Synthesis and Evaluation of Small Molecule Hydrogen Sulfide Donors

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

By

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CERTIFICATE

This is to certify that this dissertation entitled **"Synthesis and Evaluation of Small Molecule Hydrogen Sulfide Donors** "towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Indian Institute of Science Education and Research, Pune under the supervision of Dr.Harinath Chakrapani, Associate Professor, Chemistry Department during the academic year 2016-2017.

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DECLARATION

I hereby declare that the matter embodied in the report entitled "**Synthesis and Evaluation of Small Molecule 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.

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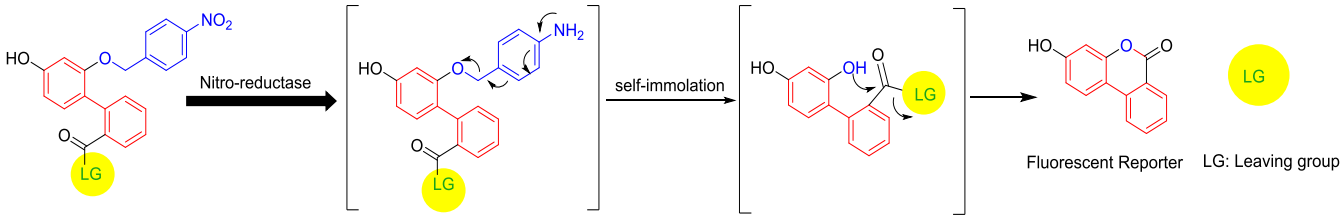
1. ABBREVATIONS

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

Hydrogen sulfide (H_2S) is an endogenous gaseous signaling molecule, which mediates cardiovascular homeostasis and exhibits antioxidant activity. When H_2S was administered along with non-steroidal anti-inflammatory drugs (NSAIDs), in animal models, the side effects associated with NSAIDs were reduced. With the growing importance of H_2S as a signaling molecule, new and effective methods are needed for its production and detection. This lead to the development of **H_2S donors**, which could release H_2S in a controlled and targeted manner inside the cells. Over the years, a number of analytical techniques for release and detection have been reported. However these techniques are associated with some limitations such as lack of selectivity between H_2S and other biological thiols, rapid oxidation on air exposure, consumption of H_2S and production of toxic by-products. Thus development of a H_2S donor with an inbuilt reporter would be useful. In this respect, aim of the project is to design of one such H_2S donor, which can give direct release of H_2S, with a concurrent fluorescence signal. It will minimize the reliability on secondary assay for H_2S detection. With nitro-reductase as the enzyme trigger and umbelliferone as fluorescence reporter, we tried to study the release of H_2S. The design also addresses the production of potential toxic by-products. Following some synthetic difficulties, we modified the fluorescence reporter to 3-hydroxy-6H-benzo[c]chromen-6-one and studied the release of H_2S.



3. INTRODUCTION

Hydrogen sulfide was first discovered by Carl Wilhelm Scheele and is a toxic air pollutant. Recently it gained attention as a biologically relevant gaseous signaling molecule similar to nitric oxide (NO)[1] and carbon monoxide (CO).

3.1 BIOLOGICAL SYNTHESIS AND METABOLISM

Biological synthesis of H_2S in mammalian cells is attributable to three enzymes namely: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST).[2] Mammalian orthologs of these enzymes are evolutionarily conserved in bacterial genome, suggesting a crucial role for H_2S, in basic cellular processes.[3] CBS and CSE are both pyridoxal 5'-phosphate (PLP) dependent and present in cytosol, whereas MST uses PLP as a co-factor and is mainly a mitochondrial enzyme. Depending on the cell type and specific stress conditions, the production of H_2S can happen in cytosol and/or mitochondria, and accordingly these enzymes can be transported from cytosol to mitochondria.[2] These enzymes act collectively to regulate H_2S production.

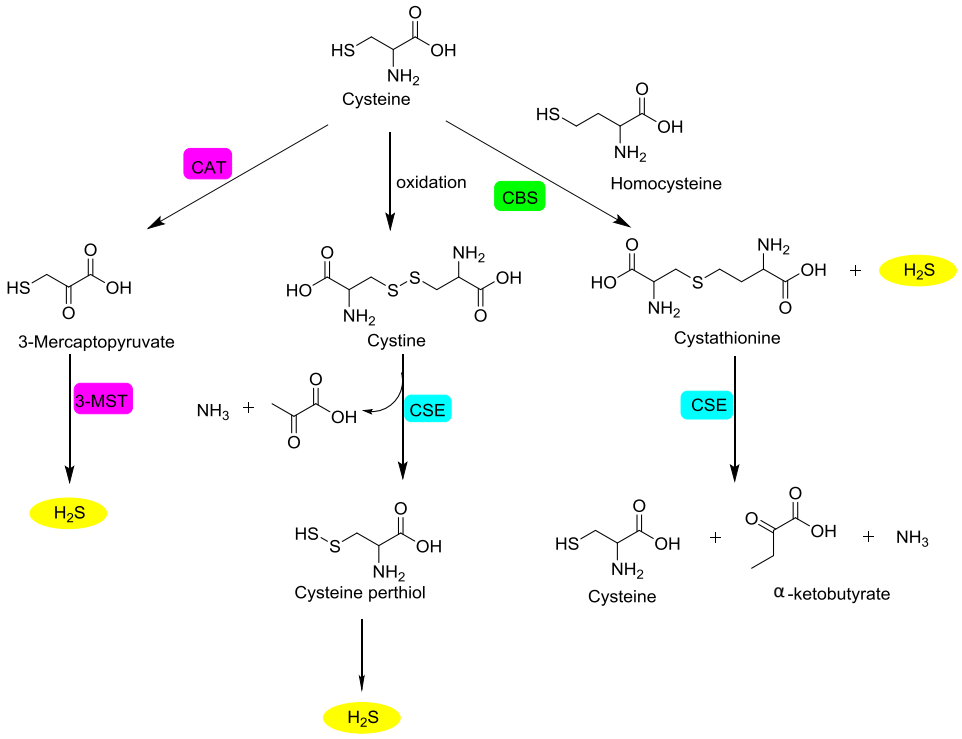


Figure 1: Enzymatic synthesis of H_2S[2]

Compared to NO, H_2S is relatively stable during circulation. It is metabolized via oxidation and methylation. Free H_2S is scavenged and stored as sulfane-sulfur and bound sulfur pools.[4]

3.2 CELLULAR AND MOLECULAR EFFECTS

Since the first report of H_2S as a neuromodulator, by Kimura and co-workers in 1996,[5] a number of studies have been carried out, revealing the various biological effects of H_2S. Its membrane permeability and unique chemical reactivity with certain macromolecules, widens the scope of its physiological or biological effects.

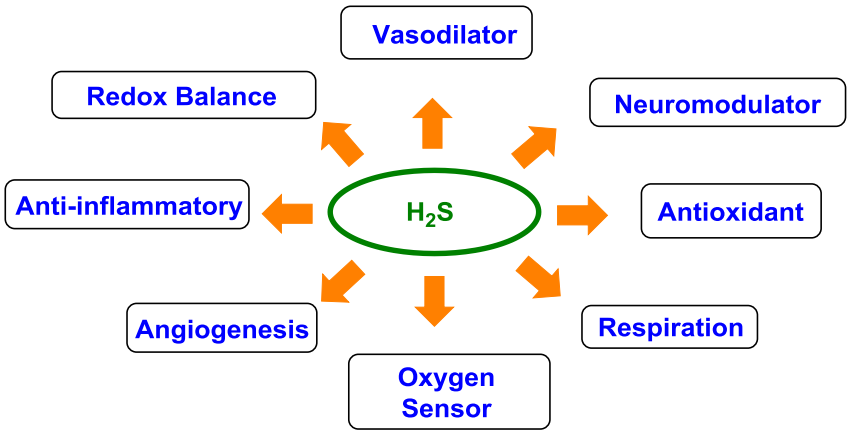


Figure 2: Biological roles of H_2S (Source: Preeti Chauhan report)

Some of the widely studied biological effects of H_2S include relaxation of blood vessels (vasodilation), protection against myocardial ischemia injury, and protection against oxidative stress (antioxidant).

The first identified molecular target of H_2S was ATP-sensitive potassium ion channels. This interaction is responsible for vasodilation mediated by H_2S.[6] It is also known to mediate the levels of secondary messengers like calcium and cyclic GMP . Although the functional consequences of these interactions are not clear, it has been suggested that the global intracellular free calcium levels will drop and vasodilation ensues.[7]Another well documented effect of H_2S is protein sulfhydration, which is mediated via the formation of polysulfides. Low-molecular-weight thiols or cysteine residues of target proteins are modified by forming a covalent persulfide.[8]

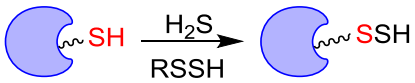


Figure 3: Protein S-sulfhydration

Oxidative stress is defined as the disruption of redox balance in the cells by an increase in equivalents of oxidizing species. In such cases, antioxidant properties of H_2S become important. For example, mitochondrion is the primary source of reactive oxygen species and is most prone to oxidative stress. It was shown that nanomolar concentrations of exogenous H_2S targeted to mitochondria has reversed the downstream effects of this oxidative stress.[4]

The plethora of new and emerging biological roles of H_2S requires improved and reliable methods to generate H_2S within cells.

3.4 H_2S DONORS

H_2S donors are small molecules capable of releasing H_2S inside the cell. There have been reports of various H_2S donors and the following section summarizes the available H_2S donors.

3.4.1 Inorganic Salts

Sodium sulfide (Na_2S) and sodium hydrogen sulfide (NaSH) are the most commonly used H_2S donors. They were used for cellular treatment, to show the protective effects of H_2S. Na_2S was used as an exogenous H_2S donor for long term therapy for attenuation of ischemia-induced heart failure in animal models.[9] It was also observed that during this therapy, there was decrease in oxidative stress associated with the heart failure. Short-term exposure has shown cardioprotective effects.[2] Despite the positive results observed, the rapid release of H_2S and the propensity of Na_2S and NaSH to undergo anaerobic oxidation are major limitations. Concentration of H_2S in between the time of preparation of stock solution and the time of experiment or the biological effect may vary. Hence usage of inorganic salts for cellular studies might not always provide accurate results.

3.4.2 Lawesson's Reagent and GYY4137

2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (Lawesson's reagent) is a sulfurization agent and an organic H_2S donor. It releases H_2S upon

hydrolysis. It was used for investigation of the effects of H_2S on inflammation and ulceration of colon during colitis. After treatment, rat models were known to show a drastic reduction in the severity of colitis. It attenuates the increase in colonic thickness, which occurs during colitis.[10]The results were comparable to the results observed with inorganic salts as donors. But the uncontrolled release and poor water solubility has limited its use.

To overcome poor water solubility, it was modified to give morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate (GYY4137). It releases H_2S, similar to Lawesson's reagent, upon hydrolysis. It was also used for some anti-inflammatory studies. Its ability to release H_2S is pH and temperature dependent, with maximum release under acidic conditions. The amount of H_2S released at physiological conditions was less than 10%, even after 7 days.[4] Therefore, its use is limited as higher yield of H_2S is desirable.

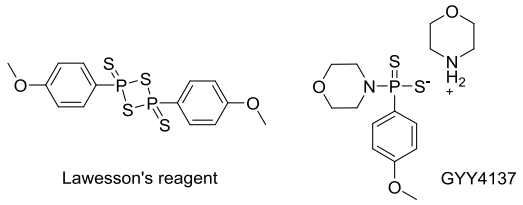


Figure 4: Structure of Lawesson's reagent and GYY4137[2]

3.4.3 1,2-Dithiole-3-thione (DTT) and derivatives

DTTs are known to release H_2S upon hydrolysis in buffers, although the underlying mechanism of release is still a question. DTTs have been used to study H_2S related effects in the alimentary system. However, since the mechanism of release of H_2S is not known, their usage in a more complex system like cells might not be very effective. Also they lack a trigger for a controllable release of H_2S, which is important for biological studies.

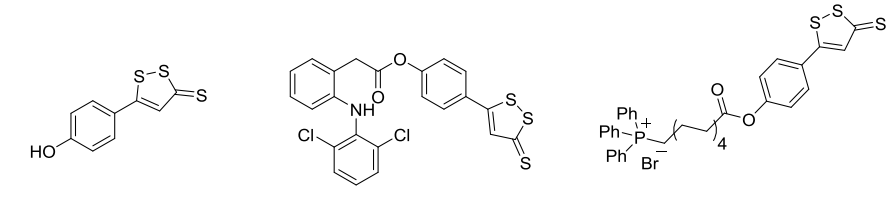


Figure 5: Structures of representative dithiolethione derivatives (Source: Dr. Vinayak Khodade thesis)

3.4.4 Thiol activated H_2S Donors

First thiol activated H_2S donors were reported by Ming Xian and co-workers in 2011.[11] N-mercapto (*N*-SH) was used as the template. These compounds were also acylated at the free SH, to increase their stability in aqueous medium. They exhibited protective effects against myocardial ischemia/reperfusion injury similar to those expected due to H_2S, suggesting their potential therapeutic benefits. But the use thiol as trigger is not very useful because of their ubiquitous nature. For example, thiols are present in both mammalian as well as bacterial cells. So using these compounds for studying antibiotic resistance might not be very effective.

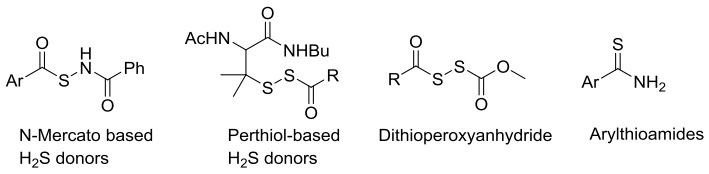


Figure 6: Structures of representative thiol activated donors[11]

3.4.5 Light activated H_2S Donors

Ming Xian and co-worker have reported light activated H_2S, by modifying *gem*-dithiol.[12] *Gem*-dithiols readily hydrolyze in aqueous medium to release H_2S as by-product. To achieve triggerable release of H_2S, the free SH was protected with 2-nitrobenzyl group. Upon irradiation with light at 350 nm, the *gem*-dithiol intermediate forms, and releases H_2S after hydrolysis *in situ*. Since hydrolysis is the mechanism of release of H_2S, the release rates cannot be controlled. Recently Nakagawa and co-workers have reported ketoprofenate based photolabile group, which releases H_2S by eliminating propenylbenzophenone and CO_2, upon irradiation with UV light of 300-360 nm.[13] Although controlled release could be achieved, the following molecules have to be irradiated in UV range, which induces phototoxicity in biological samples and can therefore interfere with the results.

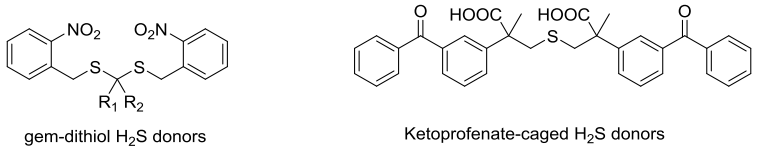


Figure 7: Structures of representative light activated donors[2]

3.4.6 Esterase-activated H_2S donors

Binghe Wang and co-workers have developed H_2S donor,[14] with esterase as the trigger. This prodrug strategy uses the concept of trimethyl lock to achieve fast rates of cyclization to release H_2S. Upon the action of esterase, the ester group cleaves to produce free hydroxyl group, which undergoes rapid lactonization to release H_2S. The modifications in the ester group will help in tuning the release rates. However the ubiquitous nature of esterase does not allow for species-specificity.

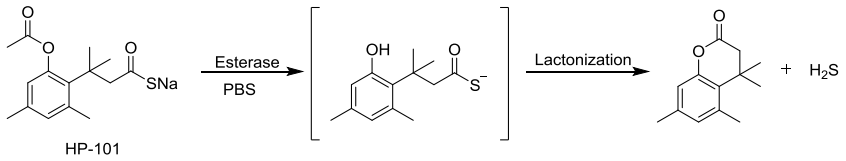


Figure 8A: Mechanism of esterase triggered release of H_2S[14]

Another esterase activated donor, reported from our lab, is pivaloyloxymethyl-based carbonothioates and carbamothioates.[15] In the presence of esterase, a free hydroxyl is formed, which undergoes a self-immolation to release carbonyl sulfide (COS). COS is substrate for an enzyme called carbonic anhydrase, which hydrolyzes it to H_2S.

Figure 8B: Mechanism of esterase triggered release of H_2S via COS[15]

3.5 Design of H_2S donors

H_2S donors discussed above are all well studied and used by researchers for biological uses. But each of them is associated with certain drawbacks. The three main problems associated with H_2S donors are as follows:

1) **Specificity of the trigger** - As shown in **Figure 8**, using esterase as a trigger will not show any species-specificity. To overcome this, Micheal Pluth and co-workers have reported a small molecule H_2S donor with boronate ester as trigger.[16]This molecule selectively gets activated under oxidative stress and releases H_2S.

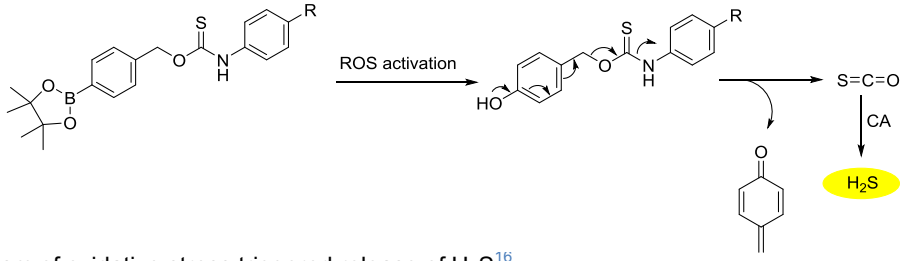


Figure 9: Mechanism of oxidative stress triggered release of H_2S[16]

2) **Cytotoxic byproducts** – As shown in the **Figure 9**, one of the byproduct is quinone methide. This molecule is a DNA alkylating agent, and is known to potentially induce cytotoxicity in cells.
3) **Real time monitoring of H_2S** – Michael Pluth and co-workers have reported a probe for H_2S, which releases a fluorescent molecule, in the presence of H_2S.[18] As shown in **Figure 10,** COS will hydrolyzed to release H_2S. However this is mainly a detection strategy and is less applicable for delivery of H_2S.

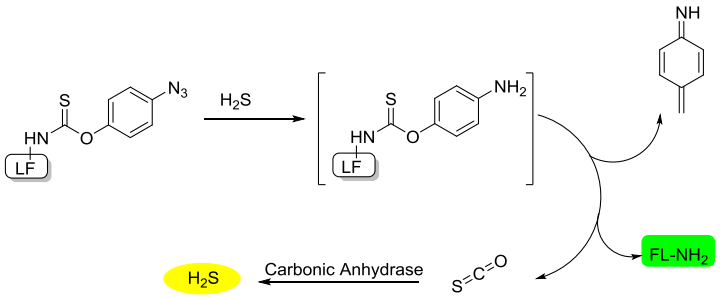


Figure 10: Mechanism of oxidative stress triggered release of H_2S[18]

We have designed a small molecule H_2S donor, which gives direct H_2S release, with concurrent fluorescence signal.

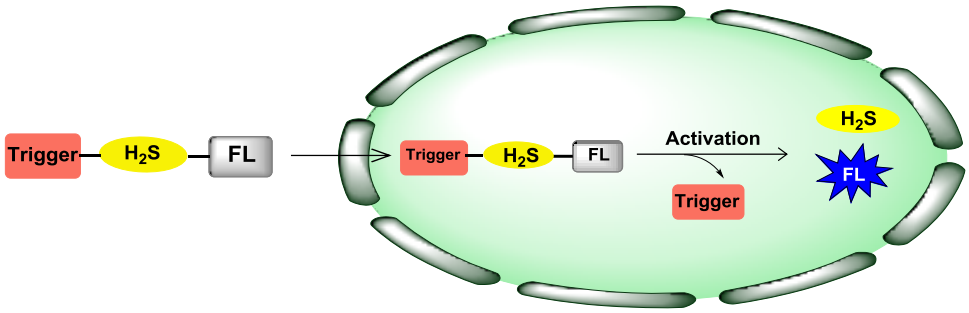


Figure 11: Schematic representation of H_2S release

The motivation for this work comes from two main literature reports.

1) Franz and co-workers have reported a prodrug strategy for release of a metal chelator.[17] In the presence of hydrogen peroxide, boronate ester is reduced to free hydroxyl group, which cyclizes to yield a fluorescence reporter, umbelliferone, with the release of metal chelator.

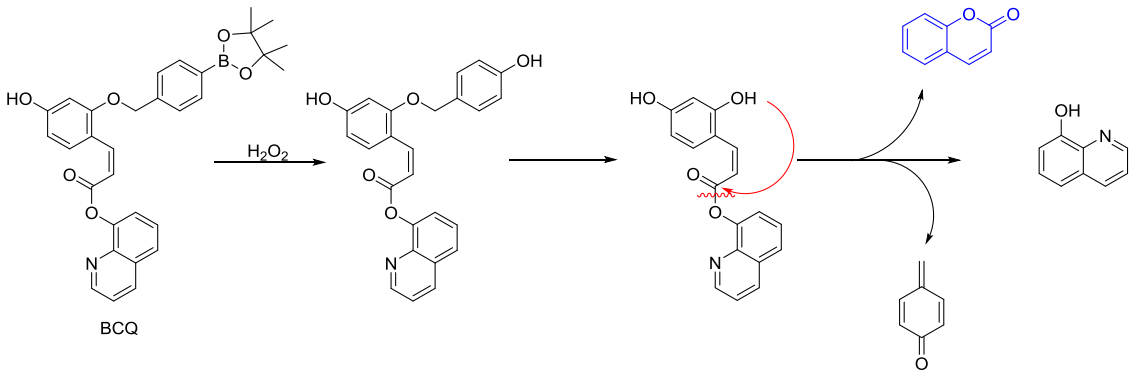


Figure 12: Activation of the fluorogenic prochelator BCQ with hydrogen peroxide[17]

2) As shown in **Figure 12**, if the metal chelator in the above result can be modified to salt of thio acid (**Figure 8A**), it can be used to release H_2S.

In order to achieve the required result, we designed a prototype with nitro-reductase as the enzyme trigger. Upon reduction by NTR, the resultant amine group will undergo self-immolation to release of free hydroxide. As shown in **Figure 12**, lactonization will happen to release H_2S and consequently form a fluorescent reporter.

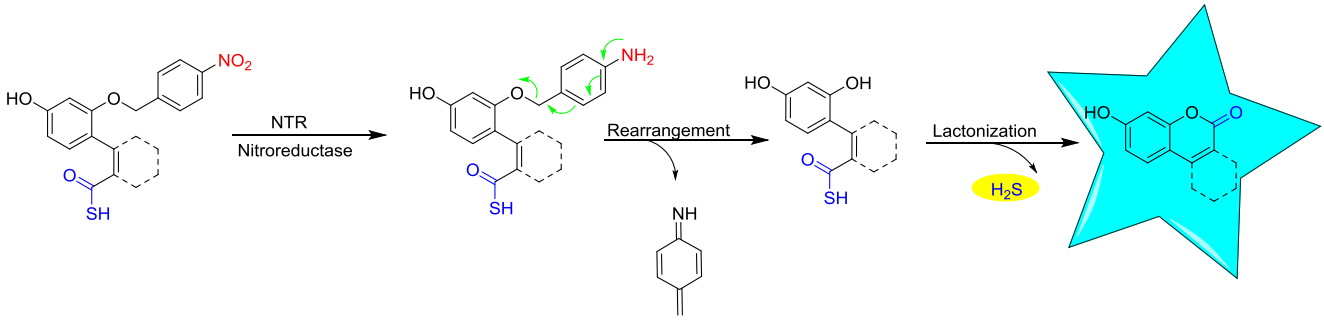


Figure 13: Proposed Experimental Design

Even though the present design does not eliminate the formation of potentially toxic byproducts, the trigger can be modified to address this issue. The free hydroxyl group can be derivatized to achieve targeted delivery of H_2S.

In the following work, attempts have been made study two such fluorescent reporters, umbelliferone and 3-hydroxy-6H-benzo[c]chromen-6-one.

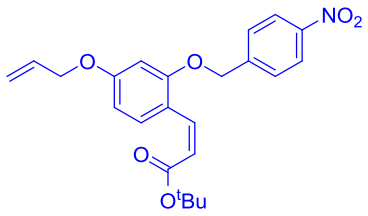
4. MATERIALS AND METHODS

All reactions were conducted under a nitrogen atmosphere. All the chemicals were purchased from commercial sources and used as received unless stated otherwise. Petroleum ether and ethyl acetate (EtOAc) for chromatography were distilled before use. Column chromatography was performed on Rankem silica gel (60–120 mesh). ^{1}H and ^{13}C spectra were recorded on JEOL 400 MHz (or 100 MHz for ^{13}C) spectrometers using either residual solvent signals as an internal standard ($CHCl_3$ δ_H, 7.26 ppm, δ_C 77.2 ppm) or an internal tetramethylsilane (δ_H = 0.00, δ_C = 0.00). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The following abbreviations are used: m (multiplet), s (singlet), d (doublet), t (triplet), dd (doublet of doublet). High-resolution mass spectra (HRMS) were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using NICOLET 6700 FT-IR spectrometer as KBr disc. High performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1260 infinity with Eclipse plus C-18 reversed phase column (250 mm × 4.6 mm, 5µm). Fluorescence and photometric measurements were performed using a Thermo Scientific Varioscan microwell plate reader.

4.1 Synthesis and Characterization

Compounds **1**[17], **5**[19], **6**[17] were prepared according to reported procedures and analytical data are consistent with reported values.

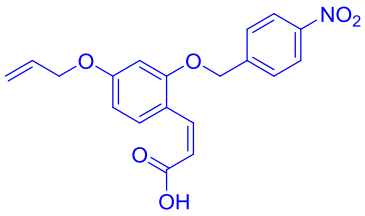
tert-butyl (Z)-3-(4-(allyloxy)-2-((4-nitrobenzyl)oxy)phenyl)acrylate (2)[17]



To an ice cold solution of **1** (100 mg, 0.49 mmol) in anhydrous DMF (5 mL), potassium tertiary butoxide (0.6 mL) was added dropwise for 5 min under N_2 atmosphere. As the reaction turned orange, para-nitrobenzylbromide was added rapidly. After stirring for 15 min, ice bath was removed and reaction was allowed to stir at room temperature for 1 h. After the consumption of starting material, as monitored by TLC, the solvent was evaporated under reduced pressure, diluted with 10 mL of water and the aqueous

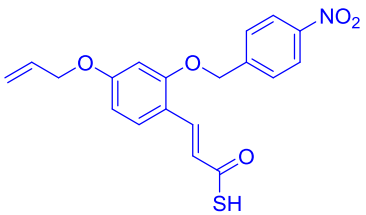
solution was extracted with EtOAc (3×5 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 (5 g), filtered and the filtrate was concentrated to give a crude compound This crude was further purified by column chromatography with EtOAc/hexane as the eluent, to obtain **2** as a yellow solid (6 mg, 2%). ^{1}H NMR (400MHz, $CDCl_3$): δ 8.24 (d, *J* = 6.9 Hz, 2H), 7.63 (d, *J* = 8.6 Hz, 1H), 7.58 (d, *J* = 8.6 Hz, 1H), 7.07 (d, *J* = 12.4 Hz, 1H), 6.53 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.45 (d, *J* = 2.3 Hz, 1H), 6.06-5.97 (m, 1H), 5.85 (d, *J* = 12.4 Hz, 1H), 5.39 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.58 (dq, *J* = 10.5, 1.0 Hz, 1H), 5.16 (s, 2H), 4.52 (td, *J* = 5.3, 1.5 Hz, 2H), 1.42 (s, 9H); ^{13}C NMR (100MHz, $CDCl_3$): δ 166.0, 160.6, 157.0, 147.7, 144.3, 137.0, 133.0, 132.2, 127.6, 127.5, 124.4, 124.0, 120.8, 118.1, 117.9, 105.8, 100.2, 80.4, 69.1, 28.2; HRMS (ESI) for $C_{23}H_{25}NO_6$ $[M+H]^+$: Calcd., 411.1682, Found., 411.1688.

(Z)-3-(4-(allyloxy)-2-((4-nitrobenzyl)oxy)phenyl)acrylic acid (3)[17]



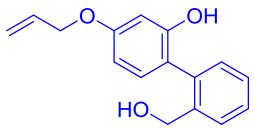
To a round bottom flask charged with **2** (200 mg, 0.48 mmol), formic acid (5 mL) was added. The reaction was stirred rigorously, until the heterogeneous mixture started to dissolve. Formic acid was removed under vacuum to give **3** as a yellow solid (153 mg, 88%). ^{1}H NMR (400MHz, $CDCl_3$): δ 8.24 (td, *J* = 8.7, 2.4 Hz, 2H), 7.07 (d, *J* = 8.6 Hz, 1H), 7.59 (td, *J* = 8.5, 2.7 Hz, 2H), 7.27 (d, *J* = 12.0 Hz, 1H), 6.52 (dd, *J* = 7.9, 2.3 Hz, 1H), 6.45 (d, *J* = 2.3 Hz, 1H), 6.07-5.97 (m, 1H), 5.91 (d, *J* = 12.5 Hz, 1H), 5.39 (dq, *J* = 17.4, 1.5 Hz, 1H), 5.29 (dq, *J* = 10.5, 1.4 Hz, 1H), 5.16 (s, 2H), 4.53 (td, *J* = 5.3, 1.5 Hz, 2H); ^{13}C NMR (100MHz, $CDCl_3$): δ 191.7, 161.2, 157.3, 147.8, 144.0, 143.8, 132.9, 132.7, 127.7, 124.0, 118.2, 117.0, 105.9, 100.1, 69.1; HRMS (ESI) for $C_{19}H_{17}NO_6$ $[M+H]^+$: Calcd., 355.1056 Found., 355.1056.

(E)-3-(4-(allyloxy)-2-((4-nitrobenzyl)oxy)phenyl)prop-2-enethioic S-acid (4)[14]



3 (70 mg, 0.19 mmol) was dissolved in 3 mL of DCM and charged in a microwave tube, followed by addition of Lawesson's reagent (40 mg, 0.09 mmol). The mixture was heated in a microwave at 100 °C for 6 min. The solvent was evaporated under reduced pressure, diluted with 10 mL of water and the aqueous solution was extracted with DCM (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 purified using flash C18 column chromatography, with methanol/water as the eluent, to give **4** as a yellow solid (32 mg, 47%).^{1}H NMR (400MHz, $CDCl_3$): δ 8.26 (dt, *J* = 8.2, 2.2 Hz, 2H), 8.05 (d, *J* = 15.8 Hz, 1H), 7.61 (d, *J* = 8.8 Hz, 2H), 7.52 (d, *J* = 8.6 Hz, 1H), 6.91 (d, *J* = 15.8 Hz, 1H), 6.57 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.47 (d, *J* = 2.2 Hz, 1H), 6.06-5.97 (m, 1H), 5.39 (dt, *J* = 17.2, 1.4 Hz, 1H), 5.31 (dq, *J* = 10.4, 1.3 Hz, 1H), 5.23 (s, 2H), 4.55 (td, *J* = 5.3, 1.6 Hz, 2H); ^{13}C NMR (100MHz, $CDCl_3$): δ 184.8, 162.7, 159.0, 147.9, 143.5, 139.0, 132.5, 131.4, 127.8, 124.2, 120.5, 118.5, 116.4, 107.3, 100.7, 69.4, 69.2; HRMS (ESI) for $C_{19}H_{17}NO_6S$ $[M+H]^+$: Calcd., 371.0827 Found., 371.0826.

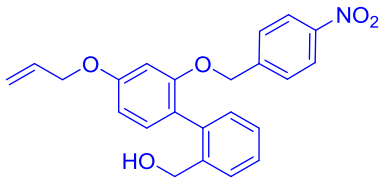
4-(allyloxy)-2'-(hydroxymethyl)-[1,1'-biphenyl]-2-ol (7)[20]



A solution of **6** (200 mg, 0.79 mmol) dissolved in anhydrous THF, was treated with lithium aluminum anhydride (90 mg, 2.38 mmol) at 0 °C. After stirring for 10 min, 1N HCl was added drop wise into the reaction, at 0 °C while stirring. Once the evolution of H_2 gas subsided, the reaction mixture was diluted with 10 mL of 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 give **7** as a white solid (200 mg, 60%). FTIR (ν, cm^{-1}): 1172, 1289, 1616, 1718, 2920, 3261; ^{1}H NMR (400MHz, $CDCl_3$): δ 7.52-7.50 (m, 1H), 7.42-7.36 (m, 2H), 7.25-2.22 (m, 1H), 7.00 (dd, *J* = 7.8, 0.8 Hz, 1H), 6.56 (s, 1H), 6.58 (d, *J* = 2.5 Hz, 1H), 6.12-6.02 (m, 1H), 5.45 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.30 (dq, *J* = 10.4, 1.4 Hz, 1H), 4.54 (td, *J* = 5.3, 1.5 Hz, 2H), 4.49 (s, 2H); ^{13}C NMR (100MHz, $CDCl_3$): δ

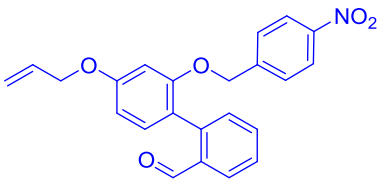
159.7, 154.0, 139.2, 136.5, 133.3, 131.5, 131.4, 129.4, 128.7, 128.6, 120.5, 118.0, 107.7, 102.9, 69.0, 63.9; HRMS (ESI) for $C_{16}H_{16}O_3$ $[M+H]^+$: Calcd., 257.1133 Found., 257.1177.

(4'-(allyloxy)-2'-((4-nitrobenzyl)oxy)-[1,1'-biphenyl]-2-yl)methanol (8)[16]



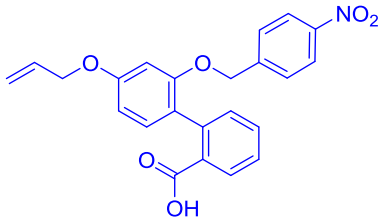
A solution of **7** (2.5 g, 9.75 mmol) and potassium carbonate (1.9 g, 13.1 mmol) dissolved in 10 mL of acetone, was treated with para-nitrobenzylbromide (2.54 g, 11.70 mmol). The reaction was refluxed at 65 °C until the starting material was completely consumed, as monitored by TLC. The solvent was evaporated under reduced pressure, diluted with 10 mL of water and the aqueous solution was 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 to give a crude compound. This crude was further purified by column chromatography with EtOAc/hexane as the eluent, to give **8** as a yellow solid (2.2 g, 57%). FTIR (ν, cm^{-1}): 1173, 1300, 1344, 1456, 1516, 1606, 2920, 3375; ^{1}H NMR (400MHz, $CDCl_3$): δ 8.11 (d, *J* = 8.7 Hz, 2H), 7.55 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.41 (td, *J* = 6.8, 1.5 Hz, 1H), 7.35 (td, *J* = 7.4, 1.52 Hz, 1H), 7.26-7.19 (m, 3H), 7.13 (d, *J* = 8.2 Hz, 1H), 6.64 (dd, *J* = 8.3, 2.4 Hz, 1H), 6.59 (d, *J* = 2.4 Hz, 1H), 6.11-6.02 (m, 1H), 5.45 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.31 (dd, *J* = 10.5, 1.4 Hz, 1H), 5.06 (d, *J* = 5.5 Hz, 2H), 4.56 (dd, *J* = 5.2, 1.5 Hz, 2H), 4.47 (s, 2H); ^{13}C NMR (100MHz, $CDCl_3$): δ 159.6, 156.0, 147.6, 144.0, 139.7, 137.0, 133.0, 131.8, 130.7, 128.4, 128.0, 127.7, 127.3, 123.9, 123.5, 118.1, 107.0, 101.9, 69.8, 69.2, 63.8; HRMS (ESI) for $C_{23}H_{21}NO_5$$[M+H]^+$: Calcd., 391.1420 Found., 391.1423.

4'-(allyloxy)-2'-((4-nitrobenzyl)oxy)-[1,1'-biphenyl]-2-carbaldehyde (9)[21]



Periodic acid (2.8 g, 7.4 mmol) was added in 10 mL of ACN and stirred rigorously for 15 min. **8** (5.1 g, 22.5 mmol) was dissolved in 10 mL of DCM and added to periodic acid solution while stirring, followed by addition of pyridinium chlorochromate (30 mg, 0.14 mmol). After the completion of the reaction, as monitored by TLC, the solvent was evaporated under reduced pressure, diluted with 10 mL of water and the aqueous solution was 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 to give a crude compound This crude was further purified by column chromatography with EtOAc/hexane as the eluent, to give **9** as a yellow solid (1.95 g, 68%). FTIR (ν, cm^{-1}): 1174, 1301, 1345, 1517, 1602, 1691, 2921, 3064; ^{1}H NMR (400MHz, $CDCl_3$): δ 9.83 (s, 1H), 8.09 (d, *J* = 8.8 Hz, 2H), 7.95 (dd, *J* = 7.7, 1.3 Hz, 1H), 7.60 (td, *J* = 7.5, 1.5 Hz, 1H), 7.45 (t, *J* = 7.0 Hz, 1H), 7.32 (d,*J* = 7.3 Hz, 1H), 7.26-7.19 (m, 3H), 6.64 (dd, *J* = 8.4, 2.3 Hz, 1H), 6.55 (d, *J* = 2.3 Hz, 1H), 6.08-5.98 (m, 1H), 5.40 (dq, *J* = 17.3, 1.4 Hz, 1H), 5.29 (dq, *J* = 10.5, 1.3 Hz, 1H), 5.04 (d, *J* = 9.1 Hz, 2H), 4.54 (td, *J* = 5.5, 1.8 Hz, 2H), 4.47 (s, 2H); ^{13}C NMR (100MHz, $CDCl_3$): δ 192.9, 160.3, 156.2, 147.6, 143.8, 141.5, 134.4, 133.8, 133.0, 132.3, 131.5, 127.9, 127.3, 127.0, 123.9, 118.3, 106.9, 101.0, 69.2, 69.1; HRMS (ESI) for $C_{23}H_{19}NO_5[M+H]^+$: Calcd., 390.1341 Found., 390.1346.

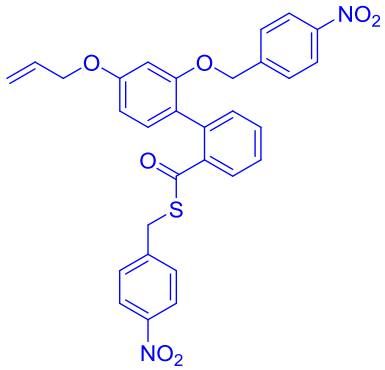
4'-(allyloxy)-2'-((4-nitrobenzyl)oxy)-[1,1'-biphenyl]-2-carboxylic acid (10)[22]



9 (1.3 g, 3.3 mmol) was dissolved in 5 mL of ACN. To the stirring solution of **9** monopotassium phosphate (0.1 g, 0.75 mmol) was added. The reaction mixture was cooled to 0 °C and was treated H_2O_2 (30% w/w) (2.15 mL, 70 mmol). After stirring for 10 min, sodium chlorite (0.66 g, 7.35 mmol) was added at 0 °C. The reaction was allowed to come to room temperature and stirred for 7 h. After completion of reaction, as monitored by TLC, it was quenched with saturated sodium metabisulfite solution. This was diluted with 10 mL of water and the aqueous solution was extracted with EtOAc

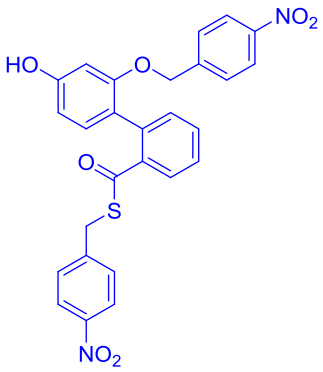
(3×15 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 (5 g), filtered and the filtrate was concentrated to give **10** as a yellow solid. (0.58 g, 42%). FTIR (ν, cm^{-1}): 1175, 1299, 1346, 1518, 1605, 1690, 3073; ^1H NMR (400MHz, $CDCl_3$): δ 8.07 (d, *J* = 8.5 Hz, 2H), 7.96 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.59 (td, *J* = 7.5, 1.4 Hz, 1H), 7.58 (td, *J* = 7.6, 1.3 Hz, 1H), 7.33-7.28 (m, 3H), 7.19 (d, *J* = 8.3 Hz, 1H), 6.61 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.46 (d, *J* = 2.3 Hz, 1H), 6.10-6.00 (m, 1H), 5.41 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.29 (dq, *J* = 10.4, 1.4 Hz, 1H), 5.00 (s, 2H), 4.53 (td, *J* = 5.4, 1.5 Hz, 2H); ^{13}C NMR (100MHz, $CDCl_3$): δ 173.1, 159.6, 155.8, 147.4, 144.2, 139.2, 133.1, 132.7, 132.0, 130.6, 127.3, 124.1, 123.7, 118.1, 106.2, 101.1, 69.3, 69.1; HRMS (ESI) for $C_{23}H_{19}NO_6[M+H]^+$: Calcd., 406.1290 Found., 406.1295.

S-(4-nitrobenzyl) 4'-(allyloxy)-2'-((4-nitrobenzyl)oxy)-[1,1'-biphenyl]-2-carbothioate (11)[23]



To a stirring solution of **10** (260 mg, 0.64 mmol) in anhydrous DCM, DMAP (40 mg, 0.64 mmol) and DCC (158 mg, 0.76 mmol) were added under N_2 atmosphere. After stirring for 5 min, the reaction mixture was treated with para-nitrobenzylthiol (110 mg, 0.64 mmol). After the completion of reaction, as monitored by TLC, the solvent was evaporated under reduced pressure, diluted with 10 mL of 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 yellow solid **11** (200 mg, 56%). Since the product showed no major impurities, it was carried out to next step, without further purification.

S-(4-nitrobenzyl) 4'-hydroxy-2'-((4-nitrobenzyl)oxy)-[1,1'-biphenyl]-2-carbothioate (12)[16]



11 (150 mg, 0.26 mmol) and tetrakis (triphenylphosphine) palladium (0) (100 mg, 0.08 mmol) were dissolved in anhydrous DCM, under N_2 atmosphere. Phenyl silane (70 µL, 0.55 mmol) was added while stirring, under the inert atmosphere. The reaction was stirred until brown colored turbid solution formed. The solvent was evaporated under reduced pressure, diluted with 5 mL of water and the aqueous solution was extracted with EtOAc (3×10 mL). The combined organic layer was washed with brine, dried over dried over Na_2SO_4 (5 g), filtered and the filtrate was concentrated to give a crude compound. This crude was initially purified by silica gel column chromatography using EtOAc/hexane as the eluent. The resulting mixture was further purified using preparative HPLC with C-18 preparative column (21.5 mm × 250 mm, 10 µm; kromasil C18), using a gradient of ACN and water (60 – 80 %), under ambient temperature with a flow rate of 20 mL/min to obtain **12** as a yellow solid (87 mg, 62%). ^{1}H NMR (400MHz, DMSO): δ 9.63 (s, 1H), 8.11 (d, *J* = 8.8 Hz, 2H), 8.09 (d, *J* = 8.6 Hz, 2H), 7.69 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.61 (td, *J* = 7.5, 1.4 Hz, 1H), 7.47-7.42 (m, 3H), 7.40-7.34 (m, 3H), 6.96 (d, *J* = 8.1 Hz, 1H), 6.43 (dd, *J* = 8.1, 2.2 Hz, 1H), 6.40 (d, *J* = 2.2 Hz, 1H), 5.02 (s, 2H), 4.24 (s, 2H); ^{13}C NMR (100MHz, DMSO): δ 192.3, 158.7, 155.5, 146.7, 146.4, 146.3, 145.0, 137.8, 136.7, 131.2, 130.0, 127.4, 127.4,127.3, 123.4, 123.2, 119.8, 107.9, 100.5, 68.1, 32.0; HRMS (ESI) for $C_{27}H_{20}N_2O_7S$ $[M+H]^+$: Calcd., 516.0991 Found., 516.0991.

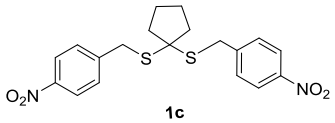
4.2 Zinc-mediated chemoreduction

Zinc mediated chemoreduction was performed according the procedure used by my colleague Dr. Kavita Sharma. A solution of the test compound (10 µL, 10 mM stock) was diluted in a water-methanol system (1:1, 980 µL), so that the final concentration of compound in the solution was 100 µM. To this 10 µL of ammonium formate (10 mM

stock in deionized water) and zinc powder (12 mg) were added and incubated at 37 ºC. At different time points, 200 µL of reaction mixture was taken out, centrifuged and the supernatant was filtered (200 µm). It was injected into the Agilent Technologies HPLC (C-18 reversed phase column, 250 mm × 4.6 mm, 5 mm; flow rate: 1 mL/min; eluent: 70% ACN/ H_2O).

4.3 BODIPY azide based study

Reaction mixture was prepared by adding **12** (4 µL, 2.5 mM), NADPH (10 µL, 10 mM), NTR (4 µL) in 172 µL of HEPES buffer (pH 7.4). The reaction mixture was incubated at 37 ºC for 15 min. Then 10 µL of **31a** was added and incubated for another 15 min. Reaction mixture was transferred into the 96-well plate and checked for fluorescence signal at excitation and emission wavelengths of 444 nm and 520 nm respectively. Compound **1c** was used as the positive control. Data represented here is an average of 3 independent experiments.

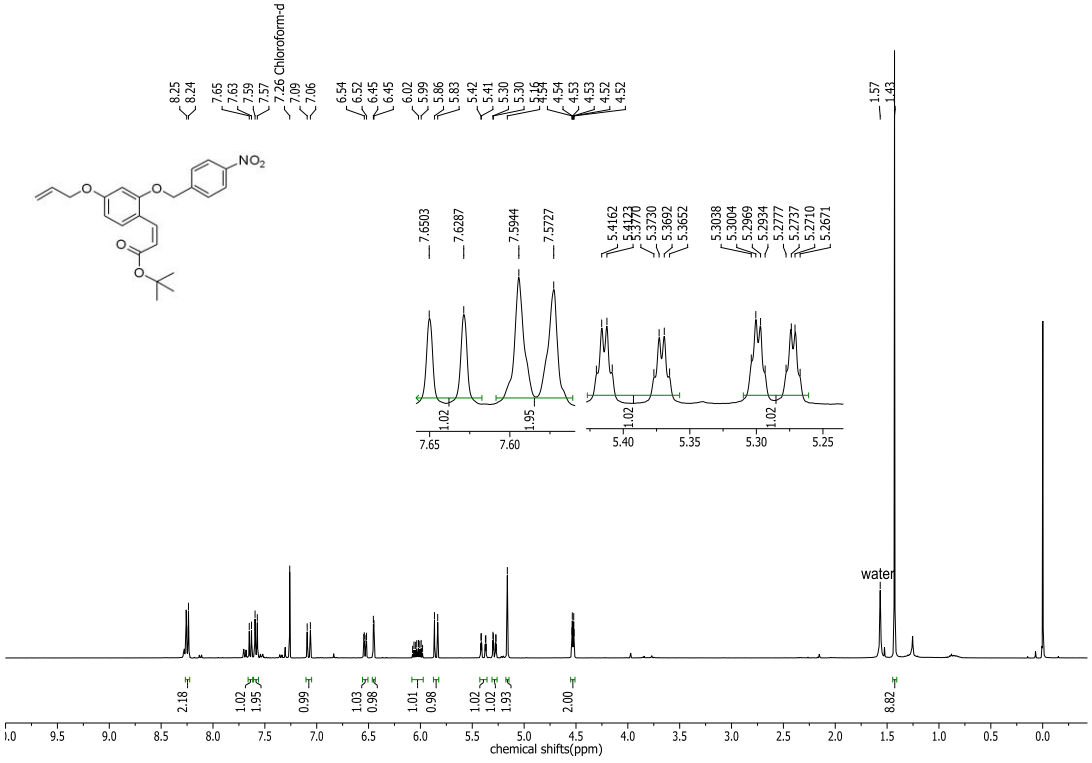


1c

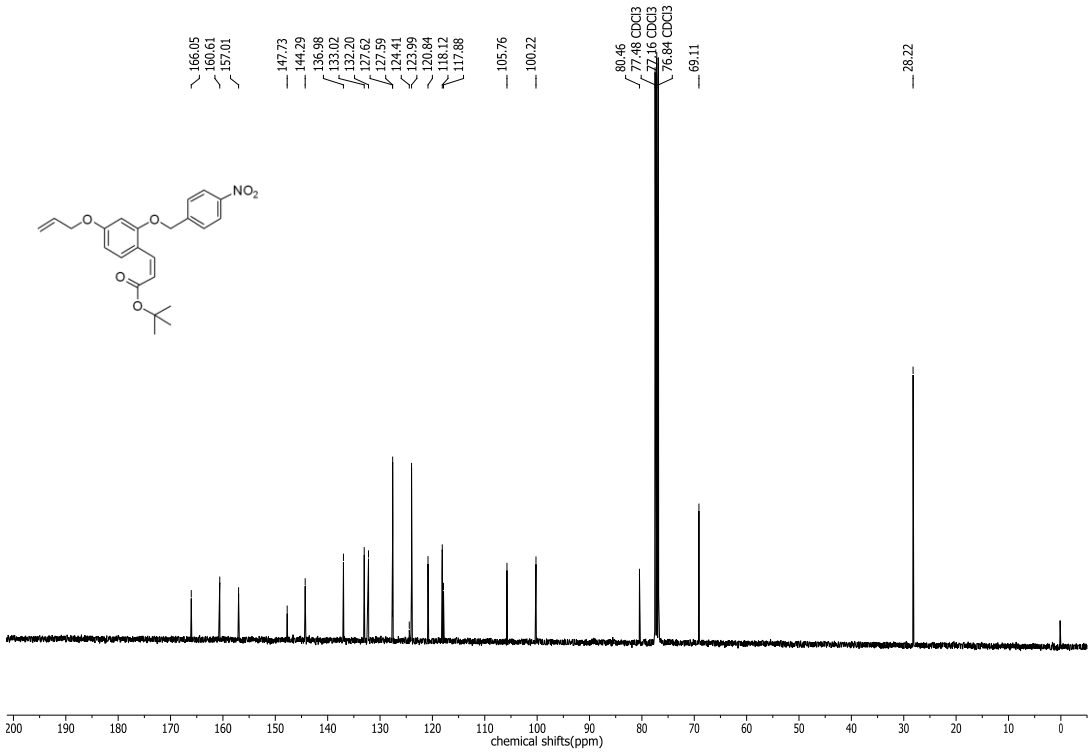
Figure 13: Positive control for BODIPY azide study

4.4 NMR Spectral Charts

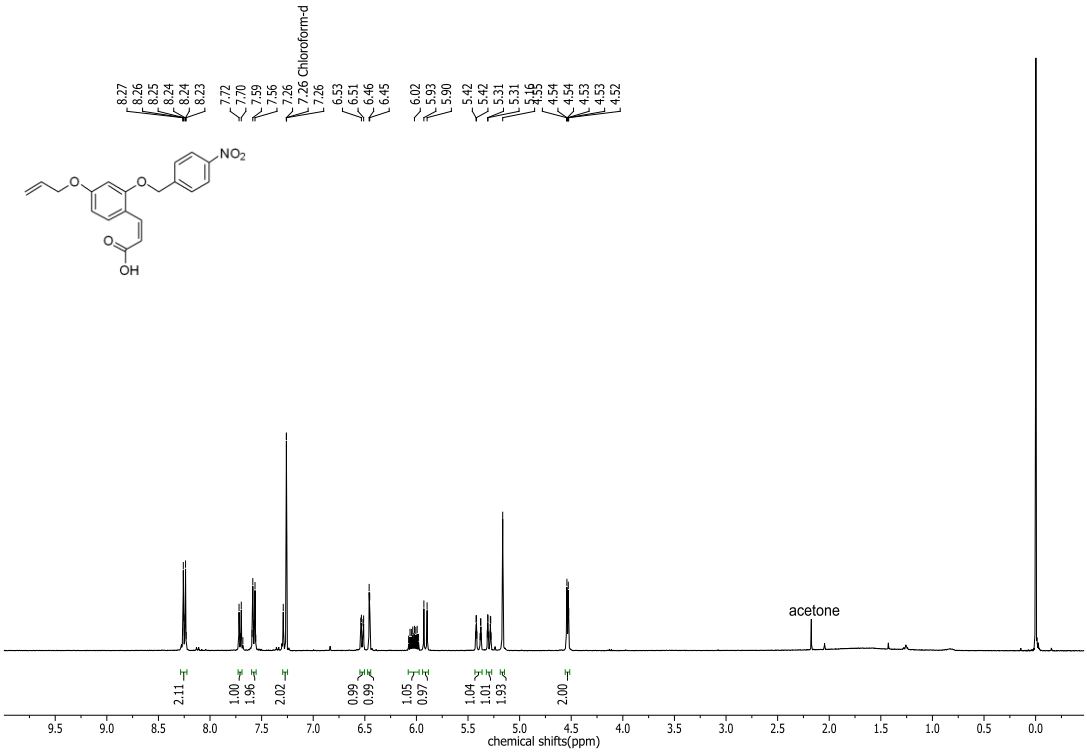
^{1}H NMR Spectrum of **2**



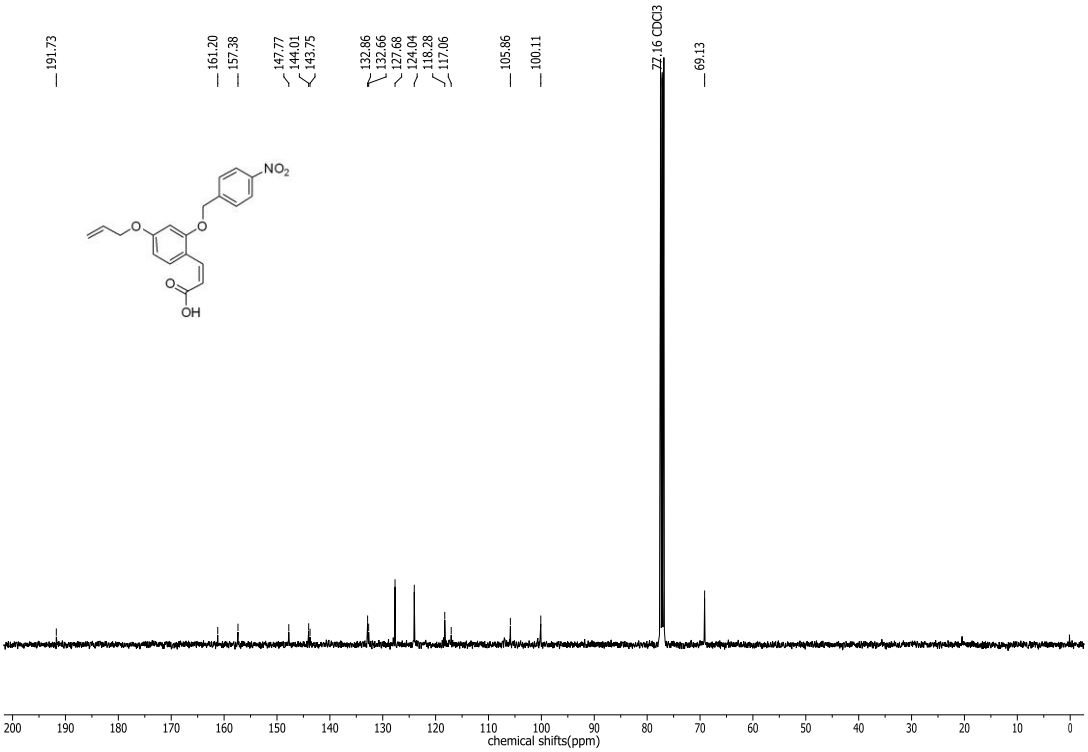
^{13}C NMR Spectrum of **2**



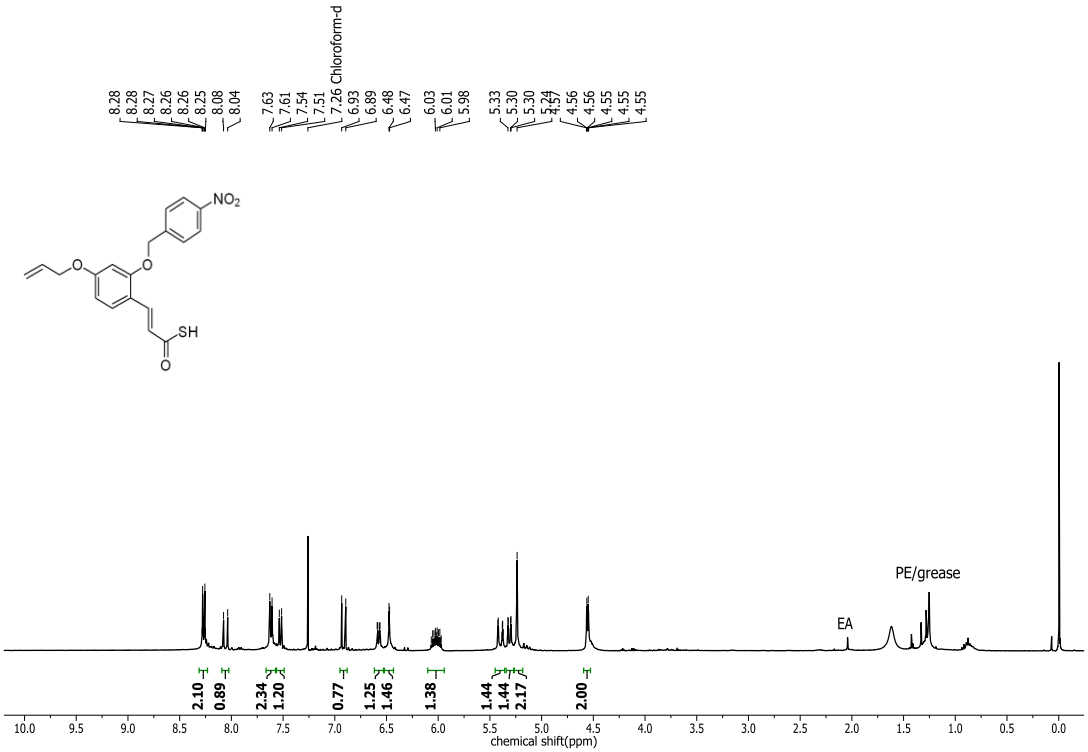
^{1}H NMR Spectrum of **3**



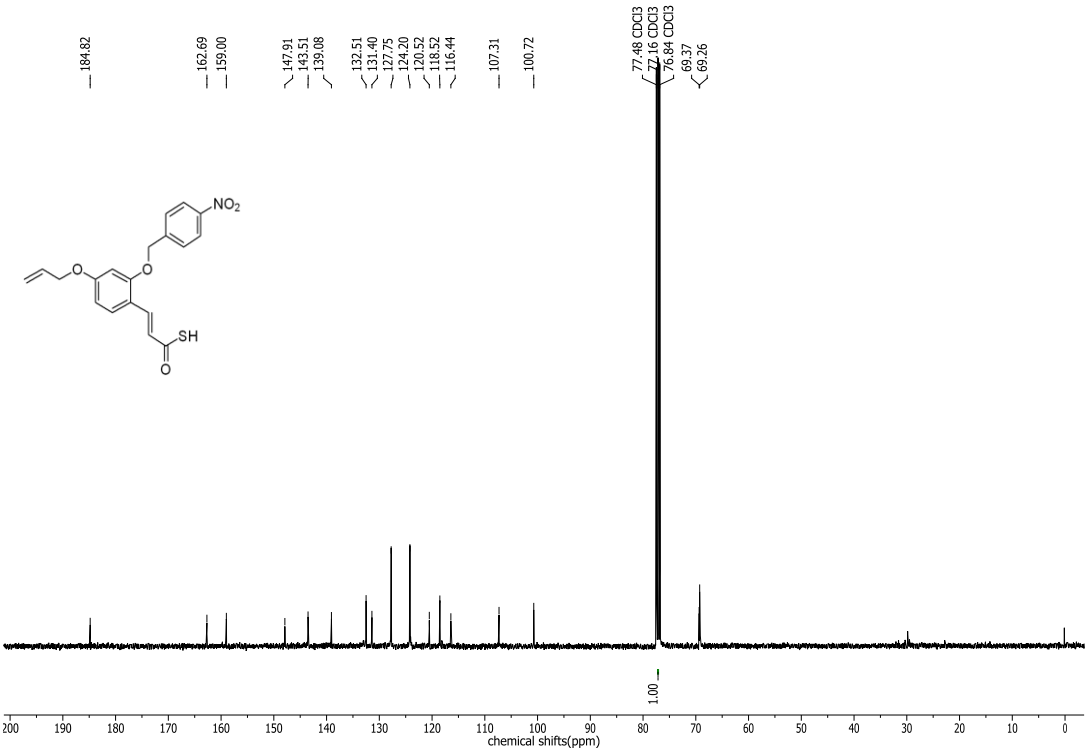
^{13}C NMR Spectrum of **3**



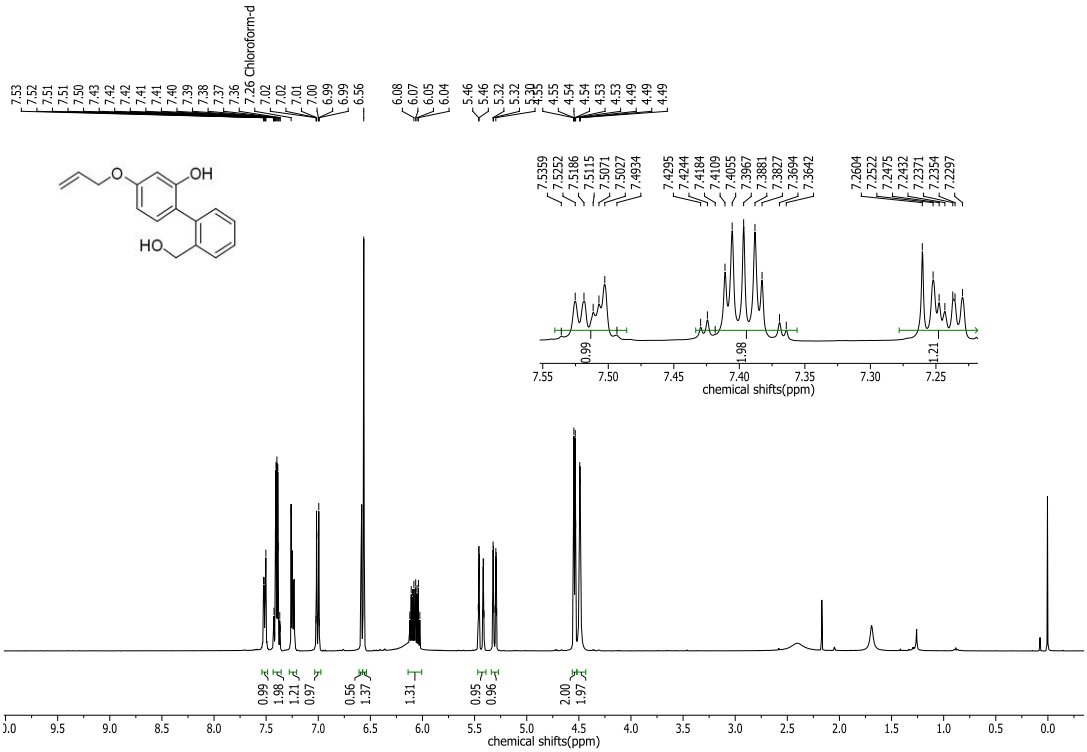
^{1}H NMR Spectrum of **4**



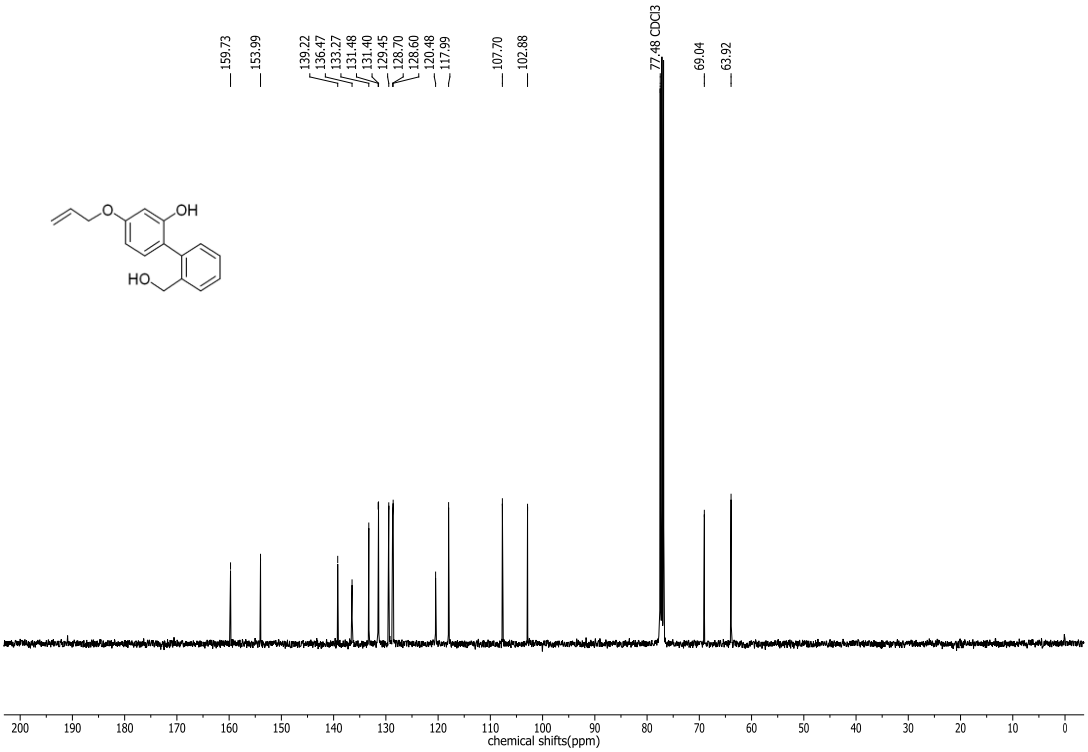
^{13}C NMR Spectrum of **4**



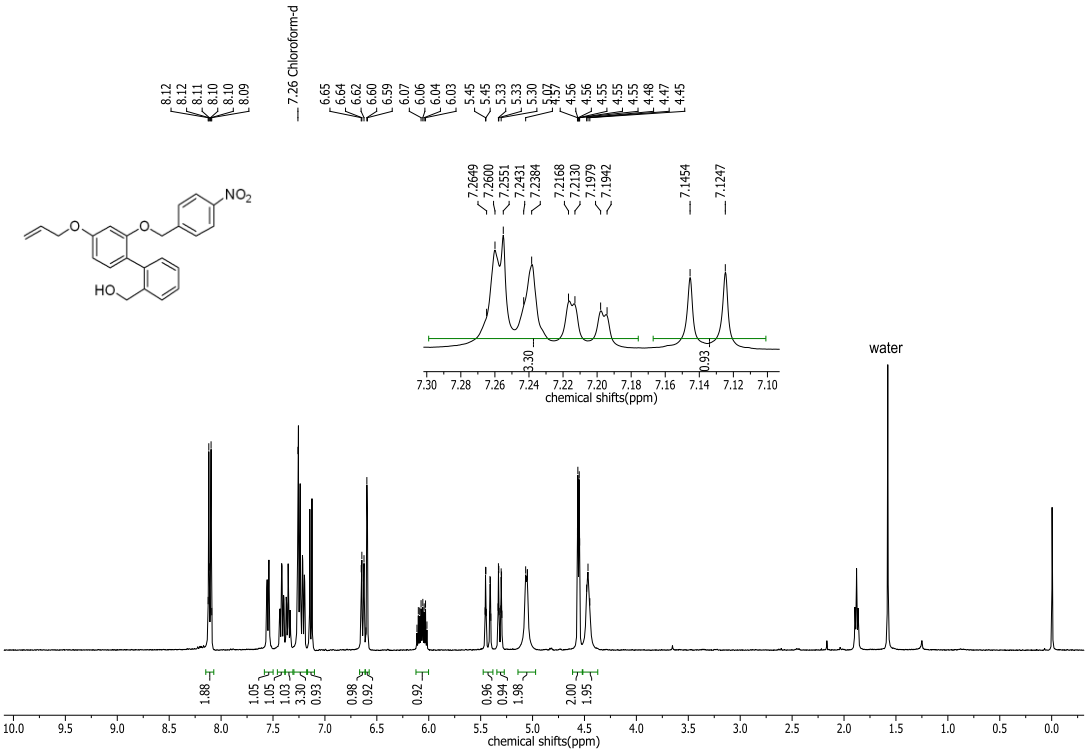
^{1}H NMR Spectrum of **7**



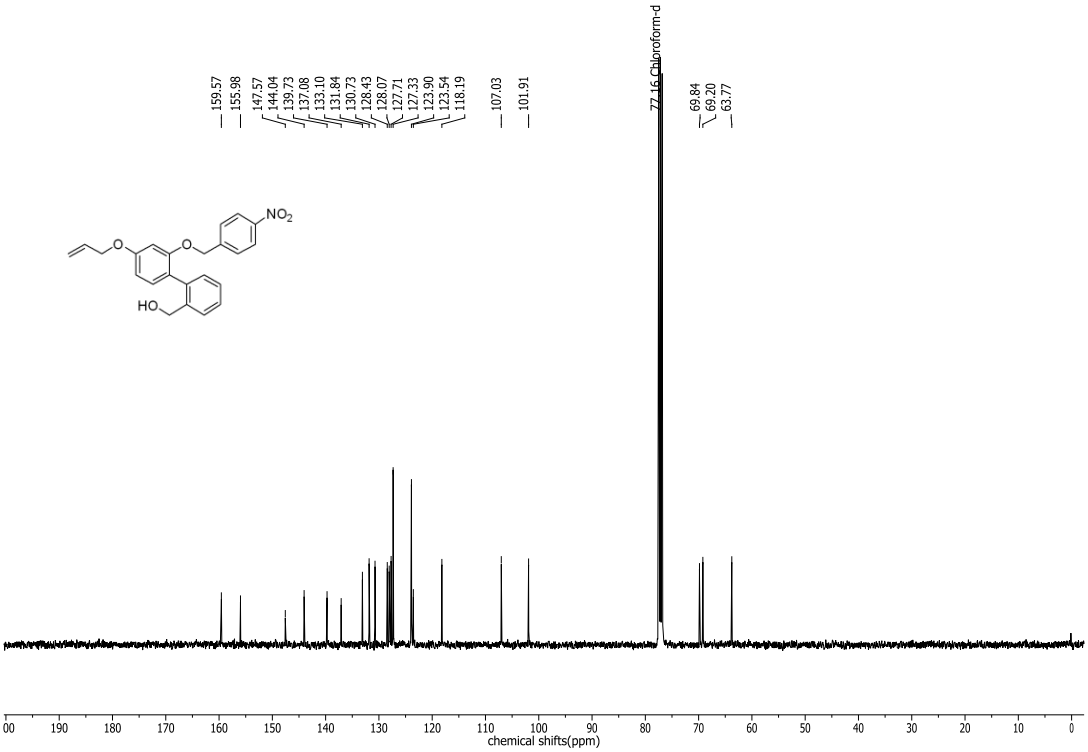
^{13}C NMR Spectrum of **7**



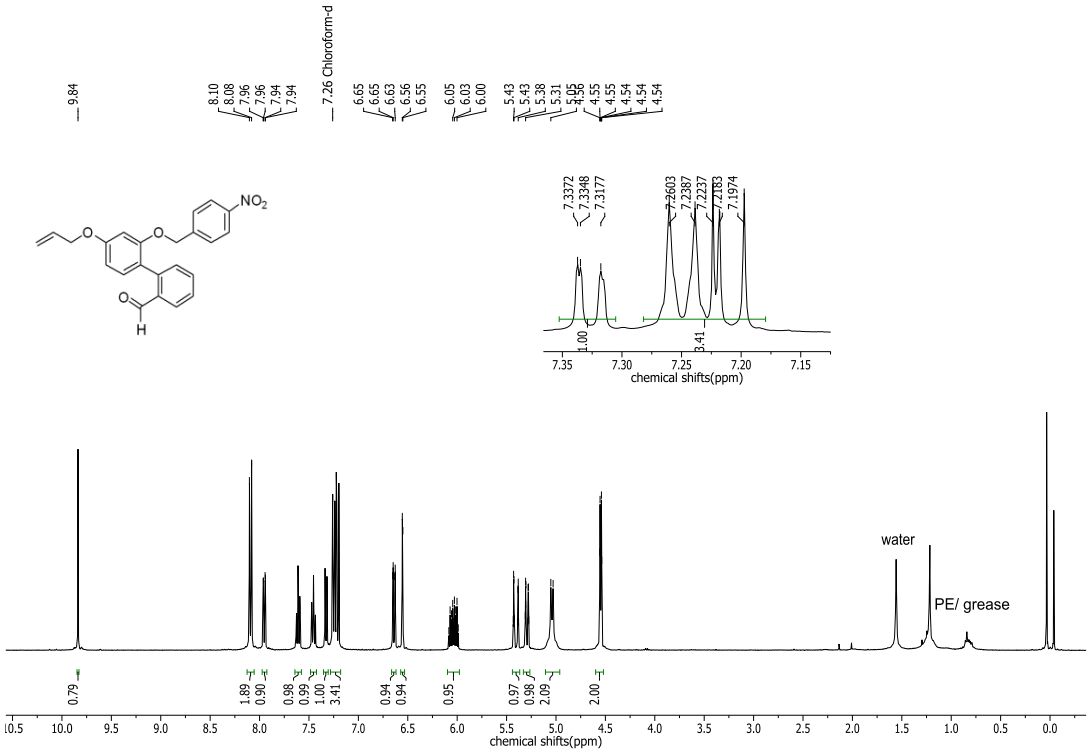
^{1}H NMR Spectrum of **8**



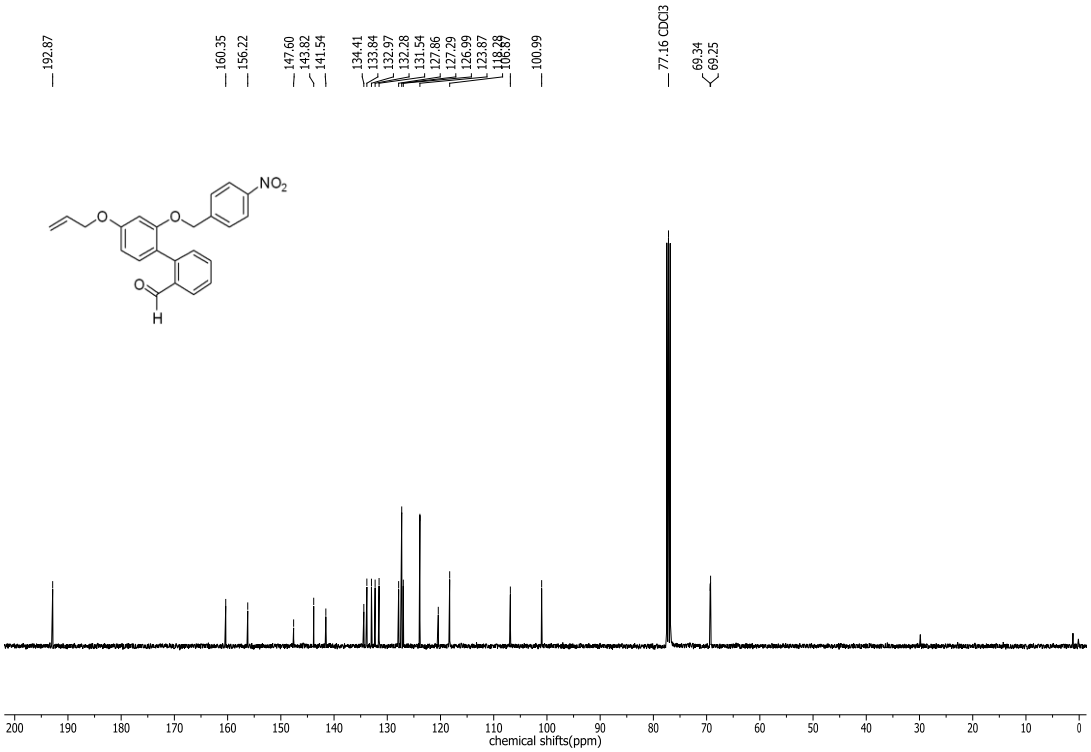
^{13}C NMR Spectrum of **8**



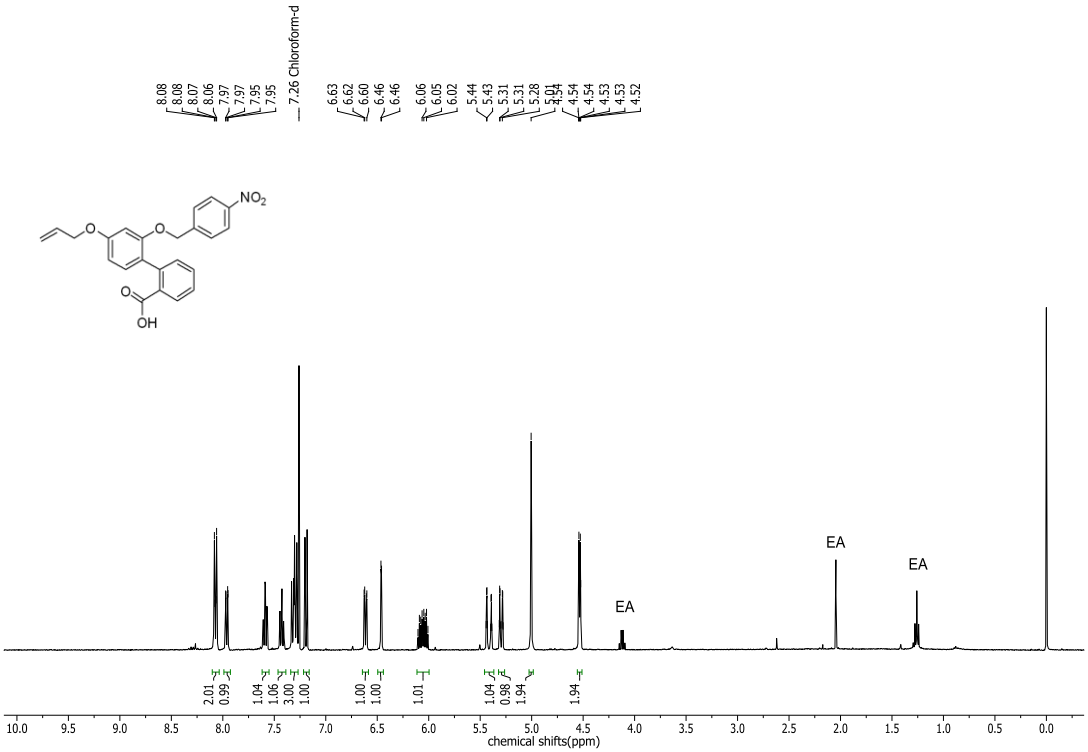
^{1}H NMR Spectrum of **9**



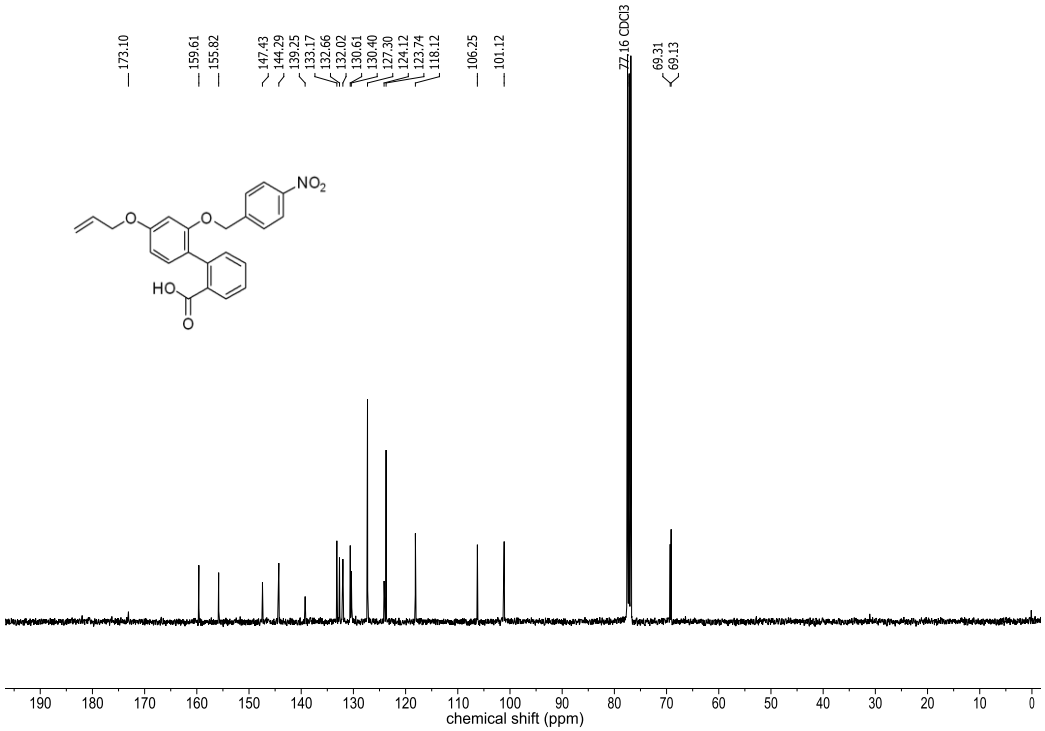
^{13}C NMR Spectrum of **9**



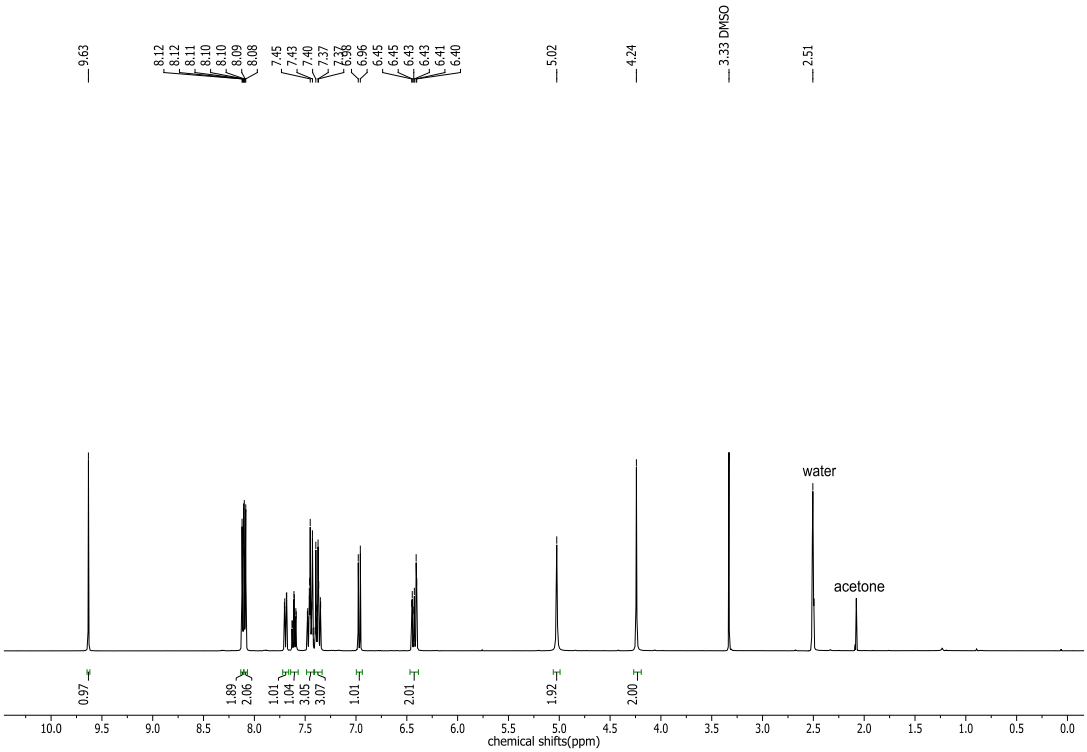
^{1}H NMR Spectrum of **10**



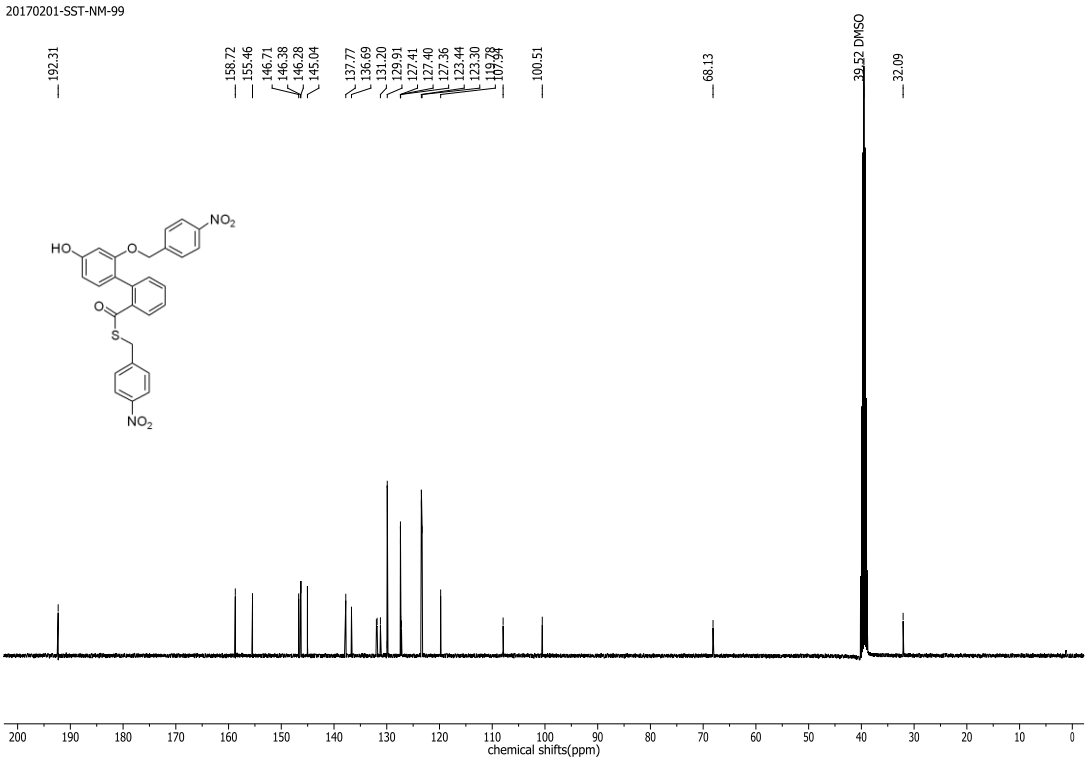
^{13}C NMR Spectrum of **10**



^{1}H NMR Spectrum of **12**



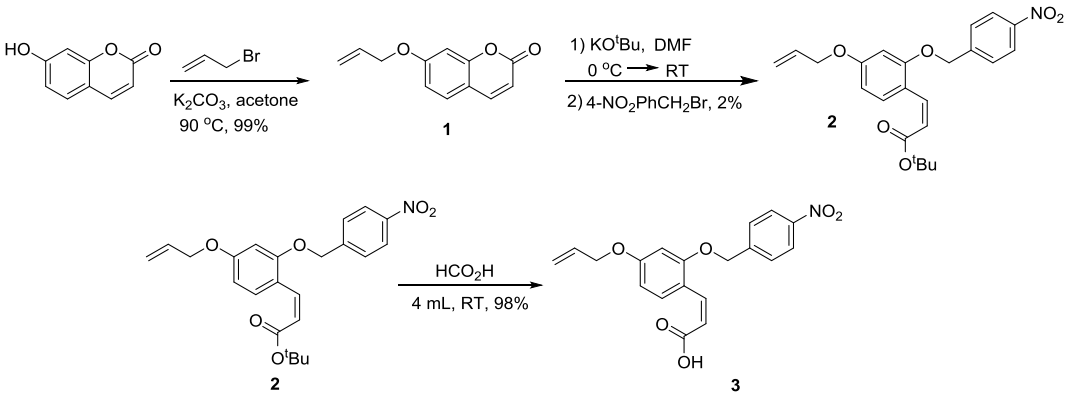
^{13}C NMR Spectrum of **12**



5. RESULTS AND DISCUSSION

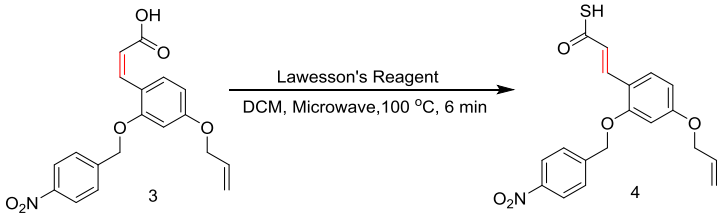
5.1 Umbelliferone as fluorescence reporter

Compound **4** was prepared in 4 steps, starting from commercially available 7-hydroxy-2H-chromen-2-one (umbelliferone). The phenol was reacted with allyl bromide to achieve **1** in quantitative yield. Following this, the lactone ring was opened up using potassium tertiary butoxide and simultaneous protection of phenolic ion with para-nitrobenzylbromide, to give **2**. Tertiary butyl group was deprotected using formic acid to give **3**.



SCHEME 1

According to the proposed **SCHEME 2**, we would expect the formation of a *cis* thio acid. However, we observed the formation of a *trans* thio acid **4**, which was inferred by the drastic difference in the coupling constants of **3** and **4**, as observed in ^{1}H NMR spectra



SCHEME 2

A coupling constant of 12.5 Hz, corresponding to a *cis* configuration was observed in case of the starting material **3**. The product showed a coupling constant of 15.8 Hz, corresponding to a *trans* configuration.

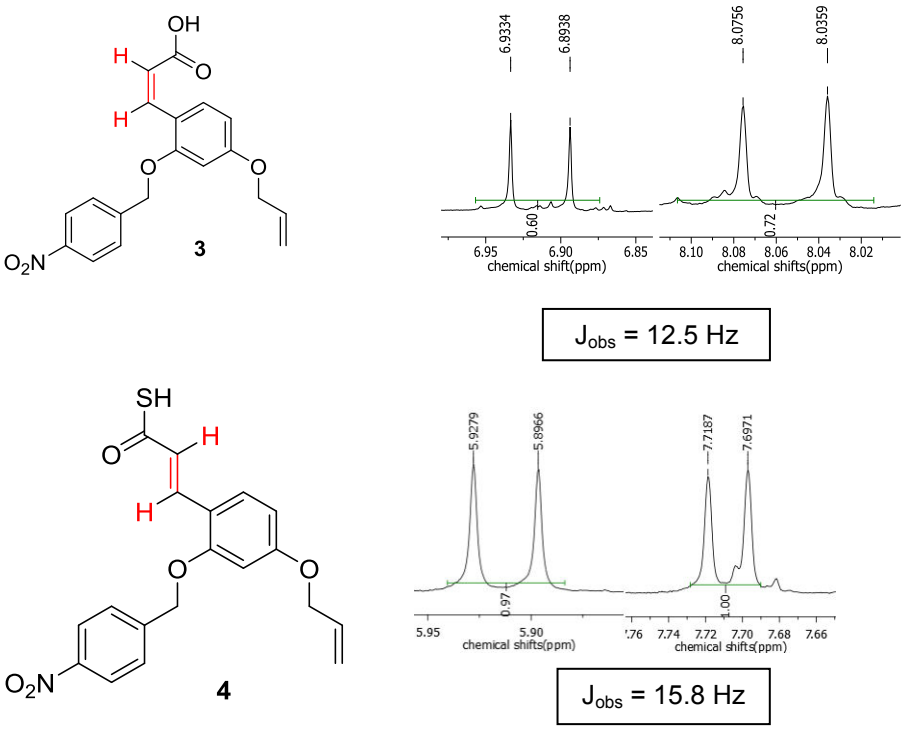


Figure 14: Evaluation of the geometrical isomer formed by comparison of coupling constants

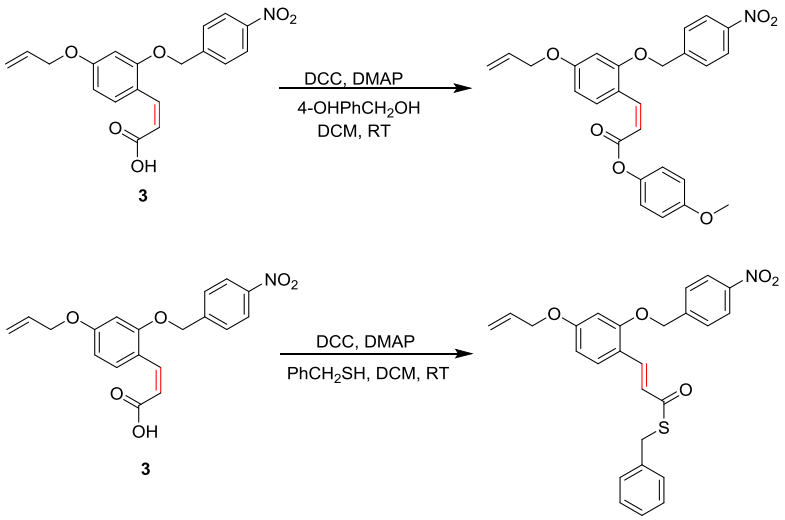
Following this result, we speculated that the high energy of microwave conditions might be giving the more stable thermodynamic product (*trans* form) as the end result. To study this possibility, we tried different reaction conditions, by varying the temperature and solvent, with and without the Lawesson's Reagent.

Table 1 : Different reaction conditions for formation of *trans* thio acid

Conditions	Solvent	Lawesson's Reagent	Result
100 ^{o}C , 6 min, MW	DCM	Yes	Trans form
100 ^{o}C, 3 min, MW	DCM	NO	No isomerization
60 ^{o}C , 3 min, MW	DCM	NO	No isomerization
60 ^{o}C, 3 min, MW	CAN	NO	No isomerization
r.t.	DCM	Yes	No reaction
Reflux	TOULENE	Yes	No reaction

MW: Microwave

The experiments concluded that the microwave conditions are required for the reaction to proceed, but cannot induce isomerization. These results suggested the role of Lawesson's reagent in this isomerization. In order to validate this speculation, we carried out the following reactions.



SCHEME 3

Reaction of **3** with para-methoxy phenol retained its *cis* configuration, whereas the reaction with benzylthiol, lead to the isomerized product. From the following experiments, we proposed a plausible mechanism for this isomerization. Since the double bond is in conjugation with carbonyl group of acid, it can act a michael acceptor. Being a soft nucleophile, sulfur can attack at this double bond and form a covalent adduct as shown in **Figure 15**. Rotation about the single bond followed by retro michael addition will then result in a more stable *trans* thio acid.

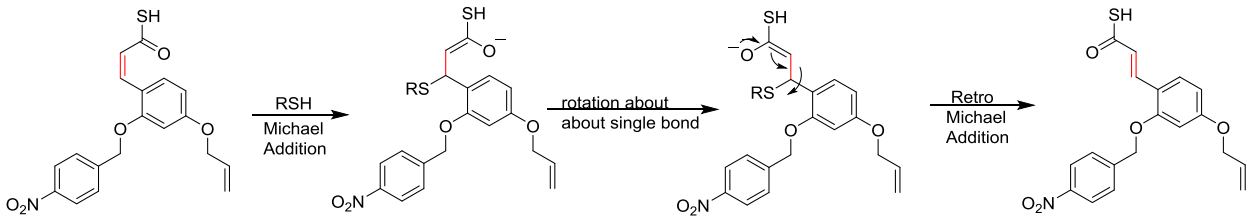
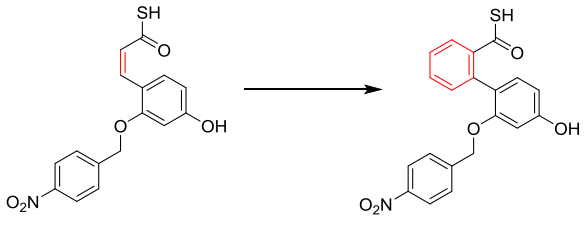


Figure 15: Proposed mechanism of *cis trans* isomerization

After some more literature search, we came across reports of photochemically induced isomerization of olefins. So we tried *trans* to *cis* isomerization by irradiating compound **4** with 375 nm light.[24] But it was observed that, a long exposure time of 1 h lead to decomposition of **4**,

while lesser exposure time of 30 min, did not show any change. In conclusion, a *cis* thio acid could not be synthesized with Lawesson's reagent as the sulfurizing agent.

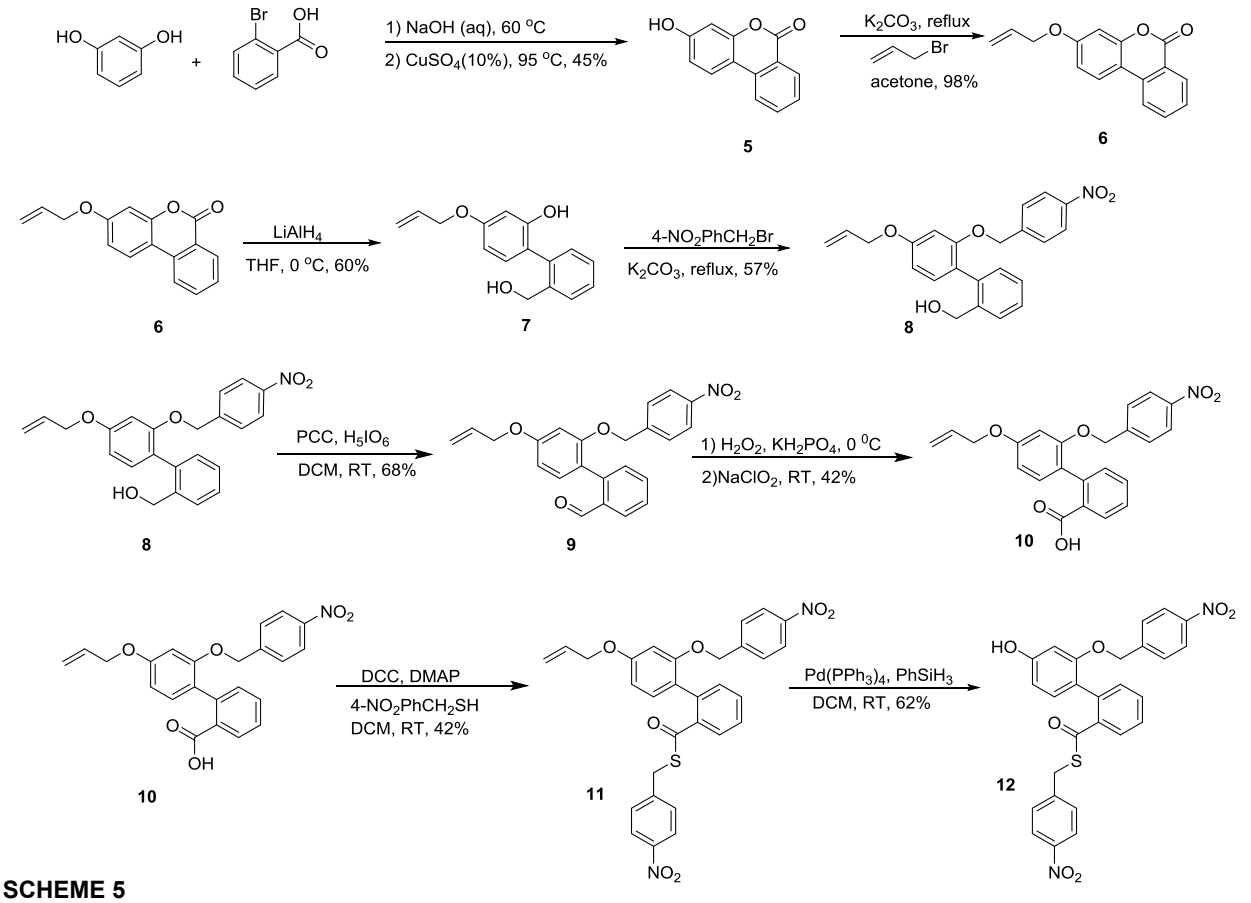
To overcome the isomerization problem, we modified the design of the molecule, so as to lock the double bond with a benzene ring as shown in **SCHEME 4**. Also this eliminated the potential for michael attack by a thiol.



SCHEME 4: Modification of the present design

5.2 3-hydroxy-6H-benzo[c]chromen-6-one as fluorescence reporter

Compound **12** was synthesized in 8 steps, using reported procedures. Starting with 2-bromo benzoic acid and resorcinol, **5** was synthesized under reflux conditions in the presence of sodium hydroxide and copper sulfate solutions. Reaction of **5** with allyl bromide afforded **6**. Using lithium aluminum hydride, lactone ring was opened to give **7**. The phenolic OH was protected with para-nitrobenzylbromide to give **8**. Further oxidation of the benzyl alcohol in 2 steps afforded **10**. Using DCC/DMAP coupling, **10** was coupled to para-nitrobenzylthiol to give **11**. Deallylation of **11**, with Palladium catalyst and phenyl silane afforded **12**.



SCHEME 5

5.3 Observation of fluorophore with HPLC based study

Compound **12** was then tested for its ability to cyclize and produces the fluorophore as shown in **Figure 16**.

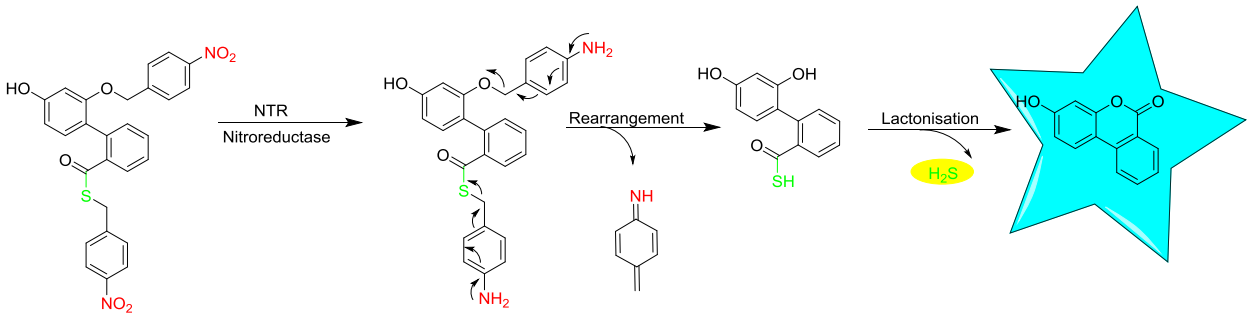


Figure 16: Proposed scheme for fluorophore formation

Compound **12** was treated with Zinc/ammonium formate and the reaction mixture was filtered and injected into the HPLC at different time intervals. After 30 min, the formation of a new peak, suggested the formation of fluorophore. Therefore the following

experiment demonstrated that the reduction of nitro group is indeed resulting in the formation of fluorophore.

Due to poor solubility of fluorophore in buffer, quantitative analysis could not be carried out.

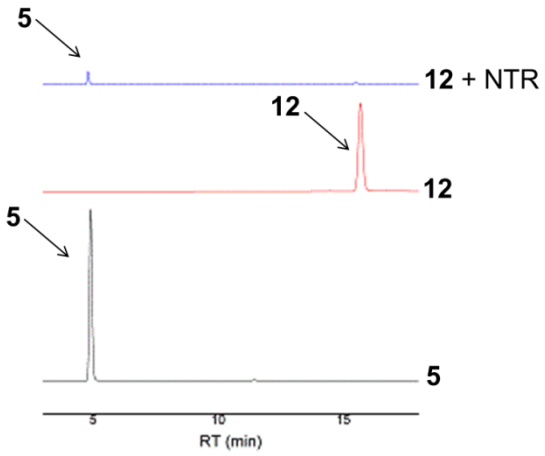
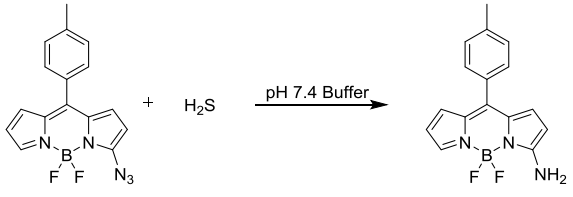


Figure 17: Formation of fluorophore

5.4 BODIPY azide study

Compound **12** was then studied for its ability to release H_2S. Organic azides are known to undergo reduction to amine, in the presence of H_2S and equivalent thiols. This property was exploited to develop fluorescence based techniques for H_2S detection. For this assay, BODIPY azide based probe **31a** was used, which was synthesized by my colleague Dr. Vinayak Khodade.



31a **31b**

λ_{ex} = 444 nm, λ_{em} = 515 nm

SCHEME 6

When sodium sulfide was incubated with **31a** a distinct fluorescence signal was observed at 520 nm, suggesting the suitability of the experiment for H_2S detection. When compound **12** was exposed to NTR and co-factor NADPH in the presence of **31a**, a distinct fluorescence signal was observed, confirming the formation of H_2S.

Compound **1c** (Figure 13), was used a positive control, which was synthesized by my colleague Dr. Vinayak Khodade. The following results demonstrate the ability of compound **12** to release H_2S.

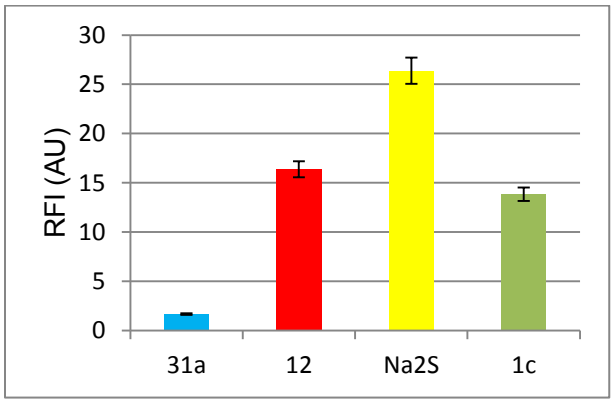


Figure 18: Estimation of H_2S

However further studies are necessary for complete characterization of H_2S formation. Poor aqueous solubility of **12** is the major limitation.

6. CONCLUSIONS AND FUTURE OUTLOOK

In summary, we have developed a prototype H_2S donor, which addresses the issue of real time monitoring of H_2S. Compound **12** showed good stability in pH 7.4 phosphate buffer. Upon incubation with NTR, it released fluorophore and H_2S, as identified by HPLC and BODIPY azide based probe studies. Biaryl lactonization can further be exploited as a prodrug strategy.

For further improvement in the design, we are working on the enhancement of fluorescence. In order increase the selectivity and eliminate the formation of potentially toxic by-products, change of the trigger is essential.

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