Rapid Electrophilic Cysteine Arylation with Pyridinium Salts

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Abstract

Here, we present a series of fluorinated cationic reagents that enable rapid arylation of cysteine under mild conditions compatible with proteins and peptides. The highly polarized C–F bond and attractive nucleophile-electrophile Coulombic interactions substantially accelerate cysteine arylation, leading to unusually high rate constants on the order of 100 $M^{-1}\cdot s^{-1}$ and allowing for equimolar labeling of substrates at micromolar concentrations. The synthetic modularity of this approach promotes the direct coupling of structurally diverse phenol-containing functional motifs to cysteine residues of biomacromolecules with high efficiency. This user-friendly chemistry enables fast bond formation between commonly used bioconjugation partners, thus greatly streamlining the synthetic chemistry workflow, and can be easily developed as convenient kits for chemical biology and medicinal chemistry applications.

Introduction

Small molecule-based bioconjugation enables *in vitro* and *in vivo* functionalization of proteins and peptides, significantly expanding the chemical space of biomacromolecules.[1-4] Among these transformations, nucleophilic aromatic substitution (S_NAr) of cysteine residues is a widely employed strategy for protein bioconjugation.[5-7]

The rational design of reagents for selective nucleophilic cysteine arylation generally follows the current mechanistic understanding of S_NAr reactions. In the classical view,[8-10] S_NAr reactions proceed through a stepwise addition-elimination mechanism. When the addition step is rate-limiting, S_NAr reactions are usually accelerated if the aromatic electrophile bears an electron-withdrawing group (EWG) as the activator and a small, electronegative substituent as the leaving group (LG).[9, 11] The EWG stabilizes the transition state (TS) of the addition step when the reactants approach each other. The barrier to this event is further lowered if the C_{ipso}–LG bond is polarized by a strongly electronegative LG, such as a halide, and the incoming nucleophile experiences reduced repulsive interactions with a small LG.[11-12] In line with this canonical mechanism, a variety of aromatic compounds, functionalized with both strong EWGs and favorable LGs, including fluoride, chloride, and sulfinate, have been identified as versatile labeling agents for nucleophilic cysteine arylation and have been used to great advantage in many biochemical studies (Fig. 1A).[13-18]

Although these reagents facilitate cysteine arylation, they are disadvantageous in two crucial aspects – limited synthetic modularity and undesired hydrophobicity. First, conventional labeling reagents are frequently activated by multiple EWGs on a single aromatic ring, leaving few synthetic handles to derivatize the reagents for advanced applications. Second, these labeling reagents are generally hydrophobic in nature and scarcely soluble in water, thus necessitating the use of organic co-solvents incompatible with certain biomolecules.[5] In addition, although the incorporation of hydrophobic tags, such as polyfluorinated arenes,[19] can potentially improve the pharmacokinetics of the resulting conjugate,[20] in other instances, it is desirable to label proteins with hydrophilic moieties that do not significantly perturb native structure and possibly preserve wild-type protein-protein interactions.[21] Overall, these challenges highlight the current limitations of cysteine arylation chemistry and reflect the increasing need for rapid, hydrophilic tools allowing for bioconjugation at low micromolar concentrations within hours.[22-24]

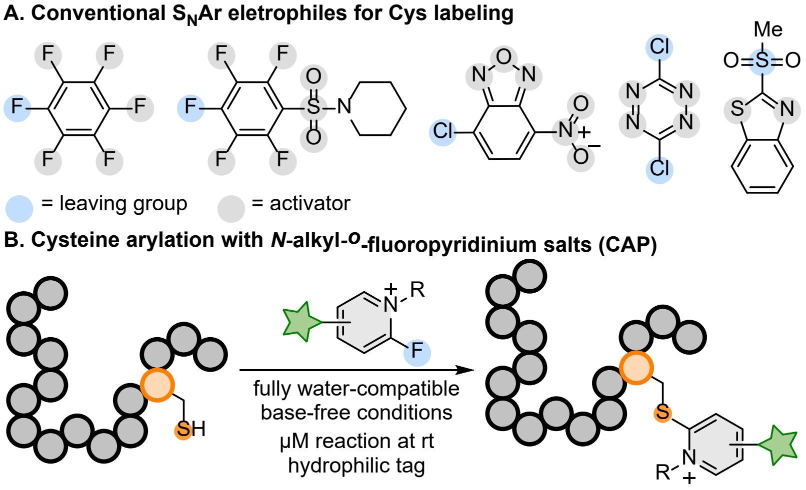


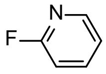
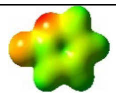
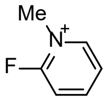
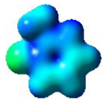
Fig. 1 A. Conventional S_NAr electrophiles for cysteine labeling. B. Rapid cysteine labeling using *N*-alkyl-*o*-fluoropyridinium salts.

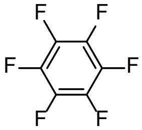
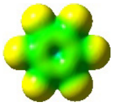
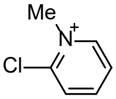
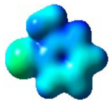
Results and Discussion

Here, we devised a novel strategy for rapid cysteine arylation using pyridinium salts (**CAP**, Fig. 1B). We hypothesized that *N*-alkylation of *ortho*-fluoropyridines would convert rather inert, hydrophobic pyridines into highly reactive, hydrophilic electrophiles, as described below.

First, because they are ionic compounds, *N*-alkylated fluoropyridinium salts should exhibit significantly improved water solubility relative to typical neutral arylating agents, such as perfluoroarenes and 4-chloro-7-nitrobenzofurazan. Furthermore, despite the fact that monohalogenation of aromatic compounds often significantly increases hydrophobicity, such effects are minimal with fluorine,[25-26] rendering fluoropyridinium salts the most water-soluble among all halopyridinium analogs. Second, *N*-alkylated pyridinium motifs are substantially electron-withdrawing, as indicated by the high Hammett σ values of *N*-methyl-*m*-pyridinium (σ = 2.10) and *N*-methyl-*p*-pyridinium (σ = 2.57) substituents.[27] In comparison, the nitro group, although widely employed as a strong activating group for SNAr electrophiles, has much lower substituent constants of $\sigma_m = 0.71$ and $\sigma_p = 0.78$.[28] In addition, both natural bond orbital (NBO) analysis[29] and electrostatic potential calculations indicate the highly positively charged *ipso* carbon of *N*-methyl-*o*-fluoropyridinium (Table 1). These results also suggest that the *o*-fluoropyridinium ion can serve as a viable electrophile in SNAr reactions. Empirically, the exceptional electron-withdrawing capacity of pyridinium motifs corresponds to the high reactivity of halogenated pyridinium salts, as exemplified by the rapid reaction between *N*-methyl-*o*-fluoropyridinium iodide (**CAP1**) and the hydroxide anion.[30] According to Mayr's reactivity scale,[31-32] the cysteine thiolate is about ten orders of magnitude more nucleophilic than hydroxide in aqueous media.[33-34] Based on these considerations, we anticipated that the *o*-fluoropyridinium-cysteine reaction pair would exhibit fast chemical kinetics suitable for bioconjugation even at low substrate concentrations.[35-36] We calculated the activation barrier of the reaction between *N*-acetylcysteine amide and a series of electrophiles in water (Table 1). The results showed a dramatic reduction in TS energy when the *o*-fluoropyridinium cation is used as the electrophile, which further validates our proposal. Compared to the chlorinated counterpart, the *o*-fluoropyridinium cation exhibits about 100-fold higher reactivity, highlighting the special ability of the polarized C–F bond to promote SNAr reactions.[37] Alternatively, the *p*- and *o*-cyano-*N*-methylpyridinium cations exhibit 30 to 50-fold higher reactivity toward piperidine in methanol, respectively, compared to the *o*-fluoropyridinium moiety.[38] Despite the promise of these electrophilic scaffolds for cysteine bioconjugation, the *o*-fluoropyridinium cation is a safer reagent that does not generate unwanted cyanide by-product. Finally, the prominent electron-withdrawing ability of pyridinium motifs facilitates the effective activation of SNAr electrophiles without introducing multiple EWGs, thus providing multiple synthetic handles on the aromatic ring. Such extraordinary synthetic modularity of the **CAP** reagents enables one-pot conjugation of a large collection of functional small molecules to proteins and peptides for rapid, operationally easy structural diversification of biomacromolecules.

Table 1. Comparison of properties of different SNAr electrophiles.

electrophile	ESP[a]	natural atomic charge of *ipso* carbon[b]	$\Delta G^{\ddagger}_{\mathrm{SNAr}}$ with *N*-acetylcysteine amide (kcal/mol)[c]
		+0.57	+26.9
		+0.65	+8.4

		+0.28	+22.7
		+0.20	+10.5

[a] The electrostatic potential (ESP) plotted on an isodensity surface of the electron density set to 0.01 e/bohr^3. Red = −33 kcal/mol, blue = +100 kcal/mol. [b] Natural atomic charge. [c] Gibbs free energy of activation of the rate-determining step of the reaction with *N*-acetylcysteine amide calculated at CPCM-M06-2X/def2-TZVP in water.

To test this hypothesis, we quantified the electrophilic reactivity of *N*-methyl-*o*-fluoropyridinium iodide (**CAP1**), the prototypical **CAP** electrophile, towards a series of common biological nucleophiles. As shown in Fig. 2A, Eq. 1, **CAP1** rapidly reacted with glutathione (GSH) at 25 °C in pH 7.0 PIPES buffer with a second-order rate constant of 1.16×10^{2} $M^{-1}\cdot s^{-1}$. The rate constants of the reaction between **CAP1** and other cysteine derivatives are analogous (Fig. S42, S43, S54, and S55). Such rapid reactions are highly desirable for stoichiometric bioconjugation at micromolar concentrations.[35-36] In pH 6.0 PIPES buffer, the rate constant decreased by 90% to 1.22×10^{1} $M^{-1}\cdot s^{-1}$ (Fig. S39). This observation is consistent with the ten-fold reduction in free thiolate, which is the ultimate nucleophile of this reaction.[39] To address the need for conducting arylation under low-salt conditions, we tested the reaction in water. Although in unbuffered aqueous media, we observed a lower reaction rate, presumably due to the significantly lower availability of the requisite thiolate nucleophile, these conditions are still effective for labeling substrates when the concentrations of both reactants are in the millimolar range (Fig. 2A, Eq. 1, Fig. S41, S61). In contrast to the high reactivity of fluorine-containing **CAP1**, *N*-methyl-*o*-chloropyridinium iodide, known as Mukaiyama reagent,[40] was 100-fold less reactive, indicating the essential role of the polarized C–F bond in activating SNAr reactions (Fig. 2A, Eq. 2, Fig. S45).[37] This observation may be explained by DFT calculations at the CPCM(water)-M062X/def2-TZVP level, which showed that the barrier to the reaction of GSH with **CAP1** was about 2 kcal/mol lower than that with Mukaiyama reagent via the classical ionic non-vicarious SNAr pathway (Fig. S66-67).[41-43]

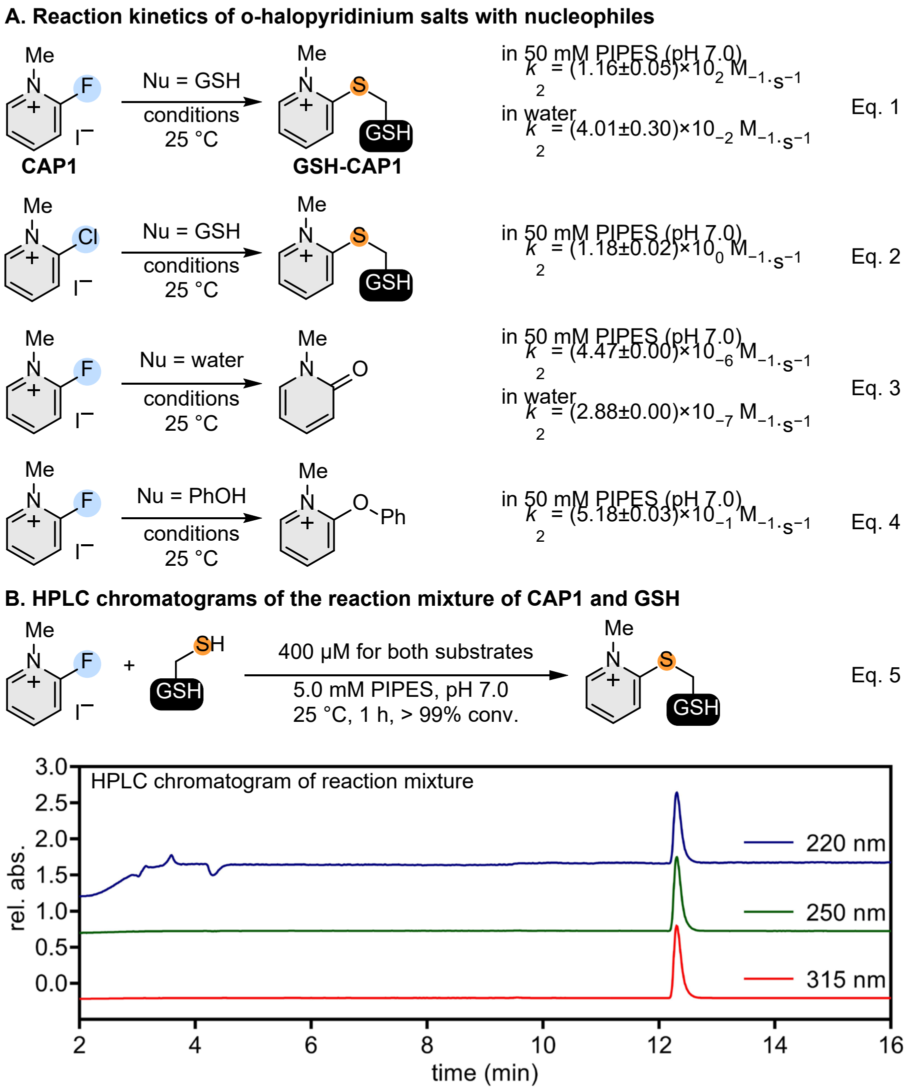
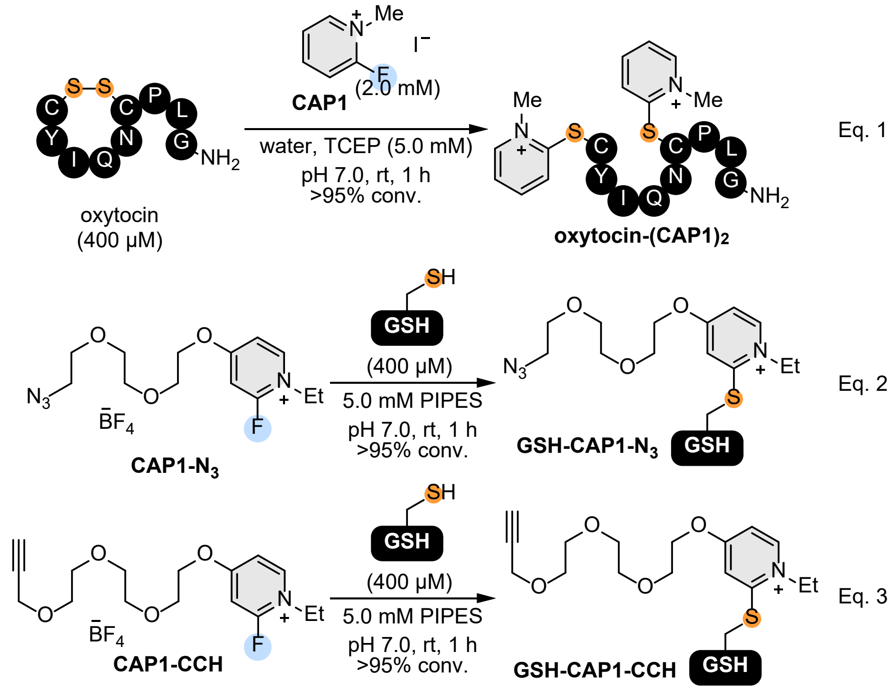


Fig. 2 A. Reaction kinetics of *o*-halopyridinium salts with various nucleophiles under different conditions. B. Preparative scale labeling of 400 μM GSH with equimolar **CAP1** in pH 7.0 PIPES buffer. HPLC chromatography of the reaction mixture shows quantitative reaction yield and remarkable selectivity.

The practicality and efficiency of this labeling strategy not only depend on the rate constant of the desired cysteine arylation but also on the side reactions between **CAP1** and other nucleophiles. To quantify the aqueous stability of **CAP1**, we determined the hydrolysis rate constant of **CAP1**. As shown in Fig. 2A, Eq 3, hydrolysis of **CAP1** was slow in both water (Fig. S49) and pH 7.0 PIPES buffer (Fig. S50) at 25 °C, corresponding to half-lives of 12 h and 45 min, respectively. Because **CAP1** is persistent in water, its aqueous solutions can be routinely handled at ambient temperatures and stored at −20 °C for two weeks without significant degradation. Despite the faster hydrolysis in PIPES buffer, this side reaction is substantially slower than the desired substitution and can be mitigated by using a slight excess of **CAP1** when cysteine is used at low micromolar concentrations. We also tested the reaction of **CAP1** with other common biological nucleophiles, including phenolic, amino, and carboxylic groups, all of which exhibited reactivity at least 200-fold lower than that with cysteine (Fig. 2A, Eq. 4, Fig. S46 and S58-60). The differential reactivities toward **CAP1** follow the general trend revealed by Mayr's quantitative nucleophilicity scale[33-34, 44] and permit rapid, selective cysteine arylation under fully biocompatible conditions. To demonstrate the high efficiency of **CAP1** labeling, we reacted 400 µM GSH with equimolar **CAP1** for 1 h in pH 7.0 PIPES buffer at 25 °C (Fig. 2B). The HPLC chromatogram of this reaction mixture showed the quantitative generation of a highly pure GSH-**CAP1** adduct, the structure of which was confirmed by tandem mass spectrometry (Fig. S1) and NMR spectroscopy (Fig. S138-146). The stability of the arylation products affects the potential scope of cysteine labeling applications. To evaluate the reversibility of cysteine arylation, we treated *N*-acetyl-*L*-cysteine methyl ester-**CAP1** adduct (11.1 mM) with 3.0 equiv. of GSH in D_2O at 25 °C and found a half-life of more 300 h under these conditions, thus demonstrating the suitability of **CAP**-based cysteine arylation for various experimental objectives (Fig. S63 and similar results in Fig. S64).



Scheme 1. Eq 1. Preparative scale arylation of cysteine residues of reduced oxytocin with **CAP1**. Eq 2. Preparative scale arylation of GSH with **CAP1-N₃**. Eq 3. Preparative scale arylation of GSH with **CAP1-CCH**.

We next used **CAP** labels to arylate a panel of cysteine-containing biomolecules. To test the chemoselectivity of **CAP1** for structurally complicated peptides, we chose to label the cysteine residues of reduced oxytocin, a nonapeptide hormone bearing a tyrosine and an unprotected *N*-terminus (Scheme 1A). In the presence of tris(2-carboxyethyl)phosphine (TCEP), a widely used reagent for reducing disulfide bonds, oxytocin was rapidly functionalized by **CAP1** under mild conditions. Tandem mass spectrometry confirmed the exclusive labeling of the cysteine residues, a result consistent with our understanding of the kinetic parameters (Fig. S3). One advantage of **CAP** labels is synthetic versatility, which allows for the easy incorporation of various functional motifs commonly employed in chemical biology. To this end, we developed **CAP1**-derived labels functionalized with an azido group (**CAP1-N₃**) or a terminal alkyne (**CAP1-CCH**), respectively (Scheme 1B and 1C, Fig. S4-

5). Both **CAP1-N$_3$** and **CAP1-CCH** were easily synthesized in quantitative yields by ethylating the corresponding *o*-fluoropyridine derivatives using Meerwein's salts,[45-46] and exhibited reactivity similar to that of the parent **CAP1**.

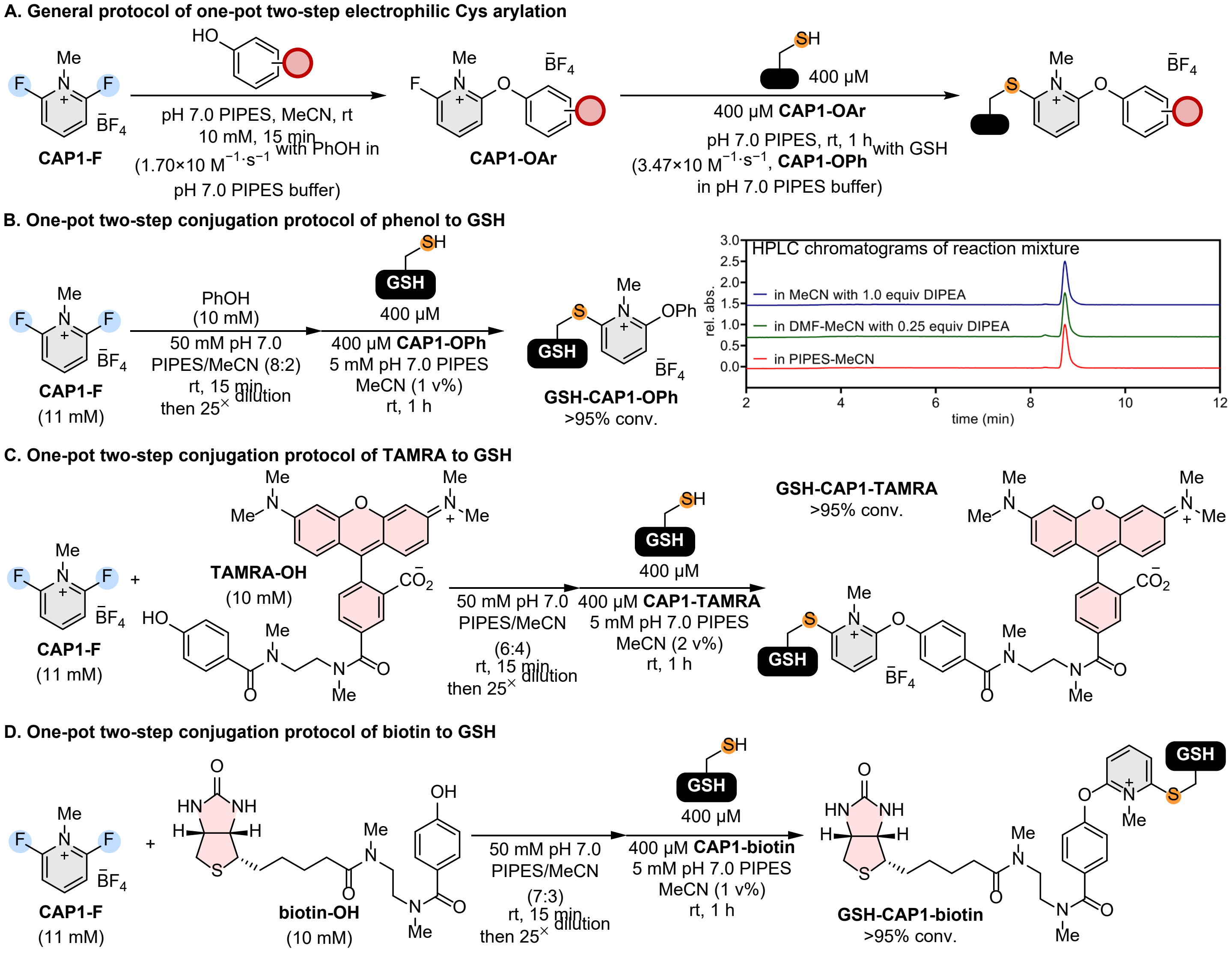
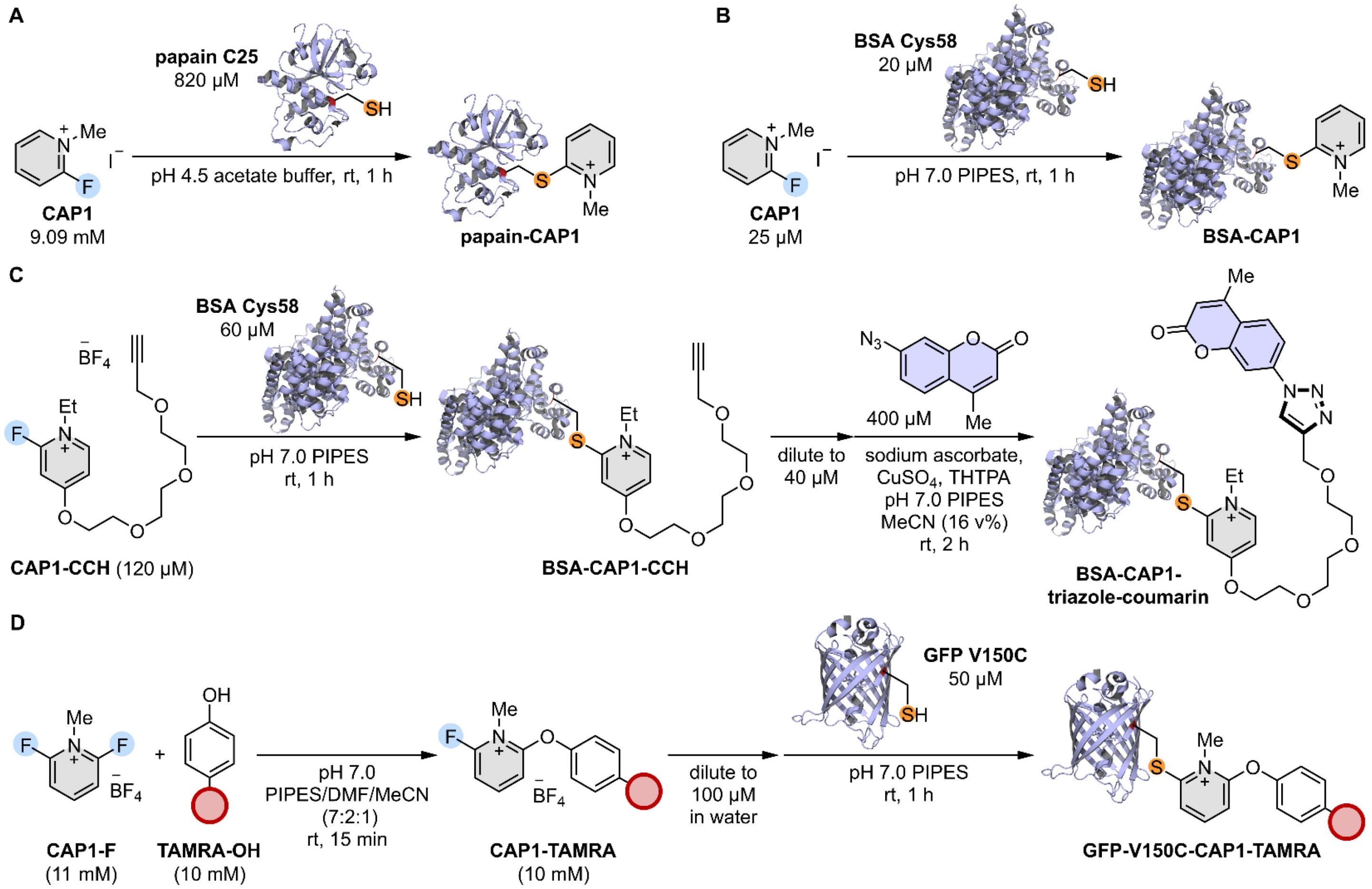


Fig. 3 One-pot two-step ligation of phenol-containing molecules to cysteines using **CAP1-F** reagent. A. General protocol for one-pot two-step electrophilic cysteine arylation and representative kinetic data. B. Preparative scale conjugation of phenol to the cysteine residue of GSH. C. Preparative scale conjugation of a TAMRA-phenol derivative to the cysteine residue of GSH. D. Preparative scale conjugation of a biotin-phenol derivative to the cysteine residue of GSH.

An ideal bioconjugation strategy should facilitate the straightforward attachment of structurally diverse functional cargo to biomacromolecules – a feature usually absent in existing electrophilic cysteine arylation methods. We considered the use of **CAP1** derivatives that possess two electrophilic reactive sites as modular linkers for connecting complicated molecular scaffolds to cysteine residues via two sequential nucleophilic substitution reactions. Accordingly, we prepared the highly electrophilic *o,o*'-difluoro-*N*-methylpyridinium tetrafluoroborate (**CAP1-F**) as an effective bioconjugation partner for relatively weak nucleophiles, such as phenols. The additional *ortho* fluorine substituent of **CAP1-F** led to a 30-fold increase in reactivity (Fig. S47), thus facilitating rapid reaction with phenols at low millimolar concentrations under mild conditions (Fig. 3A). In contrast, the electrophilicity of the resulting **CAP1-OPh** was 100 times lower than that of **CAP1-F** (Fig. 3A, Fig. S48). Such a substantial difference in reactivity was crucial for generating the labeling intermediate **CAP1-OPh** from **CAP1-F** in nearly quantitative yield while minimizing the formation of the undesired diphenolated adduct. The **CAP1-OPh** species, on the other hand, exhibited electrophilicity similar to that of **CAP1** and rapidly arylated cysteine at low concentrations (Fig. 3A and Fig. S48). Advantageously, the half-life of **CAP1-F** in pH 7.0 PIPES buffer at 25 °C is approximately three minutes. As such, any excess **CAP1-F** that does not react with the phenol rapidly decomposes to 6-fluoro-*N*-methylpyridone, which is inert toward cysteine, thus preventing unwanted cysteine-**CAP1-F** reactions. Fig. 3 (panels B-D) depicts the use of **CAP1-F** to ligate a series of functional phenol derivatives to cysteine residues in a one-pot, two-step fashion.

As demonstrated by the reaction sequence employing phenol and GSH, this **CAP1-F**-based approach afforded the desired conjugate, **GSH-CAP1-OPh**, quantitatively as determined by analytical HPLC (Fig. 3B, red trace, Fig. S6). The phenol substitution also occurred smoothly in organic solvents in the presence of bases and is suitable for incorporating substrates with low aqueous solubility (Fig. 3B, green and blue traces). The **CAP1-F** reagent also enabled efficient conjugation of complicated structural motifs, including TAMRA- and biotin-phenol derivatives, to GSH, demonstrating the excellent modularity and synthetic versatility of the method (Fig. 3C and 3D, Fig. S7-8).

We next investigated the application of **CAP** reagents for the arylation of proteins. Our initial efforts focused on the arylation of the catalytic Cys-25 of papain, a 23.4-kDa protease, using **CAP1** in pH 4.5 acetate buffer at room temperature (Scheme 2A). The reaction went smoothly, as evidenced by a functional assay revealing substantial inhibition of proteolytic activity (Fig. S9). To explore the functionalization of larger proteins using **CAP** labels, we chose the 66.5-kDa bovine serum albumin (BSA) as a model substrate, which reacted with **CAP1** rapidly (Scheme 2B, Fig. S10-S14). Additionally, we found that the alkyne-containing **CAP1-CCH** arylated the Cys-58 residue of BSA within 1 h under biocompatible conditions. The resulting functionalized **BSA-CAP1-CCH** was well-suited for routine copper-catalyzed azide-alkyne cyclization[47-50] with 7-azido-4-methylcoumarin, exhibiting the versatility of **CAP** reagents (Scheme 2C and Fig. S16-S20). We also employed the modular **CAP1-F** building block to install complicated molecular motifs to a protein in a one-pot two-step fashion. Scheme 2D depicts the fluorescent labeling of GFP V150C using an *in situ* generated **CAP1-TAMRA** synthetic intermediate, which enabled fast, convenient cysteine arylation of GFP V150C in pH 7.0 PIPES buffer at room temperature (Scheme 2D and Fig. S21). Taken together, these experiments reflect the modularity and efficiency of the **CAP** labeling strategy for the direct coupling of complicated molecules to proteins and peptides.



Scheme 2. A. Labeling papain with **CAP1**. B. Labeling BSA with **CAP1**. C. Labeling BSA with **CAP1-CCH** and the subsequent incorporation of coumarin via copper-catalyzed azide-alkyne cyclization. D. Modular synthesis of **CAP1-TAMRA** for labeling GFP V150C.

Conclusions

We developed a highly modular synthetic method for rapid electrophilic cysteine arylation using *N*-alkyl-*o*-fluoropyridinium salts (**CAP**). This particularly fast reaction was facilitated by both the electron-deficient nature of the cationic *ortho*-pyridinium construct and the unique chemical properties of the carbon–fluorine bond. The prototypical labeling reagent, **CAP1**, exhibited the reactivity toward cysteine with a rate constant of about 100 $M^{-1}\cdot s^{-1}$ under fully biocompatible conditions and enabled rapid, nearly quantitative cysteine ligation at low micromolar concentrations. Synthetically, the **CAP** strategy is highly versatile. The pyridinium scaffold can easily accommodate various functionalities and synthetic handles useful for chemical biology without significant loss in its high reactivity. The extraordinary modularity of the CAP labeling, exemplified by *o*,*o*'-difluoro-*N*-methylpyridinium tetrafluoroborate (**CAP1-F**), allowed for directly coupling phenol derivatives to cysteines in a one-pot, two-step fashion. Overall, we anticipate this chemistry to be a practical, user-friendly addition to the chemical biology toolbox.

Supporting Information

The Supporting Information is available free of charge. Supplementary figures and schemes, materials, experimental procedures; characterization data (1D and 2D NMR, LCMS, HRMS) for all small molecule compounds; HRMS, MS/MS, and MALDI for peptides and proteins; reaction kinetics; *in situ* NMR spectra for product stability studies; and DFT calculations (PDF)

Conflicts of interest

A provisional patent application has been submitted by the University of Rhode Island and Colgate University to cover this work.

Acknowledgments

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 number P20GM103430 through the Centralized Research Core facility. High-Performance Computing at the University of Rhode Island is gratefully acknowledged. This material is based upon work supported by the National Science Foundation under Grant No. CHE-2117141.

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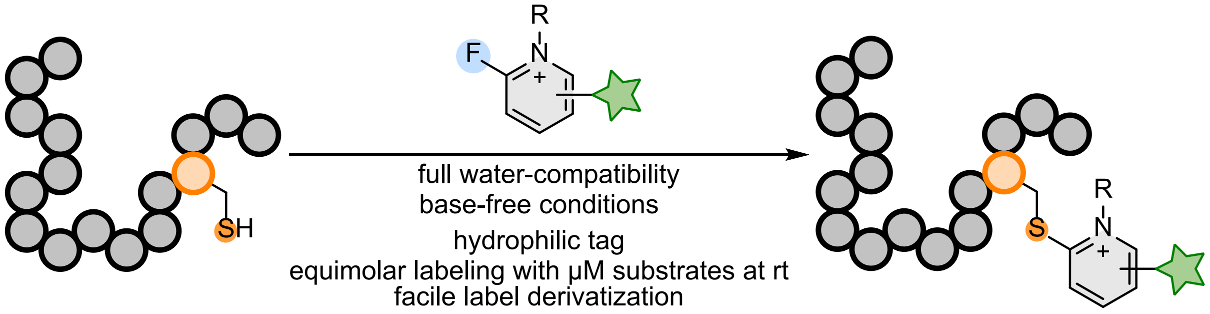
TOC Graphic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