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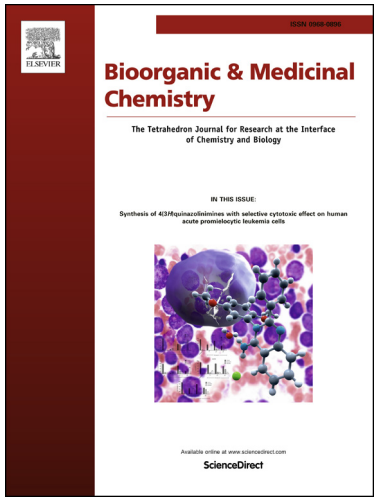

Revisiting ligation at selenomethionine: insights into native chemical ligation at selenocysteine and homoselenocysteine

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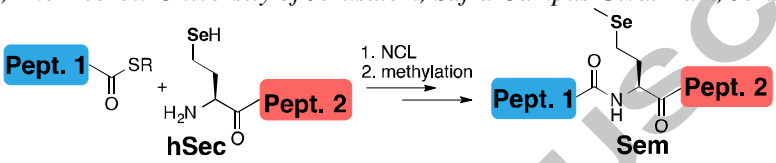

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ABSTRACT

Selenomethionine (Sem) has been incorporated recombinantly into proteins many times to elucidate their structure and function. In this paper, we revisit incorporation via chemical protein synthesis to shed light on the mechanism of native chemical ligation. The effect of chalcogen position on ligation is investigated, and selenium-containing peptide ligation is optimized. Additionally, selective methylation is performed on selenolates in a peptide in the presence of unprotected thiols.

1. Introduction

In protein chemistry, certain amino acids serve uniquely useful roles in elucidating the structure, and hence the function, of the proteins in which they reside. One such example is selenomethionine (Sem), the selenium-containing analog of methionine (Met). A product of plants' processing selenium in minerals, selenomethionine is an essential source of selenium for many forms of life.[1,2] Besides its nutritional value in nature, selenomethionine has been used by scientists for years as a tool in both NMR[3] and crystallographic studies[4] in order to uncover the structure and function of proteins. In addition, a Sem and *p*-cyanophenylalanine pair was recently used to probe protein structure, wherein Sem quenches the fluorescence of *p*-cyanophenylalanine via electron transfer, providing a sensitive fluorescent probe to uncover helical structures in proteins.[5] A recent study[6] utilizes a biocompatible, redox-based approach to apply chemoselective modifications to Met; Se's even lower redox potential makes Sem an interesting candidate for future applications.

Sem is undoubtedly a useful tool for protein study, and it is generally introduced into the protein sequence through recombinant expression. However, such an approach replaces all Met residues in the protein sequence with Sem. In addition, not all proteins are easily expressed recombinantly. For these cases, chemical protein synthesis (CPS) can be advisable. CPS, which relies heavily on solid-phase peptide synthesis (SPPS),[7] allows for the building of proteins amino acid by amino acid, and easily enables the chemist to swap out any amino acid in the sequence with natural or unnatural moieties. For shorter peptides and proteins, SPPS can be the sole method employed. For longer sequences, however, ligation of unprotected protein segments using methods such as native chemical ligation (NCL)[8] can be utilized.

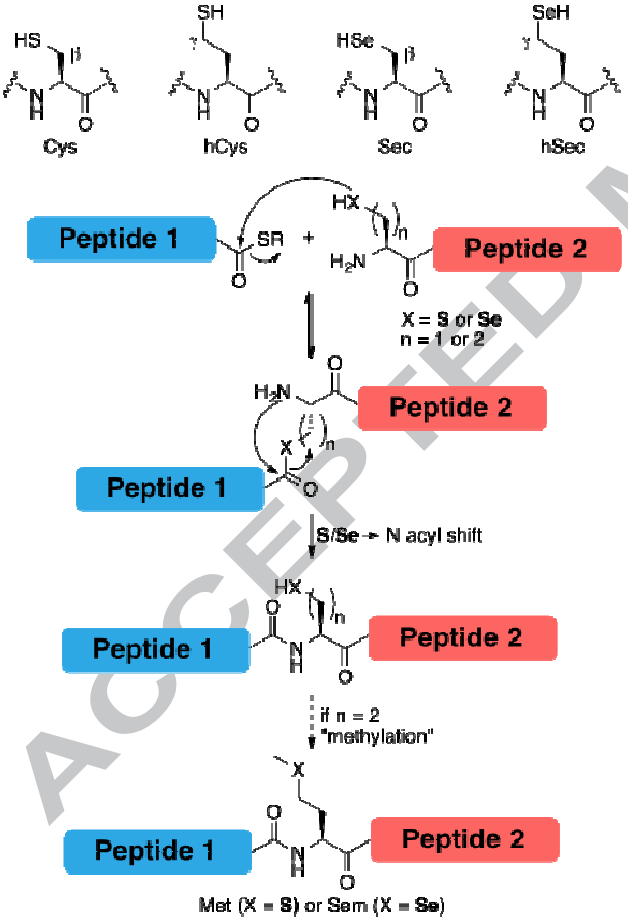
NCL allows for the joining of two unprotected peptide segments under mild conditions. In its proposed mechanism, the sulfur of an N-terminal Cys on one peptide attacks the C-terminal, labile thioester of the other segment. A subsequent, irreversible S-to-N acyl shift yields a native peptide bond (Scheme 1). Cys's close relative, selenocysteine (Sec, U)[9–11] is equally capable of participating in NCL; this discovery has since been used in the synthesis of natural and unnatural selenoproteins to shed light on protein folding[12,13] and other aspects of protein chemistry.[14] As a response to Sec and Cys's relatively low occurrence in protein sequences, a variation on NCL was developed in which the thiol of Cys was removed following ligation to yield Ala.[15,16] Similar strategies were used to enable NCL using removable, thiol-containing auxiliaries[17–23] or the use of moieties with thiolated side chains.[24–34] More complex, multistep syntheses employing thiazolidine[35] or selenazolidine[36] "masked precursors" have also been employed to access difficult-to-synthesize proteins.

In a similar vein, NCL was expanded to methionine.[37,38] The initial ligation was performed at homocysteine (hCys, hC) rather than Cys, and subsequently methylated to give the native methionine at the ligation site (Scheme 1). However, this method was not useable on proteins containing Cys residues, as Cys's lower pK_a meant it would surely undergo unwanted methylation under the described conditions. In 2003, Roelfes and Hilvert

reported successful NCL at Sem using a homoselenocysteine (hSec, hU) precursor,[39] and since that time, to the best of our knowledge, these findings were not utilized in CPS. However, we believe that with optimization, ligation at Sem can provide a much-needed tool in contemporary protein study.

Upon revisiting the paper, we chose to use it as a doorway to further insight in CPS. Firstly, we noted that in ligations at Met and Sem, the chalcogen attacking the thioester is in a γ, rather than β, position on the amino acid side-chain. We wanted to investigate further the effect this imposed on the ligation reaction, and what light it shed on the proposed mechanism of NCL. Secondly, under Roelfes and Hilvert's reported conditions, the ligation of hSec and a thioester was complete after five days.[39] In all likelihood, this can be attributed to the sensitivity of selenols to air-oxidation and formation of stable diselenides.[40] We believed that this time could be improved using recent discoveries made by our group. Thirdly, after noting the different pK_a's of selenols and thiols,[41,42] we wanted to show selective methylation of hSec to Sem was possible in the presence of unprotected thiols. This chemoselective methylation is not possible when methylating hCys to Met, making the case for performing NCL at Sem rather than Met even more powerful and useful.

In this paper, we revisit ligation at selenomethionine to address these points and better understand the nature of NCL.



***Scheme 1**. NCL at Cys, hCys, Sec, and hSec. Methylation after NCL at hCys and hSec will provide Met and Sem.*

2. Results and Discussion

In order to investigate the effects of β- vs. γ-chalcogens on the overall rate of NCL, a series of small peptides was designed. ZRAFS (Z = Cys, hCys, Sec, hSec) peptides were synthesized to allow analysis of the effect of the β- vs. γ-chalcogen position on ligation as well as to see how thiol and selenol ligations differed under our selected conditions. In addition, LYRAX-COSR (X = Gly, Leu, Val) peptides were synthesized to see how rate of reaction varied with steric hindrance of the thioester.[43] In accordance with standard protocol, all ligations were performed with 3 mM peptides at pH slightly above 7 and in the presence of thiol catalyst (250 mM MPAA).[44] Normally, TCEP is also present in reactions with Cys in order to avoid oxidation of the thiols and encourage faster reactions. However, TCEP is known to remove selenium in Sec,[9,45,46] so TCEP is not normally used in Sec-ligations. This reason, combined with Sec's comparatively low redox potential[40] in comparison to Cys, and its tendency to form stable diselenides under ambient conditions, mean that Sec ligations proceed rather slowly in ambient conditions.[9–11] Luckily, in a recent paper,[46] we reported that sodium ascorbate, a mild radical quencher, successfully hinders the deselenization reaction. For this reason, we included 50 mM TCEP and 100 mM sodium ascorbate in all ligations.

Not surprisingly, ligations involving the sterically unencumbered LYRAG-COSR proceeded the fastest, with all reactions over 90% complete in under two hours (Figures 1a, S1-S4, S17). No discernible difference in rate was apparent in any of the Gly ligations; β- and γ-chalcogens appeared to ligate at similar rates. Remarkably, both selenol and thiol containing peptides ligated at identical rates, showing that the provided conditions can successfully reduce ligation times at the previously sluggish Sec and hSec.[39]

Ligations performed at the bulkier LYRAL-COSR did take longer – most ligations were complete between two and four hours after beginning (Figures 1b, S5-S8, S18). While overall rates did seem similar, we noted that ligation at hCys was only 70% complete at two hours when compared to all other ligations, which were over 80% complete. This small but significant difference cannot be explained by different energetics of a five- vs. six-membered transition state of the reaction. Rather, the small difference in rates here is more likely due to differences in pK_a's. Cys is known to have a pK_a of 8.3,[41] whereas hCys has a slightly higher pK_a of 8.9.[42] At ligation pH of 7, a larger percentage of Cys (5.6%) compared to hCys (1.2%) will be deprotonated, allowing it to perform the necessary first step of the reaction, nucleophilic attack at the thioester, slightly faster. In this vein, Sec and hSec ligations should be significantly faster due to their low pK_a's.[41] However, their lower redox potential,[40] and resulting sensitivity to oxidation and formation of diselenides, as explained below, serves to slow their ligation rate significantly.

Our final set of ligations, performed at the quite sterically hindered, β-branched Val in LYRAV-COSR, took significantly longer (Figure 1c, S9-S12, S19).[43] Both ligations involving thiols were complete within 24 h, and, as a result, our selenol-containing peptides were left to react for similar periods of time. However, at 8 hours, some degree of deselenization was observed at Sec and hSec ligations (URAFS and hURAFS, respectively), and at 24 hours an even greater amount of deselenization was seen. Taking this as an indication that some amount of sodium ascorbate had decomposed over the longer course of the reaction, we increased the concentration of this radical quencher to 200 mM, a fourfold excess over the reductant and deselenization reagent, TCEP. We were pleased to note that this large excess of sodium ascorbate was capable of inhibiting deselenization to a satisfactory degree for 24 h. Still, selenol-containing peptides

were significantly slower in ligating when compared to thiol-containing peptides, with the selenol-containing peptides being

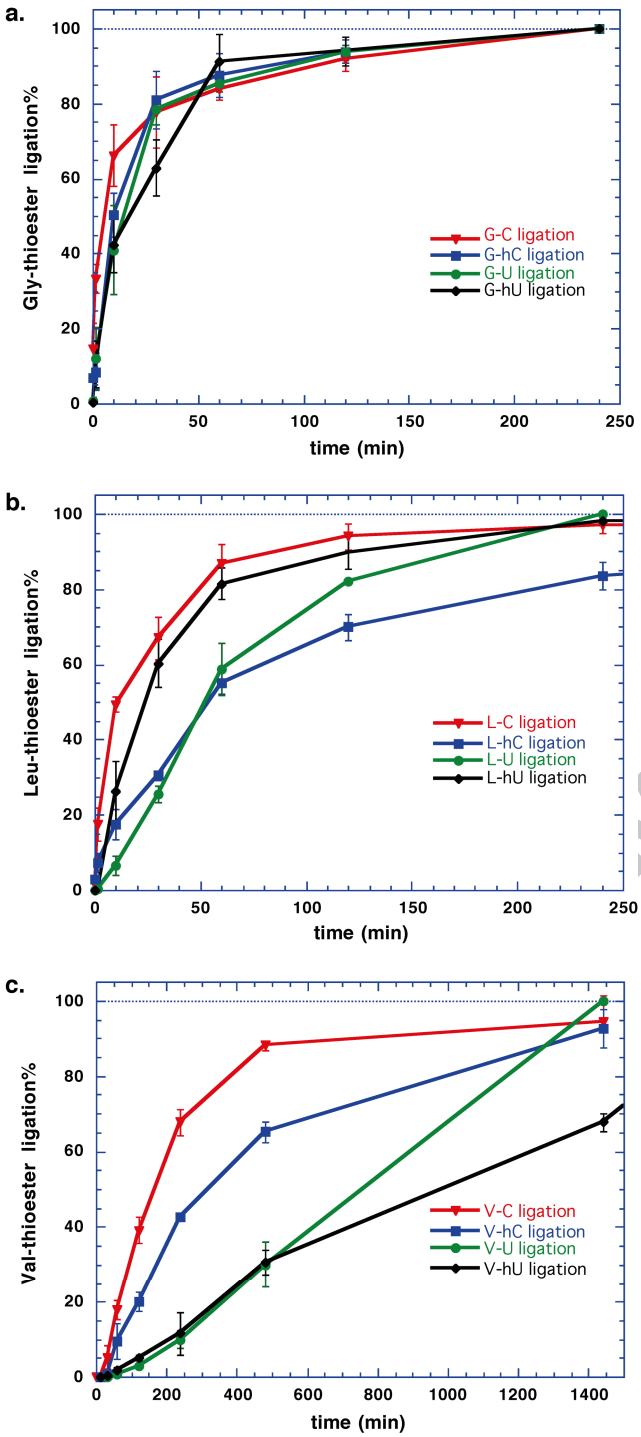


***Figure 1**. Comparison of Cys, hCys, Sec, and hSec ligations at Gly, Leu, and Val thioesters. In (**a-c**) the s.d. values were calculated from experiments done in triplicate and the lines connecting the data points are shown only for clarity and do not represent a data fit. Comparison of ZRAFS peptides over time can be seen in in Fig. S13-S16.*

only 30% consumed at 8 hours of reaction and the thiol-containing peptides, over 60% (Figure 1c).

This phenomenon may be explained when the entire system of the ligation reactions is considered. TCEP reduces the diselenide bond to a selenolate – according to the pK_a of Sec, 5.2,[41] the vast majority of selenols will be deprotonated at pH 7. The selenolate must immediately perform a nucleophilic attack on a thioester in solution to avoid being immediately re-oxidized by ambient oxygen present in the system. The rapid rate of re-oxidation is a problem only for selenolate; thiols have a much higher redox potential and are not as rapidly oxidized.[40] In relatively unhindered thioesters, such as those adjacent to Gly or Leu, the nucleophilic attack occurs rapidly enough that there is no observed difference between S and Se ligations. However, the bulky Val causes the nucleophilic attack to proceed much more slowly. Thus, the overall rate of Se ligations to Val-adjacent thioesters is doubly slow – hindered by both the difficulty for Sec or hSec to attack at the bulky, β-branched amino acid and the subsequent re-oxidation of Sec or hSec to a diselenide, which must be reduced again by TCEP to re-initiate ligation.

On the whole, a difference between the rate of ligation of chalcogens at either β- or γ-positions was observed in the two sets of longer ligations (at Leu and Val), but the difference was small and hence more easily attributed to a difference in pK_a, rather than in the kinetics of transition state. Nevertheless, we were able to reduce the ligation time of selenol-containing peptides to be similar to that of thiol-containing peptides. In addition, we found that the radical quencher sodium ascorbate has a tendency to degrade over time in traditional ligation conditions, and must be present in high concentrations to prevent deselenization in the presence of TCEP for overnight reactions.

In order to test the selectivity of selenol methylation in the presence of thiols – a property never investigated in previous works – a model peptide with sequence Ala-His-**hSec**-Ser-Tyr-Lys-Trp-Cys-Asp-Met-Ala-NH_2, which contains all potentially sensitive residues, was synthesized and subjected to various methylation conditions. All methylation studies were performed in 0.2 M phosphate buffer with 6 M guanidinium hydrochloride, 50 mM TCEP, and 300 mM sodium ascorbate. We first investigated the effect of providing different concentrations of the methylating agent, 4-methyl nitrobenzene sulfonate (MNBS) at pH 5 (Figures S20-S22). Reactions were performed in 1, 10, and 100 mM MNBS (to 1 mM peptide). As expected, the 1 mM reaction was the slowest and the 100 mM was fastest. However, unwanted side-reactions such as deselenization and Sem oxidation were observed at the highest concentration of MNBS. As a result, we chose to use 10 mM MNBS – the middle ground – in all reactions going forward.

We next studied the effect of temperature on methylation; reactions were performed at 25, 37, and 50 °C at pH 6 (Figures S25-27). Deselenization, due to the presence of TCEP in solution, was observed in all reactions. Reactions performed at 37 and 50 °C showed levels of deselenization that were unacceptable (20% deselenized at 4 h at 37 °C, and 35% deselenized at 4 h at 50 °C), while the reaction at 25 °C was more stable (8% deselenized at 4 h), although slower.

Further, we investigated selectivity of the methylation reaction as a function of pH. As methylation proceeded quite slowly at pH 5, we chose to investigate the methylation reaction at pH 6, 7, and 8 with 10 equiv MNBS and at 25 °C (Figures S23-S25). At pH 6, the peptide was approximately 5% dimethylated at 2 h; this number did not change when the reaction was checked at 4 h and 8 h. In comparison, the reaction at pH 7 showed 8% dimethylation at 8 h and 43% dimethylation at pH 8 at 8 h (Figure 2). In accordance with previous studies,[45] deselenization was also observed to increase with pH. While the peptide was only 6% deselenized at pH 6 after 8 h, it was 8% deselenized at pH 7 and 38% deselenized at pH 8 at the same time point. During

screenings for MNBS equivalents at pH 5, dimethylation (methylation of the selenol and the thiol) was not detected. Selectivity of the methylation reaction was confirmed via trypsin digest [Figure S28].

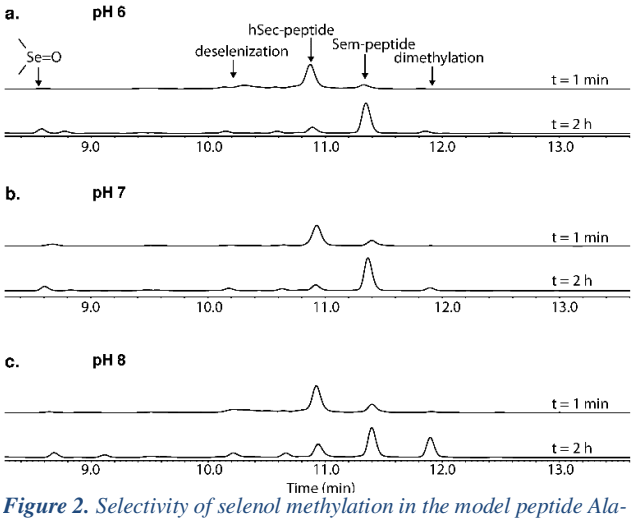


Figure 2. *Selectivity of selenol methylation in the model peptide Ala-His-**hSec**-Ser-Tyr-Lys-Trp-Cys-Asp-Met-Ala-NH_2. Comparisons of chromatograms show the dramatic effect of pH on hSec methylation in the model peptide.*

It was thus determined that pH, rather than temperature or equivalents of methylating reagent, has the most influence over the rate of methylation of hSec to Sem. This is easily explained by the different pK_a's of the amino acids. As mentioned above, Cys's pK_a of 8.3[41] and hCys's pK_a of 8.9[42] mean that the majority of both thiols are protonated, thus not susceptible to methylation, at pH's 6, 7, or 8 investigated here. The significantly lower pK_a's of Sec, 5.2,[41] and hSec, which has not been formally determined but likely is 3 units lower than hCys (~6), mean that deprotonated selenolates undergo methylation at pH's in which thiols are still protonated and thereby unreactive.

With the optimized conditions for hSec ligation and methylation in hand, we then chose to apply our findings in the synthesis of the post-translationally modifying protein, NEDD8. NEDD8 is a relatively small ubiquitin-like protein (76 amino acids) with a Met at position 50, which we chose to substitute with Sem. NEDD8 was prepared from two peptide segments: NEDD8(2-49)-COSR, which was prepared using an *N*-acylurea precursor to a thioester[47,48] and NEDD8(50-76)(Met50hSec). NEDD8(2-49)-COSR was prepared in 3% yield, and NEDD8(50-76)(Met50hSec) was prepared in similar yield, 4%, which was attributed to the poor coupling of Fmoc-hSec(Mob)-OH. Ligation was performed under conditions similar to those used on the short peptides – 3 mM of each peptide segment, 50 mM TCEP, and 100 mM sodium ascorbate in a 0.2 M phosphate buffer containing 6 M guanidinium hydrochloride and 250 mM MPAA. We realized that the ligation conditions were similar to those applied in our methylation experiments, and we were pleased to note that, following ligation, hSec50 could be methylated in a one-pot manner for a total yield of 28%. In comparison, purification of ligated product and subsequent methylation resulted in an overall yield of 26%.

3. Conclusions

In this work, we used the little-studied ligation at selenomethionine as a stepping-stone into understanding more about the reactions connected to it. We were able to determine that the number of atoms in the ring transition state of native chemical ligation has no observable impact on the overall rate of reaction. This supports the proposed mechanism, in which the rate-determining step of NCL is the attack of the thioester by thiol or selenol. Indeed, we were able to observe the impact of steric hindrance in the significantly longer ligation times at Val when compared to ligations at Leu and Gly. The impact of Val's bulkiness was compounded when ligations with the easily oxidized Sec or hSec were performed – these ligations took significantly longer than ligations at Val thioester with Cys or hCys. For less bulky amino acids, we successfully found conditions that reduced the ligation time of the easily-oxidized selenol to equal that of thiols.

Additionally, by exploiting unique properties of selenols – most importantly their lower pK_a values – we were able to determine which conditions allowed for selective methylation of hSec in the presence of unprotected Cys as well as other reactive amino acid side chains. We applied optimized ligation and methylation conditions in the synthesis of the NEDD8 protein, with satisfactory yields.

4. Experimental

1.1. Homoselenocysteine Synthesis

Boc- and Fmoc-hSec(Mob)-OH were synthesized from selenomethionine following previously published procedures.[39,49]

1.1.1. Homoselenocystine

3.06 g selenomethionine (15.6 mmol) was stirred in 150 mL liquid ammonia at -78 °C. Small pieces of 0.9 g sodium metal (39 mmol, 2.5 equiv) were added slowly over the course of 20 minutes, and the reaction was stirred for 1 h. 3.8 g solid NH_4Cl was added to neutralize any $NaNH_2$ in solution. The reaction was stirred in the fume hood at room temperature overnight to evaporate any remaining NH_3, leaving a yellow solid. 80 mL H_2O was added and stirred to form a suspension. N_2 gas was bubbled through and passed through a wash bottle filled with bleach to remove any Me_2Se. pH was lowered to 1 with concentrated HCl, then 40 mL H_2O was removed via rotary evaporation. Solution was adjusted to pH 6 with 2 N NaOH and stirred for 30 minutes. The resulting yellow precipitate was filtered and lyophilized to dryness. 1.77 g of light yellow solid homoselenocystine was retrieved (62% yield).

1.1.2. Se-p-methoxybenzyl-homoselenocysteine hydrochloride

Under Ar atmosphere, 0.5 g of homoselenocystine (1.39 mmol) was suspended in 2 mL 0.5 N NaOH and cooled to 0 °C in an ice bath. 0.45 g $NaBH_4$ (11.7 mmol, 8.4 equiv) in 4 mL H_2O was added dropwise over 15 minutes to the cooled, stirring solution. The solution was adjusted to pH 6 with glacial acetic acid, then 371 µL 4-methoxybenzyl chloride (2.71 mmol, 1.95 equiv) was added in one aliquot. The solution was stirred for 1.5 h at 0 °C. The solution was adjusted to pH 1-2 with concentrated HCl. The white precipitate was filtered and washed with a small amount of H_2O, then lyophilized to dryness. 414 mg of white solid was obtained (quantitative yield) and taken to the next step without further purification.

1.1.3. N^{α}-tBoc-Se-p-methoxybenzyl-homoselenocysteine

290 mg NH_2-hSec(Mob)-OH•HCl (0.86 mmol) was dissolved in a solution of 224 mg $NaHCO_3$ (2.66 mmol, 3 equiv) in 5.8 mL H_2O at 0 °C. A solution of 307 µL Boc-anhydride (1.33 mmol, 1.5 equiv) in 5.8 mL dioxane was added dropwise to the stirring solution over the course of 1 hour. The solution was allowed to

warm to room temperature and stir overnight. The reaction was extracted once with 12 mL ether. The ether layer was washed once with saturated aqueous $NaHCO_3$. The aqueous layers were combined and stirred over ice. 1 N HCl was added slowly, due to CO_2 formation, until pH 1. Product was extracted twice in ethyl acetate, which was dried over $MgSO_4$ and evaporated. The resulting yellowish oil was redissolved in a combination of H_2O and ACN, then lyophilized to give 202 mg yellow powder (0.503 mmol, 59% yield) of Boc-Sec(Mob)-OH.

1.1.4. N^{α}-(9H-Fluoren-9-ylmethoxycarbonyl)-Se-p-methoxybenzyl homoselenocysteine

418 mg (1.23 mmol) NH_2-hSec(Mob)-OH•HCl salt was dissolved in 5 mL of 10% m/v $NaHCO_3$ in H_2O and cooled to 0 ^{o}C. A solution of 415 mg (1.23 mmol, 1 equiv) Fmoc-ONSu was dissolved in 3.5 mL dioxane and added dropwise to the cold, stirring solution. The reaction was warmed to room temperature and stirred overnight, forming a white solid. The mixture was extracted twice with ether, and the aqueous layer was adjusted to pH 1-2 with concentrated HCl. This was extracted three times with ethyl acetate. The ethyl acetate layers were combined and washed twice with 1 N HCl and twice with H_2O, then dried over $MgSO_4$. The solvent was evaporated and the yellow oil was resuspended in H_2O and ACN, then lyophilized to give 391 mg beige powder (61% yield) of Fmoc-Sec(Mob)-OH, which was characterized by ^{1}H- and ^{13}C-NMR [Figures S34-35].

1.2. Peptide Syntheses

1.2.1. General Peptide Synthesis

All peptides were synthesized according to standard Fmoc-SPPS procedure, manually or on an automated peptide synthesizer. Unless otherwise indicated, Tentagel Rink Amide Resin was used and syntheses were performed on a 0.25 mmol scale. Standard deprotections were performed twice in 20% piperidine in DMF for five minutes each time. For manual synthesis, Fmoc-amino acids (1 mmol, 4 equiv) were activated for 3 minutes with HATU or HCTU (1 mmol, 4 equiv in 2.5 mL DMF) and DIEA (2 mmol, 8 equiv in 2.5 mL DMF), then shaken to couple for 30 minutes. For automated synthesis, Fmoc-amino acids (2 mmol, 8 equiv) were activated for 3 min with HATU or HCTU (2 mmol, 8 equiv in 5 mL DMF) and DIEA (4 mmol, 16 equiv in 5 mL DMF), then shaken to couple for 30 minutes. Amino acids containing selenium (0.375 mmol, 1.5 equiv) and OxymaPure (0.375 mmol, 1.5 equiv) were dissolved in 5 mL 1:1 DMF:DCM, cooled to 0^{o}C, and then activated with DIC (0.35 mmol, 1.4 equiv) for 5 minutes and shaken to couple for 2-4 hours.

For cleavage of 200 mg resin, 13 mL cleavage cocktail was prepared (95% TFA, 2.5% H_2O, 2.5% TIPS). If Cys or hCys was present in the sequence, a cocktail consisting of 94% TFA, 2.5% H_2O, 1% TIPS, and 2.5% EDT was used. Cocktail was added to peptidyl-resin and shaken for 3 h. The resin was removed by filtration, and N_2 gas was bubbled through to remove TFA, followed by addition of cold ether to precipitate peptide. Mixture was shaken to create one phase, then chilled at -20 ^{o}C for 30 minutes before centrifugation at 5000 RPM for 10 minutes. After decanting ether, peptide was resuspended in 50% H_2O and 50% ACN and lyophilized to dryness. At this point, peptides containing selenium underwent a second cleavage in neat TFA and 2 equiv DTNP for 3 h,[50] followed by similar treatment described above to give dry, lyophilized product. The resulting crude peptide was dissolved in either aqueous ACN or phosphate buffer containing guanidinium hydrochloride and purified via preparative RP-HPLC.

1.2.2. Peptide Thioester Synthesis

Peptide thioesters were synthesized according to the procedures outlined in the literature from an *N*-acylurea precursor.[47,48] Mono-Fmoc-(3,4)-diaminobenzoic acid (Fmoc-Dbz-OH) or Fmoc-3-amino-4-(methylamino)benzoic acid (Fmoc-MeDbz-OH) (4 equiv) was activated with HATU (4 equiv) and DIEA (8 equiv) in DMF for 3 minutes, then coupled to the deprotected resin for 2 hours. Following peptide synthesis, the peptidyl-resin was washed once in DMF, then twice in DCM. A solution of *p*-nitrophenyl chloroformate (5 equiv) in DCM (4 mL/0.1 mmol) was added, shaken for 1 hour, and then drained and washed well with DCM. This step was repeated two more times, after which a solution of 0.5 M DIEA in DMF (2 mL/0.1 mmol) was added and shaken for 30 min to complete the cyclization process. The peptide was cleaved from resin and converted to a thioester without undergoing any additional purification.

Crude peptide-Nbz was dissolved in phosphate buffer (0.2 M, 6 M GdmCl, pH ~ 7) at 3-10 mM concentration. Methyl ester of 3-mercaptopropionic acid (MMP) was added (5% v/v) and the solution was incubated at room temperature for 1-48 h and monitored via HPLC. Once reaction was deemed to be complete, the peptide-thioester was purified via RP-HPLC.

1.2.3. Synthesis of LYRAX Peptides

1.2.3.1. LYRAG-MMP

LYRAG-MMP was synthesized from an Me-Dbz precursor on a 0.1 mmol scale. Following peptide synthesis, Nbz formation was performed as described above to give LYRAG-MeNbz-resin. The resin was cleaved according to general procedure and afforded crude LYRAG-MeNbz (65 mg). The cleaved peptide was treated with MMP according to the described procedure to give the corresponding LYRAG-MMP. This peptide was purified by prep HPLC (15%-25% B over 55 min). 32.3 mg (47% yield) pure peptide was obtained, and its purity was verified by analytical HPLC.

1.2.3.2. LYRAL-MMP

LYRAL-MMP was synthesized from a Dbz precursor on a 0.25 mmol scale. Following peptide synthesis, Nbz formation was performed as described above to give LYRAL-Nbz-resin. The resin was cleaved according to general procedure and afforded crude LYRAL-Nbz (164 mg). 55.5 mg of cleaved peptide was treated with MMP according to the described procedure to give the corresponding LYRAL-MMP. This peptide was purified by prep HPLC (15%-40% B over 55 min). 23.7 mg (38% yield) pure peptide was obtained, and its purity was verified by analytical HPLC.

1.2.3.3. LYRAV-MMP

LYRAV-MMP was synthesized from a Dbz precursor on a 0.25 mmol scale. Following peptide synthesis, Nbz formation was performed as described above to give LYRAV-Nbz-resin. The resin was cleaved according to general procedure and afforded crude LYRAV-Nbz (216.4 mg). 54.6 mg cleaved peptide was treated with MMP according to the described procedure to give the corresponding LYRAV-MMP. This peptide was purified by prep HPLC (15%-40% B over 55 min). 22.7 mg (50% yield) pure peptide was obtained, and its purity was verified by analytical HPLC.

1.2.4. Synthesis of ZRAFS Peptides

1.2.4.1. CRAFS-NH_2

CRAFS-NH_2 was synthesized from TentaGel R RAM Resin on a 0.1 mmol scale and cleaved according to general procedure to give 74.3 mg crude peptide, which was purified by prep HPLC (8%-33% B over 55 min). 54 mg pure peptide was obtained (93% yield), and its purity was verified by analytical HPLC.

1.2.4.2. hCRAFS-NH_2

hCRAFS-NH_2 was synthesized from TentaGel R RAM Resin on a 0.1 mmol scale according to general procedure. 1.5 equiv Fmoc-hCys(Trt)-OH was activated with 1.5 equiv HATU and 3 equiv DIEA in DMF for 3 min, then shaken with peptidyl-resin for 1.5 h to couple. Peptidyl-resin was cleaved according to general procedure to give 74.3 mg crude peptide, which was purified by prep HPLC (8%-33% B over 55 min). 45 mg pure peptide was obtained (75% yield), and its purity was verified by analytical HPLC.

1.2.4.3. URAFS-NH_2

URAFS-NH_2 was synthesized from TentaGel R RAM Resin on a 0.1 mmol scale according to general procedure. 1.5 equiv Boc-Sec(Mob)-OH was activated with 1.5 equiv OxymaPure and 1.4 equiv DIC in 1:1 DCM:DMF for 5 minutes at 0 °C, then shaken with peptidyl-resin for 2 hours to couple. Peptidyl-resin was cleaved according to general procedure to give 102.7 mg crude peptide. This peptide was dissolved in aqueous acetonitrile and treated with 1:2 NaAsc:TCEP prior to purification in preparative RP-HPLC (3%-28% B over 55 min). 34 mg pure peptide (54% yield) was obtained, and its purity was verified by analytical HPLC.

1.2.4.4. hURAFS-NH_2

hURAFS-NH_2 was synthesized from TentaGel R RAM Resin on a 0.1 mmol scale according to general procedure. 1.2 equiv Boc-hSec(Mob)-OH was activated with 1.2 equiv OxymaPure and 1.1 equiv DIC in 1:1 DCM:DMF for 5 minutes at 0 °C, than shaken with peptidyl-resin for 2 hours to couple. Peptidyl-resin was cleaved according to general procedure to give 101.3 mg crude peptide. This peptide was dissolved in aqueous acetonitrile and treated with 1:2 NaAsc:TCEP prior to purification in preparative RP-HPLC (9.5%-34.5% B over 55 min). 35 mg pure peptide (54% yield) was obtained, and its purity was verified by analytical HPLC.

1.2.5. Synthesis of AlkPep

AlkPep (Ala-His-**hSec**-Ser-Tyr-Lys-Trp-Cys-Asp-Met-Ala-NH_2) was synthesized from TentaGel R RAM Resin on a 0.1 mmol scale according to general procedure. 1.5 equiv Fmoc-hSec(Mob)-OH was activated with 1.5 equiv OxymaPure and 1.4 equiv DIC in 1:1 DCM:DMF for 5 minutes at 0 °C, then shaken with peptidyl-resin for 2 hours to couple. Peptidyl-resin was cleaved according to general procedure to give 114.4 mg crude peptide. This peptide was dissolved in aqueous acetonitrile and treated with 1:2 NaAsc:TCEP prior to purification in preparative RP-HPLC (9.5%-34.5% B over 55 min). 20.6 mg pure peptide (15% yield) was obtained, and its purity was verified by analytical HPLC.

1.3. Ligations of Peptides – general procedure

Ligations were performed at 3 mM concentration of each peptide in 6 M GdmCl and 0.2 M phosphate buffer, with the addition of 50 mM TCEP and 200 mM sodium ascorbate (NaAsc) with 250 mM MPAA. The buffer was adjusted to pH 7.1 prior to addition of peptides. 10 uL aliquots of reaction mixture were removed at predetermined time points and quenched in 50-70 uL of 1% TFA in a 1:1 H_2O:ACN solution before HPLC analysis. The yields are calculated based upon the integration of the HPLC-traces under consideration of the extinction coefficients. (ε280 (peptide thioester) = ε280 (cysteine peptide) = 1280 $L \cdot mol^{-1} \cdot cm^{-1}$; ε280 (ligation product) = 2560 $L \cdot mol^{-1} \cdot cm^{-1}$).[51]

1.4. Methylation of AlkPep

1.4.1. Optimized Procedure

Methylations were performed at 1 mM concentration of peptide with 10 mM methyl nitrobenzene sulfonate (MNBS) in an aqueous buffer containing 6 M GdmCl and 0.2 M phosphate buffer with 10% acetonitrile and at 25 °C. 50 mM TCEP and 300 mM NaAsc were dissolved in the buffer and pH was adjusted to 6 prior to addition of peptide.

1.4.2. Equivalents MNBS Screen

Methylations were performed at 1 mM concentration of peptide at room temperature as described above with 1, 10, or 100 mM MNBS in an aqueous buffer containing 6 M GdmCl and 0.2 M phosphate buffer with 10% acetonitrile. 50 mM TCEP and 300 mM NaAsc were dissolved in the buffer and pH was adjusted to 5 prior to addition of peptide.

1.4.3. Temperature Screen

Methylations were performed at 1 mM concentration of peptide with 10 mM MNBS in an aqueous buffer at pH 6 as described above at three different temperatures: 25°, 37°, and 50°C.

1.4.4. pH Screen

Methylations were performed at 1 mM concentration of peptide with 10 mM MNBS at 25°C in an aqueous buffer as described above with the pH adjusted to 6, 7, or 8 prior to addition of peptide.

1.5. Synthesis of NEDD8(Met50Sem) Protein

NEDD8(Met50Sem) was synthesized from two segments: NEDD8(2-49)-COSR and NEDD8(50-76)(Met50hSec). The two were ligated and the product was methylated to give the final NEDD8(Met50Sem).

1.5.1. NEDD8(2-49)-COSR

NEDD8(2-49)-COSR was synthesized on a 0.125 mmol scale on an automated peptide synthesizer using *N*-acylurea precursor Dbz on TentaGel R RAM Resin, converted to Nbz, and cleaved from resin as described above. 410 mg of crude NEDD8(2-49)-Nbz was obtained. The dry peptide was dissolved in 15.2 mL buffer (6 M GdmCl, 0.2 M phosphate buffer) and 800 µL MMP was added. Reaction was observed to be complete after 5 h, and purified using preparative RP-HPLC, in 20%-45% B over 55 minutes (Figure S29). 20.7 mg pure NEDD8(2-49)-MMP (3% yield) was obtained.

1.5.2. NEDD8(50-76)(Met50hSec)

NEDD8(50-76)(Met50hSec) was synthesized on Fmoc-Gly Wang Resin at a 0.25 mmol scale on an automated peptide synthesizer according to procedures described above. The final amino acid, Fmoc-hSec(Mob)-OH, was coupled using OxymaPure and DIC as described. Cleavage was performed in two steps. First, peptidyl-resin was shaken for 2 h in 14 mL cleavage cocktail (2.5% TIPS, 2.5% H_2O, 95%TFA). Peptide was

dried and then shaken for 3 hours in TFA with 2 equiv DTNP. 200 mg of the 600 mg crude peptide was purified via prep RP-HPLC (22%-57% B over 55 minutes) giving 30 mg pure peptide (4% yield) (Figure S30).

1.5.3. Ligation

10 mg of NEDD8(2-49)-MMP and 5 mg of NEDD8(50-76)(Met50hSec) were dissolved in 577 µL (to about 3 mM of each peptide) of a buffer containing 6 M GdmCl, 0.2 M phosphate buffer, 50 mM TCEP, 100 mM NaAsc, and 250 mM MPAA. The pH was adjusted to 7.1 and the reaction was allowed to proceed overnight.

1.5.4. One-pot ligation and methylation

Following overnight ligation, 100 µL of the reaction mixture was taken and 90 µL of fresh buffer containing 6 M GdmCl, 0.2 M phosphate buffer, 50 mM TCEP, and 100 mM NaAsc were added. 12.5 µL 1 M MNBS in ACN was added to the mixture to give a final solution of 1.5 mM peptide, 75 mM MNBS, and 7.5% ACN in aqueous buffer. After 22 h, methylation was still not complete. An additional 12.5 µL 1 M MNBS was added and reaction was completed overnight (Figure S33) and purified via semi-prep RP-HPLC (Figure S32). 0.7 mg final product was obtained, for an overall yield of ligation and methylation of 28%.

1.5.5. Two-step methylation and ligation

NEDD8(2-76)(Met50hSec) was purified via semi-prep RP-HPLC (Figure S31). This was methylated according to the optimized procedure described above and again purified via RP-HPLC (Figure S32). In total, 3.2 mg of material was recovered, for an overall yield of ligation and methylation of 26%.

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Supplementary Material

Supplementary data to this article can be found in the online version.