Electronic Supporting Information (ESI)

for

An Esterase-Sensitive Persulfide/Hydrogen Sulfide Generating Fluorogenic Probe Enhances Antioxidant Response

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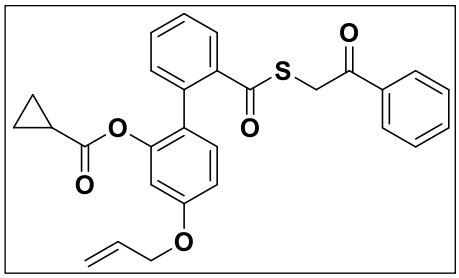
1. General methods:

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 (100 –200 mesh) as the 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 (HRMS) were obtained from HRMS-ESI-QTOF. FT-IR spectra were recorded using a BRUKER-ALPHA FT-IR spectrometer and reported in cm^{-1}. High-Performance Liquid Chromatography (HPLC) was performed on an Agilent Technologies 1260 infinity with Eclipse plus C-18 reversed-phase column (250 mm × 4.6 mm, 5μm). All measurements were done using an 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 **AS**[1], **2**[2], and **3**[2] were synthesized following previously reported protocols, and each compound's analytical data was consistent with reported values.

4-(allyloxy)-2'-(((2-oxo-2-phenylethyl)thio)carbonyl)-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (1):



A solution of **3** (0.100 g, 0.29 mmol) was taken in DCM (5 mL). To the reaction mixture, DCC (0.068 g, 0.32 mmol) and DMAP (0.008 g, 0.059 mmol) were added under an N_2 atmosphere, and the reaction was stirred for 1 h at 0 ^oC. A solution of freshly prepared phenacyl thiol **T1** (0.090 g, 0.59 mmol) in DCM (2 mL) was added to the above reaction mixture and stirred at rt for 3 h until the complete consumption of the starting material, as monitored by TLC. The solvent from the reaction mixture was evaporated, diluted residue with water (10 mL), and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, and filtered, and the filtrate was concentrated to give a crude compound. The crude residue was further purified by column chromatography using silica gel (100-200) with 4% EtOAc/hexane as eluant, to provide **1** (0.028 g, 20%) as yellowish sticky liquid. FT-IR (ν_{max}, cm^{-1}): 3308, 2958, 2917, 2850, 1730, 1700, 1678, 1617; ^1H NMR (400 MHz, $CDCl_3$): δ 7.97 (dd, *J* = 8.3, 1.2 Hz, 2H), 7.89 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.61 - 7.51 (m, 2H), 7.49 - 7.40 (m, 3H), 7.28 (dd, *J* = 7.6, 1.1 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 1H), 6.84 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.66 (d, *J* = 2.5 Hz, 1H), 6.07 (ddt, *J* = 17.6, 10.6, 5.3 Hz, 1H), 5.43 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.30 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.53 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.46 (s, 2H), 1.62 - 1.56 (m, 1H), 0.74 (d, *J* = 8.0 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 193.7, 191.7, 173.0, 159.3, 148.5, 137.4, 136.0, 135.6, 133.8, 133.1, 132.1, 131.1, 128.8, 128.7, 128.4, 127.7, 125.9, 118.0, 112.6, 108.9, 69.1, 37.2, 12.9, 9.0; HRMS (ESI-TOF) for $C_{28}H_{24}O_5S[M+Na]^+$: Calcd., 495.1237, Found, 495.1241.

3. Experimental Procedures :

Stability assessment of 1 by HPLC

A stock solution of **1** (10 mM) was prepared independently in DMSO. In a typical reaction, compound 50 μM **1** (5 μL, 10 mM stock) was added to 995 μL of phosphate buffer saline (10 mM, pH 7.4), and the reaction mixture was incubated at 37 ^oC. Aliquots were taken at determined time points (0 h and 2 h), diluted with an equal amount of acetonitrile, and then

injected in an HPLC instrument attached to a UV detector (absorbance at 250 nm). Phenomenex Luna C-18 reverse phase column, 100 Å particle size and 5 μM pore size (250 × 4.6 mm) was used. The mobile phase was water: acetonitrile with a multistep gradient starting at 35:65 → 0 min, 35:65 to 35:65 → 0 - 2 min, 35:65 to 20:80 → 2 - 5 min, 20:80 to 10:90 → 5 - 8 min, 10:90 to 5:95 → 8 - 11 min, 5:95 to 10:90 → 11 - 14 min, 10:90 to 20:80 → 14 - 17 min, 20:80 to 35:65 → 17 - 20 min, 35:65 to 35:65 → 20 - 24 min was used at a flow rate of 0.5 mL/min (**Figure S1**).

Decomposition of 1 in the presence of esterase using HPLC studies:

Stock solutions of **1** (10 mM), **AS** (10 mM), and **2** (10 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) in phosphate buffer saline (10 mM, pH 7.4) was prepared. The reaction mixture was prepared by adding 50 μM of **1** (5 μL, 10 mM) with or without 1 U/mL Es (10 μL, 100 U/mL stock), and the volume was adjusted to 1 mL using phosphate buffer saline (10 mM, pH 7.4) in an Eppendorf then incubated for 120 min at 37 ˚C on thermomixer (300 rpm). 200 μL aliquots of the reaction mixture were taken at pre-determined time points, and the reaction was quenched by adding 200 μL of acetonitrile. The samples were centrifuged at 10000 x g for 5 min at 4 ˚C; the supernatant was collected and injected (50 μL) in high-performance liquid chromatography (HPLC) attached with a UV detector (absorbance at 250 nm). Phenomenex Luna C-18 reverse phase column, 100 Å particle size and 5 μM pore size (250 × 4.6 mm) was used. The mobile phase was water: acetonitrile with a multistep gradient starting at 35:65 → 0 min, 35:65 to 35:65 → 0 - 2 min, 35:65 to 20:80 → 2 - 5 min, 20:80 to 10:90 → 5 - 8 min, 10:90 to 5:95 → 8 - 11 min, 5:95 to 10:90 → 11 - 14 min, 10:90 to 20:80 → 14 - 17 min, 20:80 to 35:65 → 17 - 20 min, 35:65 to 35:65 → 20 - 24 min was used at a flow rate of 0.5 mL/min (**Figure S2**).

Limit of detection (LOD) of 2:

A stock solution of **2** (10 mM) was prepared in DMSO. A calibration curve with varying concentrations of the lactone (**2**) (0-20 μM) was performed (**Figure S3**). The linear dynamic range and limit of detection (LOD) were determined following a reported method.[3] A good linearity (R^2 = 0.9930) between the fluorescent intensity data at 432 nm and the concentrations of lactone (**2**) in the range from 0.5 μM to 15 μM (linear dynamic range) was observed. Next, the limit of detection (LOD) was determined using the following equation:

$$LOD = 3\sigma/K$$

Where σ is the standard deviation of blank measurement, and K is the slope between the fluorescence intensity versus lactone concentration.

The linear equation was found to be Y = 43520*X + 230688 (R^2 = 0.9930), where Y is the fluorescent intensity data at 432 nm measured at a given lactone concentration, and X represents the concentration of lactone. So, the detection limit (LOD) for lactone (**2**) was calculated to be 0.3 μM (LOD = 3σ/K = 3*4199.577/ 43520 ≈ 0.3 μM) (**Figure S3**).

Monitoring the release of 2 upon esterase activation of 1[2]:

(A) Fluorometric analysis:

Stock solutions of **1** (10 mM), **2** (10 mM) in DMSO, and porcine liver esterase (Es, 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 a HORIBA Scientific Fluoromax-4 spectrofluorometer (**Figure S4A**).

(B) Fluorescence-based analysis:

Stock solutions of **1** (0.5 mM) in DMSO and Es 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 60 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 2** (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) (**Figure S4B**).

Stock solutions of PMSF (100 mM) in isopropanol and esterase (100 U/mL) in phosphate buffer saline (PBS), pH 7.4, were prepared. The reaction mixture was prepared by adding 1 mM of PMSF (7 μL, 100 mM) along with 1 U/mL esterase (70 μL, 1 U/mL stock), and the volume was adjusted to 700 μL using phosphate buffer saline (10 mM, pH 7.4) in a 96-well plate. Measurements were carried out after incubation for 30 min at 37 ˚C.

Stock solutions of **1** (0.5 mM) in DMSO and esterase (1 U/mL, pre-treated with PMSF) in phosphate buffer saline (10 mM, pH 7.4) were prepared independently. Measurements were carried out by using the above-described protocol (**Figure S5**).

Selectivity of 1:

Stock solutions of **1** and **2** (1 mM) and porcine liver esterase (100 U/mL) were prepared in DMSO and phosphate buffer saline (10 mM, pH 7.4), respectively. Stock solutions of GSH (10 mM), Cys (10 mM), vitamin-C (10 mM), arabinose (10 mM), glucose (10 mM), galactose (10 mM), *N*- acetylcysteine (NAC, 10 mM), DTT (10 mM), and H_2O_2 (30%, 10 mM) in phosphate buffer were prepared independently from commercial sources. A stock solution of 285 μM 3-MST was used.

The reaction mixture was prepared by adding 10 μM of **1** (2 μL, 1 mM), with or without 1 U/mL esterase (2 μL, 100 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 60 min at 37 ˚C. Similarly, a reaction mixture of 10 μM of **1** (2 μL, 1 mM), with or without 100 μM (10 eq.) of various analytes (2 μL, 10 mM stock), was prepared, 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 60 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 S9**).

Persulfide/polysulfide measurement using SSP2[4]:

Stock solutions of **1** (10 mM), **AS** (10 mM), **2** (10 mM), and SSP-2 (5 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) in phosphate buffer saline (10 mM, pH 7.4) was prepared.

Individual reaction samples were prepared for **1**, **AS**, and **2**. To 3-MST (1 μM), compound (10 μM) and Es (1 U/mL) were added. Volume was adjusted to 400 μL using 10 mM PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C.

A reaction was set up for compound control. Individual reaction samples were prepared for **1**, **AS**, and **2.** The reaction mixtures were prepared by adding compound (10 μM) with Es (1 U/mL), and the volume was adjusted to 400 μL using 10 mM PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C.

A reaction was set up for enzyme control. The reaction mixtures were prepared by 3-MST (10 μM), 4 μL of DMSO, Es (1 U/mL), and the volume was adjusted to 400 μL using 10 mM PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C.

A similar reaction was set up for DTT control. Individual reaction samples were prepared for **1, AS,** and **2**. The reaction mixtures were prepared by adding compound (10 μM) with Es (1 U/mL) and 3-MST (1 μM). The volume was adjusted to 360 μL using 10 mM PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C. Finally, the reaction mixture was treated with 10 mM DTT (40 μL of 100 mM stock) and further incubated at 37 °C for 1 h.

The above treatment groups were finally incubated with 5 μM SSP-2 (4 μL, 5 mM) at 37 °C for 10 min in the dark. 100 μL aliquot of each sample was transferred to a 96-well plate, and the fluorescence was recorded (λ_{ex} = 482 nm, λ_{em} = 518 nm) 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. A stock solution of GSH (10 mM) was prepared in DI water. The reaction mixture was prepared by adding 50 μM of **1** (1.5 μL, 10 mM stock), 1 U/mL esterase (3 μL, 100 U/mL stock), and 5 μM of 3-MST (19.5 μL, 77 μM stock). The volume was adjusted to 300 μL using 10 mM PBS, pH 7.4, and the reaction mixture was incubated for 60 min at 37 °C. 1 mM GSH (30 μL of 10 mM) was then added and incubated at 37 °C for 30 min, followed by 10 mM HPE-IAM (30 μL of 100 mM) and further incubation for 15 min at 37 °C. Finally, the reaction was quenched by adding 300 μL of acetonitrile. Similarly, control reactions were set up for the **1** or HPE-IAM alone and 3-MST only. The samples were centrifuged at 10,000 x g for 10 min at 4 ˚C; the supernatant was collected and assessed thereafter by LC/MS. All measurements were done using a previously established LC/MS method with slight modification.[5] 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 ($M + H^+$) 473.1417 (**1**), 305.9985 (**HPE-IAM**), 517.1421 (**GSS-HPE-AM**), 389.1530 (**Bis-S-HPE-AM**) (**Figure S10** & **S11**).

Lead acetate assay for the H_2S detection[6]:

The lead acetate assays were conducted as previously reported, with some modifications. Firstly, lead acetate paper was prepared by soaking Whatman filter paper with 10 % (w/v) lead acetate solution and dried. 392 μM stock of wild-type (wt) *b*3-MST was used for lead acetate assay. Stocks of all compounds (**1, AS,** and **2**) (10 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) in phosphate buffer saline (10 mM, pH 7.4) was prepared. Dithiothreitol (DTT, 10 mM) was prepared in deionized water.

Lead acetate assay was performed in a 96-well plate (with lid). Individual reaction samples were prepared for **1**, **AS,** and **2.** Compound (100 μM) was subsequently added to Es (1 U/mL), wt 3-MST (1 μM) and DTT (10 mM). The final volume was adjusted to 200 μL using 10 mM phosphate buffer saline (PBS) pH 7.4 buffer, covered with 10% lead acetate-soaked paper, sealed and incubated at 37 °C.

A similar protocol was followed for the substrate controls. Individual reaction samples were prepared for **1**, **AS,** and **2**. Compound (100 μM) was subsequently added to Es(1 U/mL) and DTT (10 mM). The final volume was adjusted to 200 μL using 10 mM PBS pH 7.4 buffer, covered with 10% lead acetate-soaked paper, sealed, and incubated at 37 °C.

A separate experiment was conducted to test the enzyme controls. DMSO (1%), Esterase (1 U/mL), wt 3-MST (0.5 μM), and DTT (10 mM) were sequentially added. The final volume was adjusted to 200 μL using 10 mM PBS pH 7.4 buffer, covered with 10% lead acetate-soaked paper, sealed, and incubated at 37 °C.

Lead acetate paper was carefully taken out at predetermined time points, and an image was captured using Syngene G-Box Chemi-XRQ (**Figure S12)**.

Methylene blue assay for the H_2S detection[6]:

General protocol: The methylene blue assays were conducted as previously reported with some modifications. Briefly, 392 μM stock of wt 3-MST was used for the methylene blue assay. 10 mM stocks of all compounds (**1**, **AS,** and **2**) were prepared in DMSO. Porcine liver esterase (100 U/mL) in phosphate buffer saline (10 mM, pH 7.4) was prepared. Dithiothreitol (DTT, 100 mM), NaSH (100 mM), and $Zn(OAc)_2.2H_2O$ (40 mM) were prepared in deionized water. Stock solutions of $FeCl_3$ (30 mM) and N, N–dimethyl-p-phenylenediamine sulfate (DMPPDA) (20 mM) were prepared in 1.2 M HCl and 7.2 M HCl, respectively.

Individual reaction samples were prepared for **1**, **AS,** and **2**. Compound (100 μM) was added to Es (1 U/mL), $Zn(OAc)_2.2H_2O$ (400 μM), wt 3-MST (1 μM) and DTT (10 mM). The final volume was adjusted to 0.5 mL using 10 mM PBS pH 7.4 buffer and incubated at 37 °C.

A similar protocol was followed for the substrate controls. Individual reaction samples were prepared for **1**, **AS,** and **2**. Compound (100 μM) was added to Es (1 U/mL), $Zn(OAc)_2.2H_2O$ (400 μM) and DTT (10 mM). The final volume was adjusted to 0.5 mL using 10 mM PBS pH 7.4 buffer and incubated at 37 °C.

A separate experiment was conducted to test the enzyme controls. DMSO (1%), Esterase (1 U/mL), wt 3-MST (0.5 μM) and DTT (10 mM) were sequentially added. The final volume was adjusted to 0.5 mL using 10 mM PBS pH 7.4 buffer and incubated at 37 °C.

At predetermined time points, equal volumes (200 μL) of $FeCl_3$, DMPPDA, and aliquots from the above reaction samples were mixed, and the resulting solution was 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).

Cell viability assay in 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).

Monitoring the release of 2 from 1 in C28/I2 cells:

C28/I2 cells were seeded overnight in a 96-well plate at a density of 6,000 cells per well in complete phenol red-free DMEM medium, supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% antibiotic solution (Thermo Fisher Scientific). Stock solutions of **1** and **2** were prepared in DMSO.

Once the cells reached ~70% confluence, the old medium was removed, and the cells were washed with 1× PBS. The cells were then treated with compound **1** (50 µM) in a 96-well plate and incubated at 37 °C for 24 hours. Media was removed, the cells were washed thrice with 1× PBS, and fluorescence was recorded (Excitation: 320 nm, Emission: 432 nm) (**Figure S6**).

Detection of 2 release in C28/I2 cell lysate:

C28/I2 cells were seeded overnight in a 96-well plate at a density of 6,000 cells per well in complete phenol red-free DMEM medium, supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% antibiotic solution (Thermo Fisher Scientific). Stock solutions of **1** and **2** were prepared in DMSO.

Once the cells reached ~70% confluence, the old medium was removed, and the cells were washed with 1× PBS. The cells were then lysed using 0.05% Triton X, and the lysate was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant (150 µL) was treated with compound **1** (50 µM) in a 96-well plate and incubated at 37 °C for 24 hours. Fluorescence was recorded (Excitation: 320 nm, Emission: 432 nm) (**Figure S7**).

Confocal imaging of MEF cells with $\mathbf{1}^2$:

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, the 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**/**2**, 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 a Carl Zeiss LSM710 laser scanning confocal microscope (700 nm for two-photon) with a 63x oil immersion objective. Images were analysed by ImageJ software. **(Figure S8)**.

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 cells were pre-treated for 3 hours with various concentrations of **1**, which were prepared as a DMSO stock solution to achieve a final DMSO concentration of 0.1%. Following this pre-treatment, MGR-1 was added to the cells at different concentrations and incubated for 24 hours.

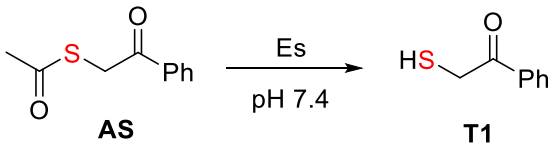
A stock solution of WST-8 containing 2 µL of WST-8 developer reagent and 2 µL of electron mediator solution per 96 µL of complete DMEM was prepared. After 24 hours of treatment, the cells were washed with 1× PBS, and 100 µL of the WST-8 solution was added to each well. After a 1-hour incubation, the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

Micromass culture assay[7]:

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 (18 µM), MGR-1(18 µM) + **1** (50 µM), and only **1** (50 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 S13**).

4. Supplementary figures:



Scheme S1. AS in the presence of Es generates phenacyl thiol **T1** in pH 7.4 buffer at 37 °C.

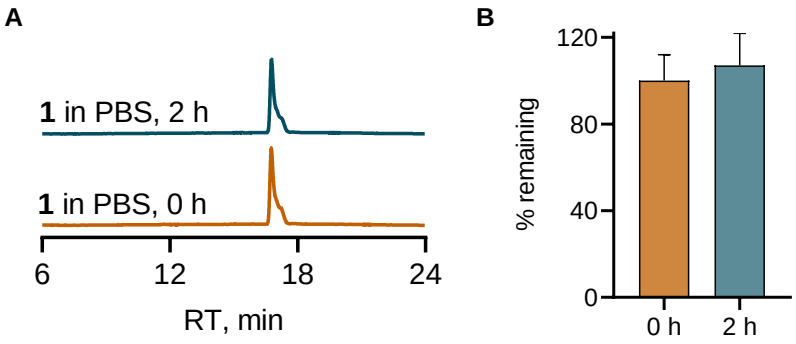


Figure S1. Stability of **1** in PBS buffer. (A) HPLC traces of stability of 1 in pH 7.4 PBS buffer (10 mM) at 37 °C. (B) Area under the curve (AUC) corresponding to **1** at 3.6 min and remaining in buffer after 2 h of incubation (Abs = 250 nm).

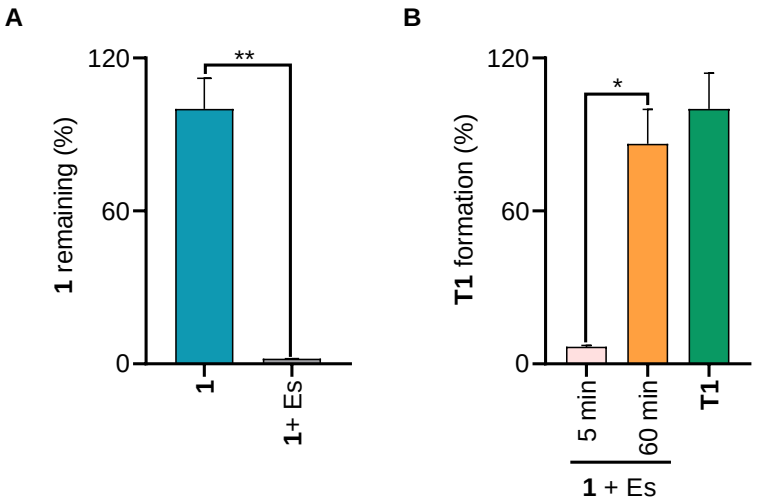


Figure S2. Area under the curve (AUC) for the peak corresponding to the **(A)** remaining of **1** and **(B)** formation of **T1** from **1** as monitored by HPLC. (Abs = 250 nm). Statistical significance was carried out using One-way ANOVA(*$p < 0.033$, **$p < 0.002$).

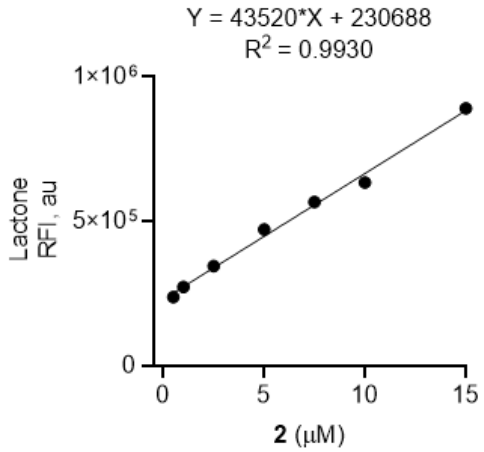


Figure S3. Calibration curve of lactone (**2**) with varying concentrations in PBS buffer, pH 7.4 (λ_{ex} = 320 nm; λ_{em} = 432 nm). A good linearity (R^2 = 0.9930) between the fluorescent intensity data at 432 nm and the concentrations of lactone (**2**) in the range from 0.5 μM to 15 μM was observed. The linear equation was found to be Y = 43520*X + 230688 (R^2 = 0.9930), where Y is the fluorescent intensity data at 432 nm measured at a given lactone concentration, and X represents the concentration of lactone. So, the detection limit (LOD) for lactone (**2**) was calculated to be 0.3 μM (LOD = 3σ/K = 3*4199.577/ 43520 ≈ 0.3 μM).

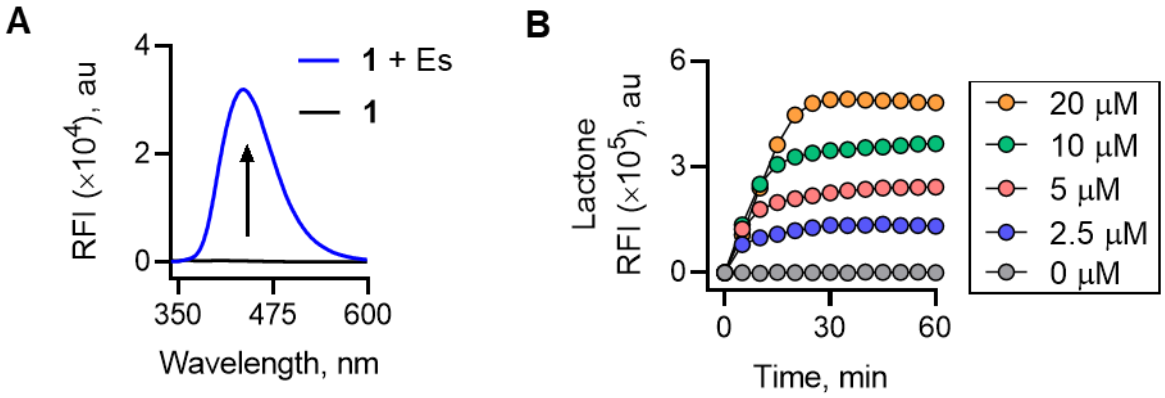


Figure S4. Monitoring the formation of lactone (**2**) from **1** by fluorescence (λ_{ex} = 320 nm and λ_{em} = 432 nm) (**A**) At 10 μM without or with Es (1 U/mL) in pH 7.4 buffer at 37 °C. (**B**) In a concentration-dependent manner (0-20 μM) with Es (1 U/mL) in pH 7.4 buffer at 37 °C.

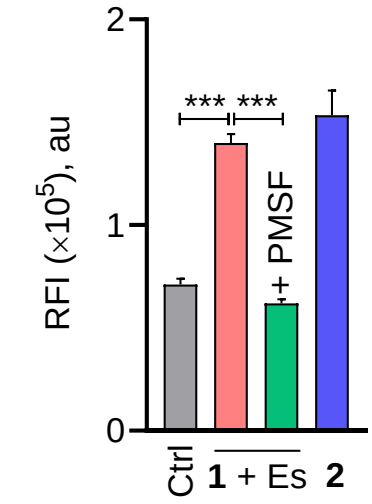


Figure S5. Monitoring the formation of lactone (**2**) from **1** (10 μM) with and without esterase (1 U/mL) inhibitor PMSF (1 mM) in PBS (pH 7.4) at 37 °C for 60 min by fluorescence (λ_{ex} = 320 nm and λ_{em} = 432 nm); ctrl refers to **1** alone; +PMSF (phenylmethanesulfonyl fluoride) refers to pre-treatment of Es with PMSF followed by compound treatment. Statistical significance was carried out using One-way ANOVA (*** $p \leq 0.001$).

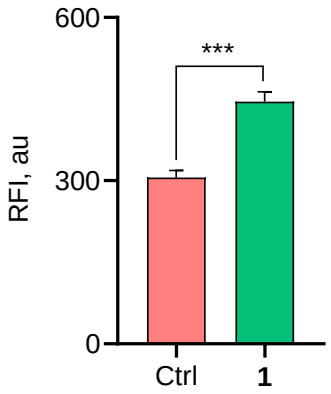


Figure S6. Monitoring the formation of lactone (**2**) from **1** (50 μM) by fluorescence (λ_{ex} = 320 nm and λ_{em} = 432 nm) in C28/I2 cells after 24 h incubation at 37 °C. Statistical significance was carried out using a student's *t*-test (*** $p < 0.001$).

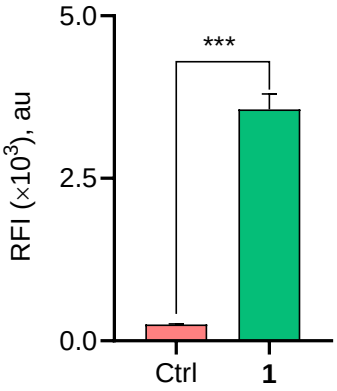


Figure S7. Monitoring the formation of lactone (**2**) from **1** (50 μM) by fluorescence (λ_{ex} = 320 nm and λ_{em} = 432 nm) in C28/I2 cell lysates after 24 h incubation at 37 °C. Statistical significance was carried out using a student's *t*-test (*** $p <$ 0.001).

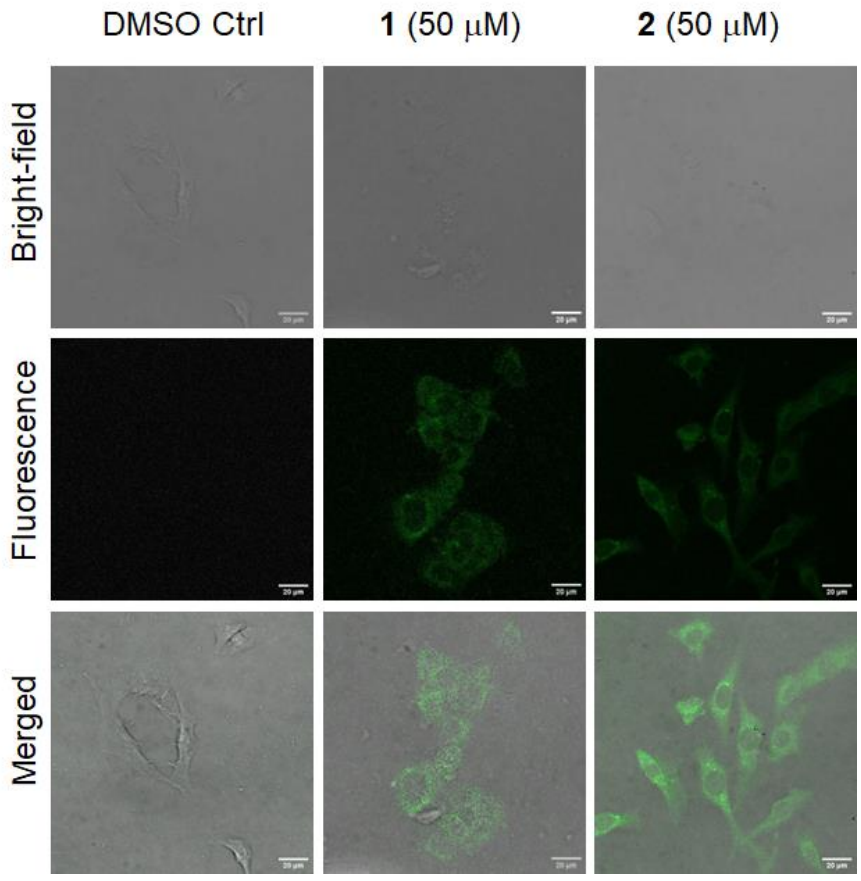


Figure S8. Two-photon confocal microscopy images of MEF cells treated with **1** and **2** (50 μM). The excitation and emission channels were 700 nm and 432 nm, respectively. Scale bar is 20 μm.

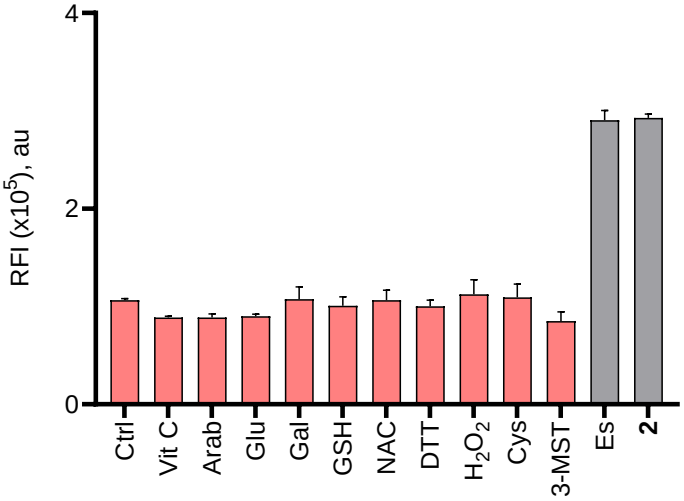
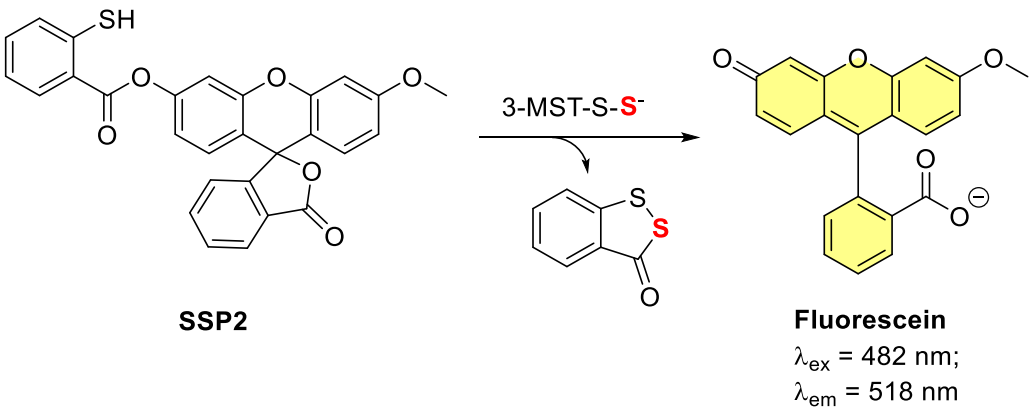


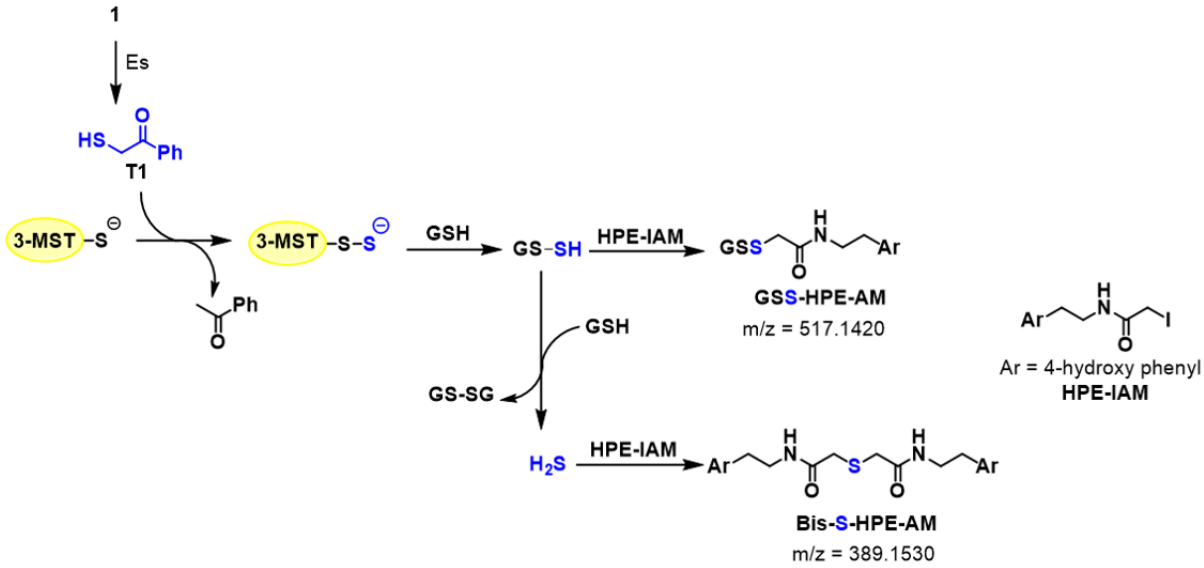
Figure S9. Fluorescence response of **1** (10 μM) to various biological analytes (100 equiv.) in PBS (pH 7.4, 10 mM) after 60 min at 37 °C. Ctrl: **1** alone; Vit C: ascorbic acid; Arab: arabinose; Glu: glucose; Gal: galactose; GSH: glutathione; NAC: *N*- acetylcysteine; DTT: dithiothreitol; H_2O_2: hydrogen peroxide; Cys: cysteine;3-MST: 3-mercaptopyruvate sulfurtransferase (10 μM); Es: esterase (1 U/mL). Results are expressed as mean ± SD (n =3/group).



SSP2

Fluorescein
λ_{ex} = 482 nm;
λ_{em} = 518 nm

Scheme S2. Detection of persulfide by SSP2.



Scheme S3. LC/MS study. Reaction scheme showing the formation and detection of persulfides/polysulfides and hydrogen sulfide/polysulfides as their HPE-AM and Bis-S-HPE-AM adducts from **1**, respectively. Ar = 4-hydroxyphenyl.

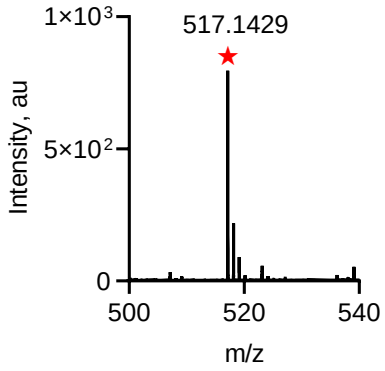


Figure S10. LC/MS study. (A) Mass spectra for **GSS-HPE-AM** (expected, m/z = 517.1420 $[M + H]^+$; observed, m/z = 517.1429).

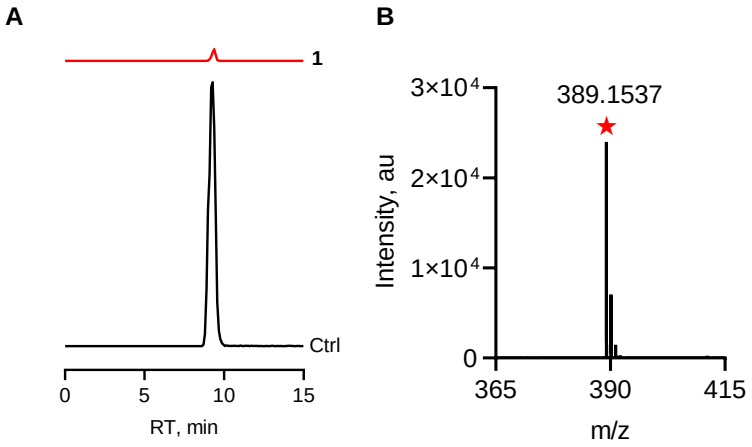
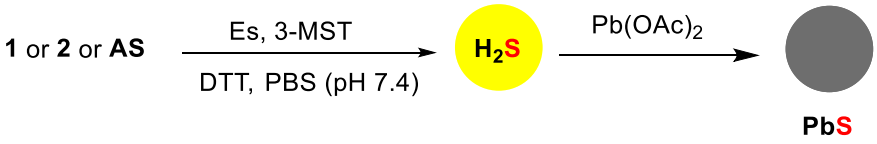


Figure S11. LC/MS study. (A) Extracted ion chromatograms from an LC/MS analysis of **Bis-S-HPE-AM** formation from **1** and ctrl;(B) Mass spectra for **Bis-S-HPE-AM** (expected, m/z = 389.1530 $[M + H]^+$; observed, m/z = 389.1537). Ctrl was prepared by reacting 200 μM GSH, 200 μM DEA/NO (sodium 2-(N, N-diethylamino)-diazenolate-2-oxide), and 200 μM NaSH at room temperature for 20 min.[8] Results are expressed as mean ± SD (n =3/group).



Scheme S4. Lead acetate assay for the detection of H_2S.

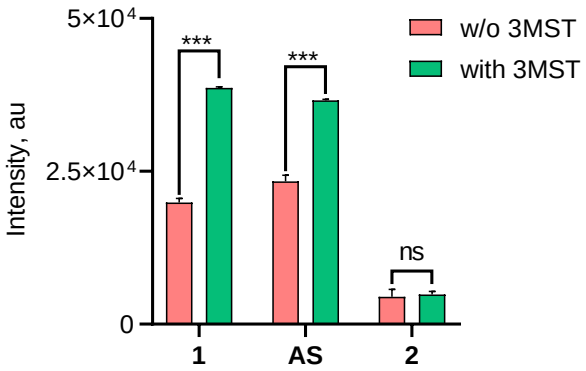
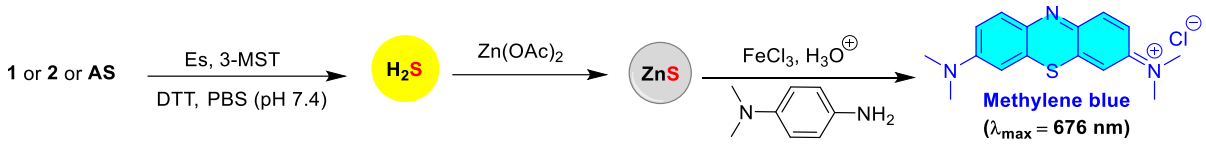


Figure S12. H_2S detection of compounds (**1**, **AS**, and **2** at 100 μM) in the presence of 3-MST (1 μM), Es (1 U/mL), and DTT (10 mM) for 2 h using lead acetate assay. PbS intensity was quantified using Image J software. Results are expressed as ± SD (n = 3). Statistical significance was established relative to w/o 3-MST using One-way ANOVA (*** $p \leq 0.001$).



Scheme S5. Methylene blue assay protocol for studying the generation of H_2S.

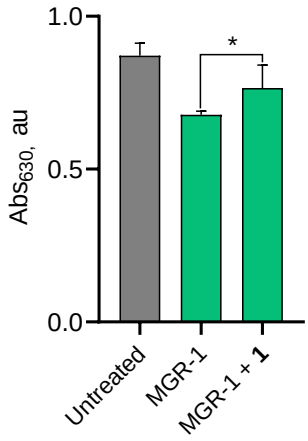


Figure S13. Micromass culture assay for sGAG formation from **1**(50 uM) in the presence of oxidative stress induced by MGR-1(18 uM). Results are expressed as mean ± SD (n =6/group). *p* value was determined by using One-way ANOVA relative to MGR-1. (**p* < 0.033).

5. References:

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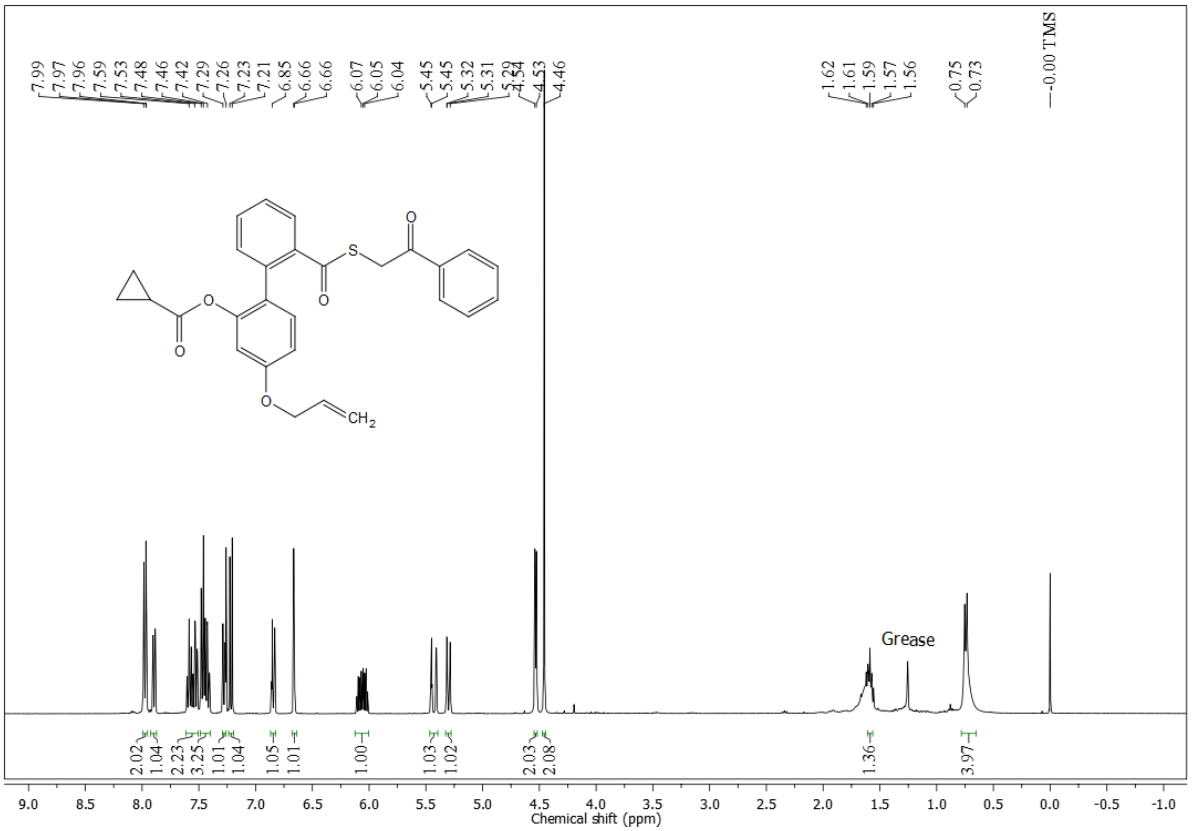
6. NMR spectra:

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



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