Mechanistic Investigation of Unnatural Substrates for Enzymatic Production of Hydrogen sulfide in Bacteria

A Thesis

submitted to

Indian Institute of Science Education and Research – Pune

in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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CERTIFICATE:

This is to certify that this dissertation entitled **"Mechanistic Investigation of Novel Unnatural Substrates for Enzymatic Production of Hydrogen sulfide in Bacteria"** towards the partial fulfilment of the B.S.-M.S. Dual Degree Programme at the Indian Institute of Science Education and Research (IISER), Pune represents study/work carried out by Mrutyunjay Nair at IISER – Pune under the supervision of Dr Harinath Chakrapani (Associate Professor, IISER – Pune) and Dr Amrita Hazra (Assistant Professor, IISER – Pune) during the academic year 2018-2019.

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Date: 20th March 2019

Place: Pune, Maharashtra, India

DECLARATION

I hereby declare that the matter embodied in the report entitled **"Mechanistic Investigation of Novel Unnatural Substrates for Enzymatic Production of Hydrogen sulfide in Bacteria"** are the results of the work carried out by me at the Department of Chemistry, IISER – Pune, under the supervision of Dr Harinath Chakrapani and Dr Amrita Hazra, and the same has not been submitted elsewhere for any other degree.

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ABBREVIATIONS:

NMR	Nuclear Magnetic Resonance
HRMS	High-Resolution Mass Spectrometry
MHz	Megahertz
DMSO	Dimethyl sulfoxide
mg	milligram
mL	millilitre
mmol	millimole
M	molar
RT	Retention time
HPLC	High-Performance Liquid Chromatography
IR	Infrared
3-MST	3-Mercaptopyruvate sulfurtransferase
*Ec*3-MST	*Escherichia coli* 3-MST
m3-MST	Mouse 3-MST
wt	Wild-type
RF cloning	Restriction-free cloning
PCR	Polymerase chain reaction
BSA	Bovine serum albumin
DTT	Dithiothreitol
GSH	Glutathione

ABSTRACT:

The mode of action of antibiotics is under investigation pertaining to the role of reactive oxygen species (ROS) that are thought to be generated as a downstream consequence of the antibiotic assault on its primary targets. Many studies have shown that hydrogen sulfide (H_2S) production in bacteria is, in some way, upregulated in periods of such oxidative stress. Since H_2S is known to act as an anti-oxidant, it serves to abate the deleterious effects of antibiotics on bacteria, conferring on it what we know, and call, as antibiotic resistance.

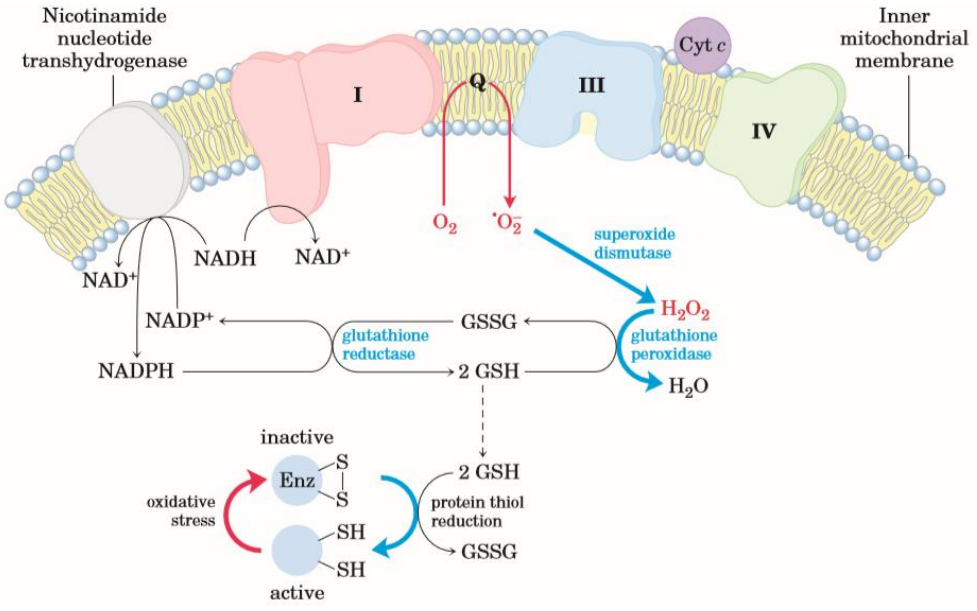
The question being investigated in this study is the extent to which H_2S provides protection to bacteria against antibiotics – i.e. if the intrinsic levels of H_2S in bacteria are changed, will it translate to changes observed in their resistance to antibiotics?

Taking *Escherichia coli* as the model, we cloned, overexpressed, and purified the only known enzyme, 3-Mercaptopyruvate sulfurtransferase (*Ec*3-MST) that produces H_2S in it. This study then takes up the systematic development of novel substrates for *Ec*3-MST that have been shown to produce H_2S at different rates *in vitro*. The mechanism of turnover of these substrates is then elucidated step by step.

The study leaves us with the prospect of testing these unnatural substrates on *E. coli* to observe if the varied levels of H_2S can be obtained *in vivo* as well. By co-incubating the bacteria with different antibiotics, this model can then be used to address our original question as to what extent is H_2S able to protect bacteria from antibiotic stress, and more importantly, bring in a more complete understanding as to how antibiotics actually work.

INTRODUCTION:

The varied classes of antibiotics that are available have numerous targets in bacteria – for example, while β-lactam-based antibiotics inhibit cell wall synthesis, fluoroquinolones inhibit DNA synthesis in bacteria. Apart from exerting their deleterious effects on bacteria by hitting their "primary target", studies have shown that the bactericidal antibiotics work by a common mechanism – by generating ROS as a downstream consequence of the primary assault[1]. The study uses, Hydroxyphenyl fluorescein, a dye that selectively gets oxidized by hydroxyl radicals ($OH^{\bullet}$), to show that there is enhanced formation of $OH^{\bullet}$ on incubation of *E. coli* with bactericidal antibiotics. The study goes on to demonstrate that strains of *E. coli* that are knock-outs of genes that code for enzymes that produce NADH/NADPH in the TCA cycle survive better under antibiotic stress due to a reduced flux of NADH/NADPH that would in turn feed into the electron-transport chain (ETC). The reason for such an effect can be explained by understanding that in ETC NADH/NADPH get oxidized to NAD^+/$NADP^+$ which result in an electron flux. Such a phenomenon is observed to occur in the plasma membrane of the prokaryotic cells, which is functionally analogous to the inner mitochondrial membrane of eukaryotic cells. This electron flux gets diverted inadvertently (with a probability of 0.1-4%) to oxygen to yield superoxide radicals ($O_2^{\bullet -}$), which can then cause bacterial cell death[2].



Challenging this proposition, another study[3] was published that demonstrated that the antibiotics kill bacteria by attacking their respective targets as there was no significant difference in cell viability seen when *E. coli* were incubated with bactericidal antibiotics in oxygen-rich or –deficient media. Furthermore, a strain of *E. coli* was developed (Hpx^-) that lacked catalase and peroxidase (ROS scavenging enzymes) activity, which showed no significant difference in cell viability as compared to its wild-type (wt) counterpart when they are challenged with antibiotics in an oxygen-rich environment. Finally, it was also observed that there is no increment in levels of hydrogen peroxide in the culture medium of *E. coli* when they receive an antibiotic challenge.

From these two studies, one can say that there exists debate pertaining to the understanding of the mechanism of action of antibiotics.

Switching gears, we move on to H_2S. This recently-found gasotrasmitter is shown to have numerous roles in physiology, the most striking of which is its ability to act as a defence mechanism for bacteria against antibiotics[4]. A study demonstrated that when challenged with *any* antibiotic, the knock-out strain of *E. coli* for H_2S-producing enzyme 3-MST (viz. *ΔMST*) survives worse as compared to wt. The survival of *ΔMST* strain augments when NaHS (an H_2S-producing salt) is supplied externally. This effect is shown not to be limited to 3-MST and *E. coli*, but extends to other bacteria such as *S. aureus*, *P. aeruginosa*, and *B. anthracis* with their respective enzymatic sources of H_2S. The study leaves us with the result showing that the activity of 3-MST under antibiotic-stress primes the activity of ROS-degrading enzymes such as catalase and superoxide dismutase.

Providing more physiological insight, another study[5] describes that under antibiotic stress conditions, H_2S is able to switch the cellular respiration in *E. coli* from the more-efficient Cytochrome *bo* oxidase to the less-efficient Cytochrome *bd* oxidase. In doing so, the bacteria now respire slowly. Connecting back to our previous discussion on respiration, the electron flux that is inadvertently lost to oxygen to form $O_2^{\bullet -}$ radicals decreases simply because the bacteria are now respiring slowly. It is in this way that H_2S is believed to save bacteria from antibiotic stress.

The question that my project has investigated is that by taking control of the enzymatic production of H_2S in bacteria, which forms the bulk of the intracellular H_2S produced, can we modulate its intracellular concentration?

There already exist compounds that can either produce H_2S in buffer conditions (**Figure 1**; **A**) or when triggered. To this end, triggers such as cellular thiols[6] (**B**), light[7] (**C**), or bacterial enzymes (such as esterase[8] (**D**) and nitroreductase[5] (**E**)) have been employed. However, these are stand-alone compounds that do not feed into the enzyme machinery, say 3-MST in *E. coli*, to produce H_2S. Such a non-enzymatic approach will allow us only to be able to generate H_2S in surplus of the already existing, and very efficient, mode of generating H_2S. To be able to hijack *Ec*3-MST and bid it to generate varied levels of intracellular H_2S would hence be desired.

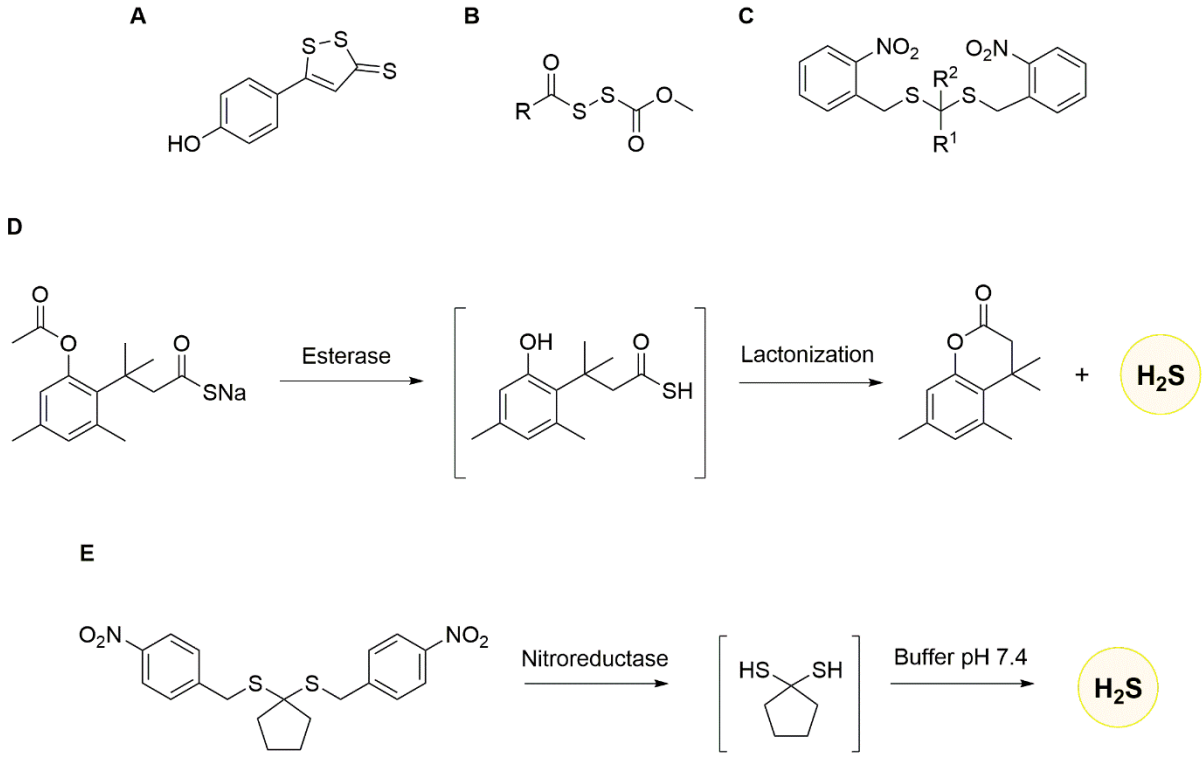


Figure 1: Some reported donors of hydrogen sulfide.

In order to address the question posed above, we decided to use *E. coli* as the model system. The major reason for this is that *E. coli* has only one known enzyme (i.e. *Ec*3-MST) that produces H_2S[4]. Furthermore, *E. coli* system has been extensively studied and is easy to work with. Also, *Ec*3-MST is well-characterized in literature (PDB code: 1URH)[9].

The physiological substrate of 3-MST, viz. 3-Mercaptopyruvate (3-MP), is synthesized from L-Cysteine by the action of a PLP-dependent enzyme, Cysteine aminotransferase (CAT)[10] (**Figure 3**; **A**). Crystal structure of m3-MST bound to an inhibitor (PDB code: 5WQJ) illustrates the position and role of the some of the active site residues[11] (**Figure 2**). Arg 188 and Arg 197 are crucial to hold the substrate in the active site; Asp 63- His 74- Ser 250 catalytic triad deprotonates Cys 248, which is the key active site residue for H_2S turnover.

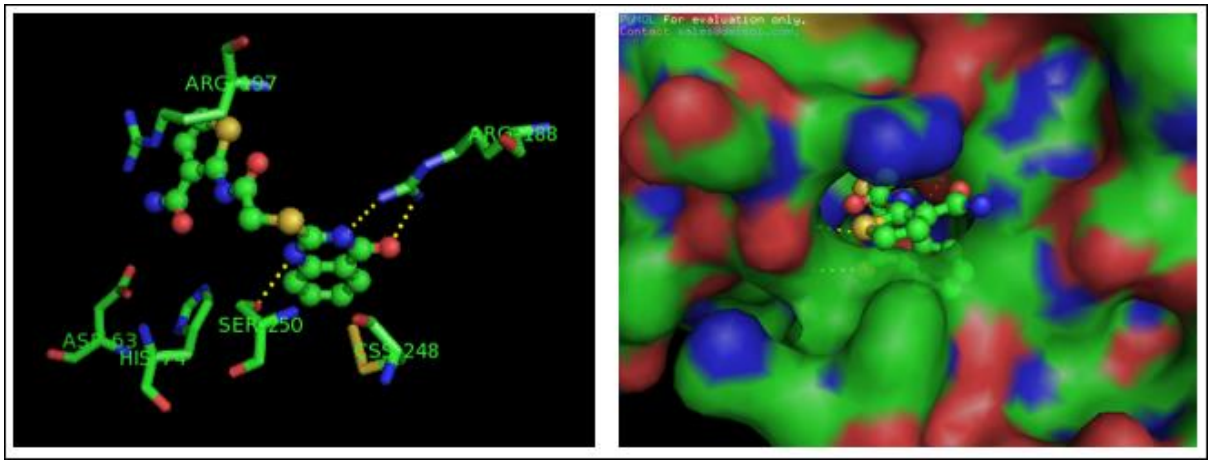


Figure 2: The major active site residues of m3-MST and the binding mode of inhibitor **1** in the active site (PDB code: 5WQJ).

The activated Cys 248 residue attacks the thiol group of the substrate, thus persulfidating the enzyme (**Figure 3**; **B**). The thiol group can then be taken up by an acceptor, which either can be another thiol (**C**) (such as Thioredoxin (**E**) or Dithiothreitol) or cyanide ion[10,12] (**D**).

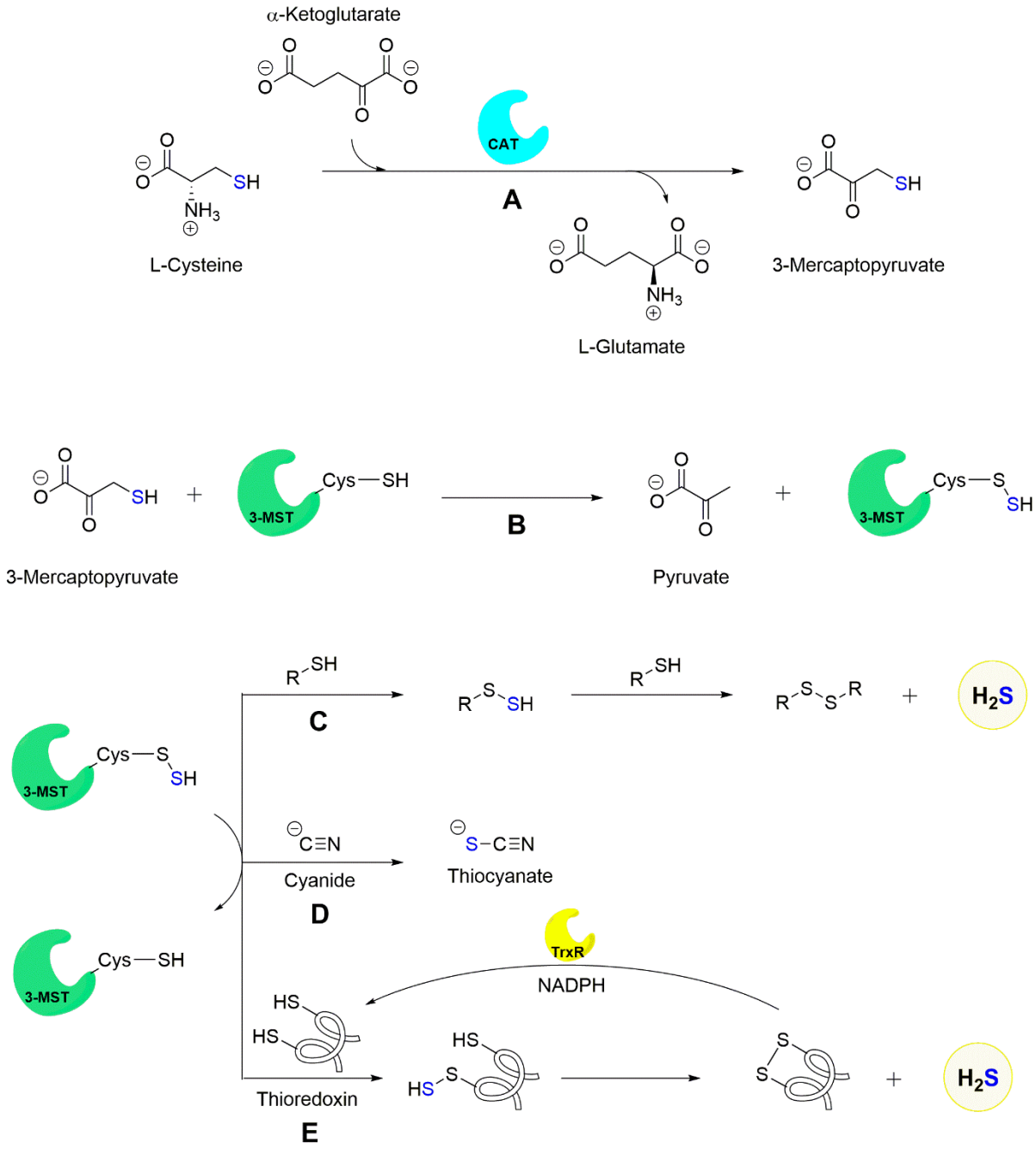


Figure 3: Reaction mechanism for turnover of its physiological substrate by *Ec*3-MST.

The motivation for the design of the compounds that would act as unnatural substrates for *Ec*3-MST originates in a previously reported study[11] carried out on mouse(m)-3MST where inhibitors were identified using high-throughput screening (**Figure 4**).

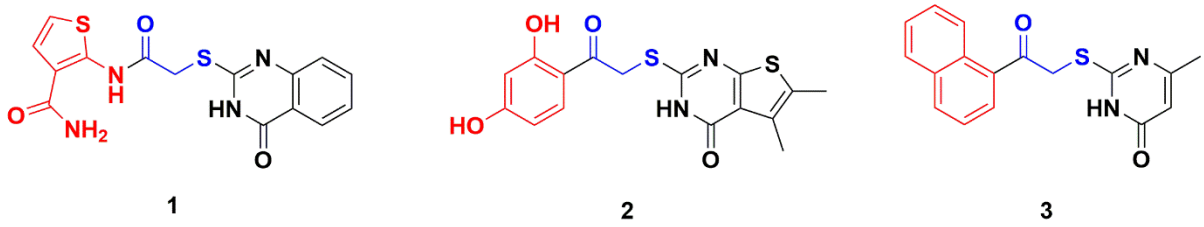


Figure 4: Some of the reported inhibitors for m3-MST.

Some of the major takeaways from this study in order to design putative compounds that could dock into *Ec*3-MST active site were as follows (**Figure 5**):

a) Although the 3-MST active site residues are conserved across species (here m3-MST and *Ec*3-MST), the active site is amenable to the docking of molecules other than the physiological substrate.
b) It would be sensible to keep the central backbone fixed (**Figure 4**; Blue highlight).
c) The functional group adjacent to the carbonyl can be chosen to be aromatic (instead of carboxyl group as in the physiological substrate).
d) Having a free thiol in the molecule would render it unstable – hence, protecting the thiol group (here, by acetylation or benzoylation) would be logical.

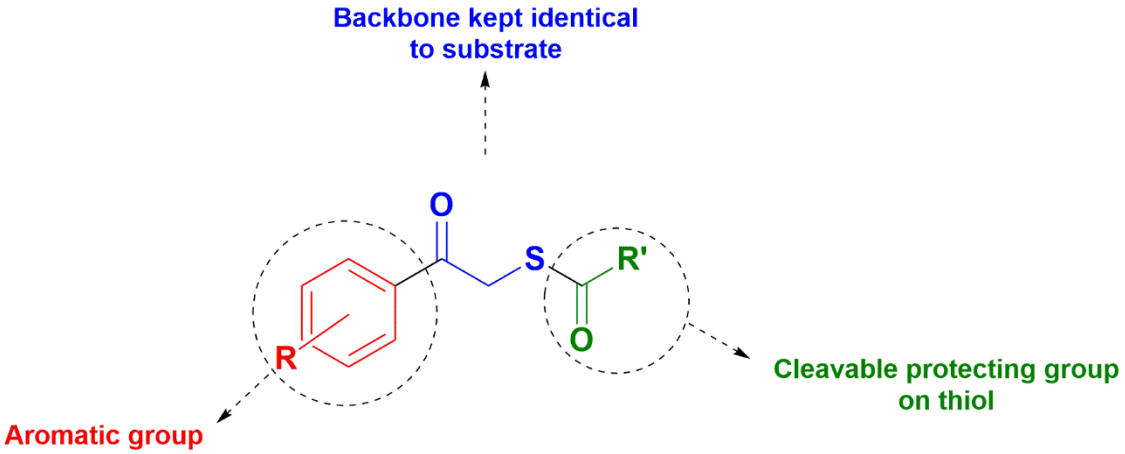


Figure 5: Conceptualisation of the unnatural substrates for *Ec*3-MST.

What this project has developed are storage-friendly compounds that are unnatural substrates for *Ec*3-MST. These unnatural substrates are turned over by *Ec*3-MST at different rates, allowing us to generate H_2S at varied rates while using the cellular machinery to our advantage.

This study then goes on to elucidate the mechanism of turnover of these compounds (**Figure 6**) *in vitro* using a variety of independent assays.

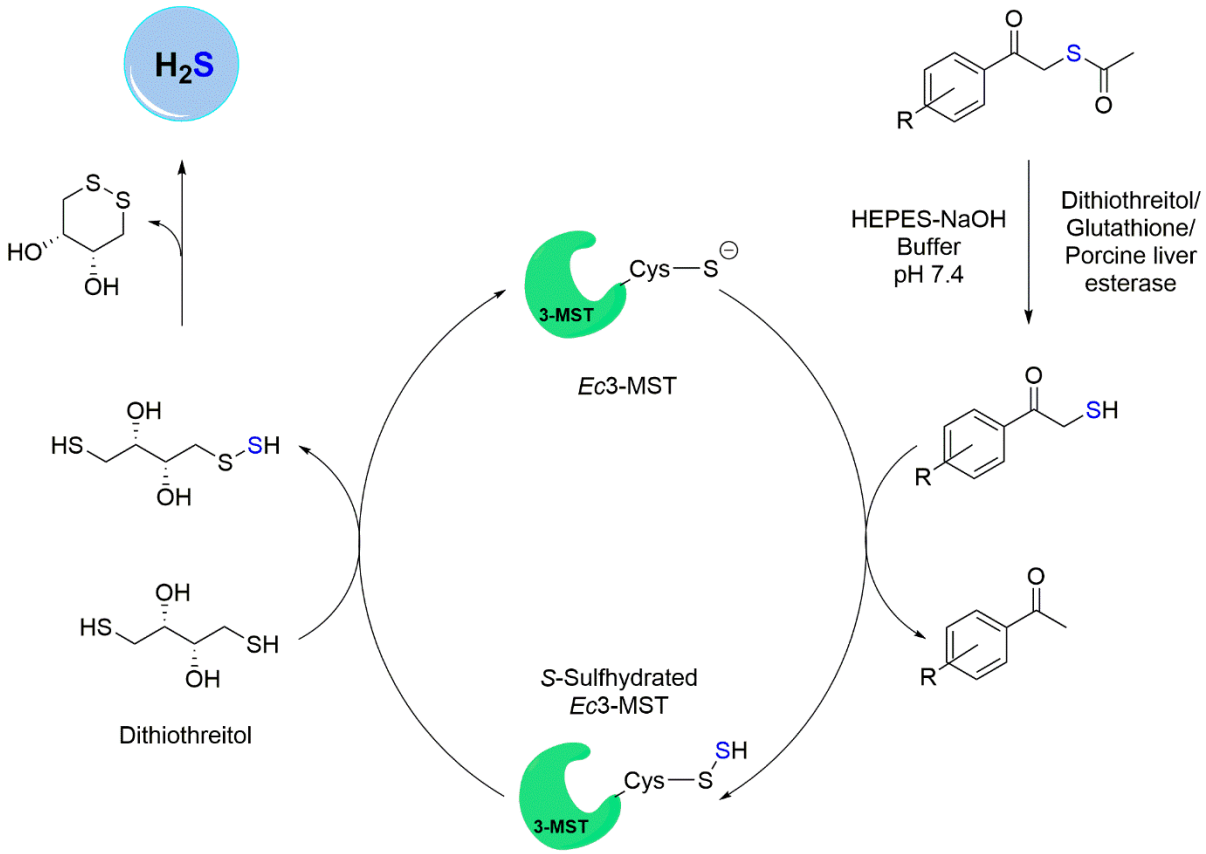


Figure 6: Proposed mechanism of turnover of the unnatural substrates designed in this study.

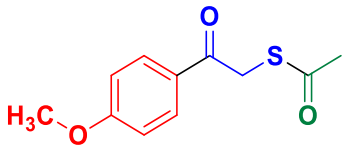
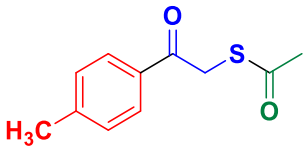
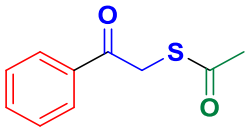
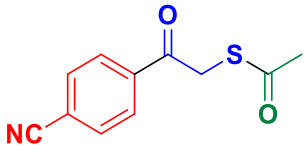
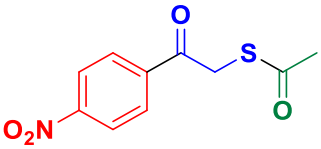
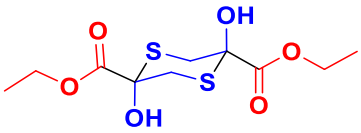
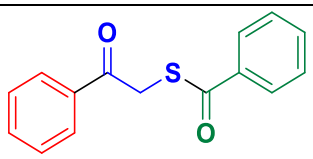
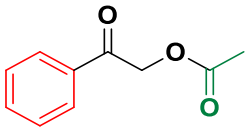
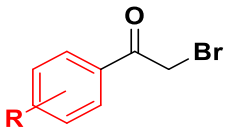
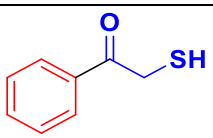
Thus, the study lays the foundation for the development of a model system allowing us to be able to create varied levels of H_2S in *E. coli*. Such a system would be an asset to be able to answer some bigger questions as to what extent is H_2S able to protect bacteria from antibiotic stress, and more importantly, bring in a more complete understanding as to how antibiotics actually work.

MATERIALS AND METHODS:

General methods:

All reactions were conducted under nitrogen atmosphere. All the chemicals and solvents were purchased from commercial sources such as Sigma-Aldrich (Merck), Spectrochem, Rankem, and Avra Synthesis, and used as received unless stated otherwise. For molecular biology, all the chemicals used were obtained either from TCI, HiMedia or Sigma-Aldrich (Merck) unless otherwise specified. The enzymes used were obtained from TaKaRa. The protein ladders used was obtained from Genetix. Column chromatography was performed using silica gel - Rankem (60–120 mesh) as the stationary phase. ^{1}H and ^{13}C spectra were recorded on a JEOL 400 MHz (or 100 MHz for ^{13}C) or a Bruker 400 MHz (or 100 MHz for ^{13}C) spectrometer unless otherwise specified using residual solvent signals – $CDCl_3$: δH= 7.26 ppm, δC = 77.2 ppm, or d_6-DMSO : δH = 2.50 ppm. Chemical shifts (δ) are reported in ppm. The following abbreviations are used: m (multiplet), and s (singlet). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS (Synapt G2, Waters). FT-IR spectra were recorded using BRUKER-ALPHA FT-IR spectrometer. Photometric measurements were performed using Thermo Scientific VarioskanFlash or PerkinElmer EnSight microtiter plate readers.

Synthesis and characterization:

Entry	Structure	Compound code
1		**1a**
2		**1b**
3		**1c**
4		**1d**
5		**1e**
6		**2**
7		**3**
8		**4**
9		**5a-e**
10		**6a**

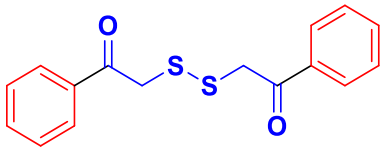
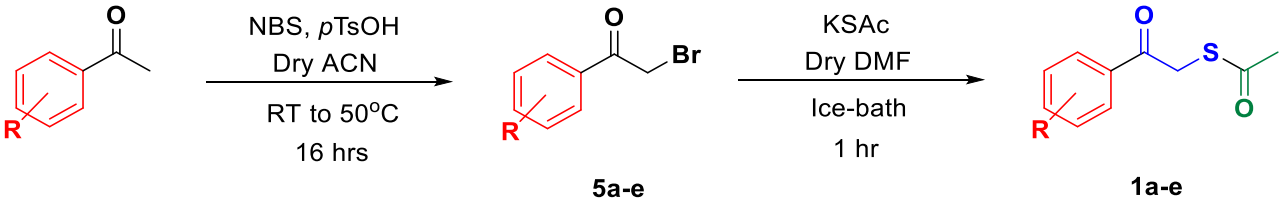
11		**6b**

Table 1: Compounds used in this study.

Compounds **2**[13], **3**[14], **4**[15], and **5a-e**[16], are synthesized as per their respective protocols that were earlier reported.

Apart from the data already available for compound **2**[13], we recorded FT-IR (ν_{max}; cm^{-1}): 3465, 1711, 1514, 1454, 1364, 1279, 1221.

Apart from the data already available for compound **4**[17], we recorded FT-IR (ν_{max}; cm^{-1}): 1747, 1698, 1595, 1427, 1373, 1212.



Scheme 1: Synthesis of compounds **1a-e**.

Synthesis of derivatives 1a-e:

From the derivatized phenacyl bromides **5a-e**, the corresponding *S*-ethanethioates were synthesized as per previously reported protocol[18,19].

Briefly, the corresponding phenacyl bromide was taken in a round-bottom flask and dissolved in dry dimethylformamide (DMF) (4 mL per millimole of phenacyl bromide used) under nitrogen atmosphere. The reaction mixture was placed on an ice bath for 10 mins, following which potassium thioacetate (KSAc) (1.5 eq.) was added. The reaction was allowed to continue for 1 hr. Workup was done by adding ethyl acetate to the reaction mixture and the transferring it to a separating funnel. The organic layer was then washed with 6 x 100 mL of water (for 10 mL DMF) to remove DMF. NaCl was also added to effect better separation. The organic layer was then dried over anhydrous Na_2SO_4, and concentrated under reduced pressure.

Purification was done by column chromatography using 60-120 mesh silica gel as the stationary phase. The column was packed in hexane, and the *S*-ethanethioates were eluted in ethyl acetate/hexane system.

Characterization of compounds **1a**[14] and **1c**[14,19] matches previous reports.

***S*-(2-Oxo-2-(*p*-tolyl)ethyl) ethanethioate (1b):** 450 mg 2-Bromo-1-(*p*-tolyl)ethan-1-one (2.11 mmol) was reacted with 362 mg KSAc (3.17 mmol) in 8 mL dry DMF. Compound **1b** was eluted in 12-17% ethyl acetate/hexane system as a pale-yellow solid obtained in 75% yield. FT-IR (ν_{max}; cm^{-1}): 2923, 2857, 1681, 1601, 1512, 1420, 1357, 1288; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90-7.87 (m, 2H), 7.28-7.26 (m, 2H), 4.38 (s, 2H), 2.41 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.4, 193.0, 144.8, 133.2, 129.6, 128.8, 36.7, 30.4, 21.8; HRMS for $C_{11}H_{12}O_2S$ $(M+Na)^+$ Calculated: 231.0457, Found: 231.0458.

***S*-(2-(4-Cyanophenyl)-2-oxoethyl) ethanethioate (1d):** 500 mg 4-(2-Bromoacetyl)benzonitrile (2.23 mmol) was reacted with 383 mg KSAc (3.35 mmol) in 8 mL dry DMF. Compound **1d** was eluted in 20% ethyl acetate/hexane system as a pale-brown solid obtained in 79% yield. FT-IR (ν_{max}; cm^{-1}): 2915, 2861, 2227, 1675, 1561, 1508, 1398, 1368, 1290; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09-8.06 (m, 2H), 7.80-7.77 (m, 2H), 4.35 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.0, 192.4, 138.7, 132.8, 129.0, 117.9, 117.1, 36.5, 30.3; HRMS for $C_{11}H_9NO_2S$ $(M+H)^+$ Calculated: 220.0428, Found: 220.0432.

***S*-(2-(4-Nitrophenyl)-2-oxoethyl) ethanethioate (1e):** 280 mg 2-Bromo-1-(4-nitrophenyl)ethan-1-one (1.15 mmol) was reacted with 197 mg KSAc (1.72 mmol) in 5 mL dry DMF. Compound **1e** was eluted in 15-17% ethyl acetate/hexane system as a brown solid obtained in 31% yield. FT-IR (ν_{max}; cm^{-1}): 2921, 2855, 1669, 1599, 1514, 1337, 1137; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35-8.32 (m, 2H), 8.17-8.13 (m, 2H), 4.38 (s, 2H), 2.42 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 194.0, 192.3, 150.7, 140.2, 129.7, 124.1, 36.7, 30.3.

Cloning, overexpression, and purification of *Ec*3-MST wt and C238A mutant: The primers used for cloning the *Ec3-MST* gene are listed in the **Table 2**. The wild-type *Escherichia coli 3-MST* gene was cloned from the genome of wild-type *E. coli*

K-12 MG1655 using gene-specific primers, and was cloned into pET28a vector by restriction-free cloning strategy[20].

For creating the point mutation C238A, a forward primer containing this mutation was used along with the T7 reverse primer to amplify the mutated part of the gene from pET28a containing the wild-type *Ec3-MST*. This amplicon was then used to clone the mutation into the pET28a containing the wild-type *Ec3-MST* using restriction-free cloning strategy. The clones were confirmed by sequencing analysis using vector-specific T7 primers. This work was completed with the help of Ms Rupali Sathe and Ms Yamini Mathur, IISER – Pune.

The wild-type or C238A *Ec3-MST*-containing pET28a was first treated with DpnI (to cleave methylated/hemi-methylated DNA) at 37^{o}C for 2.5 hrs, and then transformed into *E. coli* K-12 DH5-α strain for cloning, and subsequently into *E. coli* BL21 (DE3) for overexpression of the respective protein. Overnight primary cultures of *E. coli* BL21 (DE3) cells containing either wild-type or C238A *Ec3-MST* pET28a were grown in Luria-Bertani (LB) medium with kanamycin (25 µg/mL) at 37^{o}C at 180 rpm shaking. Then they were transferred into large secondary cultures of LB medium with kanamycin (25 µg/mL) at 1% v/v inoculum. These were grown at 37^{o}C at 180 rpm shaking to OD_{600} 0.6. Protein overexpression was then induced with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG), following which the cultures were incubated at 18°C for 18 hrs at 180 rpm shaking.

The bacteria were harvested from the large cultures by centrifugation at 6500 rpm at 4^{o}C. The cell pellets obtained were flash frozen in liquid nitrogen and stored at -80^{o}C till further use.

For protein purification, the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0) containing 300 mM NaCl, 10 mM $MgCl_2$, 50 µM Phenylmethylsulfonyl fluoride (PMSF), and 0.025% β-mercaptoethanol (BME) and were lysed by sonication (1 s “on”, 3 s “off”; 10 mins total “on” time; 60% amplitude). The cell lysate was clarified by centrifugation at 14,000 rpm at 4^{o}C for 30 mins. Then the lysate was passed through a 0.22 µm syringe filter and loaded onto a Ni-NTA column pre-equilibrated with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025% BME and 10 mM imidazole. The column was then washed with 50 mM Tris-HCl, pH 8.0, containing 300 mm NaCl, 0.025% BME and 50 mM imidazole. The protein was

eluted in 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025% BME and 250 mM imidazole. The purified proteins were buffer-exchanged in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 0.025% BME using Econo-Pac 10 DG pre-packed desalting columns (BioRad), and were stored with 15% v/v glycerol in -80°C after flash freezing in liquid nitrogen.

Sr. No.	Primer name	Purpose	Primer Sequence (5' → 3')
1.	EcMST_PCR-1_F	Cloning wild-type F	ATGTCCACGACATGGTTTGTAGGAGCCGAC
2.	EcMST_PCR-1_R	Cloning wild-type R	TTATTTCACTGGCTCAACCGGTAAATCTGC
3.	EcMST_pET28a_F	Cloning bipartite F	GTGCCGCGCGGCAGCCATATGTCCACGACATGGTTTGTAGGAGCCGAC
4.	EcMST_pET28a_R	Cloning bipartite R	CGACGGAGCTCGAATTCGGATCCTTATTTCACTGGCTCAACCGGTAAATCTGC
5.	EcMSTC238A_F	Cloning C238A F	AAACCAATTATCGTCAGCGCGGGCTCTGGTGTAACGGCA
6.	T7 Terminator_R	Cloning C238A R	GCTAGTTATTGCTCAGCGG

Table 2: Primers used for cloning.

Methylene blue assay for detection of formation of H_2S enzymatically[10,21,22]: Stocks of all compounds (in DMSO), bovine serum albumin (BSA) (in deionised water), and Dithiothreitol (DTT) (in deionised water) were prepared fresh each time just before the start of the experiment. To 1.5 mL tubes, sequentially, deionised water and 100 µL of 1 mg/mL of BSA were added first. Then 200 µL of 1 M HEPES-NaOH pH 7.4 buffer, 250 µL of 100 mM DTT, and 10 µL of 40 mM $Zn(OAc)_2.2H_2O$ (prepared in deionised water) were added. Based on desired concentration, the substrate was added from its DMSO stock, followed by addition of DMSO to keep its final concentration in each reaction mixture to 2%. Lastly, 0.5 µM of *Ec*3-MST (final concentration) (wt or C238A mutant) is added to start the reaction. The reaction mixtures (total volume of 1000 µL) were incubated at 37°C without any shaking.

At the chosen time points, aliquots of 200 µL were taken from each tube and transferred to a 0.6 mL tube. To this was added 200 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 200 µL of *N*,*N*–Dimethyl-*p*-phenylenediamine sulfate (DMPPDA) (20 mM

stock in 7.2 M HCl). The reaction mixtures were incubated at 37 °C for 30 mins to allow the formation of the methylene blue dye. After completion of the reaction, aliquots were transferred to a 96-well plate (150 µL/well) and the absorbance values were measured at 676 nm using microplate reader (Thermo Scientific VarioskanFlash).

Methylene blue assay for detection of formation of H_2S from compound 1c when incubated with esterase[23]: For attempting to hydrolyze the thioacetate group enzymatically *in situ*, porcine liver esterase (Sigma-Aldrich) stock (1000 U/mL) was prepared in deionised water and stored on ice till further use. Stocks of compound **1c** (in DMSO), bovine serum albumin (BSA) (in deionised water), and Dithiothreitol (DTT) (in deionised water) were prepared fresh each time just before the start of the experiment. To 1.5 mL tubes, sequentially, deionised water and 100 µL of 1 mg/mL of BSA were added first. Then 1 µL of esterase, 200 µL of 1 M HEPES-NaOH pH 7.4 buffer, 250 µL of 100 mM DTT, and 10 µL of 40 mM $Zn(OAc)_2.2H_2O$ (prepared in deionised water) were added. Based on desired concentration, the substrate was added from its DMSO stock, followed by addition of DMSO to keep its final concentration in each reaction mixture to 2%.

For co-incubation of **1c** with esterase, 0.5 µM of *Ec*3-MST wt (final concentration) to start the reaction immediately.

For allowing pre-incubation of **1c** with esterase, the reaction mixture was incubated at 37°C without any shaking for 30 mins, followed by addition of 0.5 µM of *Ec*3-MST wt (final concentration) to start the reaction.

The reaction mixtures (total volume of 1000 µL) were incubated at 37°C without any shaking. At the chosen time points, aliquots of 200 µL were taken from each tube and transferred to a 0.6 mL tube. To this was added 200 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 200 µL of *N*,*N*–Dimethyl-*p*-phenylenediamine sulfate (DMPPDA) (20 mM stock in 7.2 M HCl). The reaction mixtures were incubated at 37 °C for 30 mins to allow the formation of the methylene blue dye. After completion of the reaction, aliquots were transferred to a 96-well plate (150 µL/well) and the absorbance values were measured at 676 nm using microplate reader (Thermo Scientific VarioskanFlash).

Methylene blue spectroscopic profile for compound 1e, potassium thioacetate, and 4'-Nitroacetophenone: For assessing that the characteristic methylene blue signal for the unnatural substrates was not coming from thioacetate anion or from the acetophenone formed as by-product, 200 µM of each of compound **1e**, potassium thioacetate, and 4'-Nitroacetophenone were incubated with 200 mM HEPES-NaOH pH 7.4 buffer, 100 µg/mL BSA, 25 mM DTT, 400 µM $Zn(OAc)_2$, and with or without 1 µM *Ec*3-MST. The final DMSO concentration was kept to 2% in each case. The blank for the experiment had only enzyme present, with no added test compound. The reaction mixtures were incubated at 37°C without any shaking for 30 mins.

At the end of the incubation time, an aliquot of 200 µL was taken from each tube and transferred to a 0.6 mL tube. To this was added 200 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 200 µL of *N*,*N*–Dimethyl-*p*-phenylenediamine sulfate (DMPPDA) (20 mM stock in 7.2 M HCl). The reaction mixtures were incubated at 37 °C for 30 mins to allow the formation of the methylene blue dye. After completion of the reaction, aliquots were transferred to a 96-well plate (150 µL/well) and the absorption spectra were recorded from 550 nm to 800 nm using microplate reader (Thermo Scientific VarioskanFlash).

Fluorescence spectra of compounds 1e, 2, and 4, as well as DTT: For this experiment 20 mM stock solutions of **1e**, **2**, and **4** were prepared in DMSO. A 100 mM stock solution of DTT was made in deionised water. Individual reaction mixtures were then set up in 200 mM HEPES-NaOH pH 7.4 buffer each containing either 25 mM DTT or 150 µM of any of **1e**, **2**, or **4**. The blank for this experiment contained only buffer with no added compound or DTT. The final DMSO concentration was kept to 2% in each case. The samples were mixed well. Then aliquots from each reaction mixture were transferred to a 96-well plate (150 µL/well) and the emission spectrum was recorded from 300 nm to 400 nm with λ_{ex} = 287 nm, using a microplate reader (Thermo Scientific VarioskanFlash).

Intrinsic fluorescence spectra of *Ec*3-MST wt and C238A mutant when co-incubated with compounds 2, 1e, and 4[24]: For this experiment an enzyme stock of 20 µM of *Ec*3-MST was prepared in 200 mM HEPES-NaOH pH 7.4 buffer. Then,

separately, stock solutions of each of compounds **2** (5 mM), **1e** (10 mM), and **4** (10 mM) were made fresh in DMSO. A 100 mM stock solution of Dithiothreitol (DTT) was made in deionised water. Using these stocks, dilutions of each compound (150 µM final concentration for **2**; 300 µM for **1e** and **4**) were made in 200 mM HEPES-NaOH pH 7.4 buffer, both with and without 50 mM DTT. The pair of blanks for this experiment contained no added compound, one with DTT and the other without it. The final DMSO concentration was kept to 4% in each case.

From the 20 µM enzyme stock, aliquots of 75 µL/well were transferred to a 96-well plate in triplicate for each reaction mixture. After this, aliquots of 75 µL/well were transferred from each reaction mixture to the wells containing 20 µM of *Ec*3-MST. The samples were mixed well while avoiding pipetting in bubbles. Now in each well we have 10 µM of *Ec*3-MST against either just buffer or 75 µM of **2**, or 150 µM of either **1e** or **4**, with or without 25 mM DTT, all in triplicate thus allowing the enzyme three independent opportunities to interact with the additives. Using a microplate reader (PerkinElmer EnSight), emission spectra were recorded from 302 nm to 402 nm with λ_{ex} = 287 nm.

^{1}H-NMR experiment to monitor hydrolysis of compound 1a in buffer[13]: Based on a previously reported protocol, 0.1 M phosphate buffer (pH 7.4 or pD 6.99) was made in D_2O using K_2HPO_4 and KH_2PO_4 salts. Then 5-6 mg (~0.025 mmoles) of **1a** was taken in each of two 1.5 mL tubes. To a third tube was added 9-10 mg (~0.118 mmoles) of anhydrous sodium acetate. Then 700 µL of d_6-DMSO was added to each tube and their contents were dissolved in it (sodium acetate was partially miscible). To one of the tubes containing d_6-DMSO solution of **1a**, was added 300 µL of D_2O. To each of the other two tubes, one containing d_6-DMSO solution of **1a** and the other sodium acetate, was added 300 µL of the phosphate buffer made in D_2O. Finally, *tert*-Butanol was added to each tube (1/3rd of the moles of the compound taken in each tube) as an internal standard (δH = 1.65 ppm). Each sample was mixed thoroughly and submitted for ^{1}H-NMR. Since the solvent system used is 70% d_6-DMSO/D_2O, the NMR instrument was locked manually using the frequency of d_6-DMSO (that being in excess of the two deuterated solvents). The NMR spectra were

recorded 2 hrs and 20 hrs post sample preparation. Formation of acetate group on hydrolysis was monitored at δH = 2.22 ppm.

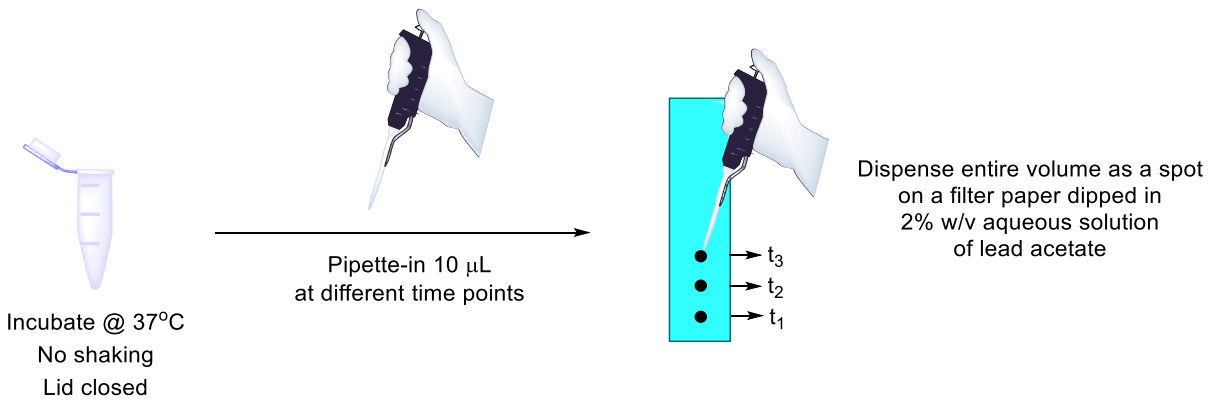
Iodoform test to qualitatively detect enzymatically produced acetophenone[25,26]**:** 2 mg of **1c** was taken in two 1.5 mL tubes and dissolved in 20 µL of DMSO. To each tube was added 250 µL of 100 mM DTT, 200 µL of 1 M HEPES-NaOH pH 7.4 buffer, 100 µL of 1 mg/mL of bovine serum albumin (prepared in deionised water) and 10 µL of 40 mM $Zn(OAc)_2.2H_2O$ solution (prepared in deionised water). To one of the tubes was added 7 µM of *Ec*3-MST (final concentration). The final volume in both tubes was made to 1000 µL by using deionised water. Both tubes were then incubated at 37ºC without shaking for 3 hrs.

After incubation, aliquots of 250 µL were taken from each tube and transferred into separate 2 mL tubes. 5-6 drops of methanol was added to each tube to allow complete dissolution of the partially miscible components. Then 330 µL of 20% NaOH and 1000 µL of 20% iodine solution (made by dissolving 10 g potassium iodide in 20 mL deionised water, followed by adding 5 g of iodine and mixing until dissolved) were added to each 2 mL tube sequentially. Add more iodine solution dropwise till deep orange color persists in both tubes. Incubate both tubes at 60ºC with shaking at 1000 rpm for 10 mins. In case the color discharges in the meanwhile, add more iodine solution dropwise till the deep orange color is restored. The color should persist during the full 10 mins of incubation. After incubation, discharge the deep orange color completely by dropwise addition of 20% $Na_2S_2O_3$ solution (made in deionised water). A yellow precipitate appears in the tube containing the sample from the reaction with the added *Ec*3-MST, while no turbidity is seen in the non-enzymatic reaction. Centrifuge both tubes at 10,000 rpm for 10 mins and observe.

Concomitantly, another pair of aliquots of 200 µL each were taken from the original reaction mixtures and added to 0.6 mL tubes. To this was added 200 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 200 µL of DMPPDA (20 mM stock in 7.2 M HCl). The reaction mixtures were incubated at 37°C for 30 min to allow the formation of the methylene blue dye. After completion of the reaction, aliquots were transferred to a 96-well plate (150 µL/well) and the absorption spectra were recorded from 550 nm to 800 nm using microplate reader (Thermo Scientific VarioskanFlash).

Lead acetate paper assay for detection of enzymatic production of H_2S[27]: In order to prepare lead acetate paper strips, 2% solution of $Pb(OAc)_2.3H_2O$ was made in deionised water. Filter paper strips (5 mm x 70 mm) were cut. Around 10 paper strips were sunk in 14 mL of the lead acetate solution in a Falcon tube and rocked for 30 mins at least. The paper strips were then fished out and allowed to air dry overnight under the laminar flow of the chemical hood. Once dried, these strips were collected and stored in a dry Falcon for later use. Caution was used while handling lead acetate as it is toxic on inhalation.

For the assay, stocks of 10 mM DTT (in deionised water), 10 mM sodium sulfide (in deionised water), and 100 mM **2** (in DMSO) were prepared fresh. Samples were prepared in 30 mM HEPES-NaOH pH 7.4 buffer such that each one has a different combination of 1 mM DTT, 500 µM **2**, and 1 µM of *Ec*3-MST in order to assess that no H_2S will be formed unless all the three components are present. Positive control for the experiment had 1 mM sodium sulfide (prepared in deionised water) added in the same buffer. The final DMSO concentration was kept to 2% in each case. The samples were then incubated at 37°C without shaking.



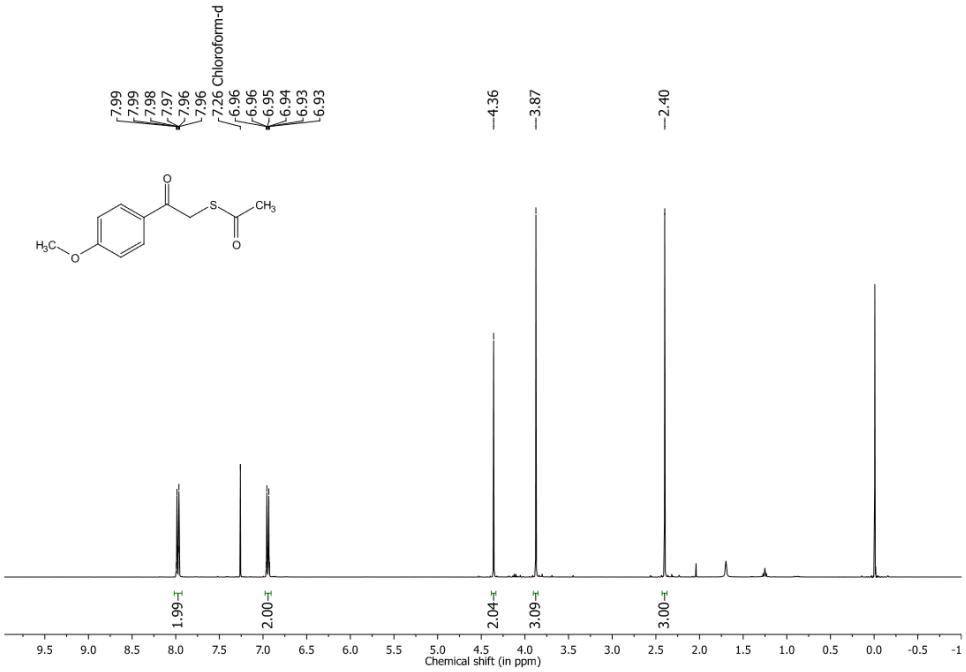
At different time points, aliquots of 10 µL were taken from each reaction mixture using a micropipette and were spot directly on the respective filter paper. If H_2S is present in the solution phase, then it should immediately react with the lead acetate of the filter paper and produce a black stain of lead sulfide.

HPLC to monitor the progress of reactions: Synthesis and characterization of compounds **6a** and **6b** was done by Ms Prerona Bora and Mr Suman Manna, IISER – Pune, respectively. Both compounds were used as is in this study. The HPLC study was carried out with both their aid.

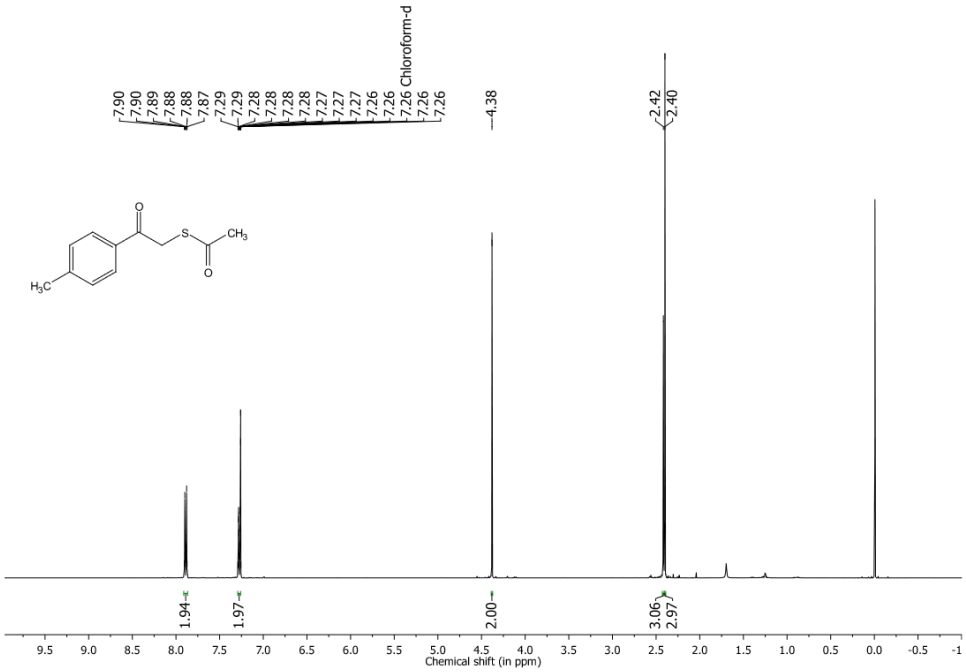
A stock solution of **1c** (10 mM) was prepared in DMSO whereas the stocks of DTT, GSH, or porcine liver esterase were prepared fresh in deionised water. The reaction mixtures (total volume 1000 µL) were prepared in 200 mM HEPES-NaOH pH 7.4 buffer as required. Typically, 100 µM of **1c**, 25 mM DTT, 25 mM GSH, 1 U/mL esterase, or 0.5 µM of *Ec*3-MST were used. Final concentration of DMSO was kept to 2%. This mixture was incubated at 37°C. The reaction mixture was filtered through a 0.22 µm syringe filter and injected (25 µL) in a high performance liquid chromatography (HPLC) attached with a UV detector (absorbance at 250 nm). C-18 reversed phase column (Phenomenex) was used. The mobile phase was water: acetonitrile with a gradient starting at 40:60 % → 0 min, 40:60 % to 30:70 % → 0 - 5 min, 30:70 % to 20:80 % → 5 - 10 min, 20:80 % → 10 - 13 min, 20:80 % to 40:60 % → 13 - 15 min, 40:60 % → 15 - 18 min was used at a flow rate of 1 mL/min.

NMR spectra:

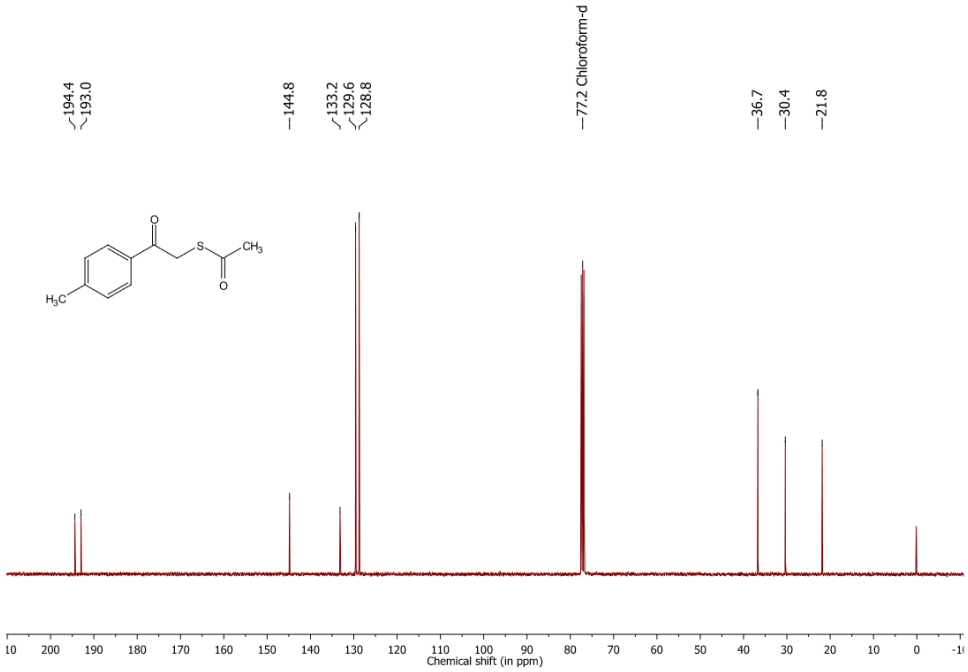
^{1}H-NMR of **1a** ($CDCl_3$; 400 MHz):



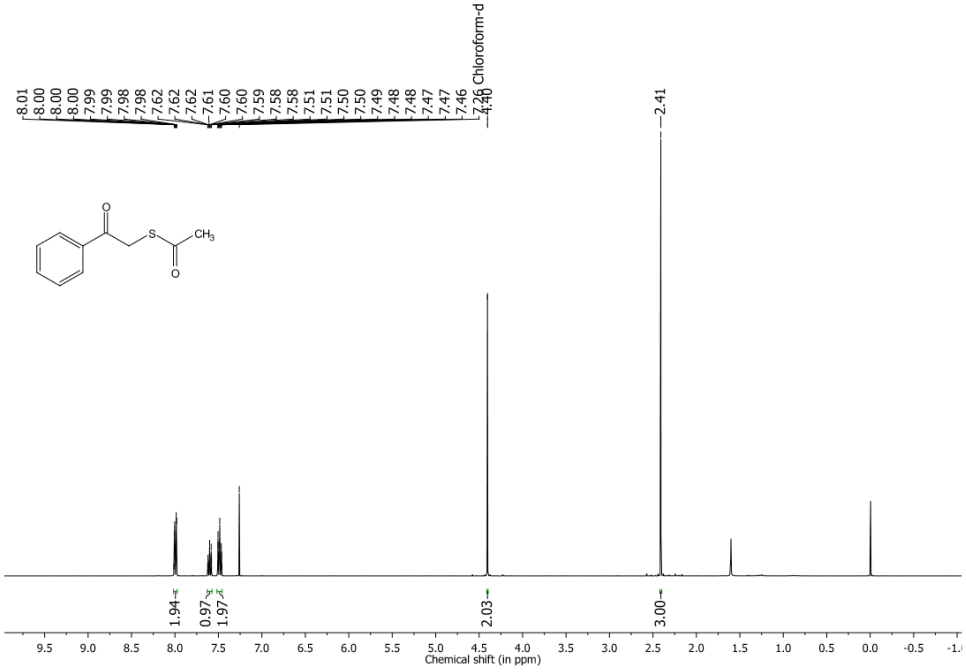
^{1}H-NMR of **1b** ($CDCl_3$; 400 MHz):



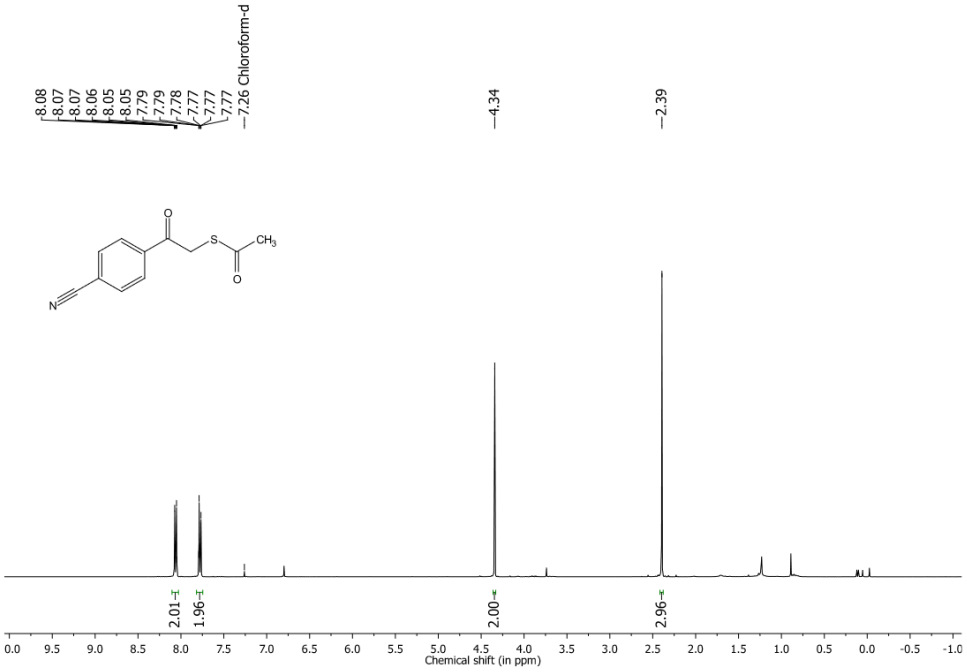
^{13}C-NMR of **1b** ($CDCl_3$; 100 MHz):



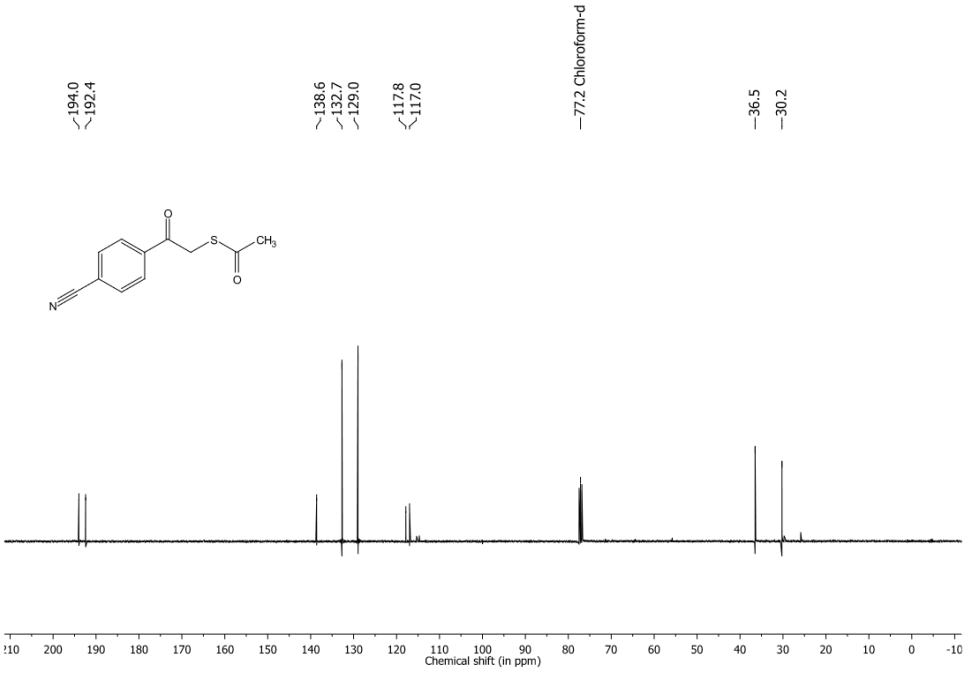
^{1}H-NMR of **1c** ($CDCl_3$; 400 MHz):



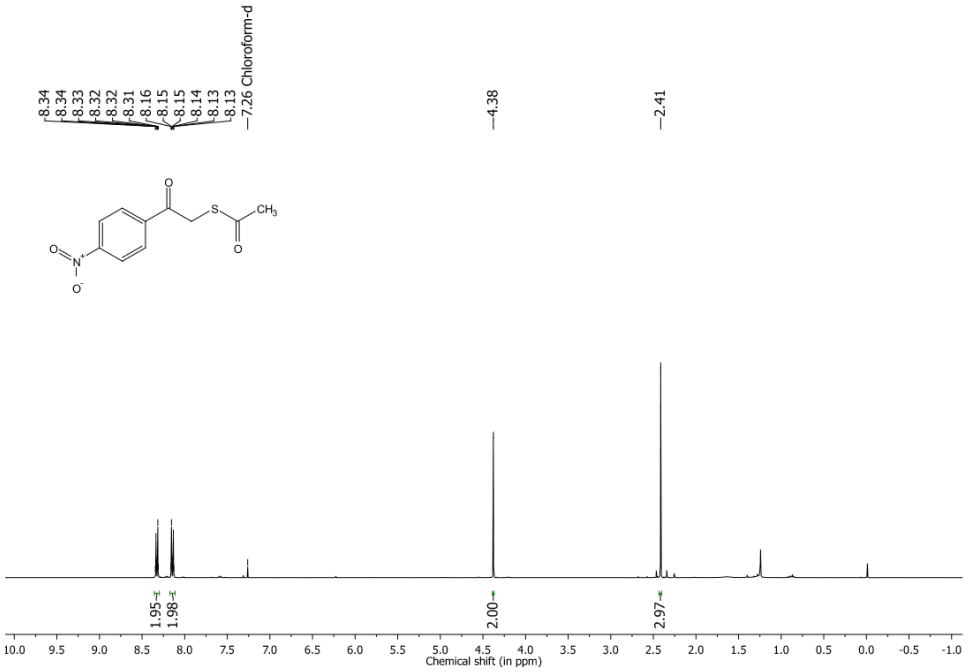
^{1}H-NMR of **1d** ($CDCl_3$; 400 MHz):



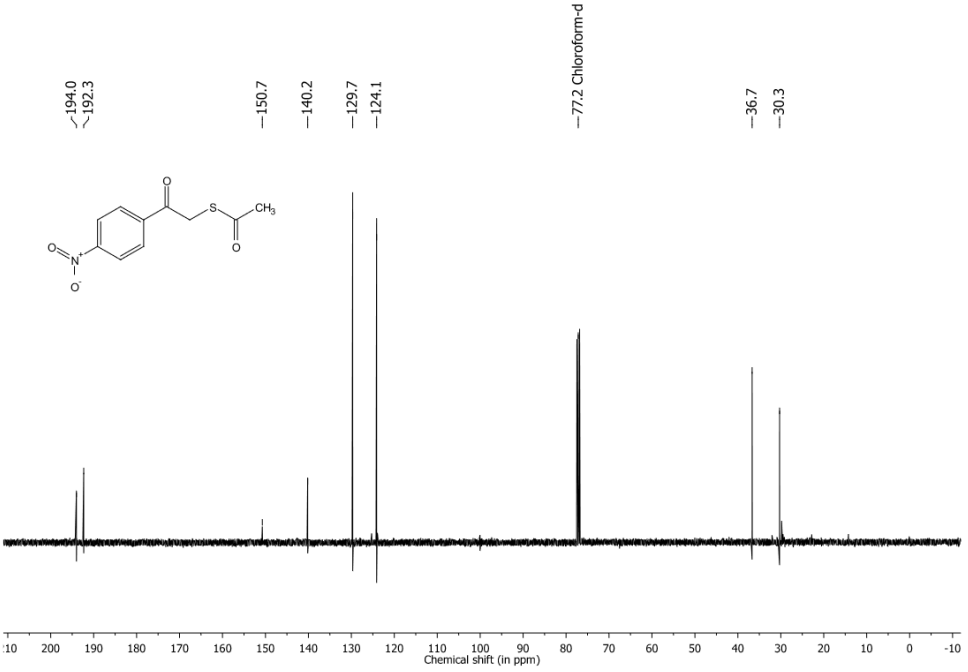
^{13}C-NMR of **1d** ($CDCl_3$; 100 MHz):



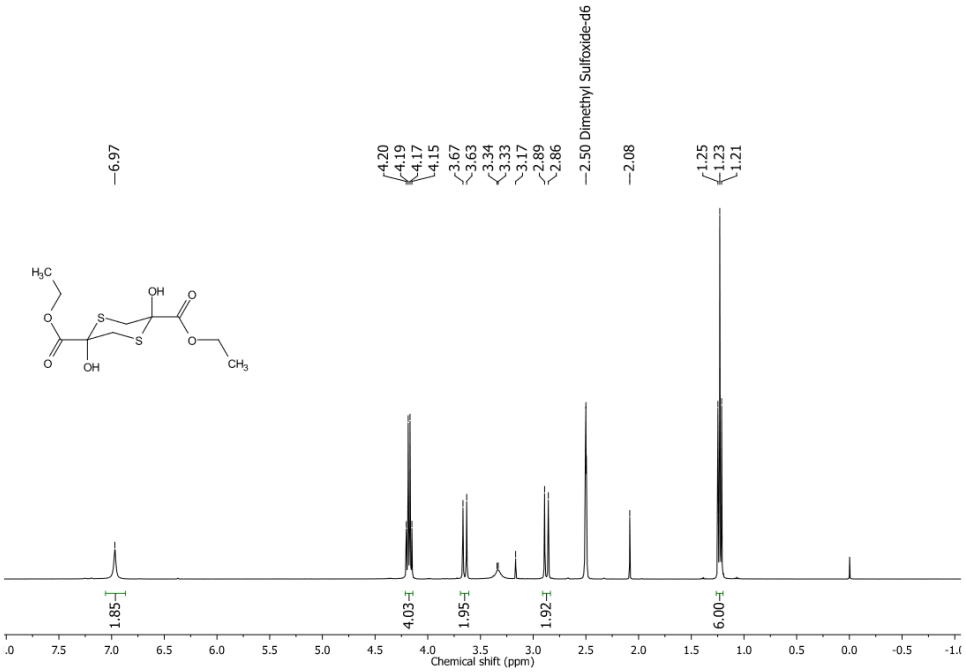
^{1}H-NMR of **1e** ($CDCl_3$; 400 MHz):



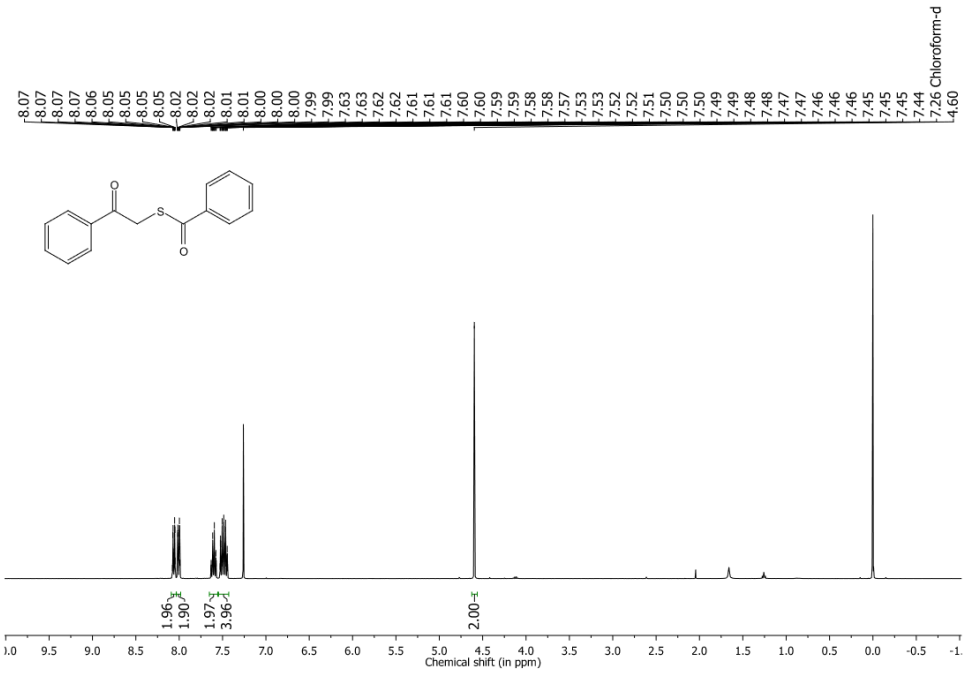
^{13}C-NMR of **1e** ($CDCl_3$; 100 MHz):



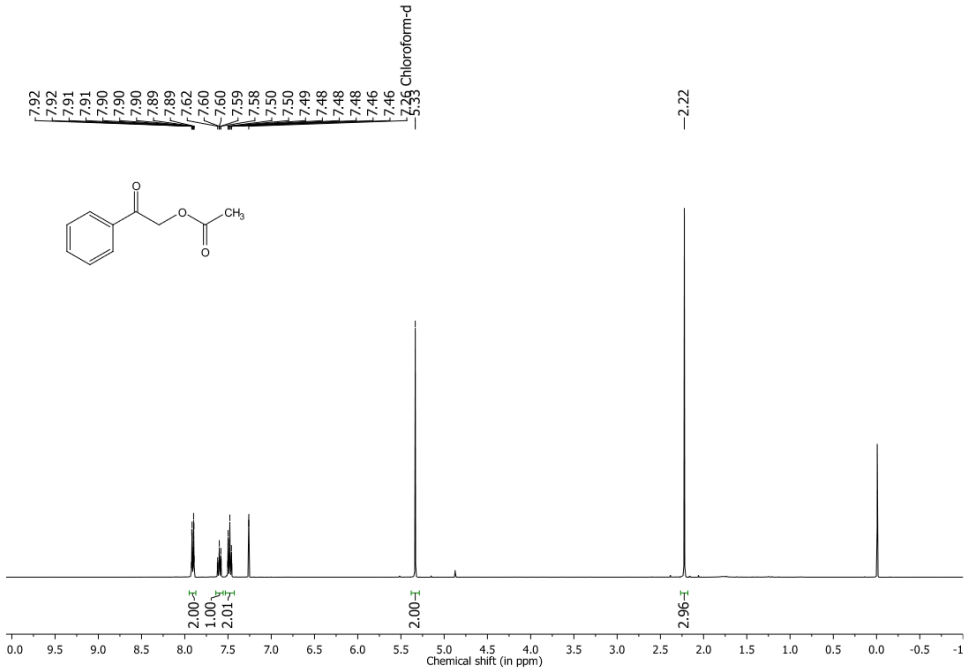
^{1}H-NMR of **2** (d_6-DMSO; 400 MHz):



^{1}H-NMR of **3** ($CDCl_3$; 400 MHz):



^{1}H-NMR of **4** ($CDCl_3$; 400 MHz):



Results and discussion:

Cloning, overexpression, and purification of proteins: *Ec*3-MST wild-type (wt) and C238A mutant were both cloned in pET28a vector system. Sequencing done using vector-specific T7 primers confirmed the clone.

After overexpression of the proteins, purification was done by Ni-NTA affinity chromatography. Post purification and desalting of the proteins, glycerol was added to make its final concentration to 15% v/v. The proteins were then divided into aliquots, flash frozen in liquid nitrogen, and then stored at -80^{o}C till further use.

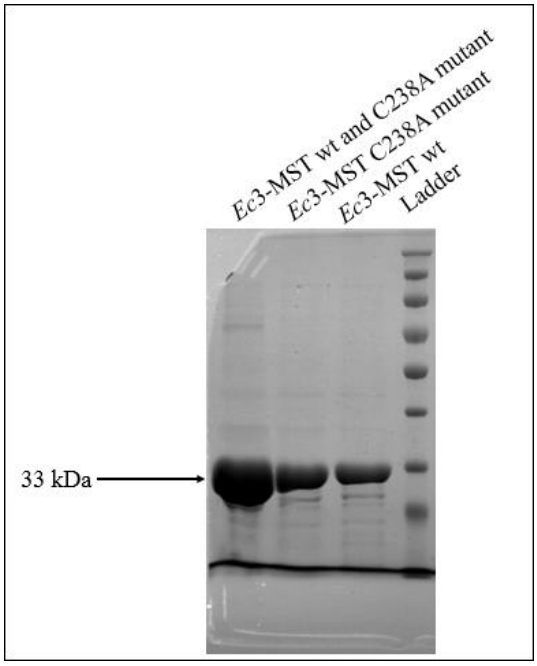
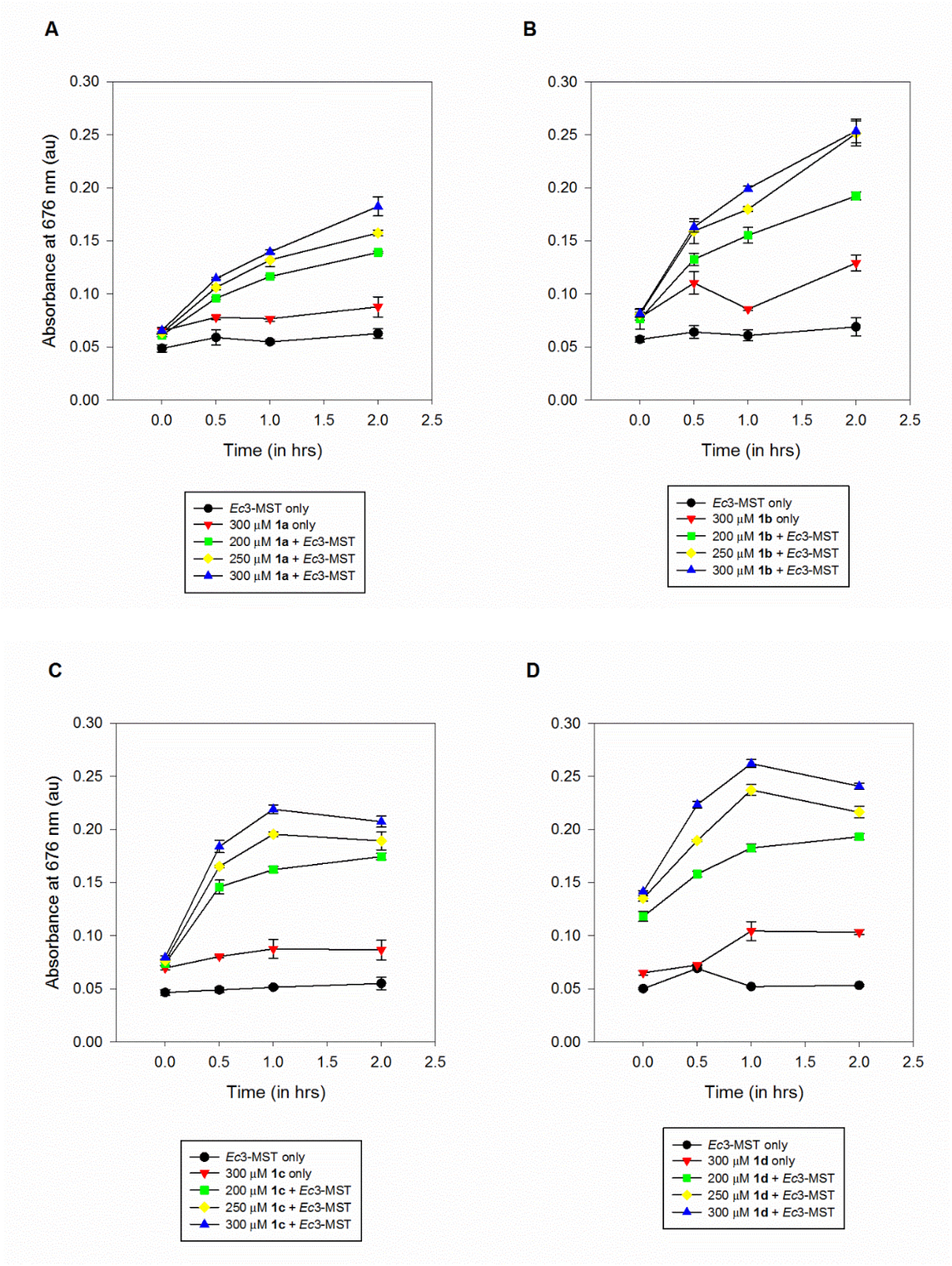


Figure 7: SDS-PAGE gel image for protein purification.

Monitoring enzymatic production of H_2S by turnover of the unnatural substrates: The compounds used in the study were synthesized and characterized thoroughly.

By conducting the methylene blue assay, it was observed that compounds **1a-e**, **2**, and **3** are turned over by *Ec*3-MST wild-type (wt) to produce H_2S, whereas **4** is not. Also, control reactions that have no *Ec*3-MST wt added to them show significantly less H_2S production from compounds **1a-e**, **2**, and **3**, indicating that it is indeed an enzymatic reaction (**Figure 8**; **A** to **H**).


A
Absorbance at 676 nm (au)
Time (in hrs)
Ec3-MST only
300 μM 1a only
200 μM 1a + Ec3-MST
250 μM 1a + Ec3-MST
300 μM 1a + Ec3-MST
B
Time (in hrs)
Ec3-MST only
300 μM 1b only
200 μM 1b + Ec3-MST
250 μM 1b + Ec3-MST
300 μM 1b + Ec3-MST
C
Absorbance at 676 nm (au)
Time (in hrs)
Ec3-MST only
300 μM 1c only
200 μM 1c + Ec3-MST
250 μM 1c + Ec3-MST
300 μM 1c + Ec3-MST
D
Time (in hrs)
Ec3-MST only
300 μM 1d only
200 μM 1d + Ec3-MST
250 μM 1d + Ec3-MST
300 μM 1d + Ec3-MST

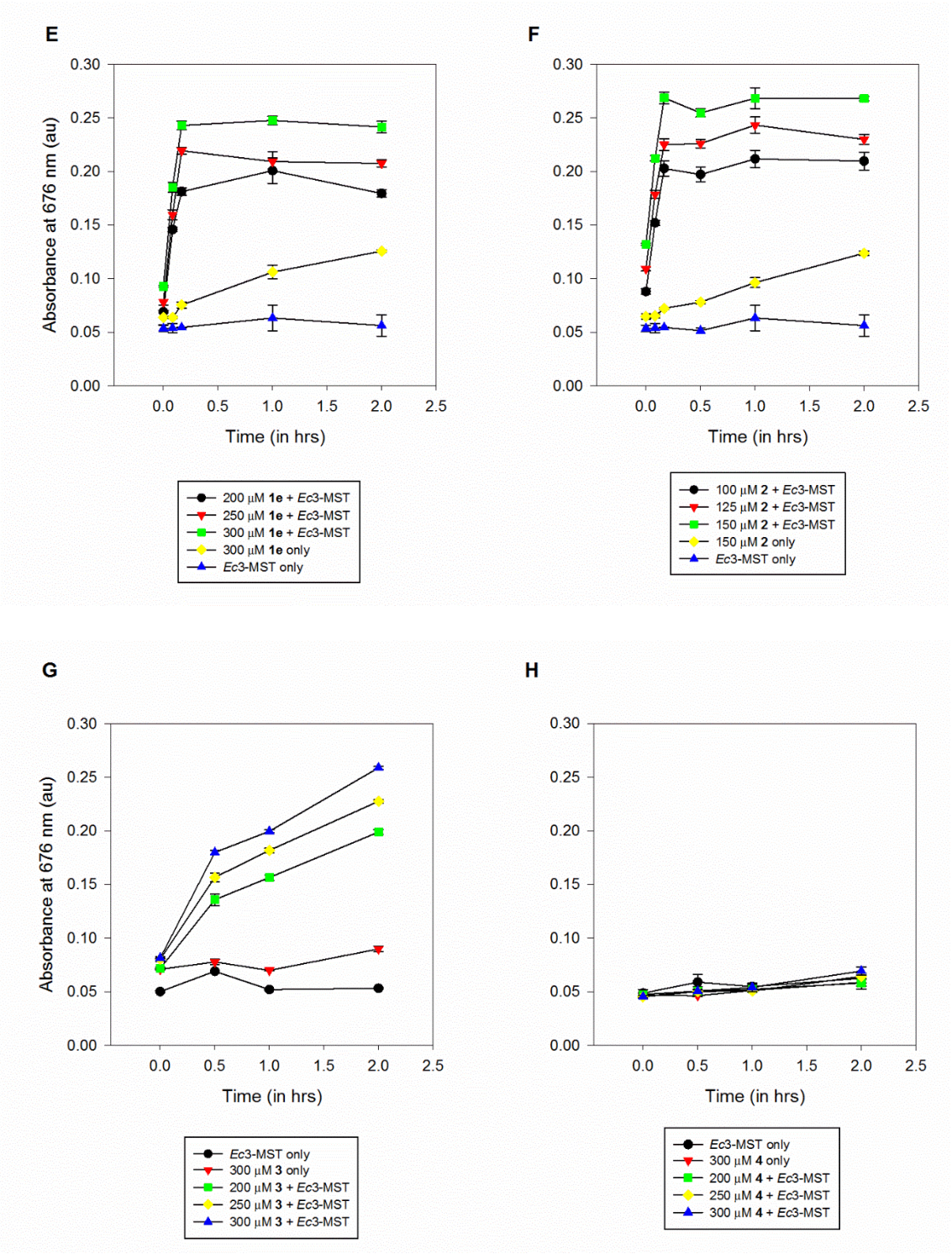


Figure 8: Methylene blue assay to assess the turnover of the compounds **1a-e**, **2**, **3**, and **4** by *Ec*3-MST.

Lead acetate assay was done using compound **2**, which independently assessed the enzymatic production of H_2S by *Ec*3-MST wt (**Figure 9**).

	Na_2S	Compound **2**	DTT	*Ec*3-MST
Conc.	1 mM	500 µM	1 mM	1 µM
A	+	-	-	-
B	-	-	+	-
C	-	+	-	-
D	-	-	-	+
E	-	-	+	+
F	-	+	-	+
G	-	+	+	-
H	-	+	+	+

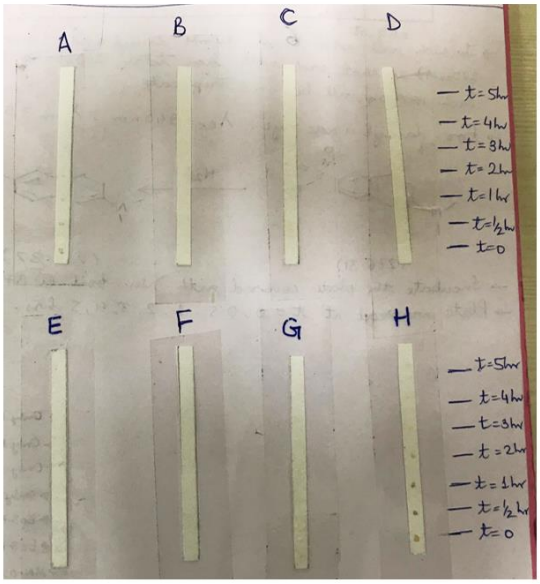
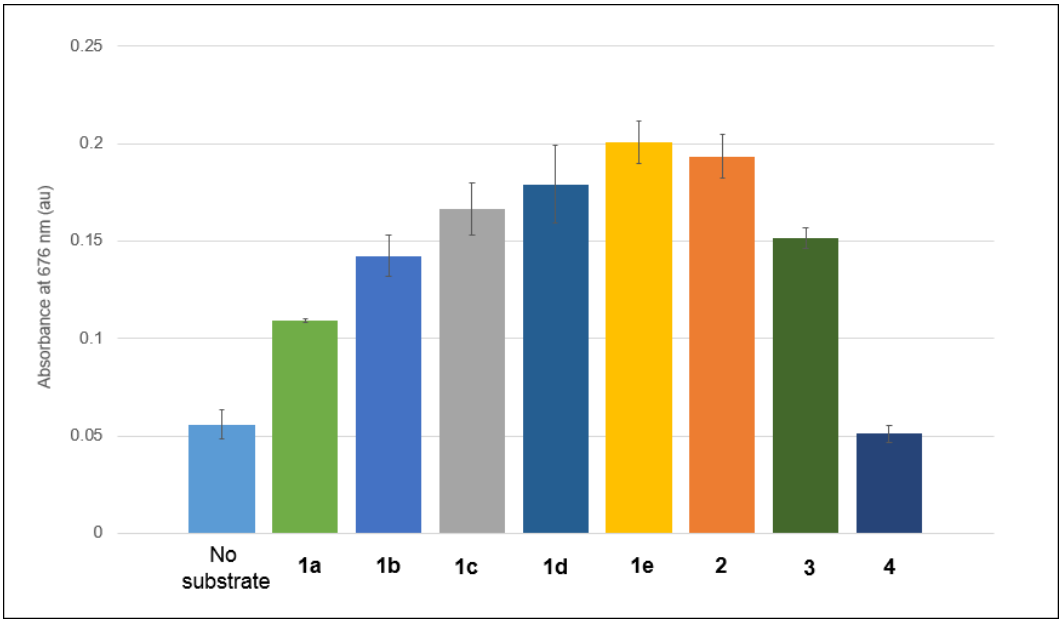


Figure 9: Lead acetate paper assay shows that the lead sulfide stain forms when *Ec*3-MST, DTT, and compound **2** are co-incubated (**H**).

Also, from their respective kinetic plots, it is apparent that the compounds (200 µM each) are turned over at varied rates by the enzyme (0.5 µM) to H_2S as monitored at 30 mins, but the rate profiles saturate beyond 1 hr. Based on the rate constant elucidated by fitting the kinetic plot to the pseudo first order rate equation (pseudo FOE) ($y = y_0 + a(1 - e^{-kx})$), it is apparent that among the unnatural substrates, the turnover of **1a** by *Ec*3-MST wt is the slowest, while that of **1e** is the fastest (**Figure 10**).



Compound code	Rate constant, k (h^{-1})	R^2 of fit to pseudo FOE ($y = y_0 + a(1 - e^{-kx})$)
1a	0.9 ± 0.1	0.9998
1b	1.0 ± 0.3	0.9941
1c	2.4 ± 0.3	0.9978
1d	1.5 ± 0.3	0.9967
1e	12.8 ± 1.8	0.9762
2	11.5 ± 2.8	0.9686
3	1.0 ± 0.4	0.9884

Figure 10: The different compounds **1a-e**, **2**, and **3** produce H_2S at different rates.

The rates of turnover of compounds **1a** and **1e** are varied. This could be due to the following reasons: (i) More electron withdrawing substitution (**1e**) on the phenyl ring is stabilising the transition state more than when an electron donating group (**1a**) is present; (ii) The enzyme active site has significantly different affinity for binding **1a** and **1e**.

To rule out the role of any other component of the compound used for enzymatic H_2S production, for example **1e**, we analyse the methylene blue profiles of 4'-Nitroacetophenone and Potassium thioacetate by recording the absorption spectra from 550 nm to 800 nm (**Figure 11**). It is observed that the methylene blue complex formed by the enzymatic turnover of **1e** is significantly different from that of 4'-Nitroacetophenone and Potassium thioacetate. This indicates that **1e** is indeed getting turned over by *Ec*3-MST wt to produce H_2S, as detected by the methylene blue assay.

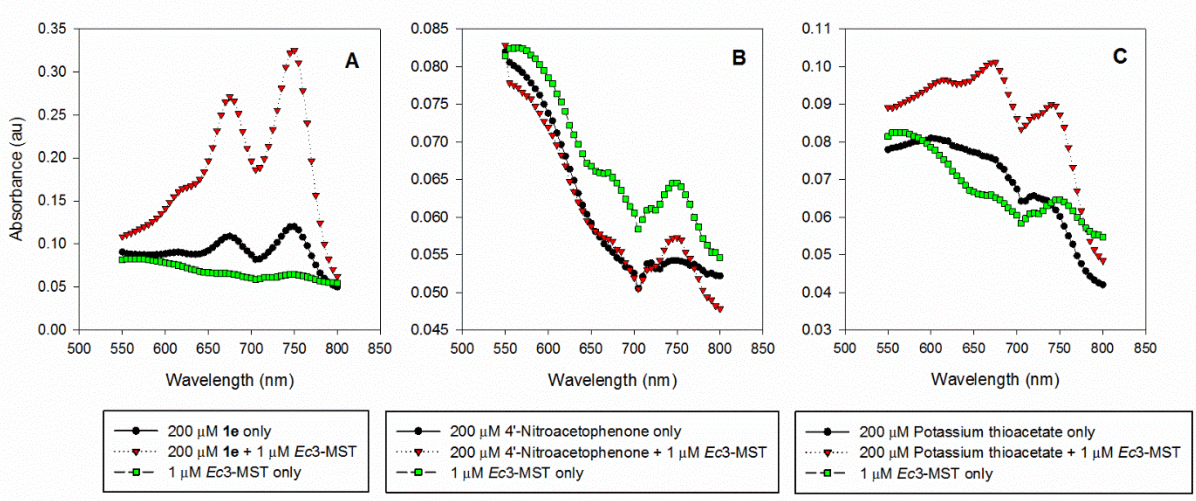


Figure 11: Methylene blue profiles of some control compounds.

Using site-directed mutagenesis, we designed the mutant *Ec*3-MST C238A that has the catalytic cysteine residue at 238th position (in wt) replaced with an alanine residue. Assessing the turnover of compounds **2**, **1c**, and **4** by *Ec*3-MST C238A mutant using the methylene blue assay, we find that only a basal level of H_2S is detected to be formed with **2** and **1c** (and none at all with **4**) as if the reaction was just proceeding *uncatalyzed* (**Figure 12**).

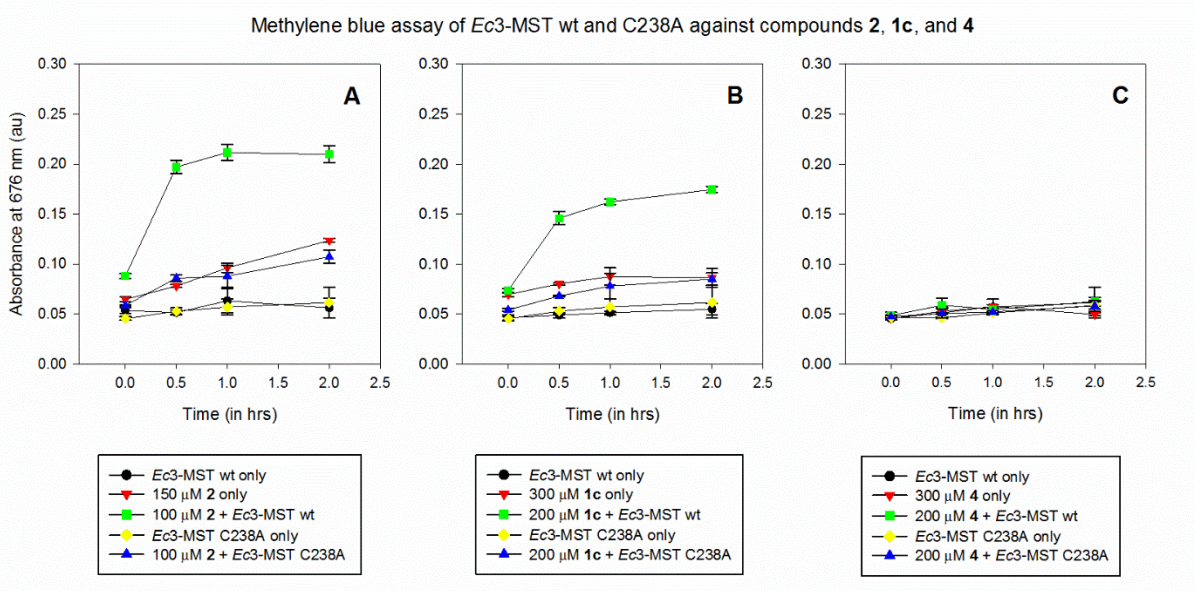


Figure 12: Methylene blue assay with *Ec*3-MST wt and C238A mutant against different compounds.

From the data discussed above, it is quite conclusive that each of compounds **1a-e**, **2**, and **3** are turned over by *Ec*3-MST wt to produce H_2S in presence of DTT. It is also clear that if any of these three components is absent, H_2S production is severely compromised. Of these, compounds **1a-e** and **3** are documented for the first time ever in this work as putative unnatural substrates for *Ec*3-MST wt. Now we will go on to elucidate the mechanism of enzymatic turnover of these unnatural substrates.

Thioacetate group is cleaved to generate the thiol *in situ*: Methylene blue assay was done using **1c** as the representative unnatural substrate of *Ec*3-MST. We wanted to test if the thioacetate group cleavage will contribute to the rate determining step. This was tested by adding porcine liver esterase to the reaction in order to cleave the thioacetate group (**Figure 13**).

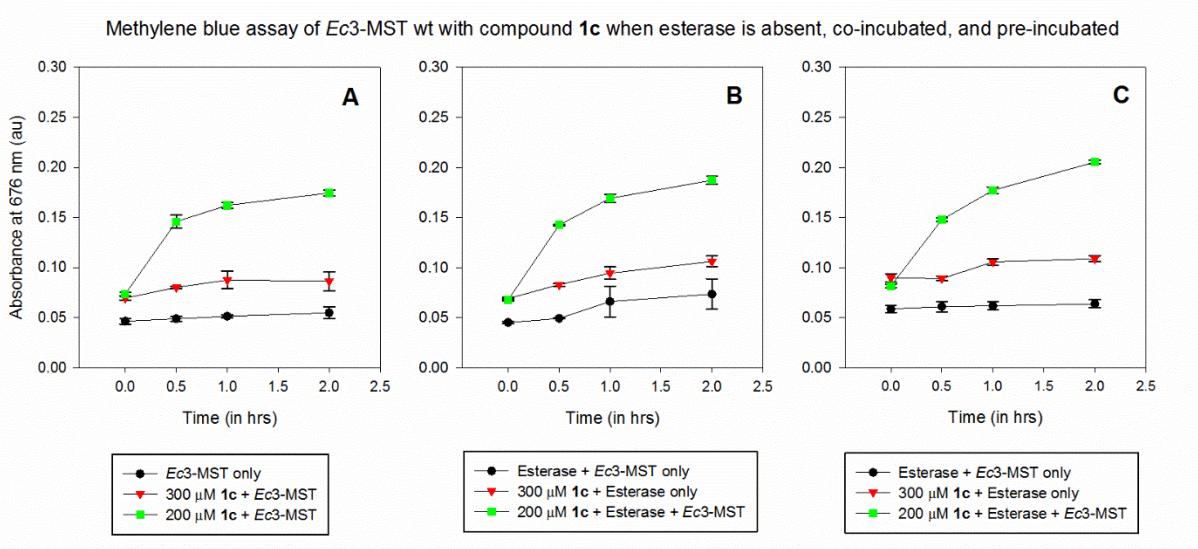


Figure 13: Methylene blue assay to study the effect of esterase on turnover rate of compound **1c**.

Whether esterase was absent (**A**; k = 2.4 ± 0.3 h^{-1}) or was co-incubated (**B**; k = 1.9 ± 0.1 h^{-1}) or was pre-incubated (**C**; k = 1.3 ± 0.1 h^{-1}) with **1c**, it was found that rate constants of turnover of **1c** by *Ec*3-MST to yield H_2S in all three cases were similar. This indicated two things: (i) Thioacetate group cleavage is perhaps *very* fast and does not contribute to determining the rate of reaction; and (ii) The components of the reaction mixture, *minus* esterase, are enough to cleave thioacetate group.

Next, we explore the possibility of a pH 7.4 buffer being sufficient to cleave off the thioacetate group by hydrolysis. For this, the progress of hydrolysis of thioacetate group to yield acetate group is monitored by ^{1}H-NMR at 400 MHz. Due to poor solubility of these unnatural substrates in water, we were compelled to use a large volume fraction of d_6-DMSO in order to solubilise the representative unnatural substrate **1a**. For the formation of acetate group, ^{1}H-NMR signal at δH = 2.22 ppm was monitored (**Figure 14**).

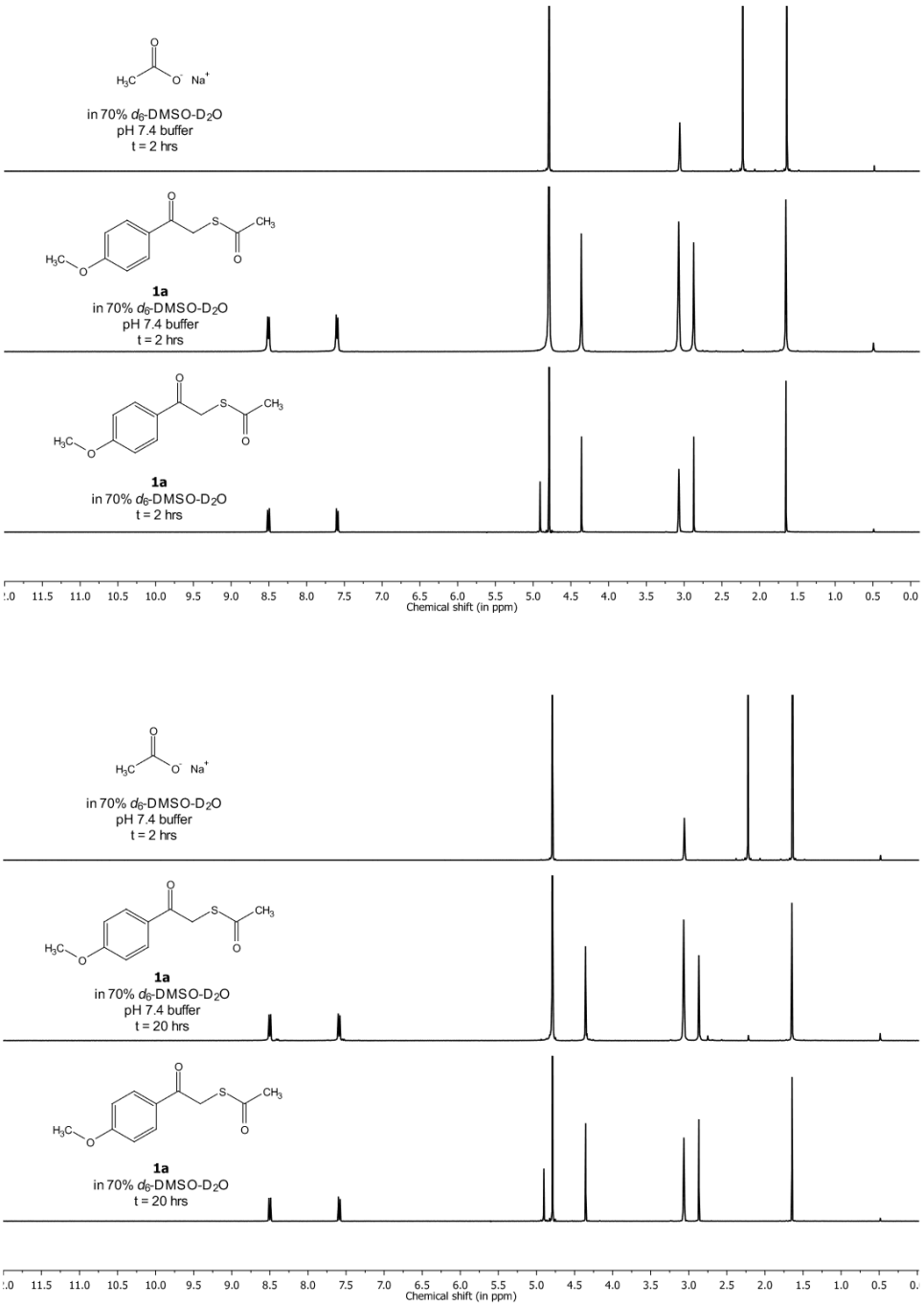
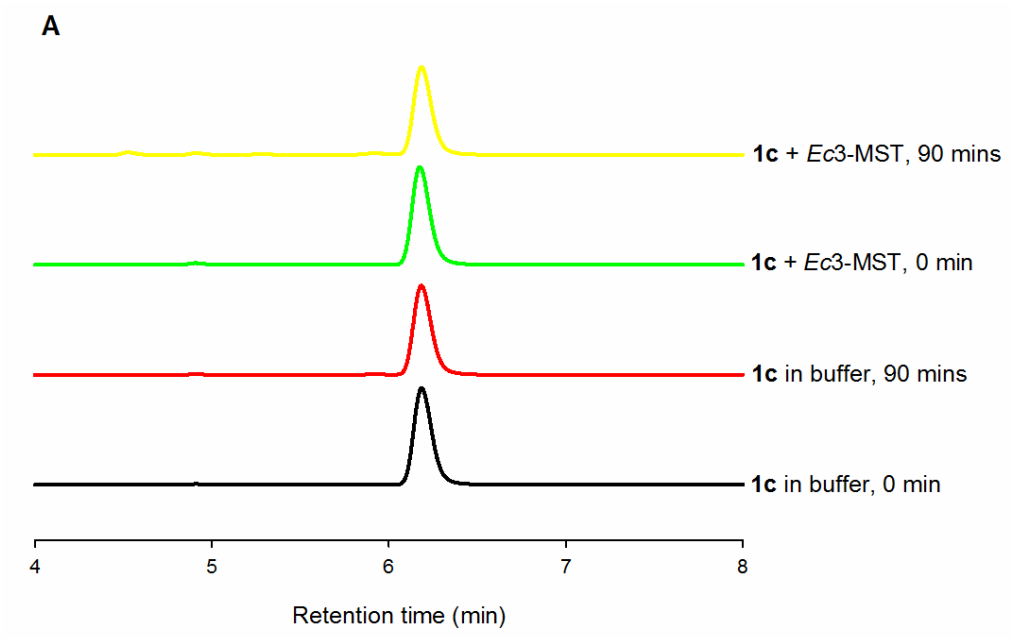


Figure 14: ^{1}H-NMR experiment to monitor the hydrolysis of thioacetate group in **1c** to acetate group under buffer conditions.

In the case where 70% d_6-DMSO/D_2O was used, no acetate formation was detected at all even after 20 hrs. However, in the case where 70% d_6-DMSO/pH 7.4 buffer in D_2O was used, acetate formation was detected at 2 hrs, which went on to increase at 20 hrs.

However, the acetate group formation in the latter case is only miniscule. This indicated that either (or both) of the two things was possible: (i) The conditions that were used for the ^{1}H-NMR experiment were significantly different than those used for the methylene blue assays (for example, 70% DMSO was used in the ^{1}H-NMR experiment versus only 2% DMSO as used in the methylene blue assays – less water, slower hydrolysis); or (ii) There is some other component that is present in the methylene blue reaction mixture (most likely, either *Ec*3-MST or DTT) that is able to speed up the thioacetate bond cleavage immensely.

Next, we will monitor the different reactions as they occur by HPLC in order to figure out what component of the reaction mixture is able to cleave off the thioacetate group. From the traces, we are able to conclude the thioacetate group of the representative compound **1c** (Retention time, RT = 6.2 min) is not cleaved significantly when only the buffer (200 mM HEPES-NaOH; pH 7.4) or 0.5 µM *Ec*3-MST is present (**Figure 15**; **A**). However, **1c** undergoes deprotection to yield the thiol (**6a**; RT = 5.9 min) almost immediately when 25 mM DTT is used (**Figure 15**; **B**).



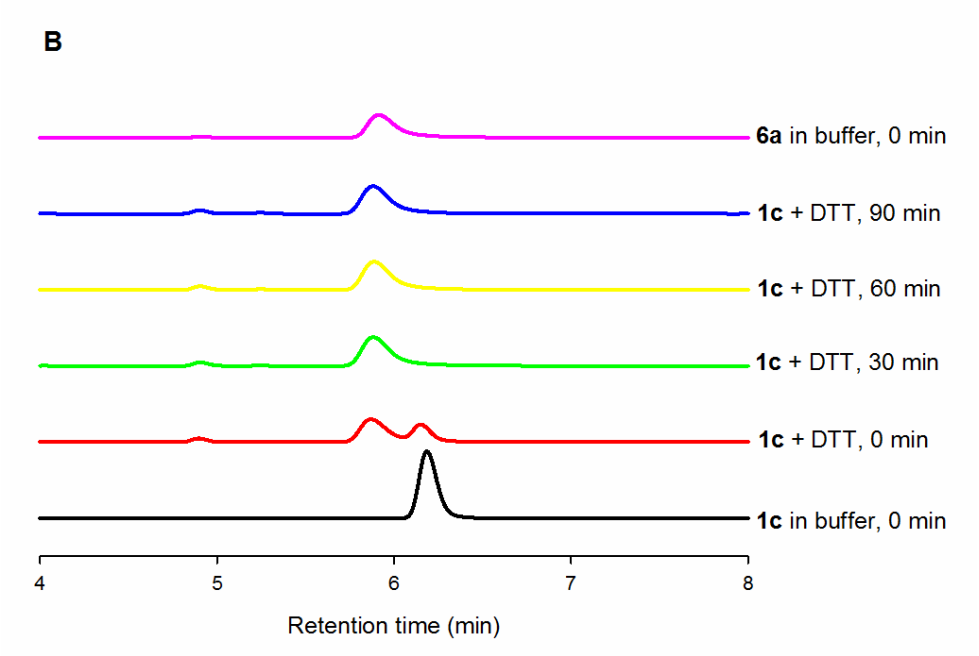
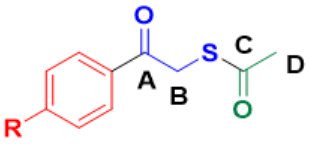


Figure 15: The thioacetate group of **1c** does not cleave significantly with only buffer or *Ec*3-MST, but undergoes immediate deprotection to yield the thiol when DTT is present.

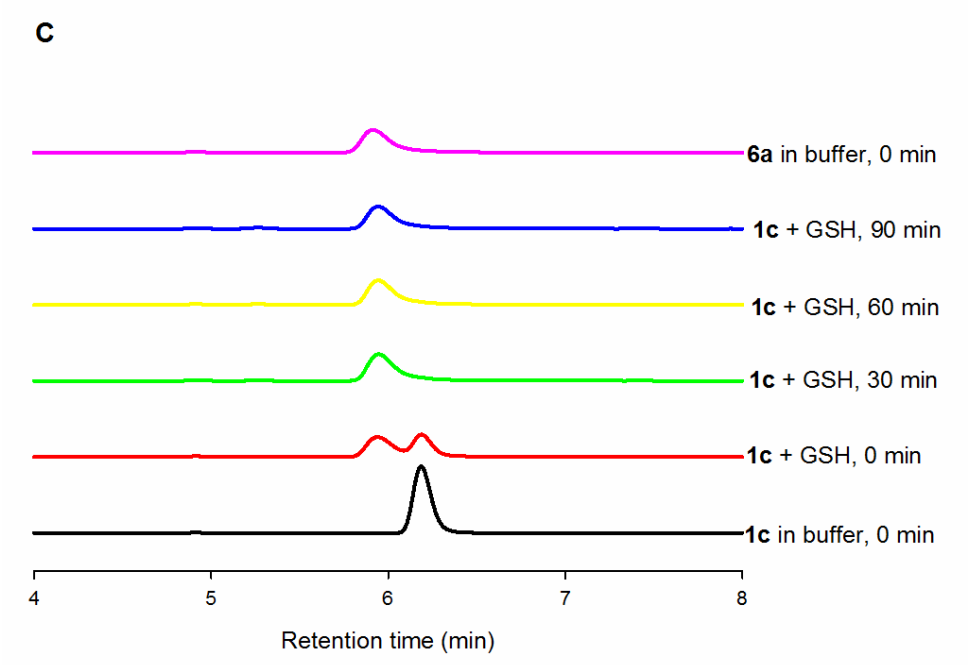
This feeds in very well with our analysis of chemical shifts of key carbon atoms in unnatural substrates **1a-e**. For this purpose, the spectra were recorded at 100 MHz in $CDCl_3$ and compiled in **Table 3**. The carbonyl group (marked as **C**) has almost identical chemical shift across all compounds **1a-e**, explaining that the substitution at the phenyl ring should not influence the rate of cleavage of the thioacetate group.



Compound code	-R	δ_A	δ_B	δ_C	δ_D
1a	$-OCH_3$	191.9	36.4	194.6	30.4
1b	$-CH_3$	193.0	36.7	194.5	30.4
1c	-H	193.4	36.8	194.4	30.4
1d	-CN	192.4	36.5	194.0	30.2
1e	$-NO_2$	192.3	36.7	194.0	30.3

Table 3: ^{13}C-NMR chemical shifts of key carbon atoms in compounds **1a-e**.

To grant that the unnatural substrates designed in this study can be used physiologically, we also established that the deprotection of the thioacetate group of **1c** happens even with 25 mM L-Glutathione (GSH) (**Figure 16**; **C**) as well as 1 U/mL porcine liver esterase (**Figure 16**; **D**) – both of which are present in abundance in a usual physiological setting. With a reducing environment absent when only esterase is present, it is further seen that the thiol eventually gets oxidized to the corresponding disulfide (**6b**; RT = 9.6 min).



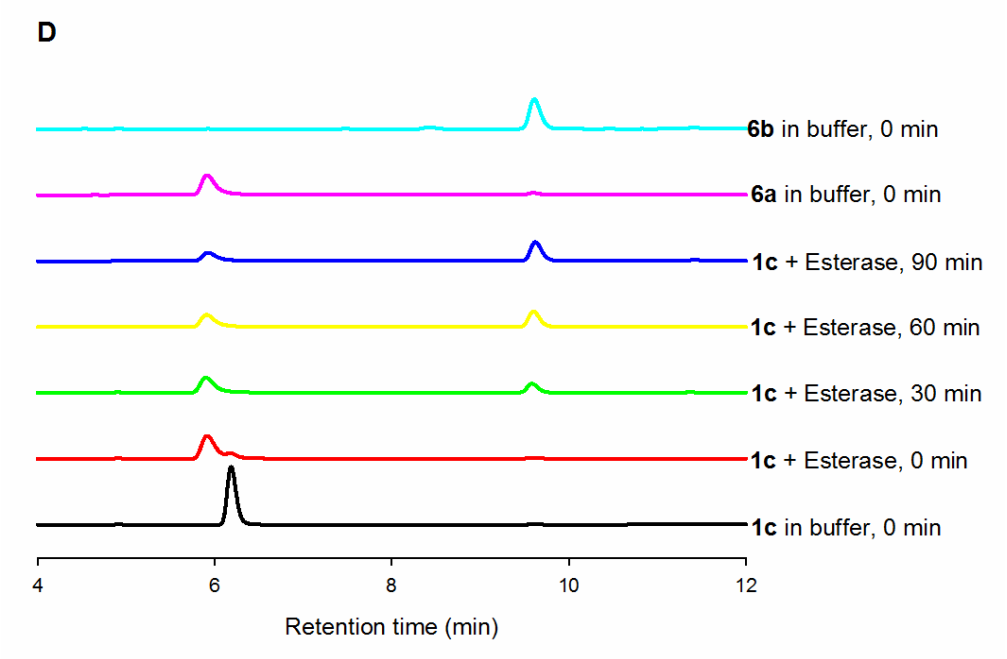
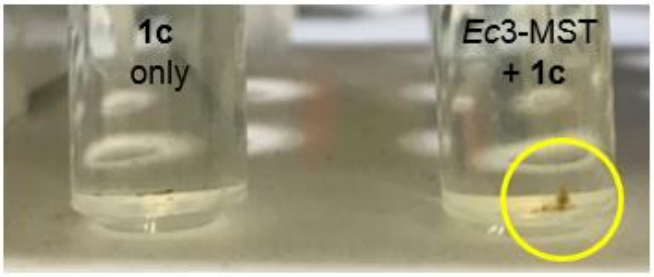


Figure 16: The thiol **6a** (or disulfide **6b**) is formed by deprotection of thioacetate group of **1c** when GSH or esterase is present.

Acetophenone is formed on turnover of 1c by *Ec*3-MST: The first qualitative indication as to the formation of acetophenone once **1c** is turned over by *Ec*3-MST came when the reaction mixture containing both **1c** and the enzyme showed a yellow precipitate of iodoform, while the reaction mixture that had no enzyme added to it gave no such precipitate (**Figure 17**; yellow circle). Methylene blue assay was carried out parallel to the iodoform test – while the former reaction mixture reacted with the methylene blue cocktail to give the characteristic absorption spectrum of the dye from 550 nm to 800 nm, the latter non-enzymatic reaction mixture yielded only miniscule levels of H_2S (**Figure 17**). This indicated that the formation of H_2S and acetophenone are concomitant and chiefly enzymatic events.



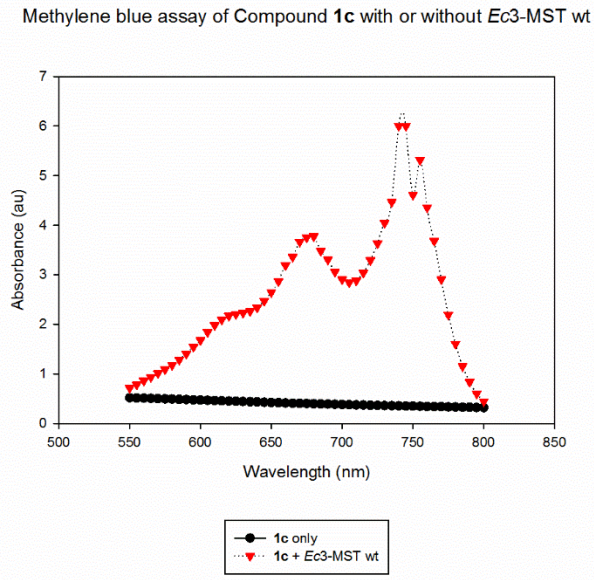


Figure 17: Iodoform test and methylene blue assay indicate that formation of acetophenone and H_2S are concomitant.

For a more quantitative estimation of acetophenone, we monitored by HPLC the turnover of the representative unnatural substrate **1c** by *Ec*3-MST. It was observed that **1c** forms the thiol **6a** almost immediately. After that, the thiol **6a** slowly gets turned over by *Ec*3-MST to yield acetophenone (RT = 5.3 min) over 90 mins (**Figure 18**).

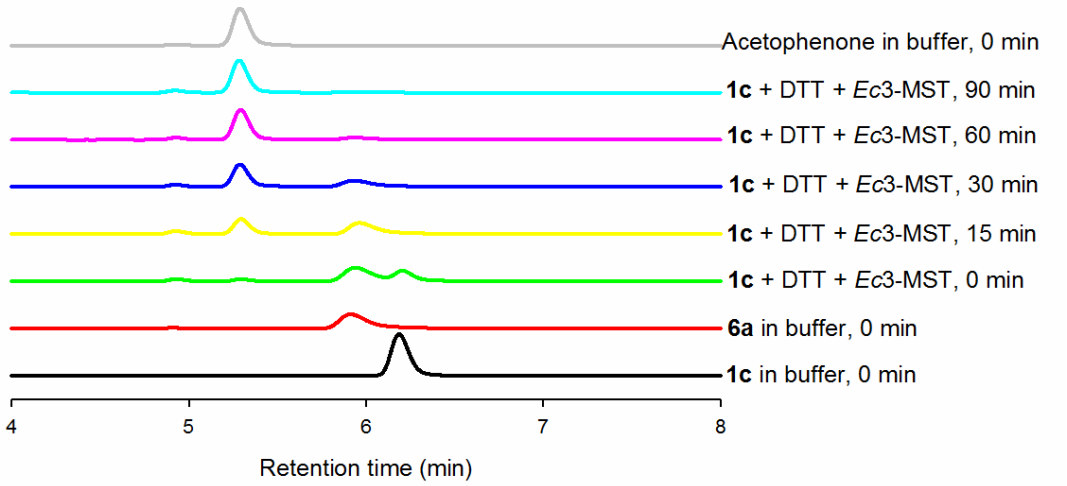


Figure 18: Compound **1c** forms thiol **6a** first, and then is turned over by *Ec*3-MST to yield acetophenone.

The area under the curve for acetophenone was monitored and compared against authentic sample. It was found that the rate constant for the formation of acetophenone is k = 2.1 ± 0.1 h^{-1}, as obtained by fitting the kinetic plot to the first order rate equation ($y = y_0 + a(1 - e^{-kx})$) (**Figure 19**). Moreover, the yield of acetophenone obtained by turnover of **1c** after 90 mins of reaction time is 86%.

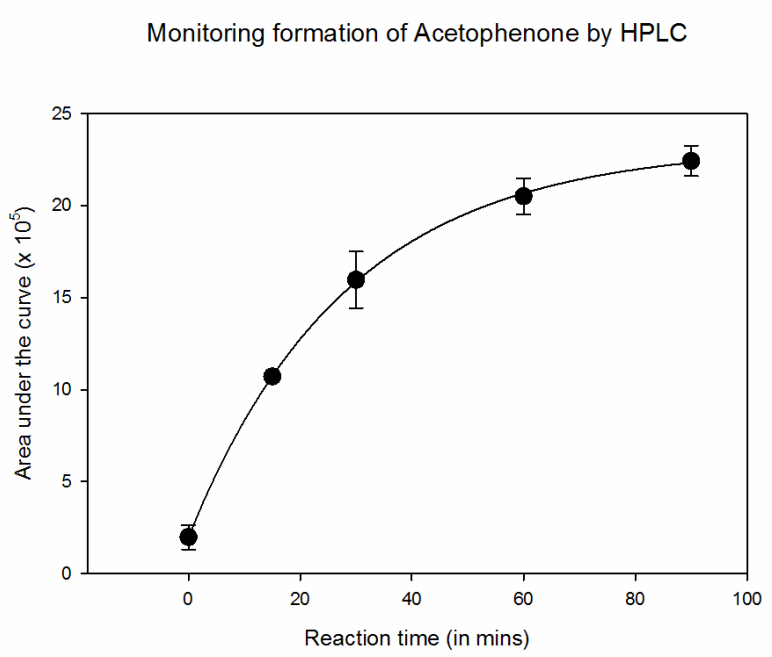
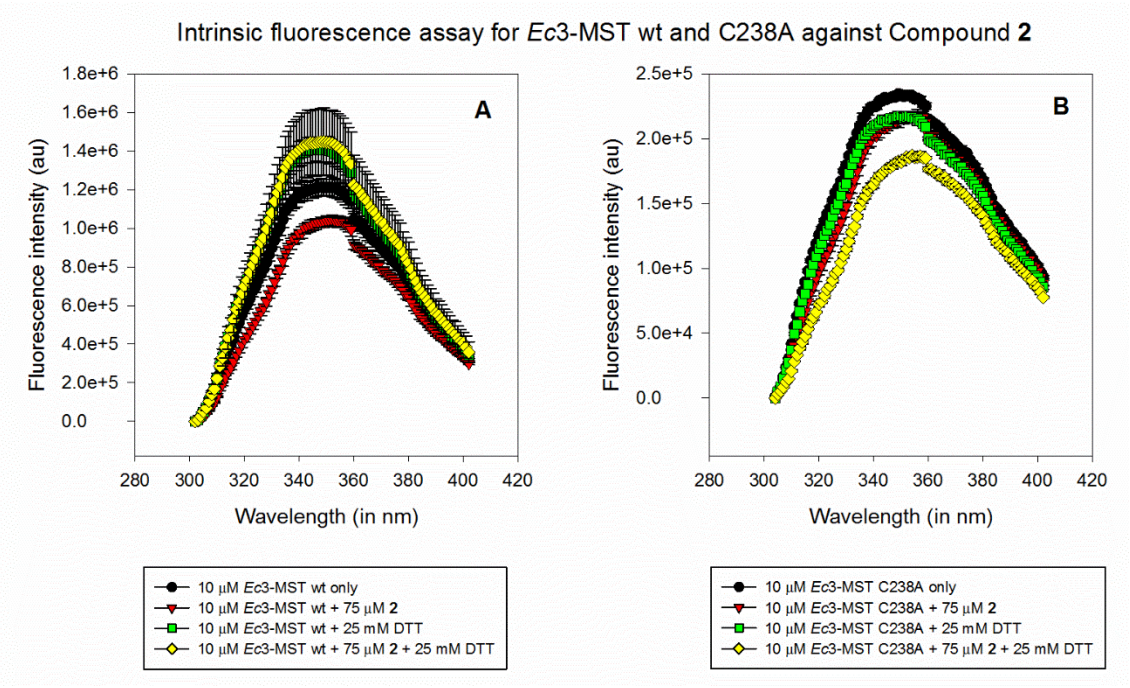


Figure 19: Kinetics of formation of acetophenone by enzymatic turnover of **1c**.

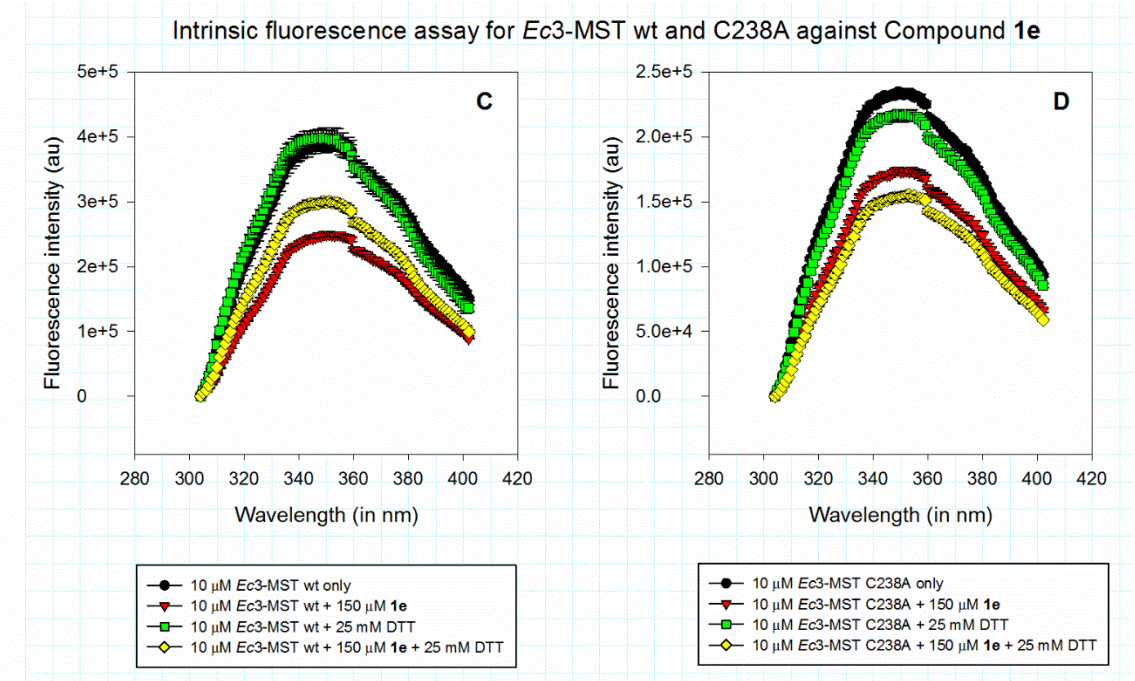
This number is good agreement with the rate constant obtained for the yield of H_2S by enzymatic turnover of **1c** as assessed by the methylene blue assay, viz. k = 2.4 ± 0.3 h^{-1}.

***S*-Sulfhydrated *Ec*3-MST is probably formed as intermediate while turning over the unnatural substrates:** To detect the presence of *S*-Sulfhydration, we monitored the intrinsic fluorescence of *Ec*3-MST wild-type (wt) and the C238A mutant when they are incubated with a substrate. A previously-reported study[24] showed that the intrinsic fluorescence drops when *Ec*3-MST is *S*-Sulfhydrated.

What our study went on to demonstrate using the physiological substrate **2**, negative control **4**, and representative unnatural substrate **1e**, is that the intrinsic fluorescence does drop when *Ec*3-MST wt is incubated with **2** or **1e**, but not with **4** (**Figure 20**; **A**, **C**, and **E**). What is more interesting is that the drop in the fluorescence (with **2** and **1e**) is *abated* when Dithiothreitol (DTT) is added, indicating that the *S*-Sulfhydrated *Ec*3-MST wt is getting reduced. The extent of drop in fluorescence of *Ec*3-MST wt, however, is different when it is incubated with **2** or **1e**.


Intrinsic fluorescence assay for Ec3-MST wt and C238A against Compound 2
A
B
Fluorescence intensity (au)
Wavelength (in nm)
10 μM Ec3-MST wt only
10 μM Ec3-MST wt + 75 μM 2
10 μM Ec3-MST wt + 25 mM DTT
10 μM Ec3-MST wt + 75 μM 2 + 25 mM DTT
10 μM Ec3-MST C238A only
10 μM Ec3-MST C238A + 75 μM 2
10 μM Ec3-MST C238A + 25 mM DTT
10 μM Ec3-MST C238A + 75 μM 2 + 25 mM DTT

1


Intrinsic fluorescence assay for Ec3-MST wt and C238A against Compound 1e
C
D
Fluorescence intensity (au)
Wavelength (in nm)
10 μM Ec3-MST wt only
10 μM Ec3-MST wt + 150 μM 1e
10 μM Ec3-MST wt + 25 mM DTT
10 μM Ec3-MST wt + 150 μM 1e + 25 mM DTT
10 μM Ec3-MST C238A only
10 μM Ec3-MST C238A + 150 μM 1e
10 μM Ec3-MST C238A + 25 mM DTT
10 μM Ec3-MST C238A + 150 μM 1e + 25 mM DTT

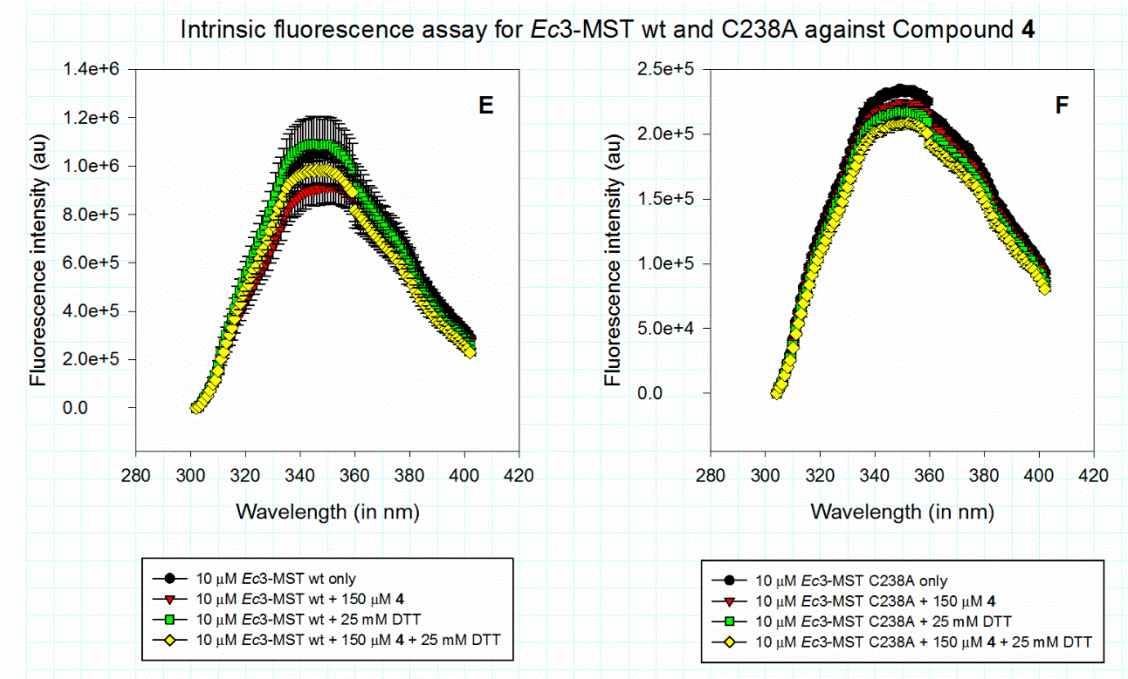


Figure 20: Intrinsic fluorescence assay with *Ec*3-MST wt and C238A mutant when incubated with compounds **2**, **1e**, and **4**.

Furthermore, we have established that none of compounds **2**, **1e**, or **4**, and DTT are fluorescent themselves (**Figure 21**).

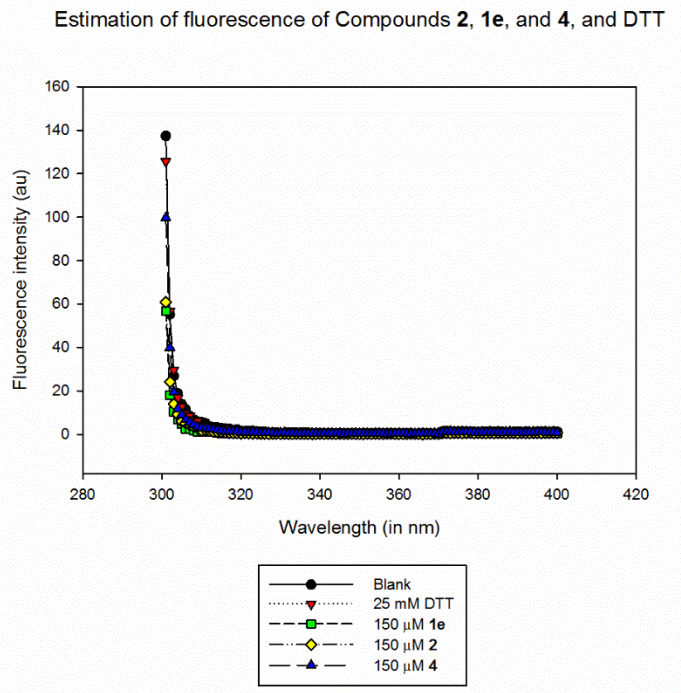


Figure 21: Fluorescence spectra of the other components of the reaction mixture indicates that nothing other the enzyme is fluorescent.

Conducting the same experiment with *Ec*3-MST C238A mutant, it is observed that fluorescence drops upon incubation with compounds **2** or **1e**, but not with **4** (**Figure 20**; **B**, **D**, and **F**). However, *no* abatement in the drop of fluorescence is observed when DTT is added, meaning that the phenomenon observed in the case of the mutant is *not* due to *S*-Sulfhydration of the enzyme; probably it was just a binding event that caused fluorescence quenching.

CONCLUSIONS:

This study, to the best of my knowledge, is the first report describing the systematic development and mechanistic investigation of storage-friendly novel unnatural substrates of *Ec*3-MST.

With the methylene blue assay, the unnatural substrates are demonstrated to be turned over by *Ec*3-MST to produce H_2S at varied rates. Quickening the cleavage of the thioacetate group of **1c** with the aid of esterase is observed *not* to influence the rate of reaction significantly, indicating that it is not the rate determining step. Data from HPLC shows that, with DTT, the thiol **6a** is formed almost immediately, followed by its eventual turnover by *Ec*3-MST to yield acetophenone (and H_2S). Put together, this means that the rate of the reaction can be *influenced* simply by changing the substitution on the phenyl ring.

The rate of production of H_2S from **1c** matches the rate of formation of its by-product, Acetophenone, which is detected by the iodoform test and quantified using HPLC.

Furthermore, during the unnatural substrate turnover, *Ec*3-MST probably undergoes *S*-Sulfhydration, as with its physiological substrate, as is studied with the intrinsic fluorescence assay.

Lastly, the thioacetate group present as a protecting group is shown to be cleaved by the action of esterase as well as by glutathione, rendering this system the potential to be taken forward to conduct cellular studies.

A recent study[28] reported the generation of a knock-out of the TcyP transporter of cysteine in *E. coli*. Given the fact that cysteine is needed to biosynthesize the physiological substrate of *Ec*3-MST, a *ΔtcyP* strain would have considerably attenuated levels of enzymatically-produced H_2S. If we now feed *ΔtcyP* strain of *E. coli* with our different unnatural substrates, it is possible to be able to culture bacteria that have varied levels of intracellular H_2S. This can then, in principle, be developed as a model system to test the *extent* to which intracellular levels of H_2S is able to protect bacteria against antibiotic stress, which can provide us further insights into the mechanism by which antibiotics actually work.

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