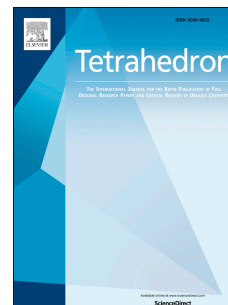


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Norbornene probes for the study of cysteine oxidation

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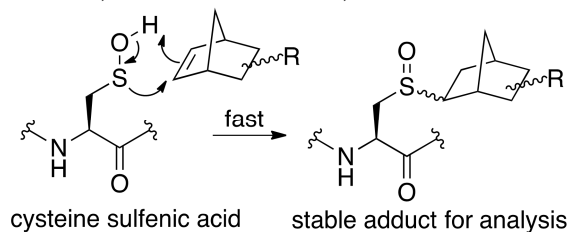
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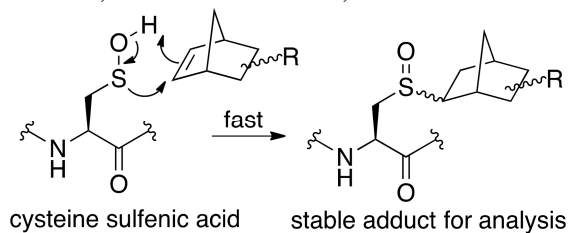
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Norbornene probes for the study of cysteine oxidation

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ABSTRACT

Cysteine residues on proteins can react with cellular oxidants such as hydrogen peroxide. While this process is important for scavenging excess reactive oxygen species, the products of this oxidation may also mediate cell signalling. To understand the role of cysteine oxidation in biology, selective probes are required to detect and quantify its occurrence. Cysteine oxidation products such as sulfenic acids are sometimes unstable and therefore short-lived. If such cysteine derivatives are to be analysed, rapid reaction with the probe is required. Here we introduce norbornene derivatives as probes for cysteine oxidation, and demonstrate their ability to trap sulfenic acids. The synthesis of norbornene derivatives containing alkyne or biotin affinity tags are also reported to facilitate the use of these probes in chemical biology and proteomics.

Introduction

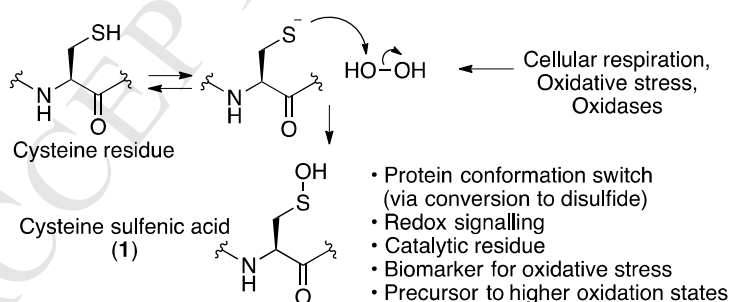


Figure 1: Cysteine reacts with hydrogen peroxide to form a sulfenic acid.

Cysteine oxidation is a critical aspect of redox homeostasis, protein folding, and intracellular signaling.¹⁻³ This oxidation can occur by reaction of the thiolate side chain of cysteine with hydrogen peroxide and other reactive oxygen or reactive nitrogen species generated in cells by the mitochondria and various oxidase enzymes.^{4,6} The immediate product of the reaction of cysteine with hydrogen peroxide is cysteine sulfenic acid (**1**, Fig. 1). Cysteine sulfenic acid may be the first product formed during the scavenging of reactive species during oxidative stress, but it is also a critical determinant of protein function in catalysis,⁷ T cell activation,⁸ redox regulation,⁹⁻¹¹ and signaling.^{10,12} Cysteine sulfenic acid is also a precursor to both inter- and intramolecular disulfides, as well as higher oxidation states of cysteine that can influence the folding and consequently the function of the protein.³ Additionally, cysteine sulfenic acid serves as a biomarker for oxidative stress and occurs with high incidence in certain types of cancer.¹³ Because of these diverse

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biological implications, it is becoming increasingly important to identify what proteins contain cysteine residues susceptible to oxidation and if they exist as functional cysteine sulfenic acids. In doing so, information about cysteine oxidation may be revealed that can help clarify its role in both healthy and diseased cells.

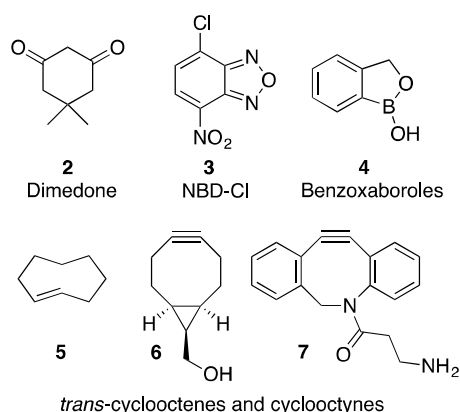
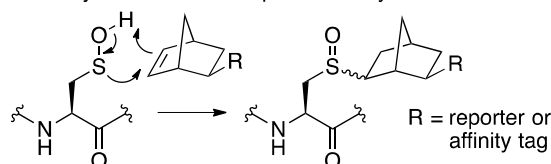


Figure 2: A selection of molecules that contain functional groups that can react with cysteine sulfenic acid. These core structures can be modified to contain a fluorophore or an affinity tag such as biotin to facilitate detection, analysis, and imaging after they have reacted with proteins.

Several functional groups are known to react with cysteine sulfenic acids on peptides and proteins (Fig. 2), but there is still a need for probes that trap short-lived cysteine sulfenic acid residues.^{3,14} Unlike some cysteine sulfenic acid residues that are persistent and stabilised by the protein microenvironment,¹⁵ many are short-lived precursors to higher oxidation states or other modifications. A comprehensive mapping of their biological function is far from complete.³ Dimedone (**2**)^{1,16} and its derivatives^{17,18} are widely used probes to trap cysteine sulfenic acids by reaction of the nucleophilic α -carbon with the sulfur atom of the sulfenic acid. While dimedone benefits from high chemoselectivity, it reacts relatively slowly with sulfenic acids. This limitation has prompted the Carroll laboratory to study other 1,3-dicarbonyls and related nucleophiles that react more rapidly with cysteine sulfenic acid.^{19,20} Indeed, subtle structural modulation of the dimedone core has led to remarkably effective probes with rate enhancements over 100-fold relative to dimedone.^{19,20} Mechanistically distinct probes such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl **3**, an electrophilic probe for cysteine sulfenic acid) is also slow to react and suffers from cross-reactivity with other cellular nucleophiles.²¹ Boronic acids and benzoxaboroles such as **4** are also electrophiles that react with cysteine sulfenic acids, but this process is reversible.²² While **4** may therefore be useful in the reversible inhibition of functional cysteine sulfenic acids, it is not suitable for proteomics applications that require a stable linkage to the cysteine residue. The strained *trans*-cyclooctene (**5**) and cyclooctyne derivative **6** (BCN) are two additional probes recently introduced that trap sulfenic acids through a cycloaddition, providing a stable sulfoxide adduct.^{23,24} The cycloaddition is driven by the release of ring strain that promotes rapid ligation to sulfenic acids—an important feature for trapping short-lived cysteine sulfenic acids. Unfortunately, this strain may also lead to off-target reactions which compromise the selectivity of the probe.^{25,26} For instance, the thiol-yne reaction of cellular thiols with strained cyclooctynes such as **6** and **7** may limit the generality of this class of molecules in detecting sulfenic acids specifically.²⁵ Additionally, compounds such as **5-7** are challenging to synthesise and, because of their strain, have limited shelf-life (especially in solution). For these reasons, we considered norbornene derivatives as alternative probes that would react rapidly with cysteine sulfenic acid due to release of strain (Fig. 3), but not be so reactive that the shelf-life and off-target reactions are concerns. Additionally, norbornene derivatives are straightforward to prepare in a modular fashion (by the Diels-Alder reaction, for instance) so the prospect of accessing functionalised probes in short-order was also attractive. Finally, norbornene compatibility with proteins has been established through its use in several selective bioconjugation methods.²⁷

This study: norbornenes as probes for cysteine sulfenic acid



- Strained alkene enhances rate relative to dimedone
- Sulfoxide adduct stable
- Norbornene derivatives easily accessible

Figure 3: The strained alkene of easily prepared norbornene derivatives is proposed here as a trap for short-lived cysteine sulfenic acids

The use of alkenes to trap cysteine sulfenic acids dates back to a report by Benitez and Allison in which water soluble cyclohexene derivatives were used to inhibit an acyl phosphatase containing a catalytically active cysteine sulfenic acid.¹⁶ This was the same study in which dimedone was initially reported to react with cysteine sulfenic acid.¹⁶ The use of norbornene specifically, as a probe for cysteine

sulfenic acid, was inspired by classic studies by Barton and co-workers in trapping the sulfenic acid formed during the thermally induced syn-elimination of the sulfoxides of penicillin.²⁸⁻³⁰ In this study we extend this concept to the amino acid cysteine and demonstrate that the short-lived sulfenic acid formed from the oxidation of *N*-acetylcysteine with hydrogen peroxide can be intercepted by cycloaddition with various norbornene derivatives.

Results and Discussion

Norbornene derivative **8** was selected as the first candidate probe for cysteine sulfenic acid. **8** contains two carboxylic acids that render it fully water-soluble after treatment with 1 equivalent of sodium carbonate, allowing these experiments to be carried out in aqueous media (H₂O and D₂O, Fig. 4A) without the need for an organic co-solvent—an important consideration for applications on biological samples. The model sulfenic acid was generated *in situ* by the oxidation of *N*-acetylcysteine (**9**) with hydrogen peroxide. Because the conversion of **9** to its disulfide **10** was very rapid (See page S3 in the Supplementary Information), the hydrogen peroxide was added to **8** first and then a solution of *N*-acetylcysteine (**9**) was added slowly and in a 3-fold excess in a second step. This protocol (excess *N*-acetylcysteine added to the solution of **8** and hydrogen peroxide) ensured that the norbornene probe would have a chance to react with the intermediate sulfenic acid before all of it was converted to the disulfide. The reaction was incubated for up to 30 minutes at room temperature and then analysed directly by ¹H NMR spectroscopy and LC-MS. The pH was measured to be 4.3 over the course of the reaction. Gratifyingly, while the major product detected by ¹H NMR and LC-MS analysis was disulfide **10**, the alkene in **8** was completely consumed in its conversion to **11** (Fig. 4A and S4-S5). Because the cycloaddition of the cysteine sulfenic acid with **8** can proceed on either face of the alkene and the sulfur in sulfoxide **11** is a stereogenic centre, there are four possible diastereomers that can be formed. All four diastereomers could be at least partially resolved in the LC-MS analysis and the observed mass spectra were consistent with the calculated value for **11** (*m/z* = 360, ESI). In control reactions, it was confirmed by ¹H-NMR spectroscopy that **8** reacted with neither hydrogen peroxide nor *N*-acetylcysteine **9** alone and that all three components were required to form **11** (S4-S5). In control experiments analysed by LC-MS, trace amounts of thiol-ene product were observed from the direct reaction of norbornene derivative **8** and *N*-acetylcysteine **9** (S6-S7), but this product was apparently below the limits of detection in the ¹H-NMR analysis (S4-S5). This result suggests that even though norbornene has less strain than cyclooctyne derivatives, **8** is not entirely inert to direct reaction with thiols (or thiol radicals generated in the presence of oxygen). With that said, the key sulfenic acid trapping in Fig. 4A was much faster, as no thiol-ene product was detected by LC-MS (S8). When a similar series of experiments was carried out with norbornene derivative **12**, the same outcomes were observed (Fig. 4B and S9-S13), with complete consumption of the alkene probe observed only when **12** and **9** were reacted in the presence of hydrogen peroxide. The pH for the reaction in Fig. 4B was slightly higher (4.6 over the course of the reaction). The key sulfoxide products **13** and **14** can each be formed as a mixture of 4 diastereomers, which could be partially resolved during the LC-MS analysis (S12). The only products observed by ¹H-NMR and LC-MS analysis of the reaction mixture from Fig. 4B were the disulfide **10** and the anticipated products formed from trapping the sulfenic acid (sulfoxides **13** and **14**, *m/z* = 316, ESI). No thiol-ene reaction was observed under these conditions.

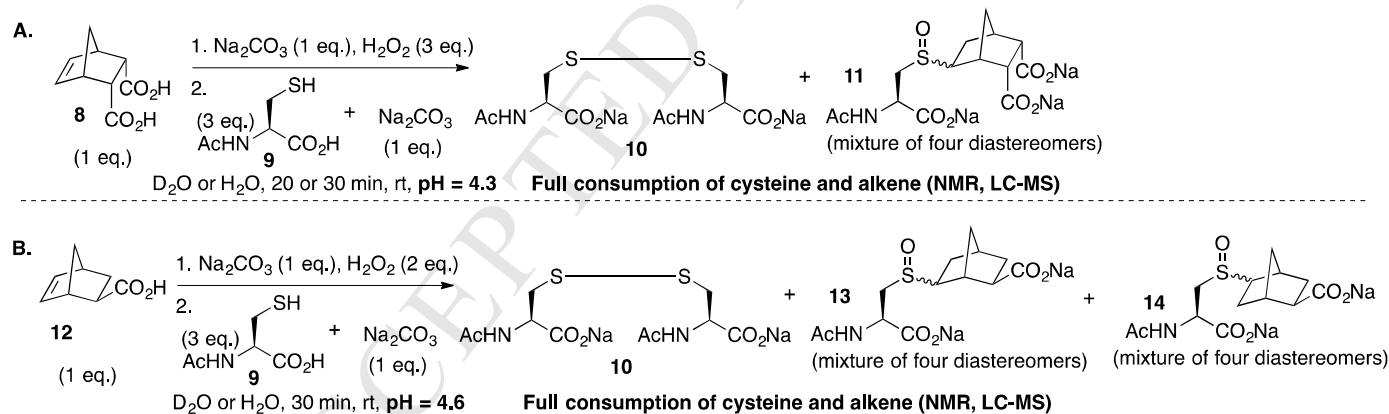


Figure 4: Norbornene probes **8** and **12** can trap the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide.

For comparison to other previously reported probes for cysteine sulfenic acid, dimedone (**2**) and cyclooctyne **6** were each used in an attempt to trap the cysteine sulfenic acid formed from the reaction of **9** with hydrogen peroxide (Fig. 5). In the attempt with dimedone, the only cysteine-derived product observed by ¹H-NMR and LC-MS was disulfide **10** (Fig. 5A and S14-S19). No evidence of **15** (the expected product of the reaction of dimedone and the sulfenic acid derived from **9**) was discovered. This result suggests the intermediate sulfenic acid formed in the reaction reacts so rapidly with **9** that dimedone is unable to trap it. This result highlights one of the limitations of dimedone, but it also illustrates how efficiently the norbornene probes **8** and **12** react with the sulfenic acid to form sulfoxide adducts **11**, **13** and **14** (Fig. 4). In control experiments in which dimedone was treated with hydrogen peroxide (and no cysteine derivative), unreacted dimedone was the major product detected by both ¹H-NMR and LC-MS, though trace amounts of another product were observed (S15-S17). While the product was not isolated, it has a mass consistent with an oxidative dimerisation (S17) and was detected by LC-MS in all experiments in which dimedone and hydrogen peroxide were present in the same reaction mixture. In the comparison to cyclooctyne **6** (BCN, Fig. 5B), the result was also a surprise: the product expected upon trapping the sulfenic acid (**16**) was not observed. The only product detected by LC-MS was disulfide **10**. This result may be the result of limited solubility of **6** in aqueous media and the need to use a mixed solvent system of water and DMSO. Precipitation of **6** throughout the course of the reaction may also complicate analysis. An additional complication is the direct reaction of **6** with **9** via the thiol-yne reaction.²⁵ In a control reaction in which **6** was treated with a 3-fold excess of **9** (and no peroxide), the thiol-yne reaction was indicated

by LC-MS (S22), however this side reaction was not observed under oxidative conditions (S23). Cyclooctyne **7** was also subjected to similar experiments, but it also suffered from limited solubility and appeared to decompose in the presence of hydrogen peroxide (S24-S27), so it was not pursued further as a probe. The thiol-yne reaction was also observed in the reaction of *N*-acetylcysteine (**9**) with the cyclooctyne **7** (S27-S28). These results corroborate concerns recently reported by others about the off-target reactions with thiols when using strained alkynes in chemical biology,^{19,25} especially when the intention is to use the alkyne to identify a specific oxidation state of cysteine such as the sulfenic acid.¹⁹

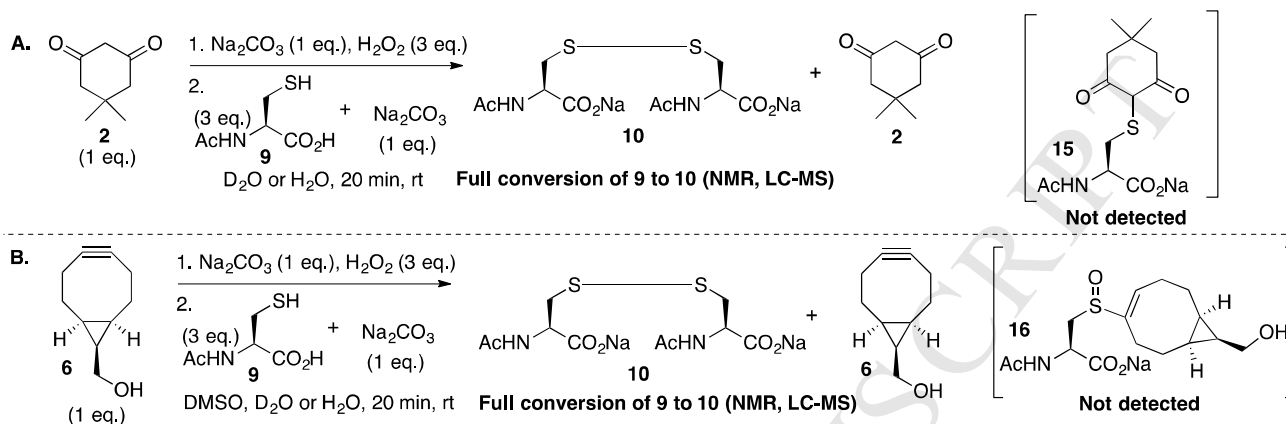


Figure 5: Using similar conditions to the experiments in Figure 4, neither dimesone (**2**) nor cyclooctyne **6** trapped the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide: neither **15** nor **16** were detected by ^1H -NMR or LC-MS analysis.

While the results in Figure 4 were a promising lead, the pH was not controlled in these experiments. Therefore these reactions were modified and carried out in a sodium acetate buffer (200 mM) at pH 5.0. The experiment with dimesone was also repeated in the same buffer (Fig. 6). The outcome was largely the same: norbornene probe **8** was completely consumed in the reaction with the sulfenic acid formed from **9** and converted into the four diastereomers of sulfoxide **11** (Fig. 6 and S29-S35). Some unreacted cysteine was detected (S29-S30) and thiol-ene adduct was also observed by LC-MS (S31-S35), but the major products in the key reaction in Fig. 6A were **10** and **11**. Importantly, control experiments also demonstrated that norbornene probe **8** reacted with neither *p*-toluenesulfinate nor *p*-toluenesulfonate (a model sulfinate and sulfonate, respectively) at pH 5.0, indicating selectivity for the sulfenic acid oxidation state (S36-S37). In contrast, the experiment with dimesone did not result in reaction with the sulfenic acid. Unreacted **9**, disulfide **10** and unreacted dimesone were the major products detected by ^1H -NMR and LC-MS analysis (S38-S45). Again, some oxidation of dimesone was observed by reaction with hydrogen peroxide (S44). In a separate control experiment, this oxidation of dimesone was shown to continue slowly over 24 hours (S40). These buffered experiments at pH 5.0 were not repeated with alkynes **6** or **7** due to complications arising from their low solubility in the reaction medium.

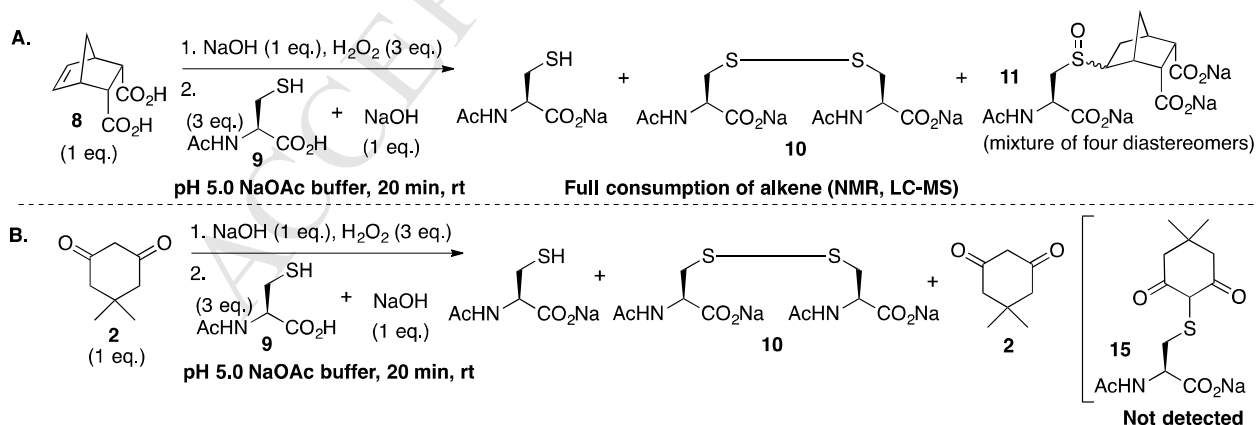


Figure 6: A. Under buffered conditions (pH 5.0, 200mM NaOAc), norbornene **8** can trap the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide. B. Under the same conditions, dimesone (**2**) did not trap the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide.

While the results in Figures 4 and 6 encourage further investigation of norbornene-based probes for cysteine sulfenic acid, this particular model system was sensitive to pH (and pD). When **8** was used to trap the sulfenic acid derived from *N*-acetylcysteine in

sodium phosphate buffer at pD 7.4, the norbornene probe did not react (Fig. 7A and S46-S47). At pD 7.4, the oxidation of *N*-acetylcysteine (**9**) is faster than at pD 5.0: full conversion to the disulfide was observed at the higher pD (Fig 7A and S46-S47) and only 60% conversion to the disulfide was observed at pD 5.0 (Fig 7B and S48-S49) over a period of 20 minutes. It is likely that both steps in the disulfide formation (nucleophilic attack of **9** on hydrogen peroxide and then nucleophilic attack of **9** on the resulting sulfenic acid) are slower at pD 5.0 because there will be a lower concentration of the more nucleophilic thiolate derived from **9** at the lower pD. The sulfenic acid in this system would be expected to be longer-lived at pD 5.0 and therefore be able to react with the norbornene probes. In contrast, the sulfenic acid formed at pD 7.5 is consumed so rapidly by the reaction with the thiolate of **9** that the norbornene cannot intercept any of the sulfenic acid intermediate. Importantly, dimedone was also unable to trap the sulfenic acid derived from **9** at pD 7.4 (S50-S51), which is consistent with a very short-lived sulfenic acid. An additional factor that may be important is the pK_a of the cysteine sulfenic acid itself. For small molecule sulfenic acids, the pK_a can vary from 4-12, and on proteins for which it has been measured, the pK_a of cysteine sulfenic acid was 6-7.^{31,14} The cycloaddition proposed in Figure 3 may require the sulfenic acid rather than its conjugate base, the sulfenate anion—another potential reason why the reaction in Figure 7A at pD 7.4 did not provide the desired product **11**. In any case, it was fortuitous that the reaction mixtures in Figure 4 were naturally between pH 4-5 without added buffer—conditions that enabled the detection of the key ligation that validated norbornene derivatives as probes for transient cysteine sulfenic acids. We also note that norbornene probe **8** was still inert to model sulfinates and sulfonates (*p*-toluenesulfinate and *p*-toluenesulfonate) at pD 7.4 (S52-S53).

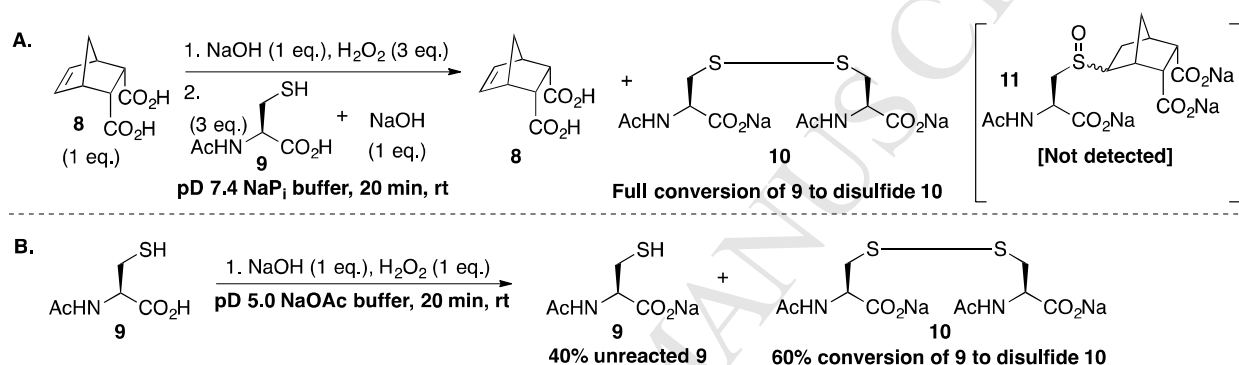


Figure 7: A. In pD 7.4 sodium phosphate buffer (200 mM), norbornene probe **8** did not trap the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide. Full conversion of *N*-acetylcysteine (**9**) to disulfide **10** was observed by ¹H-NMR at this pD and no reaction of **8** was evident. B. The oxidation of *N*-acetylcysteine (**9**) is slower at pD 5.0 than pD 7.4: only 60% conversion to disulfide **10** was observed by ¹H-NMR, with the remaining material present as unreacted **9**.

For the detection of cysteine sulfenic acid on proteins, the norbornene probe would require a reporter group—functionality that can be further labeled, visualised, and quantified during SDS-PAGE, Western blots, and cell imaging. Terminal alkynes are one such group because they can be selectively labeled with an azide-containing fluorophore or affinity tag¹² via the copper-catalysed azide-alkyne cycloaddition.^{31,32} We therefore prepared **19** by direct reaction of amine **18** with anhydride **17**, providing norbornene probe **19** in 91% yield in a single step from commercially available starting materials. The simplicity with which the reporter group could be ligated to the core norbornene structure is a valuable feature, as other probes for cysteine sulfenic acid often require multiple steps and tedious purification.^{3,14,20} The resulting carboxylic acid in **19** was also anticipated to enhance water solubility. Unfortunately, probe **19** was surprisingly unstable in pH 5 buffer and hydrolysed to **8** and **18** rather quickly, with 50% hydrolysis observed by ¹H-NMR over 20 minutes (Fig. 8 and S54-S57). For a sample incubated in buffer for 24 hours, complete hydrolysis was observed (S56). This hydrolysis is a liability for analytical techniques such as SDS-PAGE, Western blots, and cell imaging that necessitate the reporter group remain ligated to the protein throughout the analysis. We suspect that **19** is prone to hydrolysis because the adjacent carboxylic acid can participate in the reaction as a nucleophilic or acid catalyst. Analogous cases of this neighbouring group participation have been reported for arylamides on the same norbornene core,³³ so apparently this phenomenon extends to the alkylamide in **19**.

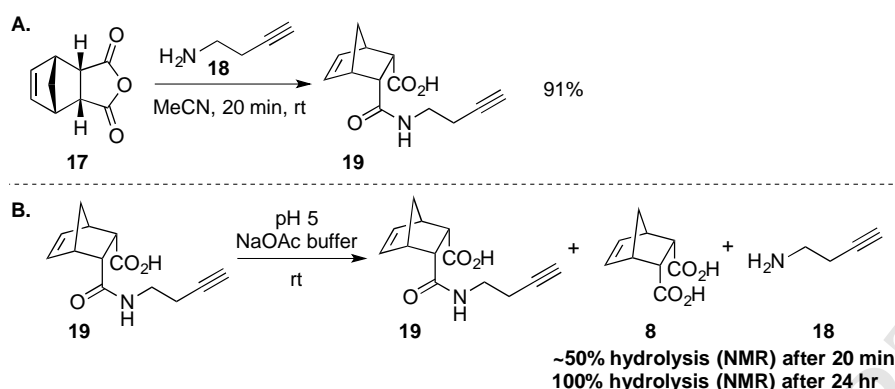


Figure 8: A. A norbornene probe for cysteine sulfenic acid containing an alkyne reporter group (**19**) was prepared in a single step by the reaction of amine **18** with anhydride **17**. B. Probe **19** hydrolysed spontaneously in water.

To avoid the hydrolysis problem associated with **19**, an alternative norbornene probe was prepared that did not contain the neighboring carboxylic acid. Norbornene derivative **12** was therefore coupled to *N*-hydroxysuccinimide (**20**) to form NHS ester **21**. Reaction of **21** with amine **18** provided the new probe **22** (Fig. 9A). The amide in this probe was stable and no hydrolysis was observed in DMSO-water mixtures. Using a similar synthetic scheme, a biotin-tagged norbornene was also synthesised (Fig. 9B). NHS ester **21** was first coupled to the diamine linker **23** in 91% yield. Separately, biotin (**25**) was converted to its NHS ester **26**. The free amine in **24** was then used as a point of ligation for the biotin NHS ester **26**. After the final coupling, the target probe **27** was isolated in 71% yield. Regarding solubility of the probes, a solution of either **22** or **27** in DMSO can be added to phosphate buffer such that the final concentration is 1 mM in **22** or **27**, with less than 1% of the total volume as DMSO. No precipitation was observed and such formulations are comparable to other probes used in pull-down assays. Norbornene derivatives **22** and **27** are now under investigation as probes for cysteine sulfenic acids on both proteins and cells.

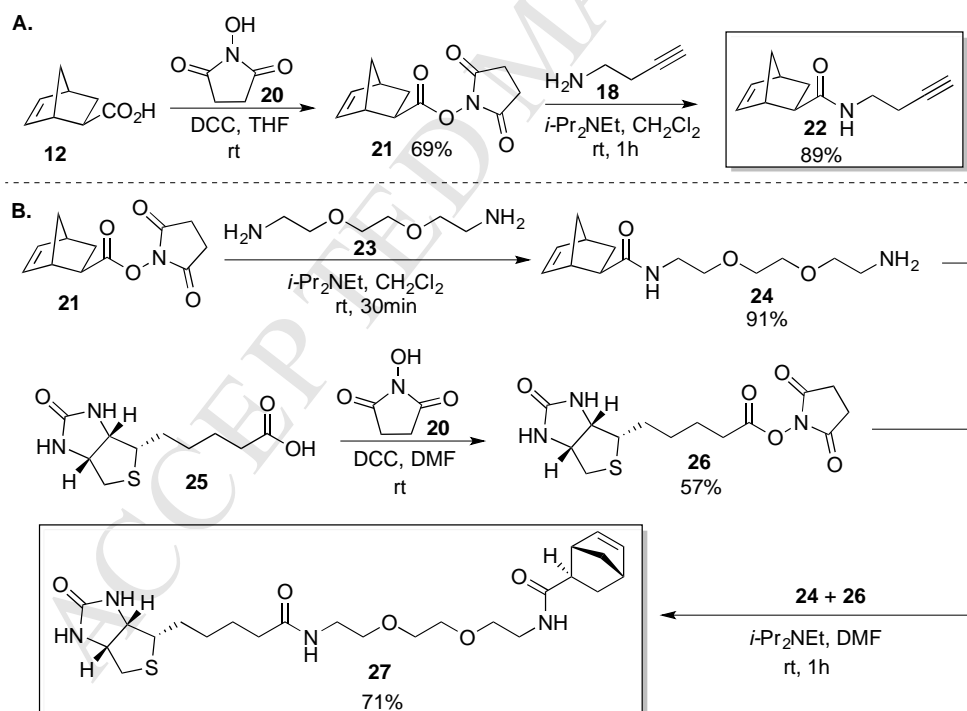


Figure 9: A. A norbornene probe for cysteine sulfenic acid containing an alkyne reporter group (**22**) was prepared in two steps from **12**. Probe **22** was not susceptible to hydrolysis in the same way as **19**. B. The synthesis of a norbornene probe for cysteine sulfenic acid containing a biotin affinity tag.

Conclusions

A current challenge in the study of cysteine redox chemistry is to detect rapid oxidation events and short-lived cysteine sulfenic acids. The oxidation of *N*-acetylcysteine to its corresponding disulfide is fast and therefore a challenging model system for any probe designed to intercept the sulfenic acid intermediate. Norbornene derivatives such as **8** and **12** were able to trap the cysteine sulfenic acid

intermediate, providing stable sulfoxide adducts that could be analysed by LC-MS. The most widely used probe for cysteine sulfenic acid, dimedone, was unable to react with the transient sulfenic acid formed in this model system. The direct reaction between *N*-acetylcysteine (**9**) and the norbornene derivatives was observed, but this off-target thiol-ene reaction was much slower than the reaction between the norbornene derivatives and the cysteine sulfenic acid. Furthermore, the product of the thiol-ene and the sulfoxide formed from trapping the cysteine sulfenic acid have distinct masses—an important point that will facilitate proteomics applications of these probes. In the synthesis of a norbornene derivative containing an alkyne reporter group (**19**), a surprisingly fast amide hydrolysis reaction was discovered that cleaved the reporter from the norbornene core structure. Revising the synthetic strategy, alkyne and biotin labeled probes **22** and **27** were prepared in short order; these compounds did not suffer the hydrolysis that occurred for **19**. With the functionalised norbornene probes in hand, we are currently evaluating them in a variety of studies on proteins and live cells with an aim to identify hitherto unknown sites and functions of cysteine sulfenic acid.

Experimental section

For additional experimental details, control experiments, LC-MS data and NMR spectra, please consult the Supplementary Information.

General considerations

All reagents were used directly from commercial suppliers without further purification. All reactions without water as a solvent were carried out under an inert atmosphere of nitrogen in flame-dried glassware. CH₂Cl₂ was distilled over CaH₂ and THF was distilled over sodium and benzophenone prior to use. All other solvents were used directly from commercial suppliers without further purification. Analytical thin layer chromatography was performed on aluminium sheets coated with silica gel containing a fluorescent indicator (0.15-0.2mm thickness, 8 μm granularity), with visualisation carried out using an ultraviolet lamp (254 nm) and/or development with potassium permanganate. Column chromatography was performed using silica gel (230–400 mesh, 60 Å pore diameter). High resolution mass spectra (HRMS) were recorded on a Waters Synapt HDMS Q-ToF by electrospray ionization (ESI) or a Perkin Elmer, AxION, DSA-ToF by atmospheric-pressure chemical ionization (APCI) and are reported as the observed molecular ion. Infrared spectra were recorded on an FTIR spectrometer with the absorptions reported in wavenumbers (cm⁻¹).

¹H and ¹³C NMR spectra were recorded on a Bruker 600 MHz Spectrometer. NMR were assigned using COSY, HMQC and HMBC where required. Where D₂O was used as the solvent and internal lock, spectra were referenced to residual solvent for HOD (δ_H 4.79 ppm) for ¹H NMR. For MeOD-d₄, spectra were referenced to residual solvent (δ_H 3.33 ppm) for ¹H NMR and (δ_C 49.0 ppm) for ¹³C NMR. For DMSO-d₆, spectra were referenced to residual solvent (δ_H 2.50 ppm) for ¹H NMR and (δ_C 39.5 ppm) for ¹³C NMR. For CDCl₃, spectra were referenced to residual CHCl₃ (δ_H 7.26 ppm) for ¹H NMR and (δ_C 77.0 ppm) for ¹³C NMR. Chemical shift values are reported in parts per million, ¹H-¹H coupling constants are reported in hertz and H multiplicity is abbreviated as; s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad signal.

Liquid chromatography mass spectrometry (LC-MS) analyses were carried out on a Waters Acquity UPLC coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer using electrospray ionization in both positive and negative mode, as specified. A kinetex C18 column with particle size 2.6 μm, and dimensions 50 × 2.1 mm length was used for all experiments. UHPLC grade solvents and milli Q water were used in these experiments. Before injection, samples were diluted to approximately 50 μg/mL in milli Q water, mixed, and then filtered through 0.2 μm nylon syringe filters. For all experiments, the mobile phases were run with water 0.1 % formic acid (solvent A) and acetonitrile (solvent B). All samples had an injection volume of 2 μL and ran with a flow rate of 0.2 ml min⁻¹. The gradient was programmed as followed: 95% A – 5% B (0 minutes) and maintained for 5 minutes (isocratic). 5% A – 95% B over 7 minutes and maintained at 5% A for a further 3 minutes. 95% A – 5% B over 0.5 minutes and held for a further 4.5 minutes to wash/equilibrate the column. The total run time was 20 minutes. The electrospray source was operated with a capillary voltage of 3 kV and cone voltage of 30 V. The source temperature was operated at 80 °C and the desolvation temperature of 350 °C. All data was analysed using Masslynx software.

***cis*-5-norbornene-endo-2,3-dicarboxylic acid (**8**) as a probe for cysteine oxidation (Figure 4A).** Two solutions were first prepared. Solution 1: In a vial, a mixture of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **8** (45 mg, 0.25 mmol), D₂O (2 mL) and Na₂CO₃ (25 mg, 0.24 mmol) was stirred or shaken until fully dissolved. Solution 2: In a vial, a mixture of *N*-acetylcysteine (125 mg, 0.77 mmol), D₂O (2 mL) and Na₂CO₃ (25 mg, 0.24 mmol) was stirred or shaken until fully dissolved. For the key reaction, solution 1 (0.4 mL, 0.05 mmol **8**) was added to a vial, followed by H₂O₂ (20 μL, 30 wt% in H₂O) and stirred for a few seconds before adding solution 2 (0.4 mL, 0.15 mmol, **9**) dropwise over 1 minute. The mixture was stirred for 20 minutes before analysing directly by NMR. Full consumption of the alkene signal (δ = 6.24 ppm) was observed. This reaction was repeated using H₂O in place of D₂O and analysed by LC-MS. Disulfide **10** was detected at 2.53 min and the four diastereomers of **11** were detected at 3.05, 3.31, 3.49 and 4.19 minutes.

***exo*-5-norbornenecarboxylic acid (**12**) as a probe for cysteine oxidation (Figure 4B).** *exo*-5-Norbornenecarboxylic acid (**12**, 30 mg, 0.22 mmol) was added to a vial and suspended in D₂O (0.4 mL). Sodium carbonate (23 mg, 0.22 mmol) was then added and the mixture was stirred to provide a homogeneous solution. Next, hydrogen peroxide (50 μL of a 30 wt% solution in H₂O, 0.44 mmol) was added to the solution of **12**. In a separate vial, *N*-acetylcysteine (**9**, 108 mg, 0.66 mmol) and sodium carbonate (23 mg, 0.22 mmol) were dissolved in D₂O (0.4 mL). The solution of **9** was then added dropwise by pipette over 1 minute at room temperature to the solution of **12**. The reaction was stirred for 30 minutes and then analysed by NMR. Full consumption of the alkene signal (δ = 6.10 ppm) was observed. This reaction was repeated using H₂O in place of D₂O and analysed by LC-MS. Disulfide **10** was detected at 2.52 min and the diastereomeric mixture of **13** and **14** were detected at 1.8-2.2, 3.4, 3.5, 3.7 and 3.8 minutes.

Dimedone as a probe for cysteine oxidation (Figure 5A). Two solutions were first prepared. Solution 1: In a vial, a mixture of dimedone (35 mg, 0.25 mmol), D₂O (2 mL) and Na₂CO₃ (36 mg, 0.34 mmol) was stirred or shaken until fully dissolved. Solution 2: In a vial, a mixture of *N*-acetylcysteine (211 mg, 1.30 mmol), D₂O (3.2 mL), and Na₂CO₃ (54 mg, 0.50 mmol) was stirred or shaken until fully dissolved. For the key reaction, solution 1 (0.4 mL, 0.05 mmol dimedone) was added to a vial, followed by H₂O₂ (20 μL, 30 wt% in H₂O). After a few seconds of stirring, solution 2 (0.4 mL, 0.16 mmol **9**) was added dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR. Only unreacted dimedone and disulfide **10** were observed by ¹H-NMR. This reaction was repeated using H₂O in place of D₂O and analysed by LC-MS. Disulfide **10** was detected at 2.61 min and unreacted dimedone at 7.34 minutes. **15** was not detected.

cyclooctyne **6 as a probe for cysteine oxidation (Figure 5B).** Two solutions were first prepared. Solution 1: In a vial, **6** (20 mg, 0.13 mmol) was dissolved in DMSO (0.4 mL) and then D₂O (1.6 mL) and Na₂CO₃ (15 mg, 0.14 mmol) were added and the mixture was stirred or shaken until fully dissolved. Note: BCN has very low solubility in aqueous media. Solution 2: *N*-acetylcysteine (60 mg, 0.37 mmol), D₂O (2 mL) and Na₂CO₃ (15 mg, 0.14 mmol) were added to a vial and stirred or shaken until fully dissolved. For the key reaction, solution 1 (0.4 mL, 0.026 mmol **6**) was added to a vial, followed by H₂O₂ (20 μL, 30 wt% in H₂O). After stirring for a few seconds, solution 2 (0.4 mL, 0.074 mmol **9**) was added dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR. Only unreacted **6** and disulfide **10** were observed. This reaction was repeated using H₂O in place of D₂O and analysed by LC-MS. Only disulfide **10** was detected. No sulfoxide adduct **16** was detected.

***cis*-5-norbornene-endo-2,3-dicarboxylic acid (**8**) as a probe for cysteine oxidation at pD or pH 5.0 (Figure 6A).** Two solutions were first prepared. Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (17 mg, 0.09 mmol **8**), D₂O (1.0 mL), and NaOH (3.8 mg, 0.09 mmol) were added to a vial and stirred or shaken until dissolved. Finally, pD 5.0 acetate buffer in D₂O (400 mM, 1.0 mL) was added and the solution was stirred. Solution 2: *N*-acetylcysteine (42 mg, 0.26 mmol **9**), D₂O (1.0 mL), and NaOH (3.3 mg, 0.08 mmol) were added to a vial and shaken or stirred until fully dissolved. Finally, pD 5.0 acetate buffer in D₂O (400 mM, 1.0 mL) was added and the solution was stirred. For the key reaction, solution 1 (0.5 mL, 0.02 mmol **8**) was added to a vial followed by H₂O₂ (7 μL, 30 wt% in H₂O). The resulting solution was stirred for a few seconds before the dropwise addition of solution 2 (0.5 mL, 0.06 mmol **9**) over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR. Full consumption of the alkene signal (δ = 6.25 ppm) was observed. This reaction was repeated using H₂O in place of D₂O in a pH 5.0 NaOAc buffer and analysed by LC-MS. Disulfide **10** was detected at 2.50 minutes and four diastereomers of **11** were detected at 2.97, 3.16, 3.39 and 4.04 minutes. The product of the thiol-ene side reaction was detected at 7.36 minutes.

Dimedone as a probe for cysteine oxidation at pD or pH 5.0 (Figure 6B). Two solutions were first prepared. Solution 1: Dimedone (13 mg, 0.09 mmol), D₂O (1.0 mL) and NaOH (3.4 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, pD 5.0 acetate buffer in D₂O (400 mM, 1.0 mL) was added and the solution was stirred. Solution 2: *N*-acetylcysteine (42 mg, 0.26 mmol), D₂O (1.0 mL) and NaOH (3.3 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, pD 5.0 acetate buffer in D₂O (400 mM, 1.0 mL) was added and the solution was stirred. For the key reaction, solution 1 (0.5 mL, 0.02 mmol) was added to a vial followed by H₂O₂ (7 μL, 30 wt% in H₂O) and stirred for a few minutes before dropwise addition of solution 2 (0.5 mL, 0.06 mmol). The solution was stirred for 20 minutes before analysing directly by NMR. Unreacted dimedone, unreacted *N*-acetylcysteine and disulfide **10** were the only products observed. **15** was not detected. This reaction was repeated using H₂O in place of D₂O in a pH 5.0 NaOAc buffer and analysed by LC-MS. Disulfide **10** was detected at 2.51 minutes and unreacted dimedone at 7.37 minutes. **15** was not detected.

***cis*-5-norbornene-endo-2,3-dicarboxylic acid (**8**) as a probe for cysteine oxidation at pD or pH 7.4 (Figure 7A).** Two solutions were first prepared. Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (20.0 mg, 0.11 mmol **8**), D₂O (1.25 mL) and NaOH (5 mg, 0.12 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, additional pD 7.4 sodium phosphate buffer (400 mM in D₂O, 1.25 mL) was added and the solution was stirred. Solution 2: *N*-acetylcysteine (49.8 mg, 0.30 mmol **9**), D₂O (1.25 mL) and NaOH (3 mg, 0.08 mmol) were added to a vial and stirred until fully dissolved. Finally, additional pD 7.4 sodium phosphate buffer (400 mM in D₂O, 1.25 mL) was added and the solution was stirred. For the key reaction, solution 1 (0.5 mL, 0.02 mmol **8**) was added to a vial followed by H₂O₂ (7 μL, 30 wt% in H₂O). The solution was stirred for a few minutes before adding solution 2 (0.5 mL, 0.06 mmol **9**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR. The only products observed were unreacted **8** and disulfide **10**. **11** was not detected.

Oxidation of *N*-acetylcysteine to its disulfide using hydrogen peroxide at pD 5.0 (Figure 7B). *N*-acetylcysteine (9.8 mg, 0.06 mmol **9**), D₂O (0.25 mL) and NaOH (2.4 mg, 0.06 mmol) were added to a vial and stirred or shaken until fully dissolved. Next, pD 5.0 sodium acetate buffer (400 mM in D₂O, 0.25 mL) was added and the solution was stirred. A solution of H₂O₂ (7 μL, 30 wt% in H₂O) was added and left to stir for 20 min. The reaction was then analysed directly by ¹H-NMR. 60% conversion to disulfide **10** and 40% unreacted **9** were observed.

Synthesis of alkyne **19 (Figure 8A).** 1-amino-3-butyne (59.2 μL, 0.723 mmol) was added to a stirred solution of *cis*-5-norbornene-endo-2,3-dicarboxylic anhydride (239 mg, 1.46 mmol) in acetonitrile (1 mL) and stirred at room temperature for 20 minutes, over which time a white precipitate formed. The resulting mixture was transferred into a centrifuge tube and pelleted by centrifugation for 10 minutes. The supernatant was decanted and the remaining pellet washed with EtOAc. The final product was isolated by filtration without further purification to give the product **19** as a white solid (154 mg, 91% yield): m.p. 129 °C; IR (ν_{max}, ATR): 3359, 2987, 1716, 1622, 1550, 1321, 1267, 1229, 1074, 846, 759, 679, 625; ¹H NMR (600 MHz, DMSO-*d*₆): δ = 11.53 (1H, br-s, COOH), 7.86 (1H, t, J =

5.9 Hz, NH), 6.15 (1H, dd, $J = 5.5, 2.9$ Hz, HC=CH), 5.95 (1H, dd, $J = 5.4$, HC=CH), 3.14 (1H, dd, $J = 10.3, 3.3$ Hz, CH=CHCHCHCOOH or CH=CHCHCHCONH), 3.08 (3H, contains CONHCH₂CH₂ and CH=CHCHCHCOOH or CH=CHCHCHCONH), 2.93 (2H, m, CHCH=CHCH), 2.80 (1H, t, $J = 2.6$, C≡CH), 2.20 (2H, m, CH₂C≡CH), 1.25 (1H, d, $J = 8.1$ Hz, CHCH_AH_BCH), 1.21 (1H, d, $J = 8.2$ Hz, CHCH_AH_BCH); ¹³C NMR (150 MHz, DMSO-d₆): 173.5, 171.2, 134.9, 133.7, 82.4, 71.9, 48.4, 48.1, 48.1, 46.7, 45.3, 37.8, 18.7; LRMS (ESI): [M-H]⁻: found 232.0, C₁₃H₁₄NO₂ requires 232.1

Hydrolysis study of alkyne probe 19 (Figure 8B). Alkyne probe **19** (24 mg, 0.10 mmol) was added to a vial and dissolved in 1.25 mL of D₂O. To this solution was added sodium acetate buffer in D₂O (1.25 mL, pD 5.0, 400 mM). After 20 minutes and 24 hours, this solution was analysed directly by ¹H NMR and LC-MS, indicating hydrolysis to **8** and **18**. After 20 minutes, 50% hydrolysis was observed by ¹H-NMR and complete hydrolysis was observed after 24 hours (as indicated by integration of the alkene signals and comparison to an authentic sample of **8**, **19**, and **18**).

Norbornene NHS derivative 21. *Exo*-5-norbornenecarboxylic acid (455 mg, 3.30 mmol), *N*-hydroxysuccinimide (357 mg, 3.40 mmol), and *N,N'*-dicyclohexylcarbodiimide (679 mg, 3.30 mmol) were dissolved in anhydrous THF (15 mL) and stirred at room temperature overnight, over which time a white precipitate formed (*N,N'*-dicyclohexylurea). The precipitate was removed by filtration and washed with THF and the filtrate was collected and concentrated under reduced pressure. The crude solid was purified by column chromatography (40% EtOAc in hexane) to give the product **21** as a white solid (537 mg, 69 %). m.p. 84-86°C; R_f (40% EtOAc:hexane) 0.37; IR (ν_{max}, ATR): 2983, 2949, 1735, 1428, 1361, 1200, 1047, 947, 841, 711, 644 cm⁻¹; ¹H NMR (600 MHz, CDCl₃): δ = 6.20 (1H, m, CH₂CHCH=CH), 6.15 (1H, m, CHCH=CH), 3.28 (1H, m, CH=CHCHCH), 3.00 (1H, m, CH=CHCHCH₂), 2.83 (4H, m, COCH₂CH₂CO), 2.51 (1H, m, COCHCH_AH_B), 2.05 (1H, m, COCHCH_AH_B), 1.58-1.50 (2H, contains COCHCH_AH_BCH and COCH), 1.45 (1H, CHCH_AH_BCH); ¹³C NMR (150 MHz, CDCl₃): δ = 171.6, 169.3, 138.5, 135.3, 47.1, 46.4, 41.8, 40.3, 31.0, 25.6.

Norbornene probe 22. Norbornene-NHS **21** (359 mg, 1.53 mmol) was dissolved in anhydrous DCM (8 mL) with stirring at room temperature and then 1-amino-4-butyne (125 μL, 1.53 mmol) and DIPEA (650 μL, 3.82 mmol) were then added successively. After 1 hour a white precipitate had formed (*N*-hydroxysuccinimide). The crude material was concentrated under reduced pressure and purified by column chromatography (40 % EtOAc in hexane) to give the product **22** as a white solid (257 mg, 89 %). m.p. 86-88 °C; R_f (40 % EtOAc in hexane) 0.47; IR (ν_{max}, ATR) 3300, 3267, 3058, 2963, 2869, 1633, 1547, 1442, 1359, 1330, 1243, 1221, 1149, 1070, 1018, 901, 864, 721, 680, 625 cm⁻¹; ¹H NMR (600 MHz, CDCl₃): δ = 6.15 (1H, dd, $J = 5.7, 3.0$ Hz, CH=CH), 6.11 (1H, dd, $J = 5.7, 3.0$ Hz, CH=CH), 5.81 (1H, br-s, CONH), 3.42 (2H, m, CONHCH₂CH₂), 2.93 (2H, m, CHCH=CHCH), 2.42 (2H, tdd, $J = 6.3, 2.7, 1.6$ Hz, CONHCH₂CH₂), 2.01 (2H, contains CH₂C≡CH and CH=CHCHCHCONH), 1.92 (1H, m, CH=CHCHCH_AH_B), 1.70 (1H, dd, $J = 8.4, 1.7$ Hz, CHCH_AH_BCH), 1.35 (2H, contains CHCH_AH_BCH and CH=CHCHCH_AH_B); ¹³C NMR (150 MHz, CDCl₃): δ = 175.6, 138.3, 136.0, 81.7, 69.9, 47.2, 46.4, 44.8, 41.6, 38.0, 30.5, 19.5; HRMS (ESI): [M+H]⁺, found 190.1228. C₁₂H₁₆NO⁺ requires 190.1226.

Norbornene amine derivative 24. 1,2-bis(2-aminoethoxy)ethane (624 μL, 4.2 mmol) and DIPEA (163 μL, 0.92 mmol) were dissolved in anhydrous DCM (5 mL) with stirring at room temperature. A solution of norbornene-NHS **21** (99 mg, 0.42 mmol) was dissolved in anhydrous DCM (1 mL) and added dropwise to the 1,2-bis(2-aminoethoxy)ethane solution and then the reaction mixture was stirred at room temperature for 30 min. After this time, the crude mixture was concentrated under reduced pressure. The resulting oil was purified by column chromatography (10% MeOH in DCM with 1% NEt₃) to give the product **24** as a yellow oil (103 mg, 91 %). R_f (10% MeOH in DCM with 1% NEt₃) 0.20; IR (ν_{max}, ATR): 3294, 3057, 2936, 2868, 1645, 1541, 1448, 1351, 1247, 1105, 905, 808, 724 cm⁻¹; ¹H NMR (600 MHz, CDCl₃): δ = 6.29 (1H, br-s, CONH), 6.13 (1H, dd, $J = 5.7, 2.9$ Hz, CH=CH), 6.09 (1H, dd, $J = 5.8, 3.1$ Hz, CH=CH), 3.63 (4H, m, 2 x CH₂ PEG), 3.56 (4H, dt, $J = 18.5, 5.1$ Hz, 2 x CH₂ PEG), 3.47 (2H, q, $J = 5.2$ Hz, CONHCH₂), 2.91 (4H, m, contains CH₂ PEG and CHCH=CHCH), 2.09 (1H, m, CHCHCONH), 1.91 (1H, dt, $J = 11.5, 4.0$ Hz, CH=CHCHCH_AH_B), 1.72 (1H, d, $J = 8.3$ Hz, CHCH_AH_BCH), 1.31 (2H, contains CH=CHCHCH_AH_B and CHCH_AH_BCH); ¹³C NMR (150 MHz, CDCl₃): δ = 175.8, 138.2, 136.0, 72.9, 70.2, 70.1, 70.0, 47.2, 46.3, 44.6, 41.6, 39.3, 30.5; HRMS (ESI): [M+H]⁺, found 269.1867. C₁₄H₂₅N₂O₃⁺ requires 269.1865.

Biotin NHS ester 26. Biotin (498 mg, 2 mmol) was dissolved in anhydrous DMF (10 mL) by heating to approximately 70 °C for 10 minutes or until fully dissolved. The reaction mixture was allowed to cool to room temperature before adding *N*-hydroxysuccinimide (240 mg, 2.1 mmol) with stirring at room temperature. A solution of *N,N'*-dicyclohexylcarbodiimide (438 mg, 2.13 mmol) in anhydrous DMF (2 mL) was added dropwise to the stirred solution. The reaction was then stirred overnight at room temperature over which time a white precipitate formed (*N,N'*-dicyclohexylurea). The precipitate was removed by filtration and washed with DMF. The filtrate was diluted with EtO₂ until a white precipitate formed. The precipitate was collected by filtration and rinsed with EtO₂ then dried to give the crude product **26** as a white solid (395 mg, 57 %): m.p. (decomp.) 178-190 °C; IR (ν_{max}, ATR): 3227, 2941, 2876, 1818, 1788, 1729, 1698, 1465, 1369, 1210, 1071, 861, 739, 656 cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆): δ = 6.43 (1H, s, NH), 6.37 (1H, s, NH), 4.30 (1H, m, HNCHCHNH), 4.14 (1H, m, HNCHCHNH), 3.10 (1H, m, SCH), 2.84-2.78 (5H, m, contains NCOCH₂CH₂ and SCH_AH_B), 2.67 (2H, t, $J = 7.7$ Hz, CH₂CH₂CO₂N), 2.57 (1H, d, $J = 12.4$ Hz, SCH_AH_B), 1.64 (3H, contains CH_AH_BCH₂CH₂CH₂CO₂N), 1.52-1.36 (3H, m, contains CH_AH_BCH₂CH₂CH₂COON); ¹³C NMR (150 MHz, DMSO-d₆): δ = 170.3, 169.0, 162.7, 61.0, 59.2, 55.3, 40.1 (overlaps with NMR solvent peak), 30.0, 27.9, 27.6, 25.5, 24.3; HRMS (ESI): M+H⁺, found 342.1128. C₁₄H₂₀N₃O₅S⁺ requires 342.1118.

Norbornene probe 27. Biotin-NHS **27** (382 mg, 1.12 mmol) was dissolved in anhydrous DMF (6 mL) with stirring at 50 °C for 5 minutes or until dissolved. Norbornene derivative **24** (398 mg, 1.5 mmol) was dissolved in anhydrous DMF (2 mL) and added dropwise to the biotin-NHS solution. DIPEA (240 μL, 1.4 mmol) was added and the reaction stirred at room temperature for 1 hr. Upon completion, EtO₂ was added until a white precipitate formed. The precipitate was collected by filtration and washed with additional EtO₂. The resulting residue was purified by column chromatography (10% MeOH in DCM) to give the product **27** as a white solid (393 mg, 71%): m.p. 126-131 °C; R_f (10 % MeOH in DCM) 0.32; IR (ν_{max}, ATR): 3291, 2942, 2869, 1703, 1639, 1551, 1464, 1309, 1248,

1214, 1129, 987, 867, 724 cm^{-1} ; ^1H NMR (600 MHz, MeOD): δ = 6.16 (2H, m, $\text{CHCH}=\text{CHCH}$), 4.51 (1H, dd, J = 8.3, 4.5 Hz, $\text{CONHCHCH}_2\text{S}$), 4.32 (1H, dd, J = 7.9, 4.4 Hz, $\text{CONHCHCH}_2\text{S}$), 3.64 (4H, m, 2 x CH_2 PEG), 3.57 (4H, m, 2 x CH_2 PEG), 3.39 (4H, m, 2 x CH_2 PEG), 3.23 (1H, m, SCH), 2.95 (1H, dd, J = 12.8, 5.0 Hz, $\text{CONHCHCH}_2\text{H}_\text{B}\text{S}$), 2.89 (2H, m, $\text{CHCH}=\text{CHCH}$), 2.73 (1H, d, J = 12.7 Hz, $\text{CONHCHCH}_2\text{H}_\text{B}\text{S}$), 2.24 (2H, t, J = 7.1 Hz, CH_2CONH), 2.15 (1H, m, $\text{CH}=\text{CHCHCH}$), 1.88 (1H, dt, J = 11.7, 4.0 Hz, $\text{CH}=\text{CHCHCH}_\text{A}\text{H}_\text{B}$), 1.79-1.59 (5H, contains 2 x CH_2 Biotin and $\text{CHCH}_\text{A}\text{H}_\text{B}\text{CH}$), 1.46 (2H, p, J = 7.5 Hz, CH_2 Biotin), 1.33 (2H, contains $\text{CH}=\text{CHCHCH}_\text{A}\text{H}_\text{B}$ and $\text{CHCH}_\text{A}\text{H}_\text{B}\text{CH}$); ^{13}C NMR (150 MHz, MeOD): δ = 178.6, 176.2, 166.1, 139.0, 137.3, 71.3, 70.7, 70.6, 63.4, 61.6, 57.0, 48.6, 47.1, 45.2, 42.8, 41.0, 40.4, 40.3, 36.7, 31.2, 29.8, 29.5, 26.8; HRMS (ESD): $\text{M}+\text{H}^+$, found 495.2641. $\text{C}_{24}\text{H}_{39}\text{N}_4\text{O}_5\text{S}^+$ requires 495.2636.

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Supplementary Material

Supplementary material is available, including additional experimental details, NMR spectra, and LC-MS data.