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Strained Cycloalkynes as New Protein Sulfenic Acid Traps

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Supporting Information

ABSTRACT: Protein sulfenic acids are formed by the reaction of biologically relevant reactive oxygen species with protein thiols. Sulfenic acid formation modulates the function of enzymes and transcription factors either directly or through the subsequent formation of protein disulfide bonds. Identifying the site, timing, and conditions of protein sulfenic acid formation remains crucial to understanding cellular redox regulation. Current methods for trapping and analyzing sulfenic acids involve the use of dimedone and other nucleophilic 1,3-dicarbonyl probes that form covalent adducts with cysteine-derived protein sulfenic acids. As a mechanistic alternative, the present study describes highly strained bicyclo[6.1.0]nonyne (BCN) derivatives as concerted traps of sulfenic acids. These strained cycloalkynes react efficiently with sulfenic acids in proteins and small molecules yielding stable alkenyl sulfoxide products at rates more than 100× greater than 1,3-dicarbonyl reagents enabling kinetic competition with physiological sulfur chemistry. Similar to the 1,3-dicarbonyl reagents, the BCN compounds distinguish the sulfenic acid oxoform from the thiol, disulfide, sulfinic acid, and *S*-nitrosated forms of cysteine while displaying an acceptable cell toxicity profile. The enhanced rates demonstrated by these strained alkynes identify them as new bioorthogonal probes that should facilitate the discovery of previously unknown sulfenic acid sites and their parent proteins.

Protein sulfenic acids (RSOH) arise from the reaction of reactive oxygen species, such as hydrogen peroxide, alkyl peroxides, and peroxynitrite, with cysteine thiols.[1,2] Sulfenic acids may further oxidize to sulfinic or sulfonic acids, condense with other sulfenic acids to form thiosulfinates, or react with thiols to yield disulfides.[3] This wide variety of sulfur-based chemistry marks protein sulfenic acids as the initial product toward potentially irreversible oxidative damage. This versatile chemistry also allows their participation in the reversible control and modulation of important cellular processes, such as transcription and enzymatic cascade pathways, which directly influence biological outcomes.[4,5] Most information regarding the biological roles of sulfenic acids comes from studies using nucleophilic 1,3-dicarbonyl-based probes, such as dimedone (Figure 1). The high reactivity of sulfenic acids limits their cellular lifetime, permitting these probes to access and label only a fraction of existing sulfenic acid sites, which ultimately constrains the understanding of the biological roles of these species.[6] Sulfenic acids also react with alkenes and alkynes via a concerted mechanism to give alkyl and alkenyl sulfoxides.[7,8] The introduction of strain energy, as shown with alkyne−azide "click" chemistry, should increase the reactivity and rate of this mechanistic alternative to current sulfenic acid trapping methods (Figure 1).[9,10] Specifically, we report the bicyclo[6.1.0]nonyne (**1**) and its biotinylated derivative (**4**) efficiently and selectively trap protein sulfenic acids at superior rates to 1,3-dicarbonyl-based probes, identifying a new group of bioorthogonal protein sulfenic acid probes (Figure 1).[11]

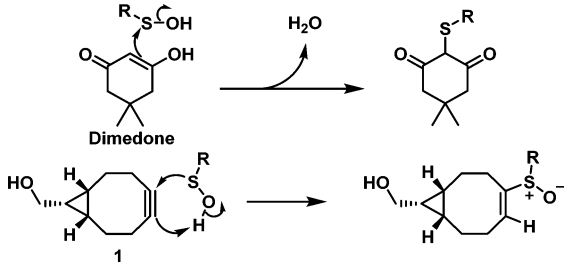

Figure 1. Sulfenic acid trapping by dimedone and 9-hydroxymethylbicyclo[6.1.0]nonyne (BCN, **1**).

The known bicyclo[6.1.0]nonyne (**1**, BCN) rapidly reacts with small molecule sulfenic acids. Treatment of a thermally generated organic-soluble cysteine-derived sulfenic acid with **1** gives a mixture of diastereomeric alkenyl sulfoxides (**2**) as determined by NMR spectroscopy and liquid chromatography-mass spectrometry in 84% isolated yield (Figure 2).[12] Similar reaction of **1** with Fries acid, a stable anthraquinone-derived sulfenic acid, also produces the diastereomeric alkenyl sulfoxide (**3**) in 99% yield (Figure 2).[13] The results clearly show for the first time that this strained cycloalkyne reacts with small molecule model sulfenic acids in organic systems. These reactions likely proceed via a concerted cycloaddition-like mechanism quite distinct from the accepted nucleophilic addition of the 1,3-dicarbonyl probes to the sulfenic acids.[7,8,14] The alkenyl sulfoxide (**2**) did not react with the

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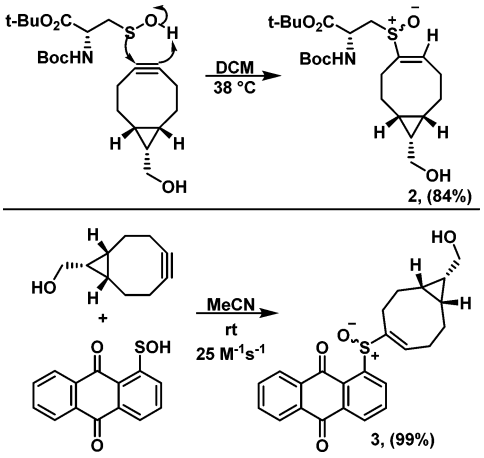

Figure 2. Reactions of **1** with a cysteine-derived sulfenic acid and Fries acid yield alkenyl sulfoxides (**2**–**3**).

nucleophilic reducing agents dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine (TCEP) demonstrating its stability.

Monitoring the decrease in absorbance at 453 nm by UV–vis spectroscopy as a function of time provides kinetic information for the reaction of **1** and Fries acid (Figure S11). A kinetic analysis of these reactions, performed under pseudo-first-order conditions in organic solvent, gives a second-order rate constant of ~25 M^{-1} s^{-1} (Figure S11), and similar experiments in a 50:50 mixture of acetonitrile:ammonium bicarbonate buffer give a second-order rate constant of ~12 M^{-1} s^{-1} (Figure S12). These rate constants are significantly greater than the reported value of 0.05 M^{-1} s^{-1} for dimedone-based probes with model protein sulfenic acids and suggest strained alkynes may act as useful protein sulfenic acid traps.[15] Similar experiments with *trans*-cyclooctene (*t*COT), a strained cyclic alkene,[16,17] give a second-order rate constant of ~0.01 M^{-1} s^{-1} and produce the corresponding alkyl sulfoxides with both the cysteine-derived sulfenic acid and Fries acid (Figure S13). Given the kinetic differences between **1** and *t*COT, further biological trapping studies focused on **1** and its derivatives. Experimental limitations including the limited aqueous solubility of the cysteine-derived sulfenic acid and the low reactivity of Fries acid prevent a direct kinetic comparison of **1** with dimedone in an aqueous environment (Figure S14). The enhanced rate of reaction provides evidence that the ring strain of **1** accelerates the reaction. This rate increase likely arises from bending of the normally 180° oriented bonds formed from the overlap of the sp and sp^3 hybridized orbitals into a conformation that approaches the geometry of the transition state.

The stabilized sulfenic acid of the C165A mutant of the alkyl hydroperoxidase AhpC protein (AhpC-SOH) provides an opportunity to measure the reaction efficiency of strained cyclooctynes with a protein sulfenic acid. This cysteine-based bacterial peroxidase forms an intersubunit disulfide bond through oxidation of C46 to the sulfenic acid followed by condensation with C165 of an adjacent AhpC monomer. Mutation of C165 to alanine or serine stabilizes the reactive SOH at C46, allowing for the evaluation of chemical probe reactions with the otherwise transient SOH. Conjugation of **1** to biotin by a DCC coupling forms the biotin ester (**4**). Monitoring the reaction of **4** and C165A AhpC-SOH (20 600 amu) by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) yields a peak at 20 976 amu corresponding to the formation of the expected alkenyl sulfoxide adduct (Figure 3A). Unreacted AhpC-SOH is observed in the gas phase as a mixture of sulfenic acid and sulfenamide (S–N

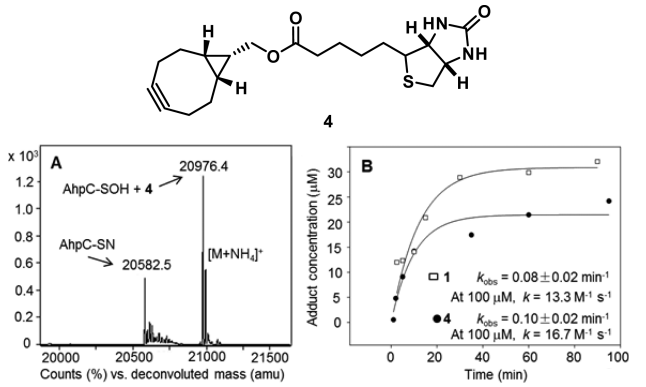

Figure 3. Reactions of C165A AhpC-SOH (40 μM) with **1** or **4** (100 μM) at rt in 50 mM NH_4HCO_3, pH 7.5. (A) ESI-TOF mass spectrum of the AhpC-SOH reaction with **4** at 30 min. (B) Time course of adduct formation. The concentration of AhpC-SOH adduct with **1** or **4** along the reaction time course was determined based on relative abundance of adduct among the total ion abundances of the prominent species in the ESI-TOF mass spectra.

condensation product, 20 582 amu) as previously described.[18] Incubation of **4** with various AhpC oxoforms including AhpC-SO_2H, AhpC-SNO and AhpC-S-S-Cys fails to yield adducts as judged by ESI-TOF MS revealing the protein sulfenic acid selectivity of **4** (Figures S16–S17). The lack of an adduct of **4** with C165A AhpC-SH (thiol) indicates that thiol–yne reactions, potentially complicating side reactions, do not occur in this system (Figure S18).[19] Lack of significant cross-reactivity of **4** with these oxoforms, particularly AhpC-SNO, that could possibly undergo radical addition or cycloaddition with **4**,[20,21] was further confirmed by Western blotting under nonreducing conditions (Figure S19).

Efficient trapping of AhpC-SOH occurs at a much lower concentration of **1** (100 μM) than reported concentrations of various 1,3-dicarbonyl-based probes (often 1–5 mM).[6,15,22] A MS-based kinetic analysis of protein sulfoxide formation from the reactions of C165A AhpC-SOH (40 μM) with both **1** and **4** (100 μM) reveals the time-dependent increase in adduct formation and gives second-order rate constants of 13.3 and 16.7 M^{-1} s^{-1}, respectively, values several hundred-fold greater than reactions with 1,3-diketone-based SOH probes (Figure 3B).[15] The increased reaction rates potentially allow **1** and **4** to trap more reactive, transient protein sulfenic acids. Experiments with higher concentrations of **4** (1–5 mM) result in protein labeling accompanied by cycloalkyne polymerization as judged by ESI-TOF MS (Figure S20). Trypsin digestion followed by tandem MS analysis verifies the addition of **1** to C46 with an XCorr of 5.9 for a 4+ charged peptide providing a high-confidence site assignment and adduct mass (Figure S21). Analogous MS^2 results were obtained by labeling AhpC-SOH with **4** followed by trypsin digestion (Figure S22). Comparatively, adducts of these cyclooctynes and AhpC-SOH produce much higher quality MS^2 spectra compared to dimedone.[23]

The chemical stability of the AhpC alkenyl sulfoxide adducts of **4** was investigated using H_2O_2 and several biochemical reductants. Of particular interest were DTT and TCEP due to their widespread use in MS-based proteomics workflows and common endogenous Michael donors like glutathione and

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cysteine. Since initial experiments did not suggest H_2O_2-mediated oxidation of the alkenyl sulfoxide to the sulfone, preparation of the AhpC-S(O)-**4** adduct (20 977 amu) in this experiment included a final step of H_2O_2 treatment to consume protein species (e.g., SH, SN, SOH) that would interfere with reductant chemistry. A small amount of SN remained. Following removal of unreacted **4** and H_2O_2, AhpC tagged with **4** (40 μM total protein) was treated with 1 mM DTT, TCEP, β-mercaptoethanol, GSH, or *N*-acetyl cysteine for 1 h at rt (Figure S23). Formation of low amounts of thiol (DTT, TCEP) and the corresponding mixed disulfides of β-ME, GSH, and NAC was observed. Since there was no significant decrease in the AhpC-SOH adduct with **4** (Figure S23 and Western blot analysis in Figure S24), we conclude these products form from the unreacted SN species. Additionally, Michael adducts of AhpC labeled with **4** were not observed under these conditions indicating the overall stability of the protein adducts of **4**.

Given the efficient reactions of **1** and **4** with AhpC-SOH, we tested the ability of **4** to trap protein sulfenic acids in cell lysates. Oxidation with increasing amounts of H_2O_2 in the presence of **4** shows concentration-dependent SOH trapping (Figure 4A). Reaction efficiency increases as the concentration

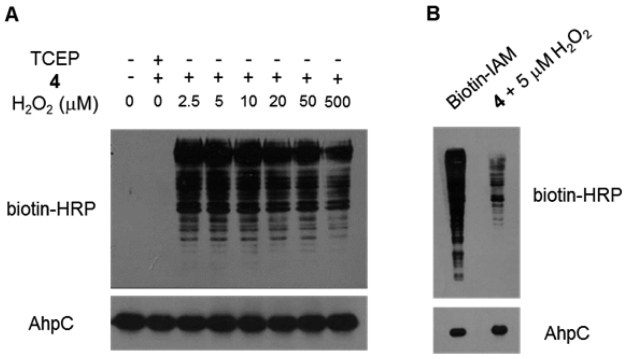

Figure 4. Reactivity of **4** with oxidized cell lysates. (A) Reduced protein lysates were spiked with C165A AhpC for loading control and aliquotted into 60 μg fractions. Fractions were treated with **4** (100 μM) and increasing concentrations of H_2O_2. A control sample of lysate (lane 2) was supplemented with TCEP then treated with **4** (100 μM). (B) Reduced protein lysate (60 μg fraction) was treated with iodoacetyl biotin (100 μM) to label all thiol content and compared to the sample in (A) treated with 5 μM H_2O_2 and 100 μM of **4**.

of H_2O_2 increases from 2.5 to 5 μM and then decreases as the concentration of H_2O_2 is raised further, likely due to the overoxidation of cysteine residues to sulfinic and sulfonic acids. Comparative analysis with iodoacetyl biotin (biotin-IAM)-treated lysate sets the level of SOH and labeling with **4** at an upper limit of 25% of total thiol content (Figure 4B).

The utility of BCN probes in labeling endogenous protein SOH was tested by lysing squamous cell carcinoma cells in a modified RIPA buffer containing **4** (100 μM) for 30 min, providing a robust level of biotinylated proteins (Figure S25). In comparison, cells lysed in buffer containing TCEP (10 mM) for 30 min then incubated with **4** (100 μM) for the same duration instead demonstrated a sharply decreased amount of protein biotinylation, demonstrating the selectivity of BCN for sulfenic acids. The small amount of BCN labeling in TCEP-treated lysates may result from incomplete reduction of SOH, particularly in buried protein microenvironments. Our studies involving the incubation of **4** with cell lysates under highly stringent reducing conditions followed by Western blot analysis show no evidence of cross-reactivity with available protein thiols or other amino acids, including their post-translational modifications (Figure 4A, lane 2).

With the intent to use **1** or **4** to label protein sulfenic acids in live cells, the cytotoxicity profile of **4** was assessed in comparison to dimedone using a human squamous cell carcinoma cell line and the MTT cell viability assay. The addition of **4** to these cells results in death with an IC_{50} of 199.3 $\pm$ 27.3 μM at 48 h (Figure 5). In comparison, dimedone

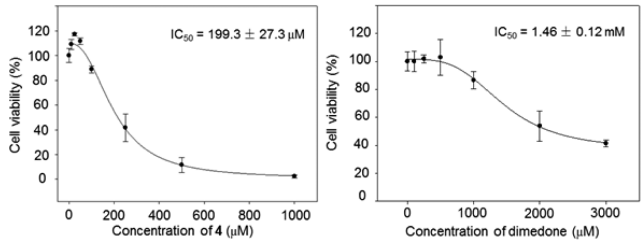

Figure 5. Viability of SCC-61 cells in the presence of **4** and dimedone as measured by the MTT assay.

induces cell death with an IC_{50} of 1.46 $\pm$ 0.12 mM at 72 h (Figure 5). While more toxic than dimedone, the high reactivity of **4** allows for use at concentrations below 100 μM and shorter incubation times that limit its toxicity.

Cell membrane permeability of the bicyclononynes along with the efficiency of in vivo SOH labeling was tested using **4** in comparison to the biotinylated dimedone-based DCP-Bio1. SCC-61 cells were incubated with media containing DCP-Bio1 (0.025−1 mM) or **4** (0.025−0.1 mM) for 30 min, washed twice with cold PBS, and then lysed. Reducing SDS-PAGE and Western blotting demonstrates the membrane permeability of both probes and increased labeling with **4** relative to DCP-Bio1 (Figure 6A). Similarly, the efficiency of SOH labeling in a

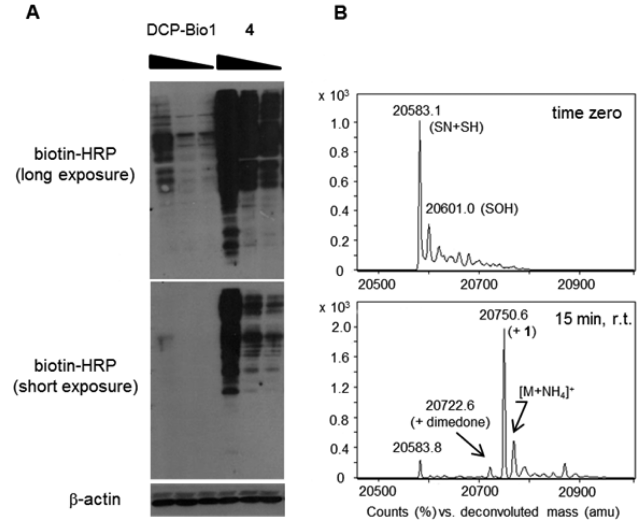

Figure 6. (A) Labeling of protein sulfenic acids in live cells using DCP-Bio1 (1, 0.1, and 0.025 mM) and **4** (0.1, 0.05, and 0.025 mM). SCC-61 cells were incubated for 30 min (37 °C, 5% CO_2) in the presence of each probe (final concentration of DMSO was 0.2%), washed twice with cold PBS, and then lysed with mRIPA buffer in the absence of probe. (B) ESI-TOF mass spectra for the labeling of C165A AhpC-SOH (20 μM, top panel) with **1** (20 μM) and dimedone (5 mM) at 15 min in 25 mM Tris pH 7.0 (rt, bottom panel).

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recombinant protein was explored using C165A AhpC-SOH (20 μM) in the presence of **1** (20 μM) and dimedone (5 mM). Quenching of the competition reaction at 15 min using size exclusion resin reveals a much higher abundance of the AhpC-SOH adduct with **1** (adduct observed at 20 750.6 amu) compared to dimedone (20 722.6 amu) (Figure 6B).

In summary, strained cyclic alkynes (**1** and **4**) rapidly react with sulfenic acids to yield alkenyl sulfoxide adducts. Using a model protein sulfenic acid, these compounds yield stable products with clear ionization states that facilitate identification and MS analysis. By reacting through concerted pathways, these traps provide a distinct mechanistic alternative to the nucleophilic 1,3-dicarbonyl compounds. Kinetic analysis with purified protein, lysates and live cells reveals reaction rates exceeding those of dimedone and dimedone-based probes by more than 2 orders of magnitude making these the fastest characterized sulfenic acid traps described to date. Cycloalkynes also demonstrate bioorthogonal reactivity with the sulfenic acid by not reacting with the thiol, disulfide, sulfinic acid, or *S*-nitroso oxoforms of cysteine. The kinetic profile of these reagents with proteins allows their use at low concentrations that minimize cell toxicity. While the rate will vary with the individual protein,[15] the excellent bioorthogonal selectivity combined with an enhanced rate and MS/MS compatible profile make strained cycloalkynes valuable new tools for detecting protein sulfenic acids in vitro or in vivo.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Poole, L. B.; Karplus, P. A.; Claiborne, A. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 325.
(2) Reddie, K. G.; Carroll, K. S. *Curr. Opin. Chem. Biol.* **2008**, *12*, 746.
(3) Roos, G.; Messens, J. *Free Radic. Biol. Med.* **2011**, *51*, 314.
(4) Paulsen, C. E.; Carroll, K. S. *ACS Chem. Biol.* **2010**, *5*, 47.
(5) Poole, L. B.; Nelson, K. J. *Curr. Opin. Chem. Biol.* **2008**, *12*, 18.
(6) Gupta, V.; Carroll, K. S. *Biochim. Biophys. Acta, Gen. Subj.* **2014**, *1840*, 847.
(7) Allison, W. S. *Acc. Chem. Res.* **1976**, *9*, 293.
(8) Cubbage, J. W.; Guo, Y.; McCulla, R. D.; Jenks, W. S. *J. Org. Chem.* **2001**, *66*, 8722.
(9) Wilson, M. R.; Taylor, R. E. *Angew. Chem., Int. Ed.* **2013**, *52*, 4078.
(10) Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272.
(11) Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, P.; van Delft, F. L. *Angew. Chem., Int. Ed.* **2010**, *49*, 9422.
(12) Aversa, M. C.; Barattucci, A.; Bonaccorsi, P.; Giannetto, P. *J. Org. Chem.* **2005**, *70*, 1986.
(13) Bruice, T. C.; Sayigh, A. B. *J. Am. Chem. Soc.* **1959**, *81*, 3416.
(14) Aversa, M. C.; Barattucci, A.; Bonaccorsi, P.; Contini, A. *J. Phys. Org. Chem.* **2009**, *22*, 1048.
(15) Klomsiri, C.; Nelson, K. J.; Bechtold, E.; Soito, L.; Johnson, L. C.; Lowther, W. T.; Ryu, S.-E.; King, S. B.; Furdui, C. M.; Poole, L. B. In *Methods in Enzymology*; Elsevier: Amsterdam, 2010; Vol. *473*, pp 77−94.
(16) Royzen, M.; Yap, G. P. A.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 3760.
(17) Blackman, M. L.; Royzen, M.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 13518.
(18) Qian, J.; Klomsiri, C.; Wright, M. W.; King, S. B.; Tsang, A. W.; Poole, L. B.; Furdui, C. M. *Chem. Commun.* **2011**, *47*, 9203.
(19) Van Geel, R.; Pruijn, G. J. M.; van Delft, F. L.; Boelens, W. C. *Bioconjugate Chem.* **2012**, *23*, 392.
(20) Talipov, M. R.; Khomyakov, D. G.; Xian, M.; Timerghazin, Q. K. *J. Comput. Chem.* **2013**, *34*, 1527.
(21) Cavero, M.; Motherwell, W. B.; Potier, P. *Tetrahedron Lett.* **2001**, *42*, 4377.
(22) Poole, L. B.; Klomsiri, C.; Knaggs, S. A.; Furdui, C. M.; Nelson, K. J.; Thomas, M. J.; Fetrow, J. S.; Daniel, L. W.; King, S. B. *Bioconjugate Chem.* **2007**, *18*, 2004.
(23) Qian, J.; Wani, R.; Klomsiri, C.; Poole, L. B.; Tsang, A. W.; Furdui, C. M. *Chem. Commun.* **2012**, *48*, 4091.