A self-immolative linker that release thiols detects penicillin amidase and nitroreductase with high sensitivity via absorption spectroscopy

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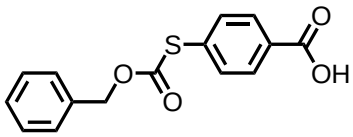
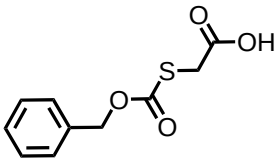
MATERIALS

General methods. All chemicals were purchased from Sigma Aldrich. The reported ^{1}H NMR and ^{13}C NMR were recorded on a Bruker Advance 400 Spectrometer at 400 and 101 MHz respectively.

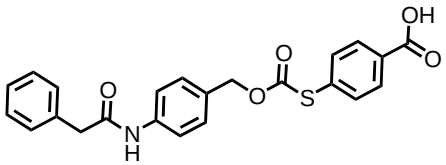
Synthesis of probes and control



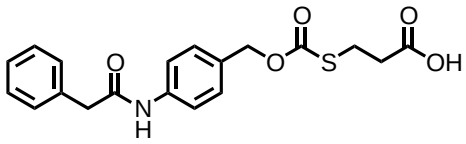
4-(((benzyloxy)carbonyl)thio)benzoic acid (Figure S1). Benzyl (4-nitrophenyl) carbonate (273 mg, 1 mmol) and 4-mercaptobenzoic acid (154 mg, 1 mmol) was placed in a round bottom flask equipped with a stir bar and purged with argon. Dichloromethane (5 mL) was added. While stirring, N,N-Diisopropylethylamine (1 mL, 5.74 mmol) was added. The solution was stirred overnight at room temperature, after which the solution was concentrated in vacuo. The solution was diluted with 25 mL of DCM and washed with water (3 × 100 mL). The organic layer was then collected, dried over Na_2SO_4, and concentrated in vacuo. Purification using flash chromatography (stationary phase: silica gel, eluent gradient: from 99% hexanes/1% acetic acid to 99% ethyl acetate/1% acetic acid) afforded 178 mg (0.62 mmol, 62%) of a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.0 (m, 2H), 7.70 (m, 2H), 7.40 (m, 5H), 5.32 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm: 167.98, 167.10, 135.39, 134.84, 132.71, 132.26, 130.45, 129.11, 129.07, 129.00, 70.06. HRMS (m/z): $[M - H]^-$ calculated for $C_{15}H_{11}O_4S^-$, 287.0384; found 287.0386.



2-(((benzyloxy)carbonyl)thio)acetic acid (Figure S2). Benzyl (4-nitrophenyl) carbonate (500 mg, 1.83 mmol) and 2-mercaptoacetic acid (128 uL, 3.66 mmol) was placed in a round bottom flask equipped with a stir bar and purged with argon. Dichloromethane (5 mL) was added. While stirring, N,N-Diisopropylethylamine (1 mL, 5.74 mmol) was added. The solution was stirred overnight at room temperature. The solution was concentrated in vacuo. The solution was diluted with 25 mL of DCM and washed with water (3 × 100 mL). The organic layer was collected, dried over Na_2SO_4, and concentrated in vacuo. Purification using flash chromatography (stationary phase: silica gel, eluent gradient: from 99% hexanes/1% acetic acid to 99% ethyl acetate/1% acetic acid and then 89% ethyl acetate/10% MeOH/1% acetic acid) afforded 115 mg (0.51 mmol, 28% column yield); 100 mg (0.44 mmol) was recrystallized from hot water to afford 70 mg (0.31 mmol, 61% recrystallization yield) of a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.49 (m, 5H), 5.26 (s, 2H), 3.73 (s, 2 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm: 169.94, 169. 83, 135.60, 129.03, 128.99, 128.88, 69.43, 33.61. HRMS (m/z): $[M + H]^+$ calculated for $C_{10}H_{11}O_4S^+$, 227.0378; found 227.2021.

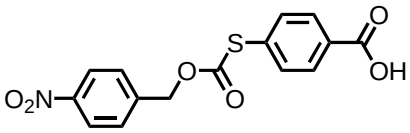


4-((((4-(2-phenylacetamido)benzyl)oxy)carbonyl)thio)benzoic acid (Figure S3). *N*-(4-(hydroxymethyl)phenyl)-2-phenylacetamide (1100 mg, 4.56 mmol) and 4-nitrophenyl chloroformate (1012 mg, 5.02 mmol) was placed in a round bottom flask equipped with a stir bar and blanked with argon. Dichloromethane (15 mL) was added. While stirring, N,N-Diisopropylethylamine (3 mL, 17.2 mmol) was added. The solution was stirred overnight at room temperature. The solution was concentrated in vacuo. The solution was diluted with 25 mL of DCM and washed with water (3 × 100 mL). The organic layer was collected, dried over Na_2SO_4, and concentrated in vacuo. Purification using flash chromatography (stationary phase: silica gel, eluent gradient: from 100% hexanes to 100% ethyl acetate) afforded 473 mg (1.16 mmol, 26%). 4-nitrophenyl 4-(2-phenylacetamido)benzyl carbonate (400 mg, 0.98 mmol) and 4-mercaptobenzoic acid (152 mg, 0.96 mmol) was placed on a round bottom flask equipped with a stir bar and blanked with argon. Dichloromethane (10 mL) was added. While stirring, N,N-Diisopropylethylamine (1 mL, 5.74 mmol) was added. The solution was stirred overnight at room temperature. The solution was concentrated in vacuo. The solution was diluted with 25 mL of DCM and washed with water (3 × 100 mL). The organic layer was collected, dried over Na_2SO_4, and concentrated in vacuo. Purification using flash chromatography (stationary phase: silica gel, eluent gradient: from 99% hexanes/1% acetic acid to 99% ethyl acetate/1% acetic acid) afforded 70 mg (0.17 mmol, 18%) of a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 10.29 (s, 1H), 7.99 (d, *J* = 7.7 Hz, 2H), 7.68 (d, *J* = 7.9 Hz, 2H), 7.62 (d, *J* = 8.1 Hz, 2H), 7.37 – 7.29 (m, 6H), 7.25 (tt, *J* = 5.9, 3.2 Hz, 1H), 5.24 (s, 2H), 3.65 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm: 169.72, 167.91, 167.10, 140.05, 136.34, 134.80, 132.76, 132.27, 130.42, 129.99, 129.96, 129.56, 128.78, 127.02, 119.51, 69.94, 43.76, 40.60, 40.39, 40.18, 39.97, 39.76, 39.55, 39.34. HRMS (m/z): $[M + H]^+$ calculated for $C_{23}H_{20}NO_5S^+$, 422.1062; found 422.1055..

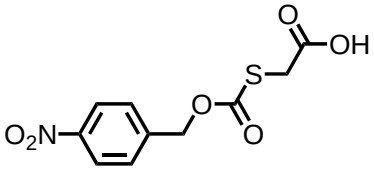


3-((((4-(2-phenylacetamido)benzyl)oxy)carbonyl)thio)propanoic acid (Figure S4). 4-nitrophenyl 4-(2-phenylacetamido)benzyl carbonate (302 mg, 0.74 mmol) and 3-mercaptopropanoic acid (503 μL, 5.79 mmol) was placed in a round bottom flask equipped with a stir bar and blanked with argon. Dichloromethane (10 mL) was added. While stirring, N,N-Diisopropylethylamine (1 mL, 5.74 mmol) was added. The solution was stirred overnight at room temperature. The solution was concentrated in vacuo. The solution was diluted with 25 mL of DCM and washed with water (3 × 100 mL). The organic layer was collected, dried over Na_2SO_4, and concentrated in vacuo. Purification using flash chromatography (stationary phase: silica gel, eluent gradient: from 99% hexanes/1% acetic acid to 99% ethyl acetate/1% acetic acid) afforded 115 mg (0.31 mmol, 42% yield) of a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 10.27

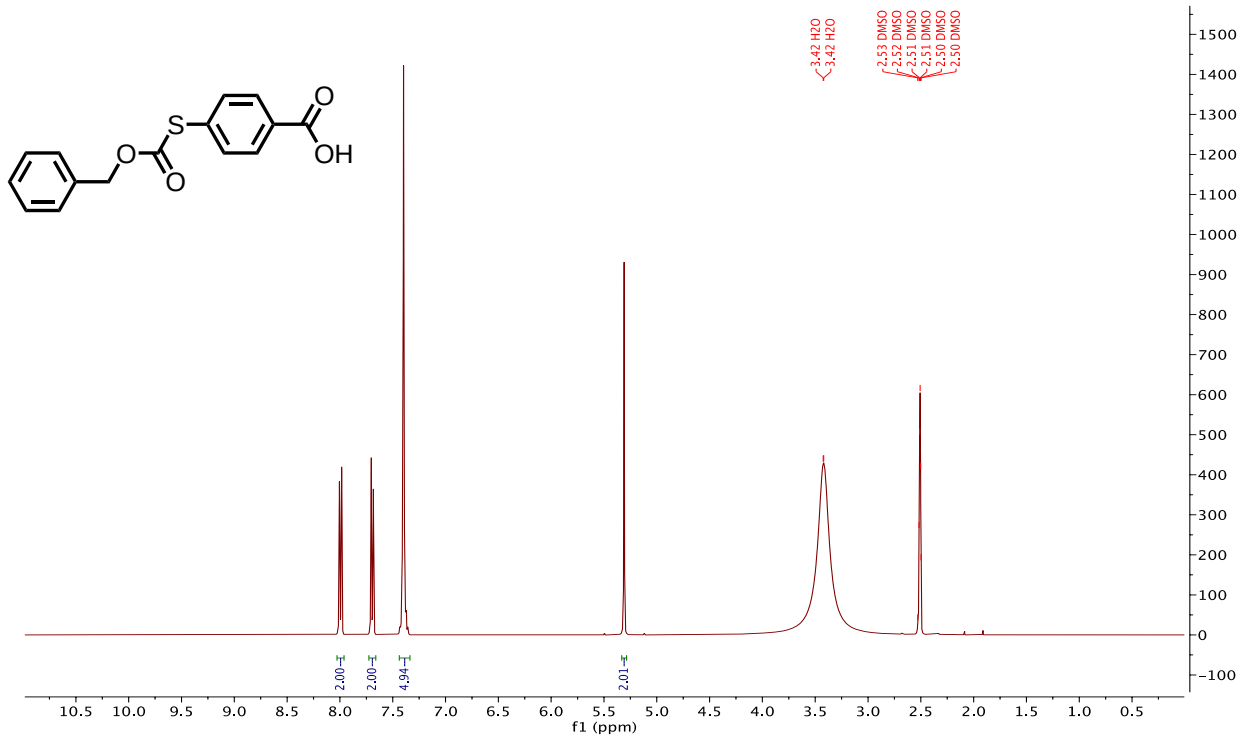
(s, 1H), 7.61 (dd, *J* = 8.6, 1.9 Hz, 2H), 7.37 – 7.31 (m, 5H), 7.31 – 7.20 (m, 2H), 5.18 (s, 2H), 3.65 (s, 2H), 3.00 (t, *J* = 6.8 Hz, 2H), 2.60 (t, *J* = 6.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ/ppm: 173.06, 170.39, 169.70, 139.92, 136.35, 130.30, 129.84, 129.56, 128.78, 127.02, 119.49, 69.04, 43.76, 40.58, 40.37, 40.16, 39.95, 39.74, 39.54, 39.33, 34.54, 26.44. HRMS (m/z): $[M - H]^-$ calculated for $C_{19}H_{18}NO_5S^-$, 372.0911; found 372.0914.



4-((((4-nitrobenzyl)oxy)carbonyl)thio)benzoic acid (Figure S5). 4-nitrobenzyl (4-nitrophenyl) carbonate (101 mg, 0.32 mmol) and 4-mercaptobenzoic acid (49 mg, 0.32 mmol) was placed in a round bottom flask equipped with a stir bar and blanked with argon. Dichloromethane (5 mL) was added. While stirring, N,N-Diisopropylethylamine (1 mL, 5.74 mmol) was added. The solution was stirred overnight at room temperature. The solution was concentrated in vacuo. Water (5 mL) was added followed by 1M HCl (2 mL) to crash out the compound. The crude compound was then collected via vacuum filtration. DCM (5 mL) was then added to the crude product and sonicated. The compound was then filtered to afford 70 mg (0.21 mmol, 66%) of an off white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, *J* = 8.4 Hz, 2H), 8.01 (d, *J* = 8.0 Hz, 2H), 7.71 (d, *J* = 8.0 Hz, 2H), 7.66 (d, *J* = 8.4 Hz, 2H), 5.46 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.05, 167.08, 147.85, 143.03, 134.87, 132.45, 132.37, 130.49, 129.50, 124.18, 68.56. HRMS (m/z): $[M - H]^-$ calculated for $C_{15}H_{10}NO_6S^-$, 332.0234; found 332.0235.



2-((((4-nitrobenzyl)oxy)carbonyl)thio)acetic acid (Figure S6). 4-nitrobenzyl (4-nitrophenyl) carbonate (103 mg, 0.33 mmol) and 4-mercaptoacetic acid (22.3 μL, 0.33 mmol) was placed in a round bottom flask equipped with a stir bar and blanked with argon. Dichloromethane (5 mL) was added. While stirring, sodium tert-butoxide (75 mg, 0.78 mmol) was added. The solution was stirred overnight at room temperature. The solution was concentrated in vacuo. Water (5 mL) was added followed by 1M HCl (2 mL) to crash out the compound. The compound was then collected via vacuum filtration to afford 29 mg (0.11 mmol, 33%) of an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (m, 2H), 7.66 (m, 2H), 5.42 (s, 2H), 3.77 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.86, 169.82, 147.83, 143.28, 129.39, 124.14, 68.00, 33.68. HRMS (m/z): $[M - H]^-$ calculated for $C_{10}H_8NO_6S^-$, 270.0078; found 270.0079.



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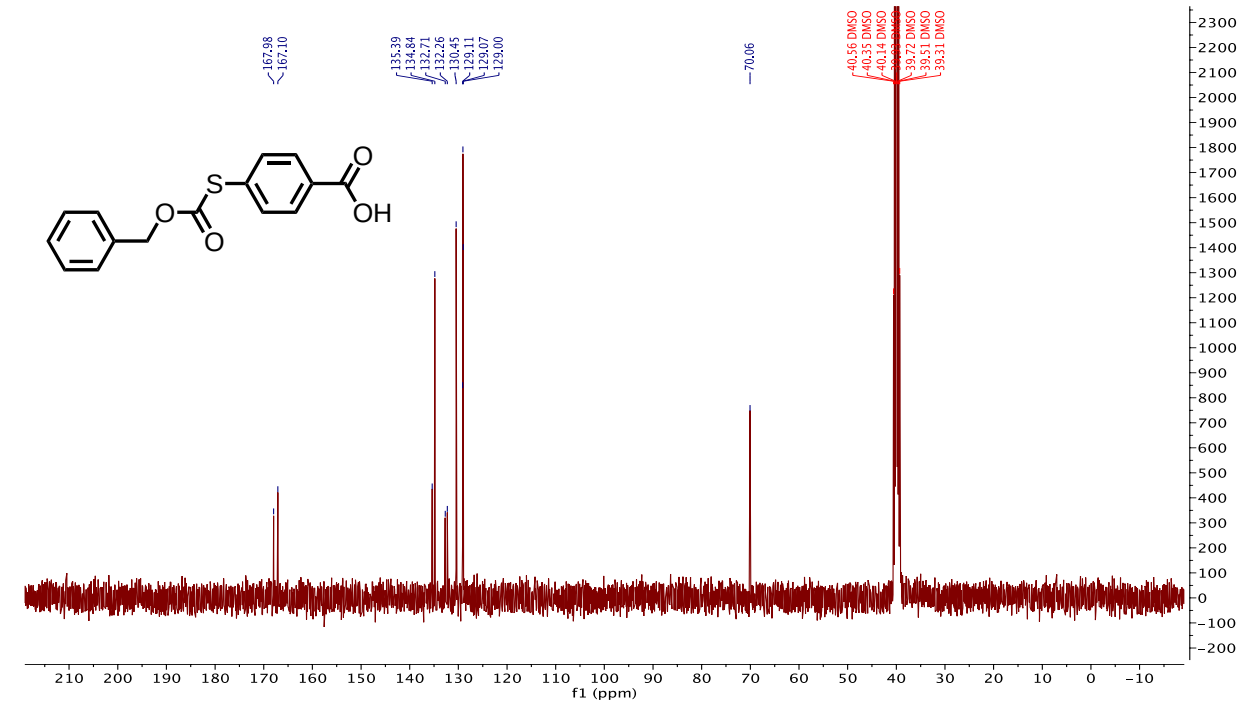
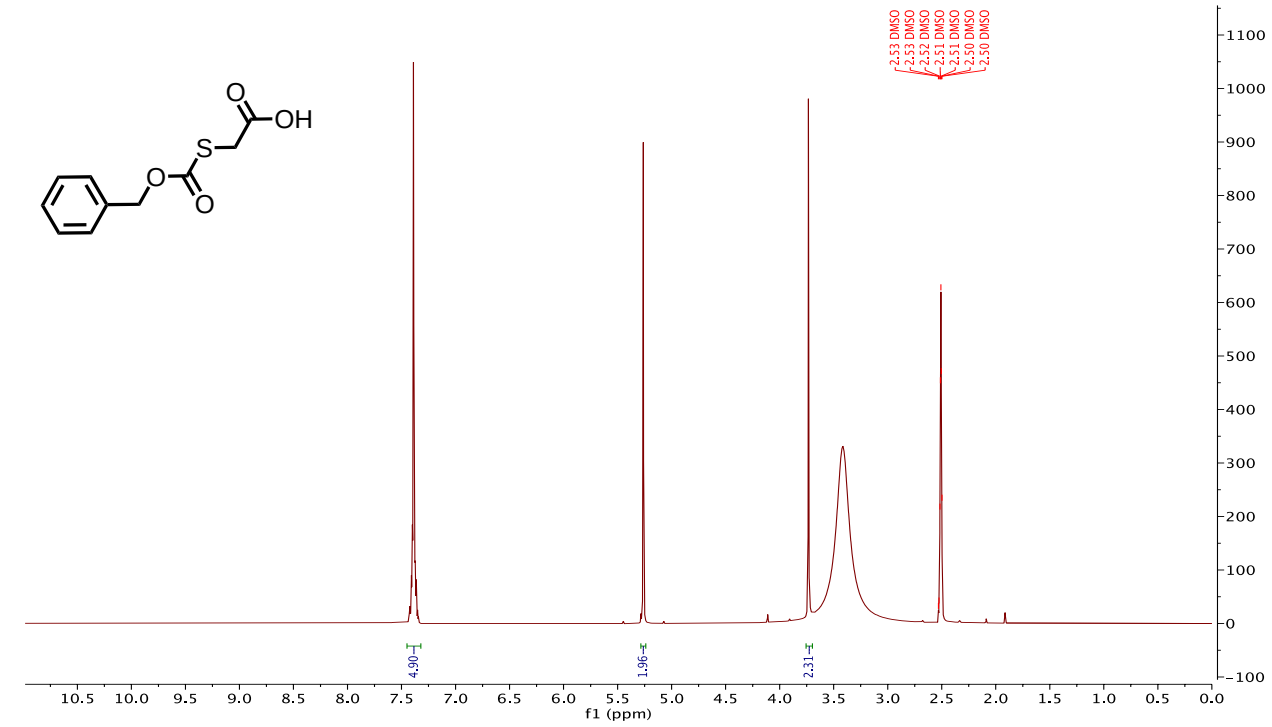


b

Figure S1. (a) ^{1}H NMR of **4-(((benzyloxy)carbonyl)thio)benzoic acid** (400 MHz, DMSO); (b) ^{13}C NMR (101 MHz, DMSO).



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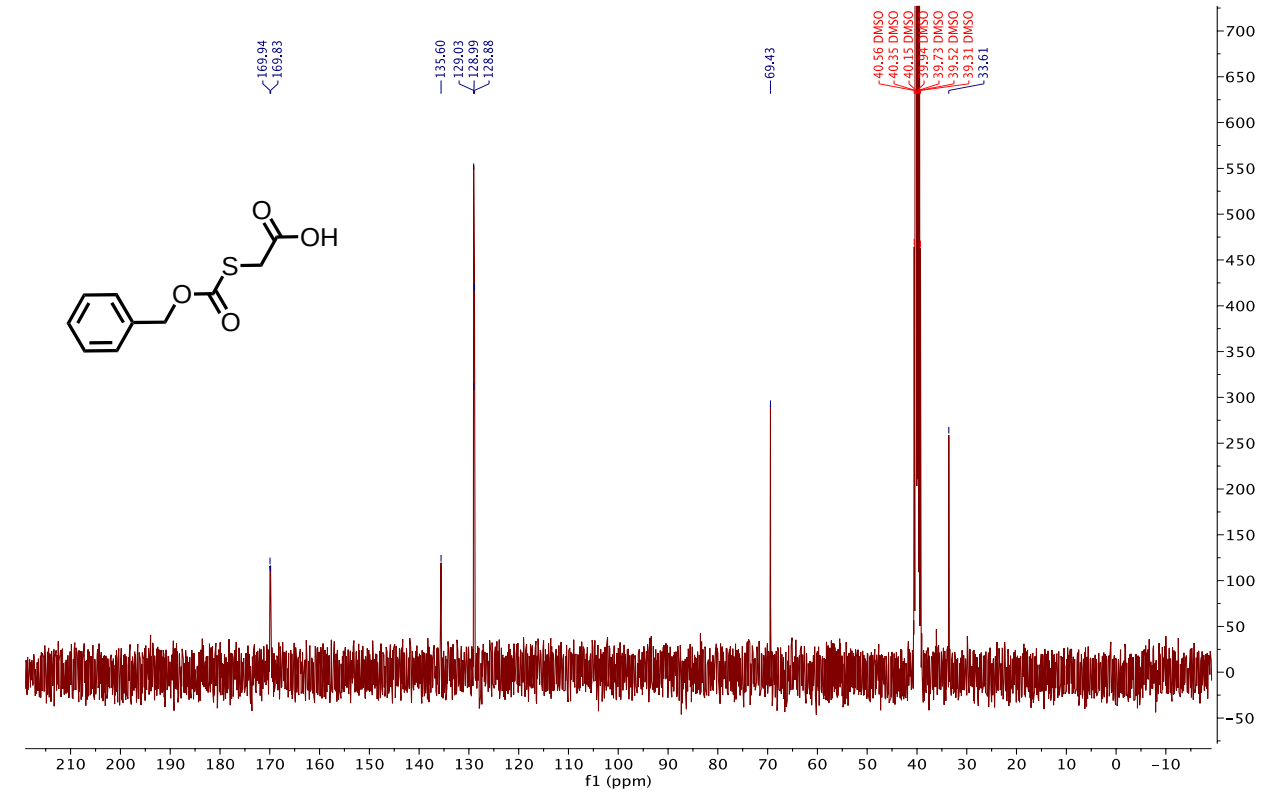

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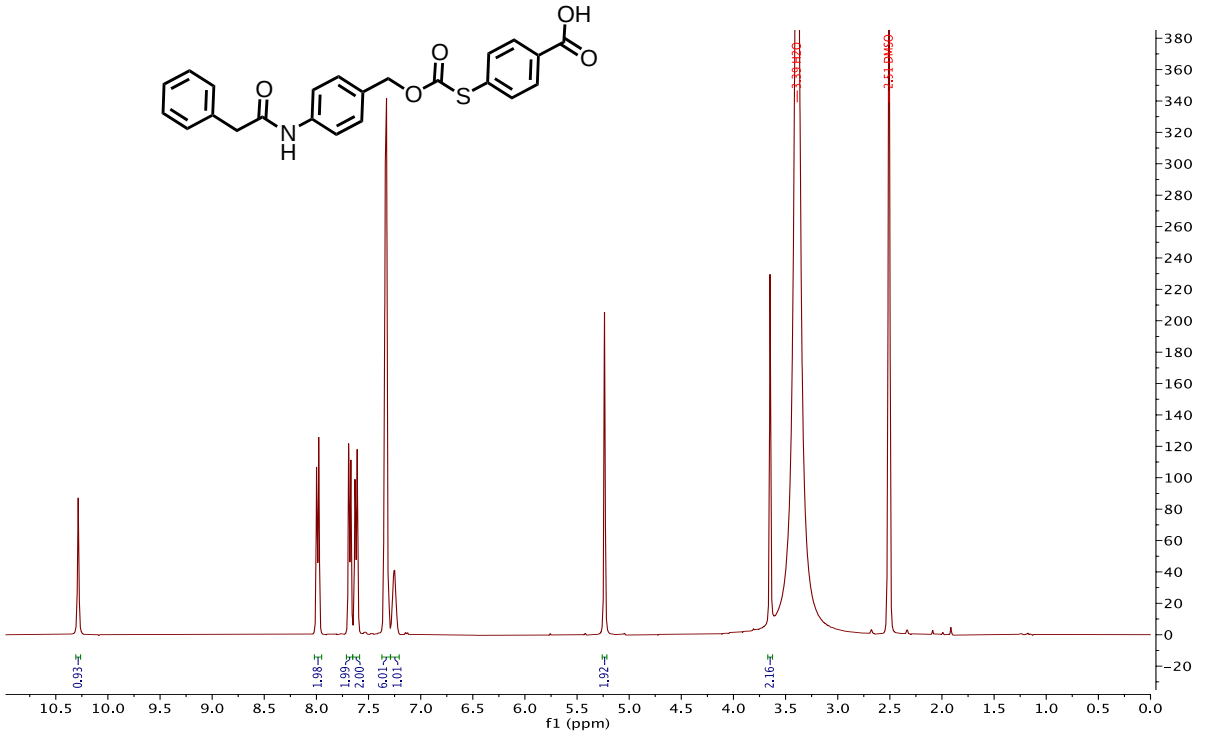
Figure S2. (a) ^{1}H NMR of **2-(((benzyloxy)carbonyl)thio)acetic acid** (400 MHz, DMSO); (b) ^{13}C NMR (101 MHz, DMSO).



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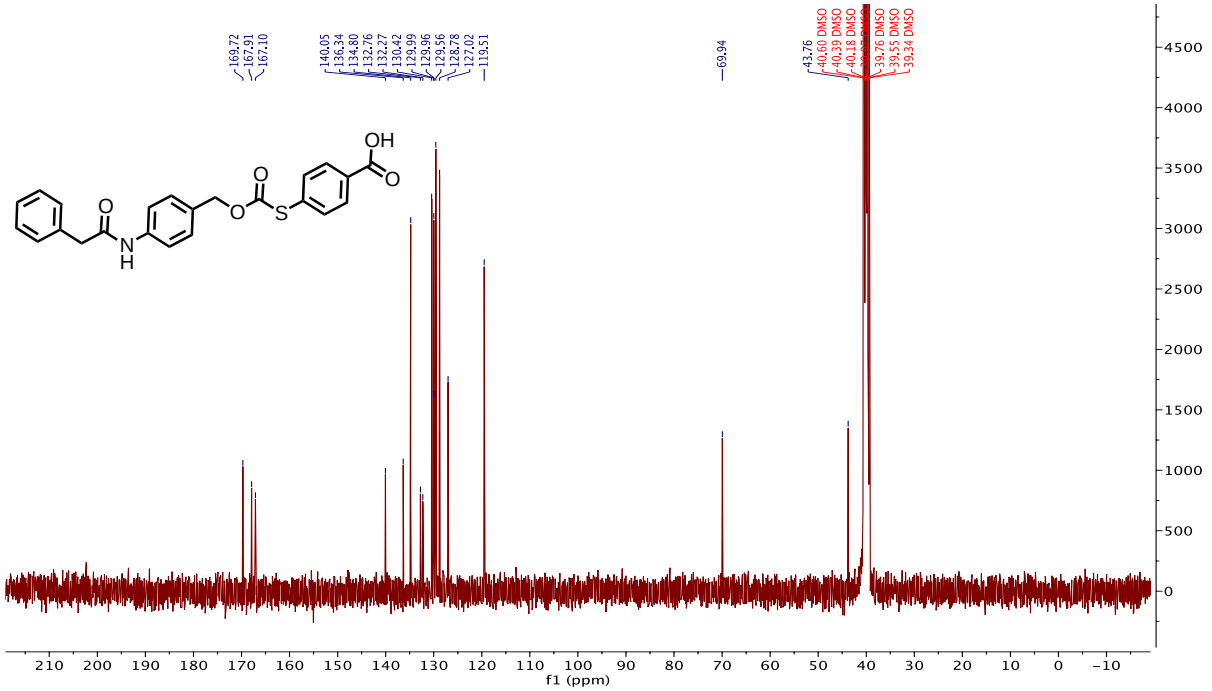
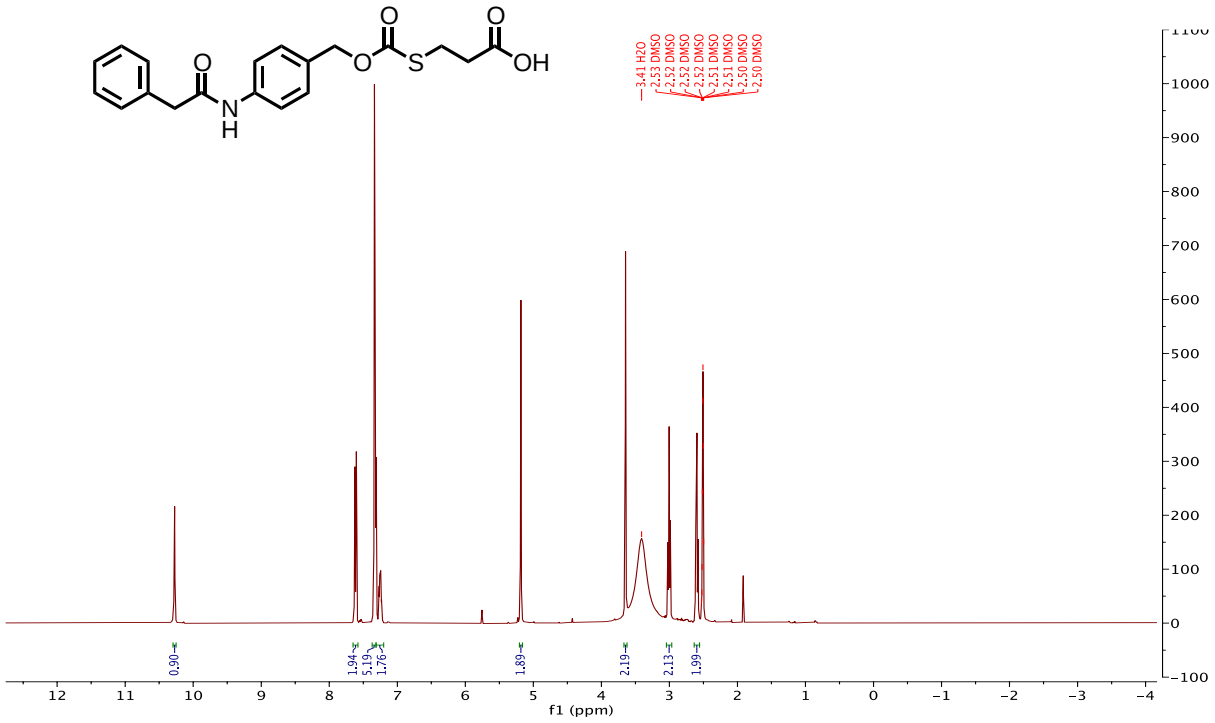


b

Figure S3. (a) ^{1}H NMR of **4-((((4-(2-phenylacetamido)benzyl)oxy)carbonyl)thio)benzoic acid** (400 MHz, DMSO); (b) ^{13}C NMR (101 MHz, DMSO).



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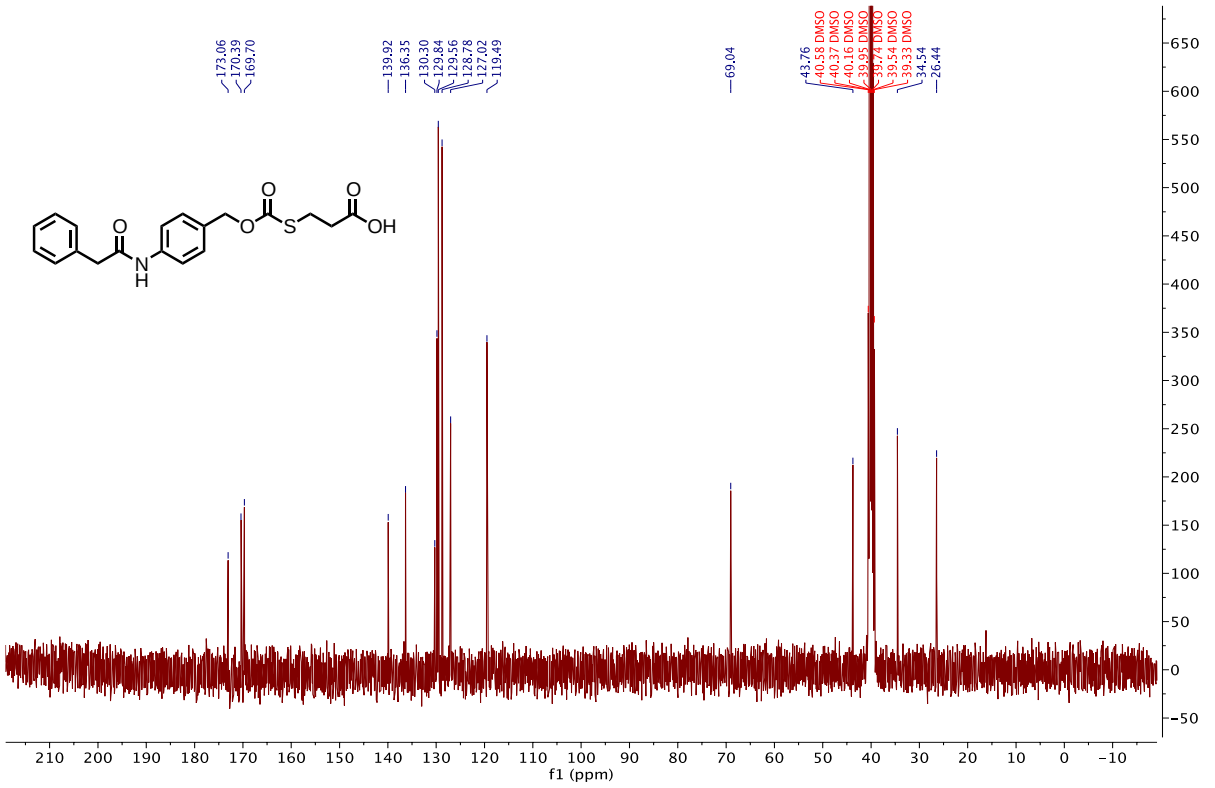
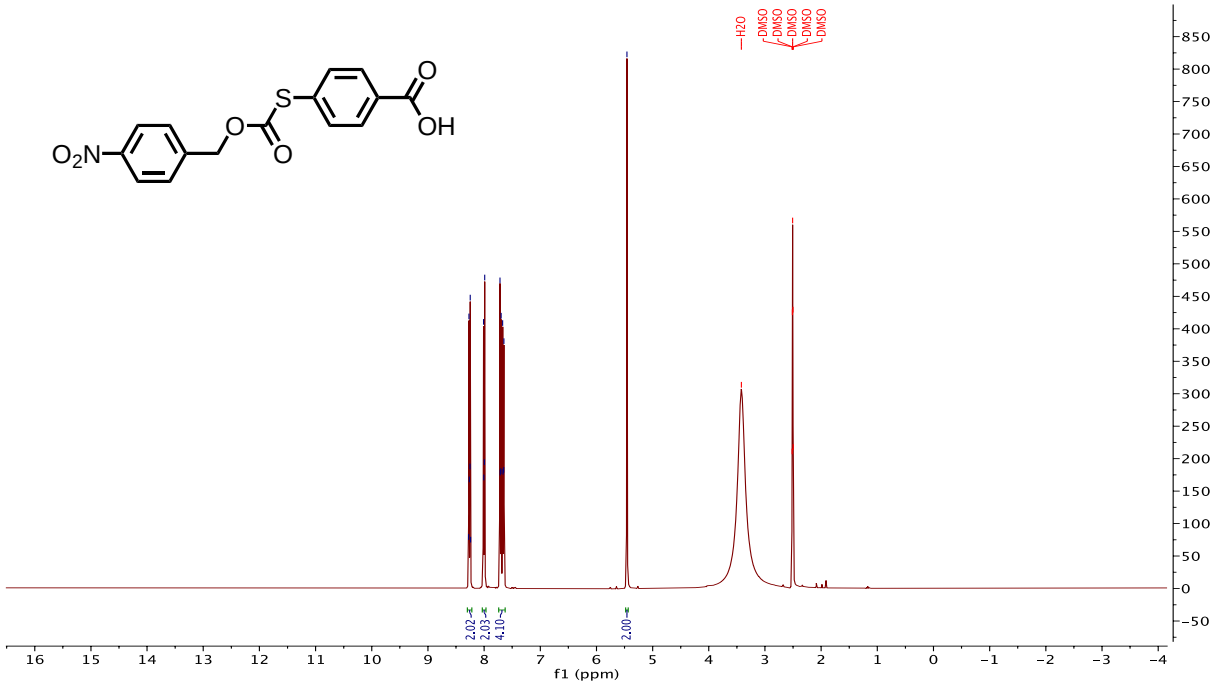


b

Figure S4. (a) ^{1}H NMR of **3-((((4-(2-phenylacetamido)benzyl)oxy)carbonyl)thio)propanoic acid** (400 MHz, DMSO); (b) ^{13}C NMR (101 MHz, DMSO).



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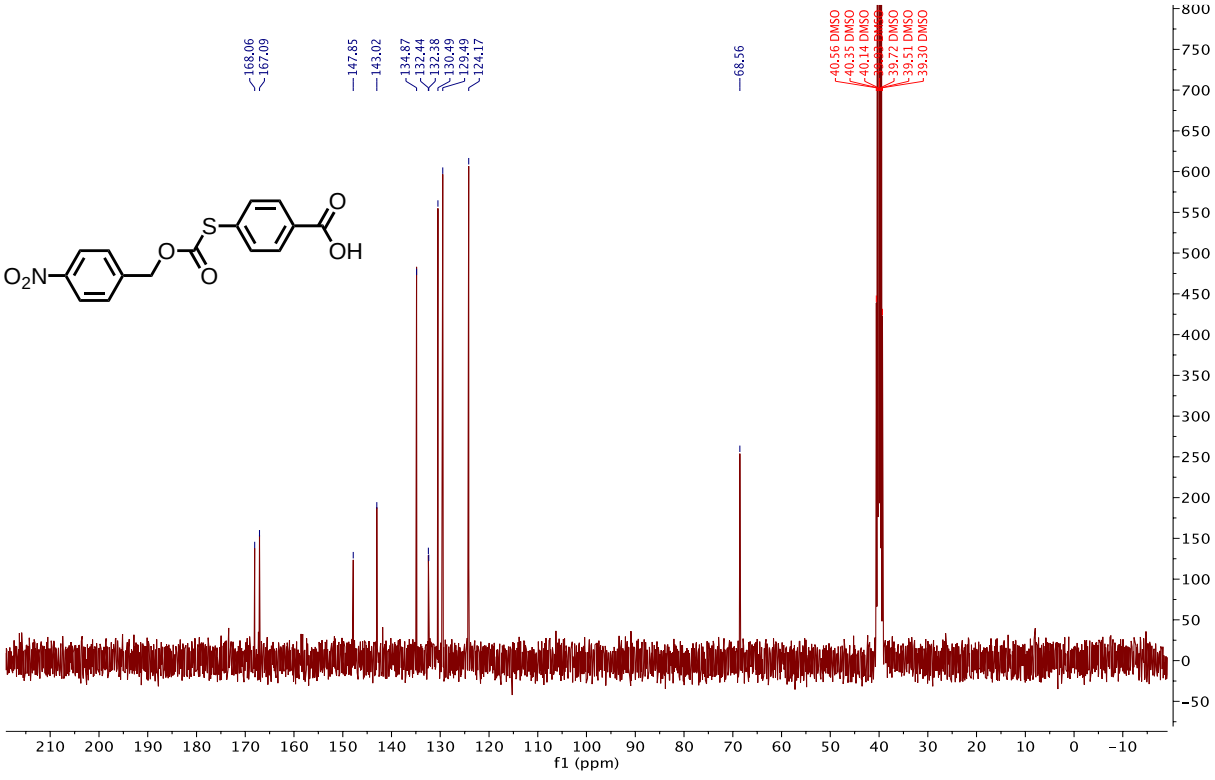

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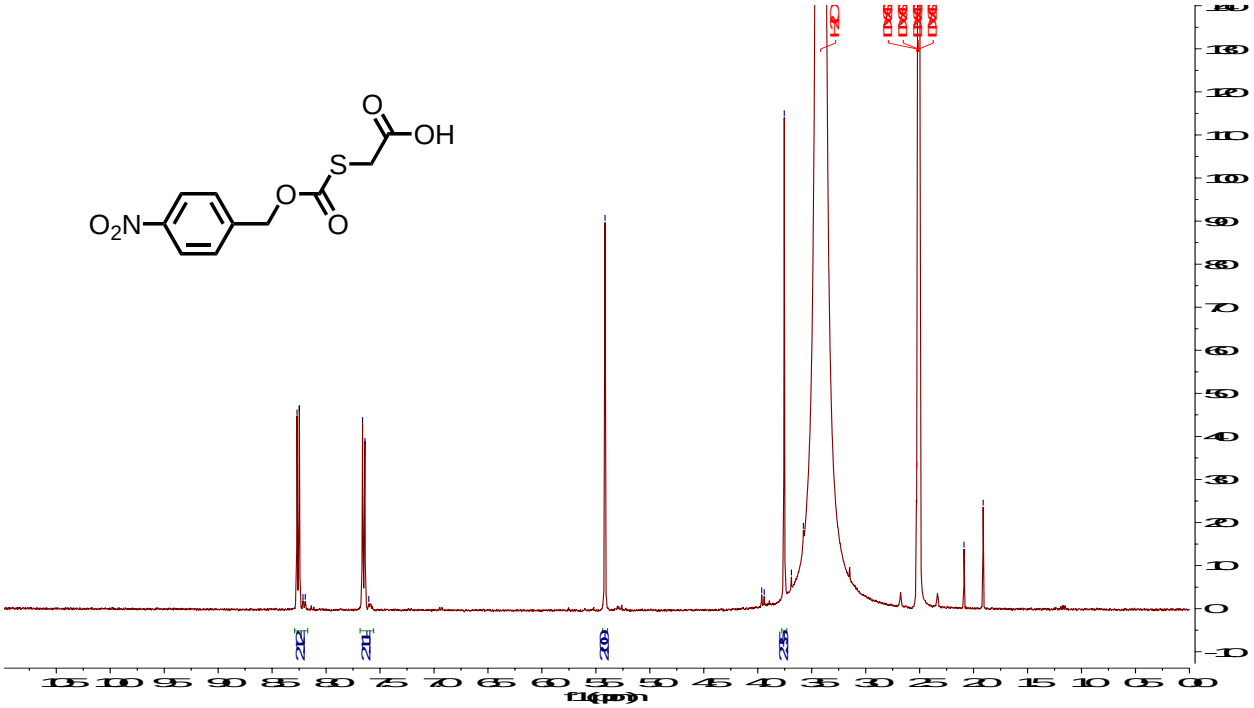
Figure S5. (a) ^{1}H NMR of **4-((((4-nitrobenzyl)oxy)carbonyl)thio)benzoic acid** (400 MHz, DMSO); (b) ^{13}C NMR (101 MHz, DMSO).



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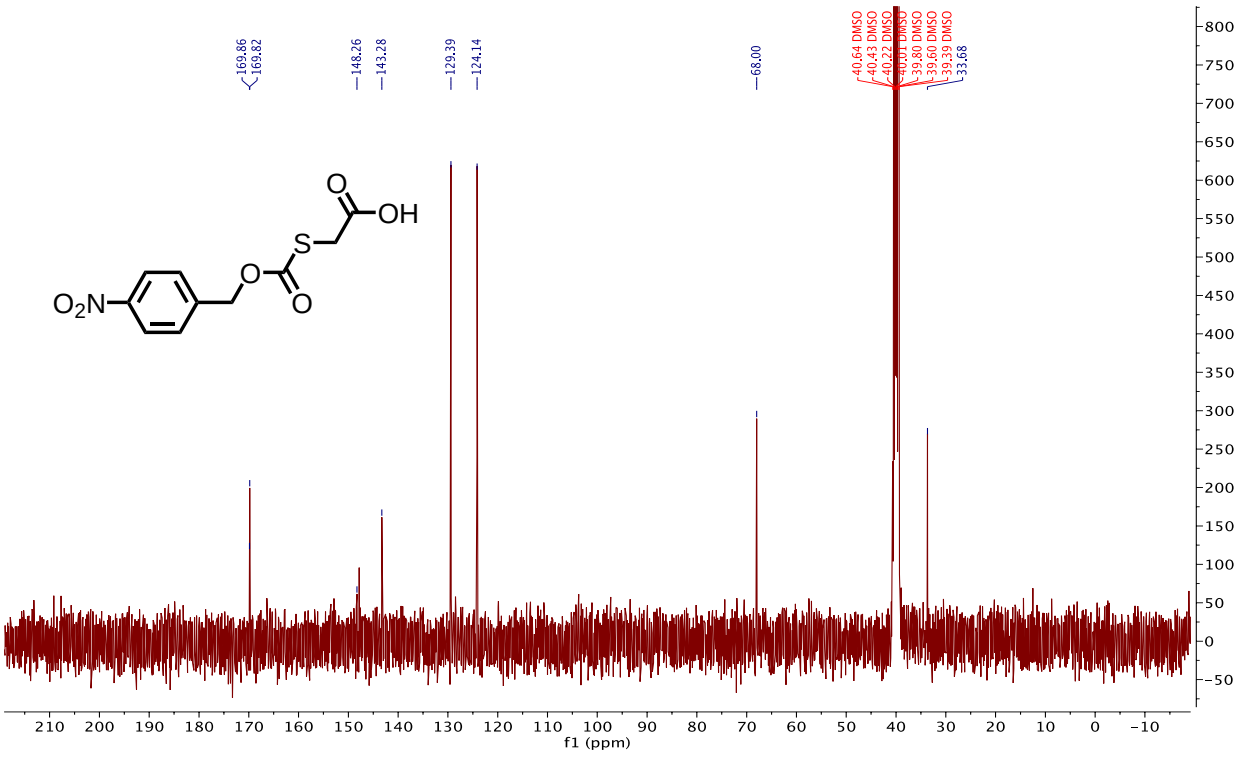


b

Figure S6. (a) ^{1}H NMR of **2-((((4-nitrobenzyl)oxy)carbonyl)thio)acetic acid** (400 MHz, DMSO); (b) ^{13}C NMR (101 MHz, DMSO).

Detection of enzyme via Ellman's Reagent. Thiol detection was performed using Ellman's reagent or (5,5'-dithio-bis-[2-nitrobenzoic acid]) (DTNB). For detection of **PGA** (Sigma Aldrich), a solution of 99 μL (500 μM) of either aromatic or alkyl thiol probe and 1 μL (2mM) of **PGA** enzyme was prepared and mixed in a 96-well plate. To this solution 22.5 μL (0.1 mM) of DTNB was added and thiol generation was monitored at 406 nm at 37°C every 10 minutes.

Detection of enzyme via DETECT. Thiol detection was performed using the Thiol and Sulfide Detection Kit (Cat. No. T-6060, Invitrogen, Carlsbad, CA) which utilizes a thiol-activatable caged papain (papain-SS-CH3) in conjunction with the papain probe, *N*α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA) to give a thiol-induced chromogenic readout. The papain-SS-CH3 and BAPA solutions were prepared according to the standard kit protocol. Thiol generation was measured using a modified protocol. For the detection of **NTR** (Abcam), a solution of 45 μL papain-SS-CH3 (0.6 mg/mL), 45 μL BAPA (4.9 mM), 4 μL of NAD(P)H (120 μM) and 5 μL of alkyl thiol probe was prepared and mixed in a 96-well plate. To this solution 1 μL of either 20.8, 15.6, 10.4 or 5.4 μM of **NTR** enzyme was then added and thiol generation was monitored at 405 nm at 37°C every 10 minutes.

For the detection of **PGA** (Sigma Aldrich), a solution of 47 μL papain-SS-CH3 (0.6 mg/mL), 47 μL BAPA (4.9mM), and 5 μL of alkyl thiol probe (500 μM) was prepared and mixed in a 96-well plate. To this solution 1 μL of either 1600, 160 or 16 μM of **PGA** enzyme was then added and thiol generation was monitored at 405 nm at 37°C every 10 minutes.

Detection of side products of thiocarbonates via Liquid Chromatography-Mass Spectrometry. For detection of side products from the thiocarbonate probes, probe **1** and **3** were used (Figure S7 and S8). A solution of 495 μL (500 μM) of **3** and 5 μL (1.6mM) of **PGA** enzyme was prepared and mixed in a vial and incubated at 37°C for 1 hr. After 1 hr, the reaction was stored in dry ice and was analyzed using intact-mass LC-MS. LC-MS data revealed that after PGA enzymatic cleavage and a 1,6 elimination the short lived 4-methylenecyclohexa-2,5-dien-1-imine intermediate reacts with the 4-mercaptobenzoic acid to produce 4-((4-aminobenzyl)thio)benzoic acid. The majority of the produced 4-mercaptobenzoic acid either dimerized to produce 4,4'-disulfanediyldibenzoic acid or reacts with the starting material to produce 4-((4-(2-phenylacetamido)benzyl)thio)benzoic acid (Scheme 1). This finding reveals why DTNB detection of free thiol was less potent for the aromatic thiol carbonate compared to the alkylated thiol carbonate.

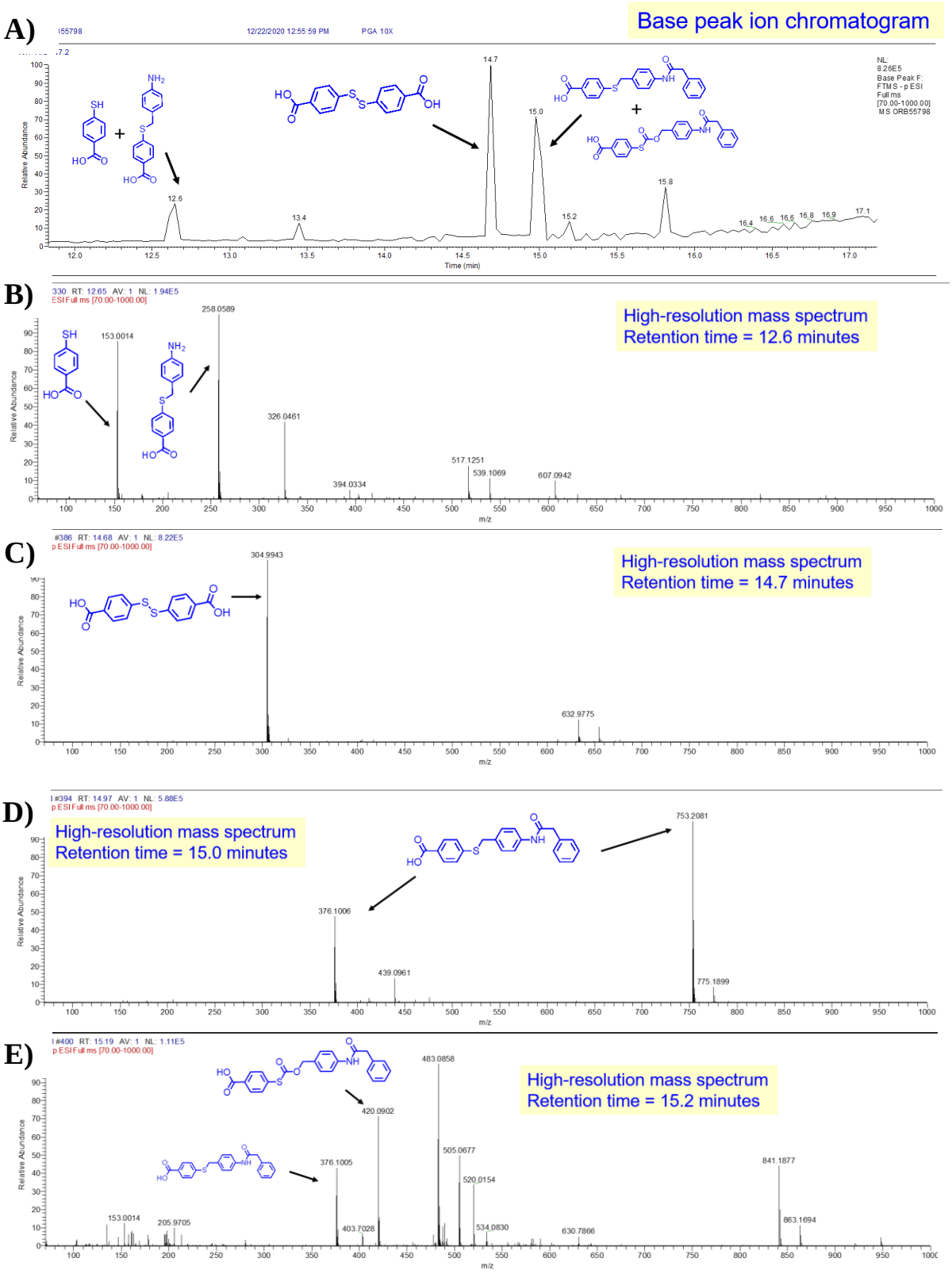


Figure S7. Liquid chromatography-mass spectrometry after 1 hr incubation of 404 μM PGA aromatic thiocarbonate probe in the presence of 16 μM PGA. A) Summary of all side products and unreacted SM and B-E) zoomed in mass spectra.

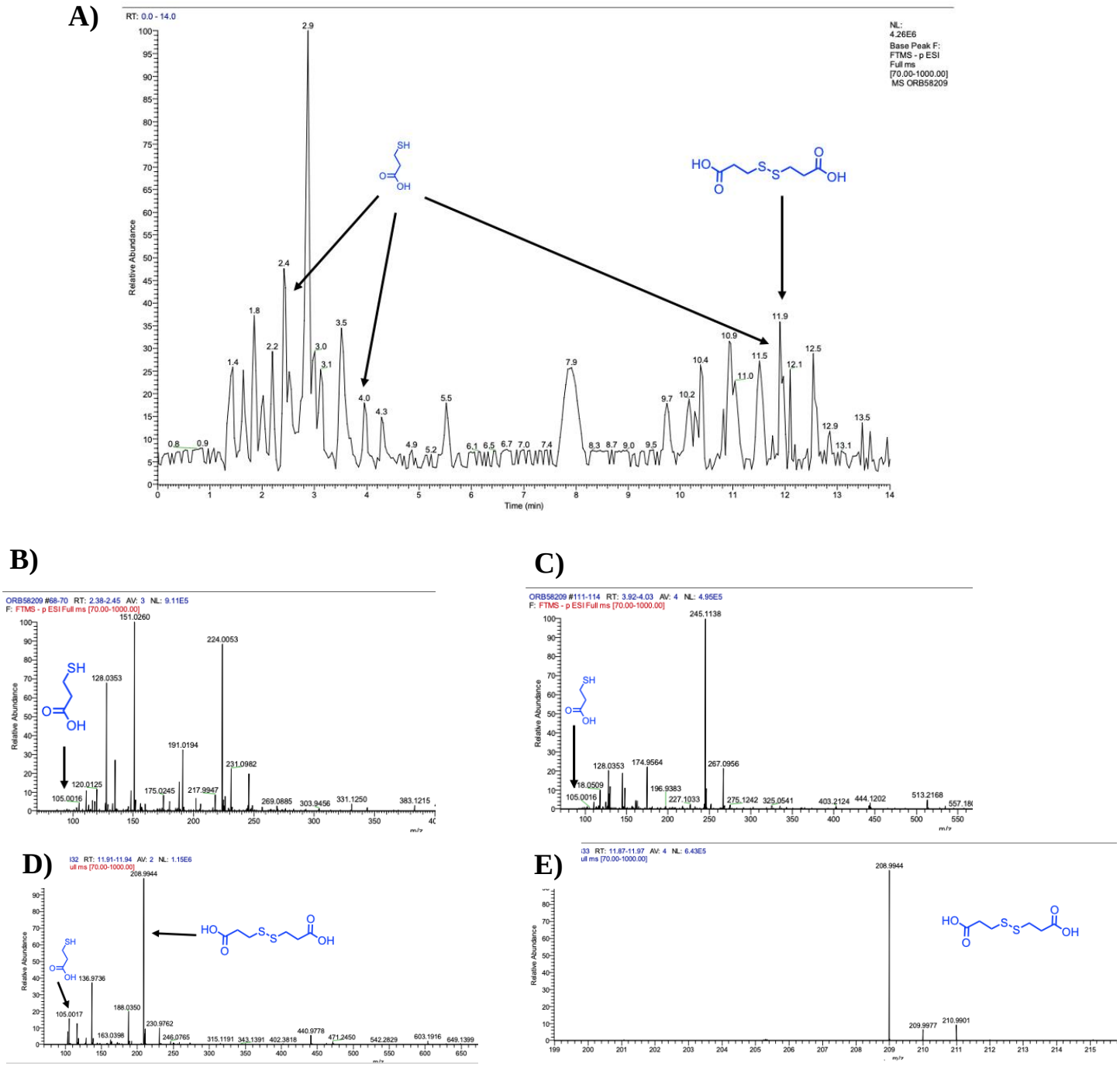
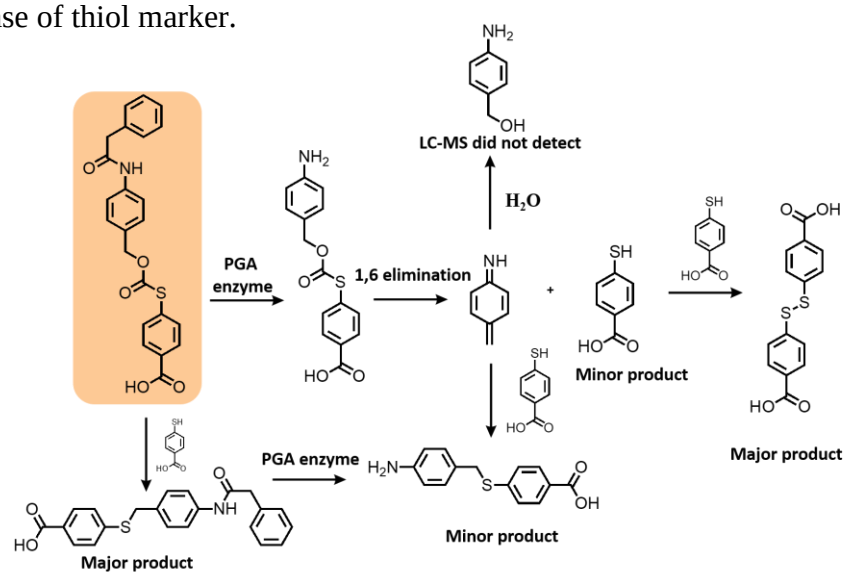


Figure S8. Liquid chromatography-mass spectrometry after 1 hr incubation of 404 μM PGA alkyl thiocarbonate probe in the presence of 16 μM PGA. A) Summary of all side products and unreacted SM and B-E) zoomed in mass spectra on relevant side products.

Scheme S1. Outline of proposed side reactions after release of aromatic thiocarbonate resulting in the decrease of thiol marker.



***in Situ* NTR assay of commercial fluorogenic probe.** A commercially available fluorogenic coumarin turn-on probe which has previously been used to detect *in vitro* NTR[1] was used for the detection of *in situ* NTR. A solution of 94 µL coumarin probe (0.076 mg/mL), 4 µL of NAD(P)H (0.1 mg/mL) was prepared and mixed in a 96-well plate. To this solution 2 µL of either 10, 7.5, 5, 2.5 µM of NTR enzyme was then added and fluorescent probe readout was measured at 440nm with an excitation wavelength of 365nm (Figure S9).

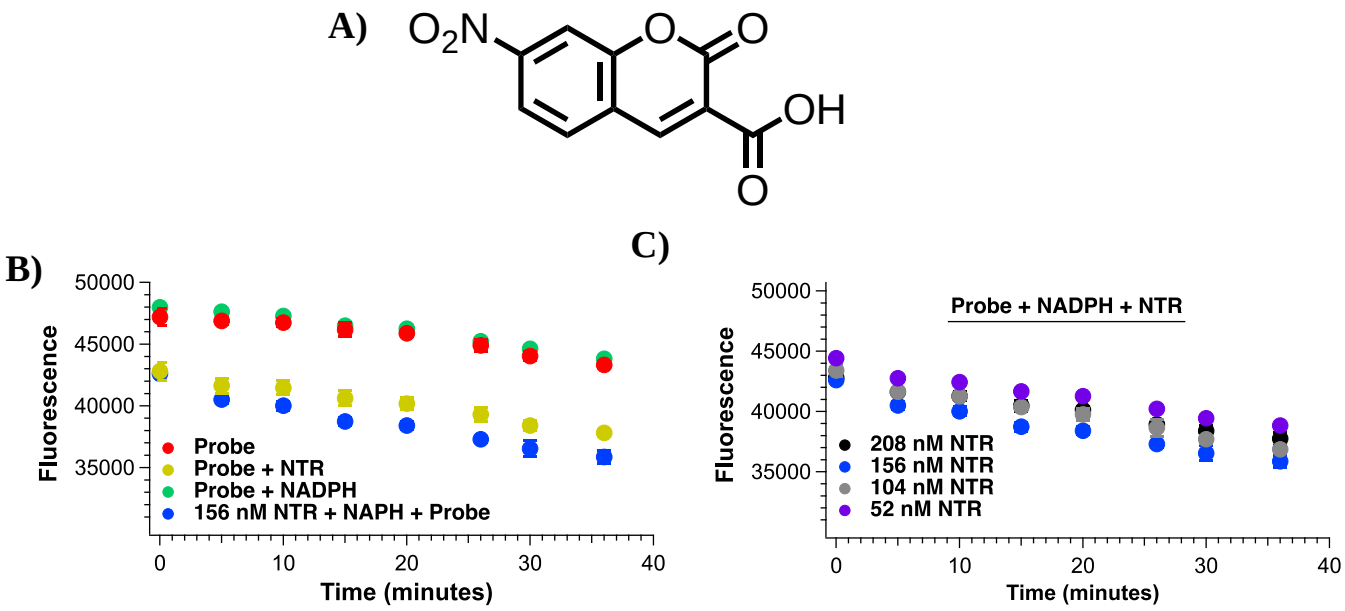


Figure S9. A) Commercially available fluorogenic probe previously used to detect the presence of NTR *in vivo*.[1] B, C) In situ assay comparing efficacy of commercially available fluorogenic probe showing 1) autofluorescence at start of experiment and 2) decrease in fluorescence, rather than an increase in fluorescence, over time.

Caged papain assay with 3-mercaptopropionic acid. 5 µL of 3-mercaptopropionic acid (500 µM, 50 µM and 5 µM) was added to a working solution of caged papain (0.3 mg/mL) and BAPA (2.45mM) to final thiol concentrations of 25 µM, 2.5 µM and 0.25 µM. The absorbance was monitored at 405 nm over 90 minutes at 37 °C.

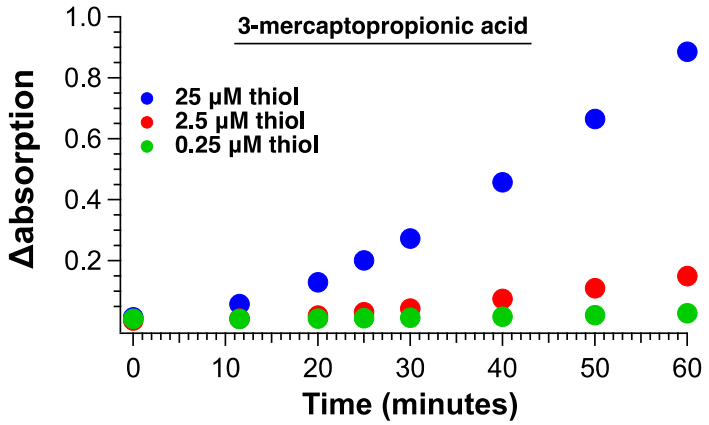


Figure S10. *in situ* activation of DETECT via standard addition of 3-mercaptobenzoic acid.

PGA detection in the presence of GSH. To a 96-well plate was added 1µL of PGA (1.6mM, 0.16mM, 0.016mM, or 0 mM), 5µL of **probe 3** (1mM), and 1µL of glutathione (GSH) (5mM, 2.5mM, 0.5mM, or 0mM), followed by the addition of 93µL of a working solution of caged papain (0.3mg/mL) and BAPA (2.45mM). The absorbance was monitored at 405 nm over 90 minutes at 37°C.

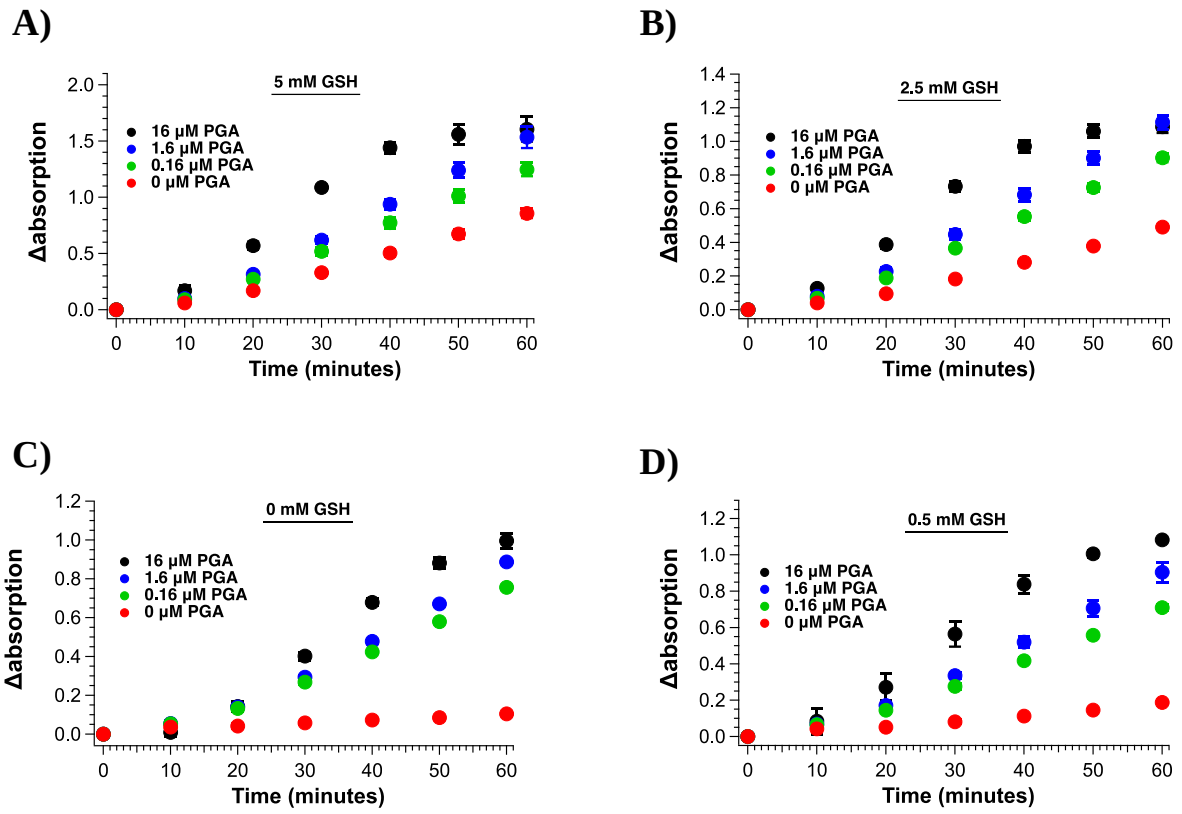


Figure S11. *in situ* detection of PGA in the presence of various concentration of GSH a) 5 mM, b) 2.5 mM, c) 0.5 mM and d) 0 mM. DETECT is able to detect the presence of various concentration of PGA.

PGA detection in biological medium. To assess the ability of the PGA probe to detect PGA in complex biological media, 1 µL of HEK 293 cell lysate (1mg/mL total protein content) were spiked with 1µL of various amounts of PGA (160µM, 16µM, 1.6µM, 160nM, 16nM, 1.6nM, 0.16nM, and 0nM) in a 96-well plate. To these wells were then added 5µL of **probe 3** and 1µL of a protease inhibitor cocktail (Protease Inhibitor Cocktail Set I, Calbiochem - MilliporeSigma, Burlington, MA, USA). The mixture was incubated at 37°C for 10 minutes before the addition of 92µL of a working solution of caged papain (0.3mg/mL) and BAPA (2.45mM). The absorbance was monitored at 405 nm over 90 minutes at 37°C. BCA assays were performed to accurately determine protein content of the cell lysate as well as PGA concentrations.

References

1 A. L. James, J. D. Perry, C. Jay, D. Monget, J. W. Rasburn and F. K. Gould, *Lett. Appl. Microbiol.*, 2001, **33**, 403–408.