

Supporting Information for

**Hydropersulfides (RSSH) Outperform Post-Conditioning and Other
Reactive Sulfur Species in Limiting Ischemia-Reperfusion Injury in the
Isolated Mouse Heart**

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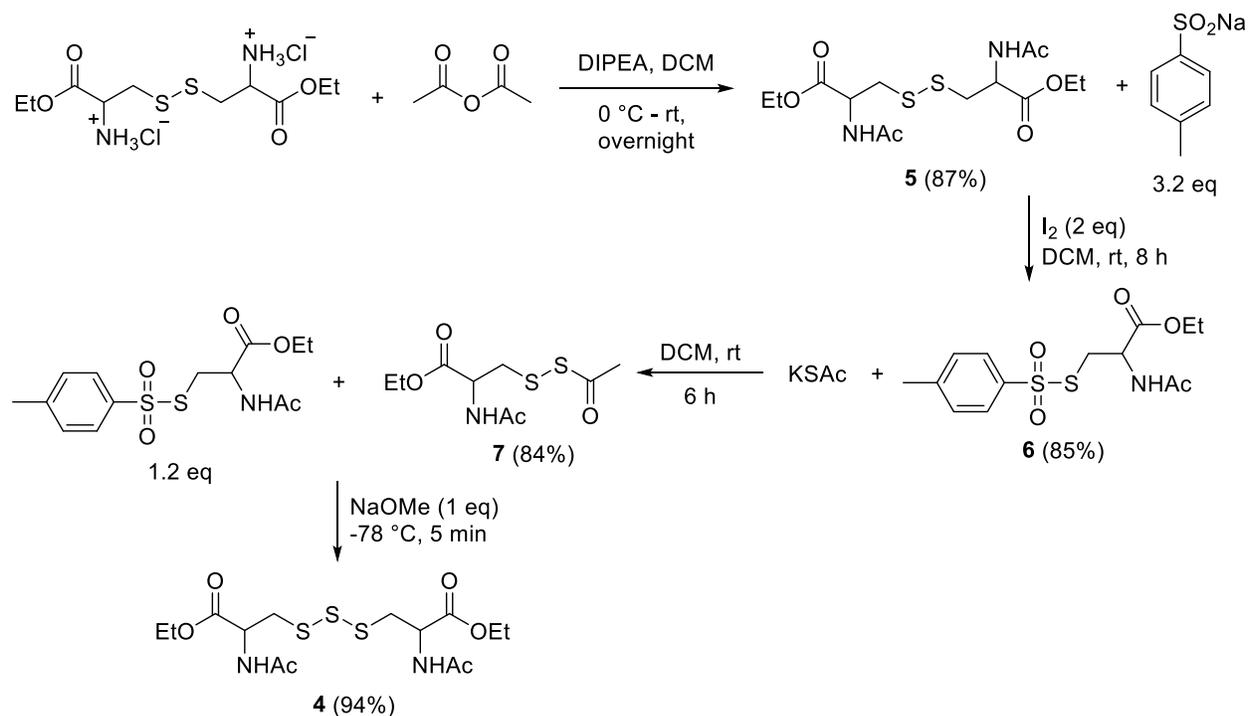
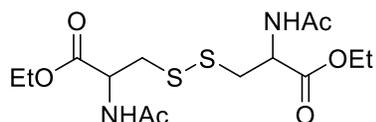
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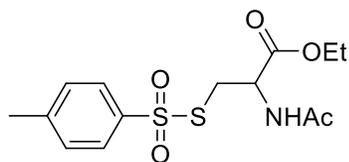
General Information

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 CDCl_3 (^{13}C NMR; $\delta = 77.16$ ppm), and $\text{DMSO}-d_6$ (^1H NMR; $\delta = 2.50$ ppm, ^{13}C NMR; $\delta = 39.52$ ppm). High-resolution mass spectra were obtained on a Waters Acquity Q-ToF MS/MS instrument. The pH measurements were performed using a Fisher Scientific Accumet AB15 pH-meter.

Scheme S1. Synthesis of *N*-acetyl-*O*-ethyl cysteine trisulfide (4)**Diethyl 3,3'-disulfanediybis(2-acetamidopropanoate) (5)**

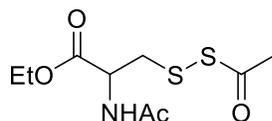
To an ice-cold suspension of *L*-cystine bis-ethyl ester dihydrochloride (2.0 g, 5.42 mmol) in dichloromethane (60 mL), diisopropylethylamine (3.01 g, 23.29 mmol) was added and the mixture was stirred at 0 °C for 5 min. Acetic anhydride (1.33 g, 13.00 mmol) was then added dropwise and the reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with dichloromethane (50 mL) and the organic phase was washed with water (3×50 mL), followed by brine. The combined organic layer was dried over Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc) to yield the desired product **5** (1.8 g, 87 %) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 6.57 (d, *J* = 7.3 Hz, 2H), 4.87 (dd, *J* = 12.5, 5.1 Hz, 2H), 4.24 (q, *J* = 7.1 Hz, 4H), 3.28 – 3.18 (m, 4H), 2.08 (s, 6H), 1.31 (t, *J* = 7.1 Hz, 6H); ¹³C NMR (101 MHz, CDCl₃) δ 170.5, 170.2, 62.1, 52.0, 40.9, 23.2, 14.2; HRMS (ESI) calc. for C₁₄H₂₄N₂O₆S₂ [M+Na]⁺: 403.0968; found: 403.0974.

Ethyl *N*-acetyl-S-tosylcysteinate (6)



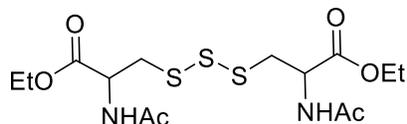
Aromatic thiosulfonate **6** was prepared according to a literature procedure.¹ Briefly, to a mixture of sodium benzenesulfinate (2.25 g, 12.62 mmol) and compound **5** (1.50 g, 3.94 mmol) in dichloromethane (40 mL), iodine (2.0 g, 7.88 mmol) was added, and the reaction mixture was stirred at room temperature for 6 h. The reaction mixture was diluted with dichloromethane (40 mL) and sodium thiosulfate (1 M) was added until the iodine color disappeared. The organic layer was separated, washed with brine, dried over Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by silica gel flash column chromatography (hexanes/EtOAc) to yield the aromatic thiosulfonate **6** (2.32 g, 85%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.79 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 6.46 (d, *J* = 7.2 Hz, 1H), 4.84 (dt, *J* = 7.4, 4.8 Hz, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.47 (dd, *J* = 6.3, 4.9 Hz, 2H), 2.45 (s, 3H), 2.02 (s, 3H), 1.27 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 170.3, 169.6, 145.4, 141.3, 130.1, 127.3, 62.4, 51.7, 37.2, 23.0, 21.7, 14.1; HRMS (ESI) calc. for C₁₄H₁₉NO₅S₂ [M+Na]⁺: 368.0579; found: 368.0602.

Ethyl *N*-acetyl-S-(acetylthio)cysteinate (7)



To a solution of aromatic thiosulfonate **6** (1.0 g, 2.89 mmol) in dichloromethane (30 mL), potassium thioacetate (0.429 g, 3.76 mmol) was added under a nitrogen atmosphere. The heterogeneous mixture was stirred at room temperature for 6 h. A white precipitate formed during the reaction was removed by filtration and filtrate was concentrated under reduced pressure. The crude was purified using silica gel flash chromatography (hexanes/ethyl acetate) to obtain the desired product **7** (0.65 g, 84%) as a colorless liquid. ¹H NMR (400 MHz, CDCl₃) δ 6.83 (d, *J* = 7.4 Hz, 1H), 4.79 (dt, *J* = 7.9, 4.8 Hz, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.40-3.07 (m, 2H), 2.43 (s, 3H), 2.07 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 195.1, 170.2, 170.1, 62.1, 51.3, 41.4, 29.0, 23.2, 14.2; HRMS (ESI) calc. for C₉H₁₅NO₄S₂ [M+Na]⁺: 288.0335; found: 288.0341.

Diethyl 3,3'-trisulfanediyldis(2-acetamidopropanoate) (4)



Compound **4** was prepared according to a literature procedure.² Briefly, *N*-acetyl-S-(acetylthio)cysteinate (0.74 g, 2.80 mmol) and aromatic thiosulfonate **6** (1.16 g, 3.36 mmol) were dissolved in THF (30 mL) under N₂ and cooled to -78 °C for 10 min. Sodium methoxide (6.17 mL, 0.5 M, 3.08 mmol) was added dropwise and the resulting solution was stirred rapidly for 5 min.

The reaction was then quenched with saturated aqueous ammonium chloride (30 mL), and the product was extracted into EtOAc (3 × 30 mL). The organic layer was washed with brine, dried over Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by reverse phase chromatography (acetonitrile/water) to afford the desired product **4** (1.09 g, 94%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 6.57 (d, *J* = 7.5 Hz, 2H), 4.91 (dt, *J* = 7.6, 4.8 Hz, 2H), 4.29 – 4.17 (m, 4H), 3.47 – 3.38 (m, 4H), 2.06 (s, 6H), 1.30 (t, *J* = 7.1 Hz, 6H); ¹³C NMR (101 MHz, CDCl₃) δ 170.3, 170.1, 62.2, 51.8, 41.5, 23.2, 14.2; HRMS (ESI) calc. for C₁₄H₂₄N₂O₆S₃ [M+H]⁺: 413.0869; found: 413.0870.

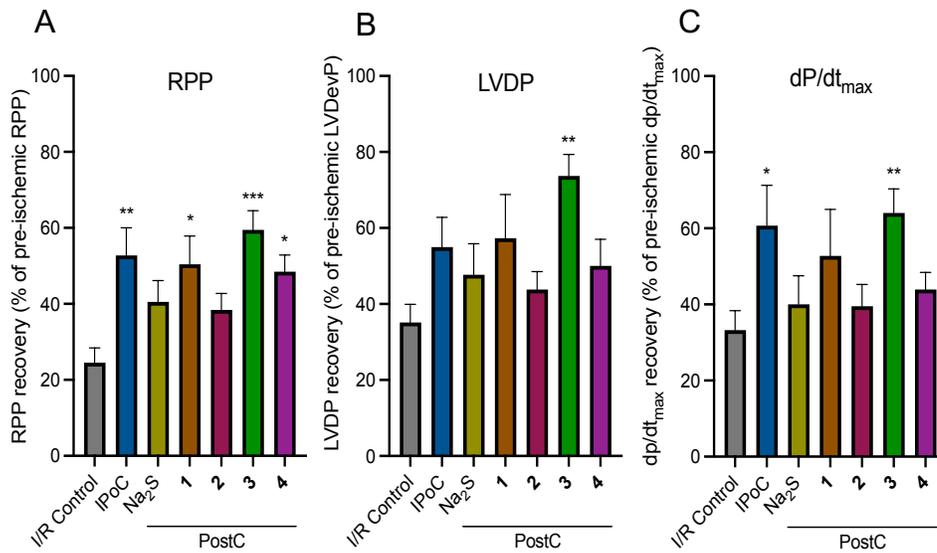


Figure S1. Cardioprotective effects induced by mechanical or pharmacological post-conditioning with different RSS at 100 μ M. (A) Post-ischemic left ventricular RPP functional recovery; (B) Post-ischemic left ventricular developed pressure recovery; (C) Post-ischemic left ventricular rate of contraction. Results are expressed as the mean \pm SEM using values obtained at 30 minutes of reperfusion normalized to the respective pre-ischemic value (n = 8 in each group) *P, 0.05, **P, 0.005, ***P, 0.001 vs. I/R-Control.

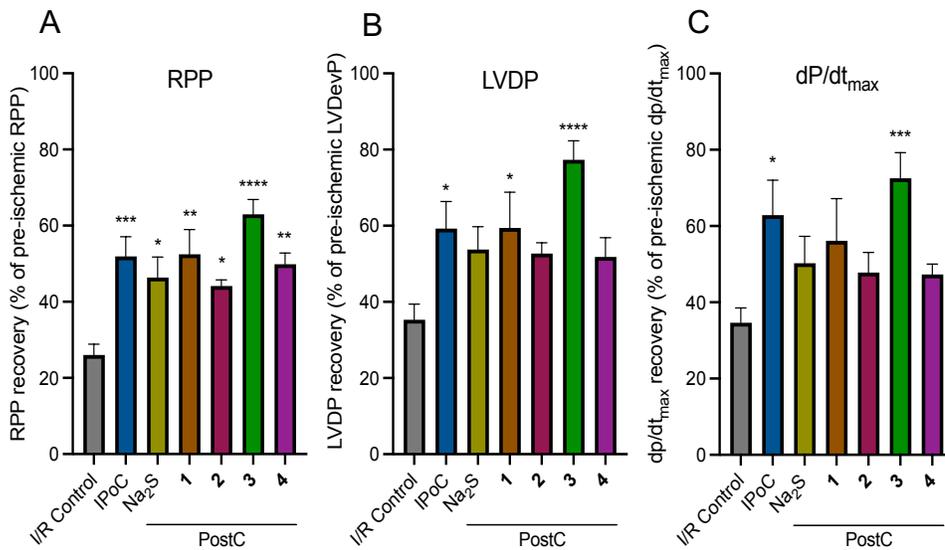


Figure S2. Cardioprotective effects induced by mechanical or pharmacological post-conditioning with different RSS at 100 μ M. (A) Post-ischemic left ventricular RPP functional recovery; (B) Post-ischemic left ventricular developed pressure recovery; (C) Post-ischemic left ventricular rate of contraction. Results are expressed as the mean \pm SEM using values obtained at 60 minutes of reperfusion normalized to the respective pre-ischemic value (n = 8 in each group) *P, 0.05, **P, 0.005, ***P, 0.001, ****P < 0.0001 vs. I/R-Control.

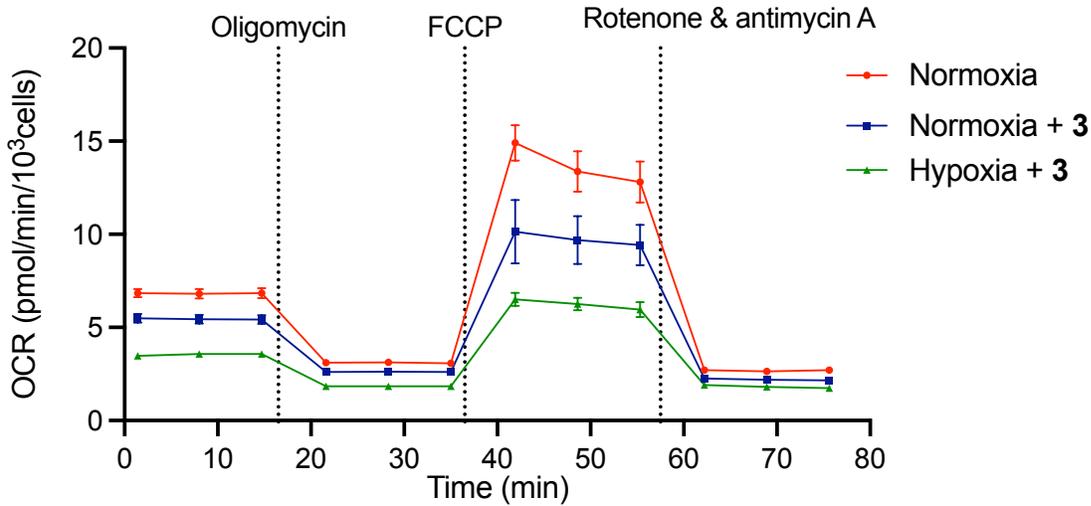


Figure S3. OCR traces for H9c2 cells undergoing 24 h incubation with RSSH precursor **3** (150 μ M) under normoxia or for 3 h following 7 h hypoxia compared to control.

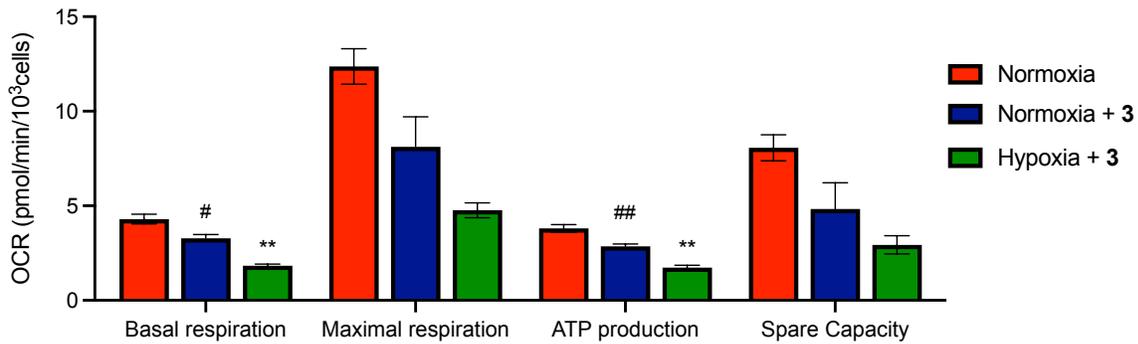


Figure S4. Calculated basal respiration, maximal respiration, ATP production, and spare capacity for H9c2 cells undergoing 24 h incubation with RSSH precursor **3** (150 μ M) under normoxia or for 3h following 7 h hypoxia compared to control ($n=3$) # p , 0.05, ## p , 0.005 vs. normoxia control; ** p , 0.005 vs. normoxia + **3** group.

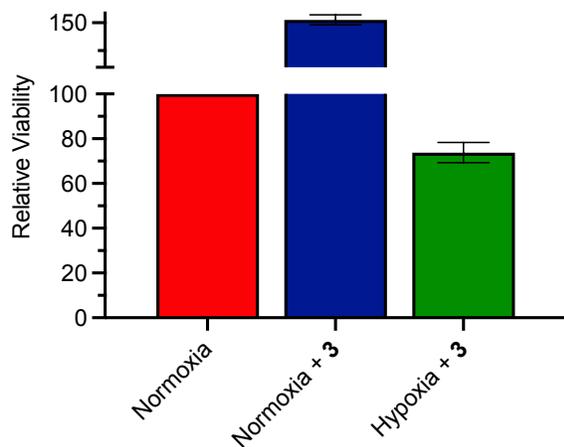
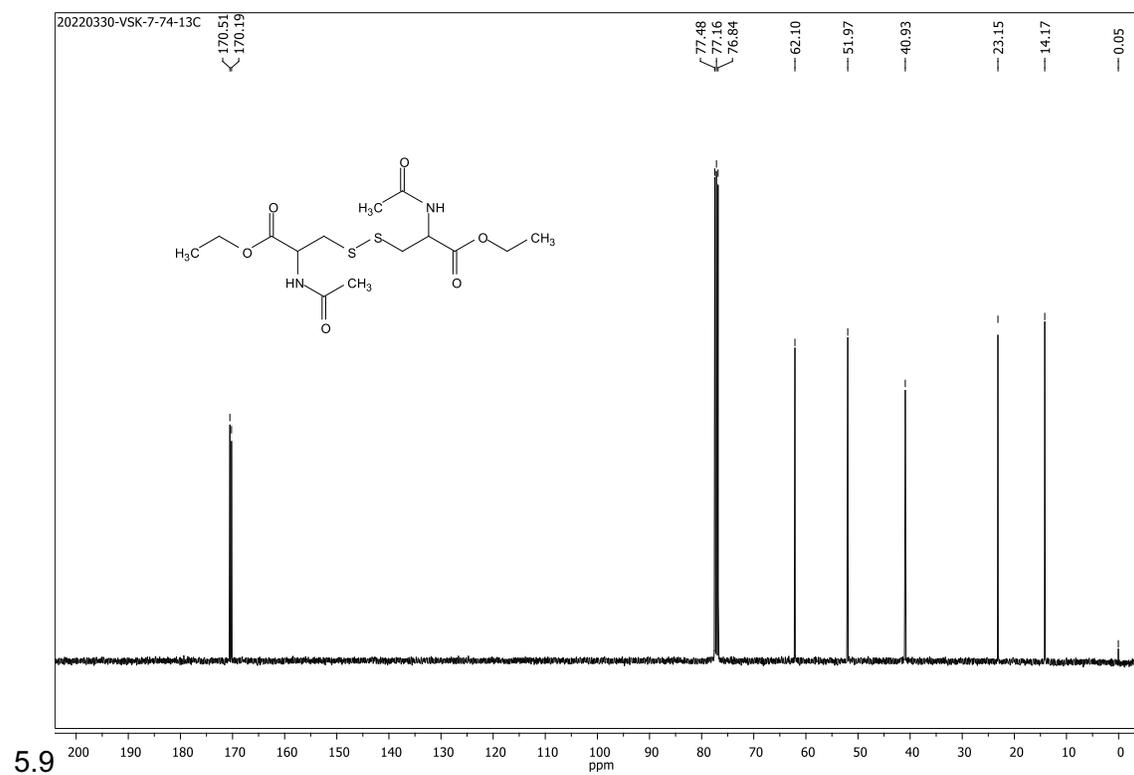
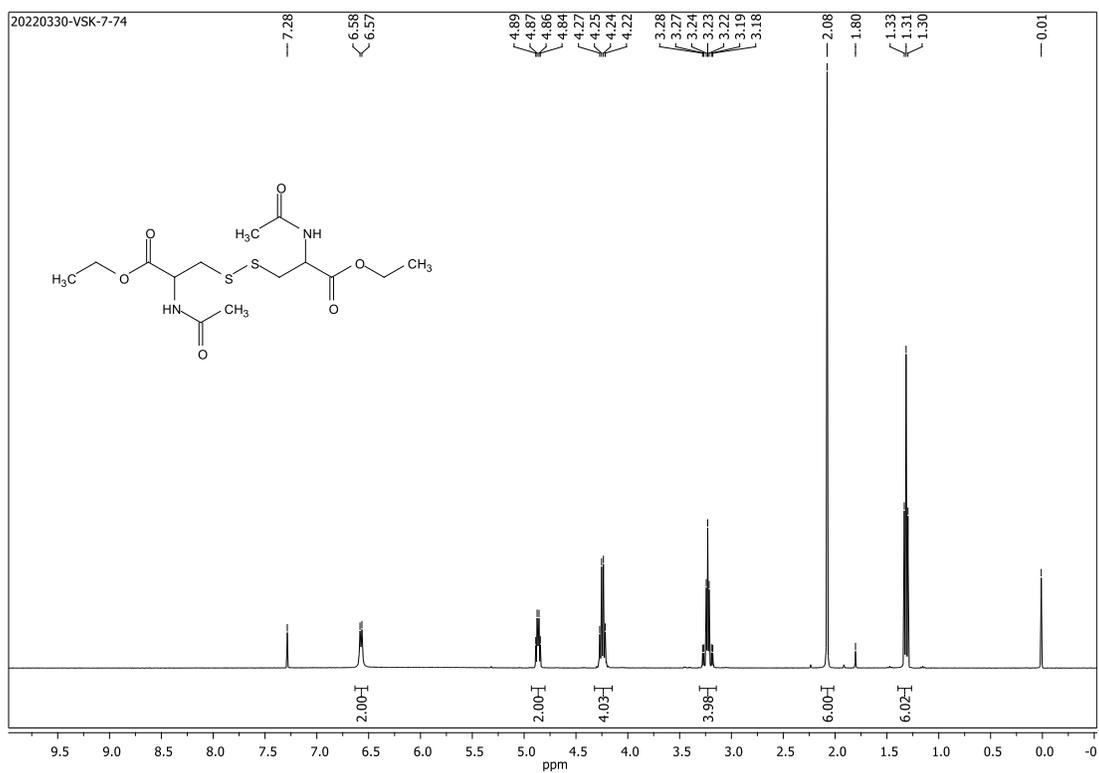
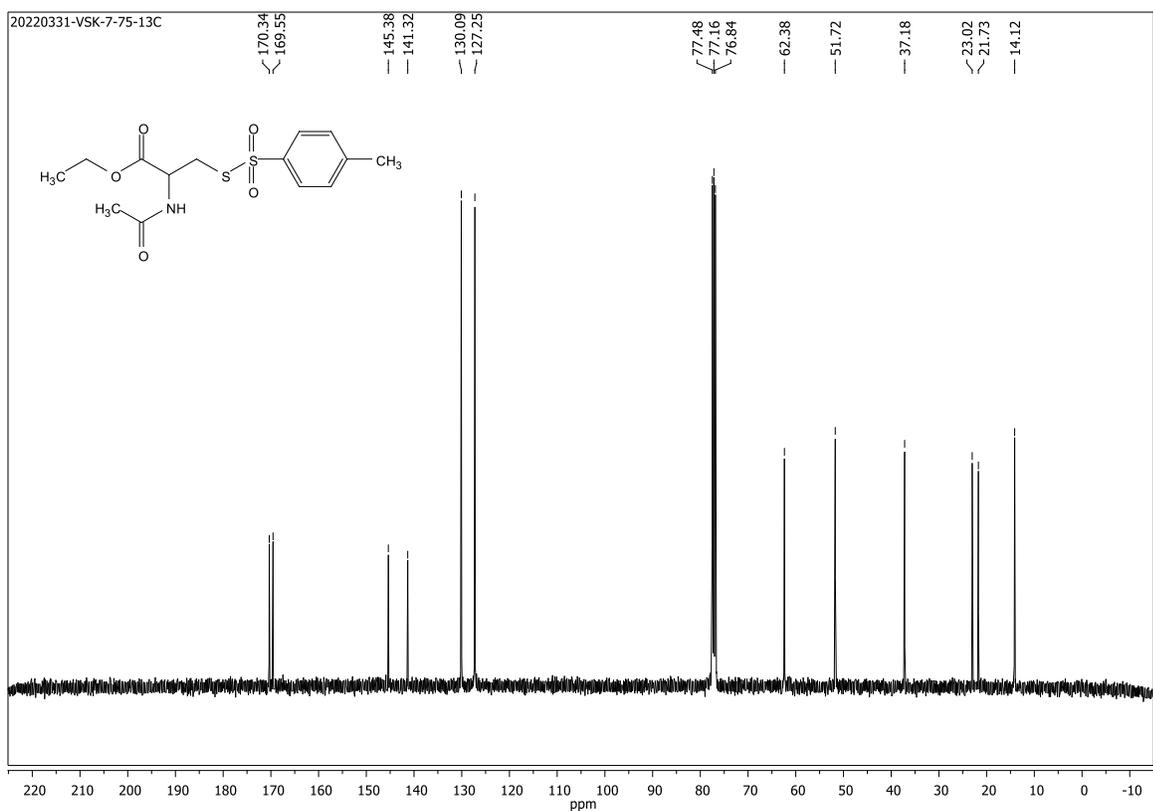
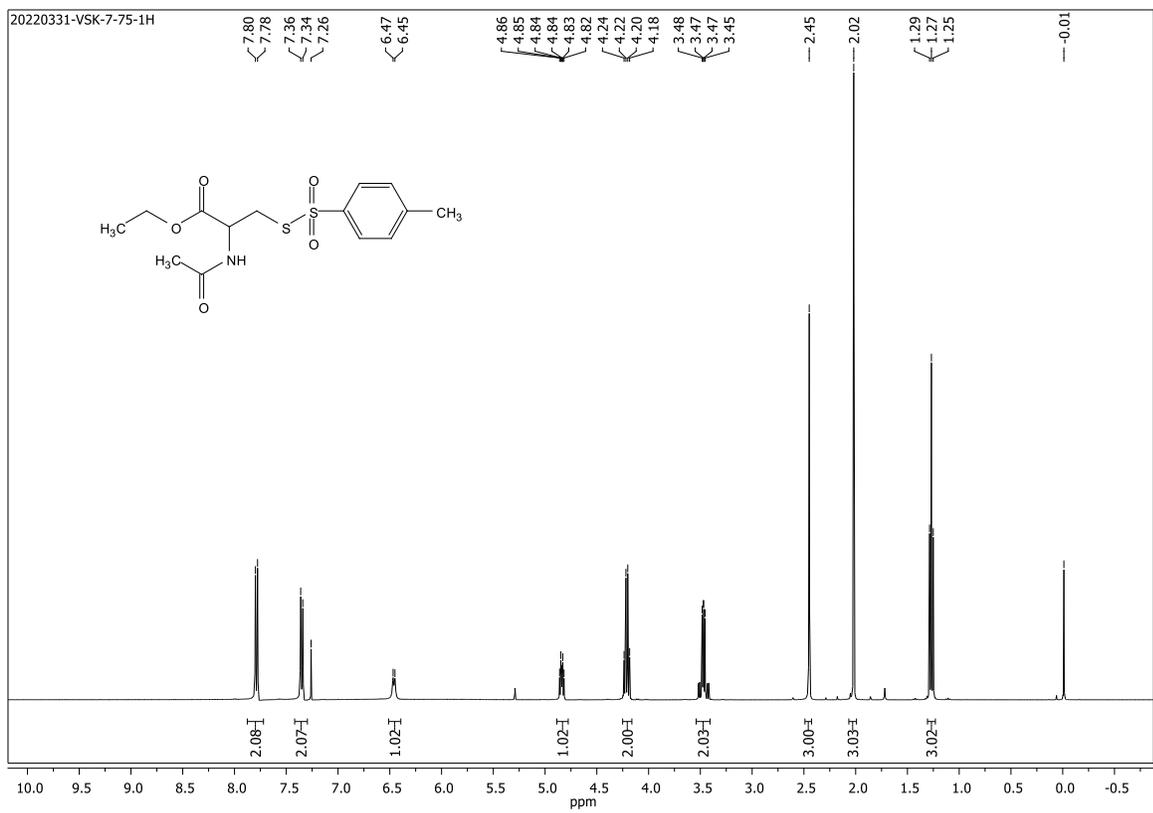


Figure S5. Comparison of cell viabilities for H9c2 cells undergoing 24 h incubation with RSSH precursor **3** under normoxia or for 3 h following 7 h hypoxia compared to control.

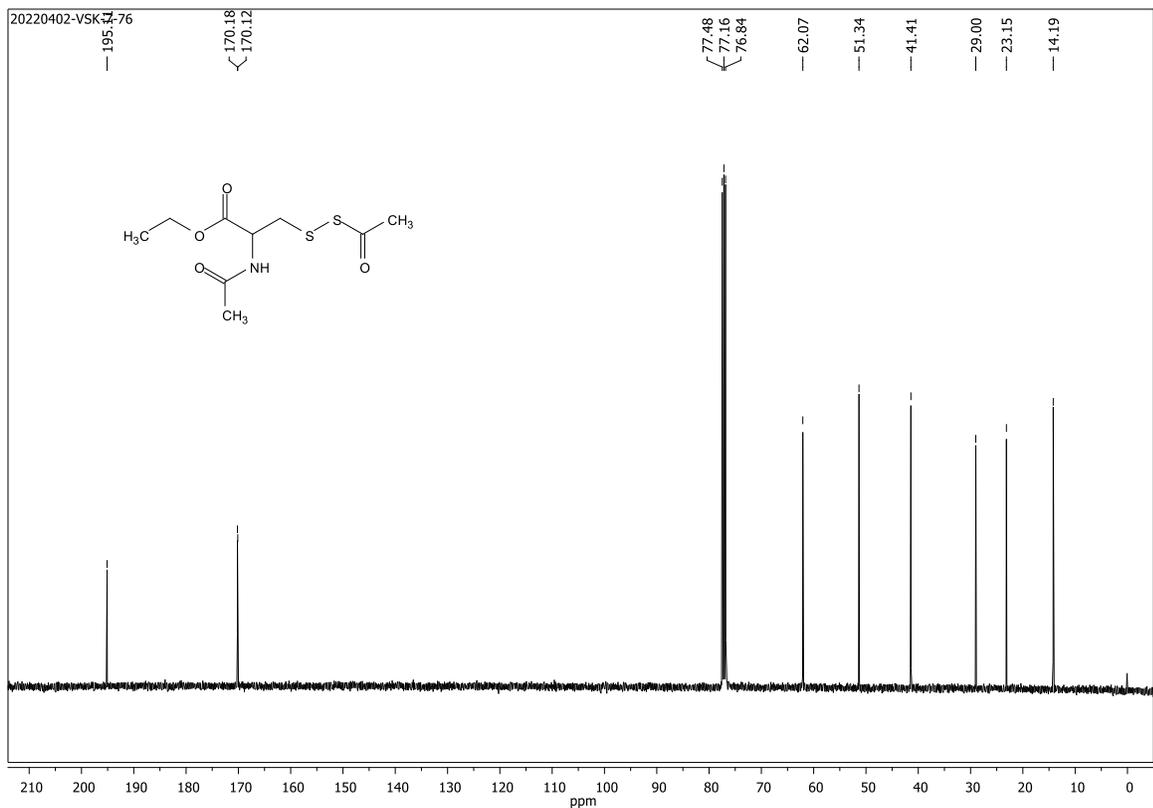
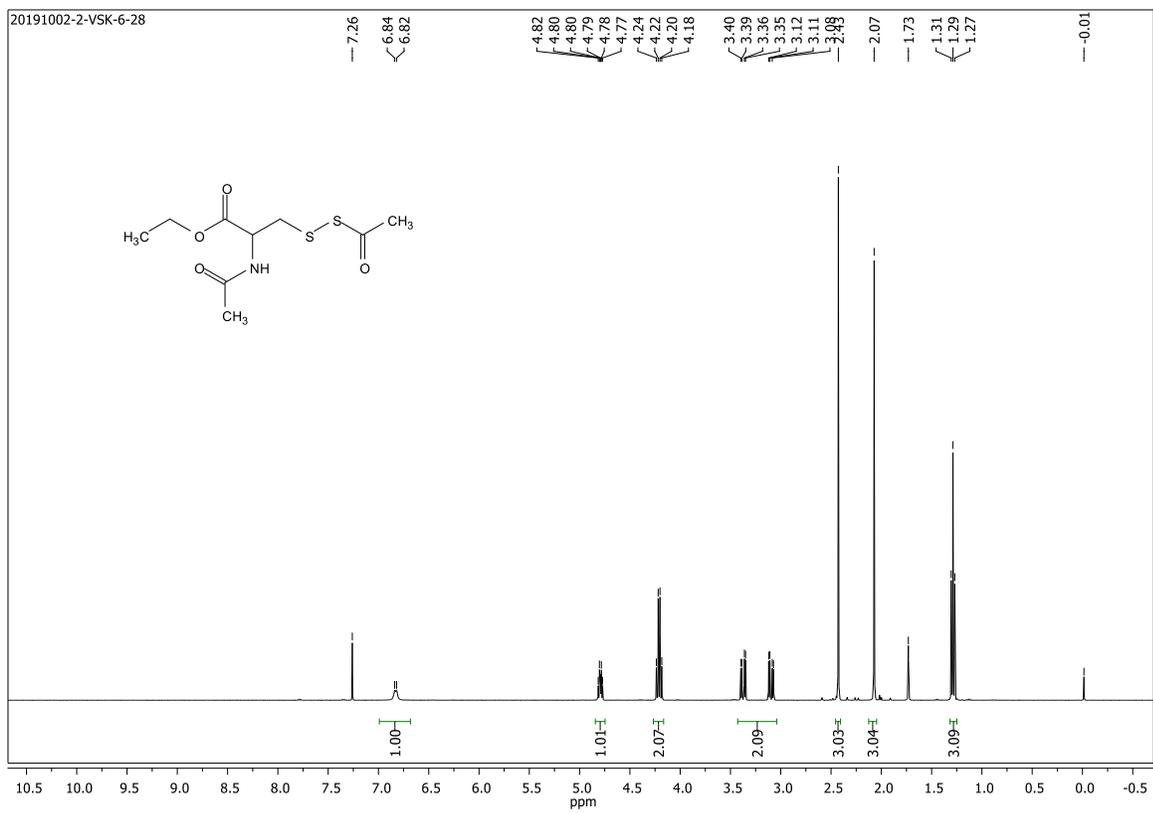
NMR spectra



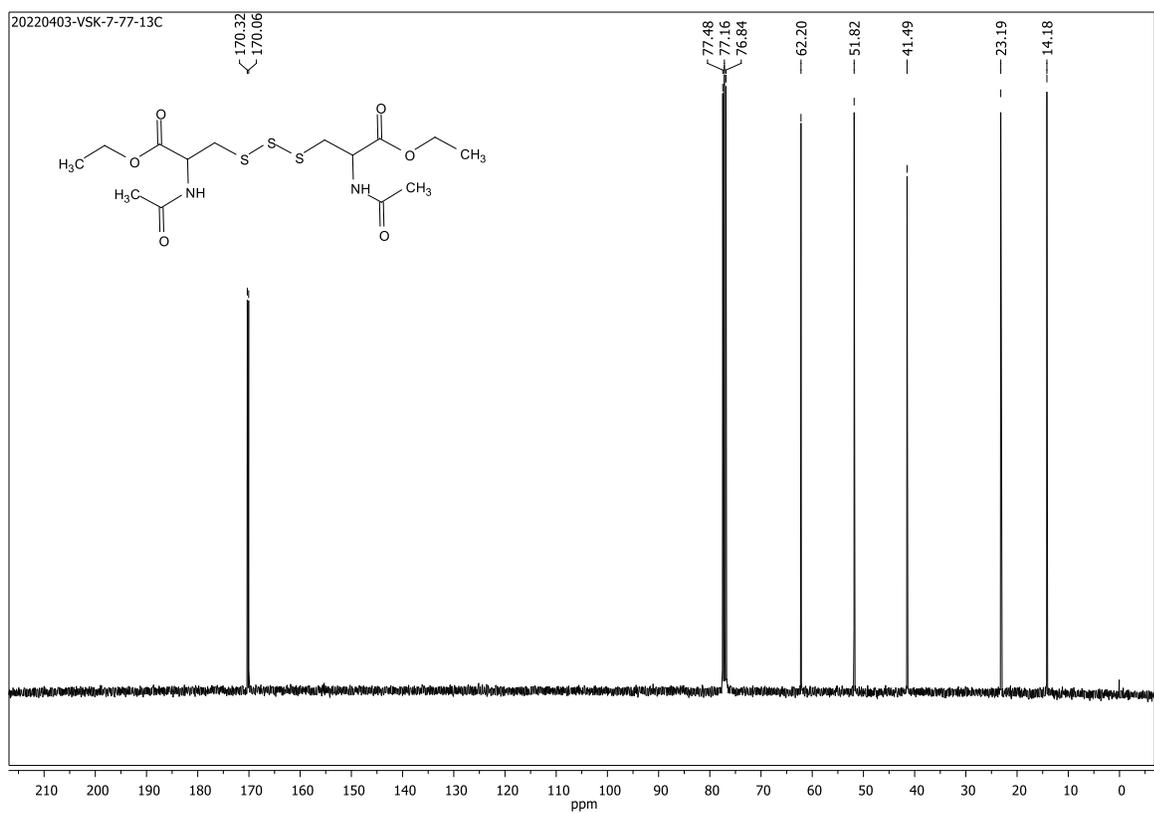
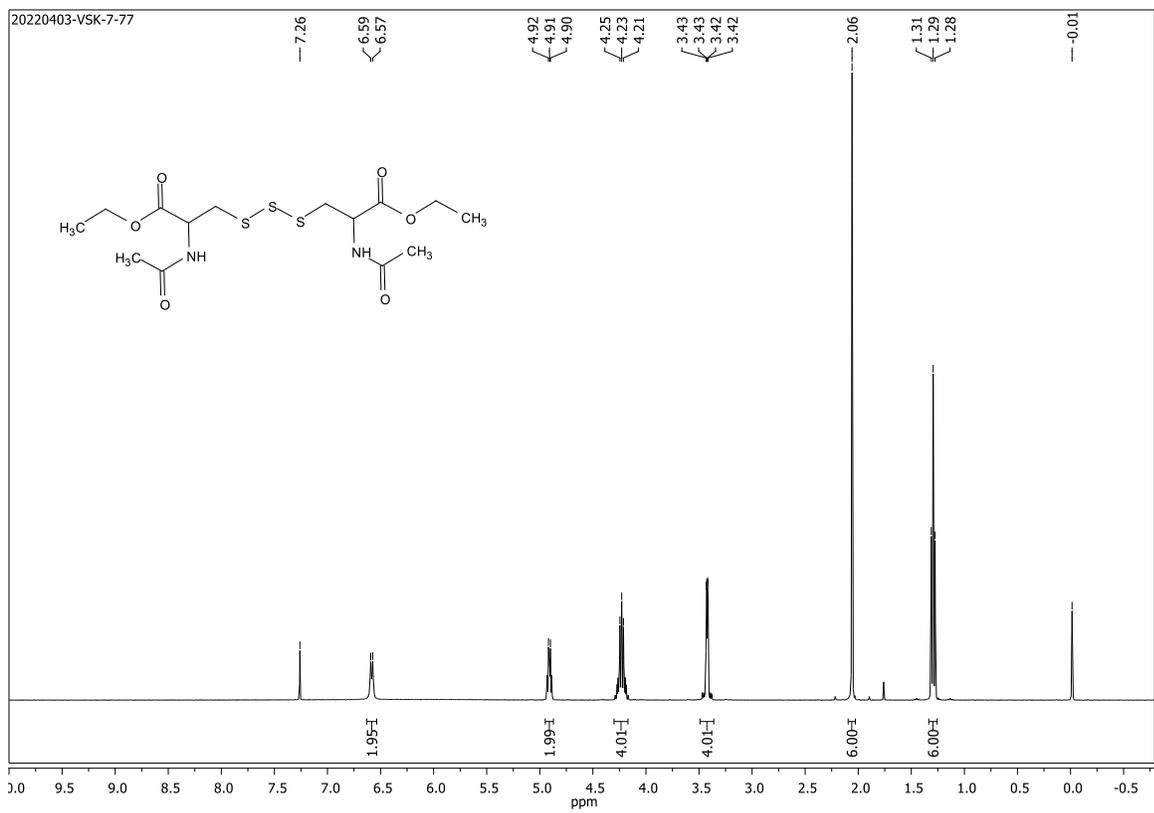
5.9 ^1H and ^{13}C NMR spectra of compound 5



^1H and ^{13}C NMR spectra of compound **6**



¹³C NMR spectra of compound 7



^1H and ^{13}C NMR spectra of compound 4