

Supporting Information for

Alkylsulfenyl Thiocarbonates: Precursors to Hydropersulfides Potently Attenuate Oxidative Stress

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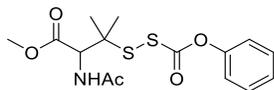
General Information

Analytical thin layer chromatography (TLC) was performed on silica gel on TLC Al foils with fluorescent indicator F254 plates (Sigma-Aldrich). Visualization was accomplished with UV light (254 nm) or staining with KMnO_4 . Starting materials, solvents, and reagents were received from commercial sources (Sigma-Aldrich, Oakwood Chemical, and TCI), unless otherwise noted and were used without purification. Deuterated solvents (Cambridge Isotope Laboratories) were used for NMR spectroscopic analyses. NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. In the case of ^1H NMR in CDCl_3 , chemical shifts are reported relative to tetramethylsilane ($\delta = 0$). The other spectra are referenced internally according to residual solvent signals of deuterated chloroform (^{13}C NMR; $\delta = 77.16$ ppm), and $\text{DMSO-}d_6$ (^1H NMR; $\delta = 2.50$ ppm, and ^{13}C NMR; $\delta = 39.52$ ppm). High-resolution mass spectra were obtained on a Waters Acquity Q-ToF MS/MS instrument. The kinetics of hydropersulfide release was monitored using a high-performance liquid chromatography (HPLC, Agilent 1100 series) system with a Phenomenex C-18 reverse phase column (250 mm \times 4.6 mm, 5 μm). UPLC-MS analysis was carried out with a Waters Acquity/Xevo-G2 UPLC-MS system equipped with ACQUITY UPLC BEH C18 column (2.1 mm \times 50 mm, 1.7 μm). The mass signals for products of RSSH trapping with *S*-methylmethanethiosulfonate (MMTS) were obtained via deconvolution using MassLynx 4.1 software. In addition to the protonated molecule $[\text{M}+\text{H}]^+$, we also observe $[\text{M}+\text{Na}]^+$ adducts during ESI-MS analysis. The pH measurements were performed using a Fisher Scientific Accumet AB15 pH-meter.

General procedure for synthesis of alkylsulfenyl thiocarbonates **1a-f**

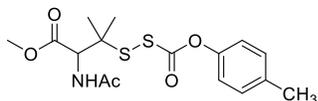
A solution of chlorocarbonylsulfenyl chloride (1.53 mmol, 1.05 equiv) in CH₂Cl₂ (5 mL) was added dropwise to a solution of *N*-acetyl penicillamine methyl ester (1.46 mmol, 1 equiv) in CH₂Cl₂ (10 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 1 h. The volatiles were removed under reduced pressure to obtain methyl-2-acetamido-3-((chlorocarbonyl)disulfaneyl)-3-methylbutanoate, which was used for the next step without further purification. To a mixture of para-substituted phenols **4a-f** (1.46 mmol, 1 equiv) and triethylamine (1.61 mmol, 1.1 equiv) in CH₂Cl₂ (10 mL), a solution of methyl-2-acetamido-3-((chlorocarbonyl)disulfaneyl)-3-methylbutanoate (1.46 mmol, 1 equiv) in CH₂Cl₂ (5 mL) was added dropwise at 0 °C. The resulting mixture was stirred at 0 °C until completion of the reaction (analyzed by TLC). The mixture was diluted with water and extracted with CH₂Cl₂ (10 mL × 3). The combined organic layer was washed with brine, dried over Na₂SO₄, and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel to afford the desired hydropersulfide precursors **1a-f**.

Methyl 2-acetamido-3-methyl-3-((phenoxy)carbonyl)disulfaneyl)butanoate (1a)



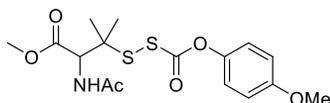
White solid (293 mg, 60%); ¹H NMR (400 MHz, CDCl₃) δ 7.42 – 7.34 (m, 2H), 7.29 – 7.25 (m, 1H), 7.20 – 7.17 (m, 2H), 6.63 (d, *J* = 9.2 Hz, 1H), 4.72 (d, *J* = 9.0 Hz, 1H), 3.77 (s, 3H), 2.03 (s, 3H), 1.51 (s, 3H), 1.42 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.4, 170.2, 169.5, 151.6, 129.7, 126.6, 121.0, 58.7, 53.5, 52.5, 26.3, 25.3, 23.2; HRMS (ESI) calcd. for C₁₅H₁₉NO₅S₂ ([M + H]⁺) 358.0777, found: 358.0779.

Methyl 2-acetamido-3-methyl-3-((p-tolyloxy)carbonyl)disulfaneyl)butanoate (1b)



White solid (454 mg, 84%); ¹H NMR (400 MHz, CDCl₃) δ 7.18 (d, *J* = 8.2 Hz, 2H), 7.06 (d, *J* = 8.5 Hz, 2H), 6.65 (d, *J* = 8.9 Hz, 1H), 4.71 (d, *J* = 8.9 Hz, 1H), 3.77 (s, 3H), 2.35 (s, 3H), 2.03 (s, 3H), 1.51 (s, 3H), 1.41 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.5, 170.3, 169.7, 149.5, 136.5, 130.2, 120.7, 58.8, 53.5, 52.5, 26.4, 25.5, 23.2, 21.0; HRMS (ESI) calcd. for C₁₆H₂₁NO₅S₂ ([M + H]⁺) 372.0934, found: 372.0934.

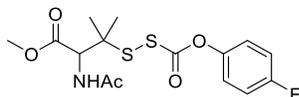
Methyl 2-acetamido-3-(((4-methoxyphenoxy)carbonyl)disulfaneyl)-3-methylbutanoate (1c)



White solid (407 mg, 86%); ¹H NMR (400 MHz, CDCl₃) δ 7.10 (d, *J* = 9.2 Hz, 2H), 6.89 (d, *J* = 9.2 Hz, 2H), 6.65 (d, *J* = 8.9 Hz, 1H), 4.71 (d, *J* = 8.9 Hz, 1H), 3.80 (s, 3H), 3.76 (s, 3H), 2.03 (s, 3H), 1.50 (s, 3H), 1.41 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.4, 170.2, 169.8, 157.9, 145.2,

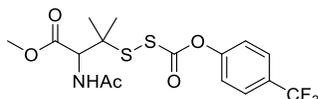
121.9, 114.7, 58.7, 55.7, 53.5, 52.5, 26.4, 25.4, 23.2; HRMS (ESI) calcd. for C₁₆H₂₁NO₆S₂ ([M + H]⁺ 388.0883, found: 388.0880.

Methyl 2-acetamido-3-(((4-fluorophenoxy)carbonyl)disulfaneyl)-3-methylbutanoate (1d)



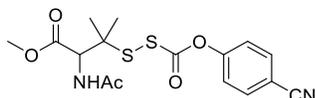
White solid (363 mg, 65%); ¹H NMR (400 MHz, CDCl₃) δ 7.18 - 7.14 (m, 2H), 7.12 - 7.03 (m, 2H), 6.60 (d, *J* = 8.9 Hz, 1H), 4.72 (d, *J* = 9.0 Hz, 1H), 3.77 (s, 3H), 2.03 (s, 3H), 1.50 (s, 3H), 1.41 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.5, 170.2, 169.7, 160.65 (d, *J* (CF) = 245.5 Hz), 147.4 (d, *J* (CF) = 3.0 Hz), 122.6 (d, *J* (CF) = 8.5 Hz), 116.4 (d, *J* (CF) = 23.7 Hz), 58.6, 53.6, 52.5, 26.4, 25.3, 23.2; HRMS (ESI) calcd for C₁₅H₁₈FNO₅S₂ ([M + H]⁺ 376.0683, found: 376.0684.

Methyl 2-acetamido-3-methyl-3-(((4-(trifluoromethyl)phenoxy)carbonyl)disulfaneyl)butanoate (1e)



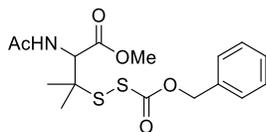
White solid (524 mg, 84%); ¹H NMR (400 MHz, CDCl₃) δ 7.67 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 8.6 Hz, 2H), 6.58 (d, *J* = 8.8 Hz, 1H), 4.75 (d, *J* = 9.1 Hz, 1H), 3.77 (s, 3H), 2.04 (s, 3H), 1.50 (s, 3H), 1.42 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.5, 170.2, 169.3, 153.8, 129.4, 129.0, 128.7, 128.4, 127.2, 125.1, 121.6, 58.5, 53.7, 52.5, 26.3, 25.2, 23.2; HRMS (ESI) calcd. for C₁₆H₁₈F₃NO₅S₂ ([M + H]⁺ 426.0651, found: 358.0.651.

Methyl 2-acetamido-3-(((4-cyanophenoxy)carbonyl)disulfaneyl)-3-methylbutanoate (1f)



White solid (317 mg, 75%); ¹H NMR (400 MHz, CDCl₃) δ 7.71 (d, *J* = 8.9 Hz, 2H), 7.35 (d, *J* = 8.9 Hz, 2H), 6.53 (d, *J* = 9.0 Hz, 1H), 4.75 (d, *J* = 9.0 Hz, 1H), 3.77 (s, 3H), 2.04 (s, 3H), 1.50 (s, 3H), 1.42 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.5, 170.2, 169.0, 134.0, 122.2, 118.0, 110.6, 58.4, 53.8, 52.5, 26.2, 25.0, 23.2; HRMS (ESI) calcd. for C₁₆H₁₈N₂O₅S₂ ([M + H]⁺ 383.0730, found: 383.0731.

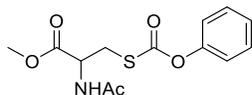
Methyl 2-acetamido-3-(((benzyloxy)carbonyl)disulfaneyl)-3-methylbutanoate (8)



This compound was prepared according to the procedure used for precursors **1a-f** synthesis.

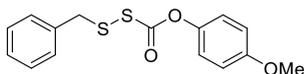
White solid (65 mg, 12%); ^1H NMR (400 MHz, CDCl_3) δ 7.46 - 7.37 (m, 5H), 6.69 (d, J = 8.4 Hz, 1H), 5.41 - 5.23 (m, 2H), 4.65 (d, J = 8.9 Hz, 1H), 3.73 (s, 3H), 2.03 (s, 3H), 1.45 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3) δ 170.44, 170.39, 170.3, 134.6, 129.1, 128.9, 128.8, 71.1, 58.9, 53.2, 52.5, 26.3, 25.5, 23.2; HRMS (ESI) calcd. for $\text{C}_{16}\text{H}_{21}\text{N}_2\text{O}_5\text{S}_2$ ($[\text{M} + \text{H}]^+$) 372.0934, found: 372.0939.

Methyl N-acetyl-S-(phenoxycarbonyl)cysteinate (9a)



To a stirred solution of *N*-acetyl cysteine methyl ester (1 g, 5.64 mmol) in dichloromethane (10 mL), phenylchloroformate (1.33 g, 8.46 mmol) and triethylamine (571 mg, 5.64 mmol) was added at 0 °C. The resulting mixture was allowed to stir at room temperature for 12 h. The reaction was quenched with saturated aq. NH_4Cl (20 mL), and the resulting mixture was extracted with EtOAc (20 mL \times 3). The organic layer was washed with water (20 mL) and brine (20 mL) sequentially. The combined organic phases were dried over anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. Purification by column chromatography afforded compound **9a** (769 mg, 46%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 7.40 - 7.36 (m, 2H), 7.27 - 7.25 (m, 1H), 7.14 - 7.12 (m, 2H), 6.47 (d, J = 7.5 Hz, 1H), 4.91 (ddd, J = 7.6, 5.8, 4.8 Hz, 1H), 3.76 (s, 3H), 3.44 (ddd, J = 20.3, 14.4, 5.3 Hz, 2H), 2.03 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3) δ 170.7, 170.1, 169.7, 151.2, 129.6, 126.4, 121.1, 52.9, 52.0, 33.2, 23.1; HRMS (ESI) calcd. for $\text{C}_{13}\text{H}_{15}\text{NO}_5\text{S}$ ($[\text{M} + \text{H}]^+$) 298.0744, found: 298.0751.

SS-benzyl O-(4-methoxyphenyl) carbonodithioperoxoate (13)



A solution of chlorocarbonylsulfenyl chloride (1.05 g, 8.05 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a solution of benzyl mercaptan (1 g, 8.05 mmol) in CH_2Cl_2 (10 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 1 h. The mixture was added dropwise to a mixture of 4-methoxyphenol (670 mg, 5.40 mmol) and *N,N*-diisopropylethylamine (1.05 g, 8.05 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The resulting mixture was stirred at 0 °C for 1 h. The mixture was quenched with water and extracted with CH_2Cl_2 (10 mL \times 3). The combined organic layer was washed with brine, dried over Na_2SO_4 , and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel to afford the compound **13** (1.37g, 83%) as a colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 7.37 - 7.26 (m, 5H), 7.03 (d, J = 9.2 Hz, 2H), 6.87 (d, J = 9.2 Hz, 2H), 4.05 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3) δ 168.9, 157.8, 145.2, 135.7, 129.7, 128.8, 128.0, 121.9, 114.6, 55.7, 43.3.

RSSH generation from **1a** in the presence of β -(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM)

RSSH precursor **1a** and HPE-IAM were dissolved in DMSO to afford a 10 mM and 100 mM stock, respectively. Briefly, HPE-IAM (30 μ L, 100 mM) was added in pH 7.4 ammonium bicarbonate buffer (50 mM, 2.94 mL) containing DTPA (100 μ M) as metal chelator. The mixture was preincubated for 10 min at 37 $^{\circ}$ C. Precursor **1a** (30 μ L, 10 mM) was added into the mixture and incubated at 37 $^{\circ}$ C for 2 h. An aliquot of the reaction mixture (500 μ L) was withdrawn and quenched with 500 μ L of 1% formic acid solution and analyzed by UPLC–MS as follows: Mobile phase: 0–1 min 90% water + 0% ACN + 10% of 0.1% formic acid (v/v) in water; 1–7.5 min gradient up to 10% water + 80% ACN + 10% of 0.1% formic acid (v/v) in water; 7.5–8.4 min 10% water + 80% ACN + 10% of 0.1% formic acid (v/v) in water; 8.4–8.5 min gradient up to 90 % water + 0% ACN + 10% of 0.1% formic acid (v/v) in water, 8.5–10 min 90% water + 0% ACN + 10% of 0.1% formic acid (v/v) in water. Flow rate = 0.3 mL/min. These studies were conducted at least in triplicate and representative spectra are presented.

HRMS spectra of RSS-HPE-AM and dialkyl trisulfide formed during incubation of **1a** with HPE-IAM

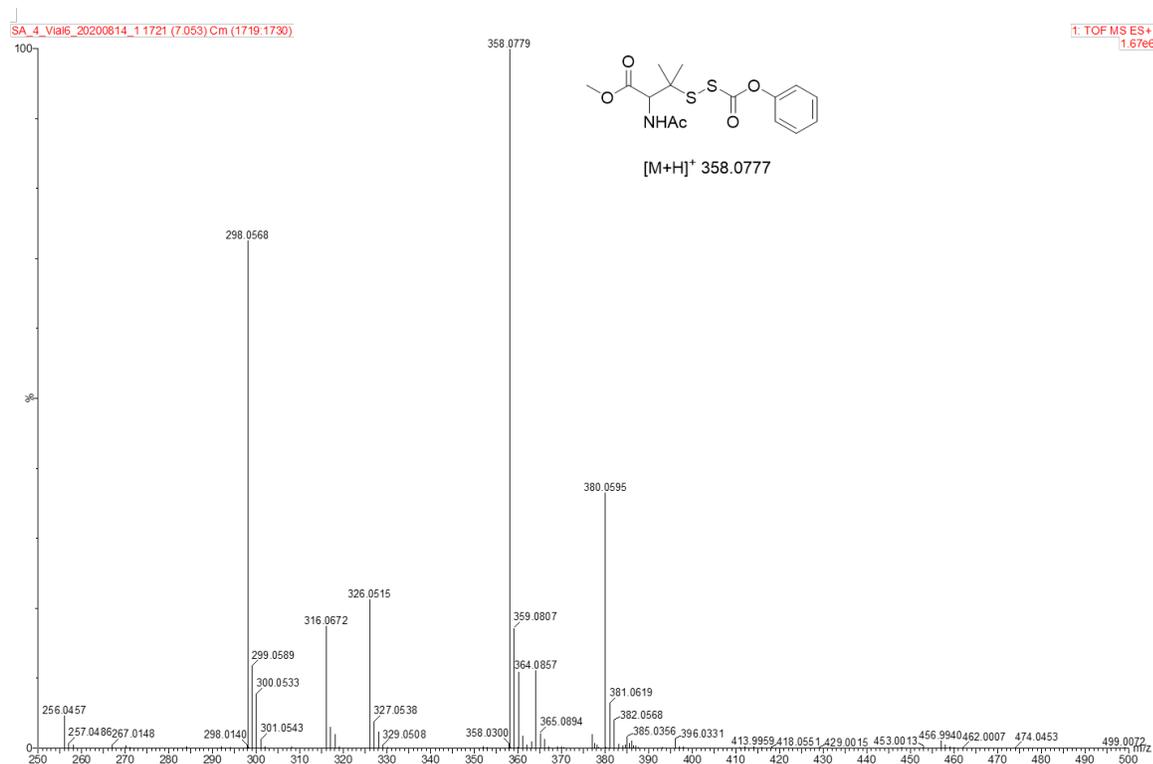


Figure S1. HRMS of the peak eluting at 7.05 min corresponding to RSSH precursor **1a**.

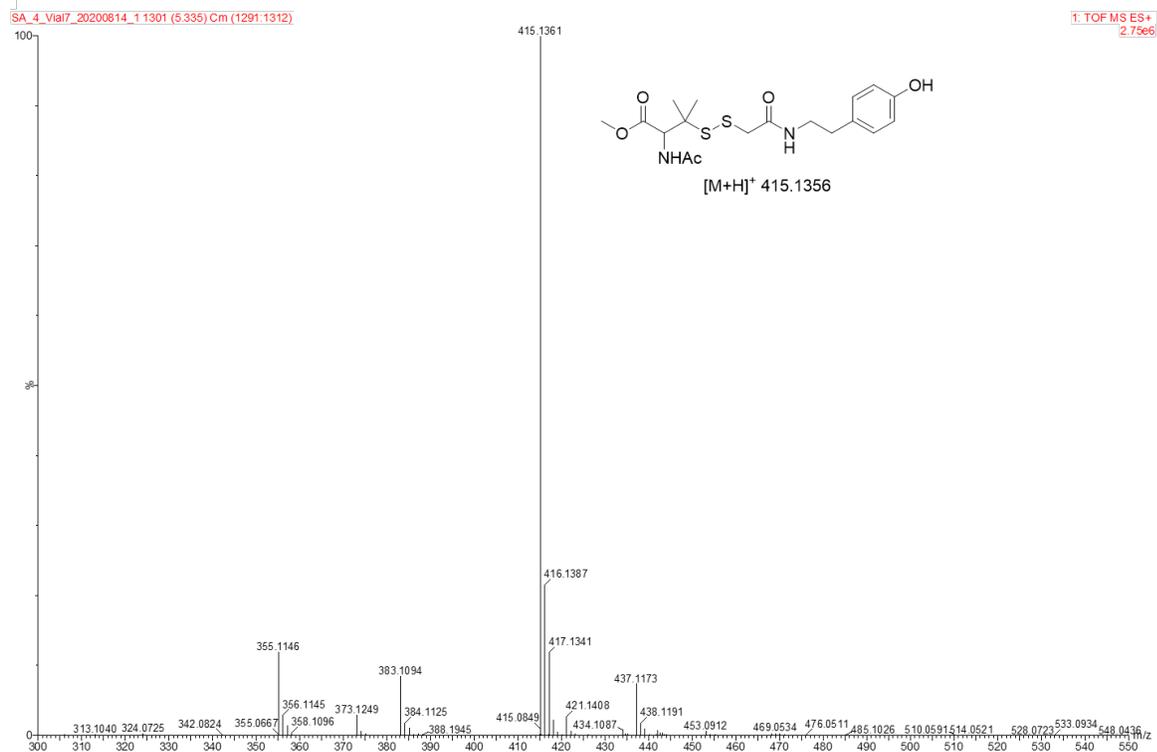


Figure S2. HRMS of peak eluting at 5.34 min corresponding to RSS-HPE-AM 5.

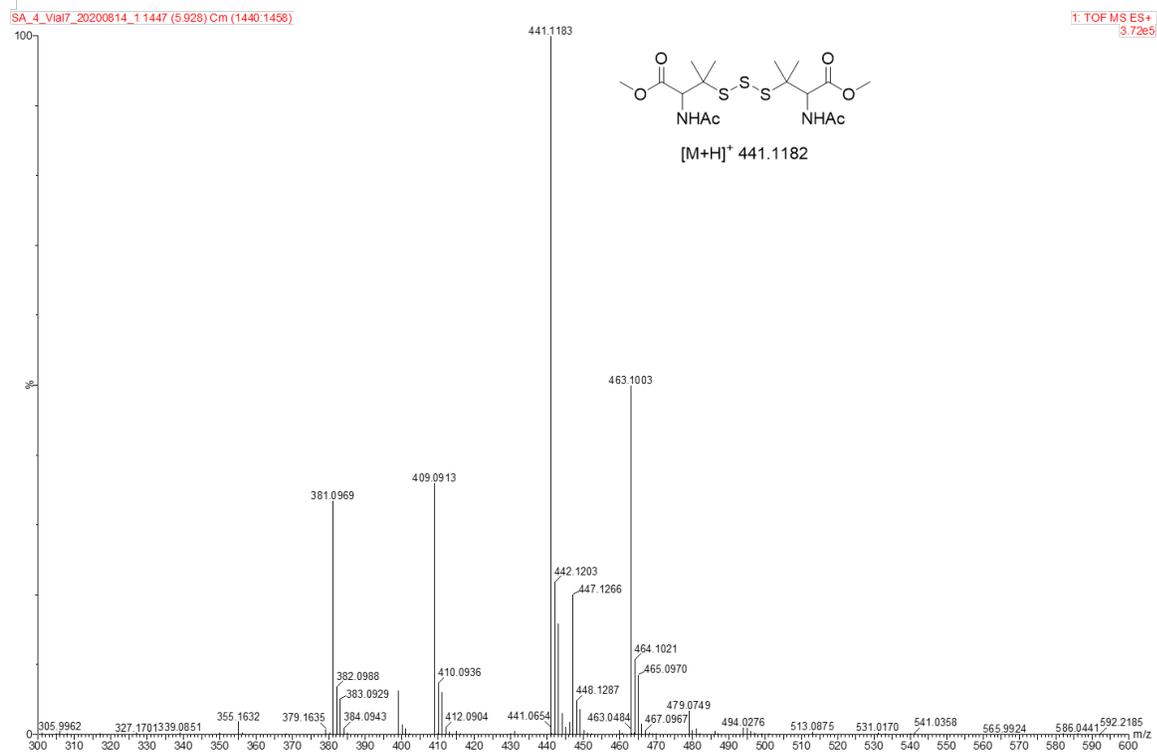
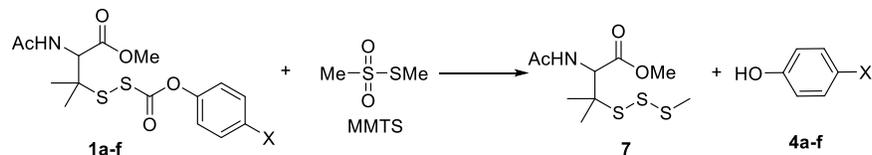


Figure S3. HRMS of the peak eluting at 5.93 min corresponding to symmetrical dialkyl trisulfide (S₃).

RSSH generation from **1a-f** in the presence of *S*-methyl methanethiosulfonate (MMTS)

In general, RSSH precursors **1a-f** and MMTS were dissolved in DMSO to afford a 10 mM and 100 mM stock, respectively unless stated otherwise. Briefly, MMTS (30 μ L, 100 mM) was added in pH 7.4 ammonium bicarbonate buffer (50 mM, 2.94 mL) containing DTPA (100 μ M) as metal chelator. The resulting solution was pre-incubated at 37 $^{\circ}$ C for 10 min. Precursor **1a-f** (30 μ L, 10 mM) were independently added into the mixture and incubated at 37 $^{\circ}$ C. An aliquot of the reaction mixture (500 μ L) was withdrawn and quenched with 500 μ L of 1% formic acid solution and analyzed using UPLC-MS.



HRMS spectra of RSS-S-Me **7** formed during incubation of **1a-f** with MMTS

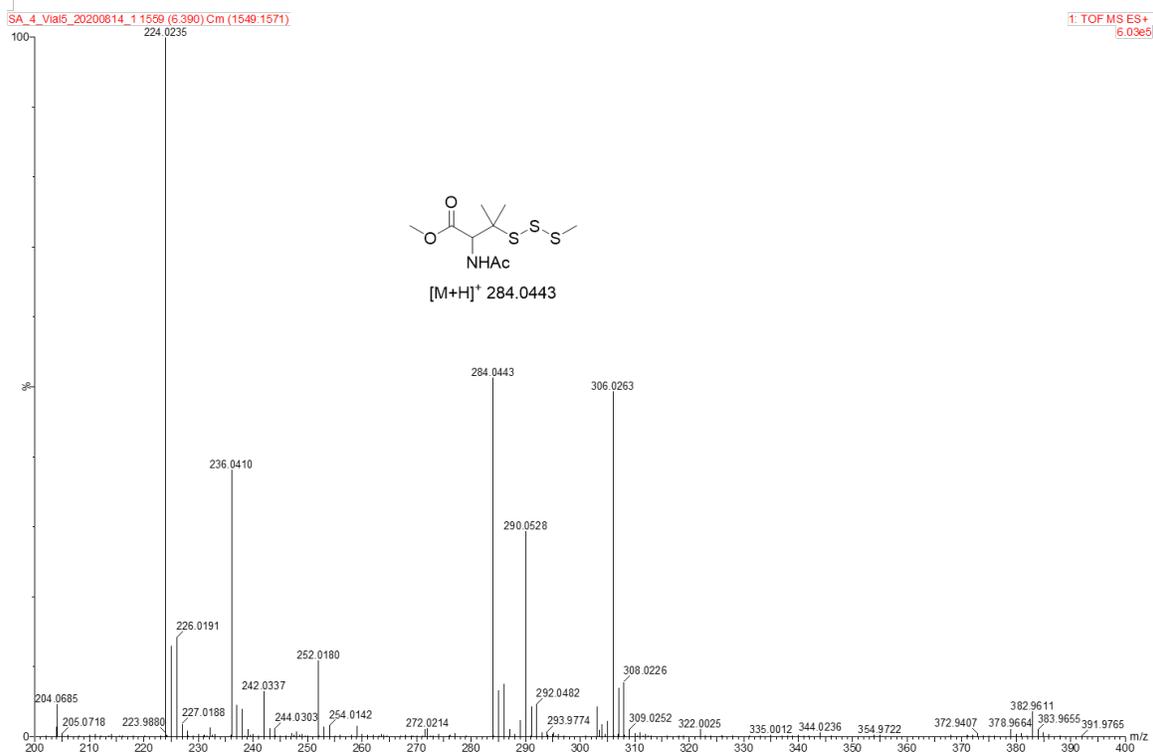


Figure S4. HRMS of peak eluting at 6.39 min corresponding to RSS-S-Me **7**.

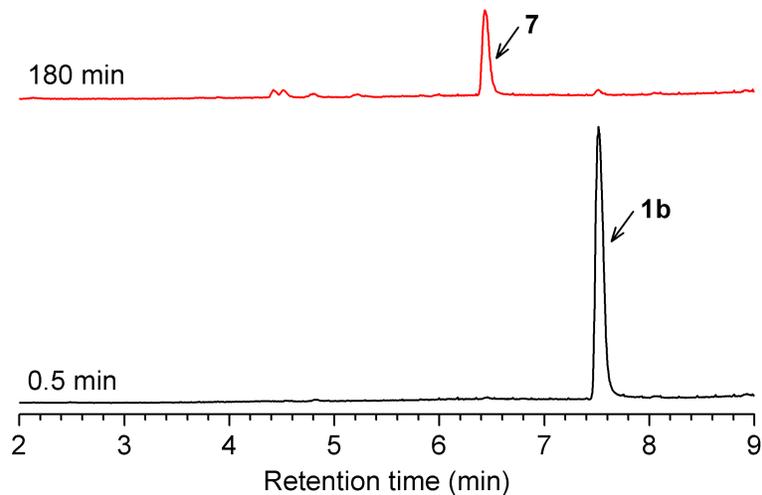


Figure S5. RSSH generation from **1b** (100 μ M) in the presence of MMTS (1 mM) in pH 7.4 ammonium bicarbonate buffer (50 mM) incubated for 180 min at 37 $^{\circ}$ C.

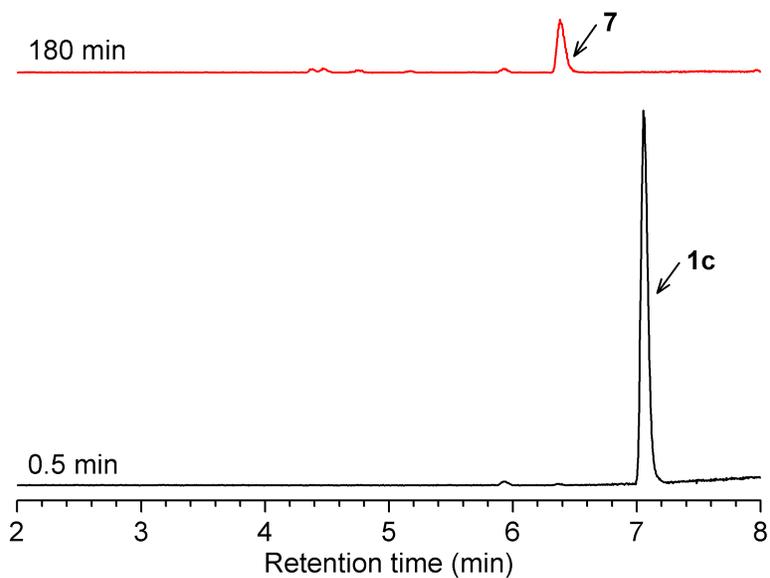


Figure S6. RSSH generation from **1c** (100 μ M) in the presence of MMTS (1 mM) in pH 7.4 ammonium bicarbonate buffer (50 mM) incubated for 120 min at 37 $^{\circ}$ C.

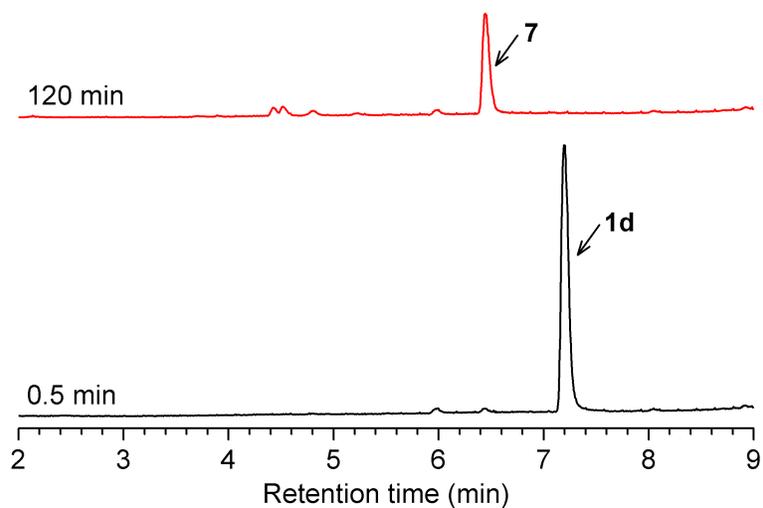


Figure S7. RSSH generation from **1d** (100 μ M) in the presence of MMTS (1 mM) in pH 7.4 ammonium bicarbonate buffer (50 mM) incubated for 120 min at 37 $^{\circ}$ C.

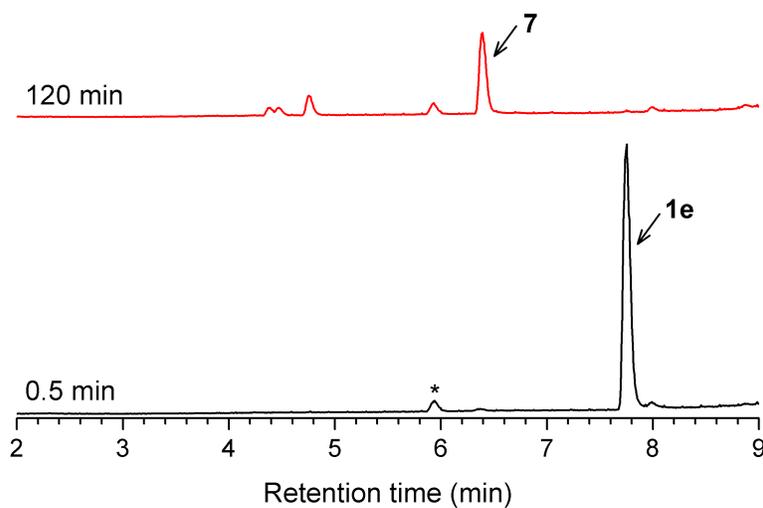


Figure S8. RSSH generation from **1e** (100 μ M) in the presence of MMTS (1 mM) in pH 7.4 ammonium bicarbonate buffer (50 mM) incubated for 120 min at 37 $^{\circ}$ C. Asterisk indicates a small amount of dialkyl trisulfide generation presumably due to the decomposition of **1e** prior to incubation with MMTS.

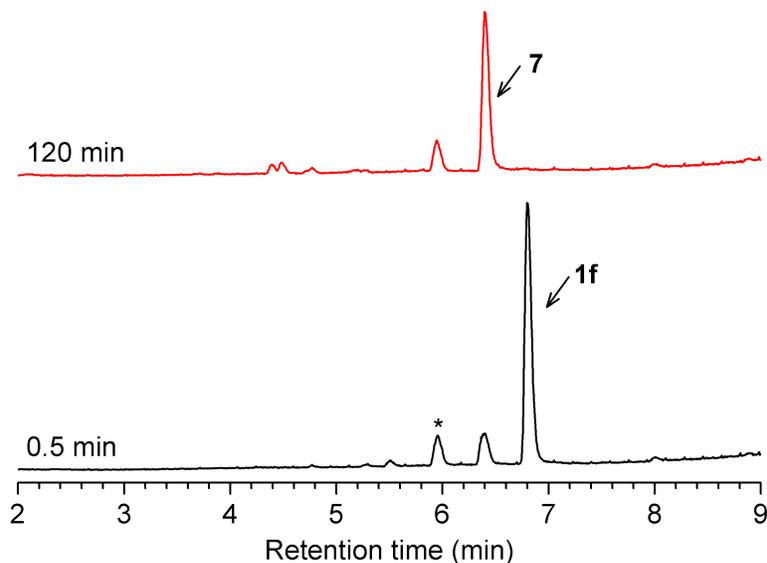


Figure S9. RSSH generation from **1f** (100 μ M) in the presence of MMTS (1 mM) in pH 7.4 ammonium bicarbonate buffer (50 mM) incubated for 120 min at 37 $^{\circ}$ C. Asterisk indicates a small amount of dialkyl trisulfide generation presumably due to the decomposition of **1f** prior to incubation with MMTS.

Kinetics of RSSH release from precursors 1a-f and 8 monitored by HPLC

MMTS (100 μ L, 50 mM) was added in pH 7.4 phosphate buffer saline (4.85 mL) containing the DTPA (100 μ M) as metal chelator. This solution was preincubated for 10 min at 37 $^{\circ}$ C and then precursor **1a-f** or **8** (50 μ L, 10 mM) were independently added into the mixture (total volume = 5 mL). The resulting solution was incubated at 37 $^{\circ}$ C. At different time points, an aliquot of 300 μ L was taken and quenched with 300 μ L 0.1% formic acid solution. These samples were stored at 0 $^{\circ}$ C until HPLC analysis was performed. The sample (20 μ L) was injected into a high-performance liquid chromatography (HPLC) equipped with Phenomenex C-18 reverse phase column (250 mm \times 4.6 mm, 5 μ m). HPLC Method: mobile phase A (H_2O) and mobile phase B (ACN), flow rate: 1 mL/min, run time: 21 min, the gradient elution method: 10% to 70% B from 0 to 10 min, 70% to 90% B from 10 to 21 min. The elution was monitored by a UV detector at 240 and 275 nm. First-order rate constants were obtained by plotting the precursor decay and phenol byproduct formation as a function of time.

Decomposition of **1a** and **8** in the presence of *N*-acetyl cysteine methyl ester

To a solution of *N*-acetyl cysteine methyl ester (500 μ M) in pH 7.4 phosphate buffer saline (100 mM) containing DTPA (100 μ M), precursor **1a** or **8** (100 μ M) was added. The resulting mixture was incubated at 37°C. At different time points, an aliquot of the reaction mixture (500 μ L) was withdrawn and transferred to pre-cooled 0.1% formic acid (500 μ L) and analyzed using HPLC. First-order rate constants were obtained by plotting the precursor decay as a function of time.

RSSH generation from **1a** in the presence of *N*-acetyl cysteine analyzed by UPLC-MS

To a solution of *N*-acetyl cysteine (500 μ M) in pH 7.4 ammonium bicarbonate buffer (50 mM) containing DTPA (100 μ M), precursors **1a** (100 μ M) was added. The resulting mixture was incubated at 37°C. An aliquot (200 μ L) of reaction mixture was withdrawn at specified time points and quenched with 1% formic acid (200 μ L). These samples were stored at 0 °C until UPLC-MS analysis was performed.

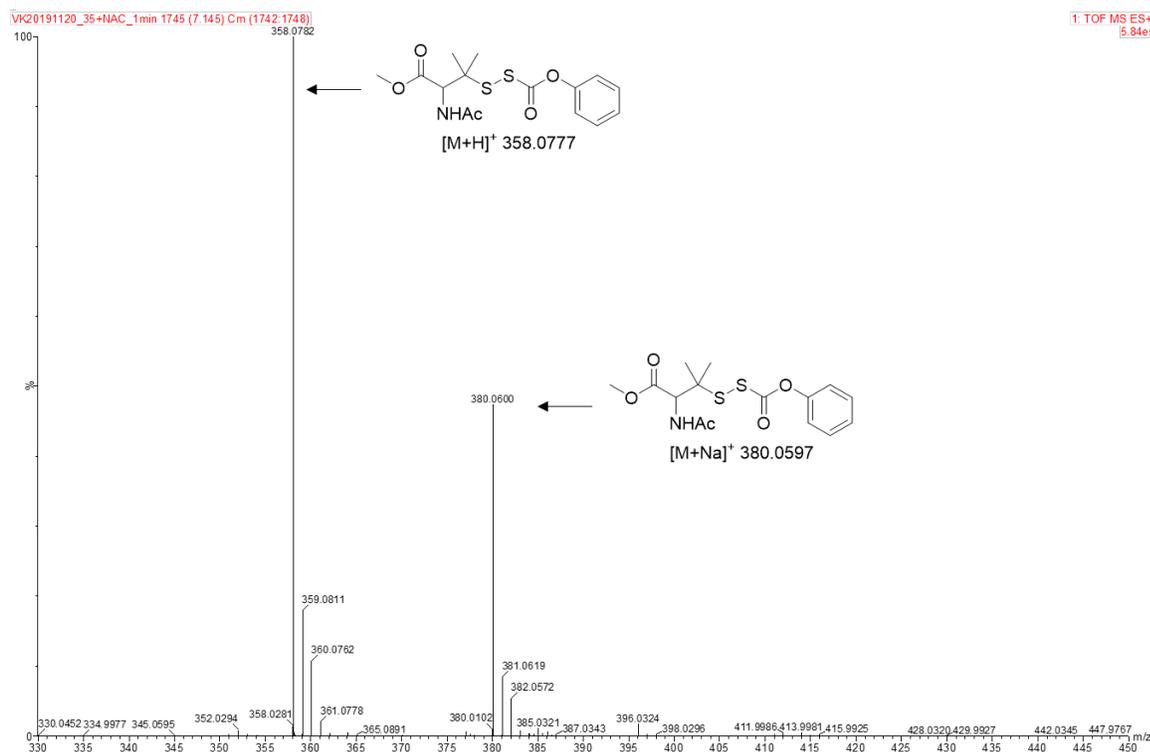


Figure S10. HRMS of the peak eluting at 7.15 min corresponding to RSSH precursor **1a**.

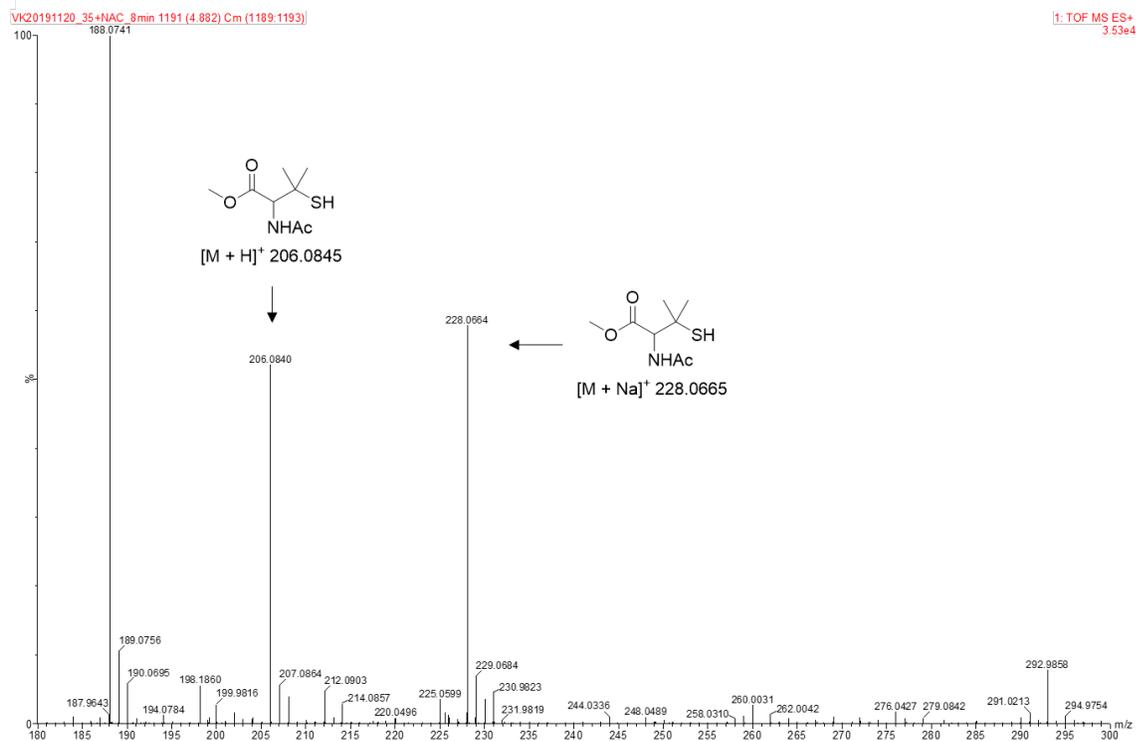


Figure S11. HRMS of the peak eluting at 4.88 min corresponding to *N*-acetyl penicillamine methyl ester.

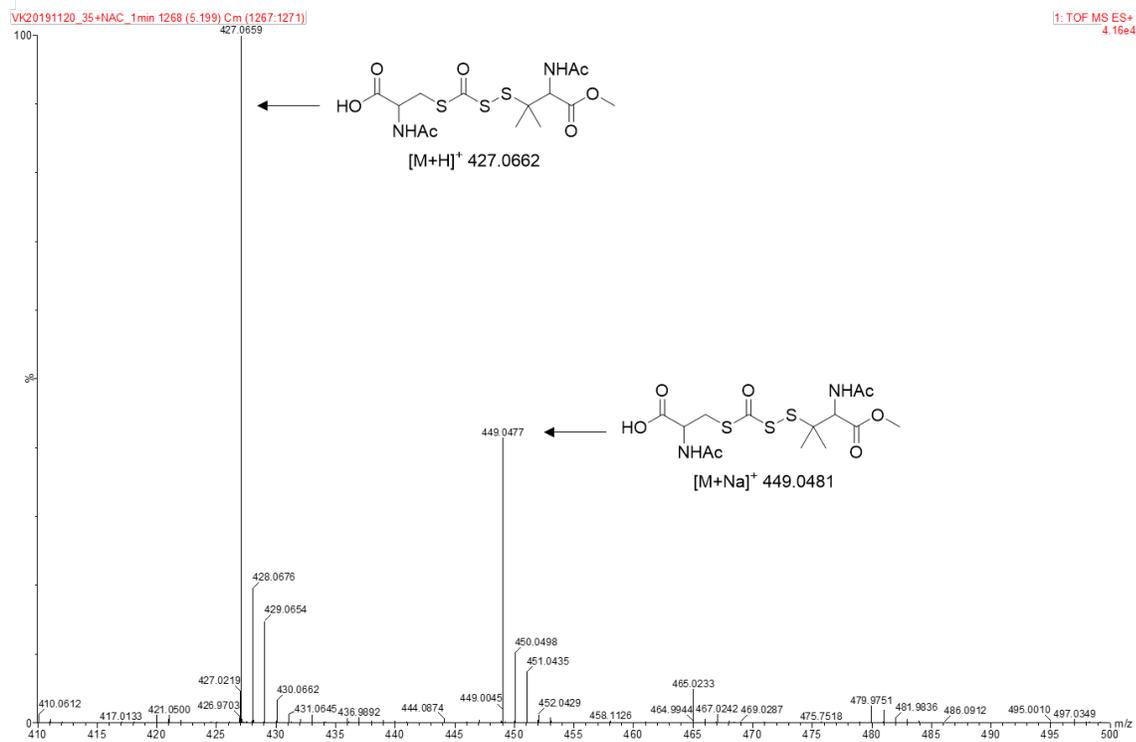


Figure S12. HRMS of the peak eluting at 5.20 min corresponding to **12**.

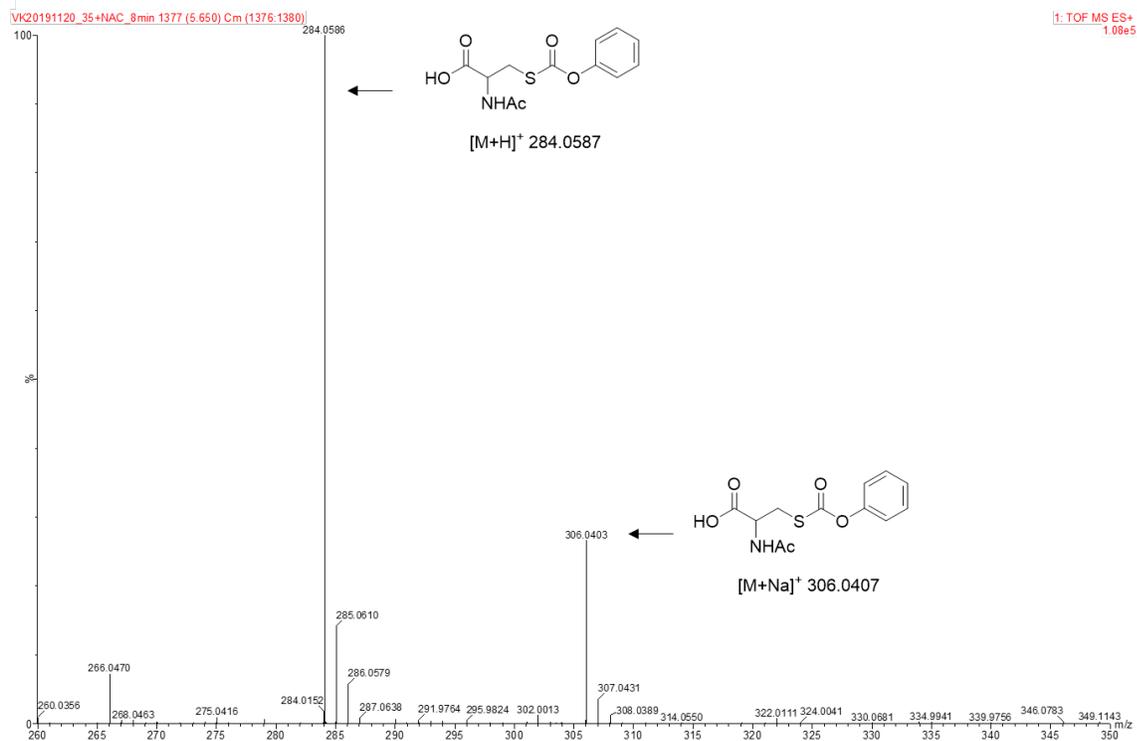


Figure S13. HRMS of the peak eluting at 5.65 min corresponding to the byproduct thiocarbonate **9a**.

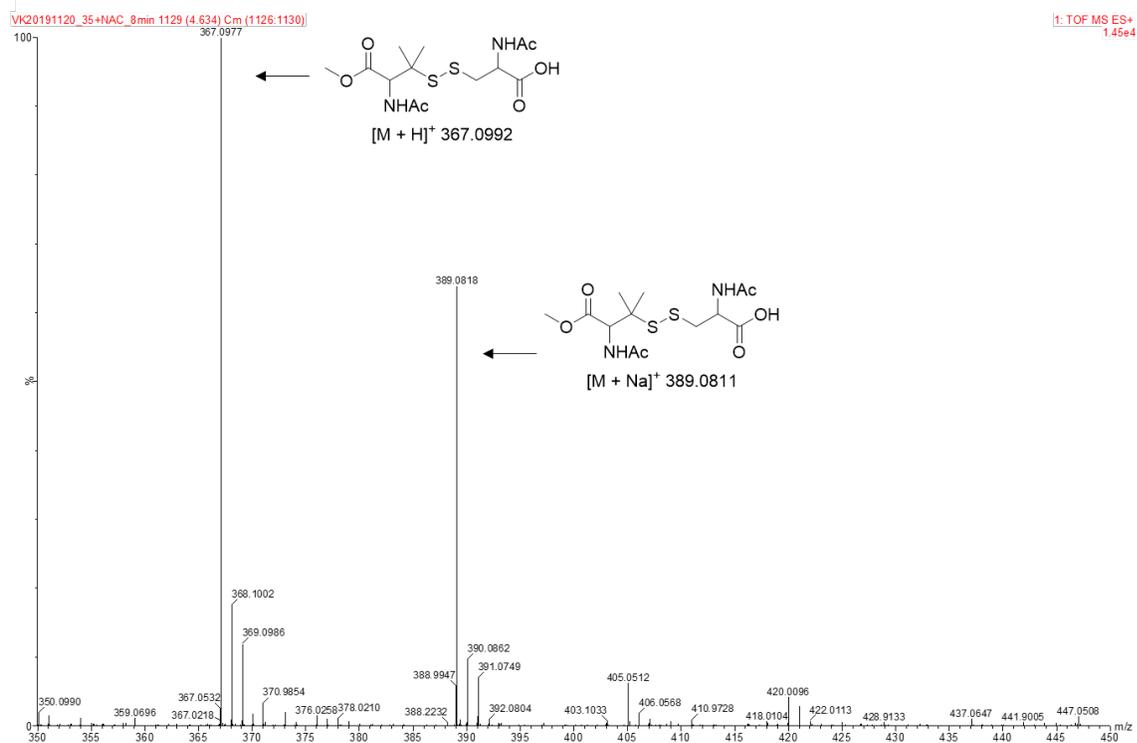


Figure S14. HRMS of the peak eluting at 4.63 min corresponding to mixed disulfide R^1S-SR^2 (S_2).

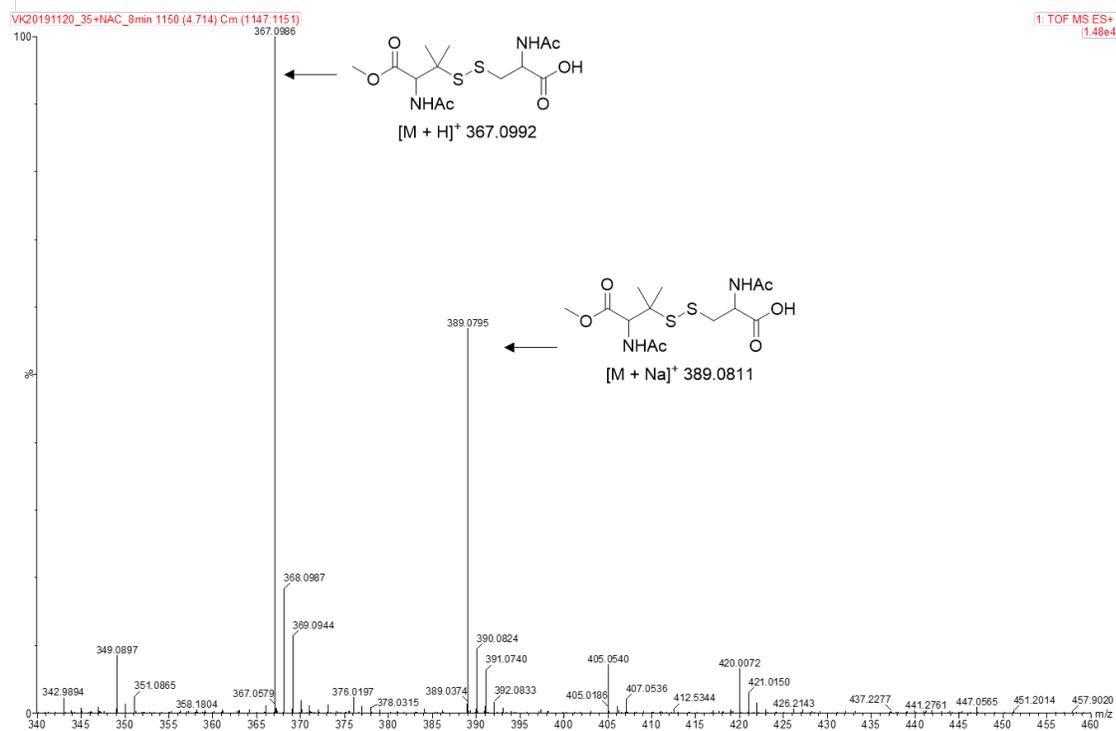


Figure S15. HRMS of the peak eluting at 4.71 min corresponding to other diastereomer of R^1S-SR^2 ($'S_2$).

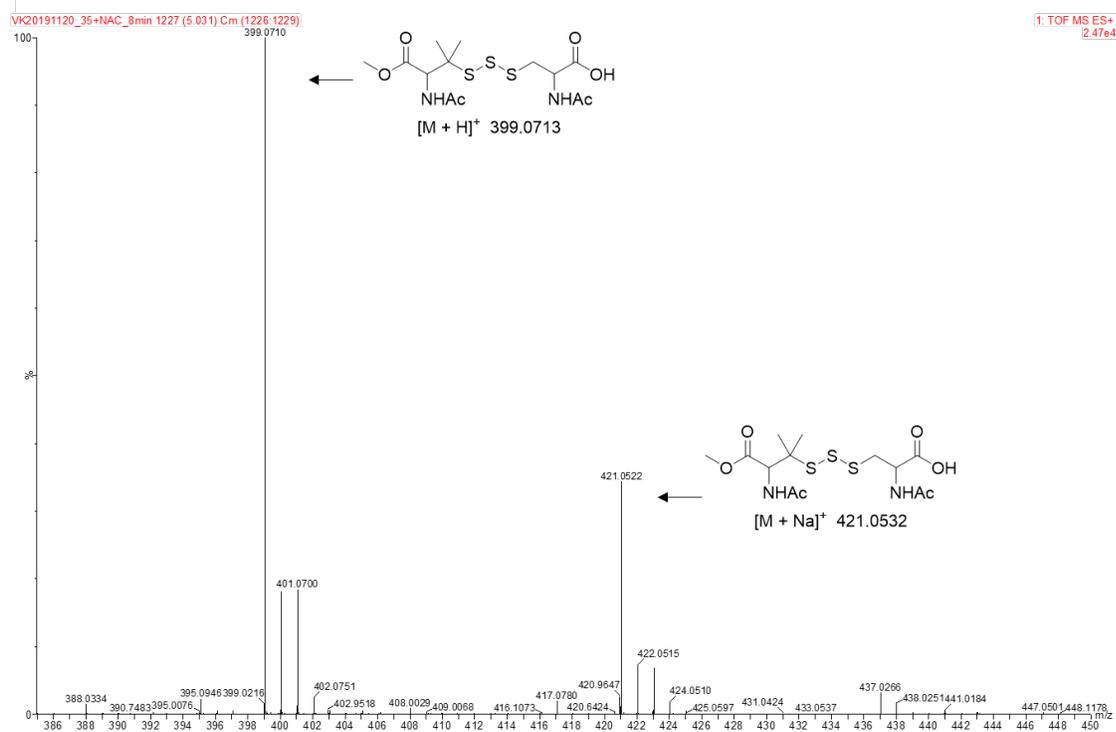


Figure S16. HRMS of the peak eluting at 5.03 min corresponding to unsymmetrical dialkyl trisulfide R^1SSSR^2 ($'S_3$).

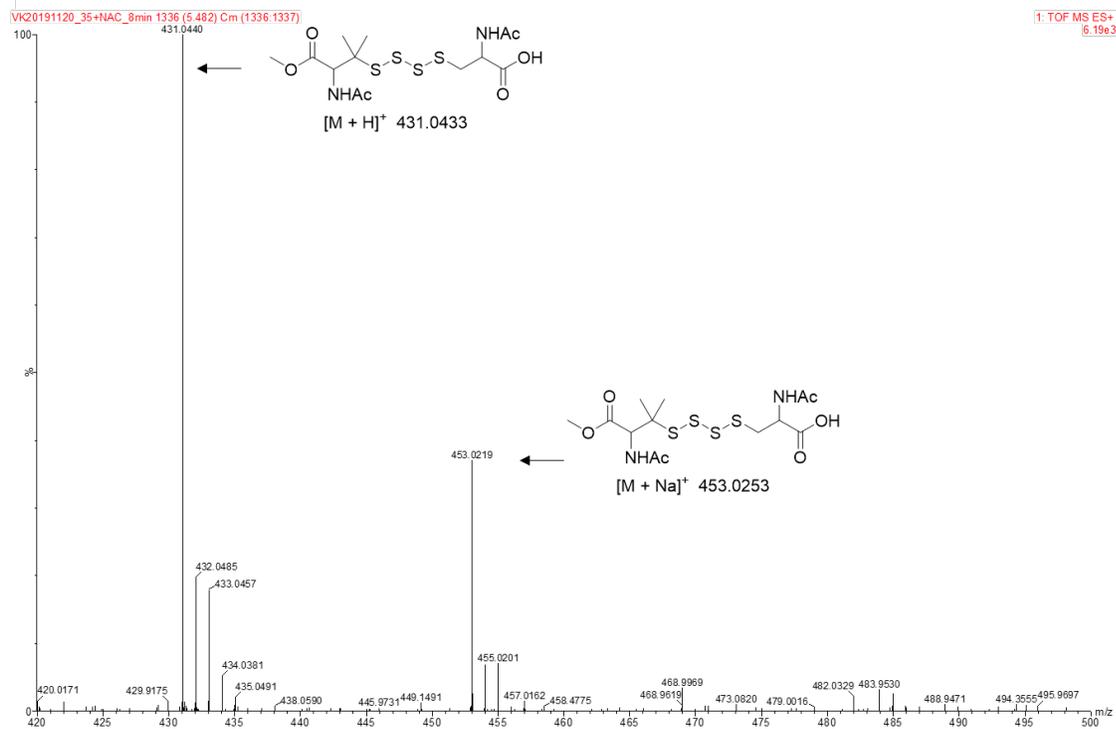


Figure S17. HRMS of the peak eluting at 5.48 min corresponding to unsymmetrical dialkyl tetrasulfide R^1SSSSR^2 (4S_4).

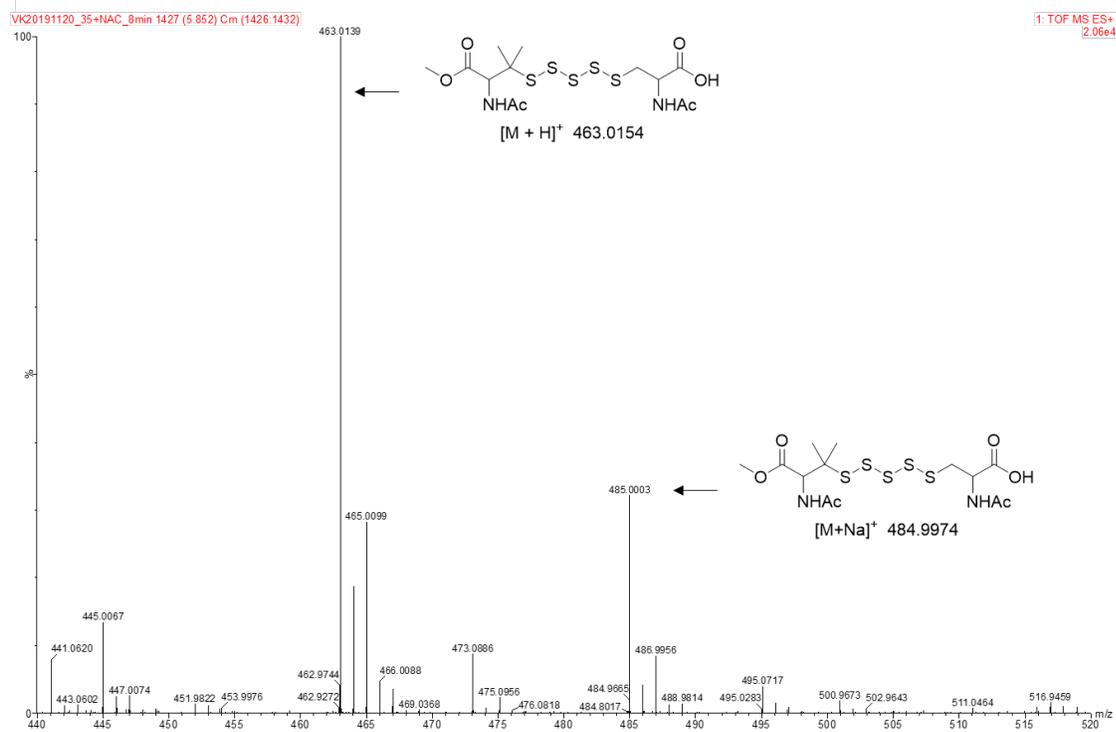


Figure S18. HRMS of the peak eluting at 5.85 min corresponding to unsymmetrical dialkyl pentasulfide $R^1SSSSSR^2$ (5S_5).

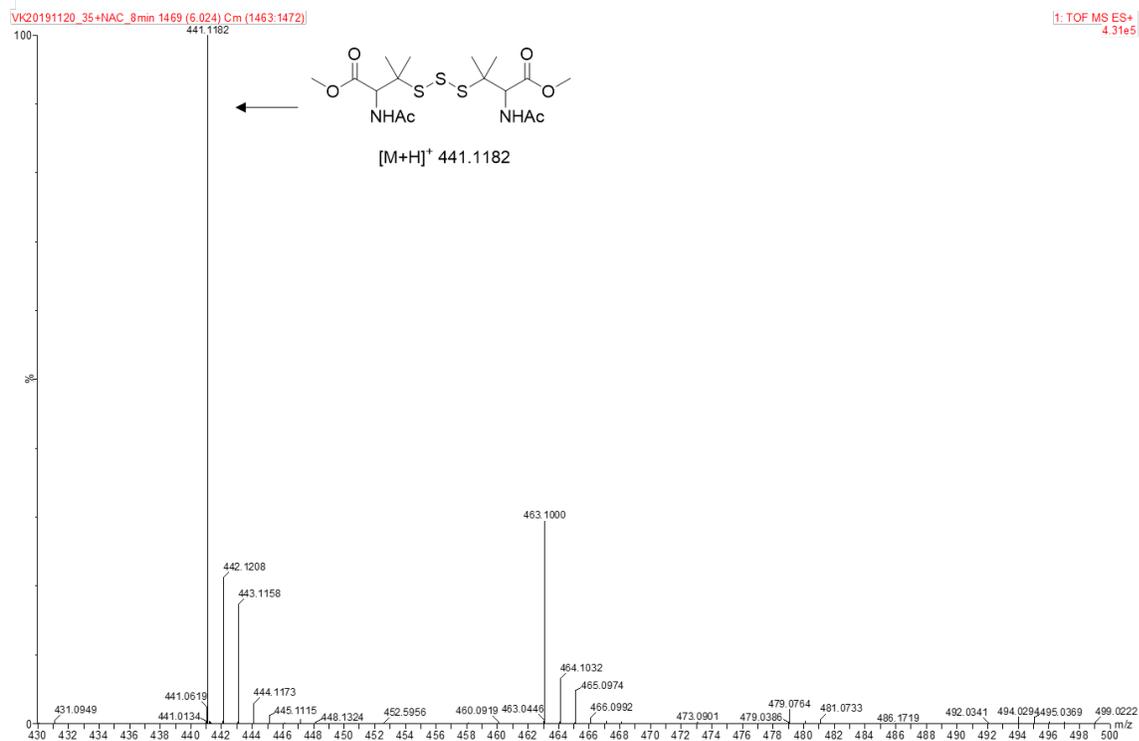


Figure S19. HRMS of the peak eluting at 6.02 min corresponding to symmetrical dialkyl trisulfide (S_3).

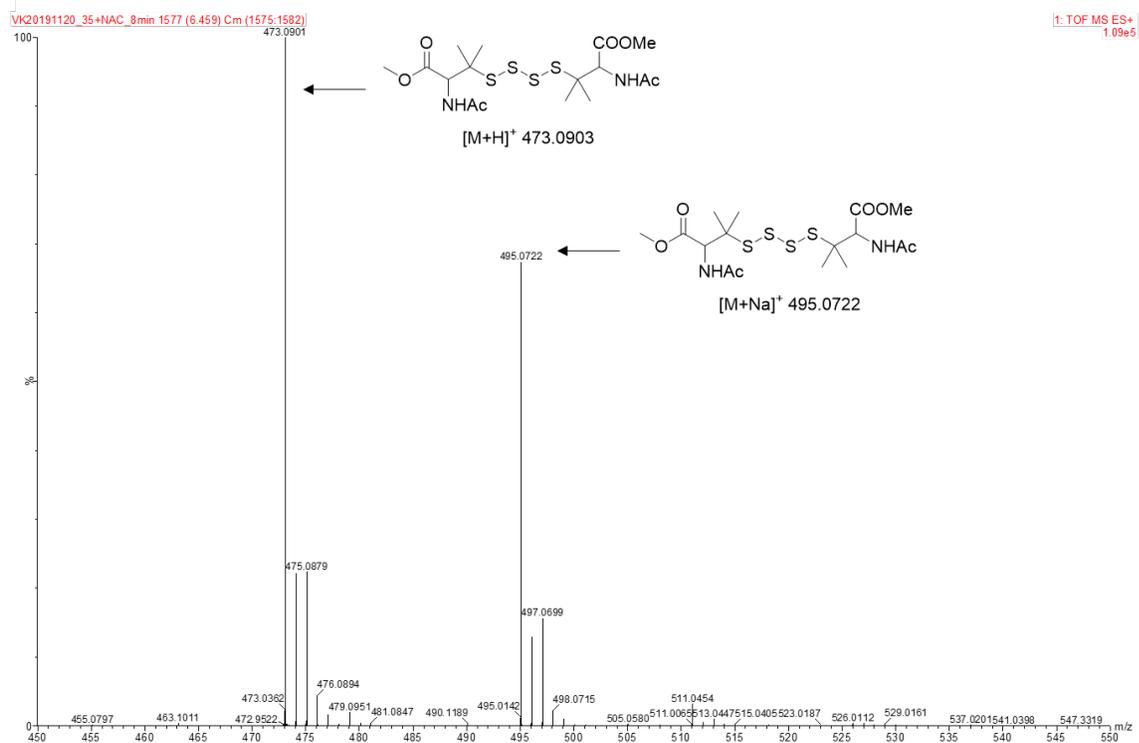


Figure S20. HRMS of the peak eluting at 6.46 min corresponding to symmetrical dialkyl tetrasulfide (S_4).

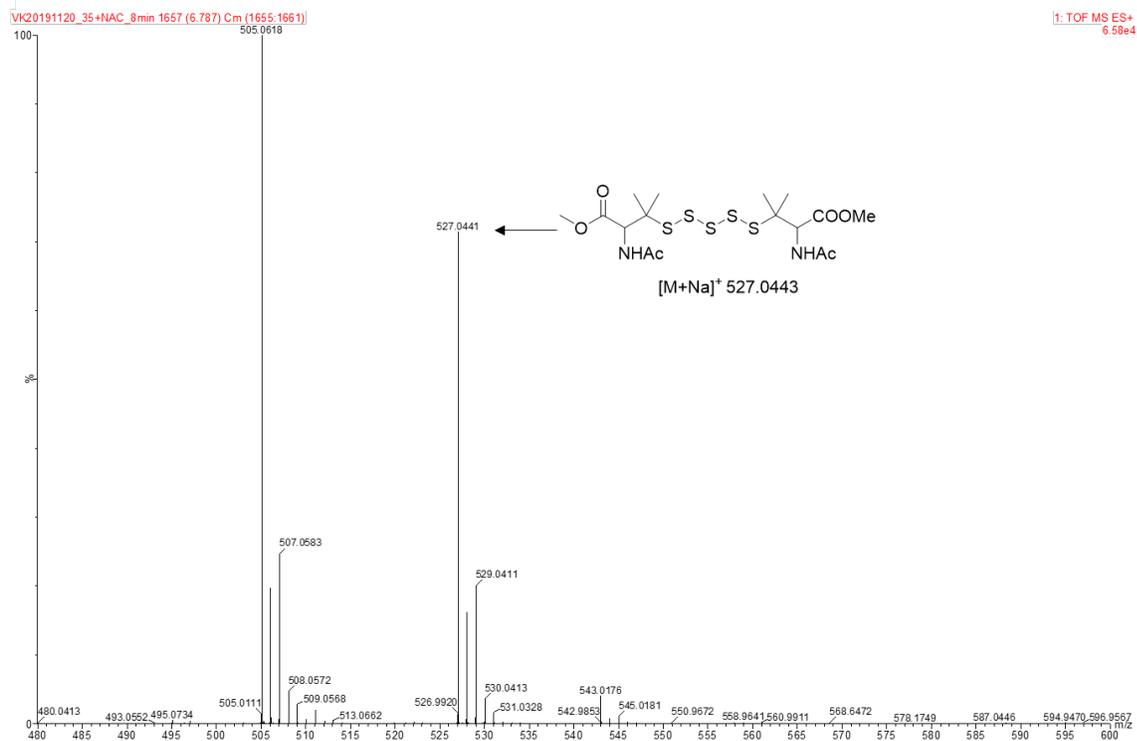


Figure S21. HRMS of the peak eluting at 6.79 min corresponding to symmetrical dialkyl pentasulfide (S₅).

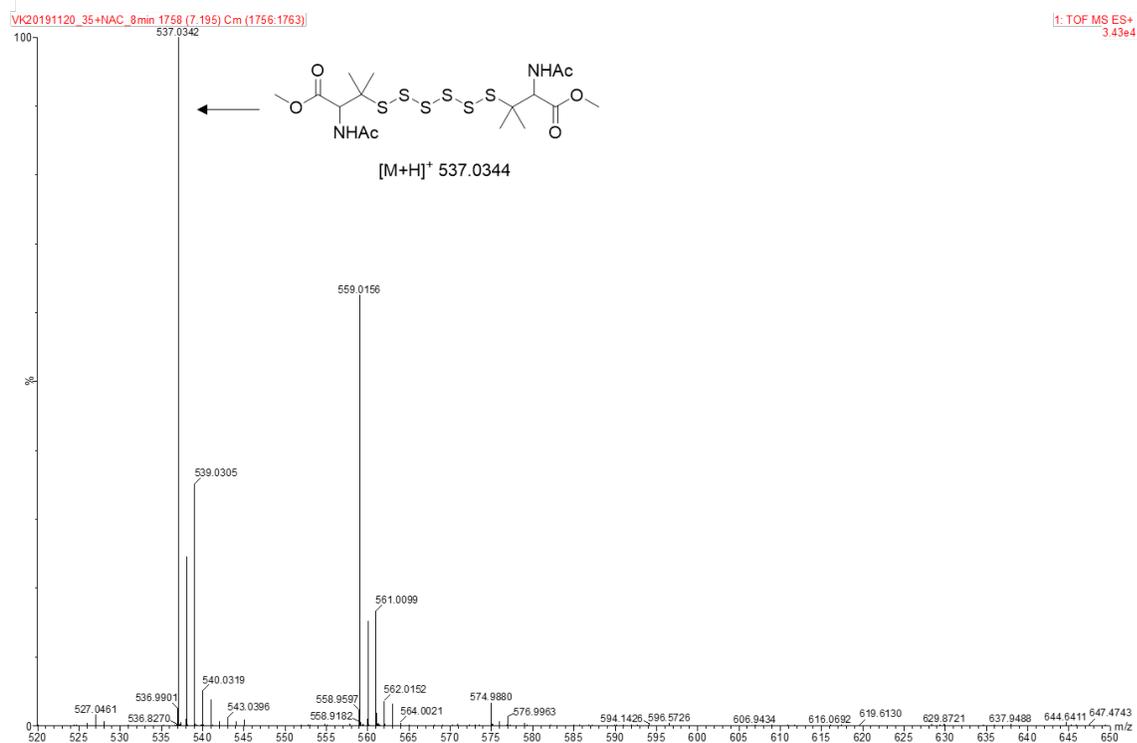
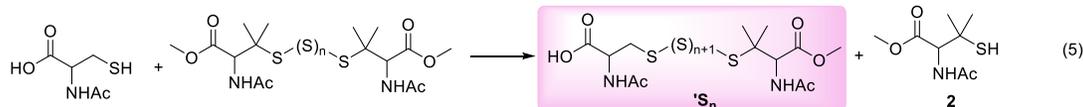
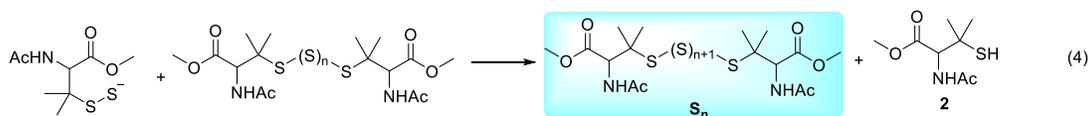
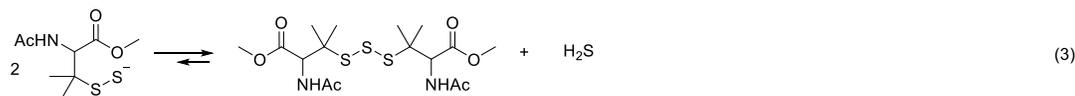
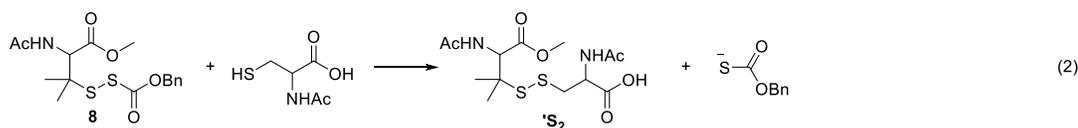
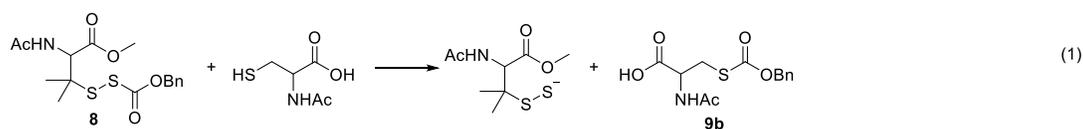


Figure S22. HRMS of the peak eluting at 7.20 min corresponding to symmetrical dialkyl hexasulfide (S₆).

RSSH generation from **8** in the presence of *N*-acetyl cysteine analyzed by UPLC-MS

To a solution of *N*-acetyl cysteine (500 μ M) in pH 7.4 ammonium bicarbonate buffer (50 mM) containing DTPA (100 μ M), precursors **8** (100 μ M) was added. The resulting mixture was incubated at 37°C. An aliquot (200 μ L) of reaction mixture was withdrawn at specified time points and quenched with 1% formic acid (200 μ L). These samples were stored at 0 °C until UPLC-MS analysis was performed.



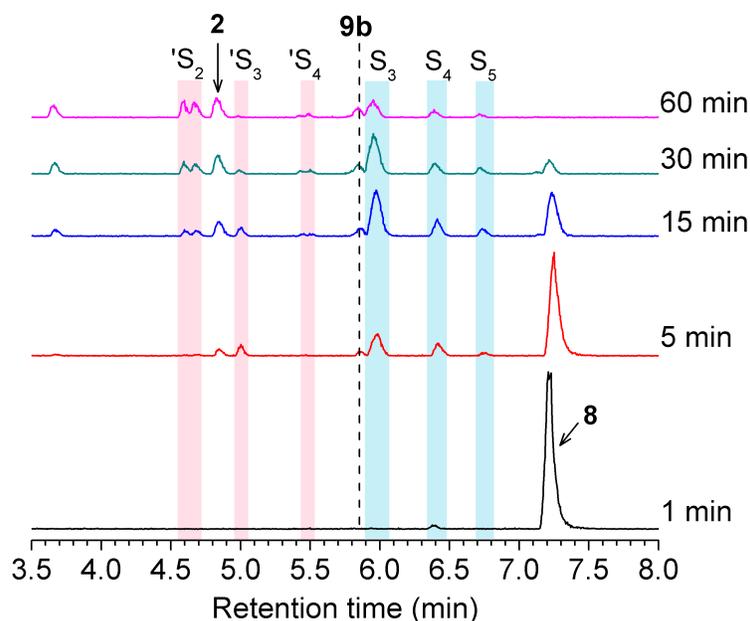


Figure S23. UPLC-MS chromatograms of RSSH generation from **8** (100 μ M) in the presence of NAC (500 μ M) incubated in pH 7.4 ammonium bicarbonate (50 mM) with the metal chelator DTPA (100 μ M) at 37 $^{\circ}$ C. RSSH-derived symmetrical dialkyl polysulfide, labeled as S_3 to S_5 ($R^1SS_nSR^1$, $n=1-3$, cyan highlight), and unsymmetrical dialkyl polysulfides labeled as $'S_2$ to $'S_5$ ($R^1SS_nSR^2$, $n=0-3$, pink highlight) formation is evident.

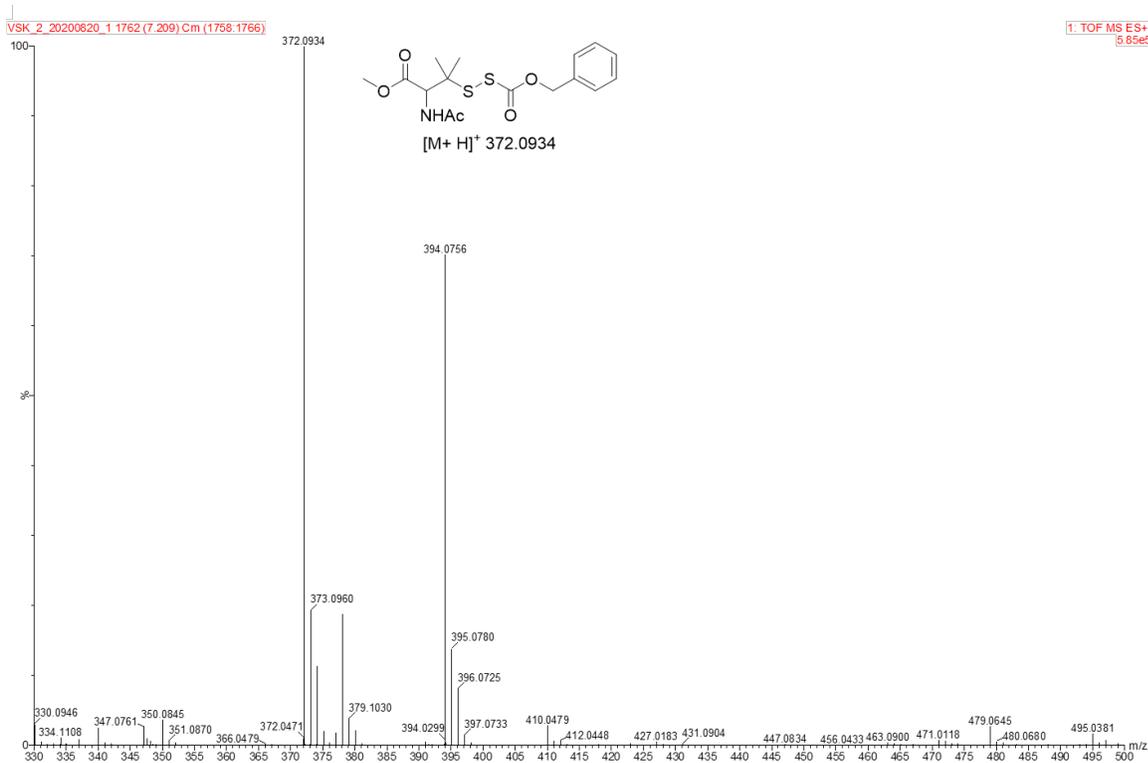


Figure S24. HRMS of the peak eluting at 7.21 min corresponding to RSSH precursor **8**.

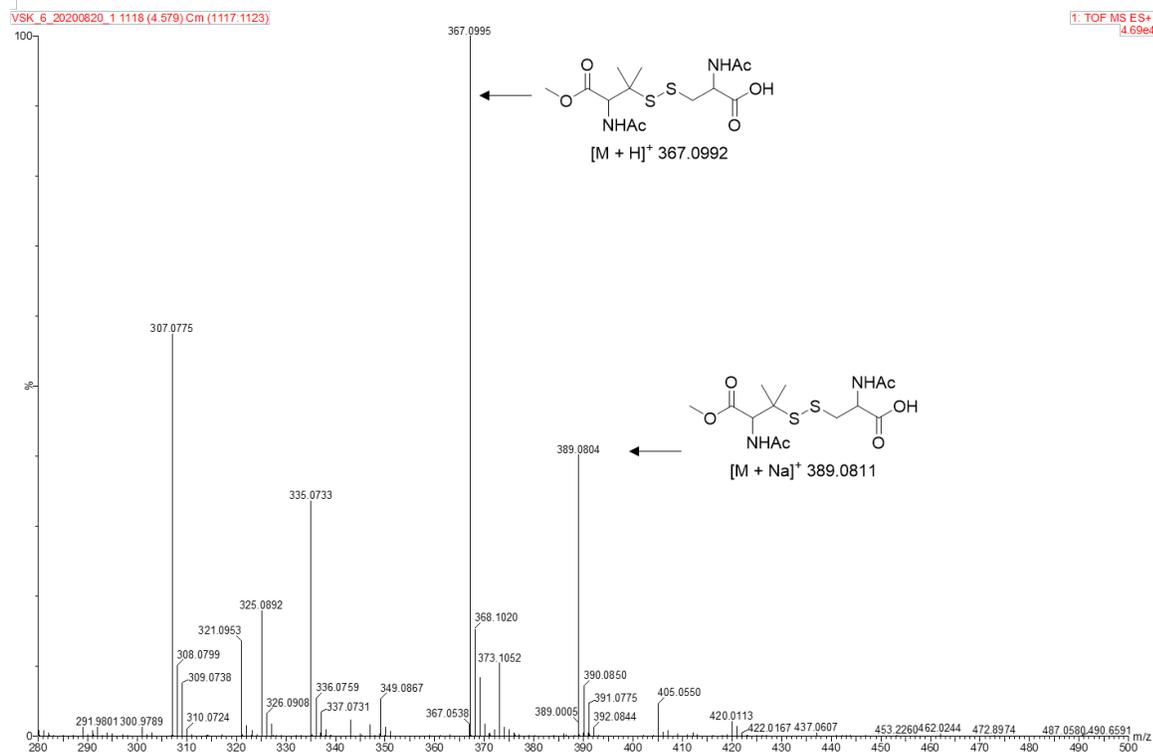


Figure S25. HRMS of the peak eluting at 4.58 min corresponding to mixed disulfide R^1S-SR^2 (S_2).

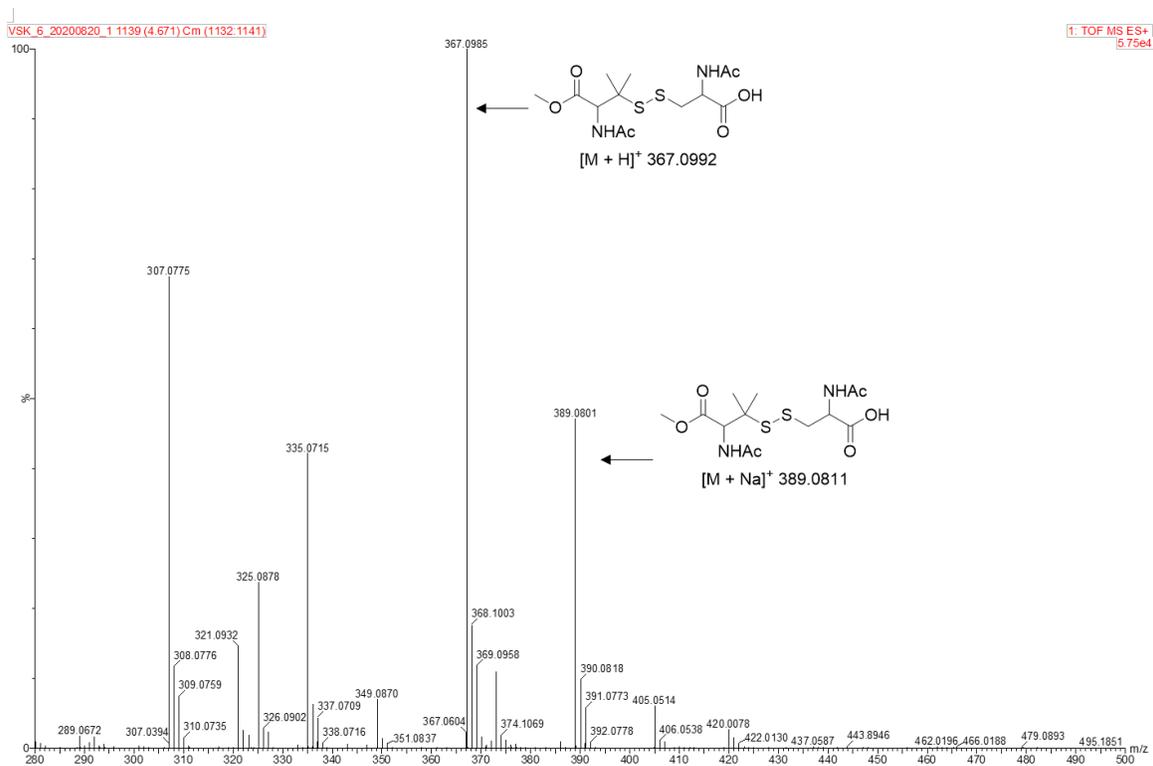


Figure S26. HRMS of the peak eluting at 4.67 min corresponding to other diastereomer of R^1S-SR^2 (S_2).

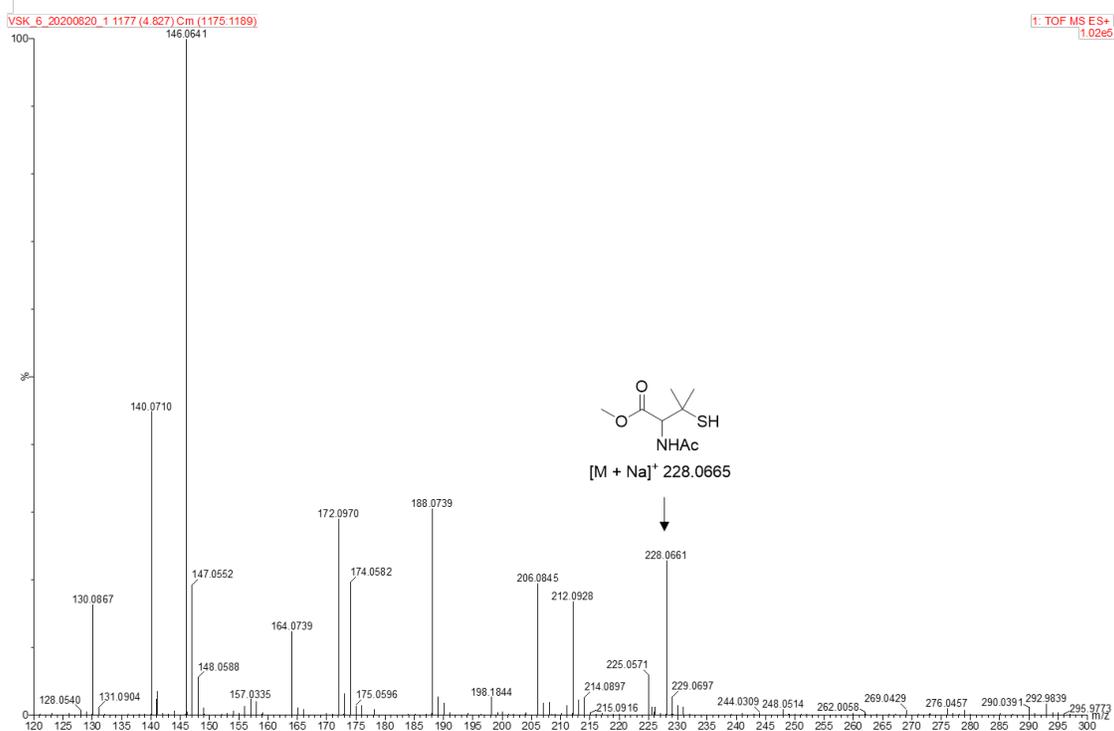


Figure S27. HRMS of the peak eluting at 4.83 min corresponding to *N*-acetyl penicillamine methyl ester.

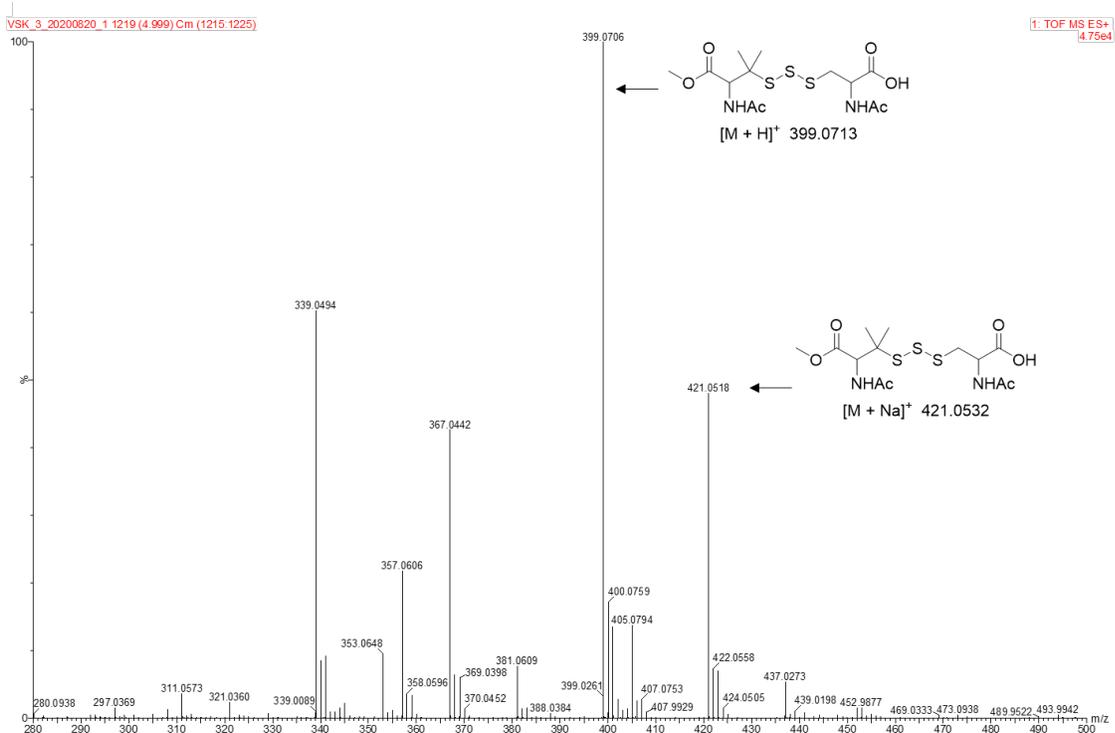


Figure S28. HRMS of the peak eluting at 4.99 min corresponding to unsymmetrical dialkyl trisulfide $R^1SSSR^2 (^{\cdot}S_3)$.

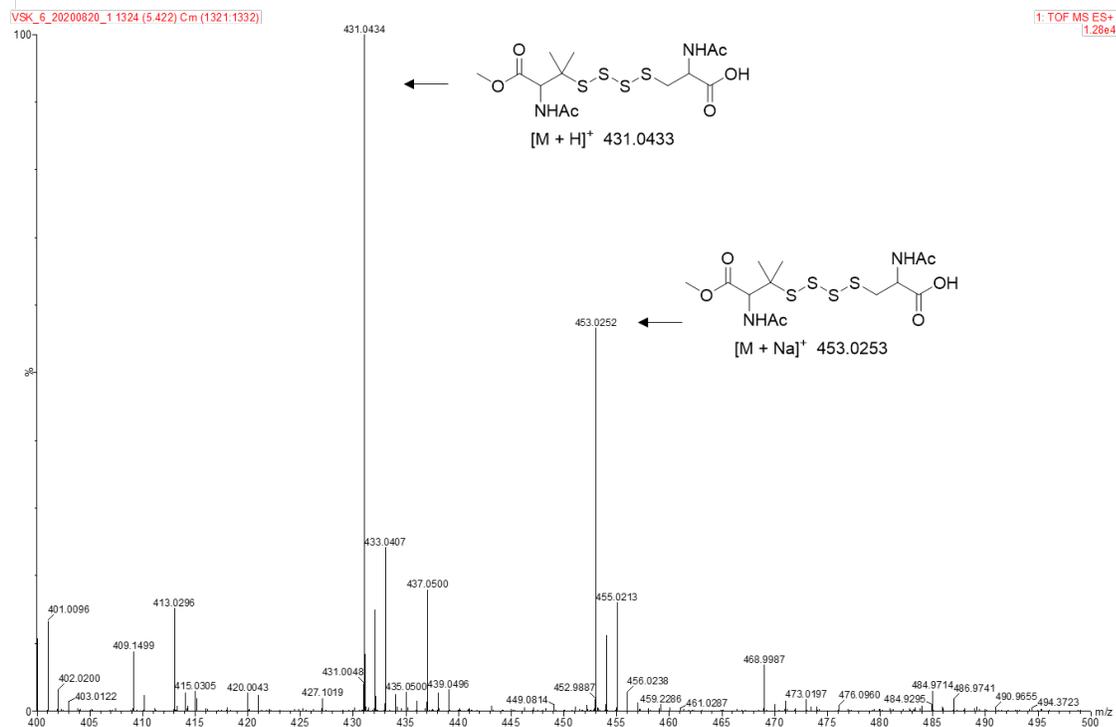


Figure S29. HRMS of the peak eluting at 5.42 min corresponding to unsymmetrical dialkyl tetrasulfide R^1SSSSR^2 (S_4).

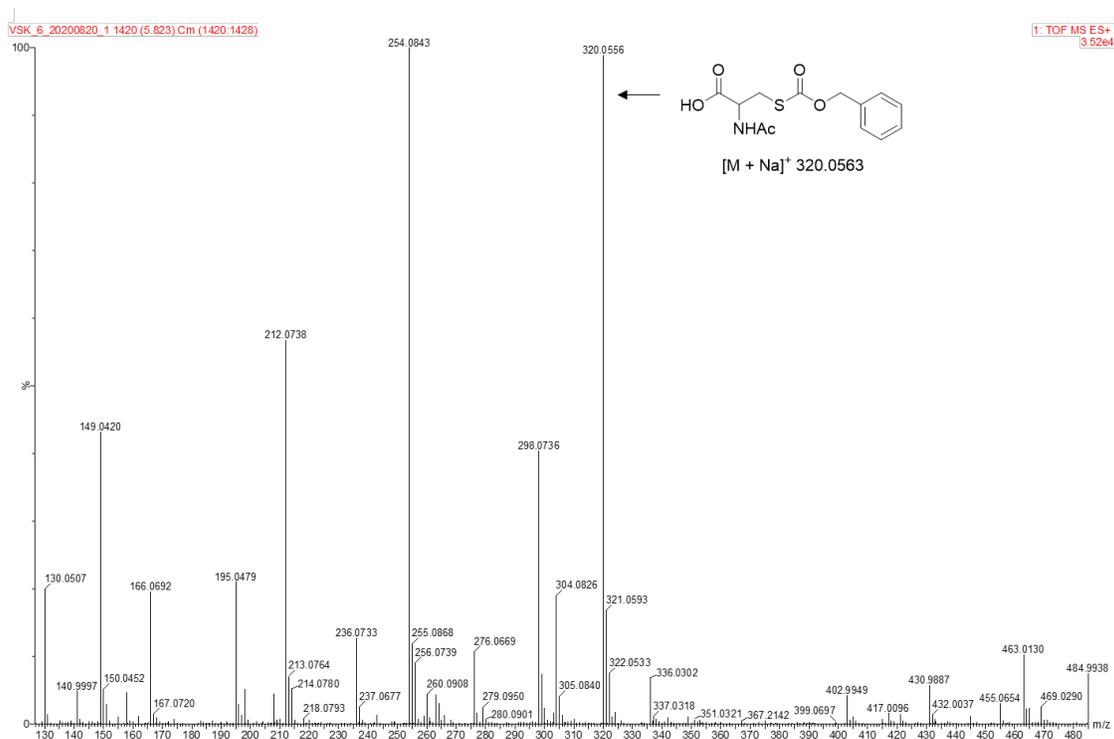


Figure S30. HRMS of the peak eluting at 5.82 min corresponding to *S*-alkyl thiocarbonate **9b**.

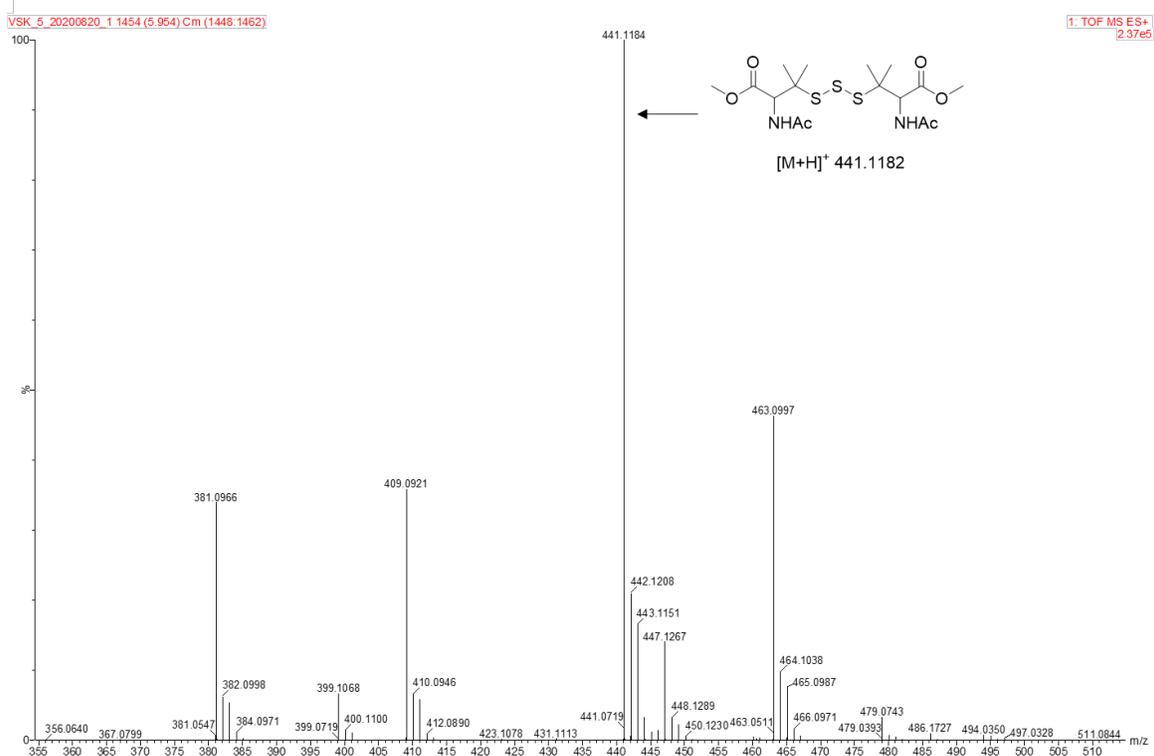


Figure S31. HRMS of the peak eluting at 5.95 min corresponding to symmetrical dialkyl trisulfide (S_3).

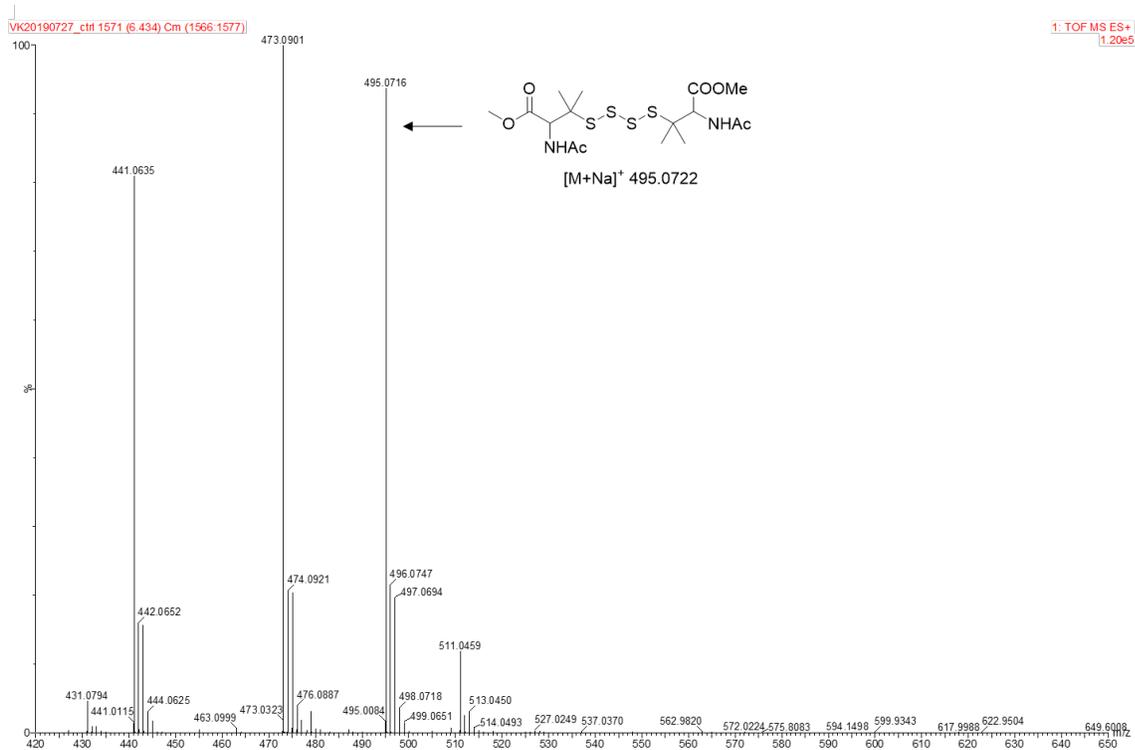


Figure S32. HRMS of the peak eluting at 6.43 min corresponding to symmetrical dialkyl tetrasulfide (S_4).

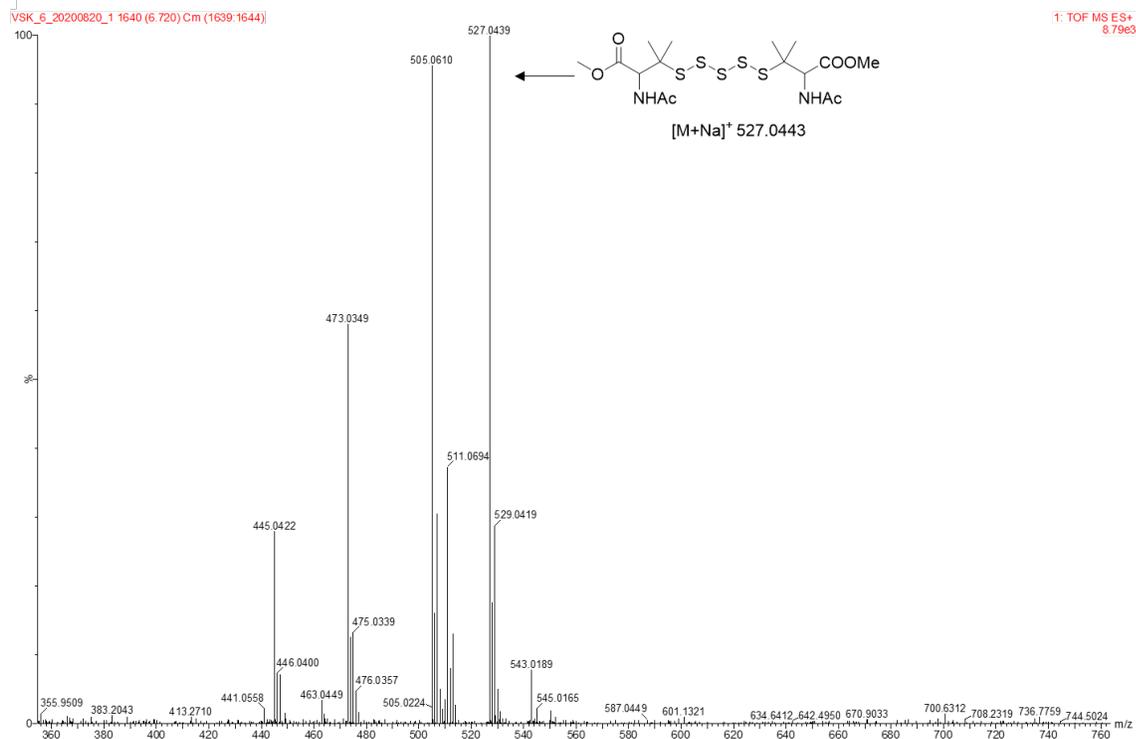
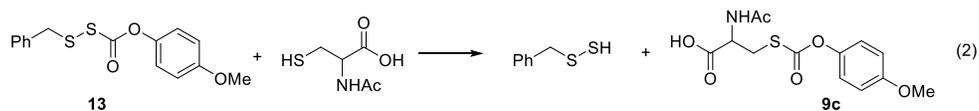
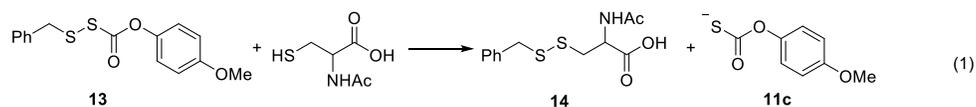


Figure S33. HRMS of the peak eluting at 6.72 min corresponding to symmetrical dialkyl pentasulfide (S_5).

Decomposition of control compound **13** in the presence of *N*-acetyl cysteine



UPLC-MS analysis of decomposition of **13** in the presence of *N*-acetyl cysteine shows exclusive unsymmetrical disulfide **14** formation, indicating that NAC selectively reacts at the disulfide bond (eq. 1). Alternatively, thiol can react at the carbonyl carbon to produce BnSSH and *S*-alkyl thiocarbonate **9c** (eq. 2). However, we do not observe evidence of **9c** formation under these conditions.

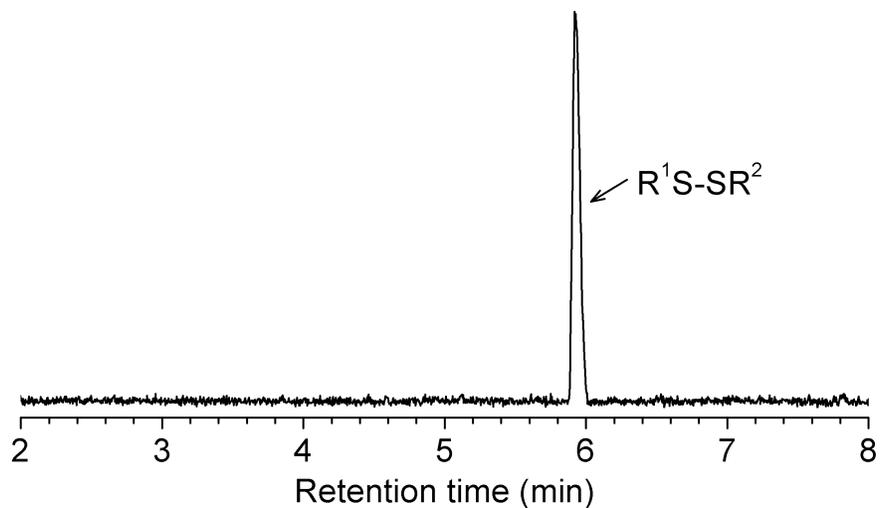


Figure S34. UPLC-MS chromatogram of compound **13** (100 μM) decomposition in the presence of NAC (500 μM) incubated in pH 7.4 ammonium bicarbonate (50 mM) with the metal chelator DTPA (100 μM) for 15 min at 37 $^{\circ}\text{C}$. A peak at 5.93 min attributed to the unsymmetrical disulfide **14** ($\text{R}^1\text{S-SR}^2$) is observed.

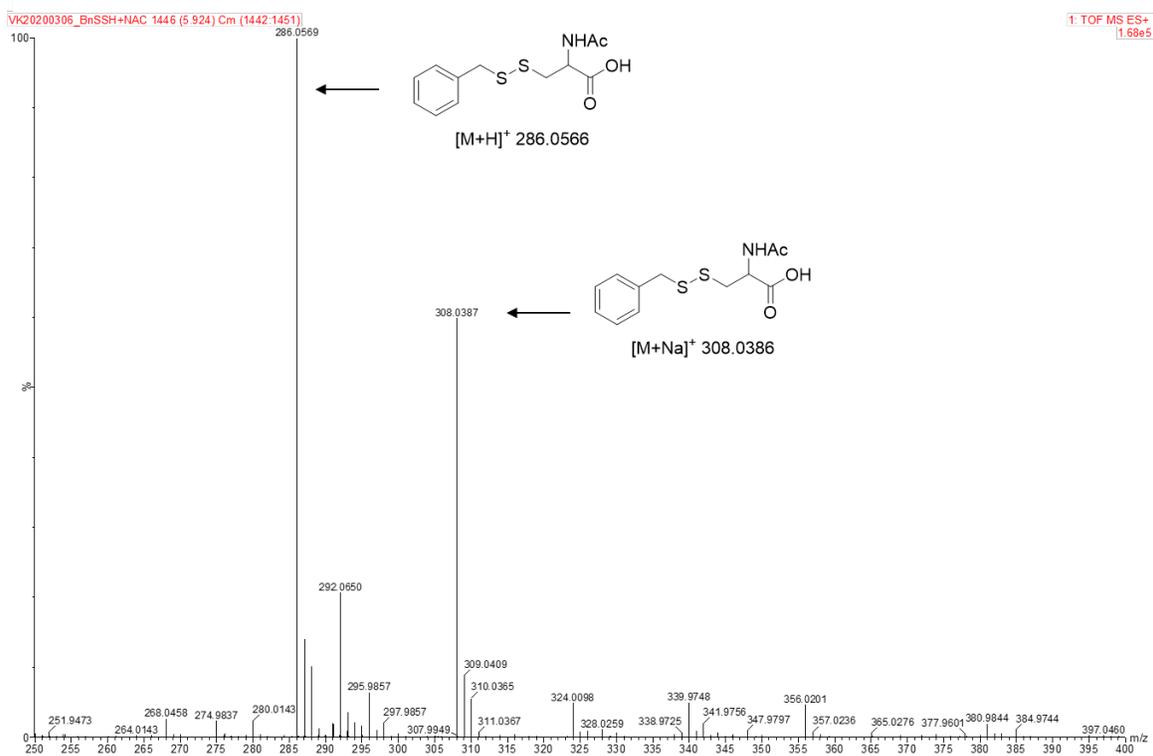


Figure S35. HRMS of the peak eluting at 5.93 min corresponding to unsymmetrical disulfide ($\text{R}^1\text{S-SR}^2$).

Analysis of COS release from RSSH precursors in the presence of thiols using MIMS

COS was analyzed using a Hiden HPR-40 MIMS system with a sample cell and membrane probe that have been optimized to detect gases dissolved in aqueous solution as described previously.¹ Stock solutions of *N*-acetyl-cysteine (25 mM) and glutathione (25 mM) were prepared in DI water. RSSH precursors stock solutions (5 mM) were prepared in DMSO. These solutions were degassed by purging with nitrogen for 10 min. Typically, 20 mL phosphate buffer solution (10 mM) was added to the sample cell, degassed and purged with a continuous flow of argon for 25 min. *N*-acetyl cysteine (200 μ L, 25 mM) or glutathione (200 μ L, 25 mM) and RSSH precursor (200 μ L, 5 mM) were then injected using a gas tight syringe and ion current at m/z 60 (COS^+) were collected (source pressure was approximately 1×10^{-7} to 5×10^{-7} Torr).

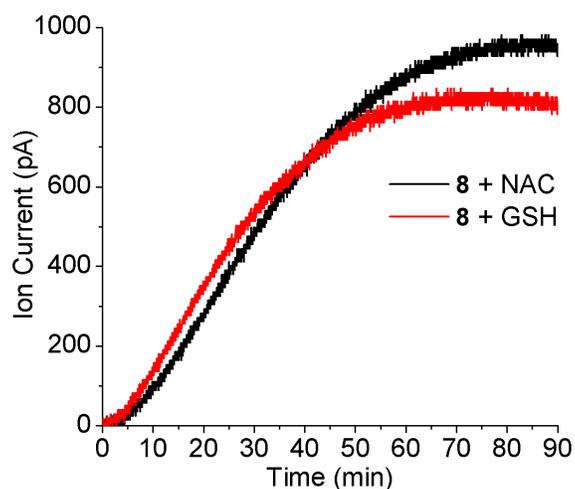


Figure S36. COS measurement using MIMS generated from the RSSH precursor **8** (50 μ M) with NAC, and GSH (0.25 mM, 5 equiv.) in PBS (pH 7.4, 10 mM) with DTPA (100 μ M) at 37 $^{\circ}$ C.

RSSH generation from **1a** in the presence of *L*-lysine analyzed by UPLC-MS

To a solution of *L*-lysine (500 μ M) in pH 7.4 ammonium bicarbonate buffer (50 mM) containing DTPA (100 μ M), precursor **1a** (100 μ M) was added. The resulting mixture was incubated at 37 $^{\circ}$ C. An aliquot (200 μ L) of reaction mixture was withdrawn at the specified time points (as shown in Figure 5 in the manuscript) and quenched with 1% formic acid (200 μ L). These samples were stored at 0 $^{\circ}$ C until UPLC-MS analysis was performed.

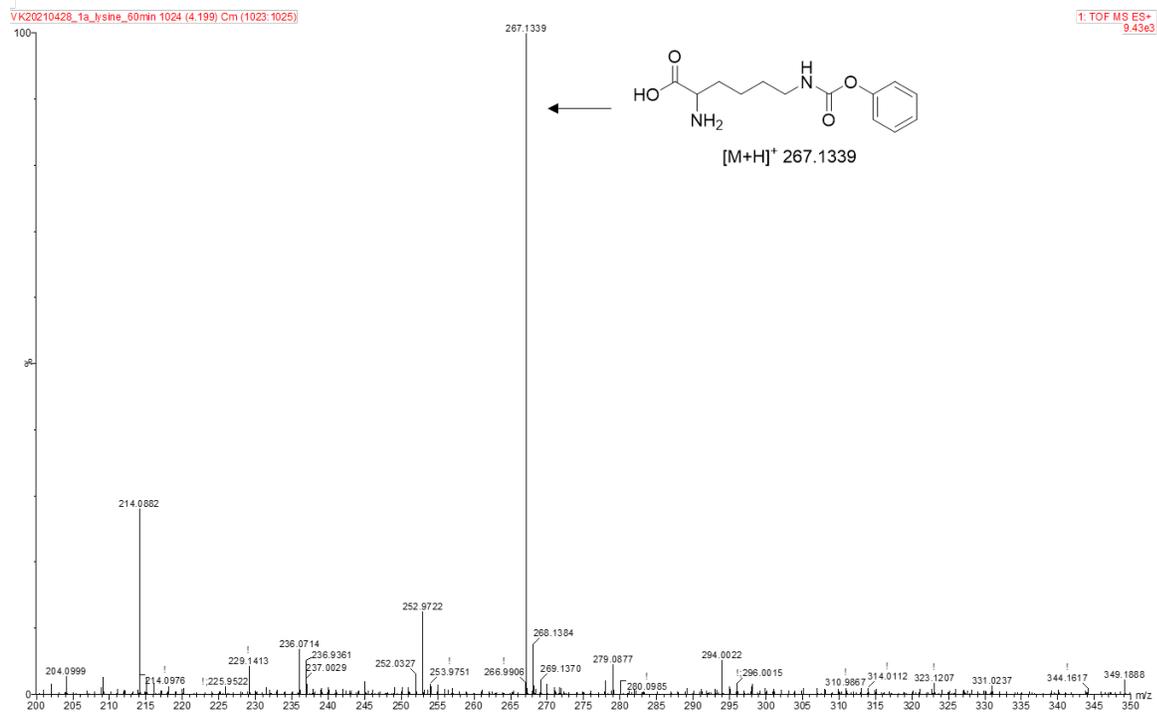


Figure S37. HRMS of the peak eluting at 4.20 min corresponding to carbamate 15.

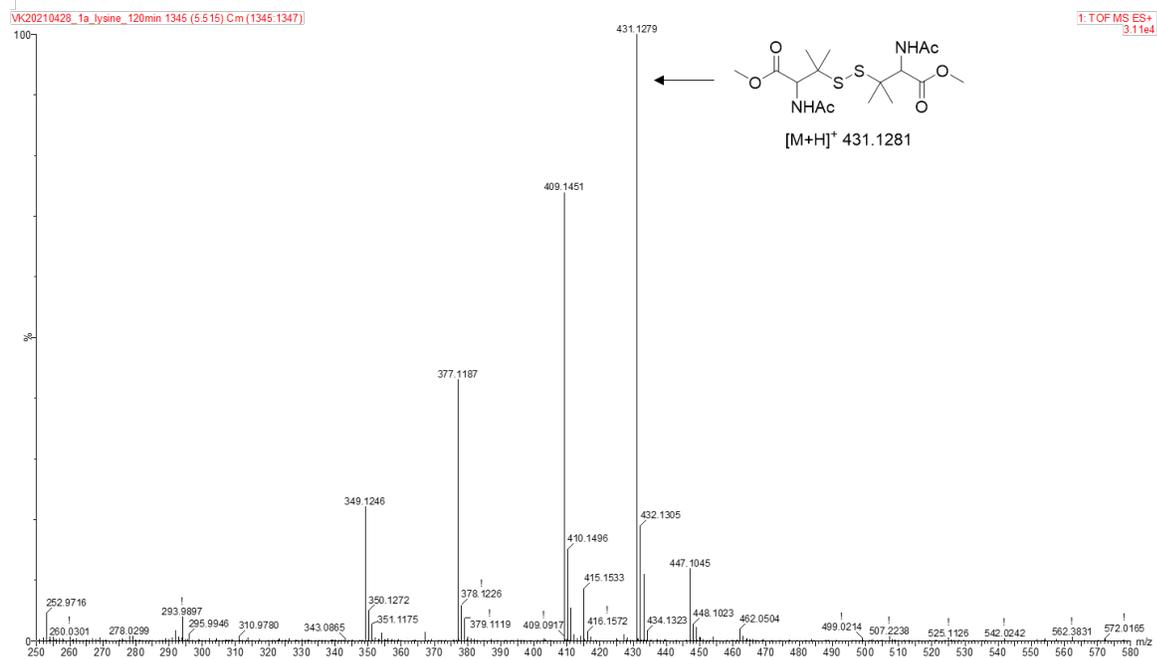


Figure S38. HRMS of the peak eluting at 5.52 min corresponding to dialkyl disulfide (S₂).

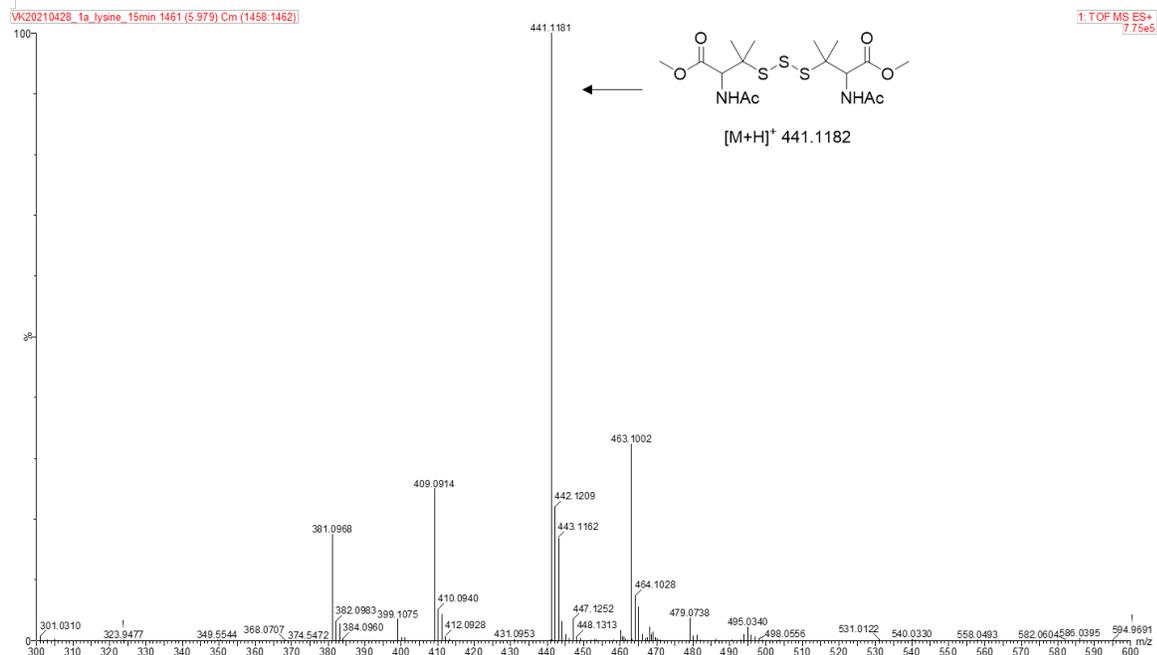


Figure S39. HRMS of the peak eluting at 5.98 min corresponding to symmetrical dialkyl trisulfide (S_3).

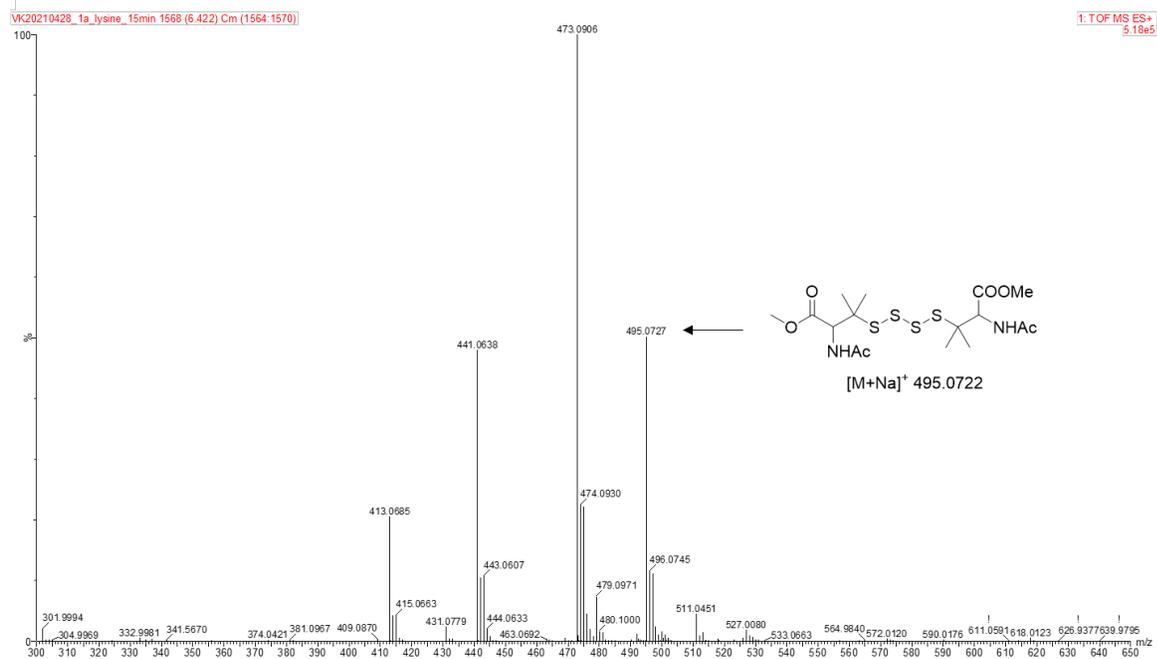


Figure S40. HRMS of the peak eluting at 6.42 min corresponding to symmetrical dialkyl tetrasulfide (S_4).

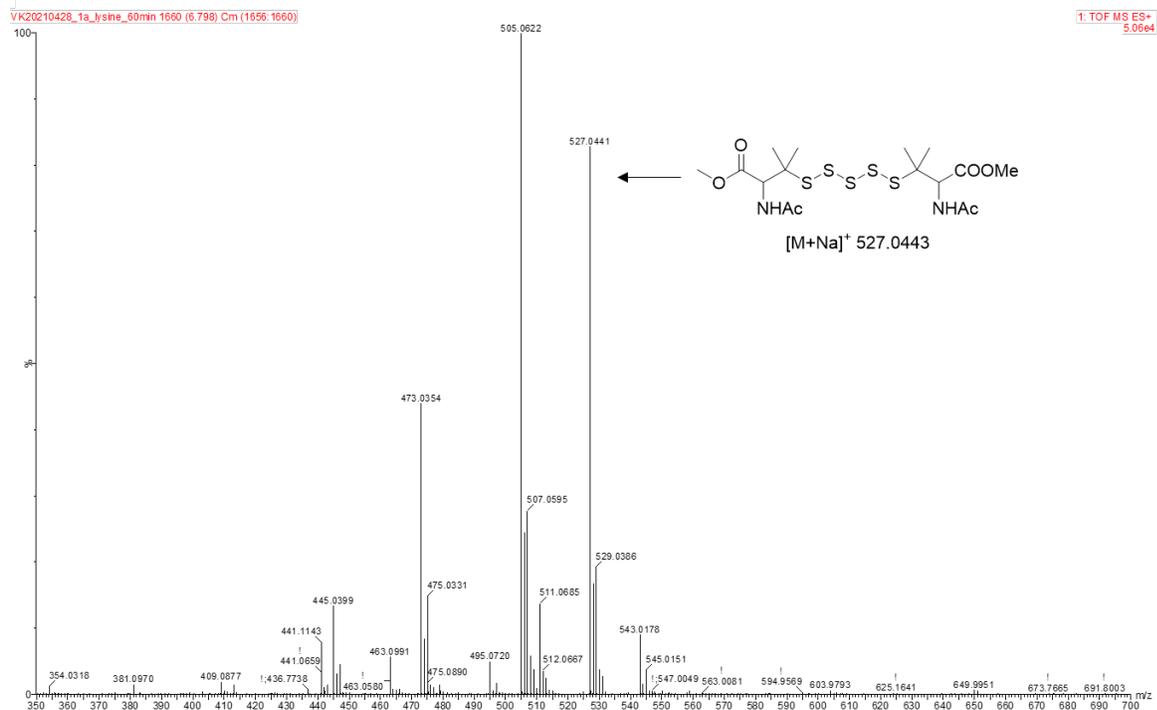


Figure S41. HRMS of the peak eluting at 6.79 min corresponding to symmetrical dialkyl pentasulfide (S_5).

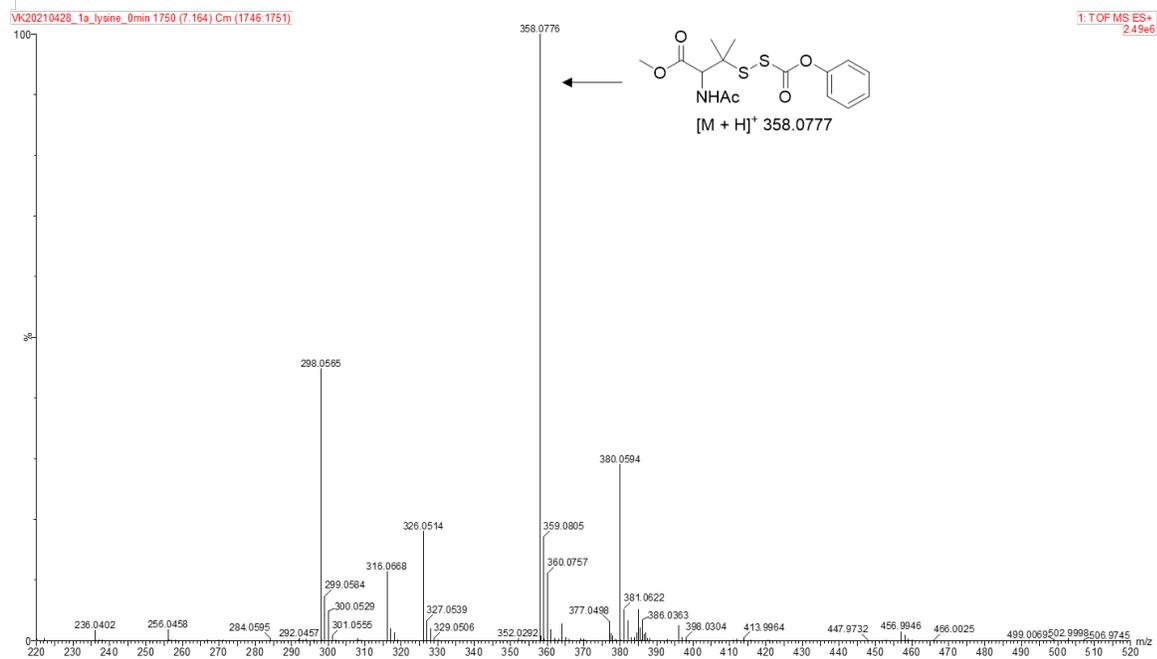


Figure S42. HRMS of the peak eluting at 7.16 min corresponding to **1a**.

Culture of cells

H9c2 embryonic rat heart myoblasts were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's minimal essential medium (DMEM), supplemented with fetal bovine serum (FBS) 10%, penicillin 100 U/mL and streptomycin 100 µg/mL. They were propagated in T75-flasks, split before reaching 70–80% confluence (usually every day or every second day), and used within 11 passages. Cells were passaged to tissue culture treated 96- well microtiter plates at the specified density in 180 µL volumes and incubated for 24 h.

Cytotoxicity study of 1a

Cells were seeded at a density of 1×10^4 cells/well. After 24 h, the media was replaced and compound added in 20 µL volumes using DMSO:H₂O (<0.01% DMSO) as the vehicle. Cells were incubated for an additional 24 h before media was removed. Then, 100 µL of media containing 3 µM Sytox Green nucleic acid stain (Invitrogen) was added and the cells were incubated for 2 h before fluorescence readings were obtained at 485_{Ex}/538_{Em} (Step 1). Finally, an additional 100 µL of media containing 3 µM Sytox and 0.2% Triton X-100 was added in order to permeabilize all cells and incubated for 1 h before fluorescence values measured (Step 2). The relative % cells surviving was calculated as a 100% minus the ratio of the fluorescence value of Step 1 over Step 2 (% cells surviving = 100% - (FL₅₃₈ (Step 1)/ FL₅₃₈(Step 2))).

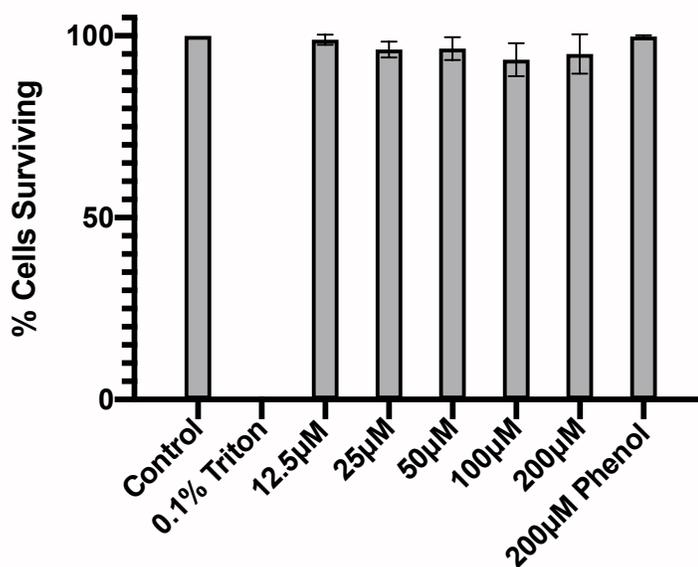


Figure S43. Cell viability assay conducted on H9c2 cells with Precursor **1a** (12.5, 50, 100 and 200 µM) and byproduct phenol (**4a**) (200 µM) using Sytox Green nucleic acid stain. Results are expressed as the mean ± SEM (n = 5 for each treatment group) with 3 independent experiments.

H9c2 cell protection by precursor 1a from H₂O₂-mediated oxidative stress Cell Counting Kit-8 (CCK-8)²

Cells were seeded at a density of 5×10^3 cells/well. After 24 h, precursor **1a** or phenol byproduct **4a** was added at 20 μ L volumes using DMSO:H₂O (<0.01% DMSO as the vehicle). Cells were incubated for 2 h before media was removed and the cells gently washed with PBS (pH 7.4). Then, 180 μ L of fresh media and 20 μ L H₂O₂ diluted into H₂O were added and cells were incubated for an additional 2 h. At the completion of H₂O₂ exposure, each well is carefully washed 3 times with PBS (pH 7.4) before adding 100 μ L of media, without-FBS, containing 10% v/v CCK-8 (Dojindo) and incubated for 3 h prior to obtaining absorbance values at 450 nm. The relative % viability was calculated as 100 times the ratio of the Abs₄₅₀ (pretreated, H₂O₂-exposed) over Abs₄₅₀(vehicle- treated, non H₂O₂-exposed).

Sytox Green nucleic acid stain³

Cells were seeded at a density of 1×10^4 cells/well. After 24 h, precursor **1a** or phenol byproduct **4a** is added in 20 μ L volumes using DMSO:H₂O (<0.01% DMSO). Cells were incubated for 2 h before media was removed and the cells gently washed with PBS (pH 7.4). Then, 180 μ L of fresh media and 20 μ L H₂O₂ diluted in H₂O were added before cells were incubated for an additional 1 h. After removing this media, 100 μ L of media containing 3 μ M Sytox Green nucleic acid stain was added, and the cells incubated for 2 h before fluorescence readings were obtained at 485_{Ex}/538_{Em} (Step 1). Finally, an additional 100 μ L of media containing 3 μ M Sytox and 0.2% Triton X-100 was added in order to permeabilize all cells and incubated for 1 h before fluorescence values measured (Step 2). The relative % cells surviving was calculated as a 100% minus the ratio of the fluorescence value of Step 1 over Step 2 (% cells surviving = 100% - (FL₅₃₈ (Step 1)/ FL₅₃₈ (Step 2))). Fluorescence values for vehicle-treated, non-H₂O₂-exposed wells were treated as background cell death and this value is added to the % cell survival for each subsequent group.

H9c2 cell protection by thiol 2 from H₂O₂-mediated oxidative stress

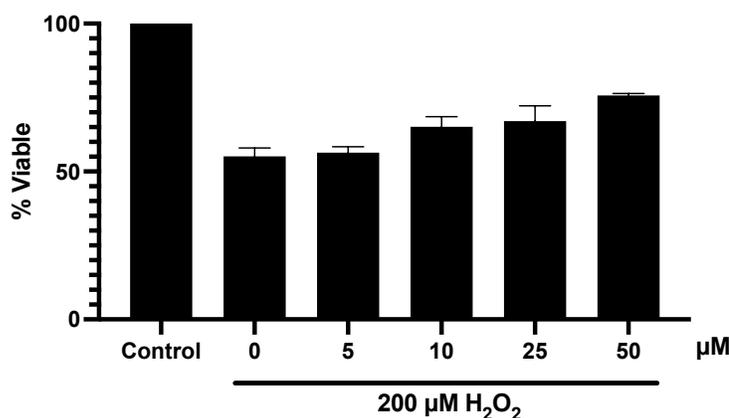


Figure S44 Cell viability of H9c2 cardiac myoblasts pretreated with *N*-acetyl-penicillamine methyl ester (**2**) at 0, 5, 10, 25, and 50 μ M for 4 h followed by exposure to H₂O₂ (200 μ M) for 2 h. Quantification of viability was carried out using Cell Counting Kit-8 (CCK-8). Results are expressed as the mean \pm SEM (n = 5 for each treatment group) with two independent experiments.

Intracellular sulfane sulfur detection using the SSP4 probe⁴

Cells were seeded at a density of 1.5×10^4 cells/well. After 24 h, the media is removed, and the cells are washed two times with fresh serum-free media. Then, 20 μM SSP4 and 500 μM CTAB are introduced to the cells in a 100 μL volume of serum-free media for 20 minutes. The SSP4/CTAB solution is removed, and the cells are washed two times with fresh serum-free media. Finally, 100 μL of serum-free media containing 50, 100, or 200 μM of **1a** is added to the wells and incubated at 37 $^\circ\text{C}$. The fluorescence readings were measured using a plate reader at the emission and excitation wavelengths of 482 nm and 515 nm, respectively. Results are normalized to the 0 μM value at each time point and expressed as the mean \pm SEM ($n = 3$ for each treatment group) with three independent experiments.

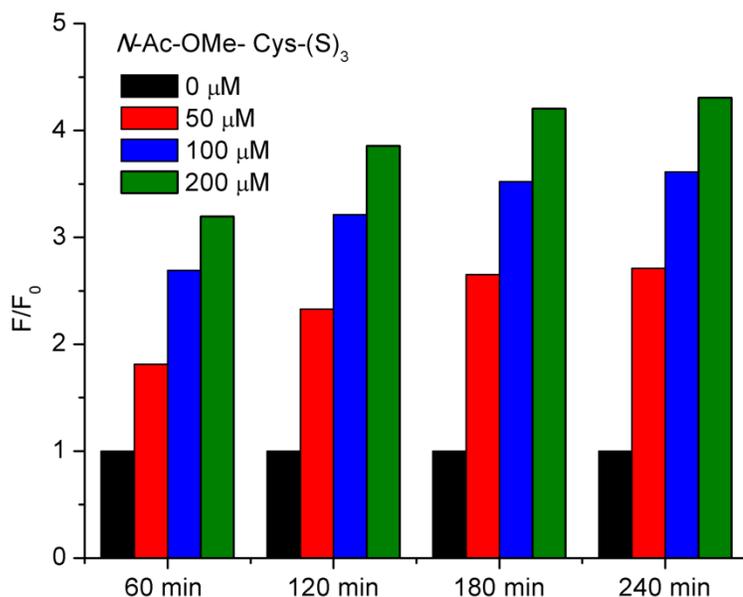


Figure S45 Intracellular RSSH release in H9c2 cardiac myoblasts. H9c2 cells were pretreated with SSP4 (20 μM) and CTAB (500 μM) for 20 min, followed by incubation with *N*-acetyl *O*-methyl cysteine trisulfide at 0, 50, 100, 25, and 200 μM . Fluorescence intensity was measured at the indicated times. Results are normalized to the 0 μM value at each time point and expressed as the mean \pm SEM ($n = 3$ for each treatment group) with three independent experiments.

SSP4 probe response from various sulfane sulfur species

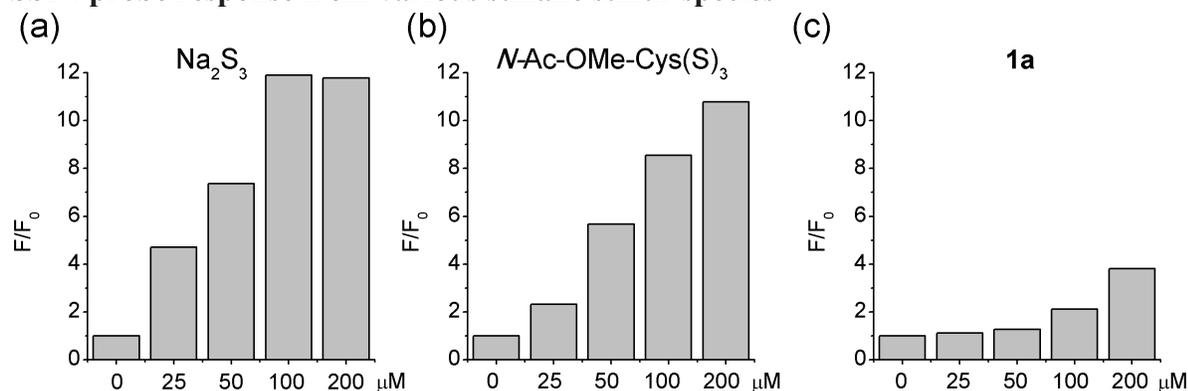


Figure S46. Fluorescence enhancement (F/F_0) of SSP4 probe in the presence of increasing concentration of (a) disodium trisulfide, (b) *N*-acetyl *O*-methyl cysteine trisulfide, and (c) **1a**. These species were first incubated at 37 °C in DMEM medium for 3 h and then the SSP4 probe (20 μM) was added. The resulting mixture was incubated for 30 min and fluorescence was measured at $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 515 \text{ nm}$.

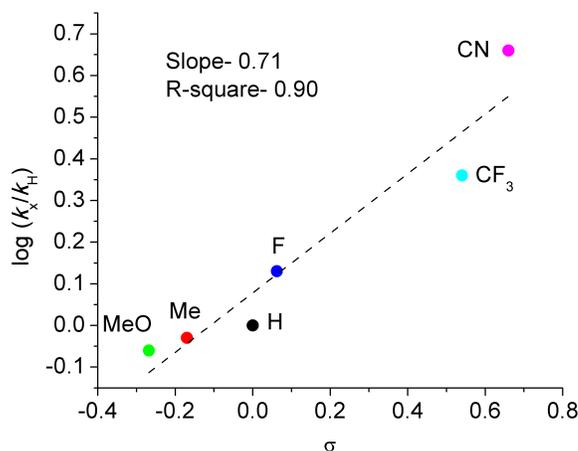


Figure S47. Plot of $\log(K_X/K_H)$ for RSSH release from **1a-f** versus the Hammett substitution constant σ (reaction constant $\rho = 0.71$; $R^2 = 0.920$).

References

1. M. R. Cline, C. Tu, D. N. Silverman and J. P. Toscano, *Free Radical Biol. Med.*, 2011, **50**, 1274-1279.
2. H. Tominaga, M. Ishiyama, F. Ohseto, K. Sasamoto, T. Hamamoto, K. Suzuki and M. Watanabe, *Anal. Commun.*, 1999, **36**, 47-50.
3. J. P. Hofgaard, K. S. Sigurdardottir and M. Treiman, *Pharmacol. Res.*, 2006, **54**, 303-310.
4. W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian, *Chem. Sci.*, 2013, **4**, 2892-2896.

NMR Spectra

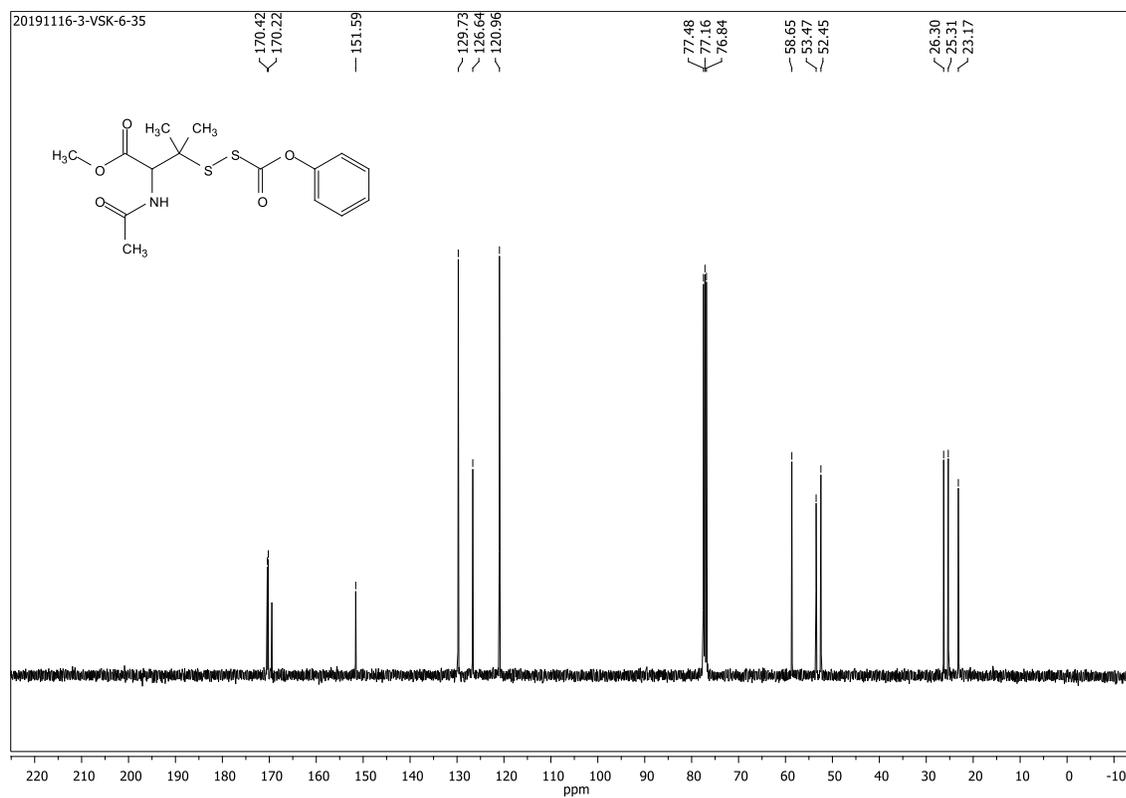
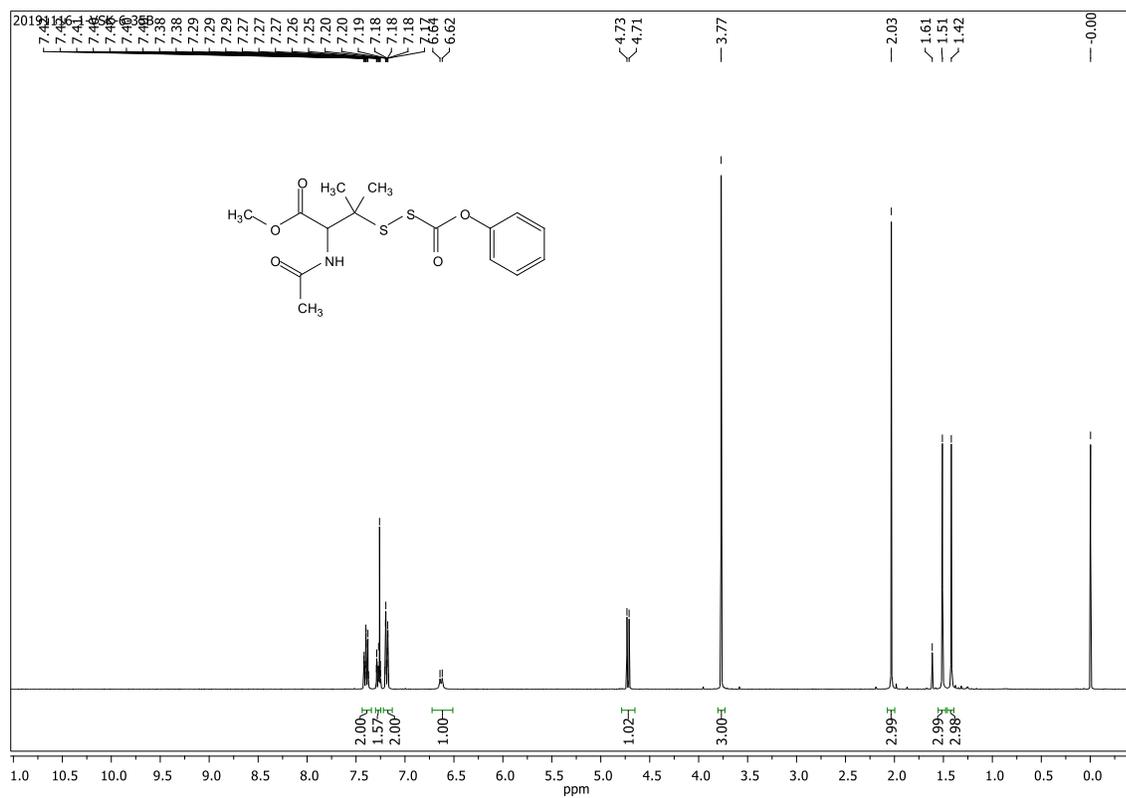


Figure S48. ¹H and ¹³C NMR spectra of 1a.

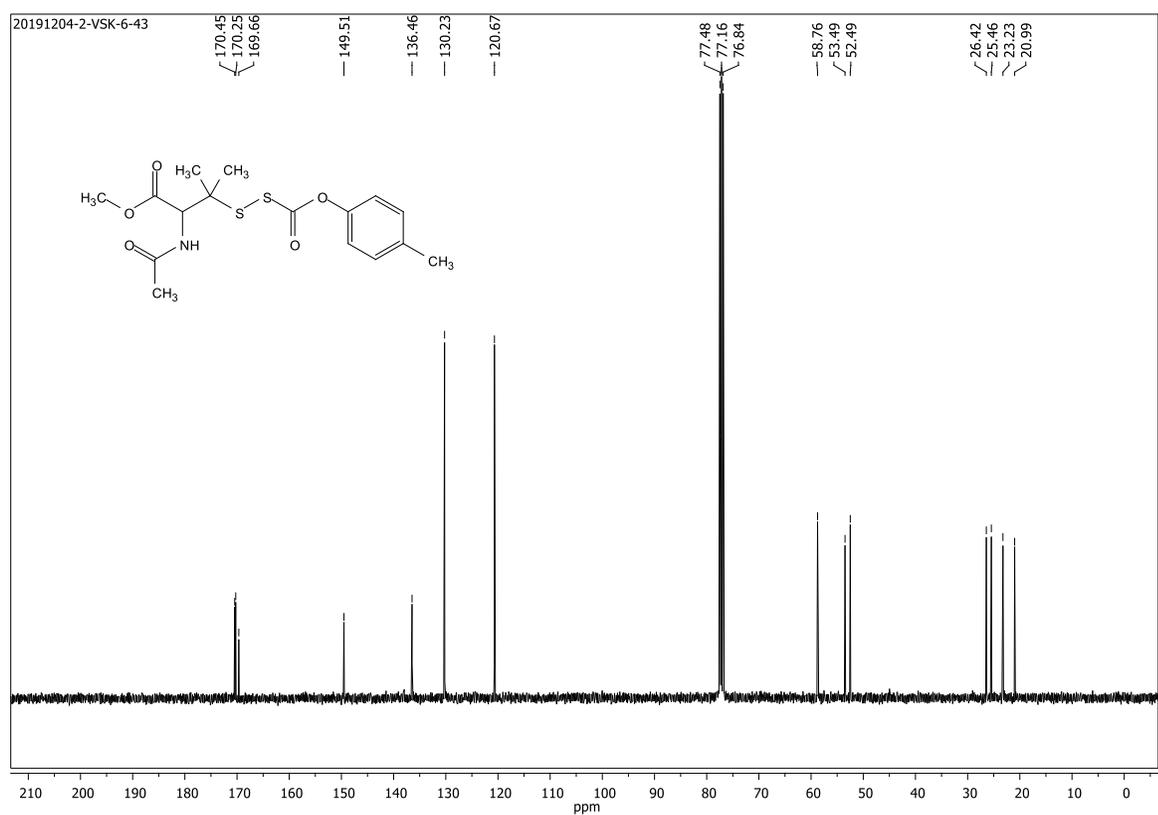
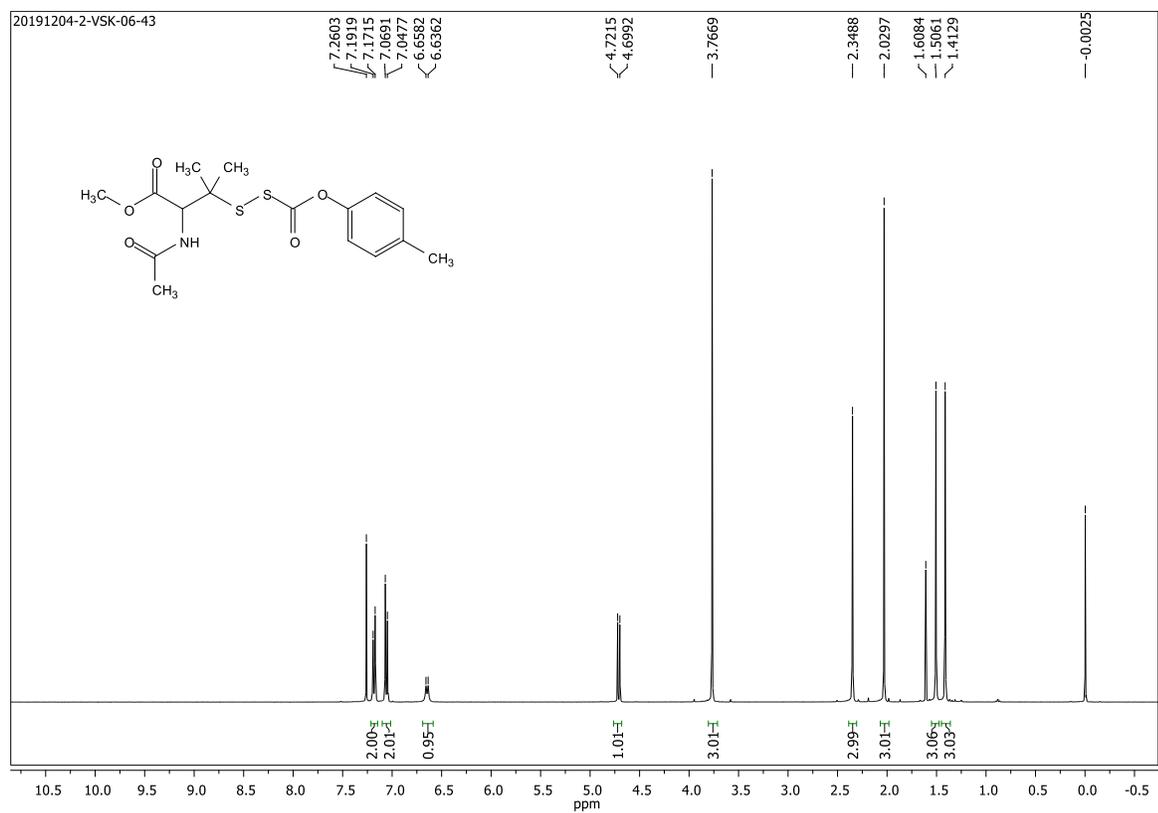


Figure S49. ¹H and ¹³C NMR spectra of **1b**.

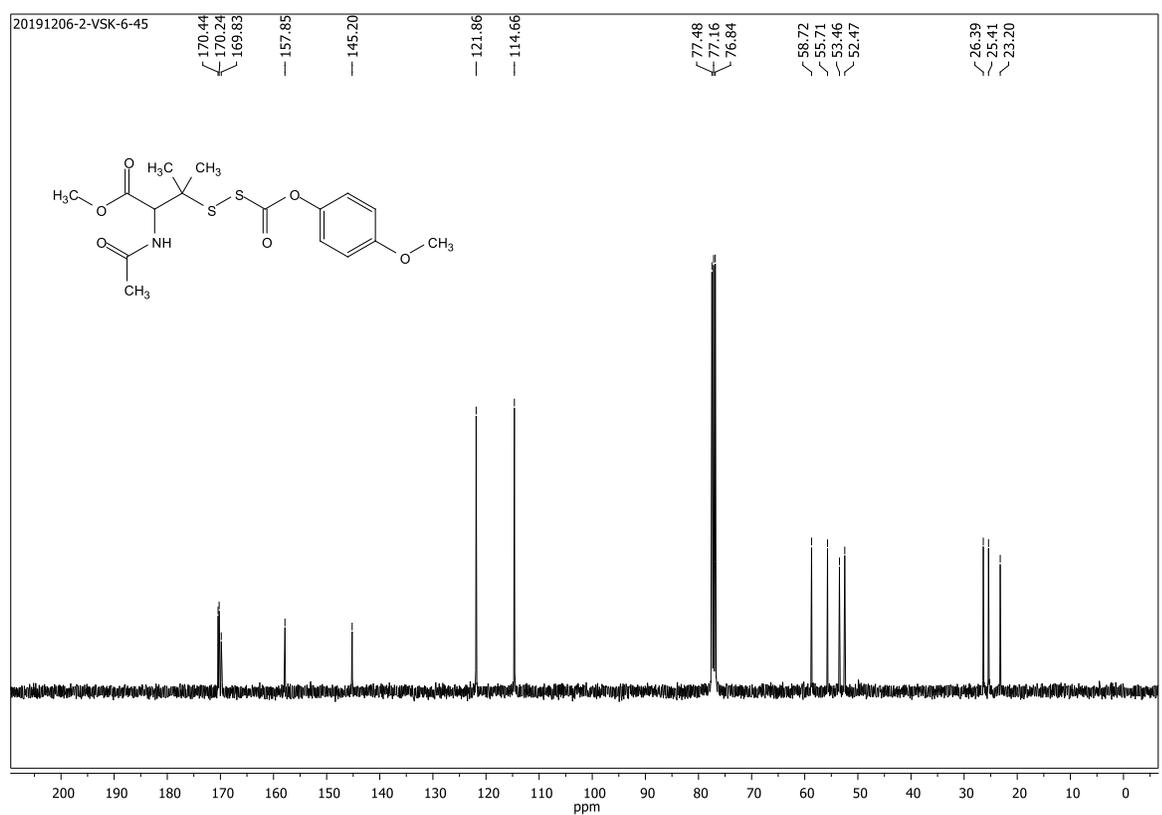
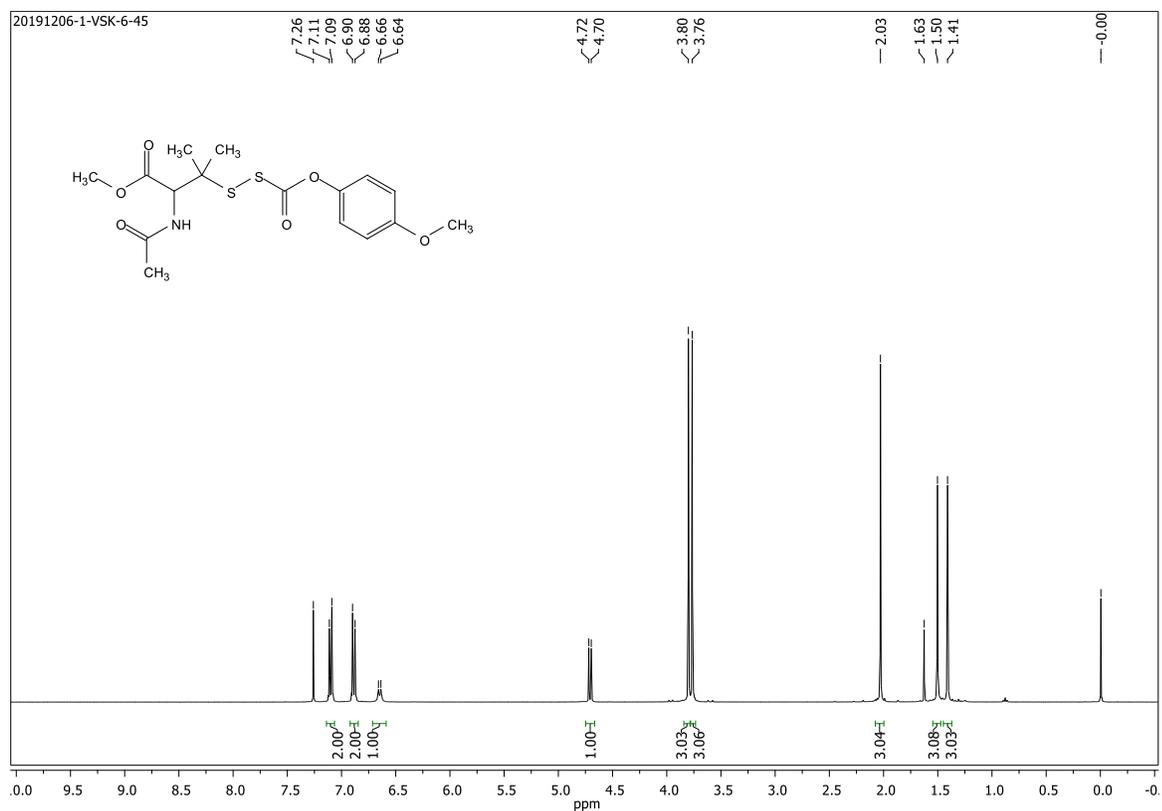


Figure S50. ^1H and ^{13}C NMR spectra of 1c.

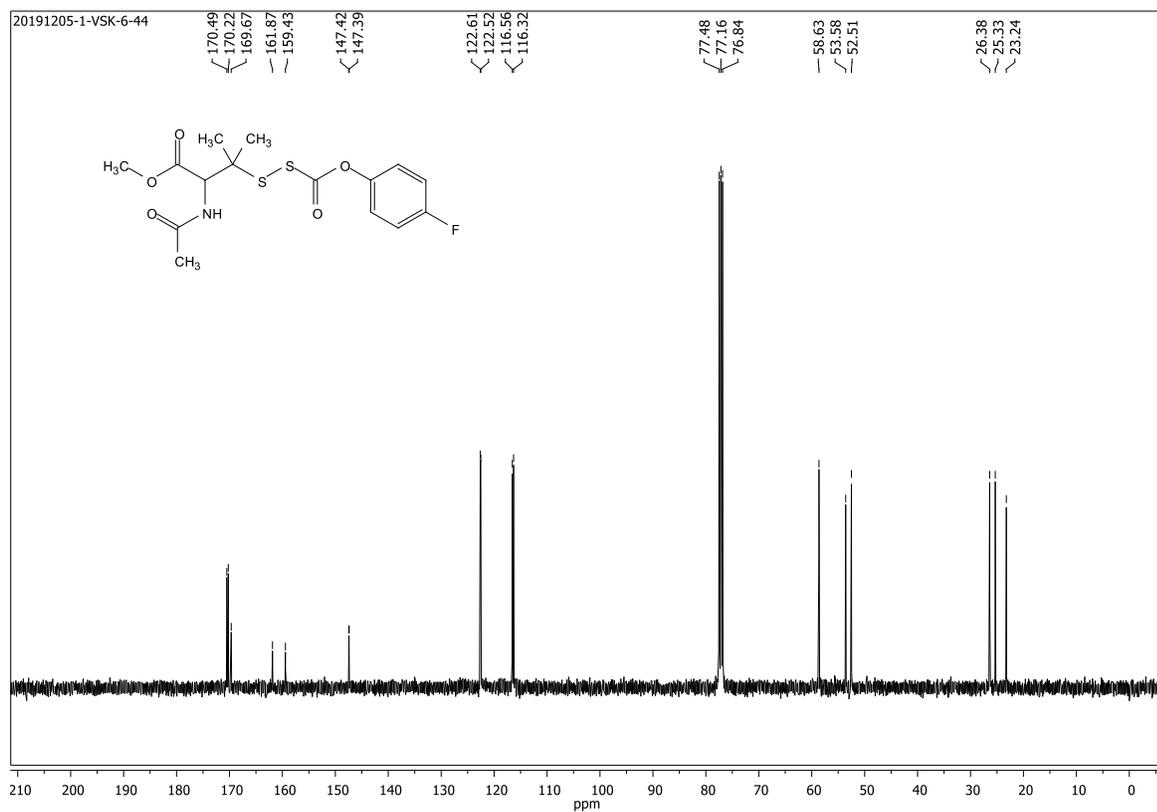
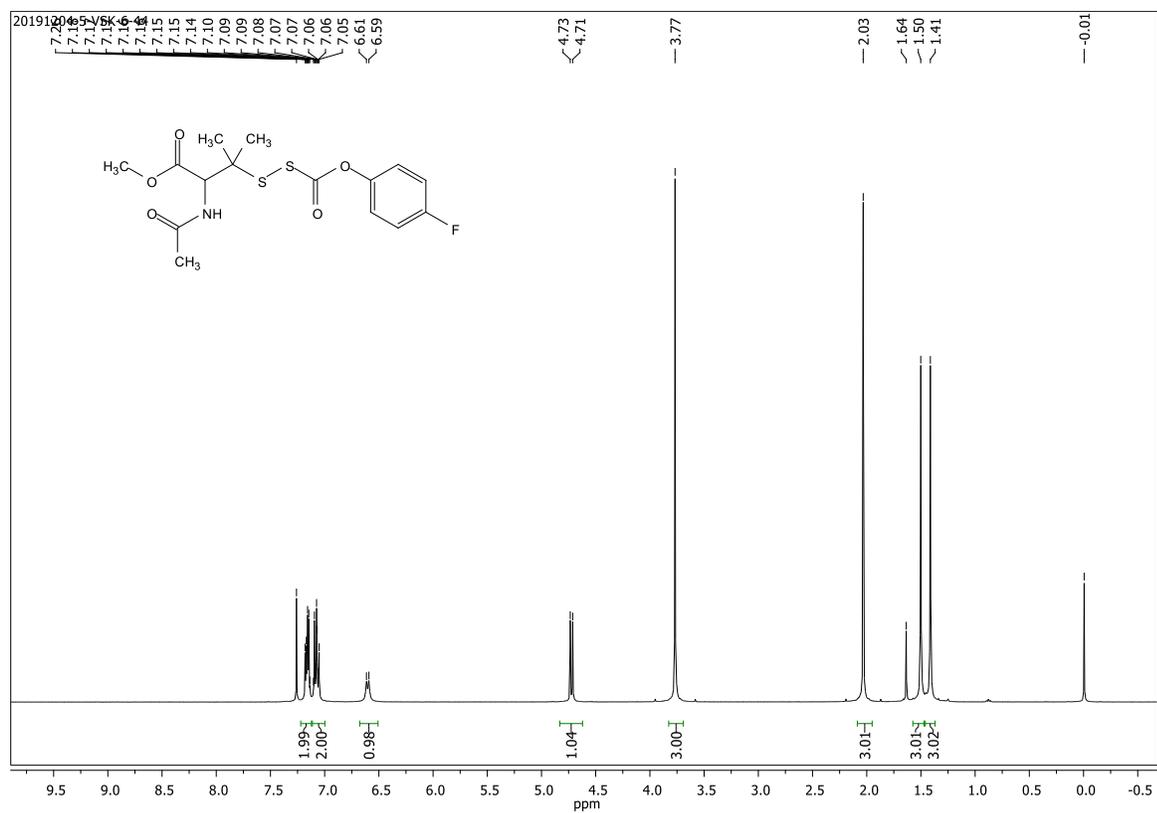


Figure S51. ^1H and ^{13}C NMR spectra of **1d**.

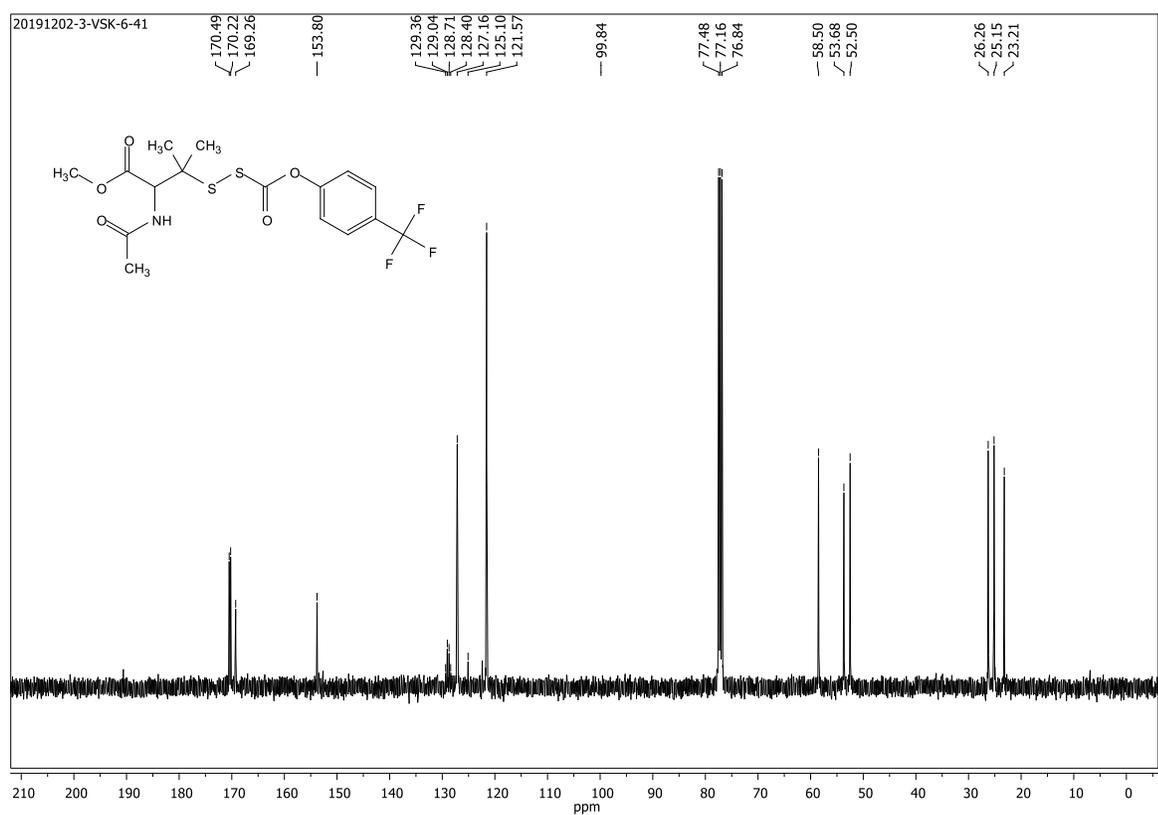
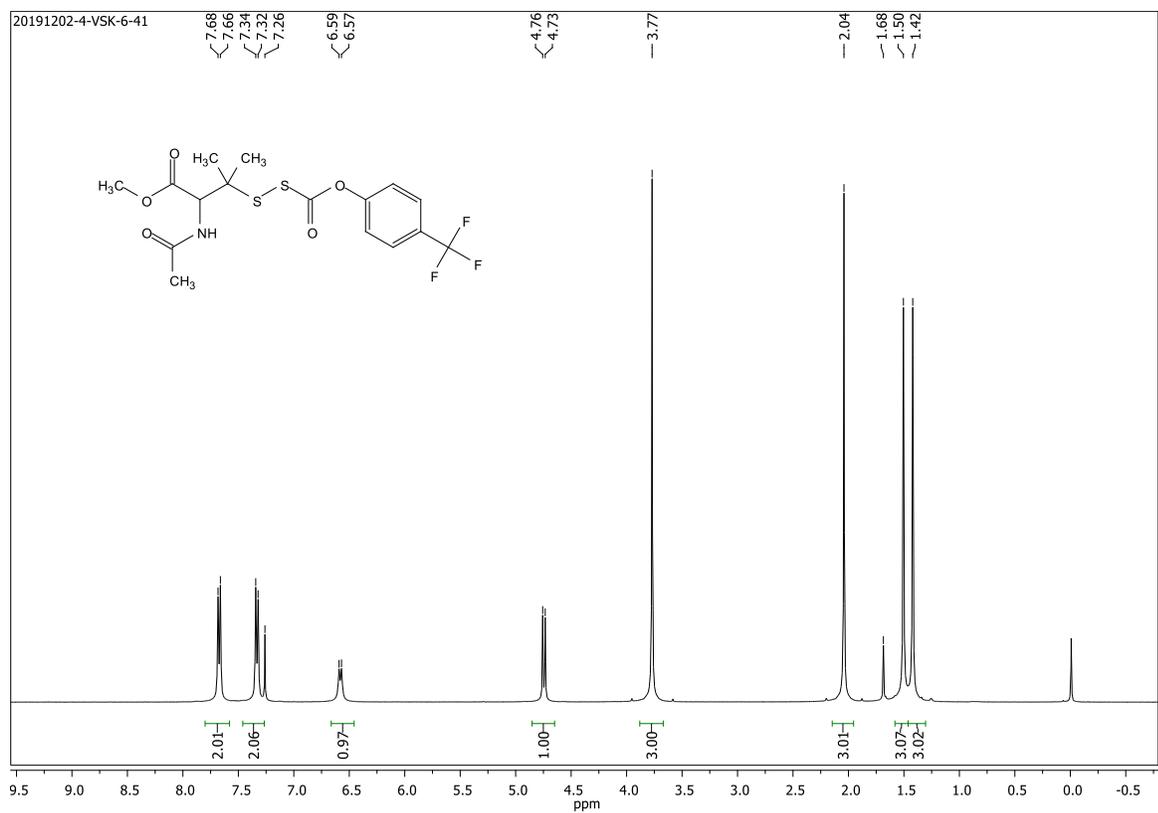


Figure S52. ^1H and ^{13}C NMR spectra of 1e.

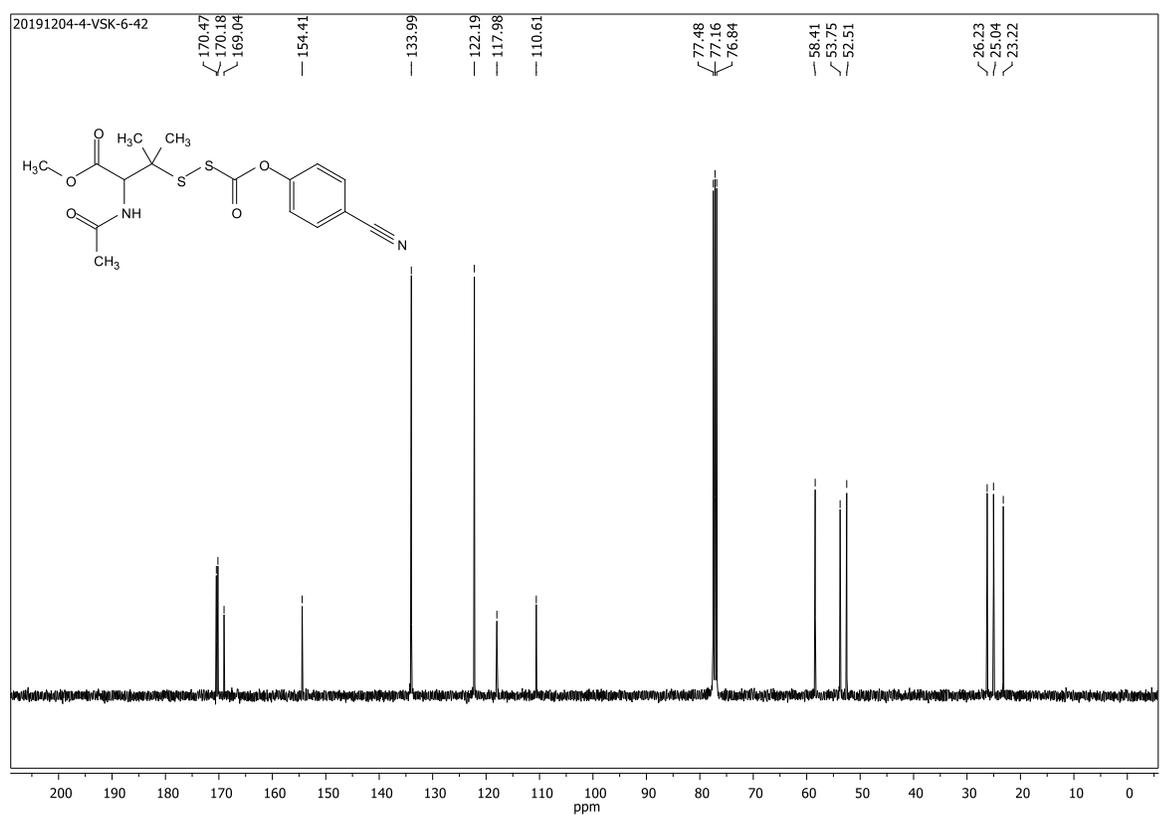
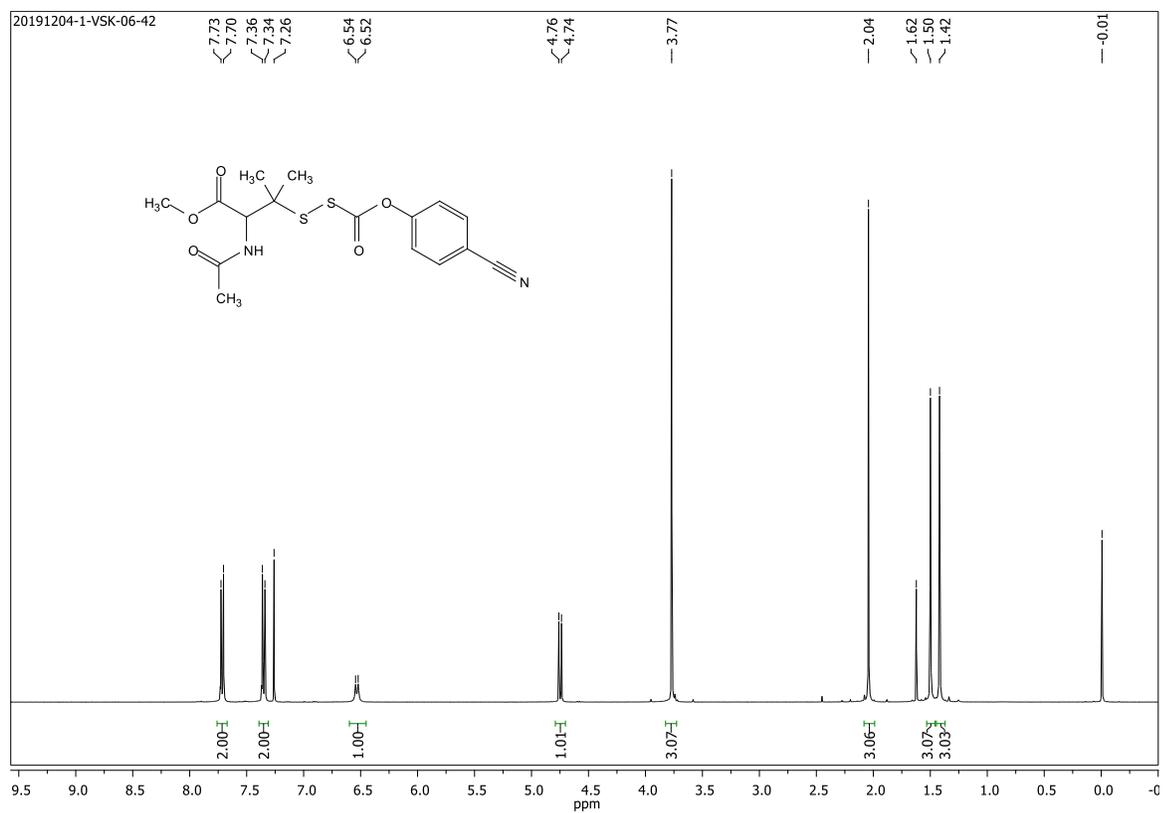


Figure S53. ^1H and ^{13}C NMR spectra of 1f.

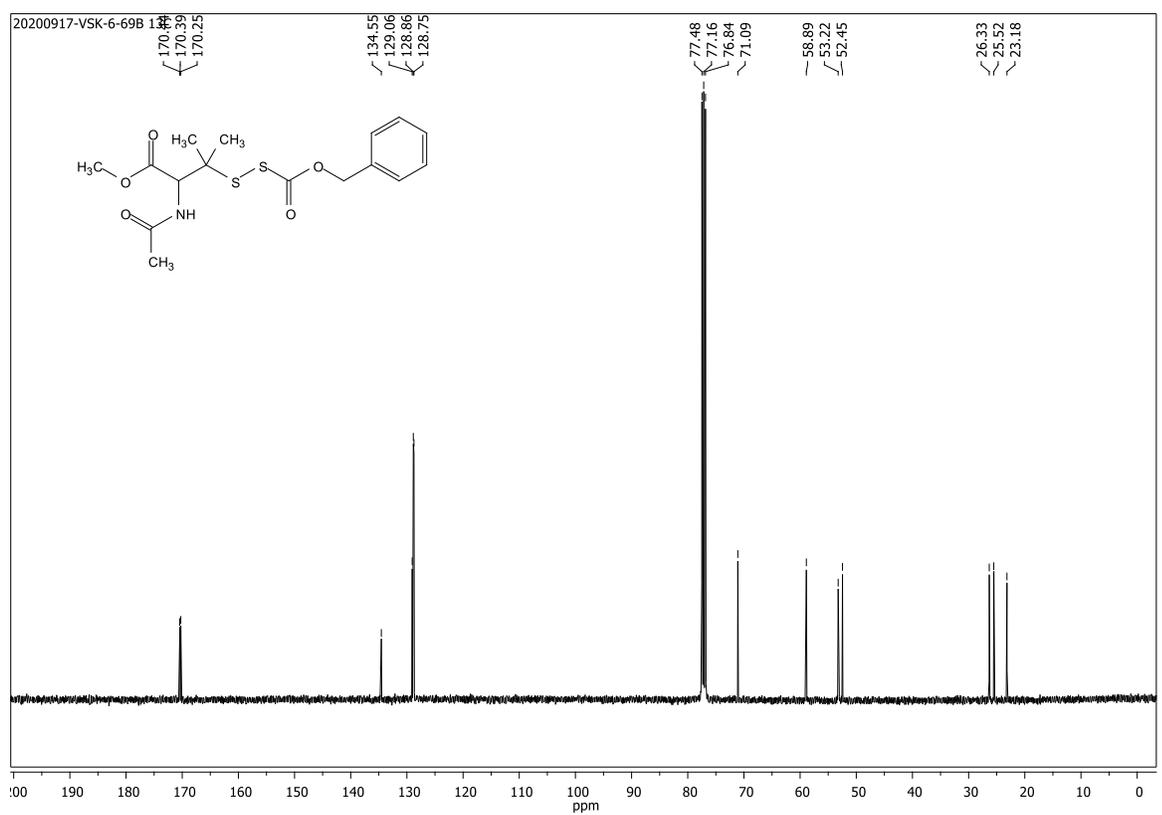
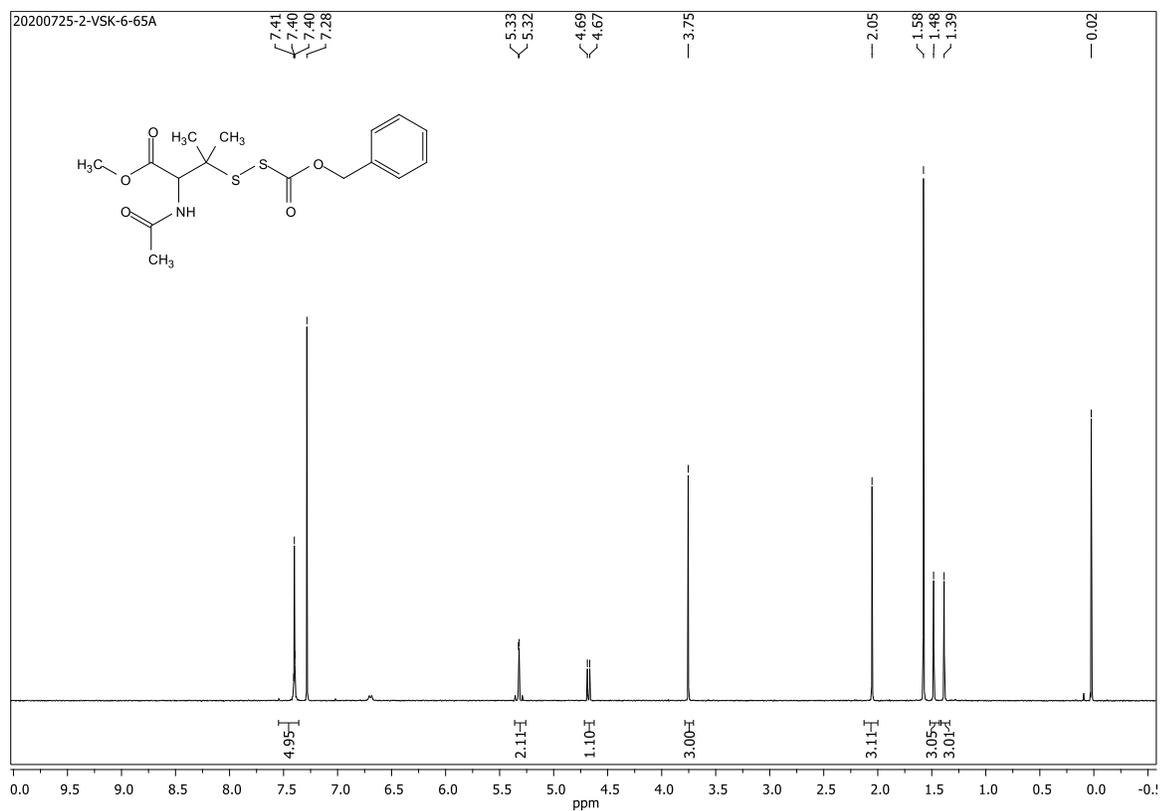


Figure S54. ^1H and ^{13}C NMR spectra of **8**.

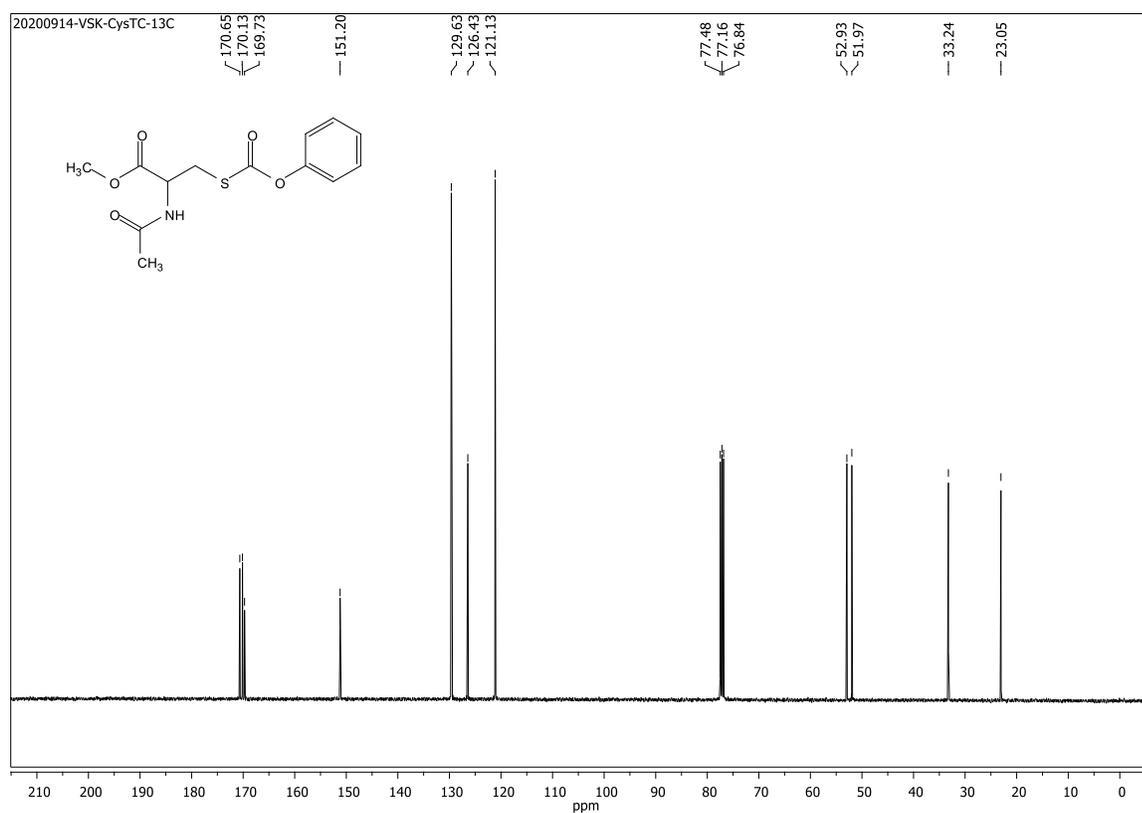
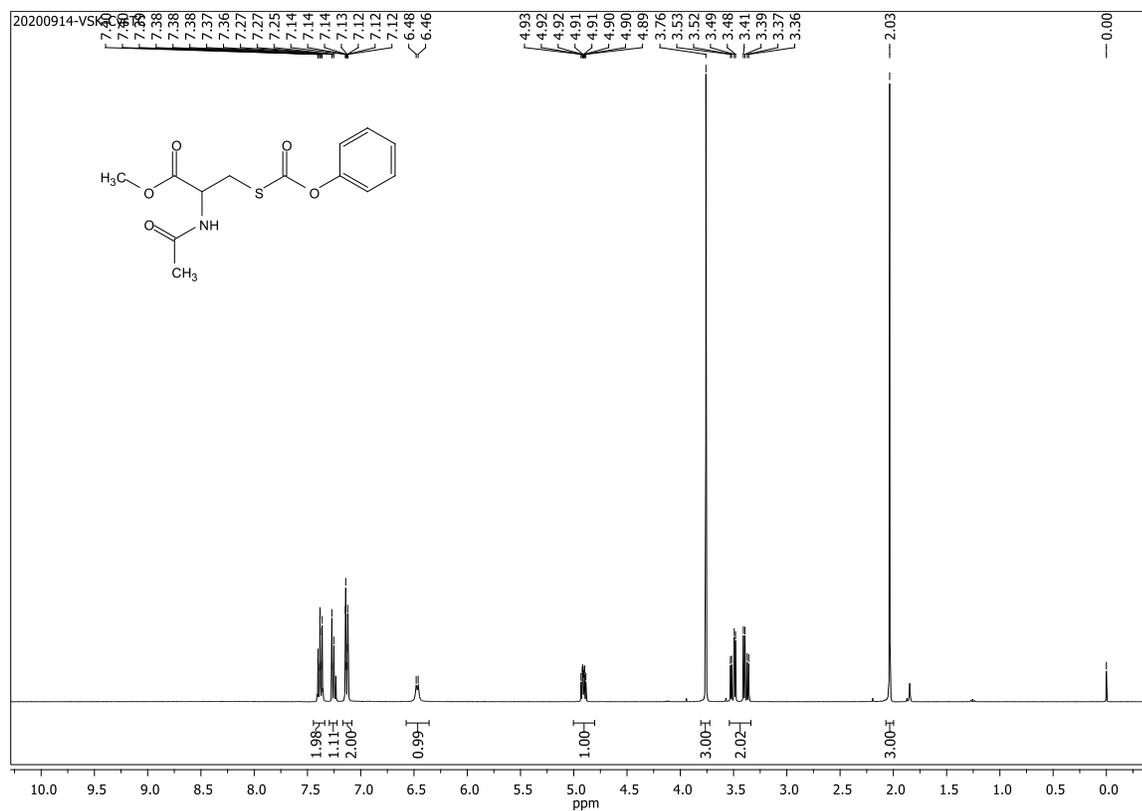


Figure S55. ^1H and ^{13}C NMR spectra of **9a**.

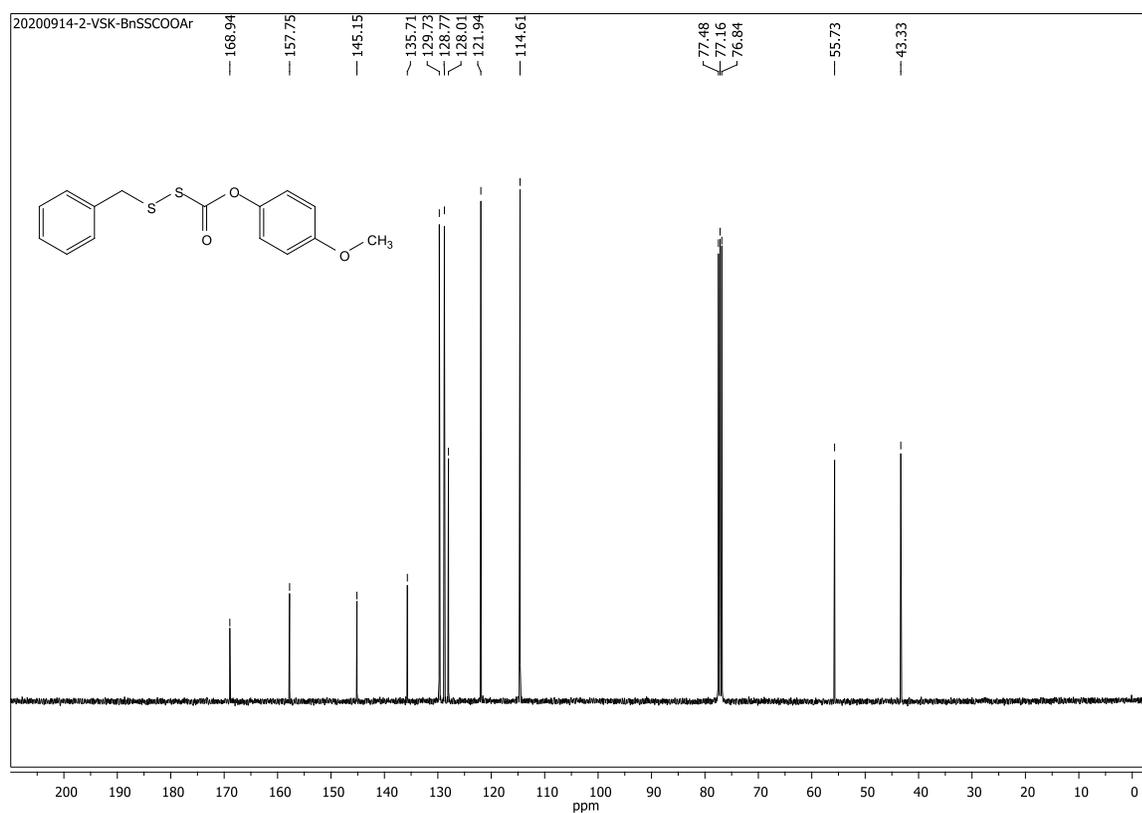
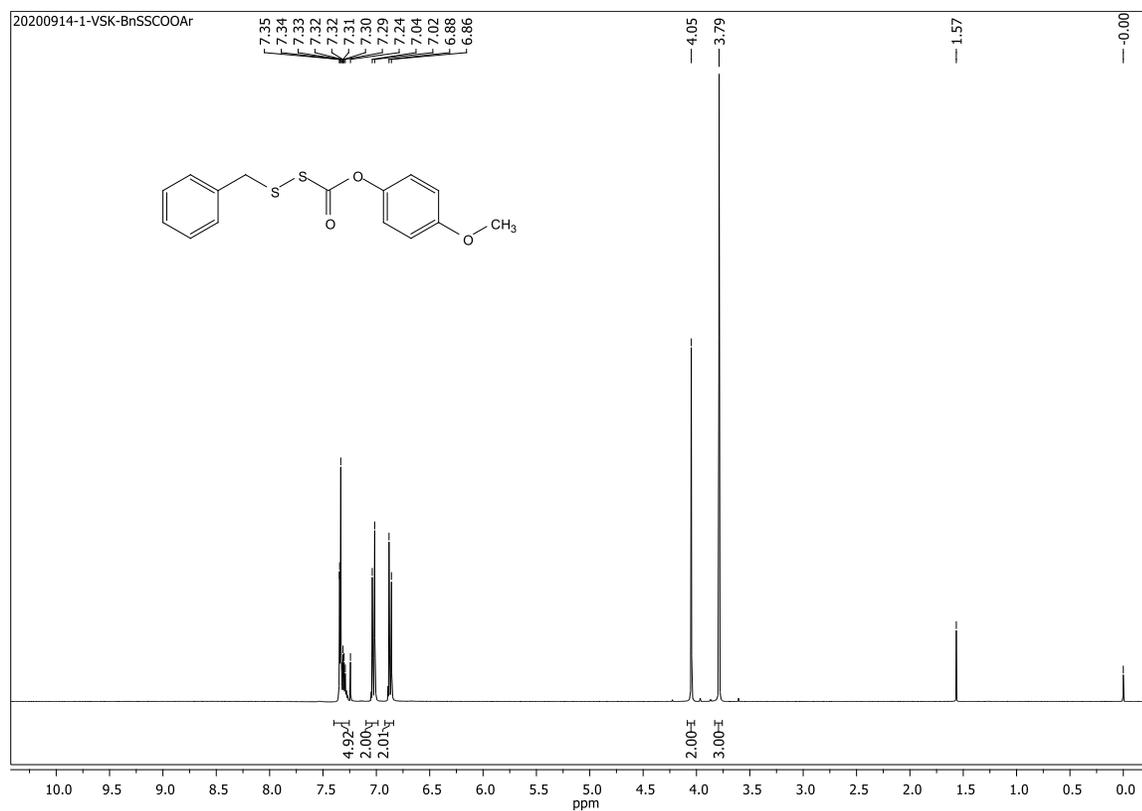


Figure S56. ^1H and ^{13}C NMR spectra of **13**.