

**ENHANCING CELLULAR PERSULFIDES THROUGH  
ARTIFICIAL SUBSTRATE OF 3-  
MERCAPTOPYRUVATE SULFURTRANSFERASE  
(3-MST)**

A THESIS  
SUBMITTED IN PARTIAL FULFILMENT OF THE  
REQUIREMENTS  
OF THE DEGREE OF  
**DOCTOR OF PHILOSOPHY**

*BY*  
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**2022**

Dedicated to.....

***My Grandfather (Dadu)***

***Late Tarak Manna***

भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान पुणे

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### CERTIFICATE

Certified that the work incorporated in the thesis entitled, "*Enhancing Cellular Persulfides Through Artificial Substrate of 3-Mercaptopyruvate Sulfurtransferase (3-MST)*" submitted by *Suman Manna* was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

Date: Dec 19, 2022

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## **DECLARATION**

I declare that this written submission represents my ideas in my own words and where others' ideas have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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### General remarks

- $^1\text{H}$  NMR spectra were recorded on JEOL ECX 400 MHz or Bruker 400 MHz spectrometer using tetramethylsilane (TMS) as an internal standard ( $\delta_{\text{H}} = 0.00$ ), unless specified otherwise. Chemical shifts are expressed in ppm units downfield to TMS.
- $^{13}\text{C}$  NMR spectra were recorded on JEOL ECX 100 MHz or Bruker 100 MHz spectrometer.
- Chemical shifts ( $\delta$ ) are reported in ppm and coupling constant ( $J$ ) in Hz
- Mass spectra were obtained using HRMS-ESI-Q-Time of Flight LC-MS (Synapt G2, Waters) or MALDI TOF/TOF Analyser (Applied Biosystems 4800 Plus).
- FT-IR spectra were obtained using Bruker Alpha-FT-IR spectrometer and reported in  $\text{cm}^{-1}$ .
- All reactions were monitored by Thin-Layer Chromatography carried out on precoated Merck silica plates (F254, 0.25 mm thickness); compounds were visualized by UV light.
- All reactions were carried out under nitrogen or argon atmosphere with freshly dried solvents under anhydrous conditions and yields refer to chromatographically homogenous materials unless otherwise stated.
- All evaporations were carried out under reduced pressure on Büchi and Heidolph rotary evaporator below 45 °C unless specified otherwise.
- Silica gel (60-120) and (100-200) mesh were used for column chromatography.
- Materials were obtained from commercial suppliers and were used without further purification.
- Preparative HPLC purification was carried out using high performance liquid chromatography (HPLC) with C-18 preparative column (21.2 mm  $\times$  250 mm, 10  $\mu\text{m}$ ; Kromasil C18).
- HPLC analysis data was obtained using Agilent Technologies 1260 Infinity, C18 reversed phase column (4.6 mm  $\times$  250 mm, 5  $\mu\text{m}$ ).
- Spectrophotometric and fluorometric measurements were performed using microwell plate reader using ThermoScientific Varioskan Flash or PerkinElmer Ensignht.
- All SDS-PAGE gels (activity-based gels and coomassie gels) were imaged on a Syngene G-Box Chemi-XRQ gel documentation system
- All statistical analysis was carried out using One-way ANOVA analysis using GraphPad Prism 8.0.1 software unless specified.

## **Abbreviations**

ACN – Acetonitrile

AcOH – Acetic acid

AD – Alzheimer's disease

ARE - Antioxidant response element

ATP – Adenosine triphosphate

au – Arbitrary unit

boc – *tert*-butyloxycarbonyl

bs – Broad singlet

BSA – Bovine serum albumin

<sup>t</sup>BuOH – Tertiary-butanol

CA – Carbonic Anhydrase

CARS – CysteinyI-<sup>t</sup>RNA synthetase

CBS – Cystathionine-β-Synthase

CDCl<sub>3</sub> – Chloroform-D

CHCl<sub>3</sub> – Chloroform

CH<sub>2</sub>Cl<sub>2</sub> – Dichloromethane

Ctrl – Control

CSE – Cystathionine-γ-Lyase

CysSH – Cysteine

CysS-SH – Cysteine persulfide

CysS-OH – Sulfenic acid

CysS-NO – Nitrosothiol

dd – Doublet of doublet

DCM – Dichloromethane

DCC – *N, N*-Dicyclohexylcarbodiimide

DIPEA – diisopropyl ethyl amine

DMAP – *N, N*-Dimethylaminopyridine

DMEM – Dulbecco's Modified Eagle's Medium

DMF – *N, N*-Dimethyl formamide

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

D<sub>2</sub>O – Deuterium Oxide

dt – Doublet of triplet

DTT – dithiothreitol

δ – Delta (in ppm)

*E. coli* – *Escherichia coli*

eq. – Equivalents

ES – Esterase

ESI – Electron spray ionization

ERK - Extracellular signal-regulated kinase

Et<sub>3</sub>N – Triethylamine

EtOH – Ethanol

EtOAc – Ethyl acetate

Et<sub>2</sub>O – Diethyl ether

FBS – Fetal bovine serum

FeCl<sub>3</sub> – Ferric Chloride

g – Gram

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GSK3 $\beta$  – Glycogen synthase kinase

GSH – Glutathione

GST – Glutathione *S*-transferase

h – Hour

HCl – Hydrochloric acid

HCysS-SH – Homocysteine persulfide

HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HO-1 – Heme oxygenase 1

H<sub>2</sub>O – Water

H<sub>2</sub>O<sub>2</sub> – Hydrogen peroxide

H<sub>2</sub>S – Hydrogen Sulfide

H<sub>2</sub>S<sub>n</sub> – Hydrogen polysulfide

HNO<sub>3</sub> – Nitric acid

HPLC – High performance liquid chromatography

HRMS – High-resolution mass spectrometry

5-HT – 5-Hydroxytryptamine

Hz – Hertz

HNO – Nitroxyl

IAM – Iodoacetamide

I $\kappa$ B – Inhibitor of  $\kappa$ B

IKK – I $\kappa$ B kinase

IR – Infrared

*J* – Coupling constant

KEAP 1 - Kelch-like ECH- associated protein 1

KCl – Potassium Chloride

Kir - Inwardly-rectifying potassium channel

K<sub>2</sub>CO<sub>3</sub> – Potassium carbonate

$\lambda_{\text{ex}}$  – Excitation wavelength

$\lambda_{\text{em}}$  – Emission wavelength

LPS – Lipopolysaccharides

LMW – Low molecular weight

m – Multiplet

MALDI – Matrix-Assisted Laser Desorption Ionization

MEF – Mouse embryonic fibroblasts

Me – Methyl

MeOH – Methanol

mg – Milligram

Min. – Minutes

MHz – Megahertz

MEK - Mitogen-activated protein kinase kinase

mL – Millilitre

mM – Millimolar

mmol – Millimoles

mRNA – Messenger ribonucleic acid

MS – Mass spectrum

MSBT-A - Methyl sulfonyl benzathiazole acid

3-MP – 3-mercaptopyruvate

3-MST – 3-Mercaptopyruvate sulfur transferase

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW – Molecular weight

m/z – Mass to charge ratio

μM – Micromolar

NAC – *N*-acetyl cysteine

NAD<sup>+</sup> – Nicotinamide adenine dinucleotide (+)

NADPH – Reduced nicotinamide-adenine-dinucleotide phosphate

NaOMe – Sodium methoxide

NFκB – Nuclear factor κB

NaHCO<sub>3</sub> – Sodium bicarbonate

NaSH - Sodium hydrosulfide

Na<sub>2</sub>SO<sub>4</sub> – Sodium sulphate

NMR – Nuclear magnetic resonances

NO – Nitric oxide

NQO1 – NAD(P)H quinone oxidoreductase 1

nM – Nanomolar

Nrf2 - Nuclear factor erythroid 2-related factor 2

HO<sup>•</sup> – Hydroxyl radical

O<sub>2</sub><sup>•-</sup> – Superoxide radical

oxPTM – oxidative post translational modification

PARPs - (poly(ADP-ribose)ation polymerases)

PB – Phosphate buffer

PBS – Phosphate buffer saline

PbS – Lead sulfide

PD - Parkinson's disease

pH – Potential of hydrogen

Ph – Phenyl

PPh<sub>3</sub> – Triphenyl phosphine

Py – Pyridine

ppm – Parts per million

% – Per cent

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute Medium

RT – Retention Time

rt – Room temperature

s – Singlet

S<sub>2</sub>O<sub>3</sub><sup>2-</sup> – Thiosulfate

SQR – Sulfide:quinone reductase

t – Triplet

TBSCl - tert-butyldimethylsilyl chloride

TEA – Triethylamine

TFA – Trifluoroacetic acid

THF – Tetrahydrofuran

TLC – Thin layer chromatography

TMS – Tetramethylsilane

TLR4 – Toll like receptor

<sup>t</sup>RNA – Transfer ribonucleic acid

TST – Thiosulfate sulfurtransferase

$t_{1/2}$  – Half-life

UV – Ultraviolet

XRCC1 - X-ray repair cross-complementing protein 1

$\mu\text{g}$  – Microgram

$\mu\text{mol}$  – Micromolar

$\mu\text{L}$  – Microliter

$\mu\text{m}$  – Micrometre

$\text{Zn}(\text{OAc})_2$  – Zinc Acetate

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**Abstract**

Hydrogen sulfide (H<sub>2</sub>S) is a major gaseous signalling molecule that is responsible for redox regulation within cells, having implications for primary metabolism, antibiotic response, health, and ageing. 3-Mercaptopyruvate sulfurtransferase (3-MST) is one of the key enzyme involved in the biosynthesis of H<sub>2</sub>S which occurs through a crucial intermediate step called as 3-MST persulfidation (MST-SSH). Persulfide generated from the 3-MST pathway is essential for regulating mitochondrial activity and transporting sulfur *via* downstream sulfide transfer to proteins, low molecular weight thiols, and Fe-S clusters. Precise mechanisms by which these effects are mediated by 3-MST remain poorly understood. Also, several studies have shown that dysfunction or dysregulation of 3-MST is linked to various pathophysiological conditions. Hence, the development of a reliable tool is required in order to have better understanding of 3-MST activity as well as 3-MST expression level.

In order to understand the biological effects of 3-MST, we hypothesized to modulate the substrate delivery to the enzyme. Owing to the limitations associated with the native substrate (3-mercaptopyruvate), it can not be used reliably to enhance 3-MST activity. In this regard, our lab identified 2-mercapto-1-phenylethan-1-one (PhCOCH<sub>2</sub>SH) as an artificial substrate for 3-MST that produces H<sub>2</sub>S/persulfide in cells. Following this, a library of PhCOCH<sub>2</sub>SH analogues was synthesized to tune the rate of H<sub>2</sub>S and persulfide production. Structure-activity study with analogues revealed that 3-MST was fairly accommodative of structural modifications. Linear free energy relationship study showed that H<sub>2</sub>S release rates from *para*-substituted aryl substrates in the presence of 3-MST had a moderate positive slope supporting a transition state with a no major charge build up. *Ortho* substituents slowed H<sub>2</sub>S generation rates likely due to the steric effects and molecular docking studies showed that this substitution pattern reduced access to the active site due to steric clash with certain residues. Next, building on these results, a substrate tethered with a mitochondrial tag at the *para*-position was synthesized since 3-MST is known to be over-expressed in mitochondria. This compound generated H<sub>2</sub>S/persulfide in the presence of 3-MST and enhanced H<sub>2</sub>S levels specifically in mitochondria. Consistent with H<sub>2</sub>S generation, a perturbation in the mitochondrial membrane potential was observed for the cells treated with the substrate. Lastly, in order to further expand the scope and improve the stability and selectivity of the artificial substrates, enzyme-activated artificial substrates were prepared. A  $\beta$ -glucosidase sensitive donor was synthesized to enhance aqueous solubility and better selectivity. Similarly, esterase-activated compounds were also synthesized to achieve a broad spectrum of applications. Upon activation, both  $\beta$ -glucosidase and esterase activated

donors were found to produce H<sub>2</sub>S/persulfide in the presence of 3-MST. Taken together, a series of artificial substrates for 3-MST were developed as a tool for enhancing persulfidation levels in cells. Hence, therapeutic utilities of these substrates with respect to 3-MST activity can be harnessed and will give a clearer understanding of persulfide biochemistry which was obsolete to date.

## CHAPTER 1. Introduction

### 1.1. H<sub>2</sub>S and persulfides in the biological system

From the beginning of life on earth, biomolecules containing sulfur atoms have served various key roles in maintaining regular functions in living organisms. Sulfur atom present in amino acids such as cysteine and methionine are known to be an important building block for creating the foundation of life.<sup>1</sup> Often, cysteine is found in the active site of an enzyme and can participate in enzyme catalysis.<sup>2</sup> On the other hand, methionine present in the protein takes part in the regulation of metabolic process, the innate immune system, and digestive functioning in mammals.<sup>3</sup> Furthermore, sulfur atoms are also part of iron-sulfur (Fe-S) clusters, coenzymes and co-substrates and mediator of several metabolic pathways.<sup>4-7</sup> In addition to amino acids containing sulfur, hydrogen sulfide (H<sub>2</sub>S), a small gaseous molecule, is found to be associated with various important intracellular signaling processes and plays a central role in cellular pathophysiology and disease.<sup>8-10</sup> H<sub>2</sub>S is known to influence the gastrointestinal, neuronal, cardiovascular, respiratory, renal, and hepatic systems.<sup>11</sup> Therefore, a variety of biological functions such as vasorelaxation, angiogenesis, apoptosis, aging, and metabolism are often linked to the activity of biological H<sub>2</sub>S.<sup>11</sup> Moreover, H<sub>2</sub>S is known to be an important antioxidant and balances cellular redox homeostasis.<sup>12</sup> An imbalance of H<sub>2</sub>S levels disturbs the aforementioned redox homeostasis and can subsequently lead to diseased states.<sup>13</sup> Therefore, sustaining a certain range of H<sub>2</sub>S levels in cells becomes important for the normal functioning of cells.

More recently, persulfides (RSSH/RSS<sup>-</sup>, where R = H or amino acid) have emerged as important player in H<sub>2</sub>S biology.<sup>14</sup> Persulfides are highly reactive, unstable species and have sulfur oxidation state -1 each, belongs to the category of reactive sulfur species (RSS) (Figure 1.1).

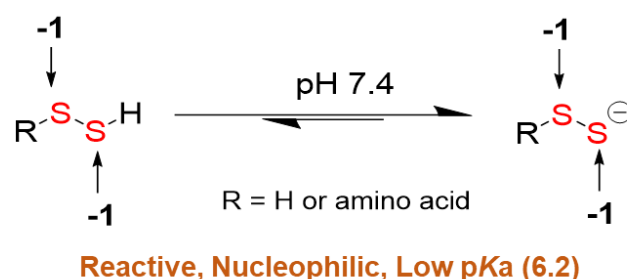


**Figure 1.1:** Persulfides in physiological pH.

There are several examples of persulfides that are formed intracellularly and take part in various biological processes (Figure 1.2).<sup>15,16</sup> Persulfides are reported to be superior antioxidants compared to  $\text{H}_2\text{S}$ , thus having more potential at countering oxidative stress and inflammation.<sup>17</sup> A multitude of studies showed, similar to  $\text{H}_2\text{S}$ , cellular persulfides are crucial in the signaling process and also relevant to many cardiovascular and neuronal diseases.<sup>17</sup> Hence, regulating the persulfide concentration in cells becomes important as well.

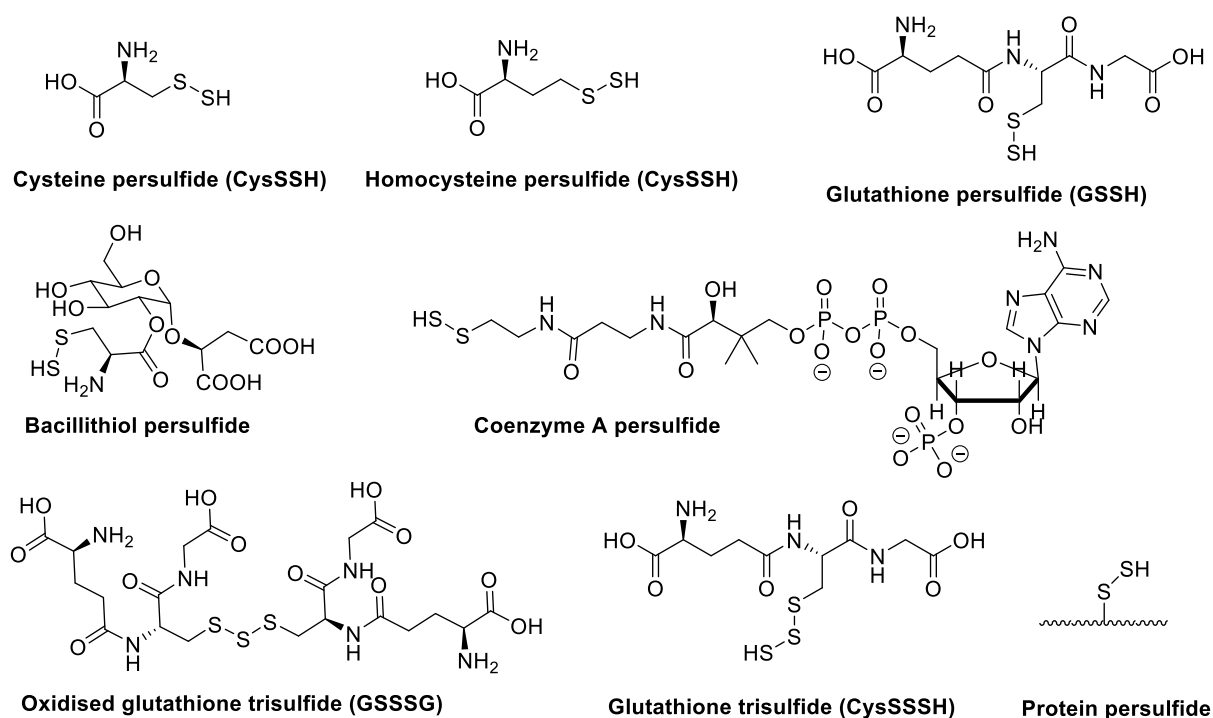


**Figure 1.2:** Examples of biological persulfides.

## 1.2. The biological relevance of protein persulfidation

Along with small molecule persulfides, the significance of protein persulfides have been reported.<sup>18</sup> Protein persulfidation or S-sulfhydration is an oxidative post-translational modification on the cysteine residue of protein where an additional sulfhydryl ( $\text{SH}/\text{S}^-$ ) group was added to a cysteine residue.<sup>18</sup> S-sulfhydration is known to be a very important regulator of several biological pathways.<sup>19</sup> Studies on protein persulfidation revealed it is a key biological transformation that directly or indirectly is involved in several pathophysiological conditions, such as protection from oxidative stress,<sup>20–22</sup> antioxidant response through KEAP1-Nrf2,<sup>12,23</sup> neurodegenerative diseases,<sup>24–26</sup> anti-inflammatory response,<sup>27–29</sup> DNA repair,<sup>30</sup> smooth muscle relaxation,<sup>31</sup> ageing,<sup>32</sup> etc. Although few reports are contradictory persulfidation has been shown to have a role in cancer,<sup>19,33,34</sup> GAPDH protein activity,<sup>35,36</sup> etc. A few of these are discussed.

### 1.2.1. Protection from oxidative stress

Protein persulfidation is particularly interesting since there have been reports which suggest that S-sulphydration protects protein thiols.<sup>37,38</sup> Protein thiols may be oxidized by ROS to sulfenic acids (R-SOH); further oxidation to sulfinic (R-SO<sub>2</sub>H) and sulfonic acids (R-SO<sub>3</sub>H) is considered an irreversible modification (Figure 1.3).<sup>20</sup> As a free thiol on the cysteine residue of protein is very important for catalytic reactions, irreversible oxidation deactivates the protein function. In such cases, H<sub>2</sub>S or a persulfide intervenes the oxidation process at the sulfenic acid stage, the sulfenic acid converts to a persulfide.<sup>21,22</sup> The newly formed persulfide would then be oxidized by ROS to form an S-sulfocysteine, from which the free thiol can be brought back *via* reduction by thioredoxin (Trx), a bioreductive enzyme.<sup>39</sup>

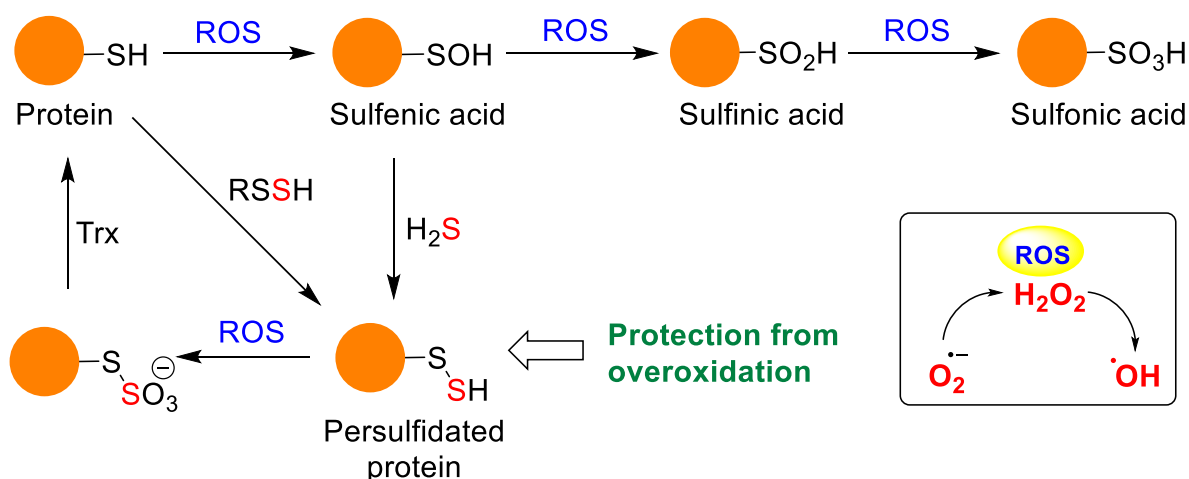
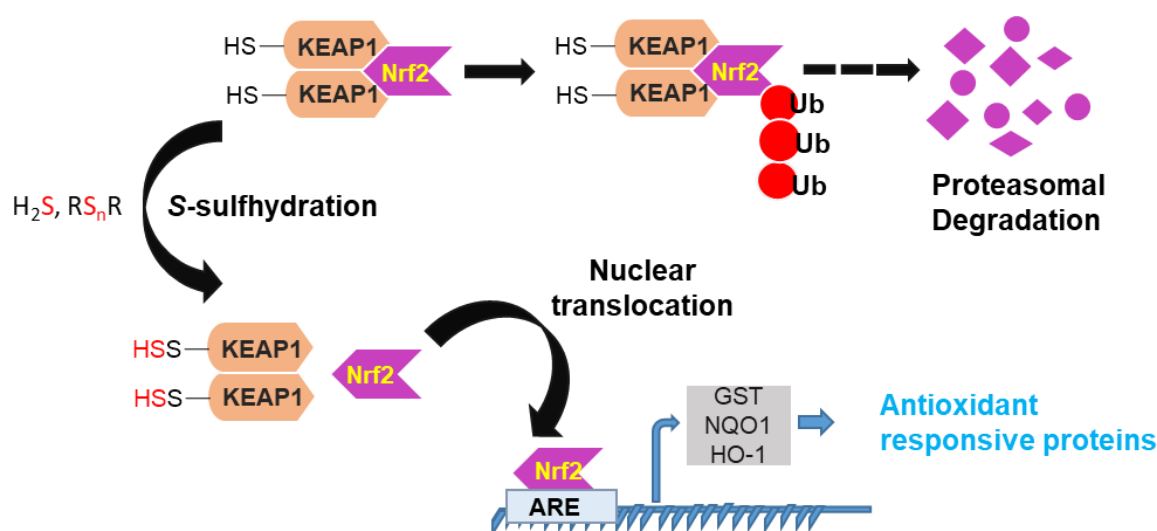


**Figure 1.3:** Oxidative stress protection *via* S-sulphydration.

### 1.2.2. Antioxidant response through KEAP1-Nrf2

One of the pathways through which persulfidation shows the antioxidant response in cells is KEAP1-Nrf2 signaling pathway.<sup>12,23</sup> KEAP1 is an oxidative stress sensor protein that forms a complex with Nrf2 under normal conditions (Figure 1.4). KEAP1, being part of ubiquitin ligase system, it causes Nrf2 complex ubiquitinated which leads to its proteasomal degradation. Under oxidative stress conditions, a free cysteine residue of KEAP1 was reported to be persulfidated *via* H<sub>2</sub>S or persulfides and simultaneously Nrf2 gets separated and translocated into the nucleus where Nrf2 binds to antioxidant-responsive elements (ARE) and activate ARE. ARE upon activation release antioxidant-responsive proteins (GST, NQO1, HO-1).<sup>40</sup>



Kelch-like ECH-associated protein 1 (KEAP1), Nuclear factor erythroid-related factor 2 (Nrf2), Ubiquitin (Ub)

**Figure 1.4:** Antioxidant response mechanism through KEAP1-Nrf2 persulfidation.

### 1.2.3. Persulfidation in neurodegenerative diseases

Parkin is a well-known neurodegenerative disorder where a protein called parkin is known to be responsible for dopamine-generating cell death.<sup>41</sup> Parkin is an E3 ligase protein that has a free cysteine residue and is available for oxidative post-translational modification (Figure 1.5).<sup>24</sup> For eg. parkin protein can be S-nitrosylated which deactivates the E3 ligase activity, therefore, protein aggregates can form which leads to Parkinson's disease.<sup>42</sup> But when the thiol group of parkin is persulfidated it leads to E3 ligase activation followed by proteasomal degradation which plays a crucial role in the clearance of damaged proteins.<sup>24,25</sup>

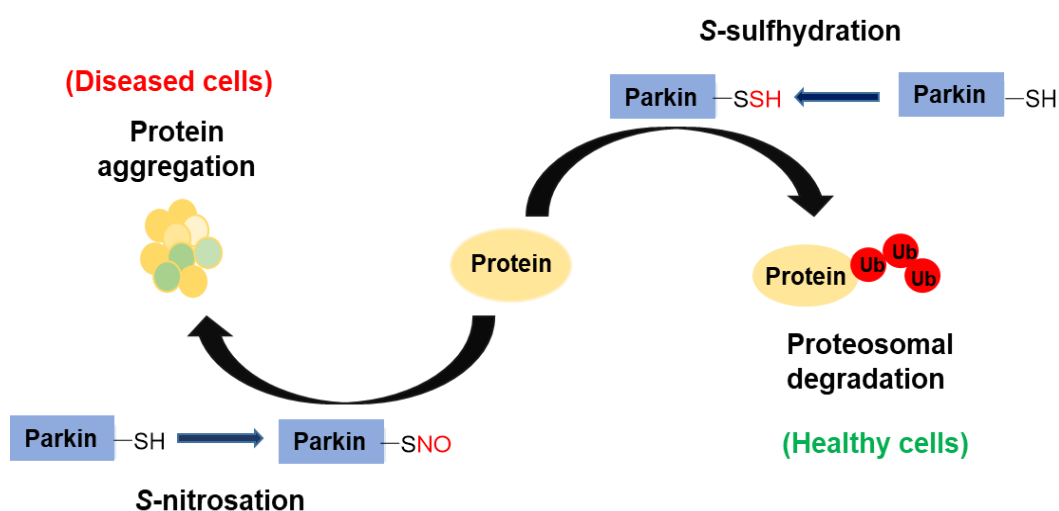


**Figure 1.5:** Regulation of parkin activity through S-sulfhydration.

Another well-known neurodegenerative disease, Alzheimer's is also linked to protein persulfidation. Alzheimer's disease is reported to be caused by abnormal aggregation of certain proteins deposited in the brain (Figure 1.6). One such protein called  $\beta$ -amyloid is known to be the main responsible factor as it aggregates plaques around the brain.<sup>43</sup> Another important protein is called tau which changes its structural conformations due to hyperphosphorylation. This hyperphosphorylation of tau is reported to be mediated by glycogen synthase kinase (GSK3 $\beta$ ) which results in Tau protein aggregation that leads to neurotoxicity, a known hallmark for Alzheimer's disease. Persulfidation on GSK3 $\beta$  has shown to have a neuroprotective effect as persulfidation inhibits its activity and thereby prevents hyperphosphorylation of Tau protein.<sup>26</sup>

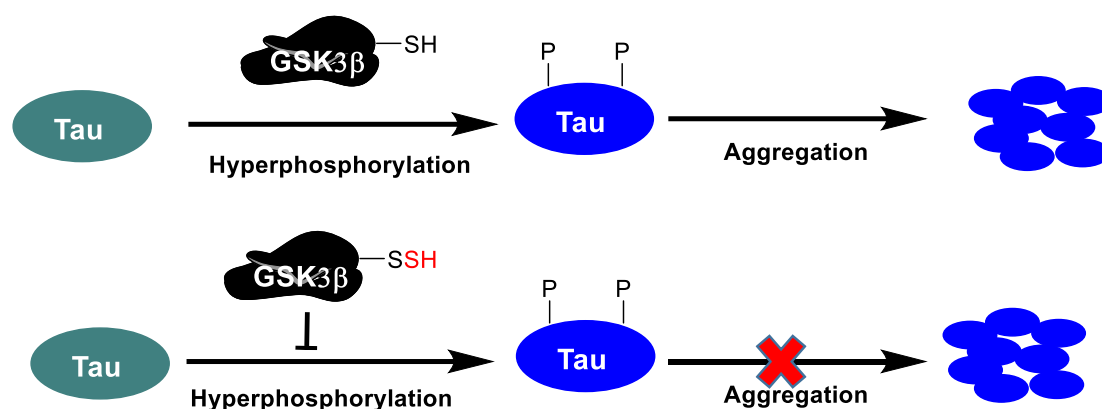


**Figure 1.6:** Regulation of GSK3 $\beta$  activity through S-sulfhydration.

#### 1.2.4. Persulfidation in DNA repair:

Another interesting case of protein persulfidation is relevant in DNA damage repair.<sup>30</sup> DNA damage stimulates a cascade of events leading to repair, part of which is the binding of PARPs (poly(ADP-ribose)ation polymerases) to DNA strand breaks.

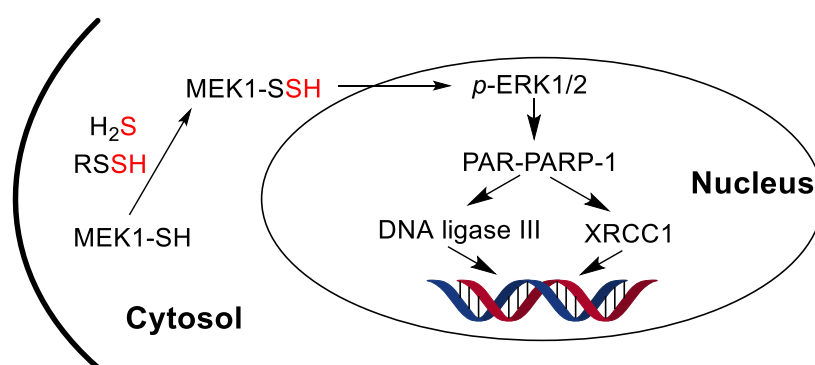


**Figure 1.7:** Role of S-sulfhydration in DNA repair.

These PARPs are activated by the MEK/ERK signalling. It has been reported that when Cys341 in MEK1 is persulfidated, as a response, the translocation of phosphorylated ERK1/2 into the nucleus is being facilitated, where it activates PARP-1 and thus, encountering damage repair (Figure 1.7).<sup>44</sup>

### 1.2.5. Persulfidation in Inflammation

Protein persulfidation also plays a key role in the immune response. The transcription factor NF- $\kappa$ B is known as a nuclear factor that regulates innate and adaptive immune functions in cells and serves as a central mediator of inflammatory response machinery.<sup>45</sup> NF- $\kappa$ B is known to express pro-inflammatory genes including the genes for various cytokines and chemokines and also participates in inflammasome regulation (Figure 1.8). NF- $\kappa$ B consists of two subunits p65 and p50 which under no stimulus condition form a complex with inhibitory  $\kappa$ B (I $\kappa$ B).

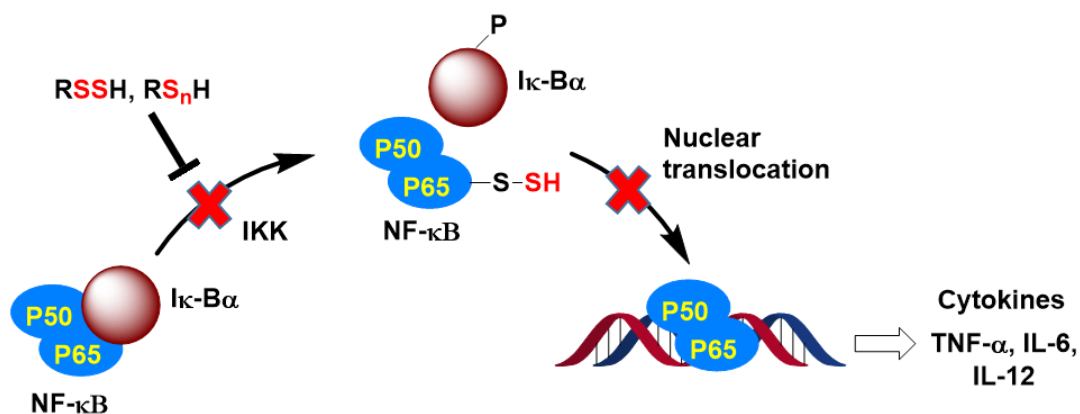


**Figure 1.8.** Role of S-sulfhydrylation in inflammation. Persulfidation of NF- $\kappa$ B triggers an anti-inflammatory effect.

In a stimulus condition, I $\kappa$ B kinase phosphorylates I $\kappa$ B and NF- $\kappa$ B is released subsequently.<sup>27</sup> NF- $\kappa$ B is then phosphorylated and after nuclear translocation induces the pro-inflammatory cytokines-releasing machinery to produce TNF- $\alpha$ , IL-6, IL-12, etc. Studies have shown that persulfidation on the Cys38 residue of the p65 subunit of NF- $\kappa$ B has an anti-inflammatory effect. Persulfidation leads to a decline in phosphorylation and reduces the rate of translocation, therefore, inhibiting DNA binding activity.<sup>28</sup> As a result, the level of cytokines level is influenced by the persulfide formation.<sup>29</sup>

### 1.2.6. Persulfidation in smooth muscle relaxation

Apart from these, persulfidation has a relevance in smooth muscle relaxation.<sup>31</sup> For instance, Cys43 in the Kir6.1 subunit of K<sub>ATP</sub> channels in smooth muscle cells is believed to get

persulfidated, leading to smooth muscle cell relaxation. Persulfidation inhibits the binding of ATP to the channel and promotes its binding with a phospholipid component (phosphatidylinositol-4,5-bisphosphate), the consequent opening of the channel to allow  $K^+$  ions to enter.

### 1.3. Biosynthesis of persulfides

Persulfides are synthesized in cells through enzymatic biosynthetic pathways. For instance, a commonly known cysteine persulfide (Cys-SSH) is formed from cystine in the presence of Cystathionine- $\beta$ -synthase (CBS) and Cystathionine  $\gamma$ -lyase (CSE) enzyme.<sup>15,46</sup> Also, it has been recently reported that Cynteinyl-*t*-RNA synthetases (CARS) can produce Cys-SSH from *L*-cysteine (Figure 1.9).<sup>47</sup>

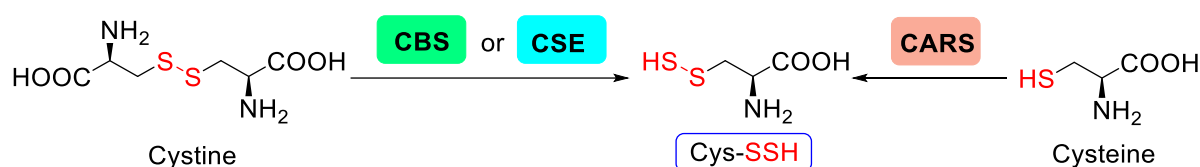


**Figure 1.9:** Cysteine persulfide formation from CBS, CSE and CARS.

Another broad class of enzymes, namely sulfurtransferases is known to be majorly responsible for persulfide biosynthesis and sulfur trafficking in the cells (Figure 1.10).<sup>48–50</sup> The catalytic cycle of this enzyme is known to follow a two-step mechanism. First, the active site cysteine residue of the native protein gets persulfidated using a donor molecule to form a transient protein-persulfide intermediate. Next, the sulfur is transferred from persulfidated intermediate to a thiophilic acceptor molecule with an instantaneous generation of the native enzyme.

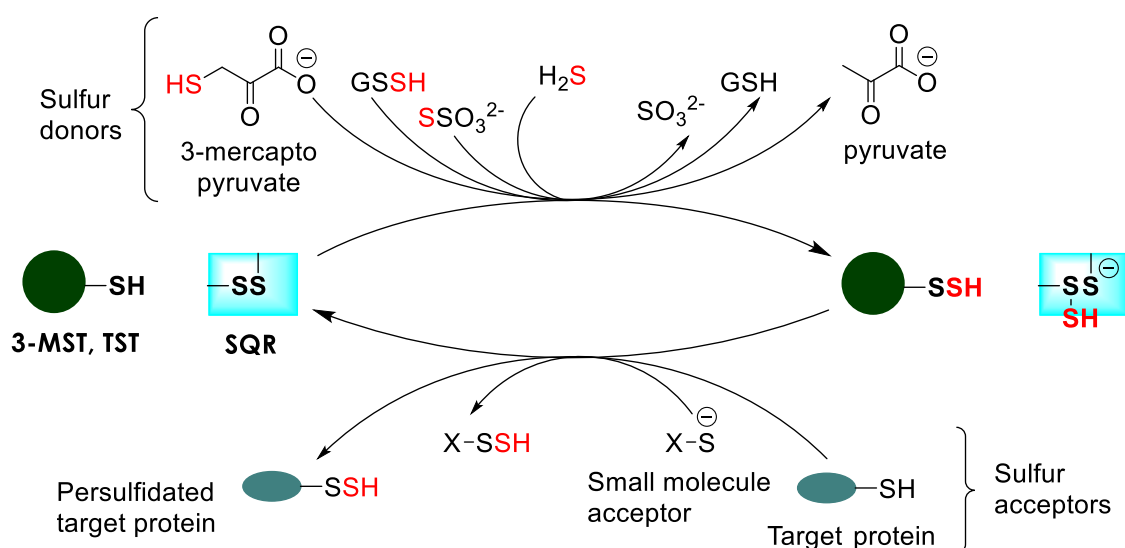


**Figure 1.10:** Catalytic turnover of sulfurtransferases.

3-Mercaptopyruvate sulfurtransferase (3-MST),<sup>49,51</sup> Thiosulfate sulfurtransferase (TST)<sup>52</sup> and sulfide:quinone reductase<sup>53</sup> are known to be primarily involved in the process of persulfide formation and sulfur transfer.

### 1.3.1. Persulfide biosynthesis through 3-mercaptopyruvate sulfurtransferase

Among all the sulfurtransferases discussed, 3-MST has gained considerable attention over the years due to its involvement in H<sub>2</sub>S, persulfide and polysulfide biosynthesis as well as in sulfur trafficking.<sup>52,54,55</sup> The natural substrate of 3-MST, 3-mercaptopyruvate (3-MP), is physiologically synthesized from *L*-cysteine by the action of cysteine aminotransferase (CAT) in presence of  $\alpha$ -ketoglutarate (Figure 1.11).<sup>51, 56</sup> During turnover, 3-MP is known to bind with 3-MST where two crucial residues Arg 188 and Arg 197 anchor the substrate in the active site whereas 3 amino acid-based catalytic triad (Asp 63 - His 74 - Ser 250) deprotonates Cys 248 residue, which reacts with 3-MP to form 3-MST persulfide (Figure 1.12).<sup>51,56</sup>

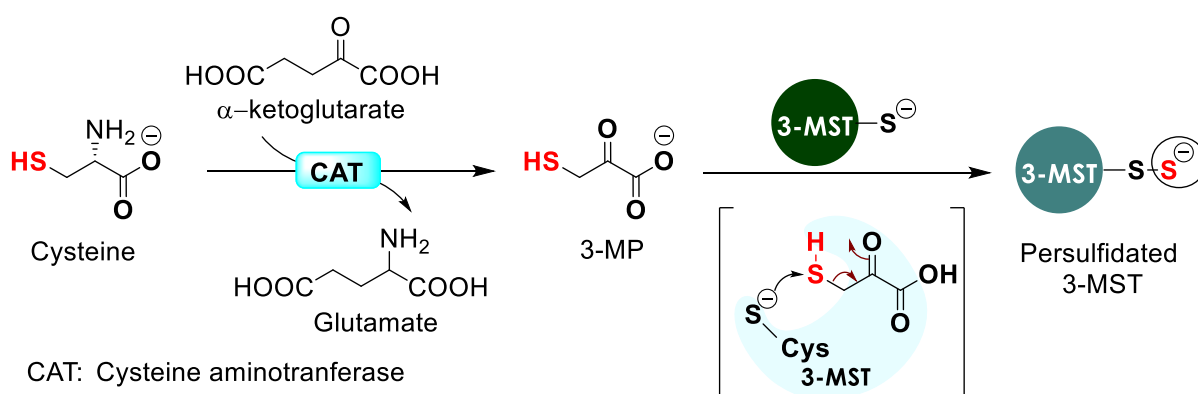


**Figure 1.11:** Catalytic turnover of 3-MST.

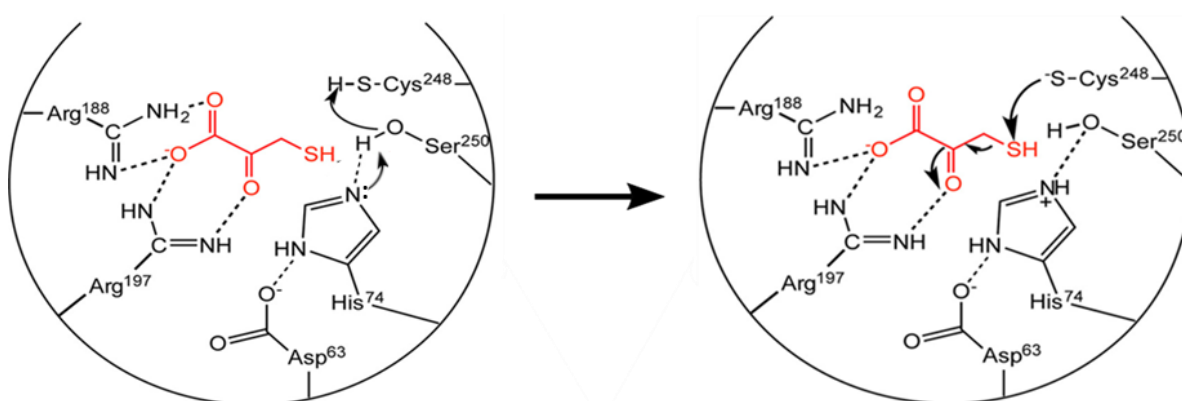


**Figure 1.12:** Turnover mechanism of 3-MP in the active site of human 3-MST.<sup>51</sup>

Formed 3-MST persulfide is known to undergo the following transformations.<sup>49,55,56</sup> 1) It can transfer the sulfur to a thiol acceptor (a protein or a small molecule) and form persulfide;<sup>49</sup> 2)

React with an excess amount of 3-MP and generate hydropersulfide and polysulfide;<sup>55</sup> 3) 3-MST bound persulfide reacts with thiols or thioredoxin (in presence of NADPH/thioredoxin reductase) to generate  $\text{H}_2\text{S}$  (Figure 1.13).<sup>56</sup>

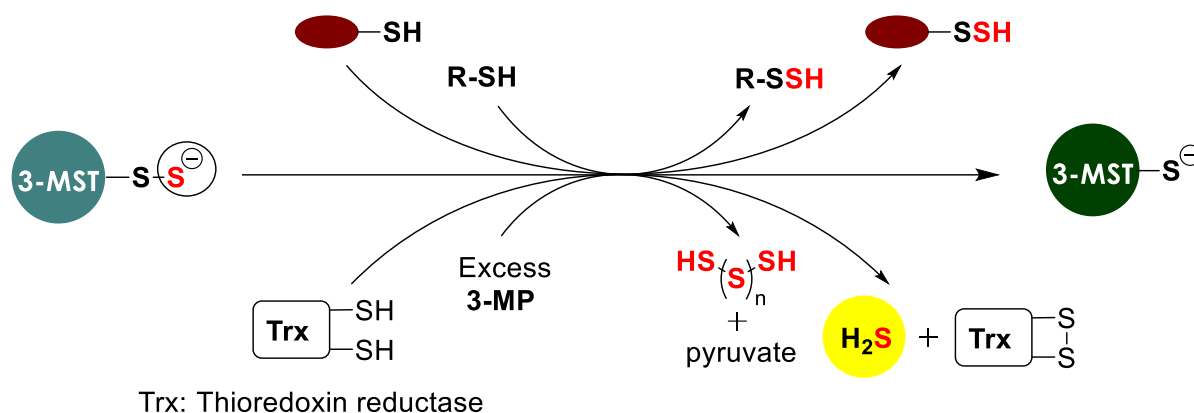


**Figure 1.13:** Enzymatic function of 3-MST in cellular  $\text{H}_2\text{S}$ , persulfide and polysulfide production.

3-MST is a widely prevalent enzyme in many living organisms. In bacteria, such as *E. coli*, 3-MST is primarily responsible for enzymatic  $\text{H}_2\text{S}$  and persulfide generation.<sup>57</sup> However, in the case of the mammalian systems, 3-MST coexists with CBS, CSE and CARS.<sup>47,50,58</sup> A comprehensive tissue distribution study has shown that 3-MST is constitutively expressed in the brain, liver and kidney (Figure 1.14).<sup>59</sup>

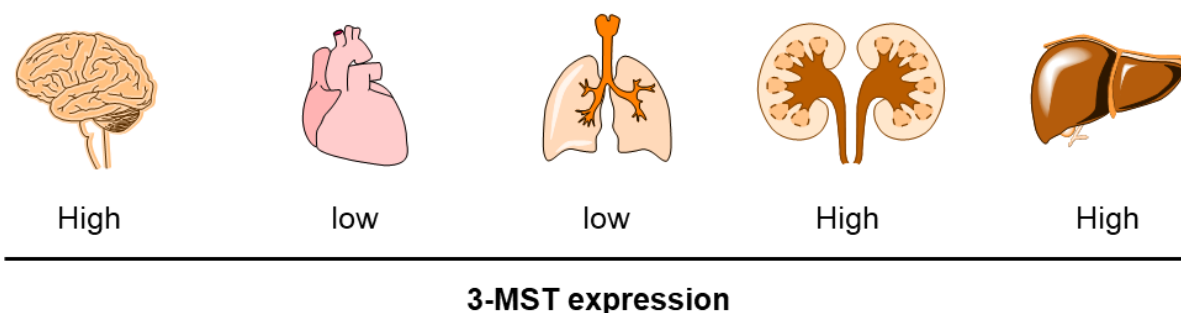


**Figure 1.14:** Biodistribution of 3-MST in tissues.

Two types of isoforms of 3-MST are found in mammalian cells.<sup>60</sup> At a subcellular level, 3-MST is present in both cytosol and mitochondria. Expression 3-MST in mitochondria (m3-MST) was found to be 2.5-3 times higher than that of cytosolic 3-MST (Figure 1.15).<sup>60,61</sup>

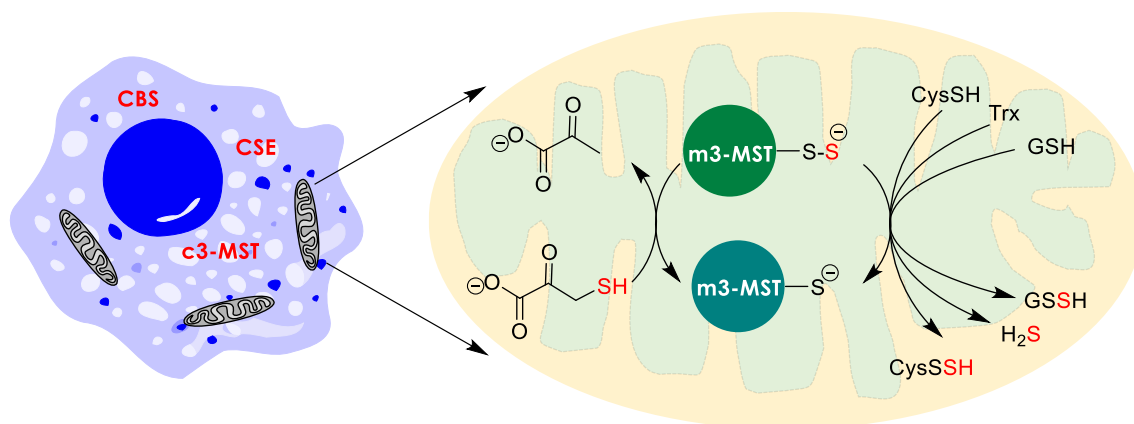


**Figure 1.15:** Subcellular distribution shows a predominant expression of mitochondrial 3-MST that generates  $H_2S$  and persulfides.

#### 1.4. Biological roles of 3-MST activity and 3-MST expression

As the name suggests, 3-MST is majorly known for sulfur transfer to the acceptor protein thiols or small molecular thiols through transpersulfidation.<sup>62,63</sup> Protein persulfides formed after 3-MST sulfur transfer are responsible for various signaling processes. Small molecular persulfides, such as CySSH or GSSH formed as a result of 3-MST transpersulfidation is very important for the antioxidant response.<sup>64</sup> Also,  $H_2S$  and persulfide generated from 3-MST contribute to the cellular sulfane sulfur pool and the biological significance of these species was discussed previously. Apart from this, 3-MST is known to have key roles in cyanide detoxification,<sup>65,66</sup> fatty acid metabolism,<sup>67</sup> synthesis of thiouridine in tRNA<sup>68</sup> and mitochondrial bioenergetics.<sup>69,70</sup> In mitochondria, 3-MST derived  $H_2S$  is known to influence the mitochondrial electron transport chain.<sup>71,72</sup> CySSH or GSSH generated in mitochondria is known to react with excess ROS to form sulfur oxides to protect mitochondria from oxidative stress.<sup>73,74</sup>

Studies have shown that variation of 3-MST expression levels correlates well with the 3-MST activity. The levels of 3-MST were found to be overexpressed in obese patients.<sup>67,75</sup> Furthermore, clinical studies have shown that patients with schizophrenia exhibited higher 3-MST protein levels in the brain.<sup>76</sup> On the contrary, the 3-MST level increases during development and then gradually declines with aging.<sup>32,59,77</sup> Another study has revealed that diabetic patients showed a tissue-dependent variation in the expression levels of 3-MST. For example, the levels were lower in cardiac and smooth muscle whereas an increase in expression was found in the skeletal muscles.<sup>78,79</sup> Studies have shown dysfunction or dysregulation of 3-MST may have a potential role in several pathophysiological conditions. A study has shown

that 3-MST knocked down (KO) mice have a higher level of 2HT<sub>2A</sub> mRNA expression which is responsible for mental retardation and anxiety-like behaviour.<sup>80</sup> Another compelling evidence demonstrated that 3-MST (KO) mice developed an exacerbated joint calcification and severe osteoarthritis.<sup>81</sup>

### 1.5. Aim and objective

3-MST has a diverse role in biological processes and diseases and hence, the control over 3-MST activity (expression) as well as persulfide generated by this pathway become important. It was hypothesized that controlling the 3-MST activity in pathophysiological conditions would eventually help us to gain control over the possible pharmacological facets such as

- 1) **Perturbation of H<sub>2</sub>S and sulfane sulfur levels in cells:** H<sub>2</sub>S and sulfane sulfur are very important for normal cellular function. By modulating 3-MST activity and catalysis, response to oxidative stress and maintenance of cellular health will be possible.
- 2) **Tracking sulfur trafficking through 3-MST:** 3-MST is known to transfer sulfur to low molecular weight thiols or protein thiols. These species are known to be involved in various downstream signaling pathways. Tracking the sulfur derived from 3-MST will enable us to understand many yet to be uncovered transsulfuration pathways.
- 3) **Controlling 3-MST-associated therapeutics:** Owing to the direct or indirect involvement in many diseases, control over 3-MST activity will provide a definite edge in 3-MST-related therapeutics.

### 1.6. Possible approaches for modulation of 3-MST activity

In principle, 3-MST activity can be modulated through genetic method and biochemical method. In a genetic method, a gene related to 3-MST expression can be modified. A knocked down cell line can be generated *via* genetic engineering to lower the expression of 3-MST which will eventually lead to lowering of 3-MST activity. On the contrary, overexpressed cells can be generated for enhancing the 3-MST activity. However, these complex methods are irreversible and involve many steps. Most importantly, genetic modification for 3-MST can alter the activity of other neighboring proteins.

On the other hand, in a biochemical method using a substrate or an inhibitor can give a similar result without harming neighboring enzyme's activity. For this, the substrate must be specific for 3-MST and the byproducts formed from the turnover must be innocuous. Similarly, an inhibitor must have high specificity towards 3-MST. The utilization of a substrate or an inhibitor can either enhance or diminish the 3-MST activity (Figure 1.16).

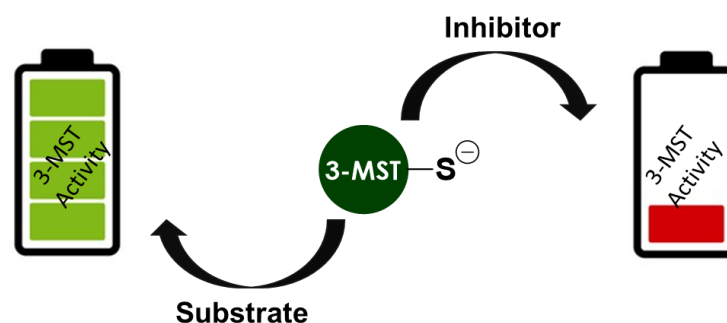


**Figure 1.16:** Modulation of 3-MST activity using a substrate and an inhibitor.

In this context, an additional substrate can be provided to enhance 3-MST enzyme activity. A substrate can be administered *via* the oral or intraperitoneal route to enhance the 3-MST activity in 3-MST deficit disorders.

### 1.7. Outline of the thesis

3-MP, a natural substrate due to the labile C-S bond generates  $H_2S$  in the absence of 3-MST which is a major limitation.<sup>82</sup> As 3-MP is not nameable for modification, the rate of catalysis cannot be tuned. Hence, a new substrate with a structural handle is required. This substrate should be synthetically accessible, stable, structurally amendable to modifications. In addition, the by-products generated during the 3-MST reaction should be benign to cells. Since, enzymes are known to be very specific for their substrates, designing a new substrate is challenging. As 3-MP is a small molecule, a similar analog needed to be designed to have efficient binding with the enzyme.

In Chapter 2, the design and development of an artificial substrate of 3-MST is described. In order to gain control over 3-MST turnover rates, we also constructed a small library of the artificial substrates. This was achieved by designing a new substrate based on an aromatic scaffold while retaining the reactive core of 3-MP. Upon turnover by 3-MST, the substrate will produce persulfides 3-MST and a benign ketone (Figure 1.17). Stability was improved by modifying the substrate to its thioacetate form. Activity of the newly developed substrate was evaluated through the efficiency of  $H_2S$  and persulfide generation. A structure activity relationship study was performed with a focussed library of artificial substrates in order to modulate turnover rates.

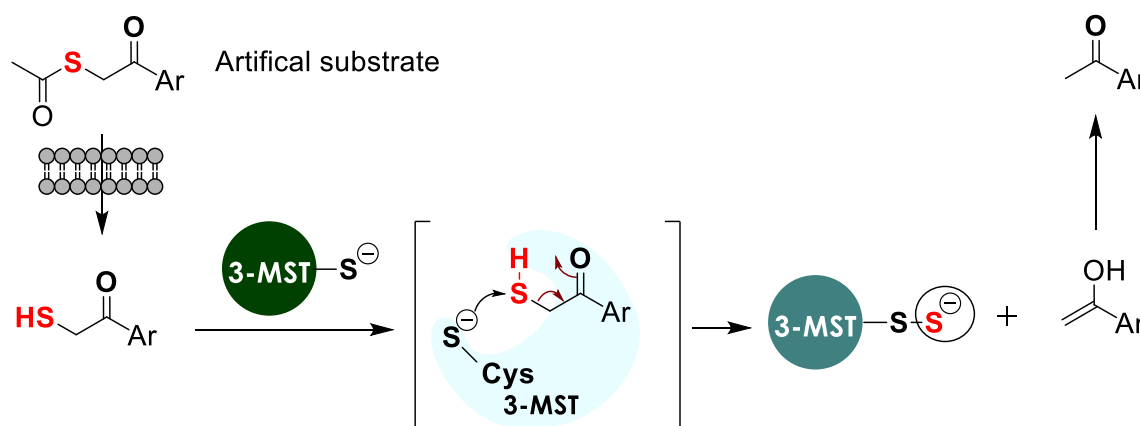


**Figure 1.17:** Design and development of an artificial substrate for 3-MST.

As 3-MST is known to be expressed to a higher degree in the mitochondria as compared with cytosol, design and synthesis of an artificial substrate tethered with a Mito-tag was investigated in Chapter 3. A modified substrate was seen to be localized into mitochondria and thereby a targeted H<sub>2</sub>S and persulfide generation was observed.

The substrate synthesized in Chapter 2 and 3 was found to be highly reactive with thiols. In order to improve the thiol stability and aqueous solubility, a glycosidase activatable prodrug of the artificial substrate was designed and synthesized in Chapter 4. The thiol group of the artificial substrates was caged with the linker-trigger moiety, cleavable by a specific glycosidase enzyme. Having an additional enzyme trigger for the generation of 3-MST substrate make this strategy highly specific.

In Chapter 5, an esterase triggered artificial substrate of 3-MST was designed and synthesized. Here a trimethyl lock linker was used for rapid substrate release during esterase-induced uncaging. Esterase, a widely prevalent enzyme, present in many organisms, will be useful to achieve a broader spectrum of applications with 3-MST.

## 1.8 Reference

- (1) Brosnan, J. T.; Brosnan, M. E. The Sulfur-Containing Amino Acids: An Overview. *J. Nutr.* **2006**, *136* (6), 1636S-1640S.
- (2) Nagahara, N. Catalytic Site Cysteines of Thiol Enzyme: Sulfurtransferases. *J. Amino Acids* **2011**, *2011*, 1–7.
- (3) Martínez, Y.; Li, X.; Liu, G.; Bin, P.; Yan, W.; Más, D.; Valdivié, M.; Hu, C.-A. A.; Ren, W.; Yin, Y. The Role of Methionine on Metabolism, Oxidative Stress, and Diseases. *Amino Acids* **2017**, *49* (12), 2091–2098.
- (4) Rouault, T. A.; Tong, W. H. Iron–Sulfur Cluster Biogenesis and Human Disease. *Trends Genet.* **2008**, *24* (8), 398–407.
- (5) Meriläinen, G.; Schmitz, W.; Wierenga, R. K.; Kursula, P. The Sulfur Atoms of the Substrate CoA and the Catalytic Cysteine Are Required for a Productive Mode of Substrate Binding in Bacterial Biosynthetic Thiolase, a Thioester-Dependent Enzyme. *FEBS J.* **2008**, *275* (24), 6136–6148.
- (6) Kessler, D. Enzymatic Activation of Sulfur for Incorporation into Biomolecules in Prokaryotes. *FEMS Microbiol. Rev.* **2006**, *30* (6), 825–840.
- (7) Leustek, T. Sulfate Metabolism. *Arab. B.* **2002**, *1*, e0017.
- (8) Gallyas, F. Involvement of Redox-Signalling in Endogenous Hydrogen Sulfide Production. *Br. J. Pharmacol.* **2012**, *166* (8), 2228–2230.
- (9) Stein, A.; Bailey, S. M. Redox Biology of Hydrogen Sulfide: Implications for Physiology, Pathophysiology, and Pharmacology. *Redox Biol.* **2013**, *1* (1), 32–39.
- (10) KIMURA, H. Hydrogen Sulfide and Polysulfides as Signaling Molecules. *Proc. Japan Acad. Ser. B* **2015**, *91* (4), 131–159.
- (11) Singh, S. B.; Lin, H. C. Hydrogen Sulfide in Physiology and Diseases of the Digestive Tract. *Microorganisms* **2015**, *3* (4), 866–889.
- (12) Xie, Z.-Z.; Liu, Y.; Bian, J.-S. Hydrogen Sulfide and Cellular Redox Homeostasis. *Oxid. Med. Cell. Longev.* **2016**, *2016* (5), 6043038.
- (13) Han, Y.; Shang, Q.; Yao, J.; Ji, Y. Hydrogen Sulfide: A Gaseous Signaling Molecule Modulates Tissue Homeostasis: Implications in Ophthalmic Diseases. *Cell Death Dis.* **2019**, *10* (4), 293.
- (14) Park, C.-M.; Weerasinghe, L.; Day, J. J.; Fukuto, J. M.; Xian, M. Persulfides: Current Knowledge and Challenges in Chemistry and Chemical Biology. *Mol. Biosyst.* **2015**, *11*

- (7), 1775–1785.
- (15) Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T. Reactive Cysteine Persulfides and S-Polythiolation Regulate Oxidative Stress and Redox Signaling. *Proc. Natl. Acad. Sci. U.S.A* **2014**, *111* (21), 7606–7611.
  - (16) Sawa, T.; Motohashi, H.; Ihara, H.; Akaike, T. Enzymatic Regulation and Biological Functions of Reactive Cysteine Persulfides and Polysulfides. *Biomolecules* **2020**, *10* (9), 1245.
  - (17) Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R. Chemical Biology of H<sub>2</sub>S Signaling through Persulfidation. *Chem. Rev.* **2018**, *118* (3), 1253–1337.
  - (18) Mustafa, A. K.; Gadalla, M. M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S. K.; Barrow, R. K.; Yang, G.; Wang, R.; Snyder, S. H. H<sub>2</sub>S Signals Through Protein S-Sulfhydration. *Sci. Signal.* **2009**, *2* (96), ra72 LP-ra72.
  - (19) Yang, C.-T.; Devarie-Baez, N. O.; Hamsath, A.; Fu, X.-D.; Xian, M. S-Persulfidation: Chemistry, Chemical Biology, and Significance in Health and Disease. *Antioxid. Redox Signal.* **2019**.
  - (20) Paulsen, C. E.; Carroll, K. S. Cysteine-Mediated Redox Signaling: Chemistry, Biology, and Tools for Discovery. *Chem. Rev.* **2013**, *113* (7), 4633–4679.
  - (21) Filipovic, M. R. Persulfidation (S-Sulfhydration) and H<sub>2</sub>S; 2015; pp 29–59.
  - (22) Dóka, É.; Ida, T.; Dagnell, M.; Abiko, Y.; Luong, N. C.; Balog, N.; Takata, T.; Espinosa, B.; Nishimura, A.; Cheng, Q.; Funato, Y.; Miki, H.; Fukuto, J. M.; Prigge, J. R.; Schmidt, E. E.; Arnér, E. S. J.; Kumagai, Y.; Akaike, T.; Nagy, P. Control of Protein Function through Oxidation and Reduction of Persulfidated States. *Sci. Adv.* **2020**, *6* (1).
  - (23) Yang, G.; Zhao, K.; Ju, Y.; Mani, S.; Cao, Q.; Puukila, S.; Khaper, N.; Wu, L.; Wang, R. Hydrogen Sulfide Protects Against Cellular Senescence via S -Sulfhydration of Keap1 and Activation of Nrf2. *Antioxid. Redox Signal.* **2013**, *18* (15), 1906–1919.
  - (24) Vandiver, M. S.; Paul, B. D.; Xu, R.; Karuppagounder, S.; Rao, F.; Snowman, A. M.; Seok Ko, H.; Il Lee, Y.; Dawson, V. L.; Dawson, T. M.; Sen, N.; Snyder, S. H. Sulfhydration Mediates Neuroprotective Actions of Parkin. *Nat. Commun.* **2013**, *4*, 1626.
  - (25) Hannula, M. J.; Myöhänen, T. T.; Tenorio-Laranga, J.; Männistö, P. T.; Garcia-Horsman, J. A. Prolyl Oligopeptidase Colocalizes with  $\alpha$ -Synuclein,  $\beta$ -Amyloid, Tau Protein and Astroglia in the Post-Mortem Brain Samples with Parkinson's and

- Alzheimer's Diseases. *Neuroscience* **2013**, 242, 140–150.
- (26) Giovinazzo, D.; Bursac, B.; Sbodio, J. I.; Nalluru, S.; Vignane, T.; Snowman, A. M.; Albacarys, L. M.; Sedlak, T. W.; Torregrossa, R.; Whiteman, M.; Filipovic, M. R.; Snyder, S. H.; Paul, B. D. Hydrogen Sulfide Is Neuroprotective in Alzheimer's Disease by Sulfhydrating GSK3 $\beta$  and Inhibiting Tau Hyperphosphorylation. *Proc. Natl. Acad. Sci.* **2021**, 118 (4).
  - (27) Aggarwal, B. B.; Gupta, S. C.; Kim, J. H. Historical Perspectives on Tumor Necrosis Factor and Its Superfamily: 25 Years Later, a Golden Journey. *Blood* **2012**, 119 (3), 651–665..
  - (28) Du, J.; Huang, Y.; Yan, H.; Zhang, Q.; Zhao, M.; Zhu, M.; Liu, J.; Chen, S. X.; Bu, D.; Tang, C.; Jin, H. Hydrogen Sulfide Suppresses Oxidized Low-Density Lipoprotein (Ox-LDL)-Stimulated Monocyte Chemoattractant Protein 1 Generation from Macrophages via the Nuclear Factor KB (NF-KB) Pathway. *J. Biol. Chem.* **2014**, 289 (14), 9741–9753.
  - (29) Zhang, T.; Ono, K.; Tsutsuki, H.; Ihara, H.; Islam, W.; Akaike, T.; Sawa, T. Enhanced Cellular Polysulfides Negatively Regulate TLR4 Signaling and Mitigate Lethal Endotoxin Shock. *Cell Chem. Biol.* **2019**, 26 (5), 686-698.e4.
  - (30) Zhao, K.; Ju, Y.; Li, S.; Altaany, Z.; Wang, R.; Yang, G. S-sulfhydration of MEK1 Leads to PARP-1 Activation and DNA Damage Repair. *EMBO Rep.* **2014**, 15 (7), 792–800.
  - (31) Kang, M.; Hashimoto, A.; Gade, A.; Akbarali, H. I. Interaction between Hydrogen Sulfide-Induced Sulfhydration and Tyrosine Nitration in the K ATP Channel Complex. *Am. J. Physiol. Liver Physiol.* **2015**, 308 (6), G532–G539.
  - (32) Zivanovic, J.; Kouroussis, E.; Kohl, J. B.; Adhikari, B.; Bursac, B.; Schott-Roux, S.; Petrovic, D.; Miljkovic, J. L.; Thomas-Lopez, D.; Jung, Y.; Miler, M.; Mitchell, S.; Milosevic, V.; Gomes, J. E.; Benhar, M.; Gonzales-Zorn, B.; Ivanovic-Burmazovic, I.; Torregrossa, R.; Mitchell, J. R.; Whiteman, M.; Schwarz, G.; Snyder, S. H.; Paul, B. D.; Carroll, K. S.; Filipovic, M. R. Selective Persulfide Detection Reveals Evolutionarily Conserved Antiaging Effects of S-Sulfhydration. *Cell Metab.* **2019**, 30 (6), 1152-1170.e13.
  - (33) Wang, R.-H.; Chu, Y.-H.; Lin, K.-T. The Hidden Role of Hydrogen Sulfide Metabolism in Cancer. *Int. J. Mol. Sci.* **2021**, 22 (12), 6562.
  - (34) Saha, S.; Reddy, C. V. R.; Xu, S.; Sankar, S.; Neamati, N.; Patro, B. Synthesis and SAR Studies of Marine Natural Products Ma'edamines A, B and Their Analogues. *Bioorg.*

- Med. Chem. Lett.* **2013**, 23 (18), 5135–5139.
- (35) Jarosz, A. P.; Wei, W.; Gauld, J. W.; Auld, J.; Özcan, F.; Aslan, M.; Mutus, B. Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) Is Inactivated by S-Sulfuration in Vitro. *Free Radic. Biol. Med.* **2015**, 89, 512–521.
- (36) Yu, B.; Zheng, Y.; Yuan, Z.; Li, S.; Zhu, H.; De La Cruz, L. K.; Zhang, J.; Ji, K.; Wang, S.; Wang, B. Toward Direct Protein S-Persulfidation: A Prodrug Approach That Directly Delivers Hydrogen Persulfide. *J. Am. Chem. Soc.* **2018**, 140 (1), 30–33.
- (37) Woo, H. A.; Chae, H. Z.; Hwang, S. C.; Yang, K.-S.; Kang, S. W.; Kim, K.; Rhee, S. G. Reversing the Inactivation of Peroxiredoxins Caused by Cysteine Sulfinic Acid Formation. *Science (80-. )*. **2003**, 300 (5619), 653–656.
- (38) Wood, Z. A.; Poole, L. B.; Karplus, P. A. Peroxiredoxin Evolution and the Regulation of Hydrogen Peroxide Signaling. *Science (80-. )*. **2003**, 300 (5619), 650–653.
- (39) Wedmann, R.; Onderka, C.; Wei, S.; Sziájtó, I. A.; Miljkovic, J. L.; Mitrovic, A.; Lange, M.; Savitsky, S.; Yadav, P. K.; Torregrossa, R.; Harrer, E. G.; Harrer, T.; Ishii, I.; Gollasch, M.; Wood, M. E.; Galardon, E.; Xian, M.; Whiteman, M.; Banerjee, R.; Filipovic, M. R. Improved Tag-Switch Method Reveals That Thioredoxin Acts as Depersulfidase and Controls the Intracellular Levels of Protein Persulfidation. *Chem. Sci.* **2016**, 7 (5), 3414–3426.
- (40) Koike, S.; Ogasawara, Y.; Shibuya, N.; Kimura, H.; Ishii, K. Polysulfide Exerts a Protective Effect against Cytotoxicity Caused by t -Buthylhydroperoxide through Nrf2 Signaling in Neuroblastoma Cells. *FEBS Lett.* **2013**, 587 (21), 3548–3555.
- (41) Shulman, J. M.; De Jager, P. L.; Feany, M. B. Parkinson's Disease: Genetics and Pathogenesis. *Annu. Rev. Pathol. Mech. Dis.* **2011**, 6 (1), 193–222.
- (42) Chung, K. K. K.; Thomas, B.; Li, X.; Pletnikova, O.; Troncoso, J. C.; Marsh, L.; Dawson, V. L.; Dawson, T. M. S -Nitrosylation of Parkin Regulates Ubiquitination and Compromises Parkin's Protective Function. *Science (80-. )*. **2004**, 304 (5675), 1328–1331.
- (43) Zhang, H.; Gao, Y.; Zhao, F.; Dai, Z.; Meng, T.; Tu, S.; Yan, Y. Hydrogen Sulfide Reduces mRNA and Protein Levels of Beta-Site Amyloid Precursor Protein Cleaving Enzyme 1 in PC12 Cells. *Neurochem. Int.* **2011**, 58 (2), 169–175.
- (44) Szczesny, B.; Marcatti, M.; Zatarain, J. R.; Druzhyna, N.; Wiktorowicz, J. E.; Nagy, P.; Hellmich, M. R.; Szabo, C. Inhibition of Hydrogen Sulfide Biosynthesis Sensitizes Lung Adenocarcinoma to Chemotherapeutic Drugs by Inhibiting Mitochondrial DNA Repair and Suppressing Cellular Bioenergetics. *Sci. Rep.* **2016**, 6 (1), 36125.

- 
- (45) Napetschnig, J.; Wu, H. Molecular Basis of NF- $\kappa$ B Signaling. *Annu. Rev. Biophys.* **2013**, *42* (1), 443–468.
- (46) YAMANISHI, T.; TUBOI, S. The Mechanism of the L-Cystine Cleavage Reaction Catalyzed by Rat Liver  $\gamma$ -Cystathionase1. *J. Biochem.* **1981**, *89* (6), 1913–1921.
- (47) Akaike, T.; Ida, T.; Wei, F.-Y.; Nishida, M.; Kumagai, Y.; Alam, M. M.; Ihara, H.; Sawa, T.; Matsunaga, T.; Kasamatsu, S.; Nishimura, A.; Morita, M.; Tomizawa, K.; Nishimura, A.; Watanabe, S.; Inaba, K.; Shima, H.; Tanuma, N.; Jung, M.; Fujii, S.; Watanabe, Y.; Ohmuraya, M.; Nagy, P.; Feelisch, M.; Fukuto, J. M.; Motohashi, H. Cysteinyl-TRNA Synthetase Governs Cysteine Polysulfidation and Mitochondrial Bioenergetics. *Nat. Commun.* **2017**, *8* (1), 1177.
- (48) Bordo, D.; Bork, P. The Rhodanese/Cdc25 Phosphatase Superfamily. *EMBO Rep.* **2002**, *3* (8), 741–746.
- (49) Nagahara, N.; Yoshii, T.; Abe, Y.; Matsumura, T. Thioredoxin-Dependent Enzymatic Activation of Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2007**, *282* (3), 1561–1569.
- (50) Melideo, S. L.; Jackson, M. R.; Jorns, M. S. Biosynthesis of a Central Intermediate in Hydrogen Sulfide Metabolism by a Novel Human Sulfurtransferase and Its Yeast Ortholog. *Biochemistry* **2014**, *53* (28), 4739–4753.
- (51) Yadav, P. K.; Yamada, K.; Chiku, T.; Koutmos, M.; Banerjee, R. Structure and Kinetic Analysis of H<sub>2</sub>S Production by Human Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2013**, *288* (27), 20002–20013.
- (52) Nagahara, N.; Katayama, A. Post-Translational Regulation of Mercaptopyruvate Sulfurtransferase via a Low Redox Potential Cysteine-Sulfenate in the Maintenance of Redox Homeostasis. *J. Biol. Chem.* **2005**, *280* (41), 34569–34576.
- (53) Jackson, M. R.; Melideo, S. L.; Jorns, M. S. Human Sulfide:Quinone Oxidoreductase Catalyzes the First Step in Hydrogen Sulfide Metabolism and Produces a Sulfane Sulfur Metabolite. *Biochemistry* **2012**, *51* (34), 6804–6815.
- (54) Nagahara, N.; Nishino, T. Role of Amino Acid Residues in the Active Site of Rat Liver Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **1996**, *271* (44), 27395–27401.
- (55) Shibuya, N.; Tanaka, M.; Yoshida, M.; Ogasawara, Y.; Togawa, T.; Ishii, K.; Kimura, H. 3-Mercaptopyruvate Sulfurtransferase Produces Hydrogen Sulfide and Bound Sulfane Sulfur in the Brain. *Antioxid. Redox Signal.* **2008**, *11* (4), 703–714.
- (56) Huang, G.-T.; Yu, J.-S. K. Enzyme Catalysis That Paves the Way for S-Sulfhydration via Sulfur Atom Transfer. *J. Phys. Chem. B* **2016**, *120* (20), 4608–4615.

- 
- (57) Li, K.; Xin, Y.; Xuan, G.; Zhao, R.; Liu, H.; Xia, Y.; Xun, L. Escherichia Coli Uses Separate Enzymes to Produce H<sub>2</sub>S and Reactive Sulfane Sulfur From L-Cysteine. *Front. Microbiol.* **2019**, *10*.
- (58) Cao, X.; Ding, L.; Xie, Z.; Yang, Y.; Whiteman, M.; Moore, P. K.; Bian, J.-S. A Review of Hydrogen Sulfide Synthesis, Metabolism, and Measurement: Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer? *Antioxid. Redox Signal.* **2019**, *31* (1), 1–38.
- (59) Tomita, M.; Nagahara, N.; Ito, T. Expression of 3-Mercaptopyruvate Sulfurtransferase in the Mouse. *Molecules* **2016**, *21* (12), 1707.
- (60) Fräsdorf, B.; Radon, C.; Leimkühler, S. Characterization and Interaction Studies of Two Isoforms of the Dual Localized 3-Mercaptopyruvate Sulfurtransferase TUM1 from Humans. *J. Biol. Chem.* **2014**, *289* (50), 34543–34556.
- (61) Pedre, B.; Dick, T. P. 3-Mercaptopyruvate Sulfurtransferase: An Enzyme at the Crossroads of Sulfane Sulfur Trafficking. *Biol. Chem.* **2021**, *402* (3), 223–237.
- (62) Hanaoka, K.; Sasakura, K.; Suwanai, Y.; Toma-Fukai, S.; Shimamoto, K.; Takano, Y.; Shibuya, N.; Terai, T.; Komatsu, T.; Ueno, T.; Ogasawara, Y.; Tsuchiya, Y.; Watanabe, Y.; Kimura, H.; Wang, C.; Uchiyama, M.; Kojima, H.; Okabe, T.; Urano, Y.; Shimizu, T.; Nagano, T. Discovery and Mechanistic Characterization of Selective Inhibitors of H<sub>2</sub>S-Producing Enzyme: 3-Mercaptopyruvate Sulfurtransferase (3MST) Targeting Active-Site Cysteine Persulfide. *Sci. Rep.* **2017**, *7* (1), 40227.
- (63) Lec, J.-C.; Boutserin, S.; Mazon, H.; Mulliert, G.; Boschi-Muller, S.; Talfournier, F. Unraveling the Mechanism of Cysteine Persulfide Formation Catalyzed by 3-Mercaptopyruvate Sulfurtransferases. *ACS Catal.* **2018**, *8* (3), 2049–2059.
- (64) Libiad, M.; Yadav, P. K.; Vitvitsky, V.; Martinov, M.; Banerjee, R. Organization of the Human Mitochondrial Hydrogen Sulfide Oxidation Pathway. *J. Biol. Chem.* **2014**, *289* (45), 30901–30910.
- (65) Chan, A.; Crankshaw, D. L.; Monteil, A.; Patterson, S. E.; Nagasawa, H. T.; Briggs, J. E.; Kozocas, J. A.; Mahon, S. B.; Brenner, M.; Pilz, R. B.; Bigby, T. D.; Boss, G. R. The Combination of Cobinamide and Sulfanegen Is Highly Effective in Mouse Models of Cyanide Poisoning. *Clin. Toxicol.* **2011**, *49* (5), 366–373.
- (66) Nagasawa, H. T.; Goon, D. J. W.; Crankshaw, D. L.; Vince, R.; Patterson, S. E. Novel, Orally Effective Cyanide Antidotes. *J. Med. Chem.* **2007**, *50* (26), 6462–6464.
- (67) Li, M.; Xu, C.; Shi, J.; Ding, J.; Wan, X.; Chen, D.; Gao, J.; Li, C.; Zhang, J.; Lin, Y.; Tu, Z.; Kong, X.; Li, Y.; Yu, C. Fatty Acids Promote Fatty Liver Disease via the

- Dysregulation of 3-Mercaptopyruvate Sulfurtransferase/Hydrogen Sulfide Pathway. *Gut* **2018**, 67 (12), 2169–2180.
- (68) Palenchar, P. M.; Buck, C. J.; Cheng, H.; Larson, T. J.; Mueller, E. G. Evidence That ThiI, an Enzyme Shared between Thiamin and 4-Thiouridine Biosynthesis, May Be a Sulfurtransferase That Proceeds through a Persulfide Intermediate. *J. Biol. Chem.* **2000**, 275 (12), 8283–8286.
- (69) Módis, K.; Coletta, C.; Erdélyi, K.; Papapetropoulos, A.; Szabo, C. Intramitochondrial Hydrogen Sulfide Production by 3-Mercaptopyruvate Sulfurtransferase Maintains Mitochondrial Electron Flow and Supports Cellular Bioenergetics. *FASEB J.* **2013**, 27 (2), 601–611.
- (70) Szabo, C.; Ransy, C.; Módis, K.; Andriamihaja, M.; Murghes, B.; Coletta, C.; Olah, G.; Yanagi, K.; Bouillaud, F. Regulation of Mitochondrial Bioenergetic Function by Hydrogen Sulfide. Part I. Biochemical and Physiological Mechanisms. *Br. J. Pharmacol.* **2014**, 171 (8), 2099–2122.
- (71) Módis, K.; Coletta, C.; Erdélyi, K.; Papapetropoulos, A.; Szabo, C. Intramitochondrial Hydrogen Sulfide Production by 3-mercaptopyruvate Sulfurtransferase Maintains Mitochondrial Electron Flow and Supports Cellular Bioenergetics. *FASEB J.* **2013**, 27 (2), 601–611.
- (72) Panagaki, T.; Randi, E. B.; Szabo, C. Role of 3-Mercaptopyruvate Sulfurtransferase in the Regulation of Proliferation and Cellular Bioenergetics in Human Down Syndrome Fibroblasts. *Biomolecules* **2020**, 10 (4), 653.
- (73) Nagahara, N. Multiple Role of 3-Mercaptopyruvate Sulfurtransferase: Antioxidative Function, H<sub>2</sub>S and Polysulfide Production and Possible SO<sub>x</sub> Production. *Br. J. Pharmacol.* **2018**, 175 (4), 577–589.
- (74) Ezeriņa, D.; Takano, Y.; Hanaoka, K.; Urano, Y.; Dick, T. P. N-Acetyl Cysteine Functions as a Fast-Acting Antioxidant by Triggering Intracellular H<sub>2</sub>S and Sulfane Sulfur Production. *Cell Chem. Biol.* **2018**, 25 (4), 447–459.e4.
- (75) Caira, S.; Iannelli, A.; Sciarrillo, R.; Picariello, G.; Renzone, G.; Scaloni, A.; Addeo, P. Differential Representation of Liver Proteins in Obese Human Subjects Suggests Novel Biomarkers and Promising Targets for Drug Development in Obesity. *J. Enzyme Inhib. Med. Chem.* **2017**, 32 (1), 672–682.
- (76) Ide, M.; Ohnishi, T.; Toyoshima, M.; Balan, S.; Maekawa, M.; Shimamoto-Mitsuyama, C.; Iwayama, Y.; Ohba, H.; Watanabe, A.; Ishii, T.; Shibuya, N.; Kimura, Y.; Hisano, Y.; Murata, Y.; Hara, T.; Morikawa, M.; Hashimoto, K.; Nozaki, Y.; Toyota, T.; Wada,

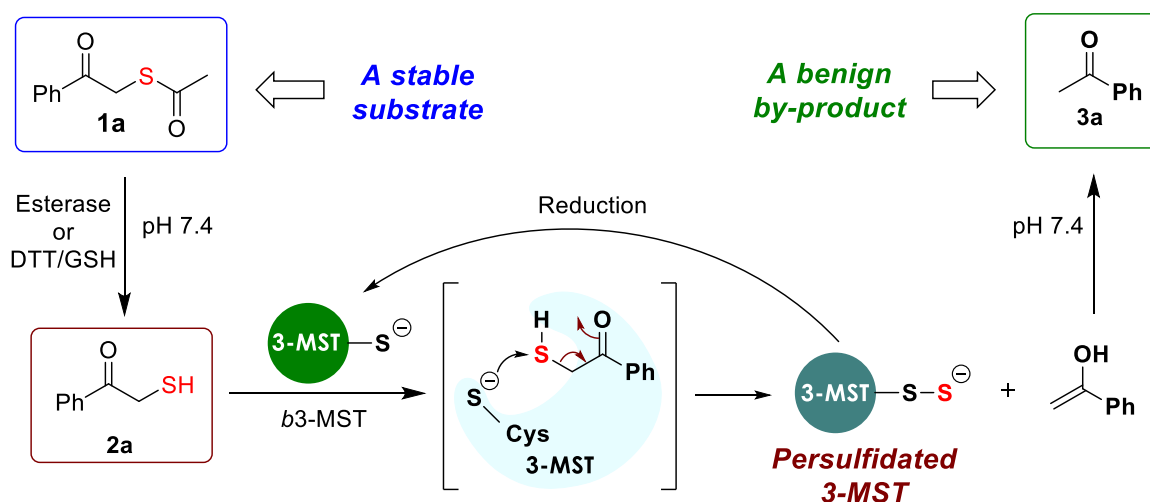
- Y.; Tanaka, Y.; Kato, T.; Nishi, A.; Fujisawa, S.; Okano, H.; Itokawa, M.; Hirokawa, N.; Kunii, Y.; Kakita, A.; Yabe, H.; Iwamoto, K.; Meno, K.; Katagiri, T.; Dean, B.; Uchida, K.; Kimura, H.; Yoshikawa, T. Excess Hydrogen Sulfide and Polysulfides Production Underlies a Schizophrenia Pathophysiology. *EMBO Mol. Med.* **2019**, *11* (12).
- (77) Nagahara, N.; Nagano, M.; Ito, T.; Suzuki, H. Redox Regulation of Mammalian 3-Mercaptopyruvate Sulfurtransferase; 2015; pp 229–254.
- (78) Jin, S.; Pu, S.-X.; Hou, C.-L.; Ma, F.-F.; Li, N.; Li, X.-H.; Tan, B.; Tao, B.-B.; Wang, M.-J.; Zhu, Y.-C. Cardiac H<sub>2</sub>S Generation Is Reduced in Ageing Diabetic Mice. *Oxid. Med. Cell. Longev.* **2015**, *2015*, 1–14.
- (79) Zhang, Y.; Yang, J.; Wang, T.; Wang, S.-G.; Liu, J.-H.; Yin, C.-P.; Ye, Z.-Q. Decreased Endogenous Hydrogen Sulfide Generation in Penile Tissues of Diabetic Rats With Erectile Dysfunction. *J. Sex. Med.* **2016**, *13* (3), 350–360.
- (80) Suwanai, Y. Functional Analysis of 3-Mercaptopyruvate Sulfurtransferase Using Knockout Mice. *Adv. Tech. Biol. Med.* **2015**, *04* (01).
- (81) Nasi, S.; Ehirciou, D.; Chatzianastasiou, A.; Nagahara, N.; Papapetropoulos, A.; Bertrand, J.; Cirino, G.; So, A.; Busso, N. The Protective Role of the 3-Mercaptopyruvate Sulfurtransferase (3-MST)-Hydrogen Sulfide (H<sub>2</sub>S) Pathway against Experimental Osteoarthritis. *Arthritis Res. Ther.* **2020**, *22* (1), 49.
- (82) Coletta, C.; Módos, K.; Szczesny, B.; Brunyánszki, A.; Oláh, G.; Rios, E. C. S.; Yanagi, K.; Ahmad, A.; Papapetropoulos, A.; Szabo, C. Regulation of Vascular Tone, Angiogenesis and Cellular Bioenergetics by the 3-Mercaptopyruvate Sulfurtransferase/H<sub>2</sub>S Pathway: Functional Impairment by Hyperglycemia and Restoration by Dl- $\alpha$ -Lipoic Acid. *Mol. Med.* **2015**, *21* (1), 1–14.



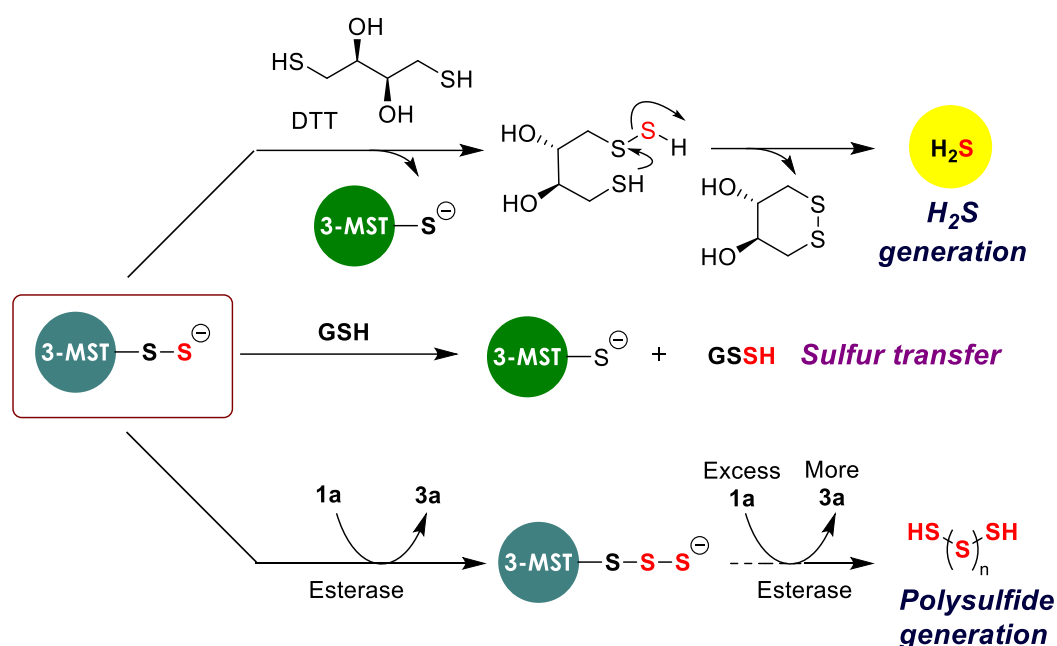
## CHAPTER 2: Structure-Activity Relationship Study of Artificial Substrates of 3-MST

### 2.1. Introduction

In Chapter 2, the development of a new artificial substrate of 3-MST is discussed, followed by the design, synthesis, and assessment of a library of synthetic substrates for modulating 3-MST activity *via* structure-activity relationships. Initial assessment towards developing a new artificial substrate of 3-MST was performed in collaboration with my colleagues, comprehensive results and discussions of this research work are included in their theses.<sup>1,2</sup> In summary, phenacyl thiol (**2a**) was identified to be an artificial substrate of *E. coli* 3-MST (*b*3-MST). The acetylated derivative of substrate **2a**, **1a** was able to generate H<sub>2</sub>S in the presence of esterase and 3-MST (Scheme 2.1). In addition to esterase, the acetyl group of **1a** can be readily cleaved in presence of small molecule thiols such as glutathione (GSH). Using **1a**, the overall 3-MST turnover mechanism was evaluated where **1a** upon esterase (or DTT/GSH) mediated cleavage form **2a** which is then turned over by 3-MST form 3-MST persulfide and acetophenone (**3a**), a benign by-product. Next, with **1a**, 3-MST mediated sulfur transfer to low molecular weight thiols (Eg. GSH) and cysteine-containing proteins were demonstrated (Scheme 2.2). As 3-MST is known to react with an excess amount of substrate to generate polysulfide, therefore in presence of **1a** the generation of persulfides was also detected.



**Scheme 2.1:** Catalytic 3-MST turnover mechanism by artificial substrate **1a**.



**Scheme 2.2:** H<sub>2</sub>S generation, sulfur transfer and polysulfide generation from 3-MST persulfide.

This compound mitigates oxidative stress and inflammation in both cellular and animal models. Representative data is described below. A known inducer of cellular ROS, MGR-1 was used and using a known ROS-sensitive NIR probe (TCF-B),<sup>3,4</sup> dose-dependent quenching ROS level was observed with a variable concentration of **1a** (Figure 2.1). Compound **1a** was also found to reduce the level of inflammatory cytokines and prostaglandins in LPS-challenged mice and thereby exhibiting protection from neuroinflammation (Figure 2.2).

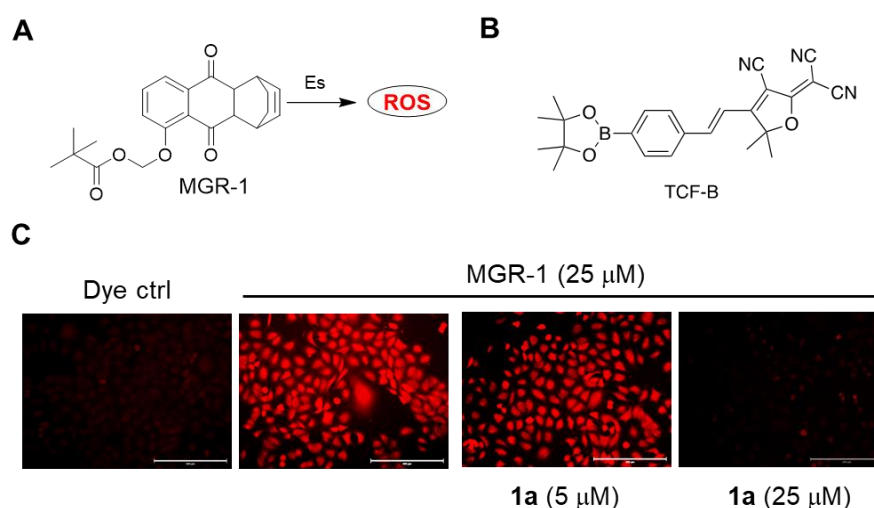


**Figure 2.1:** (A) Structure of MGR-1 that generates ROS in presence of esterase. (B) Structure of ROS-sensitive TCF-B probe. (C) Dose-dependent ROS quenching by **1a** with **MGR-1** treated cells (Credit: Dr. Prerona Bora).

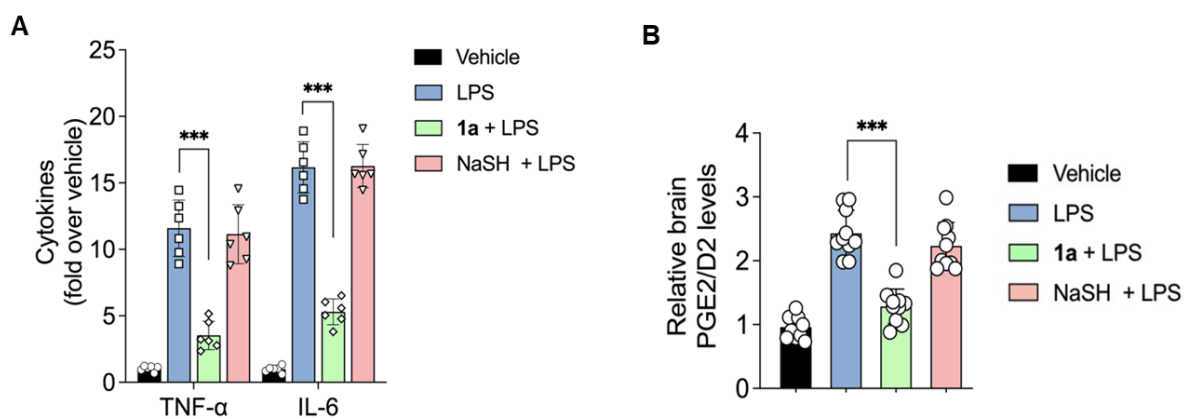


**Figure 2.2:** Mitigation in neuroinflammation in mice model (A) Shows a significant reduction of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 with **1a** ( $n = 6$  per group). (B) Shows a significant reduction of prostaglandin level with **1a** ( $n = 10$  per group). All data are presented as mean  $\pm$  SD. \*\*\* $p < 0.001$  vs LPS (Credit: Dr. Prerona Bora and Dr. Subham Singh, Dr. Siddhesh S. Kamat lab).

After validating **1a** as an excellent substrate of 3-MST, we further looked for the structural modifications on **1a** in order to establish a structure-activity relationship (SAR) in artificial substrate-enzyme catalysis.

## 2.2. Design and hypothesis for SAR

We hypothesized that incorporating a structural modification on **1a** can give more control over the 3-MST turnover rate. A substrate needs to bind to the enzyme first followed by a nucleophilic attack of a cysteine residue of 3-MST, therefore, considering a combination of electronic, steric and binding effects two series of compounds were proposed (Figure 2.3).

In series 1, a library of the artificial substrate with an electron-withdrawing group (EWG) and electron-releasing group (ERG) at the *ortho* or *para* position of the aromatic ring was proposed. A substrate with EWG is expected to stabilize the enolate and will likely have a higher rate. On the contrary, a substrate with ERG is expected to destabilize the enolate and will have a lower rate. In addition to this, series 2 compounds were designed wherein a substituent at  $\alpha$  position was introduced first. Also, it was hypothesized that a methyl group at  $\alpha$ -position to the sulfur will affect the planarity of the enolate and release the rate of H<sub>2</sub>S release. A methyl group is known to be an electron-releasing group which may reduce the electrophilicity of the  $\alpha$ -carbon. Therefore, due to both bulkiness and electron-donating properties of the methyl group, nucleophilic attack by 3-MST sulfur to the substrate is expected not to be favoured. In the same

series, a naphthyl ring was considered in the place of a phenyl ring as a reported 3-MST inhibitor with a naphthyl ring shown to have an excellent binding.

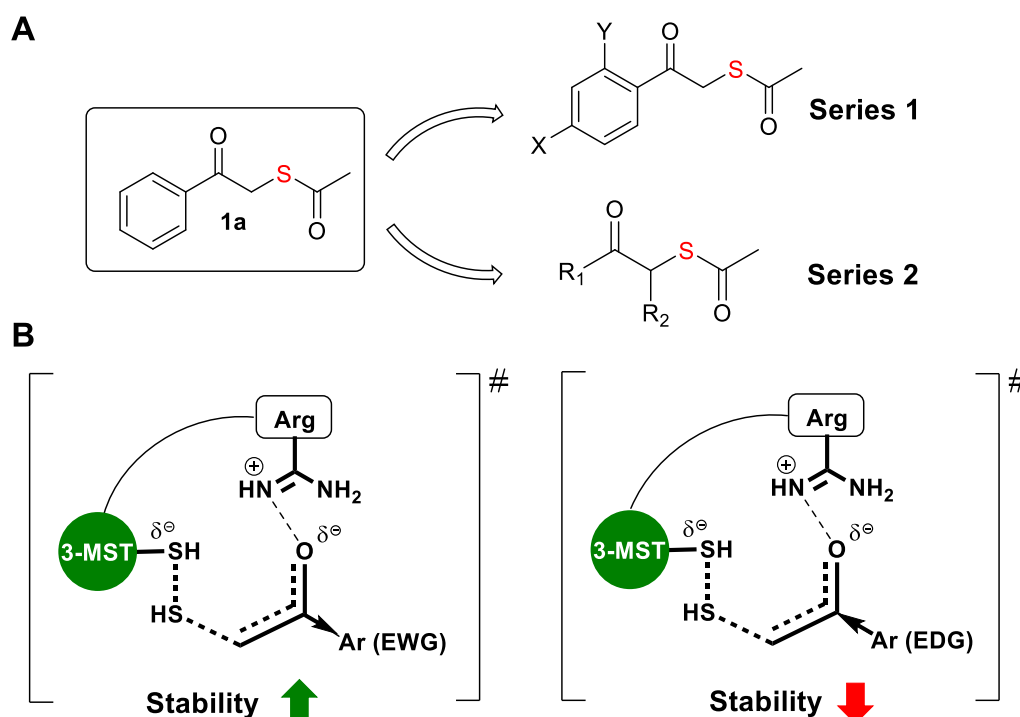


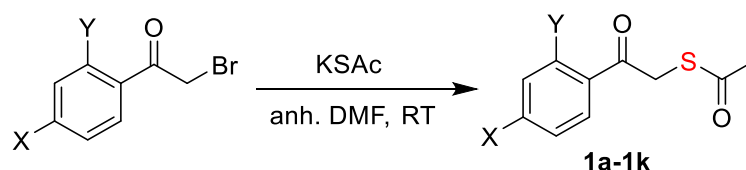
**Figure 2.3:** (A) 3-MST artificial substrates library design for SAR. (B) Expected effects of electron-withdrawing group (EWG) and electron-releasing group (ERG) in the transition state of 3-MST turnover reaction.

## 2.3. Result and discussion

### 2.3.1. Synthesis

#### 2.3.1.1 Synthesis of *ortho* and *para* substituents

Following the general protocol (Scheme 2.3), series 1 compounds with different *ortho* and *para* substituted groups were synthesized. From the derivatized phenacyl bromides, the corresponding *S*-ethanethioates (**1a-1k**) were synthesized. Derivatized phenacyl bromide reacted with potassium thioacetate (KSAc) in dry *N,N*-Dimethylformamide (DMF) to obtain compounds **1a-1k** in a good yield (Table 2.1). Of these, compounds **1b**, **1c** and **1h** were synthesized by a colleague using the aforementioned general protocol.<sup>1</sup>



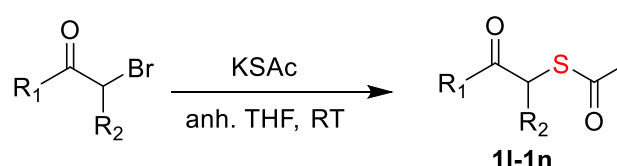
**Scheme 2.3:** Synthesis of compound **1a-1k**.

**Table 2.1:** Yield of the synthesized compounds **1a-1k**.

| Entry | Cpd.      | X                | Y | Yield, % | Entry | Cpd.      | X   | Y   | Yield, % |
|-------|-----------|------------------|---|----------|-------|-----------|-----|-----|----------|
| 1     | <b>1a</b> | H                | H | 78       | 7     | <b>1g</b> | Me  | H   | 77       |
| 2     | <b>1b</b> | NO <sub>2</sub>  | H | 86       | 8     | <b>1h</b> | OMe | H   | 81       |
| 3     | <b>1c</b> | CN               | H | 79       | 9     | <b>1i</b> | H   | F   | 85       |
| 4     | <b>1d</b> | CF <sub>3</sub>  | H | 77       | 10    | <b>1j</b> | H   | OMe | 88       |
| 5     | <b>1e</b> | OCF <sub>3</sub> | H | 73       | 11    | <b>1k</b> | H   | Me  | 80       |
| 6     | <b>1f</b> | F                | H | 83       |       |           |     |     |          |

### 2.3.1.2 Synthesis of $\alpha$ -substituted-methyl and naphthyl derivatives

Following the general protocol (Scheme 2.4), starting from derivatized phenacyl bromide and naphthyl bromides the corresponding artificial substrates (**1l-1n**) were synthesized. Potassium thioacetate (KSAc) (2 eq) was reacted with derivatized phenacyl bromide and naphthyl bromides in anhydrous tetrahydrofuran (THF) to synthesize compounds **1l-1n** in a good yield (Table 2.2).

**Scheme 2.4:** Synthesis of compound **1l-1n**.**Table 2.2:** Yield of the synthesized compounds **1l-1n**.

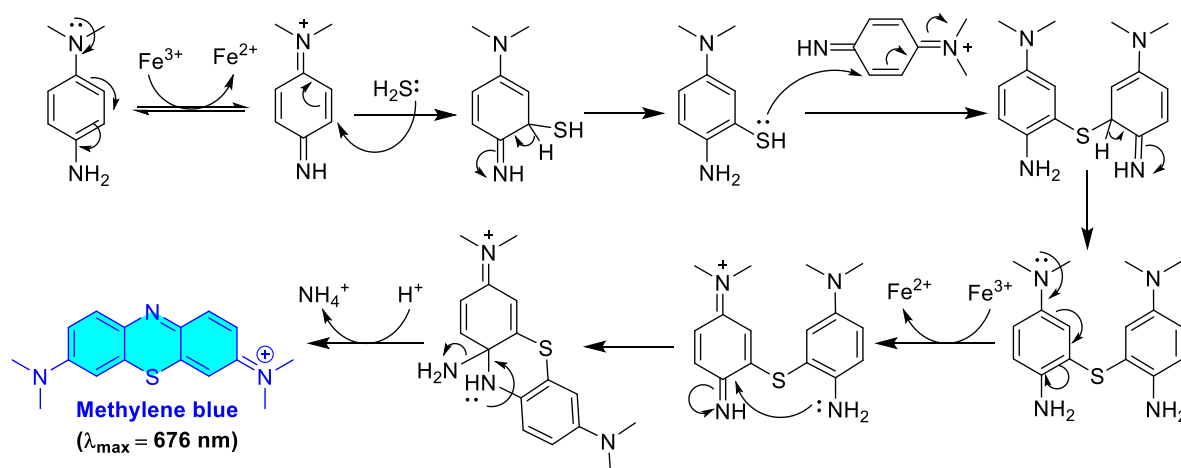
| Entry | Cpd.      | R <sub>1</sub> | R <sub>2</sub> | Yield, % |
|-------|-----------|----------------|----------------|----------|
| 1     | <b>1l</b> | H              | Me             | 78       |
| 2     | <b>1m</b> | 1-naphthyl     | H              | 86       |
| 3     | <b>1n</b> | 2-naphthyl     | H              | 79       |

### 2.3.2 Effect of *para*-substituted derivatives in 3-MST catalysis

Having synthesized the potential substrates of 3-MST, experiments were performed with *para*-substituted derivatives (with EWG and ERG) in order to establish the substituent effect in the 3-MST catalysis rate. Therefore, the efficiency of H<sub>2</sub>S generation from these compounds was studied.

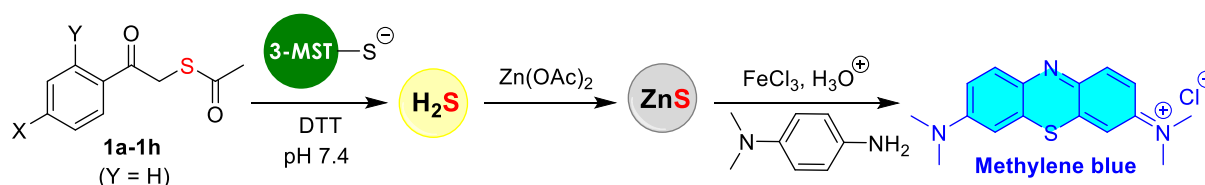
### 2.3.2.1. H<sub>2</sub>S generation

A methylene blue assay is commonly performed to measure H<sub>2</sub>S concentrations. Here, H<sub>2</sub>S released from the experiment is initially trapped by Zn(OAc)<sub>2</sub> which forms ZnS in the solution. Upon reaction with methylene blue reagents (ferric chloride and *N,N*-dimethyl-*p*-phenylene diamine), ZnS form methylene blue dye under acidic conditions (Scheme 2.5).<sup>5,6</sup> Formation of the methylene blue dye was monitored at 676 nm ( $\lambda_{\text{max}}$  of methylene blue) which accounts for the release of H<sub>2</sub>S.



**Scheme 2.5:** Mechanism of the methylene blue dye formation.

First, the methylene blue assay was performed with purified *human* 3-MST (*h3*-MST). In this assay, 100  $\mu$ M of the substrates (**1a-1h**) were incubated with *h3*-MST (0.5  $\mu$ M), Zn(OAc)<sub>2</sub> (400  $\mu$ M) and DTT (25 mM) (Scheme 2.6). Aliquots were taken at predetermined intervals from the reaction mixtures and treated with cocktail of methylene blue reagents. Then the resultant mixtures were incubated in the dark for 30 min. Finally, the formation of the methylene dye was monitored at 676 nm.



**Scheme 2.6** Methylene blue assay for studying the generation of H<sub>2</sub>S from **1a-1h**.

Formation of H<sub>2</sub>S is known to be dependent on the rate determining first step where sulfur atom was picked up from the substrate to form 3-MST persulfide. Since the amount of the substrate present in this step is excessive (200-fold), therefore rate of the reaction was calculated using pseudo first order kinetics. Hence, in order to find the rate constant for H<sub>2</sub>S generation, a time

course for H<sub>2</sub>S release was recorded following the time-dependent formation of methylene blue (Figure 2.4). Kinetic analysis revealed that 3-MST substrate with EWG has a higher rate of H<sub>2</sub>S release and substrate with ERG has a lower rate of H<sub>2</sub>S release. Relative rates for all *para* substituted derivatives compared with **1a** (unsubstituted) showed nearly a 25-fold rate difference in H<sub>2</sub>S release which suggests that substituent's affect the reaction (Table 2.3).

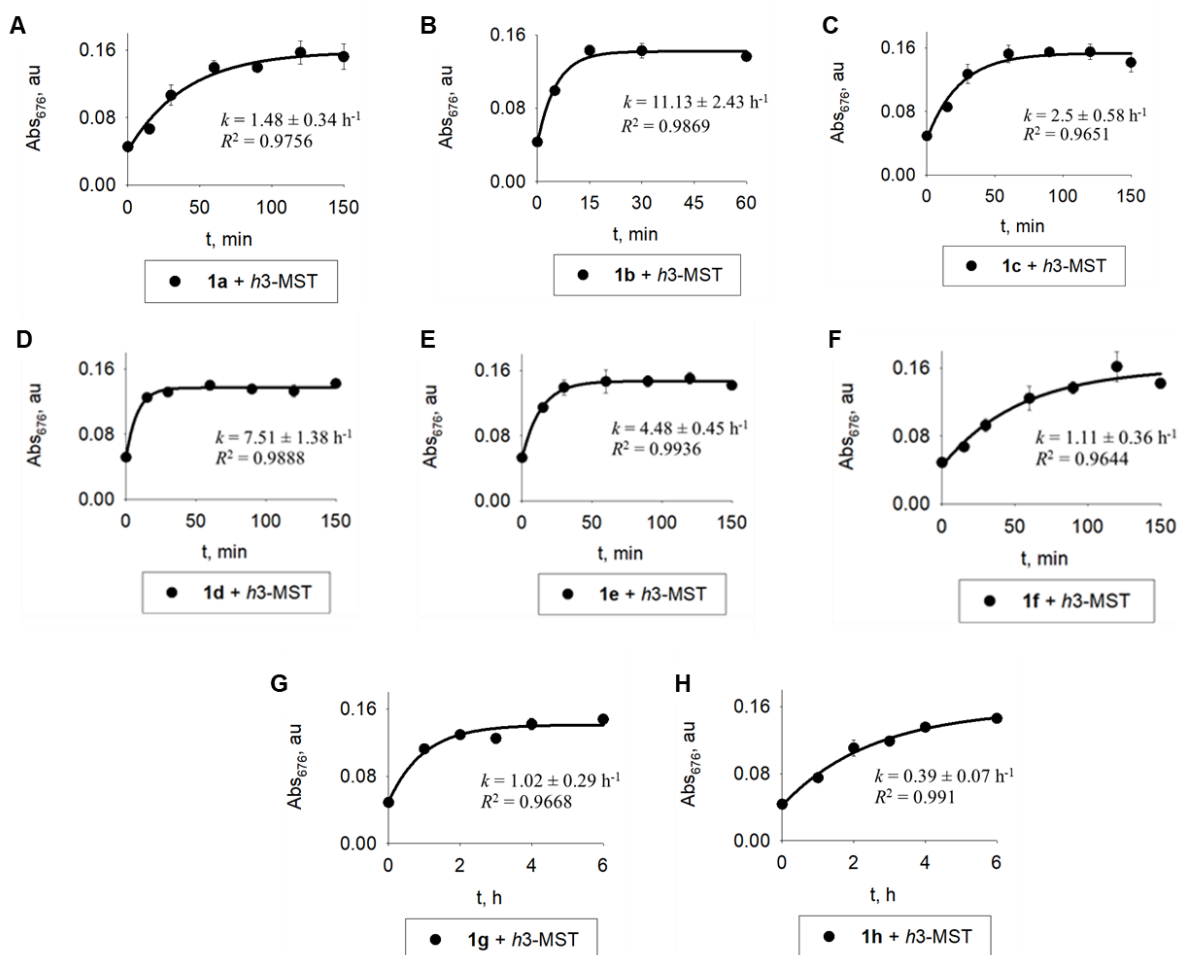


**Figure 2.4:** Detection of H<sub>2</sub>S using methylene blue assay for *para* substituted substrates in the presence of wt *h3*-MST. (A-H) Methylene blue formation during incubation of compounds **1a**-**1h** with wt *b3*-MST. Curve fitting using a pseudo-first-order equation  $y = y_0 + a(1 - e^{-kx})$  was carried out. In the absence of the enzyme 3-MST, H<sub>2</sub>S was not detected from any of the above substrates.

Next, a similar methylene blue assay was performed with purified *E. coli* 3-MST (*b3*-MST). Using the time-dependent formation of methylene blue, time course for H<sub>2</sub>S release was recorded and the rate constant for methylene blue formation (H<sub>2</sub>S generation) was calculated (Figure 2.5). Congruent with the kinetic findings obtained with *h3*-MST, 3-MST substrate with EWG displayed a higher rate of H<sub>2</sub>S release and substrate with ERG showed a lower rate of

H<sub>2</sub>S release. Finally, relative rates for all *para* substituted derivatives compared with **1a** (unsubstituted) showed nearly a 21-fold tunability in the H<sub>2</sub>S release which is similar to *h3*-MST (Table 2.3).

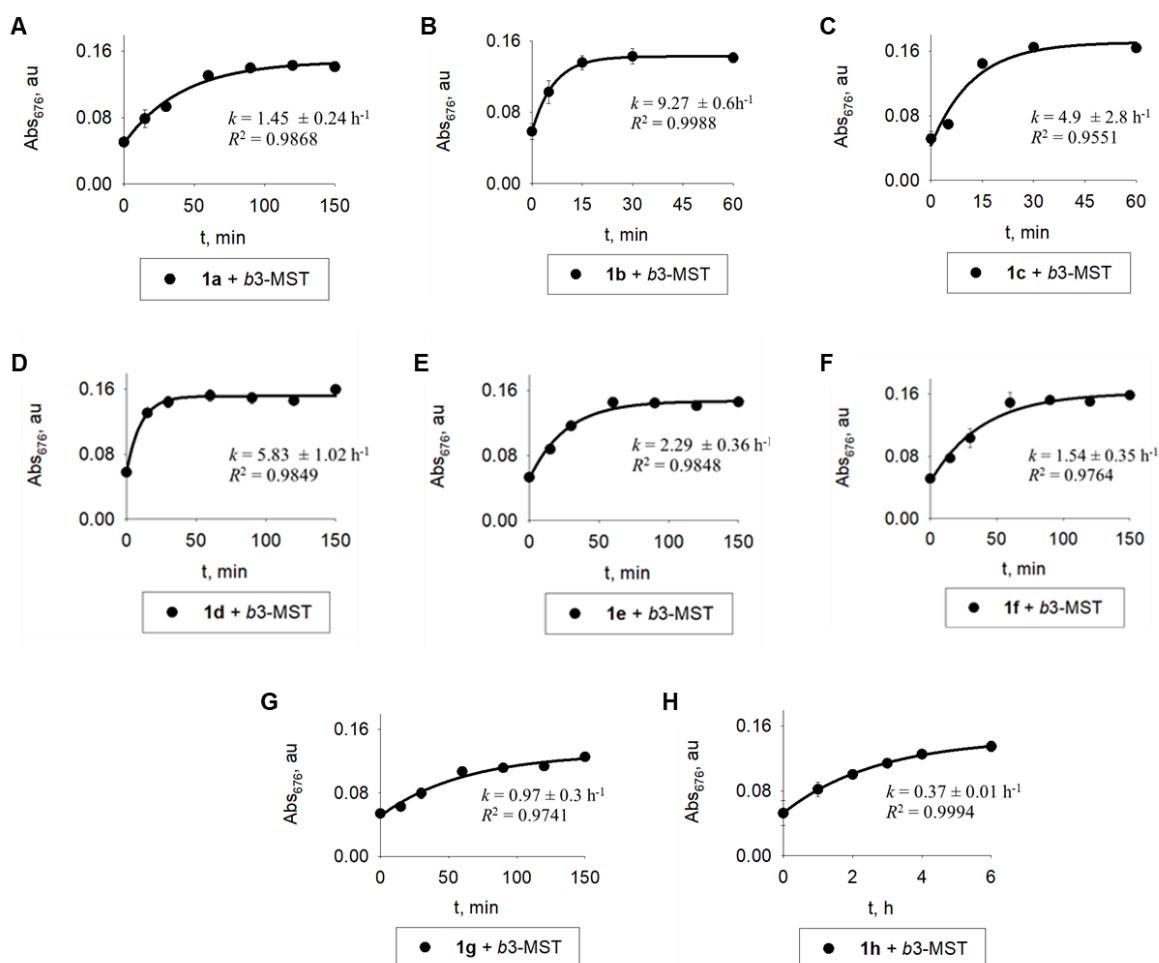


**Figure 2.5:** Detection of H<sub>2</sub>S using methylene blue assay for *para* substituted substrates in the presence of wt *b3*-MST. (A-H) Methylene blue formation during incubation of compounds **1a**–**1h** with wt *b3*-MST. Curve fitting using a pseudo-first-order equation  $y = y_0 + a(1 - e^{-kx})$  was carried out. In the absence of the enzyme *b3*-MST, H<sub>2</sub>S was not detected from any of the above substrates.

Next, relative rates of H<sub>2</sub>S release for both *h3*-MST and *b3*-MST were compared to check whether different analogues of *b3*-MST have a similar activity or not with the same artificial substrate (Table 2.3). Data showed that both enzymes had nearly identical activity towards the same substrate.

**Table 2.3:** Relative comparison of H<sub>2</sub>S release rates among the substrates with *h3*-MST and *b3*-MST.

| Cpd. | X                | Y | Rates (h <sup>-1</sup> ) |                | Relative rates (arbitrary unit) |                |                                   |
|------|------------------|---|--------------------------|----------------|---------------------------------|----------------|-----------------------------------|
|      |                  |   | <i>b3</i> -MST           | <i>b3</i> -MST | <i>h3</i> -MST                  | <i>b3</i> -MST | <i>h3</i> -MST/<br><i>b3</i> -MST |
| 1a   | H                | H | 1.48                     | 1.45           | 1                               | 1              | 1                                 |
| 1b   | NO <sub>2</sub>  | H | 11.13                    | 9.27           | 7.5                             | 6.4            | 1.2                               |
| 1c   | CN               | H | 2.5                      | 4.9            | 1.7                             | 3.3            | 0.5                               |
| 1d   | CF <sub>3</sub>  | H | 7.51                     | 5.83           | 5                               | 4              | 1.2                               |
| 1e   | OCF <sub>3</sub> | H | 4.48                     | 2.29           | 3                               | 1.6            | 1.9                               |
| 1f   | F                | H | 1.11                     | 1.54           | 0.7                             | 1.1            | 0.6                               |
| 1g   | Me               | H | 1.02                     | 0.97           | 0.7                             | 0.7            | 1                                 |
| 1h   | OMe              | H | 0.39                     | 0.37           | 0.3                             | 0.3            | 1                                 |

Next, in order to examine the electronic effect of electronics on the transition state of 3-MST turnover, Hammett analysis was carried out. Hence,  $\log(k_X/k_H)$  was calculated from the H<sub>2</sub>S releasing rates of para-substituted derivatives and plotted against the Hammett constant,  $\sigma$  (Figure 2.6). Finally, a slope ( $\rho$ ) was calculated after linear regression analysis. A positive slope (+1.11 for *h3*-MST and +1.13 for *b3*-MST) indicates a partial negative charge build in the transition state. Therefore, an electron-withdrawing substituent on the aromatic ring may possibly be stabilizing the enolate intermediate which also can be correlated with the higher rate of H<sub>2</sub>S generation. On the other hand, an electron-releasing substituent on the aromatic ring possibly destabilizes the enolate intermediate which can be correlated with the slower rate of H<sub>2</sub>S generation.

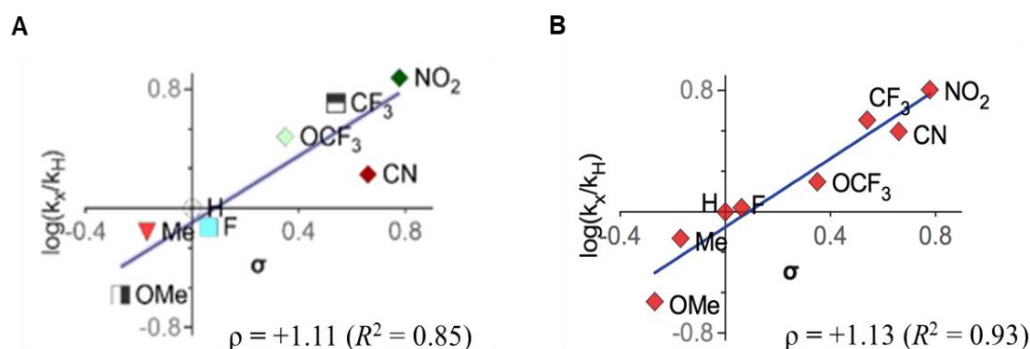


**Figure 2.6:** Hammett analysis of rate constants of H<sub>2</sub>S generation from unnatural substrates. (A) with wt *h3*-MST. Linear regression analysis yielded a slope of +1.11 ( $R^2 = 0.85$ ). (A) with wt *b3*-MST. Linear regression analysis yielded a slope of +1.13 ( $R^2 = 0.935$ ).

### 2.3.2.2. Docking analysis

In addition to the electronic effect, binding of the substrate to the enzyme can also play a key role in modulating the 3-MST turnover rate. Therefore, the extent of the substrate's binding to 3-MST was analyzed using a docking study. A reported crystal structure of *h*3-MST (PDB ID: **4JGT**) was used and *para* substituted substrates (corresponding free thiols) were docked.<sup>7</sup> For all the derivatives (**1a-1h**), a favourable binding was observed (Figure 2.7). The binding energy ( $\Delta G$ ) of the lowest energy docking conformation was evaluated. Along with this, the S-S distance (active site cysteine sulfur to sulfur of the substrate) was also calculated. Similar binding energy and S-S distance were observed in all the substrates, which suggests all 3-MST substrates bind well to the enzyme and the binding effect did not significantly affect 3-MST turnover for *para* substituted derivatives.

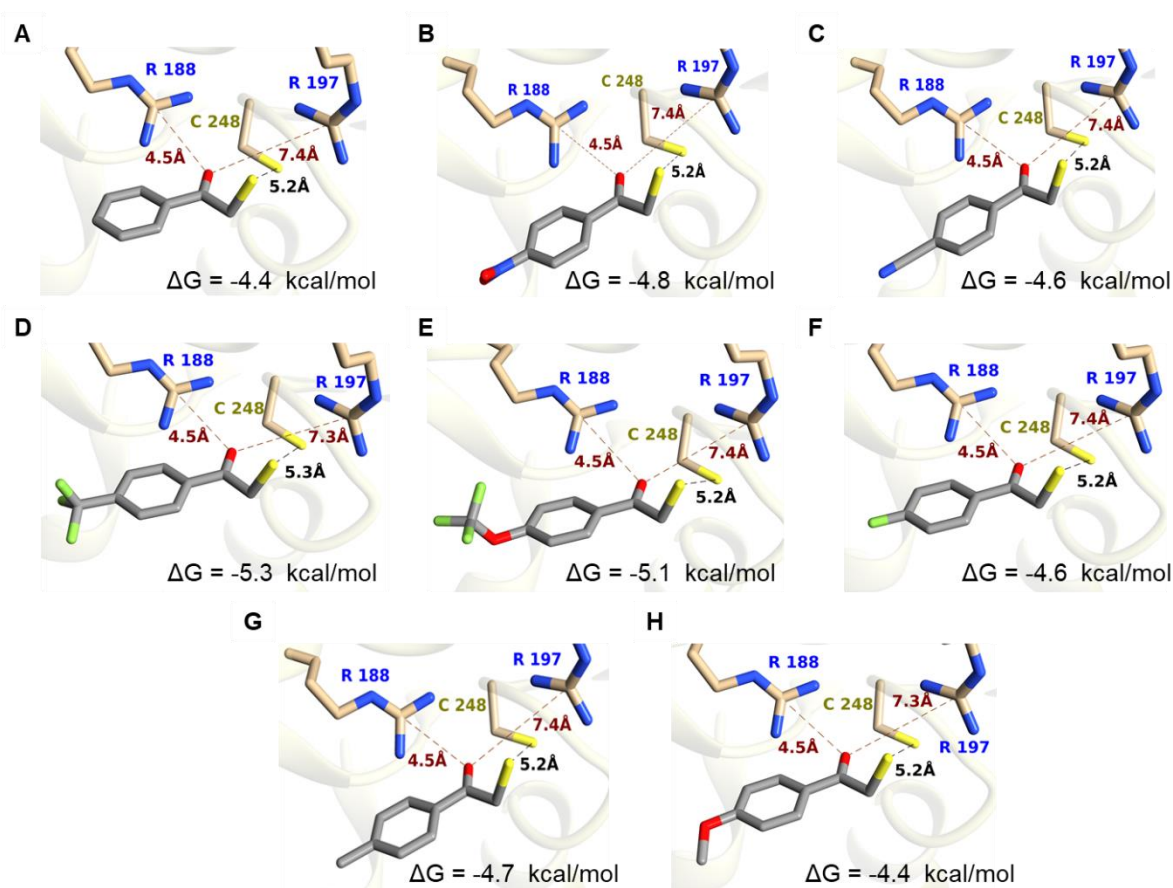


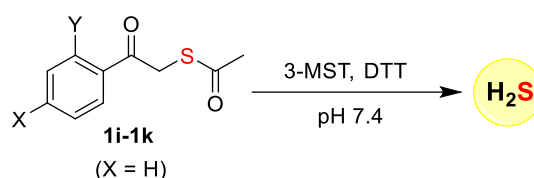
**Figure 2.7:** Lowest energy docking conformations of the *para* substituted substrates **1a-1h** (corresponding free thiols) in the active site of *h*3-MST. (Docking data provided by Venkata Sai Sreyas Adury, Dr. Arnab Mukherjee lab).

### 2.3.3. Effect of *ortho* substituted derivatives in 3-MST catalysis

Similar to *para*-substituted derivatives, the reaction rate can also be modulated using EWG and ERG substituents at the *ortho* position of the 3-MST substrates. Additionally, having a bulky substituent at the *ortho* position is expected to have steric hindrance with the enzyme as it is near the reaction centre of the substrate. Hence, a reaction rate can be slowed. Therefore, the effect of *ortho* substituted derivatives in 3-MST turnover was evaluated.

#### 2.3.3.1. H<sub>2</sub>S generation

H<sub>2</sub>S generation by *ortho* substituted 3-MST substrates was studied using similar methylene blue assay conditions as described previously. The assay was performed with both purified *h*3-MST and *b*3-MST using **1i-1k** (Scheme 2.7).



**Scheme 2.7:** Assay conditions for H<sub>2</sub>S generation from **1i-1k**.

Using the time-dependent formation of methylene blue, a kinetic plot was generated and the rate of H<sub>2</sub>S generation was calculated for **1i-1k** (Figure 2.8).

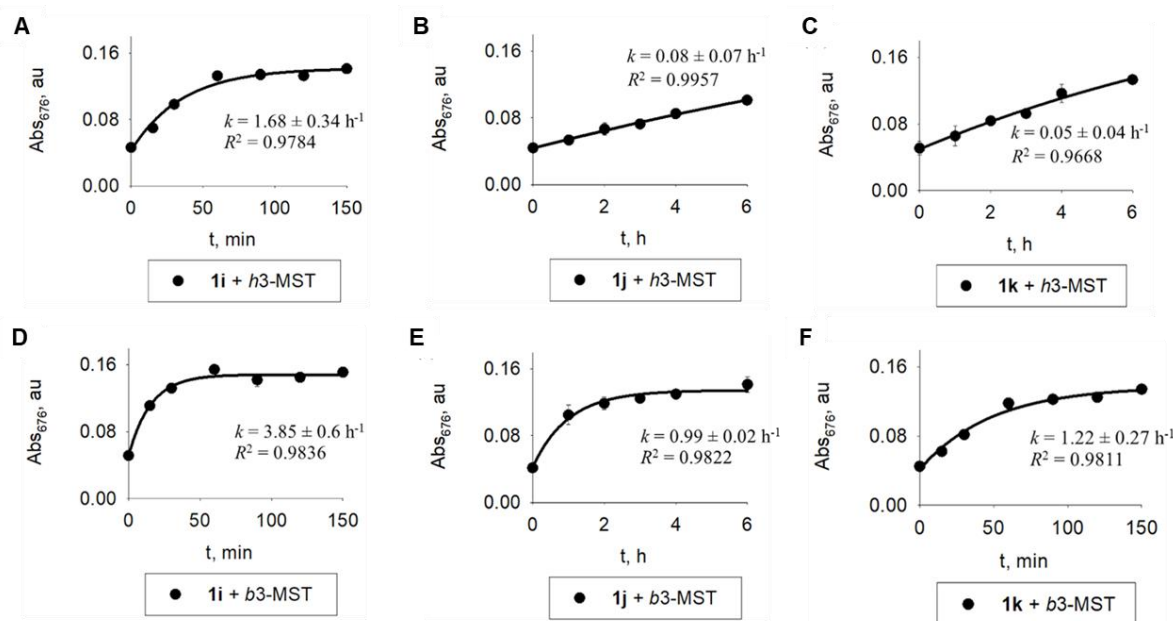


**Figure 2.8:** Detection of H<sub>2</sub>S using methylene blue assay for *ortho* substituted substrates. (A-C) Methylene blue formation during incubation of compounds **1i-1k** in the presence of wt *h*3-MST. (D-F) Methylene blue formation during incubation of compounds **1i-1k** in the presence of 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<sub>2</sub>S was not detected from any of the above substrates.

Owing to the presence of an electron-withdrawing fluorine atom at the *ortho* position, **1i** showed a moderately higher rate of H<sub>2</sub>S release than **1a** for *h3*-MST (Figure 2.8A). On the other hand, **1j** and **1k** with ERG showed an extremely slower rate of H<sub>2</sub>S release (Figure 2.8B-C). In similar conditions, kinetic data of *b3*-MST with **1i**, **1j** and **1k** showed relatively higher rate rates than *h3*-MST (Figure 2.8C-F). This result suggests that the active site of *b3*-MST is highly flexible to accommodate a larger number of modified substrates. In such cases, after binding, the electronic nature of the substituents majorly determines the H<sub>2</sub>S releasing rates.

### 2.3.3.2. Docking analysis

Next, a similar docking study was performed with *h3*-MST to rationalize the methylene blue experiment results by *ortho* substituted 3-MST substrates. Docking of the thiol corresponding to **1i** showed favourable binding with *h3*-MST having a good docking score and suitable S-S distance (Figure 2.9A). But the lowest energy conformations of the thiols corresponding to **1j** and **1k** did not show a favourable binding to the active site of *h3*-MST and S-S distance was found to be nearly 10 Å (Figure 2.9B-C).

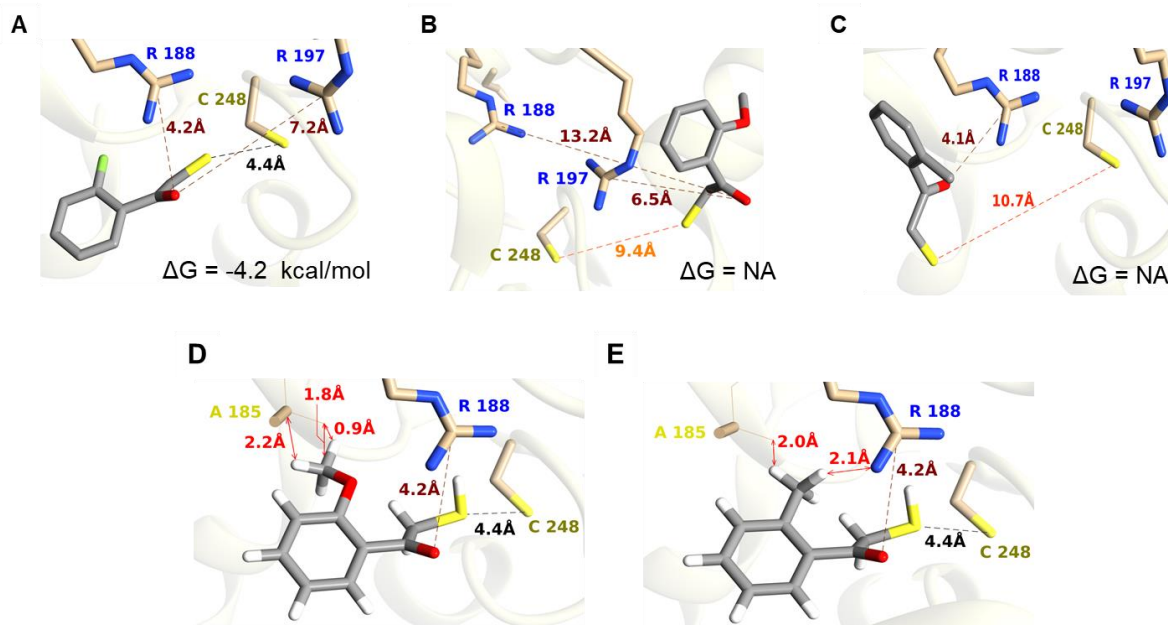


**Figure 2.9:** Docking analysis with *ortho* substituted substrates in the active site of *h3*-MST. (A-C) Lowest energy docking conformations for **1i-1k** (corresponding free thiols) (D) High energy docking conformations for **1j** (F) High energy docking conformations for **1k**. (Docking data provided by Venkata Sai Sreyas Adury, Dr. Arnab Mukherjee lab).

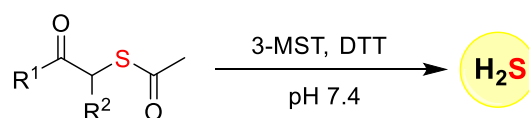
Next, further analysis for **1j** and **1k** was performed with a higher energy binding conformer (Figure 2.6D-E). Although the S-S distance seemed to be in the favourable range due to the bulkier substituents, substrates experienced a huge repulsion originating from steric clashes between substituents and active site residues. Due to these steric clashes, a bulkier substituent was expected to be very slow in the 3-MST turnover which can be correlated with extremely slow rates of H<sub>2</sub>S production.

### 2.3.4. Effect of $\alpha$ -substituted-methyl and naphthyl derivatives in 3-MST catalysis

Having a weakly electron-donating methyl substituent at the  $\alpha$ -position of the artificial substrate will reduce the electrophilicity of the  $\alpha$ -carbon. Also, due to steric effect of the bulky methyl group, reaction rate is expected to be lowered. Similarly, a lower rate of reaction is also expected for naphthyl derivatives due to bulkiness and extended conjugation. Notably, all these modifications on the substrate structure are expected to have an impact on substrate binding with the enzyme. Therefore, the effect of  $\alpha$ -substituted-methyl and naphthyl derivatives in 3-MST turnover was evaluated.

#### 2.3.4.1. H<sub>2</sub>S generation

As described previously, a similar methylene blue assay was performed with both *h*3-MST and *b*3-MST using  $\alpha$ -substituted-methyl (**1l**) and naphthyl derivatives (**1m** and **1n**) (Scheme 2.7).



**Scheme 2.8:** Assay conditions for H<sub>2</sub>S generation from **1l-1m**.

As expected, **1i** showed an extremely slow rate for H<sub>2</sub>S release for both *h*3-MST and *b*3-MST (Figure 2.10). In similar conditions, naphthyl derivatives (**1m** and **1n**) showed a lower rate of H<sub>2</sub>S release for both *h*3-MST and *b*3-MST. The higher H<sub>2</sub>S release rate for *b*3-MST indicates a more accommodative *b*3-MST active site.

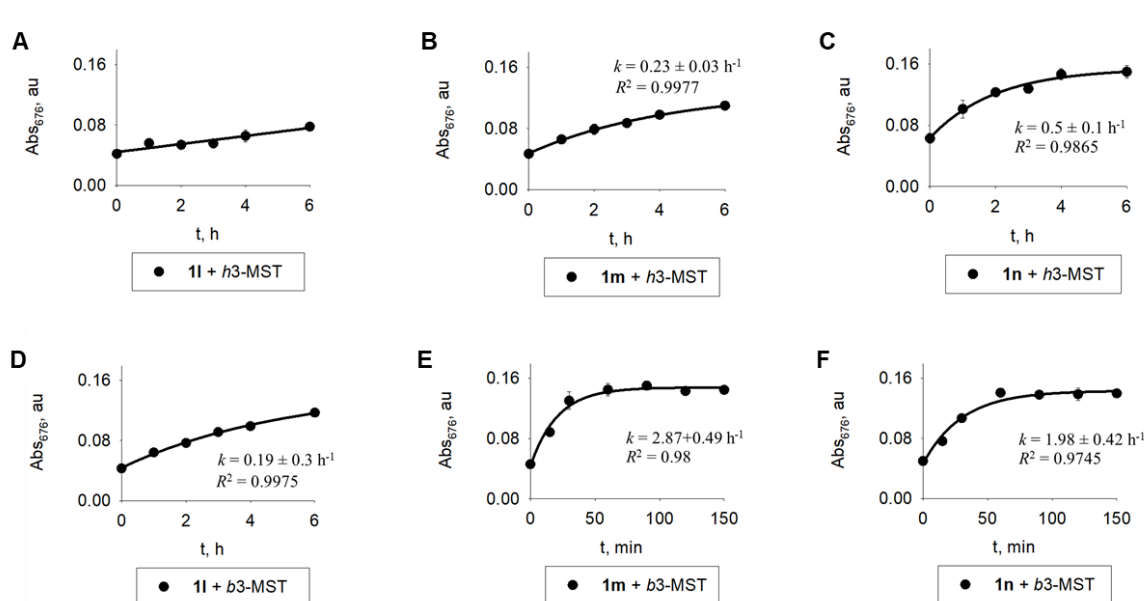


**Figure 2.10:** Detection of  $H_2S$  using methylene blue assay for  $\alpha$ -substituted-methyl and naphthyl substrates. (A-C) Methylene blue formation during incubation of compounds **1l-1n** in the presence of wt *h3*-MST. (D-F) Methylene blue formation during incubation of compounds **1l-1n** in the presence of wt *b3*-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.

### 2.3.4.3. Docking analysis

Next, a docking study was performed with *h3*-MST in order to get more insights into enzyme turnover by  $\alpha$ -substituted-methyl (**1l**) and naphthyl derivatives (**1m** and **1n**). Docking of the thiols corresponding to **1l**, **1m** and **1n** showed favourable binding with *h3*-MST having a good docking score and suitable S-S distance (Figure 2.11A-C). A marginally better docking score was obtained for **1n** than **1l** and **1m** which can be correlated with  $H_2S$  release rates. Overall analysis suggests that the electronic and steric factors influence the rate of reaction.

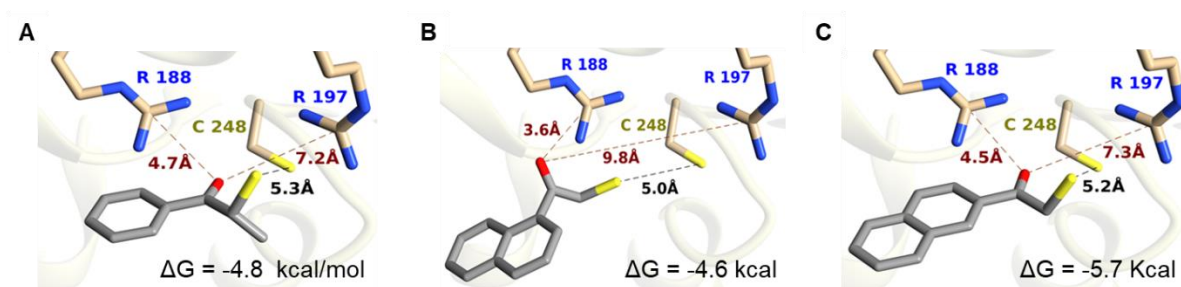


**Figure 2.11:** Docking analysis with  $\alpha$ -substituted-methyl and naphthyl derivatives in the active site of *h3*-MST. (Docking data provided by Venkata Sai Sreyas Adury, Dr. Arnab Mukherjee lab).

## 2.4. Summary

An artificial substrate (**1a**) for a biologically important enzyme 3-MST was developed. Structure-activity relationship study using a library of the artificial substrates with EWG and ERG in the *ortho/para* position was synthesized. Among the *para* substituted derivatives, linear free energy analysis suggested a significant but weak electronic effect on the H<sub>2</sub>S release rate. Molecular docking studies showed a favourable binding for all the *para*-substituted derivatives in their lowest energy conformations. Compared to *b3*-MST, *h3*-MST showed a slower rate of H<sub>2</sub>S release for *ortho* substituted bulky derivatives. Docking analysis with *h3*-MST revealed a strong steric clash that prevented the effective engagement of the substrate in the active site of *h3*-MST. Next, the H<sub>2</sub>S releasing ability for  $\alpha$ -substituted derivative was evaluated. Although docking studies showed a favourable binding of these substrates, methylene blue assay showed a negligible amount of H<sub>2</sub>S. Next, using reported 3-MST inhibitor, two 3-MST substrates with a naphthyl analogue of the 3-MST artificial substrate were synthesized. Docking studies showed that both the derivatives bound well in the active site of *h3*-MST. But the rate of H<sub>2</sub>S release was found to be slower for *h3*-MST as compared to *b3*-MST which suggests *h3*-MST is more specific towards its substrates. Among the substrate tested, a nearly 200-fold and 50-fold in rate difference was observed for *h3*-MST and *b3*-MST respectively where **1b** is the fastest and **1l** was the slowest. Taken together, we have developed a library of artificial substrates of 3-MST which showed tunability of 3-MST catalysis rate due to a combination of electronic, steric and binding effects.

## 2.5. Experimental procedures

### 2.5.1. Synthesis and characterization of compounds

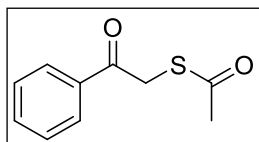
**General methods:** All reactions were conducted under a nitrogen atmosphere. All the chemicals and solvents were purchased from commercial sources and used as received unless stated otherwise. Column chromatography was performed using silica gel [Rankem (60–120 mesh) or Spectrochem (100-200 mesh)] as the stationary phase.  $^1\text{H}$  and  $^{13}\text{C}$  spectra were recorded on a JEOL 400 MHz (or 100 MHz for  $^{13}\text{C}$ ) or a Bruker 400 MHz (or 100 MHz for  $^{13}\text{C}$ ) spectrometer using tetramethylsilane ( $\delta_{\text{H}} = 0.00$  ppm,  $\delta_{\text{C}} = 0.0$  ppm) as an internal reference or residual solvent [ $\text{CDCl}_3$  ( $\delta_{\text{H}} = 7.26$  ppm,  $\delta_{\text{C}} = 77.2$  ppm) or  $\text{DMSO}-d_6$  ( $\delta_{\text{H}} = 2.50$  ppm,  $\delta_{\text{C}} = 39.6$  ppm)] signals as reference.  $^{19}\text{F}$  spectra were recorded on a Bruker (376 MHz) spectrometer using  $\alpha,\alpha,\alpha$ -trifluorotoluene ( $\delta_{\text{F}} = -63.72$  ppm) as an external reference. Chemical shifts ( $\delta$ ) 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.

#### 2.5.1.1. General protocol for the synthesis of ethanethioate derivatives (1a-1k)

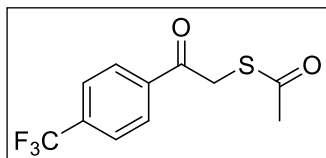
From the derivatized phenacyl bromides, the corresponding S-ethanethioates were synthesized. Phenacyl bromide (1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in dry *N,N*-Dimethylformamide (DMF, 10 mL), followed by the addition of potassium thioacetate (KSAc) (2 eq). The 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 ( $3 \times 30$  mL) and brine (30 mL) and the resulting organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , 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.

Compounds **1b**, **1c** and **1g** were synthesized using the aforementioned general protocol by Mrutyunjay Nair and detailed synthesis and characterizations are included in his MS thesis.<sup>1</sup>

**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 a 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.<sup>8</sup>

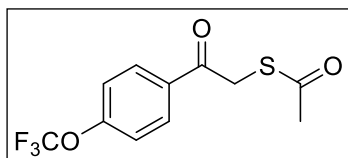


**S-(2-oxo-2-(4-(trifluoromethyl)phenyl)ethyl) ethanethioate (1d):** Following the above general synthetic protocol, 2-bromo-1-(4-(trifluoromethyl)phenyl)ethan-1-one (300 mg, 1.12 mmol) was used as a starting material. A gradient starting from 1-7% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 6-7% as the eluant. Compound **1d** was obtained as a white solid (227 mg, 77%).



FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1699, 1686;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.11 (m, 2H), 7.75 (d, 2H), 4.38 (s, 2H), 2.41 (s, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  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,  $J_{\text{C},\text{F}} = 270.5$  Hz), 36.7, 30.3;  $^{19}\text{F}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  -63.2 (s); HRMS for  $\text{C}_{11}\text{H}_9\text{F}_3\text{O}_2\text{S}$   $[\text{M}+\text{H}]^+$  Calculated: 263.0354, Found: 263.1311.

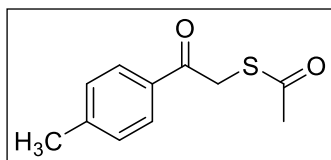
**S-(2-oxo-2-(4-(trifluoromethoxy)phenyl)ethyl) ethanethioate (1e):** Following the above general synthetic protocol, 2-bromo-1-(4-(trifluoromethoxy)phenyl)ethan-1-one (303 mg, 1.07 mmol) was used as a starting material. A gradient starting from 1-10% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 8-10% as the eluant. Compound **1e** was obtained as an off-white solid (218 mg, 73%).



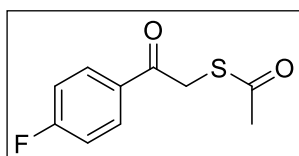
FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1687;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.04-8.07 (m, 2H), 7.30-7.33 (m, 2H), 4.36 (s, 2H), 2.41 (s, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  194.2, 192.1, 153.2, 133.8, 130.8, 120.6, 120.3 (q,  $J = 257.4$  Hz), 36.5, 30.3;  $^{19}\text{F}$  NMR (376 MHz,  $\text{CDCl}_3$ ):  $\delta$  -57.6 (s); HRMS for  $\text{C}_{11}\text{H}_9\text{NaF}_3\text{O}_3\text{S}$   $[\text{M}+\text{Na}]^+$  Calculated: 301.0122, Found: 301.0100.

**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

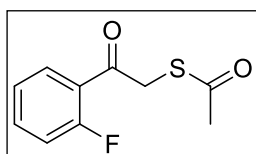
as a starting material. A gradient starting from 1-11% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 9-11% as the eluant. Compound **1h** was obtained as light brown solid (250 mg, 88%). All analytical data are consistent with reported values.<sup>9</sup>



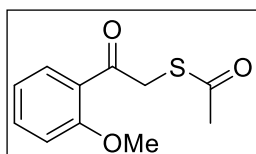
**S-(2-(4-fluorophenyl)-2-oxoethyl) ethanethioate (1f):** Following the above general synthetic protocol, 2-bromo-1-(4-fluorophenyl)ethan-1-one (270 mg, 1.27 mmol) was used as a starting material. A gradient starting from 1-8% ethyl acetate/hexane was used as the mobile phase and the desired product 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.<sup>9</sup>



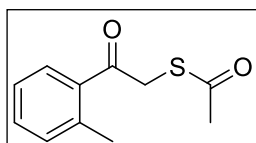
**S-(2-(2-fluorophenyl)-2-oxoethyl) ethanethioate (1i):** Following the above general synthetic protocol, 2-bromo-1-(2-fluorophenyl)ethan-1-one (270 mg, 1.24 mmol) was used as a starting material. A gradient starting from 1-8% ethyl acetate/hexane was used as the mobile phase and the desired product 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.<sup>10</sup>



**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 a 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.<sup>11</sup>



**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 a 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%).

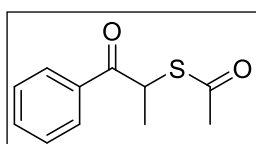


FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1686;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.73-7.75 (m, 2H), 7.39-7.42 (m, 2H), 7.26-7.31 (m, 2H), 4.30 (s, 2H), 2.49 (s, 3H), 2.40 (s, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  196.7, 194.4, 139.0, 132.2, 132.1, 128.9, 125.9, 39.2, 30.2, 21.4; HRMS for  $\text{C}_{11}\text{H}_{13}\text{O}_2\text{S}$   $[\text{M}+\text{H}]^+$  Calculated: 209.0636, Found: 209.0595.

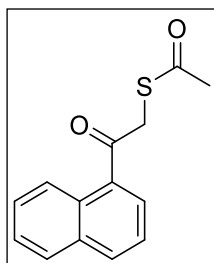
### 2.5.1.2. General synthetic procedure for **1l**, **1m** and **1n**

Corresponding bromoacetyl derivatives (1 eq) were 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  $\text{H}_2\text{O}$  and transferred to a separating funnel, where the product was extracted with ethyl acetate ( $2 \times 15$  mL). The combined organic layer was washed with brine (30 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$ , 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-(1-oxo-1-phenylpropan-2-yl) ethanethioate (1l):** Following the above general synthetic protocol, 2-bromo-1-phenylpropan-1-one (520 mg, 2.44 mmol) was used as a starting material. A gradient starting from 1-3% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 2-3% as the eluant. Compound **1j** was obtained as a yellow oil (366 mg, 72%). All analytical data are consistent with reported values.<sup>12</sup>

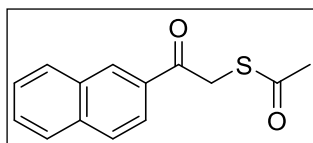


**S-(2-(Naphthalen-1-yl)-2-oxoethyl) ethanethioate (1m):** Following the above general synthetic protocol, 1-(bromoacetyl)naphthalene (500 mg, 2.01 mmol) was used as a starting material. A gradient starting from 1-12% ethyl acetate/hexane was used as the mobile phase and the desired product was obtained with 10-12% as the eluant.



Compound **1m** was obtained as a reddish-yellow oil (360 mg, 73%). FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1681;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  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);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  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  $\text{C}_{14}\text{H}_{12}\text{O}_2\text{S}$   $[\text{M}+\text{H}]^+$  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 a 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 ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1678;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  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);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  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  $\text{C}_{14}\text{H}_{12}\text{O}_2\text{S}$   $[\text{M}+\text{Na}]^+$  Calculated: 267.0455, Found: 267.0463.

### 2.5.2. Methylene blue assay for the detection of $\text{H}_2\text{S}$

The methylene blue assays were conducted as previously reported with some modifications.<sup>13,14</sup> Briefly, 10  $\mu\text{M}$  stocks of wt *b3*-MST and *h3*-MST were prepared in HEPES-NaOH pH 7.4 buffer. 10 mM stocks of all unnatural substrates (**1a-1n**, 10 mM) were prepared in DMSO. Dithiothreitol (DTT, 250 mM) and  $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$  (40 mM) were prepared in deionised water. Stock solution of  $\text{FeCl}_3$  (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  $\mu\text{M}$   $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$  (15  $\mu\text{L}$ , 40 mM stock), 100  $\mu\text{M}$  of unnatural substrates (**1a-1n**) (15  $\mu\text{L}$ , 10 mM stock), 0.5  $\mu\text{M}$  wt *b3*-MST (75  $\mu\text{L}$ , 10  $\mu\text{M}$  stock) or 0.5  $\mu\text{M}$  wt *h3*-MST (75  $\mu\text{L}$ , 10  $\mu\text{M}$  stock) and 25 mM DTT (150

$\mu\text{L}$ , 250 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. Briefly, the reaction samples were prepared by sequentially adding 400  $\mu\text{M}$   $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$  (10  $\mu\text{L}$ , 40 mM stock), 100  $\mu\text{M}$  of unnatural substrates (**1a-1n**) (10  $\mu\text{L}$ , 10 mM stock), 0.5  $\mu\text{M}$  wt *b3*-MST (75  $\mu\text{L}$ , 10  $\mu\text{M}$  stock) or 0.5  $\mu\text{M}$  wt *h3*-MST (75  $\mu\text{L}$ , 10  $\mu\text{M}$  stock) and 25 mM DTT (100  $\mu\text{L}$ , 250 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  $\mu\text{M}$   $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$  (10  $\mu\text{L}$ , 40 mM stock), 10  $\mu\text{L}$  DMSO, 0.5  $\mu\text{M}$  wt *b3*-MST (75  $\mu\text{L}$ , 10  $\mu\text{M}$  stock) or 0.5  $\mu\text{M}$  wt *h3*-MST (75  $\mu\text{L}$ , 10  $\mu\text{M}$  stock) and 25 mM DTT (100  $\mu\text{L}$ , 250 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  $\mu\text{L}$ ) of  $\text{FeCl}_3$ , 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  $\mu\text{L}$  was transferred to a 96-well plate and the absorbance values were recorded at 676 nm using a microplate reader (Thermo Scientific VarioskanFlash).

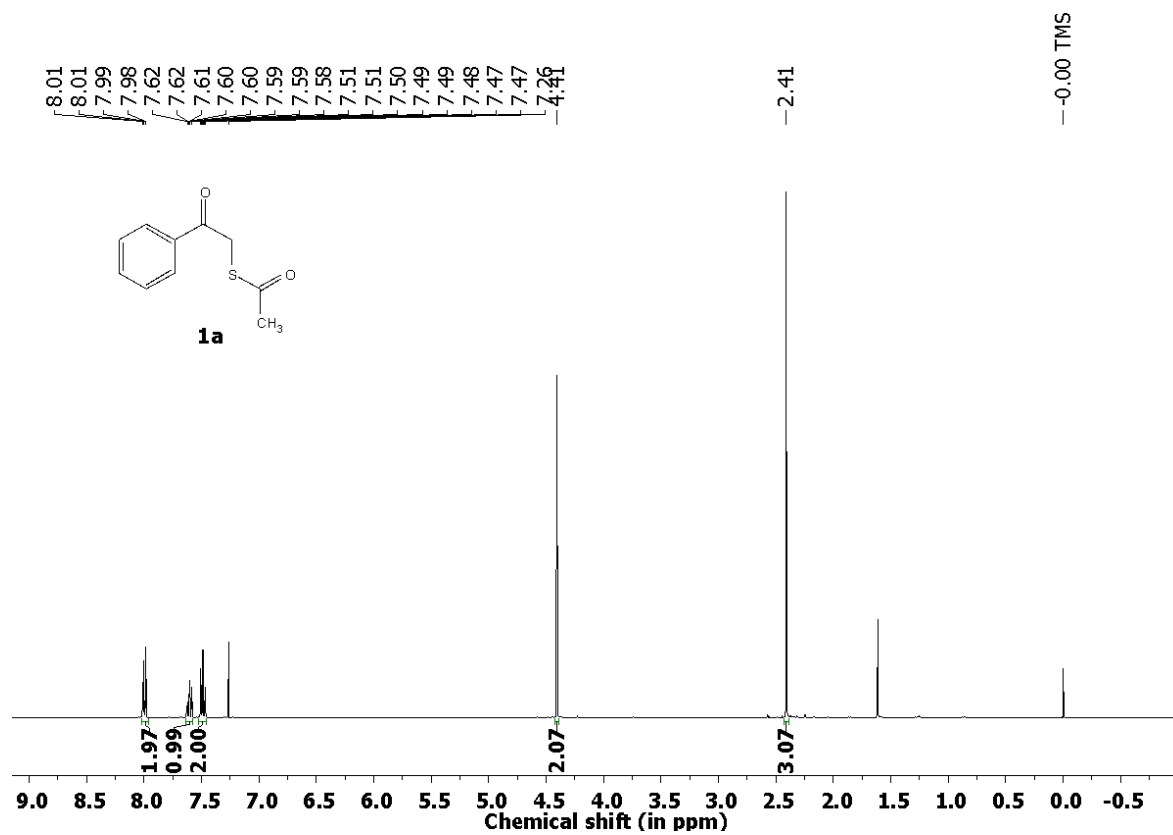
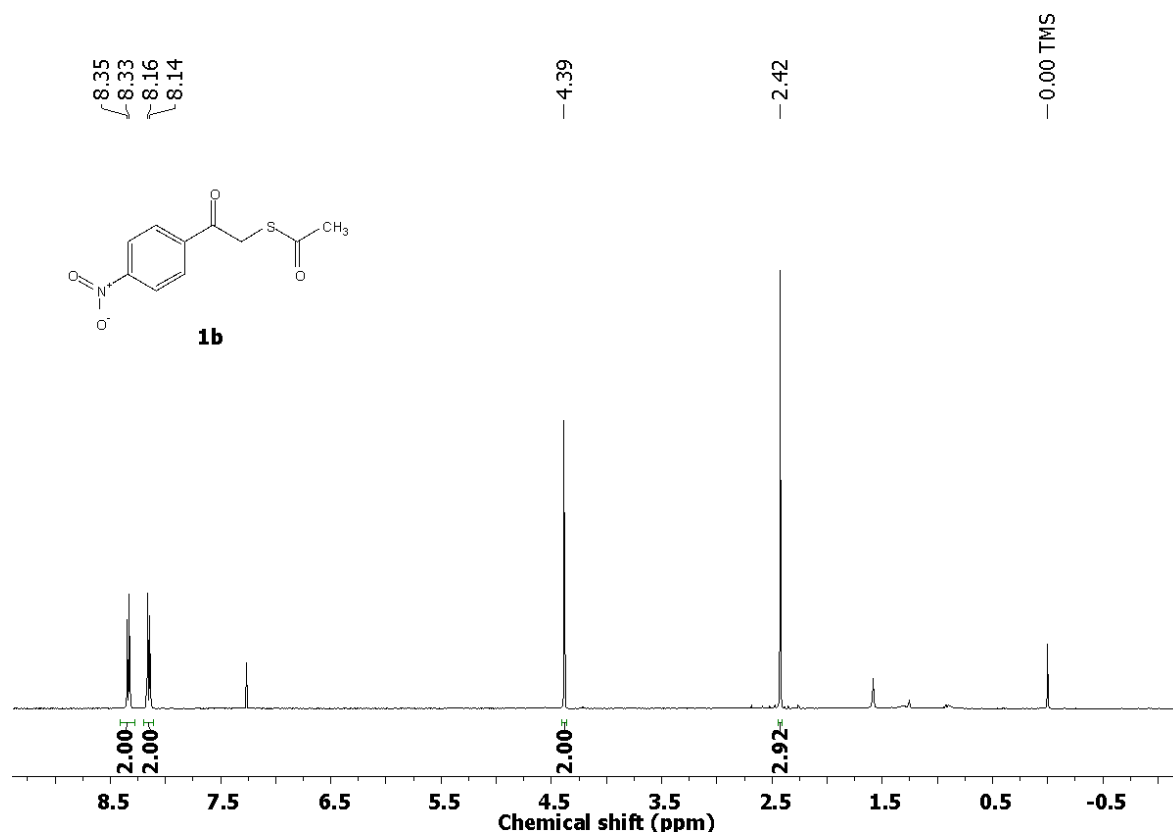
### 2.5.3. Docking protocols for *h3*-MST

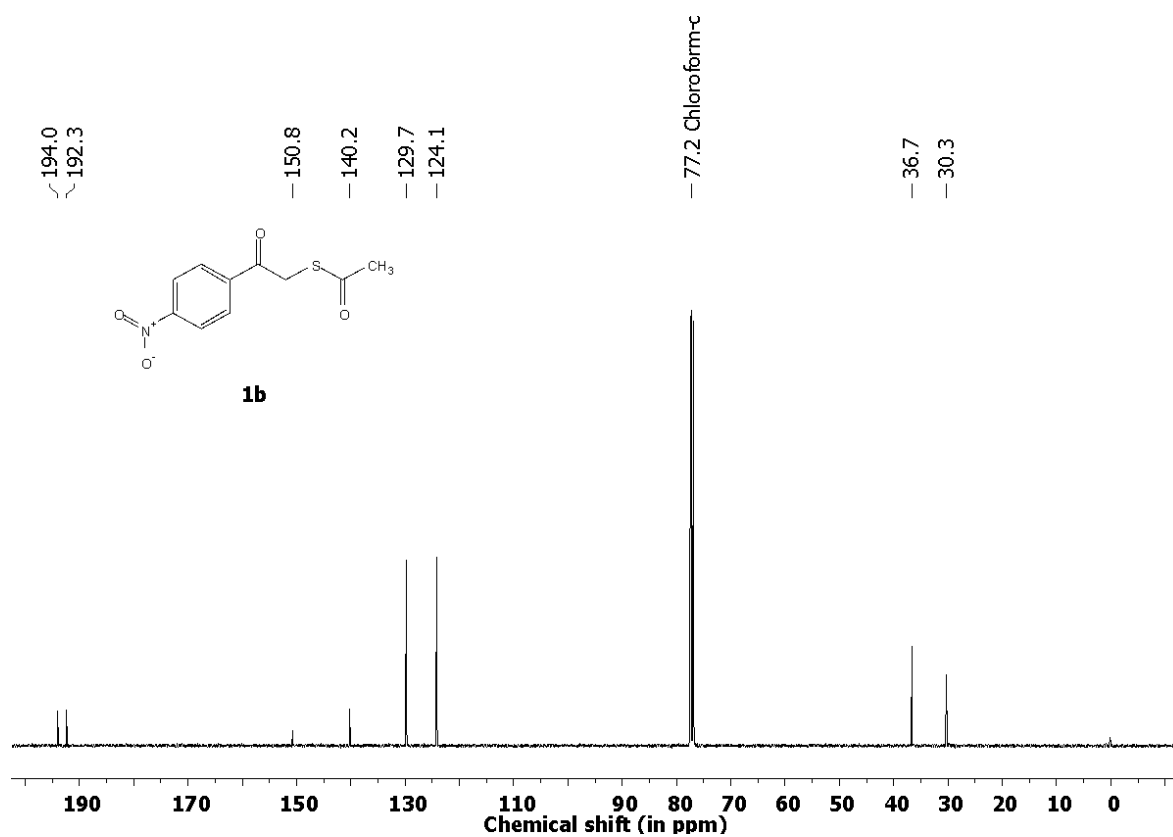
Autodock vina (V. 1.1.2) software was used to perform docking of compounds (thiol version of **1a-1n**) to the active site of ***h3*-MST** (Protein data bank (PDB) file: **4JGT**). Molecules were converted to PDB from SMILES and then from PDB to PDBQT format (as needed for docking), using OpenBabel (v 3.1.0). Docking was performed using 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**.

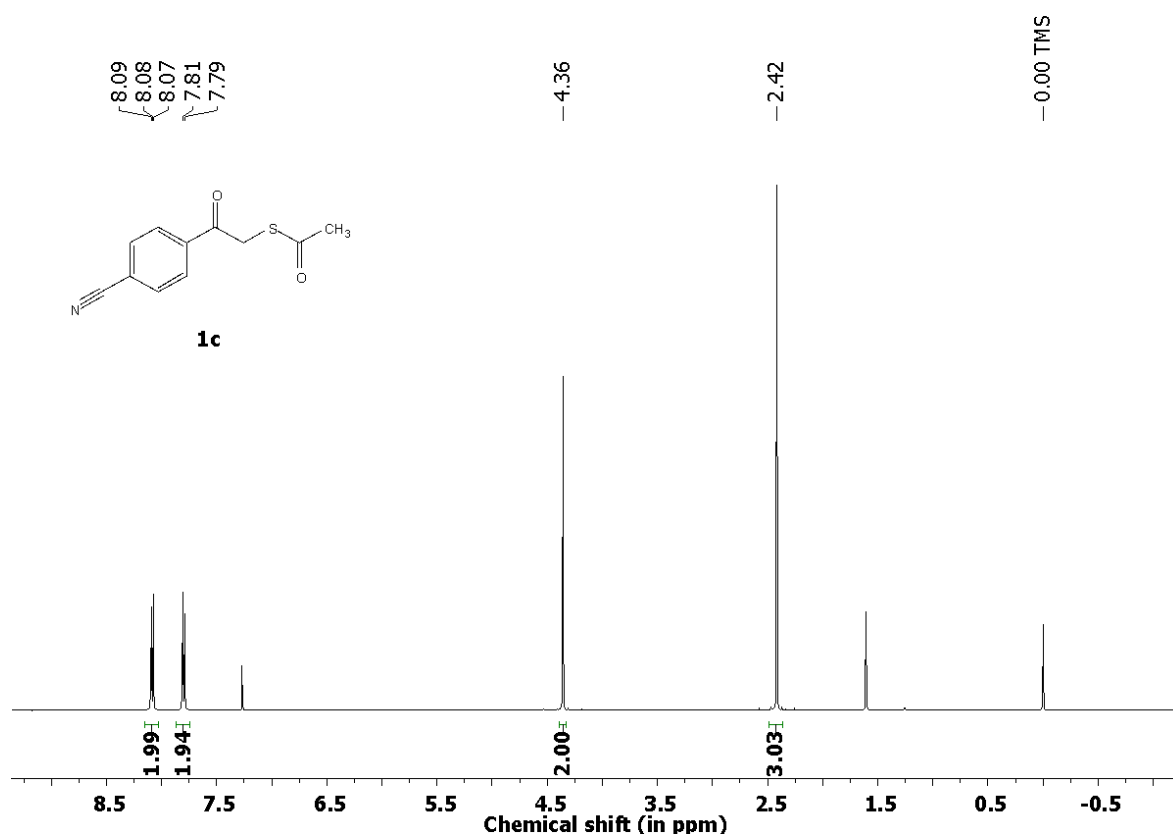
**Box Dimensions (Å):** 22 × 24 × 22.5

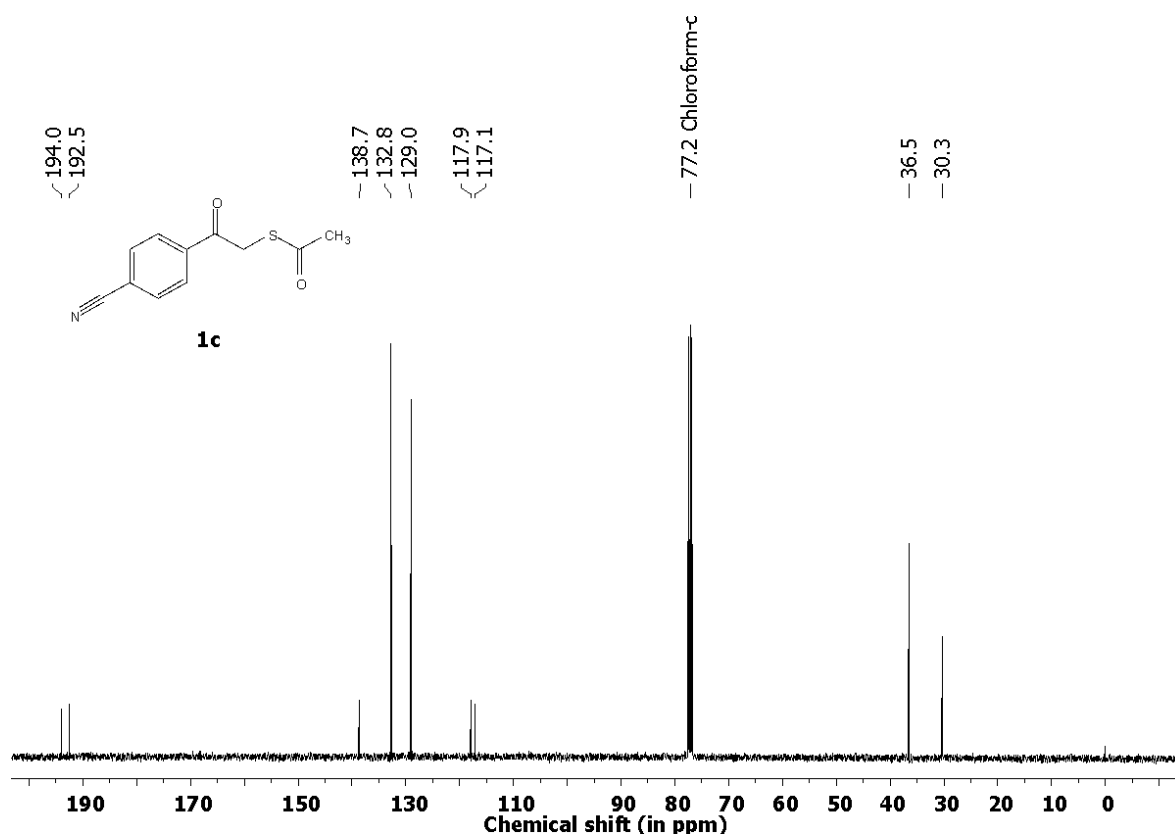
## 2.6. NMR spectra of compounds

<sup>1</sup>H NMR spectra of **1a**:<sup>1</sup>H and <sup>13</sup>C NMR spectra of **1b**:

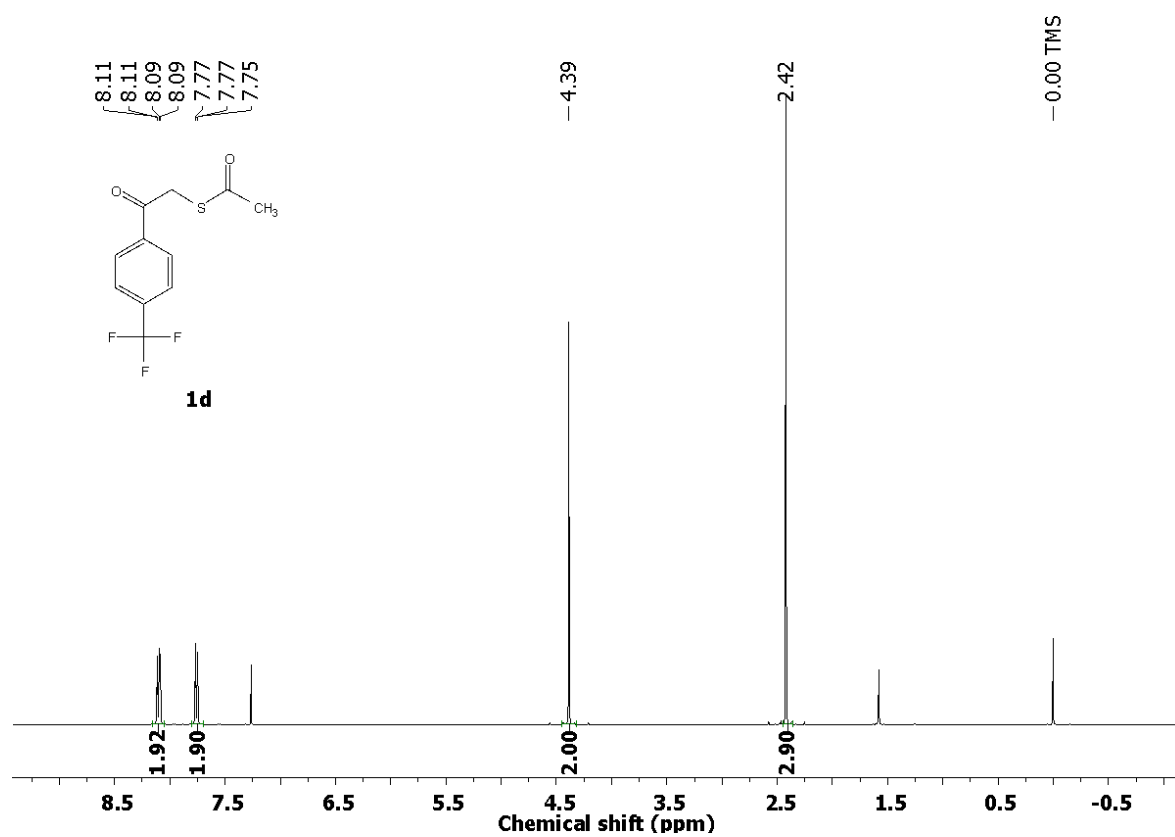


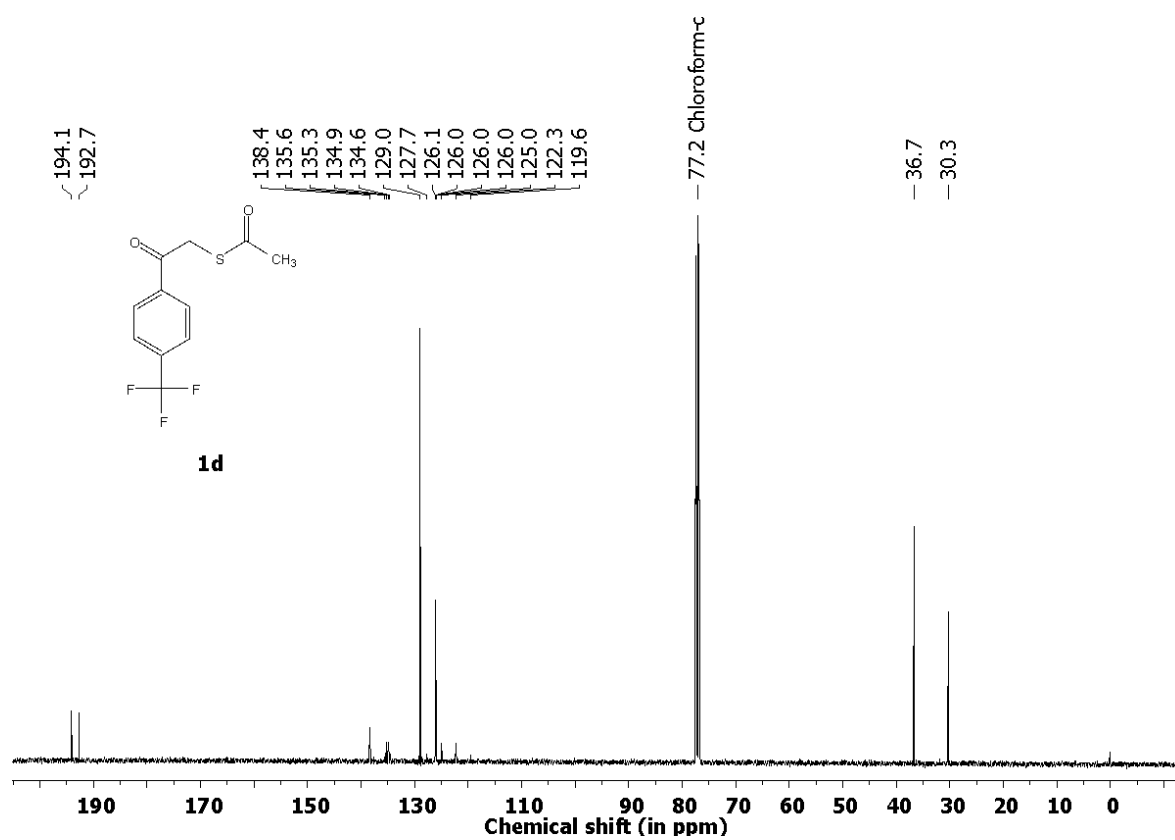
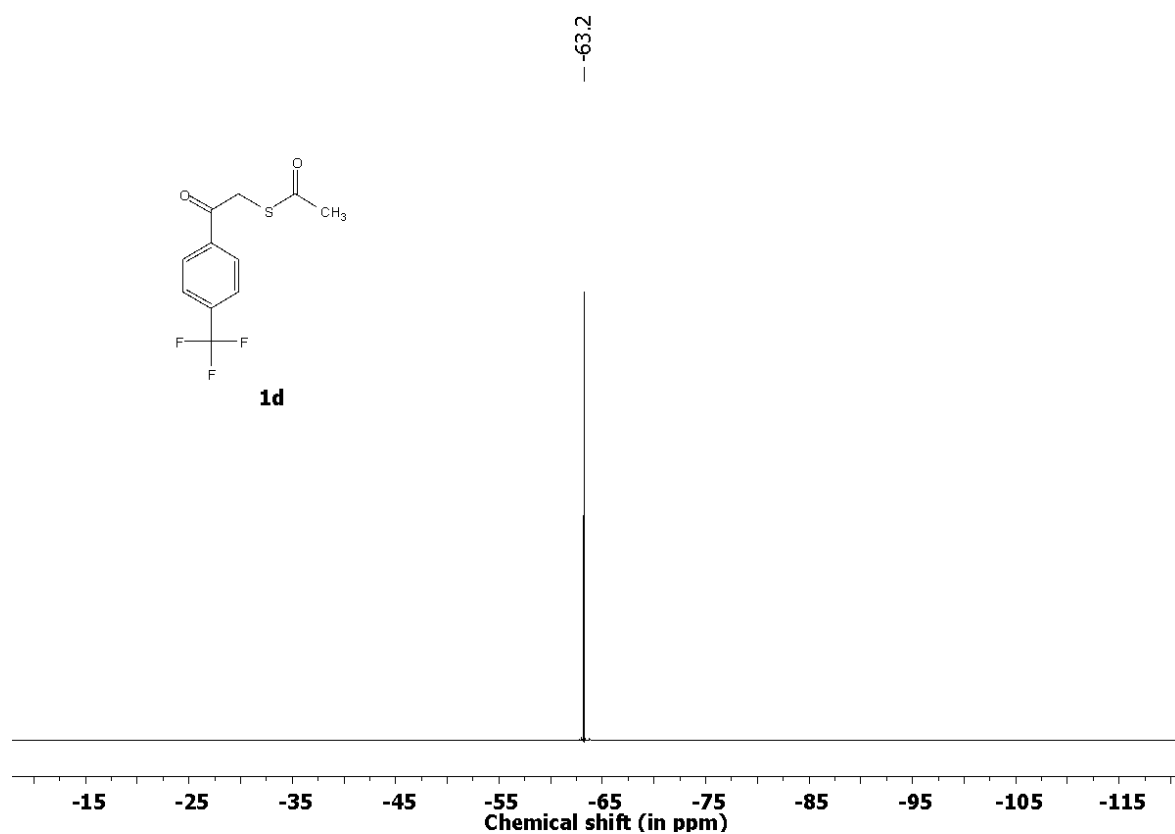
<sup>1</sup>H and <sup>13</sup>C NMR spectra of **1c**:



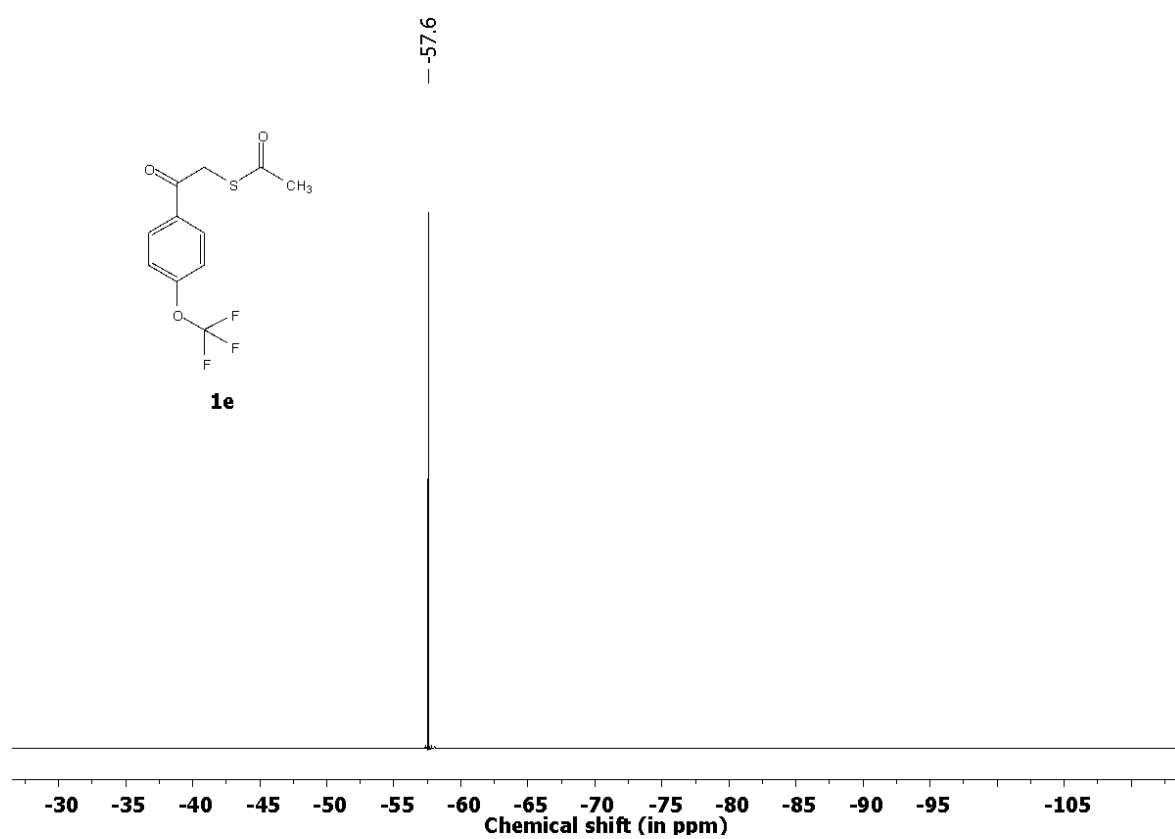
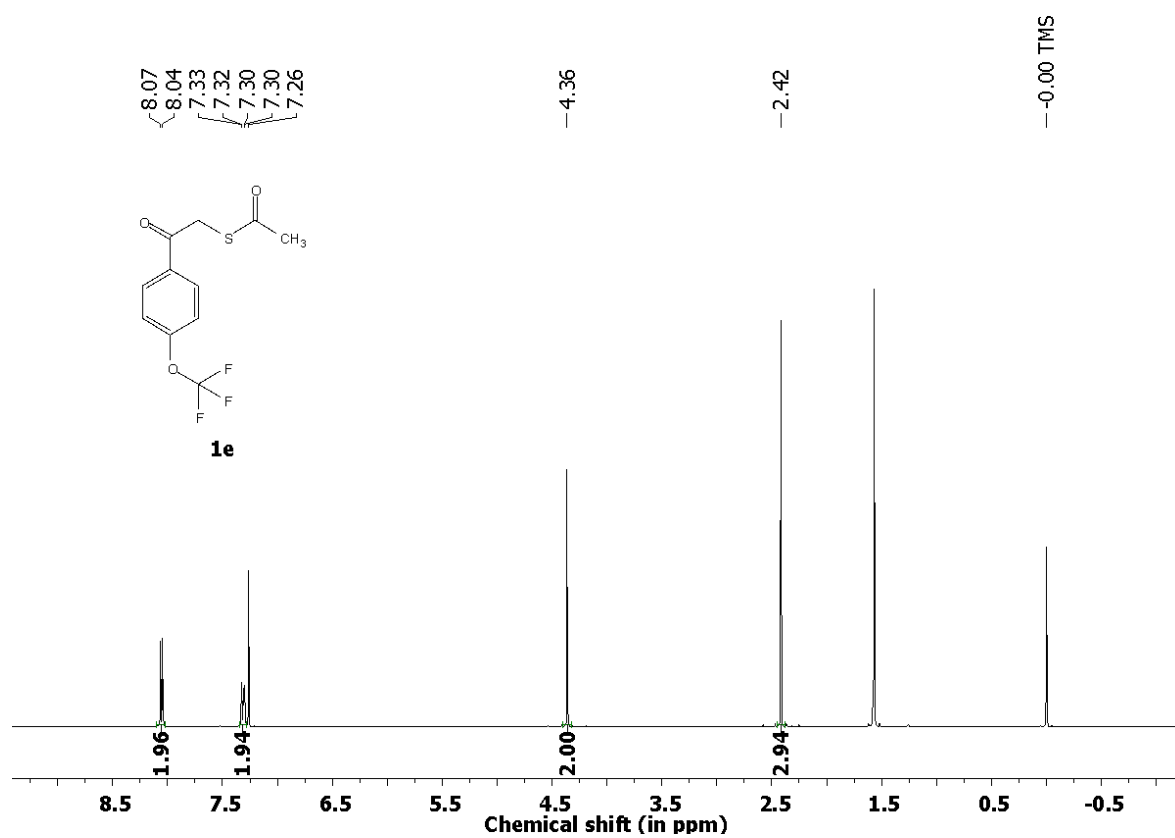


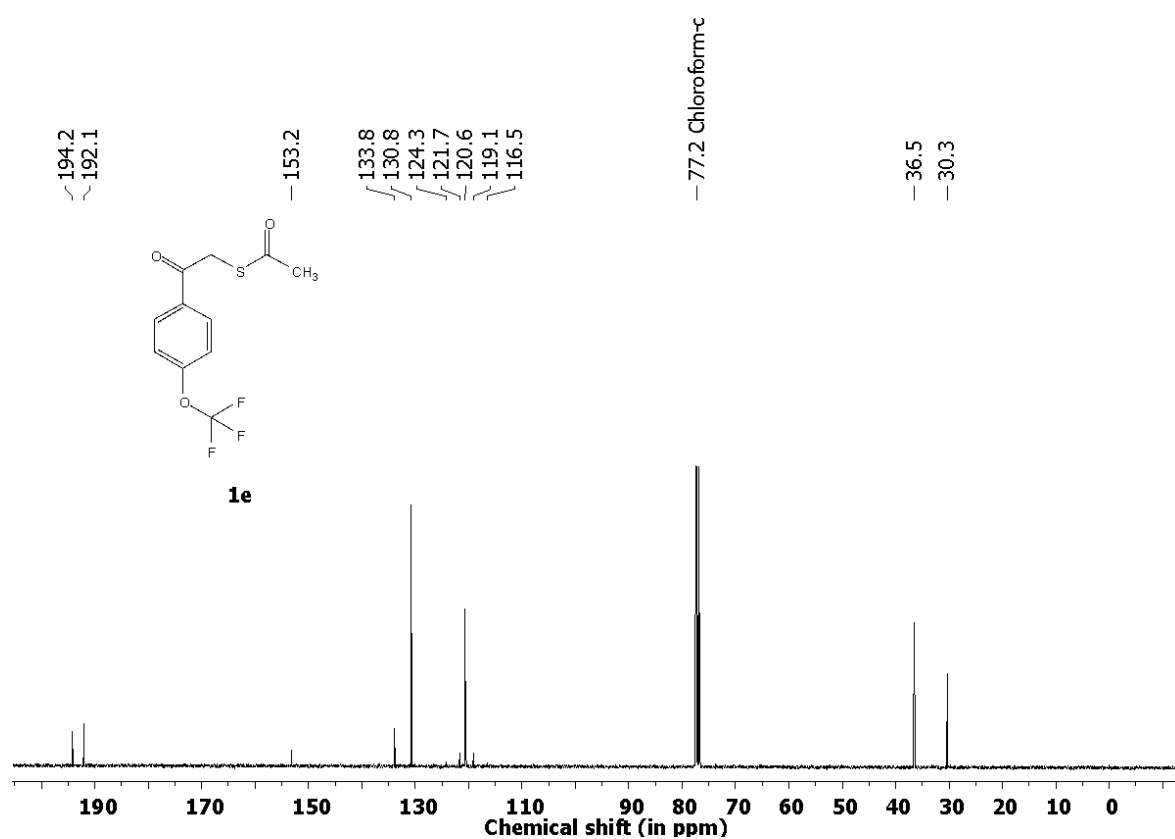
<sup>1</sup>H, <sup>19</sup>F and <sup>13</sup>C NMR spectra of **1d**:



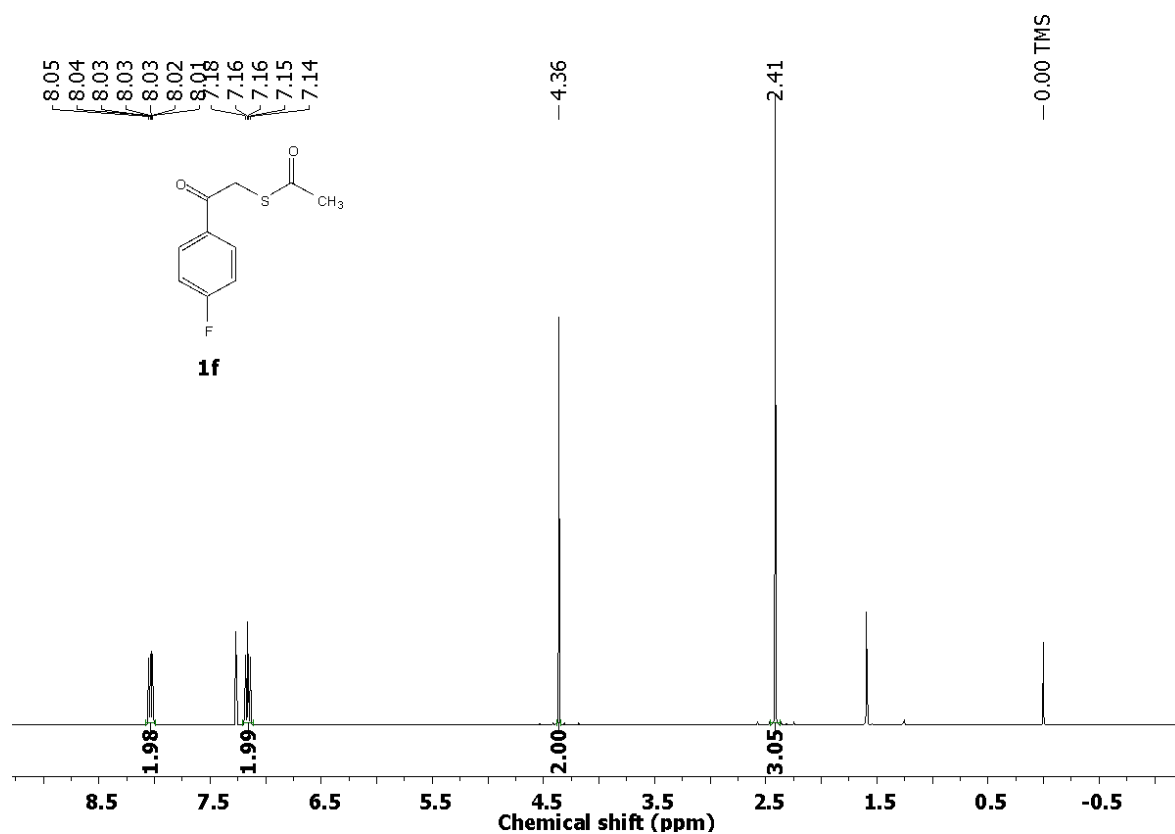


$^1\text{H}$ ,  $^{19}\text{F}$  and  $^{13}\text{C}$  NMR spectra of **1e**:

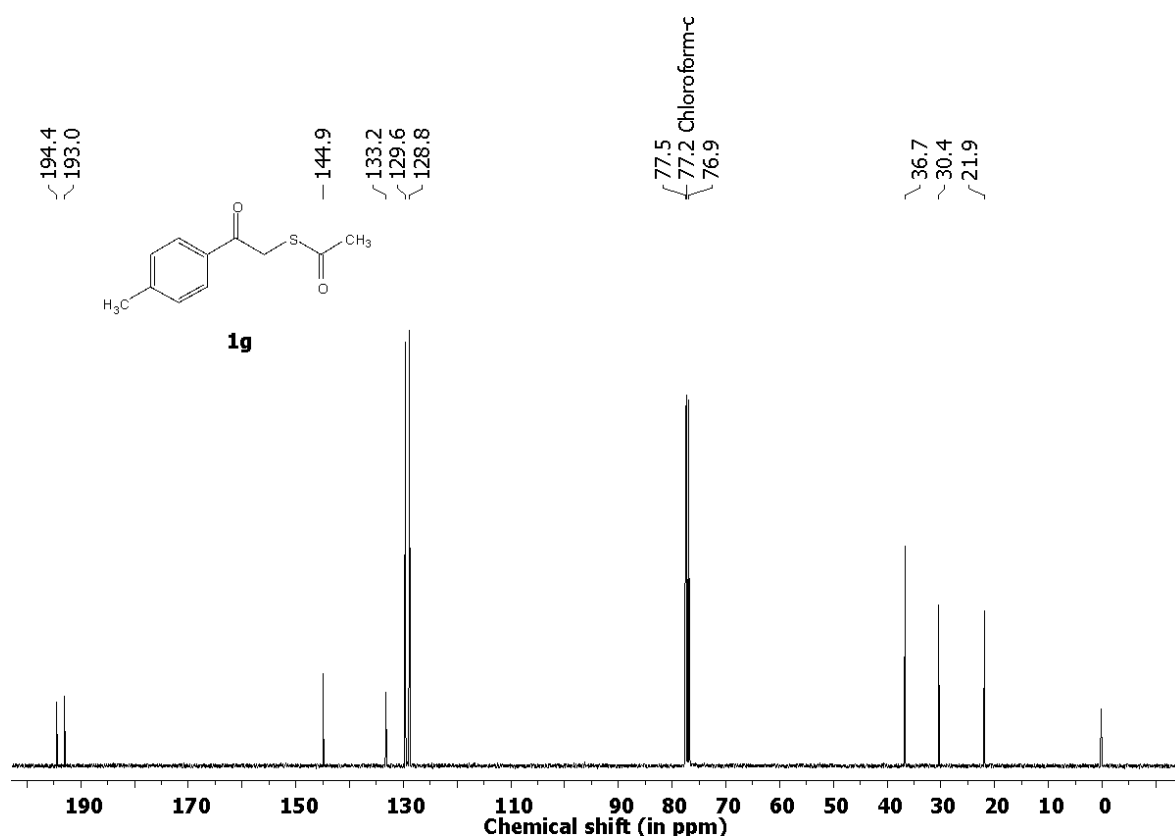
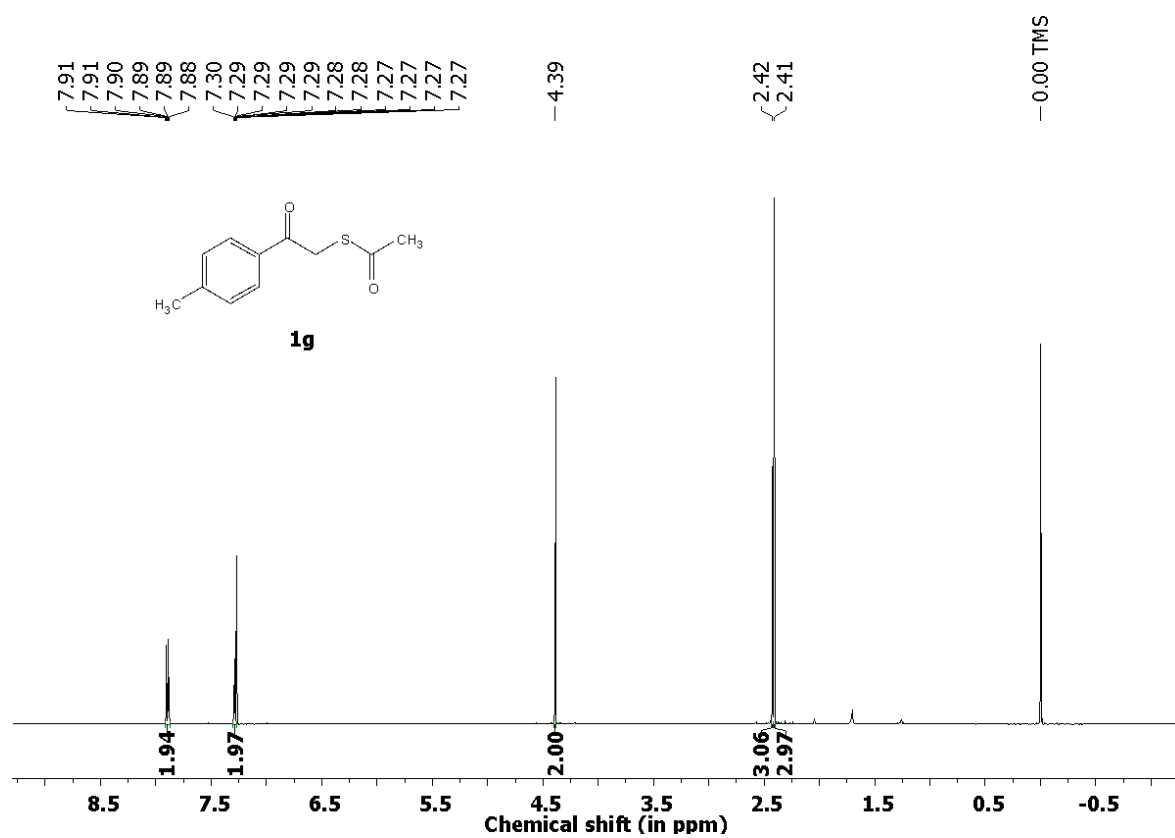




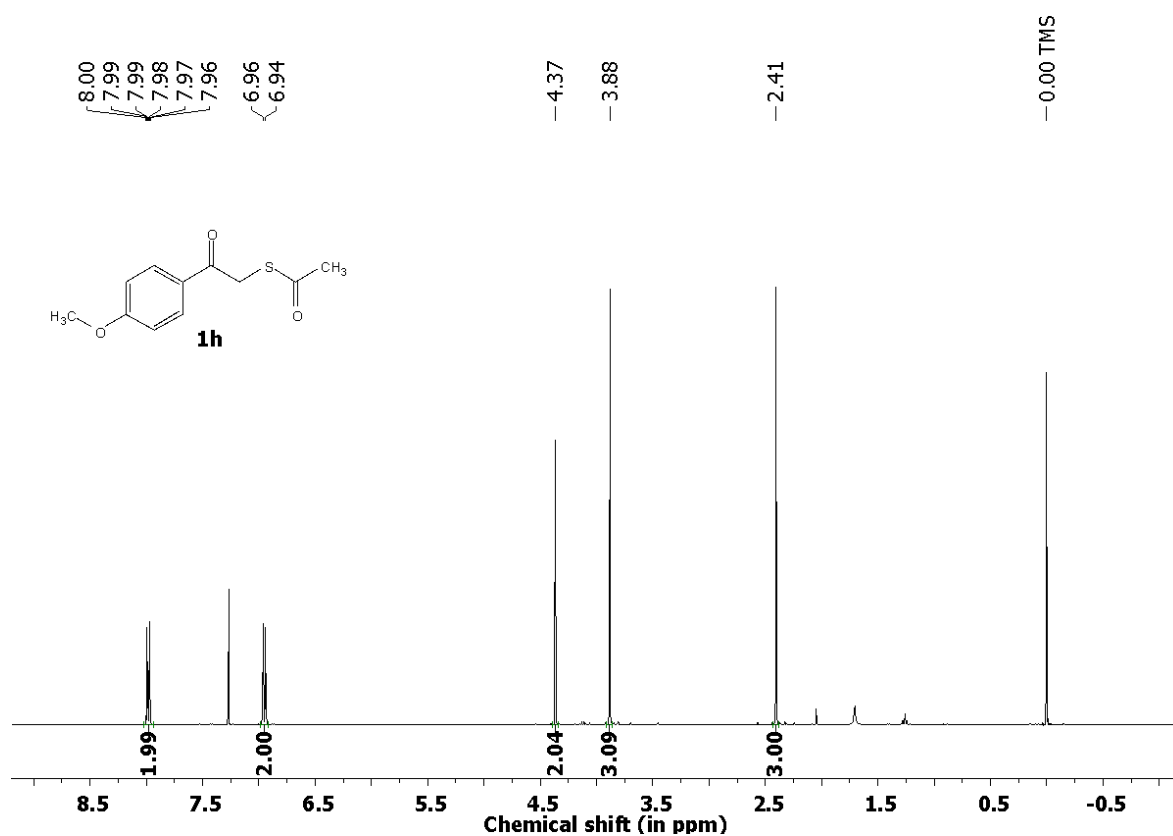
<sup>1</sup>H spectra of **1f**:



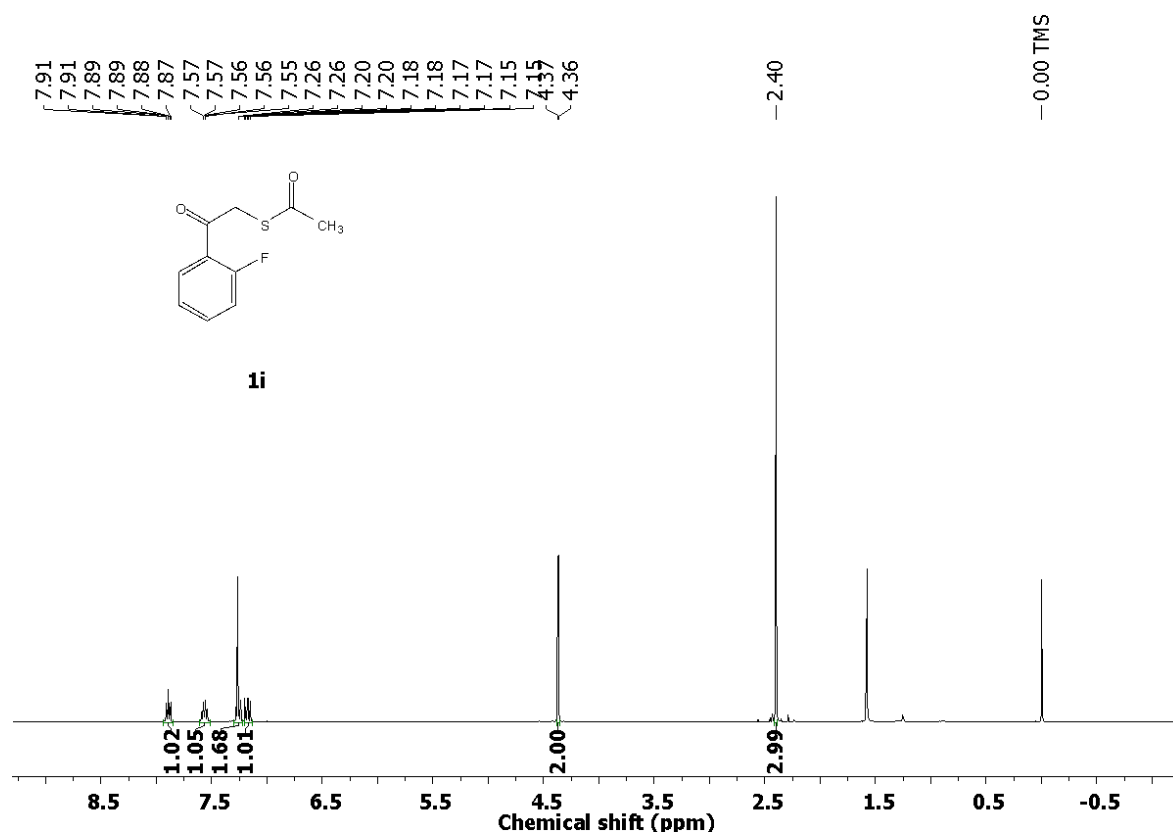
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **1g**:

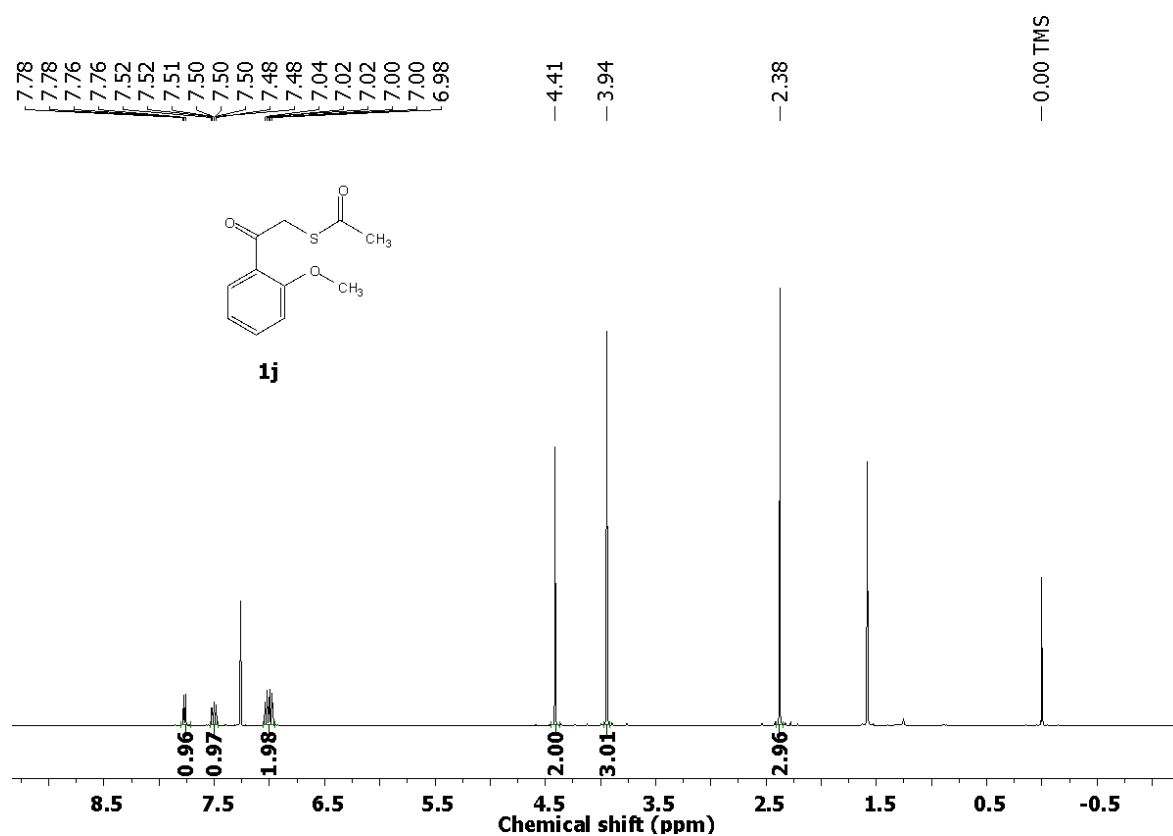
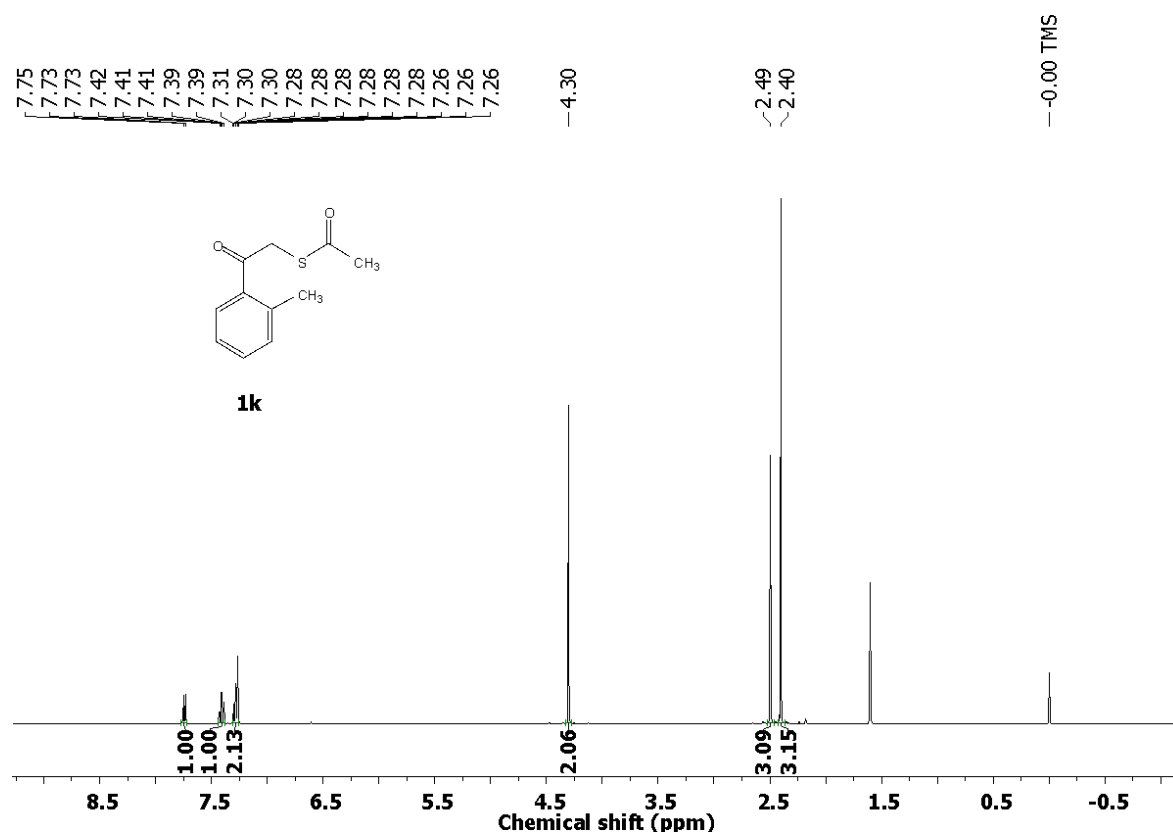


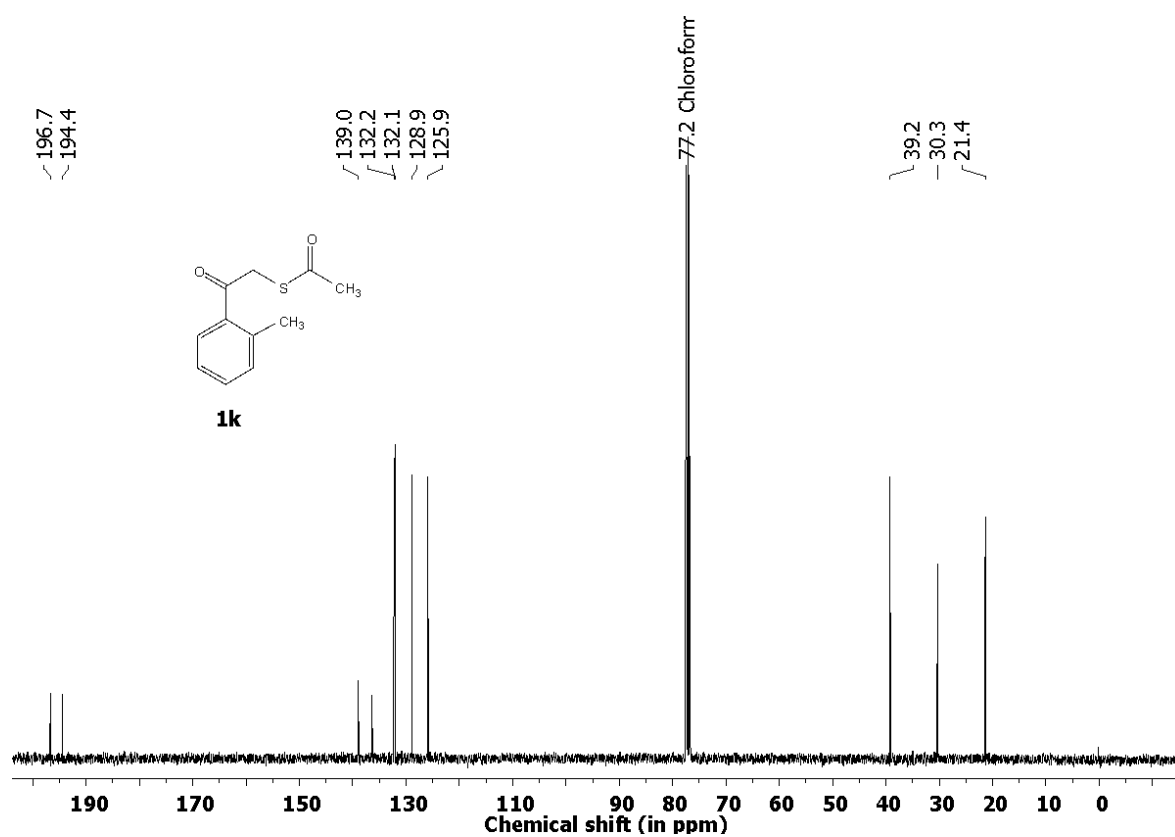
$^1\text{H}$  NMR spectra of **1h**:



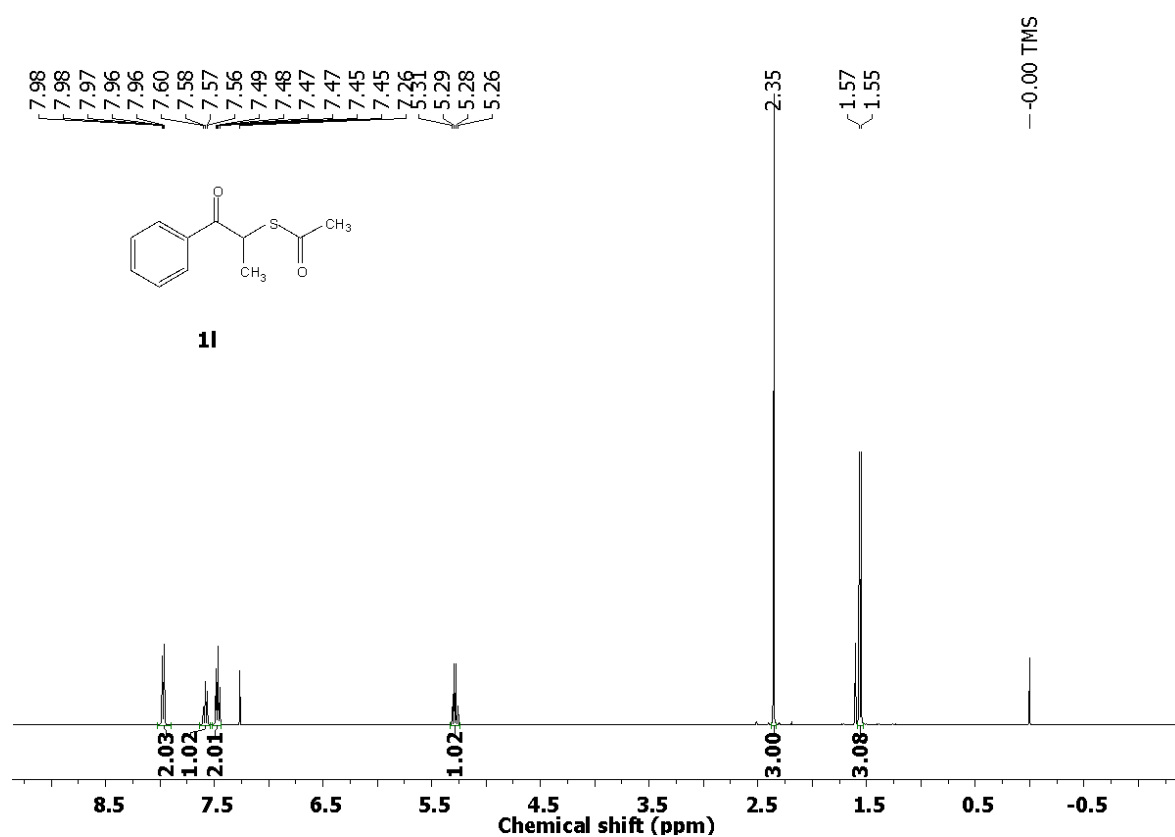
$^1\text{H}$  NMR spectra of **1i**:



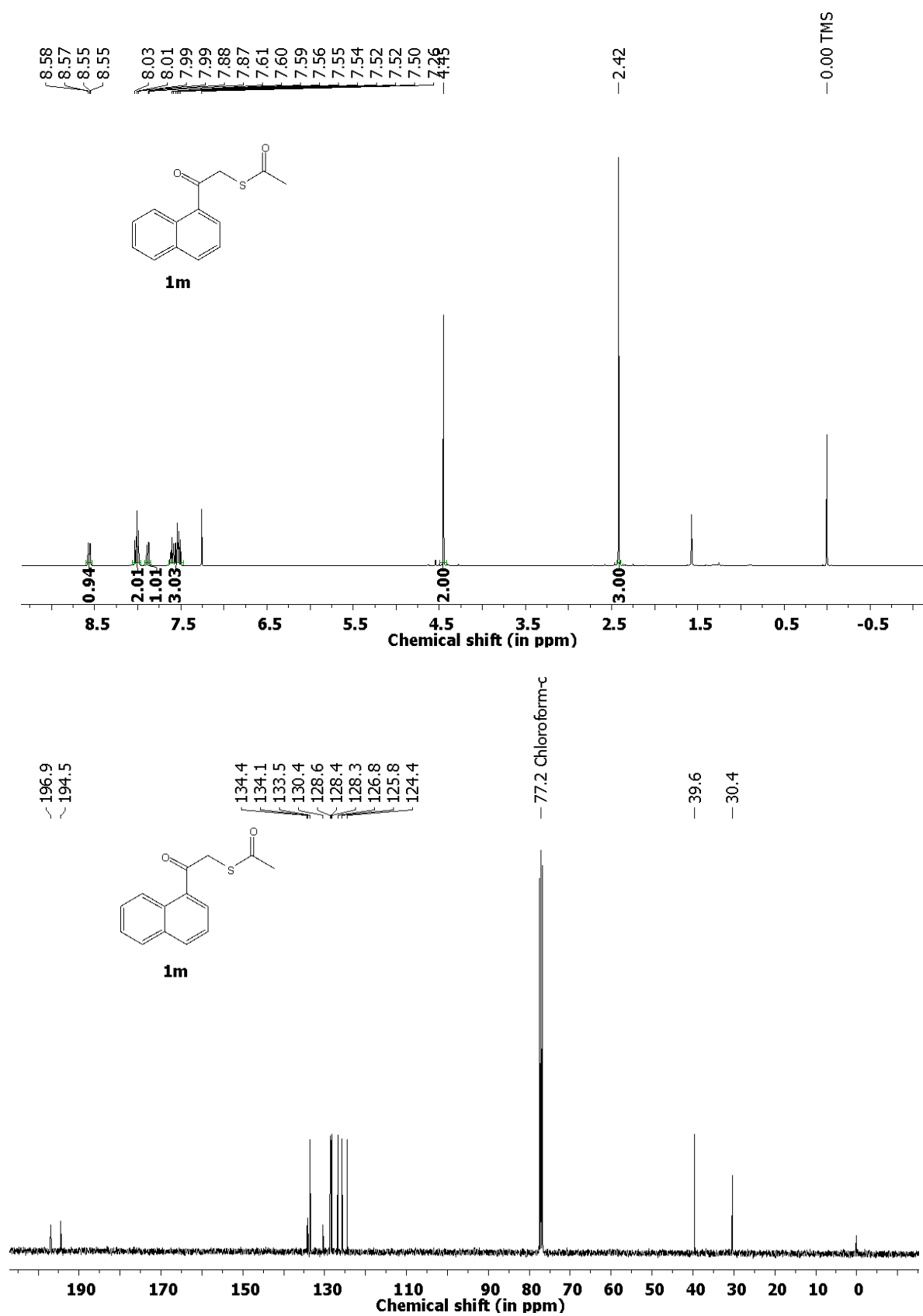
<sup>1</sup>H NMR spectra of **1j**:<sup>1</sup>H and <sup>13</sup>C NMR spectra of **1k**:

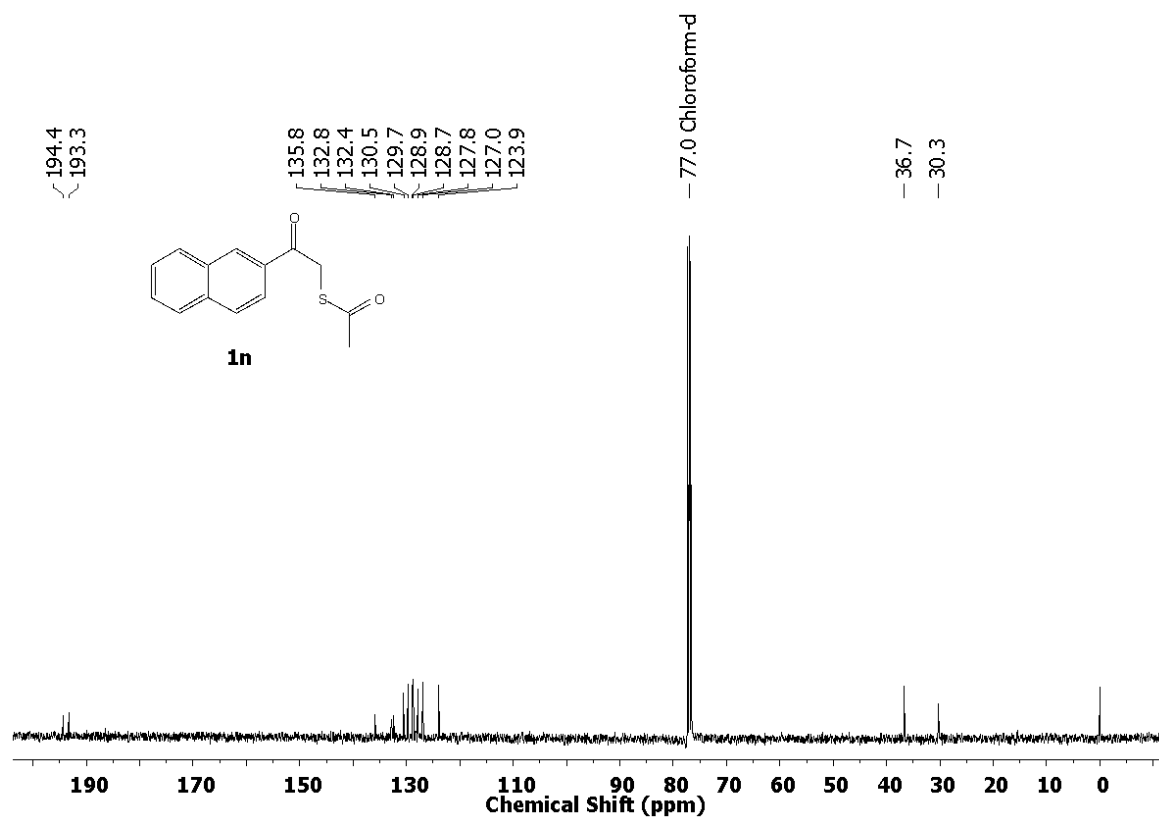
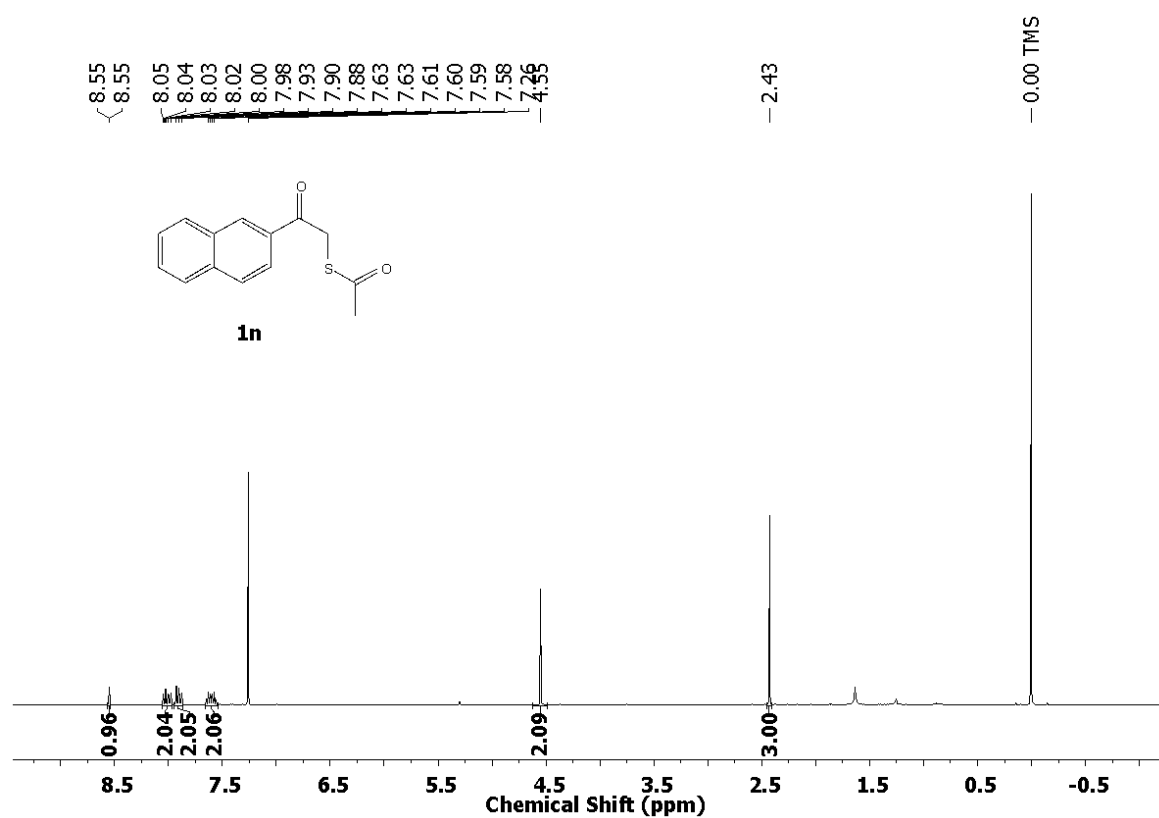


<sup>1</sup>H NMR spectra of **1l**:



$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **1m**:



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

## 2.7. References

- (1) Nair, M. Mechanistic Investigation of Unnatural Substrates for Enzymatic Production of Hydrogen Sulfide in Bacteria, IISER Pune, 2018.
- (2) Bora, P. Stimuli-Responsive Small Molecule Persulfidating Agents, IISER Pune, 2022.
- (3) Kelkar, D. S.; Ravikumar, G.; Mehendale, N.; Singh, S.; Joshi, A.; Sharma, A. K.; Mhetre, A.; Rajendran, A.; Chakrapani, H.; Kamat, S. S. A Chemical–Genetic Screen Identifies ABHD12 as an Oxidized-Phosphatidylserine Lipase. *Nat. Chem. Biol.* **2019**, *15* (2), 169–178.
- (4) Sedgwick, A. C.; Han, H.-H.; Gardiner, J. E.; Bull, S. D.; He, X.-P.; James, T. D. Long-Wavelength Fluorescent Boronate Probes for the Detection and Intracellular Imaging of Peroxynitrite. *Chem. Commun.* **2017**, *53* (95), 12822–12825.
- (5) Lawrence, N. S.; Davis, J.; Compton, R. G.; Jiang, L.; Jones, T. G. J.; Davis, S. N. Selective Determination of Thiols: A Novel Electroanalytical Approach. *Analyst* **2000**, *125* (4), 661–663.
- (6) Leggett, D. J.; Chen, N. H.; Mahadevappa, D. S. Flow Injection Method for Sulfide Determination by the Methylene Blue Method. *Anal. Chim. Acta* **1981**, *128*, 163–168.
- (7) Yadav, P. K.; Yamada, K.; Chiku, T.; Koutmos, M.; Banerjee, R. Structure and Kinetic Analysis of H<sub>2</sub>S Production by Human Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2013**, *288* (27), 20002–20013.
- (8) Heredia, A. A.; Soria-Castro, S. M.; Bouchet, L. M.; Oksdath-Mansilla, G.; Barrionuevo, C. A.; Caminos, D. A.; Bisogno, F. R.; Argüello, J. E.; Peñeñory, A. B. Stereoselective One-Pot Synthesis of  $\beta$ -Alkylsulfide Enol Esters. Base-Triggered Rearrangement under Mild Conditions. *Org. Biomol. Chem.* **2014**, *12* (33), 6516.
- (9) Lopp, J. M.; Schmidt, V. A. Intermolecular Phosphite-Mediated Radical Desulfurative Alkene Alkylation Using Thiols. *Org. Lett.* **2019**, *21* (19), 8031–8036.
- (10) Przydacz, A.; Kowalczyk, R.; Albrecht, Ł. Brønsted-Base-Catalyzed Remote Cascade Reactivity of 2,4-Dienones – Asymmetric Synthesis of Tetrahydrothiophenes. *Org. Biomol. Chem.* **2017**, *15* (45), 9566–9569.
- (11) Tatar, J.; Baranac-Stojanović, M.; Stojanović, M.; Marković, R. Reactions of Ortho-Substituted  $\alpha,\alpha$ -Dibromoacetophenones with Nucleophiles: First Examples of Combined Carbophilic and Bromophilic Attack on C–Br Bonds. *Tetrahedron Lett.* **2009**, *50* (6), 700–703.

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- (12) Hatanaka, T.; Yuki, R.; Saito, R.; Sasaki, K.  $\alpha$ -Methylphenacyl Thioesters as Convenient Thioacid Precursors. *Org. Biomol. Chem.* **2016**, *14* (45), 10589–10592.
- (13) Yadav, P. K.; Yamada, K.; Chiku, T.; Koutmos, M.; Banerjee, R. Structure and Kinetic Analysis of H<sub>2</sub>S Production by Human Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2013**, *288* (27), 20002–20013.
- (14) Sharma, A. K.; Nair, M.; Chauhan, P.; Gupta, K.; Saini, D. K.; Chakrapani, H. Visible-Light-Triggered Uncaging of Carbonyl Sulfide for Hydrogen Sulfide (H<sub>2</sub>S) Release. *Org. Lett.* **2017**, *19* (18), 4822–4825.



## CHAPTER 3. Mitochondria-targeted Artificial Substrates for 3-MST

### 3.1. Introduction

In Chapter 3, an artificial substrate for mitochondrial 3-MST that can be specifically localized into mitochondria is developed. Mitochondria, an organelle that is required for the normal functioning of cells, has been implicated as a major mediator of cellular processes related to inflammation, metabolic signaling, and cell death.<sup>1</sup> Thus, rationally designed, mitochondrion-specific drugs may be more effective for notoriously hard-to-treat diseases arising from mitochondrial gene mutations by controlling mitochondrial bioenergetics. 3-MST is expressed at higher levels in mitochondria when compared with the cytosol and it is also recognized as one of the principal contributors of H<sub>2</sub>S and sulfane sulfur in mitochondria.<sup>2,3</sup> Thus, 3-MST activity plays a major role in regulating the redox machinery and signaling processes inside mitochondria.<sup>4</sup> Therefore, targeting the cell's mitochondria in the context of 3-MST is pertinent and may prove advantageous in modulating sulfur metabolism and redox biology. As cellular membranes are made up of phospholipid bilayers, a highly hydrophilic or hydrophobic molecule can not pass through easily. Therefore, specific delivery of a drug or a fluorophore into mitochondria is challenging. Hence, in order to target mitochondria, the payload is usually attached to a lipophilic cation known as mito-tag.<sup>5</sup> There are several known examples of mito-tags (Eg. triphenylphosphonium, triethylammonium, guanidium) in the literature, of which targeting with triphenylphosphonium (TPP) is frequently employed because of its mitochondrial specificity and stability in cellular milieu.<sup>5,6</sup> Owing to the lipophilicity of TPP, a TPP-conjugated cationic drug molecule provide a balance between lipophilicity and hydrophilicity in a molecule which enhances membrane permeability. As mitochondria is negatively charged organelle, a delocalized positive charge on TPP is known to drive the molecule to localize in the mitochondria.<sup>5,6</sup> Hence, based on the SAR results obtained for the artificial substrate, we have designed two distinct mitochondria-targeted artificial substrates with TPP as a targeting group. We hypothesized that after mitochondrial localization, a TPP-tagged substrate is expected to be activated by cellular thiol/esterase and will produce an active substrate that can be turned over by 3-MST. As previously established in Chapter 2, artificial substrates in presence of 3-MST can generate H<sub>2</sub>S and sulfane sulfur, therefore same species can also be generated in mitochondria specifically. Overall, we expect that a rationally designed mito-targeted 3-MST substrate will have higher organelle specificity therefore will enhance H<sub>2</sub>S and sulfane sulfur in the cells in a targeted manner.

### 3.2. Design and hypothesis

To design a mitochondria-targeted 3-MST substrate, a TPP tag needed to be appended to a suitable position of the artificial substrate. Following this, a linker needed to be used to connect the TPP tag with the substrate. A suitable linker is very important in order to modulate the lipophilicity of the compound which will eventually help to improve the permeability of the substrate. Hence, linker with a four carbon was used to design potential mito-targeted substrate.

#### 3.2.1. Approach 1

Firstly, in order to find a suitable position to append TPP tag, we have closely analyzed the structure of the artificial substrates that were developed in Chapter 2 (Figure 3.1A). Based on SAR analysis, artificial substrate **1h** ( $k_{H_2S} = 0.39 \pm 0.07 \text{ h}^{-1}$  with *h*3-MST) and **1j** ( $k_{H_2S} = 0.08 \pm 0.07 \text{ h}^{-1}$  with *h*3-MST) were identified as the potential core for the mito-targeted substrate. Out of these two artificial substrates, **1h** was identified as the core of the mito-targeted 3-MST substrate due to its relatively faster  $H_2S$  release than **1j**. Therefore, a mitochondria-targeted substrate was designed where a TPP tag was connected to the substrate with the aforementioned linker (Figure 3.1B).

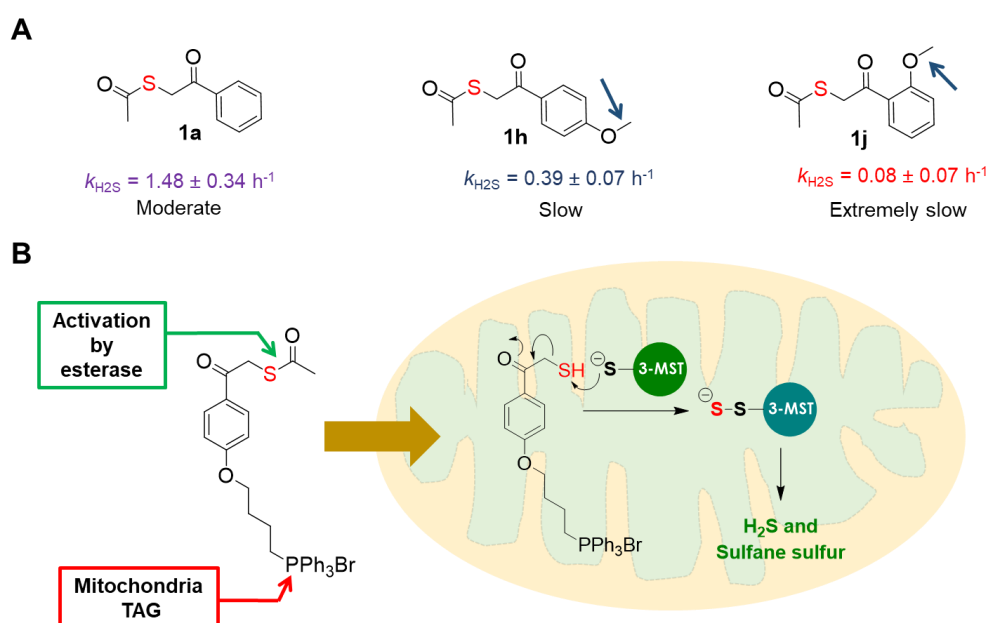


**Figure 3.1:** Design of the mitochondria-targeted artificial substrate of 3-MST using approach 1 (A) Structural analysis of 3-MST substrates to determine a suitable position for TPP tag. The directed arrow shows the possible site available for modifications. (B) Design and hypothesis of a TPP-tagged 3-MST substrate.

As the TPP tag is connected directly to the substrate, therefore, the modified substrate was hypothesized to be localized into mitochondria where the acetyl group was expected to be

hydrolyzed by esterase and release active 3-MST substrate. Owing to the direct TPP-substrate connection, once localized, the active substrate will not be able to efflux out from the mitochondria. As a result, the active substrate will be turned over by 3-MST and will generate  $\text{H}_2\text{S}$  and sulfane sulfur in a localized manner. But a possible drawback with this strategy is that, due to the ERG and presence of bulky TPP group in the modified substrate, the rate of 3-MST catalysis might be affected. Another possible drawback with this design is that the substrate activation is not very specific to esterase as the terminal acetyl group is highly labile and can get hydrolysed by cellular thiols. In such case, we hypothesized that TPP-conjugated thiol will still be able to localize in mitochondria in an efficient manner due to the lipophilicity and charge difference.

### 3.2.2. Approach 2

To overcome the drawback of the previous strategy, an alternate design of TPP-tagged compound was considered. Here, upon activation with esterase, the TPP tag will be separated from the compound which will result a free artificial substrate. Therefore, a moderate  $\text{H}_2\text{S}$  releasing substrate **1a** ( $k_{\text{H}_2\text{S}} = 1.48 \pm 0.34 \text{ h}^{-1}$  with *h3*-MST) was identified. Here, instead of connecting TPP directly to the aromatic ring, we designed an artificial substrate based on the structure of **1a** where the thiol part of the substrate is directly linked to TPP-tagged benzoic acid (Figure 3.2).

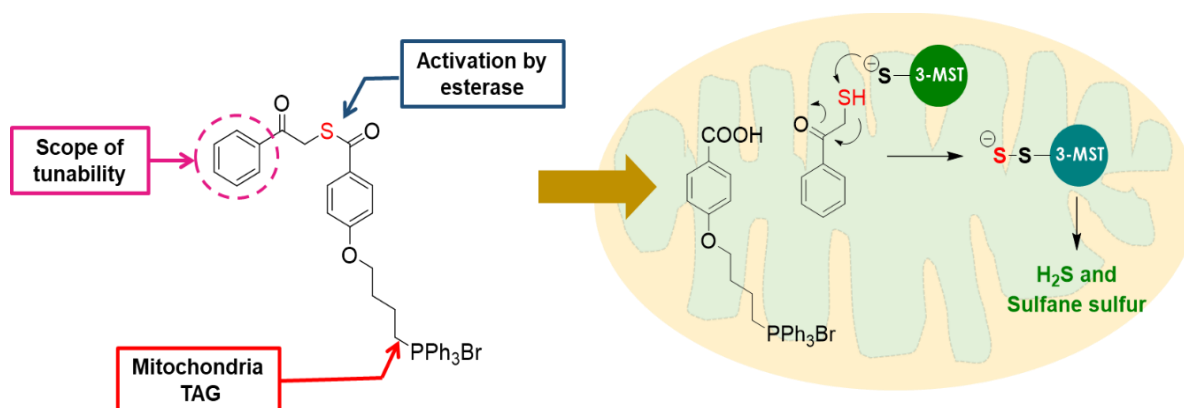


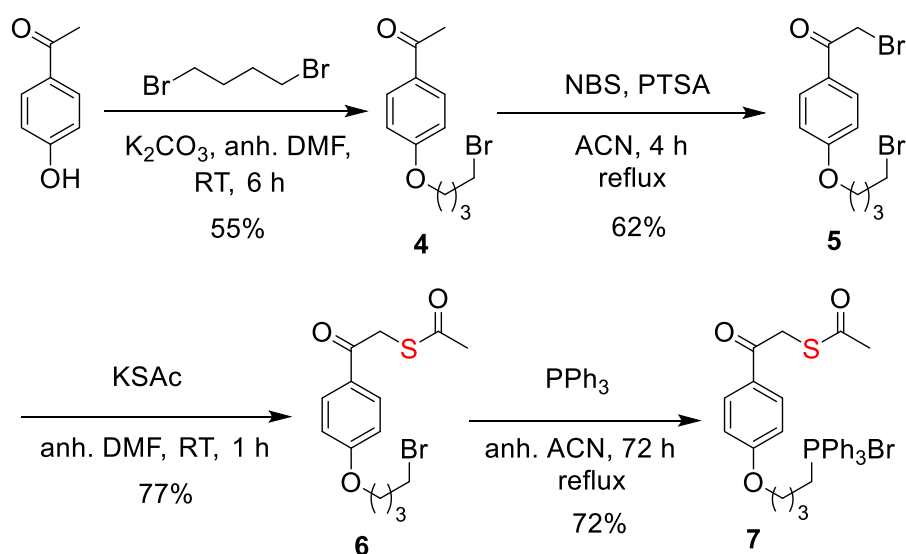
**Figure 3.2:** Design and hypothesis of the mitochondria-targeted artificial substrate of 3-MST using approach 2.

As the TPP tag is connected with the substrate, the benzoate ester of the compound upon localization into mitochondria will be hydrolyzed by esterase and release the free substrate (**2a**). Then **2a** will be turned over by 3-MST and will generate  $\text{H}_2\text{S}$  and sulfane sulfur. Here, in order to achieve additional tunability in the rate of  $\text{H}_2\text{S}$  generation, the aromatic ring of the artificial substrate can also be modified.

### 3.3. Results and discussion

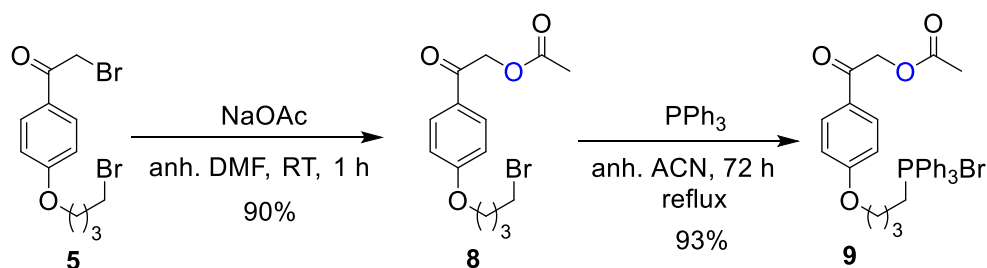
#### 3.3.1. Synthesis

Following approach **1**, mitochondria-targeted 3-MST substrate **7** was synthesized *via* a 4-step reaction sequence starting from 4-hydroxy acetophenone (Scheme 3.1). 4-Hydroxyacetophenone was reacted with 1,4-dibromobutane to provide **4** in 55% yield. Next, the  $\alpha$ -monobromination reaction was performed with **4** using NBS/PTSA to form **5** in 62% yield. Compound **5** was then treated with potassium thioacetate (KSAc) to afford **6** in 77% yield. Finally, reaction of **6** with triphenylphosphine furnished compound **7** in 72% yield.



**Scheme 3.1:** Synthesis of the mitochondria-targeted artificial substrate of 3-MST (**7**).

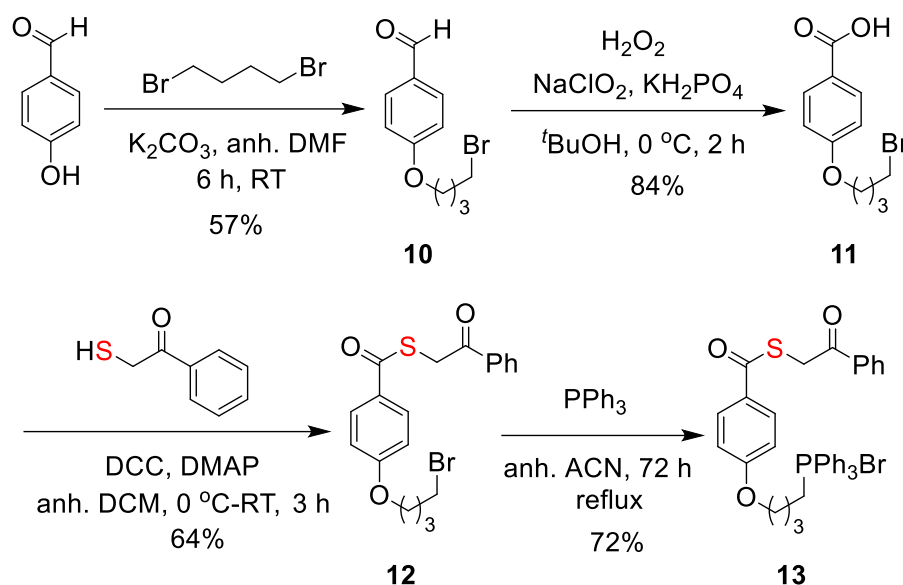
Next, a structural analog **9** was designed where sulfur was replaced with oxygen. Compound **9** was synthesized *via* 2-step reaction from **5** (Scheme 3.2). The latter compound was reacted with sodium acetate (NaOAc) and then further reacted with triphenylphosphine to afford **9** in 93% yield.



**Scheme 3.2:** Synthesis of mitochondria-targeted negative control (**9**).

Following approach **2**, mitochondria-targeted 3-MST substrate **13** was synthesized *via* a 4-step reaction sequence starting from 4-hydroxybenzaldehyde (Scheme 3.3). 4-Hydroxybenzaldehyde was reacted with 1,4-dibromobutane, and then oxidized using

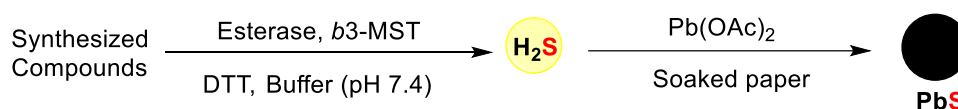
NaClO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> to give **11** in 84% yield. This was followed by DCC/DMAP mediated esterification with phenacyl thiol (**2a**) to obtain **12** in 64% yield. Finally, **12** was reacted with triphenylphosphine to synthesize the final compound **13** in 72% yield.



**Scheme 3.3:** Synthesis of the mitochondria-targeted artificial substrate of 3-MST (**13**).

### 3.3.2. Detection of H<sub>2</sub>S using lead acetate assay

Next, H<sub>2</sub>S release was studied using the lead acetate assay. In this assay, H<sub>2</sub>S released during the experiment was trapped by Pb(OAc)<sub>2</sub> soaked paper and a black spot of lead sulfide (PbS) was formed on the paper.



**Scheme 3.4:** Detection of H<sub>2</sub>S *via* lead acetate assay.

In this assay, 200 μM of **7** and **13** were incubated with porcine liver esterase (1 U/mL), along with b3-MST (1 μM) and DTT (10 mM) in pH 7.4 PBS (1×) at 37 °C. The assay was performed in 96 well plate and the plate was covered with 5% Pb(OAc)<sub>2</sub> soaked paper and the reaction was incubated for 2 h. Using a similar protocol **1a** was used as a positive control for this assay. An intense spot for PbS was observed for both **1a** and **7** which indicates H<sub>2</sub>S production (Figure 3.3A and 3.3B). A lower yield of H<sub>2</sub>S was observed for **7** indicating a slower release of H<sub>2</sub>S from **7** than **1a**. It has been observed that **13** was not able to produce a significant amount of H<sub>2</sub>S in the presence b3-MST. As expected, negative control **9** failed to produce H<sub>2</sub>S in the presence b3-MST. Only substrate control did not show a significant amount of H<sub>2</sub>S production.

Next, a dose-dependent H<sub>2</sub>S detection was conducted with varied concentrations of **7** and dose-dependent PbS formation was observed (Figure 3.3C).

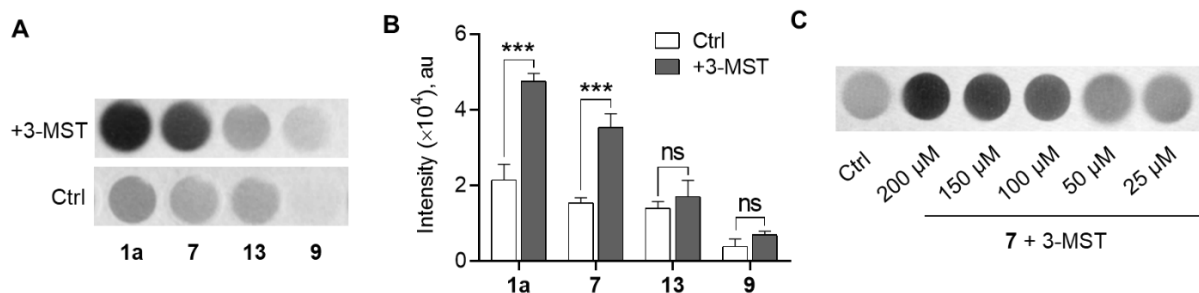


**Figure 3.3:** Lead acetate assay for H<sub>2</sub>S generation. (A) Formation of PbS as **1a**, **7** and **13** were incubated with esterase, *b3*-MST and DTT for 2 h. (B) PbS intensity was quantified using Image J software. (C) Various concentrations of **7** (200, 150, 100, 50, 25 μM) were incubated with esterase, *b3*-MST and DTT for 2 h. Ctrl represents only substrate. Results are expressed as ± SD (n = 3) [(\*\*\*), *p* < 0.001].

### 3.3.3. TLC-based experiment to study the decomposition of **13** in presence of esterase

As compound **13** was not able to produce a significant amount of H<sub>2</sub>S, a TLC-based experiment was to check the ability of **2a** formation in an esterase-mediated decomposition of **13**.

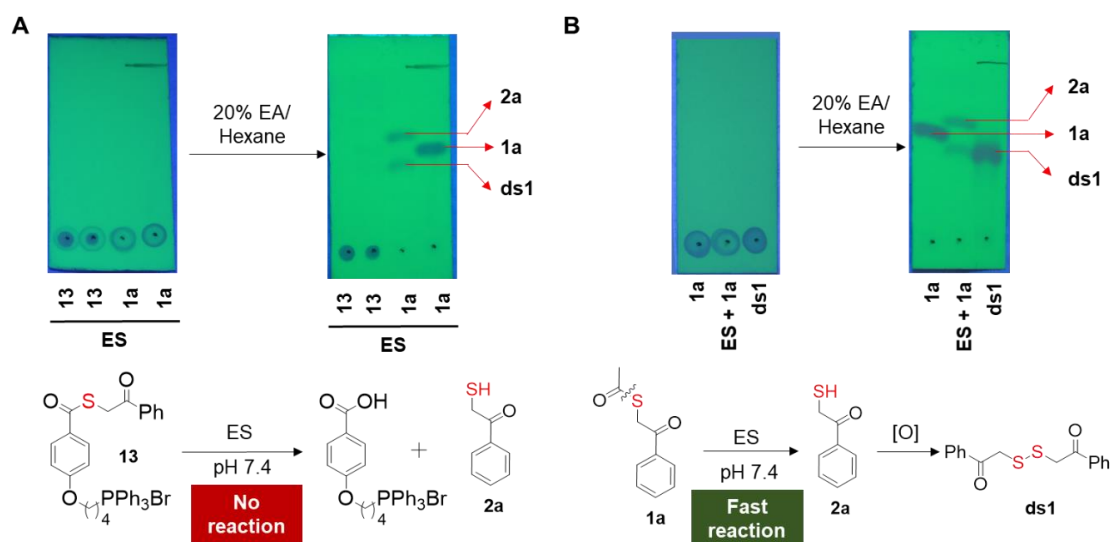


**Figure 3.4:** TLC experiment using 20% EA/Hexane as eluent. (A) **13** showed no decomposition with esterase after 1 h. (B) **1a** with esterase showed decomposition with esterase and formation of thiol (**2a**) and corresponding disulfide (**ds1**).

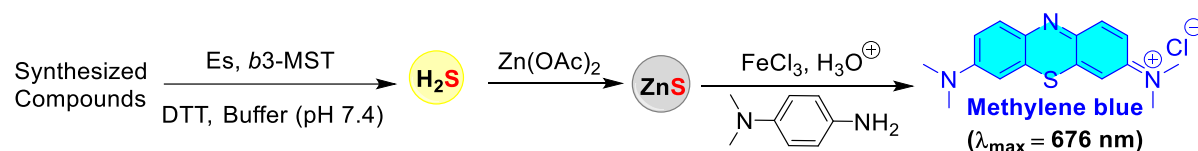
Compound **13** (100 μM) was incubated with esterase (1 U/mL) in pH 7.4 PBS (1×) at 37 °C for 1 h. Following this, DCM extraction was done and the DCM layer was spotted on a TLC plate. Along with this, a DCM layer obtained from **1a** + Es reaction was also spotted and TLC

was run in 20% ethyl acetate/hexane and the formation of **2a** was monitored. Under the experimental conditions, **13** remained largely unaffected and **2a** was not formed (Figure 3.4A). On the other hand, **1a** was able to produce **2a** in presence of esterase (Figure 3.4A and Figure 3.4B). Along with **2a**, a disulfide (**ds1**) is also formed by aerial oxidation of **2a**.

Overall, the data suggest that **13** was unable to generate **2a** in presence of esterase, therefore, it did not show significant H<sub>2</sub>S production (previously observed in the lead acetate experiment). Therefore, **13** was not used for further experiments and **7** was used as the lead compound.

### 3.3.4. Detection of H<sub>2</sub>S using methylene blue assay

Along with H<sub>2</sub>S detection for the mitochondria-targeted substrates *via* lead acetate assay, a methylene blue assay was carried out as described in Chapter 2.



**Scheme 3.5:** Detection of H<sub>2</sub>S *via* methylene blue assay.

In this assay, 200 μM of **1**, **7** and **9** were separately incubated with esterase (1 U/mL) with b3-MST (1 μM), Zn(OAc)<sub>2</sub> (400 μM) and DTT (10 mM) in pH 7.4 PBS (1×) at 37 °C for 2 h. Next, aliquots from the reaction mixtures were treated with methylene blue reagents and mixed. The final reaction mixture was then transferred to the 96-well plate and absorbance was recorded. It has been observed that **1a** has higher methylene blue absorbance of H<sub>2</sub>S compared to **7** (Figure 3.5). A lower yield of H<sub>2</sub>S from **7** indicates slower H<sub>2</sub>S production. Under similar conditions, negative control **9** did not produce H<sub>2</sub>S in presence of 3-MST. As anticipated, compound controls did not produce a significant amount of H<sub>2</sub>S in the absence of 3-MST.

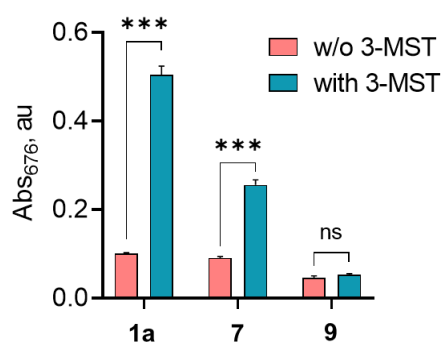


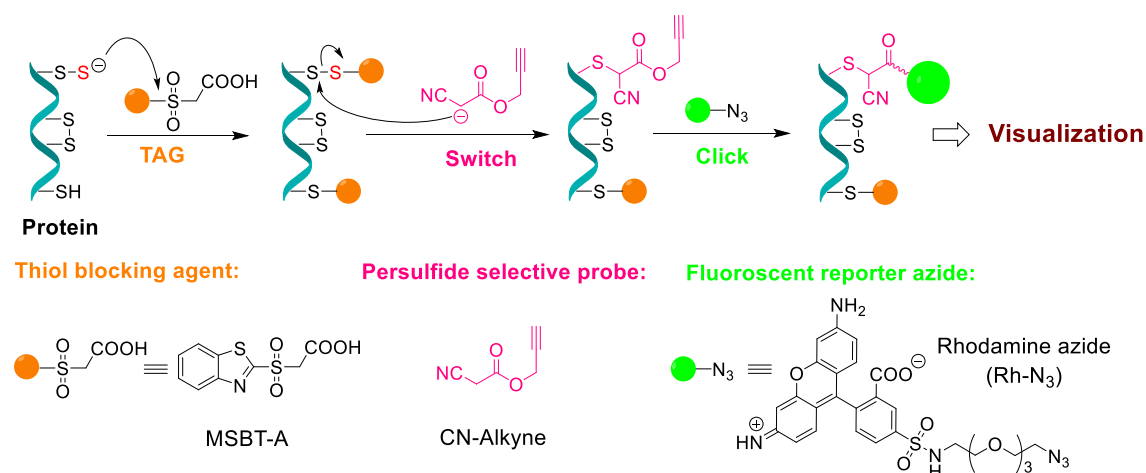
**Figure 3.5:** H<sub>2</sub>S generation by the mitochondria targeted 3-MST artificial substrates in presence of b3-MST. **1a**, **7**, **9** were separately incubated with b3-MST, esterase and DTT for 2 h. H<sub>2</sub>S generation was detected using methylene blue assay. Results are expressed as ± SD (n = 3) [(\*\*\*), *p* < 0.001 vs w/o 3-MST].

### 3.3.5. Detection of 3-MST persulfide

3-MST first reacts with its substrate **2a** and forms 3-MST persulfide which is one of the key steps in the process of H<sub>2</sub>S generation. Hence, it is crucial to detect 3-MST persulfide. However, the detection of a protein persulfidation is quite challenging due to the transient nature of cellular persulfides. A tag-switch method is often used to detect and quantify protein persulfidation.<sup>7,8</sup> In this method, free thiols and persulfides in a protein are first labeled with specific thiol-blocking agents (tag) called MSBT-A (Methyl sulfonyl benzothiazole acid). Then a nucleophilic probe (switch) connected with either biotin or a fluorescent tag is employed to react with persulfide specifically to give nucleophile-protein adduct. But this method consists of laborious synthesis of the probe, western blot transfer, and streptavidin-antibody-based visualization which increases the likelihood of experimental error. Hence, a robust, easy to use method is required in order to minimizing experimental errors. Thus, an alkyne-cyanoester-based nucleophilic probe specific for protein persulfide were designed. After the protein-probe adduct formation, free alkyne can potentially undergo an azide-alkyne-based click reaction with a reporter azide molecule. A fluorescent azide can be used and the fluorophore-protein adduct can be visualized using SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis). Similarly, a biotin azide can be used and biotinylated protein adducts can be recovered from the streptavidin column for detection and quantification of the persulfidated proteins. Hence, our proposed tools may enable for more multipurpose applications related to protein persulfidation which was lacking in the earlier methodologies.

#### 3.3.5.1. Development of clickable tag switch technique for detection of protein persulfide

The clickable tag switch assay was developed (with Dr. Pooja Kumari) to detect protein persulfidation (Scheme 3.6).



**Scheme 3.6:** Development of clickable tag switch assay for detection of protein persulfide.

We synthesized an alkyne probe (**CN-Alkyne**) and a blocking agent (**MSBT-A**) following a reported protocol.<sup>9,10</sup> In this assay, the protein persulfide can be blocked with **MSBT-A**. Then, the **CN-Alkyne** probe will be able to react with MSBT-tagged persulfide to label the persulfidated protein with a free alkyne handle. Next, a reported copper catalyzed click reaction can be performed with rhodamine azide to append a fluorophore tag on persulfidated protein.<sup>11</sup> After gel electrophoresis (SDS-PAGE), in-gel fluorescence is expected to be visualized.

Clickable tag switch assay was validated using 3-MST with its artificial substrate **1a**. 3-MST (15  $\mu$ M) was first incubated with **1a** (100  $\mu$ M) and esterase (1 U/mL) for 1 h to generate 3-MST persulfide. Then **MSBT-A** was added to block 3-MST persulfide. Next, a protease inhibitor (**PMSF**) was added to quench excess esterase. Then **CN-Alkyne** was added to label 3-MST persulfide. Next, a click reaction was performed with rhodamine azide. A SDS-PAGE was then carried out with the reaction mixtures. Finally, in-gel fluorescence imaging was done to detect 3-MST persulfide (Figure 3.6).

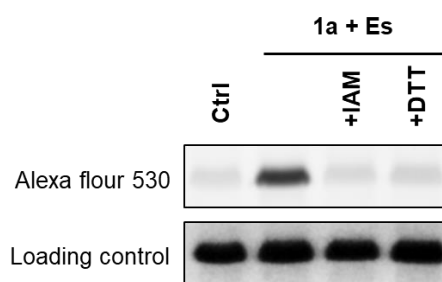


**Figure 3.6:** 3-MST persulfidation detection using a clickable tag switch assay. Compounds were incubated with 3-MST and esterase for 1 h. Coomassie brilliant blue (CBB) staining was used for loading control. Ctrl represents 3-MST only.

A similar procedure was followed where 3-MST was preincubated with iodoacetamide (IAM). IAM is an excellent thiol-blocking agent and is expected to block the active site cysteine residue of 3-MST. Therefore, 3-MST persulfide is not likely to be formed when pre-incubated of IAM followed by treatment with **1a** and esterase. On the other hand, dithiothreitol (DTT) was incubated after the reaction of **1a** with 3-MST and esterase. DTT, the persulfide was reduced by DTT, a reducing agent. Hence, the nucleophile probe may not be able to label the proteins. Consistent with our hypothesis, **1a** treated lane showed an intense band corresponding to 3-MST persulfide, whereas other controls did not show a significant level of 3-MST persulfide (Figure 3.6).

### 3.3.5.2. Detection of 3-MST persulfide with mito-substrates using clickable tag switch technique

After developing the clickable tag switch assay, further we looked for mitochondrial substrate (**7**) ability to generate 3-MST persulfide. 3-MST (15  $\mu$ M) was first incubated with **7** (25  $\mu$ M) and esterase for 2 h at 37 °C. Next, a similar clickable tag switch method was followed to detect 3-MST persulfide. For this experiment **1a** was used as a positive control and **9** was used as a negative control.

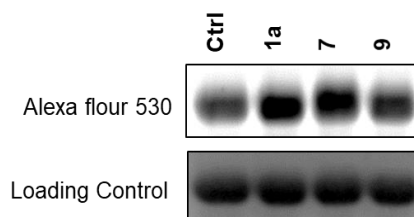
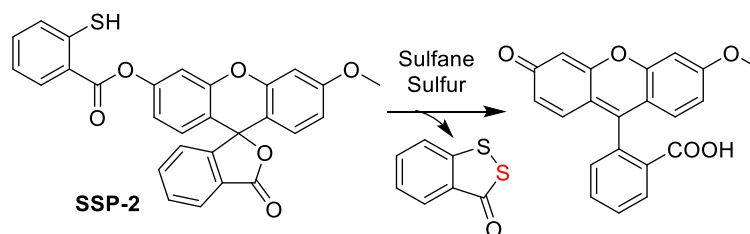


**Figure 3.7:** 3-MST persulfide detection using a clickable tag switch assay using mitochondria-targeted substrates. Compounds were incubated with 3-MST and esterase for 2 h. CBB staining was used for loading control. Ctrl represents 3-MST only.

It has been observed that similar to **1a**, **7** was able to form 3-MST persulfide significantly (Figure 3.7). As expected, negative control **9** did not show a significant 3-MST persulfide formation when compared with the treated protein.

### 3.3.6. Sulfane sulfur detection using SSP-2 assay

Persulfidated 3-MST can react with an excess amount of the substrate to generate sulfane sulfur which was detected using a reported SSP-2 probe (Scheme 3.7).<sup>12,13</sup> In a fluorescent-based assay, generated sulfane sulfur reacts with the SSP-2 probe and releases highly fluorescent fluorescein. This turn-on fluorescence enhancement arising from sulfane sulfur can be monitored using a plate reader.



**Scheme 3.7:** SSP-2 assay for the detection of sulfane sulfur. SSP-2 probe reacts with sulfane sulfur and generates highly fluorescent fluorescein.  $\lambda_{\text{ex}} = 482 \text{ nm}$  and  $\lambda_{\text{em}} = 518 \text{ nm}$ .

3-MST (15  $\mu\text{M}$ ) was incubated with **7** (25  $\mu\text{M}$ ) and esterase (0.5 U/ mL) for 1 h at 37  $^{\circ}\text{C}$ . Then, the SPP-2 probe was added and incubated for 10 min at 37  $^{\circ}\text{C}$ . It has been observed that, **7** was able to generate a significant amount of sulfane sulfur in presence of 3-MST (Figure 3.8). In another reaction, DTT was incubated after 1 h of **7** + Es + 3-MST incubation. As, DTT reduces persulfides, thereby a diminished level of fluorescent signal was observed. **9** was used as a negative control for this reaction. As expected, negative control and other controls did not show a significant sulfane sulfur formation.

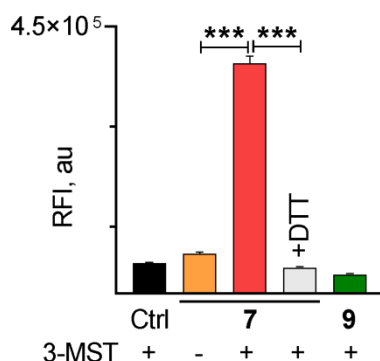
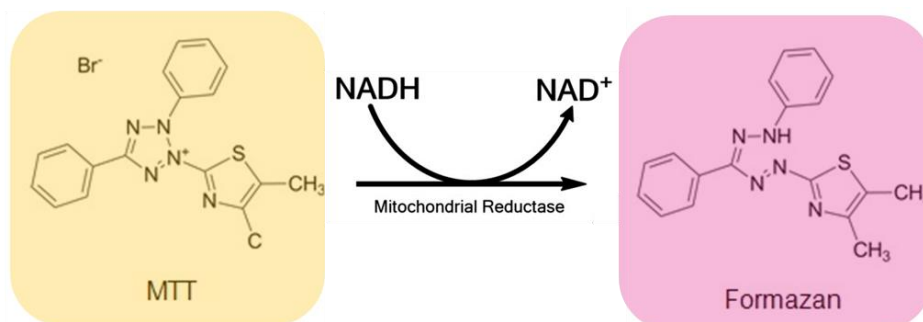


**Figure 3.8:** Sulfane sulfur detection using SSP-2 assay. Compounds were incubated with 3-MST and esterase for 1 h. Results are expressed as  $\pm$  SD ( $n = 3$ ) [(\*\*\*)  $p < 0.001$  vs ctrl]. +DTT represents the incubation of DTT with **7** + 3-MST + esterase. Ctrl represents 3-MST only.  $\lambda_{\text{ex}} = 482$  nm and  $\lambda_{\text{em}} = 518$  nm.

### 3.3.7. MTT assay for cell viability of the key compounds (**1a**, **7** and **9**)

The MTT assay is commonly used to determine the cell viability of the compounds. Live cells are known to release higher amounts of mitochondrial reductases than unhealthy/dead cells. These reductases in presence of NADH (Nicotinamide adenine dinucleotide hydrogen) react with the yellow-colored MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and gets converted to pink colored formazan dye (Scheme 3.8). The absorbance of the formazan dye was monitored at 570 nm to determine the cell viability.



**Scheme 3.8:** MTT assay for cell viability. Formation of pink-colored formazan dye from yellow-colored MTT in presence of cellular reductases and NADH.

Next, the cell viability of compounds **1a**, **7** and **9** was determined using aforementioned MTT assay. Mouse embryonic fibroblast (MEF) cells were treated with variable concentrations of **1a**, **7** and **9** and incubated for 24 h and finally, cell viability was measured. **7** was observed to be well tolerated up to 12.5  $\mu$ M and cells were viable up to 80% (Figure 3.9). **1a** and **9** were found to be well tolerated by the cells at different concentrations.

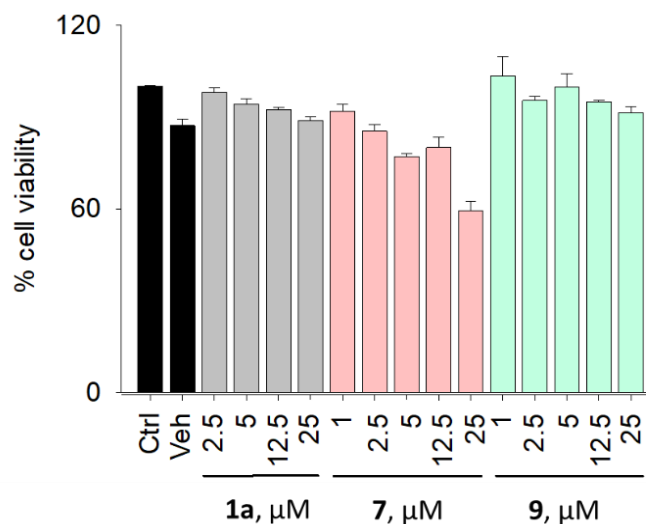
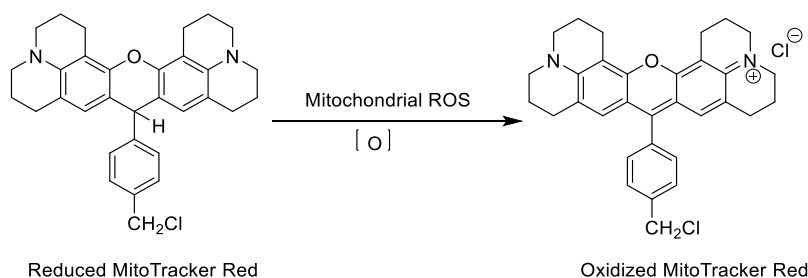


**Figure 3.9:** Cell viability assay carried out on MEF cells. The compounds **1a**, **7** and **9** were independently incubated with viable concentrations for 24 h and viable cells were determined using MTT assay. Veh = 0.5% DMSO. All data are presented as mean  $\pm$  SD (n = 3 per group).

### 3.3.8. Detection of mitochondrial H<sub>2</sub>S generation and mitochondrial membrane potential fluctuation in MEF cells using mitochondria-targeted 3-MST substrate

Having established H<sub>2</sub>S and sulfane sulfur generation in *in vitro* conditions, the effect of these species in cells were evaluated at non toxic concentrations of the compounds. H<sub>2</sub>S and sulfane sulfur known to enhance mitochondrial respiration thereby increasing mitochondrial membrane potential (MMP).<sup>14,15</sup> MMP is known to be an important biomarker that is often correlated with mitochondrial health.<sup>16</sup> An enhanced MMP indicates cells with good mitochondrial health with promoted respiration whereas a diminished MMP indicates unhealthy mitochondria with lowered mitochondrial respiration.<sup>16</sup> Therefore, to detect MMP change, a reported MitoTracker Red (MTR) dye often employed.<sup>17</sup> Once treated, a reduced form of MTR dye diffuses inside the cells and reach mitochondria where the dye get oxidized in the presence of mitochondrial ROS to form a highly fluorescent dye (Scheme 3.9).<sup>18</sup> Oxidized MTR dye with +ve charge is known to localize and label mitochondria.<sup>17,18</sup> Enhancement of fluorescent intensity was monitored to determine MMP fluctuation.<sup>18,19</sup>



**Scheme 3.9:** MitoTrackerRed (MTR) dye for studying change in mitochondrial membrane potential. Reduced MTR dye transforms into highly fluorescent oxidized MTR dye upon oxidation by mitochondrial ROS.

In order to study any effect on MMP, a control experiment was performed with varying concentrations (0, 1, 2, 3, 4 and 5  $\mu\text{M}$ ) of the compound **7** (In collaboration with Prof. Nagaraj Balasubramanian's lab, Department of Biology, IISER Pune). The experiment was carried out in wild type MEF cells using 20 nM of MTR dye for labelling mitochondria. Cells were then fixed using standard methods and finally, confocal microscopy was performed to image the cellular turn-on fluorescence. The red channel was used to image the cellular fluorescence by MTR. It has been observed that untreated cells failed to show any fluorescence change and a significant enhancement of the fluorescence signal was observed for the treated cells in a dose dependent manner (Figure 3.10A and 3.10B). This experiment suggests that compound **7** is likely promoting the mitochondrial respiration thereby showing the enhancement of the MMP.

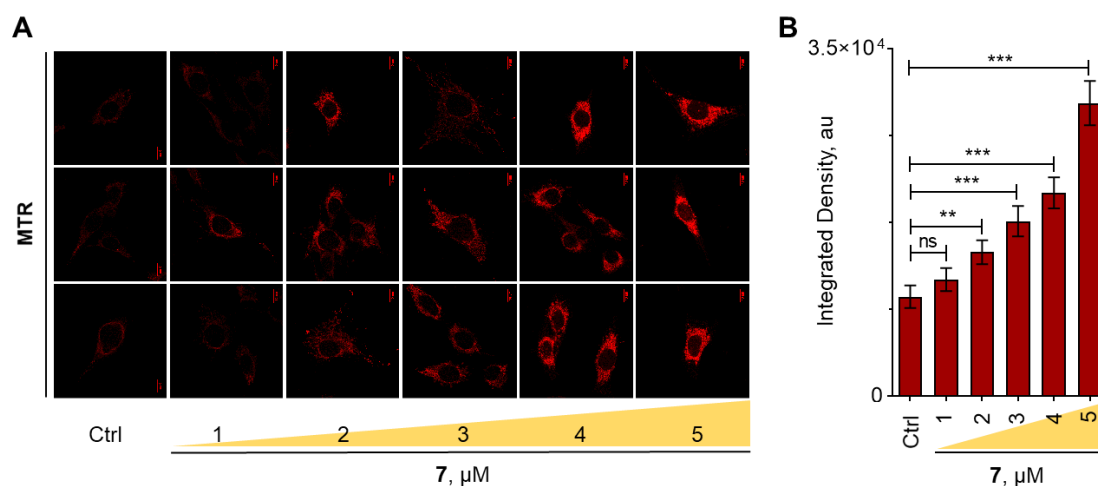


**Figure 3.10:** Mitochondrial membrane potential study with compound **7** in MTR labelled MEF cells. (A) Confocal imaging study showing dose dependent MMP enhancement when incubated with increasing concentrations of **7** (Scale bar: 10  $\mu\text{m}$ ). (B) Cellular MTR fluorescence signals corresponding to MMP were quantified using Image J software. Graph represents mean  $\pm$  SE ( $n = 3$  experiments per group). Statistical analysis was performed using Mann-Whitney test ( $***p < 0.001$ ,  $**p < 0.01$ ,  $*p < 0.05$ ).

As, a significant MMP alteration was observed for the concentration 2  $\mu\text{M}$  and onwards therefore further experiments was carried out with 2  $\mu\text{M}$  concentration.

Next, MMP fluctuation was studied using compound **7** along with control compounds (**1a** and **9**). MEF cells were co-incubated with 2  $\mu\text{M}$  of each compound. Then 20 nM of MTR dye was used to label mitochondria. Finally, a live cell confocal microscopy was performed to image the cellular turn-on fluorescence. Consistent with previous experiment, **7** was able to enhance the red fluorescence of MTR which indicates MMP was upregulated (Figure 3.11A and 3.11B). **1a** was not able to enhance MMP significantly due to lacking mitochondria targeting handle. As expected, negative control **9** which lacking of sulfur failed to enhance MMP.

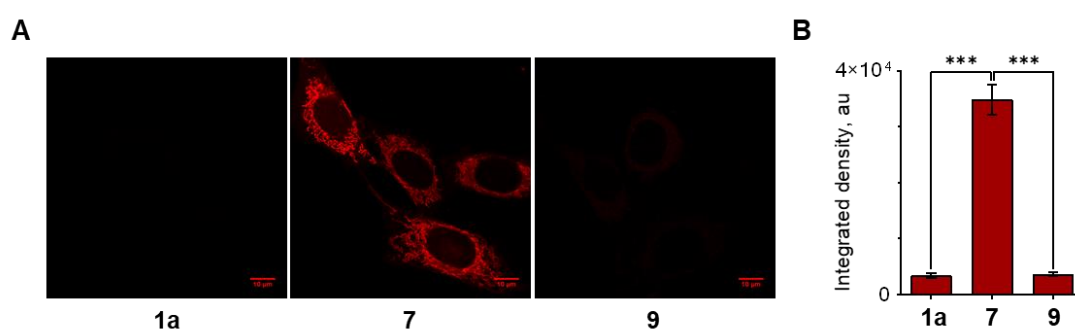
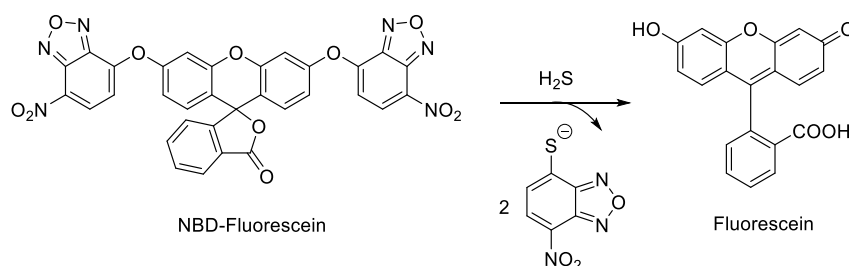


**Figure 3.11:** Mitochondrial membrane potential (MMP) study with compounds (**1a**, **7** and **9**) (A) Confocal microscopy images of MTR-labelled MEF cells showing the effect of compounds on MMP. Cells treated with **7** showed significant enhancement of red fluorescence signal (Scale bar: 10  $\mu\text{m}$ ). (B) Cellular MTR signal attributable to MMP was quantified using Image J software. All data is representative of 43 cells presented as mean  $\pm$  SEM ( $n = 3$  experiments per group). \*\*\* $p < 0.001$ .

Next, intracellular  $\text{H}_2\text{S}$  generation was studied in order to correlate the MMP change. Cellular  $\text{H}_2\text{S}$  is detected using a reported cell permeable NBD-fluorescein (NBD-F) dye.<sup>20</sup> In this assay,  $\text{H}_2\text{S}$  inside the cells reacts with NBD-F and releases fluorescent fluorescein (Scheme 3.10). Therefore, turn-on green fluorescence is monitored to determine the extent of  $\text{H}_2\text{S}$  release.



**Scheme 3.10:** NBD-fluorescein (NBD-F) dye for the detection of  $\text{H}_2\text{S}$ . NBD-F forms fluorescein upon reaction with  $\text{H}_2\text{S}$ .  $\lambda_{\text{ex}} = 482 \text{ nm}$  and  $\lambda_{\text{em}} = 518 \text{ nm}$ .

MEF cells were co-incubated with 2  $\mu$ M of each substrate (**1**, **7**, **9**) with NBD-F (10  $\mu$ M). Finally, live cell confocal microscopy was performed to image the cellular turn-on fluorescence. The green channel was used to image turn on fluorescence by NBD-F. Cells exposed to **7** gave an intense turn-on green fluorescence in the periphery of the mitochondria that indicates a mitochondrion localized H<sub>2</sub>S release by **7** (Figure 3.12A and 3.12B). As expected, **1a** and **9** failed to enhance H<sub>2</sub>S level significantly.

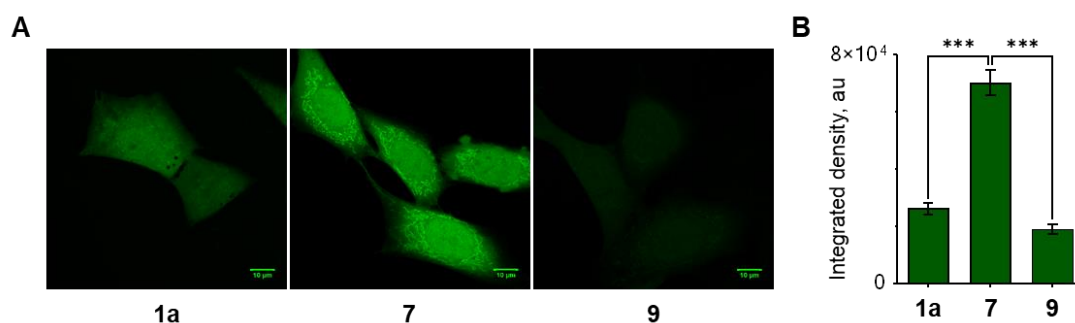


**Figure 3.12:** Mitochondrial H<sub>2</sub>S detection with compounds (**1a**, **7** and **9**) (A) Confocal microscopy images of NBD-F treated MEF cells showing H<sub>2</sub>S release in mitochondria. Cells treated with **7** showed significant enhancement of green fluorescence signal (Scale bar: 10  $\mu$ m). (B) Cellular green fluorescence signal attributable to H<sub>2</sub>S generation was quantified using Image J software. All data was representative of 43 cells presented as mean  $\pm$  SEM (n = 3 experiments per group). \*\*\* $p$  < 0.001.

Next, a co-localization analysis was performed to check whether H<sub>2</sub>S production and MMP enhancement were at the same site or not. A Mander's coefficient of co-localization was calculated and a very good correlation (Mean  $R$  = 0.89) was obtained which suggests H<sub>2</sub>S production and MMP enhancement were in a co-localized manner in the mitochondria (Figure 3.13A and 3.13B).

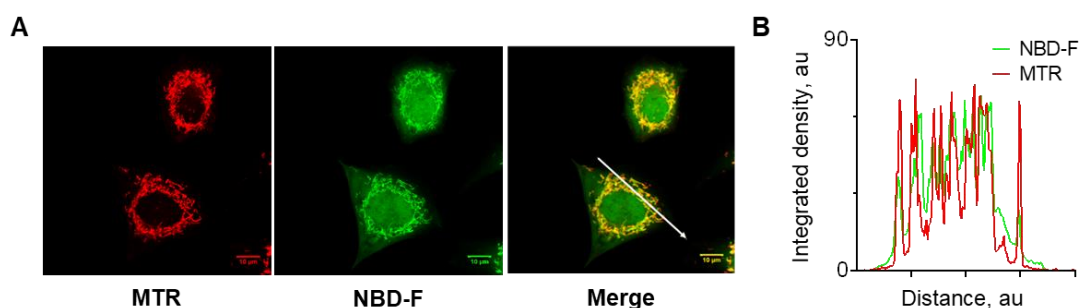


**Figure 3.13:** Confocal microscopy images of MEF cells (A) Co-localization of H<sub>2</sub>S release detected using NBD-F and MMP enhancement by **7** (Scale bar: 10  $\mu$ m). (B) A line plot (fluorescence intensity vs. distance) show a good overlap between NBD-F and MMP fluorescence.

Overall the data suggest that **7** is a mitochondria-targeted 3-MST substrate which specifically localized into mitochondria where it gets activated by the intracellular esterase or cellular thiols and releases active substrate. Active substrate upon 3-MST turnover releases H<sub>2</sub>S and enhance MMP. At the same time a cytosolic substrate **1a** and a negative control **9** failed to show such effect as former is not specifically targeted to mitochondria and later though targeted to mitochondria lacking of a sulfur.

### 3.4. Summary

To summarize, mitochondria-targeted artificial substrates of 3-MST (**7** and **13**) were designed and synthesized. Then, H<sub>2</sub>S release was monitored for both substrates using lead acetate and methylene blue assay. Compound **13** was unable to generate any significant amount of H<sub>2</sub>S as it did not get activated by esterase. Under similar condition, **7** was able to generate a significant amount of H<sub>2</sub>S as a result of esterase activation followed by a turnover by 3-MST in the presence of DTT. A clickable tag-switch assay was developed to detect 3-MST persulfide. Furthermore, using the same assay, 3-MST persulfide formation with **7** was also detected. Formation of sulfane sulfur from **7** in presence of 3-MST was observed using an SSP-2 probe. Next, cell viability of mitochondria-targeted compounds was evaluated using an MTT assay. Cell viability data showed that these substrates were benign under experimental concentrations. A significant perturbation in the MMP was observed for compound **7** which was consistent with H<sub>2</sub>S and possible sulfane sulfur generation in mitochondria. Collectively, our data suggest that **7** is a mitochondria-targeted artificial substrate of 3-MST which is specifically localizes in mitochondria and increases MMP. Having this tool in hand, novel modes of 3-MST activity can be uncovered in mitochondria which may provide additional control over 3-MST-related diseases.

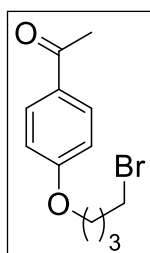
### 3.5. Experimental procedures

#### 3.5.1. Synthesis and characterization of compounds

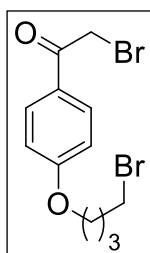
**General methods:** All reactions were conducted under a nitrogen atmosphere. All the chemicals and solvents were purchased from commercial sources and used as received unless stated otherwise. Column chromatography was performed using silica gel - Rankem (60–120 mesh) or silica gel Spectrochem (100-200 mesh) as the stationary phase.  $^1\text{H}$  NMRs were recorded on a JEOL 400 MHz (100 MHz for  $^{13}\text{C}$  or 162 MHz for  $^{31}\text{P}$ ) or a Bruker 400 MHz (100 MHz for  $^{13}\text{C}$  or 162 MHz for  $^{31}\text{P}$ ) spectrometer unless otherwise specified using either residual solvent signals  $\text{CDCl}_3$  ( $\delta_{\text{H}} = 7.26$  ppm,  $\delta_{\text{C}} = 77.2$  ppm). Chemical shifts ( $\delta$ ) are reported in ppm and coupling constants ( $J$ ) in Hz. The following abbreviations are used: m (multiplet), s (singlet), t (triplet), p (pentet) and h (sextet). 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.

Compounds **1a**,<sup>21</sup> **ds1**,<sup>22</sup> **CN-alkyne**<sup>9</sup> and **MSBT-A**<sup>10</sup> were synthesized as per their respective protocols that were earlier reported and the analytical data matches with the reported values.

**1-(4-(4-bromobutoxy)phenyl)ethan-1-one (4):** 4-Hydroxyacetophenone (1 g, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in anhydrous *N,N*-dimethylformamide (DMF) (15 mL), followed by subsequent addition of  $\text{K}_2\text{CO}_3$  (3.05 g, 3 eq) and 1,4-dibromobutane (0.97 mL, 3 eq). The reaction mixture was stirred at room temperature (RT) for 6 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 1N HCl (20 mL) and transferred to a separating funnel, where the product was extracted with EtOAc (20 mL). The organic layer was washed with brine (3×20 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was concentrated under reduced pressure to afford the crude product which was purified by silica gel column chromatography (8-10% ethyl acetate/hexane) using 60-120 mesh silica gel as the stationary phase. **4** was obtained as 1.10 g (55%). All analytical data are consistent with reported values.<sup>23</sup>

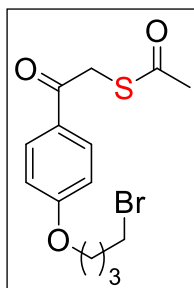


1-(4-(4-bromobutoxy)phenyl)ethan-1-one (**5**): *N*-bromosuccinimide (973 mg, 1.5 eq) and *p*-toluenesulfonic acid (317 mg, 0.5 eq) were taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL anhydrous dichloromethane (DCM). A solution of **4** (1 g, 1 eq) in 10 mL anhydrous DCM was added dropwise; the reaction mixture was then refluxed for 3 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of H<sub>2</sub>O and the product was extracted in a separating funnel with DCM (2×15 mL). The organic layer was washed with 30 mL brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate was concentrated under reduced pressure to afford the crude product which was purified by silica gel column chromatography (6-7% ethyl acetate/hexane) using 60-120 mesh silica as the stationary phase.



Yield (1.10 g, 85%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.94-7.92 (m, 2H), 6.93-6.90 (m, 2H), 4.40 (s, 2H), 4.08 (t, 2H, *J* = 5.9 Hz), 3.49 (t, 3H, *J* = 6.4 Hz), 2.12-2.05 (m, 2H), 2.02-1.95 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 190.1, 163.6, 131.5, 127.1, 114.4, 67.4, 33.4, 30.8, 29.4, 27.8; HRMS for C<sub>12</sub>H<sub>15</sub>Br<sub>2</sub>OS (M+H)<sup>+</sup> Calculated: 348.9439, Found: 348.9424

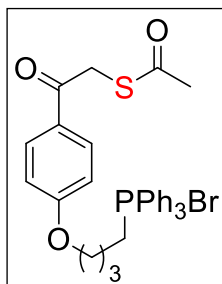
*S*-(2-(4-(4-bromobutoxy)phenyl)-2-oxoethyl) ethanethioate (**6**): Compound **5** (390 mg, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in anhydrous DMF (20 mL), followed by the addition of potassium thioacetate (KSAc) (1 g, 1.1 eq). The reaction mixture was stirred at room temperature (RT). Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of water and the crude product was extracted in a separating funnel 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<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate was concentrated under reduced pressure to afford the crude product, which was further purified by silica gel column chromatography (14-15% ethyl acetate/hexane) using 60-120 mesh silica as the stationary phase.



Yield (295 mg, 78%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.98-7.96 (m, 2H), 6.94-7.92 (m, 2H), 4.36 (s, 2H), 4.07 (t, 2H, *J* = 6.0 Hz), 3.49 (t, 2H, *J* = 6.5 Hz), 2.4 (s, 3H), 2.1-2.04 (m, 2H), 2.02-1.95 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 194.6, 191.9, 163.4, 131.1, 128.7, 114.5, 67.3, 36.5, 33.4, 30.4, 29.4, 27.8; HRMS for C<sub>14</sub>H<sub>18</sub>BrO<sub>3</sub>S (M+H)<sup>+</sup> Calculated: 345.0160, Found: 345.0145.

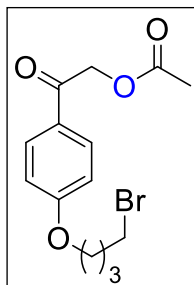
*S*-(2-(4-(4-(bromotriphenyl-15-phosphaneyl)butoxy)phenyl)-2-oxoethyl) ethanethioate (**7**): Compound **6** (230 mg, 1 eq) was taken in a round-bottom flask, purged with nitrogen and

dissolved in 10 mL anh. acetonitrile (ACN), followed by the addition of triphenylphosphine (524 mg, 3 eq). The reaction mixture was stirred at 100 °C for 72 h. Upon completion of the reaction as monitored by TLC, the solvent was evaporated under reduced pressure to afford the crude product, which was further purified by silica gel column chromatography (6-8% methanol/chloroform) using 60-120 mesh silica as the stationary phase.



Yield (290 mg, 72%). FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1677;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.95-7.93 (m, 2H), 7.88-7.76 (m, 9H), 7.69-7.65 (m, 6H), 6.90-6.88 (m, 2H), 4.34 (s, 2H), 4.21 (t, 2H,  $J = 5.6$  Hz), 4.02-3.95 (m, 2H), 2.4 (s, 3H), 2.30-2.08 (p, 2H,  $J = 6.2$  Hz), 1.91-1.82 (h, 2H,  $J = 7.7$  Hz);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  194.5, 191.9, 163.4, 135.1 (d,  $J = 2$  Hz), 133.9 (d,  $J = 10$  Hz), 130.06 (d,  $J = 13$  Hz), 131.0, 128.6, 118.5 (d,  $J = 86$  Hz), 114.6, 67.0, 36.5, 30.4, 29.2 ( $J = 17$  Hz), 22.0 ( $J = 50$  Hz), 19.3 ( $J = 3$  Hz);  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ ):  $\delta$  24.6; HRMS for  $[\text{C}_{32}\text{H}_{32}\text{O}_3\text{PS}]^+$  Calculated: 527.1810, Found: 527.1811.

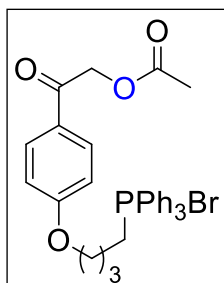
2-(4-(4-bromobutoxy)phenyl)-2-oxoethyl acetate (**8**): Compound **5** (300 mg, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in 100 mL anh. DMF, followed by the addition of potassium acetate (NaOAc) (211 mg, 3 eq). The reaction mixture was stirred at room temperature (RT) for 30 min. Upon completion of the reaction as monitored by TLC, the reaction was quenched with 30 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 ( $3 \times 30$  mL) and brine (30 mL) and the resulting organic layer was dried over anh.  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was concentrated under reduced pressure to afford the crude product, which was further purified by silica gel column chromatography (14-15% ethyl acetate/hexane) using 60-120 mesh silica as the stationary phase.



Yield (255 mg, 90%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.90-7.88 (m, 2H), 6.95-6.93 (m, 2H), 5.30 (s, 2H), 4.07 (t, 2H,  $J = 6.1$  Hz), 3.49 (t, 3H,  $J = 6.5$  Hz), 2.23 (s, 3H), 2.12-2.05 (m, 2H), 2.02-1.95 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  190.7, 170.6, 163.5, 130.2, 127.4, 114.6, 67.3, 65.9, 33.3, 29.4, 27.8, 20.8; HRMS for  $\text{C}_{14}\text{H}_{18}\text{BrO}_4$  ( $\text{M}+\text{H}$ ) $^+$  Calculated: 329.0388, Found: 329.0398.

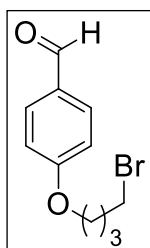
2-(4-(4-(bromotriphenyl-*l*-phosphaneyl)butoxy)phenyl)-2-oxoethyl acetate (**9**): Compound **8** (210 mg, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL anh. ACN, followed by the addition of triphenylphosphine (502 mg, 3 eq). The reaction

mixture was stirred at 100 °C for 72 h. Upon completion of the reaction as monitored by TLC, the solvent was evaporated under reduced pressure to afford the crude product, which was further purified by silica gel column chromatography (6-7% methanol/chloroform) using 100-200 mesh silica as the stationary phase.



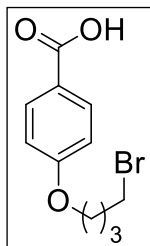
Yield (350 mg, 93%). FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1745, 1687;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.88-7.76 (m, 11H), 7.70-7.65 (m, 6H), 6.93-6.90 (m, 2H), 5.29 (s, 2H), 4.21 (t, 2H,  $J = 5.7$  Hz), 4.97-3.94 (m, 2H), 2.30-2.08 (m, 2H), 2.22 (t, 3H), 1.90-1.82 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  190.8, 170.6, 163.3, 135.1 (d,  $J = 3$  Hz), 133.7 (d,  $J = 10$  Hz), 130.6 (d,  $J = 12$  Hz), 130.2, 127.2, 118.3 (d,  $J = 85$  Hz), 114.7, 67.0, 65.9, 29.2 ( $J = 17$  Hz), 22.2 ( $J = 50$  Hz), 20.8, 19.2 ( $J = 3$  Hz);  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ ):  $\delta$  24.5; HRMS for  $[\text{C}_{32}\text{H}_{32}\text{O}_4\text{P}]^+$  Calculated: 511.2038, Found: 511.2043.

4-(4-bromobutoxy)benzaldehyde (**10**): 4-hydroxybenzaldehyde (2 g, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in 20 mL anh. DMF, followed by subsequent addition of  $\text{K}_2\text{CO}_3$  (6.78 g, 3 eq) and 1,4-dibromobutane (5.9 mL, 3 eq). The reaction mixture was stirred at RT for 2 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 1N HCl (40 mL) and transferred to a separating funnel, where the product was extracted with EtOAc (40 mL). The organic layer was washed with brine (3×30 mL), dried over anh.  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was concentrated under reduced pressure to afford the crude product which was purified by silica gel column chromatography (10-12% ethyl acetate/hexane) using 60-120 mesh silica gel as the stationary phase. **10** was obtained as 2.40 g (57%). All analytical data are consistent with reported values.<sup>24</sup>

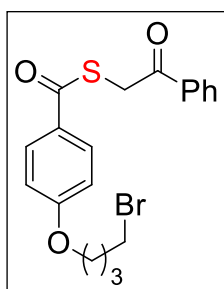


4-(4-bromobutoxy)benzoic acid (**11**): Compound **10** was taken in a round-bottom flask, purged with nitrogen and dissolved in *t*-butanol (30 mL), followed by subsequent addition of 50%  $\text{H}_2\text{O}_2$  (1.65 mL, 5 eq). Then  $\text{NaClO}_2$  (1.90 g, 3 eq) dissolved in 10 mL 0.67 M  $\text{KH}_2\text{PO}_4$  was added and the reaction mixture was stirred at RT for 2 h. Upon completion of the reaction, as

monitored by TLC, *t*-butanol was removed under reduced pressure. Then, the reaction was quenched with 1N HCl (30 mL) and transferred to a separating funnel, where the product was extracted with EtOAc (30 mL). The organic layer was washed with brine (30 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was concentrated under reduced pressure to afford the crude product which was purified by silica gel column chromatography (20-22% ethyl acetate/hexane) using 60-120 mesh silica gel as the stationary phase. **11** was obtained as 1.65 g (84%). All analytical data are consistent with reported values.<sup>25</sup>



*S*-(2-oxo-2-phenylethyl) 4-(4-bromobutoxy)benzothioate (**12**): Compound **11** (250 mg, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL anhydrous DCM. DCC (283 mg, 1.5 eq) and DMAP (56 mg, 0.5 eq) were added to the mixture at 0 °C. Then freshly synthesized phenacyl thiol (209 mg, 1.5 eq) was dissolved in anhydrous DCM and added dropwise to the reaction and stirred at 0 °C for 3 h. Upon completion of the reaction, as monitored by TLC, the reaction mixture was filtered through a sintered funnel. The filtrate was concentrated under reduced pressure to afford the crude product which was purified by silica gel column chromatography (10-12% ethyl acetate/hexane) using 60-120 mesh silica gel as the stationary phase.

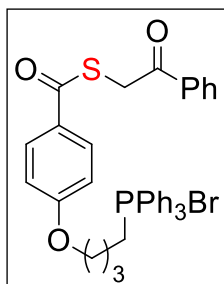


Yield (240 mg, 64%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.07-8.05 (m, 2H), 7.99-7.96 (m, 2H), 7.63-7.59 (m, 1H), 7.53-7.48 (m, 2H), 6.93-6.91 (m, 2H), 4.57 (s, 2H), 4.09 (t, 2H,  $J = 5.9$  Hz), 3.49 (t, 2H,  $J = 6.4$  Hz), 2.11-2.06 (m, 2H), 2.02-1.94 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  193.8, 188.9, 163.5, 135.8, 133.8, 129.9, 129.3, 128.9, 128.3, 114.4, 67.3, 36.6, 33.4, 29.4, 27.8; HRMS for  $\text{C}_{19}\text{H}_{20}\text{BrO}_3\text{S}$  ( $\text{M}+\text{H}$ )<sup>+</sup> Calculated: 429.0136,

Found: 429.0126.

*S*-(2-oxo-2-phenylethyl) 4-(4-(bromotriphenyl-*l*-5-phosphaneyl)butoxy)benzothioate (**13**): Compound **12** (90 mg, 1 eq) was taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL anhydrous ACN, followed by the addition of triphenylphosphine (174 mg, 3 eq). The reaction mixture was stirred at 100 °C for 72 h. Upon completion of the reaction as

monitored by TLC, the solvent was evaporated under reduced pressure to afford the crude product, which was further purified by silica gel column chromatography (7-8% methanol/chloroform) using 60-120 mesh silica as the stationary phase.



Yield (106 mg, 72%). FT-IR ( $\nu_{\max}$ ,  $\text{cm}^{-1}$ ): 1688, 1654;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.04-8.06 (m, 2H), 7.94-7.2 (m, 2H), 7.88-7.76 (m, 9H), 7.69-7.59 (m, 7H), 7.52-7.48 (m, 2H), 6.87-6.85 (m, 2H), 4.57 (s, 2H), 4.09 (t, 2H,  $J = 5.9$  Hz), 3.49 (t, 2H,  $J = 6.4$  Hz), 2.11-2.06 (m, 2H), 2.02-1.94 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  193.8, 188.8, 163.4, 135.8, 135.1 (d,  $J = 3$  Hz), 133.8 (d,  $J = 10$  Hz), 133.7, 130.6 (d,  $J = 12$  Hz), 129.8, 128.9, 128.7, 118.4 (d,  $J = 85$  Hz), 114.4, 67.0, 36.5, 29.1 (d,  $J = 17$  Hz), 22.1 (d,  $J = 50$  Hz), 19.3 (d,  $J = 4$  Hz);  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ ):  $\delta$  24.6; HRMS for  $[\text{C}_{37}\text{H}_{34}\text{O}_3\text{PS}]^+$  Calculated: 589.1966, Found: 589.1968.

### 3.5.2. Lead Acetate assay for the detection of $\text{H}_2\text{S}$

Firstly, lead acetate soaked paper was prepared. Whatman filter papers were soaked with 5% (w/v) lead acetate solution and dried. 285  $\mu\text{M}$  stock of wt 3-MST was used for lead acetate assay. Stocks of all compounds (**1a**, **7**, **9** and **13**) (10 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) and dithiothreitol (DTT, 100 mM) were prepared in deionised water.

Lead acetate assay was performed in a 96 well plate (with lid). The reaction samples were prepared by sequentially adding 200  $\mu\text{M}$  of test compounds (**1a**, **7**, **9** and **13**) (20  $\mu\text{L}$ , 10 mM), 1 U/mL of Esterase (2  $\mu\text{L}$ , 100 U/mL), 1  $\mu\text{M}$  of wt 3-MST (0.7  $\mu\text{L}$ , 285  $\mu\text{M}$  stock) and 10 mM of DTT (20  $\mu\text{L}$ , 100 mM stock). The final volume was adjusted to 200  $\mu\text{L}$  using 1 $\times$  phosphate buffer saline (PBS) pH 7.4 buffer, covered with 5% lead acetate soaked paper followed by the lid, sealed and incubated at 37  $^\circ\text{C}$ .

A similar protocol was followed for the substrate controls. Briefly, the reaction samples were prepared by sequentially adding 200  $\mu\text{M}$  of test compounds (**1a**, **7**, **9** and **13**) (20  $\mu\text{L}$ , 10 mM), 1 U/mL Esterase (2  $\mu\text{L}$ , 100 U/mL) and 10 mM DTT (20  $\mu\text{L}$ , 100 mM stock). The final volume was adjusted to 200  $\mu\text{L}$  using 1 $\times$  PBS pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37  $^\circ\text{C}$ .

A similar method was followed to detect  $\text{H}_2\text{S}$  at various concentrations of **7** (200, 150, 100, 50, 25  $\mu\text{M}$ ).

At predetermined time points, lead acetate paper was carefully taken out and image was taken using Syngene G-Box Chemi-XRQ.

### 3.5.3. TLC-based assay to study the decomposition of **13** in presence of esterase

Stock solutions of **1a** (10 mM), **13** (10 mM), and **ds1** (10 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) was prepared in deionized water. The reaction samples were prepared by sequentially adding 100  $\mu$ M of **1a** (10  $\mu$ L, 10 mM) or **13** (10  $\mu$ L, 10 mM) and 1 U/mL Esterase (10  $\mu$ L, 100 U/mL). The volume was adjusted to 1 mL with 1 $\times$  phosphate buffer saline (PBS) pH 7.4 buffer. Along with this, 100  $\mu$ M of **ds1** (10  $\mu$ L, 10 mM) with 990  $\mu$ L 1 $\times$  PBS pH 7.4 buffer. Finally, the reaction mixtures were incubated at 37  $^{\circ}$ C at 300 rpm for 1 h. At predetermined time points, 300  $\mu$ L aliquots were taken and extracted with 30  $\mu$ L DCM. Then 10  $\mu$ L of DCM extract was spotted on a TLC plate and run using 20% ethyl acetate/hexane as a mobile phase.

### 3.5.4. Methylene blue assay for the detection of H<sub>2</sub>S

The methylene blue assay was conducted as previously reported with some modifications.<sup>26,27</sup> Briefly, 285  $\mu$ M stock of wt 3-MST was used for the methylene blue assay. 10 mM stocks of all compounds (**1a**, **7**, **9**) were prepared in DMSO. Porcine liver esterase (100 U/mL), dithiothreitol (DTT, 100 mM) and Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (40 mM) were prepared in deionized water. A stock solution of FeCl<sub>3</sub> (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  $\mu$ M Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (15  $\mu$ L, 40 mM stock), 200  $\mu$ M of test compounds (**1a**, **7**, **9**) (20  $\mu$ L, 10 mM), 1  $\mu$ M wt 3-MST (3.5  $\mu$ L, 285  $\mu$ M stock) and 10 mM DTT (100  $\mu$ L, 100 mM stock). The final volume was adjusted to 1 mL using 200 mM 1 $\times$  PBS pH 7.4 buffer and incubated at 37  $^{\circ}$ C at 300 rpm.

A similar protocol was followed for the substrate controls. Briefly, the reaction samples were prepared by sequentially adding 400  $\mu$ M Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (10  $\mu$ L, 40 mM stock), 200  $\mu$ M of test compounds (**1a**, **7**, **9**) (20  $\mu$ L, 10 mM), 10 mM DTT (100  $\mu$ L, 100 mM stock). The final volume was adjusted to 1 mL using 200 mM 1 $\times$  PBS pH 7.4 buffer and incubated at 37  $^{\circ}$ C at 300 rpm.

At predetermined time points, equal volumes (200  $\mu$ L) of FeCl<sub>3</sub>, DMPPDA and aliquots from the above reaction samples were mixed and the resulting solution was incubated at 37 °C for 30 min in dark, to allow the formation of the methylene blue dye. An aliquot of 150  $\mu$ L was transferred to a 96-well plate and the absorbance values were recorded at 676 nm using a microplate reader (Thermo Scientific VarioskanFlash).

### 3.5.5. Clickable Tag-switch assay for S-sulfhydration with 3-MST

#### 3.5.5.1. Development of Clickable Tag-switch assay for S-sulfhydration with 3-MST

Stock solutions of **1a** (10 mM), MSBT-A (200 mM) and CN-Alkyne (200 mM) were prepared in DMSO. 100 mM PMSF was prepared in isopropanol. A stock of 285  $\mu$ M 3-MST was used. Click reaction stocks: TBTA (1.7 mM in DMSO), CuSO<sub>4</sub>·5H<sub>2</sub>O (50 mM in DI water), TCEP (50 mM in PBS buffer), Rhodamine azide (10 mM in DMSO).

To 15  $\mu$ M 3-MST (5.2  $\mu$ L, 285  $\mu$ M), 200  $\mu$ M of **1a** (2  $\mu$ L, 10 mM) or **5** (2  $\mu$ L, 10 mM) were added, volume was adjusted to 100  $\mu$ L by Tris buffer (pH 8.0) and incubated for 60 min. After that 5 mM MSBT-A (2.5  $\mu$ L, 200 mM) and 5  $\mu$ L 10% SDS were added and incubated for 30 min. Then 500  $\mu$ M PMSF (0.5  $\mu$ L, 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu$ L, 200 mM) was added and incubated for 1 h.

A reaction was set up for DTT control, a stock of 25 mM DTT was made in DI water. To 15  $\mu$ M 3-MST (5.2  $\mu$ L, 285  $\mu$ M), 200  $\mu$ M of **1a** (2  $\mu$ L, 10 mM) or **5** (2  $\mu$ L, 10 mM) were added, volume was adjusted to 100  $\mu$ L by Tris buffer (pH 8.0) and incubated for 60 min. Then, 250  $\mu$ M of DTT (1  $\mu$ L, 25 mM) was added and incubated for 1 h. After that 5 mM MSBT-A (2.5  $\mu$ L, 200 mM) and 5  $\mu$ L 10% SDS were added and incubated for 30 min. Then 500  $\mu$ M PMSF (0.5  $\mu$ L, 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu$ L, 200 mM) was added and incubated for 1 h.

Another reaction was set up for Iodoacetamide (IAM) control, a stock of 100 mM of IAM was made in DI water. To 15  $\mu$ M 3-MST (5.2  $\mu$ L, 285  $\mu$ M), 1 mM of IAM (1  $\mu$ L, 100 mM) was added, and volume was adjusted to 100  $\mu$ L by Tris buffer (pH 8.0) and incubated for 60 min. Then, 200  $\mu$ M of **1a** (2  $\mu$ L, 10 mM) was added and incubated for 1 h. After that 5 mM MSBT-A (2.5  $\mu$ L, 200 mM) and 5  $\mu$ L 10% SDS were added and incubated for 30 min. Then 500  $\mu$ M PMSF (0.5  $\mu$ L, 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu$ L, 200 mM) was added and incubated for 1 h.

Finally, after the reaction Click reaction cocktail: 6  $\mu\text{L}$  TBTA (from 1.7 mM stock), 2  $\mu\text{L}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (from 50 mM stock), 2  $\mu\text{L}$  TCEP (from 50 mM stock), 1  $\mu\text{L}$  Rhodamine azide (from 10 mM stock) was added to each. The reaction was stirred at 25  $^\circ\text{C}$  for 1 h. After completion of the reaction, 40  $\mu\text{L}$  of loading dye was added. For enzyme control, similar experiment was performed with DMSO in the place of compounds. Finally, the samples (40  $\mu\text{L}$ ) were loaded in a gel and SDS-PAGE was performed (run at 150 V for 1 h), the in-gel fluorescence image was taken using Syngene G-Box Chemi-XRQ.

### 3.5.5.2. Clickable Tag-switch assay for 3-MST persulfide detection

To 15  $\mu\text{M}$  3-MST (5.2  $\mu\text{L}$ , 285  $\mu\text{M}$ ), 25  $\mu\text{M}$  of **1a** (0.25  $\mu\text{L}$ , 10 mM) or **7** (0.25  $\mu\text{L}$ , 10 mM) or **9** (0.25  $\mu\text{L}$ , 10 mM) was added. 1 U/mL esterase (1  $\mu\text{L}$ , 100 U/mL) was added to individual reaction mixtures, volume was adjusted to 100  $\mu\text{L}$  by Tris buffer (pH 8.0) and incubated for 1 h. After that 5 mM MSBT-A (2.5  $\mu\text{L}$ , 200 mM) and 5  $\mu\text{L}$  10% SDS were added and incubated for 30 min. Then 500  $\mu\text{M}$  PMSF (0.5  $\mu\text{L}$ , 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu\text{L}$ , 200 mM) was added and incubated for 1 h.

A reaction was set up for enzyme control. To 15  $\mu\text{M}$  3-MST (5.2  $\mu\text{L}$ , 285  $\mu\text{M}$ ), 0.25  $\mu\text{M}$  of DMSO and 1 U/mL esterase (1  $\mu\text{L}$ , 100 U/mL) were added. The reaction volume was adjusted to 100  $\mu\text{L}$  by Tris buffer (pH 8.0) and incubated for 60 min. After that 5 mM MSBT-A (2.5  $\mu\text{L}$ , 200 mM) and 5  $\mu\text{L}$  10% SDS were added and incubated for 30 min. Then 500  $\mu\text{M}$  PMSF (0.5  $\mu\text{L}$ , 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu\text{L}$ , 200 mM) was added and incubated for 1 h.

Finally, with all the reactions, a similar copper-catalyzed rhodamine azide-based click reaction was performed. Finally, the samples (20  $\mu\text{L}$ ) were loaded in a gel and SDS-PAGE was performed (run at 150 V for 1 h), the in-gel fluorescence image was taken using Syngene G-Box Chemi-XRQ.

### 3.5.6. Sulfane sulfur detection using SSP-2 probe

Stock solutions of **7** (10 mM), **9** (10 mM) and SSP-2 (5 mM) were prepared in DMSO. DTT (100 mM) and esterase (100 U/mL) were prepared in DI water. The reaction mixtures were prepared by adding 15  $\mu\text{M}$  3-MST (21  $\mu\text{L}$ , 285  $\mu\text{M}$  stock), 25  $\mu\text{M}$  of **7** (1  $\mu\text{L}$ , 10 mM stock), 0.5 U/mL of esterase (2  $\mu\text{L}$ , 100 U/mL stock), the volume was adjusted to 400  $\mu\text{L}$  using 1 $\times$  PBS pH 7.4 buffer. A similar reaction was set up for negative control (**9**). The reaction was incubated for 1 h at 37  $^\circ\text{C}$ .

A reaction was set up for compound control. The reaction mixtures were prepared by adding 25  $\mu$ M of **7** (1  $\mu$ L, 10 mM stock), 0.5 U/mL esterase (2  $\mu$ L, 100 U/mL stock) and the volume was adjusted to 400  $\mu$ L using 1 $\times$  PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^{\circ}$ C.

A reaction was set up for enzyme control. The reaction mixtures were prepared by 10  $\mu$ M 3-MST (21  $\mu$ L, 285  $\mu$ M stock), 1  $\mu$ L of DMSO, 0.5 U/mL esterase (2  $\mu$ L, 100 U/mL stock) and the volume was adjusted to 400  $\mu$ L using 1 $\times$  PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^{\circ}$ C.

A similar reaction was set up for DTT control. The reaction mixtures were prepared by adding 15  $\mu$ M 3-MST (21  $\mu$ L, 285  $\mu$ M stock), 25  $\mu$ M of **7** (1  $\mu$ L, 10 mM stock), 0.5 U/mL esterase (2  $\mu$ L, 100 U/mL stock) and the volume was adjusted to 360  $\mu$ L using 1 $\times$  PBS pH 7.4 buffer. The reaction was incubated for 30 min at 37  $^{\circ}$ C. Finally, the reaction mixture was treated with 10 mM DTT (40  $\mu$ L of 100 mM stock) after 30 min and further incubated at 37  $^{\circ}$ C for 1 h.

The above treatment groups were finally incubated with 50  $\mu$ M SSP-2 (4  $\mu$ L, 5 mM) at 37  $^{\circ}$ C for 10 min in the dark. 100  $\mu$ L aliquot of each sample was transferred to a 96 well plate and the fluorescence was recorded ( $\lambda_{\text{ex}}$  = 482 nm,  $\lambda_{\text{em}}$  = 518 nm) using a microplate reader (PerkinElmer EnSight).

### 3.5.7. Cell viability assay

Mouse embryonic fibroblasts (MEFs) were seeded at a concentration of  $1 \times 10^4$  cells/well in a 96-well plate in complete DMEM media containing 5% fetal bovine serum (FBS) and 1% antibiotic solution and incubated in an atmosphere of 5% CO<sub>2</sub> at 37  $^{\circ}$ C for 16 h. 10 mM stock solutions of compounds (**1a**, **7**, **9**) were prepared in DMSO. Cells were treated with variable concentrations of the compounds and the final concentration of DMSO did not exceed 0.5%. The cells were incubated for 24 h at 37  $^{\circ}$ 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 was removed, 100  $\mu$ L of the MTT stock was added to each well and incubated for 4 h at 37  $^{\circ}$ C. After 4 h incubation, the media was gently removed and 100  $\mu$ L of DMSO was added and mixed carefully. Spectrophotometric analysis of each well at 570 nm using a microplate reader (Thermo Scientific Varioskan) was carried out to estimate cell viability.

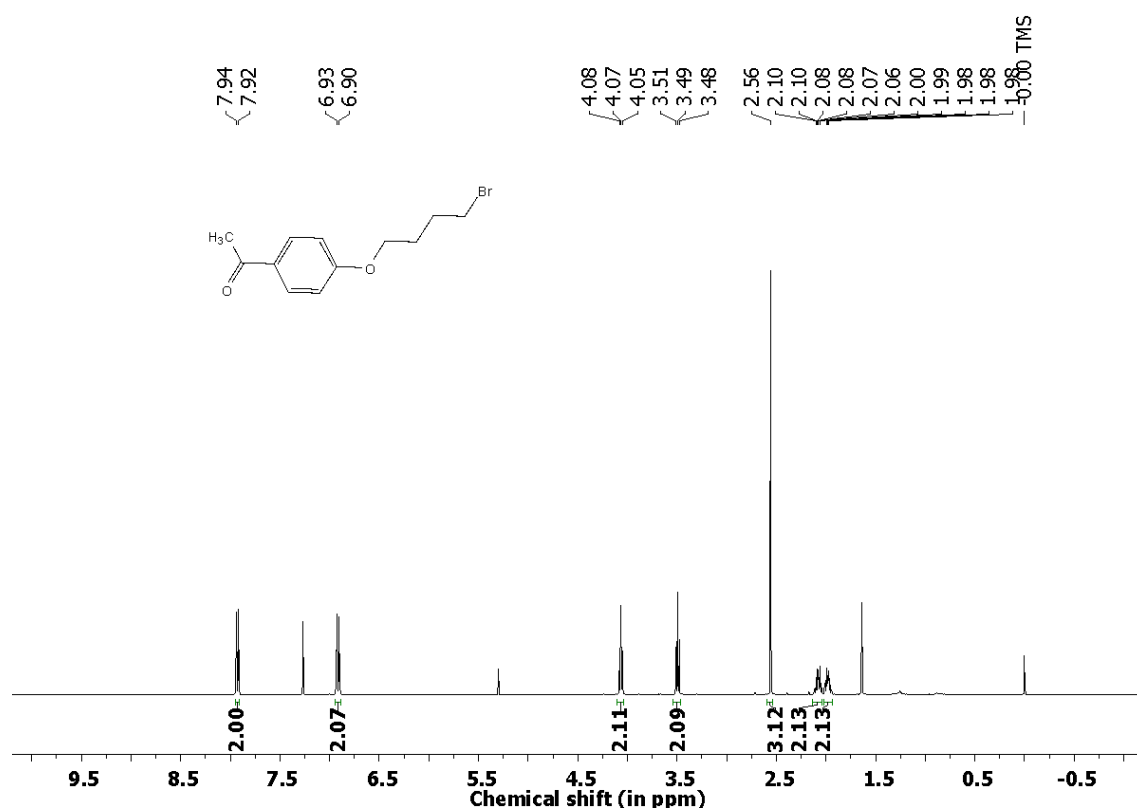
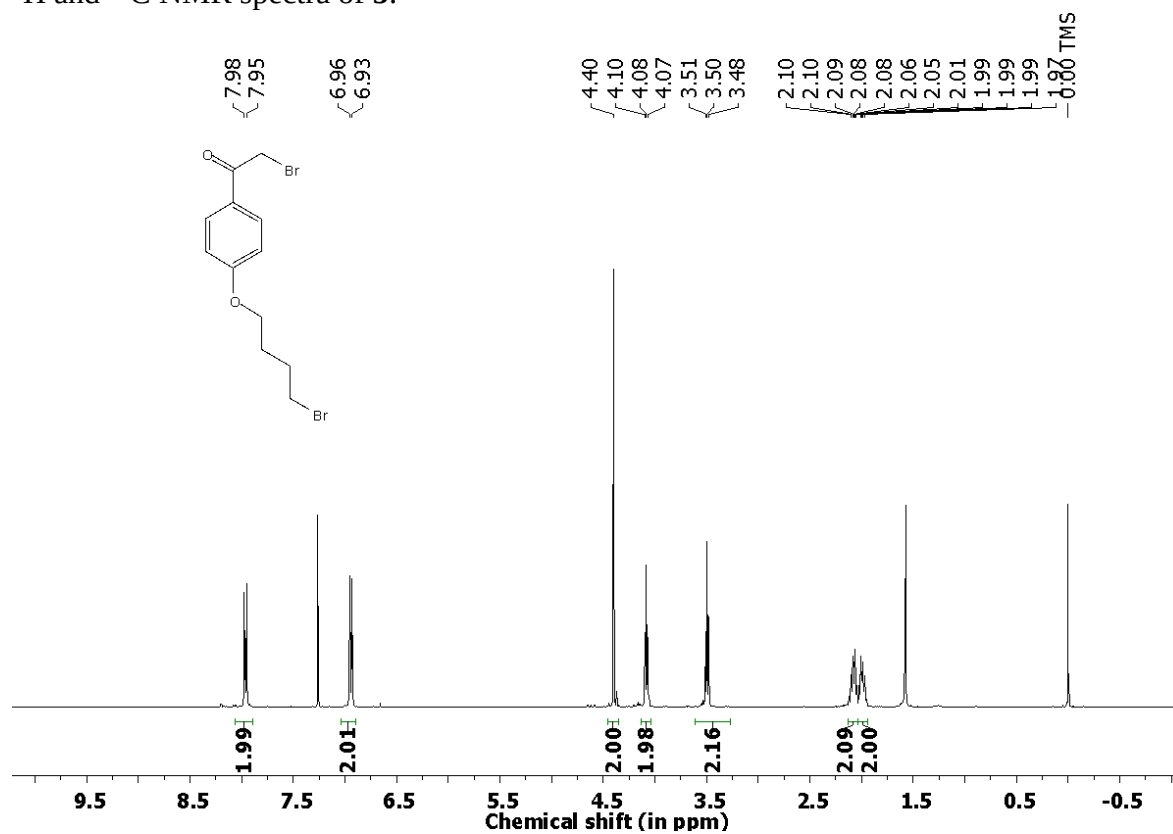
### **3.5.8. Dose dependent treatment of compound 7 for studying membrane potential change using MitoTracker red dye**

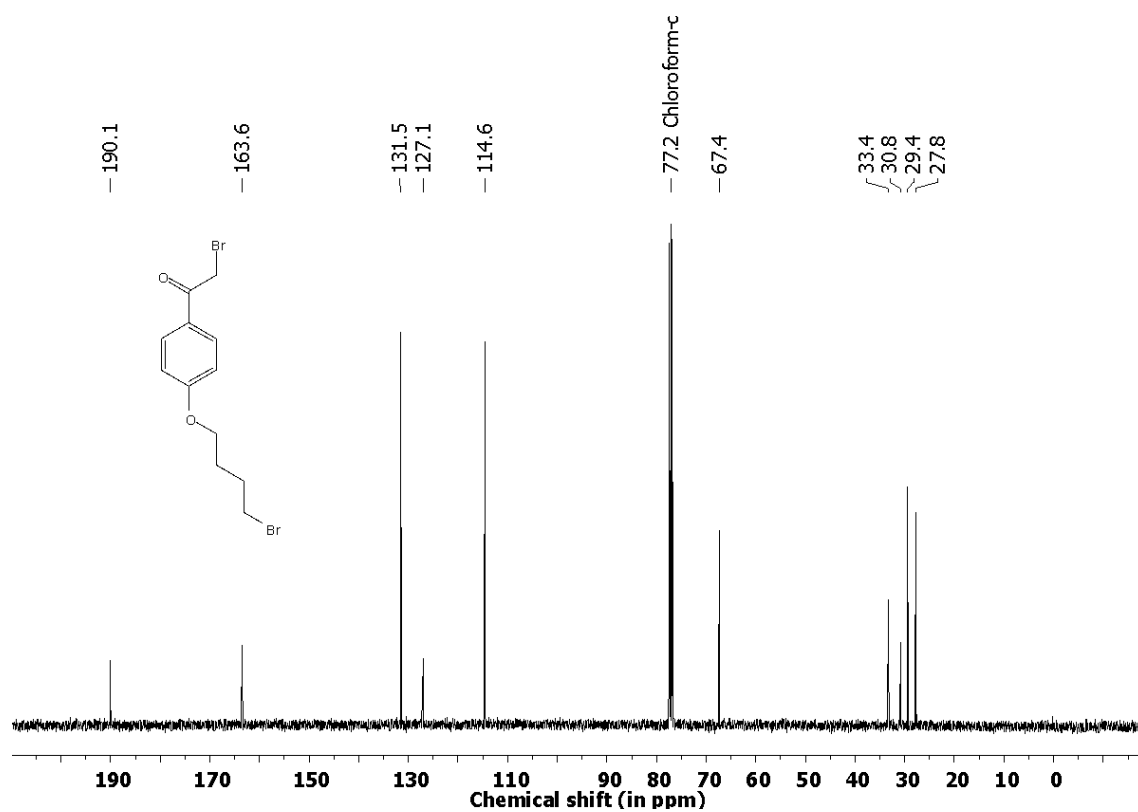
Wild type MEF cells were seeded in a 24-well plate with  $10^4$  cells/well in DMEM media containing 5% FBS and 1% antibiotic solution and cells are incubated in an atmosphere of 5% CO<sub>2</sub> at 37 °C for 24 h. Following this, the old media was removed and the cells were washed two times with 1 mL of sterile 1× PBS. This was followed by co-treatment of dose dependent concentration of **7** (1, 2, 3, 4 and 5 μM) dissolved in fresh serum-free DMEM media, incubated at 37 °C for 1 h. Next, a separate 20 nM stock solution of MitoTracker red was made in serum-free DMEM and incubated in ice for 15 min. After 1 h, media was removed and cells are co-treated with varying concentrations (1, 2, 3, 4 and 5 μM) of compound **7** in 20 nM solution of MitoTracker red stock, incubated at 37 °C for 30 min. After the incubation media was removed, cells were washed twice with 1 mL 1× PBS. Next, cells were fixed using standard paraformaldehyde-based fixation method (3.5% paraformaldehyde at room temperature). Finally, cells were imaged using a 63× confocal multiphoton fluorescence microscope.

### **3.5.9. H<sub>2</sub>S detection and mitochondrial membrane potential change imaging in cells using NBD-fluorescein and Mito-tracker red dye respectively**

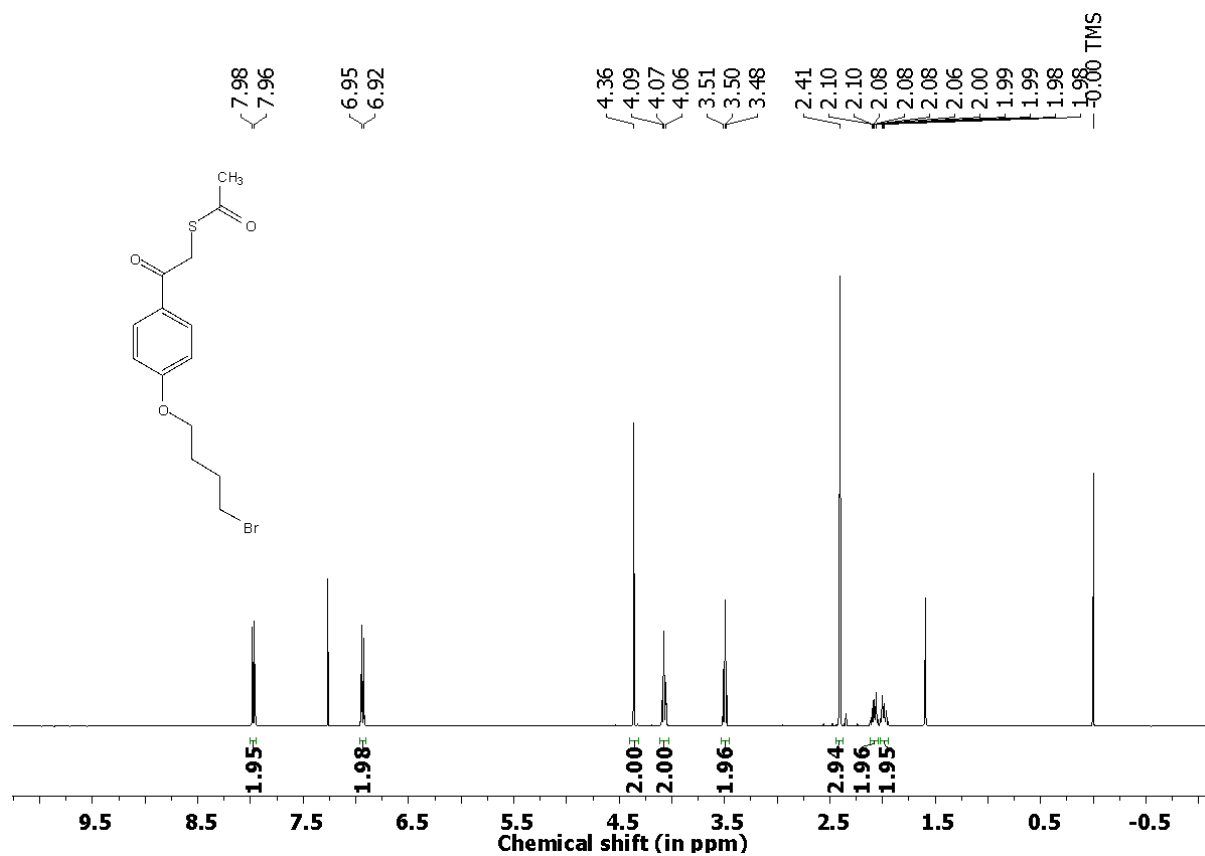
MEFs cells were seeded in a 24-well plate with  $10^4$  cells/well in DMEM media containing 5% FBS and 1% antibiotic solution and cells are incubated in an atmosphere of 5% CO<sub>2</sub> at 37 °C for 24 h. Following this, the old media was removed and the cells were washed two times with 1 mL of sterile 1× PBS. This was followed by co-treatment of 2 μM of **1a** or **7** or **9** with NBD-fluorescein (10 μM) dissolved in fresh serum-free DMEM media, incubated at 37 °C for 1 h. Next, a separate 20 nM stock solution of MitoTracker red was made in serum-free DMEM and incubated in ice for 15 min. After 1 h, media was removed and cells are co-treated with 2 μM of **1a** or **7** or **9** in 20 nM solution of MitoTracker red stock, incubated at 37 °C for 30 min. After the incubation media was removed, cells were washed twice with 1 mL 1× PBS. Finally, cells were imaged live using a 63× confocal multiphoton fluorescence microscope.

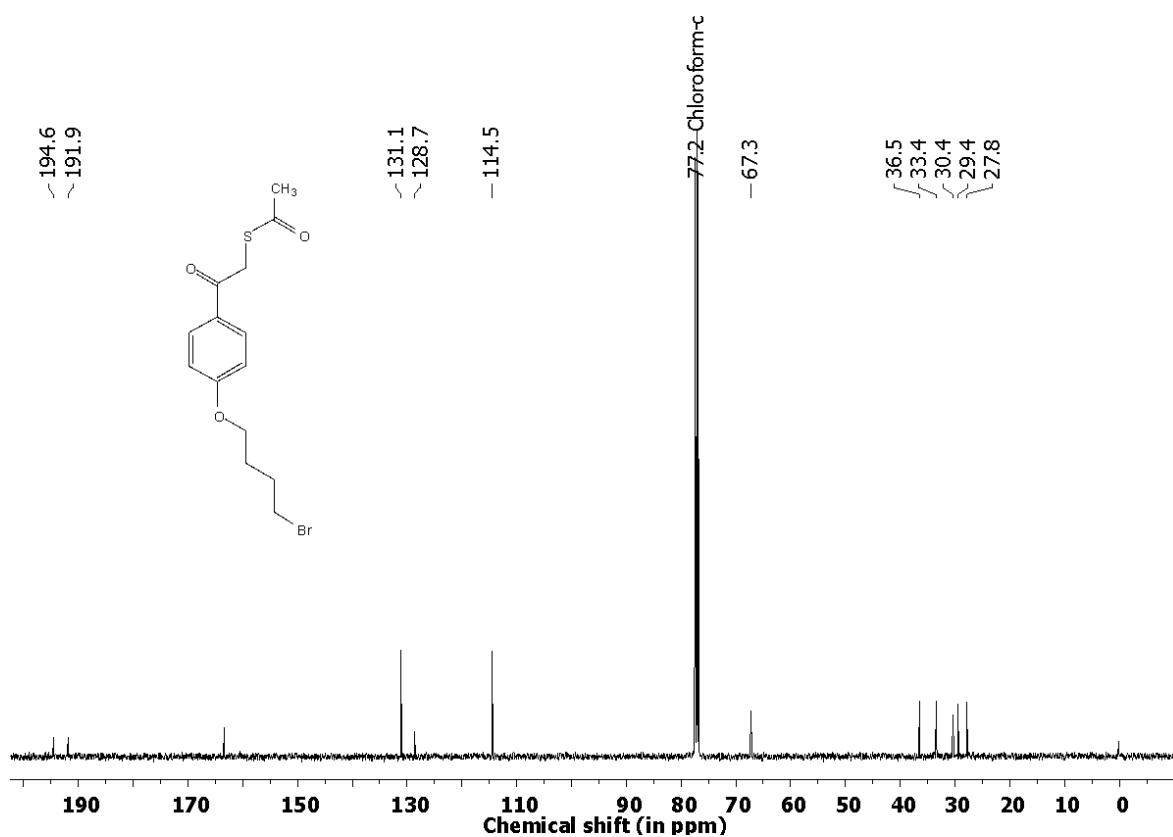
## 3.6. NMR spectra of compounds

 $^1\text{H}$  NMR spectra of 4: $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 5:

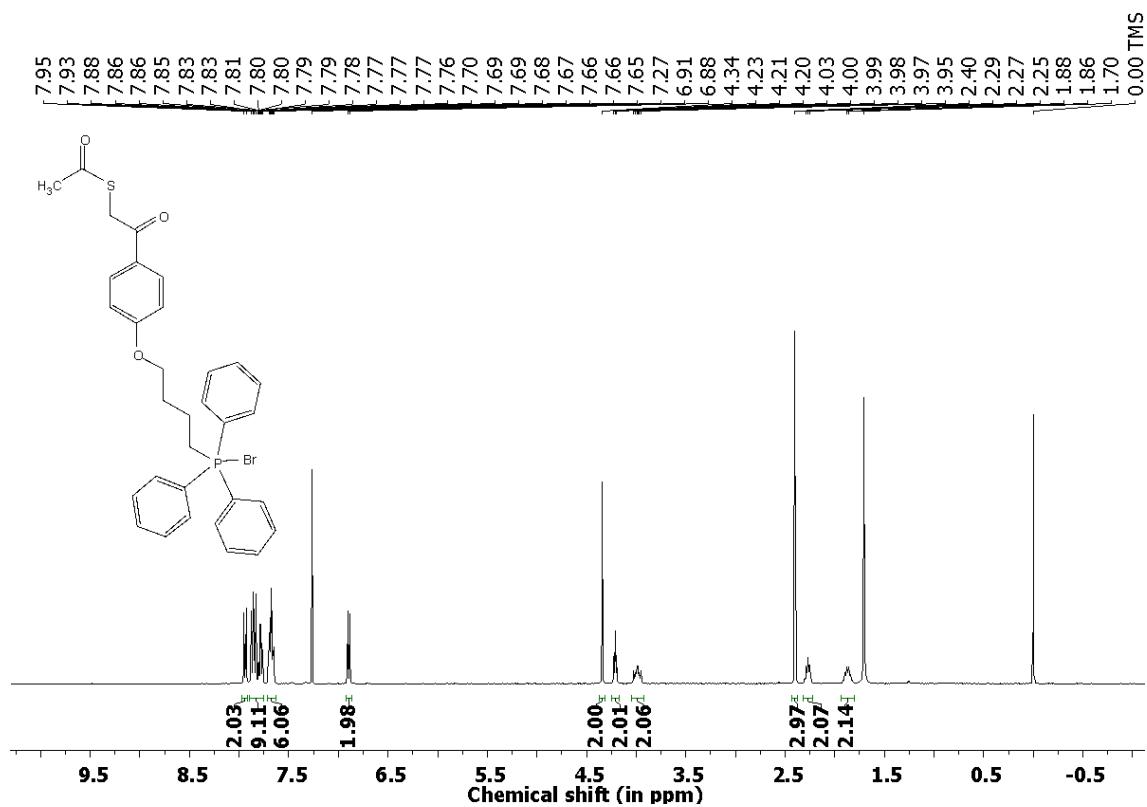


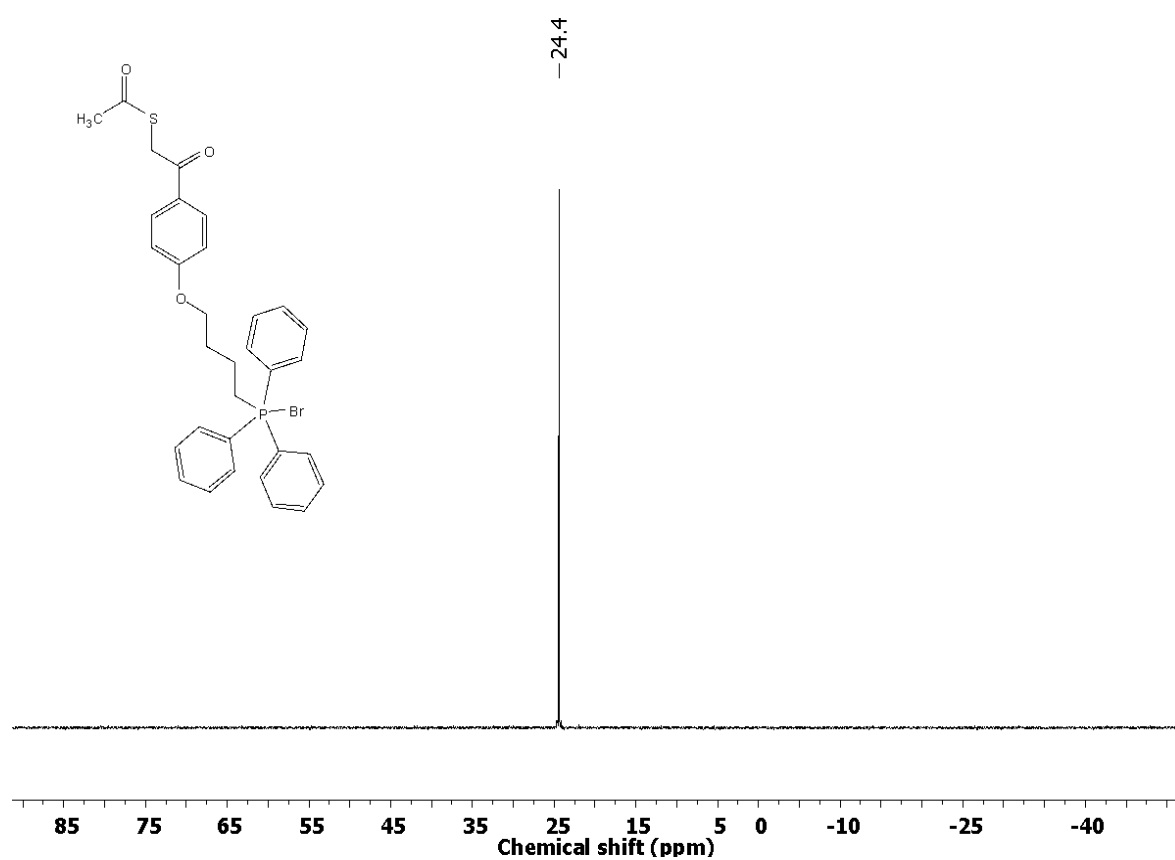
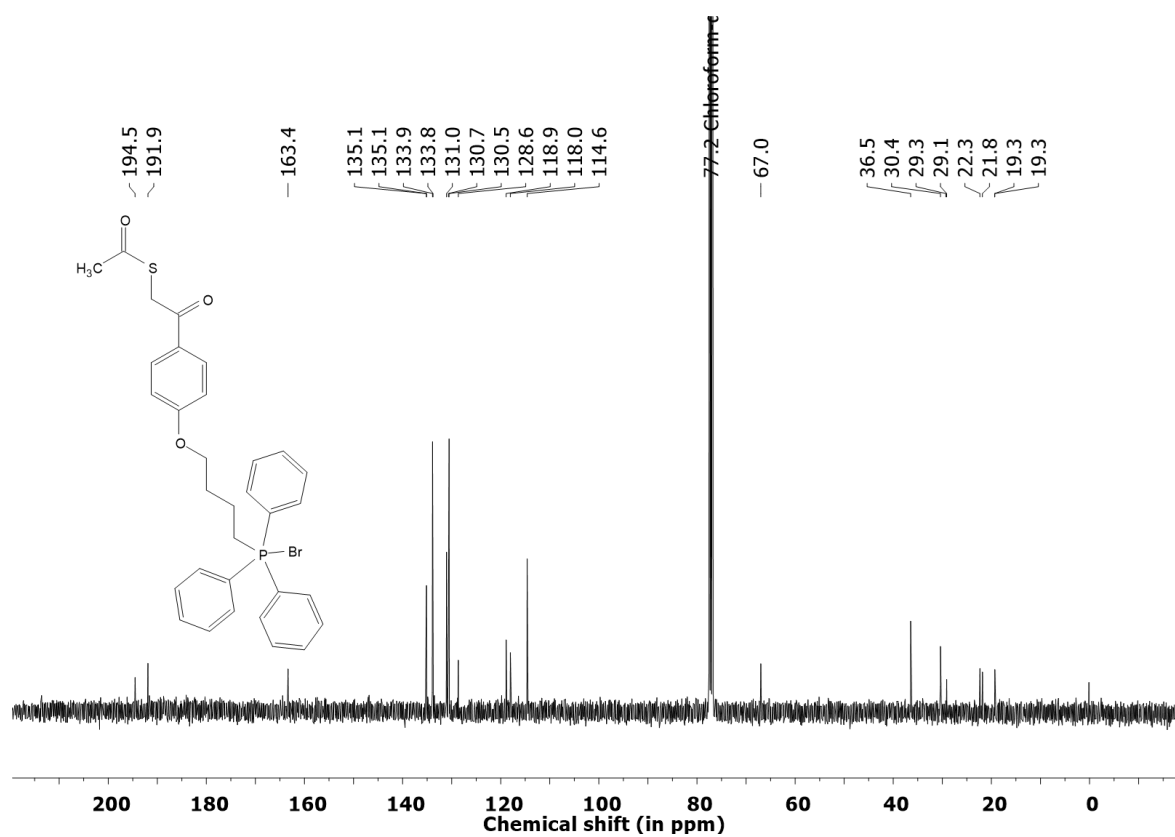
<sup>1</sup>H and <sup>13</sup>C NMR spectra of 6:



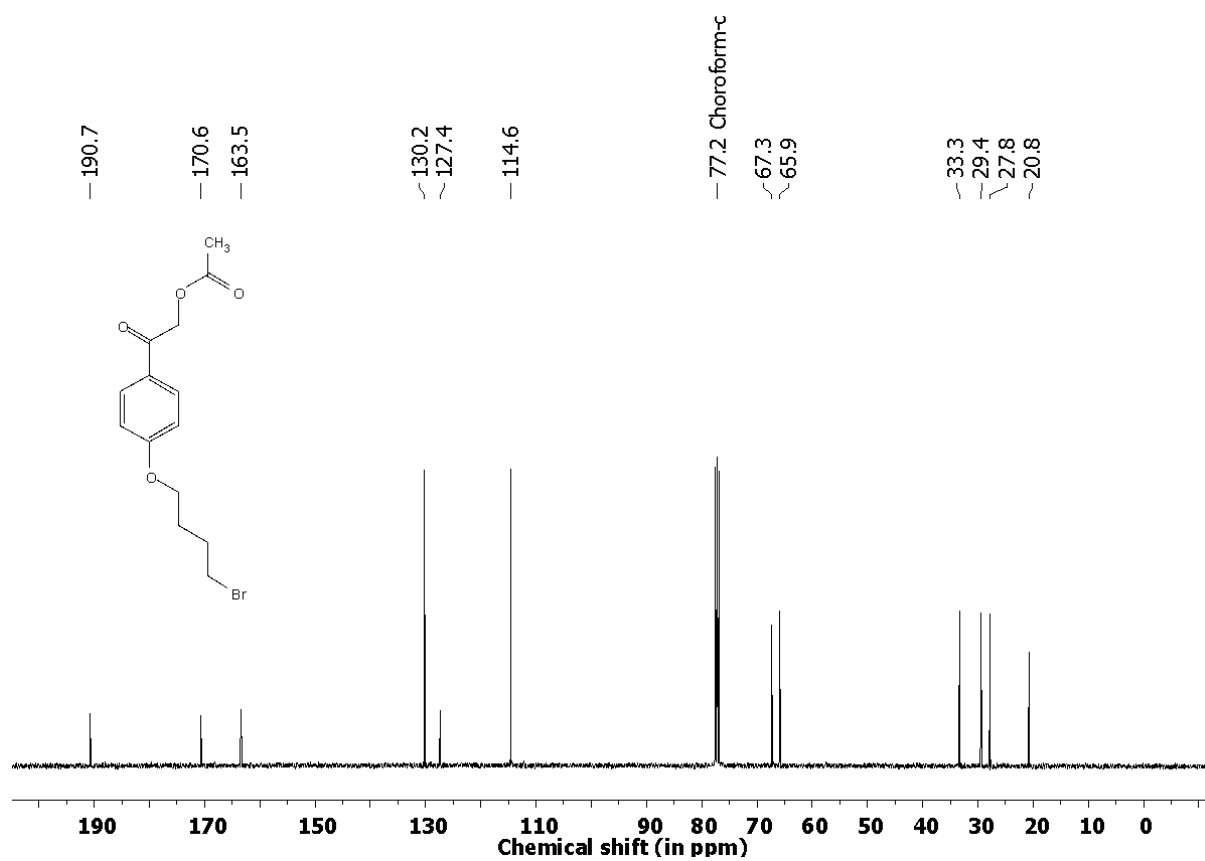
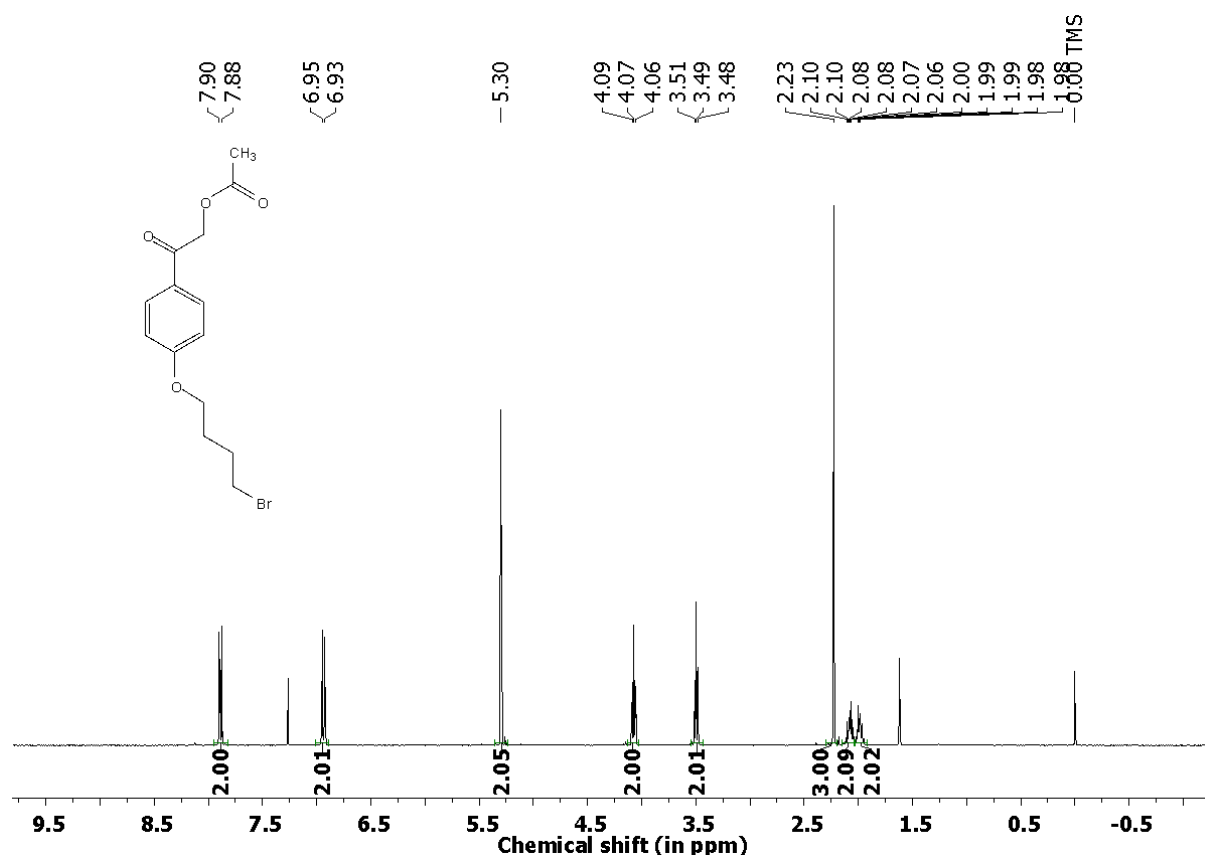


<sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra of 7:

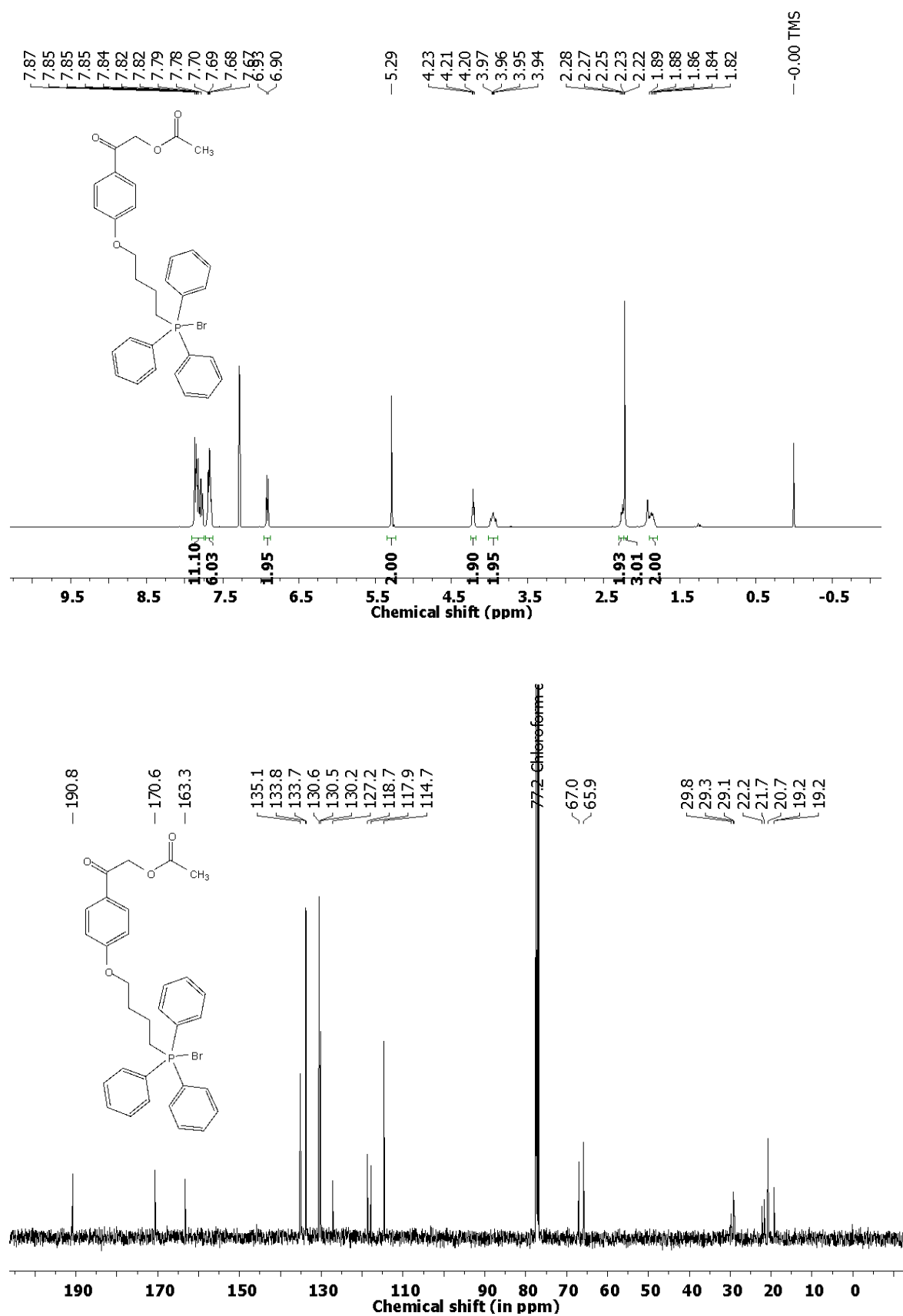


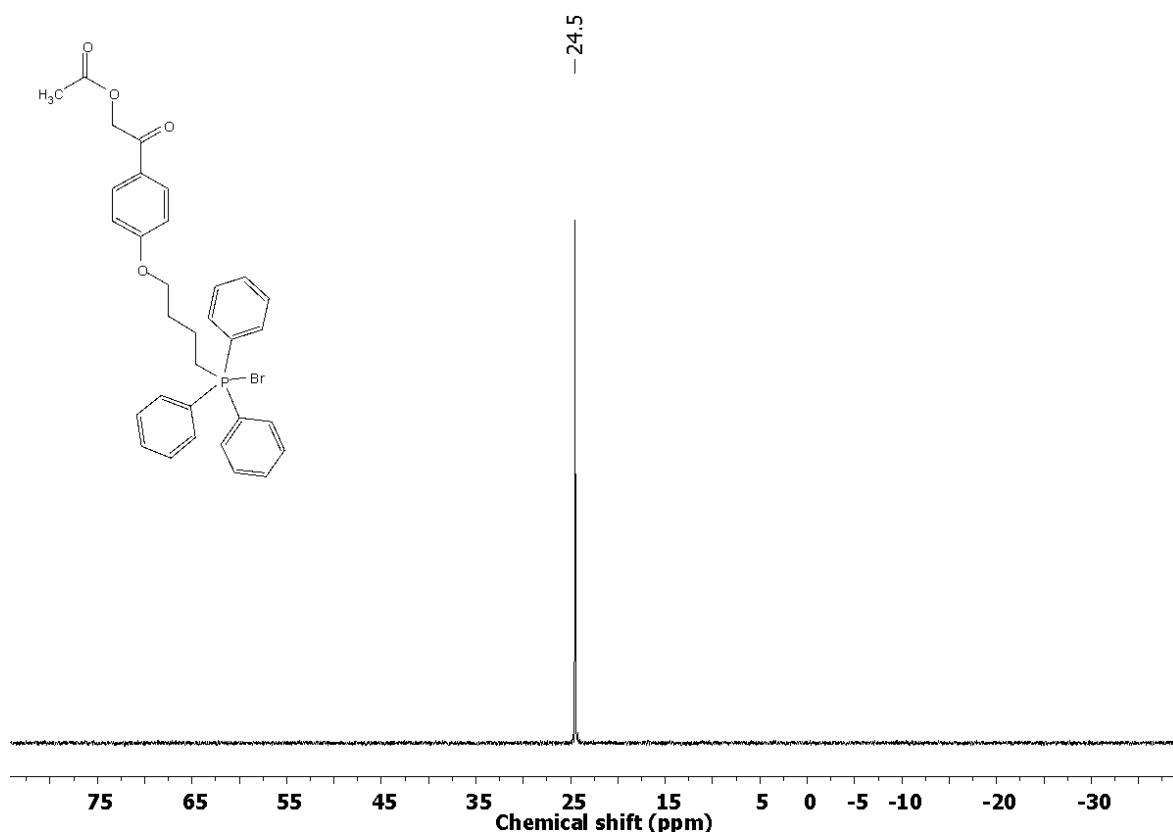


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **8**:

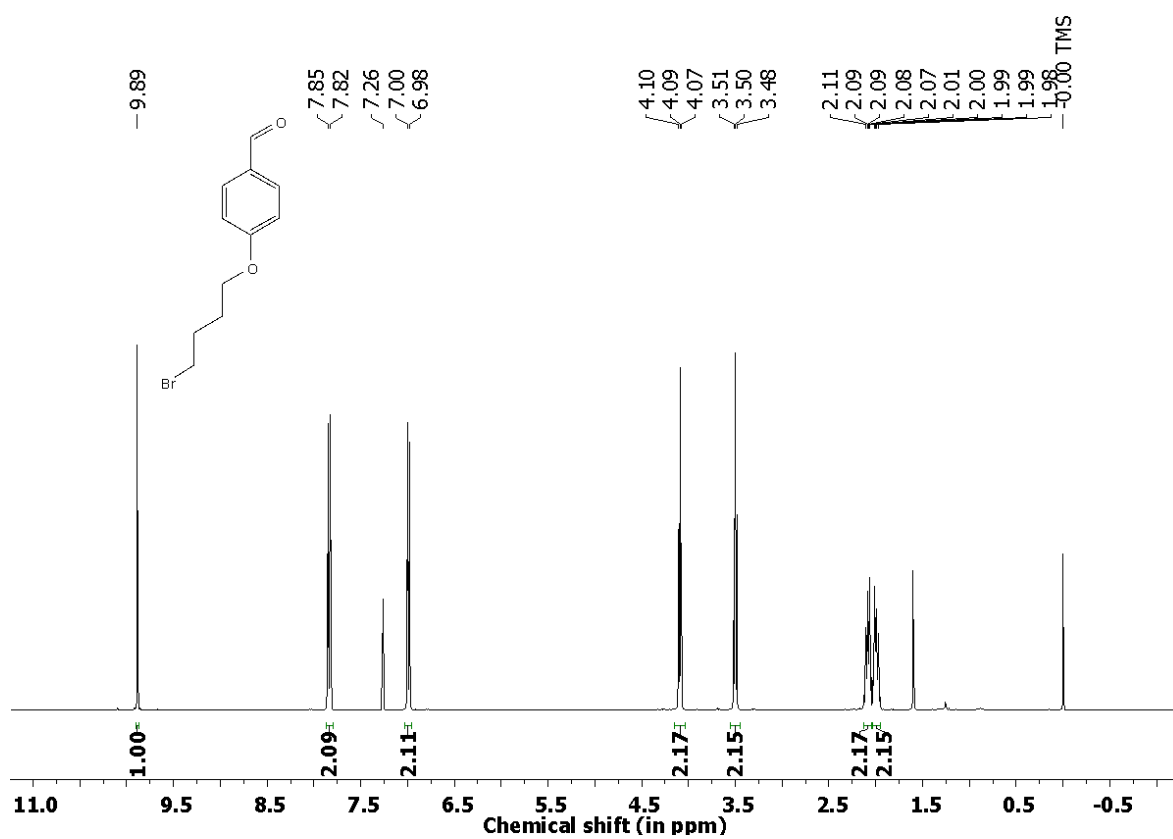


$^1\text{H}$ ,  $^{13}\text{C}$  and  $^{31}\text{P}$  NMR spectra of **9**:

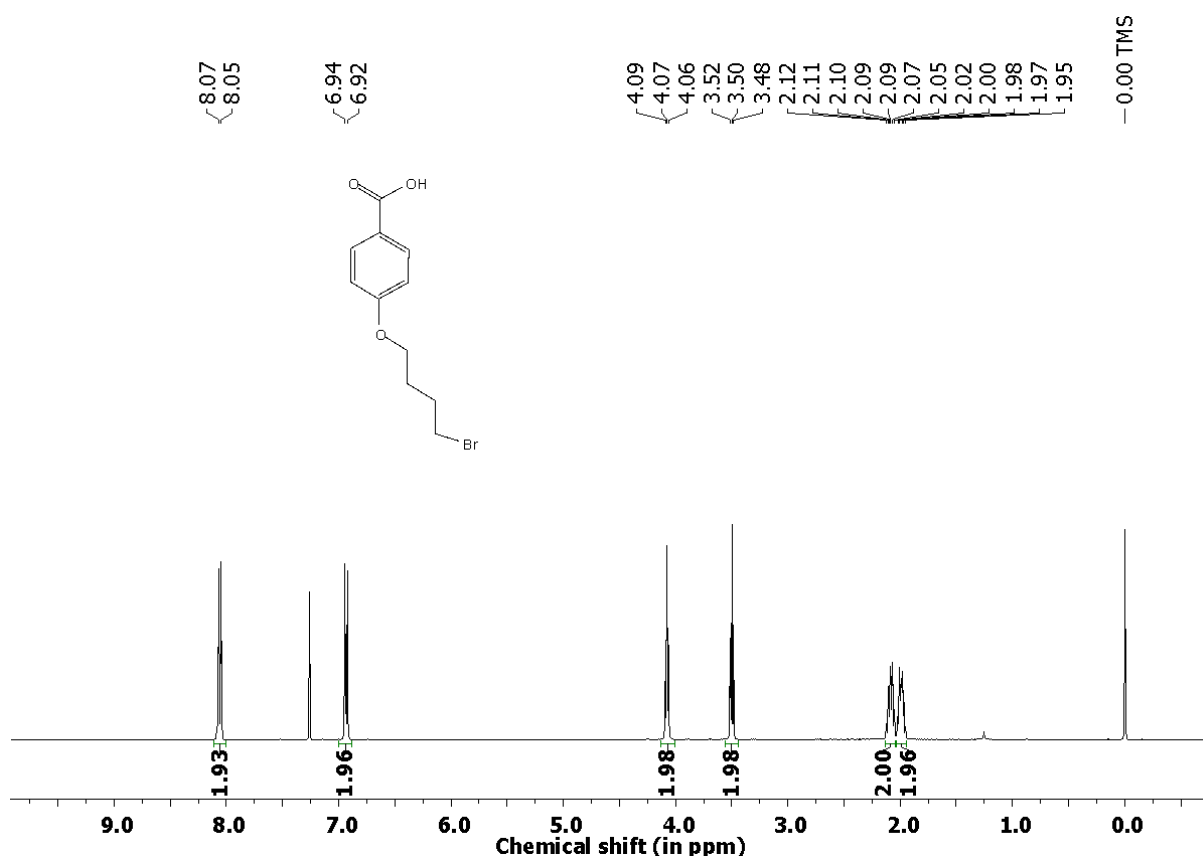




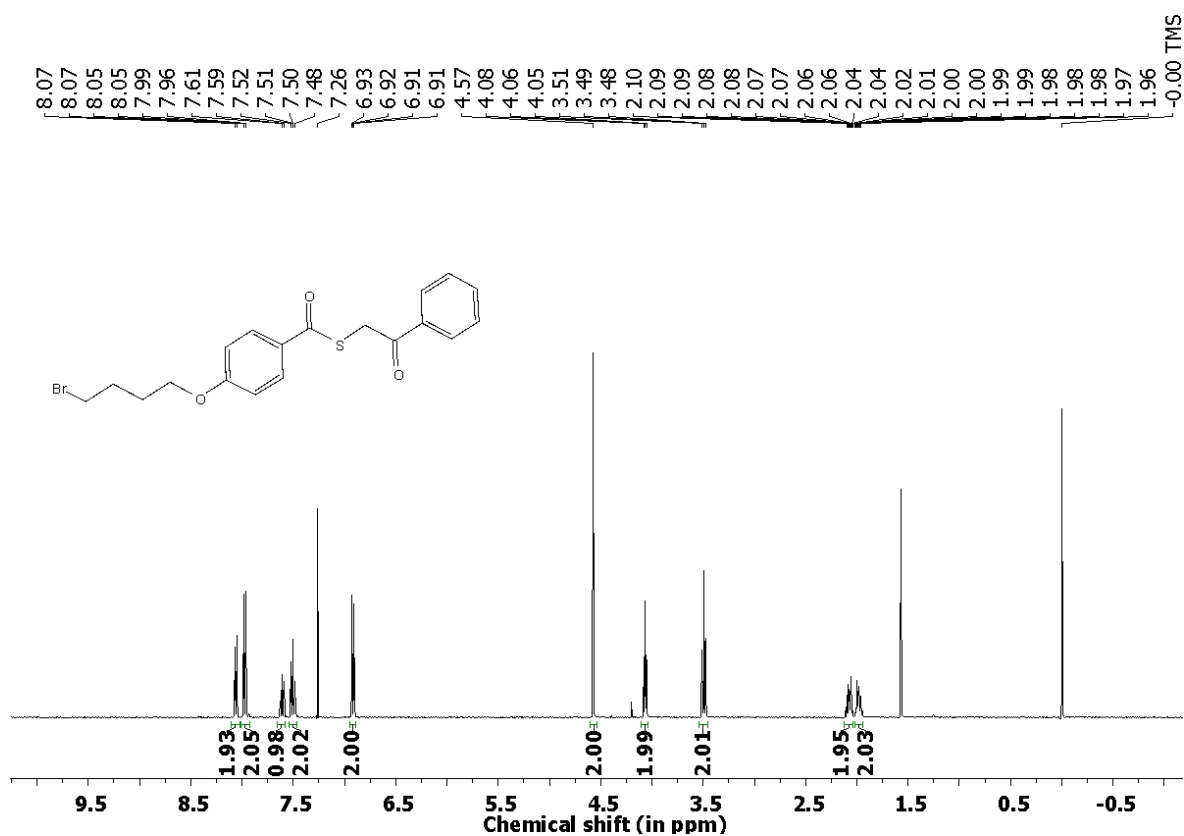
<sup>1</sup>H NMR spectra of **10**:

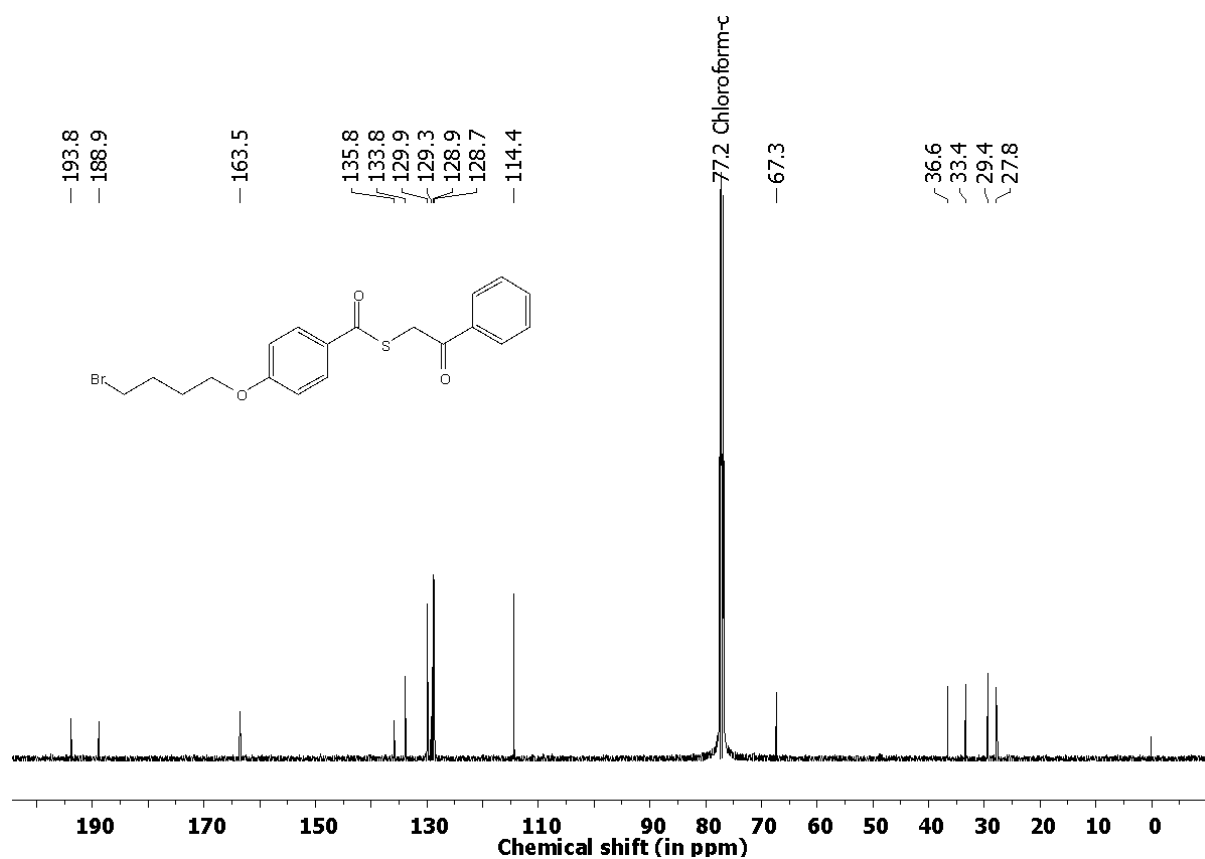


$^1\text{H}$  NMR spectra of **11**:

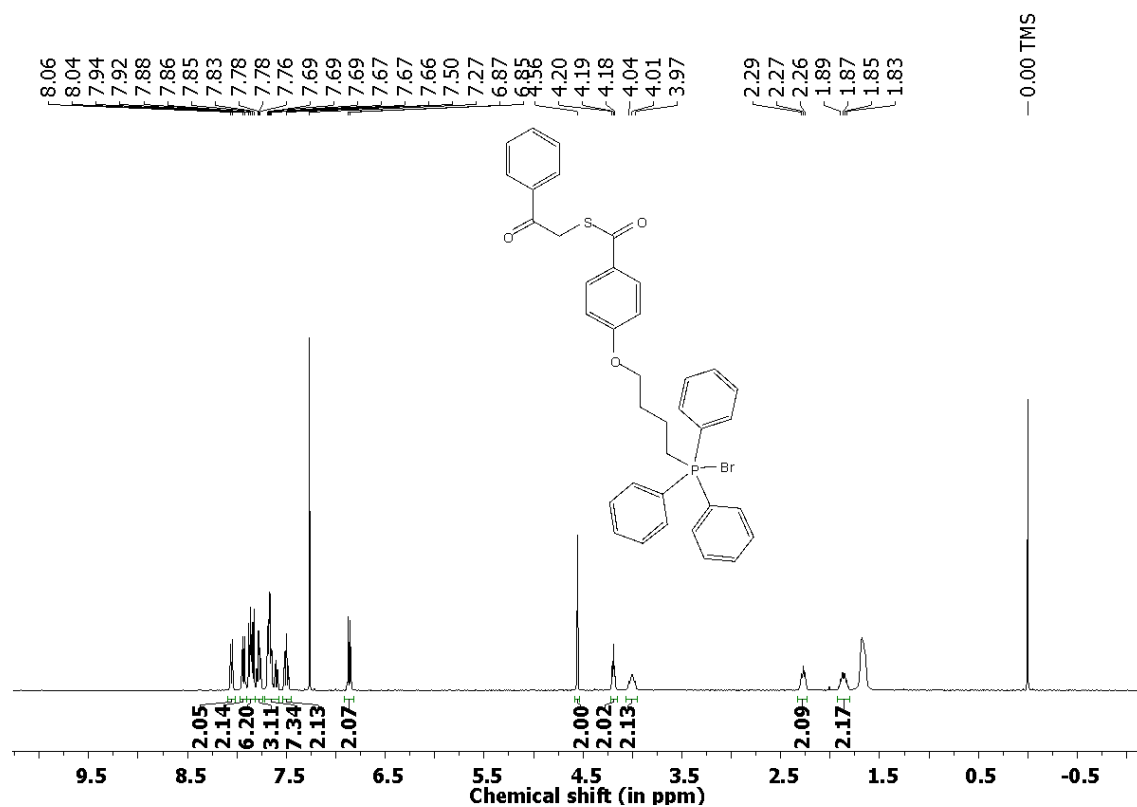


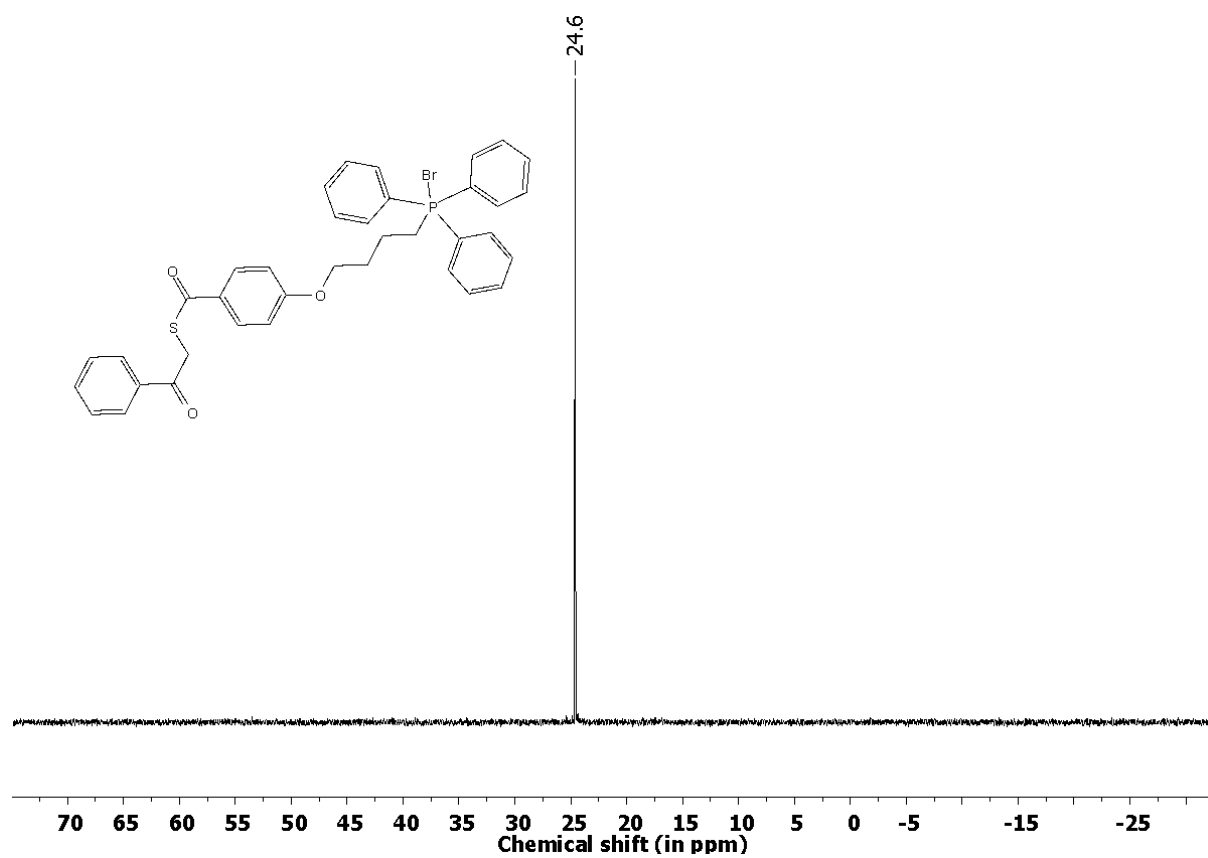
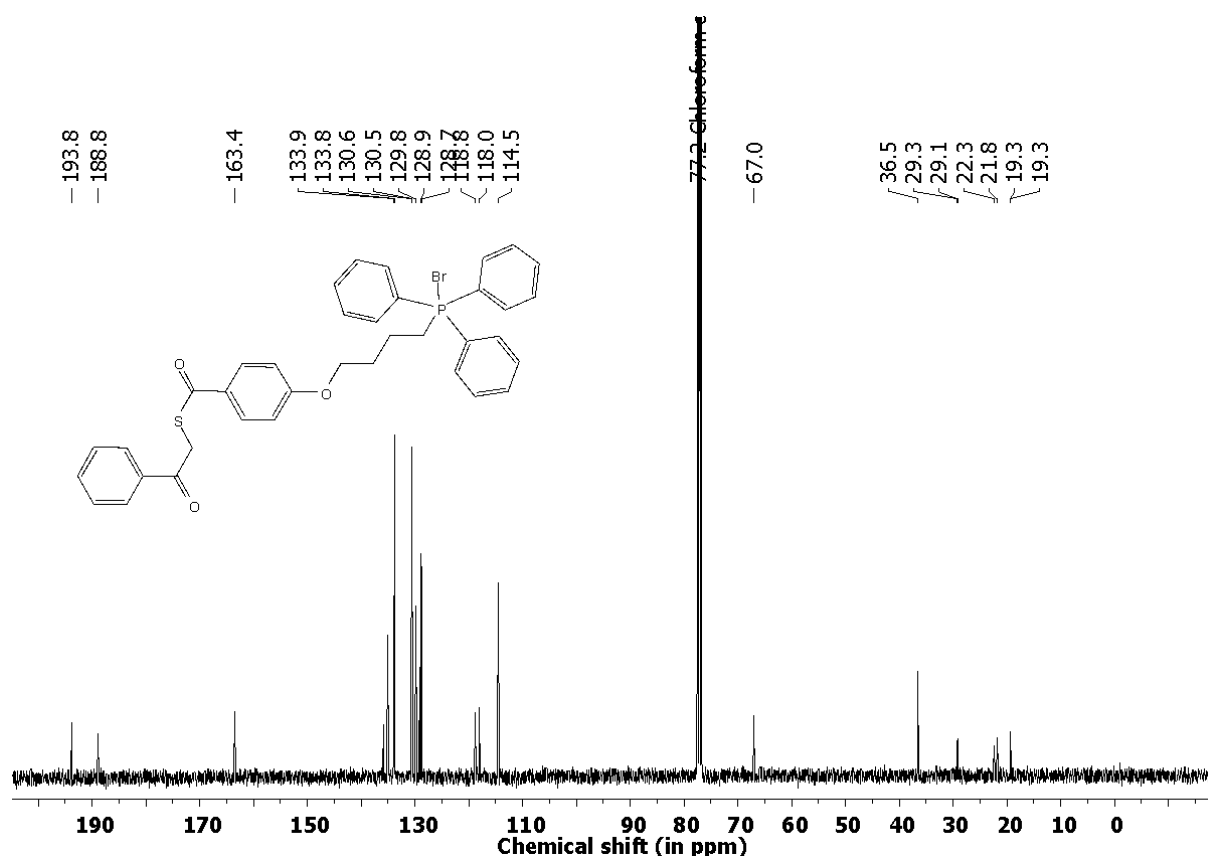
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **12**:



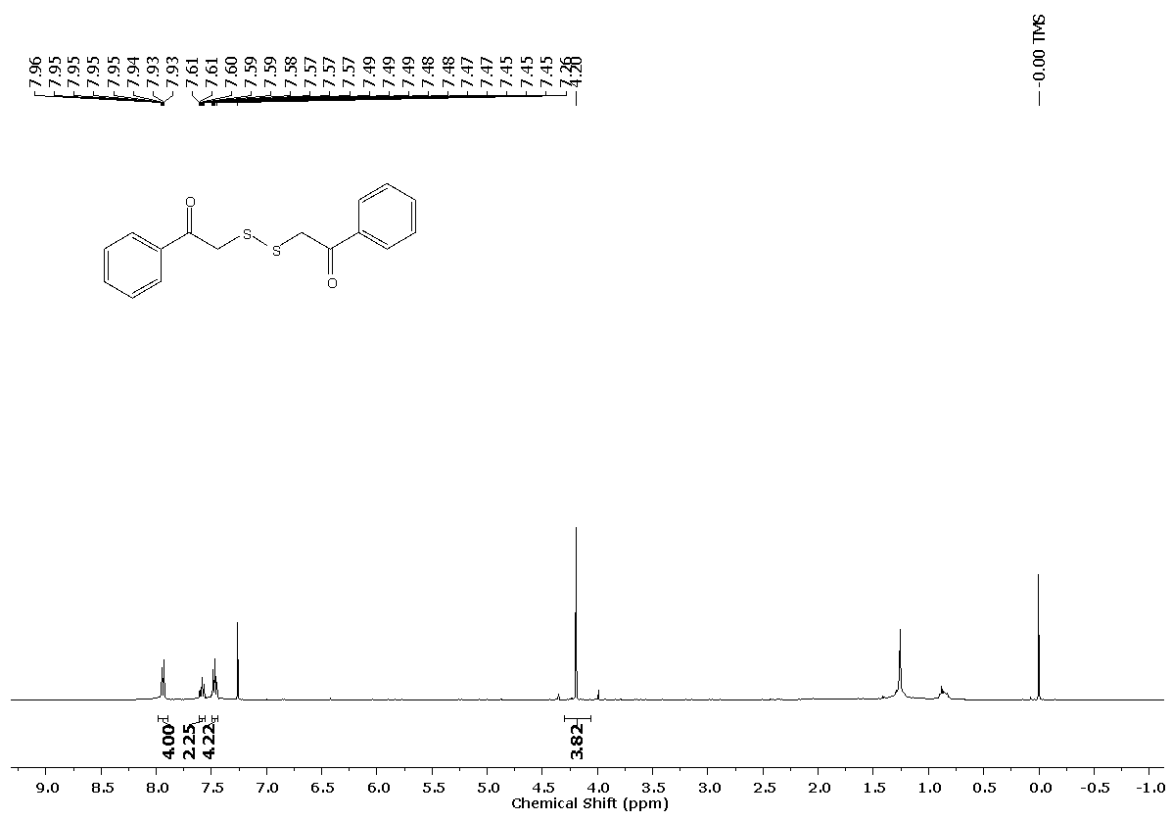


<sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra of 13:

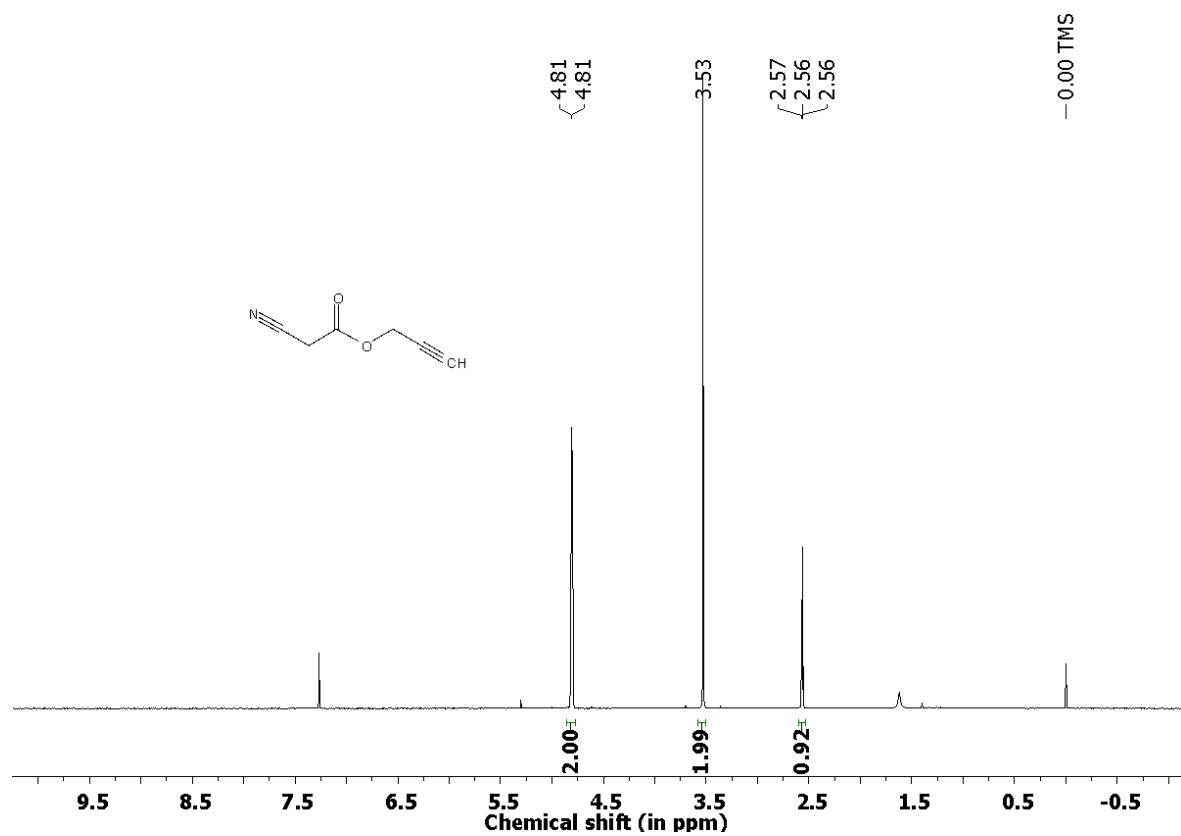


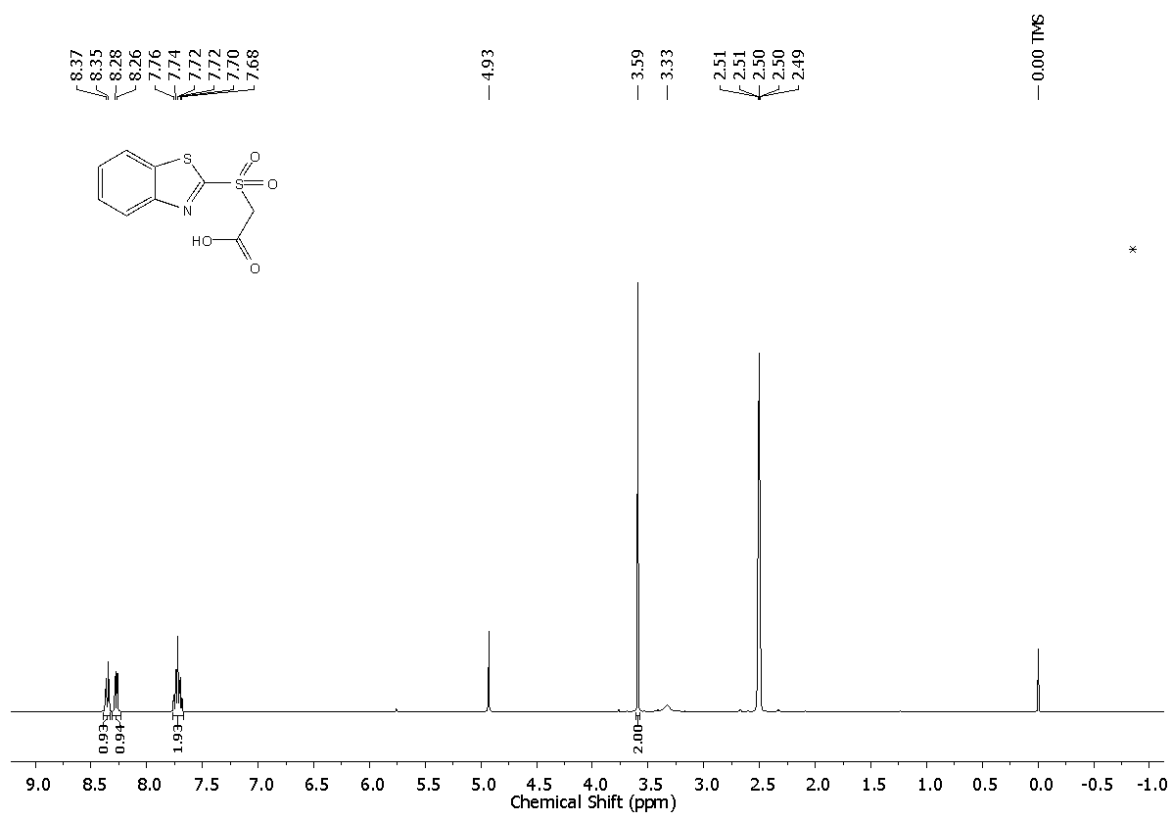


$^1\text{H}$  NMR spectra of **ds1**:



$^1\text{H}$  NMR spectra of **CN-Alkyne**:



<sup>1</sup>H NMR spectra of **MSBT-A**:

### 3.7. References:

- (1) Murphy, M. P.; Hartley, R. C. Mitochondria as a Therapeutic Target for Common Pathologies. *Nat. Rev. Drug Discov.* **2018**, 17 (12), 865–886.
- (2) Fräsdorf, B.; Radon, C.; Leimkühler, S. Characterization and Interaction Studies of Two Isoforms of the Dual Localized 3-Mercaptopyruvate Sulfurtransferase TUM1 from Humans. *J. Biol. Chem.* **2014**, 289 (50), 34543–34556.
- (3) Ezeriņa, D.; Takano, Y.; Hanaoka, K.; Urano, Y.; Dick, T. P. N-Acetyl Cysteine Functions as a Fast-Acting Antioxidant by Triggering Intracellular H<sub>2</sub>S and Sulfane Sulfur Production. *Cell Chem. Biol.* **2018**, 25 (4), 447-459.e4.
- (4) Pedre, B.; Dick, T. P. 3-Mercaptopyruvate Sulfurtransferase: An Enzyme at the Crossroads of Sulfane Sulfur Trafficking. *Biol. Chem.* **2021**, 402 (3), 223–237.
- (5) Zielonka, J.; Joseph, J.; Sikora, A.; Hardy, M.; Ouari, O.; Vasquez-Vivar, J.; Cheng, G.; Lopez, M.; Kalyanaraman, B. Mitochondria-Targeted Triphenylphosphonium-Based Compounds: Syntheses, Mechanisms of Action, and Therapeutic and Diagnostic Applications. *Chem. Rev.* **2017**, 117 (15), 10043–10120.
- (6) Battogtokh, G.; Choi, Y. S.; Kang, D. S.; Park, S. J.; Shim, M. S.; Huh, K. M.; Cho, Y.-Y.; Lee, J. Y.; Lee, H. S.; Kang, H. C. Mitochondria-Targeting Drug Conjugates for Cytotoxic, Anti-Oxidizing and Sensing Purposes: Current Strategies and Future Perspectives. *Acta Pharm. Sin. B* **2018**, 8 (6), 862–880.
- (7) Zhang, D.; Macinkovic, I.; Devarie-Baez, N. O.; Pan, J.; Park, C.-M.; Carroll, K. S.; Filipovic, M. R.; Xian, M. Detection of Protein S-Sulfhydration by a Tag-Switch Technique. *Angew. Chemie Int. Ed.* **2014**, 53 (2), 575–581.
- (8) Wedmann, R.; Onderka, C.; Wei, S.; Szijártó, I. A.; Miljkovic, J. L.; Mitrovic, A.; Lange, M.; Savitsky, S.; Yadav, P. K.; Torregrossa, R.; Harrer, E. G.; Harrer, T.; Ishii, I.; Gollasch, M.; Wood, M. E.; Galardon, E.; Xian, M.; Whiteman, M.; Banerjee, R.; Filipovic, M. R. Improved Tag-Switch Method Reveals That Thioredoxin Acts as Depersulfidase and Controls the Intracellular Levels of Protein Persulfidation. *Chem. Sci.* **2016**, 7 (5), 3414–3426.
- (9) Dabiri, M.; MaGee, D.; Salehi, P.; Torkian, L.; Fakharian, M.; Donahue, J. Combining a Click–Multicomponent Reaction: One-Pot Synthesis of 1,2,3-Triazol-4-ylmethyl 3-

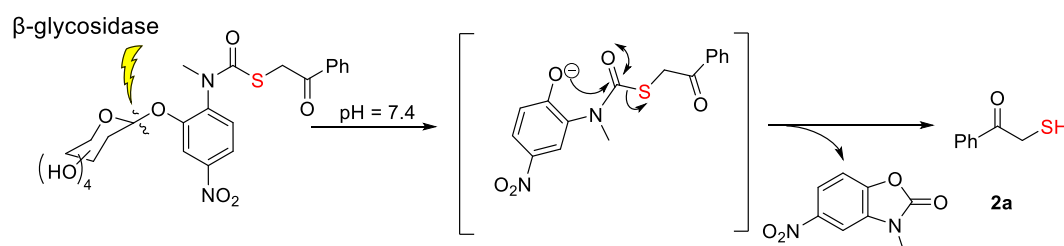
- Amino-5,10-Dihydro-5,10-Dioxo-1 H -Pyrazolo[1,2- b ]Phthalazine-2-Carboxylate Derivatives. *Synth. Commun.* **2014**, 44 (14), 2037–2044.
- (10) Chen, X.; Wu, H.; Park, C.-M.; Poole, T. H.; Keceli, G.; Devarie-Baez, N. O.; Tsang, A. W.; Lowther, W. T.; Poole, L. B.; King, S. B.; Xian, M.; Furdui, C. M. Discovery of Heteroaromatic Sulfones As a New Class of Biologically Compatible Thiol-Selective Reagents. *ACS Chem. Biol.* **2017**, 12 (8), 2201–2208.
- (11) Liang, L.; Astruc, D. The Copper(I)-Catalyzed Alkyne-Azide Cycloaddition (CuAAC) “Click” Reaction and Its Applications. An Overview. *Coord. Chem. Rev.* **2011**, 255 (23), 2933–2945.
- (12) Chen, W.; Liu, C.; Peng, B.; Zhao, Y.; Pacheco, A.; Xian, M. New Fluorescent Probes for Sulfane Sulfurs and the Application in Bioimaging. *Chem. Sci.* **2013**, 4 (7), 2892.
- (13) Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T. Reactive Cysteine Persulfides and S-Polythiolation Regulate Oxidative Stress and Redox Signaling. *Proc. Natl. Acad. Sci.* **2014**, 111 (21), 7606–7611.
- (14) Mustafina, A. N.; Yakovlev, A. V.; Gaifullina, A. S.; Weiger, T. M.; Hermann, A.; Sitdikova, G. F. Hydrogen Sulfide Induces Hyperpolarization and Decreases the Exocytosis of Secretory Granules of Rat GH3 Pituitary Tumor Cells. *Biochem. Biophys. Res. Commun.* **2015**, 465 (4), 825–831.
- (15) Sawa, T.; Motohashi, H.; Ihara, H.; Akaike, T. Enzymatic Regulation and Biological Functions of Reactive Cysteine Persulfides and Polysulfides. *Biomolecules* **2020**, 10 (9), 1245.
- (16) Zorova, L. D.; Popkov, V. A.; Plotnikov, E. Y.; Silachev, D. N.; Pevzner, I. B.; Jankauskas, S. S.; Babenko, V. A.; Zorov, S. D.; Balakireva, A. V.; Juhaszova, M.; Sollott, S. J.; Zorov, D. B. Mitochondrial Membrane Potential. *Anal. Biochem.* **2018**, 552, 50–59.
- (17) Kholmukhamedov, A.; Schwartz, J. M.; Lemasters, J. J. Mitotracker Probes and Mitochondrial Membrane Potential. *Shock* **2013**, 39 (6), 543.
- (18) Zhitomirsky, B.; Farber, H.; Assaraf, Y. G. LysoTracker and MitoTracker Red Are

- Transport Substrates of P-glycoprotein: Implications for Anticancer Drug Design Evading Multidrug Resistance. *J. Cell. Mol. Med.* **2018**, 22 (4), 2131–2141.
- (19) Johnson, L. V.; Walsh, M. L.; Chen, L. B. Localization of Mitochondria in Living Cells with Rhodamine 123. *Proc. Natl. Acad. Sci.* **1980**, 77 (2), 990–994.
- (20) Chen, W.; Matsunaga, T.; Neill, D. L.; Yang, C.; Akaike, T.; Xian, M. Rational Design of a Dual-Reactivity-Based Fluorescent Probe for Visualizing Intracellular HSNO. *Angew. Chemie Int. Ed.* **2019**, 58 (45), 16067–16070.
- (21) Heredia, A. A.; Soria-Castro, S. M.; Bouchet, L. M.; Oksdath-Mansilla, G.; Barrionuevo, C. A.; Caminos, D. A.; Bisogno, F. R.; Argüello, J. E.; Peñeñory, A. B. Stereoselective One-Pot Synthesis of  $\beta$ -Alkylsulfide Enol Esters. Base-Triggered Rearrangement under Mild Conditions. *Org. Biomol. Chem.* **2014**, 12 (33), 6516.
- (22) Chandanshive, J. Z.; Bonini, B. F.; Gentili, D.; Fochi, M.; Bernardi, L.; Franchini, M. C. Regiocontrolled Synthesis of Ring-Fused Thieno[2,3-c]Pyrazoles through 1,3-Dipolar Cycloaddition of Nitrile Imines with Sulfur-Based Acetylenes. *European J. Org. Chem.* **2010**, 2010 (33), 6440–6447.
- (23) Zhong, L.; Liu, Q.; Ting, Y. S.; Thien, V. Y.; Binti Kalong, N. S.; Yang, D.; Wang, M.-W. Adenine Derivatives Invert High Glucose-Induced Thioredoxin-Interacting Protein Overexpression. *Chem. Biol. Drug Des.* **2018**, 92 (6), 1998–2008.
- (24) Huang, Q.; Zheng, B. Facile Synthesis of Benzaldehyde-Functionalized Ionic Liquids and Their Flexible Functional Group Transformations. *Org. Chem. Int.* **2012**, 2012, 1–5.
- (25) Yanagi, T.; Somerville, R. J.; Nogi, K.; Martin, R.; Yorimitsu, H. Ni-Catalyzed Carboxylation of C(Sp<sup>2</sup>)–S Bonds with CO<sub>2</sub>: Evidence for the Multifaceted Role of Zn. *ACS Catal.* **2020**, 10 (3), 2117–2123.
- (26) Sharma, A. K.; Nair, M.; Chauhan, P.; Gupta, K.; Saini, D. K.; Chakrapani, H. Visible-Light-Triggered Uncaging of Carbonyl Sulfide for Hydrogen Sulfide (H<sub>2</sub>S) Release. *Org. Lett.* **2017**, 19 (18), 4822–4825.
- (27) Yadav, P. K.; Yamada, K.; Chiku, T.; Koutmos, M.; Banerjee, R. Structure and Kinetic Analysis of H<sub>2</sub>S Production by Human Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2013**, 288 (27), 20002–20013.

## CHAPTER 4. Development of $\beta$ -glycosidase Sensitive Artificial Substrates of 3-MST

### 4.1. Introduction

It's widely recognized that cells contain a variety of thiols. For instance, glutathione (GSH), a crucial cellular thiol, is known to exist in physiological systems at concentrations of 1-4 mM.<sup>1,2</sup> However, the series of artificial substrates that are synthesized is found to be reactive with thiols (DTT or GSH) which may be potential limitation for *in vivo* application. In order to circumvent this restriction, the design, synthesis, and evaluation of an enzyme activatable, thiol-stable hydrophilic 3-MST substrate was investigated in Chapter 4. It is anticipated that a more water-soluble 3-MST artificial substrate can be transported through the blood circulation in the physiological system and may be better absorbed from the bloodstream by the target organs. In this context, using a reported strategy from our lab as the basis, glycosidase-sensitive persulfide donors that have an attached sugar unit were considered.<sup>3</sup> Sugars are natural biomolecules with free hydroxyl groups that are expected to enhance aqueous solubility. Following the strategy, the design of a hydrophilic 3-MST artificial substrate (clogP = 1.36) linked with a hexose moiety was considered (Scheme 4.1). Firstly, we have designed a  $\beta$ -glucosidase sensitive 3-MST substrate *via* tethering a glucopyranose with an artificial substrate **2a** (An efficient H<sub>2</sub>S and sulfane sulfur generation with **2a** was demonstrated in Chapter 2).  $\beta$ -glucosidase is a ubiquitous enzyme, widely expressed across variety of cells.<sup>4</sup> Hence, the modified substrate is expected to be readily permeate into the cells and upon hydrolysis of the glycosidic bond by cellular glucosidase, a subsequent intramolecular cyclization of the phenolate ion will take place to release **2a** and a heterocyclic byproduct. A similar  $\beta$ -galactosidase sensitive 3-MST artificial substrate (**21**) was also designed and developed by a colleague.<sup>5</sup> Glycosidases in cells are found to be overexpressed under inflammatory disorders.<sup>6,7</sup> Hence, the 3-MST artificial substrates designed herein should be useful to ameliorate oxidative stress in such cells. Together these glycosidase-activated 3-MST artificial substrates may overcome the limitations of the artificial substrates which were discussed previously, and provide additional aqueous solubility, shelf stability and specificity.

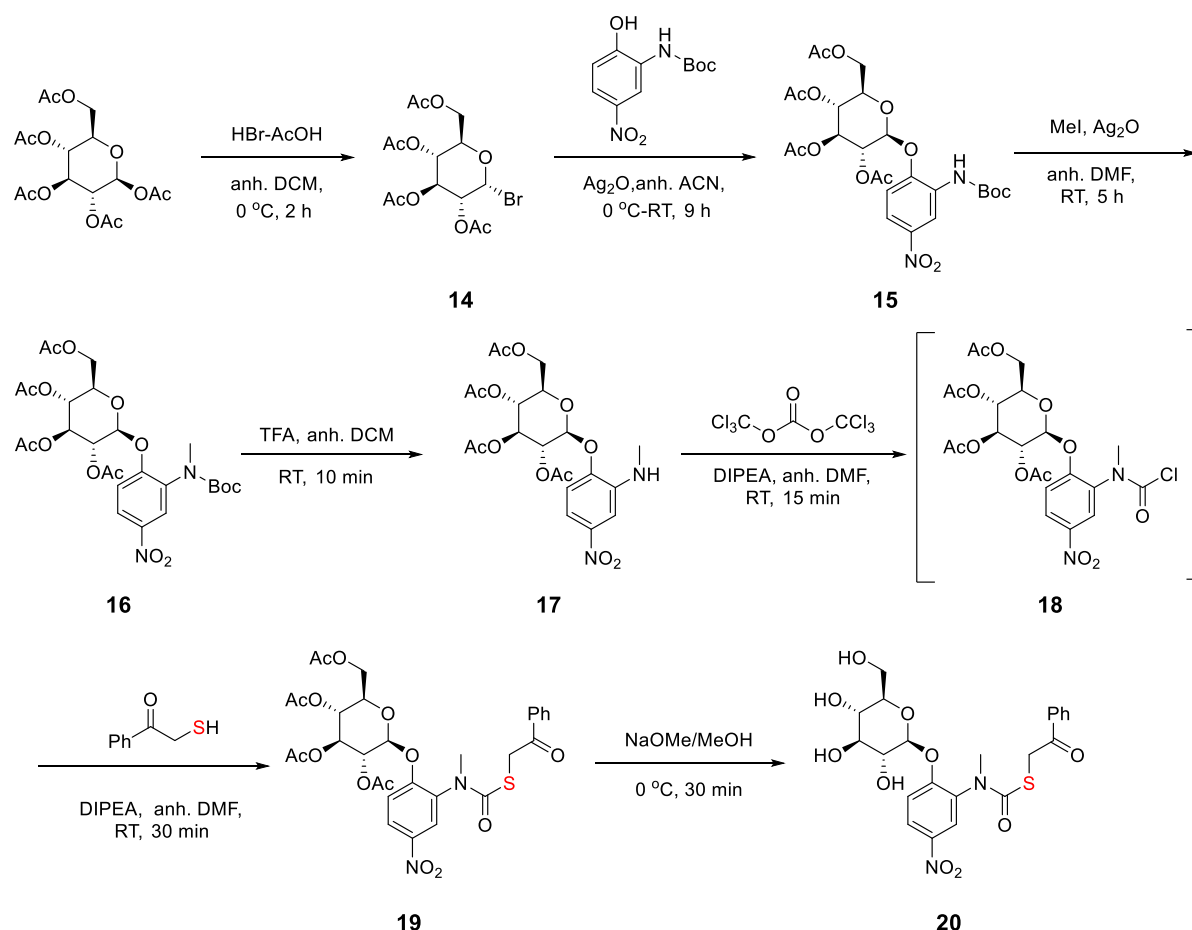


**Scheme 4.1:** Design of the  $\beta$ -glycosidase sensitive artificial substrates of 3-MST.

## 4.2. Results and Discussion

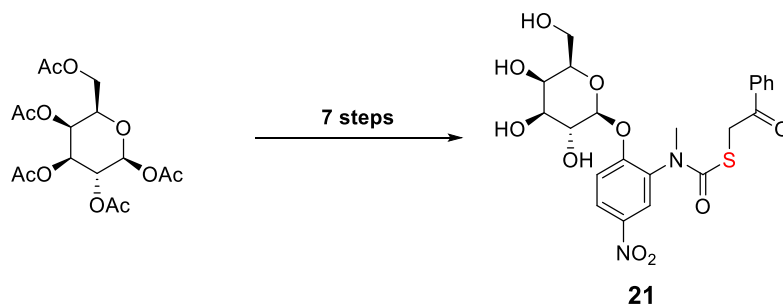
### 4.2.1. Synthesis

The glucosidase-sensitive 3-MST artificial substrate **20** was synthesized *via* 7-step reactions starting from  $\beta$ -D-glucose pentaacetate (Scheme 4.2). Tetra acetyl- $\alpha$ -D-glucopyranosyl bromide (**14**) was synthesized from corresponding  $\beta$ -D-glucose pentaacetate using HBr-AcOH using a reported method.<sup>8</sup> Compound **14** was then reacted with *tert*-butyl (2-hydroxy-5-nitrophenyl)carbamate in presence of silver oxide ( $\text{Ag}_2\text{O}$ ) to give compound **15** in 80% yield. *N*-methylation was done to **15** by using methyl iodide and  $\text{Ag}_2\text{O}$  to form **16** in 90% yield. Boc deprotection was performed with **16** to obtain **17** in 89% yield. Further, **17** was reacted with triphosgene to obtain a formyl intermediate (**18**) which was then further coupled with phenacyl thiol to obtain **19** in 59% yield. Finally, deacetylation reaction was carried out with **19** in the presence of sodium methoxide to obtain the glucosidase 3-MST artificial substrate **20** in 61% yield.



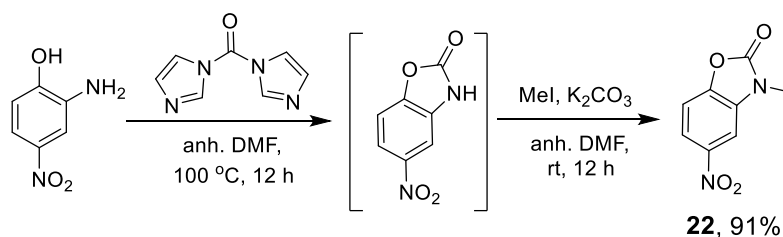
**Scheme 4.2:** Synthesis for  $\beta$ -glucosidase activated 3-MST artificial substrate **20**.

Similarly, the galactosidase-sensitive 3-MST artificial substrate **21** was synthesized by a colleague *via* a 7-step reaction scheme starting from  $\beta$ -D-galactose pentaacetate (Scheme 4.3).<sup>5</sup>



**Scheme 4.3:** Synthesis for  $\beta$ -galactosidase activated 3-MST artificial substrate **21**. (Credit: Dr. Prerona Bora and Ms. Simran Gupta).

A control compound **22** was prepared (Scheme 4.4). 2-amino 4-nitrophenol was reacted with 1,1'-Carbonyldiimidazole to form a bicyclic intermediate which was then *N*-methylated using methyl iodide.



**Scheme 4.4:** Synthesis of control compound **22**.

#### 4.2.2. Studies to determine stability, decomposition and turnover of the substrate

##### 4.2.2.1. HPLC-based decomposition study of **20** and **21** in the presence of $\beta$ -glucosidase and $\beta$ -galactosidase respectively under ambient conditions

Having both the 3-MST artificial substrates in hand, the efficiency of **2a** release from these compounds was evaluated. Compounds **20** and **21** were incubated with  $\beta$ -glucosidase (10 U/mL) and  $\beta$ -galactosidase (4 U/mL) respectively in pH 7.4 PBS (1 $\times$ ) at 37 °C. As monitored by HPLC, the disappearance of **20** (Retention time (RT) = 6.5 min) and **21** (RT = 6.5 min) in a time-dependent manner was seen (Figure 4.1). However, **20** remained even after 9 h of incubation, whereas **21** was completely consumed within 120 min. In cases of both **20** and **21**, the gradual appearance of the peak for the intermediate, **22** and released substrate (**2a**) were observed at RT of 8.7 min and 9.9 min respectively. Also, a peak for disulfide (**ds1**, RT = 13.7 min) was observed which was formed as a result of the aerial oxidation product of **2a**.

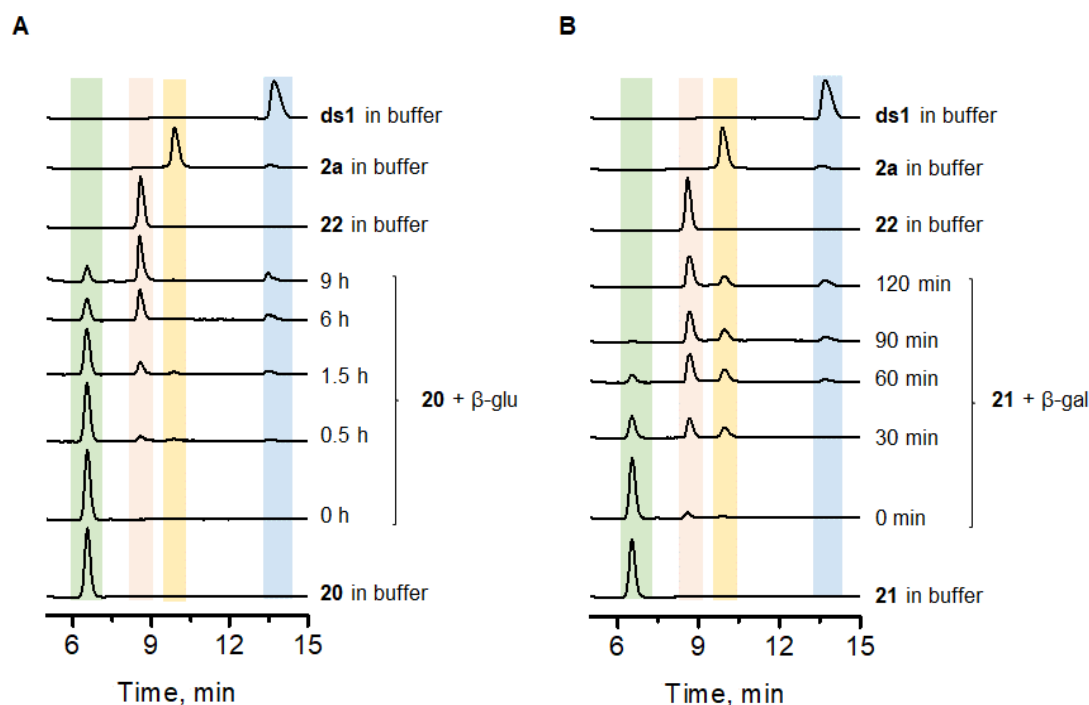


**Figure 4.1:** Decomposition of 3-MST artificial substrates and gradual formation of **2a** and **22** (Abs = 250 nm). (A) HPLC traces for the reaction mixture containing **20** in the presence of  $\beta$ -glucosidase (10 U/mL) over 9 h. (B) HPLC traces for the reaction mixture containing **21** in the presence of  $\beta$ -galactosidase (10 U/mL) over 2 h. An aerial oxidation product of **2a** (**ds1**) was also observed during the course of the reactions.

Overall HPLC data suggested that these glycosidase-sensitive donors were activated by their respective glycosidase and produced the **2a**. The rate of **2a** release from **21** was found to be higher than that of **20**.

#### 4.2.2.2. Docking analysis to rationalize **2a** release rate from **20** and **21**

In order to rationalize the difference of **2a** release rate among **20** and **21**, a docking study was performed with the substrates with their respective crystal structure of  $\beta$ -glycosidases (with T. Anand Kumar, Prof. Harinath Chakrapani Lab, IISER Pune). When **20** was docked into the active site of cyanogenic  $\beta$ -glucosidase from white clover (PDB = 1CBG), lowest energy ( $\Delta G = -9.2$  kcal/mol) conformation showed unfavourable binding (an unfavourable distance of 12.6 Å between catalytic glutamic acid (E397) residue to the glycosidic carbon) for the glycosidic bond hydrolysis reaction (Figure 4.2A). The docked ligands were shown in stick model and the active site residues are indicated by 1-letter code. The hydrogen bonding and pi-pi interactions are drawn as dotted lines and the lengths are indicated in Å unit. The figures were generated using PyMOL v 2.0. Further docking analysis suggests that a favourable (a favourable distance

of 6.8 Å between E397 residue to the glycosidic carbon) conformation for the glycosidic bond hydrolysis reaction demands relatively higher energy ( $\Delta G = -7.9$  kcal/mol) (Figure 4.2B). Using similar docking protocol, when **21** was docked into the active site of  $\beta$ -galactosidase from *E. coli* (PDB ID: 1JYN), lowest energy ( $\Delta G = -9.8$  kcal/mol) conformation shows a highly favourable binding interaction (a favourable distance of 3.8 Å between catalytic glutamine residue (Q537) to the glycosidic carbon) for the glycosidic bond hydrolysis (Figure 4.2C).

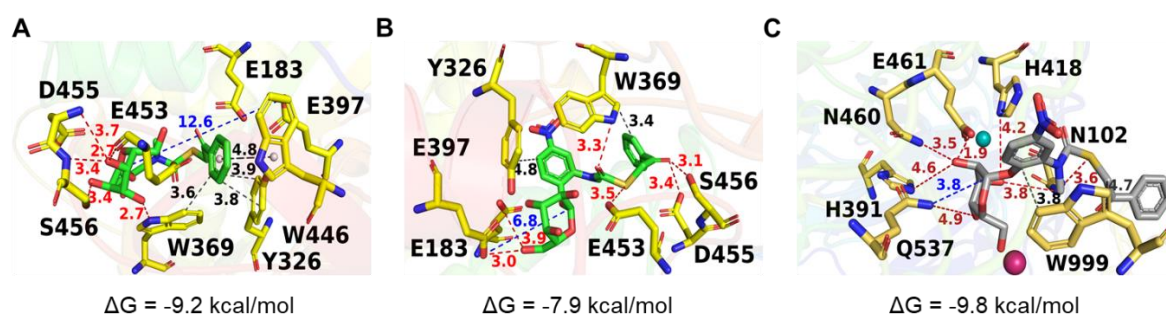


**Figure 4.2:** Cartoon representation of docked compounds. (A) **20** into the active site of  $\beta$ -glucosidase in the lowest energy conformation and (B) **20** into the active site of  $\beta$ -glucosidase in a higher energy conformation. The distance of the glycosidic carbon from the nucleophilic E397 is drawn as blue line with the indicated length. (C) **21** into the active site of cyanogenic  $\beta$ -galactosidase in the lowest energy conformation. The distance of the glycosidic carbon from the nucleophilic Q537 is drawn as blue line with the indicated length. For fig A-C, the hydrogen and pi-pi interactions are drawn as red and gray dotted lines respectively. Docking figures were provided by T. Anand Kumar (Prof. Harinath Chakrapani Lab, IISER Pune).

Overall analysis suggests that the active site of these enzymes is highly conserved and very specific to their substrate. Therefore, **2a** release from **21** is faster due to the favourable binding of the molecule with  $\beta$ -galactosidase at lowest energy conformation which is lacking in the case of **20**.

#### 4.2.2.3. HPLC-based stability study of **20** and **21** in buffer and in a presence of GSH

Next, the stability of these 3-MST artificial substrates was evaluated in pH 7.4 buffer and reducing environment over a period at 37 °C. Compounds **20** and **21** were incubated in pH 7.4 PBS (1×) at 37 °C. The peak corresponding to these 3-MST artificial substrates remained unchanged which suggests these 3-MST artificial substrates were stable in the buffer (Figure 4.2). Next, cellular stability of 3-MST artificial substrates **20** and **21** towards GSH (10 mM) was studied using HPLC. Both compounds remained unchanged supporting their stability in the presence of GSH rich environment (Figure 4.3).

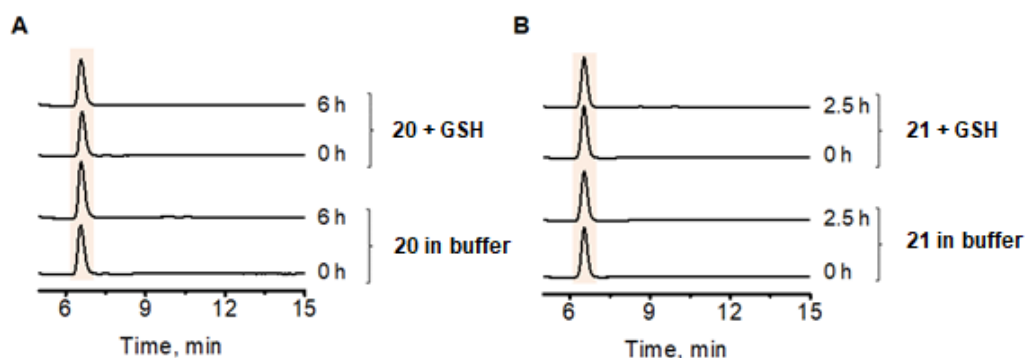
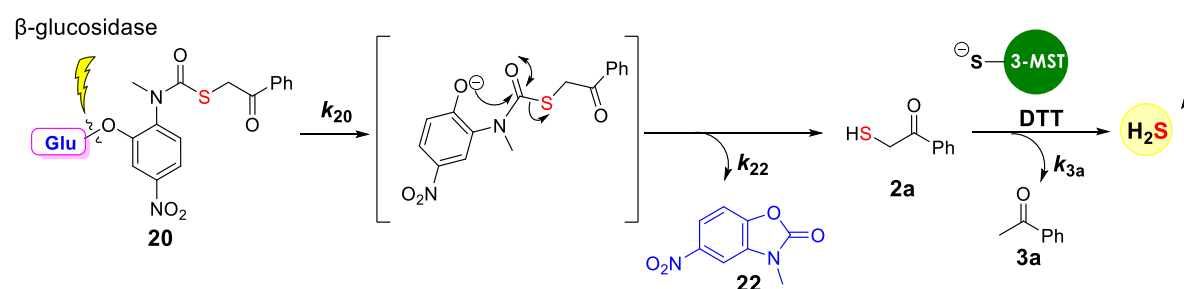


**Figure 4.3:** Stability of 3-MST artificial substrates in buffer and in presence of cellular thiol, GSH. (A) HPLC traces for the reaction mixture containing **20** in buffer and in the presence of GSH over 6 h. (A) HPLC traces for the reaction mixture containing **21** in buffer and in the presence of GSH over 2.5 h.

#### 4.2.2.4. HPLC-based study for 3-MST artificial substrates (**20** and **21**) activation followed by turnover in presence of 3-MST

After validating the 3-MST artificial substrates activation and the 3-MST artificial substrates stability the turnover of the released substrate was evaluated in the presence of 3-MST.

**Decomposition of **20** in the presence of  $\beta$ -glucosidase and substrate (**2a**) turnover under reducing condition (DTT):** Compound **20** was incubated with  $\beta$ -glucosidase (10 U/mL), *b*3-MST (1  $\mu$ M) and DTT (1 mM) in pH 7.4 PBS (1 $\times$ ) at 37  $^{\circ}$ C. As monitored by HPLC, **20** did not completely consume till 12 h (Scheme 4.5 and Figure 4.4).



**Scheme 4.5:** Decomposition of **20** in presence of  $\beta$ -glucosidase followed by **2a** turnover by 3-MST.

The rate decomposition of **20** ( $k_{20}$ ) was calculated to be  $0.27 \pm 0.03 \text{ h}^{-1}$  ( $R^2 = 0.9929$ ) which suggests slower activation by the  $\beta$ -glucosidase. The appearance of three new peaks attributable to the formation of byproduct (**22**), substrate (**2a**) release followed by turnover of **2a** and formation of acetophenone (**3a**) was observed. The rate of formation of **22** ( $k_{22}$ ) and **3a** ( $k_{3a}$ ) was calculated to be  $0.28 \pm 0.03 \text{ h}^{-1}$  ( $R^2 = 0.9951$ ) and  $0.25 \pm 0.04 \text{ h}^{-1}$  ( $R^2 = 0.9904$ ) respectively.

Overall, HPLC study suggests that **20** is specifically activated by  $\beta$ -glucosidase to yield **2a** which is then further turned over by 3-MST, and in the presence of DTT, forms **3a** and **22** as the byproducts.

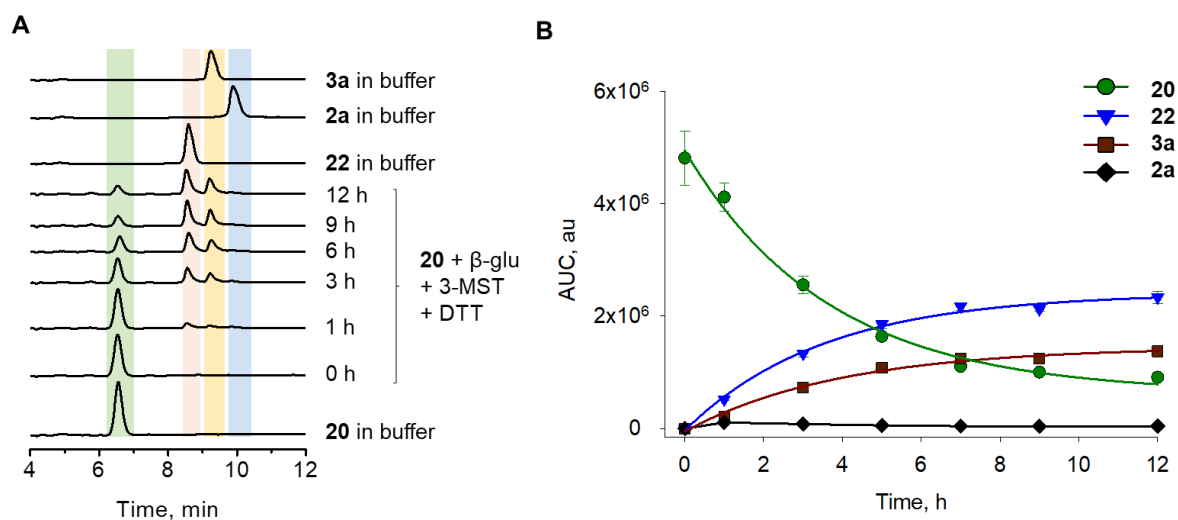
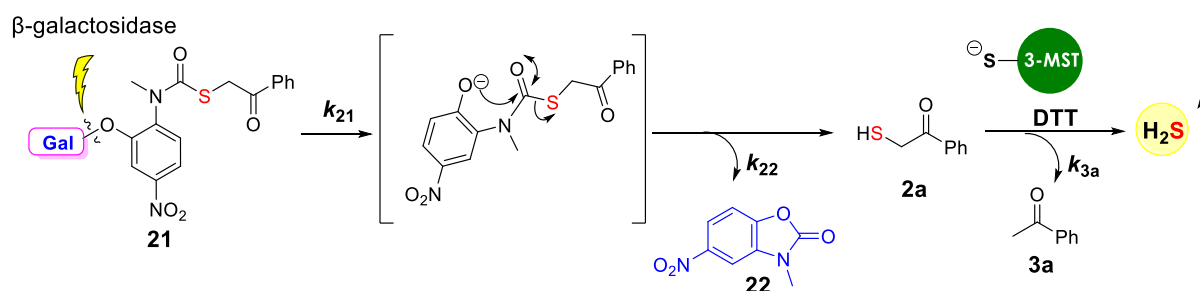


**Figure 4.4:** Decomposition of **20** and gradual formation of **2a** and **22** followed by turnover of **2a** by 3-MST and finally the formation of **3a** (Abs = 250 nm). (A) HPLC traces for: **20** +  $\beta$ -glu + b3-MST and conversion of **20** to **2a**, and the turnover of **2a** to **3a** by 3-MST in presence of DTT over 12 h. (B) Kinetic profile for decomposition of **20**, formation of **22**, formation of **2a** and turnover of **2a** to **3a**. For the reaction with **20** with 3-MST, the area under the curve for **20** was computed and was used to determine the rate constant for the decomposition of **20**. The data was fit using the pseudo first-order equation  $y = y_0 + ae^{-kx}$ . Similarly, the area under the curve for **3a** and **22** was computed and was used to determine the rate constant for **3a** and **22**. The data was fit using the pseudo first-order equation  $y = y_0 + a(1 - e^{-kx})$ .

**Decomposition of 21 in the presence of  $\beta$ -galactosidase and substrate (2a) turnover under reducing condition (DTT):** Compound **21** was incubated  $\beta$ -galactosidase (4 U/mL), b3-MST (1  $\mu$ M) and DTT (1 mM) in pH 7.4 PBS (1 $\times$ ) at 37  $^{\circ}$ C. As monitored by HPLC, **21** was consumed within 150 min (Scheme 4.6 and Figure 4.5).



**Scheme 4.6:** Decomposition of **21** in presence of  $\beta$ -galactosidase followed by **2a** turnover by 3-MST.

The rate of decomposition of **21** ( $k_{21}$ ) was calculated to be  $2.37 \pm 0.09 \text{ h}^{-1}$  ( $R^2 = 0.9994$ ) which suggests faster activation by the  $\beta$ -galactosidase. The appearance of three new peaks attributable to the formation **22**, **2a** release followed by turnover of **2a** and formation of **3a** was observed. The rate of formation of **22** ( $k_{22}$ ) and **3a** ( $k_{3a}$ ) were calculated to be  $2.17 \pm 0.37 \text{ h}^{-1}$  ( $R^2 = 0.9884$ ) and  $0.91 \pm 0.13 \text{ h}^{-1}$  ( $R^2 = 0.9955$ ) respectively. Overall HPLC study suggests that **21** specifically gets activated by  $\beta$ -galactosidase to yield **2a** which is then further turned over by 3-MST in presence of DTT and form **3a** and **22** as the byproducts.

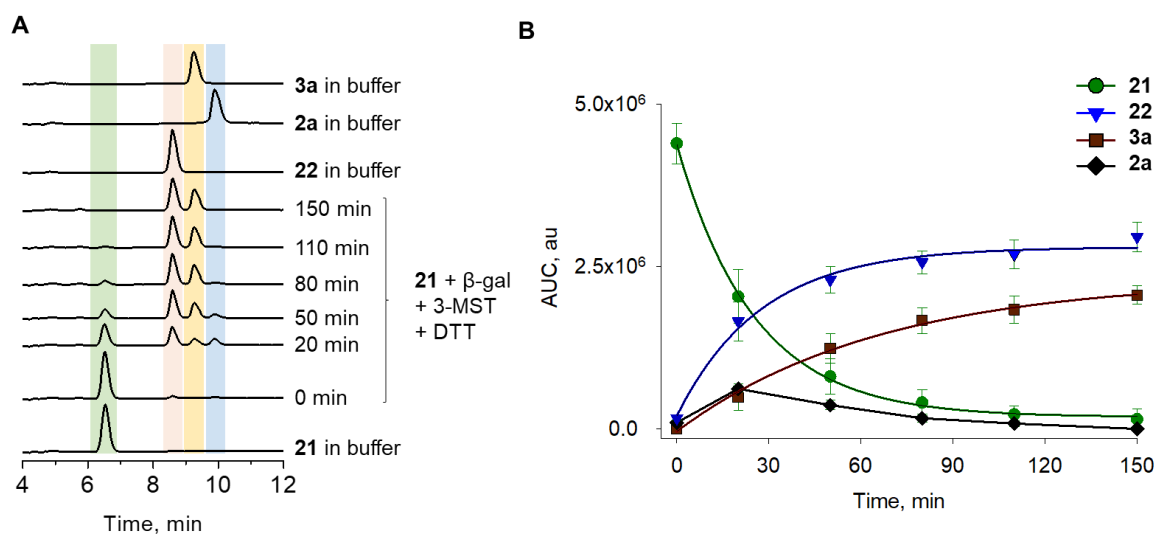


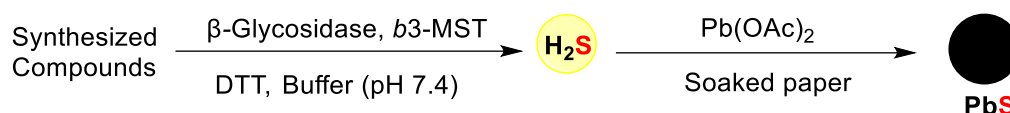
**Figure 4.5:** Decomposition of **21** and gradual formation of **2a** and **22** followed by turnover of **2a** by 3-MST and finally the formation of **3a** (Abs = 250 nm). (A) HPLC traces for: **21** +  $\beta$ -gal + b3-MST and conversion of **21** to **2a**, and the turnover of **2a** to **3a** by 3-MST in presence of DTT over 12 h. (B) Kinetic profile for the decomposition of **21**, formation of **22**, **2a** and turnover of **2a** to **3a**. For the reaction of **21** with 3-MST, the area under the curve for **21** was computed and was used to determine the rate constant for decomposition. The data was fit using the pseudo first-order equation  $y = y_0 + ae^{-kx}$ . Similarly, The area under the curve for **3a** and **22** was computed and was used to determine the rate constant for **3a** and **22**. The data was fit using the pseudo first-order equation  $y = y_0 + a(1 - e^{-kx})$ .

Overall HPLC studies suggested that 3-MST artificial substrates were specifically activated by their respective  $\beta$ -galactosidase to yield **2a** which is then further turned over by 3-MST in presence of DTT and forms **3a** and **22** as the byproducts. Also, it has been observed that 3-MST artificial substrate kinetics with **20** is much slower than that of **21**.

### 4.2.3. H<sub>2</sub>S generation from **20** and **21** in presence of 3-MST and DTT

#### 4.2.3.1. H<sub>2</sub>S detection using lead acetate assay

After demonstrating  $\beta$ -glycosidase induced 3-MST artificial substrates (**20** and **21**) activation using HPLC, next, under these conditions we attempted to detect the ability of the H<sub>2</sub>S release of the 3-MST artificial substrates using the lead acetate assay as discussed in Chapter 3.



**Scheme 4.7:** Detection of H<sub>2</sub>S via lead acetate assay.

In this assay, **20** and **21** were incubated with  $\beta$ -glucosidase (10 U/mL) and  $\beta$ -galactosidase (4 U/mL) respectively, along with *b3*-MST (1  $\mu$ M) and DTT (1 mM) in PBS (1 $\times$ ) at 37  $^{\circ}$ C. The assay was performed in a 96 well plate and the plate was covered with 5% Pb(OAc)<sub>2</sub> soaked paper and the reaction was incubated for 2.5 h. It has been observed that **20** and **21** only in the presence of their respective  $\beta$ -glycosidase and *b3*-MST release H<sub>2</sub>S (Figure 4.6). A Lower yield of H<sub>2</sub>S was observed for **20** which can be correlated with the slower rate of acetophenone formation as demonstrated previously using HPLC. **1a** was used as a positive control for this assay, therefore intense spot for PbS was observed. **22** was used as negative control which failed to produce H<sub>2</sub>S in the presence of *b3*-MST. As expected, other controls did not produce a significant amount of H<sub>2</sub>S.

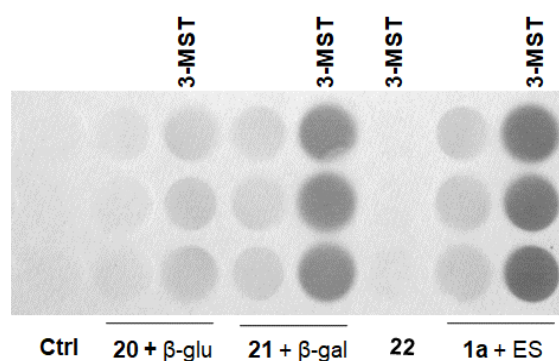
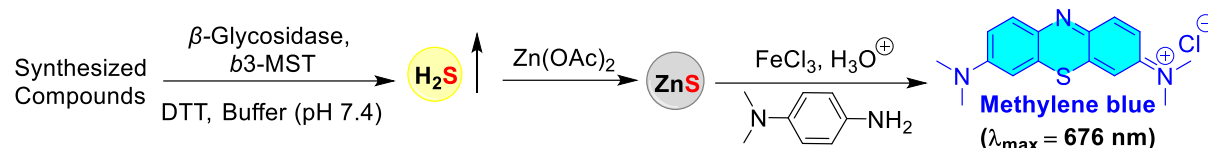


**Figure 4.6:** H<sub>2</sub>S generation by the 3-MST artificial substrates in presence of *b3*-MST. **1a** + Es, **20** +  $\beta$ -glu and **21** +  $\beta$ -gal were incubated with *b3*-MST and DTT for 2.5 h. H<sub>2</sub>S generation was detected using lead acetate assay. **22** was used as a negative control. Ctrl represents only 3-MST enzyme.

#### 4.2.3.2. H<sub>2</sub>S detection using Methylene blue assay

Along with H<sub>2</sub>S detection for the compounds via lead acetate assay, methylene blue assay was carried out as described in Chapter 2.



**Scheme 4.8:** Detection of H<sub>2</sub>S *via* methylene blue assay.

In this assay, **20** and **21** were incubated with  $\beta$ -glucosidase (10 U/mL) and  $\beta$ -galactosidase (4 U/mL) respectively, along with *b3*-MST (1  $\mu$ M), Zn(OAc)<sub>2</sub> (400  $\mu$ M) and DTT (1 mM) in PBS (1 $\times$ ) at 37 °C. At predetermined time point aliquots from the reaction mixture were treated with methylene blue reagents and mixed. The final reaction mixture was then transferred to the 96-well plate and absorbance was recorded. It has been observed that **20** has low methylene blue absorbance of H<sub>2</sub>S compared to **21** (Figure 4.7). **1a** was used as a positive control for this assay which gives a significant absorbance. Negative control **22** and other controls also did not produce a significant amount of H<sub>2</sub>S.

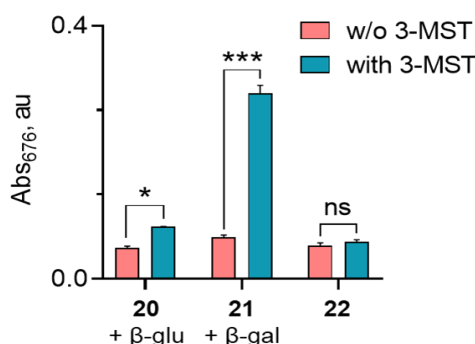


**Figure 4.7:** H<sub>2</sub>S generation by the 3-MST artificial substrates in presence of *b3*-MST. **20** +  $\beta$ -glu and **21** +  $\beta$ -gal were incubated with *b3*-MST and DTT for 2.5 h. H<sub>2</sub>S generation was detected using methylene blue assay. Results are expressed as mean  $\pm$  SD (n = 3) [(\*\*\*), p < 0.001 vs w/o MST]. **22** was used as a negative control. ns represents not significant.

#### 4.2.4. 3-MST persulfide detection using clickable tag switch assay

Next, formation of 3-MST persulfide in the presence substrate (**20** and **21**) detected using a clickable tag switch assay as described in Chapter 3. In this assay, the 3-MST artificial substrates (**20** and **21**) were incubated with respective  $\beta$ -glycosidases and 3-MST for the generation of 3-MST persulfide. Similarly, **1a** was taken as a positive control and esterase was

used for the activation. **22** was used as a negative control. An intense band corresponding to the **1a** treated lane shows 3-MST persulfidation (Figure 4.8). An insignificant band for **20** treated lane indicates 3-MST artificial substrate activation is very slow for **20** which resulted a negligible 3-MST persulfide formation. A significantly intense band was seen for **21** treated lane indicating 3-MST persulfide formation. As expected, the other control group did not show significant persulfidation.

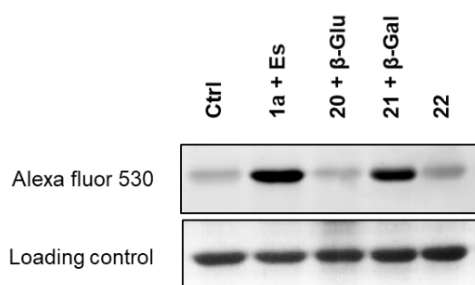


**Figure 4.8:** Persulfidation of *b3*-MST by the 3-MST artificial substrates. **1a** + Es, **20** +  $\beta$ -glu and **21** +  $\beta$ -gal were incubated with *b3*-MST, 3-MST persulfidation was detected using the clickable tag-switch assay. Ctrl represents only 3-MST enzyme. Coomassie brilliant blue (CBB) staining was used as a loading control.

#### 4.2.5. Sulfane sulfur detection using SSP-2 assay

Persulfidated 3-MST is known to react with one or more substrates and forms small molecule sulfane sulfur. Therefore, sulfane sulfur was detected using a reported fluorescent-based assay using an SSP-2 probe as described in Chapter 3.<sup>9</sup> In this assay,  $\beta$ -glycosidases and 3-MST were incubated with the 3-MST artificial substrates (**20** and **21**) for the generation of 3-MST persulfide. Similarly, **1a** was taken as positive control and esterase was used for the activation. **22** was used as a negative control. At a pre-determined time point, the SSP-2 probe was added and incubated. A turn-on fluorescence for **1a** in presence of 3-MST was observed which indicates sulfane sulfur formation (Figure 4.9). In the presence of 3-MST, a negligible enhancement of fluorescent signal was observed for **20** which implies lower sulfane sulfur formation as 3-MST artificial substrate activation is very slow. A strong fluorescence response was observed for **21** in presence of 3-MST which indicates significant sulfane sulfur formation. As DTT was known to reduce sulfane sulfur therefore sulfane sulfur formation was significantly reduced for the DTT-treated groups. As expected, other control groups did not show significant sulfane sulfur generation.

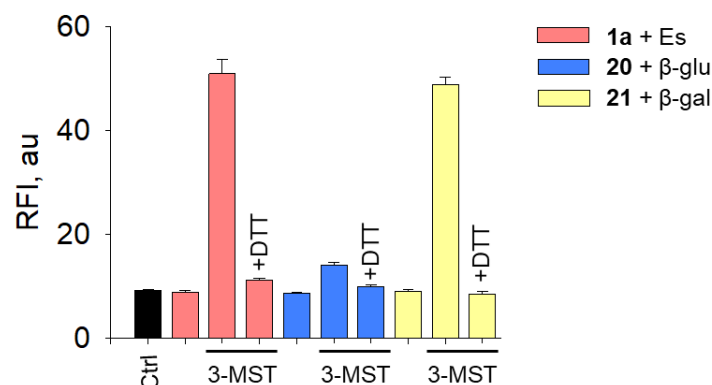


**Figure 4.9:** Sulfane sulfur detection using SSP-2 probe (Fluorescence intensities were measured;  $\lambda_{\text{ex}} = 482 \text{ nm}$ ,  $\lambda_{\text{em}} = 518 \text{ nm}$ ). **1a** + Es, **20** +  $\beta$ -glu and **21** +  $\beta$ -gal were incubated with *b*3-MST followed by treatment with the probe SSP-2. +DTT refers to the post-treatment of the enzyme with DTT to reduce sulfane sulfur at the active cysteine residue of 3-MST. Ctrl represents only enzyme.

#### 4.2.6. MTT assay for cell viability of the key compounds

The cell viability of **20**, **21** and **22** were determined using MTT assay as described in Chapter 3. Mouse embryonic fibroblast (MEF) cells were treated with variable concentrations of **20**, **21** and **22** and incubated for 24 h and finally cell viability was measured. Cells are found to be more than 80% viable for the treated concentrations of all the compounds which indicates the compounds were well tolerated by the cells for 24 h with no significant cytotoxicity (Figure 4.10).

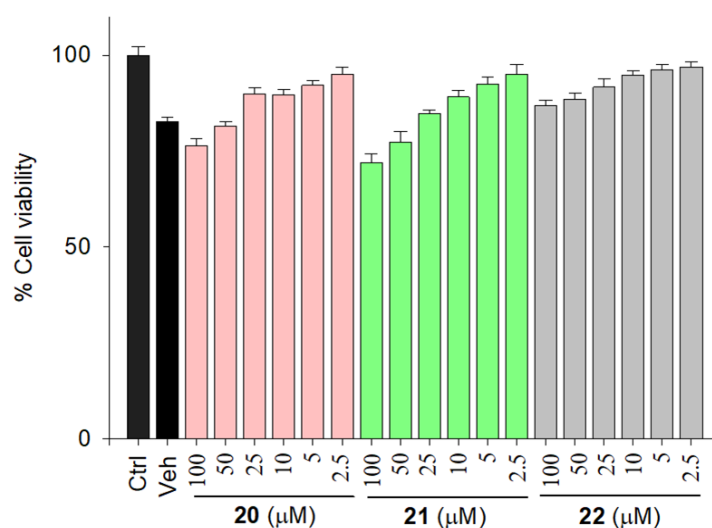


**Figure 4.10:** Cell viability was determined in MEFs cells in presence of **20**, **21** and **22** using MTT assay.

#### 4.2.7. Intracellular H<sub>2</sub>S detection using NBD-fluorescein dye

Intracellular H<sub>2</sub>S was detected using NBD-fluorescein (NBD-F) dye as described in Chapter 3.<sup>10</sup> MEF cells are co-incubated with the 3-MST artificial substrate **21** and NBD-F dye. After this, the cell's turn-on fluorescence was imaged in a fluorescence microscope. It was observed that cells treated with **21** gave an intense turn on fluorescence while **22** failed to give significant fluorescence under the assay conditions (Figure 4.11). These results indicate that **21** is permeable and is activated by the intracellular  $\beta$ -galactosidase enzyme and releases active substrate **2a**, which is turned over by 3-MST to enhance sulfane sulfur.

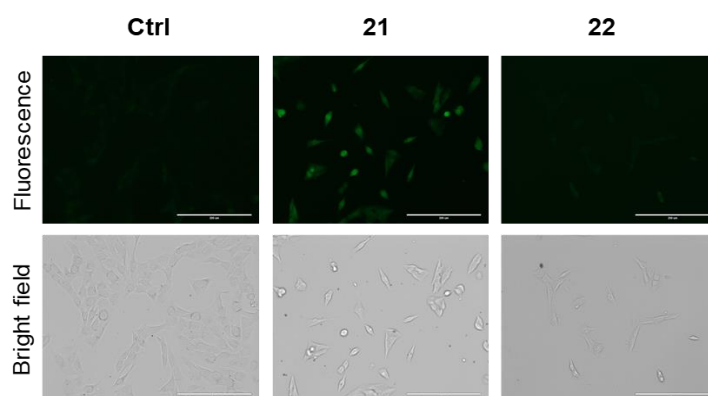
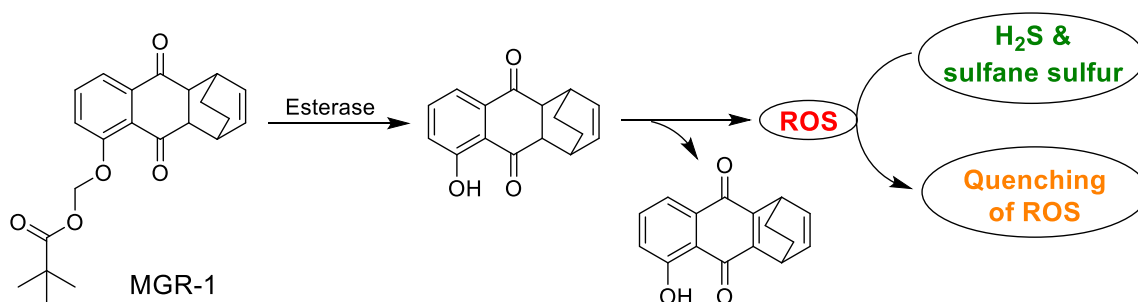


**Figure 4.11:** H<sub>2</sub>S detection in MEF cells using NBD-Fluorescein dye in presence of **21**. Enhanced fluorescence signal corresponding to H<sub>2</sub>S. Control compound **22** did not increase the fluorescence signal. Ctrl refers to untreated cells. The scale bar represents 200  $\mu$ m.

#### 4.2.8. Protection from oxidative stress using MTT assay

Since H<sub>2</sub>S and sulfane sulfur are known to mitigate oxidative stress, therefore H<sub>2</sub>S and sulfane sulfur releasing 3-MST artificial substrate's ability for attenuating oxidative stress under cellular environment was measured. A cell viability assay was performed in the presence of MGR-1, a known inducer of oxidative stress was used for the assay (Scheme 4.9).



**Scheme 4.9:** ROS production from MGR-1 in presence of esterase, which was further quenched by H<sub>2</sub>S and sulfane sulfur.

MEF cells are initially treated with 3-MST 12.5  $\mu$ M of **21** for 4 h. Next, 25  $\mu$ M of MGR-1 was added and incubated for 4 h. Finally, cell viability was evaluated using MTT assay as discussed previously. The result showed that **21** was able to protect cells from MGR-1 induced oxidative stress (Figure 4.12). No significant protection was observed for negative control **22**. Hence, it can be inferred that  $\beta$ -galactosidase present in the cells activates the 3-MST artificial substrate thereby it releases  $H_2S$  and sulfane sulfur inside cells which give protection from ROS.

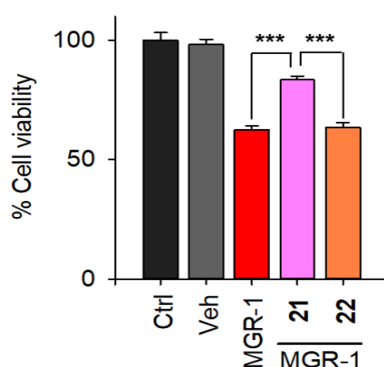


**Figure 4.12:** Cytoprotective effects of compound **21** against MGR-1 (25  $\mu$ M). Results are expressed as mean  $\pm$ SD ( $n = 3$ ) [(\*\*\*),  $p < 0.001$  vs MGR-1]. A similar assay was conducted with **22**. An insignificant effect on the percentage cell viability during incubation of **22** with MGR-1 was observed.

### 4.3. Summary

To summarize,  $\beta$ -glucosidase and  $\beta$ -galactosidase-activated artificial substrates for 3-MST (**20** and **21**) were designed and synthesized. Modified substrate activation followed by turnover of the active substrate (**2a**) by 3-MST was validated using HPLC. Furthermore,  $H_2S$  release was monitored for both **20** and **21** using lead acetate and methylene blue assay. 3-MST persulfide formation was demonstrated by clickable tag switch assay. The formation of sulfane sulfur was observed using an SSP-2 probe. Overall, **20** has a slower rate of  $H_2S$  and sulfane sulfur release when compared with **21** that may be useful for extended-release applications. Finally, the cell viability of these 3-MST artificial substrates and by-products showed that the prodrugs and by-products are benign under experimental concentrations. Compound **21** was able to enhance cellular  $H_2S$  level which was detected using NBD-fluorescein dye. Also, cells pre-treated with **21** were protected from oxidative stress-induced cell death. Collectively, our data suggest that **20** and **21** cell permeable artificial substrates of 3-MST which and these 3-MST artificial substrates can be used in oxidative stress conditions and mitigating inflammation.

## 4.4. Experimental Procedures

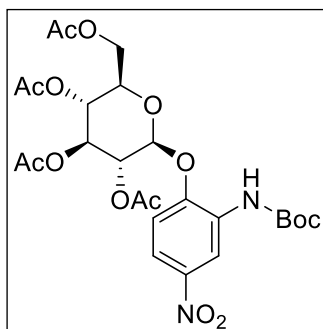
### 4.4.1. Synthesis and characterization of compounds

**General methods:** All reactions were conducted under a 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 silica gel Spectrochem (100-200 mesh) as the stationary phase.  $^1\text{H}$  NMRs were recorded on a JEOL 400 MHz (100 MHz for  $^{13}\text{C}$ ) or a Bruker 400 MHz (100 MHz for  $^{13}\text{C}$ ) spectrometer unless otherwise specified using either residual solvent signals  $\text{CDCl}_3$  ( $\delta_{\text{H}} = 7.26$  ppm,  $\delta_{\text{C}} = 77.2$  ppm). Chemical shifts ( $\delta$ ) are reported in ppm and coupling constants ( $J$ ) in Hz. The following abbreviations are used: m (multiplet), s (singlet), t (triplet). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using a BRUKER-ALPHA FT-IR spectrometer.

Compounds **1a**<sup>11</sup>, **2a**<sup>12</sup> and **ds1**<sup>12</sup> were synthesized using a previously reported protocol. All analytical data matches the reported values.

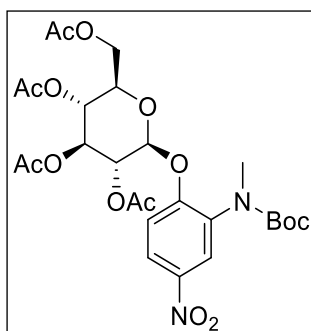
(2*R*,3*R*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(2-((*tert*-butoxycarbonyl)amino)-4-nitrophenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**15**): Firstly, *tert*-butyl (2-hydroxy-5-nitrophenyl)carbamate and (2*R*,3*R*,4*S*,5*R*,6*R*)-2-(acetoxymethyl)-6-bromotetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**14**) was synthesized using reported protocols.<sup>8,13</sup> Next, To 15 mL anhyd. acetonitrile, *tert*-butyl (2-hydroxy-5-nitrophenyl)carbamate (2 g, 7.8 mmol) and  $\text{Ag}_2\text{O}$  (1.8 g, 7.8 mmol) was added, purged with  $\text{N}_2$  and cooled in ice. Then freshly prepared compound **14** (2.2 g, 1 eq) dissolved in anhyd. ACN (15 mL) was slowly added to the reaction. The resulting mixture was stirred at room temperature for 9 h. Upon completion of the reaction as monitored by TLC, the reaction mixture was filtered through celite and washed with chloroform. The filtrate was evaporated to obtain the crude product which was diluted with  $\text{H}_2\text{O}$  (30 mL) and extracted with  $\text{CHCl}_3$  (30 mL). The organic layers were washed with brine then the resultant combined layers were passed through anhyd.  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (32-34% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.

Yield (2.5 g, 80%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  9.07 (s, 1H), 7.86 (dd,  $J = 9.0, 2.7$  Hz, 1H), 7.12 (s, 1H), 7.08 (d,  $J = 9.0$  Hz, 1H), 5.38-5.31 (m, 2H), 5.17 (m, 1H), 5.1 (m, 1H), 4.32-4.28 (m, 1H), 4.22-4.18 (m, 1H), 3.95-3.91 (m, 1H), 2.11-2.06 (12H), 1.55 (s, 9H);  $^{13}\text{C}$  NMR (100



MHz, CDCl<sub>3</sub>):  $\delta$  170.5, 170.1, 169.9, 169.5, 166.1, 152.3, 148.9, 144.1, 130.3, 130.3, 118.0, 114.3, 99.7, 81.9, 72.6, 72.0, 71.2, 68.2, 61.8, 28.4, 20.9, 20.8, 20.7; HRMS for C<sub>25</sub>H<sub>32</sub>N<sub>2</sub>O<sub>14</sub> [M+Na]<sup>+</sup>: Calculated: 607.1751, Found: 607.1766.

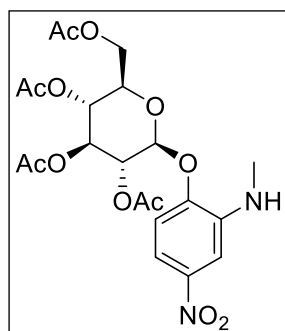
(2*R*,3*R*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(2-((*tert*-butoxycarbonyl)(methyl)amino)-4-nitrophenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**16**): Compound **33** (2.5 g, 1 eq) was dissolved in anh. DMF, then Ag<sub>2</sub>O (5.95 g, 6 eq) was added. Then methyl iodide (3.2 mL, 12 eq) was added and stirred at room temperature for 5 h. Upon completion of the reaction as monitored by TLC, the reaction mixture was filtered through celite and washed with chloroform. The filtrate was evaporated under reduced pressure to obtain the crude product. The compound was further purified by silica gel column chromatography (36-38% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.



Yield (2.3 g, 90%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  8.14-8.11 (m, 2H), 7.22 (bs, 1H), 5.30-5.17 (m, 4H), 4.29-4.19 (m, 2H), 3.94-3.90 (m, 1H), 3.12 (s, 3H), 2.07-2.06 (12H), 1.56-1.27 (m, 9H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  170.5, 170.3, 169.5, 169.1, 157.2, 143.2, 128.7, 116.3, 98.7, 98.7, 98.5, 72.5, 71.0, 68.1, 61.9, 36.8, 28.3, 20.8, 20.7; HRMS for C<sub>26</sub>H<sub>34</sub>N<sub>2</sub>O<sub>14</sub> [M+Na]<sup>+</sup>: Calculated: 621.1907,

Found: 621.1901.

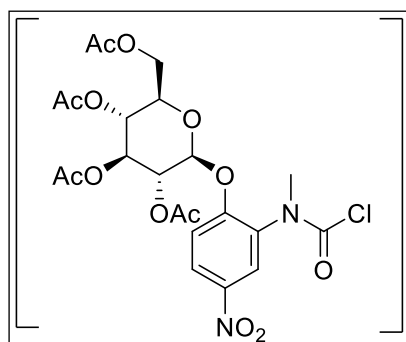
(2*R*,3*R*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(2-(methylamino)-4-nitrophenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**17**): Compound **16** (2.3 g, 1 eq) was dissolved in anh. DCM, then trifluoroacetic acid (11.8 mL, 40 eq) was added, stirred at room temperature for 10 min. Upon completion of the reaction as monitored by TLC, the reaction mixture was quenched with 4*N* NaOH to neutralize the acid. Then crude compound was extracted in DCM (30 mL). The organic layer was washed with brine (30 mL). Then resultant combined layer was passed through anh. Na<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate was evaporated under reduced pressure to obtain the crude product. The crude was further purified by silica gel column chromatography (33-35% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.



30.1, 20.9, 20.8, 20.7, 20.7

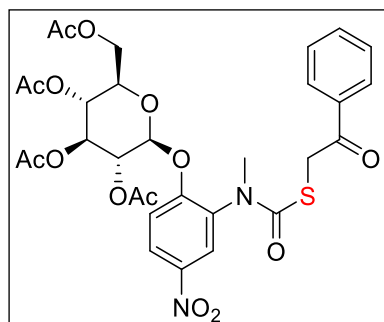
Yield (1.7 g, 89%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.53 (dd,  $J = 8.8$ , 2.6 Hz, 1H), 7.38 (d,  $J = 2.6$  Hz, 1H), 6.9 (d,  $J = 8.8$  Hz, 1H), 5.35-5.30 (m, 2H), 5.17-5.08 (m, 1H), 4.47 (m, 1H), 4.33-4.28 (m, 1H), 4.21-4.17 (m, 1H), 3.94-3.90 (m, 1H), 2.90 (d,  $J = 5.2$  Hz, 3H), 2.09-2.05 (12H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  170.6, 170.2, 170.1, 169.6, 148.1, 144.6, 140.4, 112.6, 112.0, 104.3, 99.3, 72.5, 72.1, 71.2, 68.3, 61.9,

(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-((chlorocarbonyl)(methyl)amino)-4-nitrophenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**18**): Compound **17** (250 mg, 1 eq) was dissolved in anhyd. DCM, N, N-Diisopropylethylamine (DIPEA) (0.74 mL, 5 eq) was added. Then triphosgene (446 mg, 3 eq) was added and stirred for 15 min at room temperature. Upon completion of the reaction as monitored by TLC, the reaction mixture was quenched with 1N HCl. Then crude compound was extracted in DCM (30 mL). The organic layer was washed with brine (30 mL). Then resultant combined layer was passed through anhyd.  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain cpd. **18**. The crude was taken to the next step without further purification and characterization.



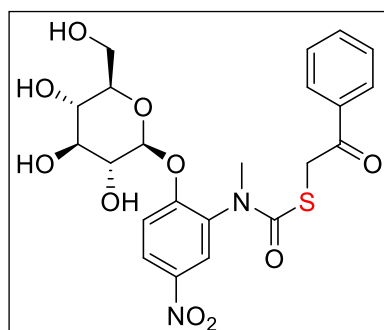
(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-(methyl((2-oxo-2-phenylethyl)thio)carbonyl)amino)-4-nitrophenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**19**): To a freshly synthesized phenacyl thiol (228 mg, 3 eq) dissolved in anhyd. DMF, DIPEA (0.74 mL, 5 eq) was added and purged with  $\text{N}_2$ . Then the synthesized cpd. **16** was dissolved in anhyd. DMF (4 mL) and slowly added to the reaction mixture and stirred at room temperature for 30 min. Upon completion of the reaction as monitored by TLC, the reaction mixture was quenched with 1N HCl. Then crude compound was extracted in ethyl acetate (30 mL). The organic layer was washed (3 $\times$ )  $\text{H}_2\text{O}$  and finally with brine (30 mL). Then resultant combined layer was passed through anhyd.  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced

pressure to obtain the crude product. The crude was further purified by silica gel column chromatography (42-45% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.



Yield (200 mg, 59%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.26 (m, 2H), 8.0 (d,  $J = 7.4$  Hz, 2H), 7.59 (m, 1H), 7.48 (m, 3H), 5.37-5.17 (m, 4H), 4.48-4.25 (m, 4H), 4.0-3.96 (m, 1H), 3.24 (s, 3H), 2.07-1.98 (12H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  193.6, 170.5, 170.2, 169.6, 169.5, 167.0, 158.6, 143.5, 135.8, 133.9, 131.9, 128.9, 128.7, 127.5, 126.3, 118.8, 107.6, 99.3, 72.5, 72.4, 70.7, 68.3, 68.1, 61.9, 38.9, 37.1, 20.8, 20.7, 20.5.

*S*-(2-oxo-2-phenylethyl) methyl(5-nitro-2-(((2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)carbamothioate (**20**): Compound 36 (90 mg, 1 eq) was dissolved in anhyd. methanol (10 mL) at 0 °C, purged with  $\text{N}_2$ . Then sodium methoxide (7.2 mg, 1 eq) was added and stirred for 30 min. Upon completion of the reaction as monitored by TLC, the reaction was quenched by adding an amberlyst-15 resin and subsequently filtered. The methanol was then evaporated and the crude was then purified using reverse phase HPLC using ACN- $\text{H}_2\text{O}$  as the eluent to afford the desired product.

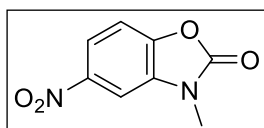


Yield (45 mg, 66%).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  8.37-8.31 (m, 2H), 8.0 (d,  $J = 7.2$  Hz, 2H), 7.67 (t,  $J = 7.2$  Hz, 1H), 7.56-7.49 (m, 3H), 5.46-5.09 (m, 4H), 4.71-4.38 (m, 3H), 3.76-3.45 (m, 3H), 3.30-3.16 (m, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  193.8, 166.4, 159.7, 141.1, 135.8, 133.8, 133.6, 129.6, 128.8, 128.3, 127.0, 126.4, 117.0, 116.4, 101.2, 100.3,

77.5, 76.7, 73.1, 69.6, 60.5, 38.3, 36.5

3-methyl-5-nitrobenzo[d]oxazol-2(3*H*)-one (**22**): Intermediate 5-nitrobenzo[d]oxazol-2(3*H*)-one was prepared from 2-amino-4-nitrophenol. 2-amino-4-nitrophenol (300 mg, 1 eq) was dissolved in anhyd. DMF then carbonyldiimidazole (405 mg, 1.5 eq) was added and the reaction was stirred at 100 °C for 2 h. Upon completion of the reaction as monitored by TLC, the reaction was quenched by  $\text{H}_2\text{O}$  (30 mL) and extracted with ethyl acetate (30 mL). The combined organic layer was washed (3 $\times$ ) with  $\text{H}_2\text{O}$  (30 mL) and brine (30 mL) to remove DMF, passed through  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to afford the crude intermediate which was taken to the next step without further purification.

The crude intermediate (270 mg, 1 eq) was dissolved in anh. DMF (5 mL) and K<sub>2</sub>CO<sub>3</sub> (228 mg, 1.1 eq) was added followed by the addition of methyl iodide (0.85 mL, 10 eq). The reaction mixture was stirred overnight at room temperature. Upon completion of the reaction as monitored by TLC, the reaction mixture was quenched by the addition of water and extracted with ethyl acetate. The combined organic layer was washed (3×) with H<sub>2</sub>O (30 mL) and brine (30 mL) to remove DMF, passed through Na<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate was evaporated under reduced pressure to afford the crude intermediate. The crude was washed with n-pentane (2×) to afford the pure product. Compound **22** was obtained as 265 mg (91%). All analytical data matches the reported values.<sup>14</sup>



#### 4.4.2. HPLC-based studies with **20** and **21**

**General method:** A stock solution of the **20**, **21**, **22**, **2a**, **3a** and **ds1** (10 mM) was prepared in DMSO whereas the stocks of DTT (100 mM) and GSH (100 mM) were prepared fresh in deionized water. The reaction mixtures were prepared in PBS (1×) as required. A stock of 285 μM of wt b3-MST was used. The final concentration of DMSO in the reaction mixture was 1%. At predetermined time points, aliquots were taken from the reaction mixture and injected (25 μL) in a High performance liquid chromatography (HPLC) attached to 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: methanol with a gradient: 35:55:10 → 0 - 1 min, 35:55:10 to 20:70:10 → 1 - 3 min, 20:70:10 → 3 - 9 min, 20:70:10 to 35:55:10 → 9 - 12 min, 35:55:10 → 12 - 17 min and the flow rate was 0.5 mL/min.

##### 4.4.2.1. Decomposition of **20** and **21** in presence of β-Glucosidase and β-Galactosidase respectively under non-reducing conditions

In PBS (1×) pH 7.4 buffer (200 μL), the reaction mixture was prepared using **20** (100 μM) with β-glucosidase (10 U/mL) or **21** (100 μM) with β-galactosidase (4 U/mL), mixed, and then incubated at 37 °C. At predetermined time points, 60 μL aliquots were taken from the reaction mixture, diluted with 60 μL ACN and 25 μL was injected in the HPLC.

#### 4.4.2.2. Stability of **20** and **21** in buffer and in presence of GSH

To determine the stability in buffer, a reaction mixture was prepared using **20** (100  $\mu$ M) or **21** (100  $\mu$ M) in PBS (1 $\times$ ) pH 7.4 buffer (200  $\mu$ L), mixed, and then incubated at 37  $^{\circ}$ C. A similar reaction was set up to check the stability of **20** and **21** in presence of glutathione (10 mM). At predetermined time points, 60  $\mu$ L aliquots were taken from the reaction mixture, diluted with 60  $\mu$ L ACN and 25  $\mu$ L was injected into the HPLC.

#### 4.4.2.3. Decomposition of **20** and **21** followed turnover of **2a** by *b3*-MST in presence of DTT to produce **3a**

In PBS (1 $\times$ ) pH 7.4 buffer (1.5 mL), the reaction mixture was prepared using **20** (100  $\mu$ M) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu$ M) with  $\beta$ -galactosidase (4 U/mL), DTT (1 mM), 3-MST (1  $\mu$ M), mixed, and then incubated at 37  $^{\circ}$ C. At predetermined time points, 60  $\mu$ L aliquots were taken from the reaction mixture, diluted with 60  $\mu$ L ACN and 25  $\mu$ L was injected into the HPLC.

#### 4.4.3. H<sub>2</sub>S generation from **20** and **21** in presence of 3-MST and DTT

##### 4.4.3.1. Lead Acetate assay for the detection of H<sub>2</sub>S

Firstly, lead acetate soaked paper was prepared. Whatman filter papers were soaked with 5% (w/v) lead (II) acetate solution and dried. 285  $\mu$ M stock of wild-type (wt) *b3*-MST is used for lead acetate assay. 10 mM stocks of all compounds (**1a**, **20**, **21** and **22**) were prepared in DMSO. Porcine liver esterase (100 U/mL),  $\beta$ -glucosidase (250 U/mL),  $\beta$ -galactosidase (400 U/mL) and dithiothreitol (DTT, 100 mM) were prepared in deionized water.

Lead acetate assay was performed in a 96-well plate (with lid). The reaction samples were prepared by sequentially adding **20** (100  $\mu$ M) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu$ M) with  $\beta$ -galactosidase (4 U/mL), wt 3-MST (1  $\mu$ M) and DTT (1 mM). A similar protocol was followed for the positive control (**1a**) and the negative control (**22**). For positive control, **1a** (100  $\mu$ M) and esterase (1 U/mL) were incubated along with 3-MST (1  $\mu$ M) and DTT (1 mM). For negative control, **22** (100  $\mu$ M) with 3-MST (1  $\mu$ M) and DTT (1 mM) were incubated. The final volume was adjusted to 200  $\mu$ L using 1 $\times$  phosphate buffer saline (PBS) pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37  $^{\circ}$ C.

A similar protocol was followed for the substrate controls. Briefly, the reaction samples were prepared by sequentially adding **1a** (100  $\mu$ M) with Esterase (1 U/mL) or **20** (100  $\mu$ M) with glucosidase (10 U/mL) or **21** (100  $\mu$ M) with galactosidase (4 U/mL) along with DTT (1 mM).

The final volume was adjusted to 200  $\mu$ L using PBS (1 $\times$ ) pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37  $^{\circ}$ C.

A separate experiment was conducted to test the enzyme controls. DMSO (1%), Esterase (1 U/mL), wt 3-MST (1  $\mu$ M) and DTT (1 mM) were sequentially added. The final volume was adjusted to 200  $\mu$ L using PBS (1 $\times$ ) pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37  $^{\circ}$ C.

At predetermined time points, lead acetate paper was carefully taken out and an image was captured using Syngene G-Box Chemi-XRQ.

#### 4.4.3.2. Methylene blue assay for the detection of H<sub>2</sub>S

The methylene blue assays were conducted as previously reported with some modifications.<sup>15,16</sup> Briefly, 285  $\mu$ M stock of wt 3-MST was used for the methylene blue assay. 10 mM stocks of all compounds (**20**, **21** and **22**) were prepared in DMSO. Porcine liver esterase (100 U/mL),  $\beta$ -glucosidase (250 U/mL),  $\beta$ -galactosidase (400 U/mL), DTT (100 mM) and Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (40 mM) was prepared in deionized water. A stock solution of FeCl<sub>3</sub> (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 **20** (100  $\mu$ M) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu$ M) with  $\beta$ -galactosidase (4 U/mL) or **22** (100  $\mu$ M) along with Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (400  $\mu$ M), wt 3-MST (1  $\mu$ M) and DTT (1 mM). The final volume was adjusted to 0.5 mL using PBS (1 $\times$ ) pH 7.4 buffer and incubated at 37  $^{\circ}$ C at 300 rpm.

A similar protocol was followed for the substrate controls. Briefly, the reaction samples were prepared by sequentially adding **20** (100  $\mu$ M) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu$ M) with  $\beta$ -galactosidase (4 U/mL) or **22** (100  $\mu$ M) along with Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (400  $\mu$ M) and DTT (1 mM). The final volume was adjusted to 0.5 mL using PBS (1 $\times$ ) pH 7.4 buffer and incubated at 37  $^{\circ}$ C at 300 rpm.

At predetermined time points, equal volumes (200  $\mu$ L) of FeCl<sub>3</sub>, DMPPDA and aliquots from the above reaction samples were mixed and the resulting solution was incubated at 37  $^{\circ}$ C for 30 min in dark, to allow the formation of the methylene blue dye. An aliquot of 150  $\mu$ L was transferred to a 96-well plate and the absorbance values were recorded at 676 nm using a microplate reader (Thermo Scientific VarioskanFlash).

#### 4.4.4. Clickable Tag-switch assay for 3-MST persulfide detection

The Clickable Tag-switch assay was conducted with 285  $\mu\text{M}$  stocks of wt 3-MST. 10 mM stocks of all compounds (**1a**, **20**, **21** and **22**) were prepared in DMSO. Porcine liver esterase (100 U/mL),  $\beta$ -glucosidase (250 U/mL),  $\beta$ -galactosidase (400 U/mL) was prepared in deionised water.

To 3-MST (15  $\mu\text{M}$ ), **1a** (100  $\mu\text{M}$ ) with esterase (1 U/mL) or **20** (100  $\mu\text{M}$ ) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu\text{M}$ ) with  $\beta$ -galactosidase (4 U/mL) or **21** (100  $\mu\text{M}$ ) was added. Volume was adjusted to 100  $\mu\text{L}$  by tris buffer (pH 8.0) and incubated for 1 h. After that 5 mM MSBT-A (2.5  $\mu\text{L}$ , 200 mM) and 5  $\mu\text{L}$  10% SDS were added and incubated for 30 min. Then 500  $\mu\text{M}$  PMSF (0.5  $\mu\text{L}$ , 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu\text{L}$ , 200 mM) was added and incubated for 1 h.

A similar reaction was set up for enzyme control. To 3-MST (15  $\mu\text{M}$ ), DMSO (1%) was added. The reaction volume was adjusted to 100  $\mu\text{L}$  by tris buffer (pH 8.0) and incubated for 60 min. After that 5 mM MSBT-A (2.5  $\mu\text{L}$ , 200 mM) and 5  $\mu\text{L}$  10% SDS were added and incubated for 30 min. Then 500  $\mu\text{M}$  PMSF (0.5  $\mu\text{L}$ , 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu\text{L}$ , 200 mM) was added and incubated for 1 h.

After the reaction click reaction cocktail: 6  $\mu\text{L}$  TBTA (1.7 mM), 2  $\mu\text{L}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (50 mM), 2  $\mu\text{L}$  TCEP (50 mM), 1  $\mu\text{L}$  Rhodamine azide (10 mM) was added to each. The reaction was stirred at 25  $^\circ\text{C}$  for 1 h. After completion of the reaction, 40  $\mu\text{L}$  of loading dye was added. The samples (20  $\mu\text{L}$ ) were loaded in a gel and SDS-PAGE was performed (run at 150 V for 1 h), the in-gel fluorescence image was taken using Syngene G-Box Chemi-XRQ.

#### 4.4.5. Sulfane sulfur measurement using SSP-2

Stock solutions of **1a** (10 mM), **20** (10 mM), **21** (10 mM), **22** (10 mM) and SSP-2 (5 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL),  $\beta$ -glucosidase (250 U/mL),  $\beta$ -Galactosidase (400 U/mL), DTT (100 mM) and esterase (100 U/mL) were prepared in DI water. To 3-MST (15  $\mu\text{M}$ ), **1a** (100  $\mu\text{M}$ ) with esterase (0.5 U/mL) or **20** (100  $\mu\text{M}$ ) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu\text{M}$ ) with  $\beta$ -galactosidase (4 U/mL). Volume was adjusted to 400  $\mu\text{L}$  using PBS (1 $\times$ ) pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^\circ\text{C}$ .

A reaction was set up for compound control. The reaction mixtures were prepared by adding **1a** (100  $\mu\text{M}$ ) with esterase (0.5 U/mL) or **20** (100  $\mu\text{M}$ ) with  $\beta$ -glucosidase (10 U/mL) or **21**

(100  $\mu$ M) with  $\beta$ -galactosidase (4 U/mL). and the volume was adjusted to 400  $\mu$ L using PBS (1 $\times$ ) pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^{\circ}$ C.

A reaction was set up for enzyme control. The reaction mixtures were prepared by 3-MST (21  $\mu$ M), 4  $\mu$ L of DMSO, esterase (0.5 U/mL) and the volume was adjusted to 400  $\mu$ L using PBS (1 $\times$ ) pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^{\circ}$ C.

A similar reaction was set up for DTT control. The reaction sample was prepared **1a** (100  $\mu$ M) with esterase (0.5 U/mL) or **20** (100  $\mu$ M) with  $\beta$ -glucosidase (10 U/mL) or **21** (100  $\mu$ M) with  $\beta$ -galactosidase (4 U/mL) along with 3-MST (15  $\mu$ M). Volume was adjusted to 360  $\mu$ L using PBS (1 $\times$ ) pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^{\circ}$ C. Finally, the reaction mixture was treated with 10 mM DTT (40  $\mu$ L of 100 mM stock) after 30 min and further incubated at 37  $^{\circ}$ C for 1 h.

The above treatment groups were finally incubated with 50  $\mu$ M SSP-2 (4  $\mu$ L, 5 mM stock) at 37  $^{\circ}$ C for 10 min in the dark. 100  $\mu$ L aliquot of each sample was transferred to a 96-well plate and the fluorescence was recorded ( $\lambda_{\text{ex}}$  = 482 nm,  $\lambda_{\text{em}}$  = 518 nm) using a microplate reader (Thermo Scientific VarioskanFlash).

#### 4.4.6. Cell viability assay with MEF cells

In a 96-well plate, Mouse embryonic fibroblasts (MEFs) were seeded in a 96-well plate at a concentration of  $1 \times 10^4$  cells/well in DMEM media supplemented with 10% FBS and 1% antibiotic solution. Cells were incubated overnight in an atmosphere of 5% CO<sub>2</sub> at 37  $^{\circ}$ C. Then, stock solutions of compounds (**20**, **21** and **22**) were prepared in DMSO and the cells were treated with varying concentrations of the compounds. For all the treatments, the final concentration of DMSO did not exceed 0.5%. The cells were incubated for 24 h at 37  $^{\circ}$ C. Following this, the old media from the cells were removed, a 0.5 mg/mL stock solution of 3-MTT was prepared in 7 mL DMEM media, 100  $\mu$ L of the MTT stock was added to each well and incubated for 4 h at 37  $^{\circ}$ C. Next, the media was carefully removed and 100  $\mu$ L of DMSO was added to each well and allowed to mix properly. Finally, absorbance for each well at 570 nm was carried out using a microplate reader (Thermo Scientific Varioskan) to estimate cell viability.

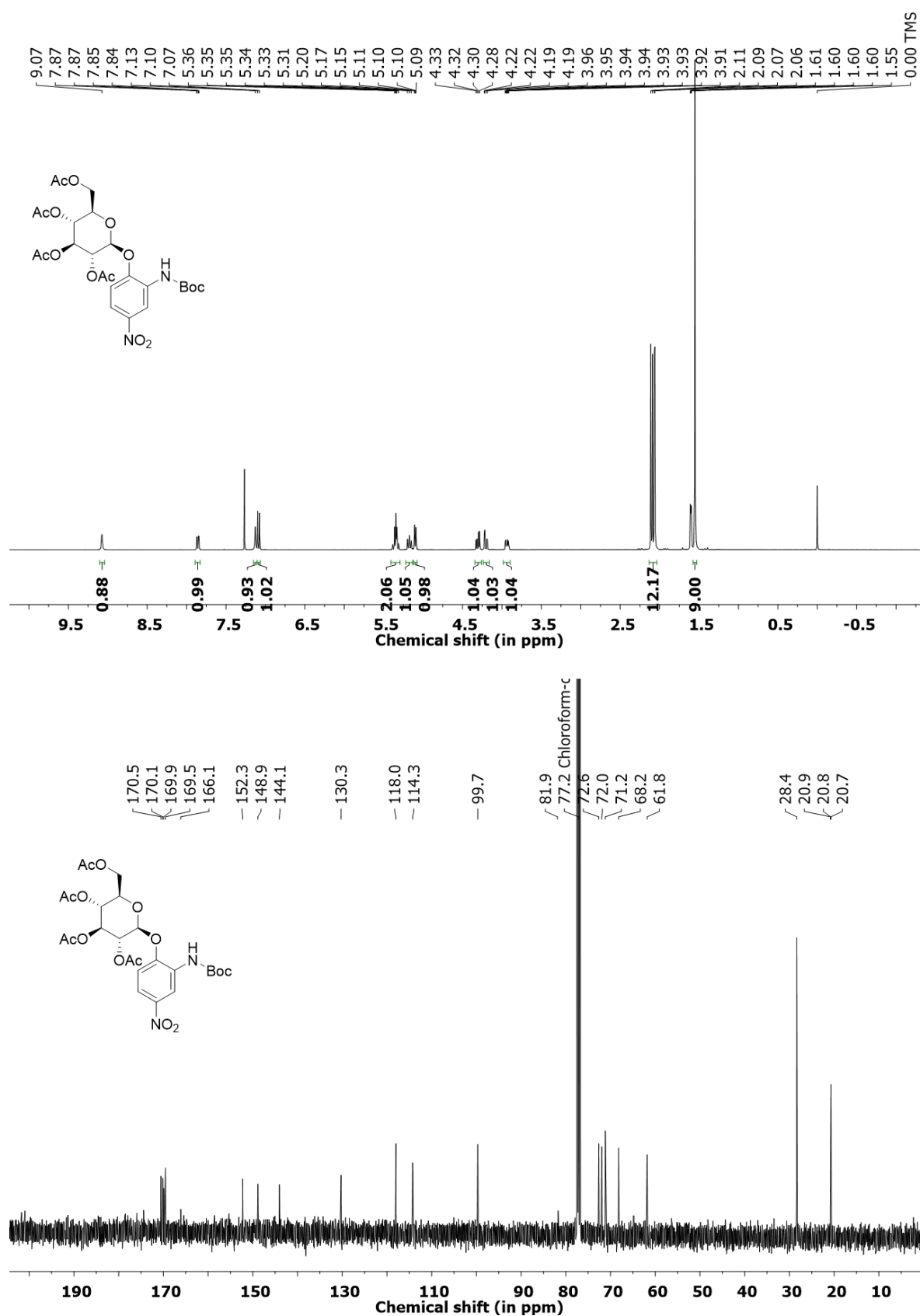
#### 4.4.7. H<sub>2</sub>S imaging in cells using NBD-fluorescein dye

MEFs cells were seeded in a 24-well plate with  $10^4$  cells/well in DMEM media containing 10% FBS and 1% antibiotic solution and cells were incubated in an atmosphere of 5% CO<sub>2</sub> at 37 °C for 24 h. Following this, the old media was removed and the cells were washed two times with 0.5 mL of sterile PBS (1×). This was followed by the addition of 1 mL of fresh DMEM media. The cells were co-treated with 100 μM of **21** or **22** and NBD-fluorescein dye (25 μM) in both the cell lines and were incubated at 37 °C for 2 h. After 2 h, cells were washed twice with 0.5 mL PBS (1×). Finally, cells were imaged on an EVOS fluorescence microscope using a 20x GFP (green fluorescence protein) filter.

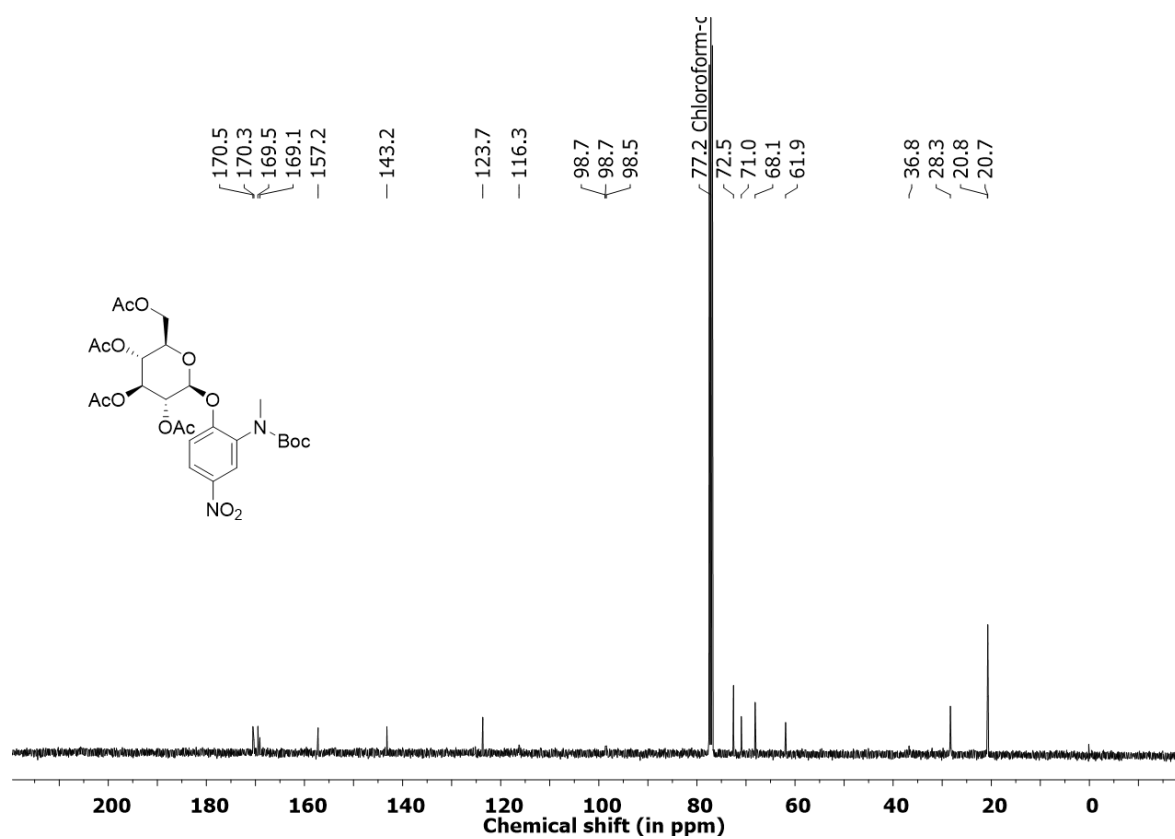
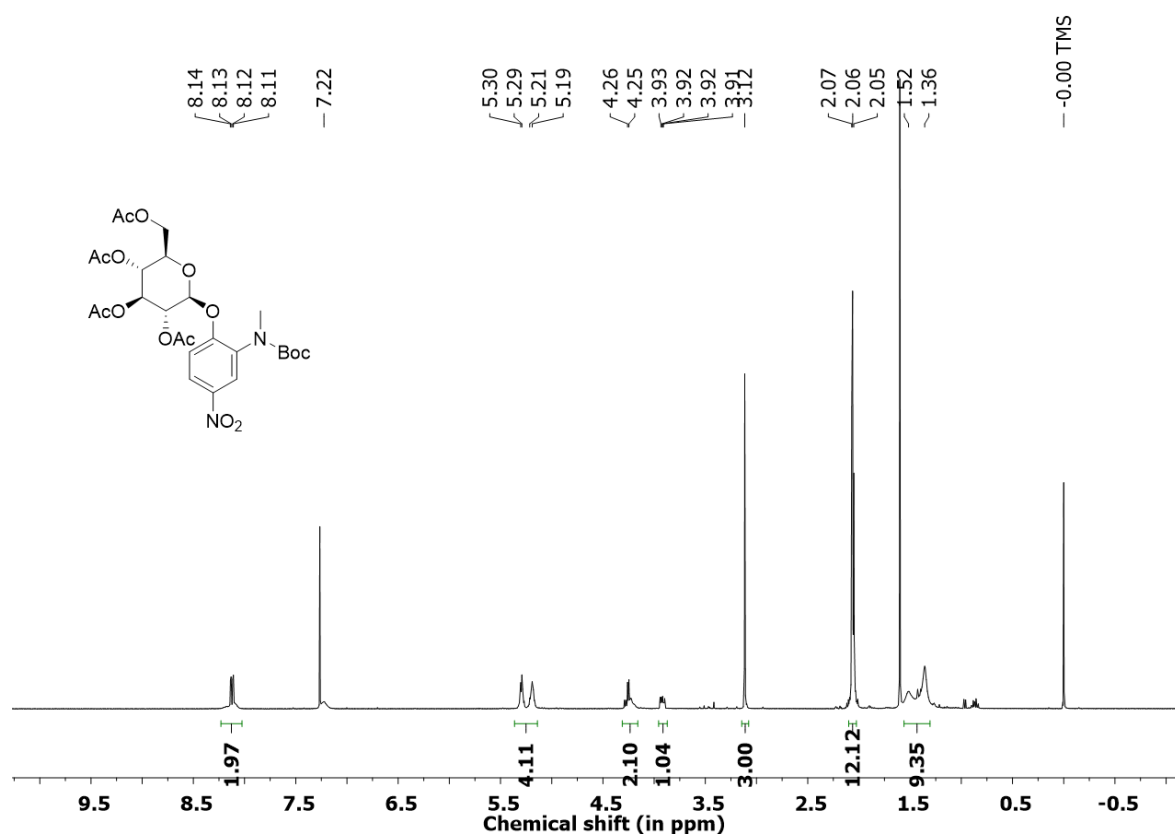
#### 4.4.8. Protection from MGR-1 induced oxidative stress

In a 96-well plate, mouse embryonic fibroblasts (MEFs) were seeded in a 96-well plate at a concentration of  $1 \times 10^4$  cells/well in DMEM media supplemented with 10% FBS and 1% antibiotic solution. Cells were incubated overnight in an atmosphere of 5% CO<sub>2</sub> at 37 °C. Then, old media was removed and 10 mM stock solutions of compounds (**21**, **22**) were prepared in DMSO. Next, cells were treated with 12.5 μM of **21** or **22**. For all the treatments, the final concentration of DMSO did not exceed 0.5%. The cells were incubated for 4 h at 37 °C. Following this, the old media from the cells were removed and MGR-1 (25 μM) in DMEM was added. The cells were incubated for 4 h at 37 °C following which the media was removed and the aforementioned MTT assay was carried out as described above to determine cell viability.

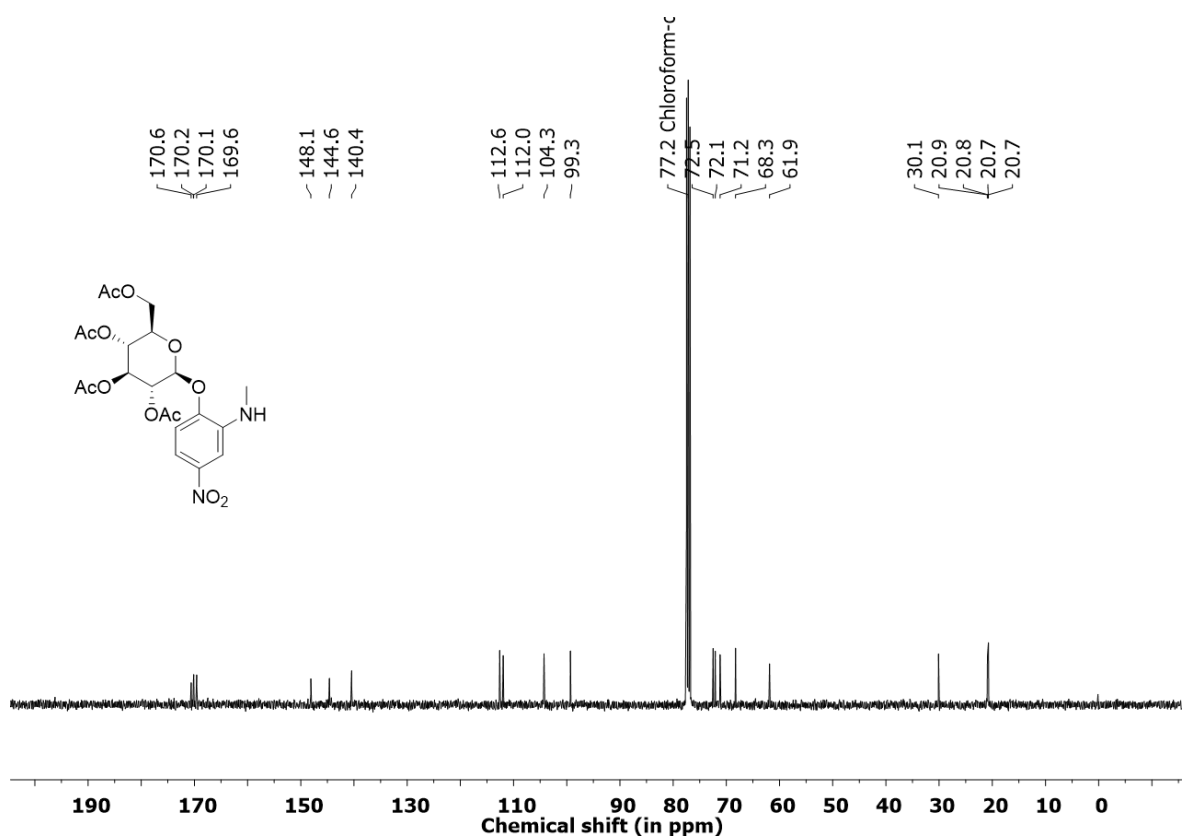
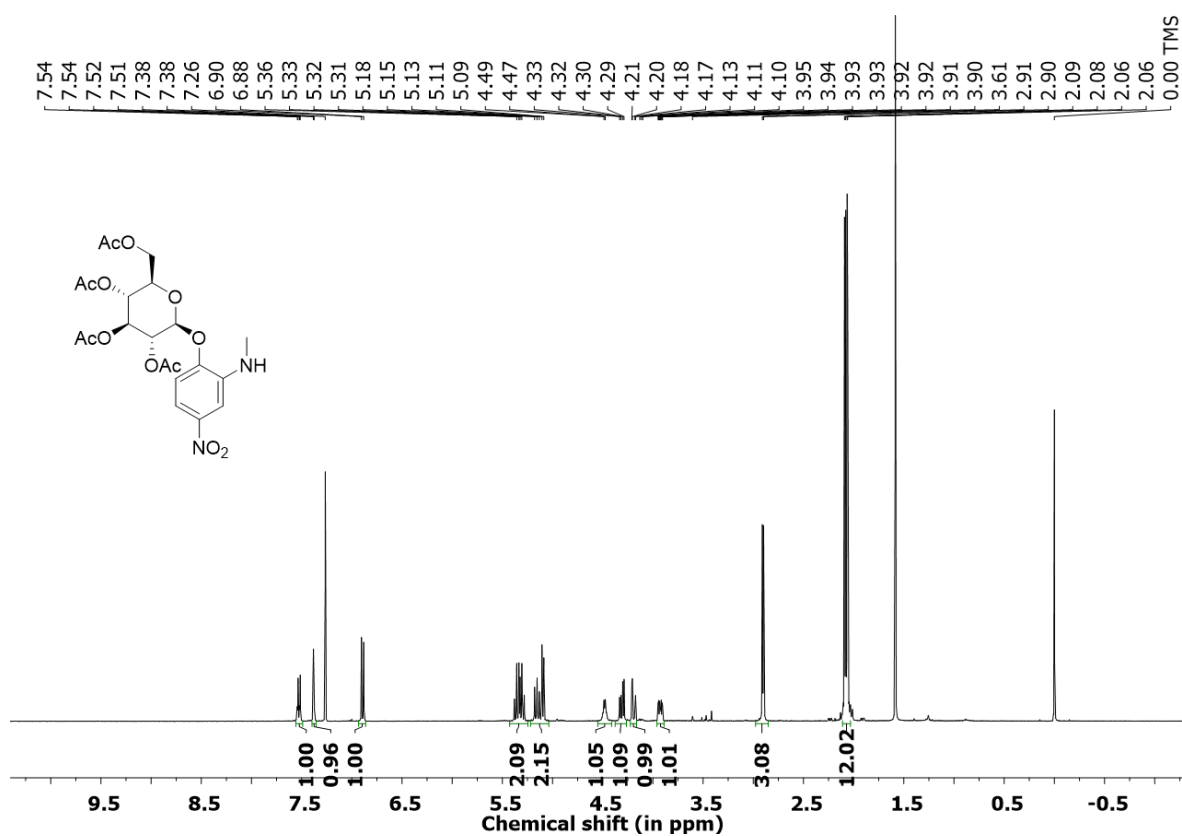
## 4.5. NMR spectra of compounds

 $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **15**:

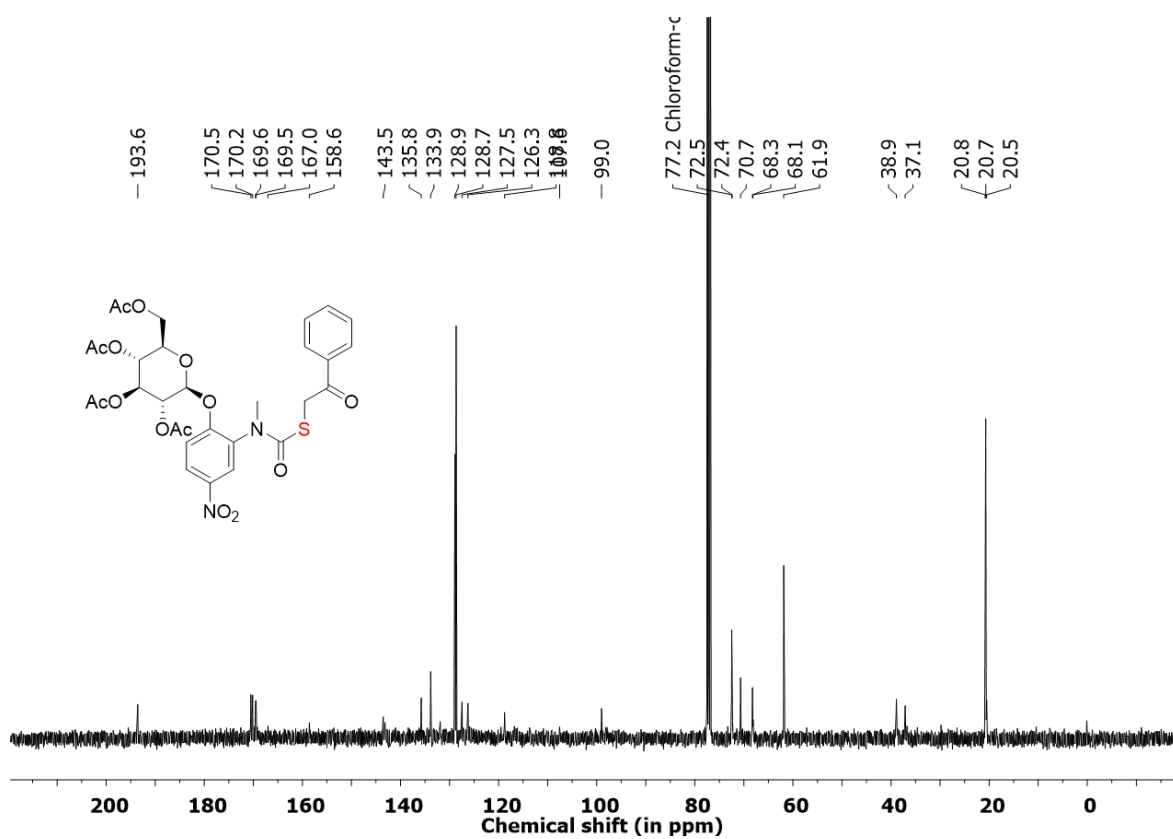
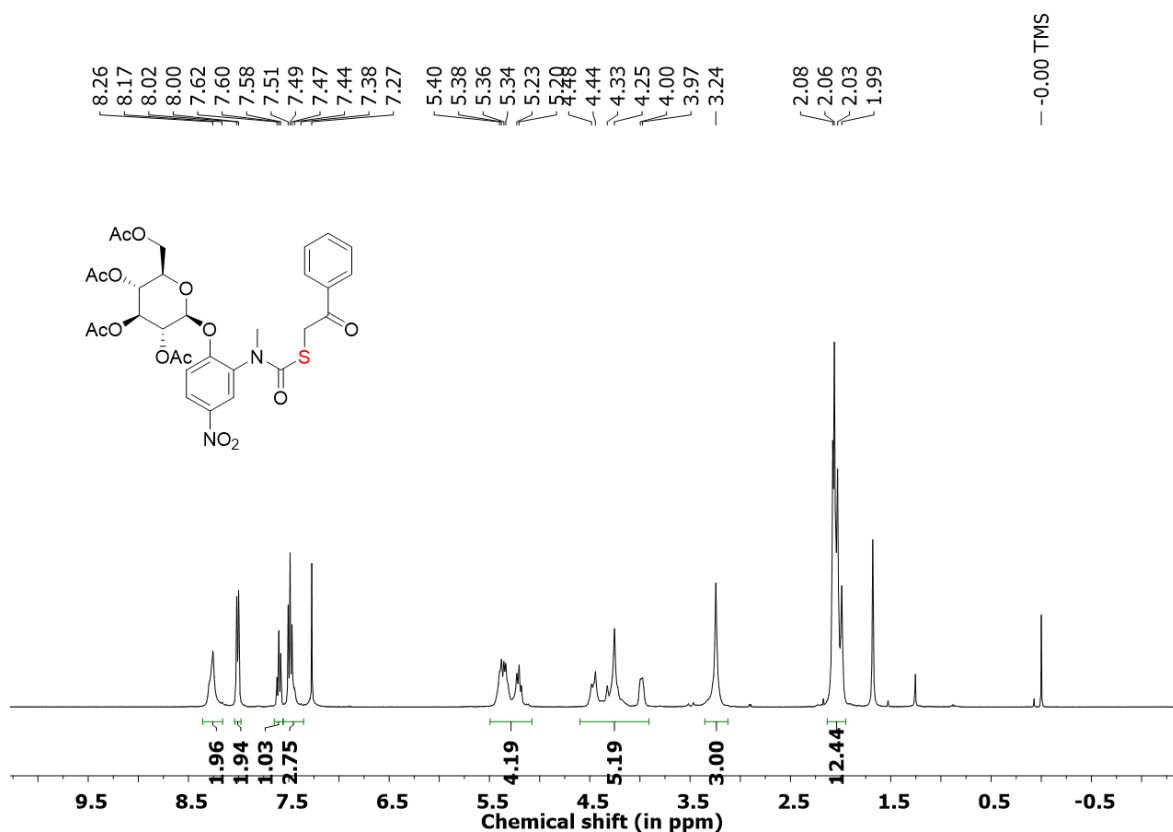
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **16**:



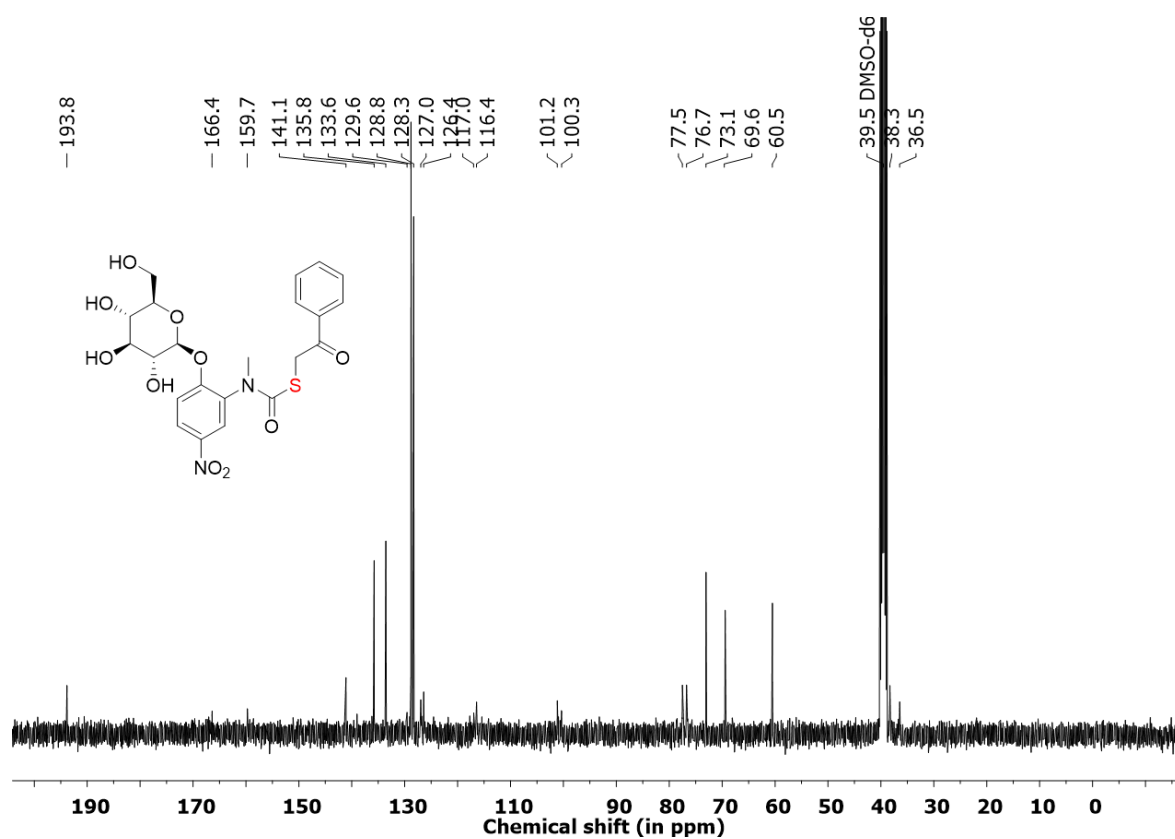
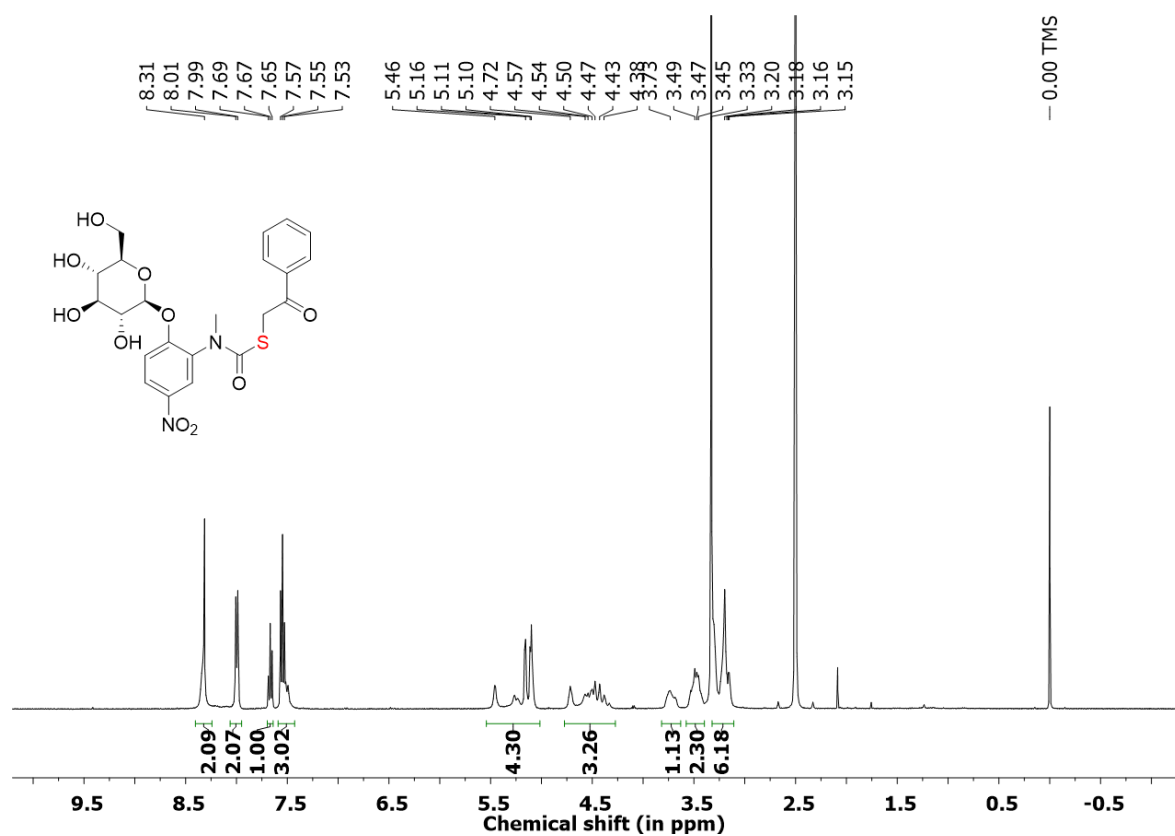
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 17:



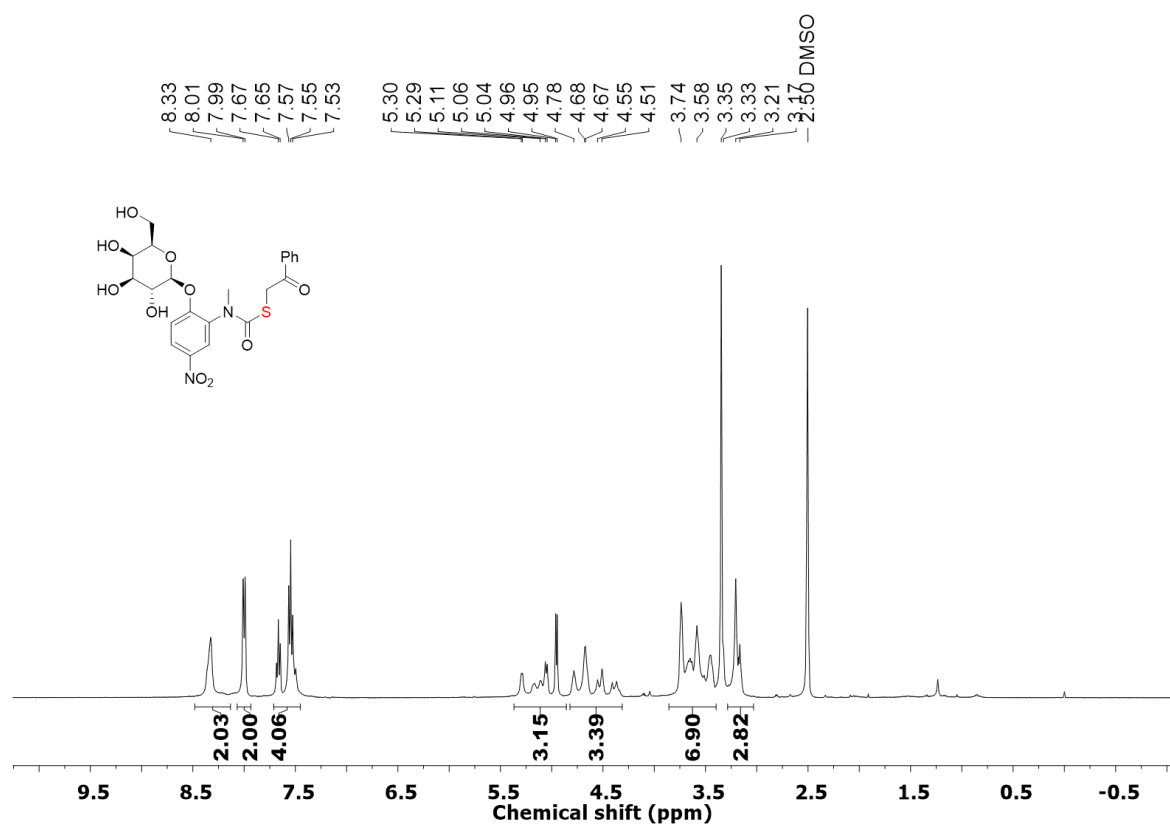
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **19**:



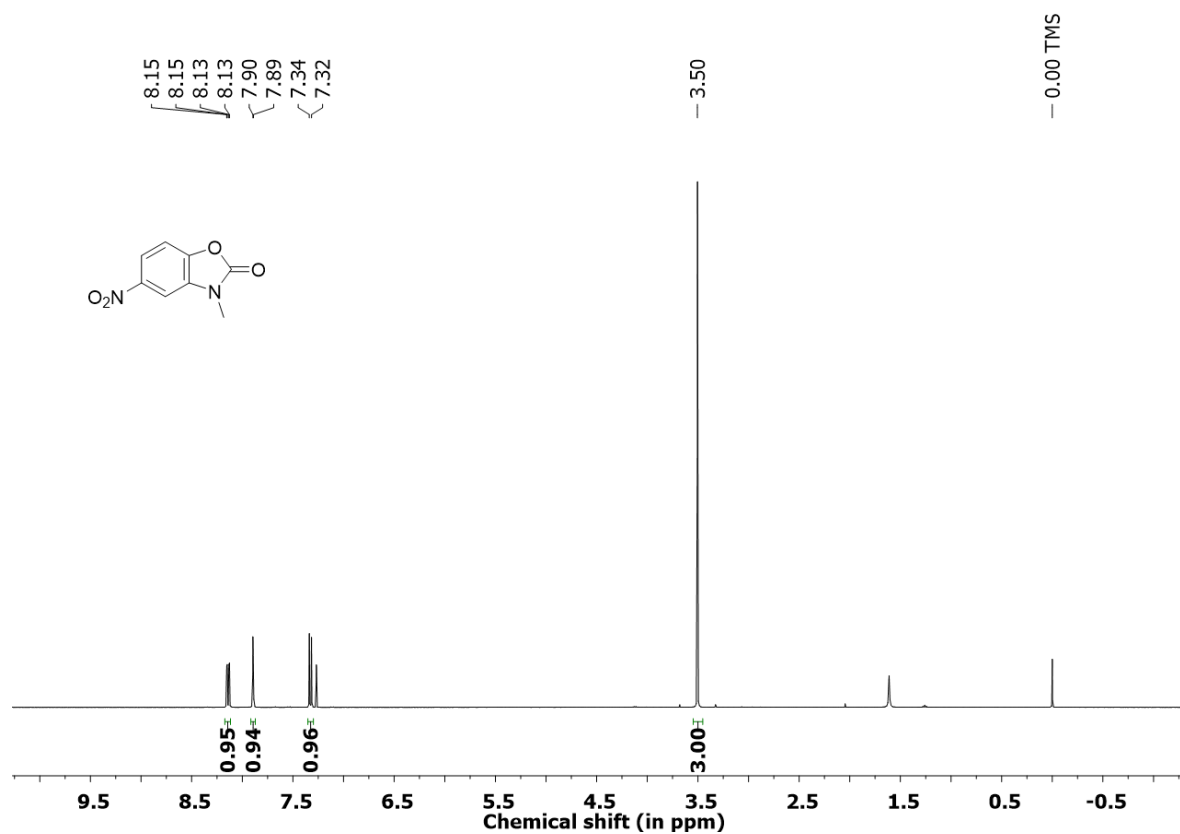
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **20**:



$^1\text{H}$  NMR spectra of **21**:



$^1\text{H}$  NMR spectra of **22**:



#### 4.6. References

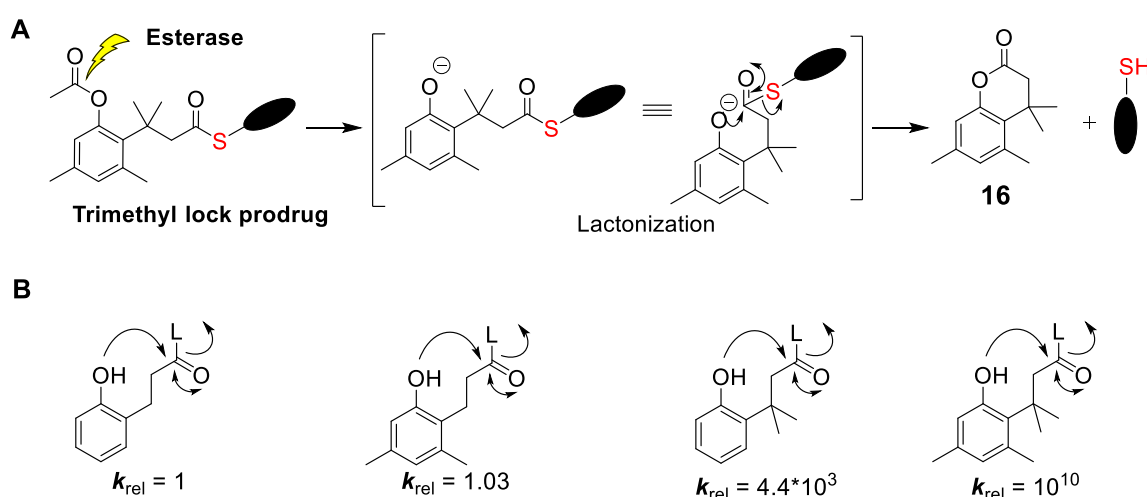
- (1) Meister, A. Glutathione Metabolism and Its Selective Modification. *J. Biol. Chem.* **1988**, 263 (33), 17205–17208.
- (2) Forman, H. J.; Zhang, H.; Rinna, A. Glutathione: Overview of Its Protective Roles, Measurement, and Biosynthesis. *Mol. Aspects Med.* **2009**, 30 (1–2), 1–12.
- (3) Bora, P.; Sathian, M. B.; Chakrapani, H. Enhancing Cellular Sulfane Sulfur through  $\beta$ -Glycosidase-Activated Persulfide Donors: Mechanistic Insights and Oxidative Stress Mitigation. *Chem. Commun.* **2022**, 58 (18), 2987–2990.
- (4) Badieyan, S.; Bevan, D. R.; Zhang, C. Probing the Active Site Chemistry of  $\beta$ -Glucosidases along the Hydrolysis Reaction Pathway. *Biochemistry* **2012**, 51 (44), 8907–8918.
- (5) Bora, P. Stimuli-Responsive Small Molecule Persulfidating Agents, IISER Pune, 2022.
- (6) Friend, D. R.; Chang, G. W. A Colon-Specific Drug-Delivery System Based on Drug Glycosides and the Glycosidases of Colonic Bacteria. *J. Med. Chem.* **1984**, 27 (3), 261–266.
- (7) Englyst, H. Polysaccharide Breakdown by Mixed Populations of Human Faecal Bacteria. *FEMS Microbiol. Lett.* **1987**, 45 (3), 163–171.
- (8) Jeremias, C. G.; Lucas, G. B.; MacKenzie, C. A. Preparation of Tetraacetyl- $\alpha$ -D-Glucopyranosyl Bromide. *J. Am. Chem. Soc.* **1948**, 70 (7), 2598–2598.
- (9) Chen, W.; Liu, C.; Peng, B.; Zhao, Y.; Pacheco, A.; Xian, M. New Fluorescent Probes for Sulfane Sulfurs and the Application in Bioimaging. *Chem. Sci.* **2013**, 4 (7), 2892.
- (10) Chen, W.; Matsunaga, T.; Neill, D. L.; Yang, C.; Akaike, T.; Xian, M. Rational Design of a Dual-Reactivity-Based Fluorescent Probe for Visualizing Intracellular HSNO. *Angew. Chemie Int. Ed.* **2019**, 58 (45), 16067–16070.
- (11) Heredia, A. A.; Soria-Castro, S. M.; Bouchet, L. M.; Oksdath-Mansilla, G.; Barrionuevo, C. A.; Caminos, D. A.; Bisogno, F. R.; Argüello, J. E.; Peñeñory, A. B. Stereoselective One-Pot Synthesis of  $\beta$ -Alkylsulfide Enol Esters. Base-Triggered Rearrangement under Mild Conditions. *Org. Biomol. Chem.* **2014**, 12 (33), 6516.
- (12) Chandanshive, J. Z.; Bonini, B. F.; Gentili, D.; Fochi, M.; Bernardi, L.; Franchini, M. C. Regiocontrolled Synthesis of Ring-Fused Thieno[2,3-c]Pyrazoles through 1,3-

- Dipolar Cycloaddition of Nitrile Imines with Sulfur-Based Acetylenes. *European J. Org. Chem.* **2010**, 2010 (33), 6440–6447.
- (13) Huvelle, S.; Alouane, A.; Le Saux, T.; Jullien, L.; Schmidt, F. Syntheses and Kinetic Studies of Cyclisation-Based Self-Immolative Spacers. *Org. Biomol. Chem.* **2017**, 15 (16), 3435–3443.
- (14) Abdelaal, S. M.; Kong, S.-B.; Bauer, L. Synthesis of 1-[3-Methyl-2(3 H )-Benzazolon-5- or 6-Yl]-4-{4-[ Cis -2-(2,4-Dichlorophenyl)-2-(1 H -Imidazol-1-Yl-Methyl)-1,3-Dioxolan-4-Yl]Methyleneoxyphenyl}piperazines. *J. Heterocycl. Chem.* **1992**, 29 (5), 1069–1076.
- (15) Yadav, P. K.; Yamada, K.; Chiku, T.; Koutmos, M.; Banerjee, R. Structure and Kinetic Analysis of H<sub>2</sub>S Production by Human Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2013**, 288 (27), 20002–20013.
- (16) Sharma, A. K.; Nair, M.; Chauhan, P.; Gupta, K.; Saini, D. K.; Chakrapani, H. Visible-Light-Triggered Uncaging of Carbonyl Sulfide for Hydrogen Sulfide (H<sub>2</sub>S) Release. *Org. Lett.* **2017**, 19 (18), 4822–4825.

## CHAPTER 5. Development of Esterase Triggered Artificial Substrates of 3-MST

### 5.1. Introduction

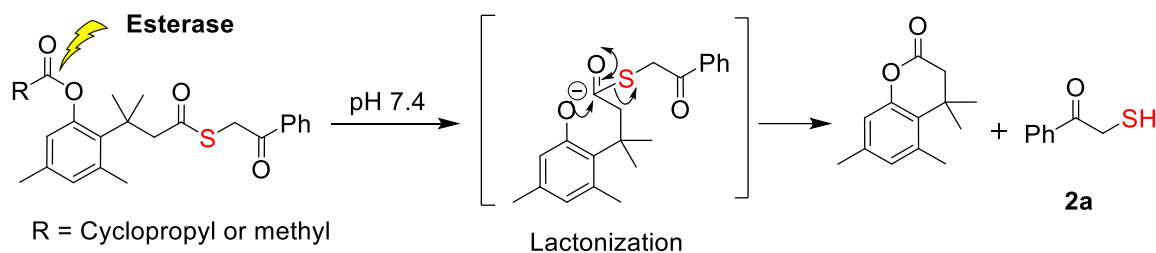
In Chapter 5, the results of the design, synthesis and evaluation of another enzyme-triggered, thiol-stable 3-MST substrate are discussed. The major limitation of the substrate developed in Chapter 2 is its reactivity with thiols. Hence, an esterase-sensitive donor that is stable to thiols would be useful. An esterase-sensitive trimethyl lock-based strategy of sulfur-based prodrugs reported by Prof. Binghe Wang's lab was considered (Scheme 5.1A).<sup>1,2</sup> Trimethyl lock-based systems are rapid and efficient in undergoing lactonization. Known as the Thorpe-Ingold effect, these are frequently used in the rapid release of payload after activation (Scheme 5.1B).<sup>3,4</sup> Hence, an esterase activated prodrug where an artificial substrate of 3-MST (**2a**) tethered to the trimethyl lock cage was designed (Scheme 5.2). Using esterase as a stimulus is advantageous as this is a prevalent enzyme, expressed in nearly all cells and would help in deciphering 3-MST related sulfur transfer.<sup>5</sup>



**Scheme 5.1:** (A) Reported esterase-activated trimethyl lock strategy (B)  $k$  relative shows steric-assisted faster cyclization due to the Thorpe Ingold effect. L corresponds to a leaving group.

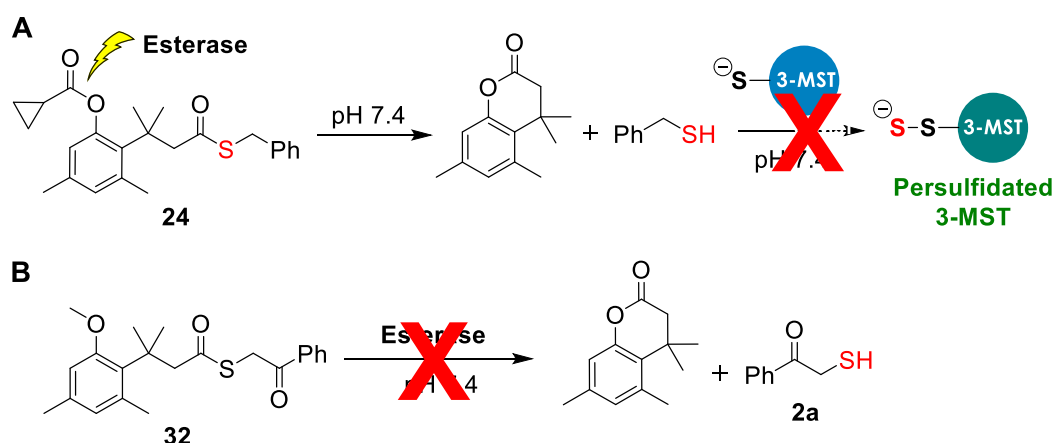
### 5.2. Design and hypothesis

Esterase sensitive artificial substrates of 3-MST were designed where free thiol group **2a** was caged using a reported trimethyl lock (Scheme 5.2). A cyclopropyl and methyl ester were considered for the design. Upon activation by esterase free phenolate ion can go through internal cyclization to form a lactone and subsequently will release free substrate **2a**. Finally, free **2a** will be then available for turnover by 3-MST.



**Scheme 5.2:** Design of esterase sensitive trimethyl-lock based artificial substrates of 3-MST.

Next, two negative control compounds were designed (Scheme 5.3A). In the first negative control (**24**), benzyl thiol was used instead of phenacyl thiol therefore upon activation benzyl thiol will be uncaged (Scheme 5.2A). As benzyl thiol is not a substrate for 3-MST, thiol control compound would help understand effects of a thiol in cellular processes. Next, a second negative control (**32**) was designed where a methyl group was introduced in the place of ester group at the phenyl ring. As the methyl ether can not be cleaved in presence of esterase, the release of **2a** is not possible (Scheme 5.3B).



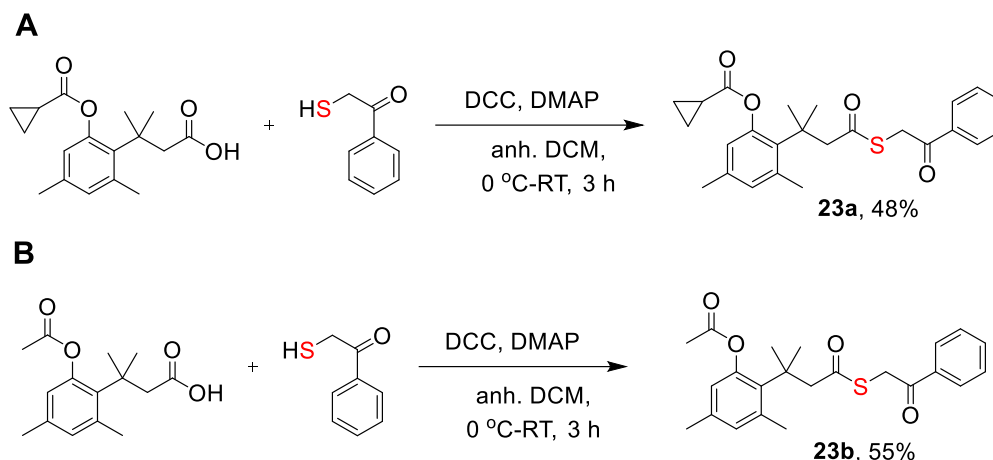
**Scheme 5.3:** Design of negative control compounds (A) Negative control **24**. (B) Negative control **32**.

### 5.3. Results and Discussion

#### 5.3.1. Synthesis

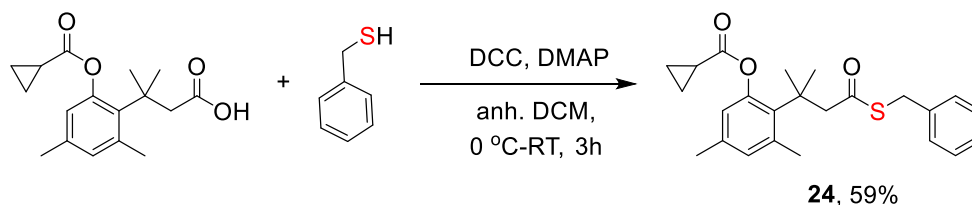
Compounds **23a** and **23b** were synthesized from their corresponding acids (Scheme 5.4). 3-(2-Cyclopropyloxy-4,6-dimethylphenyl)-3-methylbutyric acid and 3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid were synthesized using reported protocols.<sup>6</sup> 3-(2-Cyclopropyloxy-4,6-dimethylphenyl)-3-methylbutyric acid was reacted with using freshly prepared phenacyl thiol (**2a**) via a DCC based acid-thiol coupling reaction to obtain **23a** in 48%

yield (Scheme 5.4A). A similar reaction was performed with 3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid to obtain **23b** in 55% yield (Scheme 5.4B).



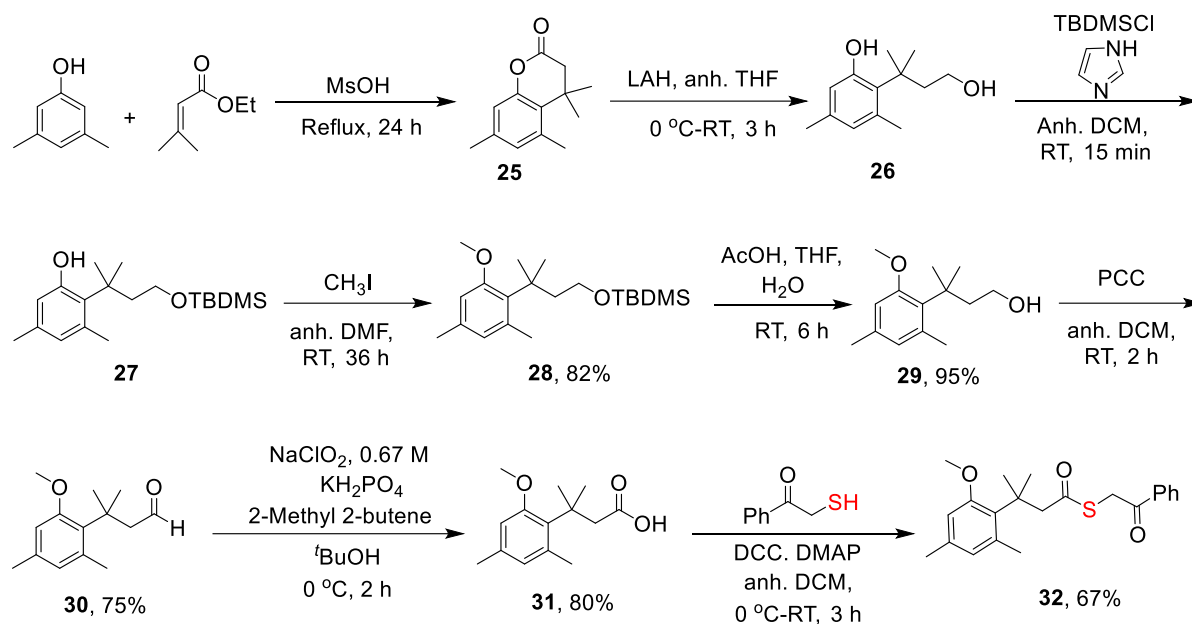
**Scheme 5.4:** Synthesis for the esterase-activated artificial substrate of 3-MST (A) Compound **23a** with a cyclopropyl ester group. (B) Compound **23b** with a methyl ester group.

Similarly, the negative control **24** was synthesized from the corresponding acid and using benzyl thiol *via* a DCC-based acid-thiol coupling reaction to obtain **24** in 59% yield (Scheme 5.5).



**Scheme 5.5:** Synthesis of negative control **24**.

Negative control **32** was synthesized *via* 8-step reactions starting from 3,5-xyleneol and ethyl 3,3-dimethylacrylate (Scheme 5.6). Compound **27** was synthesized from 3,5-xyleneol and ethyl 3,3-dimethylacrylate using a 3-step reported protocol.<sup>7</sup> The *O*-methylation of **27** was done using methyl iodide **28** in 82% yield. Then, TBDMS deprotection of **28** was performed using acetic acid to form **29** in 95% yield. Further, the primary alcoholic group of **29** was oxidized using pyridinium chlorochromate to obtain **30** in 75% yield. Compound **30** was then further oxidized using sodium chlorate (NaClO<sub>2</sub>) to obtain **31** in 80% yield. Finally, a DCC coupling was carried out with **31** and freshly synthesized phenacyl thiol (**2a**) to obtain negative control **32** in 67% yield.



**Scheme 5.6:** Synthesis of negative control **32**.

### 5.3.2. HPLC-based studies to determine stability, decomposition and turnover of the substrate

#### 5.3.2.1. Decomposition of **23a** in the presence of esterase under ambient conditions

Having synthesized esterase-sensitive artificial substrates of 3-MST, the efficiency of **2a** release from compound **23a** was evaluated. Compound **23a** was incubated with esterase (1 U/mL) in pH 7.4 phosphate buffer (PB) at 37 °C. As monitored by HPLC, the disappearance of **23a** (RT = 12.85 min) was observed in a time-dependent manner (Figure 5.1).

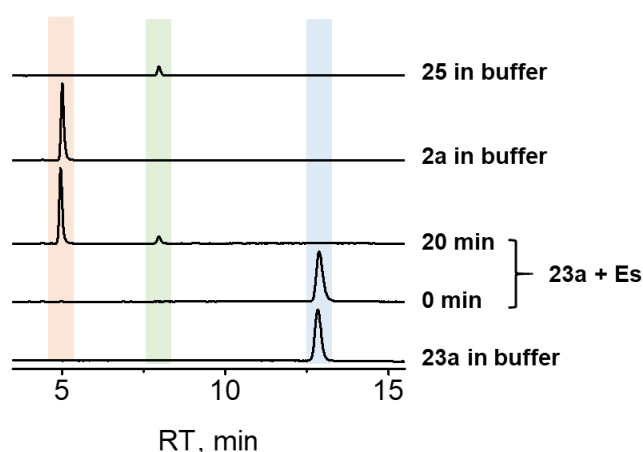


**Figure 5.1:** Decomposition of 3-MST artificial substrate **23a** and gradual formation of **2a** and **25** (Abs = 250 nm. (A) HPLC traces for the reaction mixture containing **23a** in the presence of esterase (1 U/mL).

Compound **23a** was completely consumed within 20 min and the appearance of peaks for intermediates, **25** and **2a** were observed at the retention time of 7.97 min and 4.97 min, respectively. Hence, **23a** is a substrate for esterase and efficiently releases **2a** in the presence of this enzyme.

### 5.3.2.2. Stability of **23a** in buffer and in thiols (GSH and DTT)

Next stability of these 3-MST artificial substrates was evaluated in pH 7.4 buffer and reducing environment at 37 °C. Compound **23a** was incubated in pH 7.4 PB. As cells are known to be rich in thiols, **23a** was incubated with GSH (10 mM) or DTT (10 mM) and stability was studied. The peak corresponding to **23a** remained unchanged in the aforementioned conditions which suggest these 3-MST artificial substrates were stable in the buffer as well as in the thiol-rich environment (Figure 5.2).

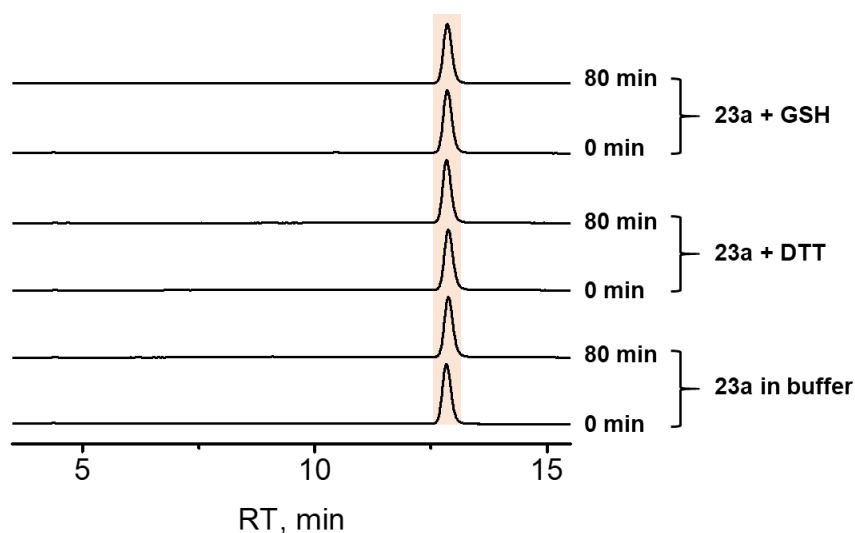
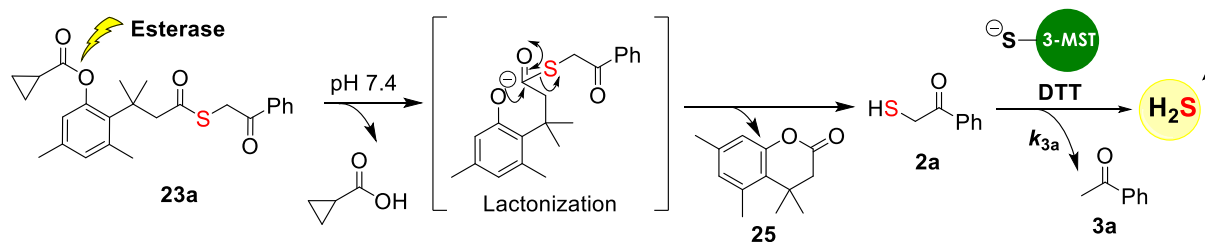


**Figure 5.2:** Stability of 3-MST artificial substrates in buffer and in presence of GSH and DTT. HPLC traces for the reaction mixture containing **20** in buffer and in the presence of GSH or DTT over 80 min.

### 5.3.3.2. Decomposition of **23a** in the presence of esterase and substrate (**2a**) turnover under reducing condition (DTT)

Compound **23a** was incubated with esterase (1 U/mL), *b*3-MST (0.5  $\mu$ M) and DTT (10 mM) in pH 7.4 PB at 37 °C. As monitored by HPLC, **23a** was consumed in 20 min and followed by formation of byproduct (**25**) and the release of the substrate (**2a**) was observed (Scheme 5.7 and Figure 5.3). Release of **2a** followed by turnover in presence of 3-MST should produce acetophenone (**3a**) (RT = 4.67 min). The yield of **3a** and **25** was found to be 89% and 90%

respectively which suggested an efficient reaction. The rate of acetophenone (**3a**) formation ( $k_{3a}$ ) was calculated to be  $2.3 \pm 0.6 \text{ h}^{-1}$  ( $R^2 = 0.9877$ ) (Figure 5.4).



**Scheme 5.7:** Decomposition of **23a** in presence of esterase followed by **2a** turnover by 3-MST.

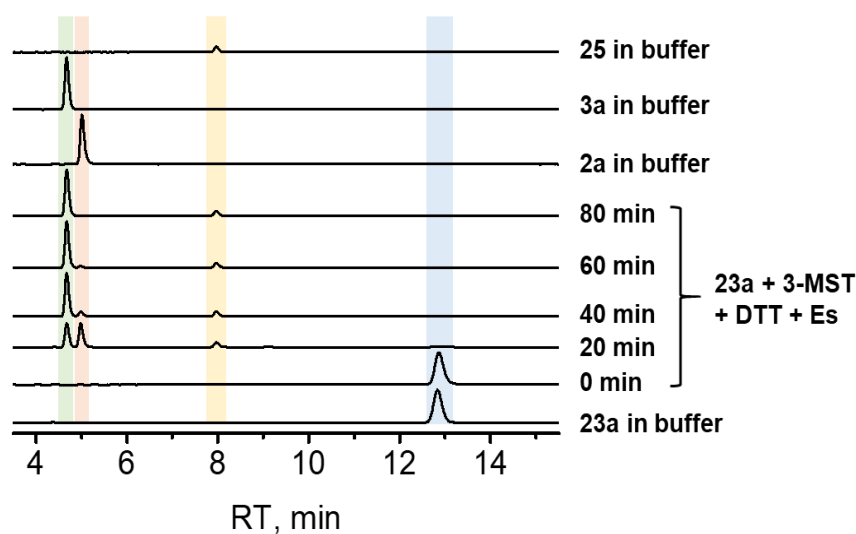


**Figure 5.3:** Decomposition of **23a** and gradual formation of **2a** and **25** followed by turnover of **2a** by 3-MST and finally the formation of **3a** (Abs = 250 nm). (A) HPLC traces for: **1a** + Es + b3-MST and conversion of **23a** to **2a**, and the turnover of **2a** to **3a** by 3-MST in presence of DTT over 80 min.

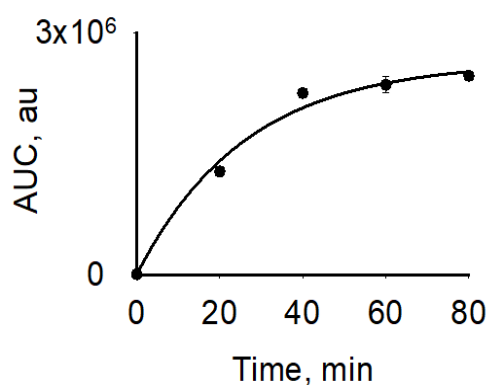


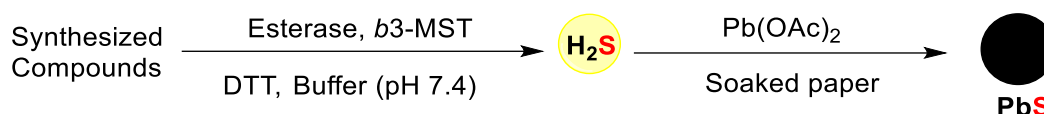
**Figure 5.4:** Kinetic profile for the formation of **3a** during the reaction of **23a** + 3-MST + Es + DTT. The area under the curve for **3a** was computed and was used to determine the rate constant for **3a**. The data were fit using the pseudo-first-order equation  $y = y_0 + a(1 - e^{-kx})$ .

Overall, HPLC study suggests that **23a** is specifically activated by esterase to yield **2a** which is then further turned over by 3-MST in presence of DTT and forms **3a** and **25** as the byproducts.

### 5.3.3. H<sub>2</sub>S generation from **23a** and **23b** in presence of 3-MST and DTT

#### 5.3.3.1. H<sub>2</sub>S detection using lead acetate assay

Next, under aforementioned conditions, H<sub>2</sub>S release was monitored using the lead acetate assay as discussed in Chapter 2 (Scheme 5.8).



**Scheme 5.8:** Detection of H<sub>2</sub>S *via* lead acetate assay.

In this assay, **20** and **21** were incubated with esterase (1 U/mL) respectively, along with *b3*-MST (0.5  $\mu$ M) and DTT (10 mM) in PB at 37 °C. The assay was performed in 96-well plate and the plate was covered with 5% Pb(OAc)<sub>2</sub> soaked paper and the reaction was incubated for 1.5 h and H<sub>2</sub>S release was seen from **23a** and **23b** only in the presence of esterase and *b3*-MST (Figure 5.5). **1a** was used as a positive control for this assay and an intense spot for PbS was observed. **24** and **32** were used as negative control, both failed to produce H<sub>2</sub>S in the presence of *b3*-MST. As expected, other controls did not produce a significant amount of H<sub>2</sub>S.

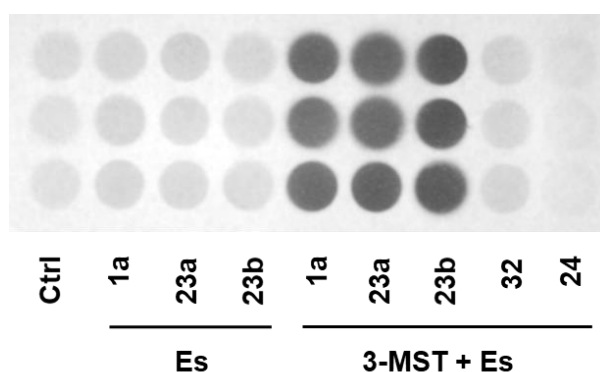
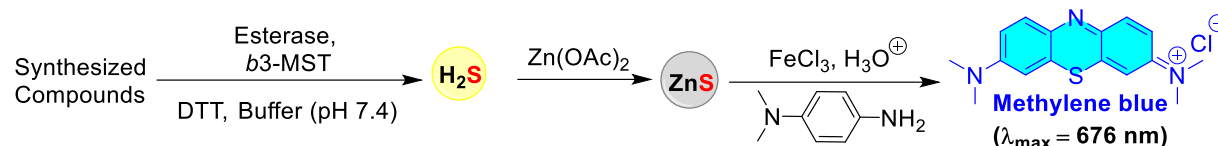


**Figure 5.5:** Lead acetate assay H<sub>2</sub>S generation by the 3-MST artificial substrates (**23a**, **23b**) in presence of *b3*-MST. Compounds were incubated with esterase *b3*-MST and DTT for 1.5 h. **1a** was used as positive control and **24** and **32** were used as a negative control. Ctrl represents only 3-MST.

### 5.3.3.2. H<sub>2</sub>S detection using methylene blue assay

Along with H<sub>2</sub>S detection for the compounds *via* lead acetate assay, methylene blue assay was carried out as described in Chapter 2.



**Scheme 5.9:** Detection of H<sub>2</sub>S *via* the methylene blue assay.

In this assay, **23a** were incubated with esterase (1 U/mL) along with b3-MST (0.5 μM), Zn(OAc)<sub>2</sub> (400 μM) and DTT (10 mM) in PB at 37 °C. At predetermined time points aliquots from the reaction mixture were treated with methylene blue reagents and mixed. The final reaction mixture was then transferred to the 96-well plate and absorbance was recorded. Both compounds **23a** and **23b** showed significant methylene blue absorbance support of H<sub>2</sub>S formation (Figure 5.6). **1a** was used as a positive control for the assay and compounds **24** and **32** as negative controls. As expected, negative controls and other controls also did not produce a significant amount of H<sub>2</sub>S.

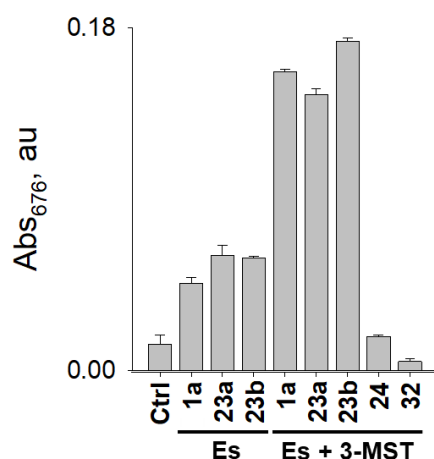


**Figure 5.6:** Methylene blue assay for H<sub>2</sub>S generation by the 3-MST artificial substrates (**23a** and **23b**) in presence of b3-MST. Compounds were incubated with esterase b3-MST and DTT for 1.5 h. **1a** was used as positive control and **24** and **32** were used as negative control. Ctrl represents only 3-MST.

### 5.3.4. 3-MST persulfide detection using clickable tag switch assay

3-MST first reacts with its substrate **2a** and forms 3-MST persulfide which is one of the key steps in the process of H<sub>2</sub>S generation. Therefore, a clickable tag switch assay was performed to detect 3-MST persulfide as described in Chapter 3. In this assay, 100  $\mu$ M of the 3-MST artificial substrates (**23a** and **23b**) were incubated with esterase (1 U/mL) and 3-MST (10  $\mu$ M) for the generation of 3-MST persulfide. **1a** was taken as a positive control and **24**, **32** were used as negative control. An intense band corresponding to the **1a** treated lane showed 3-MST persulfidation (Figure 5.7). An intense band was seen for **23a** and **23b** treated lanes indicating 3-MST persulfide formation. As expected, the other control group did not show significant persulfidation.

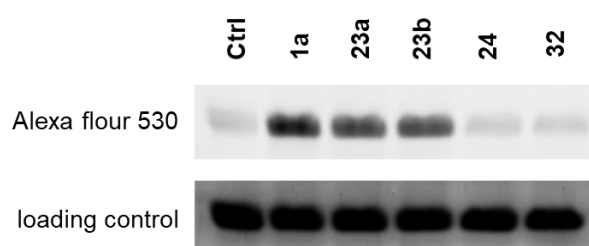


**Figure 5.7:** Persulfidation of *b3*-MST by the 3-MST artificial substrates. **1a**, **23a** and **23a** were incubated with *b3*-MST in presence of esterase. 3-MST persulfidation was detected using the clickable tag-switch assay. Ctrl represents only 3-MST enzyme. Coomassie brilliant blue (CBB) staining was used as a loading control.

### 5.3.5. Sulfane sulfur detection using SSP-2 assay

Next, formation of sulfane sulfur was detected using a reported fluorescent-based assay using an SSP-2 probe as described in Chapter 3.<sup>8</sup> In this assay, esterase (0.5 U/mL) and 3-MST (10  $\mu$ M) were incubated with 100  $\mu$ M of the 3-MST artificial substrates (**23a** and **23b**) for the generation of 3-MST persulfide. **1a** was taken as positive control and **24** and **32** were used as negative controls. At a predetermined time point, the SSP-2 probe was added and incubated. A turn-on fluorescence for **1a** in presence of 3-MST was observed which indicates sulfane sulfur formation (Figure 5.8). In the presence of 3-MST, an enhanced fluorescence was observed for both **23a** and **23b** which indicates significant sulfane sulfur formation. As DTT was known to reduce sulfane sulfur, therefore fluorescence regarding sulfane sulfur formation was significantly reduced for the DTT-treated groups. As expected, negative control and other controls did not show significant sulfane sulfur generation.

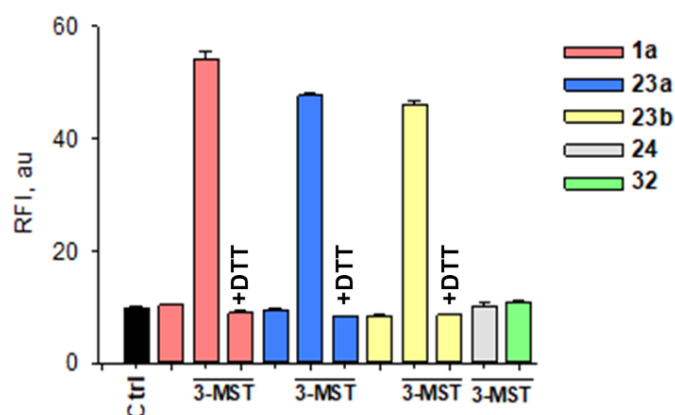


**Figure 5.8:** Sulfane sulfur detection using SSP-2 probe (Fluorescence intensities were measured;  $\lambda_{\text{ex}} = 482 \text{ nm}$ ,  $\lambda_{\text{em}} = 518 \text{ nm}$ ). **1a**, **23a** and **23b** were incubated with *b3*-MST and esterase followed by treatment with the probe SSP-2. +DTT refers to the post-treatment of the enzyme with DTT to reduce sulfane sulfur at the active cysteine residue of 3-MST. Ctrl represents only enzyme.

### 5.3.6. MTT assay for cell viability of the key compounds

Cell viability assay were conducted with **23a**, **23b**, **24** and **32** as described in Chapter 3. Mouse embryonic fibroblast (MEF) cells were treated with variable concentrations of **23a**, **23b**, **24** and **32** and incubated for 24 h and finally, cell viability was measured. Cells are found to be more than 80% viable for the highest treated concentration (100  $\mu\text{M}$ ) of all the compounds which indicates the compounds were well tolerated by the cells for 24 h with no significant cytotoxicity (Figure 5.9).

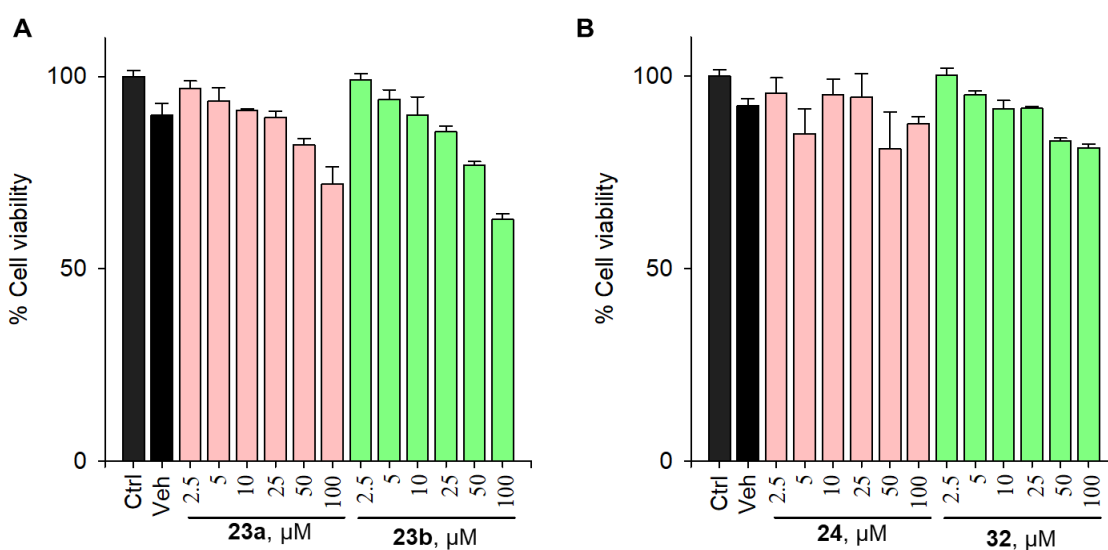


**Figure 5.9:** Cell viability was determined in MEFs cells using MTT assay. (A) in presence of **23a** and **23b**. (B) in presence of **24** and **32**.

#### 5.4. Summary

To summarize, esterase-specific artificial substrates for 3-MST (**23a** and **23b**) were designed and synthesized. Substrate activation followed by turnover of the active substrate (**2a**) by 3-MST was validated using HPLC. Furthermore, efficient H<sub>2</sub>S release from both **23a** and **23b** was detected using lead acetate and methylene blue assay. 3-MST persulfide formation was demonstrated by clickable tag switch assay. The formation of sulfane sulfur was observed using an SSP-2 probe. Finally, the cell viability of these 3-MST artificial substrates and by-products was evaluated using an MTT assay. Cell viability data shows the 3-MST artificial substrates and by-products are benign under experimental concentrations. Collectively, our data suggest that **23a** and **23b** are artificial substrates of 3-MST. Although these compounds were stable towards thiols, they are significantly more hydrophobic (ClogP = 6-7) than the parent substrates (ClogP = 1.6-2.1). It is anticipated that these compounds will find applications when encapsulated in suitable polymer-based nanoparticles for site-specific delivery, which may have applications in the mitigation of inflammation.

## 5.5. Experimental Procedures

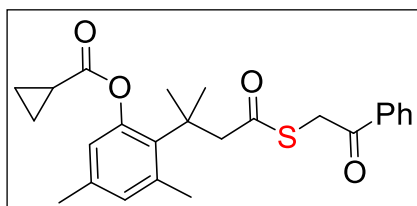
### 5.5.1. Synthesis and characterization of compounds

**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 silica gel Spectrochem (100–200 mesh) as the stationary phase.  $^1\text{H}$  and  $^{13}\text{C}$  spectra were recorded on a JEOL 400 MHz (or 100 MHz for  $^{13}\text{C}$ ) or a Bruker 400 MHz (or 100 MHz for  $^{13}\text{C}$ ) spectrometer unless otherwise specified using either residual solvent signals  $\text{CDCl}_3$  ( $\delta\text{H}$  = 7.26 ppm,  $\delta\text{C}$  = 77.2 ppm) and  $\text{d}_6$ -DMSO ( $\delta\text{H}$  = 2.50 ppm,  $\delta\text{C}$  = 39.6 ppm). Chemical shifts ( $\delta$ ) 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 VarioskanFlash or PerkinElmer EnSight microtiter plate readers.

3-(2-cyclopropyloxy-4,6-dimethylphenyl)-3-methylbutyric acid,<sup>6</sup> 3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid,<sup>6</sup> **1a**,<sup>9</sup> **2a**,<sup>10</sup> **25–27**<sup>7</sup> and **ds1**<sup>10</sup> were synthesized using a previously reported protocol. All analytical data matches the reported values.

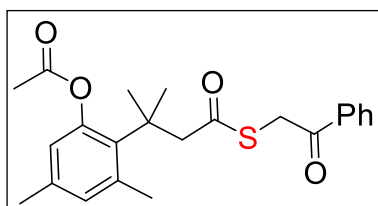
#### **3,5-dimethyl-2-(2-methyl-4-oxo-4-((2-oxo-2-phenylethyl)thio)butan-2-yl)phenyl**

**cyclopropanecarboxylate (23a):** 3-(2-Cyclopropyloxy-4,6-dimethylphenyl)-3-methylbutyric acid (200 mg, 1 eq) and freshly synthesized phenacyl thiol (105 mg, 1 eq) were taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL dry DCM, followed by subsequent addition of DCC (213 mg, 1.5 eq) and DMAP (17 mg, 0.5 eq). The reaction mixture was stirred at 0 °C for 3 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of  $\text{H}_2\text{O}$  and transferred to a separating funnel, where the product was extracted with DCM ( $2 \times 15$  mL). The organic layers were washed with brine (30 mL) then the resultant combined layers were passed through anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (7–8% ethyl acetate/hexane) using 100–200 mesh silica as a stationary phase. The compound obtained from column chromatography was subjected to prep HPLC flash chromatography to obtain pure product.



Yield (140 mg, 48%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.95-7.93 (m, 2H), 7.59-7.56 (m, 1H), 7.47-7.43 (m, 2H), 6.78 (s, 1H), 6.59 (s, 1H), 4.31 (s, 2H), 3.21 (s, 2H), 2.52 (s, 3H), 2.21 (s, 3H), 1.90-1.83 (m, 1H), 1.57 (s, 6H), 1.16-1.14 (m, 2H), 1.03-1.0 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  195.8, 193.7, 174.0, 149.8, 137.9, 136.5, 135.7, 133.7, 133.3, 132.5, 128.8, 128.6, 123.8, 56.3, 40.1, 36.6, 31.5, 25.6, 20.4, 13.7, 9.2; HRMS for  $\text{C}_{25}\text{H}_{29}\text{O}_4\text{S}$  ( $\text{M}+\text{Na}$ ) $^+$  Calculated: 447.1606, Found: 447.1607.

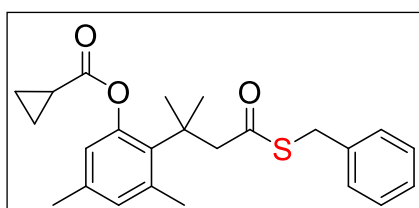
**3,5-dimethyl-2-(2-methyl-4-oxo-4-((2-oxo-2-phenylethyl)thio)butan-2-yl)phenyl acetate (23b):** 3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid (140 mg, 1 eq) and freshly synthesized phenacyl thiol (161 mg, 2 eq) were taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL dry DCM. Subsequently, DCC (164 mg, 1.5 eq), DMAP (32.5 mg, 0.5 eq) were used. The reaction mixture was stirred at 0  $^\circ\text{C}$  for 3 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of  $\text{H}_2\text{O}$  and transferred to a separating funnel, where the product was extracted with DCM ( $2 \times 15$  mL). The organic layers were washed with brine (30 mL) then the resultant combined layers were passed through anhyd.  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (9-10% ethyl acetate/hexane) using 100-200 mesh silica as a stationary phase. The compound obtained from column chromatography was subjected to prep HPLC flash chromatography to obtain pure product.



Yield (130 mg, 61%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.95-7.93 (m, 2H), 7.60-7.57 (m, 1H), 7.47-7.43 (m, 2H), 6.81-6.80 (m, 1H), 6.60-6.59 (m, 1H), 4.32 (s, 2H), 3.18 (s, 2H), 2.52 (s, 3H), 2.30 (s, 3H), 2.22 (s, 3H), 1.56 (s, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  195.7, 193.6, 170.1, 149.5, 138.0, 136.6, 135.7, 133.8, 133.2, 132.7, 128.8, 128.6, 123.3, 56.2, 39.9, 36.6, 31.5, 25.5, 22.1, 20.4; HRMS for  $\text{C}_{25}\text{H}_{28}\text{O}_4\text{S}$  ( $\text{M}+\text{Na}$ ) $^+$  Calculated: 421.1449, Found: 421.1450.

**Synthesis protocol of derivatives 2-(4-(benzylthio)-2-methyl-4-oxobutan-2-yl)-3,5-dimethylphenyl cyclopropanecarboxylate (24):** 3-(2-Cyclopropyloxy-4,6-dimethylphenyl)-3-methylbutyric acid (1 eq, 96 mg) and benzyl mercaptan (1 eq, 46 mg) were taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL dry DCM, followed by subsequent addition of DCC (1.5 eq, 113 mg) and DMAP (0.2 eq, 9 mg). The reaction mixture was stirred

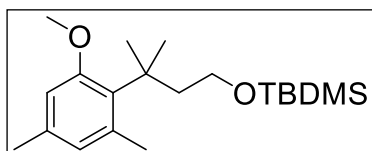
at 0 °C for 3 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of H<sub>2</sub>O and transferred to a separating funnel, where the product was extracted with DCM (2 × 15 mL). The organic layers were washed with brine (30 mL) then the resultant combined layers were passed through anh. Na<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (2-3% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase. The compound obtained from column chromatography was subjected to prep HPLC flash chromatography to obtain pure product.



Yield (80 mg, 59%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.27-7.16 (m, 5H), 6.78-6.77 (m, 1H), 6.60-6.59 (m, 1H), 4.03 (s, 2H), 3.12 (s, 2H), 2.50 (s, 3H), 2.22 (s, 3H), 1.90-1.83 (m, 1H), 1.57 (s, 6H), 1.16-1.14 (m, 2H), 1.03-1.0 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 196.8, 174.0, 149.9, 138.0, 138.0, 136.4, 133.4, 133.4, 132.5, 128.9, 128.6, 127.2, 123.2, 56.2, 40.1, 33.3, 31.7, 25.6, 20.4, 13.7, 9.1; HRMS for C<sub>24</sub>H<sub>29</sub>O<sub>3</sub>S (M+Na)<sup>+</sup> Calculated: 419.1657, Found: 419.1660.

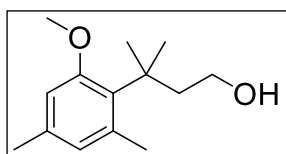
**tert-butyl(3-(2,4-dimethyl-6-(methyl-13-oxidaneyl)phenyl)-3-methylbutoxy)dimethylsilane (28):** 2-(4-((tert-butyl)dimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenol (1 eq, 1 g) and K<sub>2</sub>CO<sub>3</sub> (1.2 eq, 514 mg) were taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL dry DMF. Iodomethane (1.2 eq, 579 μL) was then added and stirred for 36 h at room temperature. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of brine and 20 mL ethyl acetate, transferred to a separating funnel, compound was allowed to distribute between both layers. Ethyl acetate layer was washed with brine (5 × 15 mL) to remove DMF. The organic layers were washed with brine (30 mL) then the resultant combined layers were passed through anh. Na<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (1-2% ethyl acetate/hexane) using 100-200 mesh silica as a stationary phase.

Yield (860 mg, 82%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.55 (m, 1H), 6.51 (m, 1H), 3.76 (s, 3H), 3.48-3.44 (m, 2H), 2.47 (s, 3H), 2.24 (s, 3H), 2.14-2.10 (m, 2H), 1.50 (s, 6H), 0.84 (s, 9H), 0.04 (s, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 159.6, 137.5, 135.8, 131.3, 127.2, 111.4,



61.7, 55.4, 45.8, 39.9, 32.2, 26.1, 25.9, 22.2, 20.9, 18.4, 12.1, -5.1 ; HRMS for  $C_{20}H_{37}O_2Si$  ( $M+H$ )<sup>+</sup> Calculated: 337.2563, Found: 337.1990.

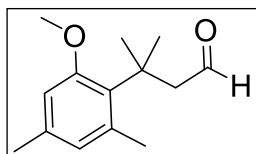
**3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutan-1-ol (29):** *tert*-butyl(3-(2,4-dimethyl-6-(methyl-13-oxidaneyl)phenyl)-3-methylbutoxy)dimethylsilane (1 g, 1 eq), water (4 mL, 80 eq) and THF (9 mL, 50 eq) were taken together in a RB, purged with nitrogen then acetic acid (8 mL, 30 eq) was added, again purged with nitrogen. The reaction was stirred at 0 °C for h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of  $NaHCO_3$  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) then the resultant combined layers were passed through anhydrous  $Na_2SO_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (20-25% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.



Yield (500 mg, 95%).  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  6.58 (bs, 1H), 6.53 (bs, 1H), 3.78 (s, 3H), 3.55-3.52 (m, 2H), 2.49 (s, 3H), 2.24 (s, 3H), 2.18-2.15 (m, 2H), 1.52 (s, 6H);  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$  159.6, 137.6, 136.2, 131.0, 127.5, 111.8, 61.5, 55.6, 45.8, 40.0, 32.3, 25.9, 22.2, 20.9; HRMS for  $C_{14}H_{23}O_2$  ( $M+H$ )<sup>+</sup> Calculated: 223.1698, Found: 223.1326.

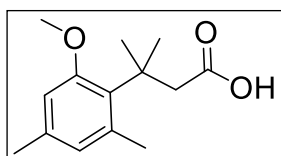
**3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutanal (30):** 3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutan-1-ol (625 mg, 1 eq) and PCC (1.08 g, 2 eq) were taken in a RB and purged with nitrogen, dissolved in dry DCM (10 mL). The reaction was stirred for 2 h at room temperature. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 30 mL of  $H_2O$  and 20 mL DCM, transferred to a separating funnel, where the product was extracted with DCM (2 × 15 mL). The combined organic layers were washed with brine (30 mL) then the resultant combined layers were passed through anhydrous  $Na_2SO_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (14-16% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.

Yield (350 mg, 75%).  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  9.50 (t,  $J=2.72$  Hz, 1H), 6.58 (bs, 1H), 6.57 (bs, 1H), 3.77 (s, 3H), 2.92 (t,  $J=2.72$  Hz, 2H), 2.51 (s, 3H), 2.25 (s, 3H), 1.57 (s, 6H);  $^{13}C$



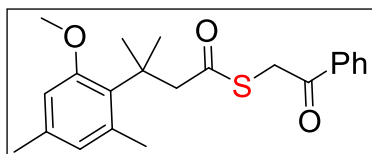
NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  204.5, 158.6, 137.5, 136.8, 129.6, 127.6, 111.5, 57.0, 55.2, 38.9, 31.8, 25.8, 20.9; HRMS for  $\text{C}_{14}\text{H}_{21}\text{O}_2$  ( $\text{M}+\text{H}$ )<sup>+</sup> Calculated: 221.1542, Found: 221.1187.

**3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutanoic acid (31):** 3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutanal (350 mg, 1 eq) taken in a RB and purged with nitrogen, dissolved in 2-methyl 2-butene (2.5 mL) and <sup>t</sup>BuOH (10 mL).  $\text{NaClO}_2$  (431 mg, 3 eq) (in 0.67 M  $\text{KH}_2\text{PO}_4$ ) was added slowly. The reaction was stirred for 2 h at room temperature. Upon completion of the reaction, as monitored by TLC, solvents were evaporated. 20 mL of  $\text{H}_2\text{O}$  and 20 mL ethyl acetate, transferred to a separating funnel, and product was extracted in ethyl acetate. The combined organic layers were washed with brine ( $2 \times 15$  mL) then the resultant combined layers were passed through anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (18-20% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase.



Yield (300 mg, 80%). <sup>1</sup>H NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  6.56 (bs, 1H), 6.53 (bs, 1H), 3.75 (s, 3H), 2.98 (s, 2H), 2.50 (s, 3H), 2.24 (s, 3H), 1.58 (s, 6H); <sup>13</sup>C NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  177.2, 158.9, 137.7, 136.2, 130.1, 127.5, 111.5, 55.4, 47.4, 39.6, 31.7, 25.6, 21.0; HRMS for  $\text{C}_{14}\text{H}_{21}\text{O}_3$  ( $\text{M}+\text{H}$ )<sup>+</sup> Calculated: 237.1491, Found: 237.1478.

**S-(2-oxo-2-phenylethyl) 3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutanethioate (32):** 3-(2-methoxy-4,6-dimethylphenyl)-3-methylbutanoic acid (1 eq, 170 mg, 1 eq), phenacyl thiol (1 eq, 110 mg) were taken in a round-bottom flask, purged with nitrogen and dissolved in 10 mL dry DCM, followed by subsequent addition of DCC (1.5 eq, 223 mg) and DMAP (0.2 eq, 18 mg). The reaction mixture was stirred at 0 °C for 3 h. Upon completion of the reaction, as monitored by TLC, the reaction was quenched with 20 mL of  $\text{H}_2\text{O}$  and transferred to a separating funnel, where the product was extracted with DCM ( $2 \times 15$  mL). The combined organic layers were washed with brine (30 mL) then the resultant combined layers were passed through anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and the filtrate was evaporated under reduced pressure to obtain the crude compound. The compound was further purified by silica gel column chromatography (5-6% ethyl acetate/hexane) using 60-120 mesh silica as a stationary phase. The compound obtained from column chromatography was subjected to Prep HPLC flash chromatography to obtain pure compound.



Yield (180 mg, 67%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.91-7.89 (m, 2H), 7.58-7.54 (m, 1H), 7.44-7.40 (m, 2H), 6.54 (bs, 1H), 6.51 (bs, 1H), 4.25 (s, 2H), 3.77 (s, 3H), 3.32 (s, 2H), 2.48 (s, 3H), 2.23 (s, 3H), 1.58 (s, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  196.6, 194.1, 158.8, 137.4, 136.3, 135.7, 133.6, 129.7, 128.8, 128.6, 127.4, 111.2, 56.4, 55.3, 40.9, 36.2, 31.8, 25.7, 21.0; HRMS for  $\text{C}_{22}\text{H}_{27}\text{O}_3\text{S}$  ( $\text{M}+\text{H}$ ) $^+$  Calculated: 371.1681, Found: 371.1496.

### 5.5.2. HPLC-based studies

**General method:** A stock solution of the **2a**, **3a**, **23a**, **25** and **ds1** (10 mM) was prepared in DMSO whereas the stocks of DTT (100 mM) and GSH (100 mM) were prepared fresh in deionized water. A stock of 32.5  $\mu\text{M}$  of wt *b3*-MST used. The reaction mixtures were prepared in pH 7.4 phosphate buffer (PB) as required. The final concentration of DMSO in the reaction mixture was kept to 1%. The reaction was mixed, and then incubated at 37°C. At pre-determined time points, reaction was quenched and half diluted by ACN, mixed, aliquots were taken from the reaction mixture, and injected (50  $\mu\text{L}$ ) in a high performance liquid chromatography (HPLC) attached with a UV detector (absorbance at 250 nm). Phenomenex Luna C-18 reverse phase column, 100 Å particle size and 5  $\mu\text{M}$  pore size (250  $\times$  4.6 mm) was used. The mobile phase was water: acetonitrile with a gradient starting at 35:65  $\rightarrow$  0 - 1 min, 35:65 to 20:80  $\rightarrow$  1 - 5 min, 20:80  $\rightarrow$  5 - 11 min, 20:80 to 35:65  $\rightarrow$  11 - 13 min, 35:65  $\rightarrow$  13 - 17 min was used at a flow rate of 1 mL/min.

#### 5.5.2.1. Decomposition of **23a** in presence of esterase under non-reducing conditions

In PB (1 mL), the reaction mixture was prepared using **23a** (100  $\mu\text{M}$ ) with esterase (1 U/mL). At pre-determined time points, 100  $\mu\text{L}$  aliquots were taken from the reaction mixture, diluted with 100  $\mu\text{L}$  ACN and 50  $\mu\text{L}$  was injected in the HPLC.

#### 5.5.2.2. Stability of **23a** in buffer and in presence of thiols (GSH and DTT)

To determine the stability in buffer, a reaction mixture was prepared using **23a** (100  $\mu\text{M}$ ) in PB (500  $\mu\text{L}$ ) and incubated at 37 °C. A similar reaction was set up to check the stability of **23a** in presence of with glutathione (10 mM) and DTT (10 mM). At pre-determined time points, 100  $\mu\text{L}$  aliquots were taken from the reaction mixture, diluted with 100  $\mu\text{L}$  ACN and 50  $\mu\text{L}$  was injected into the HPLC.

### 5.5.2.3. Decomposition of 23a followed turnover of 2a by *b3*-MST in presence of DTT to produce 3a

In PB (1 mL), the reaction mixture was prepared using **23a** (100  $\mu$ M) with esterase (1 U/mL), 10 mM DTT (10 mM) and 3-MST (0.5  $\mu$ M). At predetermined time points, 100  $\mu$ L aliquots were taken from the reaction mixture, diluted with 100  $\mu$ L ACN and 50  $\mu$ L was injected into the HPLC.

### 5.5.3. H<sub>2</sub>S generation from 23a and 23b in presence of 3-MST and DTT

#### 5.5.3.1. Lead Acetate assay for the detection of H<sub>2</sub>S

The lead acetate assays were conducted as previously reported with some modifications. Firstly, lead acetate soaked paper was prepared. Whatman filter papers were soaked with 5% (w/v) lead (II) acetate solution and dried. 285  $\mu$ M stock of wild-type (wt) *b3*-MST is used for lead acetate assay. Stocks of all compounds (**1a**, **23a**, **23b**, **24** and **32**) (10 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) and dithiothreitol (DTT, 100 mM) were prepared in deionized water.

Lead acetate assay was performed in a 96-well plate (with lid). Individual reaction samples were prepared for **1a**, **23a**, **23b**, **24** and **32**. Compound (100  $\mu$ M) was subsequently added to esterase (1 U/mL), wt 3-MST (0.5  $\mu$ M) and DTT (10 mM). The final volume was adjusted to 200  $\mu$ L using 1 $\times$  phosphate buffer saline (PBS) pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37 °C.

A similar protocol was followed for the substrate controls. Individual reaction samples were prepared for **1a**, **23a** and **23b**. Compound (100  $\mu$ M) was subsequently added to esterase (1 U/mL) and DTT (10 mM). The final volume was adjusted to 200  $\mu$ L using 1 $\times$  PBS pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37 °C.

A separate experiment was conducted to test the enzyme controls. DMSO (1%), Esterase (1 U/mL), wt 3-MST (0.5  $\mu$ M) and DTT (10 mM) were sequentially added. The final volume was adjusted to 200  $\mu$ L using 1 $\times$  PBS pH 7.4 buffer, covered with 5% lead acetate-soaked paper, sealed and incubated at 37 °C.

At predetermined time points, lead acetate paper was carefully taken out and an image was captured using Syngene G-Box Chemi-XRQ.

### 5.5.3.2. Methylene blue assay for the detection of H<sub>2</sub>S

The methylene blue assays were conducted as previously reported with some modifications. Briefly, 285  $\mu$ M stock of wt 3-MST was used for the methylene blue assay. 10 mM stocks of all compounds (**1a**, **23a**, **23b**, **24** and **32**) were prepared in DMSO. Porcine liver esterase (100 U/mL), DTT (100 mM) and Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (40 mM) was prepared in deionized water. A stock solution of FeCl<sub>3</sub> (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.

Individual reaction samples were prepared for **1a**, **23a**, **23b**, **24** and **32**. Compound (100  $\mu$ M) was added to esterase (1 U/mL), Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (400  $\mu$ M), wt 3-MST (0.5  $\mu$ M) and DTT (10 mM). The final volume was adjusted to 0.5 mL using 1× PBS pH 7.4 buffer and incubated at 37 °C at 300 rpm.

A similar protocol was followed for the substrate controls. Individual reaction samples were prepared for **1a**, **23a** and **23b**. Compound (100  $\mu$ M) was added to esterase (1 U/mL), Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (400  $\mu$ M) and DTT (10 mM). The final volume was adjusted to 0.5 mL using 1× PBS pH 7.4 buffer and incubated at 37 °C at 300 rpm.

A separate experiment was conducted to test the enzyme controls. DMSO (1%), Esterase (1 U/mL), wt 3-MST (0.5  $\mu$ M), Zn(OAc)<sub>2</sub>·2H<sub>2</sub>O (400  $\mu$ M) and DTT (10 mM) were sequentially added. The final volume was adjusted to 0.5 mL using 1× PBS pH 7.4 buffer and incubated at 37 °C at 300 rpm.

At predetermined time points, equal volumes (200  $\mu$ L) of FeCl<sub>3</sub>, DMPPDA and aliquots from the above reaction samples were mixed and the resulting solution was incubated at 37 °C for 30 min in dark, to allow the formation of the methylene blue dye. An aliquot of 150  $\mu$ L was transferred to a 96-well plate and the absorbance values were recorded at 676 nm using a microplate reader (Thermo Scientific VarioskanFlash).

### 5.5.4. Clickable Tag-switch assay for 3-MST persulfide detection

The Clickable Tag-switch assay was conducted with 285  $\mu$ M stocks of wt 3-MST. 10 mM stocks of all compounds (**1a**, **23a**, **23b**, **24** and **32**) were prepared in DMSO. Porcine liver esterase (100 U/mL) was prepared in deionized water.

Individual reaction samples were prepared for **1a**, **23a**, **23b**, **24** and **32**. To 3-MST (15  $\mu$ M), compound (100  $\mu$ M) and esterase (1 U/mL) were added. Volume was adjusted to 100  $\mu$ L by Tris buffer (pH 8.0) and incubated for 1 h. After that 5 mM MSBT-A (2.5  $\mu$ L, 200 mM) and 5  $\mu$ L 10% SDS were added and incubated for 30 min. Then 500  $\mu$ M PMSF (0.5  $\mu$ L, 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu$ L, 200 mM) was added and incubated for 1 h.

A similar reaction was set up for enzyme control. To 3-MST (15  $\mu$ M), DMSO (1%) was added. The reaction volume was adjusted to 100  $\mu$ L by Tris buffer (pH 8.0) and incubated for 60 min. After that 5 mM MSBT-A (2.5  $\mu$ L, 200 mM) and 5  $\mu$ L 10% SDS were added and incubated for 30 min. Then 500  $\mu$ M PMSF (0.5  $\mu$ L, 100 mM) was added and incubated for 30 min. Then 2 mM of CN-Alkyne (1  $\mu$ L, 200 mM) was added and incubated for 1 h.

After the reaction click reaction cocktail: 6  $\mu$ L TBTA (1.7 mM), 2  $\mu$ L CuSO<sub>4</sub>·5H<sub>2</sub>O (50 mM), 2  $\mu$ L TCEP (50 mM), 1  $\mu$ L Rhodamine azide (10 mM) was added to each. The reaction was stirred at 25 °C for 1 h. After completion of the reaction, 40  $\mu$ L of loading dye was added. The samples (20  $\mu$ L) were loaded in a gel and SDS-PAGE was performed (run at 150 V for 1 h), the in-gel fluorescence image was taken using Syngene G-Box Chemi-XRQ.

#### 5.5.5. Sulfane sulfur measurement using SSP-2

Stock solutions of **1a** (10 mM), **23a** (10 mM), **23b** (10 mM), **24** (10 mM), **32** (10 mM) and SSP-2 (5 mM) were prepared in DMSO. Porcine liver esterase (100 U/mL) and DTT (100 mM) were prepared in DI water.

Individual reaction samples were prepared for **1a**, **23a**, **23b**, **24** and **32**. To 3-MST (10  $\mu$ M), compound (100  $\mu$ M) and esterase (0.5 U/mL) were added. Volume was adjusted to 400  $\mu$ L using 1× PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C.

A reaction was set up for compound control. Individual reaction samples were prepared for **1a**, **23a** and **23b**. The reaction mixtures were prepared by adding compound (100  $\mu$ M) with esterase (0.5 U/mL). and the volume was adjusted to 400  $\mu$ L using 1× PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C.

A reaction was set up for enzyme control. The reaction mixtures were prepared by 3-MST (10  $\mu$ M), 4  $\mu$ L of DMSO, esterase (0.5 U/mL) and the volume was adjusted to 400  $\mu$ L using 1× PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37 °C.

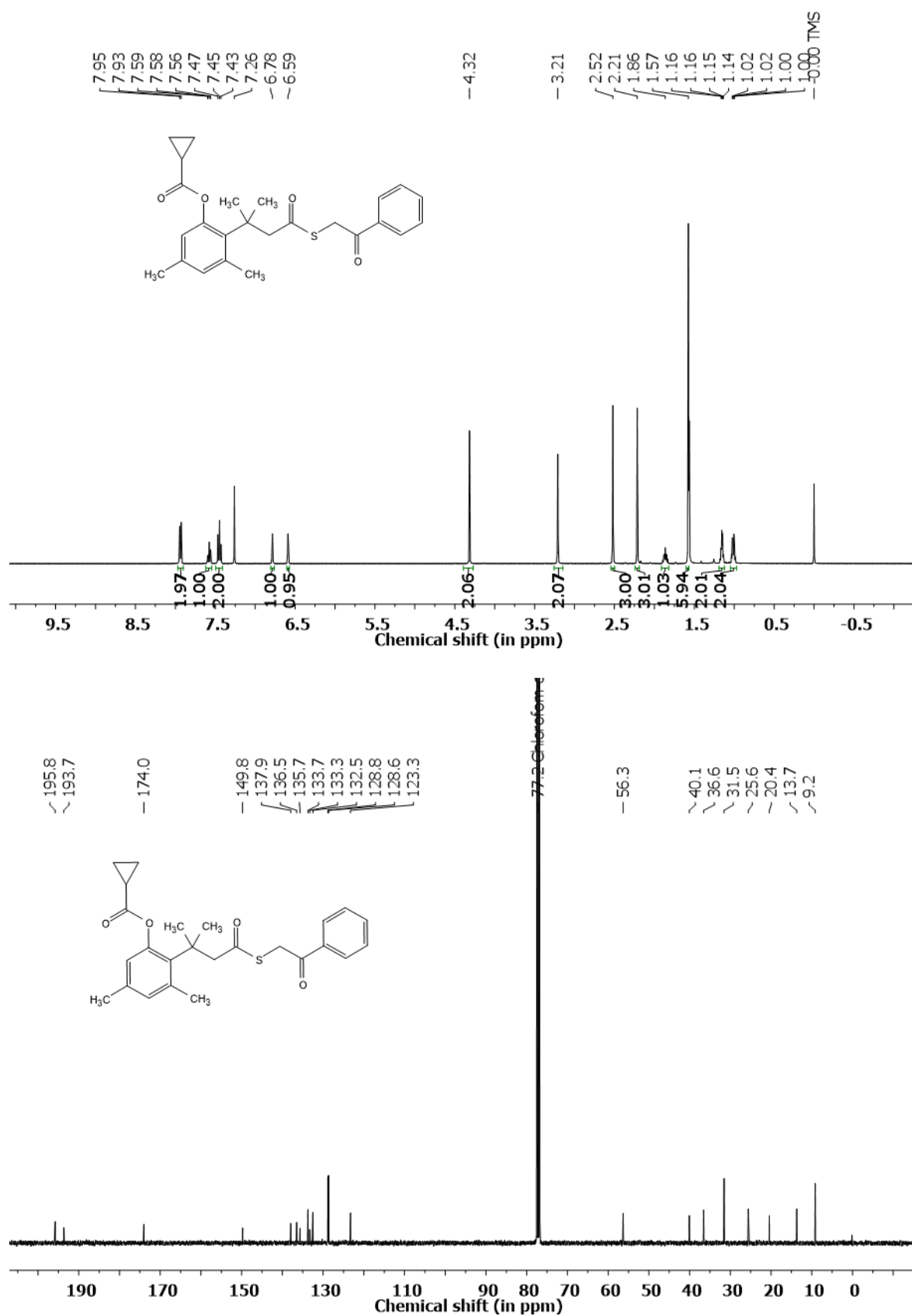
A similar reaction was set up for DTT control. Individual reaction samples were prepared for **1a**, **23a** and **23b**. The reaction mixtures were prepared by adding compound (100  $\mu$ M) with esterase (0.5 U/mL) and 3-MST (10  $\mu$ M). The volume was adjusted to 360  $\mu$ L using 1 $\times$  PBS pH 7.4 buffer. The reaction was incubated for 1 h at 37  $^{\circ}$ C. Finally, the reaction mixture was treated with 10 mM DTT (40  $\mu$ L of 100 mM stock) after 30 min and further incubated at 37  $^{\circ}$ C for 1 h.

The above treatment groups were finally incubated with 50  $\mu$ M SSP-2 (4  $\mu$ L, 5 mM) at 37  $^{\circ}$ C for 10 min in the dark. 100  $\mu$ L aliquot of each sample was transferred to a 96 well plate and the fluorescence was recorded ( $\lambda_{\text{ex}}$  = 482 nm,  $\lambda_{\text{em}}$  = 518 nm) using a microplate reader (Thermo Scientific VarioskanFlash).

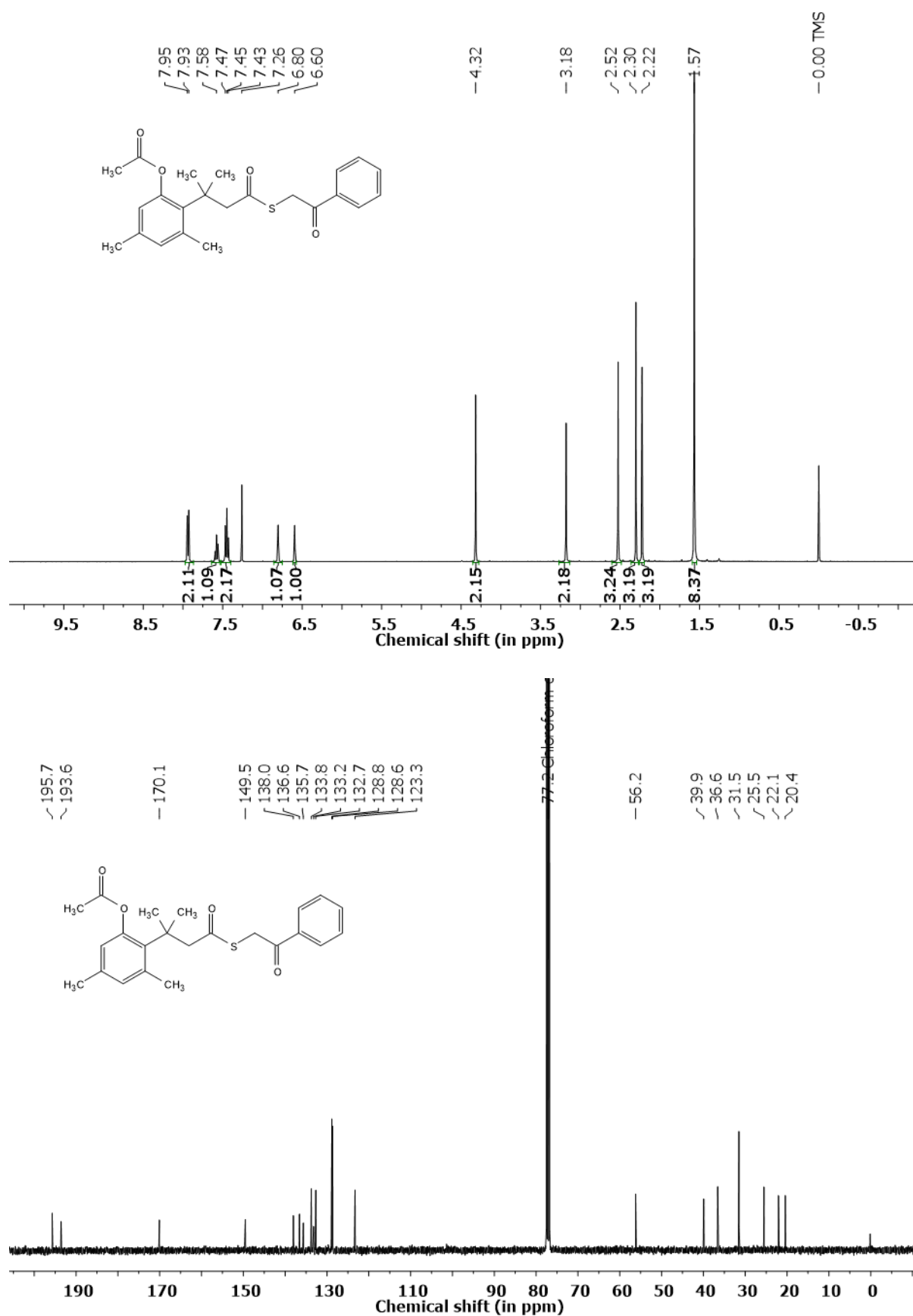
#### 5.5.6. Cell viability assay with MEF cells

In a 96-well plate, Mouse embryonic fibroblasts (MEF) were seeded in a 96-well plate at a concentration of  $1 \times 10^4$  cells/well in DMEM media supplemented with 10% FBS and 1% antibiotic solution. Cells were incubated overnight in an atmosphere of 5% CO<sub>2</sub> at 37  $^{\circ}$ C. Then, stock solutions of compounds (**23a**, **23b**, **24** and **32**) were prepared in DMSO and the cells were treated with varying concentrations of the compounds. For all the treatments, the final concentration of DMSO did not exceed 0.5%. The cells were incubated for 24 h at 37  $^{\circ}$ C. Following this, the old media from the cells were removed, a 0.5 mg/mL stock solution of 3-MTT was prepared in 7 mL DMEM media, 100  $\mu$ L of the MTT stock was added to each well and incubated for 4 h at 37  $^{\circ}$ C. Next, the media was carefully removed and 100  $\mu$ L of DMSO was added to each well and allowed to mix properly. Finally, absorbance for each well at 570 nm was carried out using a microplate reader (Thermo Scientific Varioskan) to estimate cell viability.

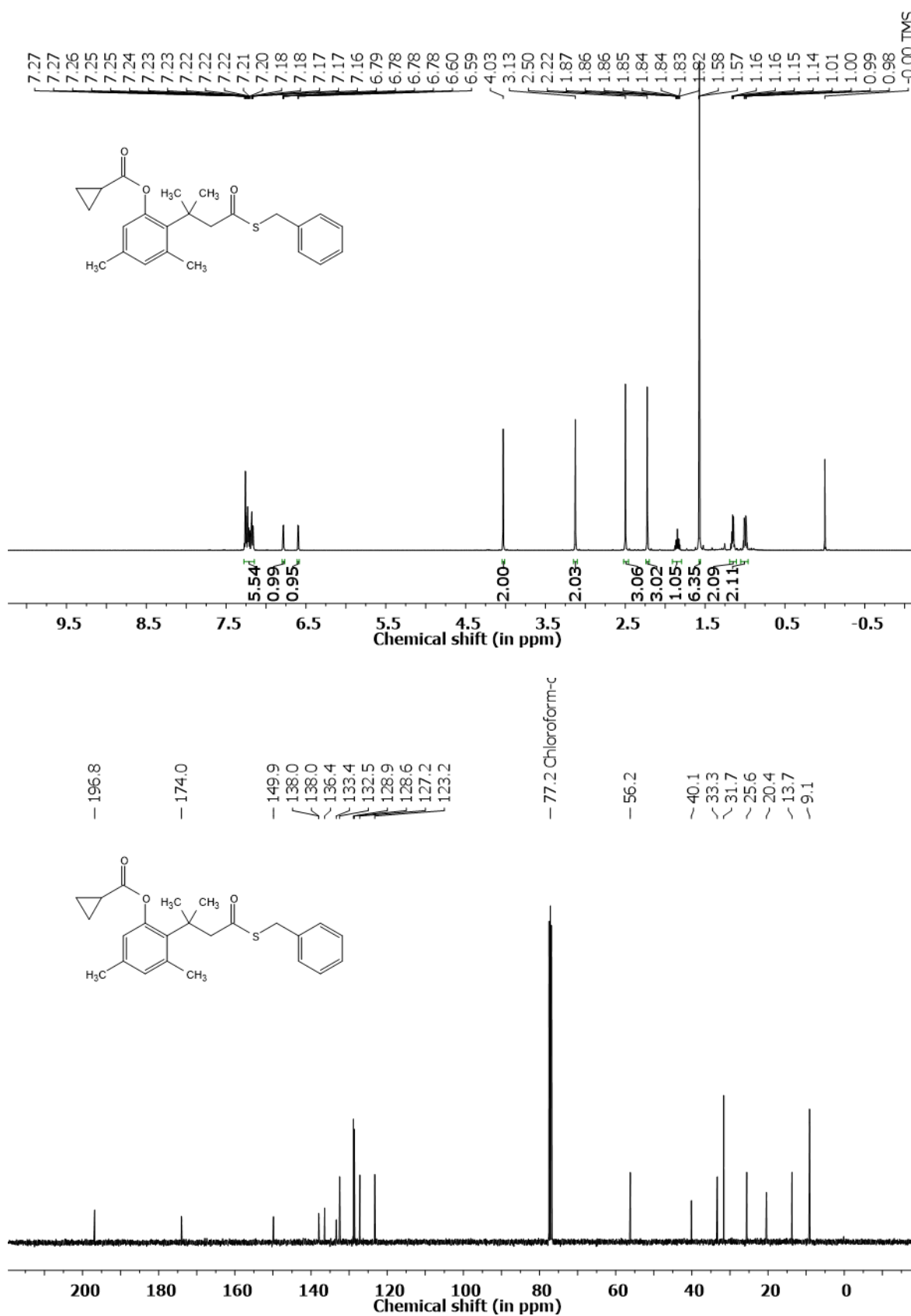
## 5.6. NMR spectra of compounds

 $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **23a**:

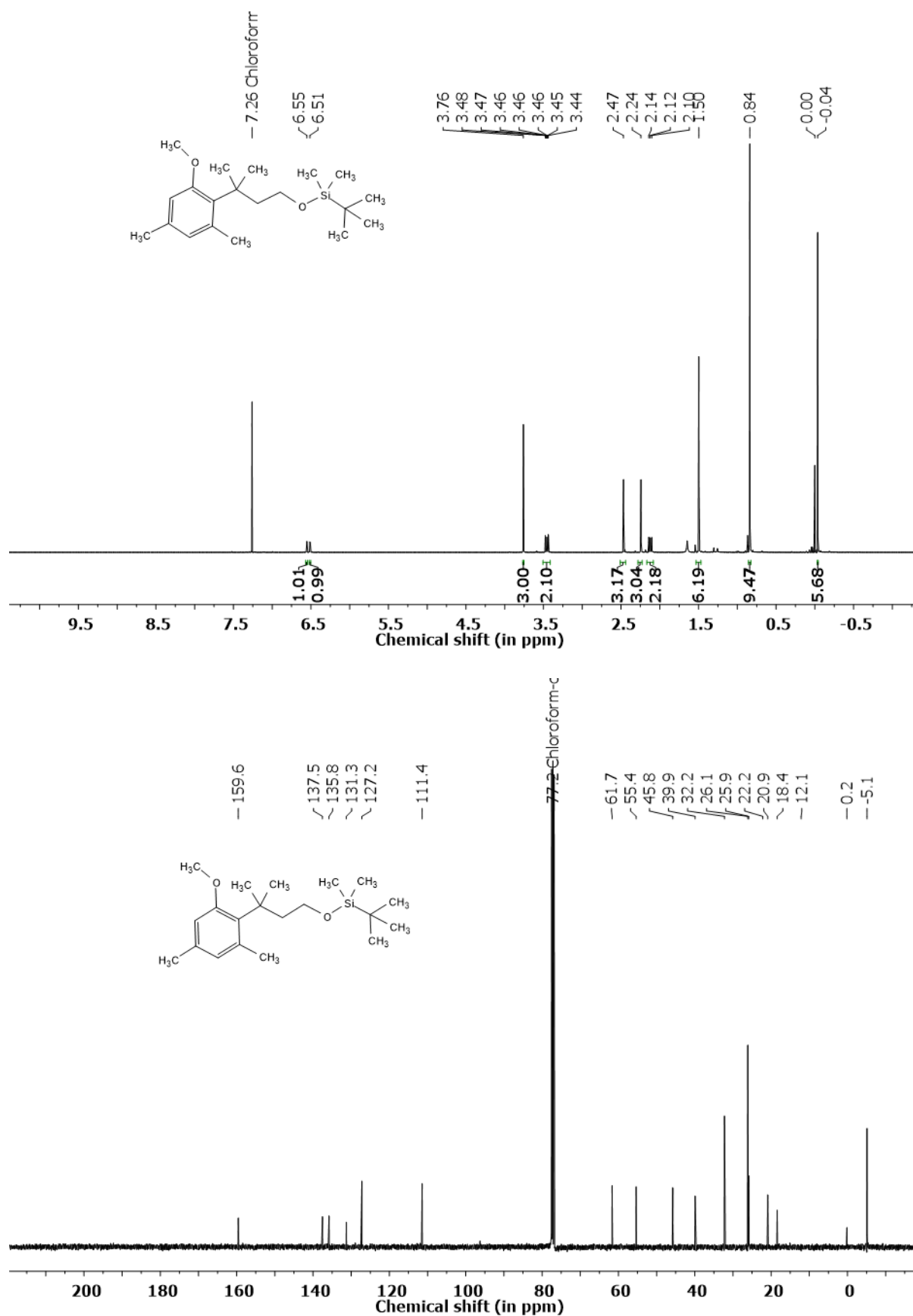
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **23b**:



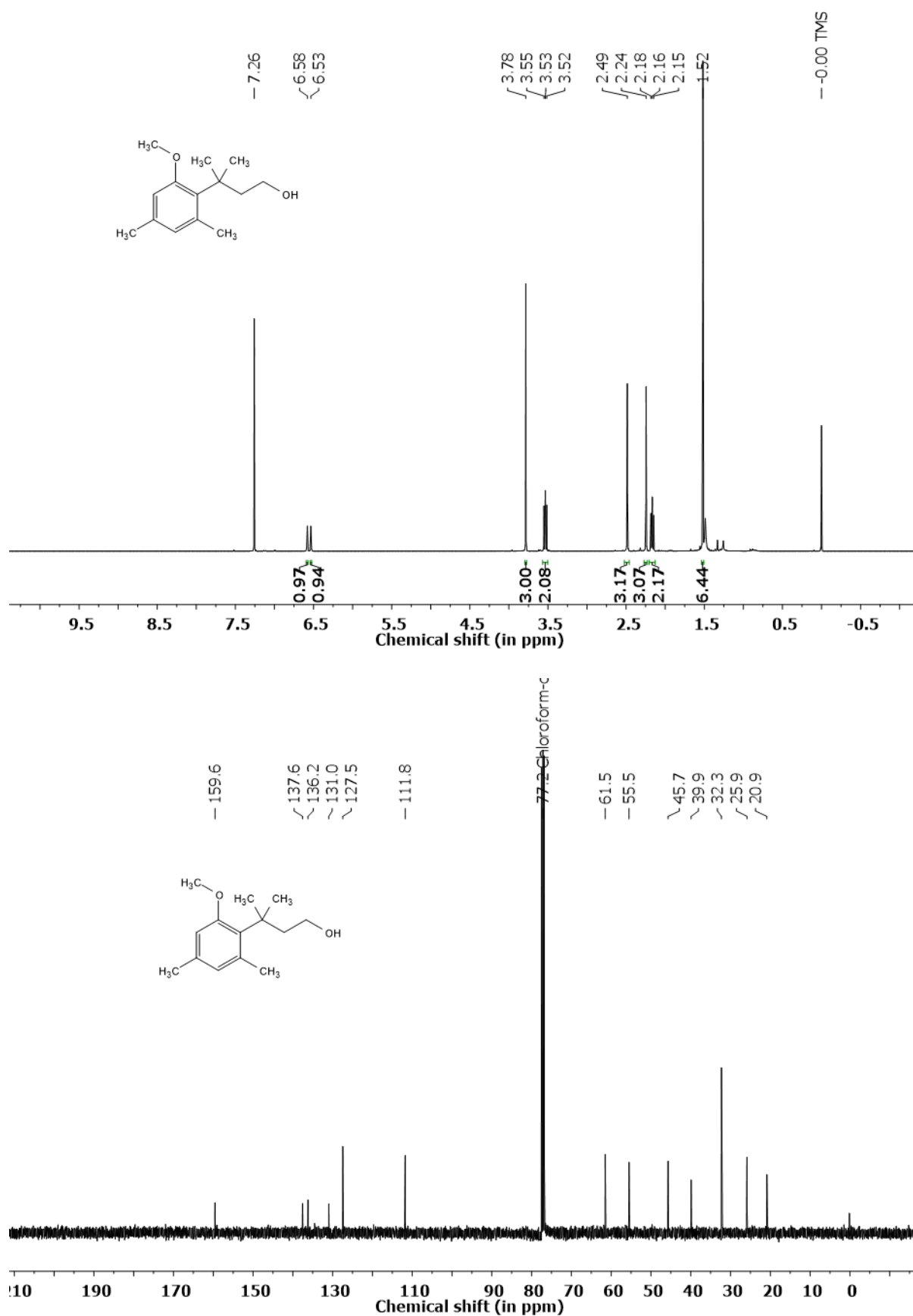
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **24**:



$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **28**:



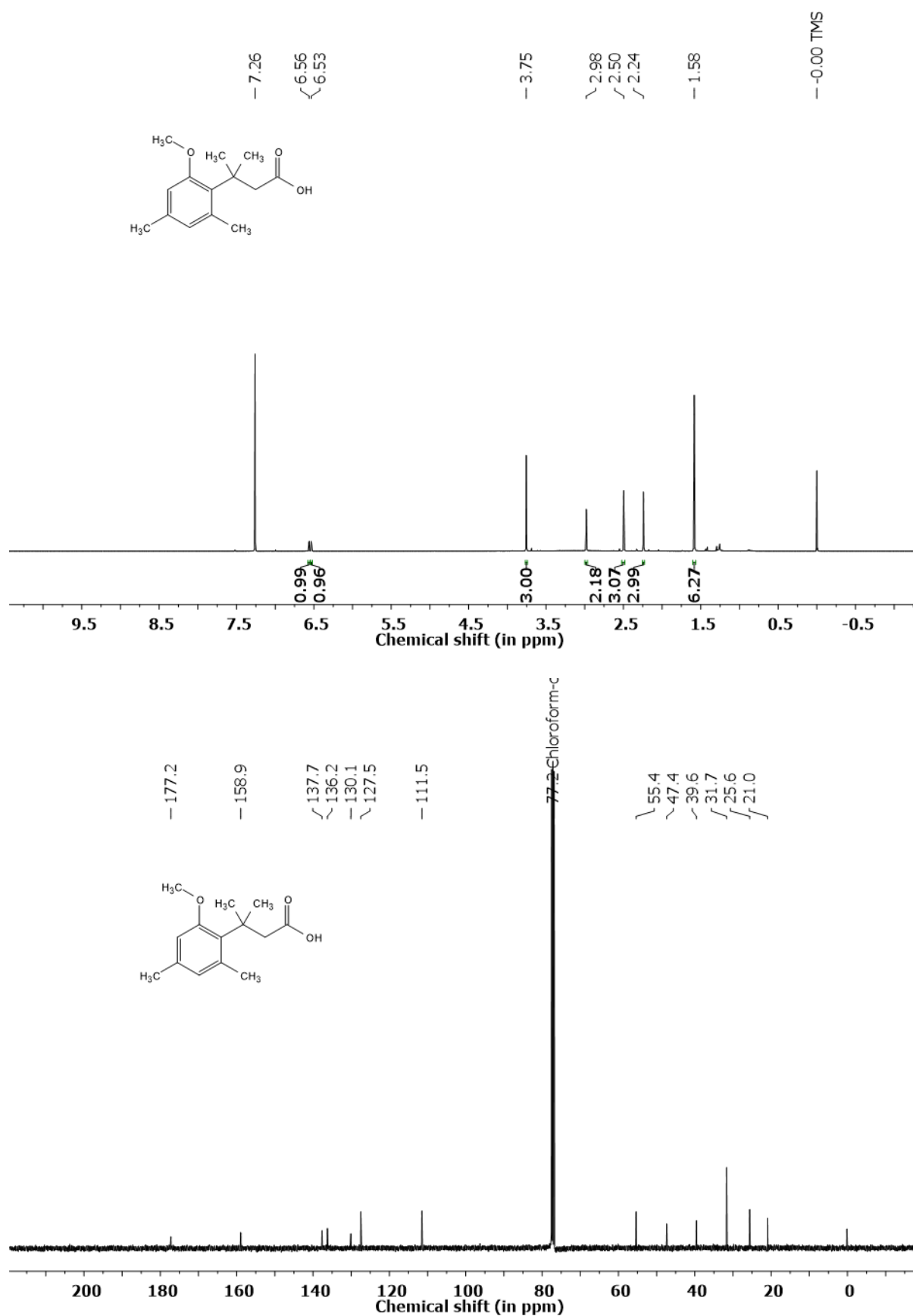
$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **29**:



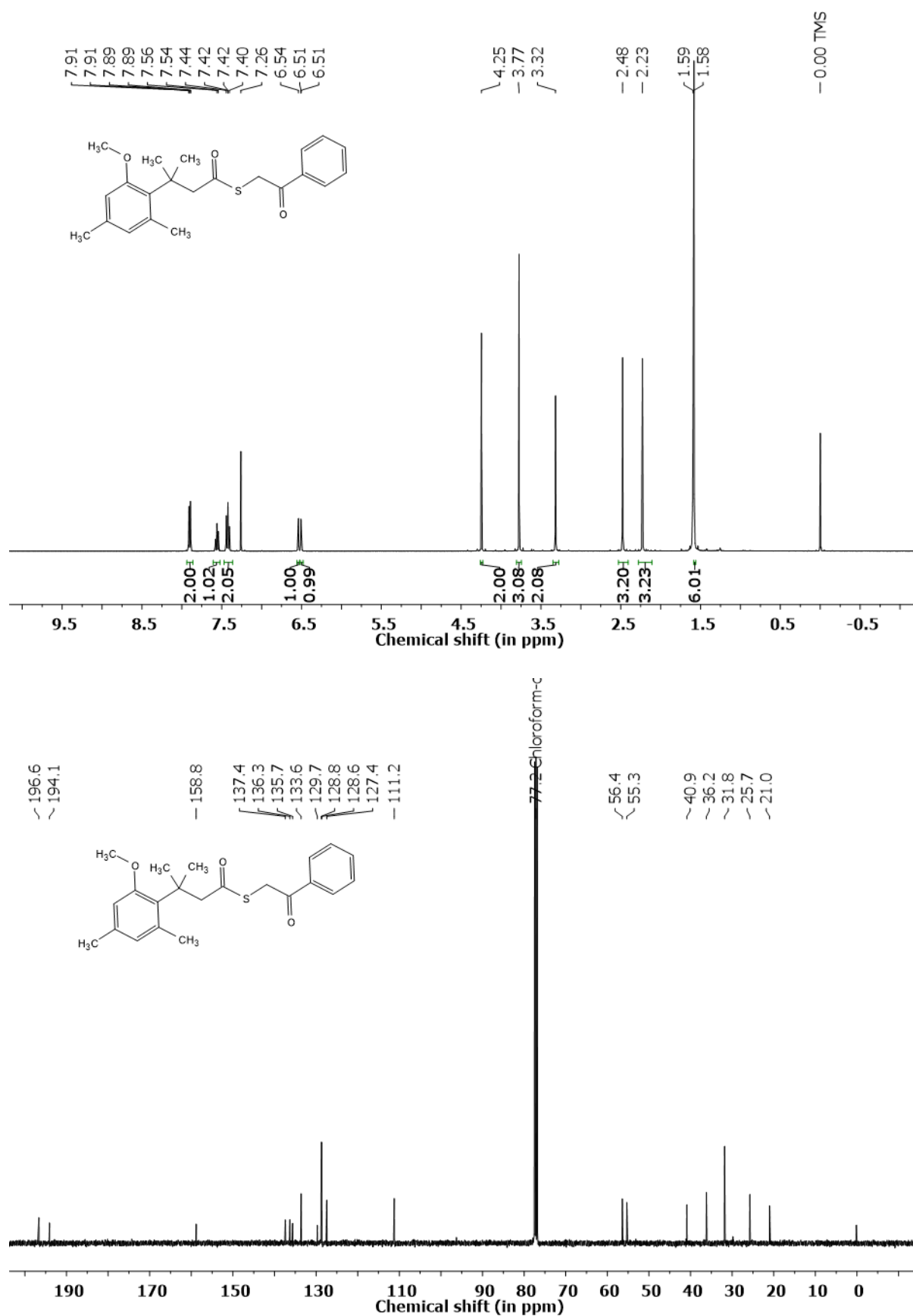
$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **30**:



$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **31**:



$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **32**:



## 5.7. References

- (1) Zheng, Y.; Yu, B.; Ji, K.; Pan, Z.; Chittavong, V.; Wang, B. Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angew. Chemie Int. Ed.* **2016**, 55 (14), 4514–4518.
- (2) Yuan, Z.; Zheng, Y.; Yu, B.; Wang, S.; Yang, X.; Wang, B. Esterase-Sensitive Glutathione Persulfide Donor. *Org. Lett.* **2018**, 20 (20), 6364–6367.
- (3) Wang, B.; Nicolaou, M. G.; Liu, S.; Borchardt, R. T. Structural Analysis of a Facile Lactonization System Facilitated by a “Trimethyl Lock.” *Bioorg. Chem.* **1996**, 24 (1), 39–49.
- (4) Levine, M. N.; Raines, R. T. Trimethyl Lock: A Trigger for Molecular Release in Chemistry, Biology, and Pharmacology. *Chem. Sci.* **2012**, 3 (8), 2412.
- (5) Wang, B.; Gangwar, S.; Pauletti, G. M.; Siahaan, T. J.; Borchardt, R. T. Synthesis of a Novel Esterase-Sensitive Cyclic Prodrug System for Peptides That Utilizes a “Trimethyl Lock”-Facilitated Lactonization Reaction. *J. Org. Chem.* **1997**, 62 (5), 1363–1367.
- (6) Zheng, Y.; Yu, B.; Li, Z.; Yuan, Z.; Organ, C. L.; Trivedi, R. K.; Wang, S.; Lefer, D. J.; Wang, B. An Esterase-Sensitive Prodrug Approach for Controllable Delivery of Persulfide Species. *Angew. Chem. Int. Ed. Engl.* **2017**, 56 (39), 11749–11753.
- (7) Huvelle, S.; Alouane, A.; Le Saux, T.; Jullien, L.; Schmidt, F. Syntheses and Kinetic Studies of Cyclisation-Based Self-Immolative Spacers. *Org. Biomol. Chem.* **2017**, 15 (16), 3435–3443.
- (8) Chen, W.; Liu, C.; Peng, B.; Zhao, Y.; Pacheco, A.; Xian, M. New Fluorescent Probes for Sulfane Sulfurs and the Application in Bioimaging. *Chem. Sci.* **2013**, 4 (7), 2892.
- (9) Heredia, A. A.; Soria-Castro, S. M.; Bouchet, L. M.; Oksdath-Mansilla, G.; Barrionuevo, C. A.; Caminos, D. A.; Bisogno, F. R.; Argüello, J. E.; Peñeñory, A. B. Stereoselective One-Pot Synthesis of  $\beta$ -Alkylsulfide Enol Esters. Base-Triggered Rearrangement under Mild Conditions. *Org. Biomol. Chem.* **2014**, 12 (33), 6516.
- (10) Chandanshive, J. Z.; Bonini, B. F.; Gentili, D.; Fochi, M.; Bernardi, L.; Franchini, M. C. Regiocontrolled Synthesis of Ring-Fused Thieno[2,3-c]Pyrazoles through 1,3-Dipolar Cycloaddition of Nitrile Imines with Sulfur-Based Acetylenes. *European J. Org. Chem.* **2010**, 2010 (33), 6440–6447.

## Synopsis

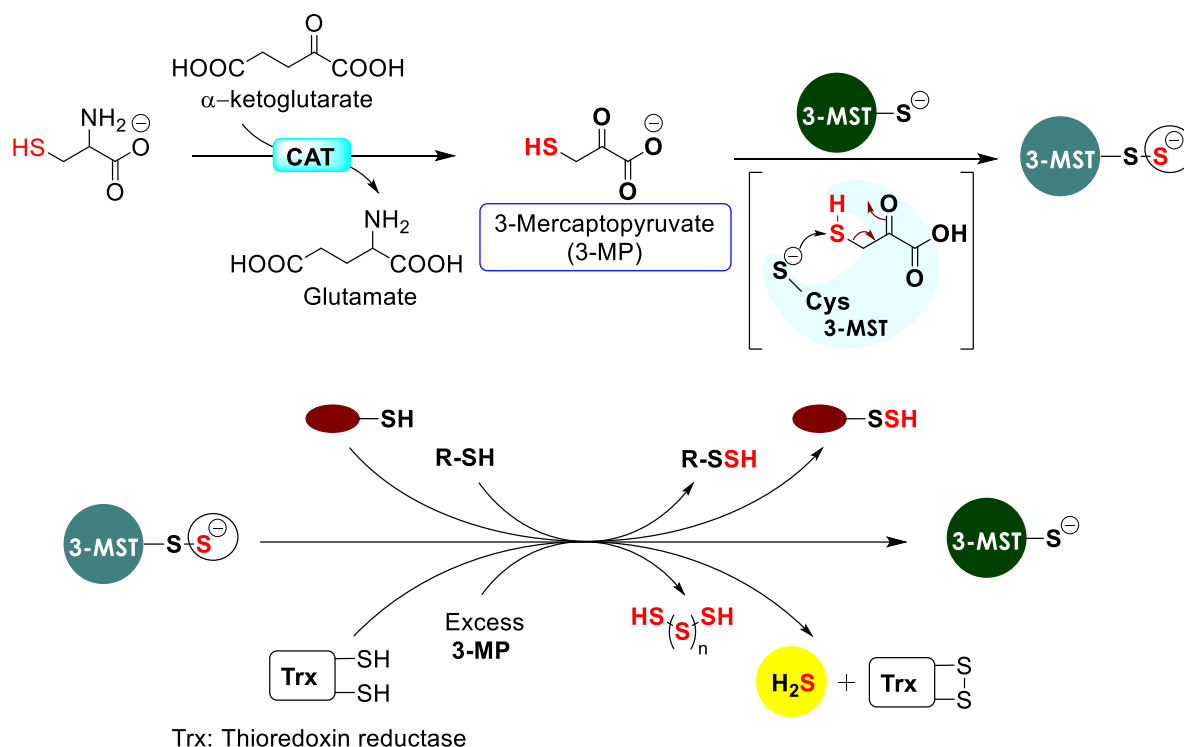
### Enhancing Cellular Persulfides through Artificial Substrate of 3-Mercaptopyruvate Sulfurtransferase (3-MST)

#### CHAPTER 1. Introduction

In the last two decades of scientific studies revealed, hydrogen sulfide ( $\text{H}_2\text{S}$ ) is a gaseous signalling molecule, known to be involved in many biological functions such as antibiotic resistance, vasorelaxation, angiogenesis, apoptosis, ageing, and metabolism.<sup>1-4</sup>  $\text{H}_2\text{S}$  is also known as an antioxidant species and plays a key role in redox homeostasis.<sup>5</sup> Along with this, persulfides ( $\text{RSSH/RSS}^-$ ) have emerged to be players of sulfur biochemistry.<sup>6,7</sup> Persulfides are highly reactive, short-lived species and have sulfur oxidation state -1. A multitude of studies showed, similar to  $\text{H}_2\text{S}$ , cellular persulfides are crucial in the redox signalling process and also relevant to cardiovascular and neuronal diseases.<sup>8,9</sup> Along with persulfides, protein persulfidation, a cysteine based oxidative post translational modification on protein (additional one  $\text{SH/S}^-$  addition on cysteine residue), has gained attention in the past few years.<sup>10,11</sup> Protein persulfidation plays a significant role in oxidative stress protection,<sup>12,13</sup> DNA repairing,<sup>14</sup> KEAP1-nrf2 antioxidant signalling<sup>15</sup> etc. These species are found to have relevance in NF- $\kappa$ B mediated inflammation machinery<sup>16</sup> and various neurodegenerative disorders.<sup>11,17</sup> Although some reports are still contradictory, instances showed the roles of persulfidation in cancer growth. Hence, regulating the persulfides concentration in cells becomes important.

In mammals, Endogenous concentrations of  $\text{H}_2\text{S}$  and persulfides are primarily governed by 4 enzymes, such as cystathionine  $\beta$ -synthase (CBS),  $\gamma$ -cystathionase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteinyl- $t$ -RNA synthetase (CARS).<sup>18-20</sup> Among these, persulfide formation through 3-MST is highly interesting due to its significant contribution in  $\text{H}_2\text{S}$ , persulfide and polysulfide biosynthesis as well as in sulfur trafficking.<sup>20-22</sup> 3-Mercaptopyruvate (3-MP), the physiological substrate of 3-MST that is synthesized from *L*-cysteine catalyzed by pyridoxal 5'-phosphate (PLP)-dependent enzyme, cysteine aminotransferase (CAT) (Scheme 1).<sup>22</sup> Catalytically active cysteine residue of 3-MST reacts with 3-MP to form 3-MST persulfide. The latter species has the capabilities to persulfidate other thiophilic acceptor proteins and metabolites.<sup>22,23</sup> 3-MST bound persulfide also reacts with thiols or thioredoxin (in presence of NADPH/thioredoxin reductase) to generate  $\text{H}_2\text{S}$ .<sup>24</sup> In addition, 3-MST is known to react with an excess amount of 3-MP to generate polysulfide.<sup>25</sup> 3-MST substantially present in brain, kidney and liver and in a subcellular level 3-MST

primarily localized in mitochondria.<sup>26,27</sup> Due to the wide distribution in tissue and subcellular level, 3-MST activity and expression level is crucial for various biological functions and disease.<sup>25,28,29</sup>



**Scheme 1:** Enzymatic functions of 3-MST in cellular H<sub>2</sub>S, persulfides and polysulfides production.

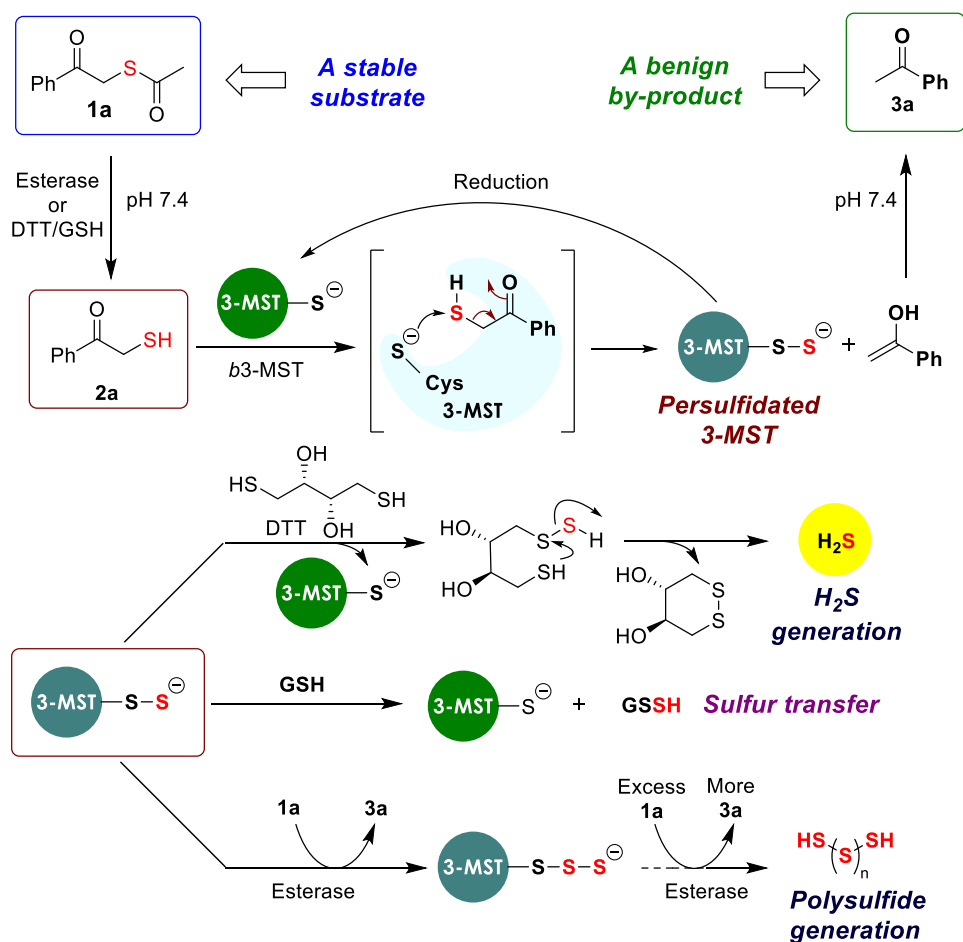
Therefore, our therapeutic interest arises to study and modulate 3-MST enzymatic activity. As 3-MP, the native substrate of 3-MST is unstable and not very amenable for modifications there is a need to develop a stable, reliable substrate with rate tuning handle. Furthermore, a structurally modified substrate having a specific trigger sensitive moiety and an organelle targeting group will be more beneficial for stimuli specific and targeted delivery into mitochondria.

In order to achieve the aforementioned goal, in Chapter 2, a stable artificial substrate of 3-MST was identified for the generation of persulfide in cells. Following this, a structure activity relationship (SAR) was studied using a library of synthesized artificial substrates to tune the rate of 3-MST turnover. In Chapter 3, based on SAR data, a mitochondrion targeted artificial substrate of 3-MST was designed to enhance persulfide in a localized manner. As the substrates synthesized in Chapter 2 and 3 were found to be reactive with thiols, a modified thiol stable substrate with a biologically relevant stimuli sensitive moiety was next synthesized. In Chapter 3, glycosidase-sensitive 3-MST substrates were synthesized in order to improve aqueous

solubility. Furthermore, in Chapter 5, thiol stable esterase-sensitive 3-MST substrates were synthesized for a wider range of application.

## CHAPTER 2. Structure-Activity Relationship Study of Artificial Substrates of 3-MST

Firstly, in collaboration with the lab colleagues, phenacyl thiol (**2a**) was identified as the substrate of 3-MST.<sup>30,31</sup> Next, in order to enhance the stability of **2a**, a stable thioester **1a** which can be cleaved by esterase (Es) or reducing thiol (GSH/DTT) to release **2a**. Using **1a** was designed. artificial substrate turnover mechanism by 3-MST was studied by high performance liquid chromatography (HPLC) and acetophenone (**3a**) was identified as a byproduct from turnover (Scheme 2). Next, the generation of H<sub>2</sub>S, persulfide and polysulfide with **1a** was evaluated using several *in vitro* experiments. Following this, cellular studies were performed and the generation of H<sub>2</sub>S and persulfide were detected using specific dyes. Finally, the antioxidant and anti-inflammatory activity of the artificial substrate **1a** was validated in both cellular and mice models.



**Scheme 2:** Catalytic 3-MST turnover mechanism by artificial substrate **1a** followed by H<sub>2</sub>S generation, sulfur transfer and polysulfide generation

Next, a structure activity relationship was studied using a library of the artificial substrates that were synthesized by considering various electron releasing and electron withdrawing substituents at *ortho* and *para* position of the phenyl ring of **2a**. All of these compounds were tested by methylene blue colorimetric assay for H<sub>2</sub>S detection using wild type *human* 3-MST (*h3-MST*) and *E. coli* 3-MST (*b3-MST*) in presence of DTT and relative rates were calculated. These substrates showed good potency and tunability in the H<sub>2</sub>S release rates (Table 1).

**Table 1.** Relative rates of H<sub>2</sub>S generation from the compounds **1a-1n**.

| <div style="display: flex; align-items: center; justify-content: space-between;"> <div style="text-align: center;"> <p><b>1</b></p> </div> <div style="border: 1px solid black; padding: 5px; text-align: center;"> <p>For <b>1a-1k</b>, R<sup>1</sup> =  X, R<sup>2</sup> = H</p> </div> </div> |                  |     |                |                                         |           |                                         |           |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|-----|----------------|-----------------------------------------|-----------|-----------------------------------------|-----------|
| Cpd.                                                                                                                                                                                                                                                                                             | R <sup>1</sup>   |     | R <sup>2</sup> | <i>h3-MST</i>                           |           | <i>b3-MST</i>                           |           |
|                                                                                                                                                                                                                                                                                                  | X                | Y   |                | <i>k<sub>h</sub></i> (h <sup>-1</sup> ) | Rel. rate | <i>k<sub>b</sub></i> (h <sup>-1</sup> ) | Rel. rate |
| <b>1a</b>                                                                                                                                                                                                                                                                                        | H                | H   | H              | 1.48                                    | 30        | 1.45                                    | 8         |
| <b>1b</b>                                                                                                                                                                                                                                                                                        | NO <sub>2</sub>  | H   | H              | 11.13                                   | 223       | 9.27                                    | 49        |
| <b>1c</b>                                                                                                                                                                                                                                                                                        | CN               | H   | H              | 2.5                                     | 50        | 4.9                                     | 26        |
| <b>1d</b>                                                                                                                                                                                                                                                                                        | CF <sub>3</sub>  | H   | H              | 7.51                                    | 150       | 5.83                                    | 31        |
| <b>1e</b>                                                                                                                                                                                                                                                                                        | OCF <sub>3</sub> | H   | H              | 4.48                                    | 90        | 2.29                                    | 12        |
| <b>1f</b>                                                                                                                                                                                                                                                                                        | F                | H   | H              | 1.11                                    | 22        | 1.54                                    | 8         |
| <b>1g</b>                                                                                                                                                                                                                                                                                        | Me               | H   | H              | 1.02                                    | 20        | 0.97                                    | 5         |
| <b>1h</b>                                                                                                                                                                                                                                                                                        | OMe              | H   | H              | 0.39                                    | 8         | 0.37                                    | 2         |
| <b>1i</b>                                                                                                                                                                                                                                                                                        | H                | F   | H              | 1.68                                    | 34        | 3.85                                    | 20        |
| <b>1j</b>                                                                                                                                                                                                                                                                                        | H                | OMe | H              | 0.08                                    | 2         | 0.99                                    | 5         |
| <b>1k</b>                                                                                                                                                                                                                                                                                        | H                | Me  | H              | 0.05                                    | 1         | 1.22                                    | 6         |
| <b>1l</b>                                                                                                                                                                                                                                                                                        | Ph               |     | Me             | H                                       | -         | 0.19                                    | 1         |
| <b>1m</b>                                                                                                                                                                                                                                                                                        | 1-naphthyl       |     | H              | H                                       | 5         | 2.87                                    | 15        |
| <b>1n</b>                                                                                                                                                                                                                                                                                        | 2-naphthyl       |     | H              | 0.50                                    | 10        | 1.98                                    | 10        |

As expected, *para* substituted derivatives showed rate tunability in accordance with the electronic nature of the substituents. For the bulky substrates (**1j-1n**), the rate of H<sub>2</sub>S release was found to be significantly slower for *h*3-MST as compared to *b*3-MST indicating that *h*3-MST is more specific while accommodating its substrates. Overall, among the compound used, nearly 200-fold and 50-fold in rate difference was observed for *h*3-MST and *b*3-MST respectively where **1b** was the fastest and **1l** was the slowest. In order to get more insights of substrate binding to 3-MST, a molecular docking study was performed with *h*3-MST (PBD: 4JGT) for compounds **2a-2n**. Overall, we find that, along with the electronic effects (for *para*) on the phenyl ring, steric effects (for *ortho*) also play a key role in substrate binding and turnover which reflects in the rate of H<sub>2</sub>S generation.

### CHAPTER 3: Mitochondria-targeted Artificial substrates for 3-MST

We expanded our goal to synthesize mitochondria-targeted 3-MST substrate that can be specifically localized into mitochondria since 3-MST is expressed at higher levels in mitochondria than in the cytosol.<sup>27</sup> Mitochondrial 3-MST is known to play an important role in regulating the redox machinery and signalling processes that keep cells functioning normally.<sup>32-34</sup> As a result, we hypothesized that a mitochondrion-specific 3-MST substrate would be more effective for notoriously difficult-to-treat diseases caused by abnormal changes in mitochondrial bioenergetics. Therefore, a well-known approach was adopted that utilizes the mitochondrial membrane potential to drive a lipophilic cation, triphenylphosphine (TPP) tagged molecules to permeate through mitochondrial phospholipid bilayer membrane resulting accumulation at mitochondria.<sup>35</sup> Hence, based on the SAR data, two mitochondria targeted artificial substrate (**7** and **13**) tethered with a TPP tag were designed and synthesized (Figure 1). In addition, we created a negative control compound **9** by replacing the sulphur in compound **7** with oxygen.

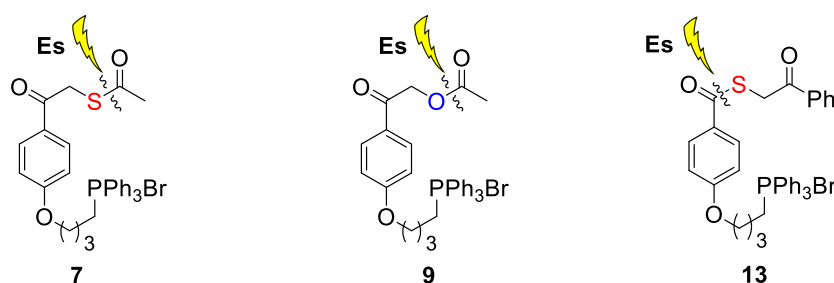


**Figure 1:** Structure of the mitochondria targeted compounds.

The H<sub>2</sub>S releasing efficiency of these compounds was screened by performing lead acetate and methylene blue experiment (Figure 2). When H<sub>2</sub>S releasing data of these compounds were compared with **1a**, **13** was not able to produce a significant amount of H<sub>2</sub>S as it did not get activated by esterase. In a similar condition, **7** was able to generate a significant amount of H<sub>2</sub>S as a result of esterase activation followed by a turnover by 3-MST in the presence of DTT.

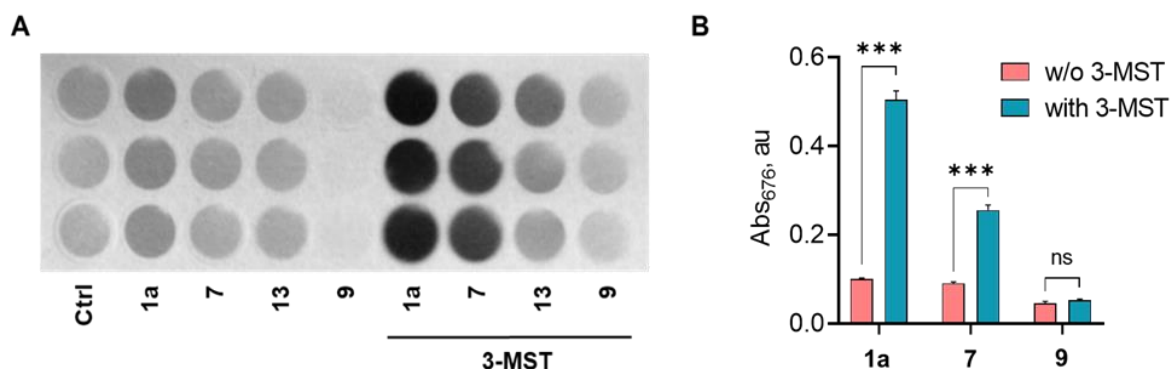


**Figure 2:** (A) Lead acetate assay for H<sub>2</sub>S generation. **1a**, **7** and **13** were incubated with esterase, b3-MST and DTT for 2 h. Ctrl represents only 3-MST enzyme. (B) Methylene blue assay for H<sub>2</sub>S generation. **1a**, **7** and **13** were incubated with esterase, b3-MST and DTT for 2 h. Results are expressed as mean  $\pm$  SD (n = 3) [(\*\*\*), p < 0.001 vs w/o 3-MST]

Then, the formation of 3-MST persulfide was detected using a newly developed clickable tag switch assay (Figure 3A). Next, generation of sulfane sulfur was also detected using SSP-2 assay (Figure 3B). For both the experiments **7** was able to produce 3-MST persulfide and sulfane sulfur significantly

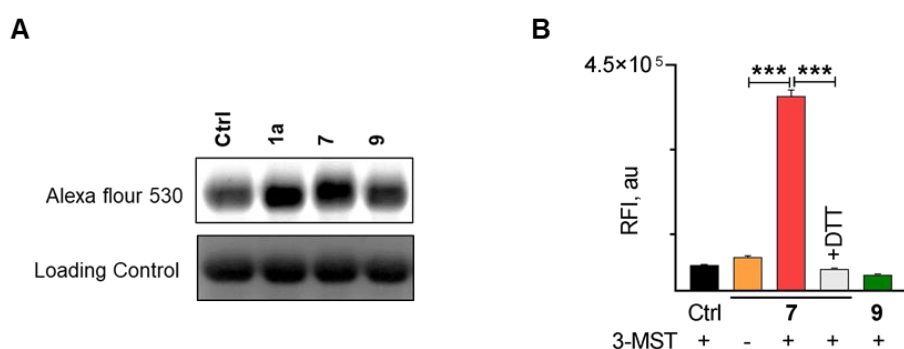


**Figure 3:** (A) 3-MST persulfide detection using a clickable tag switch assay. CBB staining was used for loading control. Ctrl represents 3-MST only. (B) Sulfane sulfur detection using SSP-2 probe (Fluorescence intensities were measured;  $\lambda_{\text{ex}}$  = 482 nm,  $\lambda_{\text{em}}$  = 518 nm). Results are expressed as mean  $\pm$  SD (n = 3) [(\*\*\*), p < 0.0001 vs ctrl]. +DTT refers to the post-treatment

of the enzyme with DTT to reduce sulfane sulfur at the active cysteine residue of 3-MST. Ctrl represents only 3-MST.

Next, mitochondrial studies were performed with MEF cells and corresponding data for these experiments were shown in figure 4. When **7** was treated to MEF cells, it gets localized in mitochondria and significantly produces H<sub>2</sub>S (enhancement green fluorescence signal of NBD-fluoresceine (NBD-F), a H<sub>2</sub>S specific dye).

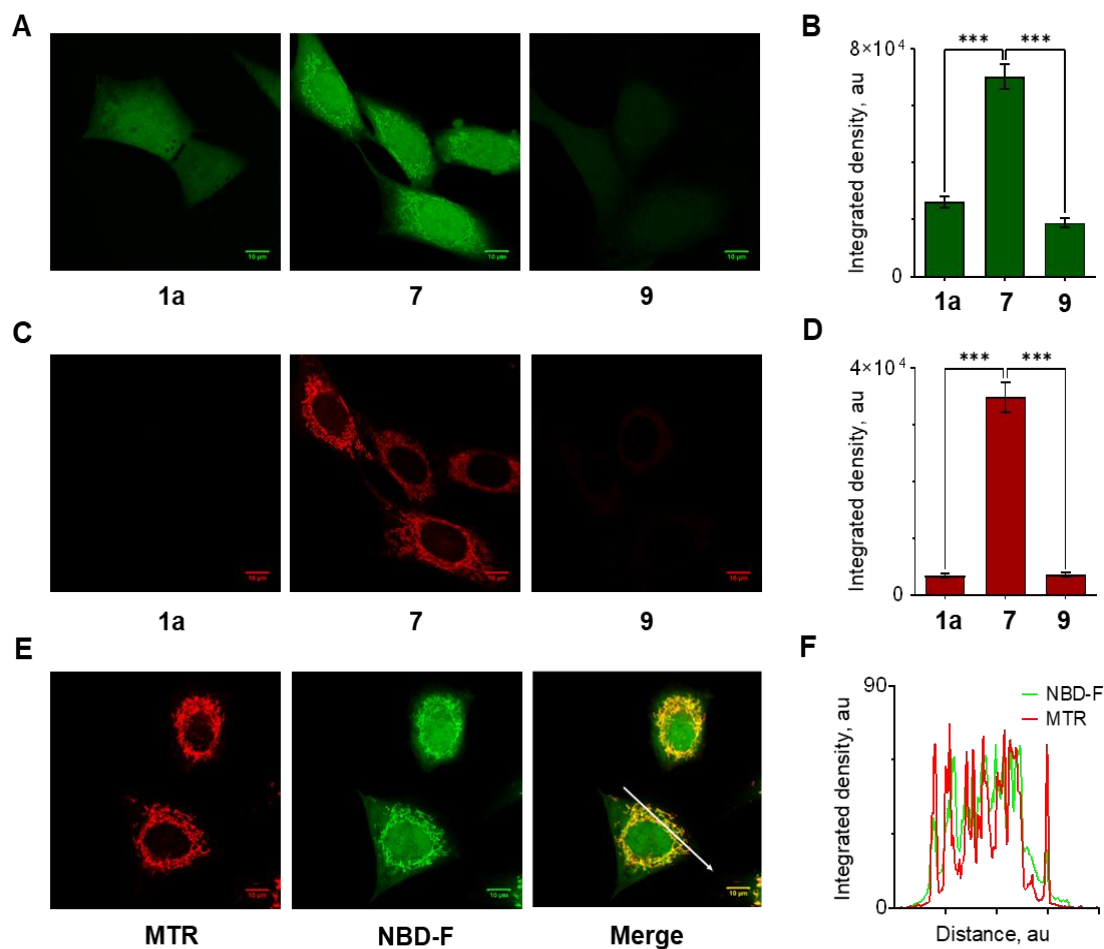
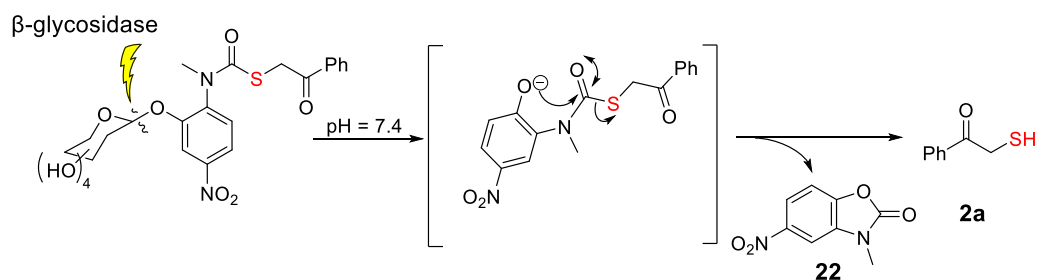


**Figure 4:** Confocal microscopy images of MEFs cells (Scale bar: 10  $\mu$ m) (A) H<sub>2</sub>S detection in cells when incubated with NBD-F dye following pretreatment with compounds **1a**, **7** and **9** respectively. (B) NBD-F fluorescence signals are quantified using IMAGE J software. (C) MMP enhancement in cells labelled with MTR dye following pretreatment with compounds **1a**, **7** and **9** respectively. (D) Cellular MTR fluorescence signals are quantified using IMAGE J software. (E) Co-localization NBD-F and MMP signals for compound **7**. (F) A line plot (fluorescence intensity vs. distance) shows an overlap between NBD-F and MTR fluorescence. All data is representative of 43 cells presented as mean  $\pm$  SEM (n = 3). \*\*\* $p$  < 0.001.

In accordance with H<sub>2</sub>S generation, an enhancement of mitochondrial membrane potential was observed (enhancement red fluorescence signal of Mito tracker red (MTR) dye). The control compounds, including the one lacking of sulfur (**9**) and another devoid of the mitochondrial targeted moiety (**1a**), were unable to produce H<sub>2</sub>S in mitochondria and as a consequence, no significant enhancement in the mitochondrial membrane potential (MMP) was observed. Taken together, we believe these substrates may be used in 3-MST related mitochondrial therapeutics.

#### CHAPTER 4. Development of $\beta$ -glycosidase Sensitive Artificial substrates of 3-MST

As compounds that were synthesized in Chapter 2 and 3 were found to be reactive with thiol, therefore in order to improve the stability, a modified artificial substrate was designed based on a reported glycosidase sensitive prodrug strategy.<sup>36</sup> Here, the free thiol group of **2a** was protected with a glycosidase sensitive trigger moiety that is expected to be thiol stable and only should be cleavable in the presence of specific glycosidase (Scheme 3).



**Scheme 3:** Design of the  $\beta$ -glycosidase sensitive artificial substrates of 3-MST.

Using the aforementioned strategy, artificial substrates, **20** with  $\beta$ -glucopyranose moiety to be activatable by glucosidase were designed and synthesized. Along with this, a previously developed 3-MST substrate **21** with a  $\beta$ -galactopyranose moiety was used.<sup>31</sup> Both the compound was selectively activated in presence of the respective glycosidase. Activation followed by substrate turnover by 3-MST was studied using HPLC. The generation of H<sub>2</sub>S was monitored for both compounds using lead acetate and methylene blue assay (Figure 5A). Next, 3-MST persulfide formation was detected using a clickable tag switch assay. Furthermore, formation of sulfane sulfur was observed using SSP-2 assay (Figure 5B). Overall experimental data of H<sub>2</sub>S release, 3-MST persulfide and sulfane sulfur generation suggest that  $\beta$ -glycosidase induced activation of **20** was slower than **21**.

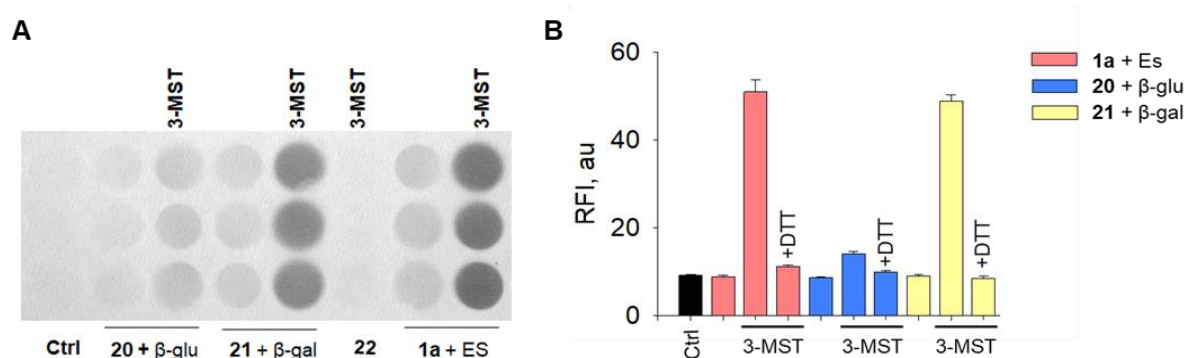


**Figure 5:** (A) Lead acetate assay for the generation of H<sub>2</sub>S. Ctrl represents only 3-MST. (B) Sulfane sulfur detection using SSP-2 probe (Fluorescence intensities were measured;  $\lambda_{\text{ex}}$  = 482 nm,  $\lambda_{\text{em}}$  = 518 nm). +DTT refers to the post-treatment of the enzyme with DTT to reduce sulfane sulfur at the active cysteine residue of 3-MST. Ctrl represents only 3-MST.

Then cellular studies were performed with NBD-F dye<sup>37</sup> and enhancement of intracellular H<sub>2</sub>S for **21** treated cells was observed (Figure 6A). Finally, the antioxidant activity of these compounds was tested in presence of MGR-1, a reported oxidative stress inducer<sup>38</sup> and compound **21** found to be protective against the stress and was able to recover cell viability (Figure 6B).

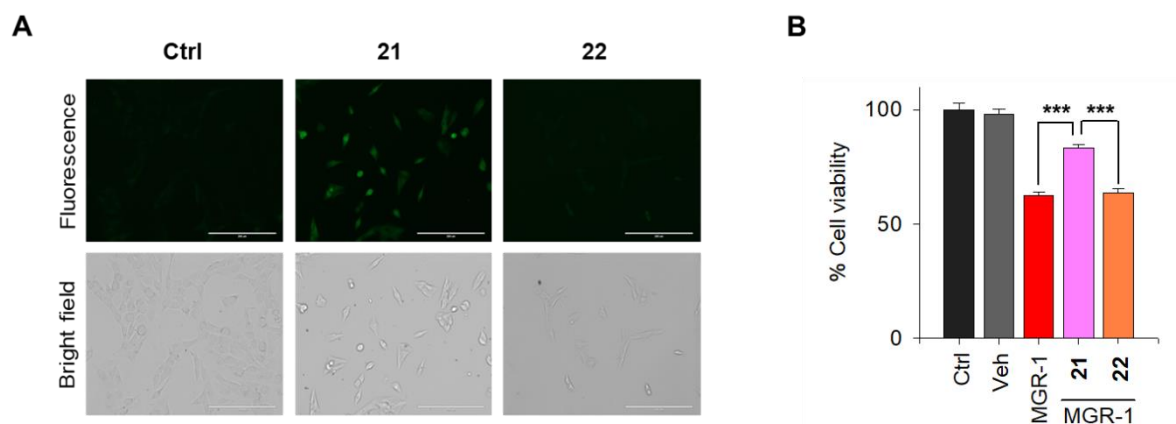
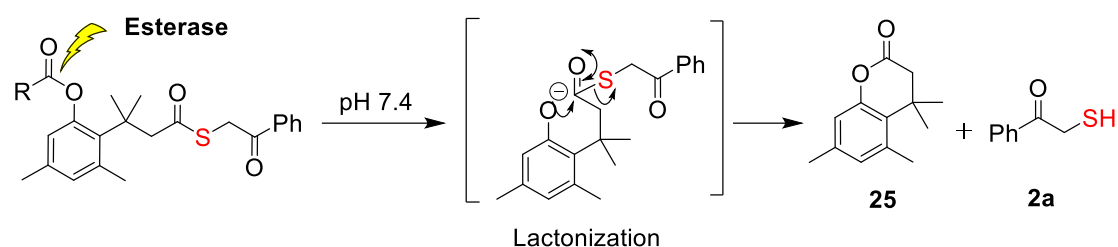


**Figure 6:** (A) H<sub>2</sub>S detection in MEF cells using NBD-F dye in presence of **21**. Enhanced fluorescence signal corresponding to H<sub>2</sub>S. Ctrl refers to untreated cells. The scale bar represents 200  $\mu$ m. (B) Cytoprotective effects of compound **21** against MGR-1 (25  $\mu$ M). Results are expressed as mean  $\pm$ SD (n = 3) [(\*\*\*),  $p < 0.001$  vs MGR-1]. Ctrl refers to untreated cells.

Collectively, our data suggest that **20** and **21** cell permeable artificial substrates of 3-MST which and these 3-MST artificial substrates can be used in oxidative stress conditions and mitigating inflammation.

## CHAPTER 5: Development of Esterase Triggered Artificial Substrates of 3-MST

In Chapter 5, we have adapted a reported esterase sensitive trimethyl lock prodrug strategy and designed an encapsulated **2a** in its thioester form. Due to the Thorpe-Ingold effect, trimethyl lock-based systems are rapid and efficient in undergoing lactonization and followed by payload release (**2a**) after esterase induced activation (Scheme 4).<sup>39–41</sup>



**Scheme 4:** Proposed mechanism for esterase mediated activation of esterase sensitive trimethyl lock based 3-MST substrate.

Conjugating different esters as protecting groups in trimethyl lock handles are expected to provide different decapsulation rates of prodrugs. Therefore, compd. **23a** and **23b** having cyclopropyl and methyl ester group respectively were synthesized (Figure 7). HPLC studies suggest that the prodrugs in these forms are stable in thiol rich environment and only could be activated by esterase only. Esterase is a biorthogonal enzyme to act independently while 3-MST turnover of the substrate. Thus, this strategy provides a selective triggerable approach for robust application. We have also synthesized two compounds, **24** (without the substrate **2a**) and **32** (without the ester group) as negative controls (Figure 7).

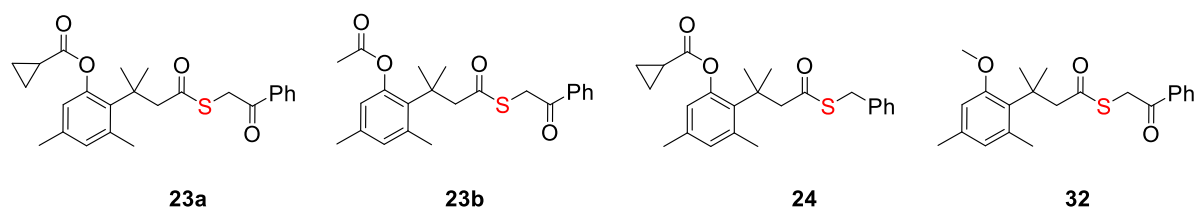


**Figure 7:** Structure of esterase sensitive 3-MST substrate (**23a** and **23b**). Structure of negative control compounds **24** and **32**.

The formation of  $\text{H}_2\text{S}$  was monitored using lead acetate and methylene blue assay (Figure 8A). Similar to **1a**, compound **23a** and **23b** showed significant  $\text{H}_2\text{S}$  response signals. As expected, negative controls didn't show significant  $\text{H}_2\text{S}$  release. 3-MST persulfide formation was demonstrated by a clickable tag switch assay. The formation of sulfane sulfur for **23a** and **23b**

was observed using an SSP-2 probe (Figure 8B). Overall reaction mechanism was studied by HPLC using compound **23a** in presence of 3-MST + ES + DTT and the hypothesis behind the selective ES triggerable 3-MST prodrug was verified.

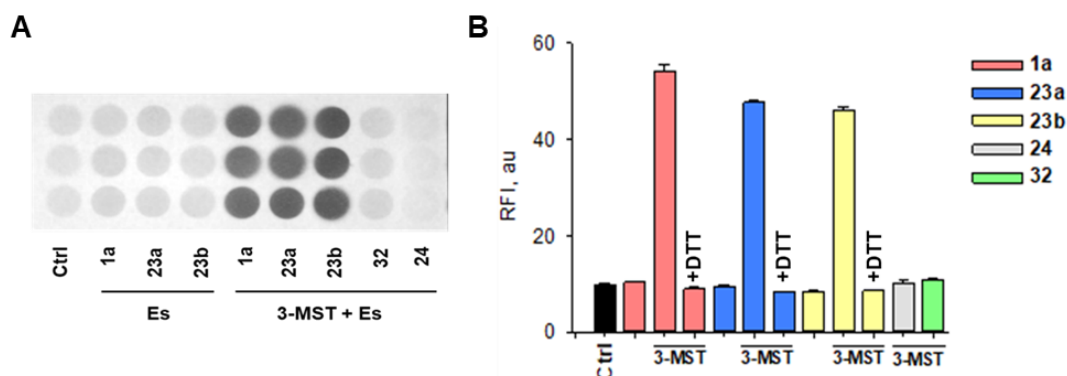


**Figure 8:** (A) Lead acetate assay for the generation of H<sub>2</sub>S. Ctrl represents only 3-MST. (B) Sulfane sulfur detection using SSP-2 probe (Fluorescence intensities were measured;  $\lambda_{\text{ex}} = 482$  nm,  $\lambda_{\text{em}} = 518$  nm). +DTT refers to the post-treatment of the enzyme with DTT to reduce sulfane sulfur at the active cysteine residue of 3-MST. Ctrl represents only 3-MST.

Cell viability data shows the 3-MST artificial substrates and by-products are benign under experimental concentrations. Collectively, our data suggest that **23a** and **23b** are artificial substrates of 3-MST. Although these compounds were stable towards thiols, they are significantly more hydrophobic ( $\text{clogP} = 6\text{--}7$ ) than the parent substrate **1a** ( $\text{clogP} = 1.7$ ). It is anticipated that these compounds will find applications when encapsulated in polymer nanoparticles for site-specific delivery, which may have applications in the mitigation of inflammation.

In summary, we present a general methodology to synthesize new prodrugs to modulate the biochemistry of a specific enzyme (3-MST). A unique class of artificial substrates of 3-MST were developed as a tool for enhancing persulfidation levels in cells. Since, this strategy takes an advantage of the cell's own enzyme machinery for improving health responses, this newly developed methodology can be implemented for enhancement of H<sub>2</sub>S and sulfane sulfur in 3-MST deficiency related diseases in future.

## Reference:

- (1) Gallyas, F. Involvement of Redox-Signalling in Endogenous Hydrogen Sulfide Production. *Br. J. Pharmacol.* **2012**, *166* (8), 2228–2230.
- (2) Stein, A.; Bailey, S. M. Redox Biology of Hydrogen Sulfide: Implications for Physiology, Pathophysiology, and Pharmacology. *Redox Biol.* **2013**, *1* (1), 32–39.
- (3) KIMURA, H. Hydrogen Sulfide and Polysulfides as Signaling Molecules. *Proc. Japan Acad. Ser. B* **2015**, *91* (4), 131–159.
- (4) Singh, S. B.; Lin, H. C. Hydrogen Sulfide in Physiology and Diseases of the Digestive Tract. *Microorganisms* **2015**, *3* (4), 866–889.
- (5) Xie, Z.-Z.; Liu, Y.; Bian, J.-S. Hydrogen Sulfide and Cellular Redox Homeostasis. *Oxid. Med. Cell. Longev.* **2016**, *2016* (5), 6043038.
- (6) Park, C.-M.; Weerasinghe, L.; Day, J. J.; Fukuto, J. M.; Xian, M. Persulfides: Current Knowledge and Challenges in Chemistry and Chemical Biology. *Mol. Biosyst.* **2015**,
- (7) Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R. Chemical Biology of H<sub>2</sub>S Signaling through Persulfidation. *Chem. Rev.* **2018**, *118* (3), 1253–1337.
- (8) Koike, S.; Ogasawara, Y.; Shibuya, N.; Kimura, H.; Ishii, K. Polysulfide Exerts a Protective Effect against Cytotoxicity Caused by t-Buthylhydroperoxide through Nrf2 Signaling in Neuroblastoma Cells. *FEBS Lett.* **2013**, *587* (21), 3548–3555.
- (9) Yang, C.-T.; Devarie-Baez, N. O.; Hamsath, A.; Fu, X.-D.; Xian, M. S-Persulfidation: Chemistry, Chemical Biology, and Significance in Health and Disease. *Antioxid. Redox Signal.* **2019**.
- (10) Mustafa, A. K.; Gadalla, M. M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S. K.; Barrow, R. K.; Yang, G.; Wang, R.; Snyder, S. H. H<sub>2</sub>S Signals Through Protein S-Sulfhydration. *Sci. Signal.* **2009**, *2* (96), ra72 LP-ra72.
- (11) Vandiver, M. S.; Paul, B. D.; Xu, R.; Karuppagounder, S.; Rao, F.; Snowman, A. M.; Seok Ko, H.; Il Lee, Y.; Dawson, V. L.; Dawson, T. M.; Sen, N.; Snyder, S. H. Sulfhydration Mediates Neuroprotective Actions of Parkin. *Nat. Commun.* **2013**, *4*, 1626.
- (12) Filipovic, M. R. Persulfidation (S-Sulfhydration) and H<sub>2</sub>S; 2015; pp 29–59.
- (13) Dóka, É.; Ida, T.; Dagnell, M.; Abiko, Y.; Luong, N. C.; Balog, N.; Takata, T.; Espinosa, B.; Nishimura, A.; Cheng, Q.; Funato, Y.; Miki, H.; Fukuto, J. M.; Prigge, J. R.; Schmidt, E. E.; Arnér, E. S. J.; Kumagai, Y.; Akaike, T.; Nagy, P. Control of Protein Function through Oxidation and Reduction of Persulfidated States. *Sci. Adv.* **2020**, *6* (1).

- (14) Zhao, K.; Ju, Y.; Li, S.; Altaany, Z.; Wang, R.; Yang, G. S-sulfhydration of MEK1 Leads to PARP-1 Activation and DNA Damage Repair. *EMBO Rep.* **2014**, *15* (7), 792–800.
- (15) Yang, G.; Zhao, K.; Ju, Y.; Mani, S.; Cao, Q.; Puukila, S.; Khaper, N.; Wu, L.; Wang, R. Hydrogen Sulfide Protects Against Cellular Senescence via S-Sulfhydration of Keap1 and Activation of Nrf2. *Antioxid. Redox Signal.* **2013**, *18* (15), 1906–1919.
- (16) Du, J.; Huang, Y.; Yan, H.; Zhang, Q.; Zhao, M.; Zhu, M.; Liu, J.; Chen, S. X.; Bu, D.; Tang, C.; Jin, H. Hydrogen Sulfide Suppresses Oxidized Low-Density Lipoprotein (Ox-LDL)-Stimulated Monocyte Chemoattractant Protein 1 Generation from Macrophages via the Nuclear Factor KB (NF-KB) Pathway. *J. Biol. Chem.* **2014**, *289* (14), 9741–9753.
- (17) Giovinazzo, D.; Bursac, B.; Sbodio, J. I.; Nalluru, S.; Vignane, T.; Snowman, A. M.; Albacarys, L. M.; Sedlak, T. W.; Torregrossa, R.; Whiteman, M.; Filipovic, M. R.; Snyder, S. H.; Paul, B. D. Hydrogen Sulfide Is Neuroprotective in Alzheimer's Disease by Sulfhydrating GSK3 $\beta$  and Inhibiting Tau Hyperphosphorylation. *Proc. Natl. Acad. Sci.* **2021**, *118* (4).
- (18) YAMANISHI, T.; TUBOI, S. The Mechanism of the L-Cystine Cleavage Reaction Catalyzed by Rat Liver  $\gamma$ -Cystathionase1. *J. Biochem.* **1981**, *89* (6), 1913–1921.
- (19) Akaike, T.; Ida, T.; Wei, F.-Y.; Nishida, M.; Kumagai, Y.; Alam, M. M.; Ihara, H.; Sawa, T.; Matsunaga, T.; Kasamatsu, S.; Nishimura, A.; Morita, M.; Tomizawa, K.; Nishimura, A.; Watanabe, S.; Inaba, K.; Shima, H.; Tanuma, N.; Jung, M.; Fujii, S.; Watanabe, Y.; Ohmuraya, M.; Nagy, P.; Feelisch, M.; Fukuto, J. M.; Motohashi, H. CysteinyI-TRNA Synthetase Governs Cysteine Polysulfidation and Mitochondrial Bioenergetics. *Nat. Commun.* **2017**, *8* (1), 1177.
- (20) Melideo, S. L.; Jackson, M. R.; Jorns, M. S. Biosynthesis of a Central Intermediate in Hydrogen Sulfide Metabolism by a Novel Human Sulfurtransferase and Its Yeast Ortholog. *Biochemistry* **2014**, *53* (28), 4739–4753.
- (21) Nagahara, N.; Yoshii, T.; Abe, Y.; Matsumura, T. Thioredoxin-Dependent Enzymatic Activation of Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2007**, *282* (3), 1561–1569.
- (22) Yadav, P. K.; Yamada, K.; Chiku, T.; Koutmos, M.; Banerjee, R. Structure and Kinetic Analysis of H<sub>2</sub>S Production by Human Mercaptopyruvate Sulfurtransferase. *J. Biol. Chem.* **2013**, *288* (27), 20002–20013.
- (23) Huang, G.-T.; Yu, J.-S. K. Enzyme Catalysis That Paves the Way for S-Sulfhydration

- via Sulfur Atom Transfer. *J. Phys. Chem. B* **2016**, 120 (20), 4608–4615.
- (24) Nagahara, N.; Katayama, A. Post-Translational Regulation of Mercaptopyruvate Sulfurtransferase via a Low Redox Potential Cysteine-Sulfenate in the Maintenance of Redox Homeostasis. *J. Biol. Chem.* **2005**, 280 (41), 34569–34576.
  - (25) Nagahara, N. Multiple Role of 3-Mercaptopyruvate Sulfurtransferase: Antioxidative Function, H<sub>2</sub>S and Polysulfide Production and Possible SO<sub>x</sub> Production. *Br. J. Pharmacol.* **2018**, 175 (4), 577–589.
  - (26) Tomita, M.; Nagahara, N.; Ito, T. Expression of 3-Mercaptopyruvate Sulfurtransferase in the Mouse. *Molecules* **2016**, 21 (12), 1707.
  - (27) Fräsdorf, B.; Radon, C.; Leimkühler, S. Characterization and Interaction Studies of Two Isoforms of the Dual Localized 3-Mercaptopyruvate Sulfurtransferase TUM1 from Humans. *J. Biol. Chem.* **2014**, 289 (50), 34543–34556.
  - (28) Coletta, C.; Módis, K.; Szczesny, B.; Brunyánszki, A.; Oláh, G.; Rios, E. C. S.; Yanagi, K.; Ahmad, A.; Papapetropoulos, A.; Szabo, C. Regulation of Vascular Tone, Angiogenesis and Cellular Bioenergetics by the 3-Mercaptopyruvate Sulfurtransferase/H<sub>2</sub>S Pathway: Functional Impairment by Hyperglycemia and Restoration by Dl- $\alpha$ -Lipoic Acid. *Mol. Med.* **2015**, 21 (1), 1–14.
  - (29) Pedre, B.; Dick, T. P. 3-Mercaptopyruvate Sulfurtransferase: An Enzyme at the Crossroads of Sulfane Sulfur Trafficking. *Biol. Chem.* **2021**, 402 (3), 223–237.
  - (30) Nair, M. Mechanistic Investigation of Unnatural Substrates for Enzymatic Production of Hydrogen Sulfide in Bacteria, IISER Pune, 2018.
  - (31) Bora, P. Stimuli-Responsive Small Molecule Persulfidating Agents, IISER Pune, 2022.
  - (32) Libiad, M.; Yadav, P. K.; Vitvitsky, V.; Martinov, M.; Banerjee, R. Organization of the Human Mitochondrial Hydrogen Sulfide Oxidation Pathway. *J. Biol. Chem.* **2014**, 289 (45), 30901–30910.
  - (33) Módis, K.; Coletta, C.; Erdélyi, K.; Papapetropoulos, A.; Szabo, C. Intramitochondrial Hydrogen Sulfide Production by 3-Mercaptopyruvate Sulfurtransferase Maintains Mitochondrial Electron Flow and Supports Cellular Bioenergetics. *FASEB J.* **2013**, 27 (2), 601–611.
  - (34) Murphy, B.; Bhattacharya, R.; Mukherjee, P. Hydrogen Sulfide Signaling in Mitochondria and Disease. *FASEB J.* **2019**, 33 (12), 13098–13125.
  - (35) Battogtokh, G.; Choi, Y. S.; Kang, D. S.; Park, S. J.; Shim, M. S.; Huh, K. M.; Cho, Y.-Y.; Lee, J. Y.; Lee, H. S.; Kang, H. C. Mitochondria-Targeting Drug Conjugates for Cytotoxic, Anti-Oxidizing and Sensing Purposes: Current Strategies and Future

- Perspectives. *Acta Pharm. Sin. B* **2018**, 8 (6), 862–880.
- (36) Bora, P.; Sathian, M. B.; Chakrapani, H. Enhancing Cellular Sulfane Sulfur through  $\beta$ -Glycosidase-Activated Persulfide Donors: Mechanistic Insights and Oxidative Stress Mitigation. *Chem. Commun.* **2022**, 58 (18), 2987–2990.
- (37) Chen, W.; Matsunaga, T.; Neill, D. L.; Yang, C.; Akaike, T.; Xian, M. Rational Design of a Dual-Reactivity-Based Fluorescent Probe for Visualizing Intracellular HSNO. *Angew. Chemie Int. Ed.* **2019**, 58 (45), 16067–16070.
- (38) Kelkar, D. S.; Ravikumar, G.; Mehendale, N.; Singh, S.; Joshi, A.; Sharma, A. K.; Mhetre, A.; Rajendran, A.; Chakrapani, H.; Kamat, S. S. A Chemical–Genetic Screen Identifies ABHD12 as an Oxidized-Phosphatidylserine Lipase. *Nat. Chem. Biol.* **2019**, 15 (2), 169–178.
- (39) Wang, B.; Nicolaou, M. G.; Liu, S.; Borchardt, R. T. Structural Analysis of a Facile Lactonization System Facilitated by a “Trimethyl Lock.” *Bioorg. Chem.* **1996**, 24 (1), 39–49.
- (40) Levine, M. N.; Raines, R. T. Trimethyl Lock: A Trigger for Molecular Release in Chemistry, Biology, and Pharmacology. *Chem. Sci.* **2012**, 3 (8), 2412.
- (41) Zheng, Y.; Yu, B.; Ji, K.; Pan, Z.; Chittavong, V.; Wang, B. Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angew. Chemie Int. Ed.* **2016**, 55 (14), 4514–4518.



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**Leveraging an enzyme/artificial substrate system to enhance cellular persulfides and mitigate neuroinflammation**

P. Bora, S. Manna, M. A. Nair, R. R. M. Sathe, S. Singh, V. S. Sreyas Adury, K. Gupta, A. Mukherjee, D. K. Saini, S. S. Kamat, A. B. Hazra and H. Chakrapani, *Chem. Sci.*, 2021, **12**, 12939 DOI: 10.1039/D1SC03828A

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**List of Publications**

- (1) Sawase, L. R.; C. V., J.; Manna, S.; Chakrapani, H. A Modular Scaffold for Triggerable and Tunable Nitroxyl (HNO) Generation with a Fluorescence Reporter. *Chem. Commun.* **2023**, 59 (23), 3415–3418.
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- (3) Bora, P.; Chauhan, P.; **Manna, S.**; Chakrapani, H. A Vinyl-Boronate Ester-Based Persulfide Donor Controllable by Hydrogen Peroxide, a Reactive Oxygen Species (ROS). *Org. Lett.* **2018**, 20 (24), 7916–7920.



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# Leveraging an enzyme/artificial substrate system to enhance cellular persulfides and mitigate neuroinflammation†

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Persulfides and polysulfides, collectively known as the sulfane sulfur pool along with hydrogen sulfide (H<sub>2</sub>S), play a central role in cellular physiology and disease. Exogenously enhancing these species in cells is an emerging therapeutic paradigm for mitigating oxidative stress and inflammation that are associated with several diseases. In this study, we present a unique approach of using the cell's own enzyme machinery coupled with an array of artificial substrates to enhance the cellular sulfane sulfur pool. We report the synthesis and validation of artificial/unnatural substrates specific for 3-mercaptopyruvate sulfurtransferase (3-MST), an important enzyme that contributes to sulfur trafficking in cells. We demonstrate that these artificial substrates generate persulfides *in vitro* as well as mediate sulfur transfer to low molecular weight thiols and to cysteine-containing proteins. A nearly 100-fold difference in the rates of H<sub>2</sub>S production for the various substrates is observed supporting the tunability of persulfide generation by the 3-MST enzyme/artificial substrate system. Next, we show that the substrate **1a** permeates cells and is selectively turned over by 3-MST to generate 3-MST-persulfide, which protects against reactive oxygen species-induced lethality. Lastly, in a mouse model, **1a** is found to significantly mitigate neuroinflammation in the brain tissue. Together, the approach that we have developed allows for the on-demand generation of persulfides *in vitro* and *in vivo* using a range of shelf-stable, artificial substrates of 3-MST, while opening up possibilities of harnessing these molecules for therapeutic applications.

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## Introduction

Hydrogen sulfide (H<sub>2</sub>S) and its redox congeners, persulfides (HSSH) and polysulfides (H<sub>2</sub>S<sub>n</sub>) act as mediators of several intracellular signaling processes.<sup>1,2</sup> Persulfides and polysulfides, collectively known as the sulfane sulfur pool, along with H<sub>2</sub>S are summoned in response to oxidative stress in cells, and dysfunctional sulfur metabolism is implicated in neurodegeneration,<sup>3,4</sup> cardiovascular disease<sup>5,6</sup> and antibiotic resistance.<sup>7,8</sup> To regulate the sulfane sulfur pool, one of the strategies

used by cells is to employ protein persulfidation, a post-translational modification where the thiol group of a reactive cysteine (Cys-SH) is modified to a persulfide (Cys-SSH).<sup>9</sup> The persulfidated protein mediates S-transfer to appropriate biomolecular targets, and not only provides protection against irreversible oxidation of cysteine residues but also regulates cellular signaling and sulfur homeostasis.<sup>10,11</sup> Persulfidation has been linked to modulation of the catalytic activity of key proteins such as parkin and GSK-3β that are involved in neurodegenerative diseases, underscoring the therapeutic relevance of exogenously controlling this post-translational modification by administering persulfide/H<sub>2</sub>S.<sup>4,9</sup> Here, we report a novel approach of developing artificial/unnatural substrates for persulfide-generating enzymes *in vivo* as a tool to study and tune sulfur metabolism in cells with applications in mitigating neuroinflammation.

In this study, we leverage natural biochemical mechanisms for generating persulfides by the development of a new class of substrates for 3-mercaptopyruvate sulfurtransferase (3-MST), a central persulfide/polysulfide generating enzyme that has been implicated in the mitigation of oxidative stress.<sup>12–14</sup> 3-MST

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operates by generating its own persulfide by the activation of a hyper-reactive active site cysteine with the sulfur atom of 3-mercaptopyruvate (3-MP) and produces pyruvate as the byproduct (Fig. 1A).<sup>15</sup> The persulfidated 3-MST (3-MST-SS<sup>−</sup>) can transfer the sulfur to low molecular weight thiols such as glutathione (GSH) to produce a persulfide (GSSH), which in turn persulfidates proteins (Fig. 1B).<sup>16</sup> Alternately, 3-MST-SS<sup>−</sup> can produce a polysulfide species (3-MST-S<sub>n</sub>S<sup>−</sup>) which subsequently persulfidates low molecular weight thiols and other proteins in the cell.<sup>2</sup> 3-MST-SS<sup>−</sup> has been found to be involved in

mitochondrial respiration and fatty acid metabolism,<sup>17,18</sup> synthesis of thiouridine in tRNA,<sup>19</sup> iron-sulfur cluster formation,<sup>20</sup> and in cyanide detoxification.<sup>21</sup> Also, 3-MST is among the major H<sub>2</sub>S-generating enzymes in the brain and is thus implicated in regulation of the sulfane sulfur pool and sulfur trafficking in neuronal cells.<sup>14</sup> Therefore, a methodology that generates 3-MST-SS<sup>−</sup> in a specific, controlled and catalytic manner would be advantageous for understanding the mechanisms of sulfur metabolism, and its role in signaling and disease.

The natural substrate 3-MP can, in principle, be used for this purpose. However, previous reports suggest that 3-MP produces H<sub>2</sub>S even in the absence of 3-MST in the cell, thus, using it may have limitations.<sup>22</sup> Hence, our strategy of developing a suite of artificial substrates specific for 3-MST as a methodology for the controlled generation of its persulfide is unique with diverse implications. Since cellular signaling is highly dependent on the concentration and rate of generation of the signaling species, it is important that the new methodology developed contains in-built characteristics for tuning these parameters. We expect that the systematic functionalization of the artificial substrates will alter their binding to the enzyme, as well as the rate of persulfide generation (Fig. 1C). Together, these factors will consequently allow for tuning the sulfur transfer reaction. Finally, since thiols are prone to oxidation, it was envisaged that the unnatural substrate could be generated *in situ* from the corresponding thioesters through ester hydrolysis that is catalyzed by a widely prevalent esterase enzyme (Fig. 1C).

## Results and discussions

### Docking studies, synthesis, enzymology, detection of persulfide and H<sub>2</sub>S

Molecular docking studies of the crystal structure of the human homolog of 3-MST (*h*3-MST, PDB ID: 4JGT) with the natural substrate 3-MP were conducted (Fig. 2A). The most energetically favorable conformation (−4.2 kcal mol<sup>−1</sup>) revealed interactions with arginine residues R188 and R197 that are consistent with previous accounts of these residues aiding in anchoring the substrate carboxylate and carbonyl groups.<sup>23,24</sup> The cysteine C248 residue was proximal to the reactive sulfhydryl group of 3-MP at a distance of 4.4 Å.

When a similar study was conducted with the proposed aryl substrate, this compound **2a** (R<sup>1</sup> = Ph, R<sup>2</sup> = H in Fig. 1C) was well accommodated in the active site and key interactions of **2a** with R188 and R197 were preserved (Fig. 2B). The distance between the C248 and the sulfur of **2a** was 5.2 Å, which was comparable with the lowest energy conformation of 3-MP in the active site of 3-MST. The binding energy of **2a** was nearly identical with 3-MP, −4.2 kcal mol<sup>−1</sup>.

We cloned and purified *h*3-MST and a bacterial 3-MST (*b*3-MST) from *Escherichia coli* (Table S3, Fig. S1†) for subsequent *in vitro* assays.<sup>23,25</sup> Compound **1a** (Fig. 1C) was synthesized by the treatment of 2-bromoacetophenone with potassium thioacetate using a reported protocol.<sup>26</sup> This compound should produce the thiol **2a** upon reaction with an esterase enzyme (ES). Compound **1a** was incubated in the presence of ES and *h*3-MST enzymes.

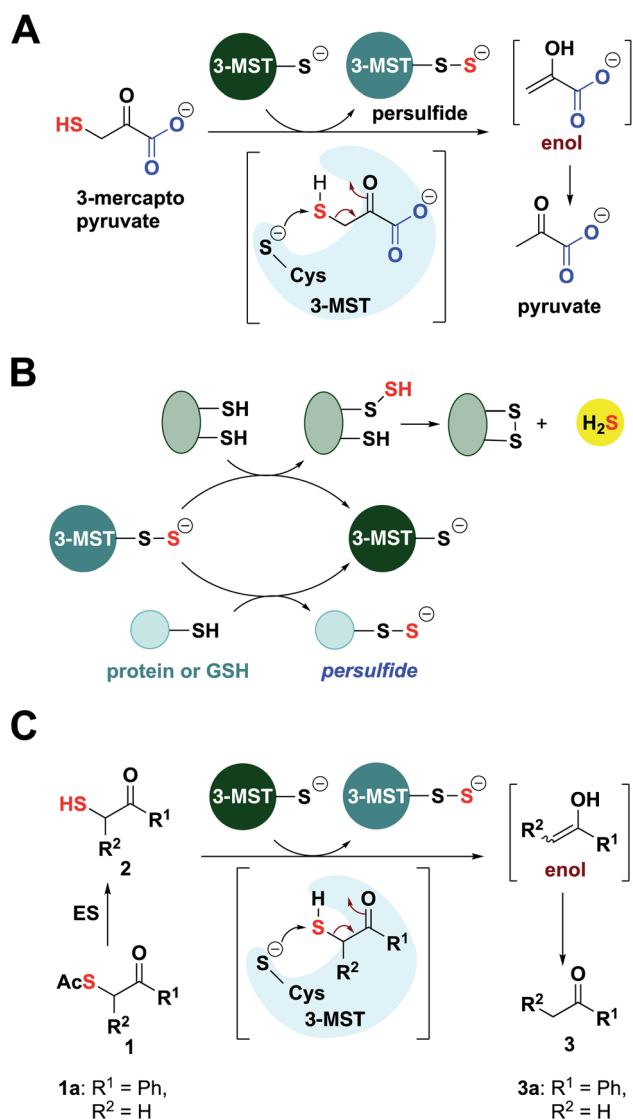


Fig. 1 (A) Catalytic cycle of 3-MST: the sulfur of 3-MP is transferred to 3-MST to produce 3-MST-SS<sup>−</sup> and pyruvate. (B) 3-MST-SS<sup>−</sup> reacts with reducing agents containing two cysteine residues such as thioredoxin (Trx) to generate H<sub>2</sub>S. 3-MST-SS<sup>−</sup> can also generate a protein persulfide through protein-protein interaction or transfer sulfur to low molecular weight thiols such as GSH to produce glutathione persulfide (GSSH). (C) Thioacetate **1** is expected to be cleaved by esterase (ES) to produce the designed 3-MST substrate **2**. This thiol is positioned to undergo a sulfur transfer reaction to produce 3-MST-SS<sup>−</sup> and a ketone **3** as the byproduct.



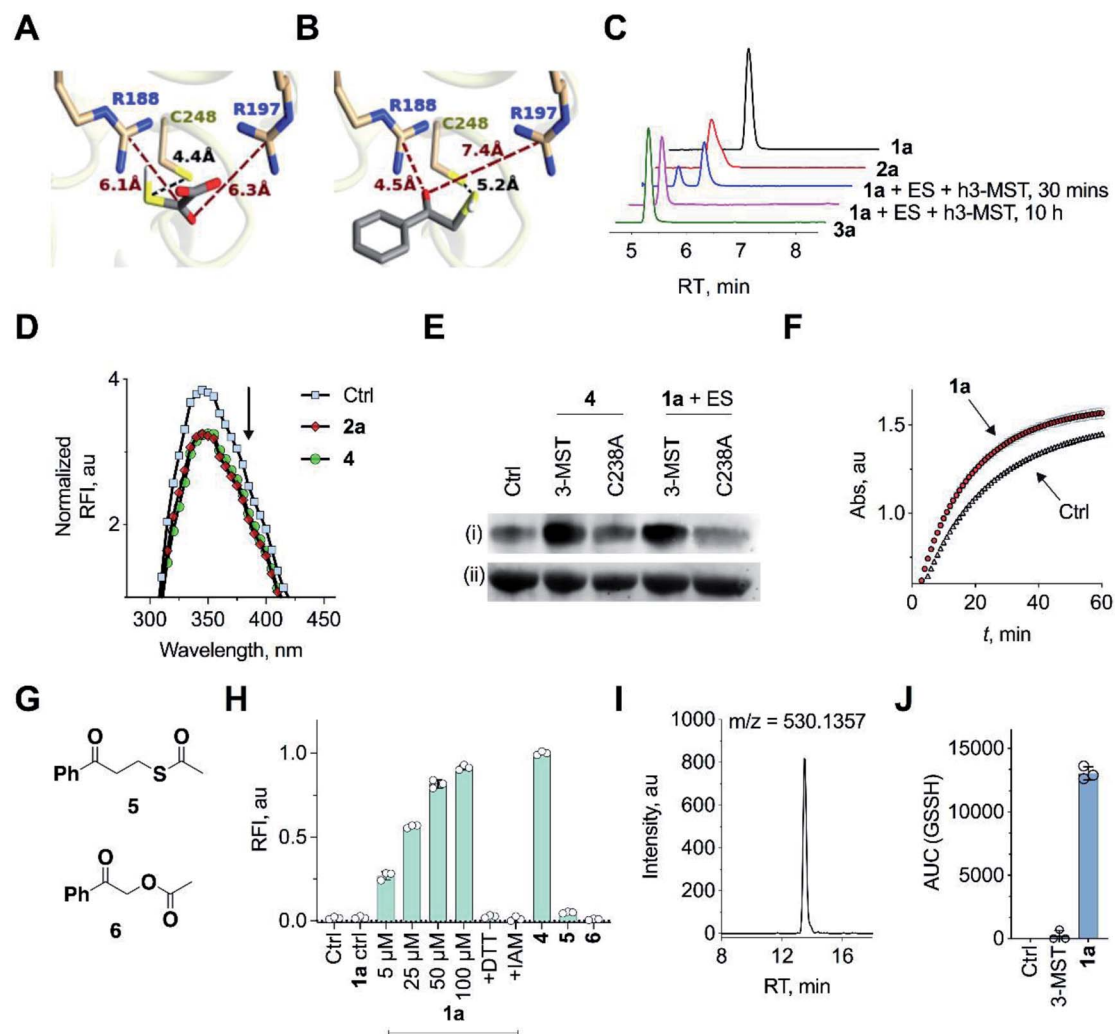


**Fig. 2** (A) Docking analysis of the active site of *h*3-MST with 3-MP shows a favorable conformation with the S–S bond distance as 4.4 Å. The R188 residue is 6.1 Å from the carboxyl group whereas the R197 residue is at a distance of 6.3 Å from the carbonyl group. (B) Docking analysis of the designed substrate **2a** ( $R^1 = \text{Ph}$ ,  $R^2 = \text{H}$  in Fig. 1C) reveals a similar anchoring of the substrate by the two arginine residues and the S–S bond distance was found as 5.2 Å. (C) HPLC analysis of **1a** + ES in the presence of *h*3-MST shows the formation of the thiol **2a** which is then subsequently converted to acetophenone **3a** during 10 h. (D) Intrinsic fluorescence assay on 3-MST with dimer of ethyl 3-mercaptopyruvate (E3-MP) **4** shows a decrease in fluorescence intensity which is consistent with the generation of 3-MST-SS<sup>-</sup>. A similar result was observed with the unnatural substrate **2a**. Ctrl refers to 3-MST alone. (E) Detection of 3-MST-SS<sup>-</sup> using the modified tag-switch technique (Fig. S7a†), conducted with **4** and **1a** + ES; The C238A 3-MST mutant treated under similar conditions showed a diminished band corresponding to the formation of 3-MST-SS<sup>-</sup>: (i) detection of 3-MST persulfide (ii) loading control. (F) Effect of persulfidation on the activity of GAPDH: GAPDH upon treatment with **1a** + ES + 3-MST enhances its activity compared to GAPDH alone presumably due to the formation of the persulfide of GAPDH. Ctrl refers GAPDH + ES + 3-MST; **1a** refers to co-incubation of **1a** + ES + 3-MST followed by addition of GAPDH (absorbance 340 nm). (G) Structures of compounds **5** and **6**. (H) Persulfide/polysulfide detection using SSP-2: Ctrl refers to 3-MST alone and **1a** ctrl refers to **1a** + ES only (100 μM); **1a** refers to co-incubation of varying concentrations of **1a**, ES and 3-MST; +DTT: addition of DTT; +IAM: addition of iodoacetamide, an electrophile that reacts with thiols; **4** refers to incubation of the compound with 3-MST; **5** and **6** refers to the incubation of the compounds with ES followed by treatment with 3-MST. (I) Extracted ion chromatogram from a mass spectrometry-based analysis of reaction products formed upon incubation of **1a** and 3-MST in the presence of ES followed by addition of GSH as the thiol acceptor. Reaction of the reactive sulfur species (GSSH) with an electrophile monobromobimane (mBBr) was employed. LC/MS analysis revealed the formation of the GSS-bimane adduct (expected  $m/z = 530.1379$ ; observed  $m/z = 530.1357$ ) when **1a** was incubated with ES and 3-MST. (J) Area under the curve (AUC) for the peak corresponding to GSS-bimane (Fig. 2I); Ctrl refers to **1a** alone while 3-MST refers to 3-MST alone and **1a** refers to **1a** + ES + 3-MST.

Gradual disappearance of **1a** and concomitant formation of the thiol **2a** was observed (Fig. 2C and S2†). After 10 hours, complete disappearance of **2a** along with the formation of acetophenone **3a** was recorded. These observations suggest that after **1a** produces **2a** upon ester hydrolysis, it is utilized by 3-MST to

produce **3a**. Under the same conditions, *b*3-MST also shows complete conversion to **3a** in 2 h (Fig. S3†).

The next series of experiments were designed to probe the intermediates during this transformation. The first intermediate that is expected to be formed in this reaction is 3-MST-SS<sup>-</sup>.

The formation of 3-MST-SS<sup>−</sup> was monitored by measurement of the intrinsic fluorescence of this enzyme using a reported protocol.<sup>27</sup> Upon incubation of 3-MST with **2a**, a significant quenching of intrinsic fluorescence of the enzyme as compared to the apo-protein was observed suggesting the formation of 3-MST-SS<sup>−</sup> (Fig. 2D and S4†).

Compound **4**, which exists as a dimer and dissociates in buffer to produce the ethyl 3-mercaptopyruvate, E3-MP, was synthesized and used as a substrate for 3-MST (Scheme 1).<sup>21</sup> Molecular docking analysis gave a binding energy for this ester as  $-4.2 \text{ kcal mol}^{-1}$  (Table S4†), identical to that of 3-MP. When 3-MST was incubated with **4**, quenching of fluorescence was observed as before supporting the generation of the protein persulfide (Fig. 2D and S4†). A similar set of results was also recorded for *h3*-MST (Fig. S5†). The compounds themselves did not show significant fluorescence under these conditions (Fig. S6†).

A reported tag-switch assay (Fig. S7†) was used for detecting persulfidated proteins (Fig. 2E).<sup>28</sup> When 3-MST was reacted with **4** or with **1a** and ES, as expected, this assay confirmed the formation of 3-MST-SS<sup>−</sup> (Fig. 2E). In contrast, under similar conditions, the mutant of 3-MST where the catalytic cysteine was replaced with alanine (3-MST C238A) showed no significant increase in the signal corresponding to the 3-MST-SS<sup>−</sup> (Fig. 2E and S7†). This result is consistent with a previous report of a similar lack of activity of the *h3*-MST cysteine mutant.<sup>29</sup>

Next, we assayed the ability of the 3-MST-SS<sup>−</sup> to transfer the sulfide to another protein. GAPDH is a redox-sensitive protein involved in the glycolytic cycle whose active site cysteine residue is susceptible to redox fluctuations and its function is influenced by this modification.<sup>30</sup> Accordingly, 3-MST-SS<sup>−</sup> was prepared by treating 3-MST with **1a** in presence of ES, following which GAPDH was added and incubated for 30 min. The activity of the enzyme was then estimated using a previously reported protocol.<sup>9</sup> Under these conditions, the GAPDH activity was found to be significantly enhanced as compared to a GAPDH only control (Fig. 2F, S8a). Treatment with dithiothreitol (DTT), which is expected to cleave the persulfide and produce the native enzyme, resulted in an activity profile that was comparable with the control GAPDH (Fig. S8b†). Thus, 3-MST-SS<sup>−</sup>, through sulfur trafficking, regulates the activity of key enzymes such as GAPDH.

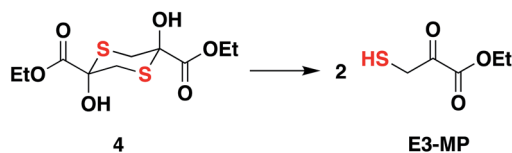
The formation of persulfide/polysulfide was then assessed using SSP-2, a fluorescence turn-on probe that is sensitive to these species.<sup>31</sup> The probe was validated using GS<sub>n</sub>SH (prepared using a reported protocol) (Fig. S9†).<sup>10</sup> When exposed to **1a** + ES, a distinct increase in fluorescence was recorded (Fig. 2H and

S10a†). When the standard reaction mixture was treated with the reducing agent DTT, a decrease in signal, likely due to the cleavage of persulfide by DTT, was observed (Fig. 2H and S10b†). Pre-treatment with iodoacetamide (IAM), a thiol alkylating agent, is expected to inactivate the enzyme. When IAM-pre-treated 3-MST was reacted with **1a** + ES, nearly complete abrogation of signal attributable to persulfide/polysulfide generation was observed (Fig. 2H). This result is consistent with the previous observation in the tag-switch persulfide assay where we found no evidence for persulfide generation in the catalytically dead 3-MST C238A mutant. Notably, compound **4** generated persulfide at levels comparable with **1a** (Fig. S11†). Next, two control compounds – **5**, the thioacetate with an additional methylene group and **6**, the analogous ester where sulfur was replaced with oxygen – were prepared (Fig. 2G). Both these analogues should not produce persulfide under the standard assay conditions. Generation of persulfide was assessed by the tag-switch assay (Fig. S12†) as well as by the fluorescence turn-on assay and no evidence for generation of persulfide from **5** or **6** was found (Fig. 2H).

The formation of persulfide/polysulfide was next studied by leveraging the ability of 3-MST-SS<sup>−</sup> to transfer its sulfane sulfur to an acceptor thiol. GSH, a thiol found abundantly in biological systems, was reacted with the 3-MST-SS<sup>−</sup> formed from the reaction of **1a** + ES with 3-MST. The formation of various reactive sulfur species was analysed using LC/MS, where monobromobimane was used as an alkylating agent (Scheme S4†).<sup>10</sup> LC/MS analysis showed the formation of the GSS-bimane adduct only in the presence of **1a** + ES and 3-MST (Fig. 2I, J and S13†). In addition, we found evidence for the formation of GSSSG and H<sub>2</sub>S as its bis-S-bimane adduct (Fig. S14 and S15†). Together, these data support the ability of our unnatural substrate–enzyme system to transfer S to low molecular weight thiols.

A standard methylene blue colorimetric assay for the detection of H<sub>2</sub>S was conducted in the presence of DTT (Fig. 1B).<sup>23,32</sup> This assay revealed the formation of H<sub>2</sub>S during incubation of **1a** with 3-MST in the presence of DTT (Fig. 3A). H<sub>2</sub>S release from compound **4** when treated with 3-MST was also observed (Fig. S16†). Based on our observation that the thioacetate **1a** could be cleaved by DTT even in the absence of the esterase to produce **2a**, the remaining experiments were conducted with **1a** + 3-MST + DTT (Fig. S17†). The rate constant  $k_h$  for H<sub>2</sub>S generation from **1a** when treated with *h3*-MST in the presence of DTT was  $1.48 \text{ h}^{-1}$  (Table 1). A similar rate constant  $k_b$  was observed with *h3*-MST (Table 1).

Having established that the substrates we designed could be used to generate persulfides as well as H<sub>2</sub>S *in vitro*, their ability to permeate cells to function as substrates for cellular 3-MST was studied. A human lung carcinoma A549 cell line with 3-MST knocked down (3-MST KD) was first generated (Fig. S18†). This cell line was used in conjunction with the corresponding scrambled cell line where 3-MST levels are not perturbed (Fig. S18†). When treated with an esterase-activated H<sub>2</sub>S donor<sup>32</sup> (Fig. S19†), significant enhancement in H<sub>2</sub>S levels in both these cell lines was observed as measured by the H<sub>2</sub>S-sensitive dye NBD-fluorescein (Fig. 3B).<sup>33</sup> However, in the presence of **1a**,



Scheme 1 Compound **4** is the dimer of E3-MP and in pH 7.4 buffer, dissociates to produce the E3-MP.



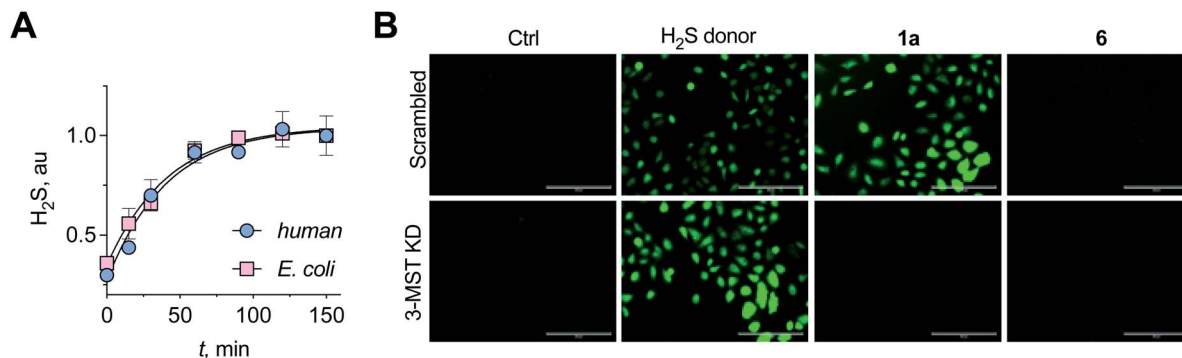


**Fig. 3** (A) A methylene blue assay was used to measure the rate of H<sub>2</sub>S generation with **1a** in the presence of *h3*-MST or *b3*-MST and DTT as the reducing agent. Nearly identical rates of H<sub>2</sub>S generation were observed. (B) 3-MST KD refers to knock-down of expression of 3-MST in A549 cells while scrambled refers to A549 cells containing non-targeting scrambled shRNA. The H<sub>2</sub>S donor used is an esterase-sensitive COS/H<sub>2</sub>S donor that has been previously characterized. H<sub>2</sub>S levels were assessed using a previously reported dye NBD-fluorescein (see ESI, Fig. S20†). Ctrl refers to untreated cells. Scale bar represents 200 μm.

**Table 1** Kinetics of H<sub>2</sub>S generation<sup>a</sup>

| Compd     | X                                               | Y   | <i>h3</i> -MST                          |                        | <i>b3</i> -MST                          |                                             |
|-----------|-------------------------------------------------|-----|-----------------------------------------|------------------------|-----------------------------------------|---------------------------------------------|
|           |                                                 |     | <i>k<sub>h</sub></i> (h <sup>-1</sup> ) | Rel. rate <sup>b</sup> | <i>k<sub>b</sub></i> (h <sup>-1</sup> ) | <i>k<sub>b</sub></i> / <i>k<sub>h</sub></i> |
| <b>1a</b> | H                                               | H   | 1.48                                    | 30                     | 1.45                                    | 1.0                                         |
| <b>1b</b> | NO <sub>2</sub>                                 | H   | 11.13                                   | 223                    | 9.27                                    | 0.8                                         |
| <b>1c</b> | CN                                              | H   | 2.5                                     | 50                     | 4.9                                     | 2.0                                         |
| <b>1d</b> | CF <sub>3</sub>                                 | H   | 7.51                                    | 150                    | 5.83                                    | 0.8                                         |
| <b>1e</b> | OCF <sub>3</sub>                                | H   | 4.48                                    | 90                     | 2.29                                    | 0.5                                         |
| <b>1f</b> | F                                               | H   | 1.11                                    | 22                     | 1.54                                    | 1.4                                         |
| <b>1g</b> | Me                                              | H   | 1.02                                    | 20                     | 0.97                                    | 1.0                                         |
| <b>1h</b> | OMe                                             | H   | 0.39                                    | 8                      | 0.37                                    | 0.9                                         |
| <b>1i</b> | H                                               | F   | 1.68                                    | 34                     | 3.85                                    | 2.5                                         |
| <b>1j</b> | H                                               | OMe | 0.08                                    | 2                      | 0.99                                    | 12.5                                        |
| <b>1k</b> | H                                               | Me  | 0.05                                    | 1                      | 1.22                                    | 25.0                                        |
| <b>1l</b> | R <sup>1</sup> = Ph; R <sup>2</sup> = Me        |     | Slow                                    | —                      | 0.19                                    | —                                           |
| <b>1m</b> | R <sup>1</sup> = 1-naphthyl; R <sup>2</sup> = H |     | 0.23                                    | 5                      | 2.87                                    | 12.5                                        |
| <b>1n</b> | R <sup>1</sup> = 2-naphthyl; R <sup>2</sup> = H |     | 0.50                                    | 10                     | 1.98                                    | 4.0                                         |

<sup>a</sup> Reaction conditions: compound + 3-MST + DTT. H<sub>2</sub>S was monitored using a methylene blue assay. <sup>b</sup> Calculated based on normalizing the rate constant with respect to **1k**.

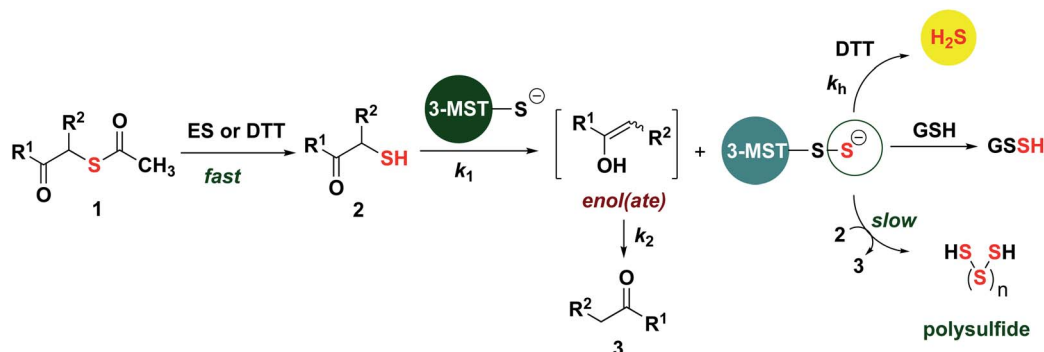
when the 3-MST KD cells were imaged, a significantly diminished signal for intracellular H<sub>2</sub>S levels was observed (Fig. 3B). In the scrambled cell line, however, where endogenous 3-MST levels are not perturbed, an enhanced signal corresponding to intracellular H<sub>2</sub>S was seen (Fig. 3B). Compound **6**, which lacks a sulfur, failed to enhance H<sub>2</sub>S in both cell lines (Fig. 3B, S20 and S21†). Together, these data suggest that **1a** is a cell-permeable persulfidating agent that is activated by 3-MST

within cells to produce H<sub>2</sub>S, putatively *via* an intracellular persulfidation mechanism, as confirmed by a separate tag-switch fluorescence reporter technique<sup>34</sup> (Fig. S22†).

Based on the above observations, we put forth the following mechanism for persulfide generation from this unnatural substrate – 3-MST system (Scheme 2). Cleavage of **1a** by esterase produces the thiol **2a**, which then reacts with 3-MST to produce the 3-MST-SS<sup>-</sup> and an enol(ate), which is expected to tautomerize to the ketone **3a**. In the absence of a reducing agent, 3-MST-SS<sup>-</sup> appears to catalyze the turnover of **2a** to produce **3a** and produces polysulfide during this transformation (Schemes 2 and S5; Fig. S15†). Under reducing conditions (DTT), 3-MST-SS<sup>-</sup> is cleaved to produce H<sub>2</sub>S (Scheme 2, Fig. 3A). HPLC analysis of the reaction mixture containing **2a**, 3-MST and DTT showed gradual disappearance of **2a** and the rate constant *k<sub>1</sub>* was found to be 0.99 h<sup>-1</sup>. The formation of **3a** was observed and a rate constant *k<sub>2</sub>* of 0.75 h<sup>-1</sup> was obtained (Fig. 4A and S23†). The rate of disappearance of **2a** and formation of **3a** was nearly identical and both parameters are comparable with the rate of H<sub>2</sub>S generation under these conditions (Table 1, see *h3*-MST data). In the absence of 3-MST, compound **2a** is prone to oxidation to its disulfide PhCOCH<sub>2</sub>S-SCH<sub>2</sub>COPh under ambient aerobic conditions (Fig. S24†). The conversion of **1a** to **2a** (and its disulfide) occurs in a nearly quantitative yield. Hence, our overall analysis demonstrates that compound **1a** is an excellent substrate for 3-MST and produces a persulfide intermediate, which is cleaved under reducing conditions to produce H<sub>2</sub>S. In the absence of esterase, **1a** is gradually consumed in the presence of 3-MST and the formation of **3a** is observed after several hours (Fig. S25 and S26†). Also, persulfide/polysulfide were detected using the probe SSP-2 under similar conditions (Fig. S27†). We find no evidence for the formation of the thiol **2a**. This may be explained by the formation of 3-MST thioacetate as a possible intermediate during the transformation of **1a** to **3a** (Scheme S5†).

### Substrate scope

In order to assess the unnatural substrate scope and the possibility of tuning sulfur transfer using this newly developed



**Scheme 2** Proposed mechanism: thioacetate **1** is cleaved by esterase or DTT (HPLC in Fig. S23, ESI†) to produce the thiol **2**, which is then turned over by 3-MST to produce 3-MST-SS<sup>-</sup> and an enol(ate). The enol(ate) in aqueous buffer is rapidly converted to the ketone **3**. 3-MST-SS<sup>-</sup> in the presence of a reducing agent produces H<sub>2</sub>S. 3-MST-SS<sup>-</sup> can react with low molecular weight thiols such as GSH to produce GSSH. Under non-reducing conditions, 3-MST-SS<sup>-</sup> can further turn over **2**, generating the ketone **3**; the likely byproduct of this reaction is polysulfide.

protocol, we synthesized a series of analogues. Arginine residues in the active site interact with the carbonyl group of 3-MP and are implicated in stabilizing the pyruvate enolate.<sup>23,24,27</sup> In order to test if the stability of the incipient enolate played a role in the rates of H<sub>2</sub>S production, compounds **1b–1h** with electron-withdrawing or electron-donating groups were synthesized. Molecular docking analysis with the corresponding thiols **2b–2h** showed nearly identical conformations (Table S5†). The rate constants of H<sub>2</sub>S release ranged from 0.39 to 7.51 h<sup>-1</sup> (Table 1, Fig. S28†). Hammett analysis of rate constants gave a positive  $\rho$  value of +1.11, that is consistent with a partial negative charge developing in the transition state in the rate limiting step of the reaction (Fig. 4B).

Due to the proximity of a substituent at the *ortho* position of the aryl ring, enolate formation is likely to be affected by stereoelectronic effects. In order to investigate these effects, compounds **1i–1k** were prepared and their H<sub>2</sub>S release was recorded (Table 1, Fig. S29†). While the rate of H<sub>2</sub>S release from **1i** was comparable with **1a**, compounds **1j** and **1k** were substantially slower in generating H<sub>2</sub>S when compared with **1a**. Molecular docking studies with the 2-fluorophenyl derivative **2i** showed a conformation and S–S distance comparable with that of **2a** bound to the active site (Table S6†). However, the lowest energy conformation of the thiol **2k** was significantly removed from the active site with the S–S bond distance of 10.7 Å (Fig. 4C). Unlike **2a**, the interaction of **2k** with the active site cysteine is restricted by steric clashes of A185 and R188 with its *ortho*-Me group, as illustrated in a higher energy conformation (Fig. 4D). A similar result was recorded for the 2-methoxy derivative **2j** (Tables S6 and S7†). These studies provide a molecular basis for the diminished H<sub>2</sub>S release rates from these analogues.

In addition to electronics, enolate stability and reactivity can also be affected by sterics. In order to study the effects of an added substituent, compound **1l** which has an  $\alpha$ -methyl substituent was synthesized. Docking analysis of **2l** with 3-MST revealed a docking score as well as a conformation that was comparable with **2a** (Table S6†). H<sub>2</sub>S release from **1l** was, however, found to be extremely slow (Table 1, Fig. S30†). The

formation of the enolate appears to be more sensitive to steric effects than electronic effects. Recently, inhibitors for 3-MST were developed and one of these inhibitors had a naphthyl ring.<sup>35</sup> Taking this cue, compounds **1m** and **1n** were synthesized (Table S2†). Our assays confirmed that these compounds were substrates for 3-MST, and H<sub>2</sub>S generation rates were moderately higher when compared with **1a** (Table 1, Fig. S30†) suggesting that a combination of binding and turnover is essential for tuning 3-MST-mediated sulfur transfer reactions.

Normalizing the rate constants with respect to **1k** revealed more than a 100-fold difference in relative rates for H<sub>2</sub>S release (Table 1). This observation suggests that this series of substrates can be used to tune rates of persulfide/polysulfide generation. Next, bacterial 3-MST was used to study the rates of H<sub>2</sub>S generation from the analogues and first-order rate constants were calculated (Table 1). Linear free energy relationship study of H<sub>2</sub>S generation rates of **1a–1h** with *b*3-MST gave a slope of +1.13 (Fig. S31†), comparable with the data from *h*3-MST. Control compounds **5** and **6** do not produce H<sub>2</sub>S under these conditions, as expected (Fig. S32†). Under the standard reaction conditions with 3-MST homologs, we found no major difference in rates of H<sub>2</sub>S release from **4** (Fig. S16†). While a majority of the substrates tested showed similar rates, compounds **1j**, **1k** and **1m** whose *k<sub>b</sub>/k<sub>h</sub>* > 10, were notable exceptions. These results indicate that our approach differentiates between human and bacterial 3-MST homologs, and is capable of achieving selective H<sub>2</sub>S generation between species. To the best of our knowledge, this is the first example of tuning persulfide/polysulfide generation, with the added advantage of species selectivity.

### Antioxidant activity

Persulfides are widely prevalent in cells as protein persulfides as well as other low molecular weight persulfide species. For example, glutathione persulfide/polysulfide has excellent *in vitro* ROS-scavenging activity when compared with glutathione or H<sub>2</sub>S alone.<sup>10</sup> These species have been previously shown to have potent antioxidant activity likely through the Nrf2-KEAP1 pathway, imparting protection from ROS-induced injury.<sup>36,37</sup> Selected analogues with varying relative rates of H<sub>2</sub>S generation



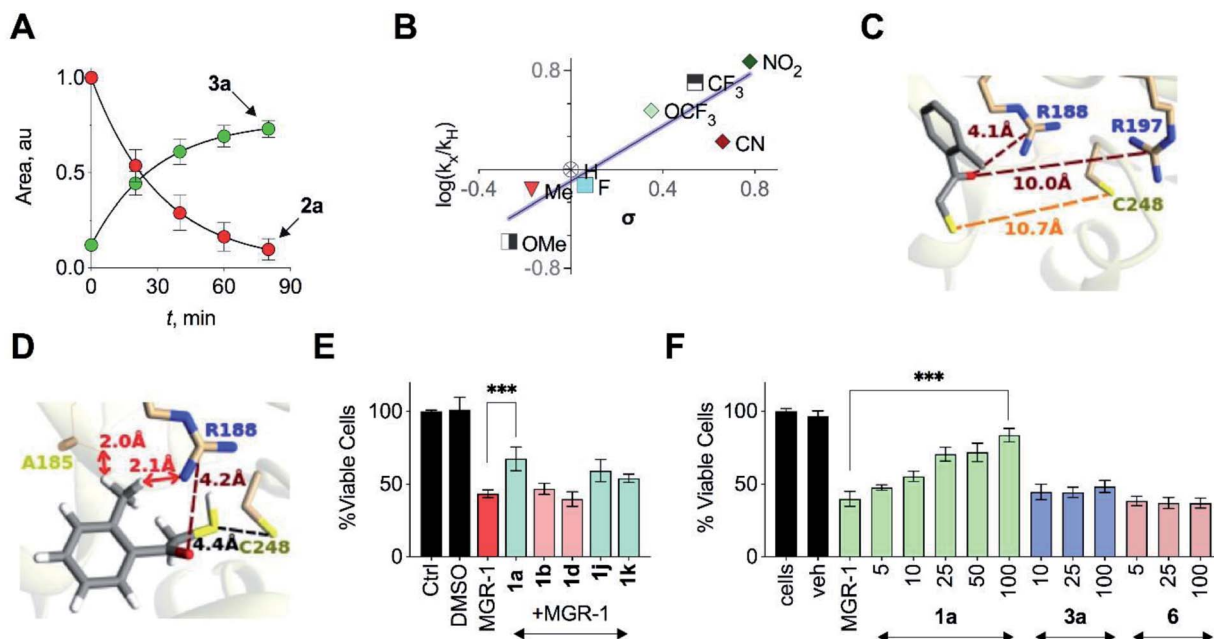


**Fig. 4** (A) HPLC analysis of a reaction mixture containing **2a** and 3-MST in the presence of DTT showed the gradual disappearance of **2a** and concomitant formation of **3a**. Curve fitting to first order kinetics gave rate constants of  $0.99 \text{ h}^{-1}$  and  $0.75 \text{ h}^{-1}$  for the disappearance of **2a** and the formation of **3a**, respectively. (B) Hammett analysis of rate constants of  $\text{H}_2\text{S}$  generation from unnatural substrates (see Table 1) with wt h3-MST. Linear regression analysis yielded a slope of +1.11 ( $R^2 = 0.8$ ). (C) Docking of **2k** in the active site of h3-MST shows S–S bond distance of 10.7 Å which is substantially larger when compared with the low energy conformation of **2a** (5.2 Å). (D) Docking of **2k** in the active site of h3-MST shows steric clashes of ortho-Me group of **2k** with A185 and R188 residues in a higher energy conformation. (E) Cell viability assay conducted on N2a cells: Cells were pre-treated with 25  $\mu\text{M}$  of compounds **1a**, **1b**, **1d**, **1j** and **1k** for 12 h and then exposed to MGR-1 (25  $\mu\text{M}$ ) for 4 h. Ctrl refers to untreated cells. Cell viability was determined using a standard MTT assay. Results are expressed as mean  $\pm$  SD ( $n = 3$ ). \*\*\* $p < 0.001$  vs. MGR-1. (F) Cell viability assay conducted on N2a cells: cells that were pre-treated with **1a**, **3a** or **6** were then treated with a cell permeable ROS generator MGR-1. A dose-dependent protection of cells from MGR-1 induced cell death by **1a** was observed. The byproduct ketone **3a** or the negative control **6** did not show any protection against the cytotoxic effects of MGR-1. All data are presented as mean  $\pm$  SD ( $n = 3$  per group). \*\*\* $p < 0.001$  vs. MGR-1.

(in the range of 1 to 223) were next tested to study their protective ability against oxidative stress-induced cell death. A mouse neuroblastoma cell line N2a was used in this experiment. MGR-1, a known cell-permeable ROS generator, was used to induce oxidative stress and cell viability was determined.<sup>38</sup> N2a cells were first independently treated with fast  $\text{H}_2\text{S}$  generators **1b** and **1d** (relative rate > 50), **1a** (relative rate = 30) as well as slow generators **1j** and **1k** (relative rate < 2). At 25  $\mu\text{M}$ , these compounds showed no significant effect on the growth of cells as determined by a cell viability assay (Fig. S33†). Cells that were independently pre-incubated with these compounds (25  $\mu\text{M}$ ) for 12 h were next exposed to MGR-1. Viable cells were determined using a standard cell viability assay. The results of this assay indicate that the fast generators did not significantly protect cells from oxidative stress-induced cell death while the other analogues **1a**, **1j** and **1k** showed remarkable protective effects (Fig. 4E). From this initial screen, we identified compound **1a** for further evaluation. The compound **1a** itself was well tolerated by cells (Fig. S34†) and the byproduct of turnover of **1a** is **3a**, which is classified as Generally Recognized as Safe (GRAS) by the Food and Drug Administration.

The lead compound **1a** was next tested against N2a cells using MGR-1, and a dose-dependent protection from lethality

was recorded (Fig. 4F). The control compound **6** and the acetophenone **3a** tested under similar conditions failed to show any protective effects (Fig. 4F). To further corroborate our results, menadione, another known ROS generator was used to induce lethality. Again, a dose-dependent protection was recorded when cells were pre-treated with **1a** (Fig. S35†). In a separate experiment with mouse embryonic fibroblasts (MEF), **1a** was similarly found to have significant protective effects (Fig. S36†). Together, these data illustrate the cytoprotective effect of the cell-permeable persulfide generator **1a**.

To validate the molecular basis of this result, the effect of **1a** in reducing hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) levels in cells was studied. Again, MGR-1 was used to increase  $\text{H}_2\text{O}_2$  as determined by the TCF-B fluorescent dye in A549 cells (Fig. 5A).<sup>39</sup> When cells pre-treated with **1a** were exposed to MGR-1, a significant decrease in  $\text{H}_2\text{O}_2$  levels was observed (Fig. 5A). Under similar conditions, control compound **6** did not affect  $\text{H}_2\text{O}_2$  levels in cells, suggesting that the ROS-quenching effects were directly mediated by persulfide/ $\text{H}_2\text{S}$  generated by **1a** (Fig. S37†).

Elevation of ROS in cells leads to perturbation of redox homeostasis and alteration of levels of various components of cellular antioxidant response.  $\text{NAD}^+/\text{NADH}$  and  $\text{GSSG}/\text{GSH}$  ratio serve as reliable markers of oxidative stress.<sup>40</sup> Three cell lines

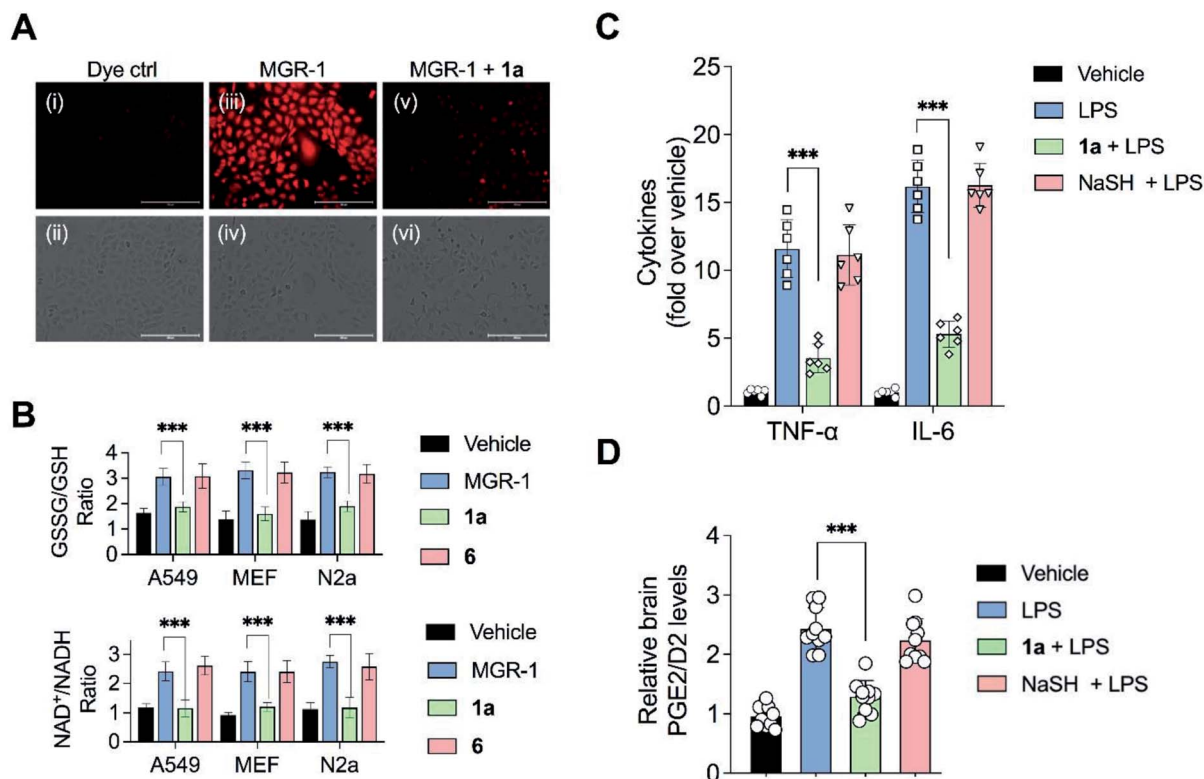


**Fig. 5** (A) Effects of **1a** on quenching hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) generated by MGR1:A549 cells treated with: (i and ii) dye control; (iii and iv) 25  $\mu\text{M}$  MGR-1; (v and vi) 25  $\mu\text{M}$  of **1a** for 12 h followed by addition of the 25  $\mu\text{M}$  MGR-1 for 1 h. Intracellular  $\text{H}_2\text{O}_2$  was detected using a reported  $\text{H}_2\text{O}_2$ -sensor TCF-B (25  $\mu\text{M}$ ). Scale bar represents 200  $\mu\text{m}$ . (B) Biomarkers for oxidative stress: Three cell lines (A549, mouse embryonic fibroblasts (MEF) and N2a) were pre-treated with vehicle, **1a** or **6** and exposed to MGR-1 following which  $\text{NAD}^+/\text{NADH}$  ratio and GSSG/GSH ratio were determined using an ELISA assay. All data are presented as mean  $\pm$  SD ( $n = 5$  per group). \*\*\* $p < 0.001$  vs. MGR-1. (C and D) Mouse endotoxin shock model: animals were treated with **1a** (20  $\text{mg kg}^{-1}$ ) or NaSH (20  $\text{mg kg}^{-1}$ ) 4 h prior to treatment with lipopolysaccharide (LPS, 5  $\text{mg kg}^{-1}$ ). 30 min post-administration of LPS, another dose of **1a** or NaSH was given. The brain tissue samples were harvested followed by measurement of: (C) Pro-inflammatory cytokines,  $\text{TNF-}\alpha$  and IL-6 using a standard ELISA assay. All data are presented as mean  $\pm$  SD ( $n = 6$  per group). \*\*\* $p < 0.001$  vs. LPS; and (D) prostaglandins  $\text{PGE}_2/\text{D}_2$  using LC/MS. All data are presented as mean  $\pm$  SD ( $n = 10$  per group). \*\*\* $p < 0.001$  vs. LPS.

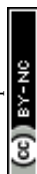
(A549, MEF and N2a) were exposed to MGR-1 and enhanced  $\text{NAD}^+/\text{NADH}$  as well as GSSG/GSH ratios were recorded (Fig. 5B). Pre-treatment of cells with **1a** followed by incubation with MGR-1 showed restoration of nearly normal ratios, and the control compound **6** was found to be ineffective in doing so. Taken together, these studies conclusively establish the efficacy of the artificial substrate **1a** that we designed to mitigate oxidative stress in both human as well as mouse-derived cell lines (Fig. 5B).<sup>35</sup>

### Mitigation of neuroinflammation

Recently, a polysulfide donor was used to study the effect of enhanced endogenous polysulfide levels on innate immune response using a mouse endotoxin shock model.<sup>41</sup> Lipopolysaccharides (LPS), a common constituent of bacterial cell walls, are recognized by toll-like receptor 4 (TLR4) and trigger the innate immune response leading to the activation of macrophages to generate pro-inflammatory cytokines that contribute to clearance of bacteria (Fig. S38<sup>†</sup>). The major finding of this study was that elevated polysulfides can negatively regulate TLR4-mediated pro-inflammatory signaling and the polysulfide

donor protects against this endotoxin shock. Taking this cue, we evaluated the ability of **1a** to mitigate neuroinflammation in a similar mouse model system. First, C57BL/6J mice were intraperitoneally injected once daily with compound **1a** over a period of seven days, and it was found to be well tolerated. Next, mice were treated with LPS alone and increased levels of pro-inflammatory cytokines  $\text{TNF-}\alpha$  and IL-6 in the brain were observed as compared with vehicle-treated mice (Fig. 5C).

In a separate experiment, mice which were treated with LPS and **1a** were found to have significantly lower levels of  $\text{TNF-}\alpha$  and IL-6 in the brain, suggesting that **1a** suppressed the inflammatory response triggered by LPS. No evidence for reduction of pro-inflammatory cytokines in mice treated with LPS and NaHS was observed, consistent with the previous report that NaHS had little or no effect on the reduction of cytokines.<sup>41</sup> LPS is also known to induce the formation of pro-inflammatory prostaglandins in the brain. Prostaglandin E2 (PGE2) has a variety of functions in the nervous system. Interaction of PGE2 with prostaglandin EP3 receptors leads to an increase in body temperature and inflammation (Fig. S39<sup>†</sup>). The use of nonsteroidal anti-inflammatory drugs (NSAIDs) blocks the activity of cyclooxygenase-2 (COX-2) which decreases PGE2 production,



resulting in remediation of fever and inflammation.<sup>42</sup> Hence, measurement of PGE2/D2 levels is a reliable inflammation biomarker. LC/MS analysis of brain homogenates was carried out to measure PGE2/D2 levels. LPS-treated mice were found to have significantly higher levels of PGE2/D2 when compared with vehicle-treated mice. Animals which were exposed to both LPS and **1a** were found to have significantly lower levels of PGE2/D2, reiterating the ability of **1a** to reduce neuro-inflammation (Fig. 5D). Again, NaSH-treated mice that were exposed to LPS did not show any effect on PGE2/D2 levels. Taken together, these assays conclusively demonstrate the ability of **1a** to act as an antioxidant as well as an anti-inflammatory agent.

Our data shows that the unnatural substrate is able to transfer sulfur to low molecular weight thiols *via* 3-MST, and induce protein persulfidation in cells. Other strategies for generating persulfides in cells have relied on installing the persulfide/polysulfide functional group in the donor.<sup>5,6,41,43–53</sup> These strategies work very well but have limitations in the ease of synthesis, poor shelf-life due to susceptibility towards decomposition, and heterogeneity of the persulfide source due to the ambiguity in the number of sulfurs in the donor. The protocol developed here involves the synthesis of a thioacetate, which is easy to prepare with high reproducibility and is stable for extended periods. For *in vitro* experiments to generate persulfide, the addition of enzymes that are readily available (esterase and 3-MST) is necessary while for cellular experiments to enhance persulfide, addition of the compound is sufficient. The substrates we have developed allow for systematic study of cellular sulfur transfer, and can be used to investigate the persulfide proteome which has been elusive till date.<sup>11</sup>

## Conclusions

The study of reactive sulfur species is often complicated by poor detection techniques, artefacts and uncharacterized cellular interactions. Thus, several facets of sulfur metabolism remain to be studied in molecular-level detail. The use of selective tools developed herein allow for systematic study of redox biology through the lens of an important enzyme, 3-MST. Our data underscores the potential of this approach as a novel therapeutic paradigm in mitigating inflammation. The importance of persulfides in ameliorating symptoms associated with neurodegenerative disorders as well as favourably impacting behavior in animals are some of the possible applications of the strategy developed here.<sup>4</sup> The tunability of rates of persulfide generation using the class of compounds that we have developed adds a new dimension to persulfide donors that has hitherto not been studied. Projecting forward, further structural optimization of the substrate would be necessary to fully exploit the translational potential of this approach. The deficits associated with diminished expression of 3-MST will likely need mapping out the 3-MST-SS<sup>−</sup> proteome and identification of proteins that depend on this enzyme for persulfidation. Given the selectivity of the substrate towards 3-MST in cells, the use of our compounds as probes for studying sulfur trafficking will provide insights into molecular mechanisms associated with

the dysfunction or deficiency of 3-MST in neuronal diseases, such as intellectual disability and Down's syndrome.<sup>54,55</sup>

## Data availability

Experimental protocols, characterization and all data pertaining to the manuscript have been uploaded in the ESI.†

## Author contributions

ABH and HC wrote the manuscript with inputs from all authors. PB, SM, MN, RRMS, SS, VSSA and KG carried out the experiments and computation under the supervision of AM, DKS, SSK, ABH and HC.

## Conflicts of interest

There are no conflicts to declare.

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## Notes and references

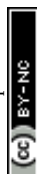
- 1 M. R. Filipovic, J. Zivanovic, B. Alvarez and R. Banerjee, *Chem. Rev.*, 2018, **118**, 1253–1337.
- 2 T. V. Mishanina, M. Libiad and R. Banerjee, *Nat. Chem. Biol.*, 2015, **11**, 457.
- 3 M. S. Vandiver, B. D. Paul, R. Xu, S. Karuppagounder, F. Rao, A. M. Snowman, H. Seok Ko, Y. Il Lee, V. L. Dawson, T. M. Dawson, N. Sen and S. H. Snyder, *Nat. Commun.*, 2013, **4**, 1626.
- 4 D. Giovino, B. Bursac, J. I. Sbodio, S. Nalluru, T. Vignane, A. M. Snowman, L. M. Albacarys, T. W. Sedlak, R. Torregrossa, M. Whiteman, M. R. Filipovic, S. H. Snyder and B. D. Paul, *Proc. Natl. Acad. Sci. U. S. A.*, 2021, **118**, e2017225118.
- 5 Y. Zheng, B. Yu, Z. Li, Z. Yuan, C. L. Organ, R. K. Trivedi, S. Wang, D. J. Lefer and B. Wang, *Angew. Chem., Int. Ed.*, 2017, **56**, 11749–11753.
- 6 V. S. Khodade, B. M. Pharoah, N. Paolocci and J. P. Toscano, *J. Am. Chem. Soc.*, 2020, **142**, 4309–4316.



- 7 K. Shatalin, E. Shatalina, A. Mironov and E. Nudler, *Science*, 2011, **334**, 986–990.
- 8 P. Shukla, V. S. Khodade, M. SharathChandra, P. Chauhan, S. Mishra, S. Siddaramappa, B. E. Pradeep, A. Singh and H. Chakrapani, *Chem. Sci.*, 2017, **8**, 4967–4972.
- 9 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. Signaling*, 2009, **2**, ra72.
- 10 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.
- 11 J. Zivanovic, E. Kouroussis, J. B. Kohl, B. Adhikari, B. Bursac, S. Schott-Roux, D. Petrovic, J. L. Miljkovic, D. Thomas-Lopez, Y. Jung, M. Miler, S. Mitchell, V. Milosevic, J. E. Gomes, M. Benhar, B. Gonzales-Zorn, I. Ivanovic-Burmazovic, R. Torregrossa, J. R. Mitchell, M. Whiteman, G. Schwarz, S. H. Snyder, B. D. Paul, K. S. Carroll and M. R. Filipovic, *Cell Metab.*, 2019, **30**, 1152–1170.
- 12 N. Nagahara and A. Katayama, *J. Biol. Chem.*, 2005, **280**, 34569–34576.
- 13 N. Nagahara, *Br. J. Pharmacol.*, 2018, **175**, 577–589.
- 14 Y. Kimura, S. Koike, N. Shibuya, D. Lefer, Y. Ogasawara and H. Kimura, *Sci. Rep.*, 2017, **7**, 10459.
- 15 N. Nagahara and T. Nishino, *J. Biol. Chem.*, 1996, **271**, 27395–27401.
- 16 M. Libiad, P. K. Yadav, V. Vitvitsky, M. Martinov and R. Banerjee, *J. Biol. Chem.*, 2014, **289**, 30901–30910.
- 17 K. Módis, C. Coletta, K. Erdélyi, A. Papapetropoulos and C. Szabo, *FASEB J.*, 2013, **27**, 601–611.
- 18 M. Li, C. Xu, J. Shi, J. Ding, X. Wan, D. Chen, J. Gao, C. Li, J. Zhang, Y. Lin, Z. Tu, X. Kong, Y. Li and C. Yu, *Gut*, 2018, **67**, 2169–2180.
- 19 P. M. Palenchar, C. J. Buck, H. Cheng, T. J. Larson and E. G. Mueller, *J. Biol. Chem.*, 2000, **275**, 8283–8286.
- 20 E. G. Mueller, *Nat. Chem. Biol.*, 2006, **2**, 185.
- 21 H. T. Nagasawa, D. J. W. Goon, D. L. Crankshaw, R. Vince and S. E. Patterson, *J. Med. Chem.*, 2007, **50**, 6462–6464.
- 22 C. Coletta, K. Módis, B. Szczesny, A. Brunyánszki, G. Oláh, E. C. S. Rios, K. Yanagi, A. Ahmad, A. Papapetropoulos and C. Szabo, *Mol. Med.*, 2015, **21**, 1–14.
- 23 P. K. Yadav, K. Yamada, T. Chiku, M. Koutmos and R. Banerjee, *J. Biol. Chem.*, 2013, **288**, 20002–20013.
- 24 G.-T. Huang and J.-S. K. Yu, *J. Phys. Chem. B*, 2016, **120**, 4608–4615.
- 25 F. van den Ent and J. Löwe, *J. Biochem. Biophys. Methods*, 2006, **67**, 67–74.
- 26 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. Peñeñory, *Org. Biomol. Chem.*, 2014, **12**, 6516–6526.
- 27 J.-C. Lec, S. Boutserin, H. Mazon, G. Mulliert, S. Boschi-Muller and F. Talfournier, *ACS Catal.*, 2018, **8**, 2049–2059.
- 28 D. Zhang, I. Macinkovic, N. O. Devarie-Baez, J. Pan, C.-M. Park, K. S. Carroll, M. R. Filipovic and M. Xian, *Angew. Chem., Int. Ed.*, 2014, **53**, 575–581.
- 29 N. Shibuya, M. Tanaka, M. Yoshida, Y. Ogasawara, T. Togawa, K. Ishii and H. Kimura, *Antioxid. Redox Signaling*, 2009, **11**, 703–714.
- 30 C. Tristan, N. Shahani, T. W. Sedlak and A. Sawa, *Cell. Signalling*, 2011, **23**, 317–323.
- 31 W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian, *Chem. Sci.*, 2013, **4**, 2892.
- 32 P. Chauhan, P. Bora, G. Ravikumar, S. Jos and H. Chakrapani, *Org. Lett.*, 2017, **19**, 62–65.
- 33 C. Wei, Q. Zhu, W. Liu, W. Chen, Z. Xi and L. Yi, *Org. Biomol. Chem.*, 2014, **12**, 479–485.
- 34 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.
- 35 K. Hanaoka, K. Sasakura, Y. Suwanai, S. Toma-Fukai, K. Shimamoto, Y. Takano, N. Shibuya, T. Terai, T. Komatsu, T. Ueno, Y. Ogasawara, Y. Tsuchiya, Y. Watanabe, H. Kimura, C. Wang, M. Uchiyama, H. Kojima, T. Okabe, Y. Urano, T. Shimizu and T. Nagano, *Sci. Rep.*, 2017, **7**, 40227.
- 36 G. Yang, K. Zhao, Y. Ju, S. Mani, Q. Cao, S. Puukila, N. Khaper, L. Wu and R. Wang, *Antioxid. Redox Signaling*, 2012, **18**, 1906–1919.
- 37 S. Koike, Y. Ogasawara, N. Shibuya, H. Kimura and K. Ishii, *FEBS Lett.*, 2013, **587**, 3548–3555.
- 38 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.
- 39 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.
- 40 F. Q. Schafer and G. R. Buettner, *Free Radical Biol. Med.*, 2001, **30**, 1191–1212.
- 41 T. Zhang, K. Ono, H. Tsutsuki, H. Ihara, W. Islam, T. Akaike and T. Sawa, *Cell Chem. Biol.*, 2019, **26**, 686–698.
- 42 J. R. Vane, *Nat. New Biol.*, 1971, **231**, 232–235.
- 43 P. Bora, P. Chauhan, S. Manna and H. Chakrapani, *Org. Lett.*, 2018, **20**, 7916–7920.
- 44 A. Chaudhuri, Y. Venkatesh, J. Das, M. Gangopadhyay, T. K. Maiti and N. D. P. Singh, *J. Org. Chem.*, 2019, **84**, 11441–11449.
- 45 R. A. Hankins, S. I. Suarez, M. A. Kalk, N. M. Green, M. N. Harty and J. C. Lukesh, *Angew. Chem.*, 2020, **132**, 22422–22429.
- 46 V. S. Khodade, S. C. Aggarwal, B. M. Pharoah, N. Paolocci and J. P. Toscano, *Chem. Sci.*, 2021, **12**, 8252–8259.
- 47 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.
- 48 Z. Yuan, Y. Zheng, B. Yu, S. Wang, X. Yang and B. Wang, *Org. Lett.*, 2018, **20**, 6364–6367.
- 49 J. Kang, S. Xu, M. N. Radford, W. Zhang, S. S. Kelly, J. J. Day and M. Xian, *Angew. Chem., Int. Ed.*, 2018, **57**, 5893–5897.
- 50 C. R. Powell, M. K. Dillon, Y. Wang, R. J. Carrazzone and J. B. Matson, *Angew. Chem., Int. Ed.*, 2018, **57**, 6324–6328.



- 51 K. M. Dillon, R. J. Carrazone, Y. Wang, C. R. Powell and J. B. Matson, *ACS Macro Lett.*, 2020, **9**, 606–612.
- 52 Y. Wang, K. M. Dillon, Z. Li, E. W. Winckler and J. B. Matson, *Angew. Chem., Int. Ed.*, 2020, **59**, 16698–16704.
- 53 V. S. Khodade and J. P. Toscano, *J. Am. Chem. Soc.*, 2018, **140**, 17333–17337.
- 54 Y. Suwanai, N. Nagahara, Z. Naito and H. Orimo, *Adv. Tech. Biol. Med.*, 2016, **4**, 167.
- 55 T. Panagaki, E. B. Randi and C. Szabo, *Biomolecules*, 2020, **10**, 653.



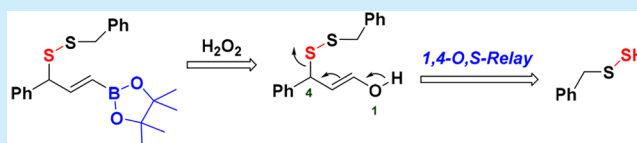
## A Vinyl-Boronate Ester-Based Persulfide Donor Controllable by Hydrogen Peroxide, a Reactive Oxygen Species (ROS)

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 Supporting Information

**ABSTRACT:** A vinyl boronate ester-based persulfidating agent that is selectively activated by hydrogen peroxide, which is a reactive oxygen species (ROS), and efficiently generated a persulfide by a hitherto unexplored 1,4-O,S-relay mechanism is reported. This donor was found to protect cells from cytotoxicity induced by oxidants, and the major byproduct is cinnamaldehyde, which is widely used in the food industry as an additive.



Hydrogen sulfide ( $\text{H}_2\text{S}$ ) has emerged as an important mediator of redox cellular processes, especially in the context of cellular responses associated with oxidative stress.<sup>1–3</sup> During several disease-like conditions, cells are exposed to increased reactive oxygen species (ROS), which contribute to neurodegenerative disorders, inflammation, diabetes, tumor progression, and aging.<sup>4–8</sup> A mechanism by which  $\text{H}_2\text{S}$  exerts its effects is protein persulfidation (or S-sulfhydration),<sup>3</sup> which is an oxidative post-translational modification where a cysteine (Cys-SH) residue is modified to Cys-SSH group.<sup>1</sup> Ambient protein persulfidation in cells is symptomatic of normal functioning in certain cells;<sup>4</sup> a corollary to this observation is that diminished persulfidation is associated with stressed or diseased states. For example, diminished persulfidation of parkin, an E3 ubiquitin ligase that contains a reactive cysteine residue, is correlated with decreased rescue of damaged neurons. Increasing parkin persulfidation appears to protect neurons by removing damaged proteins. This finding has tremendous implications in the treatment of neurodegenerative diseases such as Parkinson's disease.<sup>9</sup> However, this correlation does not hold for other proteins, underscoring the importance of developing new tools to interrogate the chemical biology of persulfidation under disease-relevant conditions.<sup>1</sup> Furthermore, since persulfides ( $\text{RSS}^-$ ) are superior reductants, compared with  $\text{RS}^-$ , these species have gained traction as important intermediates in countering oxidative stress.<sup>10,11</sup> However, since persulfides are unstable in biological milieu, it is challenging to generate these reactive sulfur species in a controllable manner,<sup>10,11</sup> hence the growing interest in small-molecule persulfidating agents as tools to interrogate redox chemical biology of this reactive sulfur species.

An ideal donor would respond to elevated ROS to produce a protein persulfidating agent. The general strategy to generate a persulfide in situ involves stimuli responsive deprotection, followed by electronic rearrangement or a relay mechanism to release a persulfide.<sup>12,13</sup> Subsequent exchange of the sulfhydryl group between the persulfidating agent and a protein occurs to induce protein persulfidation (Figure 1a). To closely mimic

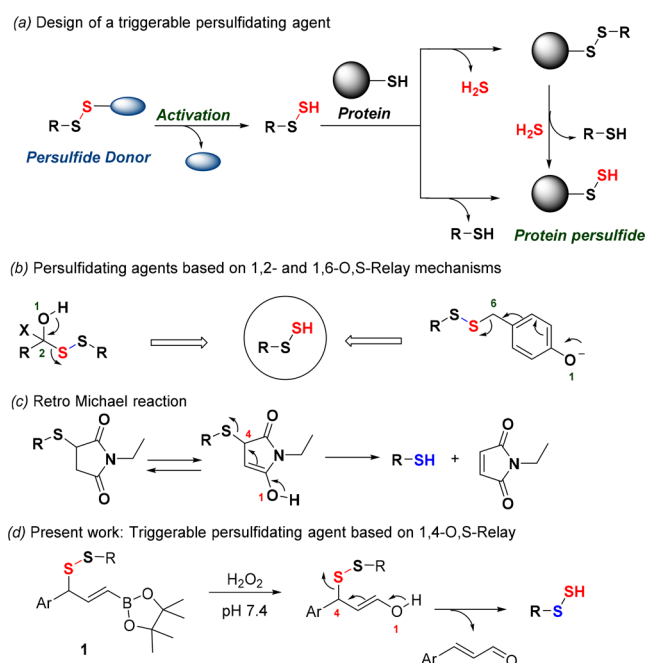


**Figure 1.** (a) Design of a triggerable protein S-sulfhydrating agent. (b) Key intermediates of some reported triggerable persulfide donors that operate via a 1,2- or a 1,6-O,S-relay mechanism. (c) An example of a retro Michael reaction: the first step is presumably the generation of an enol(ate), which generates a thiol by a 1,4-O,S-relay mechanism. (d) Design of a ROS-triggered persulfide donor that is expected to operate by a 1,4-O,S-relay mechanism.

inflammatory conditions, the donor would ideally need to be triggered by hydrogen peroxide, which is a stable ROS.<sup>14</sup> The first major class of persulfidating agents are based on a 1,2-O,S-relay mechanism, where the oxygen and sulfur are placed adjacent to each other (Figure 1b).<sup>15</sup> These donors respond to

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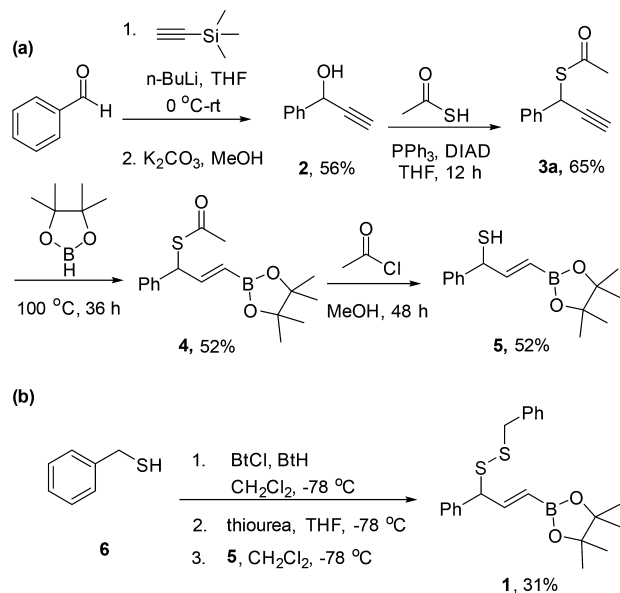
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a nucleophile,<sup>15,16</sup> enzyme,<sup>17–19</sup> or fluoride.<sup>20</sup> These donors, while useful, have limited selectivity toward oxidative stress conditions. The next class of persulfidating agents involves a 1,6-O,S-relay mechanism, where the oxygen is masked in the form of a boronate ester, which is a substrate for oxidation by ROS such as hydrogen peroxide.<sup>21</sup> As a testament to its therapeutic utility, this donor was shown to protect cells from oxidative stress. The formation of a quinone-methide<sup>22</sup> during persulfide delivery is possibly a limitation of this method.

To address these major gaps, we considered an alternate design for a ROS-triggerable persulfide donor, which was based on a hitherto unexplored 1,4-O,S-relay mechanism.<sup>12</sup> Formally, this type of rearrangement is a retro Michael reaction involving a thiol as a leaving group (Figure 1c).<sup>23,24</sup> Here, the enol undergoes a 1,4-O,S-relay mechanism to generate a thiol and an  $\alpha,\beta$ -unsaturated carbonyl compound. Since the  $pK_a$  of R-SSH (6.2) is somewhat lower than a thiol (7–9), it was envisaged that this group might depart under these conditions.<sup>11</sup> The carbonyl was masked as a vinyl boronate ester **1**, which is known to undergo oxidation in the presence of hydrogen peroxide to generate an aldehyde (Figure 1d).<sup>25,26</sup> The byproduct of decomposition of **1** is cinnamaldehyde, which is a constituent of cinnamon oil, and has been classified as Generally Recognized As Safe (GRAS). Together, **1** should respond to oxidative stress to generate a persulfide and relatively innocuous byproducts.

To synthesize **1** (Scheme 1a), benzaldehyde and TMS-acetylene were reacted in the presence of *n*-butyllithium to

**Scheme 1.** (a) Synthesis of Thiol **5**; (b) Reaction of Benzylthiol (**6**) with **5** Affords the Desired Compound **1**<sup>a</sup>



<sup>a</sup> $BtCl$  = 1-chlorobenzotriazole and  $BtH$  = benzotriazole.

produce the secondary alcohol **2**.<sup>27</sup> Under Mitsunobu reaction conditions, **2** was converted to the thioacetate **3a** in 65% yield.<sup>28</sup> Hydroboration of **3a** gave **4** as the *trans* isomer (vicinal olefinic,  $J$  = 17.2 Hz). Deprotection of the thioacetate in the presence of acetyl chloride afforded the thiol **5** in 52% crude yield. Next, using a reported protocol, the reaction of benzyl thiol **6** with **5** afforded the desired product **1** in 31% yield.<sup>29</sup>

First, to ascertain the reactivity of **1** toward ROS, **1** was incubated with  $H_2O_2$  (10 equiv) in pH 7.4 buffer at 37 °C. HPLC analysis of the reaction mixture revealed the complete disappearance of this compound in 90 min (Figure S1a in the Supporting Information). A time course of this decomposition was obtained, and curve fitting to a first-order equation gave a rate constant  $k_1$  of  $5.3 \times 10^{-2} \text{ min}^{-1}$  (Figure 2a). The

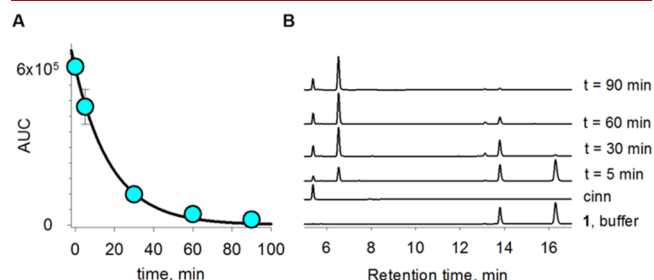


**Figure 2.** (a) Decomposition of **1**, as monitored by HPLC analysis. Curve fitting to first-order decomposition gave a rate constant ( $k_1$ ) of  $5.3 \times 10^{-2} \text{ min}^{-1}$ . (b) HPLC analysis of the reaction of **1** with hydrogen peroxide in the presence of monobromobimane (mBBR) at 37 °C in pH 7.4 buffer (containing 20%  $CH_3CN$ ).

estimated half-life of **1** under these conditions is 13 min. This value is comparable with arylboronate ester decomposition ( $0.09 \text{ min}^{-1}$ ), suggesting no significant difference in the mechanism of hydrogen peroxide-mediated oxidation. To study the potential for **1** to generate a persulfide, we used a method developed by Binghe Wang and co-workers, where they used 1-fluoro-2,4-dinitrobenzene (**7**, FDNB) to trap the persulfide.<sup>17</sup> The resulting compound **8** (Figure 3) can be detected by HPLC analysis.

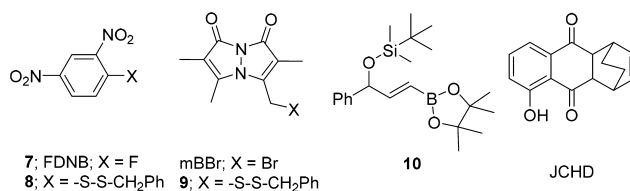
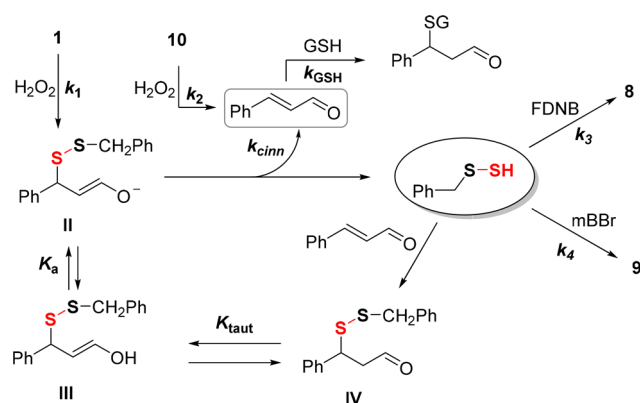


**Figure 3.** Structures of key tools and compounds used in this study.

Compound **8** was synthesized using a reported procedure and HPLC analysis showed a distinct peak at 11.3 min (Figure S3a in the Supporting Information). When **1** (retention time (RT) = 16.3 min) was coincubated with FDNB in the presence of  $H_2O_2$ , we found nearly complete decomposition in 90 min with a concomitant formation of **8** (Figure S3b in the Supporting Information). Because of the susceptibility of persulfides to decompose, this experiment was performed at room temperature. In the absence of hydrogen peroxide, we did not observe the formation of **8** (Figure S3d in the Supporting Information). These data support the generation of a persulfide when **1** was reacted with  $H_2O_2$ . The rate constant for the formation of **8** ( $k_3$ ) was  $0.15 \text{ min}^{-1}$  and is a proxy to the persulfide formation rate (Scheme 2).

An independent assay based on monobromobimane (mBBR) was next used for detecting persulfides (Figure 2b). Reaction of mBBR with a persulfide is expected to produce the disulfide **9** (Figure 3). When **1** was reacted with hydrogen peroxide at 37 °C in the presence of mBBR, we find a distinct peak that is attributable to the formation of **9** ( $m/z$  = 369.07; observed,

Scheme 2. Mechanism of Persulfide Formation during the Oxidation of **1**<sup>a</sup>

<sup>a</sup>The rate constant for decomposition of **1** was  $k_1 = 5.3 \times 10^{-2} \text{ min}^{-1}$ , whereas that for the decomposition of **10** was  $k_2 = 4.8 \times 10^{-2} \text{ min}^{-1}$ . The rate constant for the formation of cinnamaldehyde during the decomposition of **1** in the presence of FDNB was  $k_{\text{cinn}} = 12.8 \times 10^{-2} \text{ min}^{-1}$ . The rate constants for the formation of **8** ( $k_3 = 0.15 \text{ min}^{-1}$ ) and **9** ( $k_4 = 0.12 \text{ min}^{-1}$ ) were comparable.

369.25; see Figure S4 in the Supporting Information). The time course for formation of this compound was monitored, and a rate constant of  $k_4$  of  $0.12 \text{ min}^{-1}$  was obtained. This rate constant is comparable with  $k_1$ . Furthermore, the value of  $k_3$  was determined at  $25^\circ\text{C}$ , and considering rate effects on temperature, we suggest that the values of  $k_1$ ,  $k_3$ , and  $k_4$  are similar.

Next, the silylated derivative **10** was synthesized in two steps from the propargyl alcohol **2** (Scheme S1 in the Supporting Information). Compound **10** should undergo decomposition in the presence of hydrogen peroxide but does not generate a persulfide. When **10** was incubated in the presence of  $\text{H}_2\text{O}_2$ , indeed, we find evidence for the formation of cinnamaldehyde (Figure S2 in the Supporting Information).

Curve fitting yielded a rate constant ( $k_2$ ) of  $4.8 \times 10^{-2} \text{ min}^{-1}$  that was comparable in value with the rate of decomposition of **1** (Scheme 2). The yield of cinnamaldehyde under these conditions was 73%, which is comparable with the yield of **8** during incubation of **1** with hydrogen peroxide and FDNB (Figure S3b in the Supporting Information). Phillips and co-workers have previously demonstrated the use of vinyl boronate esters in the deprotection of alcohols with  $\text{pK}_a$  values of  $>11$ .<sup>26</sup> Since the estimated  $\text{pK}_a$  value of *tert*-butyldimethylsilanol is 15, this result supports the use of such vinylboronate esters for the release of poorly acidic alcohols as well.

When **1** was incubated in the presence of hydrogen peroxide, we similarly observed the formation of cinnamaldehyde, but with diminished yield (Figure S1a in the Supporting Information). This diminished yield could be due to the collateral consumption of cinnamaldehyde by the persulfide. To test this possibility, we incubated **1** in the presence of  $\text{H}_2\text{O}_2$  and mBBR, and we found that the yield of cinnamaldehyde was significantly better ( $\sim 70\%$ ; Figure 2b). The rate constant for the formation of cinnamaldehyde ( $k_{\text{cinn}}$ ) was  $12.8 \times 10^{-2} \text{ min}^{-1}$  (Scheme 2). Persulfides, being good one-electron reductants, have a high propensity to undergo oxidation to form tetrasulfides ( $\text{RSSSR}$ ) and polysulfides ( $\text{RS}_n\text{R}$ ).<sup>30</sup> Although all assays were performed in the presence of diethylenetriaminepentaacetic acid (DTPA) as a chelating

agent, to prevent the decomposition of  $\text{H}_2\text{O}_2$  and the subsequent radical-based oxidation of persulfides, the possibility of polysulfide formation cannot be ruled out. Furthermore, in cells, we would expect cinnamaldehyde to react with other thiols such as glutathione, which typically occurs in millimolar concentrations. When cinnamaldehyde was reacted with glutathione, the pseudo-first-order rate constant for this reaction was found as  $2.3 \times 10^{-2} \text{ min}^{-1}$ , which translates to a half-life of  $\sim 30 \text{ min}$  (Figure S5 in the Supporting Information). Previously, cinnamaldehyde was found to react with thiols as well as bovine serum albumin;<sup>31</sup> the half-life of the latter reaction was in the range of 3–8 min. These data suggested the possibility of cellular thiols competitively reacting with cinnamaldehyde and possibly sparing the persulfide to conduct protein persulfidation.

We next estimated the selectivity of **1** toward activation by  $\text{H}_2\text{O}_2$ . We tested **1** against a variety of oxidants in the presence of FDNB and tested if the persulfide adduct **8** was produced. We find no evidence for decomposition of **1** or the formation of **8** under these conditions (Figure S6a in the Supporting Information). In a separate assay with **10**, we found no evidence for the formation of cinnamaldehyde, except when **10** was treated with  $\text{H}_2\text{O}_2$  (Figure S6c in the Supporting Information). Together, these data support the excellent selectivity of the vinyl boronate ester functional group toward oxidation by hydrogen peroxide.

The proposed mechanism for the reaction of **1** with hydrogen peroxide involves the oxidation of the vinyl boronate ester (likely the boronic acid in pH 7.4 buffer) to produce an enolate intermediate, which decomposes to produce the persulfide and cinnamaldehyde. Since the value of  $k_{\text{cinn}}$  was comparable in magnitude with that of  $k_3$  and  $k_4$ , it is likely that the formation of the persulfide and cinnamaldehyde is concerted. Previously, retro Michael reactions involving thioethers of *N*-ethylmaleimide have been reported.<sup>23,24</sup> These reactions are extremely slow (half-lives ranging from  $\sim 1$ –7 days). Hence, the present method appears to be distinct from the previous reports due to the direct generation of an enolate **II** that rapidly rearranges to produce a persulfide. The equilibrium constant for tautomerism ( $K_{\text{taut}}$ ), which is defined as  $[\text{keto}]/[\text{enol}]$  for aliphatic aldehydes, is in the range of  $10^{-3}$ – $10^{-4}$ .<sup>32</sup>

These data suggest that, once formed, **III** would likely equilibrate to the aldehyde **IV** (Scheme 2). If the aldehyde (keto form) is indeed produced, it is likely that the generation of the persulfide might be extremely slow. Our attempts to detect this aldehyde **IV** were unsuccessful. Since the yields of products are in excess of 70%, we reasoned that tautomerism may not be a major competitive process. Thus, it is likely that the enolate **II**, once formed, would rapidly rearrange to generate the persulfide. The estimated  $\text{pK}_a$  for the enol is 9–10, likely to be deprotonated in pH 7.4.<sup>33</sup> Previously, in basic solution, the enolate was found to be the dominant form for certain aldehydes.<sup>33</sup> Although the operating pH is 7.4, it appears to stabilize the enolate sufficiently to promote the 1,4-O,S-relay.

Lastly, since persulfides are reported to mitigate oxidative stress, we tested the ability of **1** to protect colon carcinoma DLD-1 cells from cytotoxicity induced by elevated ROS. Colon cells are constantly exposed to xenobiotics and stress induced by pathogens. It has been reported that hydrogen sulfide is an important mediator of stress response in the gut.<sup>34–36</sup> Using DLD-1 colon carcinoma cells, a cell viability assay was first

conducted with increasing doses of **1**, and no significant inhibition up to 100  $\mu\text{M}$  was observed (Figure S7a in the Supporting Information). Next, using menadione, which is a known redox cycling agent and inducer of oxidative stress, we evaluated the protective effects of **1**.<sup>37,38</sup> DLD-1 cells were treated with menadione and viable cells were measured (Figure S7b in the Supporting Information). We found significant cell killing at 50  $\mu\text{M}$  (30% viable cells); this concentration was chosen to study possible cytoprotective effects of **1**. When **1** was cotreated with menadione, a dose-dependent cytoprotection was observed (Figure 4a). A cell viability assay conducted

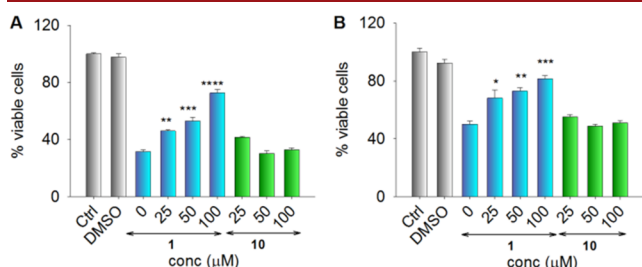


**Figure 4.** (a) Cytoprotective effects of compound **1** against menadione (50  $\mu\text{M}$ ). Results are expressed as mean  $\pm$  SEM ( $n = 3$ ). [Legend: (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.001$ , (\*\*\*\*)  $p < 0.0001$  vs menadione.] A similar assay was conducted with **10**. No significant effect on the percentage of viable cells during incubation of **10** with menadione was observed. (b) Cytoprotective effects of compound **1** against JCHD (50  $\mu\text{M}$ ). Results are expressed as mean  $\pm$  SEM ( $n = 3$ ). [Legend: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.001$ , vs JCHD.] A similar assay was conducted with **10**, and no significant effect was observed.

with **10** revealed that this compound was not significantly cytotoxic (Figure S7a). However, **10** was unable to protect cells from menadione-induced cytotoxicity, supporting the importance of the persulfide in cytoprotective effects (Figure 4a). Next, JCHD, which is a derivative of juglone, was used to simulate increased ROS within cells. This compound has been previously characterized to generate ROS in pH 7.4 buffer under ambient aerobic conditions and increase ROS levels in cells (Figure S8 in the Supporting Information). Again, we find significant cytotoxicity induced at 50  $\mu\text{M}$  by JCHD (Figure S7b).<sup>39–41</sup> The persulfide donor **1** protected cells from JCHD-induced toxicity, while **10** showed no effect (Figure 4b). Thus, the results of these cell studies demonstrate the potential for this new donor to protect cells from xenobiotics and oxidative stress.

In summary, we report a new class of 1,4-O,S-relay mechanism-based persulfide donors with a unique retro Michael reaction as the key step. Generation of persulfide from this donor was independently validated by two assays, and the mechanism is consistent with experimental data. The major byproducts that are produced appear well tolerated by cells and this observation is encouraging for further development of this donor. We found that **1** was able to protect cells from oxidative stress induced by exogenous ROS generators. Taken together, this compound is a valuable addition to the growing redox toolbox to understand the chemical biology of reactive sulfur species better while progressing toward new classes of sulfur-based therapeutic agents.

## ■ ASSOCIATED CONTENT

### § Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b03471.

Synthesis and characterization data, analytical data, and protocols (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Filipovic, M. R.; Zivanovic, J.; Alvarez, B.; Banerjee, R. *Chem. Rev.* **2018**, *118* (3), 1253.
- (2) Kabil, O.; Vitvitsky, V.; Banerjee, R. *Annu. Rev. Nutr.* **2014**, *34* (1), 171.
- (3) Mishanina, T. V.; Libiad, M.; Banerjee, R. *Nat. Chem. Biol.* **2015**, *11*, 457.
- (4) Mustafa, A. K.; Gadalla, M. M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S. K.; Barrow, R. K.; Yang, G.; Wang, R.; Snyder, S. H. *Sci. Signaling* **2009**, *2* (96), ra72.
- (5) Yang, G.; Zhao, K.; Ju, Y.; Mani, S.; Cao, Q.; Puukila, S.; Khaper, N.; Wu, L.; Wang, R. *Antioxid. Redox Signaling* **2013**, *18* (15), 1906.
- (6) Zhao, W.; Zhang, J.; Lu, Y.; Wang, R. *EMBO J.* **2001**, *20* (21), 6008.
- (7) Mustafa, A. K.; Sikka, G.; Gazi, S. K.; Steppan, J.; Jung, S. M.; Bhunia, A. K.; Barodka, V. M.; Gazi, F. K.; Barrow, R. K.; Wang, R.; Amzel, L. M.; Berkowitz, D. E.; Snyder, S. H. *Circ. Res.* **2011**, *109* (11), 1259.
- (8) Wallace, J. L.; Wang, R. *Nat. Rev. Drug Discovery* **2015**, *14*, 329.
- (9) Vandiver, M. S.; Paul, B. D.; Xu, R.; Karuppagounder, S.; Rao, F.; Snowman, A. M.; Seok Ko, H.; Il Lee, Y.; Dawson, V. L.; Dawson, T. M.; Sen, N.; Snyder, S. H. *Nat. Commun.* **2013**, *4*, 1626.
- (10) Ono, K.; Akaike, T.; Sawa, T.; Kumagai, Y.; Wink, D. A.; Tantillo, D. J.; Hobbs, A. J.; Nagy, P.; Xian, M.; Lin, J.; Fukuto, J. M. *Free Radical Biol. Med.* **2014**, *77*, 82.
- (11) Park, C.-M.; Weerasinghe, L.; Day, J. J.; Fukuto, J. M.; Xian, M. *Mol. Biosyst.* **2015**, *11* (7), 1775.
- (12) Alouane, A.; Labruere, R.; Le Saux, T.; Schmidt, F.; Jullien, L. *Angew. Chem., Int. Ed.* **2015**, *54* (26), 7492.
- (13) Artaud, I.; Galardon, E. *ChemBioChem* **2014**, *15* (16), 2361.
- (14) Li, L.; Rose, P.; Moore, P. K. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51* (1), 169.
- (15) Park, C.-M.; Johnson, B. A.; Duan, J.; Park, J.-J.; Day, J. J.; Gang, D.; Qian, W.-J.; Xian, M. *Org. Lett.* **2016**, *18* (5), 904.
- (16) Kang, J.; Ferrell, A. J.; Chen, W.; Wang, D.; Xian, M. *Org. Lett.* **2018**, *20* (3), 852.
- (17) Zheng, Y.; Yu, B.; Li, Z.; Yuan, Z.; Organ, C. L.; Trivedi, R. K.; Wang, S.; Lefer, D. J.; Wang, B. *Angew. Chem., Int. Ed.* **2017**, *56* (39), 11749.

- (18) Yu, B.; Zheng, Y.; Yuan, Z.; Li, S.; Zhu, H.; De La Cruz, L. K.; Zhang, J.; Ji, K.; Wang, S.; Wang, B. *J. Am. Chem. Soc.* **2018**, *140* (1), 30.
- (19) Yuan, Z.; Zheng, Y.; Yu, B.; Wang, S.; Yang, X.; Wang, B. *Org. Lett.* **2018**, *20* (20), 6364.
- (20) Kang, J.; Xu, S.; Radford, M. N.; Zhang, W.; Kelly, S. S.; Day, J. J.; Xian, M. *Angew. Chem.* **2018**, *130* (20), 5995.
- (21) Powell, C. R.; Dillon, M. K.; Wang, Y.; Carrazzone, R. J.; Matson, J. B. *Angew. Chem., Int. Ed.* **2018**, *57* (21), 6324.
- (22) Monks, T. J.; Jones, D. C. *Curr. Drug Metab.* **2002**, *3*, 425–438.
- (23) Baldwin, A. D.; Kiick, K. L. *Bioconjugate Chem.* **2011**, *22* (10), 1946.
- (24) Weissman, M. R.; Winger, K. T.; Ghiassian, S.; Gobbo, P.; Workentin, M. S. *Bioconjugate Chem.* **2016**, *27* (3), 586.
- (25) Brooks, A. D.; Mohapatra, H.; Phillips, S. T. *J. Org. Chem.* **2015**, *80* (21), 10437.
- (26) Brooks, A. D.; Yeung, K.; Lewis, G. G.; Phillips, S. T. *Anal. Methods* **2015**, *7* (17), 7186.
- (27) Zheng, M.; Wu, F.; Chen, K.; Zhu, S. *Org. Lett.* **2016**, *18* (15), 3554.
- (28) Yang, F.; Jin, T.; Bao, M.; Yamamoto, Y. *Tetrahedron Lett.* **2011**, *52* (8), 936.
- (29) Hunter, R.; Caira, M.; Stellenboom, N. *J. Org. Chem.* **2006**, *71* (21), 8268.
- (30) Bianco, C. L.; Chavez, T. A.; Sosa, V.; Saund, S. S.; Nguyen, Q. N. N.; Tantillo, D. J.; Ichimura, A. S.; Toscano, J. P.; Fukuto, J. M. *Free Radical Biol. Med.* **2016**, *101*, 20.
- (31) Weibel, H.; Hansen, J. *Contact Dermatitis* **1989**, *20* (3), 161.
- (32) Keeffe, J. R.; Kresge, A. J.; Schepp, N. P. *J. Am. Chem. Soc.* **1990**, *112* (12), 4862.
- (33) Chiang, Y.; Kresge, A. J.; Krogh, E. T. *J. Am. Chem. Soc.* **1988**, *110* (8), 2600.
- (34) Motta, J.-P.; Flannigan, K. L.; Agbor, T. A.; Beatty, J. K.; Blackler, R. W.; Workentine, M. L.; Da Silva, G. J.; Wang, R.; Buret, A. G.; Wallace, J. L. *Inflamm. Bowel Dis.* **2015**, *21* (5), 1006.
- (35) Fiorucci, S.; Antonelli, E.; Distrutti, E.; Rizzo, G.; Mencarelli, A.; Orlandi, S.; Zanardo, R.; Renga, B.; Di Sante, M.; Morelli, A.; Cirino, G.; Wallace, J. L. *Gastroenterology* **2005**, *129* (4), 1210.
- (36) Fiorucci, S.; Orlandi, S.; Mencarelli, A.; Caliendo, G.; Santagada, V.; Distrutti, E.; Santucci, L.; Cirino, G.; Wallace, J. L. *Br. J. Pharmacol.* **2007**, *150* (8), 996.
- (37) Criddle, D. N.; Gillies, S.; Baumgartner-Wilson, H. K.; Jaffar, M.; Chinje, E. C.; Passmore, S.; Chvanov, M.; Barrow, S.; Gerasimenko, O. V.; Tepikin, A. V.; Sutton, R.; Petersen, O. H. *J. Biol. Chem.* **2006**, *281* (52), 40485.
- (38) Loor, G.; Kondapalli, J.; Schriewer, J. M.; Chandel, N. S.; Vanden Hoek, T. L.; Schumacker, P. T. *Free Radical Biol. Med.* **2010**, *49* (12), 1925.
- (39) Dharmaraja, A. T.; Chakrapani, H. *Org. Lett.* **2014**, *16* (2), 398.
- (40) Dharmaraja, A. T.; Alvala, M.; Sriram, D.; Yogeewari, P.; Chakrapani, H. *Chem. Commun.* **2012**, *48* (83), 10325.
- (41) Tyagi, P.; Dharmaraja, A. T.; Bhaskar, A.; Chakrapani, H.; Singh, A. *Free Radical Biol. Med.* **2015**, *84*, 344.



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# A modular scaffold for triggerable and tunable nitroxyl (HNO) generation with a fluorescence reporter†

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**Nitroxyl (HNO) is a short-lived mediator of cell signalling and can enhance the sulfane sulfur pool, a cellular antioxidant reservoir, by reacting with hydrogen sulfide (H<sub>2</sub>S). Here, we report esterase-activated HNO-generators that are suitable for tunable HNO release and the design of these donors allows for real-time monitoring of HNO release. These tools will help gain a better understanding of the cross-talk among short-lived gaseous signalling molecules that have emerged as major players in health and disease.**

Gaseous species derived from nitrogen, sulfur, and even carbon (carbon monoxide) have in the past several decades become central to the understanding of redox cellular biology and the cross-talk among these biologically active gaseous species has become the focus of much attention.<sup>1,2</sup> Nitroxyl (HNO), which is a short-lived species, has been considered as an intermediate in nitrogen metabolism in cells. HNO activates soluble guanylate cyclase (sGC), a key enzyme involved in vascular smooth muscle relaxation.<sup>3,4</sup> Compounds that dissociate to produce HNO have shown promise in cardiovascular<sup>5,6</sup> and anti-cancer applications.<sup>7,8</sup> Recently, hydrogen sulfide (H<sub>2</sub>S) has emerged as a major player in redox homeostasis and the cross-talk between H<sub>2</sub>S and HNO has important implications in antioxidant response. For example, HNO can react with H<sub>2</sub>S to produce persulfide and polysulfide, which together constitute the sulfane sulfur pool.<sup>9</sup> Together, the sulfane sulfur pool is implicated in numerous cellular stress responses, including protective effects against reactive oxygen species (ROS) and inflammation.<sup>10,11</sup> Hence, platforms for reliable generation of HNO are essential for a better understanding of the chemical biology of this short-lived species.

Protected hydroxylamines were considered as HNO donors (Fig. 1A). Activation of these donors is performed to expose the

hydroxyl group of the hydroxylamine, which results in HNO generation, in either two or three steps (Fig. 1A). When an *N,O*-protected hydroxylamine (R = H; **1**, Fig. 1B) is used, the intermediate **3** can collapse to generate HNO, with the sulfinate leaving group determining the rate of HNO generation. When *N,N,O*-protected hydroxylamines (R ≠ H; **2**, Fig. 1B) are used, the product of cleavage is **3**, which does not directly produce HNO, but an additional step through the production of a common intermediate is required. The rate of collapse of the common intermediate would determine the rate of HNO generation. Else, if this collapse is rapid, the preceding steps would likely determine the rates of HNO generation. Together, by varying the protective groups, leaving groups, and intermediates, this modular scaffold can be used to tune the rate of HNO generation.

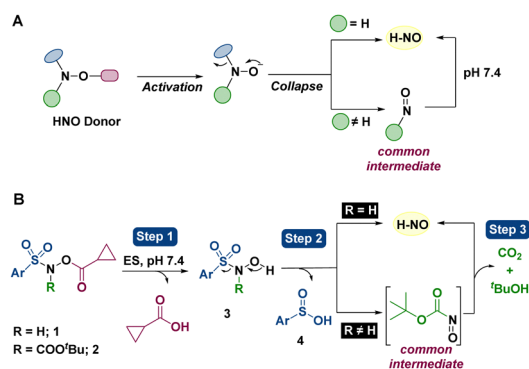


**Fig. 1** (A) A modular scaffold for HNO generation: Activation of the donor followed by collapse produces either HNO or the active HNO generating species, which dissociates to produce HNO. (B) Derivatives **1** or **2** can be cleaved by esterase to produce intermediate **3**, which can either directly produce HNO and an arylsulfonic acid **4** or lose a sulfonic acid to generate a nitroso-intermediate that collapses to produce HNO. If the sulfonic acid is fluorescent and the corresponding donor is weakly fluorescent, this design allows for a fluorescence reporter for HNO release.

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Certain *N,N,O*-protected hydroxylamines have been previously reported by Toscano and coworkers but these compounds spontaneously hydrolysed in buffer to generate HNO.<sup>12</sup> Hence, the *t*-butyl ester was the choice of *N*-protective group since the carbamate that is formed is less likely to be spontaneously hydrolysed in pH 7.4. The cyclopropylcarboxyl group, which is hydrolysed by esterases, was used as an *O*-protective group. HNO donors activated by light<sup>13,14</sup> or an enzyme<sup>15,16</sup> or chemically<sup>17</sup> are known but there are limited structural features that allow for triggerable as well as tunable release of HNO. Furthermore, given the complications associated with the detection of HNO, the incorporation of a fluorescent reporter for HNO production would be useful. Here, we designed and developed a modular scaffold that can generate HNO, and we incorporated a latent fluorophore, which upon release produces a fluorophore, hence reporting the generation of HNO.

Among the esterase-cleavable functional groups, cyclopropyl carboxylic acid-based esters are preferred due to their extended stability in pH 7.4 buffer.<sup>18,19</sup> The *N*-Boc hydroxylamine (HO-NH-Boc) was reacted with cyclopropyl carbonyl chloride to afford **5** (Scheme 1).<sup>20</sup> Reaction of **5** with phenyl sulfonyl chloride gave **2a** (Ar = Ph) (Scheme 1).<sup>12</sup>

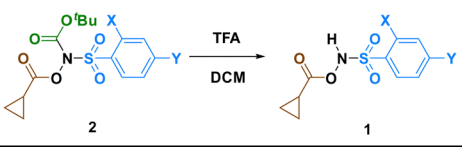
The deprotection of the Boc-group was carried out with trifluoroacetic acid (TFA) and **1a** was isolated in 56% yield (Table 1, entry 1).<sup>12</sup>

The two derivatives **1a** and **2a** were independently treated with esterase in pH 7.4 buffer and HNO generation was monitored using the reported coumarin-based fluorescent probe, PCM (**6**).<sup>21</sup> In the absence of esterase, neither compound produced significant amounts of HNO (Fig. 2A and B). In the presence of esterase, the time course of HNO generation from **1a** during 30 min was significantly different from the time course of HNO generation from **2a** and the yield of HNO from **2a** was higher when compared with **1a** (Fig. 2A and B and see ESI,† Fig. S1). The diminished yield of HNO from **1a** is consistent with a previous report where the yield of HNO from *N*-hydroxy-benzenesulfonamide was low at pH 7.4 and increasing the pH led to an increase in HNO yield.<sup>22,23</sup>

Next, in order to understand if the release of HNO depended on the nature of the leaving group, a series of analogues were prepared (Table 1, entries 2–7). When tested, all analogues **2b–2g** produced HNO at comparable levels (Fig. 2C). Among the derivatives **1b–1g**, except for the 4-nitrophenyl derivative, all compounds tested produced HNO (Fig. 2C and see ESI,† Fig. S2). The lack of HNO production from **1g** is consistent with previous reports where the yield of HNO from *N*-hydroxy-4-nitrobenzenesulfonamide was diminished at pH 7.4.<sup>6</sup>

Having established that these compounds can be triggered by esterase to generate HNO, we next proceeded to prepare the

**Table 1** Synthesis of *N,O*-protected hydroxylamines (**1**) from *N,N,O*-protected hydroxylamines (**2**)



| Entry | X               | Y               | Compd No  | Compd No  | Yield, % |
|-------|-----------------|-----------------|-----------|-----------|----------|
| 1     | H               | H               | <b>2a</b> | <b>1a</b> | 56       |
| 2     | Br              | H               | <b>2b</b> | <b>1b</b> | 53       |
| 3     | H               | Br              | <b>2c</b> | <b>1c</b> | 92       |
| 4     | CF <sub>3</sub> | H               | <b>2d</b> | <b>1d</b> | 41       |
| 5     | Br              | CF <sub>3</sub> | <b>2e</b> | <b>1e</b> | 40       |
| 6     | NO <sub>2</sub> | H               | <b>2f</b> | <b>1f</b> | 95       |
| 7     | H               | NO <sub>2</sub> | <b>2g</b> | <b>1g</b> | 65       |

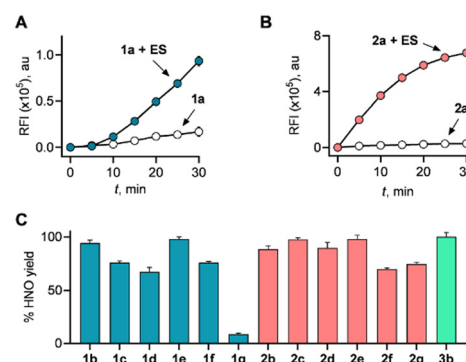
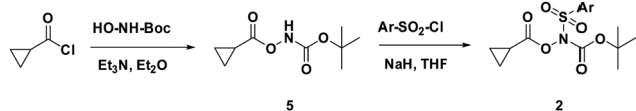


**Fig. 2** HNO detection using PCM dye **6**. Compounds were incubated with and w/o esterase (0.5 U mL<sup>-1</sup>) in PBS (pH 7.4) at 37 °C for 30 min and fluorescence was measured (excitation 370 nm and emission at 460 nm); (A) compound **1a**; (B) compounds **2a**; (C) compounds **1b–1g** and **2b–2g**. **3b**, **3b** refers to 2-bromopiloty's acid, a known HNO generator and yield of HNO from this compound was assigned as 100%.

dansyl derivatives **1h** and **2h** (Fig. 3A, for synthesis see ESI,† Scheme S1). The product formed during HNO release is dansic acid **4h**, whose fluorescence properties such as Stokes shift (189 nm, Fig. S3, ESI†), pH stability (Fig. S4, ESI†), extended lifetime ( $\tau_{\text{avg}} = 13.82$  ns, Fig. S5, ESI†), and quantum yield (83%) make it an excellent candidate for reporting fluorescence in cells.<sup>24</sup> The derivatives **1h** and **2h** are expected to be cleaved by esterase to produce HNO as well as a dansic acid-based fluorescence reporter **4h** (Fig. 3A and see ESI† Fig. S6 and S7). Esterase-catalyzed HNO release from compounds **1h** and **2h** was monitored and an increase in HNO levels was observed during 30 min (Fig. 3B). Pre-treatment with PMSF (phenylmethanesulfonyl fluoride), an inhibitor of esterase,<sup>25</sup> showed a significant decrease in HNO yield. Notably, HNO generated by **3b**, a positive control, was not affected by esterase or by the presence of PMSF (Fig. 3B). The yield of HNO from **2h** was significantly higher when compared with **1h** (Fig. 3B).

Next, the fluorescence attributable to the formation of **4h** was studied during esterase-catalysed cleavage of **1h**. Compounds **1h** and **2h** were themselves weakly fluorescent (excitation, 308 nm and emission, 497 nm; see ESI,† Fig. S7A). In the presence of esterase, in



**Scheme 1** Synthesis of **2**. Ar refers to mono or di-substituted phenyl groups.

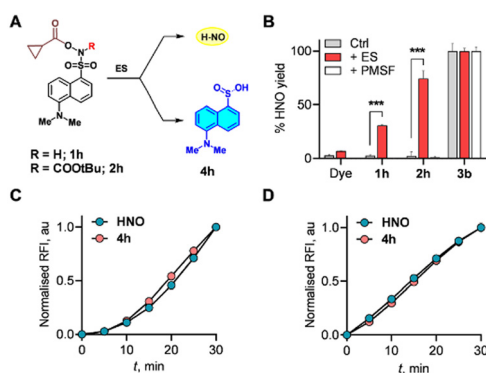


**Fig. 3** (A) Design of HNO donors with a fluorescence reporter. Compound **1h** or **2h** is expected to be cleaved by esterase to produce HNO and **4h**; (B) HNO release from compounds **1h** and **2h** upon incubation with PCM dye **6** in the presence and absence of esterase (0.5 U mL<sup>-1</sup>) with and without esterase inhibitor (**PMSF**) in PBS (pH 7.4) at 37 °C for 30 min; dye refers to PCM **6** alone (see ESI† for structure); + **ES** refers to the compounds incubated with esterase; + **PMSF** (phenylmethanesulfonyl fluoride) refers to pre-treatment of **ES** with **PMSF** followed by compound treatment; **3b** is 2-bromopyruvic acid, a known HNO generator and the yield of HNO from this compound was assigned as 100%; \*\*\**P* < 0.001; **4h** was weakly fluorescent (370 nm excitation, 460 nm emission) and does not contribute to HNO's signal in this assay (see ESI† Fig. S6B). (C) Time course of release of HNO and **4h** during incubation of **1h** with esterase in PBS (pH 7.4) at 37 °C. The rate constant for **4h** release was found to be 1.77 h<sup>-1</sup> (see ESI† Fig. S7C). (D) Time course of release of HNO and **4h** during incubation of **2h** with esterase in PBS (pH 7.4) at 37 °C. All data are presented as mean ± SD (*n* = 3 per group). The rate constant for **4h** release was found to be 1.06 h<sup>-1</sup> (see ESI† Fig. S7D).

the first 30 min, in the case of **1h**, we find a nearly concomitant release of HNO as well as **4h** (Fig. 3C) in a dose-dependent manner (see ESI† Fig. S7B). A similar profile was observed in the case of **2h** (Fig. 3D). Together, these data indicate that we have developed an HNO donor with a fluorescence reporter.

An important feature of HNO donors is the selectivity towards the generation of HNO *versus* nitric oxide (NO), which have distinct activity in cells.<sup>8</sup> Compound **3b**, for example, generates a significant amount of NO (detected as nitrite using the Griess assay; see ESI† Fig. S8) in aerobic conditions. The donors **1h** and **2h** produced significantly lower amounts of NO when compared with **3b** (see ESI† Fig. S8).

Based on the above results, a mechanism for HNO release from this scaffold was considered (Fig. 4A). Compounds belonging to series **1** were cleaved by esterase to produce the anionic intermediate, which equilibrates with **3**. LC/MS analysis of **1h** in pH 7.4 shows the stability in buffer over 300 mins (see ESI† Fig. S9), the reaction mixture of **1h** and esterase revealed complete consumption of **1h** (Fig. 4B and see ESI† Fig. S10 and S11A) and the formation of the intermediate **3h** (Fig. 4C and see ESI† Fig. S11B). The concentration of **3h** builds up over time and then disappears, to produce dansyl sulfinic acid **4h** (Fig. 4b and see ESI† Fig. S11C) and HNO (see ESI† Fig. S6A). During 30 min, the yield of HNO from **1h** is somewhat lower when compared with most other derivatives tested. Two factors determine the yield of HNO: firstly, the strength and polarization of the S–N bond, and secondly, the

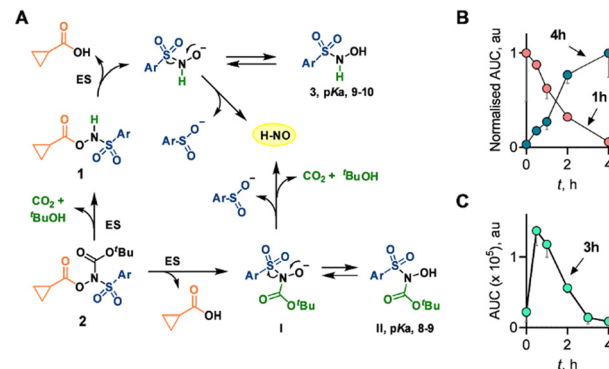


**Fig. 4** LC/MS study. (A) Proposed mechanism for the decomposition of derivatives **1** and **2** to release HNO; Ar refers to dansyl. (B) Decomposition of **1h** and formation of **4h** upon incubation with esterase (0.5 U mL<sup>-1</sup>) in PBS (pH 7.4) at 37 °C as monitored by LC/MS. **1h** (expected, *m/z* = 335.1060 [M + H]<sup>+</sup>; observed, *m/z* = 335.1058), **4h** (expected, *m/z* = 236.0739 [M + H]<sup>+</sup>; observed, *m/z* = 236.0742). (C) Formation of **3h** during the decomposition of **1h** upon incubation with esterase (0.5 U mL<sup>-1</sup>) as monitored by LC/MS. **3h** (expected, *m/z* = 267.0798 [M + H]<sup>+</sup>; observed, *m/z* = 267.0805). All data are presented as mean ± SD (*n* = 3 per group). *pK<sub>a</sub>* values were calculated by using Chemaxon software.

leaving group ability of **4h**. The calculated *pK<sub>a</sub>* of the corresponding sulfinic acid is 0.9 (see ESI† Table S1)<sup>26</sup> and is likely a good leaving group and hence, the HNO yield depends on the bond strength and polarization of the S–N bond. The diminished yield of HNO from **1h** in 30 min could be due to the slow rate of cleavage by esterase (nearly 4 h to complete consumption, Fig. 4B) as well as the electron-donating dimethylamino group that leads to a decrease in the acidity of the hydroxamic acid (see ESI† Table S2) and perhaps, steric effects of the neighboring aromatic ring.

Among the HNO donors belonging to series **2**, esterase cleavage produces intermediate **I** (Fig. 4A). Loss of sulfinate gives the common intermediate (Fig. 1B), which collapses to produce CO<sub>2</sub>, *t*BuOH and HNO. TLC analysis of **2h** in the presence of ES in pH 7.4 buffer revealed complete disappearance with **1h** being formed as a minor product suggesting the lability of the *t*-Boc group under these conditions (see ESI† Fig. S12). LC/MS analysis of **2h** + ES revealed the disappearance of this compound (see ESI† Fig. S13) and the formation of the intermediate **I** (see ESI† Fig. S14), as well as the gradual formation of **4h** (see ESI† Fig. S15C). However, due to the conditions used for LC/MS analysis (formic acid in the eluant), we also observed the loss of the *t*-Boc group and the formation of **1h** (Fig. S15A, ESI†) complicating further mechanistic analysis from this assay. However, the nearly concomitant release of HNO and **4h** in the absence of formic acid (Fig. 3D) suggests that **2h** can be used as a tool for HNO generation with a fluorescence reporter. The yield of HNO from **2h** was significantly higher than the yield of HNO from **1h**. The presence of an electron-withdrawing *t*-Boc group is likely to polarize intermediate **I** and facilitate the departure of the sulfinate group (the estimated *pK<sub>a</sub>* of **II** is 8–9, see ESI† Table S2). This observation supports a sulfinate leaving group-independent mechanism for HNO generation, which is consistent

with the formation of a neutral common intermediate (Fig. 1B) that undergoes hydrolysis to produce HNO; this hydrolysis can be accelerated by esterase. The close correlation between HNO and **4h** suggests that, under the reaction conditions, the hydrolysis of the common intermediate is fast ( $t_{1/2} < 5$  min).

Since HNO is known to react with  $\text{H}_2\text{S}$  to produce persulfide and polysulfide (Scheme S6A),<sup>18</sup> the ability of donors **1h** and **2h** to produce persulfide and polysulfide in the presence of  $\text{H}_2\text{S}$  was investigated using an established **HPE-IAM** electrophile trapping method using LC/MS detection (see ESI,† Scheme S6B).<sup>27</sup> Here, **HPE-IAM** reacts with  $\text{HS}^-$  to produce **HS-HPE-AM** ( $m/z$  212.0723), which can further react with another mole of **HPE-IAM** to produce **Bis-S-HPE-AM** ( $m/z$  = 389.1489) (see ESI,† Scheme S6B). If persulfide or polysulfide is produced, the adducts **Bis (S)<sub>n</sub>-HPE-AM** where  $n \geq 2$  are expected to be formed. The reaction mixture containing **1h** or **2h** was treated with  $\text{Na}_2\text{S}$  and esterase followed by the addition of **HPE-IAM**. Separately,  $\text{Na}_2\text{S}$  was treated with **HPE-IAM**; this mixture served as a control. LC/MS analysis showed the formation of **HS-HPE-AM** and **Bis-S-HPE-AM** in all the aforementioned reaction mixtures (see ESI,† Fig. S16, S17 and S18). Reaction mixtures containing **1h** or **2h** produced higher levels of **Bis-SS-HPE-AM** and **Bis-SSS-HPE-AM**, which is consistent with HNO's ability to produce persulfide with a higher yield of the latter species in the mixture containing **2h** as compared with **1h**, perhaps due to higher HNO yield from **2h** (see ESI,† Fig. S16C and S19). The increased formation of **Bis-SSS-HPE-AM** with **2h** when compared with **1h** is consistent with **2h**'s higher yield of HNO in 30 min when compared with **1h** (see ESI,† Fig. S16D and S20). When the HNO donor **3b** was used, a similar yield of **Bis-SS-HPE-AM** to **1h** and **2h** was seen but a significantly higher yield of **Bis-SSS-HPE-AM** was seen, suggesting that the rate of HNO production played an important role in polysulfide generation from  $\text{H}_2\text{S}$  (Fig. S21 and S22, ESI†). Hence, HNO donors can be used to enhance the sulfane sulfur pool in the presence of  $\text{H}_2\text{S}$ .

Lastly, we studied the ability of the newly developed donors to permeate cells to generate HNO as well as a fluorescence signal. Imaging data in MCF-7 breast cancer cells showed the formation of HNO by using the HNO-TCF probe (RED channel, Alexa fluor 568, see ESI,† Fig. S23) as well as a fluorescence signal attributable to the formation of **4h** (DAPI channel, see ESI,† Fig. S23, bright field images, see ESI,† Fig. S24). The formation of **4h** was also confirmed through incubation of cell lysates with **1h** or **2h** (see ESI,† Fig. S25). Both compounds were found to be well tolerated up to 25  $\mu\text{M}$  in MCF-7 cells but higher cytotoxicity in MEF and A549 cells was observed (see ESI,† Fig. S26–S28).

In summary, we report a new series of cell-permeable HNO donors that can be cleaved enzymatically to produce HNO along with a fluorophore. These probes, with a well characterized mechanism of HNO generation, enhance polysulfide levels in the presence of  $\text{H}_2\text{S}$ , and can be used to study the biology of sulfur-nitrogen species cross-talk.

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## Conflicts of interest

There are no conflicts to declare.

## Notes and references

- J. P. Marcolongo, M. F. Venâncio, W. R. Rocha, F. Doctorovich and J. A. Olabe, *Inorg. Chem.*, 2019, **58**, 14981–14997.
- J. C. Pieretti, C. V. C. Junho, M. S. Carneiro-Ramos and A. B. Seabra, *Pharmacol. Res.*, 2020, **161**, 105121.
- J. C. Wanstall, T. K. Jeffery, A. Gambino, F. Lovren and C. R. Triggle, *Br. J. Pharmacol.*, 2001, **134**, 463–472.
- M. Tare, R. S. R. Kalidindi, K. J. Bubb, H. C. Parkinson, W.-M. Boon, X. Li, C. G. Sobey, G. R. Drummond, R. H. Ritchie and B. K. Kemp-Harper, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 2017, **390**, 397–408.
- E. Cheong, V. Tumbey, J. Abramson, G. Salama and D. A. Stoyanovsky, *Cell Calcium*, 2005, **37**, 87–96.
- W. D. Gao, C. I. Murray, Y. Tian, X. Zhong, J. F. DuMond, X. Shen, B. A. Stanley, D. B. Foster, D. A. Wink, S. B. King, J. E. Van Eyk and N. Paolocci, *Circ. Res.*, 2012, **111**, 1002–1011.
- A. J. Norris, M. R. Sartippour, M. Lu, T. Park, J. Y. Rao, M. I. Jackson, J. M. Fukuto and M. N. Brooks, *Int. J. Cancer*, 2008, **122**, 1905–1910.
- E. H. Silva Sousa, L. A. Ridnour, F. S. Gouveia, C. D. Silva da Silva, D. A. Wink, L. G. de França Lopes and P. J. Sadler, *ACS Chem. Biol.*, 2016, **11**, 2057–2065.
- J. Zarekiewicz, V. S. Khodade and J. P. Toscano, *J. Org. Chem.*, 2021, **86**, 868–877.
- 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.
- K. G. Fosnacht, M. M. Cerda, E. J. Mullen, H. C. Pigg and M. D. Pluth, *ACS Chem. Biol.*, 2022, **17**, 331–339.
- A. D. Sutton, M. Williamson, H. Weismiller and J. P. Toscano, *Org. Lett.*, 2012, **14**, 472–475.
- Y. Zhou, R. B. Cink, R. S. Dassanayake, A. J. Seed, N. E. Brasch and P. Sampson, *Angew. Chem., Int. Ed.*, 2016, **55**, 13229–13232.
- M. Kawaguchi, T. Tani, R. Hombu, N. Ieda and H. Nakagawa, *Chem. Commun.*, 2018, **54**, 10371–10374.
- R. J. Holland, R. Paulisch, Z. Cao, L. K. Keefer, J. E. Saavedra and S. Donzelli, *Nitric oxide Biol. Chem.*, 2013, **35**, 131–136.
- G. Bharadwaj, P. G. Z. Benini, D. Basudhar, C. N. Ramos-Colon, G. M. Johnson, M. M. Larriua, L. K. Keefer, D. Andrei and K. M. Miranda, *Nitric oxide*, 2014, **42**, 70–78.
- Y. Long, Z. Xia, A. M. Rice and S. B. King, *Molecules*, 2022, **27**, 5305.
- Y. Zheng, B. Yu, K. Ji, Z. Pan, V. Chittavong and B. Wang, *Angew. Chem., Int. Ed.*, 2016, **55**, 4514–4518.
- K. A. Pardeshi, G. Ravikumar and H. Chakrapani, *Org. Lett.*, 2018, **20**, 4–7.
- J. T. M. Correia, G. Piva da Silva, E. André and M. W. Paixão, *Adv. Synth. Catal.*, 2019, **361**, 5558–5564.
- G. J. Mao, X. B. Zhang, X. L. Shi, H. W. Liu, Y. X. Wu, L. Y. Zhou, W. Tan and R. Q. Yu, *Chem. Commun.*, 2014, **50**, 5790–5792.
- M. N. Hughes and R. Cammack, *Methods Enzymol.*, 1999, **301**, 279–287.
- K. Aizawa, H. Nakagawa, K. Matsuo, K. Kawai, N. Ieda, T. Suzuki and N. Miyata, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 2313–2317.
- Q. Guo, Y. Wu, L. Zhang, Y. Qin, J. Bao, Y. Feng, Y. Liu and Y. Zhou, *Sens. Actuators, B*, 2022, **369**, 132309.
- P. C. Smith, A. F. McDonagh and L. Z. Benet, *J. Pharmacol. Exp. Ther.*, 1990, **252**, 218–224.
- Marvin 5.4.0.1, <https://www.chemaxon.com>.
- T. Akaike, T. Ida, F.-Y. Wei, M. Nishida, Y. Kumagai, M. M. Alam, H. Ihara, T. Sawa, T. Matsunaga, S. Kasamatsu, A. Nishimura, M. Morita, K. Tomizawa, A. Nishimura, S. Watanabe, K. Inaba, H. Shima, N. Tanuma, M. Jung, S. Fujii, Y. Watanabe, M. Ohmura, P. Nagy, M. Feelisch, J. M. Fukuto and H. Motohashi, *Nat. Commun.*, 2017, **8**, 1177.