Electronic Supplementary Information (ESI)

for

β-galactosidase-activated Nitroxyl (HNO) Donors Provide Insights into Redox Cross-Talk in Senescent Cells

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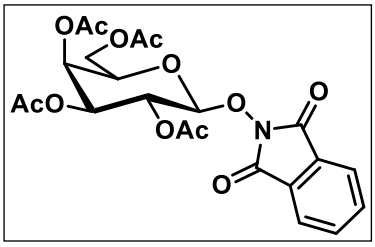
1. General methods:

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) or silica gel Spectrochem (100-200 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: br (broad signal), m (multiplet), s (singlet), d (doublet), t (triplet) and dd (doublet of doublets). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using BRUKER-ALPHA FT-IR spectrometer. Analytical HPLC was performed on an Agilent 1260-infinity with Phenomenex®C-18 reverse phase column (250 mm × 4.6 mm, 5 μm). Photometric and fluorimetric measurements were performed using Ensight microtiter plate reader (PerkinElmer, India). GC analysis was performed on an Agilent 8860 equipped with an electron capture detector (ECD) and Restek column (ShinCarbon ST 80/100, 2m, 1/8" OD),

2. Synthesis and characterization:

Compound **2** was synthesized using the reported protocol and data were consistent with reported values.[1]

(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-((1,3-dioxoisoindolin-2-yl)oxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (3):[2]



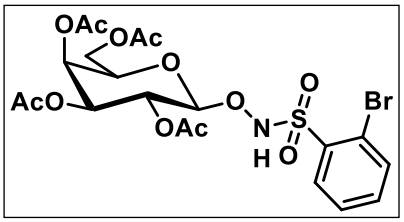
N-hydroxyphthalimide (7 g, 42.35 mmol, 3.5 eq.) and Et_3N (5 mL, 4 eq.) were dissolved in anhydrous DCM. To this solution, compound **2** (5 g, 12.1 mmol, 1 eq.) was added and the reaction mixture was stirred for 16 h at RT. Upon completion of the reaction (TLC analysis), reaction mixture was quenched by adding water and extracted with dichloromethane. The combined organic extracts were dried over Na_2SO_4 and the filtrate was concentrated under reduced pressure and the product **3** was isolated as a light yellow solid (5.4 g, 90 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 – 7.86 (m, 2H), 7.70 – 7.78 (m, 2H), 5.48 (dd, *J* = 10.4, 8.2 Hz, 1H), 5.43 (dd, *J* = 0.9, 3.4 Hz, 1H), 5.12

(dd, *J* = 10.4, 3.4 Hz, 1H), 5.0 (d, *J* = 8.2 Hz, 1H), 4.26 – 4.15 (m, 2H), 3.95 – 3.92 (m, 1H), 2.22 (s, 3H), 2.20 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H). The analytical data is consistent with the previously reported values.

Synthesis of (2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(aminooxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (4):[2]

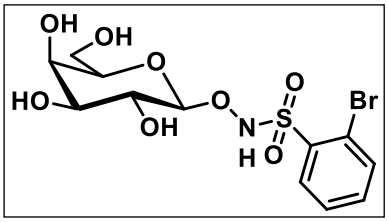
To a solution of compound **3** (1 eq.) in anhydrous DCM (5 mL), hydrazine monohydrate (1 eq.) was added dropwise at 0 ˚C. The reaction mixture was slowly warmed to room temperature and stirred for ~1 h. The white suspension was filtered off. The filtrate was washed with water and the aqueous layer was extracted with dichloromethane. The combined organic extracts were dried over Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to yield a crude product **4**, which was used for the next step without further purification.[2]

Synthesis of (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(((2-bromophenyl)sulfonamido)oxy) tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (5)[3]



To a mixture of compound **4** (300 mg, 0.82 mmol, 1 eq.) and DMAP (100 mg, 0.82 mmol, 1.2 eq.) in pyridine (5 mL) were independently added 2-bromobenzene sulfonyl chloride derivatives (210 mg, 0.82 mmol, 1.2 eq.) at 0 ˚C. The reaction mixture was warmed to RT and stirred for 2 - 3 h. Upon completion of the reaction (TLC analysis), the reaction was quenched by adding 1 N HCl and extracted with EtOAC. The combined organic extracts were dried over Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to yield crude product **5**, which was purified by silica column chromatography. The product **5** was isolated as a pale yellow solid (300 mg, 63%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35 (s, 1H), 8.07 – 8.06 (m, 1H), 7.78 – 7.76 (m, 1H), 7.53 – 7.52 (m, 1H), 5.38 – 5.37 (m, 1H), 5.05 – 5.01 (m, 2H), 4.99 – 4.97 (m, 1H), 4.14 – 4.05 (m, 2H), 3.98 – 3.95 (m, 1H), 2.11 (s, 6H), 2.07 (s, 3H), 1.97 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.4, 170.0, 169.9, 169.3, 135.4, 135.3, 135.2, 133.0, 127.8, 104.9, 71.7, 70.6, 69.9, 66.7, 61.1, 20.8, 20.7, 20.6, 20.5. HRMS (ESI): m/z for $C_{20}H_{23}BrNO_{12}S$ $[M+Na]^+$ calcd., 604.0100, found, 604.0096.

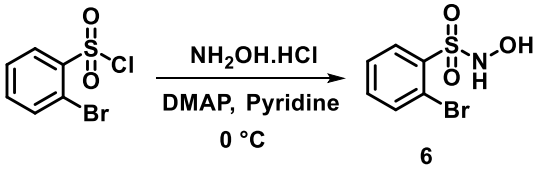
Synthesis of 2-bromo-*N*-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydro-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)benzenesulfonamide (1)[4]



Compound **5** (300 mg, 0.51 mmol, 1 eq.) was dissolved in methanol (5 mL) under a nitrogen atmosphere. Sodium methoxide (111 mg, 2.05 mmol, 4 eq.) in methanol (2 mL) was added to the solution at 0 ˚C. The mixture was warmed at RT and stirred for ~3 h. Upon completion of the reaction (TLC analysis), cation-exchange resin (H^+) was added, filtered and washed with MeOH. The solvent was evaporated under reduced pressure. The crude product **1** was purified by prep-HPLC column chromatography (MeOH/H_2O) to give the desired compounds. The product **1** was isolated as a pale yellow solid (90 mg, 43%); ^{1}H NMR (400 MHz, CD_3OD): δ 8.20 – 8.17 (m, 1H), 7.85 – 7.83 (m, 1H), 7.59 – 7.53 (m, 2H), 4.63 (d, *J* = 7.7 Hz, 2H), 3.81 (s, 1H), 3.73 – 3.65 (m, 2H), 3.54 – 3.51 (m, 1H), 3.47 – 3.46 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 135.9, 134.5, 133.9, 132.5, 127.0, 119.3, 106.6, 74.9, 72.9, 68.5, 68.0, 60.2; HRMS (ESI): m/z for $C_{12}H_{11}BrNO_6S$ $[M+H]^+$ calcd., 413.9853, found, 413.9876.

Synthesis of 2-bromo piloty's acid (6):

From 2-bromobenzenesulfonyl chloride, 2-bromo-*N*-hydroxybenzenesulfonamide (**6**) was synthesized by using the reported protocol.[3]

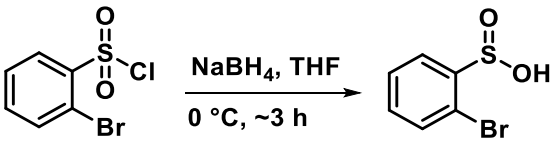


Scheme S1: Synthesis of **6**

To a mixture of hydroxylamine hydrochloride (196 mg, 2.8 mmol) and DMAP (196 mg, 2.8 mmol) in pyridine (10 mL) was added 2-bromobenzenesulfonyl chloride (360 mg, 1.4 mmol) on an ice-bath. Then, the mixture was stirred for 5 min at room temperature. The resulting suspension was poured into EtOAc (100 mL) and 1 N HCl aq. (100 mL). The EtOAc layer was separated, washed with brine (100 mL), dried over Na_2SO_4, filtered and the filtrate was concentrated. The crude product was purified by prep-HPLC (ACN:H_2O) to give 2-bromo-*N*-hydroxybenzenesulfonamide **6** as a light yellow solid. Yield (215 mg, 71%). The analytical data is consistent with previously reported values.

Synthesis of 2-bromobenzenesulfinic acid (7):

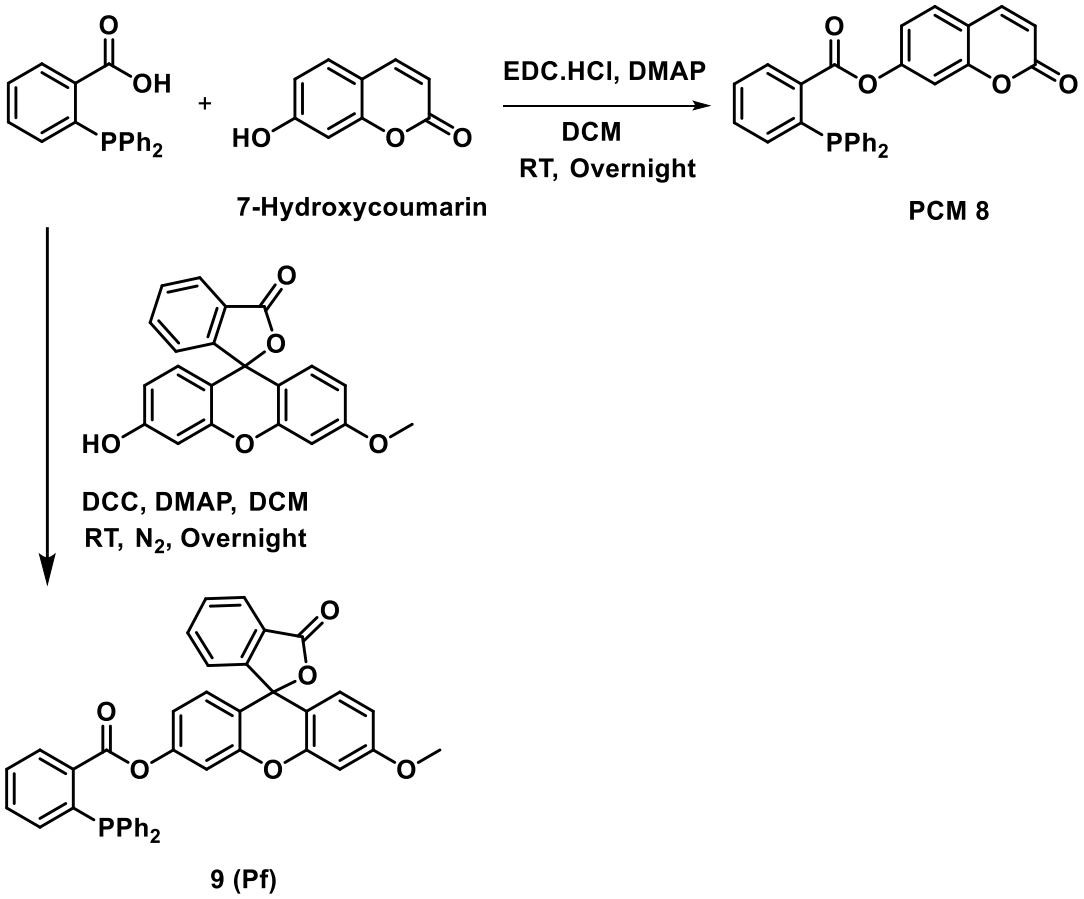
From 2-bromobenzenesulfonyl chloride, 2-bromo-sulfinic acid (**7**) was synthesized by using the reported protocol.[3]



Scheme S2: Synthesis of **7.**

To a solution of 2-bromobenzenesulfonyl chloride (100 mg, 0.39 mmol) in THF (5 mL) was added $NaBH_4$ (85 mg, 2.24 mmol) in portion at 0 ˚C. The reaction mixture was stirred for 2- 3 h. Upon completion of the reaction (TLC analysis), reaction mixture was quenched by aqueous HCl to maintained acidic pH <2 on an ice bath and resulting mixture was extracted by ethyl acetate. Organic layer was washed with brine, dried over Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to yield crude product **7** which was purified by prep-HPLC to give 2-bromobenzene sulfinic acid as white crystalline solid (60 mg, 69%): ^{1}H NMR (400 MHz, DMSO-D6): δ 7.85 – 7.82 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.70 (d, *J* = 7.7 Hz, 1H), 7.61 (t, *J* = 7.3 Hz, 1H), 7.51 – 7.47 (m, 1H); ^{13}C NMR (100 MHz, DMSO-D6): δ 147.2, 133.4, 133.2, 128.4, 125.2, 119.9; HRMS (ESI): m/z for $C_6H_5BrO_2S$ $[M+H]^+$ calcd., 220.9266, found, 220.9268.

Synthesis of HNO probes: PCM 8 and 9 (Pf) were synthesized using previously reported procedure and analytical data were consistent with reported values.[5,6]



Scheme S3: Synthesis of **PCM 8 and 9 (Pf)**

3. Experimental protocols

Analysis of Nitrous Oxide (N_2O) by Headspace Gas Chromatography:

6 and **1** stock solutions (10 mM) were prepared in DMSO and used immediately after preparation. β-Galactosidase (100 U/mL) stock solutions were prepared in pH 7.4 PBS. In a 10 mL vial sealed with a rubber septum, 4.45 mL PBS (pH 7.4, 100 mM) containing DTPA as a metal chelator (100 μM) was purged with argon for 30 min. These vials were placed in a heated cell block, which was held at 37 ˚C. **1** (100 μM) and β-galactosidase (10 U/ml) solution were added to each vial to obtain 5 mL total volume, and the resulting solutions were incubated for 15 h at 37 ˚C. A 100 μL gastight syringe with a sample lock was used for all gas injections. Headspace gas samples (60 μL) were injected into an Agilent 8860 GC with a Restek column (ShinCarbon ST 80/100, 2m, 1/8" OD) to analyze for N_2O. These experiments were carried out in triplicate with three injections performed for each vial. The N_2O yield was averaged and reported relative to the standard, 2-bromo Piloty's acid **6**.

HNO detection by using PCM 8:

Stock solutions of **PCM 8** (1 mM), **1, 6** (5 mM) in DMSO. β-galactosidase from *E. coli* (50 U/mL) stock solution in phosphate buffer saline (10 mM, pH 7.4) was prepared. The reaction mixture was prepared by adding 10 μM of **PCM 8** (2 μL, 1 mM), 50 μM of **1 or 6** (2 μL, 5 mM) along with 10 U/mL of β-galactosidase (40 μL, 50 U/mL) from stock solution and the volume was adjusted to 200 μL using phosphate buffer saline (10 mM, pH 7.4) in 96-well plate and then incubated for 4 h at 37 ˚C. The fluorescence (excitation at 370 nm; emission at 460 nm) was measured using an Ensight Multimode Plate Reader (PerkinElmer).

Nitric oxide (NO) detection by Griess assay:

A 5 mM stock solution of **1** and **6** in DMSO was prepared. A typical reaction mixture consisted of **1**, **6** and $NaNO_2$ by mixing 2 μL from 5 mM stock solution and in presence of β-galactosidase (20 μL from 100 U/mL stock solution in PBS pH 7.4) with phosphate buffer saline (pH 7.4, 10 mM) to make 200 μL final volume in 96 well plate (with lid) and incubate d for 4 h at 37 ˚C. Then, 14 μL of griess reagent was added in each well and incubated for 25 min at 37 ˚C. Absorbance was measured at 540 nm on Ensight Varioskan microtiter plate reader. The amount of NO released was estimated using a standard calibration curve generated with different concentrations of sodium nitrite ($NaNO_2$) solution (0-50 μM) using Ensight Varioskan microtiter plate reader (R^2 = 0.9969). The data represented here is average of 3 repeats.

Decomposition study by TLC:

A stock solution of **1, 6** and **7** (10 mM) were prepared in DMSO and β-galactosidase (10 U/mL from 100 U/mL stock solution in PBS pH 7.4). The reaction mixture contained **6** (5 μL, 10 mM) and **1** (5 μL, 10 mM) in the presence or absence of β-galactosidase enzyme solutions in PBS pH 7.4 (Total volume = 1 mL) and incubated at 37 ˚C for 48 h. The resulting mixture were acidified using hydrochloric acid, extracted with 100 μL of ethyl acetate and spotted on TLC plate. The eluant was 15% methanol-ethyl acetate.

Decomposition study by HPLC:

A stock solution of **1** and **7** (10 mM) were prepared in DMSO and β-galactosidase (10 U/mL from 100 U/mL stock solution in PBS pH 7.4). The reaction mixture contained **1** (5 μL, 10 mM), in the presence or absence of β-galactosidase enzyme solutions in PBS pH 7.4 (Total

volume = 1 mL) and incubated at 37 ˚C. The resulting mixture (100 μL) was taken out at predetermined time points and quenched by adding methanol (100 μL) subsequently samples were subjected to high performance liquid chromatography (HPLC Agilent Technologies 1260 Infinity). The mobile phase was water (0.1% TFA)/ Acetonitrile (0.1% TFA). The stationary phase was C-18 reverse phase column (Phenomenex, 5 μm, 4.6 x 250 mm). a multistep gradient was used with the flow rate of 1 mL/min starting with 70:30 → 0 min, 70:30 to 60: 40 → 0 - 3 min, 60:40 to 50: 50 → 3 - 6 min, 50:50 to 40: 60 → 6 – 9 min, 40:60 to 30: 70 → 9 - 10 min, 30:70 to 50: 50 → 10 - 12 min, 50:50 to 60: 40 → 12 - 13 min, 60:40 to 70: 30 → 13 - 15 min, 70:30 to 70: 30 → 15 - 18 min. Retention time for **1** is 6.9 min and for **7** is 8.9 min. The decomposition of **1** and formation of **7** was monitored at 230 nm. Authentic **7** (50 μM) in phosphate buffer saline was injected in HPLC and used as a control.

Docking study:

A. Prediction of putative ligand-binding pockets in β-galactosidase

The Computed Atlas of Surface Topography of proteins (CASTp 3.0) web server was used to predict the putative ligand-binding pockets and elucidate the amino acids lining each pocket in the protein. The protein structure of β-galactosidase from *Escherichia coli* (PDB ID: 1JYN) was submitted in a standard PDB format on the server and a probe radius of 1.4 Å was used. The CASTp server uses the weighted Delaunay triangulation and the alpha complex method to measure the area and volume of each predicted pockets or voids utilizing both the solvent accessible surface model and molecular surface model (Figure S5). The scoring results of CASTp were provided in Table S1.

B. *In silico* molecular docking studies

The structures of **1** and allolactose were built with standard bond lengths and angles using ChemDraw and then energy minimized with Chem3D using the integrated MM2 energy minimization script. The X-ray crystal structure of β-galactosidase from *E. coli* (PDB ID: 1JYN; resolution = 1.80 Å) was retrieved from PDB. The protein and ligand PDBQT files were prepared using AutoDock Tools 1.5.6 (ADT) following the standard protocol.[6] A grid box (23.56 × 23.56 × 23.56 $Å^3$) defined for Pocket 1 (Table S1, entry A) of the chain B in β-galactosidase centered at the coordinates (x = -13.527, y = 21.714, z = -27.062) was used for docking into the active site with default settings: exhaustiveness = 64, energy range = 3 kcal/mol and number of modes = 20. The best-scored docking pose with the lowest binding

energy was selected for analysis and figures were visualized using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Polysulfide measurement using LC/MS:

Stock solutions of **1** (5 mM) and HPE-IAM (100 mM) were prepared in DMSO. Stock solution of Na_2S (20 mM) was prepared in DI water. Stock solution of β-galactosidase (10 U/mL from 50 U/mL stock solution in PBS pH 7.4). The reaction mixture was prepared by adding 50 μM of **1** (5 μL, 10 mM) independently with 200 μM of Na_2S (10 μL, 20 mM) along with (10 U/mL β-galactosidase (200 μL, 50 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 15 min at 37 ˚C on thermomixer (400 rpm). 100 μL aliquots of the reaction mixture were taken and then incubated with HPE-IAM (2 μL, 100 mM) for further 15 min. The reaction was quenched by adding 100 μL of acetonitrile. The samples were centrifuged at 10000g for 10 min at 37 ˚C, the supernatant was collected and assessed thereafter by LC/MS. All measurements were done using the following protocol: Acetonitrile (A) and 0.1% formic acid in water (B) was used as the mobile phase. A gradient was used with the flow rate of 0.3 mL/min starting with 100:0 → 0 min, 100:0 to 0:100 → 0 - 30 min. The MRM-HR mass spectrometry parameters for measuring compounds are: m/z precursor ion mass ($M + H^+$) 215.0740 (HS-HPE-AM), 389.1530 (Bis-S-HPE-AM), 421.1250 (Bis-SS-HPE-AM), 453.0971 (Bis-SSS-HPE-AM) and 485.0692 (Bis-SSSS-HPE-AM).

Cell culture protocol:

Human Embryonic Kidney (HEK) 293 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.

A549 cells were grown overnight with 5% CO_2 at 37°C in the modified Dulbecco's medium DMEM containing 3.7 gm/liter sodium bicarbonate, 110 mg/liter sodium pyruvate, antibiotics (100 mg/liter penicillin and streptomycin) and 10% fetal bovine serum (FBS). All mentioned chemicals and media are from Sigma Aldrich (St. Louis, USA) and FBS from ThermoFisher

Scientific. For trypsinization 0.05% trypsin-EDTA from ThermoFisher Scientific was used. For senescence induction, A549 cells were treated with 8 Gy of ionizing radiation (Blood irradiator BI 2000), which were allowed to adhere overnight and incubated for 96 h before further analysis.

Detection of HNO release in HEK-293 cell lysate:

HEK-293 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** (5 mM), **6** (5 mM) and **9** (1 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 of **6** and 1 μM of **1** with and with β-galactosidase (10 U/mL from 100 U/mL stock solution in PBS pH 7.4) in the presence of 1 μM of probe **9** in cell lysate (88 μL, 1 mg/mL) in 96-well plate and incubated at 37 ˚C for 4 h. The fluorescence corresponding to HNO release (excitation at 465 nm; emission at 520 nm) was measured for 4 h using an Ensight Multimode Plate Reader (PerkinElmer).

Cytotoxicity Assay:

Cellular viability was determined using the resazurin assay. Briefly, A549 cells were seeded onto a 96-well plate at an initial density of 1 x 10^4 cells/well and were allowed to adhere (5% CO_2, 37 ˚C) for 24 h. Next, cells were treated with **1** and incubated for 48 h. Further, cells were incubated with 100 μl of resazurin solution (0.5 mg/ml in PBS) for 4 h at 37 ˚C. Fluorescence of each well (excitation = 560 nm and emission = 590 nm) was measured using a microplate reader.

ROS estimation using DCFDA:

A549 cells were seeded in a 24-well plate at a seeding density of 50 x 10^4 cells. After attachment, the cells were treated with **1** (50 μM) for 48 h and then cells were washed with 1× PBS. Further, cells were incubated with 10 μM of 2,7- dichlorofluorescein diacetate (DCF-H_2DA) (Sigma Aldrich, USA) in DMEM for 40 min at 37 ˚C in the dark. In control experiment, **1** was co-incubated with the 10 μM of BCA (CSE inhibitor to inhibit H_2S production) for 48 h and followed by incubation with 10 μM of 2,7- dichlorofluorescein diacetate (DCF- H_2DA) in

DMEM for 40 min at 37 ˚C in the dark. Cells were washed three times with 1× PBS, and fluorescence enhancement was measured using Infinite Pro 2000 (Tecan, Austria) (excitation at 492 nm and emission at 525 nm). Cells were counted to express DCF fluorescence per cell.

4. Supplementary figures:

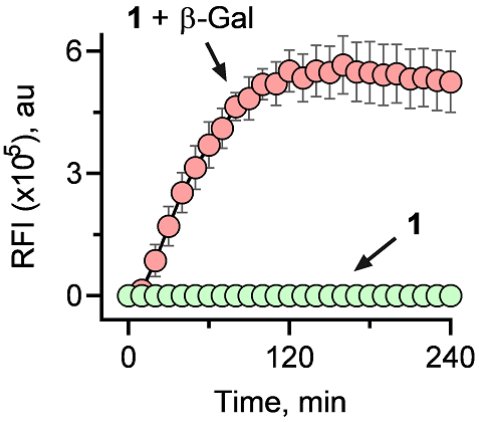


Figure S1. Time-dependent generation of HNO from **1** in the presence and absence of β-galactosidase (10 U/mL) in buffer (pH 7.4) for 4 h and the rate constant (pseudo first order kinetics) for HNO release with **1** was 0.016 min^{-1} (R^2 = 0.9855).

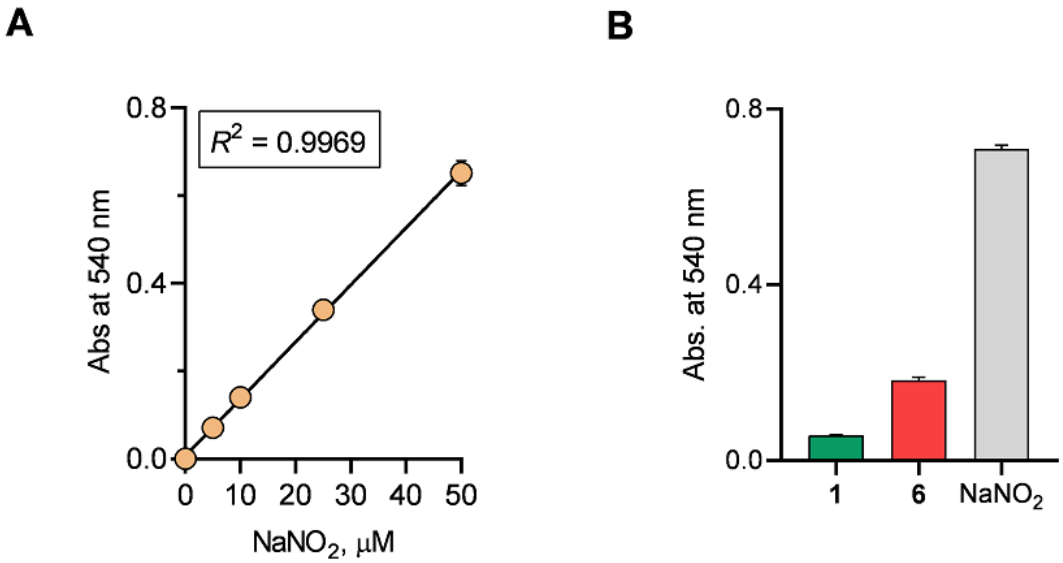


Figure S2: NO detection by Griess assay. (A) Calibration curve was generated using varying concentrations of $NaNO_2$. (B) **1** and **6** were incubated with β-galactosidase (10 U/mL) in buffer (pH 7.4) for 4 h at 37 ˚C. The NO yield was reported relative to the standard, $NaNO_2$.

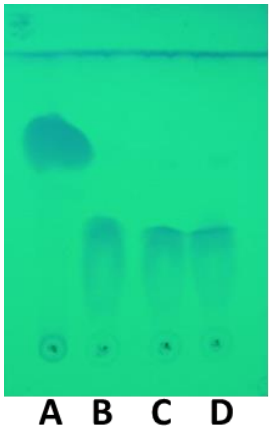


Figure S3: Formation of **7** was monitored by TLC. The solvent system used was 15% MeOH-EA. A) **1** alone; B) **1** with β-galactosidase; C) **7** alone; D) **6** alone.

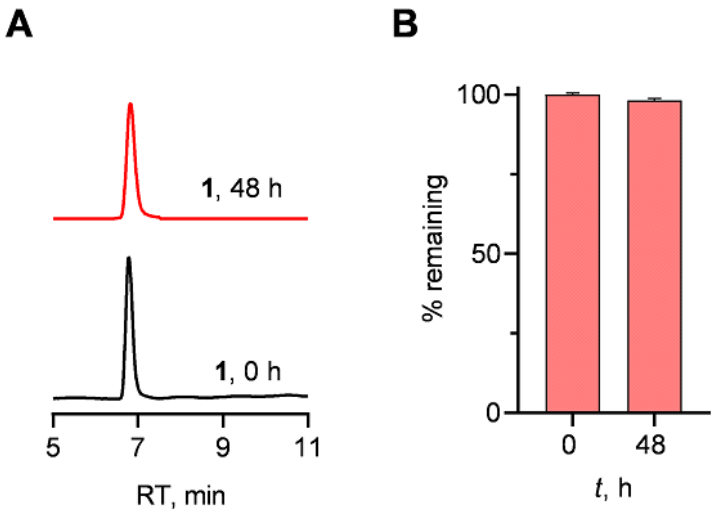


Figure S4. (A) HPLC traces of **1** (RT = 6.9 min); (B) AUC for stability of **1** in buffer (pH 7.4) for 48 h.

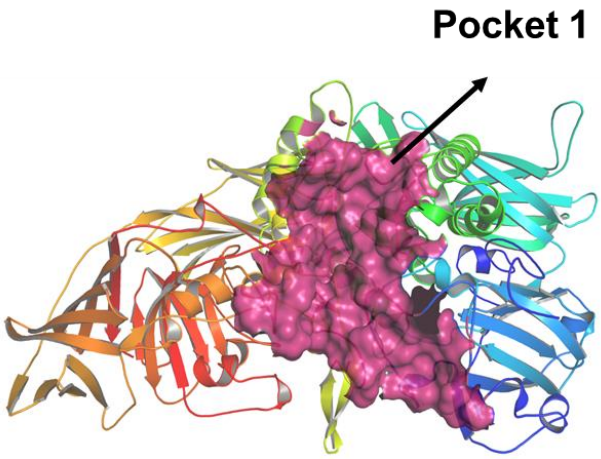


Figure S5: The ligand binding pocket of β-galactosidase (PDB: 1JYN from *E. coli*) was predicted using CASTp web server with cartoon representation of the protein and probe radius 1.4 Å. The pocket was shown as surface coloured as warm pink.

Table S1. CASTp based scoring of the identified pockets in β-galactosidase (PDB: 1JYN)

Entry	Pocket ID	Area (SA)	Volume (SA)
A	1	1173.97	1682.60
B	2	616.44	598.46
C	3	469.78	597.93
D	4	479.37	517.66
E	5	477.46	374.70

Table S2: Comparative analysis of the docking results of prodrugs **1**

Entry	Ligand/ Prodrug	Affinity (kcal/mol)	Distance from O (RCOOH) of Q537 (Å)*
1	Allolactose	-7.7	4.4
2	**1**	-8.1	3.7

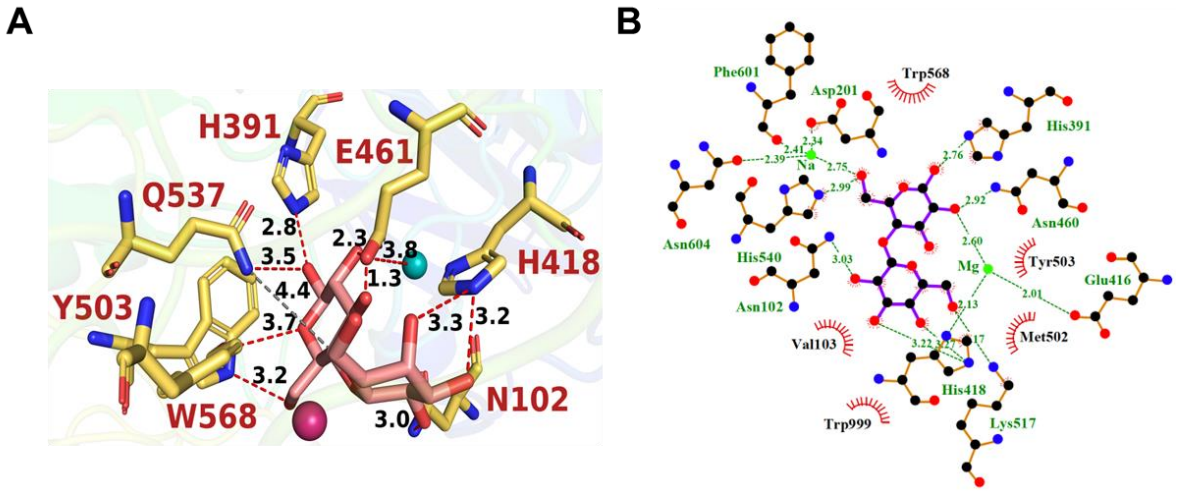


Figure S6: (A) Cartoon representation of docked allolactose into the active site of β-galactosidase from *E. coli* (PDB ID: 1JYN; resolution = 1.80 Å). The docked ligands were shown in stick model and the active site residues are indicated by 1-letter code. The hydrogen, hydrophobic and pi-pi interactions are drawn as blue, red and gray dotted lines, and the lengths are indicated. The figure was generated using PyMOL v 2.0. (B) LigPlot showing the 2D interactions of allolactose with β-galactosidase. The molecule is represented in stick model. Active site residues are labelled by 3-letter code and represented as ball and stick model. The residues forming hydrophobic interactions are shown as red arcs while the hydrogen bonds are shown as blue dashed lines with indicated bond lengths.[7]

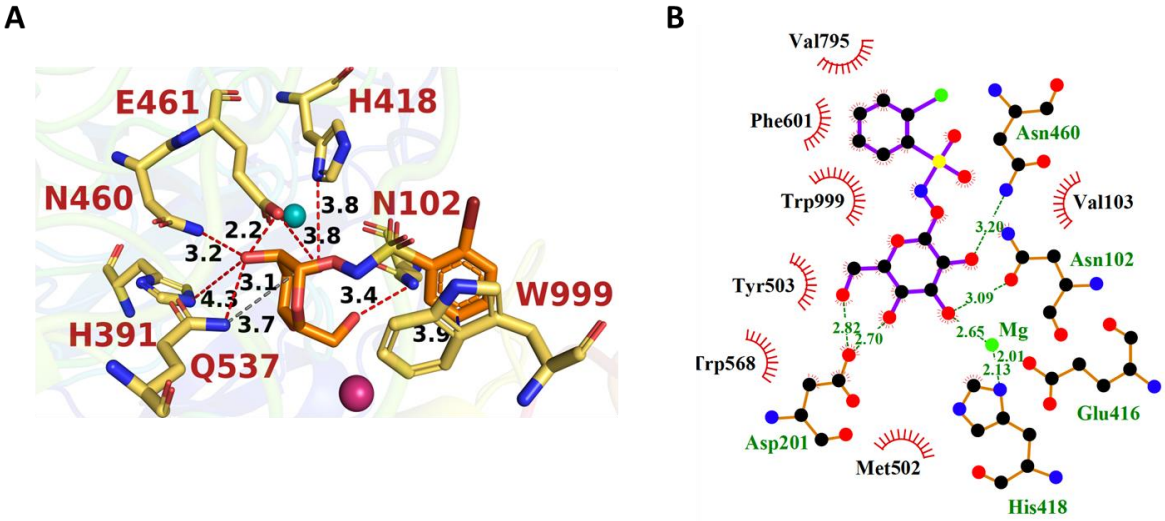
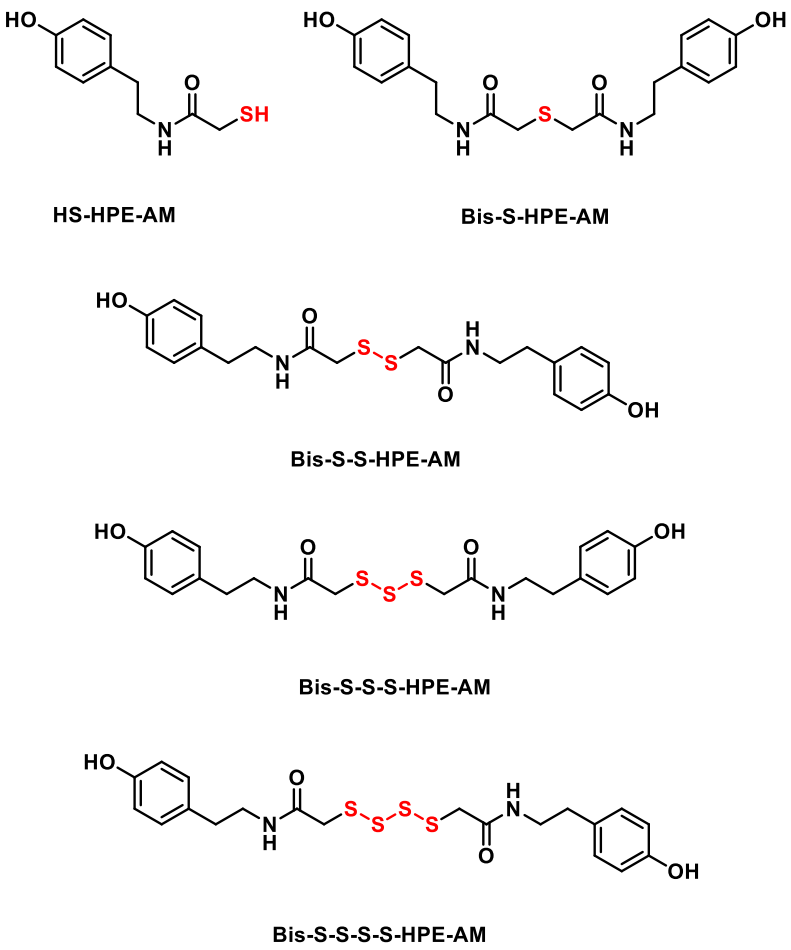


Figure S7: (A) Cartoon representation of docked compound **1** into the active site of β-galactosidase from *E. coli* (PDB ID: 1JYN; resolution = 1.80 Å). The docked ligands were shown in the stick model and the active site residues are indicated by 1-letter code. The

hydrogen, hydrophobic and pi-pi interactions are drawn as blue, red and gray dotted lines, and the lengths are indicated. The figure was generated using PyMOL v 2.0. (B) LigPlot shows the 2D interactions of **1** with β-galactosidase. The molecule is represented in a stick model. Active site residues are labelled by 3-letter code and represented as a ball and stick model. The residues forming hydrophobic interactions are shown as red arcs while the hydrogen bonds are shown as blue dashed lines with indicated bond lengths.



Scheme S4: Structures of sulfur species trapped by HPE-IAM probe

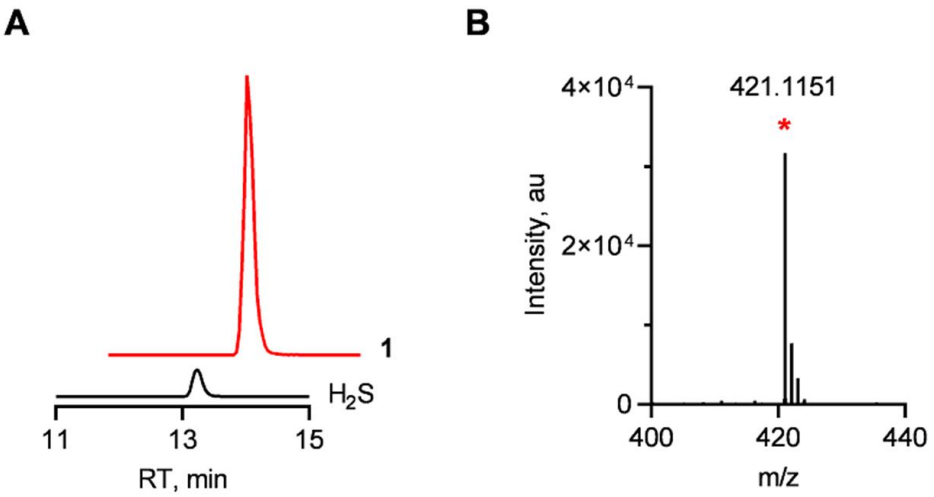


Figure S8: (A) Extracted ion chromatograms from an LC/MS analysis of Bis-SS-HPE-AM formation from Na_2S, **1** + Na_2S; (B) Mass spectra for the Bis-SS-HPE-AM (expected, m/z = 421.1250 $[M + H]^+$; observed, m/z = 421.1151).

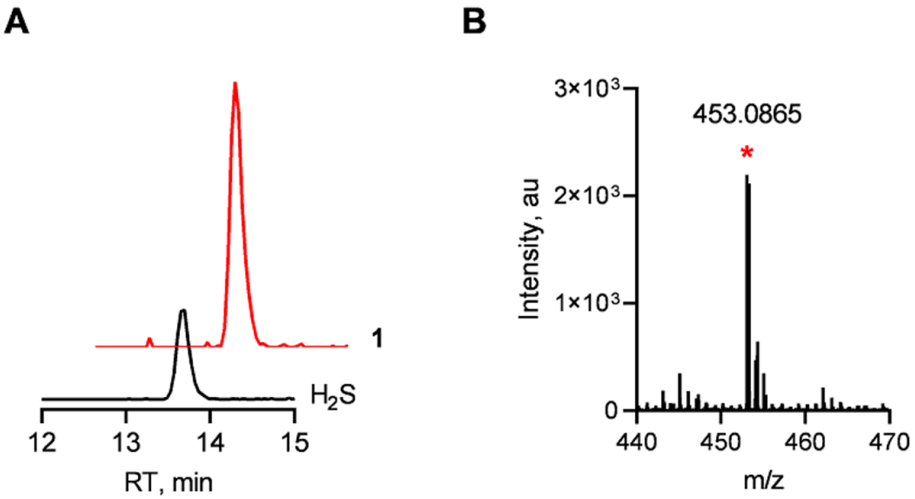


Figure S9: (A) Extracted ion chromatograms from an LC/MS analysis of Bis-SSS-HPE-AM formation from Na_2S, **1** + Na_2S; (B) Mass spectra for the Bis-SSS-HPE-AM (expected, m/z = 453.0971 $[M + H]^+$; observed, m/z = 453.0865).

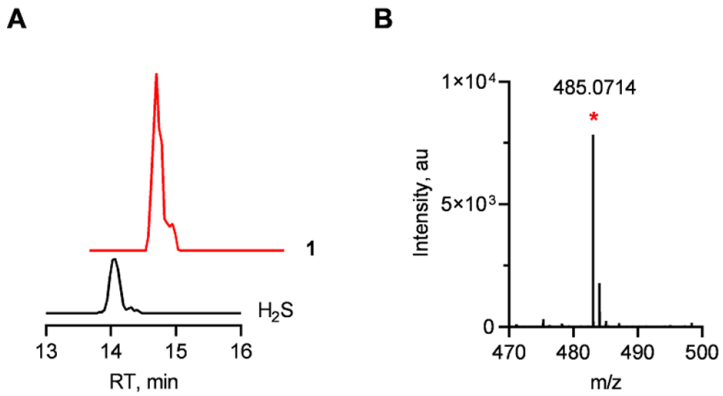


Figure S10: (A) Extracted ion chromatograms from an LC/MS analysis of Bis-SSSS-HPE-AM formation from Na_2S, **1** + Na_2S; (B) Mass spectra for the Bis-SSSS-HPE-AM (Expected, m/z = 485.0692 $[M + H]^+$; observed, m/z = 485.0714).

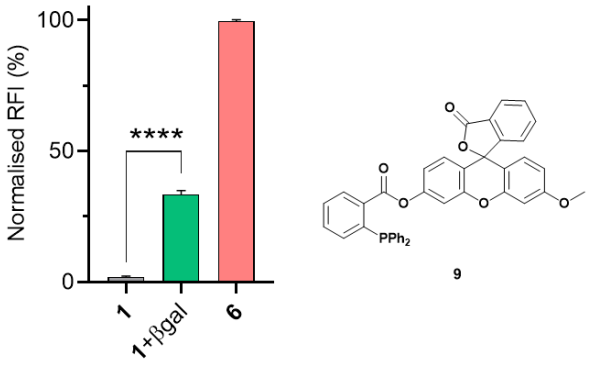


Figure S11: HNO production in human embryonic kidney (HEK) 293 cells lysate using HNO probe **9** (Pf dye). HEK-293 cell lysate (1 mg/mL) was incubated with 2-bromopiloty's acid (**6**) and compound **1** with and without β-galactosidase in the presence of probe **9** at 37 ˚C for 4 h.

5. References

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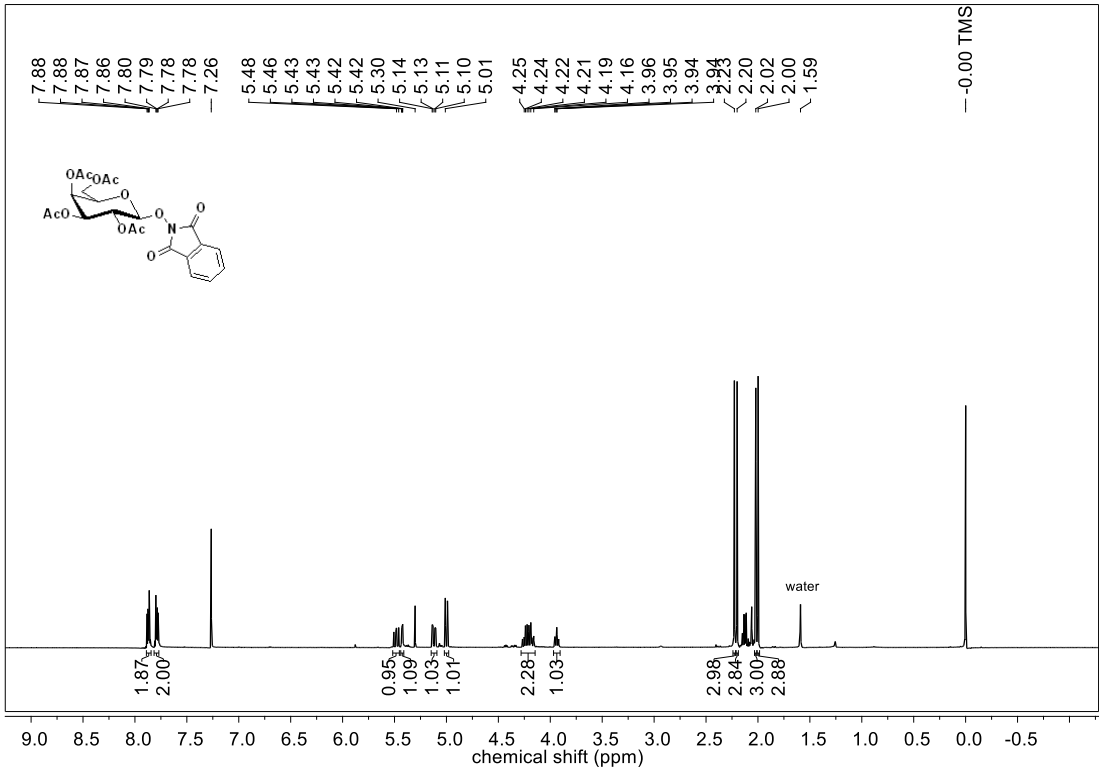
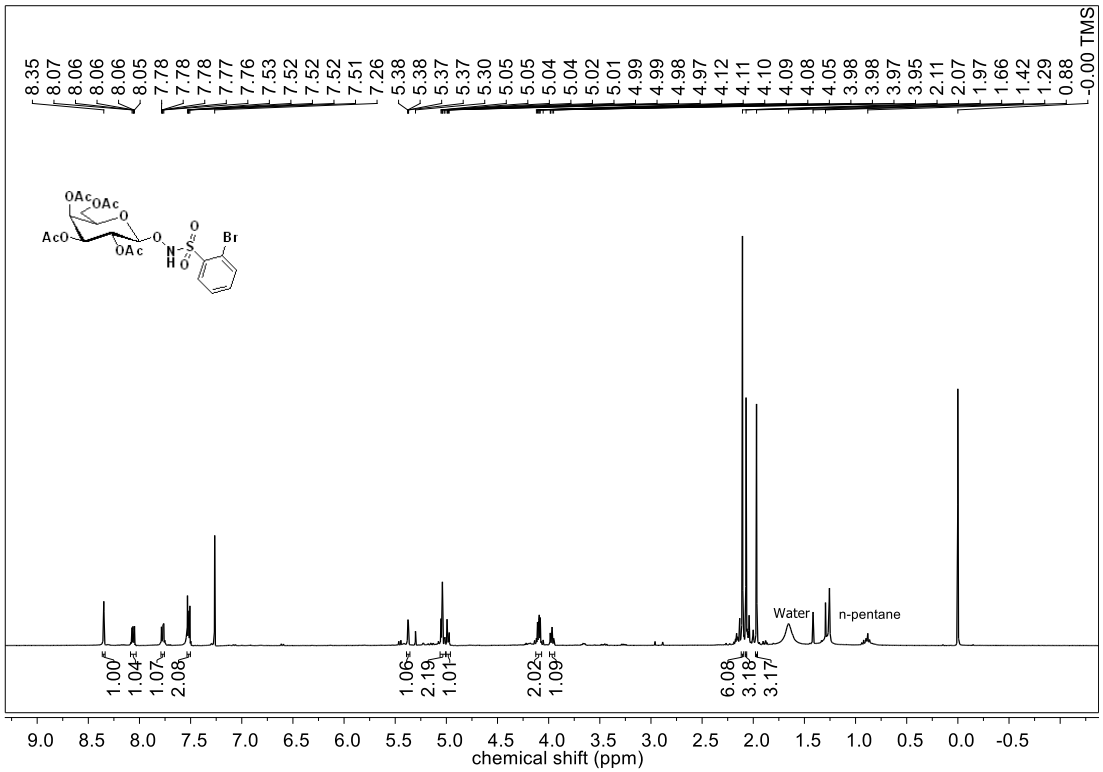
6. NMR spectra

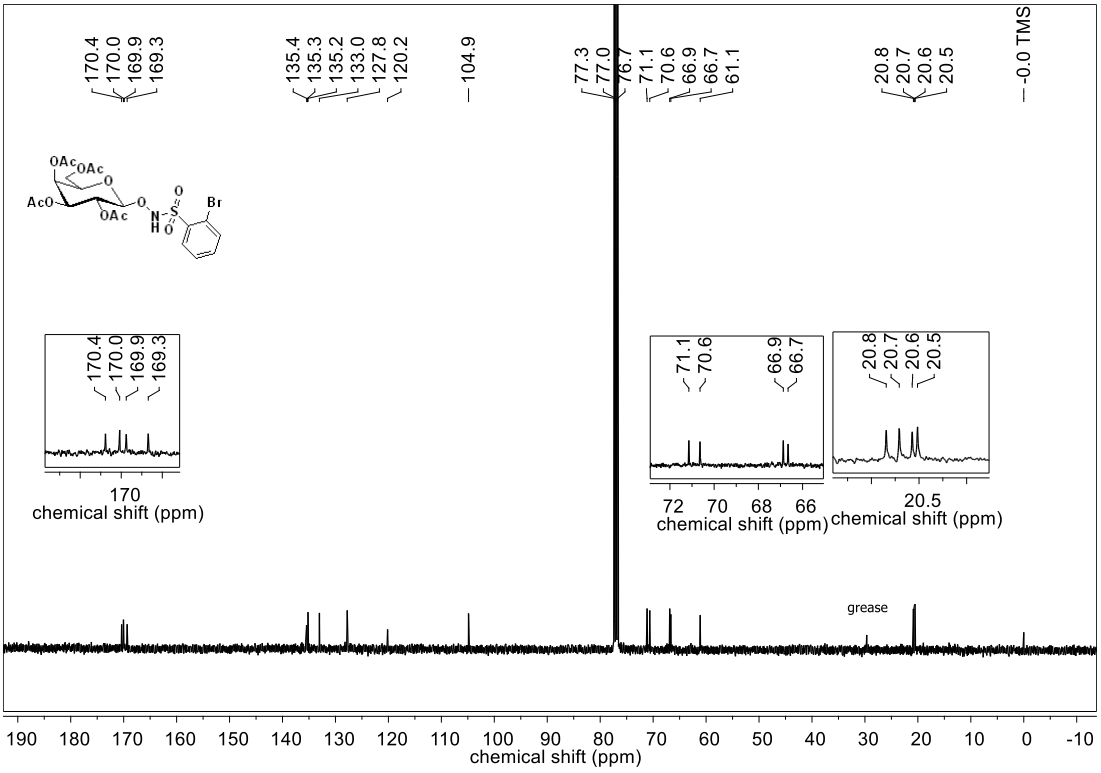
^{1}H NMR spectra for compound **3**



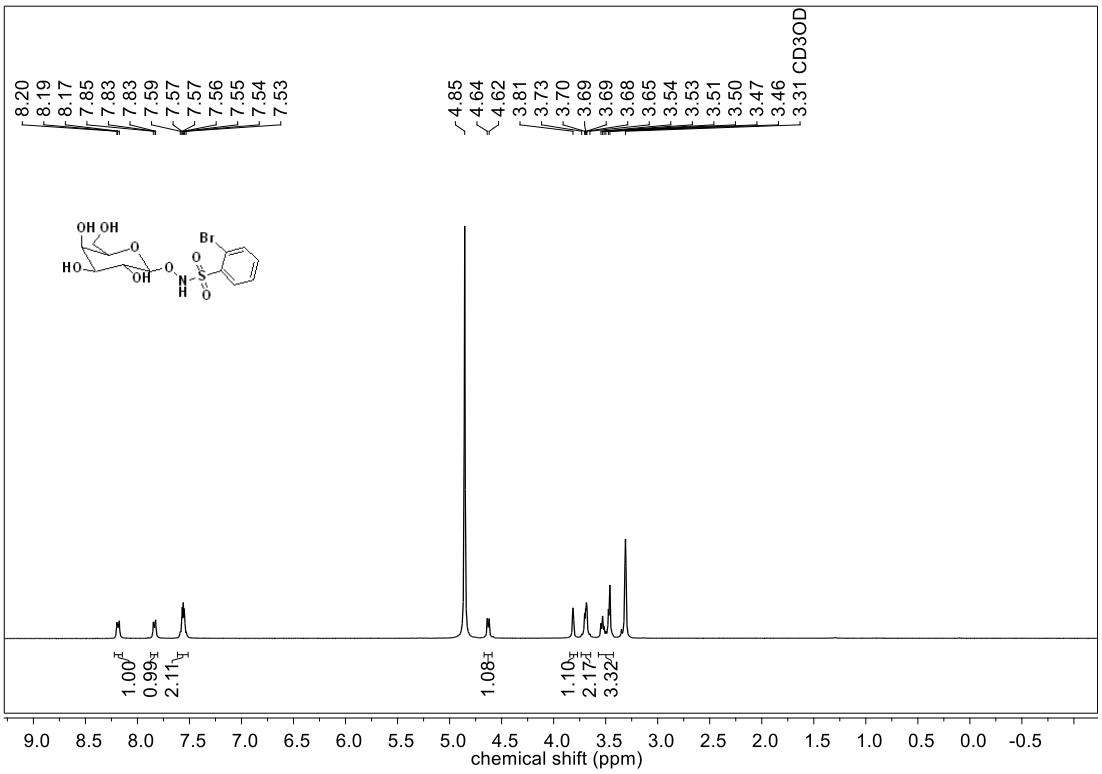
^{1}H NMR spectra for compound **5**



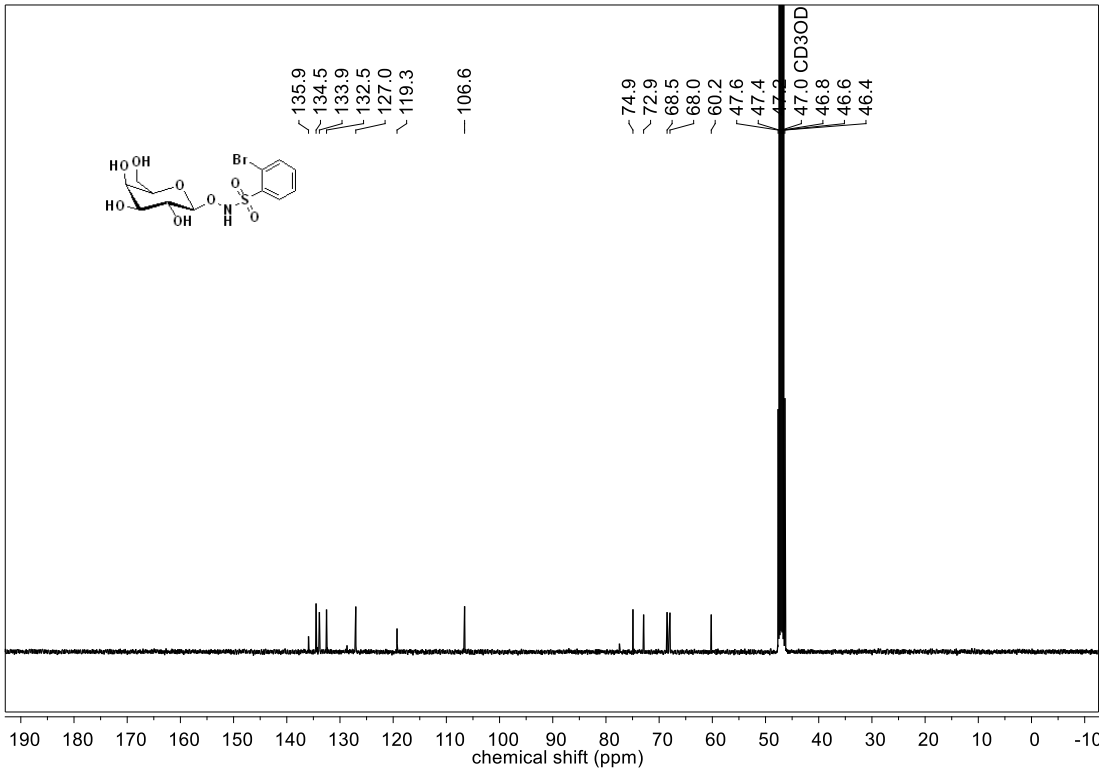
^{13}C NMR spectra for compound **5**



^{1}H NMR spectra for compound **1**



^{13}C NMR spectra for compound **1**



^{1}H NMR spectra for compound **7**