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Norbornene probes for the study of cysteine oxidation

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ABSTRACT

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

1. Introduction

Cysteine oxidation is a critical aspect of redox homeostasis, protein folding, and intracellular signaling.[1–3] This oxidation can occur by reaction of the thiolate side chain of cysteine with hydrogen peroxide and other reactive oxygen or reactive nitrogen species generated in cells by the mitochondria and various oxidase enzymes.[4–6] The immediate product of the reaction of cysteine with hydrogen peroxide is cysteine sulfenic acid (**1**, Fig. 1). Cysteine sulfenic acid may be the first product formed during the scavenging of reactive species during oxidative stress, but it is also a critical determinant of protein function in catalysis,[7] T cell activation,[8] redox regulation,[9–11] and signaling.[10,12] Cysteine sulfenic acid is also a precursor to both inter- and intramolecular disulfides, as well as higher oxidation states of cysteine that can influence the folding and consequently the function of the protein.[3] Additionally, cysteine sulfenic acid serves as a biomarker for oxidative stress and occurs with high incidence in certain types of cancer.[13] Because of these diverse biological implications, it is becoming increasingly important to identify what proteins contain cysteine residues susceptible to oxidation and if they exist as functional cysteine sulfenic acids. In doing so, information about cysteine oxidation may be revealed that can help clarify its role in both healthy and diseased cells.

Several functional groups are known to react with cysteine sulfenic acids on peptides and proteins (Fig. 2), but there is still a need for probes that trap short-lived cysteine sulfenic acid residues.[3,14] Unlike some cysteine sulfenic acid residues that are persistent and stabilised by the protein microenvironment,[15] many are short-lived precursors to higher oxidation states or other modifications. A comprehensive mapping of their biological function is far from complete.[3] Dimedone (**2**)[1,16] and its derivatives[17,18] are widely used probes to trap cysteine sulfenic acids by reaction of the nucleophilic α-carbon with the sulfur atom of the sulfenic acid. While dimedone benefits from high chemoselectivity, it reacts relatively slowly with sulfenic acids. This limitation has prompted

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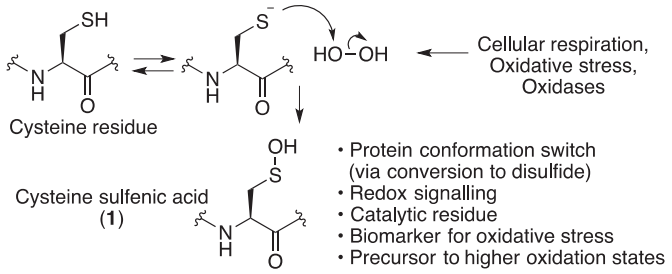

Fig. 1. Cysteine reacts with hydrogen peroxide to form a sulfenic acid.

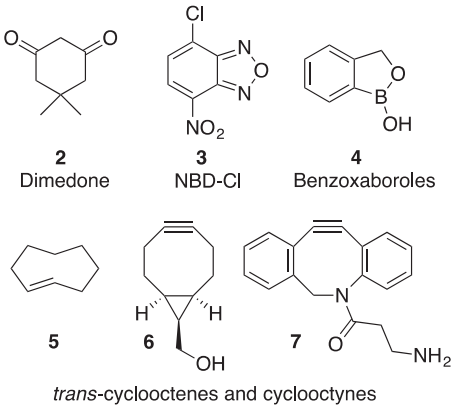

Fig. 2. A selection of molecules that contain functional groups that can react with cysteine sulfenic acid. These core structures can be modified to contain a fluorophore or an affinity tag such as biotin to facilitate detection, analysis, and imaging after they have reacted with proteins.

the Carroll laboratory to study other 1,3-dicarbonyls and related nucleophiles that react more rapidly with cysteine sulfenic acid.[19,20] Indeed, subtle structural modulation of the dimedone core has led to remarkably effective probes with rate enhancements over 100-fold relative to dimedone.[19,20] Mechanistically distinct probes such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl **3**, an electrophilic probe for cysteine sulfenic acid) is also slow to react and suffers from cross-reactivity with other cellular nucleophiles.[21] Boronic acids and benzoxaboroles such as **4** are also electrophiles that react with cysteine sulfenic acids, but this process is reversible.[22] While **4** may therefore be useful in the reversible inhibition of functional cysteine sulfenic acids, it is not suitable for proteomics applications that require a stable linkage to the cysteine residue. The strained *trans*-cyclooctene (**5**) and cyclooctyne derivative **6** (BCN) are two additional probes recently introduced that trap sulfenic acids through a cycloaddition, providing a stable sulfoxide adduct.[23,24] The cycloaddition is driven by the release of ring strain that promotes rapid ligation to sulfenic acids—an important feature for trapping short-lived cysteine sulfenic acids. Unfortunately, this strain may also lead to off-target reactions which compromise the selectivity of the probe.[25,26] For instance, the thiol-yne reaction of cellular thiols with strained cyclooctynes such as **6** and **7** may limit the generality of this class of molecules in detecting sulfenic acids specifically.[25] Additionally, compounds such as **5**–**7** are challenging to synthesise and, because of their strain, have limited shelf-life (especially in solution). For these reasons, we considered norbornene derivatives as alternative probes that would react rapidly with cysteine sulfenic acid due to release of strain (Fig. 3), but not be so reactive that the shelf-life and off-target reactions are concerns. Additionally, norbornene derivatives are straightforward to prepare in a modular fashion (by the Diels-Alder reaction, for instance) so the prospect of accessing functionalised

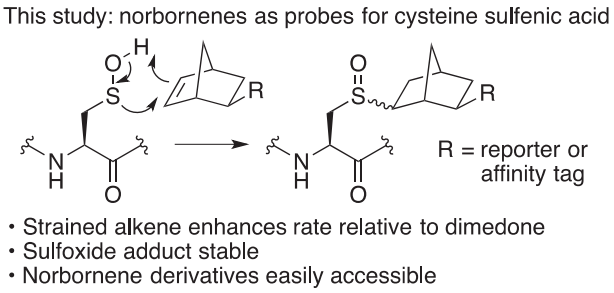

Fig. 3. The strained alkene of easily prepared norbornene derivatives is proposed here as a trap for short-lived cysteine sulfenic acids.

probes in short-order was also attractive. Finally, norbornene compatibility with proteins has been established through its use in several selective bioconjugation methods.[27]

The use of alkenes to trap cysteine sulfenic acids dates back to a report by Benitez and Allison in which water soluble cyclohexene derivatives were used to inhibit an acyl phosphatase containing a catalytically active cysteine sulfenic acid.[16] This was the same study in which dimedone was initially reported to react with cysteine sulfenic acid.[16] The use of norbornene specifically, as a probe for cysteine sulfenic acid, was inspired by classic studies by Barton and co-workers in trapping the sulfenic acid formed during the thermally induced syn-elimination of the sulfoxides of penicillin.[28–30] In this study we extend this concept to the amino acid cysteine and demonstrate that the short-lived sulfenic acid formed from the oxidation of *N*-acetylcysteine with hydrogen peroxide can be intercepted by cycloaddition with various norbornene derivatives.

2. Results and discussion

Norbornene derivative **8** was selected as the first candidate probe for cysteine sulfenic acid. **8** contains two carboxylic acids that render it fully water-soluble after treatment with 1 equivalent of sodium carbonate, allowing these experiments to be carried out in aqueous media (H_2O and D_2O, Fig. 4A) without the need for an organic co-solvent—an important consideration for applications on biological samples. The model sulfenic acid was generated *in situ* by the oxidation of *N*-acetylcysteine (**9**) with hydrogen peroxide. Because the conversion of **9** to its disulfide **10** was very rapid (see page S3 in the Supplementary Information), the hydrogen peroxide was added to **8** first and then a solution of *N*-acetylcysteine (**9**) was added slowly and in a 3-fold excess in a second step. This protocol (excess *N*-acetylcysteine added to the solution of **8** and hydrogen peroxide) ensured that the norbornene probe would have a chance to react with the intermediate sulfenic acid before all of it was converted to the disulfide. The reaction was incubated for up to 30 min at room temperature and then analysed directly by ^{1}H NMR spectroscopy and LC-MS. The pH was measured to be 4.3 over the course of the reaction. Gratifyingly, while the major product detected by ^{1}H NMR and LC-MS analysis was disulfide **10**, the alkene in **8** was completely consumed in its conversion to **11** (Fig. 4A and S4-S5). Because the cycloaddition of the cysteine sulfenic acid with **8** can proceed on either face of the alkene and the sulfur in sulfoxide **11** is a stereogenic centre, there are four possible diastereomers that can be formed. All four diastereomers could be at least partially resolved in the LC-MS analysis and the observed mass spectra were consistent with the calculated value for **11** (m/z = 360, ESI^-). In control reactions, it was confirmed by ^{1}H NMR spectroscopy that **8** reacted with neither hydrogen peroxide nor *N*-acetylcysteine **9** alone and that all three components were required to form **11** (S4-S5). In control experiments analysed by LC-MS, trace

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