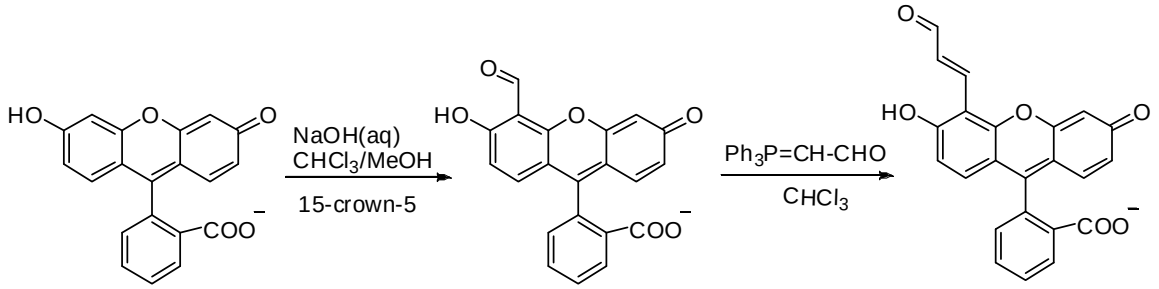
Experimental Methods and Instrumentation

All chemicals were purchased from Sigma-Aldrich and Cambridge Isotope Labs and used without further purification. NMR spectra were acquired in DMSO-d_6 or 0.1M pH 7.4 phosphate buffer/D_2O(9:1) on a Bruker AMX-400 NMR spectrometer. ESI-HRMS (high-resolution mass spectrometry) spectra were obtained at a Thermo Electron LTQ Orbitrap hybrid mass spectrometer and a Waters Micromass Q-TOF micro (ESI-Q-TOF). UV-visible measurements were collected on a UV-Vis Cary 50 and Fluorescence spectra were collected on Cary Eclipse (Varian, Inc.).



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Synthesis of Fluorescein Monoaldehyde[1]

Fluorescein (2.5 g, 7.75 mmol) and 3 mL of MeOH were placed in a 100 mL three-neck round-bottom flask and 10 g of a 50% NaOH solution along with 0.03 mL 15-crown-5 were added. 2.42 mL (30 mmol) $CHCl_3$ was added dropwise while maintaining at 55 °C temperature. The mixture was stirred at this temperature for 5 h. After cooling, the mixture was acidified with 10 M H_2SO_4 and the product precipitated. The solid was filtered and dried *in vacuo* overnight. Chromatography on silica gel (15:85 EtOAc:DCM) yielded a light yellow solid, 910 mg (32.6%). R_f =0.28 (15:85 EtOAc:DCM). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.87 (s, 1H), 10.63 (s, 1H), 10.24 (s, 1H), 8.01 (d, J = 7.4 Hz, 1H), 7.77 (dtd, J = 30.1, 7.4, 1.1 Hz, 2H), 7.31 (d, J = 7.6 Hz, 1H), 6.94 (d, J = 8.9 Hz, 1H), 6.84 (d, J = 1.9 Hz, 1H), 6.70 (d, J = 8.9 Hz, 1H), 6.61 (d, J = 2.2 Hz, 2H).

Synthesis of Compound 1 (α,β-unsaturated Monoaldehyde)

To a solution of monoaldehyde (144 mg, 0.4 mmol) in $CHCl_3$ (25 mL), triphenylphosphoranylidene acetaldehyde (152 mg, 0.5 mmol) was added resulting in a red solution. The mixture was stirred at 50 ºC under N_2 for 24 h, cooled to rt and the solvent removed *in vacuo*. Chromatography on silica gel (10:90 MeOH:DCM) yielded compound **1** (94.7 mg, 61.3%) as a red solid. ^{1}H NMR (400 MHz, 0.1M pH 7.4 phosphate buffer/D_2O 9:1) δ (ppm): 9.29 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 15.8 Hz, 1H), 7.78 (dd, J = 5.7, 3.3 Hz, 1H), 7.63 (dd, J = 5.7, 3.3 Hz, 2H), 7.30 (dd, J = 5.7, 3.3 Hz, 1H), 7.24 (dd, J = 15.8, 8.4 Hz, 1H), 7.09 (dd, J = 19.1, 9.5 Hz, 2H), 6.65 (d, J = 2.1 Hz, 1H), 6.59 (dd, J = 9.2, 2.1 Hz, 1H), 6.44 (d, J = 9.5 Hz, 1H). ^{13}C NMR (101 MHz, 0.1M pH 7.4 phosphate buffer/D_2O 9:1) δ (ppm): 199.69, 180.57, 179.09, 175.00, 158.86, 157.77, 157.58, 147.77, 139.48, 132.71, 131.20, 131.07, 130.01, 129.70, 129.44, 128.37, 127.11, 123.93, 123.17, 112.61, 111.77, 109.68, 103.51. ESI-FTMS *m/z* = 385.0706 $[M-H]^-$, calc. 385.0718 for $C_{23}H_{14}O_6$.

Preparation of the reaction product of compound 1 with thiol/analogs

Freshly prepared solutions of cysteine or homocysteine (200 μM – 20 mM in 0.1M pH 7.4 phosphate buffer) were mixed with solutions of compound **1** (2 or 4 μM in 0.1M pH 7.4 phosphate buffer) in a 1:1 volume ratio at room temperature. Other thiols and analogs (20 mM in 0.1M pH 7.4 phosphate buffer) and peptides (1 mM in 0.1M pH 7.4 phosphate buffer) were mixed with solutions of compound **1** (2 μM in 0.1M pH 7.4 phosphate buffer) in a 1:1 volume ratio at room temperature.

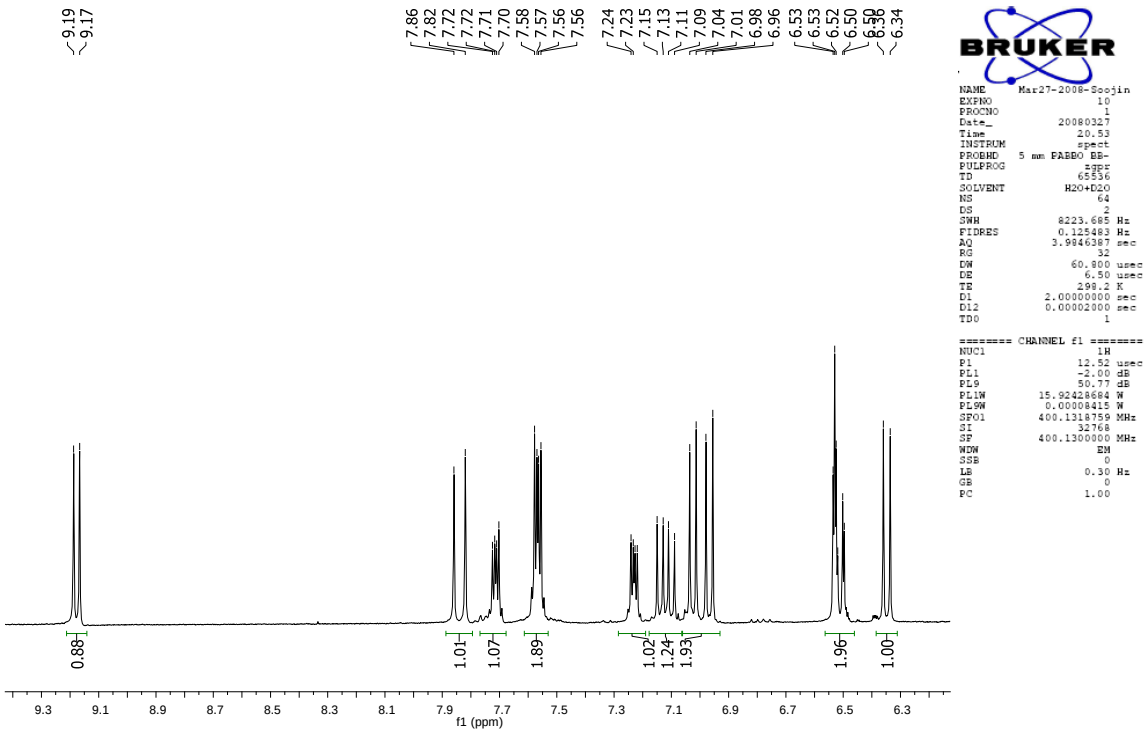

Figure S1. ^{1}H NMR of compound **1** (400 MHz, 0.1M pH 7.4 phosphate buffer/D_2O 9:1)

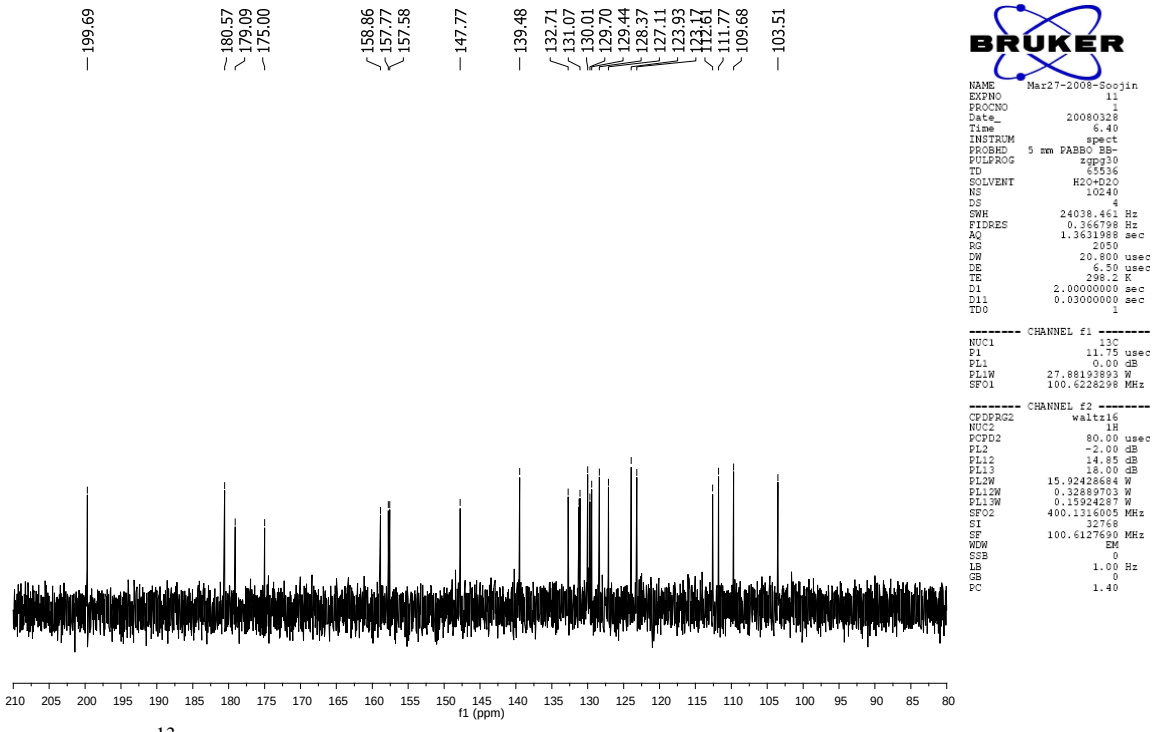

Figure S2. ^{13}C NMR of compound **1** (101 MHz, 0.1M pH 7.4 phosphate buffer/D_2O 9:1)

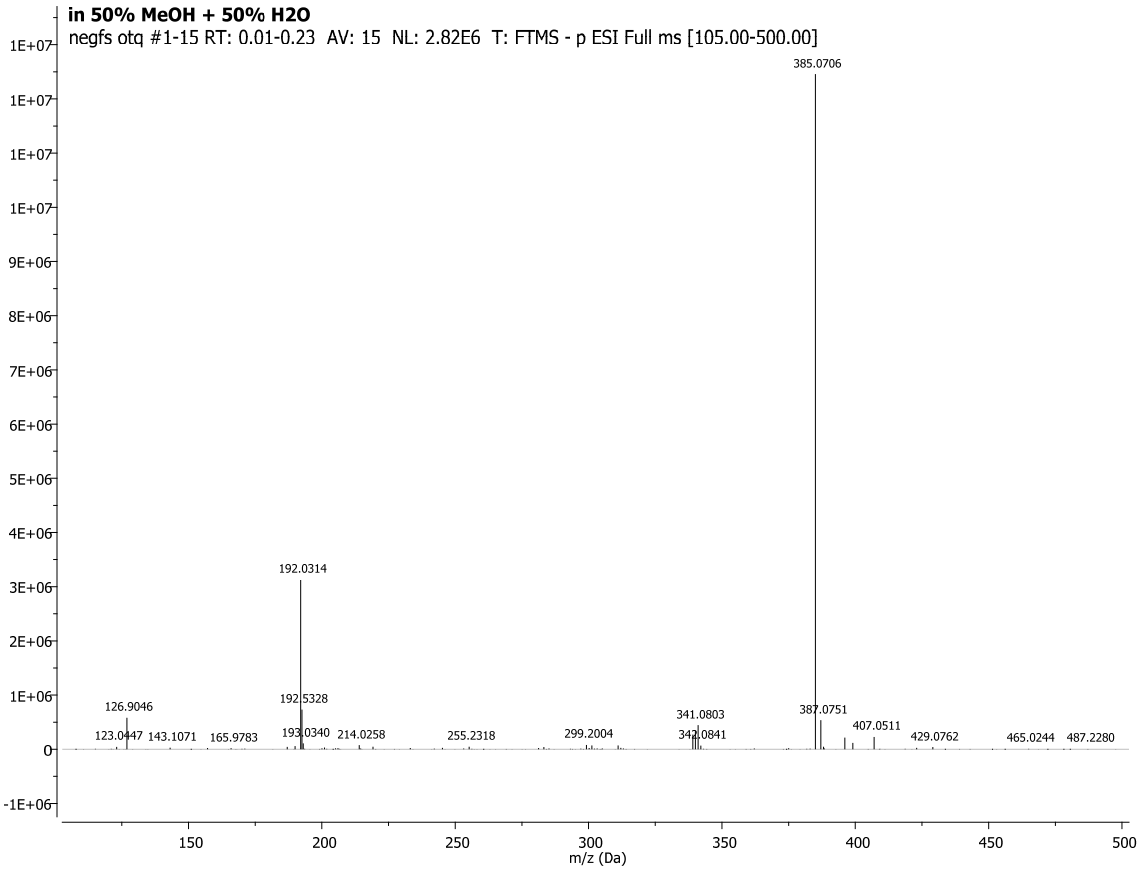

Figure S3. ESI-FTMS of compound **1**

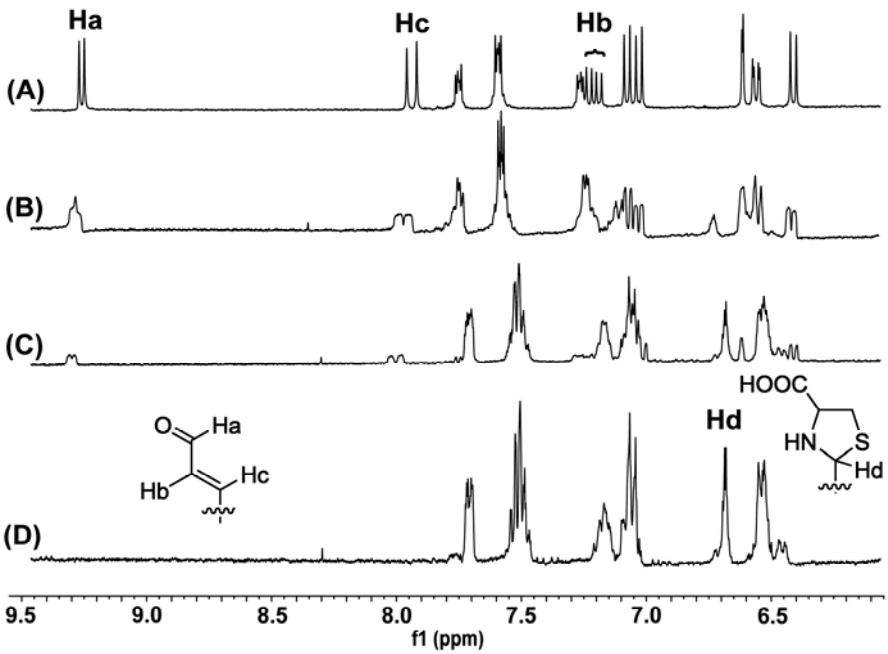

Figure S4. ^{1}H-NMR spectra vs. times in 0.1M pH 7.4 phosphate buffer/D_2O 9:1; The disappearance of the aldehyde resonance at 9.29 ppm (d, *J*= 8.4 Hz, 1H), as well as the alkene protons at 7.97 ppm (d, *J*= 15.8 Hz, 1H) and 7.24 ppm (dd, *J*= 8.4, 15.8 Hz, 1H) of **1** along with the concomitant appearance of thiazolidine resonances entered at *ca*. 6.72 ppm show, along with ESI-TOF HRMS evidence (Fig. S5), that 2 equiv Cys react with **1**; (A) compound **1** only; (B) **1** and Cys after 5 min; (C) **1** and Cys after 1 h; (D) **1** and Cys after 4 h.

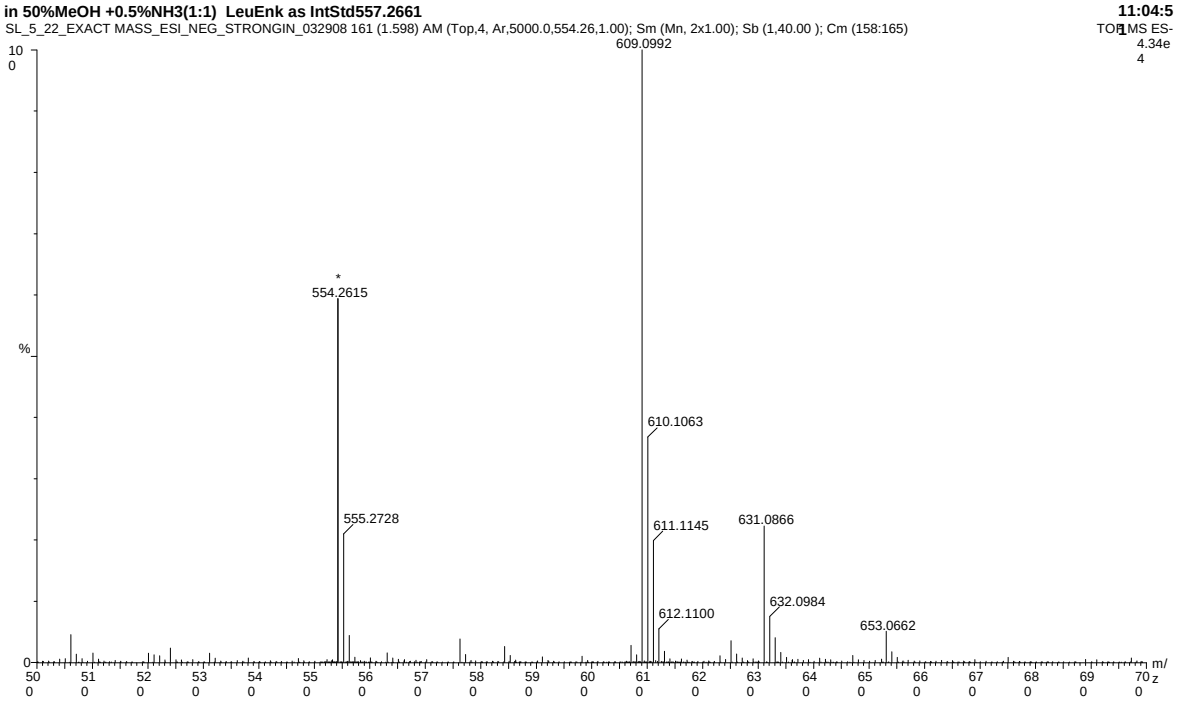

Figure S5. ESI-TOF HRMS of complex of **1** with Cys. *m/z* = 609.0992 $[M-H]^-$, calc. 609.1007 for $C_{29}H_{26}N_2O_9S_2$

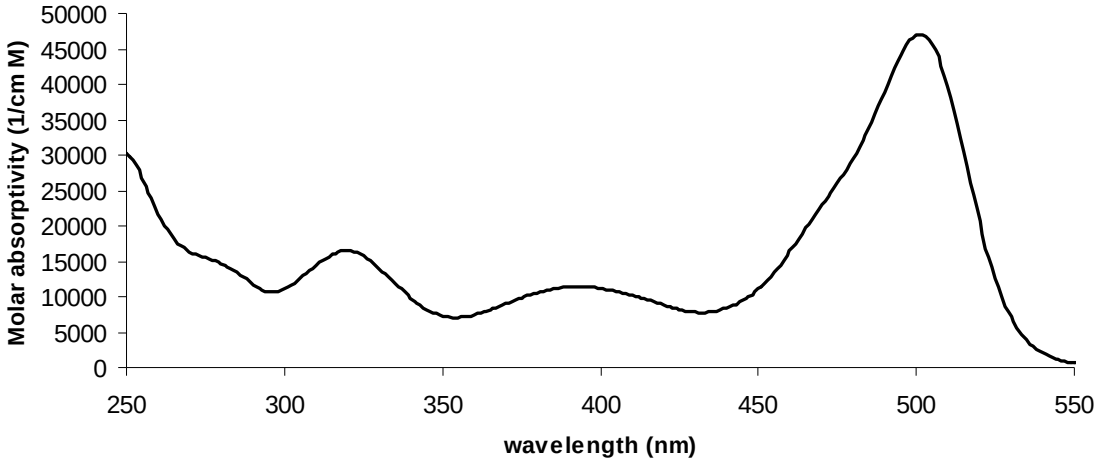

Figure S6. Absorption of **1** (ε_{501}=46981 cm^{-1} M^{-1} in 0.1 M phosphate buffer pH 7.4).

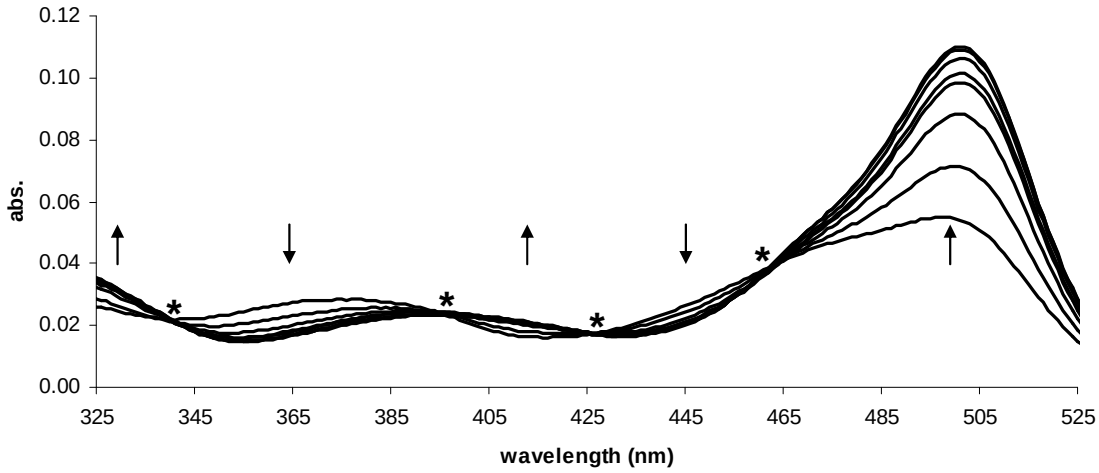

Figure S7. Absorption of **1** as a function of pH (2 μM **1** in 0.1 M phosphate buffer pH 5.5 - 9.0). Arrows indicate direction of change with increasing pH. * marks the location of well defined isosbestic points at 340 nm, 396 nm, 426 nm, 463 nm.

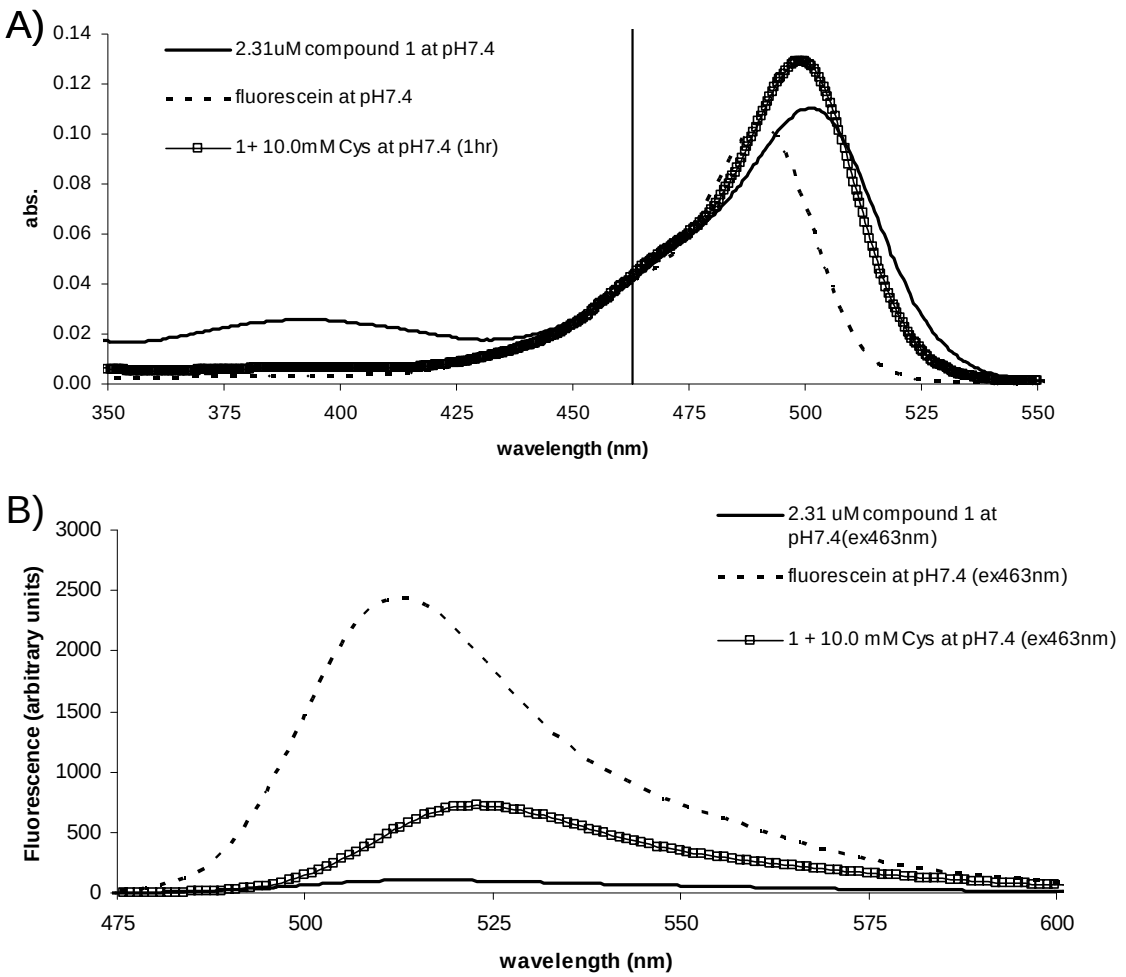

Figure S8. Spectral properties of fluorescein and compound **1** (2.31×10^{-6} M in 0.1 M phosphate buffer pH 7.4) in absence and presence of Cys (10.0 mM). (A) Absorption spectra. (B) Emission spectra excited at 463 nm. The vertical line in (A) represents the wavelength chosen for excitation in (B). All spectra were collected at room temperature. Excitation and emission bandpass = 5 nm

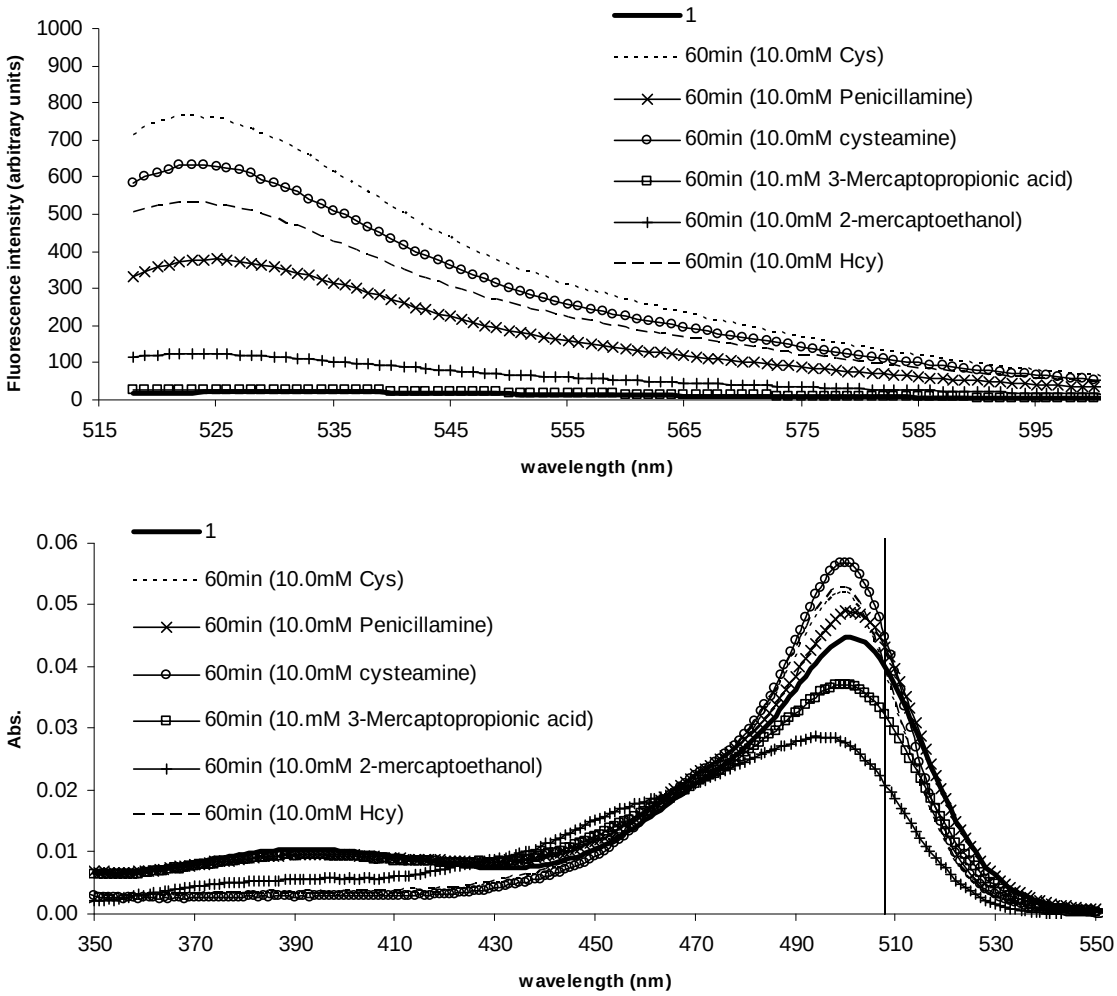

Figure S9. Response of compound **1** to thiols of interest and structural analogs (10.0 mM). (TOP) emission spectra excited at 508 nm (60 min). (BOTTOM) absorbance spectra (60 min). The vertical line represents the 508 nm excitation wavelength.

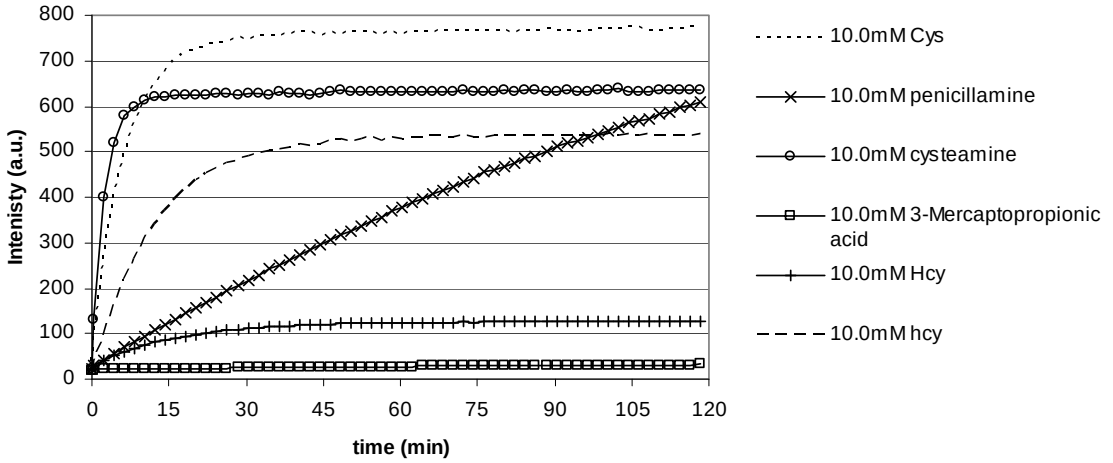

Figure S10. Evolution of fluorogenic response. Emission at 524 nm upon 508 nm excitation of 1 μM **1** in the presence of excess thiols of interest and their structural analogs (10.0 mM).

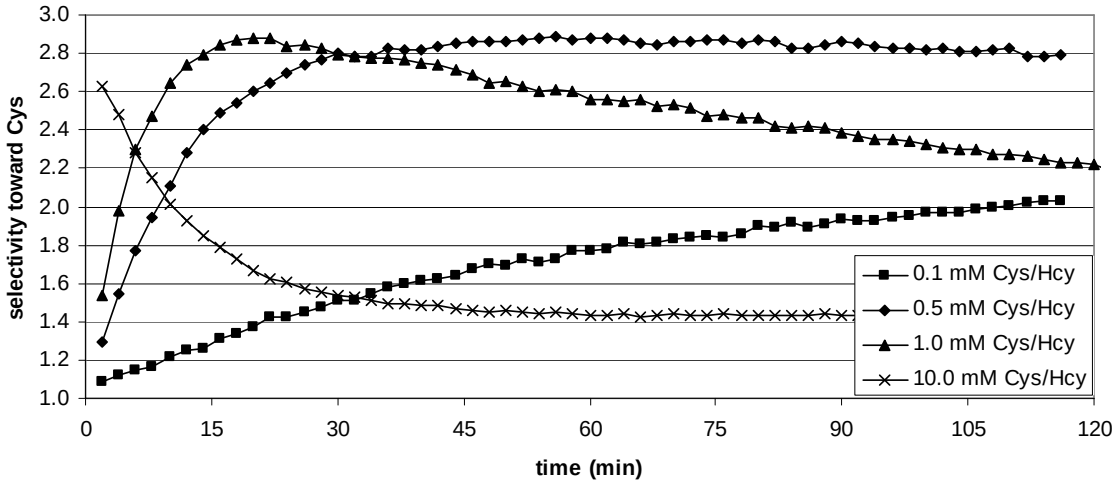

Figure S11. Selectivity towards Cys over Hcy. Ratio of fluorescence emission (λ_{em} = 524 nm upon 508 nm excitation) of Cys and Hcy reaction products with **1** as they formed over time.

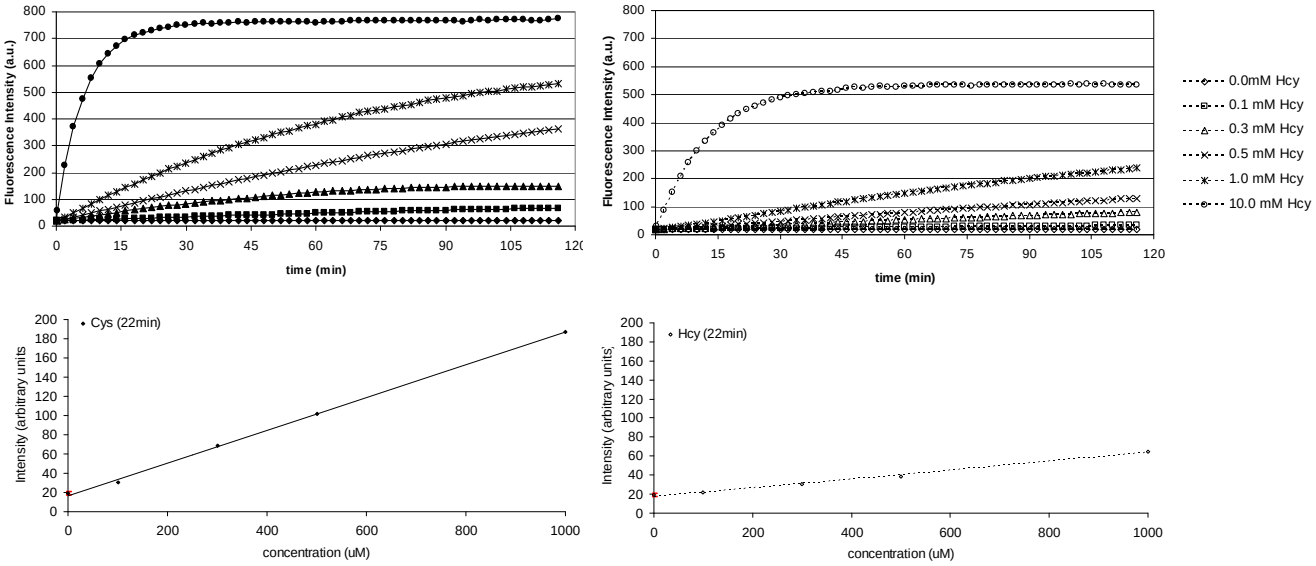

Figure S12. Fluorescence response of **1** to Cys and Hcy as a function of time and concentration. (TOP) Evolution of fluorogenic response. 1 μM **1** in the presence of excess Cys and Hcy (λ_{em} = 524 nm upon excitation at 508 nm). Filled symbols and solid lines = Cys. Open symbols and dashed lines = Hcy. (BOTTOM) Linear response of the fluorescence intensity as a function of thiol concentration (0 - 1000 μM) after 22 min of reaction time. The limit of detection for Cys was ~3-fold lower than that of Hcy (39 μM vs. 114 μM).

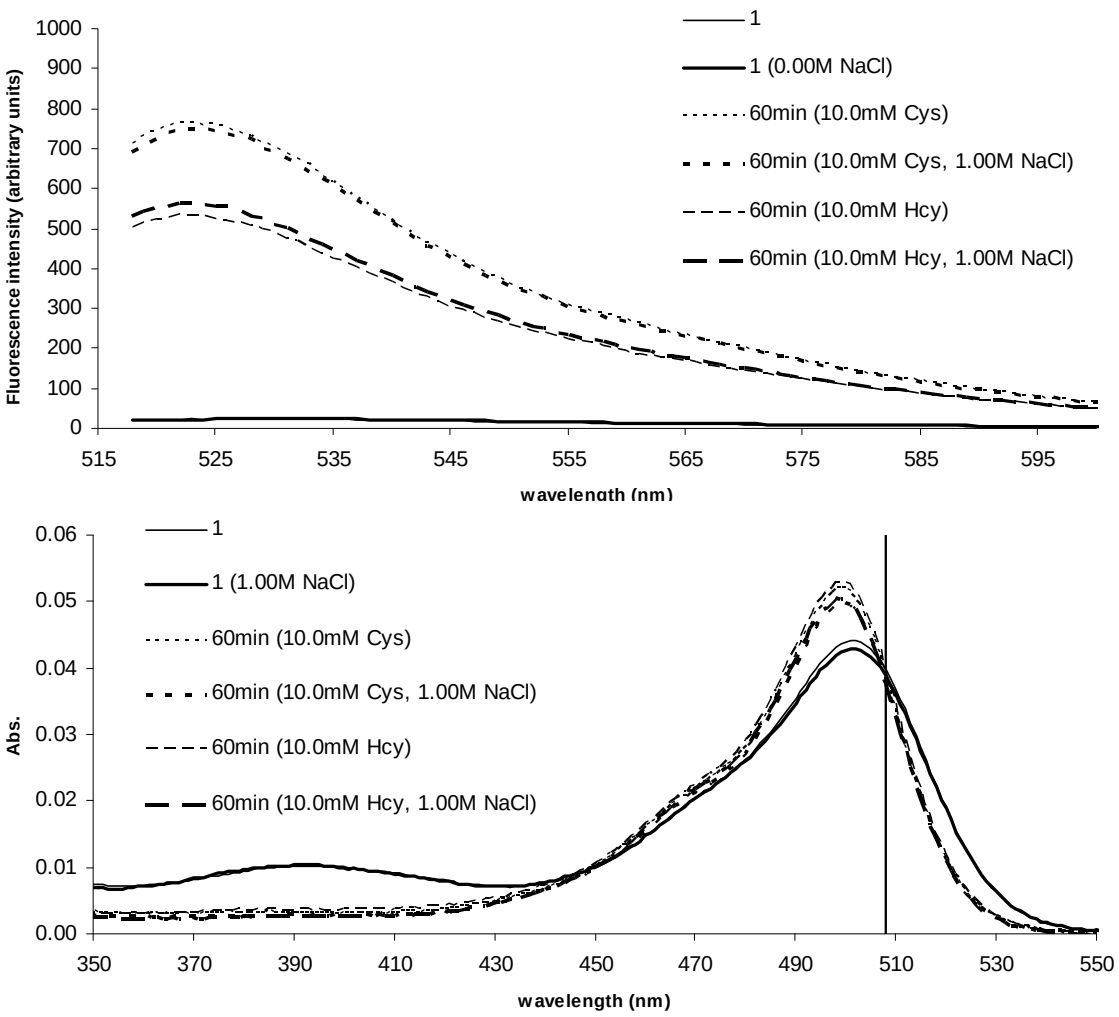

Figure S13. Effect of salt on the response of compound **1** to excess (10.0 mM) Cys and Hcy in buffered solution (0.1 M phosphate pH 7.4) with and without additional NaCl (1 M). (TOP) emission spectra excited at 508 nm (60 min). (BOTTOM) absorbance spectra (60 min). The vertical line represents the 508 nm excitation wavelength.

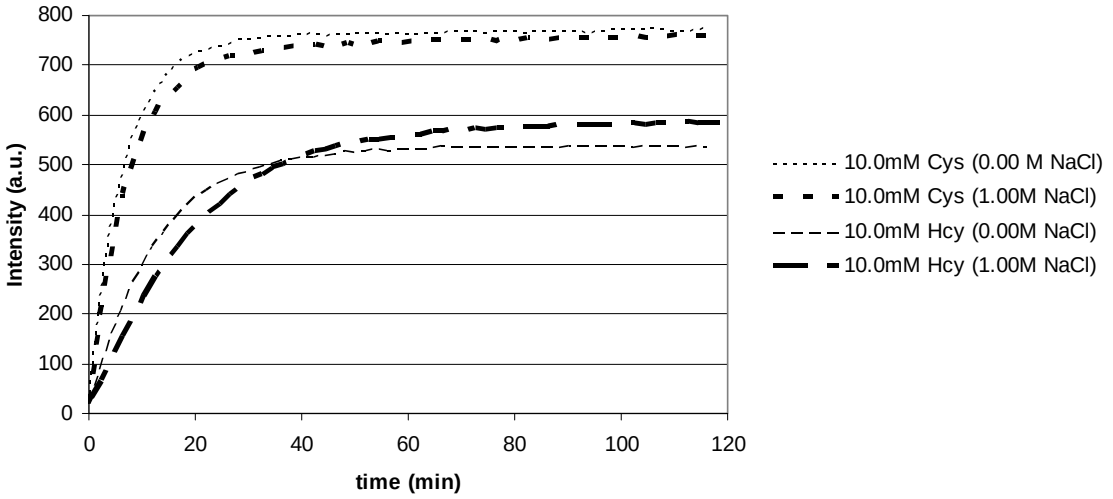

Figure S14. Effect of salt on the evolution of fluorogenic response. Emission at 524 nm upon 508 nm excitation of 1 μM **1** in the presence of excess (10.0 mM) Cys and Hcy in buffered solution (0.1 M phosphate pH 7.4) with and without additional NaCl (1 M).

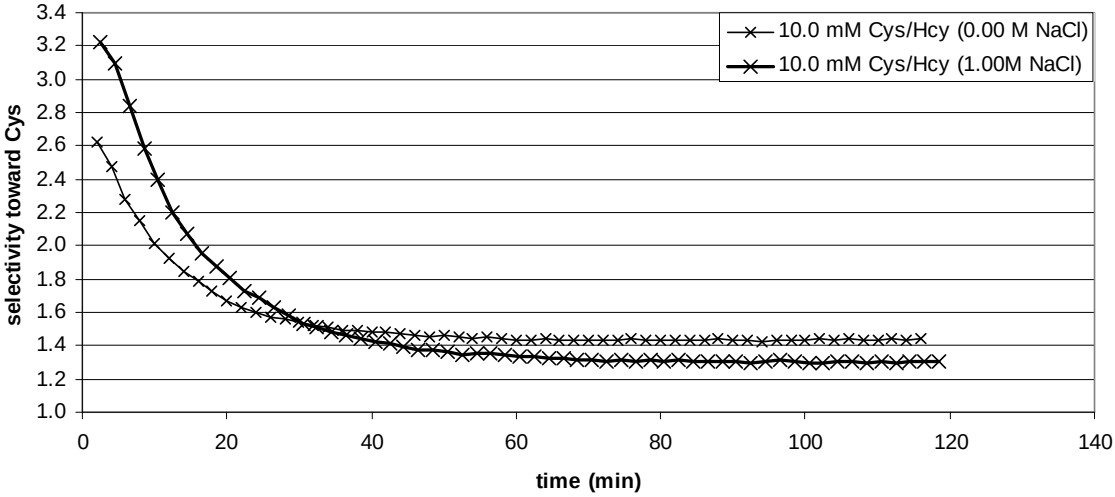

Figure S15. Effect of salt on the selectivity towards Cys over Hcy. Ratio of fluorescence emission (λ_{em} = 524 nm upon 508 nm excitation) of Cys and Hcy reaction products with **1** (1 μM 1 : 10 mM thiols) as they formed over time in buffered solution (0.1 M phosphate pH 7.4) with and without additional NaCl (1 M).

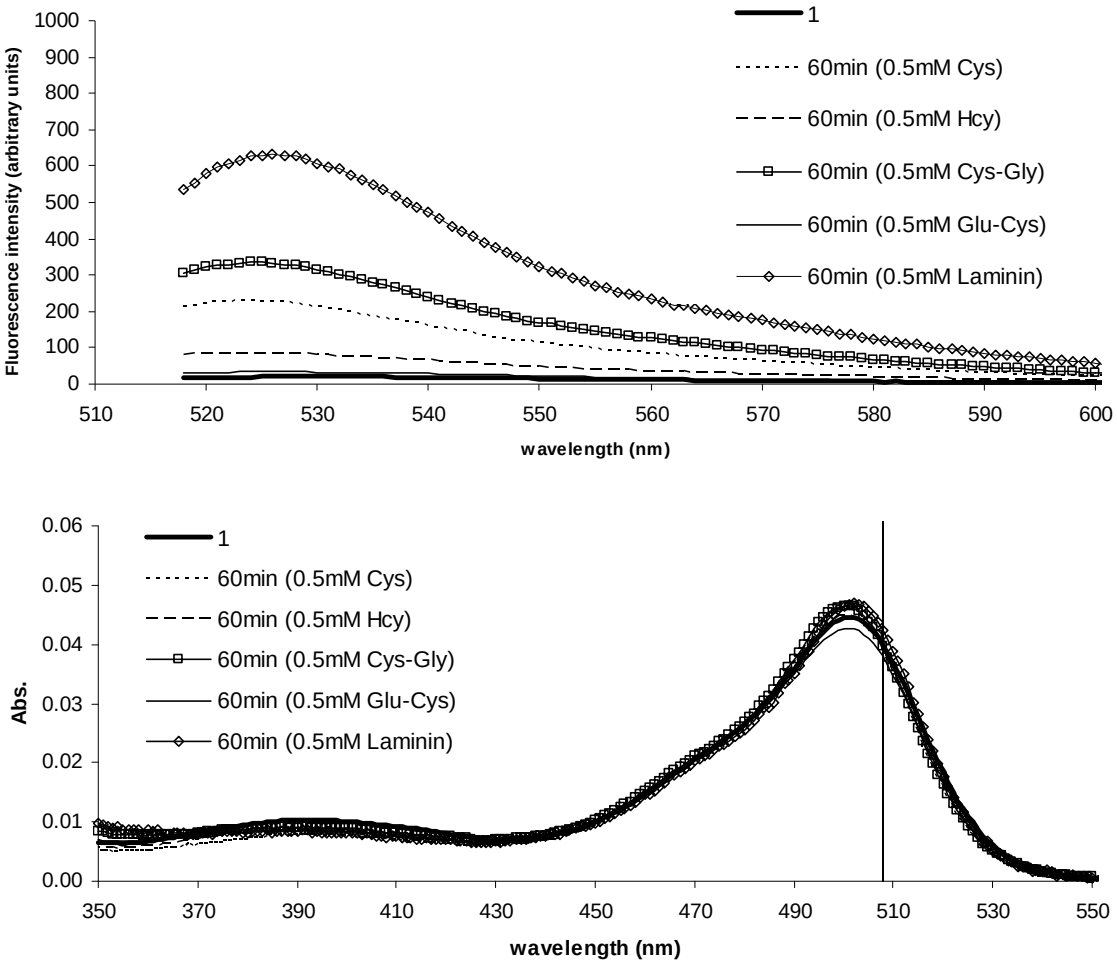

Figure S16. Response of compound **1** to thiols (0.5 mM) and peptides (0.5 mM) of interest. (TOP) emission spectra excited at 508 nm (60 min). (BOTTOM) absorbance spectra (60 min). The vertical line represents the 508 nm excitation wavelength.

References:

1 W. H. Wang, O. Rusin, X. Y. Xu, K. K. Kim, J. O. Escobedo, S. O. Fakayode, K. A. Fletcher, M. Lowry, C. M. Schowalter, C. M. Lawrence, F. R. Fronczek, I. M. Warner and R. M. Strongin, *J. Am. Chem. Soc.*, 2005, **127**, 15949.