Protein Ligation

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Oxidative Deselenization of Selenocysteine: Applications for Programmed Ligation at Serine

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Abstract: *Despite the unique chemical properties of selenocysteine (Sec), ligation at Sec is an under-utilized methodology for protein synthesis. We describe herein an unprecedented protocol for the conversion of Sec to serine (Ser) in a single, high-yielding step. When coupled with ligation at Sec, this transformation provides a new approach to programmed ligations at Ser residues. This new reaction is compatible with a wide range of functionality, including the presence of unprotected amino acid side chains and appended glycans. The utility of the methodology is demonstrated in the rapid synthesis of complex glycopeptide fragments of the epithelial glycoproteins MUC5AC and MUC4 and through the total synthesis of the structured, cysteine (Cys)-free protein eglin C.*

The advent of native chemical ligation,[1] a method for the mild and chemoselective condensation of two peptide fragments to generate larger peptides and proteins, has enabled access to numerous protein targets through chemical synthesis.[2] The methodology involves the reaction of a peptide bearing an N-terminal Cys residue with a C-terminal peptide thioester in an initial transthioesterification reaction, followed by a rapid S→N acyl shift to generate a native amide bond in aqueous media and at neutral pH. The transformation takes place in the presence of unprotected amino acid side chains and is tolerant of additional functionality, including the presence of a diverse array of post-translational modifications.[2,3]

Efforts focused on the extension of native chemical ligation to include select non-Cys ligation junctions have recently converged on ligation–desulfurization chemistry,[4] in which an amino acid bearing a reactive thiol auxiliary facilitates the ligation of two peptide fragments, with a subsequent desulfurization[5] generating a native amino acid residue. First demonstrated in the conversion of Cys to alanine (Ala),[5a] synthetic access to thiol-derived proteinogenic amino acids has recently facilitated the preparation of a variety of targets via non-Cys ligation disconnections.[6]

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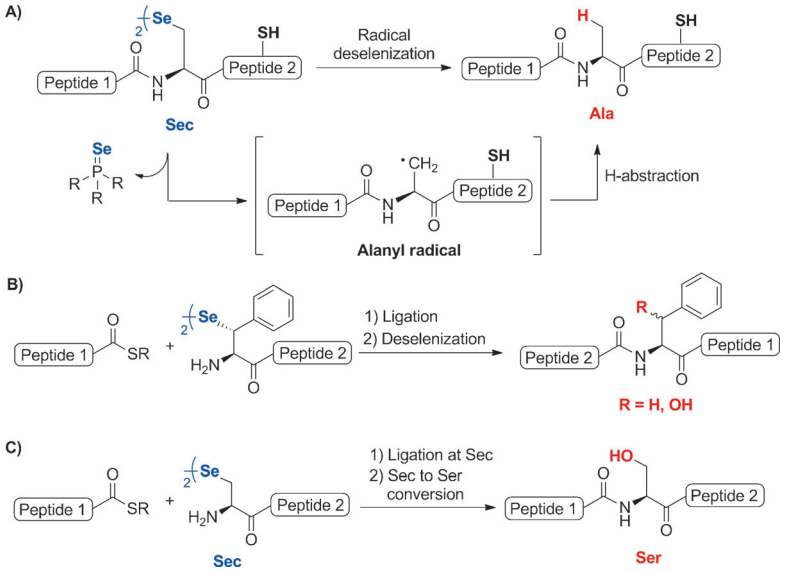
A major drawback of late-stage global desulfurization protocols is the need to protect native and structurally important Cys residues within the target sequence,[7] which would be concomitantly desulfurized to Ala if left unprotected. The recent report of peptide ligation at selenocysteine (Sec)[8] coupled with selective deselenization (in the presence of unprotected Cys residues) by treatment with tris-(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT),[9] provided, for the first time, a completely chemoselective approach to Ala residues at the ligation junction (Scheme 1 A). Mechanistically, the selective deselenization is thought to proceed via a radical mechanism (through an alanyl radical intermediate), taking advantage of the weak nature of the carbon–selenium bond as well as the ability to form selenium-centered radicals preferentially over sulfur-centered radicals.[9] This key discovery laid the intellectual framework for the development of ligation–deselenization chemistry at unnatural selenol-derived amino acids,[10] including in our laboratory at phenylalanine (Phe).[10b]

During the course of our investigations into the chemoselective deselenization of β-selenol Phe-containing peptides, we observed the formation of minor hydroxylation byproducts consistent with the formation of diastereomeric β-hydroxy Phe (Scheme 1 B).[10b,11] Invoking a similar mechanism to that proposed for the chemoselective deselenization of Sec,[9] we presumed that this intriguing byproduct resulted from reaction of a benzylic radical with dissolved oxygen in the reaction media. Although an undesirable byproduct in the context of ligation-deselenization chemistry at Phe, we hypothesized that the development of an equivalent reaction at Sec residues could provide a programmed approach to access serine (Ser) residues at the ligation junction through a ligation, followed by Sec to Ser transformation (Scheme 1 C). It should be noted that other strategies for ligation at Ser junctions have recently been explored, including the use of thiol auxiliaries linked via the Ser side-chain,[12] ligation at Ser through the rearrangement of *O*-acyl isopeptides[13] or direct ligation with activated salicylaldehyde (SAL) esters.[14] While these protocols enable ligation, there are limitations associated with each, namely lengthy reaction times[12] and a reliance on high substrate concentrations in non-aqueous media.[12b,13,14] Recently, a procedure for the conversion of Cys to Ser has been developed by Okamoto and Kajihara.[15] Although this is a powerful transformation, the requirement for multiple steps (including intermediary HPLC purifications) and the use of toxic reagents (e.g. CNBr)[15] to promote the desired substitution are considerable drawbacks. Concurrent to this work, Bode and co-workers described an effective ligation at Ser through application of an oxazetidine amino acid derivative in ketoacid–hydroxylamine ligation

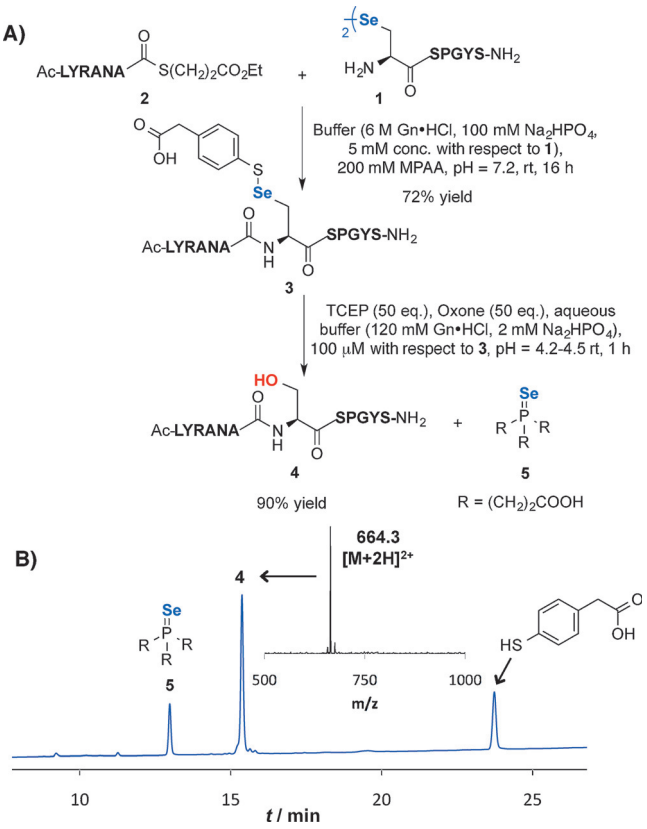


Scheme 1. Ligation–deselenization at A) Sec and B) β-selenol Phe. C) Proposed Sec-mediated ligation followed by Sec to Ser conversion.

chemistry.[16] In this study, we describe the development of an operationally simple Sec to Ser conversion under mild conditions in a single post-ligation transformation. This disclosure is an important addition to the currently available tools for peptide and protein ligation and underscores the unique chemical diversity offered by the strategic incorporation of Sec residues into target peptides and proteins.

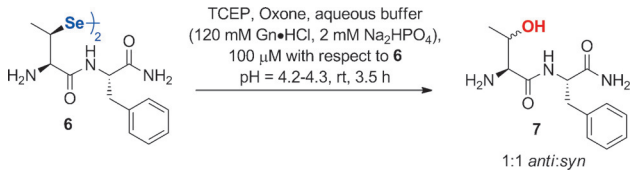
Having attributed the minor hydroxylation pathway in our β-selenol Phe work (Scheme 1B) to dissolved oxygen in the reaction media, our initial examination of the proposed Sec to Ser conversion began with the treatment of selenopeptides with TCEP in oxygen-saturated buffer (see Figure S2, Supporting Information). Under these conditions, we observed the desired transformation together with Sec to Ala conversion, akin to standard deselenization, as well as a number of backbone cleavage byproducts. Difficulties in optimizing the use of oxygen gas to maintain precise control of stoichiometry prompted the exploration of an alternative oxidant. Our focus turned to Oxone,[17] a commercially available, bench stable and water-soluble oxidant. Importantly, there is also precedence for the use of Oxone in peptide chemistry and the compatibility of the reagent with proteinogenic amino acid side-chains.[18]

We began our studies by treating model selenopeptides (on analytical scales) with excess TCEP (50 equiv) and Oxone (50 equiv) at a variety of concentrations and pH values (see Supporting Information, Figure S3–S5). We found that optimal conditions involved first solubilizing the peptide in denaturing buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, 5 mM with respect to the selenopeptide) followed by dilution with H_2O to give a final peptide concentration of 100 μM before the simultaneous addition of TCEP (50 equiv) and Oxone (50 equiv). At final concentrations of selenopeptide greater than 100 μM, we observed some deselenization to yield the corresponding Ala residue, while reactions also proceeded smoothly at peptide concentrations lower than 100 μM. The reaction was also tolerant of a wide range of pH values (pH 4–7). To probe the reaction on a preparative scale, we ligated selenopeptide **1** and thioester **2** (Ac-LYRANA-SR) in the presence of excess 4-mercaptophenylacetic acid (MPAA) to afford peptide **3** as the selenyl-MPAA sulfide in 72% isolated yield (Scheme 2A). Upon treatment of peptide **3** with the optimized Sec to Ser conditions (50 equiv TCEP, 50 equiv Oxone, aqueous buffer: 120 mM Gn·HCl, 2 mM Na_2HPO_4, final concentration of peptide **3** = 100 μM, pH 4.2–4.5), we were pleased to observe rapid ($t < 1$ h) and clean conversion to the desired Ser-containing product **4**, which was isolated in 90% yield following HPLC purification, along with the formation of the TCEP-derived phosphine selenide **5** (Scheme 2B). Importantly, the identity and stereochemical integrity of peptide **4** was confirmed by ^{1}H NMR analysis through comparison with authentic peptide standards bearing both D- and L-Ser at the ligation junction (see Figure S45, Supporting Information). It should also be noted that this operationally simple oxidative deselenization protocol proceeded in an open-air flask without the need for tedious solvent degassing.



Scheme 2. A) Ligation of selenopeptide **1** and thioester **2** and Sec to Ser conversion. B) Crude analytical HPLC trace ($t = 1$ h) of Sec to Ser conversion of peptide **3** to afford product **4** (0 to 50% MeCN over 30 min, $\lambda = 230$ nm).

Intrigued by the mechanistic considerations of this new transformation, we prepared model dipeptide dimer **6** bearing an unnatural N-terminal β-methyl L-Sec analogue (Scheme 3, see Supporting Information for synthetic details), with *R*-configuration at the β-position, to enable investigation of the stereochemical outcome at this site upon reaction with TCEP and Oxone. Treatment of **6** under the optimized Sec to Ser conditions revealed a complete loss of stereochemical integrity at the β-position to afford **7** as a 1:1 mixture of the L-Thr and L-*allo*-Thr products. The loss of stereochemistry at the β-position (but not the α-position) is consistent with an alkyl radical intermediate rather than the more common selenium oxidation-nucleophilic displacement or oxidation-elimination pathways. In addition, formation of phosphine selenide **5** (see Scheme 2) is consistent with the radical-based mechanisms proposed in the phopshine-mediated desulfurization of Cys[5b] and deselenization of Sec.[9]

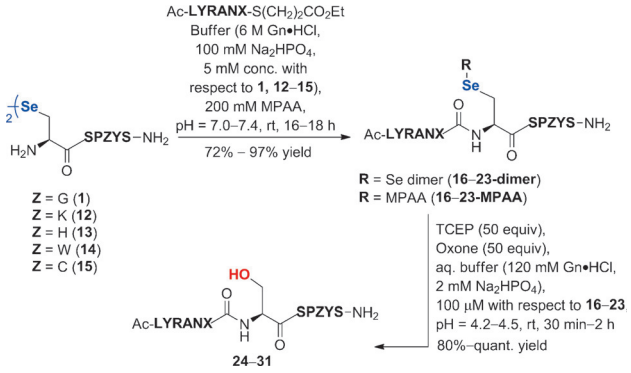


Scheme 3. Synthesis of dipeptide **6** to probe the Sec to Ser mechanism.

To probe the scope of the new reaction, we next prepared a number of selenopeptide substrates by ligation chemistry between model thioesters **2**, **8**–**11** and model selenopeptides **1**, **12**–**15** (see Supporting Information and Table 1), bearing a range of functionality and oxidation prone amino acids (e.g. Met, Phe, Trp, His, Lys and Cys). In all cases, Sec-mediated ligations proceeded in less than 18 h to afford the desired products (**16**–**23**), as the selenyl-MPAA sulfide adducts (e.g. **16-MPAA**) and/or the corresponding symmetrical diselenide dimers (e.g. **16-dimer**), in high isolated yields (72–97%, see Table 1 and Supporting information for details). Ligation products were next treated with TCEP and Oxone under the optimized conditions. Gratifyingly, reactions proceeded in high isolated yields in all cases (80%–quant.) to afford the target Ser-containing peptides (**24**–**31**, see Table 1). Both the MPAA ligation adducts and the diselenide dimers were found to be suitable substrates for the Sec to Ser conversion (see Supporting Information for details). In most cases, reactions were complete in less than 1 h after a single dose of TCEP and Oxone. Due to the competing inactivation of TCEP through oxidation by Oxone, however, some substrates required an additional dose (10 equiv TCEP and 10 equiv Oxone) to reach completion (see entries 3 and 5, Table 1).

The transformation was found to be compatible with peptides bearing internal Lys, His and Trp residues (entries 6–8), which proceeded without unwanted side-chain oxidation. Unsurprisingly, Sec to Ser conversion of ligation product **17-MPAA**, bearing an internal Met residue, led to concomitant oxidation of the Met thioether side-chain to the corresponding sulfoxide. However, facile reduction to the native Met side-chain was achieved in quantitative yield using NH_4I-dimethyl sulfide in TFA (trifluoroacetic acid), robust reduction conditions described by Hackenberger (see Supporting Information).[19] The presence of a Cys residue in the target peptide sequence (entry 9) reduced the rate of the Sec to Ser transformation, which reached only 50% conversion after the first dose of TCEP and Oxone. However, a second dose of TCEP (50 equiv) and Oxone (50 equiv) enabled complete consumption of the ligation product to yield the target Sec to Ser product **31** in 80% yield together with 20% conversion to the corresponding Ala peptide, as determined by HPLC-MS analysis. Given that a model peptide bearing a Cys residue at the ligation junction (in the absence of Sec) was completely inert to the oxidation conditions (see Figure S23, Supporting Information), we hypothesized that conversion of Sec to Ala in the presence of Cys might be proceeding through a standard deselenization pathway (see Scheme 1 A), whereby the putative alanyl radical intermediate is appropriately positioned for rapid intramolecular H-abstraction from the neighboring Cys thiol moiety. An investigation into the effect of the relative positioning of Sec and Cys within a target peptide on the outcome of the Sec to Ser transformation further supports the likelihood of an intramolecular H-abstraction event. For four model polypeptides with differential spacing between the Sec and Cys residues (spaced 4, 9, 15 or 27 amino acids apart, see Supporting Information for details), treatments with TCEP and Oxone led to increasing amounts of Ala byproduct

Table 1: Scope of the Sec to Ser conversion.



Entry	USPZYS	Thioester (X)	Ligation product/ Yield [%][a]	Sec to Ser Product/ Yield [%][a]
1	**1**: Z = **G**	**2:** X = **A**	**3:** 72	**4:** 90
2	**1**: Z = **G**	**8:** X = **G**	**16:** 93	**24:** 83
3	**1**: Z = **G**	**9:** X = **M**	**17:** 77	**25:** 97[b,c]
4	**1**: Z = **G**	**10:** X = **F**	**18:** 93	**26:** 93
5	**1**: Z = **G**	**11:** X = **L**	**19:** 76	**27:** quant.[b]
6	**12**: Z = **K**	**10:** X = **F**	**20:** 73	**28:** 93
7	**13**: Z = **H**	**2:** X = **A**	**21:** 74	**29:** 95
8	**14**: Z = **W**	**2:** X = **A**	**22:** 97	**30:** 80
9	**15**: Z = **C**	**2:** X = **A**	**23:** 85	**31:** 80[d]

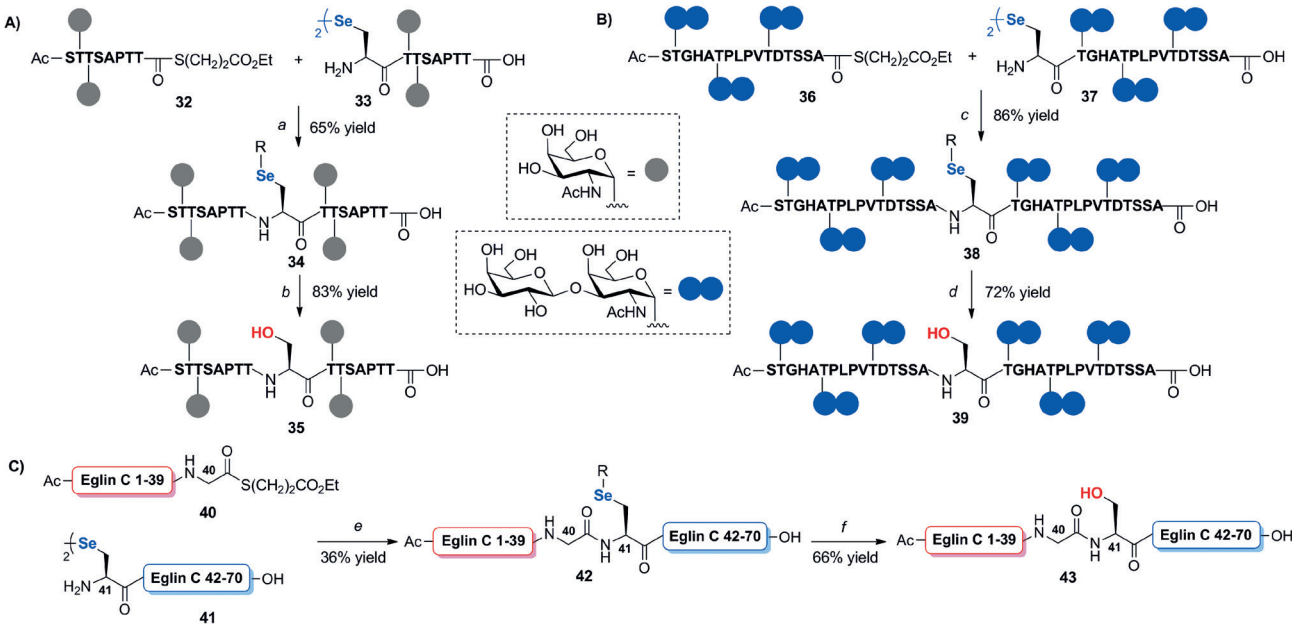
[a] Yields of isolated product. [b] Additional TCEP (10 equiv) and Oxone (10 equiv) added at $t = 1$ h. [c] Product of the Sec to Ser conversion possessed an oxidized Met residue. Reduction to the native peptide product was achieved in quant. yield under the following conditions: NH_4I (20 equiv), dimethyl sulfide (20 equiv), TFA, 0°C, 30 min.[19] [d] Additional TCEP (50 equiv) and Oxone (50 equiv) added at $t = 1$ h; product isolated along with 20% of a product corresponding to Sec to Ala conversion.

formation (relative to the target Ser product) as the internal Cys residue was placed closer along the peptide backbone to the Sec residue. Interestingly, peptides with Cys residues in close proximity to Sec also resulted in lower overall conversions of the selenopeptide starting material, suggesting that the intramolecular proximity of an H-atom donor may also inhibit the rate of the transformation. Conversely, the lack of observed Ala formation or significant effect on reaction rate in an intermolecular competition study between a Sec-containing model peptide and a Cys analogue (see Figure S24, Supporting Information) suggests that the corresponding intermolecular process is unlikely.

Given our interest in exploiting the over-expression and aberrant glycosylation of mucin epithelial proteins for the purpose of developing novel synthetic cancer vaccine candidates,[20] we were next interested in utilizing the Sec to Ser transformation to rapidly access multiple copies of the serine-rich variable number tandem repeat (VNTR) sequences of the MUC5AC and MUC4 glycoproteins. Each target peptide would bear a defined glycosylation pattern, consisting of multiple copies of the disaccharide (T) or monosaccharide (T_N) tumor-associated carbohydrate antigens (TACAs). To this end, we first synthesized, via divergent Fmoc-SPPS, the target MUC5AC thioester **32** and MUC5AC selenopeptide **33**, both of which are derived from the eight amino acid MUC5AC VNTR sequence and bear two copies each of the Thr-derived T_N antigen (see Scheme 4 and Supporting Information). Peptides **32** and **33** were then ligated to afford selenyl-MPAA sulfide **34** after 38 h and in 65 % isolated yield. Ligation product **34** was subjected to the optimized Sec to Ser conditions to afford peptide **35**, a MUC5AC VNTR dimer, containing a native Ser residue at the ligation junction, in an excellent 83 % isolated yield.

MUC4 thioester **36** and selenopeptide **37**, each decorated with three copies of the Thr-linked T antigen disaccharide, were next synthesized and ligated. The reaction was complete in 24 h to afford glycopeptide **38**, bearing a selenyl-MPAA sulfide, in an excellent 86 % isolated yield. Upon two simple treatments with Oxone and TCEP, we were pleased to observe the clean conversion of peptide **38** to the corresponding MUC4 32-mer **39** (Scheme 4 B), comprised of two MUC4 VNTRs and six copies of the Thr-derived T antigen (72 % isolated yield).

To probe the utility of the new transformation in the synthesis of a protein target, we finally embarked upon the synthesis of eglin C, a structurally ordered serine protease inhibitor derived from the leech *Hirudo medicinalis*.[21] Recombinantly, the *N*-acetylated protein is produced,[22] which is functionally equivalent to the native protein. As a Cys-free protein, eglin C maintains a three-dimensional structure through a network of electrostatic interactions rather than disulfide bridges and is highly resistant to denaturation.[21] The lack of Cys residues in this 70-amino acid target, however, also precludes its construction using traditional native chemical ligation methods. There are also no centrally placed Ala residues to facilitate a ligation-desulfurization-based assembly. As such, only Cys mutants of eglin C, in which the native Ser residue at position 41 has been replaced by a Cys residue (S41C), have been accessible using ligation chemistry.[23] Taking advantage of the centrally located Ser-41, we envisaged the first ligation-based approach to the native sequence using a tandem Sec ligation–Sec to Ser



Scheme 4. A) Synthesis of MUC5AC VNTR dimer, *a* = 6 M Gn·HCl, 0.1 M Na_2HPO_4, 5 mM conc. of peptide **33**, 200 mM MPAA, pH 7.2, 38 h, *b* = TCEP (3 × 50 equiv), Oxone (3 × 50 equiv), aqueous buffer (120 mM Gn·HCl, 2 mM Na_2HPO_4), 100 μM conc. of peptide **34**, RT, 6 h; B) Synthesis of MUC4 VNTR dimer, *c* = 6 M Gn·HCl, 0.1 M Na_2HPO_4, 5 mM conc. of peptide **37**, 200 mM MPAA, pH 7.2, 24 h, *d* = TCEP (2 × 50 equiv), Oxone (2 × 50 equiv), aqueous buffer (120 mM Gn·HCl, 2 mM Na_2HPO_4), 100 μM conc. of peptide **38**, RT, 3.5 h; C) Synthesis of eglin C, *e* = 6 M Gn·HCl, 0.1 M Na_2HPO_4, 200 mM MPAA, 70 mM TCEP, 210 mM TCEP = Se, 50 mM sodium ascorbate, 30 °C, pH 6.5, 30 h, *f* = TCEP (5 × 50 equiv), Oxone (5 × 50 equiv), 6 M Gn·HCl, 0.1 M Na_2HPO_4, 100 μM conc. of peptide **42**, pH 4.8–4.9, RT, 6 h; R = 4-mercaptophenylacetic acid.

conversion. Accordingly, we first prepared thioester **40** (eglin C 1–40) and Sec-containing peptide **41** (eglin C 41–70) using Fmoc-SPPS (Scheme 4C). Adoption of Sec-ligation conditions recently reported by Melnyk and co-workers,[24] which utilize a more reducing environment (denaturing buffer in the presence of 200 mM MPAA, 70 mM TCEP, 210 mM TCEP=Se and 50 mM sodium ascorbate), afforded the MPAA-ligation product **42** in 36% isolated yield. Initial subjection of peptide **42** to the Sec to Ser transformation resulted in substantial peptide cleavage byproducts as well as products consistent with Sec to Ala conversion and off target oxidation. However, upon carrying out the reaction entirely in denaturing buffer (6M Gn·HCl/0.1M Na_2HPO_4, pH 4.8–4.9), we were pleased to observe clean conversion of peptide **42** to the target Ser-containing product **43** upon treatment with TCEP and Oxone (5 × 50 equiv each). We hypothesize that in the case of eglin C, high concentrations of chaotropic salts serve to disrupt sequence-specific electrostatic interactions and expose the internal Sec residue, which appears to be a critical factor for efficient oxidative deselenization transformations. Following Sec to Ser conversion, the full-length protein was isolated in 66% yield. Interrogation of the structure of **43** using circular dichroism revealed structural elements consistent with an authentic sample of recombinant eglin C (see Figure S42 and S43, Supporting Information). In addition, the synthetic protein exhibited inhibitory activity against both cathepsin G and elastase, albeit less than that measured for the recombinant protein (see Figure S44, Supporting Information). Future work in our laboratory will further probe the utility of the oxidative deselenization reaction for the synthesis of folded protein targets.

In summary, we have disclosed an unprecedented and mechanistically distinct oxidative deselenization reaction for the conversion of Sec to Ser in a single-step within unprotected peptides and proteins. The high-yielding and operationally simple protocol enables programmed ligations at Ser junctions in aqueous media (with and without chaotropic salts), under mild conditions. We have demonstrated the compatibility of the transformation at a range of pH values and probed the remarkable chemoselectivity of the transformation in the presence of potentially reactive proteinogenic amino acid side chains. Extension of the methodology to MUC5AC and MUC4 VNTR glycopeptide targets and in the total synthesis of the Cys-free protein eglin C further confirms the compatibility of the reaction protocol with complex (glyco)peptide and protein systems and highlights the utility of the method as a complement to native chemical ligation for the construction of Cys-free protein targets. We further envisage that the mild reaction conditions and wide functional group tolerance may render this novel transformation generally applicable for the synthesis of functionalized small molecules. Studies of this nature are currently underway in our laboratory.

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