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Supporting Information

Cysteine-Activated Hydrogen Sulfide (H_2S) Delivery through Caged-Carbonyl Sulfide (COS) Donor Motifs

Yu Zhao,[†] Andrea K. Steiger,[†] and Michael D. Pluth[*]

Department of Chemistry and Biochemistry
Institute of Molecular Biology
Materials Science Institute
University of Oregon
Eugene, OR 97403, USA

Contact Information:

Michael D. Pluth

pluth@uoregon.edu

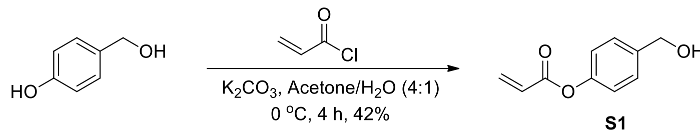
Table of Contents:

1. Material and Methods S2
2. Synthesis S2
3. H_2S Calibration Curve S4
4. Measurement of H_2S Release from **OA-CysTCM-1** S4
5. Selectivity Evaluation of H_2S Release from **OA-CysTCM-1** S4
6. Cys-Triggered H_2S Release from **OA-CysTCM-1** in the Presence of GSH S7
7. Cellular Imaging of H_2S Release from **OA-CysTCM-1** S8
8. Fluorescence Imaging of H_2S Release from **OA-CysTCM-1** S9
9. NMR Spectra of Synthesized Compounds S10
10. Reference s S13

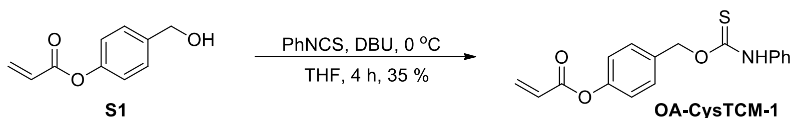
Materials and Methods

Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fisher Scientific, or VWR and used directly as received. SF7-Am was synthesized as previously reported.[1] Silica gel (SiliaFlash F60, Silicycle, 230−400 mesh) was used for column chromatography. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA). ^{1}H and ^{13}C{^{1}H} NMR spectra were recorded on Varian 300 MHz, Bruker 500 MHz or Bruker 600 MHz NMR instruments at the indicated frequencies. Chemical shifts are reported in ppm relative to residual protic solvent resonances. Methylene blue (MB) absorbances were measured using an Agilent Cary 100 UV-Vis spectrometer. b.End3 cells were purchased from ATCC (Manassas, Virginia, USA). Cell imaging experiments were performed on a Leica DMi8 fluorescence microscope, equipped with an Andor Zyla 4.2+ sCMOS detector.

Synthesis

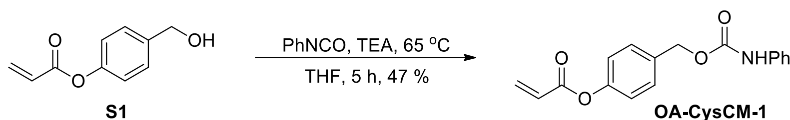


The benzyl alcohol compound **S1** was prepared using a known procedure.[2] Briefly, K_2CO_3 (207 mg, 1.50 mmol) was dissolved in acetone/H_2O (25.0 mL, v/v 4:1), followed by the addition of acryloyl chloride (90.5 mg, 1.00 mmol) at 0 °C. To this reaction mixture, a solution of 4-hydroxybenzyl alcohol (124 mg, 1.00 mmol) was added dropwise and the reaction solution was stirred at 0 °C for 4 h. Brine was then added, and the reaction mixture was extracted with DCM (3 x 25.0 mL). The organic layers were collected, dried over $MgSO_4$, and concentrated under vacuum. The product **S1** was isolated as a clear oil (42.0%) after purification by column chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.43 (d, *J* = 5.0 Hz, 2H), 7.16 (d, *J* = 5.0 Hz, 2H), 6.64 (d, *J* = 15.0 Hz, 1H), 6.36 (m, 1H), 6.05 (d, *J* = 10.0 Hz, 1H), 4.73 (s, 2H), 1.75 (br, 1H). ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ (ppm): 164.6, 150.0, 138.5, 132.7, 128.1, 127.9, 121.6, 64.8.



S1 (178 mg, 1.00 mmol) and phenyl isothiocyanate (135 mg, 1.00 mmol) were dissolved in THF (15.0 mL) and cooled to 0 °C. DBU (152 mg, 1.00 mmol) was then added, and the reaction solution was stirred at 0 °C for 4 h. The reaction mixture was then quenched by adding brine, and the aqueous solution was extracted with DCM (3 x 25.0 mL). The organic layers were collected, dried over $MgSO_4$, and concentrated under vacuum. **OA-CysTCM-1** was isolated as white solid (35.4%) after purification by column chromatography. Two sets of NMR resonances showed up due to slow rotation around the thiocarbamate functional group at room temperature. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ (ppm): 11.24 (s, 1H), 7.68 (br, 1H), 7.52 (br, 2H), 7.34 (br, 3H), 7.22 (br, 3H), 6.54 (d, *J* = 15.0 Hz, 1H), 6.42 (dd, *J* = 15.0, 5.0 Hz, 1H), 6.17 (d, *J* = 10.0 Hz, 1H), 5.55 (d, *J* = 50.0 Hz, 2H). ^{13}C{^{1}H} NMR (150 MHz, DMSO-*d*$_6$) δ (ppm): 187.8, 187.4, 164.6, 150.5, 134.2, 130.3, 129.9, 129.3, 128.9, 128.1, 125.6, 123.3, 122.3, 72.0, 70.3. IR (cm^{-1}): 3228, 3039, 2919,

1734, 1592, 1547, 1494, 1446, 1402, 1335, 1293, 1146, 1018, 968. HRMS m/z $[M + Na]^+$ calcd. for $[C_{17}H_{15}NNaO_3S]^+$ 336.0670; found 336.0673.



S1 (178 mg, 1.00 mmol) and phenyl isocyanate (119 mg, 1.00 mmol) were dissolved in THF (15.0 mL) and cooled to 0 ºC. TEA (101 mg, 1.00 mmol) was then added, and the reaction solution was heated to 65 °C and stirred for 5 h. The reaction mixture was then quenched by adding brine and the aqueous solution was extracted with DCM (3 x 25.0 mL). The organic layers were collected, dried over $MgSO_4$, and concentrated under vacuum. **OA-CysCM-1** was isolated as white solid (47.0%) after purification by column chromatography. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.43 (t, *J* = 9.0 Hz, 4H), 7.32 (t, *J* = 9.0 Hz, 2H), 7.15 (d, *J* = 9.0 Hz, , 2H), 7.09 (t, *J* = 9.0 Hz, 1H), 6.91 (br, 1H), 6.64 (dd, *J* = 15.0, 3.0 Hz, 1H), 6.35 (dd, *J* = 18.0, 9.0 Hz, 1H), 6.04 (dd, *J* = 12.0, 3.0 Hz, 1H), 5.20 (s, 2H). $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$) δ (ppm): 164.6, 153.4, 150.5, 137.8, 133.8, 132.8, 129.6, 129.1, 127.8, 123.5, 121.7, 118.8, 66.3. IR (cm^{-1}): 3367, 3064, 1735, 1709, 1634, 1598, 1532, 1502, 1447, 1407, 1318, 1298, 1234, 1198, 1167, 1071, 982, 901, 819. HRMS m/z $[M + Na]^+$ calcd. for $[C_{17}H_{15}NNaO_4]^+$ 320.0899; found 320.0897.

H_2S Calibration Curve

UV cuvettes (1.50 mL capacity) were charged with 0.500 mL of the MB cocktail (*vide infra*) and 0.500 mL of PBS buffer (pH 7.40, 10.0 mM). The resultant solution was mixed thoroughly, followed by the addition of an NaSH stock solution (1.00 mM) to make the final H_2S concentrations of 1.00, 3.00, 5.00, 10.0, 15.0, and 20.0 μM. The MB solution was allowed to react with H_2S for 1 hour before measuring the absorbance at 670 nm.

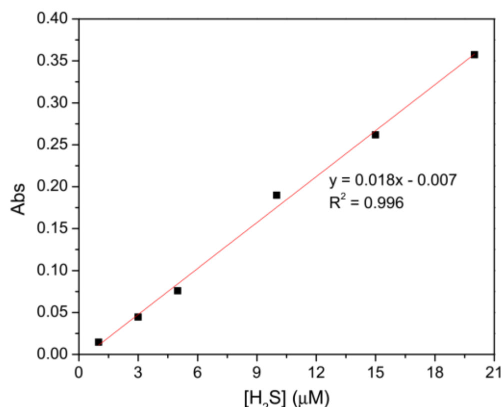


Figure S1. H_2S calibration curve for the MB assay.

H_2S Release from OA-CysTCM-1 in PBS

An **OA-CysTCM-1** stock solution (0.100 mL, 10.0 mM in DMSO) was added to 20.0 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 μg/mL) in a 25-mL scintillation vial. A Cys stock solution (0.100 M in H_2O) was then added to generate the desired Cys working concentrations as shown in Figure 3. Next, 0.300 mL aliquots of the reaction mixture were transferred to UV cuvettes containing 0.300 mL of MB cocktail (0.0600 mL zinc acetate (1.00% w/v), 0.120 mL $FeCl_3$ (30.0 mM in 1.20 M HCl), and 0.120 mL *N,N*-dimethyl-*p*-phenylene diamine (20.0 mM in 7.20 M HCl)) at different time points. The absorbance at 670 nm was then measured after 1 hour and was converted to H_2S concentration by using the H_2S calibration curve as shown in Figure S1.

Selectivity Evaluation of H_2S Release from OA-CysTCM-1 (Figure 3)

Bar 1: To 2.00 mL of PBS containing CA (25.0 μg/mL) was added Cys (10.0 μL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 μL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 2: To 2.00 mL of PBS containing CA (25.0 μg/mL) was added Cys (10.0 μL, 0.100 M in H_2O), followed by the addition of NEM (2.50 mg). After 20 min, **OA-CysTCM-1** (10.0 μL, 10.0 mM in DMSO) was added. After 3 hours of incubation at room temperature, 0.300 mL of

the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 3: To 2.00 mL of PBS was added Cys (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 4: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added Cys (10.0 µL, 0.100 M in H_2O), followed by the addition of AAA (2.00 µL, 10.0 mM in DMSO). After 20 min, **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO) was added. After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 5: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added Hcy (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 6: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added NAC (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 7: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added GSH (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 8: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added Ser (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 9: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added Lys (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 10: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added GSSG (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysTCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 11: To 20.0 mL of PBS containing CA (25.0 µg/mL) was added PLE (20.0 µL, 1.00 U/µL), followed by the addition of **OA-CysTCM-1** (0.100 mL, 10.0 mM in DMSO). 0.300 mL of the reaction aliquots were transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 12: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added Cys (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-CysCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Bar 13: To 2.00 mL of PBS containing CA (25.0 µg/mL) was added Cys (10.0 µL, 0.100 M in H_2O), followed by the addition of **OA-TCM-1** (10.0 µL, 10.0 mM in DMSO). After 3 hours of incubation at room temperature, 0.300 mL of the reaction aliquot was transferred to a UV cuvette containing 0.300 mL of the MB cocktail. The absorbance at 670 nm was then measured after 1 hour by the MB assay.

Cys-Triggered H_2S Release from OA-CysTCM-1 in the Presence of GSH

An **OA-CysTCM-1** stock solution (0.100 mL, 10.0 mM in DMSO) was added to 20.0 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 µg/mL) in a 25-mL scintillation vial. A Cys stock solution (100 µL, 100 mM in H_2O) and a GSH stock solution (50.0 µL, 100 µL, or 200 µL, 100 mM in H_2O) were then added so that the working Cys and GSH concentration ratios are 2:1, 1:1, and 1:2, respectively. Next, 0.300 mL aliquots of the reaction mixture were transferred to UV cuvettes containing 0.300 mL of MB cocktail (0.0600 mL zinc acetate (1.00% w/v), 0.120 mL $FeCl_3$ (30.0 mM in 1.20 M HCl), and 0.120 mL *N,N*-dimethyl-*p*-phenylene diamine (20.0 mM in 7.20 M HCl)) at different time points. The absorbance at 670 nm was then measured after 1 hour and was converted to H_2S concentration by using the H_2S calibration curve as shown in Figure S1.

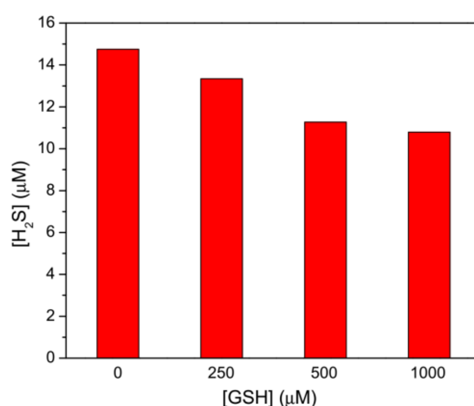


Figure S2. GSH Effects on Cys-triggered COS/H_2S release from **OA-CysTCM-1** (50 µM). The Cys concentration was 500 µM in all experiments, and H_2S concentrations were measured after 2-h incubation.

Cellular Imaging of H_2S Release from OA-CysTCM-1

b.End 3 cells were plated in poly-D-lysine coated plates (MatTek) containing 2 mL of DMEM and incubated at 37 °C under 5% CO_2 for 24 h. The confluent cells were washed with PBS and then incubated with SF7-AM (5.00 µM) for 30 min. The cells were then washed with PBS and incubated with either **OA-CysTCM-1** (100 µM) and cysteine (250 µM) or cysteine (250 µM) alone for 30 min. Prior to imaging, cells were washed with PBS and bathed in 2 mL of PBS. Cell imaging was performed on a Leica DMi8 fluorescent microscope using DIC for brightfield and a standard GFP filter cube for fluorescence imaging and 100 ms and 50 ms exposure times, respectively. The scale bar represents 100 µm.

Fluorescence Imaging of H_2S Release from OA-CysTCM-1

A stock solution of SF7-AM (1.50 µL, 20.0 mM in DMSO) was added to a vial containing PBS (pH 7.40, 3.00 mL), cysteine (250 µM), and carbonic anhydrase (25.0 µg/mL). Fluorescence measurements (λ_{ex} = 488 nm, λ_{em} = 498-700 nm) were taken either immediately upon addition of SF7-AM or after addition of **OA-CysCM-1** (15.0 µL, 20.0 mM in DMSO) or **OA-CysTCM-1** (15.0 µL, 20.0 mM in DMSO) and taken periodically throughout the course of 120 minutes.

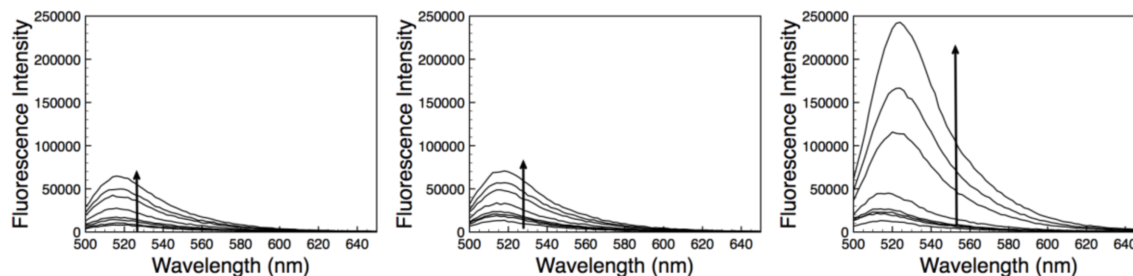
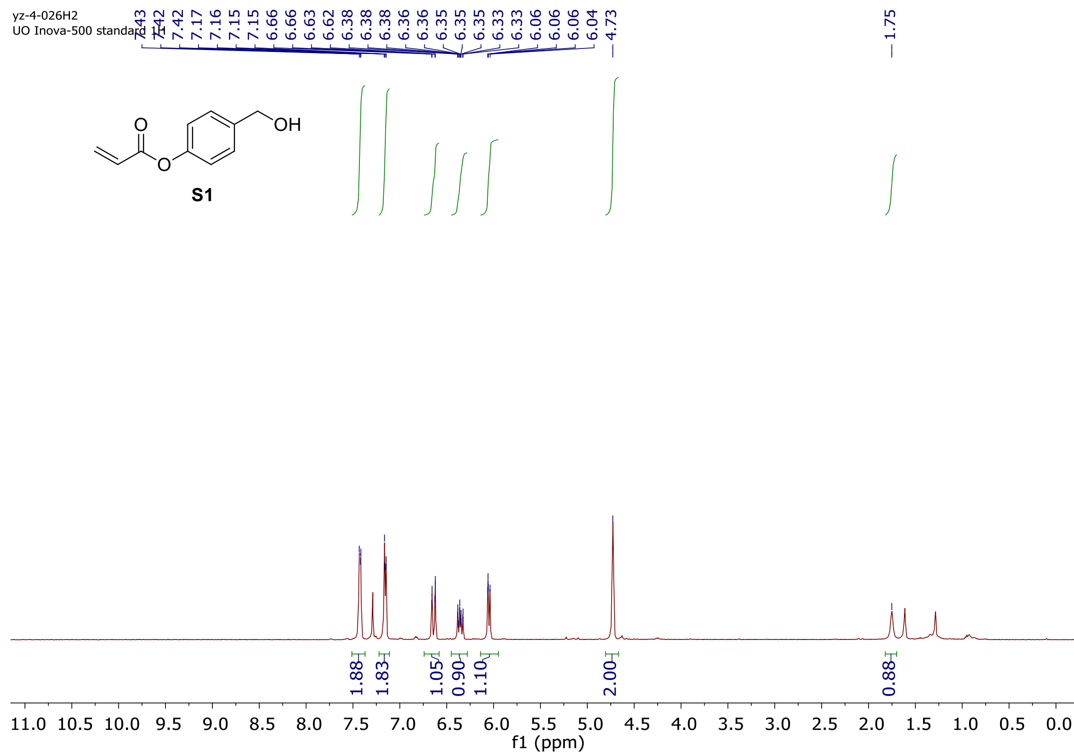
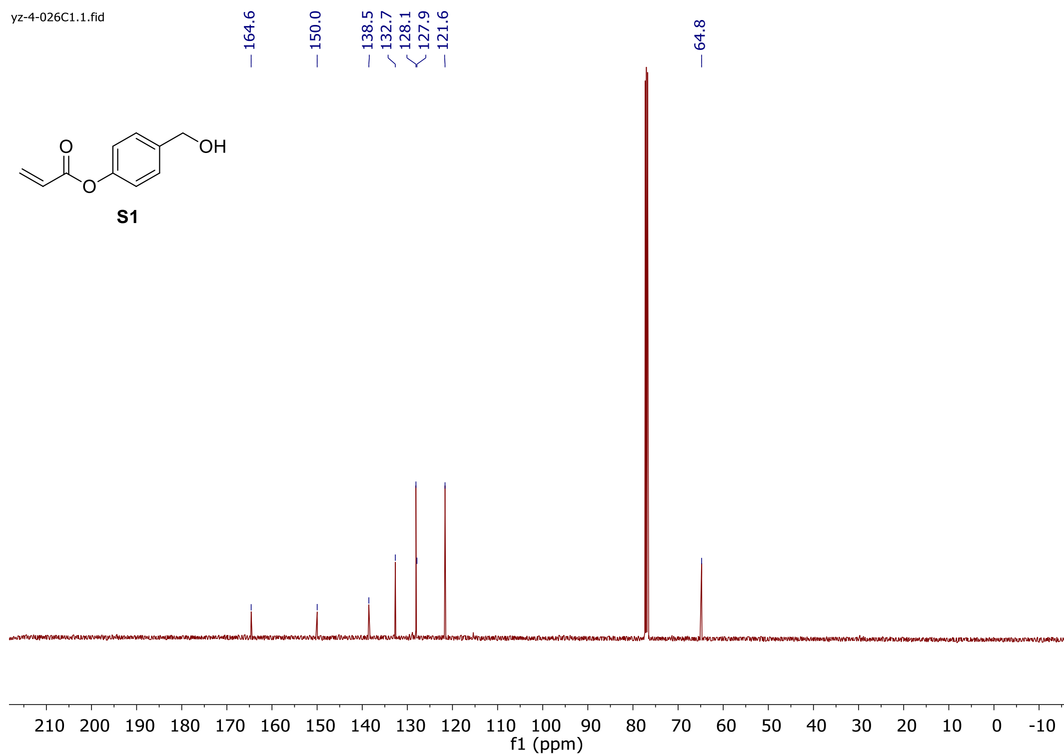


Figure S3: Fluorescence response of SF7-AM (5.00 µM) with cysteine (250 µM) and carbonic anhydrase (25.0 µg/mL) (left); upon addition of **OA-CysCM-1** (50.0 µM, middle); and upon addition of **OA-CysTCM-1** (50.0 µM, right) over the course of 120 minutes. λ_{ex} = 488 nm.

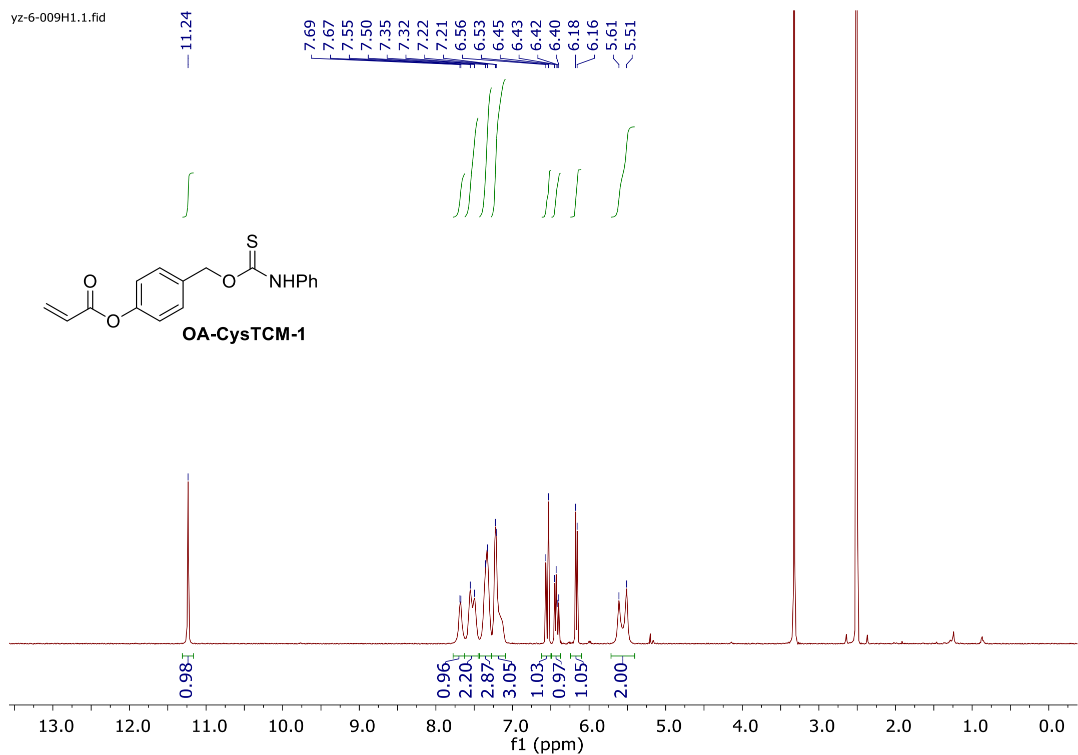
NMR Spectra

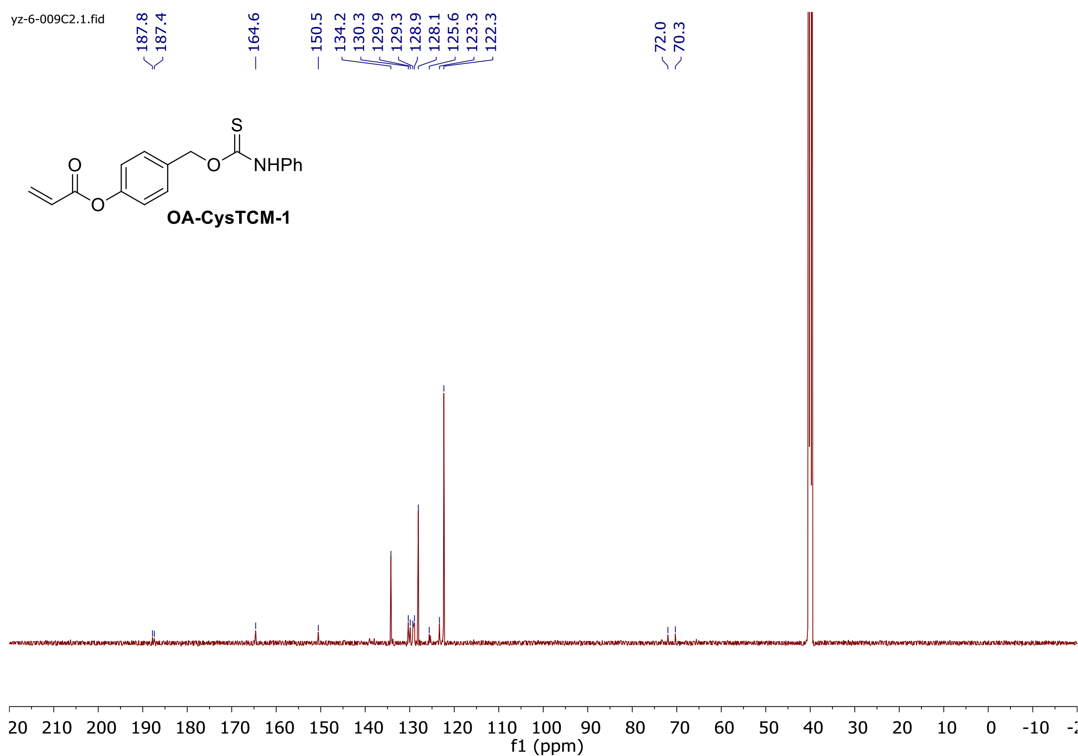
^{1}H (500 MHz, $CDCl_3$) and $^{13}C\{^{1}H\}$ (125 MHz, $CDCl_3$) NMR Spectra of **S1**.



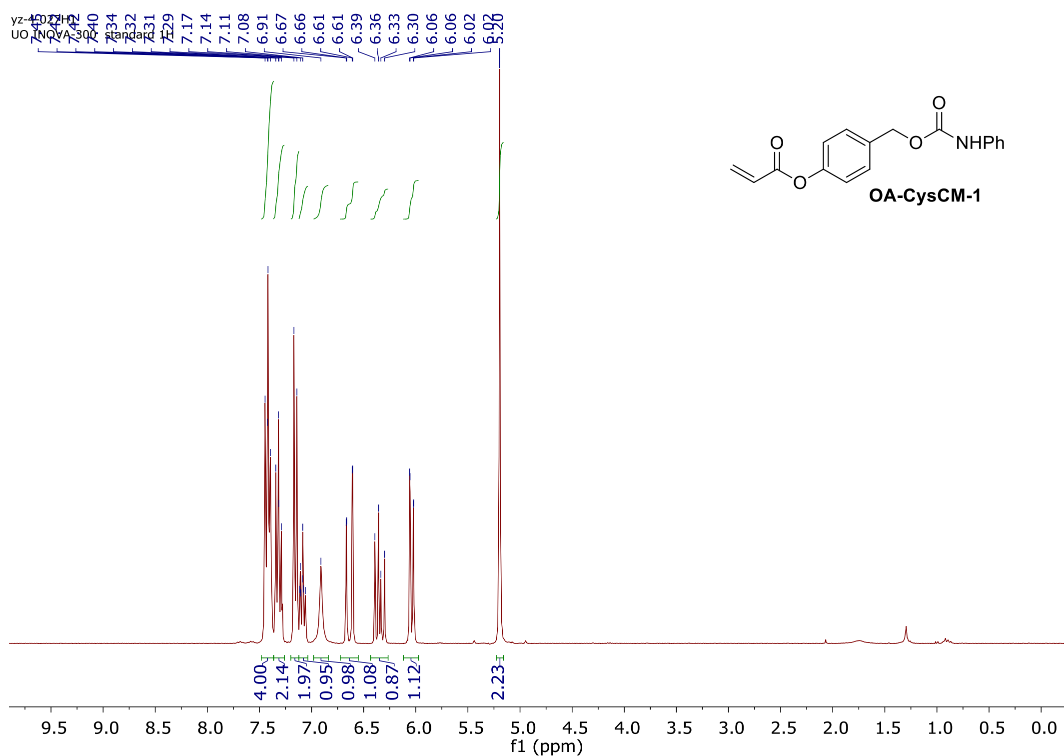


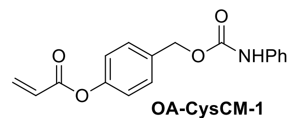
^{1}H (500 MHz, DMSO-d_6) and $^{13}C\{^{1}H\}$ (150 MHz, DMSO-d_6) NMR Spectra of **OA-CysTCM-1**. Two sets of NMR resonances showed up in ^{13}C NMR spectrum due to slow rotation around the thiocarbamate functional group at room temperature

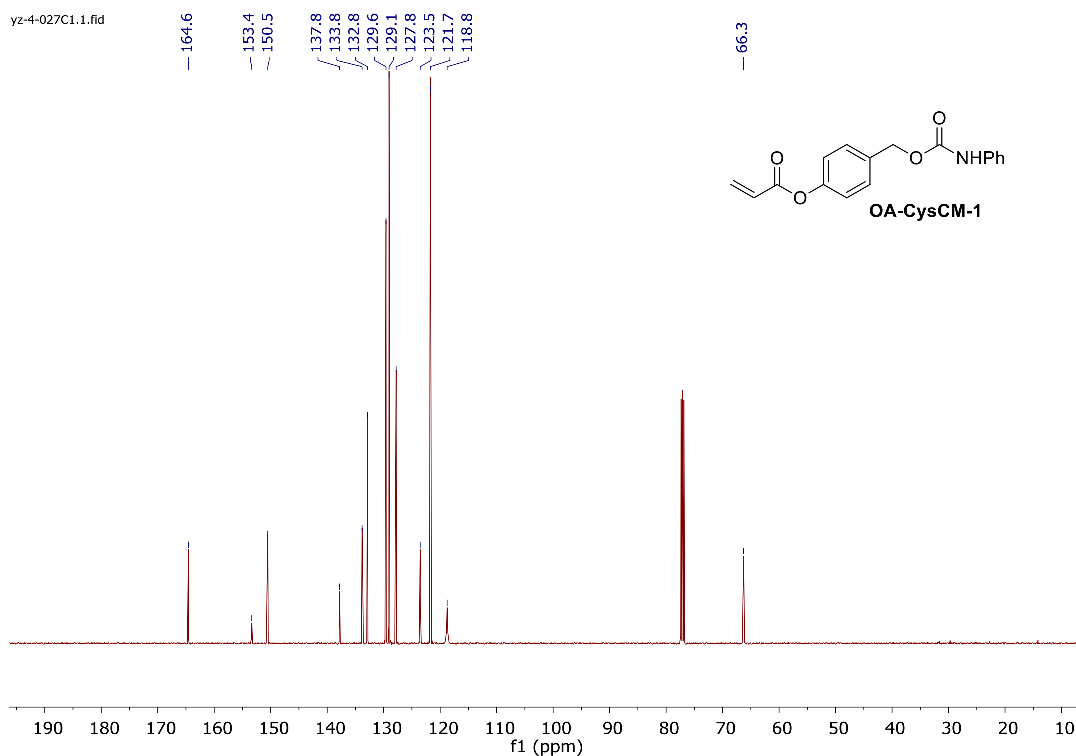


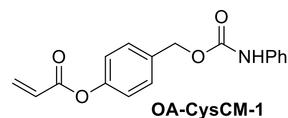


^{1}H (500 MHz, $CDCl_3$) and $^{13}C\{^{1}H\}$ (125 MHz, $CDCl_3$) NMR Spectra of **OA-CysCM-1**.









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2. A. R. Sarkar, C. H. Heo, E. Kim, H. W. Lee, H. Singh, J. J. Kim, H. Kang, C. Kang and H. M. Kim, *Chem. Commun.*, 2015, **51**, 2407.