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Communication

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Reactivity of Persulfides Toward Strained Bicyclo[6.1.0]nonyne Derivatives: Relevance to Chemical Tagging of Proteins

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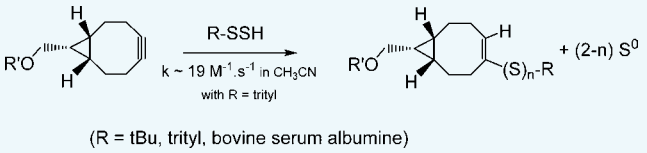
Supporting Information

ABSTRACT: Persulfides are an emerging class of cysteine oxidative post-translational modification. They react with the bioconjugation reagents bicyclo[6.1.0]nonynes (BCNs) to engender thioethers and/or disulfides. This new reactivity of BCNs with a biologically important redox-signaling species efficiently interferes with the recent usage of strained cycloalkynes to specifically trap protein sulfenic acids.

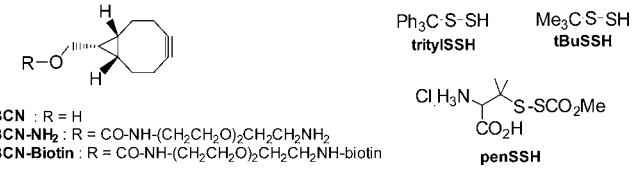


Since the first report of strained promoted [3 + 2] azide−alkyne cycloaddition in living systems,[1] strained alkynes have become important reagents in the chemical biology toolbox. In comparison with classical alkynes, strained alkynes display enhanced reactivity thanks to their severely constrained bond angles surrounding the two alkyne sp-hybridized carbon atoms. As a result, they rapidly emerged as useful tools for protein modifications, proteomics, or in vivo imaging.[2−4] To date, most of the transformations performed with strained alkynes involve dipolar cycloadditions with azide or other dipoles such as nitrile oxides, diazo derivatives, or dienes. However, a new reaction engaging strained cycloalkynes was recently introduced to efficiently trap protein sulfenic acids, a reversible cysteine oxidative post-translational modification (PTM) implicated in redox signaling and serving as a redox sensor for regulating the cell response to oxidative or nitrosative stimuli.[5,6] Hence, the reaction between bicyclo[6.1.0]nonyne (BCN)[7] and a synthetic sulfenic acid displays rate constants 2 orders of magnitude higher than those reported for standard 1,3-dicarbonyl traps.[8,5,6] Moreover, strained alkynes demonstrate interesting bioorthogonal selectivity toward some cysteine PTMs, as they are inert toward disulfide, sulfinic acids, S-nitrosothiols, or thiols, although this last point needs further clarification.[9−11] Another cysteine oxidative modification recently emerged and is related to hydrogen sulfide (H_2S)-mediated redox signaling. H_2S is the third gaseous transmitter in mammals, along with nitrogen and carbon monoxides, and part of its signaling activity is mediated by the formation of proteins[12−17] and low molecular weight persulfides (= hydrodisulfides).[18,19] Persulfide synthesis occurs under oxidative conditions and sulfenic acids might be a potential intermediate during their formation.[20,21] Importantly, while the latter are essentially transient species,[22] persulfides have been suggested to be abundant under physiological conditions. Hence, 10−25% of liver proteins might be "S-sulfhydrated"[17] and concentrations of 50 μM of glutathione persulfide have been reported in major organs,[18] although these observations should be considered with care due to conflicting reports on the methods used for persulfide detection.[20,23]

Here, we report that persulfides display reactivity toward bicyclo[6.1.0]nonyne compounds and exhibit a bimolecular rate constant similar to that reported for sulfenic acids. We discuss the implication of these results regarding the recent use of strained alkynes to trap sulfenic acids in complex biological media.

Persulfides React with BCN Derivatives in Organic or Aqueous Solutions. The synthetic persulfides tBuSSH[24] or tritylSSH[25,26] (Scheme 1) react with BCN within seconds in

Scheme 1. Strained Cyclooctynes and Organic Persulfides/Persulfides Precursors Used in This Study



chloroform, even at −40 °C, as demonstrated by the appearance of new signals in the expected region for vinylic protons in the ^{1}H NMR spectra recorded immediately after mixing the reactants. The main products of the reactions (**1** and **2**, 85% and 75% yield based on integration of the crude mixture and 64% and 54% yield after purification by flash chromatography, respectively) show pseudotriplets integrating for one proton at 6.24 and 5.48 ppm for **1** and **2**, respectively (Figures S1 and S2). This marked difference in chemical shifts is also observed for products **5** and **6** that result from the reactivity between the alkyne BCN-Biotin[7] and tBuSSH or tritylSSH (6.14 and 5.36 ppm, respectively) in chlorofom. The formation of zero valent sulfur, measured by fluorescence spectroscopy,[27] is also detected when BCN or BCN-Biotin react with tBuSSH. These data, along with the ESI-MS spectra recorded for the

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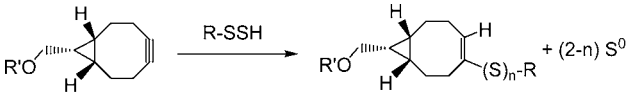
more ionizable products **5** and **6** (Figure S3 and S4), in comparison to **1** and **2**, clearly advocate for the formation of two different species depending on the starting persulfide. Accordingly, tBuSSH reacts with BCN and its derivative to generate the thioethers **1** and **5**, while tritylSSH engenders the stable disulfides **2** and **6** (Scheme 2). Importantly, no BCN

Scheme 2. Adducts Formed upon the Reaction of Various BCN-Derivatives with Synthetic Persulfides in Organic or Aqueous Buffered Solution



BCN	chloroform or acetonitrile	**1**: R = tBu n = 1 **2**: R = trityl n = 2 **3**: R = $CMe_2CH(NHCO_2Me)(CO_2H)$ n = 1 **4**: R = $CMe_2CH(NHCO_2Me)(CO_2H)$ n = 2
BCN-Biotin	chloroform	**5**: R = tBu n = 1 **6**: R = trityl n = 2
$BCN-NH_2$	Tris Buffer	**7**: R = $CMe_2CH(NHCO_2Me)(CO_2H)$ n = 1 **8**: R = $CMe_2CH(NHCO_2Me)(CO_2H)$ n = 2

modification is detected under similar conditions with an excess (10 equiv) of thiol tritSH or tBuSH, even after prolonged incubation (several hours). This confirms the increased reactivity of persulfides when compared to thiols.[18,19]

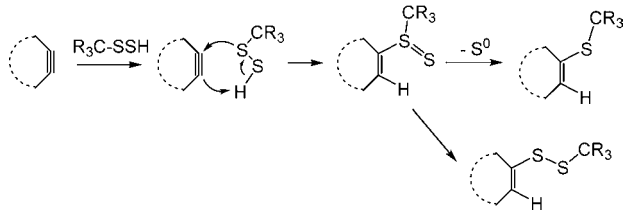
The addition of persulfides to strained alkynes proceeds similarly in buffered aqueous solution. BCN reacts in a 1:1 mixture of acetonitrile and Tris buffer (20 mM, pH = 7.4, complemented with the chelating agent DTPA (200 μM) to circumvent any metal catalyzed additions) with penSSH, a persulfide precursor recently developed in our group.[28] The latter also reacts with the water-soluble $BCN-NH_2$ in Tris buffer. The outcomes of these reactions were analyzed by HPLC-MS (Figure S5 and S'5). The detection of the two products **3**/**7** and **4**/**8** (Scheme 2) fully agrees with the results obtained in organic solution. Additionally, BCN and $BCN-NH_2$ do not suffer any transformation when reacted with N-acetyl-penicillamine, a thiol analogue to the persulfide generated from penSSH.

The addition of a persulfide to an alkyne resembles the well-known[29] and recently rediscovered thiol−yne reaction.[30,31] Although it usually requires an initiator or a catalyst, a catalyst-free version of the hydrothiolation of alkynes was recently reported. It uses thiophenol derivatives and essentially proceeds through a free-radical mechanism.[32] Interestingly, we did observe that thiophenol and other low pK_a thiols react with BCN (Figure S6). These results point out that the enhanced reactivity of persulfides vs their corresponding thiols might be related to the increased acidity of the former.[33]

Possible pathways leading to the formation of **1**/**3**/**5**/**7** and **2**/**4**/**6**/**8** include radical-based mechanisms, addition of the conjugated base of the persulfide in a similar fashion to the base-catalyzed reaction of thiols with BCN derivatives,[34] or the concerted route presented in Scheme 3. It postulates the intermediacy of a thiosulfoxide[35] followed by rearrangement to the corresponding disulfide with the trityl derivative.

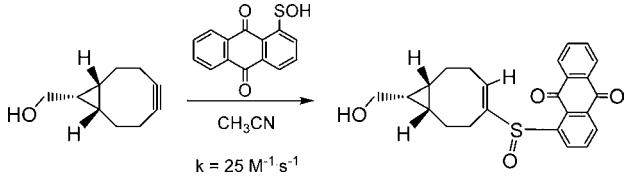
Sulfenic Acids vs Persulfides and Relevance to the Chemical Tagging of Proteins with Strained Alkynes. Despite their close relationship, the comparative reactivity of

Scheme 3. Possible Mechanistic Route to 1/3/5 and 2/4/6



sulfenic acids and persulfides remains poorly studied. To gain further insight into their relative reactivity toward strained alkyne derivatives, we determined the second-order rate constant for the reaction between tritSSH and BCN in acetonitrile. To achieve our goal, we performed competitive experiments between tritSSH and Fries acid, a stable sulfenic acid chromophore (λ_{max} = 453 nm) that reacts with BCN to form the corresponding alkenyl sulfoxide (Scheme 4).[8]

Scheme 4. Reaction of BCN and Fries Acid[8]



The global analysis with Dynafit software[36] of the kinetic traces recorded at 453 nm (Figure 1) gave second-order rate

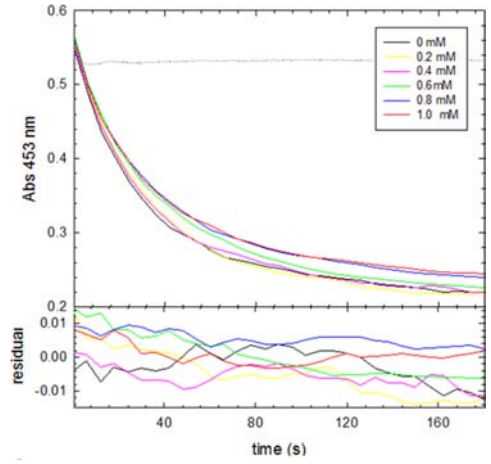


Figure 1. Time course of the absorbance recorded at 453 nm after the addition of various concentrations of tritSSH to a mixture containing BCN (2 mM) and Fries acid (0.2 mM) in acetonitrile at 25 °C (top). The gray line shows the absorbance recorded with Fries acid (0.2 mM) and 1 mM trisSSH. Residuals of the global fitting performed with Dynafit (bottom).

constants of 28.7 (±0.6) and 18.8 (±0.7) $M^{-1}s^{-1}$ for the reaction of BCN with Fries acid and tritSSH, respectively. The former value agrees perfectly with that previously reported (25 $M^{-1}.s^{-1}$) while the bimolecular rate constant determined for the persulfide clearly confirms our aforementioned experimental data. Interestingly, both sulfenic acids and persulfides exhibit second-order rate constants for BCN significantly greater than those reported for azides (~0.2−2 $M^{-1}s^{-1}$)[3,37] or nitrile oxides (~2 $M^{-1}s^{-1}$).[38]

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Despite the poor representativity of Fries acid and trityl persulfide tritSSH with respect to the biological context, these results raise the question of the selectivity of BCN as a dedicated protein sulfenic acid trap. Consequently, we carried out pull-down experiments with the biotinylated derivative BCN-Biotin and bovine serum albumin (BSA) harboring a reduced thiol (BSA-SH), a sulfenic acid (BSA-SOH),[39] or a persulfide (BSA-SSH)[20] at Cys_{34}. Protein samples (30 μM) were incubated with excess BCN-biotin (200 μM) for 90 min, desalted, concentrated, stirred with streptavidin agarose beads for an additional 30 min, and filtered. The percentage of unbound protein was then titrated in the flow-through. As shown in Figure 2, BSA-SOH and BSA-SSH are evenly trapped

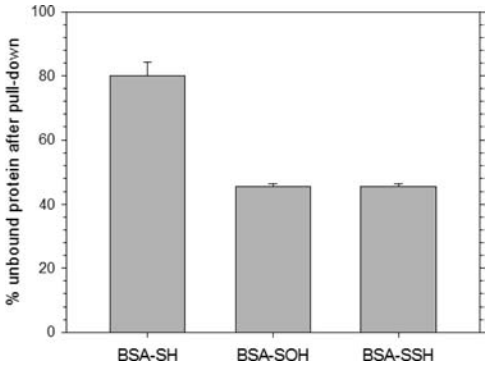

Figure 2. Unbound protein (%) remaining in the flow-through after pull-down of BCN-biotin-tagged-BSAs by streptavidin agarose beads.

by BCN-Biotin (46 ± 1% of unbound protein) while BSA-SH is far less reactive toward BCN-Biotin (78 ± 2% of unbound protein). To confirm that Cys_{34} is the target of the strained alkynes in BSA-SOH or BSA-SSH, we analyzed by MS/MS the tryptic digest of the BSA-SOH/BCN and BSA-SSH/BCN adducts. As expected, we detected in the peptide GLVLIAFS-QYLQQC$_{34}$PFDEHVK the presence of a Cys-S(O)-BCN and a Cys-S-BCN modification (see SI) for the sulfenic- and persulfide-containing protein, respectively. However, we found no evidence for the formation of a disulfide bond between Cys_{34} and BCN in the latter. Interestingly, the analysis of the BSA-SH/BCN mixture did not reveal the formation of the Cys_{34}-S-BCN adduct. Therefore, even if we cannot rule out a minimal alkylation of Cys_{34} or other residues by BCN,[10,40] we do favor nonspecific interactions between the chemical tag and BSA-SH to explain the significant amount of BSA-SH retained on the agarose resin.

This new reaction of strained alkynes with a biologically important redox-signaling species emphasizes the complexity of designing specific bioorthogonal tags. As proposed earlier,[11] the use of thiol- and persulfide-blocking reagents such as iodoacetamide[23] appears to be mandatory to improve the specificity of dipolar cycloaddition involving reactive alkynes. In addition, the use of BCN-derived reagents as sulfenic acid traps should be limited to mass spectrometry-based techniques to undoubtedly discriminate between persulfides and sulfenic acid adducts and circumvent false positives.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and additional data discussed in the manuscript. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00243.

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Notes

The authors declare no competing financial interest.

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