Oxidative stress triggered tunable Hydrogen sulfide donors

Thesis submitted towards the partial fulfillment of BS-MS dual degree program

By

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20131070

Under the guidance of

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CERTIFICATE

This is to certify that this dissertation entitled "Oxidative stress triggered tunable Hydrogen sulfide donors" towards the partial fulfilment of BS-MS dual degree programme at Indian Institute of Science Education and Research, Pune represents study/work carried out by Swetha Jos at Indian Institute Education and Research, Pune under the supervision of Dr. Harinath Chakrapani, Associate Professor, Chemistry Department during the Academic year 2017-2018.

Date: 20/3/18

C-Harinath

Dr. Harinath Chakrapani

Place: PUNE

Swetha Jos

(20131070)

DECLARATION

I hereby declare that the matter embodied in the report entitled "Oxidative stress triggered tunable Hydrogen sulfide donors" are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, under the supervision of Dr. Harinath Chakrapani, Associate Professor, Chemistry Department and the same has not been submitted elsewhere for any other degree.

C-Harinath

Dr. Harinath Chakrapani

Swetha Jos

(20131070)

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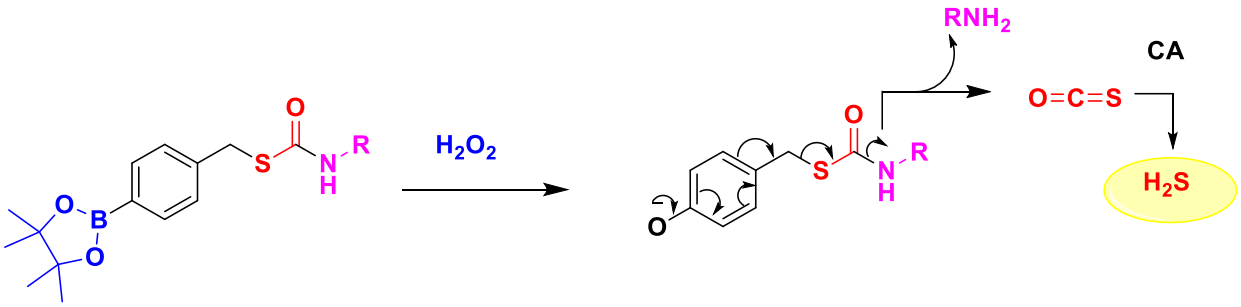
1. ABBREVIATIONS

NMR	Nuclear Magnetic Resonance
HRMS	High-Resolution Mass Spectrometry
J	Coupling constant
Hz	Hertz
MHz	Mega Hertz
EtOAc	Ethyl acetate
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
mg	milligram
g	gram
mL	millilitre
mmol	millimole
ACN	Acetonitrile
UV	Ultra violet
HPLC	High-Performance Liquid Chromatography
IR	Infra-Red

2. ABSTRACT

Hydrogen sulfide is now recognized as a gasotransmitter along with Nitric oxide (NO) and Carbon monoxide (CO) because of its involvement in signaling pathways, biological applications and the therapeutic role it plays. Most of the H_2S donors used in the biological studies do not mimic the slow and continuous release of H_2S in cells which is enzyme triggered rather, they release H_2S rapidly and in an uncontrolled manner. The biological functions of H_2S are highly correlated with the rate of release of H_2S as evidenced by the contrasting roles it plays in inflammatory pathways and in cancer therapy. The study presented here includes synthesis and evaluation of kinetics of small molecule H_2S donors which can be triggered by oxidative stress. Oxidative stress is a which is a biological state in which there is an imbalance in antioxidants and ROS (reactive oxygen species) produced as part of cellular metabolism. Upon activation by H_2O_2 the compounds undergo self-immolation to generate H_2S in the presence of carbonic anhydrase which is widely present in cells. It was hypothesized that changing the substitution on the nitrogen can vary the rate of release of H_2S. This would be dependent on the leaving group. The kinetics of release of H_2S from the donors was studied independently using methylene blue assay and HPLC. In accordance with the proposed hypothesis, varying the leaving group shows an effect on rates of release of H_2S upon activation by H_2O_2. Donors with group on the nitrogen having lower pKa showed a significantly higher rate compared to donors with higher pKa values. By varying the amines on the carbamothioate, we were able to achieve a tunable release of H_2S in the range of 13-204 min as half-lives. This study also includes small molecule H_2S donors which can release H_2S in a slow and continuous manner with half-lives comparable with macromolecule H_2S donors with reasonable yields.



3. INTRODUCTION

3.1 Biosynthesis and biological applications of H_2S

Hydrogen sulfide is now recognized as a gasotransmitter along with Nitric oxide (NO) and Carbon monoxide (CO).[1] Endogenously it is produced via the individual or concerted enzymatic activity of cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE) and cysteine aminotransferase (CAT) from cysteine and homocysteine. And 3-mercaptopyruvate sulfur transferase (3-MST), a mitochondrial enzyme produces H_2S from 3-mercapto pyruvate (**Figure 1**).[2]

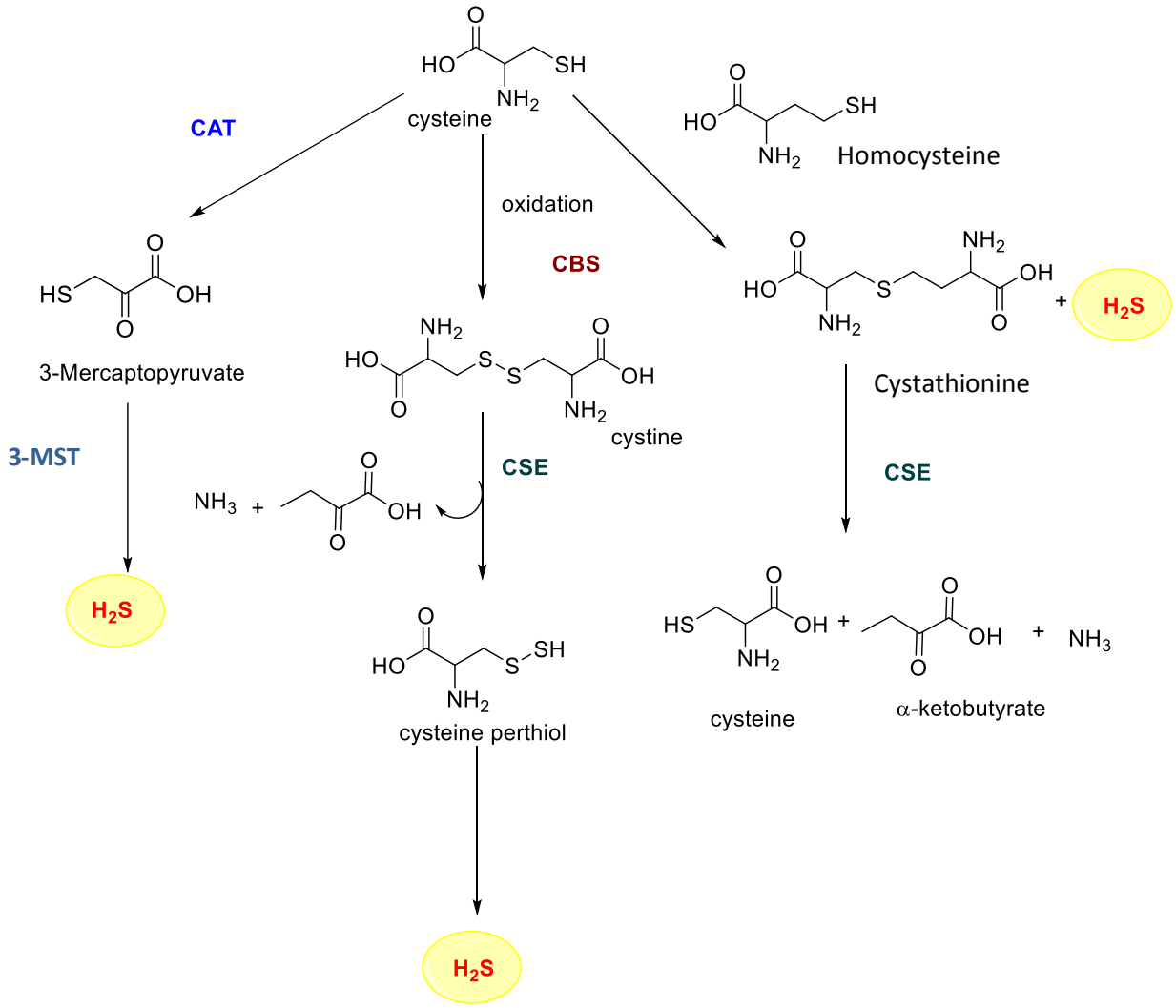


Figure 1: Biosynthesis of H_2S (Source: Sushma's Thesis)

Kimura and Coworkers discovered the neuromodulatory effects of H_2S in 1996.[3] Following this a number of studies were conducted which revealed the role of H_2S on

the relaxation of blood vessels, protection against myocardial ischemia injury. And it also showed cytoprotective effects against oxidative stress.[4] The biological applications of H_2S are summarized in **Figure 2**. The chemical and biochemical catabolic reactions are responsible for the biological roles of H_2S. For example, because of the strong reducing nature of H_2S, it can react with superoxide ($O_2^{\cdot-}$), peroxynitrite ($OONO^-$) and hydrogen peroxide (H_2O_2) produced as a part of cellular metabolism (**Figure 3a**).[2] It is also studied that the H_2S can affect protein S-sulfhydration, although the mechanisms are unclear. However, S-sulfhydration could be a potential pathway in which H_2S acts in varying the cellular functions of different proteins (**Figure 3b**).[5]

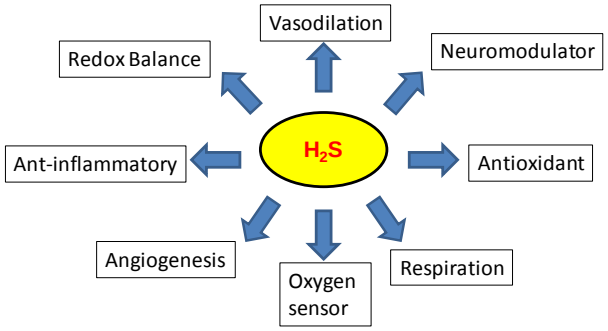


Figure 2: Biological applications of H_2S

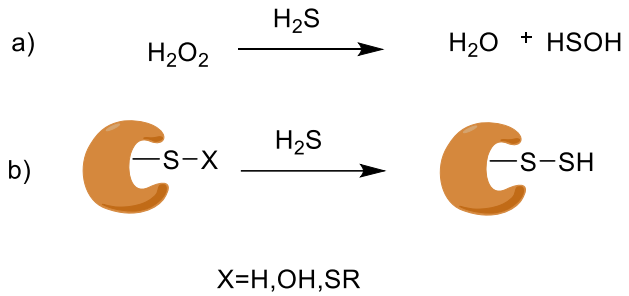


Figure 3: a) Reaction of H_2S with H_2O_2
b) S-sulfhydration by H_2S

3.2 Hydrogen sulfide donors

Inorganic salts like NaHS and Na_2S are used as sources for releasing H_2S and has shown positive effects like attenuation of ischemia-induced heart failure in animal models.[6] However, the spontaneous and burst release of H_2S can lead to cytotoxicity and misleading conclusions because of the variation in concentration of H_2S from the stock preparation to the time of the experiment.

3.3 Lawesson's reagent and GYY4137

2,4-Bis(4-methoxyphenyl)-1,3,2,4- dithiadiphosphatane-2,4-disulfide (Lawesson's reagent), a commonly used sulfurization agent is also used as an H_2S donor and it releases H_2S upon undergoing hydrolysis. It was used for studying the effects of H_2S on inflammation and ulceration of colon during colitis in a rat model and showed a

reduction in colitis.[7] But it suffers from poor water solubility and uncontrolled release of H_2S.

In order to solve the water solubility problem, Lawesson's reagent was modified to morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137). Although it releases H_2S similar to Lawesson's reagent and showed some anti-inflammatory effects the yield of H_2S was found to be only 10% even after 7 days at physiological conditions.[4] The low yield of H_2S limits its usage.

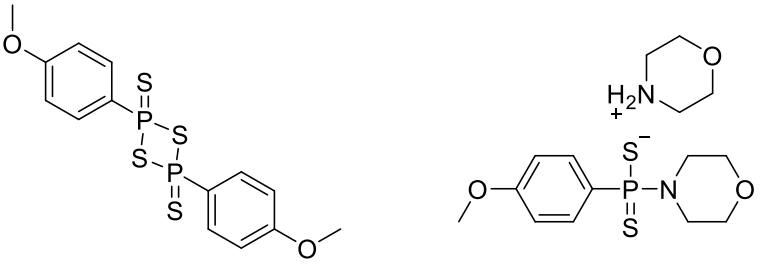


Figure 4: Structures of Lawesson's reagent and GYY4137

3.4 1,2-dithiole-3-thione (DTT) and derivatives

DTTs are known to release H_2S upon hydrolysis in buffers and have been used to study the effect of H_2S on the alimentary system. However, it lacks a trigger for the controlled release of H_2S and the mechanism of release is not very well understood, which limits its usage in complex systems like cells.

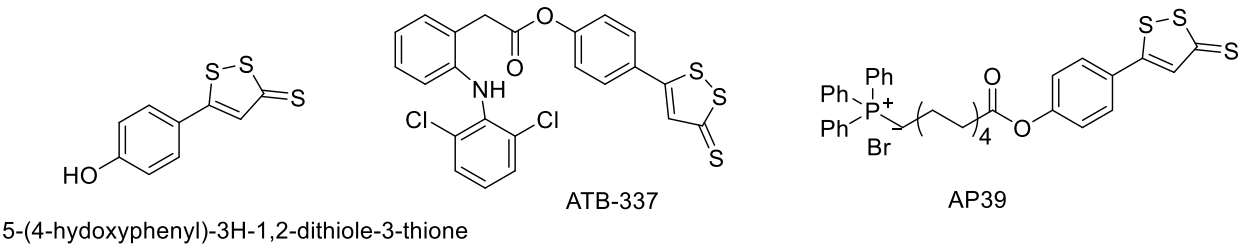


Figure 5: Structures of DTT (1,2-dithiole-3-thione) and its derivatives

3.5 Triggered release of H_2S

Triggered release of H_2S could provide a hands-on tool for controlling its release. There are many studies done in order to release H_2S on the activation by a trigger. Ming Xian and co-workers developed a thiol-activated H_2S releasing molecule with N-mercapto (N-

SH) as the base. These molecules were further modified in order to achieve better solubility. Although they exhibited protective effects against myocardial reperfusion injury, the ubiquitous nature of thiols activated H_2S donors does not provide selectivity and the control over the release is limited (**Figure 6a**).[8] The same group reported the release of H_2S on the activation by light. They modified the gem-dithiol, which released H_2S on hydrolysis in aqueous medium, to 2-nitrobenzyl group, which provided a trigger for releasing H_2S under irradiation with 350 nm light (**Figure 6b**).[9] However, the mechanism of release of H_2S was through the hydrolysis of the intermediate formed after irradiation with light and thus there was no control over release rates of H_2S. Ketoprofenate based photolabile group was used by Nakagawa and co-workers which release H_2S upon activation with UV-light of 300-360 nm accompanied by the elimination of propenylbenzophenonone and CO_2 (**Figure 6b**).[10] Since, the irradiation is in the UV range, photo-toxicity is associated with it and therefore it limits its application in biology.

Carbonyl sulfide has now emerged as a new class of H_2S releasing molecule, which is present in cells and has shown biological roles.[11] Because of the structural similarity to CO_2, the substrate for carbonic anhydrase (CA), COS is also hydrolyzed by CA. Pluth and co-workers reported a azide-based thiocarbamates for H_2S sensing based on thiocarbamate scaffold (**Figure 6c**).[12] The reduction of the azide by H2S leads to the the self-immolation which releases carbonyl sulfide, which is then hydrolyzed by CA. The same group reported a ROS (reactive oxygen species) triggered H_2S donors which also releases H_2S by thiocarbamate scaffold (**Figure 6d**).[13] They used isothiocyanates as precursors for synthesis, which limits the scope for synthesizing H_2S donors with tunability in terms of leaving group because of the lack of availability of different isothiocyanates. Matson and co-workers showed the release of carbonyl sulfide via nucleophile activation, but because of the ubiquitous nature of biological nuclepohiles, the selectivity is limited.[14] Our group also reported a thiocarbamate scaffold for release of COS/H_2S with esterase as the trigger.[15] We also reported a BODIPY (boron-dipyrromethene) based scaffold which is uncaged by visible light at 470 nm to generate carbonyl sulfide which is then hydrolyzed by carbonic anhydrase an enzyme prevalently

present in the cells.[16] However, the usage of the molecule was limited because of the usage of blue light which can induce oxidative stress in cells.

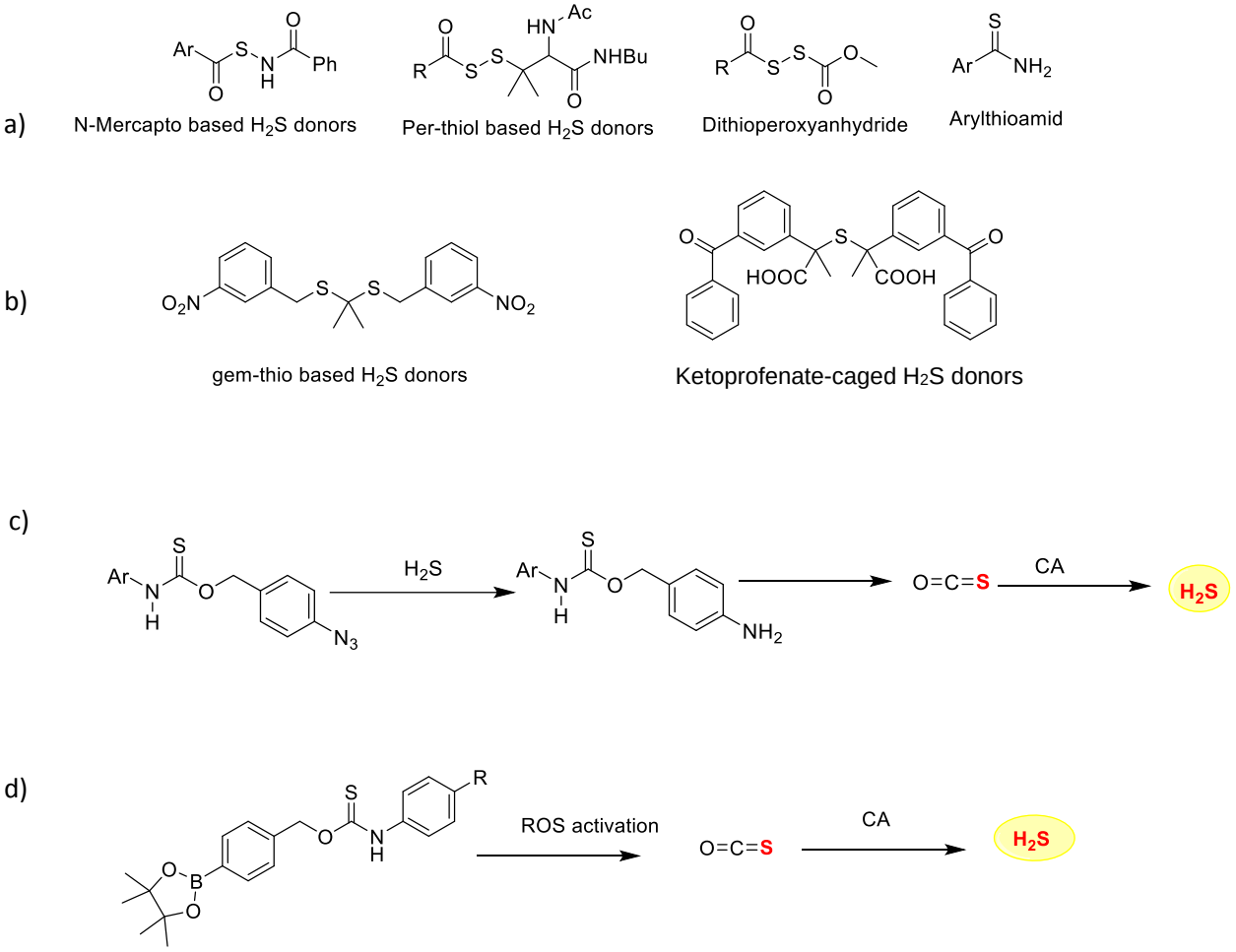


Figure 6: a) Structures of N-Mercapto based H_2S donors, Per-thiol based H_2S donors, Dithioperoxyanhydride, and Arylthioamides: b) Gem-thio based H_2S donors, and Ketoprofenate-caged H_2S donors; c)Azide- based thiocarbamates for H_2S sensing d) ROS triggered H_2S donors;

3.6 Significance of Tunable release of H_2S

The biological roles of H_2S are mainly dependent on the concentration and the rate of release of H_2S because the endogenous production of H_2S is enzyme mediated and therefore slow and continuous. Pro and anti-inflammatory effects of H_2S have always been controversial. Moore and co-workers showed that the effect of H_2S on inflammation is dependent on the rate of release of H_2S. They compared the effect of rate of release of H_2S from NaHS and GYY4137, which is comparatively slow releasing

H_2S donor, on pro- and anti-inflammatory mediators in lipopolysaccharide (LPS)-treated murine RAW264.7 macrophages. They showed that GYY4137 in concentration-dependent manner significantly inhibits LPS-induced release of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, nitric oxide (·NO), and PEG_2.In the meantime, it increased the synthesis of anti-inflammatory chemokine IL-10 through NF-κB/ATF-2/HSP-27 dependent pathways. Contrary to this, NaHS showed a biphasic effect on pro-inflammatory mediators and at high concentrations, the production of IL-1 β, IL-6, ·NO, PEG_2 and TNF-α was enhanced. This clearly shows that the effect of H_2S on inflammation is not only concentration dependent but also depends on the rate release of H_2S.

Also, GYY4137 has shown concentration-dependent selective killing of cancer cells over normal cells compared to fast releasing NaHS over a period of days.[17] But GYY4137, as mentioned earlier has a very low yield of H_2S and thus large dosage is required. Also, it is limited in usage because of the lack of trigger which can provide selectivity.

Esterase activated tunable release of H_2S was shown by Binghe Wang and co-workers using trimethyl lock based strategy.[18] On the activation by esterase, a hydroxyl group is generated by the hydrolysis of the ester group, which then lactonizes rapidly to release H_2S. They showed tunability in releasing H_2S via modifications on the ester group. However, esterase enzyme is ubiquitous in nature and thus it is limited by the lack of specificity (**Figure 7**).

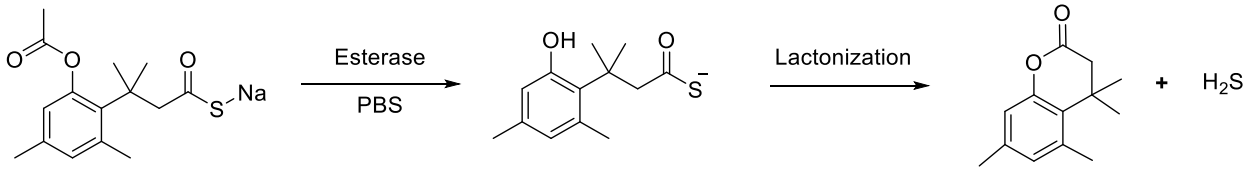


Figure 7: H_2S release via esterase trigger

Recently, Matson and co-workers have shown the anti-cancer effect of slow releasing H_2S donors via incorporating a polymeric base to the S-aroylthioxime (SATO), which releases H_2S upon attack by cysteine.[19] They showed that the amphiphilic copolymer incorporated with SATO formed micelles which release H_2S in response to cysteine with

a half-life of 3.3 h. They also showed that the polymeric SATO could kill selectively the cancer cells (HCT 116 colon cancer) and not the normal cells, compared to Na_2S, GYY4137 and the small molecule SATO. But the polymeric SATO is triggered by thiol, which is again ubiquitous in nature.

3.7 Design of scaffold

The inspiration for the work comes mainly from the work done by my lab colleague, Preeti Chauhan. Here, we showed that the carbonothioates and carbamothioates release carbonyl sulfide via self-immolation which is then hydrolyzed by carbonic anhydrase upon the activation by esterase.[15]

We showed that leaving group after self-immolation affect the rate of release of H_2S. However, the trigger was esterase and therefore it provided applicability in a broad perspective only.

In this study we modified the trigger to boronate ester, which is reported to get activated only under oxidative stress conditions and will provide selectivity to excessive ROS (reactive oxygen species) produced due to the imbalance of anti-oxidants and ROS.[20] This imbalance happens in disease states like myocardial reperfusion/injury and H_2S as shown protective effects against it.[21] Thus, an H_2S donor which can be activated selectively under oxidative stress will be useful. Changing the leaving group was hypothesized to affect the rate of release of H_2S and thus provide tunability.

If the rate limiting step for the reaction to release H_2S is the self-immolation step, then changing the leaving group will affect the rate of release of H_2S and it can provide a hands on tool to tune the release of H_2S. Changing the leaving group with different amines with a range of pKa values was thus expected to show a difference in the release kinetics of H_2S.

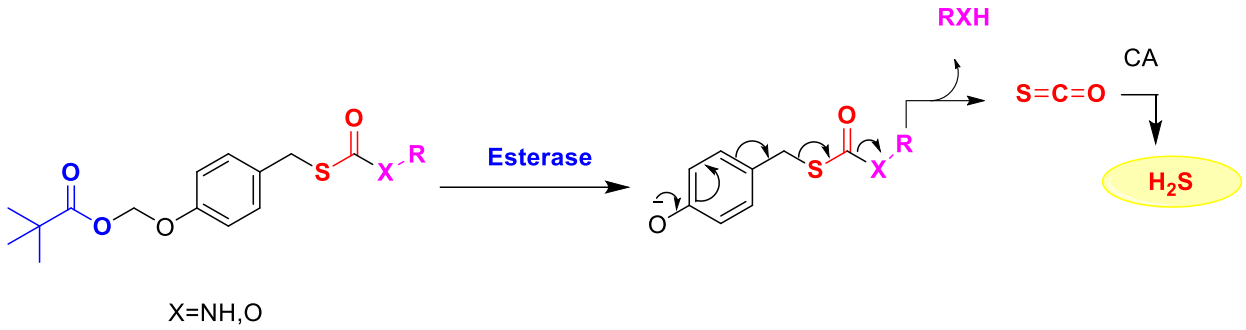


Figure 8: Esterase triggered H_2S/ COS donors

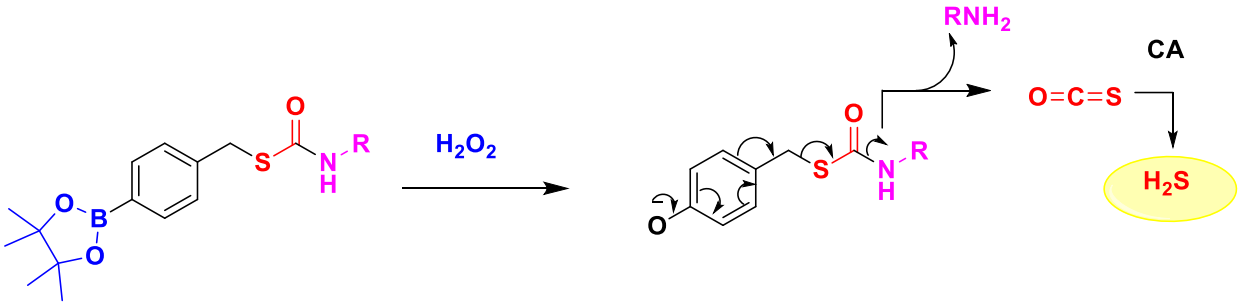


Figure 9: Scheme for the release of H_2S from carbamothioates triggered by H_2O_2

4. MATERIALS AND METHODS

4.1 General Methods:

All reactions were conducted under a nitrogen atmosphere. All the chemicals and solvents were purchased from commercial sources and used as received unless stated otherwise. Column chromatography was performed using silica gel-Rankem (60– 120 mesh) or silica gel Spectrochem (100-200 mesh) as the stationary phase. Preparative high-performance liquid chromatography (HPLC) was done using Combiflash EZ prep UV using a Kromasil®C-18 preparative column (250 mm × 21.2 mm, 5 µm). ^{1}H and ^{13}C spectra were recorded on a JEOL 400 MHz (or 100 MHz for ^{13}C) or a Bruker 400 MHz (or 100 MHz for ^{13}C) spectrometer unless otherwise specified using either residual solvent signals ($CDCl_3$ δH= 7.26 ppm, δC = 77.2 ppm) or as an internal tetramethylsilane (δH = 0.00, δC = 0.0). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The following abbreviations are used: bs (broad signal), m (multiplet), s (singlet), d (doublet), t (triplet) and dd (doublet of doublets). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using BRUKER-ALPHA FT-IR spectrometer. Analytical HPLC was

performed on an Agilent1260-infinity with Phenomenex®C-18 reverse phase column (250 mm × 4.6 mm, 5 µm). Photometric and fluorimetric measurements were performed using a Thermo Scientific Varioskan microtiter plate reader.

4.2 Synthesis and Characterization

Compounds **1**, **2**, **7**, **9**, **10**, **11**, **13** and **14** were synthesized using previously reported procedure and the analytical data that we collected were consistent with the reported values.

(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) phenyl) methanethiol (5): To a well-stirred solution of **2** (1.2 g, 5.13 mmol) in dry DCM (10 mL), PBr_3 (481 µL, 5.13 mmol) was added slowly under N_2 atmosphere. The reaction was allowed to stir for 1 h at 0 ^oC. The progress of the reaction was monitored by TLC. The reaction was quenched by adding 15 ml of saturated $NaHCO_3$ solution. The aqueous layer was extracted using DCM (3 x 10 mL). The combined organic layer was dried over Na_2SO_4, filtered and concentrated and the residue was slightly brown. This was further dissolved in dry THF (15 mL) and thiourea salt was added at room temperature and the reaction mixture was stirred overnight. After completion of the reaction, the solvent was removed under reduced pressure and salt obtained was dissolved in water (20 mL) followed by the addition of DCM (30 mL). The reaction was purged with N_2 for 5 min. To this, heterogenous solution, 4 equivalents of sodium metabisulfite salt ($Na_2S_2O_5$) were added. The resulting mixture was refluxed for 4 h under N_2 atmosphere. The solution was cooled to room temperature and washed twice with DCM (20 mL). The organic layer was combined and dried over Na_2SO_4. Filtered and concentrated. The crude obtained was purified using silica gel column chromatography (1% EtOAc/hexane). The compound was obtained as a white color crystalline solid with a pungent smell and the NMR was matched with the reported molecule. ^1H NMR (400MHz, $CDCl_3$): δ 7.76 (d, *J*=8.0 Hz, 2H), 7.28 (d, *J*=8.0 Hz, 2H), 3.36 (s, 2H), 1.35(s, 12H).

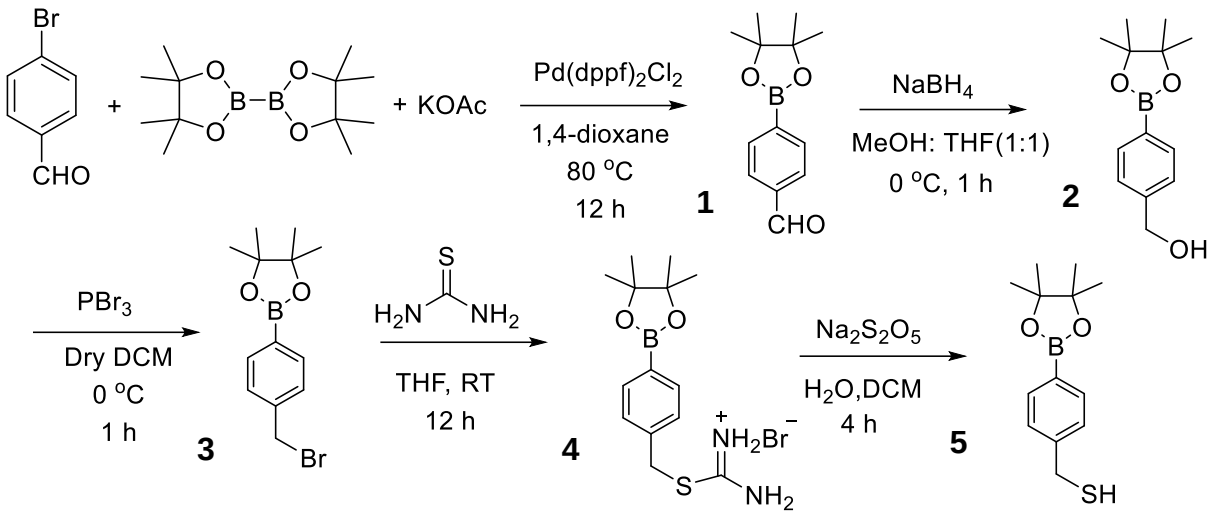


Figure 10: Synthesis scheme for boronate thiol (**5**)

General Scheme for the synthesis of the thiocarbamates:

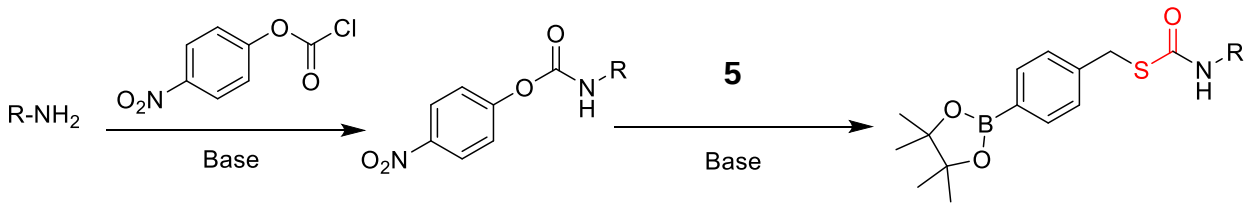
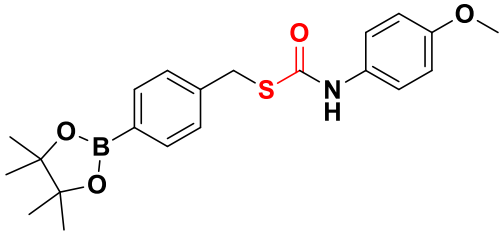


Figure 11: General scheme for the synthesis of carbamothioates

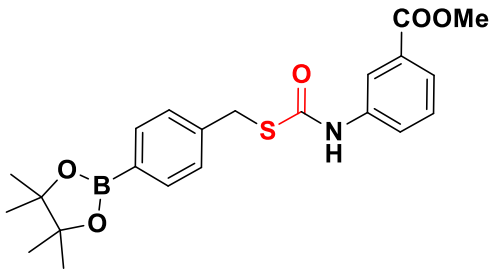
Compounds **1a**, **1b**, **1c**, **1d**, **1e**, **1f**, and **1g** were synthesized by the general scheme (**Figure 11**)

S-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzyl) (4methoxyphenyl) carbamothioate (1a):



1a was synthesized with the help of my colleague Preeti Chauhan and was used as a positive control.

Methyl3-((((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)thio)carbonyl) amino)benzoate (1b):



To a well-stirred solution of **7** (250 mg, 1.65 mmol) in Dry THF at 0 ^{o}C, $NaHCO_3$ was added. And to this *p*-nitrophenyl chloroformate (335 mg, 1.65 mmol) was added. The reaction was stirred for 1 h and the progress was monitored by TLC. To the resulting mixture was then added 10 mL of water and washed twice with DCM (3 x 10 ml). The combined organic layer was dried over Na_2SO_4, filtered and concentrated. The resulting crude was taken forward for next step without purification. To the well-stirred solution of **5** (180 mg, 0.70 mmol) in dry ACN under the N_2 atmosphere, K_2CO_3 (300 mg, 2.16 mmol) was added. Stirred for some time. The crude (230 mg, 0.70 mmol) of the previous reaction was then added. And stirred for 3 h. The reaction was monitored by TLC. To the resulting mixture was then added 10 mL of water and washed thrice with DCM (5 mL). The organic layer was combined and dried over Na_2SO_4. Filtered and concentrated. Purification was done using prep HPLC and ACN-water as the eluents. Compound **1b** was obtained as a white solid (95 mg, 28%). FT-IR ($\upsilon_{max,}$ cm^{-1}) 3471, 3064, 2983, 1723; ^{1}H NMR(400 MHz, $CDCl_3$) : δ 7.98(s, 1H), 7.79(m, 4H), 7.41(m, 1H), 7.39 (m,2H), 7.25 (s, 1H), 4.23 (s, 2H), 3.91(s, 3H), 1.32 (s,12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.4, 140.7, 140.4, 135.1, 132.3, 130.8, 128.2, 126.4, 125.4, 83.7, 52.2, 34.5, 24.7 (ppm); HRMS (ESI) for $C_{22}H_{26}BNO_5S$: Calculated $(M+H)^+$: 428.1703, Found $(M+H)^+$: 428.1708.

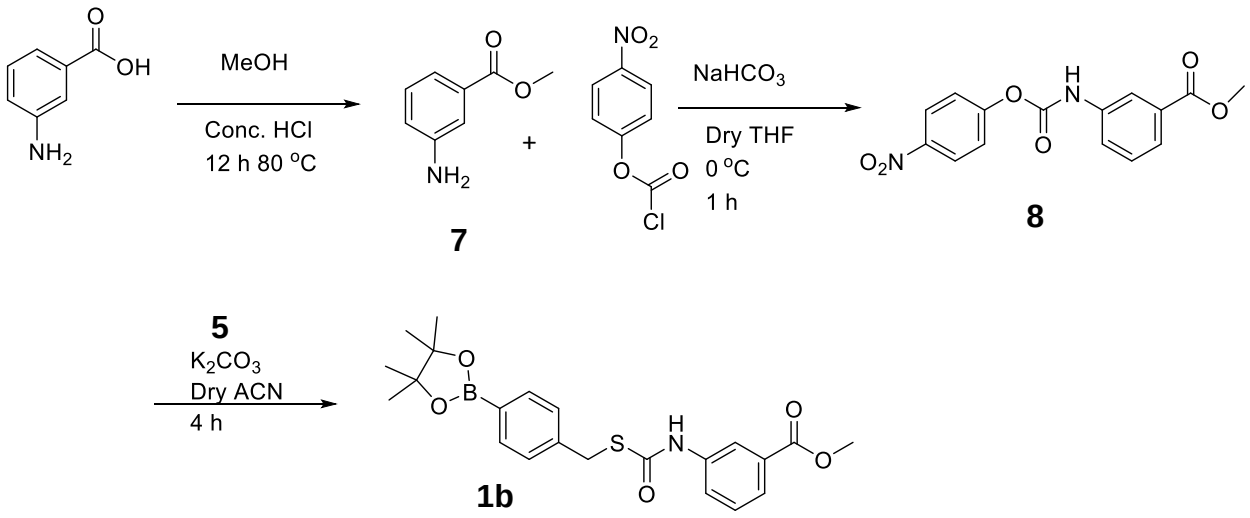
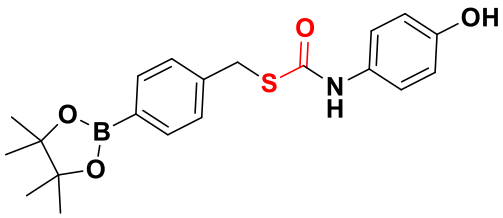


Figure 12: Scheme for the synthesis of **1b**

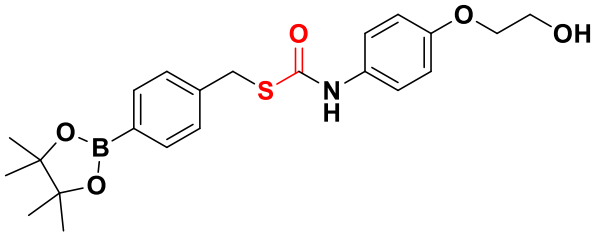
S-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)(4-hydroxyphenyl) carbamothioate (1c):



The carbamate which is the precursor for **1c** was synthesized by reacting 4-hydroxy aniline (300 mg, 2.75 mmol) with *p*-nitrophenyl chloroformate (554 mg, 2.75 mmol) with $NaHCO_3$ as the base at 0 °C and dry THF as the solvent and under N_2 atmosphere. The reaction was stirred for 30 minutes. The reaction was quenched with water, extracted in EtOAc (3x10ml), dried over Na_2SO_4, filtered and concentrated. The crude (493 mg, 1.80 mmol) was taken dissolved in Dry ACN under N_2 atmosphere and added slowly to a well stirred mixture of **5** (450 mg, 1.80 mmol) and K_2CO_3 in dry ACN under N_2 atmosphere. And stirred for 3h. The reaction was quenched with water, extracted with EtOAc (3 x 10 ml). The organic layer was combined and dried over Na_2SO_4. Filtered and concentrated. The crude was purified using Prep HPLC (ACN-water system). Compound **1c** was obtained as a white solid. (245 mg, 40%). FT-IR ($\upsilon_{max,}$ cm^{-1}): 3320, 2943, 2830, 1662, 1449, 1020; ^1H NMR (400 MHz, DMSO-d6): δ 10.10 (s, 1H), 9.29 (s, 1H), 7.62 (d,

J=8.0 Hz, 2H), 7.36 (d, *J*=8.0 Hz, 2H), 7.27 (d, *J*=8 Hz, 2H), 6.69(d, *J*=8.0 Hz, 2H), 4.14 (s,2H), 1.29(s,12 H); ^{13}C NMR(100 MHz, DMSO-d6): δ 167.8, 154.0, 143.0, 135.0, 132.0, 132.1, 129.1, 128.7, 115.7, 84.1, 38.6, 25.1 (ppm); HRMS(ESI) for $C_{28}H_{32}BNO_6S$: Calculated $(M+H)^+$:386.1597, Found $(M+H)^+$:386.1600.

S-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) (4-(2-hydroxyethoxy)phenyl)carbamothioate (1d) :



Compound **11** (100 mg, 0.60 mmol) was taken dissolved in Dry THF at 0 ^{o}C and *p*-nitro phenyl chloroformate (132 mg, 0.06 mmol) was added. The resulting mixture was stirred for 2 h and the reaction was monitored using TLC. The reaction mixture was quenched with water and washed with DCM (3 x 10 ml). And the organic layer was combined, dried over Na_2SO_4, filtered and concentrated. The resulting solid was taken forward for next step without purification. This (101 mg, 0.30 mmol) was then added to a well stirred solution of **5** (80 mg, 0.30 mmol) and K_2CO_3 (132 mg, 0.90 mmol) in Dry ACN and reaction was stirred for 4 h and monitored by TLC. The reaction mixture was quenched with water and washed with DCM (3 x 10 ml). And the organic layer was combined, dried over Na_2SO_4, filtered and concentrated. The crude was purified using Prep HPLC (ACN-water system). Compound **1d** was obtained as white solid (55 mg, 32%); FT-IR ($\upsilon_{max,}$ cm^{-1}) :3443, 3047, 1509; ^{1}H NMR (400 MHz, DMSO-d6): δ 10.17 (s, 1H), 7.61 (d, *J*=8.0 Hz, 2H), 7.37(m, 4H), 6.88 (d, *J*=9.0 Hz, 2H), 4.83 (t, *J*=5.6Hz, 1H), 4.16 (s , 2H), 3.93 (t, *J*= 4.9 Hz, 2H), 3.68 (q, J=5.3 Hz, 2H), 1.28 (s, 12H)(ppm); ^{13}C NMR (100 MHz, DMSO-d6): δ142.88, 142.31, 135.05, 128.70, 118.62, 115.06, 84.07, 70.10, 60.04, 33.32, 25.12; HRMS(ESI) for $C_{22}H_{28}BNO_5S$: Calculated $(M+H)^+$:430.1859, Found $(M+H)^+$:430.1858.

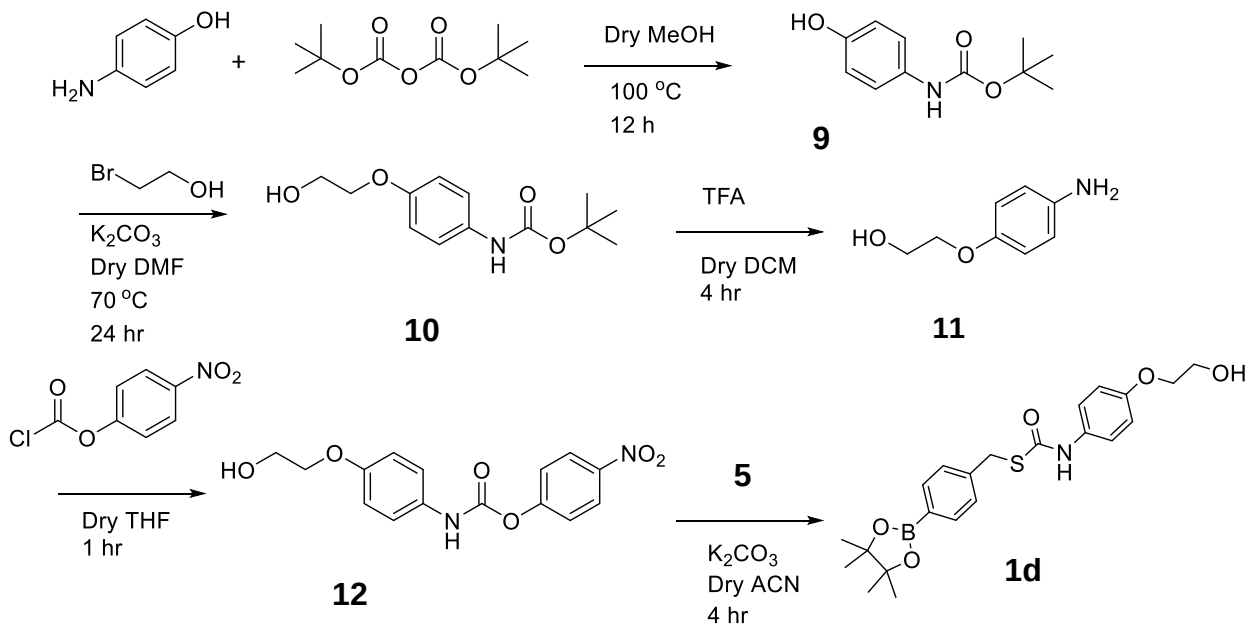
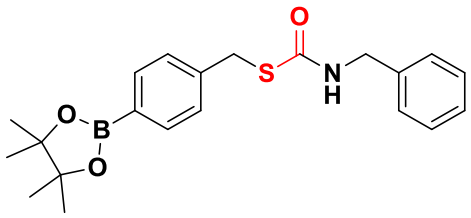


Figure 13: Scheme for the synthesis of **1d**

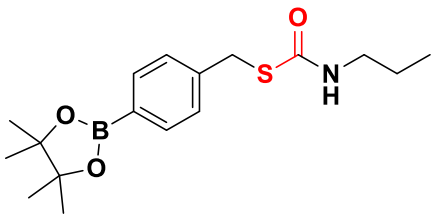
S-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)benzylcarbamothioate (1e):



To a well-stirred solution of Benzyl amine (305 µL, 3.36 mmol) in Dry THF under the N_2 atmosphere in an ice bath, Triethyl amine (585 µL, 4.2 mmol) was added, stirred. *p*-nitrophenyl chloroformate (677 mg, 3.36 mmol) was added and stirred at 0 °C for 2 h. The reaction mixture was quenched with water and washed with DCM (3 x 10 ml). And the organic layer was combined, dried over Na_2SO_4, filtered and concentrated. The resulting solid (142 mg, 0.60 mmol) was taken forward for next step without purification. **5** (130 mg, 0.50 mmol) was taken, dissolved in Dry THF(10ml), cooled in an ice bath under N_2 atmosphere and Triethylamine (217 µL, 1.56 mmol) was added. Stirred. Then the carbamate of the previous step was added. Stirred for 5h. The reaction was quenched with water and extracted with EtOAc (3 x 10 ml) and dried over Na_2SO_4, filtered and concentrated. Purified using prep HPLC with ACN-water as the eluents.

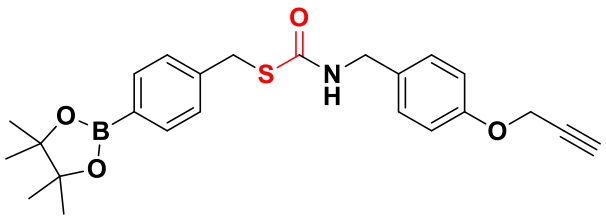
Compound **1e** was obtained as a liquid (41mg, 21%). FT-IR($\upsilon_{max,}$ cm^{-1}): 3305, 3032, 2979, 2928, 1656; ^{1}H NMR (400 MHz, CD_3OD): δ 7.68 (d, *J*=8.08 Hz, 2H), 7.31 (m, 7H), 4.41 (s, 2H), 4.16(s, 2H), 1.36(s,12H) (ppm); $^{13}CNMR$ ($CDCl_3$, 100 MHz): δ 167.8, 142.2, 138.5, 134.5, 128.2, 127.8, 127.1, 126.9, 88.7, 44.4, 33.2, 23.7 (ppm); HRMS(ESI) for $C_{21}H_{26}BNO_3S$:Calculated $(M+H)^+$:384.1804, Found :384.1806.

S-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) propylcarbamothioate (1f)



Propyl amine (278 µL, 3.38 mmol) was taken, dissolved in Dry DCM under the N_2 atmosphere, Diisopropyl amine (600 µL, 3.38 mmol) was added. Stirred for some time. To this *p*-nitro phenyl chloroformate (681 mg, 3.38 mmol) was added and stirred for 3 h. The reaction was monitored by TLC. The solvent was evaporated and washed with dilute HCl filtered and concentrated. The resulting white solid was taken forward for next step without further purification. To a well-stirred solution of **5** (250 mg, 0.90 mmol) and K_2CO_3 (414 mg, 3 mmol) in dry ACN, the carbamate (224 mg, 0.90 mmol) was added and stirred for 4 h. The reaction was monitored by TLC. The reaction was quenched with water and extracted with EtOAc (3 x 10 ml) and dried over Na_2SO_4, filtered and concentrated. Purified using prep HPLC and ACN-water as the eluent. Compound **1f** was obtained as a white solid. FT-IR ($\upsilon_{max,}$ cm^{-1}): 3317, 2974, 2925, 1613, 1320; ^{1}H NMR (400 MHz, $CDCl_3$) : δ 7.73 (d, *J*=8.0Hz, 2H), 7.33 (d, *J*=8.0 Hz, 2H), 5.26 (s, 1H), 4.15 (s, 2H), 3.25 (m, 2H), 1.55 (m, 2H), 0.90 (t, *J*=4.0 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ 167.6, 142.2, 134.7, 128.2, 127.2, 83.8, 33.2, 10.4; HRMS(ESI) for $C_{17}H_{26}BNO_3$: Calculated $(M+H)^+$:336.1804, Found :336.1808.

S-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)(4-(prop-2-yn-1-yloxy)benzyl)carbamothioate (1g):



To well-stirred solution of **14** (600 mg, 3.72 mmol) and triethyl amine (1.56 ml, 11.17 mmol) in Dry THF *p*-nitrophenyl chloroformate (750 mg, 3.72 mmol) was added and stirred for 4 h. The reaction was monitored by TLC. The reaction was quenched with water (10 ml) and extracted with EtOAc (3 x 10 ml). The resulting crude (**15**) was taken forward for next step without further purification. To a well stirred solution of **5** (150mg, 0.60 mmol) and K_2CO_3 (248.61 mg, 1.80 mmol) in dry ACN, **15** (195 mg, 0.60 mmol) was added and stirred for 4 h. Reaction was monitored by TLC. The reaction was quenched with water and extracted with EtOAc (3 x 10 ml) and dried over Na_2SO_4, filtered and concentrated. Purified using prep HPLC and ACN-water as the eluents. Compound **1g** was obtained as a white solid (90 mg, 34%). FT-IR ($\upsilon_{max,}$ cm^{-1}) : 3294, 2979, 2924, 1662, 1360; ^{1}H NMR (400 MHz $CDCl_3$): δ 7.74 (d, *J* =8.08 Hz, 2H), 7.34 (d, *J*=8.04 Hz, 2H), 7.20(d, *J*=8.72 Hz, 2H), 6.94(d, *J*=8.68 Hz, 2H), 5.51(s, 1H), 4.68 (d, *J*=2.4 Hz, 2H), 4.41 (d, *J*=5.28Hz, 2H), 4.18(s, 2H), 2.52(t, *J*=2.4Hz, 1H), 1.33(s, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): 157.2, 141.2, 135.2, 129.2, 128.3, 115.3, 63.9, 75.5, 55.9, 45.0, 34.4, 24.9 (ppm); HRMS(ESI) for $C_{24}H_{28}BNO_4S$: Calculated : 438.1910, Found :438.1913.

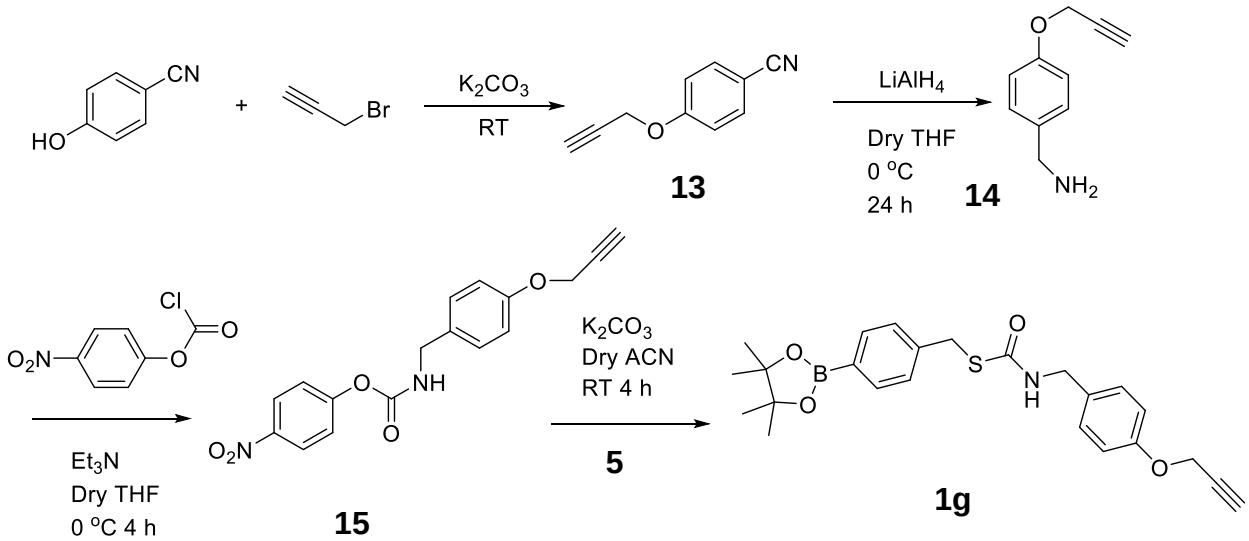


Figure 14: Scheme for synthesis of **1g**

4.3 Methylene Blue assay for H_2S detection:

a) Calibration for H_2S: Each assay described was done in triplicate in vials with closed lids. Different concentrations of Na_2S (1 mM stock in buffer) was taken along with 10 µL of $Zn(OAc)_2$ (40mM stock in H_2O) in buffer with final volume making up to 200 µL. The mixture was stirred at 37 ^oC for 15 min. 100µL of the reaction aliquot was taken out and diluted with 100 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 100 µL of N,N-dimethyl-*p*-phenylenediamine sulfate (20 mM stock in 7.2 M HCl). The reaction was again allowed to stir for 30 min. The aliquots were transferred to a 96 well plate (250 µL/well) and the absorbance spectra were collected from 500 to 800 nm wavelength.

b) H_2S release from the compounds: Each assay described was done in triplicate in vials with closed lids, containing 273 µL of PBS, 3 µL of compound (10mM stock in DMSO), 3 µL of carbonic anhydrase (1% stock in PBS buffer) ,15 µL of $Zn(OAc)_2$ (40mM stock in H_2O) and 6 µL of H_2O_2 (10 mM stock in buffer) was added. The reaction was allowed to stir at 37 ^oC for 4 h. 100µL of the reaction aliquot was taken out and diluted with 100 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 100 µL of N,N-dimethyl-*p*-phenylenediamine sulfate (20 mM stock in 7.2 M HCl). The reaction was again allowed to stir for 30 min. The aliquots were transferred to a 96 well plate (250 µL/well) and the absorbance spectra was collected from 500 to 800 nm wavelength.

c) H_2S release kinetics: Each assay described was done in triplicate in vials with closed lids, containing 732 µL of PBS, 8 µL of compound (10 mM stock in DMSO), 4 µL of carbonic anhydrase (1% stock in PBS buffer), 40 µL of $Zn(OAc)_2$ (40 mM stock in H_2O) and 16 µL of H_2O_2(10 mM stock in buffer) was added. The reaction was allowed to stir at 37 ^oC for 6h. At predetermined time points, 100 µL of the reaction aliquot was taken out and diluted with 100 µL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 100 µL of N,N-dimethyl-*p*-phenylenediamine sulfate (20 mM stock in 7.2 M HCl). The reaction was again allowed to stir for 30 min. The aliquots were transferred to a 96 well plate (250 µL/well) and the absorbance spectra were collected from 500 to 800 nm wavelength.

4.4 HPLC based kinetics study:

A stock solution of **1a** (10 mM) was prepared in DMSO. The reaction mixture contained **1a** (10 µL, 10 mM), 100 µL of H_2O_2 (10 mM in H_2O) in 990 µL of buffer and stirred at 37 ^oC for 6h. At predetermined time points, aliquots of 100 µL were taken out, filtered (0.22-micron filter) and injected (50 µL) in a high-performance liquid chromatography (HPLC Agilent Technologies 1260 Infinity). The mobile phase was H_2O/ACN. The stationary phase was C-18 reverse phased column (Phenomenex, 5 µm, 4.6 x 250 mm). A multistep gradient was used with a flow rate of 1 mL/min starting with → 0 - 5 min, 70:30 to 50:50 → 5 - 15 min, 50:50 to 10:90 → 15 - 17 min, 10:90 to 40:60 → 17-22 min, 70:30. A similar protocol was followed for the decomposition of **1f**.

4.5 Cytotoxicity assay:

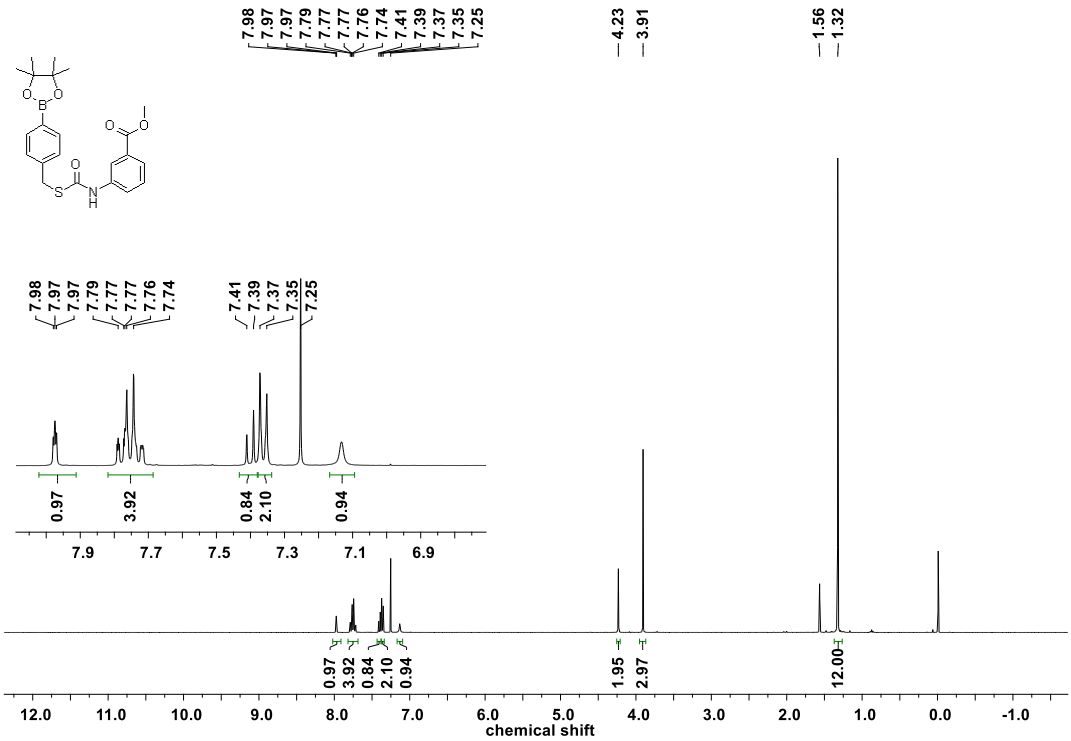
With the help of my colleague Preeti Chauhan, the cytotoxicity assay was performed. Human breast cancer cells MCF-7 were seeded at a concentration of 1 x 10^3/well overnight in a 96-well plate in complete DMEM media. Cells were exposed to varying concentrations of the test compound prepared as DMSO stock solution to make the final concentration of DMSO as 0.5%. The cells were incubated for 24 h at 37^oC. A stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared (3.5 mg in 700 µL of DMEM). The stock prepared was then diluted with 6.3 mL DMEM and 100 µL of the resulting solution was added to each well. After 4 h incubation, carefully the media was removed and 100 µL of DMSO was added. Spectrophotometric analysis of each well was performed using a microplate reader (Thermo Scientific Varioscan) at 570 nm.

4.6 pKa calculation

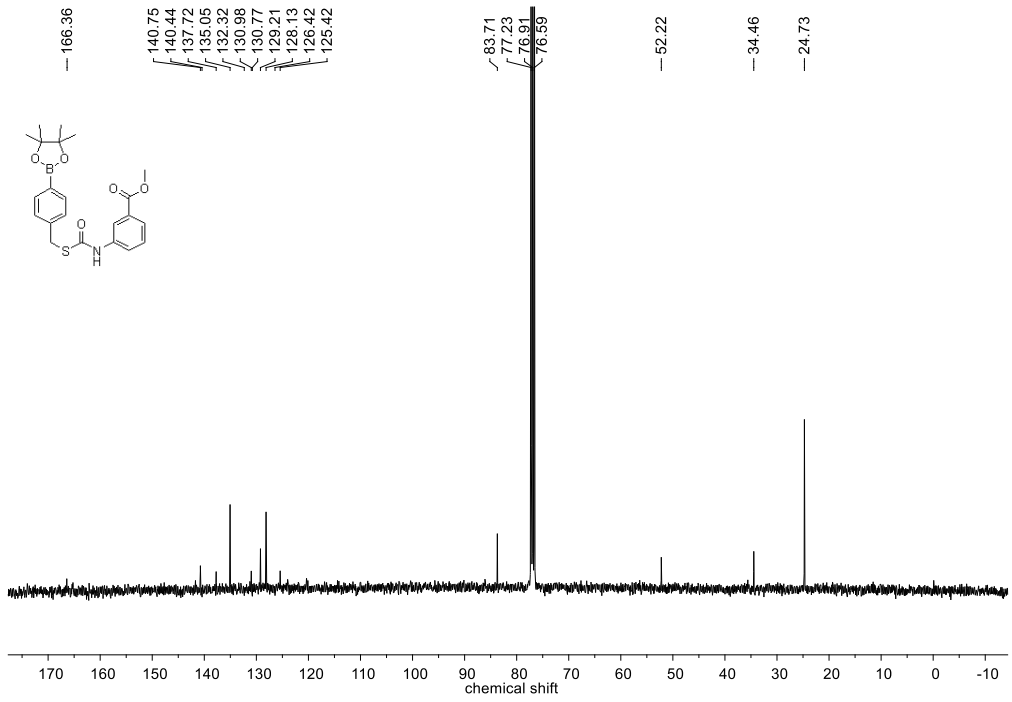
The pKa of the amines were calculated using SciFinder (https://www.cas.org/products/scifinder) .

4.7 NMR spectra of compounds

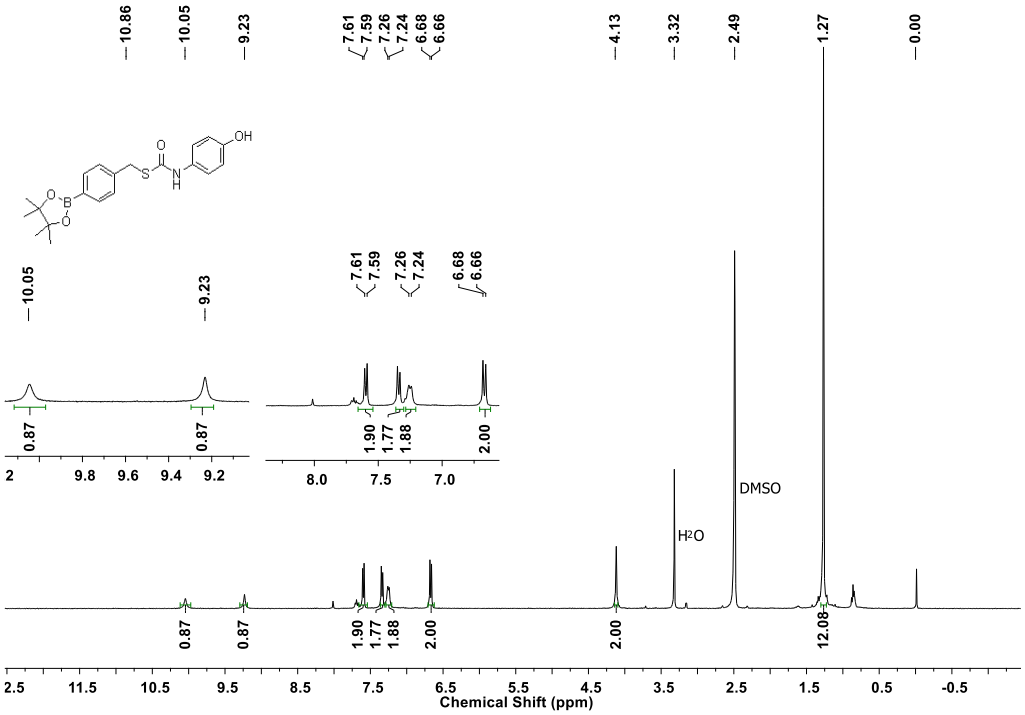
^{1}H NMR (400 MHz, $CDCl_3$) of 1b



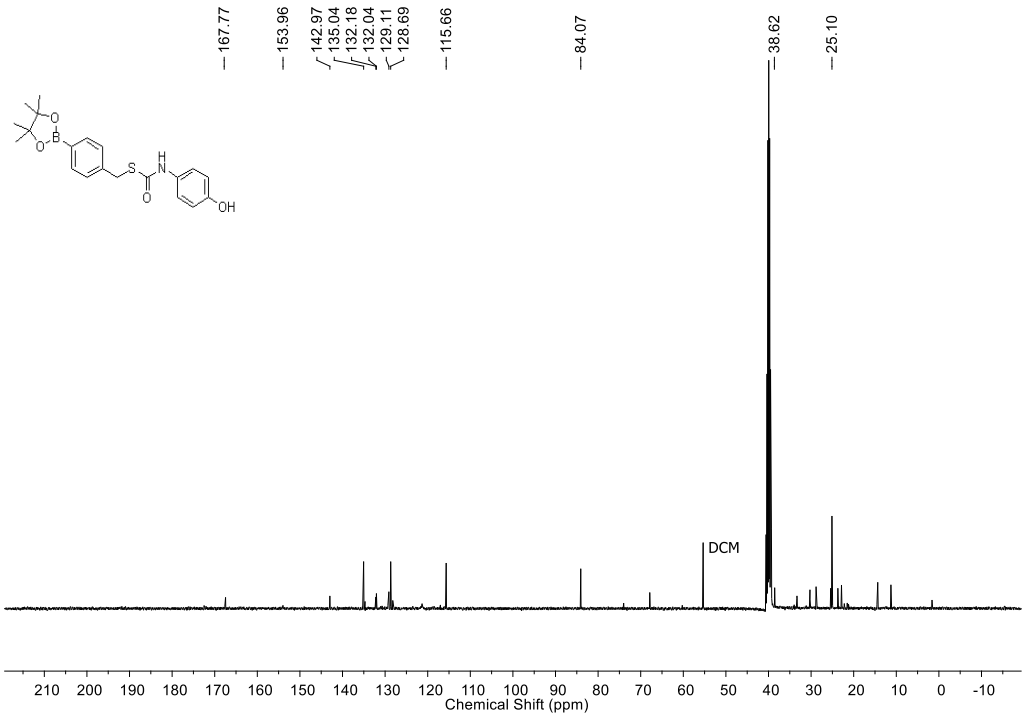
^{13}C NMR (100 MHz, $CDCl_3$) of 1b



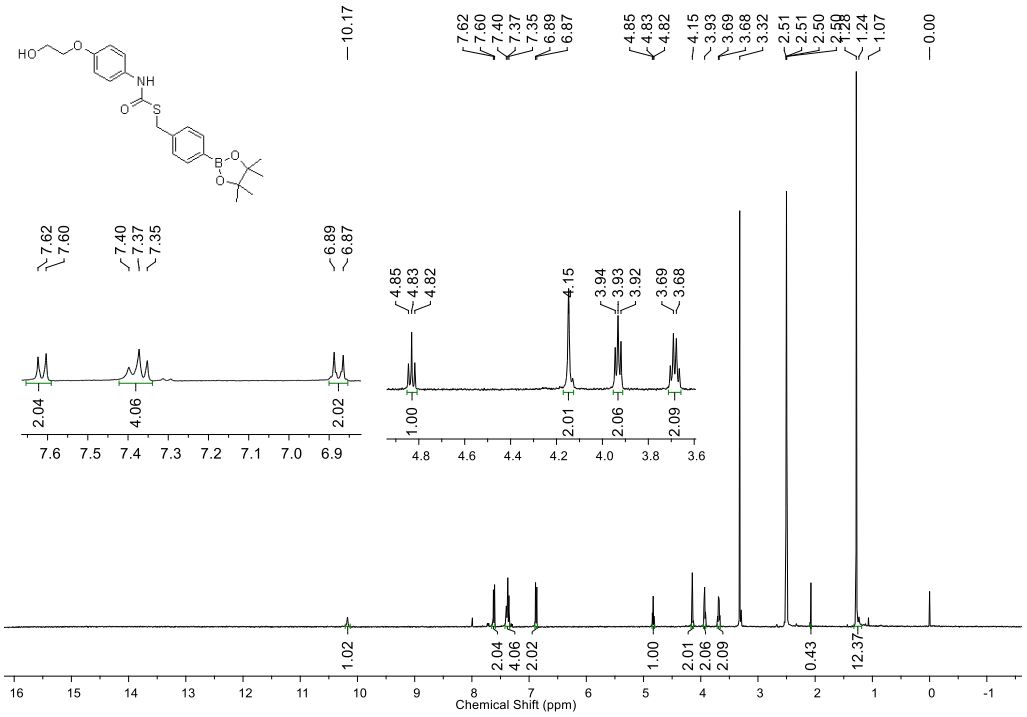
^{1}H NMR (400 MHz, DMSO-d6) of 1c



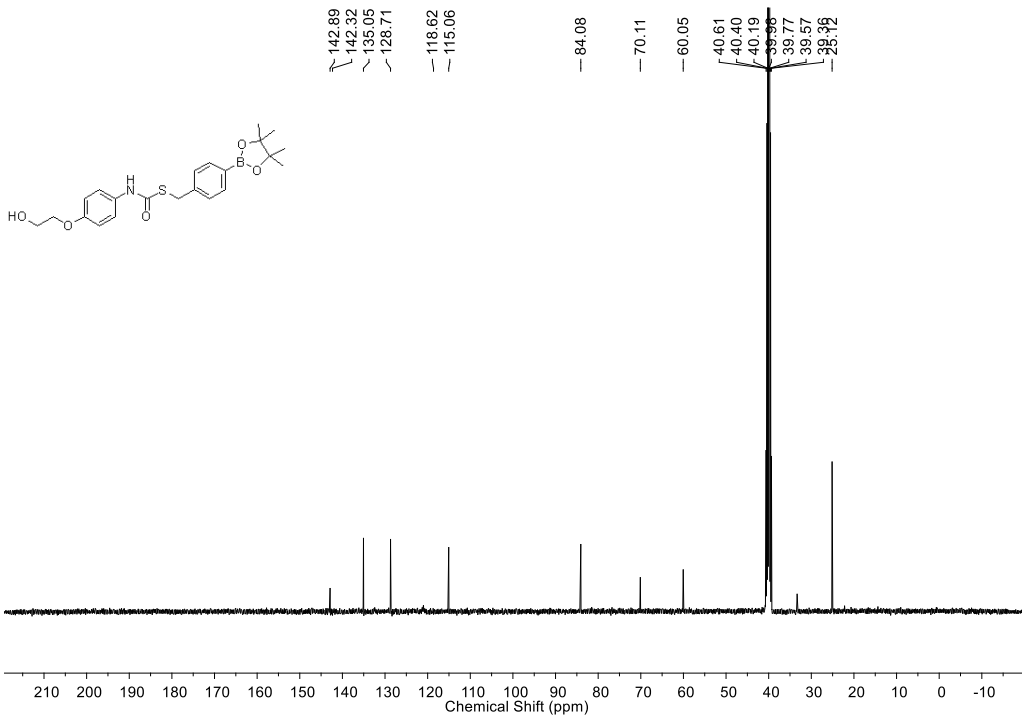
^{13}C NMR (100 MHz, DMSO-d6) of 1c



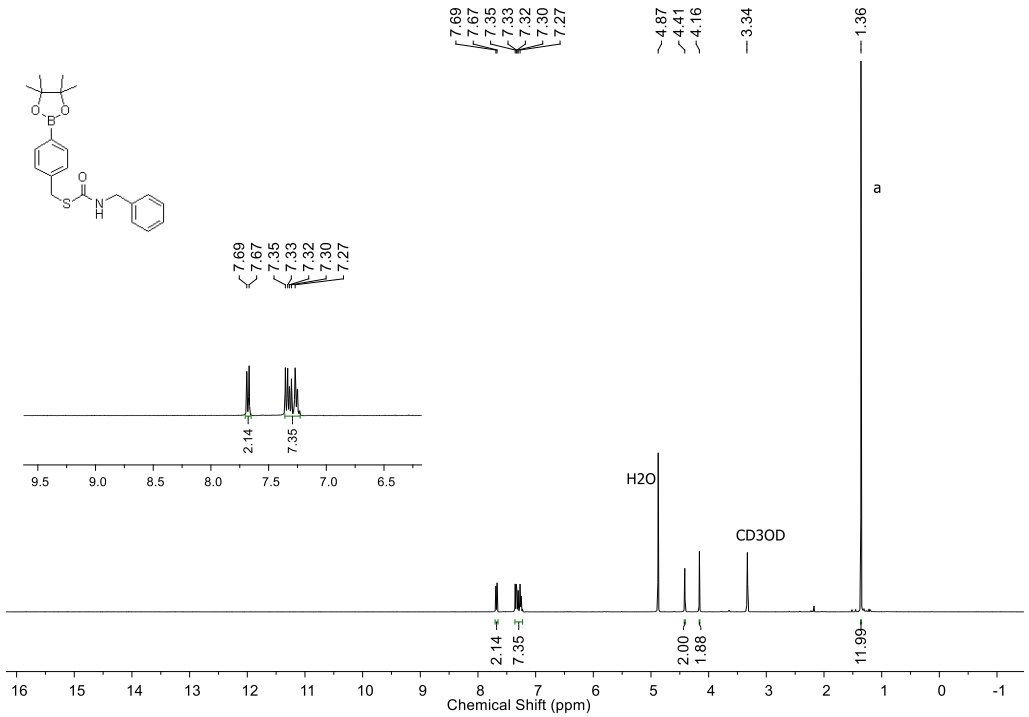
^{1}H NMR (400 MHz, DMSO-d6) of 1d



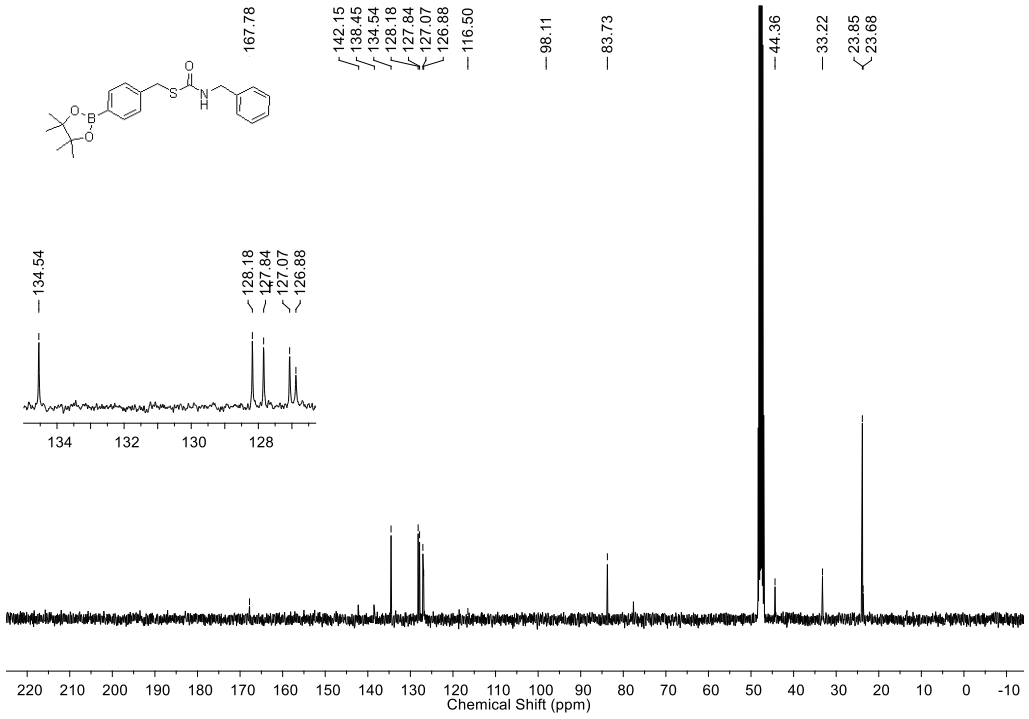
^{13}C NMR (100 MHz, DMSO-d6) of 1d



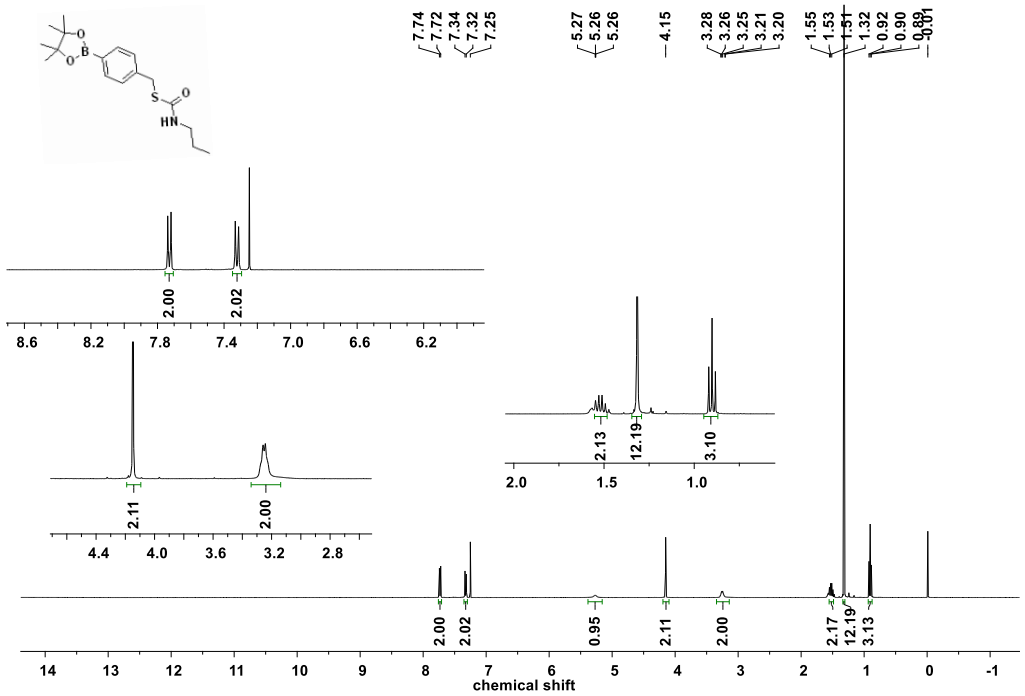
^{1}H NMR (400 MHz, CD_3OD) of 1e



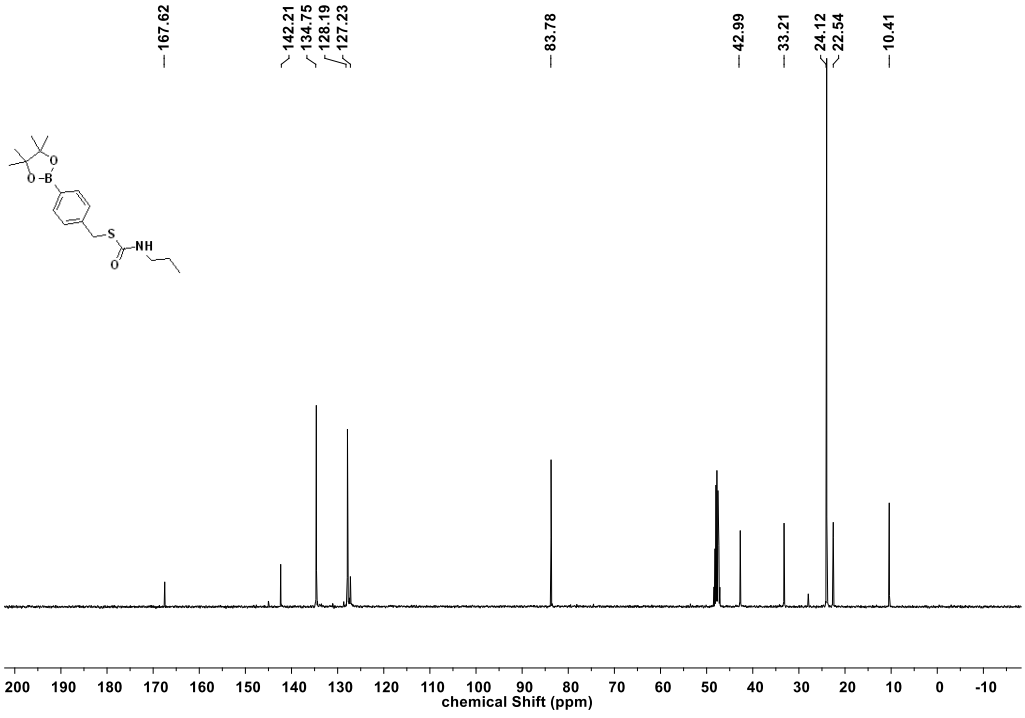
^{13}C NMR (100 MHz, CD_3OD) of 1e



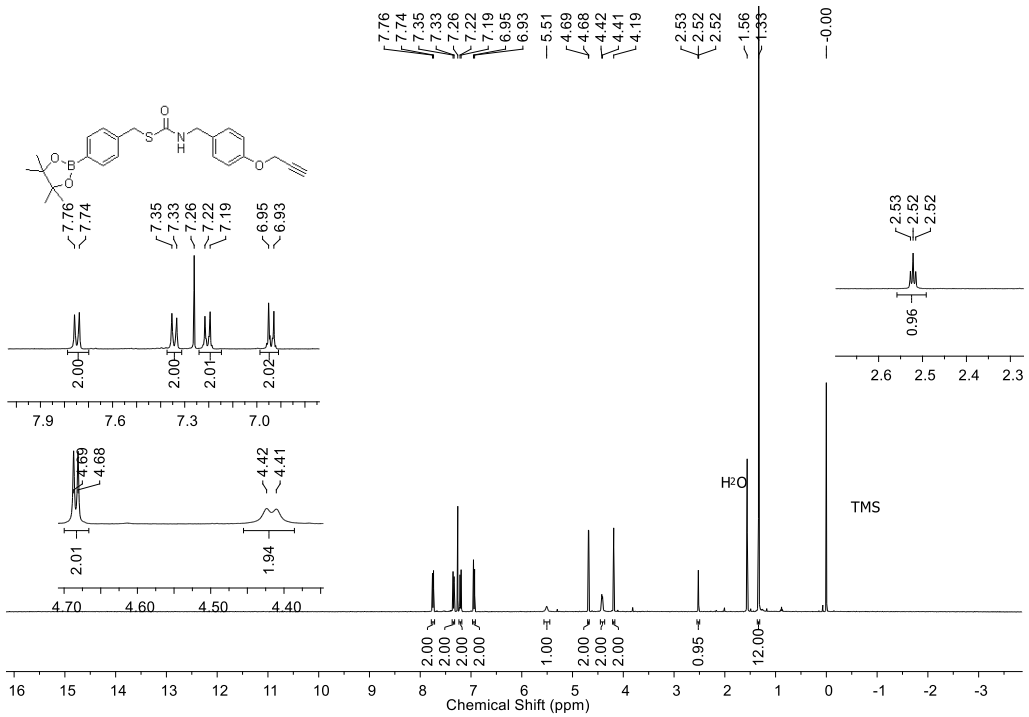
^{1}H NMR (400 MHz, $CDCl_3$) of 1f



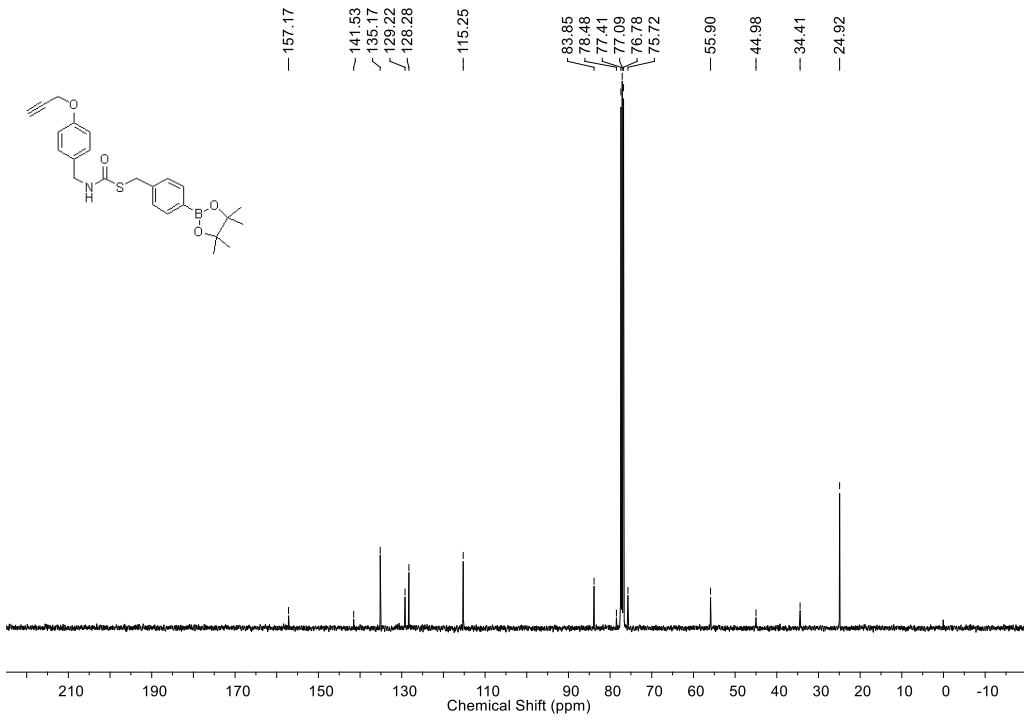
^{13}C NMR (100 MHz, $CDCl_3$) of 1f



^{1}H NMR (400 MHz, $CDCl_3$) of 1g



^{13}C NMR (100 MHz, $CDCl_3$) of 1g



5. RESULTS AND DISCUSSION:

5.1 Synthesis

In order to test our hypothesis, we synthesized a set of seven compounds **1a**-**1g** to study the effects of substituents on nitrogen on the release of H_2S upon activation by H_2O_2. In order to derivatize at the nitrogen, different amines were reacted with *p*-nitro phenyl chloroformate under basic conditions to yield the corresponding carbamate which was then reacted with compound **5** to give the corresponding thiocarbamate in yields ranging from 21-41%. (**Table 1**)

General Scheme

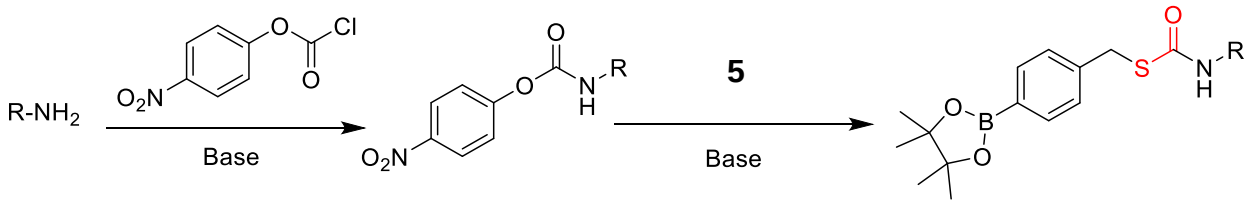
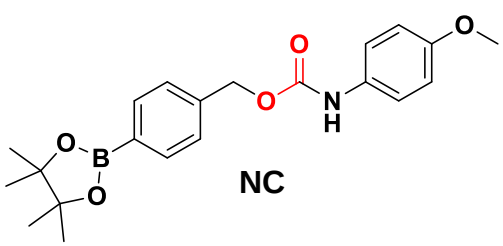


Figure 15: General Scheme for the synthesis of carbamothioates

Entry	Compound	Substituent, R	% Yield
1	**1a**	4-OMe Phenyl	27
2	**1b**	3-COOMe Phenyl	28
3	**1c**	4-OH Phenyl	24
4	**1d**	4-OCH_2CH_2OH Phenyl	32
5	**1e**	Benzyl	21
6	**1f**	$CH_2CH_2CH_3$	27
7	**1g**	4-$OCH_2CH{=}CH_2$ Benzyl	41

Table 1: Synthetic yields of different carbamothioates (unoptimized)



NC

Compound **NC**, synthesized with the help of my colleague, Preeti Chauhan was used as the negative control for the experiments. This compound is activated by H_2O_2 but does not produce COS.

5.2 Methylene Blue Assay for quantification of H_2S

Methylene blue assay, a colorimetric assay for the detection of H_2S was used for assessing the formation of the H_2S by monitoring the formation of Methylene Blue. Authentic Na_2S was used as the positive control and the absorbance profile obtained from it is shown in **Figure 16a**.

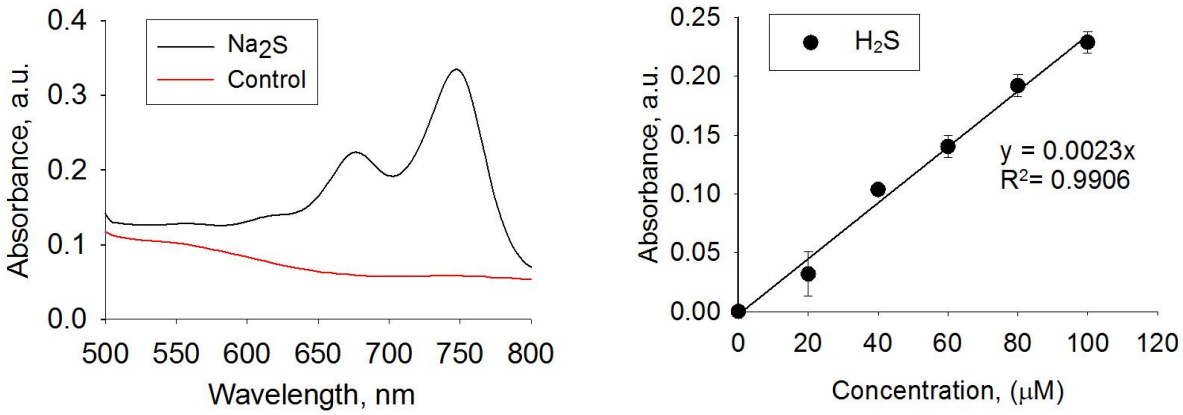


Figure 16: a) Methylene blue Absorbance profile from Na_2S b) Calibration curve for Na_2S

The characteristic peak at 676 nm accounts for methylene blue formation. Similar absorbance profile was obtained with all the compounds with thiocarbamate linkage, upon activation of H_2O_2 in the presence of carbonic anhydrase.

The calibration curve was obtained with different concentrations of Na_2S (**Figure 16b**) and the H_2S release yields from all the H_2S donors synthesized were calculated accordingly. **Table 2** shows the yields of H_2S from the compounds in 1 hour with 10 equivalents of H_2O_2. **Figure 17** shows the comparison of H_2S release from all the donors.

Entry	Compound	% yield of H_2S in 1h
1	**1a**	28
2	**1b**	38
3	**1c**	37
4	**1d**	66
5	**1e**	15
6	**1f**	16
7	**1g**	20

Table 2: H_2S release yields from the compounds in 1 h

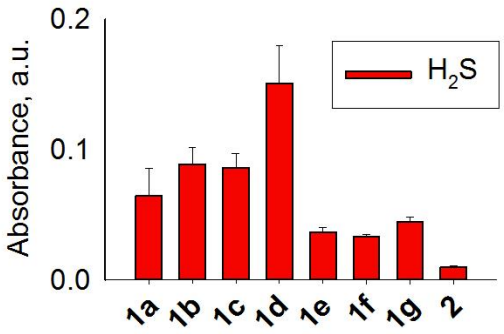


Figure 17 : Comparison of H_2S release from the compounds in 1 h

The higher yield of H_2S from **1d** may be due to the increased solubility because of the increased number of hydrogen bonding groups available. In 1 hour, **1e**, **1f** and **1g** give a lower yield of H_2S, however, the yield increases up to 64, 61 and 84% respectively in 6 hours.

5.3 Kinetics of the release of H_2S

The release kinetics of H_2S from the compounds was assessed with methylene blue assay. As expected, upon activation by H_2O_2, the compounds release H_2S in the presence of carbonic anhydrase and varying the leaving group shows variation in release rates. **Figure 18a** and **18b** shows the release kinetics of the molecules in 6 hours with 2 equivalents of H_2O_2

1a, **1b**, **1c** and **1d** reach saturation in 4 hours, whereas **1e**, **1f** and **1g** do not go to saturation even after 6 hours. This suggests that there is a correlation between the leaving group after the self-immolation on activation by H_2O_2 and the rate of release of H_2S. Further, the experiment was repeated with 10 equivalents of H_2O_2 in order to understand whether the trend persists in the presence of excess H_2O_2. **Figure 19a** and **19b** shows the data for the same. **Table 3** shows the rate of release of H_2S from the compounds and pKa of the leaving group after the activation by H_2O_2 (10 equivalents).

The rate constants are calculated taking the reaction to pseudo-first order with excess H_2O_2. The pKa were referred from literature and predicted pKa was calculated with SciFinder.[22] **Figure 20** shows the comparison of the **1d** and **1f** with 10 equivalents of H_2O_2. Even at a higher concentration of H_2O_2, the rate of release of H_2S shows correlation to the variability in the leaving group. And with excess H_2O_2, the reaction is faster compared to 2 equivalents of H_2O_2.

With 2 equivalents of H_2O_2:

a) b)

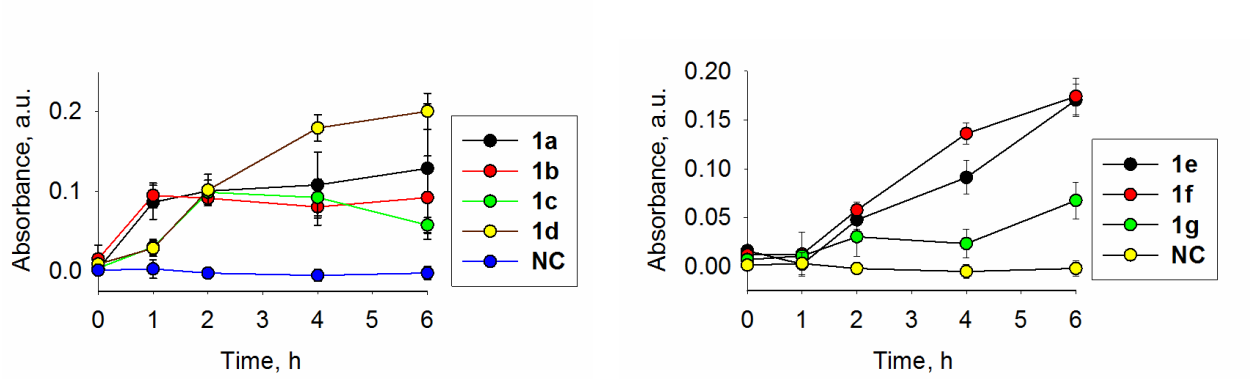


Figure 18: a) H_2S release from **1a**,**1b**,**1c**,**1d** with respect to negative control **(NC)** with 2 equivalents of H_2O_2 b) H_2S release from **1e**, **1f** and **1g** with respect to negative control (**NC**) with 2 equivalents of H_2O_2

With 10 equivalents of H_2O_2:

a) b)

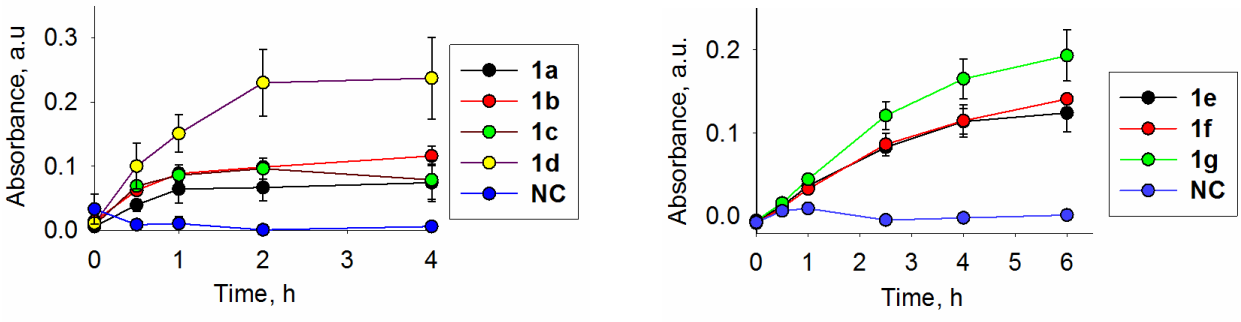


Figure 19: H_2S release from **1a**,**1b**, **1c** and **1d** with respect to negative control (**NC**) with 10 equivalents of H_2O_2. b) H_2S release from **1e**, **1f** and **1g** with respect to negative control (**NC**) with 10 equivalents of H_2O_2

The aniline derivatives of compounds with leaving groups having pKa values 3-6 show higher rate compared to benzylamine derivatives and the aliphatic amine having pKa values in the range 9-11.

Entry	Compound	Rate k, min^{-1}	Half life, min	Leaving group	pKa
1	**1a**	0.0289	23.97	4-methoxy aniline	5.21
2	**1b**	0.026	26.65	methyl 3-amino benzoate	3.45
3	**1c**	0.053	13.07	4-aminophenol	5.43
4	**1d**	0.017	40.5	2-(4-aminophenoxy)ethan-1-ol	5
5	**1e**	0.0049	141.42	phenylmethanamine	9.34
6	**1f**	0.0034	203.82	propan-1-amine	10.71
7	**1g**	0.0045	153.65	(4-(prop-2-yn-1-yloxy)phenyl)methanamine	9.18

Table 3: The rates of release of H_2S, their half-lives from the compounds and the pKa of the leaving group

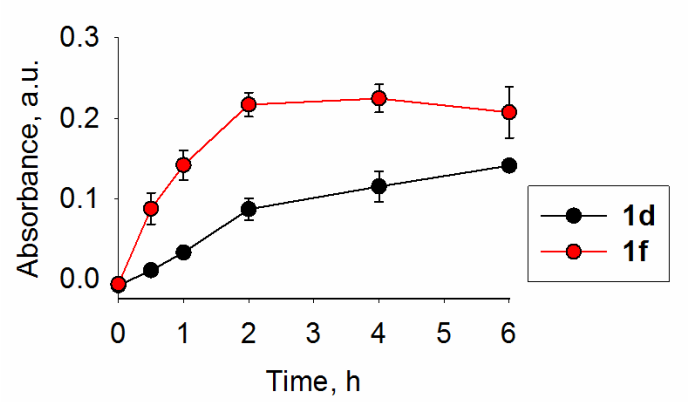


Figure 20: Comparison of H_2S from **1d** and **1f**

Comparing the rates, **1a**, **1b** and **1c** show similar rates (p-value >0.05). Whereas, **1d** is slightly different in rate from **1a**, **1b** and **1c** (p-value= 0.029). However, the difference in rates of release of H_2S from **1a**, **1b**, **1c** and **1d** is extremely statistically significant compared to **1e**, **1f** and **1g** (p-value < 0.001).

In other way, the activation barrier for the reaction to release H_2S from **1f** should be greater than, it is for **1d**.

Using Eyring-Polayani equation:

$$-\Delta G^{\pm} = RTln\, kh/\kappa K_B T$$

Where, $-\Delta G^{\pm}$ is the Gibbs free energy of activation, κ is the transmission coefficient (assumed to be one), k is the rate of reaction, K_B is the Boltzmann constant, h is the plank's constant, R is universal gas constant and T (37°C) is the temperature of the reaction. And value comes to be -90.671 KJ for **1f** and -86.522 KJ for **1d**. This indicates that the change in pKa is affecting the barrier of the reaction in a pronounced manner.

5.4 H_2S release kinetics with HPLC

The release kinetics of the compounds were further validated with HPLC. **1a** and **1f** were taken as representative compounds since the difference in rate constants for the release of H_2S was significant under the activation by H_2O_2. **Figure 22a** shows the representative plot for the release kinetics from **1a** in terms of intermediate formation. In Acetonitrile (ACN) **1a** has a retention time (R_t) of 17.62 min and with incubation in the buffer for 5 minutes, it shifts to 9.85 min. This is due to hydrolysis of boronate ester group to boronic acid group in buffer (**Figure 21**).

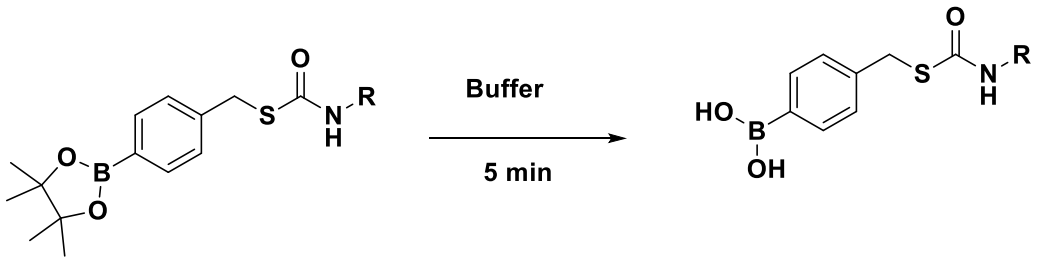


Figure 21: Hydrolysis of Boronate ester in buffer

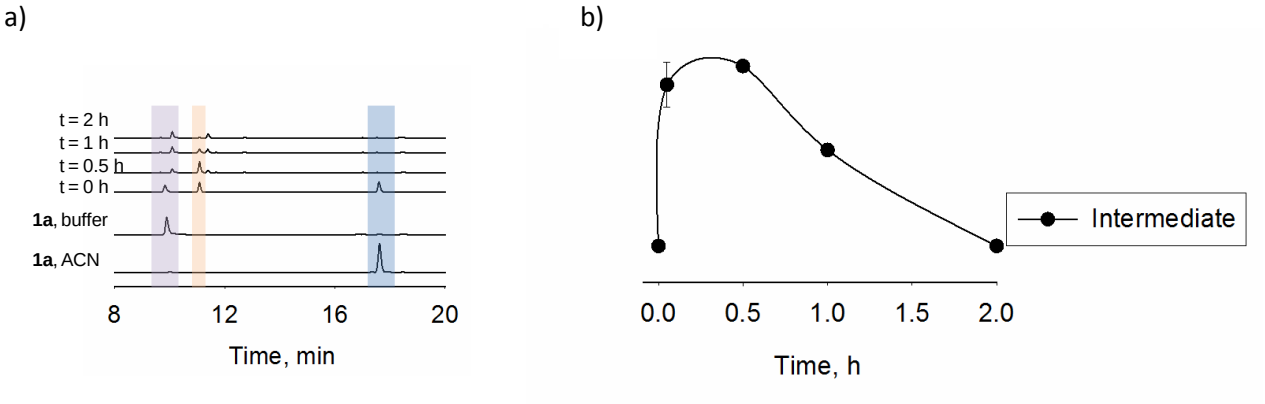


Figure 22: a) Representative plot for the kinetics of release of H_2S from **1a** based on the formation and decomposition of the intermediate. b) Formation and decomposition of intermediate formed from **1a** upon addition of H_2O_2 in buffer

On the activation by H_2O_2 (10 equivalents), **1a** converts to an intermediate (R_t = 11.06 min), which increases in intensity in 30 min and further decomposes within 2 hours (**Figure 22b**). The peak at 5.97 min which corresponds to anisidine (**3**), the product of the reaction of **1a** with H_2O_2 (R_t= 5.97 min) increases in intensity and saturates at 2 hours. (**Figure 23a** and **23b**).. The experiment was carried out in triplicate and similar results were obtained. The yield of p-ansidine from **1a** during the reaction with H_2O_2 is 45 %. And the H_2S yield from **1a** via methylene blue assay at is 33 %. The slightly less yield of H_2S might be due to the quenching of H_2S by the excess H_2O_2.

However, the rate (k = 0.3 min^{-1}) is consistent with the methylene blue assay for H_2S release kinetics data obtained from **1a** (k = 0.289 min^{-1}).

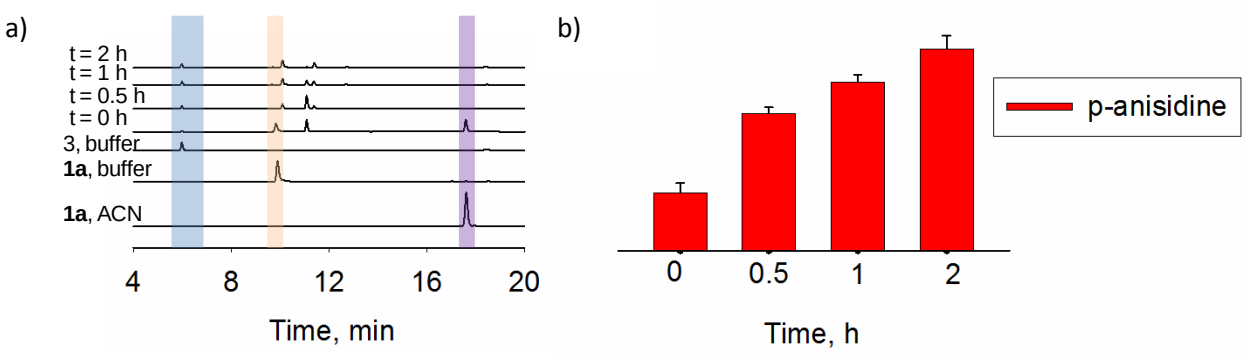


Figure 23: a) Representative plot showing the kinetics of release of H_2S from **1a** with respect to the formation of the product formed after activation by H_2O_2 via HPLC b) Formation of p-anisidine (**3**) from **1a** with time.

Further **1f**, which is a slow H_2S donor among the compounds, was taken for HPLC-based kinetics study. With the activation of H_2O_2 (10 equivalents), **1f** was converted into an intermediate (R_t= 9.31 min), which increases in intensity in 1 hour and further decomposes in 6 hours (**Figure 24a** and **24b**). Since the product formed after the self-immolation under the activation of H_2O_2 from **1f** is not UV active the product formation was not monitored. However, the formation and decomposition of the intermediate suggest consistency with the methylene blue assay kinetics for H_2S release from **1f**.

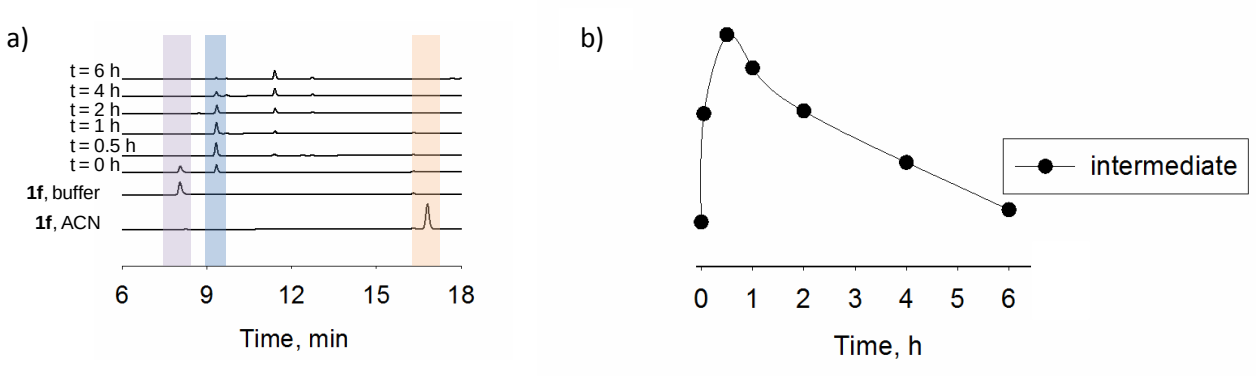
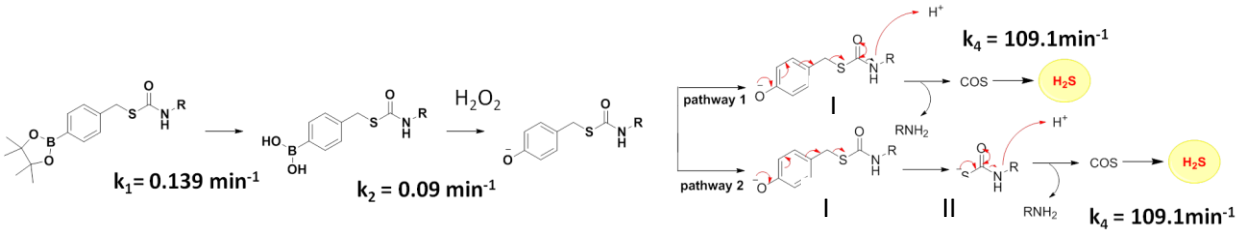


Figure 24: a) Representative plot for the decomposition of **1f** upon addition of H_2O_2 with respect to the intermediate formation. from HPLC b) Formation and decomposition of intermediate for **1f**

These experiments verify that the compounds on the activation by H_2O_2 release H_2S in the presence of carbonic anhydrase at different rates depending upon the leaving group, which is in turn dependent on the pKa.

The experiment also shines light on the mechanism of release of H_2S and into the rate limiting step of the reaction (**Figure 25**). In buffer, the compounds hydrolyzes rapidly to produce the boronic acid derivative[23] (**k = 0.1386 min^{-1}**). We see a new peak immediately after the addition of H_2O_2 which saturates over a period of 30 minutes. This indicates that the reaction of boronic acid with H_2O_2 is fast. The intermediate formed decomposes in 2 hours incase of **1a** and in 6 hours incase of **1f**. Since the compounds **1a** and **1f** vary only for the amine used, we can say that, the difference in the time of decomposition of the intermediate is dependent on the leaving group (RNH_2). The intermediate **I** is formed fast[24] (**k=0.09 min**), because we observe a new peak (11.06 min for **1a** and 9.31 min for **1f** in less than 5 minutes in both **1a** and **1f** and saturates

within 30 minutes in both case. So the formation of the intermediate **I** cannot be the rate limiting step. Also, the hydrolysis of the COS to H_2S cannot be the rate limiting step because, HPLC experiment was performed without CA, but the rate of anisidine formation with HPLC experiment (**0.03 min^{-1}**) and H_2S release from methylene blue (**0.0289 min^{-1}**) are similar. And it is reported that rate constant for COS hydrolysis by CA is **109.2 min^{-1}**.[25] This brings down to the step, where, self-immolation and protonation happens. It can happen in either via **pathway 1** or **2**. In **pathway 1** the intermediate **I** that is formed self-immolates based on the electron density on the amine and this, in turn, is dependent on the pKa. If the pKa is lower then the electron withdrawing nature of the amine is more prominent and thus the self-immolation is faster, and likewise will be the protonation of the amine. If the pKa of the amine is high, then it will be less electron withdrawing in nature, and thus the self-immolation will be slower and so the protonation is also slower. In **pathway 2** the intermediate **I** decomposes to **II** , which then gets protonated and leave. The leaving group ability is associated with the basicity. lower is the pKa , better is the leaving group ability. Probably, **pathway I** is followed, because we see only one intermediate in HPLC. In both cases as a result, carbonyl sulfide (COS), RNH_2 and quinone methide are formed. Although we were unable to characterize the intermediate, this experiment clearly shows the role of changing the amine in the kinetics of H_2S release under the activation by H_2O_2. In this way changing the amine depending on pKa, can help tune the release of H_2S.



IFigure 25 : Proposed mechanism of H_2S release from compounds with respect to the decomposition data from HPLC

5.5 Cytotoxicity study

With the help of my colleague Preeti Chauhan we did the cytotoxicity study for **1f**. Compound was not found to be significantly toxic at 25µM. (**Figure 26**).

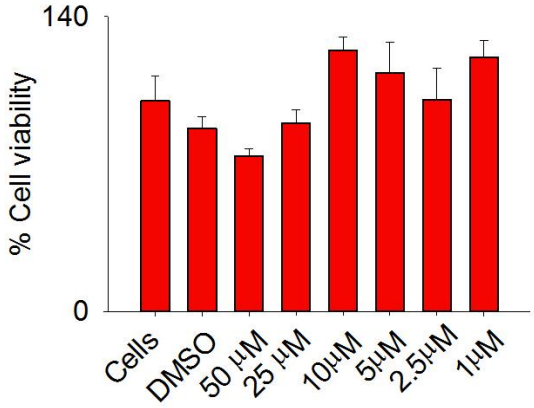


Figure 26: Cytotoxicity of **1f** at various concentrations on MCF -7 breast cancer cells with respect to the cells control

Series of boronate ester carbamothioates were synthesized, by varying the leaving group, which could be activated under oxidative stress. These compounds upon activation by H_2O_2 undergo self-immolation to release carbonyl sulfide, which is then hydrolyzed by carbonic anhydrase to release H_2S. The yields of H_2S from the molecules after saturation when triggered by H_2O_2, in the presence of carbonic anhydrase, ranged from 30-85%. The proposed hypothesis was that by changing the leaving group after self-immolation can provide a hands on tool for tunability in the release of H_2S. In accordance with this, the methylene blue studies on the kinetics of H_2S suggests that the rate of release of H_2S was dependent on the pKa of the leaving group. For the compounds with aniline derivatives as the leaving group with pKa ranging from 3-6, the rates were significantly higher compared to benzyl amine derivatives and the aliphatic amine having pKa ranging from 9-11. The release kinetics of representative molecules from each group was further validated with HPLC and we observed similar rates of release of H_2S. HPLC data provided a mechanistic view to the kinetics of release of H_2S from the donors. An intermediate was formed and the decomposition of it was followed in each case and was found to be in accordance with the results from methylene blue assay. This also provided evidence that the rate limiting step for the reaction of the compounds with H_2O_2 involves the self-immolation and the protonation of the amine which leads to the elimination of RNH_2 and thus supports our hypothesis. We were able to synthesize H_2S donors with tunability ranging from 13 - 204 min in terms of the half-life of the H_2S release, by varying the substitution at nitrogen. **1f**, which has propyl amine as the leaving group with 204 min

as half life is the first small molecule H_2S donor reported to our knowledge which can release H_2S continuously with the activation by ROS, with no significant cytotoxicity at relevant concentrations. The ROS trigger can provide selectivity and control over the H_2S release and the rates of release can be tuned via varying leaving group. Recently, Pluth and co-workers reported a series of H_2S donors with ROS trigger, via changing the COS core.[25] They included only aniline derivatives for the study and tunability that they claim to achieve is in a narrow range in terms of rates. To our knowledge, this is the first study done in terms of changing the leaving group depending on the pKa to tune the release of H_2S from carbamothioates with ROS trigger. Also, this provides further motivation for derivatizing the leaving group for organelle selectivity. For example, the leaving group can be synthetically modified to a mitochondria targeting group and could be used to release H_2S in a slow and continuous manner in mitochondria which are mainly involved in dealing with ROS, and thus pave way for utilizing H_2S based therapy for diseases related to mitochondria dysfunctioning.

7. REFERENCES

(1) Wang, R. Physiological Implications of Hydrogen Sulfide: A Whiff Exploration That Blossomed. *Physiol. Rev.* **2012**, *92*, 791–896.

(2) Zhao, Y.; Biggs, T. D.; Xian, M.; Vandiver, M. S.; Snyder, S. H.; Wang, R.; Li, L.; Moore, P. K.; Szabó, C.; Kolluru, G. K.; et al. Hydrogen Sulfide (H_2S) Releasing Agents: Chemistry and Biological Applications. *Chem. Commun.* **2014**, *50* (80), 11788–11805.

(3) Abe, K.; Kimura, H. The Possible Role of Hydrogen Sulfide as an Endogenous Neuromodulator. *J. Neurosci.* **1996**, *16* (3), 1066–1071.

(4) Wallace, J. L.; Wang, R. Hydrogen Sulfide-Based Therapeutics: Exploiting a Unique but Ubiquitous Gasotransmitter. *Nat. Rev. Drug. Discov.* **2015**, *14* (5), 329–345.

(5) Mustafa, A. K.; Sikka, G.; Gazi, S. K.; Steppan, J.; Jung, S. M.; Bhunia, A. K.; Barodka, V. M.; Gazi, F. K.; Barrow, R. K.; Wang, R.; et al. Hydrogen Sulfide as Endothelial Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels. *Circ. Res.* **2011**, *109* (11), 1259–1268.

(6) Calvert, J. W.; Elston, M.; Nicholson, C. K.; Gundewar, S.; Jha, S.; Elrod, J. W.; Ramachandran, A.; Lefer, D. J. Genetic and Pharmacologic Hydrogen Sulfide Therapy Attenuates Ischemia-Induced Heart Failure in Mice. *Circulation* **2010**, *122* (1), 11–19.

(7) Wallace, J. L.; Vong, L.; McKnight, W.; Dicay, M.; Martin, G. R. Endogenous and Exogenous Hydrogen Sulfide Promotes Resolution of Colitis in Rats. *Gastroenterology* **2009**, *137* (2), 569–578.e1.

(8) Zhao, Y.; Wang, H.; Xian, M. Cysteine-Activated Hydrogen Sulfide (H2S) Donors. *J. Am. Chem. Soc.* **2011**, *133* (1), 15–17.

(9) Devarie-Baez, N. O.; Bagdon, P. E.; Peng, B.; Zhao, Y.; Park, C.-M.; Xian, M. Light-Induced Hydrogen Sulfide Release from “Caged” Gem-Dithiols. *Org. Lett.* **2013**, *15* (11), 2786–2789.

(10) Fukushima, N.; Ieda, N.; Sasakura, K.; Nagano, T.; Hanaoka, K.; Suzuki, T.; Miyata, N.; Nakagawa, H. Synthesis of a Photocontrollable Hydrogen Sulfide Donor Using Ketoprofenate Photocages. *Chem. Commun.* **2014**, *50* (5), 587–589.

(11) Steiger, A. K.; Zhao, Y.; Pluth, M. D. Emerging Roles of Carbonyl Sulfide in Chemical Biology: Sulfide Transporter or Gasotransmitter? *Antioxid. Redox Signal.* **2017**.

(12) Steiger, A. K.; Pardue, S.; Kevil, C. G.; Pluth, M. D. Self-Immolative Thiocarbamates Provide Access to Triggered H2S Donors and Analyte Replacement Fluorescent Probes. *J. Am. Chem. Soc.* **2016**, *138* (23), 7256–7259.

(13) Zhao, Y.; Pluth, M. D. Hydrogen Sulfide Donors Activated by Reactive Oxygen Species. *Angew. Chem. Int. Ed.* **2016**, *55* (47), 14638–14642.

(14) Powell, C. R.; Foster, J. C.; Okyere, B.; Theus, M. H.; Matson, J. B. Therapeutic Delivery of H2S via COS: Small Molecule and Polymeric Donors with Benign Byproducts. *J. Am. Chem. Soc.* **2016**, *138* (41), 13477–13480.

(15) Chauhan, P.; Bora, P.; Ravikumar, G.; Jos, S.; Chakrapani, H. Esterase Activated Carbonyl Sulfide/Hydrogen Sulfide (H2S) Donors. *Org. Lett.* **2017**, *19* (1), 62–65.

(16) Sharma, A. K.; Nair, M.; Chauhan, P.; Gupta, K.; Saini, D. K.; Chakrapani, H. Visible-Light-Triggered Uncaging of Carbonyl Sulfide for Hydrogen Sulfide (H2S) Release. *Org. Lett.* **2017**, *19* (18), 4822–4825.

(17) Whiteman, M.; Li, L.; Rose, P.; Tan, C.-H.; Parkinson, D. B.; Moore, P. K. The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. *Antioxid. Redox Signal.* **2010**, *12* (10), 1147–1154.

(18) Zheng, Y.; Yu, B.; Ji, K.; Pan, Z.; Chittavong, V.; Wang, B. Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angew. Chemie Int. Ed.* **2016**, *55* (14), 4514–4518.

(19) Foster, J. C.; Radzinski, S. C.; Zou, X.; Finkielstein, C. V; Matson, J. B. H2S-Releasing Polymer Micelles for Studying Selective Cell Toxicity. *Mol. Pharm.* **2017**, *14* (4), 1300–1306.

(20) Miller, E. W.; Albers, A. E.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. Boronate-Based Fluorescent Probes for Imaging Cellular Hydrogen Peroxide. *J. Am. Chem. Soc.* **2005**, *127* (47), 16652–16659.

(21) Elrod, J. W.; Calvert, J. W.; Morrison, J.; Doeller, J. E.; Kraus, D. W.; Tao, L.; Jiao, X.; Scalia, R.; Kiss, L.; Szabo, C.; et al. Hydrogen Sulfide Attenuates Myocardial Ischemia-Reperfusion Injury by Preservation of Mitochondrial Function. *Proc. Natl. Acad. Sci.* **2007**, *104* (39), 15560 LP-15565.

(22) Hall, H. K. Correlation of the Base Strengths of Amines1. *J. Am. Chem. Soc.* **1957**, *79*, 5441–5444.

(23) Achilli, C.; Ciana, A.; Fagnoni, M.; Balduini, C.; Minetti, G. Susceptibility to Hydrolysis of Phenylboronic Pinacol Esters at Physiological pH. *Cent. Eur. J. Chem.* **2013**, *11* (2), 137–139.

(24) Zielonka, J.; Sikora, A.; Hardy, M.; Joseph, J.; Dranka, B. P.; Kalyanaraman, B. Boronate Probes as Diagnostic Tools for Real Time Monitoring of Peroxynitrite and Hydroperoxides. *Chem. Res. Toxicol.* **2012**, *25* (9), 1793–1799.

(25) Zhao, Y.; Henthorn, H. A.; Pluth, M. D. Kinetic Insights into Hydrogen Sulfide Delivery from Caged-Carbonyl Sulfide Isomeric Donor Platforms. *J. Am. Chem. Soc.* **2017**, *139* (45), 16365–16376.