An Esterase Activated Persulfide Donor with a Fluorescence Reporter

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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20151004

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CERTIFICATE

This is to certify that this dissertation entitled "*An Esterase Activated Persulfide Donor with a Fluorescence Reporter"* towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Amal S Kumar (20151004)** at Indian Institute of Science Education and Research under the supervision of **Dr. Harinath Chakrapani**, **Associate Professor, Department of Chemistry**, during the academic year **2019-2020.**

Dr. Harinath Chakrapani
Associate Professor
Department of chemistry
IISER Pune

Place:
Date:

DECLARATION

I hereby declare that the matter embodied in the report entitled "*An Esterase Activated Persulfide Donor with a Fluorescence Reporter*" are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Harinath Chakrapani and the same has not been submitted elsewhere for any other degree

Amal S Kumar

(20151004)

ACKNOWLEDGMENT

It is my immense pleasure to thank everyone who supported me and help me to make this thesis possible. I would like to express the most profound appreciation to my supervisor, Dr. Harinath Chakrapani, who continually and convincingly conveyed a spirit of adventure regarding research and excitement regarding teaching. Without his guidance and persistent help, this dissertation would not have been possible. Also, I would like to thank Prof. Srinivas Hotha for his valuable presence and suggestions during my mid-year evaluation.

I would like to express my special gratitude and thanks to my colleague Mr. T. Anand Kumar, for continuously supporting and helping me throughout my project. Also, I would like to thank my lab mates Laxman, Prerona, Suman, Gaurav, Harshit, Utsav, Bharat, Farhan, Minhaj, Subhayan, Dr. Ajay Sharma, Dr. Preeti Chauhan, Dr. Amogh Kulkarni, and Pooja for maintaining a friendly environment in the lab, and for their continuous support during my experiments. I would like to thank all the operators for all the spectroscopic data. I would like to thank my friend SwathiKrishna and Saswata Nayak, for their support and motivation during difficult times. The successful implementation of the project would not have been possible without the cutting-edge facilities provided by IISER.

Above all, I would like to thank my parents and my sister, who were very supportive throughout my career.

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1. ABBREVATIONS

NMR	Nuclear Magnetic Resonance
HRMS	High Resolution Mass Spectrometry
J	Coupling Constant
Hz	Hertz
MHz	Megahertz
EtOAC	Ethyl Acetate
DCM	Dicholoromethane
DMSO	Dimethyl Sulfoxide
mg	milligram
g	gram
mL	milliliter
mmol	milli moles
ACN	Acetonitrile

2. ABSTRACT

Persulfides are found physiologically in prokaryotes, eukaryotes and mammalian tissues, which are abundant in quantities. The persulfides are formed by a process called persulfidation or *S*-sulfhydration, which is now recognized as the primary pathway for signal transduction. Along with the H_2O_2 scavenging ability, it is also known to activate or inhibit protein activity by regulating the structures and thereby the functions. Recent signalling studies the potential importance of persulfides (RSSH) was revealed in redox biology. This prompted further investigation of their chemical properties for providing a basic understanding of their probable functioning. This led us to the development of a triggerable persulfide donor. In this project, we aim to design a persulfide (RSSH) donor, which releases persulfide (RSSH) upon activation along with the release of a fluorophore, and the fluorescent signal was used for the direct quantification of the RSSH. Here, we attempted to study the release as well as the quantification of RSSH with esterase as an enzyme trigger and 3-(allyloxy)-6H-benzo[c]chromen-6-one as a fluorescence reporter. Due to synthetic challenges, we modified the ester trigger as well as the persulfide and was characterized by NMR, Mass spectrometry, and HPLC. Upon activation with esterase, the compound undergoes lactonization to form a lactone (fluorophore), and the fluorescence was exploited for the quantification of the persulfide. The fluorescence signal that is produced should correlate with the persulfide released or is an estimate of the persulfide released. Encouragingly, HPLC analysis showed that there is a 1:1 correlation between the fluorophore and the persulfide released. Although excellent correlation between the signal and yield of persulfide was seen, the yield observed was 50%. The rationale for diminished yield is not clear, but could be due to poor solubility in buffer. The fluorescence studies also showed that the fluorophore yield was less than 50%, which was not quantitative. We hypothesize that this could be due to the solubility issues of both the persulfide donor as well as the fluorophore. The outlook of the project is to increase the solubility of the persulfide donor as well the fluorophore by changing the functional groups to achieve a quantitative yield of both.

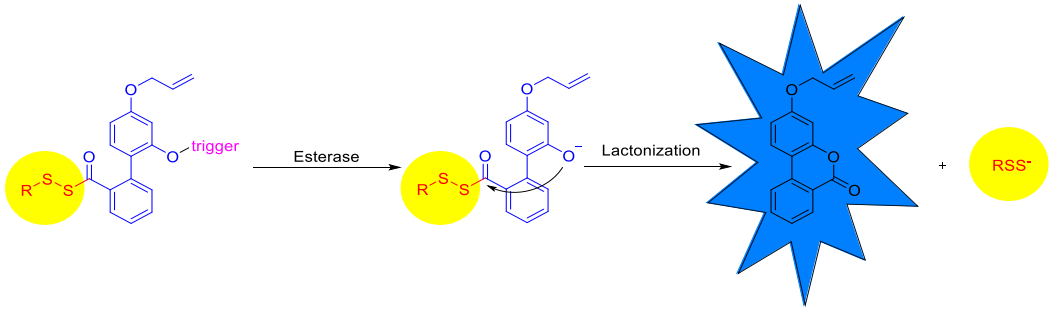


Figure 1: Proposal of a triggerable persulfide donor

3. INTRODUCTION

One of the important gaseous signaling molecule is Hydrogen sulfide (H_2S). Recently, the studies revealed that the persulfides (RSSH) have a more significant role in the hydrogen sulfide (H_2S) signaling (Park *et al.*, 2015). Even though the H_2S signaling is a sought-after research area, persulfides (RSSH) are less studied and of increased research interest.

3.1 Biosynthesis, metabolism and biological application of persulfides

Hydrogen sulfide (H_2S) is endogenously produced gaseous molecule, which is useful as harmful to both plants and animals, whereas its effect on smaller organisms is vaguely known. Therapeutic effects of H_2S includes the cytoprotection during reperfusion injury, inhibiting the proliferation of vascular smooth muscle cell, relaxation of smooth muscle cell, and inhibition of platelet aggregation(Lavu, Bhushan and Lefer, 2011). H_2S also acts as a modulator of inflammation(Li *et al.*, 2005; Zanardo *et al.*, 2006) and protects against glutamate-induced cytotoxicity(KIMURA and KIMURA, 2004; Kimura, Goto and Kimura, 2010). Although recent studies have shown the potential importance of H_2S as a signaling molecule, sulfide-based signaling is poorly understood. There are reports that the H_2S exerts its effects on protein by modifying the cysteine residues, an oxidative post-translational modification known as persulfidation or *S*-sulfhydration. Recently, this post-translational modification is accepted as one of the signaling pathways(Mustafa *et al.*, 2009).

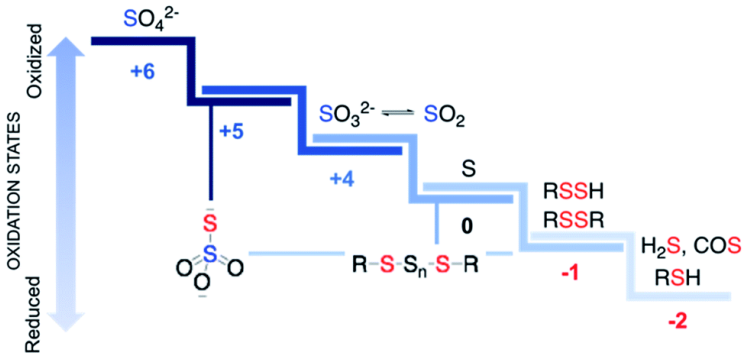


Figure 2: Some of the biologically relevant sulfur species in different oxidation states (Bora et al., 2018).

Hydrogen sulfide (H_2S) cannot directly modify thiol containing protein residues due to its limitation in the reduction potentials. The low reactivity of H_2S towards disulfides(Cuevasanta *et al.*, 2015) and other oxidised thiols led to the consideration

of alternate sulfur donors like persulfides (RSSH). Persulfides form a class of sulfur species with the oxidation state of each sulfur as -1, which are more nucleophilic when compared to their structural analog thiols (RSH)(Edwards and Pearson, 1962). *S*-sulfhydrating proteins in the physiological systems of mammals involve sulfur transferases like 3-mercaptopyruvate sulfurtransferase (3-MST), human rhodanese, and thiosulfate sulfurtransferase (TST)(Mishanina, Libiad and Banerjee, 2015). These form an intermediate with the cysteine in the active site of the protein. Small molecule persulfides like cysteine persulfide (CysSSH) and homocysteine persulfide (HcySSH) are biosynthesized by transsulfuration pathway enzymes like cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) as shown in **Figure 3.**

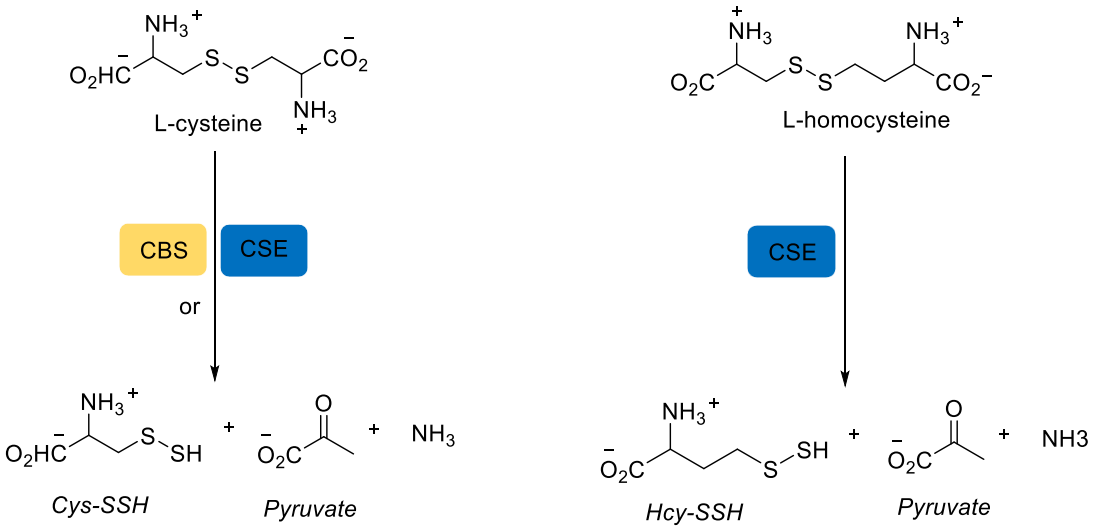


Figure 3: Persulfide generating reactions by transsulfuration enzymes(Yadav et al., 2016)

However, there is a high level of substrate uncertainty and reaction promiscuity exhibited by the transsulfuration pathway enzymes. Further studies have shown that the enzymes CBS and CSE generate significant levels of persulfides/polysulfides (i.e., CysSSH, GSSH, etc.) inside the cell. Also, the most abundant persulfide in the physiological system is glutathione persulfide (GSSH), which is identified as an essential part in the cellular antioxidant defenses(Ida *et al.*, 2014).

3.2 Cellular and molecular effects

Böhme and Zinner reported the first preparation of aryl- and alkyl-persulfides (RSSH) in 1954(H. Böhme and G. Zinner, 1954). The inherent instability of RSSH makes these species challenging to study. An essential physiological molecule for cellular survival

is Glutathione (GSH)(Trachootham *et al.*, 2008). Along with glutathione (GSH), its oxidized forms such as persulfide (GSSH) and disulfide (GSSG) were also known to play essential roles in signaling(Ida *et al.*, 2014). A redox process which regulates thioredoxin-glutathione signaling in CNS is an example of this(Ren *et al.*, 2017). Recent studies demonstrated the relevance of persulfides being a n anti-oxidative as well as prooxidative species, as shown in **Figure 4**. At physiological pH, the persulfides undergo oxidation with molecular oxygen to reactive oxygen species, which are found to be DNA damaging agents in Gates's Leinamycin model(Gates, 2000).

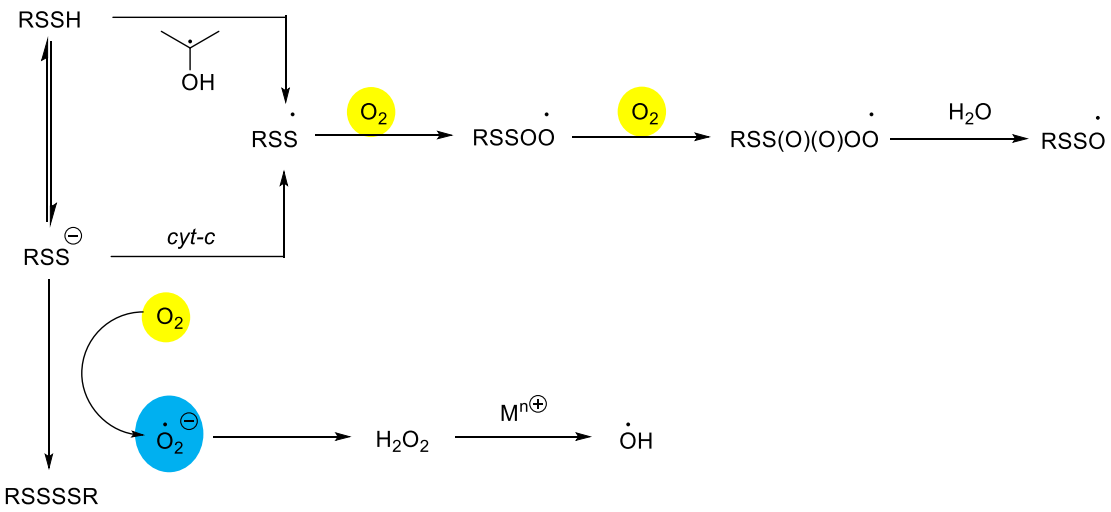


Figure 4: Persulfides as antioxidants and pro-oxidants

3.3 Persulfide donors

Horst and Gerwalt primarily reported the preparation of low molecular weight persulfides in 1954(Von Horst Bohme and Gerwalt Zinner, 1954). However, their potency for protein persulfidation and chemical biology of these persulfides was not explored. In order to study the chemical biology of persulfide, Erwan Galardon and Isabelle Artaud developed a small molecule persulfide precursor, which spontaneously rearranges by an intramolecular acyl transfer, generating the persulfide(Artaud and Galardon, 2014). Recently, various kinds of donors have been reported with tunability and specificity, which can be used for biological studies.

3.3.1 Persulfide donors based on 1,2 - O, S relay mechanisms

The first primary class of Persulfide donors was based on 1,2- O, S relay mechanism reported by Ming Xian and co-workers in 2016(Park *et al.*, 2016). In this mechanism, sulfur and oxygen are placed next to each other. They have used FmSSPy-A (Fm - 9-fluorenylmethyl) as the template. This can translate small molecules and thiols (-SH) in proteins to form persulfide adducts with Fm (-S-SFm) under mild conditions. They exhibited H_2S free method to generate persulfides. They have also demonstrated the increased nucleophilicity of persulfides towards a diversity of thiol blocking agents. However, the use of DBU impacts the reactivity studies, and for the chemical biology studies, this might not be effective.

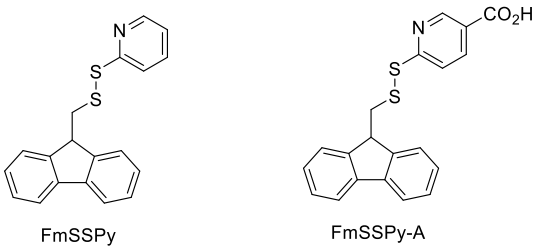


Figure 5: Structures of representative donors based on 1,2- O, S relay mechanism

3.3.2 Persulfide donors based on 1,4 - O, S-relay mechanisms

A formally introduced retro Michael reaction relating a thiol as a parting group(Baldwin and Kiick, 2011). Based on this 1,4- O, S relay mechanism, our lab developed donors of persulfides, which gets activated selectively by hydrogen peroxide, a reactive oxygen species (ROS). They exhibited protective effects against cytotoxicity induced by oxidants. Cinnamaldehyde was the major byproduct, which was known to be not toxic to the cellular environments. Nevertheless, the use of peroxide as a trigger was not useful because of their tunability nature, and the direct quantification of persulfide release was not possible using this method.

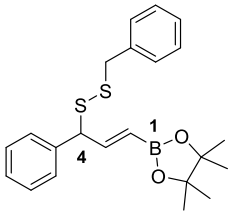
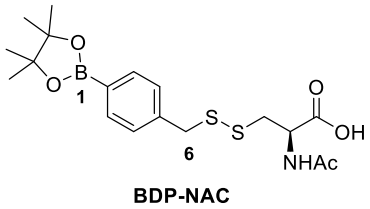


Figure 6: Structure of representative donor based on 1,4-O, S relay mechanism

3.3.3 Donors based on 1,6 - O, S relay mechanism

John B. Matson and co-workers developed a discrete persulfide donor, which is inert under normal physiological conditions (Powell *et al.*, 2018). Under oxidative conditions like in the presence of H_2O_2, it undergoes self-immolation to release a persulfide species. They have shown the cytoprotective effects under H_2O_2 conditions. Studies also illustrated that the persulfide donor rescued cells efficiently than the non-persulfide donor. In this method also, the difficulties in tunability were there since they used H_2O_2 as the trigger. Also, the direct quantification of persulfide with this method was not possible. The major drawback was that the formation of quinine methide, which is highly toxic to the cellular environment.



BDP-NAC

Figure 7: Structure of representative persulfide donor based on 1,6-O,S relay mechanism

3.3.4 Persulfide donors based on enzyme action and cyclization

Binghe Wang and co-workers developed a persulfide donor, where they have used a glutathione persulfide (GSSH) donor which is sensitive to esterase(Yuan *et al.*, 2018). They have used a trimethyl lock strategy to show that the donor gets cleaved by porcine liver esterase to release a lactone and a persulfide. They studied the release profile of the lactone, and the persulfide was trapped using reagents. They exhibited inhibitory effects towards glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cytoprotective effects under extremely oxidative conditions in a cellular environment. Although controlled release could be achieved, direct quantification of persulfide was less likely with this method.

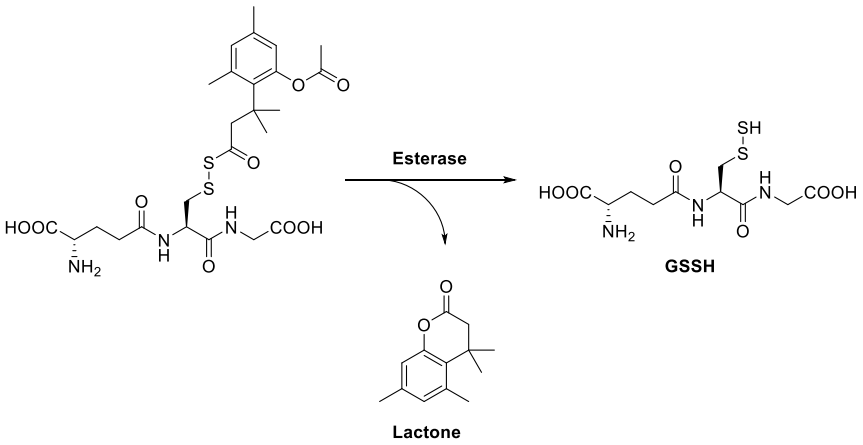


Figure 8: Mechanism of esterase triggered release of persulfide(Yuan et al., 2018).

3.3.5 pH and fluoride-triggered persulfide donors

Ming Xian and co-workers have reported library of pH and F^- activated *O*-silyl based persulfide donors, by modifying the adjacent alcohol functional groups with TMS (trimethyl silyl) or TES (triethyl silyl). They have shown the triggered release of persulfides under the presence of fluorides (**Figure 9c, 9f**) as well as in buffer with pH = 5.0 to pH 6.0 and slow release at pH 7.4 (**Figure 9a, 9b, 9d, 9e**). They have also shown the tunability with this trigger. Nevertheless, changing TMS to TES, they could attain specificity towards fluoride. However, they failed to develop a method for the direct quantification of the released persulfide. Also, *in-vivo* tunability of these triggers are challenging and the TMS or TES released into the physiological systems are probably not well tolerated by the cells.

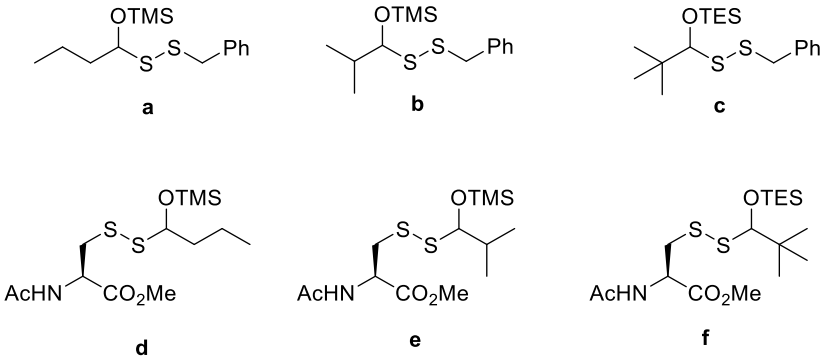


Figure 9: Structures of representative persulfide donors

3.3.6 Light activated persulfide donors

First ever light activated persulfide donors were reported by Pradeep Singh and co-workers in 2019(Chaudhuri *et al.*, 2019). They have shown the release of *N*-acetyl

cysteine persulfide (NAC-SSH) release spatiotemporally under one-photon excitation and two-photon excitations based on *O*-nitrobenzyl (ONB) photo trigger. After absorption of a photon by ONB, the reaction mechanism proceeds through Norrish – II pathway. They hypothesized that the two-photon excitation gives an upper hand in the tunability of release since a higher wavelength is used. However, the yield of persulfide from *in-vitro* studies shows comparatively very less when compared to other methods. This suggested the *in-vitro* yields could be much lower than what is expected. Apart from the tunability, the method failed to provide a piece of evidence for the direct quantification of the persulfide

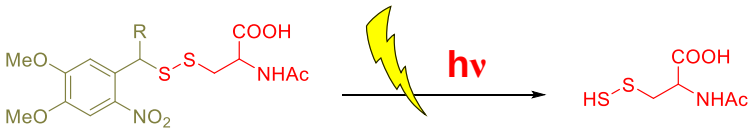


Figure 10: Mechanism of light-activated persulfide donor

3.4 Challenges in designing a persulfide donor scaffold

All the donors discussed above are well studied and used in different research areas. Nevertheless, each method has its drawbacks. Fundamental problems associated with these persulfide donors are:

- **Controlled release of persulfide:** Using oxidative stress as a trigger and using H_2O_2 as a stimulus will not give us any controlled release, but a burst release of persulfide donor is obtained. Binghe Wang and co-workers reported a persulfide donor (**Figure 11**) with esterase as a trigger(Zheng *et al.*, 2017). This molecule gets activated under esterase and releases persulfide in a controlled manner.

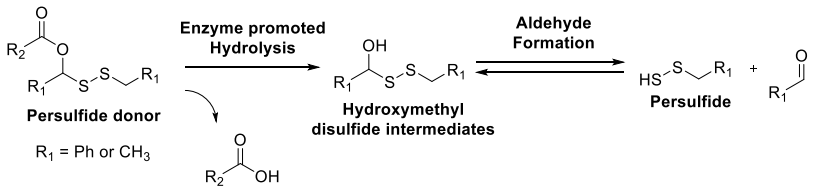


Figure 11: Mechanism of enzyme triggered persulfide donor

- **Cytotoxicity of byproducts:** In the method reported by Matson and co-workers, quinine methide is formed as one of the byproducts, as shown in

Figure 12. Studies have shown the quinine methide is a DNA alkylating agent, which is known to induce cytotoxicity in cells.

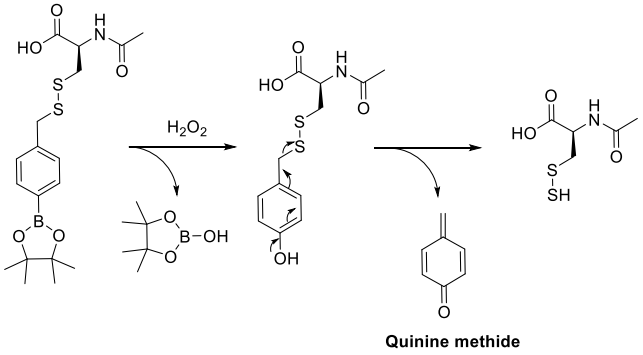


Figure 12: Formation of quinine methide upon H_2O_2 activation

- **Real-time monitoring of persulfide release:** All the methods described above show the triggerable release of a persulfide under the stimulus. The main drawback is that the real-time monitoring of persulfide release could not be achieved. So, they have used reported probes like FDNB (1-fluoro-2,4-dinitrobenzene) and mBBR (monobromobimane), where the persulfides form an adduct and the adduct is detected in order to quantify the persulfide.

3.5 Design of a scaffold

The motivation for this product was from a previous work conducted in our lab on the triggered release of Hydrogen sulfide using Nitroreductase (NTR) as a trigger, where the release of H_2S was monitored by fluorescence upon activation with Nitroreductase as a trigger. In 2018, Shuxiang Wang and co-workers had shown hypoxia-activated anticancer prodrug, which gets activated under the presence of Nitroreductase (NTR), undergoes lactonization and releases a fluorophore and a drug(Liu *et al.*, 2018). Combining these two ideas, we came up with an idea where we could release the persulfide under the presence of esterase and can release a fluorophore concurrently.

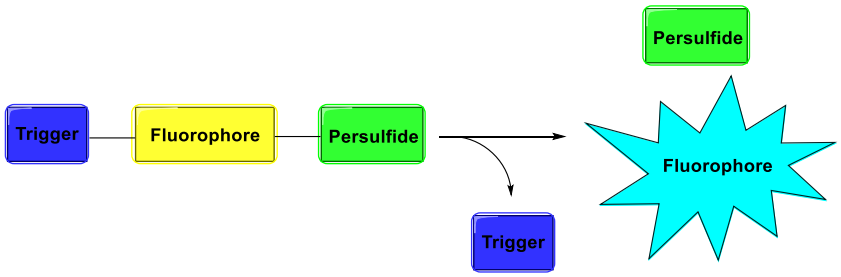


Figure 13: Schematic representation persulfide release

Based on this idea, we came up with a scaffold that could provide the proof of concept. In this scaffold, the persulfide and the esterase trigger are connected by a linker biphenyl moiety.

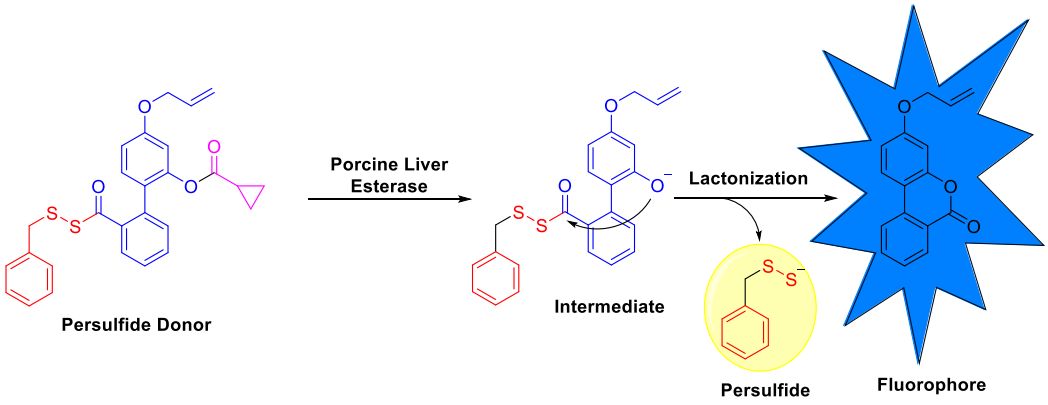


Figure 14: Proposed experimental design

Upon action with esterase, the ester bond between the cyclopropyl group and the biphenyl system gets cleaved, releasing cyclopropane carboxylic acid. The intermediate with an alkoxide undergoes lactonization to forms a biphenyl lactone, which releases a fluorophore as well as persulfide. The direct quantification of the persulfide can be done by measuring the fluorescent intensity.

4. MATERIALS AND METHODS

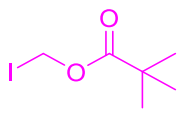
4.1 General Methods:

All reactions are conducted under a nitrogen atmosphere. All the solvents and chemicals were purchased from commercial sources and used as received unless stated otherwise. Silica gel-Rankem (60-120 mesh) or silica gel Spectrochem (100-200 mesh as the stationary phase) was sued for performing column chromatography. 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 (CDCl3 δH= 7.26 ppm, δC = 77.2 ppm) or as an internal tetramethylsilane (δH = 0.00, δC = 0.0). are reported in ppm, coupling constants (*J*) in Hz and chemical shifts (δ). Abbreviations that are used are: bs (broad signal), m (multiplet), s (singlet), d (doublet), t (triplet), dq (doublet of quartets), dt (doublet of triplets), dd (doublet of doublets), td (triplet of doublets), tt (triplet of triplets), ddd (doublet of doublet of doublets), and ddt (doublet of doublet of triplets). High-performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1260 infinity with Phenomenex®C-18 reversed-phase column (250 mm × 4.6 mm, 5µm). Fluorescence and photometric measurements were performed using an EnSight™ multimode plate reader (PerkinElmer, India). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS.

4.2 Synthesis and Characterization

Compounds **1, 2, 3, 4, 5, 17** were synthesized using previously reported procedures, and the analytical data collected were consistent with the reported values.

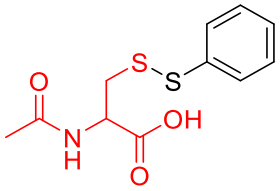
Iodomethyl pivalate (1):



To a solution of chloromethyl pivalate (1g, 6.6mmol) in dry ACN (20 mL), NaI (1.8g, 1.2mmol) was added under N_2 atmosphere and allowed to stir for 5 h at rt. Once the reaction was over (monitored *via* TLC), the reaction mixture was filtered, and the

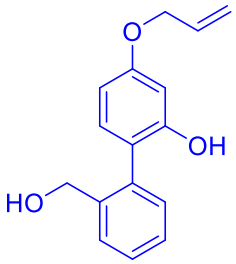
filtrate was collected and concentrated under reduced pressure. The product was used in the next step without further purification.

_N_-acetyl-S-(pyridin-2-ylthio) cysteine (2):



N-acetyl cysteine (2.0g, 12.2mmol) was added to water (20 mL). To this, aldrithiol (5.4g, 24.5mmol) in methanol (20 mL) was added to make a 1:1 (v/v) solution, and the reaction was stirred at rt for 3 h. Upon completion, as monitored by TLC, methanol was evaporated, the reaction mixture was diluted with water and extracted with ethyl acetate (3 x 15mL). The collected organic layers were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude obtained was purified by silica gel column chromatography using DCM/MeOH (9.5:0.5) to obtain compound **2** (3.10 g, 93%). ^{1}H NMR (400 MHz, DMSO) δ 8.46 (ddd, *J* = 4.8, 1.8, 0.9 Hz, 1H), 8.36 (d, *J* = 7.8 Hz, 1H), 7.82 (ddd, *J* = 8.1, 7.3, 1.8 Hz, 1H), 7.76 (dt, *J* = 8.1, 1.0 Hz, 1H), 4.46 (td, *J* = 8.8, 4.6 Hz, 1H), 3.23 (dd, *J* = 13.5, 4.6 Hz, 1H), 3.09 (dd, *J* = 13.5, 9.0 Hz, 1H), 1.86 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 172.3, 169.8, 159.2, 150.0, 138.2, 121.7, 119.7, 51.9, 22.8; HRMS (ESI) for $C_{10}H_{12}N_2O_3S_2$ [M+H]+: Calcd., 273.0323 Found., 273.0331.

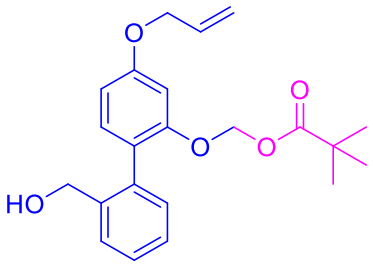
4-(allyloxy)-2'-(hydroxymethyl)-[1,1'-biphenyl]-2-ol (5):



Lithium Aluminium Hydride pellets (1.8g, 47.5mmol) were added to a solution of compound **3** (4g, 15.8mmol) in dry THF (50mL) at 0^{0}C under a N_2 atmosphere. After 15 min, following the consumption of starting material by TLC, the reaction was quenched with ice-cold water (30mL), and the resulting solution was extracted with EtOAc (3 x 10 mL). The combined organic phase was washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude product was then

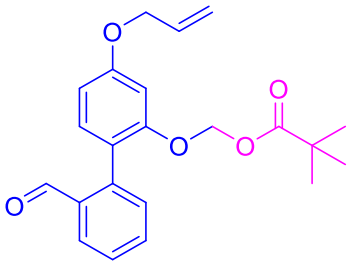
recrystallized with $CHCl_3$ to give the desired compound **5** (1.63mg, 94%) as white coloured solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.49 (m, 1H), 7.43 – 7.35 (m, 2H), 7.25 – 7.20 (m, 1H), 7.03 – 6.98 (m, 1H), 6.60 – 6.54 (m, 2H), 6.08 (ddd, *J* = 17.3, 10.5, 5.3 Hz, 2H), 5.43 (dq, *J* = 17.3, 1.6 Hz, 1H), 5.30 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.54 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.49 (s, 2H).; ^{13}C NMR (100MHz, $CDCl_3$): δ 20 159.7, 154.0, 139.2, 136.5, 133.3, 131.5, 131.4, 129.4, 128.7, 128.6, 120.5, 118.0,107.7, 102.9, 69.0, 63.9; HRMS (ESI) for $C_{16}H_{16}O_3$ [M+H]+: Calcd., 257.1133 Found., 257.1177.

((4-(allyloxy)-2'-(hydroxymethyl)-[1,1'-biphenyl]-2-yl)oxy)methyl pivalate (6):



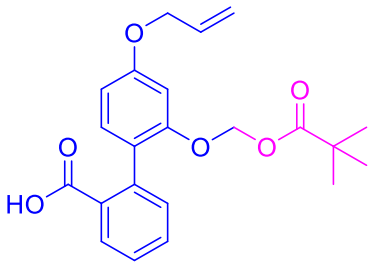
To a solution of Compound **5** (0.94g, 3.7mmol) and compound **1** (1.33g, 5.5mmol) in dry ACN (20mL), K_2CO_3 (1.4g, 10.1mmol) was added, and the reaction was stirred for 6 h at rt under a N_2 atmosphere. The reaction mixture was filtered, and the resulting filtrate was concentrated under reduced pressure to give the crude product. The crude product was purified by silica gel column chromatography using EtOAc/hexane (1:9) to afford compound **6** (1.45g, 98%) as a pale-yellow coloured liquid. ^1H NMR (400 MHz, CDCl3) δ 7.56 (dd, *J* = 7.6, 1.1 Hz, 1H), 7.39 (td, *J* = 7.5, 1.4 Hz, 1H), 7.32 (td, *J* = 7.5, 1.4 Hz, 1H), 7.17 (dd, *J* = 7.5, 1.3 Hz, 1H), 7.11 (d, *J* = 8.5 Hz, 1H), 6.75 (d, *J* = 2.4 Hz, 1H), 6.69 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.09 (ddt, *J* = 17.0, 10.6, 5.2 Hz, 1H), 5.70 (s, 1H), 5.46 (dq, *J* = 17.3, 1.5 Hz, 2H), 5.33 (dt, *J* = 10.5, 1.3 Hz, 1H), 4.58 (dt, *J* = 5.2, 1.5 Hz, 2H), 4.41 (d, *J* = 14.1 Hz, 2H), 1.18 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 159.2, 154.4, 139.8, 136.4, 133.0, 131.9, 130.4, 128.5, 127.8, 127.4, 123.5, 117.9, 108.6, 102.4, 85.9, 69.0, 63.3, 38.9, 26.9; HRMS (ESI) for $C_{22}H_{26}O_5$ [M+H]+: Calcd., 371.1814 Found., 371.1825.

((4-(allyloxy)-2'-formyl-[1,1'-biphenyl]-2-yl)oxy)methyl pivalate (7):



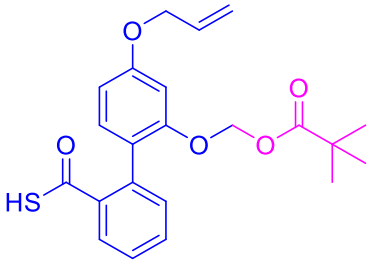
To a solution of compound **6** (1.0g, 2.7mmol) in acetone (20mL), MnO_2 (7.0g, 87.0mmol) was added. The resulting reaction mixture was stirred overnight at rt. After completion, the reaction mixture was filtered to eliminate the residual MnO_2. The filtrate was concentrated under reduced pressure to yield compound **7** (850 mg, 87 %) as a pale-yellow coloured liquid. ^{1}H NMR (400 MHz, CDCl3): δ 9.79 (s, 1H), 7.97 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.61 (td, *J* = 7.5, 1.5 Hz, 1H), 7.45 (tt, *J* = 6.8 Hz, 1H), 7.34 – 7.31 (m, 1H), 7.18 (d, *J* = 8.3 Hz, 1H), 6.75 (d, *J* = 2.3 Hz, 1H), 6.72 (dd, *J* = 8.3 Hz, 2.4 Hz, 1H), 6.08 (ddt, *J* = 17.2, 10.5, 5.3 Hz, 1H), 5.61 (s, 2H), 5.45 (dq, *J* = 17.3, 3.1, 1.6 Hz, 1H), 5.33 (dq, *J* = 7.3 Hz 1H), 4.59 (dt, *J* = 5.3, 1.5 Hz, 2H), 1.17 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.5, 177.2, 160.1, 154.9, 141.3, 134.2, 133.7, 132.9, 132.5, 131.6, 127.8, 127.2, 120.6, 118.2, 108.7, 102.0, 85.6, 69.2, 38.9, 27.0, 22.7; HRMS (ESI) for $C_{22}H_{24}O_5$ [M+H]+: Calcd., 369.1657, Found., 369.1634.

4'-(allyloxy)-2'-((pivaloyloxy)methoxy)-[1,1'-biphenyl]-2-carboxylic acid (8):



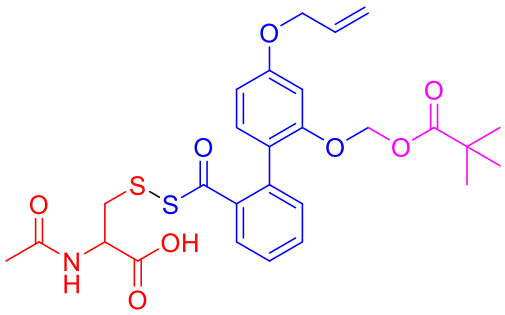
To a stirred solution of compound **7** (1.2g, 3.2mmol) in ACN (25mL) placed at rt were added KH_2PO_4 (0.137g, 1.0mmol). After 15 min, 50% H_2O_2 (1.1g, 32.3mmol) and $NaClO_2$ (0.642g, 7.1mmol) were added and the resulting reaction mixture was stirred at rt for 5 h until the complete disappearance of the starting material, as monitored by TLC. The reaction mixture was diluted with water (20 mL), extracted with ethyl acetate (3 x 15mL), washed with brine solution, dried over Na_2SO_4 and concentrated under reduced pressure. Preparative HPLC was used for purification of compound using H_2O/ACN as the mobile phase to obtain **8** (520 mg, 42%) as a pale-yellow liquid. ^{1}H NMR (400 MHz, CDCl3): δ 7.95 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.55 (td, *J* = 7.6, 1.4 Hz, 1H), 7.39 (td, *J* = 7.6, 1.2 Hz, 1H), 7.30 (dd, *J* = 7.7, 1.1 Hz, 1H), 7.16 (d, *J* = 7.9 Hz, 1H), 6.71 – 6.69 (m, 1H), 6.67 (d, *J* = 2.5 Hz, 1H), 6.09 (ddt, *J* = 17.3, 10.6, 5.3 Hz, 1H), 5.58 (s, 2H), 5.45 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.31 (dq, *J* = 10.5, 1.3 Hz, 1H), 4.57 (dt, *J* = 5.2, 1.4 Hz, 2H), 1.15 (s, 9H). ^{13}C NMR (100 MHz, CDCl3) δ 177.3, 172.7, 159.5, 154.7, 138.7, 133.2, 132.3, 131.9, 130.7, 130.3, 127.1, 124.2, 117.9, 108.4, 102.5, 86.5, 69.1, 38.9, 26.9; HRMS (ESI) for $CH_{24}O_6$ [M+H]+: Calcd., 385.1606. Found., 385.1634.

4'-(allyloxy)-2'-((pivaloyloxy)methoxy)-[1,1'-biphenyl]-2-carbothioic S-acid (9):



A mixture of compound **8** (0.20g, 0.5mmol), Lawesson's reagent (0.115g, 0.3mmol) and dry DCM (6mL), was charged to a commercial PTFE vessel (8 mL), closed and irradiated in a Monowave 400/200 oven at 10W for 15 min (final temperature 100^0C). After completion, the reaction mixture was filtered and the filtrate was concentrated under vacuum to obtain the crude compound **9** (180 mg, 92%) as a smelly liquid. The crude product was carried forward to the next step without further purification. ^{1}H NMR (400 MHz, CDCl3): δ 7.76 (dd, *J* = 7.8, 1.0 Hz, 1H), 7.53 (td, *J* = 7.6, 1.4 Hz, 1H), 7.39 (td, *J* = 7.6, 1.4 Hz, 1H), 7.30 (dd, *J* = 7.7, 0.9 Hz, 1H), 7.16 (d, *J* = 8.3 Hz, 1H), 6.73 (d, *J* = 2.3 Hz, 1H), 6.69 (dd, *J* = 8.3, 2.4 Hz, 1H), 6.08 (ddt, *J* = 17.2, 10.5, 5.3 Hz, 1H), 5.68 (s, 2H), 5.44 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.32 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.57 (dt, *J* = 5.3, 1.5 Hz, 2H), 1.19 (s, 9H). ^{13}C NMR (100 MHz, CDCl3) δ 177.1, 172.7, 159.8, 154.6, 138.6, 136.1, 133.0, 131.9, 131.4, 128.1, 127.2, 122.6, 117.9, 108.4, 102.1, 86.1, 69.0, 38.9, 26.9.

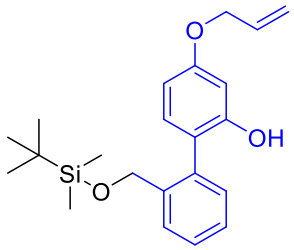
N-acetyl-S-((4'-(allyloxy)-2'-((pivaloyloxy)methoxy)-[1,1'-biphenyl]-2-carbonyl)thio)cysteine (10):



To a stirred solution of compound **9** (0.136g, 0.5mmol) in $CHCl_3$ (5mL), **2** (0.1g, 0.3mmol) was added under N_2 atmosphere. The resulting reaction mixture was stirred for 2 h at rt. After complete disappearance of starting material, as monitored by TLC, the solvent was evaporated and the crude was purified by silica gel column chromatography using hexane/EtOAc (3:7) to afford the target compound **10** as a colourless solid (32mg, 38%). ^{1}H NMR (400 MHz, DMSO) δ 12.82 (bs, 1H), 7.75 (dd,

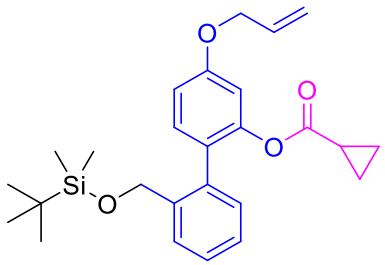
J = 5.6, 2.1 Hz, 1H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.51 (t, *J* = 7.6 Hz, 1H), 7.35 (d, *J* = 7.0 Hz, 1H), 7.13 (d, *J* = 8.2 Hz, 1H), 6.73 (dt, *J* = 5.9, 2.4 Hz, 2H), 6.07 (ddt, *J* = 17.2, 10.5, 5.2 Hz, 1H), 5.64 – 5.58 (m, 2H), 5.46 – 5.39 (m, 1H), 5.32 – 5.25 (m, 1H), 4.62 (d, *J* = 5.2 Hz, 2H), 4.38 (td, *J* = 8.3, 5.0 Hz, 1H), 3.02 (dd, *J* = 13.6, 5.0 Hz, 1H), 2.93 (dd, *J* = 13.6, 8.6 Hz, 1H), 1.85 (s, 3H), 1.08 (s, 9H). ^{13}C NMR (100 MHz, DMSO) δ 191.3, 176.6, 172.1, 169.8, 159.8, 154.4, 136.8, 136.4, 133.9, 132.8, 132.3, 131.9, 128.2, 127.9, 122.1, 118.1, 109.1, 102.3, 85.8, 79.6, 68.9, 51.9, 38.7, 26.9, 22.8; HRMS (ESI) for $C_{27}H_{31}NO_8S_2$ [M+H]+: Calcd., 562.1525. Found., 562.1543.

4-(allyloxy)-2'-(((tert-butyldimethylsilyl)oxy)methyl)-[1,1'-biphenyl]-2-ol (11):



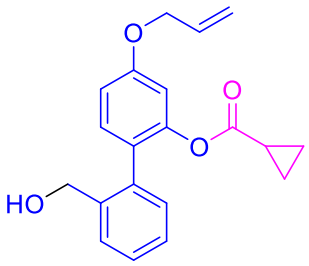
To a solution of **5** (0.256g, 1.0mmol) in anhydrous DCM (20 mL), imidazole (0.147g, 2.2mmol) was added to it at rt under N_2 atmosphere. The reaction was allowed to stir for 1 h. After stirring for 1 h the ice bath was used to bring down the temperature to 0^{o}C, and TBDMS-Cl (0.331g, 2.2mmol) was added to it. After 10 min the reaction was brought back to rt, and allowed to stir for another 2 h. After the consumption of the starting material, as monitored by TLC, the organic solvent was evaporated under reduced pressure, diluted with water, and the aqueous solution was extracted with EtOAc (3×5 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 (5 g), filtered, and the filtrate was concentrated to give a crude compound. This crude was further purified by column chromatography with EtOAc/Hexane as the eluent to obtain **11** (0.304g, 87%) as a pale yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.48 (m, 1H), 7.43 – 7.34 (m, 2H), 7.21 (dd, *J* = 7.3, 1.6 Hz, 1H), 6.98 (d, *J* = 8.3 Hz, 1H), 6.58 (dt, *J* = 8.3, 2.5 Hz, 2H), 6.09 (tq, *J* = 15.8, 5.3 Hz, 2H), 5.44 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.31 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.56 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.49 (s, 2H), 0.90 (s, 9H), 0.07 (s, 6H). HRMS (ESI) for $C_{22}H_{30}O_3Si$ [M+H]+: Calcd., 371.1998. Found., 371.2009.

4-(allyloxy)-2'-(((tert-butyldimethylsilyl)oxy)methyl)-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (12) :



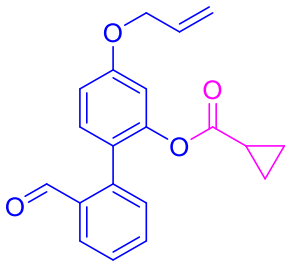
A solution of **11** (0.24g, 0.6mmol) and cyclopropane carboxylic acid (0.084g, 1.0mmol) were taken in DCM (20 mL). To that DMAP (0.008g, 64.0μmol) was added and stirred for 15 min. To the reaction mixture, DCC (0.147g, 0.712g) was added under N_2 atmosphere and the reaction was stirred at room temperature for overnight, until the starting material was completely consumed, as monitored by TLC. Upon completion of the reaction, solvent was evaporated, diluted with 10 mL of water, extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 (5 g), filtered and filtrate was concentrated to give a crude compound. Column chromatography with EtOAc:Hexane was done for the further purification to give **12** (0.261g, 92%)as a yellow liquid. ^{1}H NMR (400 MHz, CDCl3): δ 7.58 (dd, *J* = 7.7, 0.7 Hz, 1H), 7.36 (td, *J* = 7.6, 1.4 Hz, 1H), 7.23 (dd, 1H), 7.16 (d, *J* = 8.5 Hz, 1H), 7.08 (dd, *J* = 7.6, 1.2 Hz, 1H), 6.84 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.73 (d, *J* = 2.5 Hz, 1H), 6.08 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.44 (dq, *J* = 17.3, 1.6 Hz, 1H) , 5.32 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.57 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.50 (d, *J* = 7.0 Hz, 2H), 1.48 (dt, *J* = 7.8, 6.0 Hz, 1H), 0.90 (s, 9H), 0.70 (dd, 4H), 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 158.8, 148.8, 139.7, 134.7, 132.9, 131.3, 130.1, 127.6, 126.4, 126.3, 126.0, 117.9, 112.4, 108.9, 69.1, 62.7, 29.7, 18.3, 12.8, -5.4; HRMS (ESI) for $C_{24}H_{36}O_4Si$ [M+H]+: Calcd., 439.2260. Found., 439.2271.

4-(allyloxy)-2'-(hydroxymethyl)-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (13):



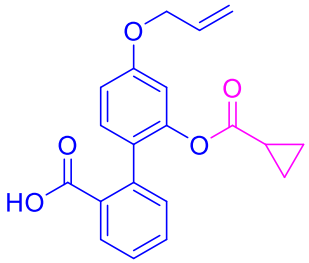
To a well stirred solution of **12** (0.24g, 0.5mmol) in H_2O:THF (1:1, 20 mL), acetic acid (60 mL) was added at room temperature and the reaction was stirred at room temperature for 2 h. Once the starting material was completely consumed as monitored by TLC, the reaction mixture was extracted with EtOAc (3×20 mL). the combined organic layer was washed with brine, dried over Na2SO4 (5 g). The organic layer was then filtered, collected and evaporated under reduced pressure to give a crude product. Column chromatography was done with EtOAc: Hexane as eluent to obtain **13** (0.091g, 52%) as a yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (dd, *J* = 7.6, 1.0 Hz, 1H), 7.36 (td, *J* = 7.5, 1.4 Hz, 1H), 7.28 (td, *J* = 7.5, 1.4 Hz, 1H), 7.17 – 7.13 (m, 2H), 6.86 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.72 (d, *J* = 2.5 Hz, 1H), 6.07 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.44 (dq, *J* = 17.3, 1.6 Hz, 1H), 5.31 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.56 (dt, *J* = 5.3, 1.5 Hz, 3H), 4.38 (d, *J* = 12.2 Hz, 1H), 1.49 (ddd, *J* = 13.0, 7.7, 5.0 Hz, 1H), 0.68 (dd, *J* = 21.2, 20.0 Hz, 4H). HRMS (ESI) for $C_{20}H_{20}O_4$ [M+H]+: Calcd., 322.1395. Found., 322.1412.

4-(allyloxy)-2'-formyl-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (14):



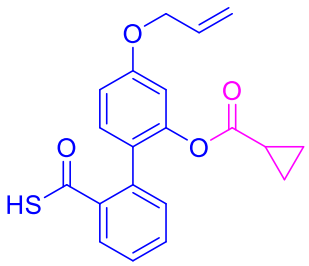
To a stirred solution of **13** (0.05g, 0.1mmol) in acetone (10 mL) at room temperature, Mn(IV)O_2 (0.67g, 7.7mmol) was added in to it under N_2 atmosphere. The reaction was stirred overnight. Once the starting material was completely consumed, as monitored by TLC, celite filtration was done using Sintered glass funnel to remove the residual Mn(IV)O_2 and the filtrate was collected, evaporated under reduced pressure to get the crude compound. This crude compound was further purified by column chromatography with EtOAc: Hexane as eluent, to obtain **14** (0.038g, 91%) as orange coloured liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.84 (s, 1H), 7.99 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.61 (td, *J* = 7.5, 1.5 Hz, 1H), 7.51 – 7.45 (m, 1H), 7.35 – 7.30 (m, 1H), 7.26 (s, 1H), 6.91 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.76 (d, *J* = 2.5 Hz, 1H), 6.07 (ddt, *J* = 17.2, 10.6, 5.3 Hz, 1H), 5.45 (ddd, *J* = 17.3, 3.1, 1.6 Hz, 1H), 5.33 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.59 (dt, *J* = 5.3, 1.5 Hz, 2H), 1.53 – 1.46 (m, 1H), 0.72 (dd, *J* = 18.9, 6.2 Hz, 4H). HRMS (ESI) for $C_{20}H_{18}O4$ [M+H]+: Calcd., 323.1239. Found., 323.1253.

4'-(allyloxy)-2'-((cyclopropanecarbonyl)oxy)-[1,1'-biphenyl]-2-carboxylic acid (15):



A solution of **14** (0.323g, 1.5mmol) was taken in dry ACN (20 mL) under N_2 atmosphere, and potassium dihydrogen phosphate (0.063g, 0.5mmol) was added and stirred for 15 min. To the reaction mixture, H_2O_2 (0.527g, 15.5mmol) and $NaClO_2$ (0.308g, 3.4mmol) were added and stirred at room temperature for 5 h. The reaction was monitored by TLC. Upon the completion, the solvent was evaporated under reduced pressure. The reaction mixture was diluted with 10 mL of water and extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 (5 g), filtered and the filtrate was concentrated under reduced pressure to give a crude compound. Column chromatography was done with EtOAc:Hexane as eluent, to give **15** (0.24g, 46%) as bright yellow colored liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (dd, *J* = 7.7, 0.9 Hz, 1H), 7.51 (td, *J* = 7.5, 1.2 Hz, 1H), 7.42 (td, *J* = 7.6, 1.1 Hz, 1H), 7.27 (d, *J* = 6.5 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 1H), 6.88 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.73 (d, *J* = 2.5 Hz, 1H), 6.06 (ddd, *J* = 22.5, 10.5, 5.3 Hz, 1H), 5.44 (dd, *J* = 17.3, 1.4 Hz, 1H), 5.32 (dd, *J* = 10.5, 1.2 Hz, 1H), 4.56 (d, *J* = 5.3 Hz, 2H), 1.65 – 1.57 (m, 1H), 0.91 – 0.48 (m, 4H). HRMS (ESI) for $C_{20}H_{18}O_5$ [M+H]+: Calcd., 339.1188. Found., 339.1195.

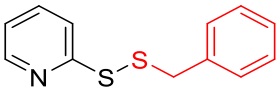
4'-(allyloxy)-2'-((cyclopropanecarbonyl)oxy)-[1,1'-biphenyl]-2-carbothioic S-acid (16):



A mixture of compound **15** (0.20g, 0.5mmol), Lawesson's reagent (0.115g, 0.3mmol) and dry DCM (6mL), was charged to a commercial PTFE vessel (8 mL), closed and irradiated in a Monowave 400/200 oven at 10W for 15 min (final temperature 100^{0}C).

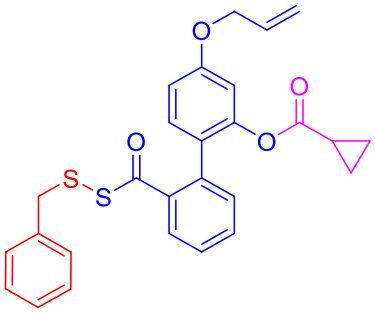
After completion, the reaction mixture was filtered, and the filtrate was concentrated under vacuum to obtain the crude compound **16** as a smelly liquid. The crude product was carried forward to the next step without further purification.

2-(benzyldisulfaneyl)pyridine (17):



Benzyl mercaptan (2.0g, 16.1mmol) was dissolved in methanol (20 mL). To this, a solution of aldrithiol (5.4g, 24.5mmol) in water (mL) was added to make a 1:1 (v/v) solution, and the reaction was stirred at rt for 3 h. Upon completion, as monitored by TLC, methanol was evaporated, the reaction mixture was diluted with water and extracted with ethyl acetate (3x15 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to obtain a crude product. The crude obtained was purified by Prep-HPLC using ACN and water as eluents to obtain compound **17** (2.45g, 65%) as a pale yellow liquid.

4-(allyloxy)-2'-(benzyldisulfannecarbonyl)-[1,1'-biphenyl]-2-yl cyclopropanecarboxylate (18):



To a well stirred solution of compound **17** (0.2g, 0.9mmol) in DCM (10 mL), **16** (0.15g, 0.4mmol) was added to it under N_2 atmosphere at rt. The solution was stirred for 2 h. Once the starting material was completely consumed, as monitored by TLC, the solvent was evaporated under reduced pressure to give crude compound. The crude compound was then purified by Prep-HPLC with ACN and water as eluents to give pure compound **18** (0.032g, 16%) as colourless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 7.7 Hz, 1H), 7.54 (t, *J* = 7.5 Hz, 1H), 7.42 (t, *J* = 7.6 Hz, 1H), 7.34 – 7.21 (m, 7H), 6.87 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.71 (d, *J* = 2.3 Hz, 1H), 6.05 (ddd, *J* = 22.4, 10.5, 5.3 Hz, 1H), 5.42 (d, *J* = 17.2 Hz, 1H), 5.29 (d, *J* = 10.5 Hz, 1H), 4.54 (d, *J* = 5.2 Hz, 2H), 3.88 (s, 2H), 1.67 – 1.50 (m, 2H), 0.75 (d, *J* = 7.5 Hz, 4H). ^{13}C NMR (100

MHz, $CDCl_3$) δ 191.9, 172.7, 159.5, 148.6, 136.9, 136.4, 135.8, 132.8, 132.0, 131.8, 131.2, 129.4, 128.5, 127.9, 127.6, 127.6, 125.4, 117.9, 112.6, 108.9, 69.1, 42.5, 12.8, 8.8; HRMS (ESI) for $C_{27}H_{24}O_4S_2$ [M+H]+: Calcd., 477.1150. Found., 477.1163.

4.3 HPLC studies:

4.3.1 Purity test

The compound **18** was purified to >95% purity, as determined by high-performance liquid chromatography (HPLC). A stock solution of compound **18** (5 mM) was prepared in DMSO. Compound **18** was added (2 μL, final conc. 50 μM), to ACN (198 μL). The mixture was then injected (25 μL) in an HPLC instrument attached with a diode array detector at 280 nm. The stationary phase used was a C-18 reverse phase column (Phenomenex, 4.6 x 250 mm, 5 μm). H_2O/ACN were used as eluents. The run time was 20 min with a flow rate of 1 mL/min with 30:70 → 0 - 3 min, 20:80 → 3 - 8 min, 10:90 → 8 - 12 min, 10:100 → 12- 15 min, 10:90 → 15- 17 min, 30:70 → 17 – 20 min.

4.3.2 Decomposition under the presence of esterase

A stock solution of **18** (10 mM) was prepared in DMSO. In a typical reaction, the compound **18** (10 μL, final conc. 50 μM), Porcine Liver Esterase (10 μL, final conc.100 U/mL) were added to phosphate buffer (980 μL). Reaction mixtures were incubated at 37ºC in pH 7.4 buffer on an Eppendorf thermomixer comfort (700 rpm). Aliquots were taken periodically, diluted with ACN (100 μL)to make a final concentration of compound **18** (25 μM) and injected (25 μL) in an HPLC instrument attached with a diode-array detector at 250 nm The stationary phase used was C-18 reverse phased column (Phenomenex, 4.6 x 250 mm, 5 μm). The mobile phase used was H_2O/ACN. The run time was 20 min with a flow rate of 1 mL/min with 30:70 → 0 - 3 min, 20:80 → 3 - 8 min, 10:90 → 8 - 12 min, 10:100 → 12- 15 min, 10:90 → 15- 17 min, 30:70 → 17 – 20 min.

4.3.3 Formation of FDNB adduct

The stock solution of compounds **18** (2.5 mM) and **19** (25 mM) were prepared independently in DMSO. In a typical reaction, the compound 18 (10 μL, final conc. 25 μM), **19** (10 μL, 25 mM) and Porcine Liver Esterase (10 μL, final conc. 100 U/mL) were added to phosphate buffer (970 μL). The reaction mixture was incubated at 37ºC in

pH 7.4 buffer on an Eppendorf thermomixer comfort (700 rpm). Aliquots were taken periodically and injected (25 μL) in an HPLC instrument attached with a diode-array detector at 250 nm. The stationary phase used was a C-18 reverse phase column (Phenomenex, 4.6 x 250 mm, 5 μm). The mobile phase used was H_2O/ACN. The run time was 20 min with a flow rate of 1 mL/min with 30:70 → 0 - 3 min, 20:80 → 3 - 8 min, 10:90 → 8 - 12 min, 10:100 → 12- 15 min, 10:90 → 15- 17 min, 30:70 → 17 – 20 min.

4.4 Quantification of fluorophore based on fluorescence study

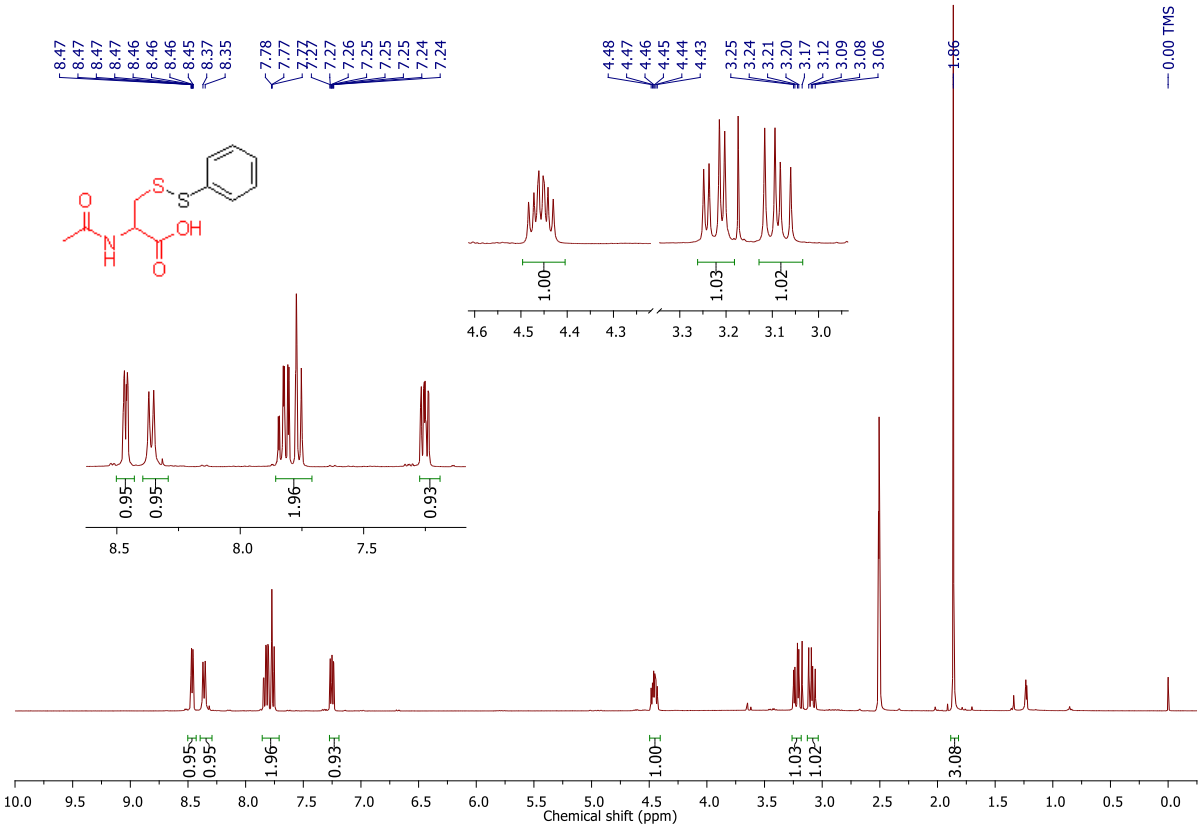
A stock solution of **18** (10 mM) and **5** (10 mM) were prepared in DMSO. The stock solutions were diluted (10-fold) in DMSO to achieve the final concentration of 1.0 mM were made in DMSO. The reaction mixture contained **18** (2 μL, 1.0 mM), 2.0 μL of Porcine Liver Esterase (100 U/mL) in 196 μL of phosphate buffer (10 mM, pH 7.4) at 37^{o}C in a 96 well plate. Readings were taken for 2 h in the EnSight™ multimode plate reader with an interval of 2 min. The excitation wavelength used for the excitation of the fluorophore was λ_{max} = 330 nm, and the emission spectrum was recorded at λ_{max} = 432 nm.

4.5 Cytotoxicity assay

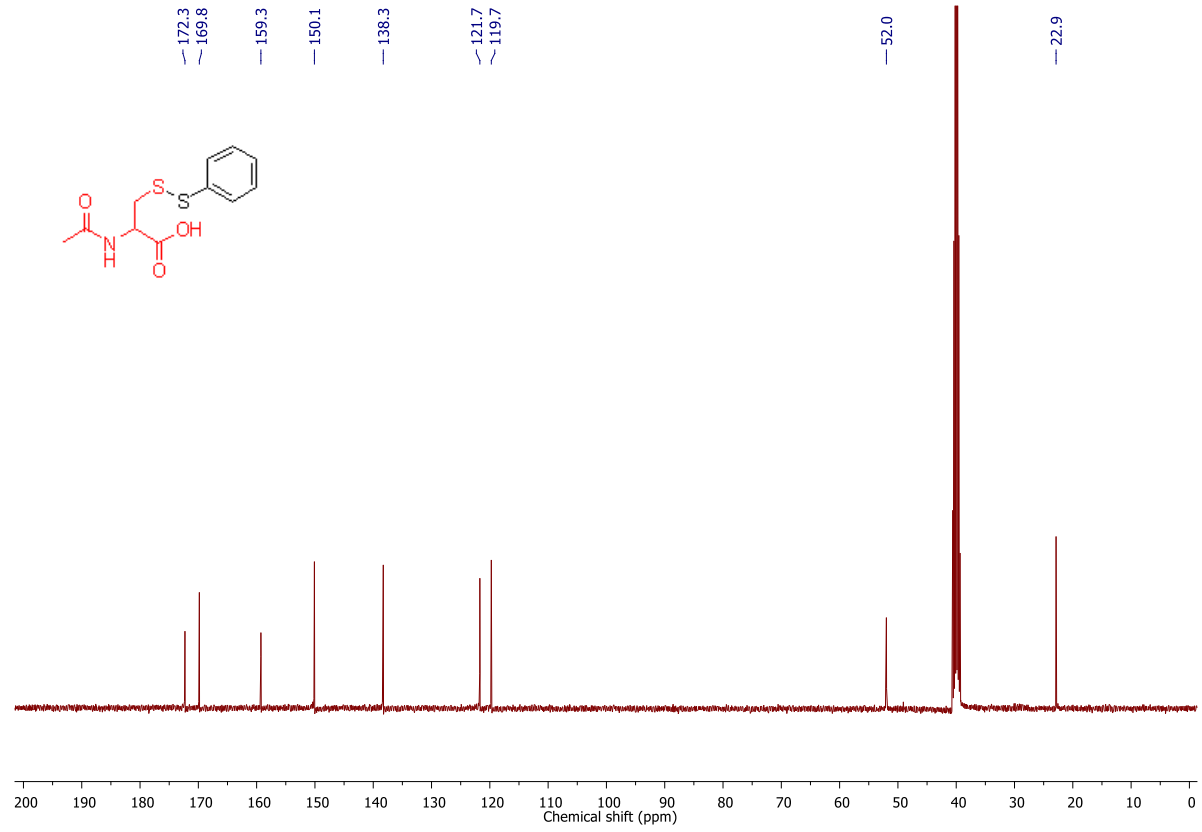
With the help of my colleague Prerona Bora, the cytotoxicity assay was performed for the compounds **18** and **4**. Adenocarcenomic human alveolar basal epithelial cells A549 cells were seeded at different concentration of 1 x 10^4 cells/well overnight in a 96-well plate in RPMI 1640 media. The cells were exposed to varying concentrations of the test compound **18** and **4** prepared as DMSO stock solutions and made the final concentration of DMSO to 1.0%. The cells were incubated at 37^{o}C for 24 h. The stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared (3.5 mg in 700 μL of RPMI 1640) diluted with RPMI 1640 (6.3 mL), and then the resulting solution (100 μL) was added to each well. After 4 h of incubation, the media was removed from the wells, and 100 μL of DMSO was added into each well. Spectrophotometric analysis was done to measure the absorbance using a microplate reader (ThermoFisher Varioskan) at an absorption wavelength of 570 nm.

4.6 NMR Spectra of compounds

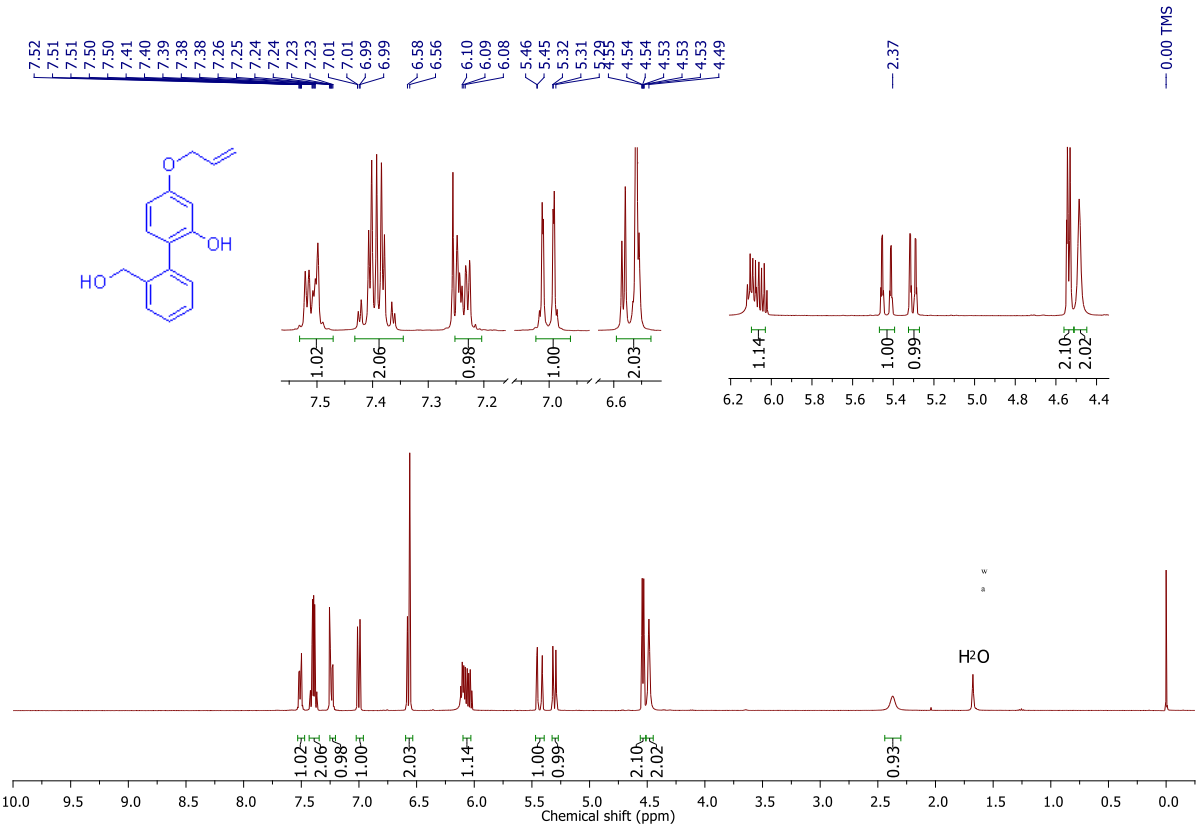
^{1}H NMR (400 MHz, DMSO-d$_6$) of 2



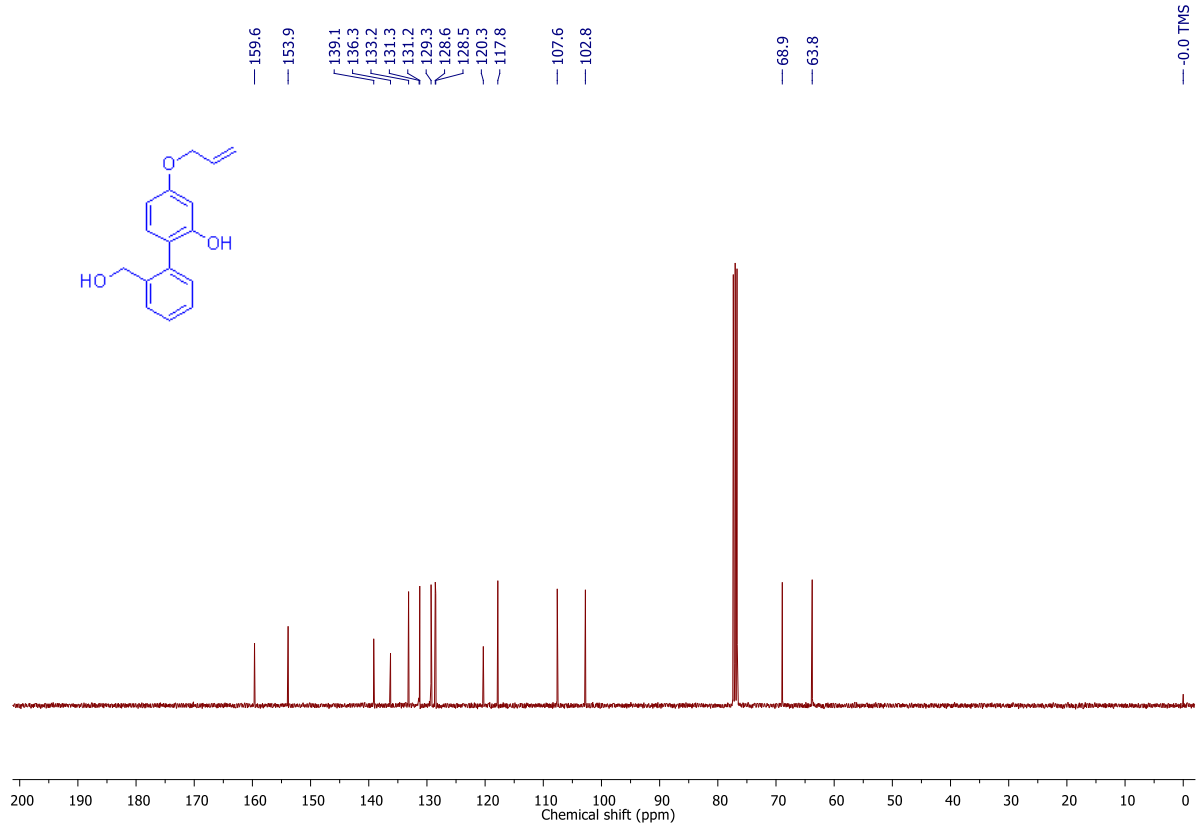
^{13}C NMR (100 MHz, DMSO-d$_6$) of 2



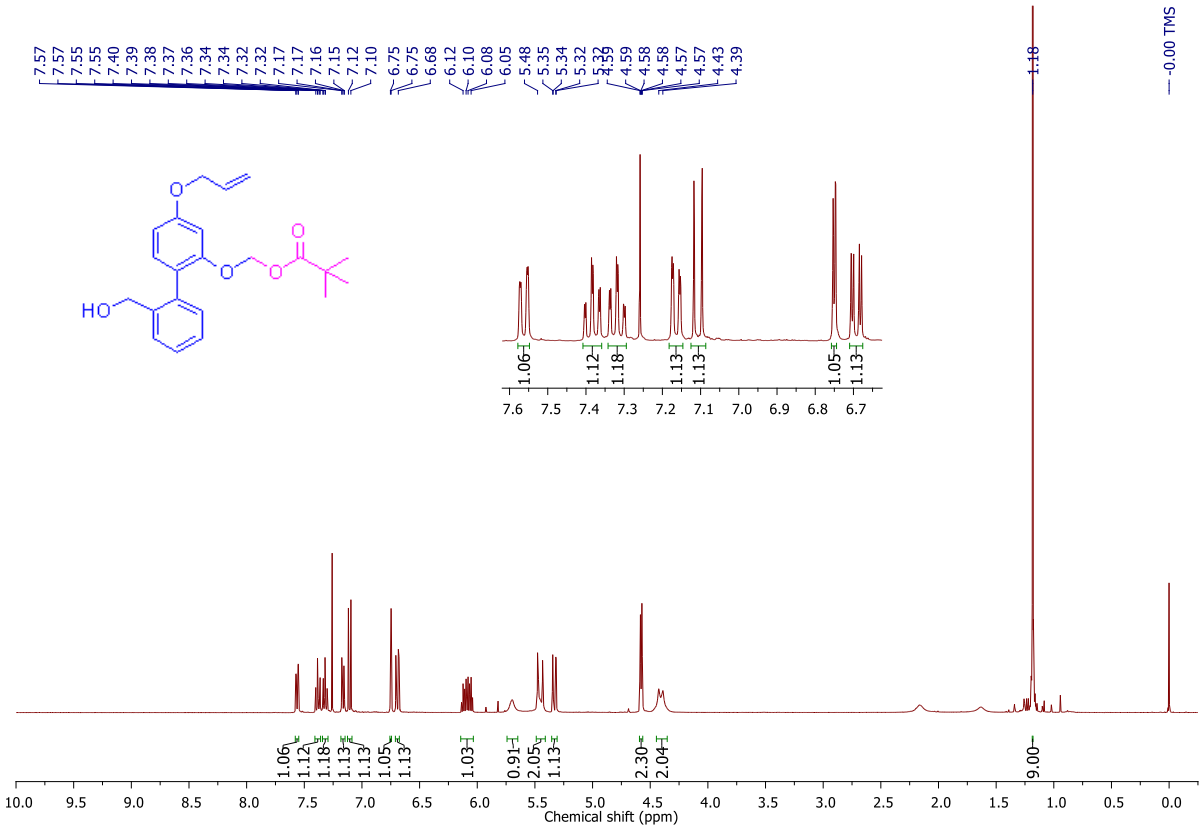
^{1}H NMR (400 MHz, $CDCl_3$) of 5



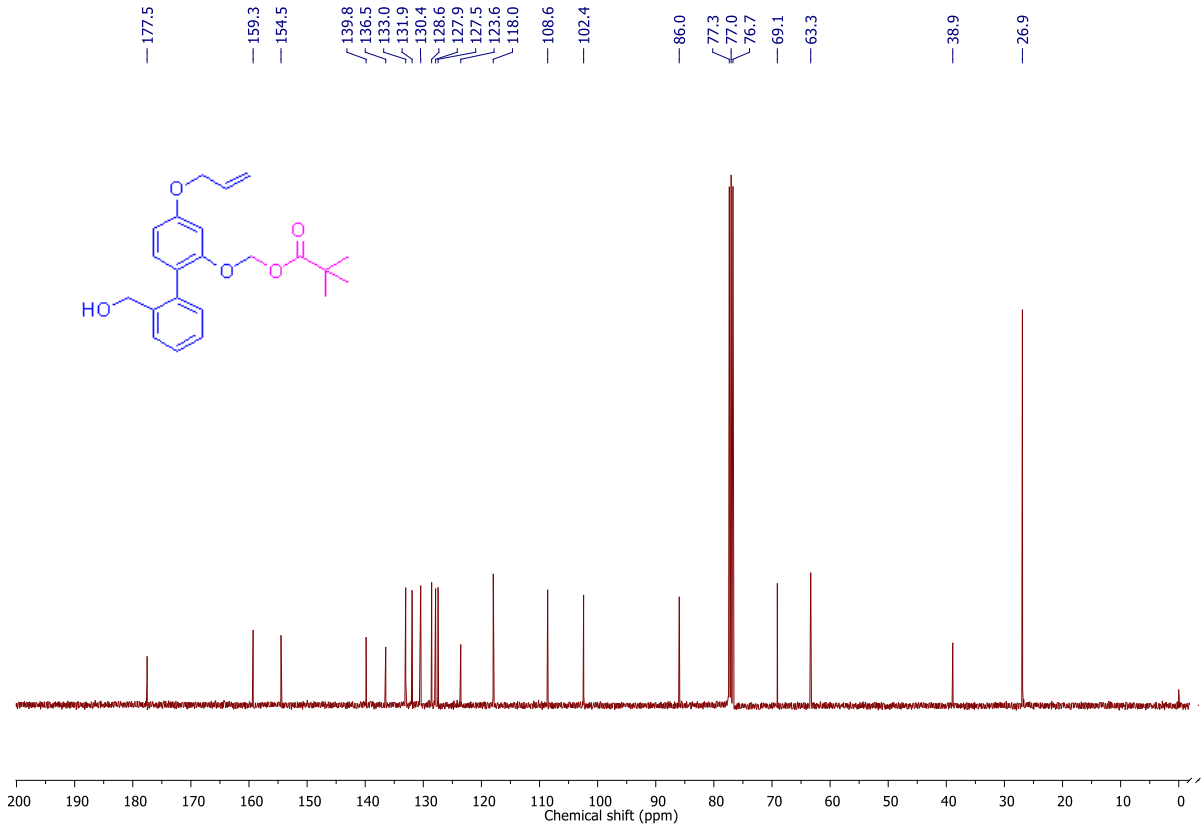
^{13}C NMR (100 MHz, $CDCl_3$) of 5



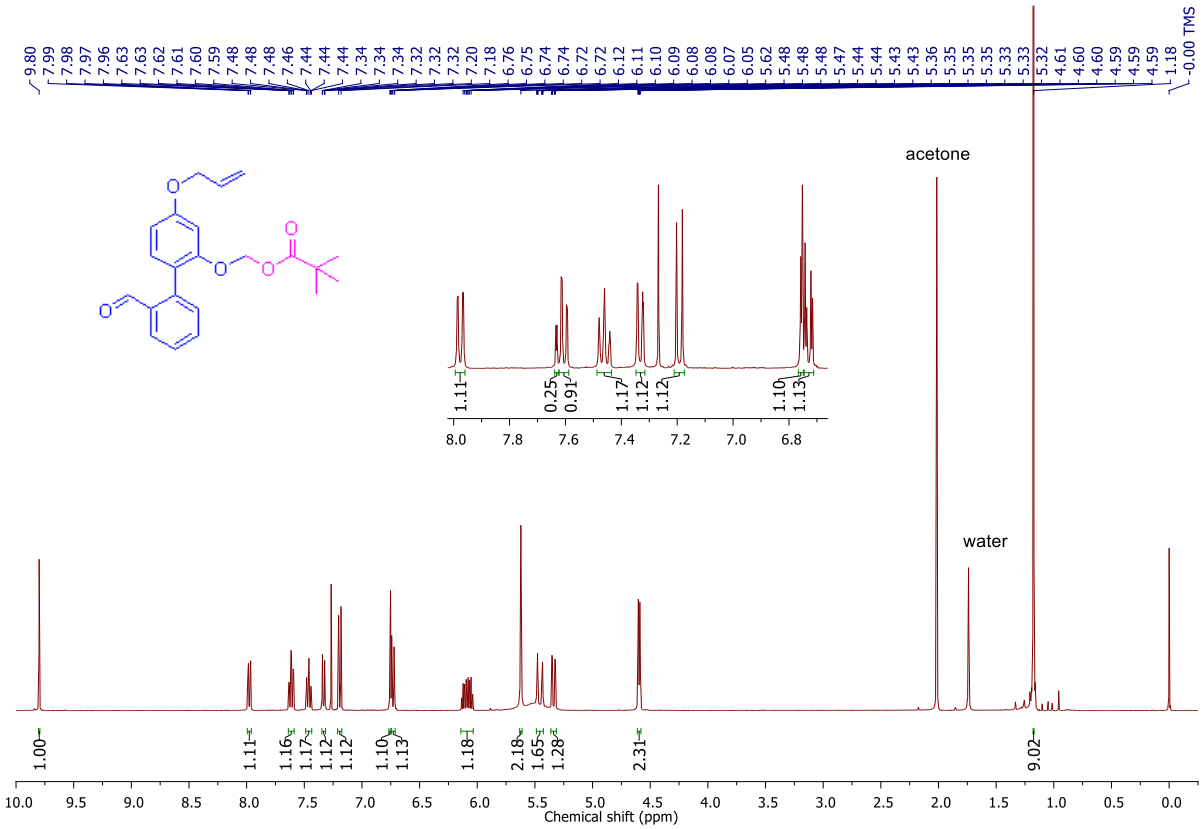
^{1}H NMR (400 MHz, $CDCl_3$) of 6



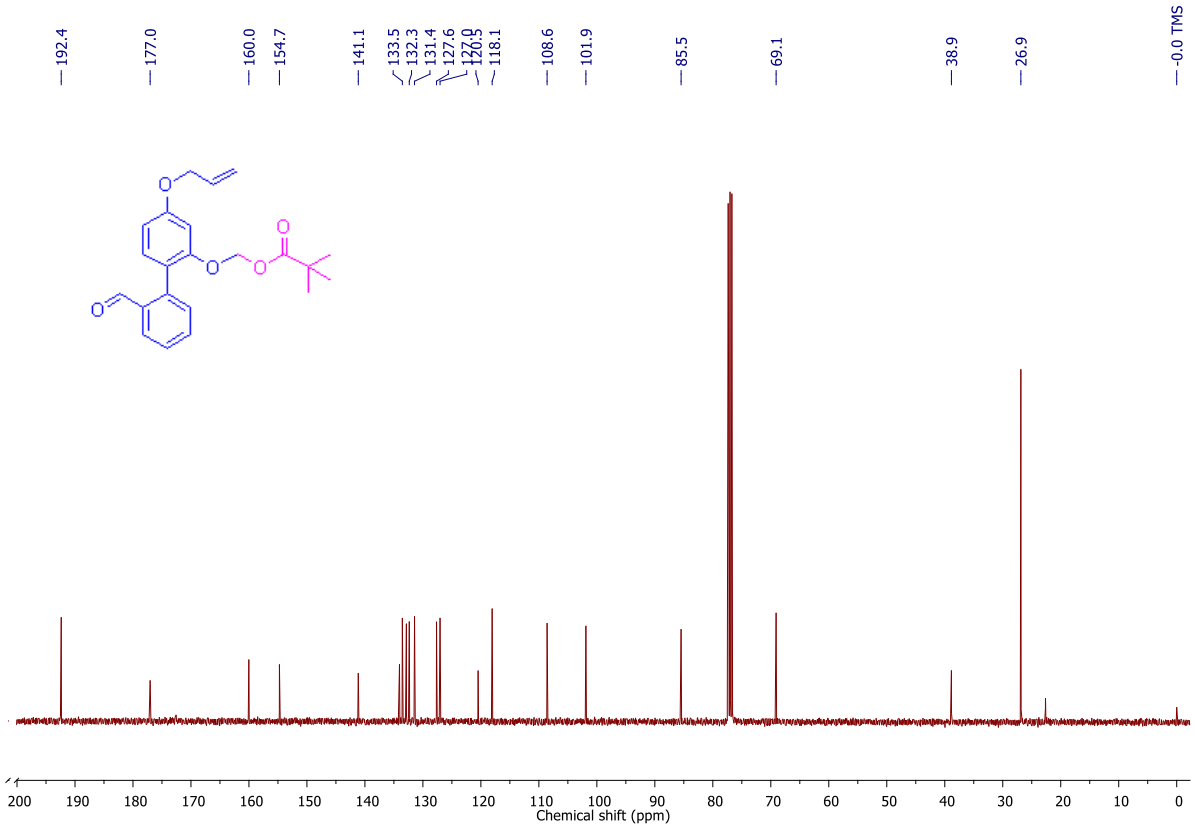
^{13}C NMR (100 MHz, $CDCl_3$) of 6



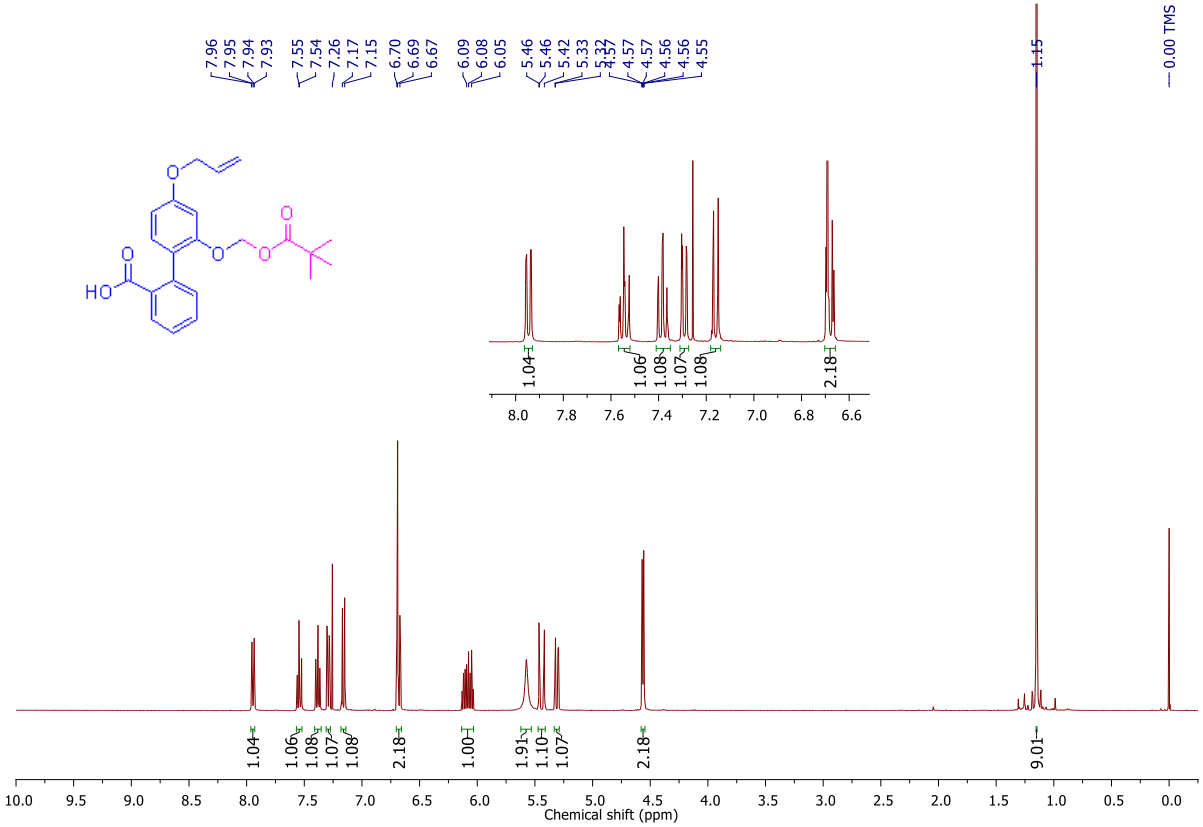
^{1}H NMR (400 MHz, $CDCl_3$) of 7



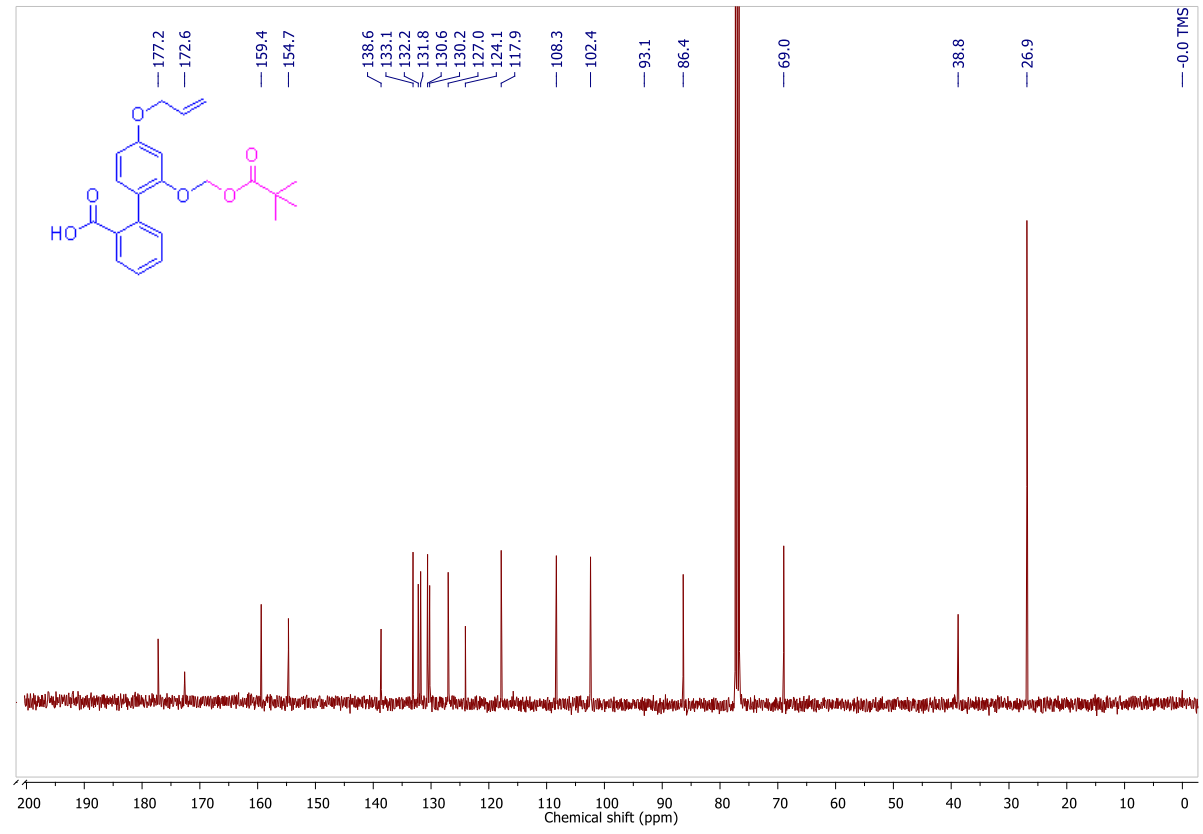
^{13}C NMR (100 MHz, $CDCl_3$) of 7



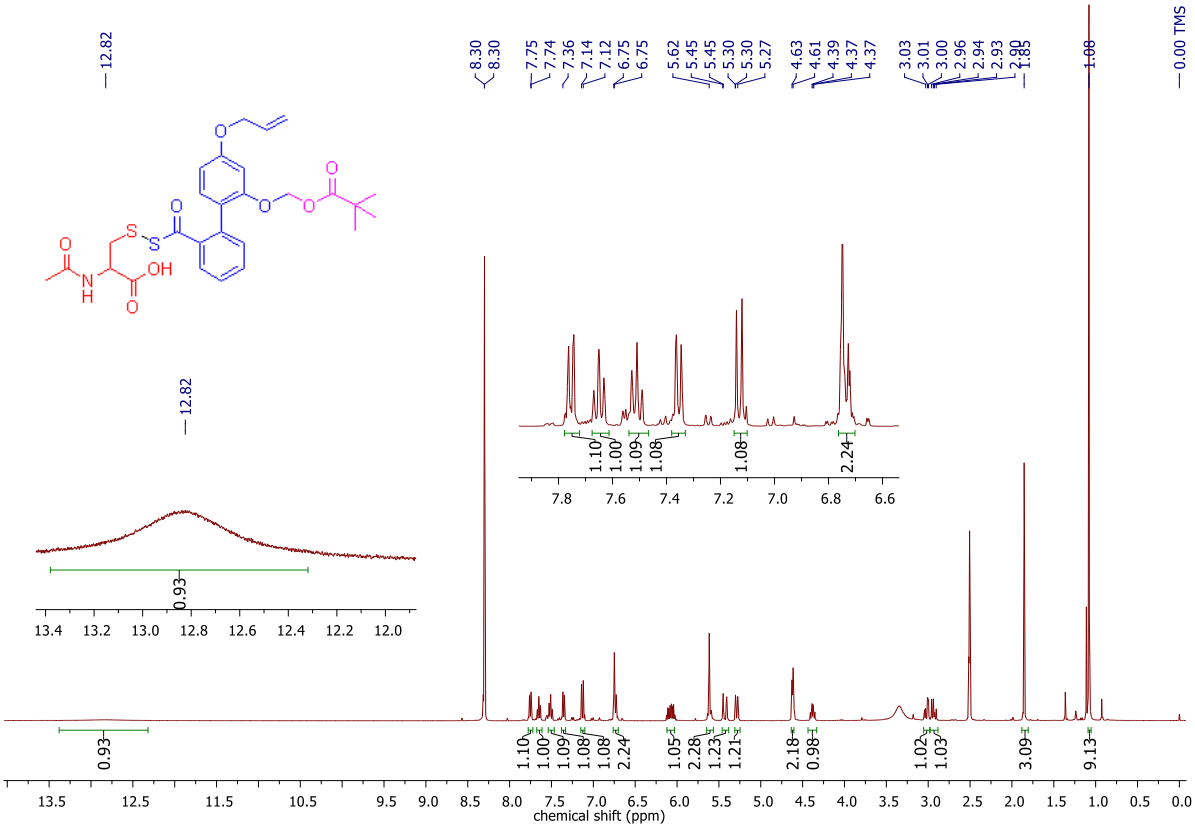
^{1}H NMR (400 MHz, $CDCl_3$) of 8



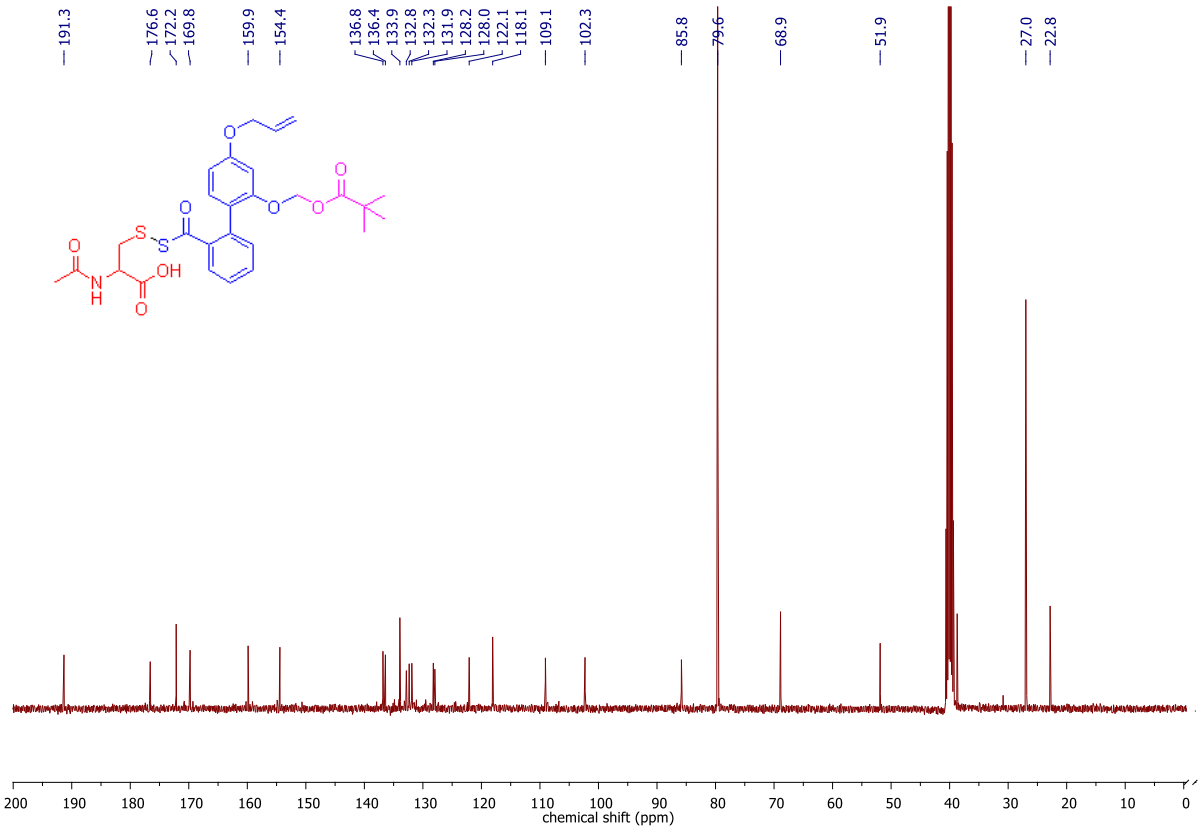
^{13}C NMR (100 MHz, $CDCl_3$) of 8



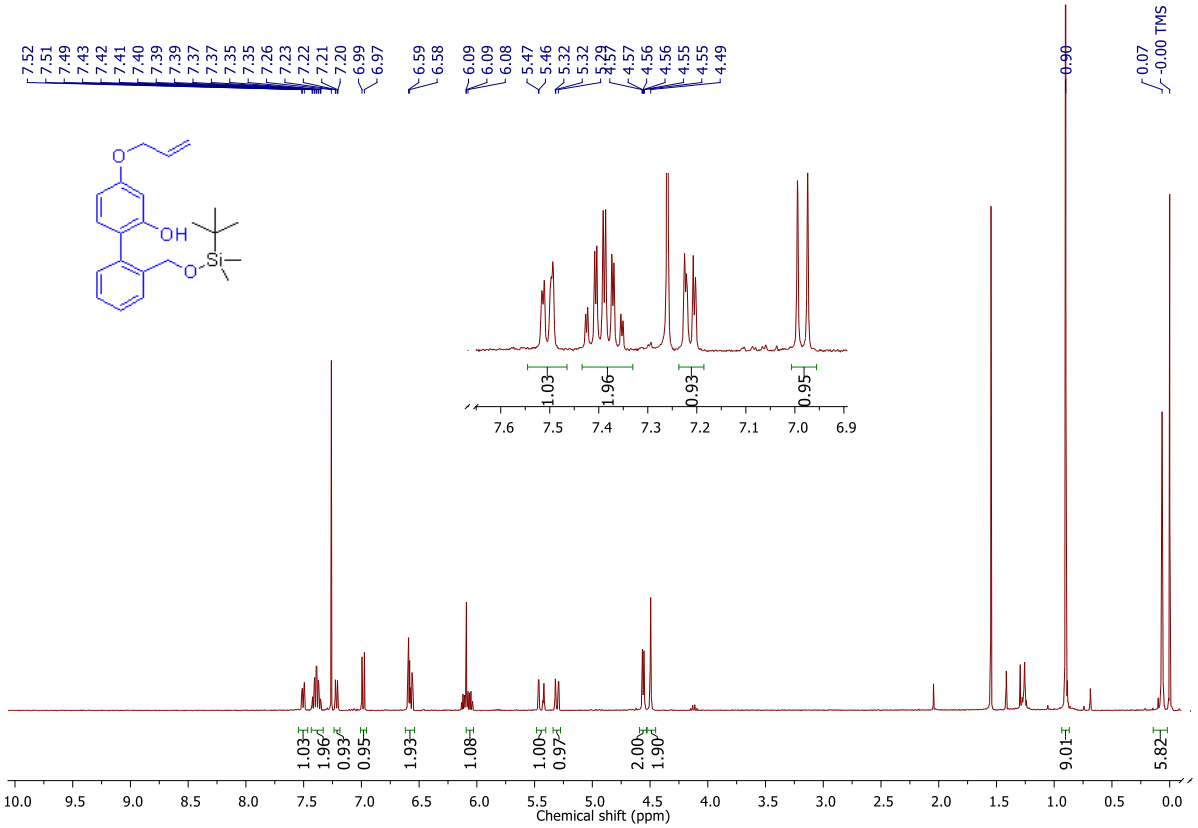
^{1}H NMR (400 MHz, DMSO-d_6) of 10



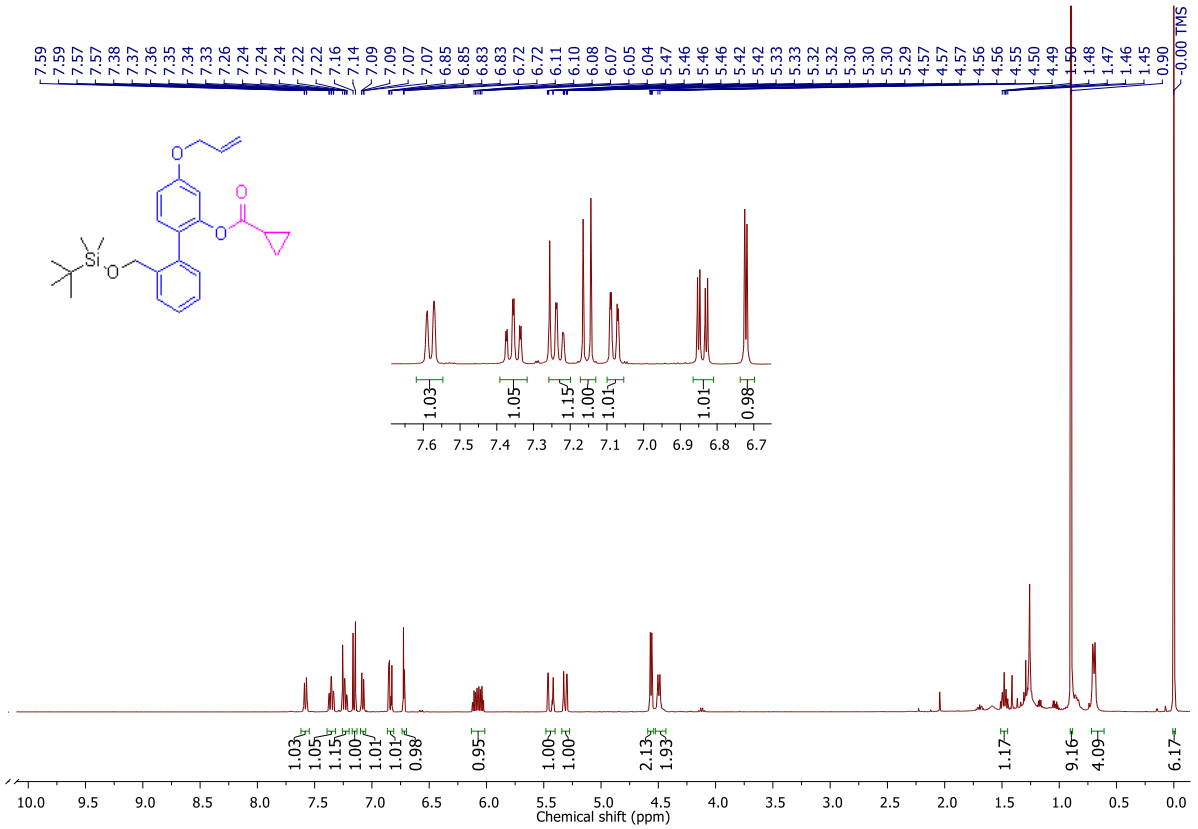
^{13}C NMR (100 MHz, DMSO-d_6) of 10



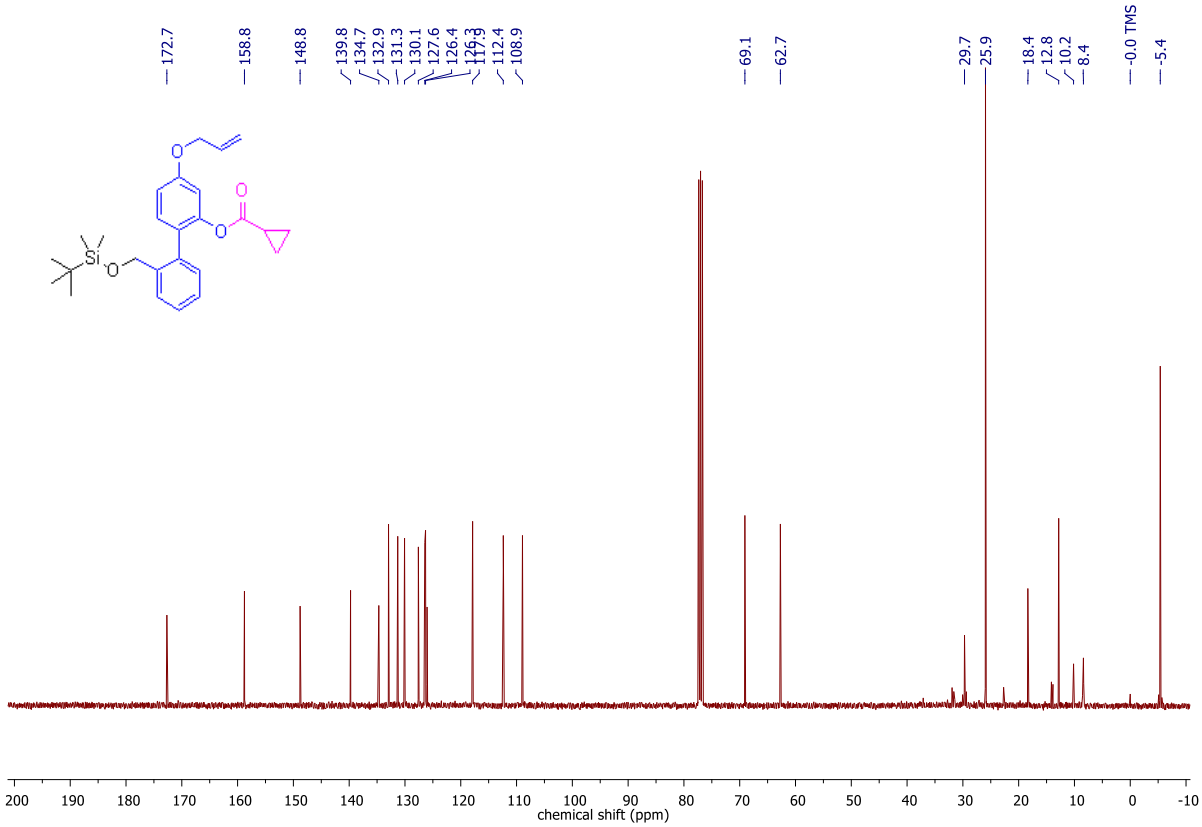
^{1}H NMR (400 MHz, $CHCl_3$) of 11



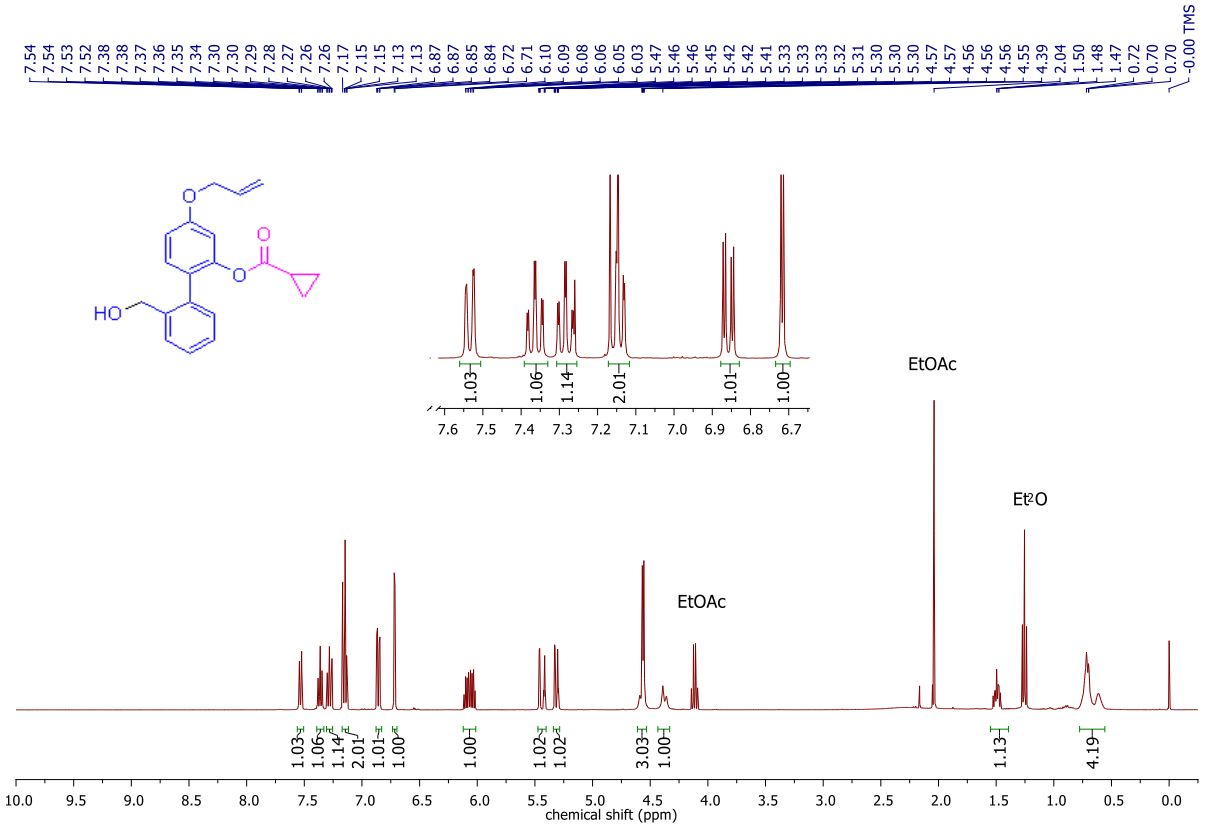
^{1}H NMR (400 MHz, $CHCl_3$) of 12



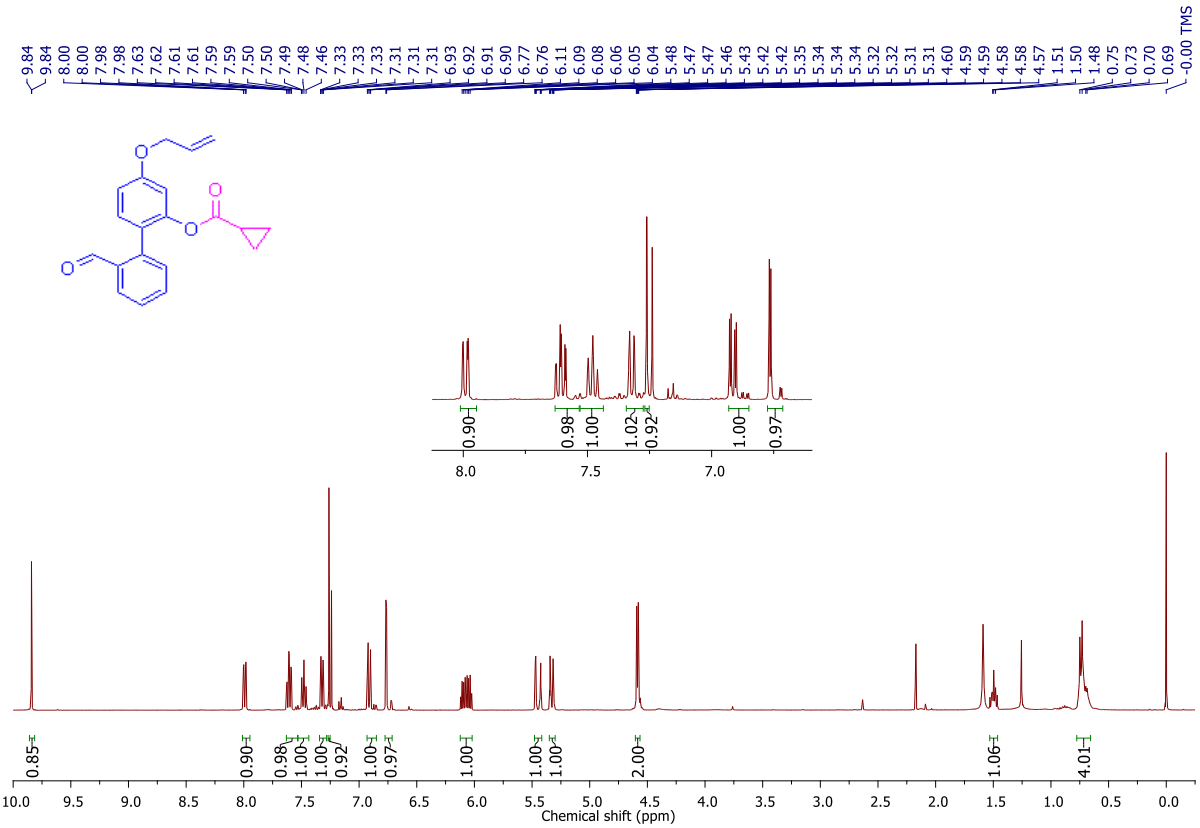
^{13}C NMR (100 MHz, $CHCl_3$) of 12



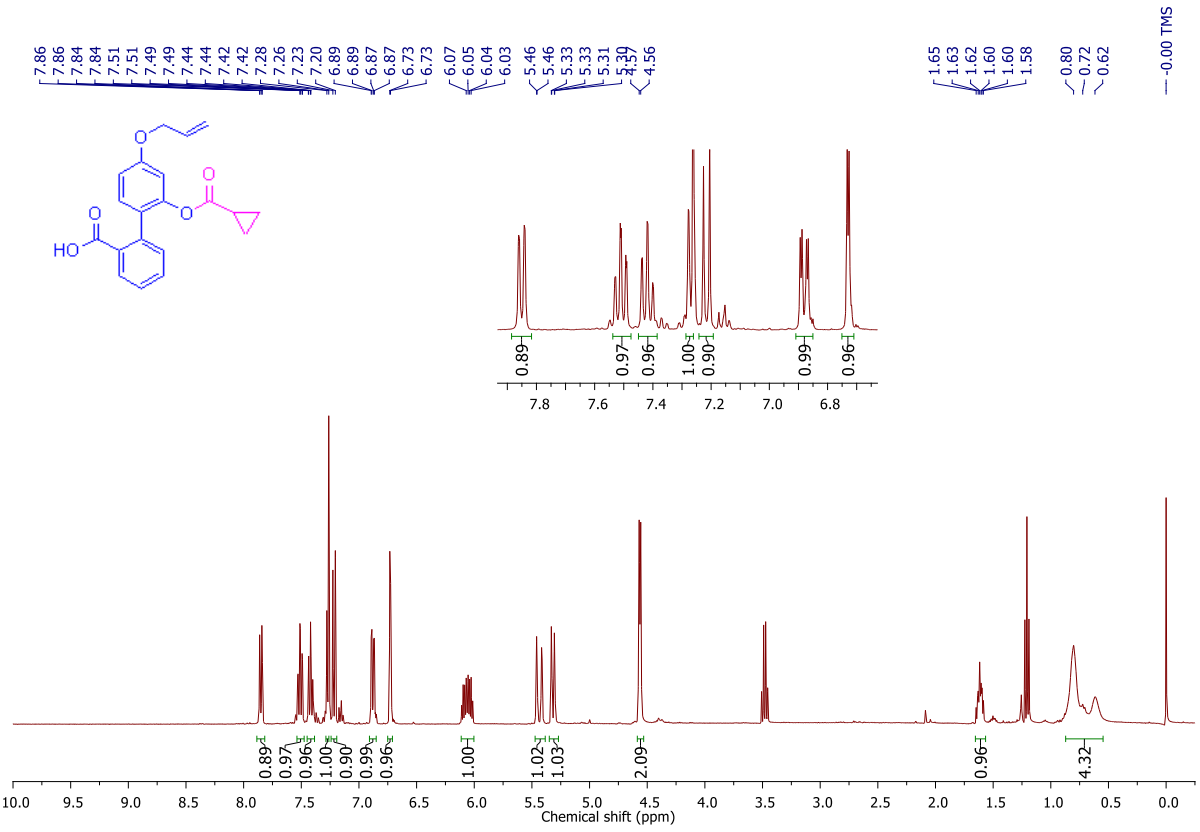
^{1}H NMR (400 MHz, $CHCl_3$) of 13



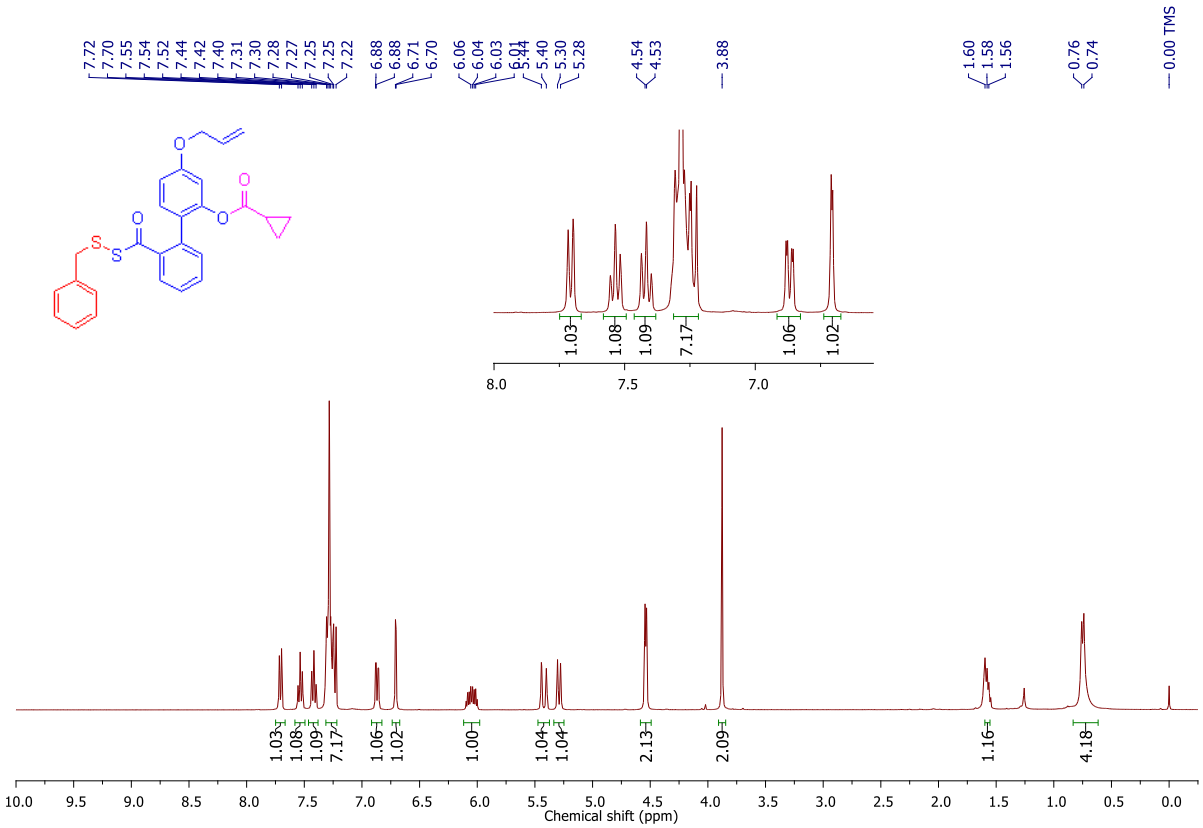
^{1}H NMR (400 MHz, $CHCl_3$) of 14



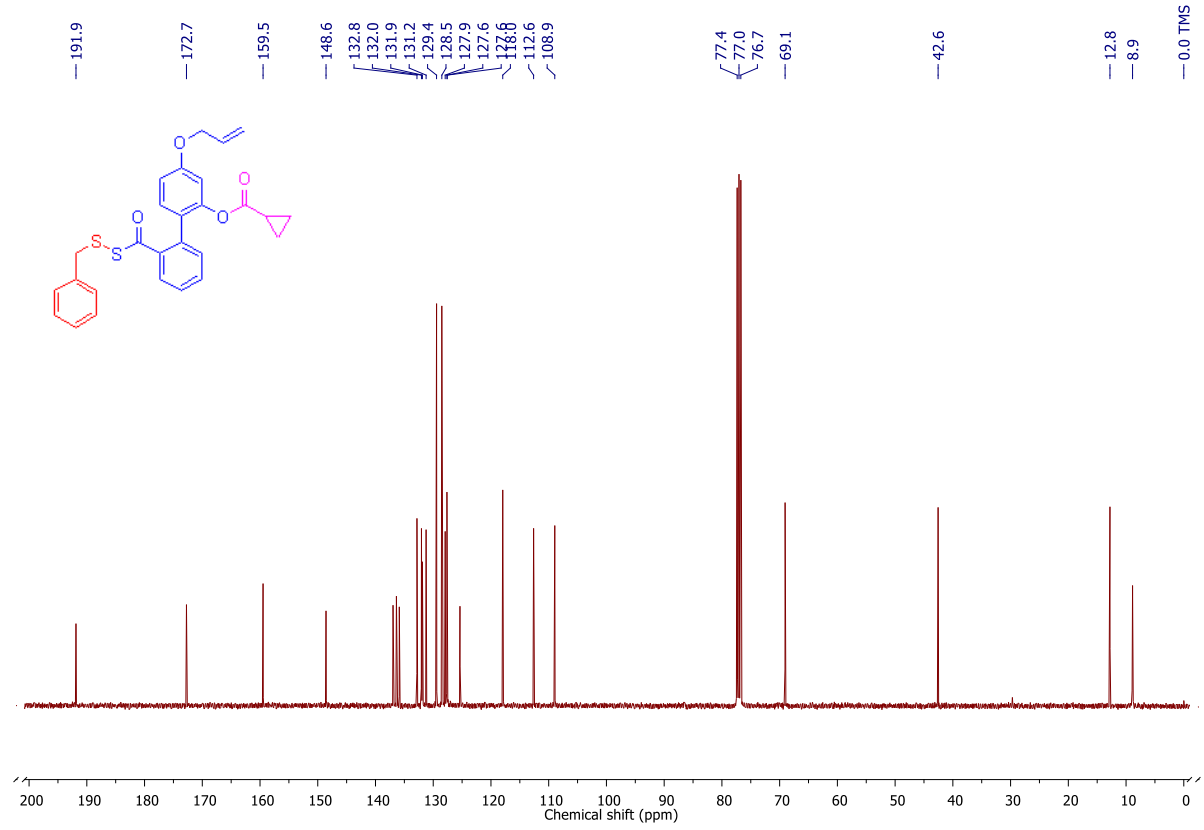
^{1}H NMR (400 MHz, $CHCl_3$) of 15



^{1}H NMR (400 MHz, $CHCl_3$) of 18



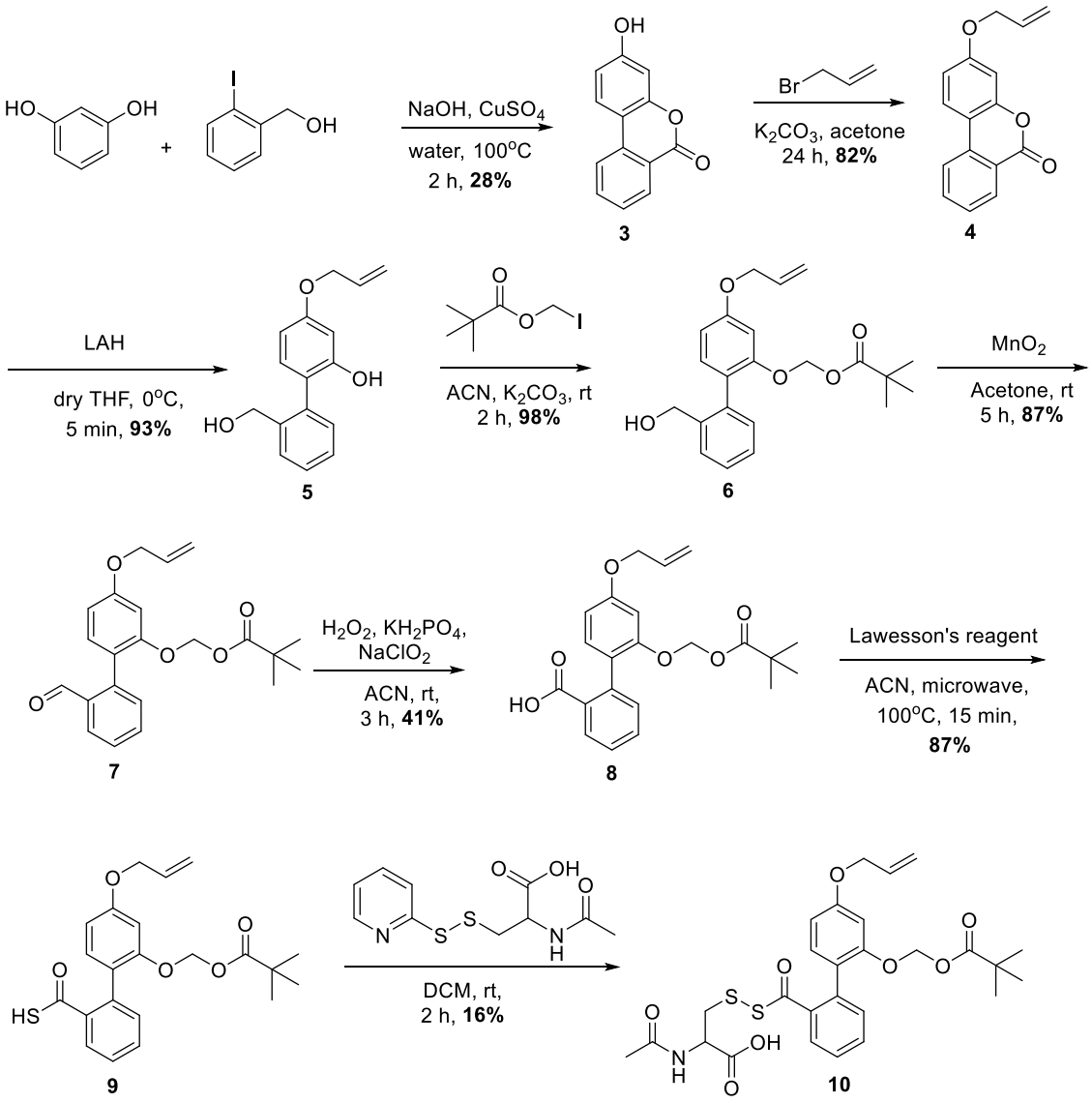
^{13}C NMR (100 MHz, $CHCl_3$) of 18



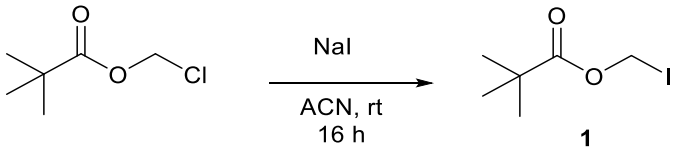
5. RESULTS AND DISCUSSION

5.1 3-(allyloxy)-6H-benzo[c]chromen-6-one as a fluorescence reporter

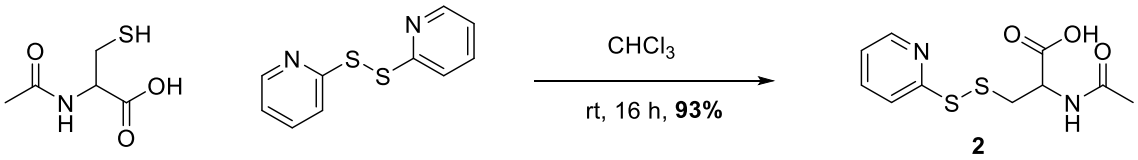
Compound **10** was prepared in 10 steps, using reported procedures. From the commercially available resorcinol and 2-iodobenzoic acid, **2** synthesized under reflux conditions in the presence of copper sulfate and sodium hydroxide solutions. Allyl bromide then reacted with **2** to give **3** (allylated lactone). The allylated lactone was opened up by using lithium aluminium hydride to give **5**. The phenolic alcohol was then protected with the Iodomethyl pivalate **1** to give **6**. The benzyl alcohol was then further oxidized in two steps to give **8**. Using Lawesson's reagent, **8** was converted into thio acid **9**. Compound **9** then underwent sulfur exchange reaction with the *N*-acetyl cysteine pyridine disulfide to for the persulfide **10**.



SCHEME 1



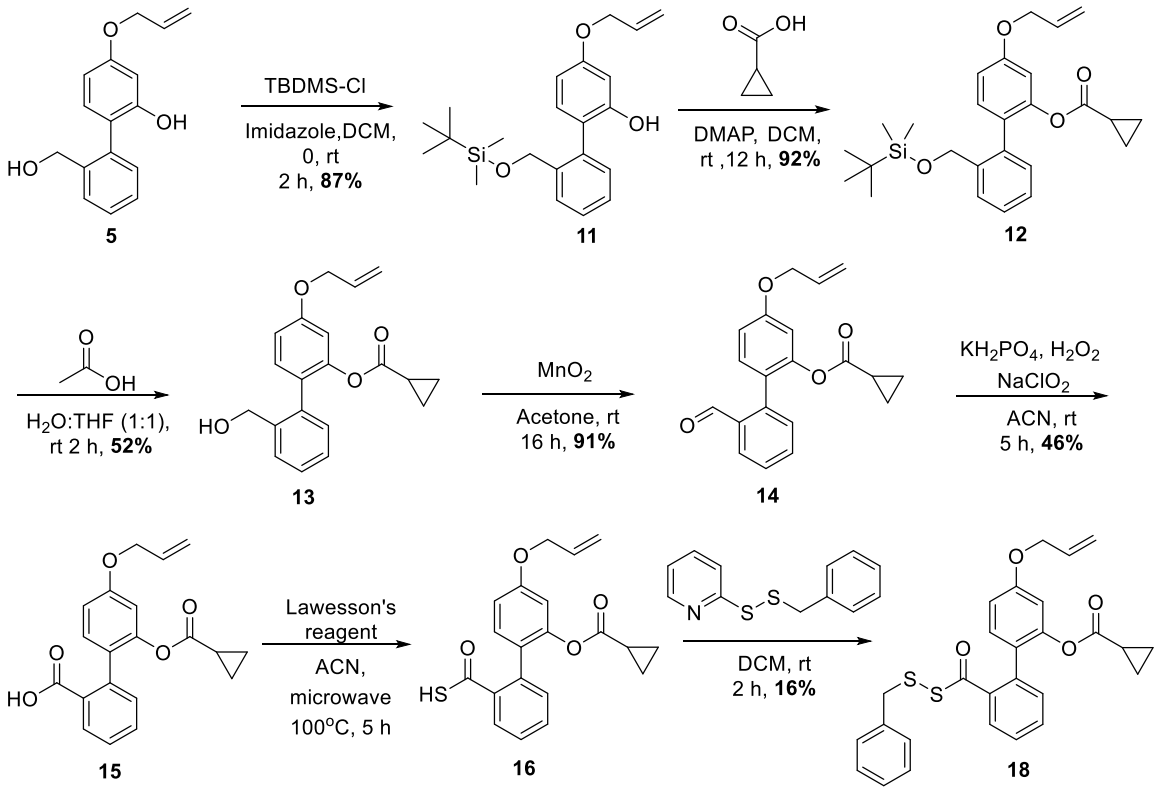
SCHEME 2



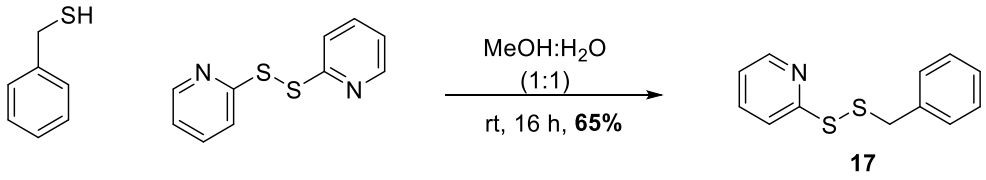
SCHEME 3

According to the proposed scheme 1, we expected the formation of persulfide **10**. However, the purification of **10** was not successful, and we could not attain a purity, which is higher than 85%. Its instability hindered the attempts for the purification of the compound.

To overcome these problems, we came up with a structure where the pivalate moiety substituted with a cyclopropane carboxylic acid, with a similar mechanism. The modified scheme is as follows:



SCHEME 4



SCHEME 5

According to the proposed scheme 4, we synthesized compound **18** in 8 steps. The benzylic alcohol of **5** was protected with TBDMS-Cl to give **11**. The phenolic alcohol of **5** then esterified with cyclopropane carboxylic acid to afford **12**—deprotection of the benzylic alcohol using acetic acid to give **13**. The benzylic alcohol was oxidized into carboxylic acid in 2 steps to afford **15**, and then carboxylic acid was converted into thio acid in the presence of Lawesson's reagent to give **16**. The compound **16** underwent sulfide exchange reaction with **17** to afford the desired persulfide **18** in a quantitative yield.

5.2 Observation of fluorophore by HPLC study

The ability of Compound **18** to cyclize and form lactone, as shown in the figure, was tested under the presence of porcine liver esterase.

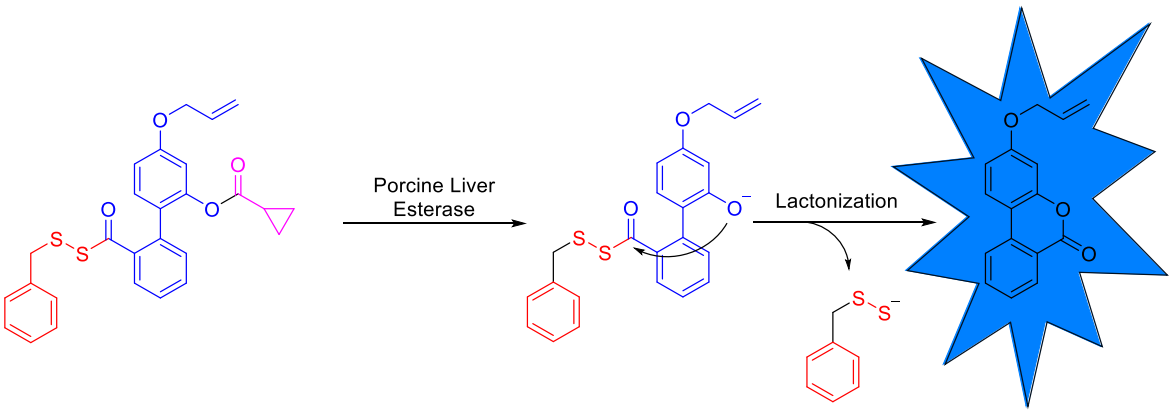


Figure 15: Scheme for fluorophore formation

The compound **18** was treated with porcine liver esterase, and the reaction mixture was injected into the HPLC at different time intervals. The formation of a new peak after 30 min, suggested the formation of a fluorophore **4**. It was detected in the fluorescence detector. The following experiment was done as a proof of concept to show the cleavage of the ester bond. The quantitative yield of the fluorophore was not obtained, presumably due to solubility issues.

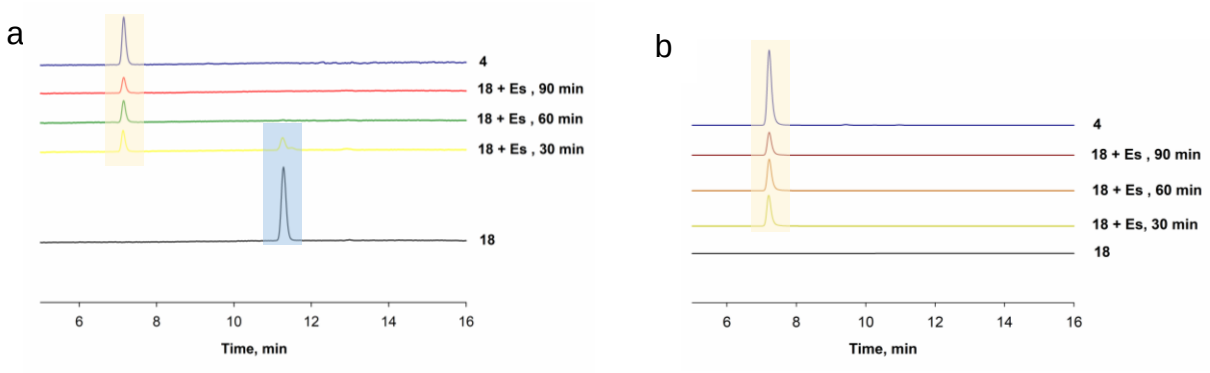


Figure 16: HPLC traces showing the formation of fluorophore **4** at different time intervals at 37 °C in pH 7.4 buffer (a) 250 nm wavelength detector. (b) Fluorescence detector (432 nm)

Compound **18** showed a peak, which corresponds to a retention time of 11.13 min. Upon activation with porcine liver esterase (1 U/mL), **18** converted into the fluorophore **4** (R_t = 7.14 min). The intensity of peak increases in 30 min, and the maximum intensity observed was **37%**, at 30 min. Further incubation showed a decreased intensity likely due to the decreased solubility of the lactone. This trend was observed in both the detectors (**Figure 16a and Figure 16b)**. The complete disappearance of the peak corresponding to **18** was observed at 60 min (**Figure 16a)**. The experiment was carried out in triplicate, and the results were reproducible.

5.3 Quantifications of FDNB adduct formation by HPLC study

The compound **18** upon decomposition with esterase releases fluorophore as well as benzyl persulfide. The persulfide release by compound **18** was studied by using 1-fluoro-2,4-dinitrobenzene (FDNB) to trap the benzyl persulfide, a method developed by Binghe Wang and co-workers, to form a disulfide adduct as shown in **Figure 17**.

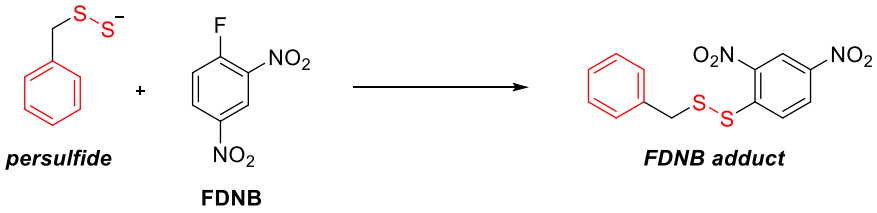


Figure 17: Proposed scheme for FDNB adduct formation

The compound **18** incubated with porcine liver esterase and FDNB was injected into the HPLC at predetermined time points. Controls of **18**, fluorophore **4**, and FDNB adduct were also injected to compare with the formed new peaks.

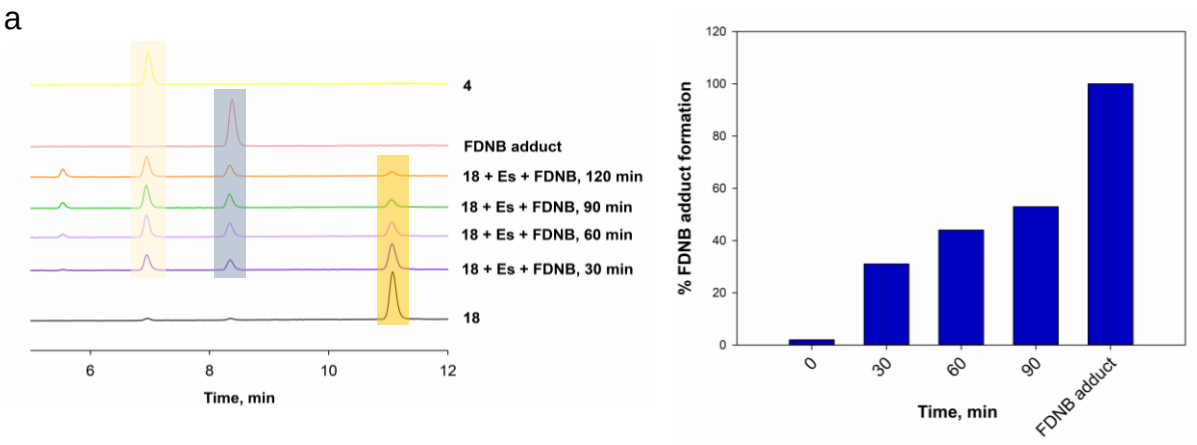


Figure 18: (a) HPLC traces showing the formation of FDNB adduct and fluorophore in the presence of esterase and FDNB, respectively, at 37 °C in pH 7.4 buffer. (b) Quantification of FDNM adduct, as monitored by HPLC analysis.

The experiment further provided a piece of evidence that compound **18** upon activation by esterase released fluorophore and benzyl persulfide, which is detected by the HPLC. Compound **18** showed a peak corresponding to a retention time of 11.13 min. When esterase was acted on **18**, it gets cleaved and forms the fluorophore **4** (R_t = 7.14 min), and a new peak was formed corresponding to the FDNB adduct (R_t = 8.56 min). After 120 min, 3% of **18** was remaining, where the kinetics was slower when compared to the reaction without FDNB. The maximum amount of FDNB adduct observed was **52%** at 90 min (**Figure 18b)**. A new uncharacterized peak (R_t = 5.41 min) was observed. This could be due to the formation of disulfides of persulfides that are being released. Further incubation shows a decreased amount of FDNB adduct formation (**Figure 18a)**, which could be due to the decomposition of FDNB adduct in the buffer. The experiments were carried out in triplicate, and reproducible results were obtained.

5.4 Quantification of fluorophore based on fluorescence studies

5.4.1 Fluorimeter based studies

The compound **18** (10 μM) was incubated with porcine liver esterase at 37 °C in pH 7.4 buffer, and the emission spectra at λ_{max} = 432 nm were recorded in the fluorimeter at an excitation wavelength of λ_{max} = 330 nm. Varying concentrations of the compound

4 were taken, and a calibration plot was generated **(Figure 19a)**, and the yield of the fluorophore from **18 (Figure 19b)** was calculated accordingly. **Figure 19** showed the release of the fluorophore from compound **18**.

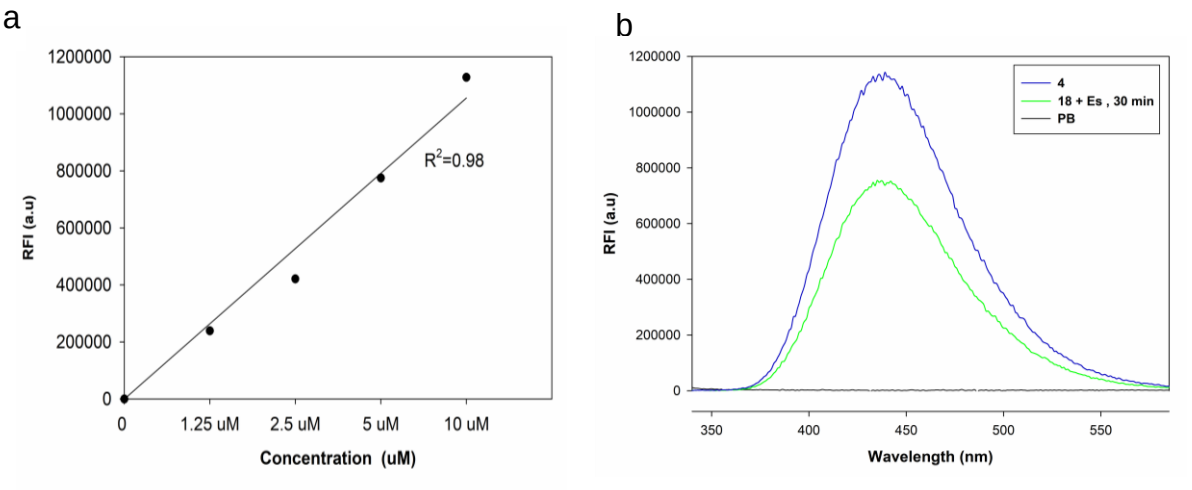


Figure 19: (a) Calibration curve fitting for the lactone. (b) Decomposition of 18, as monitored by fluorimeter in the presence of esterase at 37°C in pH 7.4 buffer.

The fluorophore **4,** which was generated as a result of the action of esterase on **18,** has a characteristic excitation at λ_{max} = 330 nm and emission at λ_{max} = 432 nm. Based on this fluorescence profile, the excitation spectrum was generated, and the fluorophore yield was obtained from that. It was observed that the maximum amount of fluorophore formed was **48%**, at 30 min. Upon further incubation of **18** with esterase, a reduction in the fluorophore intensity was observed. This is likely due to the reduced solubility of fluorophore in the buffer.

5.4.2 Fluorescence studies - EnSight™ multimode plate reader (PerkinElmer, India)

Compound **18** of different concentrations were incubated with porcine liver esterase and the **Figure 20a** and **20b** showed the release of the fluorophore **4** from **18**. It was observed that the fluorescence signal from fluorophore **4** which has a characteristic excitation wavelength of λ_{max} = 330 nm and emission wavelength of λ_{max} = 432 nm reaches saturation in 40 min, and even after 120 min, there was no significant enhancement in the fluorescence signal.

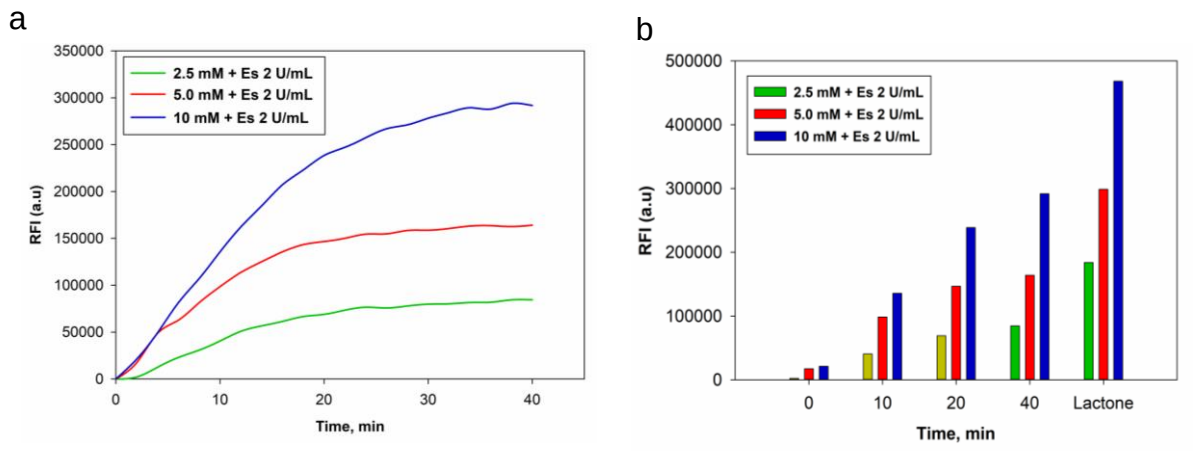


Figure 20: (a) Dose-dependent decomposition of 18 under the presence of esterase at 37°C in pH 7.4 buffer with an excitation wavelength of λ_{max} = 330 nm and emission wavelength of λ_{max} = 432 nm. (b) Formation of fluorophore 4 at different time intervals.

5.5 Cell lysate experiment

With the help of my colleague Anand Kumar, we performed the activation of compound 18 by the cell lysate. We hypothesize that the presence of esterase enzymes in the cell lysate will result in the decomposition of **18**, resulting in the enhancement of fluorescence. Consistent with this hypothesis, an increase in the fluorescence was observed upon incubation of cell lysate with compound **18**. The fluorescence intensity was measured at a wavelength of 430nm at specified time intervals.

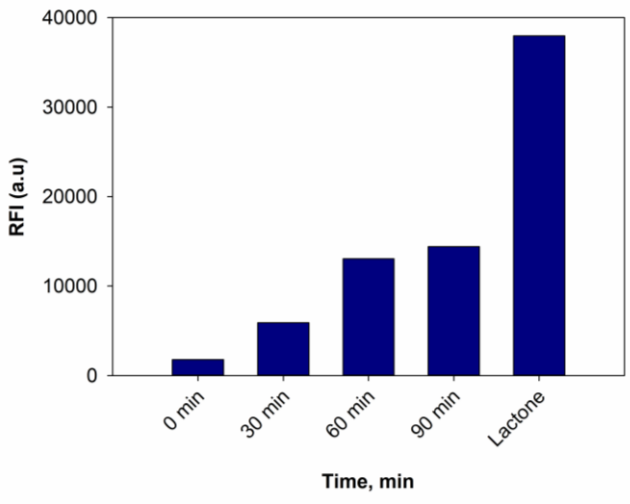


Figure 21: Esterase mediated decomposition of 18 in cell lysate at different time intervals.

Lactone **4** (fluorophore) was used as a control. The data showed that the maximum amount of fluorophore **4** was observed at 90 min, which was approximately 38% when

compared to that of control. The quantitative yield was not obtained presumably due to reduced solubility of **18**.

5.6 Cytotoxicity study

With the help of my colleague Prerona Bora, cytotoxicity study for **4** and **18** were done. The fluorophore was not found to be significantly toxic at 25 μM, but at a higher concentration of 50 μM, it was moderately toxic (**Figure 22a)**. However, Compound **18** was found to be well tolerated, even at 50 μM (**Figure 22b)**.

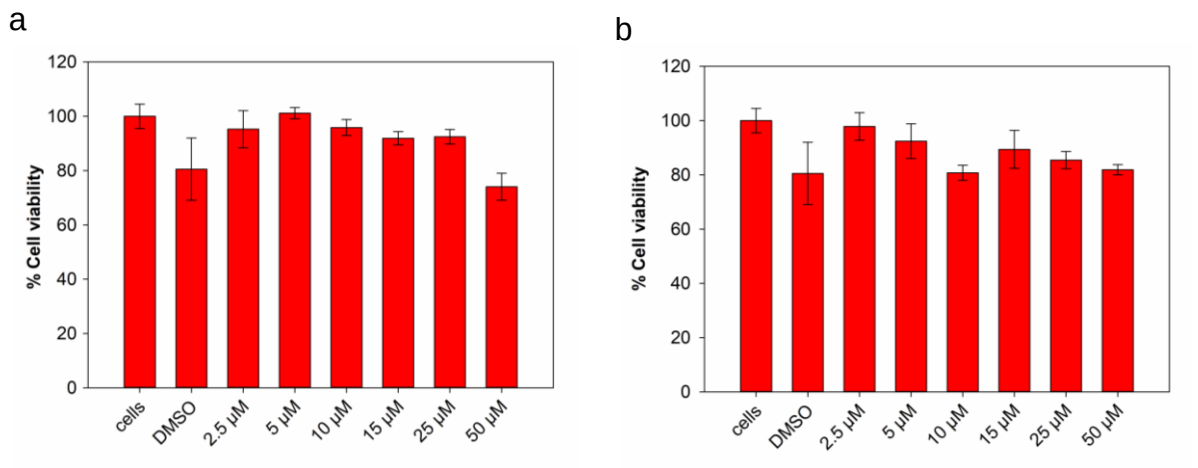


Figure 22: Cytotoxicity at various concentrations Adenocarcenomic human alveolar basal epithelial cells A549 to the control of the cell. (a) Various concentrations of fluorophore 4. (b) Various concentrations of 18.

6 CONCLUSIONS AND OUTLOOK

In summary, we have developed a novel persulfide (RSSH) donor, which addressed the issue of real time-monitoring of persulfide. Also, we have tackled the issue of nucleophile generation during the reaction by using biphenyl moiety as our linker. Upon incubation with porcine liver esterase, we found that it released a persulfide and a simultaneous release of a lactone, which is fluorescent. By exploiting this, we tried to quantify the persulfide release. Due to some limitations, quantitative yields could not be obtained.

In the future, we are planning to work to improve the design for the enhancement of fluorescence as well as the solubility of the persulfide donor and the fluorophore. We are further planning to increase the selectivity by changing the trigger.

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