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Chemoselective Copper-Mediated Modification of Selenocysteines in Peptides and Proteins

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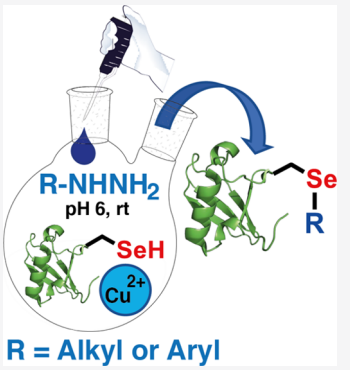
ABSTRACT: Highly valuable bioconjugated molecules must be synthesized through efficient, chemoselective chemical modifications of peptides and proteins. Herein, we report the chemoselective modification of peptides and proteins via a reaction between selenocysteine residues and aryl/alkyl radicals. *In situ* radical generation from hydrazine substrates and copper ions proceeds rapidly in an aqueous buffer at near neutral pH (5−8), providing a variety of Se-modified linear and cyclic peptides and proteins conjugated to aryl and alkyl molecules, and to affinity label tag (biotin). This chemistry opens a new avenue for chemical protein modifications.



INTRODUCTION

Protein post-translational modifications (PTMs) play a prominent role in expanding proteins function and enable the precise modification of proteins with a diverse range of functional moieties.[1] Although PTMs are normally facilitated by enzymatic processes, chemists have turned their attention to the development of regio- and chemoselective chemical modifications of proteins based on metal or organic catalysis. In recent years we have witnessed significant progress in the field, as many research groups have contributed to a rich chemical toolbox with precise and highly chemoselective reactions,[2] most of which work in aqueous solutions and under ambient conditions.[3−7] Among the 20 canonical amino acids, cysteine (Cys) is still the most extensively targeted for chemical modifications owing to its high nucleophilicity and low abundance (1.7% of known sequences).[8−12] This includes classical thiol−ene chemistry,[13] typical nucleophilic substitution reactions with electrophiles,[8−10,14,15] or transition-metal-mediated modifications.[16−19] Because these reactions require a free Cys residue, site selectivity among multiple free Cys residues in the protein sequence has eluded protein chemists (Figure 1a).[20] Therefore, it is important to develop complementary protein modification methods based on other amino acid residues that are chemoselective, even in the presence of Cys residues.

Selenocysteine (Sec, U), the 21st natural amino acid,[21] is Cys's isostere and shares many of its properties, with notable differences including lower pK_a[22,23] and reduction potential.[24−26] These differences have been explored in chemical protein synthesis,[27−29] modification,[30,31] and folding.[32−40] Similar to Cys, Sec's inherent nucleophilicity makes it a good target for chemical modification in peptides and proteins.[9,11,15,30,41,42] As a result, Sec is readily oxidized to diselenide (Se−Se) or selenylsulfide (Se−S); to generate active selenol for downstream modification, a reducing reagent, such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP), is typically needed (Figure 1a). However, both reagents reduce disulfide cross-links as well, and TCEP is

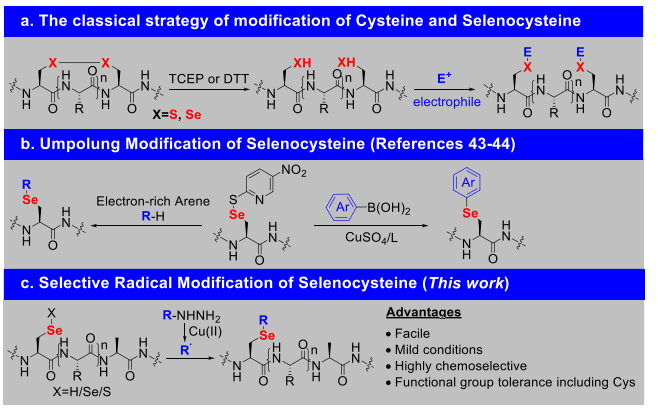


Figure 1. Modification of Sec/Cys in peptides and proteins.

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known to cause undesired deselenization.[27] Both outcomes would interfere with the intended modification reactions. Recently, Pentelute, Buchwald, and co-workers exploited the electrophilicity of oxidized Sec, and using arylboronic acids with either copper catalysts or electron-rich aromatic conjugates, they showed regio- and chemoselective modification of oxidized Sec in peptides and proteins (Figure 1b).[43,44] Yet, in these reports the modification was not tested in the presence of other reactive side chains (such as disulfide bonds). Furthermore, it is extremely desirable to develop highly selective bioconjugation techniques that are based on new chemistries.

Here, we report a previously unexplored, chemoselective Sec modification with a series of hydrazine compounds in the presence of copper ions. This strategy exploits the efficient generation of an aryl/alkyl radical from hydrazine substrates in the presence of Cu(II),[45−48] which readily reacts with Sec in peptides and proteins to provide the desired corresponding conjugates (Figure 1c).

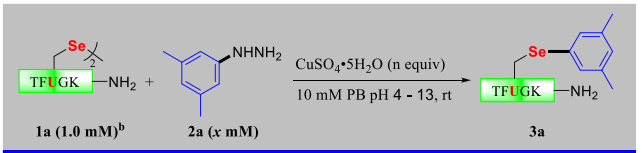
Recently, we reported on the use of Cu(II) ions for the deprotection of selenazolidine (Sez) and thiazolidine (Thz) during chemical protein synthesis[49−51] and proposed that Cu(II) acts as a Lewis acid that binds the selenium of Sez. In addition, Arsenyan's group reported the use of Cu(II) to bind and oxidize the Se atom of Ph_2Se_2, converting it to the electrophilic $PhSe^+$, which in turn reacted with triple-bond-containing molecules.[52] Almost half a century ago, it was reported that Cu(II) (and other metal ions) can oxidize phenylhydrazine to generate a phenyl radical, which can react with biomolecules *in vivo*.[45,53,54] Of note, the deselenization reaction of Sec to Ala[27,28] (and Ser)[28,55] in the presence of TCEP was proposed to go through a radical mechanism, owing to the ability of Se atoms to form radicals under mild conditions.[56] Combining these observations, we envisioned generating radical intermediates from hydrazine reagents with Cu(II), enabling efficient, chemoselective modification of Sec residues in peptides and proteins (Figure 1c).

RESULTS AND DISCUSSION

Optimization of the Reaction Conditions. To test our hypothesis, a Sec-containing model peptide, **TFUGK-NH$_2$** dimer (**1a**), was prepared by standard Fmoc-SPPS,[57−59] and 3,5-dimethylphenylhydrazine (**2a**) was employed as the model substrate. Combining model peptide (**1a**, 1.0 mM for the selenol monomer) with 2 mM **2a** and 0.5 equiv of $CuSO_4 \cdot 5H_2O$ in phosphate buffer (PB, 10 mM, pH 6) at room temperature provided the desired product **3a** in 70% yield in 10 min (Table 1, entry 1). The yield increased to 95% with 1.0 equiv of $CuSO_4 \cdot 5H_2O$ (entry 2), but was not improved further with more Cu(II) (entry 2, Figure S2, Supporting Information). Expectedly, the yield of **3a** decreased to 60% (entry 4) and 80% (entry 5) when the concentration of hydrazine **2a** was decreased to 1.2 and 1.5 mM, respectively (Figure S3). Varying pH conditions (entries 2, 6−10, Figure S4) showed that slightly acidic conditions (pH 6, entry 2) gave the optimized yield of **3a**.

Scope of Hydrazines in the Reaction. With the optimized reaction conditions in hand, we next investigated the scope of hydrazine derivatives tolerated in the modification reaction. As shown in Figure 2, we tested the reaction between the model peptide **1a** and aromatic hydrazine substrates with electron-donating groups on the phenyl ring, such as 3,5-dimethyl (**2a**) and *p*-methoxyl (**2b**) substituents (Figure 2a).

Table 1. Optimization of the Reaction Conditions[a]



entry	$CuSO_4$ (equiv)	[hydrazine] (mM)	pH	yield[c] (%)
1	0.5	2.0	6	70
2	1.0	2.0	6	95
3	2.0	2.0	6	95
4	1.0	1.2	6	60
5	1.0	1.5	6	85
6	1.0	2.0	4	65
7	1.0	2.0	5	91
8	1.0	2.0	7	91
9	1.0	2.0	10	87
10	1.0	2.0	13	24

[a]Further details can be found in the Supporting Information. [b]1.0 mM of reduced peptide. [c]Yields calculated according to integrated areas of HPLC peaks (at 220 nm).

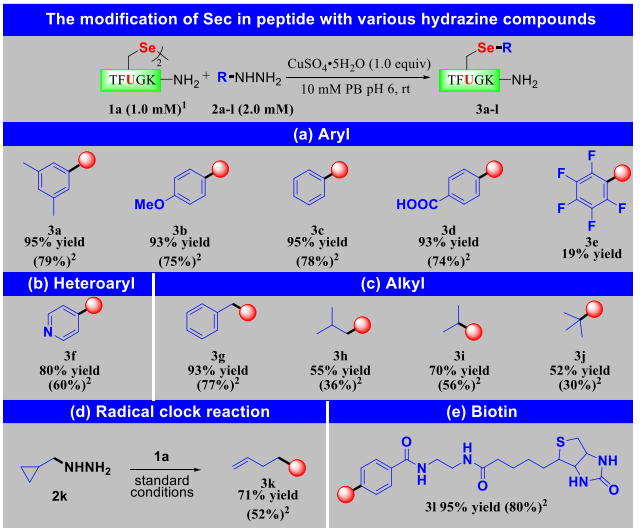


Figure 2. Substrate scope of hydrazines. [1]1.0 mM with respect to the selenol monomer. (a) Arylhydrazines: The reactions for **3a**−**3d** were performed at room temperature, and the yields were obtained after 10 min, whereas strong electron-deficient **3e** was obtained after 3 h. (b) Heteroarylhydrazine: The reaction performed at 37 °, and the yield was obtained after 2 h. (c) Benzyl and alkylhydrazines: The reaction performed at 37 °C; the yields of **3g** and **3h** were obtained after 1 h, and for **3i**, **3j**, and **3k** they were obtained after 2 h. (d) Radical-clock reaction. (e) Biotinylation of the model peptide **1a** under optimized conditions. All yields presented were determined by integrated areas of HPLC peaks (at 220 nm), [2]whereas isolated yields are in parentheses.

Both reactions were completed within 10 min and generated the corresponding products with excellent yields (**3a** and **3b**, Figures S4 and S5). Unsubstituted phenylhydrazine (**2c**) reacted with model peptide **1a** to form the modified product (**3c**) with 95% conversion (Figure S6), implying that the

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electron-donating groups on the phenyl ring are not required for the modification of Sec residue in peptides. Furthermore, 4-hydrazineylbenzoic acid (**2d**), which contained a weak electron-withdrawing group (*p*-carboxyl), was also found to be effective under optimized conditions (Figure S7). However, when the extremely electron-poor perfluorophenylhydrazine (**2e**) was used as a substrate, the strong electron-withdrawing substituents slowed down the reaction, and only 19% conversion of modified product (**3e**) was observed after prolonged incubation (3 h, Figure S8). As a heteroarene, 4-hydrazineylpyridine (**2f**) was also tolerated in this reaction system and afforded the desired product (**3f**) in good yield within 2 h (Figure 2b and Figure S9).

Given that the diversity of substrates is one of the most challenging targets in *in vitro* peptide modification, we decided to test the applicability of alkyl hydrazine substrates, which are less reactive than aromatic hydrazine because of the unstable radical intermediates[60] in this reaction. We were delighted to find that all tested hydrazines including benzyl, isobutyl, isopropyl, and *tert*-butyl hydrazines proceeded smoothly to form the modified products in moderate-to-good yields (52−93%, **3g**−**3j**) and within 1−3 h (Figure 2c and Figures S10−S13). Notably, the reaction of the model peptide **1a** with isobutylhydrazine (**2h**) provided the isobutyl-modified product **3h**, whereas the reaction with *tert*-butylhydrazine (**2j**) provided the *tert*-butyl product **3j**. This was supported by HPLC data (Figure S13) and unequivocally confirmed with NMR analysis (Figures S11 and S13) of the two products, **3h** and **3j**, suggesting that the radical combination reaction was so rapid that any undesired 1,2-rearrangement of isobutyl radical, which could occur to generate stabilized *tert*-butyl radical,[61] had not taken place. Furthermore, (cyclopropylmethyl)hydrazine (**2k**, Figure 2d) reacted with the model peptide **1a** to afford the exclusive cyclopropane-opened modified product **3k** (Figure S14), which supports the formation of radical intermediates in this transformation.[62] Lastly, the biotin affinity tag (**2l**, see Scheme S2 for synthesis details) was successfully introduced to the model peptide (**1a**) within 10 min by our developed protocol (Figure 2e and Figure S15).

Mechanistic Study by Electron Paramagnetic Resonance (EPR). For further support of the likely radical mechanism, we followed the arylation reaction by EPR. The clean formation of a DMPO-Ph spin adduct was obtained when the model seleno-peptide **TFUGK-NH$_2$** dimer (**1a**) reacted with phenylhydrazine (**2c**) and $CuSO_4$ (Figure 3), as well as when just **2c** and $CuSO_4$ were used alone (Figure S17). The hyperfine values (g = 2.00552, a_N = 15.9227 G, and a_H = 24.6433 G) are consistent with those of a carbon radical being trapped by the DMPO and agree with Hill and Thornalley, who ascribed them to the phenyl radical.[45] In addition, we studied three peptides containing Se (**TFUGK-NH$_2$**, **1a**), S (**LKFCAG-NH$_2$**, **1c**), and neither Se nor S (**ALKFAG-NH$_2$**, **1b**), first analyzing the effect of varied molar ratios of peptide/hydrazine/$CuSO_4$, 1:2:1 and 1:1:1 (Figure S20, top row and middle row, respectively), in which DMPO was added last. No obvious difference of the signal of the DMPO-Ph spin adduct was observed in these cases. However, when the ratio of 1:0.5:1 was tested, the signal of the DMPO-Ph spin adduct was largest with **1a** (Figure 3c, green) and then with **1c** (Figure 3e, green) and was smallest with **1b** (Figure 3d, green). Furthermore, the signal intensity of the DMPO-Ph spin adduct increased dramatically (approximately 100%) when **2c** was the last component added in the cases of **ALKFAG-NH$_2$** (**1b**, Figure 3d, black) and **LKFCAG-NH$_2$** (**1c**, Figure 3e, black), whereas the signal intensity increase of the DMPO-Ph spin adduct was much smaller than that for **TFUGK-NH$_2$** (**1a**, Figure 3c) (approximately 30%) when **2c** was the last component added. These results suggest that the Se atom interacts with the formed Ph radical and perhaps partially stabilizes it, thus increasing the radical lifetime until it is able to react with DMPO to form the DMPO-Ph spin adduct. Sulfur is partially able to stabilize the Ph radical, whereas when no Se or S are present, the Ph radical lifetime is very short because of the reactions with water and other radicals (further EPR experiments can be found in the Supporting Information).

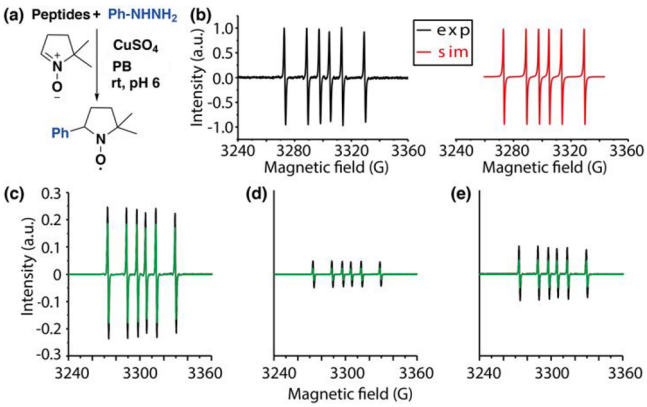

Figure 3. EPR experiments support the radical reaction. (a) Scheme of the trapping of phenyl radical by DMPO. EPR spectrum of DMPO-Ph spin adduct, formed during the reaction of the peptides **1a**−**1c**, phenylhydrazine **2c**, and $CuSO_4$, with a ratio of 1:2:1 (b) and 1:0.5:1 (c−e). (b) Peptide: **TFUGK**, **1a**, black for experimental, red for simulated. (c) Peptide: **TFUGK**, **1a**. (d) Peptide: **ALKFAG**, **1b**. (e) Peptide: **LKFCAG**, **1c**. Black lines in (c−e): Phenylhydrazine **2c** was added last. Green lines in (c−e): DMPO was added last.

In light of the above results, we propose the mechanism shown in Scheme 1. R-NHNH$_2$ (**2**) is oxidized to R$^\bullet$ by Cu^{2+}, which binds to the Se of Sec in the peptide.[45−48,52] The formed R$^\bullet$ reacts with diselenide (or selenylsulfide) to generate the intermediate trivalent selenium radical (**3**),[63,64] which converts to the final product (**4**) and another Cys peptide (**3-II**) or another Sec radical (**3-I**), which could in turn form dimer (**1**) for the next reaction.

Chemoselectivity Study. Next, we turned to study the tolerance of this modification on unprotected amino acid side chains. First, in the absence of Sec residue, the peptide **ALKFAG-NH$_2$** (**1b**) was inert to the reaction with phenylhydrazine (**2c**) under optimized conditions (Figure 4a and Figure S21). Because of similar properties of sulfur and selenium, the chemoselectivity of this reaction was tested. Thus, peptide **LKFCAG-NH$_2$** (**1c**) showed 10% Cys-modified products (Figure S22), whereas 13% of the Met modification product was obtained for **LKMAG-NH$_2$** (**1d**) (Figure 4a and Figure S23), both of which were observed only after an extended time (2−18 h) when compared to that of the Sec reaction (<10 min).

To establish the versatility of this methodology, the modification of more complex peptide substrates (**1e** and **1f**, Figure 4b), which contained various functional groups, was also evaluated. When the modification of peptide **1e** was conducted under standard conditions, the modified product (**4e**) was obtained in 16% yield. Yet, the yield of product **4e** increased to 73% when the concentration of phenylhydrzaine

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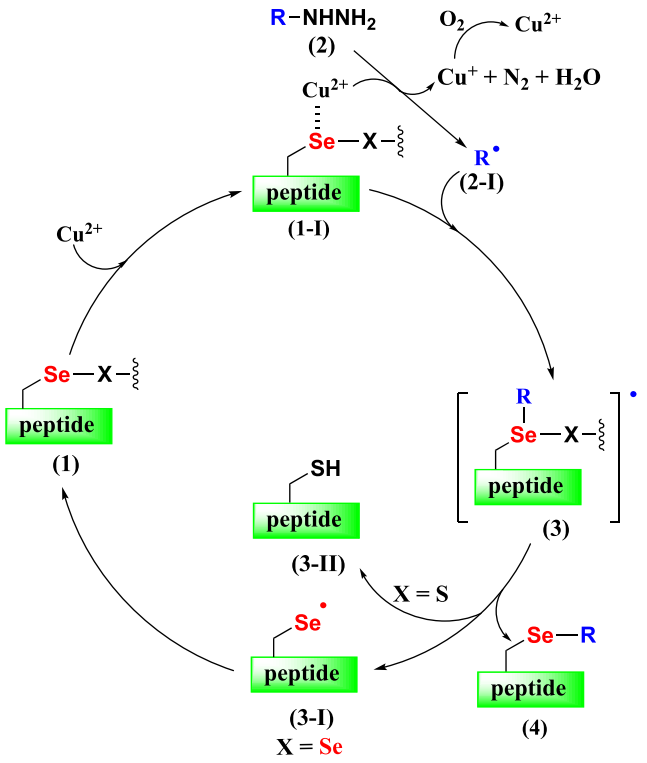
Scheme 1. Proposed Mechanism for Radical Modification of Sec by Hydrazine in the Presence of Cu Ions



(**2c**) was increased to 5 mM, the amount of $CuSO_4$ was increased to 2 equiv, and the reaction was incubated for 30 min (Figure 4b). Note that even with excess phenylhydrazine (**2c**) used in this reaction, only a minor degree of arylation at the Cys residue (~3%) was observed (Figure S24). Moreover, trypsin digestion of **4e** confirmed the site-selective modification of Sec residue of the peptide (Figure S28). The radical conjugation was similarly successful in a variety of more unusual circumstances. Because peptide **1f** contains a Met residue at its N-terminus, which can be easily oxidized (to Met(O)), we decided to do the reaction under argon. Hence, Cu(II) was provided in excess, to compensate for the absence of molecular oxygen (typically required for the hydrazine oxidation step, Scheme 1). Therefore, using 8 equiv of $CuSO_4$, peptide **1f** reacted with phenylhydrazine (**2c**) to afford the corresponding monoarylated product (**4f**) in 93% yield (Figure 4b and Figure S25), with no side reactions observed on other functional groups in the peptide. Furthermore, the modification of head-to-tail cyclic peptide **1g** proceeded smoothly to form the product (**4g**) in 88% yield (Figure 4c and Figure S26), which further demonstrates the potential of this transformation in the late-stage modification of biomolecules. Finally, peptide **1h**, which possessed a selenylsulfide between selenocysteine and 2-thiol-5-nitropyridine (TNP), reacted readily with phenylhydrazine (**2c**), giving 96% yield of Sec-modified peptide (**4h**, which has the same structure as **3c**) within 10 min under our standard conditions (Figure 4d and Figure S27).

Intramolecular Cyclization of Sec-Contained Peptide. With an efficient intermolecular peptide modification method established, we envisioned applying this technique to provide a cyclic peptide through the construction of an intramolecular Se-phenyl linkage (Figure 5a). To this end, we further

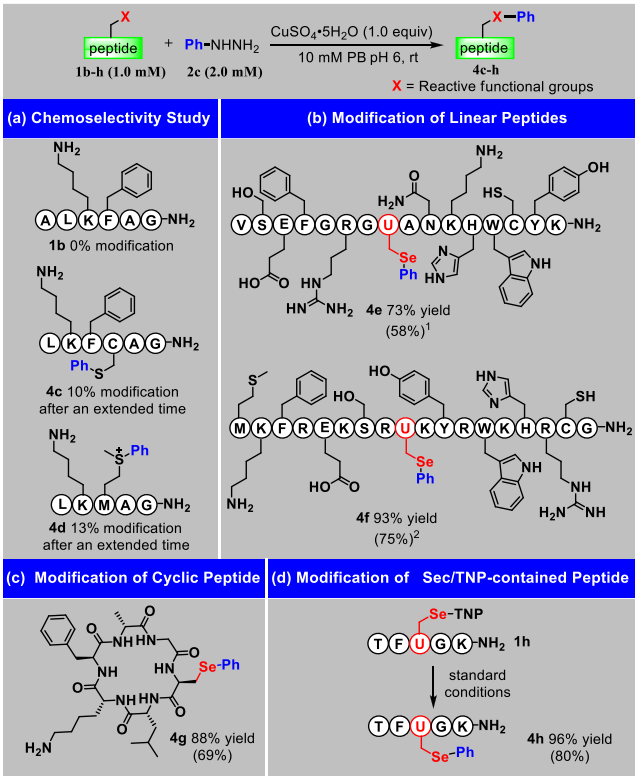


Figure 4. Systematic investigation of Sec-specific modification in the presence of other reactive residues. The yields were determined by HPLC, whereas the values in parentheses were the isolated yields. (a) Chemoselectivity study. (b) Modification of linear peptides. [1]2 equiv of $CuSO_4$ and 5 mM phenylhydrazine were used. [2]8 equiv of $CuSO_4$ and 1.5 mM phenylhydrazine under an Ar atmosphere. (c) Modification of cyclic peptide: 0.5 mM peptide dimer (**1g**), 1.0 mM with respect to the selenol monomer. (d) Modification of TNP-Sec-containing peptide, the structure of **4h** is the same as **3c**. TNP = 2-thiol-5-nitropyridine.

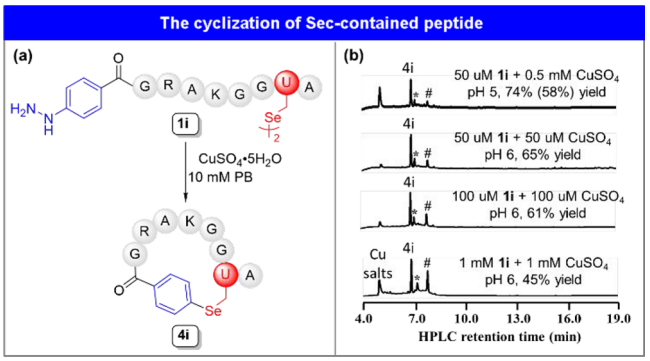


Figure 5. Intramolecular modification. (a) Cyclization of Sec-containing peptide (**1i**) bearing phenylhydrazine at the N-terminus. (b) HPLC traces of the cyclization of **1i** under different conditions (at room temperature and after 10 min). The yields were determined by HPLC; yield in parentheses was isolated yield. * is a side product formed by hydrogen transfer to the phenyl radical intermediate; # is a side product formed by self-coupling of the phenyl radical intermediate (dimer) (Figure S30).

optimized conditions for peptide cyclization with a linear Sec-containing peptide (**1i**) bearing phenylhydrazine at the N-terminus (Figure S29). The cyclization of peptide **1i** (1 mM) under the standard conditions described above (10 mM PB,

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1 equiv of $CuSO_4$, pH 6) provided the desired cyclic product (**4i**) in 45% yield after 10 min. Two side products were observed (assigned as * and #, the chromatogram in Figure 5b and Figure S30) formed by hydrogen transfer to the phenyl radical intermediate and self-coupling of phenyl radical intermediate (to give the diphenyl dimer peptide, Figure S30), respectively. Increased yield of the desired cyclization product could be obtained when diluted peptide solutions were used.[65,66] Ultimately, this transformation was further improved to obtain 74% yield of **4i** within 10 min by using 50 μM peptide and 10 equiv of $CuSO_4$ at slightly more acidic conditions (pH 5, Figure S30), which decrease the percentage of both side products.

Biotinylation of Sec-Containining Proteins. Encouraged by these results, we focused on expanding our method toward larger protein domains. The ubiquitin(G47U) variant **5a** was prepared by Fmoc-SPPS[57−59] and native chemical ligation (NCL)[67] (Figure S33), in which the solvent-exposed Gly47 was substituted with Sec. With ubiquitin(G47U) in hand, the biotin-containing phenylhydrazine (**2l**) (Scheme S2) was utilized for the modification of **5a**. To prevent Met oxidation in the presence of Cu(II) and O_2,[68,69] the reaction of **5a** was carried out under an argon atmosphere. Gratifyingly, almost quantitative biotinylation of protein **5a** was observed at 100 μM concentration within 30 min in the presence of 4 equiv of $CuSO_4$ (Figure 6 and Figure S35) and the modified

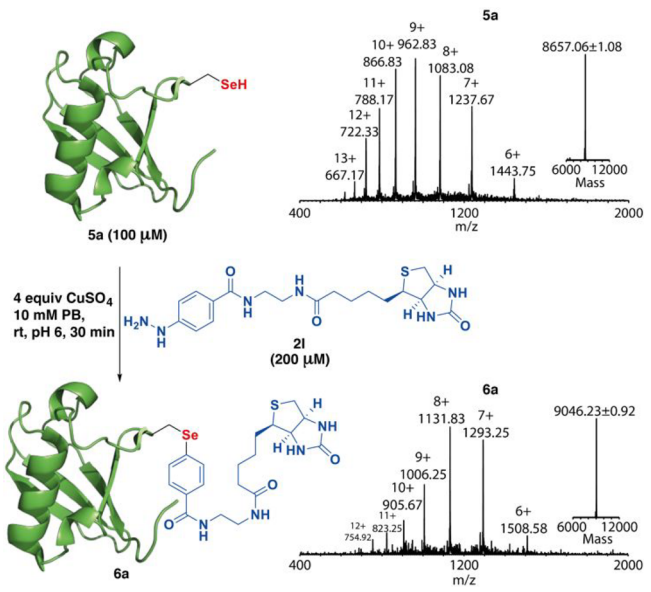

Figure 6. Modification of ubiquitin(G47U) **5a** with **2l**, and the mass spectra of **5a** and modified product **6a** (see Figures S73 and S74 for HR-MS data).

product **6a** was isolated in 60% yield by semiprep HPLC. In contrast, the G47A variant of ubiquitin (**5b**), which was prepared by the deselenization of **5a**,[27,28] was inert to the reaction with **2l** (see Figure S36 for the details).

Furthermore, we decided to confirm the reaction's chemoselectivity with a Cys-rich protein, stromal cell-derived factor-1 (SDF1), which contains two disulfide bonds and all reactive side chains.[70] The chemical synthesis of SDF1(S25U), in which the solvent-exposed Ser25 was substituted with Sec, was accomplished by SPPS and NCL (see Figures S37−S39 for further details). With SDF1(S25U) in hand, the oxidative folding of the synthetic protein was accomplished in the presence of reduced and oxidized glutathione (GSH and GSSG, respectively) to provide the folded protein **7a**, containing two disulfide bonds and glutathionylated Sec25 (Sec25-SG)[71] (Figures S40 and S70). The modification of **7a** with **2l** also proceeded under an argon atmosphere to prevent undesired oxidation at the Met residues. For the SDF1(S25U) modification, we employed 12 equiv of **2l** and 4 equiv of $CuSO_4$ (excess reagent was used in this case to minimize the possible electrostatic repulsion of Cu(II) with the solvent-exposed cluster of positive charges on the protein surface). Within 30 min, we observed more than 70% biotinylation of protein **7a**, and the modified product **8a** was isolated in 43% yield (Figure 7 and Figures S41 and S72). Gratifyingly, the

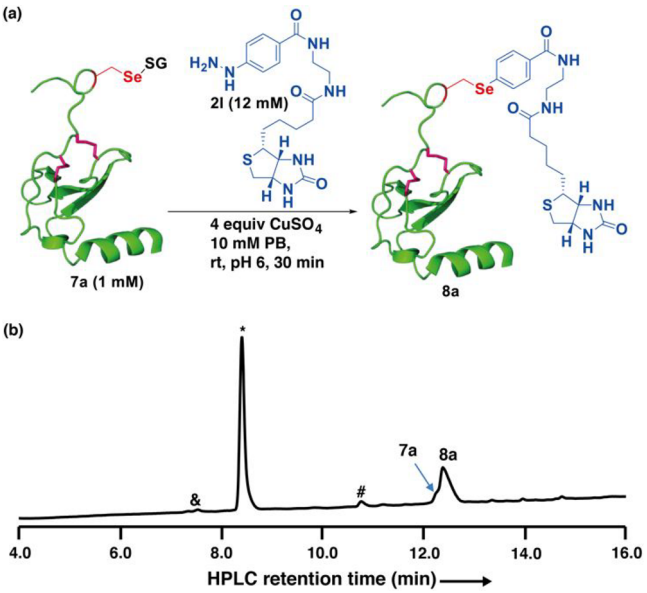

Figure 7. Modification of disulfide-containing protein SDF1(S25U). (a) Scheme and (b) HPLC trace of the modification of SDF1(S25U) **7a** with **2l** to form the modified product **8a** (see Figures S76 and S77 for HR-MS data). & is a side product from hydroxylation of **2l** radical intermediate; * is a side product from hydrogen transfer to **2l** radical intermediate; and # is a side product from self-coupling of **2l** radical intermediate (dimer).

reaction was tolerant to the two disulfide bonds of SDF1(S25U), and all other amino acid side chains in the product **8a**. For further support of the site-selective modification at the Sec residue, we tested the deselenization reaction of SDF1(S25U) (**7a**) in comparison to the biotinylated protein **8a**. Under deselenization reaction conditions (50 mM TCEP, pH 5), SDF1(S25U)-SG (**7a**) was converted into fully reduced SDF1(S25A) (the Sec was converted into Ala, as expected, and the two disulfide bonds were reduced to dithiols). On the other hand, in the biotinylated SDF1(S25U) case, **8a**, under the same reaction conditions, only the two disulfide bonds were reduced to dithiols, whereas the modified biotinylated-Sec was unaffected. These results implied that the modification of Sec by our approach has a negligible effect on disulfide bonds or other functional groups in the protein.

CONCLUSION

In summary, we have developed a widely applicable and efficient protocol for chemoselective modification of peptides

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and proteins based on a radical-mediated arylation and alkylation of selenocysteine using Cu(II) and a corresponding hydrazine. Mechanistic analysis, specifically EPR and radical clock reaction, supports a radical mechanism. The reported facile transformation can be carried out without any reducing agent, at a near-neutral pH (5−8), in aqueous solution, without organic cosolvents, and under mild reaction conditions. This reaction displays a broad scope of hydrazine substrates, including aromatic, heteroaryl, and even alkyl hydrazines, and is highly selective, even in the presence of other reactive amino acids. In addition, oxidized and reduced selenocysteine both perform well in this transformation, which greatly expands the range of application of this technique. Furthermore, we demonstrated the high efficiency of this strategy in the preparation of cyclic peptides and the modification of larger and more complex peptides and proteins (ubiquitin and SDF1) at micromolar concentrations. Although all tested peptides and proteins were prepared by chemical synthesis, this methodology can be applied to selenoproteins that are prepared using expression systems.[72−76] This new approach is complementary to the reported umpolung method by Cohen et al.,[43,44] as our radical reaction is highly chemoselective, does not require ligands for copper ions, works in neat aqueous solutions on peptides and proteins, and tolerates all functional groups. Further work to explore the labeling and fine-tuning of more complex biological molecules *in vivo* and *in vitro* with this technology is ongoing in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c06101.

Materials and methods, characterization data, HPLC, LCMS, and HRMS, ^{1}H- and ^{13}C NMR spectra, and synthesis procedures (PDF)

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Notes

The authors declare no competing financial interest.

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