Ultra-Rapid Electrophilic Cysteine Arylation

Bradley M. Lipka,[a] Daniel S. Honeycutt,[a] Gregory M. Bassett,[a] Taylor N. Kowal,[a] Max Adamczyk,[a] Zachary C. Cartnick,[b] Vincent M. Betti,[b] Jacob M. Goldberg,*[b] and Fang Wang*[a]

[a]Department of Chemistry, University of Rhode Island, 140 Flagg Rd, Kingston, Rhode Island, 02881, United States

[b]Department of Chemistry, Colgate University, 13 Oak Drive, Hamilton, New York, 13346, United States

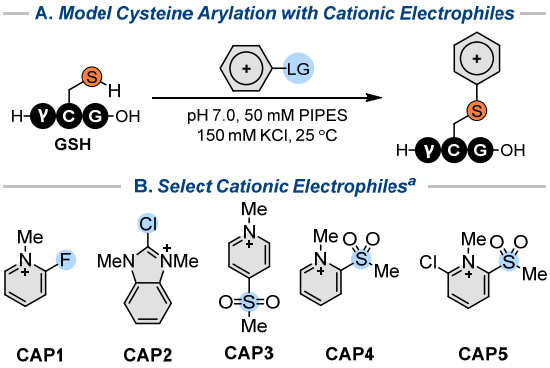
Supporting Information Placeholder

ABSTRACT: Rapid bond forming reactions are crucial for efficient bioconjugation. We describe a simple and practical strategy for facilitating ultra-rapid electrophilic cysteine arylation. Using a variety of sulfone-activated pyridinium salts, this uncatalyzed reaction proceeds with exceptionally high rate constants, ranging from 9800 to 320,000 $M^{-1}\cdot s^{-1}$, in pH 7.0 aqueous buffer at 25 °C. Such reactions allow for stoichiometric bioconjugation of micromolar cysteine within minutes or even seconds. Even though the arylation is extremely fast, the chemistry exhibits excellent selectivity, thus furnishing functionalized peptides and proteins with both high conversion and purity.

Fast chemoselective reactions are critical for efficient chemical synthesis, particularly bioconjugation chemistry.[1-4] These transformations frequently entail rapid functionalization of unprotected biomacromolecules at low concentrations to afford products with high selectivity.

In the context of protein and peptide modification, many synthetic approaches employ the cysteine side chain as a reactive handle due to its high nucleophilicity, wide distribution, but low abundance.[5-6] Over the past decades, many classes of electrophiles have been discovered for cysteine-based functionalization of proteins and peptides, substantially expanding the biomacromolecular chemical space and allowing for advanced proteomic investigations.[7-29]

Despite important advances in this area, limitations remain. A major challenge in bioconjugation chemistry is identifying reactions that can efficiently operate at micromolar or lower concentrations under biocompatible conditions: neutral, aqueous solutions at ambient temperatures. To achieve 97% conversion within one hour, a minimum second-order rate constant (k_2) of 100 $M^{-1}\cdot s^{-1}$ is required for stoichiometric conjugation at 100 μM.[2-4] Extrapolating from this analysis, efficient equimolar labeling of 1 μM target substrates needs reactions with an even higher k_2 of 10,000 $M^{-1}\cdot s^{-1}$, a value rarely achievable in the absence of enzymes or other catalysts. A number of elegant reagents have been developed that exhibit cysteine reactivity approaching such rate constants, ranging from 100 to 5,500 $M^{-1}\cdot s^{-1}$ under physiologically relevant conditions. Examples include 4-chloro-7-nitrobenzofurazan,[9] heteroaromatic sulfones,[20] palladium-aryl complexes,[30] and 2-formyl phenylboronic acid derivatives.[31-32] Here, we present a straightforward method for ultra-rapid, highly selective cysteine arylation with second-order rate constants around 100,000 $M^{-1}\cdot s^{-1}$ in pH 7.0 aqueous buffers at ambient temperatures.



C. *Pyridinium Salts Reactivity toward GSH and H_2O*

Electrophile	k_2 with GSH ($M^{-1}\cdot s^{-1}$)	97% conv. with equimolar GSH at 100 μM	$t_{½}$ in pH 7 PIPES at rt
Mukaiyama reagent	1.2×10^{0}	3.7 days	23 days
CAP1	1.2×10^{2}	45 min	47 min
CAP2	2.4×10^{1}	3.7 h	59 h
CAP3	9.8×10^{3}	33 s	17 h
CAP4	1.2×10^{5}	2.7 s	39 min
CAP5	3.2×10^{5}	1.0 s	5.6 min

Figure 1. A. Model arylation reaction between **CAP** reagents and glutathione (GSH, γ-glutamylcysteinylglycine). B. Structures of select **CAP** reagent cations. Iodide or tetrafluoroborate counter anions are omitted. C. Half-lives of electrophilic pyridinium salts in pH 7.0 50 mM PIPES buffer containing 100 mM KCl at 25 °C. The half-lives in water at 25 °C were 8–15 times longer. **CAP5** reacts with GSH and water via sulfone substitution and chlorine replacement, respectively.

We hypothesize that two major factors limit the application of conventional organic electrophiles for rapid cysteine bioconjugation – (i) insufficient thiol reactivity and (ii) general water incompatibility, particularly poor aqueous solubility. To circumvent these issues, we previously demonstrated a convenient method for cysteine arylation with pyridinium (**CAP**) salts, including *N*-methyl-*o*-fluoropyridinium iodide (**CAP1**, Fig. 1B) and its derivatives.[33] The advantageous reactivity of these water-soluble organic salts arises from the cationic nature of the electrophiles and highly polarized C–F bonds.[34-35] Specifically, the reaction between **CAP1** and glutathione (GSH) showed a

high second-order rate constant of 116 $M^{-1}\cdot s^{-1}$ in pH 7.0 aqueous buffers at 25 °C and thus allowed for efficient functionalization of cysteine in the 100 μM concentration range (Fig. 1C).

Based on these results, we speculated that pyridinium salts that are even more electron-deficient would enable ultra-rapid cysteine arylation under mild conditions. We thus chose to functionalize the pyridinium ring with a strongly electron-withdrawing methyl sulfone group,[36] which can serve both as an effective activator for the electrophilic aromatic system and a good leaving group during the substitution.[12, 19-20, 37-41] Accordingly, two water-soluble pyridinium salts were synthesized, including *N*-methyl-4-methylsulfonyl pyridinium (**CAP3**) and *N*-methyl-2-methylsulfonyl pyridinium (**CAP4**) tetrafluoroborates (Fig. 1B). The kinetic study with GSH revealed the remarkable reactivity of **CAP3** and **CAP4**, with respective k_2 values of 9,800 and 120,000 $M^{-1}\cdot s^{-1}$ in pH 7.0 PIPES buffer at 25 °C (Fig. 1C, S1, and S15). In pH 7.4 PBS, **CAP4** reacted with GSH at 200,000 $M^{-1}\cdot s^{-1}$; in pH 8.0 Tris, the rate constant reached 870,000 $M^{-1}\cdot s^{-1}$ (Fig. S3 and S4). Such extreme reactivities enable ultra-rapid arylation of sub-micromolar cysteine within minutes. Even so, **CAP3** and **CAP4** showed a long half-life of approximately 140 h and 18 h in water at 25 °C, respectively (Fig. 1C, S14, and S17). Such persistence allows for the convenient storage and handling of aqueous stock solutions, significantly simplifying experimental protocols. Compared to **CAP1-2**, **CAP3-5** show an even greater preference for cysteine arylation over hydrolysis, ensuring the high chemoselectivity of the bioconjugation. Moreover, the cysteine arylation also proceeds very rapidly even at low pH or in water, conditions occasionally encountered but not typically preferred due to the low reactive thiolate availability. These attributes demonstrate the versatility of this chemistry for different synthetic applications (Fig. S2, S7, S304, and S311). In addition to these two prototypical pyridinium salts, we further synthesized a ditopic labeling reagent, *N*-methyl-2-chloro-6-methylsulfonyl pyridinium tetrafluoroborate (**CAP5**), bearing a chloride and the methyl sulfone moiety as the leaving groups. Reactivity investigation suggested that **CAP5** also undergoes ultra-rapid reaction with equimolar GSH at approximately 320,000 $M^{-1}\cdot s^{-1}$ through sulfonyl group substitution, an extremely fast reaction under very mild conditions without any catalyst (Fig. 1C, S18, S317–322). Together with *N*-methyl-*o*-chloropyridinium iodide, commonly known as Mukaiyama reagent,[42] and other **CAP** reagents previously reported (Fig. 1B-1C),[33] these substituted pyridinium salts comprise a library of convenient cysteine arylating reagents with rate constants that span five orders of magnitude and that are amenable for a multitude of synthetic tasks.

We next investigated synthetic applications of **CAP3** and **CAP4**. As revealed by NMR and high-performance liquid chromatography (HPLC) studies, the reaction of GSH with 1.1 equiv. of **CAP3** and **CAP4** afforded the corresponding sulfur arylated product in both high conversion (>99%) and purity (73-98%, Fig. S25–26 and 29–30). For more structurally complicated peptides containing other nucleophilic residues, **CAP3** and **CAP4** also enabled efficient cysteine bioconjugation with remarkable chemoselectivity. We first arylated oxytocin, a nonapeptide hormone containing a disulfide bond, which can be reduced by tris(2-carboxyethyl)phosphine (TCEP). With 100 μM reduced oxytocin, 2.1 equiv. of the pyridinium salts labeled both cysteine residues in pH 7.0 PIPES buffer at room temperature, yielding essentially pure product in 99% conversion within 5 min (Fig. 2C, S46–47, and S51–52). Similarly, the disulfide-containing 14-mer peptide somatostatin also rapidly reacted with **CAP3** or **CAP4** under mild conditions to give doubly arylated products in both high conversion and purity (Fig. 2C, S34–35, and S39–40). The reaction of **CAP4** with structurally diverse substrates, including ADH-1 and the 22-mer C-type natriuretic peptide, also exhibited near quantitative conversion, underscoring the broad scope of this chemistry. Notably, as confirmed by HPLC and tandem mass spectrometry (MS) studies, both **CAP3** and **CAP4** exclusively arylated the cysteine side chains of all peptides investigated, highlighting the marked preference of these pyridinium electrophiles with the thiol moiety over other nucleophiles, including lysine, histidine, tyrosine, serine, threonine, and the unprotected *N*-terminus (Fig. 2C, S62–79). This observation is in good agreement with our kinetic studies of **CAP** reagents with common biological nucleophiles and can also be predicted by Mayr's nucleophilicity parameters.[43-45] For example, the reaction of **CAP4** with cysteine is about five to six orders of magnitude faster than that with histidine, lysine, or tyrosine analogs (Fig. S10–12).

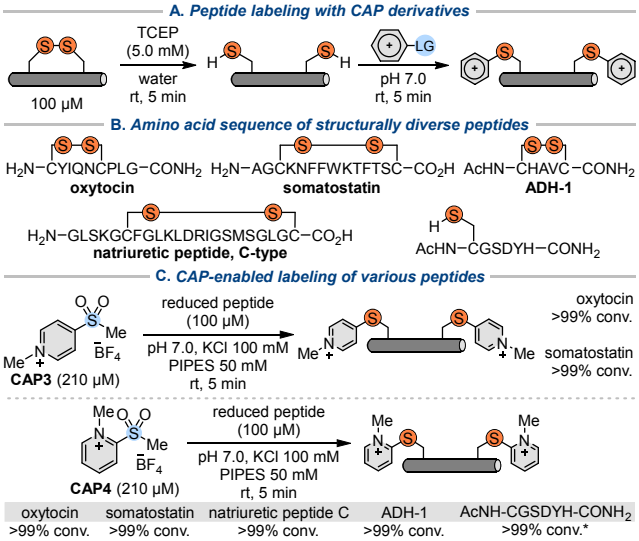


Figure 2. A. Typical protocol for dual labeling of disulfide-containing peptides using **CAP** reagents. B. The amino acid sequence of structurally diverse peptides. C. Labeling of reduced various peptides using **CAP3** or **CAP4**. *Directly arylated at 100 μM with 105 μM **CAP4** without TCEP.

Prompted by these results, we utilized the **CAP**-based strategy for constructing macrocyclic peptides[46-49] through cysteine stapling reactions. Our initial effort focused on using this pyridinium salt to arylate the reduced disulfide bond in oxytocin and somatostatin. The presence of two electrophilically distinct carbon atoms with S_NAr reactivity separated by five orders of magnitude makes **CAP5** particularly suitable for this stapling chemistry (Fig. 1C). The exceedingly more reactive sulfone-substituted carbon can initiate an ultra-rapid intermolecular C–S bond forming reaction at high substrate dilution, a condition usually necessary in biomacromolecule cyclization reactions.[50-51] In contrast, the substantially lower electrophilicity of the chlorinated carbon disfavors similar intermolecular processes to prevent undesired peptide oligomerization or polymerization but still allows for a feasible intramolecular cysteine arylation reaction, exclusively rendering monomeric cyclized peptides. The stapling reaction of oxytocin and somatostatin with **CAP5** experimentally supports the high efficiency of this chemistry (Fig. 3B). In both cases, the macrocyclic peptides were obtained within 5 min under very mild conditions with high conversion as indicated by HPLC analysis (Fig. S80–81, S86, and S105–106). Tandem MS studies further confirmed that the cyclization results from the desired dual cysteine arylations.

We next developed bipyridinium salts that featured two independent electrophilic aromatic systems, respectively bearing a chlorine and a sulfone leaving group. A series of regioisomers, **CAP6-8**, were synthesized to allow for the precise modification of the structural parameters of the cyclized peptides. Analogous to **CAP5**, **CAP6-8** also facilitated ultra-rapid, highly selective macrocyclization of reduced somatostatin in pH 7.0 PIPES buffer at 25 °C within 5 min, generally furnishing the anticipated products in high purity (Fig. 3B, S89, S94–95, and S100). The remarkable efficiency of the transformation was observed across all three regioisomeric stapling reagents and the reaction concentrations tested (25-100 μM), indicating the broad scope and universal applicability of this strategy for different synthetic purposes.

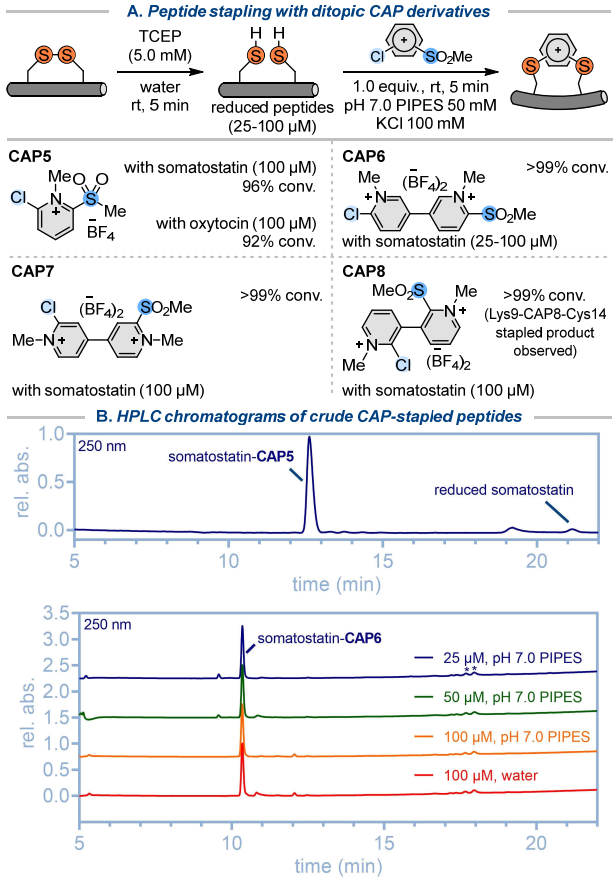


Figure 3. A. Typical protocol for stapling reaction of disulfide-containing peptides using ditopic **CAP** reagents. Reaction efficiencies of oxytocin and somatostatin stapling using various **CAP** reagents are shown. B. Select HPLC chromatograms of the reaction mixture of **CAP**-enabled peptide stapling, indicating the high purity of the crude product (asterisks indicate impurities from the HPLC mobile phase).

We investigated the applications of these sulfone-based **CAP** reagents for protein bioconjugation (Fig. 4). We explored the reaction of two prototypical sulfone-containing pyridinium salts, **CAP3** and **CAP4**, with bovine serum albumin (BSA). MALDI and intact protein MS studies indicated that both reagents efficiently labeled BSA in pH 8.0 Tris or pH 7.0 PIPES at room temperature within 15 min, revealing their remarkably high reactivity toward the thiol group in not only small peptides but also large proteins (Fig. 4A, 4B, and S127–S139). We further investigated **CAP**-enabled arylation of KRAS and *Yersinia* tyrosine phosphatase (YopH) proteins, which bear multiple free cysteine residues. Mass spectrometry analysis of **CAP3**-arylated KRAS revealed efficient functionalization of all three free cysteines (Fig. S140–S156). Despite its relatively higher reactivity with small cysteine-containing peptides, **CAP4** exhibited comparatively lower efficiency of cysteine arylation in KRAS (Fig. S157–S168). Whereas we observed arylation of up to all five free cysteines in YopH with **CAP3** (Fig. S170–S182), the mass spectrometry data of **CAP4**-labeled YopH only indicate arylation of three cysteines. Signals corresponding to labeling at the remaining two cysteine residues (Cys93 and Cys262) are near the detection limit, perhaps due to inefficient reaction at these sites (Fig. S183–S195).

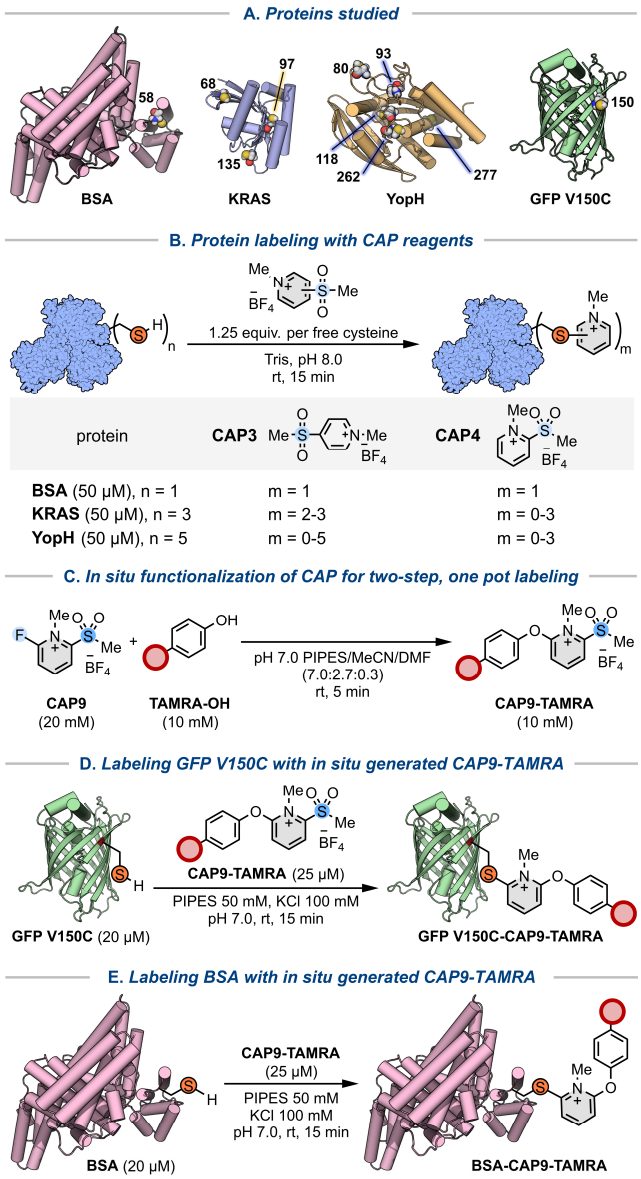


Figure 4. (A) Protein structures; (B) Summary of protein labeling experiments with **CAP3** and **CAP4**; (C) Generation of **CAP9-TAMRA** cysteine arylating reagent with **CAP9** and phenol-containing TAMRA *in situ*; (D) Modular arylation of GFP V150C with **CAP9-TAMRA**; (E) Modular arylation of BSA with **CAP9-TAMRA**.

We next performed modular one-pot, two-step functionalization of proteins using the ditopic **CAP9** reagent, which bears *ortho* fluorine and an *ortho* methyl sulfone as leaving groups (Fig. 4C). Our NMR and HPLC studies indicated nucleophile-specific reactivity of these two leaving groups toward hydroxylation and thiolation: the C–F moiety preferentially reacts

with the hydroxyl group, but the sulfone group is rapidly substituted by cysteine (Fig. S22, S122–123, and S323–328). In particular, the high hydroxyl reactivity of the C–F bond facilitates quantitative phenol arylation in pH 7.0 PIPES buffer with **CAP9**, the excess of which is converted into the rather thiol-inert methylsulfonyl pyridone, thus avoiding undesired cysteine labeling with unreacted **CAP9**. Following this strategy, we reacted **CAP9** with a phenol-containing tetramethylrhodamine (TAMRA) derivative **TAMRA-OH** under very mild conditions (Fig. 4C). The resulting red-fluorescent pyridinium salt, **CAP9-TAMRA**, was generated *in situ* and promoted rapid arylation of both BSA and cysteine-containing green fluorescent protein (GFP V150C) in pH 7.0 PIPES buffer at room temperature, demonstrating the versatility and high synthetic modularity of this one-pot two-step chemistry (Fig. 4D, 4E, and S196–197).

In conclusion, the synthetic strategy presented here provides a simple and user-friendly solution for ultra-rapid cysteine functionalization under biologically compatible conditions. Pairing cationic pyridinium systems with strongly electron-withdrawing methyl sulfone moiety, this unique chemistry substantially enhances the efficiency of cysteine arylation, with exceedingly large rate constants of up to 870,000 $M^{-1}{\cdot}s^{-1}$ under very mild conditions without a catalyst. Significantly, their high reactivity with cysteine does not lead to low chemoselectivity.[52] Derivatization of the **CAP** compounds with other reaction handles, such as phenol motifs, facilitates the construction of complex biomacromolecular scaffolds, including stapled peptides and labeled proteins, generally in minutes – or even seconds – with excellent purity. Given the facile accessibility of **CAP** reagents, the operational ease of the method, and the ultra-rapid and selective transformations, we expect wide application of this synthetic platform in practical biomacromolecule modifications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supplementary figures and schemes, materials, experimental procedures; characterization data (1D and 2D NMR, LCMS, HRMS) for all small molecule compounds; mass spectrometry of peptides and proteins; reaction kinetics (PDF)

AUTHOR INFORMATION

Corresponding Author

Jacob M. Goldberg - Department of Chemistry, Colgate University, Hamilton, New York 13346, United States; orcid.org/0000-0002-8004-3769; Email: jgoldberg@colgate.edu
Fang Wang - Department of Chemistry, University of Rhode Island, Kingston, Rhode Island 02881, United States; orcid.org/0000-0002-9192-6858; Email: fangwang@uri.edu

Notes

The authors declare the following competing financial interest(s): A patent application has been submitted by the University of Rhode Island and Colgate University to cover this work.

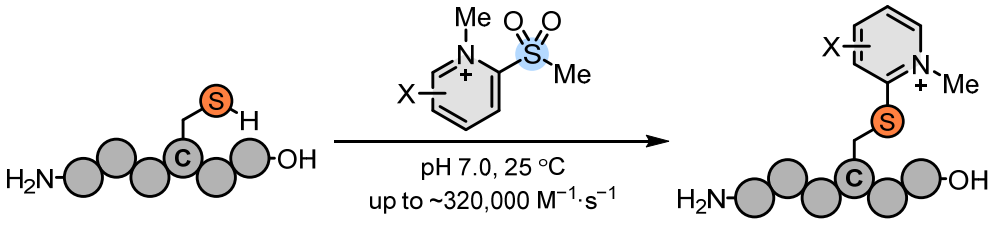
ACKNOWLEDGMENT

This research was made possible by the use of equipment available through the Rhode Island Institutional Development Award (IDeA) Network of Biomedical Research Excellence from the National Institute of General Medical Sciences of the National Institutes of Health under Grant No. P20GM103430 through the Centralized Research Core facility. High-Performance Computing at the University of Rhode Island is gratefully acknowledged. T.N.K. and M.A. thank the Office of Undergraduate Research and Innovation at the University of Rhode Island. This material is based upon work supported by the National Science Foundation under Grant No. CHE-2117141. We gratefully acknowledge the mass spectrometry-based analysis of intact proteins conducted by Jennifer C. Liddle and Jeremy L. Balsbaugh of the UConn Proteomics & Metabolomics Facility, a component of the Center for Open Research Resources and Equipment at the University of Connecticut.

REFERENCES

1. Hermanson, G. T., In *Bioconjugate Techniques (Third Edition)*, Academic Press: Boston, 2013.
2. Dirksen, A.; Dawson, P. E., Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling. *Bioconjugate Chem.* **2008,** *19*, 2543-2548.
3. Kalia, J.; Raines, R., Advances in Bioconjugation. *Curr. Org. Chem.* **2010,** *14*, 138-147.
4. Saito, F.; Noda, H.; Bode, J. W., Critical Evaluation and Rate Constants of Chemoselective Ligation Reactions for Stoichiometric Conjugations in Water. *ACS Chem. Biol.* **2015,** *10*, 1026-1033.
5. Miseta, A.; Csutora, P., Relationship Between the Occurrence of Cysteine in Proteins and the Complexity of Organisms. *Mol. Biol. Evol.* **2000,** *17*, 1232-1239.
6. Go, Y. M.; Chandler, J. D.; Jones, D. P., The cysteine proteome. *Free Radical Biol. Med.* **2015,** *84*, 227-245.
7. Chen, F.-J.; Gao, J., Fast Cysteine Bioconjugation Chemistry. *Chem. Eur. J.* **2022**, *28,* e202201843.
8. Smythe, C. V., The Reaction of Iodoacetate and of Iodoacetamide with Various Sulfhydryl Groups, with Urease, and with Yeast Preparations. *J. Biol. Chem.* **1936,** *114*, 601-612.
9. Price, N. C.; Cohn, M.; Schirmer, R. H., Fluorescent and Spin Label Probes of the Environments of the Sulfhydryl Groups of Porcine Muscle Adenylate Kinase. *J. Biol. Chem.* **1975,** *250*, 644-52.
10. Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G., Chemical Modification of Proteins at Cysteine: Opportunities in Chemistry and Biology. *Chem. Asian J.* **2009,** *4*, 630-640.
11. Tucker, M. J.; Courter, J. R.; Chen, J.; Atasoylu, O.; Smith III, A. B.; Hochstrasser, R. M., Tetrazine Phototriggers: Probes for Peptide Dynamics. *Angew. Chem. Int. Ed.* **2010,** *49*, 3612-3616.
12. Zhang, D.; Devarie-Baez, N. O.; Li, Q.; Lancaster, J. R.; Xian, M., Methylsulfonyl Benzothiazole (MSBT): A Selective Protein Thiol Blocking Reagent. *Org. Lett.* **2012,** *14*, 3396-3399.
13. Toda, N.; Asano, S.; Barbas III, C. F., Rapid, Stable, Chemoselective Labeling of Thiols with Julia–Kocieński-like Reagents: A Serum-Stable Alternative to Maleimide-Based Protein Conjugation. *Angew. Chem. Int. Ed.* **2013,** *52*, 12592-12596.
14. Martínez-Sáez, N.; Sun, S.; Oldrini, D.; Sormanni, P.; Boutureira, O.; Carboni, F.; Compañón, I.; Deery, M. J.; Vendruscolo, M.; Corzana, F., et al., Oxetane Grafts Installed Site-Selectively on Native Disulfides to Enhance Protein Stability and Activity In Vivo. *Angew. Chem. Int. Ed.* **2017,** *56*, 14963-14967.
15. Sun, S.; Oliveira, B. L.; Jiménez-Osés, G.; Bernardes, G. J. L., Radical-Mediated Thiol-Ene Strategy: Photoactivation of Thiol-Containing Drugs in Cancer Cells. *Angew. Chem. Int. Ed.* **2018,** *57*, 15832-15835.
16. Embaby, A. M.; Schoffelen, S.; Kofoed, C.; Meldal, M.; Diness, F., Rational Tuning of Fluorobenzene Probes for Cysteine-Selective Protein Modification. *Angew. Chem. Int. Ed.* **2018,** *57*, 8022-8026.
17. Shindo, N.; Fuchida, H.; Sato, M.; Watari, K.; Shibata, T.; Kuwata, K.; Miura, C.; Okamoto, K.; Hatsuyama, Y.; Tokunaga, K., et al., Selective and Reversible Modification of Kinase Cysteines with Chlorofluoroacetamides. *Nat. Chem. Biol.* **2019,** *15*, 250-258.
18. Matos, M. J.; Navo, C. D.; Hakala, T.; Ferhati, X.; Guerreiro, A.; Hartmann, D.; Bernardim, B.; Saar, K. L.; Compañón, I.; Corzana, F., et al., Quaternization of Vinyl/Alkynyl Pyridine Enables Ultrafast Cysteine-Selective Protein Modification and Charge Modulation. *Angew. Chem. Int. Ed.* **2019,** *58*, 6640-6644.
19. Zambaldo, C.; Vinogradova, E. V.; Qi, X.; Iaconelli, J.; Suciu, R. M.; Koh, M.; Senkane, K.; Chadwick, S. R.; Sanchez, B. B.; Chen, J. S., et al., 2-Sulfonylpyridines as Tunable, Cysteine-Reactive Electrophiles. *J. Am. Chem. Soc.* **2020,** *142*, 8972-8979.

20. Motiwala, H. F.; Kuo, Y.-H.; Stinger, B. L.; Palfey, B. A.; Martin, B. R., Tunable Heteroaromatic Sulfones Enhance in-Cell Cysteine Profiling. *J. Am. Chem. Soc.* **2020,** *142*, 1801-1810.
21. Tang, K.-C.; Cao, J.; Boatner, L. M.; Li, L.; Farhi, J.; Houk, K. N.; Spangle, J.; Backus, K. M.; Raj, M., Tunable Amine-Reactive Electrophiles for Selective Profiling of Lysine. *Angew. Chem. Int. Ed.* **2022,** *61*, e202112107.
22. Shannon, D. A.; Banerjee, R.; Webster, E. R.; Bak, D. W.; Wang, C.; Weerapana, E., Investigating the Proteome Reactivity and Selectivity of Aryl Halides. *J. Am. Chem. Soc.* **2014,** *136*, 3330-3333.
23. Banerjee, R.; Pace, N. J.; Brown, D. R.; Weerapana, E., 1,3,5-Triazine as a Modular Scaffold for Covalent Inhibitors with Streamlined Target Identification. *J. Am. Chem. Soc.* **2013,** *135*, 2497-2500.
24. Zhang, C.; Vinogradova, E. V.; Spokoyny, A. M.; Buchwald, S. L.; Pentelute, B. L., Arylation Chemistry for Bioconjugation. *Angew. Chem. Int. Ed.* **2019,** *58*, 4810-4839.
25. Kubota, K.; Dai, P.; Pentelute, B. L.; Buchwald, S. L., Palladium Oxidative Addition Complexes for Peptide and Protein Cross-linking. *J. Am. Chem. Soc.* **2018,** *140*, 3128-3133.
26. Fadzen, C. M.; Wolfe, J. M.; Cho, C.-F.; Chiocca, E. A.; Lawler, S. E.; Pentelute, B. L., Perfluoroarene–Based Peptide Macrocycles To Enhance Penetration across the Blood–Brain Barrier. *J. Am. Chem. Soc.* **2017,** *139*, 15628-15631.
27. Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B. D.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F., Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010,** *468*, 790-795.
28. Bechtel, T. J.; Li, C.; Kisty, E. A.; Maurais, A. J.; Weerapana, E., Profiling Cysteine Reactivity and Oxidation in the Endoplasmic Reticulum. *ACS Chem. Biol.* **2020,** *15*, 543-553.
29. Verhoog, S.; Kee, C. W.; Wang, Y.; Khotavivattana, T.; Wilson, T. C.; Kersemans, V.; Smart, S.; Tredwell, M.; Davis, B. G.; Gouverneur, V., 18F-Trifluoromethylation of Unmodified Peptides with 5-^{18}F-(Trifluoromethyl)dibenzothiophenium Trifluoromethanesulfonate. *J. Am. Chem. Soc.* **2018,** *140*, 1572-1575.
30. Vinogradova, E. V.; Zhang, C.; Spokoyny, A. M.; Pentelute, B. L.; Buchwald, S. L., Organometallic palladium reagents for cysteine bioconjugation. *Nature* **2015,** *526*, 687-691.
31. Bandyopadhyay, A.; Cambray, S.; Gao, J., Fast and selective labeling of N-terminal cysteines at neutral pH via thiazolidino boronate formation. *Chem. Sci.* **2016,** *7*, 4589-4593.
32. Faustino, H.; Silva, M. J. S. A.; Veiros, L. F.; Bernardes, G. J. L.; Gois, P. M. P., Iminoboronates are efficient intermediates for selective, rapid and reversible *N*-terminal cysteine functionalisation. *Chem. Sci.* **2016,** *7*, 5052-5058.
33. Lipka, B. M.; Betti, V. M.; Honeycutt, D. S.; Zelmanovich, D. L.; Adamczyk, M.; Wu, R.; Blume, H. S.; Mendina, C. A.; Goldberg, J. M.; Wang, F., Rapid Electrophilic Cysteine Arylation with Pyridinium Salts. *Bioconjugate Chem.* **2022,** *33*, 2189-2196.
34. Amii, H.; Uneyama, K., C–F Bond Activation in Organic Synthesis. *Chem. Rev.* **2009,** *109*, 2119-2183.
35. O'Hagan, D., Understanding Organofluorine Chemistry. An Introduction to the C-F Bond. *Chem. Soc. Rev.* **2008,** *37*, 308-319.
36. Hansch, C.; Leo, A.; Taft, R. W., A survey of Hammett substituent constants and resonance and field parameters. *Chem. Rev.* **1991,** *91*, 165-195.
37. Miller, J., *Aromatic nucleophilic substitution*. Elsevier Pub. Co.: Amsterdam, 1968.
38. Terrier, F., *Modern Nucleophilic Aromatic Substitution*. Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2013; p 409.
39. Prakash, G. K. S.; Ni, C.; Wang, F.; Zhang, Z.; Haiges, R.; Olah, G. A., Difluoro(sulfinato)methylation of *N*-Sulfinyl Imines Facilitated by 2-Pyridyl Sulfone: Stereoselective Synthesis of Difluorinated b-Amino Sulfonic Acids and Peptidosulfonamides. *Angew. Chem. Int. Ed.* **2013,** *52*, 10835-10839.
40. Prakash, G. K. S.; Ni, C.; Wang, F.; Hu, J.; Olah, G. A., From Difluoromethyl 2-Pyridyl Sulfone to Difluorinated Sulfonates: A Protocol for Nucleophilic Difluoro(sulfonato)methylation. *Angew. Chem. Int. Ed.* **2011,** *50*, 2559-2563.
41. Zhao, Y.; Huang, W.; Zhu, L.; Hu, J., Difluoromethyl 2-Pyridyl Sulfone: A New gem-Difluoroolefination Reagent for Aldehydes and Ketones. *Org. Lett.* **2010,** *12*, 1444-1447.
42. Mukaiyama, T., New Synthetic Reactions Based on the Onium Salts of Aza-Arenes [New synthetic methods (29)]. *Angew. Chem. Int. Ed. Engl.* **1979,** *18*, 707-721.
43. Brotzel, F.; Mayr, H., Nucleophilicities of Amino Acids and Peptides. *Org. Biomol. Chem.* **2007,** *5*, 3814-3820.
44. Mayer, R. J.; Ofial, A. R., Nucleophilicity of Glutathione: A Link to Michael Acceptor Reactivities. *Angew. Chem. Int. Ed.* **2019,** *58*, 17704-17708.
45. Mayer, R. J.; Breugst, M.; Hampel, N.; Ofial, A. R.; Mayr, H., Ambident Reactivity of Phenolate Anions Revisited: A Quantitative Approach to Phenolate Reactivities. *J. Org. Chem.* **2019,** *84*, 8837-8858.
46. Räder, A. F. B.; Weinmüller, M.; Reichart, F.; Schumacher-Klinger, A.; Merzbach, S.; Gilon, C.; Hoffman, A.; Kessler, H., Orally Active Peptides: Is There a Magic Bullet? *Angew. Chem. Int. Ed.* **2018,** *57*, 14414-14438.
47. Vinogradov, A. A.; Yin, Y.; Suga, H., Macrocyclic Peptides as Drug Candidates: Recent Progress and Remaining Challenges. *J. Am. Chem. Soc.* **2019,** *141*, 4167–4181.
48. Li, X.; Chen, S.; Zhang, W.-D.; Hu, H.-G., Stapled Helical Peptides Bearing Different Anchoring Residues. *Chem. Rev.* **2020,** *120*, 10079-10144.
49. Li, X.; Craven, T. W.; Levine, P. M., Cyclic Peptide Screening Methods for Preclinical Drug Discovery. *J. Med. Chem.* **2022,** *65*, 11913–11926.
50. Martí-Centelles, V.; Pandey, M. D.; Burguete, M. I.; Luis, S. V., Macrocyclization Reactions: The Importance of Conformational, Configurational, and Template-Induced Preorganization. *Chem. Rev.* **2015,** *115*, 8736-8834.
51. Collins, J. C.; James, K., Emac – A Comparative Index for the Assessment of Macrocyclization Efficiency. *MedChemComm* **2012,** *3*, 1489-1495.
52. Mayr, H.; Ofial, A. R., The Reactivity–Selectivity Principle: An Imperishable Myth in Organic Chemistry. *Angew. Chem. Int. Ed.* **2006,** *45*, 1844-1854.


Me
O
O
N
S
Me
X
S
H
C
H2N
OH
pH 7.0, 25 °C
up to ~320,000 M−1·s−1
X
N
Me
S
C
H2N
OH