Electronic Supporting Information (ESI)

for

An Esterase-Cleavable Persulfide Donor with No Electrophilic Byproducts and a Fluorescence Reporter

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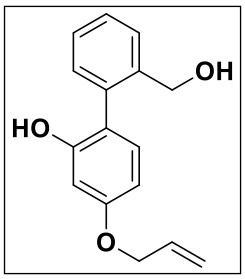
1. General methods:

All the chemicals and solvents were purchased from commercial sources and used as received unless stated otherwise. Column chromatography was performed using silica gel-Rankem (60–120 mesh) as stationary phase. Preparative high-performance liquid chromatography (HPLC) was done using Combiflash EZ prep UV using a Kromasil®C-18 preparative column (250 mm × 21.2 mm, 5 μm). ^{1}H and ^{13}C spectra were recorded on a JEOL 400 MHz (or 100 MHz for ^{13}C) or a Bruker 400 MHz (or 100 MHz for ^{13}C) spectrometer unless otherwise specified using either residual solvent signals ($CDCl_3$ δH = 7.26 ppm, δC = 77.2 ppm), or as an internal tetramethylsilane (δH = 0.00, δC = 0.0). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The following abbreviations are used: m (multiplet), s (singlet), d (doublet), t (triplet), ddt (doublet of doublet of triplet) and dq (doublet of quartet). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using a BRUKER-ALPHA FT-IR spectrometer and reported in cm^{-1}. All measurements were done using a LC/MS method in the positive ion mode using high-resolution multiple reaction monitoring (MRM-HR) analysis on a Sciex X500R quadrupole time-of-flight (QTOF) mass spectrometer fitted with an Exion UHPLC system. Photometric measurements were performed using an Ensight Multimode Plate Reader (PerkinElmer). Fluorometric measurements were performed using a Thermo Scientific Varioscan microplate reader and a HORIBA Scientific Fluoromax-4 spectrofluorometer.

2. Synthesis and characterization:

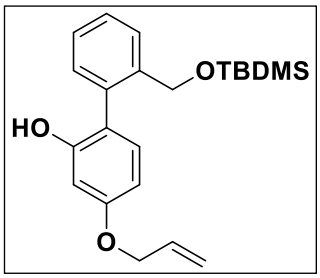
Compounds **2a**[1,2], **2b**[2], and **9**[3] were synthesized following previously reported protocols, and each compound's analytical data was consistent with reported values.

4-(allyloxy)-2'-(hydroxymethyl)-[1,1'-biphenyl]-2-ol (3)[4]:



Lithium aluminium hydride (LAH) pellets (1.8g, 47.5 mmol) were added to a solution of compound **2a** (4g, 15.8 mmol) in dry THF (50 mL) at 0 ˚C under N_2 atmosphere. After 15 min, following the consumption of starting material by TLC, the reaction was quenched with ice-cold water (30 mL), and the resulting solution was extracted with EtOAc (3 × 10 mL). The combined organic phase was washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude product was then recrystallized with $CHCl_3$ to give the desired compound **3** (1.63 g, 94%) as white colored solid. FT-IR (ν_{max}, cm^{-1}): 3273, 1616; ^1H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.51 (m, 1H), 7.43 – 7.37 (m, 2H), 7.25 – 7.23 (m, 1H), 7.02 – 6.99 (m, 1H), 6.58 – 6.57 (m, 2H), 6.08 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.99 (s, 1H), 5.46 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.32 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.55 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.50 (s, 2H), 2.26 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.7, 154.0, 139.2, 136.5, 133.3, 131.5, 131.4, 129.4, 128.7, 128.6, 120.5, 118.0, 107.7, 102.9, 69.0, 63.9; HRMS (ESI-TOF) for $C_{16}H_{16}O_3[M+H]^+$: Calcd., 257.1172, Found, 257.1177.

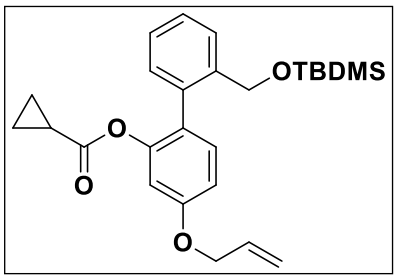
4-(allyloxy)-2'-(((*tert*-butyldimethylsilyl)oxy)methyl)-[1,1'-biphenyl]-2-ol (4)[4]:



To a solution of **3** (0.256 g, 1.0 mmol) in anhydrous DCM (20 mL), imidazole (0.147 g, 2.2 mmol) was added at RT under N_2 atmosphere. After stirring for 1 h at RT the ice bath was used to bring down the temperature to 0 ˚C, and TBDMS-Cl (0.331 g, 2.2 mmol) was added to it. After 10 min , the reaction was brought back to RT and stirred for another 2 h. After the consumption of the starting material, as monitored by TLC, the organic solvent was evaporated under reduced pressure, diluted with water, and the aqueous solution was extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and the filtrate was concentrated to give a crude compound. This crude was further purified by column chromatography using silica gel (60 - 120) with 8% EtOAc/hexane as the eluent to obtain **4** (0.304 g, 87%) as a pale-yellow liquid. FT-IR (ν_{max}, cm^{-1}): 2928, 2856, 1618; ^1H NMR (400 MHz, $CDCl_3$) δ 7.52 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.36 (td, *J* = 6.8, 1.7 Hz, 2H), 7.3 - 7.21 (m, 1H), 7.00 (d, *J* = 8.3 Hz, 1H), 6.59 (dt, *J* =

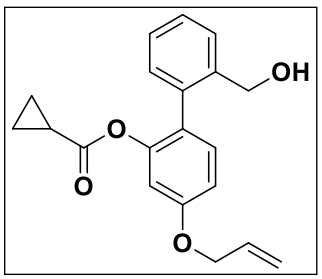
8.3, 2.5 Hz, 2H), 6.09 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.45 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.31 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.56 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.50 (s, 2H), 0.91 (s, 9H), 0.07 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.7, 158.8, 148.8, 139.7, 134.7, 132.9, 131.3, 130.1, 127.6, 126.4, 126.3, 117.9, 112.4, 108.9, 69.1, 62.7, 29.7, 18.3, 12.8, -5.4; HRMS (ESI-TOF) for $C_{22}H_{30}O_3Si[M+Na]^+$: Calcd., 393.1856, Found, 393.1863.

4-(allyloxy)-2'-(((*tert*-butyldimethylsilyl)oxy)methyl)-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (5)[4]:



A solution of **4** (0.24 g, 0.6 mmol) and cyclopropane carboxylic acid (CPCA) (0.084 g, 1.0 mmol) were taken in DCM (20 mL). To the above reaction mixture DMAP (0.008 g, 0.064 mmol) was added and stirred for 15 min. To the reaction mixture, DCC (0.147g, 0.71 mmol) was added under N_2 atmosphere and the reaction was stirred at RT for overnight. Upon completion of the reaction, as monitored by TLC solvent was evaporated, diluted with 10 mL of water, extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and filtrate was concentrated to give a crude compound. This crude was further purified by column chromatography using silica gel (60 - 120) with 6% EtOAc/hexane as the eluent to obtain **5** (0.261 g, 92%) as a yellow liquid. FT-IR (ν_{max}, cm^{-1}): 2928, 2856, 1751, 1617; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (dd, *J* = 7.7, 0.7 Hz, 1H), 7.36 (td, *J* = 7.6, 1.4 Hz, 1H), 7.23 (d, *J* = 6.2 Hz, 1H), 7.16 (d, *J* = 8.5 Hz, 1H), 7.09 (dd, *J* = 7.6, 1.2 Hz, 1H), 6.84 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.72 (d, *J* = 2.5 Hz, 1H), 6.08 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.44 (dq, *J* = 17.3, 1.6 Hz, 1H) , 5.32 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.57 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.50 (d, *J* = 7.0 Hz, 2H), 1.51-1.45 (m, 1H), 0.90 (s, 9H), 0.70 (d, *J* = 8.0 Hz, 4H), 0.00 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.8, 158.9, 148.9, 139.9, 134.8, 133.1, 131.4, 130.2, 127.7, 126.6, 126.5, 126.2, 118.0, 112.5, 109.1, 69.2, 62.8, 26.1, 18.5, 13.0, 8.5, -5.2; HRMS (ESI-TOF) for $C_{24}H_{34}O_4Si[M+Na]^+$: Calcd., 461.2123, Found, 461.2119.

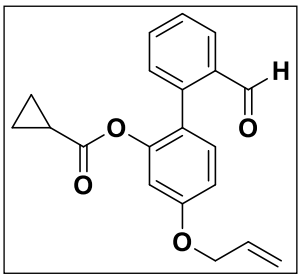
4-(allyloxy)-2'-(hydroxymethyl)-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (6)[4]:



To a well-stirred solution of **5** (0.24 g, 0.5 mmol) in H_2O:THF (1:1 v/v, 20 mL), AcOH (60 mL) was added and the reaction was stirred at RT for 18 h. Once the starting material was completely consumed, as monitored by TLC, the reaction mixture was extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and the filtrate was concentrated to give a crude product. This

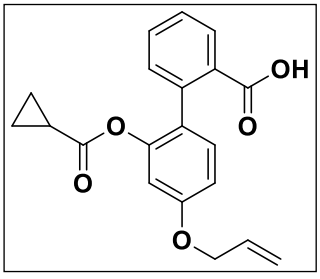
crude was further purified by column chromatography using silica gel (60 - 120) with 15% EtOAc/hexane as the eluent to obtain **6** (0.091 g, 52%) as a yellow liquid. FT-IR (ν_{max}, cm^{-1}): 3434, 1742, 1616; ^1H NMR (400 MHz, $CDCl_3$): δ 7.53 (dd, *J* = 7.7, 1.1 Hz, 1H), 7.37 (td, *J* = 7.5, 1.4 Hz, 1H), 7.28 (td, *J* = 7.5, 1.4 Hz, 1H), 7.17 – 7.13 (m, 2H), 6.86 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.72 (d, *J* = 2.5 Hz, 1H), 6.07 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.44 (dq, *J* = 17.3, 1.6 Hz, 1H), 5.31 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.56 (dt, *J* = 5.3, 1.5 Hz, 3H), 4.38 (d, *J* = 12.2 Hz, 1H), 1.52-1.47 (m, 1H), 0.70 (d, *J* = 8.0 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.4, 159.1, 149.1, 139.7, 135.8, 133.0, 131.6, 130.7, 129.0, 128.2, 127.2, 126.2, 118.1, 112.6, 109.0, 69.2, 63.1, 34.0, 12.9, 8.9, 8.8; HRMS (ESI-TOF) for $C_{20}H_{20}O_4[M+Na]^+$: Calcd., 347.1254, Found, 347.1259.

4-(allyloxy)-2'-formyl-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (7)[4]:



To a stirred solution of **6** (0.05 g, 0.15 mmol) in DCM (10 mL) at room temperature, PCC (0.07 g, 0.31 mmol) was added in to it under inert atmosphere. The reaction was stirred at RT for 40 min. The reaction mixture was filtered through celite and the filtrate was evaporated. The residue was further purified by column chromatography using silica gel (60-120) with 10% EtOAc/hexane as eluent, to provide **7** (0.038 g, 76%) as yellowish coloured solid. FT-IR (ν_{max}, cm^{-1}): 2921, 1750, 1694, 1616; ^1H NMR (400 MHz, $CDCl_3$) δ 9.84 (d, *J* = 0.7 Hz, 1H), 7.99 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.61 (td, *J* = 7.5, 1.5 Hz, 1H), 7.48 (t, *J* = 7.6 Hz, 1H), 7.32 (dd, *J* = 7.7, 0.8 Hz, 1H), 7.25 (d, *J* = 8.9 Hz, 1H), 6.91 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.76 (d, *J* = 2.5 Hz, 1H), 6.07 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.45 (dq, *J* = 17.3, 1.6 Hz, 1H), 5.33 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.57 (dt, *J* = 5.3, 1.5 Hz, 2H), 1.53 – 1.46 (m, 1H), 0.72 (d, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.5, 172.7, 159.8, 149.3, 140.8, 134.1, 133.7, 132.8, 131.5, 128.1, 127.0, 118.3, 113.0, 109.1, 69.3, 12.8, 9.0, 8.8; HRMS (ESI-TOF) for $C_{20}H_{18}O_4[M+Na]^+$: Calcd., 345.1097, Found, 345.1102.

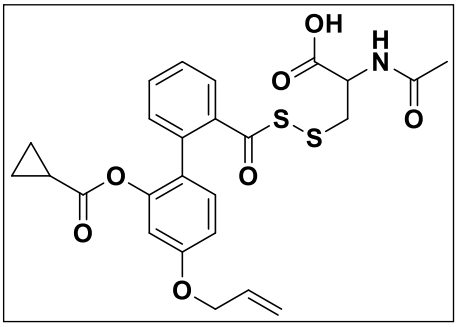
4'-(allyloxy)-2'-((cyclopropanecarbonyl)oxy)-[1,1'-biphenyl]-2-carboxylic acid (8)[4]:



A solution of **7** (0.520 g, 1.61 mmol) was taken in dry ACN (30 mL) under N_2 atmosphere, and KH_2PO_4 (0.074 g, 0.53 mmol) was added and stirred for 15 min. To the reaction mixture, 30% H_2O_2 (1.3 mL, 16.66 mmol) and $NaClO_2$ (0.332 g, 3.66 mmol) were added and stirred at room temperature for 2 h. Upon the completion of reaction, as monitored by TLC the solvent was evaporated under reduced

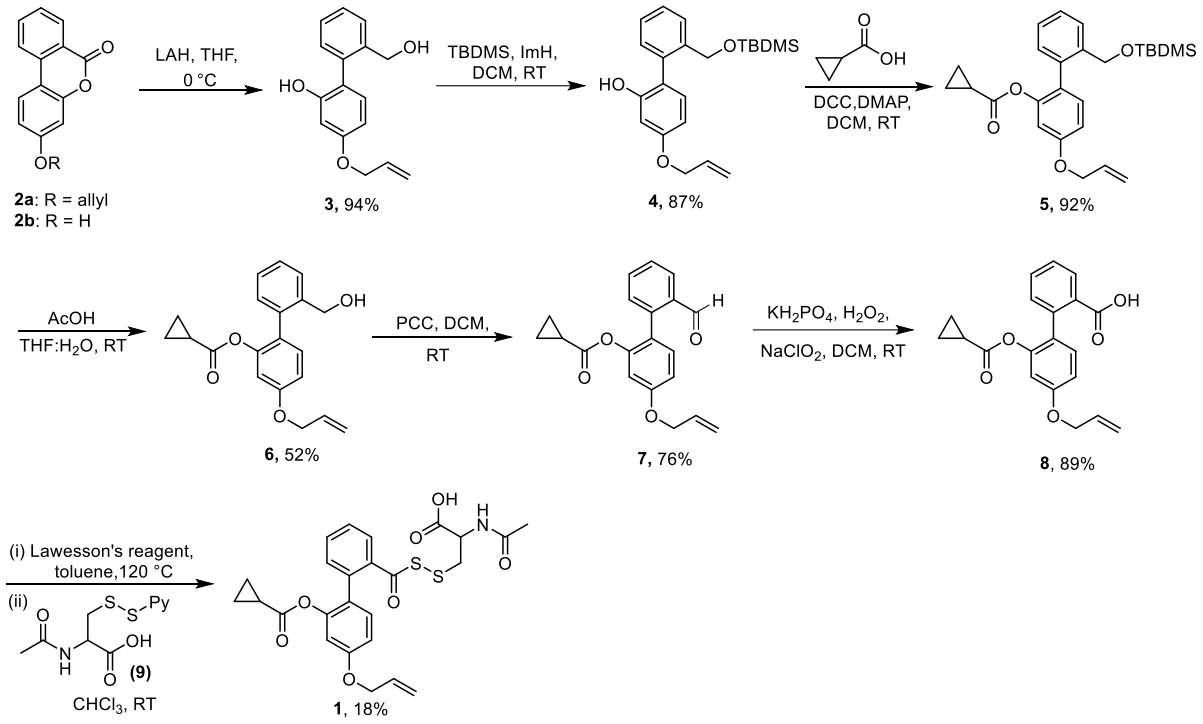
pressure. The reaction mixture was diluted with 10 mL of water and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to give a crude compound. The residue was further purified by column chromatography using silica gel (60-120) with 30% EtOAc/hexane as eluent, to give **8** (0.486 g, 89%) as bright yellow colored solid. FT-IR (ν_{max}, cm^{-1}): 2922, 1745, 1699, 1618; ^1H NMR (400 MHz, $CDCl_3$): δ 7.84 (d, *J* = 7.7 Hz, 1H), 7.51 (td, *J* = 7.5, 1.2 Hz, 1H), 7.42 (td, *J* = 7.6, 1.1 Hz, 1H), 7.27 (d, *J* = 6.5 Hz, 1H), 7.21 (d, *J* = 8.5 Hz, 1H), 6.88 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.73 (d, *J* = 2.5 Hz, 1H), 6.06 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.44 (dd, *J* = 17.3, 1.4 Hz, 1H), 5.32 (dd, *J* = 10.5, 1.2 Hz, 1H), 4.57 (d, *J* = 5.3, 1.5 Hz, 2H), 1.65 – 1.57 (m, 1H), 0.80 (d, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.6, 170.3, 159.7, 148.2, 136.2, 132.8, 132.5, 131.4, 131.3, 130.9, 130.3, 127.9, 125.9, 118.3, 113.2, 108.8, 69.3, 13.0, 9.7, 9.2; HRMS (ESI-TOF) for $C_{20}H_{18}O_5[M+Na]^+$: Calcd., 361.1046, Found, 361.1050.

***N*-acetyl-S-((4'-(allyloxy)-2'-((cyclopropanecarbonyl)oxy)-[1,1'-biphenyl]-2-carbonyl)thio)cysteine (1)[5]:**



A mixture of compound **8** (0.1 g, 0.28 mmol), lawesson's reagent (0.057 g, 0.14 mmol), and dry toluene (6 mL), was charged to a pressure tube and heated at 120 °C for 3 h. After consumption of the starting material, as monitored by TLC, the solvent was evaporated under reduced pressure. The reaction mixture was diluted with DCM (10 mL), washed with 1 N HCl and brine, and dried over Na_2SO_4. The filtrate was concentrated under reduced pressure to give a crude oily compound which was carried forward to the next step without further purification. The oily product was dissolved in $CHCl_3$ (10 mL), **9** (NAC-SS-Py) (0.078 g, 0.28 mmol) was added under N_2 atmosphere at RT. The solution was stirred for 8 h. Once the starting material was completely consumed, as monitored by TLC, the solvent was evaporated under reduced pressure to give crude compound. The residue was purified by preparative HPLC using Kromasil®C-18 column at ambient temperature under the gradient elution with H_2O:ACN (40:60, v/v) as mobile phase at a flow rate of 12 mL/min to afford pure product **1** (0.027 g, 18%) as a brownish solid. FT-IR (ν_{max}, cm^{-1}): 3348, 2922, 2855, 1736, 1707, 1647, 1618; ^1H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, *J* = 7.6 Hz, 1H), 7.72 (s, 1H), 7.58 (t, *J* = 7.1 Hz, 1H), 7.45 (t, *J* = 7.3 Hz, 1H), 7.33 (d, *J* = 7.6 Hz, 1H), 7.21 (d, *J* = 8.5 Hz, 1H),

6.86 (dd, *J* = 8.5, 2.4 Hz, 1H), 6.69 (d, *J* = 2.5 Hz, 1H), 6.06 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.42 (dd, *J* = 17.3, 1.1 Hz, 1H), 5.30 (dd, *J* = 10.5, 1.2 Hz, 1H), 4.54 (d, *J* = 5.2 Hz, 2H), 3.44 (s, 1H), 2.96 (d, *J* = 12.3 Hz, 1H), 1.96 (s, 3H), 1.61 - 1.54 (m, 1H), 0.78 (d, *J* = 6.7 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.0, 171.7, 159.7, 136.2, 136.1, 132.8, 132.3, 131.5, 128.3, 127.9, 125.3, 118.2, 109.0, 69.2, 52.5, 41.8, 22.6, 12.9, 9.1; HRMS (ESI-TOF) for $C_{25}H_{25}NO_7S_2[M+H]^+$: Calcd., 516.1145, Found, 516.1150.



Scheme S1: Synthesis of NAC-SSH persulfide donor **1.**

3. Experimental protocols

Monitoring the release of 2a upon esterase activation of 1:

(A) Fluorometric analysis:

Stock solutions of **1** (10 mM), **2a** (10 mM) in DMSO, and porcine liver esterase (100 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by adding 10 μM of **1** (10 μL, 1 mM), with or without 1 U/mL Es (esterase;10 μL, 100 U/mL stock) and the volume was adjusted to 1000 μL using phosphate buffer saline (10 mM, pH 7.4) in a 1.5 mL eppendorf tube and incubated for 60 min at 37 ˚C then transferred into a micro-fluorescence cell (Hellma, path length 1.0 cm). Fluorescence spectra (λ_{ex} = 320 nm and λ_{em} = 432 nm) were recorded using HORIBA Scientific Fluoromax-4 spectrofluorometer with an excitation and an emission slit width of 2 nm.

(B) Fluorescence-based analysis:

Stock solutions of **1** (0.5 mM) in DMSO and porcine liver esterase (10 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by adding 10 μM of **1** (4 μL, 0.5 mM) with or without 1 U/mL esterase (20 μL, 10 U/mL stock) and the volume was adjusted to 200 μL using phosphate buffer saline (10 mM, pH 7.4) in a 96-well plate and then incubated for 120 min at 37 ˚C. The fluorescence (λ_{ex} = 320 nm and λ_{em} = 432 nm) was measured using an Ensight Multimode Plate Reader (PerkinElmer).

Stock solutions of **1 or 2a** (0.125, 0.25, 0.5, 1 mM) in DMSO and porcine liver esterase (10 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by varying concentrations (0-20 μM) of **1** (4 μL from respective stocks (0.125-1 mM)), with or without 1 U/mL esterase (20 μL, 10 U/mL stock) and the volume was adjusted to 200 μL using phosphate buffer saline (10 mM, pH 7.4) in a 96-well plate. The time-dependent fluorescence increment (λ_{ex} = 320 nm and λ_{em} = 432 nm) was recorded at 37 ˚C for a period of 120 min using an Ensight Multimode Plate Reader (PerkinElmer).

Photophysical properties:

Measurement of absorbance:

Stock solutions of **2a** and **2b** (10 mM) in DMSO were prepared. The solution was prepared by adding 100 μM of **2a** or **2b** (10 μL, 10 mM) with 990 μL phosphate buffer saline (10 mM, pH 7.4) into a cuvette. UV/vis spectra were recorded using a SHIMADZU, UV-2600 UV-Vis spectrophotometer at room temperature. (**Figure S1A**)

Measurement of fluorescence:

Stock solutions of **2a** and **2b** (10 mM) in DMSO were prepared. The solution was prepared by adding 5 μM of **2a** or **2b** (0.5 μL, 10 mM) with 990 μL phosphate buffer saline (10 mM, pH 7.4) into a cuvette. Fluorescence spectra were recorded by using a HORIBA Scientific Fluoromax-4 spectrofluorometer at room temperature. (**Figure S1B**)

Persulfide measurement using monobromobimane (mBBr) [6]:

(A) Fluorometric analysis:

Time-dependent fluorescence analysis:

Stock solutions of **1** (10 mM), monobromobimane (mBBr, 10 mM) in DMSO, and porcine liver esterase (100 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by adding 100 μM of **1** (10 μL, 10 mM),

100 μM of mBBr (10 μL, 10 mM), with or without 1 U/mL esterase (10 μL, 100 U/mL stock), and the volume was adjusted to 1000 μL using phosphate buffer saline (10 mM, pH 7.4) in a 1.5 mL eppendorf tube and then transferred into a micro-fluorescence cell (Hellma, path length 1.0 cm). Fluorescence spectra (λ_{ex} = 380 nm and λ_{em} = 455 nm) were recorded over 60 min using a HORIBA Scientific Fluoromax-4 spectrofluorometer with an excitation and an emission slit width of 1 nm.

Concentration-dependent fluorescence analysis:

Stock solutions of **1** (10 mM), monobromobimane (mBBr, 10 mM) in DMSO, and porcine liver esterase (100 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by varying concentrations (0-100 μM) of **1** (1-10 μL from 10 mM), with or without 1 U/mL esterase (20 μL, 10 U/mL stock), and the volume was adjusted to 1000 μL using phosphate buffer saline (10 mM, pH 7.4) in a 1.5 mL eppendorf tube. The reactions were incubated for 60 min at 37 ˚C and then transferred into a micro-fluorescence cell (Hellma, path length 1.0 cm). Fluorescence spectra (λ_{ex} = 380 nm and λ_{em} = 455 nm) were recorded using a HORIBA Scientific Fluoromax-4 spectrofluorometer with an excitation and an emission slit width of 1 nm.

(B) Fluorescence-based analysis:

Concentration-dependent fluorescence analysis:

Stock solutions of **1** (0.25, 0.5, 1, 2 mM), mBBr (2 mM) in DMSO, and porcine liver esterase (10 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by varying concentrations (0-20 μM) of **1** (2 μL from respective stocks (0.25-2 mM)), mBBr (2 μL, 2 mM stock), with or without 1 U/mL esterase (20 μL, 10 U/mL stock) and the volume was adjusted to 200 μL using phosphate buffer saline (10 mM, pH 7.4) in a 96-well plate. The time-dependent fluorescence increment (λ_{ex} = 380 nm and λ_{em} = 455 nm) was recorded at 37 ˚C for a period of 120 min using an Ensight Multimode Plate Reader (PerkinElmer).

Persulfide/polysulfide measurement using SSP2[7] :

(A) Fluorometric analysis:

Stock solutions of **1** (10 mM), SSP-2 (10 mM) in DMSO, and porcine liver esterase (100 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction

mixture was prepared by adding 100 μM of **1** (10 μL, 10 mM), 50 μM of SSP2 (5 μL, 10 mM), with or without 1 U/mL of esterase (10 μL, 100 U/mL stock), and the volume was adjusted to 1000 μL using phosphate buffer saline (10 mM, pH 7.4) in a 1.5 mL eppendorf tube and then transferred into a micro-fluorescence cell (Hellma, path length 1.0 cm). Fluorescence spectra (λ_{ex} = 482 nm and λ_{em} = 518 nm) were recorded over 120 min using a HORIBA Scientific Fluoromax-4 spectrofluorometer with an excitation and an emission slit width of 1 nm.

(B) Fluorescence-based analysis:

Stock solutions of **1** (0.25, 0.5, 1, 2 mM), SSP2 (1 mM) in DMSO, and porcine liver esterase (10 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by varying concentrations (0-20 μM) of **1** (2 μL from respective stocks (0.25-2 mM)), SSP-2 (2 μL, 1 mM stock), with or without 1 U/mL esterase (20 μL, 10 U/mL stock) and the volume was adjusted to 200 μL using phosphate buffer saline (10 mM, pH 7.4) in a 96-well plate. The time-dependent fluorescence increment (λ_{ex} = 482 nm and λ_{em} = 518 nm) was recorded at 37 ˚C for a period of 120 min using an Ensight Multimode Plate Reader (PerkinElmer).

Persulfide/polysulfide measurement from 1 using LC/MS:

Stock solutions of **1** (10 mM) and HPE-IAM (100 mM) were prepared in DMSO. A stock solution of porcine liver esterase (100 U/mL; Sigma Aldrich, E3019) was prepared in PBS pH 7.4. The reaction mixture for **1** was prepared by adding 50 μM of **1** (2 μL, 10 mM stock) along with 10 mM HPE-IAM (4 μL, 100 mM) and 1 U/mL esterase (4 μL, 100 U/mL stock). The volume was adjusted to 400 μL using 10 mM PBS, pH 7.4, and the reaction mixture was incubated at 37 °C. 100 μL aliquots of the reaction mixture were taken at pre-determined time points, and the reaction was quenched by adding 100 μL of acetonitrile. The samples were centrifuged at 10, 000 x *g* for 10 minat 4 ˚C, the supernatant was collected and assessed thereafter by LC/MS. All measurements were done using a previously established LC/MS method[8] with slight modification. All measurements were done using the following protocol: Acetonitrile (A) and 0.1% formic acid in water (B) were used as the mobile phase. A multistep gradient was used with the flow rate of 0.2 mL/min starting with 0:100 → 0 min 0:100 to 5:95 → 0.10 -1 min, 5:95 to 90:10→ 1 - 15 min, 90:10 to 0:100 → 15 – 15.10 min, and 0:100 → 15 – 22 min. Measurements were carried out in the positive ion mode using high-resolution

multiple reaction monitoring (MRM- HR) analysis on a Sciex X500R quadrupole time-of-flight (QTOF) mass spectrometer fitted with an Exion UHPLC system using a Kinetex 2.6 mm hydrophilic interaction liquid chromatography (HILIC) column with 100 Å particle size, 150 mm length and 3 mm internal diameter (Phenomenex). Nitrogen was the nebulizer gas, with the nebulizer pressure set at 50 psi, declustering potential = 80 V, entrance potential = 10 V, collision energy = 20 V, and collision exit potential = 5 V. The MRM-HR mass spectrometry parameters for measuring compounds are: m/z precursor ion mass ($M + H^+$) 516.1145 (**1**), 305.9985 (**HPE-IAM**), 373.0891 (**NAC-SS-HPE-AM**), 389.1530 (**Bis-S-HPE-AM**). (**Figure S9, S10 and S11**)

Methylene blue assay for the H_2S detection:

General protocol: The methylene blue assay was conducted as previously reported with some modifications.[9,10] Stock solutions of **1** (10 mM), **2a** (10 mM) in DMSO, and porcine liver esterase (100 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. Dithiothreitol (DTT, 100 mM), NaSH (100 mM), and $Zn(OAc)_2.2H_2O$ (40 mM) were prepared in deionized water. Stock solution of $FeCl_3$ (30 mM) was prepared in 1.2 M HCl and *N*, *N*–dimethyl-*p*-phenylenediamine sulfate (DMPPDA) (20 mM) was prepared in 7.2 M HCl.

The reaction samples were prepared by sequentially adding 400 μM $Zn(OAc)_2.2H_2O$ (10 μL, 40 mM stock), varying concentrations of **1 or 2a** from 25-100 μM (10 μL from respective stocks (1-4 mM)) with or without 1 U/mL esterase (10 μL, 100 U/mL stock) and the volume was adjusted to 1000 μL using phosphate buffer saline (10 mM, pH 7.4) in a 1.5 mL eppendorf tube and placed in a static incubator maintained at 37 ˚C.

207 μL aliquot of the reaction mixture was taken at pre-determined time points and 10 mM DTT (23 μL, 100 mM stock) was added, from which 200 μL was added to a 1.5 mL eppendorf containing equal volumes (200 μL) of $FeCl_3$, DMPPDA and incubated at 37 ˚C for 30 min in dark, to allow the formation of the methylene blue dye. An aliquot of 150 μL was transferred to a 96-well plate and the absorbance values were recorded at 676 nm using a microplate reader (Thermo Scientific VarioskanFlash). (**Figure S12**)

Lead acetate assay for the H_2S detection:

Firstly, lead acetate paper was prepared by soaking Whatman filter paper with 10 % (w/v) lead acetate solution and dried. Stock solutions of **1** (10 mM), **2a** (10 mM) in DMSO, and porcine

liver esterase (100 U/mL; Sigma Aldrich, E3019) in phosphate buffer saline (10 mM, pH 7.4) were prepared. Dithiothreitol (DTT, 10 mM) was prepared in deionized water.

Lead acetate assay was performed in a 96-well plate (with lid). The reaction samples were prepared by sequentially adding 200 μM of **1 or 2a** (14 μL from 10 mM), 10 mM of DTT (7 μL, 10 mM stock), with or without 1 U/mL esterase (7 μL, 100 U/mL stock) and the volume was adjusted to 700 μL using phosphate buffer saline (10 mM, pH 7.4) in a 1.5 mL Eppendorf tube. Aliquoted 200 μL from each reaction (in triplicate) in a 96-well plate, covered with 10 % lead acetate-soaked paper followed by the lid, sealed, and placed in a static incubator maintained at 37 ˚C for 3 h. After 3 h, lead acetate paper was carefully removed and the image was taken using Syngene G-Box Chemi-XRQ. (**Figure S13)**

Cell viability assay:

MEF cells: Mouse embryonic fibroblasts (MEF) cells were seeded at a concentration of 1×10^4 cells/well overnight in a 96-well plate in complete DMEM medium supplemented with 5% FBS (fetal bovine serum) and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37 ˚C. Cells were exposed to varying concentrations of the compound **1** prepared as a DMSO stock solution so that the final concentration of DMSO was 0.5%. The cells were incubated for 24 h at 37 ˚C. A 0.5 mg/mL stock solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was prepared in DMEM and 100 μL of the resulting solution was added to each well. After 4 h incubation, the media was removed carefully and 100 μL of DMSO was added. Spectrophotometric analysis of each well using a microplate reader (Thermo Scientific Varioscan) at 570 nm was carried out to estimate cell viability (**Figure S5**).

C28/I2 cells: Cell metabolic activity was assessed using the water-soluble tetrazolium salt-8 (WST-8) proliferation assay Kit (Cayman). Human chondrocytes, C28/I2 cells, were seeded overnight in a 24-well plate at a concentration of 6000 cells/well in complete DMEM medium (Gibco) supplemented with 5% FBS (fetal bovine serum: Sigma Aldrich) and 1% antibiotic solution (Thermo Fisher Scientific). The cells were incubated with different concentrations of **1** prepared as a DMSO stock solution, resulting in a final DMSO concentration of 0.1%.

A stock solution of WST-8 containing 2 μL of WST-8 developer reagent and 2 μL of electron mediator solution per 96 μL of complete DMEM was prepared. After 24 hours, the cells were washed with 1X PBS, and 100 μL of the WST-8 solution was added to each well. After a 1-

hour incubation, the cell metabolic activity was estimated by measuring the absorbance at 450 nm using a microplate reader (Tecan Spark). (**Figure S14**).

Protection from oxidative stress:

C28/I2 cells were seeded overnight in a 24-well plate at a concentration of 6000 cells/well in complete DMEM medium (Gibco) supplemented with 5% FBS (fetal bovine serum: Sigma Aldrich) and 1% antibiotic solution (Thermo Fisher Scientific). The cell were either pre-treated or co-treated with MGR-1/95 for 3 hours with various concentrations of drugs, which were prepared as a DMSO stock solution to achieve a final DMSO concentration of 0.1%. Following pre-treatment with either MGR or 95, the cells were further treated with either 95 or MGR-1, respectively for 21 hours.

A stock solution of WST-8 containing 2 µL of WST-8 developer reagent and 2 µL of electron mediator solution per 96 µL of complete DMEM was prepared. After 24 hours of treatment, the cells were washed with 1X PBS, and 100 µL of the WST-8 solution was added to each well. After a 1-hour incubation, the cell metabolic activity was estimated by measuring the absorbance at 450 nm using a microplate reader (Tecan Spark). (**Figure S15**).

Detection of 2a release in N2a cell lysate:

N2a cells were cultured in a 10 cm plates in complete DMEM medium supplemented with 5% FBS (fetal bovine serum) and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37 ˚C. When the cells were 70% confluent, old media was removed and the cells were washed with serum free DMEM media. The cells were trypsinized and subsequently resuspended in DMEM. The cells were harvested by centrifugation at 1000 rpm/min at 4 ˚C. Pellets were washed twice with PBS (1x), resuspended in PBS (1x, 2 mL) and transferred to a microcentrifuge tube. Cells were lysed by sonication using (130 W ultrasonic processor, VX 130W) stepped microtip for 2 min (with 3 sec. ON and 3 sec. OFF pulse, 60% amplitude) under ice cold conditions. The total protein concentration of the whole cell lysate was determined by Bradford assay and further adjusted to 1 mg/mL with PBS (1x).

Stock solutions of **1, 2a** (0.05 mM, 0.125 mM, 0.25 mM, 0.5 mM) independently in DMSO and 1 mg/mL stock solution of cell lysate in phosphate buffer saline (10 mM, pH 7.4) were prepared. The reaction mixture was prepared by adding 1 µM, 2.5 µM, 5 µM and 10 µM **1** or **2a** (2 µL from 0.05 mM, 0.125 mM, 0.25 mM, 0.5 mM) independently along with cell lysate (98 µL, 1 mg/mL) in 96-well plate and incubated at 37 ˚C. The fluorescence corresponding to

2a release (excitation at 320 nm; emission at 432 nm) was measured for 3 h using an Ensight Multimode Plate Reader (PerkinElmer). (**Figure S4)**

Micromass culture assay[11]**:**

C28/I2 cell suspension was prepared, with a concentration of 25 million cells per ml in growth media. The growth media consisted of DMEM medium (Gibco) supplemented with 5% FBS (fetal bovine serum: Sigma Aldrich) and 1% antibiotic solution (Thermo Fisher Scientific). Micromasses were obtained by adding 15 µL of the cell suspension into individual wells of a 24-well plate. These micromasses were allowed to adhere for 3 hours in the absence of growth media. After this initial 3-hour period, 1 mL of growth media was added, and the plate was incubated for 24 hours.

After this 24-hour incubation period, the growth media was substituted with phenol-free DMEM media (Gibco) containing various supplements, including 2% FBS, 1% antibiotic solution, 1x insulin-transferrin-selenium (Gibco), 10 ng mL^{-1} TGF-β (PeproTech), 50µg/ ml ascorbic acid (Sigma), and 200 mM L-Glutamine (Sigma). The micromasses, now in differentiation media, were divided into groups for treatment. These groups included MGR-1 (15 µM), MGR-1(15 µM) + **1** (75 µM), and only **1** (75 µM). Following 48 hours of incubation with these treatments, the cells were fixed using 4% formaldehyde followed by Alcian blue (Sigma) staining at pH < 1 to stain the sGAG overnight. The next day, the micromasses were washed to remove any non-specific stains, followed by Alcian blue stain extraction using guanidine HCl (Sigma). The absorbance of the extracted guanidine HCl was read at 630 nm using a plate reader (Tecan Spark) to quantify the proteoglycans present in the micromasses.(**Figure S17**)

Confocal imaging of MEF cells with 1:

MEF cells were seeded at 1×10^5 cells/well in 6-well Corning plate (on cover slip) for overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37 ˚C. After incubation, media was removed and the cells were washed with 1 mL of PBS. Then 1 mL of fresh DMEM media was added along with 50 µM of **1**/**2a**, and the cells were incubated for 4 h at 37 ˚C. After 4 h, the media was removed, cells were washed twice with 1 mL of PBS and then cells were imaged on Carl Zeiss LSM710 laser scanning confocal microscope (700 nm for two photon) with 63x oil immersion objective. Images were analysed by ImageJ software. **(Figure S6).**

ROS quenching in MEF cells with 1:

MEF cells were seeded at 1×10^5 cells/well in 6-well Corning plate for overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37 ˚C. The old media was removed and the cells were washed with 1 mL of PBS. Then 1 mL of fresh DMEM media was added along with 25 and 50 μM of **1**, and the cells were incubated for 4 h at 37 ˚C. After 4 h, the media was removed, cells were washed with 1 mL of PBS and treated with MGR-1 (25 μM) for 1 h. After MGR-1 treatment, the media was removed, cells were washed with 1× PBS and then treated cells with H_2DCF-DA-probe (10 μM) and cells were incubated further for 10 min at 37 ˚C. The cells were washed twice with 1 mL of PBS and then were imaged on an EVOS fluorescence microscope using a 20x GFP filter.(**Figure S16**)

Stability and reactivity of 2a towards nucleophiles:

Stock solution of **2a** (0.5 mM) in DMSO was prepared. Stock solutions of 10 mM of L-cysteine (Cys), *N*- acetyl cysteine (NAC), L-glutathione (GSH), L-histidine (His), L-threonine (Threo), L-serine (Ser), L-lysine (Lys), L-proline (Pro), sodium chloride (NaCl), sodium nitrate ($NaNO_3$) were prepared in de-ionised water. Stock solution of 10 mM of L-tyrosine (Tyr) was prepared in DMSO. The reaction mixture was prepared by adding 10 μM of **2a** (4 μL, 0.5 mM), with or without 100 eq. of nucleophiles (20 μL, 10 mM stock) and the volume was adjusted to 200 μL using phosphate buffer saline (10 mM, pH 7.4) in a 96-well plate and then incubated for 120 min at 37 ˚C. The fluorescence (λ_{ex} = 320 nm and λ_{em} = 432 nm) was measured using an Ensight Multimode Plate Reader (PerkinElmer).(**Figure S18**)

Table S1: Rate constants and rates of **2a** formation and persulfide generation from **1**

1 Conc. (μM)	**2a** formation ($\times10^{-1}$ min^{-1})	Persulfide generation ($\times10^{-1}$ min^{-1})		Rate of **2a** formation (μM min^{-1})	Rate of persulfide generation (μM min^{-1})	
		SSP-2	mBBr		SSP-2	mBBr
2.5	0.73	0.17	0.18	0.18	0.04	0.05
5	0.56	0.24	0.22	0.28	0.12	0.11
10	0.51	0.23	0.25	0.51	0.24	0.25
20	0.47	0.27	0.35	0.94	0.55	0.70

4. Supplementary figures:

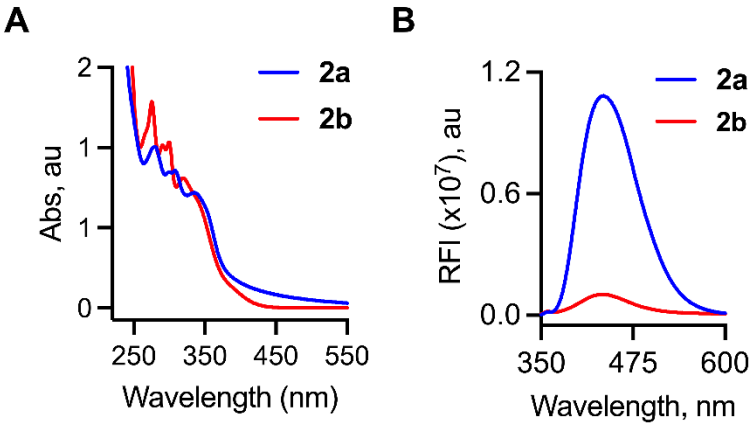


Figure S1. (A) Absorbance spectrum for compounds **2a** and **2b** (100 µM) were measured in PBS (pH 7.4); (B) Fluorescence spectrum for compounds **2a** and **2b** (5 µM) were measured in PBS (pH 7.4); (λ_{ex} = 320 nm; λ_{em} = 432 nm).

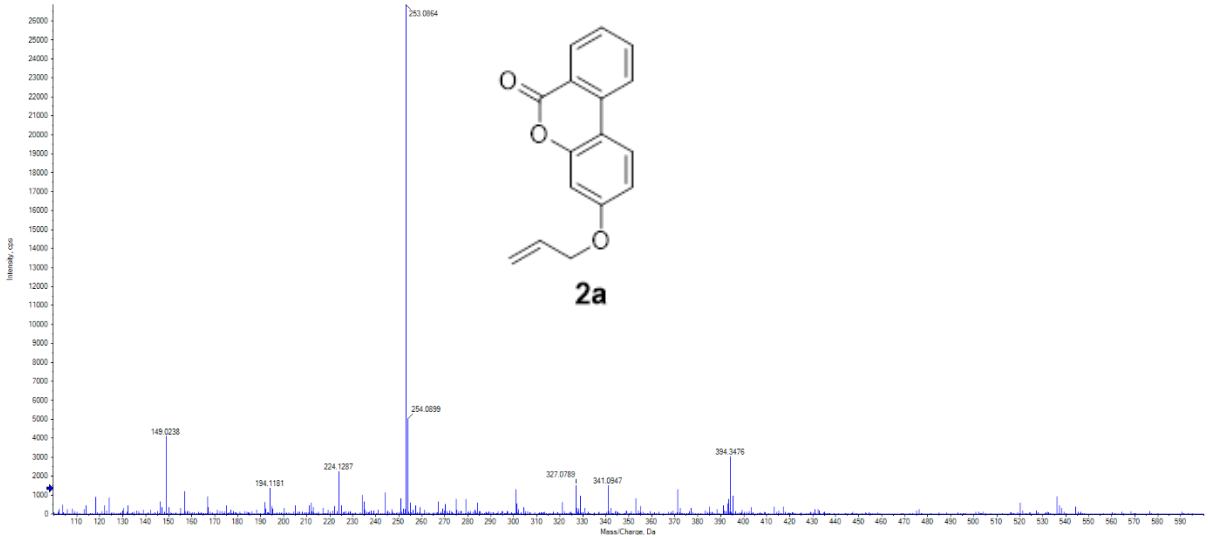


Figure S2. Mass spectrometry analysis of **1**(10 µM) upon incubation with Es (2.5 U/mL) for 30 min. For **2a**, (expected, m/z =253.0859, $[M+H]^+$; observed, m/z = 253.0864).

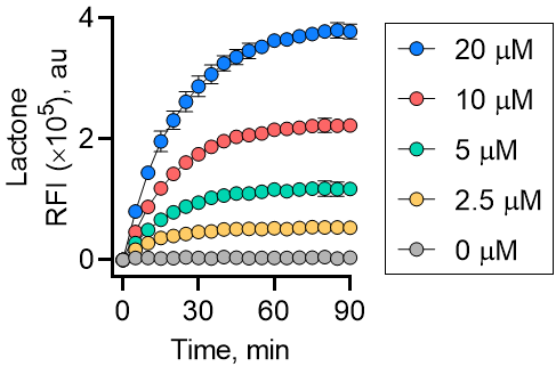


Figure S3. Monitoring the formation of lactone (**2a**) from **1** by fluorescence in a concentration-dependent manner (0-20 µM) with Es (1 U/mL) in pH 7.4 buffer at 37 °C.

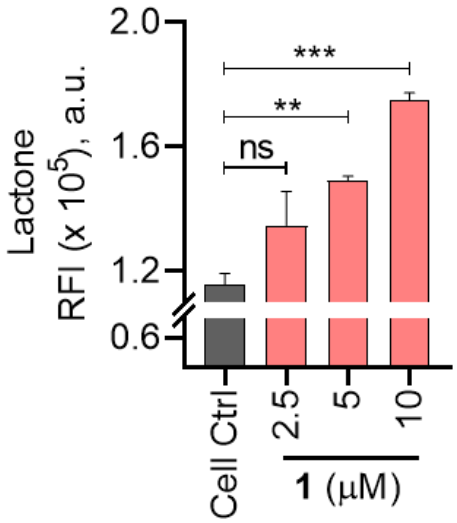


Figure S4. Compound **1** was incubated in N2a cell lysate (1 mg/mL) for 2 hours; cell ctrl refers to untreated cell lysate; Fluorescence measurement (λ_{ex} = 320 nm; λ_{em} = 432 nm) was carried out by varying concentrations of **1.** Results are expressed as mean ± SD (n =3/group). *p* value was determined by using Two-way ANOVA relative to cell ctrl. (** $p \leq 0.002$; *** $p \leq 0.001$ and ns indicates not significant).

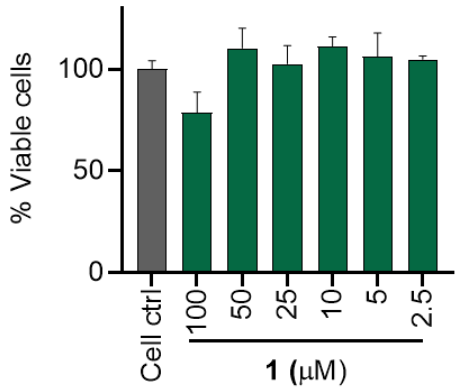


Figure S5: Cell viability assay was conducted on MEF cells with compound **1** for 24 h.

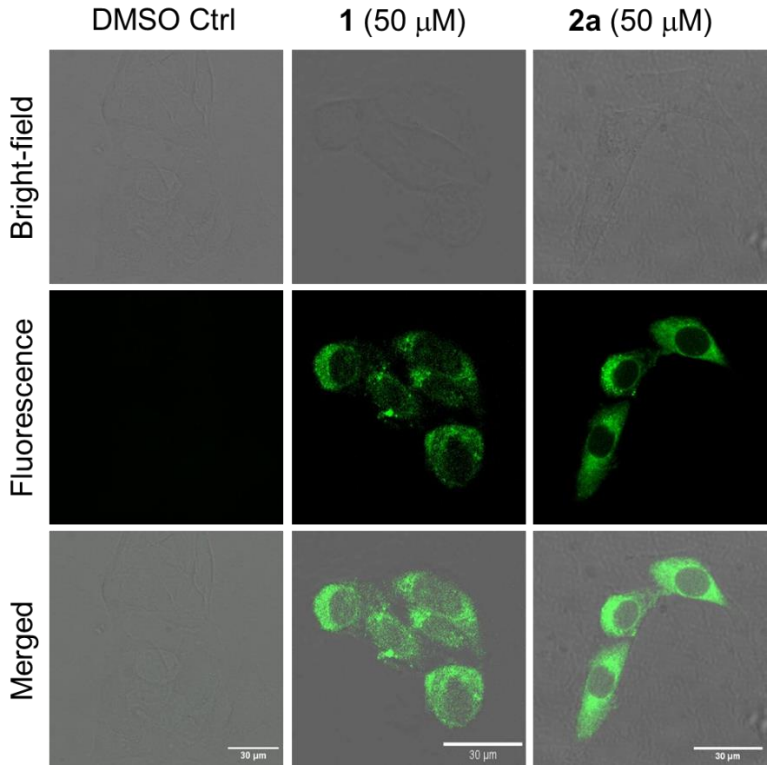
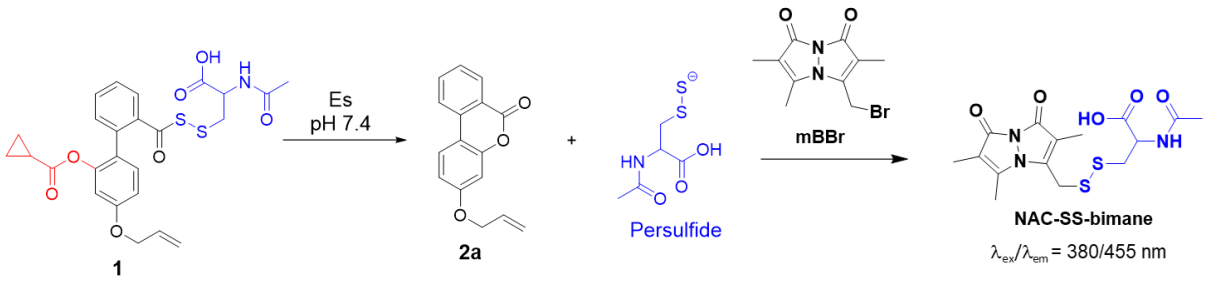


Figure S6. Two-photon confocal microscopy images of MEF cells treated with **1** and **2a** (50 μM). The excitation and emission channels were 700 nm and 432 nm, respectively. Scale bar is 30 μm.



Scheme S2: Formation of the **NAC-SS-bimane** adduct when **1** incubated with Es and mBBr.

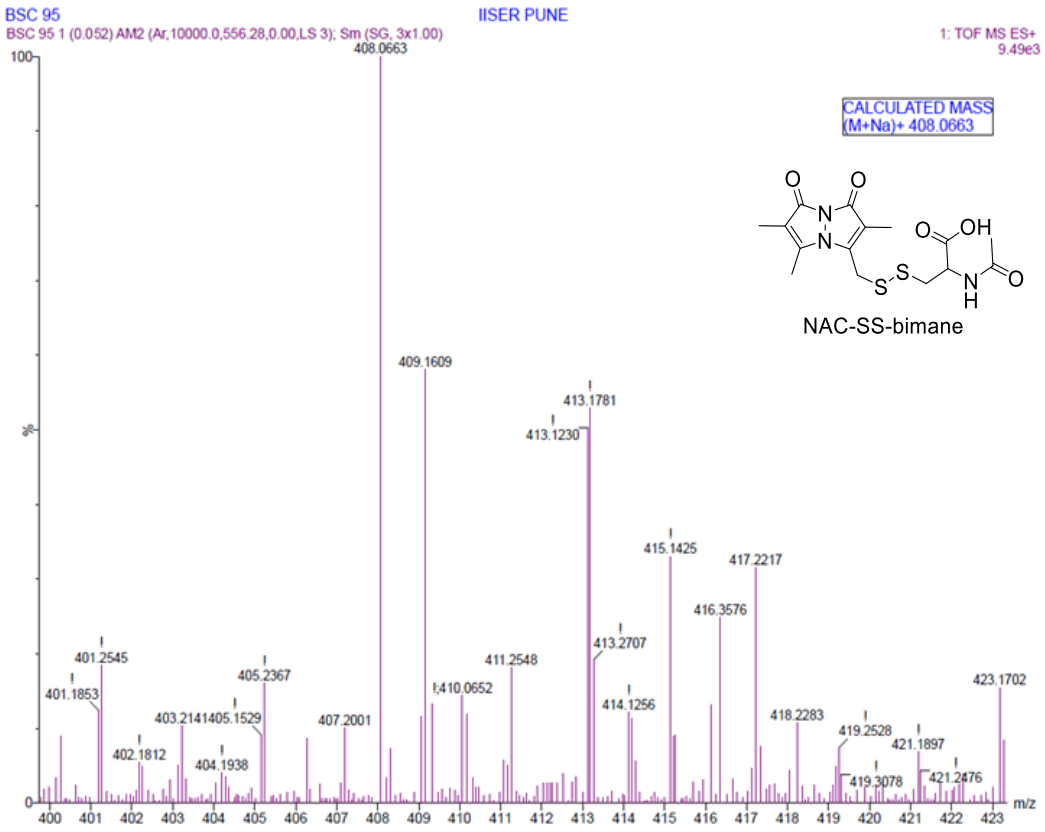


Figure S7. Mass of the **NAC-SS-bimane** adduct formation when **1** incubated with Es and mBBr (expected, m/z = 408.0658, $[M+Na]^+$; observed, m/z = 408.0663).

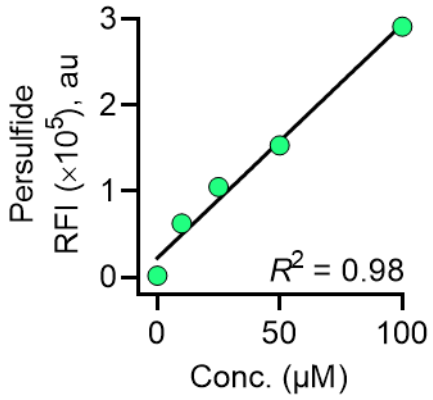
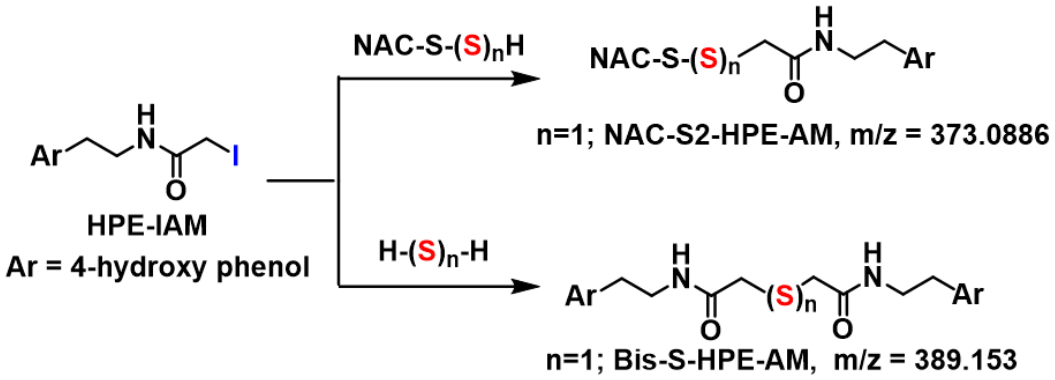


Figure S8. Detection of persulfide released from **1** (0-100 μM) in a concentration dependent manner following treatment with Es (1 U/mL) and mBBr (100 μM) after 60 min.



Scheme S3: LC/MS study. Reaction scheme showing detection of persulfides/polysulfides and hydrogen sulfide/polysulfides as their HPE-AM and bis-S-HPE-AM adducts, respectively. Ar = 4-hydroxyphenyl

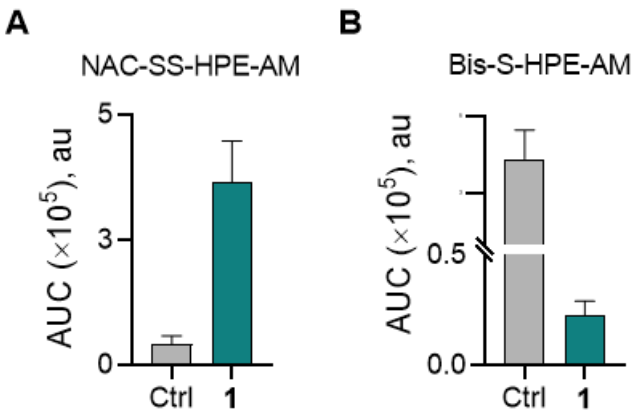


Figure S9. LC/MS study. (A) Persulfide **(NAC-SS-HPE-AM**) and (B) H_2S **(Bis-S-HPE-AM**) formation were measured by the detection of trapped HPE-IAM species from **1** and ctrl. The ctrl was prepared by reacting 200 μM NAC, 200 μM DEA/NO (sodium 2-(*N*, *N*-diethylamino)-

diazenolate-2-oxide) and 200 μM NaSH at room temperature for 20 min[12]. Results are expressed as mean ± SD (n =3/group).

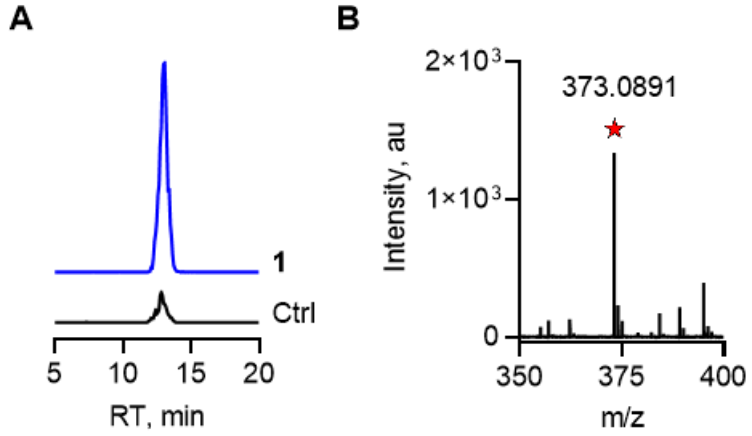


Figure S10. A) Extracted ion chromatograms from an LC/MS analysis of **NAC-SS-HPE-AM** formation from **1** and ctrl; (B) Mass spectra for **NAC-SS-HPE-AM** (expected, m/z = 373.0886 $[M + H]^+$; observed, m/z = 373.0891).

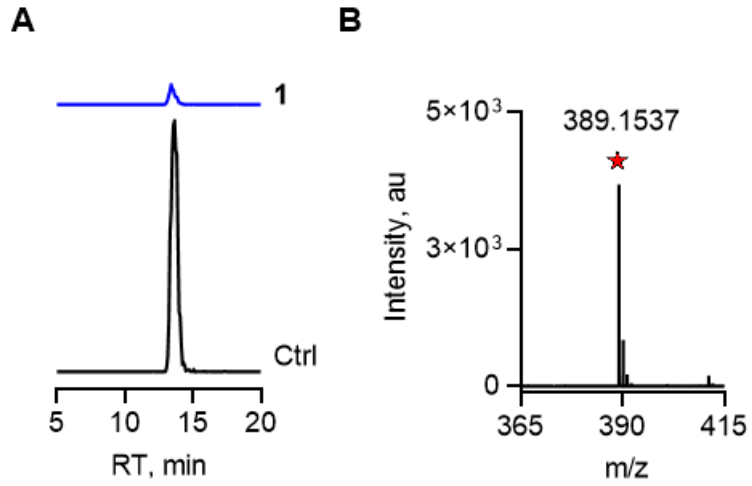


Figure S11. A) Extracted ion chromatograms from an LC/MS analysis of **Bis-S-HPE-AM** formation from **1** and ctrl ; (B) Mass spectra for **Bis-S-HPE-AM** (expected, m/z = 389.1530 $[M + H]^+$; observed, m/z = 389.1537).

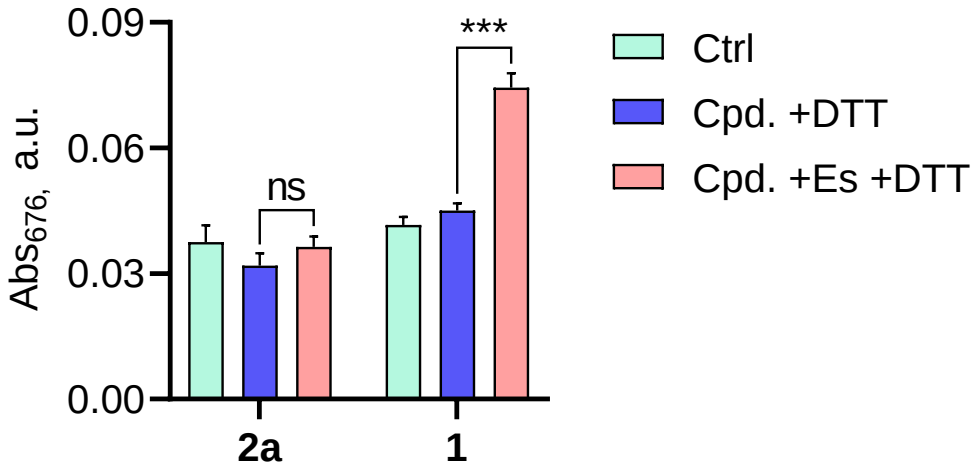


Figure S12. Methylene blue assay for H_2S generation from **1** in the presence of Es and DTT for 3 h. Results are expressed as mean ± SD (n =3/group). *p* value was determined by using Two-way ANOVA relative to w/o Es. (***$p \leq 0.001$ and ns indicates not significant). **2a** was used as a negative control.

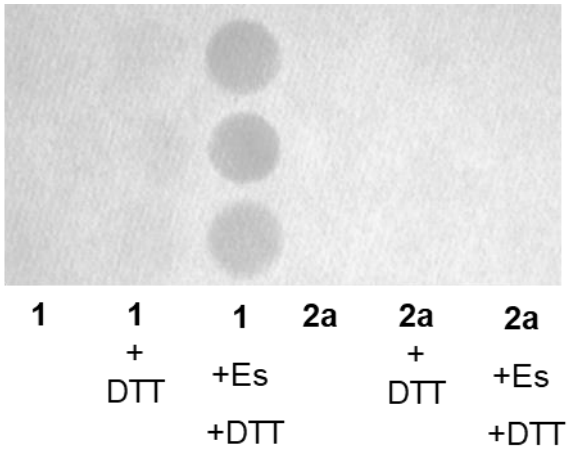


Figure S13. Lead acetate assay for H_2S generation from **1** (200 μM) in the presence of Es and DTT for 3 h. **2a** was used as a negative control.

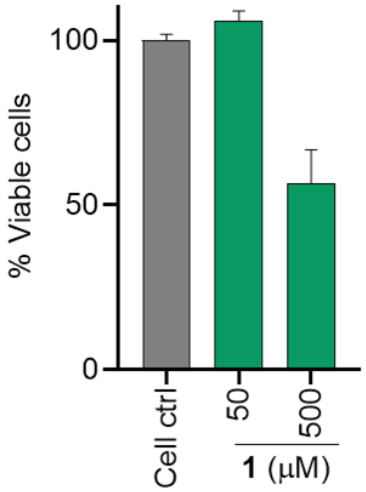
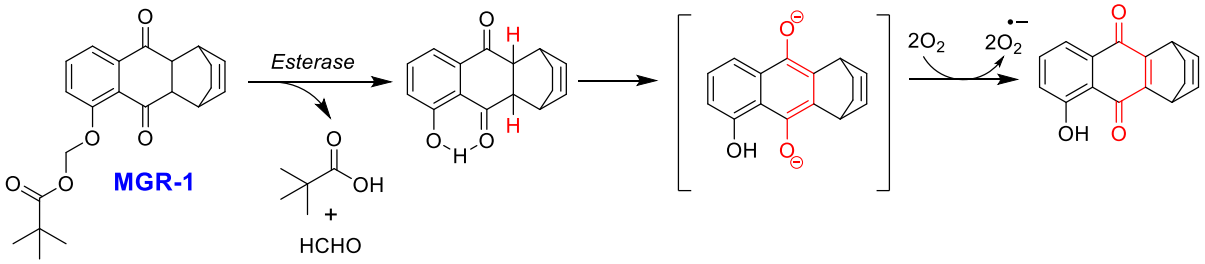


Figure S14: Cell viability assay was conducted on C28/I2 cells with varying concentrations of compound **1** for 24 h. The LD50 of **1** was found to be 500 μM.



Scheme S4. Structure and generation of ROS from MGR-1.

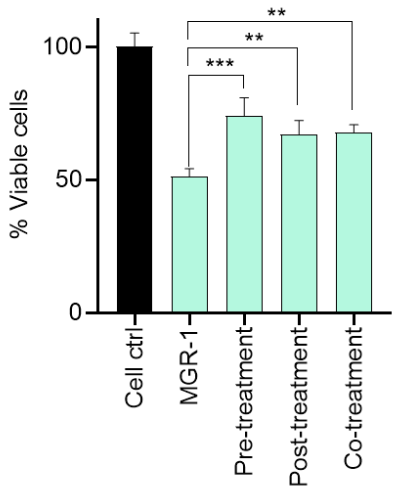


Figure S15. Cell viability assay on C28/I2 cells upon pre, post, or co-treatment of **1**(50 μM) with MGR-1 (15 μM).

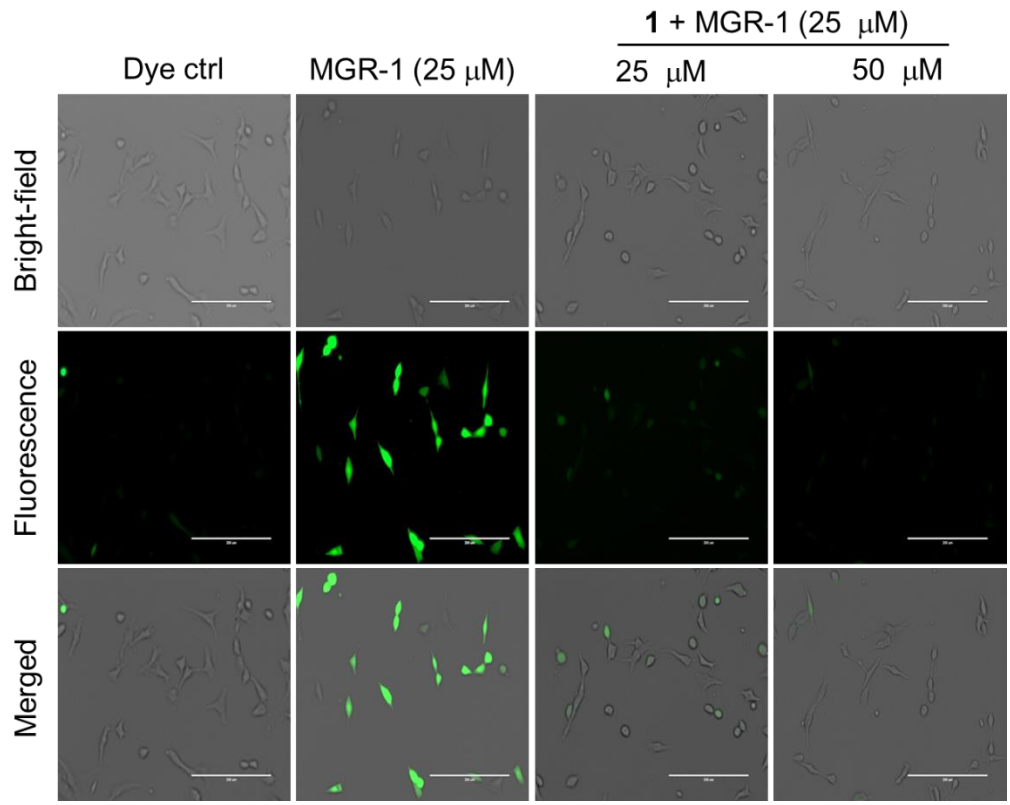


Figure S16. MEF cells were treated with varying concentrations of **1** (25 μM and 50 μM) for 4 h, followed by treatment with MGR-1 (25 μM) for 1 h. Intracellular ROS levels were detected using DCF dye (10 μM). The cells were imaged in the 20× GFP filter. Scale bar is 200 μm.

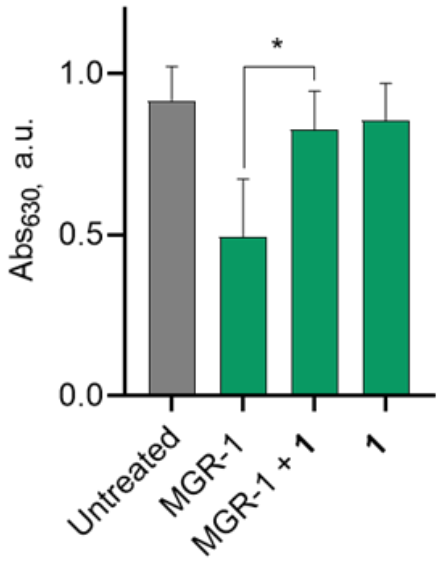


Figure S17. Micromass culture assay for sGAG formation from **1**(75 μM) in the presence of oxidative stress induced by MGR1(15 μM). Results are expressed as mean ± SD (n =6/group). *p* value was determined by using One-way ANOVA relative to MGR-1. (**p* < 0.033).

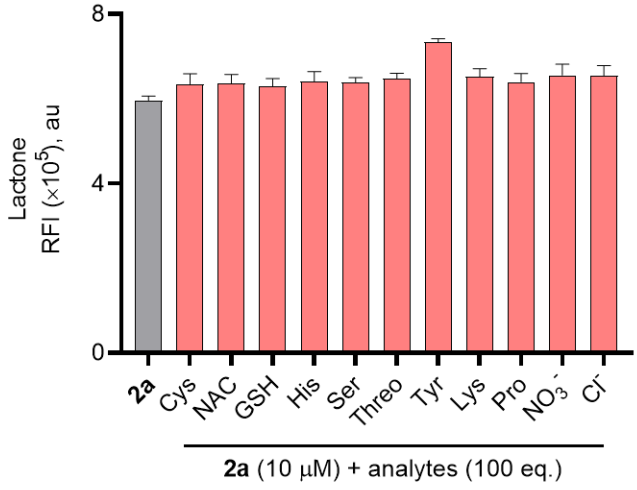


Figure S18. Stability and reactivity of **2a** (10 μM) towards nucleophiles (100 equiv.) for 120 min. Cys: cysteine; NAC: *N*- acetylcysteine; GSH: glutathione; His: histidine; Ser: serine; Threo: threonine; Tyr: tyrosine; Lys: lysine; Pro: proline; NO_3^-: sodium nitrate; Cl^- : sodium chloride. Results are expressed as mean ± SD (n =3/group).

5. References:

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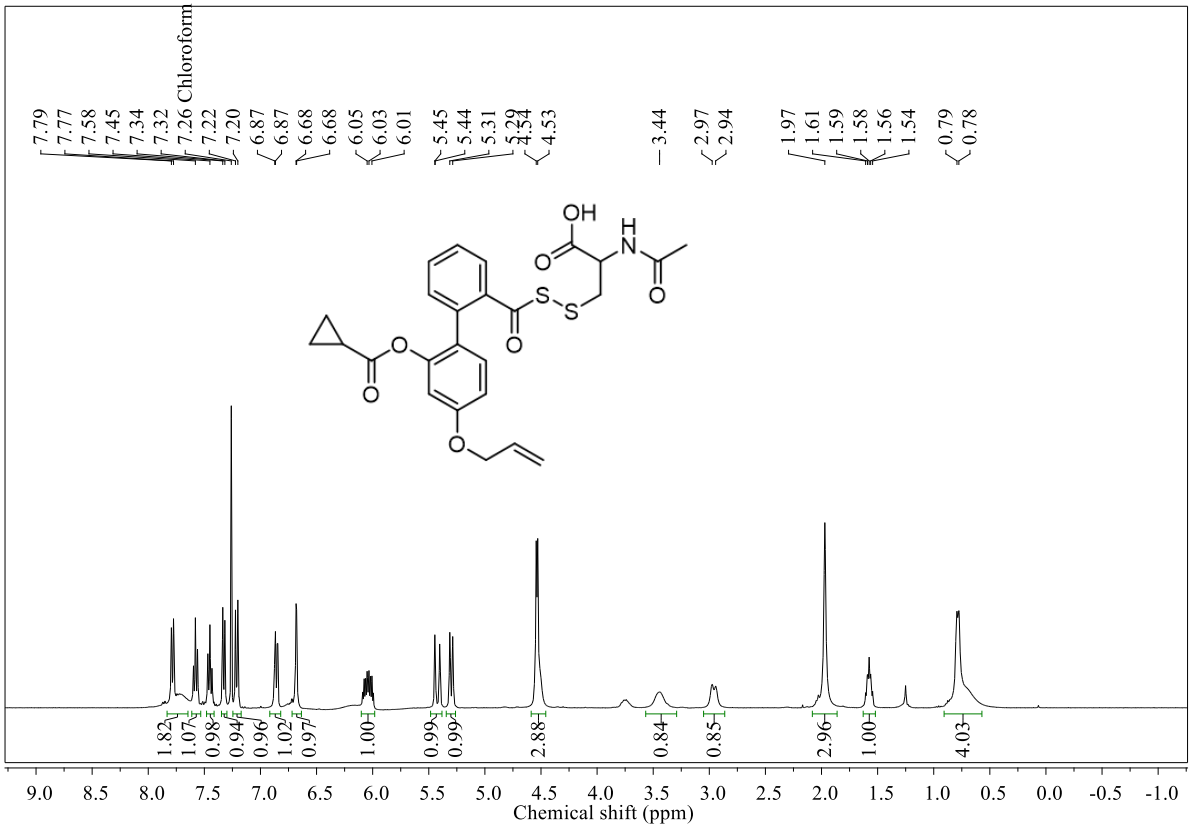
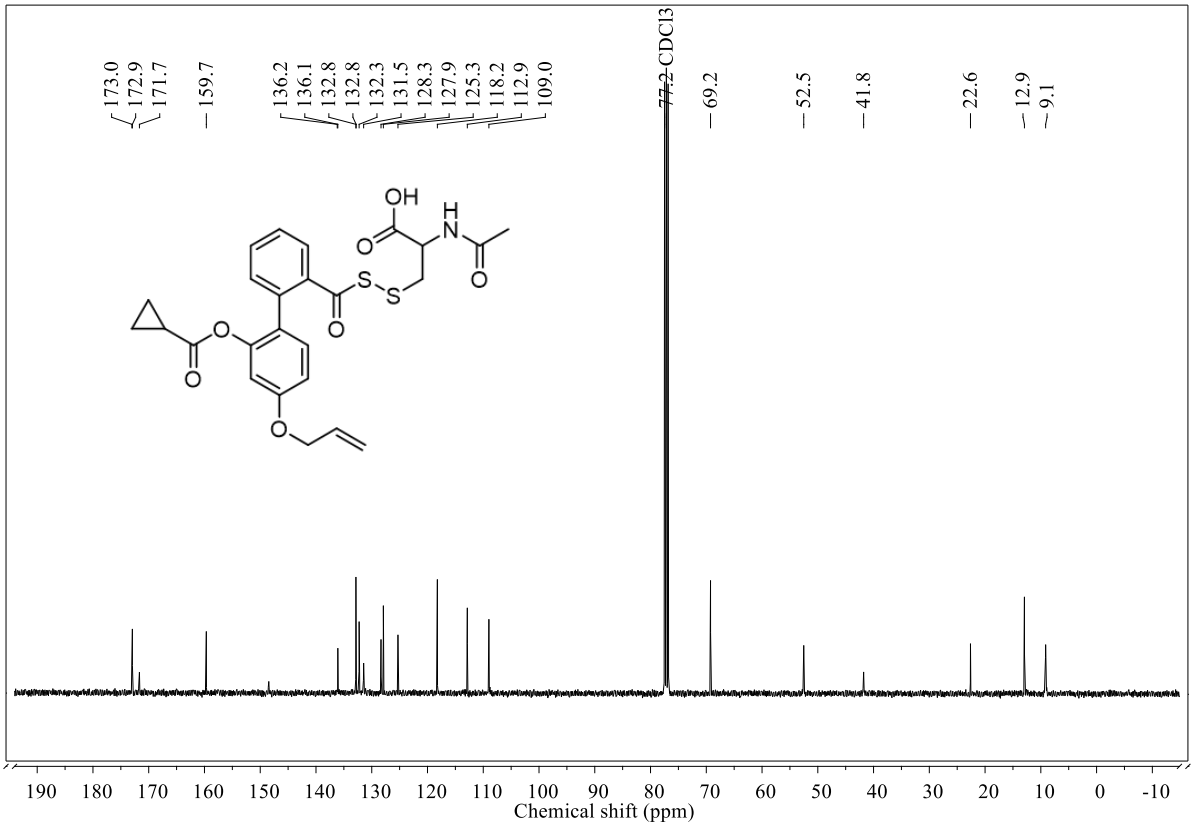
6. NMR spectra:

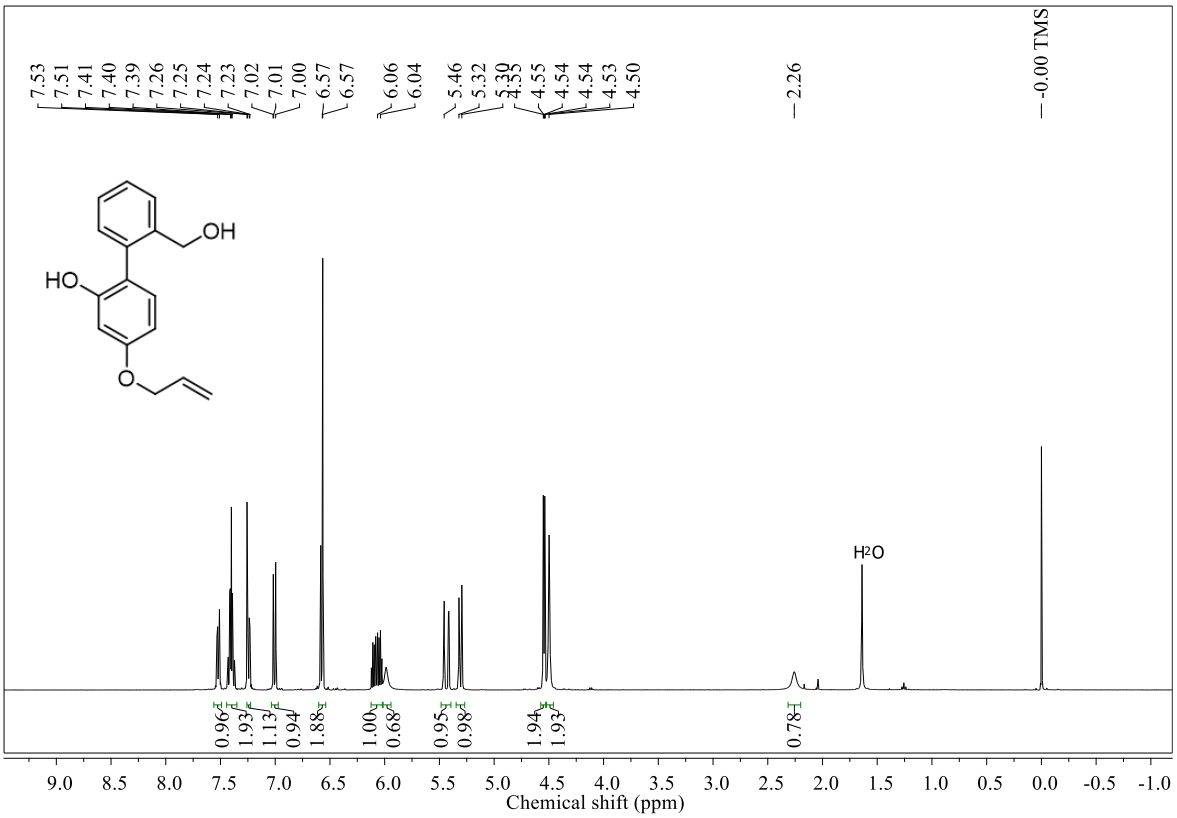
^{1}H NMR spectra of **1**



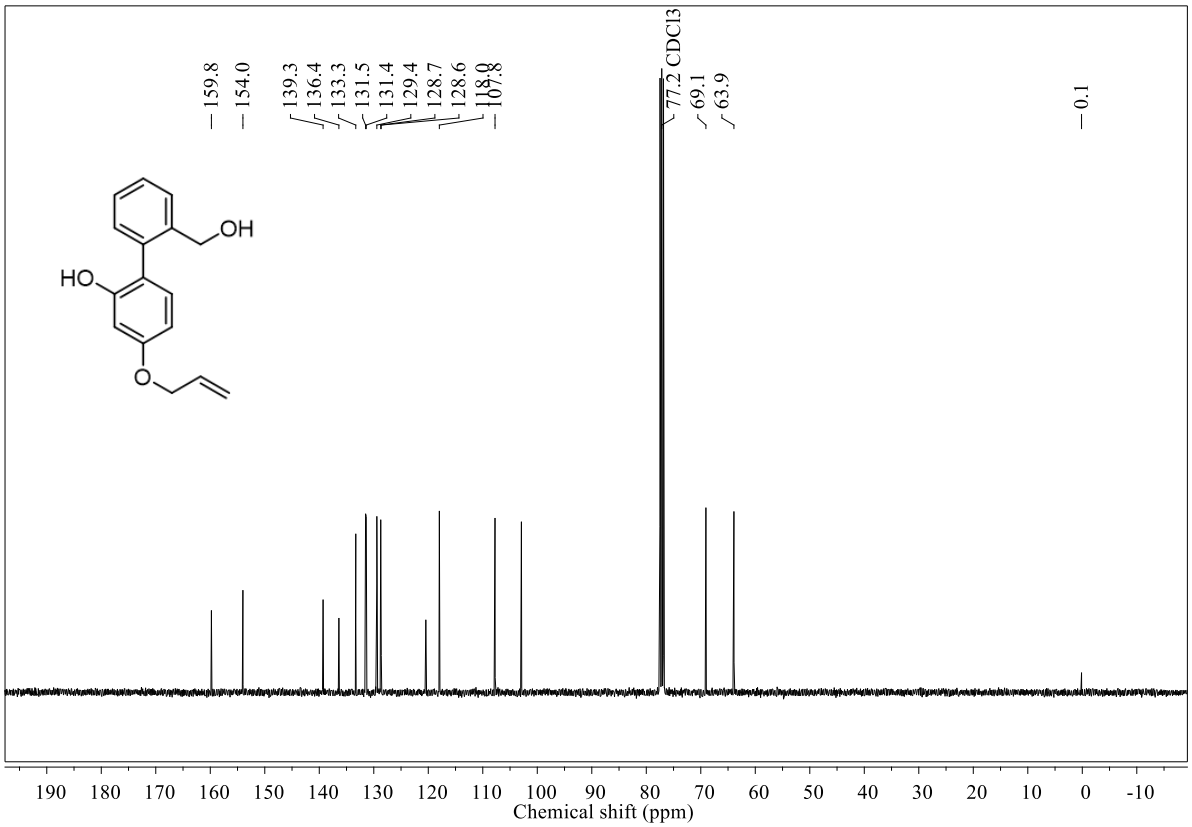
^{13}C NMR spectra of **1**



^{1}H NMR spectra of **3**



^{13}C NMR spectra of **3**



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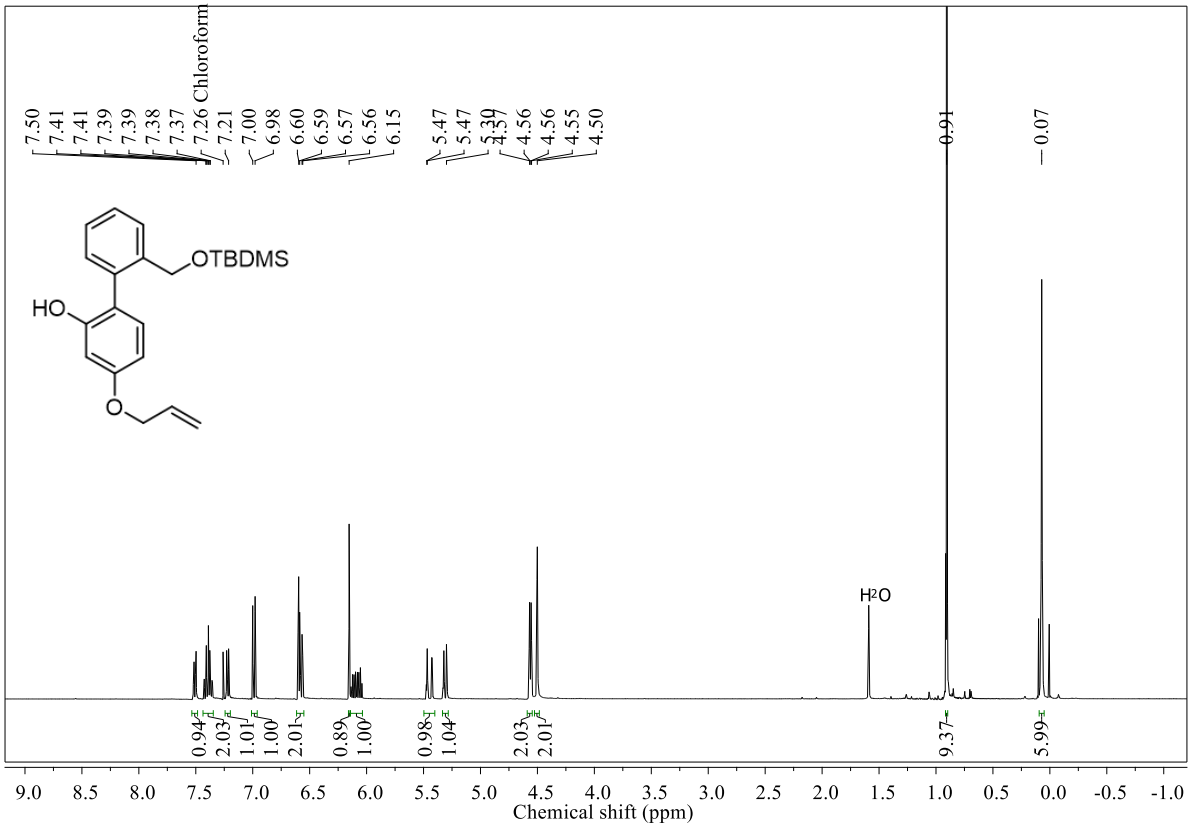
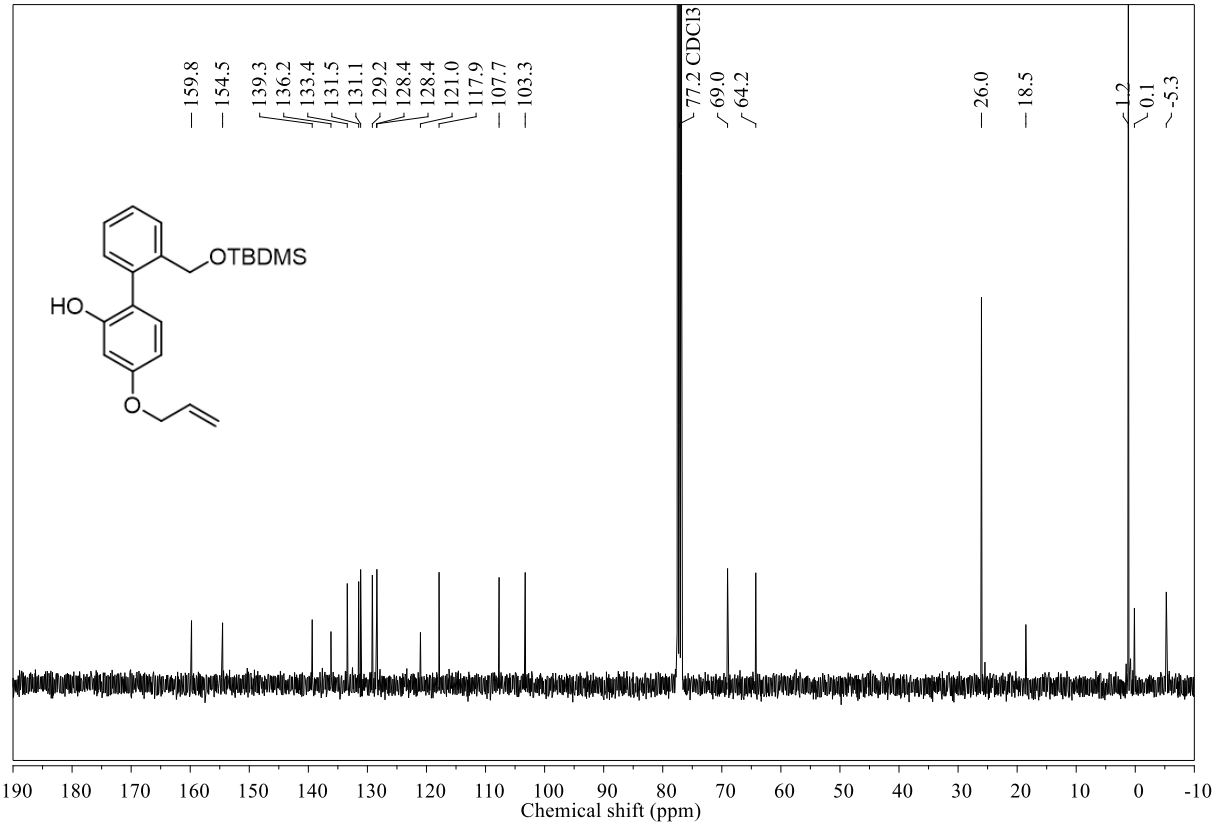
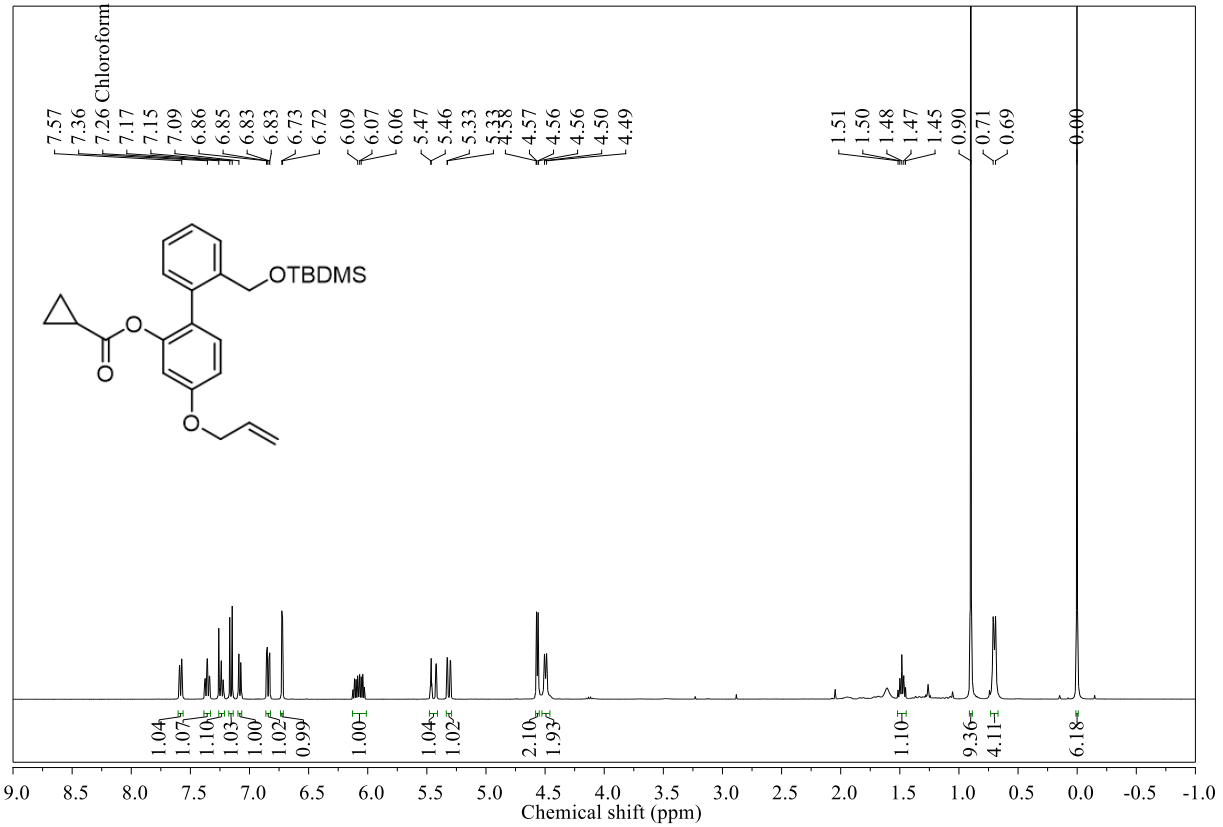


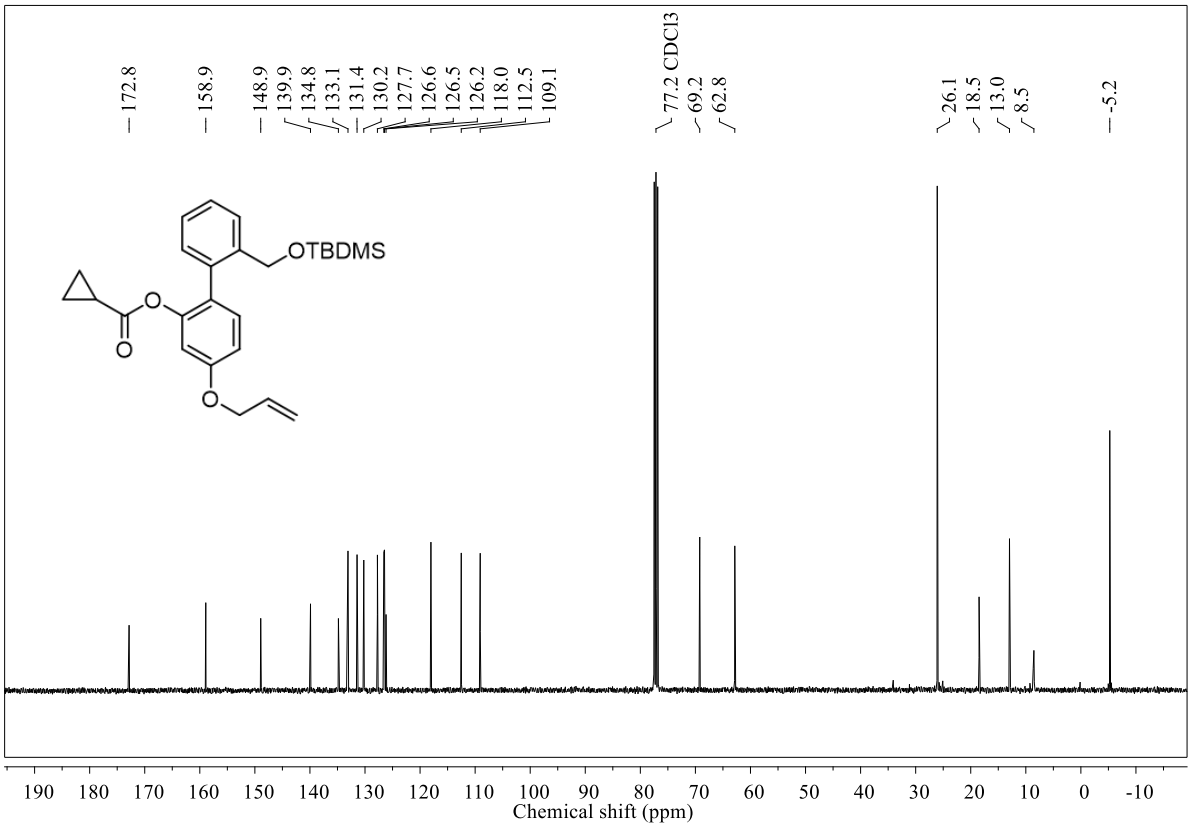
^{13}C NMR spectra of **4**



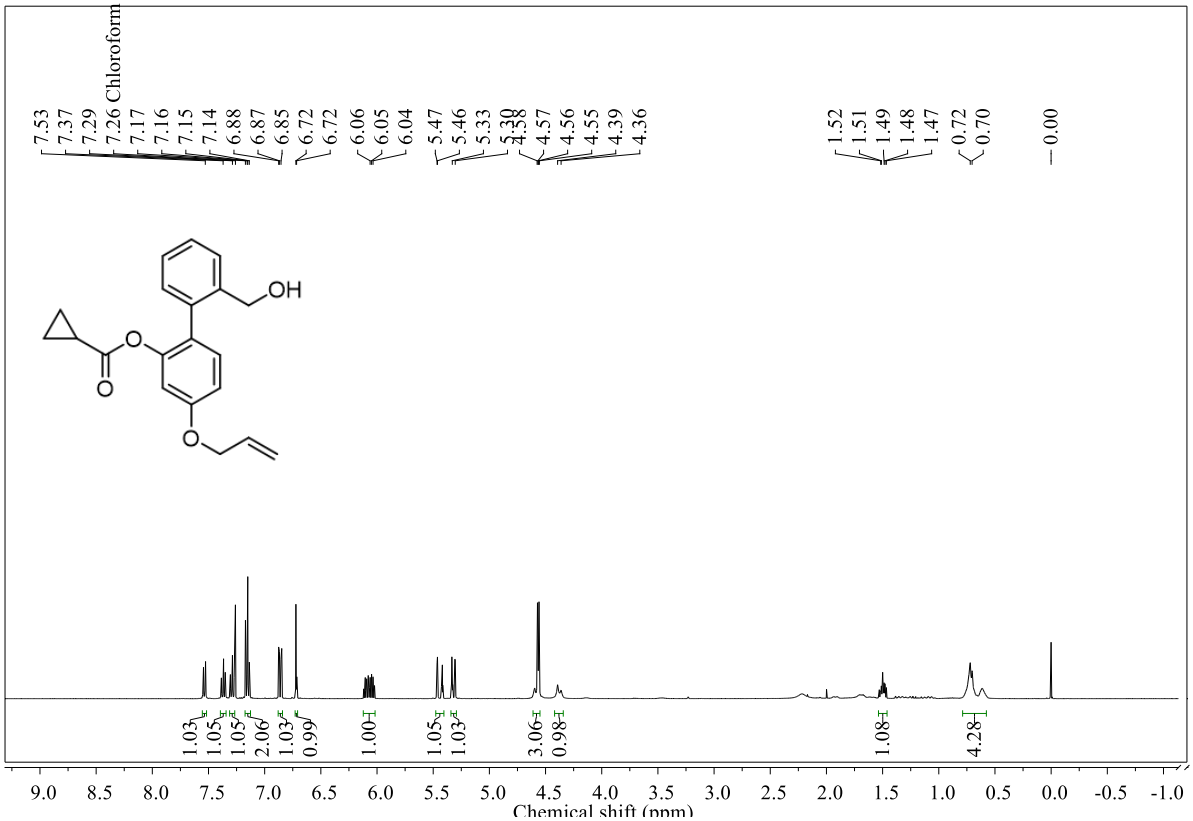
^{1}H NMR spectra of **5**



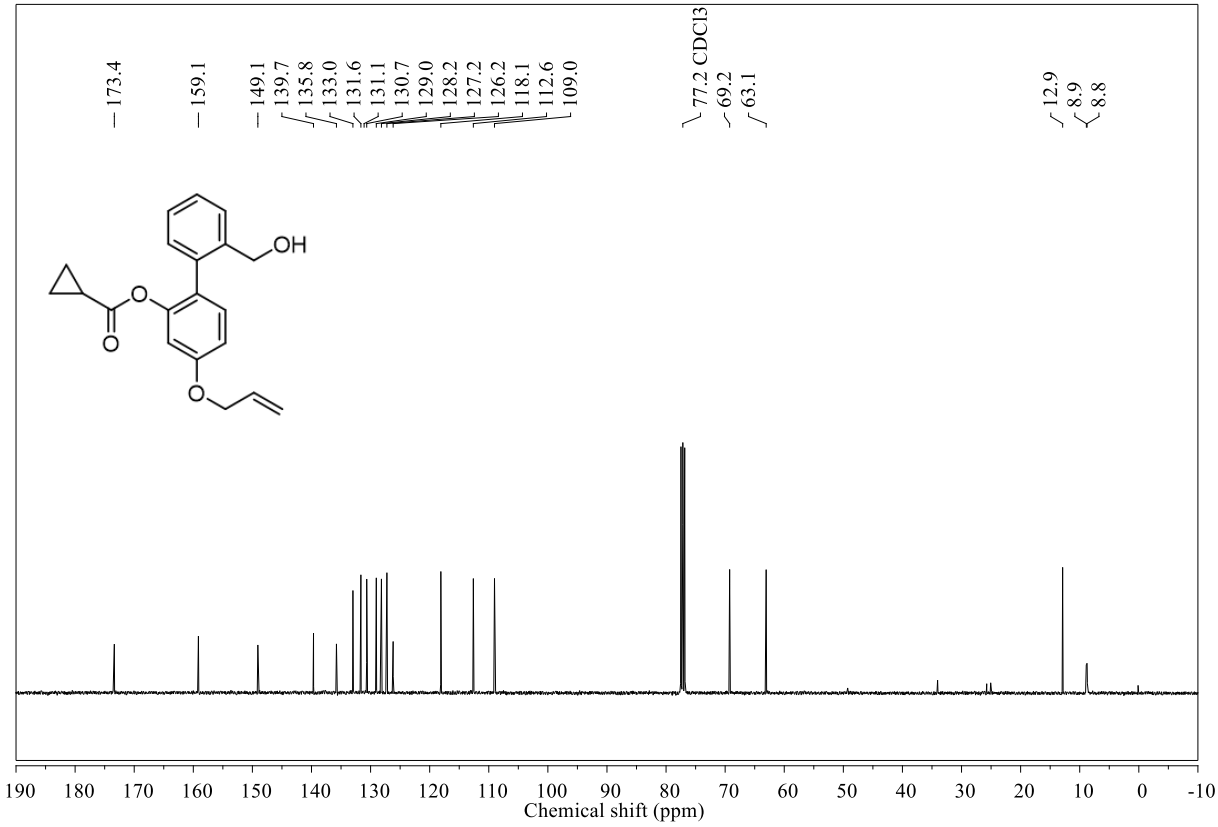
^{13}C NMR spectra of **5**



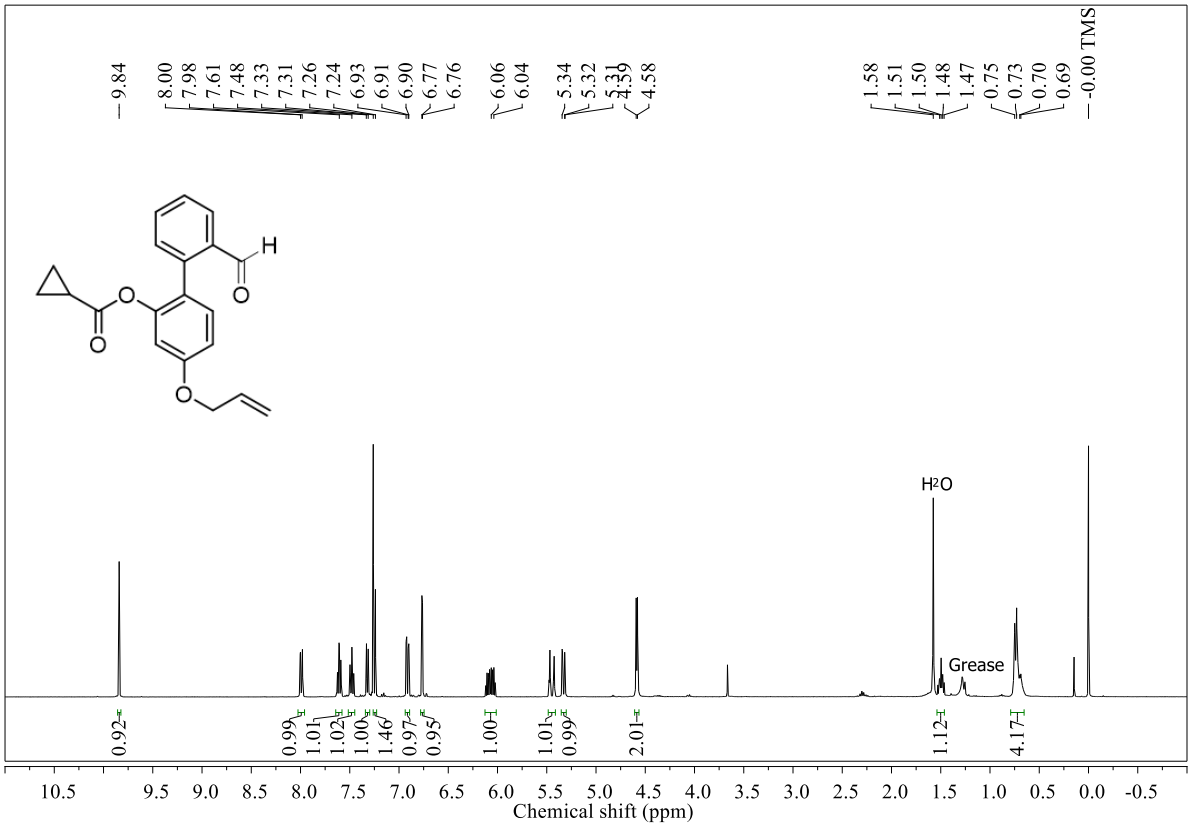
^{1}H NMR spectra of **6**



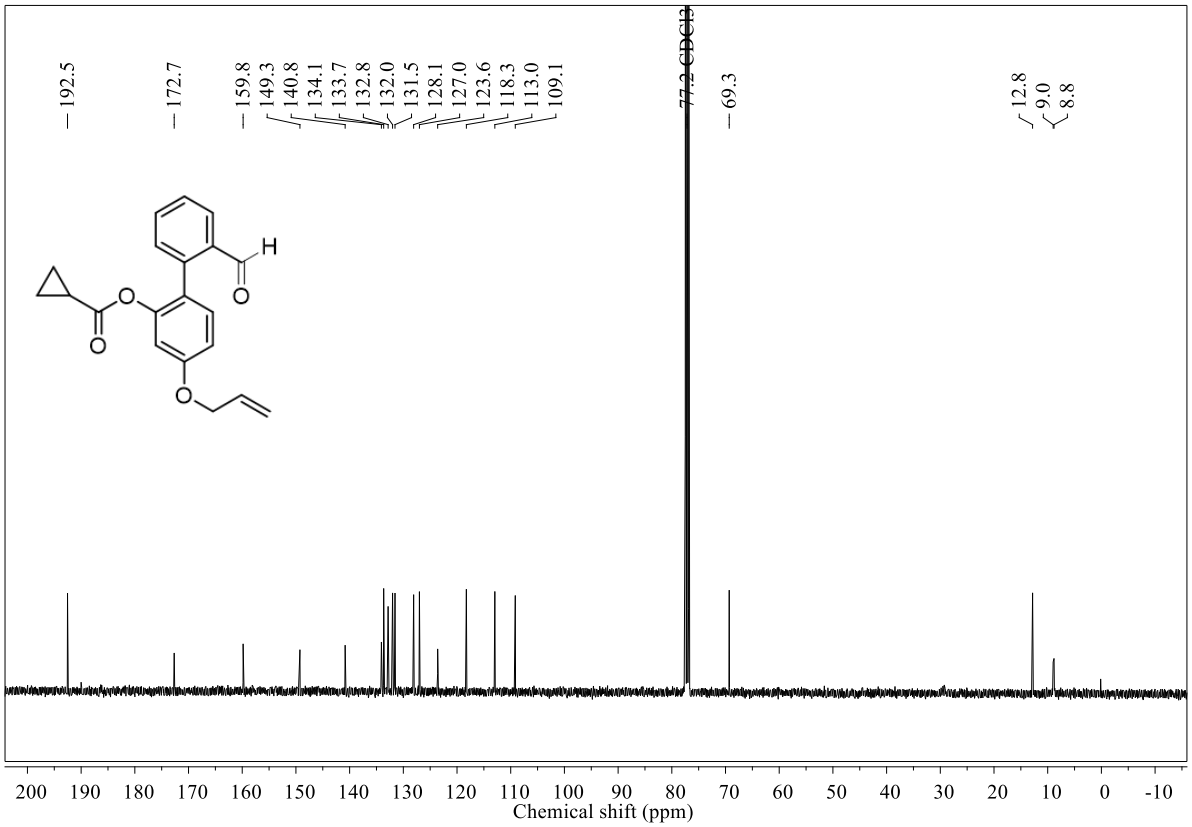
^{13}C NMR spectra of **6**



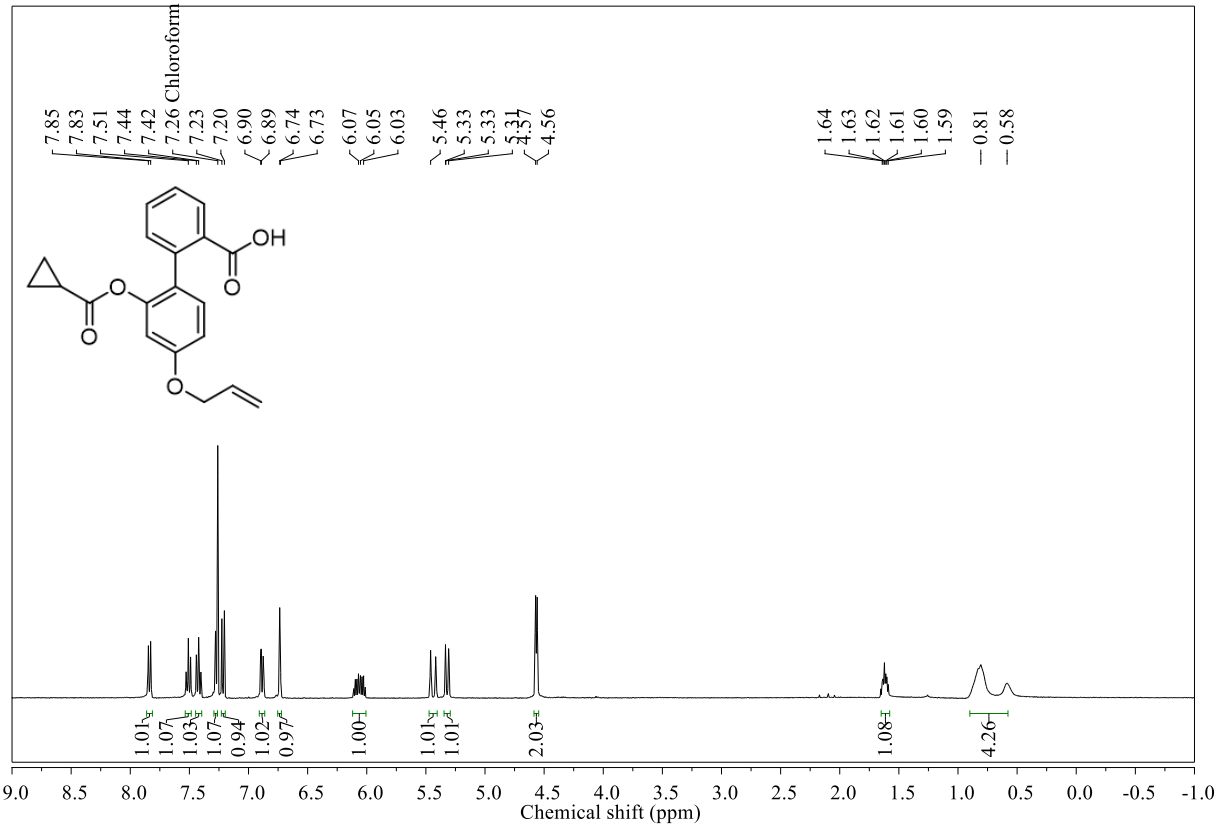
^{1}H NMR spectra of **7**



^{13}C NMR spectra of **7**



^{1}H NMR spectra of **8**



^{13}C NMR spectra of **8**