Small Molecule Modulators of Sulfur/Selenium Transfer

A Thesis

submitted to

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by

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Certificate

This is to certify that this dissertation entitled Small Molecule Modulators of Sulfur/Selenium Transfer towards the partial fulfilment of the MS degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Mahima Rana at Indian Institute of Science Education and Research under the supervision of Prof. Harinath Chakrapani, Professor, Department of Chemistry, during the academic year 2023-2024.

C. Harinath

Prof. Harinath Chakrapani

Committee:

Prof. Harinath Chakrapani

Dr. Amrita Hazra

This thesis is dedicated to my parents

Declaration

I hereby declare that the matter embodied in the report entitled "Small Molecule Modulators of Sulfur/Selenium Transfer" are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. Harinath Chakrapani and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Mahima Rana
20212011

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The completion of this project would not have been possible without the continuous and sincere efforts of many individuals and organizations. I am deeply grateful to all who contributed.

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Abstract

Hydrogen Sulfide (H_2S) and its congener Hydrogen Selenide (H_2Se) exhibit high toxicity, but are indispensable for maintaining sulfur and selenium homeostasis respectively. Both being a part of the chalcogen family, they share close chemical and biochemical similarities; however, they exhibit significant differences. Our study provides an overview to understand the biochemical variances between these two reactive species by modulating sulfur/selenium transfer within the biological milieu through the development of small molecules. We developed an inhibitor **7** targeting a key enzyme responsible for H_2S production, 3-Mercaptopyruvate sulfurtransferase (3-MST). Additionally, we designed a self-immolating selenourea donor **8** equipped with a fluorophore reporter which can be utilised to develop a direct H_2Se donor **10.** The Prospect of our research extend beyond providing insights into the intricate sulfur and selenium transfer processes *in cellulo*; it also enables us to modulate their activities.

Chapter 1 **Introduction**

1.1 Background

Cell function is governed by numerous signaling molecules. Among them, lie the "gasotransmitter" or "gaseous signaling molecules", which can freely diffuse through cell membranes triggering diverse responses independent of transporters and membrane receptors. Currently, only nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) are accepted as gasotransmitter.[1] However, recent evidences also postulate the inclusion of hydrogen selenide (H_2Se) as the fourth endogenous gasotransmitter.[2]

Of these small molecules, H_2S and H_2Se both belong to the chalcogen family with selenium being located below sulfur on the periodic table. As a result, they possess close similarities but also major differences in terms of their biochemistry and chemistry. Both the species are enzymatically produced, play a significant role in normal physiology and pathophysiology and exhibit high reactivity and toxicity at higher doses.[2] However, selenium is much more polarizable than sulfur and has a significantly lower pKa value. Our study focuses on expanding the understanding of these biochemical differences between sulfur and selenium species by modulating sulfur/selenium transfer in the biological milieu.

1.2 Hydrogen sulfide in Biochemistry

H_2S is implicated in various processes within mammalian physiology, including vasodilation, regulation of inflammation and control of intracellular redox homeostasis. Additionally, H_2S produced by several bacteria contributes to antibiotic resistance by alleviating the oxidative stress inflicted by antibiotics.[3]

Till now, three major enzymes involved in producing H_2S have been characterized in mammals: 3-mercaptopyruvate sulfurtransferase (3-MST), cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). CSE and CBS generate H_2S predominantly from *L*-cysteine, and 3-MST does so by utilizing 3-Mercaptopyruvate as a substrate.[4] The schematic representation of these H_2S-producing pathways is depicted in figure 1.[5]

In order to understand the redox biology of H_2S and its physiological functions, it is essential to develop inhibitors that can regulate the functions of the aforementioned three enzymes.[3] Since *E. coli* possesses the 3-MST enzyme but lacks CBS/CSE, development of a selective inhibitor targeting 3-MST enzyme, which can be used as antibiotic adjuvant, would be highly

beneficial for studying H_2S, persulfides/polysulfides, and for countering antibacterial resistance.

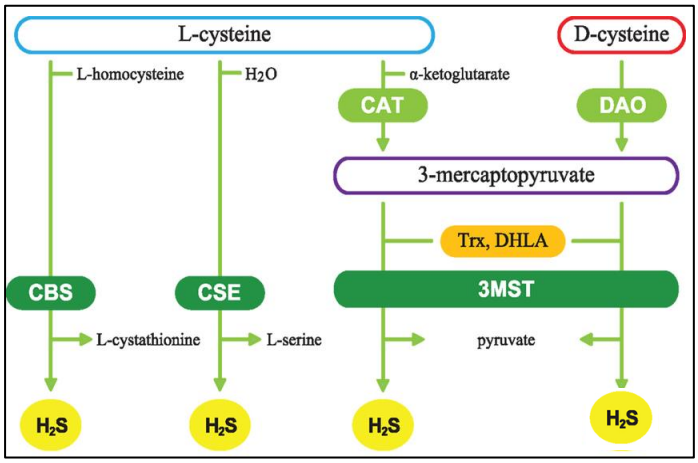
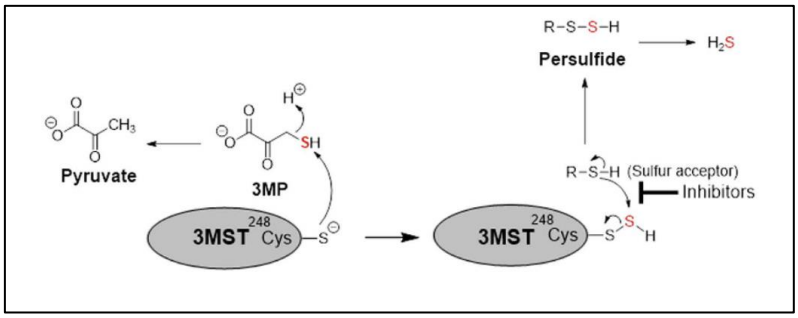


Figure 1: H_2S biosynthesis pathways.[4]

Motivation and Design of the work:

In 2017, Tetsuo Nagano and co-workers reported selective inhibitors of mouse 3-MST through a High throughput screen (HTS) of a large compound library.[3] Three of the four identified inhibitors contain a *β*-keto thioether moiety, with their target site revealed to be a persulfidated cysteine residue situated in the active loop of 3-MST. The turnover mechanism by 3-MST has been depicted in scheme 1.



Scheme 1: Enzymatic reaction mechanism of 3-MST enzyme.[3]

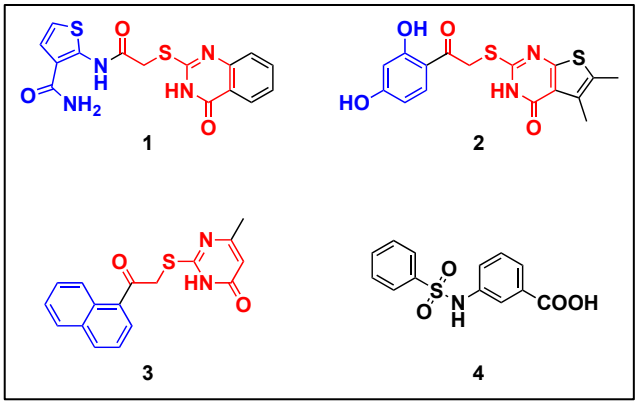


Figure 2: Structures of the four potent inhibitors for mouse 3-MST.[3]

Based on their findings, our group designed inhibitors for the bacterial 3-MST enzyme with the β-keto thioether moiety, and successfully identified moderate reversible inhibitors for the enzyme. In order to improve potency and/or selectivity of the inhibitors, we are investigating conformational restriction as one of the approaches in our study. This involves introducing structural limitations in the lead molecule to reduce total possible conformations and promoting the adoption of the bioactive conformation, thus, improving the recognition of the inhibitor by the target enzyme (Figure 3).[6]

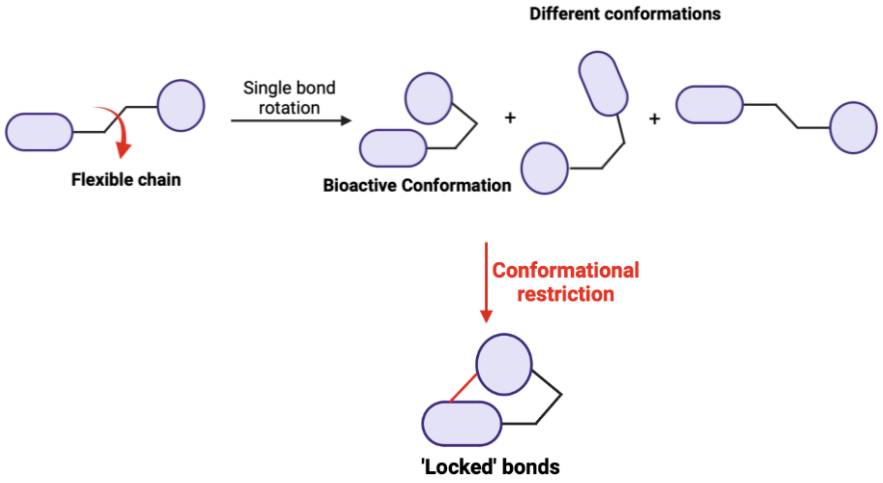


Figure 3: Rigidification or conformational restriction of the lead molecule to improve efficacy.

Further, since the enzyme contains a highly reactive cysteine residue in the catalytic loop, we considered the possibility of developing irreversible inhibitors that can form a covalent bond with the cysteine residue to become irreversibly bound to the active site. Thereby, preventing the enzyme from catalysing its enzymatic reaction (Figure 4).[7] The potential applications of our study extends beyond merely neutralizing bacterial defence mechanisms against antibiotics, it could also offers insights into the intricate processes of sulfur transfer within the biological environment.

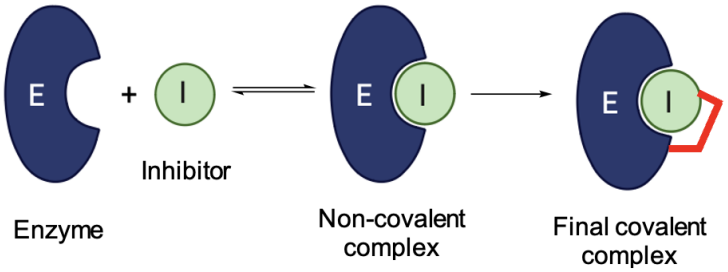
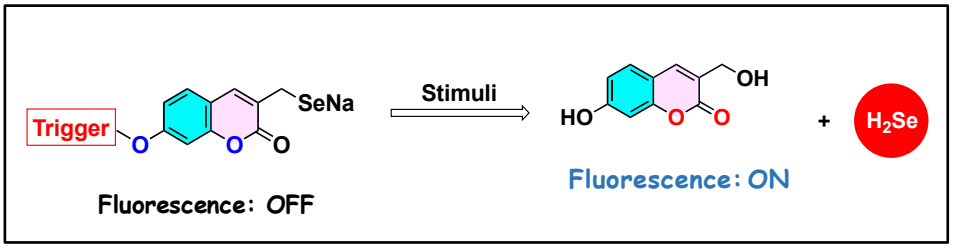


Figure 4: Mechanism of binding of a covalent inhibitor to the target enzyme.

1.3 Hydrogen selenide in Biochemistry

Similar to the early stages of contemporary research on biological H_2S, originally recognised as a toxin but later acknowledged for its vital role in various mammalian and bacterial processes, there is a growing interest in investigating the chemical biology of its counterpart H_2Se.[8] Parallel to its lighter congener H_2S, H_2Se exhibits high reactivity and toxicity at doses above 400 μg/L, but it is indispensable for maintaining selenium homeostasis, thyroid function and proper operation of intrinsic antioxidant systems at lower concentrations.[9] Recent evidences indicate the crucial role of H_2Se as a regulator of endogenous redox by directly detoxifying reactive oxygen species (ROS). Reports also highlight how H_2Se may induce protein modifications (protein–SSeH) that impact cellular functions, even at considerably lower concentrations when compared to its congener H_2S.[10] Despite the significance of H_2Se and its related reactive selenium species (RSeS), only a few chemical tools are available that enable the direct transportation of these species into biological settings.[8]

Our study focuses on the development of an enzyme-activated H_2Se donor based on the ester motif to enable direct delivery of H_2Se and to broaden our understanding of the biochemical distinctions and similarities between reactive sulfur and selenium species. Further, to bridge the gap in the lack of suitable methods to detect H_2Se, we have designed a self-immolating H_2Se donor with a fluorophore reporter. Our study will not only expand the repertoire of tools for H_2Se delivery but also pave the way for future explorations into directly detecting H_2Se in various biological scenarios.



Scheme 2: Stimuli responsive hydrogen selenide donor with fluorescence reporter

Chapter 2 **Materials and Methods**

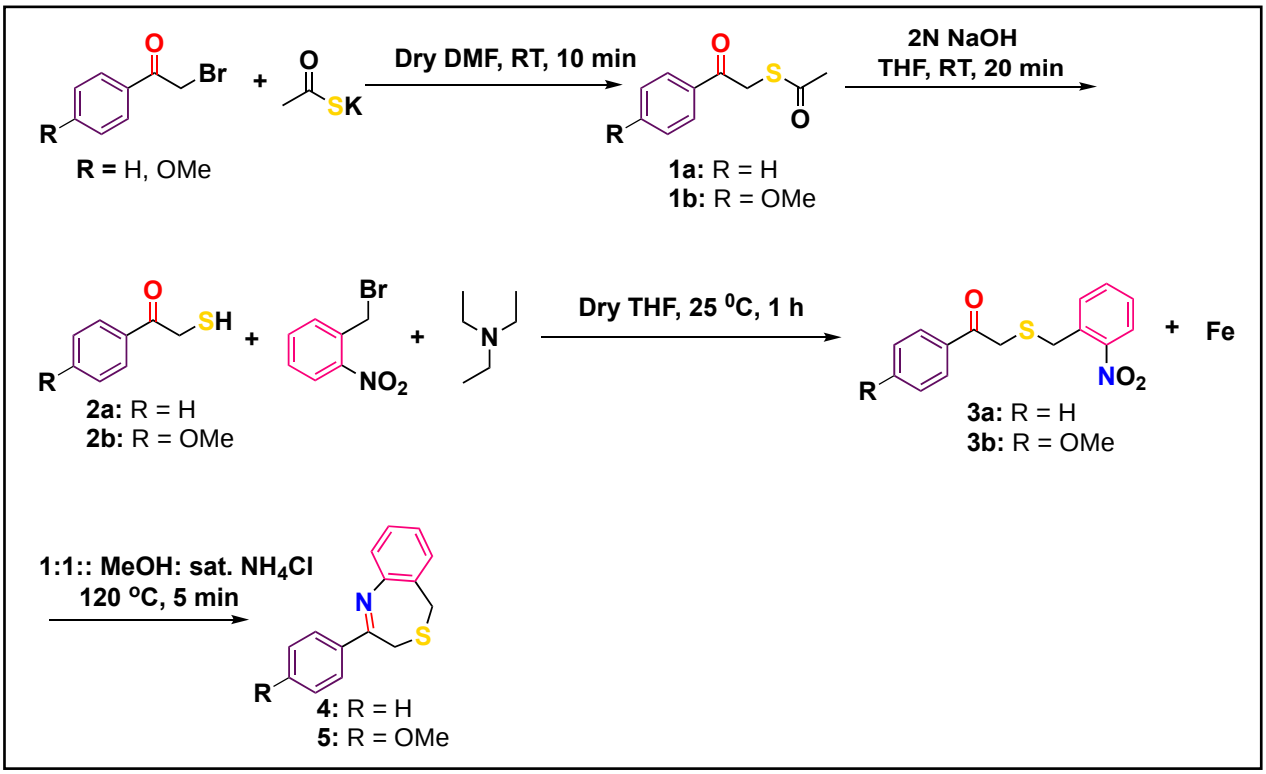
2.1 General methods

Unless otherwise specified, all reagents, starting materials, and dry solvents were procured from commercial suppliers and utilized without further purification. All reactions were conducted under a nitrogen atmosphere. Column chromatography employed Spectrochem (100–200 mesh) silica gel as the stationary phase.

^{1}H and ^{13}C spectra were acquired on either a BRUKER 400 MHz (or 100 MHz for ^{13}C) or JEOL 400 MHz (or 100 MHz for ^{13}C) spectrometer, referencing tetramethylsilane (δ_H = 0.00 ppm, δ_C = 0.0 ppm) as the internal standard or residual solvent signals ($CDCl_3$, δ_H= 7.26 ppm, δC = 77.2 ppm) as references. Chemical shifts (δ) are denoted in parts per million (ppm), and coupling constants (*J*) in Hz. Abbreviations such as: s (singlet), t (triplet) and m (multiplet) are utilized. High-resolution mass spectra (HRMS) were obtained from HRMS-ESI-Q-Time of Flight LC/MS. Infrared Spectra (IR) were recorded using a BRUKER-ALPHA FT-IR spectrometer. Fluorescence and other spectrophotometric measurements were conducted using either a Thermo Scientific Varioscan microplate reader or EnSight Multimode Plate Reader under the Perkin Elmer-IISER Pune, Centre of Excellence facility.

2.2 Synthesis and characterization

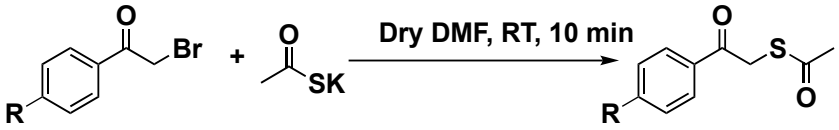
*2.2.1 General Scheme for Synthesis of compound **4** and **5***



Scheme 3: Synthetic scheme for compounds **4** and 5

Compounds **1a**, **1b**[11] and **2a**, **2b**[12] were synthesized using reported protocol.

General protocol for synthesis of ethanethioate derivatives (1a and **1b):**[11]



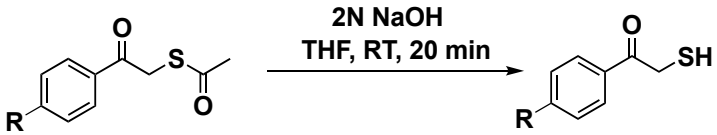
1a: R = H
1b: R = OMe

The synthesis of the corresponding ethanethioates was achieved from the derivatized phenacyl bromides. Phenacyl bromide (1 equivalent) was dissolved in *N*,*N*-Dimethylformamide (DMF, 10 mL) and stirred, followed by the addition of potassium thioacetate (KSAc) (573.75 mg, 5.02 mmol). The reaction mixture was stirred at room temperature under N_2 atmosphere, and the progress of the reaction was monitored by TLC (10-15 min). Upon completion, the solvent was evaporated in vacuo, diluted with 10 mL of water and extracted with ethyl acetate (2×10 mL). Afterward, the combined organic layers were washed with brine solution (10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product. Further purification was accomplished through column chromatography using 100-200 mesh silica gel.

***S*-(2-oxo-2-phenylethyl) ethanethioate (1a):** Utilising the above outlined synthetic protocol, 2-bromo-1-phenylethan-1-one (500 mg, 2.51 mmol) served as the starting material. A gradient ranging from 2% ethyl acetate/hexane was employed as the mobile phase, and the target product was collected with 5-6% as the eluent. Compound **1a** was isolated as a brown-yellow liquid (272mg, 56% yield). The analytical data is consistent with previously reported values.

***S*-(2-(4-methoxyphenyl)-2-oxoethyl) ethanethioate (1b):** Utilising the above outlined synthetic protocol, 2-bromo-1-(4-methoxyphenyl) ethan-1-one (500 mg, 2.18 mmol) served as the starting material. A gradient ranging from 5% ethyl acetate/hexane was employed as the mobile phase, and the target product was collected with 8-9% as the eluent. Compound **2a** was isolated as a brown-yellow liquid (311 mg, 64% yield). The analytical data is consistent with previously reported values.

General protocol for synthesis of 2-mercapto-ethan-1-one derivatives (2a and **2b):**[12]

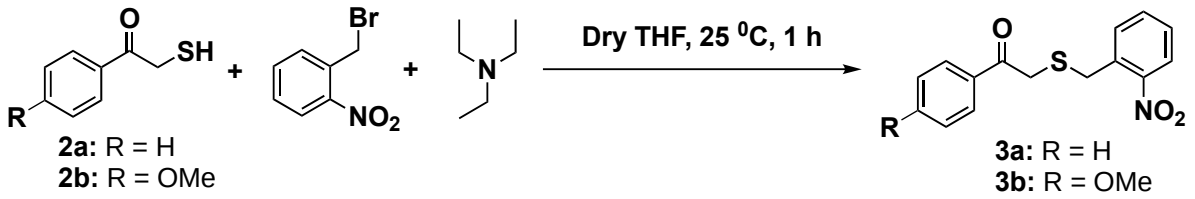


1a: R = H
1b: R = OMe

2a: R = H
2b: R = OMe

A solution of ethanethioate **1a** or **1b** (150 mg, 772.21 mmol) in tetrahydrofuran (THF, 4 mL) was treated with 2M aqueous solution of NaOH (4 mL), and the resulting biphasic mixture was stirred vigorously at 25 ^{o}C. The reaction progress was monitored for completion using TLC (20–25 min), following which the solvent was evaporated in vacuo, acidified with 1M HCl and then extracted with ethyl acetate (3×10 mL). Afterward, the combined organic layers were washed with brine solution (10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product, which was adequately pure and directly utilized for the subsequent step without requiring further purification.

General protocol for synthesis of (2-nitrobenzyl)thioethan-1-one derivatives (3a and **3b):**



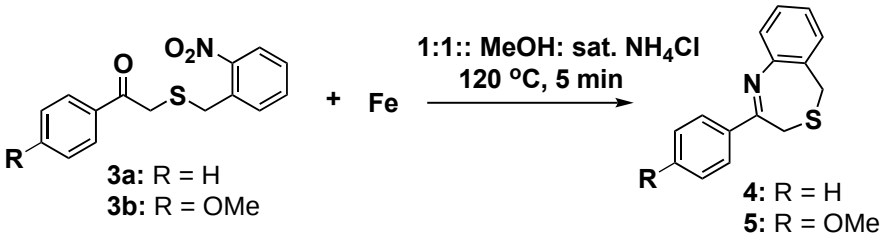
Compounds **3a** and **3b** were synthesized using modified literature procedure.[12] Briefly, 2-mercapto-ethan-1-one **2a** or **2b** (1 eq) was dissolved in tetrahydrofuran (THF, 5 mL) under N_2 atmosphere. Subsequently, 2-nitrobenzyl bromide (1 eq) was added at room temperature. NEt_3 (2.5 equivalents) was then introduced to the stirring solution. The reaction mixture was left to stir for 1 h, with the progress monitored by TLC. Upon completion, the solvent was evaporated in vacuo, diluted with 10 mL of water and extracted with $CHCl_3$ (3×10 mL). The combined organic layers were washed with brine solution (10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product. Further purification was accomplished through column chromatography using 100-200 mesh silica gel.

2-((2-nitrobenzyl)thio)-1-phenylethan-1-one (3a): Utilising the above outlined synthetic protocol, 2-mercapto-1-phenylethan-1-one (117 mg, 772.22 μmol) served as the starting material. A gradient ranging from 1% ethyl acetate/hexane was employed as the mobile phase and the target product was collected with 5-6% as the eluent. Compound **3a** was isolated as a light-yellow sticky solid (161mg, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (d, 1H, *J* = 8 Hz), 7.94-7.91 (m, 2H), 7.60-7.55 (m, 3H), 7.48-7.40 (m, 3H), 4.11 (s, 2H), 3.66 (s, 2H)

1-(4-methoxyphenyl)-2-((2-nitrobenzyl)thio)ethan-1-one (3b): Utilising the above outlined synthetic protocol, 2-mercapto-1-(4-methoxyphenyl)ethan-1-one (121 mg, 663.97 μmol) served as the starting material. A gradient ranging from 5% ethyl acetate/hexane was employed as the mobile phase and the target product was collected with 8-15% as the eluent. Compound

3b was isolated as a light-yellow sticky solid (144 mg, 69% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (d, 1H, *J* = 8 Hz), 7.91 (d, 2H, *J* = 8 Hz), 7.57-7.55 (m, 2H), 7.44-7.40 (m, 1H), 6.93 (d, 2H, *J* = 8 Hz) 4.12 (s, 2H), 3.87 (s, 3H), 3.62 (s, 2H)

Procedure for synthesis of 1,4-thiazepine derivatives (4 and **5):**



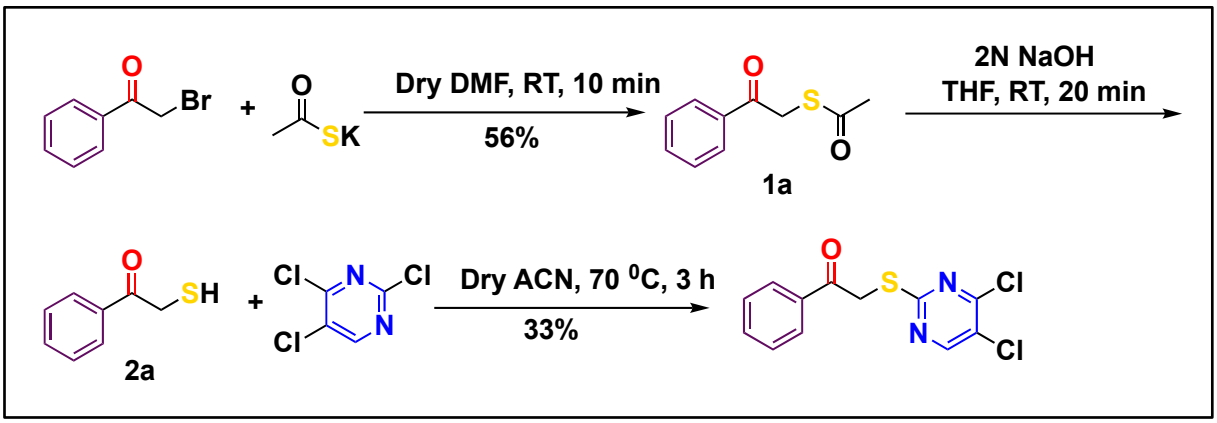
Compounds **4** and **5** were synthesized using modified literature procedure.[13] Nitrobenzene derivative **3a** or **3b** (100 mg, 348.03 μmol) and iron powder (116.61 mg, 2.09 mmol) were added to a sealed tube containing 1:1 solution of MeOH (2 mL) and saturated NH_4Cl (2 mL). Reaction was left to stir for 5 min at a temperature of 120 ^{0}C. Once the reaction time was complete, the resulting residue was dissolved in ethyl acetate (EtOAc) and filtered through celite before extracting it with $CHCl_3$ (3×10 mL). Afterward, the combined organic layers were washed with brine solution (10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product. Further purification was accomplished through column chromatography using 100-200 mesh silica gel.

2-phenyl-3,5-dihydrobenzo[*e*][1,4]thiazepine (4): Utilising the above outlined synthetic protocol, 2-((2-nitrobenzyl)thio)-1-phenylethan-1-one (100 mg, 348.03 μmol) served as the starting material. A gradient ranging from 0.5% ethyl acetate/hexane was employed as the mobile phase and the target product was collected with 1-2% as the eluent. Compound **4** was isolated as a dark-yellow sticky solid (21 mg, 17% yield). FT-IR (υ_{max}, cm^{-1}): 1593; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05-8.03 (m, 2H), 7.53-7.46 (m, 3H), 7.37-7.3 (m, 1H), 7.26-7.24 (m, 1H), 7.15-7.11 (m, 1H), 7.05 (d, 1H, *J* = 8 Hz), 3.53 (s, 2H), 3.42 (s, 2H); HRMS for $C_{15}H_{13}NS$ $[M+H]^+$ Calculated: 240.0849, Found: 240.0847.

2-(4-methoxyphenyl)-3,5-dihydrobenzo[*e*][1,4]thiazepine (5): Utilising the above outlined synthetic protocol, 1-(4-methoxyphenyl)-2-((2-nitrobenzyl)thio)ethan-1-one (100 mg, 315.1 μmol) served as the starting material. A gradient ranging from 2% ethyl acetate/hexane was employed as the mobile phase and the target product was collected with 5% as the eluent. Compound **4** was isolated as a dark-yellow sticky solid (61 mg, 67% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (d, 2H, 8 Hz), 7.35-7.22 (m, 2H), 7.13-7.09 (m, 1H), 7.04-6.97 (m, 3H),

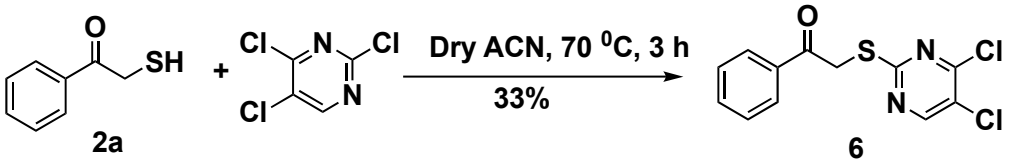
3.88 (s, 3H), 3.52 (s, 2H), 3.40 (s, 2H); HRMS for $C_{15}H_{13}NS$ $[M+H]^+$ Calculated: 270.0952, Found: 270.0952

*2.2.2 General Scheme for Synthesis of compound **6***



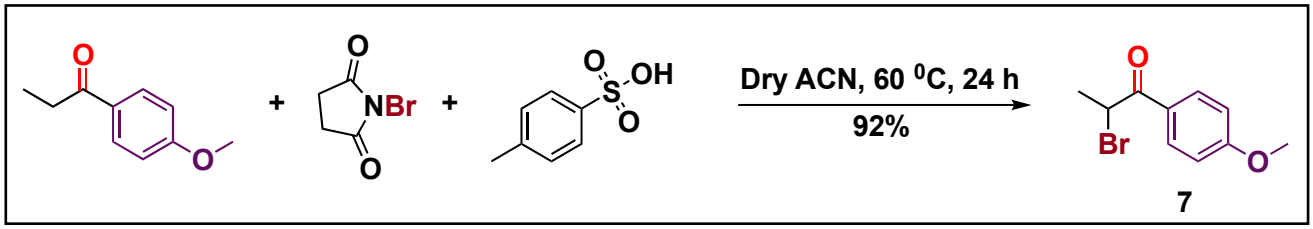
Scheme 4: Synthetic scheme for compound **6**

Procedure for synthesis of 2 2-((4,5-dichloropyrimidin-2-yl)thio)-1-phenylethan-1-one (5)



Compound **6** was synthesized using modified literature procedure.[14] To a stirred suspension of 2-mercaptoactophenone **2a** (117mg, 768.67 µmol) in CH_3CN (7 mL) were sequentially added 2,4,5-Trichloropyrimidine (88.1 µL, 768.67 µmol) and Na_2CO_3 (244.42 mg, 2.31 mmol) at 25 ^{0}C. The reaction mixture was refluxed at a temperature of 70 ^{0}C for 3 h. Once the reaction was complete, the solvent was evaporated *in vacuo*, diluted with 10 mL of water and extracted with EtOAc (3×10 mL). Afterward, the combined organic layers were washed with brine solution (10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product. Further purification was accomplished through column chromatography using 100-200 mesh silica gel (PE/EtOAc = 20/1) to obtain **5** (76 mg, 33% yield) as a white solid. FT-IR (υ_{max}, cm^{-1}): 1592, 1688; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.25 (s, 1H), 8.08-8.06 (m, 2H), 7.66-7.63 (m, 1H), 7.55-7.52 (m, 2H), 4.71 (s, 2H); HRMS for $C_{12}H_8Cl_2N_2OS$ $[M+H]^+$ Calculated: 298.9814, Found: 298.9814.

2.2.3 *General Scheme for Synthesis of compound 7*



Scheme 5: Synthetic scheme for compound **7**

Procedure for synthesis of 2-bromo-1-(4-methoxyphenyl)propan-1-one (7)

To a solution of *p*-methoxypropiophenone (53.36 µL, 304.5 µmol) in CH_3CN was added *N*-bromosuccinimide (NBS) (74.79 mg, 420.21 µmol) and *p*-toluenesulfonic acid (PTSA) (7.53 mg, 39.58 µmol) at once. The reaction mixture was stirred at 60 ^{0}C under N_2 atmosphere for 24 h and the progress of the reaction was monitored by TLC. Upon completion, the solvent was evaporated in vacuo, diluted with 10 mL of water and extracted with EtOAc (3×10 mL). Afterward, the combined organic layers were washed with brine solution (10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude product. Further purification was accomplished through column chromatography using 100-200 mesh silica gel (PE/EtOAc = 20/1) to obtain **6** (68 mg, 92% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03-8.00 (m, 2H), 6.97-6.95 (m, 2H), 5.26 (q, 1H, *J* = 8 Hz), 3.89 (s, 3H), 3.76 (s, 2H), 1.89 (d, 3H, *J* = 8 Hz); HRMS for $C_{10}H_{11}BrO_2$ $[M+K]^+$ Calculated: 243.0022, Found: 243.0020.

2.3 Lead Acetate assay for qualitative detection of H_2S:[15,16]

20 mM stocks of the synthesized covalent inhibitors (**4**, **5**, **6,** and **7)**, ethyl 3-bromopyruvate (EBP, 20 mM) and pro-substrate *i.e.* ethyl 3-mercaptopyruvate (mimic of the natural substrate 3-Mercaptopyruvate, 5mM) were prepared in DMSO. Dithiothreitol (DTT, 10 mM) was prepared in 10 mM PBS buffer (pH 7.4).

The reaction mixtures were prepared through subsequent addition of 1 µM of *E. coli* 3-MST (10 µL, 104 µM stock), 200 µM of the inhibitors (10 µL, 20 mM stock) or EBP (10 µL, 20 mM stock) and the volume was adjusted to 1 mL using 10 mM PBS pH 7.4 buffer in 1.5 mL mini centrifuge tubes. These mixtures were then incubated for 3 h at 37°C in a shaker incubator (300 rpm) before adding 50 µM of proS (10 µL, 5 mM stock). A second incubation of 1 h at 37 ^{0}C in a shaker incubator (300 rpm) was followed by the addition of 1 mM DTT (100 µL, 10 mM stock). A 200 µL portion of each sample was dispensed into a 96-well plate and sealed with a

filter paper saturated in lead acetate solution (10% w/v). Subsequently, the plate was placed in a static incubator set to a temperature of 37°C.

In a second group, 1 μM of *E. coli* 3-MST (10 μL, 104 μM) was pre-incubated with the inhibitor **7** at various concentrations (10 μM, 25 μM, 50 μM, 100 μM and 200 μM) for 3 h at 37 ^{0}C in a shaker incubator (300 rpm), followed by the addition of 50 μM of ProS (10 μL, 5 mM). 1 mM DTT (100 μL, 10 mM stock) was added after a second incubation for 1 h at 37 ^{0}C in a shaker incubator (300 rpm). A 200 μL portion of each sample was dispensed into a 96-well plate and sealed with a filter paper saturated in lead acetate solution (10% w/v). Subsequently, the plate was placed in a static incubator set to a temperature of 37°C.

2.4 Persulfide/polysulfide measurement using SSP-2:[17]

Stock solutions of inhibitors (**4, 5, 6, 7,** 10 mM), EBP (10 mM), ProS (5 mM) and SSP-2 (5 mM) were made in DMSO. SSP-2 probe has been synthesized and purified in our lab by Dr. Kavita Sharma.

The reaction mixtures were prepared by sequentially adding 1 μM of *E. coli* 3-MST (4 μL, 104 μM stock), 100 μM of the test compounds (4 μL, 10 mM stock) or EBP (4 μL, 10 mM stock) and the volume was adjusted to 1 mL using 10 mM PBS pH 7.4 buffer in 1.5 mL mini centrifuge tubes. These mixtures were then incubated for 3 h at 37 ^{0}C in a shaker incubator (300 rpm) before adding 50 μM of ProS (4 μL, 5 mM stock). Further incubation of 1 h at 37°C in a shaker incubator (300 rpm) was followed by the addition of 50 mM SSP-2 (4 μL, sourced from a 5 mM stock) and another 10-min incubation in the dark. A 100 μL aliquot of each sample was then transferred to a 96-well plate, and the fluorescence was measured (λex = 482 nm, λem = 518 nm) using a microplate reader (PerkinElmer EnSight multimode plate reader).

A similar procedure was employed for the pro-substrate control reaction. In brief, the reaction sample was prepared by adding 1 μM of *E. coli* 3-MST (4 μL, 104 μM stock) in a total reaction volume of 400 μL adjusted using 10 mM PBS pH 7.4 buffer. The reaction mixture was then incubated for 3 h at 37°C in a shaker incubator (300 rpm) before adding 50 μM of ProS (4 μL, 5 mM stock). Following a subsequent 1-hour incubation at 37°C and 300 rpm, 50 mM SSP-2 (4 μL, 5 mM stock) was added, and the mixture was incubated for an additional 10 min in the dark. A 100-μL aliquot of each sample was transferred to a 96-well plate, and the fluorescence was measured using a microplate reader (PerkinElmer EnSight multimode plate reader) with excitation and emission wavelengths set at 482 nm and 518 nm, respectively.

2.5 Methylene blue assay for quantitative detection of H_2S:[18–20]

The methylene blue assays were performed utilising previously reported methods with few adjustments.[19,20,21] 30 mM stocks of the inhibitors (**4**, **5**, **6,** and **7**), EBP (30 mM) and ProS (15mM) were prepared in DMSO. DTT (10 mM) and $Zn(OAc)_2.2H_2O$ (40 mM) was prepared in deionized water. Stock solution of $FeCl_3$ (30 mM) was prepared in 1.2 M HCl and *N*,*N*–dimethyl-*p*-phenylenediamine sulfate (DPDHS) (20 mM) was prepared in 7.2 M HCl.

The reaction samples were prepared through sequent addition of 400 mM $Zn(OAc)_2.2H_2O$ (10 µL, 40 mM stock), 1 µM of *E. coli* 3-MST (10 µL, 104 µM stock), 300 µM of the inhibitors (10 µL, 30 mM stock) or EBP (10 µL, 30 mM stock) and the volume was adjusted to 1 mL using 30 mM HEPES-NaOH pH 7.4 buffer in 1.5 mL mini centrifuge tubes. These mixtures were then incubated for 3 h at 37 ^{0}C in a shaker incubator (300 rpm) before adding 150 µM of ProS (10 µL, 15 mM stock). Following a subsequent 1-hour incubation at 37°C in a shaker incubator (300 rpm), 1 mM DTT (100 µL, sourced from a 10 mM stock) was added, and the reaction was placed in a static incubator set to a temperature of 37°C.

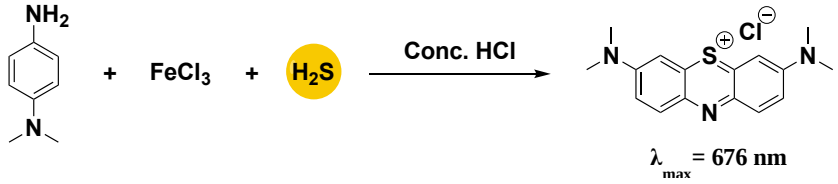
A similar procedure was employed for the pro-substrate control reaction. In Brief, the reaction sample was prepared by adding 400 µM $Zn(OAc)_2.2H_2O$ (10 µL, 40 mM stock), 10 µL DMSO, 1 µM of *E. coli* 3-MST (10 µL, 104 µM stock) in a total reaction volume of 1.0 mL, adjusted with 30 mM HEPES-NaOH pH 7.4 buffer. The reaction mixture was then incubated in a shaker incubator (300 rpm) for 3 h at 37°C before adding 150 µM of ProS (10 µL, 15 mM stock). Subsequently, the samples were incubated for an additional 1 hour at 37°C in a shaker incubator (300 rpm), followed by the addition of 1 mM DTT (100 µL, sourced from a 10 mM stock), and then placed in a static incubator set to a temperature of 37°C.

At specified intervals, equal volumes (200 µL) of DPDHS, $FeCl_3$, and aliquots from the aforementioned reaction samples were combined and incubated at 37°C for 30 minutes in the dark to facilitate the formation of the methylene blue dye. 150 µL of these samples were then transferred to a 96-well plate, and the absorbance values were measured at 676 nm using a microplate reader (Thermo Scientific Varioskan Microplate Reader).

Chapter 3 **Results and Discussion**

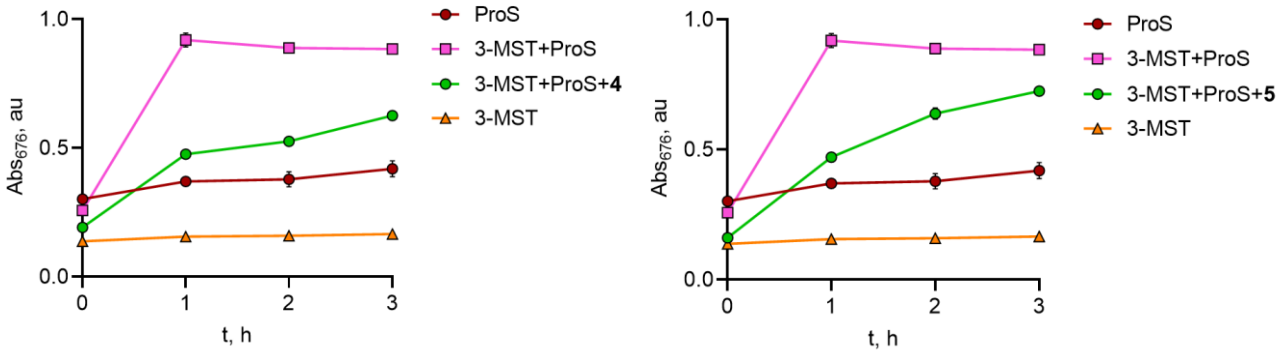
3.1 Methylene blue assay for quantitative H_2S detection

In vitro methylene blue assay is used to quantitively measure H_2S concentration in solution. The reaction produces a dye called methylene blue that has absorbance maxima at 676 nm and 750 nm, attributed respectively to the MB cation (MB^+) and the protonated di-cation (MBH^{2+}).[13-14]



Scheme 4: Methylene blue complex formation

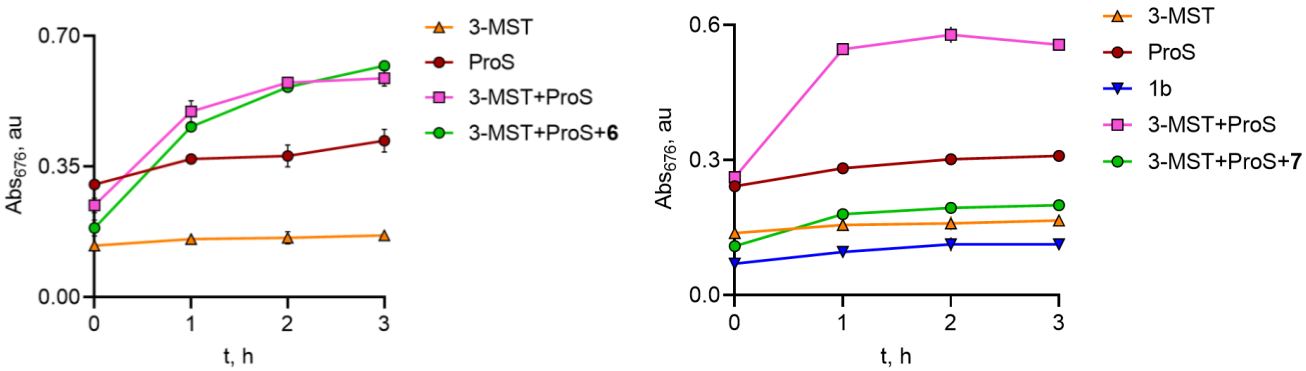
Figure 3.1.1: ***Methylene blue assay of conformationally restricted inhibitors 4 and 5 with E. coli 3-MST enzyme in the presence of pro-substrate showing a slight inhibition in H_2S production.***



Assay conditions: 30 mM HEPES NaOH buffer, $Zn(OAc)_2$ = 400 µM. 2% DMSO, *E. coli* 3-MST = 1 µM, Inhibitor = 300 µM. Incubate for 3 h, 37 ºC. ProS = 150 µM. 1h incubation at 37 ºC. DTT = 1 mM

The above studies show a slight decrease in H_2S production with the conformationally restricted compounds **4** and **5**. However, we did not see a significant improvement in the inhibitory potency of these compounds when compared to their conformationally unrestricted counterparts previously synthesised by our lab, indicating that conformation of the thioether moiety has little or no role to play in binding to the active site of the enzyme. Therefore, we proceeded to synthesise covalent inhibitors for the enzyme and evaluated them for their inhibitory potency using this assy.

Figure 3.1.2: ***Methylene blue assay of covalent inhibitors 6 and 7 with E. coli 3-MST enzyme in the presence of pro-substrate showing a diminished band corresponding to treatment of E. coli 3-MST with 7.***



Assay conditions: 30 mM HEPES NaOH buffer, $Zn(OAc)_2$ = 400 µM. 2% DMSO, *E. coli* 3-MST = 1 µM, Inhibitor = 300 µM. Incubate for 3 h, 37 °C. ProS = 150 µM. 1h incubation at 37 °C. DTT = 1 mM

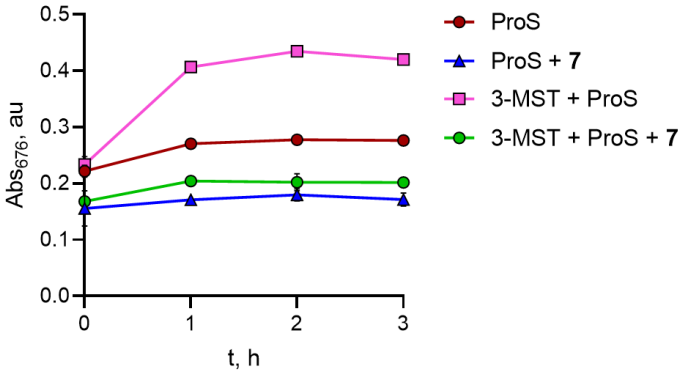
There is a significant decrease in the H_2S signal upon pre-treatment of *E. coli* 3-MST enzyme with compound **7**; however, with compound **6,** no inhibition in H_2S signal is observed.

Based on the above experiments, compound **7** appears to be a potent inhibitor for *E. coli* 3-MST enzyme. However, a peculiar observation in the methylene blue assay is the further decrease in the H_2S signal on treatment of 3-MST with compound **7** (Figure 3.3.1, Graph-2, green circle) when compared to the ProS alone signal (Figure 3.3.1, Graph-2, brown circle). E3-MP released from ProS in pH 7.4 buffer is capable of dimerising to form a disulfide bond which can be broken by DTT, resulting in the release of H_2S and thereby giving a background absorbance signal at 0.2 – 0.3 (Graph-2, brown circle).

A possible explanation for this further decrease in H_2S signal beyond the ProS background could be the reaction of compound **7** with ProS itself and hence rendering the ProS unavailable to be turned over by the enzyme.

To test this hypothesis, a control experiment was designed where inhibitor **7** was treated with ProS with and without the enzyme.

Figure 3.1.3: ***Methylene blue assay of inhibitor 7 with and without E. coli 3-MST enzyme in the presence of ProS to check for direct reaction of compound 7 with ProS.***

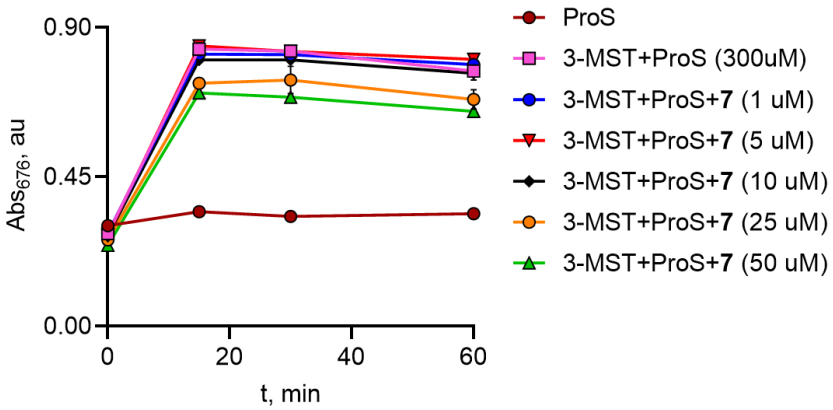


Assay conditions: 30 mM HEPES NaOH buffer, $Zn(OAc)_2$ = 400 µM. 2% DMSO, *E. coli* 3-MST = 1 µM, Inhibitor = 300 µM. Incubate for 3 h, 37 ºC. ProS = 150 µM. 1h incubation at 37 ºC. DTT = 1 mM

From the control experiment designed above, it can be seen that quenching in the absorbance signal of ProS (black square) when treated with compound **7** (blue triangle) takes place even without the enzyme, indicating the ability of compound **7** to directly react with ProS.

Hence, for further experiments lower concentrations of compound **7** were chosen to avoid this side reaction and to test for its inhibitory potency against *E. coli* 3-MST enzyme at those concentrations.

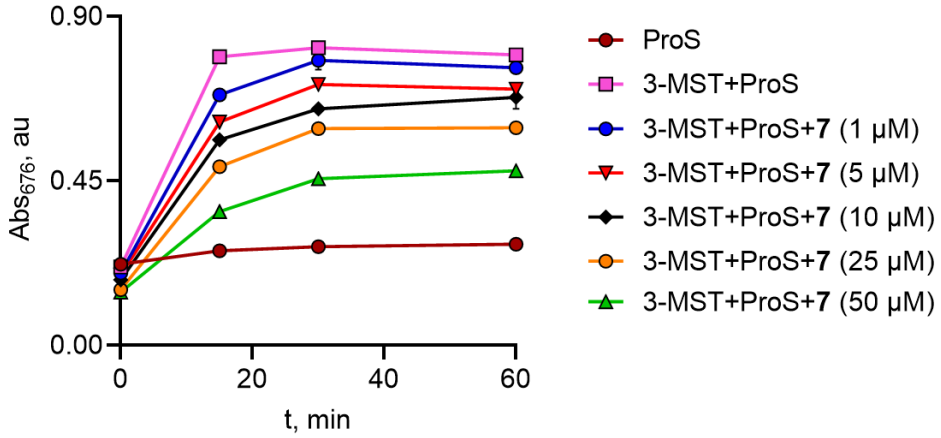
Figure 3.1.4: ***Methylene blue assay of inhibitor 7 (in a dose dependent manner) upon co-incubation with E. coli 3-MST enzyme and ProS for detection of H_2S***



Assay Conditions: HEPES buffer (pH= 7.4), **7** (varying concentration), $Zn(OAc)_2$ (400 µM), *E. coli* 3-MST (1 µM), 2% DMSO, ProS (150 µM), 37 ^{o}C, 30 min. DTT (1 mM), aliquot into Methylene Blue cocktail)

The above studies do not show any significant inhibition in H_2S signal for co-incubation of *E. coli* 3-MST enzyme with inhibitor **7** at lower concentrations as compared to only ProS. Inhibitor **7** was then subjected to preincubation with *E. coli* 3-MST enzyme and evaluated for its inhibitory potency.

Figure 3.1.5: ***Methylene blue assay of inhibitor 7 (in a dose dependent manner) upon pre-incubation with E. coli 3-MST enzyme for detection of H_2S***

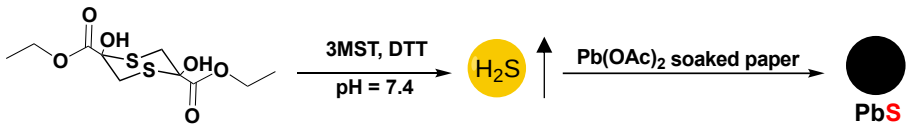


Assay Conditions: HEPES buffer (pH= 7.4), **7** (varying concentration), $Zn(OAc)_2$ (400 µM), *E. coli* 3-MST (1 µM), 2% DMSO, 37 °C, 1 h. ProS (150 µM), 37 °C, 30 min. DTT (1 mM), aliquot into Methylene Blue cocktail)

Upon pre-incubation of *E. coli* 3-MST enzyme with **7** at 25 µM and 50 1µM, a significant decrease in H_2S production can be seen. Based on the above experiments, compound **7** appears to be a potent inhibitor for *E. coli* 3-MST enzyme at these concentrations. The compound **7** was then evaluated for its ability to inhibit the enzyme using other biochemical assays.

3.2 H_2S detection using lead acetate assay

Lead acetate method is a standard *in vitro* assay which provides a qualitative measure of H_2S produced in a solution. A dark black or brown stain appears on the lead acetate paper as a result of the reaction between evolved H_2S and lead acetate. [18, 19]



ProS (dimer of ethyl 3-mercaptopyruvate), which dissociates in pH 7.4 buffer to give Ethyl 3-mercaptopyruvate (E3-MP), is a mimic of the natural substrate 3-mercaptopyruvate.

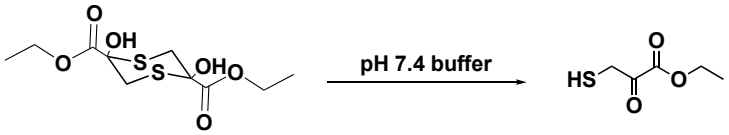
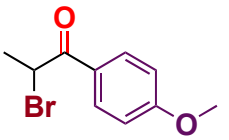


Figure 3.2.1: ***Lead acetate experiment of inhibitor 7 (in a dose dependent manner) with E. coli 3-MST enzyme showing inhibition in H_2S signal at 200 µM of the inhibitor***



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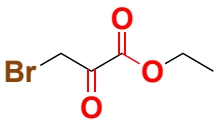
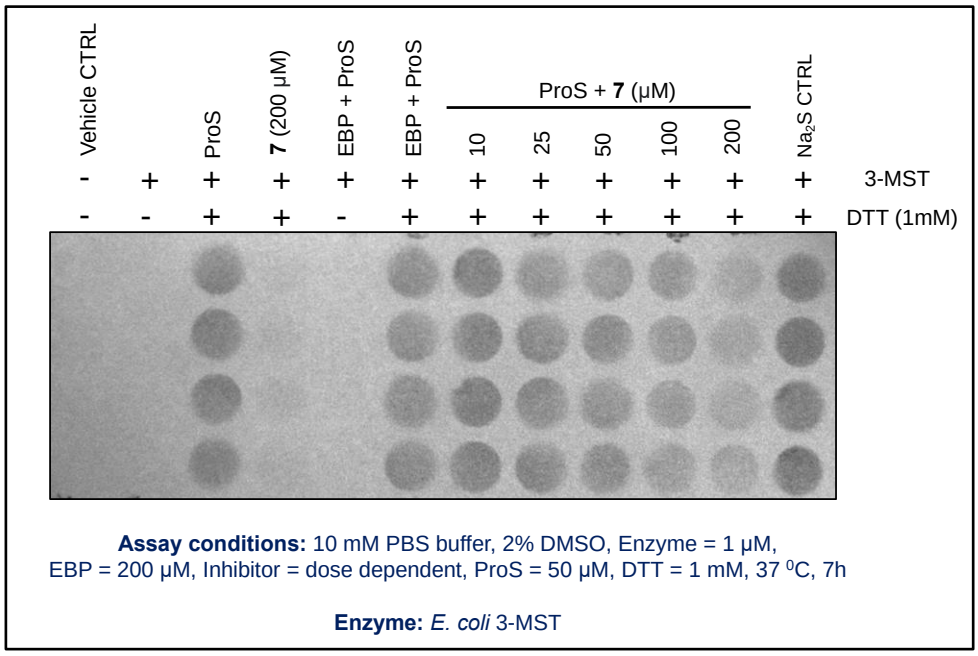


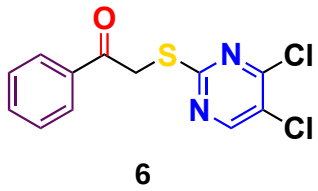
Ethyl 3-bromopyruvate (EBP)



Upon pre-incubation of *E. coli* 3-MST with **7** (200 µM, lane-11), a significant decrease in PbS signal, corresponding to H_2S, as compared to only ProS (lane-3) was observed, suggesting the ability of **7** in quenching H_2S production from *E. coli* 3-MST by inhibiting the enzyme.

A similar set of experiments was carried out with the other synthesized inhibitors (**4** and **6**).

Figure 3.2.2: ***Lead acetate assay of inhibitors 4,6 and 7 with E. coli 3-MST enzyme in the presence of pro-substrate showing a diminished band corresponding to treatment of E. coli 3-MST with 6.***



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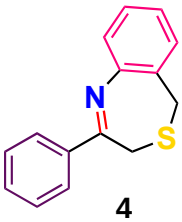

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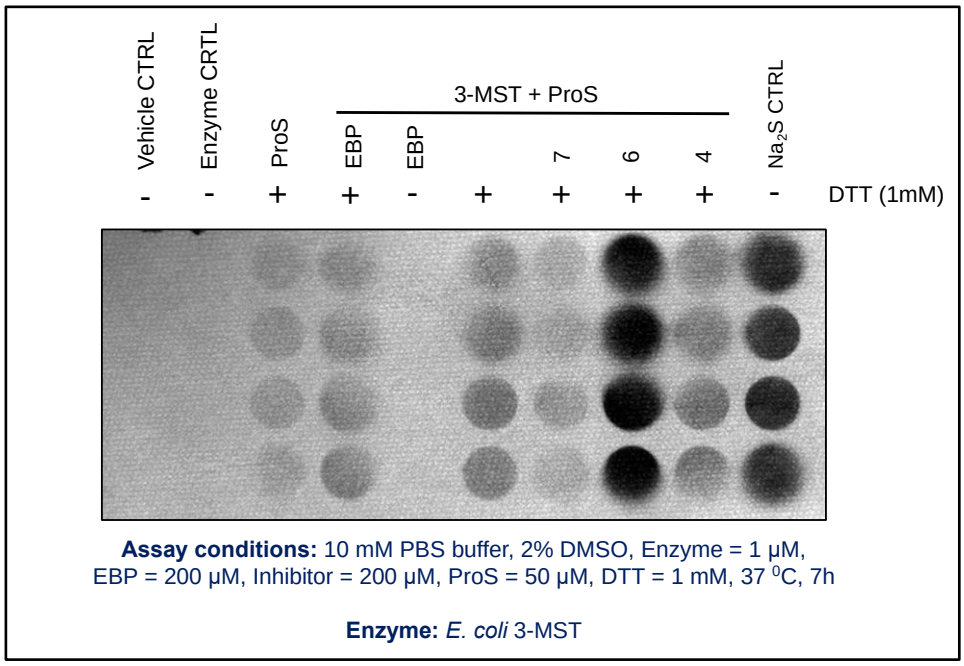


Assay conditions: 10 mM PBS buffer, 2% DMSO, Enzyme = 1 µM, EBP = 200 µM, Inhibitor = 200 µM, ProS = 50 µM, DTT = 1 mM, 37 ^{0}C, 7h

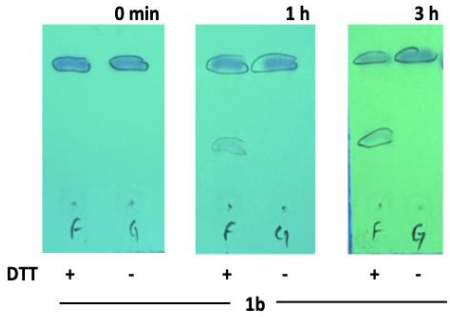
Enzyme: *E. coli* 3-MST

The above studies do not show any significant inhibition in H_2S signal for *E. coli* 3-MST enzyme with inhibitor **4** (lane-9) and **6** (lane-8) even after three hours of pre-incubation as compared to only ProS (lane-6).

An interesting observation here is the increase in H_2S signal upon treatment of the enzyme with compound **6** (lane-8) suggesting its ability to act as a possible substrate, perhaps by the release of 2-mercapto-1-phenylethan-1-one (through the reaction of **6** with either DTT or ProS), which has been reported as an artificial substrate for 3-MST by our group in 2021.

To test this hypothesis, a TLC experiment was done where 300 μM of compound **6** (10 μL, 30 mM stock) was added to 1mM of DTT (100 μL, 10 mM stock) and the volume was adjusted to 1 mL using 30 mM HEPES-NaOH pH 7.4 buffer in a 1.5 mL mini centrifuge tube.

Figure 3.2.3: ***TLC experiment: Reaction of compound 6 with DTT.***



A new spot formation can be seen within an hour of the reaction, confirming, that compound **6** reacts directly with DTT in the conditions used for lead acetate experiment. [21]

3.4 Persulfide/polysulfide measurement using SSP-2

SSP-2 is fluorescent probe to visualise and quantify sulfane sulfur. The nonfluorescent SSP-2 probe's nucleophilic thiol undergoes a reaction with sulfane sulfur, resulting in the formation of a persulfide intermediate. This intermediate subsequently reacts with the electrophilic ester group of SSP-2, inducing spontaneous cyclization and fluorescence release.[21]

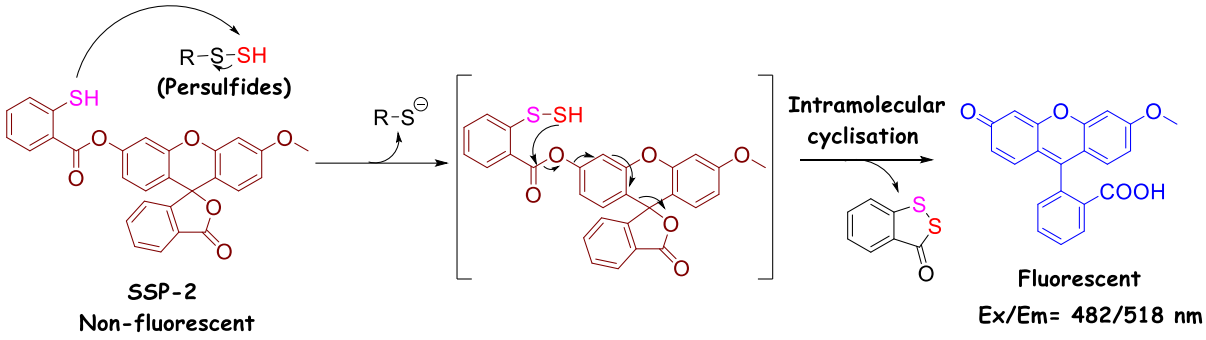
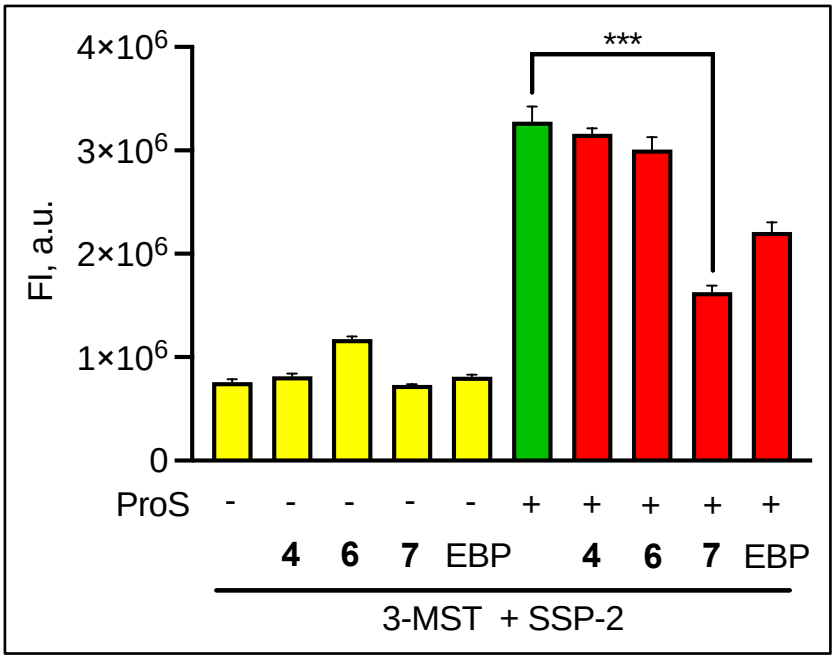


Figure 3.3.1: ***SSP-2 assay of inhibitors 4, 6 and 7 with E. coli 3-MST enzyme showing a decrease in persulfide level corresponding to treatment of E. coli 3-MST with 7.***

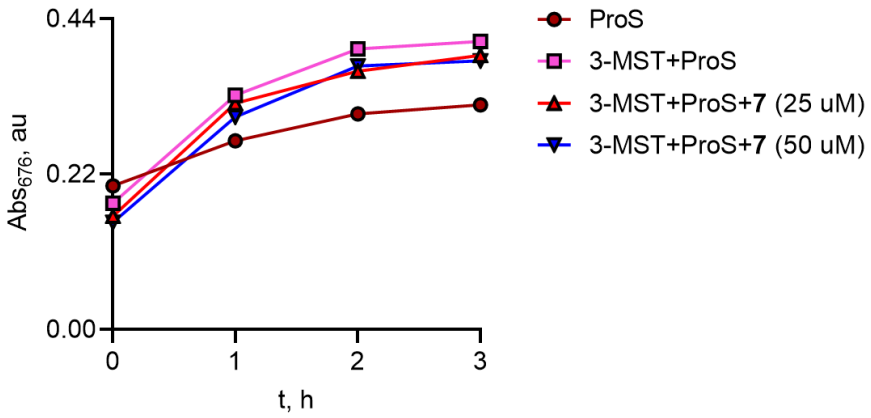


A decrease in persulfide level for *E. coli* 3-MST enzyme can be seen with compound **7** (lane-8, Green**)**, however, no such decrease can be seen with compounds **4** (lane-7, Green) and **6** (lane-9, Green) even after three hours of pre-incubation as compared to only ProS (lane-6, Red).

3.4 Accessing selectivity of the inhibitor using methylene blue assay.

Having established that compound **7** is a potent inhibitor of *E. coli* 3-MST enzyme, the compound was further assessed for its selectivity towards bacterial 3-MST enzyme over mammalian 3-MST enzyme.

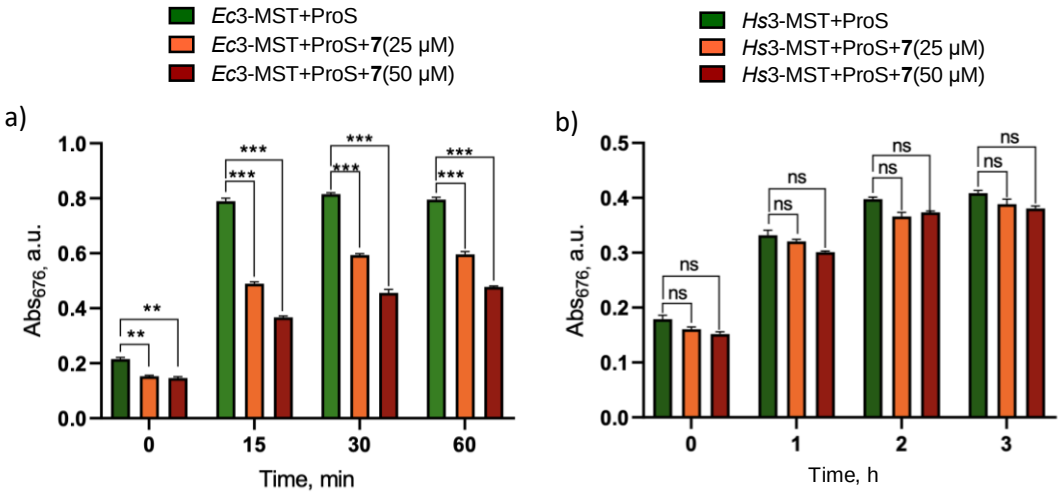
Figure 3.4.1: ***Methylene blue assay of inhibitor 7 upon pre-incubation with homo sapiens 3-MST enzyme to check for selectivity of the inhibitor against bacterial 3-MST enzyme***



Assay Conditions: HEPES buffer (pH= 7.4), **7** (varying concentration), $Zn(OAc)_2$ (400 µM), *homo sapiens* 3-MST (1 µM), 2% DMSO, 37 °C, 1 h. ProS (150 µM), 37 °C, 30 min. DTT (1 mM), aliquot into Methylene Blue cocktail)

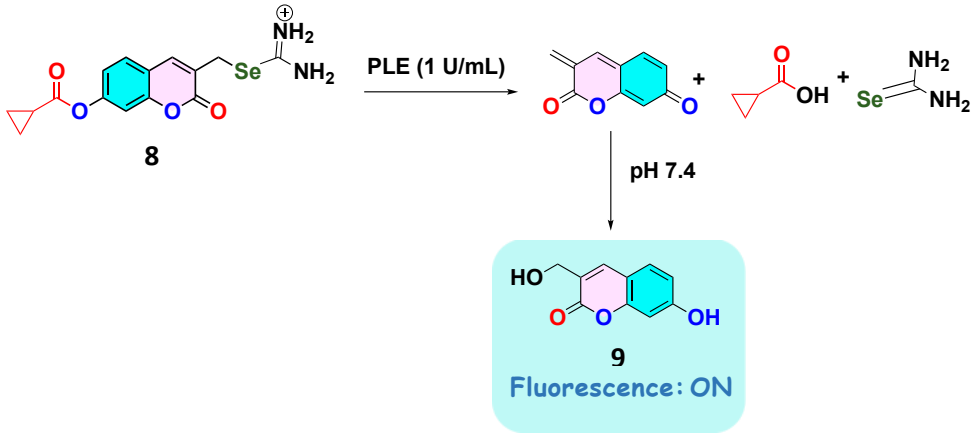
No significant inhibition in H_2S signal for *homo sapiens* 3-MST enzyme was observed even after one hour of preincubation of inhibitor **7** with the enzyme. The inhibition for bacterial versus mammalian 3-MST enzyme has also been quantified below indicating that the inhibitor is selective for bacterial 3-MST enzyme.

Figure 3.4.2: ***Quantification of H_2S formation upon incubation of inhibitor 7 with a) E. coli 3-MST enzyme b) Homo sapiens 3-MST enzyme***



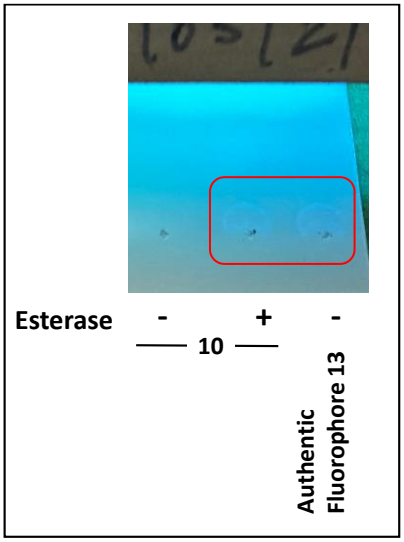
After successfully developing a bioactive covalent inhibitor selective for bacterial 3-MST enzyme that may have applications not only in disarming bacterial defense mechanisms to antibiotics but also in understanding the mechanisms of sulfur transfer within the biological milieu, we designed a H_2Se donor to enhance our fundamental understanding of the biochemical variances between reactive sulfur and selenium species. For this, a Selenourea donor **8** with a fluorophore reporter was designed, synthesised and assessed for lability of its carbon-selenium bond by monitoring the release of authentic fluorophore **9** which was synthesised using reported protocol.[22]

3.5 Assessing the lability of C-Se bond in compound 8 by monitoring the release of Authentic Fluorophore 9.



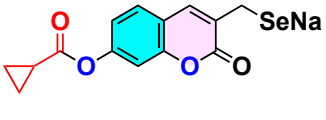
To check the lability of C-Se bond in compound **8**, a TLC experiment was done where 100 µM of compound **8** (10 µL, 10 mM stock) was added to 1 U/mL of Esterase (10 µL, 100 U/mL stock) and the volume was adjusted to 1 mL using 1X PBS pH 7.4 buffer in a 1.5 mL mini centrifuge tube.

Figure 3.5.1: ***TLC Experiment: Reaction of compound 8 with Esterase to monitor the release of Authentic Fluorophore 9***



A new Fluorescent spot formation corresponding to the authentic fluorophore can be seen within 15 minutes of the reaction, confirming, that compound **8** is readily cleaved by esterase to release Selenourea and the authentic fluorophore.

Building upon the findings from the aforementioned experiments, we propose the synthesis of a hydrogen selenide donor **10.** Furthermore, a ratiometric fluorescent probe **HBT-Se** designed for the highly selective detection of gaseous H_2Se was synthesised as per reported protocol.[23] The compound **10** will be further accessed for it antioxidant and anti-inflammatory properties. Collectively, our study aims to expand the repertoire of tools available for investigating reactive selenium species, while also facilitating future research endeavors aimed at directly detecting H_2Se in various biological contexts.



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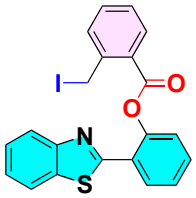

HBT-Se

Chapter 4 **Conclusion**

In this present study, we characterised thioether/selenoester class of molecules to understand the enzymatic sulfur/selenium transfer under physiological conditions. Bacterial 3-MST, an enzyme responsible for generating H_2S, is found in over 500 species of gram-negative bacteria and has been suggested as a novel target in the fight against antibiotic resistance. In order to selectively target this enzyme in *Escherichia coli*, an inhibitor **7** was developed. Conformational restrictions of the active fragment "thioether" were achieved and shown to have negligible effects on binding with the target enzyme. The high reactivity of amino acid cysteine located in the active loop of the enzyme was utilised to develop covalent inhibitors for the enzyme. Next, the H_2S inhibitory effects of the synthesised inhibitors were evaluated. Compared to the mammalian 3-MST, the lead compound **7** selectivity inhibited H_2S production in bacterial 3-MST enzyme. Additionally, a selenourea donor **8** with a fluorophore reporter was developed which could be further used to develop a corresponding H_2Se donor **10** allowing for simultaneous delivery as well as detection of H_2Se. Collectively, our findings refer to a set of small molecules which allow us to tune sulfur and selenium transfer and to study their chemical biology.

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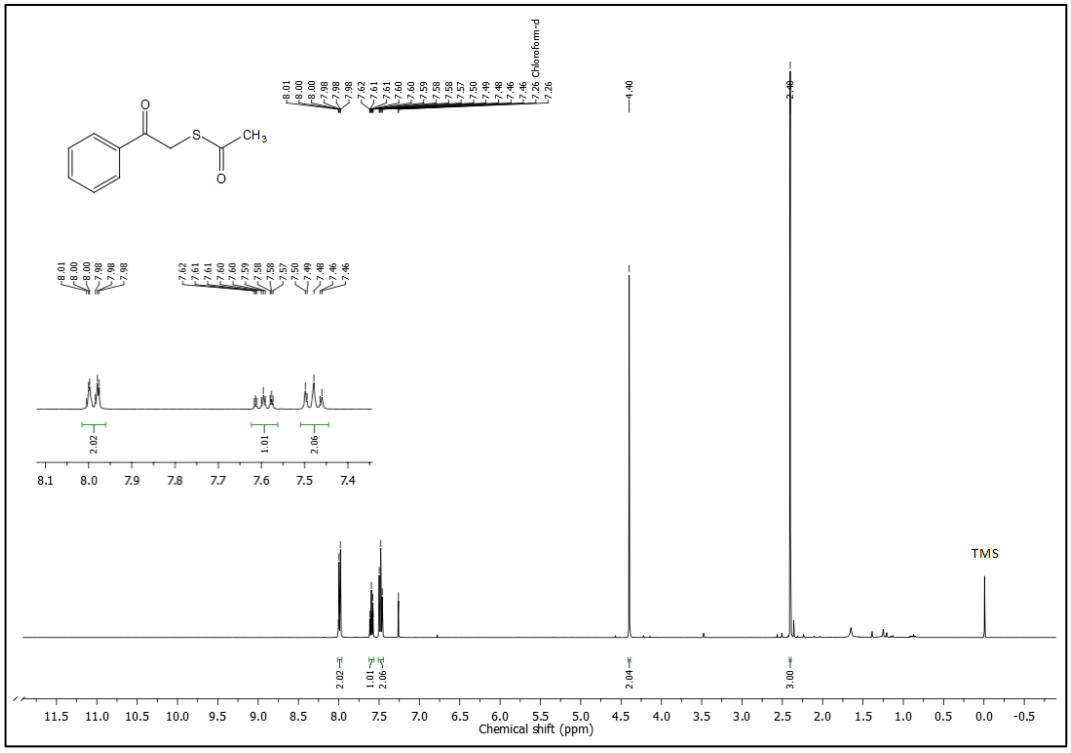
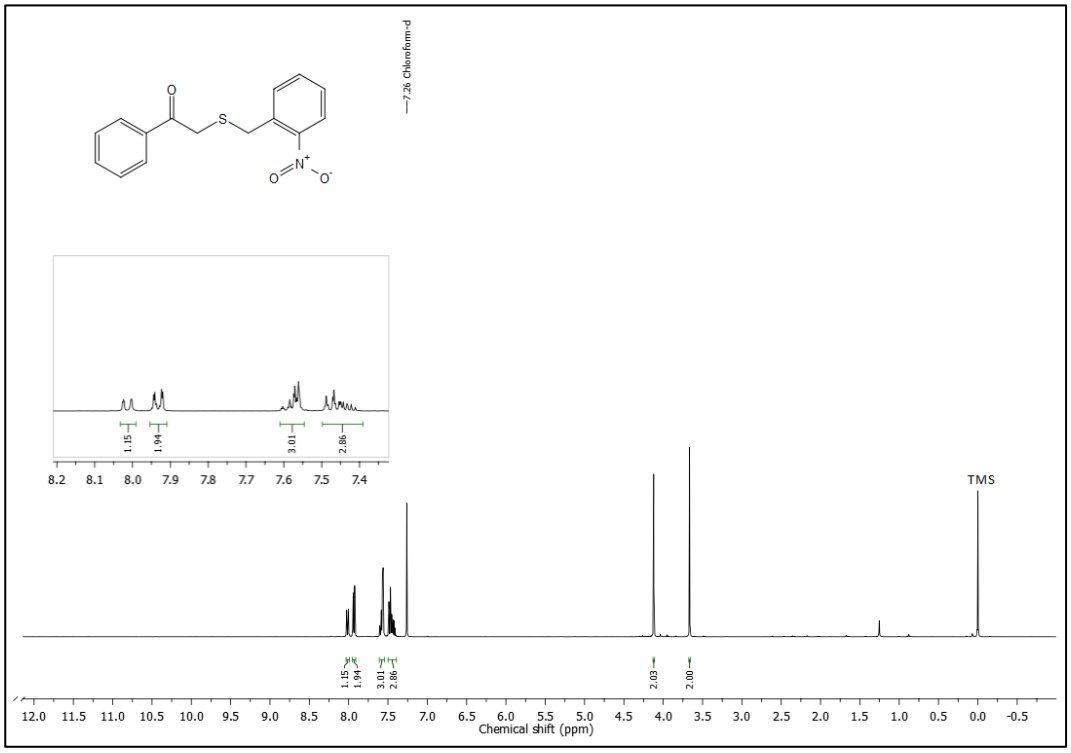
Supporting Data

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



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



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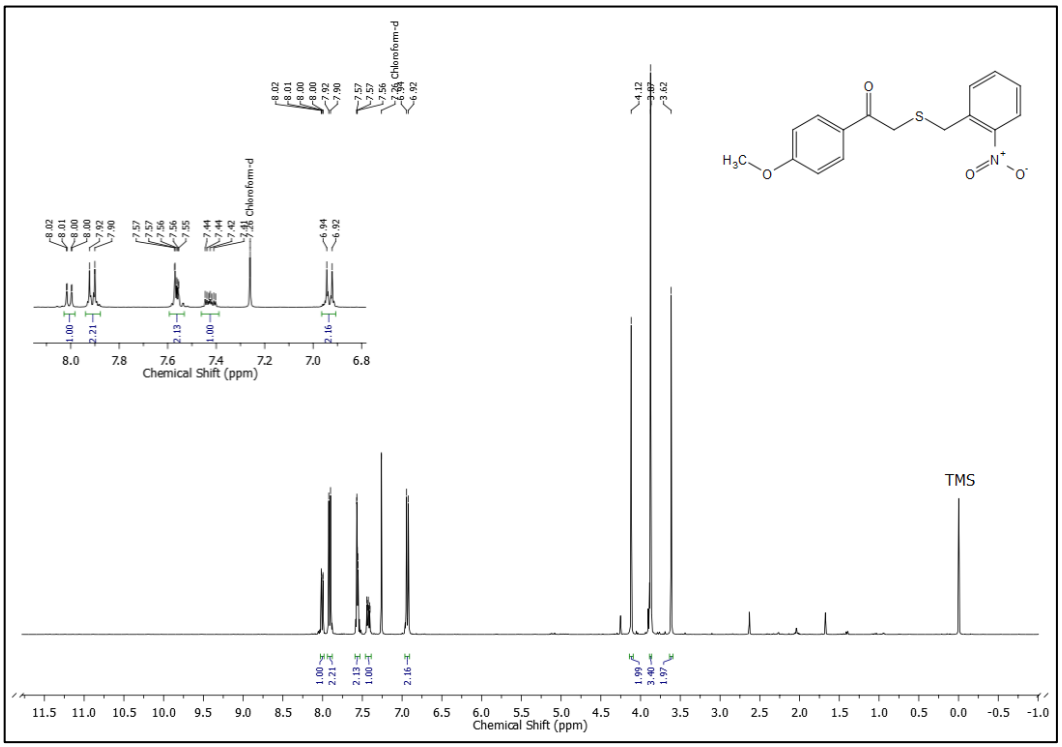
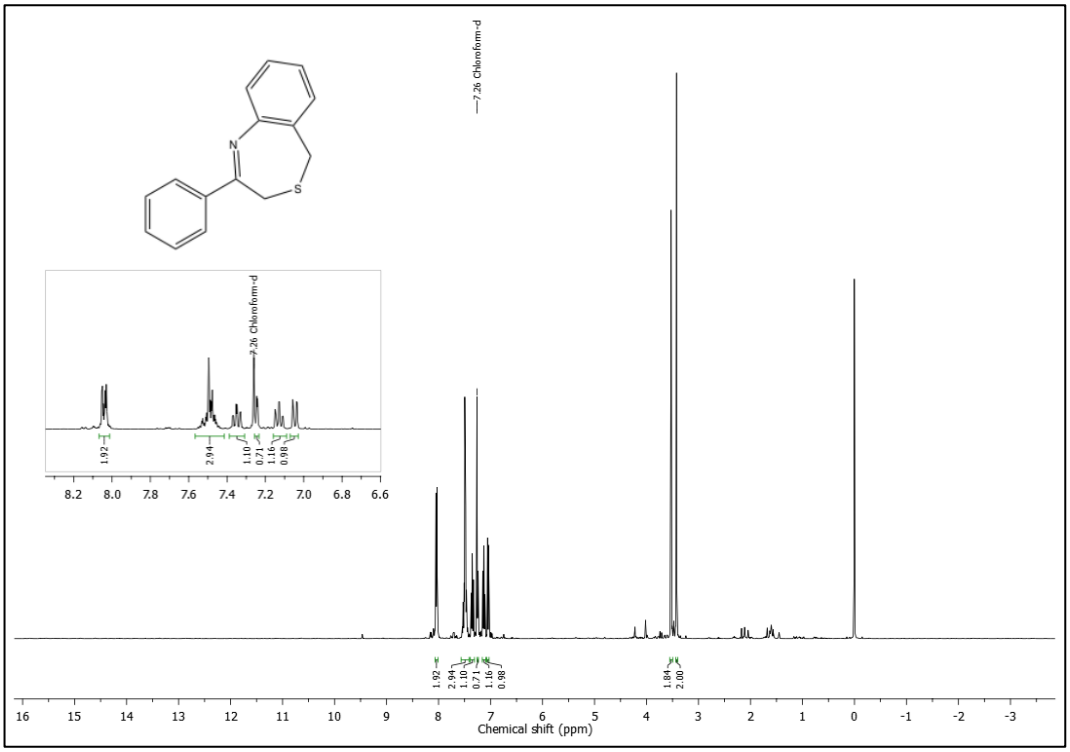
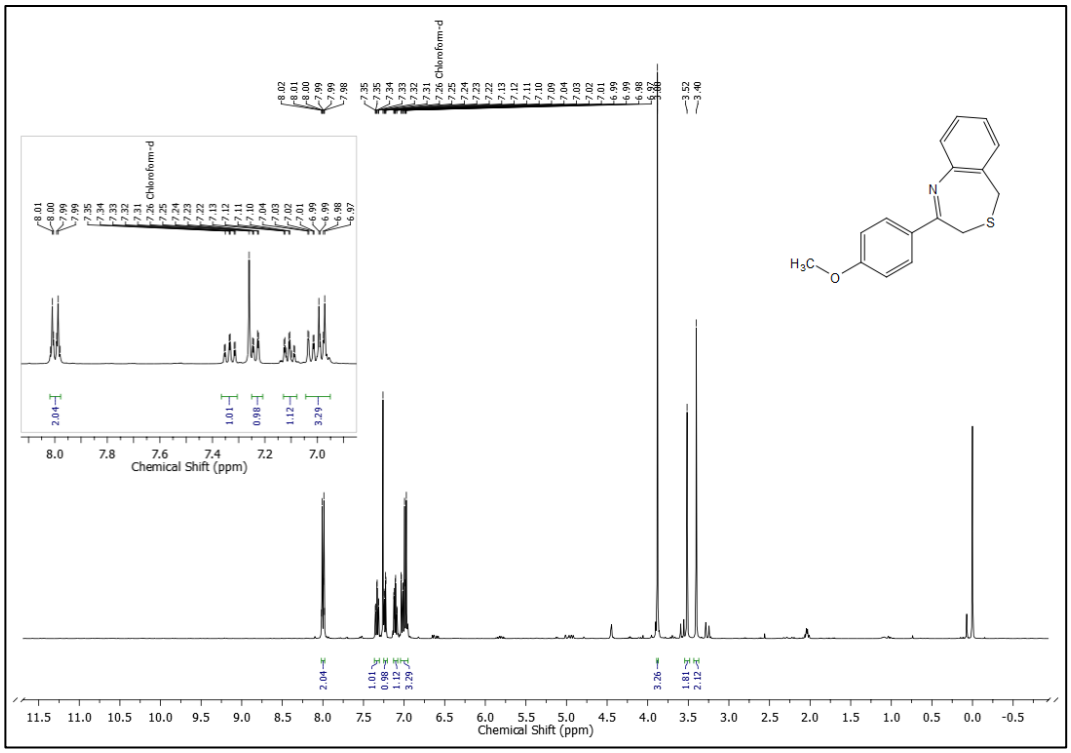


^{1}H NMR of **4**



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



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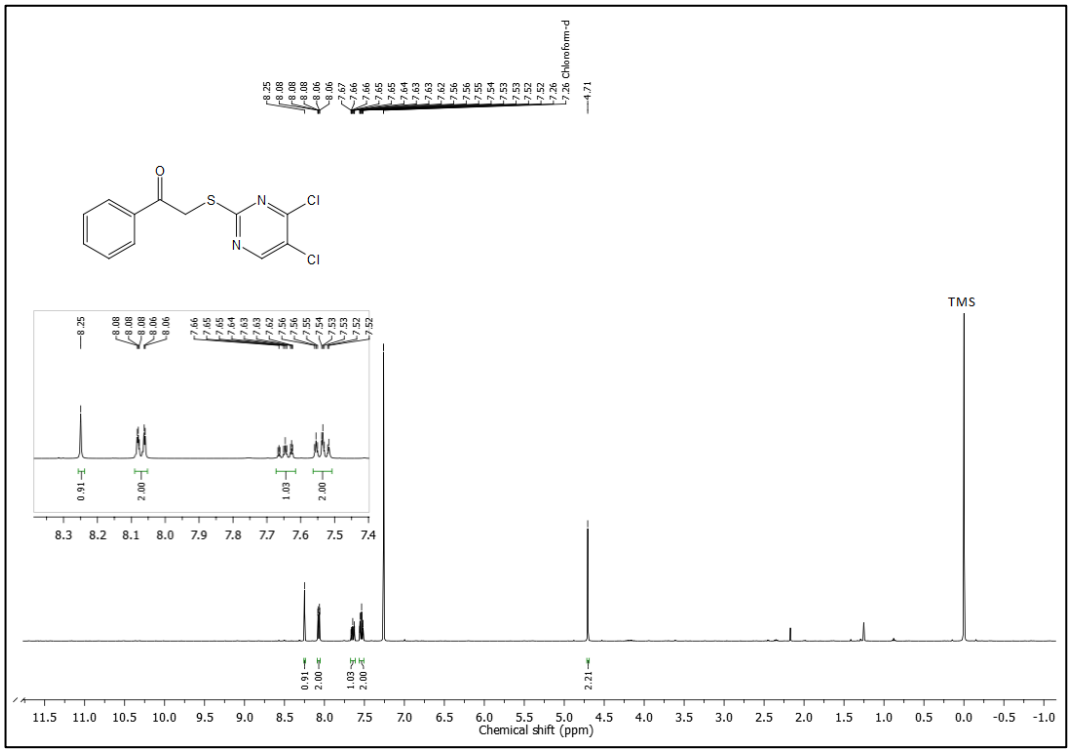

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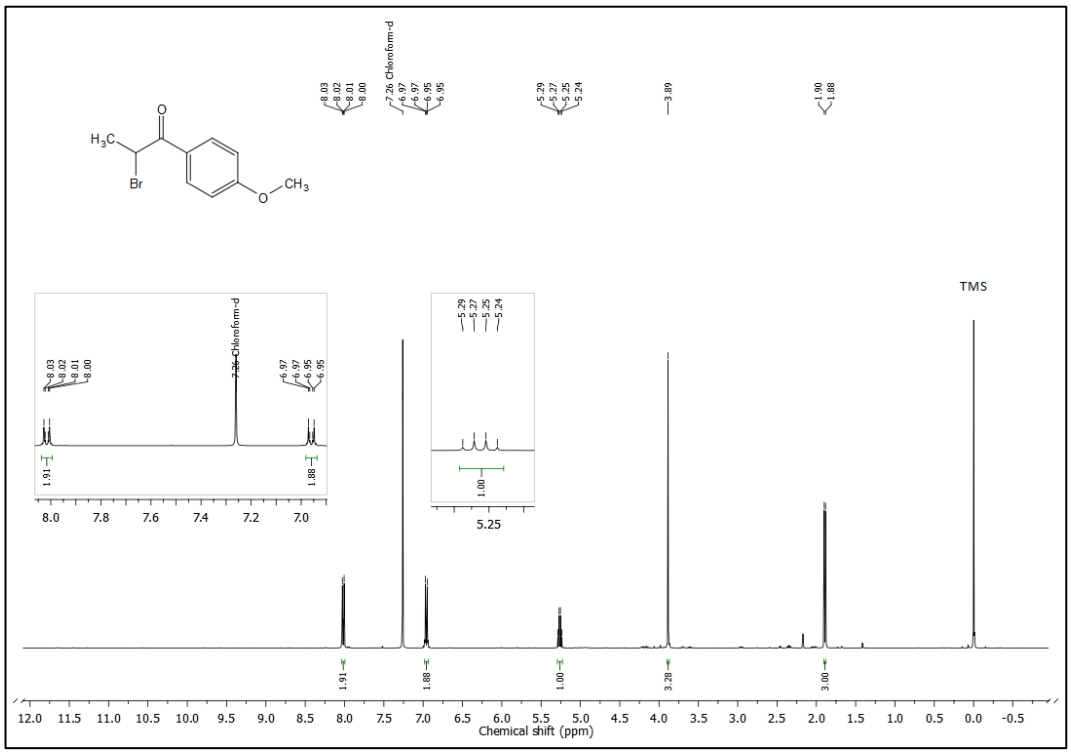

^{13}C NMR of **7**