

# Site-Selective Functionalization of Methionine Residues via Photoredox Catalysis

Junyong Kim, Beryl X. Li, Richard Y.-C. Huang, Jennifer X. Qiao, William R. Ewing, and David W. C. MacMillan\*



Cite This: *J. Am. Chem. Soc.* 2020, 142, 21260–21266



Read Online

ACCESS |



Metrics & More



Article Recommendations



Supporting Information

**ABSTRACT:** Bioconjugation technologies have revolutionized the practice of biology and medicine by allowing access to novel biomolecular scaffolds. New methods for residue-selective bioconjugation are highly sought to expand the toolbox for a variety of bioconjugation applications. Herein we report a site-selective methionine bioconjugation protocol that uses photoexcited lumiflavin to generate open-shell intermediates. This reduction-potential-gated strategy enables access to residues unavailable with traditional nucleophilicity-based conjugation methods. To demonstrate the versatility and robustness of this new protocol, we have modified various proteins and further utilized this functional handle to append diverse biological payloads.

The development of general strategies for the selective modification of proteins remains an important challenge in chemical biology.<sup>1</sup> Especially, the emergence of small-molecule–protein conjugates as a new class of pharmaceuticals has inspired a wealth of research efforts aimed at the discovery of new methods to achieve site- and chemoselective bioconjugation of wild-type proteins. To date, a number of elegant strategies have been devised that permit the direct functionalization of endogenous amino acids.<sup>2</sup> However, despite impressive advances in this area, there remain significant selectivity challenges due to the inherent structural complexity of proteins.

Traditional bioconjugation methods rely on substitution reactions between nucleophilic amino acid residues and small-molecule electrophiles to covalently link the biological payload at specific sites on the protein. Early bioconjugation protocols had been focused on highly nucleophilic residues, such as lysine<sup>3</sup> and cysteine,<sup>4</sup> in order to ensure chemoselective reactivity under the requisite aqueous conditions. Furthermore, under a nucleophilicity-based reactivity paradigm, it is difficult to control the structural homogeneity of the modified protein mixture. The surface abundance of lysine residues renders site-selective bioconjugation challenging, while cysteine-based modifications sometimes require pretreatment of the native protein to reduce disulfide bonds where free cysteine residues are unavailable.<sup>4a</sup> In light of these challenges, recent efforts have focused on achieving site selectivity through the use of less abundant residues, such as tyrosine,<sup>5</sup> tryptophan,<sup>6</sup> and histidine.<sup>7</sup>

Given the aforementioned complexities in implementing nucleophilicity-based bioconjugation strategies, our laboratory has been pursuing an alternative approach wherein site selectivity is attained by exploiting differences in the inherent reduction potentials of the native amino acid residues. According to our photoredox-based strategy, photonic energy from visible light is harvested by a photocatalyst and delivered as an electrochemical potential (Figure 1).<sup>8</sup> Single electron

transfer (SET) events, gated by amino acid oxidation potentials, can selectively target specific residues to yield reactive open-shell radical intermediates, leaving other residues intact. Crucially, the mild and highly selective nature of photoredox catalysis, as well as its compatibility with biological conditions, renders it an ideal platform for the development of new bioconjugation technologies.<sup>9</sup> Along these lines, our group recently reported the selective C-terminal alkylation of proteins via photoredox catalysis,<sup>10</sup> wherein the C-terminal carboxylate group is selectively oxidized in preference to aspartate and glutamate residues owing to subtle differences in oxidation potential.

In an effort to expand the reach of our photoredox bioconjugation platform, we aim to develop new conjugation techniques targeting other redox-active residues. Under the traditional nucleophilicity-based reactivity paradigm, bioconjugation of methionine has posed a particular challenge because of the pronounced hydrophobicity and weak nucleophilicity of the thioether side chain.<sup>11</sup> However, methionine is readily oxidizable,<sup>12</sup> rendering it potentially susceptible to functionalization via photoredox catalysis. Along these lines, the Chang and Toste groups<sup>13</sup> and the Gaunt group<sup>14</sup> recently reported methionine-selective bioconjugation methods that employ chemoselective sulfur-oxidizing reagents to convert the thioether moiety to a sulfilimine or  $\alpha$ -azo sulfonium ion, respectively, seminal studies that are likely to have a broad range of pharmaceutical applications, including antibody–drug conjugates,<sup>15</sup> stapled peptides,<sup>16</sup> and positron emission tomography<sup>17</sup> and optical<sup>18</sup> imaging and diagnostics.

Received: September 16, 2020

Published: December 8, 2020



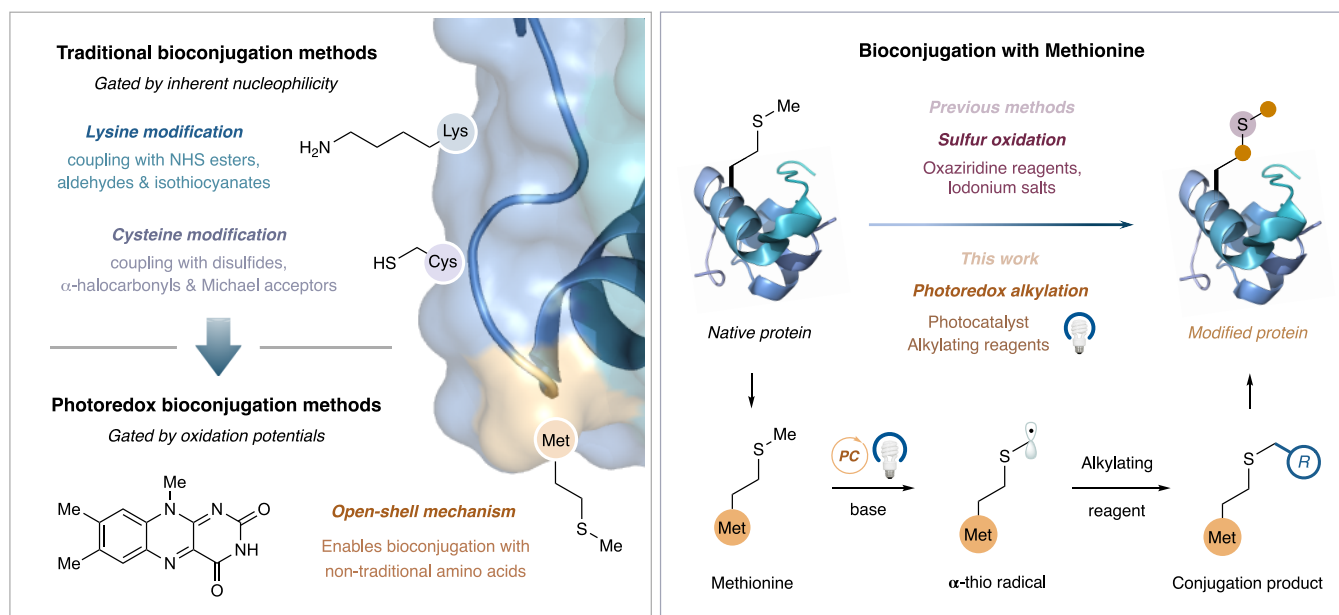


Figure 1. Development of a methionine-selective bioconjugation method via photoredox catalysis.

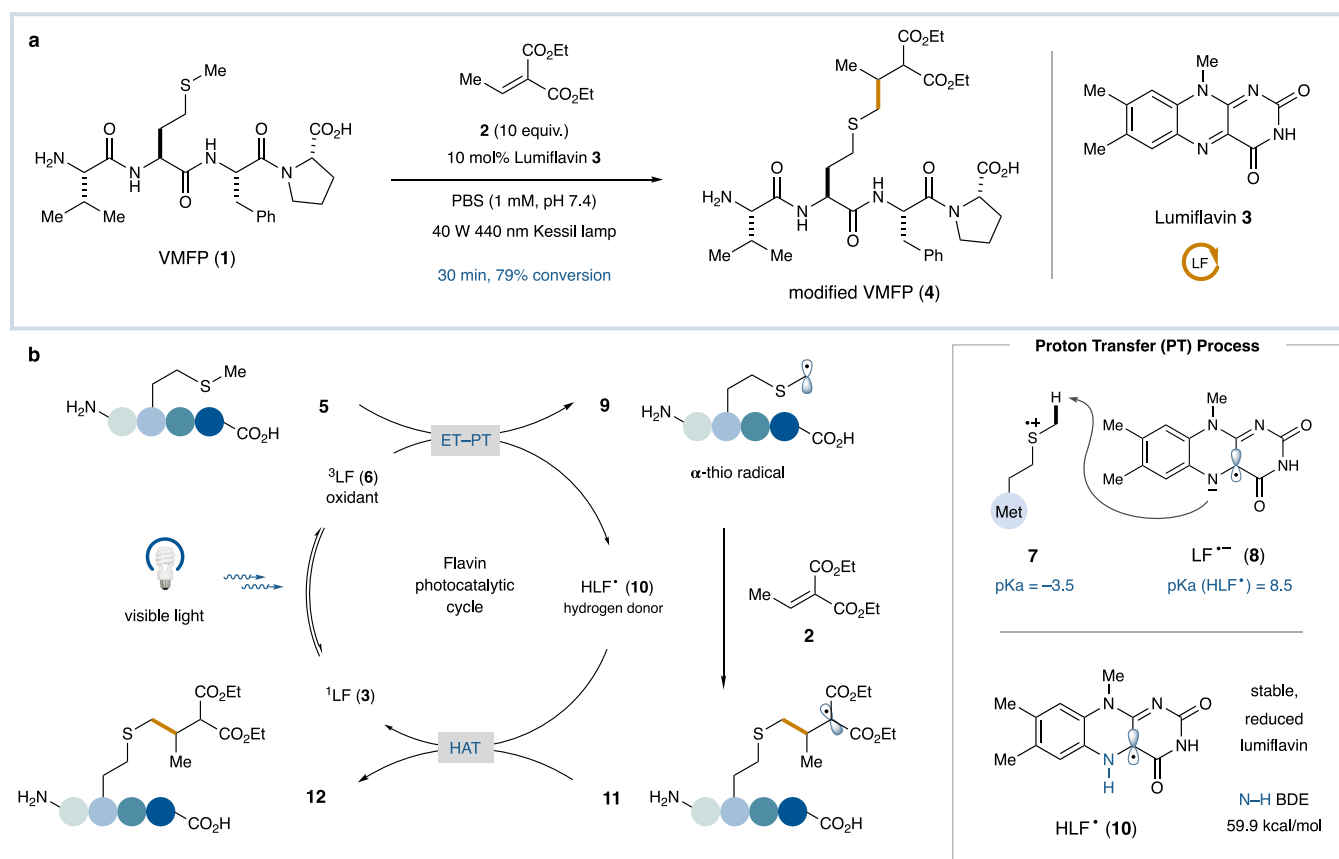
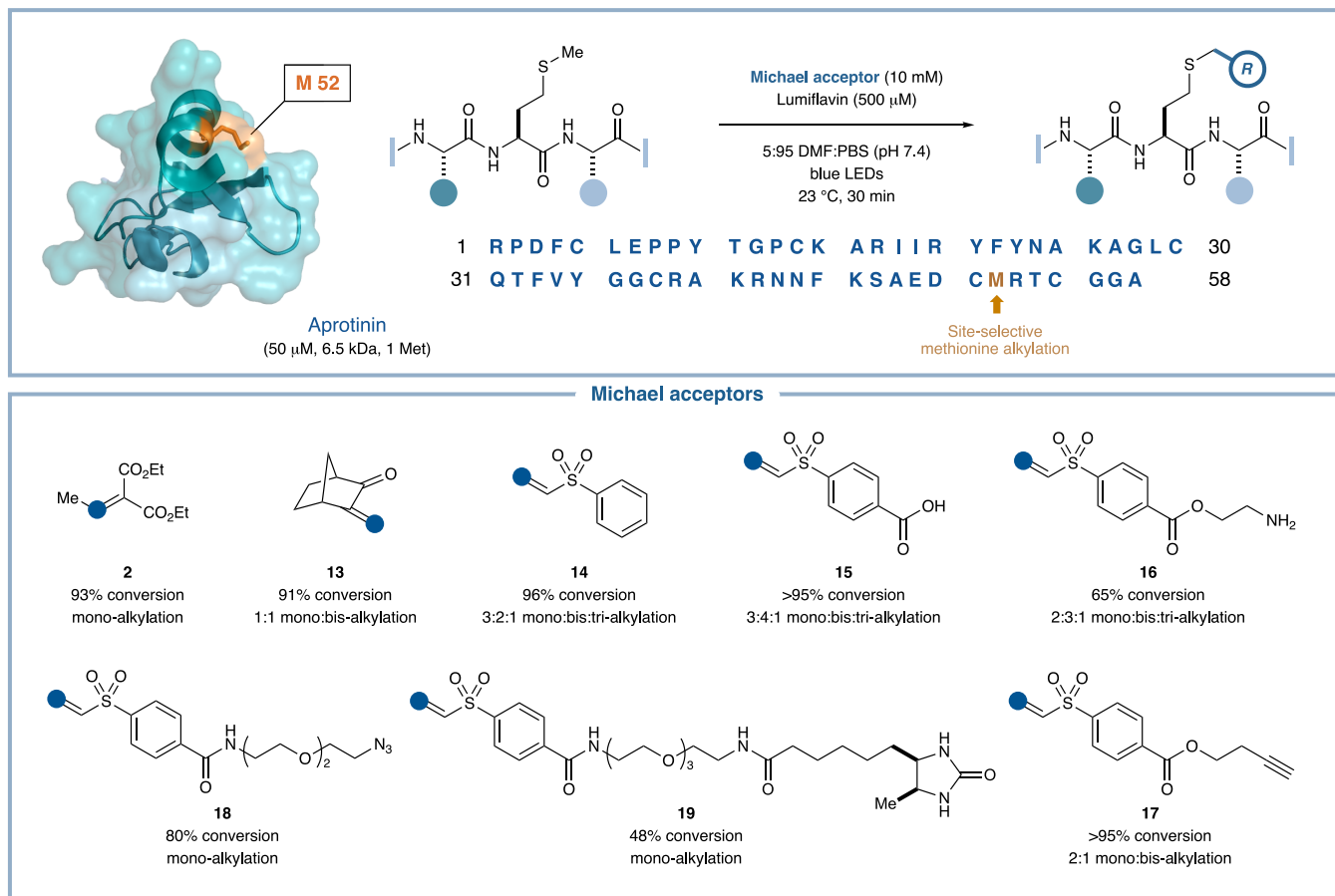


Figure 2. Optimized conditions and proposed mechanism for the alkylation of methionine with lumiflavin photocatalyst.

Alternatively, using our photoredox-based strategy, we envisioned achieving single-electron oxidation of sulfur by an excited photocatalyst, with subsequent  $\alpha$ -deprotonation to generate a carbon-centered  $\alpha$ -thio radical. This nucleophilic radical species could then react with SOMOphilic alkylating reagents (Figure 1b). In contrast to other methionine-based methods, in which thioether modification occurs on the sulfur

atom, our proposed method involves conjugation to the adjacent carbon to irreversibly afford a stable conjugate product. The successful development of such a methionine conjugation protocol would underscore the utility of photoredox catalysis in facilitating the mild, selective, and robust functionalization of complex biomolecules.

Table 1. Scope of Michael Acceptors for the Photoredox Methionine-Selective Alkylation<sup>a</sup>

<sup>a</sup>Conditions: aprotinin (50  $\mu\text{M}$ ), **3** (500  $\mu\text{M}$ ), and Michael acceptor (10 mM) in PBS (pH 7.4)/DMF (19:1) under irradiation at 440 nm for 30 min. See the Supporting Information for experimental details.

In our initial studies, we examined the conjugation of the tetrapeptide VMFP (**1**) with diethyl ethylidenemalonate (**2**) in phosphate-buffered saline (PBS) (pH 7.4) under blue light (Kessil, 440 nm) in the presence of a water-soluble photocatalyst. A number of photocatalysts were explored; iridium- and ruthenium-based complexes, as well as organic dyes, resulted in exclusive formation of an undesired methionine sulfoxide product (Table S1), presumably as a result of sluggish  $\alpha$ -deprotonation following sulfur single-electron oxidation.<sup>19</sup> We thus turned our attention to the flavin family of photocatalysts. We postulated that after quenching of the flavin triplet excited state by methionine via SET, the resulting reduced flavin radical anion could act as a base<sup>20</sup> to deprotonate the position adjacent to the sulfur radical cation, thereby generating the desired  $\alpha$ -thio radical species. Gratifyingly, lumiflavin (**3**) efficiently catalyzed the transformation under the photocatalytic conditions, producing the desired methionine conjugate product **4** with 79% conversion without observable formation of the methionine sulfoxide byproduct (Figure 2a and Table S2).

The proposed mechanism of flavin-catalyzed methionine bioconjugation is illustrated in Figure 2b. Initially, photoexcitation of ground-state lumiflavin (<sup>1</sup>LF, **3**) ( $\lambda_{\text{max}}^{\text{abs}} = 444 \text{ nm}$ ) produces a long-lived ( $\tau = 20 \mu\text{s}$ ) triplet excited state (<sup>3</sup>LF, **6**).<sup>20b</sup> This highly oxidizing excited state ( $E_{1/2}^{\text{red}}[\text{}^3\text{LF/LF}^-] = 1.5 \text{ V vs SCE}$ )<sup>21</sup> is capable of undergoing SET with the methionine thioether ( $E_{\text{pa}} = 1.36 \text{ V vs SCE}$ ; see the Supporting

Information). Deprotonation at the  $\alpha$ -position of the resultant methionine radical cation **7** ( $\text{p}K_{\text{a}} = -3.5$ )<sup>22</sup> by lumiflavin radical anion (<sup>1</sup>LF<sup>-</sup>, **8**) ( $\text{p}K_{\text{a}}(\text{HLF}^{\bullet}) = 8.5$ )<sup>23</sup> furnishes  $\alpha$ -thio radical **9** and the stable reduced form of lumiflavin (HLF<sup>•</sup>, **10**). The nucleophilic carbon-centered radical intermediate **9** then undergoes addition to Michael acceptor **2** to afford  $\alpha$ -acyl radical **11**. Subsequent hydrogen atom transfer (HAT) between radical intermediate **11** and hydrogen atom donor **10** (N–H bond dissociation energy (BDE) = 59.9 kcal/mol)<sup>23</sup> regenerates the ground-state lumiflavin **3** and furnishes the methionine conjugate product **12**.

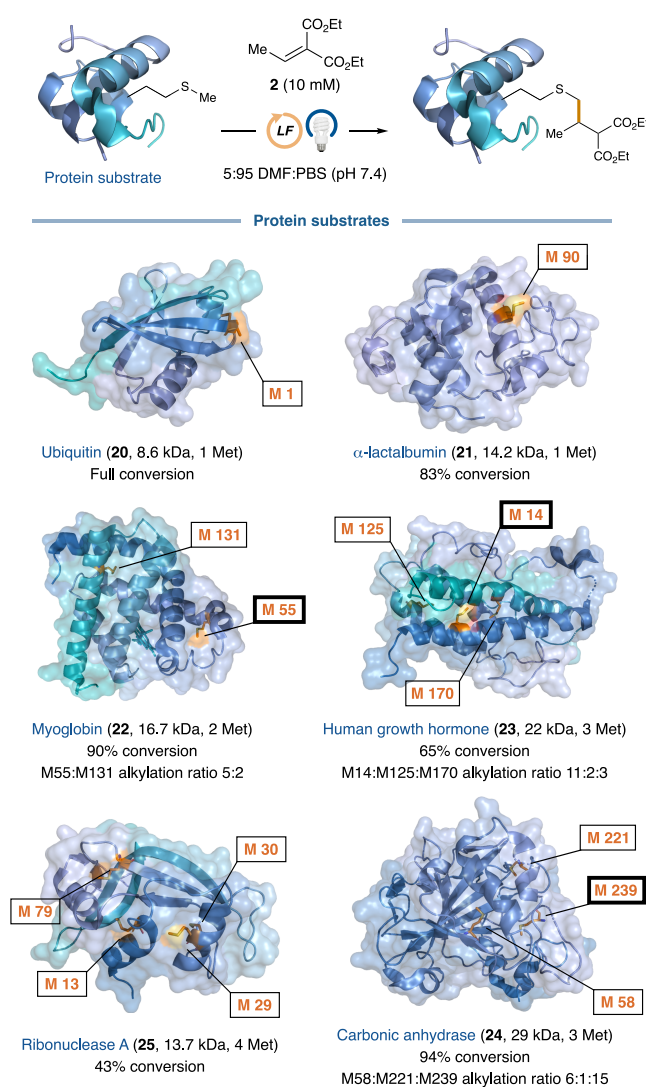
It should be noted that tyrosine, tryptophan, and histidine residues can also undergo sequential electron transfer–proton transfer (ET–PT) with excited lumiflavin; however, the corresponding radical intermediates are electrophilic in nature and expected to favor HAT reaction with **10** to regenerate the parent residues rather than addition to electrophilic Michael acceptor **2**.<sup>24</sup> Accordingly, we expected this method to permit selective modification of the methionine residue. To evaluate amino acid selectivity in the conjugation, we subjected a wide array of tetrapeptide substrates that did not contain methionine residues to the reaction conditions; in each case, the substrates were recovered (>90%) with no observable conjugate product formation (Table S3). One caveat that needs to be noted is that thiol radicals generated from free cysteine residues can readily react with Michael acceptors under the reaction conditions (see Table S4). In contrast to

free cysteine residues, however, cysteine disulfide bonds did not undergo photocatalyzed modification.

With a methionine-selective bioconjugation protocol in hand, we turned our attention to exploring the scope of the Michael acceptor component (Table 1). Aprotinin, a serine protease inhibitor containing one methionine residue, was selected as the model protein substrate. Under our optimal conditions, **2** was found to afford the monoalkylation product with 93% conversion. Similarly, 3-methylene-2-norbornanone (**13**) and phenyl vinyl sulfone (**14**) provided the conjugated products with 91% and 96% conversion, respectively. Notably, when acceptors **13** and **14** were employed, mixtures of mono-, bis-, and trisalkylation products were obtained, presumably as a result of subsequent  $\alpha$ -thio radical formation. Proteomic analysis of the products confirmed multialkylation on the single methionine residue. Importantly, various functional groups and biological payloads could be attached to the vinyl sulfone acceptor and readily incorporated into the protein via our methionine conjugation protocol. Notably, carboxylic acid and primary amine moieties were well-tolerated under the reaction conditions (**15** and **16**, >95% and 65% conversion, respectively). Vinyl sulfones containing bio-orthogonal azide and alkyne handles for copper-catalyzed click chemistry were also readily employed (**17** and **18**, >95% and 80% conversion, respectively). Finally, a substrate bearing a desthiobiotin affinity tag was successfully conjugated with useful efficiency (**19**, 48% conversion). Thus, the photocatalytic conjugation can accommodate a range of functional groups on the Michael acceptor component, allowing the introduction of useful functional handles that are not compatible with traditional nucleophilicity-based bioconjugation protocols.

We next sought to evaluate the generality and site selectivity of the protocol across a diverse range of protein substrates of varying molecular weights. As shown in Figure 3, proteins with one methionine, including ubiquitin (**20**) and  $\alpha$ -lactalbumin (**21**), underwent conjugation with high conversions. Notably, an extended reaction time of 90 min was required for the ubiquitin conjugation, which we attributed to the hindered placement of the methionine in the protein. In general, we anticipated that the rate of conjugation would be mediated by the degree of methionine surface exposure. To test this hypothesis, we exposed substrates with multiple methionines to the bioconjugation conditions. Following reaction, labeled protein products were digested with trypsin and then analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) to determine the relative amounts of peptides with modified methionines. Myoglobin (**22**) is a muscular oxygen-binding protein with a surface-exposed M55 residue and an internal M131 residue. Under standard bioconjugation conditions, myoglobin was alkylated with 90% conversion. Proteomic analysis of modified myoglobin revealed a 5:2 alkylation ratio at positions M55 and M131. It should be noted the alkylation ratio was based solely on the relative ion count and does not account for possible differences in the ionization efficiencies of different peptides.

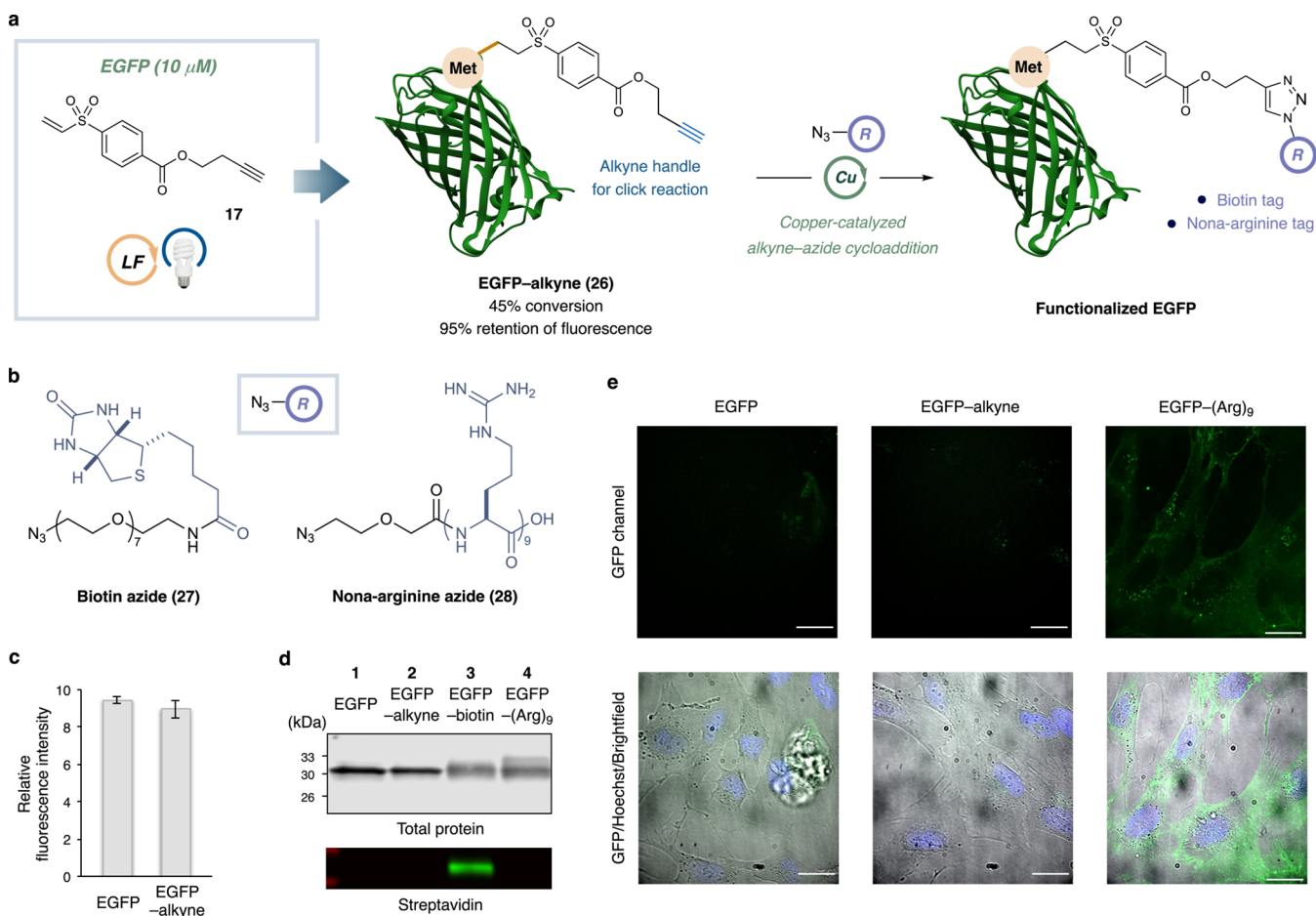
However, the ratio partly supports the hypothesis that the surface-exposed (M55) residue should be alkylated to a higher degree than the internal (M131) amino acid. Similar selectivity was observed in the conjugation of recombinant human growth hormone (**23**). Three methionine residues on the protein—M14, M125, and M170—were labeled in a ratio of 11:2:3, with conjugation primarily occurring on the most surface-exposed M14 residue. Surprisingly, carbonic anhydrase



**Figure 3.** Scope of proteins and site selectivity of the methionine bioconjugation methodology. The reaction conditions are the same as those shown in Table 1. See the Supporting Information for experimental details.

(**24**), which contains no surface-exposed methionines, was conjugated with nearly complete conversion, with an M58:M221:M239 alkylation ratio of 6:1:15. Finally, we were also able to label ribonuclease A (**25**) with greater than 43% conversion.

We then set out to evaluate the photoredox bioconjugation in the context of a biological system to probe whether the protocol is capable of modifying proteins under mild conditions without altering the tertiary structure. Enhanced green fluorescent protein (EGFP) was selected as a model substrate for this study. As illustrated in Figure 4a, we aimed to install an alkyne functional group by conjugating Michael acceptor **17** to EGFP. The alkyne would then serve as a handle for further functionalization of the protein via copper-catalyzed alkyne–azide cycloaddition (CuAAC). In practice, exposure of 10  $\mu$ M EGFP to the standard reaction conditions resulted in the formation of EGFP–alkyne conjugate **26** with 45% conversion. Because EGFP is known to lose its activity as a result of denaturation or photobleaching,<sup>25</sup> the fluorescence level of the product was measured to verify the integrity of



**Figure 4.** Functionalization of EGFP via the photoredox methionine bioconjugation protocol. (a) EGFP was initially labeled with 17, and then azides containing biological motifs were appended to the modified EGFP via CuAAC. (b) Azides bearing a biotin tag and a nonaarginine peptide. (c) After functionalization of EGFP via the photoredox methionine bioconjugation protocol, the product retained 95% of the fluorescence relative to wild-type EGFP ( $n = 3$ , mean  $\pm$  SD). (d) Western blot images of wild type EGFP and modified EGFPs. (e) Confocal microscopy images of SJS-A-1 cells treated with EGFP, EGFP-alkyne, and EGFP-(Arg)<sub>9</sub>. Scale bars = 20  $\mu$ m.

protein function. Notably, we observed 95% retention of fluorescence relative to wild-type EGFP (Figure 4c).<sup>26</sup> EGFP-alkyne conjugate 26 readily underwent CuAAC, which served to install the target biological motifs. Both biotin azide 27 and nonaarginine azide 28 were successfully employed in this transformation, affording the corresponding EGFP-biotin and EGFP-(Arg)<sub>9</sub> adducts (Figure 4b). We confirmed the attachment of the biotin tag to EGFP by streptavidin immunohistological staining (Figure 4d, lane 3). To verify attachment of the nonaarginine tag, SJS-A-1 cells were treated with EGFP-(Arg)<sub>9</sub> and visualized using confocal microscopy. Since the nonaarginine peptide facilitates delivery of cargo proteins into cells,<sup>27</sup> only EGFP-(Arg)<sub>9</sub> conjugate would be internalized (Figure 4e). As expected, no significant internalization was observed in the cells treated with unmodified EGFP or EGFP-alkyne conjugate 26. These results highlight the capacity of the photocatalytic bioconjugation method to enable rapid methionine-selective conjugation and delivery of biological payloads into cells.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c09926>.

Experimental procedures, NMR spectra, and CV data (PDF)

Proteomics analysis (ZIP)

## ■ AUTHOR INFORMATION

### Corresponding Author

David W. C. MacMillan – Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United States; [orcid.org/0000-0001-6447-0587](https://orcid.org/0000-0001-6447-0587); Email: [dmacmill@princeton.edu](mailto:dmacmill@princeton.edu)

### Authors

Junyong Kim – Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United States; [orcid.org/0000-0001-7037-8621](https://orcid.org/0000-0001-7037-8621)

Beryl X. Li – Merck Center for Catalysis at Princeton University, Princeton, New Jersey 08544, United States

Richard Y.-C. Huang – Pharmaceutical Candidate Optimization, Research and Development, Bristol-Myers Squibb Company, Princeton, New Jersey 08542, United States; [orcid.org/0000-0002-7172-110X](https://orcid.org/0000-0002-7172-110X)

Jennifer X. Qiao – Discovery Chemistry, Research and Development, Bristol-Myers Squibb Company, Princeton, New Jersey 08542, United States

William R. Ewing – Discovery Chemistry, Research and Development, Bristol-Myers Squibb Company, Princeton, New Jersey 08542, United States; [orcid.org/0000-0002-9434-8053](https://orcid.org/0000-0002-9434-8053)

Complete contact information is available at:  
<https://pubs.acs.org/10.1021/jacs.0c09926>

## Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors are grateful for financial support provided by the National Institute of General Medical Sciences (NIGMS), the NIH (under Award R35GM134897-01), the Princeton Catalysis Initiative, and kind gifts from Merck, Janssen, BMS, Genentech, Celgene, and Pfizer. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIGMS.

## REFERENCES

- (1) Hermanson, G. T. *Bioconjugate Techniques*, 3rd ed.; Academic Press: San Diego, CA, 2013.
- (2) For selected reviews, see: (a) Boutureira, O.; Bernardes, G. J. L. Advances in Chemical Protein Modification. *Chem. Rev.* **2015**, *115*, 2174. (b) Stephanopoulos, N.; Francis, M. B. Choosing an Effective Protein Bioconjugation Strategy. *Nat. Chem. Biol.* **2011**, *7*, 876. (c) Schumacher, D.; Hackenberger, C. P. R. More than Add-on: Chemoselective Reactions for the Synthesis of Functional Peptides and Proteins. *Curr. Opin. Chem. Biol.* **2014**, *22*, 62. (d) deGruyter, J. N.; Malins, L. R.; Baran, P. S. Residue-Specific Peptide Modification: A Chemist's Guide. *Biochemistry* **2017**, *56*, 3863.
- (3) Chen, X.; Muthoosamy, K.; Pfisterer, A.; Neumann, B.; Weil, T. Site-Selective Lysine Modification of Native Proteins and Peptides via Kinetically Controlled Labeling. *Bioconjugate Chem.* **2012**, *23*, 500.
- (4) (a) Kim, Y.; Ho, S. O.; Gassman, N. R.; Korlann, Y.; Landorf, E. V.; Collart, F. R.; Weiss, S. Efficient Site-Specific Labeling of Proteins via Cysteines. *Bioconjugate Chem.* **2008**, *19*, 786. (b) Smith, M. E. B.; Schumacher, F. F.; Ryan, C. P.; Tedaldi, L. M.; Papaioannou, D.; Waksman, G.; Caddick, S.; Baker, J. R. Protein Modification, Bioconjugation, and Disulfide Bridging Using Bromomaleimides. *J. Am. Chem. Soc.* **2010**, *132*, 1960. (c) Morales-Sanfrutos, J.; Lopez-Jaramillo, F. J.; Hernandez-Mateo, F.; Santoyo-Gonzalez, F. Vinyl Sulfone Bifunctional Tag Reagents for Single-Point Modification of Proteins. *J. Org. Chem.* **2010**, *75*, 4039.
- (5) (a) Gavriluk, J.; Ban, H.; Nagano, M.; Hakamata, W.; Barbas, C. F., III Formylbenzene Diazonium Hexafluorophosphate Reagent for Tyrosine-Selective Modification of Proteins and the Introduction of a Bioorthogonal Aldehyde. *Bioconjugate Chem.* **2012**, *23*, 2321. (b) Zhang, J.; Ma, D.; Du, D.; Xi, Z.; Yi, L. An Efficient Reagent for Covalent Introduction of Alkynes into Proteins. *Org. Biomol. Chem.* **2014**, *12*, 9528. (c) Joshi, N. S.; Whitaker, L. R.; Francis, M. B. A Three-Component Mannich-Type Reaction for Selective Tyrosine Bioconjugation. *J. Am. Chem. Soc.* **2004**, *126*, 15942. (d) Mileo, E.; Etienne, E.; Martinho, M.; Lebrun, R.; Roubaud, V.; Tordo, P.; Gontero, B.; Guigliarelli, B.; Marque, S. R. A.; Belle, V. Enlarging the Panoply of Site-Directed Spin Labeling Electron Paramagnetic Resonance (SDSL-EPR): Sensitive and Selective Spin-Labeling of Tyrosine Using an Isoindoline-Based Nitroxide. *Bioconjugate Chem.* **2013**, *24*, 1110. (e) Guo, H.-M.; Minakawa, M.; Ueno, L.; Tanaka, F. Synthesis and Evaluation of a Cyclic Imine Derivative Conjugated to a Fluorescent Molecule for Labeling of Proteins. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1210. (f) Ban, H.; Gavriluk, J.; Barbas, C. F., III Tyrosine Bioconjugation through Aqueous Ene-Type Reactions: A Click-Like Reaction for Tyrosine. *J. Am. Chem. Soc.* **2010**, *132*, 1523.
- (6) (a) Antos, J. M.; Francis, M. B. Selective Tryptophan Modification with Rhodium Carbenoids in Aqueous Solution. *J. Am. Chem. Soc.* **2004**, *126*, 10256. (b) Seki, Y.; Ishiyama, T.; Sasaki, D.; Abe, J.; Sohma, Y.; Oisaki, K.; Kanai, M. Transition Metal-Free Tryptophan-Selective Bioconjugation of Proteins. *J. Am. Chem. Soc.* **2016**, *138*, 10798. (c) Foettinger, A.; Melmer, M.; Leitner, A.; Lindner, W. Reaction of the Indole Group with Malondialdehyde: Application for the Derivatization of Tryptophan Residues in Peptides. *Bioconjugate Chem.* **2007**, *18*, 1678. (d) Ruiz-Rodríguez, J.; Albericio, F.; Lavilla, R. Postsynthetic Modification of Peptides: Chemoselective C-Arylation of Tryptophan Residues. *Chem. - Eur. J.* **2010**, *16*, 1124. (e) Gu, C.-X.; Bi, Q.-W.; Gao, C.-K.; Wen, J.; Zhao, Z.-G.; Chen, Z. Post-Synthetic Modification of Tryptophan Containing Peptides via NIS Mediation. *Org. Biomol. Chem.* **2017**, *15*, 3396. (f) Tower, S. J.; Hetcher, W. J.; Myers, T. E.; Kuehl, N. J.; Taylor, M. T. Selective Modification of Tryptophan Residues in Peptides and Proteins Using a Biomimetic Electron Transfer Process. *J. Am. Chem. Soc.* **2020**, *142*, 9112.
- (7) (a) Ohata, J.; Minus, M. B.; Abernathy, M. E.; Ball, Z. T. Histidine-Directed Arylation/Alkenylation of Backbone N-H Bonds Mediated by Copper(II). *J. Am. Chem. Soc.* **2016**, *138*, 7472. (b) Kapanidis, A. N.; Ebright, Y. W.; Ebright, R. H. Site-Specific Incorporation of Fluorescent Probes into Protein: Hexahistidine-Tag-Mediated Fluorescent Labeling with (Ni<sup>2+</sup>:Nitrilotriacetic Acid)<sub>n</sub>-Fluorochrome Conjugates. *J. Am. Chem. Soc.* **2001**, *123*, 12123. (c) Lai, Y.-T.; Chang, Y.-Y.; Hu, L.; Yang, Y.; Chao, A.; Du, Z.-Y.; Tanner, J. A.; Chye, M.-L.; Qian, C.; Ng, K.-M.; Li, H.; Sun, H. Rapid Labeling of Intracellular His-Tagged Proteins in Living Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 2948. (d) Jia, S.; He, D.; Chang, C. J. Bioinspired Thiophosphorodichloridate Reagents for Chemoselective Histidine Bioconjugation. *J. Am. Chem. Soc.* **2019**, *141*, 7294.
- (8) (a) Shaw, M. H.; Twilton, J.; MacMillan, D. W. C. Photoredox Catalysis in Organic Chemistry. *J. Org. Chem.* **2016**, *81*, 6898. (b) Staveness, D.; Bosque, I.; Stephenson, C. R. J. Free Radical Chemistry Enabled by Visible Light-Induced Electron Transfer. *Acc. Chem. Res.* **2016**, *49*, 2295.
- (9) Bottecchia, C.; Noël, T. Photocatalytic Modification of Amino Acids, Peptides, and Proteins. *Chem. - Eur. J.* **2019**, *25*, 26.
- (10) Bloom, S.; Liu, C.; Kölmel, D. K.; Qiao, J. X.; Zhang, Y.; Poss, M. A.; Ewing, W. R.; MacMillan, D. W. C. Decarboxylative Alkylation for Site-Selective Bioconjugation of Native Proteins via Oxidation Potentials. *Nat. Chem.* **2018**, *10*, 205.
- (11) (a) Gundlach, H. G.; Moore, S.; Stein, W. H. The Reaction of Iodoacetate with Methionine. *J. Biol. Chem.* **1959**, *234*, 1761. (b) Kramer, J. R.; Deming, T. J. Reversible Chemoselective Tagging and Functionalization of Methionine Containing Peptides. *Chem. Commun.* **2013**, *49*, 5144.
- (12) Bergès, J.; de Oliveira, P.; Fourré, I.; Houé-Levin, C. The One-Electron Reduction Potential of Methionine-Containing Peptides Depends on the Sequence. *J. Phys. Chem. B* **2012**, *116*, 9352.
- (13) Lin, S.; Yang, X.; Jia, S.; Weeks, A. M.; Hornsby, M.; Lee, P. S.; Nichiporuk, R. V.; Iavarone, A. T.; Wells, J. A.; Toste, F. D.; Chang, C. J. Redox-based Reagents for Chemoselective Methionine Bioconjugation. *Science* **2017**, *355*, 597.
- (14) Taylor, M. T.; Nelson, J. E.; Suero, M. G.; Gaunt, M. J. A Protein Functionalization Platform Based on Selective Reactions at Methionine Residues. *Nature* **2018**, *562*, 563.
- (15) Elledge, S. K.; Tran, H. L.; Christian, A. H.; Steri, V.; Hann, B.; Toste, F. D.; Chang, C. J.; Wells, J. A. Systematic Identification of Engineered Methionines and Oxaziridines for Efficient, Stable, and Site-Specific Antibody Bioconjugation. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 5733–5740.
- (16) Christian, A. H.; Jia, S.; Cao, W.; Zhang, P.; Meza, A. T.; Sigman, M. S.; Chang, C. J.; Toste, F. D. A Physical Organic Approach to Tuning Reagents for Selective and Stable Methionine Bioconjugation. *J. Am. Chem. Soc.* **2019**, *141*, 12657–12662.
- (17) Lin, D.; Wallace, M.; Allentoff, A. J.; Donnelly, D. J.; Gomes, E.; Voronin, K.; Gong, S.; Huang, R. Y.-C.; Kim, H.; Caceres-Cortes, J.; Bonacorsi, S., Jr. Chemoselective Methionine Bioconjugation: Site-Selective Fluorine-18 Labeling of Proteins and Peptides. *Bioconjugate Chem.* **2020**, *31*, 1908–1916.

(18) Ohata, J.; Krishnamoorthy, L.; Gonzalez, M. A.; Xiao, T.; Iovan, D. A.; Toste, F. D.; Miller, E. W.; Chang, C. J. *ACS Cent. Sci.* **2020**, *6*, 32–40.

(19) Emmanuel, N.; Mendoza, C.; Winter, M.; Horn, C. R.; Vizza, A.; Dreesen, L.; Heinrichs, B.; Monbaliu, J.-C. M. *Org. Process Res. Dev.* **2017**, *21*, 1435.

(20) (a) Heelis, P. F. The Photophysical and Photochemical Properties of Flavins (Isoalloxazine). *Chem. Soc. Rev.* **1982**, *11*, 15.

(b) Grodowski, M. S.; Veyret, B.; Weiss, K. Photochemistry of Flavins. II. Photophysical Properties of Alloxazines and Isoalloxazines. *Photochem. Photobiol.* **1977**, *26*, 341.

(21) Lu, C.; Lin, W.; Wang, W.; Han, Z.; Yao, S.; Lin, N. Riboflavin (VB2) Photosensitized Oxidation of 2'-Deoxyguanosine-5'-Monophosphate (dGMP) in Aqueous Solution: a Transient Intermediates Study. *Phys. Chem. Chem. Phys.* **2000**, *2*, 329.

(22) Hiller, K. O.; Masloch, B.; Goebel, M.; Asmus, K. D. Mechanism of the Hydroxyl Radical Induced Oxidation of Methionine in Aqueous Solution. *J. Am. Chem. Soc.* **1981**, *103*, 2734.

(23) Warren, J. J.; Tronic, T. A.; Mayer, J. M. Thermochemistry of Proton-Coupled Electron Transfer Reagents and its Implications. *Chem. Rev.* **2010**, *110*, 6961.

(24) Heelis, P. F.; Parsons, B. J.; Phillips, G. O.; McKellar, J. F. The Photoreduction of Flavins by Amino Acids and EDTA. A Continuous and Flash Photolysis Study. *Photochem. Photobiol.* **1979**, *30*, 343.

(25) Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y. A Guide to Choosing Fluorescent Proteins. *Nat. Methods* **2005**, *2*, 905.

(26) We speculate that the presence of excess lumiflavin ( $\lambda_{\text{max}}^{\text{abs}} = 444$  nm) in the reaction mixture inhibits photoexcitation of EGFP ( $\lambda_{\text{max}}^{\text{abs}} = 488$  nm), thereby preventing photobleaching.

(27) Böhmová, E.; Machová, D.; Pechar, M.; Pola, R.; Venclíková, K.; Janoušková, O.; Etrych, T. Cell-Penetrating Peptides: A Useful Tool for the Delivery of Various Cargoes into Cells. *Physiol. Res.* **2018**, *67*, S267.