Chemical tools for detecting cysteine sulfenic acid

by

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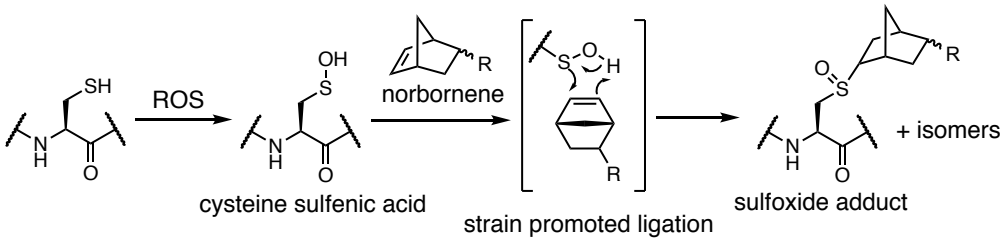
SUMMARY

The study of oxidative stress is a complex problem in biological systems. Cysteines are susceptible to oxidation by reactive oxygen species (ROS) to the cysteine sulfenic acid, which is often considered a biomarker of oxidative stress. Oxidation of cysteine residues has become recognised as a major regulatory route for protein function as well as a key player in signalling pathways. Discovering these nuances in cysteine reactivity, particularly sulfenic acids, will assist in exploring their purpose within the cellular environment. An arsenal of chemical probes for the detection of cysteine sulfenic acids is at the disposal of researchers, however issues such as chemoselectivity, reactivity, and reversibility have hindered their potential for sulfenic acid detection. This research explores the application of a new chemical probe, norbornene, to further study cysteine sulfenic acid formation in cells and address the current caveats in detection methods.

In this thesis, norbornene derivatives were found to react selectively with the sulfenic acid functional group on a small molecule cysteine model as analysed by NMR and LC-MS. This reaction proceeded with fast kinetics through a strain promoted ligation to give a stable and distinguishable sulfoxide product. Other reported cysteine sulfenic acid probes failed to trap the sulfenic acid generated in the small molecule cysteine model, highlighting the superior kinetics and reactivity of the norbornene scaffold. These results provided the foundational evidence for the continued pursuit of norbornene probes as a contender in the study of sulfenic acid formation in living cells.



Norbornene probes were able to react selectively with protein sulfenic acids in a similar manner and with broad reactivity across a range of proteins. Using both commercially available and synthesised norbornene derivatives, this demonstrated for the first time the ability of norbornene to react with sulfenic acids on purified proteins and protein mixtures. Comparison to a commonly used dimedone derivative revealed some interesting questions about its selectivity. Extending this further, norbornene probes were applied to live cell systems and found to selectively trap only the sulfenic acid modified proteins. This result provided the information needed to introduce these norbornene probes as tools to study proteome-wide cellular oxidative stress.

Application of the norbornene probe to living cells under oxidative stress revealed new protein targets of cysteine oxidation along with numerous previously identified protein sulfenic acids through proteomics analysis. Further examination of these protein hits in a future study could provide useful insight into how they are affected during oxidative stress, and how this may play a wider role in regulation or disease.

The work presented in this thesis has provided the core experimental reasoning for implementing norbornene as chemical tools to study cysteine sulfenic acid formation on the proteome-wide cellular scale. The results presented provide constructive insight for the field of cysteine redox chemistry and could lead to further discoveries into unravelling the specific roles of the identified protein sulfenic acids regarding oxidative stress.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Lisa Alcock

16 August 2019

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ABBREVIATIONS

The following is a list of non-standard abbreviations used throughout this thesis. Other abbreviations not included in this list have been included in-text for clarity.

APCI	atmospheric pressure chemical ionisation
ATR	attenuated total reflectance
Cat.	catalytic
CBZ	Benzyl chloroformate
COSY	homonuclear correlation spectroscopy
CuAAC	Copper catalysed azide-alkyne cycloaddition reaction
Cys	cysteine residue
d	days
Da	daltons
δ_c	chemical shift of carbon nuclei (ppm) for NMR
DCC	N, N'-Dicyclohexylcarbodiimide
DCE	dichloroethane
DCM	dichloromethane
δ_h	chemical shift of hydrogen nuclei (ppm) for NMR
DIPEA	diisopropyl ethylamine
DMAP	4-Dimethylaminopyridine
DMEDA	1,2-Dimethylethylenediamine
DMEM	Dulbecco's modified eagles medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DSA-ToF	direct sample analysis – time of flight mass spectroscopy
DTT	dithiothreitol
EDC.HCl	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ESI	electrospray ionisation
EtO_2	diethyl ether
EtOAc	ethyl acetate
FBS	fetal bovine serum
FTIR	fourier transform infrared spectroscopy
GSH	glutathione
h	hours
HI	heat inactivated
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlatio
HRMS	high resolution mass spectroscopy
i-Pr_2NEt	diisopropylethylamine
IAM	iodoacetamide
IR	infrared spectroscopy
J	coupling constant
LC	liquid chromatography

LRMS	low resolution mass spectrocsopy
m.p.	melting point (°C)
m/z	mass to charge ratio
*m*CPBA	meta-Chloroperoxybenzoic acid
MeCN	acetonitrile
MeOH	methanol
min	minutes
MMTS	*S*-Methyl methanethiosulfonate
MS	mass spectroscopy
MWCO	molecular weight cut off
NEM	N-ethylmaleimide
NEt_3	triethylamine
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance spectroscopy
Nu	nucleophile
oxPTM	oxidative post-translational modifications
PBS	phosphate buffered saline
pD	pH when deuterated solvents used
PEG	polyethylene glycol
pK_a	acid dissociation constant
PVDF	polyvinylidene fluoride
Py	pyridine
Q-ToF	quadrupole – time of flight mass spectroscopy
R_f	retention factor
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSS	reactive sulfur species
rt	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UHPLC	ultra-high pressure liquid chromatography
UV-Vis	ultraviolet -visible light
ν_{max}	wavenumber of maximum absorption (cm^{-1})

PUBLISHED WORK

The following list of peer-reviewed articles and conference presentations were conducted during the duration of this doctoral study.

Peer-reviewed articles related to the work presented in this thesis

1. L. J. Alcock, K. D. Farrell, M. T. Akol, G. H. Jones, M. M. Tierney, H. B. Kramer, T. L. Pukala, G. J. L. Bernardes, M. V. Perkins, J. M. Chalker, "Norbornene probes for the study of cysteine oxidation", *Tetrahedron,* **2018**, 74 (12), 1220-1228.
2. L. J. Alcock, M. V. Perkins, J. M. Chalker, "Chemical methods for mapping cysteine oxidation", *Chemical Society Reviews*, **2018**, 47 (1), 231-268.
3. L. J. Alcock, B. L. Oliveira, M. J. Deery, T. L. Pukala, M. V. Perkins, G. J. L. Bernardes, J. M. Chalker, "Norbornene probes for the detection of cysteine sulfenic acid in cells", ACS Chemical Biology, **2019**, 14 (4), 594-598.

Articles submitted for publication related to the work presented in this thesis

4. L. J. Alcock, M. Langini, K. Stühler, M. Remke, M. V. Perkins, G. J. L. Bernardes, J. M. Chalker, "Proteome-wide survey reveals norbornene is complementary to dimedone and related nucleophilic probes for cysteine sulfenic acid", submitted, **2019**. Preprint Available on ChemRxiv (DOI: 10.26434/chemrxiv.8874077.v1)

Peer-reviewed articles not related to the work presented in this thesis but published during the candidature

1. L. J. Alcock, M. D. Norris, M. V. Perkins, "Total synthesis and structural elucidation of spongosoritin A", *Organic and Biomolecular Chemistry*, **2018**, 16, 1351-1358.
2. K. R. Scroggie, L. J. Alcock, M. J. Matos, G. J. L. Bernardes, M. V. Perkins, J. M. Chalker, "A silicon-labelled amino acid suitable for late-stage fluorination and unexpected oxidative cleavage reactions in the preparation of a key intermediate in the Strecker synthesis", *Peptide Science*, **2018**, 110 (3), e24069.

Conference presentations and programs

1. L. J. Alcock, B. L. Oliveira, T. L. Pukala, M. V. Perkins, G. J. L. Bernardes, J. M. Chalker, "Chemical tools for detecting cysteine sulfenic acids in cells", JSPS (Japan Society for the Promotion of Science) 10th HOPE Meeting with Nobel Laureates, poster presentation, Yokohama, Japan (March **2018**).
2. L. J. Alcock, M. V. Perkins, and J. M. Chalker, "Chemical tools for detecting cysteine sulfenic acid", RACI (Royal Australian Chemical Institute) Adelaide Synthesis Symposium, oral presentation, Adelaide, Australia (December **2017**).
3. L. J. Alcock, B. L. Oliveira, T. L. Pukala, M. V. Perkins, G. J. L. Bernardes, J. M. Chalker,

"Chemical tools for detecting cysteine sulfenic acids in cells", SciFinder (American Chemical Society) Participant of SciFinder Future leaders Program, poster presentation, Columbus, Ohio (August **2017**).

4. L. J. Alcock, M. V. Perkins, and J. M. Chalker, "Chemical tools for detecting cysteine sulfenic acids in proteins", RACI (Royal Australian Chemical Institute) Centenary Congress, poster presentation organic chemistry #404, Melbourne, Australia (July **2017**).
5. L. J. Alcock and M. V. Perkins, "Total synthesis and structural elucidation of spongosoritin A", RACI (Royal Australian Chemical Institute) Adelaide Synthesis Symposium, oral presentation, Adelaide, Australia (December **2016**).
6. L. J. Alcock, M. V. Perkins, and J. M. Chalker, "Chemical tools for detecting cysteine sulfenic acids in proteins", RACI (Royal Australian Chemical Institute) Medicinal Chemistry and Chemical Biology Meeting, poster presentation, Coogee Beach Sydney, Australia (November **2016**).

1. DETECTING CYSTEINE SULFENIC ACIDS AND THEIR BIOLOGICAL ROLE

1.1 Overview

Cysteine redox chemistry is a broad and important area of chemical biology given its involvement in numerous cellular pathways. The amino acid residue cysteine plays many functional roles in proteins given both its diverse reactivity and its ability to be oxidised by reactive oxygen species (ROS). Not only do cysteines serve functional roles in proteins, they also serve regulatory roles in many signalling pathways. Since cysteine residues are sensitive to changes in the redox balance (ROS levels) of the cell, they are greatly affected during episodes of oxidative stress. In this way, the study and detection of cysteine oxidation is vital for understanding the broader impacts of oxidative stress on cellular function and disease and remains a key area of interest for researchers from several related fields.

In this Chapter, a comprehensive overview of the literature concerning cysteine sulfenic acids, their detection, and biological role will be discussed. Current chemical methods for mapping cysteine sulfenic acids will be explored to address the successes and pitfalls of current detection methods with selected examples of what biochemistry was learned in these studies. Finally, the future challenges and opportunities will be discussed in relation to the relevance of the research to be discussed in this thesis for developing a new cysteine sulfenic acid selective chemical probe.

Portions of this Chapter have been adapted from a review article titled 'Chemical methods for mapping cysteine oxidation' as published in *Chemical Society Reviews*, The Royal Society of Chemistry on 15th December 2017 (DOI: 10.1039/C7CS00607A). Only relevant sections of the above-mentioned review article are included in this Chapter for clarity and have been reformatted to align with the Chapter. All literature was researched, reviewed, and prepared by the candidate unless otherwise stated in-text and was listed as the primary author of the manuscript.

1.2 Oxidative stress and disease

A prevalent area of study for the biological and medical community are the impacts of oxidative stress on cellular function and survival. Oxidative stress has been associated with many diseases such as heart disease,[1-2] diabetes,[3-4] neurodegenerative diseases, [5-6] and cancer[7-8] as well as the ageing process.[9-10] Oxidative stress is classically described as the imbalance between the build-up of ROS and the ability of the cell to remove reactive species or repair the resulting damage. When the cells antioxidant defence mechanisms against ROS are exhausted, the cell is under oxidative stress. ROS can impart damage to most biological macromolecules with eventual cell death if left unchecked. Several sources of ROS include the mitochondrial electron transport chain, where $O_2^{\bullet -}$

is generated,[11] and enzymatic generation of $O_2^{\bullet -}$ by various oxidases.[12] As such, generation of ROS occurs under normal metabolic conditions. It is the regulation and removal of these species that can be problematic. ROS are known to act as secondary messengers in redox signalling pathways.[13] Small increases in ROS act as signalling molecules that induce biological responses through reversible oxidative post-translational modifications (oxPTMs) of biomolecules. Too large an increase in ROS results in oxidative stress and may construe these signalling pathways and result in damage leading to an array of complications.[14-15] Covalent modification of proteins represents a major molecular mechanism for oxidant signalling and relaying a biological response caused by ROS. Cysteine residues are a key target of these oxidative post-translational modifications due to their broad range of reactivities which will be discussed below. Putting this in context, the study and detection of cysteine oxidation is therefore a critical component of the general study of oxidative stress as a whole. In this way, the generation of ROS, its regulation, signalling, and the effect it has on proteins and other macromolecules within the cell is crucial to understanding the impacts of oxidative stress and its wider involvement in diseases.[16-18]

1.3 Overview of cysteine chemistry

The amino acid cysteine (**1**) exhibits diverse redox chemistry in proteins. For instance, the thiol group of the cysteine side chain is subject to a variety of oxPTMs and can be converted to *S*-nitrosothiols **2**, sulfenic acids **3**, sulfinic acids **4**, sulfonic acids **5**, sulfenamides **6**, persulfides **7**, and various disulfides (**8-9**) including intramolecular disulfide bridges and intermolecular disulfides with small molecules such as glutathione (**9**) (Fig. 1). Each oxidation state of cysteine exhibits different reactivity which, in turn, may determine function. The formation of each of these oxPTMs is often in response to various stimuli within cells, such as the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS).[19] Since cysteine can react with these species and adopt a variety of oxidation states, cysteine residues in proteins are key regulators of redox homeostasis and signalling.[20] Interestingly, only certain cysteine residues are susceptible to oxidation due to variations in their protein microenvironment such as solvent accessibility, pK_a, and polarity of neighbouring residues. The type of oxidant (ROS, RNS, RSS) may also influence which cysteine residue is modified. Understanding these issues of selectivity is important for understanding the biological roles of cysteine oxidation.[21-23]

Cysteine is found in relatively low abundance in proteins (~2 % of all residues are cysteine),[24] but its versatile chemistry makes it a critical determinant of protein structure and function. The cysteine sulfur atom can adopt a variety of oxidation states (oxidation numbers can range from –2 to +6), with diverse chemistry observed across this series.[25] For example, the cysteine thiol can behave as a potent nucleophile or reducing agent, while its corresponding disulfide might behave as an electrophile or oxidising agent. The sulfur of cysteine can also exist as a thiyl radical, opening entirely

different modes of reactivity. The specific reactivity of each cysteine thiol is governed by its microenvironment in the protein, with its pK_a and redox potential influenced by the local polarity and interactions with neighbouring residues. For example, most thiols have a pK_a around 8-9 making them almost fully protonated at physiological pH and therefore less susceptible to oxidation or reaction with electrophiles in those scenarios. However, the pK_a of protein thiols can vary dramatically, and values ranging from 2.5 to 12 have been reported.[26-29] This diverse chemistry and array of oxidation states (and the ability to interconvert between several of these oxidation states *in vivo*) is one of the reasons cysteine was selected by nature for diverse roles in cellular redox regulation and signalling.

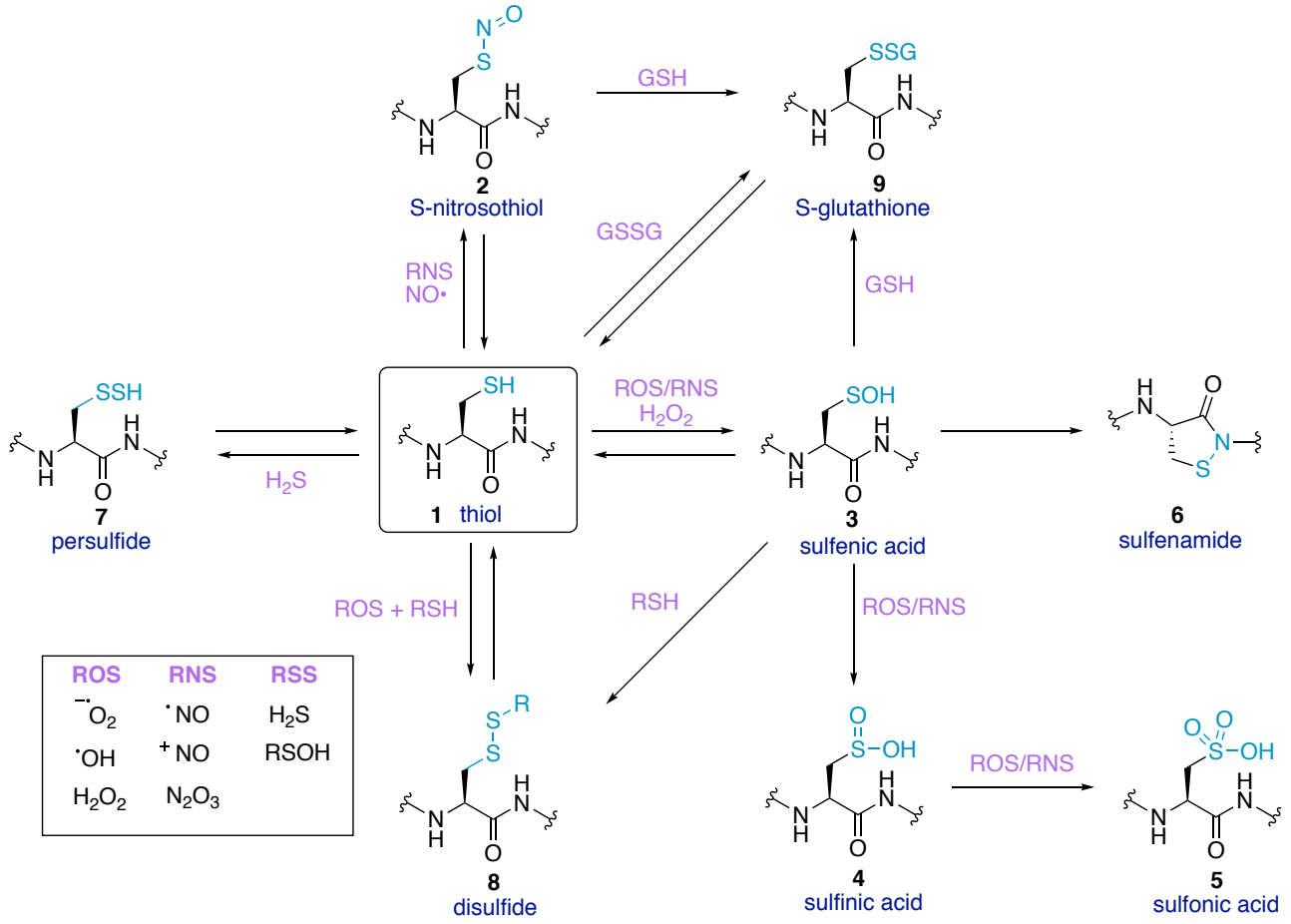


Figure 1. A selection of biologically relevant oxidative post-translational modifications of cysteine.

1.4 Mapping the function of cysteine

Within a given protein, not all cysteine residues are equal. Due to subtle changes in the protein microenvironment, different cysteine residues will exhibit unique reactivity, including the susceptibility towards oxidation by reactive oxygen, nitrogen, and sulfur species. In many cases, these differences in reactivity may be the basis for cysteine redox signalling. Therefore, it is imperative to understand the impact of these subtle differences by locating oxidisable cysteine

residues in proteins. The selectivity for oxidation of particular cysteines is not well understood or easily predicted, although several studies have looked at factors that may influence selectivity.[18, 30-32]

Understanding the type and level of oxidant is also important in cysteine redox chemistry. Some cysteine oxPTMs occur at the basal levels of oxidants, suggesting that oxidative modification of certain cysteines is expected, perhaps playing an essential regulating role in healthy cells. These oxidative modifications increase in number and type, however, during oxidative stress. In such events, an influx of ROS, RNS, and/or RSS may lead to cysteine modifications that are harmful to the cell. Understanding the different oxPTM profiles that occur in healthy and diseased states in humans is one reason why profiling cysteine chemistry is important, but redox regulation is important in a variety of species and often conserved. The most biologically relevant cysteine oxidation states are shown in Figure 1. Many of these cysteine oxidation states have been implicated in some form of redox-based regulation in proteins.[33-35] Several modes of cysteine redox regulation have been identified that influence signal transduction, often by way of structural changes that influence enzymatic activity. For example, protein tyrosine phosphatase activity,[21, 36] epidermal growth factor receptor (EGFR) signalling,[37-38] and thioredoxin activity[39-40] are all influenced by cysteine oxidation. It is also important to note that oxPTMs at cysteine can be deactivating (e.g. reduced structural stability and activity of *M. tuberculosis* tyrosine phosphatase A upon *S*-nitrosylation of a non-catalytic cysteine),[41] activating (e.g oxidative activation of OxyR transcription factor in *E. coli* by disulfide formation),[42-43] or part of a normal catalytic cycle (e.g the active site of thioredoxins features interconversion of thiols and disulfides in the active site).[39, 44-45]

1.5 Dual roles of reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Reactive oxygen and reactive nitrogen species such as hydrogen peroxide, superoxide, nitric oxide, and peroxynitrite[20] have been viewed traditionally as destructive oxidants that compromise the structure and activity of proteins and genetic material. However, while elevated levels of ROS and RNS may be associated with disease, it is becoming increasingly apparent that these species also act as second messengers in healthy cells. For example, hydrogen peroxide has been shown to have a proliferative effect on cells and can promote wound healing.[34] There are many sources of ROS and RNS within cells. For example, production of H_2O_2 occurs in the mitochondria during the electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produces superoxide in the neutrophil-based defence against bacteria, and the production of nitric oxide by nitric oxide synthase is also critical as a defence mechanism.[23, 34, 46] Since these ROS and RNS are produced as by-products of normal metabolic function, it is not surprising that they might have gained a functional role in signalling.

These reactive species can be categorised into two main types, radical and non-radical, and this reactivity profile may influence which cysteines are oxidised for a given protein. For non-radical or two electron reactions, cysteine thiolates may act as nucleophiles, as in the case of the direct reaction of the thiolate with hydrogen peroxide to form a sulfenic acid. The rate and site of such modifications often depends on the steric hindrance and pK_a of the cysteine thiol.[23] What is important to note is that not all cysteine residues are susceptible to oxidation. Some ROS/RNS/RSS react far faster with thiolates (the deprotonated thiol), and more slowly with the thiol in its neutral, protonated form which is often the predominant form at physiological pH. This differential reactivity of ROS and RNS with cysteines is often the basis for selective redox-regulation. Another factor that can be considered is the proximity of a protein to the ROS/RNS/RSS source, where ROS/RNS/RSS can exist in concentration gradients within cellular compartments based on their production. Therefore, proteins in closer proximity to ROS/RNS/RSS sources through various inter-protein interactions are more likely to become oxidised.

Antioxidant molecules are also present in the intracellular environment that contribute to maintenance of the overall redox homeostasis. These antioxidants include glutathione, superoxide dismutase, peroxiredoxins, thioredoxins, and glutaredoxins.[13] These antioxidant molecules assist in redox-regulation presumably as a defence against overoxidation or as a way to restore pre-oxidation function, making them active players in redox signalling.

Overall, the important message here is that cysteine oxidation is integral to signalling in healthy cells, but also a hallmark of diseases associated with oxidative stress. Understanding the delicate balance between these two seemingly opposing functions motivates the development of tools that help detect the proteins involved in these processes.

1.6 Detecting oxidative modifications at cysteine: challenges and limitations

Since it has been established that oxidation of certain protein thiols is involved in redox-regulation events, it is important to develop strategies for detecting the level and site of these oxidations *in vivo*. This is not a simple task. In order to detect specific oxidation states of cysteine, chemical probes must react selectively (or better yet, specifically) with the oxPTM of interest and not any other oxidation state. Furthermore, the probe must be compatible with the hundreds of other reactive groups within a protein and cellular environment. Most cysteine oxidation states are themselves reactive and may react further to higher oxidation states. In cases where a modification of interest is short-lived, the probe must react rapidly.

When testing a chemical probe for a particular oxidation state of cysteine in cells, it is critical to use conditions relevant to the cellular redox environment. For instance, subjecting cells to extreme oxidative stress could result in false positives or overoxidation of key sites that are not normally

present in these states. Another important consideration is the source of the ROS/RNS/RSS used in experiments. Since not all cysteines can be oxidised equally by different ROS/RNS/RSS, this should be taken into consideration. Additionally, it is critical to consider if cell lysis is required, as this process will immediately change the redox environment of key proteins and perhaps lead to a different outcome than if the probe acted inside the cell. Finally, many of the oxPTMs of cysteine react similarly with common reagents (sulfenic acids, nitrosothiols and disulfides can all react with strong reducing agents, for instance) so it is critical to evaluate the outcomes of mapping experiments with these considerations in mind. In the following sections, a discussion of sulfenic acids and factors that contribute to their stabilisation as well as specific chemical methods for their detection will be highlighted from the current literature. The many challenges in selectivity and reactivity will be discussed where specific issues have been identified in the literature.

1.7 Sulfenic acids (RSOH)

Sulfenic acids (**3**) have garnered increasing attention since Allison and Benitez trapped the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with dimedone (**19**) and various alkenes, thereby inhibiting the enzyme.[47-48] Poole and Claiborne also provided evidence for a stabilised cysteine sulfenic acid in the redox-centre of the streptococcal NADH peroxidase,[49] and Denu and Tanner revealed the reversible inactivation of protein tyrosine phosphatases by cysteine sulfenic acid formation several years later.[36] Together these studies indicated that cysteine sulfenic acid is not merely an intermediate oxidation state of cysteine (though it can be) but rather it is a key regulator of protein function, catalysis and signalling. Protein sulfenic acids are also intriguing because they are often an early oxidation product of the reaction of cysteine with ROS and RNS. For this reason, cysteine sulfenic acids may also be a biomarker for oxidative stress and play a key role in redox-regulation. Commonly, sulfenic acids are formed in proteins through oxidation of the thiolate side chain of cysteine by H_2O_2 (Fig. 2A). Acidic cysteine residues are particularly reactive, as the nucleophilic thiolate (RS^-) can attack H_2O_2 directly via an S_N2 reaction.[50] The pK_a of cysteine residues is therefore an important factor in assessing its susceptibility to oxidation. Most protein thiols have a pK_a of 8-9, making them largely protonated at physiological pH and hence less reactive.[32] However, the pK_a is not the only determinant and the protein microenvironment (varying in polarity, charge and steric congestion) can modulate reactivity. For example, the rate constant determined for the reaction of the active site cysteine of peroxiredoxin 2 with H_2O_2 was 1.3×10^7 $M^{-1}s^{-1}$ (pK_a 5-6),[51] compared to tyrosine phosphatases (PTPs) which was only 10-20 $M^{-1}s^{-1}$ (pK_a 5.4).[36]

H_2O_2 has been described as both a second messenger and a toxic by-product of metabolic processes. The mitochondrial electron transport chain[52] and NADPH oxidases[53] generate superoxide as a by-product of oxygen reduction. Superoxide ($O_2^{\bullet-}$) can undergo dismutation to H_2O_2 and O_2 with a rate constant of $\approx 10^5$ $M^{-1}s^{-1}$, or when catalysed by superoxide dismutase, $\approx 10^9$ $M^{-1}s^{-}$

[1].[54-55] Other ROS and RNS can form sulfenic acids by reaction with thiolates such as organic hydroperoxides (ROOH),[56] peroxynitrous acid (ONOOH), and the peroxynitrite anion ($ONOO^-$) (Fig. 2A).[57] Comparative rate constants for the oxidation of cysteine and BSA by H_2O_2 and $ONOO^-$ showed the latter to be a stronger oxidant at pH 7.4. The reported rate constants for $ONOO^-$ oxidation of cysteine and BSA were 5.9×10^3 and 2.7×10^3 $M^{-1}s^{-1}$ respectively, with H_2O_2 oxidation proceeding with rate constants of 4.69 and 1.14 $M^{-1}s^{-1}$ respectively.[57] It was also found that cysteine can react with $ONOO^-$ as either the thiol or thiolate.

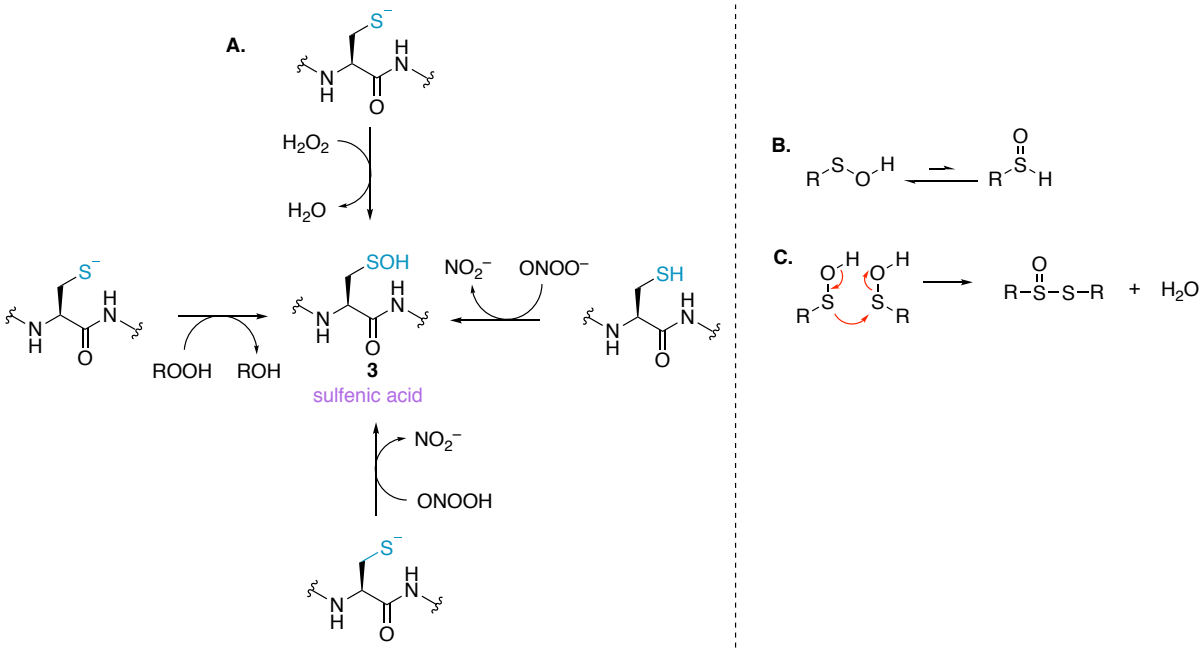


Figure 2. A. The formation of cysteine sulfenic acids in proteins. B. Tautomeric forms of sulfenic acids. C. Self-condensation of sulfenic acid to form a thiosulfinate.

Sulfenic acids can exist in two tautomeric forms, with the proton bound to either the oxygen or the sulfur (Fig. 2B). Early spectroscopic work by Bruice showed that the tautomer with the proton bound to the oxygen is favoured almost exclusively, likely due to the stronger O-H bond versus the weaker S-H bond.[58] The sulfur atom in sulfenic acids exhibits both electrophilic and weak nucleophilic character. This is evident in the self-condensation of sulfenic acids to form thiosulfinates (Fig. 2C). While this frequently occurs in small molecule sulfenic acids, the occurrence of thiosulfinates in proteins has not been reported. Due to its reactive nature, sulfenic acids are a challenge to detect and isolate and the mechanisms by which they are trapped are often unclear.[18, 45, 59]

An important factor for formation of cysteine sulfenic acids is the initial sulfenic acid stabilisation within the protein microenvironment. In some cases, an assisting microenvironment is essential for oxidation of thiols.[60] A suggested major factor in protein sulfenic acid stabilisation is the structural geometry where the sulfenic acid is embedded in a cavity shielding it from other reactive groups.[45] The sulfenylated cysteine can be stabilised by electrostatic interactions and H-bonding or protected

through reversible reaction (disulfide or sulfenamide formation), preventing over-oxidation to higher oxidation states that are irreversible. So far, no enzyme has been described which has general sulfinic or sulfonic acid reductase properties, although very recently a cysteine sulfinic acid reductase was found to have more than 55 possible targets.[60-62] Various forms of sulfenic acid stabilisation shown through small molecule examples will be discussed in the following section.

Sulfenic acids can also react with carbon nucleophiles such as enolates displaying its electrophilic behavior.[26] Another important reaction of sulfenic acids is the concerted addition to both alkenes and alkynes forming sulfoxides.[45] The electrophilic nature of the sulfenic acid group leads to one of the most important biological reactions, condensation with a nearby protein thiol forming disulfide linkages.[63-64] Formation of these disulfide bridges can modulate protein structure and function, protect against irreversible inactivation, modify structures to create, destroy or modulate functional sites and ultimately regulate enzymatic function.[46, 64] Disulfide bonds can be reversed through cellular reductants (eg. glutathione and thioredoxin). The sulfur atom in a sulfenic acid can also react with a nearby nitrogen of an amide forming a cyclic sulfenamide serving as a protective role by preventing over-oxidation to sulfinic or sulfonic acids.[26] The sulfenic acid modification has been observed in more than 30 protein crystal structures, but no direct estimate for the number of sulfenic acid modified proteins in cells has been made.[63]

Cysteine sulfenic acids have been suggested to play key roles in the immune response, stem cell development, pathogenesis of cancers, neurodegeneration, and growth factor signalling.[65] Sulfenic acids are involved in the protein tyrosine phosphatase (PTP) signal transduction pathways,[36] activation and function of T-cells (cell growth, calcium flux, proliferation and phosphorylation),[66] redox-switching to regulate kinase activity (formation of the sulfenic acid represses or enhances certain kinase activity),[67-68] and regulation of intracellular signalling of the EGFR by oxidation of a key cysteine residue.[37] With such ranging function attributed to this intriguing oxidation state of cysteine, it is critical to understand its incidence and mechanism in biology.

1.8 Small molecule Sulfenic acids (RSOH)

1.8.1 Sulfenic acids stabilised by H-bonding

Since the first direct evidence for the involvement of sulfenic acids as transient intermediates in the oxidation of thiols noted by Davis in 1981,[69] sulfenic acids have gained increasing interest as tools for understanding cysteine redox chemistry. Over the last hundred years, only a few stabilised sulfenic acids have been isolated and characterised to date, highlighting the difficulty associated with synthesising this class of compounds attributed to their instability.[70-71] Little is known about the physiochemical properties of this functional group, largely due to the difficulty of preparation, isolation and purification of sulfenic acids.[72]

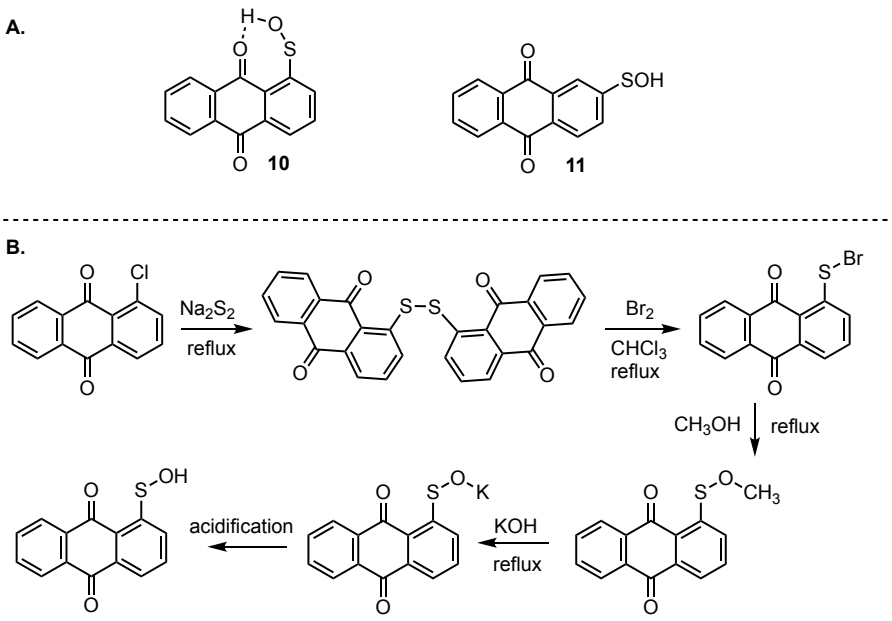


Figure 3. A. Structure of Fries acid **10**. Structure of 2-anthraquinonesulfenic acid **11** which could not be isolated. B. Synthesis of Fries acid.

The first reported stable sulfenic acid was that synthesised by Fries[73] in 1912, named 'Fries acid' **10** (Fig. 3). The stability of this arenesulfenic acid has been attributed to the hydrogen bonding present, postulated by Kharasch[74] in 1946 upon the observation that earlier attempts by Fries and Schurmann[75] to isolate the 2-anthraquinonesulfenic acid **11** had failed, suggesting some special structural feature of **10** resulted in reduced likelihood of conversion to the thiosulfinate, sulfinic acid or other products.

Attempts by Fries to obtain **10** directly from the sulfenyl bromide were unsuccessful due to formation of the disulfide when treated with aqueous sodium hydroxide.[74-75] Several other attempts by Fries and co-workers to synthesise similar sulfenic acids in the anthraquinone series did not prove as effective, with most considerably less stable than the original. However, some years later Bruice and Markiw[76] successfully synthesised anthraquinone-1,4-disulfenic acid in a similar manner to **10**.

In order to test the hypothesis of Fries acid stability being attributed to H-bonding, Kharasch and Bruice[77] attempted to synthesise 1-fluorenone sulfenic acid, but were unable to isolate the compound and instead mostly reported formation of the disulfide. Based on this observation, they concluded that the stability of Fries acid could not be explained on the basis of expected slight differences in H-bonding stabilisation between Fries acid and the 1-fluorenone sulfenic acid. Lecher[78] later postulated that a more probable explanation for the stability of Fries acid would be conversion to the phenolic lactone, a resonance structure not possible for the 1-fluorenone sulfenic acid. Barltrop and Morgan[79] however found this structure not in agreement with the IR spectra of Fries acid which clearly showed carbonyl as well as hydroxyl absorption. Sometime later, Bruice and Sayigh[58] were able to confirm the original structural assignment of Fries to be correct based on agreeance with the spectral data.[79-

[80] Of interest is the lack of other spectral data available for Fries acid, including NMR and X-ray crystallography. This is most likely due to its instability in solution and hence difficulty with purification.

Another sulfenic acid prepared more than 50 years after the anthraquinone series was the first pyrimidine sulfenic acid **12**, synthesised and characterised by Cohn in 1969[81] (Fig. 4A). The stability of this sulfenic acid was also postulated to be due to H-bonding, with three different tautomeric forms possible (Fig. 4B).[81] A similar sulfenic acid **13** synthesised by Heckel[82] in 1983 adopted similar stabilisation through hydrogen bonding with the adjacent ring nitrogen, also existing in three tautomeric forms (Fig. 4C-D).

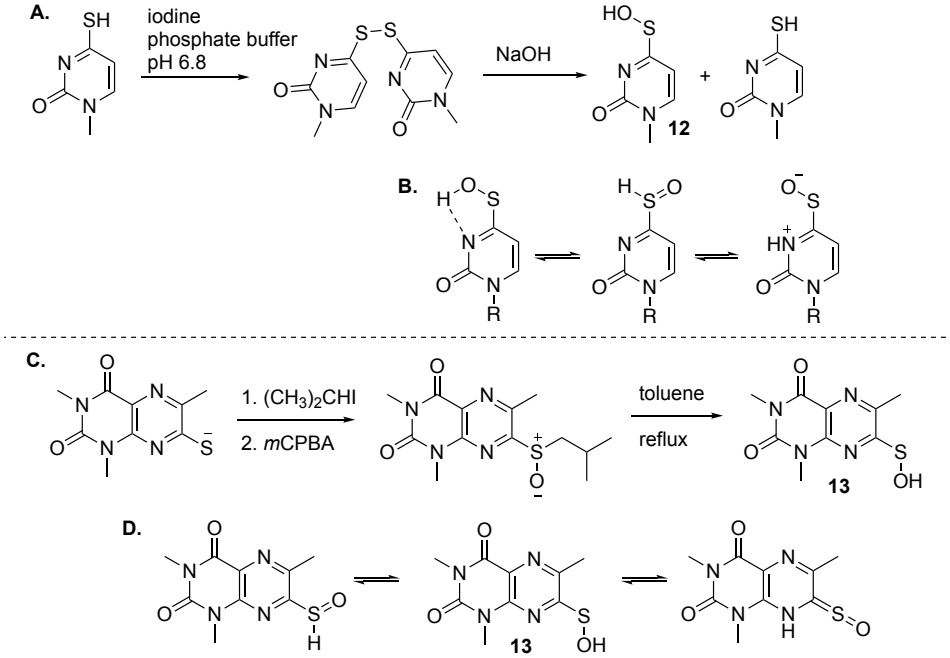


Figure 4. A. Synthesis of pyrimidine sulfenic acid **12** and B. tautomers of **12**. C. Synthesis of lumazine sulfenic acid **13** and D. the tautomers of **13**.

While stabilisation through hydrogen bonding proved to be an efficient method, typical sulfenic acid reactions could still occur over time such as condensation and sulfinic acid formation, but provides insight into potential stabilisation routes for sulfenic acids.

1.8.2 Sulfenic acids stabilised by sterics

A different type of stable sulfenic acid **14** was synthesised by Nakamura[83] in 1983 using the steric nature of the triptycene skeleton, which essentially prevents dimerisation and has been used in studies looking at reaction of sulfenic acids with peroxy radicals (Fig. 5A).[84] Using a similar idea, Yoshimura[85] synthesised a stable trans-9-decalin sulfenic acid **15**, where it was proposed that the

four axial protons would offer steric hindrance and prevent dimerisation, representing the first stabilised sulfenic acid without the aid of unsaturated bonds or heteroatoms (Fig. 5B).

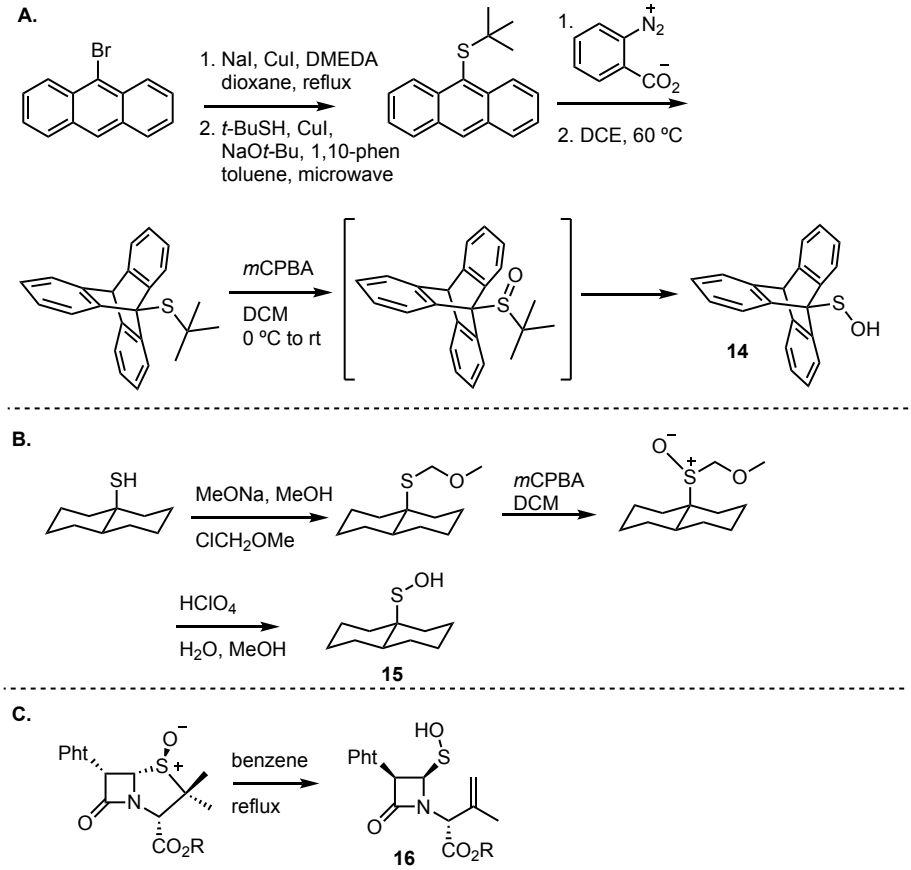


Figure 5. A. Synthesis of triptycene sulfenic acid **14**. B. Synthesis of trans-9-decalin sulfenic acid **15**. C. Synthesis of azetidinone sulfenic acid **16**.

A more recent (1998) somewhat stable sulfenic acid are those of the azetidinone variety. Schofield[86] demonstrated the ease with which the (2S,5S,6S)-penam sulfoxides can form stable sulfenic acids **16** (Fig. 5C). The stability can be attributed to both the steric bulk of the modified azetidinone moiety which prevents dimerisation, as well as the relative thermodynamic instability of the substrates, and may also have internal H-bonding.

1.8.3 Sulfenic acids as models

Of the synthesised stable sulfenic acids, several have been used as models for the sulfenic acid functional group to test chemical probes. However, using these small molecule models as a substitute for protein sulfenic acids has some immediate drawbacks. Firstly, most have limited solubility with aqueous physiological conditions and hence would not be a good representation of the conditions of a sulfenic acid group in a protein environment. Secondly, due to their enhanced

stability, the sulfenic acid group may not be as readily reactive with chemical probes compared to highly reactive short-lived cysteine sulfenic acids that the probes are designed to detect in proteins.

Other transient sulfenic acid models have been used to study sulfenic acid formation in proteins such as a study by Gates and co-workers using a small organic model to characterise the redox regulation of protein tyrosine phosphatase 1B.[87] The ortho substituted β-sulfinyl propionic acid ester on a benzamide derivative **17** was used for *in situ* sulfenic acid generation in aqueous buffer solution (Fig. 6A). However, the overall reaction is extremely slow (~36 h) limiting its utility.

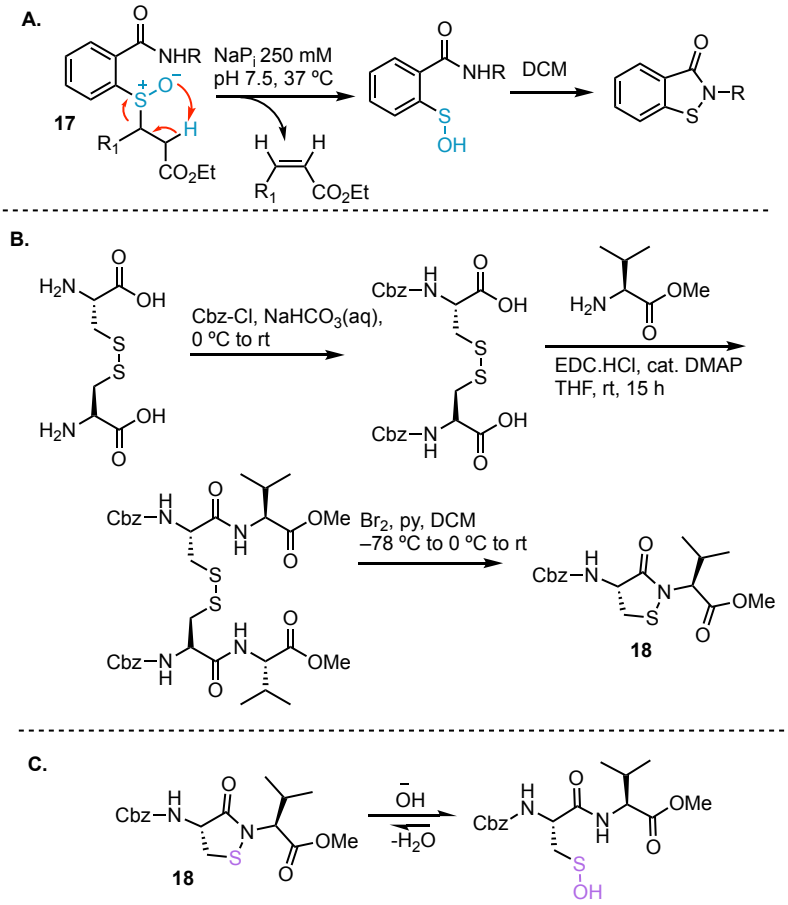


Figure 6. A. Formation of sulfenic acid from benzamide derivative **17**. B. Synthesis of a sulfenamide model **18** and C. the equilibrium of the sulfenamide **18** to the cysteine derived sulfenic acid under aqueous conditions.

Very recently (2016), the Carroll[88] group devised a dipeptide based cyclic sulfenamide model **18** for use with comparing the relative reactivity of various sulfenic acid probes (Fig. 6B). The model is advantageous as it presents a dipeptide (cysteine) structural core similar to those experienced in proteins, unlike the other synthetic sulfenic acids described above. Secondly, the reaction is able to be performed at physiological pH, mimicking the environment of proteins in cells. The sulfenamide model is expected to exist in equilibrium with the corresponding sulfenic acid under aqueous

conditions, allowing the sulfenic acid to be liberated and react with the probes where relative reactivity can be compared (Fig. 6C).

These few examples of stabilised sulfenic acid highlights both the difficulty of preparation of such a species for study, but also emphasises the key features that may allow these transient species to be stabilised or prolonged. Although stable enough for analysis, most of these stabilised sulfenic acids still have relatively short half-lives especially in solution. The same concepts such as stabilisation by H-bonding or sterics should be applicable to stabilisation of sulfenic acid in proteins, and thus these considerations may help to understand and interpret the occurrence of sulfenic acid formation in proteins. Several other examples of stabilised and transient small molecule sulfenic acids have been reported and utilised for study of this unique functional group.[45, 89]

1.9 Protein sulfenic acids (RSOH)

Because cysteine sulfenic acids are most often the first oxidation product formed during oxidative stress events, they likely play an intermediate role in downstream redox signalling. Because of its potential role in diverse pathways, the oxidation of cysteine to its sulfenic acid is sometimes compared to (and also linked to) universal signalling mechanisms such as protein phosphorylation. For example, protein tyrosine phosphatases (PTP), a family of about 80 proteins, are inactivated by oxidation of the cysteine to its sulfenic acid.[36, 90] This reversible oxidation to the sulfenic acid can promote phosphorylation-dependent signalling cascades as seen in the epidermal growth factor receptor pathway (EGFR).[38] The resultant sulfenic acid may also react with a backbone amide to form a cyclic sulfenamide,[44] or react with an adjacent thiol to form an intramolecular disulfide.[91] This reversible conversion prevents overoxidation to the sulfinic and sulfonic acids.

Using A431 cell lines, Carroll and co-workers investigated epidermal growth factor (EGF)-induced protein sulfenylaton.[38] Binding of epidermal growth factor (EGF) to the epidermal growth factor receptor (EGFR) induces the production of H_2O_2 through the enzyme Nox2. The H_2O_2 generated can directly oxidise the EGFR Cys-797 to the sulfenic acid, which enhances the tyrosine kinase activity up to 5-fold. H_2O_2 may also oxidise and deactivate PTPs, which regulate the level of EGFR phosphorylation.[37] Together these events lead to an increase in receptor autophosphorylation which promotes downstream signalling pathways related to proliferation, differentiation, growth, migration and survival. Using a histochemical approach, Carroll and Seo found that EGFR was overexpressed in Her2 breast cancer cell lines that exhibited increased levels of H_2O_2 and protein sulfenylation.[92] More recently, further work on EGFR from A431 cell lines has confirmed the Cys-797 as a functional redox switch that stimulates EGFR autophosphorylation.[93] Using computational modelling, oxidation of Cys-797 to the sulfenic acid may influence the catalytic loop through stabilisation, stimulating kinase activity.

The monoacylglycerol lipase (MGL) is a serine hydrolase that can deactivate the endocannabinoid 2-arachidonoyl-*sn*-glycerol (2-AG). Two regulatory cysteines Cys-201 and Cys-208 control entry of substrates into the active site. Studies by Piomelli and co-workers explored the effect of H_2O_2 induced oxidation of these two cysteine residues using site-directed mutagenesis and dimedone **19** (a chemoselective probe for cysteine sulfenic acids).[94] They found that H_2O_2 inhibited the enzyme MGL through an allosteric mechanism involving the two redox-sensitive cysteines Cys-201 and Cys-208. This in turn regulates 2-AG-mediated signalling in neurons, which may serve as a presynaptic control point.

Recent work by the Toledao and Rahuel-Clermont groups have also demonstrated the intricate ways in which sulfenic acid signalling can be mediated. [95] In this study it was shown that a key cysteine sulfenic acid residue in the peroxidase Orp1 is protected through binding to the Yap1-binding protein (Ybp1). These two proteins then form a ternary complex with Yap1. This ternary complex leads to a directed and rate-enhanced formation of disulfide on Yap1 through reaction with the Orp1 cysteine sulfenic acid. Without Ybp1, this process is slow and unselective. These results indicate that Yap1-binding protein operates as a sulfenic acid chaperone and facilitates this redox signalling.

Another protein, Src kinase, was found to be regulated by redox-dependent mechanisms at Cys-185 and Cys-277 using various chemical probes.[96] Src kinase are tyrosine kinases involved in signalling pathways that promote cell survival and motility through protein interactions and tyrosine phosphorylation. Oxidation of these residues to the sulfenic acids result in structural alterations that contribute to the activation of Src activity and may promote autophosphorylation. It is also thought that both sulfenic acids become *S*-glutathionylated which permanently obstructs refolding of Src extending the activity. These observations are particularly intriguing for their potential to develop Src-selective inhibitors for treatment of diseases where Src is activated, such as cancers.

With growing evidence for the intermediate role of sulfenic acids in diverse signalling pathways, the importance of defining these pathways has become clear. Next, detection methods and chemical tools employed to probe cysteine sulfenic acids will be discussed extensively.

1.10 Detection methods

1.10.1 Detecting the sulfenic acid functional group

Due to the lack of spectroscopic information obtainable from a sulfenic acid group, it can be rather difficult to detect, especially in protein environments and cells where thousands of other components are present. Detection of sulfenic acids is usually difficult due to its high reactivity and instability, often further oxidising making analysis using tools such as mass spectrometry difficult, without considering the additional oxidation products and adducts that could form during the analysis.[97] Analysis with NMR is also challenging to differentiate between sulfenic, sulfonic and sulfinic acids,

as the chemical shifts are often too similar to unambiguously assign, especially when the effects of the protein microenvironment are taken into account.[97]

Detection by chemical binding is much more effective for unknown sulfenic acids, or sulfenic acids present in mixtures. Commonly, a chemical probe is used to trap cysteine sulfenic acids followed by analysis of changes in mass to the protein by mass spectrometry.[97-98] This is particularly useful for chemical probes which do not contain a tag detectable by other means and has been implemented in a wide number of studies testing the applicability of chemical probes for use as sulfenic acid traps.[88, 99-101] However, not all sulfenic acids are equivalent in their reactivity towards oxidants or electrophiles, requiring a large subset of potential chemical probes with different reactivities as well as different modes of reaction to ensure all sulfenic acids are captured.

For studying protein oxidation in cells, mapping of the cysteine proteome can occur through indirect or direct methods. Indirect methods generally involve conversion of the modification of interest into a detectible group, such as one labelled with biotin or a fluorophore. These methods often require masking the reactivity of all other cysteines (for instance by alkylation), followed by selective reduction and labelling of the cysteine bearing the oxPTM of interest. In contrast, direct methods for detecting oxPTMs feature chemistry that is highly selective for the modification of interest, ligating the reporter group directly to the modified cysteine (and no other residues). Direct methods are attractive because, in principle, they can be applied to living cells without the need for tricky reduction and alkylation steps which would disrupt normal cellular function and processes.

1.10.2 General detection methods

Addition of detectable tags such as fluorophores has allowed for the use of UV-Vis and fluorescence as a method for monitoring binding of chemical probes to sulfenic acids. Typically, changes in the fluorescent/visible properties are observed when the probe binds to the sulfenic acid, allowing binding to be monitored and determination of reaction rates possible.[99,102-105] However, due to the large size, these kinds of chemical probes are not always suitable for cell analysis or have reduced reactivity.

Affinity tools are also useful in detection of sulfenic acids. A common example is biotin, a water-soluble B-vitamin. Biotin is known to bind very strongly with streptavidin, one of the strongest non-covalent interactions in nature. Streptavidin, a bacterial protein, can be conjugated to inexpensive fluorophores or enzymes allowing the probe containing the biotin to be detected easily, with amplification of the signal possible (Fig. 7).[103, 106]

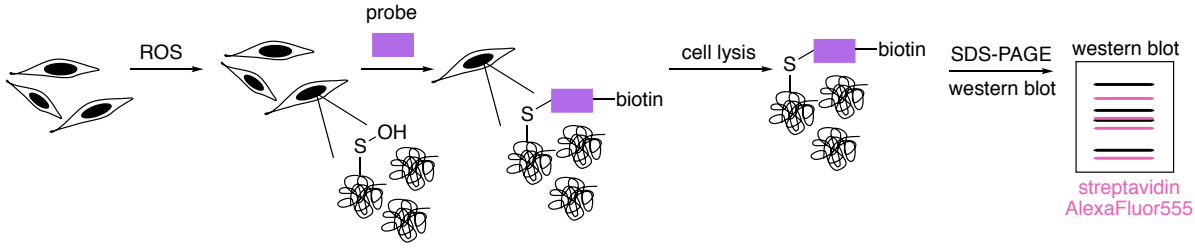


Figure 7. Process for detecting biotinylated probes using western blot techniques.

Another form of identification utilises click chemistry, such as azide or alkyne handles which can be conjugated *ex vivo* for identification. These tools have proven very useful for global studies, as their small size allows ready uptake into cells with less hindrance for binding. Probes such as **33** and **34** (Fig. 9) have utilised this technique, whereby the protein is incubated with the azide tagged probe then labelled with phosphine-biotin via the Staudinger ligation reaction, then analysed by western blot (Fig. 8).[67, 100, 107] In a similar manner, the alkynyl chemical handle can be used as a small chemical probe and analysed by western blot through a Cu-catalysed azide-alkyne cycloaddition reaction with the corresponding biotin (Fig. 8).[38, 101] Both of these techniques have proven useful in cells for global analysis of protein sulfenic acid formation due to increased cell permeability and small size of the probe, coupled with the ability to then detect these probes via *ex vivo* modification. This facilitates live cell studies but does not compromise on reactivity or detectability.[101]

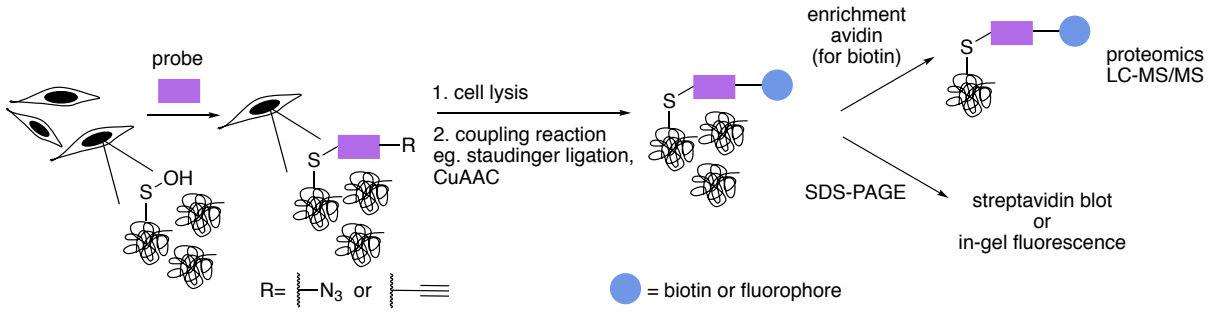


Figure 8. Process of detecting sulfenic acid sites in cells using the azide or alkyne handle, followed by cell lysis and coupling reactions to add detectable tags to the probes, and analysis with LC-MS/MS or western blot.[18]

1.11 Chemical methods for detecting RSOH

Locating sites of cysteine oxidation is important for understanding these functions and meeting this goal is highly challenging. For instance, many early studies used exogenous sources of oxidants or performed experiments with purified proteins which may not fully represent the redox process *in vivo*. Many cysteine sulfenic acid residues are thought to be short-lived species. Therefore, prolonged manipulation may lead to degradation of the sulfenic acid (by over oxidation or reaction with a

nucleophile, for instance). Therefore, direct and rapid methods are required to detect this oxidation state of cysteine presenting a significant challenge in chemical biology. This next section will discuss the current state of chemical methods for detecting sulfenic acids that are available and highlight their advantages and disadvantages, with examples of their use to study biological systems.

1.11.1 Modified Biotin Switch Technique for detecting cysteine sulfenic acids

A method similar to the biotin switch technique initially developed for *S*-nitrosothiols[108] was used to detect sulfenic acids, exploiting the selective reduction of the sulfenic acid by arsenite (Fig. 10A).[109] Free thiols are first blocked with maleimide in the presence of sodium dodecyl sulfate (SDS), followed by reduction of SOH by sodium arsenite in the presence of biotin-maleimide. However, several limitations restrict the overall efficiency of this indirect approach, particularly the long time-scale over which short-lived sulfenic acids will not persist. The method is also performed under denaturing conditions, which could compromise sulfenic acids that rely on the protein microenvironment for stability. The selectivity of the reduction by sodium arsenate has also been debated and because arsenic is toxic, the disruption to normal cellular function must also be considered. Nevertheless, this study provided motivation for further study of cysteine sulfenylation.

Since a key component of the biotin switch technique is subsequent reaction or prior blocking of the reduced thiols with detectable reagents, several recent studies have explored the potential cross-reactivity of many thiol-reactive reagents and their use in various strategies. Reactivity of electrophilic thiol-blocking reagents such as iodoacetamide (IAM), *N*-ethylmaleimide (NEM), and *S*-methyl methanethiosulfonate (MMTS) have been reported with sulfenic and sulfinic acids in addition to thiols, with MMTS also reacting with persulfides and nitrosothiols.[110-112] This presents many concerns for this detection strategy where cross-reactivity of the initial thiol blocking reagent may be reacting with the oxidation state of interest, preventing detection and an under-representation.

1.11.2 1,3-Diketone chemical probes for cysteine sulfenic acids

Detection of the sulfenic acid modification has become increasingly more versatile due to a large set of chemical probes that contain an enolisable 1,3-diketone which can react with sulfenic acids directly. The most commonly used sulfenic acid trap is dimedone **19** (5,5-dimethyl-1,3-cyclohexanedione) and its derivatives. The reaction of dimedone with cysteine sulfenic acids was first discovered by Allison during studies of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that paved the way for the majority of current sulfenic acid detection methods.[47] The exact mechanism for the reaction of **19** with sulfenic acids has not been established but several hypotheses have been advanced to account for the formation of the putative adduct **22** including

hydrogen-bond assisted substitution (via the enol **20**) or direct substitution (via the enolate **21**) (Fig. 9A). Other mechanisms in which dimedone acts as an electrophile have also been proposed.[26]

Because dimedone does not contain a detectable handle, many derivatives have been synthesised that contain fluorophores, affinity probes, or functionalisable groups such as azides and alkynes (Fig. 9). King *et al.* first attached fluorescent labels in the form of isatoic acid derivative **23** and 7-methoxy coumarin derivative **24** (Fig. 9B).[99] Testing these probes on a cysteine-dependent peroxidase enzyme from *Salmonella typhimurium*, small changes in the excitation and emission wavelength maxima were observed upon protein sulfenic acid adduct formation. But, due to bleaching of the fluorophore, monitoring the protein reaction was technically difficult. King and co-workers then disclosed a new library of dimedone probes containing fluorescein (**25**-**26**), rhodamine (**27**-**28**) and biotin (**29**-**31**).[103] Charles *et al.* also disclosed a similar biotin probe (**32**) to study oxidation in rat hearts (Fig. 9C).[106] Both **28** and **29** were further utilised to study sulfenic acid formation during lysophosphatidic acid-mediated cell signalling.[113] The probe **29** has been used in several recent studies focussed on different proteins such as, endoplasmic reticulum (ER) transmembrane protein IRE-1,[114] ERK-1/2 kinase,[68] and c-Jun N-terminal kinase 2 (JNK2).[115]

While these probes offered a convenient means for detection through fluorescence analysis or affinity enrichment using the biotin tag, the steric bulk of the probes might limit the detection of more hindered cysteine sulfenic acids. Smaller probes were therefore prepared to address this potential issue. Accordingly, azide and alkyne handles were explored as they can be imaged through azide-alkyne cycloadditions and Staudinger ligation reactions that install a biotin group or fluorophore after the dimedone derivative has trapped the sulfenic acid.[116] Using this strategy, Carroll *et al.* devised a dimedone probe DAz-1 (**33**) containing an azide chemical handle (Fig. 9D).[107, 117] Using this probe, a new method for detecting sulfenic acids directly in un-manipulated, intact cells was achieved using Jurkat cells as a model. This study also revealed the important differences between labelling cell lysates and live cells, with significantly more oxidation occurring during the lysis process. However, **33** was less reactive relative to dimedone (**19**), and the amide linkage was thought to reduce cell permeability and accessibility making the probe non-optimal for global proteomic studies. Improving upon this probe, Carroll *et al.* synthesised derivative DAz-2 (**34**) which lead to increased labelling *in vitro* and in live cell studies, providing the first global analysis of the sulfenome (Fig. 10B).[100, 118] These studies on tumour cell lines validated DAz-2 as a general sulfenic acid probe demonstrating membrane permeability and reaction with cysteine sulfenic acids in cells. Importantly, **34** was not toxic and did not appear to trigger oxidative stress or cell death.

In a related approach using an alkyne handle on dimedone, probes DYn-1 (**35**) and DYn-2 (**36**) were found to offer superior sensitivity (Fig. 9D).[38] Detection of sulfenic acids was also dependent on probe dose and incubation time with no loss of cell viability and redox-balance was maintained for cells treated with **36**. This probe was integral in the discovery that a key cysteine residue in epidermal growth factor receptor, when oxidised to its sulfenic acid, enhances the kinase activity of the protein.

Development of this direct detection method revealed the molecular details of at least one way in which hydrogen peroxide can act as a signalling molecule,[119] and how oxidation of cysteine can be regarded as a method of signalling akin to phosphorylation.

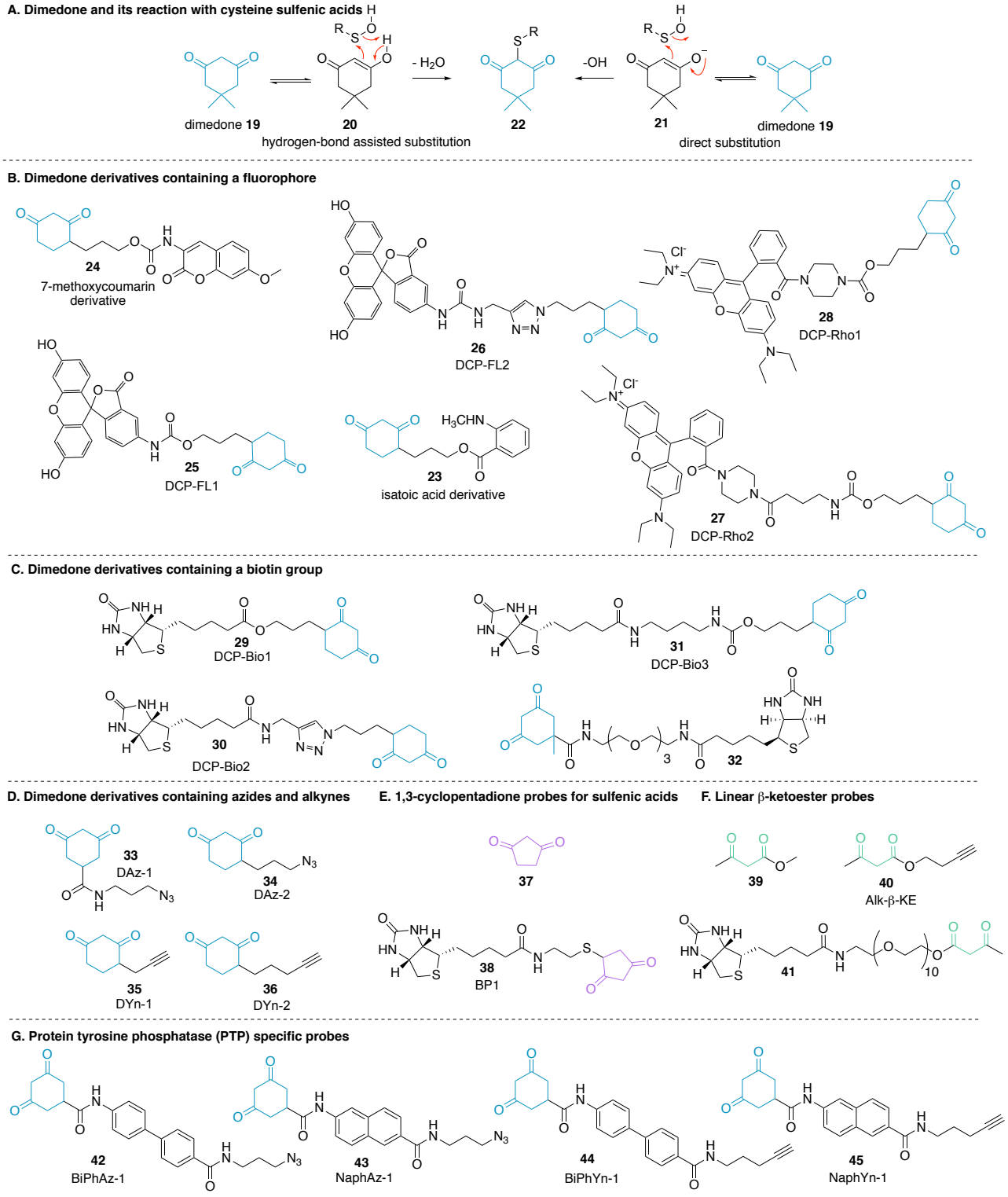


Figure 9. Sulfenic acid probes containing 1,3-diketone functionalities with various detectable tags.

Furdui *et al.* developed 1,3-cyclopentadione sulfenic acid probes (**37** and **38**, Fig. 9E). These probes exhibited reasonable reactivity however some pH-dependence was seen, where lowering the pH

increased reactivity (suggesting the enol form as the reactive species).[120] Additionally, linear β-ketoesters (**39**-**41**) were also shown to trap sulfenic acids with improved reactivity at physiological pH (Fig. 9F).[121] The β-ketoester is cell permeable and does not cause cell death. An advantage of **40** and **41** is their ability to react with NH_2OH, cleaving the ester and removing the affinity tag, ultimately generating a 3-methyl-5-isoxazolone at the key cysteine, a modification readily integrated with mass spectrometry. However, these probes still possess modest reaction rates, a key issue with most sulfenic acid probes to date.

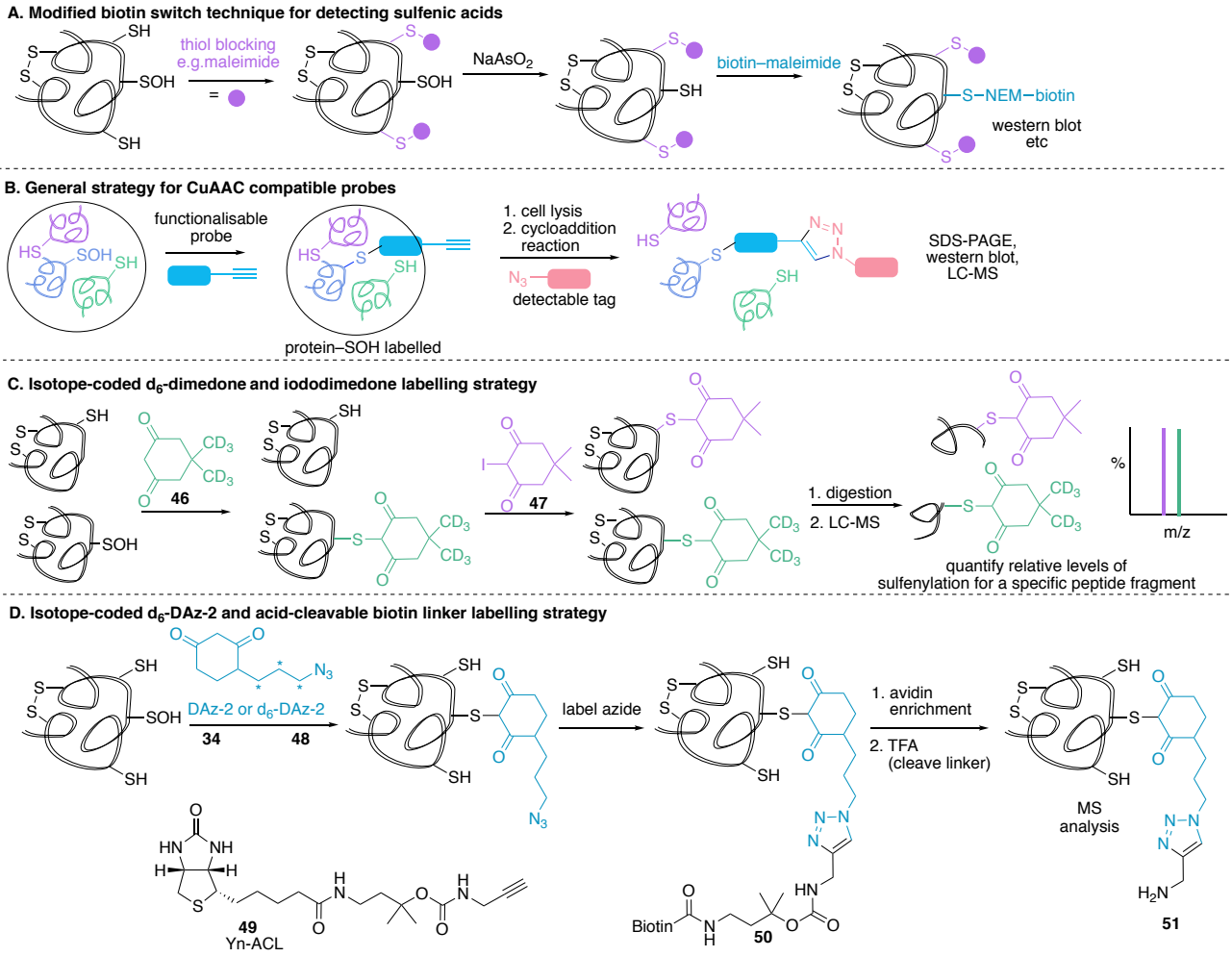


Figure 10. A. Modified biotin switch technique for sulfenic acid detection. Free thiols are blocked, then remaining SOH reduced with arsenate to the thiol and detected with biotin-maleimide. B. General strategy for using alkyne-labelled sulfenic acid probes. After ligation to the sulfenic acid, probes are visualised through the Cu-catalysed azide-alkyne cycloaddition reaction which can attach a fluorophore or affinity tag. C. Isotope labelling method using d_6-dimedone (**46**). Sulfenic acids are labelled with **46** then thiols are labelled with iododimedone (**47**). Proteolytic digestion and mass spectrometric analysis can reveal the ratio of thiol and sulfenic acid for a specific cysteine. D. DAz-2 (**34**) (and its isotopically labelled derivative, **48**) can trap sulfenic acids and then be isolated after conjugation to a biotin affinity tag. Cleavage of the biotin tag facilitates analysis by mass spectrometry.

In order to further study protein tyrosine phosphatases (PTPs) specifically, Carroll and co-workers designed several probes (**42**-**43**) for direct detection of PTP oxidation (Fig. 9G).[122] Oxidative inhibition of PTPs have been shown to promote phosphorylation-dependent signalling cascades, but low cellular abundance has hindered further investigation. The trifunctional probes consisted of a cyclic 1,3-diketone group to form the covalent adduct with the PTP sulfenic acids, a module that directs binding to target the PTP (such as naphthalene and biphenyl), and a reporter tag used for detection. Compounds containing the binding module showed increase in labelling for PTPs compared to DAz-1 **33**. Related probes were also prepared so that they contained an alkyne handle (**44**-**45**). The key cycloaddition reaction used to visualise the alkyne appeared to be enhanced in comparison to the azide analogue.[123] The study examined protein tyrosine phosphatase 1B (PTP1B) which is a negative regulator in the insulin signalling pathway, which acts by dephosphorylating tyrosine residues on the insulin receptor. Probes **44** and **45** were able to bind with oxidised and inactivated PTP, which prolonged signalling by preventing this dephosphorylation.

Based on the cysteine-specific acid-cleavable isotope-coded affinity tag (ICAT) approach used to quantify oxidant-sensitive thiols,[124-125] Carroll *et al.* developed a similar dimedone-based strategy for detection and quantification of sulfenic acids in proteins.[126] The isotope-coded dimedone (**46**) and iododimedone (**47**) were used to quantify the extent of sulfenic acid modification for a given residue (Fig. 10C). In the event, sulfenic acids are first labelled with **46** and then free thiols are labelled with **47**. The protein samples are then digested and analysed by LC-MS. The extent of cysteine oxidation for a specific site on a protein is then determined by the relative peak intensities of the heavy and light dimedone labels in the mass spectrum. Using isotope-coded dimedone/iododimedone-labelling strategies, other proteins such as the heme-thiolate cysteine (Cys-457) of cytochrome P450 4A11 was found to be selectively oxidised to the sulfenic acid, leading to loss of activity.[127]

A complementary strategy couples an isotope-coded DAz-2 derivative **48** with an acid-cleavable biotinylated tag **49**.[128] In this method, protein sulfenic acids are trapped by **34** or **48** and then labelled with Yn-ACL **49** to give the triazole **50** (Fig. 10D). Biotinylated proteins are enriched on avidin beads and then cleaved from biotin by treatment with acid to give **51**. The advantage of this method is that after enrichment of the proteins of interest, the biotin label can be removed. This is important as biotin can, in some circumstances, complicate protein MS analysis. Additionally, the isotope pair **34** and **48** can be used to monitor relative changes in protein oxidation. DYn-2 **36** has also been used in development of proteomic methods for global identification and quantitation of sulfenic acid modified sites using light and heavy DYn-2 derivatives.[129]

1.11.3 Alternative chemical probes

Other chemical probes for detection of cysteine sulfenic acids that are complementary to dimedone (**19**) have been explored. The reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, **52**) was

studied by Poole *et al*. as an electrophilic sulfenic acid trap, which is complementary to the nucleophilic dimedone (Fig. 11A). **52** had previously been shown to react with thiols, the phenolic group of tyrosine, and amino groups under basic conditions, which can present challenges with selectivity. However, each product had a unique absorbance maximum (420, 382 and 480 nm respectively) which can provide a way to potentially distinguish products.[102] **52** was also found to react with sulfenic acids with a characteristic absorbance maximum at 347 nm allowing it to be distinguished spectroscopically from other adducts such as **55**. Importantly, the reaction of **52** with cysteine gives a product with a different mass than when it reacts with cysteine sulfenic acid. With that said, there is some uncertainty as to whether sulfoxide **53** or sulfenate ester **54** (or both) are formed when **52** reacts with a sulfenic acid. In any event, **52** has been used in several studies including the reversible inactivation of protein tyrosine phosphatases,[36] OhrR,[56] and the Orp1 protein.[130]

Benkovic *et al.* explored boronic acids as sulfenic acid traps (Fig. 11B).[104] Arylboronic acids and cyclic benzoxaboroles such as **56** can reversibly form adducts such as **57** with sulfenic acids under aqueous conditions. The benzoxaborole **56** was the most effective probe for reversibly binding to sulfenic acids. While this may not be an effective sulfenic acid trap for proteomics analysis, boronic acids may be useful for reversible inhibition of proteins that rely on cysteine sulfenic acid formation for function.

The main limitation of most sulfenic acid probes is the slow rate of reaction, possibly preventing the detection of shorter-lived sulfenic acids. As a consequence, there are likely protein sulfenic acids that remain undiscovered. Sulfenic acids are known to react with alkenes and alkynes via a concerted mechanism to give sulfoxide adducts.[47-48] Early work by Allison found that the olefins 3-cyclohexene-1-carboxylate, dihydropyran and tetrahydrophthalimide inactivated GAPDH, presumably by covalent inhibition due to the ligation of the sulfenic acid and the alkene. King *et al.* revisited this concept in their study of strained alkenes and alkynes and their reaction with cysteine sulfenic acids. The key advance was to increase the rate of reaction by virtue of strain relief during the reaction.[105] Using bicyclo[6.1.0]nonyne derivatives (**58**), they demonstrated rapid reaction with small molecule sulfenic acids as well as sulfenic acids on proteins. The sulfoxide adduct **59** was formed with reaction rates up to 2 orders of magnitude greater than dimedone (Fig. 11C). The strained alkene *trans*-cyclooctene (**60**) was also tested and verified to form sulfoxide **61** after reaction with sulfenic acids, though the rate was less than that of the cyclooctyne (Fig. 11D). Other variations to **58** have been implemented to contain a biotin tag **62**[105] and also a triphenylphosphonium (TPP) ion **63** to direct the probe towards the mitochondria for targeted labelling.[131] Other dimedone based probes have also been employed for mitochondrial targeting.[132] Biscyclooctyne **64** has also been explored recently in mapping sulfenic acids. After the sulfenic acid reacts with one alkyne in **64**, the other alkyne can be labelled through a cycloaddition with an azide (Fig. 11E).[133]

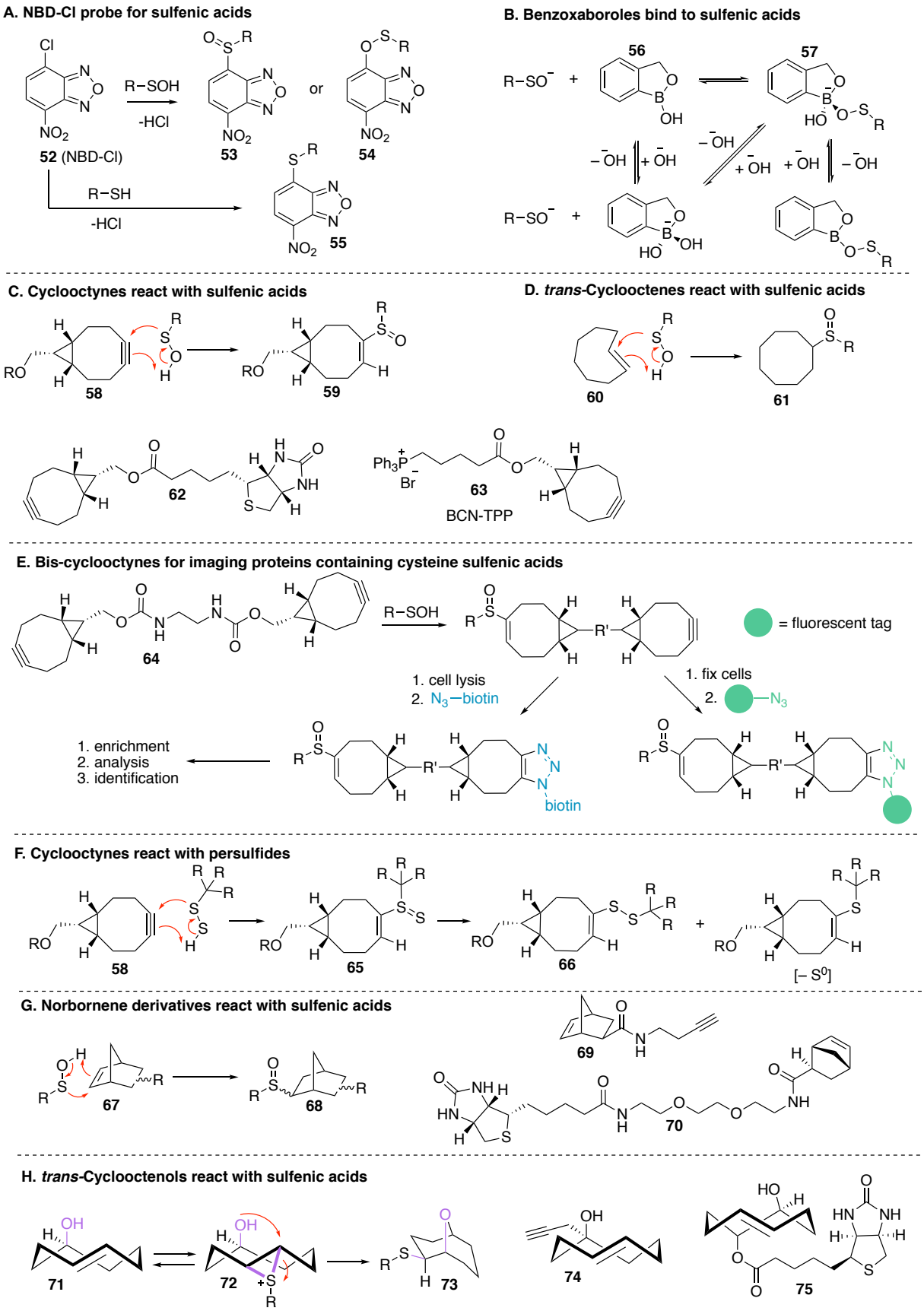


Figure 11. A. NBD-Cl (**52**) is an electrophile that reacts with cysteine sulfenic acid. B. Boronic acids and benzoxaboroles such as **56** bind reversibly to sulfenic acids. C. and D. Strained alkynes and alkenes react with sulfenic acids. E. A biscyclooctyne probe **64** can react with protein sulfenic acids.

The second alkyne can be used to ligate a reporter group for affinity purification or imaging. F. In addition to sulfenic acids, strained alkynes also react with persulfides. G. Norbornene derivatives react with short-lived sulfenic acids. Two norbornene probes for cysteine sulfenic acid detection are shown, one labelled with an alkyne (**69**) and the other with biotin (**70**). This work will be discussed in this thesis. H. *Trans*-cyclooctenol **71** reacts with sulfenic acids via a transannular thioetherification.

While cyclooctynes have been shown to react rapidly with cysteine sulfenic acids, this highly strained system can also react with other groups such as thiols and persulfides. For example **58** reacts rapidly with synthetic persulfides, another functional group that may have biological importance (Fig. 11F).[134] The reaction of **58** with persulfides proceeds analogously to the reaction with sulfenic acids, providing adduct **65**, which can undergo subsequent rearrangement to disulfide **66** or the corresponding thioether after extrusion of sulfur (Fig. 11F). The thiol-yne reaction is another well-documented reaction between an alkyne and thiyl radical that should be considered when using cyclooctynes in a biological context. Indeed the direct reaction of strained alkynes and cysteines has been demonstrated for peptides and proteins in live cells,[135] which prompts an issue of caution when using cyclooctynes to map sulfenic acids. In such cases, clear mass spectrometric analysis and hit validation is required to eliminate such off-target reactions.

Our group has recently explored other strained alkenes as cysteine sulfenic acid traps and will be discussed in this thesis.[136] It was demonstrated that easily accessible norbornene probes (**67**) could trap the short-lived sulfenic acid formed by oxidation of *N*-acetylcysteine with H_2O_2 through a strain-promoted ligation mechanism to form sulfoxide adduct **68** (Fig. 11G). This work was inspired by the early work of Allison and Benitez using water soluble cyclohexene derivatives to trap cysteine sulfenic acids and later Barton and co-workers who specifically used norbornene derivatives to trap the sulfenic acid formed during thermally induced *syn*-elimination of the sulfoxides of penicillin.[47, 137-139] The strain of the norbornene was sufficient to lead to rapid reaction with the unstable sulfenic acid. Dimedone was far less reactive and unable to trap the same short-lived cysteine sulfenic acid. The use of moderately strained alkenes, as opposed to highly strained alkynes, was designed to help overcome off-target reactions such as direct reaction with the cysteine thiol. Some thiol-ene chemistry was still observed with norbornene, but the rapid reaction with short-lived sulfenic acids is a promising and complementary feature to the chemistry of dimedone. The preparation of the probes **69** and **70** in Figure 11G allowed application of norbornene probes to study sulfenic acid formation in proteins and living cells. Both probes **69** and **70** labelled a broad range of proteins in cell lysates with apparent better selectivity for the sulfenic acid when compared to probe **29**.[140] Probe **70** was also applicable to live cell analysis, providing a new means for live cell sulfenic acid detection.

Other use of strained alkenes as sulfenic acid traps has very recently been reported through use of *trans*-cyclooctenol's such as **71**,[141] similar to the probe **60** employed by King and coworkers.[105] Although containing a strained alkene, it is postulated that the reaction occurs via a transannular

thioetherification whereby the TCO (**71**) rapidly forms thiiranium ion intermediates **72** with the sulfenic acid before irreversibly capturing the thiiranium ion via an intramolecular thioetherification from the hydroxyl nucleophile positioned for attack to give thioether product **73**. This represents a new mechanism for capture of sulfenic acids. Control reactions in which the hydroxyl group was omitted or positioned unfavourably resulted in no or reduced reaction. Two functionalised probes **74**, with an alkyne, and **75**, with a biotin tag were also synthesised and applied to cell lysates, live cell studies, and proteomics analysis. Additionally, it was shown that the reactivity of the sulfenic acid probe could be quenched by addition of a tetrazine to undergo bioorthogonal reaction with the strained alkene of **71**. This was suggested as a way to quench the probe before cell lysis, which is known to induce oxidation and may introduce false positives. This bioorthogonal quenching could be applied to other strained probes such as **58** or **67** to employ the same temporal control and prevent false positives during lysis-induced oxidation.

1.11.4 Other carbon-centred nucleophiles

Carroll *et al.* recently conducted a wide ranging study on various carbon-centred nucleophiles (inspired by dimedone) to improve their rate of reaction with sulfenic acids (Fig. 12).[88] Using pseudo first-order conditions with a dipeptide-based sulfenic acid model **18** (Fig. 12A), rate constants were compared across a wide range of potential probes. It was found that increasing the ring size from a 6 to 7-membered ring such as **76** increased the rate of reaction slightly (Fig. 12B). Rate enhancements relative to dimedone were also seen for linear 1,3-diketone **77**. Substitution at the C-4 or C-5 position of 6-membered ring 1,3-carbonyls (**78**) resulted in relatively small effects, but *N*-alkyl analogues such as **79** proved remarkably reactive with a 100-fold increase in reaction rate relative to dimedone. Other heteroatom substitutions such as **80** and **81** also resulted in increased rates in comparison to dimedone. Replacement of one of the carbonyls with a sulfonamide moiety (**82** and **83**) gave dramatic rate enhancements up to 2000-fold greater than dimedone. The increased nucleophilicity of the anion derived from deprotonation of **82** and **83** is thought to arise from the half-boat conformation of the heterocyclic ring that decreases resonance stabilisation, making the anion more reactive.[142]

A range of sulfenic acid probes were also designed based on linear *C*-nucleophiles (Fig. 12C).[143] Rather than the 1,3-dicarbonyl groups, these probes contain the electron withdrawing sulfone and nitrile or nitro groups that render the α-carbon acidic and nucleophilic (**84**-**86**). Analogues of these probes were also prepared with the reactive α-carbon, and an alkyne handle for detection (**87**-**88**). It was also found that under reducing conditions (addition of DTT, GSH or TCEP), the thioether adduct of some linear *C*-nucleophiles such as **88** were unstable. Other comparative studies have also identified next generation probes which aim to improve the slow reaction kinetics of current probes.[144]

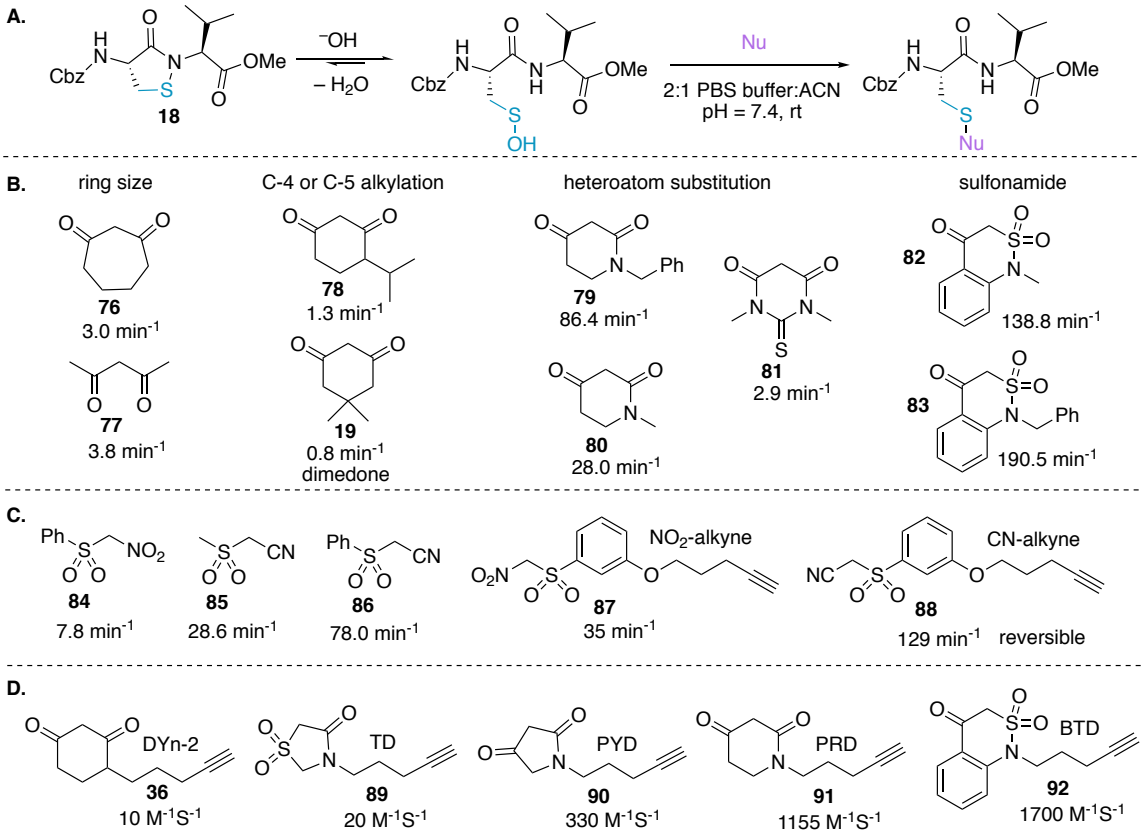


Figure 12. A. A dipeptide-based sulfenic acid model **18**. B. Various 1,3-diketones and related derivatives that react with sulfenic acid/sulfenamide probe **18** (k_{obs} shown for pseudo first-order conditions with >10-fold excess of nucleophile). C. Linear carbon-centred nucleophiles that can react with sulfenic acid/sulfenamide probe **18** (k_{obs} shown for pseudo first-order conditions with >10-fold excess of nucleophile). D. Selection of probes with the highest reaction rates with cysteine sulfenic acids (second order rate constants shown for the reaction with **18**). These five probes were used to identify cysteine sulfenic acids in RKO colon adenocarcinoma cells. Only nine of the 761 proteins identified in the screen were detected by all five nucleophilic probes.

Extending on these discoveries, Carroll *et al*. developed additional *C*-nucleophiles containing an alkyne reporter group (**89**-**92,** Fig. 12D) and compared them with **36**.[145] Interestingly, when all five probes were used to map the sulfenic acid sites in RKO cells, only nine of the 761 proteins detected were labelled by all five probes. This result suggests that each sulfenic acid has a unique reactivity and it also highlights how different probes can display complementary reactivity towards protein sulfenic acids. On one hand, this means that it might actually be challenging to generate a general probe for cysteine sulfenic acids. On the other hand, this discovery opens the opportunity for selective manipulation of proteins containing sulfenic acids which could be a useful capability in chemical biology and perhaps medicinal chemistry. Future research will clarify the parameters that match a particular probe with its protein target. The probe BTD **92** has further been detailed for proteome-wide protein sulfenic acid analysis.[146] Probes based on **92** such as **93** have been

implemented in an isotopic tagging of cysteine oxidation states in cells.[147] The method uses isotopically distinct benzothiazine and halogenated benzothiazine probes to sequentially alkylate sulfenic acids, free thiols, then after reduction, disulfides and other reducible cysteine states. This method incorporates methodology from both direct and in-direct labelling techniques quantitatively. The method termed isotopic tagging of oxidised and reduced cysteines (iTORC) was applied to mouse hepatocyte lysates to identify known cysteine oxidation states validating the method (Fig. 13).

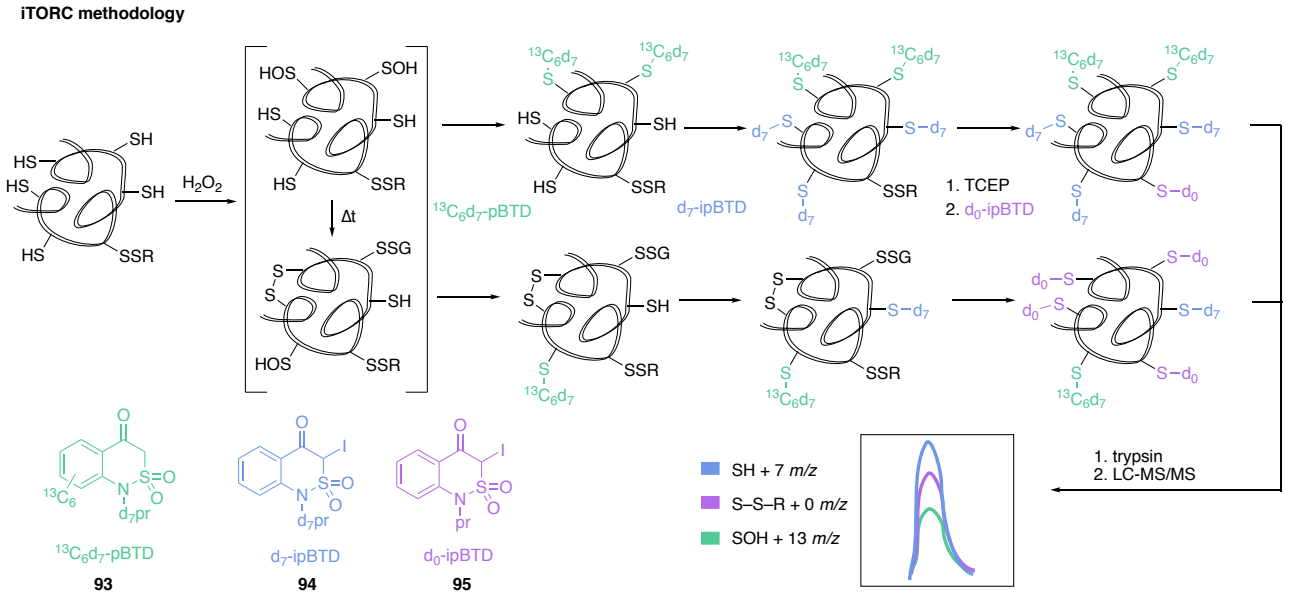


Figure 13. Methodology for iTORC labelling of cysteine oxidation states. Sulfenic acids are covalently labelled with **93**, followed by free thiol labelling with **94**. After reduction with TCEP, remaining free thiols from other reduceable states are alkylated with **95** and relative quantitation performed.

Very recently, a report on the reactivity and selectivity of dimedone (**19**) was published by Ursini *et al.* in which the authors provide evidence that dimedone can react directly with certain sulfenamides.[148] For example, dimedone was shown to cleave the S-N bond of the sulfenamide 2-methyl-4-isothiazolin-3-one and provide the dimedone adduct in which a C-S bond is formed. This result means that dimedone derivatives might, in some cases, react with sulfenamides of the type found in **18**. The product of the reaction of dimedone with the sulfenamide or the sulfenic acid is the same, which makes it challenging to distinguish whether the probe reacted with the sulfenamide or sulfenic acid. In cases where the site of oxidation is the primary interest, this distinction might not matter as the sulfenamide likely is derived reversibly from the sulfenic acid. However, when this distinction is important, the validation by mechanistically distinct probes (e.g. strain promoted ligation of the putative sulfenic acid with a strained alkyne or alkene vs. no reaction with the sulfenamide) may prove useful.

1.12 Concluding remarks

The oxidation of cysteine in cells features diverse chemistry. The thiol of this unique amino acid residue can be oxidised to the sulfenic, sulfinic and sulfonic acids or converted to a disulfide, persulfide or nitrosylated cysteine. These oxidative modifications are formed in response to oxidative stress. However, these modifications are also integral to several signalling pathways in healthy cells that control enzyme activity, protein structure, and protein-protein interactions. This Chapter has recounted common chemical methods for mapping cysteine sulfenic acids with spotlights on a few of the important biological processes that have been elucidated through the use of various chemical probes.

A reoccurring theme after investigation of the current literature seems to highlight the difficulties experienced with specificity and selectivity of chemical probes for only the sulfenic acid oxidation state of cysteine. After introduction to small molecule sulfenic acids and the study of this functional group, it was also apparent that this highly reactive species is difficult to detect and isolate by conventional methods. Both direct and in-direct detection methods have been employed for their detection with varying success. In-direct methods require a number of blocking steps which introduces numerous pathways for false positives and/or negatives, diminishing selectivity as a result. Additionally, several of these key thiol blocking reagents essential to these methods have come into question since strong evidence has been put forth demonstrating that these key electrophilic thiol-blocking reagents are not selective for thiols. For such strategies, development of truly thiol-specific reagents would be greatly beneficial. In contrast, direct detection methods are highly sought after as many of the difficulties associated with in-direct detection methods (such as blocking steps) are eliminated by use of chemical probes which react directly and selectively with only the sulfenic acid. This is clearly challenging as several accounts of cross-reactivity have been documented which is not surprising given the similarities of many cysteine oxidation states. This in part, can be accounted for by testing for potential off-target reactivity to help researchers anticipate false positives and validate hits. Small off-target labelling does not necessarily render a probe incompatible provided the knowledge is there and the off-target labelling can be distinguished from real hits. Many of the chemical probes discussed in this Chapter demonstrated off-target reactions with other cysteine oxidation states that were not always addressed in initial studies. This warrants careful consideration when assessing the outcomes of these experiments. Equally as important, the rate of reaction of chemical probes needs to be considered alongside selectivity. Sulfenic acids are short-lived, and cells respond rapidly to oxidative stress and second messengers such as H_2O_2 and NO. Therefore, chemical probes which react on the same timescale are useful for monitoring real-time changes to oxidation states. Increasing reactivity, however, can often come at the expense of selectivity so this is not a trivial task. Additionally, cell-compatible probes are desirable so that cysteine sulfenic acids can be detected under native conditions so as not to disrupt normal cellular interactions or processes.

Several examples were provided of proteins identified using these chemical methods during oxidative stress and other signalling events. In some cases, identifying a protein sulfenic acid site allowed hypotheses to be generated about the involvement of sulfenic acids in a specific signalling pathway. From this, opportunities exist for translating this information into useful strategies for chemical biology and medicine. For example, specific protein targets of cysteine oxidation could be identified which play significant roles in a disease pathway or unwanted events. This sulfenic acid site could then be targeted to prevent the unwanted pathway from occurring presenting a new opportunity for targeted drug therapies for diseases linked to oxidative stress. Considering this possibility further, there is substantial opportunity to identify how these molecular probes can be modified to react not just with a specific cysteine modification, but with that modification on a specific protein. If these probes are to be developed into pharmaceuticals, protein-specific probes will likely be required. Some accounts of research into developing protein-specific sulfenic acid probes was addressed.

Chemical methods for detecting cysteine sulfenic acids have made remarkable strides over the last decade. In particular, cell-compatible probes have revealed the importance of this oxidative modification in cellular signalling and redox regulation. With suggestions that cysteine sulfenic acids may play as critical a role as phosphorylation, there are clearly exciting opportunities ahead in mapping and understanding its precise role in living organisms. Developing sulfenic acid specific probes is a challenge in chemoselective labelling, with many more opportunities to improve upon current methods and develop new strategies with enhanced or streamlined selectivity. This presents a unique opportunity and challenge for the chemical community to contribute to this area.

1.13 Doctoral research contributions

Despite the growing number of chemical probes available for cysteine sulfenic acid detection, various issues remain unmet, with the potential of undetected cysteine sulfenic acids and therefore a gap in our understanding of cellular response during oxidative stress. To address these issues, this PhD thesis will concern the design, synthesis, and validation of a new chemical probe utilising the norbornene scaffold for detecting cysteine sulfenic acid on small molecules, proteins, and in living cells. This new cysteine sulfenic acid probe will address the current caveats highlighted in Chapter one from the literature on the reactivity and selectivity of current sulfenic acid probes to improve upon chemical detection methods. Finally, application of this new probe will be applied to study cellular mechanism and the role of cysteine sulfenic acids in cellular function.

The work presented herein will describe the candidate's contribution to the field of cysteine sulfenic acid detection in proteins and cells. Chapter one presented a comprehensive review of the current literature and status of cysteine sulfenic acid detection methods, placing the candidate's own contribution in context to the available literature. Chapter two explores the chemistry and reactivity of norbornene probes with small molecule model cysteine sulfenic acids to assess their suitability as

sulfenic acid probes. The norbornene probes are also compared to current probes such as dimedone and strained alkynes for rate and selectivity. Chapter three then explores the applicability of using functionalised norbornene probes as cysteine sulfenic acid probes on purified proteins, protein mixtures and in living cells. Comparison to currently used dimedone derivatives highlights additional selectivity issues with current methods and places norbornene probes as viable candidates. This also demonstrates the first use of norbornene probes to label protein sulfenic acids in living cells. Chapter four addresses the application of norbornene probes to map the sulfenic acid proteome in HeLa cells and identify potential protein sulfenic acids of biological interest. Additional cell lines are also probed using alternative oxidative conditions to highlight the widespread applicability of norbornene probes to study oxidative stress and cellular mechanism. Finally, Chapter five highlights the current stance of the research discussed and future directions using this newly developed cysteine sulfenic acid probe.

2. SMALL MOLECULE SULFENIC ACID STUDIES AND NORBORNENE PROBE SYNTHESIS

2.1 Overview

As researchers strive to discover and understand new disease-causing pathways in the human body associated with oxidative stress, the chemical toolbox of available probes grows ever so steadily. No one chemical probe can provide all the answers required to solve the intricate details of disease-causing pathways, or even metabolic pathways in general. Subtle differences in chemical reactivity of probes can be what provides the understanding by highlighting a previously unknown feature, or lead to specific targeting. Since we might not know what we are looking for specifically, we might not know exactly what we need to find it. Therefore, design and synthesis of new generation chemical probes with different reactivity profiles to those traditionally used will always be needed to better our understanding of the complicated process of oxidative stress.

In an effort to add to the ever-expanding toolbox of oxidative stress-related chemical probes, this PhD thesis set-out to discover a new cysteine sulfenic acid probe. Guided by the literature as depicted in Chapter one, our attention was turned to strained alkenes as potential cysteine sulfenic acid traps. A few reports had been documented in the literature detailing the reaction of strained alkenes with sulfenic acids, however these mostly focussed on small molecule degradation studies and not proteins or cells. This Chapter details the evaluation and application of small, commercially available norbornenes as cysteine sulfenic acid probes using a small molecule sulfenic acid model. The synthesis of norbornene derivatives functionalised with detectable tags are also reported.

Portions of this Chapter have been adapted from an article titled 'Norbornene probes for the study of cysteine oxidation' as published in *Tetrahedron*, Elsevier on 4th November 2017 (DOI: 10.1016/j.tet.2017.11.011). Some work detailed in this Chapter was not included in the above-mentioned publication, but has been incorporated in with the published work as this demonstrates additional findings that contribute to the overall understanding of the topic. Work included from the publication has been reformatted to align with this Chapter, but the original findings and conclusions remain the same. All work presented in this Chapter was prepared by the candidate unless otherwise stated in-text and was listed as the primary author of the manuscript.

2.2 Introduction

Examination of the current literature on detection of cysteine sulfenic acids highlights both the successes and pitfalls of current detection methods for cysteine sulfenic acids. Several successful chemical probes with a wide range of reactive functional groups have been designed and implemented to study oxidative stress in cells. Classic examples include dimedone (**19**) and its derivatives,[47-48, 100, 107] various carbon nucleophiles (**92** and **87**),[88, 145] strained cyclooctenes (**60**),

cyclooctenol (**71**),[141] and cyclooctynes (**96**-**97**),[105, 133] and other mechanistically distinct probes such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (**52**)[102] and benzoxaboroles (**56**) (Fig. 14).[104] Caveats aside, whilst all of these probes are known to react with cysteine sulfenic acids on peptides and proteins, they likely do not cover the full diverse range of reactivities of cysteine microenvironments which exist in the proteome. Some of these probes are very good general probes, exhibiting high reactivity for a broad range of cysteine sulfenic acids such as probe **92**, while others are much more selective as demonstrated in a study by Carroll and co-workers.[145] Cysteine sulfenic acid probes could be designed to therefore serve different functions depending on whether one is interested in mapping the cysteine proteome as a whole, or selectively targeting specific cysteine residues. Some cysteine sulfenic acid residues are persistent and stabilised by the protein microenvironment, whereas others are short-lived precursors to higher oxidation states or further modifications.[149] Others exist as surface cysteines, while others may be buried in binding pockets or folds. With this in mind, there is still a need to develop new chemical probes with differing reactivity profiles so that a comprehensive and more complete picture of the biological functions of this oxidation state can be mapped and further understood.

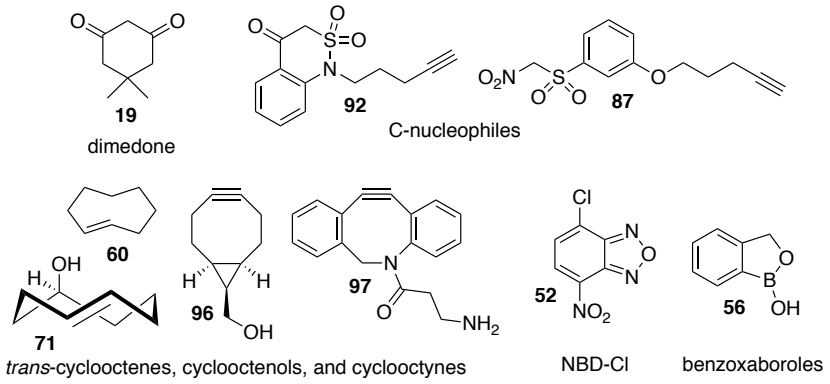


Figure 14. Reported cysteine sulfenic acid probes.

Intrigued by the reactivity of *trans*-cyclooctenes and cyclooctynes with cysteine sulfenic acids,[105, 131, 133] we wondered whether other strained ring systems could be utilised as cysteine sulfenic acid traps. Surveying the literature, reports of norbornene compounds reacting with generated sulfenic acids dating back to the 1970's piqued our interest. Barton and co-workers explored the reactivity of several strained alkenes (including norbornadiene) with the intermediate sulfenic acid formed from heating penicillin sulfoxides.[137-139] We envisioned that norbornene compounds would have less strain than previously tested cyclooctynes therefore less off-target reactions, but enough release of strain energy so as to rapidly react with sulfenic acids. Another key consideration of our strategy was the ease with which potential probes could be functionalised in straight-forward and easily accessible synthesis. Various norbornene compounds are commercially available with easily functionalised handles such as carboxylic acid or alcohol groups. Lastly, norbornene's compatibility with proteins

has been established through its use in several selective bioconjugation methods, so we anticipated that off-target reactions would be minimal as no such issues had been reported as far as we could tell.[150-153]

2.3 Proposed mechanism for reaction of norbornene with sulfenic acids

The reaction between norbornene and sulfenic acid is expected to proceed through a strain promoted group transfer reaction, with the strain release being the driving force behind the enhanced reactivity (Fig. 15). This is the same mechanism to what has been reported for the addition of cyclooctynes (**96**) to sulfenic acids, with the same strain-promoted driving force behind the reaction. With this, an estimated 100 fold increase in the reaction with sulfenic acids compared to the commonly used dimedone (**19**) was reported on a model system.[105] With its strain, norbornene is estimated to be 100 times more reactive toward dipoles than unstrained olefins.[154] The bond angles of an unsubstituted norbornene at the double bond has been measured as 107.3 °, where a typical sp^2 hybridised orbital will exhibit a bond angle of 120 °.[155] The ring strain energy for norbornene was calculated at 19.2[156] and 21.6[157] kcal/mol compared to norbornane which was 14.4[156] and 16.6[157] kcal/mol in two independent studies respectively.[156-157] These figures highlight the potential of norbornene, with 4.8 or 5.0 kcal/mol release of strain energy in each reported data respectively. Of course, this does not consider addition of sulfoxide to the norbornene structure upon reaction, so actual strain release values would differ in reality. Nonetheless, this provides an insight into the capabilities of norbornene compounds. The utility of strain-promoted ligation reactions has already been recognised as a strategy for bioconjugation using norbornene compounds, particularly in its application in click chemistry for labelling due to the large thermodynamic driving force.[154, 158] Therefore, given the high strain energy present, and subsequent relieving of that strain energy upon reaction, norbornenes present promising candidates as rapidly reacting cysteine sulfenic acid traps. Addition of various functional groups on the norbornene compounds at different positions affects this strain energy in model studies,[157] so there is potential for fine tuning the reactivity of these compounds in later studies. The proposed mechanism for reaction is likely to occur via a strain promoted ligation, but has not been confirmed (Fig. 15).

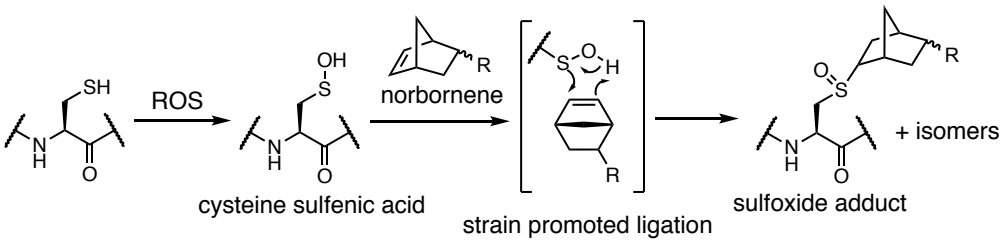


Figure 15. Proposed mechanism for reaction of norbornene with sulfenic acids. Reactive oxygen species (ROS) could include; hydrogen peroxide, peroxynitrite etc.

2.4 Designing a small molecule sulfenic acid model

To confirm whether norbornene compounds could react with sulfenic acids, a small molecule sulfenic acid model would be required. Many forms of small molecule sulfenic acids have been reported in the literature as discussed extensively in Chapter one. Despite this, several issues exist with synthesising small molecule, stabilised sulfenic acids as model compounds for assessing probe reactivity. In order for the sulfenic acid functional group to react with the prospective chemical probes, it needs to be somewhat stabilised so as to remain in existence long enough for reactions to be assessed and characterised. Often, this enhanced stability from H-bonding or sterics dramatically reduces the reactivity of the sulfenic acid and therefore does not necessarily exhibit the same reactivity of transient sulfenic acids that would be found in proteins. Additionally, the majority of these small molecule sulfenic acids are not water soluble, which brings into play possible solvent effects, once again complicating the assessment of the reactivity of chemical probes as candidates. Many studies also use these small molecule sulfenic acids as models to calculate rates of reactions of various probes, which may then not be a true representation of how the probe will react with proteins given the reasons listed above.

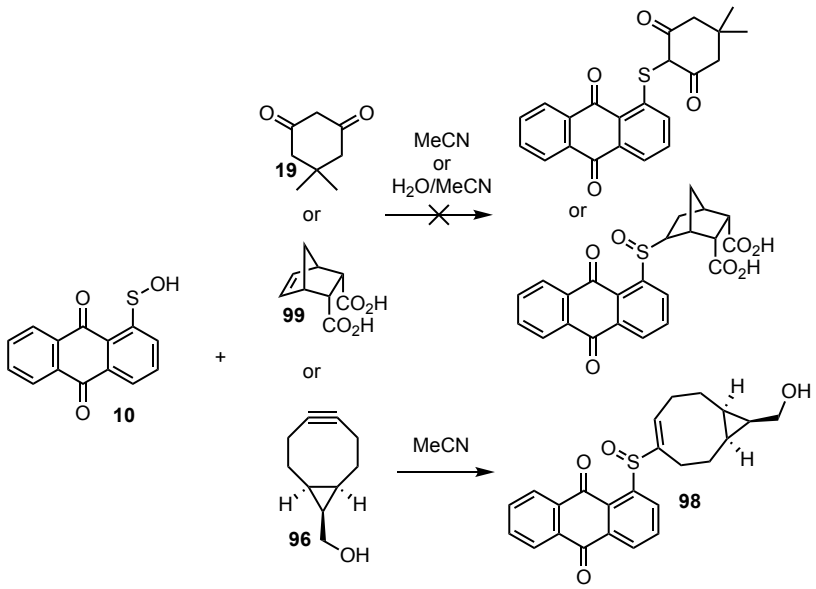


Figure 16. Fries acid does not react with dimedone[105] or norbornene. The predominant reaction is an acid/base reaction. BCN does appear to react with Fries acid as reported.[105]

For example, the stabilised sulfenic acid Fries acid (Fig. 16, **10**) has been used in several studies as a model sulfenic acid to assess the reactivity of prospective sulfenic acid probes.[104-105] However, as highlighted by one of these studies,[105] the main reaction of Fries acid with sulfenic acid probe dimedone (**19**) appears to be an acid/base reaction, not an addition reaction as reported in other

studies.[104-105] This is probably due to the enhanced stability of the sulfenic acid through H-bonding. Other probes such as BCN (**96**) did appear to react with Fries acid to form the expected sulfoxide product **98** as evidenced by characterisation of the isolated product.[105] In our own study, Fries acid was synthesised and reacted with *cis*-norbornene-endo-2,3-dicarboxylic acid (**99**), dimedone (**19**), and BCN (**96**) (data not shown). As predicted, no reaction was observed with dimedone or norbornene (**99**), supporting these claims. Limited reactivity was observed with BCN (**96**), but could not be confirmed. Based on the reported literature and these initial findings, it was concluded that the use of any stabilised small molecule sulfenic acid would probably exhibit similar loss of reactivity and therefore not accurately depict reactivity of protein sulfenic acids and was not pursued further.

However, we still desired to assess the chemistry of norbornene compounds on small molecule sulfenic acids before pursuing their reactivity with complicated biological systems. In order to more closely resemble the formation of sulfenic acids that would occur *in situ*, we decided to use a cysteine-derived model, *N*-acetylcysteine (**100**). This compound contains the cysteine group found in proteins, is water soluble (with assistance of base), and a small molecule for ease of characterisation. Upon oxidation with hydrogen peroxide, the short-lived *N*-acetylcysteine sulfenic acid (**101**) is generated before rapidly reacting with unreacted thiols to form the disulfide product (**102**). It was anticipated that the short-lived sulfenic acid intermediate could be intercepted by strain promoted ligation with various norbornene derivatives to give the resultant sulfoxide (**103**), before disulfide formation (Fig. 17). This would demonstrate rapid reaction of the norbornene probe in a manner similar to what would occur on proteins in cells during episodes of oxidative stress. Inclusion of hydrogen peroxide as the oxidising agent also mimics cellular oxidative stress, rather than a pre-formed or stabilised sulfenic acid. This model is a similar concept to the cysteine derivatives used in other studies such as that conducted by Carroll and co-workers[88] that generates reactive sulfenic acids *in situ*, rather than using stabilised small molecules. However, this model requires an organic co-solvent and the sulfenic acid is formed in solution from its protected form as the sulfenamide.

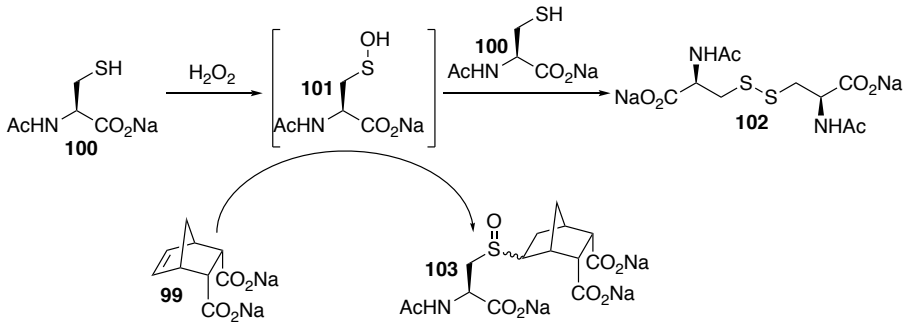


Figure 17. *N*-acetylcysteine as a model for sulfenic acid formation and trapping with norbornene.

2.5 Trapping *N*-acetylcysteine sulfenic acid with norbornene derivatives

Now that a suitable small molecule sulfenic acid model had been selected with *N*-acetylcysteine, the norbornene derivative *cis*-norbornene-endo-2,3-dicarboxylic acid **99** was selected as the first candidate to trap cysteine sulfenic acids. **99** contains two carboxylic acid groups that upon treatment with 1 equivalent of base (eg. sodium carbonate or sodium hydroxide), make it fully water soluble. This is advantageous over previously used small molecule sulfenic acid models as it allows the experiments to be conducted in 100 % aqueous buffer, as opposed to the use of organic solvents or co-solvents necessary in previous examples.[88, 104-105, 143-144] Preliminary studies on the reactivity of norbornene **99** and other alkenes screened on *N*-acetylcysteine were initially performed by Mawey Akol in the Chalker lab. These experiments were then repeated, refined, and optimised by the candidate, and only these methods and data will be presented and discussed.

Firstly, both the norbornene derivative **99** and *N*-acetylcysteine **100** were solubilised separately by addition of 1 equivalent sodium carbonate in D_2O. Since the formation of the disulfide **102** of *N*-acetylcysteine occurred rapidly upon addition of hydrogen peroxide, the hydrogen peroxide was added to the solution of norbornene **99**, mixed, then the solution of *N*-acetylcysteine was added dropwise in a 3-fold excess (Fig. 18). This protocol was designed to ensure the norbornene probe was present when the sulfenic acid was generated, allowing it to react before rapid disulfide formation (a matter of seconds, rate not determined). The reaction was then incubated for 20 minutes at room temperature with stirring and analysed directly by ^{1}H-NMR spectroscopy. The pH of the reaction was not controlled in these experiments, as these initial studies were to first establish if the chemistry was sound before pursuing further. The pH was measured at 4.3 after addition of *N*-acetylcysteine, norbornene **99**, and hydrogen peroxide solutions.

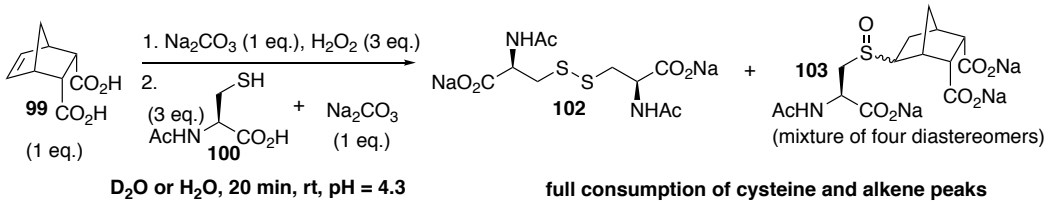


Figure 18. Norbornene probe **99** can trap the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide.

After ^{1}H-NMR analysis, the major product detected was disulfide **102** (since **100** was added in 3-fold excess), with complete consumption of the alkene peaks of **99**, in its conversion to suspected sulfoxide product **103**. A single product peak could not be observed likely due to the possibility of four diasteromers that could be formed during the reaction. The strain promoted ligation reaction that

is suspected to occur between norbornene and sulfenic acids can proceed at either face of the alkene, with the sulfur in the expected sulfoxide product **103** a stereogenic centre. To further confirm the formation of the sulfoxide product (which could not be fully characterised based on NMR alone, see Appendix A), LC-MS experiments were performed. The exact same experimental conditions were maintained, with the only difference being the use of H_2O instead of D_2O. The concentrations of reagents were also adjusted for suitable detection by LC-MS. All equivalencies of reagents were maintained. Upon LC-separation and detection of products by MS (compounds were not UV-active at wavelengths monitored by the photodiode array detector), remarkably all four diastereomers could be partially resolved in the LC trace, with some overlap of peaks. The mass spectra observed were consistent with the calculated values for the expected sulfoxide products **103** (m/z = 360, ESI^-). As additional confirmation, several important control experiments were conducted. In ^{1}H-NMR spectroscopy, norbornene **99** was stable in the aqueous solution, did not react with hydrogen peroxide itself, nor did it react with the pre-formed disulfide product **102**. These results were confirmed by LC-MS with the mass of norbornene **99** detected in all control experiments and no detectable unexpected products. Evidence of the reaction of norbornene **99** with *N*-acetylcysteine **100** (the free thiol) was not clear by ^{1}H-NMR spectroscopy, but the alkene peak of **99** was still strong. However, the thiol-ene product (reaction of norbornene with the free thiol of *N*-acetylcysteine **100**) was observed in the LC-MS analysis in varying amounts (m/z = 344, ESI^-).

This result suggests caution in the use of these norbornene probes as sulfenic acids traps, as it is possible that a reaction can occur between the thiol and norbornene. The reaction of thiols with enes is known to proceed through a radical reaction termed thiol-ene (Fig. 19) and are typically rapid, efficient, and can occur under ambient conditions.[159-161] Specific examples of thiol-ene reactions between norbornene and thiols have been reported extensively, but usually require an initiator (typically photo-initiator) and/or photo-activation.[160, 162-165] These reactions are between the thiyl radical (not directly with the thiol) and the ene, so perhaps would be more negligible in a biological context such as a cell. Indeed, similar reactions have been observed with the strained cyclooctynes through the equivalent thiol-yne reaction, supporting this claim.[135, 166]

Regardless, the key distinction is that the products of reaction of norbornene with either the thiyl or the sulfenic acid differ by inclusion of an oxygen for the latter. This observation is important, as it allows the products to be distinguishable by MS as demonstrated in our study. Other commonly used chemical probes such as dimedone do not retain the oxygen from the sulfenic acid in the resultant product (see Chapter one for mechanism). Evidence has been put forth that dimedone can actually react with other cysteine oxidation states such as sulfenamides to yield the same product, therefore there is limited way to tell what species the probe actually reacted with, as the product would be the same in all cases.[148] While this particular example may not be as significant because sulfenic acids can rapidly form sulfenamides, and thus could be an indirect detection of sulfenic acids, it is still important to know and understand the reactivity of the chemical probes used for detection to prevent

false positives/negatives. Despite the apparent cross-selectivity of norbornene with thiols (rather thiyls), the reaction of norbornene with sulfenic acids is presumably much more rapid than the formation of the thiol-ene, as little to no thiol-ene product is detected by LC-MS in the key reaction when hydrogen peroxide is added. This also eliminates the possibility that the thiol-ene reaction occurs first, then is oxidised to the sulfoxide by the hydrogen peroxide. If this were to be the case, the thiol-ene reaction should occur as rapidly as formation of the sulfoxide product, which is not observed by ^{1}H-NMR.

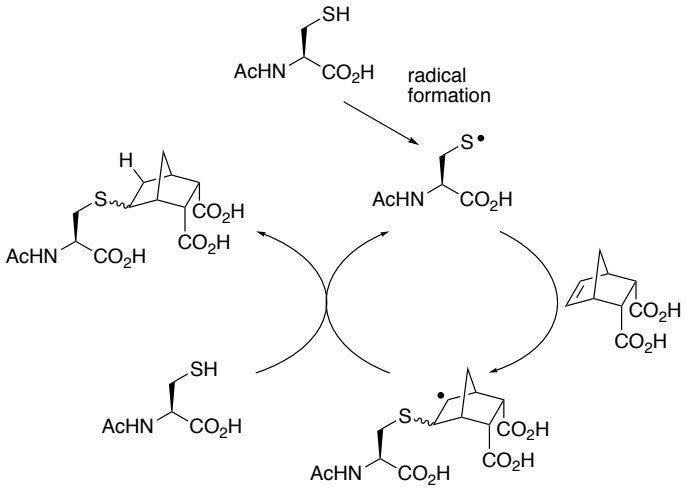


Figure 19. Mechanism for thiol-ene reaction. This could be initiated by oxygen in open systems.

2.6 Comparison to dimedone and cyclooctynes

Since the reactivity of norbornene with a model cysteine sulfenic acid had been confirmed, a direct comparison with previously reported sulfenic acid probes was sought to gauge the initial performance of the newly confirmed probe. Both dimedone and two cyclooctyne's were chosen as both probes have been used extensively. Using identical reaction conditions as those used for norbornene, with the exception of the cyclooctyne's which required DMSO as a co-solvent for solubility, all experiments and control experiments were repeated.

When dimedone (**19**) was tested, no expected thioether product **104** was observed by ^{1}H-NMR or LC-MS (Fig. 20). This result suggests that perhaps dimedone cannot react rapidly enough with the generated sulfenic acid **101** upon oxidation of *N*-acetylcysteine with hydrogen peroxide, before it forms the disulfide product. In control experiments where dimedone is treated with hydrogen peroxide (with no cysteine), a trace amount of an unknown product was observed (see Appendix A). While the product was not isolated, as it was barely detectable by NMR, it has a mass consistent with an oxidative dimerisation and was detected in all LC-MS experiments that contained both dimedone and hydrogen peroxide, suggesting it was not just an artefact of the LC-MS, but a product

formed during the reaction. This result raises some interesting questions about the stability of dimedone in the presence of hydrogen peroxide.

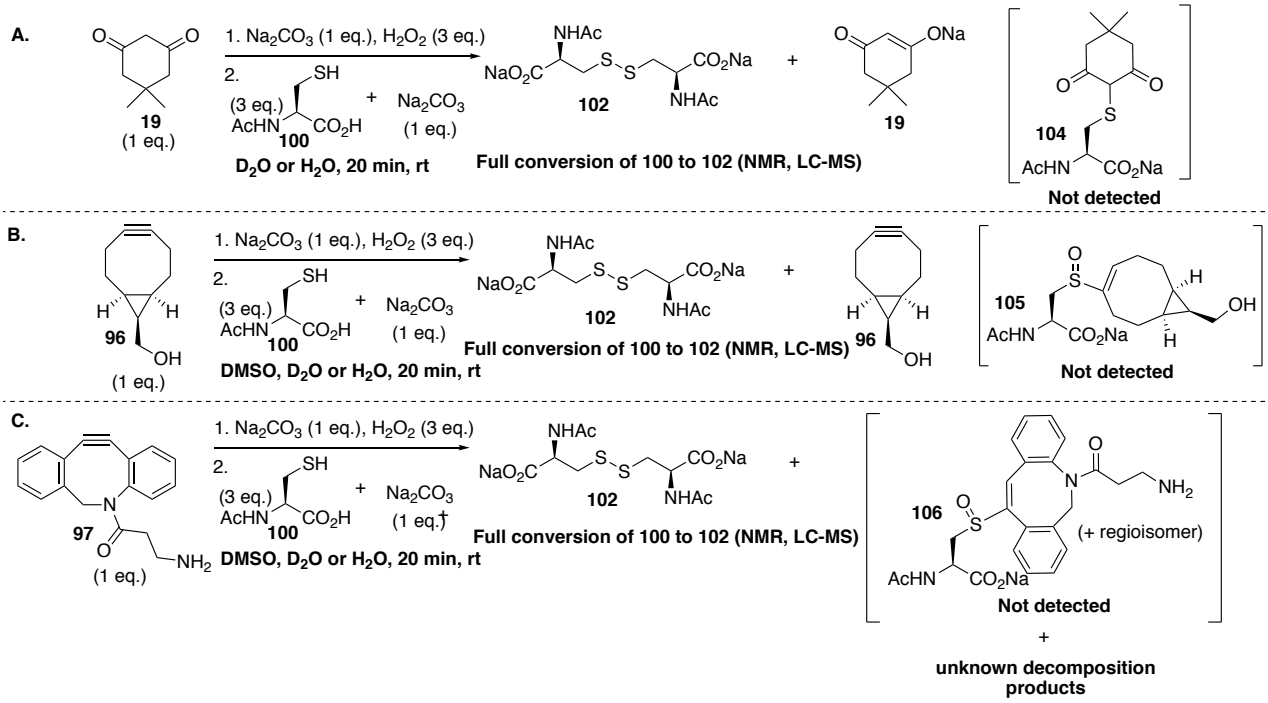


Figure 20. Under the same conditions in Figure 18, neither dimedone **19** (A.), BCN **96** (B.), nor DBCO **97** (C.) was able to trap the sulfenic acid in detectable quantities by NMR or LC-MS under these conditions.

When compared to BCN **96**, another interesting result was observed. The expected sulfoxide product **105** upon reaction of BCN with the generated *N*-acetylcysteine sulfenic acid was not detected by ^{1}H-NMR or LC-MS (Fig. 20). This could be related to the limited solubility of **96** in aqueous media as DMSO was required as a co-solvent. The precipitation of **96** throughout the course of the reaction could also impact on its reactivity. This could also be an issue with the ability of the probe **96** to ionise effectively in MS analysis, as the probe itself (unreacted) could not be detected. Cyclooctyne DBCO **97** was also tested under the same conditions with similar results. No expected adduct **106** between the *N*-acetylcysteine sulfenic acid and DBCO were observed (Fig. 20). Additionally, decomposition products were observed whenever DBCO and hydrogen peroxide were reacted together which could explain this result. These decomposition products could not be identified (see Appendix A). Precipitation was also an issue for DBCO, as well as ionisation and decomposition complicating the detection of species. Moreover, the only observed reaction of either cyclooctyne **96** or **97** was the direct reaction with the thiol of *N*-acetylcysteine by LC-MS, probably through the thiol-yne (see Appendix A).[135] Interestingly, this side reaction was not observed under oxidative conditions. These results agree with recent reports stating the apparent off-target reactions of strained alkynes with thiols when utilised in chemical biology.[88, 135]

Based on these data, both cyclooctyne probes were not pursued for analysis any further due to solubility issues and lack of detectable adduct formation with the sulfenic acid, deeming these probes unsuitable for use as sulfenic acid probe comparisons. This result was surprising since **96** has been used specifically as a sulfenic acid probe with none of these issues highlighted.[105] If these probes were to be utilised further, addition of water solubilising tags would be needed to assist with the analysis.

2.7 Effect of pH on sulfenic acid reactivity with norbornene

The results obtained with norbornene in previous sections were promising, however, the pH in these experiments was not controlled. This is an important consideration as any chemical probe to be used in a biological system must still function at physiological pH (pH 7.4). The above experiments were measured in an unbuffered system measured at pH 4.3. Since at this pH the solution is acidic, experiments were initially repeated for norbornene **99** and dimedone in a sodium acetate buffer (200 mM) at pH 5. Buffer concentration was optimised for the minimum concentration required to ensure buffering capacity was maintained throughout the experiment. For the experiment, when norbornene **99** was treated with hydrogen peroxide and *N*-acetylcysteine, the alkene peak was completely consumed as demonstrated by ^{1}H-NMR and the expected sulfoxide product **103** (m/z = 360, ESI^{-}) detected as four diastereomers by LC-MS (Fig. 21A and Fig. 22). Control experiments showed that **99** was stable in the buffer for the duration of the experiment and in the presence of hydrogen peroxide. **99** also did not react with pre-formed disulfide. Limited evidence of thiol-ene addition was observed in ^{1}H-NMR (Fig. 22), but once again, some thiol-ene product (m/z = 344, ESI^{-}) was observed in LC-MS analysis (see Appendix A). As an additional control, we wanted to ensure that norbornene was inert towards other higher cysteine oxidation states such as the sulfinic and sulfonic acids. These over-oxidation products of sulfenic acids are highly likely to be present in any of our future analyses and as such we wanted to ensure they were inert towards our probe. Using model compounds *p*-toluenesulfinate and *p*-toluenesulfonate, norbornene **99** was reacted with each under identical conditions as used previously in the pH 5 buffer system. No reaction was observed, even after 24 h indicating selectivity of norbornene for the sulfenic acid as expected based on mechanistic restrictions.

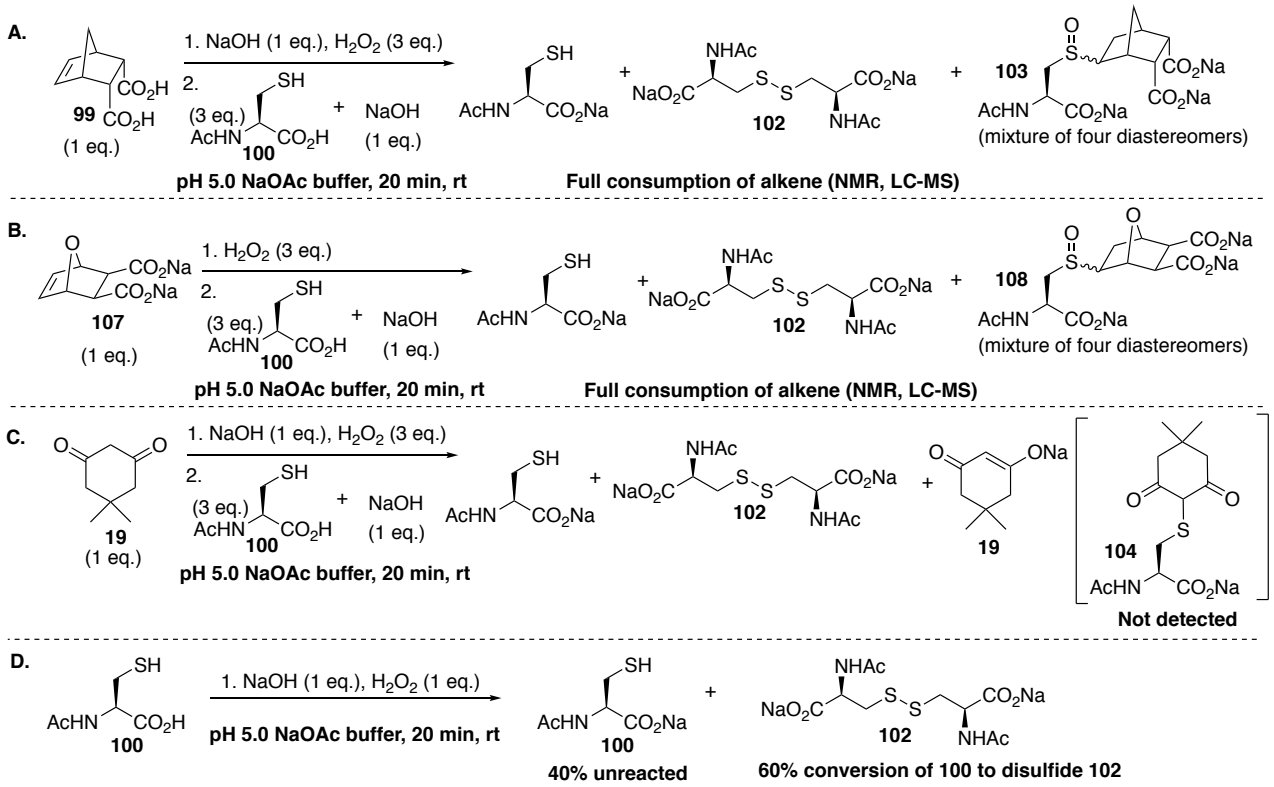


Figure 21. A-B. In buffer (pH 5.0, 200mM NaOAc), norbornene **99** (A.) and oxa-norbornene **107** (B.) can trap the sulfenic acid intermediate **101** formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide. C. Under the same conditions, dimedone (**19**) did not trap the sulfenic acid intermediate formed upon oxidation of *N*-acetylcysteine with hydrogen peroxide at pH 5. D. Under buffered conditions (pH 5.0, 200 mM NaOAc), *N*-acetylcysteine **100** is not fully converted to the disulfide **102** after 20 min at rt.

It was also noted that for the duration of the experiment, when *N*-acetylcysteine was treated with hydrogen peroxide using the same conditions as previously, not all the thiol was converted to the disulfide. Based on ^{1}H-NMR integration of the Hα-signals, only 60 % conversion to the disulfide was observed over the 20 min experimental conditions (Fig. 21D). This was interesting but could be explained by the pK_a of the thiol. Small molecule sulfenic acids typically have a pK_a from 4-12, whereas protein cysteine sulfenic acid has generally been measured at pK_a of 6-7.[18, 45] Hydrogen peroxide oxidises the thiol group much faster when in its deprotonated form, therefore lowering the pH would increase the abundance of protonated thiols, resulting in slower oxidation rates of cysteine, and therefore less disulfide formation. Likely both steps in this process, nucleophilic attack of the thiol on hydrogen peroxide, and nucleophilic attack of unreacted thiol (since the protonated form is less nucleophilic than the thiolate anion) on the sulfenic acid, are slower at lower pH. This observation was important to keep in mind for the reactivity of *N*-acetylcysteine under various pH conditions.

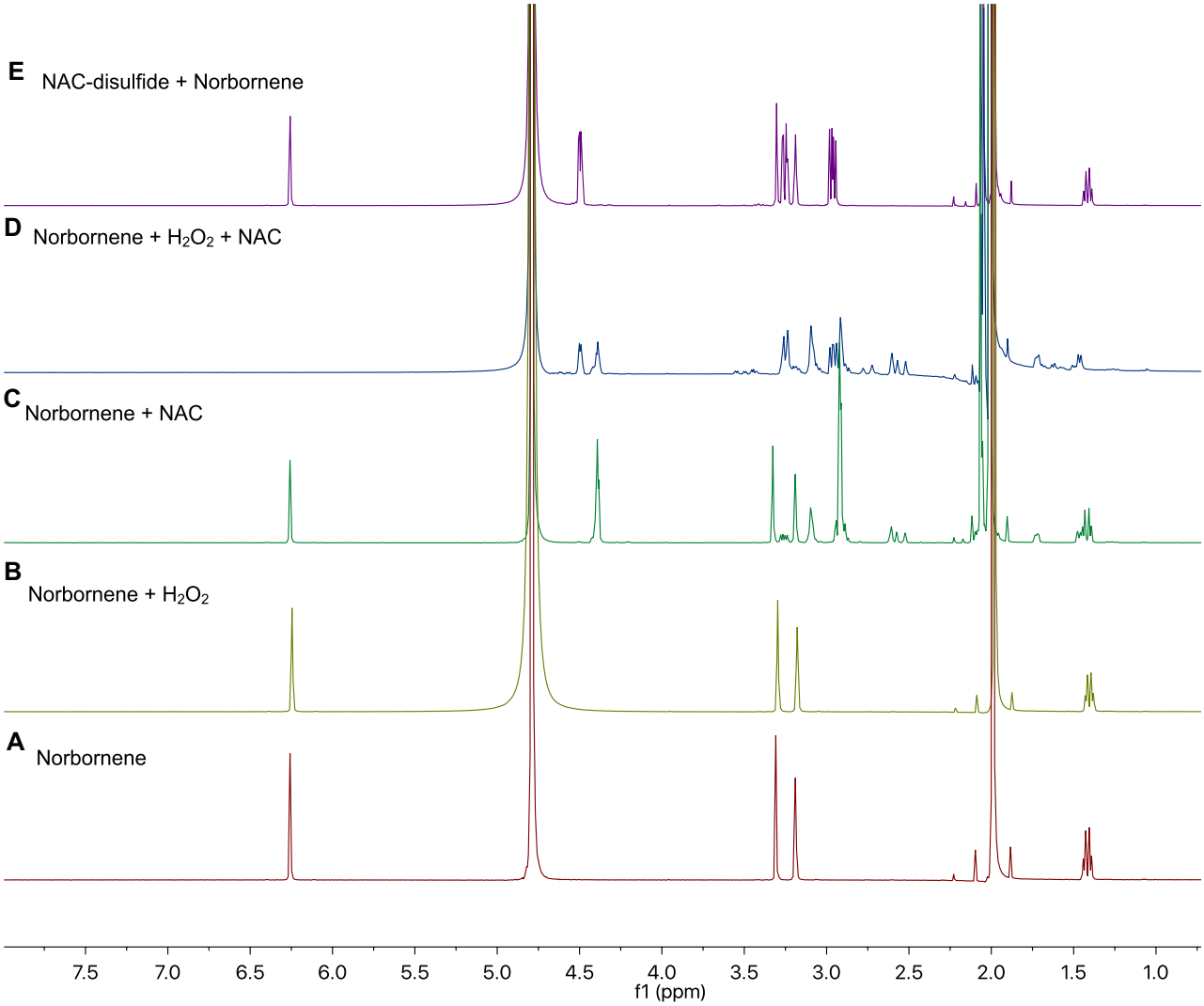


Figure 22. NMR studies using cis-5-norbornene-endo-2,3-dicarboxylic acid (**99**) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM). A. **99** is stable in pH 5 buffer. B. **99** is unreactive towards hydrogen peroxide. C. **99** does not reactive with *N*-acetylcysteine (NAC) in appreciable quantities. D. **99** traps the sulfenic acid **101** with complete consumption of the alkene peak. E. **99** does not react with the disulfide **102**. Full experimental detailed in 2.9.5.

A major issue in chemical probe application to biological systems is solubility. We were fully aware that any addition of detectable tags that we would add to the norbornene scaffold for later downstream detection could dramatically reduce the solubility. Currently, we were using a norbornene derivative with a carbon bridge-head (derived from cyclopentadiene), but were curious to see if the oxa-norbornene **107** with an oxygen bridge-head (derived from furan) would react in the same way, possibly increasing solubility for future applications. The oxa-norbornene probe was synthesised from the reaction of furan and maleic anhydride, followed by treatment with sodium hydroxide to open up the ring forming the dicarboxylic acid derivative in 100 % *exo*. This ring-opening also mimics the initial step for solubilisation of the norbornene probe **99**. **107** was then treated under

the exact same conditions as **99** at pH 5. As anticipated, the alkene of **107** was also fully consumed under the reaction conditions (Fig. 21B) as observed by ^{1}H-NMR and the expected sulfoxide product **108** (m/z = 362, ESI^-) detected by LC-MS (see Appendix A). Control experiments yielded the same outcome as norbornene **99**. The thiol-ene product (m/z = 346, ESI^-) was also observed in LC-MS analysis (see Appendix A). This was promising that both norbornene variations seemed to display similar reactivity towards sulfenic acids. Detection of the thioether adduct was concerning as this could interfere and complicate downstream analysis when determining hits from proteins and cells. As an additional reassurance, the reaction mixtures containing only *N*-acetylcysteine **100** and either norbornene **99** or oxa-norbornene **107** were left for an additional 3 days in pH 5 buffer at room temperature and analysed by ^{1}H-NMR. Even after this time the alkene peaks of both norbornene compounds was present, confirming this thiol-ene side reaction to be largely insignificant under these conditions.

The experiments were then repeated at pH 5 with dimedone to directly compare the reactivity under the exact same conditions (ie. controlled pH). The pH could have a significant impact on the reactivity thus it is vital all probes are treated to identical labelling conditions. Still no reaction between dimedone and the sulfenic acid was observed (Fig. 21C). Once again, oxidation of dimedone treated with hydrogen peroxide appeared to occur (see Appendix A). To further investigate formation of this unknown product, the sample was left for a further 24 h and assessed by ^{1}H-NMR. Formation of the unknown product had increased with time, confirming this oxidation product was not an artefact of LC-MS but a product forming during the reaction. This is obviously problematic for dimedone if the probe is consumed by hydrogen peroxide during the course of the reaction, and could present a competing reaction. It should be noted that the formation of this unknown oxidation product during the 20 min reaction time is very minimal, so likely does not have a great impact on the reaction overall, but should be highlighted nonetheless.

Back to norbornene, the experiments had been successfully repeated at buffered pH 5 indicating the chemistry was reliable. The next logical step was to determine whether the reaction could occur at physiological pH 7.4. A phosphate buffer (200 mM) was prepared for all experiments. Before conducting the experiments, oxidation of *N*-acetylcysteine by hydrogen peroxide to the disulfide was tested at pH 7.4 and found to proceed to completion within 20 min. This supports the earlier assumption that deprotonation to the thiolate dramatically increases the rate for sulfenic acid and disulfide formation. Once established, the control and experimental conditions used previously were repeated on norbornene **99** and oxa-norbornene **107** at pH 7.4. Interestingly, no adduct formation was observed by ^{1}H-NMR (Fig. 23A-B and see Appendix A). The most reasonable explanation for this occurrence is that at higher pH, more thiolate anion exists in solution which reacts far more rapidly with both hydrogen peroxide and sulfenic acids to form the disulfide. This rate could exceed the rate of reaction of norbornene with the intermediate sulfenic acid, resulting in no interception and trapped sulfoxide product. Another consideration for the lack of reactivity could relate to the proposed

mechanism between norbornene and sulfenic acids. The reaction mechanism may require the sulfenic acid in its protonated form, rather than the sulfenate anion. Therefore, at higher pH, more of the sulfenic acid would exist in the deprotonated form, hindering its reaction with norbornene long enough for unreacted thiol to intercept and rapidly form the disulfide. This was not confirmed and remains speculative. All control experiments yielded the same results as previous. Norbornene **99** was also assessed for its cross-reactivity with the model sulfinic and sulfonic acids using the same conditions as previously used for the pH 5 test. No cross-reactivity was observed at pH 7.4 either.

This result of no reaction at pH 7.4 was somewhat discouraging as the ultimate fate of the norbornene probes was intended for protein and cell analysis which must occur at pH 7.4. But, this was not to say the norbornene probes could not react at pH 7.4. If disulfide formation at pH 7.4 is too rapid for the norbornene to trap, this is likely only a problem for the small molecules as proteins are much larger with possible steric affects and other contributing sources of stabillisation. Lack of reactivity of norbornene with *N*-acetylcysteine at pH 7.4 in this model study was most likely a limitation of the model itself and the sensitivity of *N*-acetylcysteine to pH. The fact that norbornene could trap any of the sulfenic acid intermediate at any pH was remarkable. Therefore, testing these norbornene probes on proteins at pH 7.4 is still a valuable experiment to perform, as these results do not rule out their reactivity entirely.

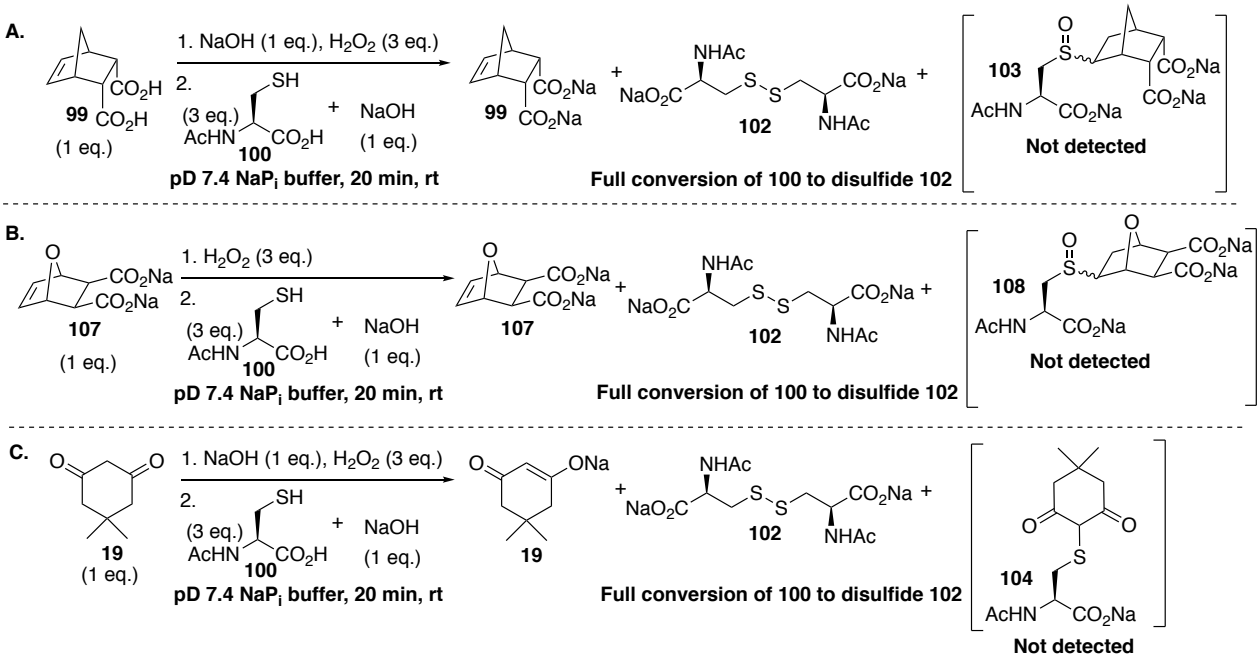


Figure 23. Under buffered conditions (pD 7.4, 200mM sodium phosphate), neither norbornene or dimedone was able to trap the sulfenic acid in detectable quantities as determined by ^{1}H-NMR.

Dimedone was also tested for its reactivity with *N*-acetylcysteine under these conditions at pH 7.4 but the same result was concluded. Dimedone did not appear to trap the sulfenic acid as observed

by NMR (Fig. 23C and see Appendix A). This result was important as typically dimedone labelling experiments are performed at near physiological pH. Earlier experiments involving dimedone were conducted in acidic pH, which could affect the chemistry depending on whether the mechanism proceeds through the direct substitution or hydrogen-bond assisted substitution (see Chapter one for mechanism). Therefore, the lack of reaction of dimedone at pH 7.4 (and pH 5) suggests it does not react fast enough with the sulfenic acid **101** to out-compete its reaction to form disulfide **102** in this model. This result is very promising as norbornene probes did trap the sulfenic acid **101** intermediate where dimedone could not, albeit at pH 5.

Whilst the comparable reactivity of oxa-norbornene **107** was encouraging, such compounds are known to undergo retro Diels-Alder reactions under certain conditions, for example, high heat (100 ºC).[167-168] Although these extreme conditions are unlikely to occur under physiological conditions, cell experiments are still conducted at elevated temperatures of 37 ºC. The risk of the probe reversing is present and thus it was concluded that initially only norbornene probes with CH_2 bridge-heads would be pursued further for convenience. No retro Diels-Alder reactions had been observed in any of the experiments. Regardless, knowing that oxa-norbornene probes react in the same way as norbornene probes could be useful for second generation design of norbornene probes for different purposes. The use of oxa-norbornene probes could have potential future use as target and release molecules with careful design.[169]

2.8 Synthesis of norbornene derivatives

Now that norbornene probes had been validated as cysteine sulfenic acid probes on small molecules, functionalised norbornene probes would be required for downstream analysis of protein and cell samples. This additional functionality would need to allow visualisation and/or affinity capture of the probe-bound proteins. Surveying the literature, common examples include biotin,[103, 106] fluorophores,[99, 103] or azide[100, 107]/alkyne[38, 145] handles for downstream labelling. Both biotin and fluorophores are convenient as they already contain the detectable portion and do not require any conjugation reactions. Biotin can be detected selectively through several means by its high affinity to streptavidin (which can be functionalised with a fluorophore for visualisation) or used for affinity purification with a streptavidin resin for example. However, both these reporter groups are bulky and may interfere with binding of the probe to buried or hindered cysteine residues. Alternatively, terminal alkynes or azides can be utilised by the copper-catalysed azide-alkyne cycloaddition (CuAAC) reaction.[170-171] Both groups are initially much smaller, reducing interference with binding in the initial reaction. Then, fluorophore or biotin tags containing the opposite alkyne or azide can be conjugated post-reaction and probe-bound proteins detected.[38, 107, 170-171]

Other key considerations of probe design include solubility, toxicity, membrane permeability and compatibility with downstream processing such as western blot and proteomics. Generally, considerably high concentrations of probes are used in sulfenic acid labelling experiments, therefore solubility and toxicity are extremely important. Initial synthesis of probes containing different detectable tags was attempted such as those containing a fluorophore and azide handle. However, many issues were encountered with solubility, reactivity, and compatibility with detection methods such as SDS-PAGE and western blot. Consequently, these syntheses were not pursued. Instead, it was concluded that the most ideal detectable tags to have would be a biotin and alkyne. Biotin-functionalised probes are attractive since they are readily detectable without the need for additional functionalistion, with applicability to many methods. Addition of the biotin group would also improve solubility, an issue that had been faced when trying to incorporate other functional groups (data not shown). It had also been reported in the literature that the CuAAC reaction where the alkyne was conjugated to the biomolecule (and azide conjugated to the added tag) were more effective. Azides can be prone to reduction under some conditions, and hence the alkyne handle was chosen over the azide.[123] This probe was designed with downstream proteomic applicability in mind.

Another key consideration for the synthesis design was simplicity to avoid cumbersome and long synthetic routes to allow the probes to be easily accessible by other researchers.[18, 45, 145] A probe **111** was prepared by the direct reaction of amine **110** with anhydride **109** via a single step from commercially available starting materials (Fig. 24A). These experiments were initially performed by Kyle Farrell with methods developed by the candidate using other amine derivatives for alternative probe synthesis. Synthesis of **111** was repeated by the candidate for further analysis and this data is included. The resulting carboxylic acid **111** was anticipated to enhance water solubility. Unfortunately, probe **111** was unstable in water (including pH 5 buffer) and hydrolysed 50 % to products **99** and **110** after 20 min as analysed by ^{1}H-NMR (Fig. 24B). After 24 h, complete hydrolysis was observed by both ^{1}H-NMR and LC-MS (see Appendix A). This hydrolysis was obviously not ideal as it removes the detectable portion of the probe. Surveying the literature, reports of arylamides on the same norbornene core undergoing hydrolysis have been observed.[172] In these cases, the adjacent carboxylic acid can participate in the reaction as a nucleophile or acid catalyst. The alkylamide of probe **111** could be susceptible to hydrolysis via this same neighbouring group participation. To try and prevent this hydrolysis, a ring closing reaction was performed on the carboxylic acid **111** to form an imide. This was successful and no hydrolysis appeared to occur after leaving for 24 h in a DMSO-water mixture. However, solving the hydrolysis problem caused a new insolubility issue and hence this synthetic route was abandoned.

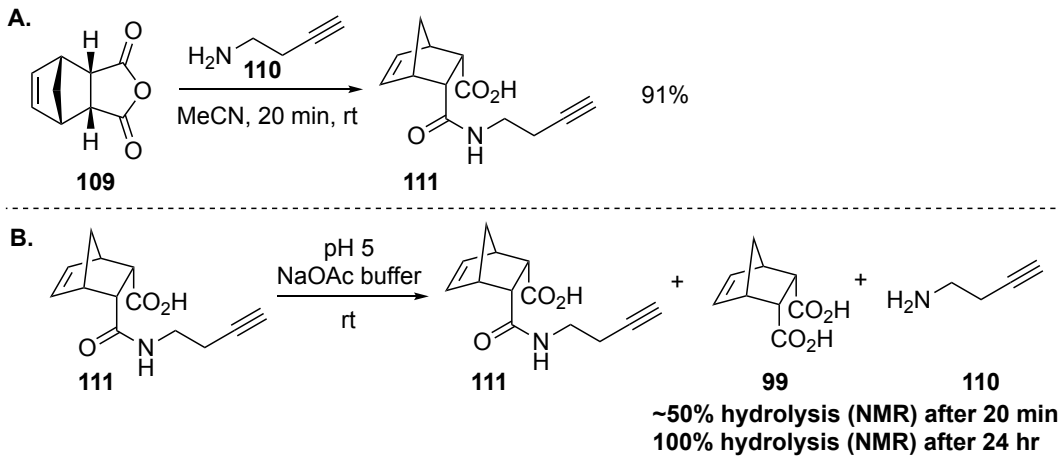


Figure 24. A. A norbornene probe for cysteine sulfenic acid containing an alkyne reporter group (**111**) was prepared in a single step by the reaction of amine **110** with anhydride **109**. B. Probe **111** hydrolysed spontaneously in water, losing the alkyne handle. Hydrolysis studies performed in part by Kyle Farrell.

Finally, an alternative probe **69** was synthesised to avoid hydrolysis from the neighbouring carboxylic acid group, but also containing an amide functional group to assist with solubility. Norbornene derivative **112** was therefore coupled to *N*-hydroxysuccinimide **113** to form NHS ester **114** in 69 % yield. Reaction of **114** with amine **110** provided the new probe **69** in 89 % yield (Fig. 25A). This probe was tested for hydrolysis and appeared to be stable in DMSO-water mixtures and had a solubility of at least 1 mM containing less than 1 %v/v DMSO. Using a similar synthetic scheme, a biotin-tagged norbornene was also synthesised. NHS ester **114** was first coupled to the diamine linker **115** to give **116** in 91 % yield. Separately, biotin **117** was converted to its NHS ester **118** via the same method. The free amine of **116** was then coupled to the biotin-NHS ester to give the target probe **70** in 71 % yield (Fig. 25B). The solubility was also tested and again could be dissolved in water/buffer to at least 1 mM with less than 1 %v/v DMSO (probes first dissolved in DMSO then added to water). Rather than pursuing the synthesis of additional probes (a time-consuming process), it was then decided the best course of action was to test whether the reaction of norbornene with sulfenic acids would translate to proteins both as purified mixtures and in living cells.

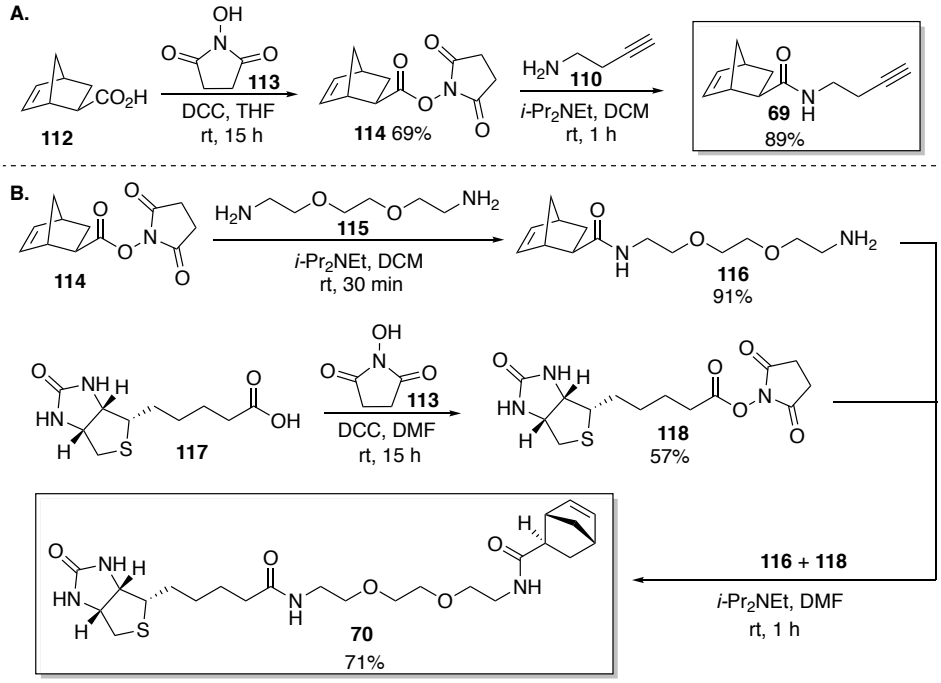


Figure 25. A. Norbornene probe for cysteine sulfenic acid containing an alkyne reporter group (**69**) was prepared in two steps from **112**. Probe **69** was not susceptible to hydrolysis in the same way as **111**. B. The synthesis of a norbornene probe for cysteine sulfenic acid containing a biotin affinity tag.

For future applications and probe design, the hydrolysis observed with probe **111** could be utilised as a target and release strategy. Since norbornene targets sulfenic acids, which are in high abundance in cells under oxidative stress, the probes could bind and release a bound drug for example, accumulating in disease areas. This would depend on whether the hydrolysis could be influenced by the neighbouring group to have a longer half-life so as to reach the target site before hydrolysis occurred. Nonetheless, this was an interesting observation that could have potential future applications.

2.8 Concluding remarks

Norbornene probes were successfully shown to react with short-lived, intermediate sulfenic acids generated *in-situ* by hydrogen peroxide. The model sulfenic acid generated from *N*-acetylcysteine reacted with norbornene compounds to give the expected sulfoxide adduct as analysed by both ^{1}H-NMR and LC-MS. The reactions were found to be pH dependent, where norbornene only reacted at pH 5 and not physiological pH 7.4. However, this is expected to be a consequence of the model sulfenic acid, not the reaction, as it was noted the oxidation of *N*-acetylcysteine was also affected by pH. Comparison to currently used sulfenic acid probes dimedone and two cyclooctynes

demonstrated the superior reactivity and selectivity of norbornene compared to these other probes which were all unsuccessful in capturing the sulfenic acid of *N*-acetylcysteine under any of the conditions tested. Despite its caveats, *N*-acetylcysteine was a good model system as it provided a transient sulfenic acid generated *in-situ*, similar to what would actually occur in proteins in cells. Finally, two functionalised norbornene probes were synthesised to contain a terminal alkyne **69** and biotin **70**. These were designed for protein and cell compatibility for detecting cysteine sulfenic acids to be discussed in Chapter three.

2.9 Experimental procedures

General experimental 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. CH_2Cl_2 was distilled over CaH_2 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, 60Å pore diameter). High resolution mass spectra (HRMS) were recorded on a Waters Synapt HDMS Q-ToF by electrospray ionisation (ESI) or a Perkin Elmer, AxION, DSA-ToF by atmospheric-pressure chemical ionisation (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 ^{13}C NMR spectra were recorded on a Bruker 600 MHz Spectrometer. NMR were assigned using COSY, HMQC and HMBC where required. Where D_2O 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-d_4, spectra were referenced to residual solvent (δ_H 3.33 ppm) for ^1H NMR and (δ_C 49.0 ppm) for ^{13}C NMR. For DMSO-d_6, spectra were referenced to residual solvent (δ_H 2.50 ppm) for ^1H NMR and (δ_C 39.5 ppm) for ^{13}C NMR. For $CDCl_3$, spectra were referenced to residual $CHCl_3$ (δ_H 7.26 ppm) for ^1H NMR and (δ_C 77.0 ppm) for ^{13}C 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 ionisation 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 capiliary 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.

2.9.1 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation (not buffered)

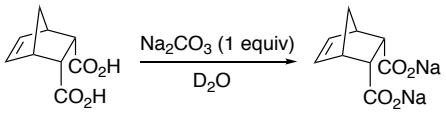
NMR studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation (not buffered)

General experimental procedure for NMR experiments A-D: For reactions A-D, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1) and *N*-acetylcysteine (solution 2):

Solution 1: In a vial, a mixture of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** (45 mg, 0.25 mmol), D_2O (2 mL) and Na_2CO_3 (25 mg, 0.24 mmol) was stirred or shaken until fully dissolved.

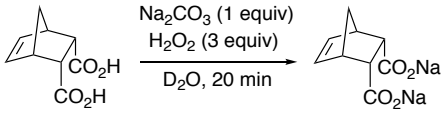
Solution 2: In a vial, a mixture of *N*-acetylcysteine (125 mg, 0.77 mmol), D_2O (2 mL) and Na_2CO_3 (25 mg, 0.24 mmol) was stirred or shaken until fully dissolved.

A: Stability of *cis*-5-norbornene-endo-2,3-dicarboxylic acid disodium salt in water



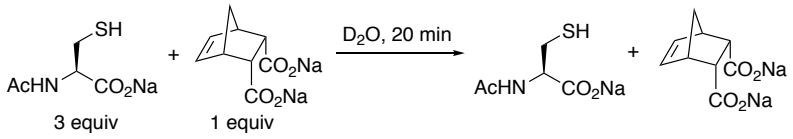
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.05 mmol **99**) and stirred for 20 minutes before analysing directly by NMR.

B: Stability of *cis*-5-norbornene-endo-2,3-dicarboxylic acid disodium salt in presence of hydrogen peroxide



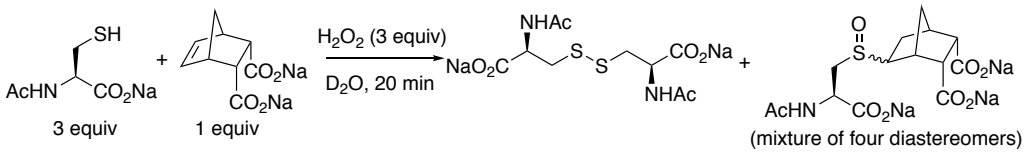
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.05 mmol **99**) and stirred for a few seconds before the addition of H_2O_2 (20 μL, 30 wt% aq. solution). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction between **99** and *N*-acetylcysteine (**100**)



To a vial, solution 1 (0.4 mL, 0.05 mmol **99**) was added followed by solution 2 (0.4 mL, 0.15 mmol **100**) and stirred for 20 minutes before analysing directly by NMR.

D: Reaction of norbornene **99** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.4 mL, 0.05 mmol **99**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O) and stirred for a few seconds before adding solution 2 (0.4 mL, 0.15 mmol, **100**) dropwise over 1 minute. The mixture was stirred for 20 minutes before analysing directly by NMR. The pH of this reaction mixture was 4.3.

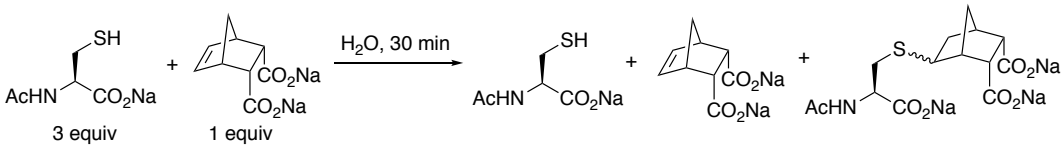
LC-MS studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation (not buffered)

General experimental procedure: For each reaction, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, a mixture of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** (18 mg, 0.10 mmol), H_2O (0.8 mL), and Na_2CO_3 (13 mg, 0.12 mmol) was stirred or shaken until fully dissolved.

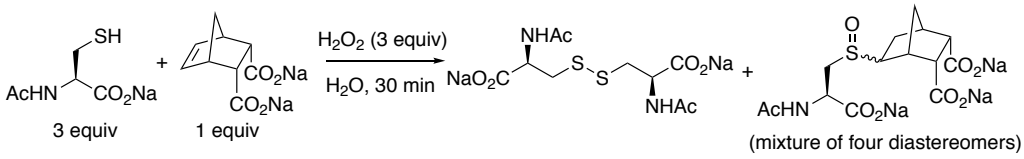
Solution 2: In a vial, a mixture of *N*-acetylcysteine (36 mg, 0.22 mmol), H_2O (0.6 mL) and Na_2CO_3 (9.8 mg, 0.09 mmol) was stirred or shaken until fully dissolved.

C: LC-MS data for control experiment in which **99** and *N*-acetylcysteine were reacted (in the absence of hydrogen peroxide)



To a vial, solution 1 (0.2 mL, 0.025 mmol, **99**) was added followed by solution 2 (0.2 mL, 0.073 mmol, **100**) and stirred for 30 minutes before analysing directly by LC-MS.

D: LC-MS data for reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic (**99**) and *N*-acetylcysteine sulfenic acid **101** at pH 4.3



To a vial, solution 1 (0.2 mL, 0.025 mmol **99**) was added followed by H_2O_2 (10 μL, 30 wt% in H_2O) and stirred for a few seconds before adding solution 2 (0.2 mL, 0.073 mmol **100**) dropwise over 1 minute. The mixture was stirred for 30 minutes before analysing by LC-MS. The pH of this reaction was 4.3.

2.9.2 Dimedone (19) as a probe for cysteine oxidation (not buffered)

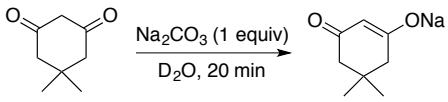
NMR studies using dimedone as a probe for cysteine oxidation (not buffered)

For each reaction, stock solutions were prepared for both dimedone (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, a mixture of dimedone (35 mg, 0.25 mmol **19**), D_2O (2 mL) and Na_2CO_3 (36 mg, 0.34 mmol) was stirred or shaken until fully dissolved.

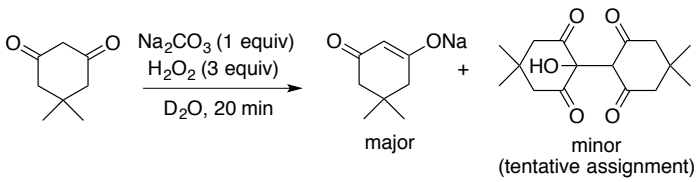
Solution 2: In a vial, a mixture of *N*-acetylcysteine (211 mg, 1.30 mmol **100**), D_2O (3.2 mL), and Na_2CO_3 (54 mg, 0.50 mmol) was stirred or shaken until fully dissolved.

A: Stability of dimedone enolate in water



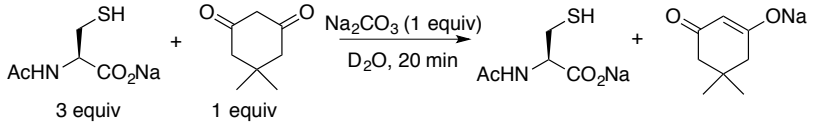
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.05 mmol **19**). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of dimedone enolate in presence of hydrogen peroxide



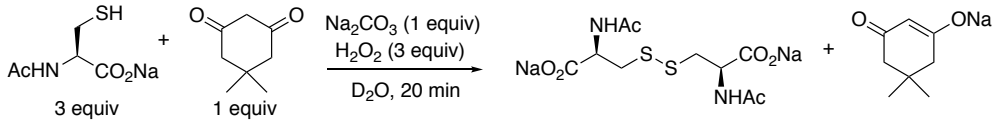
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.05 mmol **19**) with stirring followed by addition of H_2O_2 (20 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of dimedone **19** and *N*-acetylcysteine **100**



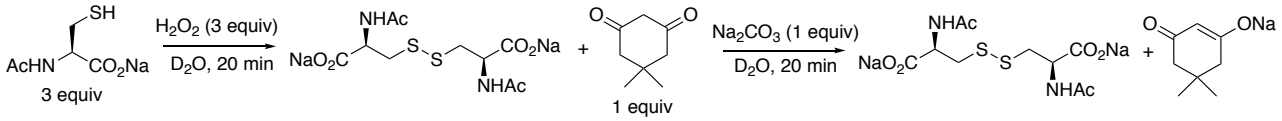
To a vial, solution 1 (0.4 mL, 0.05 mmol **19**) was added followed by solution 2 (0.4 mL, 0.16 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of dimedone **19** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.4 mL, 0.05 mmol **19**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O). After a few seconds of stirring, solution 2 (0.4 mL, 0.16 mmol **100**) was added dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of dimedone enolate with disulfide **102**



To a vial, solution 2 (0.4 mL, 0.16 mmol **100**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O) and stirred for 20 minutes before adding solution 1 (0.4 mL, 0.05 mmol **19**) dropwise over 1 minute. The mixture was left to stir for 20 minutes.

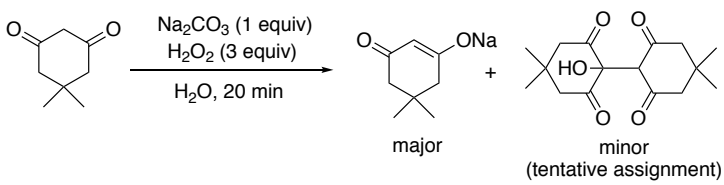
LC-MS studies using dimedone as a probe for cysteine oxidation (not buffered)

For each reaction, stock solutions were prepared for both dimedone (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, a mixture of dimedone (16 mg, 0.11 mmol **19**), H_2O (0.8 mL) and Na_2CO_3 (12 mg, 0.11 mmol) was stirred until fully dissolved.

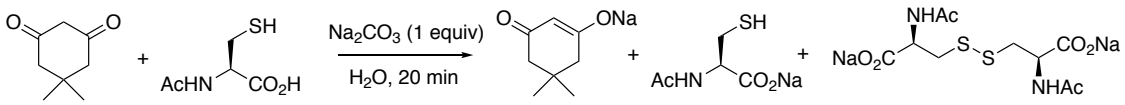
Solution 2: In a vial, a mixture of *N*-acetylcysteine (38 mg, 0.23 mmol **100**), H_2O (0.6 mL) and Na_2CO_3 (9.1 mg, 0.09 mmol) was stirred until fully dissolved.

B: Stability of dimedone enolate in presence of hydrogen peroxide



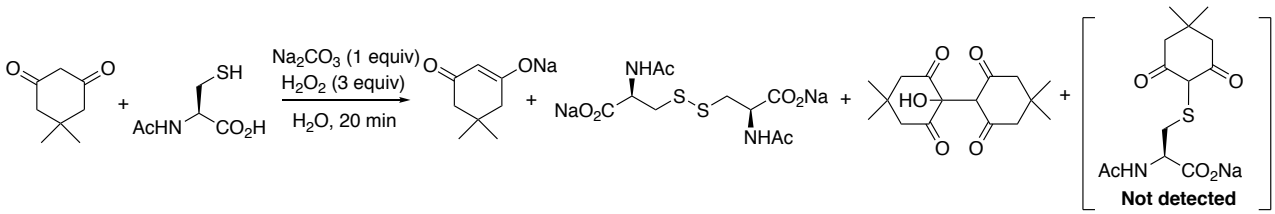
To a vial, H_2O (0.2 mL) was added to solution 1 (0.2 mL, 0.028 mmol **19**) with stirring followed by addition of H_2O_2 (10 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing by LC-MS.

C: Control reaction of dimedone **19** and *N*-acetylcysteine **100**



To a vial, solution 1 (0.2 mL, 0.028 mmol **19**) was added followed by solution 2 (0.2 mL, 0.077 mmol **100**). The solution was stirred for 20 minutes before analysing by LC-MS.

D: Reaction of dimedone **19** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.2 mL, 0.028 mmol **19**) was added followed by H_2O_2 (10 μL, 30 wt% in H_2O). After stirring for a few seconds, solution 2 (0.2 mL, 0.077 mmol **100**) was added dropwise over 1 minute. The reaction mixture was stirred for 20 minutes before analysing by LC-MS.

2.9.3 Cyclooctyne BCN (96) as a probe for cysteine oxidation (not buffered)

NMR studies using cyclooctyne BCN (96) as a probe for cysteine oxidation (not buffered)

For each reaction, stock solutions were prepared for both BCN (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, BCN (20 mg, 0.13 mmol **96**) was dissolved in DMSO-d_6 (0.4 mL) and then D_2O (1.6 mL) and Na_2CO_3 (15 mg, 0.14 mmol) were added and the mixture was stirred or shaken until fully dissolved.

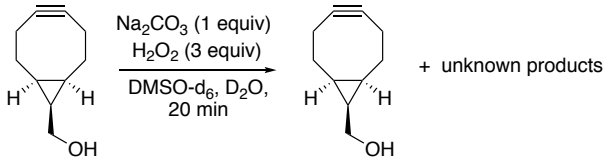
Note: BCN has very low solubility in aqueous media.

Solution 2: *N*-acetylcysteine (60 mg, 0.37 mmol **100**), D_2O (2 mL) and Na_2CO_3 (15 mg, 0.14 mmol) were added to a vial and stirred or shaken until fully dissolved.

A: Stability of BCN **96** in water

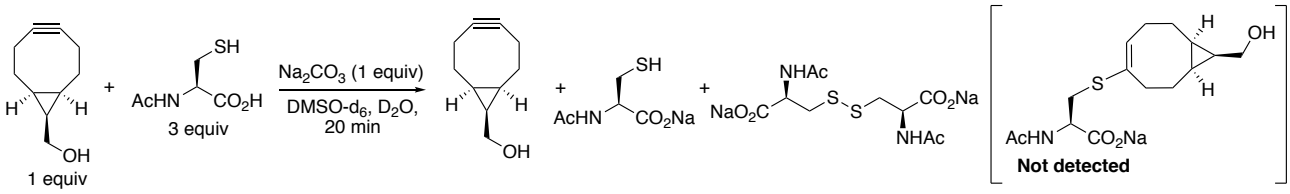
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.026 mmol **96**). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of BCN **96** in presence of hydrogen peroxide



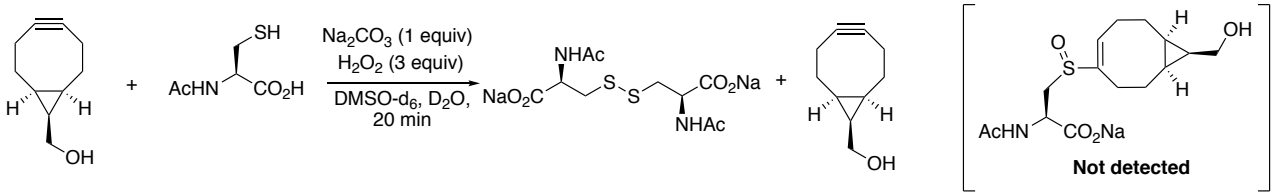
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.026 mmol **96**) with stirring followed by addition of H_2O_2 (20 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of BCN **96** and *N*-acetylcysteine **100**



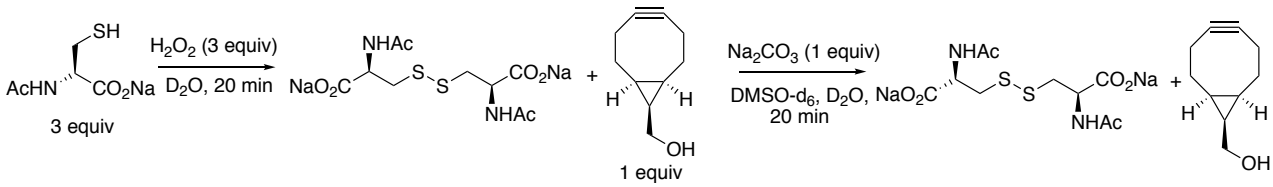
To a vial, solution 1 (0.4 mL, 0.026 mmol **96**) was added followed by solution 2 (0.4 mL, 0.074 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of BCN **96** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.4 mL, 0.026 mmol **96**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O). After stirring for a few seconds, solution 2 (0.4 mL, 0.074 mmol **100**) was added dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of BCN **96** with disulfide **102**



To a vial, solution 2 (0.4 mL, 0.074 mmol **100**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O) and stirred for 20 minutes before adding solution 1 (0.4 mL, 0.026 mmol **96**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

LC-MS studies using cyclooctyne BCN (96) as a probe for cysteine oxidation (not buffered)

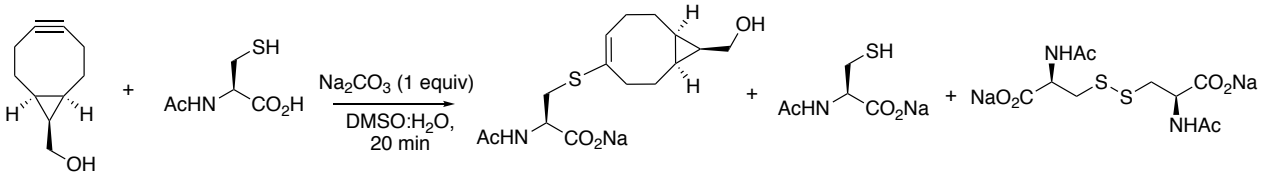
For each reaction, stock solutions were prepared for both BCN (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, BCN (13 mg, 0.09 mmol **96**) was dissolved in DMSO (0.2 mL), followed by the addition of H_2O (0.6 mL) and Na_2CO_3 (12 mg, 0.11 mmol). The mixture was stirred or shaken until fully dissolved.

Note: BCN has very low solubility in aqueous media.

Solution 2: *N*-acetylcysteine (38 mg, 0.23 mmol **100**), H_2O (0.6 mL), and Na_2CO_3 (10 mg, 0.09 mmol) were added to a vial and stirred or shaken until fully dissolved.

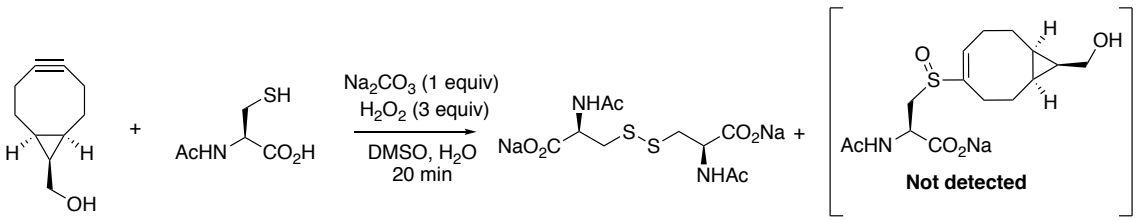
C: Control reaction of BCN **96** and *N*-acetylcysteine **100**



To a vial, solution 1 (0.2 mL, 0.023 mmol **96**) was added followed by solution 2 (0.2 mL, 0.077 mmol **100**). The solution was stirred for 20 minutes before analysing by LC-MS.

Note: BCN itself was not detectable by ESI under the conditions of this experiment.

D: Reaction of BCN **96** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.2 mL, 0.023 mmol **96**) was added followed by H_2O_2 (10 μL, 30 wt% in H_2O). The mixture was stirred for a few seconds before adding solution 2 (0.2 mL, 0.077 mmol **100**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing by LC-MS.

2.9.4 Cyclooctyne DBCO (97) as a probe for cysteine oxidation (not buffered)

NMR studies using cyclooctyne DBCO 97 as a probe for cysteine oxidation (not buffered)

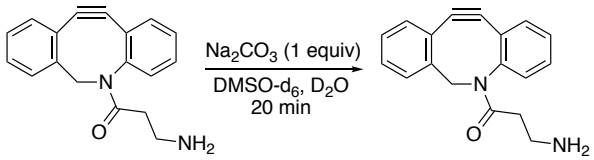
For each reaction, stock solutions were prepared for both DBCO (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, DBCO (35 mg, 0.13 mmol **97**) was dissolved in DMSO-d_6 (0.4 mL) and then D_2O (1.6 mL) and Na_2CO_3 (13 mg, 0.12 mmol) were added. The mixture was stirred or shaken until fully dissolved.

Note: DBCO has very low solubility.

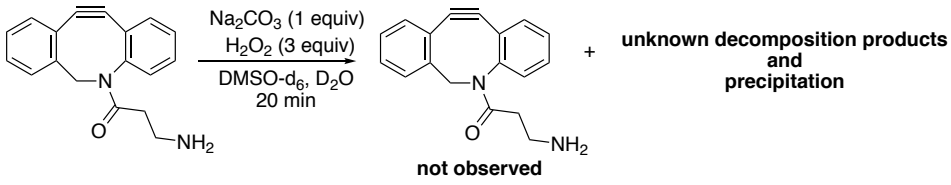
Solution 2: *N*-acetylcysteine (63 mg, 0.39 mmol **100**), D_2O (2 mL) and Na_2CO_3 (13 mg, 0.12 mmol) were added to a vial and stirred or shaken until fully dissolved.

A: Stability of DBCO **97** in water



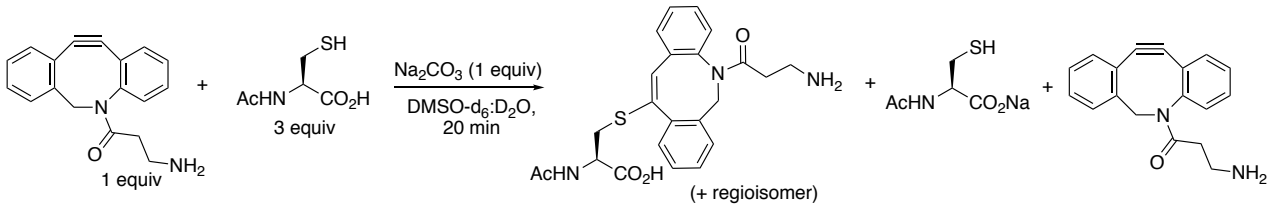
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.026 mmol **97**). The mixture was stirred for 20 minutes before analysing directly by NMR.

B: Stability of DBCO **97** in presence of hydrogen peroxide



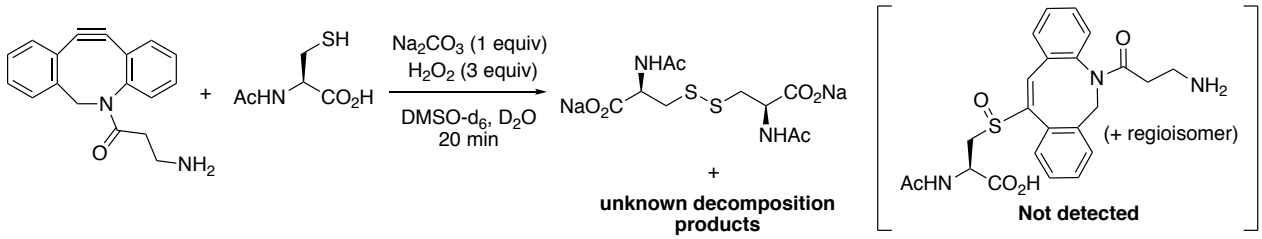
To a vial, D_2O (0.4 mL) was added to solution 1 (0.4 mL, 0.026 mmol **97**) with stirring followed by addition of H_2O_2 (20 μL, 30 wt% in H_2O). The reaction mixture was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of DBCO **97** and *N*-acetylcysteine **100**



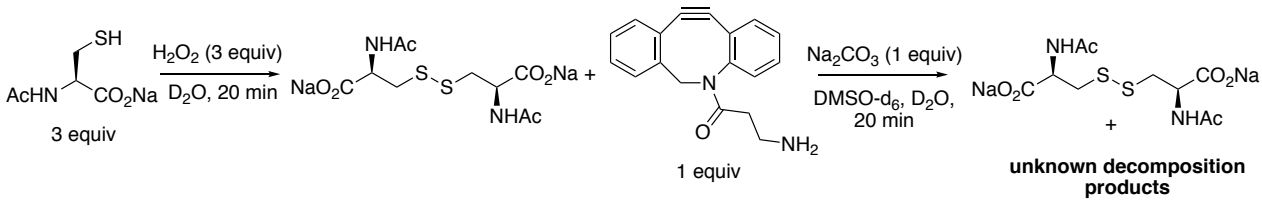
To a vial, solution 1 (0.4 mL, 0.026 mmol **97**) was added followed by solution 2 (0.4 mL, 0.078 mmol **100**). The reaction mixture was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of DBCO **97** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.4 mL, 0.026 mmol **97**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O) and stirred for a few seconds before adding solution 2 (0.4 mL, 0.078 mmol **100**) dropwise over 1 minute. The reaction mixture was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of DBCO **97** with disulfide **102**



To a vial, solution 2 (0.4 mL, 0.026 mmol **100**) was added followed by H_2O_2 (20 μL, 30 wt% in H_2O). The reaction was then stirred for 20 minutes before adding solution 1 (0.4 mL, 0.078 mmol **97**) dropwise over 1 minute. The reaction mixture was stirred for 20 minutes before analysing directly by NMR.

LC-MS studies using cyclooctyne DBCO 97 as a probe for cysteine oxidation (not buffered)

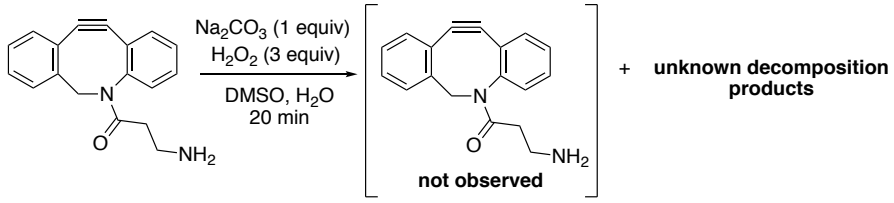
For each reaction, stock solutions were prepared for both the DBCO (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: In a vial, DBCO (28.6 mg, 0.10 mmol **97**) was dissolved in DMSO (0.2 mL) and then H_2O (0.6 mL) and Na_2CO_3 (11 mg, 0.10 mmol) were added. The resulting mixture was stirred or shaken until fully dissolved.

Note: solubility of DBCO was very low.

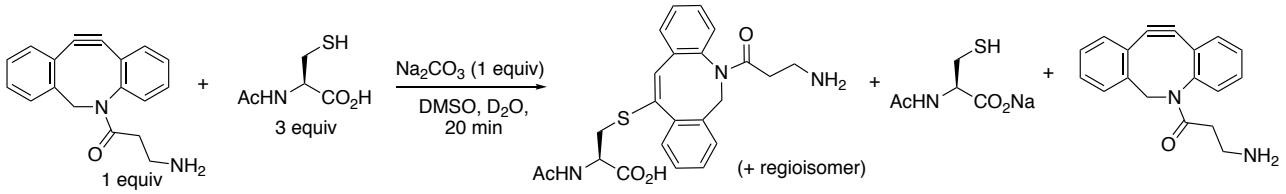
Solution 2: *N*-acetylcysteine (38 mg, 0.24 mmol **100**), H_2O (0.6 mL) and Na_2CO_3 (9.5 mg, 0.09 mmol) were added to a vial and the mixture was stirred or shaken until fully dissolved.

B: Stability of DBCO **97** in presence of hydrogen peroxide



In a vial, H_2O (0.2 mL) was added to solution 1 (0.2 mL, 0.025 mmol **97**) and stirred followed by addition of H_2O_2 (10 μL, 30 wt% in H_2O). The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

C: Control reaction of DBCO **97** and *N*-acetylcysteine **100**



To a vial, solution 1 (0.2 mL, 0.025 mmol **97**) was added followed by solution 2 (0.2 mL, 0.077 mmol **100**). The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

2.9.5 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

NMR studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

Preparation of buffer: In a beaker, sodium acetate trihydrate (1.35 g, 0.010 mol) was dissolved in approx. 20 mL of D_2O. The pH was adjusted by adding acetic acid until a reading of pH = 4.60 was measured using a pH meter, which corresponds to a pD = 5.0. The solution was then diluted to 25 mL with D_2O using a volumetric flask so that the concentration of the buffer was 400 mM.

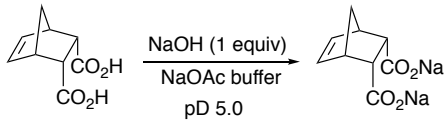
For each reaction, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1), *N*-acetylcysteine (solution 2), and *N*-acetylcysteine disulfide (solution 3).

Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (17 mg, 0.09 mmol **99**), D_2O (1.0 mL), and NaOH (3.8 mg, 0.09 mmol) were added to a vial and stirred or shaken until dissolved. Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1.0 mL) was added and the solution was stirred.

Solution 2: *N*-acetylcysteine (42 mg, 0.26 mmol **100**), D_2O (1.0 mL), and NaOH (3.3 mg, 0.08 mmol) were added to a vial and shaken or stirred until fully dissolved. Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1.0 mL) was added and the solution was stirred.

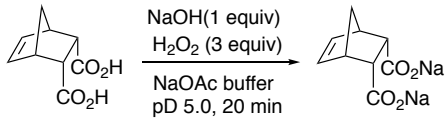
Solution 3: *N*-acetylcysteine (9.8 mg, 0.06 mmol **100**), D_2O (0.25 mL), and NaOH (2.4 mg, 0.06 mmol) were added to a vial and stirred or shaken until fully dissolved. A solution of H_2O_2 (15 μL, 30 wt% in H_2O) was then added and the reaction was stirred for 1 hour or until *N*-acetylcysteine was fully converted to the disulfide (as monitored by ^{1}H-NMR). Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 0.25 mL) was added and the solution was stirred.

A: Stability of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** in water



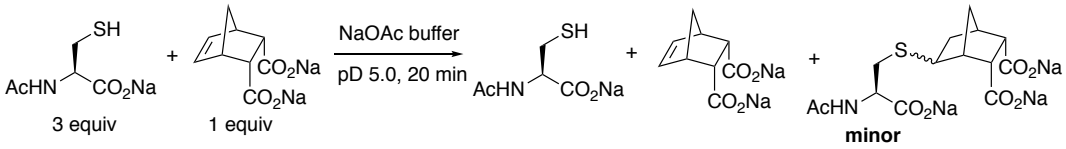
To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by pD 5.0 acetate buffer (0.5 mL, 200 mM). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** in presence of hydrogen peroxide



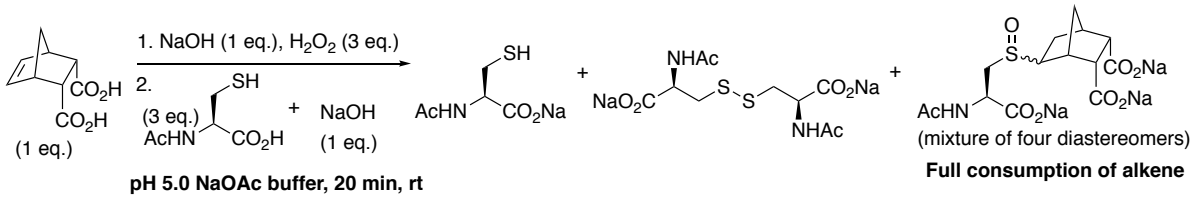
To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by pD 5.0 acetate buffer (0.5 mL, 200 mM) and H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** and *N*-acetylcysteine **100**



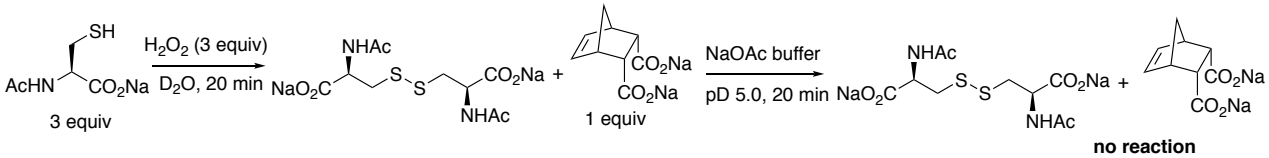
To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The resulting solution was stirred for a few seconds before the dropwise addition of solution 2 (0.5 mL, 0.06 mmol **100**) over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** with disulfide **102**



To a vial, solution 3 (0.5 mL, 0.06 mmol **100**) was added followed by dropwise addition of solution 1 (0.5 mL, 0.02 mmol **99**). The solution was stirred for 20 minutes before analysing directly by NMR.

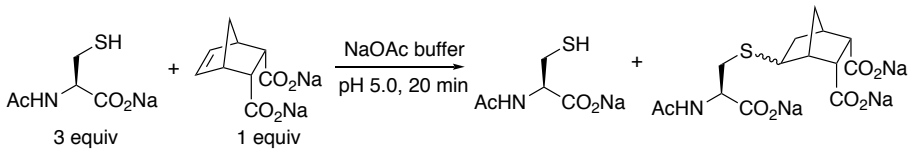
LC-MS studies using cis-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation at pH 5.0 (NaOAc buffer, 200 mM)

For each reaction, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (14.8 mg, 0.08 mmol **99**), H_2O (1.0 mL) and NaOH (3.8 mg, 0.09 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, pH 5.0 sodium acetate buffer in H_2O (400 mM, 1.0 mL) was added and the solution was stirred.

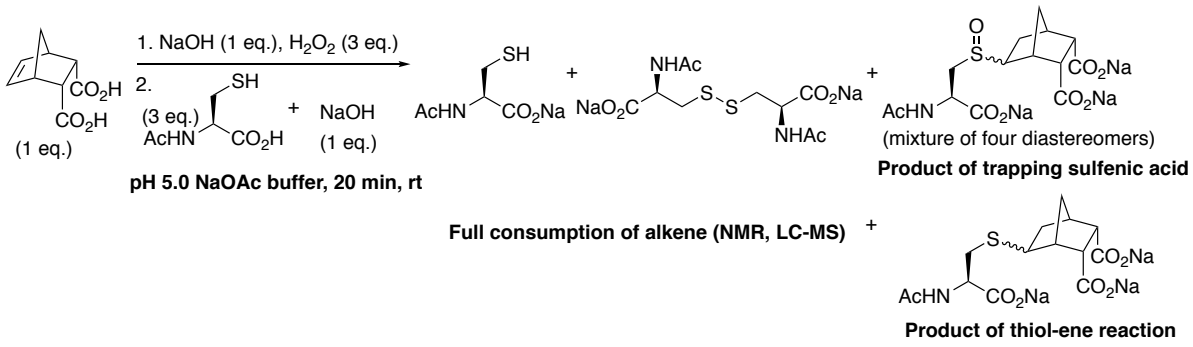
Solution 2: *N*-acetylcysteine (38.1 mg, 0.23 mmol **100**), H_2O (1.0 mL), and NaOH (3.1 mg, 0.08 mmol) were added to a vial and stirred to dissolve. Finally, pH 5.0 sodium acetate buffer in H_2O (400 mM, 1.0 mL) was added and the solution was stirred.

C: Control reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** and *N*-acetylcysteine **100**



To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

D: Reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was then stirred for a few seconds before dropwise addition of solution 2 (0.5 mL, 0.06 mmol **100**) over 1 minute. The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

2.9.6 NMR studies showing *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) does not react with sulfinates or sulfonates at pD 5.0 (NaOAc buffer, 200 mM)

Preparation of buffer: In a beaker, sodium acetate trihydrate (0.53 g, 0.004 mol) was dissolved in approximately 8 mL of D2O. The pH was adjusted by adding acetic acid until a reading of pH = 4.60 was measured using a pH meter, which corresponds to a pD = 5.0. The solution was then diluted to 10 mL with D2O using a volumetric flask so that the concentration of the buffer was 400 mM.

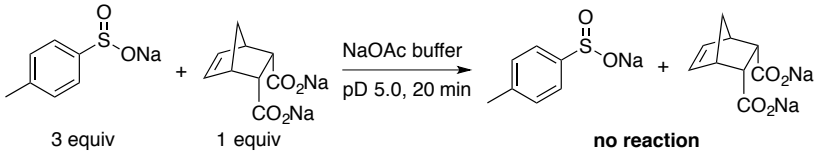
For each reaction, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1), sodium *p*-toluenesulfinate (solution 2), and sodium *p*-toluenesulfonate (solution 3).

Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (17.4 mg, 0.1 mmol **99**), D2O (1.0 mL), and NaOH (3.3 mg, 0.08 mmol) were added to a vial and stirred or shaken until dissolved. The resulting solution was then diluted with the pD 5.0 acetate buffer in D2O (400 mM, 1.0 mL).

Solution 2: sodium *p*-toluenesulfinate (43.2 mg, 0.24 mmol) and D2O (1.0 mL) were added to a vial and shaken or stirred until fully dissolved. The resulting solution was then diluted with the pD 5.0 acetate buffer in D2O (400 mM, 1.0 mL).

Solution 3: *p*-toluenesulfonic acid (48.9 mg, 0.23 mmol), D2O (1.0 mL), and NaOH (3.2 mg, 0.08 mmol) were added to a vial and stirred or shaken until dissolved. The resulting solution was then diluted with the pD 5.0 acetate buffer in D2O (400 mM, 1.0 mL).

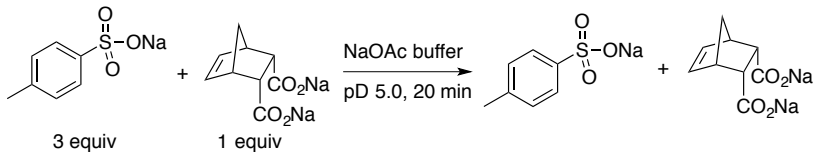
A: No reaction between **99** and sodium *p*-toluenesulfinate



3 equiv 1 equiv **no reaction**

To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 2 (0.5 mL, 0.06 mmol sodium *p*-toluenesulfinate). The solution was stirred for 20 minutes before analysing directly by NMR.

B: No reaction between **99** and sodium *p*-toluenesulfonate



3 equiv 1 equiv

To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 3 (0.5 mL, 0.06 mmol sodium *p*-toluenesulfonate). The solution was stirred for 20 minutes before analysing directly by NMR.

2.9.7 oxa-norbornene dicarboxylic acid (107) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

NMR studies using oxa-norbornene dicarboxylic acid (107) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

Preparation of buffer: as previous.

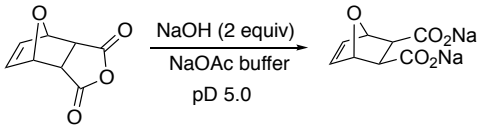
For each reaction, stock solutions were prepared for both oxa-norbornene dicarboxylic acid (solution 1), *N*-acetylcysteine (solution 2), and *N*-acetylcysteine disulfide (solution 3). These solutions were used for all experiments.

Solution 1: oxa-norbornene anhydride (14.1 mg, 0.085 mmol), D_2O (1.0 mL), and NaOH (6.5 mg, 0.16 mmol) were added to a vial and stirred or shaken until dissolved. Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1.0 mL) was added and the solution was stirred.

Solution 2: *N*-acetylcysteine (64.7 mg, 0.40 mmol **100**), D_2O (1.5 mL), and NaOH (5.2 mg, 0.13 mmol) were added to a vial and shaken or stirred until fully dissolved. Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1.5 mL) was added and the solution was stirred.

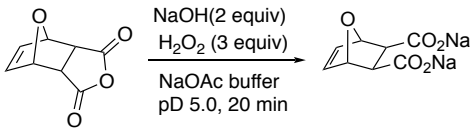
Solution 3: *N*-acetylcysteine (42.3 mg, 0.06 mmol **100**), D_2O (1 mL), and NaOH (2.4 mg, 0.06 mmol) were added to a vial and stirred or shaken until fully dissolved. A solution of H_2O_2 (28 μL, 30 wt% in H_2O) was then added and the reaction was stirred for 1 hour or until *N*-acetylcysteine was fully converted to the disulfide (as monitored by ^{1}H-NMR). Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1 mL) was added and the solution was stirred.

A: Stability of oxa-norbornene dicarboxylic acid **107** in pH 5



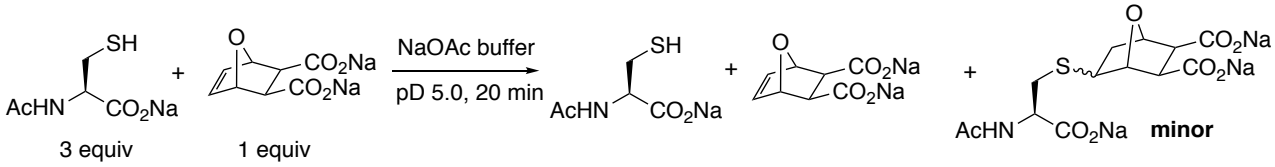
To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by pD 5.0 acetate buffer (0.5 mL, 200 mM). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of oxa-norbornene dicarboxylic acid **107** in presence of hydrogen peroxide



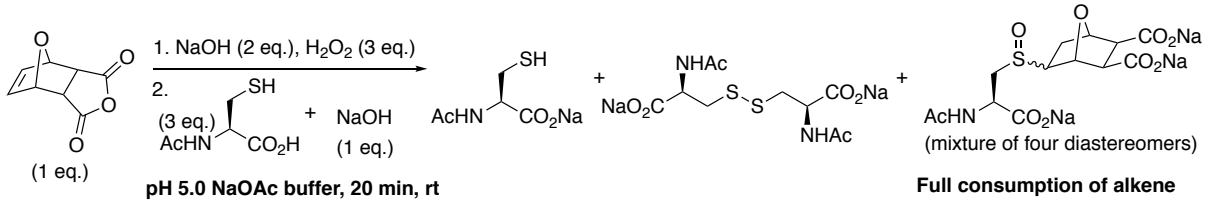
To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by pD 5.0 acetate buffer (0.5 mL, 200 mM) and H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of oxa-norbornene dicarboxylic acid **107** and *N*-acetylcysteine **100**



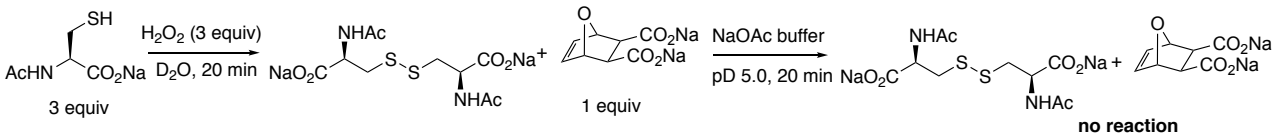
To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of oxa-norbornene dicarboxylic acid **107** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The resulting solution was stirred for a few seconds before the dropwise addition of solution 2 (0.5 mL, 0.06 mmol **100**) over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of oxa-norbornene dicarboxylic acid **107** with disulfide **102**



To a vial, solution 3 (0.5 mL, 0.06 mmol **100**) was added followed by dropwise addition of solution 1 (0.5 mL, 0.02 mmol **107**). The solution was stirred for 20 minutes before analysing directly by NMR.

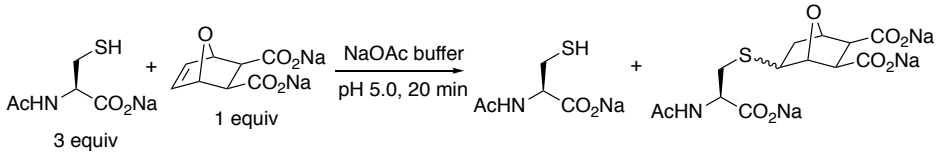
LC-MS studies using oxa-norbornene-dicarboxylic acid 107 as a probe for cysteine oxidation at pH 5.0 (NaOAc buffer, 200 mM)

For each reaction, stock solutions were prepared for both oxa-norbornene dicarboxylic acid (solution 1) and *N*-acetylcysteine (solution 2) and used for all experiments.

Solution 1: oxa-norbornene anhydride (13.7 mg, 0.074 mmol), H_2O (1.0 mL) and NaOH (6.5 mg, 0.16 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, pH 5.0 sodium acetate buffer in H_2O (400 mM, 1.0 mL) was added and the solution was stirred.

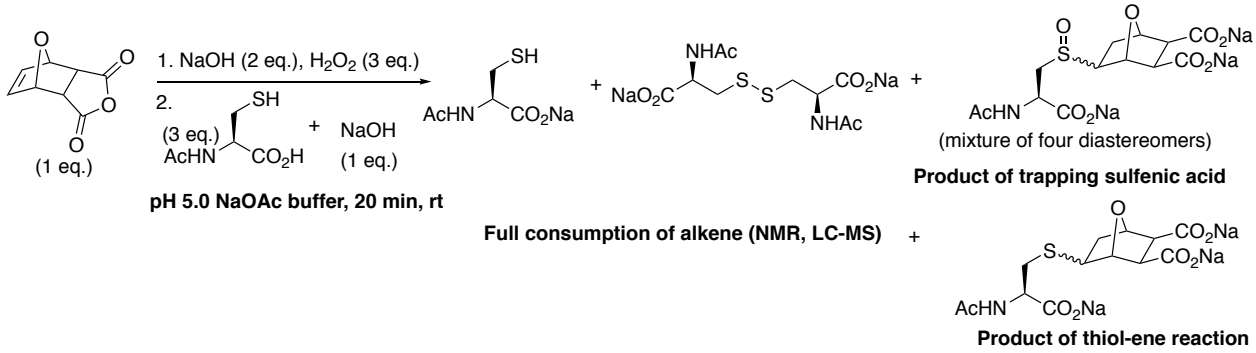
Solution 2: *N*-acetylcysteine (30.2 mg, 0.19 mmol **100**), H_2O (1.0 mL), and NaOH (3.0 mg, 0.075 mmol) were added to a vial and stirred to dissolve. Finally, pH 5.0 sodium acetate buffer in H_2O (400 mM, 1.0 mL) was added and the solution was stirred.

C: Control reaction of oxa-norbornene dicarboxylic acid **107** and *N*-acetylcysteine **100**



To a vial, solution 1 (0.5 mL, 0.018 mmol **107**) was added followed by solution 2 (0.5 mL, 0.046 mmol **100**). The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

D: Reaction of oxa-norbornene dicarboxylic acid **107** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.018 mmol **107**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was then stirred for a few seconds before dropwise addition of solution 2 (0.5 mL, 0.046 mmol **100**) over 1 minute. The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

2.9.8 Thiol-ene test at pH 5

For each reaction, stock solutions were prepared for *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 2), oxa-norbornene dicarboxylic acid (solution 3), and *N*-acetylcysteine (solution 4). These solutions were used for all experiments.

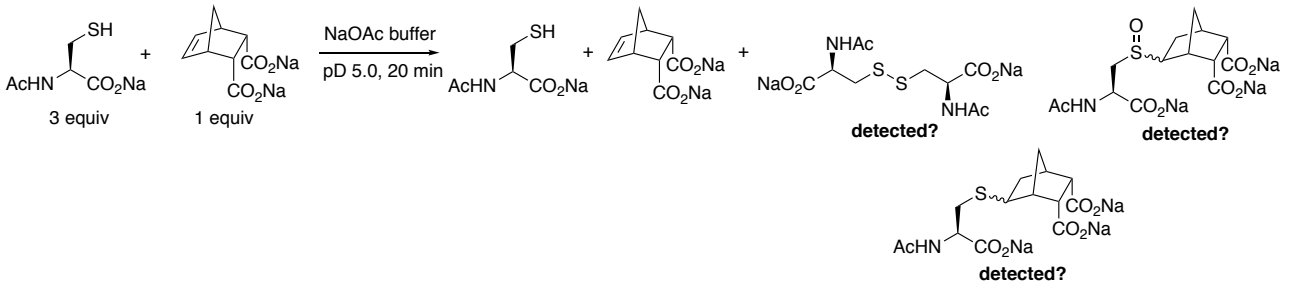
Solution 1: NaOH (4.6 mg, 0.12 mmol) was dissolved in D_2O (1 mL).

Solution 2: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (4.2 mg, 0.02 mmol **99**) was dissolved in solution 1 (0.25 mL) before adding pD 5.0 acetate buffer in D_2O (400 mM, 0.25 mL).

Solution 3: oxa norbornene anhydride (3.6 mg, 0.02 mmol) was dissolved in solution 1 (0.25 mL) before adding pD 5.0 acetate buffer in D_2O (400 mM, 0.25 mL).

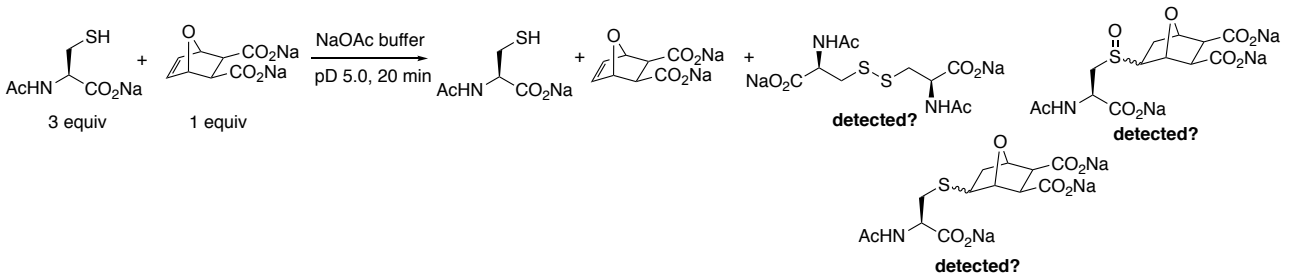
Solution 4: *N*-acetylcysteine (19.6 mg, 0.12 mmol **100**) was dissolved in solution 1 (0.5 mL) before adding pD 5.0 acetate buffer in D_2O (400 mM, 0.5 mL).

***cis*-5-norbornene-endo-2,3-dicarboxylic acid 99 thiol-ene test**



To a vial, solution 2 (0.5 mL, 0.02 mmol **99**) was added followed by drop-wise addition of solution 4 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 3 days before analysing directly by ^{1}H-NMR.

Oxa norbornene dicarboxylic acid 107 thiol-ene test



To a vial, solution 3 (0.5 mL, 0.02 mmol **107**) was added followed by drop-wise addition of solution 4 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 3 days before analysing directly by ^{1}H-NMR.

2.9.9 Dimedone (19) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

NMR studies using dimedone (19) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

Preparation of buffer: as previous.

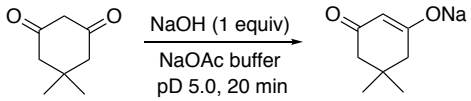
For each reaction, stock solutions were prepared for both dimedone (solution 1), *N*-acetylcysteine (solution 2), and *N*-acetylcysteine disulfide (solution 3). These solutions were used for all experiments.

Solution 1: Dimedone (13 mg, 0.09 mmol **19**), D_2O (1.0 mL) and NaOH (3.4 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1.0 mL) was added and the solution was stirred.

Solution 2: *N*-acetylcysteine (42 mg, 0.26 mmol **100**), D_2O (1.0 mL) and NaOH (3.3 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 1.0 mL) was added and the solution was stirred.

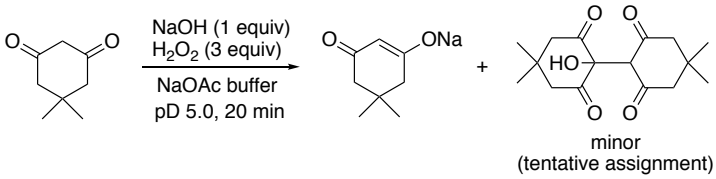
Solution 3: *N*-acetylcysteine (9.8 mg, 0.06 mmol **100**), D_2O (0.25 mL) and NaOH (2.4 mg, 0.06 mmol) were added to a vial and stirred or shaken until fully dissolved. A solution of H_2O_2 (15 μL, 30 wt% in H_2O) was then added and stirred for 1 hr or until *N*-acetylcysteine was fully converted to the disulfide (as monitored by ^{1}H-NMR). Finally, the pD 5.0 acetate buffer in D_2O (400 mM, 0.25 mL) was added and the solution was stirred.

A: Stability of dimedone **19** in pH 5



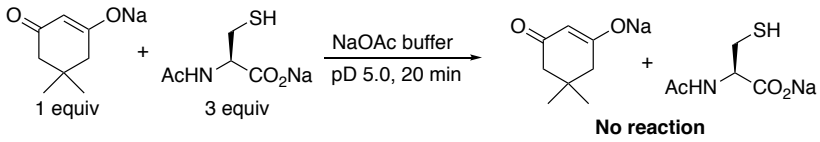
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by pD 5.0 acetate buffer in D_2O (0.5 mL, 200 mM). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of dimedone **19** in presence of hydrogen peroxide



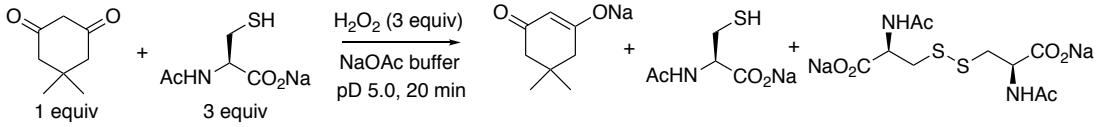
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by pD 5.0 acetate buffer in D_2O (0.5 mL, 200 mM) and H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of dimedone **19** and *N*-acetylcysteine **100**



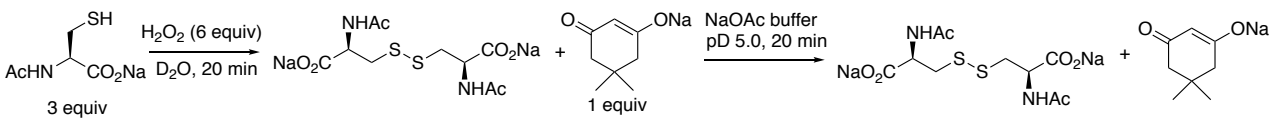
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of dimedone **19** with *N*-acetylcysteine sulfenic acid **101**



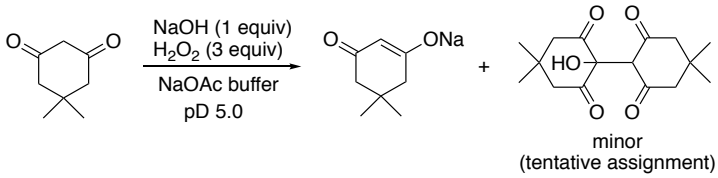
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O) and stirred for a few minutes before dropwise addition of solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of dimedone **19** with disulfide **102**



To a vial, solution 3 (0.5 mL, 0.06 mmol **100**) was added followed by dropwise addition of solution 1 (0.5 mL, 0.02 mmol **19**). The solution was stirred for 20 minutes before analysing directly by NMR.

NMR comparison for the reaction of dimedone enolate with H_2O_2 from 20 mins to 24 hrs.



Reaction B. was then left for a further 24 h at room temperature and analysed directly by NMR.

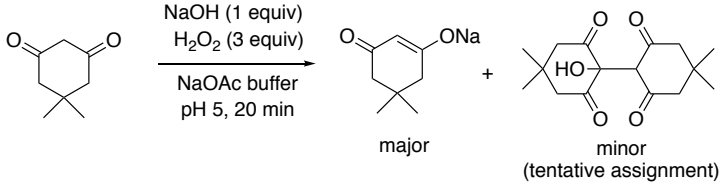
LC-MS studies using dimedone as a probe for cysteine oxidation at pH 5.0 (NaOAc buffer, 200 mM)

For each reaction, stock solutions were prepared for both dimedone (solution 1) and *N*-acetylcysteine (solution 2).

Solution 1: dimedone (12 mg, 0.09 mmol **19**), H_2O (1.0 mL), and NaOH (3.0 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, pH 5.0 acetate buffer in H_2O (400 mM, 1.0 mL) was added and the solution was stirred.

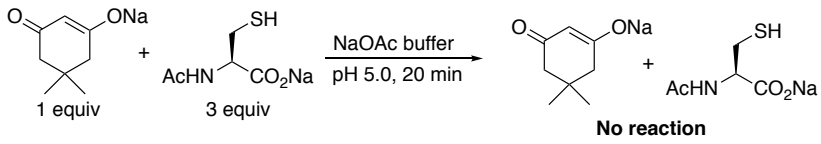
Solution 2: *N*-acetylcysteine (31.0 mg, 0.19 mmol **100**), H_2O (1.0 mL), and NaOH (3.0 mg, 0.08 mmol) were added to a vial and stirred or shaken until fully dissolved. Finally, the pH 5.0 acetate buffer in H_2O (400 mM, 1.0 mL) was added and the solution was stirred.

B: Stability of dimedone **19** in presence of hydrogen peroxide



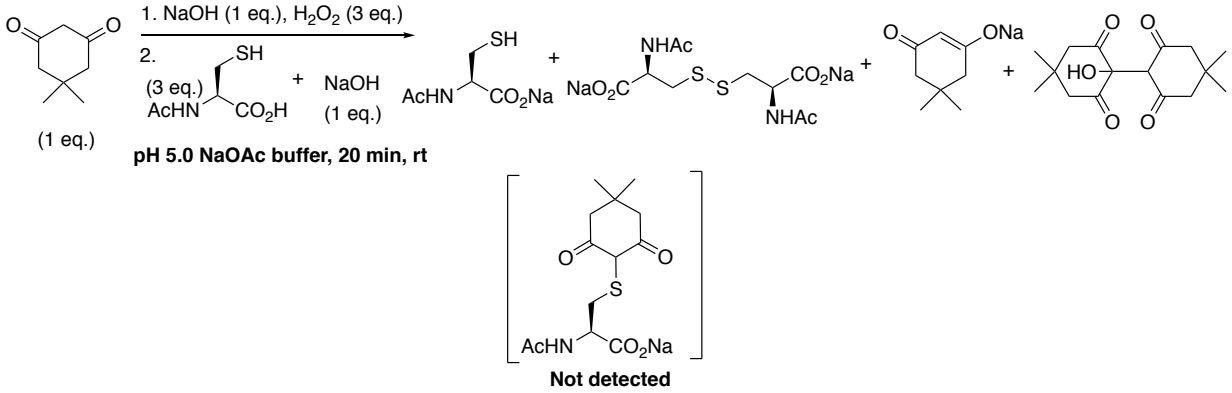
To a vial, solution 1 (0.5 mL, 0.02 mmol, **19**) was added followed by acetate buffer (0.5 mL, 200 mM) and H_2O_2 (7 μL, 30 wt% in H_2O). The reaction mixture was stirred for 20 minutes before analysing directly by LC-MS.

C: Control reaction of dimedone **19** and *N*-acetylcysteine **100**



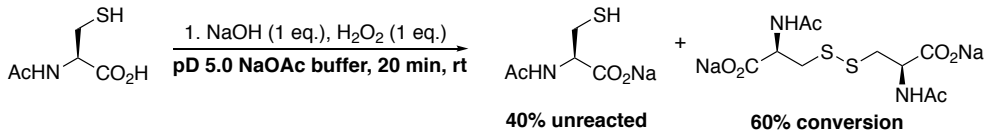
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by solution 2 (0.5 mL, 0.05 mmol **100**). The resulting solution was stirred for 20 minutes before analysing directly by LC-MS.

D: Reaction of dimedone **19** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The resulting solution was stirred for a few minutes before dropwise addition of solution 2 (0.5 mL, 0.05 mmol **100**). The resulting solution was stirred for 20 minutes before analysing directly by LC-MS.

2.9.10 Oxidation of N-acetylcysteine 100 to its disulfide 102 using hydrogen peroxide at pD 5.0 (sodium acetate buffer)



N-acetylcysteine (9.8 mg, 0.06 mmol **100**), D_2O (0.5 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 D_2O, 0.5 mL) was added and the solution was stirred. Finally, a solution of H_2O_2 (7 μL, 30 wt% in H_2O) was added and the reaction was stirred for 20 minutes before analysing directly by ^{1}H-NMR.

2.9.11 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

NMR studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

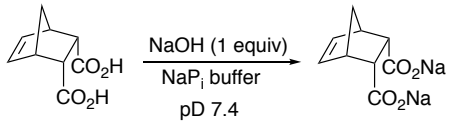
For each reaction, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1) and *N*-acetylcysteine (solution 2).

Buffer: In a beaker, sodium phosphate monobasic (0.63 g, 0.004 mol) was dissolved in approximately 8 mL of D2O. The pH was adjusted by adding acetic acid until a reading of pH = 7.0 was measured using a pH meter, which corresponds to a pD = 7.4. The solution was then diluted to 10 mL with D2O using a volumetric flask so that the concentration of the buffer was 400 mM.

Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (20.0 mg, 0.11 mmol **99**), D_2O (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_2O, 1.25 mL) was added and the solution was stirred.

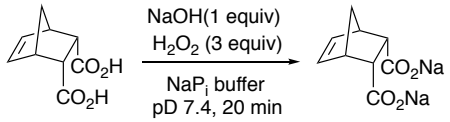
Solution 2: *N*-acetylcysteine (49.8 mg, 0.30 mmol **100**), D_2O (1.25 mL) and NaOH (3 mg, 0.08 mmol) were added to a vial and stirred until fully dissolved. Finally, additional pD 7.4 sodium phosphate buffer (400 mM in D_2O, 1.25 mL) was added and the solution was stirred.

A: Stability of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** in pH 7.4



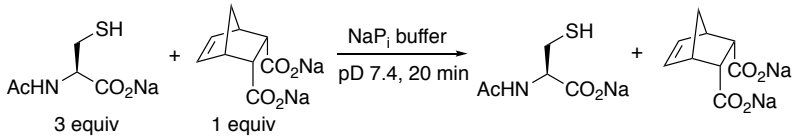
To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by pD 7.4 phosphate buffer (0.5 mL, 200 mM). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** in presence of hydrogen peroxide



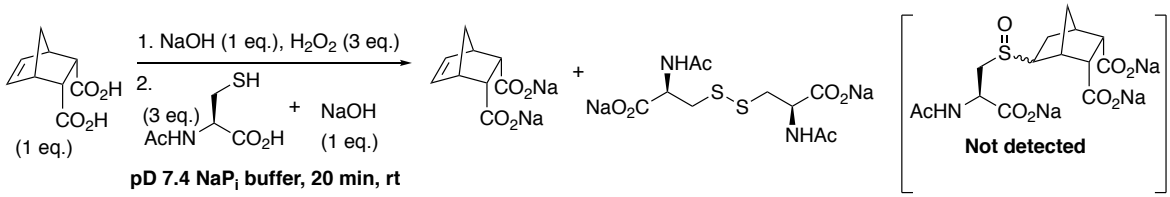
To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by pD 7.4 phosphate buffer (0.5 mL, 200 mM) and H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** and *N*-acetylcysteine **100**



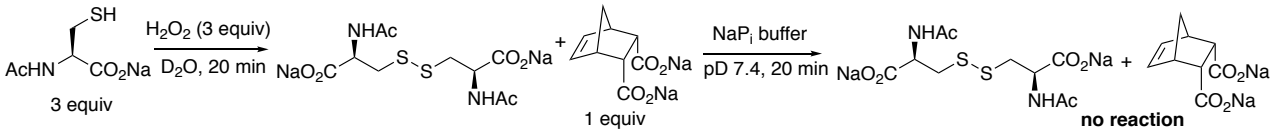
To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for a few minutes before adding solution 2 (0.5 mL, 0.06 mmol **100**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** with disulfide **102**



To a vial, solution 2 (0.5 mL, 0.06 mmol **100**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 1 hour to ensure complete conversion to disulfide **102** before adding solution 1 (0.5 mL, 0.02 mmol **99**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

2.9.12 oxa-norbornene dicarboxylic acid 107 as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

NMR studies using oxa-norbornene dicarboxylic acid 107 as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

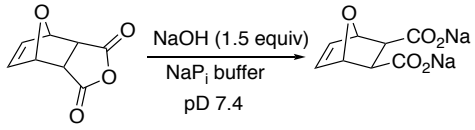
For each reaction, stock solutions were prepared for both oxa-norbornene dicarboxylic acid (solution 1) and *N*-acetylcysteine (solution 2).

Buffer: as previous.

Solution 1: oxa-norbornene anhydride (17.6 mg, 0.11 mmol), D_2O (1.25 mL) and NaOH (6.4 mg, 0.16 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_2O, 1.25 mL) was added and the solution was stirred.

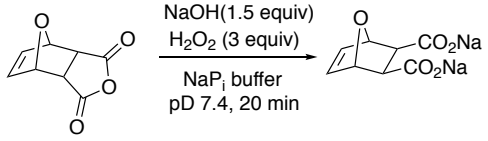
Solution 2: *N*-acetylcysteine (31 mg, 0.19 mmol **100**), D_2O (0.75 mL) and NaOH (3 mg, 0.08 mmol) were added to a vial and stirred until fully dissolved. Finally, additional pD 7.4 sodium phosphate buffer (400 mM in D_2O, 5 mL) was added and the solution was stirred.

A: Stability of oxa-norbornene dicarboxylic acid **107** in pH 7.4



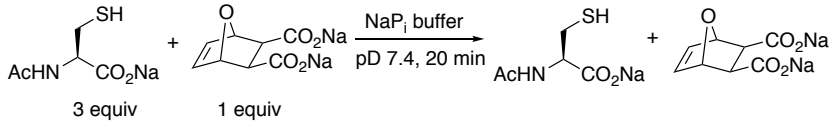
To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by pD 7.4 phosphate buffer (0.5 mL, 200 mM). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of oxa-norbornene dicarboxylic acid **107** in presence of hydrogen peroxide



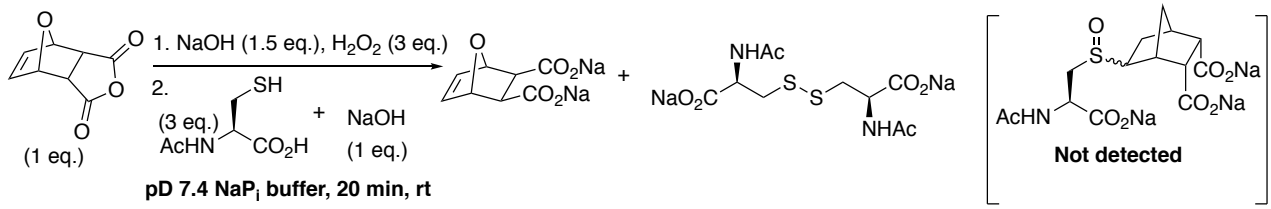
To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by pD 7.4 phosphate buffer (0.5 mL, 200 mM) and H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of oxa-norbornene dicarboxylic acid **107** and *N*-acetylcysteine **100**



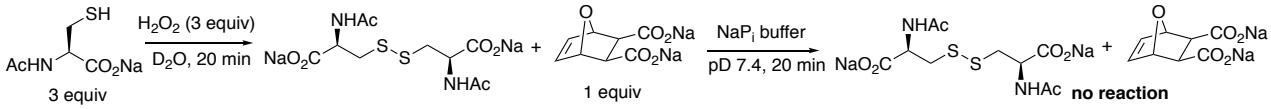
To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of oxa-norbornene dicarboxylic acid **107** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **107**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for a few minutes before adding solution 2 (0.5 mL, 0.06 mmol **100**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of oxa-norbornene dicarboxylic acid **107** with disulfide **102**



To a vial, solution 2 (0.5 mL, 0.06 mmol **100**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for 1 hour to ensure complete conversion to disulfide **102** before adding solution 1 (0.5 mL, 0.02 mmol **107**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

2.9.13 Dimedone (19) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

NMR studies using dimedone (19) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

Buffer: as previous

For each reaction, stock solutions were prepared for dimedone (solution 1), *N*-acetylcysteine (solution 2), and *N*-acetylcysteine disulfide (solution 3).

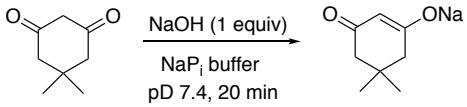
Solution 1: Dimedone (19 mg, 0.13 mmol **19**), D_2O (1.6 mL), and NaOH (5.1 mg, 0.13 mmol) were

added to a vial and stirred or shaken until dissolved. Finally, the pD 7.4 phosphate buffer in D_2O (400 mM, 1.6 mL) was added and the solution was stirred.

Solution 2: *N*-acetylcysteine (38 mg, 0.23 mmol **100**), D_2O (0.75 mL), and NaOH (2.4 mg, 0.06 mmol) were added to a vial and shaken or stirred until fully dissolved. Finally, the pD 7.4 phosphate buffer in D_2O (400 mM, 0.75 mL) was added and the solution was stirred.

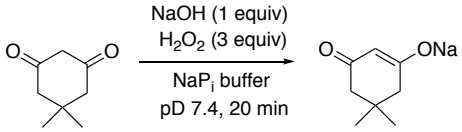
Solution 3: *N*-acetylcysteine (12.6 mg, 0.08 mmol **100**), D_2O (0.25 mL), and NaOH (1 mg, 0.03 mmol) were added to a vial and stirred or shaken until dissolved. A solution of H_2O_2 (15 µL, 30 wt% in H_2O) was then added and stirred for 1 hr or until *N*-acetylcysteine was fully converted to the disulfide (as monitored by ^{1}H-NMR). Finally, the pD 7.4 phosphate buffer in D_2O (400 mM, 0.25 mL) was added and the solution was stirred.

A: Stability of dimedone **19** in pH 7.4



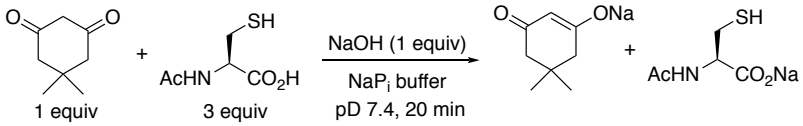
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by pD 7.4 phosphate buffer (0.5 mL, 200 mM). The solution was stirred for 20 minutes before analysing directly by NMR.

B: Stability of dimedone **19** in presence of hydrogen peroxide



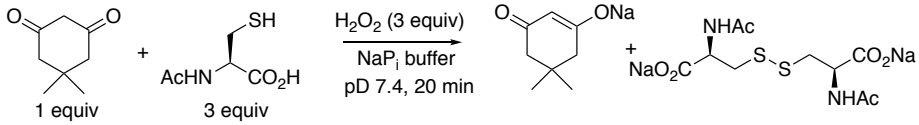
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by pD 7.4 phosphate buffer (0.5 mL, 200 mM) and H_2O_2 (7 µL, 30 wt% in H_2O). The solution was stirred for 20 minutes before analysing directly by NMR.

C: Control reaction of dimedone **19** and *N*-acetylcysteine **100**



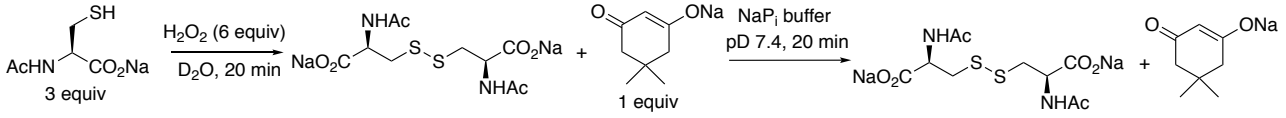
To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by solution 2 (0.5 mL, 0.06 mmol **100**). The solution was stirred for 20 minutes before analysing directly by NMR.

D: Reaction of dimedone **19** with *N*-acetylcysteine sulfenic acid **101**



To a vial, solution 1 (0.5 mL, 0.02 mmol **19**) was added followed by H_2O_2 (7 μL, 30 wt% in H_2O). The solution was stirred for a few minutes before adding solution 2 (0.5 mL, 0.06 mmol **100**) dropwise over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

E: Control reaction of dimedone **19** with disulfide **102**



To a vial, solution 3 (0.5 mL, 0.06 mmol **100**) was added followed by dropwise addition of solution 1 (0.5 mL, 0.02 mmol **19**) over 1 minute. The solution was stirred for 20 minutes before analysing directly by NMR.

2.9.14 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) does not react with sulfinates or sulfonates at pD 7.4 (NaH_2PO_4 buffer, 200 mM)

NMR studies showing *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) does not react with sulfinates or sulfonates at pD 7.4 (NaH_2PO_4 buffer, 200 mM)

Preparation of buffer: as previous.

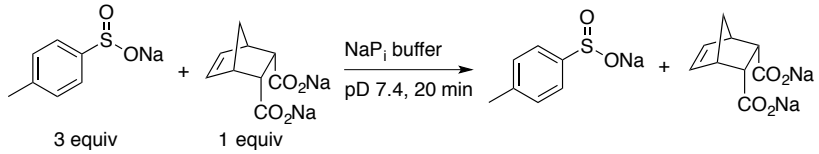
For each reaction, stock solutions were prepared for both *cis*-5-norbornene-endo-2,3-dicarboxylic acid (solution 1), sodium *p*-toluenesulfinate (solution 2), and sodium *p*-toluenesulfonate (solution 3).

Solution 1: *cis*-5-norbornene-endo-2,3-dicarboxylic acid (17.4 mg, 0.1 mmol **99**), D2O (1.0 mL), and NaOH (3.3 mg, 0.08 mmol) were added to a vial and stirred or shaken until dissolved. Finally, this solution was diluted with the pD 7.4 phosphate buffer in D2O (400 mM, 1.0 mL).

Solution 2: sodium *p*-toluenesulfinate (43.2 mg, 0.24 mmol) and D2O (1.0 mL) were added to a vial and shaken or stirred until fully dissolved. Finally, this solution was diluted with the pD 7.4 phosphate buffer in D2O (400 mM, 1.0 mL).

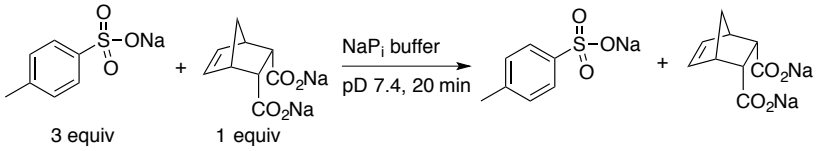
Solution 3: *p*-toluenesulfonic acid (48.9 mg, 0.23 mmol), D2O (1.0 mL), and NaOH (3.2 mg, 0.08 mmol) were added to a vial and stirred or shaken until dissolved. Finally, this solution was diluted with the pD 7.4 phosphate buffer in D2O (400 mM, 1.0 mL).

A: No reaction between **99** and sodium *p*-toluenesulfinate



To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 2 (0.5 mL, 0.06 mmol sodium *p*-toluenesulfinate). The solution was stirred for 20 minutes before analysing directly by NMR.

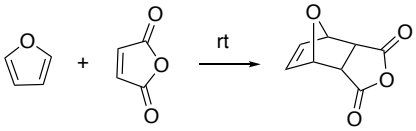
B: No reaction between **99** and sodium *p*-toluenesulfonate



To a vial, solution 1 (0.5 mL, 0.02 mmol **99**) was added followed by solution 3 (0.5 mL, 0.06 mmol sodium *p*-toluenesulfonate). The solution was stirred for 20 minutes before analysing directly by NMR.

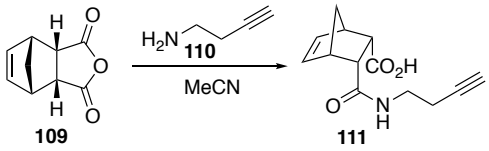
2.9.15 Probe synthesis

Synthesis of oxa-norbornene anhydride (known compound)



Furan (3 mL, 0.04 mol) was added slowly to maleic anhydride (4.05 g, 0.04 mol) and the reaction stirred until all the maleic anhydride dissolved. The reaction mixture was stirred for a further 1 hr or until the reaction mixture solidified. The solid was diluted with DCM and filtered to give the crude white solid. The crude was recrystallised from acetone until just dissolved, followed by slow addition of hexane until recrystallisation occurred, filtered and dried to give the title compound as a white solid (1.8 g, 26 %); m.p. 109-112 °C (lit. 116 °C); δ_H (600 MHz, $CDCl_3$) 6.58 (2H, s, HC=CH), 5.46 (2H, s, CHCHOCH), 3.17 (2H, s, C=OCH); δ_C (150 MHz, 150) 169.8, 137.0, 82.2, 48.7. reported values match literature.[173]

Synthesis of alkyne probe 111 (synthesis initially performed by Kyle Farrell)



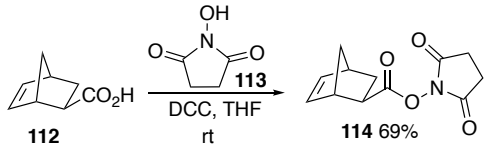
1-amino-3-butyne (60 μL, 0.72 mmol) was added to a stirred solution of *cis*-5-norbornene-*endo*-2,3-dicarboxylic anhydride (234 mg, 1.45 mmol) in acetonitrile (2 mL) and stirred at room temperature for 20 minutes, over which time a white precipitate formed. The resulting mixture was transferred into 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 **111** as a white solid (110mg, 65 % yield): m.p. 129 ºC; R_f (2.5 % MeOH:DCM) 0.19; IR (ν_{max}, ATR): 3359, 2987, 1716, 1622, 1550, 1321, 1267, 1229, 1074, 846, 759, 679, 625; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 11.54 (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 CONHCH_2CH_2 and

CH=CHCHC<u>H</u>COOH or CH=CHCHC<u>H</u>CONH), 2.93 (2H, m, C<u>H</u>CH=CHC<u>H</u>), 2.80 (1H, t, *J* = 2.6, C≡C<u>H</u>), 2.28-2.14 (2H, m, C<u>H</u>$_2$C≡CH), 1.25 (1H, d, J = 8.1 Hz, CHC<u>H$_A$</u>H$_B$CH), 1.21 (1H, d, J = 8.2 Hz, CHCH$_A$<u>H$_B$</u>CH); ^{13}C NMR (150 MHz, DMSO-d$_6$): δ = 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, $C_{13}H_{14}NO_2$ requires 232.1.

Hydrolysis study of alkyne probe 111 (performed in part by Kyle Farrell)

Alkyne probe **111** (24 mg, 0.10 mmol) was added to a vial and dissolved in 1.25 mL of D_2O. To this solution was added sodium acetate buffer in D_2O (1.25 mL, pD 5.0, 400 mM). After 20 minutes and 24 hours, this solution was analysed directly by ^{1}H NMR, indicating hydrolysis (performed by Kyle Farrell). The experiment was repeated in non-deuterated solvents for LC-MS analysis (performed by the candidate). A NMR of the alkyne probe in D_2O without hydrolysis product (t = 0) could not be obtained as the hydrolysis appears to occur rapidly under these conditions.

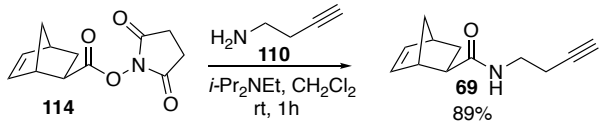
Synthesis of norbornene NHS derivative 114



112 **114** 69%

This method was adapted from previously published procedures.[174] *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 **114** 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}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 6.20 (1H, dd, J = 5.7, 3.0 Hz, CH_2CHC<u>H</u>=CH), 6.15 (1H, dd, J = 5.7, 3.1 Hz, CHCH=C<u>H</u>), 3.28 (1H, m, CH=CHC<u>H</u>CH), 3.00 (1H, m, CH=CHC<u>H</u>C<u>H$_2$</u>), 2.83 (4H, m, COC<u>H$_2$</u>C<u>H$_2$</u>CO), 2.51 (1H, m, COCHC<u>H$_A$</u>H$_B$), 2.05 (1H, m, COCHCH$_A$<u>H$_B$</u>), 1.58-1.50 (2H, contains CHC<u>H$_A$</u>H$_B$CH and COC<u>H</u>), 1.45 (1H, CHCH$_A$<u>H$_B$</u>CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 171.6, 169.3, 138.5, 135.3, 47.1, 46.4, 41.8, 40.3, 31.0, 25.6.

Synthesis of norbornene probe 69 containing an alkyne reporter group

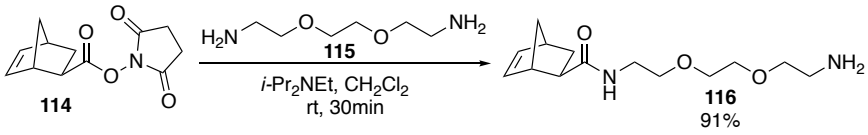


114 **69** 89%

Norbornene-NHS **114** (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, 3.73 mmol)

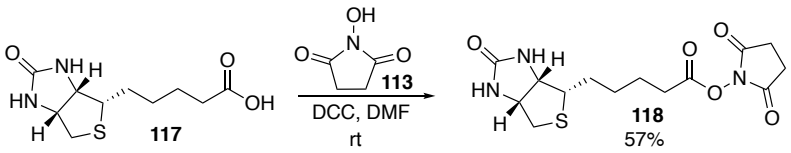
were then added successively. After 1 hour a white precipitate had formed (N-hydroxysuccinimide). The crude material was concentrated under reduced pressure and purified by column chromatography (40 % EtOAc in hexane) to give the product **69** as a white solid (257 mg, 89 %). m.p. 86-88 °C; R_f (40 % 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, $CDCl_3$): δ = 6.15 (1H, dd, J = 5.7, 3.0 Hz, C<u>H</u>=CH), 6.11 (1H, dd, J = 5.7, 3.0 Hz, CH=C<u>H</u>), 5.81 (1H, br-s, CON<u>H</u>), 3.42 (2H, m, CONHC<u>H_2</u>CH_2), 2.93 (2H, m, C<u>H</u>CH=CHC<u>H</u>), 2.42 (2H, tt, J = 6.1, 2.1 Hz, CONHCH_2C<u>H_2</u>), 2.01 (2H, contains $CH_2C{\equiv}C$<u>H</u> and CH=CHCHC<u>H</u>CONH), 1.92 (1H, m, CH=CHCHC<u>H_A</u>H_B), 1.70 (1H, d, J = 8.1 Hz, CHC<u>H_A</u>H_BCH), 1.35 (2H, contains CHCH_A<u>H_B</u>CH and CH=CHCHCH_A<u>H_B</u>); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 175.7, 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. $C_{12}H_{16}NO^+$ requires 190.1226.

Synthesis of norbornene amine derivative 116



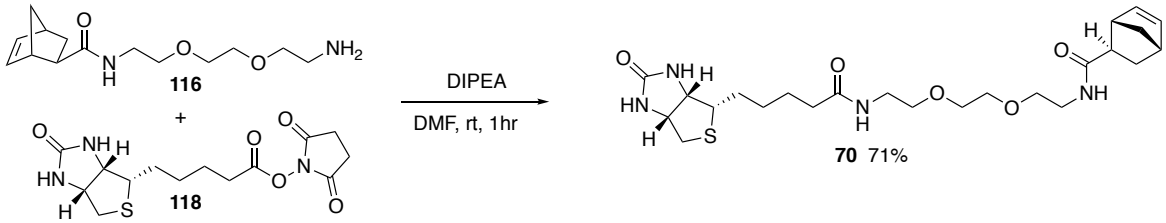
1,2-bis(2-aminoethoxy)ethane (624 μL, 4.2 mmol) and DIPEA (163 μL, 0.92 mmol) were dissolved in anhydrous DCM (5 mL) with stirring at room temperature. A solution of norbornene-NHS **114** (99 mg, 0.42 mmol) was dissolved in anhydrous DCM (1 mL) and added dropwise to the 1,2-bis(2-aminoethoxy)ethane solution and then the reaction mixture was stirred at room temperature for 30 min. After this time, a white precipitate formed (N-hydroxysuccinimide), which was removed by filtration, washed with DCM, and the crude filtrate concentrated under reduced pressure. The resulting oil was purified by column chromatography (10 % MeOH in DCM with 1 % NEt_3) to give the product **116** as a yellow oil (103 mg, 91 %). R_f (10 % MeOH in DCM with 1 % NEt_3) 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, $CDCl_3$): δ = 6.29 (1H, br-s, CON<u>H</u>), 6.13 (1H, dd, J = 5.7, 2.9 Hz, C<u>H</u>=CH), 6.09 (1H, dd, J = 5.8, 3.1 Hz, CH=C<u>H</u>), 3.63 (4H, m, 2 x C<u>H_2</u> PEG), 3.56 (4H, dt, J = 18.5, 5.1 Hz, 2 x C<u>H_2</u> PEG), 3.47 (2H, q, J = 5.2 Hz, CONHC<u>H_2</u>), 2.91 (4H, contains C<u>H_2</u> PEG and C<u>H</u>CH=CHC<u>H</u>), 2.03 (1H, m, CHC<u>H</u>CONH), 1.91 (1H, dt, J = 11.5, 4.0 Hz, CH=CHCHC<u>H_A</u>H_B), 1.72 (1H, d, J = 8.3 Hz, CHC<u>H_A</u>H_BCH), 1.31 (2H, contains CH=CHCHCH_A<u>H_B</u> and CHCH_A<u>H_B</u>CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 175.8, 138.2, 136.0, 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. $C_{14}H_{25}N_2O_3^+$ requires 269.1865.

Synthesis of biotin NHS-ester 118



This method was adapted from previously published procedures.[175] Biotin (498 mg, 2 mmol) was dissolved in anhydrous DMF (10 mL) by stirring at 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 EtO_2 until a white precipitate formed. The precipitate was collected by filtration and rinsed with EtO_2 then dried to give the crude product **118** 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-d_6): δ = 6.40 (1H, s, NH), 6.35 (1H, s, NH), 4.30 (1H, m, HNCHCHNH), 4.14 (1H, m, HNCHCHNH), 3.10 (1H, m, SCH), 2.84-2.78 (5H, contains $NCOCH_2CH_2$ and SCH_AH_B), 2.67 (2H, t, J = 7.7 Hz, $CH_2CH_2CO_2N$), 2.57 (1H, d, J = 12.4 Hz, SCH_AH_B), 1.64 (3H, contains $CH_AH_BCH_2CH_2CH_2CO_2N$), 1.52-1.36 (3H, contains $CH_AH_BCH_2CH_2CH_2COON$); ^{13}C NMR (150 MHz, DMSO-d_6): 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. $C_{14}H_{20}N_3O_5S^+$ requires 342.1118.

Synthesis of norbornene probe 70 containing a biotin reporter group



Biotin-NHS **118** (382 mg, 1.12 mmol) was dissolved in anhydrous DMF (6 mL) by stirring at 50 °C for 5 minutes or until dissolved. Norbornene derivative **116** (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, EtO_2 was added until a white precipitate formed. The precipitate was collected by filtration and washed with additional EtO_2. The resulting residue (an oily solid) was purified by column chromatography (10 % MeOH in DCM) to give the product **70** 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 = 6.14 (2H, m, CHCH=CHCH), 4.49 (1H, dd, J = 8.3, 4.5 Hz, CONHCHCH$_2$S), 4.30 (1H, dd, J = 7.9, 4.4 Hz, CONHCHCHS), 3.62 (4H, m, 2 x CH_2

PEG), 3.55 (4H, m, 2 x C$\underline{H_2}$ PEG), 3.37 (4H, m, 2 x C$\underline{H_2}$ PEG), 3.20 (1H, m, SC$\underline{H}$), 2.93 (1H, dd, J = 12.8, 5.0 Hz, CONHCHC$\underline{H_A}H_BS$), 2.87 (2H, m, C$\underline{H}$CH=CHC$\underline{H}$), 2.71 (1H, d, J = 12.7 Hz, CONHCHC$H_A\underline{H_B}$S), 2.22 (2H, t, J = 7.1 Hz, C$\underline{H_2}$CONH), 2.13 (1H, m, CH=CHCHC$\underline{H}$), 1.87 (1H, dt, J = 11.7, 4.0 Hz, CH=CHCHC$\underline{H_A}H_B$), 1.77-1.57 (5H, contains 2 x C$\underline{H_2}$ Biotin and CHC$\underline{H_A}H_B$CH), 1.44 (2H, p, J = 7.5 Hz, C$\underline{H_2}$ Biotin), 1.31 (2H, contains CH=CHCHC$H_A\underline{H_B}$ and CHC$H_A\underline{H_B}$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 (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. $C_{24}H_{39}N_4O_5S^+$ requires 495.2636.

3. PROTEIN MODEL STUDIES AND LIVE CELL EVALUATION OF NORBORNENE PROBES

3.1 Overview

A new cysteine sulfenic acid probe was introduced in Chapter two that detailed the rapid reaction of the norbornene olefin with *N*-acetylcysteine sulfenic acid. This result was especially encouraging as the commonly used dimedone probe was not able to trap the *N*-acetylcysteine sulfenic acid. This prompted us to shift our research efforts directly towards proteins and cells since this was the overall target of our strategy. To confirm the norbornene probe as a suitable and useful tool to study oxidative stress, its reactivity and selectivity must be demonstrated on proteins and cells, preferably under physiological conditions.

Chapter three details the application of norbornene probes, both commercially available and those synthesised as described in Chapter two, as cysteine sulfenic acid traps on purified proteins, protein mixtures, and live cells. Equivalent commercially available dimedone probes are also tested as comparison studies against the norbornene probes.

Portions of this Chapter have been taken from an article titled 'Norbornene probes for the detection of cysteine sulfenic acid in cells' as published in *ACS Chemical Biology*, American Chemical Society on 20th March 2019 (DOI: 10.1021/acschembio.8b01104). Some work detailed in this Chapter was not included in the above-mentioned publication, but has been incorporated in with the published work as this demonstrates additional findings that contribute to the overall understanding of the work. Work included in the publication has been reformatted to align with this Chapter, but the original findings and conclusions remain the same. The data presented in this Chapter (section 3.4-3.5) on cell lysates and live cells were collected during a research visit at The Department of Chemistry, University of Cambridge, England. The candidate was supported by an Endeavour Postgraduate Scholarship for the duration of this 12-month research visit. The candidate prepared the Chapter with full intellectual and practical contribution unless otherwise stated in-text and was listed as the primary author of the manuscript.

3.2 Introduction

Within proteins, the amino acid residue cysteine has a low representation compared to other amino acids predicted to comprise only ~2 % of all protein residues.[24] As discussed in Chapter one, this low abundance coupled with the diverse reactivity of the thiol group, could be what fosters cysteine residues as key structural, functional, and regulatory residues in proteins. As such, these cysteine residues have extremely varied ability to be oxidised and subsequently react depending on a number

of interplaying factors of the protein microenvironment. These include: neighbouring amino acid residues such as those containing amines which may deprotonate the thiol, increasing its reactivity; location of the cysteine residue, whether it is a surface or buried residue impacts its accessibility; the pK_a of the thiol determines whether it is protonated or deprotonated at physiological pH; and lastly the oxidation state of the thiol under basal conditions, for example whether it is oxidised to a disulfide. Therefore, not all cysteine residues in all proteins can be oxidised under normal cellular or oxidative stress conditions. These factors contributing to cysteine oxidation and also its subsequent reactivity with probes should be considered for all protein and cell experiments as examples of this varied reactivity have been reported in the literature.[145, 176] This also means the choice of model protein is essential, as well as the conditions used for the duration of the experiment.

3.3 Protein model experiments with papain

3.3.1 Activity assay for papain inhibition studies

For the first assessment of norbornene as a cysteine sulfenic acid probe on proteins, a suitable model was required. Papain was chosen for the model protein since it has a free cysteine in its active site (Cys-25) that is known to be readily oxidised with hydrogen peroxide[177-178] to generate a cysteine sulfenic acid.[47, 103, 107] Given papain is a cysteine protease, it contains a cysteine (Cys-25) and histidine (His-159) residue in the active site. Within the pH range of 3.5-8.0,[179] both residues exist as an ion pair in the catalytic pocket, where the deprotonated thiol is more susceptible to oxidation by hydrogen peroxide and also accounts for the activity of papain (Fig. 26). Moreover, papain has been used previously as a model to study cysteine sulfenic acid probe reactivity and therefore presented a good reference to assess whether norbornene could also react with protein cysteine sulfenic acids.[47, 107] For initial evaluation of the reaction, an activity assay was used. This would allow convenient and straightforward detection of any changes to papain's activity (and in turn, its thiol) and provide a means to optimise the various reaction parameters. The commercially available tripeptide L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA, **119**) was chosen as the substrate for the activity assay as upon cleavage of the amide bond by papain, would release a coloured nitroanilide product which absorbs at 410 nm (Fig. 26). Increase in monitored absorbance at 410 nm would correlate to increased papain activity and vice versa. Any modification to papain's active site thiol (such as oxidation or covalent linkage) would result in loss of activity. Reduction back to the thiol would restore activity.

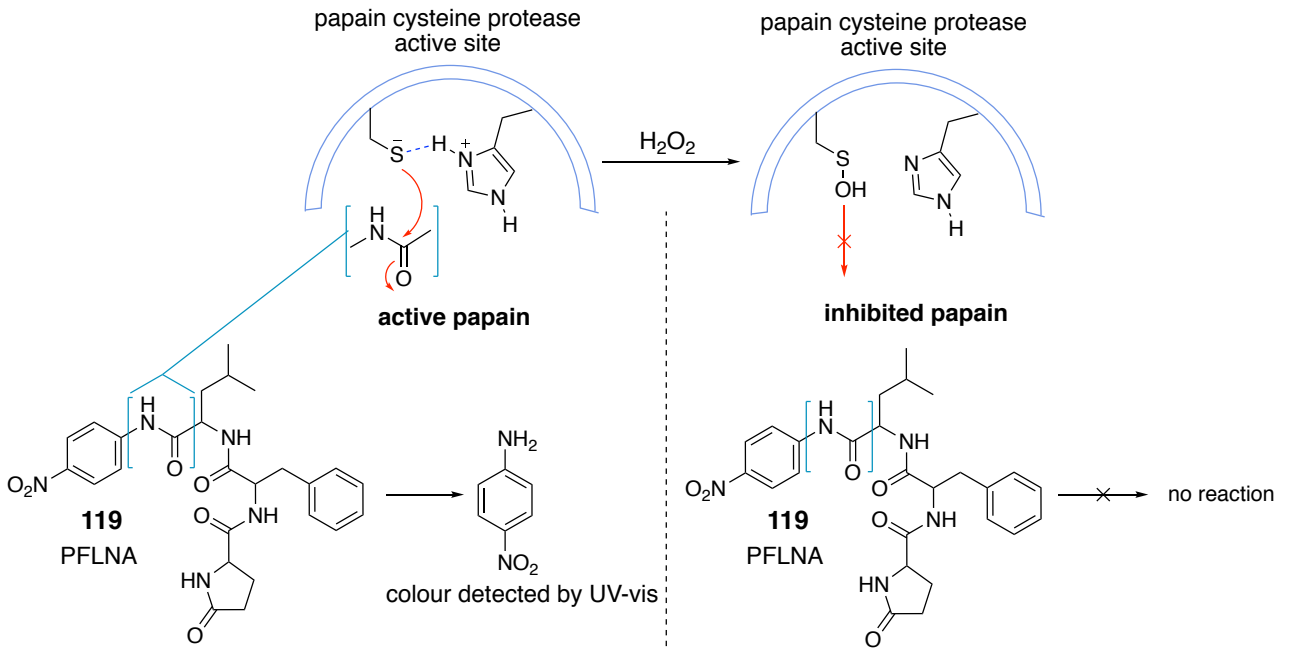


Figure 26. Papain active site. Papain contains a cysteine protease which can cleave the amide bond in the tripeptide PFLNA **119** to generate the *p*-nitroanilide product. Production of *p*-nitroanilide can be monitored by UV-Vis at 410 nm where increased absorbance correlates to increased papain activity. When Cys-25 of papain is either oxidised (to SOH) or covalently modified, papain activity is inhibited.

As an initial control, the oxidation and reduction of papain was assessed. This was to optimise the reaction conditions for concentration of hydrogen peroxide, papain, and DTT. After careful optimisation, papain (7 μM) was treated with H_2O_2 (2.8 mM) leading to oxidation and complete loss of enzyme activity (Fig. 27), monitored by UV-Vis spectroscopy at 410 nm using a 10 min kinetics program (Fig. 28A). The oxidised papain solution was then treated with DTT (10 mM) to reduce the sulfenic acid back to the thiol, recovering activity. Compared to the papain-only control (Fig. 28A), only partial recovery of activity was achieved under the conditions trialed. A portion of the thiol that is oxidised to the sulfenic acid may further be oxidised to the sulfinic or even sulfonic acid during the course of the experiment. Both of these higher oxidation states are irreversible by DTT reduction, and would account for reduction in papain activity under these conditions. The conditions used to oxidise papain were carefully optimised as the amount of hydrogen peroxide required to completely oxidise papain (resulting in no activity), but still recover some activity after DTT reduction, was a narrow window. Adding significantly less hydrogen peroxide so as to prevent overoxidation led to incomplete inhibition in the first instance. Increasing the amount of hydrogen peroxide resulted in no recoverable activity.

With optimised papain conditions (Fig. 27), norbornene derivative **99** was chosen as the first norbornene probe to be tested on papain. Initially, papain (7 μM) was treated with **99** (27 mM)

followed by H_2O_2 (2.8 mM) for 1 h at room temperature resulting in total inhibition as expected. After reduction with DTT, minimal recovery of papain activity was observed (Fig. 28B), at least far less than had been recovered in the papain-only control (Fig. 28A). Importantly, papain exhibited no loss in activity when treated with only **99** (27 mM), indicating the norbornene did not react with the reduced thiol. To ensure **99** was actually responsible for the inability of papain to regain its activity, the concentration of **99** was doubled. Papain (7 µM) was treated with **99** (53 mM) followed by H_2O_2 (2.8 mM) for 1 h at room temperature and resulted in no recovery of papain activity after oxidation followed by DTT reduction (Fig. 28C). This indicates total, irreversible inhibition of papain with concentration dependence for labelling. Once again, papain treated only with **99** (53 mM) and no hydrogen peroxide displayed full activity. Together these results indicate covalent binding of **99** with only the sulfenic acid of papain, and not the thiol. The fact that total inhibition of papain could be achieved with norbornene (albeit at high concentrations) only when papain was oxidised was noteworthy.

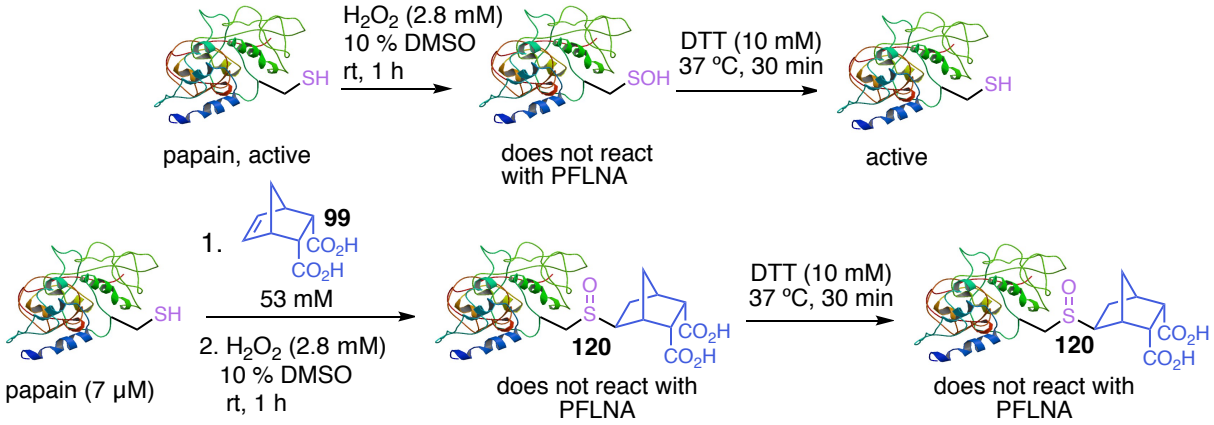


Figure 27. Papain activity assay experimental set-up. Treating papain with hydrogen peroxide results in complete loss of activity. Reduction with DTT can restore papain activity. Under the same conditions, treating oxidised papain with norbornene **99** inhibits papain irreversibly. Subsequent reduction with DTT does not recover papain activity due to the covalently bound probe.

For comparison, the probe dimedone **19** was also tested on the papain model. Using the same optimised conditions for complete inhibition, papain (7 µM) was treated with **19** (59 mM) followed by H_2O_2 (2.8 mM) for 1 h at room temperature. Complete inhibition of papain activity was observed with no papain activity recovered when oxidised papain was treated with DTT as expected (Fig. 28D). This also indicates covalent binding of dimedone to the papain sulfenic acid, consistent with previous studies of dimedone derivatives.[107] When papain was treated only with dimedone (59 mM), no loss in activity was observed indicating no reaction between the thiol and dimedone.

These experiments were performed in phosphate buffer (100 mM) at pH 7.4, however due to the high concentration of norbornene **99** required to cause total inhibition of papain, and the fact **99**

contains two acidic groups, the final pH of solutions containing **99** at 53 mM were measured as pH 6.22. This was interesting for two reasons: in the small molecule studies conducted in Chapter two, norbornene only trapped sulfenic acid at pH 5; and papain activity has an optimal pH range of 6.0-7.0 as indicated in manufacturers guidelines. The concentration of buffer required to effectively buffer the **99** (53 mM) containing solution at pH 7.4 was phosphate 500 mM. This extremely high salt concentration then caused insolubility issues for papain and **99**, with visible precipitation observed over time. This high salt concentration and precipitation of papain also caused interference under the conditions for the UV-vis measurement. Regardless, the experiment was repeated with phosphate buffer (500 mM, pH 7.4) and norbornene **99** (53 mM) but no conclusive results were obtained. The activity of papain appeared to be affected in some way but it was not entirely clear the cause of these unusual results.

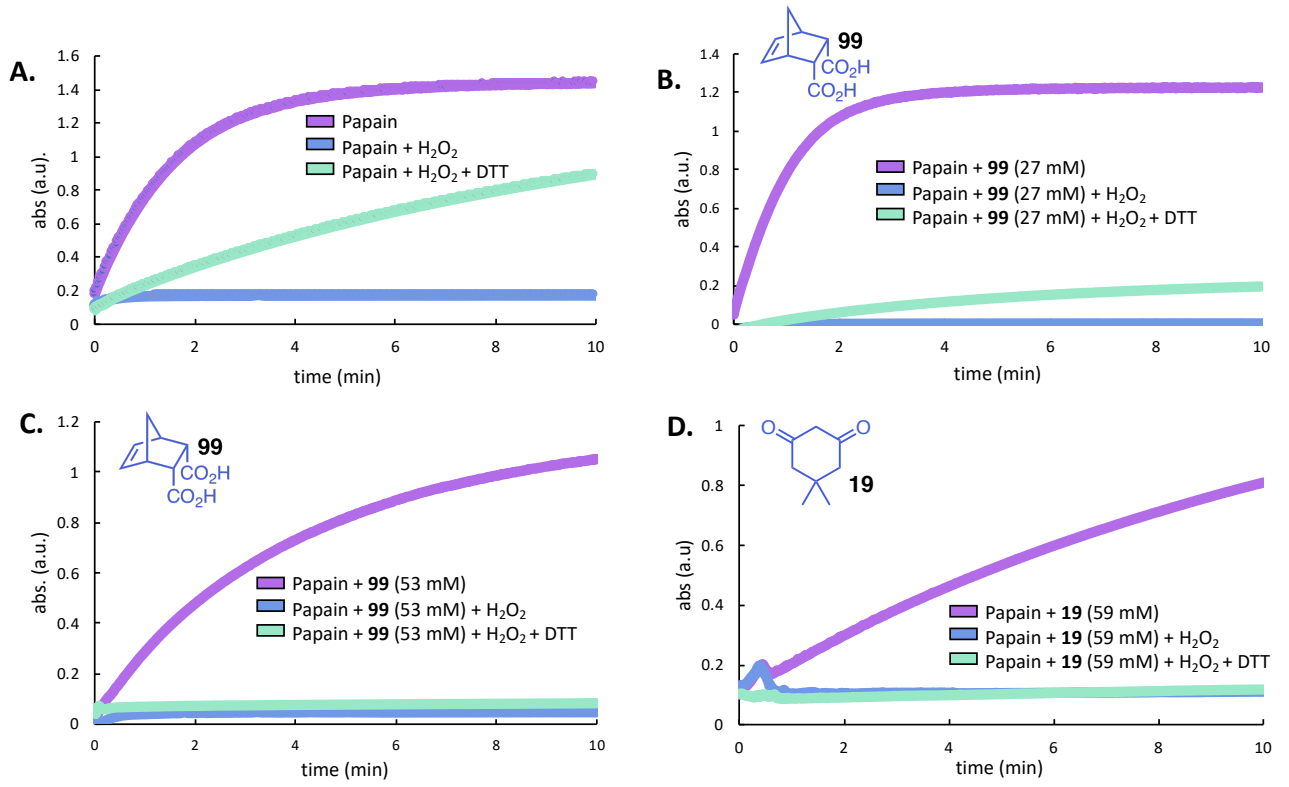


Figure 28. Papain activity profiles. A. Papain control experiment. B. Papain reaction with **99** (27 mM). C. Papain reaction with **99** (53 mM). D. Papain reaction with dimedone **19** (59 mM).

Several key pieces of information can be taken from this initial papain study. It is apparent that labelling of papain sulfenic acid is highly concentration dependent, with unexpectedly high concentration of probe required for significant labelling. In the same way, papain's cysteine is sensitive to oxidant concentration, to both ensure oxidation to the sulfenic acid, but reduce overoxidation to the sulfinic or sulfonic acids. Both of these factors are important considerations for all future studies on protein labelling. Most importantly, the norbornene probe only reacts with

oxidised papain and not the thiol, encouraging the use of norbornene as a selective sulfenic acid trap.

3.3.2 LC-MS analysis of sulfoxide formation

Next, we sought direct evidence of the reaction of norbornene **99** with papain sulfenic acid. While the activity assay provided useful insight into the oxidation and trapping of papain sulfenic acid, it could not reveal how the papain was being inhibited directly. LC-MS was sought to evaluate the reactions. Using the UV-vis experimental conditions as a guide, the reaction and various control experiments were performed. A few adjustments were made to the reaction conditions to be more suitable for LC-MS analysis. Since the buffering at pH 7.4 had been an issue due to the high concentration of **99** required, initial attempts were conducted using lower equivalents of norbornene **99** (10 mM) to assist with adequate buffering. Even though lower equivalents of **99** resulted in incomplete inhibition in the activity assay, it was thought that total inhibition might not be required to see the product peak in MS. Unfortunately, no detectable product peak **120** was found. The most reasonable explanation for this occurrence is that at lower concentrations of **99**, fewer papain is labelled with the probe such that the abundance of labelled protein compared to unlabelled protein falls below the detection limits of the MS.

After several optimisation attempts for both probe and hydrogen peroxide concentration, papain (15 μM) was treated with norbornene **99** (53 mM) before adding H_2O_2 (280 μM) (Fig. 29A). After incubating for 1 hour at room temperature, the excess probe and oxidant were removed using a centrifugal concentrator to prevent interference during the LC-MS analysis. Samples containing **99** (53 mM) were measured at pH 4.90 in phosphate buffer (50 mM, pH 7.4). The protein mixture was then analysed by LC-MS. The LC trace (UV) revealed what appeared to be two distinct, but slightly overlapping peaks. Taking the combined MS of the second peak, two clear signals were observed in the deconvoluted mass spectrum (Fig. 29B). The signal at 23619 Da is consistent with sulfoxide adduct **120**, expected to form after reaction of the probe **99** with the sulfenic acid of papain. This result is significant as it documents the first direct evidence of norbornene trapping a protein cysteine sulfenic acid. The signal at 23457 Da is consistent with conversion of the active site cysteine to the sulfinic acid **121**, a product of over-oxidation.

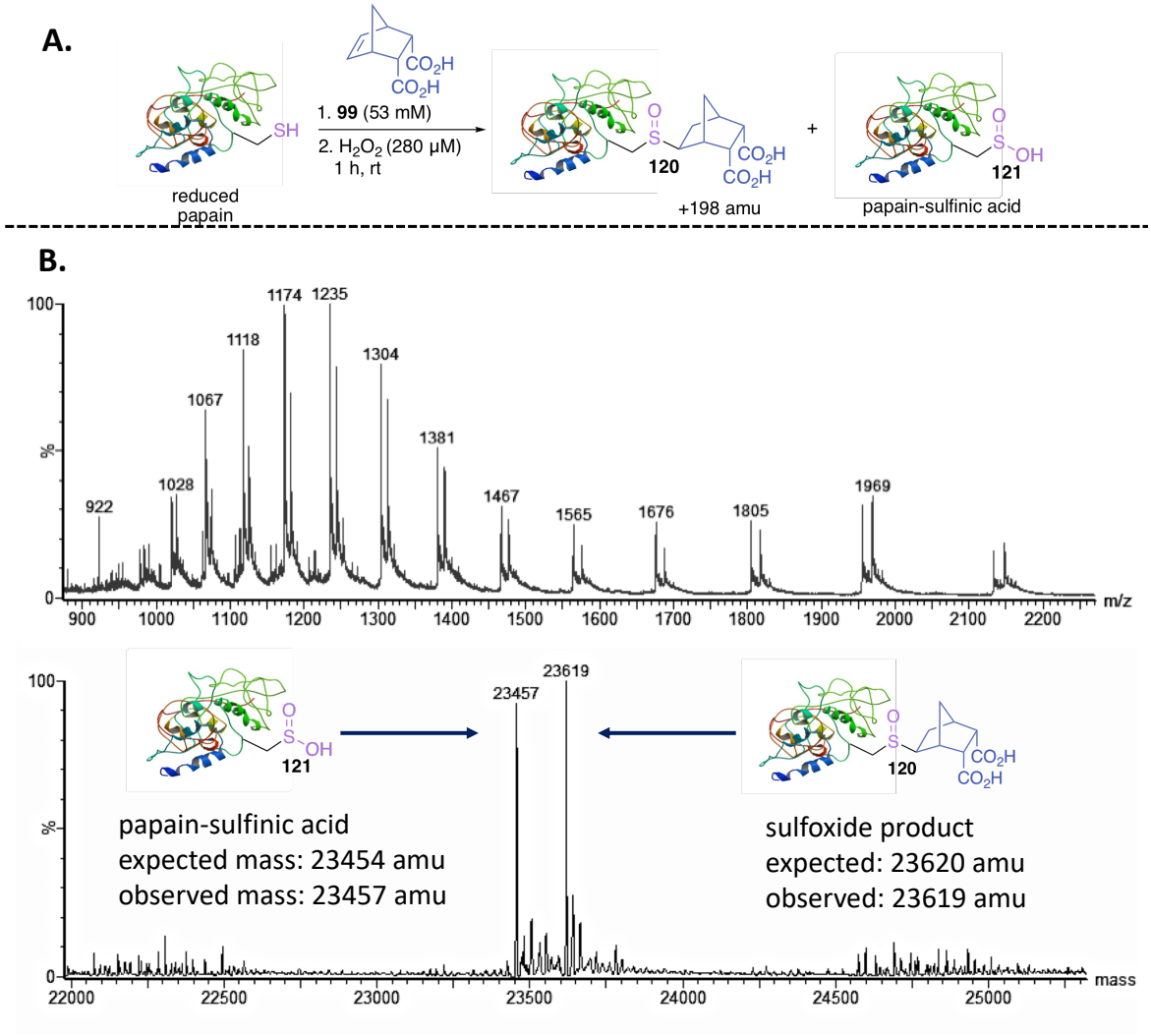


Figure 29. A. LC-MS analysis of reaction of norbornene **99** with papain-sulfenic acid revealing the sulfoxide product **120**. B. Top: Ion series of the reaction at the selected peak. Bottom: deconvoluted mass of the above ion series revealing the sulfoxide **120** and sulfinic acid **121**.

Several control experiments were also conducted. A sample of just papain was treated to the same reaction conditions and analysed by LC-MS. Interestingly, it was found that even though no hydrogen peroxide had been included, the overoxidation products of the sulfinic and sulfonic acids of papain were observed, along with the reduced papain at 23423 Da (see Appendix B). This result indicated that papain might be susceptible to oxidation in air, something which was not apparent from the activity assay. The most unusual observation was the presence of an unidentified peak in the deconvoluted mass spectrum at 26539 Da. Papain was then treated with just hydrogen peroxide (no probe) and the deconvoluted mass spectrum showed two peaks, one for the sulfinic and sulfonic acids (see Appendix B). This sample also contained an unidentified peak at 26537 Da, except the intensity of the peak was much smaller than for the papain-only sample, where it was the most intense signal. Appearance of this peak was consistent throughout the remainder of the experiments

where it seemed the peak appeared in larger quantities when the papain was in its reduced form (no added hydrogen peroxide). This alluded to the possibility that this may be a product of papain's activity. For the control reaction of papain treated with **99** without hydrogen peroxide, no thiol-ene product was observed. However, degradation of the protein itself was experienced with an unidentified peak at 23358 Da (see Appendix B). This could possibly be a result of auto-proteolysis of active papain. Both sulfinic and sulfonic acids were also identified despite no addition of hydrogen peroxide. Finally, the last control involved treating papain with hydrogen peroxide for 30 min before addition of probe **99** to generate the overoxidised products. A small peak in the deconvoluted mass spectrum of 23617 Da was observed which could correspond to the sulfoxide product **120**, but mostly the sulfinic and sulfonic acid products were observed. This suggests it is best to add the probe first so it can rapidly react with the sulfenic acid as it is formed, otherwise overoxidation is probable. Overall it seemed that papain was susceptible to oxidation in air and suffered degradation during the course of the experiment.

Another experiment was repeated using increased hydrogen peroxide to treat papain in the presence of **99** to assess whether **99** could still trap the sulfenic acid before overoxidation. Treating papain (15 μM) with **99** (53 mM) followed by H_2O_2 (2.8 mM) did not result in detection of the sulfoxide product **120** mass (Fig. 30A). Interestingly, when the concentration of hydrogen peroxide was increased, overoxidation of the sulfoxide adduct **120** was apparently observed. A mass of 23636 Da appeared which could be **120** with the addition of an oxygen (sulfoxide adduct 23620 Da + 16 Da) as well as 23471 Da which is consistent with the overoxidised sulfonic acid **122** (Fig. 30B). This result highlights the care which must be taken to avoid overoxidation during the course of the reaction and during sample preparation and analysis. Whether this overoxidation product is the sulfone (addition of oxygen to the sulfoxide) was not determined.

Despite the successful detection of the anticipated papain-sulfoxide product **120**, several issues were encountered with papain during the course of the experiments, particularly its apparent degradation. Several unknown masses were observed in most samples, with higher degradation rates observed when the active site thiol was in its reduced form (suggesting auto-proteolysis). Additionally, a high concentration of probe was required to achieve a high enough labelling ratio to be observed in the mass spectrum. This result was consistent with the UV-vis experiments. However, what this meant was that the experiments conducted for LC-MS were also not buffered with a measured pH of 4.90. As had been observed with the UV-vis experiments, increasing the buffer concentration to a concentration that maintained buffering capacity (500 mM), compromised the integrity of the protein in some way, whether by precipitation or denaturation from the high salt content. This unfortunately prevented monitoring of the reaction at pH 7.4. Since the oxidisable cysteine is contained in the active site of papain, it might not be as easily accessible, and thus requires the extremely high equivalent of probe to ensure trapping of the sulfenic acid. Regardless,

direct evidence of the papain sulfoxide adduct **120** had been obtained by LC-MS at pH 4.90, representing the first instance of norbornene labelling cysteine sulfenic acid on a protein.

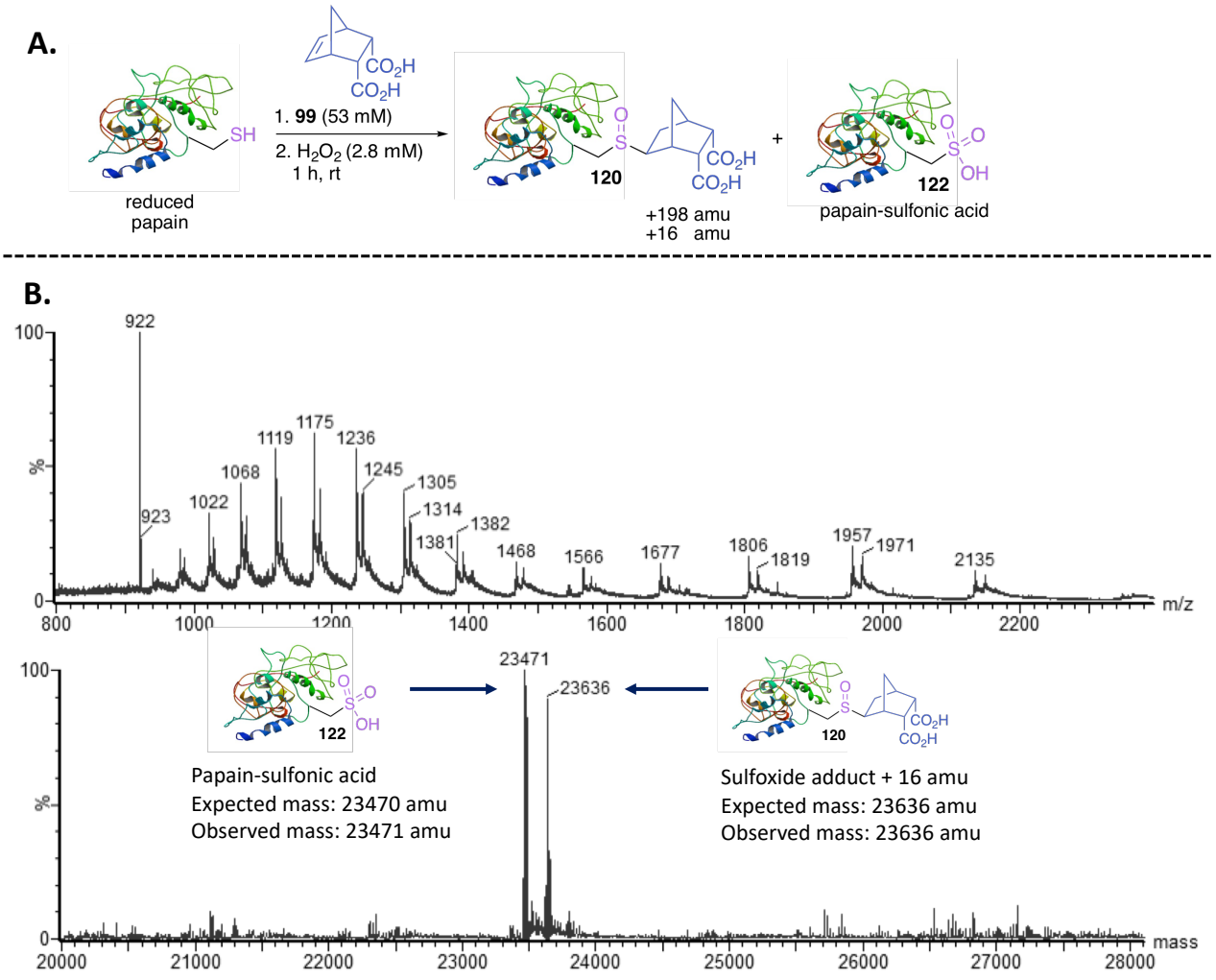


Figure 30. A. LC-MS analysis of reaction of norbornene **99** with papain-sulfenic acid revealing the sulfoxide product **120** plus overoxidation. B. Top: Ion series of the reaction at the selected peak. Bottom: deconvoluted mass of the above ion series revealing products **120** plus overoxidation and **122**.

3.3.3 Western blot analysis of norbornene probes

Given the success of labelling papain sulfenic acid with **99** to inhibit activity, and the sulfoxide product **120** detected by MS, the next step was to determine if the two synthesised probes norb-bio **70** and norb-yne **69** (detailed in Chapter two) would react in the same way. Both probes could also be tested for their applicability for western blot analysis. Both the activity assay and LC-MS analysis had demonstrated the necessity of high concentration of probe for sufficient labelling of papain to occur. However, both norb-bio **70** and norb-yne **69** were not soluble at 50 mM in phosphate buffer (pH 7.4)

with DMSO (10 %v/v) so the conditions that had been successfully used previously were not possible. Since western blot analysis is typically very sensitive, labelling of papain might still be observable as total inhibition would not be required. The conditions were altered accordingly after several optimisation attempts for protein loading and reactivity. Ultimately, papain (15 μM) was incubated with norb-bio **70** (9 mM) both with and without hydrogen peroxide (1 mM) for 1 h at room temperature (Fig. 31A). Papain was also treated with and without hydrogen peroxide with DMSO control. These reactions were carried out open to air. Samples were also labelled with the thiol alkylating reagent iodoacetamide (IAM) to block free thiols after labelling with the probe and before SDS-PAGE. This step was performed as it was noted that when the thiol was active, degradation products were observed on the gel stained with coomassie. This observation is consistent with that observed from the LC-MS experiments, where unknown degradation products were detected, especially when the thiol was in its reduced (active) form. Samples were resolved by SDS-PAGE and transferred to a western blot PVDF membrane where biotin signals were detected with streptavidin-alkaline phosphatase (AP) developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) for visualisation. Upon SDS-PAGE and western blot analysis, a biotin-streptavidin-AP signal was detected for both the papain samples treated with norb-bio **70** with and without hydrogen peroxide (Fig. 31B). This was perplexing as no indication of a reaction between norbornene and the free thiol of papain had been observed in the activity assay nor was the thioether adduct detected in LC-MS. Perhaps if a small amount of thioether adduct had formed, it could be insignificant by activity assay or LC-MS, but visible by western blot given the increased sensitivity. An alternative explanation could be that a small portion of papain thiol was being oxidised to the sulfenic acid by air during the reaction, something that is known to occur.[180] In fact, when papain alone was analysed by LC-MS, oxidation to the sulfinic and sulfonic acid were observed without hydrogen peroxide addition, confirming this proposal. Once again, this small amount of product formation might not be significant enough for detection by activity assay or LC-MS. A number of experimental processes could account for this apparent reactivity. During the SDS-PAGE sample preparation, the samples are heated (70 ºC) under reducing conditions in the presence of SDS and DTT. It is possible this elevated temperature enhances the off-target thiol-ene reaction or oxidation of papain by air. To test this, samples were prepared for SDS-PAGE without heating (but inclusion of SDS loading buffer) but still a signal was observed for the control lane (papain treated with probe without hydrogen peroxide). Samples were then purified with a zeba size exclusion column (7 kDa MWCO) to remove any unbound probe before preparation for SDS-PAGE so as to prevent any post-reaction labelling. This also yielded a signal in the control lane. This indicated the labelling was probably occurring during the time course of the experiment.

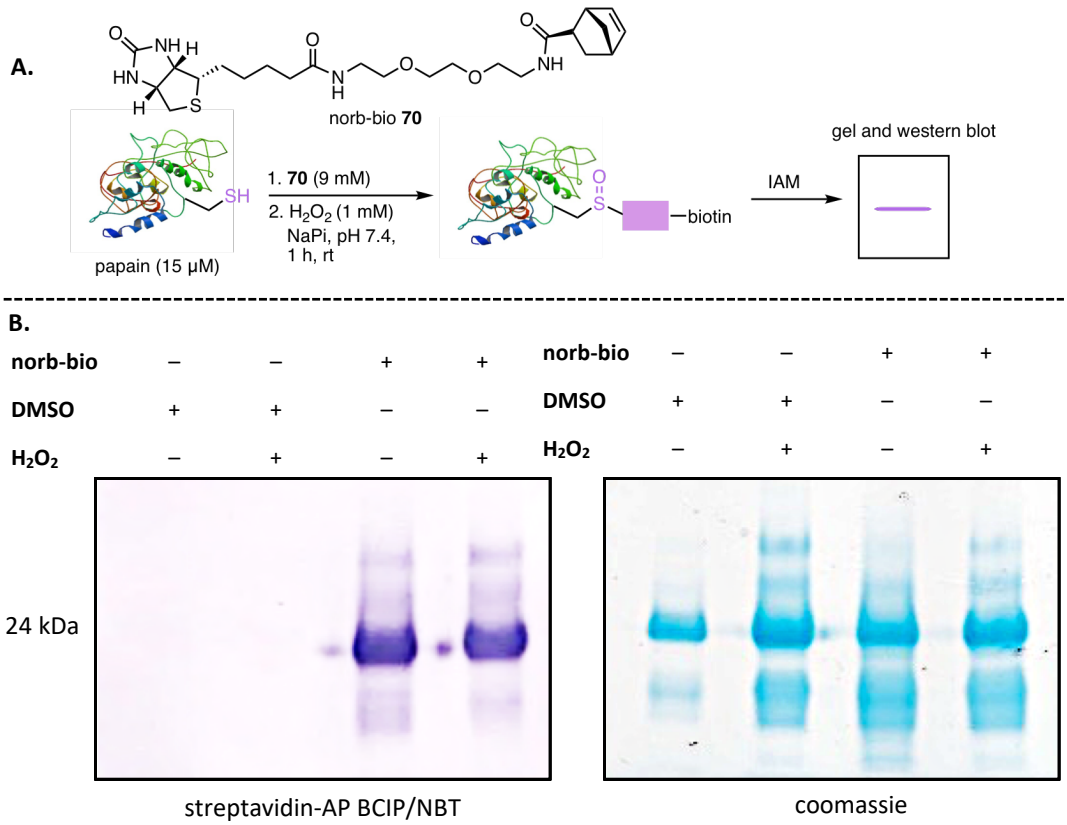


Figure 31. A. Reaction of norb-bio **70** with papain open to air. B. Western blot analysis of reaction of papain with norb-bio. Left: Western blot treated with streptavidin-alkaline phosphatase and developed with BCIP/NBT to show biotinylated proteins. Right: Coomassie stain of reactions showing total protein. Samples were treated with iodoacetamide to alkylate free cysteines before running on SDS-PAGE. This was to prevent auto-proteolysis of papain.

The exact same experiments where then repeated on papain using norb-yne **69** (Fig. 32A) as the probe. The experimental conditions were identical to Figure 31 with the addition of the CuAAC step to ligate the biotin-azide to the alkyne so the probe could be detected by western blot with streptavidin. The exact same result was observed. Both the oxidised and non-oxidised papain samples treated with **69** gave a biotin-streptavidin signal (Fig. 32B). Although, this experiment demonstrated that the norb-yne probe could effectively be ligated to a biotin-azide through the CuAAC reaction for detection by western blot.

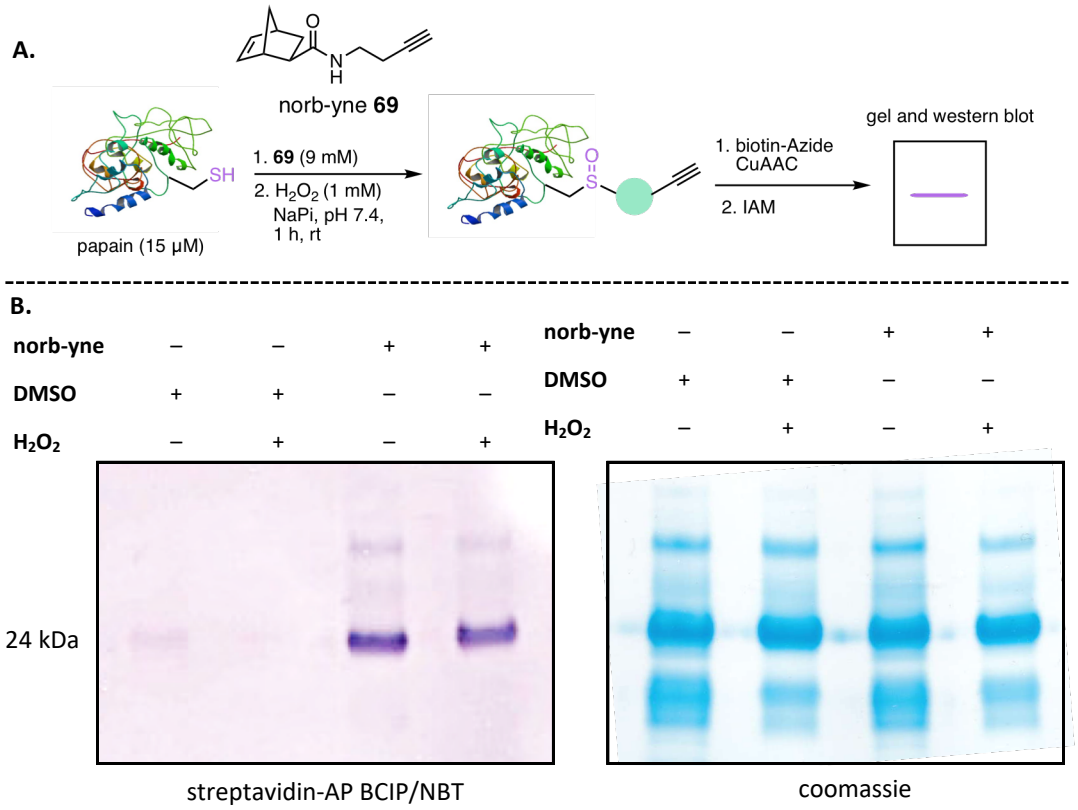


Figure 32. A. Reaction of norb-yne **69** with papain open to air. B. Western blot analysis of reaction of papain with norb-yne. Left: Western blot treated with streptavidin-alkaline phosphatase and developed with BCIP/NBT to show biotinylated proteins. Right: Coomassie stain of reactions showing total protein. Samples were treated with iodoacetamide to alkylate free cysteines before running on SDS-PAGE. This was to prevent auto-proteolysis of papain.

To get to the bottom of this unexplained reactivity, the reactions were then carried out under an atmosphere of N_2 or in the presence of DTT to prevent air oxidation of Cys-25 of papain. The N_2 experiments would eliminate dissolved oxygen, whereas the DTT could reduce the oxidised sulfenic acid back to the thiol as it is formed. Under both conditions, papain (15 µM) was treated with and without hydrogen peroxide (2 mM) in the presence of norb-bio **70** (10 mM) (Fig. 33A). Samples were not alkylated with IAM before SDS-PAGE as we were curious to see if the protein degraded under these conditions. After analysis by SDS-PAGE and western blot, only papain treated with hydrogen peroxide produced a biotin-streptavidin signal under both conditions (Fig. 33B). This result was welcome news as it agreed with both the activity assay and LC-MS experimental outcomes. Another interesting observation was the degradation of papain visible on the gel. Both samples which were not treated with hydrogen peroxide had severely degraded compared to those that had been oxidised, which still had a distinguishable band at the expected molecular weight. This once again agreed with the earlier LC-MS observations on the degradation of papain. Interestingly, the biotin-streptavidin signal on the BCIP/NBT stained western blot gives a distinct band compared to the

corresponding coomassie stain of total protein. This indicates the probe only labels the intact protein, presumably when the active site is intact, and does not react with the degradation products. Successful labelling of a protein sulfenic acid with the synthesised probe **70** and its analysis by western blot confirms these probes as suitable candidates for labelling sulfenic acids in cells.

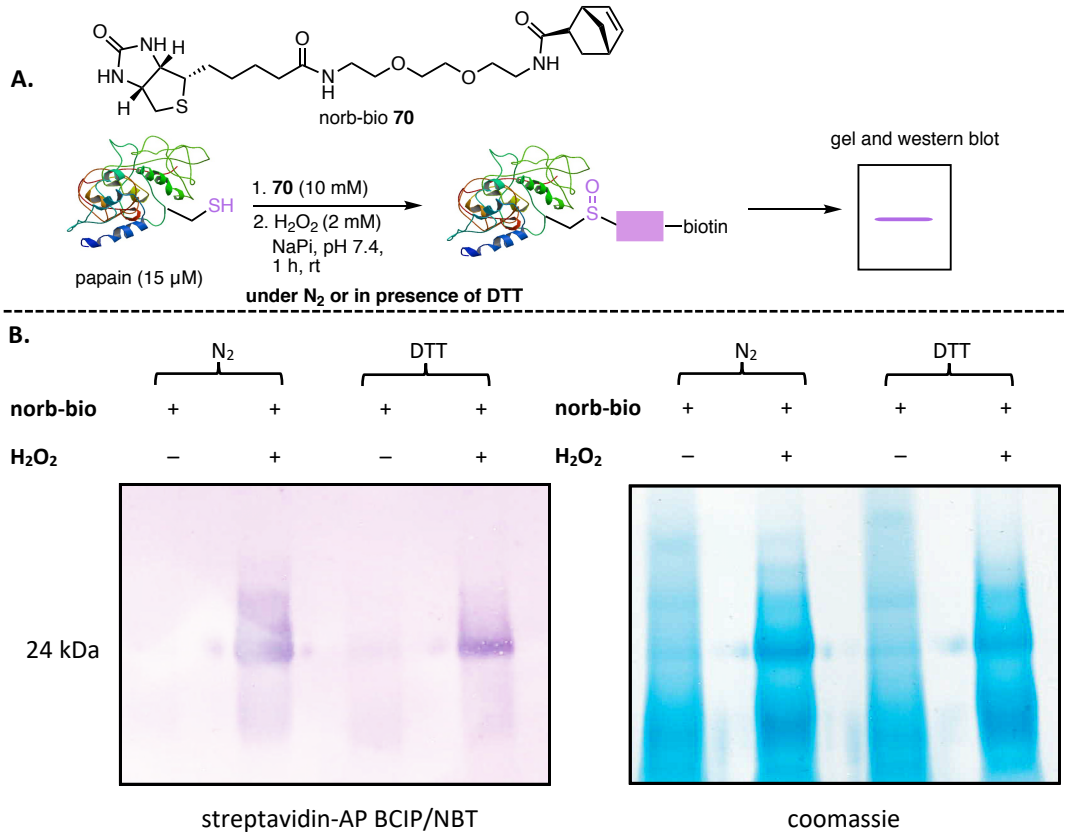


Figure 33. A. Reaction of norb-bio **70** with papain either under N_2 or in the presence of DTT. B. Western blot analysis of reaction of papain with norb-bio either under an atmosphere of N_2 or in the presence of DTT. Left: Western blot treated with streptavidin-alkaline phosphatase and developed with BCIP/NBT to show biotinylated proteins. Right: Coomassie stain of reactions showing total protein. Samples were not treated with iodoacetamide prior to SDS-PAGE analysis. Therefore, degradation products from auto-proteolysis can be observed in the coomassie gel on the right.

3.4 Cell lysate experiments

3.4.1 Norbornene probes

The next approach was to then examine the reactivity of both our probes on cell lysates which would contain the full range of proteins. This way, we could gauge the general reactivity of our probes across the whole proteome rather than searching for individual proteins. The HeLa cell line was chosen as the first target. This is a human cervical cancer cell line and as such an appropriate model

for evaluating oxidative stress. HeLa cells have been treated with hydrogen peroxide and shown to produce ROS when under oxidative stress, leading to apoptosis.[100, 181] Other studies have also used HeLa cells to examine the reactivity of cysteine sulfenic acid probes by identifying the protein hits.[100] This would provide a good platform to directly compare our protein hits in later analyses.

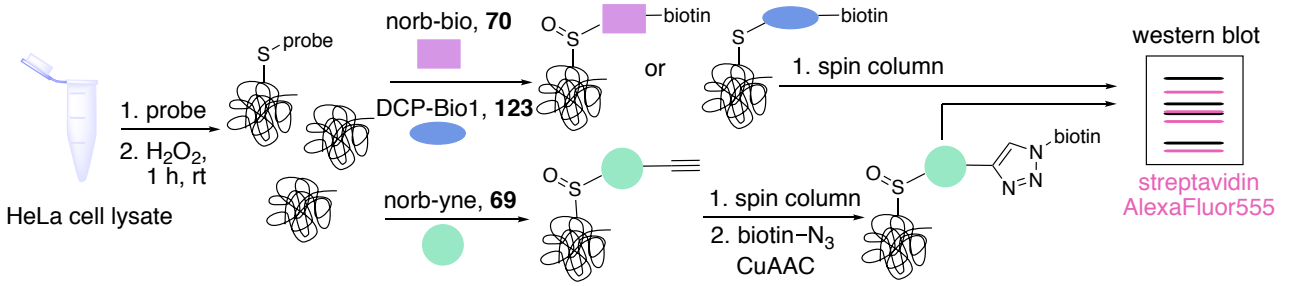


Figure 34. Outline for testing probes on HeLa cell lysate.

HeLa cell lysates were generated and conditions optimised for several factors including protein concentration, protein loading for SDS-PAGE, and western blot analysis. Initially, lysates were treated with either probe **70** or **69** (1 mM), while varying the concentration of hydrogen peroxide (0 to 2 mM) (Fig. 34). As a control, the lysate treated with no hydrogen peroxide was pre-treated with tris(2-carboxyethyl)phosphine (TCEP) so as to keep protein thiols in their reduced state. This precaution was applied since the earlier protein experiments had shown that cysteine thiols can be oxidised in air, but inclusion of a reducing agent prevents this (Fig. 33B). Additionally, lysates were pre-treated with hydrogen peroxide (2 mM) for 30 min before probe addition. This was to effectively overoxidise the sulfenic acids to sulfinic or sulfonic acids, which do not react with norbornene probes based on results presented in Chapter two. Pre-treatment with hydrogen peroxide had shown to dramatically reduce labelling in the papain LC-MS experiments. Lysates were also treated with varying concentration of probes **70** and **69** (0.1 to 3 mM) followed by treatment with hydrogen peroxide (0.1 mM). This was so the effects of both probe concentration and hydrogen peroxide concentration could be assessed. All reactions were left for 1 h at room temperature. Samples were then purified with size exclusion spin columns (Zeba 7 kDa MWCO) to remove excess probe so as to prevent further reaction during sample preparation. For norb-bio **70**, samples were analysed directly by SDS-PAGE and western blotting. For norb-yne **69**, samples were first ligated to an azide-tagged biotin derivative using CuAAC prior to SDS-PAGE. Biotinylated proteins were detected in western blot using streptavidin conjugated to the fluorescent AlexaFluor555 and visualised. Total protein was assessed using Sypro ruby blot stain to ensure equal protein loading in each lane.

For both norbornene probes, an increase in labelling could be observed with an increase in both probe concentration and hydrogen peroxide concentration (Fig. 35 and Fig. 36). Additionally, protein labelling was seen across the full proteome, confirming the broad reactivity of the norbornene

probes. For the control treated with TCEP, no labelling was seen for either probe (Fig. 35 and 36, far left lane). This result was significant as it suggests that norbornene probes do not react with cellular thiols under the conditions of the assay. Finally, the control pre-treated with hydrogen peroxide also had no labelling for either norbornene probe (Fig. 35 and 36, far right lane), confirming their selectivity for the sulfenic acids. Figure 35 and Figure 36 are representative western blots showing the optimised conditions of probe **70** and **69** respectively.

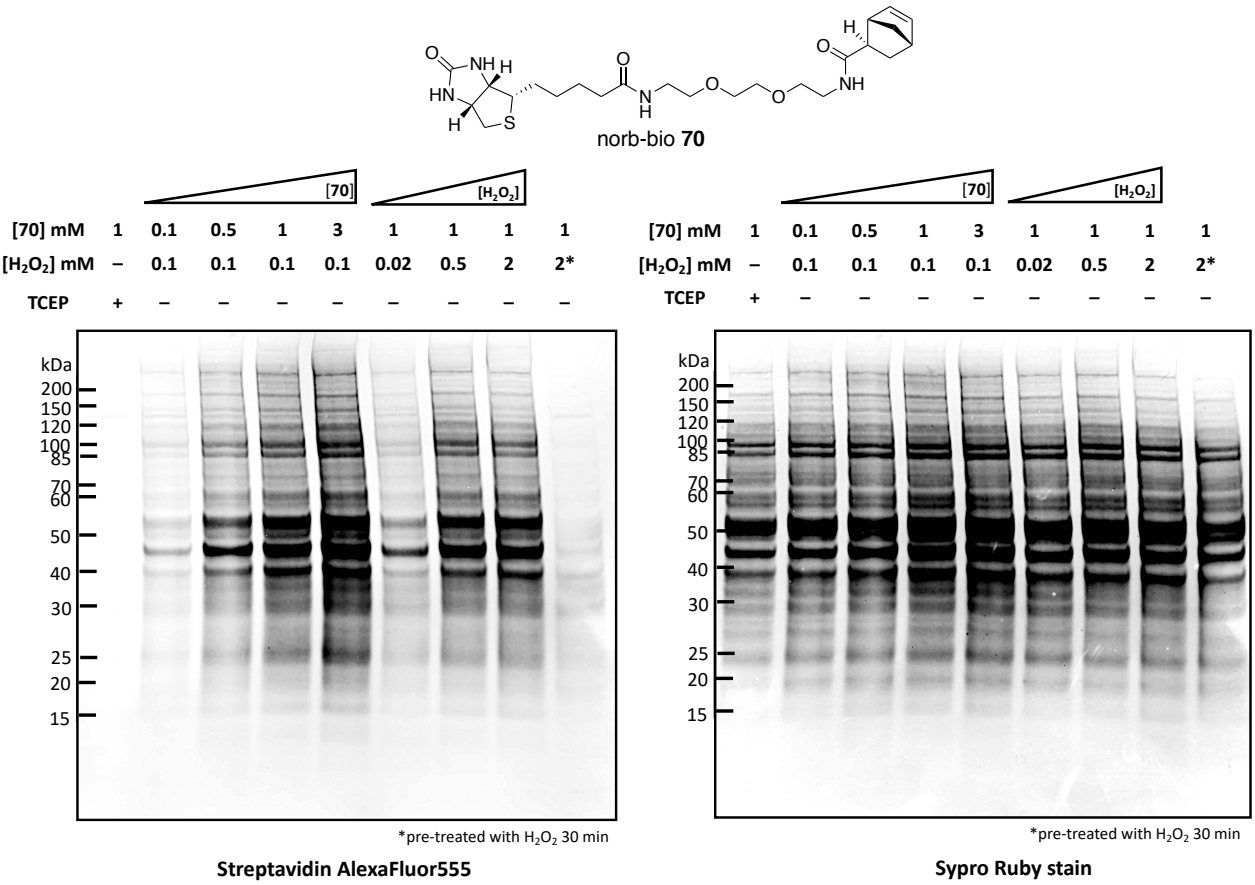


Figure 35. Western blot analysis of the reaction of probe **70** with HeLa cell lysates treated with hydrogen peroxide. Left: Western blot analysed with streptavidin AlexaFluor555 to show biotinylated proteins. Right: Western blot treated with Sypro Ruby total protein stain to show total protein loading. Each lane was loaded with approximately 15 μg total protein.

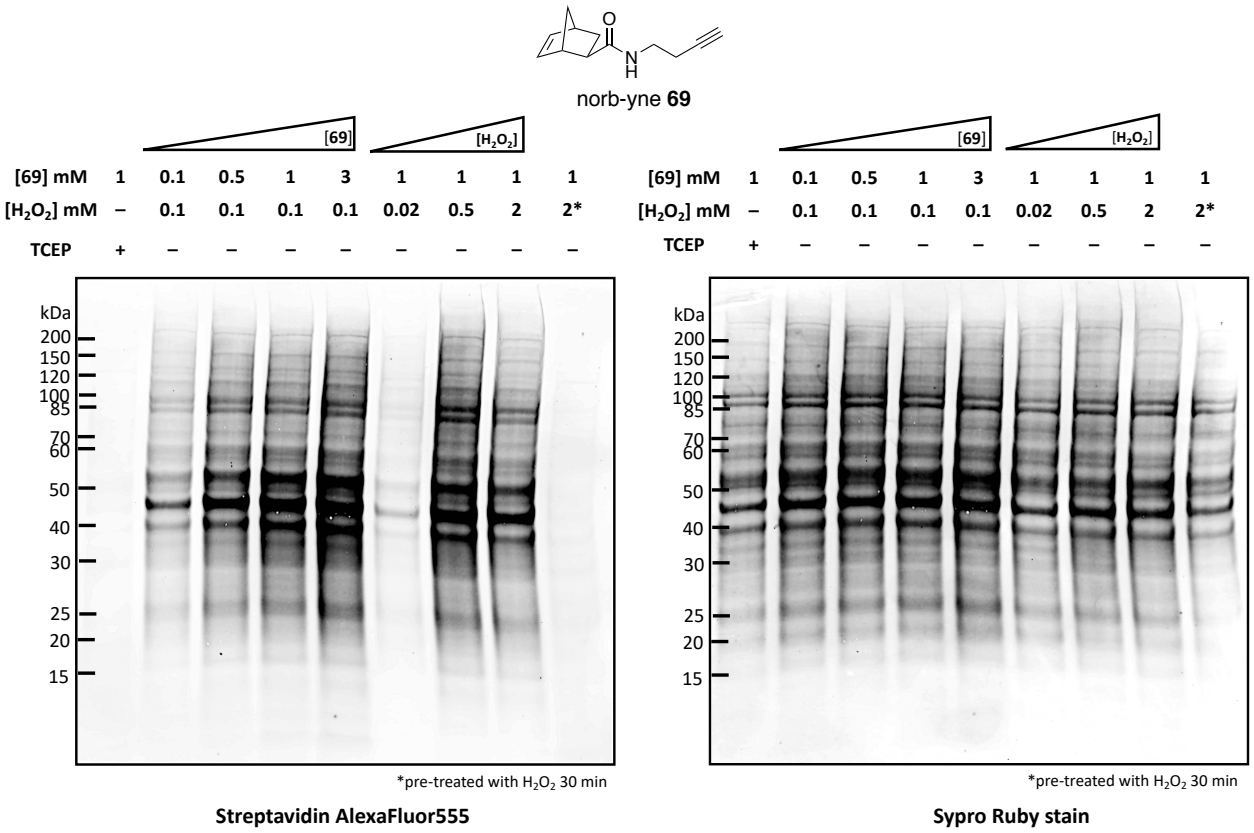


Figure 36. Western blot analysis of the reaction of probe **69** with HeLa cell lysates treated with hydrogen peroxide. Left: Western blot analysed with streptavidin AlexaFluor555 to show biotinylated proteins. Right: Western blot treated with Sypro Ruby total protein stain to show total protein loading. Each lane was loaded with approximately 8 μg total protein.

The results depicted in Figure 35 and 36 were obtained after careful optimisation of all conditions, taking into consideration the results of the papain model experiments. For these figures, the samples were first purified with a size exclusion spin column before preparation for SDS-PAGE. When excess norbornene probe **70** was not removed prior to SDS-PAGE, minimal off-target labelling could be observed in the control lane treated with TCEP and no hydrogen peroxide, as well as the control pre-treated with hydrogen peroxide (Fig. 37, far left and right lanes respectively). Since this off-target labelling could be eliminated simply by removing excess probe before SDS-PAGE, it was concluded that this off-target reactivity was occurring during the sample preparation, not during the course of the reaction. The conditions of SDS-PAGE sample preparation include SDS buffer under reducing conditions (DTT) with heating to 95 ºC. Since the probe is added in high excess, it is not so surprising this reactivity could occur. Although, based on Figure 37 and the relative intensity of each lane, the off-target labelling is minimal, certainly not labelled to the degree of the hydrogen peroxide treated samples. While this off-target reactivity is possible, it clearly does not out-compete the reaction of the norbornene olefin with sulfenic acids, considering it also requires extreme conditions (95 ºC) to

occur. Whether this was occurring through the thiol-ene reaction is uncertain. Regardless, it was clear that effective removal of any excess probe seemed to alleviate this cross-reactivity, deeming it not an issue if care was taken with sample preparation. Overall, both norbornene probes displayed a broad reactivity with good selectivity across the proteome.

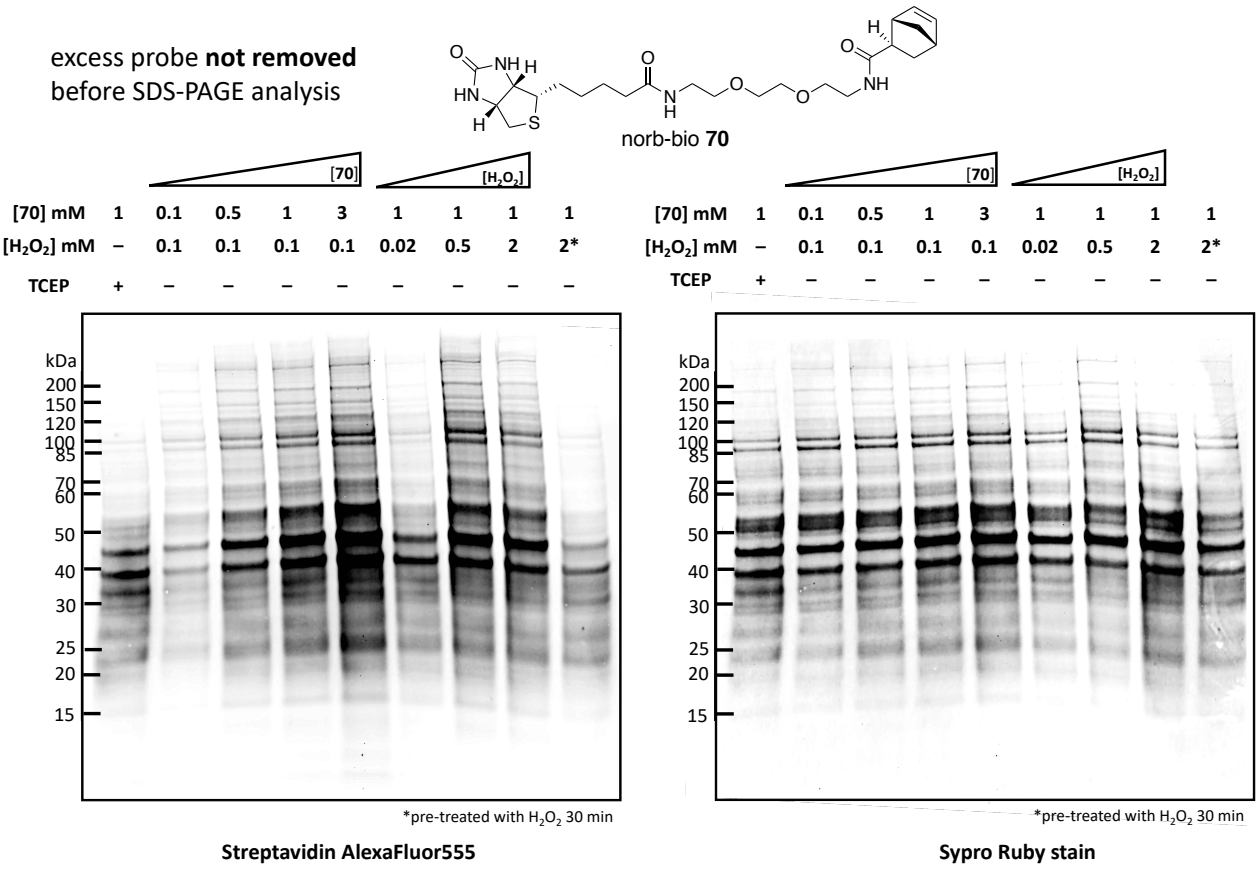


Figure 37. Western blot analysis of the reaction of probe **70** with HeLa cell lysates treated with hydrogen peroxide without removal of excess probe prior to SDS-PAGE. Left: Western blot analysed with streptavidin AlexaFluor555 to show biotinylated proteins. Right: Western blot treated with Sypro Ruby total protein stain to show total protein loading. Each lane was loaded with approximately 15 μg total protein.

3.4.2 Dimedone probe (DCP-Bio1)

Encouraged by these promising results, we sought to compare our probe to a commonly used dimedone probe that was commercially available. We opted for dimedone with a biotin tag (DCP-Bio1, Kerafast) as this appeared to be the most widely used, and was similar to our norbornene biotin probe.[103, 176, 182-183] Using the exact same conditions as for our norbornene study, cell lysates were treated with DCP-Bio1 **123** with varying concentration of probe (0.1 to 3 mM), and varying concentration of hydrogen peroxide (0 to 2 mM) as well as the TCEP and pre-treated hydrogen peroxide control. The results obtained were intriguing. DCP-Bio1 appeared to show increase in

labelling with increase in both probe concentration or hydrogen peroxide concentration as expected (Fig. 38). It also seemed **123** reacted with a slightly different labelling profile when compared alongside both norbornene probes, based on the intensity of various bands. These differing protein hits could arise from the distinct reaction mechanisms of the norbornene and dimedone probes, preferring different subsets of proteins, or the differences in sterics of the probes due to size and molecular geometry. The most interesting result was the control experiments. For the control experiment where lysates were pre-treated with TCEP to reduce all thiols, significant labelling was seen for **123**, with equivalent intensity to the labelling observed when the lysates were treated with hydrogen peroxide (Fig. 38, far left lane). For the second control experiment where lysates were pre-treated with hydrogen peroxide before addition of any probe, significant labelling was also observed with **123** (Fig. 38, far right lane)**,** almost as intense as the TCEP control. Under these conditions, most cysteines (but perhaps not all) would presumably be oxidised to the sulfinic or sulfonic acids. Therefore, it is unclear what the DCP-bio1 **123** may be unselectively reacting with, since in the TCEP control, all cysteines would presumably be reduced to the thiols. Previous studies have indicated that DCP-Bio1 **123** can label proteins non-selectively if cysteine thiols are not alkylated.[21] It is also known, for instance, that dimedone can react with amines even at room temperature under certain conditions. [99, 184] It is not clear what is causing the non-selective labelling in this experiment, but is certainly worth following up in further studies to determine if this off-target reactivity is unique to probe **123**, or applies to all dimedone-based probes. This would assist with interpreting the results that have been obtained with probe **123** so far, especially given the product of the reaction of **123** with either the sulfenic acid or the thiol is the same.

To further evaluate the scope of this off-target labelling, the conditions of the reduced control were altered. It was possible that TCEP itself may be enhancing the off-target reactivity, although not likely as there was still considerable off-target labelling in the overoxidised control sample which did not contain TCEP. An alternative reducing agent, DTT, as well as a non-treated lysate sample were analysed against the TCEP and hydrogen peroxide treated samples under the same conditions. In all cases, including the non-treated samples, off-target labelling was seen (Fig. 39). Most intriguing was the intensity of the labelling. The TCEP treated sample had the most labelling, followed by DTT and non-treated. This could possibly be explained by the availability of free thiols. In the non-treated samples, some thiols may exist in their oxidised forms (disulfide or even sulfenic acid etc) reducing the overall availability of free thiols. Whereas for the samples treated with DTT and TCEP, the availability of free thiols is increased. Since TCEP is a stronger reducing agent, and all samples were treated with identical conditions so as not to introduce bias, it's likely the TCEP sample contained more free thiols. This increase in available thiols seemed to correspond to increased labelling, suggesting the thiols could be the culprit of the non-selective binding, but this was not confirmed.

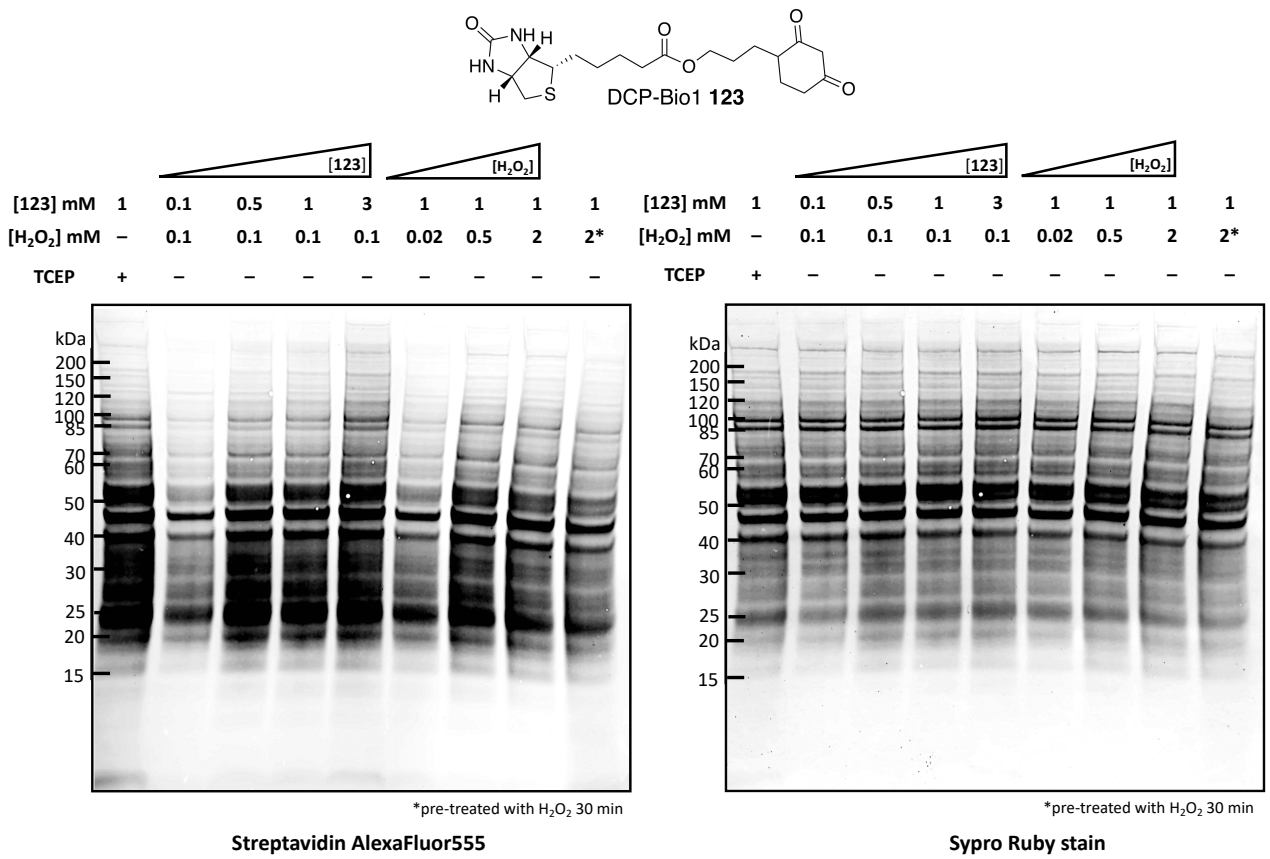


Figure 38. Western blot analysis of the reaction of probe **123** with HeLa cell lysates treated with hydrogen peroxide. Left: Western blot analysed with streptavidin AlexaFluor555 to show biotinylated proteins. Right: Western blot treated with Sypro Ruby total protein stain to show total protein loading. Each lane was loaded with approximately 15 μg total protein.

All of these experiments were initially conducted in the same way as Figure 35 and 36, by first removing excess **123** before SDS-PAGE sample preparation and analysis. Unlike norb-bio **70**, which only experienced off-target labelling if excess probe was not removed, DCP-Bio1 **123** seemed to experience this off-target labelling whether or not excess probe was removed. This strongly suggests the off-target labelling is occurring during the course of the experiment, not during the preparation as appeared to be the case for norb-bio **70**. Out of curiosity, the experiment was repeated under the same conditions, but excess probe was not removed prior to SDS-PAGE. In these experiments, an enormous increase in labelling was observed equally across all reaction conditions, regardless of the cysteine oxidation state (Fig. 39). While this extreme off-target labelling was only observed under the SDS-PAGE sample preparation conditions (SDS buffer, DTT, 95 ºC) when excess probe was not removed, it is a very important consideration. It demonstrates the capability of **123** to react vigorously and non-selectively under certain conditions. While this off-target labelling was also observed with norb-bio **70** when excess probe was not removed, the intensity of labelling observed for **70** was incredibly minimal when compared to **123**, indicating the greater ability of **123** to react non-selectively

under certain conditions. From the results of Figure 38 and 39, it could be concluded that perhaps DCP-Bio1 **123** is not as selective as norbornene probes under the conditions tested. These results prompt a cautionary note that false positives are possible when using DCP-Bio1 in the presence of free thiols or in experiments in which reducing conditions are established by adding TCEP or DTT to protein mixtures.

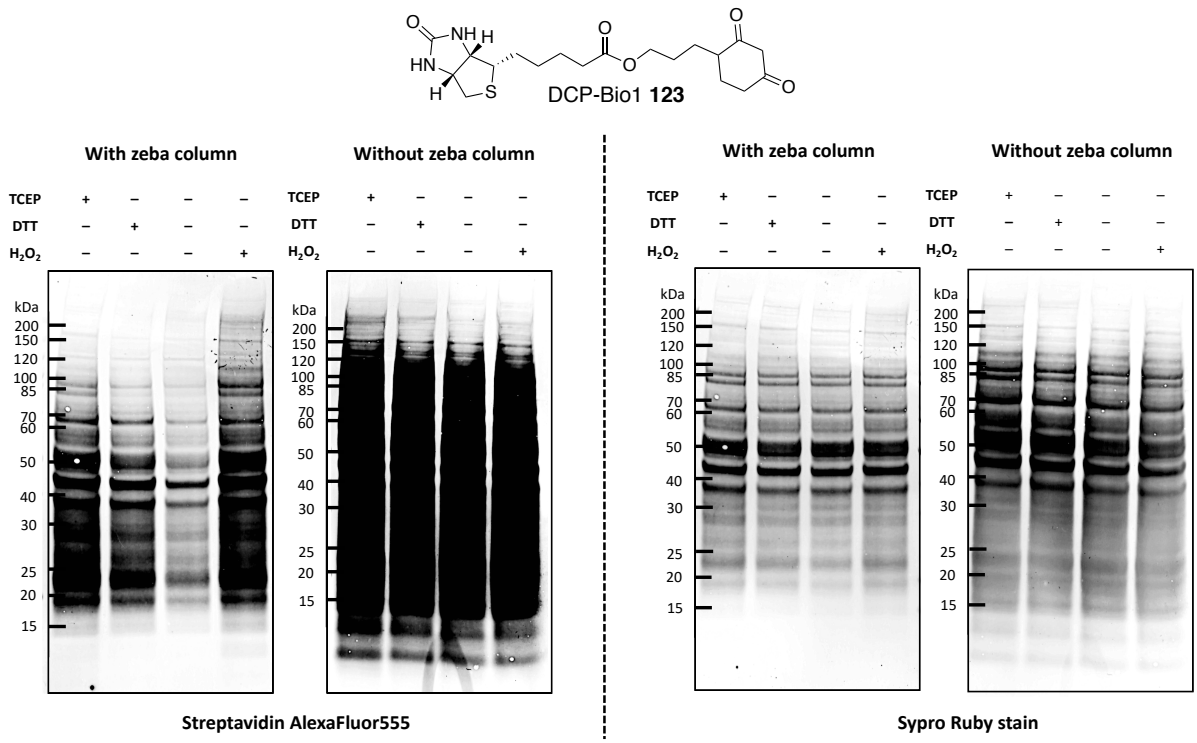


Figure 39. Western blot analysis of the reaction of probe **123** (1 mM) with HeLa cell lysates under reducing conditions. Analysis is shown both with prior removal of excess probe and without as labelled. Left: Western blot analysed with streptavidin AlexaFluor555 to show biotinylated proteins. Right: Western blot treated with Sypro Ruby total protein stain to show total protein loading. Each lane was loaded with approximately 13 μg total protein.

3.4.3 Proteomics for site of modification

The initial studies on cell lysates were exciting as they confirmed the general reactivity of the norbornene probes across the thiol proteome while maintaining selectivity. In addition to these results, we wanted confirmation that the norbornene was indeed reacting at the cysteine sulfenic acid site in proteins. Proteomics analysis was sought to confirm the site of labelling for representative hits. The norb-yne **69** probe was chosen as the example in the first instance. In order to prevent over-complication and detect the exact probe on the peptide fragments, rather than one which had been modified, no additional ligation (of the biotin-azide through CuAAC) or purification steps were performed. Cell lysates were labelled with norb-yne (1 mM, **69**) and treated with hydrogen peroxide

(0.1 mM) for 1 h at room temperature as these conditions seemed to give the best labelling based on Figures 35 and 36. The sample was then directly prepared for SDS-PAGE and the gel submitted for proteomics analysis. All proteomic sample preparation and analysis was performed by the Cambridge Centre for Proteomics at the University of Cambridge, England. The band at 45 kDa was excised, digested with chymotrypsin, and analysed by LC-MS/MS. This band was selected because it was a prominent signal for both probes in the western blots of Figures 35 and 36. The expected product formed from the reaction of norb-yne **69** with cysteine sulfenic acid was indeed detected on two separate actin protein peptide fragments, providing for the first-time confirmation of the site of reaction being at the cysteine residue (Fig. 40). Actin proteins were chosen as the representative hits as their high abundance in cells allowed significant labelling to be achieved. This meant a significant amount of labelled actin peptides were present so as to be detectable in LC-MS/MS without additional purification to enrich labelled proteins. This experiment could be refined by the use of affinity purification to only extract the labelled proteins and increase their abundance to enhance detection. In this case, however, the site of reaction had been confirmed which was the key purpose of this experiment. Affinity purification for proteomics analysis will be discussed further in Chapter four.

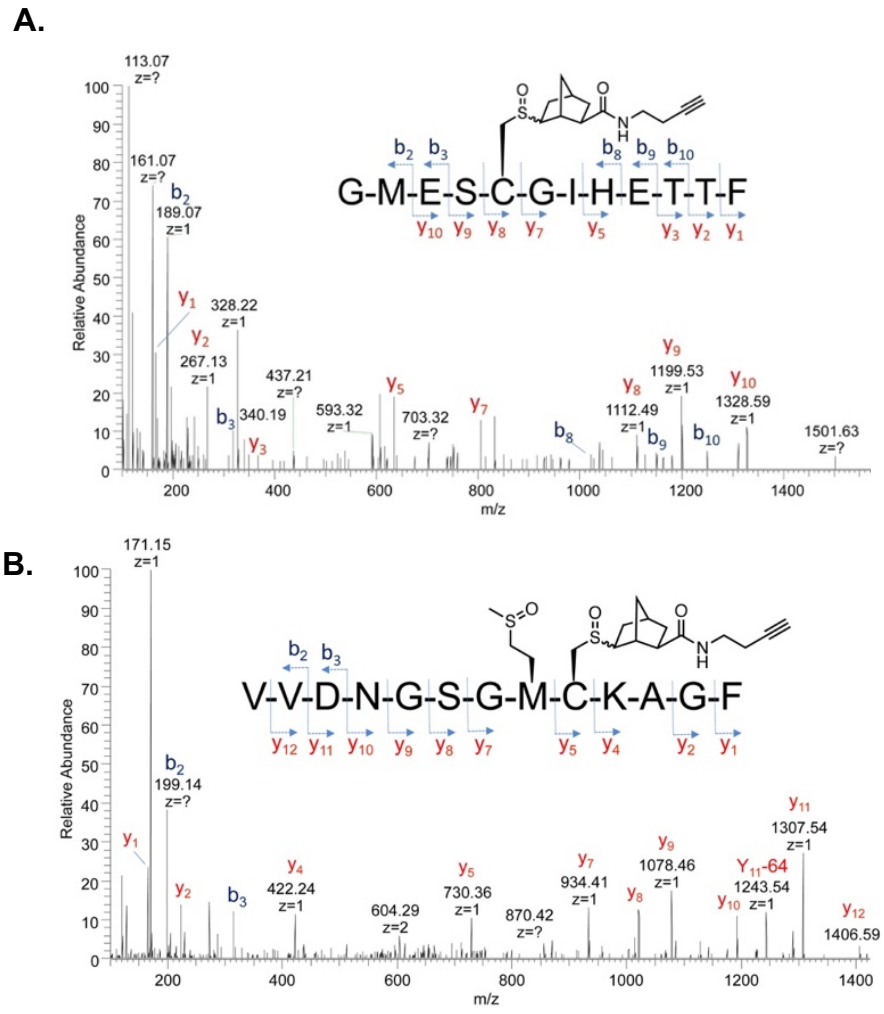


Figure 40. A. MS/MS spectrum of the m/z 758.83 doubly charged ion of the chymotryptic peptide GMESCGIHETTF (actin cytoplasmic 2), containing the modification **69** at the cysteine residue (Cys-

271). B. MS/MS spectrum of the peptide VVDNGSGMCKAGF, a modified fragment of the protein actin cytoplasmic 1. The parent ion of m/z = 753.34 corresponds to the doubly charged ion of this peptide fragment, containing an oxidised methionine and the ligation of probe **69** to the sulfenic acid of Cys-17. The y_{11}-64 fragment ion represents loss of CH_3SOH from Met-16. This figure was provided by the Cambridge Centre for Proteomics.

3.5 Live cell experiments

3.5.1 Norbornene probe toxicity

With the results of the cell lysate experiments confirming the selectivity and broad protein reactivity of the norbornene probes, we next tested their applicability in living cells. For these probes to be effective as cysteine sulfenic acid probes in cells, they would need to be relatively non-toxic and able to cross the cell membrane. Firstly, the toxicity of both probes, DMSO vehicle, and hydrogen peroxide was assessed in HeLa cells. The solubility of the probes appeared to be problematic. In preparing the probe **70** stock solution in DMSO, increasing the concentration greater than 300 mM resulted in eventual precipitation of the probe. Since the live cell labelling experiments would likely require high concentration of probe, this would mean high concentrations of DMSO would need to be employed to perform the experiment. Initially both probes were screened at low concentrations (0.012 to 1 mM) in DMSO (0.33 %v/v) and found to have no apparent toxicity effects compared to the DMSO 0.33 %v/v control after incubation for 72 h (Fig. 41A). Increasing the probe concentration beyond 1 mM then required increase in DMSO concentration. Generally, DMSO concentrations higher than 0.5 % by volume can be toxic to cells.[185] This was problematic as the DMSO toxicity would likely mask the probe toxicity if any were experienced. However, many live cell sulfenic acid studies have used DMSO concentrations up to 2 %, so perhaps would not be an issue because the duration of the experiment would be a few hours.[38, 100] To account for this DMSO toxicity, the cells were incubated with probe (1 to 6 mM), DMSO vehicle (0.33 to 2 %v/v), or hydrogen peroxide (0.037 to 3 mM) for only 5 h before removing and replacing the media and allowing the cells to continue to grow for 72 h. This was to prevent total cell death by DMSO (which was found to be the case if cells were left in high concentrations of DMSO for the total 72 h). Rather than assessing cell viability after the initial 5 h incubation, cells were left longer to incubate so that long term effects (ie. cell proliferation) could be assessed and differences between conditions more pronounced. The concentration of norb-bio **70** was tolerated up to 3 mM with 75-80 % cell viability in DMSO 1 %v/v (Fig. 41B). At 6 mM of **70**, toxic effects were observed with 30-40 % viability, however this was consistent with the DMSO control at 2 %v/v which only had 40-50 % viability suggesting that most of the toxic effects were associated with DMSO, not the probe. Unfortunately, the same could not be said for norb-yne **69**. At 3 mM and DMSO 1 %v/v, **69** had limited solubility and was toxic to cells leading to total loss of cell viability (Fig. 41B). The same result was obviously seen at 6 mM. Both probes were also tested at

probe concentrations of 0.5 to 6 mM, all with DMSO 2 %v/v so the DMSO concentration was held constant, while probe concentration was varied. In this case, the norb-bio **70** probe had similar viability to the DMSO control in all cases, but the norb-yne **69** was completely toxic at 3 mM, with reasonable viability at 1 mM (Fig. 41C). This was consistent with the toxicity data of **69** when DMSO concentration was not constant, indicating the toxicity was in fact coming from **69**.

The toxicity of hydrogen peroxide was also readily apparent. It was tolerated up to 0.11 mM with total cell death observed at concentrations of >1 mM after 5 h exposure and replenishing media followed by 72 h additional incubation (Fig. 41D). Similar toxicity levels of hydrogen peroxide have been reported previously, but the values vary widely.[186] In these cases, cells are most likely dying due to extreme oxidative stress conditions. Treating cells with hydrogen peroxide is known to induce apoptosis even at relatively low concentrations so it was not surprising that total cell death was observed.[181, 187]

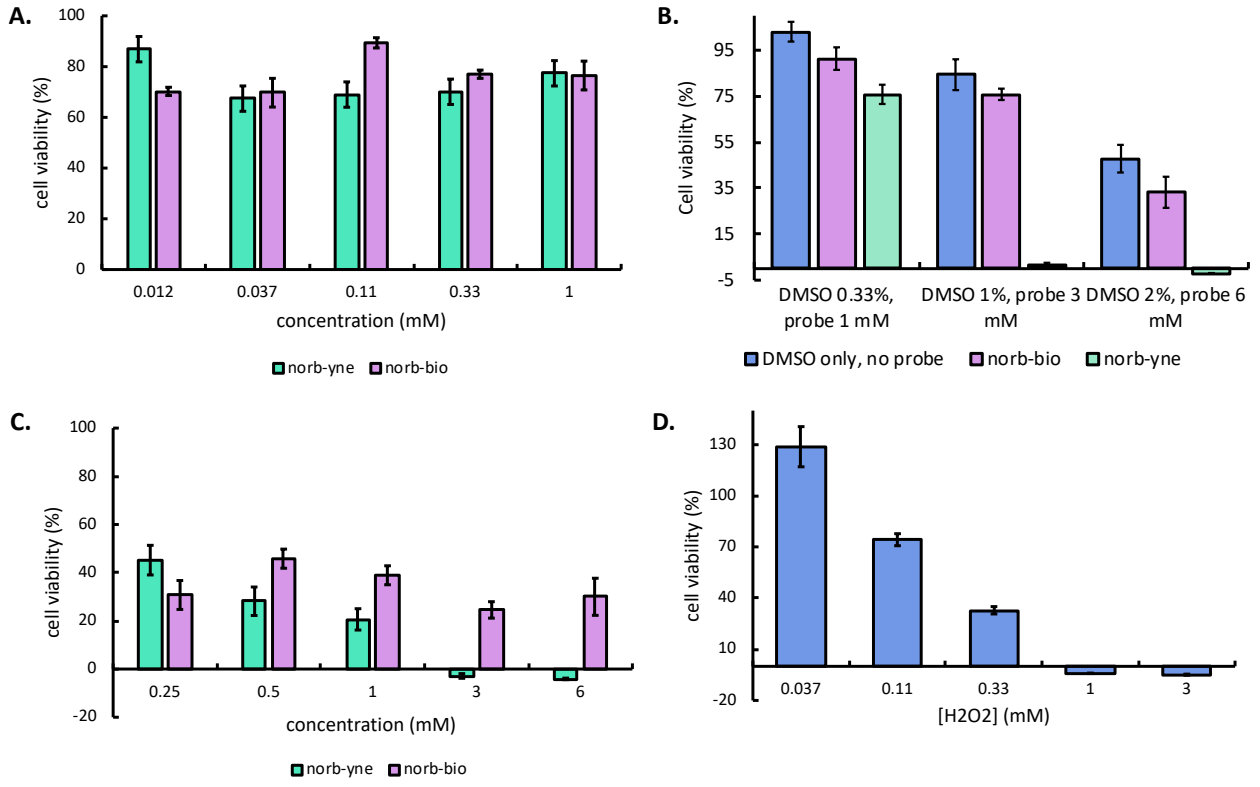


Figure 41. A. Toxicity comparison of both probes **69** and **70** at varying concentrations in DMSO 0.33 %v/v. B. Toxicity comparison of probes **69** and **70** at different concentrations and different DMSO concentrations. C. Toxicity comparison of both probes **69** and **70** at varying concentrations in DMSO 2 %v/v. D. Toxicity of hydrogen peroxide.

3.5.2 Labelling sulfenic acids in live cells

Based on these toxicity data for an appropriate probe concentration, the labelling of cysteine sulfenic acids was tested in live HeLa cells. Several optimisation experiments were performed for growth media suitability, cell number, protein loading, and concentration of probe and hydrogen peroxide. DMEM (low glucose) with only 0.5 % FBS was chosen as the experimental media to lessen the likelihood of the probe reacting with components of the media. DMEM, unlike other media such as McCoy's does not contain free cysteine (instead, contains cystine). Low FBS was also used as some of the components of FBS are potential oxidation targets, and could consume hydrogen peroxide and/or the probe before it reaches the cell. In fact, when the experiments were performed with regular DMEM (high glucose, 10 % FBS), no labelling was observed after SDS-PAGE and western blot (data not shown).

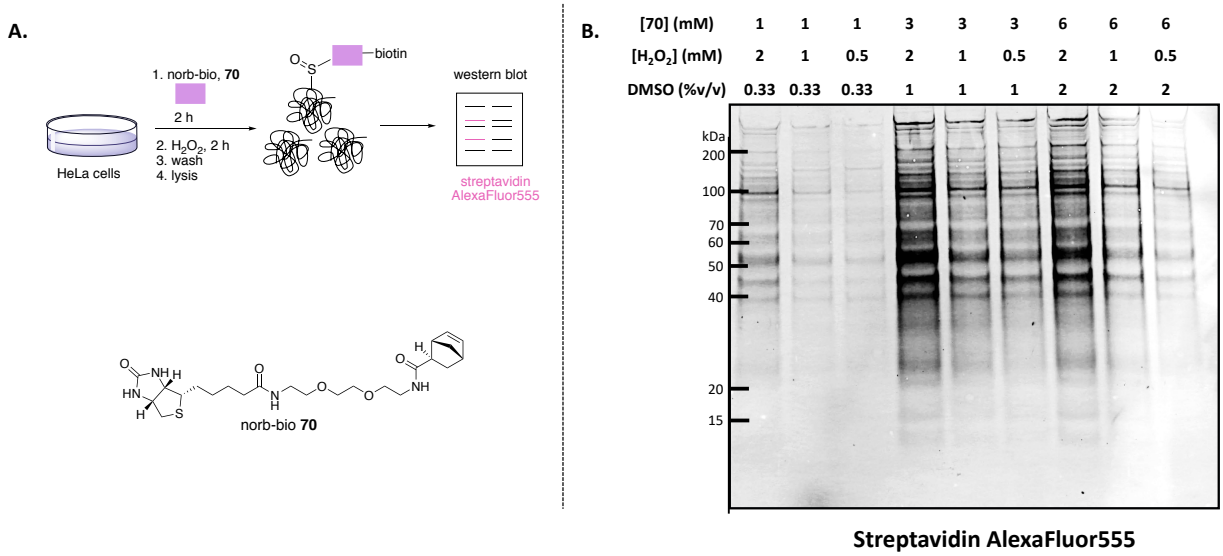


Figure 42. A. Live Hela cells were treated with norb-bio **70** under oxidising conditions. B. Western blot analysis of live HeLa cells treated with varying concentrations of **70** and hydrogen peroxide. Optimal labelling conditions were found to be probe **70** (3 mM) treated with H_2O_2 (2 mM).

For the experiment, HeLa cells (DMEM, low glucose, 0.5 % FBS) were treated with a solution of probe in DMSO so that the probe concentration was 1, 3, or 6 mM and the final concentration of DMSO was 0.33 %, 1 %, or 2 % by volume, respectively. The cells were then incubated for 2 hours at 37 °C before the addition of H_2O_2 (0.5-2 mM) (Fig. 42A). This initial incubation period was designed to allow the probe to enter the cell and reach equilibrium before inducing oxidative stress so that sufficient concentration of probe would be ready and waiting in the cellular compartments. After the addition of the hydrogen peroxide, the cells were incubated for an additional 2 hours before they were harvested, washed, and lysed. The protein lysate was then analysed by SDS-PAGE and western blotting. For norb-bio **70**, protein labelling increased with increasing concentration of probe

and hydrogen peroxide (Fig. 42B). This result was remarkable as it demonstrated for the first time the application of norbornene probes as cysteine sulfenic acid traps in living cells. A broad range of proteins were once again labelled, indicating both the general reactivity of the probe for cellular proteins, and also its ability to cross the cell membrane. In control samples treated only with **70** and no hydrogen peroxide, no labelling was observed (Fig. 43B). This once again confirmed the selectivity of the norbornene probe and more importantly in the cellular environment.

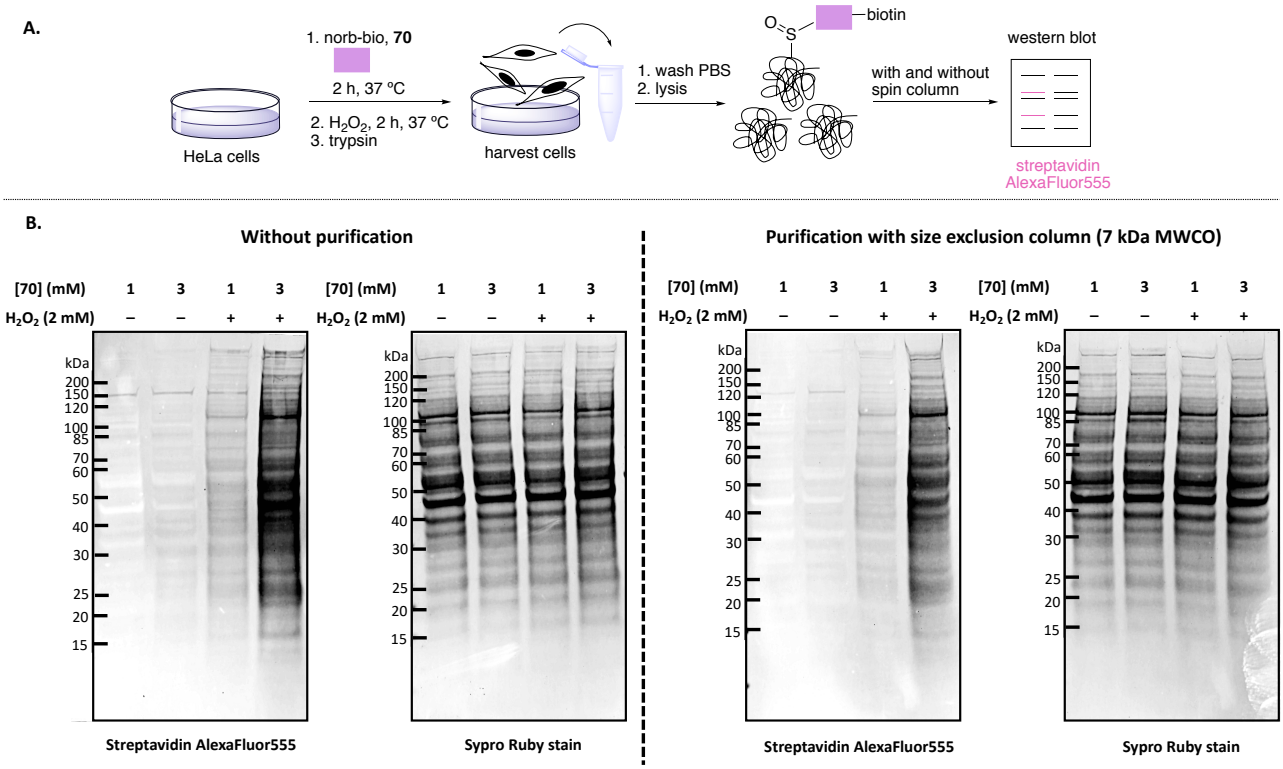


Figure 43. A. Outline of live HeLa cell experiments. B. Live HeLa cells were treated with norb-bio **70** both with and without hydrogen peroxide. Left: Samples were not purified prior to SDS-PAGE. Each lane was loaded with approximately 15 μg total protein. Right: Samples were purified prior to SDS-PAGE. Each lane was loaded with approximately 12 μg total protein.

The most efficient labelling conditions were achieved with norb-bio 3 mM and hydrogen peroxide 2 mM. Figure 43 shows a representative western blot of the optimised conditions for live cell labelling. At lower concentrations of hydrogen peroxide, very little labelling was observed. This was a difficult situation as the higher concentrations were extremely toxic to the cells, but any less was not labelling sufficient protein to be detected. It was unclear if the hydrogen peroxide was being consumed by the components of the cell media before reaching the cell, and perhaps could account for reduced labelling. Since this was an initial screening of conditions, it was decided that using the highest concentration of hydrogen peroxide to give good labelling was beneficial purely for testing the ability of the norbornene probe to act as a sulfenic acid probe in cells in the first instance. It was clearly

noted that these extreme oxidative conditions did not therefore accurately depict the type of oxidative stress environment a cell would naturally experience. This observation was also noted for the use of an external source of hydrogen peroxide, rather than generated endogenously by the cells own metabolic pathways to mimic real conditions of oxidative stress. This could cause bias in the types of proteins detected by this method as there would exist a different concentration gradient of hydrogen peroxide across the cell compared to endogenously generated hydrogen peroxide in localised cellular compartments. Other sulfenic acid studies had also used concentrations of hydrogen peroxide up to 1 mM to stimulate acute oxidative stress,[92] with other studies using hydrogen peroxide concentrations (> 0.5 mM) that although lower than the 2 mM used here, are still likely considered toxic. However, these studies fail to report their own toxicity of the hydrogen peroxide concentration used under their conditions, so direct comparison cannot be made.[100, 107]

Unfortunately, all attempts to label HeLa cells with norb-yne **69** were unsuccessful. HeLa cells were treated under the exact same conditions as norb-bio **70** but failed to trap any protein. This could be due to a number of reasons. Firstly, **69** was more toxic than **70** at concentrations of 3 mM, with total cell death experienced. It was also far less soluble at this higher concentration which could mean the probe was unable to successfully cross the cell membrane, and hence no labelling observed. Whatever the case, the increased toxicity, insolubility and lack of labelling deemed the norb-yne probe **69** as unsuitable for live cell studies. This result was disappointing as this probe was designed with downstream analysis in mind such as proteomics processing, where the convenience of ligating desirable groups to the alkyne depending on the detection required would be extremely useful. Improvements to both the solubility and toxicity might be introduced through addition of short ethylene glycol linkers, or additional amide linkages. Possible improvements to the probe design will be discussed further in Chapter five.

From lysate experiments, it was noted that removing excess probe before SDS-PAGE was critical to prevent post-experiment non-selective reactions. This was tested on live cell experiments using the optimised conditions both with and without hydrogen peroxide (Fig. 43B). In these cases, it was noted that the same labelling was observed whether or not the lysate from the live cell experiment was purified with a size exclusion column before SDS-PAGE. This is likely due to the wash steps and effective concentration of the probe inside the cell being much lower than the concentration added, with additional loss of unbound probe possible during the wash steps. This result suggests it is not as critical to purify samples with a spin column prior to SDS-PAGE for live cell experiments. Overall these results were extremely gratifying as the norb-bio probe had now been used to successfully trap cysteine sulfenic acids in living cells.

3.6 Concluding remarks

Overall, the reactivity of norbornene **99** was confirmed on the sulfenic acid of papain by LC-MS to give the sulfoxide product **120**. Reactivity with the active site, and by inference the cysteine residue, was confirmed by UV-vis activity assay. The synthesised probes norb-bio **70** and norb-yne **69** were then reacted with papain in a similar manner and analysed by SDS-PAGE and western blot, demonstrating their utility for later use. Several issues were experienced with the papain model, mainly its apparent degradation likely from auto-proteolysis, and relative ease with which it seemed to oxidise in air. Nonetheless, a clear reactivity of norbornene probes with papain sulfenic acid was consistently observed. Moving on to lysate studies, both norbornene probes **70** and **69** were found to react with a broad range of proteins, demonstrating good selectivity for the oxidised samples. Comparison to a dimedone-based probe **123** found subtle differences in reactivity profile as well as selectivity issues for **123** especially when reducing conditions were used. This highlighted the superior selectivity of norbornene probes as cysteine sulfenic acid traps. Lastly, norbornene probes were analysed in live HeLa cells to assess their function as cysteine sulfenic acid probes for future studies on cellular mechanisms within live cells. Gratifyingly, the norb-bio probe **70** was able to trap sulfenic acids only when cells were treated with hydrogen peroxide, ultimately confirming both its selectivity and reactivity in the complex cellular environment. Unfortunately, the norb-yne probe **69** was not compatible with live cell studies. Successful labelling of cysteine sulfenic acids with norb-bio sets the scene for the further use of norbornene probes as chemical tools to study cysteine oxidation in living cells.

3.7 Experimental procedures

General considerations

All purchased chemicals were used as received without further purification. All UV-Vis spectroscopy was performed on a Cary 50 UV-Vis spectrophotometer (Agilent Technologies). All fluorescence and UV-Vis spectroscopy in a 96-well plate format was performed on a SpectraMax i3x plate reader (Molecular Devices). All western blot images were recorded on a Typhoon Trio Variable Mode Imager (GE Healthcare). Liquid chromatography–mass spectrometry (LC-MS) solvents were UHPLC grade. All LC-MS protein MS was run on an Agilent 6230 TOF LC-MS with 1260 Infinity LC system (Agilent) coupled to a TOF mass spectrometer (Agilent). An Acquity UPLC BEH column (Waters Corporation, C4, 1.7 μm particle size, 300A pore size, 2.1 × 50 mm) was used for all protein experiments. Proteomics of peptide fragments were submitted to Cambridge Centre for Proteomics and performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific) system and a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Separation of peptides

was on a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 μm particle size, 100A pore size, 75 μm i.d. x 50 cm length).

3.7.1 Papain activity assays

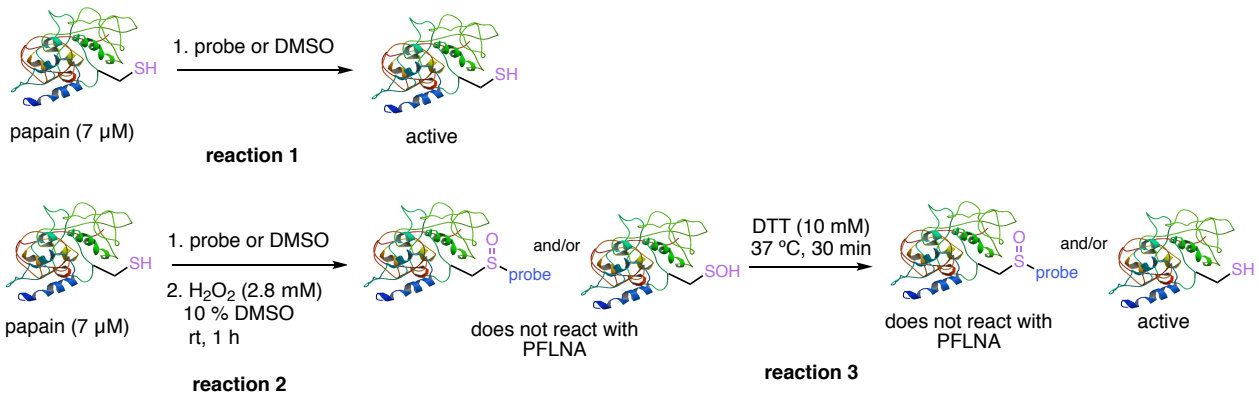
Preparation of papain stock

Papain (118 mg crude powder, Sigma) was dissolved in phosphate buffer (5 mL, pH 7.4, 100 mM) by vortexing for several minutes until mostly dissolved (note: not all material will dissolve). The mixture was then centrifuged and the supernatant collected. Dithiothreitol (DTT) (18.2 mg, 0.11 mmol) was added and the mixture incubated at 37 °C for 30 min. The resulting reduced protein solution (1 mL) was filtered through PD-midi de-salting columns pre-equilibrated with phosphate buffer (2 mL, 7 kDa MWCO, GE Healthcare). The sample was eluted with 1.5 mL phosphate buffer, split into aliquots and flash frozen. To confirm the concentration of papain in solution, the absorbance was measured at 280 nm on a Cary 50 Bio UV-Vis spectrometer using the extinction coefficient 52,770 $M^{-1}cm^{-1}$ from protein database entry 1PPN (molecular weight 23,428 Da). The concentration was then calculated using Beer's law to give an approximate concentration of 66 μM.

Stock solutions

DTT (3.2 mg, 0.021 mmol) was dissolved in phosphate buffer (0.2 mL, pH 7.4, 100 mM) to give a concentration of 100 mM and prepared fresh each time immediately prior to use. L-pyroglutamyl-L-phenylalanyl-L-leucine-p-nitroanilide (PLFNA) (1.3 mg, 0.002 mmol) was dissolved in DMSO (1.5 mL). H_2O_2 (10 μL, 33 wt% in H_2O, 9.8 M) was diluted in H_2O (990 μL) to give a concentration of 98 mM. *Cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** (88 mg, 0.48 mmol) was dissolved in DMSO (1 mL) to give a concentration of 480 mM. Alternatively, *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** (46.2 mg, 0.25 mmol) was dissolved in DMSO (1 mL) to give a concentration of 250 mM. Dimedone (39 mg, 0.28 mmol) was dissolved in DMSO (0.5 mL) to give a concentration of 540 mM.

General protocols:



Reaction 1: A solution of papain stock (100 μL, 66 μM stock) was diluted in phosphate buffer (700 μL, pH 7.4, 100 mM). A solution of probe (100 μL) or DMSO (100 μL) was then added and the reaction vortexed and left to stir for 1 h at room temperature.

Reaction 2: A solution of papain (100 μL, 66 μM stock) was diluted in phosphate buffer (700 μL, pH 7.4, 100 mM). A solution of probe (100 μL) or DMSO (100 μL) was then added and the reaction vortexed. A solution of H_2O_2 (27 μL, 98 mM in H_2O) was added, vortexed, and left to stir for 1 h at room temperature.

Reaction 3: A solution of papain (100 μL, 66 μM stock) was diluted in phosphate buffer (700 μL, pH 7.4, 100 mM). A solution of probe (100 μL) or DMSO (100 μL) was then added and the reaction vortexed. A solution of H_2O_2 (27 μL, 98 mM in H_2O) was added, vortexed, and left to stir for 1 h at room temperature. After this time, a solution of DTT (100 μL, 100 mM stock) was added and left to incubate at 37 °C for 30 min.

All samples were then analysed by UV-vis spectroscopy at 410 nm using Cary bio 50 kinetics program for the duration of 10 min. Immediately before analysis of each sample, PFLNA stock (100 μL) was added and quickly mixed before recording.

3.7.2 Papain LC-MS experiments

LC-MS parameters

All LC-MS experiments were performed using an Agilent 6230 TOF LC-MS with 1260 Infinity LC system (Agilent Technologies) coupled to a TOF/Q-TOF mass spectrometer (Agilent technologies). Separation was performed by reverse-phase chromatography at a flow rate of 0.2 mL/min and an Acquity UPLC BEH column (Waters Corporation, C4, 1.7 μm particle size, 300A pore size, 2.1 × 50 mm) used with a standard injection volume of 5 μL unless otherwise specified. The mobile phases were run with water (solvent A) and acetonitrile (solvent B), with both containing 0.1 % formic acid. The gradient was programmed as follows: 95-5 % A over 20 min, hold for 5 min, then 5-95 % A over 10 min as post equilibration and wash. All m/z values of eluting ions were measured with a TOF with Dual AJS ESI ion source (Agilent technologies) in positive ion mode and scanned between m/z 500-3200.

Papain stock

Papain (131 mg crude powder, Sigma) was dissolved in phosphate buffer (6.55 mL, pH 7.4, 50 mM) by vortexing for several minutes until mostly dissolved (note: not all material will dissolve). The mixture was then centrifuged and the supernatant collected. Dithiothreitol (DTT) (7.5 mg, 0.05 mmol) was added and the mixture incubated at 37 °C for 30 min. The resulting reduced protein solution (1

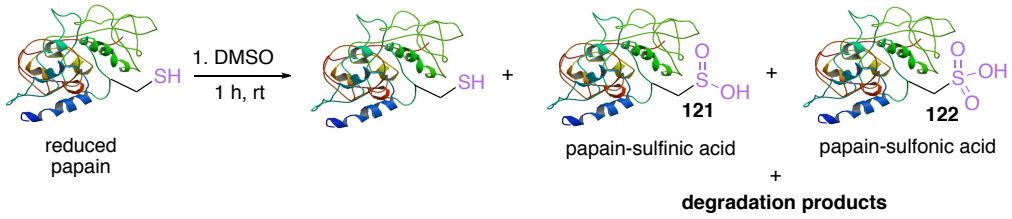
mL) was filtered through PD-midi de-salting columns pre-equilibrated with phosphate buffer (2 mL, 7 kDa MWCO, GE Healthcare). The sample was eluted with 1.5 mL phosphate buffer, aliquoted and flash frozen. To confirm the concentration of papain in solution, the absorbance was measured at 280 nm on a Cary 50 Bio UV-Vis spectrometer using the extinction coefficient 52,770 $M^{-1}cm^{-1}$ from protein database entry 1PPN (molecular weight 23,428 Da). The concentration was then calculated using Beer's law to give an approximate concentration of 66 μM.

Stock solutions

H_2O_2 (10 μL, 33 wt% in H_2O, 9.8 M) was diluted in H_2O (990 μL) to give a concentration of 98 mM. This 98 mM H_2O_2 solution was further diluted (100 μL) in H_2O (900 μL) to give a concentration of 9.8 mM. *Cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** (88 mg, 0.48 mmol) was dissolved in DMSO (1 mL) to give a concentration of 480 mM.

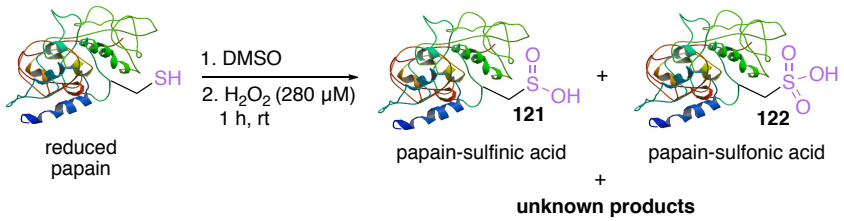
Procedures for papain LC-MS experiments:

Papain control



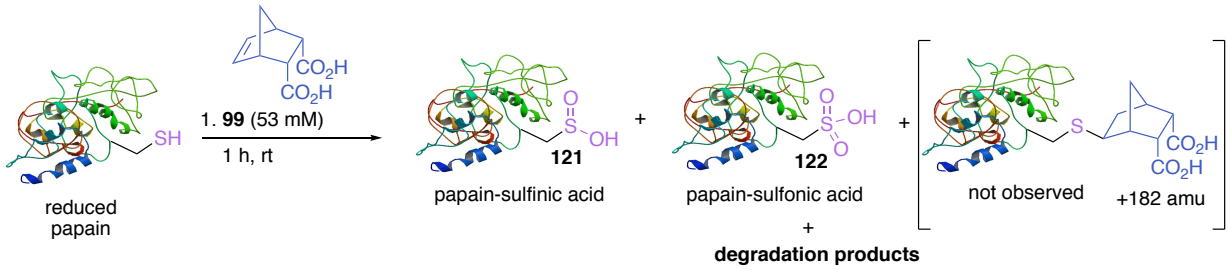
A solution of papain (50 μL, 66 μM stock) was diluted in phosphate buffer (350 μL, 50 mM, pH 7.4) for a final protein concentration of ~7 μM. A solution of DMSO (50 μL) was added and vortexed to mix. A solution of H_2O (13.5 μL) was added, vortexed and left to react for 1 h at room temperature with shaking.

Papain oxidation control



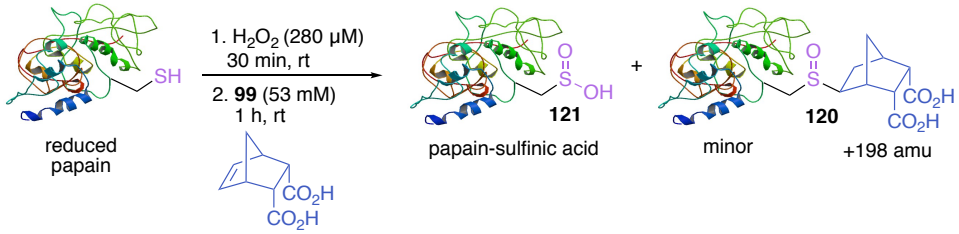
A solution of papain (50 μL, 66 μM stock) was diluted in phosphate buffer (350 μL, 50 mM, pH 7.4) for a final protein concentration of ~7 μM. A solution of DMSO (50 μL) was added and vortexed to mix. A solution of H_2O_2 (13.5 μL, 9.8 mM in H_2O) was added, vortexed and left to react for 1 h at room temperature with shaking.

Papain treated with 99 control



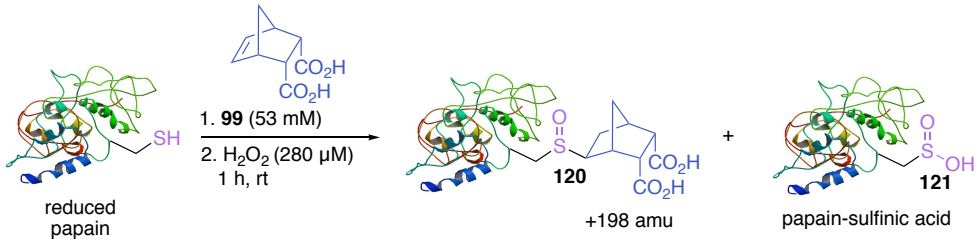
A solution of papain (50 μL, 66 μM stock) was diluted in phosphate buffer (350 μL, 50 mM, pH 7.4) for a final protein concentration of ~7 μM. A solution of **99** (50 μL, of a 480 mM stock) was added and vortexed to mix. A solution of H_2O (13.5 μL) was added, vortexed and left to react for 1 h at room temperature with shaking. Final pH of the reaction was pH 4.90.

Papain pre-treated with hydrogen peroxide then 99 control



A solution of papain (50 μL, 66 μM stock) was diluted in phosphate buffer (350 μL, 50 mM, pH 7.4) for a final protein concentration of ~7 μM. A solution of H_2O_2 (13.5 μL, 9.8 mM in H_2O) was added, vortexed and left to react for 30 min at room temperature with shaking. Then, a solution of **99** (50 μL, of a 480 mM stock) was added, vortexed and left to react for 1 h at room temperature with shaking. Final pH of the reaction was pH 4.90.

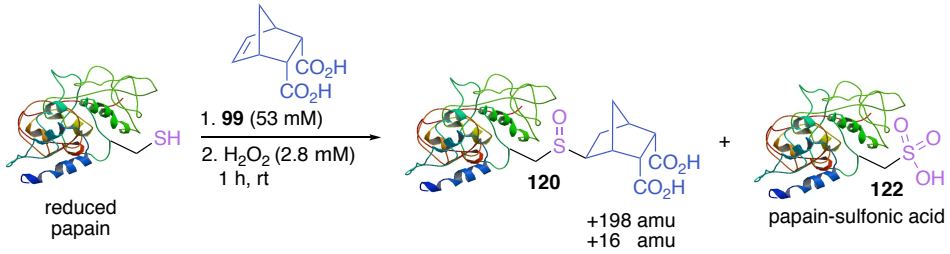
Papain treated with 99 and hydrogen peroxide



A solution of papain (50 μL, 66 μM stock) was diluted in phosphate buffer (350 μL, 50 mM, pH 7.4) for a final protein concentration of ~7 μM. A solution of **99** (50 μL, of a 480 mM stock) was added and vortexed to mix. A solution of H_2O_2 (13.5 μL, 9.8 mM in H_2O) was added, vortexed and left to react for 1 h at room temperature with shaking. Final pH of the reaction was pH 4.90.

All samples were then concentrated (Vivaspin 500, 5 kDa MWCO, Sartorius) to remove unreacted probe and excess H_2O_2 and analysed by LC-MS.

Papain treated with 99 and excess hydrogen peroxide leading to overoxidation



A solution of papain (40 μL, 66 μM stock) was diluted in phosphate buffer (280 μL, 50 mM, pH 7.4) for a final protein concentration of ~7 μM. A solution of **99** (40 μL, of a 480 mM stock) was added and vortexed to mix. A solution of H_2O_2 (10.8 μL, 98 mM in H_2O) was added, vortexed and left to react for 1 h at room temperature with shaking. Samples were then purified with a Zeba size exclusion spin column (0.5 mL, 7 kDa MWCO, ThermoFisher) to remove excess probe and analysed by LC-MS.

Total ion series and deconvoluted mass spectrum of selected peaks are shown in Appendix B for all papain experiments listed above.

3.7.3 Papain western blot experiments

Gel electrophoresis and western blot protocol

Samples were separated by SDS-PAGE using Bolt 4-12 % Bis-Tris pre-cast protein gels (Invitrogen) with MES running buffer (200 V) and either stained with SimplyStain Safeblue (Invitrogen) or transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μM, iBlot, ThermoFisher) at 20 V. After transfer, the PVDF membrane was first washed with water (3 × 1 min) then blocked with BSA (3 % in tris-buffered saline Tween-20 (TBST)) for 1 h at room temperature with gentle rocking. The membrane was washed with TBST (3 × 5 min) then incubated with 1:2,000 streptavidin-alkaline phosphatase (1 mg/mL stock, Rockland) in TBST for 30 min at room temperature with gentle rocking. The PVDF membrane was washed with TBST (2 × 5 min) and water (2 × 5 min) and incubated with BCIP/NBT (2-4 mL, Sigma) until purple bands developed (usually a few minutes) then immediately diluted and washed with water. Images were obtained by standard scanning.

Stock solutions

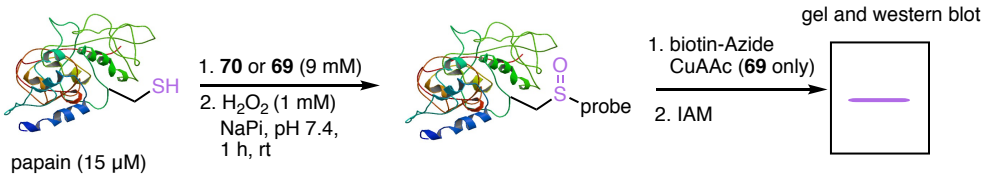
Norb-bio **70** (20 mg, 0.04 mmol) was dissolved in DMSO (404 μL) to give a concentration of 100 mM. Norb-yne **69** (8 mg, 0.042 mmol) was dissolved in DMSO (420 μL) to give a concentration of

100 mM. Iodoacetamide (8.8 mg, 0.048 mmol) was dissolved in 170 μL water to give a concentration of 280 mM. H_2O_2 (10 μL, 33 wt% in H_2O, 9.8 M) was diluted in H_2O (990 μL) to give 98 mM. Papain solutions were prepared as above. Biotin-PEG3-azide (4.9 mg, 0.011 mmol, Kerafast) was dissolved in DMSO (1.1 mL) to give a final concentration of 10 mM. TCEP (3.1 mg, 0.012 mmol) was dissolved in H_2O (270 μL) to give a final concentration of 50 mM. $CuSO_4.5H_2O$ (4.5 mg, 0.018 mmol) was dissolved in H_2O (380 μL) to give a final concentration of 50 mM. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] (TBTA) (0.6 mg, 0.0011 mmol) was dissolved in *tert*-butanol (480 μL) and DMSO (120 μL) to give a final concentration of 1.7 mM.

CuAAC reaction

After removing unreacted probe (Zeba spin column, 7 kDa MWCO, ThermoFisher), to each sample was added the azide-biotin (0.1 mM from 10 mM solution in DMSO), TCEP (1 mM from a 50 mM solution in H_2O), TBTA (0.1 mM from 1.7 mM in 1:4 DMSO:*tert*-butanol) and the samples vortexed for 10 seconds. A solution of $CuSO_4$ (1 mM from a 50 mM solution in H_2O) was added and the samples vortexed for 10 seconds to initiate the reaction. Samples were left for 1 h at room temperature with constant shaking.

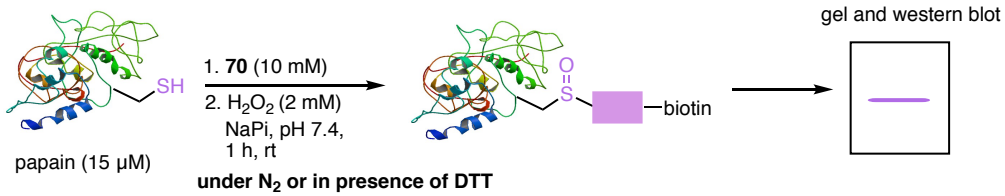
Trapping the sulfenic acid of papain (open to air)



A solution of papain (20 μL) was diluted in phosphate buffer (60 μL, 50 mM, pH 7.4) to give a concentration of papain ~15 μM. A solution of probe **70** or **69** (8 μL, 100 mM stock) or DMSO (8 μL) was added and vortexed to mix. A solution of H_2O_2 (1 μL, 98 mM in H_2O) or H_2O (1 μL) was added, vortexed, and left to react for 1 h at room temperature with shaking. Samples were then filtered through a Zeba size exclusion spin column (0.5 mL. 7 kDa MWCO, ThermoFisher) to remove unreacted probe if necessary. For norb-yne **69**, samples were subject to CuAAC with biotin-azide. **69**-treated samples were incubated with azide-biotin (100 μM, 1 μL of a 10 mM stock), tris-triazolyl ligand (TBTA) (100 μM, 6 μL of a 1.7 mM stock in 1:4 DMSO:*tert*-butanol,), TCEP (1 mM, 2 μL of a 50 mM stock in H_2O), samples vortexed, then $CuSO_4$ (1 mM, 2 μL of a 50 mM stock in H_2O) added to initiate the reaction. Samples were vortexed and allowed to react for 1 h at room temperature before purifying with a zeba size exclusion column. To each sample was then added iodoacetamide (10 μL, 280 mM stock), and the reactions were vortexed and left for 1 h at room temperature in the dark. Samples were then prepared for SDS-PAGE by mixing sample (30 μL) with lithium dodecyl

sulfate (LDS 4×) (10 μL, Invitrogen) without heating. The proteins were then resolved by SDS-PAGE and analysed by streptavidin-alkaline phosphatase western blot as described above.

Trapping papain sulfenic acid with norb-bio 70 – under N_2



Preparation of papain stock: Papain (38 mg, crude powder, Sigma) was dissolved in phosphate buffer (1.5 mL, pH 7.4, 50 mM) pre-purged with N_2 gas for 5 min. DTT (40 μL of 120 mM stock in purged phosphate buffer) was added and incubated at 37 °C for 30 min. The solution was centrifuged and 130 μL purified with a Zeba size exclusion spin column (0.5 mL, 7 kDa MWCO, ThermoFisher) and the collected eluent immediately purged with N_2 upon collection to generate the papain stock. Both reactions were conducted in a vial fitted with a rubber seal with constant flow of N_2. For each reaction, phosphate buffer (44 μL, pH 7.4, 50 mM) and **70** (10 μL, 100 mM stock) was added and purged for 2 min with N_2. Papain stock (44 μL) was then added. To one reaction, H_2O_2 (2 μL, 98 mM in H_2O) was added and to the other H_2O (2 μL) and purged with N_2 for 2 min. Both reactions were sealed under N_2 and left to react for 1 h at room temperature. After this time, both reactions were purified with a Zeba size exclusion spin column (0.5 mL, 7 kDa MWCO, ThermoFisher) to remove unreacted probe and prevent reaction with oxidised protein once exposed to air. Samples were then prepared for SDS-PAGE by mixing sample (30 μL) with lithium dodecyl sulfate (LDS 4×) (10 μL, Invitrogen) without heating. The proteins were then resolved by SDS-PAGE and analysed by streptavidin-alkaline phosphatase western blot as described above.

Trapping papain sulfenic acid with norb-bio 70 – in the presence of DTT

Reactions were prepared open to air. Using the same papain stock as the N_2 experiment without purification by spin column (to retain DTT), papain (44 μL) was diluted in phosphate buffer (44 μL, pH 7.4, 50 mM) followed by addition of **70** (10 μL, 100 mM stock). To one reaction was added H_2O_2 (2 μL, 98 mM in H_2O) and to the other H_2O (2 μL) and left to react for 1 h at room temperature. Samples were then prepared for SDS-PAGE by mixing sample (30 μL) with lithium dodecyl sulfate (LDS 4×) (10 μL, Invitrogen) without heating. The proteins were then resolved by SDS-PAGE and analysed by streptavidin-alkaline phosphatase western blot as described above.

3.7.4 HeLa cell experiments general procedures

Cell culture of HeLa cells

HeLa cells (derived from cervical cancer cells, ATCC) were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C and cultured in high glucose DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS) (Gibco), and 1 % penicillin-streptomycin (PS) (Gibco). Cells were grown to ~80-90 % confluency, trypsinised (0.25 %, trypsin-EDTA, Gibco), then neutralised with complete media prior to use.

Preparation of HeLa cell lysates

Cells were cultured as described above. Approximately 1.5 $\times 10^7$ cells were pelleted (150 $\times$g, 5 min) and washed with PBS (3 $\times$ 10 mL). Cell extraction buffer with protease inhibitor (700 μL, Invitrogen) was then added and incubated on ice for 45 min. The resulting solution was centrifuged (10,000 $\times$g, 10 min) to clarify the lysate and then purified using a Zeba size exclusion spin column (2 mL, 7 kDa MWCO, ThermoFisher) pre-equilibrated with PBS. Protein concentration was determined using Protein 660 nm assay (Pierce) and typically gave around 2-3 mg/mL protein. The eluent was aliquoted and frozen until use.

CuAAC reaction

After removing unreacted probe (Zeba spin column, 7 kDa MWCO, ThermoFisher), to each sample was added the azide-biotin (0.1 mM from 10 mM solution in DMSO), TCEP (1 mM from a 50 mM solution in H_2O), TBTA (0.1 mM from 1.7 mM in 1:4 DMSO:*tert*-butanol) and the samples vortexed for 10 seconds. A solution of $CuSO_4$ (1 mM from a 50 mM solution in H_2O) was added and the samples vortexed for 10 seconds to initiate the reaction. Samples were left for 2 h at room temperature with constant shaking. Occasionally, an off-white precipitate could be observed, but did not appear to interfere with the reaction. Samples were then analysed by SDS-PAGE and western blot as described below.

Gel electrophoresis and western blot

Samples were separated by SDS-PAGE using NuPage 4-12 % Bis-Tris protein gels (Invitrogen) with MES running buffer (200 V) and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μM, iBlot, ThermoFisher) at 20 V. After transfer, the PVDF membrane was first washed with water (3 $\times$ 5 min) then blocked with BSA (3 % in tris-buffered saline Tween-20 (TBST)) for 1 h at room temperature with gentle rocking. The membrane was washed with TBST (3 $\times$ 5 min) then incubated with 1:1,000 Alexa Fluor® 555 streptavidin (1 mg/mL stock in PBS, Invitrogen) in TBST for 30 min at room temperature with gentle rocking. The PVDF membrane was washed with TBST (2 $\times$ 5 min), water (2 $\times$ 5 min), and imaged by fluorescence (filter: 580, laser: 532 nm, PMT: 550 V) using Typhoon

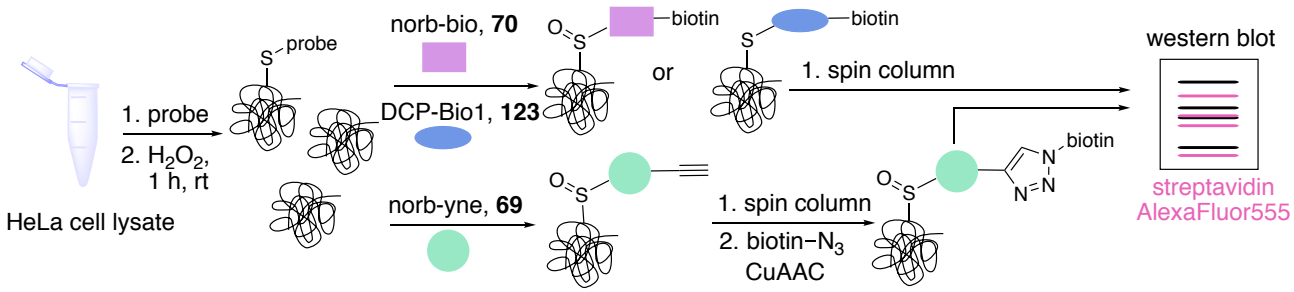
Trio imager. To assess equal protein loading, PVDF membranes were stained with Sypro Ruby blot stain (Invitrogen) according to manufacturer's instructions and imaged by fluorescence (filter: 610, laser: 532 nm, PMT: 550 V) using Typhoon Trio imager.

3.7.5 HeLa cell lysate experiments

Preparation of stock solutions

Probes **70** (14.8 mg, 0.03 mmol) in DMSO (100 µL), **69** (11.4 mg, 0.06 mmol) in DMSO (200 µL), and **123** (2 mg, 0.005 mmol, Kerafast) in DMSO (17 µL), were prepared to give final concentrations of 300 mM. Each 300 mM stock was then diluted to concentrations of 200, 100, and 20 mM in DMSO. H_2O_2 (1.1 µL, 50 wt.% in H_2O, 17.6 M) was diluted in H_2O (1 mL) to give a final concentration of 20 mM. This H_2O_2 (20 mM) solution was then diluted to 5, 1, and 0.2 mM solutions in H_2O. TCEP (13.2 mg, 0.046 mmol) was dissolved in H_2O (920 µL) to give a final concentration of 50 mM. HeLa cell lysate was prepared as described above and diluted to a final concentration of 1.2 mg/mL in PBS. Azide biotin (6 mg, 0.015 mmol, Kerafast) was dissolved in DMSO (1.5 mL) to give a final concentration of 10 mM. $CuSO_4.5H_2O$ (11 mg, 0.044 mmol) was dissolved in H_2O (880 µL) to give a final concentration of 50 mM. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] (TBTA) (3.1 mg, 0.0058 mmol) was dissolved in *tert*-butanol (2.7 mL) and DMSO (0.7 mL) to give a final concentration of 1.7 mM.

Labelling of protein sulfenic acids in HeLa cell lysates with probes 70, 69, and 123



Variation of probe concentration: HeLa cell lysate was prepared as described above. To the cell lysate (45 µL, 1.2 mg/mL total protein) in PBS, probe 0.1 mM (0.25 µL, 20 mM stock), 0.5 mM (0.25 µL, 100 mM stock) or 3 mM (0.5 µL, 300 mM stock) in DMSO was added and the reaction vortexed. To each reaction H_2O_2 (0.1 mM, 5 µL of a 1 mM stock) was added, and the samples vortexed and left to react for 1 h at room temperature.

Variation of H_2O_2 concentration: To the cell lysate (45 µL) in PBS, probe 1 mM (0.25 µL, of a 200 mM stock) in DMSO was added and the reaction vortexed. Then H_2O_2 0.02 mM, (5 µL, of a 0.2 mM stock), 0.5 mM (5 µL, of a 5 mM stock), or 2 mM (5 µL, of a 20 mM stock), was added, the samples were vortexed and left to react for 1 h at room temperature.

Pre-treatment with H_2O_2: The cell lysate (45 µL) was treated with H_2O_2 (2 mM, 5 µL of a 20 mM stock) for 30 min at room temperature. Probe (1 mM, 0.25 µL of a 200 mM stock) was then added, vortexed and left to react for a further 1 h at room temperature.

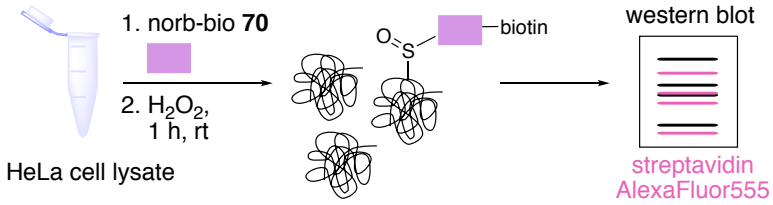
Pre-reduction with TCEP: The cell lysate (45 µL) was treated with TCEP (2 mM, 2 µL of a 50 mM stock) and water (3 µL) and then incubated for 30 min at room temperature, followed by addition of probes (1 mM, 0.25 µL of a 200 mM stock). The reaction was vortexed and allowed to react for a further 1 h at room temperature.

All samples were then quenched after 1 h reaction by purification through a Zeba size exclusion spin column (0.5 mL, 7 kDa MWCO, ThermoFisher) to remove any unbound probe.

For HeLa cell lysates treated with norb-yne **69**, reactions were subject to CuAAC before SDS-PAGE. Samples were treated with azide-biotin (100 µM, 0.55 µL of a 10 mM stock), tris-triazolyl ligand (TBTA) (100 µM, 3.2 µL of a 1.7 mM stock in 1:4 DMSO:*tert*-butanol,), TCEP (1 mM, 1.1 µL of a 50 mM stock in H_2O), samples vortexed, then $CuSO_4$ (1 mM, 1.1 µL of a 50 mM stock in H_2O) added to initiate the reaction. Samples were vortexed and allowed to react for 2 h at room temperature in the dark.

For all reactions, protein concentration was assessed by Protein 660 nm assay reagent (Pierce) after purification by spin column. Samples were then prepared for SDS-PAGE by mixing sample (17 µL, after protein concentration normalisation) with lithium dodecyl sulfate (LDS 4×) (6.5 µL, Invitrogen), and DTT NuPAGE sample reducing agent (2.5 µL, Invitrogen) and heating to 95 °C for 5 min. Protein samples were then resolved by electrophoresis and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

Labelling of protein sulfenic acids in HeLa cell lysates without removing excess probe by spin column before SDS-PAGE and western blot analysis



Variation of probe concentration: HeLa cell lysate was prepared as described above. To the cell lysate (45 µL, 1.2 mg/mL total protein) in PBS, probe 0.1 mM (0.25 µL, of a 20 mM stock), 0.5 mM (0.25 µL, of a 100 mM stock) or 3 mM (0.5 µL, of a 300 mM stock) in DMSO was added and the reaction vortexed. To each reaction H_2O_2 (0.1 mM, 5 µL of a 1 mM stock) was added, the samples were vortexed, and then left to react for 1 h at room temperature.

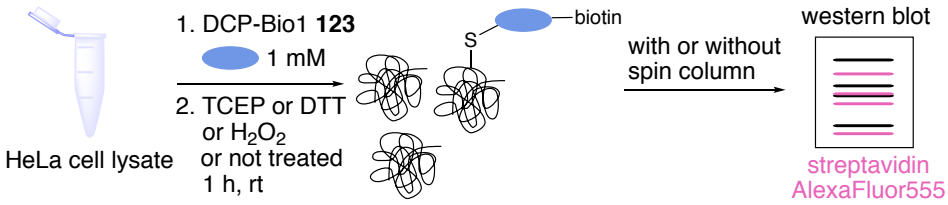
Variation of H_2O_2 concentration: To the cell lysate (45 µL) in PBS, probe 1 mM (0.25 µL, of a 200 mM stock) in DMSO was added and the reaction vortexed. Then H_2O_2 0.02 mM (5 µL, of a 0.2 mM stock), 0.5 mM (5 µL, of a 5 mM stock), or 2 mM (5 µL, of a 20 mM stock) was added, the samples were vortexed and then left to react for 1 h at room temperature.

Pre-treatment with H_2O_2: The cell lysate was treated with H_2O_2 (2 mM, 5 µL of a 20 mM stock) for 30 min at room temperature. Probe (1 mM, 0.25 µL of a 200 mM stock) was then added, the sample was vortexed and then left to react for a further 1 h at room temperature.

Pre-reduction with TCEP: The cell lysate (45 µL) was treated with TCEP (2 mM, 2 µL of a 50 mM stock) and water (3 µL) and then incubated for 30 min at room temperature, followed by addition of probes (1 mM, 0.25 µL of a 200 mM stock). The samples were vortexed and then allowed to react for a further 1 h at room temperature.

All samples were quenched by addition of DTT (2.5 µL, 500 mM stock). Samples were then prepared for SDS-PAGE by mixing sample (17 µL) with lithium dodecyl sulfate (LDS 4×) (6.5 µL, Invitrogen), and DTT NuPAGE sample reducing agent (2.5 µL, Invitrogen) and heating to 95 °C for 5 min. Samples were then resolved by electrophoresis and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

Labelling of protein sulfenic acids in HeLa cell lysates with dimedone probe DCP-Bio1 123 with different reducing conditions



Pre-treatment with TCEP: HeLa cell lysate was prepared as described above. To the cell lysate (45 µL, 1.2 mg/mL total protein) in PBS, TCEP (2 mM, 2 µL of a 50 mM stock) and water (3 µL) were added and the mixture was incubated for 30 min at room temperature, followed by addition of probes (1 mM, 0.25 µL of a 200 mM stock). The reactions were vortexed and allowed to react for a further 1 h at room temperature.

Pre-treatment with DTT: To the cell lysate (45 µL) in PBS, DTT (2 mM, 2 µL of a 50 mM stock) and water (3 µL) were added and the mixture was incubated for 30 min at room temperature, followed by addition of probes (1 mM, 0.25 µL of a 200 mM stock). The reactions were vortexed and allowed to react for a further 1 h at room temperature.

No reducing agent, no H_2O_2: To the cell lysate (45 µL) in PBS, water (5 µL) and probes (1 mM, 0.25 µL of a 200 mM stock) were added. The reactions were vortexed and allowed to react for a further 1 h at room temperature.

Oxidation with H_2O_2: To the cell lysate (45 µL) in PBS, probe (1 mM, 0.25 µL of a 200 mM stock) was added and the reaction vortexed. H_2O_2 (0.1 mM, 5 µL of a 1 mM stock) was added, the samples were vortexed and then left to react for 1 h at room temperature.

Before analysis by SDS-PAGE and western blot, 30 µL of each sample was purified using a Zeba size exclusion spin column (0.5 mL, 7 kDa MWCO, ThermoFisher) to remove any unbound probe before preparing for SDS-PAGE. The remaining 20 µL was prepared for western blot analysis without purification. Samples were then prepared for SDS-PAGE by mixing sample (17 µL) with lithium dodecyl sulfate (LDS 4×) (6.5 µL, Invitrogen), and DTT NuPAGE sample reducing agent (2.5 µL, Invitrogen) and heating to 95 °C for 5 min. Samples were then resolved by electrophoresis and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

3.7.6 Proteomics analysis

Proteomics gel preparation

Gels were prepared for proteomics analysis using NuPage 4-12 % Bis-Tris protein gels (Invitrogen) with MES running buffer (200 V). After separation, gels were fixed (1 % acetic acid, 45 % methanol in water) for 30 min then washed in water (2 × 5 min). After fixing, gels were incubated with coomassie overnight at 4 °C with gentle rocking. Gels were then washed with water for 1-2 h. The appropriate bands were excised for LC-MS/MS analysis and protein identification performed by Cambridge Centre for Proteomics. 1D gel bands were transferred into a 96-well PCR plate. The bands were cut into 1 mm^2 pieces, destained, reduced (DTT) and alkylated (iodoacetamide) and subjected to enzymatic digestion with chymotrypsin overnight at 25 °C. After digestion, the supernatant was pipetted into a sample vial and loaded into an autosampler for automated LC-MS/MS analysis.

Proteomics parameters (performed at Cambridge Centre for Proteomics)

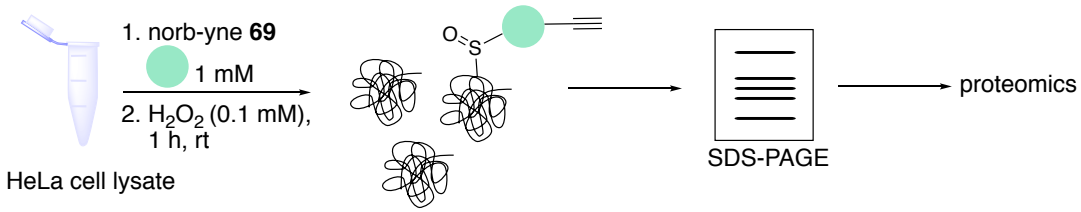
All LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific) system and a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Separation of peptides was performed by reverse-phase chromatography at a flow rate of 300 nL/min and a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 µm particle size, 100A pore size, 75 µm i.d. x 50 cm length). Peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5 µm particle size, 100A pore size, 300 µm

i.d. x 5 mm length) from the Ultimate 3000 autosampler with 0.1 % formic acid for 3 minutes at a flow rate of 10 μL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1 % formic acid and solvent B was 80 % acetonitrile, 20 % water + 0.1 % formic acid. The linear gradient employed was 2-40 % B in 30 minutes. The LC eluent was sprayed into the mass spectrometer by means of an Easy-Spray source (Thermo Fisher Scientific). All m/z values of eluting ions were measured in an Orbitrap mass analyser, set at a resolution of 70000 and was scanned between m/z 380-1500. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD, Normalised Collision Energy: 25 %) in the HCD collision cell and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17500. Singly charged ions and ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic exclusion window of 20 seconds was employed.

Proteomics data processing (performed at Cambridge Centre for Proteomics)

Post-run, all MS/MS data were converted to mgf files and the files were then submitted to the Mascot search algorithm (Matrix Science) and searched against the UniProt human database (93609 sequences; 37041084 residues) and a common contaminant sequences database. Variable modifications of oxidation (M), deamidation (NQ) and probe **69** (C) and a fixed modification of carbamidomethyl (C) were applied. The peptide and fragment mass tolerances were set to 20 ppm and 0.1 Da, respectively. A significance threshold value of $p < 0.05$ and a peptide cut-off score of 20 were also applied.

Proteomics analysis of HeLa cell lysate treated with norb-yne 69



HeLa cell lysate was prepared as described above. Protein concentration was assessed using BCA assay (Pierce) and determined to be approximately 1.4 mg/mL. To the cell lysate (89 μL) in PBS, norb-yne **69** (1 mM, 0.5 μL of a 200 mM stock) was added and the reaction vortexed. H_2O_2 (0.1 mM, 10 μL of a 1 mM stock) was added, the samples were vortexed, and then left to react for 1 h at room temperature. The reaction was quenched by addition of DTT (13 μL NuPage reducing agent). Samples were then prepared for SDS-PAGE by mixing sample (85 μL) with lithium dodecyl sulfate (LDS 4×) (25 μL, Invitrogen) and heating to 95 °C for 5 min. Samples were then resolved by SDS-PAGE as described above. The gel was prepared for proteomics analysis as described above. The

band at 45 kDa was excised and analysed by LC-MS/MS at Cambridge Centre for Proteomics as described above.

3.7.7 Live HeLa cell experiments

Cell viability assay preparation of stock solutions

Probes **70** (14.8 mg, 0.03 mmol) in DMSO (100 µL) and **69** (11.4 mg, 0.06 mmol) in DMSO (200 µL) were prepared to give final concentrations of 300 mM. These 300 mM stock solutions of probe were diluted to 150, 50, 25, and 12.5 mM in DMSO. These 300 mM stock solutions of probe were also diluted to 100, 33, 11, and 3.7 mM in DMSO. H_2O_2 (51 µL, 50 wt.% in H_2O, 17.6 M) was diluted in H_2O (949 µL) to give a final concentration of 900 mM.

Cell viability assay protocol

HeLa cells were cultured as described above. In a 96–well plate, HeLa cells were seeded to 2.5 $\times 10^3$ cells/well and incubated overnight in 200 µL DMEM (10 % FBS, 1$\times$ Pen-Strep) in a humidified atmosphere of 5 % CO_2 at 37 °C to recover.

Probe screen at low concentration: To each well, the appropriate concentration of probe (**70** or **69**) was added as follows: 1 mM (0.67 µL of a 300 mM stock), 0.33 mM (0.67 µL of a 100 mM stock), 0.11 mM (0.67 µL of a 33 mM stock), 0.037 mM (0.67 µL of a 11 mM stock), 0.012 mM (0.67 µL of a 3.7 mM stock) in DMSO 0.33 %v/v. As a control, DMSO (0.67 µL, 0.33 %v/v) was added to the appropriate wells. Samples were performed in triplicate.

Probe screen at high concentration: To each well, the appropriate concentration of probe (**70** or **69**) was added as follows: 6 mM (4 µL of a 300 mM stock), 3 mM (2 µL of a 300 mM stock) or 1 mM (0.67 µL of a 300 mM stock) in DMSO. This gave respective DMSO concentrations as 2, 1 and 0.33 % v/v. As controls, DMSO was added to each appropriate well as follows: 2 % v/v (4 µL), 1 % v/v (2 µL) and 0.33 % v/v (0.67 µL). Samples were performed in six replicates.

Probe screen at high concentration with constant DMSO concentration (2 %v/v): To each well, the appropriate concentration of probe (**70** or **69**) was added as follows: 6 mM (4 µL of a 300 mM stock), 3 mM (4 µL of a 150 mM stock), 1 mM (4 µL of a 50 mM stock), 0.5 mM (4 µL of a 25 mM stock), 0.25 mM (4 µL of a 12.5 mM stock) in DMSO 2 %v/v. As a control, DMSO (4 µL, 2 %v/v) was added to the appropriate wells. Samples were performed in triplicate.

Hydrogen peroxide screen: H_2O_2 (1 µL of a 900 mM stock) was added to appropriate wells with an additional 100 µL of media (300 µL total media per well) and a serial dilution performed by removing 100 µL of H_2O_2-containing media and diluting it in adjacent wells containing 200 µL media (and so

on) to give additional concentrations of 1, 0.33, 0.11 and 0.037 mM. Samples were performed in triplicate.

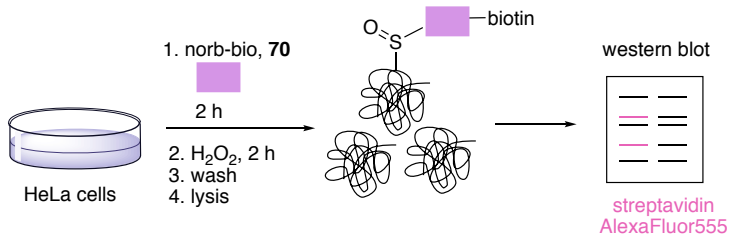
Both a cell only and media only control were included and all left to incubate at 37 °C. After 5 h, the media was removed and replaced with fresh complete DMEM and left to incubate for a total time of 72 h. After this incubation period, CellTiter Blue (Promega) (1:5 dilution) was added to all wells and left to incubate for a further 3-4 h at 37 °C or until complete. The cell viability was assessed by fluorescence emission intensity (590 nm) using a SpectraMax i3x plate reader. Cell viability was calculated as a percentage of viable cells compared to the cell-only control. Error bars represent the standard deviation of each sample with three or six replicates as stated.

3.7.8 Assessment of norb-bio probe 70 in live HeLa cells

Preparation of stock solutions

Probe **70** (14.8 mg, 0.03 mmol) was dissolved in DMSO (100 µL) to give a final concentration of 300 mM. H_2O_2 (34 µL, 50 wt. % in H_2O, 17.6 M) was diluted in H_2O (966 µL) to give a final concentration of 600 mM. This 600 mM solution of H_2O_2 was then diluted to 300 and 150 mM.

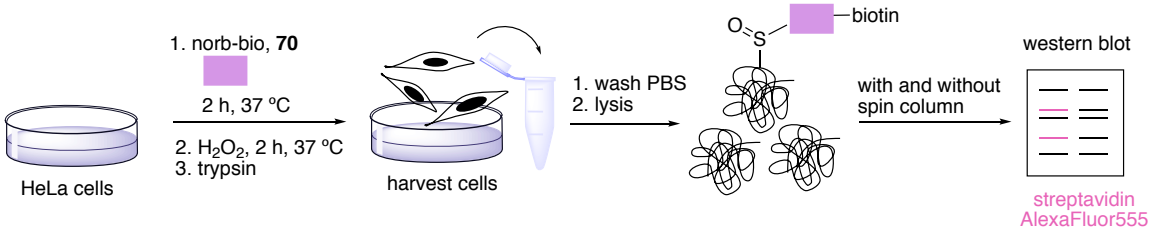
Assessment of norb-bio probe 70 in live HeLa cells



HeLa cells were cultured as described above. In a 24–well plate, HeLa cells were seeded to approximately 6 $\times 10^4$ cells/well and incubated overnight in DMEM (10 % FBS, 1× Pen-Strep) at 37 °C to recover. After 24 h, the media was removed and cells washed (2 × 200 µL) with fresh DMEM (low-glucose, 0.5 % FBS) with a final volume of 300 µL added. To each well, norb-bio **70** (1, 3 or 6 mM, 1, 3 or 6 µL respectively of a 300 mM stock, giving a DMSO concentration of 0.33, 1, and 2 %v/v respectively) was added as appropriate and cells left to incubate for 2 h at 37 °C. H_2O_2 (2, 1, or 0.5 mM, 1 µL from 600, 300, or 150 mM stock respectively) was added as appropriate and cells left to incubate for a further 2 h at 37 °C. After this time, the media was removed, cells washed with PBS (× 3) and cell extraction buffer with protease inhibitor (Invitrogen) (200 µL per well) added. Cells were left to incubate on ice for 45 min before collecting and centrifuging (10,000 ×g, 10 min). Protein concentration was assessed by Protein 660 nm assay reagent (Pierce). Reactions were then prepared directly for electrophoresis without any further purification. Samples were prepared for

SDS-PAGE by mixing sample (17 μL) with lithium dodecyl sulfate (LDS 4×) (6.5 μL, Invitrogen), and DTT NuPAGE sample reducing agent (2.5 μL, Invitrogen) and heating to 95 °C for 5 min. Samples were then resolved by electrophoresis and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

Optimised labelling of protein sulfenic acids in HeLa cells and assessing if a spin column is required before SDS-PAGE and western blotting



HeLa cells were cultured as described above. In a 6–well plate, HeLa cells were seeded to approximately 6 ×10^5 cells/well and incubated overnight in DMEM (10 % FBS, 1× Pen-Strep) at 37 °C to recover. After 24 h, the media was removed and washed (2 × 1 mL) with fresh DMEM (low-glucose, 0.5 % FBS) and a final volume of 1.2 mL added to each well. To each well, norb-bio **70** was added (1 or 3 mM, 4 or 12 μL, respectively, of a 300 mM stock solution in DMSO. The final DMSO concentration was 0.33 or 1 %v/v, respectively). The cells were then incubated for 2 h at 37 °C. For cells treated with H_2O_2, the oxidant (2 mM, 4 μL of a 600 mM stock) was added as appropriate and cells left to incubate for a further 2 h at 37 °C. Cells not treated with H_2O_2 were also left to incubate for a further 2 h at 37 °C. After this time, the media was removed, and trypsin-EDTA (0.25 %, 1 mL) was added to each well and incubated for 5 min at 37 °C. Detached cells were collected, centrifuged (150 ×g, 5 min) then washed with cold PBS (3 × 1 mL). To each sample cell extraction buffer (150 μL) was added and left on ice for 45 min. After this time, samples were centrifuged (10,000 ×g, 10 min) and half (75 μL) taken and purified with Zeba size exclusion spin columns (0.5 mL, 7 kDa MWCO, ThermoFisher) to remove any unreacted probe. The other half was used without further purification. Protein concentration was assessed by Protein 660 nm assay reagent (Pierce). Samples were then prepared for SDS-PAGE by mixing sample (17 μL, after protein concentration normalisation) with lithium dodecyl sulfate (LDS 4×) (6.5 μL, Invitrogen), and DTT NuPAGE sample reducing agent (2.5 μL, Invitrogen) and heating to 95 °C for 5 min. Samples were then resolved and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

4. APPLICATION OF NORBORNENE PROBES TO STUDY CELLULAR PROCESSES

4.1 Overview

In Chapter three, the norbornene probes were tested on proteins, cell lysates, and living cells to evaluate their reactivity and selectivity for sulfenic acids in a biological context. In all cases, norbornene was found to be selective for the sulfenic acid and applicable to live cell studies. This was encouraging as it meant these norbornene probes could be used further to delve into the cellular processes at work. Identification of new protein targets of cysteine oxidation could provide insight into new disease-causing pathways or identify new protein targets for selective drug strategies to combat diseases. Adapting various methods to incorporate labelling with norbornene probes alongside cellular stimulation (or oxidative stress) would be required to facilitate these studies.

Chapter four details the application of norbornene probes to profile which proteins contain sulfenic acid when oxidative stress is induced by exposure to hydrogen peroxide. Additionally, alternative methods to induce endogenous oxidative stress *inside* the cell were explored in contrast to adding hydrogen peroxide exogenously. This includes a model macrophage cell-line to mimic the immune response and the effect of ROS-generating drugs on cancer cell destruction.

This Chapter, at the time of submission, contains work submitted for publication (section 4.2) in an article titled 'Proteome-wide survey reveals norbornene is complementary to dimedone and related nucleophilic probes for cysteine sulfenic acid' and a preprint is available on ChemRxiv, on 16th July 2019, (DOI: 10.26434/chemrxiv.8874077.v1). Work included in the manuscript has been reformatted to align with this Chapter, but the original findings and conclusions remain the same. The remaining sections of the Chapter contain work not previously published and represent preliminary work performed by the candidate for future studies. All data presented in this Chapter was collected during a research visit at The Department of Chemistry, University of Cambridge, England. The candidate was supported by an Endeavour Postgraduate Scholarship for the duration of this 12-month research visit. The candidate prepared the Chapter with full intellectual and practical contribution unless otherwise stated in-text and was the co-primary author of the submitted manuscript.

4.2 Proteomics identification of sulfenylated proteins

4.2.1 Introduction

Detection of cysteine sulfenic acid formation in response to oxidative stress is essential for understanding how oxidation might contribute to disease pathways. It has become increasingly apparent in the literature that oxidation of certain cysteine residues to the sulfenic acid plays an

active role in many pathways of redox regulation, perhaps akin to the role phosphorylation plays in post-translational regulation of protein function.[188] Developing new chemical methods with distinct reaction mechanisms could allow detection of previously unknown cysteine sulfenic acid containing proteins through differing reactivity profiles. Identifying the proteins susceptible to cysteine oxidation could lead to identifying key proteins with redox sensitive cysteine residues that potentially regulate certain pathways or cellular signalling. If these happen to be involved in disease-causing pathways or events, this could alert us to potential new targeted drug strategies to treat that disease. This could allow drugs to be delivered more selectively to regions experiencing high oxidative stress levels through selective reaction with sulfenic acids, for example. These types of drug strategies might also require features that increase the binding to specific proteins or target locations, something which has already been explored in a few cases.[122, 131]

The diverse range of chemical probes for detecting cysteine sulfenic acids was recounted in Chapter one, with many of these probes advancing our understanding of the role of cysteine oxidation during oxidative stress.[47, 88, 100, 102, 104-105, 107, 129, 133, 145-146] However, a comprehensive assessment of cysteine oxidation and its cellular role remains elusive. Chapter three discussed the application of norbornene probes to study cysteine sulfenic acid formation on proteins and in live cells, validating these probes as viable tools for the cellular study of cysteine oxidation. This section will report the next advance in the use of norbornene probes to study cells under oxidative stress through the first proteome-wide survey to identify proteins susceptible to cysteine oxidation and detection by norbornene probes.

4.2.2 Optimising proteomics methods in HeLa cells

With labelling of cysteine sulfenic acids successfully achieved in live cells (Chapter three), we next sought to identify these protein hits to compare to known cysteine sulfenic acid containing proteins. To conduct this proteomics study, affinity purification to extract only the labelled proteins would be required to facilitate their identification by MS. Since the probe **70** contained biotin, streptavidin agarose beads could be employed to extract biotin-labelled proteins. Firstly, the quantity of beads required to capture sufficient probe-bound proteins to visualise a signal by SDS-PAGE was optimised on cell lysates and found to be between 100-200 μL (using a 50 % bead slurry) (Fig. 44A). Additionally, the number of live cells required to obtain enough probe-bound protein for detection was optimised (Fig. 44B). After several attempts and using live cell labelling conditions optimised in Chapter three, it was found that a minimum of 5-6 $\times 10^6$ cells treated with norb-bio (3 mM, **70**) and hydrogen peroxide (2 mM) was required to observe a coomassie signal by SDS-PAGE that would provide sufficient protein for identification by LC-MS/MS.

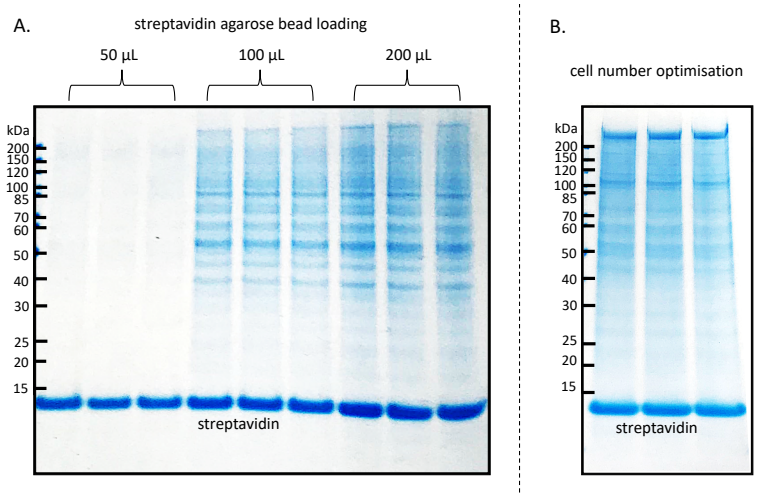


Figure 44. Optimisation of streptavidin agarose bead quantity on cell lysates (A.) and cell number on living cells (B.) to capture and release a detectable amount of probe-bound protein for LC-MS/MS analysis.

From the whole cell analysis (Fig. 44B), one lane was divided into 10 bands, digested with trypsin, and peptides analysed by LC-MS/MS. This analysis was performed by Cambridge Centre for Proteomics at the University of Cambridge, England. Unfortunately, peptide fragments carrying the expected probe modification from reaction with **70** were not detected by MS/MS, despite the samples undergoing affinity purification before analysis. A large quantity of proteins were identified by MS/MS, however it could not accurately be determined which were real hits and which were false positives since no bound probe was detected and no control employed. A number of reasons could be responsible for this detection issue. The norb-bio probe itself is large (494 g/mol) which can increase difficulty of detecting the modification on the peptide fragments by LC-MS/MS, especially for large peptides. Biotin itself can also cause issues in MS detection which is known to occur.[189] Addition of the polar modification could increase retention of those peptide fragments on the LC column, increasing the difficulty of detection. Finally, it is possible that the modification, due to its size, undergoes fragmentation during the MS analysis. It is suspected the difficulties with detection are specifically due to the presence of the biotin, as the norb-yne probe **69** was identified on peptide fragments in a previous cell lysate study with no apparent issues (Chapter three).

To overcome this, a modified approach was designed so that detection of the probe modification on the peptide fragments was not necessary for protein identification. This approach was to compare any protein hits identified in a negative control sample of HeLa cells not treated with hydrogen peroxide to a sample of HeLa cells treated with hydrogen peroxide. Comparison of these two sets of protein hits would allow quantification of enrichment due to oxidation (e.g. sulfenic acid formation). In other words, proteins that are susceptible to oxidation will increase in abundance in the hydrogen peroxide treated samples compared to the control, allowing these proteins to be identified. This

strategy would also help filter out any false-positives enriched in the negative control, or those not significantly enriched in either (e.g. endogenously biotinylated proteins). This strategy also provides additional information regarding individual protein enrichment, which can highlight the proteins most susceptible to cysteine oxidation and capture by probe **70**. In this way, changes to the oxidation state of cysteines in response to stimuli can also be monitored and compared.

4.2.3 Identifying protein hits by proteomics in HeLa cells

Guided by the optimisation experiments in Figure 44B, we chose the conditions which would provide the best opportunity to label, enrich, and identify proteins containing cysteine sulfenic acid using our enrichment strategy. In the event, HeLa cells were incubated with norb-bio (3 mM, **70**) in DMSO 1 %v/v for 2 h at 37 ºC in 5 % CO_2 humidified atmosphere. After the initial 2 h of incubation with probe **70**, H_2O_2 (2 mM) was added to the set of cells designated for oxidation. This high concentration of oxidant was used to ensure significant protein oxidation, so that changes between the control and treated samples were more pronounced. Both the control samples and the oxidised samples were prepared in biological triplicates. All samples were then left to incubate for a further 2 h at 37 ºC in a 5 % CO_2 atmosphere. After this time, the media was removed, and the cells were trypsinised, collected, washed, and lysed. Samples were then purified using a size exclusion spin column to remove any unbound probe (Fig. 45).

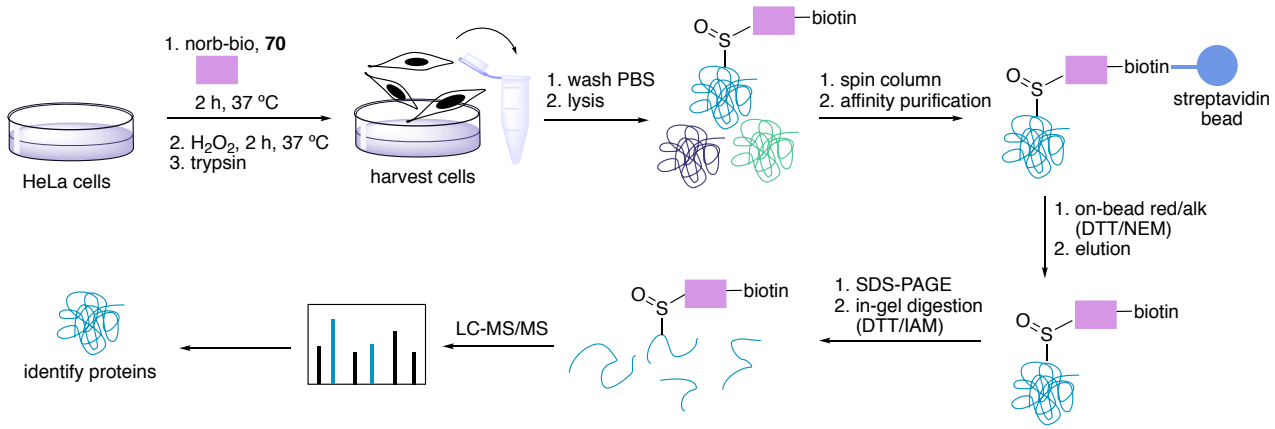


Figure 45. Summary of protocol for using norb-bio **70** to trap, enrich, and identify proteins containing cysteine sulfenic acid. HeLa cells were used in this study and oxidative stress was simulated by treating the cells with 2 mM H_2O_2.

Before affinity purification, a small portion of each sample was analysed by SDS-PAGE and western blot to ensure adequate enrichment of oxidised proteins (e.g. those that contain cysteine sulfenic acid). Only the oxidised samples displayed a streptavidin signal, indicating presence of the norb-bio probe **70** (Fig. 46A-B). This result also demonstrated that little to no protein was captured by **70** in the control (non-oxidised samples) emphasising the earlier observations from Chapter three that **70**

only labels protein when hydrogen peroxide is added, indicative of sulfenic acid formation. Of course, basal levels of ROS may oxidise some protein cysteines in the control, but this is very minimal compared to the number of proteins oxidised when hydrogen peroxide is added, indicated by the results in Figure 46A-B. All samples were then subject to affinity purification using streptavidin agarose beads. After incubation of the samples with the beads for 2 h at 4 ºC, the supernatant was removed and beads washed. The supernatant of the hydrogen peroxide treated samples was analysed by SDS-PAGE and western blot to asses affinity capture of the probe-bound proteins. After affinity capture, no streptavidin signal was observed by western blot, indicating efficient capture of the biotinylated proteins bound to probe **70** (Fig. 46A, far right lanes). After affinity capture on the beads, all samples were subject to on-bead reduction with dithiothreitol (DTT) and alkylation with *N*-ethylmaleimide (NEM) to block free thiols. This blocking with NEM was designed to prevent disulfide bond formation or other reactions of cysteine thiols during the remainder of the sample preparation. Blocking free thiols at this stage with NEM would also provide an identifier for cysteine residues which do not contain probe **70**, since these cysteine residues are bound by the probe and captured by the streptavidin beads. Samples were then eluted from the beads in SDS-PAGE sample buffer containing free biotin. Addition of free biotin in the elution buffer increased protein recovery presumably by blocking and/or exchanging with the probe-bound proteins during elution. These samples as prepared were then sent to Maike Langini at Molecular Proteomics Laboratory (MPL), Biomedical Research Center (BMFZ) at Heinrich-Heine-University Düsseldorf in Germany for proteomics analysis. All LC-MS/MS sample preparations, sample runs, and protein identification, enrichment and statistical analysis in section 4.2.3 was performed by Maike Langini.

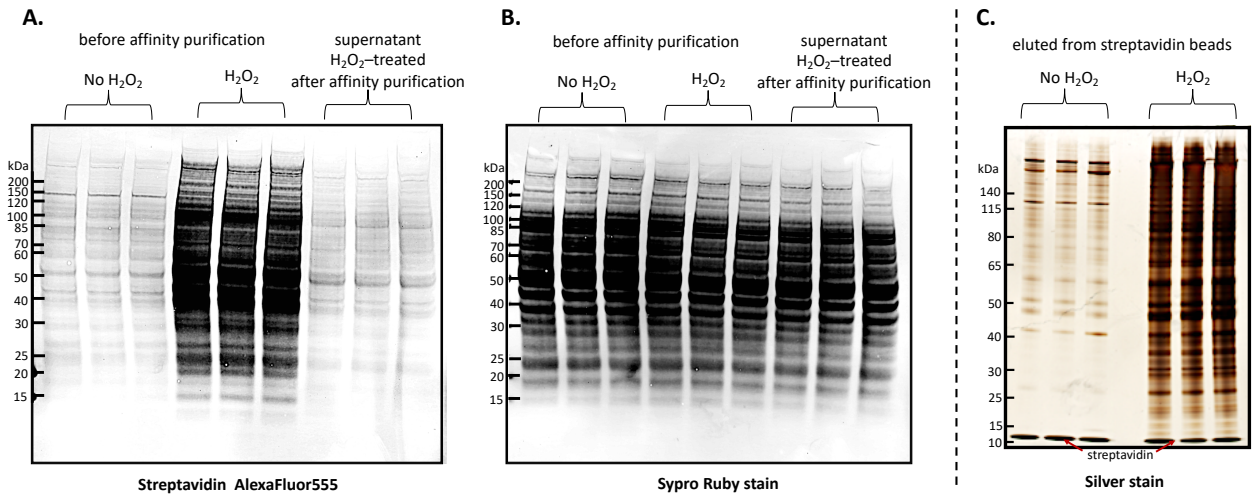


Figure 46. A. Western blot analysed with streptavidin AlexaFluor555 showing the enrichment of proteins labelled with **70** in live HeLa cells after treatment with and without H_2O_2. Streptavidin signals are only observed for the H_2O_2–treated samples (middle three lanes). After affinity purification with streptavidin agarose beads, the supernatant (non-bound proteins) was analysed for the H_2O_2–treated samples and no streptavidin labelling was observed, indicating efficient extraction of probe-bound proteins (right three lanes). B. Western blot treated with Sypro Ruby total protein stain to show

total protein loading across all experiments. Each lane was loaded with approximately 20 μg total protein. C. SDS-PAGE analysis of total proteins eluted from streptavidin agarose beads after affinity purification of samples treated with and without H_2O_2. Silver stain was used to visualise total proteins. Significant enrichment is observed for H_2O_2–treated samples.

After this affinity purification and elution of bound proteins from the streptavidin agarose beads, a small portion of the sample was analysed by SDS-PAGE. Silver staining was used to assess the total protein extracted during the affinity purification and release step. Once again, significant enrichment in protein loading was only observed for the oxidised samples compared to the control (Fig. 46C). This result is consistent with previous Chapters that indicate, at 37 ºC or lower, norbornene derivatives only react significantly with cysteine when conversion to the sulfenic acid occurs.

Eluted proteins were then prepared for proteomics analysis by desalting (SDS-PAGE), reduction (DTT) and alkylation with iodoacetamide (IAM), trypsin digestion, and peptide extraction. Samples were not fractionated but ran as a single run. At this stage of analysis, free cysteines were blocked with IAM to distinguish these free cysteines from those blocked in the on-bead reduction/alkylation step with NEM. Therefore, any cysteine containing an NEM modification could not be a sulfenic acid site. Initial LC-MS/MS analysis revealed a broad spectrum of proteins, with 1056 hits identified across both sample sets based on one unique peptide match. After applying a stringent set of selection criteria (proteins identified with at least two unique peptides, a peptide FDR < 0.01, a protein FDR < 0.01, and a search engine score > 20 for Mascot and > 100 for MS Amanda), 635 proteins were quantified from both sample sets. This data set was further filtered by applying selection criteria for those proteins that were significantly enriched in the oxidised sample compared to the control (fold change > 2 and ANOVA score < 0.01) to give a narrower set of 482 potential protein hits. A graphical representation of the comparison between the control (not treated with H_2O_2) and oxidised (treated with H_2O_2) sample sets and statistical analysis of the identified proteins is shown in Figure 47.

These 482 protein hits were further filtered to eliminate any proteins which do not contain a cysteine residue in their amino acid chain (remaining protein analysis performed by the candidate). This provided 473 proteins that may contain the sulfenic acid modification, and therefore constitute a class of proteins that are modified during oxidative stress. It was interesting to note that 9 of the significantly enriched proteins did not contain cysteine residues. Of these 9 proteins, most were histone or protein subunits, so their enrichment might be due to their strong interaction with a separate protein that contains the cysteine sulfenic acid modification. For example, one of the 9 proteins, keratin-18, is known to bind with 14-3-3 proteins, one of which, 14-3-3 protein theta, was

also identified as one of the enriched proteins. In this way, the assay provides a way to identify proteins that interact with the sulfenome, without necessarily containing a cysteine residue. Other studies have reported similar occurrences of proteins identified in sulfenic acid enrichment studies which do not contain cysteines.[109] This study also suggests that enrichment of these protein hits without cysteine residues may be due to protein-protein interactions with other cysteine-containing proteins. The list of 9 proteins is shown in Table 1. Further exploration of the relationship between these proteins without cysteines with those that contain oxidisable cysteines may provide interesting observations.

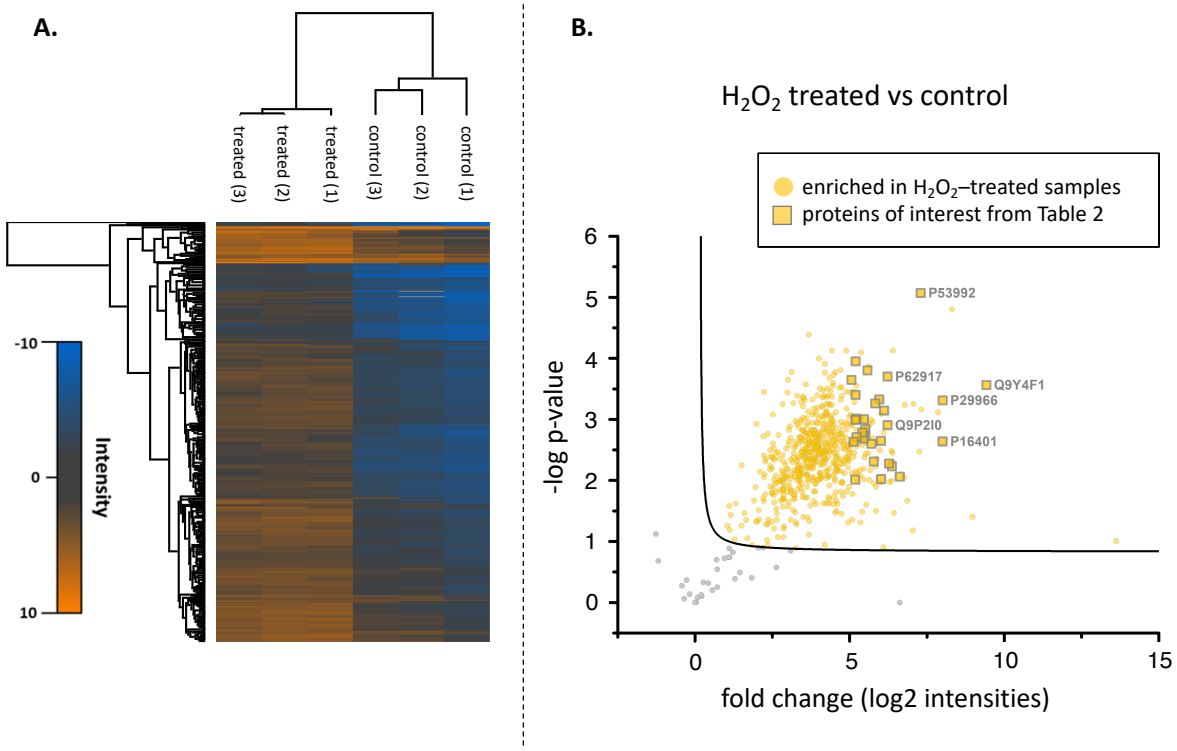


Figure 47. A. Heatmap showing the similarity between samples in both the negative control (no treatment with H_2O_2) and oxidised samples (treated with H_2O_2). The difference between samples is represented by the length of the connecting line, with a greater difference resulting in longer connecting lines. The heatmap was generated with Perseus 1.6.0.7 on log2 transformed protein intensities and clustered hierarchically with Euclidean distance. B. Volcano plot depicting the protein significance (y-axis) compared to the fold change (x-axis). Proteins towards the top right of the plot display large magnitude fold changes that are also statistically significant. The black line depicts the significance border which was corrected for multiple testing in Perseus with S0 value = 0.1 and FDR = 0.01, performed as a two-sided Student's T-test. The 30 proteins in Table 2 are highlighted to show their significant enrichment, with labels for a select few. This figure was prepared by Maike Langini.

Table 1. List of 9 proteins significantly enriched in the hydrogen peroxide treated samples which do not contain a cysteine residue in their amino acid chain. Details of their cellular location, biological process (if known), and comments on their route of enrichment are provided using entries from the UniProt database.

Uniprot ID	Protein name	Cellular location#	Biological Process	Notes
Q99623	Prohibitin-2	C, PM, M, N	transcription, transcription regulation	mitochondrial chaperone protein
P16401	Histone H1.5	N	DNA binding	
Q96A08	Histone H2B type 1-A	N	DNA binding	
P61353	60S ribosomal protein L27	C, ER	ribonucleoprotein, ribosomal protein	component of large ribosomal subunit
P06576	ATP synthase subunit beta, mitochondrial	M	ATP synthesis, hydrogen ion transport, ion transport, transport	component of ATP synthase complex
P05783	Keratin, type I cytoskeletal 18	C, N	cell cycle, host-virus interaction	binds with 14-3-3 proteins
P05787	Keratin, type II cytoskeletal 8	C, N	host-virus interaction	
P16402	Histone H1.3	N	DNA binding	
P62081	40S ribosomal protein S7	C, CS	ribonucleoprotein, ribosomal protein	component of small ribosomal subunit

#Cellular locations: C = cytoplasm, PM = plasma membrane, N = nucleus, ER = endoplasmic reticulum, CS =cytoskeleton, G = golgi apparatus, E = endosome, S = secreted, M = mitochondrion

It should be noted that these (473) hits require additional validation to verify the site of the cysteine sulfenic acid formation and also to establish a cellular role of this protein oxidation. Despite sequential blocking attempts with NEM and IAM, the sulfenic acid site of each protein could not be identified for certain. Since biotin is known to cause issues in MS detection this was not entirely surprising.[189] With that said, it should be reiterated that these 473 proteins all have cysteine residues and only react efficiently with norbornene in the presence of hydrogen peroxide. This is consistent with sulfenic acid formation and trapping with the norbornene probe. Therefore, this set of proteins should be of interest to the community of researchers investigating the sulfenome and broader aspects of protein oxidation and redox regulation. A full list of all 482 proteins identified is provided in Appendix C. This includes protein description, confidence score, Anova (p), and max fold change for each replicate.

4.2.4 Analysis and comparison of identified proteins from HeLa cells

These 482 enriched protein hits were then assessed for their involvement in biological pathways and cellular locations (remaining protein analysis in section 4.2.4 performed by the candidate) (Fig. 48). Regarding the latter, a large number of proteins in the cytosol or nucleus were detected. This is not surprising given both the nature of the oxidising agent (introduced exogenously) and that the cytosol and nucleus typically contain the most abundance of proteins. With respect to biological pathways, the majority of protein hits were associated with generalised cellular metabolism or gene expression which was also to be expected. This includes well documented proteins such as alpha-enolase, GAPDH, lactate dehydrogenase (A chain), and fructose-bisphosphate aldolase A, all of which are involved in glycolysis and have been shown to be susceptible to cysteine oxidation, some with implications in maladies such as Alzheimer's disease.[190-195]

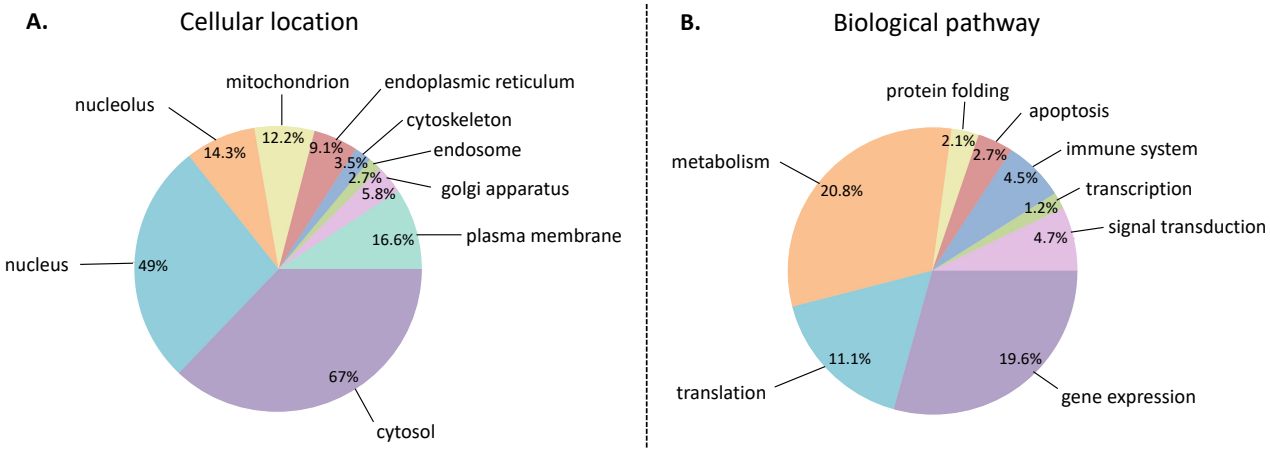


Figure 48. A. 482 enriched proteins identified using norb-bio probe **70**, categorised into generalised cellular locations. B. 482 enriched proteins identified using norb-bio probe **70**, categorised into generalised biological pathways. In both cases, some proteins may have multiple cellular locations or participate in multiple cellular pathways and is reflected in the number of proteins assigned to each category. As such, total protein components or pathways percentages reflect the percentage of proteins in the dataset which exhibit the particular feature. The 9 enriched proteins without cysteine residues were included in this analysis since they may play a role by association with other cysteine-oxidisable proteins.

The data set of 473 enriched proteins with cysteine residues was then compared to protein hits identified in a previous cysteine sulfenic acid survey on HeLa cells using dimedone-based probe **34**.[100] Of the 193 reported proteins, and the 59 able to be cross-referenced in the UniProt (Swiss-Prot) database, only 26 proteins were common with this current study. This difference in profile could relate to the distinct mechanisms of the two probes (Fig. 49) or steric differences. This observation

is not unexpected as previous studies by Carroll and co-workers have demonstrated how subtle differences in probe design can lead to interactions with different proteins.[144-145] In fact, cell lysate experiments in Chapter three using a similar dimedone-based probe (**123**) appeared to display a different reactivity profile to our norb-bio probe (**70**) by western blot, which is consistent with the outcome observed in this proteomics study.[140]

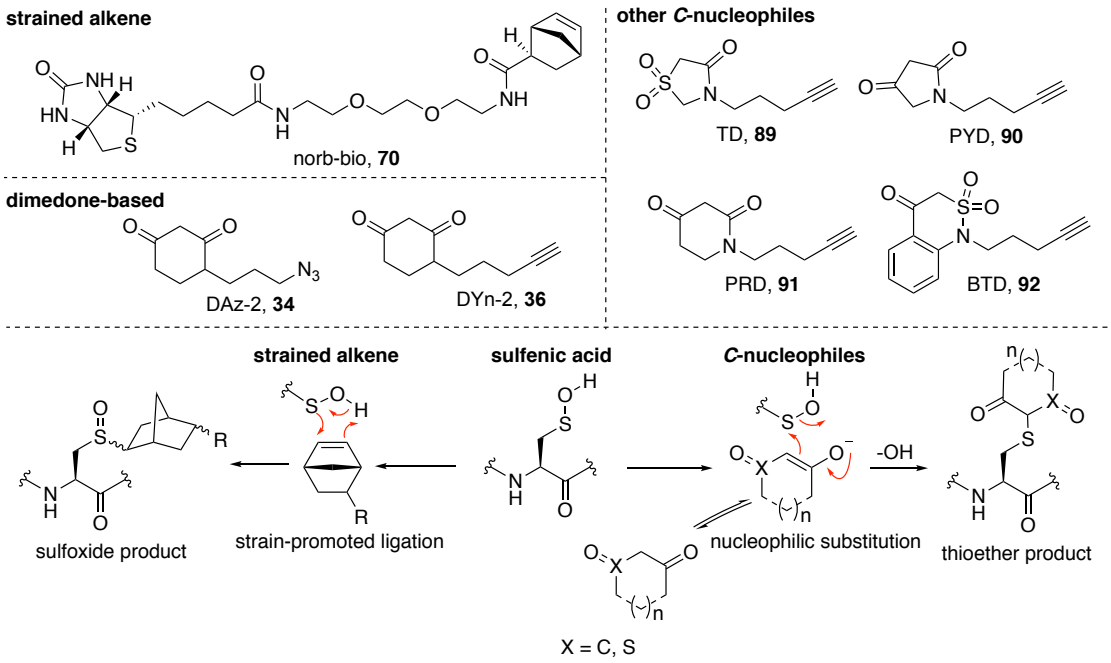


Figure 49. Probes used to detect cysteine sulfenic acid in cell lysates and live cell studies. Probe **70** was used in the current study and reacts with sulfenic acids through a strain-promoted group transfer reaction.[140] Dimedone-based probes **34**[100] and **36**[129] have been used extensively in previous studies.[38, 67, 118, 196-197] Other *C*-nucleophilic probes such as **89-92** as well as **36** were compared in a cell lysate study.[145] Probe **92** has also independently been analysed for a proteome-wide survey.[146]

We next compared the hits in our sulfenome survey with those reported for other probes. These studies all used different chemical probes such as **36** (human RKO cell line, 778 protein hits),[129] **92** (human RKO cell line, 1202 protein hits),[146] and a comparison of probes **36, 89-92** (human RKO cell lysates, 761 protein hits combined),[145] some of which have reported greater reactivities than typical dimedone-based probes. These comparisons should be taken with due care as the conditions and methods differ slightly across each study, which could account for the differences in protein identification. These differences can include the cell line used in the analysis (differences in protein expression), the concentration and method of adding the oxidant (ROS gradient across the cell), and whether the analysis was performed on cell lysates (all proteins equally accessible) or live cells (compartmentalisation of proteins to consider). Despite this, we still thought it would be valuable to investigate if any of the 473 protein hits we had identified with **70** had been previously reported to contain cysteine sulfenic acids, and also to determine if there are any new proteins identified in the

sulfenome. We found that of the 473 enriched proteins we identified as plausible hits, 148 (31 %) appear to be previously unknown sulfenic acid sites in the studies we compared. It was also reassuring that 325 of the 473 hits (69 %) had been previously identified in other studies, indicating our probe is reacting with a similar subset of proteins as other probes. This high level of similarity between our protein sulfenic acid hits and other studies also confirms the validity of our proteomics strategy for identifying proteins enriched in the hydrogen peroxide treated samples. A graphical representation of the number of common proteins between the datasets is shown in Figure 50. The 148 previously unknown sulfenic acid proteins are highlighted in Appendix C.

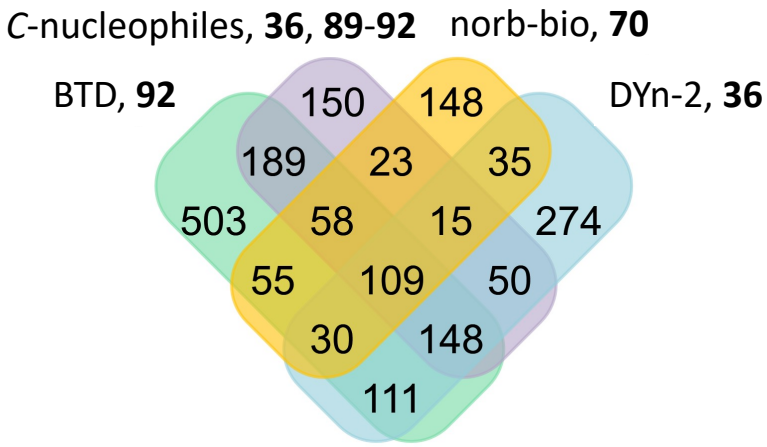


Figure 50. Venn diagram comparing different datasets of identified protein sulfenic acids. Orange represents the 473 proteins identified in this data set using norb-bio **70**. Blue represents hits found using **36** (772/778 proteins matched).[129] Purple represents a combined dataset of proteins found using probes **36, 89**-**92** (742/761 proteins matched).[145] Green represents hits found using probe **92**.[146] Note: not all proteins were able to be cross-referenced in the database for comparison and this is indicated by proteins matched out of total proteins reported in the original dataset. The 9 enriched proteins without cysteine residues were excluded from this comparison since they do not contain sulfenic acids.

From these 148 previously unknown hits, 28 proteins were analysed which appeared to have the greatest fold change (>30) in enrichment and therefore proteins that react significantly with norb-bio **70** in the presence of hydrogen peroxide. These selected proteins are compiled in Table 2. These protein hits represent potential leads for future investigation of cysteine sulfenic acid in redox regulation and other cellular functions. The last two entries of Table 2 represent two of the 9 proteins which do not contain cysteines but were significantly enriched in the analysis. These proteins may be of interest for their interactions with proteins that do contain oxidisable cysteine residues. All 30 protein hits in Table 2 are highlighted in Figure 47B to emphasise their statistical significance and magnitude of enrichment in the comparison between cells treated with hydrogen peroxide and those not subjected to oxidative stress.

Table 2. List of 30 selected proteins, their cellular location, and biological process (if known) from UniProt database. Proteins were selected from the 148 previously unknown hits and chosen based on largest fold change (>30). The last two entries represent proteins that do not contain cysteine residues but were significantly enriched in the analysis when treated with hydrogen peroxide.

UniProt ID	Protein name	Cellular location[#]	Biological process
A2RRP1	Neuroblastoma-amplified sequence	ER	protein transport
P30530	Tyrosine-protein kinase receptor UFO	PM	differentiation, immunity
Q12802	A-kinase anchor protein 13	C, N	
Q9C0D5	Protein TANC1	PM	
P49756	RNA-binding protein 25	N, C	mRNA processing, mRNA splicing
Q8NI27	THO complex subunit 2	N	mRNA processing, mRNA splicing, mRNA transport, transport
Q9H3S7	Tyrosine-protein phosphatase non-receptor type 23	E, N, CS	cilium biogenesis/degradation, protein transport, transport
P49589	Cysteine--tRNA ligase, cytoplasmic	C	protein biosynthesis
P29966	Myristoylated alanine-rich C-kinase substrate	CS, PM	
P33527	Multidrug resistance-associated protein 1	M	transport
Q9ULH0	Kinase D-interacting substrate of 220 kDa	E, PM	neurogenesis
Q9Y4F1	FERM, ARHGEF and pleckstrin domain-containing protein 1	C, PM	
P11274	Breakpoint cluster region protein	PM	
Q9Y3P9	Rab GTPase-activating protein 1	C, CS	cell cycle
Q6KC79	Nipped-B-like protein	N	cell cycle, transcription, transcription regulation
Q15276	Rab GTPase-binding effector protein 1	E, C	apoptosis, endocytosis, protein transport, transport
Q8TF05	Serine/threonine-protein phosphatase 4 regulatory subunit 1		
Q96SU4	Oxysterol-binding protein-related protein 9	E, G	lipid transport, transport
Q9P2I0	Cleavage and polyadenylation specificity factor subunit 2	N	mRNA processing
P46781	40S ribosomal protein S9	C	
P46821	Microtubule-associated protein 1B	CS, C	
P46379	Large proline-rich protein BAG6	C, N, S	apoptosis, differentiation, immunity, transport
P62917	60S ribosomal protein L8	C	
Q5SRE5	Nucleoporin NUP188 homolog	N	mRNA transport, protein transport, translocation, transport
P53992	Protein transport protein Sec24C	ER, C	ER-golgi transport, protein transport, transport

Q27J81	Inverted formin-2	C	actin binding
Q6YHU6	Thyroid adenoma-associated protein	C, ER	
Q99570	Phosphoinositide 3-kinase regulatory subunit 4	E, M	kinase, serine/threonine-protein kinase, transferase
P16401*	Histone H1.5	N	DNA-binding
P16402*	Histone H1.3	N	DNA-binding

[#]Cellular locations: C = cytoplasm, PM = plasma membrane, N = nucleus, ER = endoplasmic reticulum, CS =cytoskeleton, G = golgi apparatus, E = endosome, S = secreted

*These proteins do not contain a cysteine residue but are significantly enriched in the analysis.

Additionally, 109 proteins were identified as common across all studies compared in this analysis (Fig. 50). The 109 proteins are highlighted in Appendix C. Because these proteins are consistently detected across multiple studies with different probes and labelling conditions, these proteins clearly contain oxidisable cysteine residues that can be readily converted into cysteine sulfenic acid. Due to this, we suspect these proteins may also be of particular interest for further investigation. For example, the protein cofilin-1 identified as one of the 109 common proteins has previously been shown to undergo oxidation at Cys-139 and Cys-147 which reduces actin binding and severing through inhibition.[198]

For future studies, specific proteins of interest could be tested to determine what function, if any, the sulfenic acid residue plays in redox regulation or signalling. For instance, a protein of interest could be assessed for its activity before and after treatment with hydrogen peroxide and probe **70** to establish whether oxidation of the cysteine directly effects activity. If so, downstream events influenced by that particular protein could be assessed. For example, if this protein of interest was involved in glycolysis, the expected products of glycolysis (eg. lactate) could be monitored before and after hydrogen peroxide treatment to determine if the cysteine oxidation results in any downstream alterations to the glycolysis pathways.

Additionally, a total of 1056 proteins were identified in this study before applying stringent selection criteria, so there is a possibility of an even broader population of proteins in the sulfenome yet to be detected. To assess these proteins, development of modified norbornene probes to facilitate more sensitive and general detection of proteins containing cysteine sulfenic acid would be needed. This will be discussed further in Chapter five.

4.2.5 Concluding remarks

This proteomics study has provided new insight into members of the sulfenome. A total of 148 out of 473 proteins were detected that had not been previously identified in other studies. This result

reveals that the norbornene probes for cysteine sulfenic acid are complementary to dimedone and related nucleophilic probes. From these comparisons between different probes, 109 proteins were found to be common across all studies, indicating a selection of proteins with high susceptibility to cysteine oxidation. These newly identified proteins with high enrichment in the hydrogen peroxide treated samples represent promising leads to further investigate proteins with redox-sensitive cysteines. Identification of 9 proteins with significant enrichment in hydrogen peroxide treated samples but no cysteine residues was intriguing as this may suggest protein-protein interactions with oxidation-sensitive cysteine containing proteins. Further investigation into the sulfenome using improved norbornene probes could reveal more previously unknown sulfenic acid proteins which, along with the 148 new protein hits already identified, will be of value to both the biochemical and medical community.

4.3 Induction of oxidative stress through endogenous generation of ROS

4.3.1 Introduction

Throughout this study, small molecules, proteins and cells have been subject to oxidative stress through application of exogenous hydrogen peroxide sources. This method of introducing hydrogen peroxide to the cells has been used extensively in previous sulfenic acid studies for testing chemical probes. Since this method is common, we decided to initially test our norbornene probes through exogenous introduction of hydrogen peroxide so as to compare to previous studies as demonstrated in previous Chapters. While this has provided a means for testing the initial applicability of norbornene as cysteine sulfenic acid probes, it does not necessarily mimic oxidative stress events that would occur in real living cells or organisms. Several factors can determine whether a particular cysteine residue can be oxidised from a chemical standpoint, but other external factors such as proximity of the protein to the ROS source (e.g. compartmentalisation and ROS concentration gradient) also become important in a cellular context. As such, we sought an alternative way in which we could induce oxidative stress within the cell to generate endogenous ROS to mimic the biological responses associated. In doing so, this may help to identify biologically relevant cysteine sulfenic acids as not all cysteine sulfenic acids necessarily play a biological role. Other studies have used various methods for inducing oxidative stress, such as stimulating cells with epidermal growth factor (EGF) to induce ROS,[38, 129] or using hydrogen peroxide as a stimulant to induce further production of ROS, rather than an oxidising species itself.[38] Other oxidants such as tertiary butyl hydroperoxide have also been used,[199] but express the same concerns as with hydrogen peroxide treatments of introducing an oxidising species exogenously. Therefore, we wanted to investigate a new model system for generating ROS endogenously to better reflect the ROS gradient within the cell, and also

show that the norbornene probes could equally capture cysteine sulfenic acids from endogenously generated ROS species.

4.3.2 RAW264.7 cell line as model system

The cell line RAW264.7 (ECACC), a macrophage-like murine cell line, was chosen as a model to test alternative generation of ROS. Macrophages are a type of white blood cell that participate in phagocytosis for the uptake and degradation/removal of infectious particles or senescent cells which do not display specific surface proteins associated with healthy cells. This process by macrophages is involved in development, tissue remodelling, the immune response, and inflammation.[200-201] It is well known that macrophages generate a large amount of ROS upon activation as part of their defence against invading entities, including the RAW264.7 cell line and including hydrogen peroxide through superoxide production.[202-204] Since ROS species are generated, it is likely that cysteine residues are oxidised during the process. This encouraged us to investigate whether we could detect any sulfenic acid formation using our norbornene probe upon stimulating the RAW264.7 cell line to generate ROS. Lipopolysaccharide (LPS) is an endotoxin from bacteria which is known to activate monocyte and macrophage cells. LPS triggers the release of cytokines which are small signalling proteins that induce inflammation as part of the immune response by conferring instructions and mediating communication between immune and non-immune cells.[205-207] Upon activation (induced by this signalling), macrophages increase production of ROS, a key component of their defence mechanism against invading microorganims.[208] Therefore, we thought this could be an alternative model system to study formation of cysteine sulfenic acids, specifically associated with the immune response. The use of the RAW264.7 cell line has been used previously to study formation of other cysteine oxidation states and was therefore promising for detecting sulfenic acids specifically.[209-210]

4.3.3 RAW264.7 stimulation with LPS

Before carrying out the sulfenic acid labelling experiments, the activation and production of ROS by the RAW264.7 cells was optimised. Toxicity was tested over a concentration range of 0.05-15 μg/mL for 24 h, after which the media was replaced and further incubated for 48 h. Generally, LPS was quite toxic. After a concentration of 0.5 μg/mL, no further increase in toxicity was observed, regardless of increase in LPS concentration (Fig. 51A). LPS is suspected to be toxic to RAW264.7 cells as once activated by the endotoxin, cells typically enter apoptosis.[211]

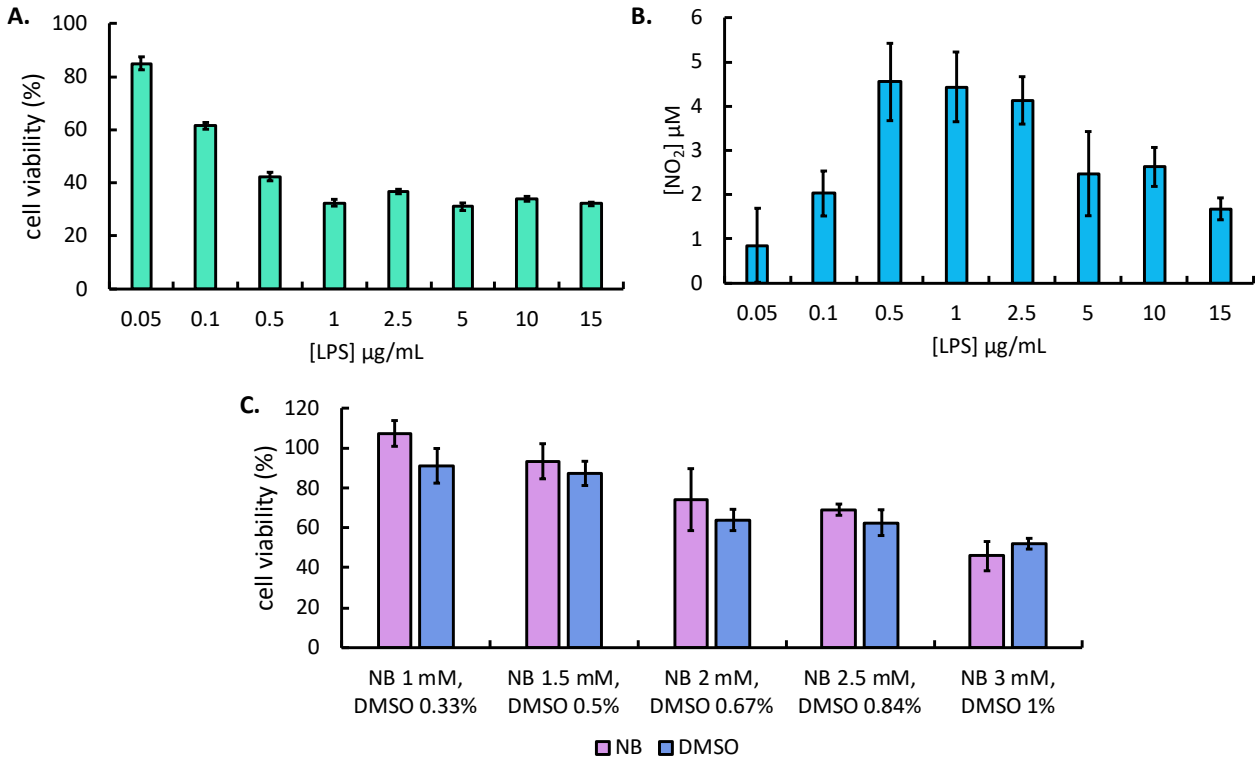


Figure 51. A. LPS toxicity in RAW264.7 cells. Cells were treated with LPS (0.05-15 µg/mL) for 72 h. B. Activation of RAW264.7 cells by LPS stimulation was optimised and measured using the Greiss assay for NO_2 readout. C. Toxicity of norb-bio (1-3 mM, **70**) and DMSO (0.33-1 %v/v) were tested on RAW264.7 cells. Cells were treated with probe for 24 h before replacing media and left for a further 48 h.

RAW264.7 cells were then assessed for their activation. Activation was measured using the Griess assay, which is an indirect measure of the concentration of NO in the media (measured through the oxidised form, NO_2). Under acidic conditions, NO_2 converts sulfanilamide to the corresponding diazonium salt, which couples to naphthylethylenediamine forming an azo dye, which has an absorbance at 548 nm. When RAW264.7 cells are activated, they will increase production of ROS in response, including NO to provide a read-out for activation levels. Various ROS are produced however detection of NO (via oxidised NO_2) was chosen as the Griess assay provides a simple and inexpensive measure, ideal for initial assessment. Increase in NO_2 concentration relative to a control should indicate greater activation of RAW264.7 cells. Treating RAW264.7 cells with varying concentration of LPS (0.05-15 µg/mL) found an optimal range of 0.5-1 µg/mL (Fig. 51B). Increasing LPS to greater concentrations did not result in greater activation, probably due to increased cell death at an accelerated rate.

Finally, the toxicity of **70** and DMSO was tested on RAW264.7 cells by incubating for 24 h, removing and replacing the media, then incubating for a further 48 h. This cell line was found to be sensitive

to DMSO, with only 50 % viability when treated with DMSO 1 %v/v (Fig. 51C). Acceptable conditions were found to be norb-bio 2 mM, and DMSO 0.67 %v/v with 75-85 % viability observed after the incubation period. The requirement to use lower concentrations of DMSO, and in turn, lower concentrations of **70** due to solubility constraints, was concerning. Based on results from HeLa cell experiments outlined in Chapter three, lower concentrations of **70** does not give adequate labelling as it seems to be concentration dependent. This observation could impact upon labelling efficiency.

4.3.4 Labelling RAW264.7 cells with norb-bio 70

Using the optimised conditions for RAW264.7 activation, the labelling experiment was performed. Cells were treated with varying concentrations of norb-bio (1-3 mM, **70**) and LPS (0.5-1 μg/mL) and left to incubate for 24 h at 37 °C in 5 % CO_2 humidified atmosphere. After this time, cells were harvested, washed, lysed, and analysed by SDS-PAGE and western blot. Unfortunately, no streptavidin signal was observed under any conditions, indicating no sulfenic acid had been captured. This was intriguing as the Griess assay had shown activation of the cells, and previous experiments in HeLa cells had shown significant labelling at norb-bio 3mM. It was unknown whether the cause of no-labelling related to production of hydrogen peroxide to oxidise the thiols, the relative abundance of oxidised species, or the probes ability to trap sulfenic acids under the conditions. When the Griess assay was initially performed to optimise LPS concentration, the possible effects of DMSO or **70** were not considered as the Griess assay was not performed under the conditions used for the labelling experiment (eg. no DMSO and no **70**). Therefore, either or both of these components could affect the activation of the RAW264.7 cells.

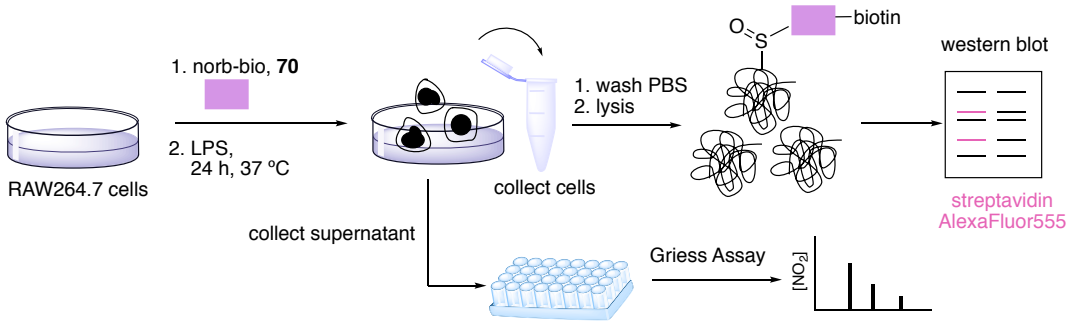


Figure 52. Workflow for RAW264.7 labelling experiments with norb-bio **70** and LPS stimulation. The supernatant can also be collected and analysed by the Griess assay for assessing activation.

The labelling experiment was then repeated but this time under conditions necessary to also perform the Griess assay on the supernatant before cells were harvested, washed, and lysed for western blot (Fig. 52). This was to assess whether under the labelling conditions, the RAW264.7 cells were still being activated to the same extent. The results obtained were interesting (Fig. 53A). The LPS

only controls had the expected output of NO_2. However, when the cells were treated either with DMSO and LPS or **70** and LPS, the read-out of NO_2 decreased with increasing concentration of both DMSO and **70**. Since the **70**-treated samples also contain DMSO, it could be reasoned that the DMSO may be acting as a radical scavenger. DMSO is known to be a radical scavenger,[212] but is usually used at such low concentrations (0.1 %v/v) that the effects are probably masked. As noted previously, increasing the concentration of **70** is not feasible without increasing the DMSO %v/v. Moreover, at lower concentrations of **70** in the cell culture media, little to no labelling is detected as it is concentration dependent. Other possible solvents were considered to replace DMSO, but the solubility of **70** is limited. Additionally, equally high concentrations of an alternative solvent would be required, causing its own issues.

As a final assessment, RAW264.7 cells were treated with and without DMSO at varying concentrations of LPS (1-15 μg/mL) (Fig. 53B). The same trend was observed regardless of the concentration of LPS. Increasing the DMSO percentage decreased the NO_2 read-out in all cases. Therefore, increasing the LPS concentration did not seem to counteract the antioxidant effects of DMSO. Based on these results, it was concluded that due to technical difficulties, the RAW264.7 cell line was not an ideal model to detect sulfenic acids using **70** under these conditions.

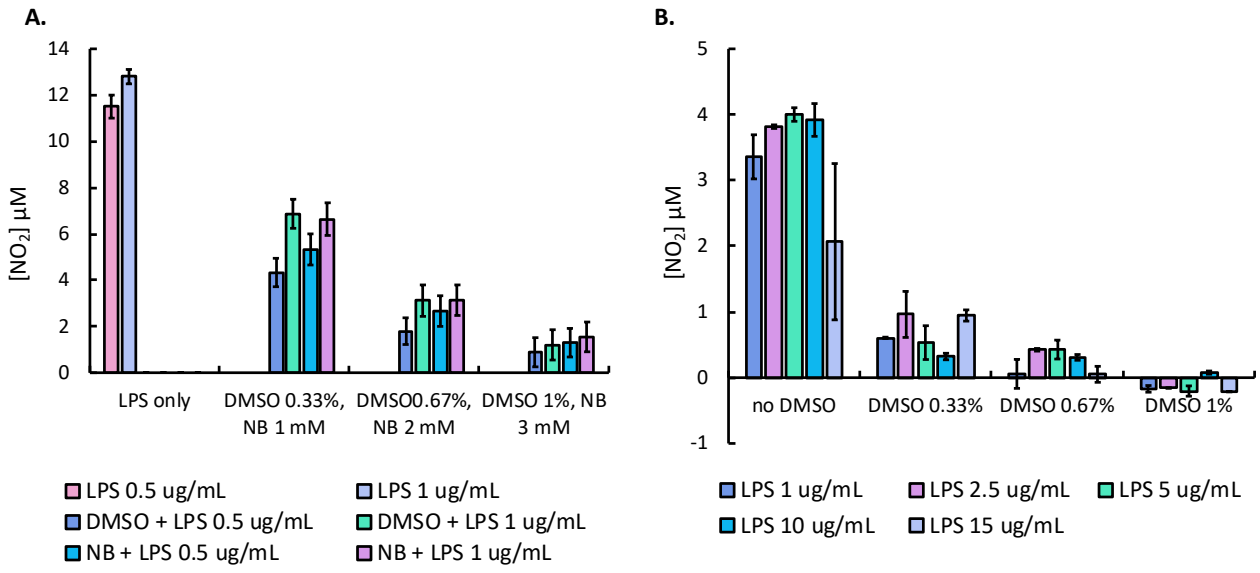


Figure 53. A. Activation of RAW264.7 cells by LPS (0.5 or 1 μg/mL) under various treatment conditions including LPS only, DMSO and LPS, and norb-bio **70** and LPS. Activation was assessed using the Griess assay for concentration of NO_2 detected. B. Activation of RAW264.7 cells by LPS (1-15 μg/mL) both with and without varying amounts of DMSO (0.33-1 %v/v). Activation was assessed using the Griess assay for concentration of NO_2 detected. Cell-only controls had no NO_2 detected. DMSO and norb-bio-only controls also had no NO_2 detected.

This heightened effect of the DMSO radical scavenger ability compared to other cell lines tested is likely due to the endogenous nature of the ROS (generated by the cell). In the HeLa cell experiments detailed in Chapter three, hydrogen peroxide was added directly, which is not scavenged by DMSO and directly oxidises thiols. This model relies on the production of superoxide by stimulation with LPS, where this superoxide is then converted to hydrogen peroxide for reaction with cysteine and formation of sulfenic acids. It is therefore likely that the superoxide is scavenged well before it is converted to hydrogen peroxide, meaning sulfenic acids are not formed in appreciable amounts above basal levels. Given this consideration, it is not surprising that **70** is unable to capture any sulfenic acids. The gradual production of the ROS (rather than flooding with hydrogen peroxide) may also contribute to the scavenging of these ROS species by DMSO.

4.3.5 Concluding remarks

Sulfenic acids generated through stimulation of the RAW264.7 cell line with LPS were unable to be detected with the norb-bio probe **70**. However, this appears to be an issue of the model and conditions required for labelling, rather than the reaction of norb-bio with the protein sulfenic acids in cells. The high concentration of DMSO required for sufficient labelling (due to solubility of **70**) appeared to act as a radical scavenger, preventing efficient formation/detection of protein sulfenic acids by **70**. Together, this prevented adequate detection of cysteine sulfenic acids in RAW264.7 cells stimulated with LPS due to labelling conditions.

While unsuccessful in this attempt, the RAW264.7 cell line could still be a viable model for future studies if correct labelling conditions could be met. Future redesign of a more water-soluble norbornene probe may alleviate the issues experienced in respect to the apparent antioxidant effects of DMSO. By reducing the DMSO concentration needed for solubility, this could allow more efficient oxidation of the thiols and their detection. In which case the results presented here provide a foundation for developing this model in future studies. This cell line could provide valuable information regarding redox regulation events during inflammation and the immune response, which could further be applied to human macrophages.

4.4 Investigating the effect of ROS-generating drugs and sulfenic acid formation

4.4.1 Introduction

Norbornene probes have proven useful in the detection of cysteine sulfenic acids in live cells with promising protein hits identified through a proteome-wide whole cell analysis outlined earlier in this Chapter. This study focused on identifying proteins which are oxidised during oxidative stress

conditions and could therefore play a functional role in disease-causing pathways associated with oxidative stress. However, oxidative stress conditions can also play functional roles in treating some diseases. A large body of work exists around the use of ROS-generating drugs to treat cancer.[213-214] This piqued our interest as often it is known that ROS-generating drugs help destroy cancer, but it is not always clear through which metabolic pathway this occurs or whether different ROS-generating drugs target different mechanisms. Cancer cells typically operate under elevated levels of oxidative stress compared to normal cells. Addition of exogenous agents to increase ROS generation or decrease ROS removal can therefore cause accumulation of ROS in cancer cells (compared to normal cells) raising the oxidative stress levels above the toxic threshold (overcoming the antioxidant defence) leading to cell death.[214-216] These ROS-generating drugs aim to increase the ROS levels more selectively in cancer cells since these cells are already undergoing a redox imbalance and thus are more susceptible to any perturbations in redox levels.

Since generation of ROS causes cysteine oxidation, we thought our norb-bio probe **70** may provide insight into sulfenic acid generation during the drug treatment, and what role this plays in the drug's efficacy. Understanding what proteins are targeted during the drug treatment and how their oxidation may affect the events leading to cell death could better our understanding of the drugs action and identify new protein targets for improved treatment.

4.4.2 ROS-generating drugs

A common ROS-generating drug is β-lapachone **124**. This compound generates superoxide through a redox-pathway with assistance of the enzyme NQO1 and NADPH (Fig. 54).[217-219] **124** is reduced to the corresponding alcohol **125** alongside oxidation of NADPH, catalysed by NQO1. **125** can undergo a radical reaction with oxygen, producing **126** and superoxide as a by-product. Superoxide can dismutate to hydrogen peroxide with assistance of enzymes and leads to accumulation of ROS. The β-lapachone **124** is regenerated by production of a second superoxide molecule through a radical reaction of **126** with a second molecule of oxygen and thus is recycled. β-lapachone (**124**) has been studied as an anti-cancer drug in several studies. Specifically, it has been shown that NQO1 has a key role in the activation of **124**, but the detailed mechanism for its toxic efficacy remains for investigation.[217, 220-221] For example, cancer cells have increased expression of NQO1 which may account for the increased cytotoxicity of **124** specifically in cancer cells.[217] Several targets of **124** have been identified, but their exact involvement in cell death is still unclear.[222-224] We sought to investigate whether our norb-bio probe **70** could capture any protein sulfenic acids generated by treatment with **124**. This would provide a new platform in which to study the effects of ROS-generating drugs, and also show the ability of **70** to capture protein sulfenic acids formed through generation of ROS within the cell rather than applying external hydrogen peroxide.

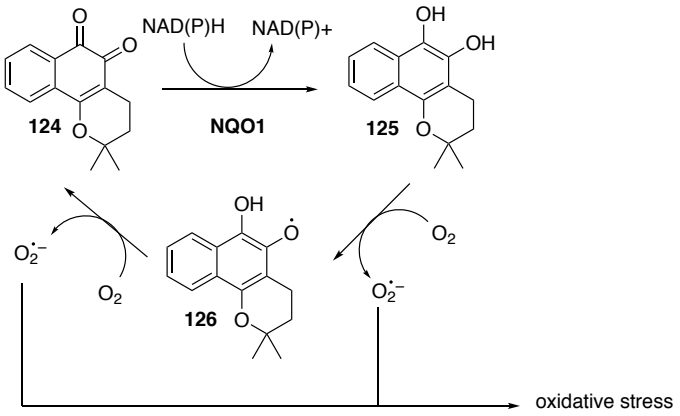


Figure 54. Mechanism for the bioactivation of the drug β-lapachone **124** by NQO1. The enzyme system reduces the ketone groups to alcohols (**125**), which undergo a radical reaction with oxygen, forming the ROS superoxide. A second radical reaction of **126** with oxygen regenerates the ketone, forming a redox cycle. The superoxide molecules contribute to oxidative stress.

The cell line HL-60 (human leukemia cell line) represents a good model for this experiment as this cell line responds well to treatment with **124** to produce ROS. Therefore, we sought to treat HL-60 cells with **124** in the presence of **70** to detect cysteine sulfenic acid formation. Several considerations were taken into account for experimental design. HL-60 cells are a suspension cell line which could affect concentration of probe required. HL-60 cells are also known to differentiate in DMSO.[225-227] This could be an issue given the high concentration of DMSO required for the labelling experiment. Although this likely will not be an issue given the time frame of the experiment (few hours), but should be considered nonetheless.

4.4.3 Toxicity of norb-bio 70 and DMSO

First, the toxicity of the norb-bio probe **70** and DMSO was tested. Guided by the results obtained with the HeLa cell experiments discussed in Chapter three, we wanted to test viable labelling conditions. Since DMSO can cause differentiation in HL-60 cells, we sought to keep the concentration to a minimum. Typical conditions to induce differentiation are usually >1 %v/v DMSO. Therefore, we decided to decrease the probe concentration to 1 and 2 mM to keep the DMSO >1 %v/v, since it had already been established that increasing the probe **70** concentration without increasing the DMSO concentration was not viable. The compound **124** was also tested for toxicity. Since this is also added in DMSO (at 0.1 %v/v) this was taken into consideration to ensure all conditions accounted for all sources of DMSO to be used in the labelling experiments.

HL-60 cells were incubated with either **70**, **124**, or DMSO for a total of 24 h at 37 ºC in 5 % CO_2 humidified atmosphere before performing the cell toxicity assay. For cells treated with **124** at any

concentration, significant toxicity and cell death was observed compared to the DMSO control (Fig. 55A). Toxicity is expected for cells treated with **124** as this compound generates ROS in a redox-recycled manner, and purpose is to kill cancer cells. Samples were tested at two different concentrations of DMSO, one to correspond to the concentration of DMSO required for labelling **70** at 1 mM (0.33 %v/v) and the other at 2 mM (0.67 %v/v). The DMSO-only controls seemed to have minimal toxic effects. Importantly, when the cells were treated with just **70** at both 1 and 2 mM, no increase in toxicity was observed compared to the DMSO-only control (Fig. 55B). This suggests that under these conditions, **70** is non-toxic and suitable for use in the labelling experiment.

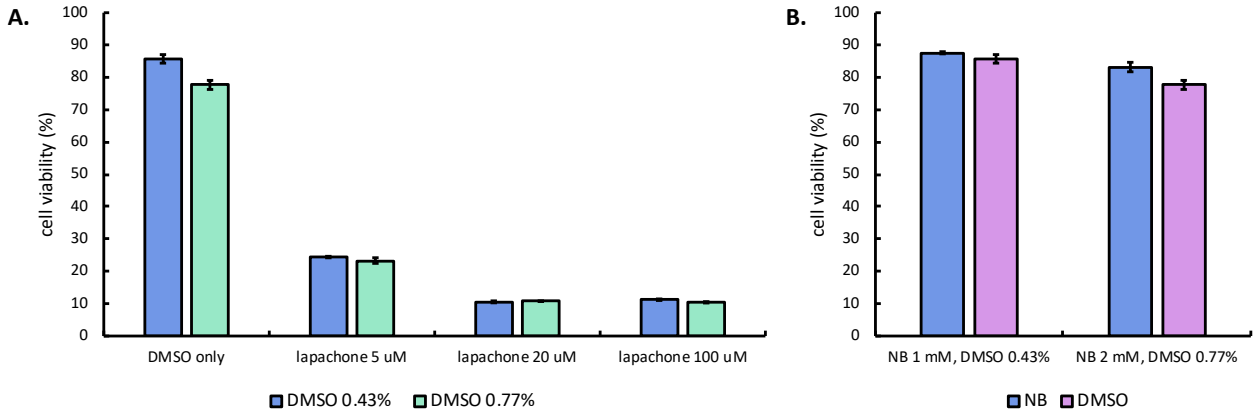


Figure 55. A. Toxicity of **124** and DMSO in HL-60 cells over 24 h. B. Toxicity of **70** and DMSO in HL-60 cells over 24 h.

4.4.4 Labelling of sulfenic acids by norb-bio 70

Before performing the labelling experiment, the appropriate conditions were assessed. The HL-60 cells were cultured in RPMI media which does not contain free cysteine and therefore suitable for the labelling experiment. Based on the HeLa cell experiments addressed in Chapter three, a low FBS media is also required. Immediately before the labelling experiment, cells were centrifuged and washed twice with RPMI (no FBS) media and diluted to the appropriate concentration. For the experiment, HL-60 cells were incubated with norb-bio (1 or 2 mM, **70**) for 2 h before addition of β-lapachone (0-100 μM, **124**) and left to incubate for a further 2 h at 37 ºC in 5 % CO_2 humidified atmosphere (Fig. 56A). These conditions were based on the conditions used for the HeLa cell experiments in Chapter three, but were not further optimised. Since **124** is redox-recycled, the incubation time could be vital for effective labelling and may provide optimisation of the labelling conditions in future studies. After this incubation time, cells were collected, washed, lysed, and analysed by SDS-PAGE and western blot. A streptavidin signal was only detected when cells were treated with both **70** and **124**, indicating that **124** was required for sulfenic acid labelling (Fig. 56B). This also highlighted that lack of an oxidant producing species prevented labelling, further implying

the selectivity of the norb-bio probe **70** for cysteine sulfenic acids. This result also confirms that **70** is able to label cysteine sulfenic acids even when hydrogen peroxide is not added directly, demonstrating its ability to trap sulfenic acids formed by endogenous generation of ROS. This shows that at least for the current conditions, **70** can react with sulfenic acids on the same time-scale that ROS are generated and subsequently react. This is very encouraging for the future application of **70** to study cysteine sulfenic acids in other cell-types with varying modes of ROS production. Both increasing concentration of **70** and increasing concentration of **124** resulted in increased streptavidin labelling. Even at low concentrations of β-lapachone (5 μM, **124**), with norb-bio (2 mM, **70**), detectable labelling was observed. This outcome is also promising as this method of introducing ROS is distinct from most previous studies which employ either hydrogen peroxide or EGF for formation of sulfenic acids. This result is extremely promising and warrants further investigation for future projects.

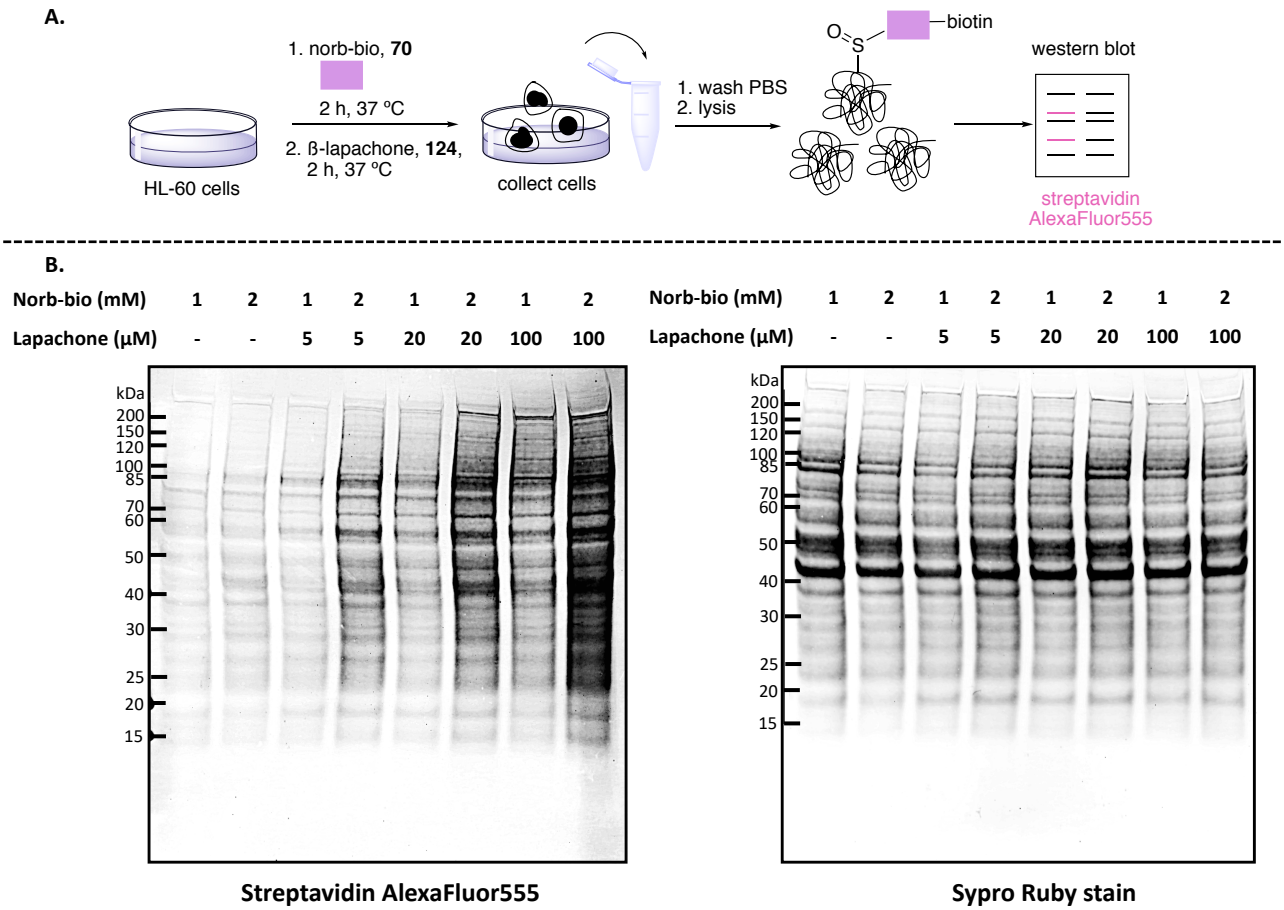


Figure 56. A. Outline for labelling of sulfenic acids with **70** in HL-60 cells treated with β-lapachone. B. Left: Western blot analysis of HL-60 cells treated with norb-bio (1 or 2 mM, **70**) with varying concentration of β-lapachone (0-100 μM, **124**). Biotinylated proteins were detected with streptavidin AlexaFluor555. Right: Total protein loading assessed using Sypro Ruby total protein stain. Protein loading was normalised to 30 μg per lane.

For future studies, the trapped proteins could be identified. This would provide information about the types of proteins that have oxidisable cysteine residues when treated with the drug **124**. Using quantified proteomics and comparison to a control (not treated with **124**), the most enriched proteins could be identified as key proteins of interest in a similar strategy employed in section 4.2. Further evaluation of the protein hits may provide insight into the mechanism of how this ROS-generating drug causes cell death, and whether there are any distinct proteins or pathways responsible for this. Further to this, it could be interesting to compare the effects of **124** to other known ROS-generating drugs to see if similar proteins are targeted by different drugs, or whether they may act via different mechanisms. This could also be compared across different cell-types. Identifying how drugs such as **124** cause cell death may help improve their efficacy, and reduce the unwanted side-effects often associated with these types of drugs.

4.4.5 Concluding remarks

Overall, norb-bio (**70**) was able to detect cysteine sulfenic acid formation in HL-60 cells only when treated with the ROS-generating drug **124**. Labelling was found to be concentration dependent for both norb-bio and β-lapachone. The endogenous generation of ROS within the cell also highlights the ability of **70** to capture sulfenic acids even when cells are not treated directly with exogenous hydrogen peroxide. This distinction is important as it means this class of norbornene probes could be applied to study a range of biological processes related to oxidative stress, not just treatment with hydrogen peroxide. These preliminary studies reveal the applicability of **70** to further study the effect of ROS-generating drugs in the treatment of cancers. Future studies to detect, isolate and identify the trapped proteins will reveal what proteins are targeted during treatment with ROS-generating drugs such as **124**. This will provide new insight into improving the efficacy and reducing the toxicity of these drugs for clinical use.

4.5 Experimental procedures

4.5.1 General procedures

General considerations

All purchased chemicals were used as received without further purification. All fluorescence and UV-Vis spectroscopy in a 96-well plate format was performed on a SpectraMax i3x plate reader (Molecular Devices). All western blot images were recorded on a Typhoon Trio Variable Mode Imager (GE Healthcare). Proteomics of peptide fragments were performed using a RSLCnano U3000 HPLC coupled to a QExactive plus mass spectrometer via a nano-electrospray ion source.

Separation of peptides was on an Acclaim PepMap RSLC C_{18}, 25 cm x 75 µm x 2 µm particle size, 100 Å pore size column.

Cell culture of HeLa cells

HeLa cells (derived from cervical cancer cells, ATCC) were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C and cultured in high glucose DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS) (Gibco), and 1 % penicillin-streptomycin (PS) (Gibco). Cells were grown to ~80-90 % confluency, trypsinised (0.25 %, trypsin-EDTA, Gibco), then neutralised with complete media prior to use.

Gel electrophoresis and western blot

Samples were separated by SDS-PAGE using NuPage 4-12 % Bis-Tris protein gels (Invitrogen) with MES running buffer (200 V) and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 µM, iBlot, ThermoFisher) at 20 V. After transfer, the PVDF membrane was first washed with water (× 3) then blocked with BSA (3 % in tris-buffered saline Tween-20 (TBST)) for 1 h at room temperature with gentle rocking. The membrane was washed with TBST (3 × 5 min) then incubated with 1:1,000 Alexa Fluor® 555 streptavidin (1 mg/mL stock in PBS, Invitrogen) in TBST for 30 min at room temperature with gentle rocking. The PVDF membrane was washed with TBST (2 × 5 min), water (2 × 5 min), and imaged by fluorescence (filter: 580, laser: 532 nm, PMT: 550 V) using Typhoon Trio imager. To assess equal protein loading, PVDF membranes were stained with Sypro Ruby blot stain (Invitrogen) according to manufacturer's instructions and imaged by fluorescence (filter: 610, laser: 532 nm, PMT: 550 V) using Typhoon Trio imager.

4.5.2 Proteomics optimisation of affinity purification

Preparation of stock solutions

Norb-bio **70** (13.5 mg, 0.027 mmol) was dissolved in DMSO (91 µL) to give a final concentration of 300 mM. H_2O_2 (5.7 µL, 50 wt. % in H_2O, 17.6 M) was diluted in H_2O (994 µL) to give a final concentration of 100 mM. This was further diluted 10 µL in H_2O (990 µL) to give a final concentration of 1 mM. Alternatively, H_2O_2 (34 µL, 50 wt. % in H_2O, 17.6 M) was diluted in H_2O (966 µL) to give a final concentration of 600 mM. Biotin (24 mg, 0.1 mmol) was dissolved in DMSO (1 mL) to give a 100 mM stock solution.

Preparation of HeLa cell lysates

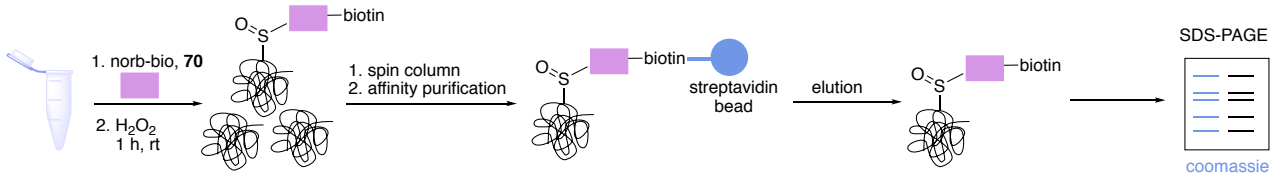
Cells were cultured as described above. Approximately 1.5 $\times 10^7$ cells were pelleted (150 ×g, 5 min) and washed with PBS (3 × 10 mL). Cell extraction buffer with protease inhibitor (700 µL, Invitrogen)

was then added and incubated on ice for 45 min. The resulting solution was centrifuged (10,000 ×g, 10 min) to clarify the lysate and then purified using a Zeba size exclusion spin column (2 mL, 7 kDa MWCO, ThermoFisher) pre-equilibrated with PBS. Protein concentration was determined using Protein 660 nm assay (Pierce) and typically gave around 2-3 mg/mL protein. The eluent was aliquoted and frozen until use.

Proteomics gel preparation

Gels were prepared for proteomics analysis using NuPage 4-12 % Bis-Tris protein gels (Invitrogen) with MES running buffer (200 V). Gels were fixed (1 % acetic acid, 45 % methanol in water, 30 min) then washed in water (× 2, 5 min). After fixing, gels were incubated with coomassie overnight at 4 °C. Gels were then washed with water for 1-2 h and imaged.

Labelling of protein sulfenic acids in HeLa cell lysates

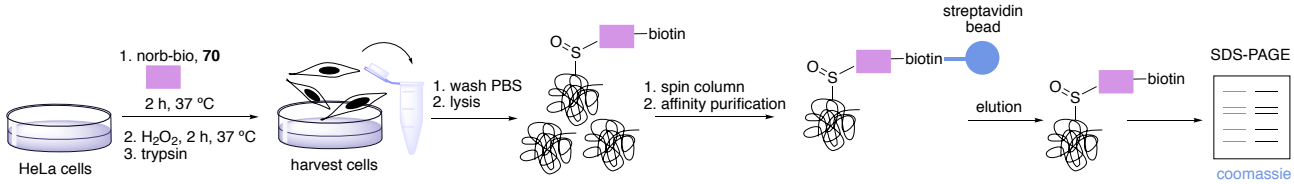


HeLa cell lysate was prepared as described above. Protein concentration was assessed using BCA assay (Pierce) to be approximately 1400 µg/mL. To the cell lysate (200 µL) in PBS, norb-bio **70** (1.65 µL, of 300 mM in DMSO) was added and the reaction vortexed. H_2O_2 (30 µL of 1 mM stock) was added, samples vortexed, then left to stir for 1 h at room temperature. The samples were then quenched by purification with a Zeba spin column (0.5 mL, 7 kDa MWCO, ThermoFisher) to remove any unbound probe and frozen until further analysis.

Affinity purification

Reactions were conducted according to the above labelling protocol. After removing unbound probe (zeba spin column), samples were then collected and incubated with pre-washed (3 × 1 mL PBS) streptavidin agarose bead conjugates (1.2 mg/mL as a 50 % slurry in PBS, Merck). Cell lysate reaction (~90 µg protein, 65 µL), was added to 50, 100 and 200 µL of beads with additional PBS (500 µL total volume) to aid in mixing. The beads were left to incubate for 2 h at 4 °C with gentle rocking. The beads were collected by centrifugation (10,000 g, 30 sec) and the supernatant removed (retained for analysis). The beads were washed with PBS (3 × 1 mL) then heated at 95 °C for 10 min in elution buffer (LDS sample buffer (25 µL, 0.5 % LDS), DTT sample reducing agent (5 µL), and free biotin (2 mM, 2 µL of 100 mM stock in DMSO) with PBS (25 µL)). The supernatant was ready for direct loading onto gel for separation by SDS-PAGE as described above. For each lane, 20 µL was loaded.

Proteomics experiment on whole cell optimisation



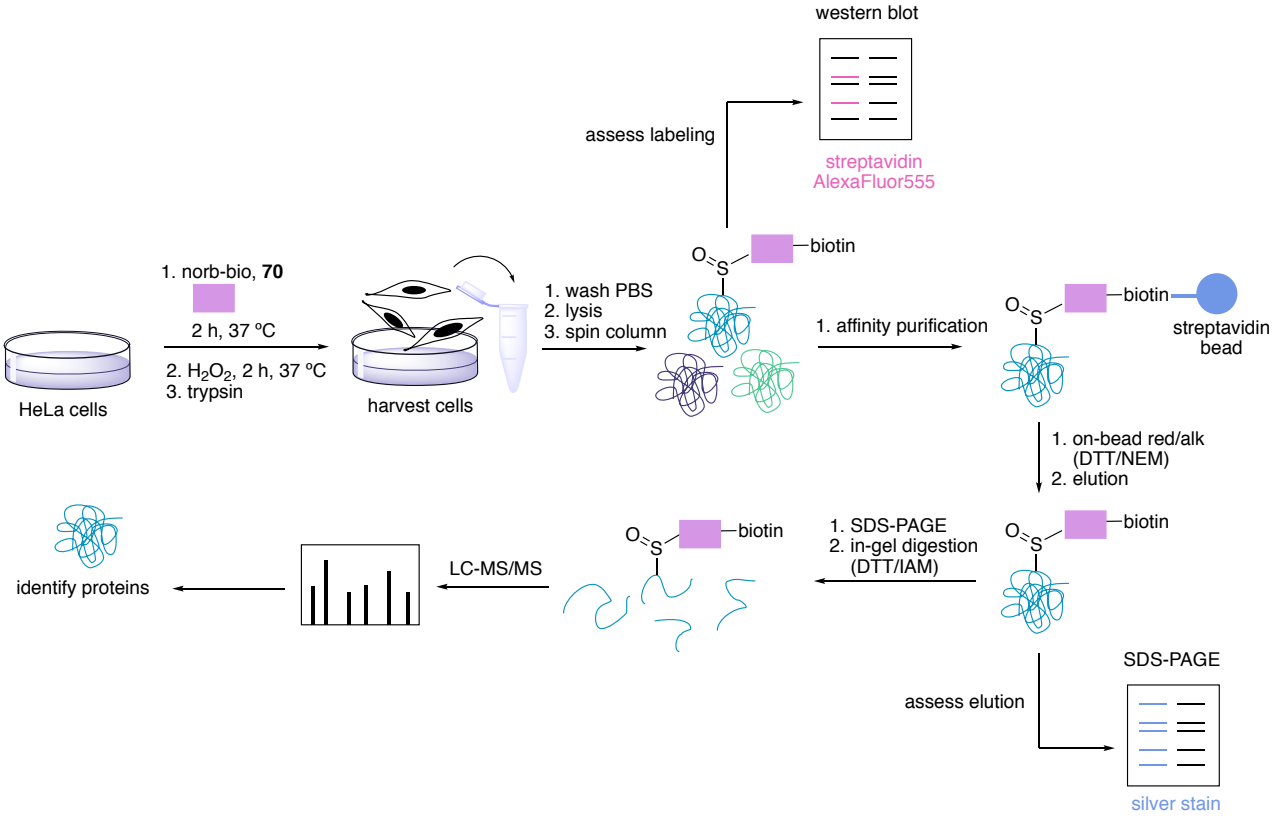
Labelling HeLa cells with norb-bio 70

Cells were cultured as described above. Cells were seeded in 2 × T25 flasks and grown until ~90 % confluency (approximately 2.8×10^6 cells per flask at confluency). Prior to experiment, the media was removed and cells washed with DMEM low glucose 0.5 % FBS media (2 × 3 mL), then 3 mL added to each T25 flask. Norb-bio **70** (30 µL, from 300 mM stock) was added to each flask and incubated at 37 ºC for 2 h. After this time, H_2O_2 (10 µL, from 600 mM stock) was added to each flask and further incubated at 37 ºC for 2 h. After 2 h, the media was removed and trypsin (3 mL) added for 5 min. Cells from both flasks were collected and combined. Cells were gently centrifuged (160 × g, 5 min) and trypsin removed before washing with cold PBS (3 × 5 mL). Cell extraction buffer (500 µL) was added to the pellet and incubated on ice for 45 min. The cell lysate was centrifuged (10000 × g, 5 min) then purified with a zeba spin column (2 mL, 7 kDa MWCO, ThermoFisher) pre-equilibrated with PBS and stored at –80 ºC until further use.

Affinity purification

Reactions were conducted according to the above labelling protocol. After removing unbound probe (zeba spin column), cell lysis (500 µL) was collected and incubated with pre-washed (3 × 1 mL PBS) streptavidin agarose bead conjugates (300 µL, of 1.2 mg/mL as a 50 % slurry in PBS, Merck). The beads were left to incubate for 2 h at 4 °C with gentle rocking. The beads were collected by centrifugation (14,000 g, 30 sec) and the supernatant removed. The beads were washed (PBS 3 × 1 mL, cell extraction buffer 1 × 1 mL, PBS 1 × 1 mL) then heated at 95 °C for 10 min in elution buffer (LDS sample buffer (40 µL, 0.5 % LDS), DTT sample reducing agent (9 µL), and free biotin (2 mM, 2 µL of 100 mM stock in DMSO) with PBS (40 µL)). The eluted supernatant was ready for direct loading onto gel for separation by SDS-PAGE as described above. For each lane, 25 µL was loaded.

4.5.3 Proteomics experiment whole cell analysis for quantification



Preparation of stock solutions

Norb-bio **70** (74 mg, 0.150 mmol) was dissolved in DMSO (500 µL) to give a final concentration of 300 mM. H_2O_2 (34 µL, 50 wt. % in H_2O, 17.6 M) was diluted in H_2O (966 µL) to give a final concentration of 600 mM. Dithiothreitol (DTT) (30.2 mg, 0.19 mmol) was dissolved in H_2O (982 µL) to give a 200 mM stock solution. *N*-Ethylmaleimide (NEM) (54.5 mg, 0.44 mmol) was dissolved in PBS (4.355 mL) to give a 100 mM solution. Biotin (24 mg, 0.1 mmol) was dissolved in DMSO (1 mL) to give a 100 mM stock solution.

Labelling experiment on HeLa cells

Cells were cultured as described above. Cells were seeded in 6 × T75 flasks and grown until ~90 % confluency (approximately 8.4×10^6 cells per flask at confluency). Prior to experiment, the media was removed and cells washed with DMEM low glucose 0.5 % FBS media (2 × 8 mL). A stock solution of norb-bio probe in media was prepared by adding norb-bio **70** (500 µL of a 300 mM stock in DMSO) to DMEM low glucose 0.5 % FBS media (50 mL). After removing wash media from cells, the norb-bio (3 mM, **70**) media (8 mL) was added to each flask and incubated at 37 ºC for 2 h. After this time, H_2O_2 (27 µL, 600 mM stock) was added to three flasks (oxidised samples) and further incubated at 37 ºC for 2 h. The remaining three flasks were also further incubated for 2 h at 37 ºC. After 2 h, the media was removed and trypsin (4 mL) added for 5 min. Trypsin was then neutralised with complete DMEM 10 % FBS media (6 mL). Cells were collected and gently centrifuged (160 × g, 5 min) and

trypsin removed before washing with cold PBS (3 × 10 mL). Cell extraction buffer with protease inhibitor (500 µL, Pierce) was added to the pellet and incubated on ice for 45 min with regular vortexing. After this time, cell lysis was centrifuged (10,000 × g, 5 min) then purified with a zeba spin column (2 mL, 7 kDa MWCO, ThermoFisher) pre-equilibrated with PBS and stored at –80 ºC until further use.

Affinity purification

Reactions were conducted according to the above labelling protocol. After removing unbound probe (zeba spin column), cell lysis (500 µL) was collected and incubated with pre-washed (3 × 1 mL PBS) streptavidin agarose bead conjugates (200 µL, of 1.2 mg/mL as a 50 % slurry in PBS, Merck). The beads were left to incubate for 2 h at 4 ºC with gentle rocking. The beads were collected by centrifugation (14,000 g, 30 sec) and the supernatant removed and retained for analysis. The beads were washed (PBS 2 × 1 mL, cell extraction buffer 1 × 1 mL, PBS 2 × 1 mL) each for 5 min with mixing between steps. After the final wash, a solution of DTT (50 µL, 200 mM stock in water) was added to the beads with 450 µL PBS to give 20 mM DTT. The beads were incubated at 37 ºC for 1.5 h with shaking in the dark. The supernatant was removed and retained, then a solution of NEM (500 µL, of 100 mM in PBS) was added to each sample and incubated at 37 ºC for 1.5 h with shaking in the dark. The supernatant was removed and retained and the beads washed with PBS (1 mL). Biotinylated proteins were then eluted in 50 µL elution buffer (prepared PBS (160 µL), LDS (160 µL), sample reducing agent (36 µL), 2 mM free Biotin (8 µL from 100 mM stock in DMSO)) by first incubating at room temperature for 20 min, followed by heating to 95 ºC for 10 min. The supernatant was collected and stored at –80 ºC until further use.

LC-MS sample preparation (performed by Maike Langini)

20 µL protein eluate per sample were desalted through electrophoretic migration at 50 V for 10 min on a 4–12 % Bis-Tris polyacrylamide gel (Novex NuPAGE, Thermo Scientific). After silver staining, protein bands were cut out, reduced, alkylated and digested with trypsin before peptide extraction via sonication. Peptides were dissolved and diluted with 0.1 % TFA (v/v).

LC-MS analysis (performed by Maike Langini)

For mass spectrometric analysis, 15 µL peptide solution per sample were analysed on a nano-high-performance liquid chromatography electrospray ionisation mass spectrometer. 20 fmol Pierce Peptide Retention Time Calibration Mixture (Thermo Fischer Scientific) were added to each sample before injection. The analytical system was composed of a RSLCnano U3000 HPLC coupled to a QExactive plus mass spectrometer via a nano-electrospray ion source (Thermo Fischer Scientific). Injected peptides were concentrated and desalted at a flow rate of 6 µL/min on a trapping column (Acclaim PepMao C_{18}, 2 cm x 100 µm x 3 µm particle size, 100 Å pore size, Thermo Fischer

Scientific) with 0.1 % TFA (v/v) for 10 min. Subsequently, peptides were separated at a constant flowrate of 300 nL/min over a 60 min gradient on an analytical column (Acclaim PepMap RSLC C_{18}, 25 cm x 75 µm x 2 µm particle size, 100 Å pore size, Thermo Fischer Scientific) at 60 ºC. Separation was achieved through a gradient from 4 to 40 % solvent B (solvent A: 0.1 % (v/v) formic acid in water, solvent B: 0.1 % (v/v) formic acid, 84 % (v/v) acetonitrile in water). Afterwards, peptides were ionised at a voltage of 1,500 V and introduced into the mass spectrometer operated in positive mode. MS scans were recorded in profile mode in a range from 200-2000 *m/z* at a resolution of 70,000 while tandem mass spectra were recorded at a resolution of 17,500. Tandem mass spectra were recorded with a data dependent Top20 method and 30 % normalised collision energy. Dynamic exclusion was activated.

Computational mass spectrometric data analysis (performed by Maike Langini)

Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific) was applied for peptide/protein identification with Mascot and MS Amanda as search engines employing the UniProt database (human; including isoforms; date 2019-02-13). A false discovery rate of 1 % ($p \leq 0.01$) on peptide level was set as identification threshold. Proteins were quantified with Progenesis QI for Proteomics (Version 2.0, Nonlinear Dynamics, Waters Corporation).

Analysis of proteins

All pie charts, Venn diagrams, and comparison lists were generated using FunRich analysis tool.[228-229] The UniProt database (human; Swiss-Prot; isoforms not included) was used for all individual protein comparison and compartment assignments within the FunRich tool. The FunRich database was used for biological pathway assignments. Data from previously published surveys were taken from provided supplementary files and converted to their corresponding UniProt accession for use in comparison studies.

4.5.4 RAW 264.7 experimental procedures

Preparation of stock solutions

Norb-bio **70** (15.7 mg, 0.032 mmol) was dissolved in DMSO (106 µL) to give a final concentration of 300 mM. LPS (1 mg/mL, sigma) was diluted to 0.1 mg/mL (5 µL of 1 mg/mL in 45 µL media) and 0.01 mg/mL (5 µL of 0.1 mg/mL in 45 µL media).

Cell culture of RAW264.7

Macrophages (RAW246.7) were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C and cultured in DMEM (high glucose, pyruvate, Gibco) supplemented with 10 % heat inactivated fetal

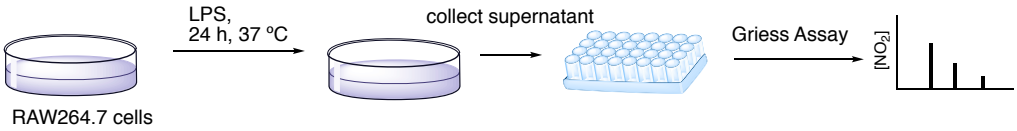
bovine serum (FBS) (Gibco), and 1 % penicillin-streptomycin (Gibco). Cells were grown to ~70-80 % confluency, and scraped to suspend before use in experiments. For Griess assay experiments, media was replaced with phenol red free DMEM (high glucose, pyruvate, HEPES, Gibco).

Cell viability assay protocol

RAW264.7 were cultured as described above. In a 96-well plate, cells were seeded to approximately 5,000 cells/well and incubated overnight in 200 µL complete media at 37 °C. To appropriate wells norb-bio probe **70** was added as follows: 1 mM (0.67 µL of a 300 mM stock), 1.5 mM (1 µL of a 300 mM stock), 2 mM (1.33 µL of a 300 mM stock), 2.5 mM (1.67 µL of a 300 mM stock), 3 mM (2 µL of a 300 mM stock). As controls, DMSO was added as follows: 0.67 µL (0.33 %v/v), 1 µL (0.5 %v/v), 1.33 µL (0.67 %v/v), 1.67 µL (0.84 %v/v), 2 µL (1 %v/v). To appropriate wells LPS was added as follows: 0.05 µg/mL (1 µL from 0.01 mg/mL stock), 0.1 µg/mL (2 µL from 0.01 mg/mL stock), 0.5 µg/mL (1 µL from 0.1 mg/mL stock), 1 µg/mL (2 µL from 0.1 mg/mL stock), 2.5 µg/mL (0.5 µL from 1 mg/mL stock), 5 µg/mL (1 µL from 1 mg/mL stock), 10 µg/mL (2 µL from 1 mg/mL stock), 15 µg/mL (3 µL from 1 mg/mL stock).

All cells were left to incubate for 24 h at 37 °C. Both a cell only and media control were performed. After this time, media was replaced with fresh media and allowed to further incubate for 48 h. After this incubation period, CellTiter Blue (Promega) (1:5 dilution) was added to all wells and left to incubate for a further 3-4 h at 37 °C or until complete. The cell viability was assessed by fluorescence emission intensity (590 nm) using a SpectraMax i3x plate reader. Cell viability was calculated as a percentage of viable cells compared to the cell-only control. Error bars represent the standard deviation of each sample with six replicates.

Griess Assay

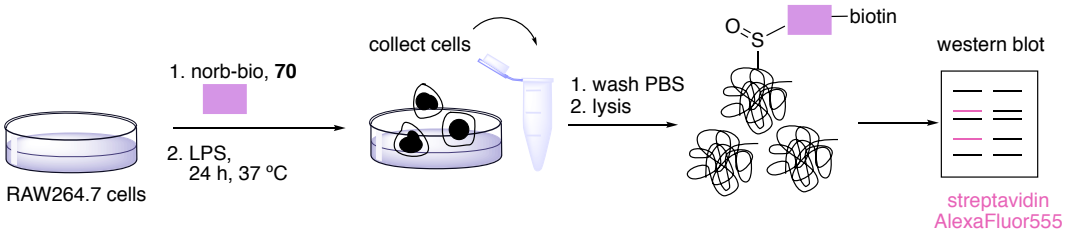


RAW264.7 were cultured as described above. In a 96-well plate, cells were seeded to approximately 40,000 cells/well and incubated overnight in 200 µL complete media at 37 °C. After this time, media was replaced with 200 µL fresh phenol red free complete media. To appropriate wells LPS was added as follows: 0.05 µg/mL (1 µL from 0.01 mg/mL stock), 0.1 µg/mL (2 µL from 0.01 mg/mL stock), 0.5 µg/mL (1 µL from 0.1 mg/mL stock), 1 µg/mL (2 µL from 0.1 mg/mL stock), 2.5 µg/mL (0.5 µL from 1 mg/mL stock), 5 µg/mL (1 µL from 1 mg/mL stock), 10 µg/mL (2 µL from 1 mg/mL stock), 15 µg/mL (3 µL from 1 mg/mL stock). Both a cell only and media only control were also performed. cells were left to incubate for 24 h at 37 °C. After this incubation period, 100 µL of the cell media supernatant was removed and added to a new 96-well plate. In the new 96-well plate, a standard curve for $NaNO_2$ was prepared by adding 200 µL of $NaNO_2$ (100 µM) and performing a

serial dilution by taking 100 µL of the $NaNO_2$ (100 µM) and adding to phenol red free media to give concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µM. To each well was then added 100 µL of Griess reagent prepared according to manufacturers instructions. The solution was allowed to react for 15 min before NO_2 concentration was assessed by absorbance (540 nm) using a SpectraMax i3x plate reader. NO_2 concentration was determined using the $NaNO_2$ standard curve. Error bars represent the standard deviation of each sample with six replicates.

For DMSO vs. LPS experiments: In a 96-well plate, cells were seeded to approximately 40,000 cells/well and incubated overnight in 200 µL complete media at 37 °C. After this time, media was replaced with 200 µL fresh phenol red free complete media. To appropriate wells LPS was added as follows: 1 µg/mL (2 µL from 0.1 mg/mL stock), 2.5 µg/mL (0.5 µL from 1 mg/mL stock), 5 µg/mL (1 µL from 1 mg/mL stock), 10 µg/mL (2 µL from 1 mg/mL stock), 15 µg/mL (3 µL from 1 mg/mL stock). Then, to appropriate wells, DMSO was added as follows: 0.67 µL (0.33 %v/v), 1.33 µL (0.67 %v/v), and 2 µL (1 %v/v). Combinations of each LPS concentration with each DMSO concentration were performed including an LPS only control. Both a cell only and media only control were also performed. cells were left to incubate for 24 h at 37 °C. After this incubation period, 100 µL of the cell media supernatant was removed and added to a new 96-well plate. The Griess assay was performed as described above. Error bars represent the standard deviation of each sample with three replicates.

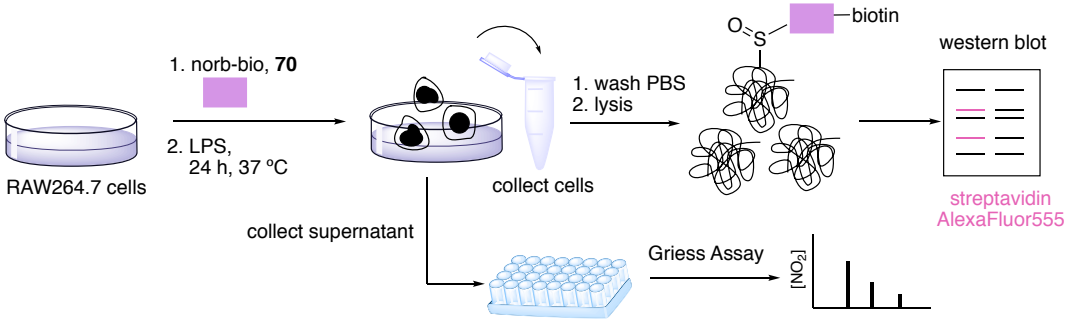
Labelling of protein sulfenic acids in RAW264.7



RAW264.7 were cultured as described above. In a 6–well plate, RAW264.7 were seeded to approximately 300,000 cells/well and incubated overnight in 3 mL complete DMEM media (10 % HI FBS, 1 % penicillin-streptomycin) in a humidified atmosphere of 5 % CO_2 at 37 °C. After 24 h, the media was replaced with fresh complete media (1.2 mL). To appropriate wells, norb-bio **70** was added as follows: 1 mM (4 µL from 300 mM stock in DMSO), 2 mM (8 µL from 300 mM stock in DMSO), 3 mM (12 µL from 300 mM stock in DMSO). After gently swirling to mix, LPS was added to appropriate wells as follows: 0.5 µg/mL (6 µL from 0.1 mg/mL stock), and 1.0 µg/mL (12 µL from 0.1 mg/mL stock) and left to incubate for 24 h in a humidified atmosphere of 5 % CO_2 at 37 °C. After this time, media was removed and replaced (DMEM, 1 mL), cells were scraped, collected, centrifuged (150 ×g, 5 min), and cells washed with PBS (3 × 1 mL). Each sample was then lysed with cell extraction buffer with protease inhibitor (100 µL, Pierce) on ice for 45 min. Cell lysates were clarified

by centrifugation (14,000 ×g, 10 min), and stored at –80 °C until analysis by western blot. Protein concentration was determined by Protein 660 nm assay (Pierce).

Labelling of protein sulfenic acids with norb-bio 70 in RAW264.7 and Griess assay



RAW264.7 were cultured as described above. In a 6–well plate, cells were seeded to approximately 600,000 cells/well and incubated overnight in 3 mL complete DMEM media (10 % HI FBS, 1 % penicillin-streptomycin) in a humidified atmosphere of 5 % CO_2 at 37 °C. After 24 h, the media was replaced with fresh phenol red free complete DMEM media (1.5 mL). To appropriate wells, norb-bio **70** was added as follows: 1 mM (5 µL from 300 mM stock in DMSO), 2 mM (10 µL from 300 mM stock in DMSO), 3 mM (15 µL from 300 mM stock in DMSO). To appropriate wells (control), DMSO was added as follows: 5 µL (0.33 %v/v), 10 µL (0.67 %v/v), 15 µL (1 %v/v). After gently swirling to mix, LPS was added to appropriate wells as follows: 0.5 µg/mL (7.5 µL from 0.1 mg/mL stock), and 1.0 µg/mL (15 µL from 0.1 mg/mL stock). Cells were treated with **70**, DMSO and LPS only as controls. Cells were also treated with **70** and LPS, and DMSO and LPS with the above conditions. In these cases, LPS was added last. All cells were left to incubate for 24 h in a humidified atmosphere of 5 % CO_2 at 37 °C. After this time, 100 µL of the supernatant was removed and transferred to a 96-well plate in triplicate and analysed by Griess assay as described above. The remaining media in each well containing **70** was replaced (1 mL), cells were scraped, collected, centrifuged (150 ×g, 5 min), and cells washed with PBS (3 × 1 mL). Each sample was then lysed (100 µL) on ice for 45 min. Cell lysates were clarified by centrifugation (14,000 ×g, 10 min) and stored at –80 °C until analysis by western blot. Protein concentration was determined by Protein 660 nm assay (Pierce).

4.5.5 HL-60 ROS production experimental

Preparation of stock solutions

Norb-bio **70** (14.9 mg, 0.03 mmol) was dissolved in DMSO (99 µL) to give a final concentration of 300 mM. β-lapachone **124** was dissolved in DMSO to give a final concentration of 100 mM (provided by Lavinia Dunsmore). This 100 mM solution of **124** was then diluted to 20 and 5 mM in DMSO.

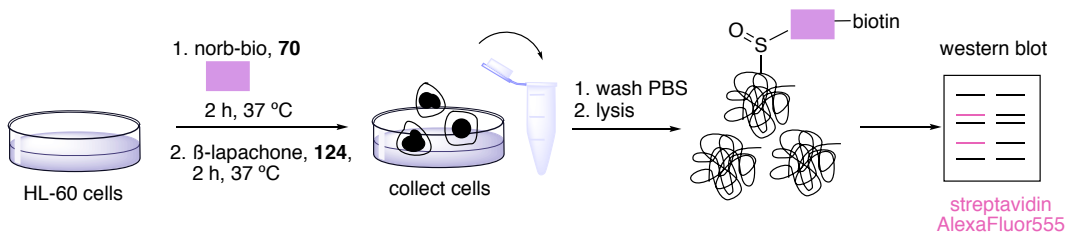
Cell culture of HL-60

HL-60 cells were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C and cultured in RPMI (Gibco) supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Gibco), and 1 % penicillin-streptomycin (Gibco). Cells were grown to a maximum of 1 × 10^6 cells/mL confluency. HL-60 cells used in these experiments were cultured by Lavinia Dunsmore, but all experiments performed by the candidate.

Cell viability assay protocol

HL-60 cells were cultured as described above. In a 24–well plate, cells were seeded to 7×10^5 cells/well in RPMI (0.5 mL, 10 % HI FBS, 1× Pen-Strep). To each well, the appropriate concentration of norb-bio **70** was added as follows: 3 mM (3.35 µL of a 300 mM stock) or 1 mM (1.7 µL of a 300 mM stock) in DMSO. As controls, DMSO was added to each appropriate well as follows: 0.67 % v/v (3.35 µL) and 0.33 % v/v (1.7 µL). To all wells (both norb-bio **70** and DMSO treated), an additional portion of DMSO (0.5 µL) was added to simulate the DMSO contribution from **124**. This gave a total DMSO concentration of either 0.43 and 0.77 %v/v. In a separate set of experiments, the appropriate concentration of β-lapachone **124** was added in duplicate as follows: 5 µM (0.5 µL of a 5 mM stock), 20 µM (0.5 µL of a 20 mM stock), and 100 µM (0.5 µL of a 100 mM stock). To one set of 5-100 µM of β-lapachone **124** treated cells, an extra portion of DMSO (1.7 µL) was added and to the other DMSO (3.35 µL) added to simulate contribution from added **70**. This gave a total DMSO concentration of 0.43 and 0.77 %v/v respectively. Both a cell only and media only control were included and all left to incubate at 37 °C for 24 h. After this incubation period, CellTiter Blue (Promega) (1:5 dilution) was added to all wells and left to incubate for a further 6-7 h at 37 °C or until complete. The cell viability was assessed by fluorescence emission intensity (590 nm) using a SpectraMax i3x plate reader. Cell viability was calculated as a percentage of viable cells compared to the cell-only control. Error bars represent the standard deviation of each sample with three replicates.

HL-60 cell ROS production with β-lapachone 124 and labelling with norb-bio 70



HL-60 cells were cultured as described above. HL-60 cells were washed twice with FBS free RPMI media then diluted to 4×10^6 cells/mL in RPMI (FBS free). To a 6-well plate, 1.5 mL of the HL-60 cells were added to give 6×10^6 cells. To each well, norb-bio **70** (1 or 2 mM, 5 or 10 µL respectively of a

300 mM stock, giving a DMSO concentration of 0.33 or 0.67 %v/v respectively) was added as appropriate and cells left to incubate for 2 h in a humidified atmosphere of 5 % CO_2 at 37 °C. β-lapachone **124** (5, 20 or 100 μM, 1.5 μL from 5, 20, or 100 mM stock respectively) or DMSO control (1.5 μL) was added as appropriate and cells left to incubate for a further 2 h at 37 °C. After this time, the cells were collected, centrifuged (160×g, 5 min), media decanted, and cells washed with PBS (3 × 2 mL) and RiPA buffer with protease inhibitor (Invitrogen) (100 μL per well) added to the cell pellets. Cells were left to incubate on ice for 45 min before collecting and centrifuging (10,000 g, 10 min). Protein concentration was assessed by BCA protein assay (Pierce) with an average of 2.5 mg/mL total protein. Reactions were then prepared directly for electrophoresis without any further purification. Samples were prepared for SDS-PAGE by mixing normalised sample (17 μL) with lithium dodecyl sulfate (LDS 4×) (6.5 μL, Invitrogen), and DTT NuPAGE sample reducing agent (2.5 μL, Invitrogen) and heating to 70 °C for 10 min. Samples were then resolved by electrophoresis and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Overview

The work presented in this thesis details the application of norbornene probes as cysteine sulfenic acid traps. Presented is the first reported instance of using norbornene compounds to trap cysteine sulfenic acids on purified proteins, cell lysates and most significantly living cells. This work establishes norbornene as a new chemical tool to study oxidative stress that differs mechanistically from the commonly used dimedone derivatives. Comparison of these norbornene probes to dimedone equivalents has shown norbornene to have superior selectively and reactivity in each case. Finally, the application of norbornene probes to study cellular mechanism was implemented in several scenarios including proteomics identification of protein hits, endogenous generation of ROS in macrophages, and investigation of the effect of ROS-generating cancer drugs in HL-60 cells. These studies are involved in on-going investigations to gain further biological insight. The further testing and improvement of these norbornene probes will provide a new means with which to study oxidative stress in cells and compare the findings to known outcomes in the hope of bettering our understanding of these disease-causing pathways associated with oxidative stress.

Several opportunities exist for continuation and improvement of the outcomes of this project as highlighted briefly throughout this thesis. This Chapter will discuss the issues faced during the development of the norbornene probes and their testing, as well as provide suggested improvements. The future application of norbornene probes to not only detect sulfenic acid formation, but also as treatment strategies will also be discussed.

5.2 Design of norbornene probes

The next logical direction for this project would be design of second generation norbornene probes to address the restrictions experienced throughout this project. This would include higher aqueous solubility, reduction in size, decreased toxicity, and better compatibility with proteomics analysis (namely MS/MS). These features are somewhat difficult to predict and often contradictory. For example, the bulky biotin group adds solubility, but increases difficulty in MS/MS detection. Similarly, for the alkyne functional group, this seems to increase toxicity and decrease solubility, but is more suited for proteomics analysis. Additionally, other water solubility enhancing groups, such as amides and carboxylic acids can apparently reduce membrane permeability while groups such as ethylene glycol increase difficulty in MS/MS. Other connectivity such as ester groups are best avoided as these may experience hydrolysis. Ultimately, a norbornene probe with an alkyne handle is the most desirable for implementing this probe to study proteome-wide cysteine oxidation in living cells. Unfortunately, the alkyne probe discussed in this thesis was not cell compatible. Additionally, ease

of synthesis is also a contributing factor and thus probes should extend from commercially available norbornene derivatives to allow readily accessible synthesis for other researchers.

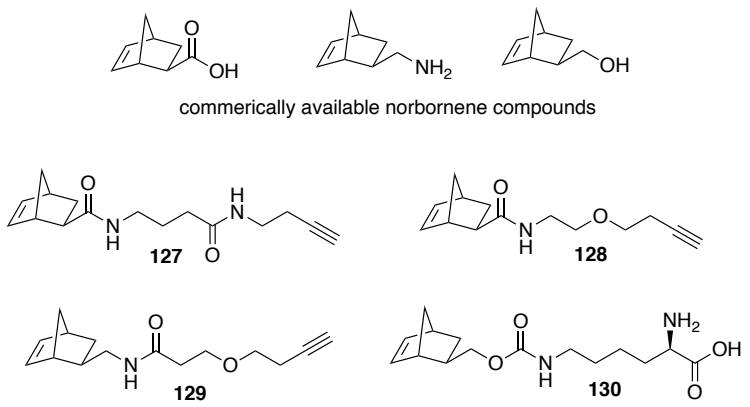


Figure 57. Potential structures of second generation norbornene-alkyne probes and current water-soluble norbornene derivatives.

Figure 57 highlights a few possible examples of the types of probes that could be synthesised to aid in water solubility. All probes are synthesised from commercially available norbornene compounds such as 5-norbornenecarboxylic acid or 5-norbornene-2-methylamine. Compound **127** could be assembled from commercially available compounds gamma amino butyric acid and 1-amino-pentyne through simple amide coupling chemistry. The alkyne handle of **128** could be synthesised through coupling of ethanolamine to 5-norbornenecarboxylic acid, followed by nucleophilic substitution of 4-bromo-1-butyne as an example, with **129** produced in a similar manner using a different norbornene starting material. Whether or not these additional functional groups increase solubility enough while decreasing toxicity would need to be tested on a case-by-case basis. Other norbornene containing amino acids such as **130** have been reported with increased solubility and an alkyne handle could be added to the amine or carboxylic acid for detection.[151-152] Alternatively, norbornene probes with biotin and a cleavable linker (eg. UV-cleavable or acid cleavable) could be designed to allow removal of biotin before proteomics analysis to avoid detection issues. However, incorporation of a cleavable linker might introduce opportunities for loss of the biotin tag during analysis and the bulky group may hinder reactivity.

5.3 The study of oxidative stress

Design of a new norbornene probe with increased water solubility, decreased toxicity, and an alkyne handle would facilitate improved proteomics analysis. Currently, the norb-bio probe **70** experiences difficulties in MS/MS, but could be corrected for by careful design of the experiment as outlined in Chapter four, including quantification and comparison to a control sample to determine enriched

proteins. Design of the norb-yne probe **69** was to take into account MS compatibility, however this compound was incredibly toxic to cells and had low solubility in the aqueous media at concentrations needed for labelling, making it unsuitable for live cell study of oxidative stress. Nonetheless, the norb-bio probe exhibited in this study displayed the capacity of these class of compounds as effective and reliable sulfenic acid probes for live cell study, and examination of oxidative stress induced events. Improving the probes suitability to these conditions would further enhance the detection capabilities of norbornene probes, allowing better detection of cysteine sulfenic acids, and investigation of redox sensitive pathways. This would likely lead to identification of even more previously unknown sulfenic acid containing proteins, and provide confirmation of hits already identified.

Application of the norbornene probes (and future improvements) discussed could be extended to other cell models to study oxidative stress under different conditions. For example, the use of the immune cell macrophages was discussed in Chapter four and could be extended upon once the water-solubility issue of the probe is addressed. Other cell lines could be included in the study to determine if the same types of proteins are oxidised across these cells and whether their redox function is conserved. Comparison of various norbornene probes (with different functional groups) for their protein hits could reveal differing reactivities for the types of cysteine residues targeted by the probes. For example, whether the probe reacts with surface residues, buried, or whether certain neighbouring groups enhance reactivity. These types of selectivity factors have been observed in previous studies concerning probe design for cysteine sulfenic acid detection.[145] These improved norbornene probes could be applied to numerous studies to enhance our understanding of oxidative stress further.

5.4 Norbornene probes as drug strategies

While most cysteine sulfenic acid probes, and indeed our own, react with a generally wide range of sulfenic acids in the proteome, it is possible to target specific types of cysteine residues. This was highlighted in a recent study by Carroll and co-workers around the differing reactivities of probes with similar mechanistic approaches, but structurally varied.[145] This idea coupled with improved norbornene probe design could lead to a class of norbornene probes with selective functions for certain types of cysteine sulfenic acids. This selectivity could arise from the probes reactivity itself (low or high reactivity) or addition of groups which act to direct binding.[122, 131] This opens up the possibility of using identified protein sulfenic acids as targeted drug strategies, where binding would only occur under oxidative stress conditions thus targeting diseased cells. There exist numerous opportunities to fine-tune the reactivity of these norbornene probes to not only study and understand what happens to key proteins and pathways during oxidative stress, but also devise ways to use the selectivity of these probes for sulfenic acids to treat or prevent the diseases that arise from oxidative

stress. This type of study was initiated with the experiments on HL-60 cells and the ROS-generating drug β-lapachone **124** as highlighted in Chapter four, whereby using the norb-bio probe **70** to detect which cysteine residues become oxidised during treatment, could provide information to improve the drug's efficacy or new proteins to target.

5.5 Concluding remarks

The research presented in this thesis has provided a new chemical probe for cysteine sulfenic acid detection that has good selectivity and reactivity with the sulfenome. The norbornene probes can be used on small molecules, proteins, and even live cells making them applicable to most applications for study of oxidative stress. While further exploration of the ideas discussed in this Chapter would provide interesting insight into the study of oxidative stress, the work presented in this thesis has provided the core research needed to allow the widespread use of norbornene probes to study cysteine oxidation in living cells.

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7. APPENDICES

The appendices herein contain additional supporting material which has been referenced throughout the body of the text to accompany the results presented in Chapters two-four. For additional supporting information, please refer to the publisher's website for the corresponding publication associated with each Chapter as described.

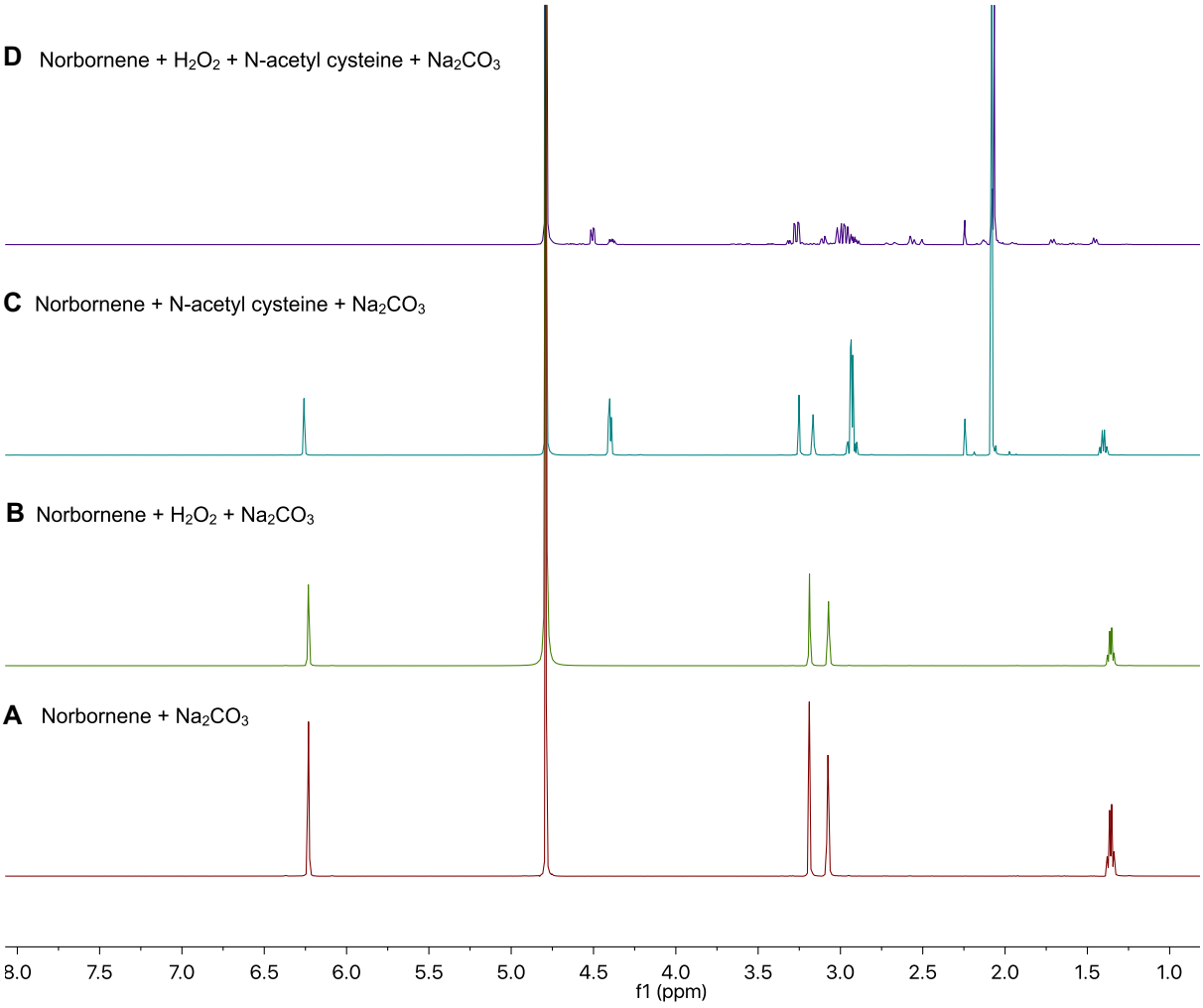
Appendix A: NMR and LC-MS data for Chapter two

The following are the NMR and LC-MS data for the reactions of norbornenes **99** and **107**, dimedone **19**, and cyclooctynes **96** and **97** as presented in Chapter two. The ^{1}H and ^{13}C NMR data for the synthesised probes **69** and **70** presented in Chapter two are also provided as supporting evidence.

2.9.1 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation (not buffered)

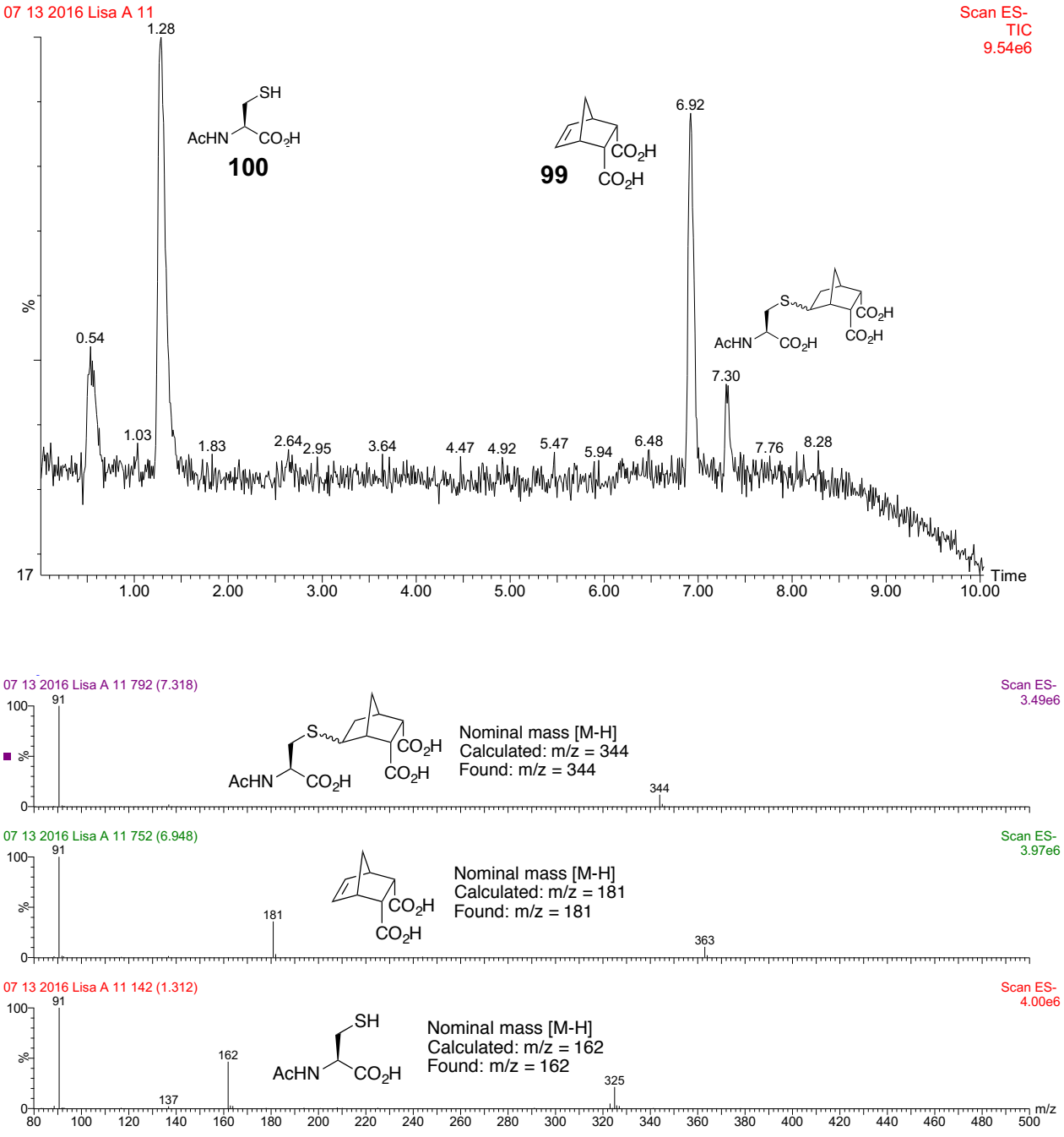
NMR studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation (not buffered)

Complete consumption of the alkene peaks can be observed in D as compared to C where no thiol-ene reaction is observed.

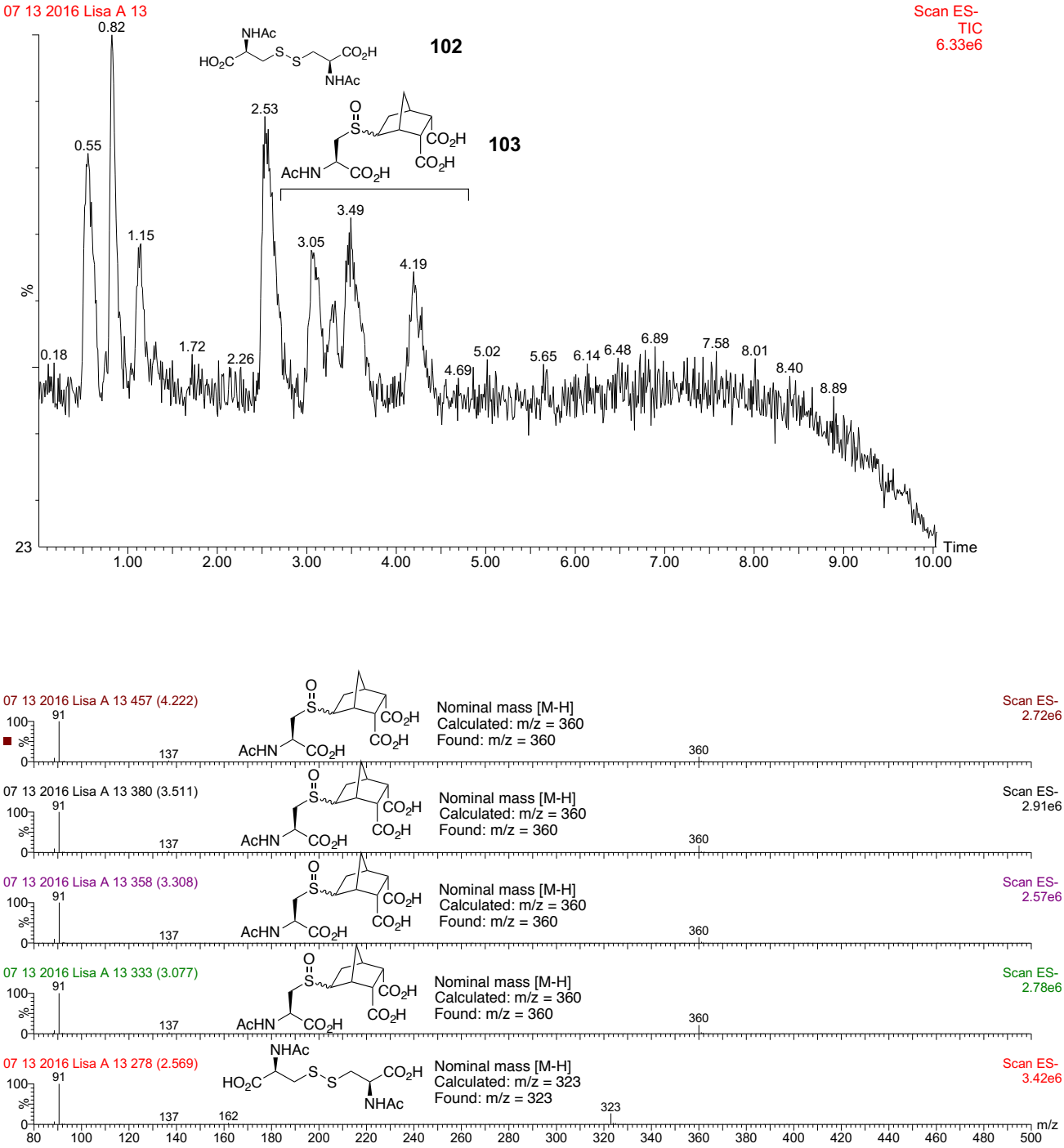


LC-MS studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation (not buffered)

LC-MS data for control experiment in which **99** and *N*-acetylcysteine **100** were reacted (in the absence of hydrogen peroxide). Some thiol-ene product is detected.



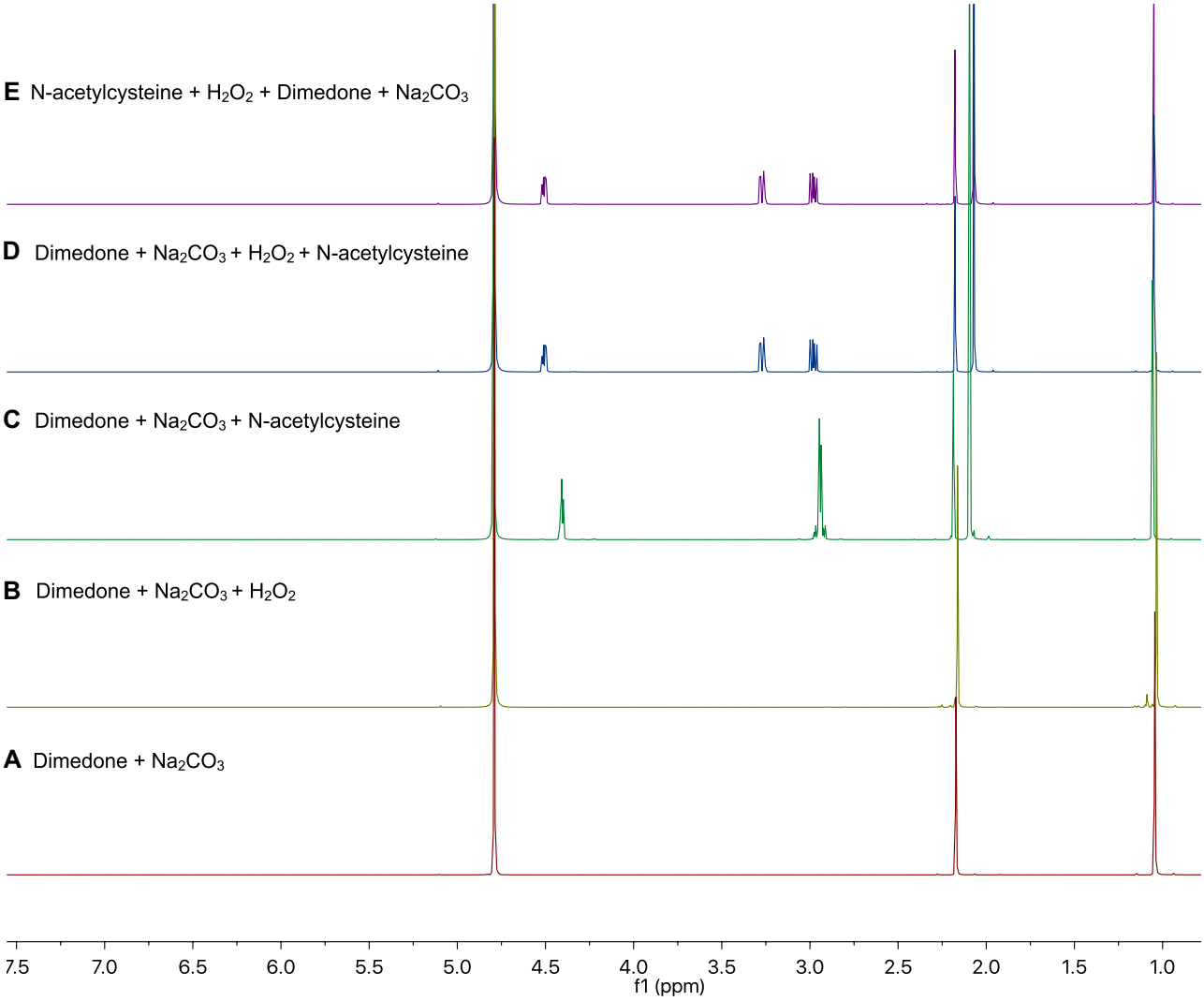
LC-MS study indicating *cis*-5-norbornene-endo-2,3-dicarboxylic **99** can trap the sulfenic acid of *N*-acetylcysteine **100** at pH 4.3. No thiol-ene product was detected. The separate peaks corresponding to the four diastereomers can be observed in the TIC.



2.9.2 Dimedone (19) as a probe for cysteine oxidation (not buffered)

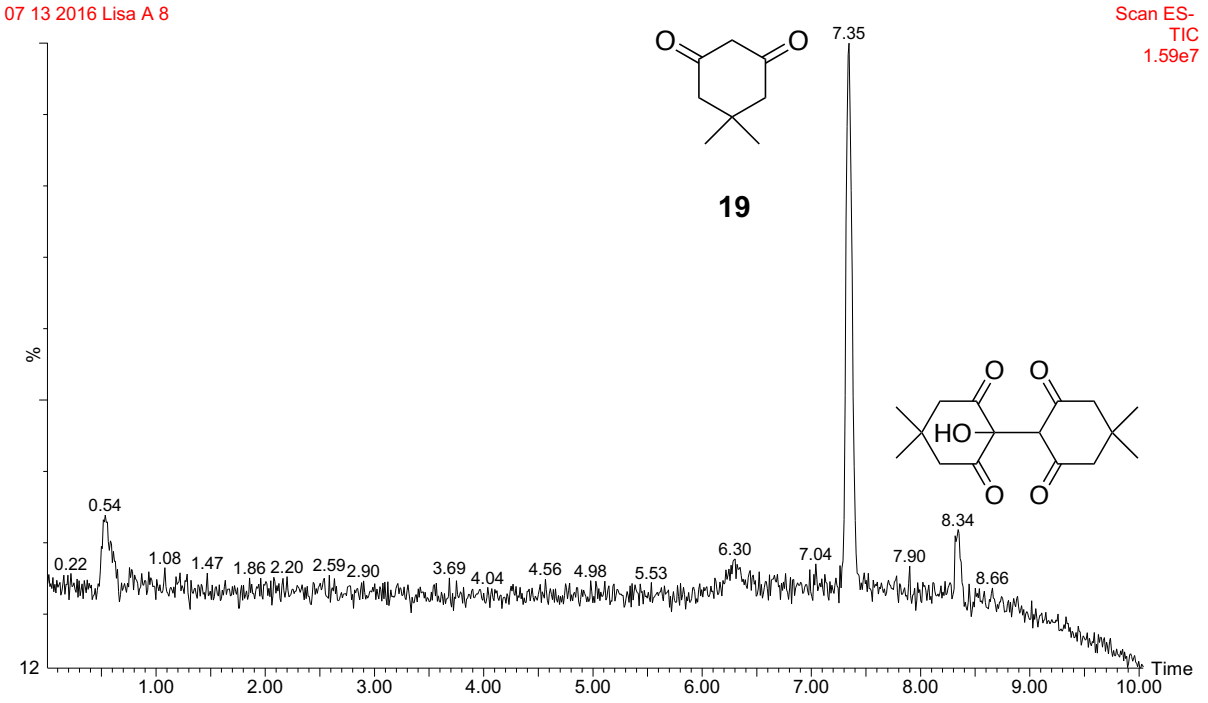
NMR studies using dimedone 19 as a probe for cysteine oxidation (not buffered)

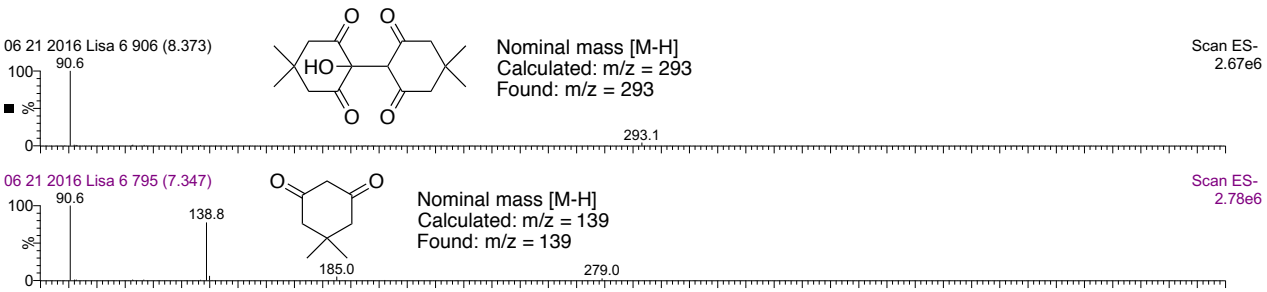
No reaction can be observed under any of the conditions A-E.



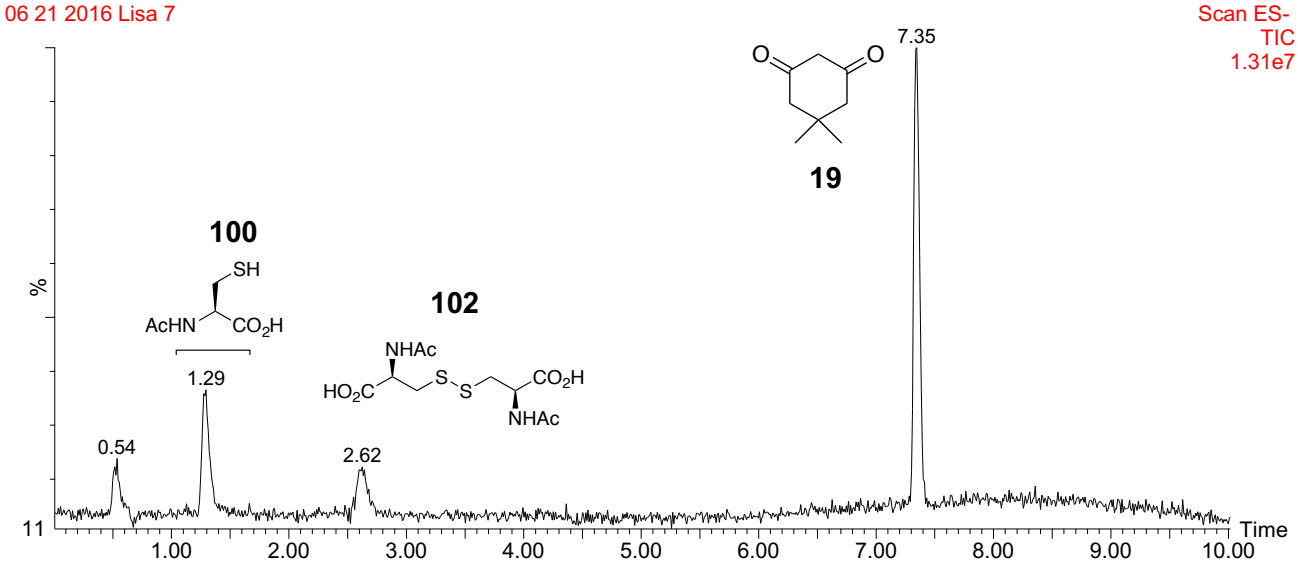
LC-MS studies using dimedone 19 as a probe for cysteine oxidation (not buffered)

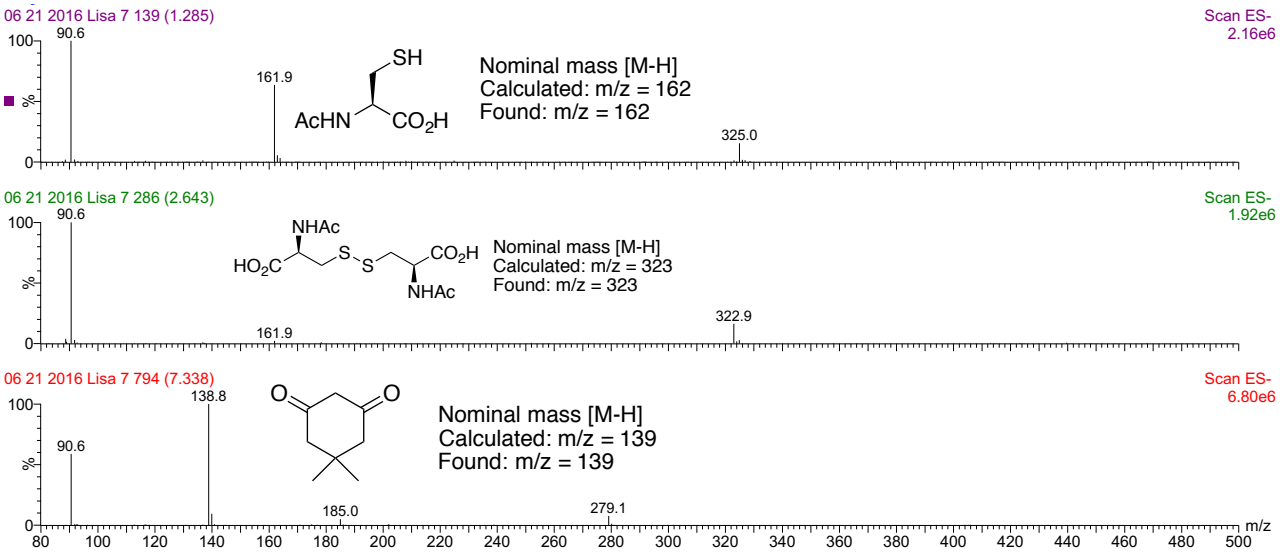
Dimedone reacts slowly with hydrogen peroxide (LC-MS). A tentative structure is shown.



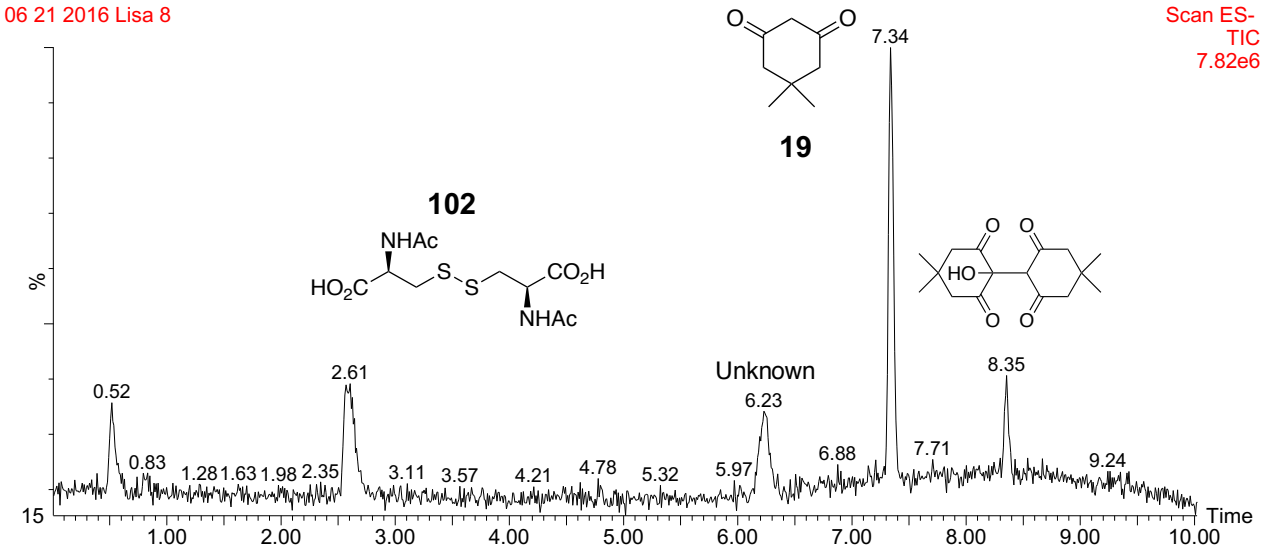


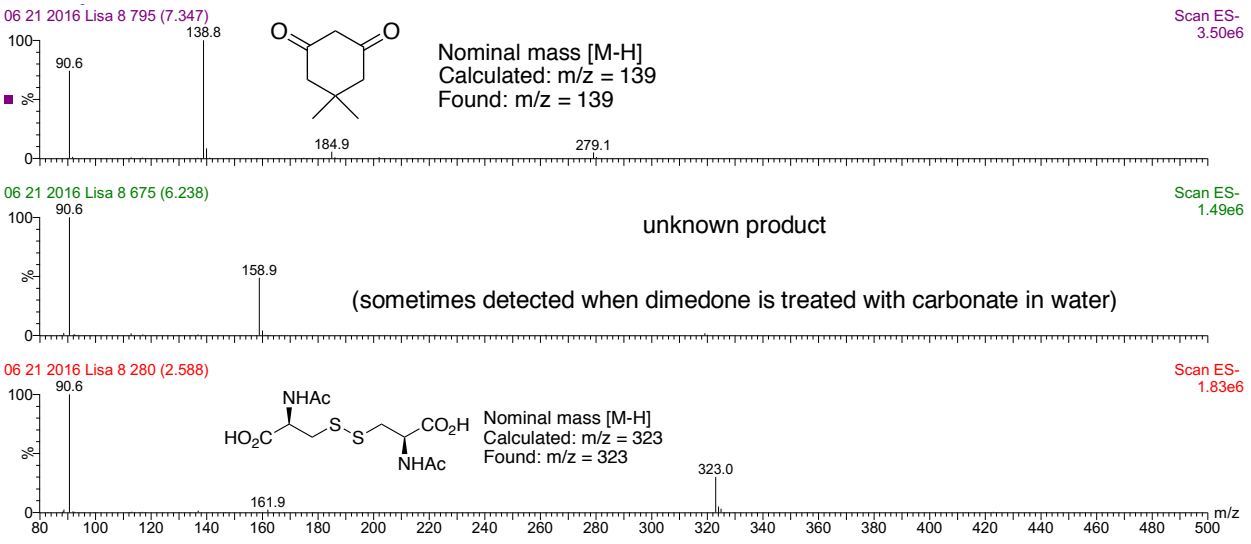
Dimedone **19** does not react with *N*-acetylcysteine **100**. Some oxidation of **100** is seen.





Dimedone **19** does not trap the *N*-acetylcysteine sulfenic acid **101**. Unknown degradation products are also observed.

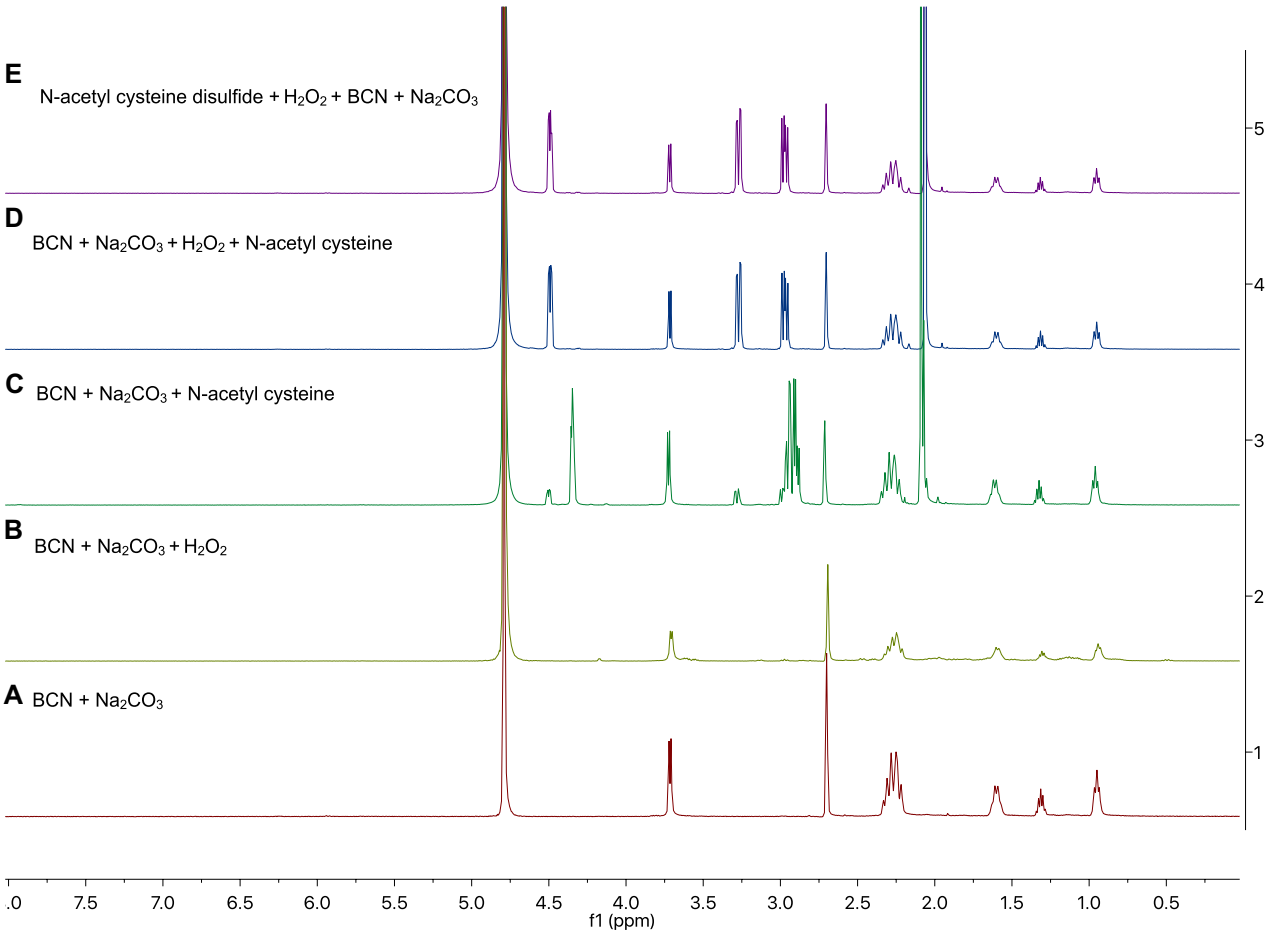




2.9.3 Cyclooctyne BCN (96) as a probe for cysteine oxidation (not buffered)

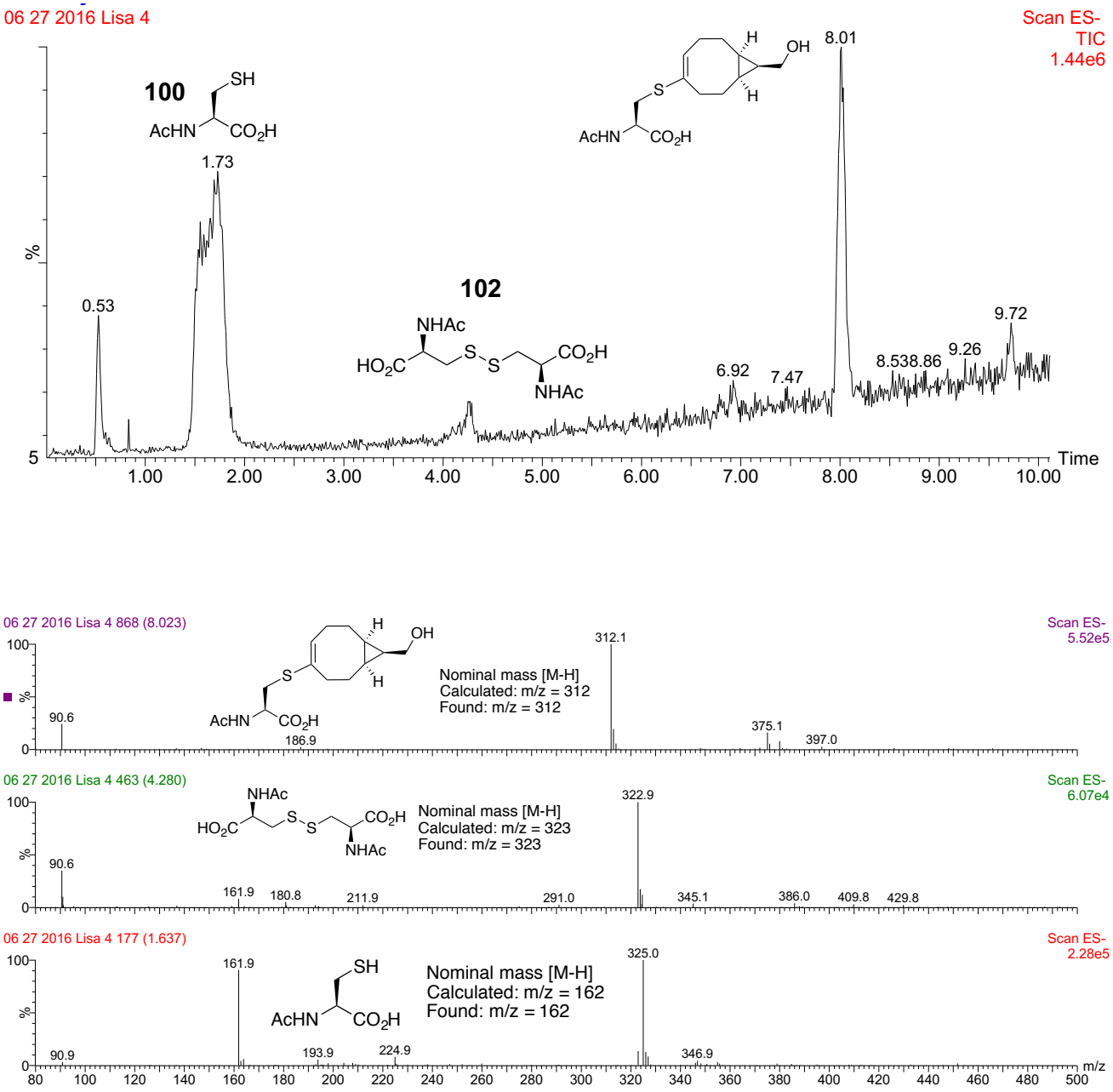
NMR studies using cyclooctyne BCN 96 as a probe for cysteine oxidation (not buffered)

No reactions are observed across conditions A-E.

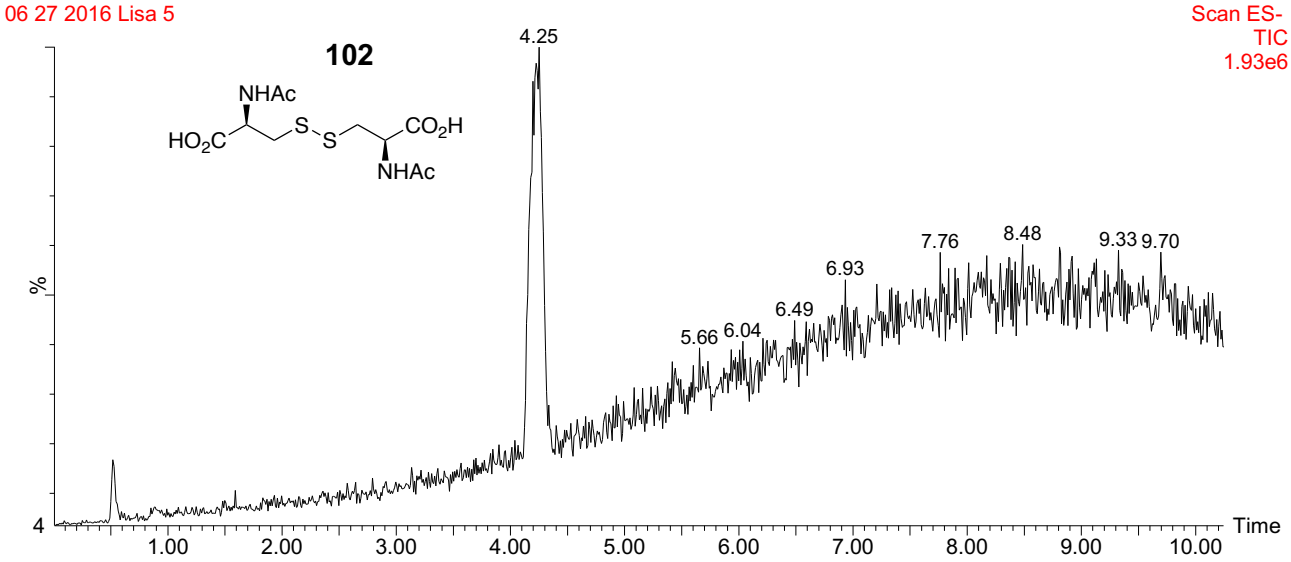


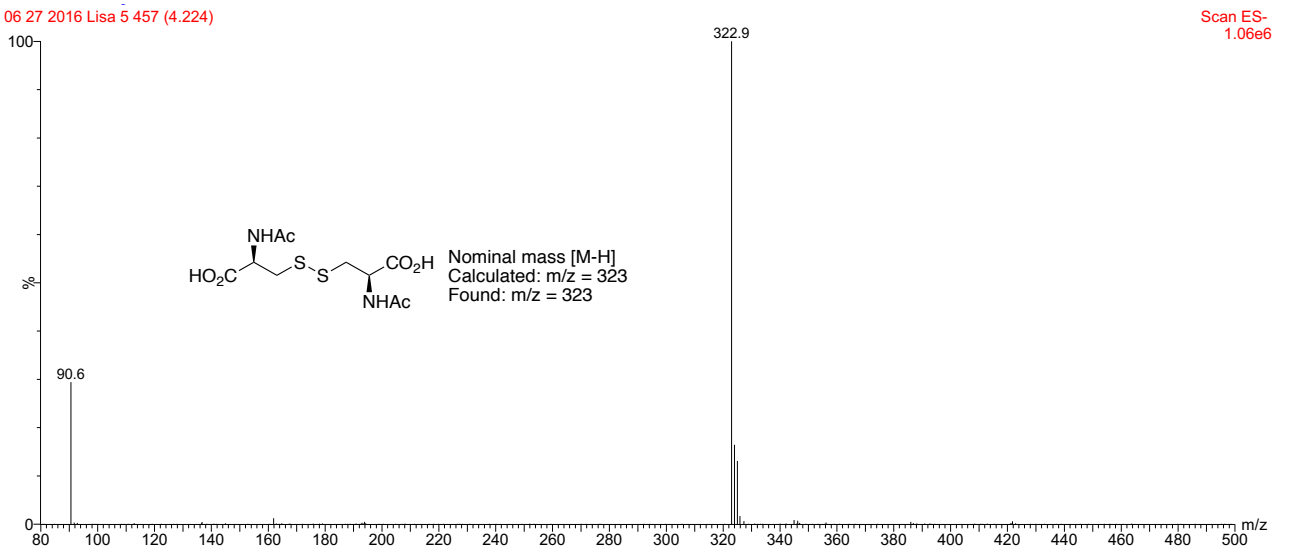
LC-MS studies using cyclooctyne BCN (96) as a probe for cysteine oxidation (not buffered)

The direct reaction of cyclooctyne BCN **96** and *N*-acetylcysteine **100** is observable by LC-MS.



Note: BCN **96** itself was not detectable by ESI under the conditions of this experiment.

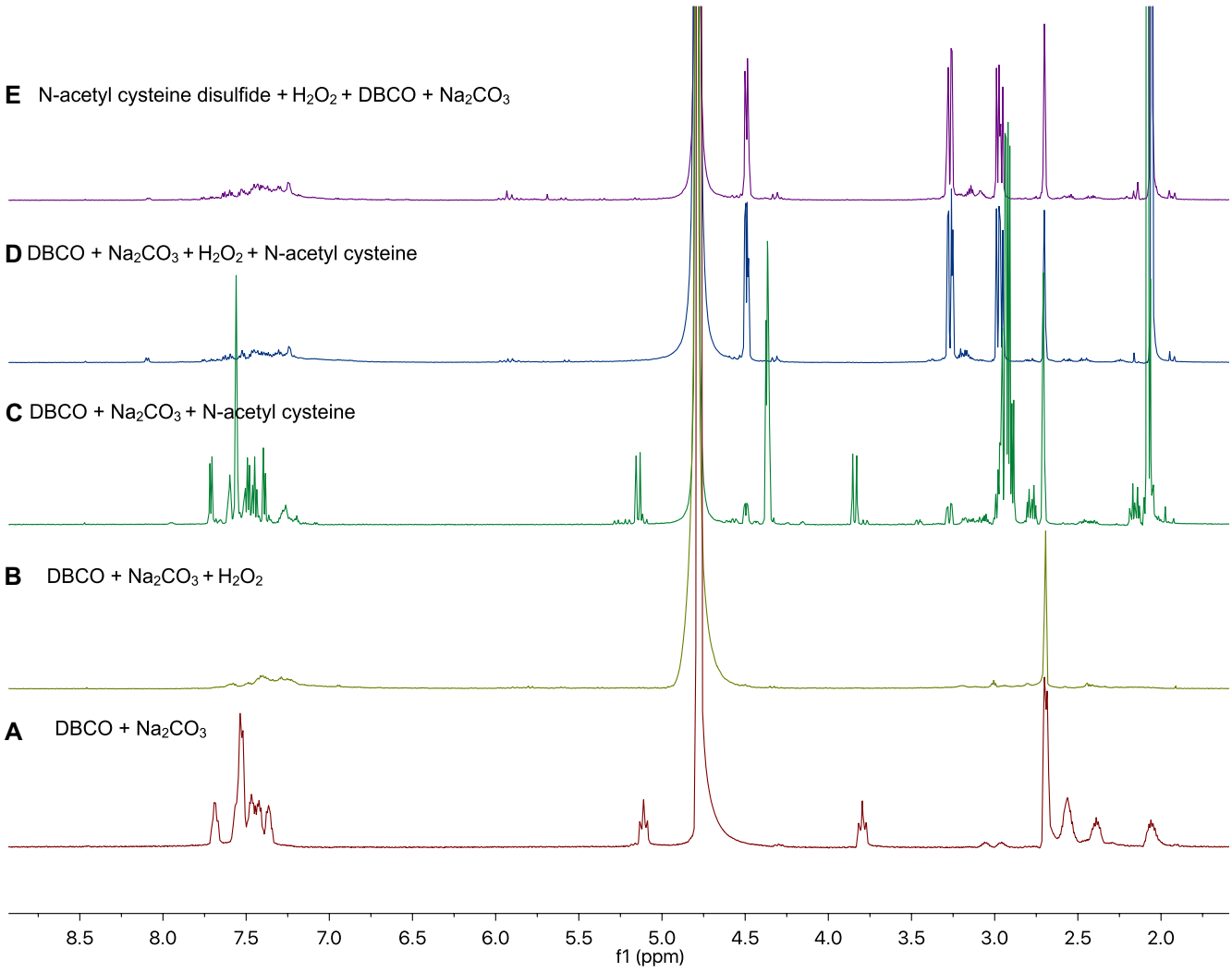




2.9.4 Cyclooctyne DBCO (97) as a probe for cysteine oxidation (not buffered)

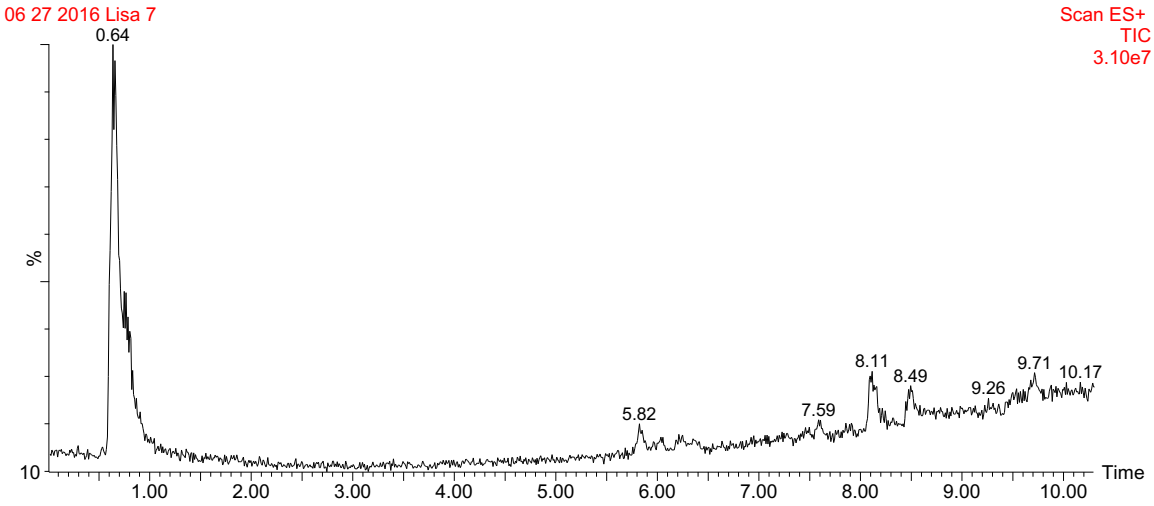
NMR studies using cyclooctyne DBCO 97 as a probe for cysteine oxidation (not buffered)

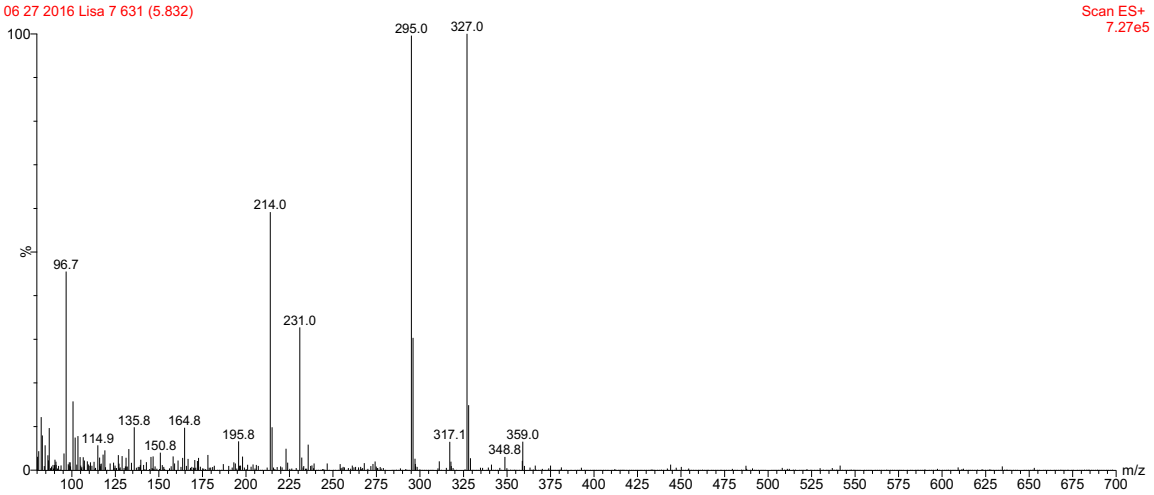
No reaction was observed under any of the conditions A-E.



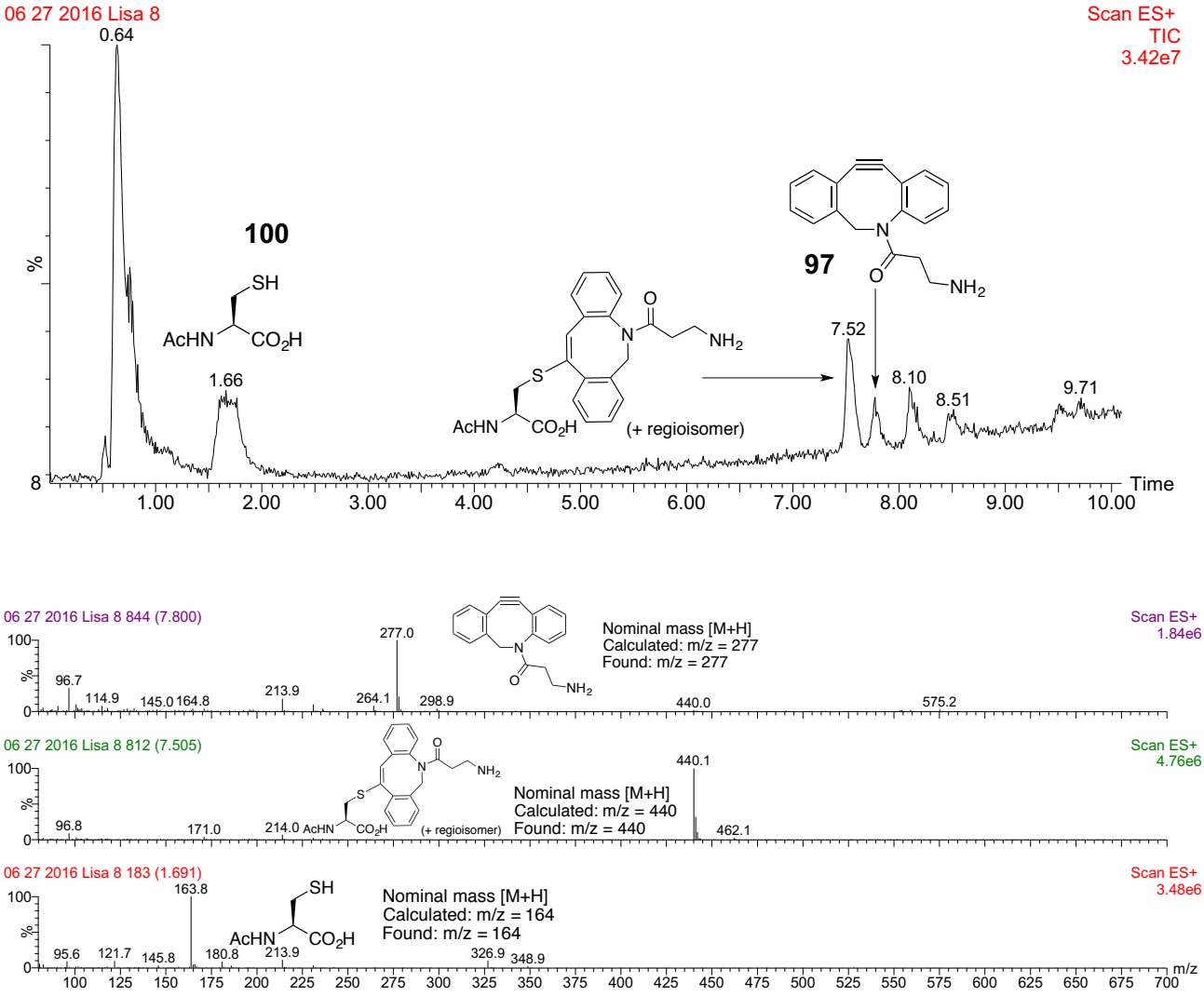
LC-MS studies using cyclooctyne DBCO 97 as a probe for cysteine oxidation (not buffered)

The cyclooctyne DBCO **97** reacts with hydrogen peroxide. Unknown degradation products are observed.





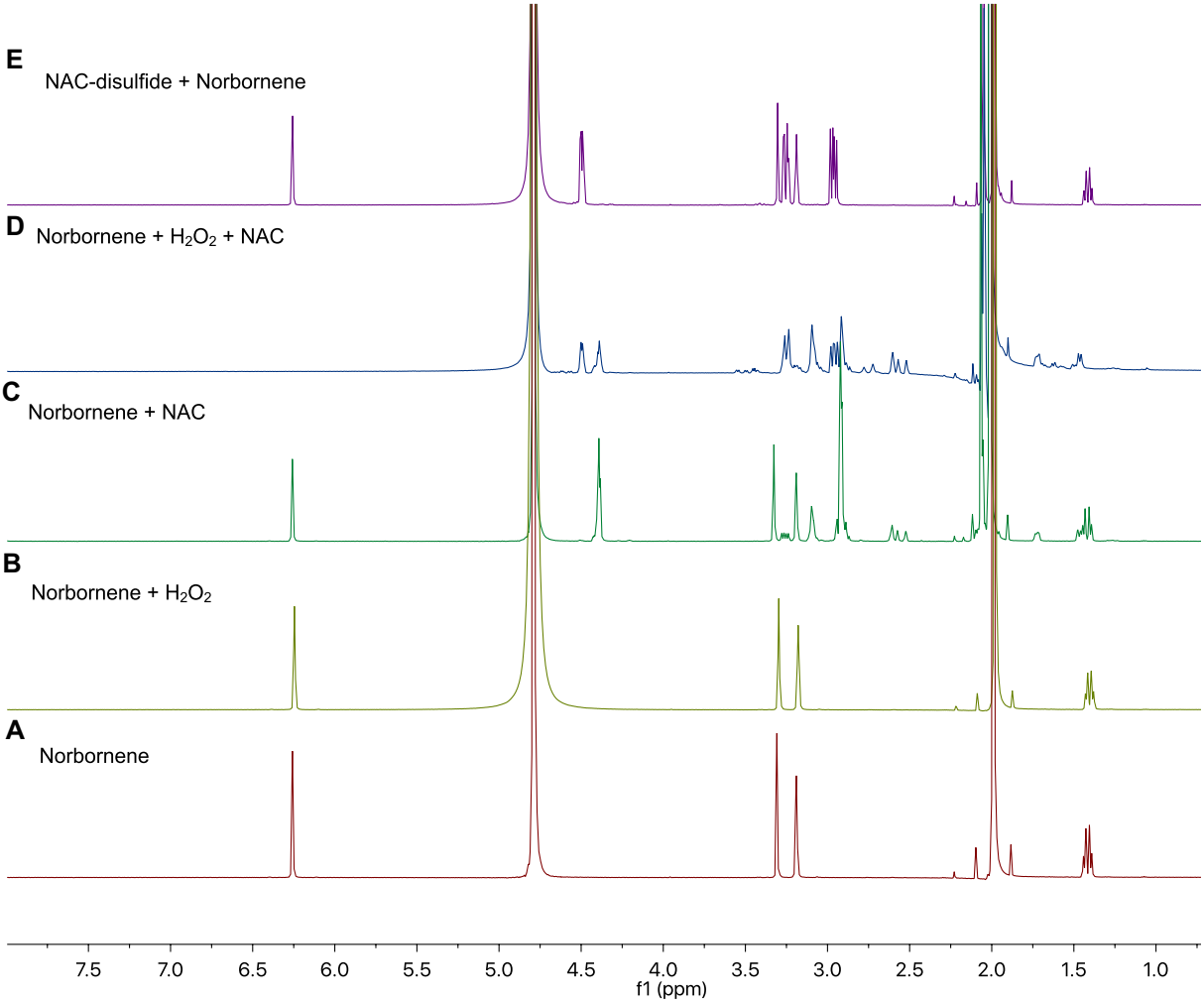
The cyclooctyne DBCO **97** reacts directly with cysteine **100**.



2.9.5 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

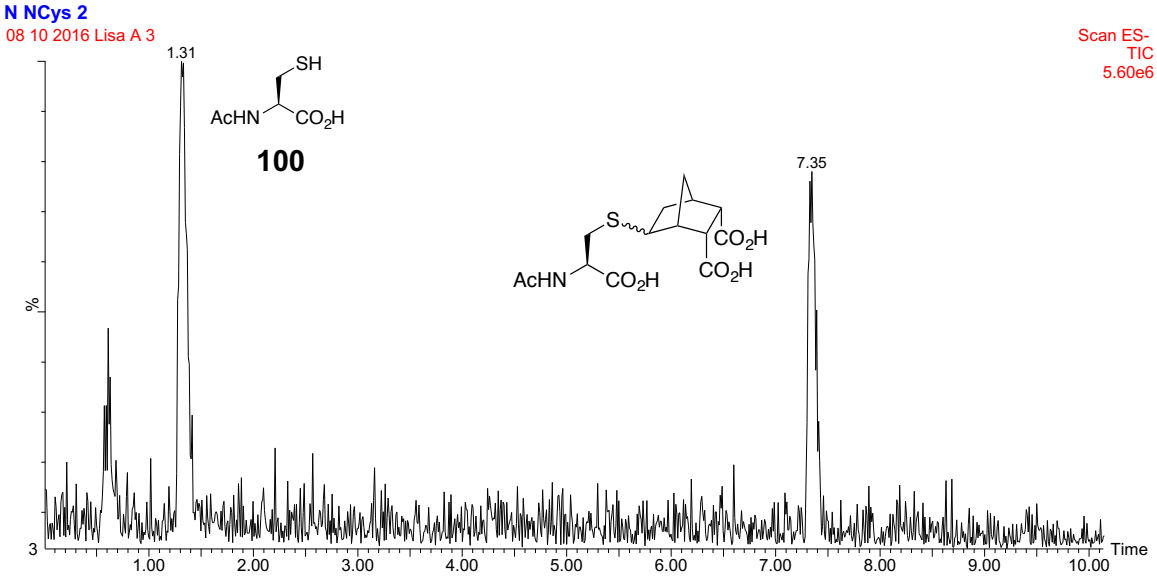
NMR studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid 99 as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

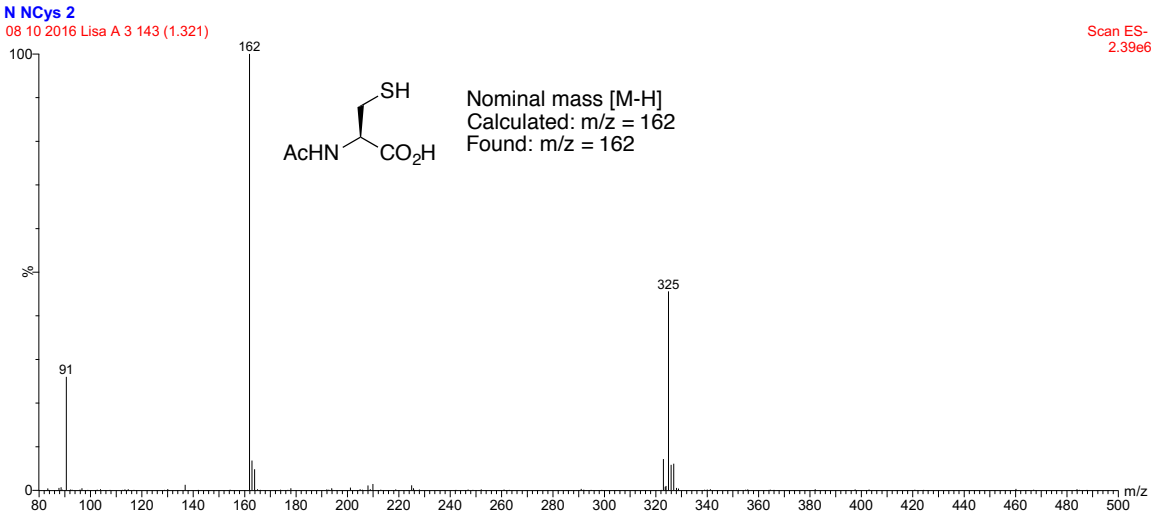
Complete consumption of the alkene peak is observed in reaction D. No apparent reactivity is observed in A-C or E.

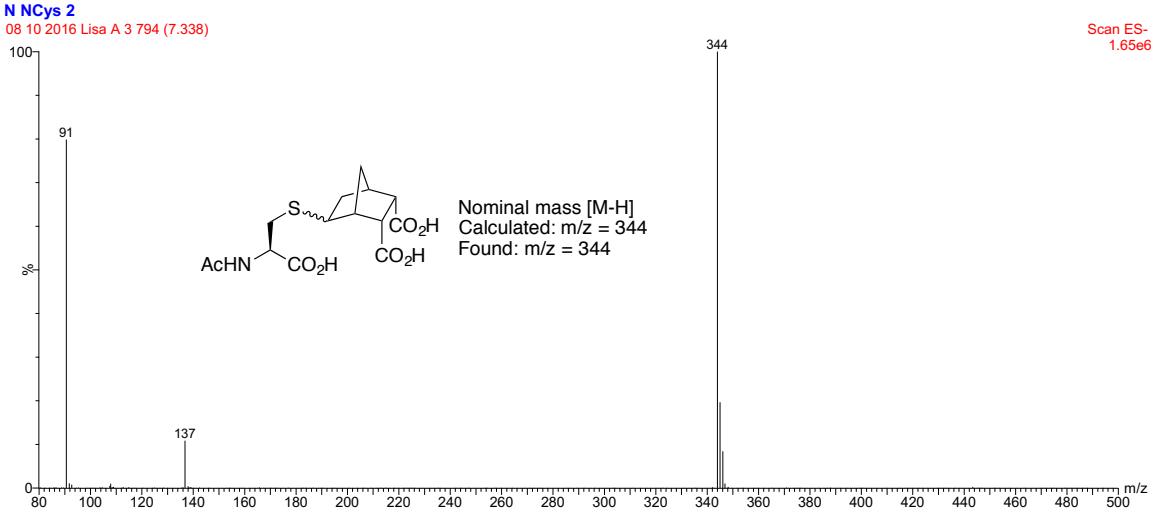


LC-MS studies using cis-5-norbornene-endo-2,3-dicarboxylic acid 99 as a probe for cysteine oxidation at pH 5.0 (NaOAc buffer, 200 mM)

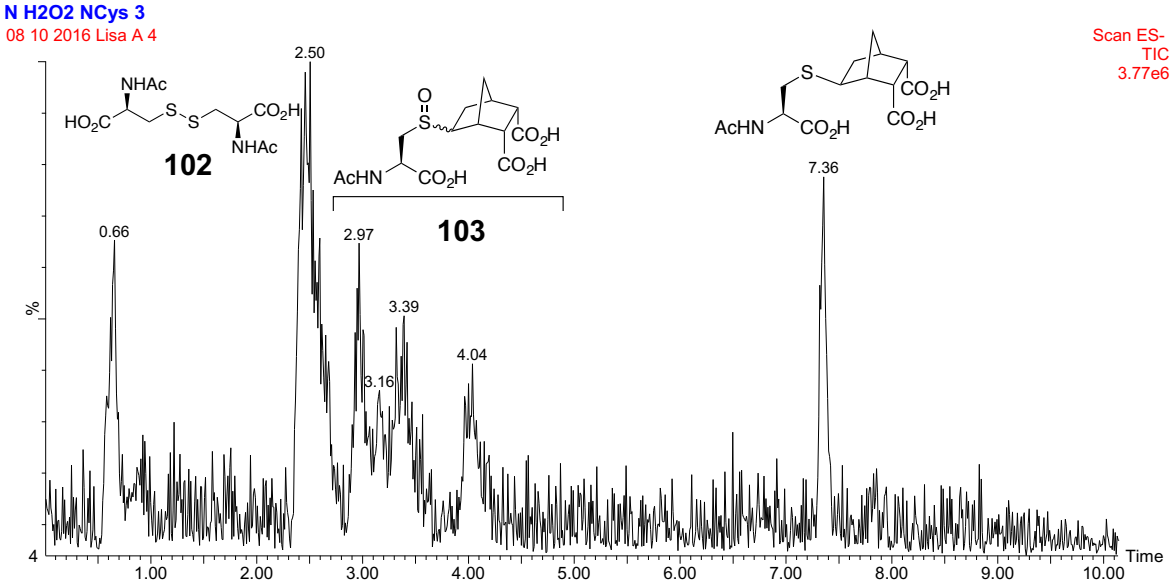
The thiol-ene product is observed when no hydrogen peroxide is added.

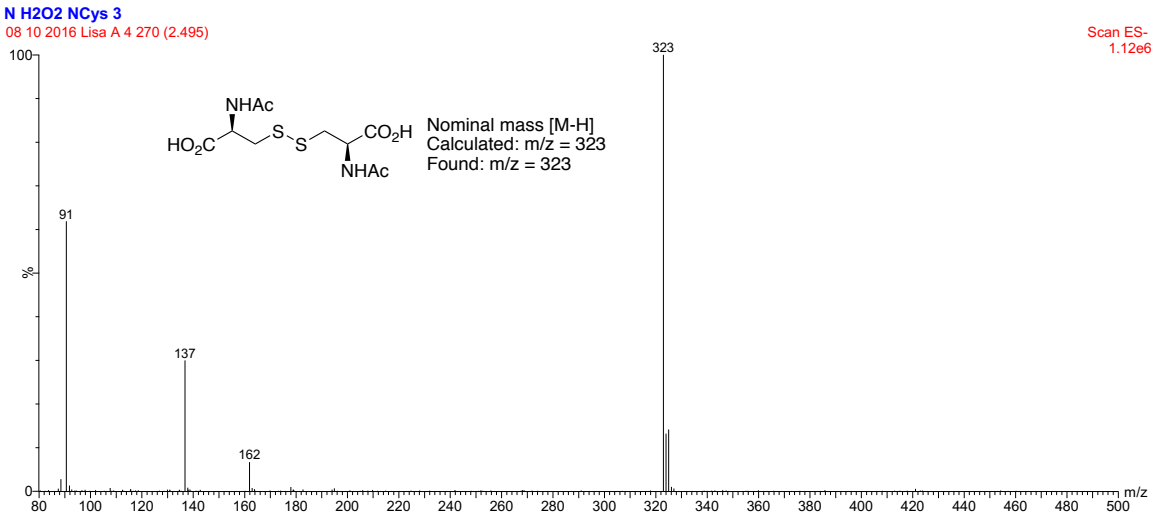


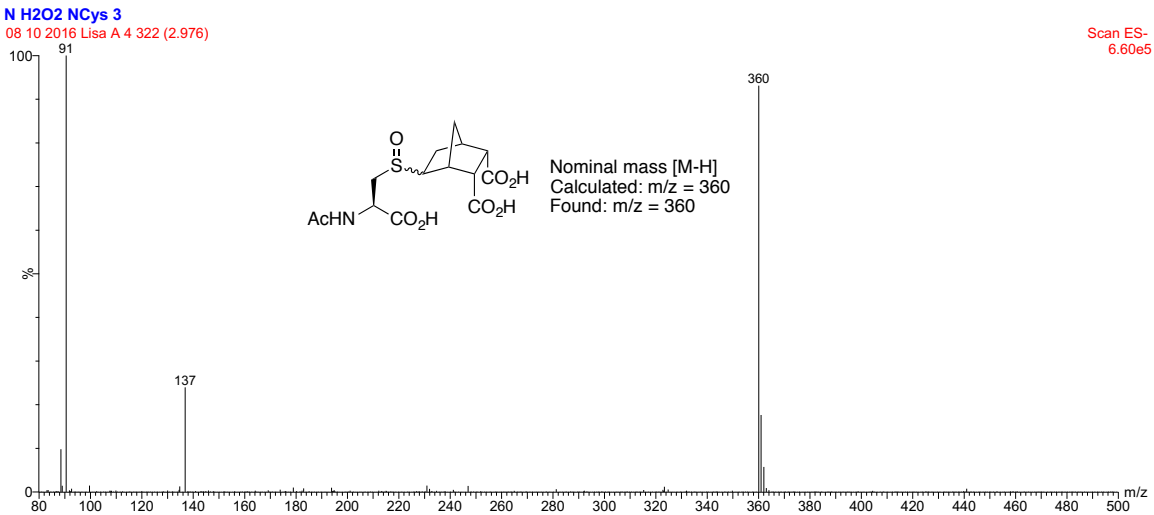




LC-MS study indicating *cis*-5-norbornene-endo-2,3-dicarboxylic acid **99** can trap the sulfenic acid of *N*-acetylcysteine at pH 5.0. The four diastereomers can be observed.







N H2O2 NCys 3

08 10 2016 Lisa A 4 437 (4.039)

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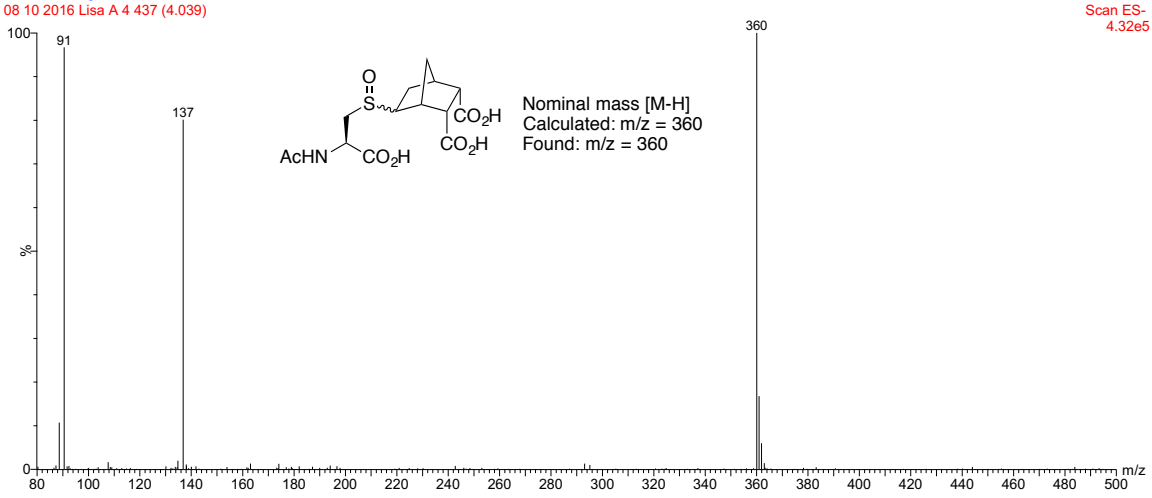
4.32e5



Nominal mass [M-H]
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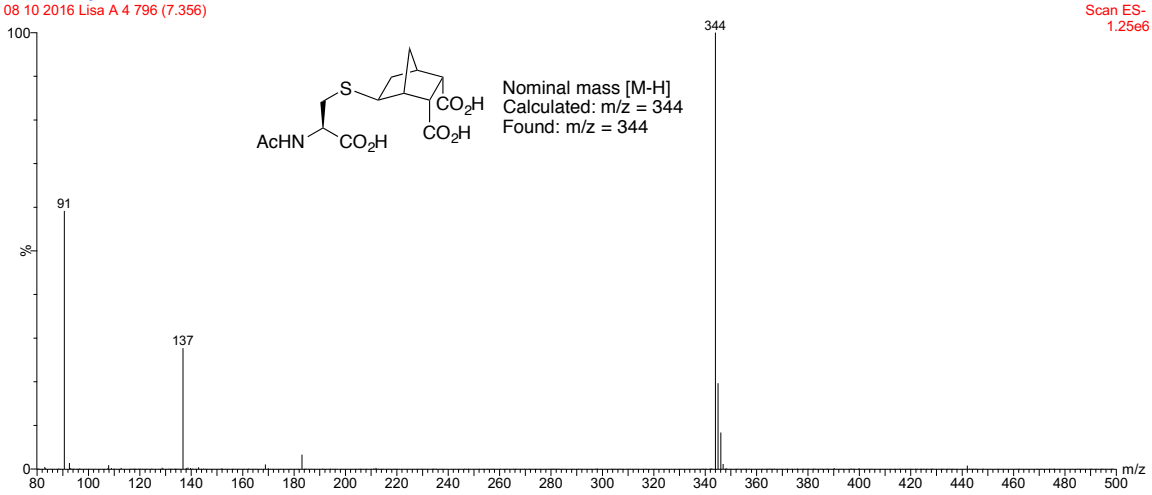
N H2O2 NCys 3

08 10 2016 Lisa A 4 796 (7.356)

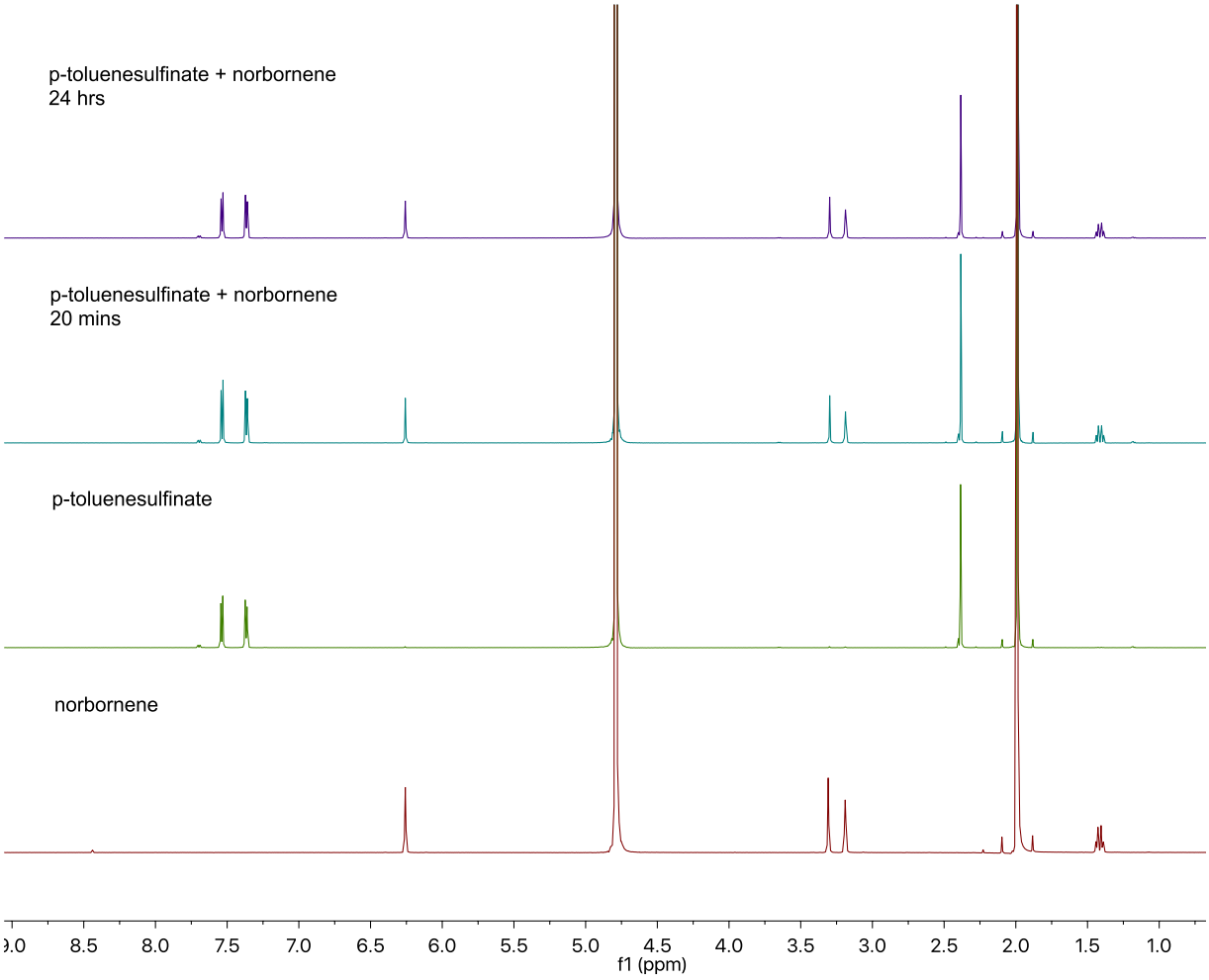
Scan ES-
1.25e6

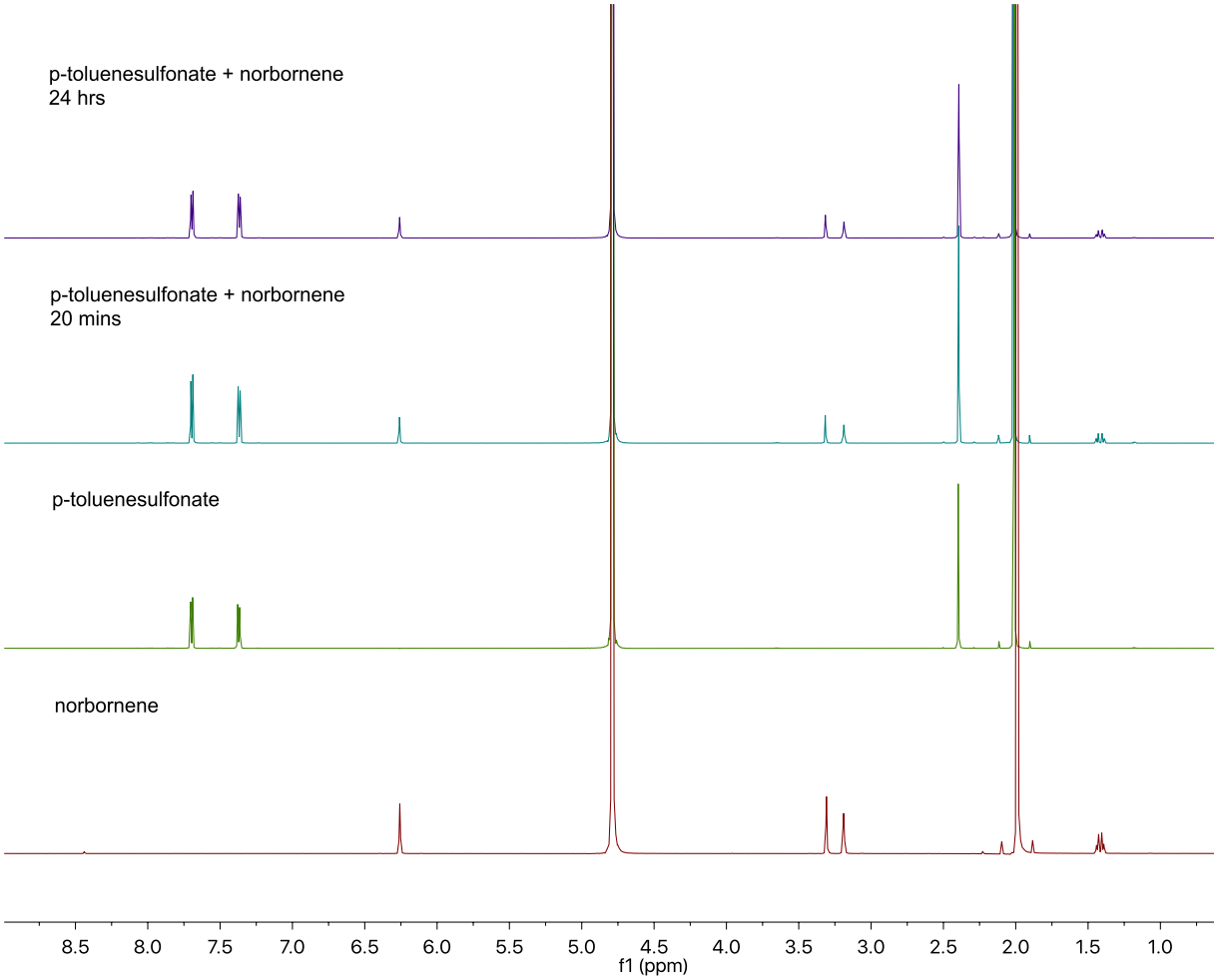


Nominal mass [M-H]
Calculated: m/z = 344
Found: m/z = 344

2.9.6 NMR studies showing *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) does not react with sulfinates or sulfonates at pD 5.0 (NaOAc buffer, 200 mM)

No reaction is observed.

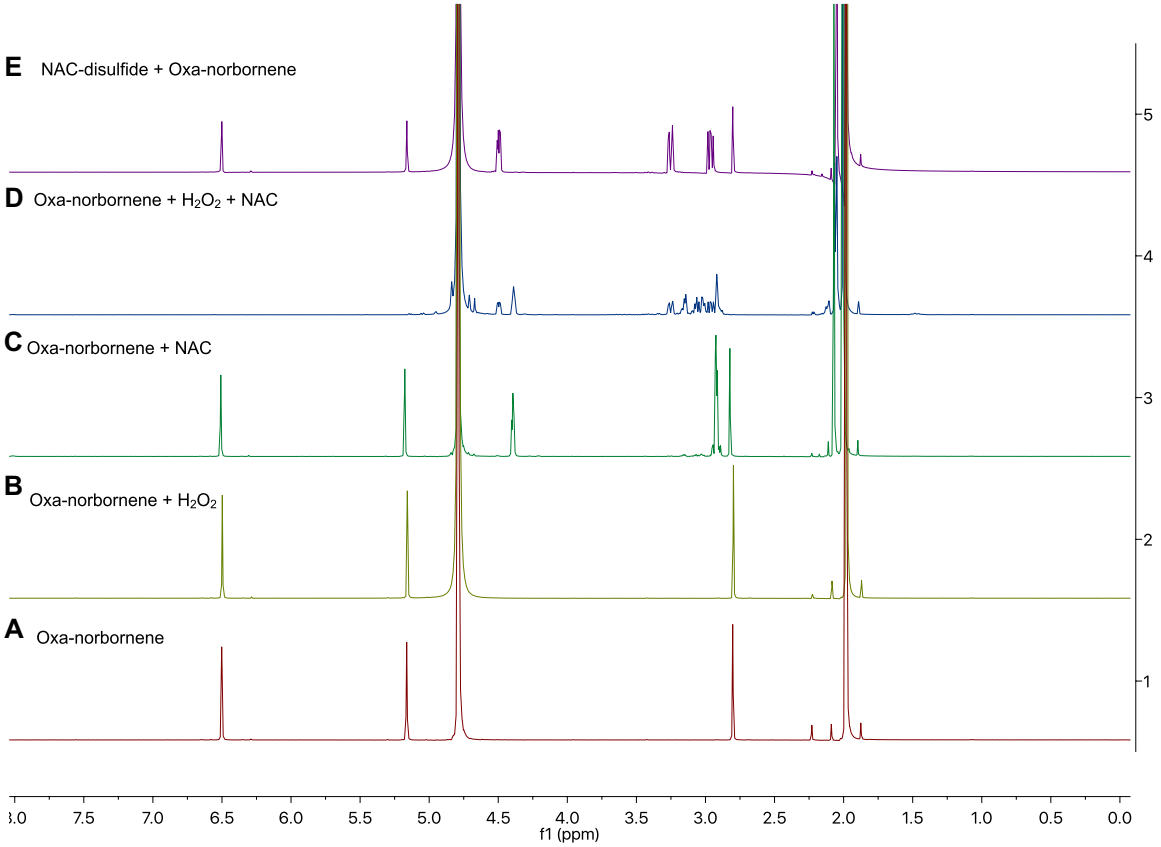



p-toluenesulfonate + norbornene
24 hrs
p-toluenesulfonate + norbornene
20 mins
p-toluenesulfonate
norbornene
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)

2.9.7 oxa-norbornene dicarboxylic acid (107) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

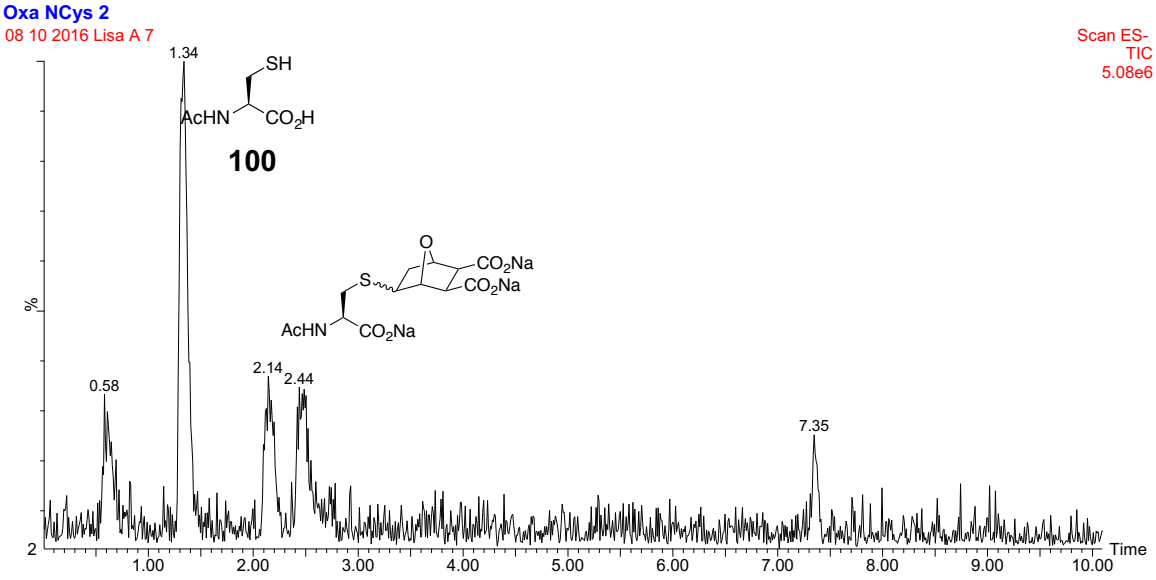
NMR studies using oxa-norbornene dicarboxylic acid 107 as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

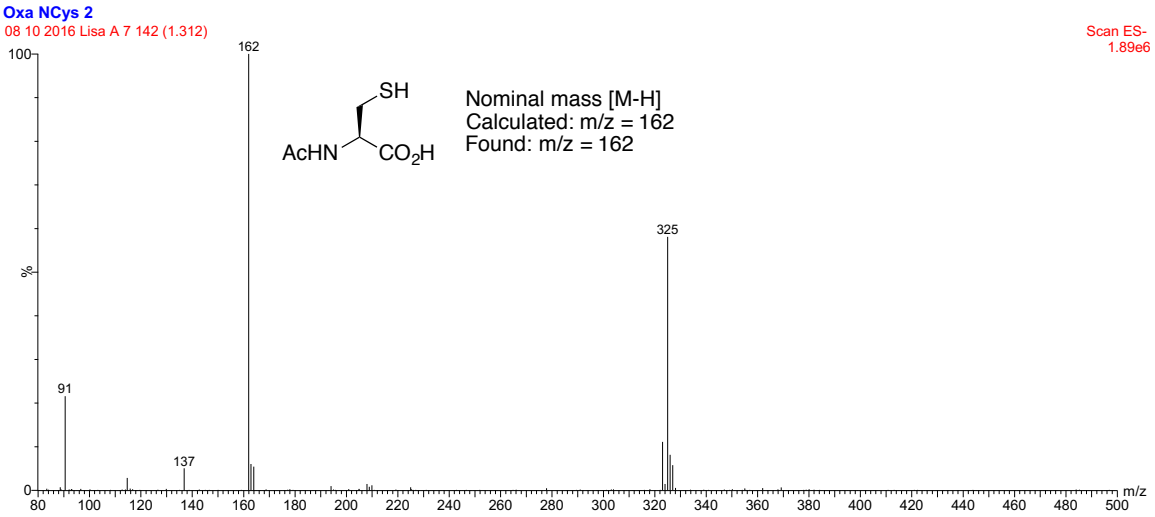
Complete consumption of the alkene peak is observed in D upon oxidation of the cysteine. No reaction is observed under conditions A-C or E.

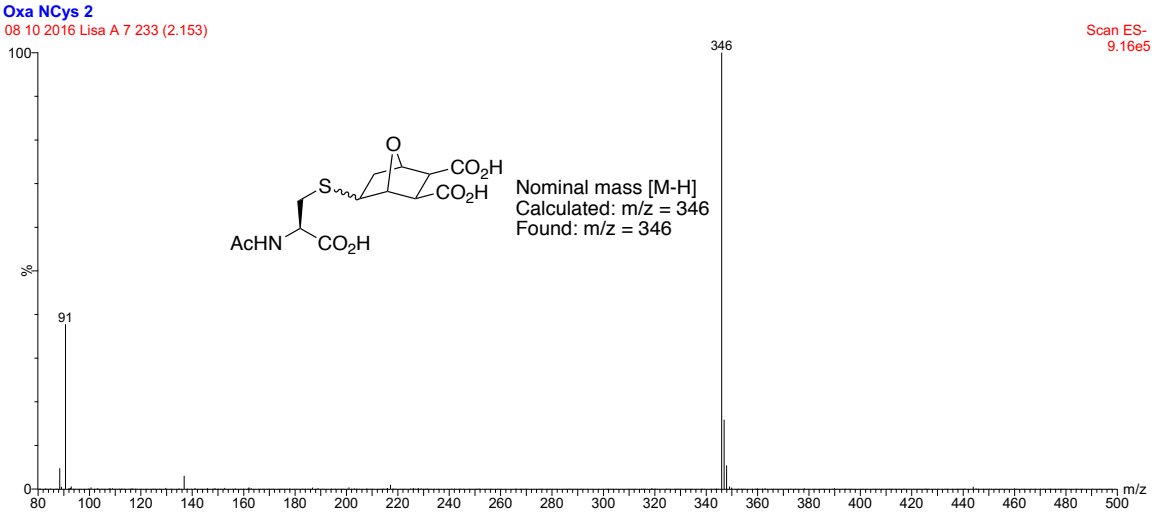


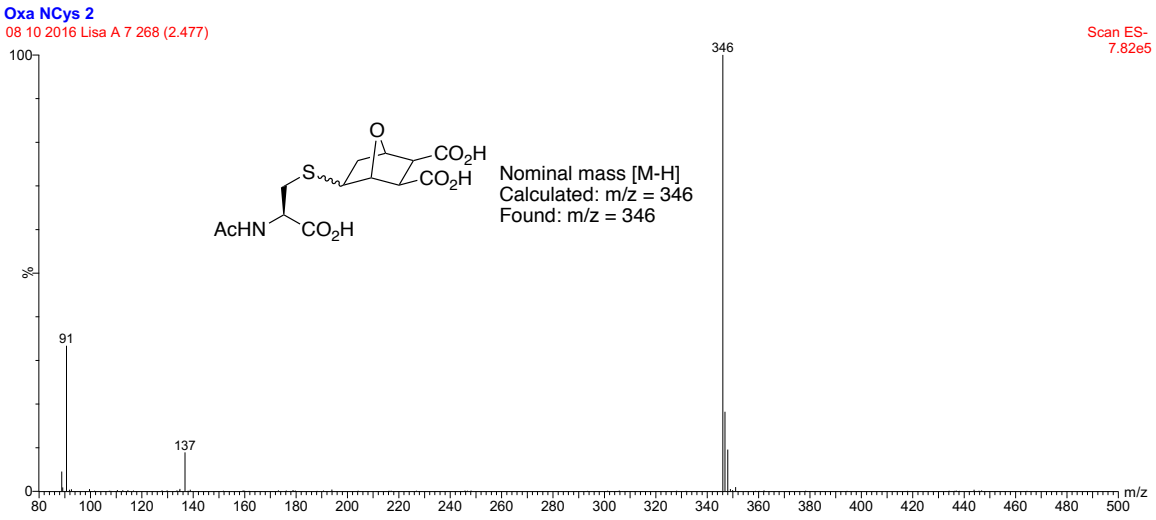
LC-MS studies using oxa-norbornene-dicarboxylic acid 107 as a probe for cysteine oxidation at pH 5.0 (NaOAc buffer, 200 mM)

Some thiol-ene product is observed with two diastereomers.

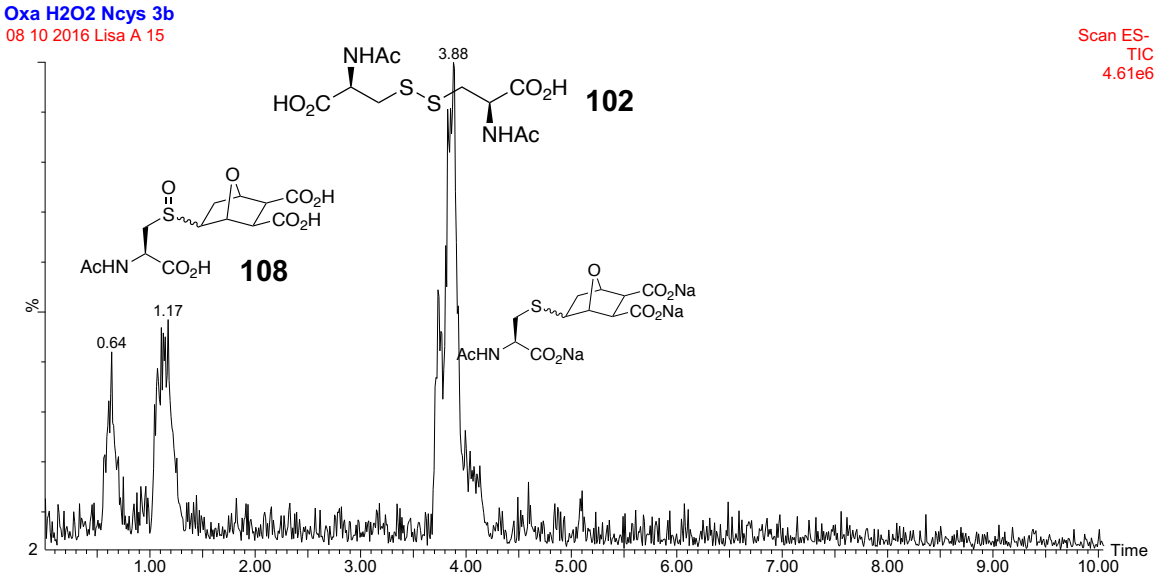


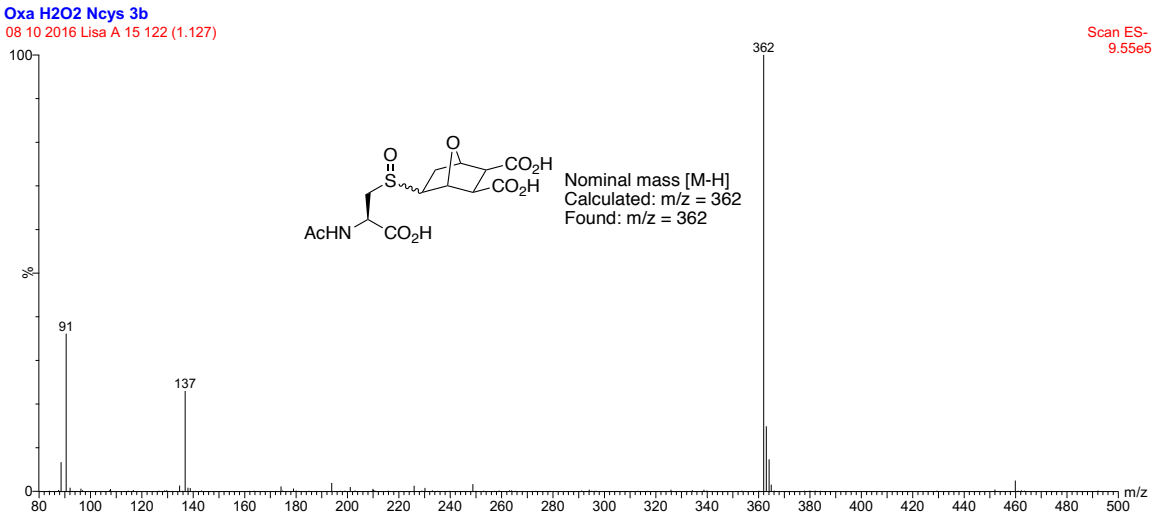


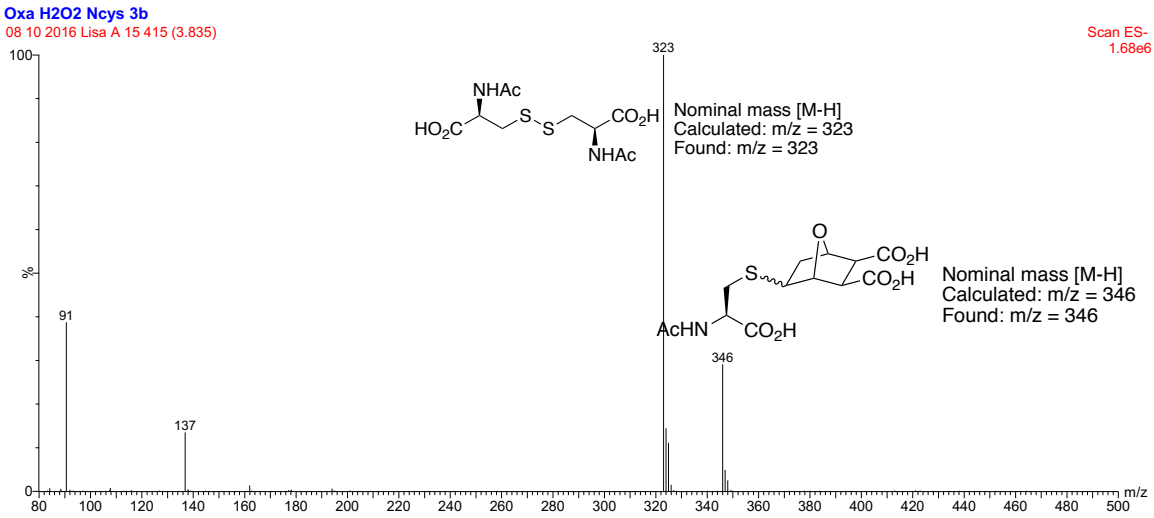




LC-MS study oxa-norbornene dicarboxylic acid **107** traps the *N*-acetylcysteine sulfenic acid **101** at pH 5.0. The products of disulfide and the potential thiol-ene product overlap.

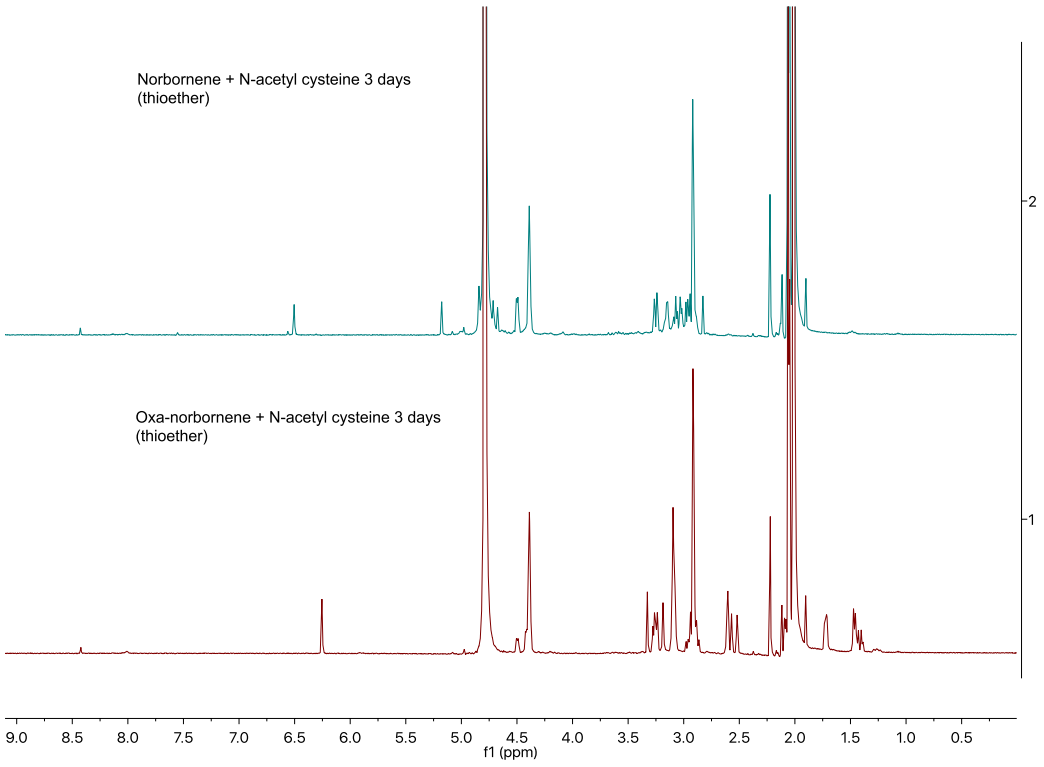






2.9.8 Thiol-ene test at pH 5

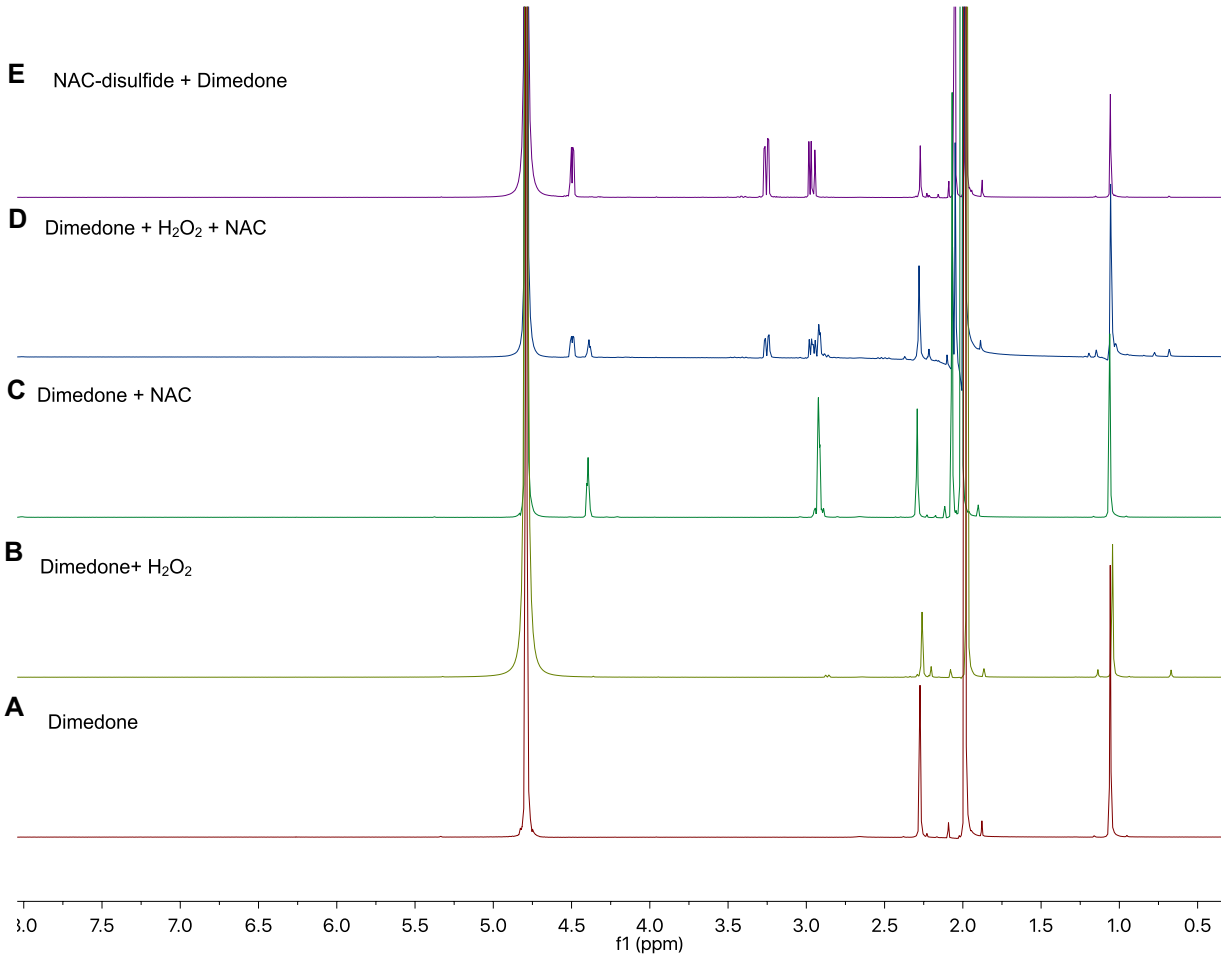
Even after 3 days, complete consumption of the alkene peak is not observed. Some thiol-ene product might be observed, but is not characterisable by the NMR.



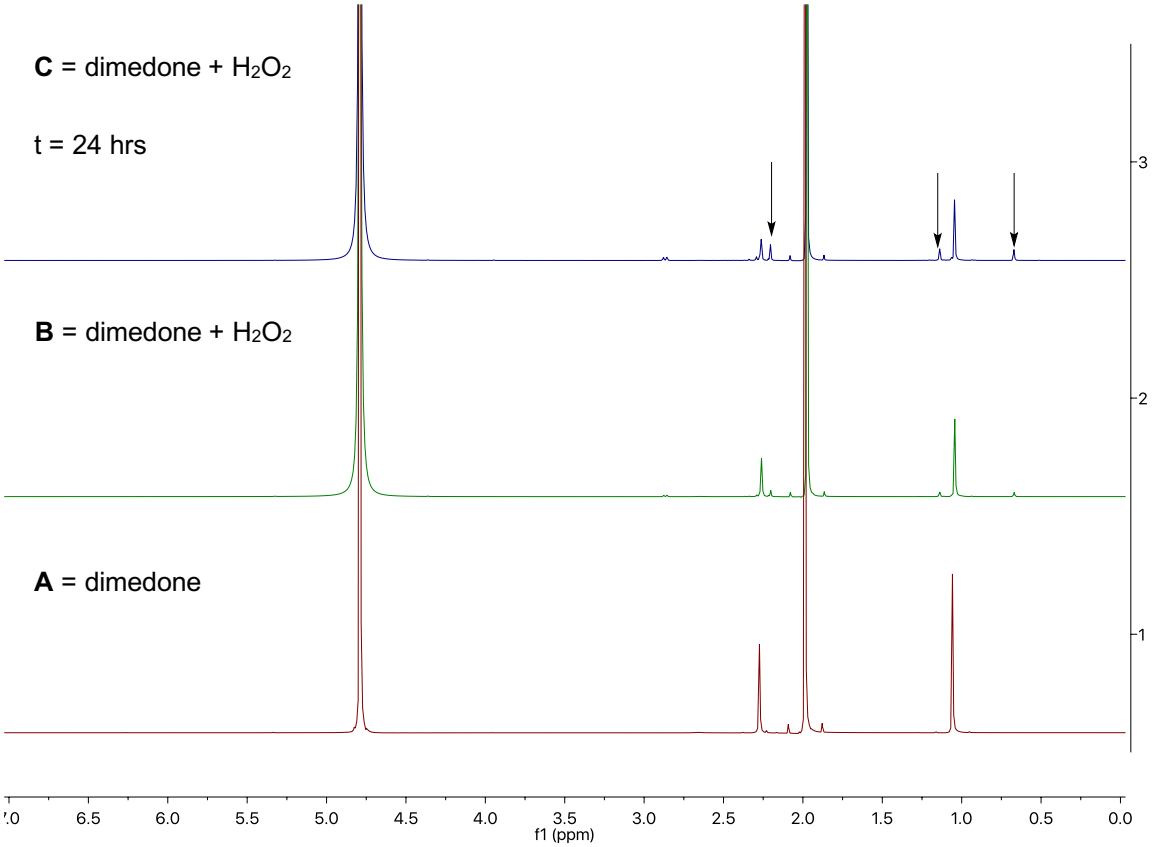
2.9.9 Dimedone (19) as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

NMR studies using dimedone 19 as a probe for cysteine oxidation at pD 5.0 (NaOAc buffer, 200 mM)

No reactions are observed under conditions A-E.

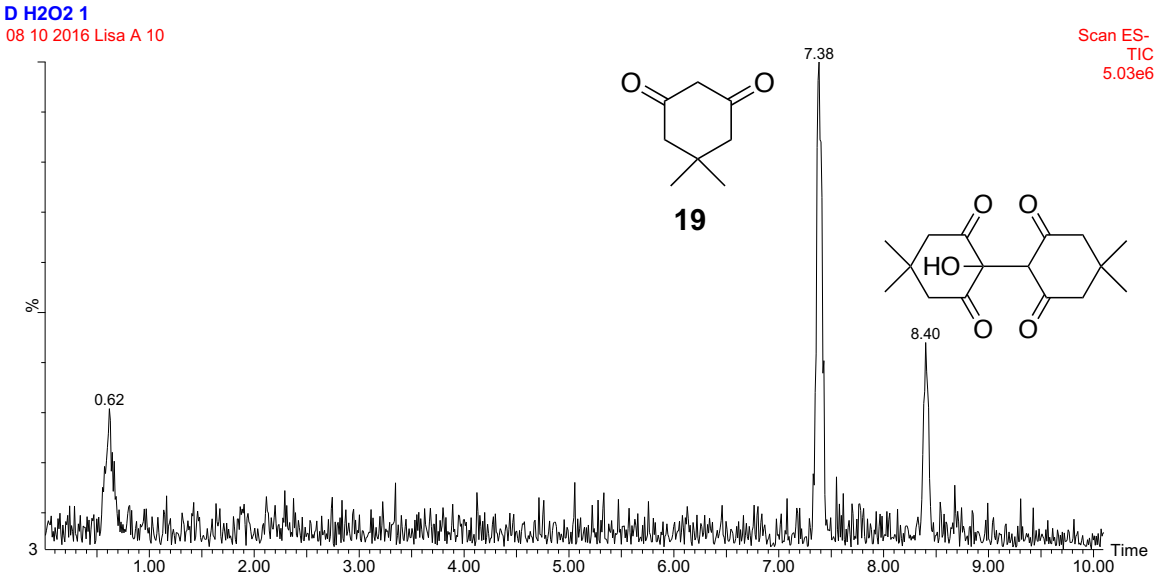


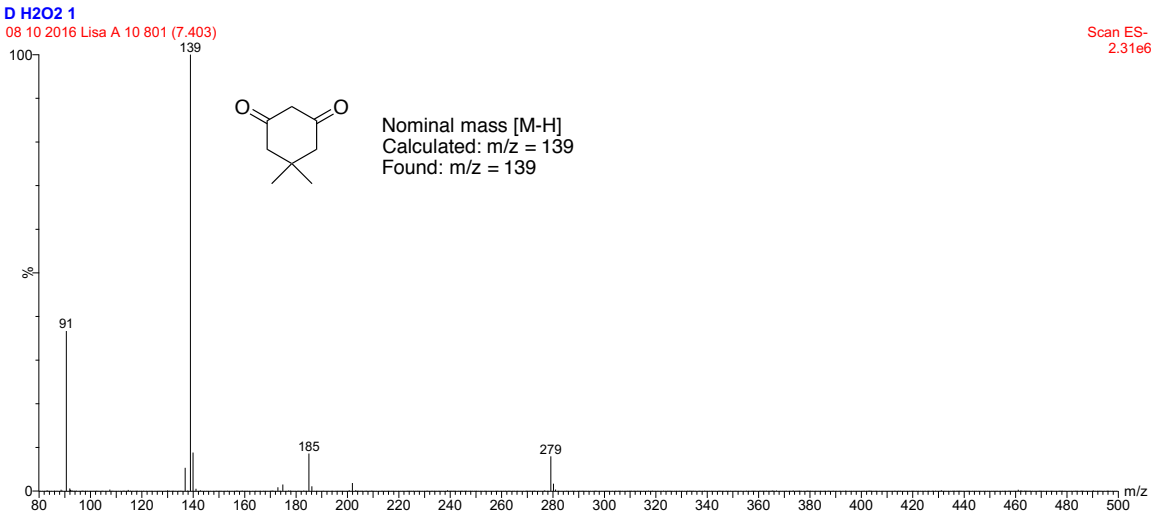
NMR comparison for the reaction of dimedone enolate with H_2O_2 from 20 mins to 24 hrs.

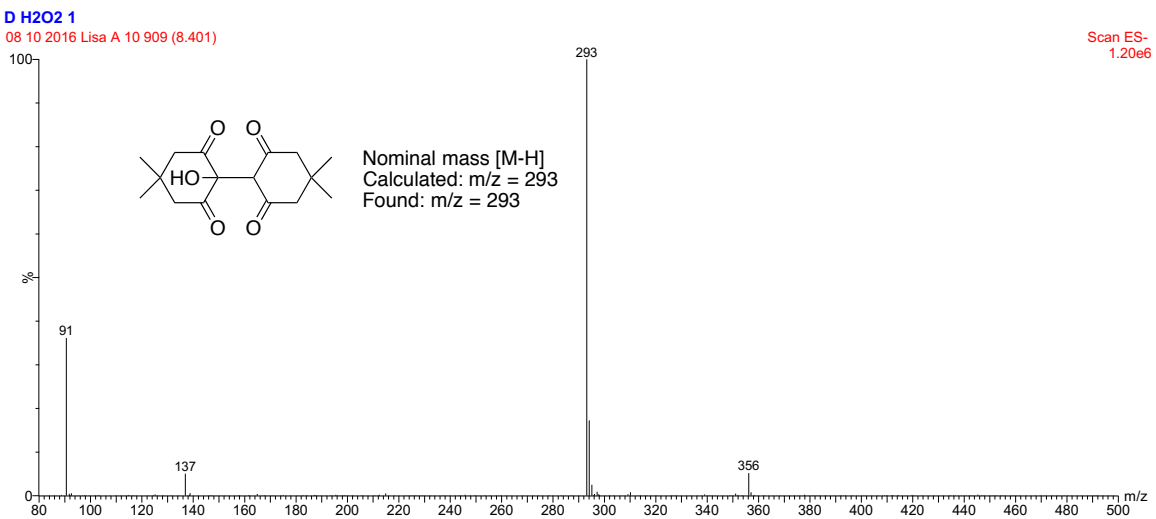


LC-MS studies using dimedone 19 as a probe for cysteine oxidation at pH 5.0 (NaOAc buffer, 200 mM)

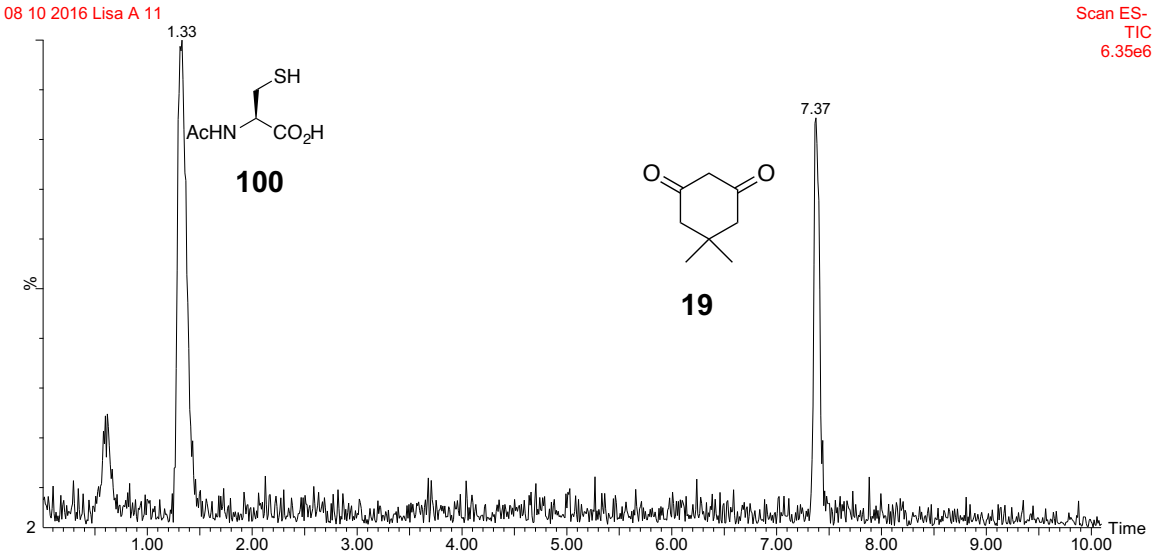
Dimedone **19** reacts slowly with hydrogen peroxide.

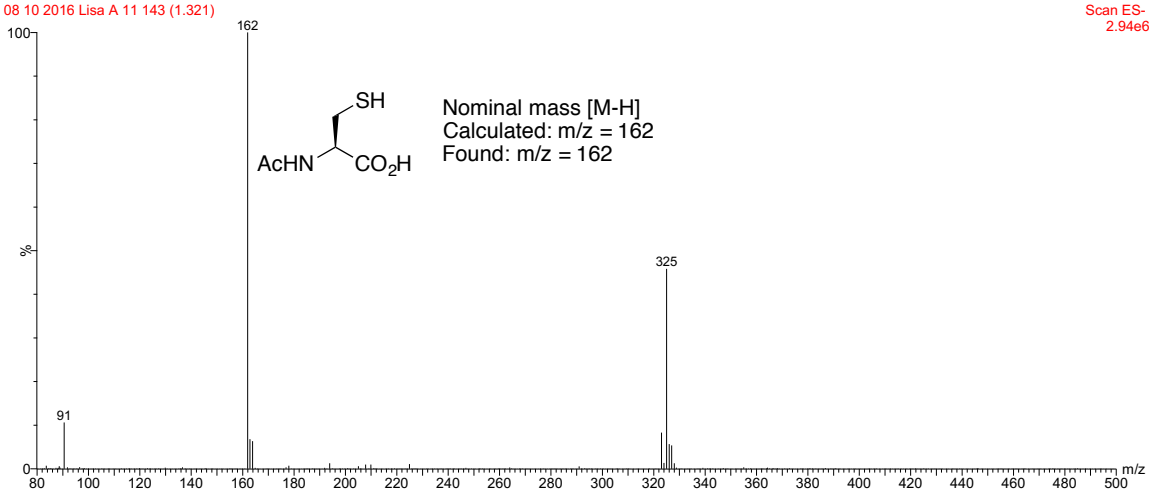


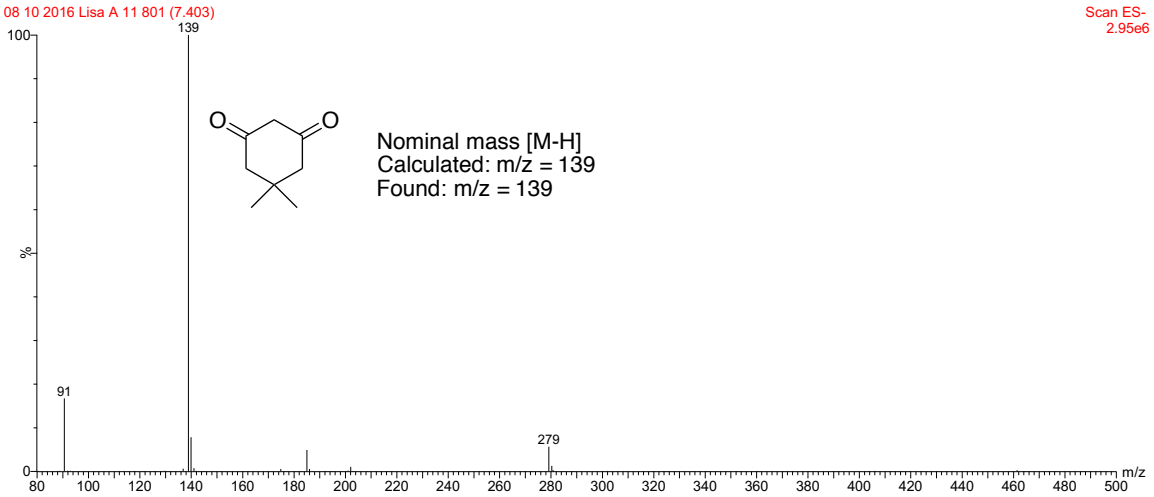




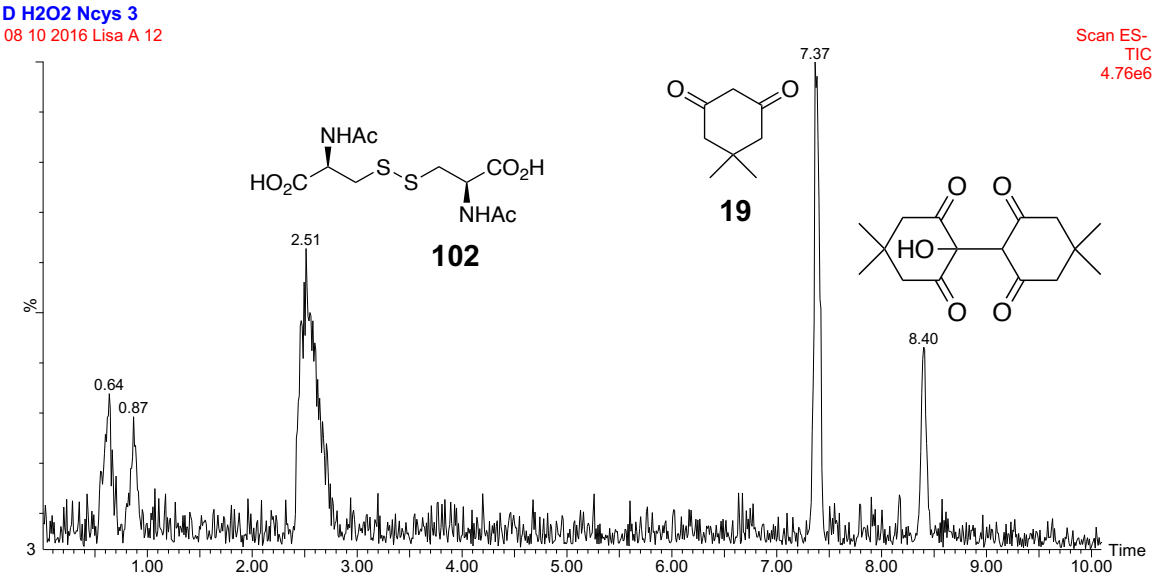
Dimedone **19** does not react with *N*-acetylcysteine **100**.

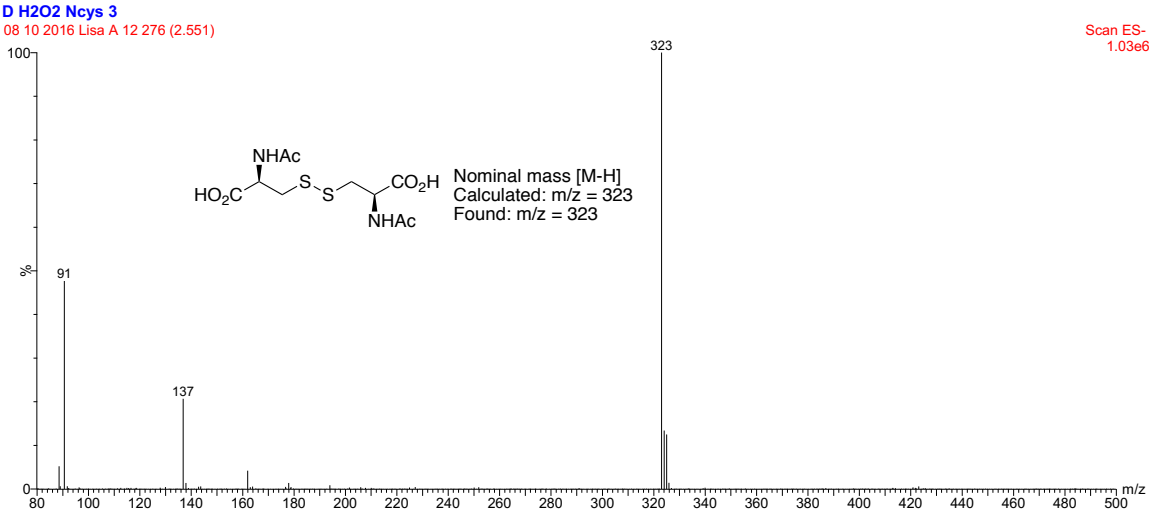


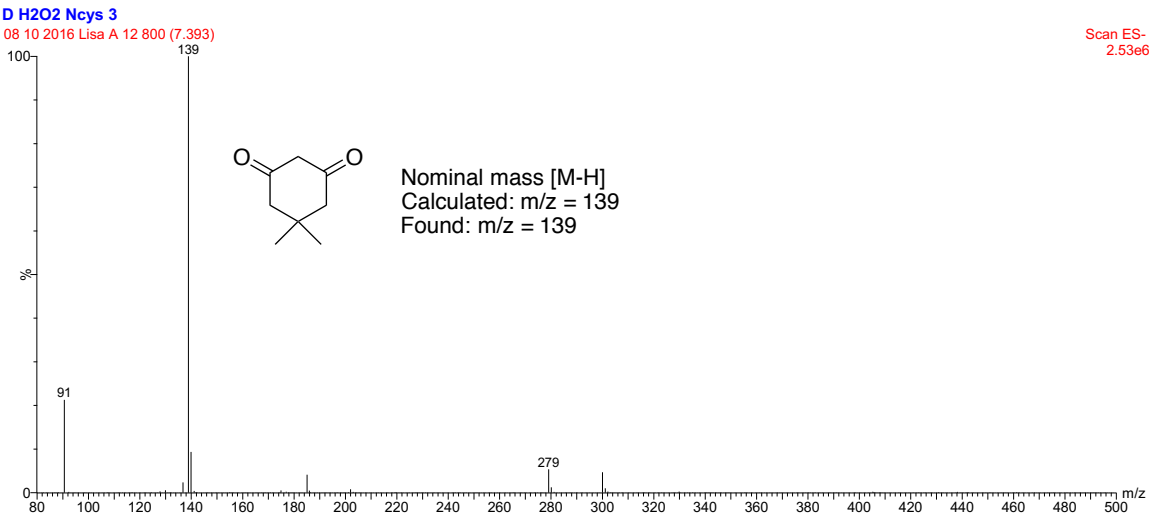


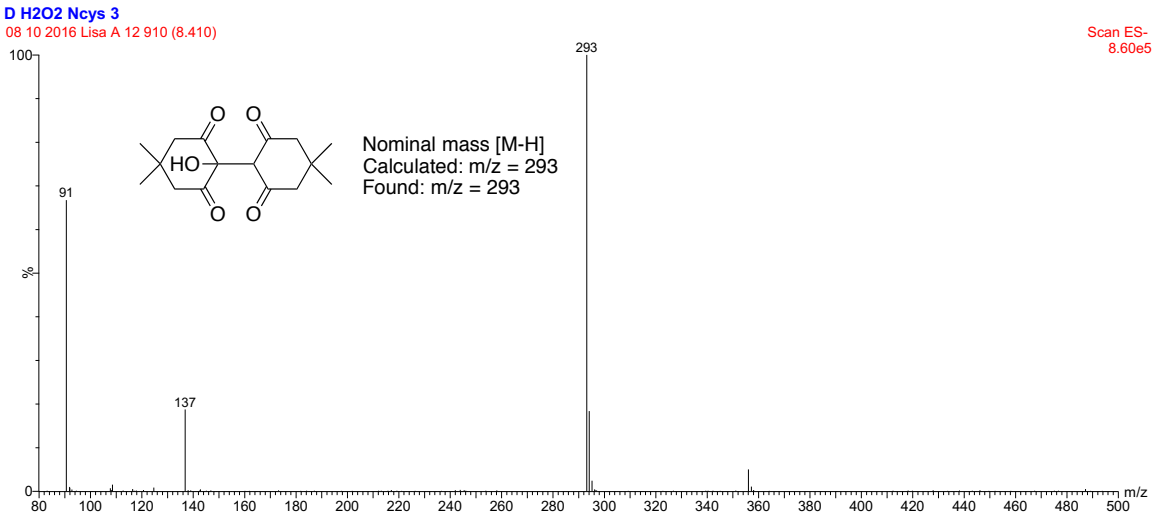


Dimedone **19** does not trap the *N*-acetylcysteine sulfenic acid **101**.

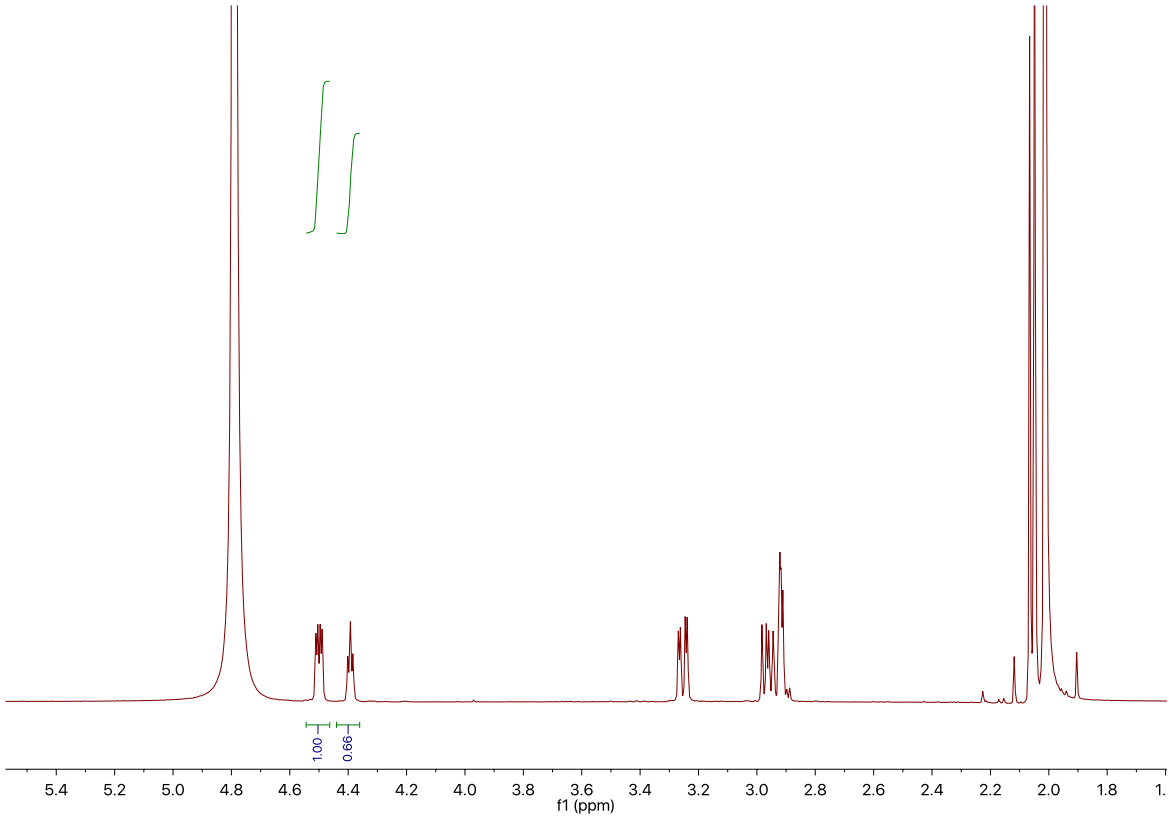


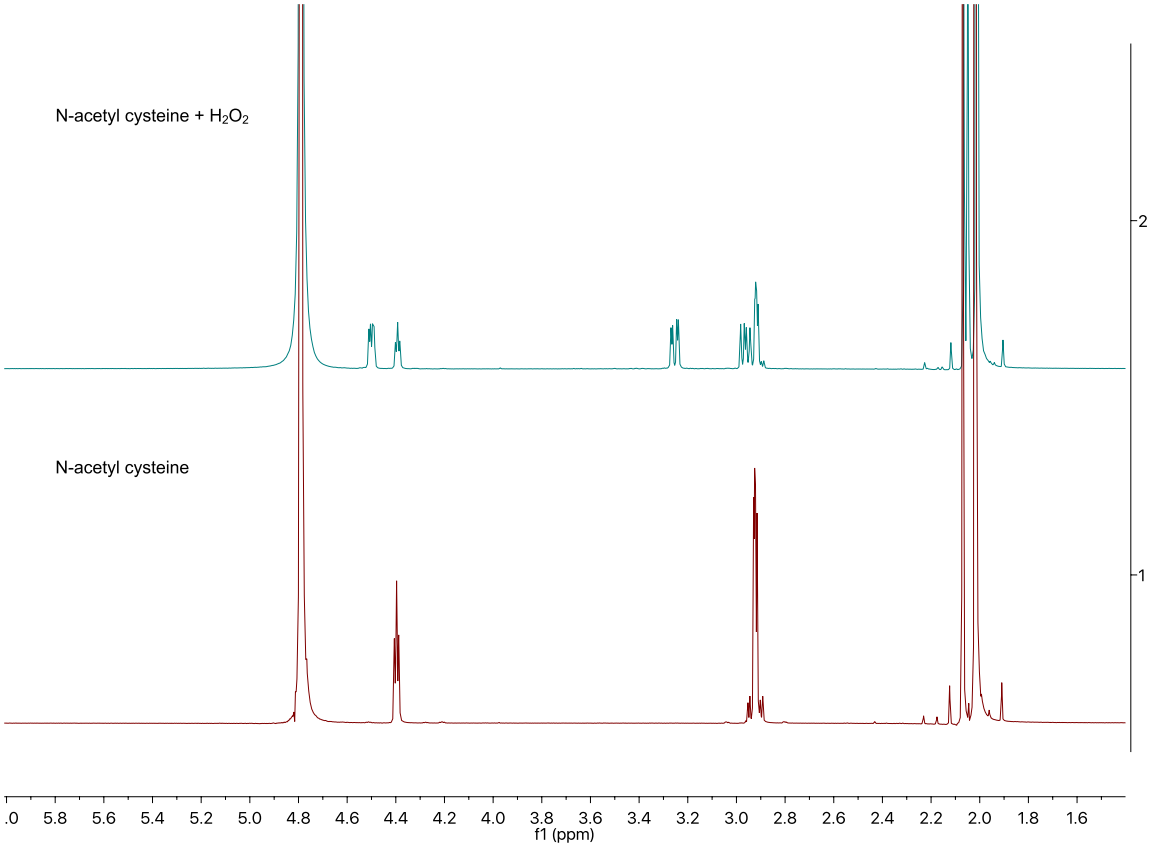






2.9.10 Oxidation of N-acetylcysteine 100 to its disulfide 102 using hydrogen peroxide at pD 5.0 (sodium acetate buffer)

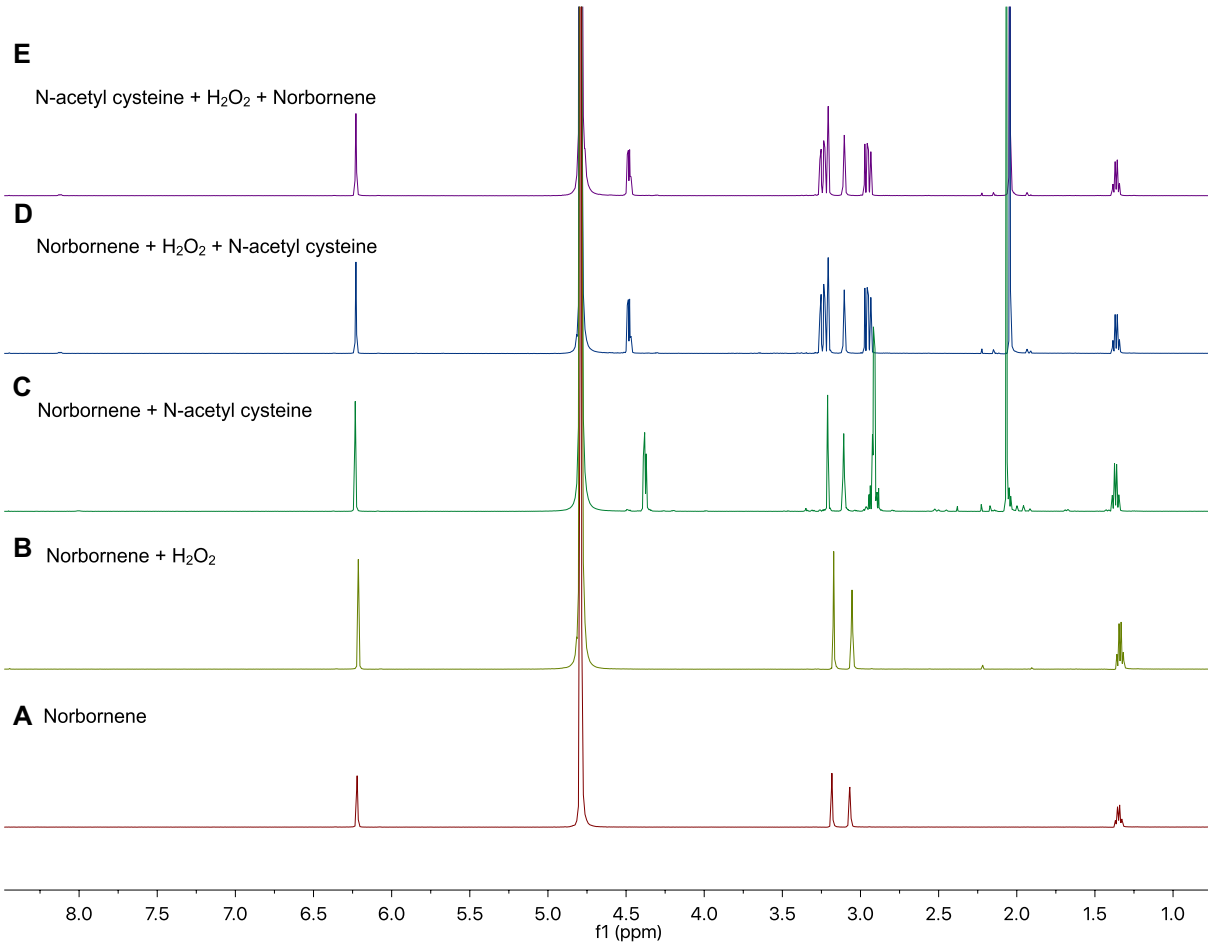



N-acetyl cysteine + H_2O_2
N-acetyl cysteine
2
1
.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6
f1 (ppm)

2.9.11 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

NMR studies using *cis*-5-norbornene-endo-2,3-dicarboxylic acid 99 as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

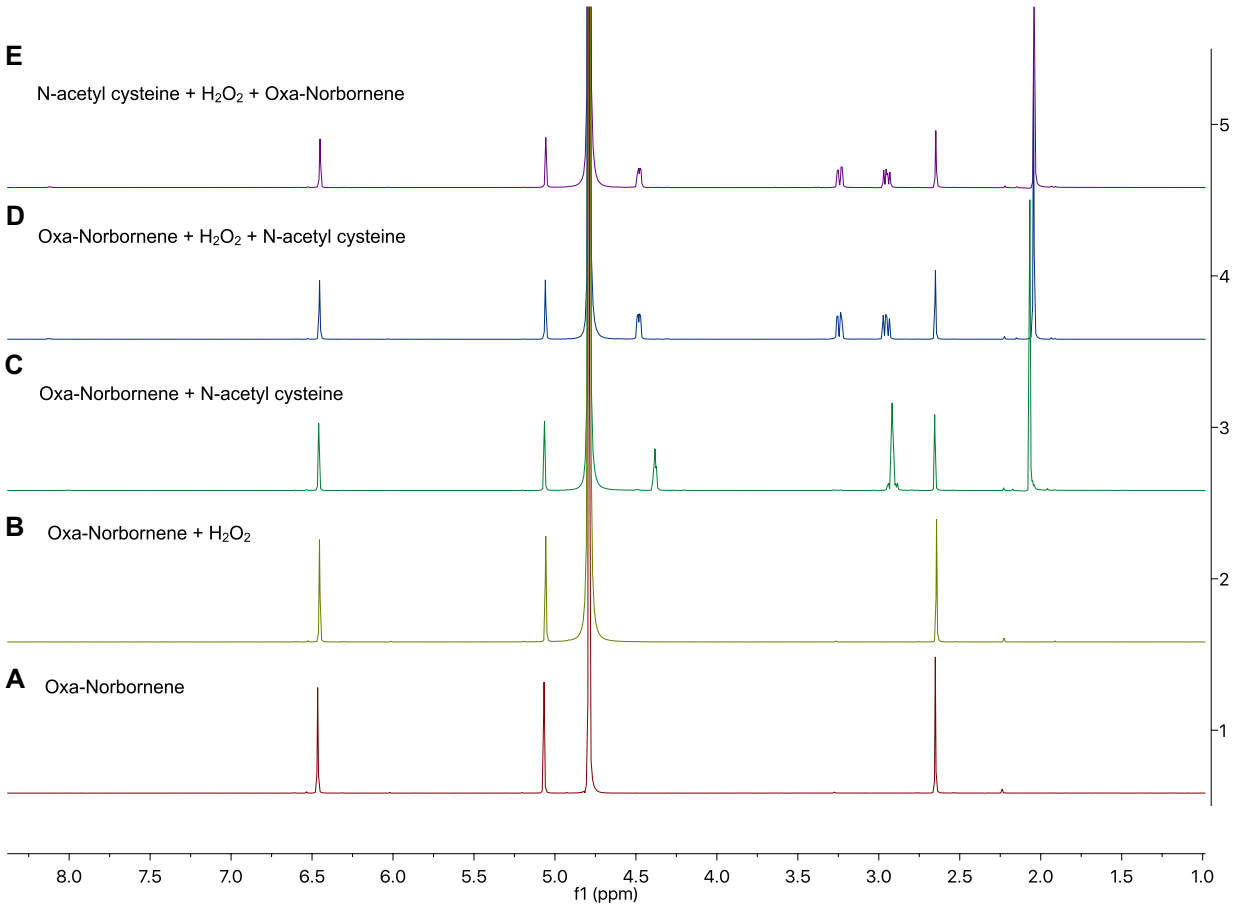
No reactions are observed across conditions A-E.



2.9.12 oxa-norbornene dicarboxylic acid (107) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

NMR studies using oxa-norbornene dicarboxylic acid 107 as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

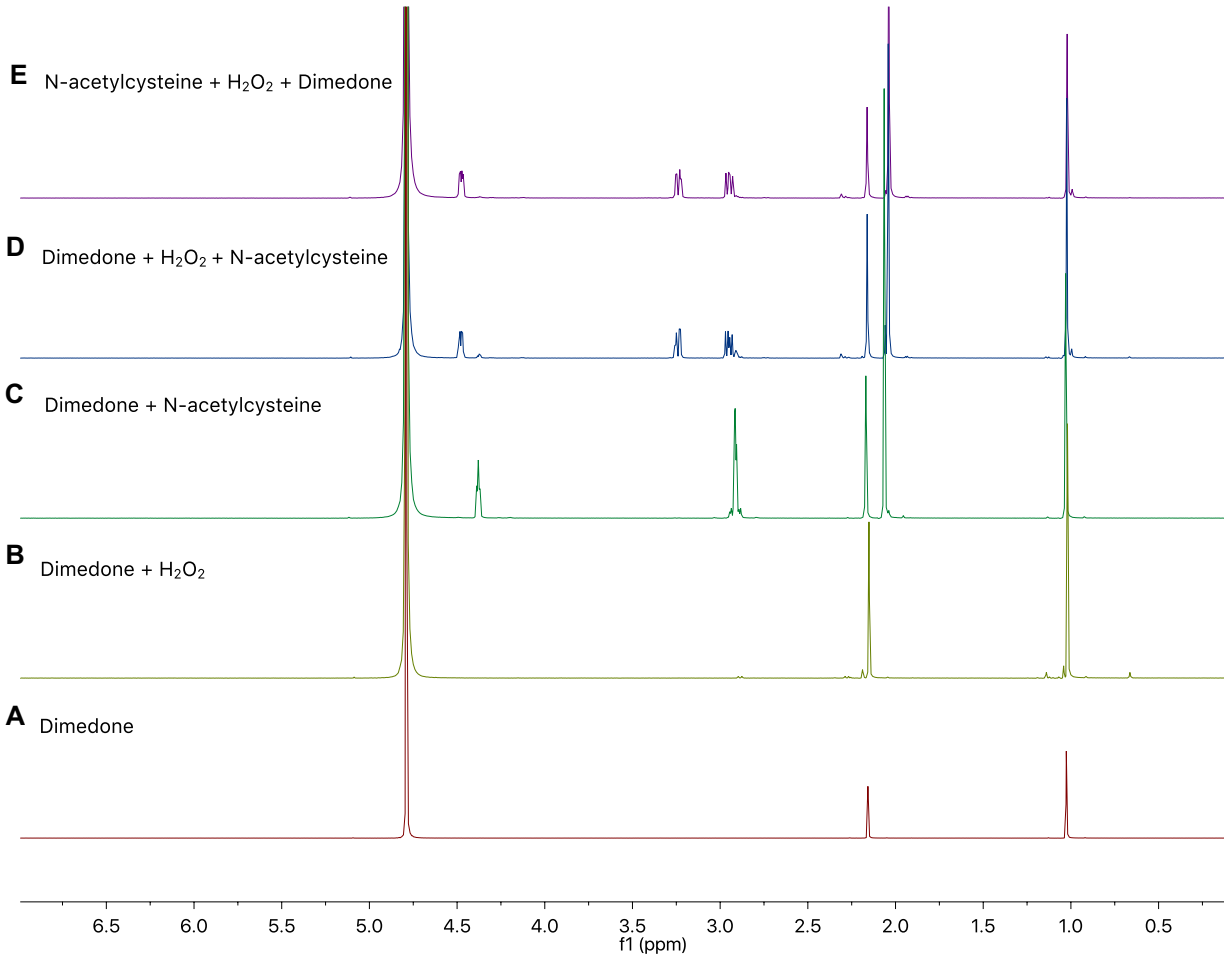
No reaction is observed across conditions A-E.



2.9.13 Dimedone (19) as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

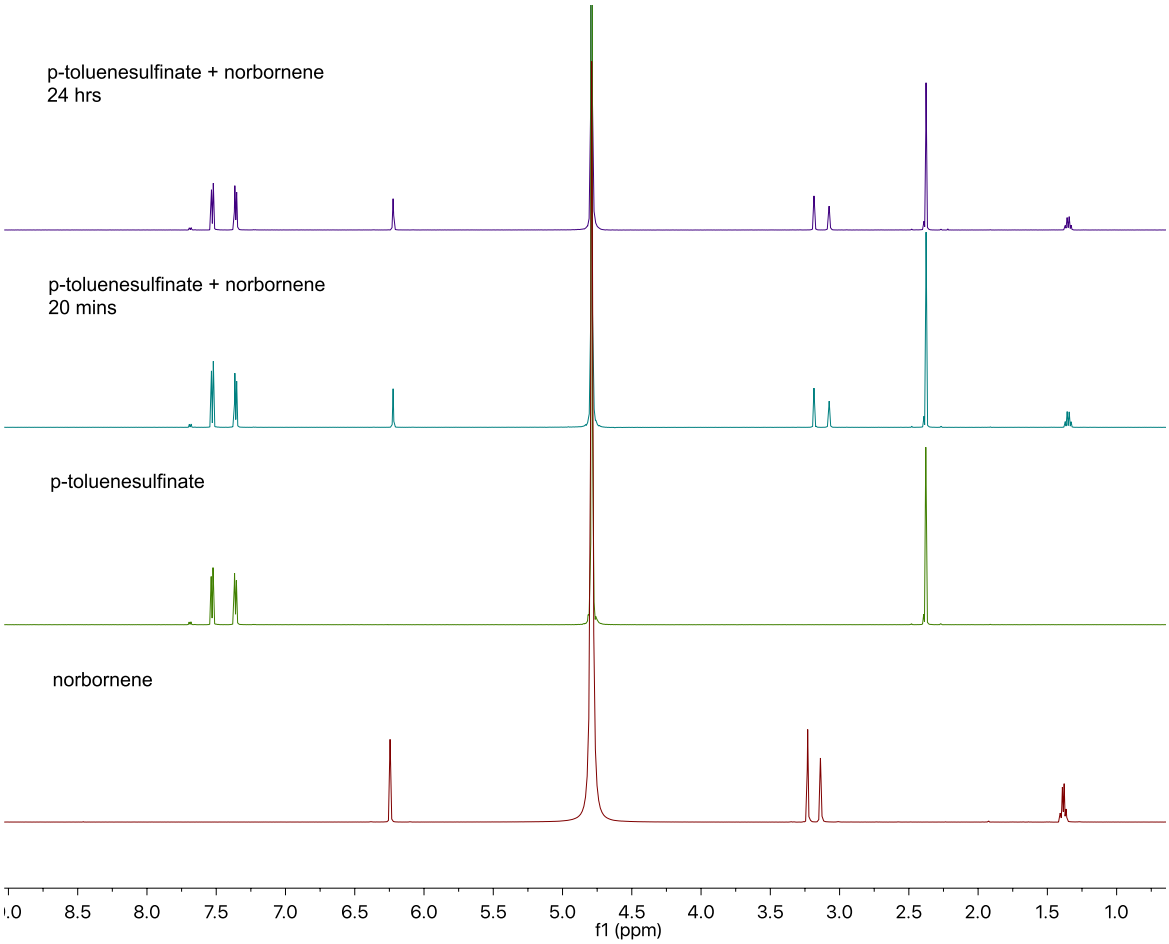
NMR studies using dimedone 19 as a probe for cysteine oxidation in pD 7.4 (sodium phosphate buffer, 200 mM)

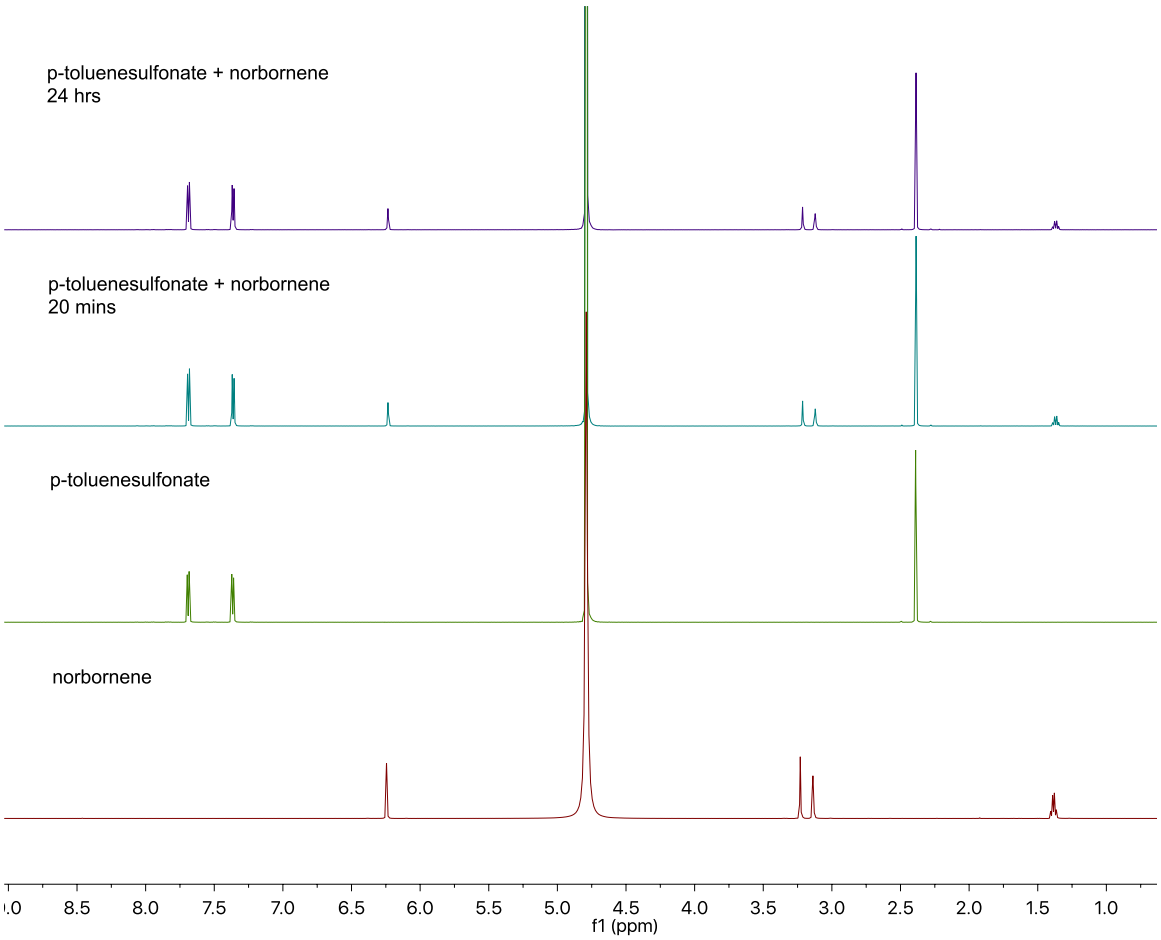
No reaction is observed across conditions A-E.



2.9.14 *cis*-5-norbornene-endo-2,3-dicarboxylic acid (99) does not react with sulfinates or sulfonates at pD 7.4 (NaH_2PO_4 buffer, 200 mM)

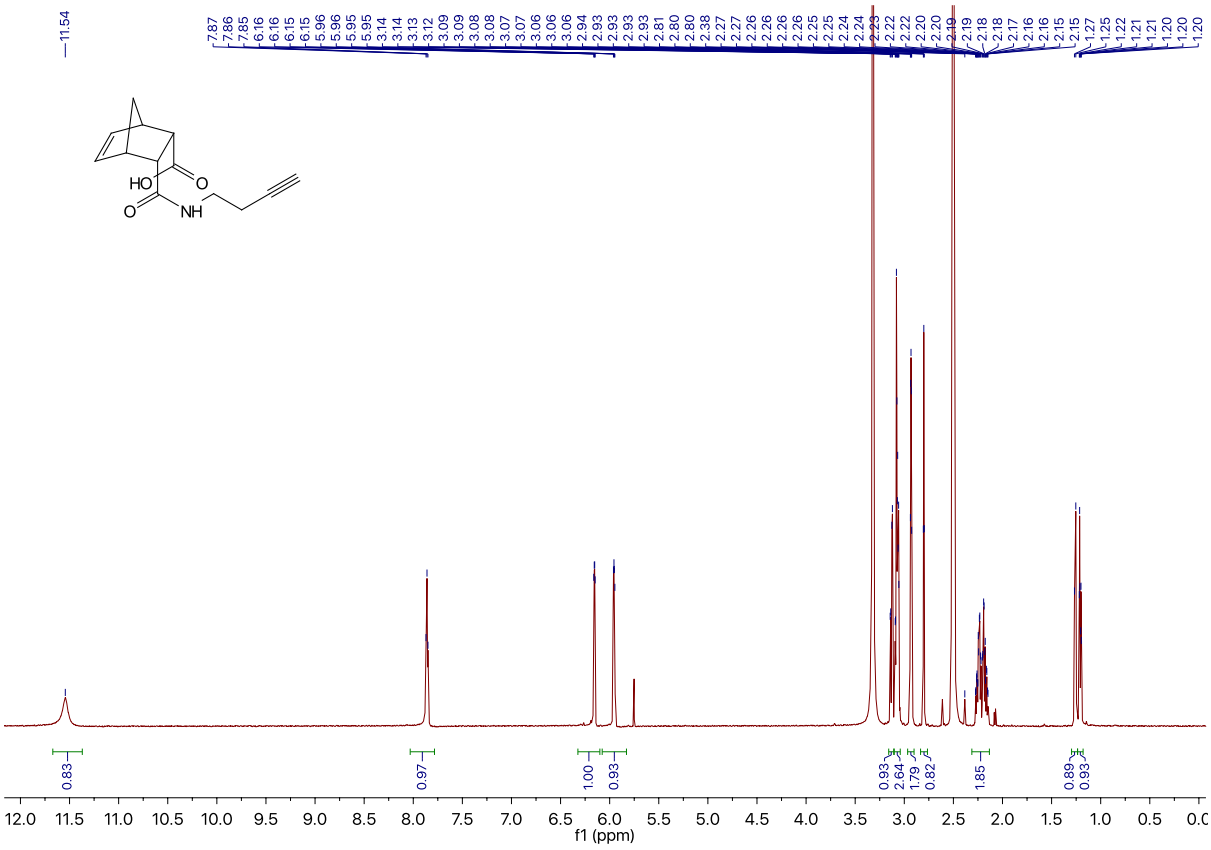
NMR studies showing *cis*-5-norbornene-endo-2,3-dicarboxylic acid 99 does not react with sulfinates or sulfonates at pD 7.4 (NaH_2PO_4 buffer, 200 mM)

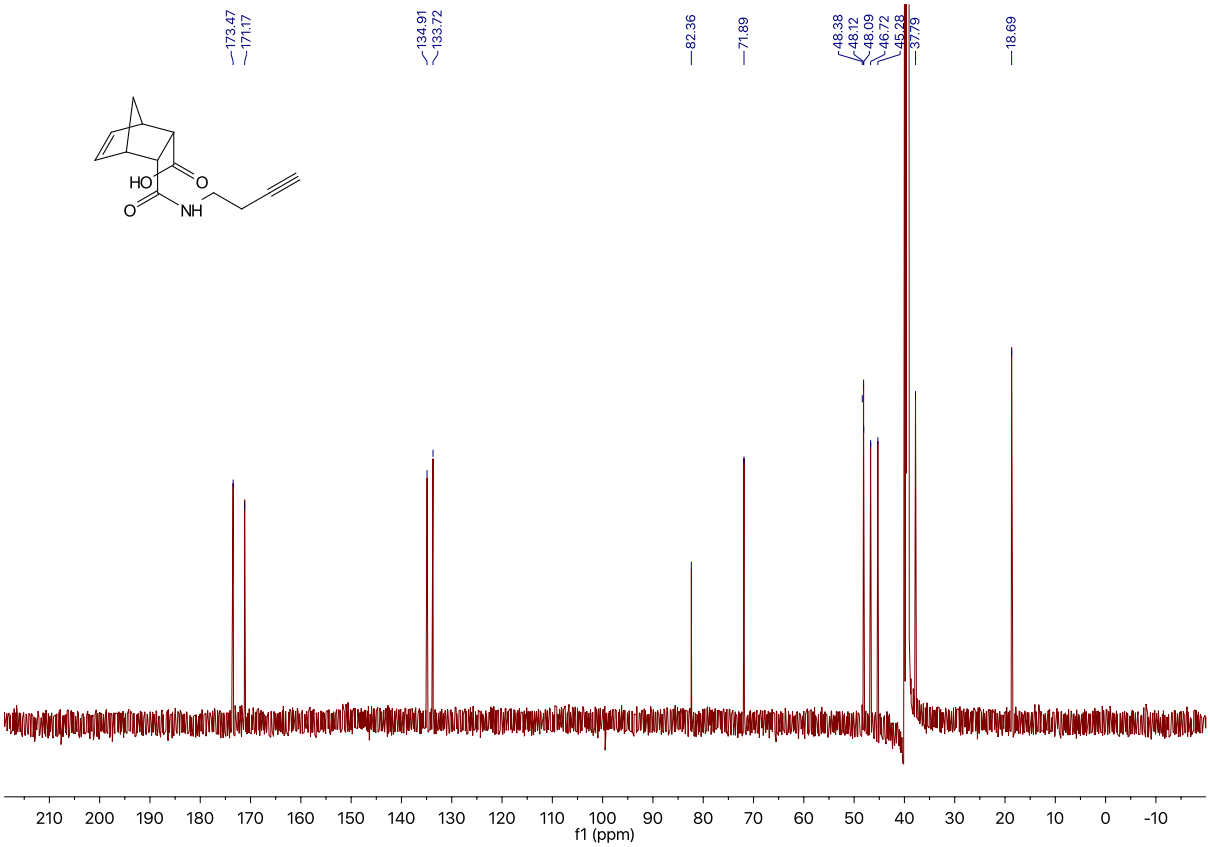



p-toluenesulfonate + norbornene
24 hrs
p-toluenesulfonate + norbornene
20 mins
p-toluenesulfonate
norbornene
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)

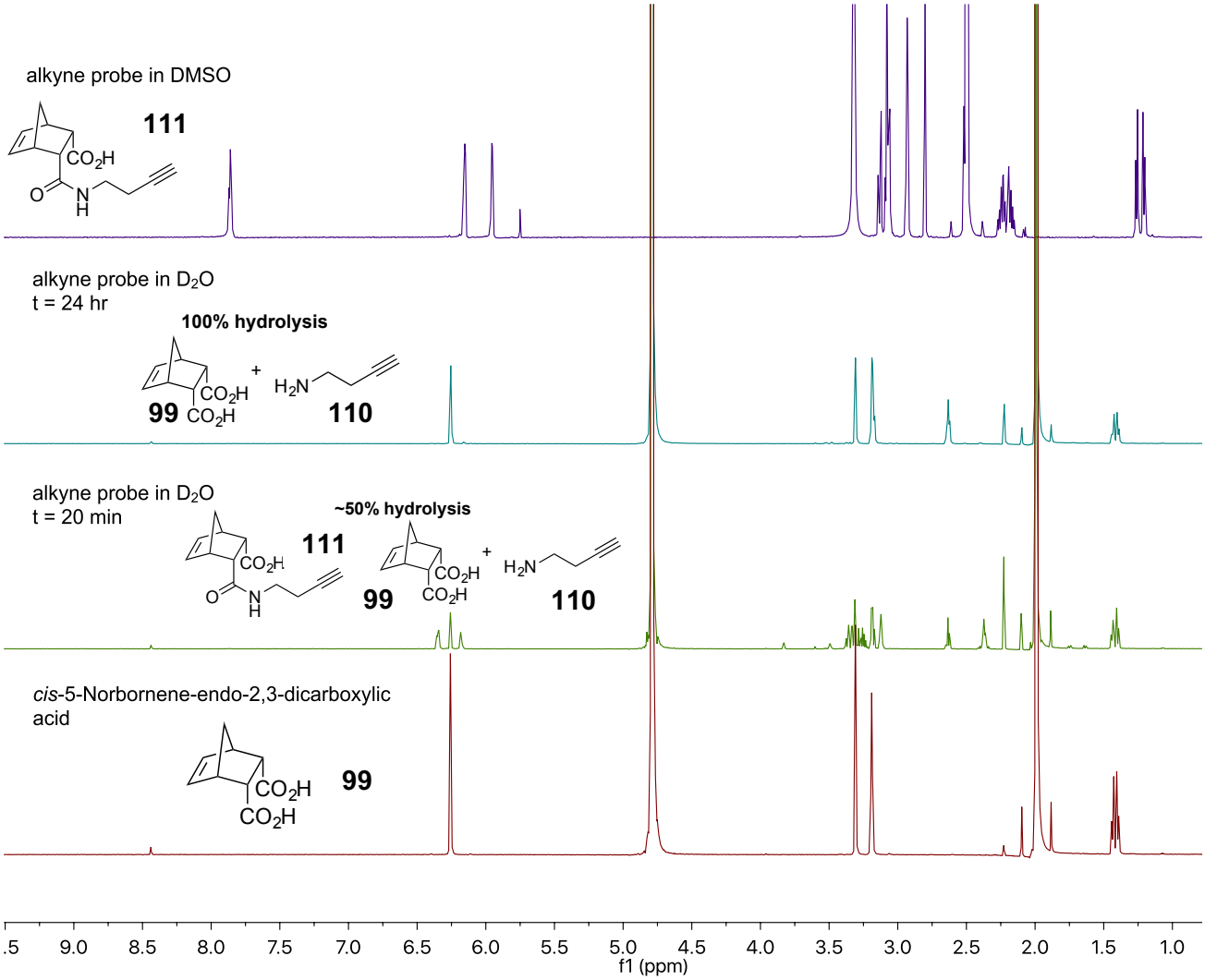
2.9.15 Probe synthesis

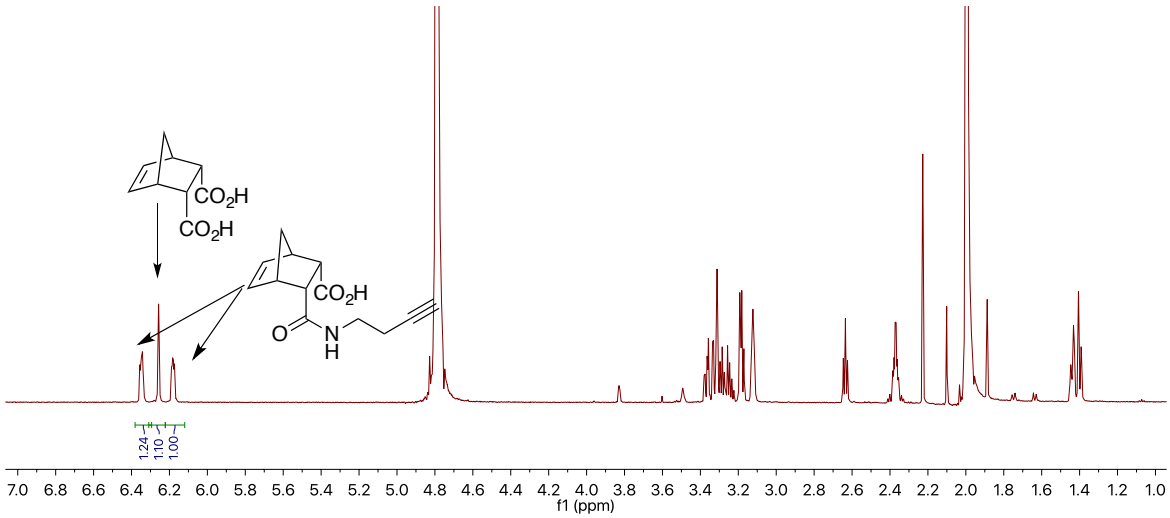
Synthesis of alkyne probe **111** (initially performed by Kyle Farrell)





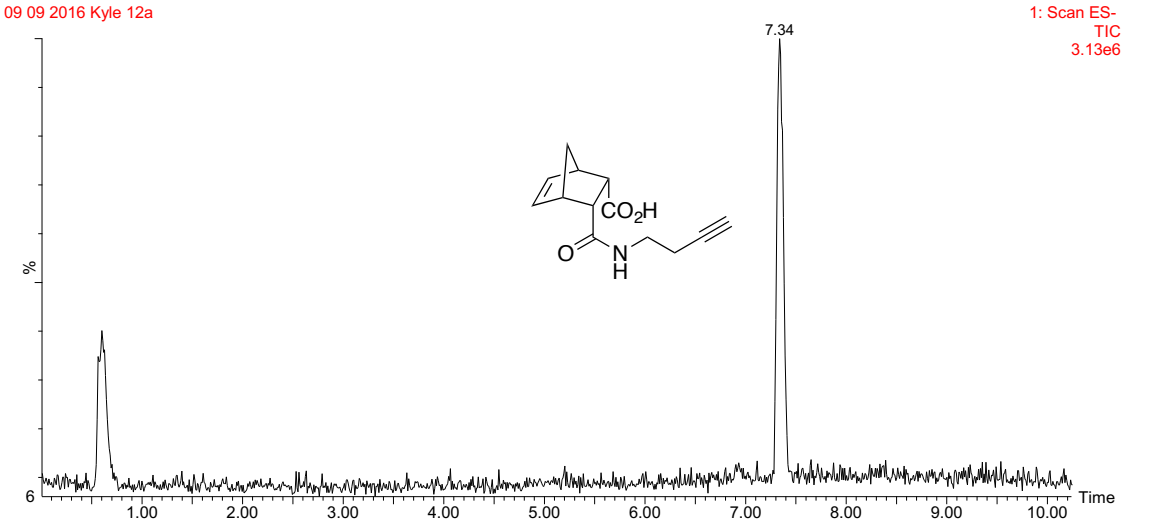
Hydrolysis study of alkyne probe **111** (NMR performed by Kyle Farrell)

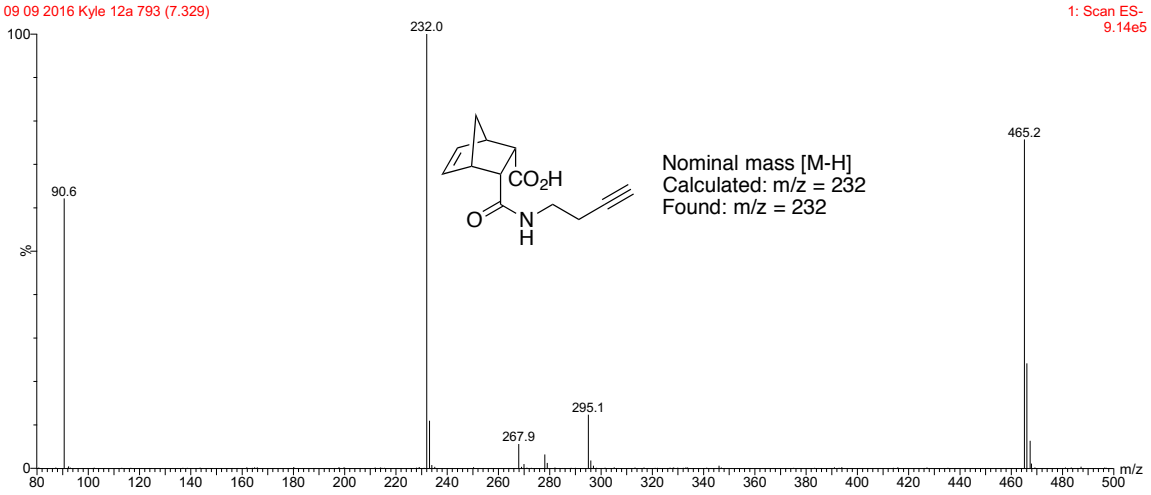




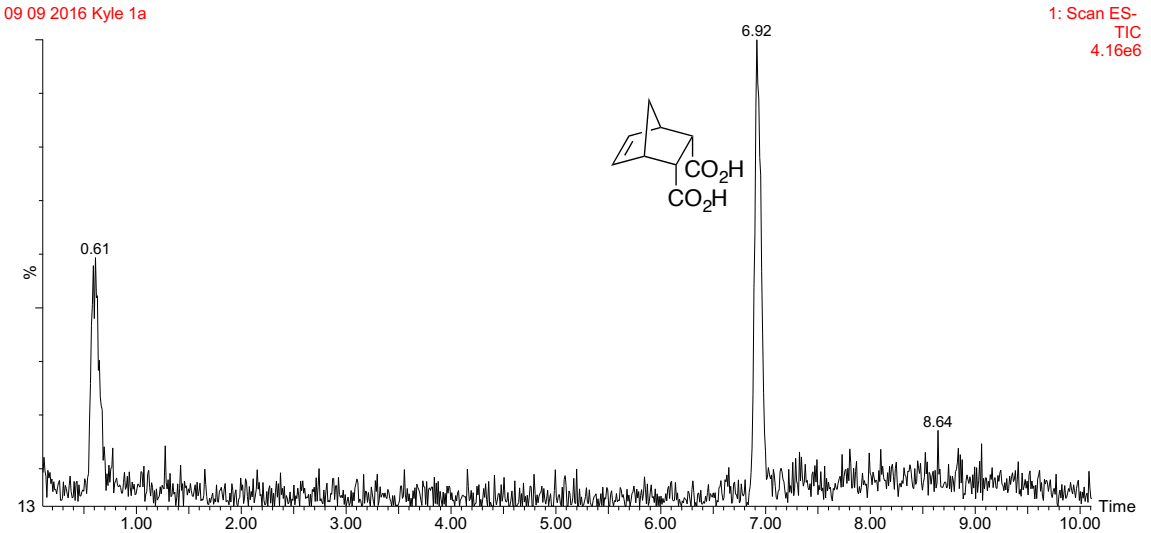
LC-MS traces (ESI^-) for hydrolysis of alkyne probe **111:**

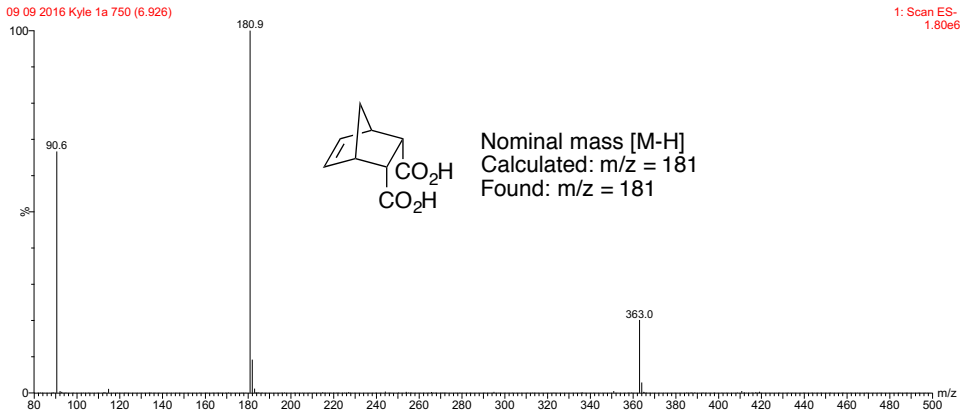
t = 0



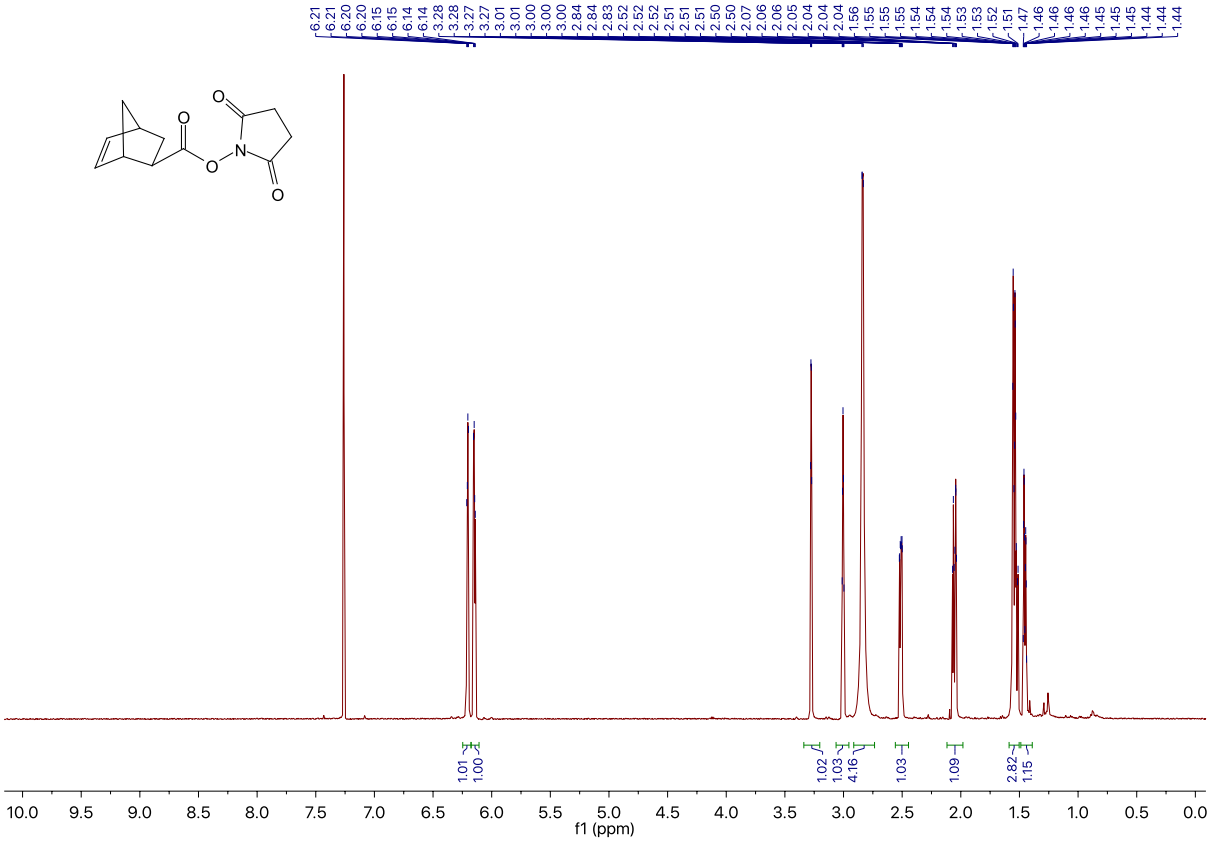


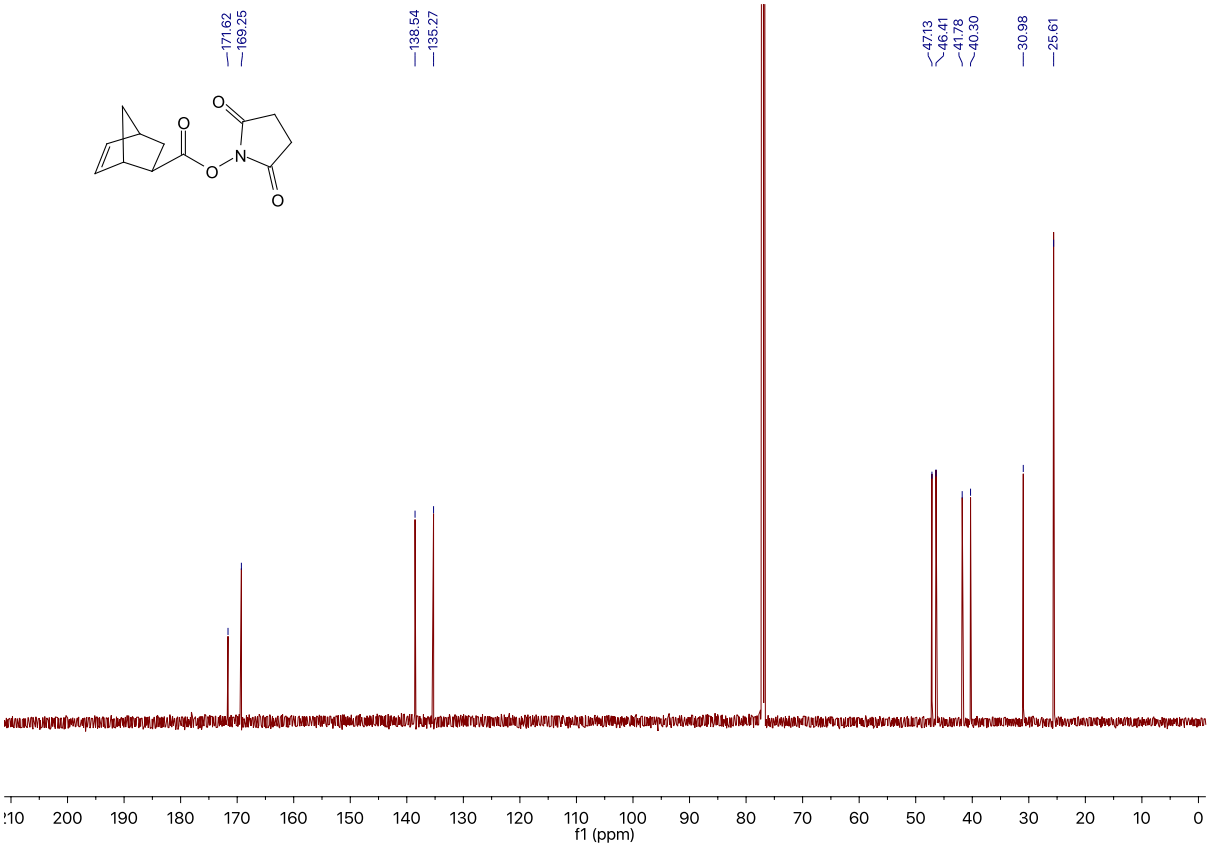
t = 24 hours



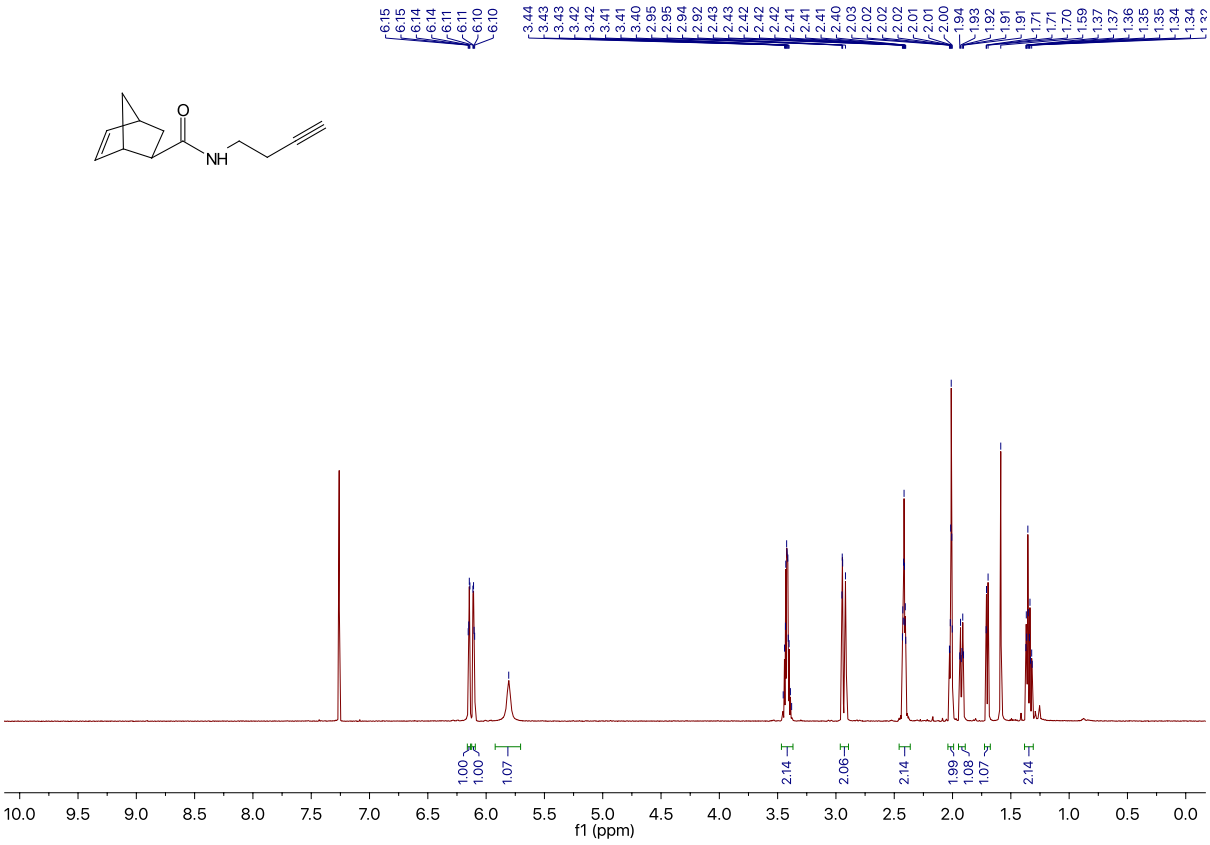


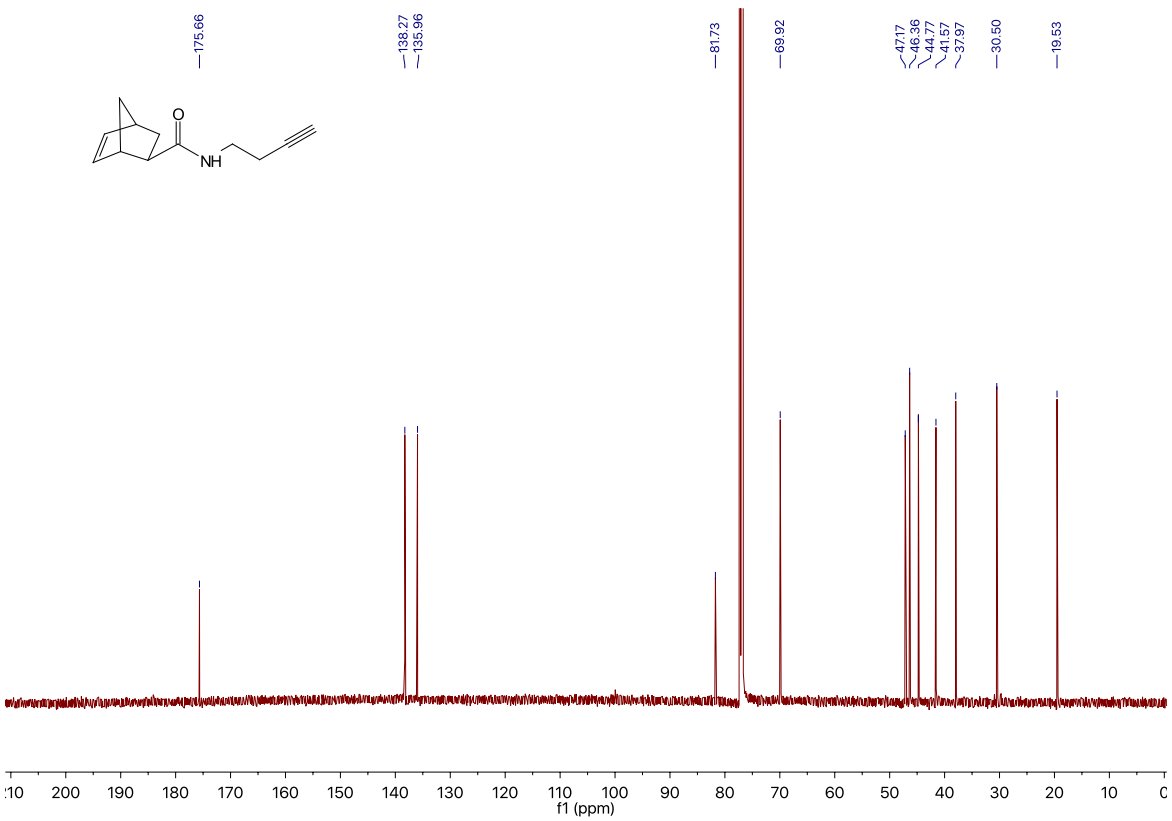
Synthesis of norbornene NHS derivative **114**



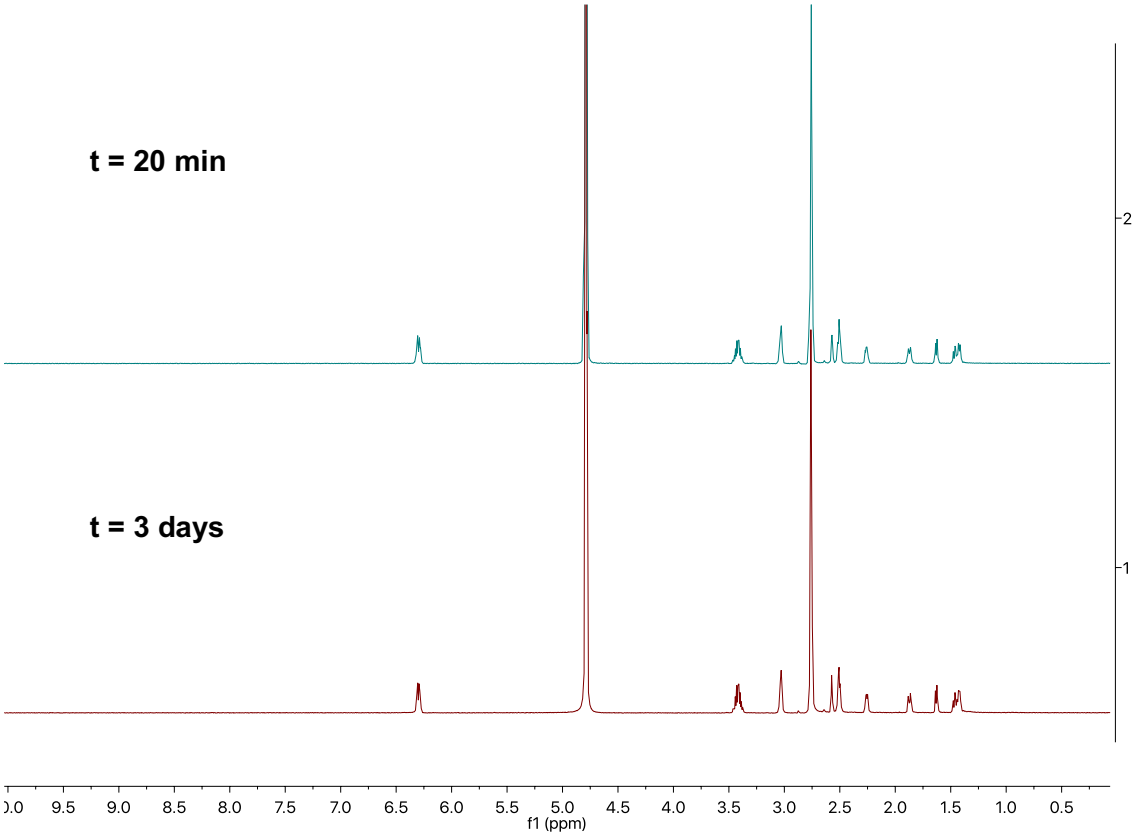


Synthesis of norbornene probe **69** containing an alkyne reporter group

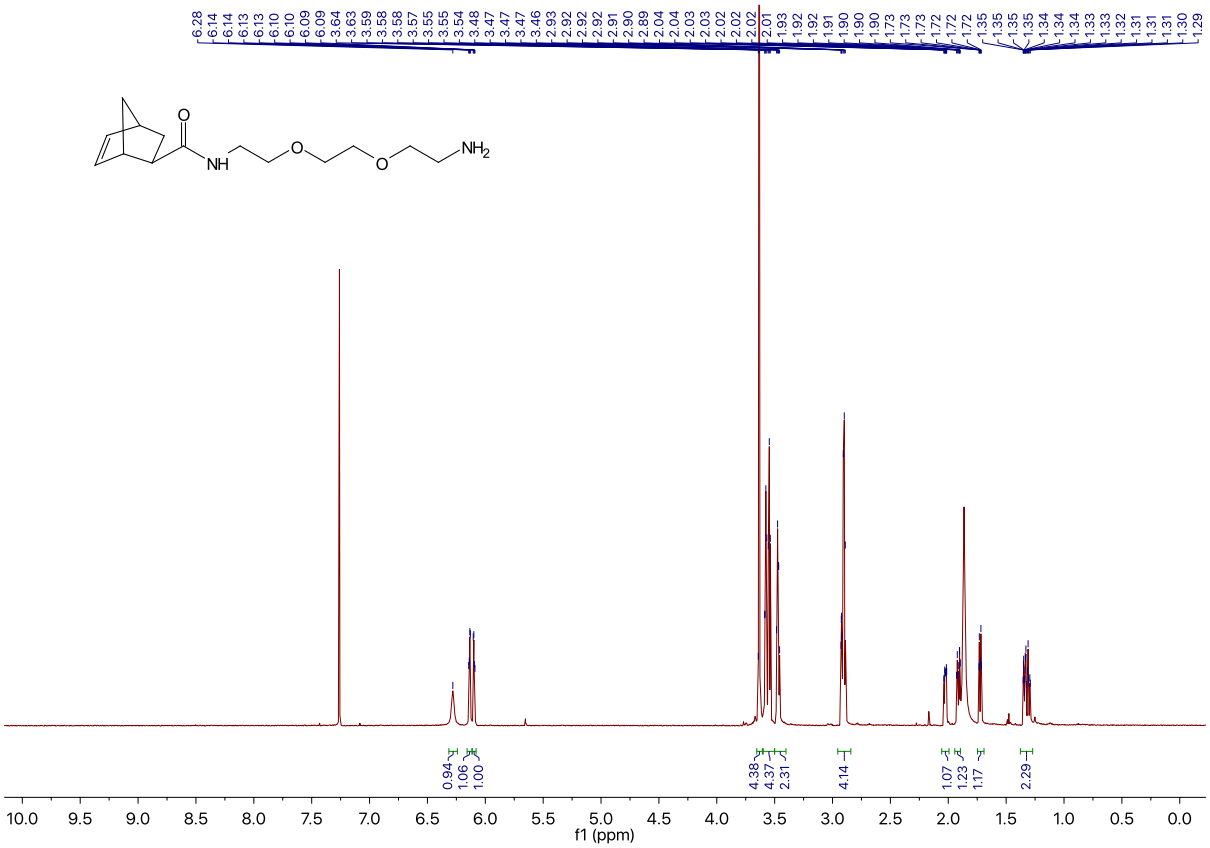


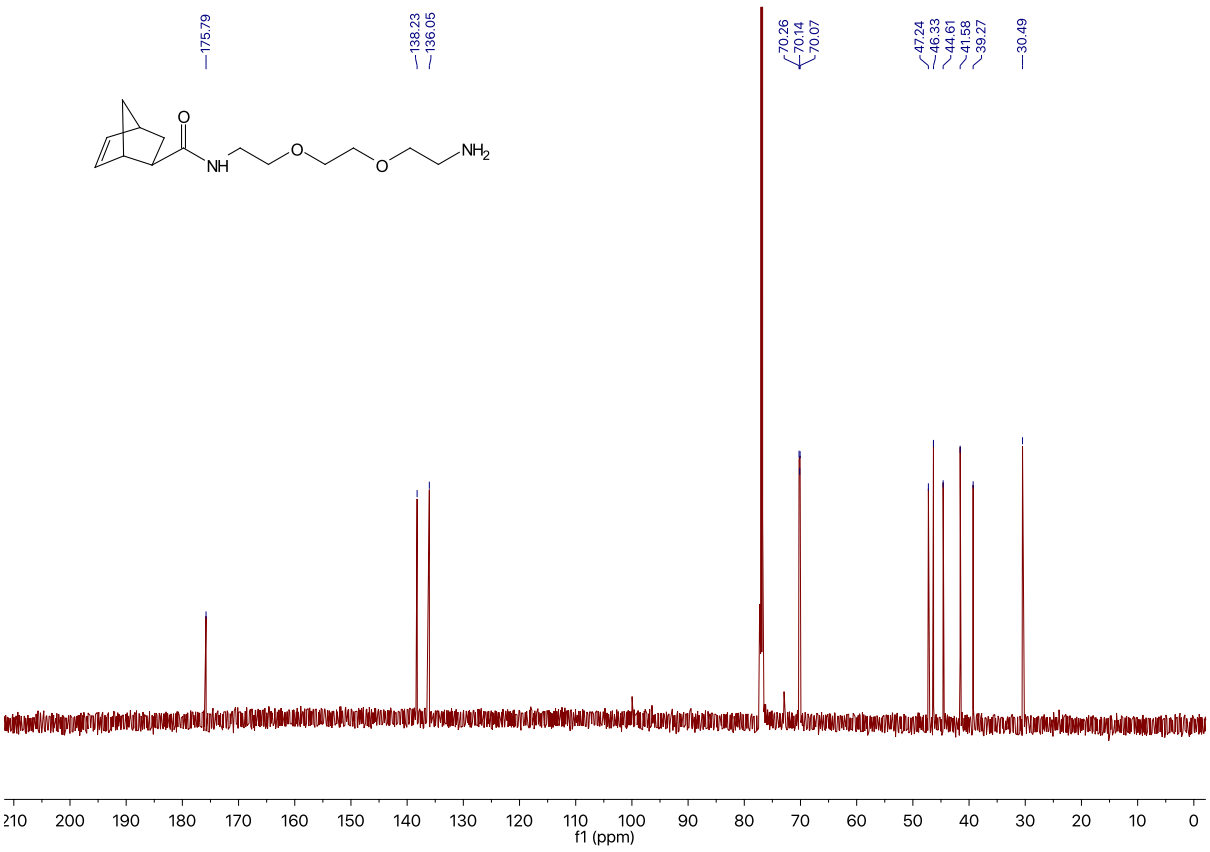


Stability of norbornene probe in DMSO/Water mix

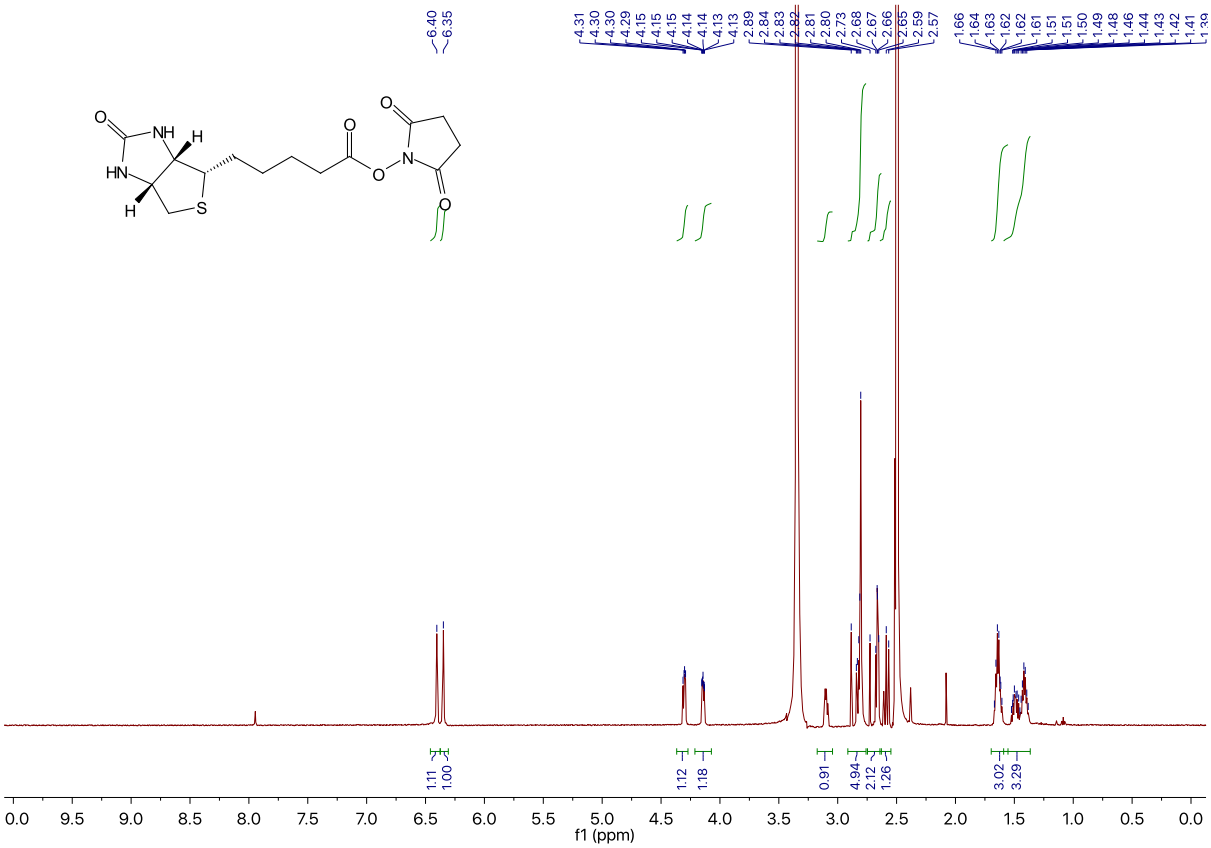


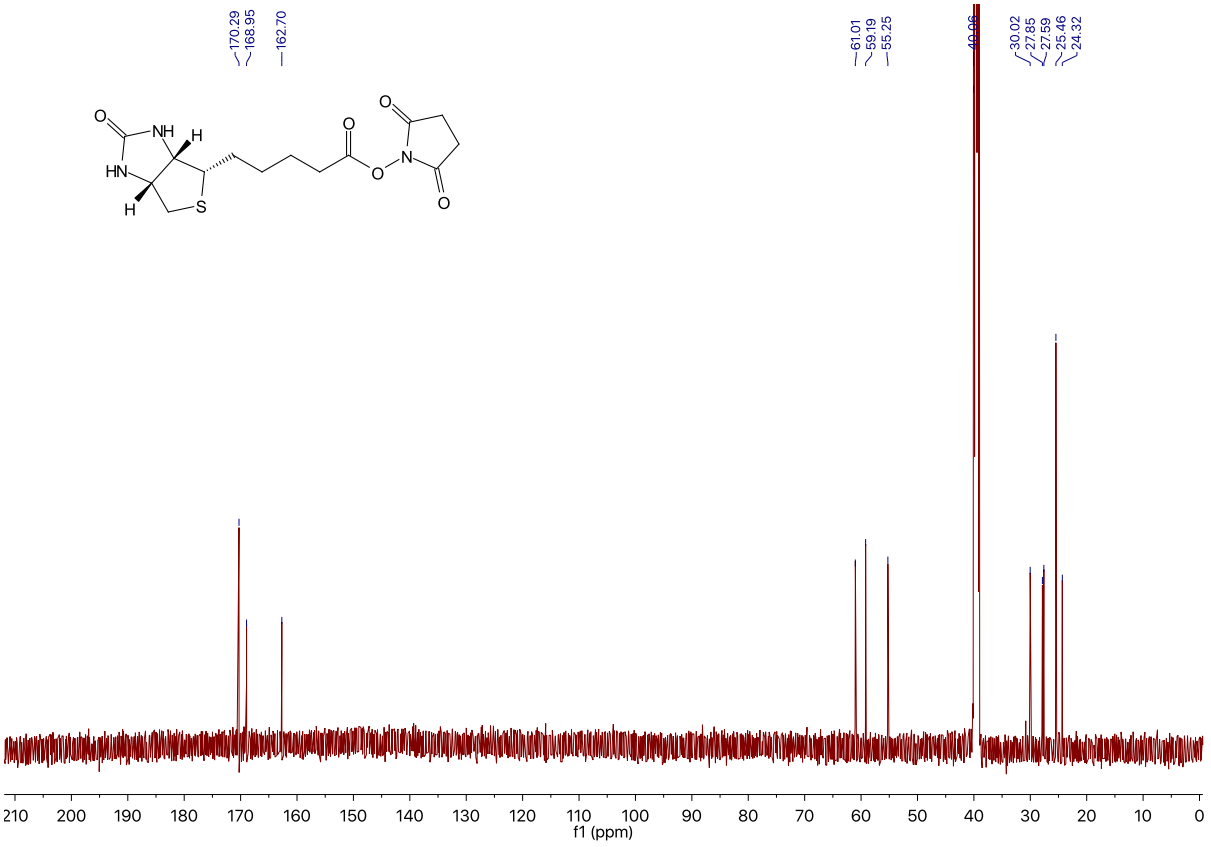
Synthesis of norbornene amine derivative **116**



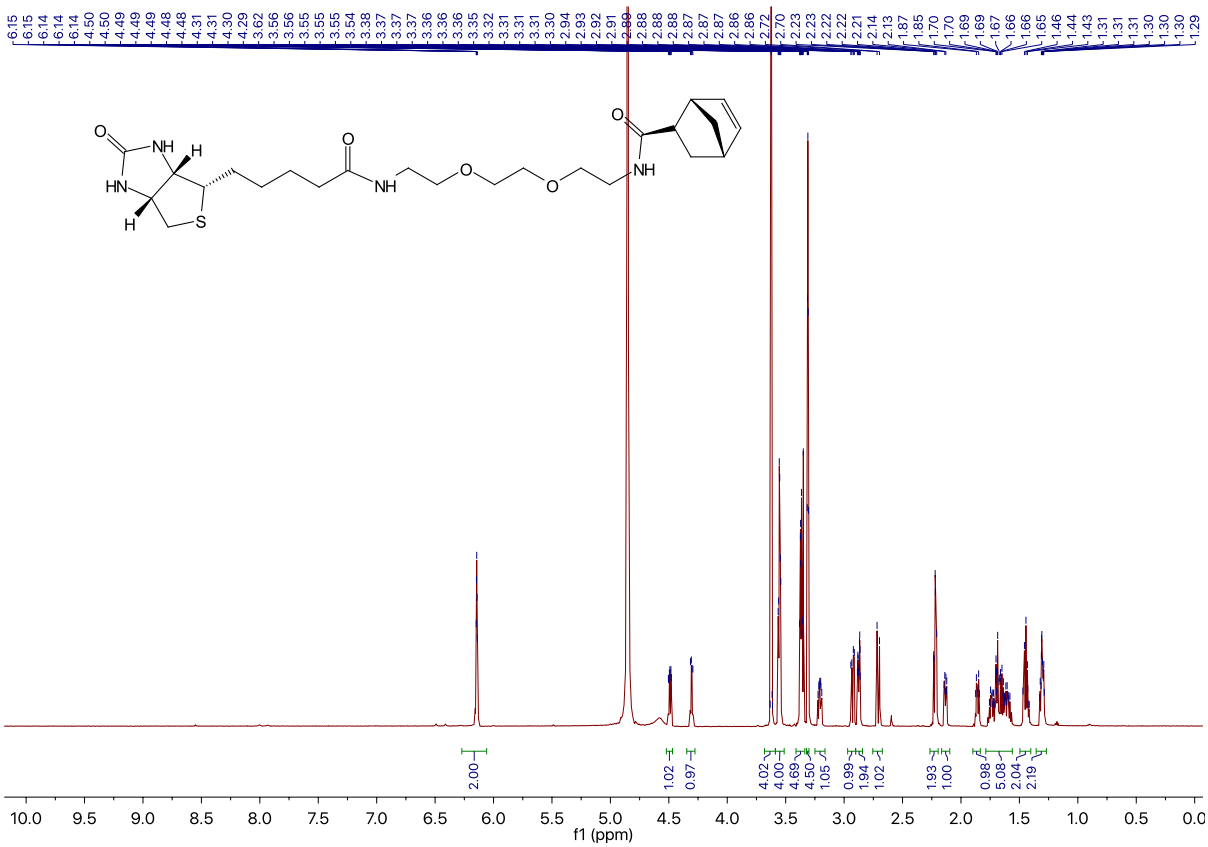


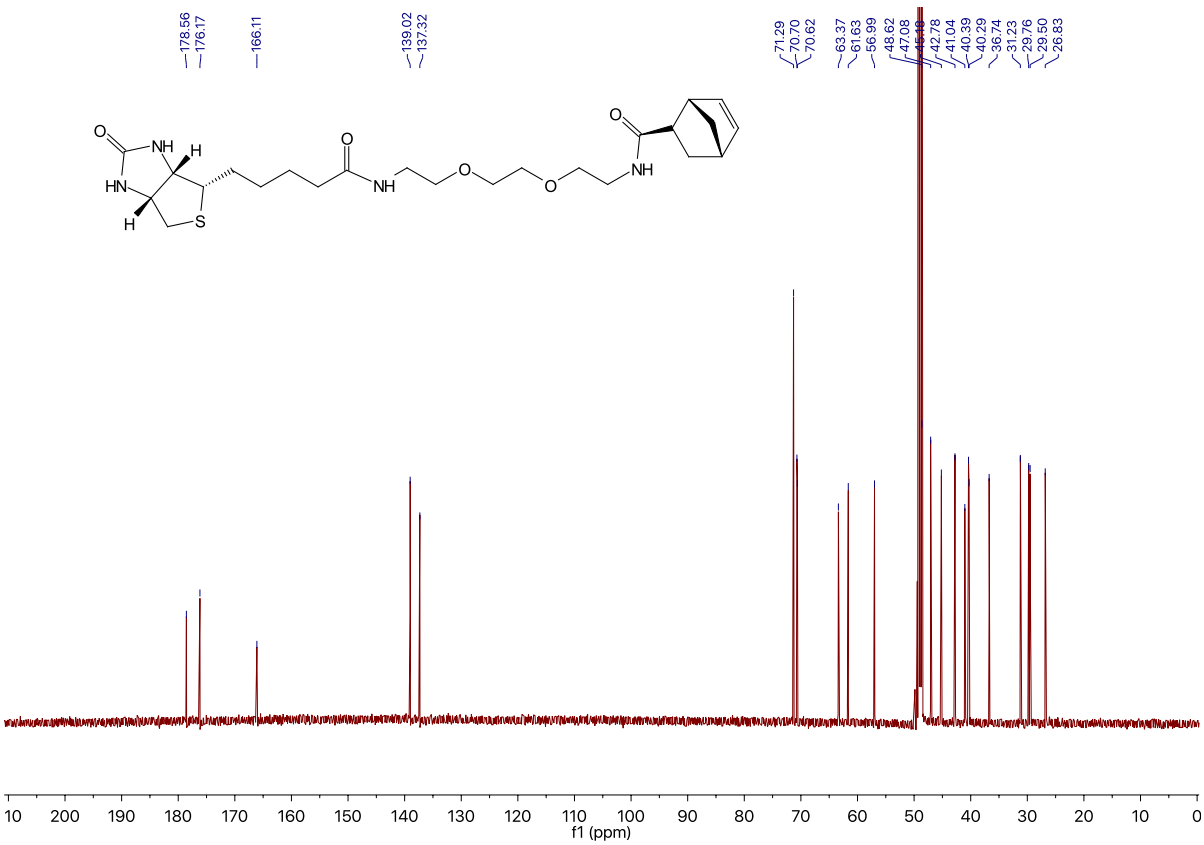
Synthesis of biotin NHS-ester **118**





Synthesis of norbornene probe **70** containing a biotin reporter group





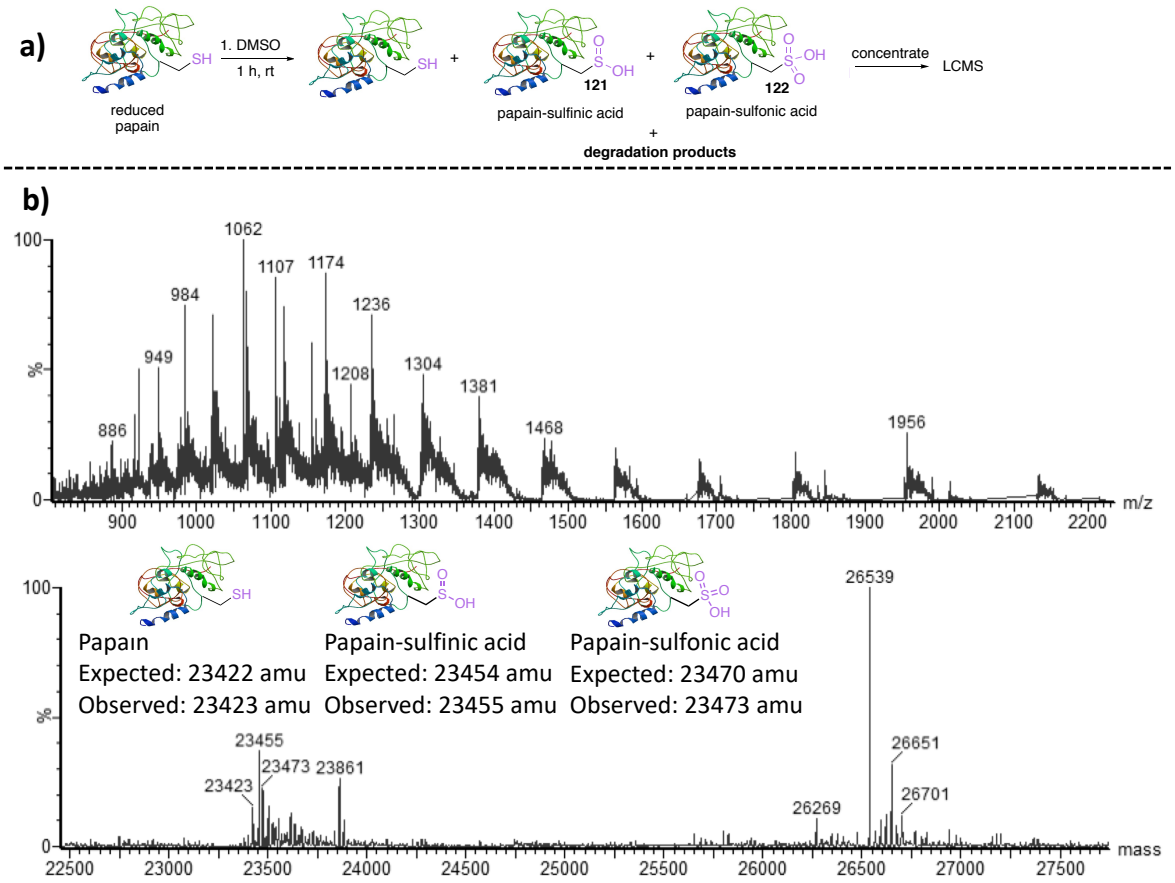
Appendix B: Protein LC-MS data for Chapter three

The following are the mass spectrum for the papain experiments treated with **99** presented in Chapter three, section 3.3.

3.3.2 LC-MS analysis of sulfoxide formation

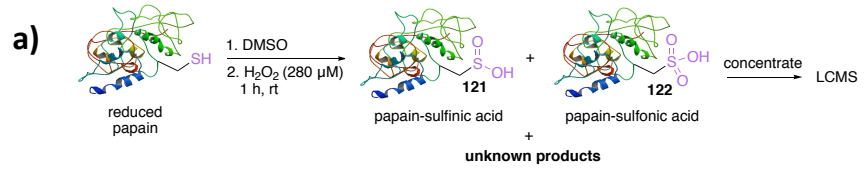
Negative control: no probe 99, no H_2O_2

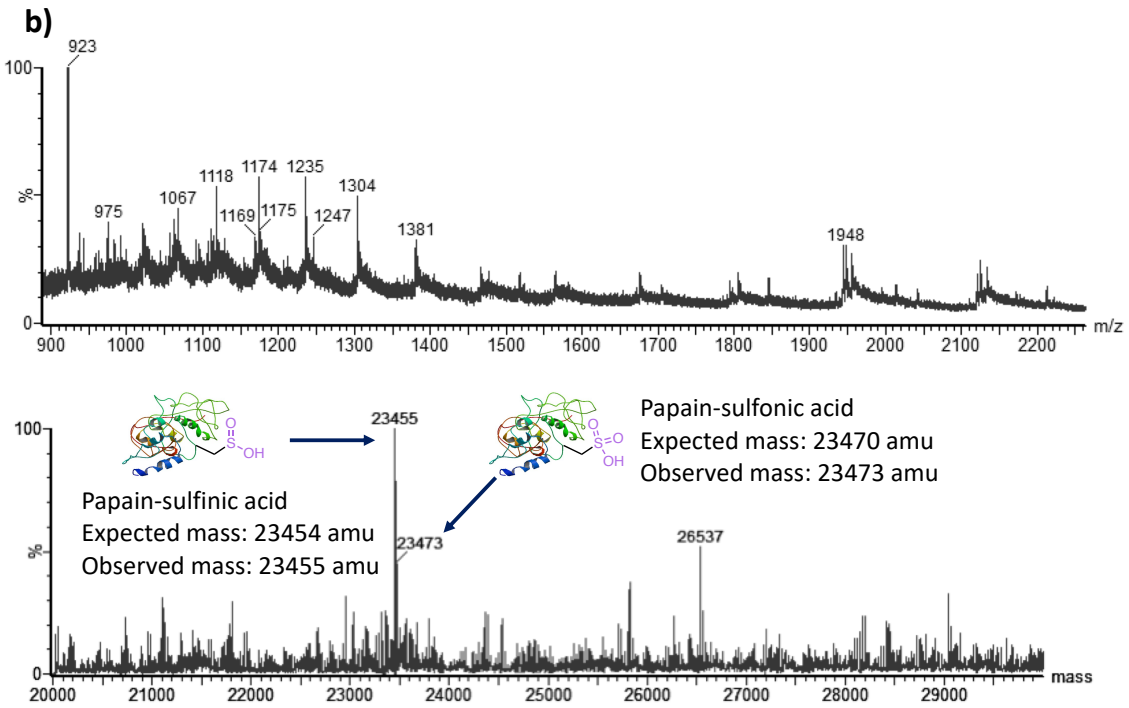
Papain is oxidised by air to give the sulfinic and sulfonic acid products. Several unknown products are also observed.



Control in which papain treated with H_2O_2, but not probe 99.

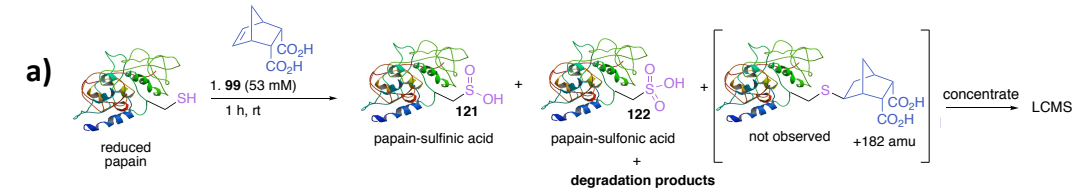
H_2O_2 oxidises papain to the sulfinic and sulfonic acids.

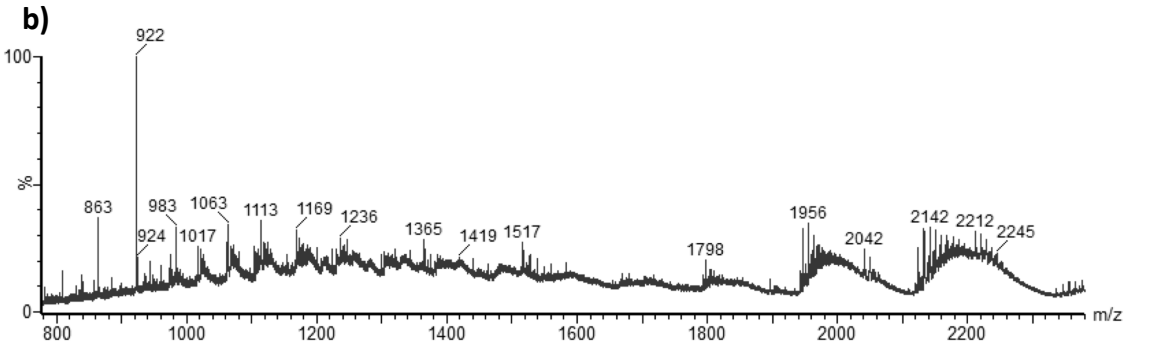


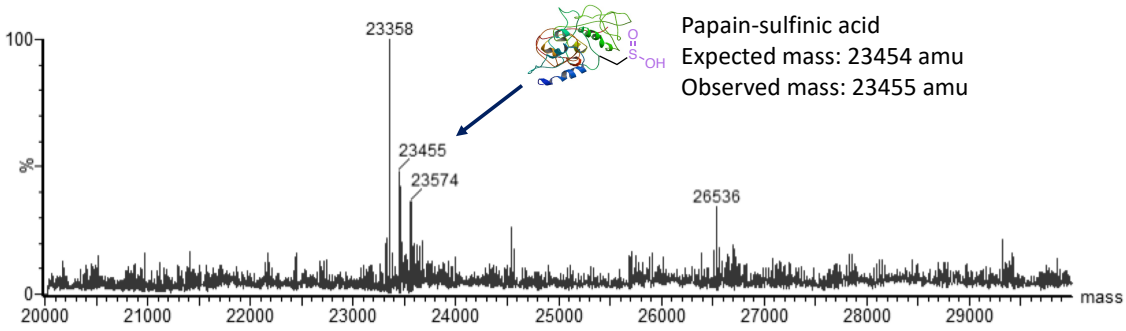


Control reaction in which papain is treated with 99, but not H_2O_2.

No thiol-ene reaction was observed, with some overoxidised sulfinic and sulfonic acids due to air oxidation. Degradation is observed.

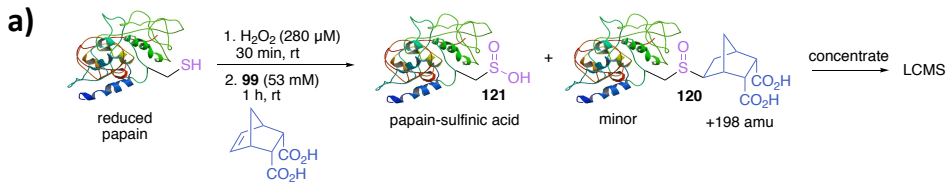


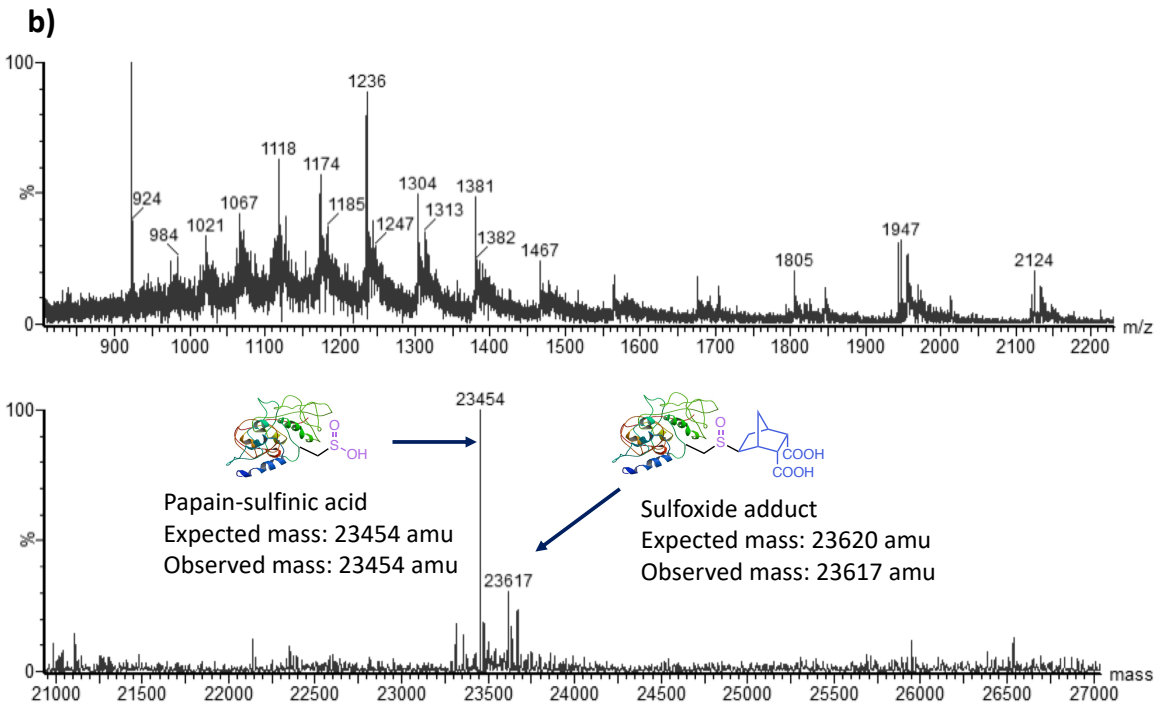




Reaction in which probe 99 is added after papain is pre-treated with H_2O_2.

Probe **99** was able to trap the sulfenic acid, but the conversion is low because of over oxidation.





Appendix C: Protein hits identified in Chapter four

The following are the protein hits identified by probe **70** in the proteomics study presented in Chapter four, section 4.2.3. Protein identification by LC-MS/MS was performed by Maike Langini.

4.5.4 Proteomics experiment whole cell analysis for quantification

List of 482 proteins enriched during treatment of HeLa cells with norb-bio **70** and hydrogen peroxide compared to a control (not treated with hydrogen peroxide). Protein name, peptide count, number of unique peptides identified, confidence score, Anova, and max fold change (between control and treated samples) are displayed. All samples were performed in biological triplicates. More detailed evaluation of protein hits can be found in the supporting information section of the manuscript preprint 'Proteome-wide survey reveals norbornene is complementary to dimedone and related nucleophilic probes for cysteine sulfenic acid' on ChemRxiv (DOI: 10.26434/chemrxiv.8874077.v1).

148 proteins previously unknown to form sulfenic acids

9 proteins which do not contain a cysteine residue

109 proteins common across all sulfenic acid studies

Accession	Protein name	Peptide count	Unique peptide	Confidence score	Anova (p)	Max fold change
Q9Y4F1	FERM, ARHGEF and pleckstrin domain-containing protein 1	2	2	46.82	1.2E-03	560.65
Q99459	Cell division cycle 5-like protein	2	2	78.01	9.7E-04	317.02
Q5JTH9	RRP12-like protein	3	3	97.36	5.5E-04	280.11
P29966	Myristoylated alanine-rich C-kinase substrate	2	2	89.22	7.9E-05	199.55
P53992	Protein transport protein Sec24C	2	2	3.42	4.4E-04	156.93
P16401	Histone H1.5	2	2	15.96	1.8E-03	155.10
Q86UP2	Kinectin	9	9	124.16	5.9E-06	118.59
Q12906	Interleukin enhancer-binding factor 3	2	2	28.7	5.5E-04	113.87
O15042	U2 snRNP-associated SURP motif-containing protein	3	3	57.06	1.3E-03	91.05
P04083	Annexin A1	2	2	27.17	2.5E-03	85.57
O60841	Eukaryotic translation initiation factor 5B	2	2	78.31	2.8E-04	80.32
P13798	Acylamino-acid-releasing enzyme	3	3	67.86	5.1E-06	79.73
P62917	60S ribosomal protein L8	4	4	191.98	9.3E-04	75.23
Q9P2I0	Cleavage and polyadenylation specificity factor subunit 2	2	2	89.52	5.5E-04	61.15
P54886	Delta-1-pyrroline-5-carboxylate synthase	3	2	125.85	1.6E-03	59.60
P27816	Microtubule-associated protein 4	18	17	767.94	3.8E-04	59.10

Q01970	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3	3	3	83.29	4.4E-03	58.37
P49756	RNA-binding protein 25	3	3	51.64	3.3E-04	56.87
Q96SU4	Oxysterol-binding protein-related protein 9	2	2	66.75	1.9E-03	53.33
P11274	Breakpoint cluster region protein	2	2	19.11	8.0E-06	52.78
P41252	Isoleucine--tRNA ligase, cytoplasmic	33	32	1403.5	1.7E-03	51.59
Q8NI27	THO complex subunit 2	4	4	84.26	1.2E-03	49.73
Q16531	DNA damage-binding protein 1	5	3	89.92	3.1E-05	49.45
Q6KC79	Nipped-B-like protein	2	2	72.85	4.4E-03	48.79
Q9C0D5	Protein TANC1	3	3	68.82	3.6E-03	47.75
P26640	Valine--tRNA ligase	5	5	59.29	2.6E-05	46.30
Q14008	Cytoskeleton-associated protein 5	4	4	78.67	1.5E-03	45.71
Q96QK1	Vacuolar protein sorting-associated protein 35	2	2	72.46	3.4E-04	44.66
O94925-3	Isoform 3 of Glutaminase kidney isoform, mitochondrial	2	2	0	7.6E-05	44.38
Q9ULH0	Kinase D-interacting substrate of 220 kDa	2	2	40.21	4.6E-03	44.03
P62979	Ubiquitin-40S ribosomal protein S27a	6	6	335.23	7.9E-04	43.96
Q5SRE5	Nucleoporin NUP188 homolog	6	6	209.37	4.3E-04	41.89
P62753	40S ribosomal protein S6	3	3	139.2	4.5E-05	40.67
A2RRP1	Neuroblastoma-amplified sequence	3	3	105.15	6.3E-03	40.43
P49589	Cysteine--tRNA ligase, cytoplasmic	4	4	7.76	7.1E-03	40.11
Q8TEQ6	Gem-associated protein 5	13	13	578.71	4.5E-04	40.07
Q9NR30	Nucleolar RNA helicase 2	7	7	185.66	2.1E-05	39.74
O75369-8	Isoform 8 of Filamin-B	107	91	0	6.8E-03	39.06
Q02878	60S ribosomal protein L6	5	5	187.54	3.2E-04	38.01
Q12802	A-kinase anchor protein 13	3	3	50.72	3.3E-03	37.76
P30530	Tyrosine-protein kinase receptor UFO	3	3	119.51	2.5E-07	37.46
Q9UQE7	Structural maintenance of chromosomes protein 3	2	2	72.66	2.2E-03	37.35
Q8TF05	Serine/threonine-protein phosphatase 4 regulatory subunit 1	2	2	72.28	4.9E-03	37.13
P06748	Nucleophosmin	3	3	181.49	6.2E-04	37.04
Q9H3S7	Tyrosine-protein phosphatase non-receptor type 23	3	2	76.58	3.1E-03	36.80
P61978	Heterogeneous nuclear ribonucleoprotein K	6	6	219.61	6.9E-04	36.59
P46379	Large proline-rich protein BAG6	5	5	206.73	9.6E-06	35.18
P33527	Multidrug resistance-associated protein 1	2	2	52.72	1.8E-03	34.30
Q9BR76	Coronin-1B	2	2	72.11	5.0E-03	34.24
P30876	DNA-directed RNA polymerase II subunit RPB2	4	4	106.17	1.2E-06	34.10
Q15276	Rab GTPase-binding effector protein 1	2	2	9.01	2.5E-04	34.08
P50914	60S ribosomal protein L14	3	3	188.29	1.6E-03	33.76
Q09666	Neuroblast differentiation-associated protein AHNAK	58	57	2158.8	1.1E-03	33.35
Q9Y5L0	Transportin-3	5	5	85.17	5.3E-04	33.27
Q99570	Phosphoinositide 3-kinase regulatory subunit 4	4	4	132.29	5.9E-04	33.11
P46781	40S ribosomal protein S9	8	7	255.79	1.3E-04	33.06
P15880	40S ribosomal protein S2	6	6	77.72	4.8E-04	32.88
P16402	Histone H1.3	4	4	158.89	1.2E-04	32.45

P46777	60S ribosomal protein L5	2	2	22.27	3.6E-04	32.08
Q13347	Eukaryotic translation initiation factor 3 subunit I	3	3	66.2	5.4E-06	31.97
Q9Y3P9	Rab GTPase-activating protein 1	2	2	10.82	1.5E-05	31.68
P46821	Microtubule-associated protein 1B	20	20	763.48	5.5E-03	31.51
P09382	Galectin-1	5	5	130.75	3.1E-04	31.48
Q27J81	Inverted formin-2	5	5	243.83	5.1E-04	30.60
Q15149	Plectin	57	56	1926.5	1.2E-03	30.24
Q6YHU6	Thyroid adenoma-associated protein	2	2	33.55	7.7E-03	30.19
P51114	Fragile X mental retardation syndrome-related protein 1	3	3	220.87	7.4E-04	29.91
P20810	Calpastatin	5	5	137.22	8.7E-05	29.51
Q8IVT2	Mitotic interactor and substrate of PLK1	2	2	95.27	1.6E-03	29.40
P18621	60S ribosomal protein L17	4	4	54.49	3.7E-03	29.33
Q04206	Transcription factor p65	3	3	76.73	2.1E-03	29.15
O00425	Insulin-like growth factor 2 mRNA-binding protein 3	2	2	25.91	9.8E-04	28.76
P61221	ATP-binding cassette sub-family E member 1	3	3	2.71	4.2E-03	28.42
P46977	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	3	3	48.79	4.4E-05	28.14
A5YKK6	CCR4-NOT transcription complex subunit 1	8	8	258.33	1.5E-03	28.14
P55786	Puromycin-sensitive aminopeptidase	7	7	114.43	1.8E-04	28.05
P46779	60S ribosomal protein L28	4	4	124.55	8.8E-04	27.54
P04075	Fructose-bisphosphate aldolase A	7	7	264.68	2.5E-03	27.37
O75400	Pre-mRNA-processing factor 40 homolog A	3	3	70.05	2.8E-04	27.35
P24666	Low molecular weight phosphotyrosine protein phosphatase	2	2	127.82	3.7E-03	27.19
Q8N726	Tumor suppressor ARF	2	2	39.76	3.3E-03	27.18
O95239	Chromosome-associated kinesin KIF4A	3	3	62.6	2.5E-03	27.16
P11940	Polyadenylate-binding protein 1	7	7	230.43	2.5E-03	27.07
P53621	Coatomer subunit alpha	9	9	327.82	3.5E-05	26.95
P20700	Lamin-B1	5	4	77.03	4.7E-04	26.75
P00374	Dihydrofolate reductase	2	2	68.42	7.0E-04	26.72
O00303	Eukaryotic translation initiation factor 3 subunit F	3	3	168.59	2.1E-03	26.59
P36578	60S ribosomal protein L4	10	10	283.74	8.7E-04	26.58
Q96DV4	39S ribosomal protein L38, mitochondrial	3	2	71.41	4.7E-03	25.81
P63241	Eukaryotic translation initiation factor 5A-1	3	3	89.07	5.2E-03	25.49
O00159	Unconventional myosin-Ic	5	5	119.66	1.4E-04	25.25
Q15477	Helicase SKI2W	7	7	151.47	1.1E-03	25.22
Q9H583	HEAT repeat-containing protein 1	5	5	164.51	7.2E-03	25.20
Q8N163	Cell cycle and apoptosis regulator protein 2	4	4	181.11	8.3E-04	25.15
P33992	DNA replication licensing factor MCM5	4	3	73.78	3.1E-03	25.06
O60610	Protein diaphanous homolog 1	11	11	252.39	2.9E-04	25.06
O95373	Importin-7	7	7	262.76	1.2E-05	24.94
O43707	Alpha-actinin-4	26	26	1405.8	2.7E-04	24.77
Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	2	2	129.95	6.5E-06	24.67

P46087	Probable 28S rRNA (cytosine(4447)-C(5))-methyltransferase	2	2	106.99	1.5E-04	24.55
Q5VYK3	Proteasome adapter and scaffold protein ECM29	5	5	128.93	1.0E-03	24.45
Q9BQA1	Methylosome protein 50	2	2	65.14	3.2E-04	24.38
Q86TG7	Retrotransposon-derived protein PEG10	2	2	181.29	1.0E-03	24.29
Q6PGP7	Tetratricopeptide repeat protein 37	2	2	37.39	1.3E-03	24.23
P83731	60S ribosomal protein L24	4	4	78.63	5.9E-04	23.95
B5ME19	Eukaryotic translation initiation factor 3 subunit C-like protein	9	9	223.26	2.8E-06	23.64
P51532	Transcription activator BRG1	5	5	164.64	5.2E-04	23.52
P35658	Nuclear pore complex protein Nup214	7	7	75.58	4.9E-03	23.45
Q9UBB4	Ataxin-10	3	3	88.93	4.5E-05	23.41
Q14C86	GTPase-activating protein and VPS9 domain-containing protein 1	5	5	240.57	3.0E-04	23.14
P35268	60S ribosomal protein L22	2	2	55.14	8.3E-04	22.99
Q14204	Cytoplasmic dynein 1 heavy chain 1	83	82	2639.4	1.9E-03	22.93
P18085	ADP-ribosylation factor 4	2	2	79.95	8.3E-04	22.88
P62266	40S ribosomal protein S23	2	2	32.41	4.9E-03	22.64
O95782	AP-2 complex subunit alpha-1	4	4	79.94	9.6E-06	22.14
P06733	Alpha-enolase	14	14	554.14	1.4E-03	22.07
Q9NQX3	Gephyrin	3	3	60.63	3.5E-03	22.01
P12814	Alpha-actinin-1	25	25	1004.4	1.4E-04	22.01
Q9HB07	UPF0160 protein MYG1, mitochondrial	2	2	8.86	3.6E-05	21.74
Q92621	Nuclear pore complex protein Nup205	9	8	321.46	2.1E-03	21.70
P61353	60S ribosomal protein L27	2	2	82.93	1.5E-04	21.57
Q13200	26S proteasome non-ATPase regulatory subunit 2	5	5	273.01	1.9E-03	21.40
P40763	Signal transducer and activator of transcription 3	5	5	41.97	3.2E-04	21.40
Q00341	Vigilin	11	11	340.07	1.7E-04	21.39
P11388	DNA topoisomerase 2-alpha	6	6	89.17	1.7E-04	21.35
P62280	40S ribosomal protein S11	10	10	203.83	2.4E-04	21.19
Q9Y224	RNA transcription, translation and transport factor protein	3	3	29.3	1.1E-03	21.09
P22314	Ubiquitin-like modifier-activating enzyme 1	16	16	789.77	1.6E-05	21.00
P08670	Vimentin	19	18	690.08	4.0E-04	21.00
P62424	60S ribosomal protein L7a	7	7	175.93	3.3E-04	20.99
Q9H3U1	Protein unc-45 homolog A	4	4	175.68	3.1E-03	20.76
Q9P035	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	2	2	24.63	4.7E-04	20.73
P62906	60S ribosomal protein L10a	3	3	77.44	4.7E-04	20.66
P26599	Polypyrimidine tract-binding protein 1	4	4	216.28	6.2E-03	20.59
Q99460	26S proteasome non-ATPase regulatory subunit 1	2	2	57.05	1.0E-03	20.54
Q96GQ7	Probable ATP-dependent RNA helicase DDX27	2	2	39.68	5.3E-03	20.49
P61026	Ras-related protein Rab-10	4	3	192.49	3.1E-03	20.42
P29401	Transketolase	7	7	451.5	1.3E-03	20.32
P27348	14-3-3 protein theta	3	3	130.23	4.1E-04	20.25

Q14974	Importin subunit beta-1	6	6	235.34	1.6E-04	20.22
Q15365	Poly(rC)-binding protein 1	5	5	170.23	6.5E-04	20.08
P78527	DNA-dependent protein kinase catalytic subunit	81	79	3512	6.7E-03	20.02
P39023	60S ribosomal protein L3	5	5	192.77	1.2E-05	19.97
P62241	40S ribosomal protein S8	8	8	241.44	1.1E-03	19.91
O95155	Ubiquitin conjugation factor E4 B	2	2	62.28	2.2E-04	19.83
P07814	Bifunctional glutamate/proline--tRNA ligase	57	57	2743.2	3.6E-03	19.53
P50991	T-complex protein 1 subunit delta	9	9	381.4	8.3E-04	19.47
P17980	26S proteasome regulatory subunit 6A	3	3	58.48	9.8E-03	19.44
Q92888	Rho guanine nucleotide exchange factor 1	3	3	128.97	3.0E-04	19.44
Q29RF7	Sister chromatid cohesion protein PDS5 homolog A	4	4	59.94	4.7E-04	19.42
Q9P2R3	Rabankyrin-5	3	2	42.98	1.2E-04	19.42
Q96TA1	Niban-like protein 1	3	3	11.09	3.6E-03	19.32
Q13501	Sequestosome-1	4	4	185.41	3.5E-03	19.29
P40429	60S ribosomal protein L13a	2	2	50.13	9.2E-05	19.13
P13639	Elongation factor 2	32	31	1632	1.1E-03	19.12
Q5T4S7	E3 ubiquitin-protein ligase UBR4	22	22	614.54	2.7E-03	19.09
Q9Y4L1	Hypoxia up-regulated protein 1	2	2	88.52	1.2E-03	19.00
Q99873	Protein arginine N-methyltransferase 1	3	3	71.8	1.0E-03	18.98
P17812	CTP synthase 1	7	7	330.88	2.2E-03	18.95
P07355	Annexin A2	19	18	857.1	8.7E-04	18.87
P31948	Stress-induced-phosphoprotein 1	2	2	40.38	5.3E-05	18.82
Q9P2E9	Ribosome-binding protein 1	3	3	133.35	2.1E-04	18.80
Q99715	Collagen alpha-1(XII) chain	6	6	121.87	3.2E-04	18.78
Q93009	Ubiquitin carboxyl-terminal hydrolase 7	4	4	90.36	1.7E-05	18.71
O14980	Exportin-1	6	6	207.92	5.1E-05	18.59
P08243	Asparagine synthetase [glutamine-hydrolyzing]	2	2	86.45	5.1E-03	18.58
Q12769	Nuclear pore complex protein Nup160	7	6	257.1	2.2E-03	18.49
P50990	T-complex protein 1 subunit theta	13	13	520.87	8.2E-04	18.40
Q9P2J5	Leucine--tRNA ligase, cytoplasmic	18	18	745.67	1.3E-04	18.37
Q8TEX9	Importin-4	10	10	530.29	1.4E-04	18.36
P49588	Alanine--tRNA ligase, cytoplasmic	10	10	378.43	9.0E-05	18.08
P62701	40S ribosomal protein S4, X isoform	7	6	206.76	3.9E-04	18.08
Q92974	Rho guanine nucleotide exchange factor 2	4	3	130.64	1.1E-03	18.07
Q9NZM1	Myoferlin	10	10	305.18	8.7E-04	18.04
Q02543	60S ribosomal protein L18a	7	7	290.39	3.8E-04	17.96
P52292	Importin subunit alpha-1	9	9	334.29	1.3E-03	17.89
Q9NSV4	Protein diaphanous homolog 3	3	3	87.33	3.2E-05	17.85
Q92900	Regulator of nonsense transcripts 1	2	2	12.48	4.8E-04	17.81
P62244	40S ribosomal protein S15a	4	3	82.79	2.3E-05	17.79
Q9BTW9	Tubulin-specific chaperone D	2	2	50.29	6.6E-05	17.68
Q14683	Structural maintenance of chromosomes protein 1A	5	3	118.4	1.1E-04	17.67
O00410	Importin-5	6	5	240.63	3.5E-04	17.65
Q9H0A0	RNA cytidine acetyltransferase	5	5	144.88	2.5E-06	17.63
P62249	40S ribosomal protein S16	3	3	128.08	1.1E-03	17.63

P61204	ADP-ribosylation factor 3	3	3	85.03	1.4E-04	17.33
Q8TAQ2	SWI/SNF complex subunit SMARCC2	4	4	126.74	5.4E-04	17.23
Q9NR12	PDZ and LIM domain protein 7	5	5	112.55	3.5E-04	17.21
P26641	Elongation factor 1-gamma	5	5	267.71	4.5E-04	17.19
Q96T88	E3 ubiquitin-protein ligase UHRF1	2	2	58.98	6.5E-04	17.16
Q86VP6	Cullin-associated NEDD8-dissociated protein 1	17	17	585.86	4.2E-04	17.14
P30050	60S ribosomal protein L12	5	5	124.84	2.4E-03	17.03
Q8TCJ2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	3	3	55.17	1.5E-03	16.98
Q8TD19	Serine/threonine-protein kinase Nek9	3	3	68.79	5.4E-05	16.88
A0AVT1	Ubiquitin-like modifier-activating enzyme 6	3	3	86.07	1.6E-05	16.72
P31327	Carbamoyl-phosphate synthase [ammonia], mitochondrial	24	24	751.14	1.4E-03	16.71
P25205	DNA replication licensing factor MCM3	3	3	122.07	3.3E-04	16.70
Q96N67	Dedicator of cytokinesis protein 7	2	2	28.02	5.1E-05	16.65
P62829	60S ribosomal protein L23	4	4	170.32	1.7E-03	16.64
Q32MZ4	Leucine-rich repeat flightless-interacting protein 1	3	3	56.99	5.4E-04	16.60
P23528	Cofilin-1	3	3	56.23	2.9E-03	16.55
P56192	Methionine--tRNA ligase, cytoplasmic	16	15	455.94	1.5E-04	16.55
P04406	Glyceraldehyde-3-phosphate dehydrogenase	2	2	124.49	5.5E-04	16.54
P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	7	7	172.82	7.7E-05	16.42
P41091	Eukaryotic translation initiation factor 2 subunit 3	4	4	78.1	1.0E-03	16.19
P68104	Elongation factor 1-alpha 1	12	11	386.7	5.8E-04	16.17
Q00610	Clathrin heavy chain 1	68	68	4178.6	9.7E-05	16.04
Q07020	60S ribosomal protein L18	3	3	149.86	5.8E-04	16.04
P62888	60S ribosomal protein L30	3	3	118.06	1.8E-03	16.01
P60866	40S ribosomal protein S20	2	2	70.98	6.5E-05	15.95
Q9BXJ9	N-alpha-acetyltransferase 15, NatA auxiliary subunit	4	4	104.72	6.5E-04	15.92
P49368	T-complex protein 1 subunit gamma	8	8	309.46	1.7E-03	15.90
Q68CZ2	Tensin-3	2	2	21.52	1.4E-03	15.87
P49792	E3 SUMO-protein ligase RanBP2	28	28	727.68	1.0E-03	15.78
Q14203	Dynactin subunit 1	6	6	205.03	4.8E-04	15.70
O60506	Heterogeneous nuclear ribonucleoprotein Q	3	3	65.36	3.0E-03	15.69
P27635	60S ribosomal protein L10	2	2	78.89	2.7E-03	15.67
Q9Y2L1	Exosome complex exonuclease RRP44	3	3	78.7	3.2E-04	15.65
Q32P28	Prolyl 3-hydroxylase 1	2	2	53.98	9.8E-05	15.64
P23526	Adenosylhomocysteinase	5	4	160.35	3.3E-04	15.63
P48507	Glutamate--cysteine ligase regulatory subunit	2	2	51.07	2.2E-04	15.62
Q09028	Histone-binding protein RBBP4	3	3	69.63	7.3E-04	15.44
P27824	Calnexin	4	4	166.64	1.5E-04	15.41
P11413	Glucose-6-phosphate 1-dehydrogenase	4	4	162.3	4.8E-03	15.40
Q99829	Copine-1	5	5	226.58	1.3E-03	15.11
Q9UMS4	Pre-mRNA-processing factor 19	3	3	45.33	7.2E-03	15.10

Q63ZY3	KN motif and ankyrin repeat domain-containing protein 2	5	5	175.63	1.1E-04	14.97
P52597	Heterogeneous nuclear ribonucleoprotein F	2	2	64.52	9.0E-03	14.94
O15160	DNA-directed RNA polymerases I and III subunit RPAC1	2	2	2.91	2.2E-03	14.89
Q04695	Keratin, type I cytoskeletal 17	28	27	1576.3	2.5E-03	14.88
P07900	Heat shock protein HSP 90-alpha	17	16	785.37	1.9E-03	14.86
Q15393	Splicing factor 3B subunit 3	5	5	106.21	4.8E-05	14.81
Q9NQW6	Anillin	4	4	134.96	3.7E-04	14.81
Q13637	Ras-related protein Rab-32	2	2	61.79	1.5E-03	14.78
P53396	ATP-citrate synthase	37	36	1925	5.9E-04	14.76
P18124	60S ribosomal protein L7	4	4	135.84	1.5E-03	14.75
Q5H9R7	Serine/threonine-protein phosphatase 6 regulatory subunit 3	4	4	90.31	2.8E-04	14.71
P42224	Signal transducer and activator of transcription 1-alpha/beta	6	4	119.29	3.3E-03	14.70
O75821	Eukaryotic translation initiation factor 3 subunit G	2	2	31.88	1.2E-03	14.59
Q9NR09	Baculoviral IAP repeat-containing protein 6	8	8	126.74	2.4E-03	14.51
P39880	Homeobox protein cut-like 1	2	2	3.19	4.7E-04	14.50
Q9Y2D5	A-kinase anchor protein 2	2	2	102.91	9.5E-04	14.49
P0DMV9	Heat shock 70 kDa protein 1B	15	15	621.04	2.3E-03	14.46
P62913	60S ribosomal protein L11	4	3	138.34	9.8E-04	14.45
Q15046	Lysine--tRNA ligase	4	4	104.23	2.8E-04	14.36
Q13084	39S ribosomal protein L28, mitochondrial	2	2	0	8.6E-04	14.33
P62263	40S ribosomal protein S14	6	5	346.59	2.4E-04	14.32
P62081	40S ribosomal protein S7	7	7	104.63	7.0E-03	14.31
P50570	Dynamin-2	3	3	97.14	9.1E-05	14.24
Q12888	TP53-binding protein 1	3	3	109.18	4.4E-04	14.15
Q8IVF2	Protein AHNAK2	7	7	148.49	6.9E-04	14.06
Q92598	Heat shock protein 105 kDa	9	9	318.04	1.9E-04	14.05
Q04637	Eukaryotic translation initiation factor 4 gamma 1	16	16	388.65	6.2E-04	14.01
Q9Y4W2	Ribosomal biogenesis protein LAS1L	4	4	140.98	4.8E-05	14.00
O15357	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2	2	2	17.48	1.2E-03	13.95
Q8WUM4	Programmed cell death 6-interacting protein	9	9	181.92	2.1E-04	13.95
P36542	ATP synthase subunit gamma, mitochondrial	2	2	30.91	5.6E-03	13.90
Q9UJS0	Calcium-binding mitochondrial carrier protein Aralar2	3	3	124.31	1.7E-03	13.90
P04792	Heat shock protein beta-1	10	10	617.27	3.0E-03	13.81
Q14166	Tubulin--tyrosine ligase-like protein 12	4	4	98.36	1.6E-04	13.74
O60343	TBC1 domain family member 4	9	9	365.62	1.3E-04	13.74
Q9HAV4	Exportin-5	5	5	288.79	3.4E-04	13.73
P47712	Cytosolic phospholipase A2	3	3	168.56	1.4E-03	13.64
O43795	Unconventional myosin-Ib	2	2	39.89	3.7E-04	13.56
Q9ULT8	E3 ubiquitin-protein ligase HECTD1	6	6	50	4.3E-03	13.55
Q14152	Eukaryotic translation initiation factor 3 subunit A	13	12	422.59	8.3E-04	13.52

Q01813	ATP-dependent 6-phosphofructokinase, platelet type	12	11	251.22	8.9E-04	13.51
P55072	Transitional endoplasmic reticulum ATPase	22	22	1028.4	1.2E-03	13.45
O60264	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	6	6	128.01	3.4E-05	13.28
Q8NB46	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C	2	2	11.39	2.5E-04	13.26
Q8N0X7	Spartin	2	2	51.23	1.5E-04	13.21
Q92973	Transportin-1	10	10	257.33	8.9E-04	13.21
Q14839	Chromodomain-helicase-DNA-binding protein 4	5	4	72.63	9.6E-03	13.10
O75489	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	2	2	19.3	4.2E-04	12.84
Q9Y678	Coatomer subunit gamma-1	8	8	311.62	2.1E-03	12.74
O00469	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	10	9	319.05	9.6E-05	12.72
Q9BPX3	Condensin complex subunit 3	3	3	139.62	1.9E-05	12.67
O43847-2	Isoform 2 of Nardilysin	3	2	0	5.5E-05	12.67
Q7L576	Cytoplasmic FMR1-interacting protein 1	6	6	198.22	1.7E-04	12.60
Q9NZB2	Constitutive coactivator of PPAR-gamma-like protein 1	6	6	115.34	1.2E-04	12.58
P05388	60S acidic ribosomal protein P0	4	4	131.72	1.1E-03	12.58
P02545	Prelamin-A/C	20	20	1224.4	5.9E-04	12.53
P29692	Elongation factor 1-delta	7	7	252.14	5.5E-04	12.52
Q92841	Probable ATP-dependent RNA helicase DDX17	6	6	209.91	2.5E-04	12.51
Q92576	PHD finger protein 3	2	2	34.38	8.6E-03	12.49
P17987	T-complex protein 1 subunit alpha	8	8	330.43	2.1E-03	12.49
O94776	Metastasis-associated protein MTA2	3	3	150.58	2.5E-04	12.47
P55265	Double-stranded RNA-specific adenosine deaminase	3	2	48.62	5.2E-04	12.37
Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	2	2	45.81	8.3E-05	12.31
P12956	X-ray repair cross-complementing protein 6	2	2	42.73	7.7E-04	12.31
Q02218	2-oxoglutarate dehydrogenase, mitochondrial	4	4	119.13	2.6E-04	12.28
Q96P70	Importin-9	3	3	212.54	1.0E-03	12.17
P12268	Inosine-5'-monophosphate dehydrogenase 2	11	11	277.77	6.8E-04	12.17
P49736	DNA replication licensing factor MCM2	2	2	0	3.0E-03	12.15
P15170-3	Isoform 3 of Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	3	3	0	1.2E-04	12.14
P35998	26S proteasome regulatory subunit 7	2	2	29.27	3.5E-03	12.09
Q12931	Heat shock protein 75 kDa, mitochondrial	9	8	593.92	3.1E-03	12.04
Q13155	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	5	5	221.75	1.2E-03	12.01
Q9H0D6	5'-3' exoribonuclease 2	2	2	33.31	2.1E-04	11.99
Q16881	Thioredoxin reductase 1, cytoplasmic	12	12	491.02	2.4E-03	11.94
P50552	Vasodilator-stimulated phosphoprotein	2	2	26.76	1.1E-04	11.94
P34897	Serine hydroxymethyltransferase, mitochondrial	7	7	191.13	5.2E-03	11.92
Q01433	AMP deaminase 2	6	6	141.9	6.4E-05	11.83
P13010	X-ray repair cross-complementing protein 5	6	6	125.49	6.2E-04	11.75

P43686	26S proteasome regulatory subunit 6B	5	5	79.82	1.9E-03	11.73
Q93008	Probable ubiquitin carboxyl-terminal hydrolase FAF-X	10	10	260.61	2.1E-03	11.69
P30044	Peroxiredoxin-5, mitochondrial	2	2	7.7	2.1E-03	11.68
O43390	Heterogeneous nuclear ribonucleoprotein R	3	3	139.19	1.1E-03	11.67
P23921	Ribonucleoside-diphosphate reductase large subunit	10	10	163.36	6.6E-04	11.58
O14744	Protein arginine N-methyltransferase 5	8	7	145.22	4.3E-03	11.58
P08238	Heat shock protein HSP 90-beta	14	14	496.79	4.9E-03	11.55
Q00839	Heterogeneous nuclear ribonucleoprotein U	14	13	594.03	2.4E-04	11.52
P32969	60S ribosomal protein L9	7	7	218.36	9.4E-03	11.46
O75691	Small subunit processome component 20 homolog	5	5	182.79	4.4E-04	11.46
Q13557	Calcium/calmodulin-dependent protein kinase type II subunit delta	4	4	78.46	1.6E-03	11.43
P55884	Eukaryotic translation initiation factor 3 subunit B	8	8	281.11	7.9E-04	11.39
Q9UBB6	Neurochondrin	2	2	42.98	7.4E-04	11.36
O14617	AP-3 complex subunit delta-1	2	2	119.5	3.1E-04	11.24
Q9BVP2	Guanine nucleotide-binding protein-like 3	3	3	41.93	6.4E-05	11.22
P05141	ADP/ATP translocase 2	2	2	116.46	8.8E-04	11.14
Q14566	DNA replication licensing factor MCM6	5	5	132.61	2.7E-04	11.14
P11142	Heat shock cognate 71 kDa protein	18	18	1146.3	2.8E-03	11.12
P42166	Lamina-associated polypeptide 2, isoform alpha	6	6	145.93	4.7E-04	11.07
P57740	Nuclear pore complex protein Nup107	4	4	90.2	1.2E-04	10.96
O95248	Myotubularin-related protein 5	2	2	60.16	3.5E-03	10.88
Q7L2E3	Putative ATP-dependent RNA helicase DHX30	4	4	227.73	2.9E-04	10.88
P12236	ADP/ATP translocase 3	8	8	297.6	1.7E-03	10.84
P45974	Ubiquitin carboxyl-terminal hydrolase 5	4	4	119.45	2.3E-05	10.84
P22102	Trifunctional purine biosynthetic protein adenosine-3	16	16	388.37	4.7E-04	10.78
Q6PJG6	BRCA1-associated ATM activator 1	3	3	103.41	2.3E-03	10.70
P36957	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	4	4	146.77	3.0E-03	10.70
Q9NYU2	UDP-glucose:glycoprotein glucosyltransferase 1	3	3	46.69	2.4E-03	10.66
O43592	Exportin-T	3	3	102.84	1.4E-03	10.65
Q9Y6D5	Brefeldin A-inhibited guanine nucleotide-exchange protein 2	3	3	69.19	2.4E-03	10.65
O75533	Splicing factor 3B subunit 1	4	4	38.55	1.1E-05	10.65
P11021	Endoplasmic reticulum chaperone BiP	13	13	623.54	2.6E-03	10.64
Q7KZF4	Staphylococcal nuclease domain-containing protein 1	11	10	267.68	1.4E-04	10.62
P62826	GTP-binding nuclear protein Ran	9	9	400.53	9.5E-04	10.62
Q8WWM7	Ataxin-2-like protein	3	3	110.48	1.1E-03	10.60
P11166	Solute carrier family 2, facilitated glucose transporter member 1	2	2	77.17	2.2E-04	10.53
P62736	Actin, aortic smooth muscle	2	2	45.14	7.2E-03	10.51
P61313	60S ribosomal protein L15	5	5	187.98	2.0E-03	10.50
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	9	9	316.43	6.5E-04	10.41
Q6P2Q9	Pre-mRNA-processing-splicing factor 8	18	18	404.69	8.4E-03	10.39

P00390	Glutathione reductase, mitochondrial	2	2	61.25	2.7E-03	10.39
Q9BUF5	Tubulin beta-6 chain	7	7	249.58	4.1E-03	10.38
Q5UIP0	Telomere-associated protein RIF1	7	7	105.01	5.9E-05	10.36
Q99714	3-hydroxyacyl-CoA dehydrogenase type-2	3	3	142.04	5.2E-03	10.36
P05787	Keratin, type II cytoskeletal 8	14	14	479.5	1.9E-03	10.33
P08729	Keratin, type II cytoskeletal 7	28	25	1507.6	7.9E-03	10.30
Q96CX2	BTB/POZ domain-containing protein KCTD12	2	2	52.31	1.8E-03	10.29
P14625	Endoplasmin	13	13	440.74	1.2E-03	10.20
P07195	L-lactate dehydrogenase B chain	6	6	183.96	1.0E-03	10.20
Q92945	Far upstream element-binding protein 2	6	6	257.54	1.2E-03	10.16
Q15020	Squamous cell carcinoma antigen recognized by T-cells 3	4	4	106.5	4.8E-04	10.16
P42704	Leucine-rich PPR motif-containing protein, mitochondrial	14	14	504.68	4.2E-03	10.09
P68371	Tubulin beta-4B chain	6	5	242.3	4.8E-03	10.04
P10515	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	5	5	219.22	5.8E-04	10.00
P33176	Kinesin-1 heavy chain	10	10	265.93	3.9E-04	9.98
Q99567	Nuclear pore complex protein Nup88	3	3	82.19	1.0E-03	9.97
O15294	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit	3	3	63.68	1.1E-04	9.97
P22234	Multifunctional protein ADE2	16	16	1094.1	7.8E-03	9.93
Q08379	Golgin subfamily A member 2	7	7	191.37	2.9E-03	9.93
Q01780	Exosome component 10	2	2	62.64	1.2E-03	9.87
P11717	Cation-independent mannose-6-phosphate receptor	2	2	59.75	7.7E-03	9.87
Q9UNF1	Melanoma-associated antigen D2	2	2	11.34	6.9E-03	9.76
P21980	Protein-glutamine gamma-glutamyltransferase 2	2	2	22.91	2.0E-04	9.75
P40227	T-complex protein 1 subunit zeta	12	12	505.56	3.6E-03	9.71
P45880	Voltage-dependent anion-selective channel protein 2	4	4	175.87	2.3E-03	9.70
P17844	Probable ATP-dependent RNA helicase DDX5	6	6	159.2	2.5E-04	9.69
P52732	Kinesin-like protein KIF11	2	2	86.83	2.0E-04	9.68
Q8IWZ3	Ankyrin repeat and KH domain-containing protein 1	6	6	206.83	8.7E-03	9.64
Q12797	Aspartyl/asparaginyl beta-hydroxylase	4	4	37.35	9.5E-04	9.58
P48643	T-complex protein 1 subunit epsilon	5	5	153.16	5.9E-03	9.58
P68366	Tubulin alpha-4A chain	6	6	409.62	7.2E-03	9.53
P11586	C-1-tetrahydrofolate synthase, cytoplasmic	12	11	488.81	4.5E-04	9.47
O60568	Multifunctional procollagen lysine hydroxylase and glycosyltransferase LH3	2	2	72.37	2.6E-04	9.44
P06858	Lipoprotein lipase	2	2	98.79	1.6E-03	9.42
O75643	U5 small nuclear ribonucleoprotein 200 kDa helicase	16	16	694.92	4.9E-03	9.40
P63244	Receptor of activated protein C kinase 1	10	10	442.91	4.3E-03	9.39
O43175	D-3-phosphoglycerate dehydrogenase	8	8	285.76	5.5E-03	9.32
P46940	Ras GTPase-activating-like protein IQGAP1	32	30	1038.2	9.4E-03	9.21
P08195	4F2 cell-surface antigen heavy chain	14	14	508.29	1.9E-03	9.17

P05783	Keratin, type I cytoskeletal 18	9	9	180.08	3.3E-03	9.12
Q06210	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	6	6	149.45	1.0E-03	9.10
Q9BWH6	RNA polymerase II-associated protein 1	3	3	79.61	2.4E-03	9.04
P54136	Arginine--tRNA ligase, cytoplasmic	13	13	566.32	2.8E-03	9.00
O75116	Rho-associated protein kinase 2	10	10	470.96	2.6E-03	8.89
P33991	DNA replication licensing factor MCM4	5	5	237.25	2.3E-04	8.88
P29144	Tripeptidyl-peptidase 2	3	3	29.06	4.7E-03	8.82
O95347	Structural maintenance of chromosomes protein 2	18	17	390.72	1.3E-03	8.78
Q14694	Ubiquitin carboxyl-terminal hydrolase 10	5	5	206.38	1.3E-04	8.68
Q9NU22	Midasin	7	7	108.41	2.1E-04	8.68
P43243	Matrin-3	4	4	89.82	9.1E-05	8.68
Q9Y6Y8	SEC23-interacting protein	2	2	0.92	3.6E-04	8.40
Q9NXH9	tRNA (guanine(26)-N(2))-dimethyltransferase	2	2	62.53	2.0E-03	8.34
P78347	General transcription factor II-I	5	5	80.08	7.2E-03	8.28
P23396	40S ribosomal protein S3	11	11	357.47	4.1E-03	8.17
Q96P48	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1	4	4	67.86	1.3E-03	8.08
P07437	Tubulin beta chain	16	15	969.57	9.1E-03	8.06
Q14697	Neutral alpha-glucosidase AB	10	10	230.57	2.0E-03	8.06
P84103	Serine/arginine-rich splicing factor 3	3	3	17.07	3.0E-03	8.03
O60664	Perilipin-3	2	2	77.5	4.2E-04	8.02
P25705	ATP synthase subunit alpha, mitochondrial	12	12	735.04	7.0E-03	8.02
O95433	Activator of 90 kDa heat shock protein ATPase homolog 1	2	2	19.01	5.8E-04	7.99
O95071	E3 ubiquitin-protein ligase UBR5	6	5	170.62	9.1E-03	7.97
O43865	S-adenosylhomocysteine hydrolase-like protein 1	2	2	48.11	2.0E-04	7.95
Q6PKG0	La-related protein 1	9	8	218.93	7.7E-03	7.91
P00338	L-lactate dehydrogenase A chain	7	7	246.27	7.1E-04	7.81
O75962	Triple functional domain protein	18	16	521.92	8.2E-03	7.80
Q8NBJ5	Procollagen galactosyltransferase 1	5	5	146.96	2.4E-04	7.79
Q71U36	Tubulin alpha-1A chain	17	16	1294	8.9E-03	7.76
P06576	ATP synthase subunit beta, mitochondrial	9	7	462.75	9.5E-03	7.71
O94979	Protein transport protein Sec31A	4	4	104.56	6.5E-04	7.60
O00139	Kinesin-like protein KIF2A	4	4	124.36	2.8E-03	7.57
O75534	Cold shock domain-containing protein E1	7	6	271.77	7.3E-04	7.54
P06753-2	Isoform 2 of Tropomyosin alpha-3 chain	7	7	0	6.7E-03	7.25
P26373	60S ribosomal protein L13	3	3	100.86	4.1E-03	7.24
Q8N1F7	Nuclear pore complex protein Nup93	5	5	121.38	3.3E-04	7.14
Q9C0C2	182 kDa tankyrase-1-binding protein	6	6	182.94	4.4E-04	7.08
O43242	26S proteasome non-ATPase regulatory subunit 3	3	3	126.94	9.5E-03	7.08
Q08J23	tRNA (cytosine(34)-C(5))-methyltransferase	10	10	350.61	3.5E-03	7.02
Q8IY17	Neuropathy target esterase	2	2	149.49	4.0E-03	7.00
Q92922	SWI/SNF complex subunit SMARCC1	3	3	85.07	6.8E-03	6.98
Q92878	DNA repair protein RAD50	7	7	101.47	3.5E-04	6.91

P41250	Glycine--tRNA ligase	7	7	419.28	1.8E-03	6.91
P78371	T-complex protein 1 subunit beta	7	7	305.44	2.7E-03	6.88
Q9Y570	Protein phosphatase methylesterase 1	2	2	36.65	2.8E-03	6.88
O15084	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A	2	2	83.8	7.6E-04	6.79
Q13813	Spectrin alpha chain, non-erythrocytic 1	23	22	820.47	5.7E-03	6.69
Q86X55	Histone-arginine methyltransferase CARM1	2	2	0	5.1E-03	6.60
O75153	Clustered mitochondria protein homolog	2	2	50.35	2.0E-03	6.59
P06493	Cyclin-dependent kinase 1	4	3	108.35	3.7E-03	6.54
Q9NVI7	ATPase family AAA domain-containing protein 3A	18	18	600.65	4.4E-03	6.53
P12081	Histidine--tRNA ligase, cytoplasmic	5	5	153.81	2.1E-03	6.50
O15067	Phosphoribosylformylglycinamidine synthase	11	10	579.32	4.3E-03	6.48
Q15084	Protein disulfide-isomerase A6	3	3	118.02	5.1E-03	6.48
Q13310	Polyadenylate-binding protein 4	9	9	259.56	6.5E-03	6.38
P04843	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	5	5	171.2	5.0E-03	6.32
Q96A08	Histone H2B type 1-A	2	2	46.67	2.3E-03	6.30
P55060	Exportin-2	7	7	114.51	1.7E-03	6.21
Q6UB35	Monofunctional C1-tetrahydrofolate synthase, mitochondrial	8	7	314.42	9.4E-04	6.06
P62937	Peptidyl-prolyl cis-trans isomerase A	2	2	56.45	8.2E-03	6.05
P33993	DNA replication licensing factor MCM7	11	11	442.72	2.7E-03	6.02
Q92667	A-kinase anchor protein 1, mitochondrial	2	2	3.1	6.7E-04	6.02
P30837	Aldehyde dehydrogenase X, mitochondrial	2	2	75.62	2.8E-03	5.95
P14868	Aspartate--tRNA ligase, cytoplasmic	9	9	265.98	5.9E-03	5.94
Q08499	cAMP-specific 3',5'-cyclic phosphodiesterase 4D	2	2	71.97	5.0E-03	5.87
Q8WX93	Palladin	7	7	116.58	1.1E-03	5.84
Q96T76	MMS19 nucleotide excision repair protein homolog	4	4	69.54	6.7E-03	5.82
P28340	DNA polymerase delta catalytic subunit	8	7	220.4	1.3E-03	5.67
Q13045	Protein flightless-1 homolog	8	8	270.56	5.0E-04	5.61
P06737	Glycogen phosphorylase, liver form	3	3	95.06	9.9E-04	5.54
P43246	DNA mismatch repair protein Msh2	4	4	49.22	4.3E-03	5.53
P57678	Gem-associated protein 4	5	5	87.49	3.8E-04	5.50
P08240	Signal recognition particle receptor subunit alpha	2	2	106.05	8.4E-04	5.45
P24534	Elongation factor 1-beta	2	2	62.04	1.6E-03	5.39
O00203	AP-3 complex subunit beta-1	2	2	58.37	5.4E-03	5.31
P38646	Stress-70 protein, mitochondrial	10	10	440.69	5.0E-03	5.31
P17655	Calpain-2 catalytic subunit	4	4	95.43	2.3E-03	5.30
Q14980	Nuclear mitotic apparatus protein 1	20	20	575.69	7.0E-03	5.28
O95163	Elongator complex protein 1	8	8	154.05	7.2E-04	5.25
Q12789	General transcription factor 3C polypeptide 1	6	6	78.58	2.0E-03	5.20
Q9BYD1	39S ribosomal protein L13, mitochondrial	2	2	48.21	2.7E-03	5.19
Q99623	Prohibitin-2	4	4	50.52	7.6E-04	5.10
Q96PK6	RNA-binding protein 14	2	2	31.26	4.1E-03	5.06
O00411	DNA-directed RNA polymerase, mitochondrial	2	2	61.55	8.1E-04	5.04

P35908	Keratin, type II cytoskeletal 2 epidermal	20	20	804.06	4.5E-03	4.93
O43143	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	10	9	268.46	7.1E-03	4.85
P28331	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	2	2	56.97	6.9E-03	4.78
O00571	ATP-dependent RNA helicase DDX3X	13	13	629.35	4.4E-03	4.77
P04264	Keratin, type II cytoskeletal 1	31	29	1855.9	1.9E-03	4.75
P35637	RNA-binding protein FUS	3	3	62.91	3.0E-04	4.71
P19338	Nucleolin	6	6	106.96	9.5E-03	4.62
O75694	Nuclear pore complex protein Nup155	19	18	775.18	3.6E-03	4.46
P07384	Calpain-1 catalytic subunit	2	2	62.99	1.1E-03	4.39
Q08211	ATP-dependent RNA helicase A	11	11	544.86	7.1E-03	4.27
Q5JSZ5	Protein PRRC2B	2	2	24.14	5.5E-03	4.26
Q9H857-2	Isoform 2 of 5'-nucleotidase domain-containing protein 2	6	6	0	8.1E-03	4.17
P61106	Ras-related protein Rab-14	2	2	6.34	1.2E-03	3.98
Q13263	Transcription intermediary factor 1-beta	2	2	75.43	2.4E-03	3.98
Q15029	116 kDa U5 small nuclear ribonucleoprotein component	6	6	147.05	6.7E-03	3.60
O94906	Pre-mRNA-processing factor 6	2	2	40.46	4.0E-03	2.75