Bioorganic & Medicinal Chemistry Letters 19 (2009) 356–359

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Facile synthesis and biological evaluation of a cell-permeable probe to detect redox-regulated proteins

Young Ho Seo [a], Kate S. Carroll [a,b,*]

[a] Life Sciences Institute, University of Michigan, 210 Washtenaw Ave. #4401, Ann Arbor, MI 48109-2216, USA
[b] Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-2216, USA

ARTICLE INFO

Article history:
Received 15 October 2008
Revised 19 November 2008
Accepted 20 November 2008
Available online 24 November 2008

Keywords:
Redox regulation
Cysteine oxidation
Sulfenic acids
Tyrosine phosphatase
Chemical probes
Staudinger ligation
Click chemistry

ABSTRACT

We have developed an improved synthesis for the cell-permeable, sulfenic acid probe DAz-1. Using DAz-1, we detect sulfenic acid modifications in the cell-cycle regulatory phosphatase Cdc25A. In addition, we show that DAz-1 has superior potency in cells compared to a biotinylated derivative. Collectively, these findings set the stage for the development of activity-based inhibitors of Cdc25 cell-cycle phosphatases, which are sensitive to the redox state of the active-site cysteine and demonstrate the advantage of bioorthogonal conjugation methods to detect protein sulfenic acids in cells.

The reversible oxidation of cysteine is a widespread mechanism in the regulation of protein function.[1] In mammalian cells, activation of cell surface receptors can trigger enzymatic production of reactive oxygen species (ROS). These oxidants function as second messengers and modify signaling proteins through cysteine oxidation.[2,3] For example, oxidation of a critical active-site cysteine inhibits protein tyrosine phosphatases (PTPs) and results in increased phosphorylation levels.[4] Redox-based signal transduction plays an important role in many normal biological events, including cell proliferation, differentiation, migration and programed cell death.[3] In addition, abnormal ROS production correlates with cancer and aging-associated degenerative diseases.[2,3] Because of the central role that cysteine oxidation plays in cell signaling and human pathology, there has been considerable interest in developing small-molecule probes to detect these post-translational modifications directly in cells.

Sulfenic acid (RSOH) is the simplest cysteine oxoform and is formed by reaction of a thiolate anion (RS^-) with cellular oxidants such as hydrogen peroxide (H_2O_2). Sulfenic acids can oxidize further to sulfinic (RSO_2H) and sulfonic (RSO_3H) acid, condense with a neighboring thiolate to form a disulfide, or be stabilized by the protein microenvironment. The electrophilic sulfur atom in sulfenic acid reacts with carbon nucleophiles such as alkenes and enolates, including 5,5-dimethyl-1,3-cyclohexadione (**1**; dimedone, Figure 1). Under aqueous conditions, dimedone reacts selectively with cysteine sulfenic acids and not with other protein functional groups.[5–7] This chemoselective reaction has been exploited to detect sulfenic acid modifications *via* mass analysis; fluorescent-dimedone conjugates have also been reported.[3] However, these reagents are not suitable for proteomic studies since they lack an affinity handle for isolating tagged proteins. To extend the utility of the dimedone scaffold as a probe for protein sulfenic acid modifications we recently developed DAz-1 (**2**; Figure 1).[7] DAz-1 covalently modifies sulfenic acid-modified proteins directly in cells and

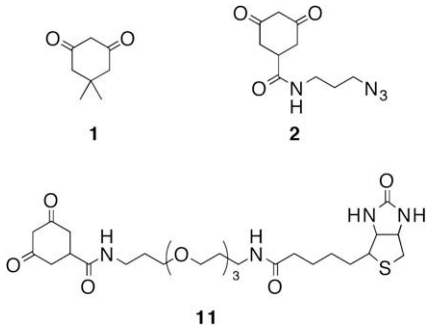


Figure 1. Structures of small-molecule probes for sulfenic acids.

* Corresponding author. Tel.: +1 734 615 2739.
E-mail address: katesc@umich.edu (K.S. Carroll).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.11.073

has an azide chemical handle, which can be functionalized with a wide variety of phosphine or alkyne-based reporter tags for detection and isolation.[8] In this report, we describe an improved synthesis of DAz-1 and investigate sulfenic acid formation in the cell-cycle regulatory phosphatase Cdc25A. In addition, we compare the activity of DAz-1 to a biotinylated derivative in vitro and in HeLa cells.

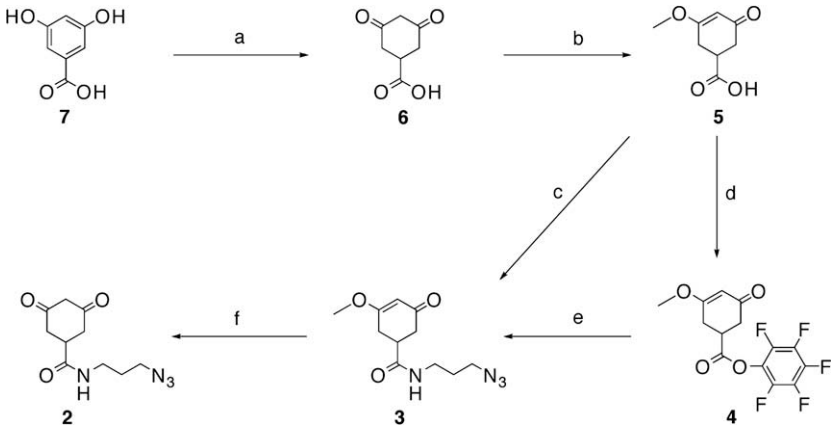
In a previous report, we pursued the synthesis of DAz-1 *via* route A (Scheme 1, a → b → c → f).[7] Ni-catalyzed hydrogenation of 3,5-dihydroxybenzoic acid (**7**) gave diketone **6** in 85% yield.[9] Initial attempts to couple the carboxylic acid functional group on **6** with 3-azidopropylamine (**8**)[10] using standard peptide coupling reagents such as PyBop/TEA, DIC/4-DMAP or TBTU/DIEA were not successful and produced nucleophilic substitution products at the carbonyl carbon of compound **6**. To prevent these undesired reactions we protected the ketone **6** as the methyl ether. PTSA catalyzed protection was achieved in MeOH at room temperature to give compound **5** in 97% yield. The amide coupling reaction of carboxylic acid **5** with amine **8** was carried out with TBTU and DIEA in DMF to provide amide **3** in 99% yield. The synthesis of DAz-1 was completed through deprotection of methyl vinyl ether with 2 N HCl in THF. The overall yield of DAz-1 through route A was 64%.

In small-scale reactions amide coupling with TBTU, an HOBt-based aminium salt, afforded DAz-1 in excellent yield. However, when the reaction was scaled up compound DAz-1 and HOBt were not completely resolved by silica gel chromatography. Although reverse phase HPLC could be used to purify DAz-1 the procedure is time consuming and reduced the overall yield to 20%. For this reason, we explored an alternate synthetic route B (Scheme 1, a → b → d → e → f). Pentafluorophenyl trifluoroacetate (TFAPfp) can activate carboxylic acids as Pfp esters in good yields and volatile side-products TFA and pentafluorophenol are easily removed during workup.[11] Therefore, we used TFAPfp as the activating reagent for the carboxylic acid functional group on **5**. Esterification of carboxylic acid **5** was achieved with TFAPfp and DIEA in DMF in quantitative yield. Pfp ester **4** was then coupled with 3-azidopropylamine (**8**) to furnish compound **3** in high purity. For the final step of the synthesis, we used cerium (IV) ammonium nitrate (CAN) to deprotect **3**. Non-acidic deprotection using catalytic amount of CAN provided DAz-1 in 96%. Route B did not require HPLC purification and furnished DAz-1 over five steps in 52% yield.

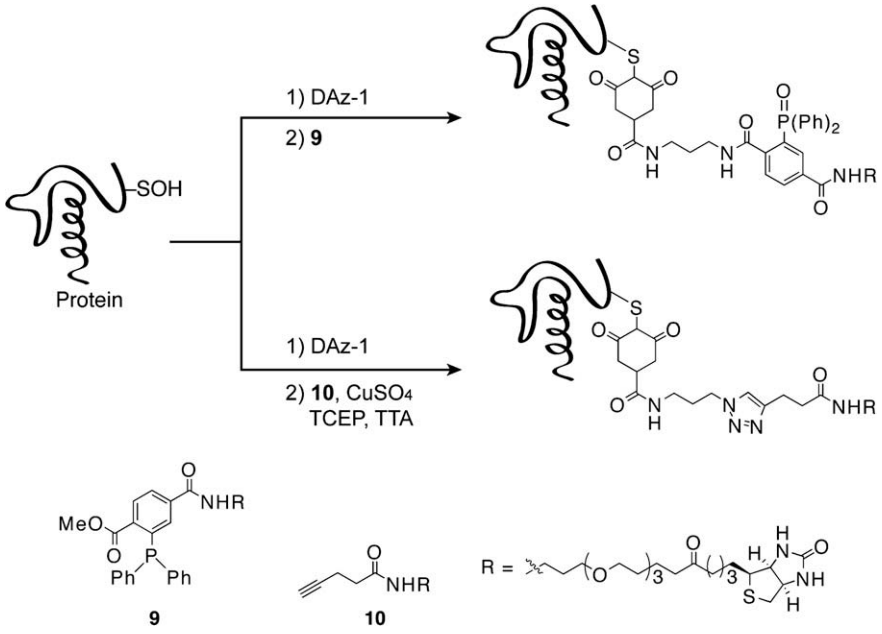
Having established an efficient synthesis for DAz-1, we next investigated sulfenic acid formation in the cell-cycle regulatory phosphatase Cdc25A (Scheme 2). In humans, three different Cdc25 family members exist with Cdc25A required for the G_1/S transition and Cdc25B/Cdc25C involved in the G_2/M.[12] Like all tyrosine phosphatases, members of the Cdc family contain an active-site cysteine involved in formation of a phosphocysteine intermediate. The pK_a of this active-site residue is significantly perturbed from the typical 8.5 to ~6.[13] Cdc25 phosphatases also possess a second conserved cysteine located ~5 Å from the active-site residue. This additional cysteine is not required for activity, but can form an intramolecular disulfide with the catalytic cysteine under mild oxidizing conditions.[14] The proposed function of this disulfide is to prevent overoxidation of the catalytic cysteine to irreversible sulfinic and sulfonic acid forms.

Biochemical studies indicate that the active-site cysteine in Cdc25B and Cdc25C is sensitive to oxidants and that sulfenic acid formation inhibits phosphatase activity.[15] However, it is not known whether Cdc25A—the only essential Cdc25 isoform—is susceptible to oxidation. Therefore, we treated the recombinant soluble catalytic domain of Cdc25A C384S[16] with DAz-1 and conjugated it to biotin reporter tags **9** or **10** using the Staudinger ligation or click chemistry, respectively (Scheme 2). Western blot analysis shows DAz-1-dependent labeling of Cdc25A C384S (Fig. 2). Pre-treatment of the phosphatase with the reducing agent dithiothreitol (DTT) significantly reduced labeling, as expected (Fig. S1a). Trapping the sulfenic acid modification also blocked formation of disulfide-linked Cdc25A homodimers (Fig. S1b). Taken together, these data indicate that the active-site cysteine in Cdc25A can oxidize to a sulfenic acid, which can be trapped by DAz-1. Since Cdc25A expression is elevated in a wide variety of cancers,[12] small-molecules inhibitors, which target the phosphatase active-site and are sensitive to the redox state of the catalytic cysteine, may inhibit proliferation of transformed cells that are associated with high levels of ROS.

In recent work, Charles and colleagues reported a biotinylated dimedone derivative to monitor protein sulfenic acid formation in peroxide-treated rat ventricular myocytes.[17] In their studies, Charles et al. observed protein labeling only when primary cells were treated with hydrogen peroxide.[17] One interpretation of these data is that the basal level of cellular sulfenic acids in primary myocytes is below the threshold of detection. Alternatively, since oxidants stimulate programed cell death and necrosis in cultured cells,[18,19] it is possible that treatment compromised membrane integrity and allowed the dimedone-biotin derivative to enter the dying cell. Consistent with the latter proposal, several recent reports demonstrate that direct conjugation of a reporter tag such as biotin or a fluorophore to an inhibitor reduces potency and prevents passive diffusion across cell membranes.[20,21] For



Scheme 1. Synthesis of DAz-1 (**1**). Reagents and conditions: (a) T1 Raney nickel, NaOH, 750 psi, 70 °C, 85%; (b) PTSA, MeOH, rt, 10 min, 97%; (c) 3-azidopropylamine (**8**), TBTU, DIEA, DMF, rt, 15 min, 99%; (d) TFAPfp, TEA, DMF, rt, 2 h, 100%; (e) 3-azidopropylamine (**8**), DIEA, DMF, rt, 3 min, 89%; (f) 2 N HCl, THF, rt, 1 h, 78% or CAN, H_2O-MeCN, reflux, 1 h, 96%.



Scheme 2. Strategy to detect sulfenic acid-modified Cdc25A with biotinylated reporter tags and the Staudinger ligation (top) or click chemistry (bottom) methods.

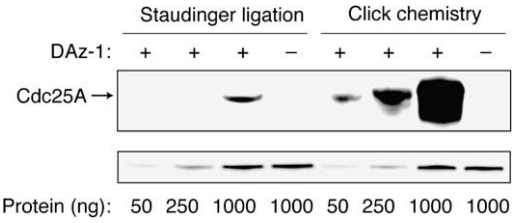


Figure 2. DAz-1 detects sulfenic acid modifications in the cell-cycle regulatory phosphatase Cdc25A. Cdc25A C384S was reacted with 10 mM DAz-1, separated from excess probe and conjugated to **9** or **10** using standard procedures.[8] Samples were analyzed by Western blot using HRP-conjugated streptavidin (top). Equivalent protein loading for the Staudinger and click reactions was verified by SDS–PAGE (bottom).

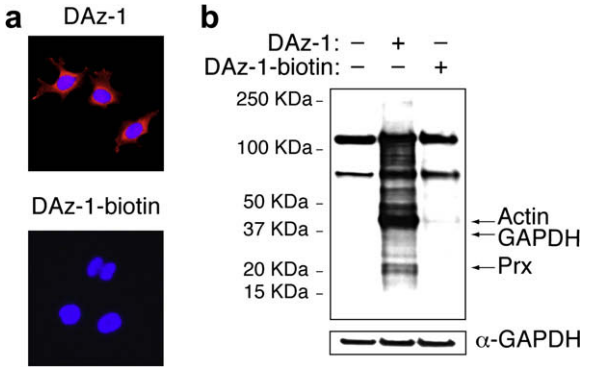


Figure 3. Immunofluorescence microscopy and Western blot showing sulfenic acid-modified proteins labeled by DAz-1 in HeLa cells. (a) HeLa cells were incubated in media containing 0.1 mM DAz-1 (top) or DAz-1-biotin (bottom). After fixation, DAz-1-modified proteins were conjugated to p-biotin. Biotinylated proteins from DAz-1 or DAz-1-biotin treated cells were detected by streptavidin-Alexa Fluor 555 (red). Nuclei were counterstained with DAPI (blue). (b) Protein was isolated from cells incubated in media containing 10 mM DAz-1 or DAz-1-biotin. Protein lysate from DAz-1 treated cells was conjugated to p-biotin. Biotinylated proteins from DAz-1 or DAz-1-biotin treated cells were resolved by SDS–PAGE and detected by Western blot analysis using HRP-conjugated streptavidin (top). Equal protein loading was verified by reprobing the blot with an antibody against GAPDH (bottom).

these reasons, we hypothesized that direct conjugation of the biotin reporter tag could decrease cellular activity.

To test this hypothesis, we synthesized DAz-1-biotin **11** (Fig. 1 and Scheme S1) and conducted a side-by-side comparison of sulfenic acid labeling in vitro and in cells. To generate DAz-1-biotin, Pfp ester **5** was coupled to biotin-PEO_3-amine (**13**). Subsequently, methyl vinyl ether **12** was deprotected with 1 N HCl in THF. The DAz-1-biotin **11** probe differs from the reagent reported by Charles et al. only in the absence of a methyl substitutent at the C-5 position of the 1,3-cyclohexadione scaffold. In biochemical experiments, DAz-1 and DAz-1-biotin covalently tagged oxidized glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), as demonstrated by Western blot detection (Fig. S2a). In competition experiments, 250 μM DAz-1 was sufficient to block 90% of protein labeling by 1 mM DAz-1-biotin (Fig. S2). Differences in the extent and pattern of protein labeling by DAz-1-biotin and DAz-1 were also observed in HeLa cell lysate (Fig. S2c). In particular, more individual sulfenic acid-modified proteins were visualized in DAz-1-treated samples. Since the specificity of DAz-1 and dimedone derivatives has been rigorously established in prior work[5–7] the simplest explanation for the observed differences is that the large, hydrophobic reporter tag on DAz-1-biotin interfered with protein binding. When tested in intact HeLa cells, DAz-1 was significantly more potent than DAz-1-biotin as shown by immunofluorescence microscopy (Fig. 3a) and Western blot (Fig. 3b). Using specific antibodies we confirmed the identities of several DAz-1 labeled proteins as GAPDH, actin and peroxiredoxin (Fig. 3b), which are well known to contain sulfenic acid modifications.[1,3] Furthermore, no significant differences in cell viability were observed among DMSO, DAz-1-biotin and DAz-1-treated cells (Fig. S3). These data demonstrate the advantage of DAz-1 and bioorthogonal conjugation methods for detecting protein sulfenic acids directly in living cells.

In summary, we have reported an improved synthesis for the sulfenic acid probe DAz-1. This probe was successfully applied to detect sulfenic acid modifications in the cell-cycle regulatory phosphatase Cdc25A. These findings set the stage for development of activity-based tyrosine phosphatase inhibitors that are sensitive to the redox state of the active-site cysteine. Furthermore, we show that DAz-1 and bioorthogonal conjugation with reporter tags is the method of choice for detecting protein sulfenic acids under physi-

ological conditions in cells. Bi-functional probes such as DAz-1 should facilitate determination of the specific roles played by sulfenic acid modifications in redox-based cell signaling and in regulatory mechanisms that involve oxidation of cysteine residues.

Acknowledgments

We thank Prof. Mark Saper for the generous gift of Cdc25A C384S and Prof. Howard Hang for alkyne-biotin reporter **10**. We also thank the Life Sciences Institute and the Leukemia & Lymphoma Society Special Fellows Award #3100-07 and the American Heart Association Scientist Development Grant #0835419N to K.S.C. for support of this work.

Supplementary data

Synthetic procedures, analysis data, and protocols for biochemical studies. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.073.

References and notes

1. Reddie, K. G.; Carroll, K. S. *Curr. Opin. Chem. Biol.* **2008**. doi:10.1016/j.cbpa.2008.07.028. and references therein.
2. Miller, E. W.; Chang, C. J. *Curr. Opin. Chem. Biol.* **2007**, *11*, 620. and references therein.
3. Poole, L. B.; Nelson, K. J. *Curr. Opin. Chem. Biol.* **2008**, *12*, 18. and references therein.
4. Tonks, N. K. *Nat. Rev.* **2006**, *7*, 833. and references therein.
5. Benitez, L. V.; Allison, W. S. *J. Biol. Chem.* **1974**, *249*, 6234.
6. Poole, L. B.; Zeng, B. B.; Knaggs, S. A.; Yakubu, M.; King, S. B. *Bioconjug. Chem.* **2005**, *16*, 1624.
7. Reddie, K. G.; Seo, Y. H.; Muse, W. B.; Leonard, S. E.; Carroll, K. S. *Mol. Biosyst.* **2008**, *4*, 521.
8. Agard, N. J.; Baskin, J. M.; Prescher, J. A.; Lo, A.; Bertozzi, C. R. *ACS Chem. Biol.* **2006**, *1*, 644.
9. Van Tamelen, E. E.; Hildahl, G. T. *J. Am. Chem. Soc.* **1956**, *78*, 4405.
10. Hatzakis, S. N.; Engelkamp, H.; Velonia, K.; Hofkens, J.; Christianen, P. C. M.; Svendsen, A.; Patkar, S. A.; Vind, J.; Maan, J. C.; Rowan, A. E.; Nolte, R. J. M. *Chem. Commun. (Camb)* **2006**, 2012.
11. Green, M.; Berman, J. *Tet. Lett.* **1990**, *31*, 5851.
12. Boutros, R.; Dozier, C.; Ducommun, B. *Curr. Opin. Cell Biol.* **2006**, *18*, 185. and references therein.
13. Rudolph, J. *Antioxid. Redox Signal.* **2005**, *7*, 761.
14. Rudolph, J. *Biochemistry* **2007**, *46*, 3595.
15. Sohn, J.; Rudolph, J. *Biochemistry* **2003**, *42*, 10060.
16. The soluble catalytic domain of Cdc25A consists of residues 336–523; the back-door cysteine, which is not required for phosphatase activity, has been changed to serine (Cdc25A C384S).
17. Charles, R. L.; Schroder, E.; May, G.; Free, P.; Gaffney, P. R.; Wait, R.; Begum, S.; Heads, R. J.; Eaton, P. *Mol. Cell. Proteomics* **2007**, *6*, 1473.
18. Kwon, S. H.; Pimentel, D. R.; Remondino, A.; Sawyer, D. B.; Colucci, W. S. *J. Mol. Cell. Cardiol.* **2003**, *35*, 615.
19. Troyano, A.; Sancho, P.; Fernandez, C.; de Blas, E.; Bernardi, P.; Aller, P. *Cell Death Differ.* **2003**, *10*, 889.
20. Cohen, M. S.; Hadjivassiliou, H.; Taunton, J. *Nat. Chem. Biol.* **2007**, *3*, 156.
21. Hang, H. C.; Loureiro, J.; Spooner, E.; van der Velden, A. W.; Kim, Y. M.; Pollington, A. M.; Maehr, R.; Starnbach, M. N.; Ploegh, H. L. *ACS Chem. Biol.* **2006**, *1*, 713.