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.

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1. Introduction

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.10,11 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.1 In addition, cysteine sulfenic acid serves as a biomarker for oxidative stress and occurs with high incidence in certain types of cancer.12 Because of these diverse 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.

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)16 and its derivatives17,18 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. Indeed, subtle structural modulation of the dimedone core has led to remarkably effective probes with rate enhancements over 100-fold relative to dimedone. Mechanistically distinct probes such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, an electrophilic probe for cysteine sulfenic acid) is also slow to react and suffers from cross-reactivity with other cellular nucleophiles. Boronic acids and benzoxaboroles such as 4 are also electrophiles that react with cysteine sulfenic acids, but this process is reversible. 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. The cycloaddition is driven by the release of ring 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.

The use of alkynes 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. This was the same study in which dimedone was initially reported to react with cysteine sulfenic acid. 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. 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.

2. 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 min 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 spectroscopy and LC-MS was disulphide 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 $^1$H 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 thiyl radicals generated in the presence of oxygen). With that said, the key sulfinic 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 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 $^1$H NMR and LC-MS analysis of the reaction mixture from Fig. 4B were the disulfide 10 and the anticipated products formed from trapping the sulfinic acid (sulfoxides 13 and 14, $m/z = 316$, ESI$^-$). No thiol-ene reaction was observed under these conditions.

For comparison to other previously reported probes for cysteine sulfinic acid, dimedone (2) and cyclooctyne 6 were each used in an attempt to trap the cysteine sulfinic acid formed from the reaction of 9 with hydrogen peroxide (Fig. 5). In the attempt with dimedone, the only cysteine-derived product observed by $^1$H NMR and LC-MS analysis of the reaction mixture from Fig. 4B was disulfide 10 (Fig. 5A and S14-S19). No evidence of 15 (the expected product of the reaction of dimedone and the sulfinic acid derived from 9) was discovered. This result suggests the intermediate sulfinic acid formed in the reaction reacts so rapidly with 9 that dimedone is unable to trap it. This result further highlights one of the limitations of dimedone, but it also illustrates how efficiently the norbornene probes 8 and 12 react with the sulfinic 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 $^1$H 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 sulfinic 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

While the results in Fig. 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 dimeredone 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. This experiment validated norbornene derivatives as probes for transient cysteine sulfinic and sulfenic acids. However, probe 8 did not trap the sulfenic acid intermediate formed upon oxidation of N-acetylcysteine (9) at pH 5.0 and therefore be able to react with the norbornene probes.

In contrast, the sulfenic acid formed at pH 7.4 is consumed so rapidly by the reaction with the thiolate of 9 that the norbornene probe would require a reporter group because they can be selectively labeled with an azide-containing fluorophore or affinity tag 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.33,34,20 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 min (Fig. 8 and S54-S57). For a sample incubated in buffer for 24 h, complete hydrolysis was observed (S56). This hydrolysis is a liability for analytical techniques such as 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 tag 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.33,34,20 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 min (Fig. 8 and S54-S57). For a sample incubated in buffer for 24 h, complete hydrolysis was observed (S56). This hydrolysis is a liability for analytical techniques such as 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 tag via the copper-catalysed azide-alkyne cycloaddition.31,32

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 tag 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.33,34,20 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 min (Fig. 8 and S54-S57). For a sample incubated in buffer for 24 h, complete hydrolysis was observed (S56). This hydrolysis is a liability for analytical techniques such as 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 tag via the copper-catalysed azide-alkyne cycloaddition.31,32

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core, so apparently this phenomenon extends to the alkylamide in 19.

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. 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.

3. 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.

4. Experimental section

For additional experimental details, control experiments, LC-MS data and NMR spectra, please consult the Supplementary Information.
4.1. 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 (0.15–0.2 mm 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 by electrospray ionization (ESI) on a Waters Synapt HDMS Q-ToF by atmospheric-pressure liquid chromatography was performed using silica gel (45–74 μm) with solvents and milli Q water. Before injection, samples were passed through 0.2 μm nylon syringe filters. All reactions without water as a solvent 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 keinetex 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 follows: 95% A – 5% B (0 min) and maintained for 5 min (isocratic). 5% A – 95% B over 7 min and maintained at 5% A for a further 3 min. 95% A – 5% B over 0.5 min and held for a further 4.5 min to wash/equilibrate the column. The total run time was 20 min. For the LC-MS analysis of reactions employing cyclooctynes 6 and 7, methanol was used as solvent B to ensure solubility. Before injection, methanol was also used to dilute samples to approximately 50 μg/mL and then the samples were passed through 0.2 μm nylon syringe filters. The gradient was programmed as follows: 95% A – 5% B (0 min) and maintained for 2 min (isocratic). 5% A – 95% B over 10 min and maintained at 5% A for a further 3 min. 95% A – 5% B over 0.5 min and held for a further 4.5 min to wash/equilibrate the column. 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 at 350 °C. All data was analysed using Masslynx software.

4.2. cis-5-Norbornene-endo-2,3-dicarboxylic acid (8) as a probe for cysteine oxidation (Fig. 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), D_{2}O (2 mL) and Na_{2}CO_{3} (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), D_{2}O (2 mL) and Na_{2}CO_{3} (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 H_{2}O_{2} (20 μL, 30 wt% in H_{2}O) and stirred for a few seconds before adding solution 2 (0.4 mL, 0.15 mmol, 9) dropwise over 1 min. The mixture was stirred for 20 min before analysing directly by NMR. Full consumption of the alkene signal (δ = 6.24 ppm) was observed. This reaction was repeated using H_{2}O in place of D_{2}O 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 min.

4.3. exo-5-Norbornenecarboxylic acid (12) as a probe for cysteine oxidation (Fig. 4B)

exo-5-Norbornenecarboxylic acid (12, 30 mg, 0.22 mmol) was added to a vial and suspended in D_{2}O (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 H_{2}O, 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 D_{2}O (0.4 mL). The solution of 9 was then dropped by pipette over 1 min at room temperature to the solution of 12. The reaction was stirred for 30 min and then analysed by NMR. Full consumption of the alkene signal (δ = 6.10 ppm) was observed. This reaction was repeated using H_{2}O in place of D_{2}O 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 min.

4.4. Dimedone as a probe for cysteine oxidation (Fig. 5A)

Two solutions were first prepared. Solution 1: In a vial, a mixture of dimedone (35 mg, 0.25 mmol), D_{2}O (2 mL) and Na_{2}CO_{3} (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), D_{2}O (3.2 mL), and Na_{2}CO_{3} (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 H_{2}O_{2} (20 μL, 30 wt% in H_{2}O). After a few seconds of stirring, solution 2 (0.4 mL, 0.16 mmol 9) was dropped over 1 min. The solution was stirred for 20 min before analysing directly by NMR. Only unreacted dimedone and disulfide 10 were observed by ^1H NMR. This reaction was repeated using H_{2}O in place of D_{2}O and analysed by LC-MS. Disulfide 10 was detected at 2.61 min and unreacted dimedone at 7.34 min. 15 was not detected.

4.5. Cyclooctyne 6 as a probe for cysteine oxidation (Fig. 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 D_{2}O (1.6 mL) and Na_{2}CO_{3} (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), D_{2}O (2 mL) and Na_{2}CO_{3} (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 H_{2}O_{2} (20 μL, 30 wt% in H_{2}O). After stirring for a few seconds, solution 2 (0.4 mL, 0.074 mmol) 9 was added dropwise over 1 min. The solution was stirred for 20 min after analysing directly by NMR. Only unreacted 6 and disulfide 10 were observed. This reaction was repeated using H_{2}O in place of D_{2}O and analysed by LC-MS. Only disulfide 10 was detected. No sulfoxide adduct 16 was detected.

4.6. cis-5-Norbornene-endo-2,3-dicarboxylic acid (8) as a probe for cysteine oxidation at pH 5.0 (Fig. 6A)

Two solutions were first prepared. Solution 1: cis-5-norbornene-endo-2,3-dicarboxylic acid (17 mg, 0.09 mmol 8), D_{2}O (1.0 mL), and NaOH (3.8 mg, 0.09 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, pD 5.0 acetate buffer in D_{2}O (400 mM, 1.0 mL) was added and the solution was stirred. Solution 2: N-acetylcysteine (42 mg, 0.26 mmol 9), D_{2}O (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 D_{2}O (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 H_{2}O_{2} (7 μL, 30 wt% in H_{2}O). 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 min. The solution was stirred for 20 min before analysing directly by NMR. Full consumption of the alkene signal (δ = 6.25 ppm) was observed. This reaction was repeated using H_{2}O in place of D_{2}O in a pH 5.0 NaOAc buffer and analysed by LC-MS. Disulfide 10 was detected at 2.50 min and four diastereomers of 11 were detected at 2.97, 3.16, 3.39 and 4.04 min. The product of the thiol-ene side reaction was detected at 7.36 min.

4.7. Dimedone as a probe for cysteine oxidation at pH 5.0 (Fig. 6B)

Two solutions were first prepared. Solution 1: Dimedone (13 mg, 0.09 mmol), D_{2}O (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 D_{2}O (400 mM, 1.0 mL) was added and the solution was stirred. Solution 2: N-acetylcysteine (42 mg, 0.26 mmol), D_{2}O (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 D_{2}O (400 mM, 1.0 mL) was added and the solution was stirred. For the key reaction, solution 1 (0.5 mL, 0.02 mmol) 9 was added to a vial followed by H_{2}O_{2} (7 μL, 30 wt% in H_{2}O) and stirred for a few minutes before dropwise addition of solution 2 (0.5 mL, 0.06 mmol 9) over 1 min. The solution was stirred for 20 min 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 H_{2}O in place of D_{2}O in a pH 5.0 NaOAc buffer and analysed by LC-MS. Disulfide 10 was detected at 2.51 min and unreacted dimedone at 7.37 min. 15 was not detected.

4.8. cis-5-Norbornene-endo-2,3-dicarboxylic acid (8) as a probe for cysteine oxidation at pH 7.4 (Fig. 7A)

Two solutions were first prepared. Solution 1: cis-5-norbornene-endo-2,3-dicarboxylic acid (20.0 mg, 0.11 mmol 8), D_{2}O (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 D_{2}O, 1.25 mL) was added and the solution was stirred. Solution 2: N-acetylcysteine (49.8 mg, 0.30 mmol 9), D_{2}O (1.25 mL) and NaOH (3 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, additional pD 7.4 sodium phosphate buffer (400 mM in D_{2}O, 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 H_{2}O_{2} (7 μL, 30 wt% in H_{2}O). The solution was stirred for a few minutes before adding solution 2 (0.5 mL, 0.06 mmol 9) dropwise over 1 min. The solution was stirred for 20 min before analysing directly by NMR. The only products observed were unreacted 8 and disulfide 10. 11 was detected.
was not detected.

4.9. Oxidation of N-acetylcyesteine to its disulfide using hydrogen peroxide at pH 5.0 (Fig. 7B)

N-acetylcyesteine (9.8 mg, 0.06 mmol), D₂Ο (0.25 mL) and NaOH (2.4 mg, 0.06 mmol) were added to a vial and stirred or shaken until fully dissolved. Next, pH 5.0 sodium acetate buffer (400 mM in D₂Ο, 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.

4.10. Synthesis of alkyne 19 (Fig. 8A)

1-aminoo-3-butene (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 min, over which time a white precipitate formed. The resulting mixture was transferred into a centrifuge tube and pelleted by centrifugation for 10 min. 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 (v_max, ATR): 3359, 2987, 1716, 1622, 1531, 1267, 1229, 1074, 846, 759, 679, 625; ¹H NMR (600 MHz, DMSO-d₆): 4.12, 4.11, 2.91 (4H, contains CH₂CH₂), 1.58–1.50 (2H, contains COCH₂H₂CH₂CH₂ and COCH₂), 1.45 (1H, CH₂CH₂H₂), 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.

4.13. 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-aminoo-4-butyne (125 µL, 1.53 mmol) and DIPEA (650 µL, 3.73 mmol) were then added successively. After 1 h 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; Rf(40% EtOAc in hexane) 0.47; IR (v_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, CONH–CH₂), 2.93 (2H, m, CH=CH–CH₂), 2.42 (2H, t, J = 6.1, 2.1 Hz, CONH–CH₂), 2.01 (2H, contains CH=CH–ciclopentadienone), 1.72 (1H, d, J = 8.1 Hz, CH=CHCH₂), 1.35 (2H, contains CH=CH–ciclopentadienone, CH=CH–CH₂), 13C NMR (150 MHz, CDCl₃); δ = 175.7, 173.3, 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. C₁₂H₂₅N₂O requires 190.1226.


1-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-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%), Rf (10% MeOH in DCM with 1% NEt₃) 0.20; IR (v_max, ATR): 3294, 3057, 2936, 1645, 1544, 1428, 1351, 1247, 1105, 905, 808, 724 cm⁻¹; ¹H NMR (600 MHz, CDCl₃); δ = 6.29 (1H, br-s, CONH), 6.13 (1H, d, 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 × CH₂–PEG), 3.56 (4H, dt, J = 18.5, 5.1 Hz, 2 × CH₂–PEG), 3.47 (2H, q, J = 5.2 Hz, CONH–CH₂), 2.91 (4H, contains CH₂–PEG and CH=CH–CH₂), 2.03 (1H, m, CH=CHCONH), 1.91 (TH, d, J = 11.5, 4.0 Hz, CH=CH–CH₂H₂), 1.72 (1H, d, J = 8.3 Hz, CH=CHCH₂H₂), 1.31 (2H, contains CH=CH–CH₂H₂H₂ and CH=CH–CH₂H), 13C NMR (150 MHz, CDCl₃); δ = 175.8, 138.2, 136.0, 70.2, 70.7, 70.0, 47.2, 46.3, 44.6, 41.6, 39.3, 30.5; HRMS (ESI): [M+H⁺]; found 269.1867. C₁₂H₂₅N₂O requires 269.1865.

4.15. Biotin NHS ester 26

Biotin (498 mg, 2 mmol) was dissolved in anhydrous DMF (10 mL) by heating to approximately 70 °C for 10 min 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'-dicyclohexylcarbodiimide). The precipitate was removed by filtration and washed with DMF. The filtrate was diluted with Et₂O until a white precipitate formed. The precipitate was collected by filtration and rinsed with Et₂O then dried to give
the crude product 26 as a white solid (389 mg, 57%): m.p. (decomp.) 178–190 °C; IR (Pmax, ATR): 3227, 2941, 2876, 1818, 1788, 1729, 1698, 1465, 1369, 1210, 1071, 861, 739, 656 cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆): δ = 6.60 (1H, s, NH), 6.35 (1H, s, NH), 4.30 (1H, m, NHCHNH), 4.14 (1H, m, NHCHNH), 3.10 (1H, m, SCH), 2.84–2.78 (5H, contains NOCH₂CH₃ and SCH₂H₆), 2.67 (2H, t, J = 7.7 Hz, CH₂CH₂CO₂N), 2.57 (1H, d, J = 12.4 Hz, SCH₂H₆), 1.64 (3H, contains CH₃CH₂CH₂CH₂CO₂N), 1.52–1.36 (3H, contains CH₃CH₂CH₂CH₂COON); 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.

4.16. Norbornene probe 27

Biotin–NHS 26 (382 mg, 1.12 mmol) was dissolved in anhydrous DMF (6 mL) with stirring at 50 °C for 5 min 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; Rₜ (10% MeOH in DCM) 0.32; IR (Pmax, ATR): 3291, 2942, 2869, 1703, 1639, 1464, 1309, 1248, 1214, 1129, 987, 867, 724 cm⁻¹; ¹H NMR (600 MHz, MeOD): δ = 6.14 (2H, m, CHCH=CHCH), 4.49 (1H, dd, J = 8.3, 4.5 Hz, CONHCH₂S), 4.30 (1H, dd, J = 7.9, 4.4 Hz, CONHCH₂S), 3.62 (4H, m, 2 × CH₂PEG), 3.55 (4H, m, 2 × CH₂PEG), 3.37 (4H, m, 2 × CH₂PEG), 3.20 (1H, m, SCH), 2.93 (1H, dd, J = 12.8, 5.0 Hz, CONHCH₂CH₂S), 2.87 (2H, m, CHCH=CHCH), 2.71 (1H, d, J = 12.7 Hz, CONHCH₂CH₂S), 2.22 (2H, t, J = 7.1 Hz, CH₂CONH), 2.13 (1H, m, CH=CHCH=CHCH), 1.86 (1H, dt, J = 11.7, 4.0 Hz, CH=CHCH=CHCH), 1.77–1.57 (5H, contains 2 × CH₂ Biotin and CHCH₂CH₂H), 1.44 (2H, p, J = 7.5 Hz, CH₂ Biotin), 1.31 (2H, contains CH=CHCH₂H and CHCH₂HCH), 1.30 (11C 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 (overlaps with NMR solvent peak), 47.1, 45.2, 42.8, 41.0, 40.4, 40.3, 36.7, 31.2, 29.8, 29.5, 26.8; HRMS (ESI): M+H⁺, found 495.2641. C₂₄H₃₉N₃O₅S requires 495.2636.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tet.2017.11.011.

References