Fluorescent and Affinity-Based Tools To Detect Cysteine Sulfenic Acid Formation in Proteins

Leslie B. Poole,*[,†] Chananat Klomsiri,[†] Sarah A. Knaggs,[‡] Cristina M. Furdui,[||] Kimberly J. Nelson,[†] Michael J. Thomas,[†] Jacquelyn S. Fetrow,[§] Larry W. Daniel,[†] and S. Bruce King*[,‡]

Department of Biochemistry and Section on Molecular Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, and Departments of Chemistry, Physics and Computer Science, Wake Forest University, Winston-Salem, North Carolina 27109. Received July 10, 2007; Revised Manuscript Received September 3, 2007

Cysteine sulfenic acid formation in proteins results from the oxidative modification of susceptible cysteine residues by hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite. This species represents a biologically significant modification occurring during oxidant signaling or oxidative stress, and it can modulate protein function. Most methods to identify such oxidatively modified proteins rely on monitoring the loss of one or more thiol group(s) or on selective labeling of nascent thiol groups following reduction of oxidized proteins. Our previous work reported the direct labeling of these chemically distinct modifications with a dimedone analogue, 1,3-cyclohexadione, to which a linker and functional group (an alcohol) had been added; further addition of a fluorescent isatoic acid or methoxycoumarin reporter allowed detection of the incorporated tag by fluorescence techniques (Poole, L. B., Zeng, B. B., Knaggs, S. A., Yakubu, M., and King, S. B. (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. *Bioconjugate Chem. 16*, 1624–1628). We have now expanded our arsenal of tagging reagents to include two fluorescein-, two rhodamine-, and three biotin-conjugated probes based on the original approach. The new tools provide readily detectable fluorescent and affinity probes to identify sulfenic acid modifications in proteins and have been used in subsequent mass spectrometric analyses to confirm covalent attachment of the conjugates and directly determine the site of modification.

INTRODUCTION

Given the significant role played by formation of cysteine sulfenic acid (*S*-hydroxycysteine, R–SOH) in the redox regulation of enzymes and transcription regulators (*1–3*) and its general instability toward protein analytical methods (*4*), there is a critical need for better reagents to trap and identify these modifications in proteins. On the basis of a known alkylator of R–SOH, dimedone (5,5-dimethyl-1,3-cyclohexanedione), we previously designed, synthesized, and validated the use of two fluorescent reagents linked to the reactive core of dimedone, 1,3-cyclohexadione, as detectable markers of R–SOH formation in proteins (*5*). These reagents were shown to specifically trap only the R–SOH modification in a test protein, AhpC (a cysteine-based peroxidase from bacteria), leaving underivatized the other protein functional groups (including amines), as well as the thiol, disulfide, or hyperoxidized forms of AhpC. Other oxidized, sulfur-containing functional groups such as *S*-nitrosothiols and sulfoxides were similarly unreactive toward these reagents and dimedone. A limitation of the fluorophores chosen for these initial studies, isatoic acid and 7-methoxycoumarin, is their poor detectability in polyacrylamide gels; they are also not useful as affinity reagents for the isolation of R–SOH forming proteins.

To provide an expanded array of chemical tools to identify and isolate proteins that undergo oxidative modification to form R–SOH, we have implemented a strategy similar to that previously used to incorporate isatoic acid and methoxycoumarin into our dimedone-like reagent. Here, we describe the generation of seven new reagents through the attachment of fluorescein, rhodamine, or biotin moieties to an alcohol functional group on a linker attached to 1,3-cyclohexadione. In related work, generation of a biotinylated, dimedone-like reagent was also very recently reported by another group (*6*); this latter reagent was used to detect and isolate sulfenic acid-modified proteins from cells treated with hydrogen peroxide. Our new compounds are all useful for detecting labeled proteins in polyacrylamide gels; with the fluorescent reagents, a major advantage is that the labeled proteins are directly observable both in solution and after gel electrophoresis. Our data also demonstrate that all of our reagents are compatible with mass spectrometric approaches to confirm covalent attachment and identify the specific site of modification. Three of the reagents with biotin reporter groups offer advantages not only of being detectable through Western blotting type approaches but also of enabling affinity techniques for isolating such modified proteins.

EXPERIMENTAL PROCEDURES

General Synthetic Methods. Glassware was oven-dried before use and cooled to room temperature under N_2. All reagents and solvents were obtained from a commercial source and used without further purification unless noted otherwise. Analytical thin layer chromatography was performed on 250 μm silica gel 60 plates (DC-Fertigplatten Krieselgel 60 F254). Visualization was accomplished with UV light, ethanolic phosphomolybdic acid solution, ethanolic ninhydrin solution,

* To whom correspondence should be addressed. Address: Department of Biochemistry, Center for Structural Biology, Medical Center Boulevard, Winston-Salem, NC 27157. Telephone: 336-716-6711(L.B.P.), 336-758-5774(S.B.K.). Fax: 336-777-3242 (L.B.P.), 336-758-4656 (S.B.K.). E-mail: lbpoole@wfubmc.edu (L.B.P.), kingsb@wfu.edu (S.B.K.).

[†] Department of Biochemistry, Wake Forest University School of Medicine.

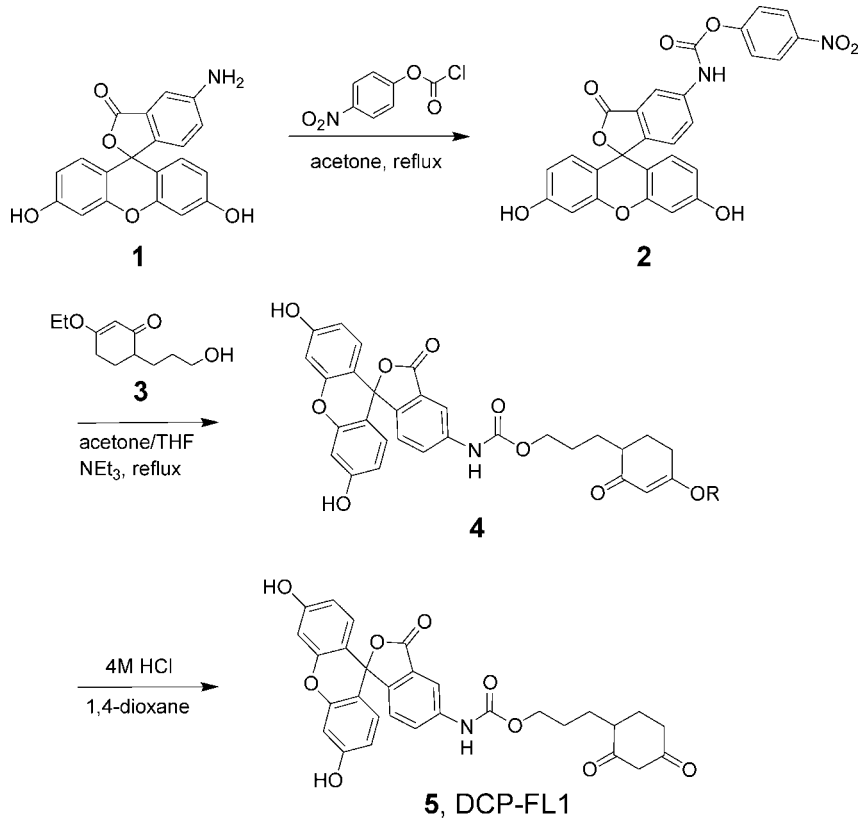
[‡] Department of Chemistry, Wake Forest University.

[||] Section on Molecular Medicine, Wake Forest University School of Medicine.

[§] Departments of Physics and Computer Science, Wake Forest University.

10.1021/bc700257a CCC: $37.00
Published on Web 11/21/2007

Scheme 1



or aqueous potassium permanganate solution followed by heating. Purification of the reaction products was carried out by flash column chromatography using silica gel 60 (32–63 μm). NMR spectra were recorded on a Bruker DPX 300 spectrometer, operating at 300 MHz (^{1}H NMR) and 75 MHz (^{13}C NMR), with chemical shifts referenced to the residual solvent peak. ^{1}H NMR data are reported as follows: chemical shift, integration, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), and coupling constants (Hz). Melting points were determined on a Thomas Hoover Uni-melt capillary melting point apparatus and are uncorrected. Mass spectra of synthesized compounds were obtained as a service by HT Laboratories (San Diego, CA) and are reported as *m/z*. Elemental combustion analysis was performed as a service by Atlantic Microlab Inc. (Atlanta, GA). For log *P* analyses, UV–vis spectra were recorded on a Cary 100 Bio UV–visible spectrophotometer.

Fluoresceinamine 5′-*N*-(*p*-Nitrophenyl)carbamate (2). To a solution of fluoresceinamine isomer I (**1**) (1.0 g, 2.88 mmol) in acetone (40 mL) was added *p*-nitrophenyl chloroformate (638 mg, 3.17 mmol) (Scheme 1). The resultant mixture was refluxed for 4 h and then stirred at room temperature for 24 h. The crude product was filtered from the reaction mixture and washed with dichloromethane (DCM)[1]/acetone (3/1 v/v, 40 mL) to yield a yellow-orange solid (1.42 g, 96.3%). TLC analysis and NMR indicated adequate purity for use in the next step: mp 186–189 °C (dec); R_f = 0.30 (hexanes/EtOAc/MeOH, 5/4/1); ^{1}H NMR (300 MHz, DMSO-d_6, δ) 11.05 (1H, bs), 8.34 (2H, d, J = 9.3 Hz), 8.18 (1H, s), 7.87 (1H, dd, J = 1.5, 8.1 Hz), 7.60 (2H, d, J = 9.0 Hz), 7.29 (1H, d, J = 8.4 Hz), 6.77 (2H, d, J = 1.5 Hz), 6.67–6.60 (4H, m); ^{13}C NMR (75 MHz, DMSO-d_6, δ) 168.7, 160.7, 155.7, 152.7, 151.2, 145.1, 140.4, 129.6, 127.9, 126.2, 125.7, 125.5, 123.3, 113.9, 113.5, 110.4, 102.6; ESI MS *m/z* 513 $(M + H)^+$.

Fluoresceinamine 5′-*N*-[3-(4-Ethoxy-2-oxocyclohex-3-enyl)-propyl)]carbamate (4). To a suspension of fluoresceinamine-5′-*N*-(*p*-nitrophenyl)carbamate (**2**) (500 mg, 0.91 mmol) in THF (10 mL) was added NEt_3 (130 μL, 0.91 mmol) and a solution of 3-ethoxy-6-(3-hydroxypropyl)-cyclohex-2-enone (**3**) (343 mg, 1.73 mmol) in THF (2 mL) (Scheme 1). Alcohol **3** was prepared by previously reported methods (*5*). The resultant mixture was stirred at reflux for 48 h. When the mixture was cooled, MeOH (10 mL) was added. The mixture was filtered, and the filtrate was reduced to dryness to yield an orange oil that was purified by flash column chromatography (hexanes/EtOAc/MeOH, 4/2/1). The pure product (**4**) was obtained as an orange solid (350 mg, 68.4%): mp 172–174 °C (dec); R_f = 0.28 (hexanes/EtOAc/MeOH, 4/2/1); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 7.96 (1H, s), 7.52 (1H, dd, J = 2.9, 8.5 Hz), 6.90 (1H, d, J = 8.4 Hz), 6.46–6.41 (4H, m), 6.32 (2H, dd, J = 2.3, 8.7 Hz), 5.13 (1H, s), 4.40 (2H, t, J = 6.3 Hz), 3.75 (2H, q, J = 6.3 Hz), 2.29 (2H, t, J = 5.7 Hz), 2.23–2.04 (1H, m), 1.96–1.87 (1H, dq, J =

[1]Abbreviations used: DCM, dichloromethane; DMSO, dimethyl sulfoxide; DMAP, 4-(dimethylamino)pyridine; THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; BOC, *tert*-butoxycarbonyl; GABA, γ-aminobutyric acid; HOBt, 1-hydroxybenzotriazole; DCP-FL1, fluoresceinamine 5′-*N*-[3-(2,4-dioxocyclohexyl)propyl)]carbamate; DCP-FL2, fluoresceinamine-5′-*N*-[3-((1-(3-(2,4-dioxocyclohexyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl]urea; DCP-Bio1, 3-(2,4-dioxocyclohexyl)propyl 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanoate; DCP-Bio2, 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)-*N*-((1-(3-(2,4-dioxocyclohexyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)pentanamide; DCP-Bio3, 3-(2,4-dioxocyclohexyl)propyl 4-(5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-d]imidazol-6-yl)-pentanamido)butylcarbamate; DCP-Rho1, rhodamine B [4-[3-(2,4-dioxocyclohexyl)propyl]carbamate]piperazine amide; DCP-Rho2, rhodamine B 3-(2,4-dioxocyclohexyl)propyl 4-oxo-4-(piperazin-1-yl)-butylcarbamate; TOF, time of flight; ESI-MS, electrospray ionization mass spectrometry.

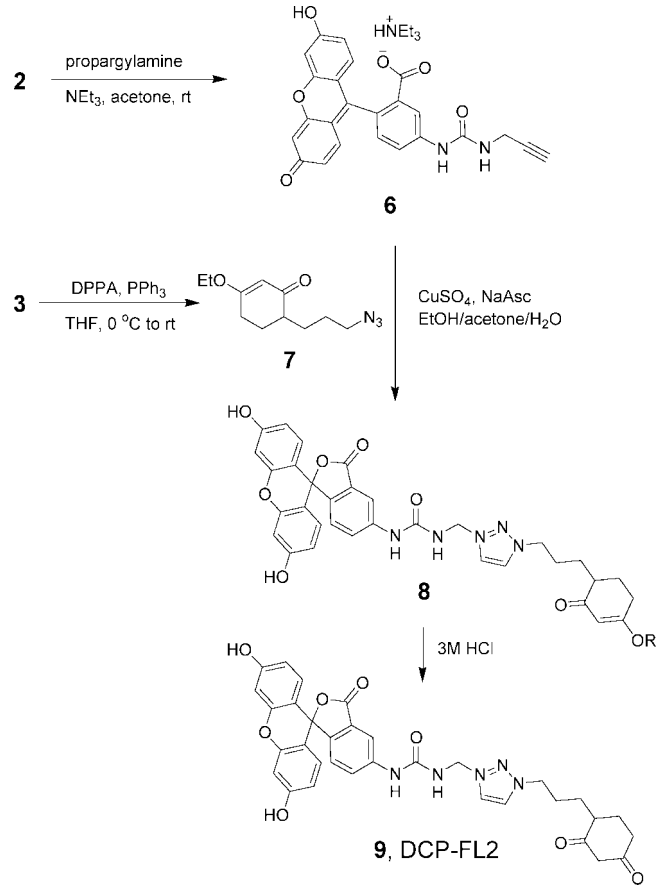
5.0 Hz), 1.79–1.68 (1H, m), 1.65–1.44 (3H, m), 1.38–1.20 (1H, m), 1.14 (3H, t, $J = 7.1$ Hz); ^{13}C NMR (75 MHz, acetone-d_6, δ) 199.9, 177.0, 169.4, 160.2, 154.6, 153.4, 147.6, 142.1, 142.0, 130.2, 128.9, 126.1, 126.0, 125.2, 113.4, 113.2, 111.9, 103.2, 102.5, 83.6, 65.9, 64.8, 45.5, 27.4, 27.3, 26.8, 14.4; ESI MS *m/z* 594 $(M + Na)^+$, 572 $(M + H)^+$. Anal. Calcd for $C_{32}H_{29}NO_9 \cdot H_2O$: C, 65.19; H, 5.30; N, 2.38. Found: C, 65.08; H, 5.30; N, 2.40.

Fluoresceinamine 5′-*N*-[3-(2,4-Dioxocyclohexyl)propyl)]-carbamate (5, DCP-FL1). Fluoresceinamine 5′-*N*-[3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl)]carbamate (**4**) (60 mg, 0.1 mmol) was stirred in a mixture of acetone (10 mL) and 3 N HCl (200 μL) (Scheme 1) until TLC analysis showed complete conversion. The reaction mixture was carefully neutralized (with either 10% NaOH solution or solid Na_2CO_3 followed by filtration) and reduced to dryness. The crude product (**5**) was purified by flash column chromatography (DCM/Et_2O/^{i}PrOH, 8/8/1) to yield the pure product as a yellow solid (54 mg, 94.7%): mp 239 °C (dec); $R_f = 0.19$ (hexanes/EtOAc/MeOH, 4/2/1); log *P* (octan-1-ol/phosphate buffer, pH 7.2) $= -0.89 \pm 0.20$; ^{1}H NMR (300 MHz, MeOH-d_4, δ) 8.23 (1H, s), 7.75 (1H, dd, $J = 1.9$, 8.4 Hz), 7.14 (1H, d, $J = 8.3$ Hz), 6.73–6.69 (4H, m), 6.58 (2H, dd, $J = 2.3$, 8.7 Hz), 4.27 (2H, t, $J = 6.2$ Hz), 2.45 (2H, q, $J = 6.1$ Hz), 2.41–2.34 (1H, m), 2.20–2.10 (1H, m), 2.00–1.75 (3H, m), 1.67–1.57 (1H, m); ^{13}C NMR (75 MHz, acetone-d_6, δ) 202.7, 169.8, 160.6, 155.1, 153.8, 148.1, 142.5, 130.6, 129.3, 126.6, 126.5, 125.7, 113.6, 112.4, 105.1, 103.7, 84.0, 66.3, 66.2, 50.0, 40.7, 27.8, 27.6, 26.1, 24.7; ESI MS *m/z* 544 $(M + H)^+$. Anal. Calcd for $C_{30}H_{25}NO_9 \cdot i$PrOH: C, 65.66; H, 5.51; N, 2.32. Found: C, 64.84; H, 5.38; N, 2.34.

Fluoresceinamine-5′-*N*-(3-propargyl)urea Triethylamine Salt (6). To a suspension of fluoresceinamine 5′-*N*-(*p*-nitrophenyl)carbamate (**2**) (160 mg, 0.31 mmol) in acetone/THF (2/1 v/v, 8 mL) was added propargylamine (21 μL, 0.34 mmol) and NEt_3 (44 μL, 0.31 mmol) (Scheme 2). The resultant solution was stirred at room temperature for 3 h during which the product (**6**) precipitated from the reaction mixture. This was filtered off and washed with DCM/acetone (2/1 v/v, 30 mL) to yield **6** as an orange solid (132 mg, 80.0%). TLC analysis and NMR indicated adequate purity for use in the next step: mp 187–189 °C (dec); $R_f = 0.56$ (EtOAc/hexanes/MeOH, 6/3/1); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 7.96 (1H, d, $J = 2.0$ Hz), 7.79 (1H, dd, $J = 2.1$, 8.3 Hz), 7.13 (1H, d, $J = 8.3$ Hz), 7.06 (2H, d, $J = 8.9$ Hz), 6.64 (2H, dd, $J = 2.2$, 5.4 Hz), 6.61 (2H, d, $J = 2.2$ Hz), 4.03 (2H, q, $J = 2.4$ Hz), 3.07 (6H, q, $J = 7.3$ Hz), 2.62 (1H, t, $J = 2.4$ Hz), 1.22 (9H, t, $J = 7.3$ Hz); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 173.0, 157.9, 157.4, 142.4, 132.1, 129.6, 122.6, 119.8, 118.9, 114.5, 103.9, 81.6, 72.0, 47.6, 30.2; ESI MS *m/z* 429 $(M + H)^+$.

3-Ethoxy-6-(3-azidopropyl)cyclohex-2-enone (7). To a cooled solution of 3-ethoxy-6-(3-hydroxylpropyl)cyclohex-2-enone (**3**) (2.0 g, 10.0 mmol) in THF (40 mL) at 0 °C was added triphenylphosphine (2.91 g, 11.0 mmol), diisopropyl azodicarboxylate (DIAD, 2.15 mL, 11.0 mmol), and diphenylphosphorylazide (DPPA, 2.4 mL, 11.0 mmol) sequentially (Scheme 2). The resultant mixture was stirred at 0 °C and then at room temperature for 16 h before being partitioned between DCM (100 mL) and water (50 mL). The aqueous phase was extracted with DCM (100 mL), and the organic phases were combined, washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and reduced to dryness. The resultant oil was purified by flash column chromatography (2×, hexanes/EtOAc, 2/1, and hexanes/EtOAc/MeOH, 12/2/1) to yield the pure product (**7**) as a pale-yellow liquid (1.6 g, 71.3%). $R_f = 0.31$ (EtOAc/hexanes/MeOH 12/2/1); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 5.31 (1H, s), 3.89 (2H, q, $J = 6.9$ Hz), 3.30 (2H, dt, $J = 1.6$, 6.8 Hz), 2.44 (2H, t, $J = 5.9$ Hz), 2.25–2.16 (1H, dpentet, $J = 1.8$, 5.2 Hz), 2.12–2.03 (1H, dq, $J = 5.0$, 13.2 Hz), 1.95–1.80 (1H, m), 1.78–1.59 (3H, m), 1.53–1.41 (1H, m), 1.36 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.2, 117.2, 102.5, 64.6, 51.9, 45.1, 28.5, 27.2, 26.9, 14.5.

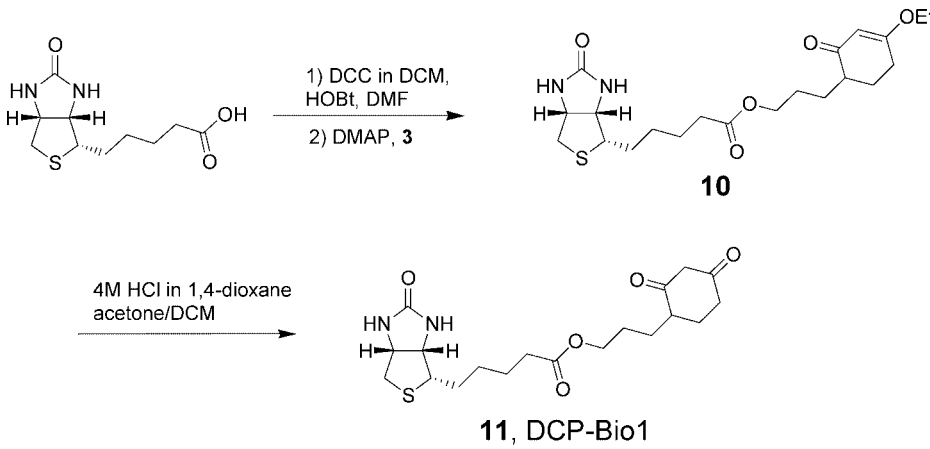
Scheme 2



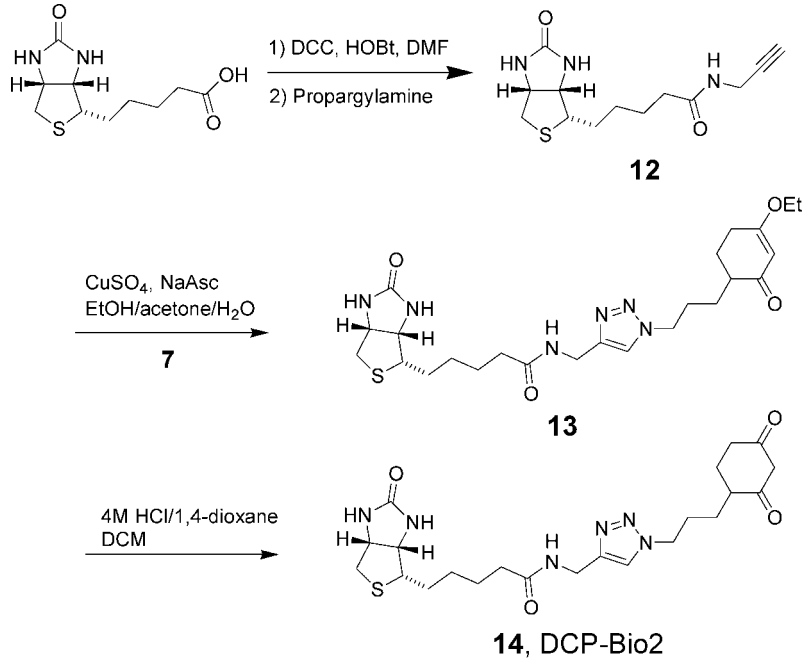
Fluoresceinamine-5′-*N*-[3-((1-(3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl]urea (8). Fluoresceinamine-5′-*N*-(3-propargyl)urea triethylamine salt (**6**) (132 mg, 0.25 mmol) and 3-ethoxy-6-(3-azidopropyl)cyclohex-2-enone (**7**) (139 mg, 0.62 mmol) were solubilized in a mixture of acetone/EtOH/H_2O (1/2/1 v/v, 4 mL). Sodium ascorbate (12 mg, 0.062 mmol) and $CuSO_4 \cdot 5H_2O$ (156 μL of a 0.1 M solution in H_2O, 0.0156 mmol) were added, and the resultant solution stirred at room temperature for 16 h (Scheme 2). The solvent was removed under reduced pressure and the resultant orange oil purified by flash column chromatography (gradient elution with EtOAc/hexanes/MeOH, 6/3/1, to EtOAc/MeOH, 7/1) to yield the pure product (**8**) as a bright-yellow foam (151 mg, 71.4 %): mp 157 °C (dec); $R_f = 0.14$ (EtOAc/hexanes/MeOH 6/3/1); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 8.10 (1H, s), 7.85 (1H, s), 7.56 (1H, d, $J = 8.3$ Hz), 6.96 (1H, d, $J = 8.3$ Hz), 6.59 (2H, d, $J = 2.0$ Hz), 6.53 (2H, d, $J = 8.7$ Hz), 6.44 (2H, dd, $J = 2.0$, 8.7 Hz), 5.19 (1H, s), 4.43 (2H, s), 4.28 (2H, t, $J = 6.7$ Hz), 3.80 (2H, q, $J = 7.1$ Hz), 2.38–2.21 (2H, m), 2.16–2.08 (1H, m), 1.97–1.78 (3H, m), 1.72–1.61 (1H, m), 1.59–1.48 (1H, m), 1.22 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (75 MHz, acetone-d_6) δ 200.8, 177.9, 170.5, 160.6, 156.5 (x2), 156.4, 153.8, 147.0, 143.6 (x2), 130.6, 129.1, 126.5, 126.4, 125.4, 123.7, 113.6, 112.4, 103.7, 102.9, 84.1, 65.4, 51.2, 45.6, 36.6 (×2), 29.1, 27.7, 14.9; ESI MS *m/z* 652 $(M + H)^+$, 674 $(M + Na)^+$. Anal. Calcd for $C_{35}H_{33}N_5O_8 \cdot 2$MeOH: C, 62.09; H, 5.77; N, 9.78. Found: C, 62.07; H, 5.46; N, 9.65.

Fluoresceinamine-5′-*N*-[3-((1-(3-(2,4-dioxocyclohexyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl]-urea (DCP-FL2, 9). Fluoresceinamine-5′-*N*-[3-((1-(3-(4-ethoxy-2-oxocyclohex-3-enyl)-

Scheme 3



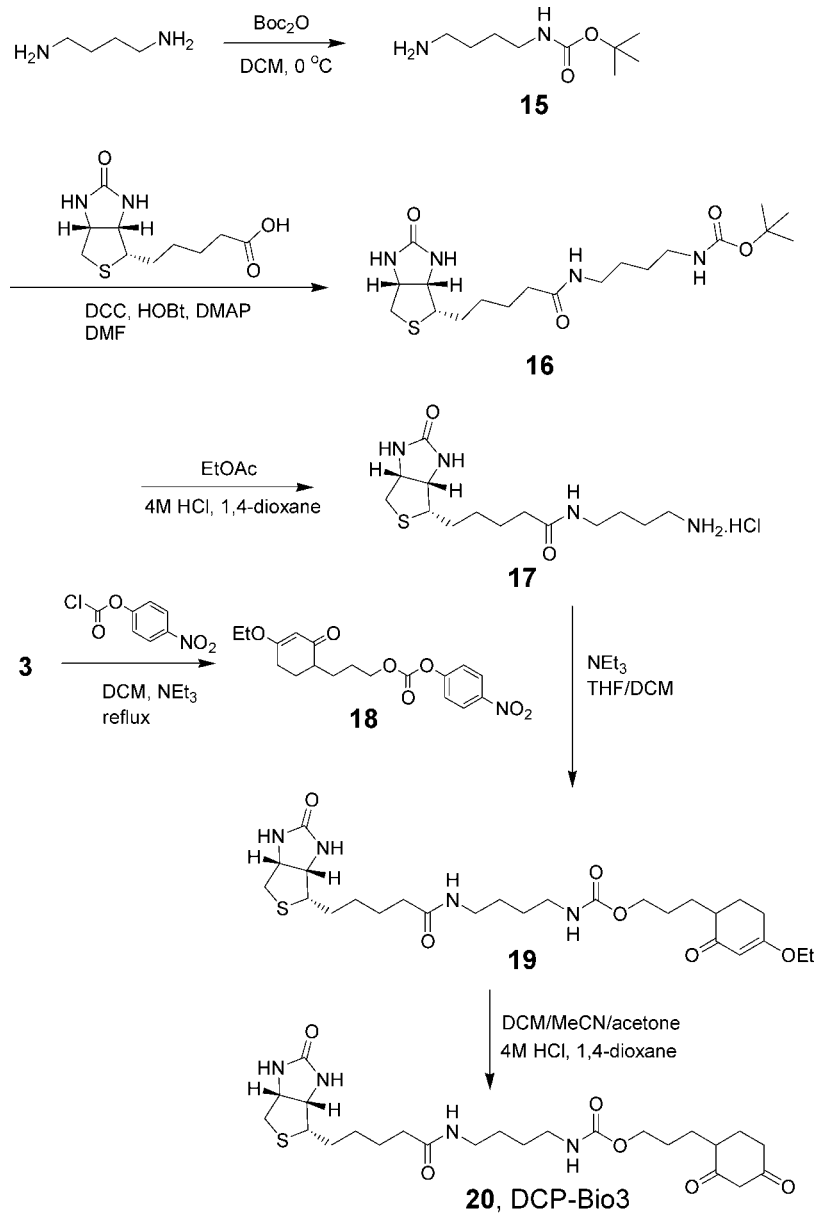
Scheme 4



propyl)-1*H*-1,2,3-triazol-4-yl)methyl]urea (**8**) (70 mg, 0.11 mmol) was stirred in a mixture of THF/acetone/H_2O (4/4/1 v/v/v, 9 mL) and treated with 3 N HCl (500 μL) (Scheme 2). The solution was stirred until TLC analysis indicated near complete conversion of **8** to **9** (3 h to 3 days). The reaction mixture was neutralized and then reduced to dryness. The resulting solids were washed with MeOH and filtered, and the filtrate was concentrated. The crude product was purified by flash column chromatography (gradient elution with DCM/MeOH, 8/2 to 6/4) to yield the pure product (**9**) as a yellow solid (64 mg, 95.5%): mp 224 °C (dec); $R_f = 0.31$ (DCM/MeOH 8/2); log *P* (octan-1-ol/phosphate buffer, pH 7.2) = -1.61 ± 0.24; ^{1}H NMR (300 MHz, MeOH-d_4, δ) 8.10 (1H, d, $J = 1.7$ Hz), 7.86 (1H, s), 7.55 (1H, dd, $J = 2.0$, 8.4 Hz), 6.95 (1H, d, $J = 8.3$ Hz), 6.57 (4H, d, $J = 2.2$ Hz), 6.45 (2H, dd, $J = 2.2$, 8.7 Hz), 4.40 (2H, s), 4.30 (2H, t, $J = 6.9$ Hz), 2.26 (2H, t,$J = 7.0$ Hz), 2.21–2.13 (1H, m), 2.05–1.80 (3H, m), 1.69–1.55 (2H, m), 1.37–1.28 (1H, m), 1.27–1.17 (2H, m); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 204.4, 181.6, 173.0, 172.2, 170.9, 168.5, 167.3, 161.2, 160.8, 144.6, 144.5, 134.7, 134.5, 133.2, 132.4, 131.9, 127.5, 123.6, 122.0, 121.6, 118.9, 118.6, 103.7, 52.5, 52.2, 36.3, 29.2, 29.0, 27.9, 27.7, 27.2; ESI MS *m/z* 624 $(M + H)^+$, 646 $(M + Na)^+$.

3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanoate (10). D-(+)-Biotin (600 mg, 2.46 mmol) and hydroxybenzotriazole (HOBt, 66 mg, 0.49 mmol) were suspended in anhydrous DMF (20 mL) with 4 Å molecular sieves and heated until a clear solution was obtained. When the mixture was cooled, a solution of dicyclohexylcarbodiimide (DCC) in DCM (2.7 mL of a 1.0 M solution in DCM, 2.70 mmol) was added dropwise and the mixture stirred at room temperature for 3 h (solution goes cloudy). 3-Ethoxy-6-(3-hydroxypropyl)cyclohex-2-enone (584 mg, 2.95 mmol) and *N,N*-dimethylaminopyridine (DMAP, 30 mg, 0.025 mmol) were added, and the mixture was stirred at 60 °C for 4 h and then at room temperature for 24 h. The mixture was filtered and washed with DCM/MeOH (1/1 v/v, 20 mL), and the filtrate was reduced to dryness and purified by column chromatography (2×, DCM/MeOH, 92/8, then DCM/MeOH, 95/5). The pure product was obtained as white foam (805 mg, 77.2%): mp 164–166 °C; $R_f = 0.43$ (DCM/MeOH 9/1); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 6.14 (1H, s), 5.70 (1H, s), 5.29 (1H, s), 4.49–4.45 (1H, m), 4.27 (1H, dd, $J = 4.7$, 7.2 Hz), 4.04 (2H, t, $J = 6.5$ Hz), 3.85 (2H, q, $J = 7.0$ Hz), 3.12 (1H, q, $J = 4.6$, 7.2 Hz), 2.87 (1H, dd, $J = 4.9$, 12.8 Hz), 2.71

Scheme 5

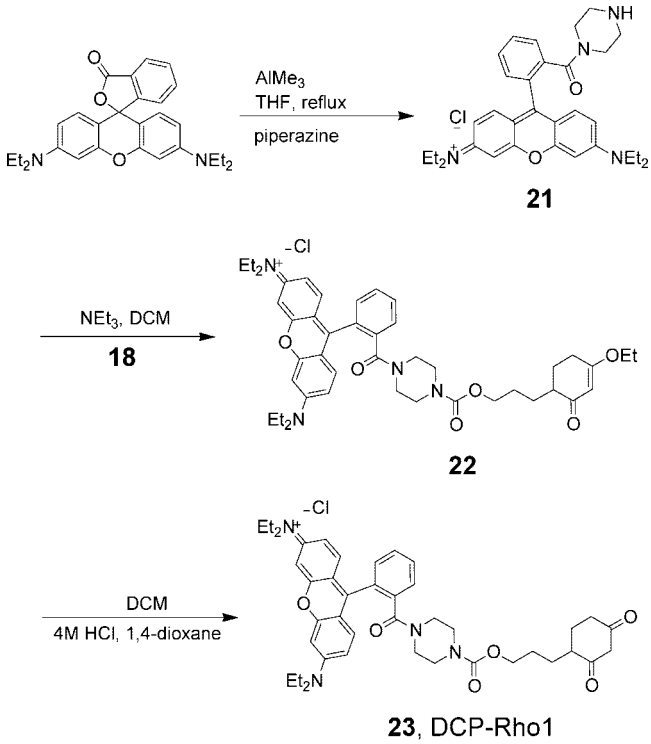


(1H, d, J = 12.7 Hz), 2.40 (2H, t, J = 5.8 Hz), 2.29 (2H, t, J = 7.4 Hz), 2.23–2.12 (1H, m), 2.09–2.00 (1H, dq, J = 5.0, 13.1 Hz), 1.93–1.81 (1H, m), 1.76–1.57 (6H, m), 1.46–1.36 (4H, m), 1.32 (3H, t, J = 7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.5, 177.3, 174.2, 164.3, 102.6, 64.7 (×2), 62.3, 60.5, 55.9, 50.4, 45.1, 34.4, 28.7, 28.6, 28.5, 26.8, 26.6 (×2), 26.4, 25.2, 14.5; ESI MS m/z 425 $(M + H)^+$.

3-(2,4-Dioxocyclohexyl)propyl 5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanoate (DCP-Bio1, 11). 3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanoate (**10**) (600 mg, 1.41 mmol) was solubilized in acetone/DCM (3/1 v/v, 24 mL) and treated with 4 M HCl in 1,4-dioxane (2 mL) (Scheme 3). The mixture was stirred at room temperature for 5 h and then carefully neutralized by the addition of solid Na_2CO_3. The inorganic solids were filtered off and washed with 10% MeOH in DCM, and the combined filtrates were reduced to dryness. The crude yellow oil was purified by flash column chromatography (gradient elution, DCM/MeOH, 90/10 to 89/11) to yield the pure product (**11**) as a glassy solid (390 mg, 69.6% [83.5% based on recovery of **3**]): mp 90–91 °C; R_f = 0.25 (DCM/MeOH 9/1); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 4.74 (1H, dd, J = 5.0, 7.4 Hz), 4.55 (1H, dd, J = 4.4, 7.6 Hz), 4.35 (2H, t, J = 6.1 Hz), 3.46 (1H, m), 3.18 (1H, dd, J = 4.9, 12.8 Hz), 2.96 (1H, d, J = 12.7 Hz), 2.69–2.52 (5H, m), 2.44–2.29 (1H, m), 2.16–1.68 (12H, m); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 204.8, 204.2, 174.1, 164.4 (163.9), 104.6, 64.6 (64.5), 62.5 (62.4), 60.7 (60.6), 58.7, 55.8 (×2), 53.8, 51.2, 49.3, 40.9, 40.1, 34.5 (34.3), 33.5, 30.1 (29.7), 28.7, 27.1, 26.7, 26.6, 26.4, 26.0, 25.3 (25.1); ESI MS m/z 419 $(M + Na)^+$. Anal. Calcd for $C_{19}H_{28}N_2O_5S \cdot 0.5$MeOH: C, 56.77; H, 7.33; N, 6.79. Found: C, 56.91; H, 7.27; N, 6.78.

5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)-*N*-(prop-2-ynyl)pentanamide (12). When D-(+)-biotin (400 mg, 1.64 mmol), HOBt (44 mg, 0.33 mmol), DCC (1.8 mL of a 1.0 M solution in DCM), and propargyamine (170 μL, 2.46 mmol) were used, the pure product (**12**, Scheme 4) was obtained after flash column chromatography (gradient elution, DCM/MeOH, 92/8 to 90/10) as a white solid (395 mg, 85.7%): mp 169–170 °C; R_f = 0.24 (DCM/MeOH, 9/1); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 4.28 (1H, dd, J = 4.9, 7.9 Hz), 4.10 (1H, dd, J = 4.4, 7.9 Hz), 3.04–2.97 (1H, m), 2.73 (1H, dd, J = 4.9, 12.7 Hz), 2.50 (1H, d, J = 12.7 Hz), 2.38 (2H, t, J = 2.5 Hz), 2.02 (2H, t, J = 7.4 Hz), 1.60–1.31 (4H, m), 1.25 (2H, q, J =

Scheme 6



7.3 Hz); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 176.1, 166.5, 81.1, 72.5, 63.8, 62.0, 57.4, 41.5, 36.9, 30.1, 29.9, 29.8, 27.1; ESI MS *m/z* 282 $(M + H)^+$, 304 $(M + Na)^+$.

***N*-((1-(3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamide (13).** 5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)-*N*-(prop-2-ynyl)pentanamide (**12**) (500 mg, 1.78 mmol) and 3-ethoxy-6-(3-azidopropyl)cyclohex-2-enone (**7**) (397 mg, 1.78 mmol) were solubilized in a mixture of acetone/EtOH/H_2O (1/2/2 v/v/v, 20 mL) (Scheme 4). Sodium ascorbate (70 mg, 0.35 mmol) and $CuSO_4$ (890 μL of a 0.1 M solution in H_2O) were then added, and the resultant solution was stirred at room temperature for 16 h. During this time the product (**13**) precipitated out of solution and was filtered off and washed with acetone to yield a white solid (690 mg). The filtrate was reduced to partial dryness, and EtOAc was added and filtered to yield a second crop (144 mg). Combined yield was 834 mg, 93%: mp 215–217 °C; R_f = 0.5 (DCM/MeOH 8/2); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 7.97 (1H, s), 5.43 (1H, s), 4.64–4.58 (1H, m), 4.54 (2H, s), 4.49 (2H, d, *J* = 7.0 Hz), 4.40 (1H, dd, *J* = 4.4, 7.9 Hz), 4.04 (2H, q, *J* = 7.0 Hz), 3.34–3.27 (1H, m), 3.03 (1H, dd, *J* = 4.9, 12.9 Hz), 2.81 (1H, d, *J* = 12.7 Hz), 2.62–2.56 (2H, m), 2.42–2.33 (3H, m), 2.23–2.12 (1H, m), 2.11–2.02 (3H, m), 1.92–1.63 (6H, m), 1.58–1.50 (2H, m), 1.46 (3H, t, *J* = 7.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 203.8, 180.1, 176.4, 167.1, 104.7, 64.5 (64.4), 62.9 (62.8), 58.4, 54.1, 47.1, 43.2, 38.3 (×2), 37.2 (37.1), 35.5 (35.3), 32.3, 30.9 (30.8), 30.6, 30.5, 29.3 (29.2), 28.1, 27.1, 16.8; ESI MS *m/z* 539 $(M - Na)^-$.

5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)-*N*-((1-(3-(2,4-dioxocyclohexyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)pentanamide (DCP-Bio2, 14). *N*-((1-(3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-5-((3a *R*,6 *S*,6a *S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamide (**13**) (88 mg, 0.174 mmol) was solubilized in hot DCM (15 mL) and treated with a 4 M solution of HCl in 1,4-dioxane (0.1 mL) and stirred at room temperature for 72 h. The mixture was then neutralized with solid Na_2CO_3, washed with MeOH, filtered, and reduced to dryness. The pure product (**14**) was obtained after column chromatography (DCM/MeOH, 8/2) as a tan foamy solid (69 mg, 83.1%): mp 176 °C (dec); R_f = 0.21 (DCM/MeOH, 8/2); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 8.02 (1H, s), 4.64 (1H, dd, *J* = 4.4, 7.8 Hz), 4.54 (2H, bs), 4.43 (2H, dd, *J* = 4.4, 7.8 Hz), 3.36–3.30 (1H, m), 3.06 (1H, dd, *J* = 4.9, 12.7 Hz), 2.83 (1H, d, *J* = 12.7 Hz), 2.53 (2H, t, *J* = 5.9 Hz), 2.48–2.44 (1H, m), 2.38 (2H, t, J = 7.3 Hz), 2.24–2.05 (2H, m), 1.95–1.64 (6H, m), 1.62–1.52 (4H, m); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 198.5, 189.0, 176.4, 166.4, 146.8, 124.7, 104.7, 63.8, 62.1 57.4, 51.8, 43.5, 41.5, 37.0, 36.0, 31.1, 30.1, 29.9, 29.4, 28.5, 27.2; ESI MS *m/z* 499 $(M + Na)^+$.

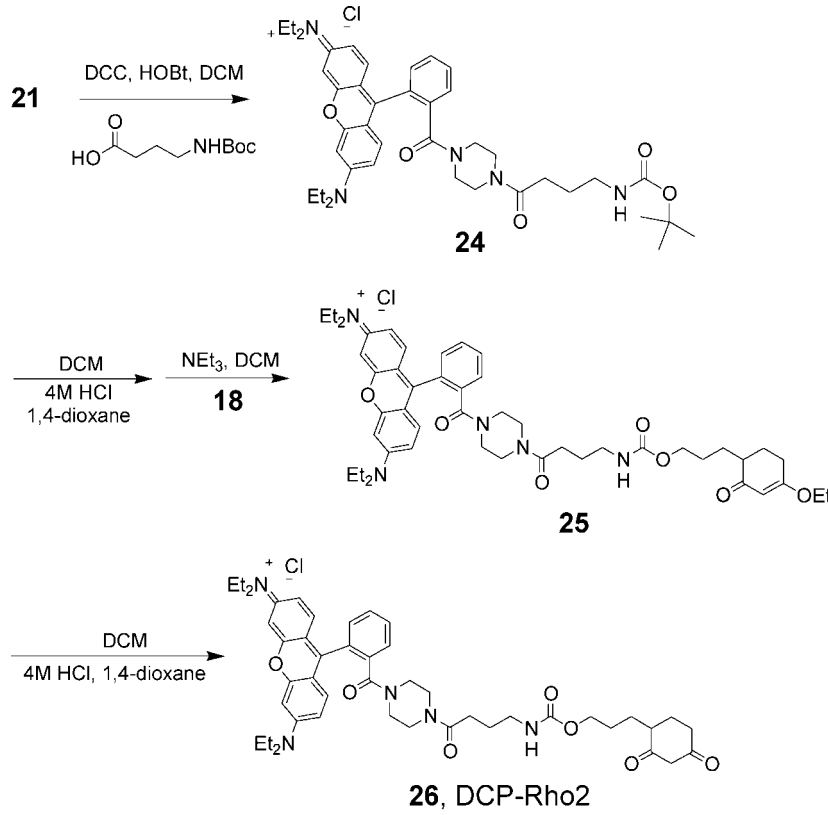
***tert*-Butyl 4-Aminobutylcarbamate (15).** To a solution of 1,4-diaminobutane (11.17 g, 0.127 mol) in DCM (170 mL) at 0 °C was added a solution of di-*tert*-butyl dicarbonate (2.77 g, 12.7 mmol) in DCM (50 mL) dropwise over 50 min (Scheme 5). The mixture was stirred at 0 °C for 2 h and then at room temperature for 16 h before being washed with water (100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4, and reduced to dryness. The crude oil was purified by column chromatography (gradient elution with a 1% NEt_3 saturated solution of DCM/MeOH, 9/1 to 8/2) to yield the pure product (**15**) as a waxy yellow solid (2.217 g, 92.8%). R_f = 0.16 (DCM/MeOH/NEt_3, 90/10/1); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 4.91 (1H, bs), 3.01 (2H, t, *J* = 6.0 Hz), 2.62 (2H, t, *J* = 6.6 Hz), 1.65 (2H, bs), 1.55–1.41 (4H, m), 1.32 (9H, s); ^{13}C NMR (300 MHz, $CDCl_3$, δ) 156.01, 78.9, 41.6, 40.4, 30.5, 28.4, 27.5.

***tert*-Butyl 4-(5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)butylcarbamate (16).** When D-(+)-biotin (500 mg, 2.05 mmol), HOBt (55 mg, 0.41 mmol), DCC (2.25 mL of a 1.0 M solution in DCM), and *tert*-butyl 4-aminobutylcarbamate (**15**) (578 mg, 3.07 mmol) were used, the pure product (**16**) was obtained after flash column chromatography (gradient elution with DCM/MeOH, 9/1 to 85/15) as a white solid (656 mg, 77.4 %; Scheme 5): mp 178–180 °C; R_f = 0.13 (DCM/MeOH, 9/1); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 4.48 (1H, dd, J = 7.9, 4.2 Hz), 4.30 (1H, dd, *J* = 4.5, 7.9 Hz), 3.23–3.14 (3H, m), 3.03 (2H, t, *J* = 6.5 Hz), 2.91 (1H, dd, *J* = 5.0, 12.7 Hz), 2.69 (1H, d, *J* = 12.7 Hz), 2.18 (2H, t, *J* = 7.3 Hz), 1.76–1.55 (4H, m), 1.53–1.45 (6H, m), 1.42 (9H, s); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 176.4, 166.5, 159.0, 80.3, 63.8, 62.0, 57.4, 41.5, 40.5, 37.3, 30.2, 29.9 (×2), 28.8, 28.1, 27.3, 26.6; ESI MS *m/z* 415 $(M + H)^+$, 437 $(M + Na)^+$.

***N*-(4-Aminobutyl)-5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamide Hydrochloride Salt (17).** To a suspension of *tert*-butyl 4-(5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)butylcarbamate (**16**) (370 mg, 0.89 mmol) in EtOAc (15 mL) was added a solution of HCl in 1,4-dioxane (2 mL of a 4 M solution) (Scheme 5). The mixture was stirred at room temperature for 1 h and then reduced to dryness. The crude product (**17**) was recrystallized to purity (MeOH/Et_20) to yield an off-white solid (289 mg, 92.3%): mp 50–51 °C (hygroscopic); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 4.62 (1H, dd, *J* = 4.7, 7.9 Hz), 4.43 (1H, dd, *J* = 4.4, 7.9 Hz), 3.31–3.23 (3H, m), 3.03–2.95 (3H, m), 2.77 (1H, d, *J* = 12.9 Hz), 2.27 (2H, t, *J* = 7.4 Hz), 1.84–1.54 (8H, m), 1.45 (2H, q, *J* = 7.1 Hz); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 174.0, 166.2, 64.7, 63.1, 57.3, 41.1, 40.8, 40.2, 36.9, 30.2, 29.8, 27.7, 27.3, 26.3.

3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 4-Nitrophenylcarbonate (18). To a solution of 3-ethoxy-6-(3-hydroxylpropyl)cyclohex-2-enone (**3**) (500 mg, 2.52 mmol) in anhydrous DCM (10 mL) was added NEt_3 (0.39 mL, 2.77 mmol) and *p*-nitrophenyl chloroformate (559 mg, 2.77 mmol) (Scheme 5). The mixture was stirred at reflux for 4 h, then at room temperature for 72 h, before being washed with water (20 mL)

Scheme 7



and brine (20 mL). The organic phase was dried, reduced to dryness, and purified by column chromatography (gradient elution with hexanes/EtOAc, 6/4 to 1/1) to yield the pure product (**18**) as a white solid (706 mg, 77.0%): mp 83–85 °C; R_f = 0.35 (hexanes/EtOAc, 1/1); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 8.27 (2H, dt, J = 9.3, 3.2, 2.3 Hz), 7.40 (2H, dt, J = 9.3, 3.2, 2.3 Hz), 5.33 (1H, s), 4.31 (2H, t, J = 6.5 Hz), 3.88 (2H, q, J = 6.9 Hz), 2.48–2.43 (2H, dt, J = 5.6Hz), 2.29–2.18 (1H, m), 2.14–2.05 (1H, dq, J = 4.9 Hz), 2.01–1.71 (4H, m), 1.60–1.47 (1H, m), 1.36 (3H, t, J = 7.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.2, 177.2, 156.0, 152.9, 145.7, 125.7, 122.2, 102.6, 69.9, 64.7, 45.0, 28.6, 26.9, 26.5, 26.2, 14.5; ESI MS *m/z* 364 (M + H)$^+$, 386 (M + Na)$^+$.

3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 4-(5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)butylcarbamate (19). To a suspension of *N*-(4-aminobutyl)-5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamide hydrochloride salt (**17**) (300 mg, 0.83 mmol) in THF/DCM (1/1 v/v, 8 mL) was added 3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl 4-nitrophenylcarbonate (**18**) (245 mg, 0.78 mmol) and NEt_3 (110 μL, 0.83 mmol) (Scheme 5). The mixture was stirred at room temperature for 72 h and filtered, and the solids were washed with MeOH/DCM (1/1 v/v, 20 mL). The filtrate was reduced to dryness to yield a yellow oil that was purified by column chromatography (DCM/MeOH, 85/15). The pure product (**19**) was obtained as a white solid (200 mg, 48.8%): mp 191–192 °C; R_f = 0.13 (DCM/MeOH, 9/1); ^{1}H NMR (300 MHz, $CDCl_3$/MeOH-d_4, δ) 5.05 (1H, s), 4.05 (1H, dd, J = 4.5, 7.8 Hz), 3.78 (2H, t, J = 6.3 Hz), 3.69 (2H, q, J = 7.1 Hz), 2.96–2.91 (3H, m), 2.87 (2H, t, J = 6.8 Hz), 2.67 (1H, dd, J = 4.9, 12.9 Hz), 2.47 (1H, d, J = 12.9 Hz), 2.21 (2H, t, J = 5.8 Hz), 2.00–1.98 (1H, m), 1.94 (2H, t, J = 7.2 Hz), 1.89–1.81 (1H, m), 1.66–1.52 (1H, m), 1.50–1.33 (6H, m), 1.30–1.18 (6H, m), 1.12 (3H, t, J = 7.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$/MeOH-d_4, δ) 207.2, 205.5, 182.8, 178.7, 168.6, 162.0, 105.9, 105.6, 68.8, 66.3, 64.5, 61.7, 59.9, 48.9, 44.5, 43.2, 40.0, 32.7, 32.4, 32.2, 31.4, 30.8, 30.7, 30.4, 30.2, 29.8, 21.9, 18.1, 12.8; ESI MS *m/z* 561 (M + Na)$^+$.

3-(2,4-Dioxocyclohexyl)propyl 4-(5-((3a*R*,6*S*,6a*S*)-Hexahydro-2-oxo-1 *H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)butylcarbamate (DCP-Bio3, 20). 3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 4-(5-((3a*R*,6*S*,6a*S*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-6-yl)pentanamido)butylcarbamate (**19**) (110 mg, 0.20 mmol) was solubilized in DCM/MeCN/acetone (2/1/1 v/v/v, 12 mL) and treated with a 4 M solution of HCl in 1,4-dioxane (0.5 mL) (Scheme 5). The mixture was stirred at room temperature for 4 h and then neutralized by the addition of solid sodium carbonate. The mixture was then filtered and washed with DCM/MeOH (3/1 v/v, 20 mL). The filtrate was reduced to dryness and purified by flash column chromatography (gradient elution with DCM/MeOH, 90/10 to 80/20) to yield the pure product (**20**) as a tan foamy solid (82 mg, 78.8%): mp 155 °C (dec); R_f = 0.42 (DCM/MeOH, 80/20); ^{1}H NMR (300 MHz, MeOH-d_4, δ) 4.60 (1H, dd, J = 4.8, 7.4 Hz), 4.40 (1H, dd, J = 4.4, 7.8 Hz), 4.13 (2H, t, J = 5.8 Hz), 3.28 (2H, bd, J = 3.7 Hz), 3.20 (2H, m), 3.02 (1H, dd, J = 4.9, 12.7 Hz), 2.80 (1H, d, J = 12.7 Hz), 2.50 (2H, m), 2.42–2.39 (1H, m), 2.30 (2H, t, J = 7.2 Hz), 2.25–2.15 (1H, m), 1.96–1.50 (16H, m); ^{13}C NMR (75 MHz, MeOH-d_4, δ) 176.5, 166.4, 159.7, 66.2, 63.8, 62.1, 57.4, 53.0, 43.6, 41.8, 41.5, 40.4, 37.3, 34.3, 31.1, 30.2, 29.9, 28.7, 28.3, 28.1, 27.7, 27.3, 26.7, 26.6; ESI MS *m/z* 533 (M + Na)$^+$.

Rhodamine B Piperazine Amide (21). To a solution of piperazine (4.67 g, 54 mmol) in anhydrous DCM (50 mL) was added trimethylaluminum (13.6 mL of a 2 M solution in toluene) dropwise. The resulting mixture was stirred at room temperature for 1 h, and then a solution of rhodamine B base (6.0 g) in DCM (15 mL) was added dropwise (Scheme 6). The reaction mixture was refluxed for 72 h. Upon cooling, the mixture was carefully neutralized by the dropwise addition of a 0.1 M HCl aqueous solution. The heterogeneous mixture was filtered, and the inorganics salts were washed sequentially with DCM (100

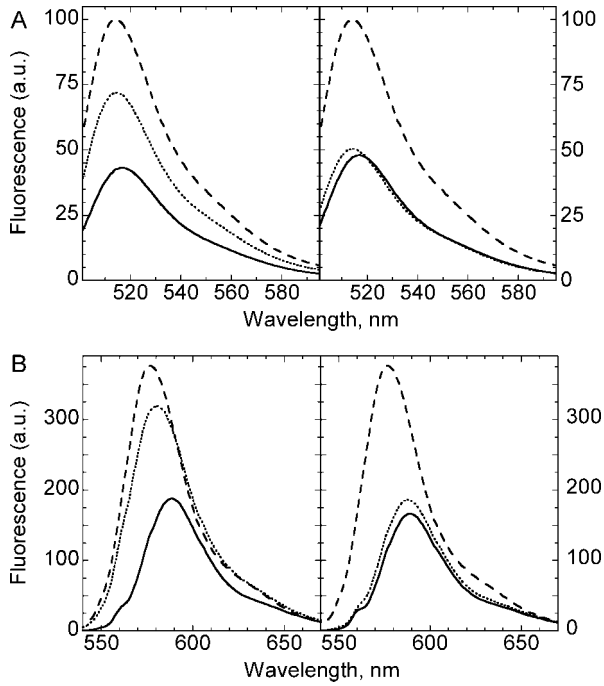


Figure 1. Fluorescence spectra of fluorescein- (A) and rhodamine-linked (B) reagents before (dotted) and after (solid) reaction with cysteine sulfenic acid-containing AhpC. Free fluorescein (A) and rhodamine B (B) are shown as the dashed spectra for comparison. Labeled C165S AhpC was prepared as described in the Experimental Procedures with the cysteine sulfenic acid form of the protein incubated anaerobically with 5 mM **5** (DCP-FL1), **9** (DCP-FL2), **23** (DCP-Rho1), or **26** (DCP-Rho2) for 60 min and then washed free of the unreacted reagent and buffer components into a final buffer of 50 mM Tris-HCl at pH 8 (fluorescein) or 50 mM potassium phosphate buffer at pH 7 (rhodamine) using an Apollo concentrator. Samples in panel A at ~0.5 absorbance (491 nm excitation) were analyzed on a Tecan Safire 2 monochromator-based microplate reader with a gain of 68. Samples in panel B at an absorbance of 0.14 (560 nm excitation) were analyzed on a Varian Cary Eclipse fluorescence spectrophotometer at medium sensitivity, with 5 nm bandwiths for both excitation and emission wavelengths in both instruments.

mL) and DCM/MeOH (4/1 v/v, 150 mL). The filtrate was reduced to partial dryness and partitioned between EtOAc (3 × 200 mL) and saturated $NaHCO_3$ solution (300 mL). The aqueous phase was saturated with NaCl, acidified with 1 M HCl, and extracted with *i*PrOH/DCM (2/1 v/v, 5 × 150 mL). The organic phase was dried over $MgSO_4$, filtered, and reduced to dryness to yield a purple oil. This was solubilized in a minimal amount of MeOH and added dropwise to Et_2O (500 mL), and the crude product (**21**) was filtered off and obtained as a purple-gold solid (5.88 g, 79.3%). ^{1}H NMR (300 MHz, $CDCl_3$, δ) 7.59–7.53 (2H, m), 7.46–7.41 (1H, m), 7.25–7.17 (1H, m), 7.10 (1H, d, J = 9.5 Hz), 7.02 (1H, d, J = 9.5 Hz), 6.84–6.79 (2H, m), 6.64 (2H, dd, J = 2.3, 6.2 Hz), 3.78 (2H, bd, J = 14.1 Hz), 3.63–3.40 (8H, m), 3.32–3.18 (4H, bs), 3.25 (4H, m), 2.98 (2H, bd, J = 20.4 Hz), 2.42 (1H, bs), 1.20 (12H, t, J = 7.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 167.5, 158.0, 156.0, 155.9, 155.8, 134.6, 132.4, 131.7, 131.4, 131.0, 130.7, 130.6, 130.4, 130.3, 127.9, 114.7, 114.4, 96.7, 58.1, 50.4, 46.6, 46.5, 18.7, 13.0; ESI MS *m/z* 511 $(M + H)^+$.

Rhodamine B [4-[3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl]carbamate]piperazine Amide (22). To a solution of rhodamine B piperazine amide (**21**) (434 mg, 0.79 mmol) in anhydrous DCM (10 mL) was added NEt_3 (180 μL, 1.32 mmol) and 3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl 4-nitrophenylcarbonate (**18**) (240 mg, 0.66 mmol) (Scheme 6). The reaction mixture was stirred at reflux for 6 h and then at room temperature for 16 h, reduced to dryness, and purified by flash column chromatography (2×, DCM/MeOH, 90/10 and then 91/9) to yield the pure product (**22**) as a purple foam (300 mg, 58.8%). R_f = 0.25 (DCM/MeOH 9/1); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 7.64 (2H, m), 7.48 (1H, dd, J = 2.8, 6.8 Hz), 7.30 (1H, dd, J = 2.6, 5.9 Hz), 7.16 (2H, q, J = 9.5 Hz), 6.89 (2H, d, J = 7.6 Hz), 6.74 (2H, bs), 5.24 (1H, s), 3.99 (2H, t, J = 6.5 Hz), 3.80 (2H, q, J = 7.0 Hz), 3.59 (8H, q, J = 7.1 Hz), 3.32 (4H, bs), 3.25 (4H, bs), 2.34 (2H, d, J = 5.3 Hz), 2.15–2.06 (1H, m), 2.04–196 (1H, m), 1.84–1.75 (1H, m), 1.69–1.49 (3H, m), 1.38–1.18 (16H, m); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.4, 201.3, 177.4, 168.0, 158.0, 156.0, 155.9, 155.5, 135.3, 132.3, 131.0, 130.8, 130.6, 127.9, 114.5, 114.0, 102.4, 96.8, 66.1, 64.6, 53.9, 46.5, 45.0, 28.5, 26.8, 26.7, 26.2, 14.5, 13.0.

Rhodamine B [4-[3-(2,4-Dioxocyclohexyl)propyl]carbamate]piperazine Amide (DCP-Rho1, 23). Rhodamine B [4-[3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl]carbamate]piperazine amide (**22**) (280 mg, 0.36 mmol) was solubilized in DCM (16 mL) and treated with 4 M HCl in 1,4-dioxane (1 mL) (Scheme 6). The mixture was stirred at room temperature for 16 h, then carefully neutralized by the addition of solid Na_2CO_3. The inorganic solids were filtered off and washed with 10% MeOH in DCM, and the combined filtrates were reduced to dryness. The crude purple oil was purified by flash column chromatography (gradient elution, DCM/MeOH, 92/8 to 90/10) to yield the pure product (**23**) as a purple solid (215 mg, 79.6%): log P (octan-1-ol/phosphate buffer, pH 7.2) = 1.026 ± 0.113; ^{1}H NMR (300 MHz, $CDCl_3$, δ) 7.62 (2H, d, J = 3.3 Hz), 7.48 (1H, d, J = 3.0 Hz), 7.29 (1H, d, J = 3.0 Hz), 7.17 (2H, d, J = 9.0 Hz), 6.90 (2H, bs), 6.75 (2H, bs), 5.24 (1H, bs), 3.99 (2H, t, J = 6.0 Hz), 3.82 (8H, t, J = 7.8 Hz), 3.32 (4H, bs), 3.26 (4H, bs), 2.35 (2H, d, J = 5.4 Hz), 2.14–2.08 (1H, m), 2.03–1.98 (1H, m), 1.79–1.75 (1H, m), 1.74–1.62 (3H, m), 1.61–1.44 (1H, m), 1.26 (12H, t, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.3, 178.2, 168.1, 158.1, 156.1, 155.6, 135.4, 132.4, 131.1, 130.8, 130.6, 128.0, 114.6, 114.1, 102.4, 96.9, 66.2, 66.0, 56.1, 46.6, 45.1, 28.4, 27.7, 27.1, 26.8, 26.7, 26.4, 26.2, 13.1; ESI MS *m/z* 708 $(M + H)^+$.

Rhodamine B *tert*-Butyl 4-oxo-4-(piperazin-1-yl)butylcarbamate (24). *N*-Boc GABA-OH (149 mg, 0.73 mmol) in anhydrous DCM/THF/acetone (1/1/3 v/v/v, 10 mL) was treated with DCC (0.8 mL of 1.0 M solution in DCM). After the mixture was stirred at room temperature for 2 h, rhodamine B piperazine amide (**21**) (400 mg, 0.73 mmol) was added. The mixture was stirred at room temperature for 16 h and reduced to dryness. The residue was purified by flash column chromatography (DCM/MeOH, 90/10) to yield the pure product (**24**) as a purple foam (351 mg, 65.6%). ^{1}H NMR (300 MHz, $CDCl_3$, δ) 7.76 (2H, bs), 7.63 (1H, bs), 7.41–7.01 (5H, m), 6.98 (1H, bs), 6.78 (1H, bs), 3.82–3.57 (10H, m), 3.51–3.36 (5H, m), 3.25–3.18 (3H, m), 2.62–2.57 (1H, m), 1.92–1.88 (4H, m), 1.51 (9H, d, J = 5.0 Hz), 1.42 (12H, t, J = 7.0Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 172.6, 168.0, 158.1, 156.7, 156.6, 156.1, 135.6, 132.5, 131.4, 131.3, 130.6, 130.5, 128.0, 115.3, 114.2, 96.9, 96.4, 79.2, 78.9, 53.9, 48.5, 46.5 (46.3, 42.1, 41.3, 40.5, 32.6, 31.5, 30.7), 28.8, 25.8, 13.0.

Rhodamine B 3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 4-Oxo-4-(piperazin-1-yl)butylcarbamate (25). Rhodamine B *tert*-butyl 4-oxo-4-(piperazin-1-yl)butylcarbamate (**24**) (350 mg, 0.48 mmol) in DCM (6 mL) was treated with 4 M HCl in 1,4-dioxane (0.5 mL). The mixture was stirred at room temperature until TLC showed no starting material remaining (5 h), and the mixture was reduced to dryness to yield the crude product as a purple solid (320 mg, 100%). This was directly used in the next step without purification, resolubilized in DCM (10 mL), and treated with NEt_3 (133 μL, 0.96 mmol) and 3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl 4-nitrophenylcarbonate (**18**) (174 mg, 0.48 mmol). The mixture was stirred at room temperature

Table 1. Mass and Spectral Information for Sulfenic Acid-Directed Reagents and Their Adducts with AhpC

reagent	empirical formula	monoisotopic molecular mass	average molecular weight (g/mol)	characteristic ion mass ([M + H]$^+$, Da)[a]	mass of AhpC protein adduct (Da)[b]	$\lambda_{max,ex}$ and $\lambda_{max,em}$ of free reagent (nm)	λ_{max} and $\lambda_{max,em}$ of protein adduct (nm)
DCP-FL1	$C_{30}H_{25}NO_9$	543.15	543.5	576.13	21142.9 ± 2.1	490, 514	493, 517
DCP-FL2	$C_{33}H_{29}N_5O_8$	623.20	623.6	656.18	21223.3 ± 2.1	491, 514	494, 517
DCP-Bio1	$C_{19}H_{28}N_2O_5S$	396.17	396.5	429.14	20996.1 ± 2.1	NF[c]	NF[c]
DCP-Bio2	$C_{22}H_{32}N_6O_4S$	476.22	476.6	509.20	21076.6 ± 2.1	NF[c]	NF[c]
DCP-Bio3	$C_{24}H_{38}N_4O_6S$	510.25	510.7	543.23	21112.5 ± 2.7	NF[c]	NF[c]
DCP-Rho1	$C_{42}H_{51}N_4O_6$	707.38	707.9	739.38[d]	21304.9 ± 2.1	560, 581	570, 588
DCP-Rho2	$C_{46}H_{58}N_5O_7$	792.43	793.0	824.38[d]	21389.6 ± 2.1	570, 588	570, 589

[a] *m/z* value of ion released during MS–MS analysis of the labeled peptide from AhpC, as described in the text. [b] Mass of adduct by electrospray ionization mass spectrometry in a triple quadrupole mass spectrometer. For comparison, the underivatized sulfenic acid form of the C165S mutant of AhpC analyzed under these conditions gave a major peak at 20648.7 Da, accounting for the addition of three oxygen atoms to the protein (presumably the sulfonic acid form at the active site cysteine). The errors listed reflect the hardware accuracy of ±0.01%. Where the MaxEnt error was greater than the instrument error, this number was used instead. [c] NF = nonfluorescent. [d] [M$^+$], positively charged without the addition of H$^+$.

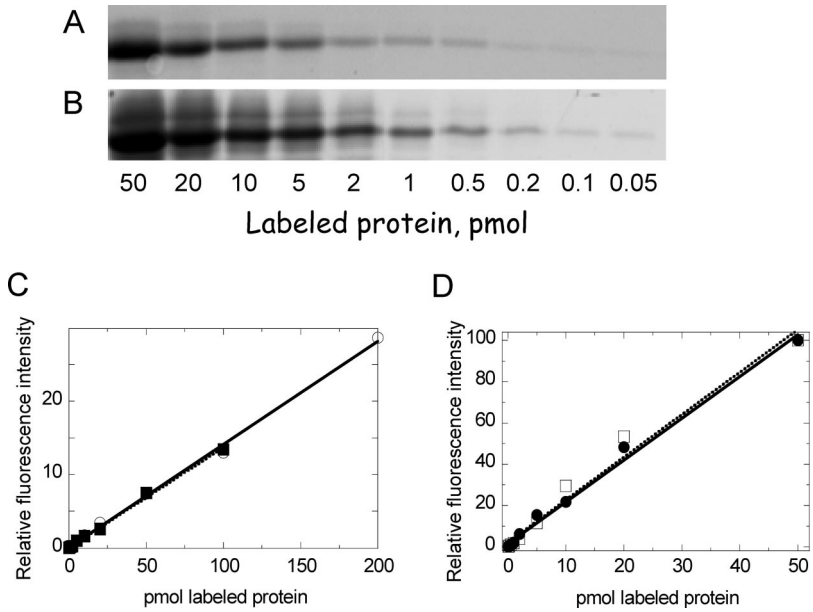


Figure 2. Visualization of labeled AhpC proteins after 1D gel electrophoresis. Varying amounts of AhpC C165S labeled with DCP-FL1 (A) or DCP-Rho1 (B) were loaded onto SDS–polyacylamide gels and resolved by electrophoresis. Gels were washed in deionized water for 30 min and then imaged on an Amersham Storm 840 for fluorescein reagents (using a 450 nm LED at normal sensitivity) or on a Typhoon 8600 fluorescence imager for rhodamine reagents (with a 633 nm red laser and Tamara 580 BP 30 filter at normal sensitivity). Relative fluorescence intensity was determined using ImageQuant software and plotted vs pmol of labeled protein for (C) DCP-FL1 (■) and DCP-FL2 (○) or for (D) DCP-Rho1 (●) and DCP-Rho2 (□).

for 16 h, reduced to dryness, and purified by flash column chromatography (gradient elution, DCM/MeOH, 93/7 to 90/10) to yield the pure product (**25**) as a purple foam (229 mg, 55.9%). R_f = 0.35 (DCM/MeOH 9/1); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 7.61 (2H, bs), 7.48 (1H, bs), 7.27–7.16 (4H, m), 6.85–6.37 (3H, m), 5.20 (1H, s), 3.93 (2H, bs), 3.79 (2H, q, J = 7.0 Hz), 3.57–3.50 (10H, m), 3.34–3.23 (6H, m), 3.13 (2H, bs), 2.68 (2H, bs), 2.48 (1H, bs), 2.35 (2H, t, J = 5.7 Hz), 2.10–1.97 (2H, m), 1.75–1.57 (5H, m), 1.30–1.23 (12H, m); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.7, 177.3, 172.7, 168.0, 158.0, 157.5, 156.3, 156.0, 135.6, 132.5, 131.4, 130.5, 130.4, 130.3, 128.0, 115.2, 114.4, 114.1, 102.4, 96.8, 96.4, 64.8, 64.5, 48.5, 46.5, 46.3, 45.1, (42.1, 41.3, 40.9, 31.6, 31.3, 30.0), 28.4, 27.0, 26.6, 26.2, 25.7, 14.5, 13.0; ESI MS *m/z* 821 (M + H)$^+$.

Rhodamine B 3-(2,4-Dioxocyclohexyl)propyl 4-Oxo-4-(piperazin-1-yl)butylcarbamate (DCP-Rho2, 26). Rhodamine B 3-(4-ethoxy-2-oxocyclohex-3-enyl)propyl 4-oxo-4-(piperazin-1-yl)butylcarbamate (**25**) (200 mg, 0.23 mmol) was solubilized in DCM (10 mL) and treated with 4 M HCl in 1,4-dioxane (1 mL). The mixture was stirred at room temperature for 6 h and then carefully neutralized by the addition of solid Na_2CO_3. The inorganic solids were filtered off and washed with 10% MeOH in DCM, and the combined filtrates were reduced to dryness. The residue was purified by flash column chromatography (2×, DCM/MeOH, 86/14 and then 88/12) to yield the product (**26**) as a purple foam (99 mg, 51.3%). ^{1}H NMR (300 MHz, $CDCl_3$, δ) 7.64 (2H, m), 7.48 (1H, dd, J = 2.8, 6.8 Hz), 7.30 (1H, dd, J = 2.6, 5.9 Hz), 7.16 (2H, q, J = 9.5 Hz), 6.89 (2H, d, J = 7.6 Hz), 6.74 (2H, bs), 5.24 (1H, s), 3.99 (2H, t, J = 6.5 Hz), 3.80 (2H, q, J = 7.0 Hz), 3.59 (8H, q, J = 7.1 Hz), 3.32 (4H, bs), 3.25 (4H, bs), 2.34 (2H, d, J = 5.3 Hz), 2.15–2.06 (1H, m), 2.04–196 (1H, m), 1.84–1.75 (1H, m), 1.69–1.49 (3H, m), 1.38–1.18 (16H, m); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 201.7, 177.3, 172.7, 168.0, 158.0, 157.5, 156.3, 156.0, 135.6, 132.5, 131.4, 130.5, 130.4, 130.3, 128.0, 115.2, 114.4, 114.1, 102.4, 96.8, 96.4, 64.8, 64.5, 48.5, 46.5, 46.3, 45.1, (42.1, 41.3, 40.9, 31.6, 31.3, 30.0), 28.4, 27.0, 26.6, 26.2, 25.7, 14.5, 13.0; ESI MS *m/z* 793 (M + H)$^+$.

Generation and Labeling of Sulfenic Acid-Containing C165S AhpC and Papain for Spectral and Mass Spectrometric Analysis. The C165S mutant of AhpC, stored in 5 mM 1,4-dithiothreitol (DTT) but washed free of DTT prior to use, was purified essentially as described previously (*7*). Papain was purchased from Sigma-Aldrich and subjected to concentration and redilution in Apollo concentrators (Orbital Biosciences, Topsfield, MA; 20 kDa cutoff) before use to remove low

molecular weight contaminants. Generation of the sulfenic acid form of AhpC followed earlier procedures (*5*). The corresponding oxidized form of papain was generated by addition of 2 equiv of hydrogen peroxide under anaerobic conditions (in a 10 mL pear-shaped flask subjected to a gentle vacuum and flushed with argon multiple times).

Labeling procedures for generating the alkylated forms of AhpC and papain for subsequent mass spectrometric (MS) analysis were performed with approximately 6 nmol of the sulfenic acid-containing protein under anaerobic conditions. Reactions in either an anaerobic flask or argon-flushed Eppendorf tubes were started with the addition of 5 mM (final concentration) of the reagent of choice in a final volume of 50–100 μL. Reagents were added from a 250 mM stock predissolved in dimethyl sulfoxide (DMSO). Following incubation at room temperature for 60 min or overnight, samples were concentrated and rediluted (at least 3 times, 6 times or more for sticky fluorescein and rhodamine-based reagents) into the buffer of choice using 20 kDa cutoff Apollo ultrafiltration devices. Samples were stored at −20 °C until further use.

Spectral and Gel Analysis of Reagents and Labeled Proteins. UV–visible absorbance spectra were acquired with either an Agilent HP8452A UV–vis diode array spectrophotometer or a Beckman DU-7500 diode array spectrophotometer. Fluorescence measurements were made with either a Varian Cary Eclipse fluorescence spectrophotometer or a Tecan Safire 2 monochromator-based microplate reader. Approximate 100 or 250 mM stock solutions of DCP-FL1 or DCP-FL2 (**5** or **9**, respectively) in DMSO were in some cases standardized using the published extinction coefficient of ∼75 000 M^{-1} cm^{-1} for free fluorescein in pH 9 buffer at 491 nm (*8*). By use of this value and by comparison of the free reagents in pH 9 ammonium bicarbonate and pH 7 phosphate buffers, these reagents and presumably their protein-bound counterparts exhibited average extinction coefficients of ∼67 000 M^{-1} cm^{-1} for both fluorescein reagents. For the rhodamine-linked reagents DCP-Rho1 and DCP-Rho2 (**23** and **26**, respectively), the extinction coefficient for free rhodamine B in methanol (90 000 M^{-1} cm^{-1} at 554 nm) was used (Sigma-Aldrich data sheet); in neutral pH phosphate buffer, this extinction coefficient decreases to about 80 000 M^{-1} cm^{-1} for rhodamine B. Again, assuming the same extinction coefficient for both rhodamine reagents as for rhodamine B in methanol, DCP-Rho1 and DCP-Rho2 exhibited extinction coefficients of approximately 70 000 and 61 000 M^{-1} cm^{-1} in neutral pH phosphate buffer, respectively.

One-dimensional 12% SDS–polyacrylamide gels were electrophoresed using a BioRad Mini-PROTEAN 3 system and standard procedures. Two-dimensional gels used the Bio-Rad Protean IEF cell and Criterion gel systems from Bio-Rad, including 11 cm, pH 3–10 NL IPG strips and 8–16% gradient gels, and methods recommended by the manufacturer. Various amounts of DCP-FL1 labeled C165S AhpC were loaded onto separate strips and focused for ∼5½ h. After focusing, each strip was trimmed to within about 2 cm of the protein band (as estimated from one control strip that was stained with Coomassie blue) and then three different strips were loaded onto the top of each Criterion gel to run the second dimension. All gels were soaked in water for 30 min and then imaged for fluorescence on either an Amersham Storm 840 or a Typhoon 8600 fluorescence imager. Data were analyzed either with ImageQuant software (for 1D gels) or by direct analysis of the images with MATLAB (for 2D gels). Amounts of labeled protein loaded onto each gel were determined using the absorbance of the chromaphores attached to the protein (at 491 and 570 nm for fluorescein- and rhodamine-labeled samples, respectively) and the approximate extinction coefficients, given above. A completely independent calculation was conducted in each case using the known amount of protein and the percent modified based on mass spectral analysis of the intact protein (and an assumed recovery of 100% from the Apollo concentrators) and gave a difference in amounts of ∼10% or less.

Mass Spectrometric Analysis of Labeled Proteins and Peptides. To confirm the size and covalent attachment of each adduct, 1–2 nmol of labeled or unlabeled protein (∼60 μL) in 10 mM ammonium bicarbonate was submitted to the Analytical Chemistry Core Laboratory at Wake Forest University School of Medicine for infusion analysis on a Micromass Quattro II triple quadrupole mass spectrometer equipped with a Z-spray source. Just prior to analysis, samples were diluted 1:1 with acetonitrile and 1% formic acid was added. The data were processed and analyzed using MassLynx, version 3.5.

For MS–MS analysis of alkylated peptides, 0.5 nmol of labeled AhpC proteins was digested in 25 mM ammonium bicarbonate with 50 ng of AspN for ∼16 h at 37 °C. Peptide mixtures were analyzed with a Waters/Micromass Q-TOF API US tandem mass spectrometer equipped with a nanospray source interfaced to a Waters CapLC to detect labeled peptides and reagent-specific ions released by fragmentation. AhpC labeled with DCP-Bio1 and digested as above was also analyzed on a Thermo LTQ ion-trap mass spectrometer coupled with an Ultimate 3000 nano-HPLC system. A typical gradient was run for 60 min from 0% to 100% solvent B (80% acetonitrile, 20% H_2O, and 0.1% formic acid). Solvent A consists of 5% acetonitrile, 95% H_2O, and 0.1% formic acid. The flow rate was set at 200 nL/min on a 75 μm × 10 cm fused silica capillary column (New Objectives) in-house-packed with Michrom Magic C18AQ (200 Å, 5 μm). The analysis was performed in SIM mode where the DCP-Bio1-labeled peptide (*m*/*z* 811.77, +2 charge) was isolated for further fragmentation. Peptide identification was performed using the SEQUEST module in Bioworks suite 3.1 software.

Kinetic Studies of Reagents Reacting with Sulfenic Acid-Containing Proteins. To 5 nmol of papain in 25 mM potassium phosphate buffer at pH 7.0, with 1 mM EDTA, was added DCP-Bio1 (from a 250 mM stock solution in DMSO) at final concentrations of 0.5–5 mM and a total volume of 50 μL. The reaction was initiated with the addition of 2 equiv of hydrogen peroxide. At the appropriate incubation time, each sample was applied to a Bio-Gel P6 spin column to remove small molecules and exchange the buffer into 50 mM ammonium bicarbonate, and then the mass spectra were obtained after rapid addition of acetonitrile and formic acid and injection into an Agilent electrospray ionization (ESI)-TOF mass spectrometer. Fits of the data were carried out using KinTekSim using the model A → B → C, where A is the R–SH form, B the R–SOH form, and C the biotinylated form of papain. The pseudo-first-order rate of A → B was considered unchanging at all concentrations of DCP-Bio1 used, and the rate of B → C varied with the amount of reagent added.

RESULTS AND DISCUSSION

Synthesis of Two Fluorescein Derivatives, DCP-FL1 and DCP-FL2. Initial synthetic approaches involving the synthesis of 5(6)carboxyfluorescein did not yield separable derivatives, which prompted us to explore a different synthetic strategy utilizing commercially available fluoresceinamine isomer I (**1**). Treatment of **1** with *p*-nitrophenyl chloroformate in refluxing acetone gave carbamate derivative (**2**) in near-quantitative yield (Scheme 1). The product could be filtered from the reaction mixture and separated from unreacted starting material. TLC and NMR analyses indicated that this material could be used without additional purification. Reaction with our protected alcohol derivative of 1,3-cyclohexadione (**3**) yielded the protected fluorescein–carbamate conjugate (**4**) in moderate

yield (Scheme 1). Deprotection of the enol ether proceeded cleanly using 4 M HCl in 1,4-dioxane without concomitant carbamate cleavage to give the desired fluorescein derivative (DCP-FL1, **5**; note that the use of this abbreviation is carried over from the nomenclature previously introduced (*5*)).

Synthesis of a more stable urea derivative (DCP-FL2, **9**) involved the introduction of a urea "linker", which began with the reaction of the *p*-nitrophenyl carbamate of fluoresceinamine (**2**) with propargylamine (Scheme 2). Careful addition of 1 equiv of triethylamine induced salt formation, and the product (**6**) could be simply filtered from the reaction mixture, thus obviating the need for purification by column chromatography. Utilization of "click" chemistry (*9*) enabled us to couple the propargylurea (**6**) with the azide derivative (**7**) of our alcohol (synthesized using Mitsunobu conditions; Scheme 2) to form triazole (**8**, Scheme 2). Acid deprotection yielded the urea-linked fluorescein conjugate (DCP-FL2, **9**) in near-quantitative yield.

Synthesis of Three Biotin Derivatives: DCP-Bio1, DCP-Bio2, and DCP-Bio3. To generate the first biotinylated derivative, D-(+)-biotin was coupled with alcohol (**3**) under standard dicyclohexylcarbodiimide (DCC) coupling conditions to yield ester **10** in good yield (Scheme 3). Acid deprotection using anhydrous HCl followed by silica gel column chromatography reproducibly gave the deprotected biotin probe (DCP-Bio1, **11**) in >60% yield (Scheme 3).

Other synthetic strategies were tried to link D-(+)-biotin to our sulfenic acid-reactive compound, as the lability of the ester linkage in **11** was expected to preclude its use in cellular assays because of nonspecific esterase cleavage. On the basis of the "click" chemistry utilized in the synthesis of the fluorescein triazole conjugate (**9**), we applied this strategy to the synthesis of a biotin amide derivative (DCP-Bio2, **14**, Scheme 4). Finally, a further biotin derivative incorporating a longer linker was synthesized as depicted in Scheme 5. Mono *N*-Boc protection of 1,4-diaminobutane was carried out as previously described (*10*), followed by DCC coupling to D-(+)-biotin and Boc deprotection to give the amine hydrochloride salt (**17**, Scheme 5). Coupling of the free amine of **17** with the *p*-nitrophenyl carbonate derivative (**18**) of alcohol **3** gave the protected version of this biotin derivative (**19**, Scheme 5). Similar deprotection methods yielded the extended biotin derivative as before (DCP-Bio3, **20**, Scheme 5).

Synthesis of Two Rhodamine Derivatives: DCP-Rho1 and DCP-Rho2. The utility of cell-permeable fluorophores with spectral profiles different from that of fluorescein may permit future multiplex, differential labeling of protein sulfenic acids. The synthesis of rhodamine B derivatives was explored because rhodamine B is known to be cell-permeable (*11*), absorbs and emits at a longer wavelength than fluorescein, and has a large extinction coefficient.

The synthesis and spectral properties of rhodamine B piperazine amide (**21**) have previously been described (*12*), and this was used as a starting point in our syntheses of rhodamine B probes. Rhodamine B piperazine amide was synthesized as described and isolated as a crude iridescent purple-gold solid in 57–79% yield (**21**, Scheme 6). Coupling of compound **18** and deprotection proceeded smoothly to give the rhodamine derivative (DCP-Rho1, **23**, Scheme 6). The synthesis of a second rhodamine B derivative was undertaken as outlined in Scheme 7. The piperazine amide (**21**) was coupled, using DCC, to *N*-Boc GABA to give the extended protected derivative (**24**, Scheme 7). Deprotection of the *N*-Boc group and further coupling to the carbonate derivative (**18**) followed by enol ether deprotection yielded the second rhodamine probe (DCP-Rho2, **26**, Scheme 7).

Reactivity of Cyclohexadione-Linked Reagents toward Cysteine Sulfenic Acid. Spectroscopic Properties of Free Reagents and Protein Adducts. As described previously, the C165S mutant of AhpC, a cysteine-based peroxidase enzyme (peroxiredoxin) from *Salmonella typhimurium*, is a good test protein because of its rapid formation of the sulfenic acid intermediate at the active site (Cys46) upon oxidation by peroxides and the relative stability of this species under anaerobic condtions when the resolving cysteine (Cys165) is not present to generate the disulfide bond (*4, 13*). By use of AhpC, all seven conjugates linked to the alcohol functional group of **3** were shown to possess approximately the same reactivity and specificity toward the cysteine sulfenic acid form of C165S AhpC as dimedone and the two conjugates previously synthesized (*5*). Papain (a cysteine-based protease) has also been used as a test protein in the studies described below because of its known propensity to form sulfenic acid at the active site cysteine (*14, 15*).

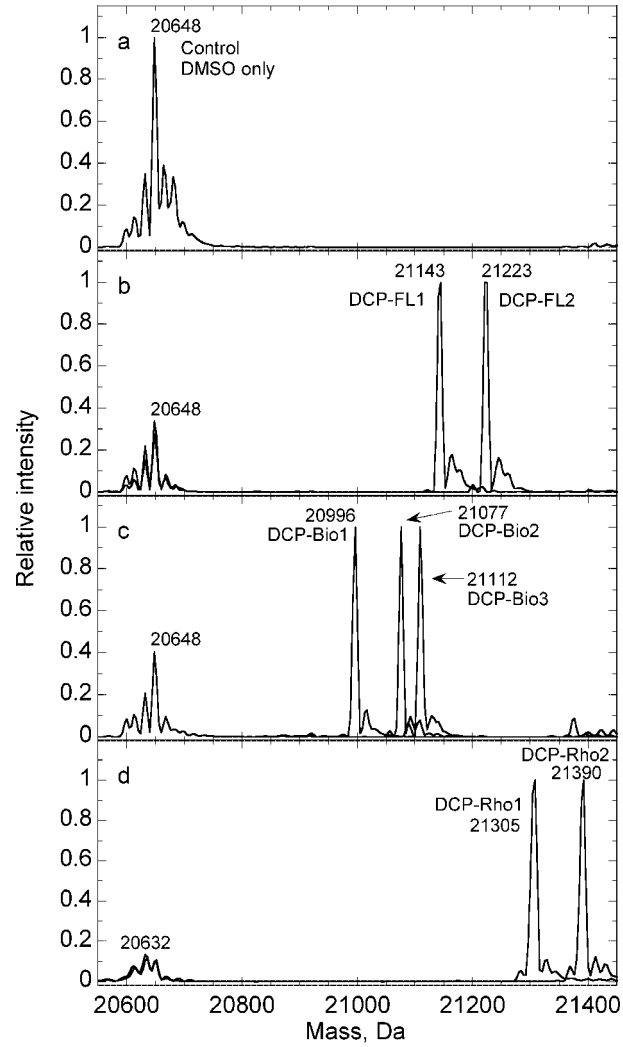


Figure 3. Electrospray ionization mass spectrometry analysis of adducts with the sulfenic acid form of C165S AhpC. The mutant of the bacterial peroxidase AhpC (C165S) containing only the peroxidatic cysteine (Cys46) but not the resolving cysteine (Cys165) that participates in disulfide bond formation was treated with hydrogen peroxide to yield the relatively stabilized sulfenic acid form of the protein under anaerobic conditions, then incubated with DMSO (a) or DMSO containing DCP-FL1 or DCP-FL2 (b), DCP-Bio1, DCP-Bio2, or DCP-Bio3 (c), or DCP-Rho1 or DCP-Rho2 (d) to yield the covalent protein adduct. The treated protein samples were washed, diluted with acetonitrile and formic acid, and analyzed by electrospray ionization mass spectrometry. Shown are the transformed data that represent the relative abundance of the prominent species of C165S AhpC. The peaks observed at 20 632 and 20 648 amu represent the protein with the active site Cys46 in the sulfinic and sulfonic acid states, respectively. Exact masses of the covalent adducts are listed in Table 1.

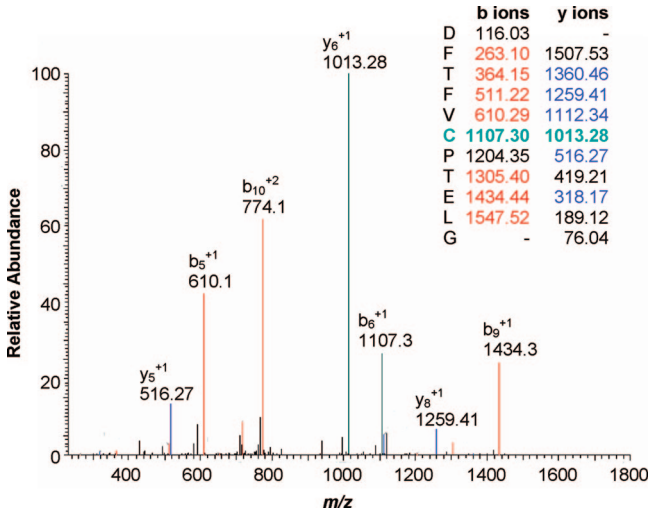


Figure 4. Nano-LC–MS/MS spectrum of the DCP-Bio1-labeled peptide of C165S AhpC containing an adduct at Cys46. The covalent adduct with DCP-Bio1 was prepared from the R–SOH form of the AhpC mutant and digested with AspN in order to generate an 11-residue peptide. The AspN digest was separated by nano-HPLC coupled to a Thermo LTQ ion trap mass spectrometer. The analysis was performed in SIM mode where the DCP-Bio1-labeled peptide (*m/z* 811.77, +2 charge) was isolated for further fragmentation. Cleavage of the amide bond results in N-terminal fragments designated as "b" and C-terminal fragments designated as "y". The masses of both sets of ions are consistent with DCP-Bio1 linked covalently to Cys46 (b6 – b5 = y6 – y5 = 497.1 *m/z*).

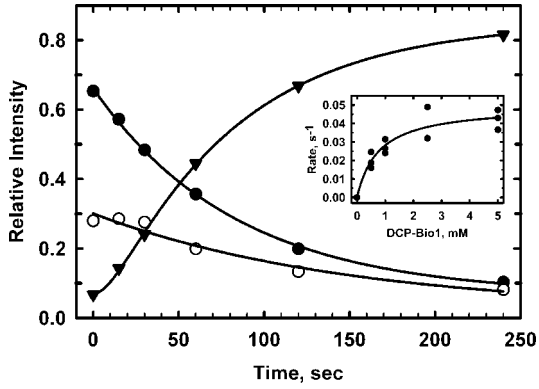


Figure 5. Kinetic studies of modification rates of sulfenic acid-containing papain with DCP-Bio1. Papain in 25 mM potassium phosphate buffer, pH 7, with 1 mM EDTA was incubated with DCP-Bio1 and 2 equiv of hydrogen peroxide, with the time of reaction taken after addition of the oxidant. At each time point a 50 μL sample containing 5 nmol of papain was applied to a Bio-Gel P6 spin column to remove small molecules and exchange the buffer into 10 mM ammonium bicarbonate. Then the mass spectra were obtained after rapid addition of acetonitrile and formic acid and injection into an an Agilent LC/MSD TOF mass spectrometer. Data plotted from one reaction with 5 mM DCP-Bio1 show the loss of the thiol form of papain (closed circles), the appearance of the covalent adduct with DCP-Bio1 (inverted triangles), and the putative sulfenic acid form (open circles) over time. Data from multiple concentrations of reagent and three independent experiments were fit to a A → B → C kinetic model as described in the text, giving a single first-order rate in this experiment of 0.024 ± 0.007 s^{-1} for formation of the sulfenic acid form of papain, and a second, saturable step with a maximal rate of 0.050 ± 0.005 s^{-1} and apparent K_m = 0.77 ± 0.24 mM for reaction of DCP-Bio1 with the sulfenic acid of papain.

The fluorescein- and rhodamine-linked reagents possess highly fluorescent chromophores that are incorporated into the target protein upon modification. Compared with free fluorescein, the DCP-FL1 and DCP-FL2 (**5** and **9**, respectively) reagents in 50 mM Tris-HCl buffer, pH 8, are partially quenched in their emission (Figure 1A) but are not shifted in their absorbance maxima (Table 1). Upon covalent attachment to protein, DCP-FL1 yields a conjugate that is further quenched in emission intensity at the peak by about 40% relative to the free reagent; under these same conditions, DCP-FL2 is not further quenched upon attachment to protein (Figure 1A). Reaction of DCP-FL1 with papain leads to even greater quenching of the fluorophore (by about 75% compared with free DCP-FL1, data not shown). For rhodamine compounds compared with rhodamine B, DCP-Rho1 (**23**) is slightly quenched in its free form but significantly quenched when covalently attached to AhpC (Figure 1B, left panel) and papain (data not shown). DCP-Rho2 (**26**) fluorescence is already significantly quenched (by about 50%) for the free reagent and largely unchanged upon attachment to either AhpC or papain proteins (Figure 1B, right panel and not shown). The quenching of fluorescence in bound DCP-Rho1 and in free and bound DCP-Rho2 is accompanied by a shift in the λ_{max} of both the excitation and emission to longer wavelengths (Table 1).

To evaluate the limit of detection and the linearity of response for the fluorophore-labeled AhpC protein, various amounts of C165S AhpC protein labeled with the different fluorescein- and rhodamine-linked reagents were analyzed in 1D and 2D polyacrylamide gels. For fluorescein, amounts as low as 0.1–0.2 pmol of protein were still detectable using a Storm 840 imager with a 450 nm LED for excitation (Figure 2A) (no improvement in sensitivity was observed using the Typhoon 8600 imager with a 532 nm laser for excitation). Furthermore, quantitation of the signal indicated linearity over at least 4 orders of magnitude (Figure 2C), even though the protein on the gel was significantly overloaded at 100 and 200 pmol (~2 and 4 μg). For comparison, 0.5 pmol was readily detected in a two-dimensional gel spot with good linearity up to at least 5 pmol (see Supporting Information, Figure S1). Gels with rhodamine-labeled AhpC showed similar, but perhaps slightly enhanced, sensitivity of detection (with a Typhoon 8600 imager and a 633 nm laser for excitation) and a reasonable degree of linearity of the signal with the amount of labeled protein loaded (parts B and D of Figure 2).

Because the biotin reagents cannot be directly observed, biotinylated protein was visualized by a Western blotting-like technique that involved transfer of the labeled proteins in the gel to nitrocellulose, addition of a 1:20000 dilution of HRP-conjugated streptavidin, and detection of HRP using the Pico chemiluminescence kit from Pierce. The limit and range of linearity for detection are thus highly dependent on the efficiency of transfer, the chemiluminescence substrate added, and the amount of time the blot is exposed to film. As with Western blots, linearity of signal with the applied biotinylated protein is over a limited range for any one exposure to a chemiluminescent reporter substrate but might be extended by obtaining multiple different exposures of a single gel. In our studies, an approximate lower limit of 0.2–0.5 pmol is typically achievable (e.g., with the Supersignal West Dura HRP detection kit from Pierce), but linearity of response must be established each time (data not shown).

Mass Spectrometry Analysis of Labeled Protein Conjugates, Intact and Proteolytically Digested. Collision-Induced Fragmentation of Label to Trigger MS–MS Sequencing. Covalent adducts of each of the probes were observed only with the sulfenic acid form of AhpC and were not generated with sulfhydryl or disulfide-containing forms of the protein; the covalent modification of AhpC with each probe was confirmed using ESI-MS analysis of the intact, labeled proteins (Figure 3 and Table 1). In each case, the higher molecular weight adduct gave a mass within 1–2 Da of the calculated masses of reduced protein (20 600 Da) plus reagent minus 2 H atoms (as expected

when the covalent bond forms) (Table 1). As shown previously, if not trapped with one of these reagents, the sulfenic acid form of AhpC undergoes further oxidation to sulfinic and sulfonic acids, presumably during incubation of the sample in acetonitrile and formic acid before MS analysis or during the analytical procedure itself (*5, 13*).

To confirm the site of AhpC modification with different probes, AspN digestion of the labeled AhpC was also carried out to generate an 11-residue peptide containing the expected cysteine site susceptible to oxidation. The peptide mixtures were analyzed using liquid chromatography and either a Q-TOF or an LTQ MS. As shown in Figure 4 and in Figure S2 of the Supporting Information, MS–MS analysis of the parent peptides gives high-quality spectra that accurately sequence the labeled peptide and confirm the presence of the covalent adduct on Cys46 of this AhpC peptide. Furthermore, for each of the fluorescein-, biotin- and rhodamine-linked adducts, a characteristic ion is detected of a mass that suggests fragmentation during collision-induced decay between the sulfur and β-carbon of the adducted cysteinyl residue (Table 1). For appropriately equipped mass spectrometers, this specific ion can be used to trigger MS–MS analysis, as was carried out for the DCP-FL1-modified peptide analyzed in the Q-TOF (Supporting Information, Figure S2). Alternatively, several analyses can be conducted with different collision energies, first to identify those peptides giving rise to the characteristic ion of interest and then in a second pass to carry out MS–MS analysis of that set of peptides.

MS-Linked Kinetic Studies of the Reactivity of DCP-Bio1 with Sulfenic Acid-Containing Proteins. To determine the rate of reaction of our reagents with the sulfenic acid forms of AhpC or papain, we used an Agilent ESI-TOF spectrometer to monitor the appearance of sulfenic acid and alkylated protein after the addition of DCP-Bio1 (or dimedone) and hydrogen peroxide. Our initial studies of C165S AhpC indicated a surprisingly slow reaction of the sulfenic acid-containing protein with dimedone; in the presence of 5 mM dimedone, the alkylated protein was generated at an initial rate of about 0.1 min^{-1}, slowing to about 0.009 min^{-1} in a second phase. During this extended incubation, the aerobic sample was also generating the further oxidized sulfinic and sulfonic acid-containing protein, complicating the kinetic analysis. On the basis of what is already known about the active site cysteine group of AhpC, the reactivity of the R–SOH group may be restricted in its "fully folded" form where the active site pocket acts as a shield against modification (*16, 17*).

We then used the same approaches to monitor the reaction of commercially available papain with hydrogen peroxide and DCP-Bio1 and discovered the alkylation reaction to be far faster than that observed between dimedone and AhpC (Figure 5). The reaction in this case is essentially complete within 5 min in the presence of 5 mM DCP-Bio1 and a 2-fold excess of hydrogen peroxide. While additional oxidized species are formed to a limited extent, these species are present at levels of less than 10% at the start of the reaction and at less than 20% at the final time point for concentrations of DCP-Bio1 down to 0.5 mM. The kinetic fits of the data are not significantly affected by ignoring these additional species; from these, the rate constant for formation of the sulfenic acid was determined to be 0.024 $\pm$ 0.007 s^{-1} in 200 μM hydrogen peroxide and potassium phosphate buffer at neutral pH and room temperature (from three independent experiments). This value (translating to a second-order rate constant of 7.2×10^3 M^{-1} min^{-1}) is in very good agreement with a previous determination, where activity loss of papain in the presence of hydrogen peroxide at pH ~6 and 23 °C was found to occur with a second-order rate constant of 3.7×10^3 M^{-1} min^{-1} (*15*). As shown in Figure 5 (inset), the rate constant for modification of the sulfenic acid of papain by DCP-Bio1 increased with reagent concentration to a maximal value of ~0.05 s^{-1} at 5 mM reagent. These data, from multiple reagent concentrations and three independent experiments, fit a Michaelis–Menten type of saturation curve, as is sometimes observed with irreversible inhibitors, with an apparent K_m of 0.77 ± 0.24 mM for DCP-Bio1 and a maximal rate of 0.050 ± 0.005 s^{-1} (Figure 5, inset). This implies a reversible binding step prior to irreversible modification of papain. If this represents pre-equilibrium binding followed by a slow chemical step, 0.77 mM is the K_i of the inhibitor. We had anticipated a simple bimolecular process of sulfenic acid alkylation but instead saw saturation kinetics; whether or not saturable kinetics are typically observed or are unique to papain and/or other cysteine-based proteases remains to be determined. Regarding the differences we observed between AhpC and papain reactivity, it is not surprising to find different rates of reaction in different proteins because the accessibility and stability of the sulfenic acid will vary greatly depending on its microenvironment; this species in papain appears to be far more accessible and/or reactive than that in C165S AhpC.

CONCLUSIONS

Seven new reagents bearing fluorescein, biotin, or rhodamine as detectable tags have now been generated for detection and isolation of proteins or peptides that form sulfenic acids at susceptible cysteinyl residues. These labeled species can be detected at high sensitivity upon gel analysis or can be shown by mass spectrometric analyses to incorporate the covalent label in each case. A further feature of these conjugates is their ability to release specific, detectable fragments upon fragmentation during mass spectrometric analyses that can be used to trigger MS–MS analysis for sequence determination of labeled peptides. Modification of sulfenic acids in the presence of 5 mM reagents can occur within as little as 5 min, as shown in this study for papain. These sulfenic acid-reactive reagents provide a diverse set of new tools with which we can greatly enhance our understanding of the contributions of sulfenic acid formation to protein function and cellular processes like redox signaling.

ACKNOWLEDGMENT

The authors thank Laura Soito for technical assistance and Mike Samuel for mass spectrometry analyses and acknowledge financial support from the American Heart Association Established Investigatorship program (to both L.B.P., Grant 0140175N, and S.B.K., Grant 0140020N), from the Cross Campus Collaborative Research Program at Wake Forest University (to S.B.K. and L.B.P.), and from the National Institutes of Health to L.B.P., with S.B.K., L.W.D., and J.S.F. as coinvestigators (Grant R21 CA112145). Support from the NSF-NIGMS Program in Mathematical Biology through a grant to J.S.F., with L.B.P. and L.W.D. as coinvestigators (Grant RO1 GM075304), is also acknowledged. The Quattro II tandem mass spectrometer was purchased and updated with funds from National Science Foundation (Grant BIR-9414018), the North Carolina Biotechnology Center (Grant 9903IDG-1002), and the Winston-Salem Foundation. The Waters Q-TOF mass spectrometer was purchased with funds from the National Institutes Health (Grant 1S10RR017846). The maintenance of these instruments was supported in part by the Comprehensive Cancer Center of Wake Forest University. A donation from the Dorothy Rhyne and Willard Duke Kimbrell family supported the purchase of the mass spectrometers in the CMF laboratory. The NMR spectrometers used in this work were purchased with partial support from NSF (Grant CHE-9708077) and the North Carolina Biotechnology Center (Grant 9703-IDG-1007).

Supporting Information Available: Experimental details for 2D gel analyses of DCP-FL1 modified AhpC, MS–MS analysis of the labeled peptide from that protein, and log *P* analyses for the compounds synthesized. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Claiborne, A., Yeh, J. I., Mallett, T. C., Luba, J., Crane, E. J., 3rd, Charrier, V., and Parsonage, D. (1999) Protein–sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry 38*, 15407–15416.

(2) Poole, L. B. (2003) Formation and function of sulfenic acids in proteins. In *Current Protocols in Toxicology* (Maines, M. D., Ed.) pp 17.1.1–17.1.17, John Wiley & Sons, Inc., New York.

(3) Poole, L. B., Karplus, P. A., and Claiborne, A. (2004) Protein sulfenic acids in redox signaling. *Annu. Rev. Pharmacol. Toxicol. 44*, 325–347.

(4) Poole, L. B., and Ellis, H. R. (2002) Identification of cysteine sulfenic acid in AhpC of alkyl hydroperoxide reductase. *Methods Enzymol 348*, 122–136.

(5) Poole, L. B., Zeng, B. B., Knaggs, S. A., Yakubu, M., and King, S. B. (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. *Bioconjugate Chem. 16*, 1624–1628.

(6) Charles, R. L., Schröder, E., May, G., Free, P., Gaffney, P. R., Begum, S., Wait, R., Heads, R. J., and Eaton, P. (2007) Protein sulfenation as a redox sensor: proteomic studies using a novel biotinylated dimedone analogue, *Mol. Cell Proteomics*. *6*, 1473–1484.

(7) Poole, L. B., and Ellis, H. R. (1996) Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry 35*, 56–64.

(8) Haugland, R. P. (2002) *Handbook of Fluorescent Probes and Research Products*, 9th ed., Molecular Probes, Inc., Eugene, OR.

(9) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. 40*, 2004–2021.

(10) Pons, J.-F., Fauchère, J.-L., Lamaty, F., Molla, A., and Lazaro, R. (1998) A constrained diketopiperazine as a new scaffold for the synthesis of peptidomimetics. *Eur. J. Org. Chem.* 853–859.

(11) Toropainen, E., Ranta, V. P., Talvitie, A., Suhonen, P., and Urtti, A. (2001) Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest. Ophthalmol. Visual Sci. 42*, 2942–2948.

(12) Nguyen, T., and Francis, M. B. (2003) Practical synthetic route to functionalized rhodamine dyes. *Org. Lett. 5*, 3245–3248.

(13) Ellis, H. R., and Poole, L. B. (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. *Biochemistry 36*, 15013–15018.

(14) Allison, W. S. (1976) Formation and reactions of sulfenic acids in proteins. *Acc. Chem. Res. 9*, 293–299.

(15) Lin, W. S., Armstrong, D. A., and Gaucher, G. M. (1975) Formation and repair of papain sulfenic acid. *Can. J. Biochem. 53*, 298–307.

(16) Wood, Z. A., Poole, L. B., and Karplus, P. A. (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science 300*, 650–653.

(17) Parsonage, D., Youngblood, D. S., Sarma, G. N., Wood, Z. A., Karplus, P. A., and Poole, L. B. (2005) Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. *Biochemistry 44*, 10583–10592.

BC700257A