Supporting information

Sense and Release: A Thiol-Responsive Flavonol-Based Photonically Driven Carbon Monoxide-Releasing Molecule That Operates via a Multiple-Input AND Logic Gate

Livia S. Lazarus,[†] Hector J. Esquer,[‡] Abby D. Benninghoff, [‡] and Lisa M. Berreau[*†]

[†]Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322-0300, United States.
[‡]Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah 84322-4815, United States.

Contact Information:
Lisa M. Berreau
lisa.berreau@usu.edu

Table of Contents: **Page**

Experimental Details

Chemicals and Reagents. All chemicals and reagents were obtained from commercial sources and used as received unless otherwise specified.

Physical Methods. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra (in ppm) were collected using a Brüker AvanceIII HD Ascend-500 (proton at 500 MHz) spectrometer and are referenced to the residual solvent peak in $CDCl_3$ (^{1}H 7.26 (singlet) ppm; ^{13}C 77.16 (triplet) ppm). *J* values are given in Hz. IR spectra were collected using a Shimadzu FTIR-8400 spectrometer. UV-vis spectra were recorded at ambient temperature or 37 °C using a CARY 50Bio spectrophotometer. Fluorescence emission spectra were collected using a Shimadzu RF-530XPC spectrometer in the range of 367-800 nm, with the excitation wavelength corresponding to the absorption maxima of the molecules. The excitation and emission slit widths were set at 1.5 nm for all the experiments except for the determination of detection limits for which the slit width was set at 3.0 nm. Mass spectral data were collected at the Mass Spectrometry Facility, University of California, Riverside. ESI/APCI mass spectra were recorded on an Agilent LCTOF (2006) with a Windows XP operating system. A Rayonet photoreactor equipped with RPR-4190A lamps was used for all photochemical reactions. Apogee Instruments MQ-500 full spectrum quantum meter was used to determine the photon flux of the illumination set ups. Modulus™ microplate reader was used for the MTT colorimetric assay. A Carl Zeiss LSM-710 AxioObserver live cell imaging system was used for fluorescence microscopy studies on live cells.

Synthesis:

3-acryloyl-2-phenyl-benzo[g]chromen-4-one (3). 2-hydroxy-2-phenyl-benzo[g]chromen-4-one (**1** [1], 0.2500 g, 0.8672 mmol), potassium carbonate (0.1439 g, 1.041 mmol) were combined in 17.50 mL of dry acetone and then cooled in an ice-water bath. Then, acryloyl chloride (84.10 µL, 1.041 mmol) in 8.75 mL dry acetone was slowly added to the suspension. The reaction mixture was stirred for 12 hours. Reaction completion was monitored using TLC in dichloromethane as mobile phase. The acetone was evaporated and the remaining white-yellow solid was redissolved in dichloromethane and passed through a silica/celite pipette column. The collected eluent was rotary evaporated to a solid that was subsequently recrystallized from pentane/Et_2O obtaining a white, brittle solid **3** (0.2700 g, 91%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.86 (s, 1H), 8.07 (d, *J* = 8.5 Hz, 1H), 8.02 (s, 1H), 7.97-7.90 (m, 3H), 7.63 (t, *J* = 8.5 Hz, 1H), 7.57-7.50 (m, 4H), 6.66 (d, *J* = 18.5 Hz, 1H), 6.43-6.38 (dd, *J* = 10.5 Hz, *J* = 10.5 Hz 1H), 6.07 (d, *J* = 11.5 Hz, 1H). $^{13}C\{^{1}H\}$ NMR ($CDCl_3$, 125 MHz) 173.0, 163.4, 157.2, 152.0, 136.1, 133.8, 132.3, 131.5, 130.4, 129.8, 129.1, 128.8, 128.6, 127.5, 127.4, 127.1, 126.3, 122.6, 114.5 ppm (19 signals expected and observed). FTIR (KBr, cm^{-1}) 1763 ($\nu_{C=O}$), 1734 ($\nu_{C=O}$). UV-vis (CH_3CN, nm) (ε, $M^{-1}cm^{-1}$) 366 (11,928), 329 (22,491). Melting point 192-193 °C. ESI/APCI-MS (relative intensity) calcd. for $C_{22}H_{14}O_4$ $[MH]^+$: 343.0900; found: 343.1000 (100%).

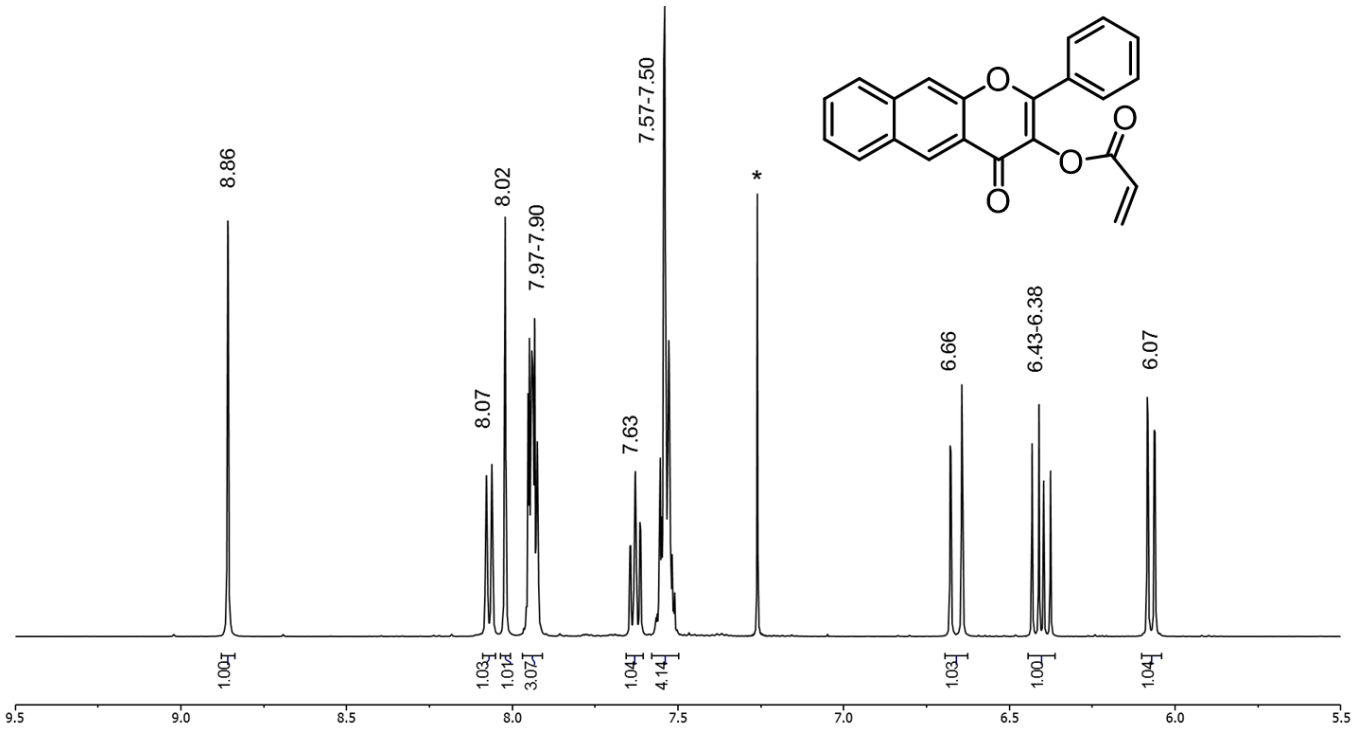


Figure S1. ^{1}H NMR spectrum of **3** in $CDCl_3$. The * indicates the residual signal of the solvent ($CHCl_3$).

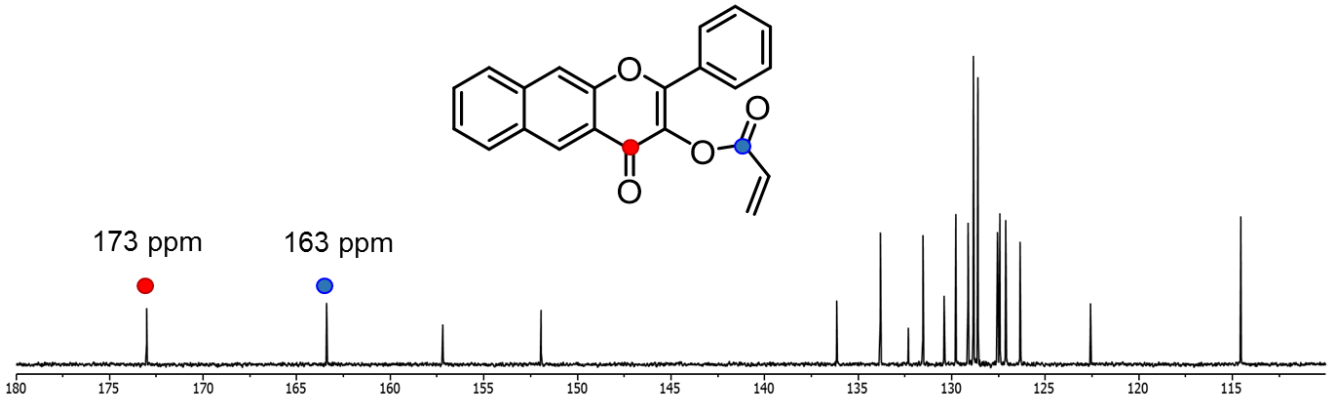


Figure S2. ^{13}C NMR spectrum of **3** in $CDCl_3$. 19 signals expected and observed.

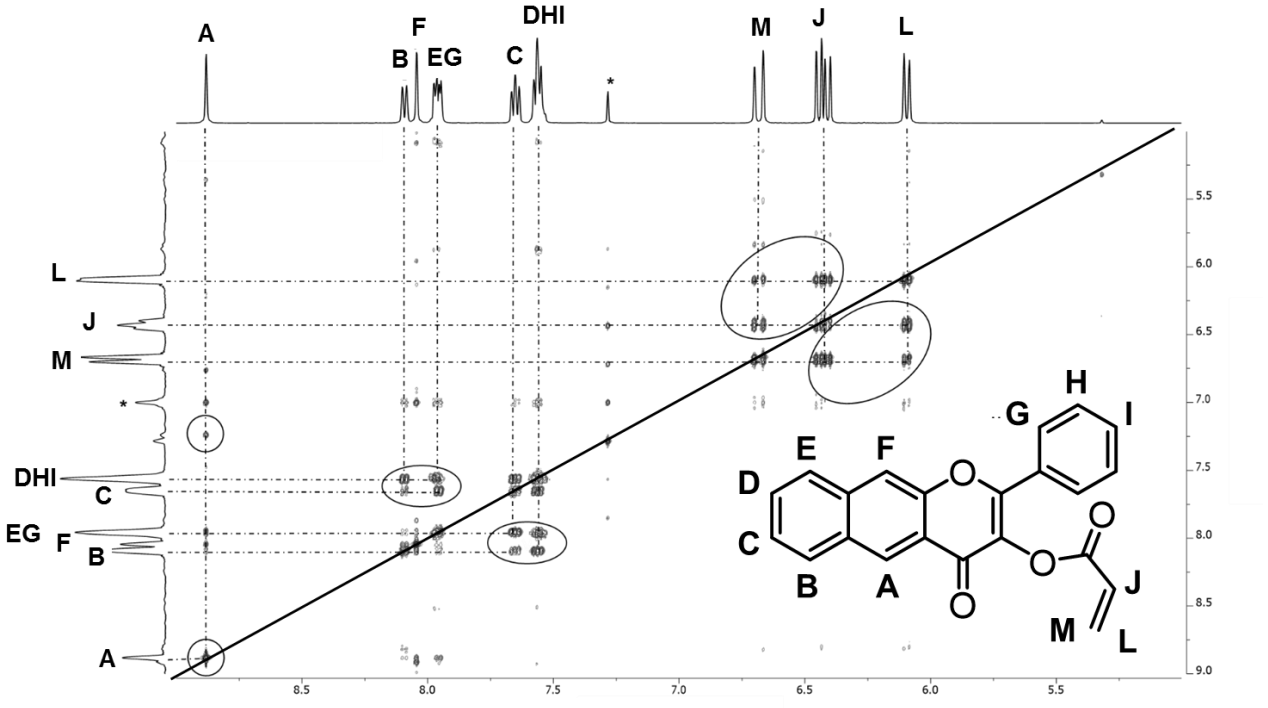


Figure S3. 2D-COSY spectrum of **3** in $CDCl_3$. The * indicates the residual signal of the solvent ($CHCl_3$).

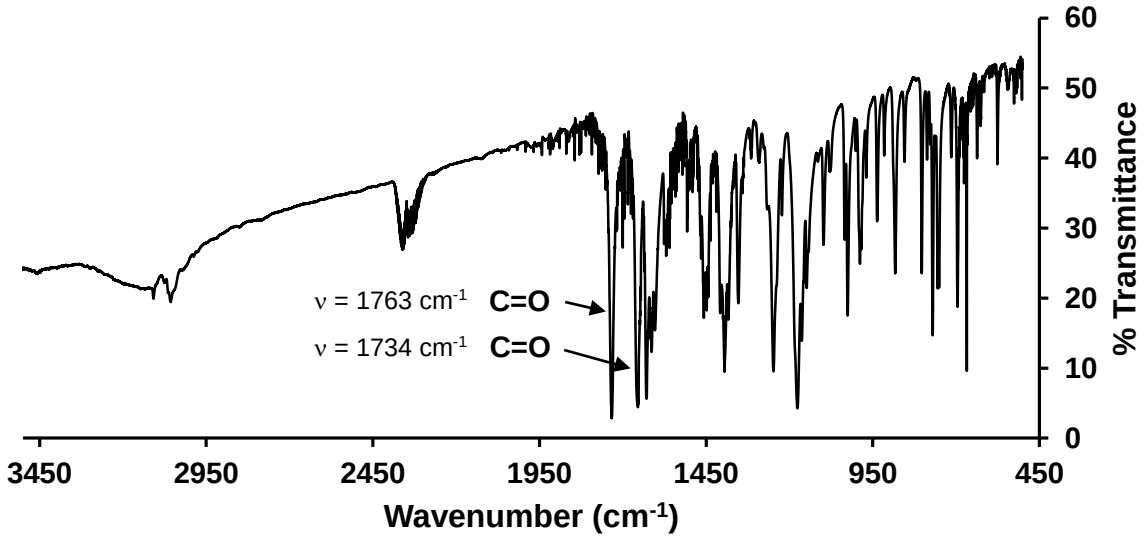
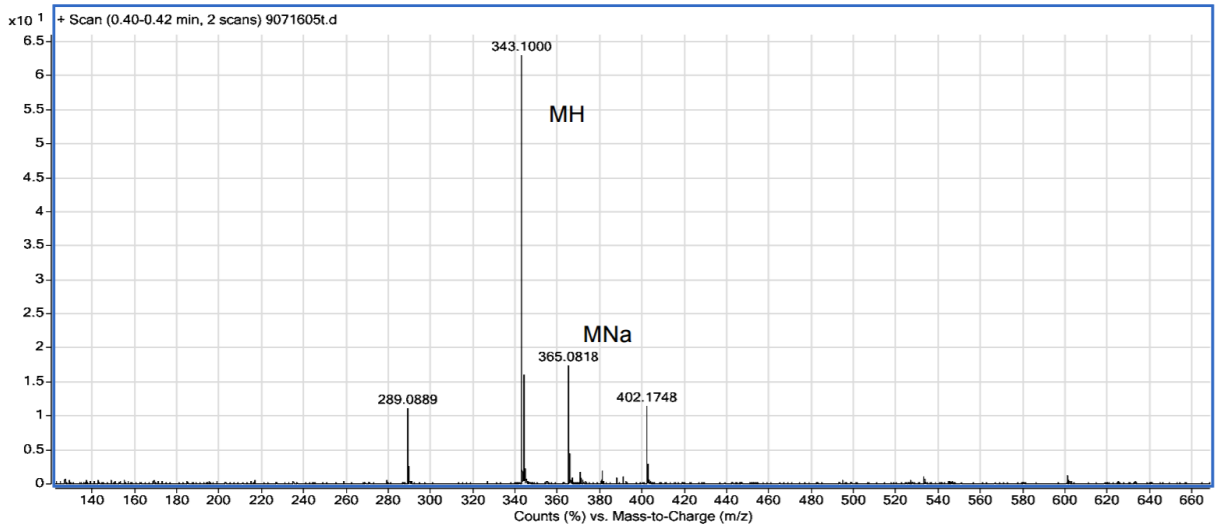
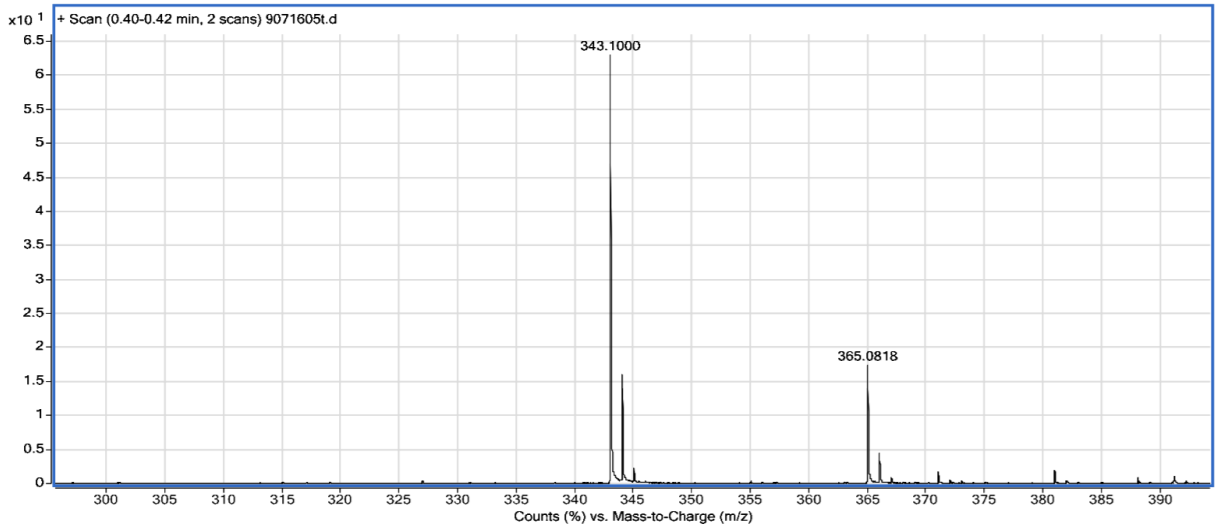


Figure S4. FT-IR spectrum of **3** in KBr.





Measured Mass	342.1			
Element	Low Limit	High Limit		
C	15	35		
H	5	20		
O	2	6		
Formula	Calculated Mass	mDaError	ppmError	RDB
C19 H18 O6	342.1103	-10.3	-30.2	11
C22 H14 O4	342.0892	10.8	31.5	16

Figure S5. ESI/APCI-MS of **3**.

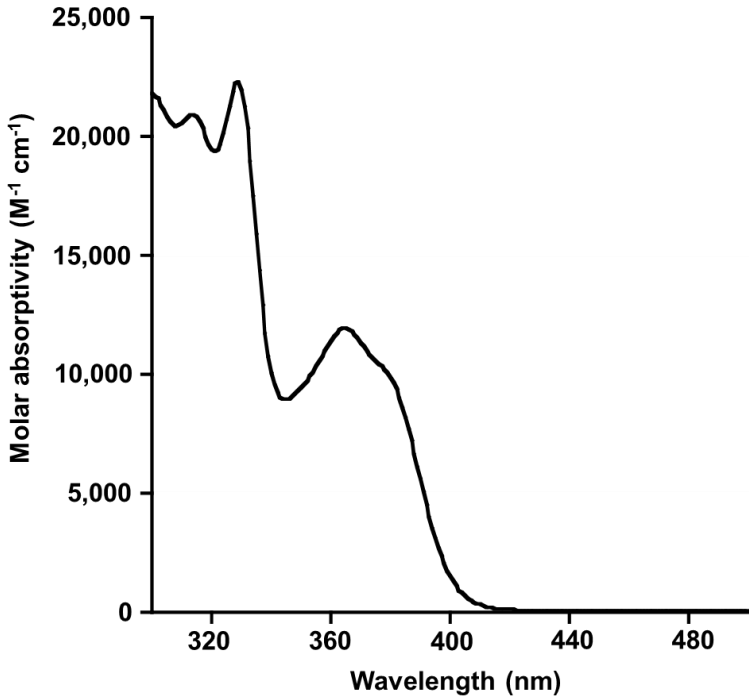


Figure S6. Molar absorptivity **3** in acetonitrile.

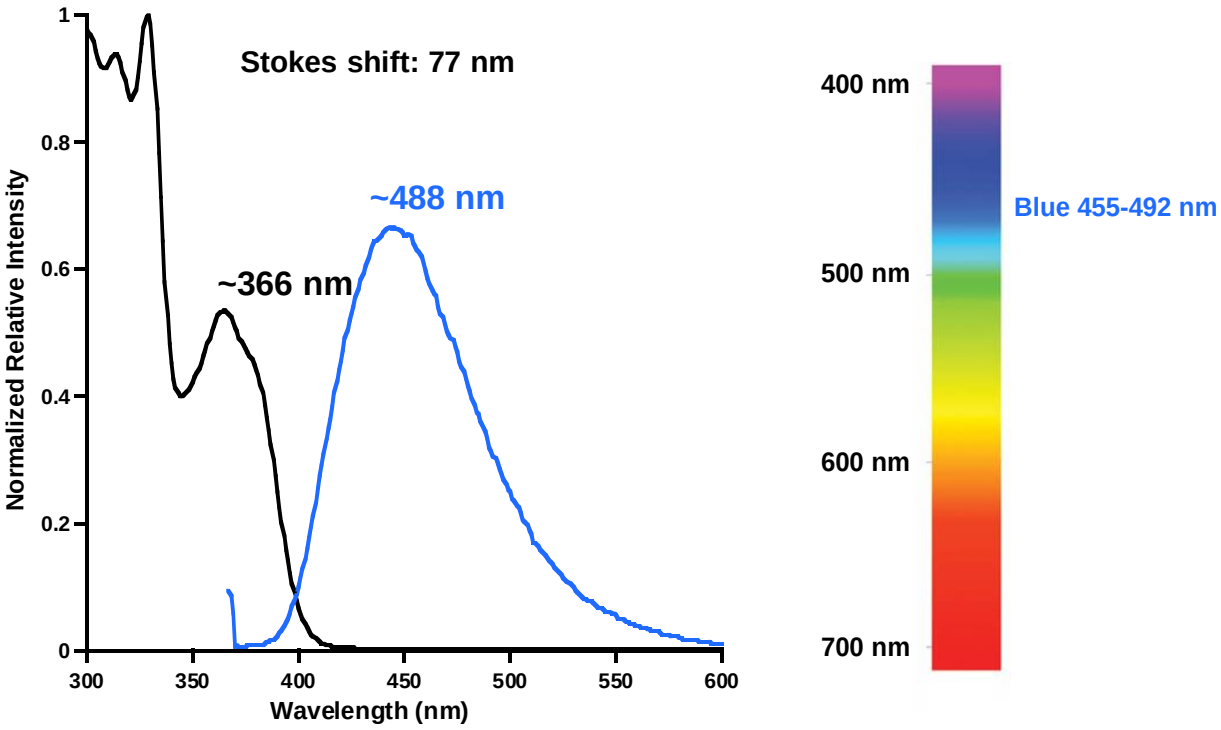


Figure S7. Normalized overlay of the lowest energy absorption feature of **3** (shown in black) with the emission spectrum (shown in blue) in acetonitrile.

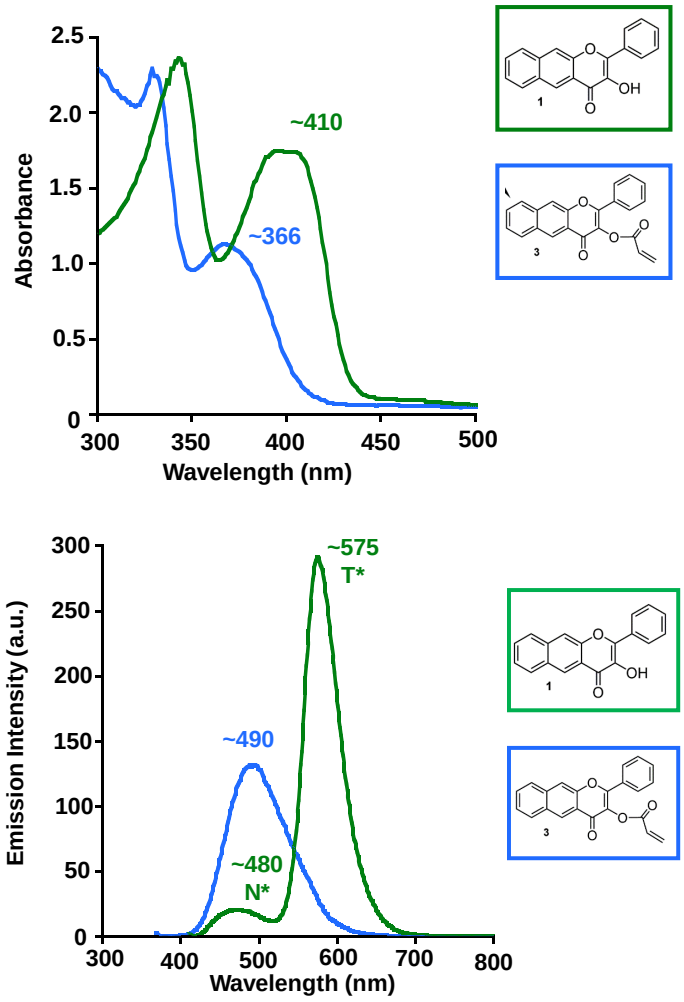


Figure S8. Absorption (top) and emission (bottom) spectra of **1** and **3** in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4). The emission spectrum of **1** contains components from normal (N*) and tautomeric (T*) excited state species.

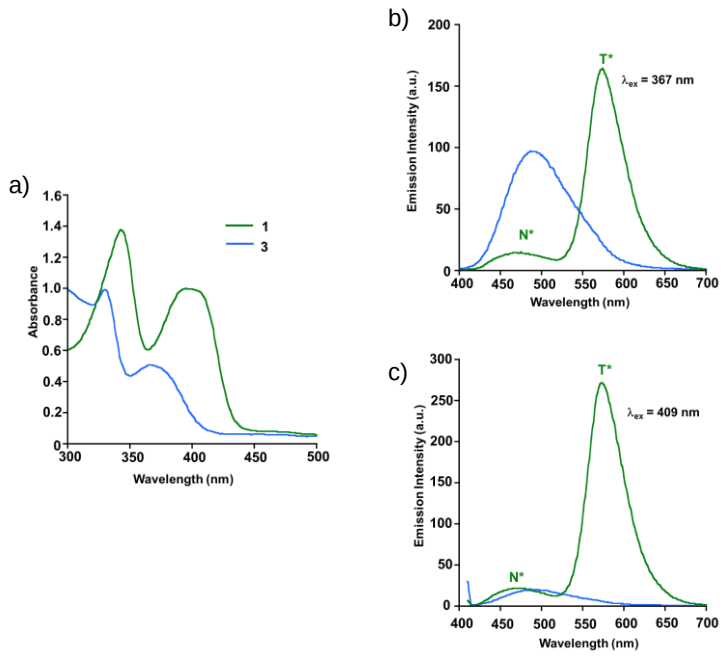


Figure S9. Absorption (a) and emission (b,c) spectra of the reaction of **3** with a 10-fold excess Cys. The spectra were acquired after 1 hour of incubation at 37 °C in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4).

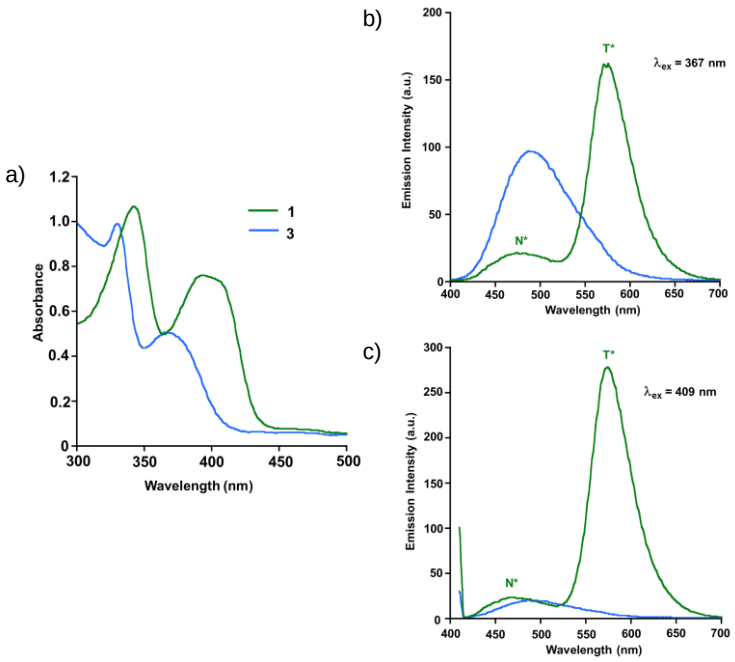


Figure S10. Absorption (a) and emission (b,c) spectra of the reaction of **3** with a 10-fold excess Hcy. The spectra were acquired after 1 hour of incubation at 37 °C in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4).

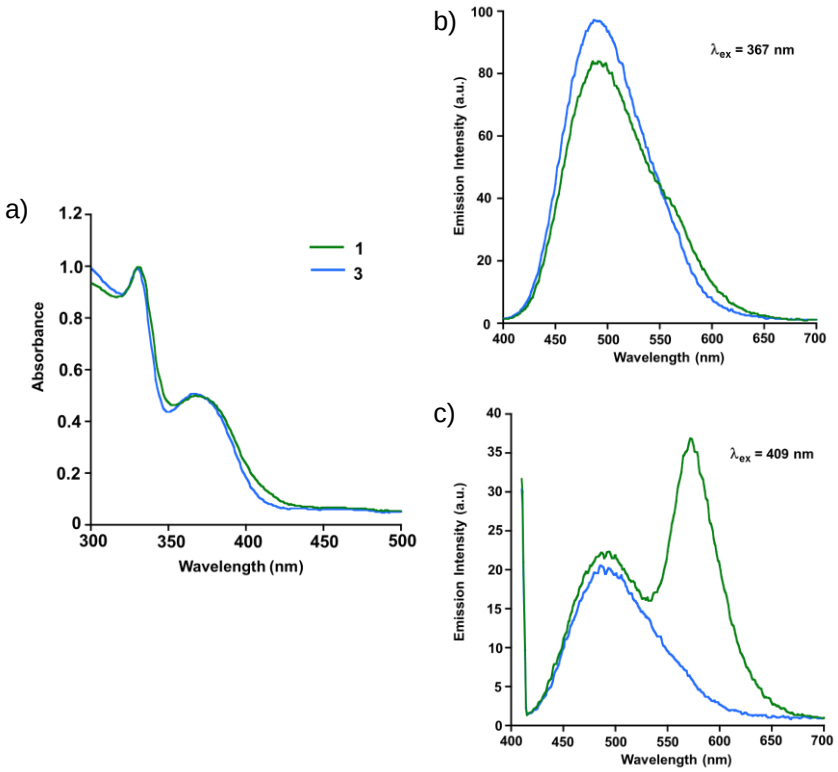


Figure S11. Absorption (a) and emission (b,c) spectra of the reaction of **3** with a 10-fold excess GSH. The spectra were acquired after 1 hour of incubation at 37 °C in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4).

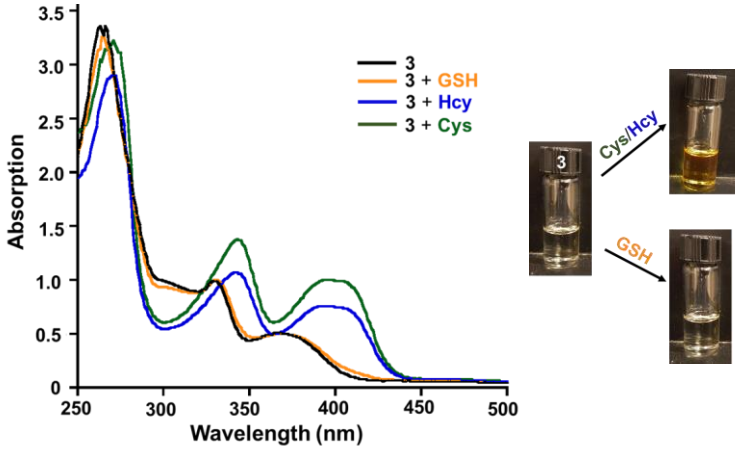


Figure S12. (Left) Overlaid absorption spectra of the reactions of **3** with GSH, Hcy, and Cys after 1 hour of incubation at 37 °C in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4). (Right) Solution of **3** shown after incubation with thiols emphasizing the color change (clear-to-yellow) that can be observed by naked eye in the case of Cys and Hcy.

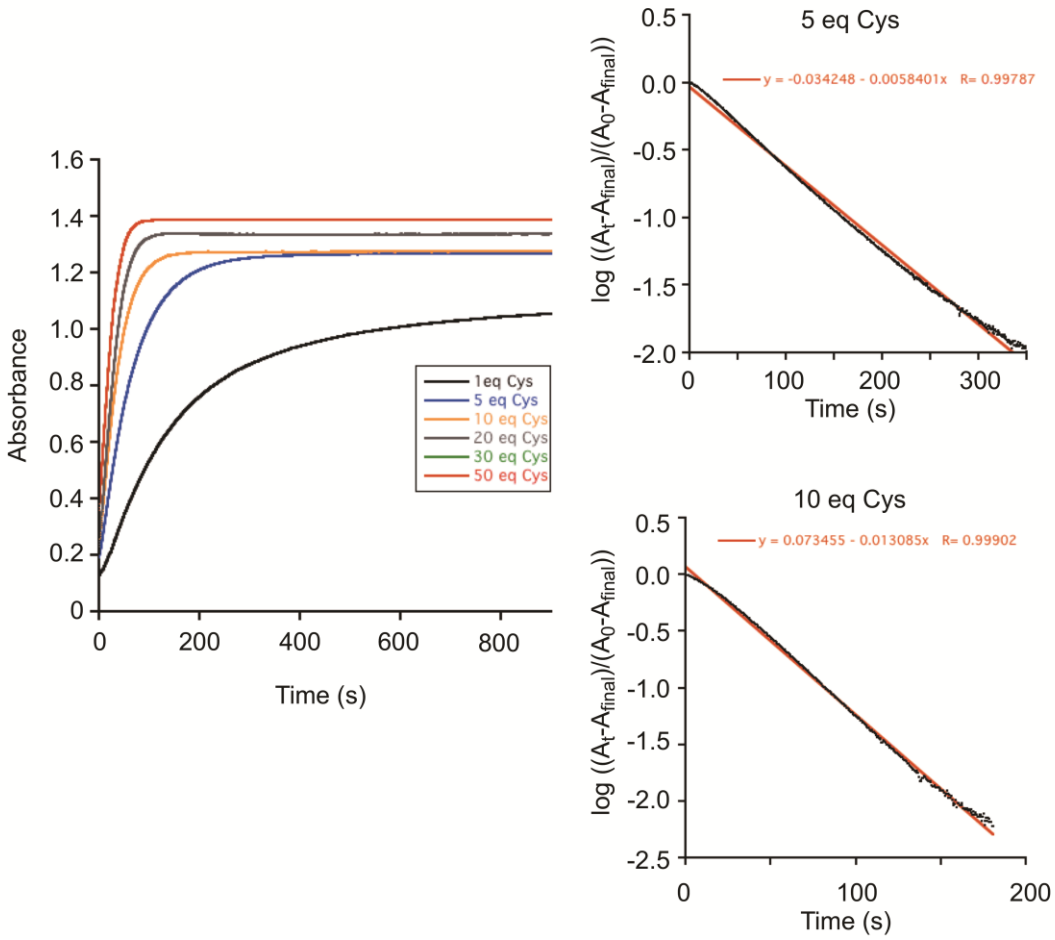


Figure S13. Kinetic studies of the reaction of **3** with varying amounts of cysteine at 37 ºC in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4).

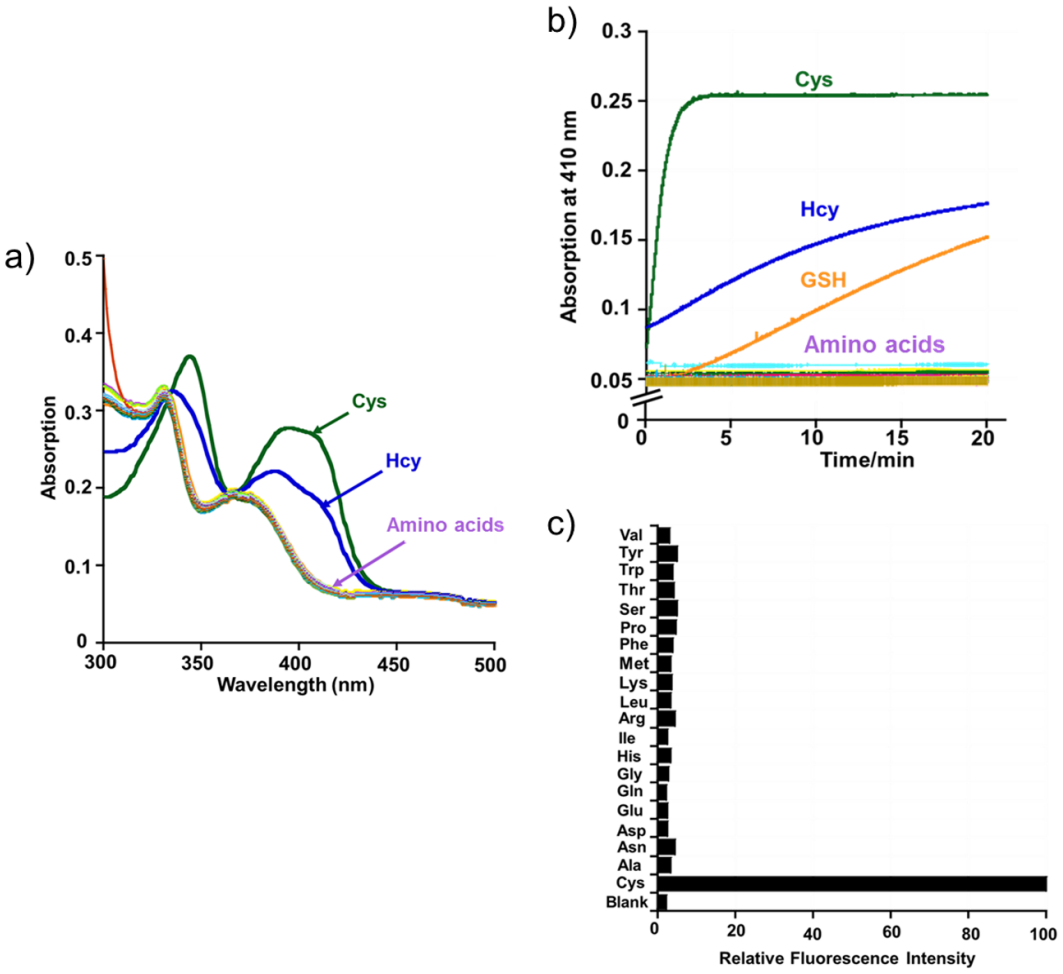


Figure S14. Amino acid selectivity studies of **3** as determined by absorption (a,b) and fluorescence emission (c). Spectra were acquired in CH_3CN:PBS (1:1 v/v, w/ 3% DMSO, 10 mM, pH = 7.4) at 37 °C after 20 minutes of incubation. The analyte concentration was 1:10 **3**:amino acid tested.

Linear response and detection limit (DL) determination:

The detection limit (DL) was calculated based on the fluorescence intensity upon titration of 10 µM **3** with various Cys concentrations. The measurements of emission were performed in triplicate. The linear response was determined to range from 1.7 – 10.0 µM of added Cys. Average emission values were plotted against Cys concentration to determine the slope. The detection limit was then calculated with the following equation: DL= 3 σb_l/m. Where σ_{bl} is the standard deviation of the blank measurements (the blank was measured 30 times) and m is the slope of the linear response. The detection limit of **3** for Cys was determined to be 24 nM.

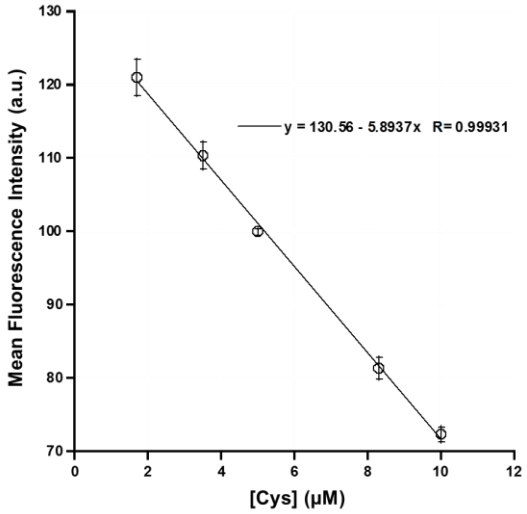


Figure S15. Detection limit determination.

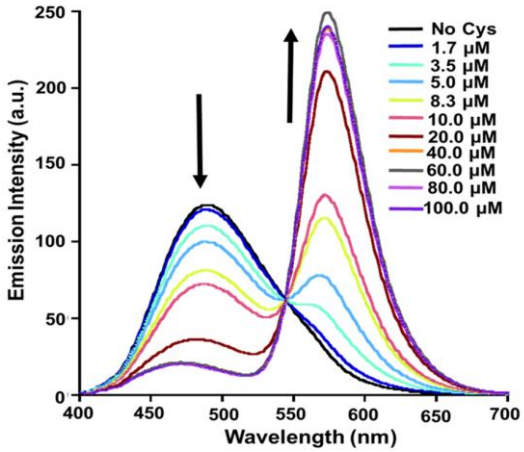


Figure S16. Overlaid emission spectra of **3** reacted with varying concentrations of Cys (1.7 µM – 100 µM) used in the determination of the linear response range and detection limit of **3** for Cys. Spectra were acquired in CH_3CN:PBS (1:1 v/v, w/ 0.33% DMSO, 10 mM, pH = 7.4) incubated for 5 minutes each at 37 °C.

Comparison of compound 3 among known thiol sensors and their detection limits[4]:

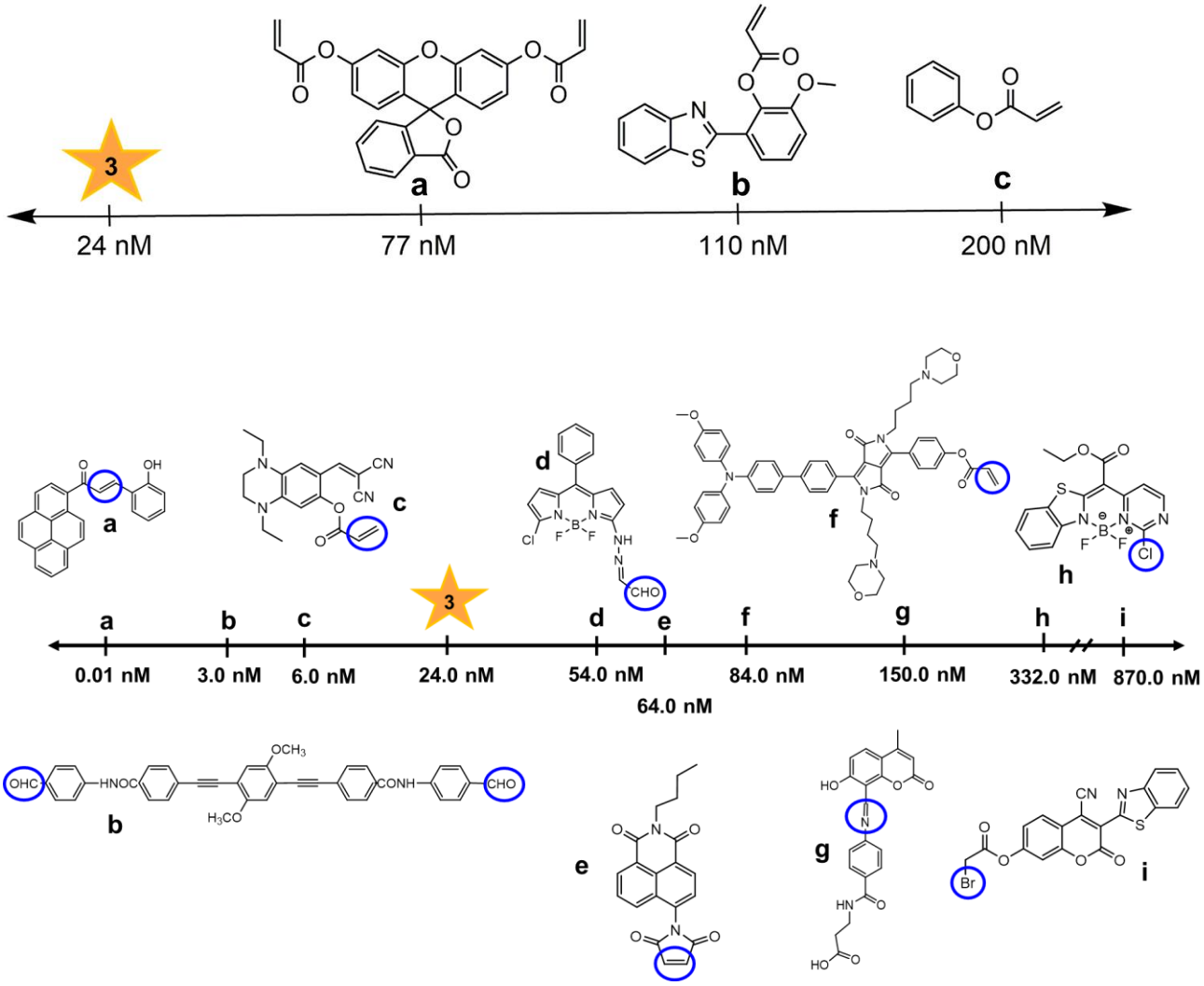


Figure S17. (Top) Range of known organic structural motifs with acryloyl tail as sensing unit for thiol detection and their detection limits. (Bottom) Extended list of organic frameworks with various sensing units for thiol detection and their detection limits. The displayed detection limits are related to Cys detection. Circled in blue are the thiol-reactive units. The yellow star represents the DL found for compound **3**.

Cytotoxicity Assay:

A549 human adenocarcinoma cells (ATCC, Manassas, VA) were grown in DMEM/Ham's F12 1:1 mixture medium, supplemented with 10% charcoal-stripped, heat-inactivated fetal bovine serum (FBS; Caisson Laboratories, Logan, UT) in a humidified incubator at 37 °C with 5% CO_2.[2] Cytotoxicity was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as previously described[3], with a starting cell density of 31,250 cells/cm^2. Briefly, a stock solution of **3** was prepared in DMSO and then added to culture media for a maximum final DMSO concentration of less than 0.4% (v/v). A serial dilution was performed to obtain final concentration ranges of 0.08 µM - 100 µM and the cells were incubated for 24 h in the dark. Upon incubation, the cells were treated with MTT to assess cell viability. All experiments were done in triplicate.

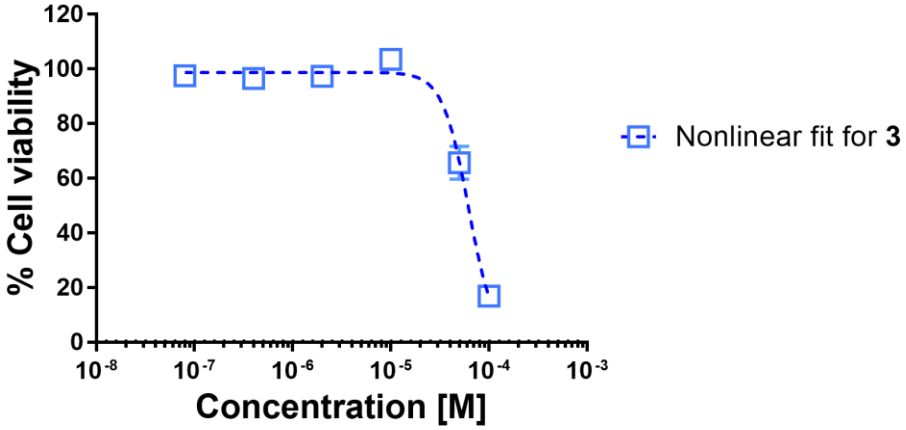


Figure S18. Plot of A549 cell viability expressed in % cell viability versus concentration of **3**. The IC_{50} value was determined using a four-parameter nonlinear regression for assays wherein at least 50% reduction in cell viability was observed (IC_{50} = 62 µM). Values displayed represent the average ±SEM (standard error of the mean) of three independently replicated experiments. Some data points shown in the plot have error bars that are smaller than the size of the symbol, thus, are not seen.

Fluorescence Microscopy:

A549 cells (passage 3) were maintained in culture as described above. Cells were seeded into Millicell E-Z-Slide culture chambers (EMD Millipore, Billerica, MA) at an initial density of 1 x 10^5 cells/cm^2 and were allowed to adhere to the chamber slides for 24 hours in an incubator. Compound **3** dissolved in DMSO was added to a final concentration of 25 µM per chamber not exceeding 0.4% final DMSO concentration in the chamber. The cells were incubated for 1 hour in media, protected from light. Upon incubation for 1 hour, the cells were washed three times with Dulbecco's PBS buffer and treated with CellMask Deep Red Plasma Membrane dye (final concentration of 1X) for 15 minutes, followed by three washes with PBS buffer to remove all the residual dye.

The cells were imaged using a Carl Zeiss LSM-710 AxioObserver live cell imaging system equipped with 34 spectral detection channels, five laser lines (458, 488, 514, 561, and 633 nm) and a 405 nm laser kit. Images were acquired using a 63x objective/1.40 Oil DIC M27, a beam splitter at 488/561/633 and the following excitation and emission parameters: for CellMask Deep Red Plasma Membrane dye (channel 1) λ_{ex} = 633 nm and a detection wavelength range λ_{em} = 649-699 nm; for compound **1** (channel 2) λ_{ex} = 405 nm and a detection wavelength range λ_{em} = 543-640 nm; for compound **3** (channel 3) λ_{ex} = 405 nm and a detection wavelength range λ_{em} = 415-515 nm. To evaluate CO release, the cells were illuminated for 5 and 10 minutes using 405 nm laser system. Acquired images were processed by universal adjustment to enhance contrast levels (same settings were applied for all acquired images in each detection channel) using ZEISS ZEN 2.3 Lite software (Figure 3).

Several control experiments were performed to confirm intracellular thiol detection. One of them included cell culture media pretreatment with 200 μM NEM (N-ethylmalemide, an efficient thio-reactive compound) for 30 minutes followed by the addition of **3** (25 μM final concentration per chamber). The subsequent procedures were performed as previously described (Figure 3). Another control experiment included addition of excess Cys to the media followed by incubation with **3** (25 μM final concentration per chamber). The subsequent procedures were performed as previously described (Figure 3).

Normoxia/Hypoxia experimental design:

Each UV-vis cuvette was volume calibrated prior to any quantitative studies.

For the normoxic condition studies, a solution of DMSO:H_2O (1:1,v/v) containing **1** at a concentration of $1.0 \cdot 10^{-4}$ M was degassed by purging with nitrogen gas in a sealed quartz cuvette (total volume 3 mL). O_2 gas was then injected through the septum to obtain a final O_2 concentration of 20% by volume. The obtained system was illuminated with 419 nm wavelength light (8 lamps) over the course of 1350 seconds with an interval of 50 seconds for periodically measuring absorption spectra of the solution mixture.

For the hypoxic condition studies, a solution of DMSO:H_2O (1:1,v/v) containing **1** at a concentration of $1.0 \cdot 10^{-4}$ M was degassed by purging with nitrogen gas in a sealed quartz cuvette (total volume 3 mL). O_2 gas was then injected through the septum to obtain a final O_2 concentration of 1% by volume. The obtained system was illuminated with 419 nm wavelength light (8 lamps) over the course of 1350 seconds with an interval of 50 seconds for periodically measuring absorption spectra of the solution mixture.

For hypoxic condition studies, a solution of DMSO:H_2O (1:1,v/v) containing $1.0 \cdot 10^{-4}$ M concentration of compound **1** was degassed in a sealed quartz cuvette (total volume 3 mL). Then O_2 gas was injected through septum to obtain a final O_2 concentration of 1% by volume (with respect to the total volume of the calibrated cuvette). The obtained system was illuminated with 419 nm wavelength light (8 lamps) in the course of 1350 seconds (with an interval of 50 seconds) measuring periodically the absorption spectra of the solution mixture.

An additional experiment was performed in the case of hypoxic condition using a lower concentration of **1** (25 μM). This experiment was designed to test the reaction completion at the scale of compound concentration used in cellular studies (Figure 4).

References:

1. Anderson S.N.; Richards, J.M.; Esquer, H.J.; Benninghoff, A.D.; Arif, A.M.; Berreau, L.M. *ChemistryOpen* **2015**, *4*, 590.
2. Shorey, L.E.; Hagman, A.M.; Williams, D.E.; Ho, E.; Dashwood, R.H.; Benninghoff, A.D. *Plos One* **2012**, *7*.
3. Saraf, S.L.; Fish, T.J.; Benninghoff, A.D.; Buelt, A.A.; Smith, R.C.; Berreau, L.M. *Organometallics* **2014**, *33*, 6341.
4. a) Wang, H.; Zhou, G.; Gai, H.; Chen, X. *Chem. Commun.* **2012**, *48*, 8341.
   b) Yang, X.; Guo, Y.; Strongin, R.M. *Angew. Chem. Int. Ed.* **2011**, *50*, 10690.
   c) Yang, X.; Guo, Y.; Strongin, R.M. *Org. Biomol. Chem.* **2012**, *10*, 2739.
   d) Rani, B.K.; John, S.A. *Biosens. Bioelectron.* **2016**, *83*, 237.
   e) Yang, C.; Wang, X.; Shen, L.; Deng, W.; Liu, H.; Ge, S.; Yan, M.; Song, X. *Biosens. Bioelectron.* **2016**, *80*, 17-23.
   f) Liu, X.; Yang, D.; Chen, W.; Yang, L.; Qi, F.; Song, X. *Sens. Actuators B Chem.***2016**, *234*, 27.
   g) Gong, D.; Tian, Y.; Yang, C.; Iqbal, A.; Wang, Z.; Liu, W.; Qin, W.; Zhu, X.; Guo, H. *Biosens. Bioelectron.* **2016**, *85*, 178.
   h) Liu, T.; Huo, F.; Li, J.; Chao, J.; Zhang, Y.; Yin, C. *Sens. Actuators B Chem.* **2016**, *237*, 127.
   i) Zhang, X.; Hang, Y.; Qu, W.; Yan, Y.; Zhao, P.; Hua, J. *RSC Adv.* **2016**, 20014.
   j) Yan, L.; Kong, Z.; Shen, W.; Du, W.; Zhou, Y.; Qi, Z. *Anal. Biochem.* **2016**, *500*, 1.
   k) Liu, Q.; Zhang, C.; Wang, X.; Gong, S.; He, W.; Liu, Z. *Chem. Asian J.* **2016**, *11*, 202.
   l) Manibalan, K.; Chen, S. -M.; Mani, V.; Huang, T. –T.; Huang, S. –T. *J. Fluoresc.* **2016**, *26*, 1489.