	**12**	78	76
5	Val	**13**	75	71

Ligation conditions: **8** (5 mM) 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: ligation product (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. [a] Yields of isolated products after HPLC purification.

Having successfully prepared **1**, the building block was smoothly incorporated into model peptide **8** using standard Fmoc-strategy solid-phase peptide synthesis (SPPS; see the Supporting Information for synthetic details). Ligation reactions between peptide **8** and a number of peptide thioesters (**9**–**13**) that bear a representative selection of C-terminal residues were next carried out to determine the scope of the reaction (Table 1, see the Supporting Information). Ligations were conducted in a denaturing buffer comprising guanidine hydrochloride (Gn·HCl, 6 M), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, 200 mM) and tris-(2-carboxyethyl)phosphine (TCEP, 50 mM) at 37°C and a pH range of 7.3–7.5. Gratifyingly, each peptide ligation proceeded to completion with rates comparable to those reported for native chemical ligation of peptides that bear N-terminal cysteine residues (see the Supporting Information).[11] Interestingly, while it has been shown that the rate of ligation at β-mercapto leucine is faster with the *threo* diastereoisomer,[8g] we determined that ligations at β-mercapto Asp were equally facile with either diastereoisomer (see the 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 reaction[4] 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).

Although ligation–desulfurization reactions have greatly expanded the scope of ligation chemistry, a major limitation is the inability to chemoselectively desulfurize the installed thiol auxiliary in the presence of free sulfhydryl side chains of native Cys residues. This problem 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 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 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 the strengths of C–S bonds, that is, the energy necessary to generate the β-carbon-centered radical, and that the propensity for 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 the 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

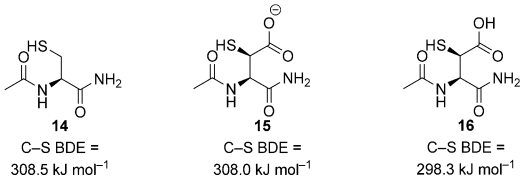
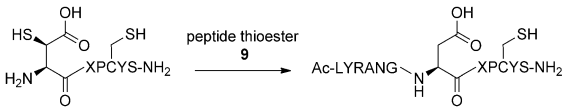


Figure 1. Calculated C–S BDEs of model peptides **14**–**16**.

approximately 10 kJ mol^{-1} lower BDE of **16** compared with **14** and **15** corresponds to an increase in rate of roughly two orders of magnitude 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.

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



17, **19–27** **9**: Ac-LYRANG-S(CH$_2$)$_2$CO$_2$Et **18**, **28–36**

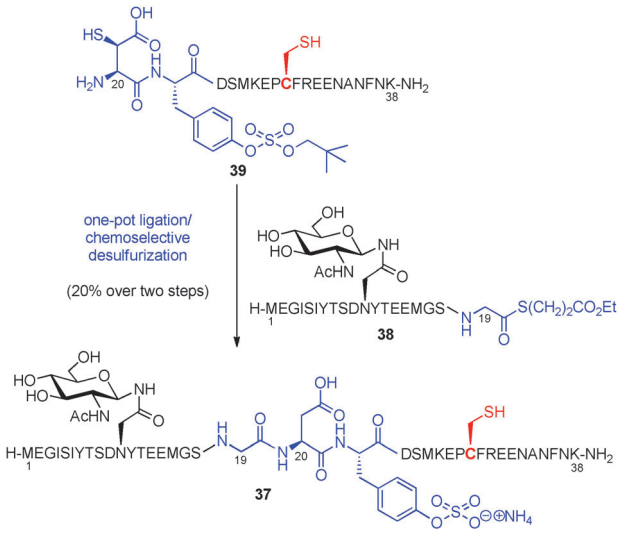
Entry	X	Peptide	Product	Yield[a] [%]
1	Ser	**17**	**18**	48
2	Gly	**19**	**28**	< 5[b]
3	Pro	**20**	**29**	0[b]
4	Ala	**21**	**30**	45
5	His	**22**	**31**	59
6	Lys	**23**	**32**	47
7	Glu	**24**	**33**	57
8	Asn	**25**	**34**	50
9	Phe	**26**	**35**	63
10	Ile	**27**	**36**	58

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

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**, which bears 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**, which bears a C-terminal Gly residue, under identical conditions to those described previously (Table 1). 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 the aryl thiol from hindering the desulfurization reaction rate.[19] Following removal of the aryl thiol, the mixture was immediately treated with TCEP (250 mM) and DTT (50 mM), and reactions at a variety of temperatures and pH values were assessed. The most rapid rate of desulfurization and complete selectivity was observed when the reaction was conducted at pH 3 at 65 °C. Increasing the pH value 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 the 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 (pK_a of β-CO$_2$H of Asp = 3.86). After incubating for 20 hours under the optimized conditions, HPLC–MS analysis showed complete consumption of the ligation product and only the singly desulfurized **18** as the major product, together with two minor by-products. Gratifyingly, purification by reverse-phase HPLC provided **18** (48% yield over the two steps), the identity of which was confirmed by spectroscopic comparison to an authentic sample of **18** (entry 1, Table 2).

Investigation into the identity of the minor by-products showed 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 values, 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**) that bear 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 two hours the reactions were treated with TCEP (250 mM) and DTT (50 mM) at pH 3 to effect the selective desulfurization. After incubation for 20 hours, 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 amounts of peptide cleavage by-products (see the Supporting Information for all analytical HPLC data). An exception was the reaction of peptide **19**, which bears 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, which possess 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 the desired singly desulfurized products (**30**–**36**) in excellent yields 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 group has an interest in understanding the role of glycosylation and sulfation of N-terminal receptor domains on chemokine binding,[22] and in this instance the presence of three Asp residues and one Cys residue within the sequence of 38 amino acids provided a perfect target to test the synthetic utility of our method. We proposed to assemble doubly modified CXCR4(1–38) **37** through ligation between glycopeptide **38**, which bears a C-terminal Met thioester, and neopentyl-protected sulfopeptide **39**, which possesses an N-terminal β-mercapto Asp moiety (Scheme 3, see the Supporting Information for the synthesis



Scheme 3. Synthesis of CXCR4(1-38) **37** through a one-pot Asp ligation–chemoselective desulfurization reaction. For reaction conditions of ligation (thioester **39** as starting material) and desulfurization, see Table 2.

of **38** and **39**). Ligation between **38** and **39** was carried out under the same conditions described for the model systems. After 24 hours, LC–MS analysis indicated that the ligation reaction had proceeded to completion, with concomitant deprotection of the neopentyl ester (because of 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 hours to effect chemoselective desulfurization. After 24 hours 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 by 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 reaction 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 possibility of a pH-dependent polar pathway. 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 described one-pot ligation–desulfurization protocol, it is anticipated that this methodology will find widespread use in the chemical synthesis of peptides and proteins.

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