Electronic Supplementary Information (ESI)

for

Leveraging an Enzyme/ Artificial Substrate System to Enhance Cellular Persulfides and Mitigate Neuroinflammation

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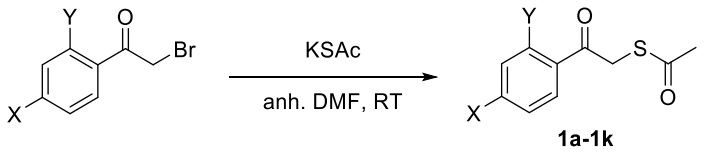
1. General methods

All reactions were conducted under nitrogen atmosphere. 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 Spectrochem (100-200 mesh)] as the stationary phase. ^{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 using tetramethylsilane (δH = 0.00 ppm, δC = 0.0 ppm) as an internal reference or residual solvent [$CDCl_3$ (δ_H= 7.26 ppm, δ_C = 77.2 ppm) or DMSO-d_6 (δ_H= 2.50 ppm, δ_C = 39.6 ppm)] signals as reference. ^{19}F spectra were recorded on a Bruker (376 MHz) spectrometer using α,α,α-trifluorotoluene (δ_F = -63.72 ppm) as an external reference. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The following abbreviations are used: m (multiplet), s (singlet), and t (triplet). 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. Photometric measurements were performed using a Thermo Scientific Varioskan Flash or PerkinElmer EnSight microtiter plate readers.

2. Synthesis and characterization

Compounds **1l**,[1] **2a**,[2] $(PhCOCH_2S)_2$ [disulfide formed by aerobic oxidation of **2a**],[2] **4**,[3] **6**,[4] methylsulfonyl benzothiazole (MSBT-A),[5] CN-BOT,[6] MGR-1[7] were synthesized as per reported protocols and their analytical data were consistent with literature values.

Table S1: Synthesis of ethanethioate derivatives



1a-1k

Entry	Product	X	Y	Yield, %
1	**1a**	H	H	78
2	**1b**	NO_2	H	86
3	**1c**	CN	H	79
4	**1d**	CF_3	H	77
5	**1e**	OCF_3	H	73
6	**1f**	F	H	83
7	**1g**	Me	H	77
8	**1h**	OMe	H	81
9	**1i**	H	F	85
10	**1j**	H	OMe	88
11	**1k**	H	Me	80

General protocol for synthesis of ethanethioate derivatives (1a-1k): From the derivatized phenacyl bromides, the corresponding *S*-ethanethioates were synthesized. Phenacyl bromide (1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in dry *N,N*-Dimethylformamide (DMF, 10 mL), followed by the addition of potassium thioacetate (KSAc) (2 eq). The reaction mixture was stirred at room temperature (RT). Upon completion of the reaction (TLC analysis), the reaction was quenched with 20 mL of water and transferred to a separating funnel where the crude product was extracted with ethyl acetate (30 mL). The combined organic layers were washed with water (3 × 30 mL) and brine (30 mL) and the resulting organic layer was dried over anhydrous Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to afford the crude product, which was further purified by silica gel column 60-120 mesh silica gel chromatography, unless otherwise specified.

***S*-(2-oxo-2-phenylethyl) ethanethioate (1a):** Following the above general synthetic protocol, 2-bromo-1-phenylethan-1-one (300 mg, 1.50 mmol) was used as starting material. A gradient starting from 1-17% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 15-16% as the eluant. Compound **1a** was obtained as a light brown solid (230 mg, 78%). All analytical data are consistent with reported values.[8]

***S*-(2-(4-Nitrophenyl)-2-oxoethyl) ethanethioate (1b):** Following the above general synthetic protocol, 2-bromo-1-(4-nitrophenyl)ethan-1-one (280 mg, 1.15 mmol) was used as starting material. A gradient starting from 1-17% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 15-17% as the eluant. Compound **1b** was obtained as a brown solid (85 mg, 31%). FT-IR (υ_{max}, cm^{-1}): 1669; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35-8.32 (m, 2H), 8.17-8.13 (m, 2H), 4.38 (s, 2H), 2.42 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.0, 192.3, 150.7, 140.2, 129.7, 124.1, 36.7, 30.3. HRMS for $C_{11}H_9NO_2S$ $[M+K]^+$ Calculated: 277.9889, Found: 277.1816.

***S*-(2-(4-Cyanophenyl)-2-oxoethyl) ethanethioate (1c):** Following the above general synthetic protocol, 4-(2-bromoacetyl)benzonitrile (500 mg, 2.23 mmol) was used as starting material. 100-200 mesh silica gel was used. A gradient starting from 1-17% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 15-17% as the eluant. Compound **1c** was obtained as a pale-brown solid (388 mg, 79%). FT-IR (υ_{max}, cm^{-1}): 2227, 1675; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09-8.06 (m, 2H), 7.80-7.77 (m, 2H), 4.35 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.0, 192.4, 138.7, 132.8, 129.0,

117.9, 117.1, 36.5, 30.3; HRMS for $C_{11}H_9NO_2S$ $[M+H]^+$ Calculated: 220.0428, Found: 220.0432.

S-(2-oxo-2-(4-(trifluoromethyl)phenyl)ethyl) ethanethioate (1d): Following the above general synthetic protocol, 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (300 mg, 1.12 mmol) was used as starting material. A gradient starting from 1-7% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 6-7% as the eluant. Compound **1d** was obtained as a white solid (227 mg, 77%). FT-IR (υ_{max}, cm^{-1}): 1699, 1686; ^1H NMR (400 MHz, $CDCl_3$): δ 8.11 (d, 2H, J = 8.16 Hz), 7.75 (d, 2H, J = 8.16 Hz), 4.38 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.2, 192.7, 138.4, 135.6 (q, J = 32.7 Hz), 129.0, 126.0 (q, J = 3.7 Hz), 123.5 (q, $J_{C,F}$ = 270.5 Hz), 36.7, 30.3; ^{19}F NMR (376 MHz, $CDCl_3$): δ -63.2 (s); HRMS for $C_{11}H_{10}F_3O_2S$ $[M+H]^+$ Calculated: 263.0354, Found: 263.1311.

S-(2-oxo-2-(4-(trifluoromethoxy)phenyl)ethyl) ethanethioate (1e): Following the above general synthetic protocol, 2-bromo-1-(4-(trifluoromethoxy)phenyl)ethan-1-one (303 mg, 1.07 mmol) was used as starting material. A gradient starting from 1-10% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 8-10% as the eluant. Compound **1e** was obtained as an off-white solid (218 mg, 73%). FT-IR (υ_{max}, cm^{-1}): 1687; ^1H NMR (400 MHz, $CDCl_3$): δ 8.05 (m, 2H), 7.30 (m, 2H), 4.36 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.2, 192.1, 153.2, 133.8, 130.8, 120.6, 120.3 (q, J = 257.4 Hz), 36.5, 30.3; ^{19}F NMR (376 MHz, $CDCl_3$): δ -57.6 (s); HRMS for $C_{11}H_9NaF_3O_3S$ $[M+Na]^+$ Calculated: 301.0122, Found: 301.0100.

***S*-(2-(4-fluorophenyl)-2-oxoethyl) ethanethioate (1f):** Following the above general synthetic protocol, 2-bromo-1-(4-fluorophenyl)ethan-1-one (270 mg, 1.27 mmol) was used as starting material. A gradient starting from 1-8% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 6-8% as the eluant. Compound **1f** was obtained as a brown solid (220 mg, 83%). All analytical data are consistent with reported values.[9]

***S*-(2-Oxo-2-(*p*-tolyl)ethyl) ethanethioate (1g):** Following the above general synthetic protocol, 2-bromo-1-(*p*-tolyl)ethan-1-one (450 mg, 2.11 mmol) was used as starting material. A gradient starting from 1-17% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 15-17% as the eluant. Compound **1g** was obtained as a pale-yellow solid (330 mg, 75%). FT-IR (υ_{max}, cm^{-1}): 1681; ^1H NMR (400 MHz, $CDCl_3$): δ 7.90-7.87 (m, 2H), 7.28-7.26 (m, 2H), 4.38 (s, 2H), 2.41 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (100 MHz,

$CDCl_3$): δ 194.4, 193.0, 144.8, 133.2, 129.6, 128.8, 36.7, 30.4, 21.8; HRMS for $C_{11}H_{12}O_2S$ $[M+Na]^+$ Calculated: 231.0457, Found: 231.0458.

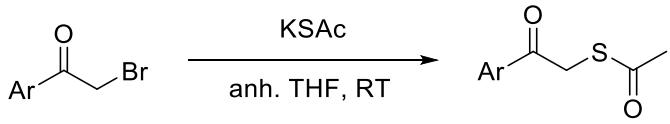
***S*-(2-(4-methoxyphenyl)-2-oxoethyl) ethanethioate (1h):** Following the above general synthetic protocol, 2-bromo-1-(4-methoxyphenyl)ethan-1-one (250 mg, 1.57 mmol) was used as starting material. A gradient starting from 1-11% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 9-11% as the eluant. Compound **1h** was obtained as light brown solid (250 mg, 88%). All analytical data are consistent with reported values.[9]

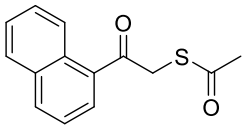
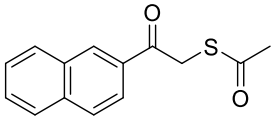
***S*-(2-(2-fluorophenyl)-2-oxoethyl) ethanethioate (1i):** Following the above general synthetic protocol, 2-bromo-1-(2-fluorophenyl)ethan-1-one (270 mg, 1.24 mmol) was used as starting material. A gradient starting from 1-8% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 6-8% as the eluant. Compound **1i** was obtained as a yellow oil (224 mg, 85%). All analytical data are consistent with reported values.[10]

***S*-(2-(2-methoxyphenyl)-2-oxoethyl) ethanethioate (1j):** Following the above general synthetic protocol, 2-bromo-1-(2-methoxyphenyl)ethan-1-one (295 mg, 1.29 mmol) was used as starting material. A gradient starting from 1-11% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 9-11% as the eluant. Compound **1j** was obtained as light brown solid (250 mg, 88%). All analytical data are consistent with reported values.[11]

***S*-(2-oxo-2-(o-tolyl)ethyl) ethanethioate (1k):** Following the above general synthetic protocol, 2-bromo-1-(o-tolyl)ethan-1-one (330 mg, 1.55 mmol) was used as starting material. A gradient starting from 1-8% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 6-8% as the eluant. Compound **1k** was obtained as a yellow oil (258 mg, 80%). FT-IR (υ_{max}, cm^{-1}): 1686; ^1H NMR (400 MHz, $CDCl_3$): δ 7.73 (m, 2H), 7.40 (m, 2H), 7.27 (m, 2H), 4.30 (s, 2H), 2.49 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.7, 194.4, 139.0, 132.2, 132.1, 128.9, 125.9, 39.2, 30.2, 21.4; HRMS for $C_{11}H_{13}O_2S$ $[M+H]^+$ Calculated: 209.0636, Found: 209.0595.

Table S2: Synthesis of naphthyl derivatives of ethanethioates

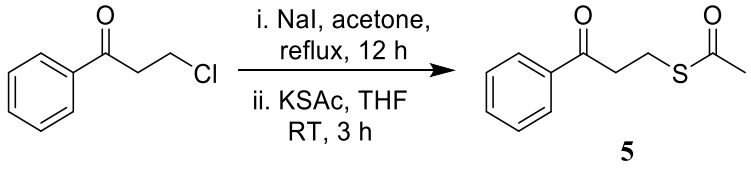


Entry	Ar	Product	Yield, %
1	1-Naphthyl	 **1m**	73
2	2-Naphthyl	 **1n**	96

General Synthesis of 1m and 1n: Bromoacetylnaphthalene (1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL dry tetrahydrofuran (THF), followed by the addition of KSAc (3 eq). The reaction mixture was stirred at RT for 3 h. Upon completion of the reaction (TLC analysis), the reaction mixture was quenched with 20 mL of H_2O and transferred to a separating funnel, where the product was extracted with ethyl acetate (2 × 15 mL). The combined organic layers were washed with brine (30 mL), dried over anhydrous Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to afford the crude product which was further purified by silica gel column chromatography using 60-120 mesh silica gel as the stationary phase.

***S*-(2-(Naphthalen-1-yl)-2-oxoethyl) ethanethioate (1m):** Following the above general synthetic protocol, 1-(bromoacetyl)naphthalene (500 mg, 2.01 mmol) was used as starting material. A gradient starting from 1-12% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 10-12% as the eluant. Compound **1m** was obtained as a reddish-yellow oil (360 mg, 73%). FT-IR (υ_{max}, cm^{-1}): 1681; ^1H NMR (400 MHz, $CDCl_3$): δ 8.57-8.55 (m, 1H), 8.03-8.00 (m, 2H), 7.89-7.87 (m, 1H), 7.63-7.50 (m, 3H), 4.45 (s, 2H), 2.42 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.9, 194.5, 134.3, 134.1, 133.5, 130.4, 128.6, 128.4, 128.3, 126.8, 125.8, 124.4, 39.6, 30.4; HRMS for $C_{14}H_{12}O_2S$ $[M+H]^+$ Calculated: 245.0636, Found: 245.0637.

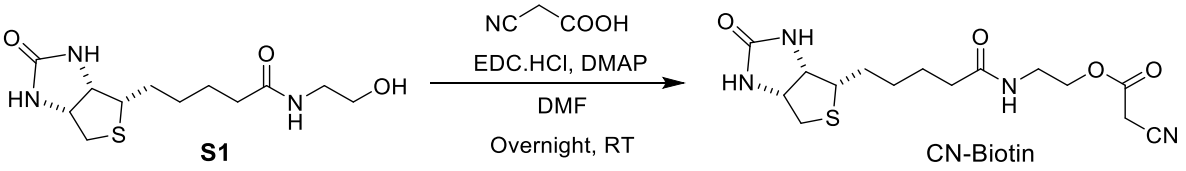
***S*-(2-(Naphthalen-2-yl)-2-oxoethyl) ethanethioate (1n):** Following the above general synthetic protocol, 2-(bromoacetyl)naphthalene (200 mg, 0.80 mmol) was used as the starting material. A gradient starting from 1-12% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 10-12% as the eluant. Compound **1n** was obtained as brown solid (190 mg, 96%). FT-IR (υ_{max}, cm^{-1}): 1678; ^1H NMR (400 MHz, $CDCl_3$): δ 8.55-8.54 (m, 1H), 8.05-7.98 (m, 2H), 7.92-7.88 (m, 2H), 7.65-7.55 (m, 2H), 4.55 (s, 2H), 2.43 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.5, 193.4, 136.0, 133.0, 132.6, 130.6, 129.9, 129.0, 128.8, 128.0, 127.1, 124.1, 36.9, 30.4; HRMS for $C_{14}H_{12}O_2S$ $[M+Na]^+$ Calculated: 267.0455, Found: 267.0463.



5

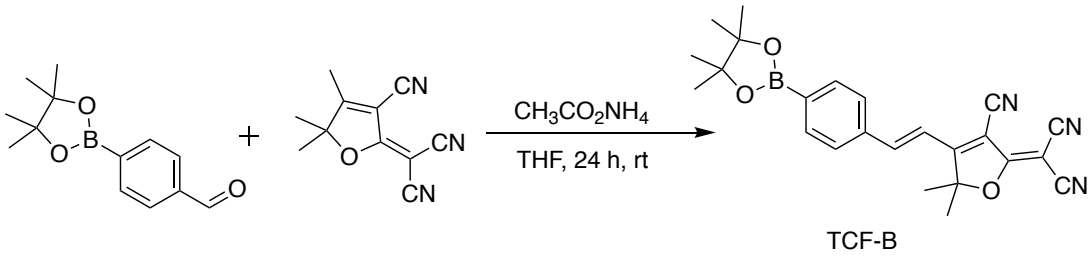
Scheme S1: Synthesis of *S*-(3-Oxo-3-phenylpropyl) ethanethioate **(5)**

***S*-(3-Oxo-3-phenylpropyl) ethanethioate (5):** From 3-chloropropiophenone, 3-iodo-1-phenylpropan-1-one was synthesized using a reported protocol.[12] 3-iodo-1-phenylpropan-1-one (920 mg; 3.56 mmol) was taken in a round-bottom flask, purged with nitrogen and dissolved in 15 mL dry THF. Potassium thioacetate (1.21 g; 10.61 mmol) was then added and the reaction mixture was again purged with nitrogen. The reaction was stirred at RT for 3 h. Upon completion of the reaction (TLC analysis), the reaction was quenched with 30 mL of water. The product was extracted in ethyl acetate (2 × 20 mL). The combined organic layers were washed with brine (30 mL), dried over anhydrous Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to afford the crude product, which was further purified by column chromatography using 100-200 mesh silica gel as the stationary phase. A gradient starting from 1-8% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 6-8% as the eluant. Compound **5** was obtained as a brown solid (550 mg, 75%): FT-IR (υ_{max}, cm^{-1}): 1675; ^1H NMR (400 MHz, $CDCl_3$): δ 7.97-7.95 (m, 2H), 7.60-7.56 (m, 1H), 7.49-7.45 (m, 2H), 3.33 (m, 2H), 3.25 (m, 2H), 2.34 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 198.1, 196.4, 136.5, 133.5, 128.8, 128.2, 38.8, 30.7, 23.7; HRMS for $C_{11}H_{12}O_2S$ $[M+Na]^+$ Calculated: 231.0455, Found: 231.0448.



Scheme S2: Synthetic scheme for CN-Biotin

CN-Biotin: The intermediate **S1** was prepared using a reported protocol.[13] This compound (300 mg, 1.04 mmol) was taken in dry DMF (20 mL). Cyanoacetic acid (266 mg 3.13 mmol,). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC.HCl) (640 mg, 4.2 mmol) was then added followed by DMAP (64 mg, 0.32 mmol) and reaction mixture was stirred overnight at RT. Upon completion of the reaction (TLC analysis), the solvent was removed and concentrated under reduced pressure to afford the crude product which was further purified by column chromatography using 60-120 mesh silica gel as the stationary phase. A gradient starting from 1-8% methanol/dichloromethane was used as the mobile phase and the desired product was obtained with 6-8% as the eluant. CN-Biotin was obtained as a white solid (120 mg, 32%). All analytical data are consistent with reported values.[13]



Scheme S3: Synthetic scheme for TCF-B

(*E*)-2-(3-Cyano-5,5-dimethyl-4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)furan-2(5*H*)-ylidene) malononitrile (TCF-B): TCF[14] and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde[15] were prepared as per previously reported protocol. To a solution of 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (175 mg, 0.75 mmol) in anhydrous THF was added TCF (100 mg, 0.5 mmol) followed by ammonium acetate (58 mg, 0.75 mmol). The resulting mixture was stirred at RT for 24 h. Upon completion of the reaction (TLC analysis), the reaction was quenched by addition of water (3 mL). The product was extracted using ethyl acetate (3 × 50 mL). The combined organic layers were washed with brine (30 mL), dried over anhydrous Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure to afford the crude product. The crude was purified by reverse phase HPLC

using ACN-H_2O as the eluent to afford the desired product TCF-B (35 mg, 20% yield) as a red powder. All analytical data are consistent with reported values.[16]

3. Experimental protocols

Docking methods for *h*3-MST:

Autodock vina (V. 1.1.2) software was used to perform docking of compounds (**3-MP**, **E3-MP**, **2a-2n**) to the active site of ***h*3-MST** (Protein data bank (PDB) file: **4JGT**). Molecules were converted to PDB from SMILES and then from PDB to PDBQT format (as needed for docking), using OpenBabel (v 3.1.0). Docking was performed using an exhaustiveness parameter of 40, and keeping all other parameters to the vina default. The parameters defining the docking site are:

Box Coordinates: Centre**:** (127, -35.318, 5.183) which is nearest (<1.6 Å) to the CG side-chain carbon of the ARG-184 residue of **4JGT**.

Box Dimensions (Å): 22 × 24 × 22.5

Cloning of wild-type (wt) *Escherichia coli* 3-MST (wt *b*3-MST), and *Escherichia coli* 3-MST C238A (*b*3-MST C238A) mutant and wt *Homo sapiens* 3-MST (wt *h*3-MST):

Table S3: Primers used for cloning of wt *b*3-MST, *b*3-MST C238A mutant and wt *h*3-MST

S. No.	Primer name	Plasmid constructed	Primer Sequence (5′ → 3′)
1	EcMST_PCR-1_F	wt *Ec*3-MST	ATGTCCACGACATGGTTTGTAGGAGCCGAC
2	EcMST_PCR-1_R	wt *Ec*3-MST	TTATTTCACTGGCTCAACCGGTAAATCTGC
3	EcMST_pET28a_F	wt *Ec*3-MST	GTGCCGCGCGGCAGCCATATGTCCACGACATGGTTTGTAGGAGCCGAC
4	EcMST_pET28a_R	wt *Ec*3-MST	CGACGGAGCTCGAATTCGGATCCTTATTTCACTGGCTCAACCGGTAAATCTGC
5	EcMSTC238A_F	*Ec*3-MST C238A	AAACCAATTATCGTCAGCGCGGGCTCTGGTGTAACGGCA

6	T7 Terminator_R	*Ec*3-MST C238A	GCTAGTTATTGCTCAGCGG
7	HsMPST_PCR1_FP	*Hs*3-MST	ATGGCTTCGCCGCAGCTCTG
8	HsMPST_PCR1_RP	*Hs*3-MST	TCAGTGGGTCTTCCCCCGGC
9	HsMPST_PCR2_FPnew	*Hs*3-MST	AGCCATATGGCTAGCATGGCTTC GCCGCAGCTCTG
10	HsMPST_PCR2_RPnew	*Hs*3-MST	AGCTCGAATTCGGATCCTCAGTG GGTCTTCCCCCGGC

All the primers used for cloning the 3-MST genes are listed in **Table S3**. The 3-MST gene was amplified from the genomic DNA of *E. coli* strain K-12 substrain MG1655 using gene-specific primers, and were each cloned in pET28a vector using restriction-free cloning strategy.[17,18]

For creating the *b*3-MST C238A mutant, a forward primer containing the altered sequence was used along with the T7 reverse primer to amplify the mutated part of the gene from pET28a containing the wt *Ec*3-MST. This amplicon was then used to synthesize the *b*3-MST C238A mutant in the pET28a vector with wt *b*3-MST using restriction-free cloning as previously described.

The plasmid pcDNA3.1+/C-(K)DYK containing wild-type human 3-MST gene (wt *Hs*3-MST) was obtained from Genscript via BiotechDesk (Genscript accession IDs are: Clone ID: OHu09558, RefSeq Accession NM_001130517.2). The gene was then subcloned into the pET28a vector using digestion by NdeI and BamHI enzymes at 37 ºC for 2 h, followed by PCR purification, and ligation using T4 DNA ligase at room temperature for 5 h. All three recombinant plasmids were further transformed into *E. coli* DH5-α cells, selected for kanamycin resistance and the clones were confirmed by sequencing using vector-specific T7 primers.

General protocols for protein expression and purification protocols for wt *b*3-MST, *b*3-MST C238A and wt *h*3-MST:

All three pET28a constructs of 3-MST were transformed into *E. coli* BL21 (DE3) cells for overexpression of the respective proteins. Overnight primary cultures of *E. coli* BL21 (DE3) cells containing the respective 3-MST pET28a plasmids were grown in Luria-Bertani (LB) medium with kanamycin (25 μg/mL) at 37 °C, 180 rpm. These cultures were transferred into large secondary cultures of LB-Kan medium at 1% v/v inoculum and were grown at 37 °C, 180 rpm to an OD_{600} of 0.6. Protein overexpression was achieved with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), following which the cultures were incubated at 18 °C for 18 h at 180 rpm. The cells were harvested from the large cultures by centrifugation at 6500 rpm, 4 °C and the pellets were flash frozen in liquid nitrogen and stored at -80 °C till further use.

The cell pellets obtained were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0) containing 300 mM NaCl, 10 mM $MgCl_2$, 0.05 mM phenylmethylsulfonyl fluoride and 0.025% β-mercaptoethanol (BME) and were lysed by sonication. The cell lysate was loaded onto a Ni-NTA column pre-incubated with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025% BME and 10 mM imidazole. The column was then washed with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025% BME and 50 mM imidazole, and the proteins were eluted in 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025% BME and 250 mM imidazole. The eluted protein fractions were run in sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to check purity (**Fig S1**). The purified proteins were buffer-exchanged in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 0.025% BME using Econo-Pac 10DG prepacked desalting columns (Bio-Rad), and were stored with 15% v/v glycerol in -80 °C.

HPLC based studies for wt *h*3-MST and *b*3-MST activity:

a) **General method:**

A stock solution of the substrate (10 mM) was prepared in DMSO whereas the stocks of DTT (100 mM), GSH (100 mM), and porcine liver esterase (100 U/mL) were prepared fresh in deionised water. The reaction mixtures (total volume 1000 μL) were prepared in 200 mM HEPES-NaOH pH 7.4 buffer as required. Typically, 100 μM of the substrate, 25 mM DTT, 25 mM GSH, 1 U/mL esterase, or 0.5 μM of wt *b*3-MST or h3-MST were used. Final concentration of DMSO in the reaction mixture was 2%. The reaction mixture was filtered through a 0.22 μm syringe filter, mixed, and then

incubated at 37 °C. At predetermined time points, aliquots were taken from the reaction mixture, and injected (25 µL) in a high performance liquid chromatography (HPLC) attached with a UV detector (absorbance at 250 nm). A Phenomenex Luna C-18 reverse phase column, 100 Å particle size and 5 µM pore size (250 × 4.6 mm) was used as the stationary phase. The mobile phase used was water: acetonitrile with a gradient: 40:60 → 0 min, 40:60 to 30:70 → 0 - 5 min, 30:70 to 20:80 → 5 - 10 min, 20:80 → 10 - 13 min, 20:80 to 40:60 → 13 - 15 min, 40:60 → 15 - 18 min and the flow rate was 1 mL/min.

b) **Sub-stoichiometric reaction of 1a with *h*3-MST and its turnover to 3a in the presence of porcine liver esterase:** 10 mM stock solution of **1a** was prepared in DMSO. The reaction mixture contained 390 µL of 200 mM HEPES-NaOH pH 7.4 buffer, 100 µM of **1a** (5 µL, 10 mM stock), 1 U/mL esterase (5 µL, 100 U/mL stock), 10 µM *h*3-MST (100 µL, 50 µM stock). At predetermined time points, aliquots were taken from the reaction mixture, and injected in the HPLC. The data from this experiment can be found in Figure 2C, S2.

c) **Sub-stoichiometric reaction of 1a with *h*3-MST and its turnover to 3a in the absence of porcine liver esterase:** 10 mM stock solution of **1a** was prepared in DMSO. The reaction mixture contained 395 µL of 200 mM HEPES-NaOH pH 7.4 buffer, 100 µM of **1a** (5 µL, 10 mM stock), 10 µM *h*3-MST (100 µL, 50 µM stock). At predetermined time points, aliquots were taken from the reaction mixture, and injected in the HPLC. The data from this experiment can be found in Figure S24.

d) **Sub-stoichiometric reaction of 1a with *b*3-MST and its turnover to 3a in the presence of porcine liver esterase:** 10 mM stock solution of **1a** was prepared in DMSO. The reaction mixture contained 156 µL of 200 mM HEPES-NaOH pH 7.4 buffer, 100 µM of **1a** (2 µL, 10 mM stock), 1 U/mL esterase (2 µL, 100 U/mL stock) 10 µM *b*3-MST (40 µL, 50 µM stock). At predetermined time points, aliquots were taken from the reaction mixture, and injected in the HPLC. The data from this experiment can be found in Figure S3.

e) **Sub-stoichiometric reaction of 1a with *b*3-MST and its turnover to 3a in the absence of porcine liver esterase:** 10 mM stock solution of **1a** was prepared in DMSO. The reaction mixture contained 158 µL of 200 mM HEPES-NaOH pH 7.4 buffer, 100

μM of **1a** (2 μL, 10 mM stock), 10 μM *b*3-MST (40 μL, 50 μM stock). At predetermined time points, aliquots were taken from the reaction mixture, and injected in the HPLC. The data from this experiment can be found in Figure S25.

f) Cleavage of 1a in presence of DTT, GSH and porcine liver esterase:

10 mM stock solution of **1a** was prepared in DMSO. The reaction mixture contained 200 mM HEPES-NaOH pH 7.4 buffer (200 μL, 1M stock) or 1 U/mL esterase (10 μL, 100 U/mL stock), 100 μM of **1a** (10 μL, 10 mM stock), 25 mM DTT (250 μL, 100 mM stock) or 25 mM GSH (250 μL, 100 mM stock) the final volume was adjusted to 1 mL using deionized water with 2% DMSO. At predetermined time points, aliquots were taken from the reaction mixture, and injected in the HPLC. The data from this experiment can be found in Figure S16, S23.

g) Turnover of 2a by *b*3-MST in presence of DTT to produce 3a

10 mM stock solution of **2a** was prepared in DMSO. The reaction mixture contained 200 mM HEPES-NaOH pH 7.4 buffer (200 μL, 1 M stock), 25 mM DTT (250 μL, 100 mM stock), 100 μM of **1** (10 μL, 10 mM stock), 0.5 μM 3-MST (11 μL, 46 μM stock) and the final volume was adjusted to 1 mL using deionized water with 2% DMSO. At predetermined time points, aliquots were taken from the reaction mixture, and injected in the HPLC. The data from this experiment can be found in Figure S27.

The area under the curve for acetophenone **3a** during the reaction of **2a** with 3-MST was computed and was used to determine the rate constant for the formation of acetophenone (Figure 5c). The data was fit using the pseudo first order equation $y = y_0 + a(1 - e^{-kx})$.

Intrinsic fluorescence experiment with wt *h*3-3MST and *b3*-MST: The intrinsic fluorescence assays were conducted as previously reported.[19] 50 μM enzyme stocks of wt *h*3-3MST or *b3*-MST were prepared in HEPES-NaOH pH 7.4 buffer. Stock solutions of **2a** (10 mM) and **4** (5 mM) were freshly prepared in DMSO before each experiment. Individual reaction mixtures were set up in 200 mM HEPES-NaOH pH 7.4 buffer, pre-incubated at 37 °C. The final DMSO concentration was 1% and final reaction volume was adjusted to 700 μL.

a) Emission spectra of compounds: 100 μM of **2a** (7 μL, 10 mM stock) or 50 μM of **4** (7 μL, 5 mM stock) was added to 693 μL of 200 mM HEPES-NaOH pH 7.4 buffer.

b) **Emission spectra of wt *h*3-3MST or *b3*-MST:** 7 µL DMSO, 10 µM of enzyme (140 µL, 50 µM stock) was added to 553 µL of 200 mM HEPES-NaOH pH 7.4 buffer.

c) **Emission spectra of 3-MST on incubation with 2a or 4:** To 553 µL of 200 mM HEPES-NaOH pH 7.4 buffer, 10 µM of enzyme (140 µL, 50 µM stock), 100 µM of **2a** (7 µL, 10 mM stock) or 50 µM of **4** (7 µL, 5 mM stock) was added.

From the above reaction samples, aliquots of 200 µL were transferred to a 96-well plate and emission spectrum was recorded at excitation wavelength (λ_{ex}) of 287 nm using a microplate reader (PerkinElmer EnSight).

Tag-Switch technique for persulfidation of *b*3-MST:[13,20] Stock solutions of compounds, MSBT-A and CN-biotin were prepared in DMSO. To 100 µL of 3-MST (0.4 mg/mL; wt or C238A mutant), compounds (2 µL,10 mM stock) were independently added followed by addition of 1 U/mL esterase (1 µL,100 U/mL stock) and the reaction mixture was incubated for 1 h. This was followed by addition of 10 µL of 25% SDS (sodium dodecyl sulfate) and MSBT-A (6 µL, 200 mM stock) and further incubated for 30 min at 37 °C. The reaction mixtures were desalted using Amicon centrifugal filters. The supernatants were collected and treated with CN-biotin (4 µL, 50 mM stock) and further incubated for 1 h at 37 °C. The reaction mixtures were desalted again using Amicon centrifugal filters.

The supernatants were collected and mixed with 4× Laemmli Buffer and resolved on a 12% polyacrylamide gel. Proteins were transferred onto a PVDF membrane, blocked using 3% BSA for 1 h and incubated overnight at 4 °C with anti-biotin antibody produced in goat followed by HRP-conjugated anti-goat secondary antibody. The signal was visualized using Advansta WestBright ECL chemiluminescent HRP substrate.

Persulfide/polysulfide measurement using SSP-2:[21,22]

GS_nH was prepared by reacting 10 mM GSH, 10 mM DEA/NO (sodium 2-(*N*, *N*-diethylamino)-diazenolate-2-oxide) and 10 mM NaSH at room temperature for 20 min.

Stock solutions of **1a** (10 mM), **4** (10 mM), **5** (10 mM), **6** (10 mM) and SSP-2 (5 mM) were prepared in DMSO. DTT (100 mM), iodoacetamide (IAA, 100 mM) and esterase (100 U/mL) were prepared in DI water. The reaction mixture was prepared by adding 10 µM 3-MST (30 µL, 106 µM stock), **4** or **1a**, **5**, **6** along with 1 U/mL esterase (3 µL, 100 U/mL stock) and the

volume was adjusted to 300 μL using 20 mM tris-HCl pH 7.4 buffer. The reaction was incubated for 30 min at 37 °C.

A similar reaction was set up wherein the above reaction mixture was treated with 10 mM DTT (30 μL of 100 mM stock) after 30 min and further incubated at 37 °C for 30 min.

In a separate group, 3-MST was pre-incubated with 1 mM IAA (3 μL, 100 mM stock) for 30 min followed by addition of 100 μM **1a** or 100 μM **4** and 1U/mL esterase. The reaction was incubated for 30 min at 37 °C.

The above treatment groups were finally incubated with 50 μM SSP-2 (3 μL, 5 mM) at 37 °C for 10 min in the dark. 200 μL aliquot of each sample were transferred to a 96 well plate and the fluorescence was recorded (λ_{ex} = 482 nm, λ_{em} = 518 nm) using a microplate reader (Thermo Scientific VarioskanFlash).

Persulfide/polysulfide measurement using LC-MS:[22] Stock solutions of **1a** (10 mM), **5** (10 mM) and **6** (10 mM) were prepared in DMSO. GSH (10 mM) and esterase (100 U/mL) were prepared in DI water. The reaction mixture was prepared by adding 10 μM 3-MST (10 μL, 106 μM stock), **1a**, **5** or **6** along with 1U/mL esterase (1 μL, 100 U/mL) and the volume was adjusted to 100 μL using 20 mM tris-HCl pH 7.4 buffer. The reaction mixture was incubated for 30 min at 37 °C. 1 mM GSH (10 μL of 10 mM) was then added and incubated at 37 °C for 30 min. 50 μL aliquot of the above reaction samples were added to a 50 μL methanol solution of 20 mM monobromobimane (mBBr, final concentration 10 mM) and incubated in the dark at 37 °C for 15 min. The samples were centrifuged at 10000g for 10 min at 4 °C and the supernatants were collected and assessed thereafter by LC/MS. All measurements were done using a previously established LC/MS method[22] 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. The MRM-HR mass spectrometry parameters for measuring polysulfides are: GSS-bimane precursor ion mass (Q1, $M + H^+$) = 530, product ion mass (Q3, $M + H^+$) = 192, declustering potential =130 V, entrance potential = 10 V, collision energy = 33 V, and collision exit potential = 10 V; GSSSG precursor ion mass (Q1, $M + H^+$) = 645, product ion mass (Q3, $M + H^+$) = 387, declustering potential =170 V, entrance potential

= 10 V, collision energy = 21 V, and collision exit potential = 10 V; bis-S-bimane precursor ion mass (Q1, M + H^+) = 415, product ion mass (Q3, M + H^+) = 193, declustering potential =130 V, entrance potential = 10 V, collision energy = 13 V, and collision exit potential = 10 V.

GAPDH activity:[20,23] 1 mg/mL stock of GAPDH was prepared in pH 7.4 phosphate buffer (50 mM). The GAPDH was reduced by incubating it with 10 mM DTT for 1 h at room temperature. The DTT was removed by desalting using Amico Ultracel 10K tube and washing with phosphate buffer. Finally, the concentration of GAPDH was adjusted to 1 mg/mL.

The reaction mixture was prepared by adding 2 μM 3-MST (20 μL, 32 μM stock), 200 μM **1a** (6 μL, 10 mM), 1U/mL esterase (3 μL, 100 U/mL) and the final volume was adjusted to 300 μL using pH 7.4 phosphate buffer (50 mM). After 30 min of incubation at 37 °C, 20 μg/mL GAPDH (5.6 μL, 1 mg/mL) was added and further incubated at 37 °C for 30 min. GAPDH activity was studied as described below. GAPDH activity was tested using a previously reported protocol.[23] Briefly, 100 μL aliquot of each treatment group was mixed with equal volume of GAPDH assay buffer. GAPDH assay buffer is constituted of 20 mM tris-HCl buffer (pH 7.8), 100 mM NaCl, 0.1 mg/mL bovine serum albumin (BSA), 20 mM sodium arsenate, 10 mM sodium pyrophosphate, 6 mM glyceraldehyde-3-phosphate and 1 mM NAD^+ (nicotinamide adenine dinucleotide). The formation of NADH was monitored spectrophotometrically in real time at 340 nm and 37 °C using a microplate reader (Thermo Scientific VarioskanFlash).

For the experiment carried out with DTT, all reaction conditions were similar, except that 150 μL aliquot from the standard reaction mixture was taken and treated with 2 mM DTT (3 μL, 100 mM) and incubated at 37 °C for 1 h. 100 μL of this reaction mixture was used for further analysis.

Methylene blue assay for the detection of H_2S

General protocol: The methylene blue assays were conducted as previously reported with some modifications.[18,24,25] Briefly, 10 μM stocks of wt *b3*-MST and *h3*-MST were prepared in HEPES-NaOH pH 7.4 buffer. Stocks of all unnatural substrates (**1a-1m**, **5**, **6,** 10 mM) and **4** (5 mM) were prepared in DMSO. Dithiothreitol (DTT, 100 mM) and $Zn(OAc)_2.2H_2O$ (40 mM) was prepared in deionised 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 $Zn(OAc)_2.2H_2O$ (15 μL, 40 mM stock), 50 μM of **4** (15 μL, 5 mM stock) or 100 μM of unnatural substrates (15 μL, 10 mM stock), 0.5 μM wt *b*3-MST (75 μL, 10 μM stock) or 0.5 μM wt *h*3-MST (75 μL, 10 μM stock) and 25 mM DTT (150 μL, 100 mM stock). The final volume was adjusted to 1.5 mL using 200 mM HEPES-NaOH pH 7.4 buffer and placed in a static incubator maintained at 37 °C.

A similar protocol was followed for the substrate controls **5** and **6**. Briefly, the reaction samples were prepared by sequentially adding 400 μM $Zn(OAc)_2.2H_2O$ (10 μL, 40 mM stock), 50 μM of **4** (10 μL, 5 mM stock) or 100 μM of test compounds (**1a**-**1m**, **5** or **6**) (10 μL, 10 mM stock), 0.5 μM wt *b*3-MST (75 μL, 10 μM stock) or 0.5 μM wt *h*3-MST (75 μL, 10 μM stock) and 25 mM DTT (100 μL, 100 mM stock). The final volume was adjusted to 1.0 mL using 200 mM HEPES-NaOH pH 7.4 buffer and placed in a static incubator maintained at 37 °C.

In a separate experiment conducted to test the enzyme controls, 400 μM $Zn(OAc)_2.2H_2O$ (10 μL, 40 mM stock), 10 μL DMSO, 0.5 μM wt *b*3-MST (75 μL, 10 μM stock) or 0.5 μM wt *h*3-MST (75 μL, 10 μM stock) and 25 mM DTT (100 μL, 100 mM stock) were sequentially added. The final volume was adjusted to 1.0 mL using 200 mM HEPES-NaOH pH 7.4 buffer and placed in a static incubator maintained at 37 °C.

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

Generation of 3-MST knockdown cell line

Virus generation: Human embryonic kidney cell line expressed with SV40 large T-antigen (HEK293T cells) were seeded at a density of 0.8×10^4 in 24 well plate and incubated with Dulbecco's modified eagle's media (DMEM) supplemented with 10% FBS (fetal bovine serum) along with 1% antibiotic solution (penicillin and streptomycin) in an atmosphere of 5% CO_2 at 37 °C overnight. Next day, psPAX2, pMD2.G and specific shRNA plasmid (from

Sigma TRC shRNA library from Broad Institute) targeting human MST were added in 100 µl DMEM in a ratio of 4:1:4 after mixing with Turbofect transfection reagent (Thermo Fischer Scientific) and incubated at room temperature for 30 min. Before transfection, cells were washed twice with 1× PBS and 2% FBS containing DMEM was added followed by incubation with 5% CO_2 at 37 °C for 30 min. After 30 min incubation, transfection mixture was added dropwise in the plate and further incubated with 5% CO_2 at 37 °C for 72 h followed by supernatant collection.

Knockdown generation: For the generation of 3-MST expression knocked down human lung carcinoma cell line, A549 cells were used. The cells were seeded at a density of 0.5 × 10^4 cells/well in a 12-well plate in DMEM supplemented with 10% FBS along with 1% antibiotic solution (penicillin and streptomycin) and incubated in an atmosphere of 5% CO_2 at 37 °C overnight. Next day, scrambled and MST shRNA were introduced in the by lentiviral transduction (prepared above) followed by selection of stable knockdown cells in presence of puromycin (5µg/ml) for 24 h. The knockdown efficiency was analysed in both scrambled and MST shRNA containing cells by RT-PCR analysis.

Detection of persulfidation in cells:[6] A549 cells containing scrambled or MST shRNA were seeded on cover slips at a density of 0.5 × 10^4 cells/well in 12 well plate in DMEM supplemented with 10% FBS along with 1% antibiotic solution and incubated at 5% CO_2 at 37 °C overnight. Next day, the cells were treated with the 3-MST substrates **1a** or **4** (10 µM) and Na_2S (200 µM) for 1 h. The cells were washed twice with sterile PBS after the treatment and fixed by incubating with ice cold methanol at -20 °C for 20 min, followed by permeabilization using ice cold acetone -20 °C for 5 min. The cells were then treated with 50 mM HEPES containing 1% Triton X-100 and 10 mM MSBT-A overnight at room temperature. The cells were washed three times with PBS and further incubated with CN-BOT (25 µM) in PBS for 1 h at 37 °C. The cells were then washed 3 times with PBS and imaged in GFP channel (λ_{ex} = 488 nm, λ_{em} = 507 nm) using IX83 fluorescence microscope (Olympus, Japan).

H_2S imaging in cells using NBD-fluorescein:[26] A549 scrambled and 3-MST KD cells were seeded in a 12-well plate with 10^4 cells/well in DMEM media supplemented with 10% FBS and 1% antibiotic solution and incubated in an atmosphere of 5% CO_2 at 37 °C for 48 h. After incubation, the old media was removed and the cells were washed with 1 mL of sterile 1× PBS. This was followed by the addition of 1 mL fresh DMEM media. The cells were co-treated with a COS/H_2S donor (50 µM) or the unnatural substrate **1a** (100 µM) and NBD-fluorescein dye

(10 μM) in both the cell lines and were incubated at 37 °C for 1 h. After 1 h, cells were washed twice with 1× PBS and then imaged on an EVOS fluorescence microscope using 20x GFP (green fluorescence protein) filter.

Cell viability assay: A549 cells, mouse embryonic flibroblasts (MEFs) and mouse neuroblastma cells (N2a) were seeded at a concentration of 1×10^4 cells/well overnight in a 96-well plate in complete DMEM media. Following this, the cells were exposed to varying concentrations of the compounds. Stock solutions of compounds were prepared in DMSO and the final concentration of DMSO did not exceed 0.5%. The cells were incubated for 12 h or 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. After incubation, the old media from the cells were removed, 100 μL of the MTT stock was added to each well and incubated for 4 h at 37 °C. After 4 h incubation, the media was carefully removed and 100 μL of DMSO was added. Spectrophotometric analysis of each well at 570 nm using a microplate reader (Thermo Scientific Varioskan) was carried out to estimate cell viability.

ROS quenching: A549 cells were seeded in a 12-well plate with 10^5 cells/well in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and 1% antibiotic solution and incubated in an atmosphere of 5% CO_2 at 37 °C for 48 h. The cells were treated with **1a** (0, 10, 25 and 50 μM) or **6** (50 μM) and incubated for 12 h at 37 °C after which MGR-1 (25 μM) and TCF-B (25 μM) were co-incubated for 1 h. After 1 h, the media was removed, cells were washed twice with 1× PBS and the cells were imaged on an EVOS fluorescence microscope using a 20x TxRed filter.

Measuring intracellular GSSG/GSH and NAD^+/NADH: A549, MEF and N2a cells were grown in 100 mm petri plates in an atmosphere of 5% CO_2 at 37 °C. The cells were pre-treated with compounds **1a** (50 μM) or **6** (50 μM) for 12 h followed by treatment with MGR-1 (25 μM) for 1 h. Each group had 5 replicates. The cells from each treatment groups were harvested and the ratio of GSSG/GSH and NAD^+/NADH was measured using ELISA kits from Abcam (ab239709 for GSSG/GSH and ab65348 for NAD^+/NADH) as per manufacturer's protocol.

Protection from oxidative stress:

MEF cells: MEF were seeded in a 96-well plate with 10^4 cells/well in DMEM media supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution and incubated in an atmosphere of 5% CO_2 at 37 °C for 16 h. Stock solutions of compounds were prepared in

DMSO with final concentration of DMSO not exceeding 0.5%. After 16 h, the cells were pre-treated with different concentrations of the compound for 24 h followed by treatment with MGR-1 (25 μM). The cells were incubated for 4 h at 37 °C following which the media was removed and MTT assay was carried out as described above to determine cell viability.

N2a cells: N2a were seeded in a 96-well plate with 2.5 x 10^4 cells/well in DMEM media supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution and incubated in an atmosphere of 5% CO_2 at 37 °C for 24 h. Stock solutions of compounds were prepared in DMSO with final concentration of DMSO not exceeding 0.5%. After 24 h, the cells were pre-treated with different concentrations of the compound for 12 h followed by treatment with menadione (15 μM) or MGR-1 (25 μM). The cells were incubated for 4 h at 37 °C following which the media was removed MTT assay was performed as described above to determine cell viability.

Mouse studies: All mouse studies described in the manuscript received formal approval from the Indian Institute of Science Education and Research, Pune-Institutional Animal Ethics Committee (protocol no: IISER_Pune IAEC/2019_2/07), constituted as per the guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All mice were maintained at National Facility for Gene Function in Health and Disease (NFGFHD) at IISER Pune, supported by a grant from the Department of Biotechnology, Govt. of India (BT/INF/22/SP17358/2016). All mice used in the study were generated by breeding wild type C57BL/6J mice and had *ad libitum* access to water and food. All mice used for experiment were age and gender matched. For compound toxicity studies in mice, the test compound was intraperitoneally injected at 20 mg/kg body weight (o.d.) for 7 days and the mice were monitored for any changes in their routine cage behavior. After 7 days, all mice were euthanized, their tissues were harvested, and examined for any signs of inflammation and/or toxicity. For the experiments where neuroinflammation was studied, an intraperitoneal injection of lipopolysaccharide (LPS) in 1× PBS (vehicle) at a dose of 5 mg/kg body weight was used to generate systemic inflammation. The test compounds (NaSH or **1a**) were also injected intraperitoneally in two doses – the first at 20 mg/kg body weight 4 h before the LPS administration and second dose of 20 mg/kg body weight 30 min after the LPS administration, following which, the mice were kept overnight.

Prostaglandin extraction and measurements: Mice were deeply anesthetized with isoflurane and euthanized by cervical dislocation. The brains of the mice were dissected within 15 s post decapitation, divided into two sagittal equal halves, weighed, washed with cold phosphate buffered saline (PBS) and flash frozen in liquid nitrogen. The prostaglandins were extracted and measured using protocols described previously.[7,27] Briefly, half brains were re-suspended in 2 mL cold 1× PBS and the lipids from them were extracted using 6 mL of 1:1 (vol/vol) ethyl acetate: hexane containing 1 nmol of 17:1 free fatty acid (FFA) (internal standard) per sample by dounce homogenization. The resulting mixture was vortexed vigorously and centrifuged at 2,500 g for 15 min to separate the aqueous and organic layers. Thereafter, the top (organic) layer containing the lipids (prostaglandins) was collected in a glass vial and dried under an inert N_2 stream at room temperature. Dried extracts were re-solubilized in 200 mL of 2:1 (v/v) chloroform: methanol (MeOH), and 20 mL was injected into liquid chromatography coupled mass spectrometry (LC/MS) instrument for prostaglandin measurements. All the prostaglandin measurements were done as per as previously established multiple reaction monitoring high resolution (MRM-HR) methods[7] set on Sciex X500R QTOF mass spectrometer coupled to an Exion-series UHPLC with a quaternary pump. Briefly, the lipids were separated using a Gemini 5U C18 Phenomenex column (5 μm, 50 × 4.6 mm) using LC solvents as follows: Solvent A: 95:5 (vl/v) water: MeOH + 0.1% (vol/vol) ammonium hydroxide and Solvent B: 60:35:5 (v/v/v) isopropanol: MeOH: water + 0.1 % (vol/vol) ammonium hydroxide. The LC runs were for 30 min with gradient of 0% solvent B for 5 min, linear gradient of solvent B (0% to 100%) for 20 min followed by 100% solvent B for 5 min all at a flow rate of 0.3 mL per min. MS was calibrated in negative mode and samples were analyzed with the following parameters: Mode: Electrospray ionization (ESI), curtain gas 1 = 60 psi, curtain gas 2 = 40 psi, ion spray voltage = - 4.5 kV, and temperature = 500 °C. All quantifications were done by normalizing the metabolite area under the curve to the area under the curve for the internal standard, followed by normalization to weight of the tissue.

4. Supplementary figures

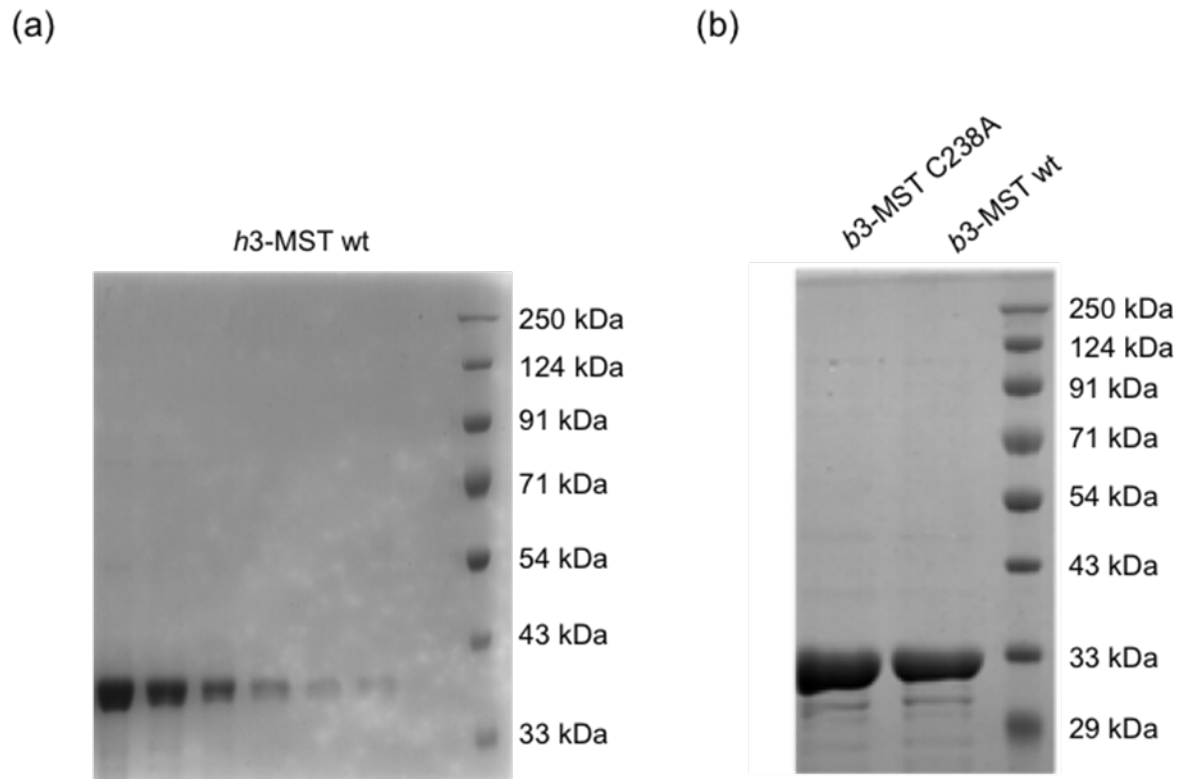


Figure S1: (a) SDS-PAGE gel image for the purified protein wt *h*3-MST. (b) SDS-PAGE gel image for the purified proteins: wt *b*3-MST and *b*3-MST C238A mutant.

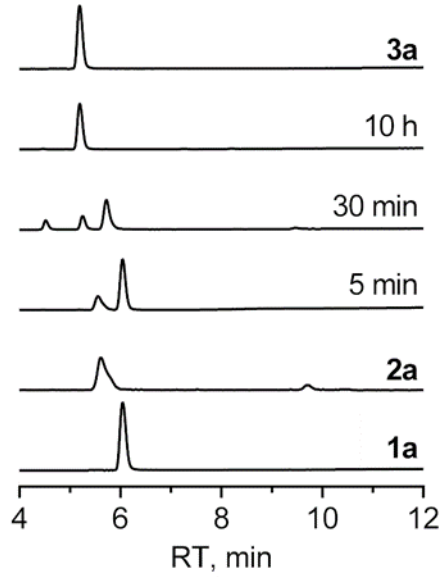


Figure S2: HPLC traces for: **1a** + ES + *h*3-MST. Conversion of **1a** to **2a**, and the turnover of **2a** to **3a** by *h*3-MST was observed. The time points considered were 5 min, 30 min and 10 h. (Abs 250 nm).

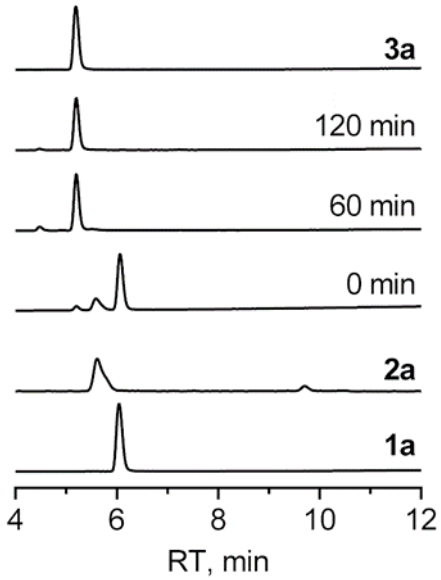


Figure S3: HPLC traces for: **1a** + ES + *b*3-MST Conversion of **1a** to **2a**, and the turnover of **2a** to **3a** by *b*3-MST was observed. The time points considered were 0 min, 60 min and 120 min. (Abs 250 nm).

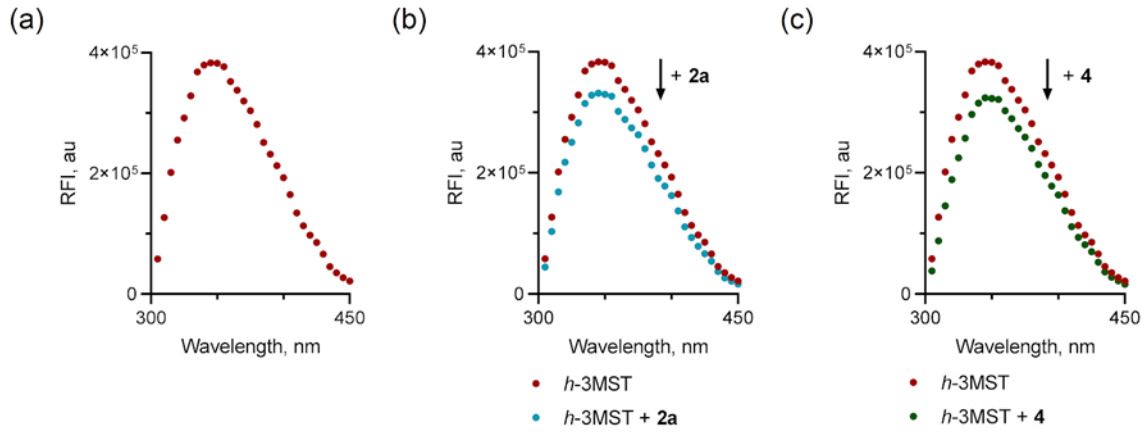


Figure S4: Intrinsic fluorescence study: (a) Emission spectrum of *h*3-MST alone (b) Comparison of emission spectra of *h*3-MST alone and upon addition of **2a** (100 μM) (c) Comparison of emission spectra of *h*3-MST alone and upon addition of **4** (50 μM). (λ_{ex} = 287 nm). No detectable fluorescence was observed for **2a** or **4** under these conditions (**Figure S6**).

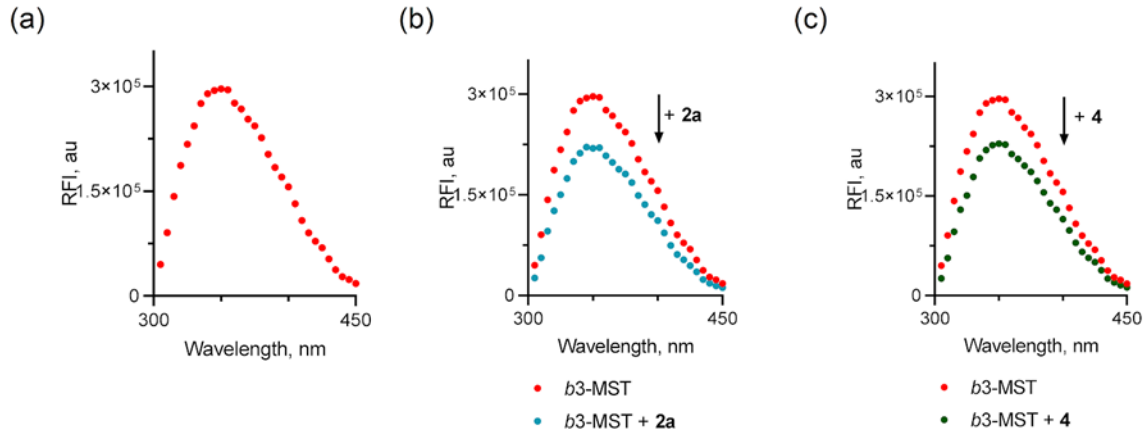


Figure S5: Intrinsic fluorescence study: (a) Emission spectrum of *b*3-MST alone (b) Comparison of emission spectra of *b*3-MST alone and upon addition of **2a** (100 μM) (c) Comparison of emission spectra of *b*3-MST alone and upon addition of **4** (50 μM). (λ_{ex} = 287 nm). No detectable fluorescence was observed for **2a** or **4** under these conditions (**Figure S6**).

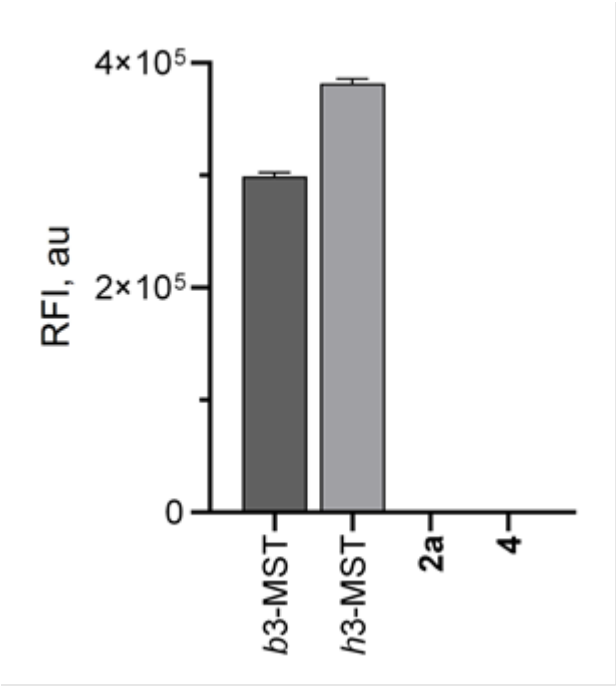
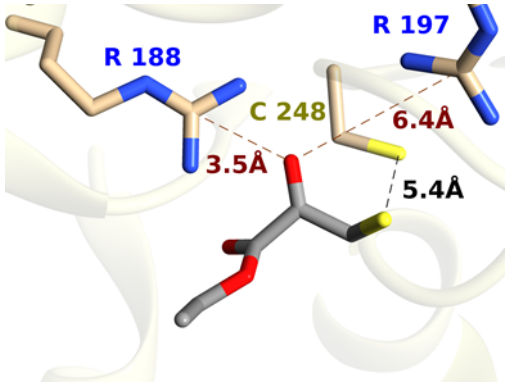


Figure S6: Emission maximum ((λ_{ex} = 287 nm; λ_{em} = 435 nm) values of *b*3-MST and *h*3-MST. Substrates **2a** (100 µM) and **4** (50 µM) alone are not fluorescent.

Table S4: Docking score of **E3-MP** in the active site of *h*3-MST and the lowest energy conformation

Entry	Comp.	Docking score	Lowest energy conformation
1	**E3-MP**	-4.2 kcal/mol	

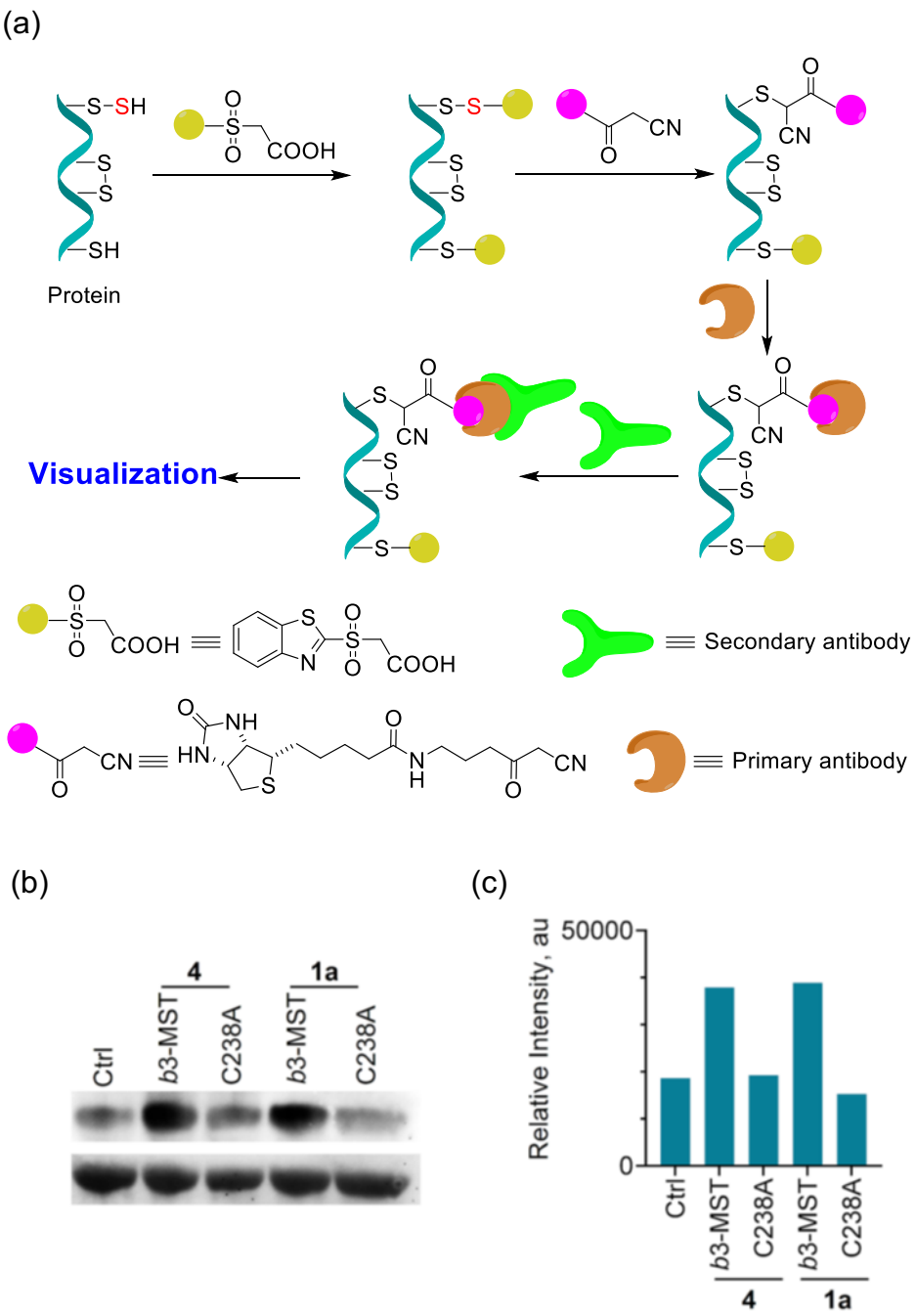


Figure S7: (a) Schematic of a reported protocol for detection of persulfides that was used.[13] The protein persulfide was reacted with MSBT-A followed by reaction with CN-biotin. The biotinylated protein was then visualized using western blotting technique. (Refer to page S17 for the detailed protocol) (b) Persulfidation of wt *b*3-MST and *b*3-MST C238A mutant by compounds **1a** in the presence of esterase (ES) and **4** were studied using the modified tag-switch technique. PonceauS staining was used as a loading control. (c) Relative quantification of the bands was done using ImageJ.

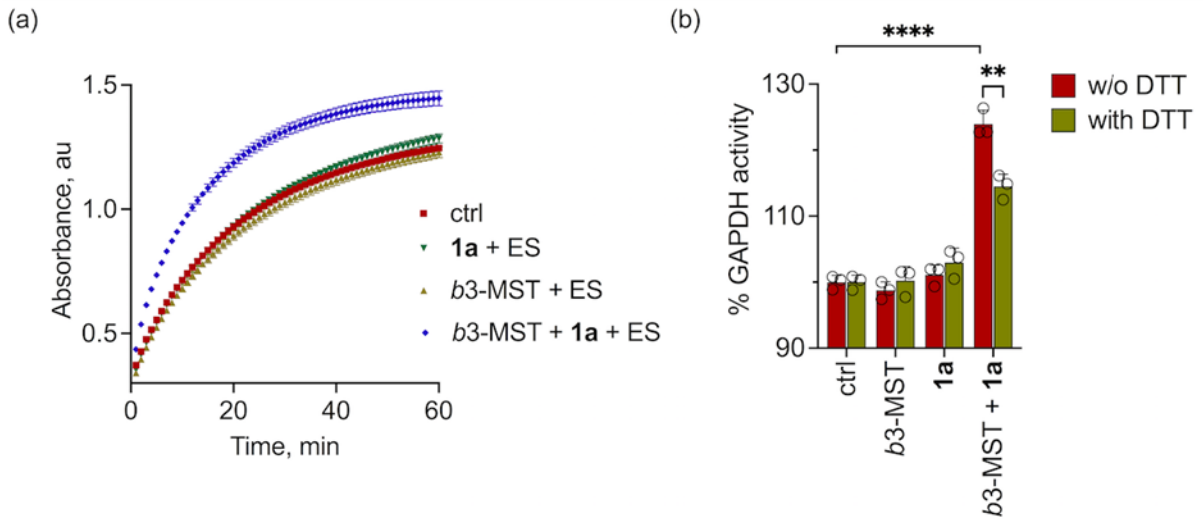


Figure S8: (a) Activity of GAPDH was measured by monitoring the formation of NADH, real-time at 340 nm. A significant increment in the activity of GAPDH was observed when treated with 3-MST-SS^{-} generated by reacting **1a** with *b*3-MST in the presence of esterase (blue) compared to GAPDH alone (ctrl, red). GAPDH and ES were present across all groups. (b) Each group was treated with 2 mM DTT following which the GAPDH activity was measured after 1 h. Addition of DTT to the reaction sample containing **1a** and 3-MST led to a decrease in the % activity of GAPDH. Ctrl represents GAPDH alone. GAPDH and ES were present across all groups. All data are presented as mean ± SD (n =3/group). Student's two-tailed unpaired parametric *t*-test was carried out to determine significance: **p < 0.01 for the comparison between the sample with DTT and the one w/o DTT, ****p < 0.0001 for w/o DTT versus ctrl.

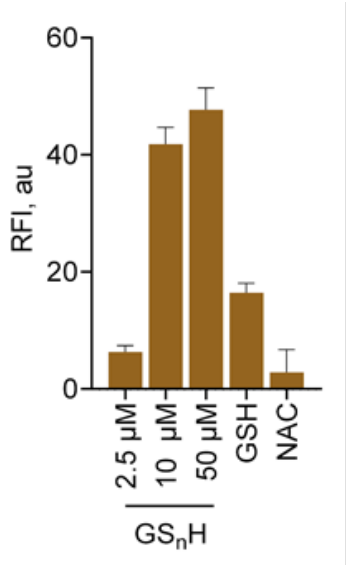


Figure S9: Validation of the probe SSP-2 using varying concentrations of GS_nH. GS_nH was prepared by reacting 10 mM GSH, 10 mM DEA/NO (sodium 2-(*N*, *N*-diethylamino)-diazenolate-2-oxide) and 10 mM NaSH at room temperature for 20 min. Fluorescence measurements (λ_{ex} = 482 nm; λ_{em} = 518 nm) were carried out at various concentrations of GS_nH, GSH (10 mM) and *N*-acetylcysteine (NAC, 10 mM).

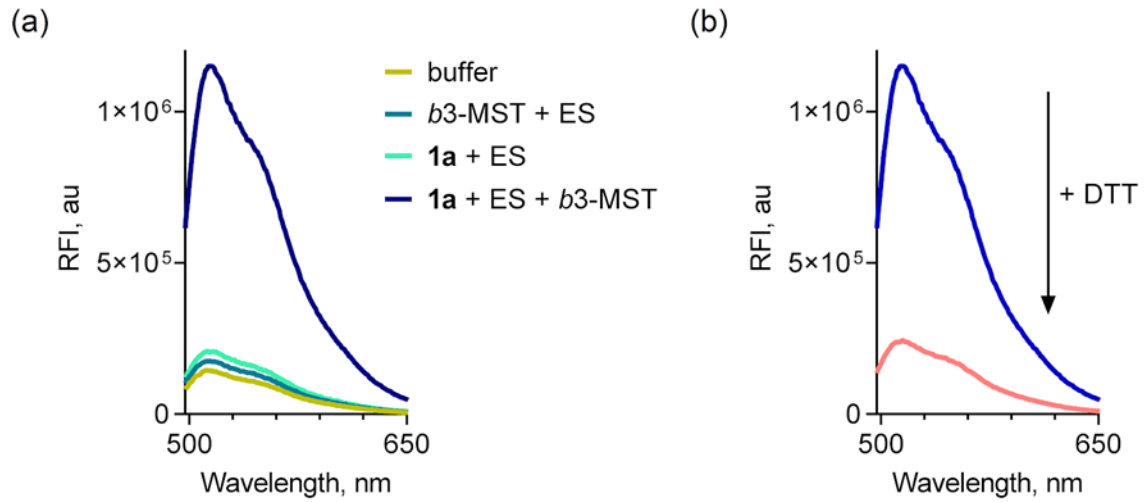


Figure S10: a) Persulfide/polysulfide detection using probe SSP-2. Fluorescence emission spectra of SSP-2 (λ_{ex} = 482 nm) upon treatment with *b*3-MST and **1a** in the presence of esterase. b) Fluorescence quenching of SSP-2 upon addition of DTT to the reaction sample containing *b*3-MST, **1a** and esterase. A significant decrease in signal was observed likely due to the reduction of persulfide/polysulfide to the native thiol.

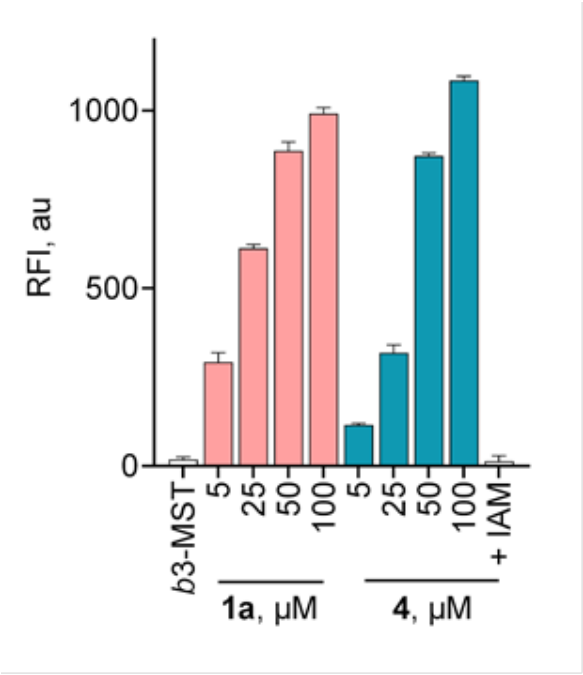


Figure S11: Persulfide/polysulfide detection using probe SSP-2 (Fluorescence intensities were measured; λ_{ex} 482 nm, λ_{em} 518 nm). Varying concentrations of **1a** and **4** were incubated with *b*3-MST followed by treatment with the probe SSP-2. +IAM refers to pre-treatment of the enzyme with iodoacetamide to block the active cysteine residue.

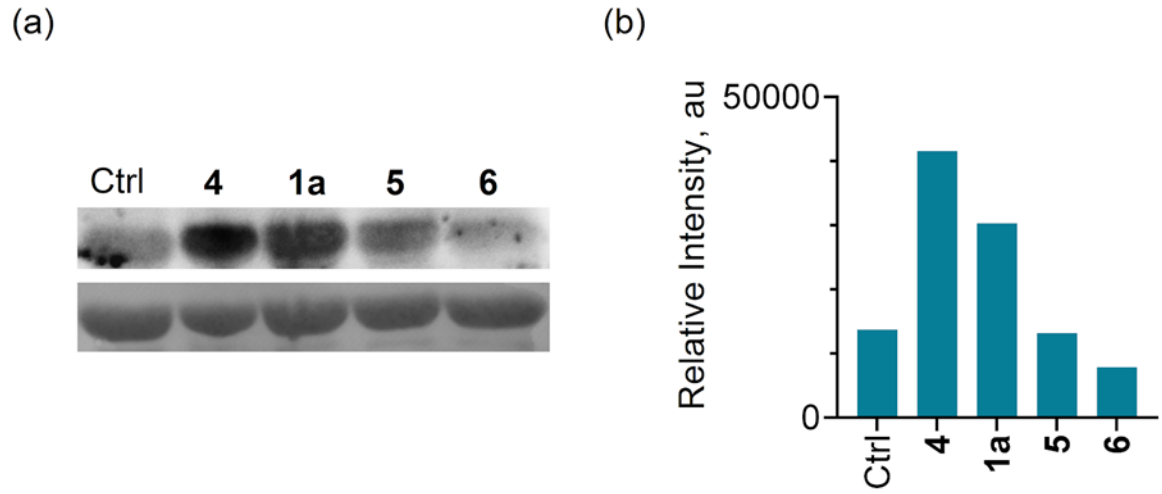
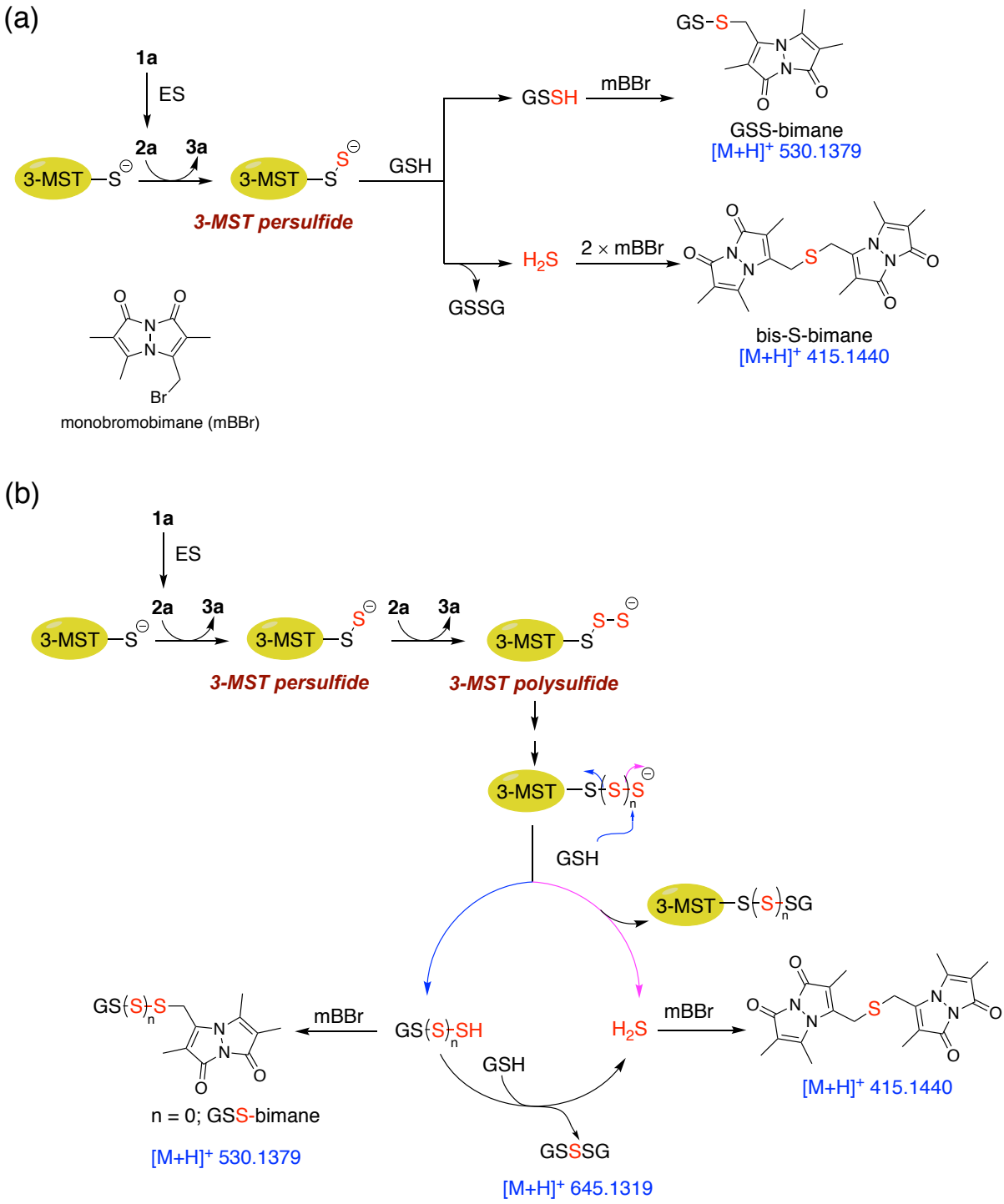


Figure S12: (a) Persulfidation of *b*3-MST by the ethyl ester of 3-mercaptopyruvate (**4**) and the unnatural substrate (**1a**) in presence of ES, detected using the modified tag-switch technique. Compounds **5** and **6** were used as negative controls. PonceauS staining was used as a loading control. (b) Relative quantification of the bands using ImageJ.



Scheme 4: (a) A schematic diagram of the various reactive sulfur species formed when 3-MST persulfide, generated by reacting *b*3-MST and **1a** is treated with GSH. Due to the short half-life of persulfide GSSH under the reaction conditions, this species is detected as its bimane adduct by reacting it with monobromobimane (mBBr) (GSS-bimane $[M+H]^+ = 530.1379$). H_2S is detected as its bis-S-bimane adduct ($[M+H]^+ = 415.144$). (b) A schematic for the generation

of 3-MST polysulfide and its degradation products. GSSSG is formed by the reaction of glutathione with glutathione polysulfide was also observed ($[M+H]^+$ = 645.1319). In the presence of mBBr, formation of adducts GSS-bimane and bis-S-bimane as depicted above is also possible.

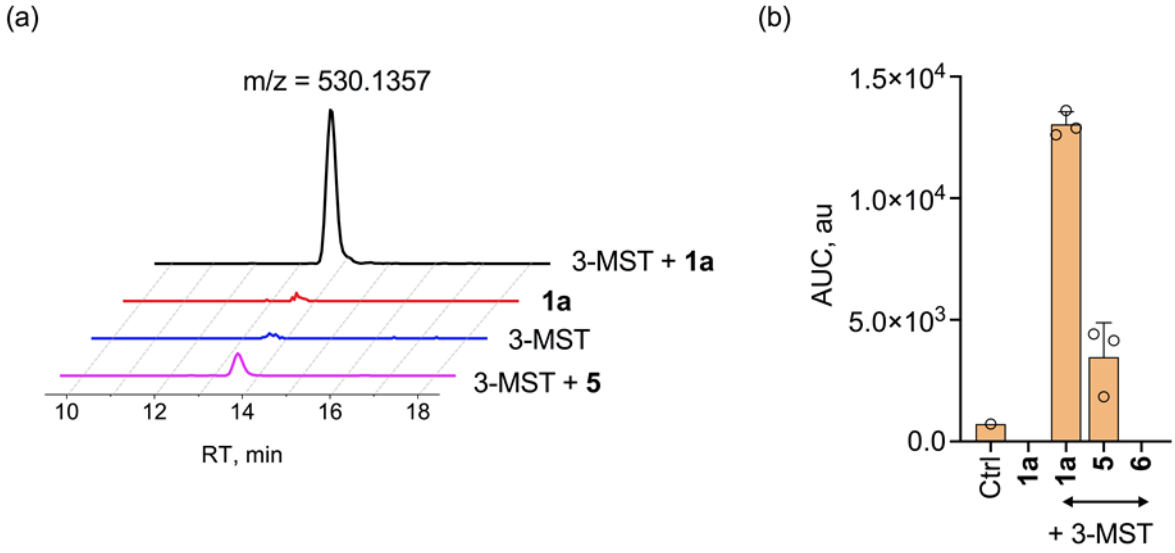


Figure S13: (a) Extracted ion chromatograms from an LC/MS analysis of GSS-bimane (expected, $[M+H]^+$ = 530.1379; observed $[M+H]^+$ = 530.1357) showing the formation of GSSH upon reacting **1a** with *b*3-MST in the presence of esterase followed by treatment with GSH. b) Area under the curve (AUC) for the peak corresponding to GSS-bimane. During the incubation of **5** with 3-MST, the formation of GSS-bimane adduct was observed at diminished levels when compared with a similar experiment conducted with **1a** + ES + 3-MST. While the origin of this result is yet to be characterized, it appears that **5** undergoes decomposition to produce a persulfide or H_2S as a minor pathway. Ctrl refers to enzyme alone.

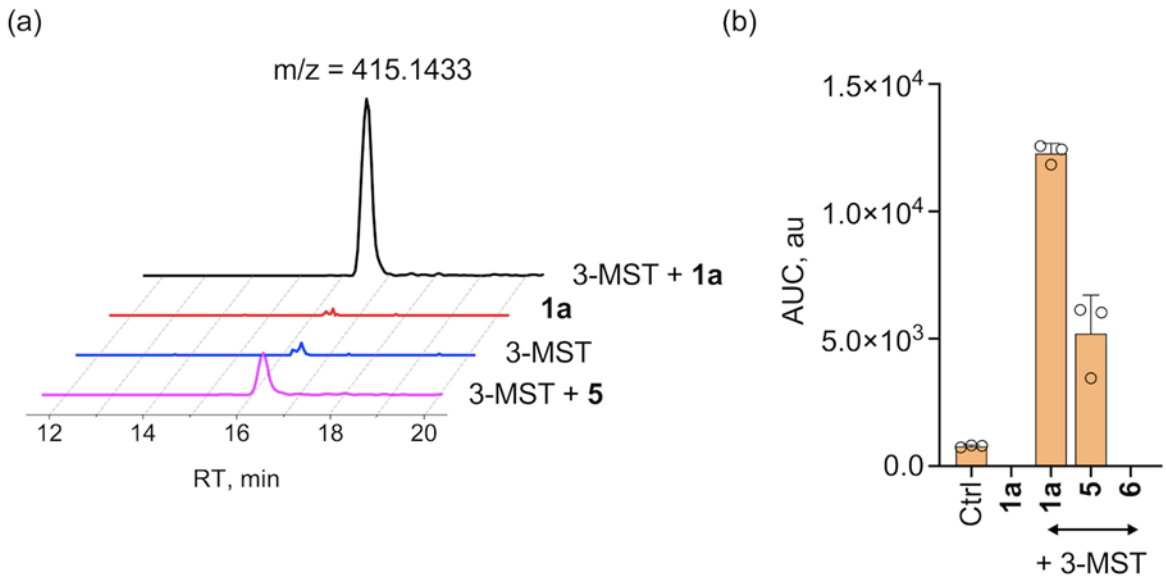


Figure S14: a) Extracted ion chromatograms from an LC-MS analysis of bis-S-bimane (expected, $[M+H]^+ = 415.144$; observed $[M+H]^+ = 415.1433$) indicating the formation of H_2S upon reacting **1a** with *b*3-MST in the presence of esterase followed by treatment with GSH. b) Area under the curve (AUC) for the peak corresponding to bis-S-bimane after incubation of the compound in the presence of ES and/or 3-MST followed by addition of mBBr. Again, **5** appears to undergo non-specific decomposition under the assay conditions to produce the bis-S-bimane adduct as a minor product. Ctrl refers to enzyme alone.

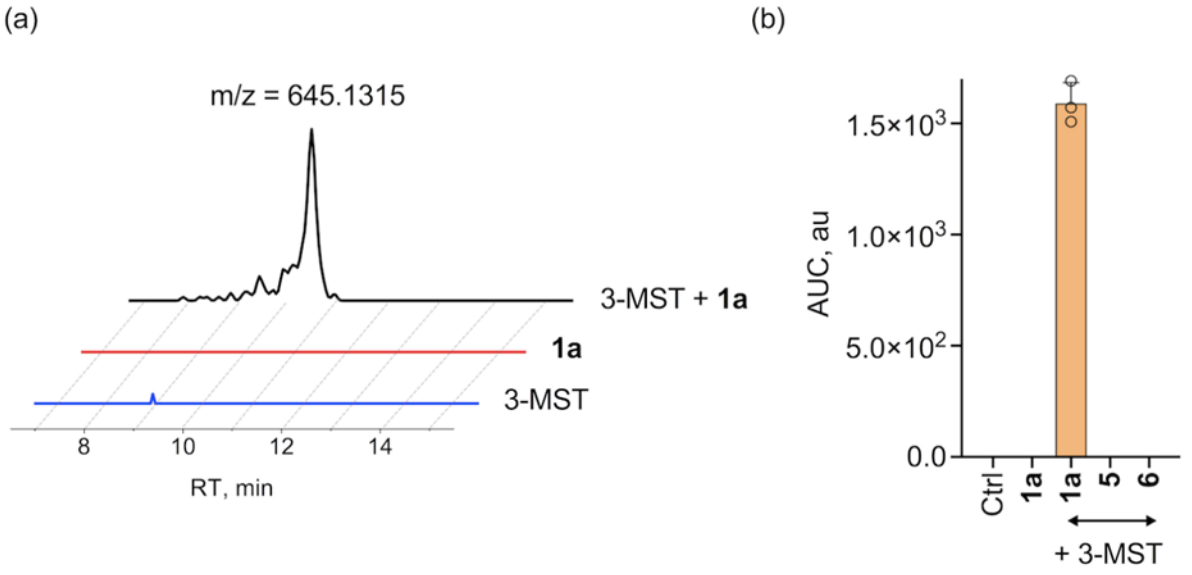


Figure S15: a) Extracted ion chromatograms from an LC/MS analysis of GSSSG (expected $[M+H]^+ = 645.1319$; observed $[M+H]^+ = 645.1315$) indicating the formation of GSSSG upon reacting **1a** with *b*3-MST in the presence of ES followed by treatment with GSH. b) Area under

the curve (AUC) for the peak corresponding to GSSSG after incubation of the compound in the presence of ES and/or 3-MST. Ctrl refers to enzyme alone.

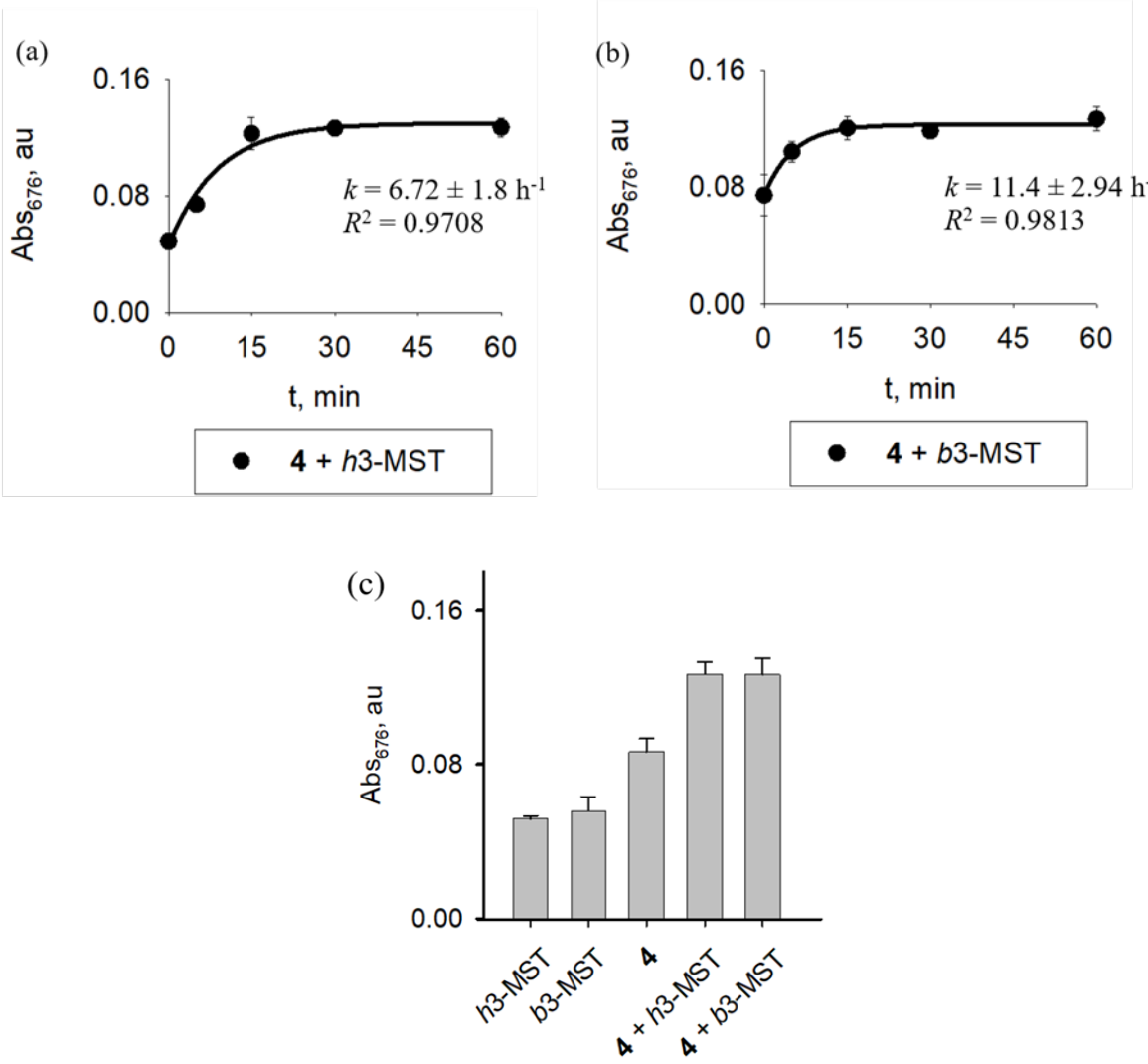


Figure S16: (a) Formation of methylene blue during incubation of **4** in the presence of wt *h*3-MST. (b) Formation of methylene blue during incubation of **4** in the presence of wt *b*3-MST. Curve fitting using a pseudo first order equation $y = y_0 + a(1 - e^{-kx})$ was carried out. (c) Collated methylene blue data for 3-MST alone, **4** alone as well as **4** in the presence of 3-MST. All measurements were done after 60 min. All data are presented as mean ± SD (n =3/group).

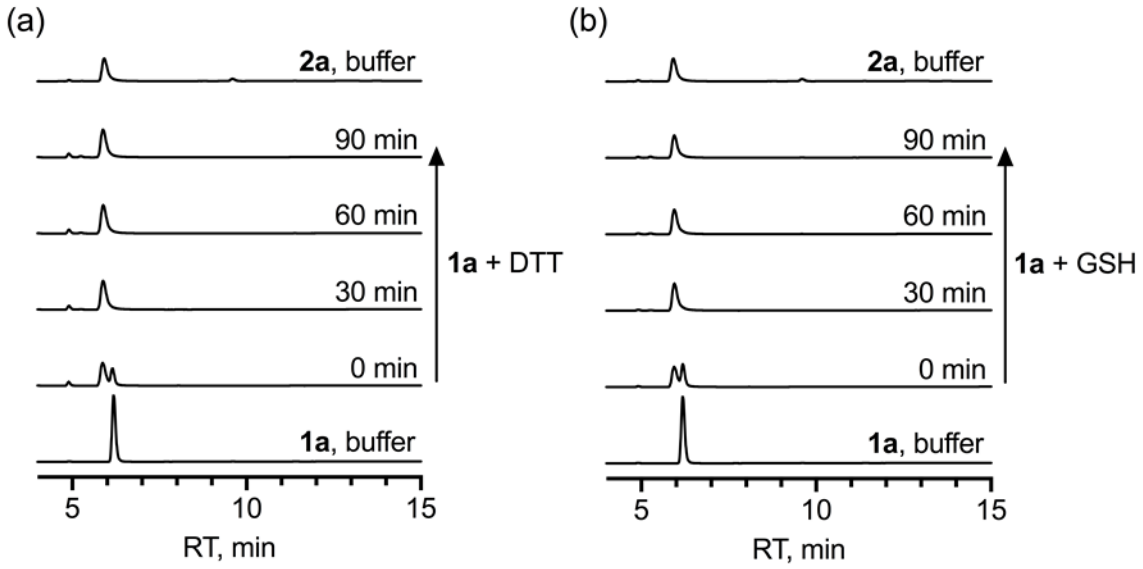


Figure S17: HPLC traces for the conversion of **1a** to **2a** during incubation with (a) 25 mM DTT and (b) 25 mM GSH. The time points considered were 0 min, 30 min, 60 min and 90 min.

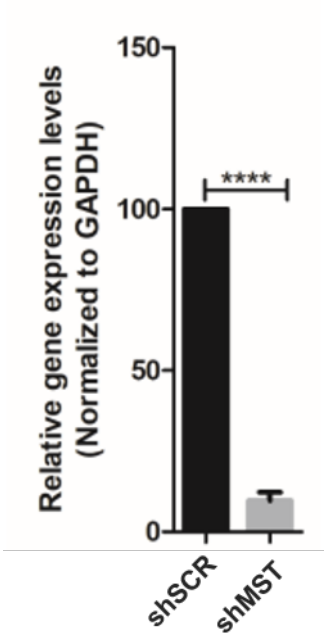
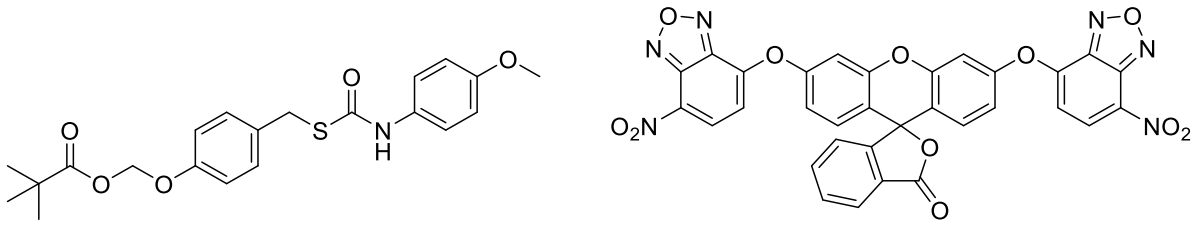


Figure S18: Validation of knockdown of 3-MST in A549 cells by real time PCR analysis carried out on scrambled cells (shSCR) as well as 3-MST KD cells (shMST). All data are presented as mean ± SD (n =3/group). ****$p < 0.0001$ as determined by Student's two-tailed unpaired parametric *t*-test.



Esterase activated COS/H_2S donor

NBD-Fluorescein

Figure S19: Structures of esterase activated COS/H_2S donor and the H_2S dye NBD-Fluorescein

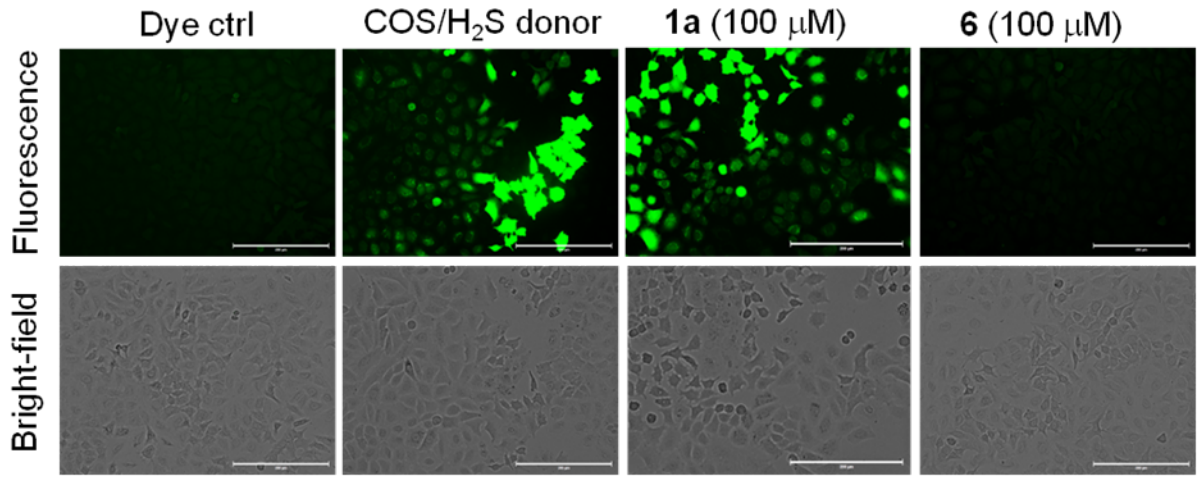


Figure S20: H_2S detection using the NDB-Fluorescein dye in A549 cells. The cells were imaged in the 20x GFP filter. Scale bar is 200 μm.

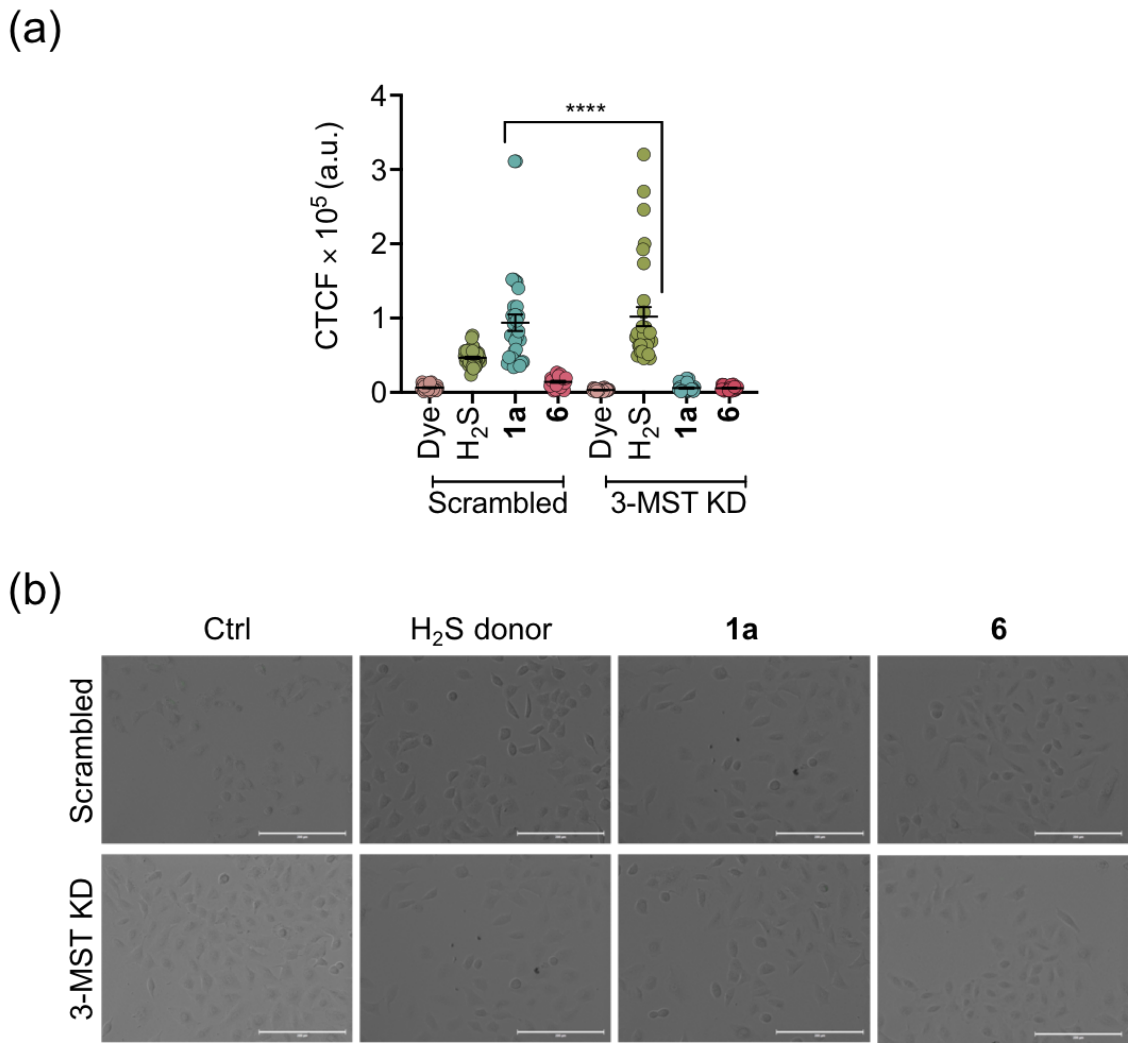


Figure S21: (a) H_2S levels were assessed using a previously reported dye NBD-Fluorescein. Quantification of the fluorescence signal generated by the dye NBD-fluorescein upon reaction with H_2S. All data are presented as mean ± SD (n = 3/group). ****$p < 0.0001$ scrambled versus 3-MST KD group. (b) Representative bright field images of H_2S detection using the NDB-Fluorescein assay in scrambled and 3-MST KD A549 cells. Scale bar is 200 μm.

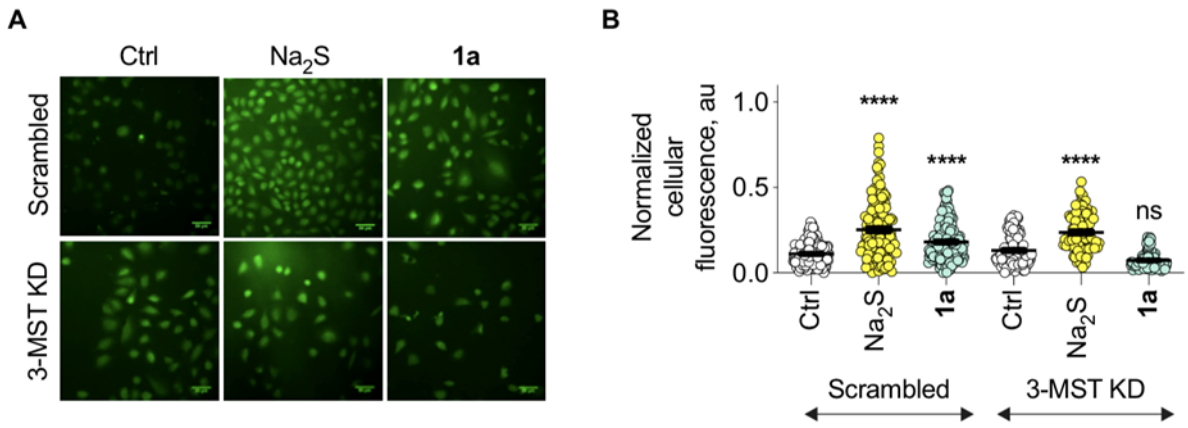
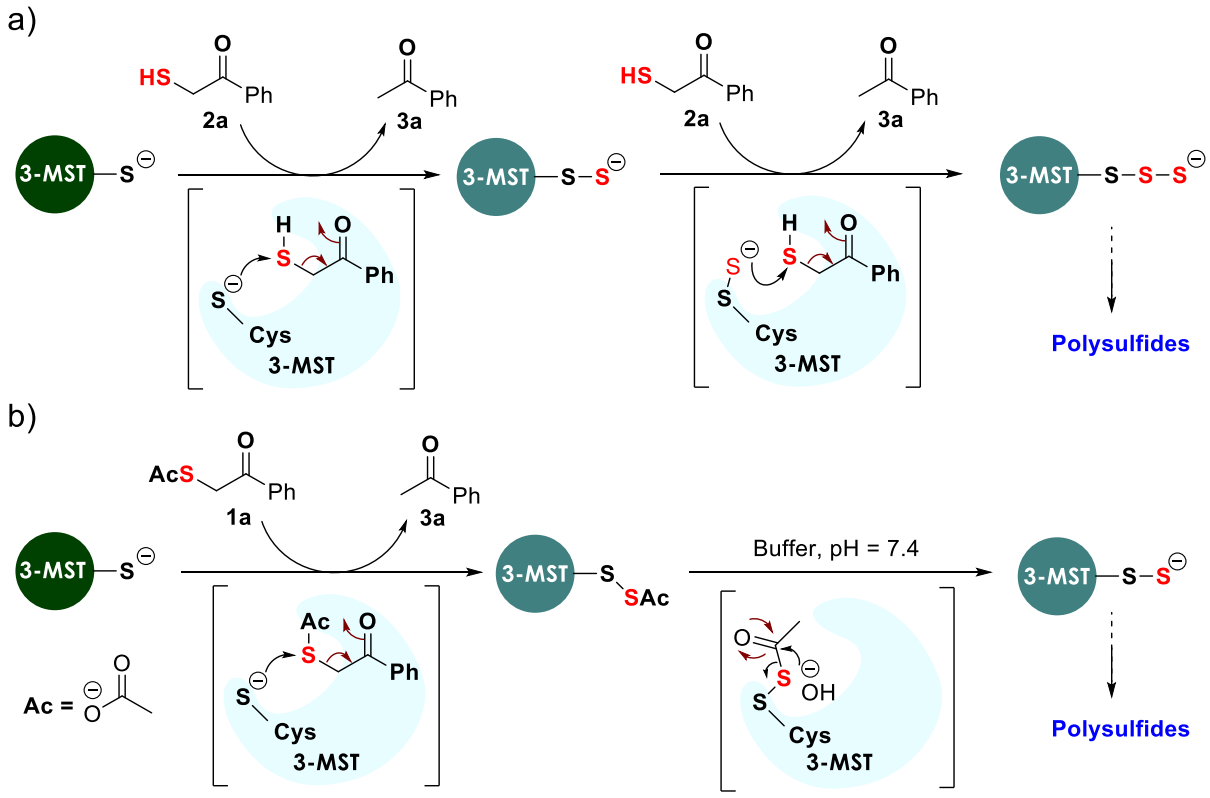


Figure S22: (a) Representative images of persulfidation induced by **1a** and Na_2S in A549 scrambled and 3-MST KD cell lines using the modified tag-switch technique. The cells were imaged in the 20x GFP filter. Scale bar is 50 μm. (b) Quantification of the persulfidation signal induced by **1a** and Na_2S in A549 scrambled and 3-MST KD cell lines using the modified tag switch technique. All data are presented as mean ± SD (n =3/group). Significance was determined with respect to control in each cell line by Student's two-tailed unpaired parametric *t* test: ****$p < 0.0001$ versus ctrl group while ns = non-significant.



Scheme S5: (a) Proposed mechanism for consumption of **1a** during incubation with 3-MST in the presence of esterase. In the presence of ES, **1a** is converted to **2a**, which reacts with 3-MST to produce the 3-MST persulfide. Further reaction of 3-MST persulfide with **2a** should give 3-MST polysulfide. (b) Proposed mechanism for consumption of **1a** during incubation with 3-MST in the absence of esterase. The formation of a thioacetylated 3-MST is proposed, which can undergo hydrolysis to produce 3-MST persulfide. This pathway for polysulfide generation is likely minor since the reaction of **1a** with ES is fast.

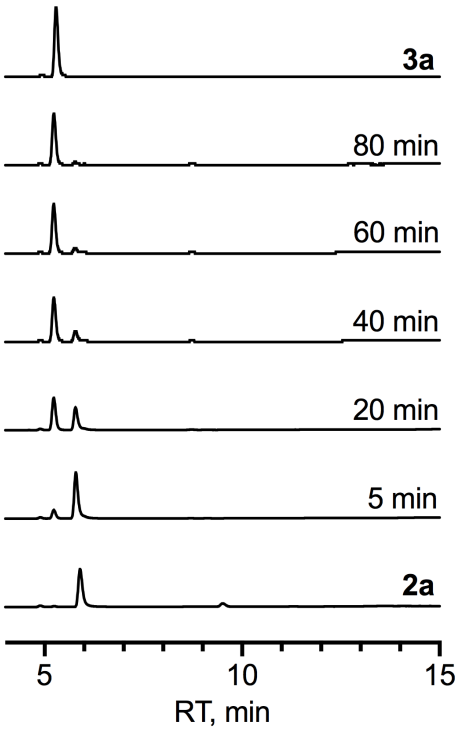


Figure S23: HPLC traces for the formation of acetophenone **3a** from **2a** in the presence of *b*3-MST and DTT over a period of 80 min. The rate constant k_1 for the decomposition of **2a** was found as 0.99 h^{-1}. The rate constant k_2 for the formation of acetophenone during this time was obtained as 0.75 h^{-1}.

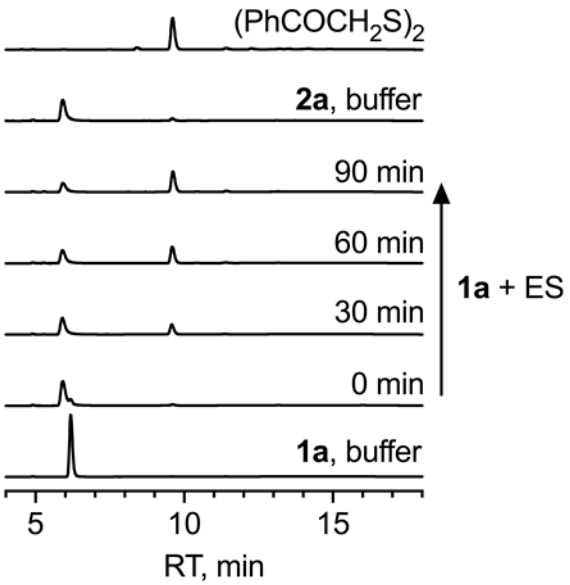


Figure S24: HPLC traces for the conversion of **1a** to **2a** during incubation with esterase (ES). The time points considered were 0 min, 30 min, 60 min and 90 min. In the absence of DTT or GSH, evidence for the formation of the disulfide $(PhCOCH_2S)_2$ (likely due to aerobic

oxidation) was observed. Based on injection of the corresponding standards, the combined yield of **2a** and its disulfide $(PhCOCH_2S)_2$ was 96%.

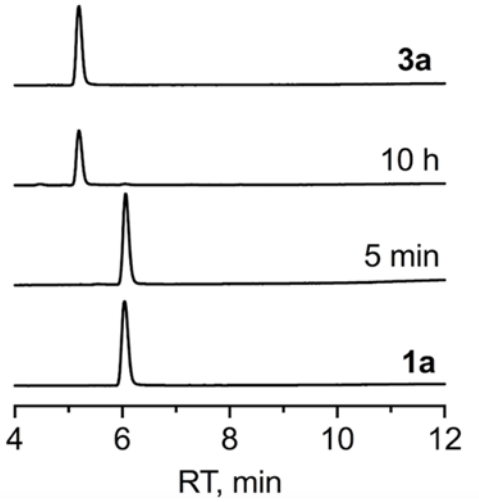


Figure S25: HPLC traces for the reaction mixture containing **1a** and *h*3-MST in the absence of esterase. The gradual formation of acetophenone (**3a**) was observed during 10 h (Abs = 250 nm).

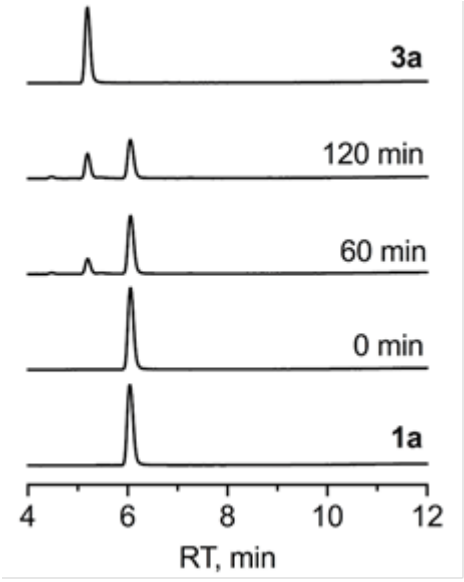


Figure S26: HPLC traces for the reaction mixture containing **1a** and *b*3-MST in the absence of esterase. The time points considered were 5 min, 60 min and 120 min. (Abs = 250 nm).

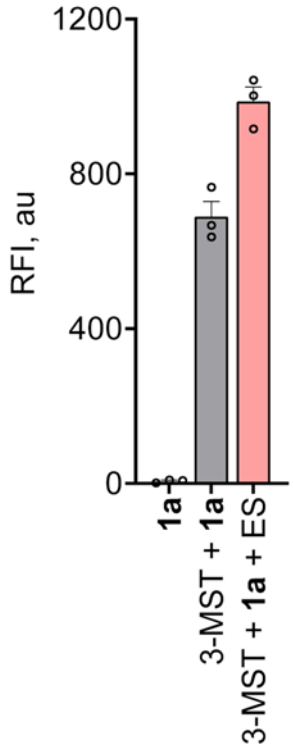
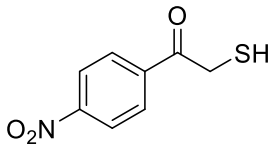
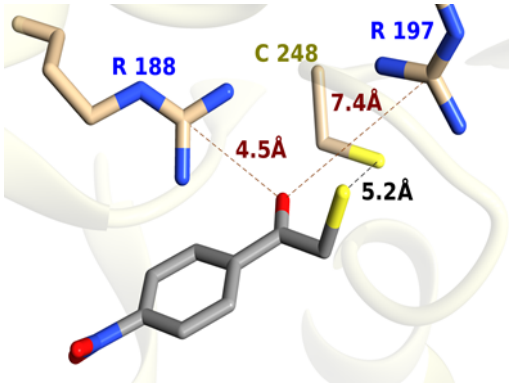
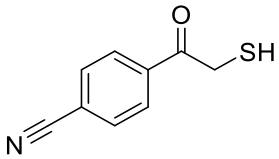
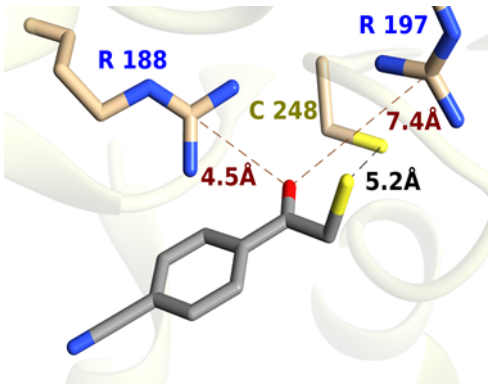
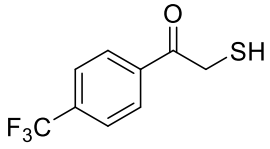
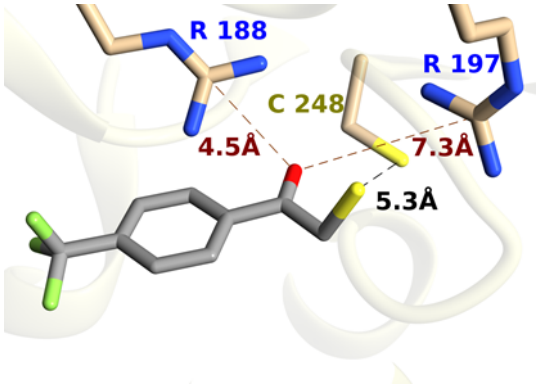
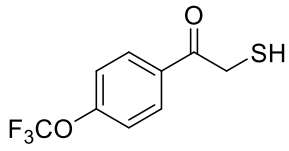
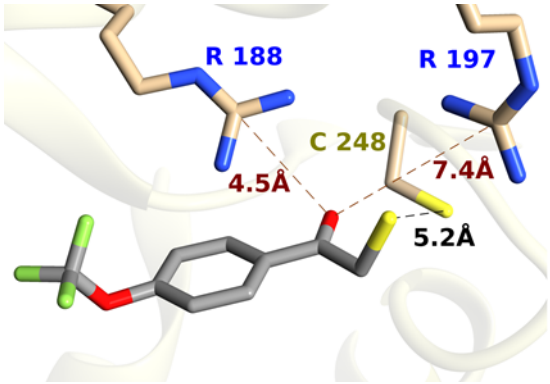
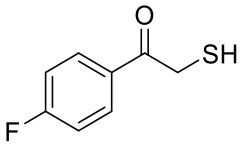
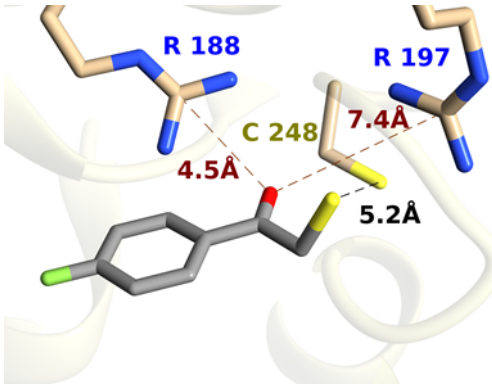
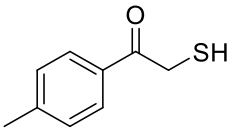
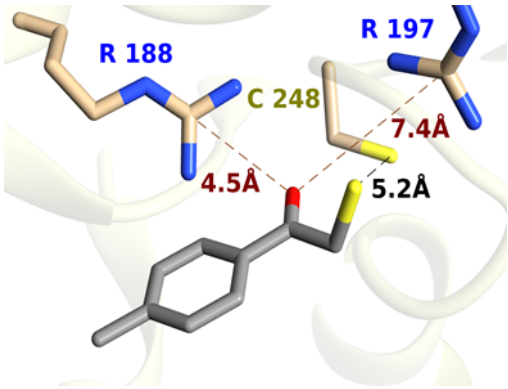
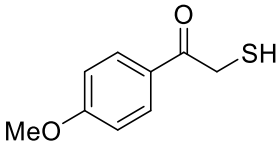
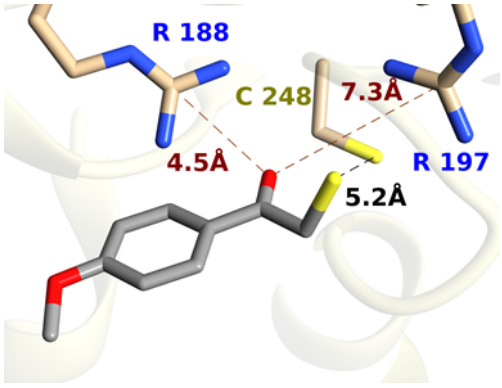
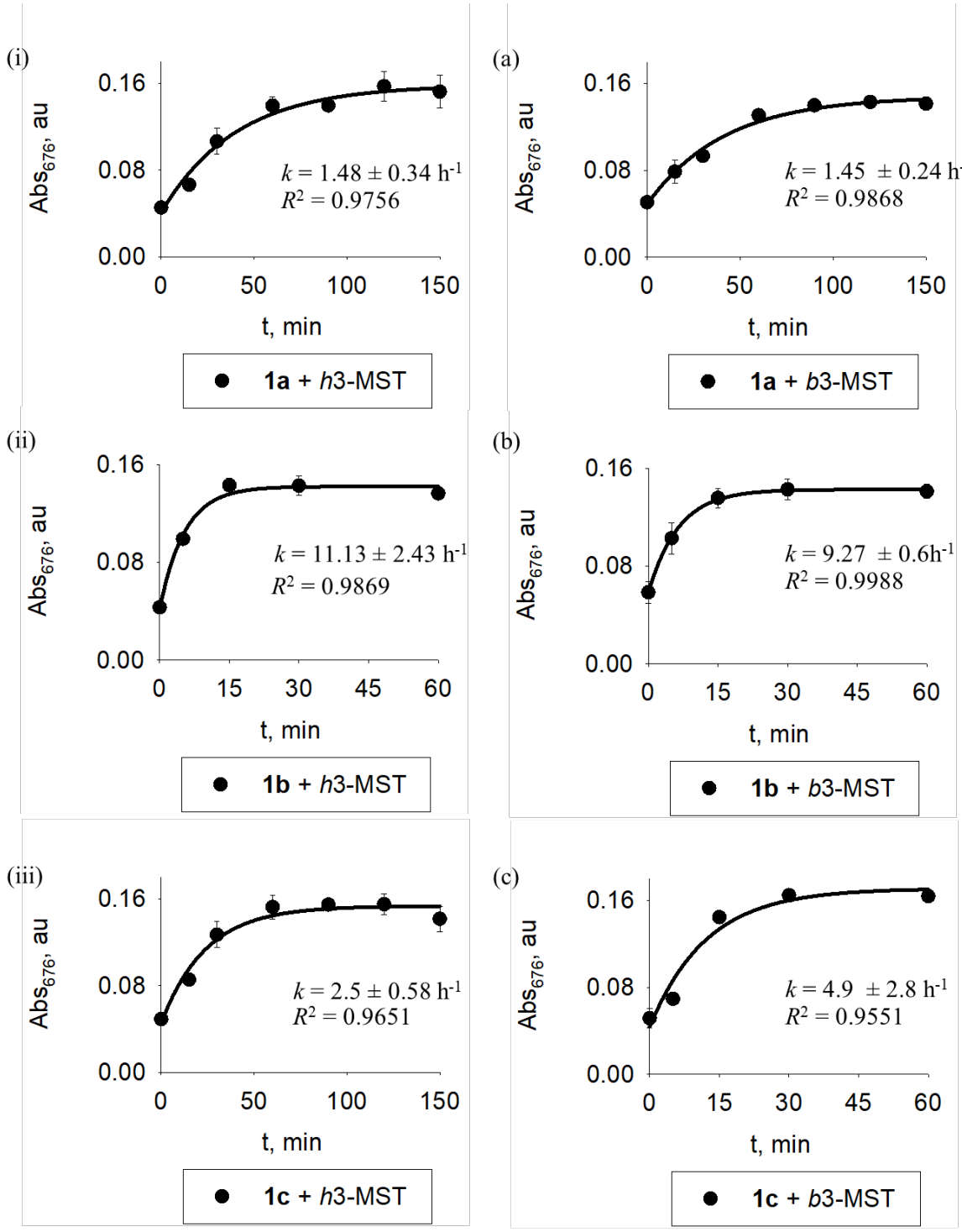


Figure S27: Fluorescence intensity data: Persulfide/polysulfide detection during incubation of **1a** alone, **1a** + *b*3-MST and **1a** + *b*3-MST in the presence of ES using the probe SSP-2 (λ_{ex} 482 nm, λ_{em} 518 nm). Analysis was carried out after 1 h.

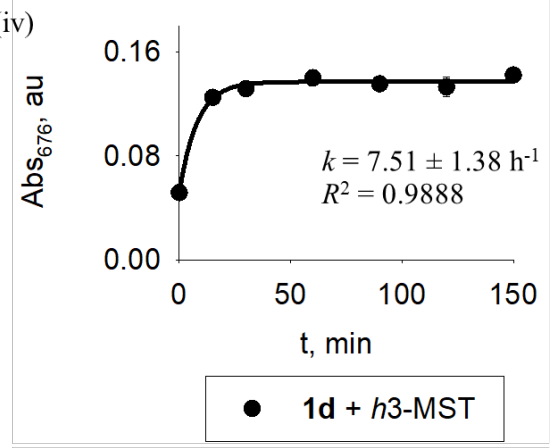
Table S5: Docking scores of **2b-2h** in the active site of *h*3-MST and the lowest energy conformations

Structure	Comp. No.	Docking score (kcal/mol)	Lowest energy conformation
	**2b**	-4.8	
	**2c**	-4.6	
	**2d**	-5.3	

	**2e**	-5.1	
	**2f**	-4.6	
	**2g**	-4.7	
	**2h**	-4.4	


(i)
Abs676, au
0.16
0.08
0.00
0
50
100
150
t, min
k = 1.48 ± 0.34 h-1
R2 = 0.9756
1a + h3-MST
(a)
Abs676, au
0.16
0.08
0.00
0
50
100
150
t, min
k = 1.45 ± 0.24 h-1
R2 = 0.9868
1a + b3-MST
(ii)
Abs676, au
0.16
0.08
0.00
0
15
30
45
60
t, min
k = 11.13 ± 2.43 h-1
R2 = 0.9869
1b + h3-MST
(b)
Abs676, au
0.16
0.08
0.00
0
15
30
45
60
t, min
k = 9.27 ± 0.6h-1
R2 = 0.9988
1b + b3-MST
(iii)
Abs676, au
0.16
0.08
0.00
0
50
100
150
t, min
k = 2.5 ± 0.58 h-1
R2 = 0.9651
1c + h3-MST
(c)
Abs676, au
0.16
0.08
0.00
0
15
30
45
60
t, min
k = 4.9 ± 2.8 h-1
R2 = 0.9551
1c + b3-MST

(iv)


0.16
0.08
0.00
Abs676, au
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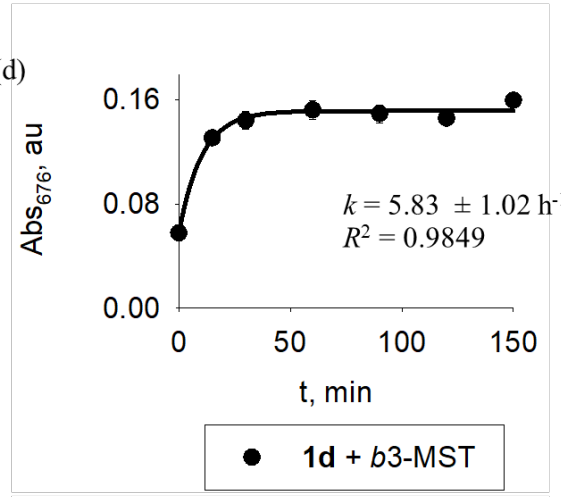
R2 = 0.9888
0
50
100
150
t, min
1d + h3-MST

(d)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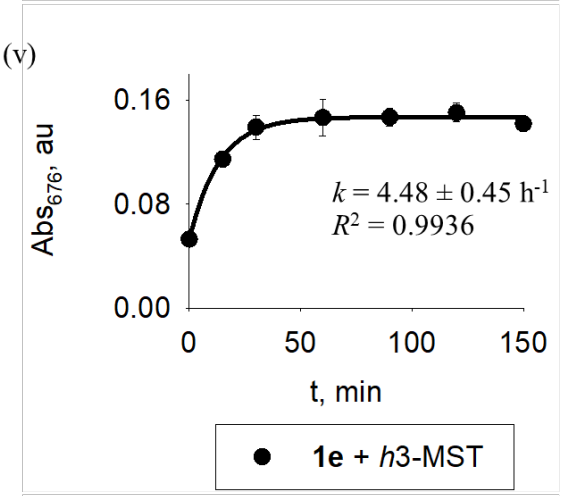
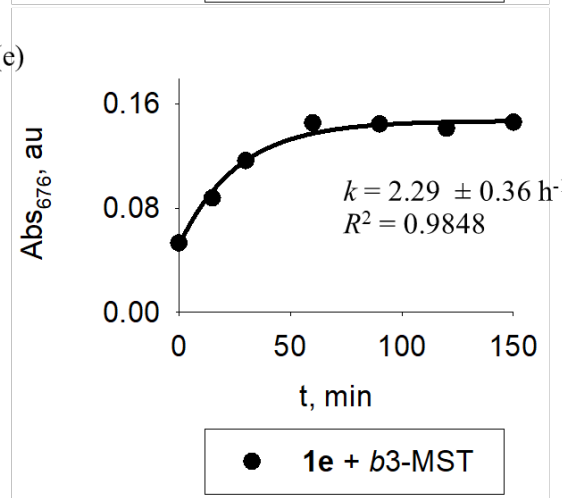
R2 = 0.9849
0
50
100
150
t, min
1d + b3-MST

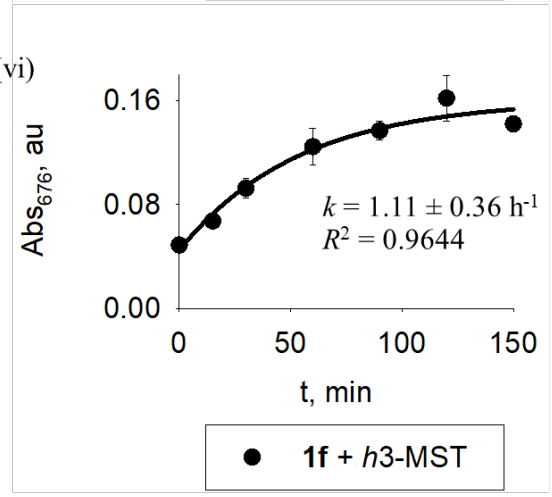
(v)


0.16
0.08
0.00
Abs676, au
k = 4.48 ± 0.45 h-1
R2 = 0.9936
0
50
100
150
t, min
1e + h3-MST

(e)


0.16
0.08
0.00
Abs676, au
k = 2.29 ± 0.36 h-1
R2 = 0.9848
0
50
100
150
t, min
1e + b3-MST

(vi)


0.16
0.08
0.00
Abs676, au
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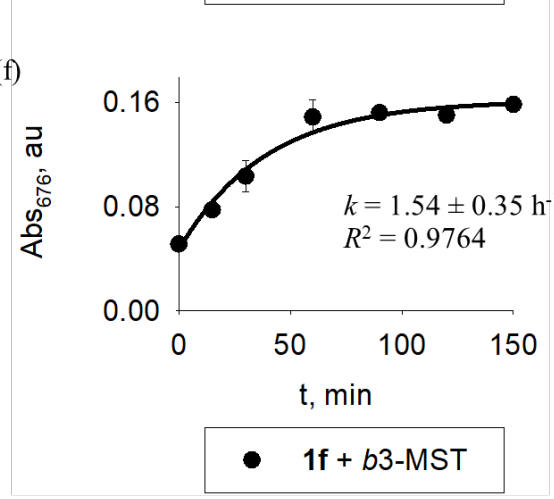
R2 = 0.9644
0
50
100
150
t, min
1f + h3-MST

(f)


0.16
0.08
0.00
Abs676, au
k = 1.54 ± 0.35 h-1
R2 = 0.9764
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50
100
150
t, min
1f + b3-MST

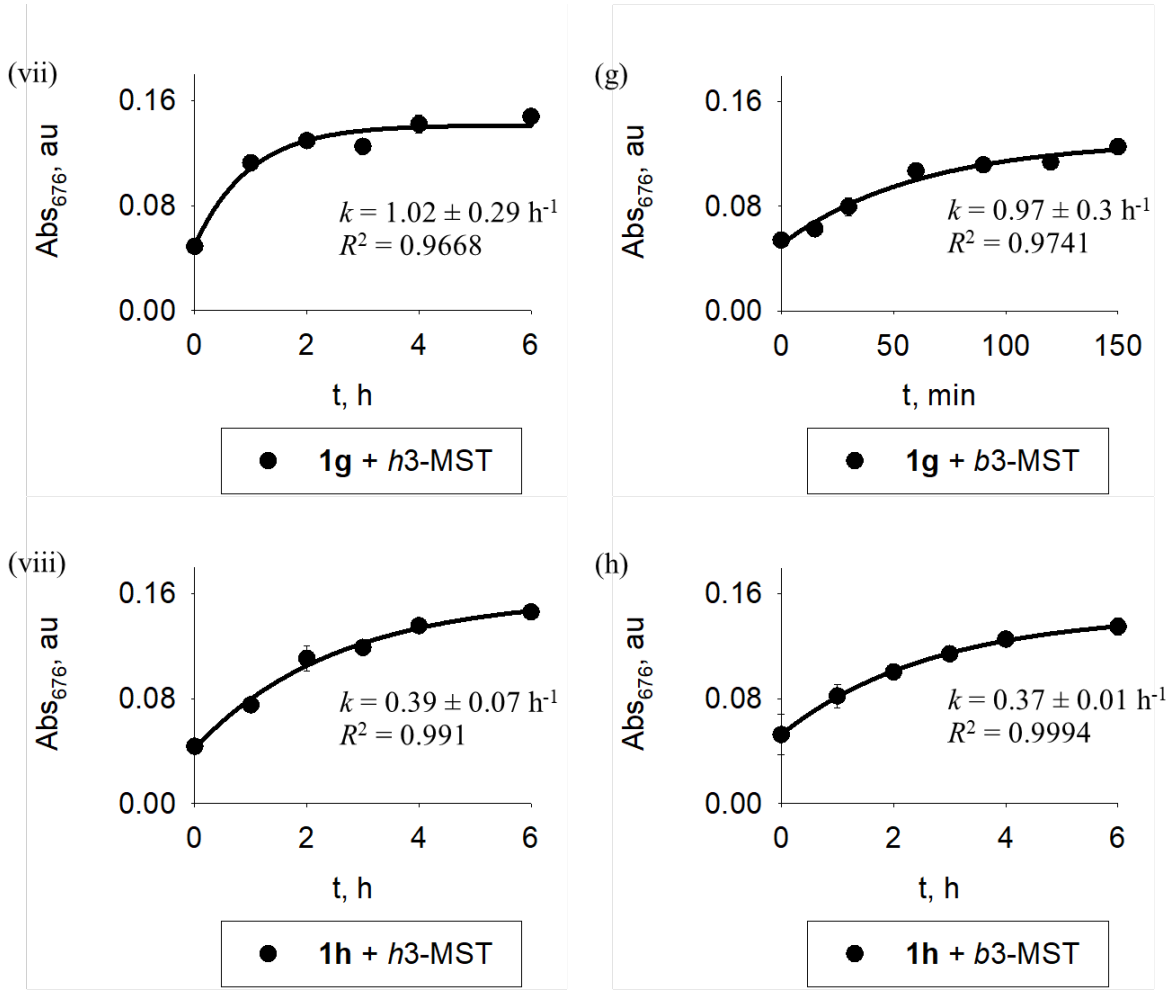


Figure S28: (i-vii) Formation of methylene blue during incubation of compounds **1a-1h** in the presence of wt *h*3-MST. (a-h) Formation of methylene blue during incubation of compounds **1a-1h** in the presence of wt *b*3-MST. Curve fitting using a pseudo first order equation $y = y_0 + a(1 - e^{-kx})$ was carried out. In the absence of the enzyme 3-MST, H_2S was not detected from any of the above substrates,

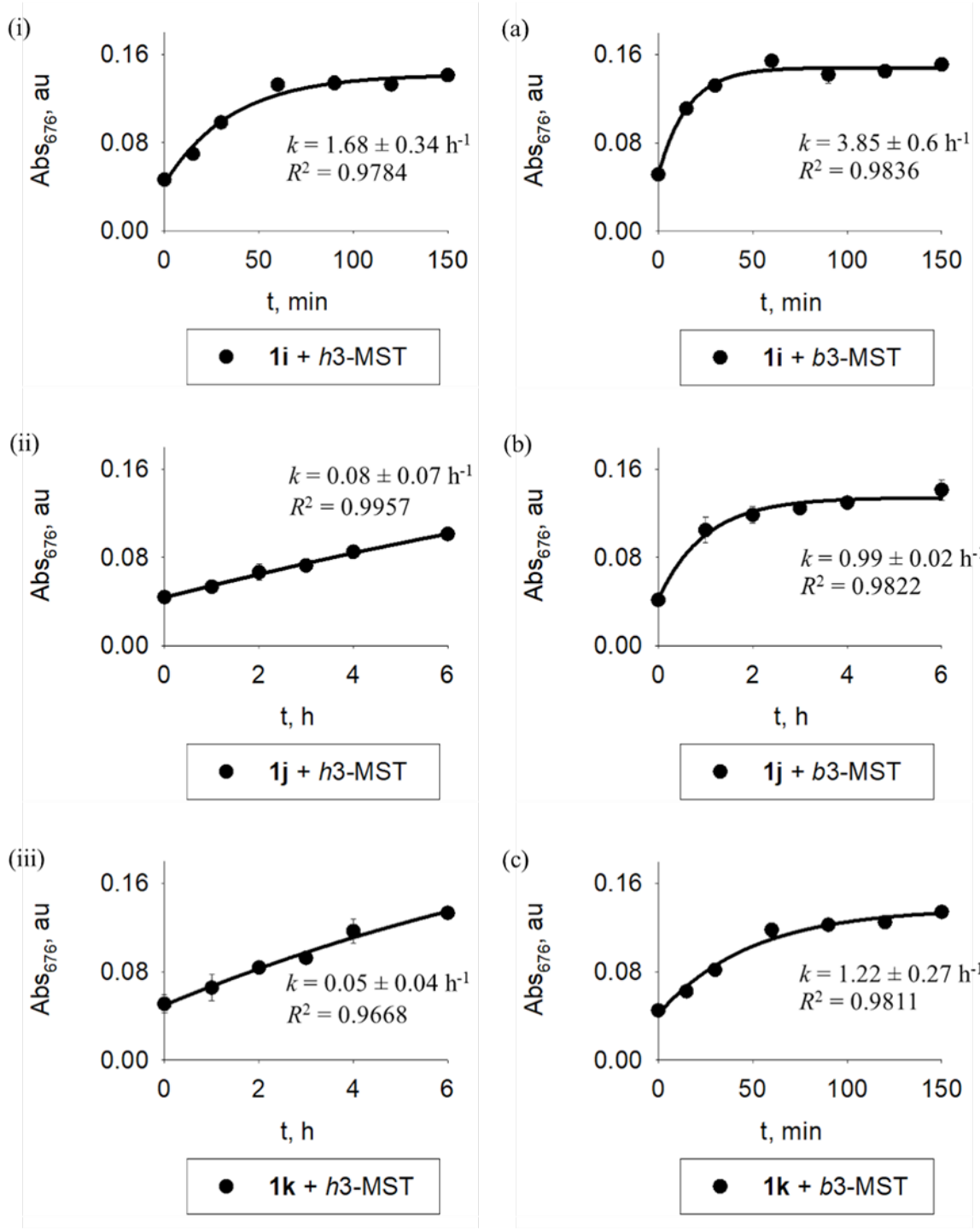
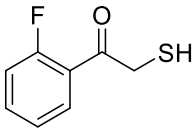
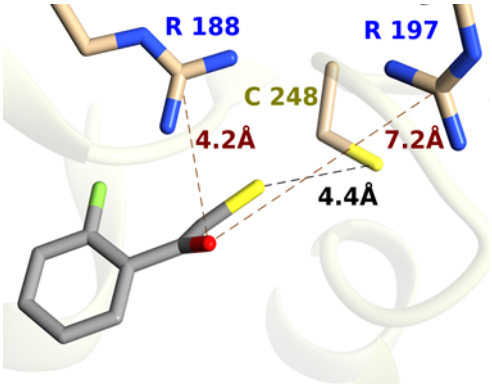
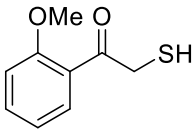
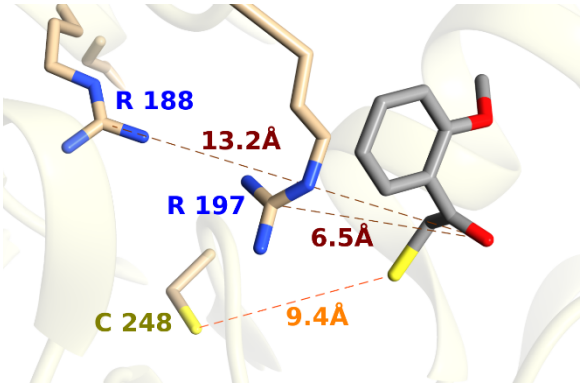
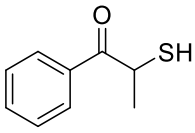
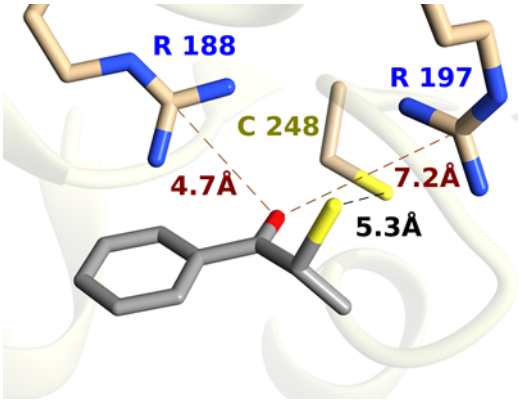


Figure S29: (i-iii) Formation of methylene blue during incubation of compounds **1i-1k** in the presence of wt *h*3-MST. (a-c) Formation of methylene blue during incubation of compounds **1i-1k** in the presence of wt *b*3-MST. Curve fitting using a pseudo first order equation $y = y_0 + a(1 - e^{-kx})$ was carried out. In the absence of the enzyme 3-MST, H_2S was not detected from any of the above substrates.

Table S6: Docking scores of compounds **2i, 2j, 2l, 2m** and **2n** in the active site of *h*3-MST and the lowest energy conformations

Structure	Comp. No.	Docking score (kcal/mol)	Lowest energy conformation
	**2i**	-4.2	
	**2j**	NA	
	**2l**	-4.8	

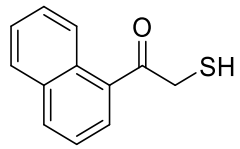
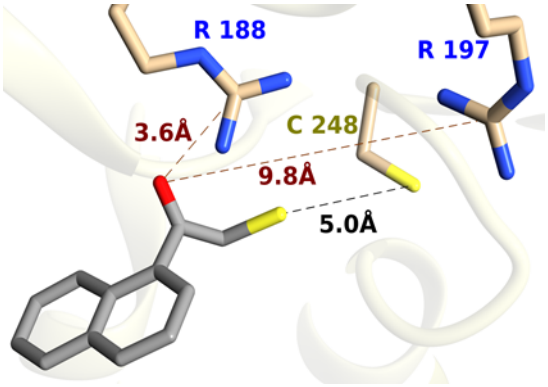
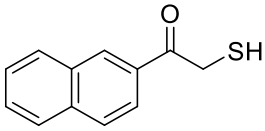
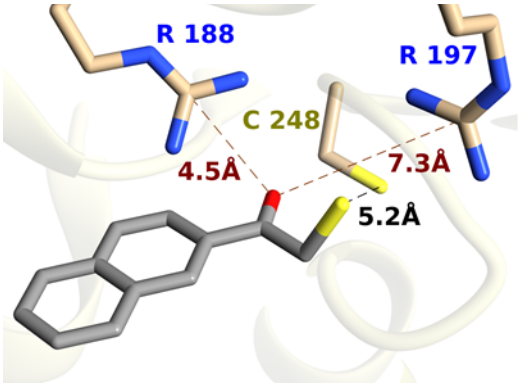
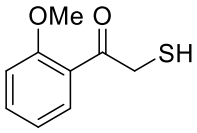
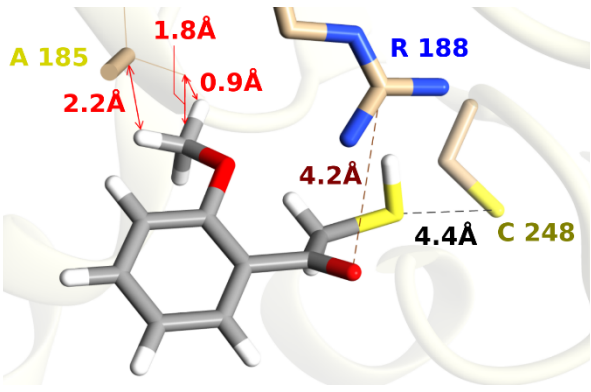
	**2m**	-4.6	
	**2n**	-5.7	

Table S7: Docking of thiol **2j** in the active site of *h*3-MST in the high energy conformation

Structure	**Comp. No.**	**Docking score (kcal/mol)**	**High energy conformation**
	**2j**	NA	

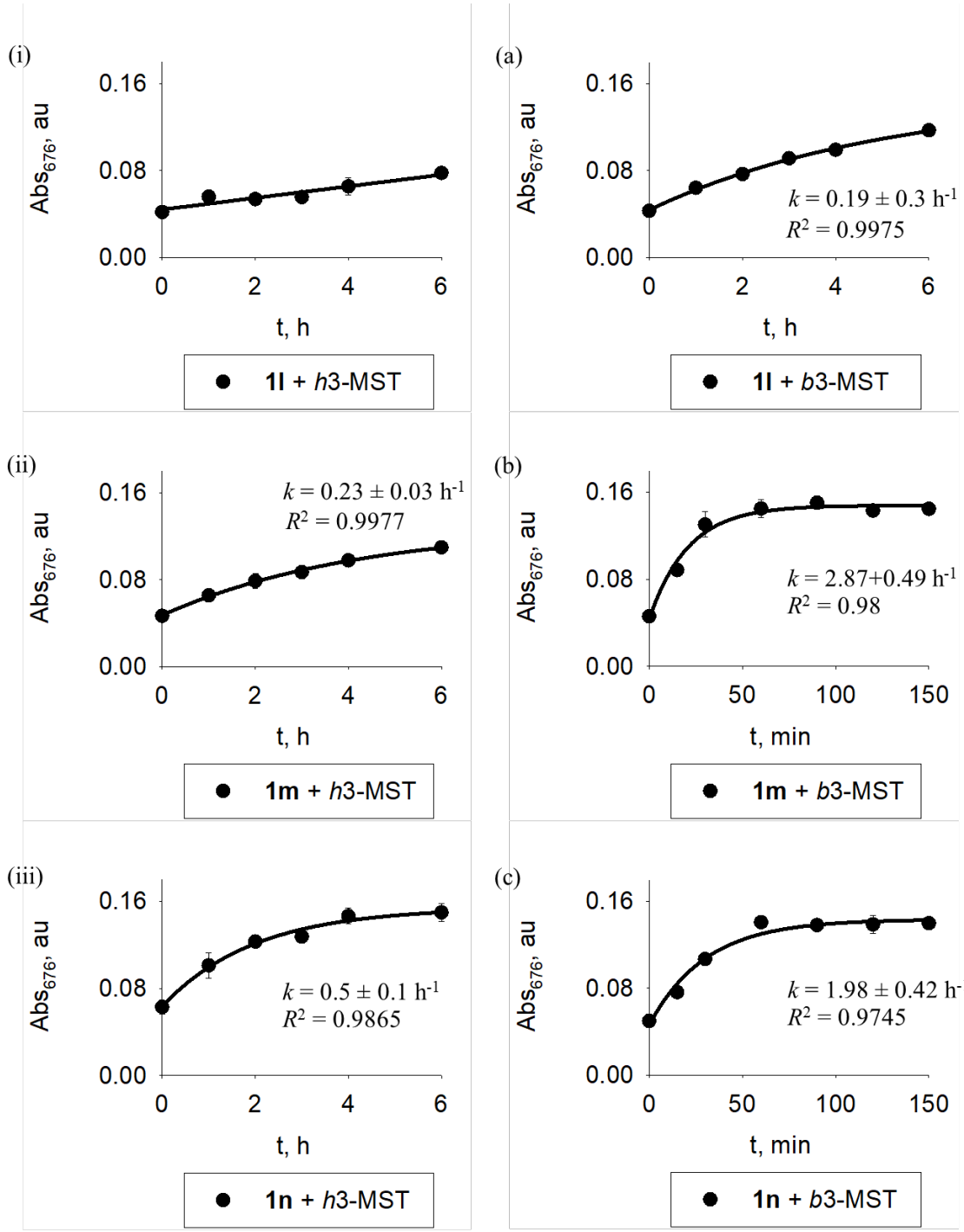


Figure S30: (i-iii) Formation of methylene blue during incubation of compounds **1l-1n** in the presence of wt *h*3-MST. (a-c) Formation of methylene blue during incubation of compounds **1l-1n** in the presence of wt *b*3-MST. Curve fitting using a pseudo first order equation $y = y_0 + a(1 - e^{-kx})$ was carried out. In the absence of the enzyme 3-MST, H_2S was not detected from any of the above substrates.

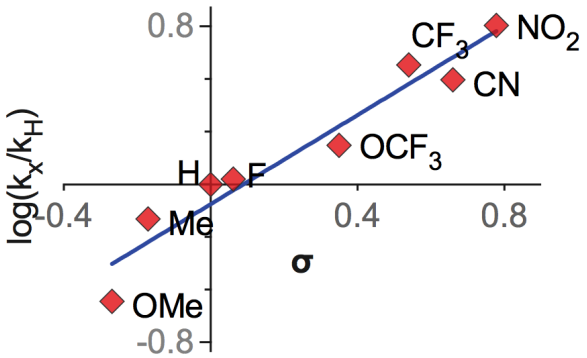


Figure S31: Hammett analysis of rate constants of H_2S generation from unnatural substrates (see Table 1) with wt *b*3-MST. Linear regression analysis yielded a slope of +1.13 ($R^2 = 0.935$).

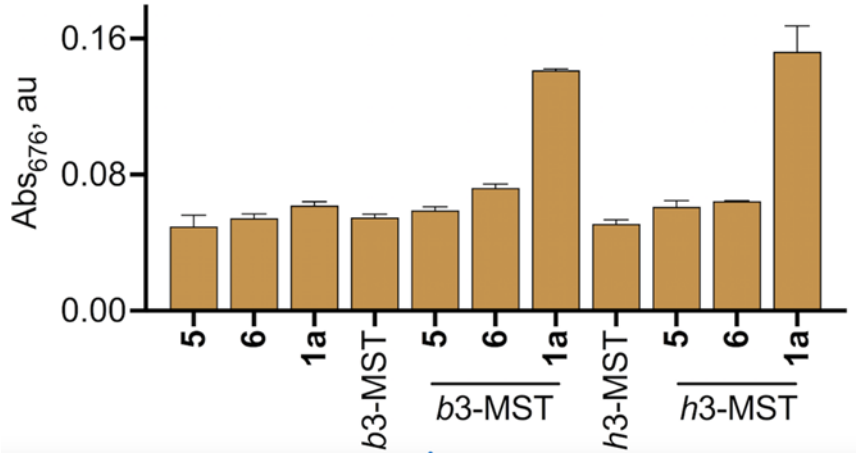


Figure S32: Comparison of absorbance corresponding to the formation of methylene blue during the incubation of **1a**, **5** or **6** in the presence of wt *b*3-MST or *h*3-MST. Compound **1a** in the absence of either enzymes do not generate H_2S. Negative controls **5** and **6** do not generate H_2S in the presence or absence of either *b*3-MST or *h*3-MST. The time point for carrying out the methylene blue assay was 150 min. All data are presented as mean ± SD (n =3/group).

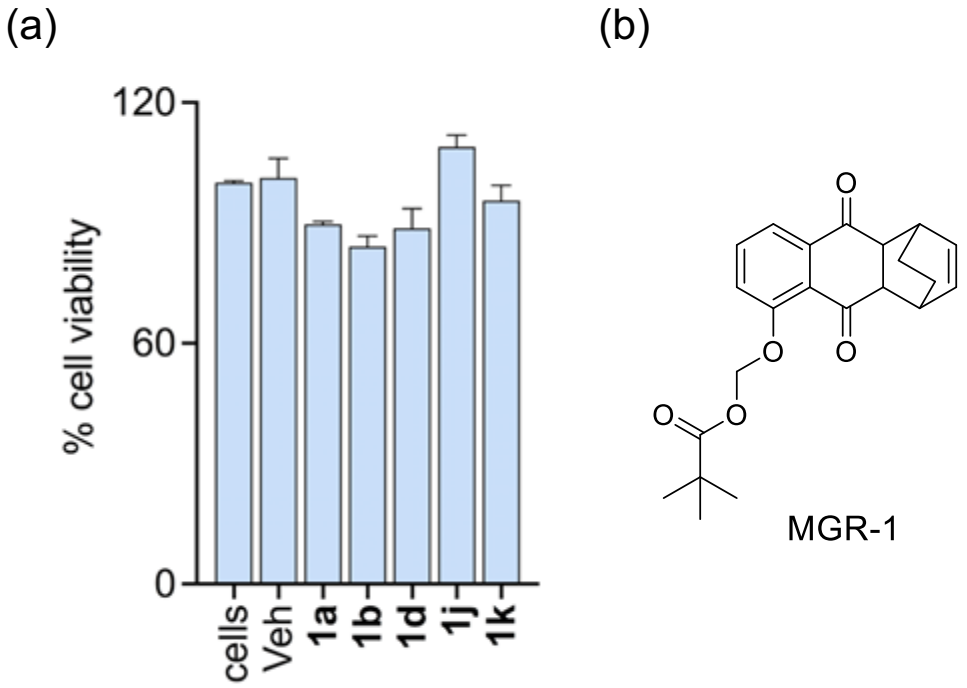


Figure S33: (a) Cell viability assay carried out on N2a cells. The compounds **1a**, **1b**, **1d**, **1j** and **1k** were independently incubated at 25 μM for 12 h and viable cells were determined using a standard cell viability assay. Veh = 0.5% DMSO. All data are presented as mean ± SD (n =3 per group). (b) Structure of MGR-1, a cell-permeable ROS generator used in this study.

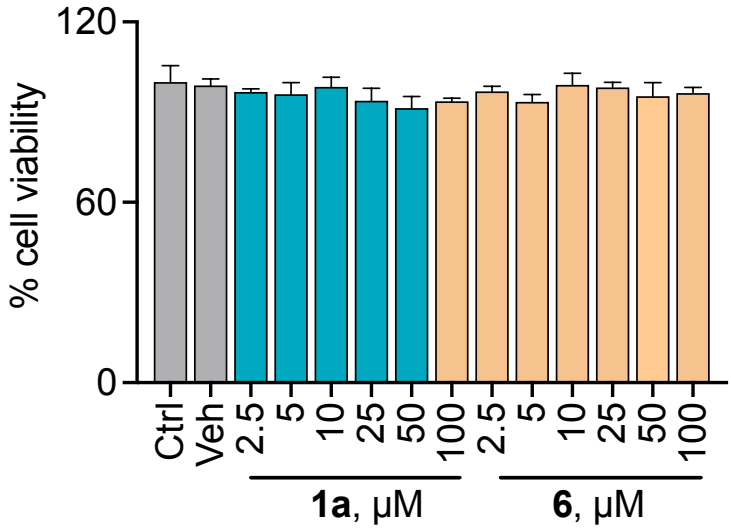


Figure S34: Cell viability assay carried out with substrate **1a** as well as negative control **6** on N2a cells for 12 h. No significant change in viable cells upon treatment with these compounds when compared with control was found. All data are presented as mean ± SD (n =3/group).

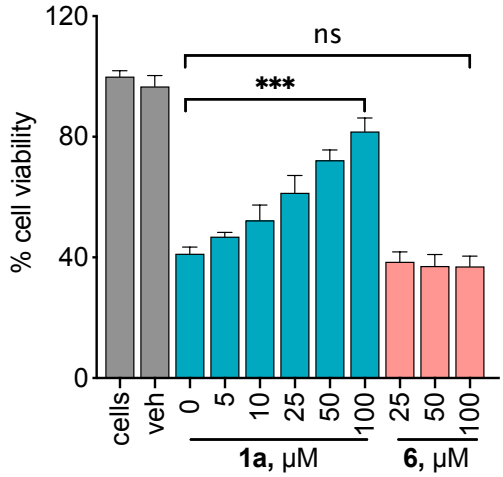


Figure S35: Cell viability assay conducted on N2a cells: Cells were first treated with **1a** or **6** followed by treatment with menadione, a known inducer of oxidative stress in cells. Dose-dependent protection of cells from menadione-induced cell death by **1a** was observed. Negative control **6** did not show any protective effect in this assay. All data are presented as mean ± SD (n =3/group). Student's two-tailed unpaired parametric *t*-test was carried out to determine significance: All data are presented as mean ± SD (n =3/group). ***$p < 0.001$ vs menadione; ns = non-significant compared to menadione only.

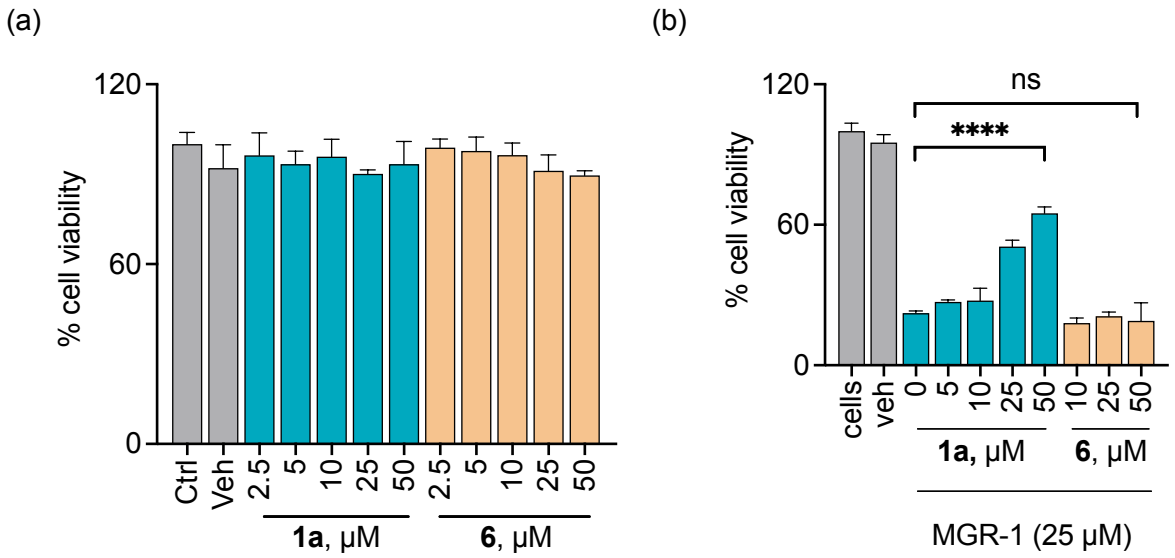


Figure S36: Cell viability assay conducted on mouse embryonic fibroblast (MEF) cells: a) Cells were treated with **1a** or **6** and a standard cell viability assay was used to assess the number of viable cells after 24 h. No significant effect of **1a** or **6** on MEF cell viability was observed. b) MEF cells were first treated with **1a** or **6** followed by treatment with MGR-1, a known inducer of oxidative stress in cells. Dose-dependent protection of cells from MGR-1-induced cell death by **1a** was observed. Negative control **6** did not show any significant cytoprotective effect under similar conditions. Student's two-tailed unpaired parametric *t*-test was carried out to determine significance: All data are presented as mean ± SD (n =3/group). ****$p < 0.0001$ vs MGR-1; ns = non-significant versus MGR-1 only.

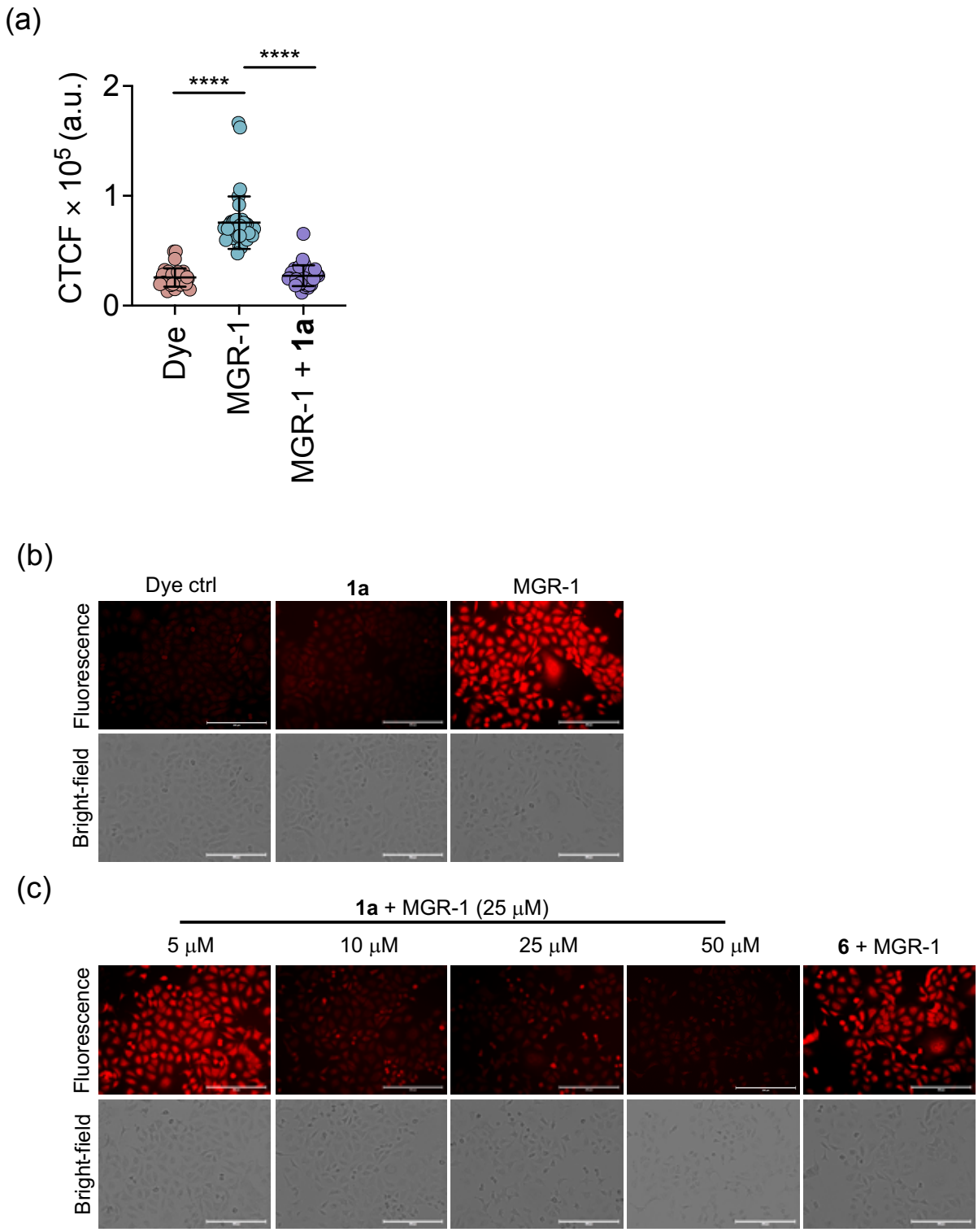


Figure S37: (a) Quantification of intracellular H_2O_2 generated by MGR-1, measured using the dye TCF-B (Figure 5A). A549 cells were treated with: Dye control; 25 μM MGR-1; 25 μM of **1a** for 12 h followed by addition of the 25 μM MGR-1 for 1 h. All data are presented as mean ± SD (n =3/group). ****p < 0.0001 vs MGR-1.

(b) A549 cells were treated with veh control (DMSO), **1a** (50 μM) and MGR-1 (25 μM)

(c) A549 cells treated with varying concentrations of **1a** (5 μM, 10 μM, 25 μM and 50 μM) or **6** (50 μM) for 12 h followed by treatment with MGR-1 (25 μM). Intracellular H_2O_2 was detected using the H_2O_2-sensitive turn-on fluorescence sensor TCF-B. Cells were imaged using a 20x TxRed filter. Scale bar is 200 μm.

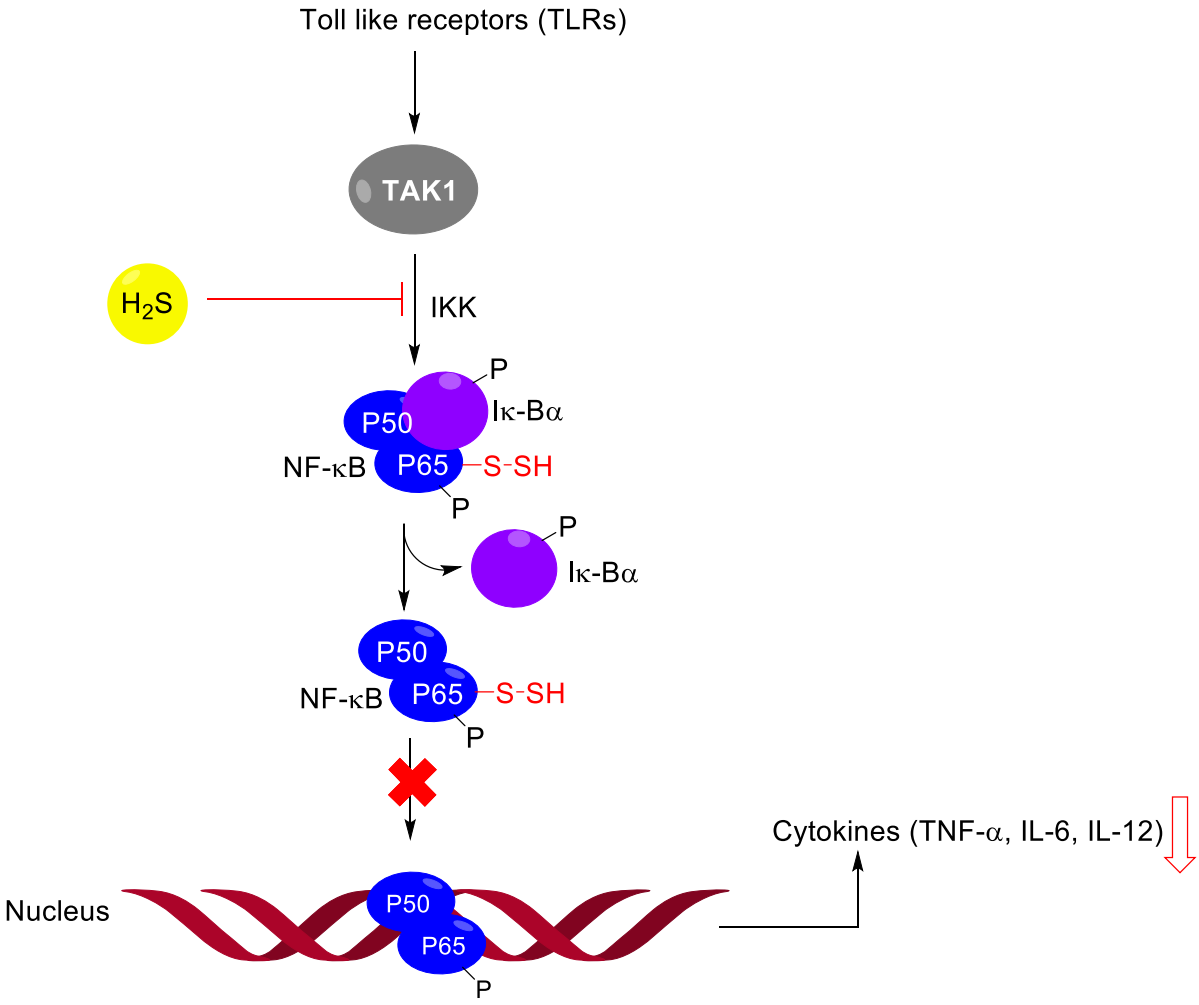


Figure S38: Some mechanistic insights into the anti-inflammatory roles of persulfides. Previous studies have shown that polysulfides inhibit the phosphorylation of Iκ-Bα and NF-κB resulting in a deactivation of the Iκ-Bα/ NF-κB axis and ultimately supressing the production of cytokines (red arrow indicates downregulation).[28] Another report suggests that H_2S can persulfidate the Cys38 residue in the p65 subunit of NF-κB which not only inhibited its activation but also inhibited the phosphorylation of Iκ-Bα and its subsequent degradation.[29] H_2S was also found to inhibit the phosphorylation of NF-κB, its nuclear translocation and its DNA binding activity.

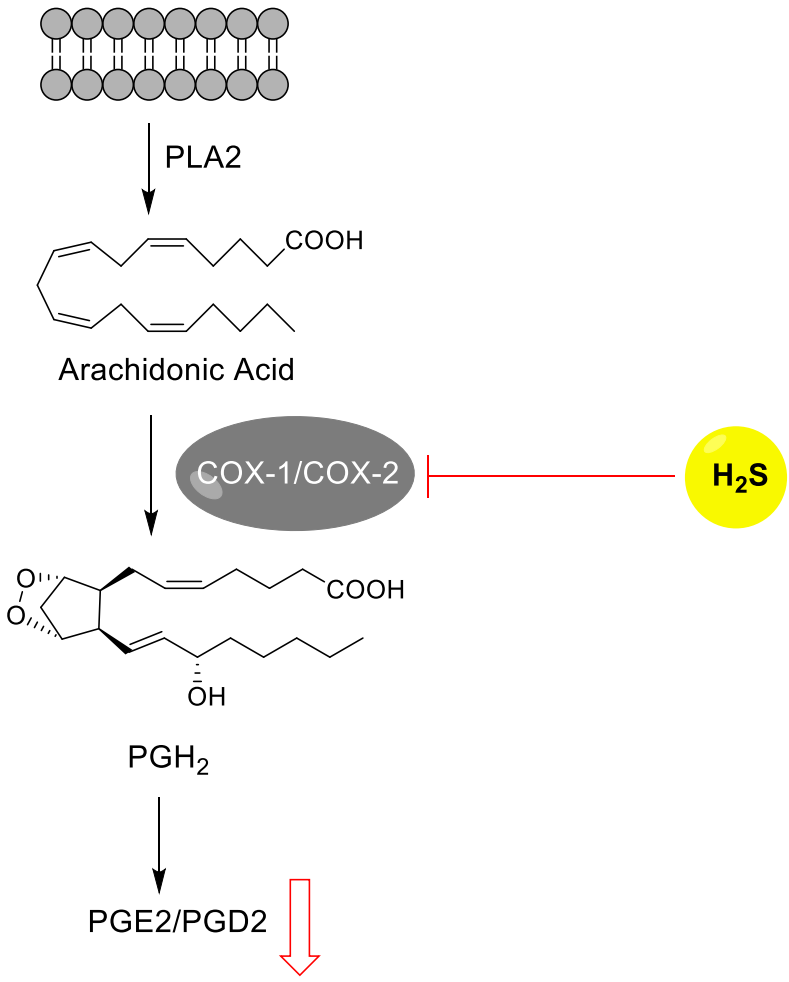


Figure S39: Mechanistic insights into pro-inflammatory prostaglandins formation. LPS, a component of the gram-negative bacteria induces the expression of cytosolic phospholipase A2 (PLA2). Arachidonic acid (AA) is then released from the cell membrane, catalysed by PLA2 which can then be converted to cyclic endoperoxides PGH_2 by COX enzymes, leading to the downstream production of pro-inflammatory prostaglandins. Several reports suggest that H_2S-NSAID hybrids can exhibit potent anti-inflammatory effects by inhibiting the expression of COX-2, which is an inducible enzyme, leading to the inhibition of PG production.

5. References

1 T. Hatanaka, R. Yuki, R. Saito and K. Sasaki, *Org. Biomol. Chem.*, 2016, **14**, 10589–10592.

2 J. Z. Chandanshive, B. F. Bonini, D. Gentili, M. Fochi, L. Bernardi and M. C. Franchini, *European J. Org. Chem.*, 2010, **2010**, 6440–6447.

3 H. T. Nagasawa, D. J. W. Goon, D. L. Crankshaw, R. Vince and S. E. Patterson, *J. Med. Chem.*, 2007, **50**, 6462–6464.

4 G. Crank and H. R. Khan, *Formation of Thioamide Derivatives from Reactions of Isothiocyanates with Oxazol-2-amines*, 1985, vol. 38.

5 D. Zhang, N. O. Devarie-Baez, Q. Li, J. R. Lancaster and M. Xian, *Org. Lett.*, 2012, **14**, 3396–3399.

6 R. Wedmann, C. Onderka, S. Wei, I. A. Szijártó, J. L. Miljkovic, A. Mitrovic, M. Lange, S. Savitsky, P. K. Yadav, R. Torregrossa, E. G. Harrer, T. Harrer, I. Ishii, M. Gollasch, M. E. Wood, E. Galardon, M. Xian, M. Whiteman, R. Banerjee and M. R. Filipovic, *Chem. Sci.*, 2016, **7**, 3414–3426.

7 D. S. Kelkar, G. Ravikumar, N. Mehendale, S. Singh, A. Joshi, A. K. Sharma, A. Mhetre, A. Rajendran, H. Chakrapani and S. S. Kamat, *Nat. Chem. Biol.*, 2019, **15**, 169–178.

8 A. A. Heredia, S. M. Soria-Castro, L. M. Bouchet, G. Oksdath-Mansilla, C. A. Barrionuevo, D. A. Caminos, F. R. Bisogno, J. E. Argüello and A. B. Peñéñory, *Org. Biomol. Chem.*, 2014, **12**, 6516–6526.

9 J. M. Lopp and V. A. Schmidt, *Org. Lett.*, 2019, **21**, 8031–8036.

10 A. Przydacz, R. Kowalczyk and Ł. Albrecht, *Org. Biomol. Chem.*, 2017, **15**, 9566–9569.

11 J. Tatar, M. Baranac-Stojanović, M. Stojanović and R. Marković, *Tetrahedron Lett.*, 2009, **50**, 700–703.

12 V. S. Velingkar and V. D. Dandekar, *Chinese J. Chem.*, 2011, **29**, 504–510.

13 D. Zhang, I. Macinkovic, N. O. Devarie-Baez, J. Pan, C.-M. Park, K. S. Carroll, M. R. Filipovic and M. Xian, *Angew. Chemie Int. Ed.*, 2014, **53**, 575–581.

14 M.-Y. Wu, K. Li, C.-Y. Li, J.-T. Hou and X.-Q. Yu, *Chem. Commun.*, 2014, **50**, 183–185.

15 P. Chauhan, S. Jos and H. Chakrapani, *Org. Lett.*, 2018, **20**, 3766–3770.

16 A. C. Sedgwick, H.-H. Han, J. E. Gardiner, S. D. Bull, X.-P. He and T. D. James, *Chem. Commun.*, 2017, **53**, 12822–12825.

17 F. van den Ent and J. Löwe, *J. Biochem. Biophys. Methods*, 2006, **67**, 67–74.

18 P. K. Yadav, K. Yamada, T. Chiku, M. Koutmos and R. Banerjee, *J. Biol. Chem.*, 2013, **288**, 20002–13.

19 J.-C. Lec, S. Boutserin, H. Mazon, G. Mulliert, S. Boschi-Muller and F. Talfournier, *ACS Catal.*, 2018, **8**, 2049–2059.

20 B. Yu, Y. Zheng, Z. Yuan, S. Li, H. Zhu, L. K. De La Cruz, J. Zhang, K. Ji, S. Wang and B. Wang, *J. Am. Chem. Soc.*, 2018, **140**, 30–33.

21 W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian, *Chem. Sci.*, 2013, **4**, 2892.

22 T. Ida, T. Sawa, H. Ihara, Y. Tsuchiya, Y. Watanabe, Y. Kumagai, M. Suematsu, H. Motohashi, S. Fujii, T. Matsunaga, M. Yamamoto, K. Ono, N. O. Devarie-Baez, M. Xian, J. M. Fukuto and T. Akaike, *Proc. Natl. Acad. Sci. U.S.A*, 2014, **111**, 7606–7611.

23 A. K. Mustafa, M. M. Gadalla, N. Sen, S. Kim, W. Mu, S. K. Gazi, R. K. Barrow, G. Yang, R. Wang and S. H. Snyder, *Sci. Signal.*, 2009, **2**, ra72 LP-ra72.

24 D. J. Leggett, N. H. Chen and D. S. Mahadevappa, *Anal. Chim. Acta*, 1981, **128**, 163–168.

25 A. K. Sharma, M. Nair, P. Chauhan, K. Gupta, D. K. Saini and H. Chakrapani, *Org. Lett.*, 2017, **19**, 4822–4825.

26 C. Wei, Q. Zhu, W. Liu, W. Chen, Z. Xi and L. Yi, *Org. Biomol. Chem.*, 2014, **12**, 479–485.

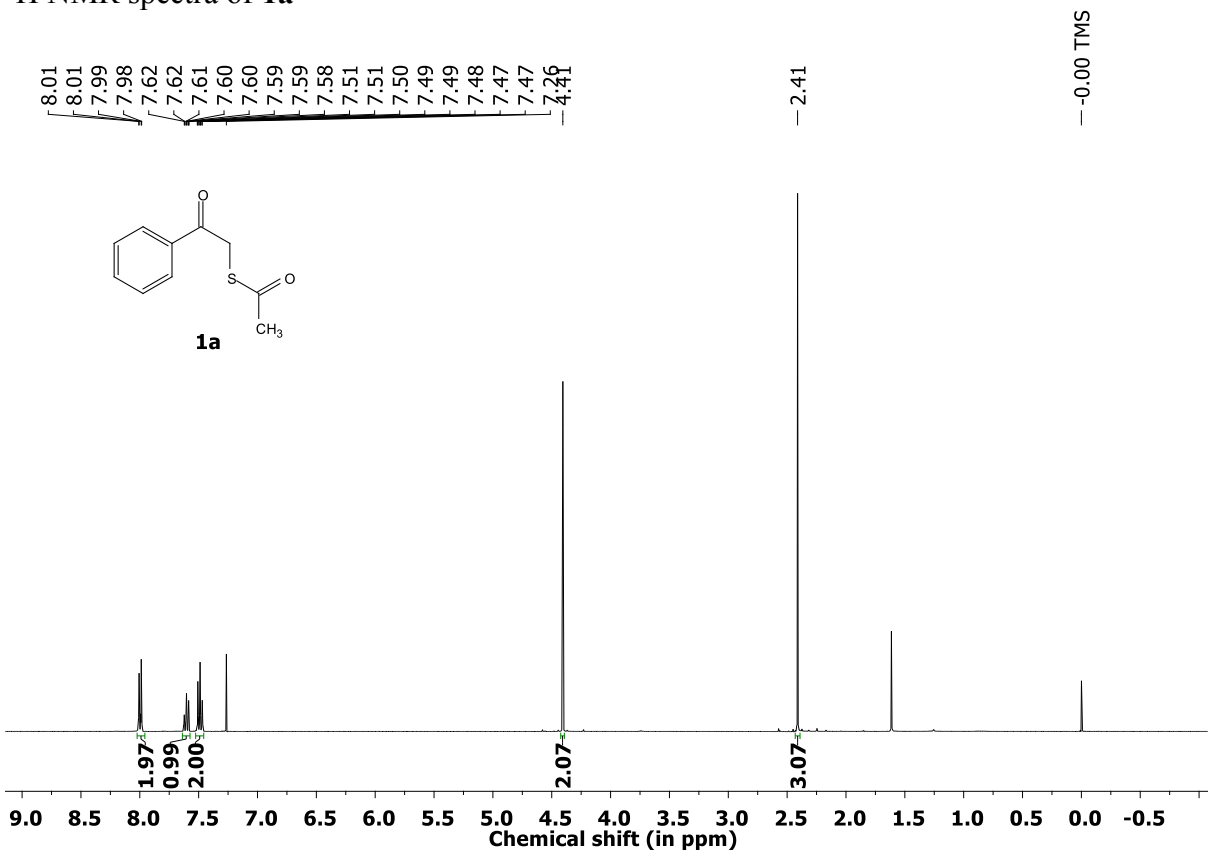
27 D. K. Nomura, B. E. Morrison, J. L. Blankman, J. Z. Long, S. G. Kinsey, M. C. G. Marcondes, A. M. Ward, Y. K. Hahn, A. H. Lichtman, B. Conti and B. F. Cravatt, *Science (80-.).*, 2011, **334**, 809–813.

28 T. Zhang, K. Ono, H. Tsutsuki, H. Ihara, W. Islam, T. Akaike and T. Sawa, *Cell Chem. Biol.*, 2019, **26**, 686-698.e4.

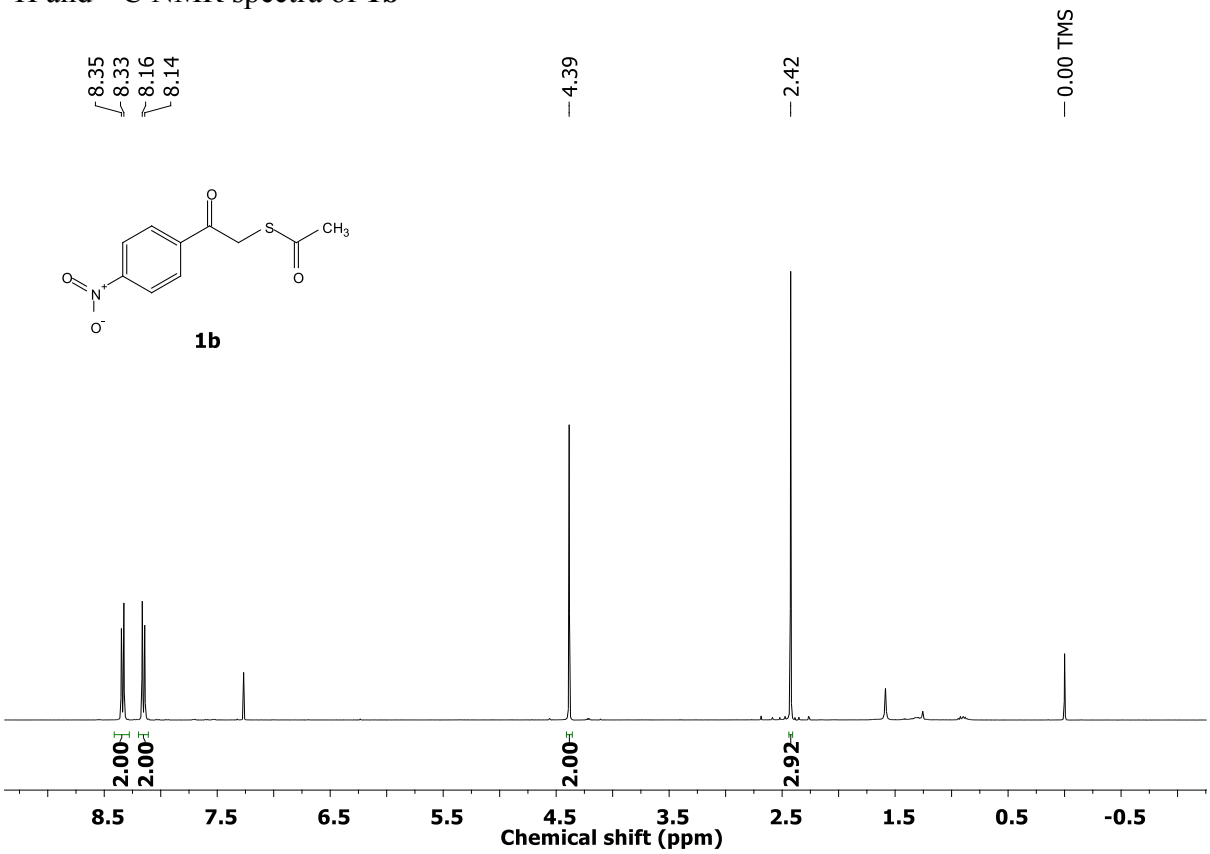
29 J. Du, Y. Huang, H. Yan, Q. Zhang, M. Zhao, M. Zhu, J. Liu, S. X. Chen, D. Bu, C. Tang and H. Jin, *J. Biol. Chem.*, 2014, **289**, 9741–9753.

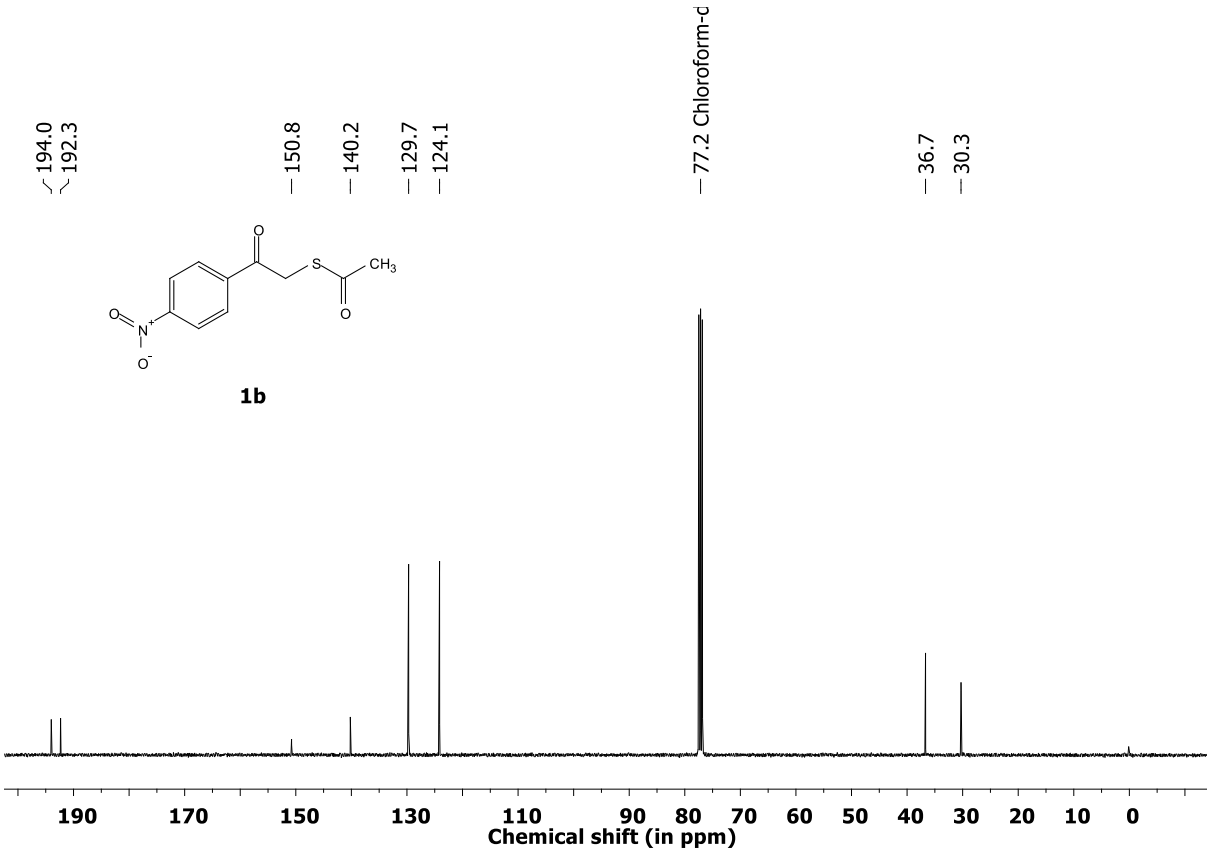
6. NMR spectra of compounds

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



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





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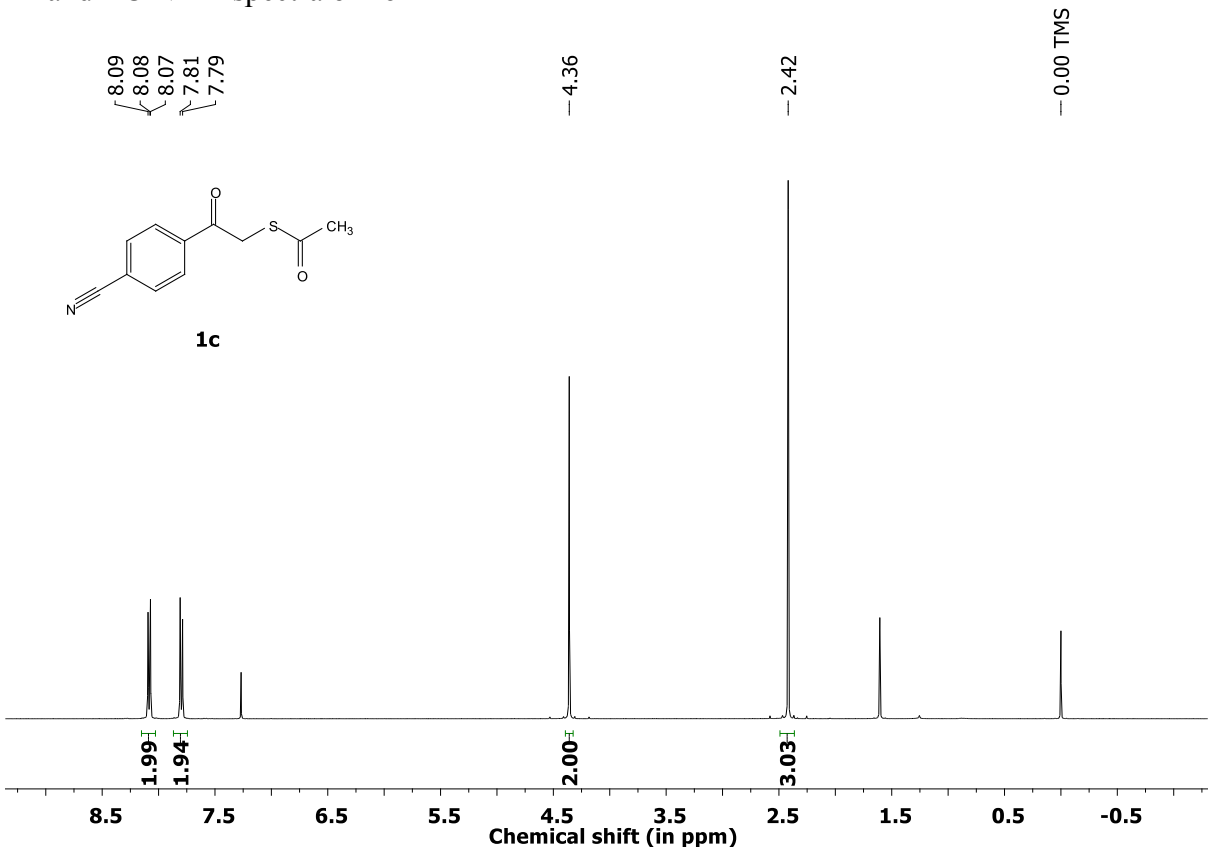
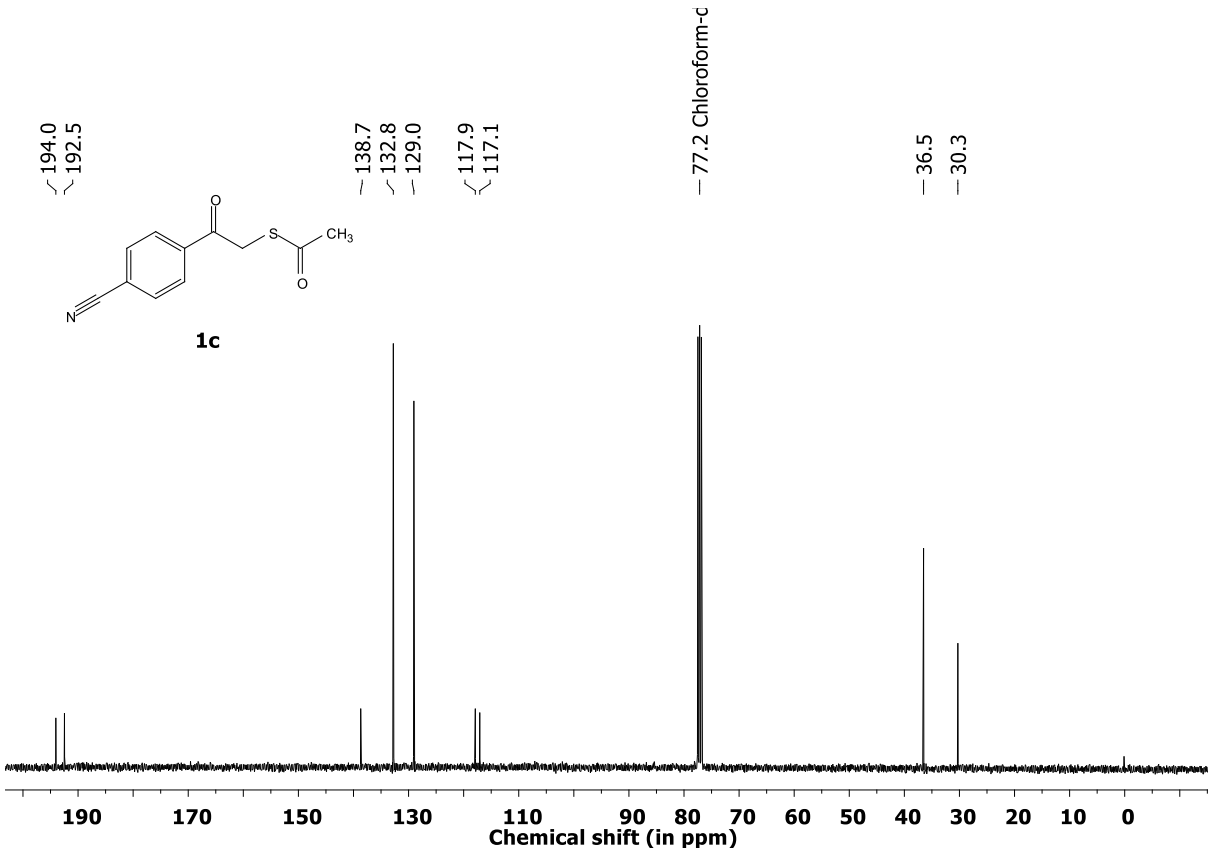
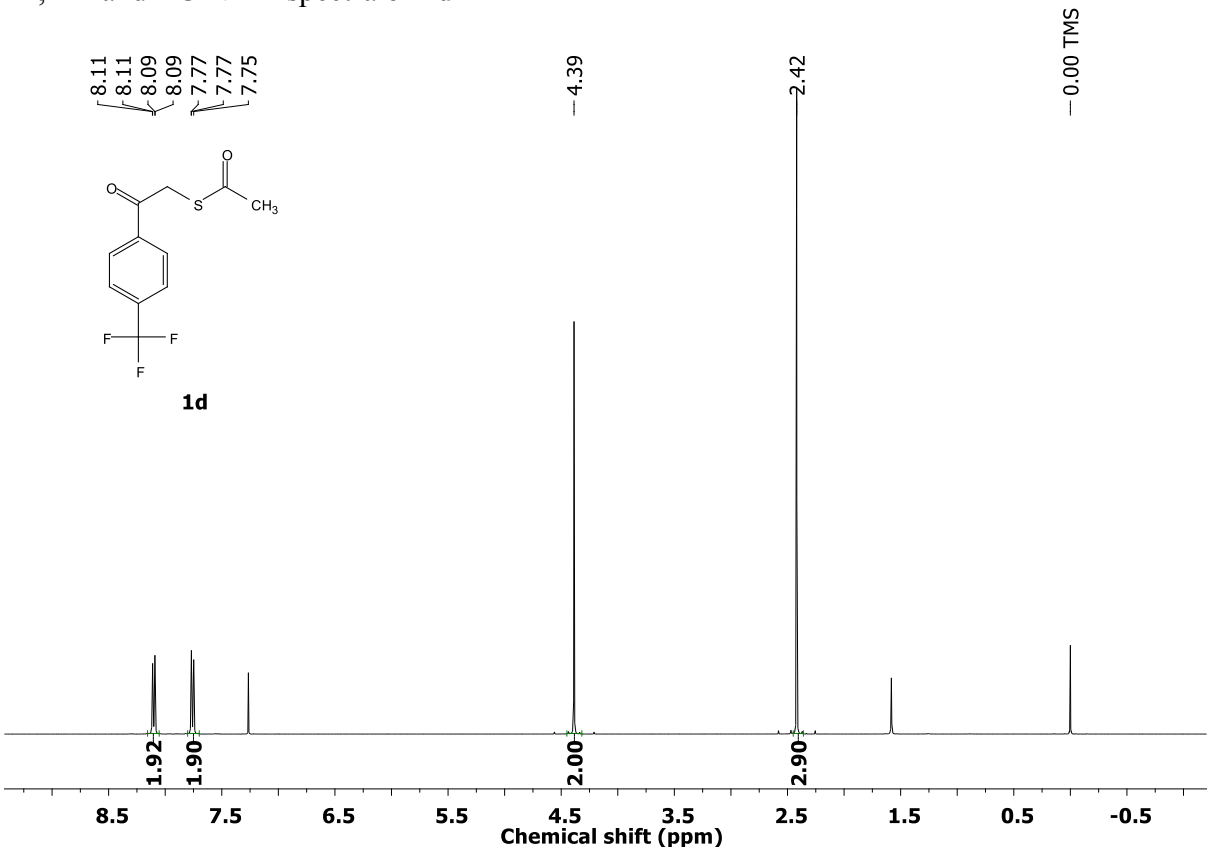
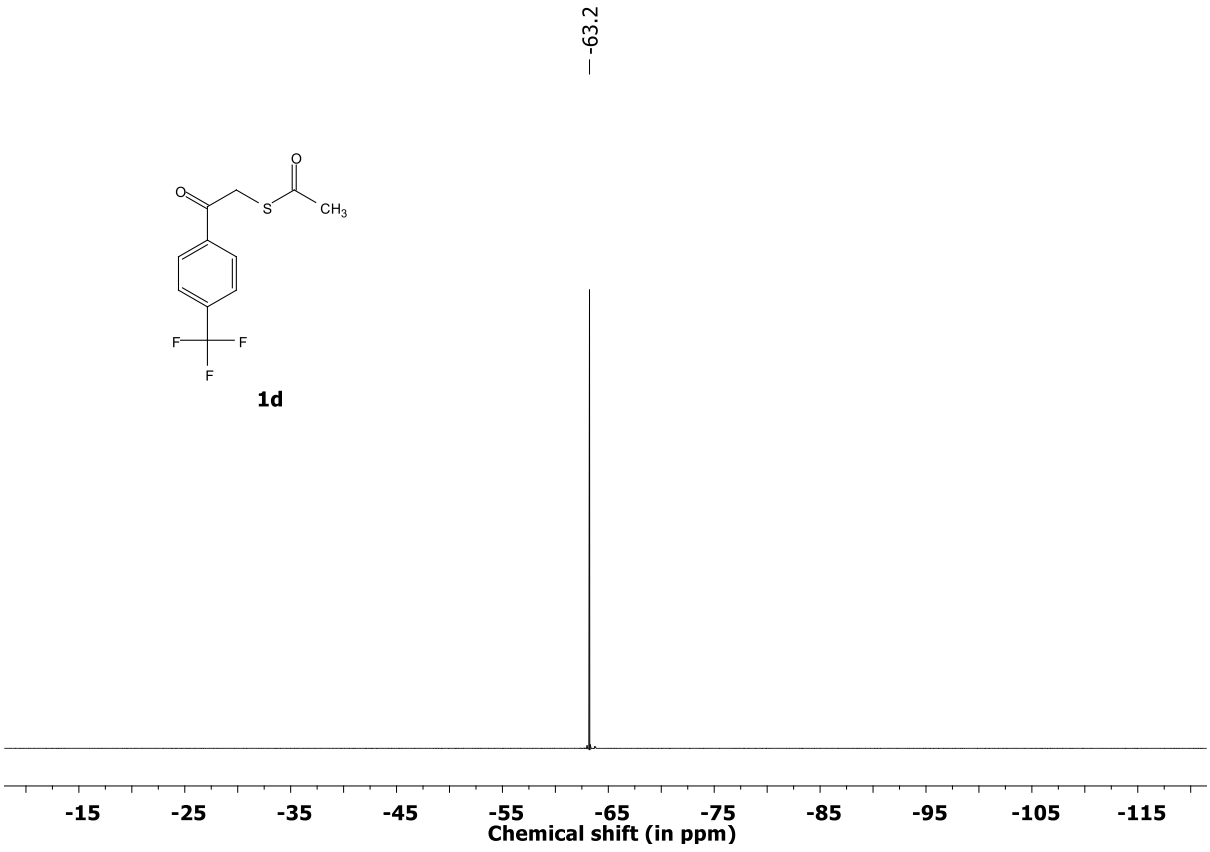
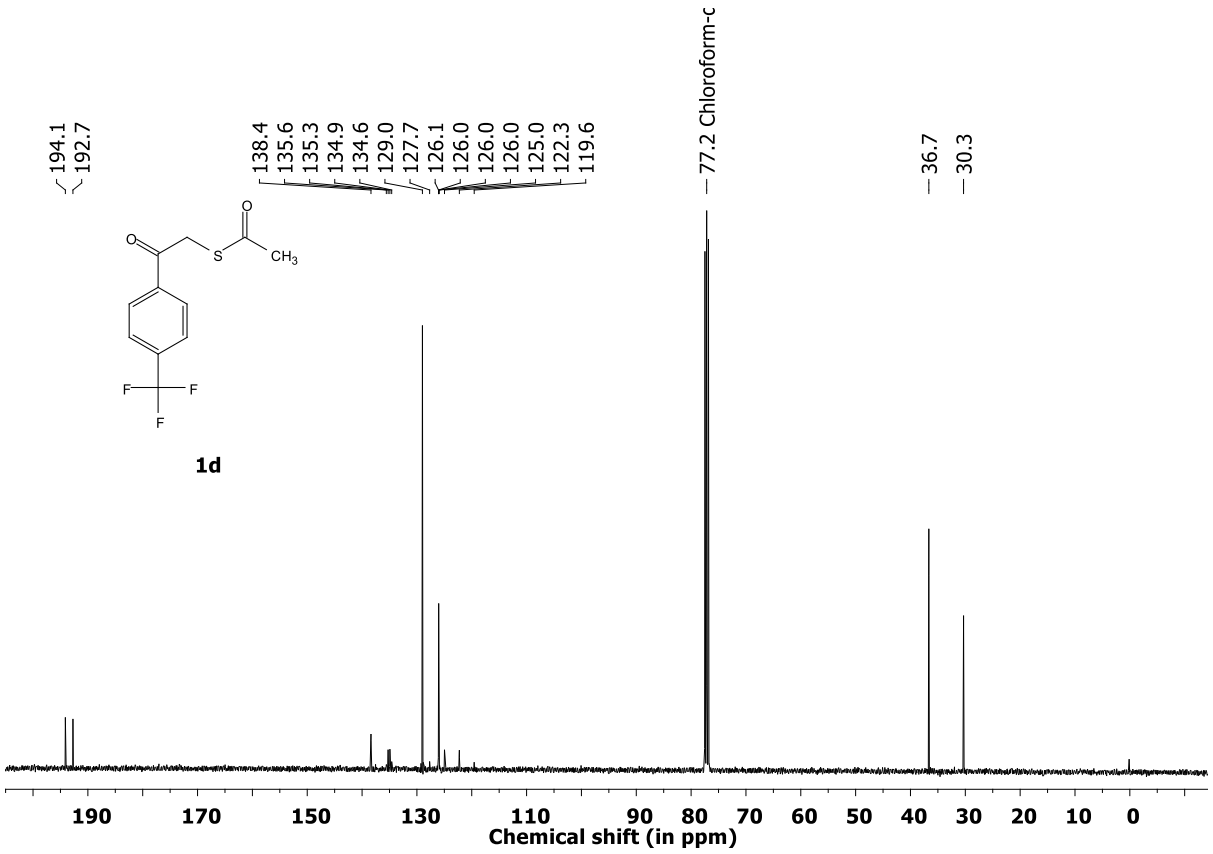




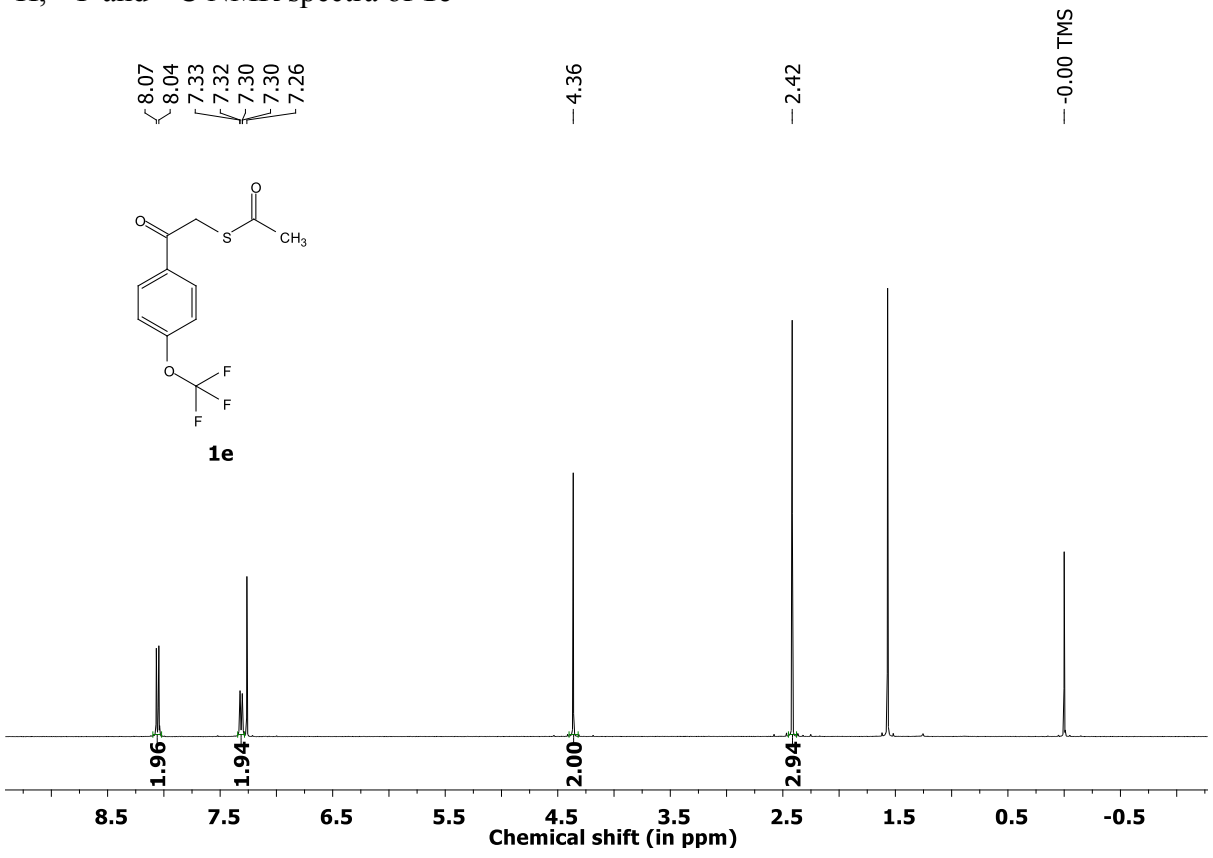
^{1}H, ^{19}F and ^{13}C NMR spectra of **1d**

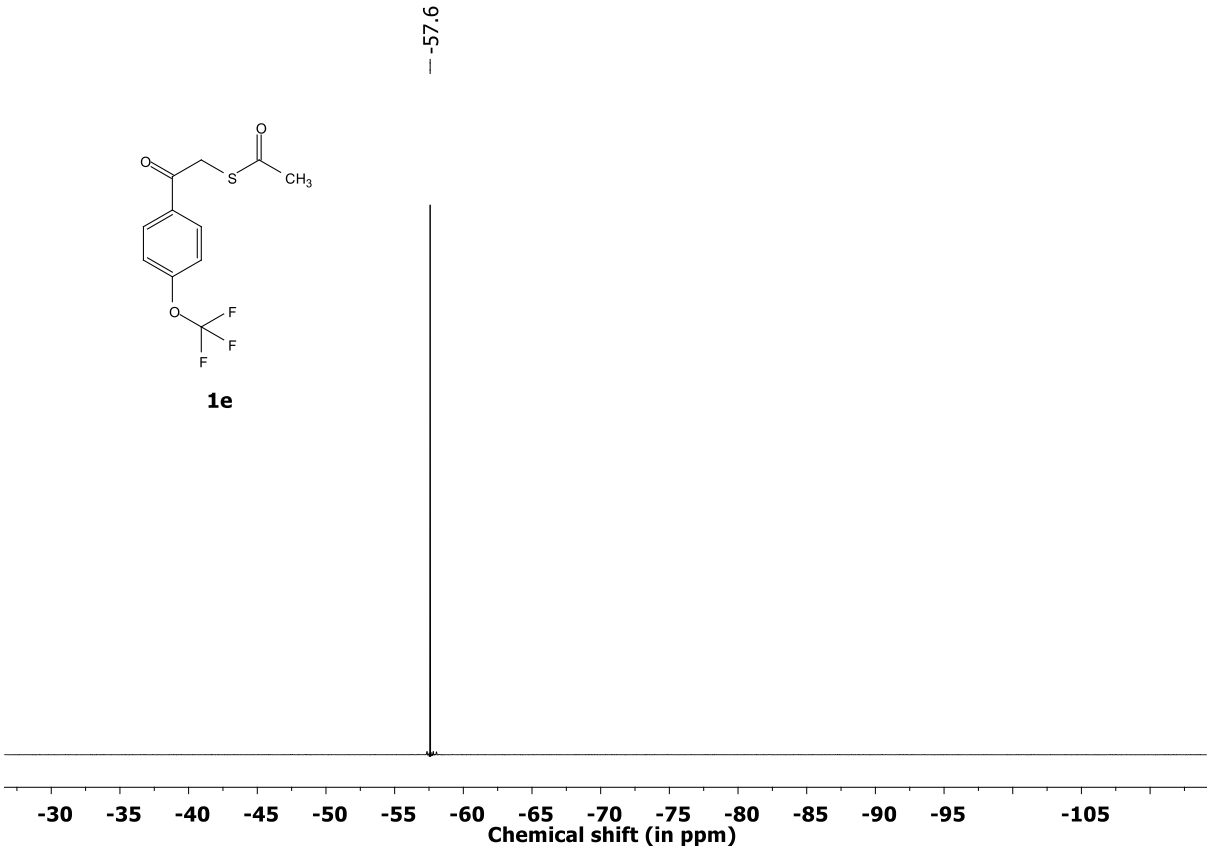


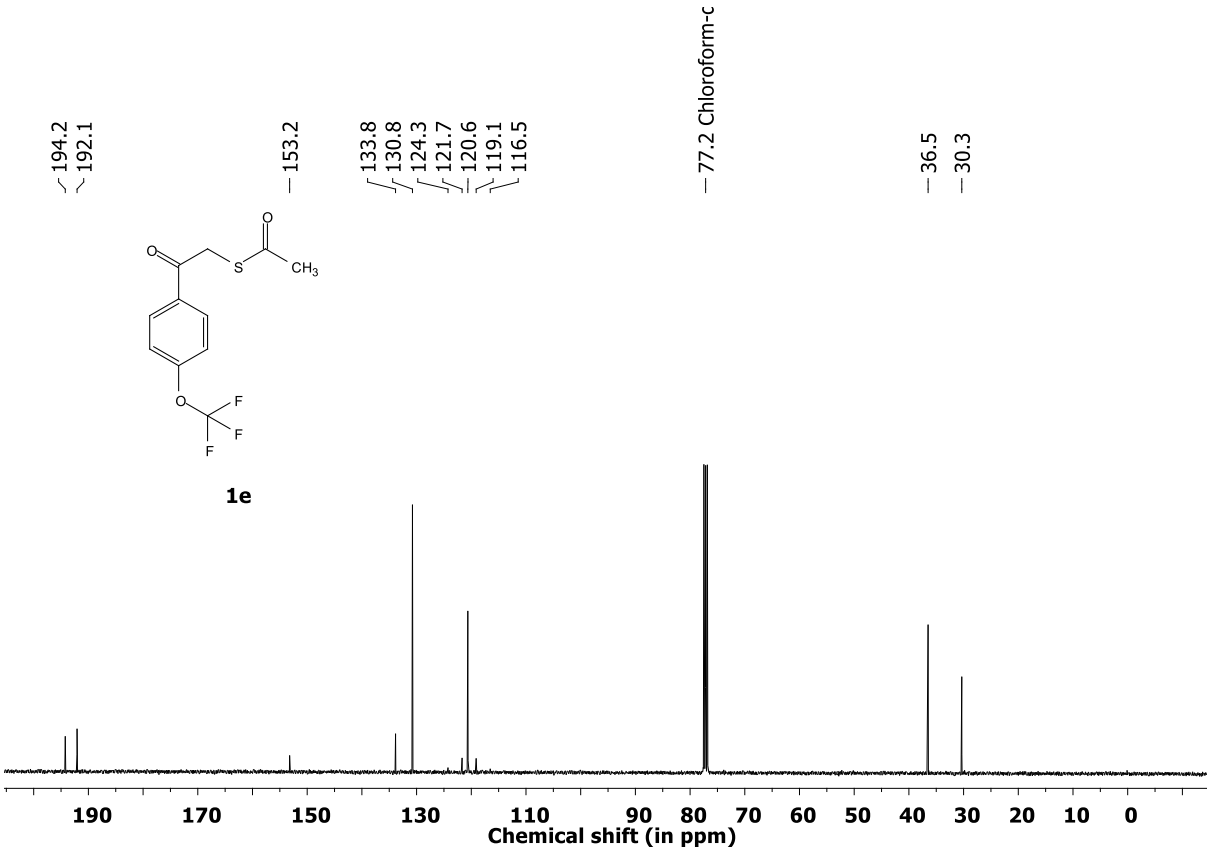




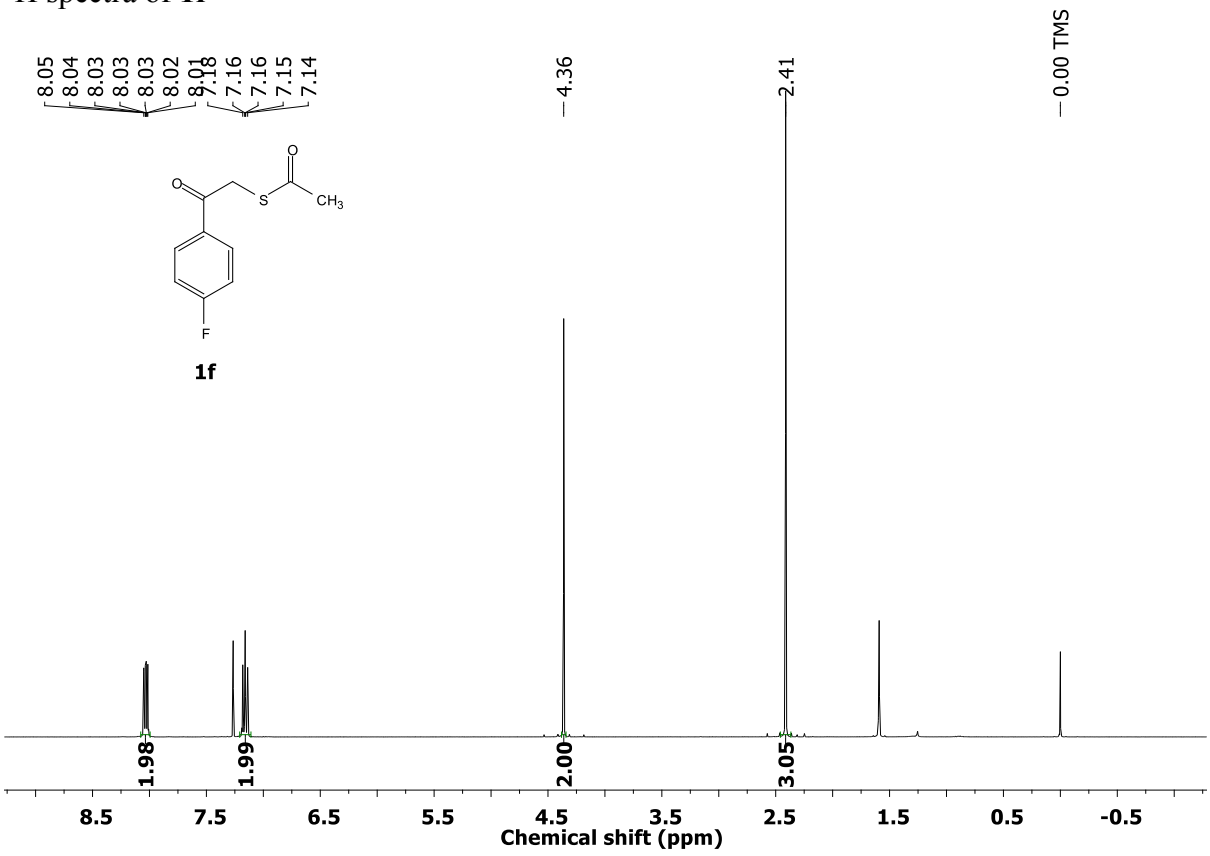
^{1}H, ^{19}F and ^{13}C NMR spectra of **1e**



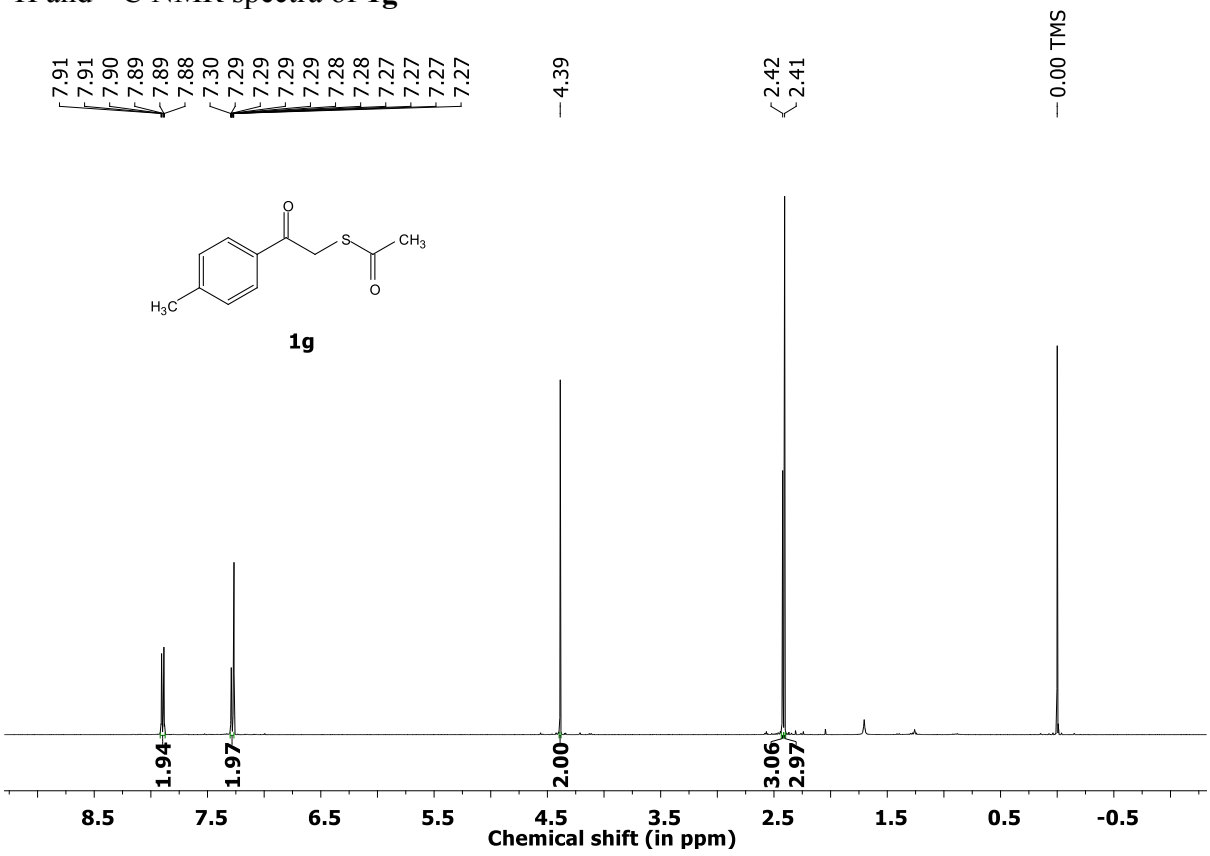

-57.6
1e
-30 -35 -40 -45 -50 -55 -60 -65 -70 -75 -80 -85 -90 -95 -105
Chemical shift (in ppm)

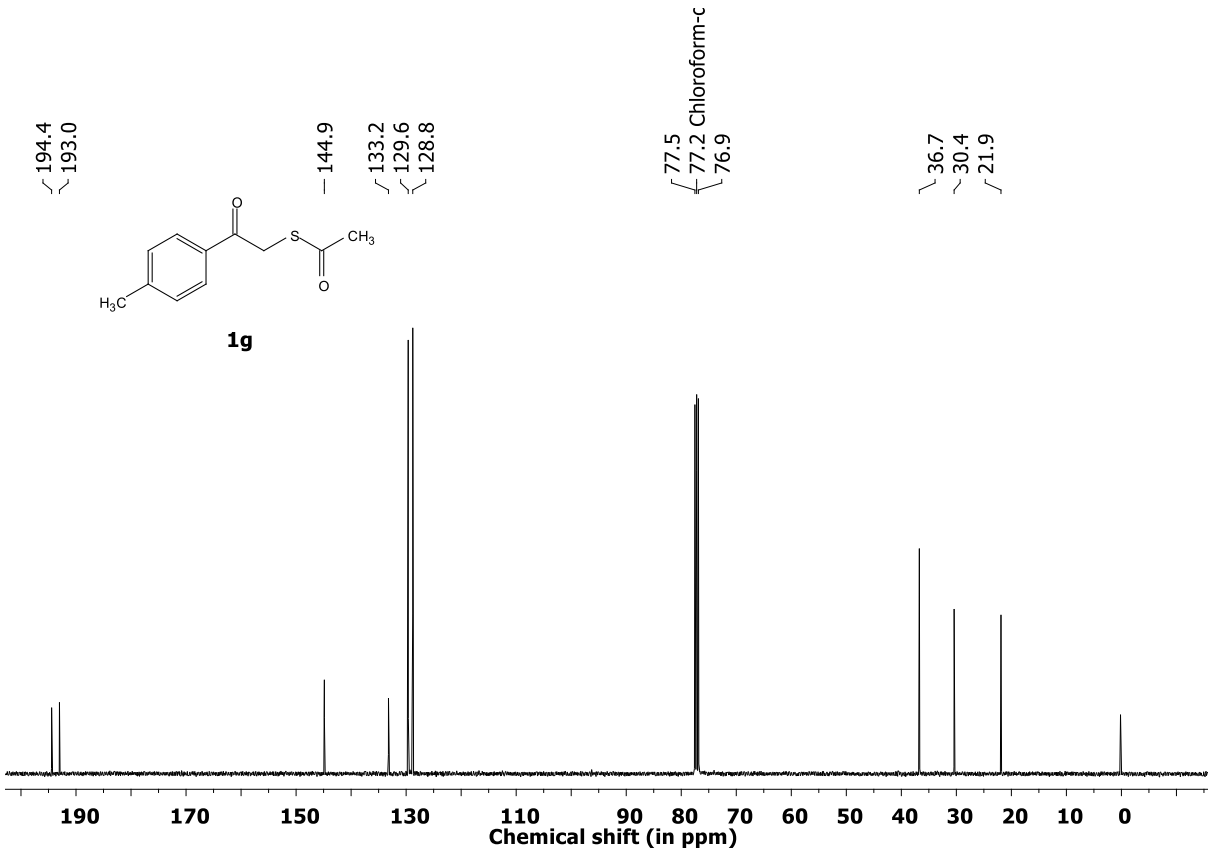

194.2
192.1
153.2
133.8
130.8
124.3
121.7
120.6
119.1
116.5
77.2 Chloroform-c
36.5
30.3
1e
190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
Chemical shift (in ppm)

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

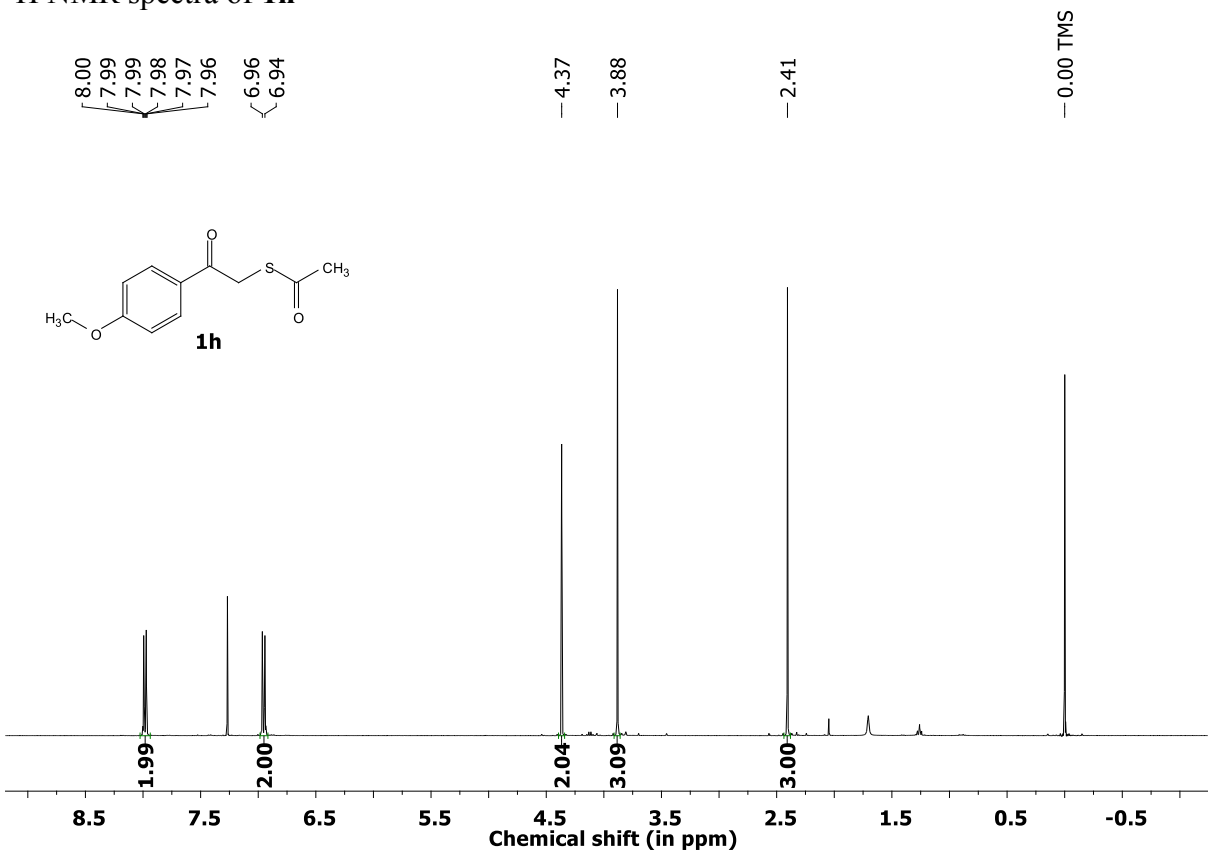


^{1}H and ^{13}C NMR spectra of **1g**





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



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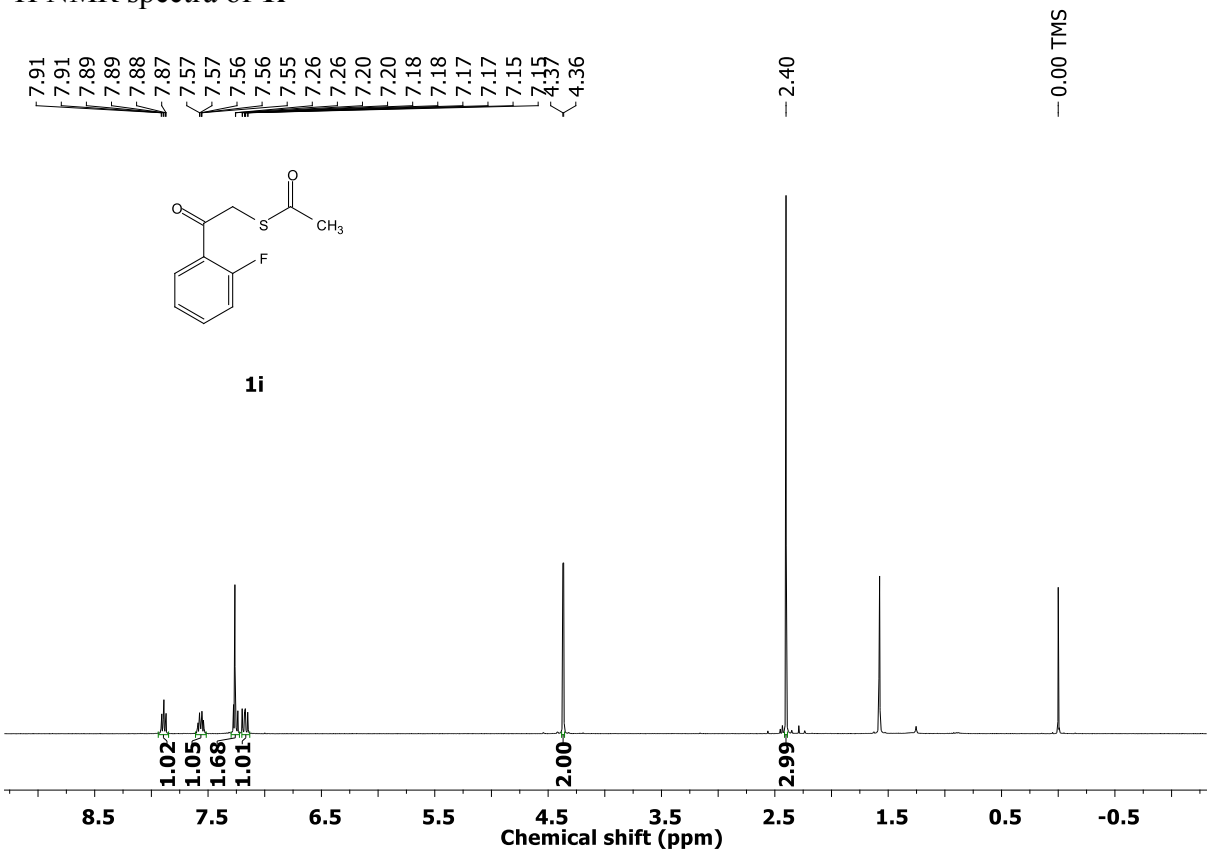
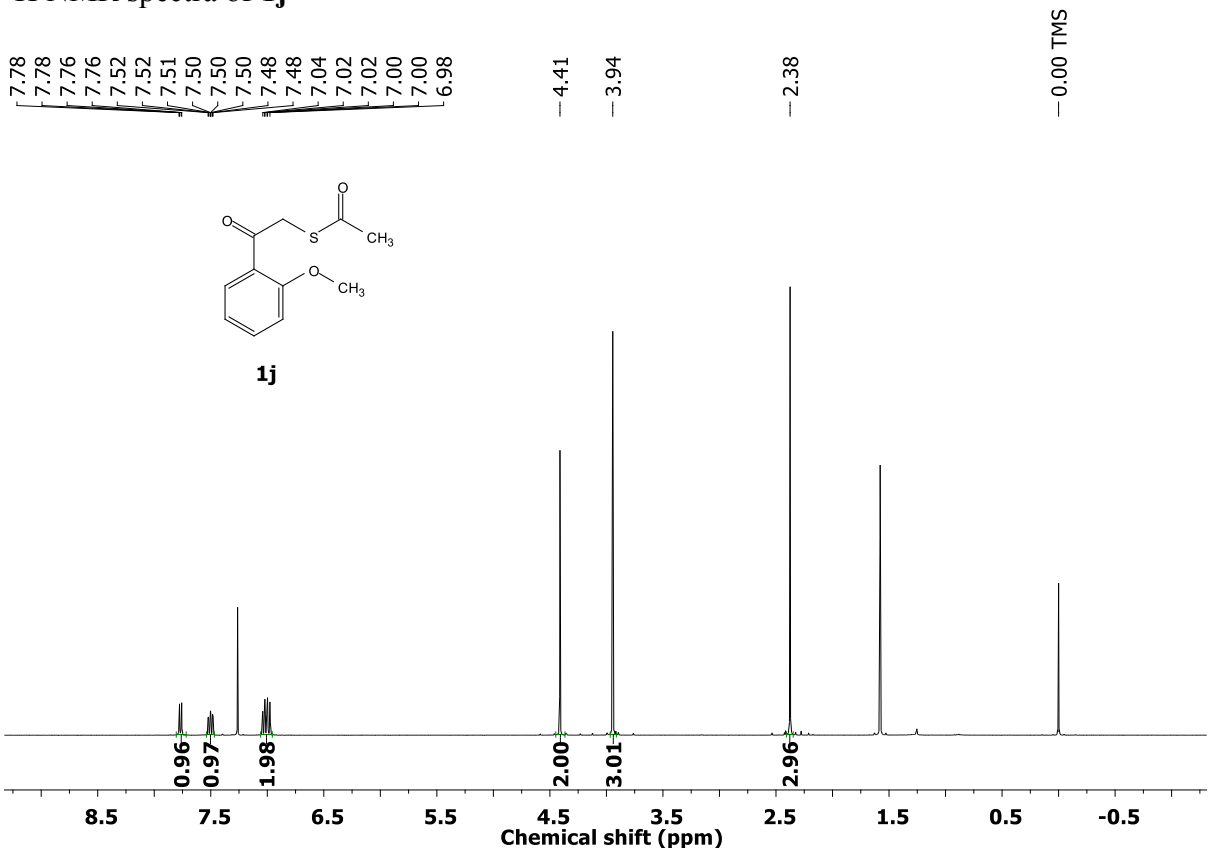


1i

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



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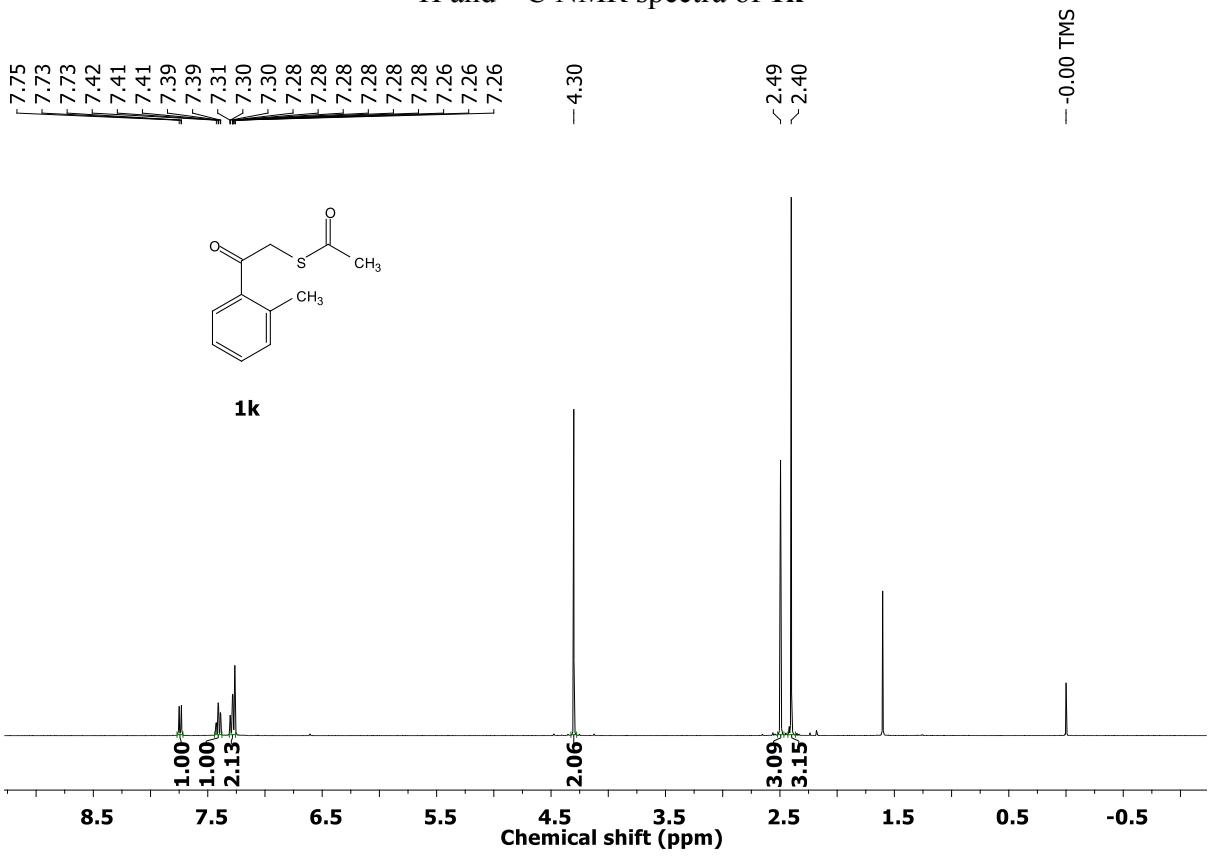
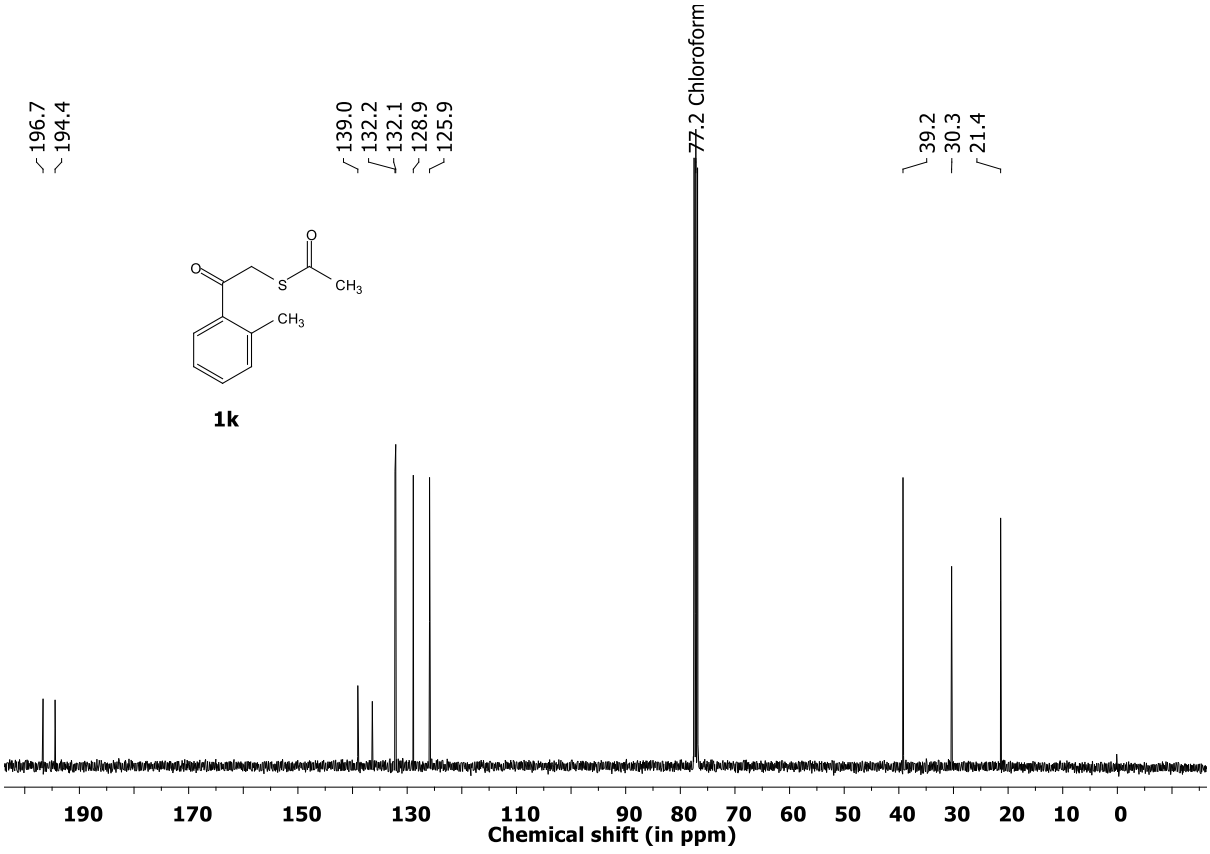
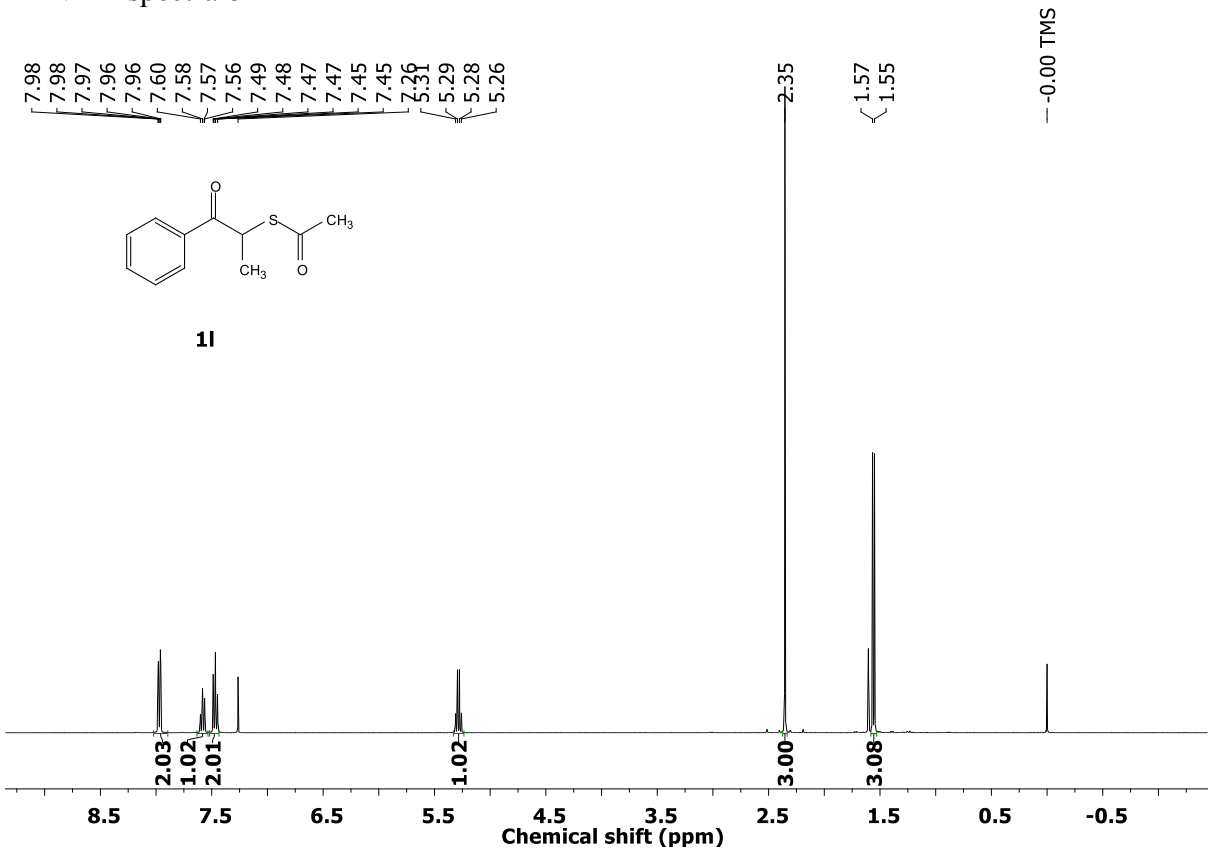
^{1}H and ^{13}C NMR spectra of **1k**

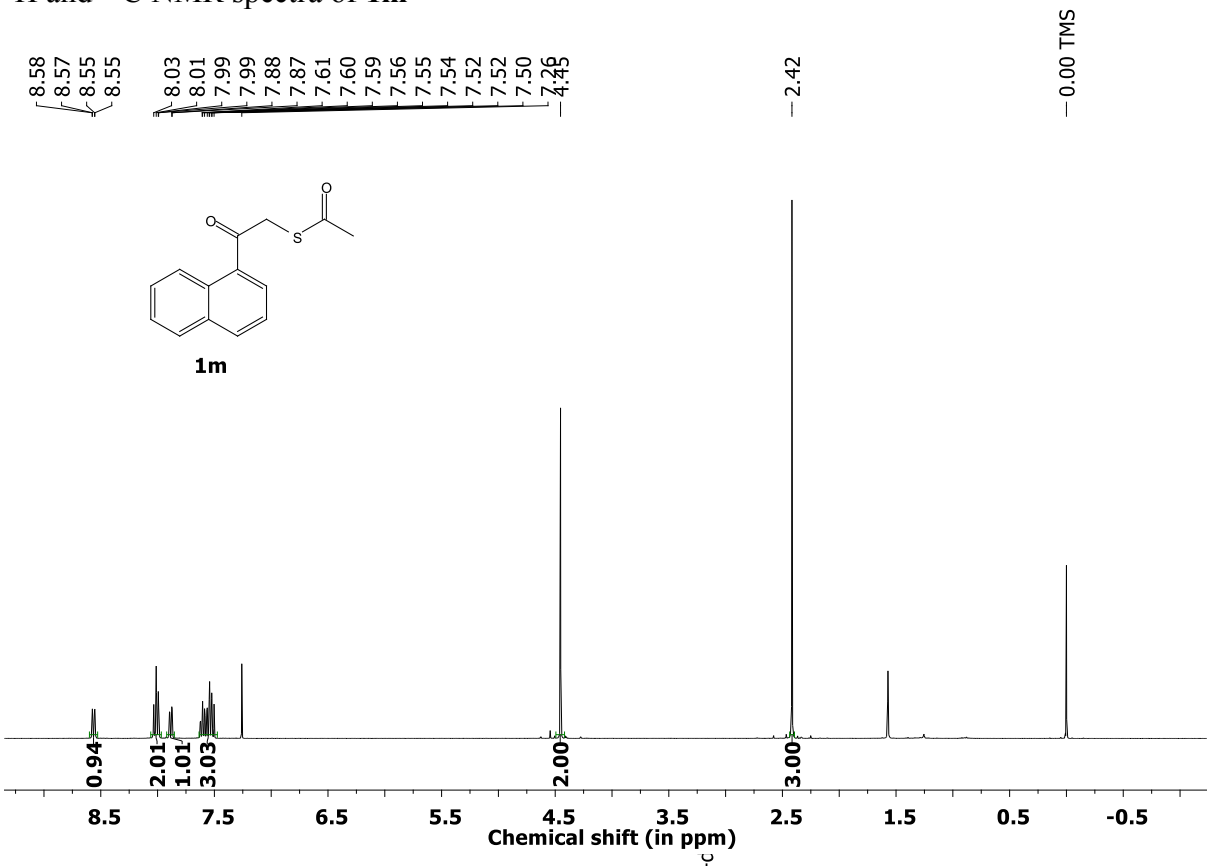


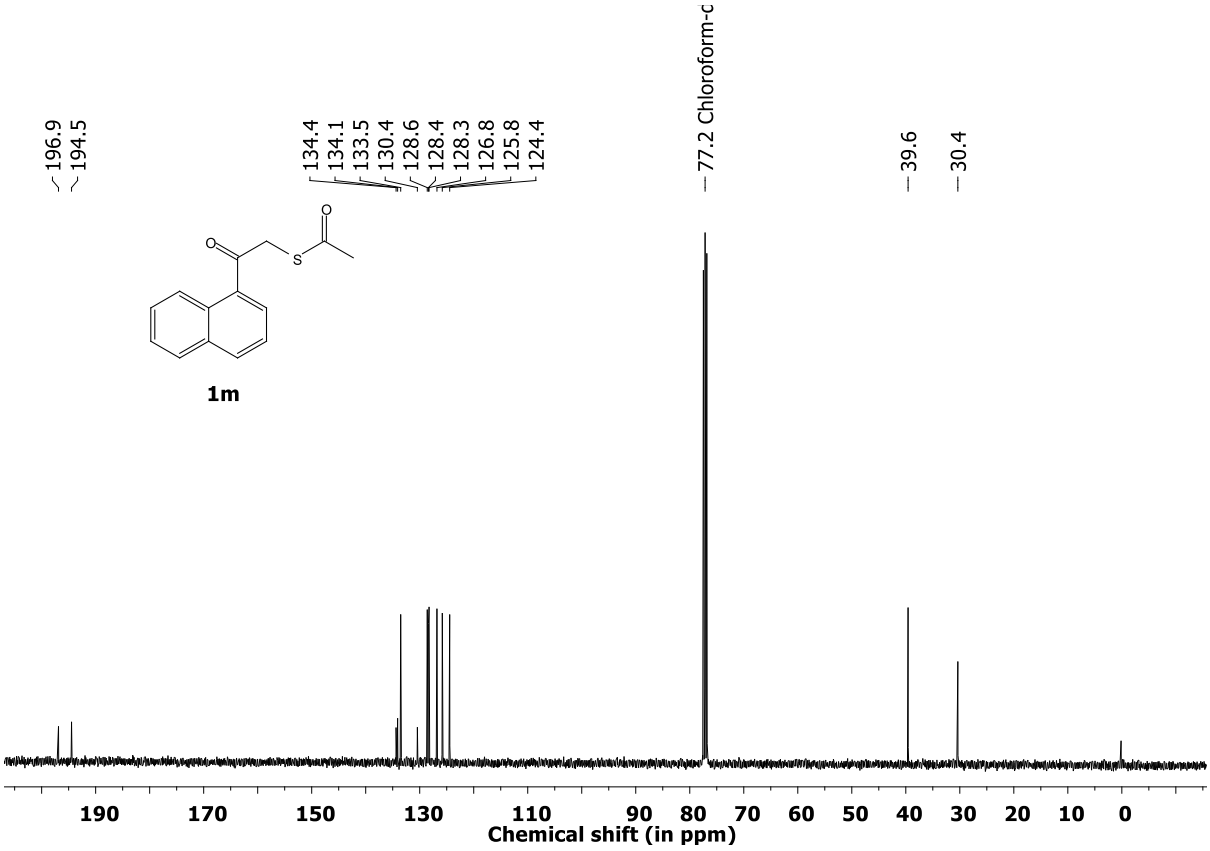


^{1}H NMR spectra of **1l**

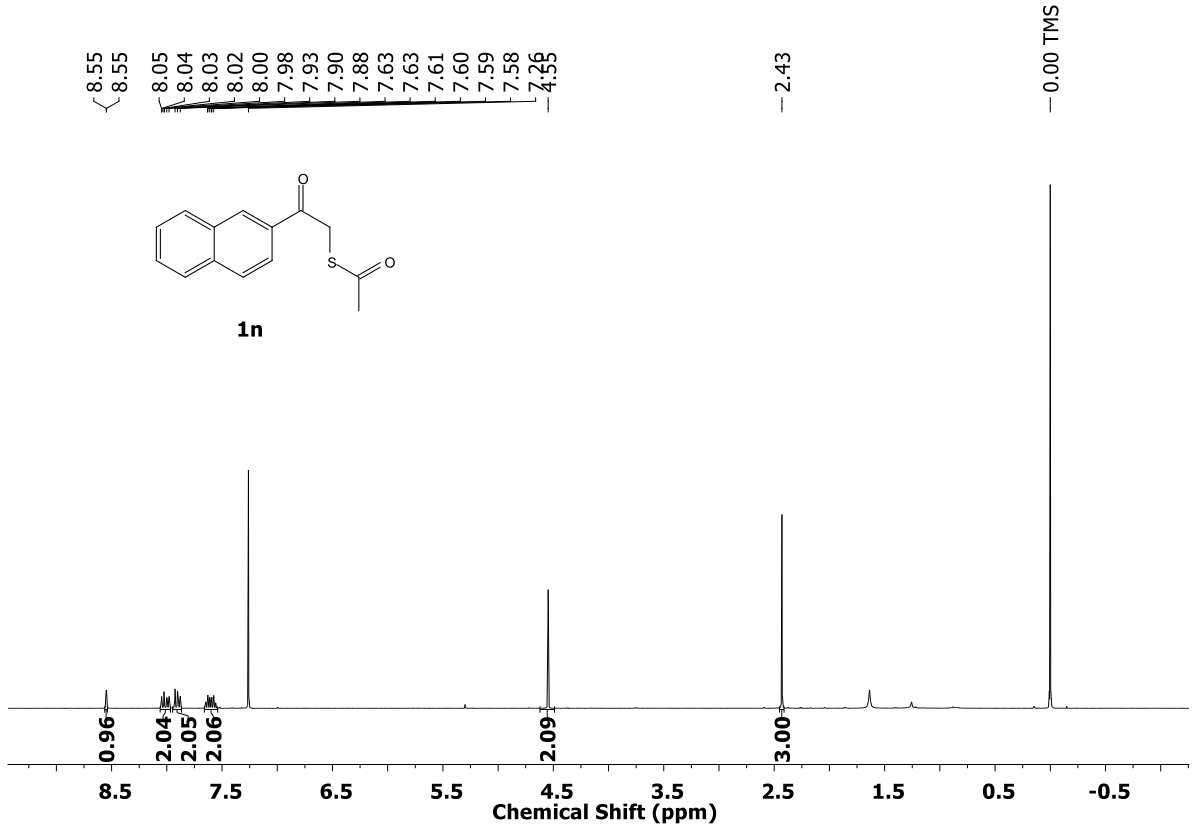


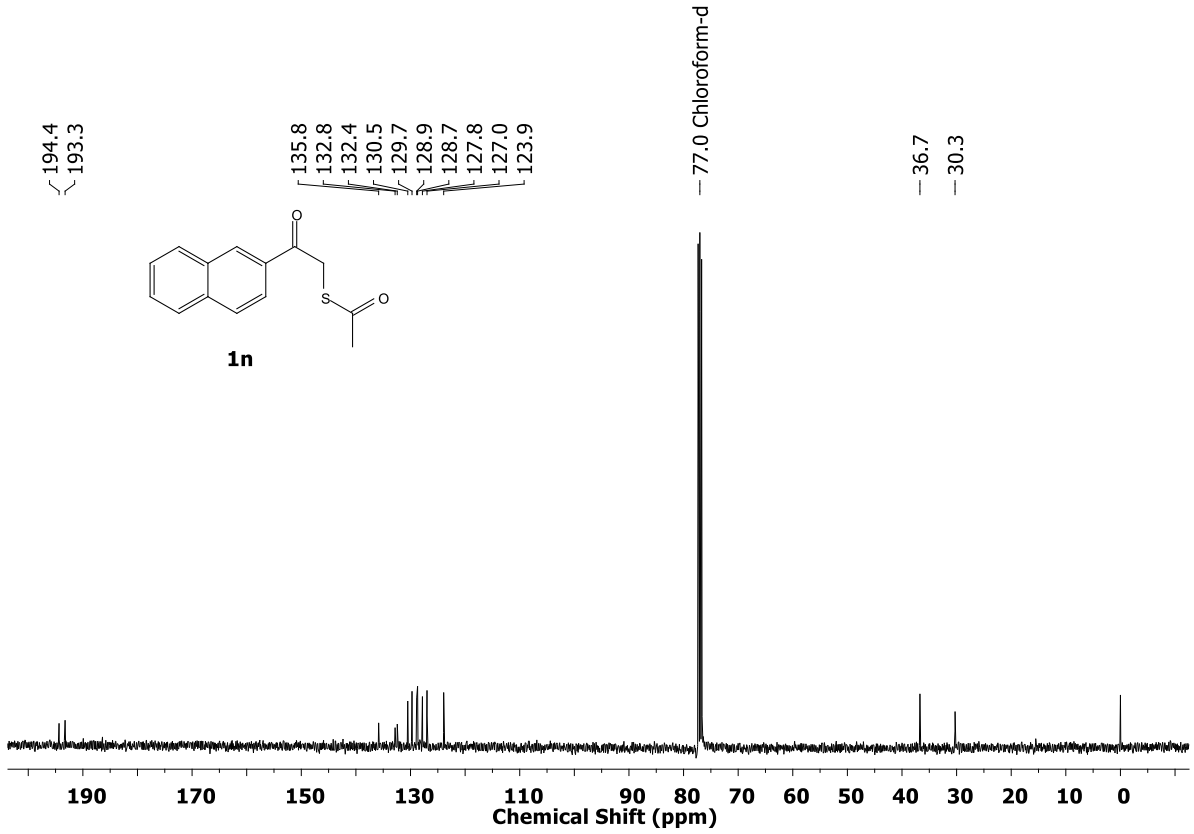
^{1}H and ^{13}C NMR spectra of **1m**



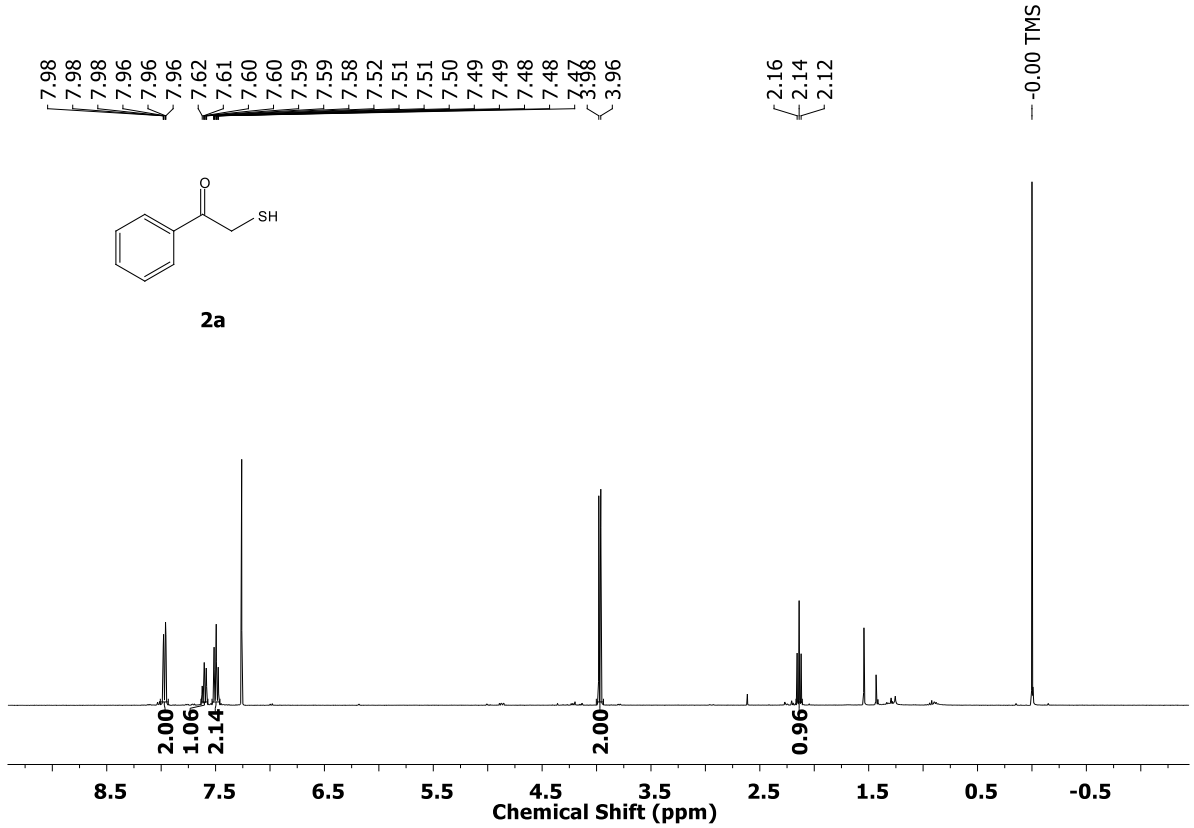


^{1}H and ^{13}C NMR spectra of **1n**

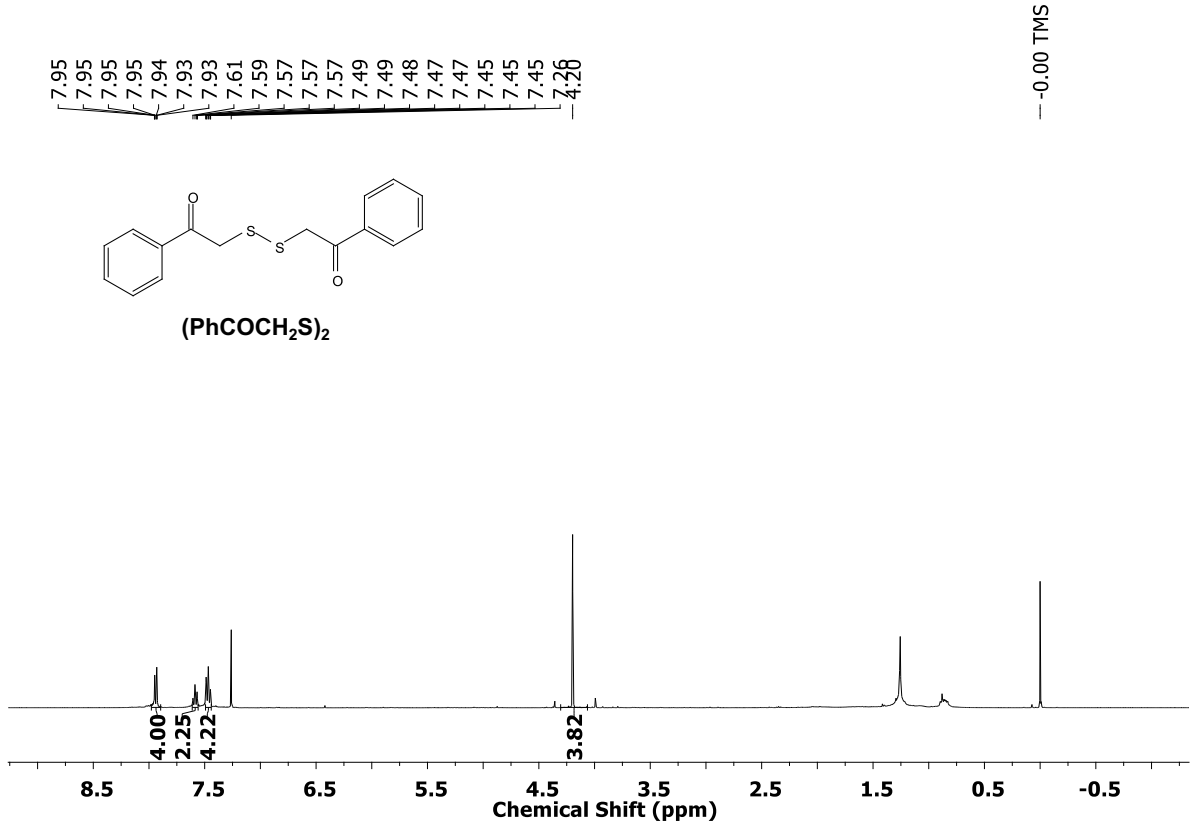




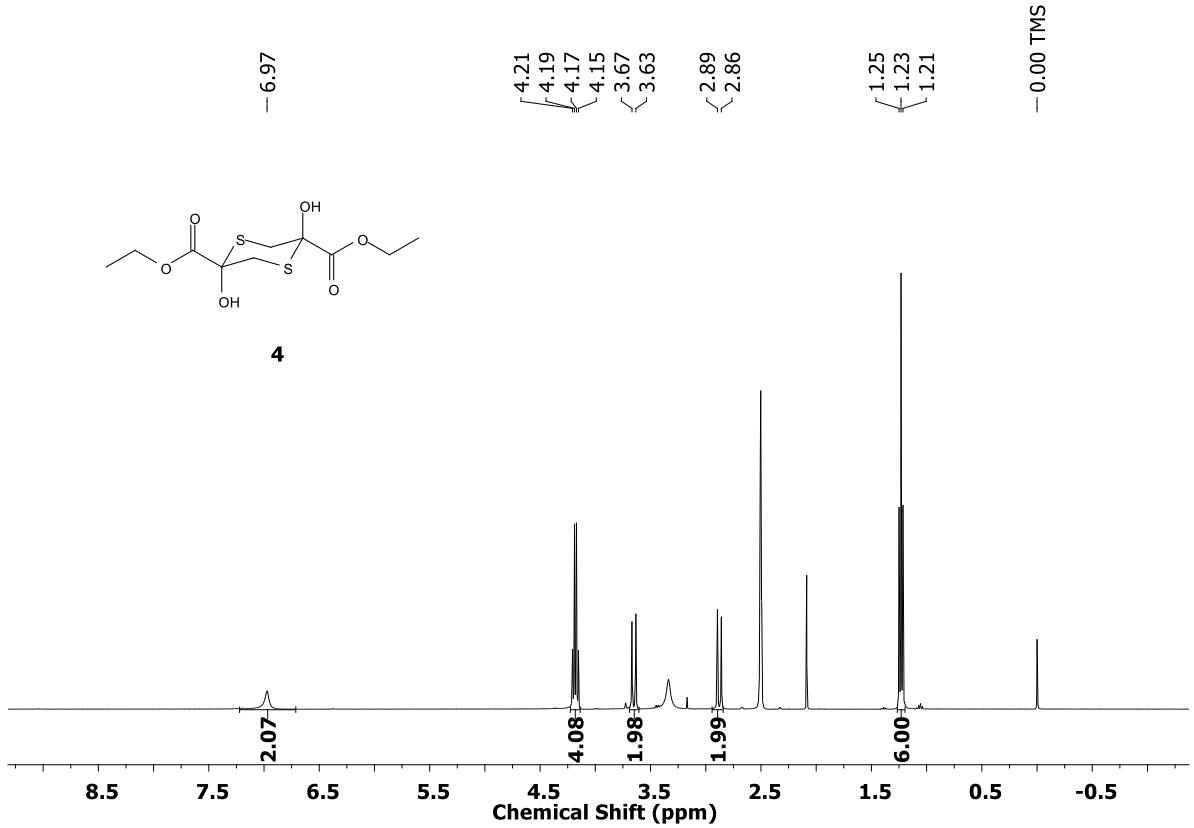
^{1}H NMR spectra of **2a**



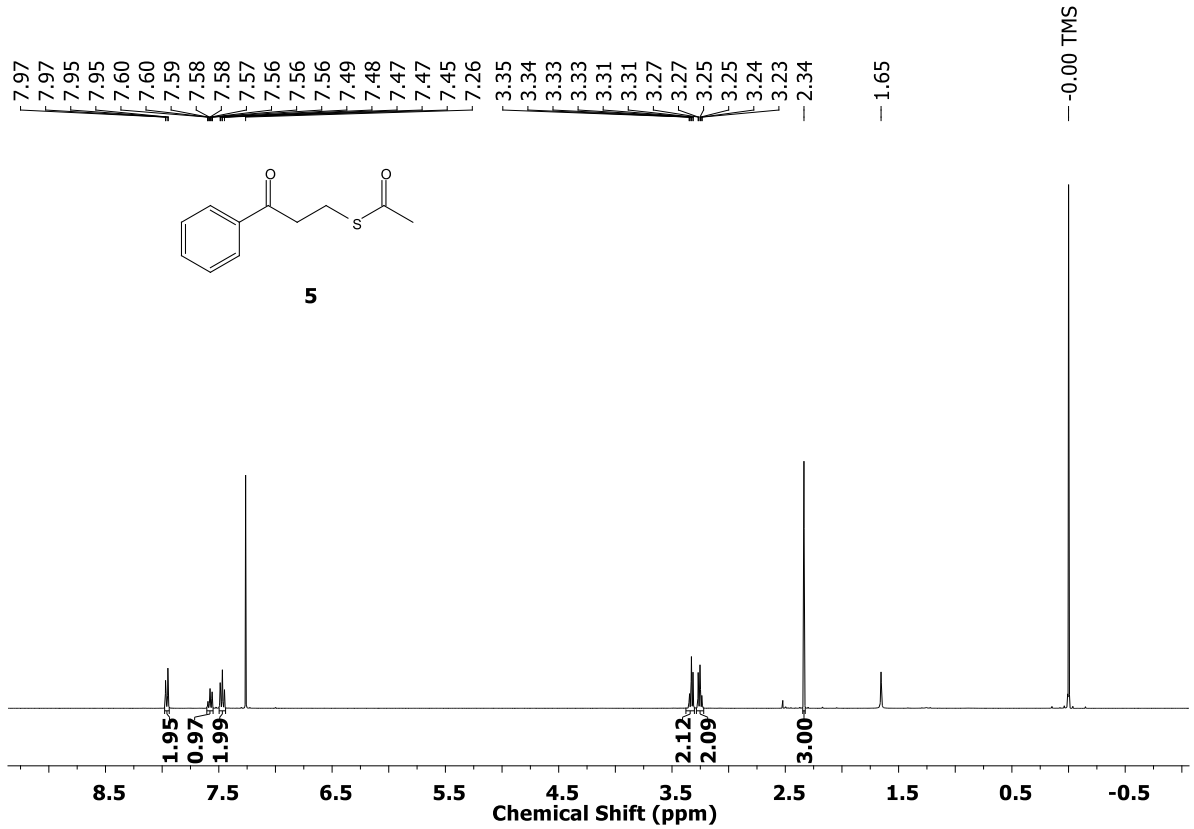
^{1}H NMR spectra of $(PhCOCH_2S)_2$

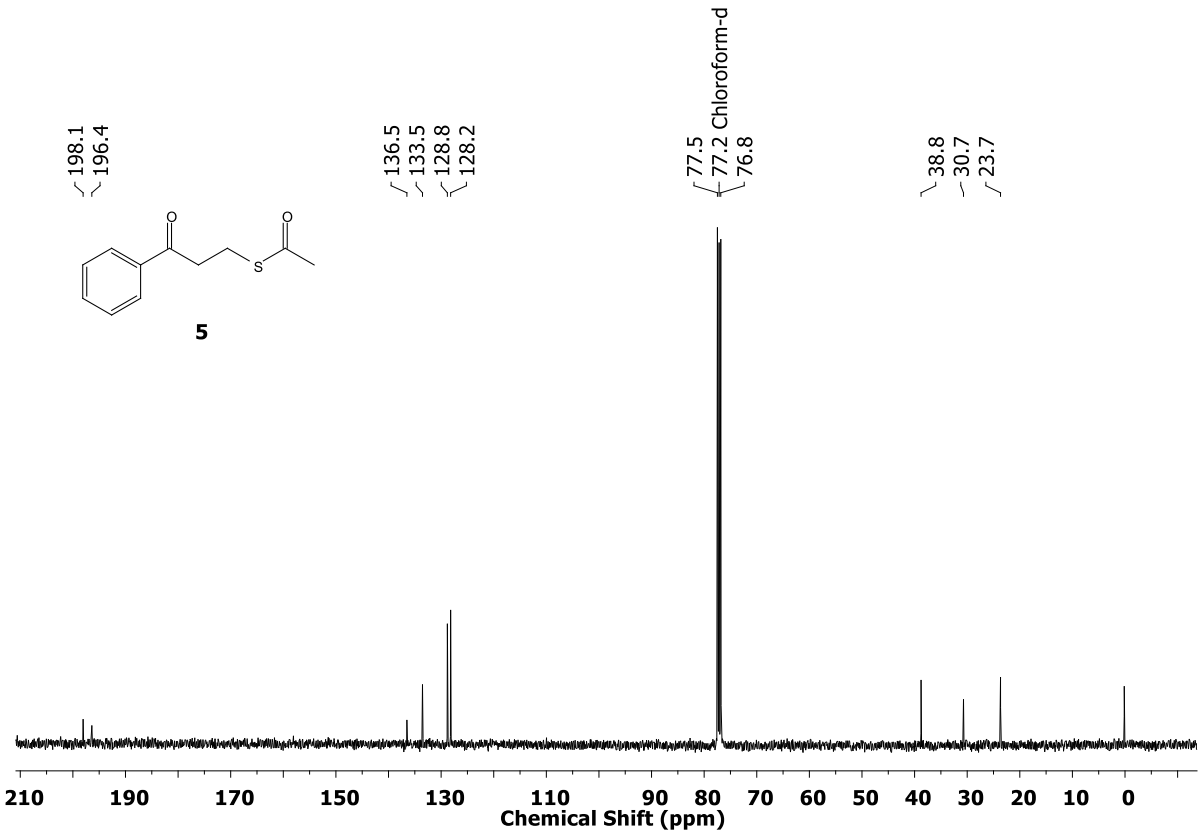


^{1}H NMR spectra of **4**

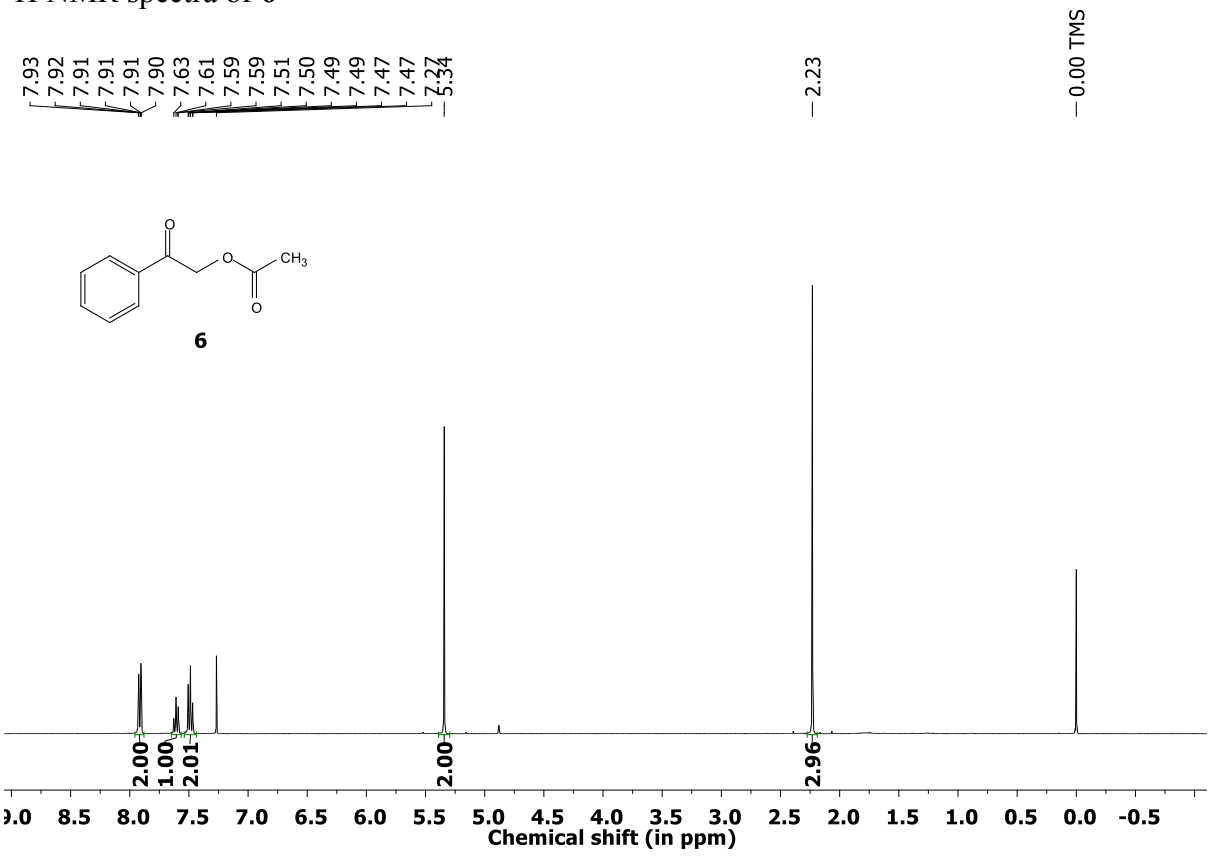


^{1}H and ^{13}C NMR spectra of **5**

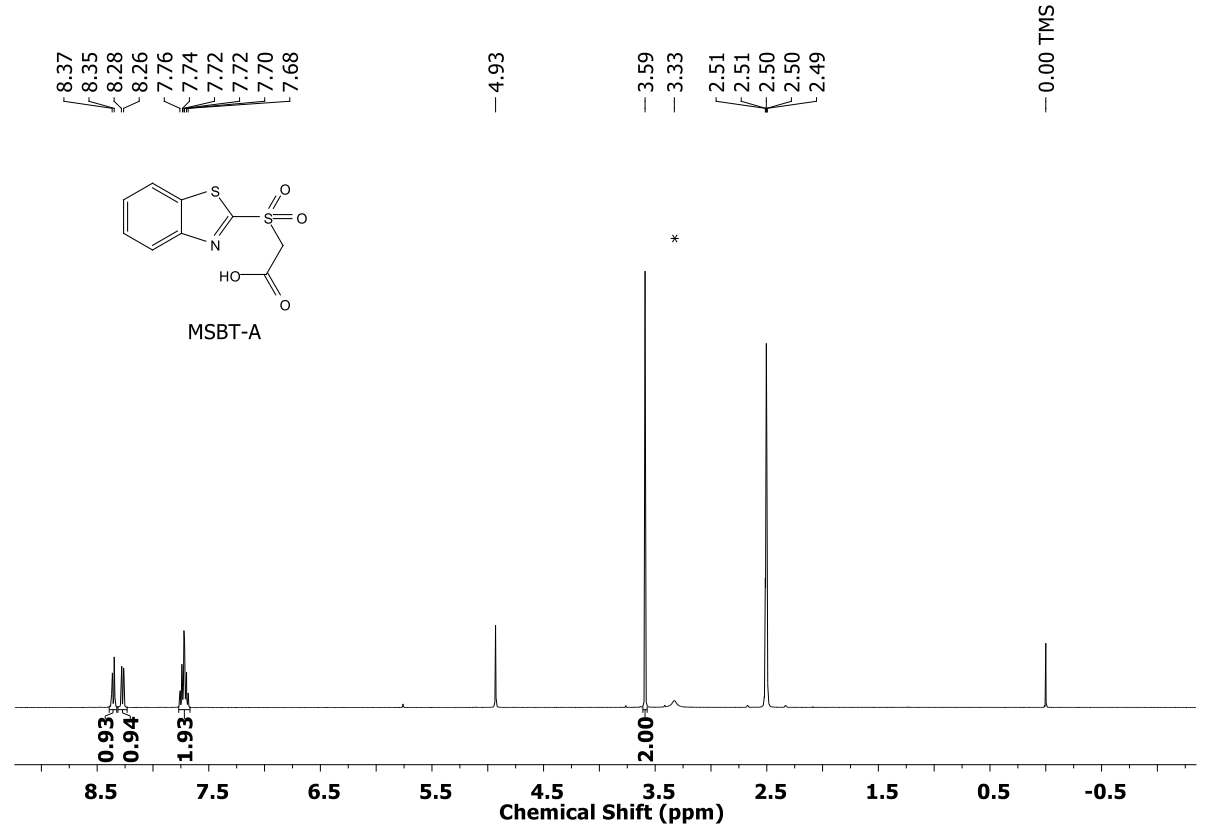




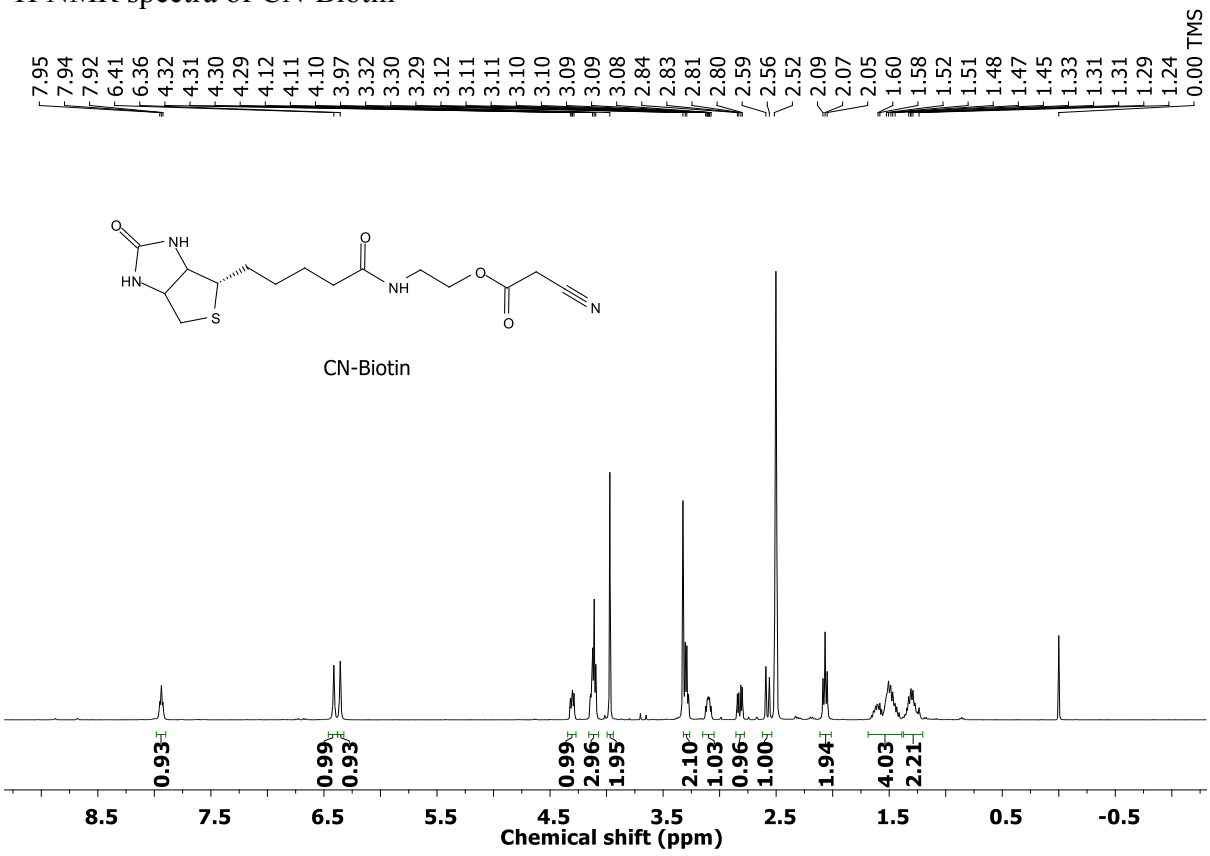
^{1}H NMR spectra of **6**



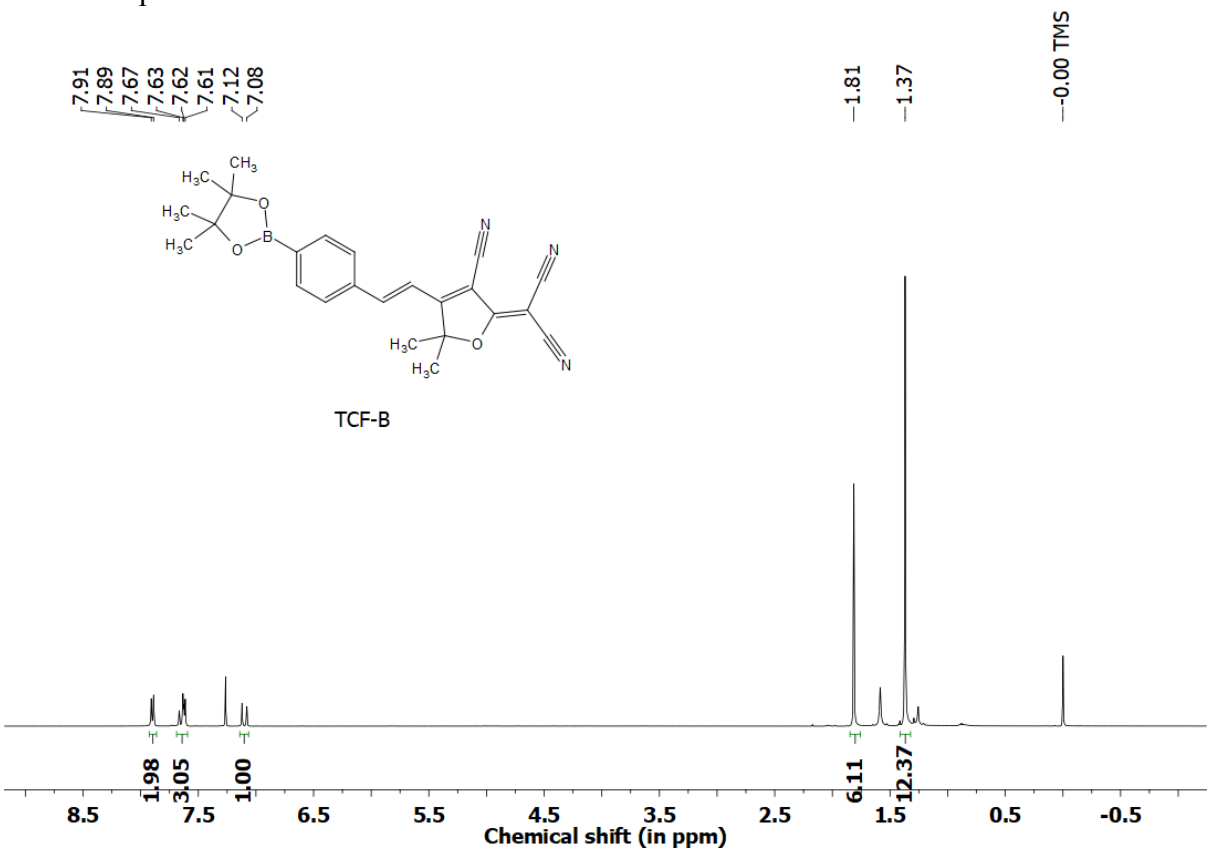
^{1}H NMR spectra of MSBT-A



^{1}H NMR spectra of CN-Biotin



^{1}H NMR spectra of TCF-B



Link to NMR and Mol Files:

https://drive.google.com/drive/folders/1cmEOVwTPUnFA3kKKuKOHe2mKCGpqyRN8?usp=sharing