Selenomethionine as an Expressible Handle for Bioconjugations

Dillon T. Flood, [a] Jordi C. J. Hintzen, [a] Chenxi Lu,[b] Philip A. Cistrone, [a] Jason S. Chen, [c] Takanori Otomo, [a] and Philip. E. Dawson [a]

[a] *Department of Chemistry, Scripps Research, 10550 N. Torrey Pines Road, La Jolla, CA 92037*

[b] *Department of Chemistry, Nankai University, 300071, Tianjin, China*

[c] *Automated Synthesis Facility, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037*

Site-selective chemical protein ligation reactions are enabling tools for chemical biology. Herein, we employ a physical organic study to refine the selenomethionine (SeM) benzylation as a practical protein bioconjugation strategy. SeM is readily introduced through auxotrophic expression and exhibits unique nucleophilic properties that allow it to be selectively modified even in the presence of cysteine. The resulting benzylselenonium adduct is stable at physiological pH, selectively labile to glutathione and embodies a broadly tuneable reactivity profile. Guided by a mechanistic analysis of the reaction, a 4-bromomethylphenylacetyl linker is identified for efficient conjugations of complex organic molecules to SeM containing proteins. This optimized benzyl linker exhibits a rate constant of $3x10^{-1}$ $M^{-1}s^{-1}$, facilitating efficient conjugation at micromolar concentrations. The selenonium conjugate is further advanced through a linker that can be selectively photo-locked or reductively cleaved on demand. This tool-kit of selenonium forming reagents have broad potential in the development of chemically enhanced proteins.

The development of site-specific and chemoselective protein modification techniques (1–6) are critical to the field of chemical biology as they allow the elucidation and perturbation of the complex molecular systems surrounding human health.(3, 7–10) Chemically modified biomolecules have been used to create probes to elucidate the pathways underlying human disease as well as several important classes of established therapeutics, such as constrained peptides, antibody-drug conjugates (ADCs) and PEGylated proteins.(11, 12) These bioactive molecules rely on the precise chemical modification of native peptide and protein structure to achieve efficacy, for example, the location of drug conjugates on an antibody has been shown to strongly affect the effectiveness of ADCs.(11, 12) The most useful protein modification strategies are chemoselective, site-specific, and have fast reaction kinetics since biomolecules are usually modified at high μM concentrations.(1, 2, 5) Further, the most robust bioconjugation approaches facilitate direct conjugation of complex molecules in a single chemical step, without incorporating an intermediate chemical handle which is ultimately functionalized. While proteins can be assembled

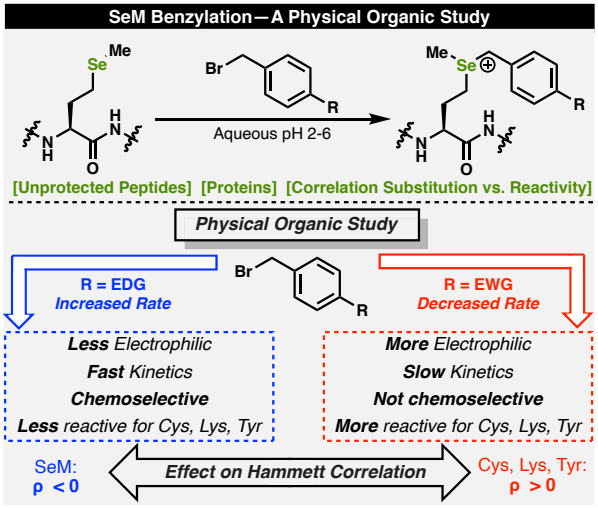


Figure 1: A Physical Organic Study of the SeM Benzylation

synthetically or semisynthetically, the ability to modify proteins produced through biological expression allows efficient bioproduction of large, chemically modified protein domains.(6, 7, 13, 14) Most bioconjugation strategies target the nucleophiles of naturally-occurring amino acids, such as the primary amines present at the protein N-terminus or on lysine residues, and the cysteine thiol. Less commonly used strategies target tyrosine(15, 16), arginine,(1, 17) and methionine,(18) but these procedures are usually sluggish or dependent on complex reagents.(18–20) More recently, a redox based linker has been developed for methionine-based sulfilimide bioconjugation, demonstrating the potential utility of this residue.(21, 22)

To address the issues surrounding natural amino acid specificity, unnatural amino acids can be introduced through auxotrophic expression or amber codon suppression for bioorthogonal conjugations.(23–25) For example, methionine can be substituted in auxotrophic systems with homologs such as azidohomoalanine and propargyl glycine for copper-catalyzed alkyne-azide cycloadditions (CuAAC, “click”

chemistry). In precedent to our current work, Palmer and coworkers labelled selenomethione with a variety of alkyl halides at high concentration but this method has yet to evolve as a protein conjugation strategy.(26, 27) Alternatively, unnatural amino acids can be introduced through amber codon suppression.(23–25) While this technique allows for the incorporation of unique and orthogonal groups, such as ketones and azides, for use in oxime ligations and CuAAC respectively, it requires transfections of multiple specifically engineered plasmids and is generally inefficient on production scale.(6)

Selenomethionine is easily and efficiently incorporated into proteins and peptides though either chemical protein synthesis or auxotrophic expression.(28, 29) Methionine (which is replaced by selenomethionine in auxotrophic systems) is the second rarest amino acid in vertebrates, where most residues are not surface exposed due to its hydrophobicity, thereby allowing surface exposed residues to be engineered for selective chemical modification.(21) Selenomethionine auxotroph expression,(6, 30) is an efficient and robust technique that has been fine-tuned and utilized by crystallographers for decades in the production of numerous heavy atom labelled proteins.(30–32) The selenoether moiety of selenomethionine displays interesting chemical properties, as selenoethers are uniquely nucleophilic and sensitive to mild redox transformations.(33) These unique chemical properties and the facile introduction into expressed proteins make selenomethionine a prime target for the development of new biorthogonal chemistries.

Selenomethionine can be selectively modified with benzyl bromide(26, 27, 34) and the selenonium intermediate used to introduce backbone engineering modifications(34) into proteins. In order to better realize the potential of SeM conjugation, a detailed study of the selenomethionine benzylation reaction has been performed, leading to the identification of labelling reagents that form the basis of a highly chemoselective and tunable bioconjugation strategy. The robust ligation kinetics and conditional cleavage properties of the selenonium conjugate suggest a broad utility of the method.

Results and Discussion

pH Independence of the Selenomethionine Benzylation. To determine the pH dependence of benzylation with proteinogenic amino acids and SeM, model peptide **1** (2.5mM) **(Figure 2A)** in a solution of 30% ACN, 5% DMSO, and 10mM ascorbic acid was exposed to 2 equiv. BnBr over varying pH **(Figure 2B)**. The consistent reaction profiles observed for conversion of **1** to the corresponding benzylated species **2** suggests that the SeM benzylation is pH independent, over the range of pH 2-7.4 **(Figure 2 C)**. In contrast, most proteogenic

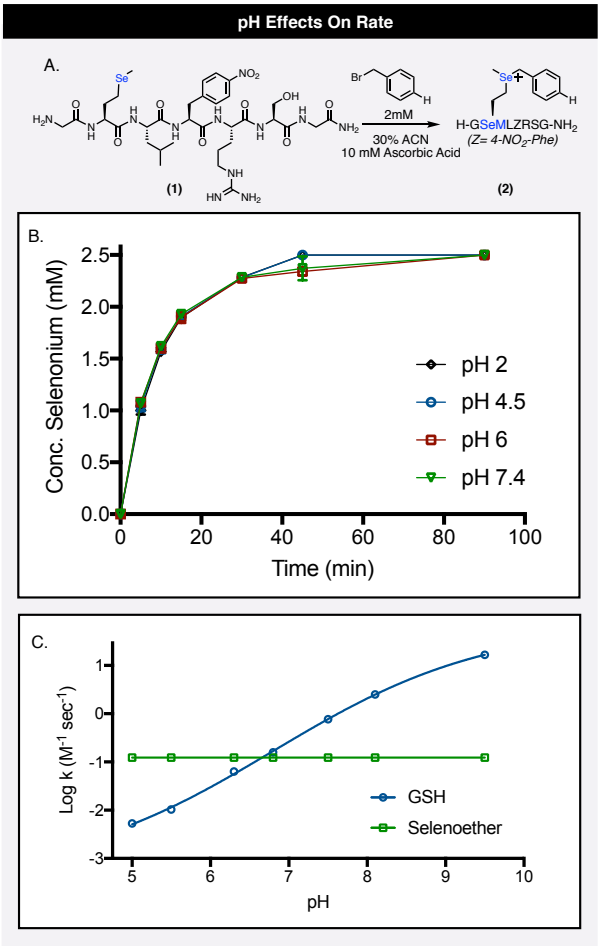


Figure 2: **A** Selenomethionine benzylation **B** pH dependence of SeM Benzylation (2.5 mM peptide **1**, 5 mM BnBr, 30% ACN, 5% DMSO, 1 mM Boc-Tyr-OH as internal standard). **C** Benzylation of GSH data taken and replotted from Boyer et. al. 1976 (18) and SeMet (this study).

nucleophiles targeted for bioconjugations become less reactive at low pH as they become protonated. In contrast, the selenoether of SeM is not protonatable, resulting in its nucleophilicity being unaffected by pH **(Figure 2)**.

Selenomethionine orthogonality. In order for the SeM benzylation to be a useful bioconjugation strategy, SeM must be orthogonal to other common bioconjugation strategies, especially thiol conjugation reagents.(1) Upon treating peptide **1** with maleimidopropioic acid, under standard thiol labeling conditions (pH 6),(1) no SeM conjugation is observed at pH 6 while only minor reactivity is observed at pH 7.5, suggesting that maleimide-Cys and benzyl bromide-SeM conjugations can occur simultaneously (**SI**).(26) Also, in a previous study we found that selenomethionine was unreactive towards iodoacetamide, a common thiol labeling reagent, in the time frames tested.(34)

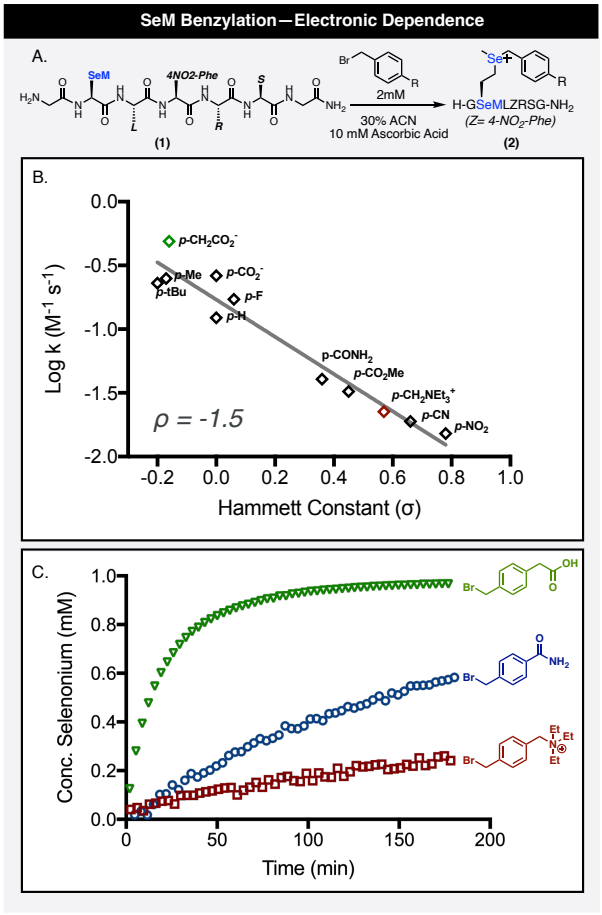


Figure 3: **A** The Selenomethionine Benzylation **B** Hammett correlation of the selenomethionine benzylation (the Hammett constant for *p*-$CH_2NEt_3^+$ was estimated from *p*-$CH_2N(Me)_3^+$). **C** Conversion of **1** to the corresponding selenonium species of representative linkers (1 mM **1**, 2 mM R-BnBr, 1 mM Boc-Try-OH (internal standard), 10 mM ascorbic acid, 40 mM MES pH 6, 30% ACN, 5% DMSO in water).

Hammett Study of the Selenomethionine Benzylation. Further optimizing this reaction, benzyl bromides with electron donating and withdrawing substituents were evaluated. An interesting phenomenon was observed in preliminary experiments when 4-nitrobenzyl bromide benzylated SeM more slowly than benzyl bromide under identical conditions. This experiment suggested a reactivity profile that is opposite of that observed in the reaction of a Cys-thiolate(18) for which appending electron withdrawing groups increase the reaction rate constant. A full Hammett study revealed how electronic perturbations about the phenyl ring effect the reaction rate **(Figure 3B)**.(18)

The resulting plot shows a clear inverse Hammett correlation, with a ρ of -1.5, implying significant S_N1 reaction character **(Figure 3B)**. (38–40) This finding is consistent with reports of thioether sulfonium formation, perhaps acting through a benzyl ion-pair intermediate.(18) This correlation is significant as it indicates that the rate of reaction improves upon introduction of electron donating substituents. This results in a less electron deficient benzylic position that is correspondingly less susceptible to attack by endogenous nucleophiles. Therefore, the electronic demands and pH dependence of the SeM nucleophile are the opposite of most other nucleophiles present in biological macromolecules, which should result in high chemoselectivity towards SeM.

DFT Calculations of the Selenomethionine Benzylation. The experimental observations were supported by mechanistic calculations of the benzylation reaction of a selenomethionine containing fragment and various bezyl bromides in water at room temperature. Density functional theory (DFT) calculations confirmed that the activation energies of the necessary intermediates for each pathway, a benzyl cation for S_N1 and a pentacoordinate benzyl transition state for S_N2, favored a reaction pathway with S_N1 charecter. These calculations revealed that a strictly S_N2 reaction would be too slow at room temperature to recapitulate the experimental results, while a benzyl cation intermediate (the necessary transition state for a S_N1 pathway) could be formed more readily **(SI)**. Calculations also supported the experimental observations that electron donating substituents increase reaction rate **(SI)**.

Towards Practical Bioconjugations Next, the benzylation rate of representative linkers were compared. These linkers, included 4-bromomethylphenylacetic acid (BrMePAA), 4-bromo methyl benzamide, and a 4-bromomethyl benzyltriethyl ammonium, an analog of the previously reported SeM conjugation linker (27) **(Figure 3C)** for ability to benzylate the SeM of peptide **1.** It is clear that the electron donating nature of BrMePAA greatly increases conjugation speed **(Figure 3C)** compared to either of the other two representative linkers. BrMePAA significantly outperforms the simple linker choice, the benzamide, and is ~20-fold faster than the previously described quaternary amine based linker. (26) These observations guided linker choice for further bioconjugation reactions.

Anticipating that cysteine/benzyl bromide cross reactivity may present a problem in the selective benzylation of SeM, we consulted the literature to compare the rates of thiol and selenoether benzylation as a function of pH **(Figure 2C)**.(18) A classic study, conducted by Boyer in 1976, utilized glutathione (GSH) as a cysteine thiol surrogate to probe the pH dependence of the thiol benzylation with benzyl bromide. We compared the SeM benzylation rate constants against the previously published thiol benzylation rate constants. As expected, GSH thiol benzylation is highly pH dependent, and since SeM benzylation is pH independent, we expected to

employ a pH range in which SeM can be selectively modified in the presence of Cys. Utilizing BrMePAA at pH 6, the rate of SeM benzylation should be greater than 1.5 logs faster than Cys **(Figure 2C)**. If Cys cross reactivity is encountered in a specific system, even more selectivity for SeM can be achieved by lowering the pH of the reaction. For example, at pH 5, benzylation of SeM is expected to be over 2.75 logs faster than Cys **(Figure 3B)**.

Benzyl-selenonium conjugate stability. Useful bioconjugation chemistry must be chemoselective, kinetically fast (to be employed employ at high µM concentrations), and form a stable conjugate.(1, 2, 4, 17) Thus, the stability of the benzyl-selenonium adduct under various physiological conditions was profiled. First, the long-term stability in the human blood plasma mimic (PBS, pH 7.4) was explored with two distinct benzyl-selenonium derivatives, of **1**. The benzyl-selenonium adduct is surprisingly stable under these conditions, with >95% remaining after 3 days **(Figure 4A)**. The 4-methylbenzyl (an electron donating substituent) containing adduct degraded faster than the benzylated construct, yet still retained >90% of the conjugate after 3 days (**Figure 4A)**. These costructs even exhibited good long-term stability with half-lives of 43 and 24 days for the benzyl bromide and 4-Me benzylbromide adduct, respectively. This data is interesting as it shows that electronic perturbations to the phenyl ring have great effects not only on the benzylation rate but also on the various degradation pathways. The benzyl selenonium linkage is highly tunable, allowing one to use electronic perturbation of the phenyl ring to conjugation and cleavage rates of the desired conjugate.

Next, the stability of three benzyl selenonium adducts of **1** in a cytosol mimic (7.5 mM GSH in PBS pH 7.4) was probed.(41, 42) Interestingly, the conjugate with an electron withdrawing group ($4NO_2$) degraded the fastest. Less than 5% of the 4-nitrobenzyl bromide adduct remained after 2 hours, while about 50% of the 4-bromomethylphenylacetic acid adduct remained at the same time point **(Figure 4B)**. This electronic correlation is opposite from that observed above in the PBS experiment and is easy to rationalize. The electron withdrawing adducts result in a more electrophilic benzylic carbon which is more susceptible to the negatively charged thiolate nucleophile of GSH. Since these benzylselenonium linkers are expected to exhibit opposite degradation kinetics in serum versus in cytosol, an interesting opportunity is presented. One can envision creating a tunable linker which exhibits a long, stable half-life while in circulation but upon cell internalization is readily cleaved by intracellular GSH to release a payload.

The SeM Benzylation as a strategy for bioconjugation. Insight gained from the initial biophysical study led to the selected linker **(Figure 5A).** BrMePAA is readily available, shelf-stable

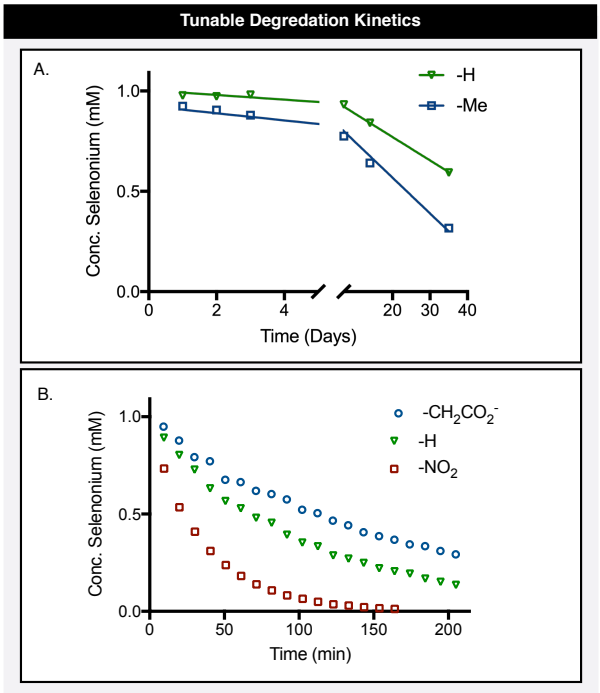
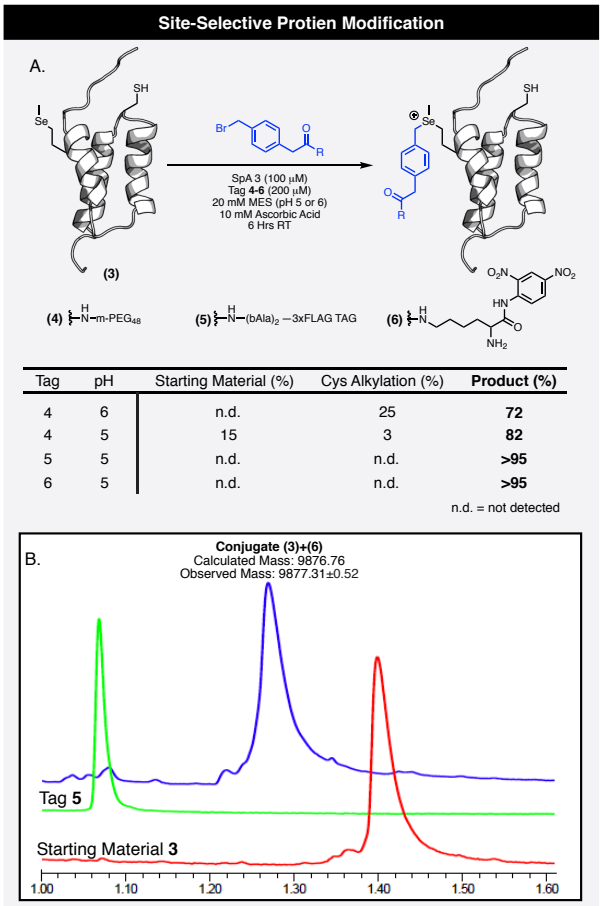


Figure 4: Selenonium stability. **A** Benzyl-selenonium stability in plasma mimic (1 mM peptide, in PBS pH 7.4, monitored by HPLC). **B** Benzyl selenonium stability in cytosol mimic (1 mM peptide, in PBS pH 7.4, with 7.5 mM GSH, monitored by HPLC).

and easily incorporated into tags of interest by either solution or solid phase methods.

To test the utility of this linker, the albumin-binding, three helix bundle SpA, **3**, was selected due to its utility as a half-life extending moiety *in vivo* **(Figure 5A)**.(44, 45) The natural solvent exposed methionine was replaced with a SeM and a free Cys **(C22)** was introduced to probed chemoselectivity and to explore the potential for dual protein labeling. BrMePAA was coupled to $mPeg_{48}$-amine through a succinimidyl ester intermediate, yielding compound **4**. In the first protein labeling experiment, **3** was exposed to tag **4** at pH 6, 6 h. This reaction resulted in 75% of the desired (SeM conjugated) product and 25% of a side product in which both the SeM and the Cys were labeled **(Figure 5A)**. In a subsequent reaction, the pH was adjusted to 5 while leaving all other parameters constant. The mono selenonium product (82%) was observed along with, 15% starting material, and only traces of the doubly lableled material **(Figure 5A)**. Two other tags, a 3xFlag tag **5** and a chromophore containing tag **6**, were used to label protein **3** (**Figure 5A**) These tags completely label the SeM residue, resulting in complete conversion to the tagged conjugate with no detectable double labeled side product or starting material **(Figure 5A)**. This dual labelling route was explored to test SeM benzylation selectivity under highly challenging conditions. In practice, this level of selectivity is unncesseary as any cysteine residues can be selectively modified by haloacetamide or



Tag	pH	Starting Material (%)	Cys Alkylation (%)	**Product (%)**
4	6	n.d.	25	**72**
4	5	15	3	**82**
5	5	n.d.	n.d.	**>95**
6	5	n.d.	n.d.	**>95**

n.d. = not detected

Figure 5: **A** Modification of SpA protien **3** with various tags at various pH (0.15 mM **3,** 0.2 mM tag **4, 5,** or **6,** 20 mM MES, 10 mM ascorbic acid). **B** HPLC Chromatogram of reaction of **3** and **5** (0.15 mM **3,** 0.2 mM tag **6,** 20 mM MES buffer pH 5). Deconvolution performed manually from centroid collapsed peaks between m/z 600-1250.

maleimide reagents prior to SeM labelling. SeM benzylation allows for complex protein systems to be quickly and selectively modified at SeM, with tags that are easily synthesized. Chemoselectivity over even thiol functional groups, suggest a broad utility in bioconjugation.

Drawing inspiration from Palmer's early report, (26) we strove to exploit the unique reactivity profiles of SeM and Cys in protein **3**, to develop a dual conjugation strategy **(Figure 6A)**. By combining SpA protein **3** (150 μM), BrMePAA tag **6** (200 μM), and maleimide tag **7** (200 μM), protein **3** was simultaneously modified with no detectable side products or starting material **(Figure 6A)**. Although compounds **3** and **7** co-elute we were able to confirm by mass spectrometry that no starting material **3** is present. This approach allows facile access to dual site specific labeling of expressible protein with unique labels.

To evaluate conjugation to a large biological macromolecule, the maltose binding protein (MBP) construct

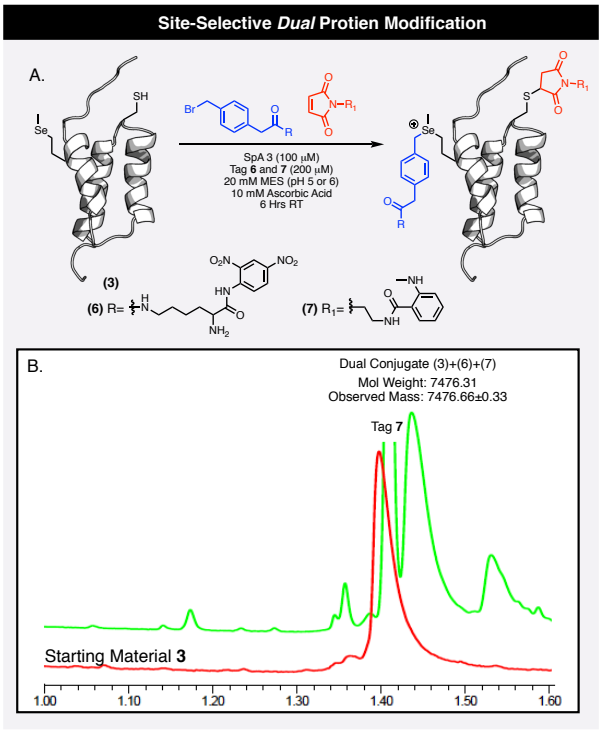


Figure 6: **A** Dual Modification of SpA protien **3** with tags **6** and **7** at pH 6 (0.15 mM **3,** 0.2 mM tag **4, 5,** or **6,** 20 mM MES, 10 mM ascorbic acid). **B** HPLC Chromatogram of dual labeling of **3** with **6** and **7** (0.15 mM **3,** 0.2 mM tag **6,** 0.2 mM tag **7,** 20 mM MES buffer pH 6). Deconvolution performed manually from centroid collapsed peaks between m/z 600-1250.

(SeM-Hisx6-MBP-Tev) **8** was expressed in a methionine auxotroph. This protein contains a total of seven SeMs, four of which are completely buried in the core of the protein, two of partially exposed, and one completely solvent exposed. MBP construct **8** was treated with tag **7** at pH 6 **(Figure 7A)**. Only one SeM was efficiently modified (~80%) with a trace of double modification observed by LC-MS **(Figure 7B)**. This suggests that only solvent exposed SeMet residues are reactive and that folding can protect buried residues from modification. (27)

Chemoselective locking and cleaving of selenonium conjugates. Anticipating the need for an irreversible linkage, for use in a long lifetime biologics,(11, 12, 46) we created a bifunctional reagent by introducing an azide moiety to the ortho- position of a bromomethyl benzoic acid. The amino group of 3-amino-4-methyl benzoic acid **11** was oxidized to the diazonium salt and displaced by the addition of sodium azide to yield 3-azido-4-methyl benzoic acid **12 (Figure 8A).** Phenylazide **12** was then converted to the corresponding benzyl bromide **13** by radical bromination **(Figure 8A)**. The initial design was that when a selenonium conjugate of **13** was exposed to a phosphine source, the aryl-azide would decompose in a Staudinger-like mechanism (6, 47, 48) to form a highly nucleophilic aza-ylide that would attack the SeM γ-

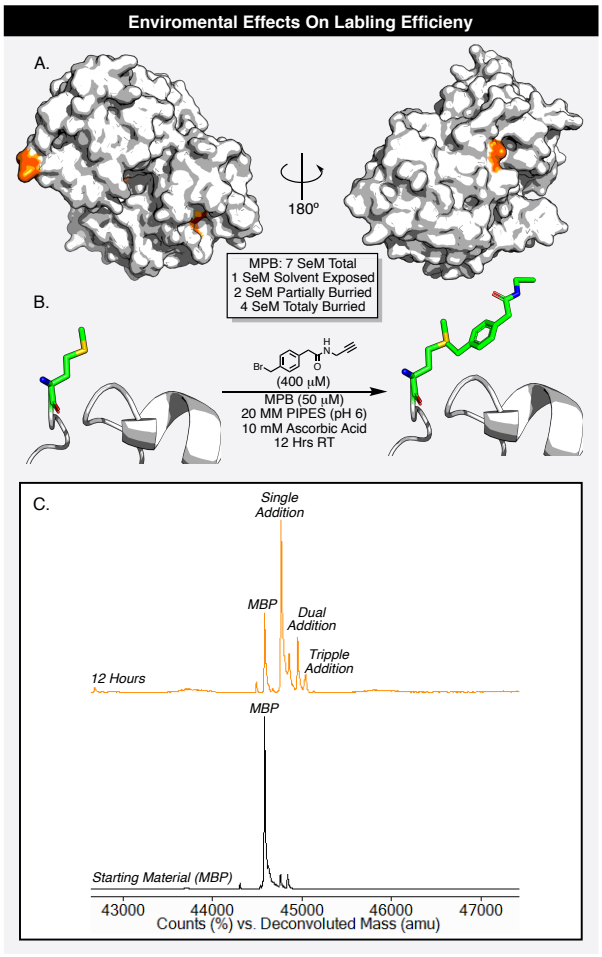


Figure 7: Environment determines reactivity: **A** MBP Surface rendering showing the one solvent exposed SeM (orange) and two partially buried SeM residues (orange). **B** Modification of MBP **8** with tag **9** (0.05 mM **8,** 0.4 mM tag **9,** 20 mM PIPES pH 6, 10 mM ascorbic acid). **C** Deconvoluted mass spectrum of MBP tagging reaction at time points.

carbon and push-out the benzyl-selenonium leaving group. (6, 47, 48) Unfortunately, upon exposing **15** to a phosphine source (TCEP) and subsequent Staudinger decomposition, (6, 47, 48) the highly nucleophilic aza-ylide attacks the most electrophilic position accessible, the benzylic carbon. This results in self-cleavage of the benzyl adduct yielded the corresponding benzazetidine, and original unmodified model peptide **14 (Figure 8B)**. Although this was not the desired reaction, it still provides the opportunity to create a traceless self-cleavable linker. Alternatively, selenonium conjugate **15** was exposed to UV, (254 nm) from a handheld lamp for 30 minutes to generate an electrophilic nitrene species (49–54) that can undergo C-H insertion (49–54) to form a stable C-N linked conjugate, **16 (Figure 8B)**. Thus, this linker allows exogenously triggered access to two divergent transition states, which dictate the subsequent transformations **(Figure 8B)** leading to cleavage or irreversible conjugation.

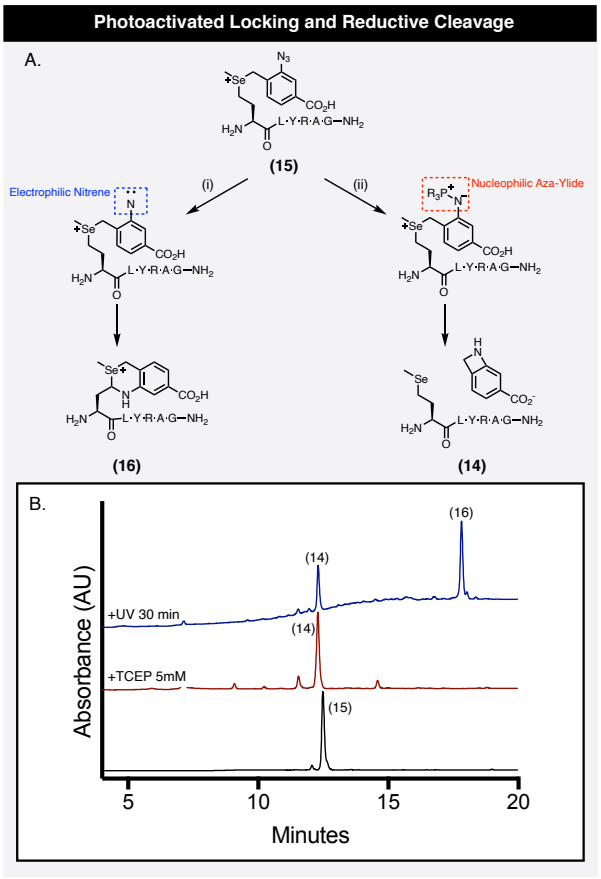


Figure 8: A Dual Functional Labeling Reagent: **A** Generation of divergent transitions states. **(i)** UV irradiation: 254 nm, 30 minutes from handheld lamp. **(ii)** Phosphine source: TCEP HCl. **B** HPLC Chromatograms of **15** after exposed to UV irradiation and phosphine source.

While this preliminary linker shows phosphine induced cleavage and photochemical C-N bond formation are possible, we anticipate that further optimization may create a more general linker. An electron withdrawing group placed para to the azide should increase the triplet nitrene lifetime and thus increase reaction conversions (52) while an electron donating group placed para to the leaving group will increase conjugation rate.

In summary, we present a detailed study of the SeM benzylation reaction and demonstrate its utility towards the bioconjugation of proteins. The orthogonality of the reaction towards other common thiol labeling regents is demonstrated under physiological through mildly acidic buffers. We have also outlined the stability of the selenonium conjugate under physiological conditions and suggested its lability to cytosolic glutathione could act as a release mechanism for the conjugate inside cells. Compatibility of the approach with large SeM proteins generated though auxotrophic expression suggests a broad applicaation of SeM benzylation, which can be used in

concert with traditional thiol-maleimide chemistry to yield dual modified proteins. Finally, a second generation linker is described that facilitates subsequent cleavage or conversion to an irreversible conjugate. SeM benzylation has significant potential as a user friendly addition to the chemical biology toolbox.

Methods

Peptide and Protein Synthesis

Peptides were chain assembled by Fmoc-SPPS by previously reported methods. Chemically synthesized proteins were created by native chemical ligation of two corresponding fragments (See SI).

Kinetic Profiling:

Peptide **1** (at 1 mM) was exposed to 2 equivalents of substituted benzyl bromide (X-BnBr) in a solution of 30% acetonitrile, 5% DMSO, 40mM MES buffer pH 6, and 10 mM ascorbic acid. Consumption of peptide **1** was monitored by UV absorbance on UPLC-MS every ~3 minutes over approximately three hours. Ascorbic acid was added, as it is known to selectively inhibit and reverse the oxidation of SeM to the corresponding selenoxide which would deactivate the SeM towards alkylation, while not interfering with sulfoxides or disulfides.(35) UV absorbances at 277nm were auto-integrated against internal standard (Boc-Tyr-OH). Assuming mass balance (as no peptidic side products are observed) the change in concentration of benzylated product was plotted against time.(36, 37) The reaction conversion traces clearly indicate that electron donating substituents increase the rate of SeM benzylation (**SI**). The starting material consumption rate was fitted to exponential decay functions, and the derivative of these functions were taken to derive instantaneous rates. Utilizing this, the instantaneous rate over concentration benzyl bromide reactant was plotted verse the concentration of peptide starting material. From these plots a line was fitted and the rate constants k (mM/sec) were derived.(36, 37) Finally, these rate constants were plotted against Hammett constants from the literature.(38, 39)

DFT Calculations of the Selenomethionine Benzylation

All structures are calculated at the (CPCM)-M06-2X/def2-TZVP level of theory in water at room temperature with *Gaussian 09* program. Monte Carlo conformational searches were carried out with the Merck molecular force field (MMFF) implemented in Spartan '18. Conformers (in total 22) were generated and the five with lowest energy were further calculated with QM (See SI), Transition states were based on the structure with lowest energy (See SI).

Conjugate Stability

Plasma Mimic: Disappearance of peptide benzyl-selenonium adducts of **1** were monitored in plasma every few days for 36 days, as monitored by UPLC-MS via UV absorbance.

GSH Mediated: Disappearance of peptide benzyl-selenonium adducts of **1** were monitored in cytosol mimic every 12 minutes for 3 hours, as monitored by UPLC-MS via UV absorbance.

Significance: **The ability to manipulate protein structure in a site selective manner underpins much of modern protein science. A careful mechanistic evaluation of the benzylation of selenomethionine led to the development of a highly efficient halobenzyl linker. This selenonium forming ligation reaction can efficiently link proteins to other complex molecules such as synthetic peptides, drugs or fluorescent dyes. The selenonium product is sufficiently stable at physiological pH to support *in vivo* applications, yet is rapidly cleaved by glutathione at concentrations present inside cells. A second-generation linker facilitates the photo-locking of the selenonium product for applications demanding a more stable linkage. The mechanistically guided development of these reagents should be broadly applicable for the engineering of complex macromolecules.**

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