Biosynthesis of Branched Alkoxy Groups: Iterative Methyl Group Alkylation by a Cobalamin-Dependent Radical SAM Enzyme

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Supporting Information

Materials and Abbreviations:

Methylcobalamin (MeCbl), 5′-deoxyadenosine (5′-dA), methyl viologen (MV), nicotinamide adenine dinucleotide phosphate hydrate (NADPH), isopropyl β-D-1-thiogalactopyranoside (IPTG), 4-(2-hydroxyethyl)-1-piperazineethane- sulfonic acid (HEPES), S-adenosylhomocysteine (SAH), tris(hydroxymethyl)aminomethane hydrochloride (Tris•HCl), and other reagents were purchased from commercial sources unless otherwise noted. LB medium (Miller), HPLC solvents and LCMS solvents were purchased from EMD chemicals (Philadelphia, PA). Solvents used for HPLC analysis were of HPLC grade, while solvents used for LCMS analysis were of LCMS grade and were used without further purification. HisTrap-HP columns were purchased from GE Healthcare (Piscataway, NJ). Amicon Ultra centrifugal filter units were purchased form Merck-Millipore (Darmstadt, Germany). P-10DG desalting columns were purchased from BioRad (Hercules, CA). Kanamycin and IPTG were obtained from Lab Scientific Inc. Chloramphenicol was from Fisher Scientific. [^{13}C-methyl]-*L*-methionine was purchased from Campro Scientific. M medium (10 g/L peptone (Bacto), 10 g/L maltose, 1 g/L $CaCl_2 \cdot 2H_2O$, 1 g/L $MgSO_4 \cdot 7H_2O$, 8 mg/L Fe-EDTA, 50 mM HEPES, adjusted to pH 7.4 with 10 N KOH). XAD-7 was purchased from Sigma-Aldrich.

^{13}C labeled methionine feeding and metabolite LC-ESI-MS analysis:

25 mL M-medium was inoculated with 1mL of a high density overnight culture of Cbv34. 0.5 mL of XAD-7 was added on day 1 and 12.5 mg of [^{13}C-methyl]-*L*-methionine was fed over 4 days. On day 5 the cultures were centrifuged and the pellet and XAD were extracted with 2×10 mL methanol. The solvent was removed through rotary evaporation and the extract was dissolved in 2 mL methanol.

LC-ESI- MS was performed using a Thermo Scientific Dionex Ultimate 3000 UHPLC system coupled with a Bruker Amazon speed iontrap mass spectrometer using an ESI in positive mode. LC was performed on a Waters Acquity UPLC BEH C18 50 mm 1.7 µm column.

LC conditions:

A-Water + 0.1% formic acid

B-Acetonitrile + 0.1% formic acid

0min – 95% A 5% B, 0.5 min – 95% A 5% B, 9.5 min – 5% A 95% B, 10.5 min – 5% A 95% B, 10.8 min – 95% A 5% B, 11.8 min – 95% A 5% B.

Over-expression and purification of CysS:

The CysS gene[1] was cloned into the pET28b vector and was co-expressed with a plasmid encoding the *suf* operon in *E.coli* BL21 (DE3) for *in vivo* assembly of the [4Fe-4S] cluster. A 15 mL overnight culture was grown in LB medium in the presence of 40µg/mL kanamycin and 30µg/mL chloramphenicol. This was then added to 1.5 L of LB medium containing 40µg/mL kanamycin and 30µg/mL chloramphenicol. The cultures were incubated at 37°C with shaking (180 rpm) until the OD_{600} reached 0.45. The culture was then incubated at 4°C without shaking for 2 hrs. Then ferrous ammonium sulfate 120 mg and cysteine 120 mg were added. This was followed by induction of the culture with 100 µM IPTG. The culture was then incubated at 15°C with shaking (110 rpm) overnight. The cells were harvested by centrifugation and stored in liquid nitrogen until further use. All steps for protein purification were carried out in an anaerobic chamber (COY laboratories). Cell pellets were thawed and resuspended in lysis buffer (100 mM Tris-HCl, pH 7.5) in the presence of lysozyme (0.2 mg/mL) and benzonase (100 units). This mixture was then cooled in an ice-bath with continuous stirring for 1-2 hrs. The suspension was further sonicated to lyse the cells. Cell debris was removed by centrifugation and the lysate was loaded onto a Histrap column pre-equilibrated with lysis buffer. The column was washed with 5-10 column volumes of wash buffer (100 mM Tris-HCl, 30 mM Imidazole, pH 7.5). The protein was then eluted using elution buffer (100 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 7.5). Brown colored fractions containing the desired protein were pooled and buffer exchanged to final buffer (100 mM Tris-HCl, pH 7.5 at 25 °C, 30% glycerol) via Econo-Pac 10DG size exclusion chromatography. The purified enzyme was stored in liquid nitrogen. Protein concentration was measured by the absorbance at 280 nm (A280) with an extinction coefficient calculated using the ProtParam tool of the ExPASy proteomics server.

Iron quantification of CysS

Iron content was analyzed using the ferene assay.[2] The following reagents were prepared: A, 1.35 g SDS, 30 mL water, 450 µL saturated sodium acetate; B, 540 mg L-ascorbic acid, 18 mg sodium metabisulfite, 11.2 mL water, 800 µL saturated sodium acetate; C, 18 mg ferene in 1 mL water. To 300 µL of assay solution containing CysS, 300 µL of 8M guanidine hydrochloride, 300 µL Reagent A, 300 µL Reagent B were added. After mixing by inversion, the samples were incubated for 15 min at 37 °C and then 15 µL of Reagent C was added. The samples were incubated for 5 min at 25 °C, and centrifuged for 5 min at 25 °C. The absorbance at 593 nm of the supernatant was recorded using a Varian Cary Bio 300 UV-Visible Spectrophotometer. The iron content was finally determined by comparing the reading to a standard curve that was generated under identical conditions using $FeCl_3$ in 2% HNO_3 with a concentration range from 0 to 200 µM.

Sulfide quantification of CysS

Sulfide content was analyzed using the methylene blue assay.[3] To 300 µL of assay solution containing CysS, 1 ml of 1% (w/v) zinc acetate was added followed by 50 µL of 3 M NaOH. This mixture was agitated gently and 250 µL of 0.1 % N,N-dimethyl-p-phenylenediamine (DMPD) monohydrochloride in 5 M HCl and 50 µL of 23 mM $FeCl_3$ in 1.2 M HCl were added. The resulting solution was mixed vigorously for 30 mins. The samples were then centrifuged for 10 min at 25 °C. The supernatant was collected and the absorbance at 670 nm was recorded using a Varian Cary Bio 300 UV-Visible Spectrophotometer. The sulfide content was determined by comparing this reading to a standard curve that was generated under identical conditions using a fresh solution of sodium sulfide (Na_2S; Fisher Scientific) in 0.1 M NaOH with a concentration range from 0 to 100 µM.

***In vitro* reconstitution assay with CysS:**

All CysS enzymatic reactions were carried out in an anaerobic chamber containing 95% nitrogen and 5% hydrogen. A typical enzymatic reaction was performed in 100 mM phosphate buffer, pH 7.5 containing 55 µM of CysS with 0.75 mM MeCbl, 2.5 mM SAM, 0.9 mM substrate with different reducing systems, flavodoxin, flavodoxin reductase system (25 µM FldA, FPR; 50 µM FAD, FMN; 3 mM NADPH); 1 mM methyl viologen, 4 mM NADPH; 10 mM dithionite, 10 mM dithiothreitol (DTT); 1 mM methyl viologen, 4 mM NADPH, 50

mM DTT; 1 mM methyl viologen, 4 mM NADPH, 50 mM DTT. The reaction was incubated at room temperature for 12-15 hours. For LC-MS analysis the enzyme was removed by ultrafiltration using a 10kDa cut-off filter (VWR).

LC-MS parameters for the analysis of the CysS reaction mixture

LC-ESI-TOF-MS was performed using an Agilent 1260 HPLC system equipped with a binary pump and a 1200 series diode array detector followed by a MicroToF-Q II mass spectrometer (Bruker Daltonics) using an ESI source in positive mode. Analysis was performed on a LC-18-T column (15 cm x 3 mm, 3 μm particles, Supelco).

LC conditions:

A- 5 mM Ammonium acetate buffer, pH 6.6

B- 75 % Methanol and 25 % Water.

LC method:

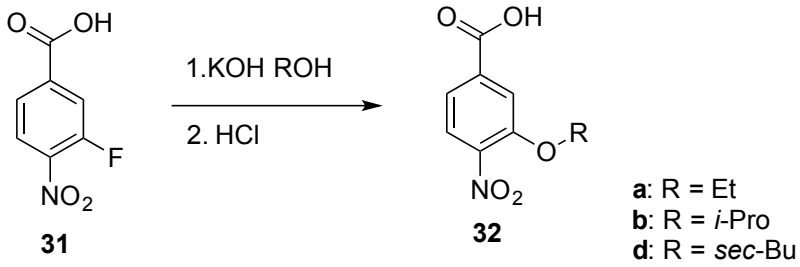
0min – 100% A, 2 min – 100% A, 4 min – 80% A 20% B, 27 min – 100% B, 29 min – 100% B, 30 min – 100% A, 40 min – 100% A.

Quantification of enzymatic products in the CysS reaction mixture

The CysS assay, using methyl ether **21** was carried out under the above conditions with flavodoxin, flavodoxin reductase or methyl viologen, NADPH as reducing systems. Calibration curves were constructed by measuring the peak intensity of 5′-dA, SAH, synthetic ether **19**, **22a** and **22b** with known concentration using LC-MS. Then the calibration curves were used to measure the concentration of 5′-dA, SAH, **19**, **22a** and **22b** in the enzymatic reaction. Control reactions without CysS were used to assess the quantity of non-enzymatically

produced SAH and this amount was subtracted out of the enzymatic reaction to give the final result. With our current enzyme preparation, 55 μM of CysS with 0.75 mM MeCbl, 2.5 mM SAM, 0.9 mM substrate and flavodoxin, flavodoxin reductase system (25 μM FldA, FPR; 50 μM FAD, FMN; 3 mM NADPH) produced 111 μM of 5′-dA, 109 μM of SAH, 78 μM of ethyl ether **22a**, 15 μM of isopropyl ether **22b**. The relative rate of **18** and **21** was measured by the amount of product formations of **19** and **22** by CysS with 1:1 ratio of **18** to **21**. 11 μM CysS was incubated with 0.3 mM MeCbl, 0.5 mM SAM, 0.5 mM **18**, 0.5 mM **21**, and 1 mM methyl viologen, 3 mM NADPH produced 0.4 uM of **19**, 15.8 μM of **22a**, 3.2 μM of **22b**.

General procedure for the synthesis of the 4-nitrobenzoic acids (32a, b and d)



31 **32**

a: R = Et
b: R = *i*-Pro
d: R = *sec*-Bu

Potassium hydroxide (5.0 mmol) was added to a solution of 3-fluoro-4-nitrobenzoic acid (2.0 mmol) **31** in excess of alcohol (10 ml) and the reaction mixture was slowly heating to reflux for several hours and monitored by TLC (Hexane and EtOAc solvent system). Then it was acidified using 2N HCl and extracted with ethyl acetate. The extracts were combined, washed with water, dried with $MgSO_4$ and evaporated to give 4-nitro-benzoic acids **32a, b** and **d**.

3-ethoxy-4-nitrobenzoic acid (32a)

Compound **32a** was prepared by the above procedure using ethanol in 63% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.80 (m, 2H), 7.68 (d, J = 9.8 Hz, 1H), 4.26 (q, J = 7.0 Hz, 2H), 1.43 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 152.59, 136.72, 125.79, 122.58, 116.71, 106.43, 66.77, 14.72.

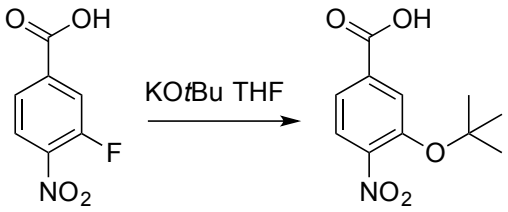
3-isopropoxy-4-nitrobenzoic acid (32b)

Compound **32b** was prepared by the above procedure using isopropyl alcohol in 90% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.81 (s, 1H), 7.76 (d, *J* = 8.3 Hz, 1H), 7.66 (d, *J* = 8.3 Hz, 1H), 4.80 (m, 1H) 1.36 (d, *J* = 6.0 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ 172.96, 151.57, 136.51, 125.75, 122.57, 118.13, 74.13, 22.03, 20.85.

3-(*sec*-butoxy)-4-nitrobenzoic acid (32d)

Compound **32d** was prepared by the above procedure using 2-butanol in 37% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.87 – 7.72 (m, 2H), 7.66 (d, *J* = 8.3 Hz, 1H), 4.68 – 4.51 (m, 1H), 1.82 – 1.60 (m, 2H), 1.33 (d, *J* = 6.1 Hz, 3H), 0.99 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 167.76, 151.82, 136.52, 125.75, 122.40, 117.79, 106.38, 78.82, 30.03, 19.21, 9.67.

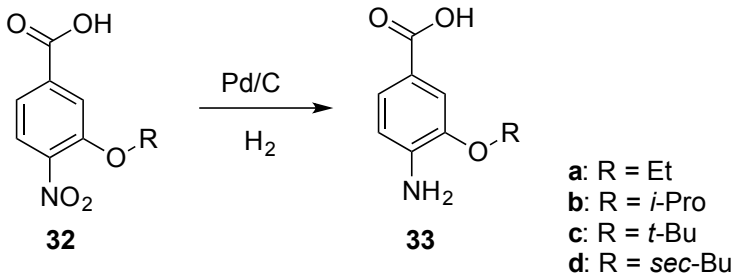
3-(*tert*-butoxy)-4-nitrobenzoic acid (32c)



31 **32c**

3-fluoro-4-nitrobenzoic acid **31** (0.5 mmol) was dissolved in 5 ml anhydrous THF in a flame-dried flask under argon and cooled to 0 °C. A 1.0 M solution of potassium *tert*-butoxide (1.5 mmol) was added dropwise. This solution was stirred and allowed to warm from 0 °C to room temperature overnight and then diluted with ethyl acetate and washed with saturated aqueous NH_4Cl. The aqueous layer was back-extracted with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, and concentrated. Purification of the crude mixture by flash chromatography (silica gel, 15:1 CH_2Cl_2: MeOH) afforded **32c** in 90% isolated yield. ^{1}H NMR (400 MHz, MeOD) δ 7.90 (s, 1H), 7.80 (d, *J* = 8.3 Hz, 1H), 7.74 (d, *J* = 8.3 Hz, 1H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 167.63, 149.76, 125.76, 125.37, 124.96, 84.11, 29.08.

General procedure for synthesis of 4-aminobenzoic acids (33a-d)



Using 10% Pd/C as catalyst, 50 mL of 4-nitrobenzoic acid (2.0 mmol) in methanol was pumped through the H-Cube®. The pressure of the system was set to full hydrogen mode, and the temperature to 35 °C. The flow rate was 0.8 mL/min. The fraction was analyzed using TLC, which showed complete conversion to the product, and the solvent was reduced to dryness, affording the 4-aminobenzoic acids **33a-d**.

4-amino-3-ethoxybenzoic acid (33a)

Compound **33a** was prepared by the above procedure from 3-ethoxy-4-nitrobenzoic acid **32a** in 98% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.57 – 7.30 (m, 2H), 6.71 (d, J = 8.2 Hz, 1H), 4.09 (q, J = 7.0 Hz, 2H), 1.43 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 170.56, 146.83, 143.50, 125.45, 120.08, 114.36, 113.49, 65.07, 15.11.

4-amino-3-isopropoxybenzoic acid (33b)

Compound **33b** was prepared by the above procedure from 3-isopropoxy-4-nitrobenzoic acid **32b** in 98% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.54 – 7.40 (m, 2H), 6.70 (s, 1H), 4.58 (m, 1H), 1.34 (d, J = 6.0 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ 171.77, 145.15, 126.99, 115.39, 113.88, 112.36, 72.35, 26.60, 22.32.

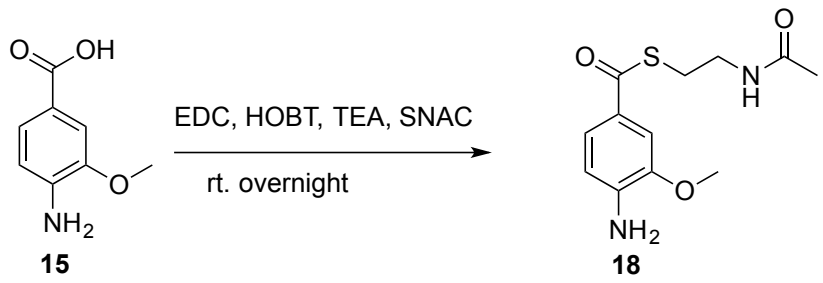
4-amino-3-(*tert*-butoxy) benzoic acid (33c)

Compound **33c** was prepared by the above procedure from 3-(*tert*-butoxy)-4-nitrobenzoic acid **32c** in 99% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.56 (s, 1H), 7.54 (d, J = 8.3 Hz, 1H), 6.71 (d, J = 8.3 Hz, 1H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 170.48, 148.85, 142.48, 127.64, 125.04, 119.07, 114.84, 81.01, 29.10.

4-amino-3-(*sec*-butoxy) benzoic acid (33d)

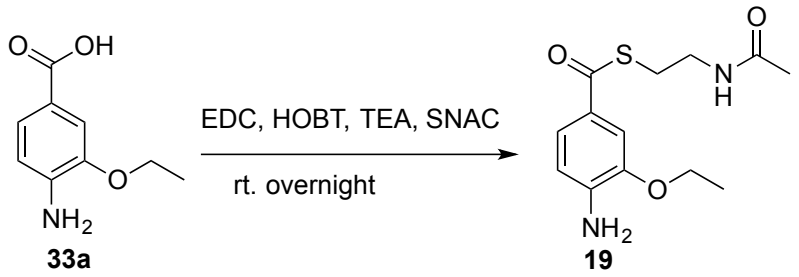
Compound **33d** was prepared by the above procedure from 3-(*sec*-butoxy)-4-nitrobenzoic acid **32d** in 97% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.51 – 7.35 (m, 2H), 6.69 (d, *J* = 8.2 Hz, 1H), 4.36 (m, 1H), 1.86 – 1.57 (m, 2H), 1.30 (d, *J* = 6.1 Hz, 3H), 1.00 (t, *J* = 7.5 Hz, 3H).

S-(2-acetamidoethyl) 4-amino-3-methoxybenzothioate (18)



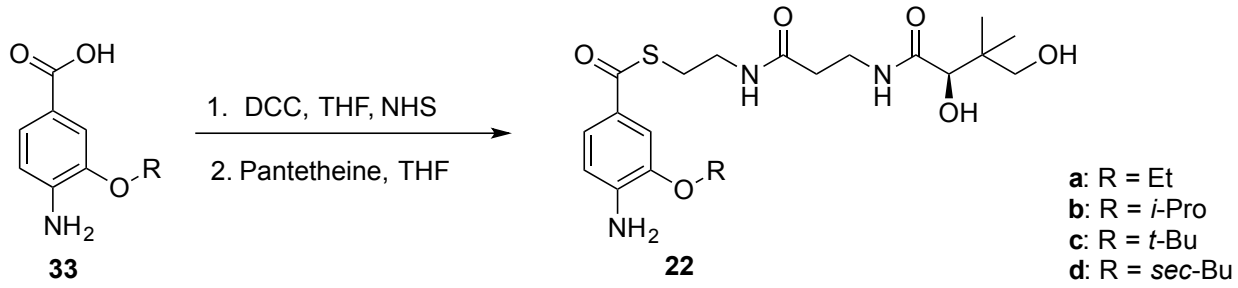
To a solution of triethylamine (TEA) (2.80 mmol) in dichloromethane (10 mL) was added 4-amino-3-methoxybenzoic acid **15** (1.40 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (1.40 mmol), 1-hydroxybenzotriazole (HOBt) (1.40 mmol) and N-acetylcysteamine (SNAC) (1.4 mmol). The reaction mixture was stirred overnight under argon. The organic layer was washed with saturated $NaHCO_3$ solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate and concentrated under vacuum. Purification of the crude mixture by flash chromatography (silica gel, 1:1 to 2:1 of hexanes:EtOAc) afforded **18** in 20% isolated yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.2, 1H), 7.36 (s, 1H), 6.63 (d, *J* = 8.2 Hz, 1H), 3.89 (s, 3H), 3.51 (dd, *J* = 12.2, 5.9 Hz, 2H), 3.18 (t, *J* = 6.3 Hz, 2H), 1.95 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.29, 170.29, 146.21, 142.34, 126.60, 122.85, 112.78, 108.72, 55.63, 40.08, 28.31, 23.21.

S-(2-acetamidoethyl) 4-amino-3-ethoxybenzothioate (19)



Compound **19** was prepared from 4-amino-3-methoxybenzoic acid **33a** in the same manner as the above procedure in 30% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (d, *J* = 8.2 Hz, 1H), 7.33 (s, 1H), 6.62 (d, *J* = 8.2 Hz, 1H), 4.09 (q, *J* = 7.0 Hz, 2H), 3.48 (dd, *J* = 12.3, 5.9 Hz, 2H), 3.15 (t, *J* = 6.3 Hz, 2H), 1.93 (s, 3H), 1.42 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.30, 170.29, 145.49, 142.45, 126.56, 122.69, 112.80, 109.63, 77.34, 77.03, 76.71, 64.05, 40.09, 28.30, 23.21, 14.77.

General procedure for synthesis of S-pantetheinyl-4-aminobenzothioate (22a-d)



To a stirred solution of 4-aminobenzoic acid **33** (2.0 mmol) in 10 mL of dry THF was added N-hydroxysuccinimide (NHS) (2.0 mmol), followed by N, N'-Dicyclohexylcarbodiimide (DCC) (2.0 mmol) at room temperature. The resulting mixture was stirred overnight at room temperature. The white precipitate was filtered and then washed with EtOAc. The filtrate was concentrated under reduced pressure. The residue was triturated with CH_2Cl_2 to give the NHS ether of **33** as a pale brown powder. Pantethine (0.075 mmol), sodium bicarbonate (1.9 mmol), and 1,4-dithiothreitol (DTT) (0.08 mmol) were dissolved in 3 mL of water, and left to stand for 10 minutes to allow reduction of the pantethine to pantetheine to take place. The NHS ester (0.54 mmol) was dissolved in 7 mL of tetrahydrofuran (THF) and added to the 3 mL water mixture. The reaction

mixture was stirred overnight and then the solvent was evaporated under reduced pressure. The crude product was then dissolved in water and the insoluble residue was removed by filtration. The resulting solution was lyophilized and purified by flash chromatography (silica gel, 20:1 to 10:1 CH_2Cl_2: MeOH). The fraction was further purified by Prep HPLC using a SPLC-18DB column (10 x 250 mm, 5 µm, Supelco) at a flow rate of 2 mL/min on an Agilent 100 HPLC system with a quaternary pump and an automatic injector.

(R)-S-pantetheinyl-4-amino-3-methoxybenzothioate (21)

Compound **21** was prepared by the above procedure from 4-amino-3-methoxybenzoic acid **15**. ^{1}H NMR (400 MHz, D_2O) δ 7.52 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.35 (d, *J* = 1.9 Hz, 1H), 6.79 (d, *J* = 8.3 Hz, 1H), 3.91 (s, 1H), 3.88 (s, 3H), 3.47 – 3.29 (m, 6H), 3.17 (t, *J* = 6.3 Hz, 2H), 2.44 (t, *J* = 6.5 Hz, 2H), 0.83 (d, *J* = 13.9 Hz, 6H). ^{13}C NMR (101 MHz, D_2O) δ 193.17, 175.04, 174.02, 146.63, 126.36, 123.04, 114.04, 109.43, 75.86, 68.49, 55.92, 38.93, 38.58, 35.50, 35.33, 28.03, 22.30, 20.46, 19.11.

(R)-S-pantetheinyl-4-amino-3-ethoxybenzothioate (22a)

Compound **22a** was prepared by the above procedure from 4-amino-3-ethoxybenzoic acid **33a**. ^{1}H NMR (400 MHz, D_2O) δ 7.56 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.41 (d, *J* = 1.8 Hz, 1H), 6.84 (d, *J* = 8.3 Hz, 1H), 4.15 (q, *J* = 7.0 Hz, 2H), 3.91 (s, 1H), 3.54 – 3.25 (m, 6H), 3.20 (t, *J* = 6.2 Hz, 2H), 2.44 (t, *J* = 6.5 Hz, 2H), 1.40 (t, *J* = 7.0 Hz, 3H), 0.83 (d, *J* = 13.8 Hz, 6H). ^{13}C NMR (101 MHz, D_2O) δ 193.40, 175.05, 174.08, 145.68, 143.76, 126.41, 123.11, 114.25, 111.11, 75.86, 68.48, 65.24, 38.90, 38.58, 35.51, 35.34, 28.06, 20.45, 19.09, 14.02.

(R)-S-pantetheinyl-4-amino-3-isopropoxybenzothioate (22b)

Compound **22b** was prepared by the above procedure from 4-amino-3-isopropoxybenzoic acid **33b**. ^{1}H NMR (400 MHz, D_2O) δ 7.54 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.45 (d, *J* = 1.8 Hz, 1H), 6.82 (d, *J* = 8.4 Hz, 1H), 4.62 (m, 1H), 3.88 (s, 1H), 3.48 – 3.21 (m, 6H), 3.17 (t, *J* = 6.2 Hz, 2H), 2.41 (t, *J* = 6.5 Hz, 2H), 1.30 (d, *J* = 6.1 Hz, 6H), 0.79 (d, *J* = 14.0 Hz, 6H). ^{13}C NMR (101 MHz, D_2O) δ 193.36, 175.05, 174.07, 144.96, 144.19, 126.43, 123.38, 114.65, 114.13, 75.86, 73.18, 68.48, 38.90, 38.59, 35.50, 35.35, 28.07, 21.21, 20.47, 19.11.

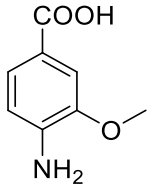
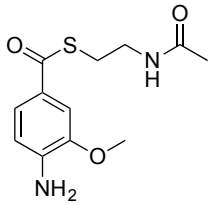
(R)-S-pantetheinyl-4-amino-3-(*tert*-butoxy)benzothioate (22c)

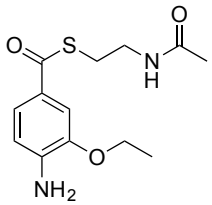
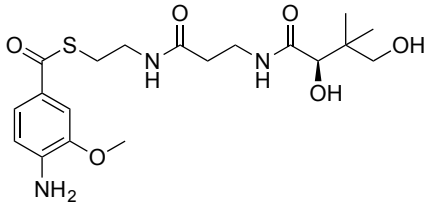
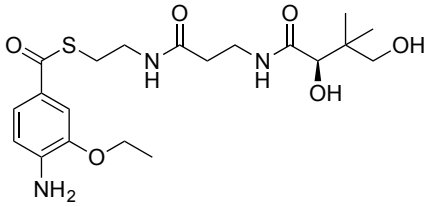
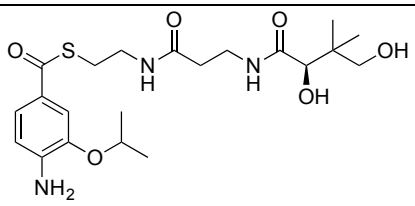
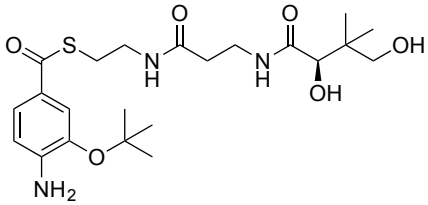
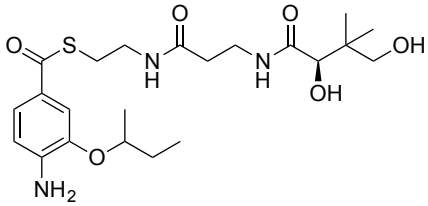
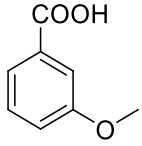
Compound **22c** was prepared by the above procedure from 4-amino-3-(*tert*-butoxy)benzoic acid **33c**. ^{1}H NMR (400 MHz, MeOD) δ 7.65 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.59 (d, *J* = 2.0 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 1H), 3.92 (s, 1H), 3.49 – 3.32 (m, 6H), 3.20 (t, *J* = 6.2 Hz, 2H), 2.44 (t, *J* = 6.5 Hz, 2H), 1.41 (s, 9H), 0.83 (d, *J* = 14.1 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ 193.19, 175.05, 174.06, 148.38, 140.87, 125.93, 125.41, 122.52, 115.25, 82.68, 75.84, 68.48, 38.89, 38.61, 35.51, 35.36, 28.08, 27.86, 20.50, 19.14.

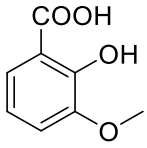
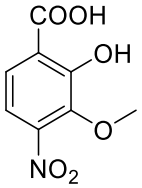
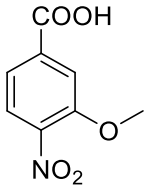
(R)-S-pantetheinyl-4-amino-3-(*sec*-butoxy)benzothioate (22d)

Compound **22d** was prepared by the above procedure from 4-amino-3-(*sec*-butoxy)benzoic acid **33d**. ^{1}H NMR (400 MHz, MeOD) δ 7.48 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.38 (d, *J* = 1.7 Hz, 1H), 6.71 (d, *J* = 8.3 Hz, 1H), 4.40 (m, 1H), 3.92 (s, 1H), 3.58 – 3.37 (m, 6H), 3.16 (t, *J* = 6.6 Hz, 2H), 2.44 (t, *J* = 6.6 Hz, 2H), 1.82-1.64 (m, 2H), 1.33 (d, *J* = 6.1 Hz, 3H), 1.03 (t, *J* = 7.5 Hz, 3H), 0.94 (s, 6H). ^{13}C NMR (101 MHz, MeOD) δ 189.63, 174.62, 172.51, 144.87, 144.07, 125.29, 122.32, 112.41, 111.11, 75.98, 75.79, 69.01, 39.11, 38.95, 35.03, 34.36.95, 28.76, 27.51, 19.89, 19.53, 18.15, 8.63.

Table S1: Compounds tested as possible CysS substrates:

Compound	Structure	Product
15		–
18		**19**

19		**20**
21		**22a**
22a		**22b**
22b		**22c,d**
22c		–
22d		–
41		–

42		–
43[4]		–
44		–

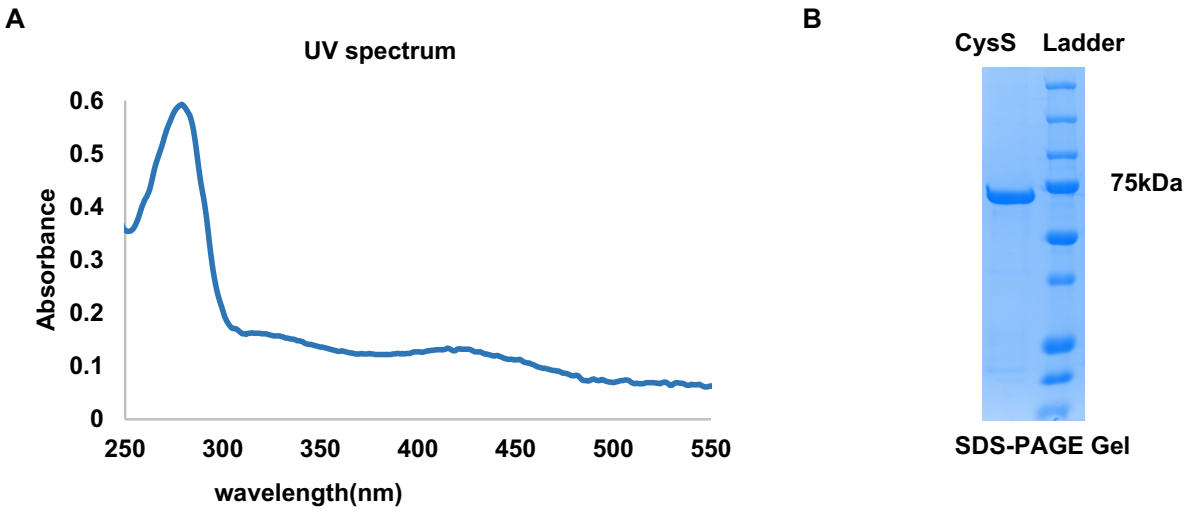


Figure S1. (A) UV spectrum of purified CysS (B) SDS/PAGE analysis of purified CysS.

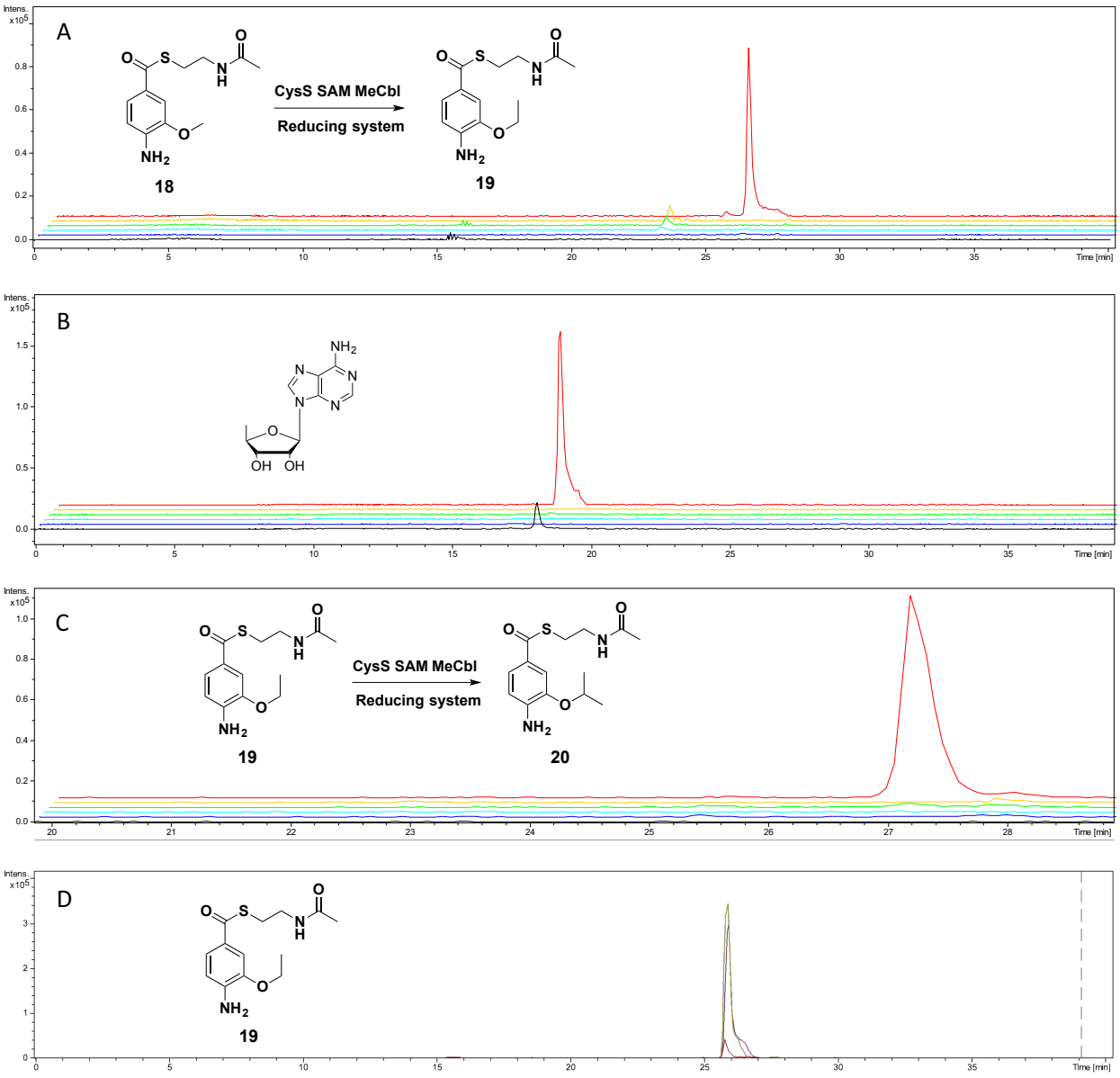


Figure S2. LC-MS detection of the CysS reaction products using methyl ether **18** and ethyl ether **19**. Red trace is the full reaction where all the components are present. Orange, cyan, blue, black and green traces respectively are for reaction mixtures where either CysS, MeCbl, SAM, substrate, or reducing system flavodoxin/flavodoxin reductase/NADPH) is absent. (A) EIC of compound **19** $[M+H]^+$ (283.11±0.02) (B) EIC of 5′-dA $[M+H]^+$ (252.10±0.02) (C) EIC of compound **20** $[M+H]^+$ (297.13±0.02) (D) EIC of compound **19** co-elution with an authentic sample $[M+H]^+$ (283.11±0.02). Red trace is the full enzymatic reaction. Purple trace is compound **19**. Green trace is the co-elution of the enzymatic product and the synthetic standard.

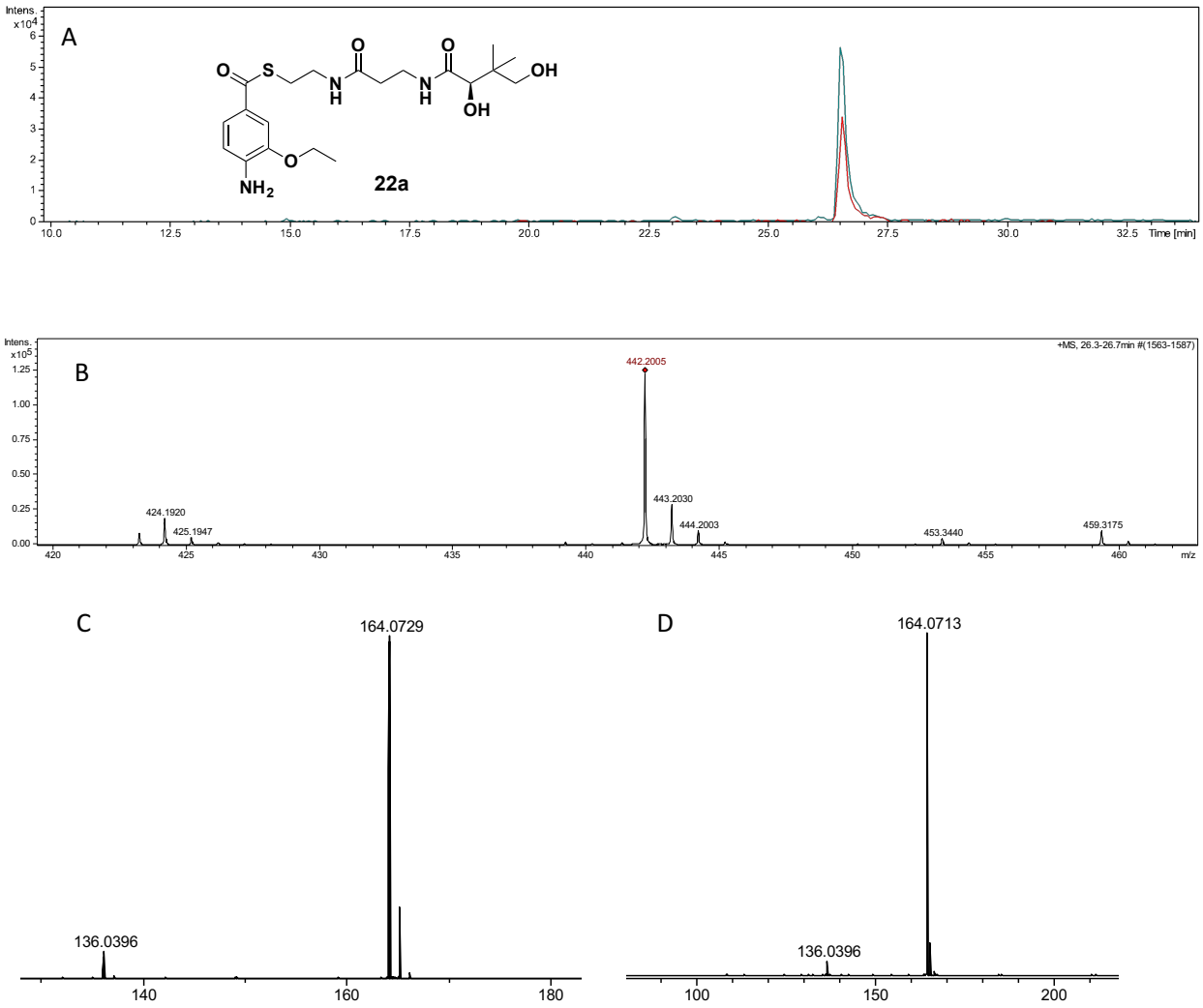


Figure S3. LC-MS detection of the CysS reaction products using methyl ether **21** as substrate. Red trace is the enzymatic full reaction. Green trace is the co-elution of the enzymatic product and synthetic standard of the ethyl ether **22a** (A) EIC of ethyl ether **22a** $[M+H]^+$ (442.20±0.02). (B) Mass spectrum of ethyl ether **22a** (exact mass: 442.2021, Accuracy: <1.0 ppm). (C) Mass fragments of the ethyl ether **22a** produced in the enzymatic reaction. (D) Mass fragments of synthetic standard of the ethyl ether **22a**.

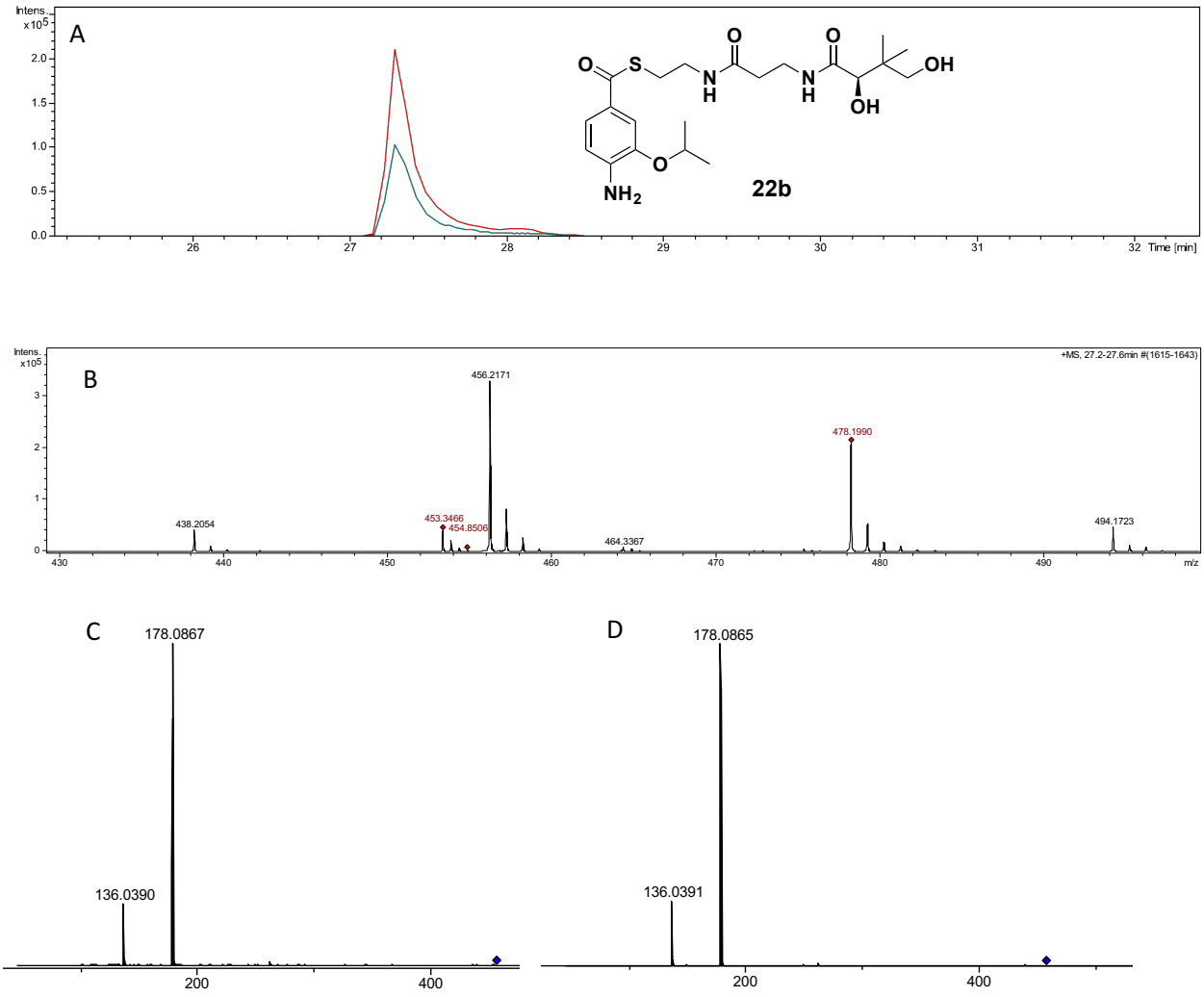


Figure S4. LC-MS detection of the CysS reaction products using methyl ether **21** as substrate. Red trace is enzymatic full reaction. Blue trace is synthetic standard of isopropyl ether **22b** (A) EIC of isopropyl ether **22b** $[M+H]^+$ (456.21±0.02). (B) Mass spectrum of isopropyl ether **22b** (exact mass: 456.2171, Accuracy: 1.7 ppm). (C) Mass fragments of the isopropyl ether **22b** produced in the enzymatic reaction. (D) Mass fragments of synthetic standard of isopropyl ether **22b**.

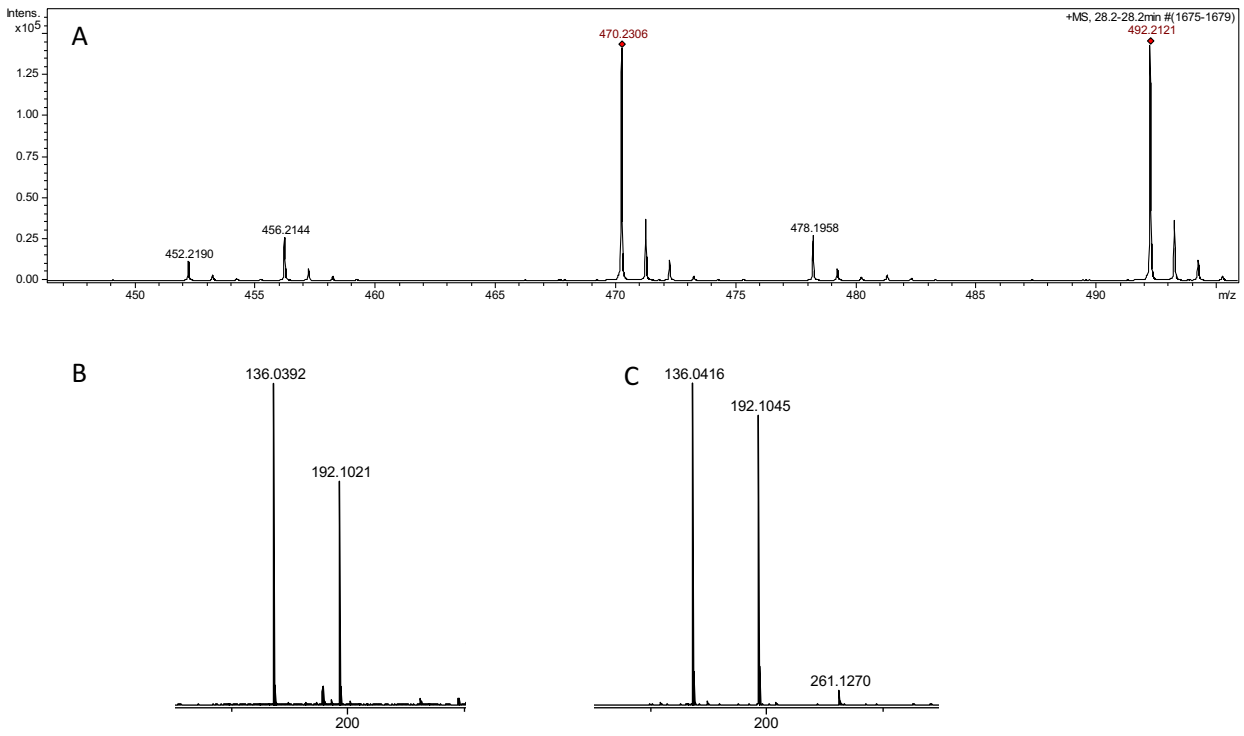


Figure S5. LC-MS detection of the CysS reaction products using methyl ether **21** as substrate. (A) Mass spectrum of *t*-butyl ether **22c** (exact mass: 470.2319, Accuracy: 3.0 ppm). (B) Mass fragments of *t*-butyl ether **22c** produced in the enzymatic reaction. (C) Mass fragments of synthetic standard of *t*-butyl ether **22c**.

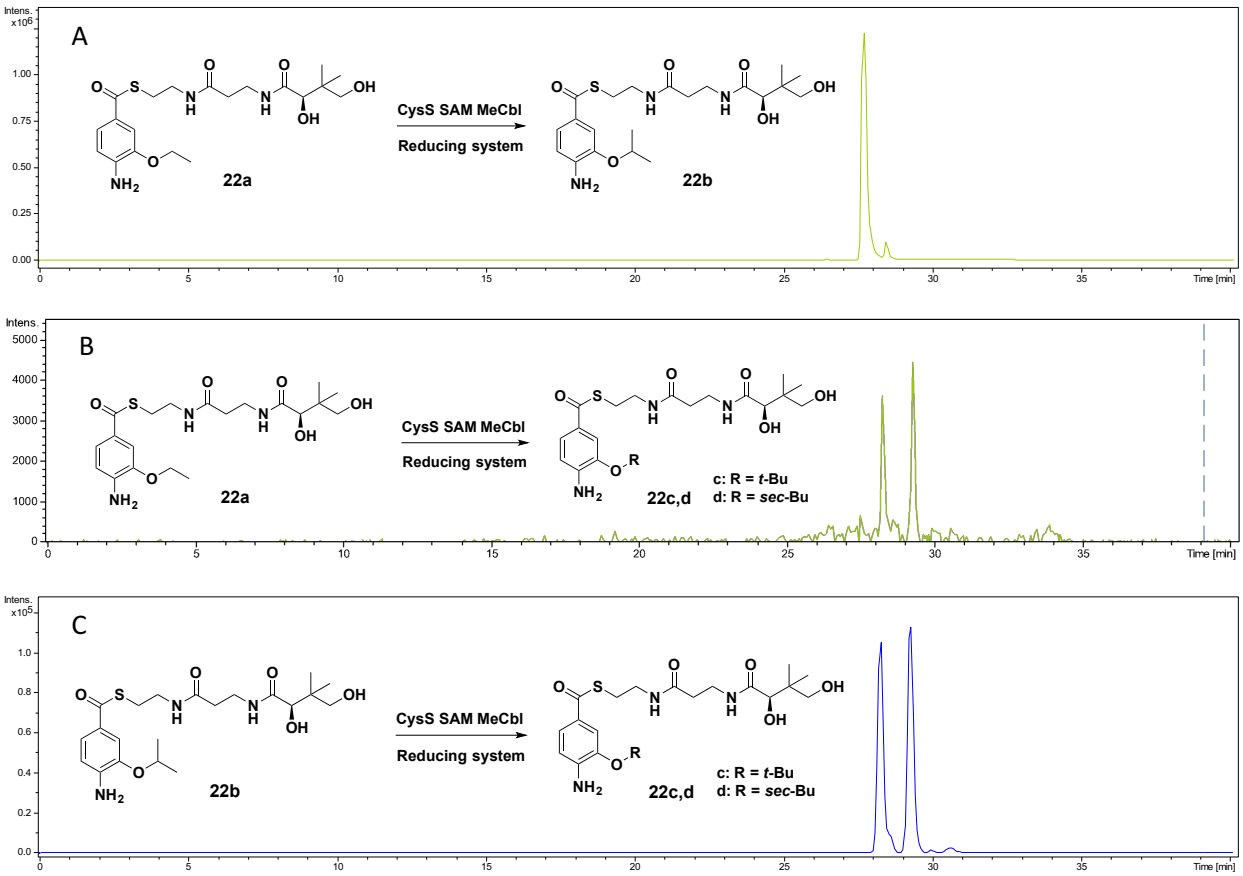


Figure S6. LC-MS detection of the CysS reaction products using ethyl ether **22a** (A), (B) or isopropyl ether **22b** (C). (A) EIC of isopropyl ether **22b** $[M+H]^+$ (456.21±0.02). (B), (C) EIC of butyl ethers **22c, 22d** $[M+H]^+$ (470.23±0.02).

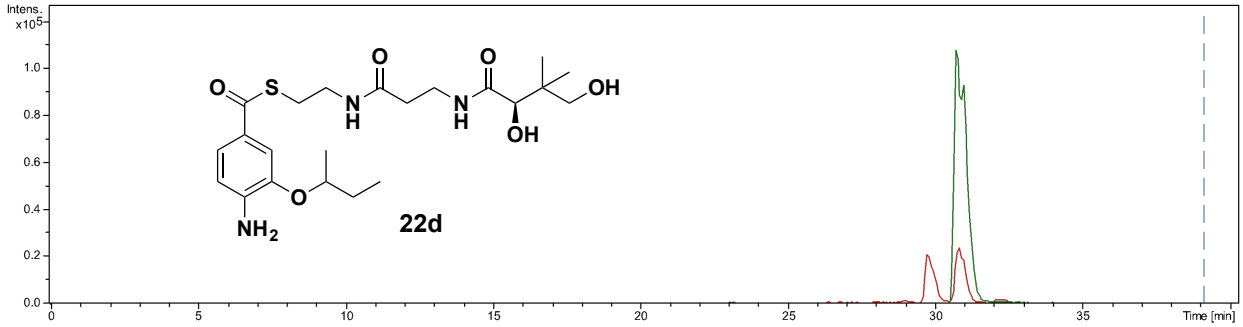


Figure S7. LC-MS detection of the CysS reaction products using isopropyl ether **22b** as substrate. The second peak was co-migrated with an authentic standard of *sec*-butyl ether **22d**. Red trace is EIC of mass (470.23±0.02) for enzymatic full reaction. Green trace is synthetic standard of *sec*-butyl ether **22d**.

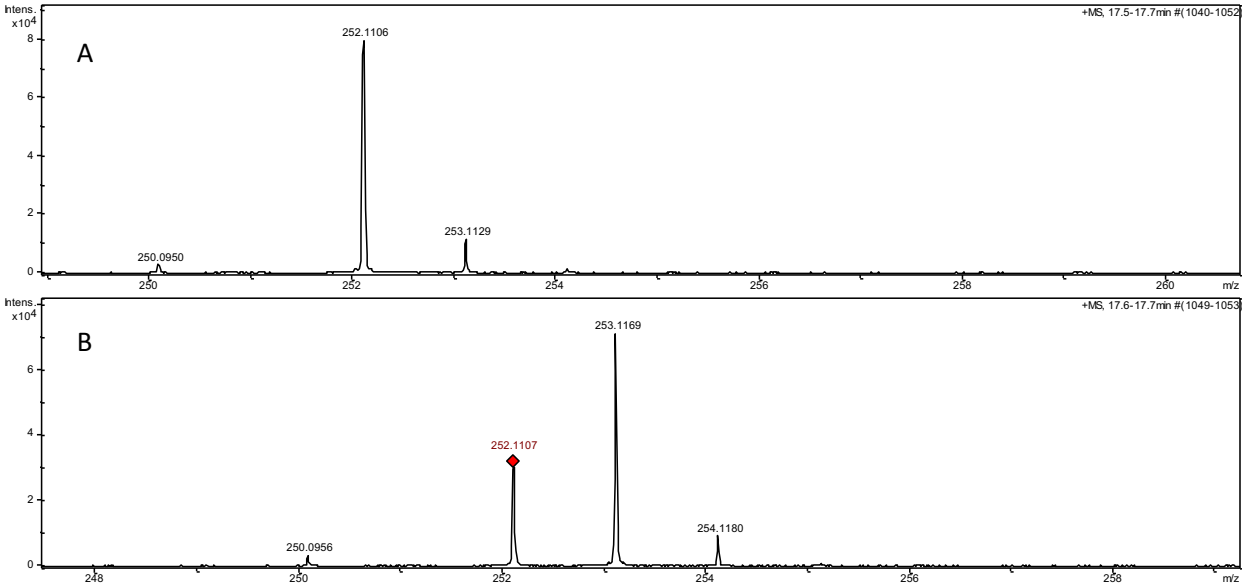


Figure S8. LC-MS detection of 5′-dA produced in the CysS reaction products using (A) methyl ether **21** or (B) CD_3-methyl ether **CD_3-21** as substrate.

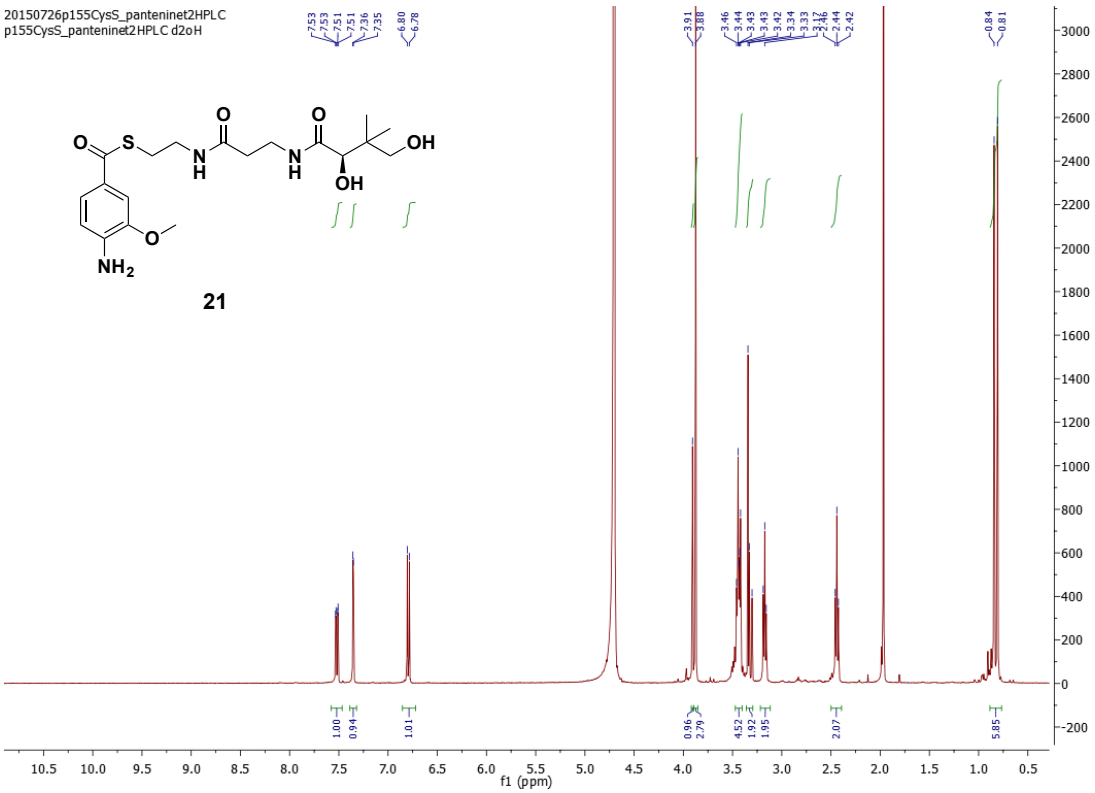


Figure S9. ^{1}H NMR (400 MHz, D_2O) spectrum of compound **21**

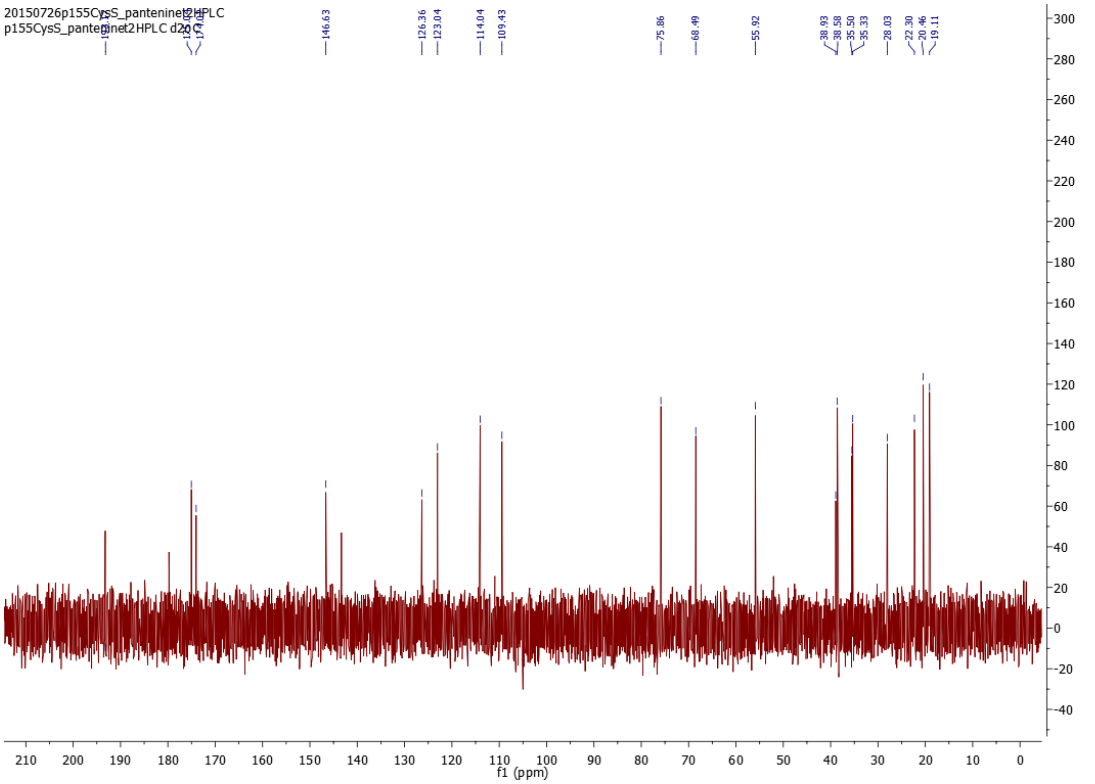


Figure S10. ^{13}C NMR (400 MHz, D_2O) spectrum of compound **21**

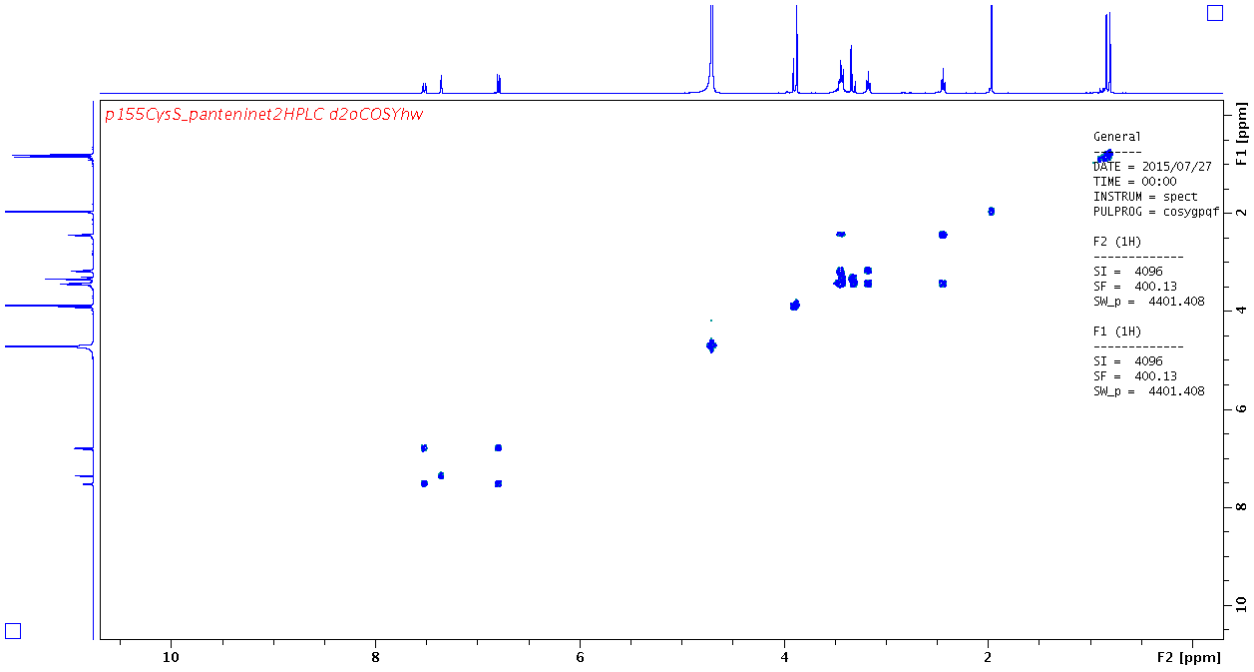


Figure S11. ^{1}H-COSY spectrum of compound **21**

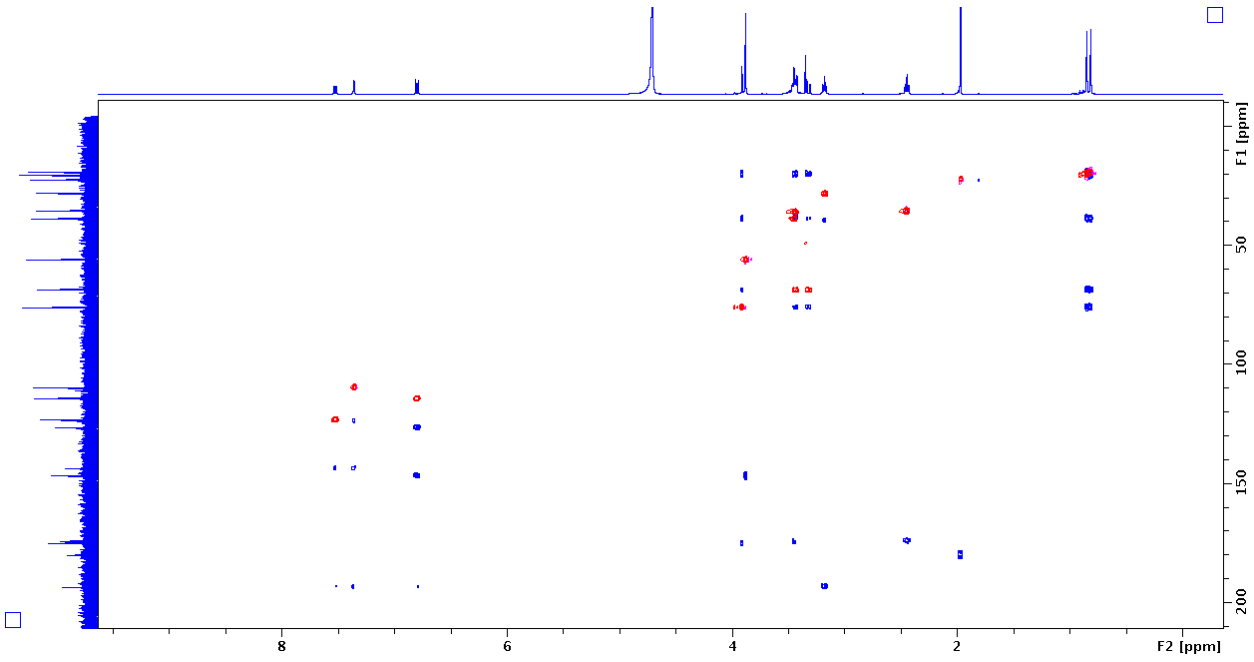


Figure S12. HSQC (red) and HMCB(blue) spectrum of compound **21**

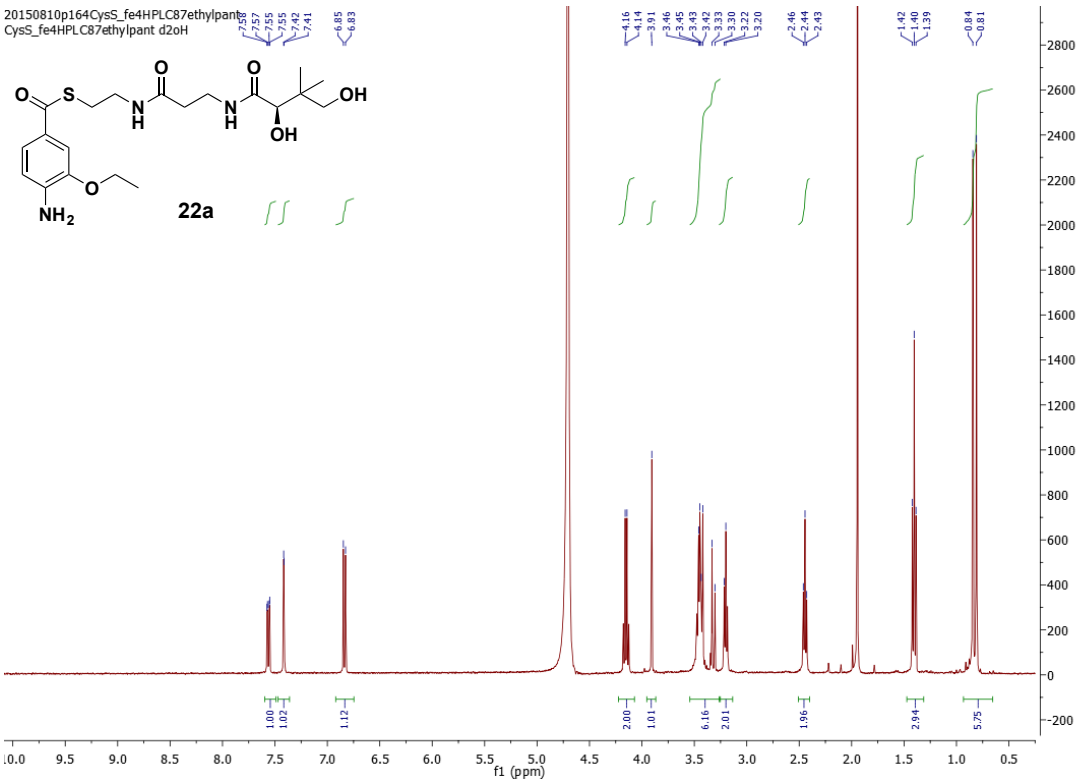


Figure S13. ^{1}H NMR (400 MHz, D_2O) spectrum of **22a**

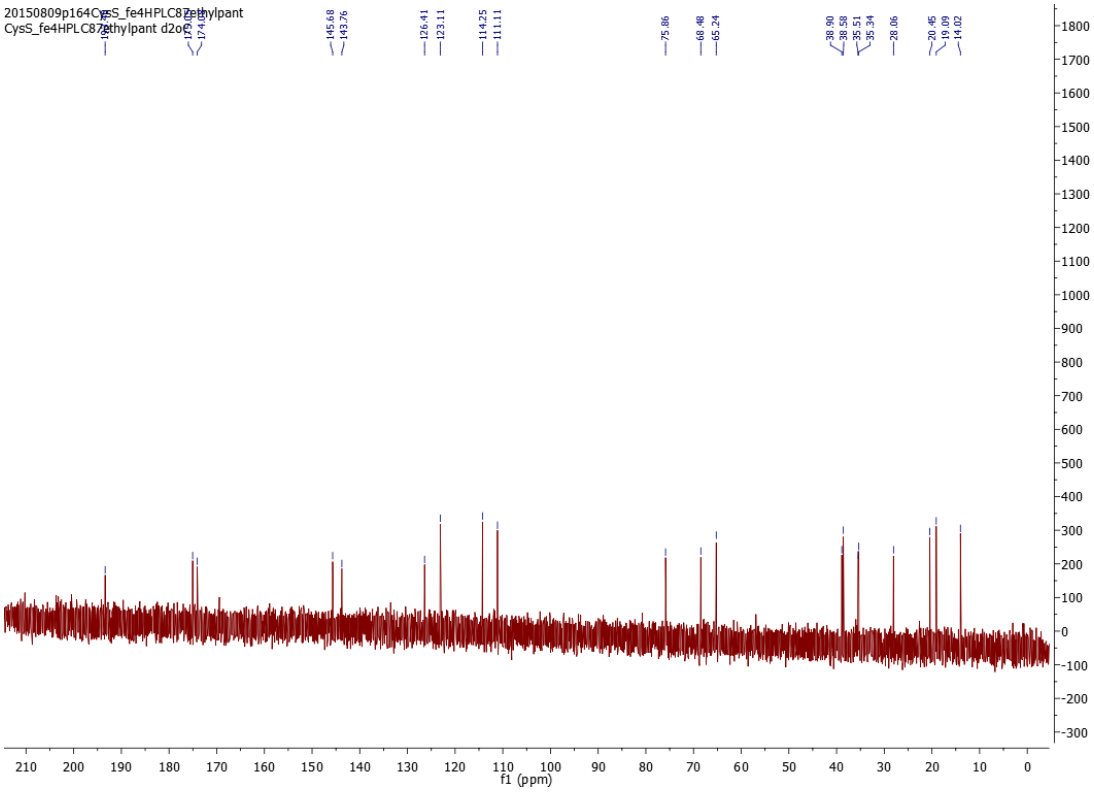


Figure S14. ^{13}C NMR (400 MHz, D_2O) spectrum of **22a**

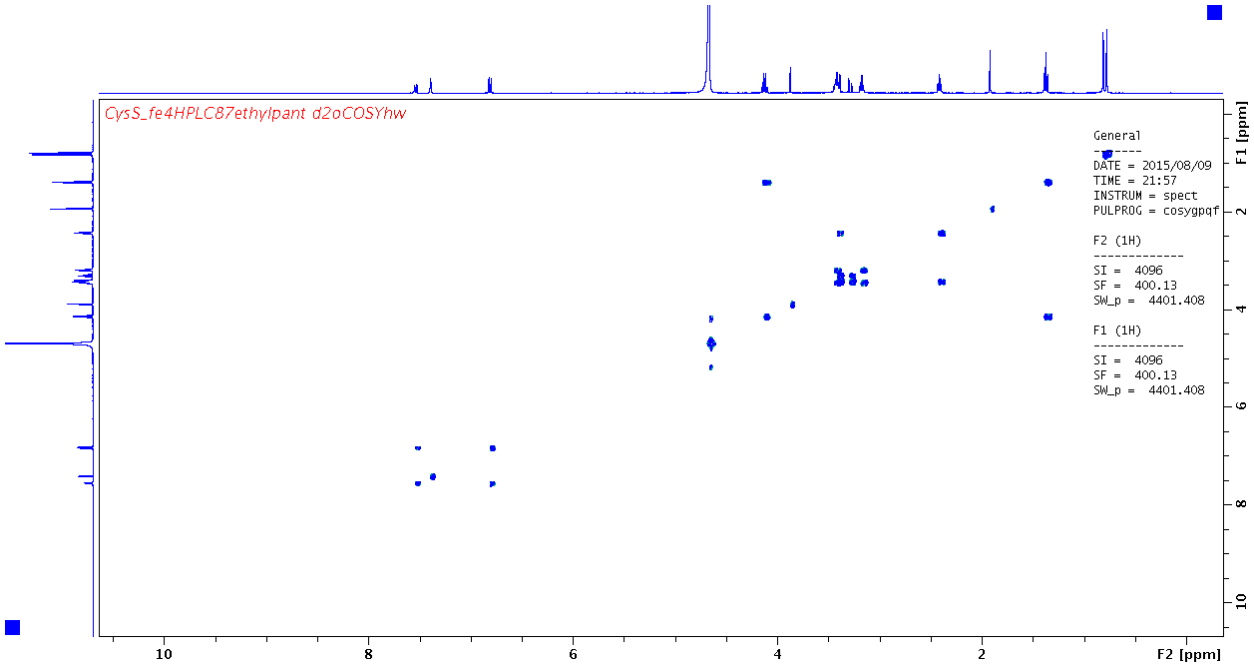


Figure S15. ^{1}H-COSY spectrum of compound **22a**

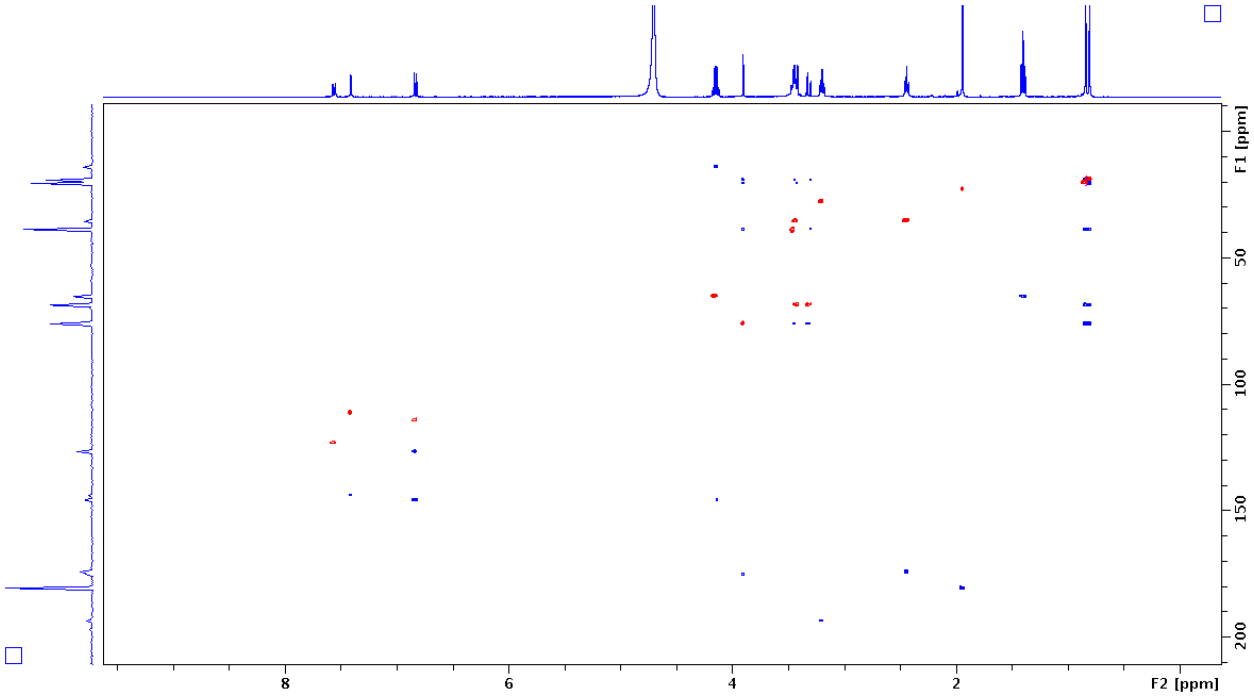


Figure S16. HSQC (red) and HMCB(blue) spectrum of compound **22a**

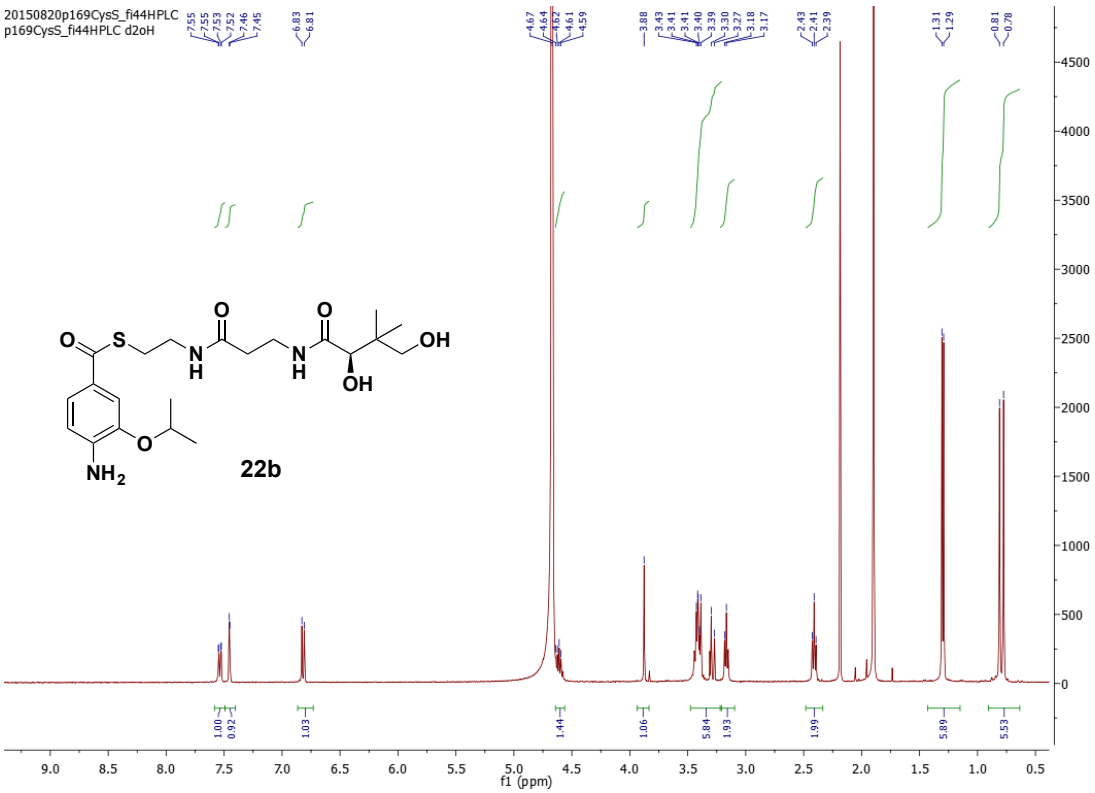


Figure S17. ^{1}H NMR (400 MHz, D_2O) spectrum of **22b**

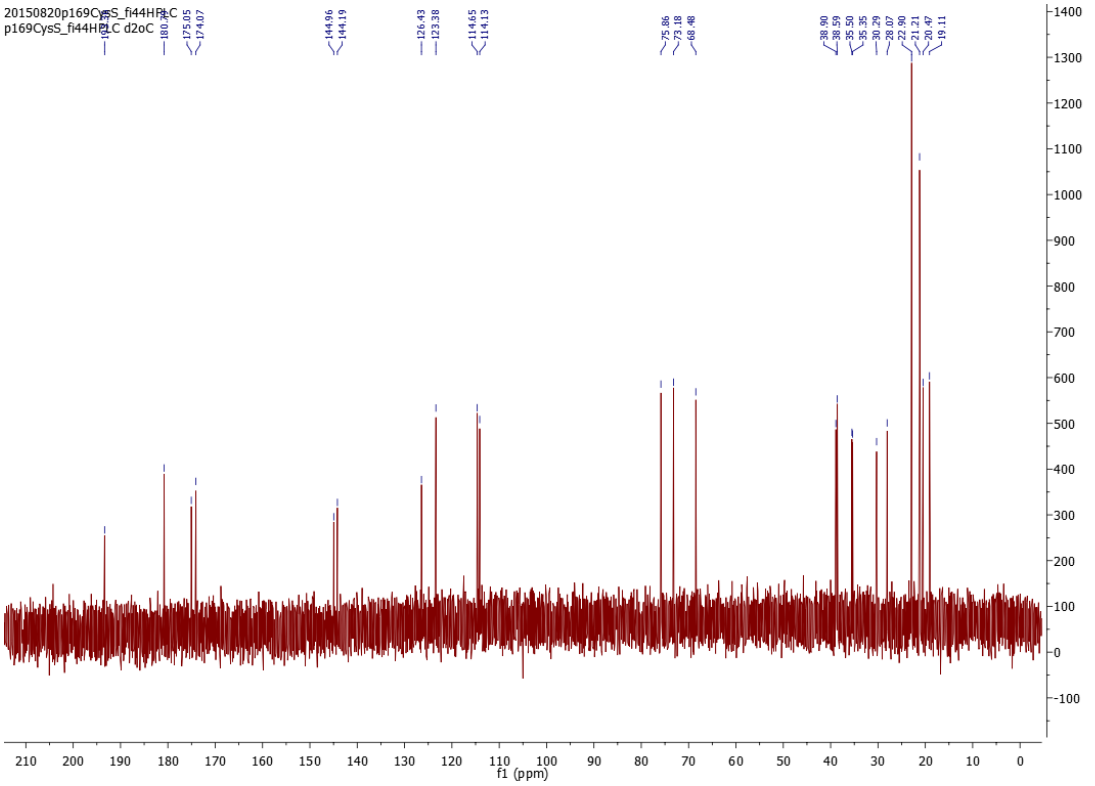


Figure S18. ^{13}C NMR (400 MHz, D_2O) spectrum of compound **22b**

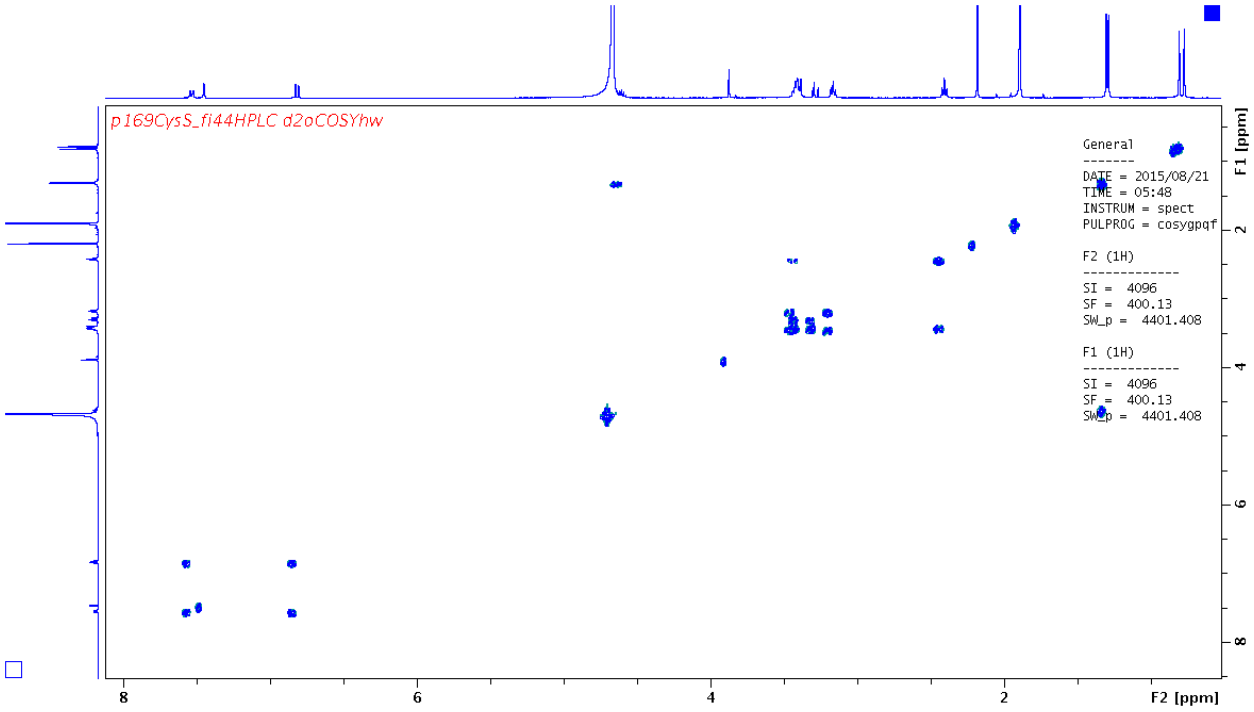


Figure S19. ^{1}H-COSY spectrum of compound **22b**

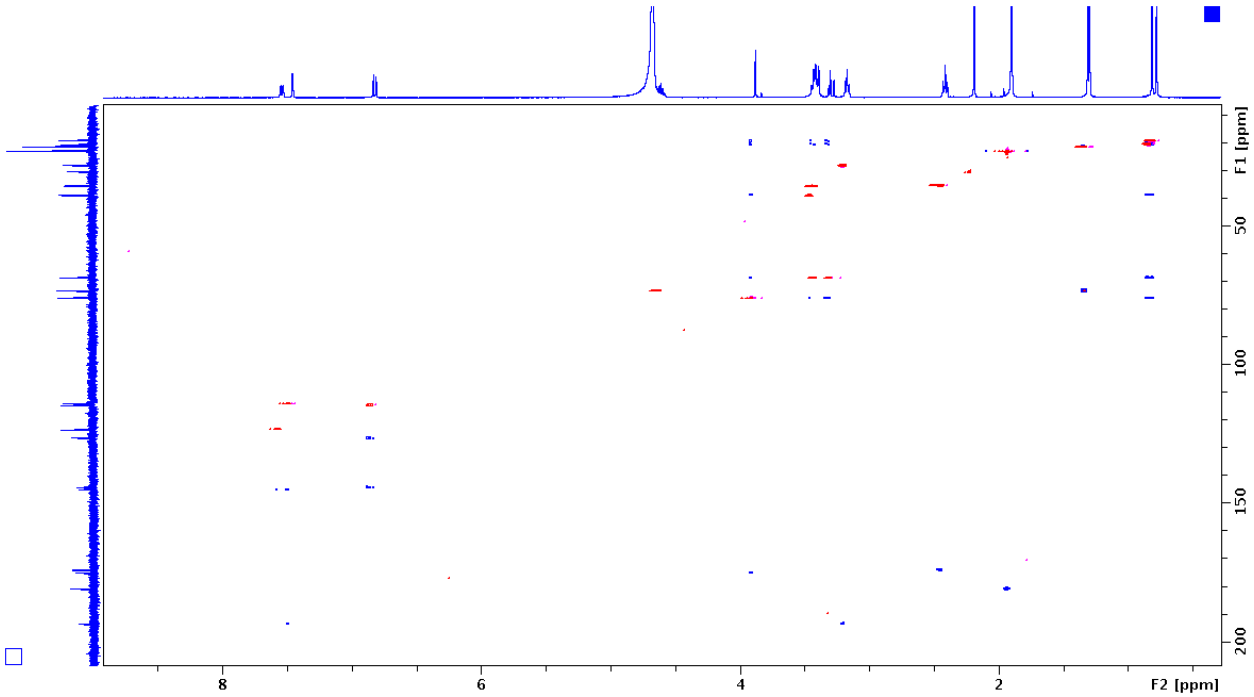


Figure S20. HSQC (red) and HMCB(blue) spectrum of compound **22b**

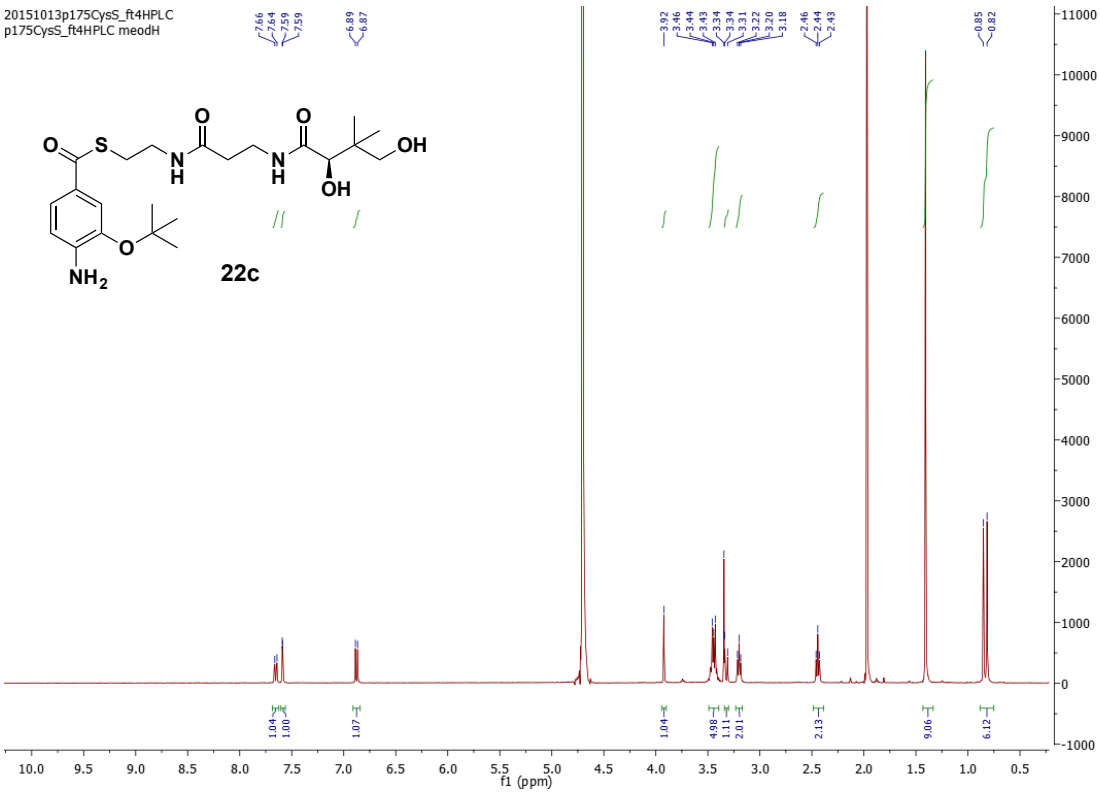


Figure S21. ^{1}H NMR (400 MHz, CD_3OD) spectrum of compound **22c**

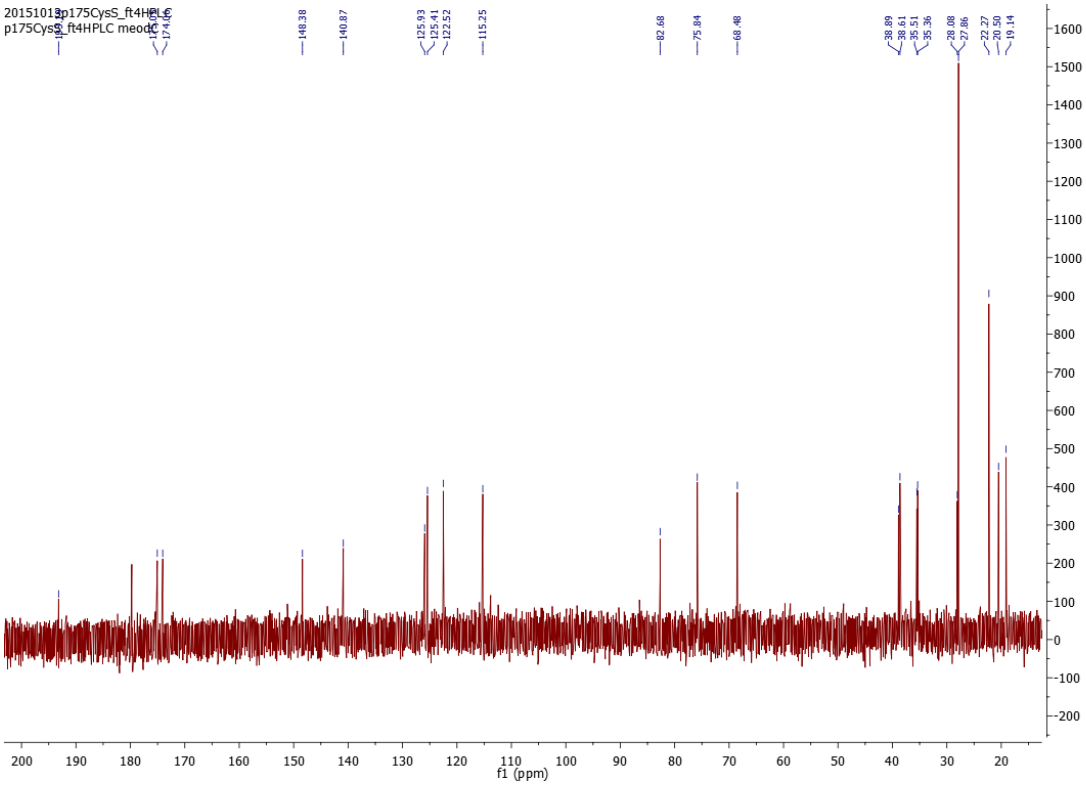


Figure S22. ^{13}C NMR (400 MHz, CD_3OD) spectrum of compound **22c**

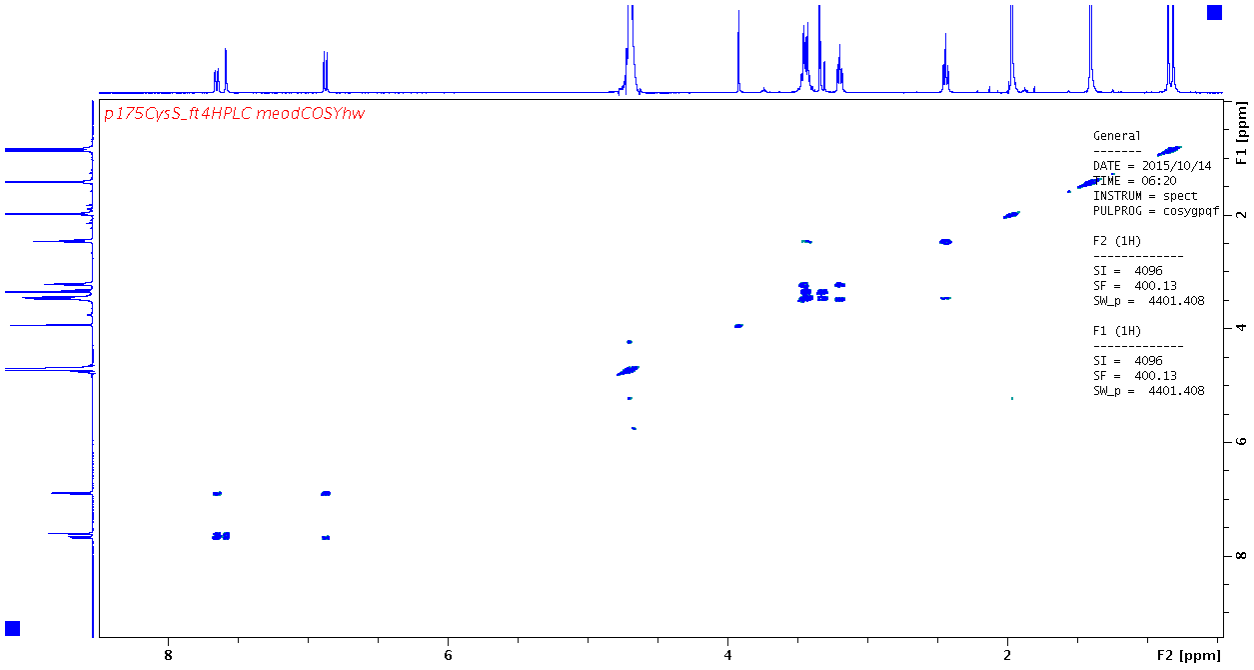


Figure S23. ^{1}H-COSY spectrum of compound **22c**

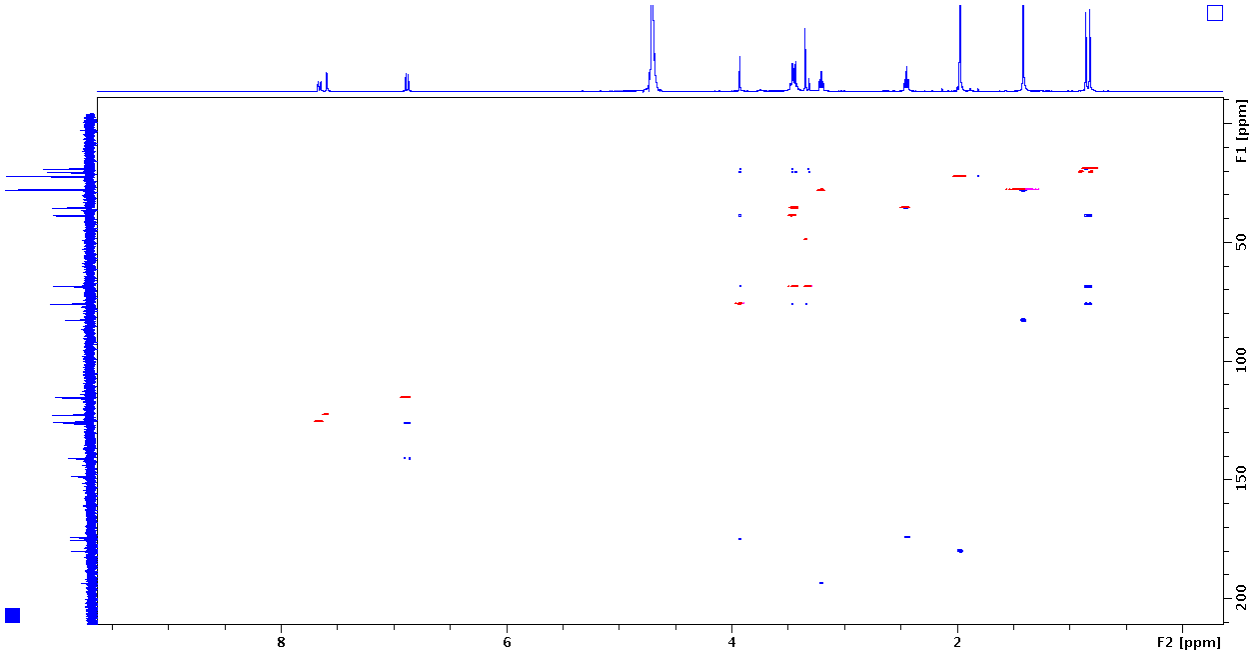


Figure S24. HSQC (red) and HMCB(blue) spectrum of compound **22c**

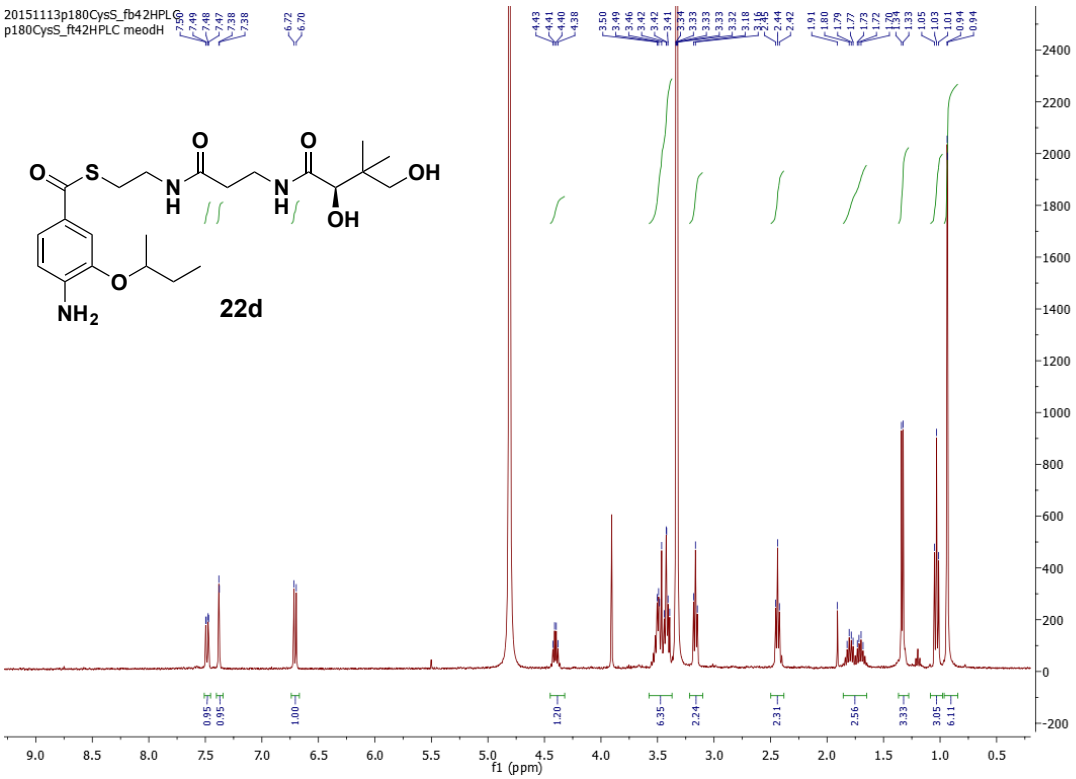


Figure S25. ^{1}H NMR (400 MHz, CD_3OD) spectrum of compound **22d**

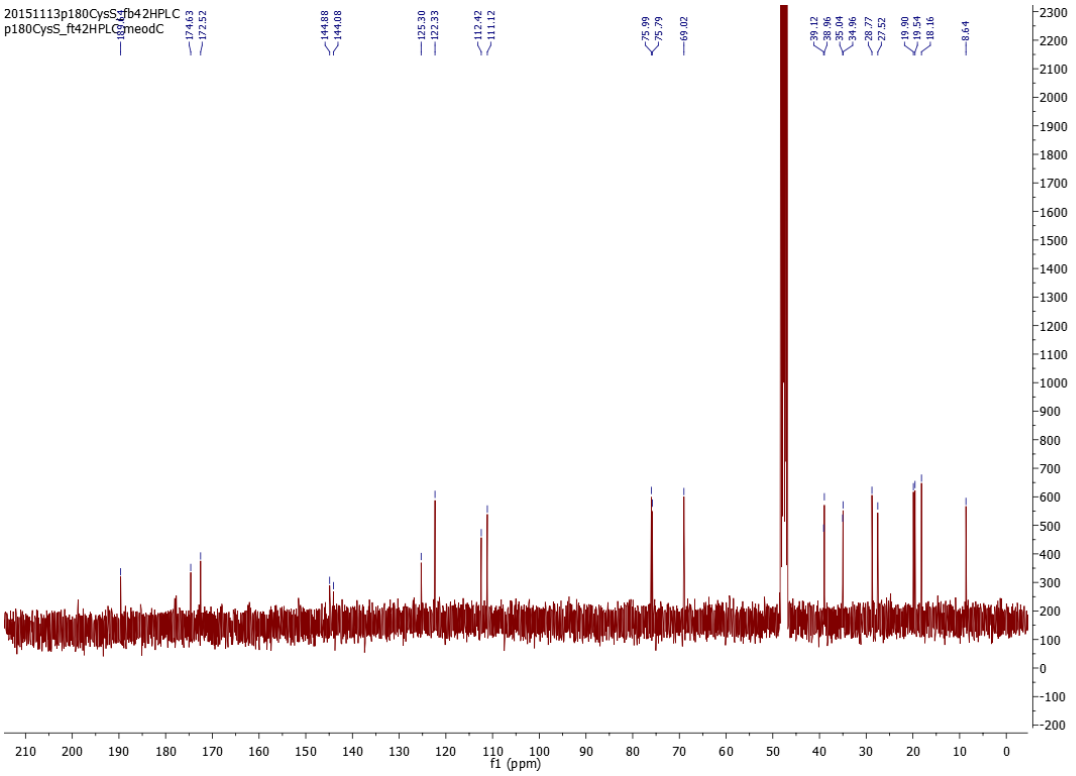


Figure S26. ^{13}C NMR (400 MHz, CD_3OD) spectrum of compound **22d**

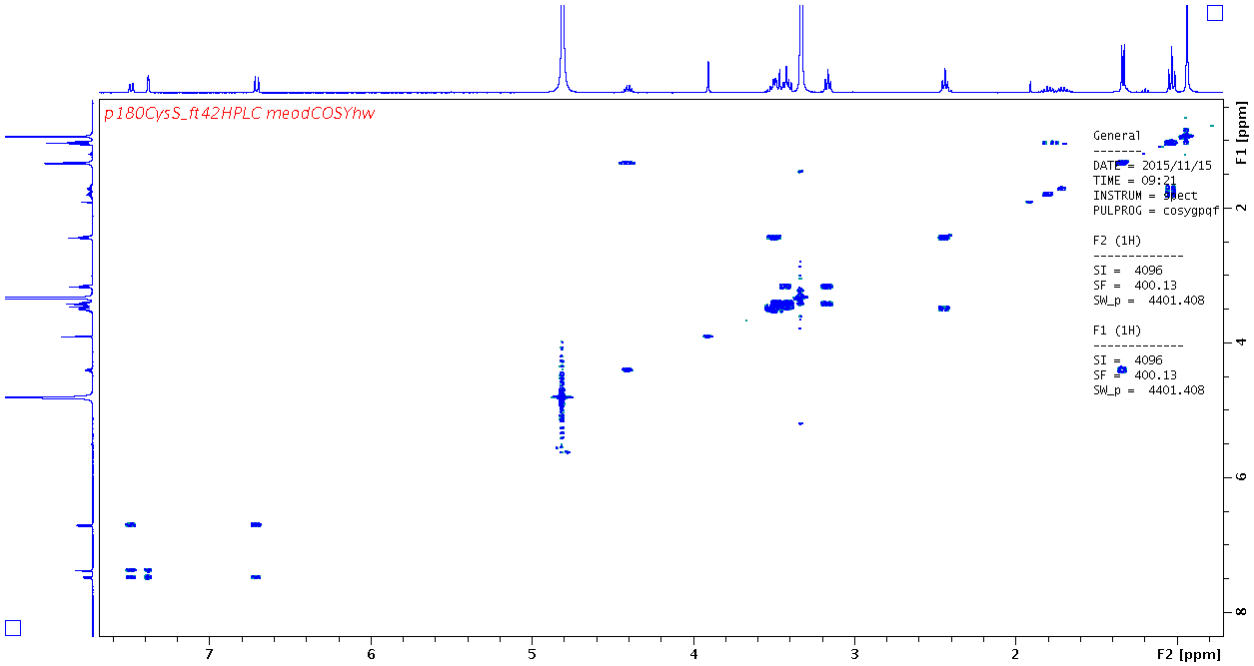


Figure S27. ^{1}H-COSY spectrum of compound **22d**

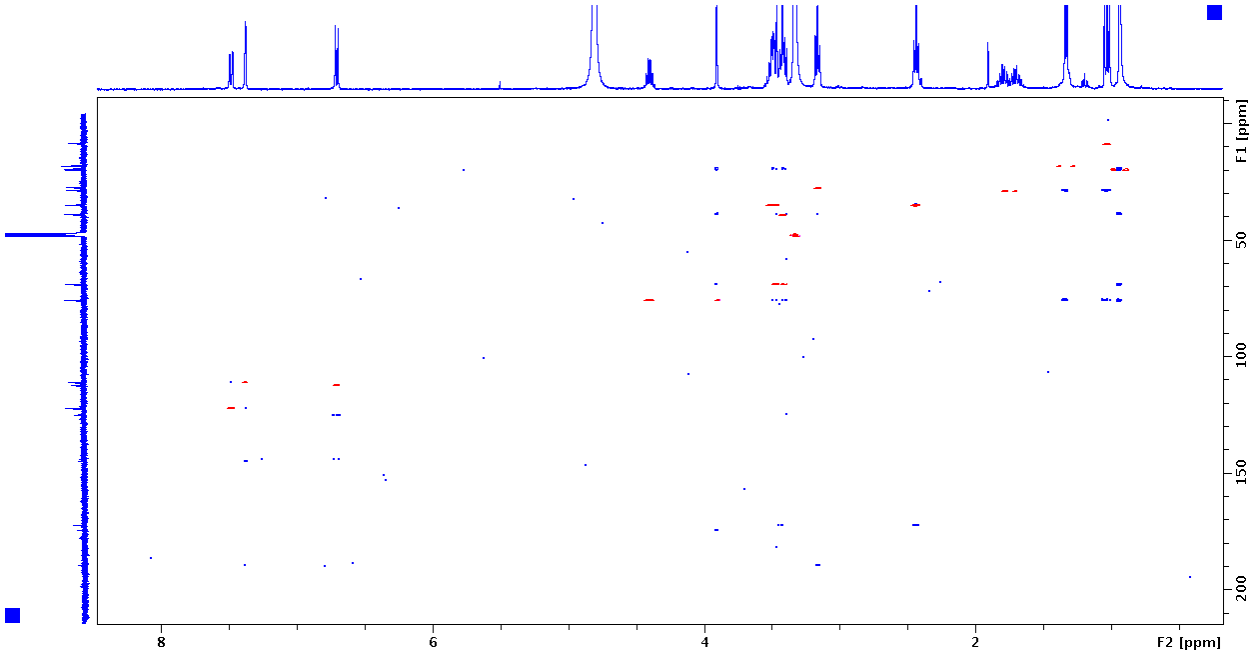


Figure S28. HSQC (red) and HMCB(blue) spectrum of compound **22d**

Reference:

(1) Baumann, S.; Herrmann, J.; Raju, R.; Steinmetz, H.; Mohr, K. I.; Huttel, S.; Harmrolfs, K.; Stadler, M.; Müller, R. *Angew. Chem., Int. Ed.* **2014,** *53*, 14605-9.
(2) Cooper, L. E.; Fedoseyenko, D.; Abdelwahed, S. H.; Kim, S. H.; Dairi, T.; Begley, T. P. *Biochemistry* **2013,** *52*, 4592-4.
(3) Mahanta, N.; Fedoseyenko, D.; Dairi, T.; Begley, T. P. *J. Am. Chem. Soc.* **2013,** *135*, 15318-15321.
(4) Moreno, M.; Elgaher, W. A. M.; Herrmann, J.; Schlager, N.; Hamed, M. M.; Baumann, S.; Müller, R.; Hartmann, R. W.; Kirschning, A. *Synlett* **2015,** *26*, 1175-1178.