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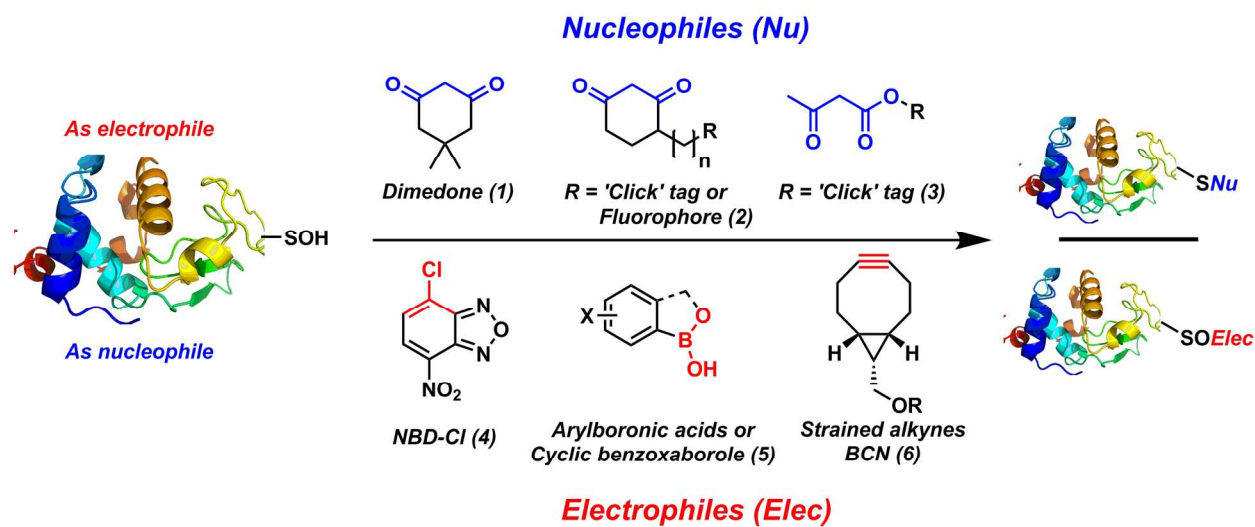
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Reactivity, Selectivity and Stability in Sulfenic Acid Detection:
A Comparative Study of Nucleophilic and Electropilic Probes

Vinayak Gupta,^a Hanumantharao Paritala^a and Kate S. Carroll^{a, *}

Graphical Abstract



Abstract

The comparative reaction efficiencies of currently used nucleophilic and electrophilic probes towards cysteine sulfenic acid have been thoroughly evaluated in two different settings – (i) a small molecule dipeptide based model and, (ii) a recombinant protein model. We further evaluated the stability of corresponding thioether and sulfoxide adducts under reducing conditions which are commonly encountered during proteomic protocols and in cell analysis. Powered by the development of new cyclic and linear C-nucleophiles, the unsurpassed efficiency in the capture of sulfenic acid under competitive conditions is achieved and thus holds great promise as highly potent tools for activity-based sulfenome profiling.

Introduction

Owing to the intrinsic nucleophilicity and oxidation susceptibility, cysteine thiols (Cys-SH) are prime targets for the redox-based modulation of protein activity. Oxidation of a protein cysteine thiol (Cys-SH) to sulfenic acid (Cys-SOH) by reactive oxygen species (ROS) (e.g. H_2O_2), termed S-sulfenylation, is a reversible post-translational modification that plays a crucial role in regulating several protein functions.¹⁻⁶ With an estimated half-life in minutes, Cys-SOH is considered a transient species whose ultimate fate depends upon factors such as presence of neighbouring thiol, level/duration of ROS and protein microenvironment.⁵ Essentially, absence of proximal thiols capable of forming intramolecular disulfide bond, limited solvent access and proximal hydrogen bond acceptors contribute towards Cys-SOH stabilization. High/chronic oxidative stress may cause –SOH to undergo further oxidation to irreversible sulfinic (Cys-SO₂H) or sulfonic acid (Cys-SO₃H). Due to the abundance of biological thiols (mM levels), an important and facile biological reaction of Cys-SOH is the disulfide formation. The nascent disulfide may undergo thiol-disulfide exchange to regenerate initial Cys-SH. Cys-SOH may undergo intramolecular reaction with adjacent amide nitrogen resulting in the formation of a cyclic species known as cyclic sulfenamide which may be reduced back to thiol via disulfide formation. Due to the central role of Cys-SOH in reversible/irreversible pathway, it serves as an important hub within the redox milieu.⁵ Indeed, over the past decade several groups have reported the role of Cys-SOH in regulation of several proteins such as transcription factors, kinases, phosphatases, ion channels, peroxidases and

cysteine proteases, sirtuins, human serum albumin and many others.^{3, 6-21} Moreover, aberrant Cys-SOH formation has been shown to correlate with disease state.^{12, 22} The aforementioned examples and many other reports establish protein S-sulfenylation as a global signaling mechanism akin to phosphorylation and a potential drug target.^{5, 6, 15, 23-27}

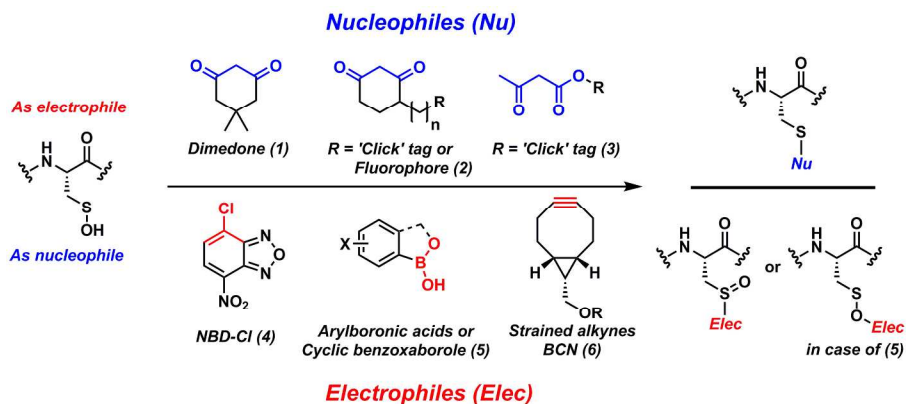


Fig. 1 Dual chemical reactivity of sulfenic acid.

Due to the electrophilic as well as nucleophilic character of sulfur atom in Cys-SOH, the detection methods exploiting both have been reported (**Fig. 1**).^{5, 10-13, 19, 20, 25, 26, 28-43} Briefly, due to higher electrophilicity (than nucleophilicity) of Cys-SOH, vast majority of probes are nucleophilic in nature and based on cyclic 1,3-carbonyl scaffold such as dimedone (**1**). These nucleophilic probes react with Cys-SOH to give thioether adducts. Such nucleophilic probes are extensively employed to study qualitative and quantitative capture of Cys-SOH.^{1, 5, 9-11, 13-16, 18-22, 29-32, 34-39, 42-51} Commonly used repertoire of C-nucleophilic probes consist of commercially available 1,3-dicarbonyl-based 'clickable' probes such as DYn-2 (**2a**),¹⁵ DAz-2 (**2b**),¹⁰ Alk- β -KE (**3**),⁴⁶ BP1 (**7**),⁴⁴ and under development 6-hydroxy derivatives of phenalene-1,3-dione (**8**) (**Fig. 2**). Enrichment-ready/imaging-ready

reagents such as DCP-Bio1 (**2c**) and DCP-Rho1 (**2d**) have also been made commercially available and used extensively.³⁰ Although these nucleophilic probes are selective for protein sulfenic acid under aqueous physiological conditions, they suffer from poor reaction kinetics ($k_{obs} = 0.008 \text{ M}^{-1}\text{s}^{-1} - 0.05 \text{ M}^{-1}\text{s}^{-1}$) when compared to other biological reactions of Cys-SOH (such as disulfide formation or over-oxidation to sulfinic/sulfonic acid).⁵ To overcome this limited reactivity problem, we recently developed and reported a facile mass-spectrometry-based assay and a repurposed dipeptide-based sulfenic acid model using which we screened a library of cyclic and linear C-nucleophiles for reactivity towards sulfenic acid under aqueous conditions. We obtained the reaction rate constants of more than 150 C-nucleophiles and from this collection we identified novel classes of compounds with more than 200-fold enhanced reactivity towards Cys-SOH.^{25, 26}

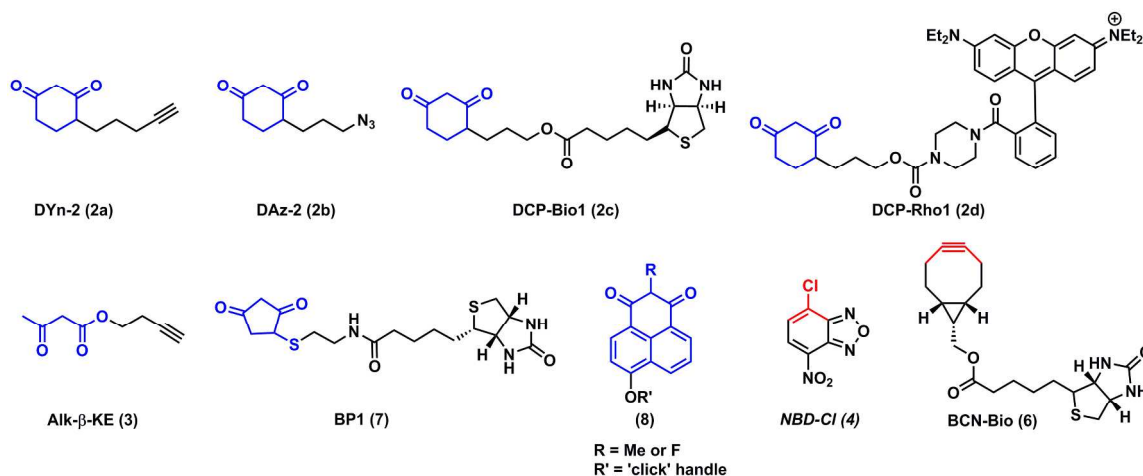


Fig. 2 Nucleophilic and electrophilic probes reported for detecting sulfenic acid.

Sulfenic acid can also act as a nucleophile and react with electrophiles to produce a sulfoxide adduct (**Fig. 1**). Given the nucleophilic properties of thiols and several other biological species, it is generally considered that the selectivity

towards Cys-SOH maybe difficult to achieve with electrophilic detection tools.⁴⁷ Currently reported examples of electrophilic probes are commercially available compounds such as (i) low reactive NBD-Cl (**4**) which also lack the chemoselectivity and react with several biological nucleophiles including sulfenic acid (but the resulting adducts are distinguishable due to difference in their spectroscopic properties);^{5, 28, 38} (ii) arylboronates (**5**) which react with Cys-SOH in a reversible manner and thus have limited utility in proteomic-based sulfenic acid detection⁵² and; (iii) strained cycloalkynes (**6**) which were recently reported as new, highly reactive sulfenic acid traps ($k_{obs} = 13.3 \text{ M}^{-1}\text{s}^{-1} - 16.7 \text{ M}^{-1}\text{s}^{-1}$) with > 100-fold higher reactivity than 1,3-dicarbonyls.⁴⁰ Not surprisingly, over the years strained cycloalkynes have been shown to react with other sulfur species such as protein- and small molecule thiols (present in mM concentration inside the cells) and persulfides.⁵³⁻⁵⁵ Moreover, the product of the reaction of Cys-SOH and strained cycloalkyne **6** is an activated vinylic sulfoxide which is a Michael-acceptor and may readily react with biological thiols as well.⁵⁶ As a consequence of the promiscuous reactivity of strained cycloalkynes and the resultant sulfoxide adduct, an additional thiol-blocking step is required to increase the selectivity for sulfenic acid. As mentioned above, even though over the past decade several nucleophilic and electrophilic probes for sulfenic acid detection have been developed and commercialized, the issue of modest reactivity of such probes is well documented.^{5, 26, 40} Several subsequent publications reported probes with superior reactivity compared to dimedone-based probes at lower or higher pHs.^{44, 46, 47} However, such reports generally compared the reactivities of these probes

towards Cys-SOH in specific model protein systems where the reactivity could be biased by the protein microenvironment surrounding Cys-SOH. For this reason a direct comparison of the reactivity of various nucleophilic/electrophilic probes towards Cys-SOH and the stability of resulting thioether/sulfoxide adduct under exact same conditions is needed for the better understanding of the reactivity profile of each probe. In order to do so, we chose to utilize our peptide-based sulfenic acid model with subsequent verification in a protein sulfenic acid model. The peptide-based sulfenic acid model exists in the masked form as a cyclic sulfenamide (**9**) which rearranges to dipeptide sulfenic acid (**10**) under aqueous conditions (**Fig. 3**).²⁶ Since this model is not influenced by a complex protein microenvironment, the observed reaction rates are independent of factors such as steric hindrance and stabilization/destabilization of sulfenic acid.

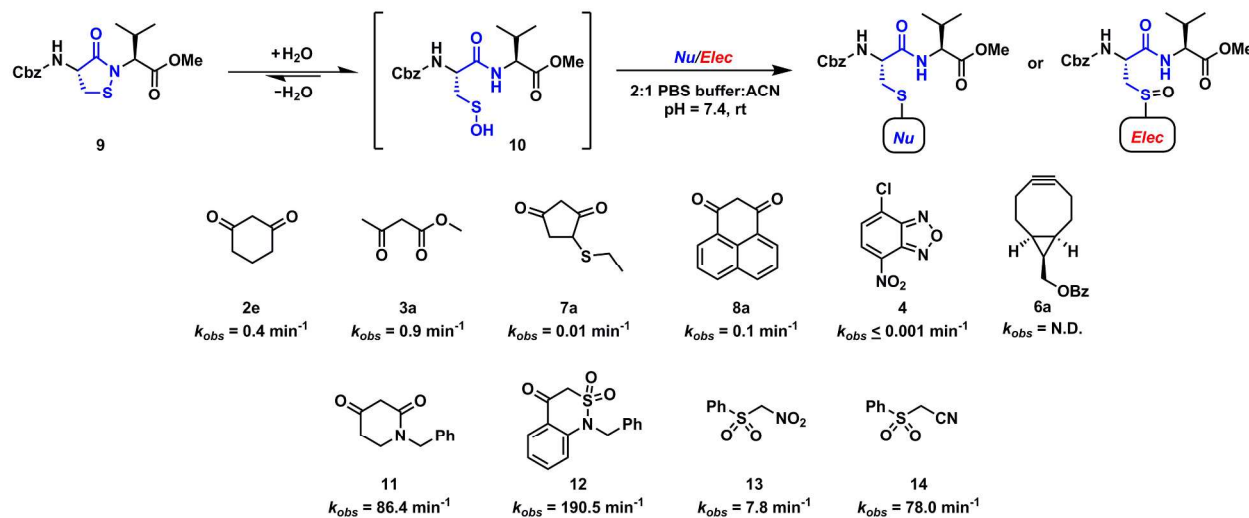


Fig. 3 Nucleophiles and electrophiles tested for reactivity with sulfenic acid **10**.

Results and Discussion

Using the peptide-based model and the previously developed LC/MS assay, we initially obtained the pseudo 1st order rate constants ($[\text{Nu/Elec}] = 1$

mM, [10] = 100 μ M) of three nucleophilic (**2e**, **3a**, **7a**) and two electrophilic (**4**, **6a**) compounds (**Fig. 3**). These compounds constitute the reactive component of prevalent and commercially available Cys-SOH probes such as **2a-d**, **3**, **7**, **4** and **6** (**Fig. 2**). Nucleophilic cyclohexane-1,3-dione (**2e**, $k_{obs} = 0.4 \text{ min}^{-1}$), methyl acetoacetate (**3a**, $k_{obs} = 0.9 \text{ min}^{-1}$) and 4-(ethylthio)cyclopentane-1,3-dione (**7a**, $k_{obs} = 0.01 \text{ min}^{-1}$) successfully reacted with dipeptide-SOH **10** to form corresponding thioether adduct. While **2e** and **3a** had similar reactivity under physiological conditions, it is noteworthy that **7a** was 40-folds less reactive compared to **2e** towards sulfenic acid **10**. Electrophilic 4-chloro-7-nitrobenzo[c][1,2,5]oxadiazole (**4**, $k_{obs} \leq 0.001 \text{ min}^{-1}$) was extremely slow to react and only a small amount of sulfoxide adduct could be detected even after 48 h (**ESI, Fig. S4**). Conversely, while the benzoyl derivative of strained cycloalkyne ((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl benzoate (**6a**, $k_{obs} = \text{N.D.}$) was highly reactive, the corresponding sulfoxide adduct was unstable and quickly rearranged to disulfide (Cbz-Cys-Val-OMe)₂ (**ESI, Fig. S5**). Next, we obtained the pseudo 1st order rate constants of 1*H*-phenalene-1,3(2*H*)-dione (**8a**, $k_{obs} = 0.1 \text{ min}^{-1}$), recently developed cyclic C-nucleophiles 1-benzylpiperidine-2,4-dione (**11**, $k_{obs} = 86.4 \text{ min}^{-1}$), 1-benzyl-1*H*-benzo[c][1,2]thiazin-4(3*H*)-one 2,2-dioxide (**12**, $k_{obs} = 190.5 \text{ min}^{-1}$), linear C-nucleophiles ((nitromethyl)sulfonyl)benzene (**13**, $k_{obs} = 7.8 \text{ min}^{-1}$), and 2-(phenylsulfonyl)acetonitrile (**14**, $k_{obs} = 78.0 \text{ min}^{-1}$) for the reaction with dipeptide-SOH **10**. Due to the opposing effect of resonance stabilization and electron donating naphthyl group, **8a** showed a reduced rate of reaction towards sulfenic acid **10**. Cyclic and linear C-nucleophiles **11-14** showed

elevated reactivity towards **10**. In brief, the reactions of various nucleophiles and electrophiles towards dipeptide-SOH **10** clearly demonstrated the substantially enhanced reactivity of **11-14** compared to currently prevalent probes (**Fig. 3**).

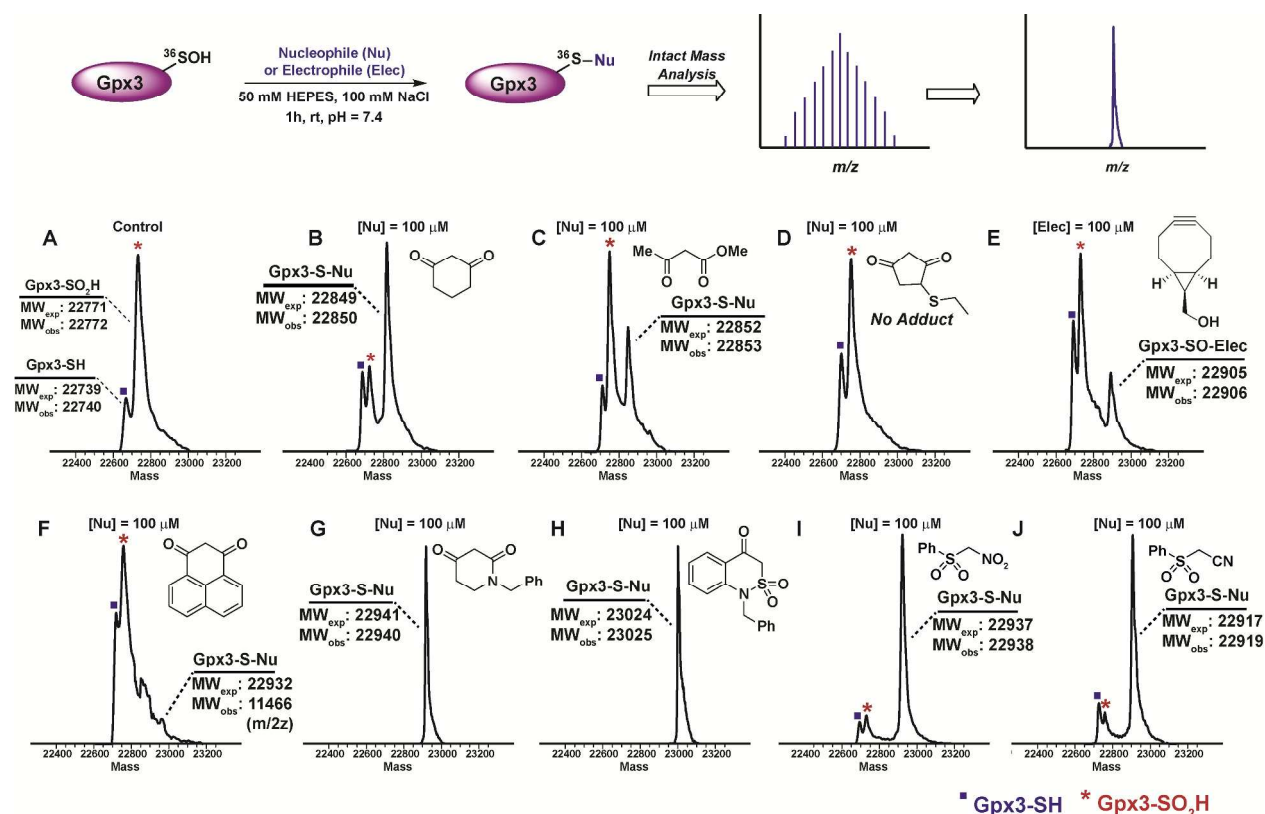


Fig. 4 Reactivity of nucleophiles and electrophiles with Gpx3-SOH.

Subsequently, we examined the reactivity of above-tested nucleophiles and electrophiles towards a protein Cys-SOH. For these studies, we used our well-established Cys64Ser Cys82Ser variant of the recombinant thiol peroxidase, Gpx3.¹¹ Incubation of nucleophiles and electrophiles at 100 μM concentration with Gpx3-SOH led to the covalent modification of Gpx3-SOH. Of note, while trapping with **2e** was not quantitative, it still captured ~ 50% Gpx3-SOH (**Fig. 4B**). Interestingly, even though β-ketoester **3a** showed a higher rate of reaction in the

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3 dipeptide-SOH model, the amount of Gpx3-SOH captured was lower than **2e**,
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5 highlighting the influence of protein microenvironment on reactivity as well as the
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7 tendency of the thioether adduct of **3a** to undergo loss of a methylketo (-COMe)
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9 group (**Fig. 4C**).^{25, 57} Owing to poor reaction rate, **7a** failed to capture Gpx3-SOH,
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11 a finding that was consistent with data obtained in the dipeptide-SOH model (**Fig.**
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13 **4D**). In contrast to data reported using the C165A AhpC-SOH,⁴⁰ the hydroxyl
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15 version of strained cycloalkyne **6** captured only a small amount of Gpx3-SOH
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17 (**Fig. 4E**). This result correlates well with the observation that the sulfoxide adduct
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19 of **6a** with dipeptide-SOH **10** quickly degrades (**ESI, Fig. S5**). Phenalene-1,3-
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21 dione **8a** also showed extremely poor reactivity towards Gpx3-SOH, also
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23 consistent with results obtained in the dipeptide-SOH model (**Fig. 4F**). Finally,
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25 akin to their superior reactivity towards the dipeptide-SOH, cyclic C-nucleophiles
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27 **11** and **12** quantitatively trapped Gpx3-SOH (**Fig. 4G-H**). Linear C-nucleophiles
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29 **13** and **14** also tagged the majority of Gpx3-SOH (**Figure 4I-J**). These results
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31 gratifyingly confirmed the order of reactivity observed in our LC/MS assay using
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33 the dipeptide-SOH model system.
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Reducing conditions
GSH (5 mM)

Nucleophile

| # | Structure | $t_{1/2}$ | # | Structure | $t_{1/2}$ |
|----|-----------|-----------|----|-----------|-----------|
| 15 | | Stable* | 19 | | > 39 h |
| 16 | | 1.4 h | 20 | | > 433 h |
| 17 | | Stable* | 21 | | > 216 h |
| 18 | | Stable* | 22 | | 0.4 h |

* Stable adduct formation is defined as an adduct in which no detectable cleavage has occurred within 48 h.

Table 1 Reversibility of dipeptide-S-Nu adducts under reducing conditions.

We previously showed that due to the difference in reactivity, pK_a , and carbanion stability of C-nucleophiles, the resulting thioether adducts also display a range of stability under reducing conditions.^{25, 26} As cell contains millimolar concentrations of glutathione (GSH) we tested the ability of this low-molecular weight thiol to reduce the nascent covalent thioether bond formed between **10** and nucleophiles. Initially thioether adducts of linear C-nucleophiles were shown to be reversible under reducing conditions – a view strengthened by the stability of thioether adducts **15**, **17** and **18** and unstable nature of thioether adducts **16**

($t_{1/2} = 1.4$ h) and **22** ($t_{1/2} = 0.4$ h) (**Table 1**, ESI Fig. S6 – S21 and Fig. S34 – S37).^{25, 26} However, the instability of thioether adduct **19** ($t_{1/2} \geq 39$ h) formed by cyclic C-nucleophile **11** (ESI, Fig. S22 – S25), and the stability of thioether adduct **21** ($t_{1/2} \geq 9$ days) of linear C-nucleophile **14** (ESI, Fig. S30 – S33), indicates that the cyclic/linear nature of nucleophiles is not the determining factor with respect to the stability/instability of corresponding adducts (**Table 1**). Interestingly, enhanced reactivity of a nucleophile is also not the sole criterion for reversibility, as illustrated by the stability of thioether adduct **20** ($t_{1/2} \geq 18$ days) which results from trapping by the highly reactive cyclic C-nucleophile **12** (**Table 1**, ESI Fig. S26 – S29). Thioether adducts showing $t_{1/2} \leq 48$ h were then tested for reversibility in the Gpx3 model. Gpx3-SOH was first labeled with nucleophiles **3a**, **11** and **14**. Subsequently, each Gpx3-Nu adduct was subjected to mM glutathione (GSH), dithiothreitol (DTT) or the phosphine-based reductant, TCEP. Correlating with the reversibility trends observed in the reduction of dipeptide-based model, Gpx3-S-Nu adducts formed by β -ketoester **3a** and phenylsulfonylacetonitrile **14** were almost completely reversible while the adduct corresponding to **11** showed some reversibility under reducing conditions (ESI Fig. S50).

In light of the aforementioned stability data, we subsequently explored the effect of C-2 substitution on the reactivity of nucleophiles towards sulfenic acid. In this regard, one key issue is that a tertiary carbanion is less stable than a secondary carbanion. Moreover, upon reaction with sulfenic acid, a C-2 substituted nucleophile would be 'locked' in the keto tautomer. Such a conformational lock could potentially result in

unique spectroscopic properties (*i.e.*, UV/Vis or fluorescence) of the nucleophile before and after reaction with sulfenic acid. Such difference in UV/Vis absorption or fluorescence may be exploited to develop a 'turn-on/turn-off switch' based sulfenic acid detection strategy. A representative example of a nucleophile undergoing such change in spectroscopic properties is 2-substituted derivatives of phenalene-1,3-dione (**8a**). Keto-enol tautomerism is facile for compound **8a** readily and, upon reaction with Cys-SOH, keto product **18** can easily rearrange to give enol **18'** (**Fig. 5A**), whereas the thioether derivative of 2-substituted- phenalene-1,3-dione (**23**) would be locked in the keto form **24** (**Fig. 5B**) resulting in turn-on fluorescence.⁵⁸ Although new spectral properties of the resulting thioether adduct is an attractive possibility, the effect of C-2 substitution by an electron-donating group (EDG) or electron-withdrawing group (EWG) on nucleophile reactivity has not been studied.

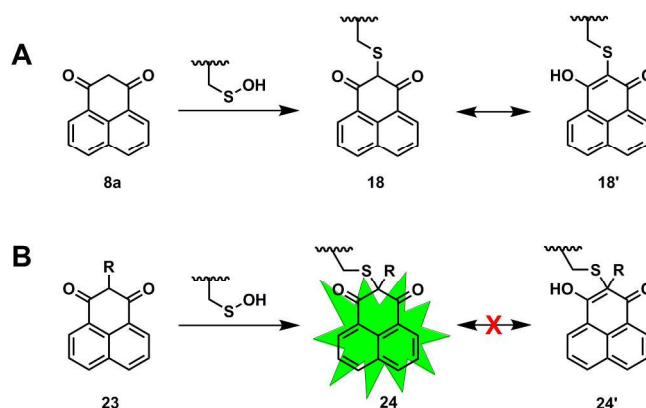


Fig. 5 Effect of C-2 substitution on the UV-vis and fluorescence properties of the nucleophiles and corresponding thioether adducts.

To examine the issue of C-2 substitution, we studied the reactivity of dipeptide sulfenic acid **10** with C-2 methylated (EDG) and C-2 fluorinated (EWG) derivatives of

three nucleophiles – (a) cyclohexane-1,3-dione (**2e**), (b) 1*H*-phenalene-1,3(2*H*)-dione (**8a**), and (c) diethylmalonate (**29**). The analogs were prepared following literature reported procedures.⁵⁹ The kinetic studies data are summarized in **Table 2**. Briefly, while 2-methylcyclohexane-1,3-dione (**25**, $k_{obs} \geq 0.4 \text{ min}^{-1}$) showed slightly higher reactivity than **2e** ($k_{obs} = 0.4 \text{ min}^{-1}$), 2-fluorocyclohexane-1,3-dione (**26**, $k_{obs} < 0.001 \text{ min}^{-1}$) was very slow to react (**Table 2A**). Conversely, both 2-methyl-1*H*-phenalene-1,3(2*H*)-dione (**27**, $k_{obs} = 0.002 \text{ min}^{-1}$) and 2-fluoro-1*H*-phenalene-1,3(2*H*)-dione (**28**, $k_{obs} < 0.001 \text{ min}^{-1}$) showed reduced reactivity, as compared to **8a** ($k_{obs} = 0.1 \text{ min}^{-1}$) (**Table 2B**). Diethylmalonate (**29**, $k_{obs} = 0.1 \text{ min}^{-1}$) exhibited similar reactivity to **8a** and its C-2 substituted derivatives diethyl 2-methylmalonate (**30**, $k_{obs} < 0.001 \text{ min}^{-1}$) and diethyl 2-fluoromalonnate (**31**, $k_{obs} < 0.001 \text{ min}^{-1}$) were extremely slow to react as well (**Table 2C**).

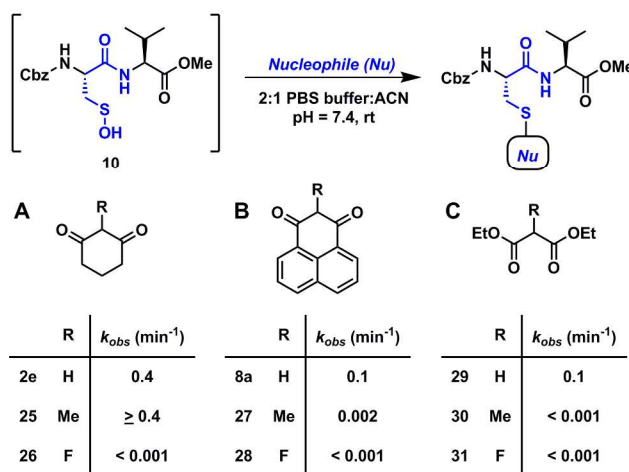


Table 2 Effect of C-2 substitution on the reactivity of nucleophiles towards dipeptide-SOH.

The poor reactivity of C-2 fluorinated analogs **26**, **28** and **31** may be attributed to the strongly electron-withdrawing effect resulting in hydration of carbonyl group. However, the reactivity of C-2 methylated analogs towards dipeptide-SOH **10** is more

complex to explain. While **25** showed slightly enhanced reactivity compared to **2e** the resulting thioether adduct quickly underwent carbonyl hydration due to the electron-withdrawing effect of -SR group (**ESI Fig. S51 – S52**). Similar carbonyl hydration was observed in case of thioether adduct of **27** (**ESI Fig. S53**). Another consequence of hydration is that the diketo nucleophile becomes a poorer leaving group and the corresponding thioether adducts are stable under reducing conditions (**ESI Fig. S38 – S41**). The hydration effect is not observed in case of the thioether adduct **15** of **2e** or **18** of **8a** because under aqueous conditions they readily undergo keto-enol tautomerism and favor the enol form. Diethylmalonate **29** has a comparatively high pK_a value of 13 and its C-2 methyl derivative **30** is expected to have an even higher pK_a , which would result in reduced reactivity towards sulfenic acid **10**, as was observed. In general, C-2 substitution is detrimental for the reactivity of C-nucleophiles towards sulfenic acid and reduced the reaction rates by 100-folds or more.

Like many other critical post-translational modifications, such as phosphorylation and methylation, protein sulfenylation is reversible with an estimated half-life occurring on the timescale of minutes.⁵ In the case of sulfenic acid, its transient nature is often attributed to its reaction with GSH and protein thiol group and these reactions compete with chemical probes. We illustrate this issue by calculating the percent of Human Serum Albumin sulfenic acid (HSA-SOH) and oxidized MAP Kinase Phosphatase 3 (oxMKP3) trapped by various nucleophiles in the presence of mM GSH (**ESI, Fig. S54**). It is clear from the calculations that in both cases, the majority of C-nucleophiles on which current probes are based would capture only a fraction of sulfenic acid (**Table 3, entries 2e, 3a, 7a and 8a**). By contrast, the same calculations with more recently

developed cyclic and linear C-nucleophiles predict near quantitative capture of both HSA-SOH and oxMKP3, even at low nucleophile concentration ($[\text{Nu}] = 100 \mu\text{M}$) (**Table 3, entries 11-14**). Collectively, these data highlight key differences among chemical probes for detecting sulfenic acid in the face of competing biological reactions.

| # | Nucleophiles | k_2 ($\text{M}^{-1}\text{s}^{-1}$) | % HSA-SOH trapped by Nu | % oxMKP3 trapped by Nu | # | Nucleophiles | k_2 ($\text{M}^{-1}\text{s}^{-1}$) | % HSA-SOH trapped by Nu | % oxMKP3 trapped by Nu |
|----|--------------|---|----------------------------|---------------------------|----|--------------|---|----------------------------|---------------------------|
| 2e | | 6.7 | 10% | 14% | 11 | | 1442.8 | 96% | 97% |
| 3a | | 15.0 | 20% | 27% | 12 | | 3175.6 | 98% | 99% |
| 7a | | 0.17 | 0.3% | 0.4% | 13 | | 130.0 | 69% | 76% |
| 8a | | 1.7 | 3% | 4% | 14 | | 1300 | 96% | 97% |

Table 3 Amount of protein-SOH captured by nucleophiles in the presence of GSH at steady state.

The impact of the slow reaction rates of currently used probes in the detection and quantitative analysis of protein-SOH is countered by using large amount of starting biological material ($> 30 \text{ mg}$), higher probe concentrations ($5 \text{ mM} - 10 \text{ mM}$) and longer incubation periods.^{19, 41} It is speculated that higher probe concentrations and longer incubation times increase the rate of false positive identification.²¹ Thus, an ideal probe for proteomic studies would have the following characteristics: (i) sufficiently fast rate of reaction with protein-SOH at low probe concentrations; (ii) form stable adducts with protein-SOH; and (iii) retain chemoselectivity for sulfenic acid. Many of the commercially available probes do not satisfy one or more of these criteria. For example, most

commonly used probes such as DYn-2 (**2a**), DAz-2 (**2b**), DCP-Bio1 (**2c**), DCP-Rho1 (**2d**) and many more are based on cyclohexane-1,3-dione (**2e**) core, which has modest reactivity towards SOH ($k_{obs} = 0.4 \text{ min}^{-1}$). Factors such as the poor reactivity of cyclopentane-1,3-dione ($k_{obs} = 0.01 \text{ min}^{-1}$), strongly anionic nature and presence of a biotin group (poor cell permeability and increased complexity for peptide identification) make BP1 (**7**) less attractive for proteomic analysis of protein-SOH. On the other hand, while β -ketoester-based Alk- β -KE (**3**) gives reaction rates ($k_{obs} = 0.9 \text{ min}^{-1}$) similar to **2e**, the corresponding thioether adduct is susceptible to reduction as well as deacetylation. Recently reported strained cycloalkyne BCN-Bio (**6**) was highly reactive towards small molecule SOH (**10**), but the corresponding sulfoxide adduct was highly unstable under the conditions of LC/MS analysis. Moreover, BCN-bio (**6**) showed poor reactivity towards Gpx3-SOH and is not chemoselective for sulfenic acid.⁵³⁻⁵⁵ The concept of exploiting 2-substituted-phenalene-1,3(2*H*)-diones (**8**, **27**, **28**) as fluorescent turn-on probes using conformational-lock is attractive, however such compounds suffered from poor reactivity towards dipeptide-SOH **10** ($k_{obs} < 0.001 \text{ min}^{-1}$) as well as Gpx3-SOH.

In an effort to address above limitations and develop “next-generation” sulfenic acid probes, we performed side-by-side comparisons with recently developed cyclic (**11**, **12**) and linear C-nucleophiles (**13**, **14**). With k_{obs} ranging from 7.8 min^{-1} to 190.5 min^{-1} , C-nucleophiles **11-14** showed 20- to 500-fold rate enhancement, compared to **2e**. Moreover, these nucleophiles quantitatively trapped Gpx3-SOH at low concentrations (100 μM). Thioether adducts resulting from reaction of cyclic **11** and linear **14** with sulfenic acid **10** (i.e., **19** and **22**) exhibited varying stability under reducing conditions. With a $t_{1/2} = 39 \text{ h}$, thioether **19** was substantially more stable than **22** ($t_{1/2} = 0.4 \text{ h}$),

indicating that cyclic C-nucleophile **11** may be suitable for future probe development. Thioether adducts **20** and **21** showed robust stability under reducing conditions, suggesting that C-nucleophiles **12** and **13** are excellent candidates for “next-generation” sulfenic acid probes.

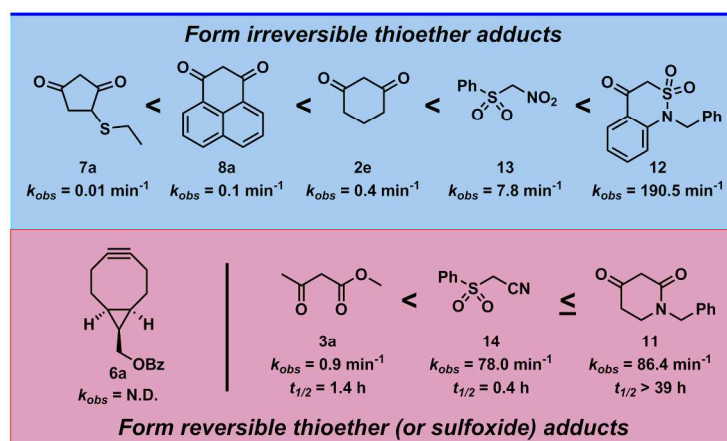


Fig. 6 Ratification of various nucleophiles and electrophiles on the basis of sulfenic acid reactivity and stability/reversibility of corresponding thioether (or sulfoxide) adduct.

Conclusion

In conclusion, we have compared the reactivity of commonly used/commercially available nucleophilic and electrophilic probes with recently developed cyclic and linear C-nucleophiles with sulfenic acid. Using the rate constants obtained from our dipeptide-SOH model, and comparing them with known rates of glutathionylation for protein-SOH, our data indicate that while current probes are sufficient for qualitative analysis, they are not capable of quantitative trapping and may not be well-suited to detect more short-lived sulfenic acid modifications. By extension, “next-generation” probes with superior reaction rates and sufficient stability will enable more comprehensive analysis of the sulfenylome. Another important consideration is the effect of probe/protein structure on

chemical reactivity. Along these lines, to obtain deep coverage of the sulfenylome, it may be valuable to explore multiple probes with varying reactivity and distinct structural features. Further, we have established the reversibility of the thioether adduct resulting from β -ketoester-based Alk- β -KE (**3**) and other nucleophiles, such as **14**, under reducing conditions encountered during trapping or in common use during proteomic sample preparation. Such reversibility can result in false negative results and, thus, it is critical to evaluate the stability of thioether (or sulfoxide) adducts formed by the reaction of nucleophilic (or electrophilic) probes (**Fig. 6**). Finally, we highlight the utility of dipeptide sulfenic acid **10** as model for screening and a facile way to compare the reactivity of nucleophiles and electrophiles with sulfenic acid. Moreover, dipeptide-SOH **10** provides a straightforward way to prepare the corresponding thioether (or sulfoxide) adducts in order to study their stability under various conditions.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Experimental procedures, ^1H NMR, ^{13}C NMR, and LC-MS data including stability studies.

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Notes

The authors declare no competing financial interest.

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