Design, Synthesis and Evaluation of Nitroreductase Activated Hydrogen Sulfide Donors

Thesis Submitted towards the partial fulfillment of

BS-MS dual degree programme

By

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CERTIFICATE

This is to certify that this dissertation entitled **Design, Synthesis and Evaluation of Nitroreductase Activated Hydrogen Sulfide Donors** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Sharath Chandra Mallojjala**, IISER Pune under the supervision of **Dr Harinath Chakrapani**, Assistant Professor – Department of Chemistry, IISER Pune during the academic year 2014-2015.

Date: 25.03.2015
Place: Pune

Dr Harinath Chakrapani
IISER Pune

DECLARATION

I hereby declare that the matter embodied in the report entitled **Design, Synthesis and Evaluation of Nitroreductase Activated Hydrogen Sulfide Donors** are the results of the investigations carried out by me at the Department of Chemistry, IISER Pune, under the supervision of Dr Harinath Chakrapani and the same has not been submitted elsewhere for any other degree.

Date: 25.03.2015
Place: Pune

Sharath Chandra Mallojjala
IISER Pune.

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Sharath Chandra Mallojjala

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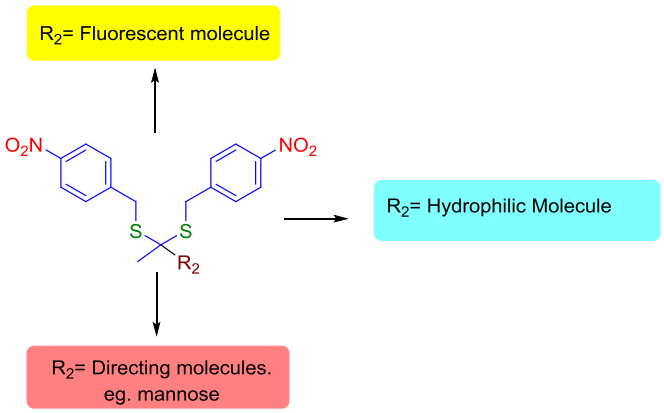
Abbreviations

TLC	Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance
HRMS	High Resolution Mass Spectrometry
J	Coupling Constant
Hz	Hertz
MHz	Megahertz
EtOAc	Ethyl acetate
Bz	Benzoyl
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
mg	milligram
g	gram
h	hour
M	molar
mL	millilitre
mol	mole

Abstract

Hydrogen sulfide (H_2S) was long considered as a toxic gas, but recently found to play key roles in the physiological processes. H_2S is now recognized as a "gasotransmitter" along with carbon monoxide and nitric oxide. Endogenous production of H_2S in mammals and most bacteria is mainly attributed to Cystathionine-β-synthase (CBS), Cystathionine-γ-lyase (CSE) 3-Mercaptopyruvate sulfurtransferase (3MST) and their bacterial orthologs. H_2S was recently found to play protective role in bacteria against stress induced by antibiotics. The precise mechanism by which this occurs is yet to be understood. Methodologies to relatively enhance H_2S within cells are required. Commercial sources of H_2S mainly include various inorganic sulfide salts like Na_2S and NaHS, which have poor bio-availability and lack of selectivity. A variety of organic H_2S donor molecules have been reported as well but have drawbacks such as: Thiol activated H_2S donors may not be a suitable strategy as thiols are targets of H_2S; Light activated H_2S donors require the use of high intensity light which may not be compatible with certain cellular studies. Our laboratory has developed a series of nitroreductase (NTR) activated H_2S donors that may be suited for reliably enhancing H_2S levels within bacterial cells. However, these donors were found to have low water solubility. In this work, using levulinic acid as the starting material, we developed a general strategy to functionalize these H_2S donors with: (a) a 2-(2-ethoxyethoxy)ethyl group that increased aqueous solubility; (b) a fluorophore that facilitates tracking of this compound within cells; and (c) a sugar which might improve site-directed delivery of H_2S.



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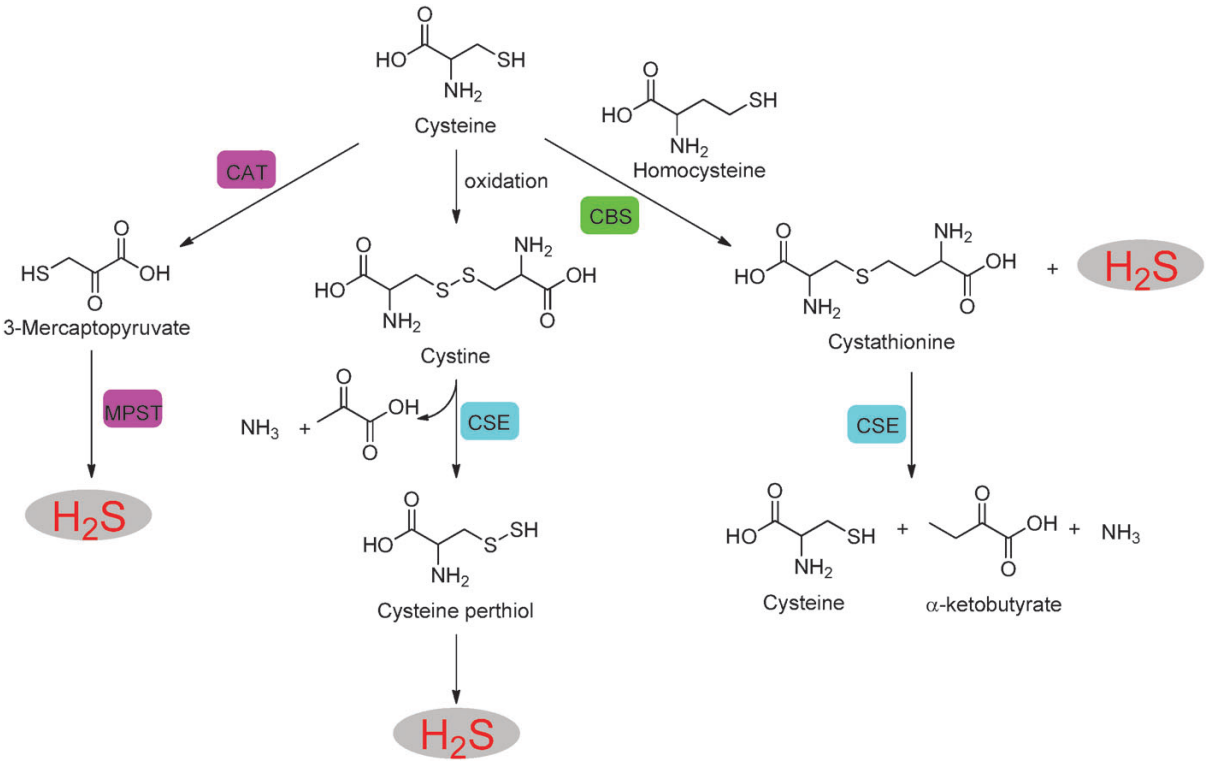
Introduction

Hydrogen sulfide (H_2S), a colourless poisonous gas, was first discovered by Carl Wilhelm Scheele in 1777. It is known as an air pollutant and has a characteristic rotten eggs odour. However, recent studies establish that H_2S acts as a gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO).[1] Gasotransmitters are endogenously synthesized small gaseous molecules which play an important role in signalling pathways. Endogenous production of H_2S in mammalian cells is mainly attributed to three enzymes namely Cystathionine-β-synthase (CBS), Cystathionine-γ-lyase (CSE) and 3-Mercapto pyruvate sulphur transferase (3-MPST).[2] CBS, CSE and 3-MPST mainly use cysteine and cysteine derivatives as substrates to produce H_2S (Figure 1). These enzymes play a critical role in regulating the H_2S levels inside the cells.[2] Apart from playing a role in signalling pathways, H_2S was also known to show vasodilatory and cytoprotective effects.[3-5]

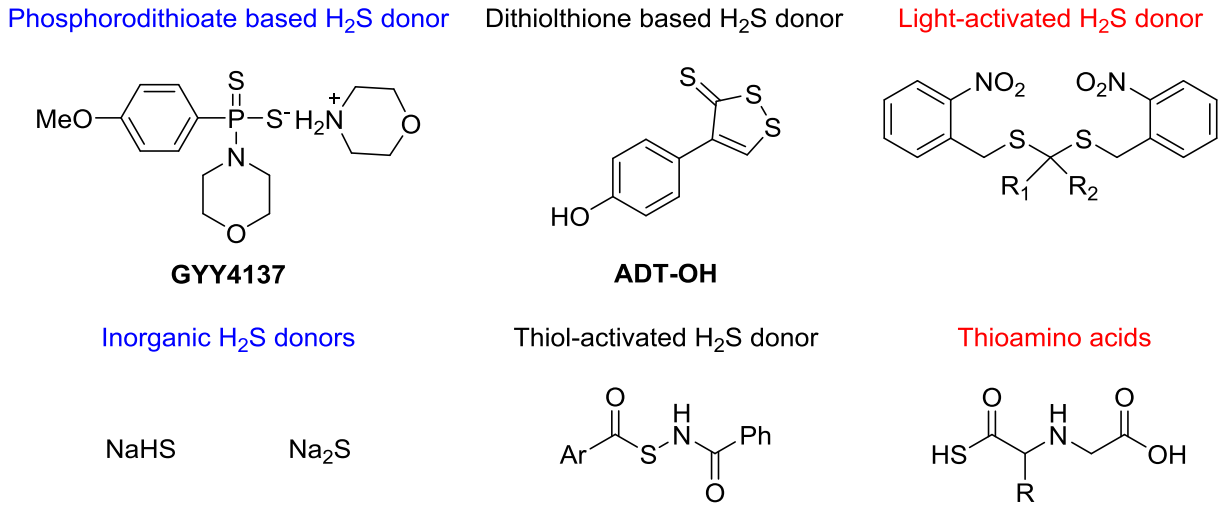
Figure 1: Biosynthesis of H_2S.[1]



H_2S production in bacteria has been known since a very long time and was assumed to be only a by-product of sulphur metabolism. Recently, Shatalin *et al*. have reported that H_2S plays an important role in the defence mechanism of bacteria

against anti-bacterial agents.[4] After treating the wild-type strains and bacterial strains lacking 3-MPST with antibacterial agents, the authors observed that the mutant strains were more susceptible towards the antibacterial agents. It was also observed that upon addition of NaHS, a H_2S donor, resistance towards antibacterial agents was restored. This data along with others was used to suggest a cytoprotective role of H_2S, but the precise molecular mechanism of action is not understood. In order to study the effects of H_2S in bacterial physiology there is a need for H_2S donors with spatiotemporal control under physiological conditions.

Figure 2: Available H_2S donors.



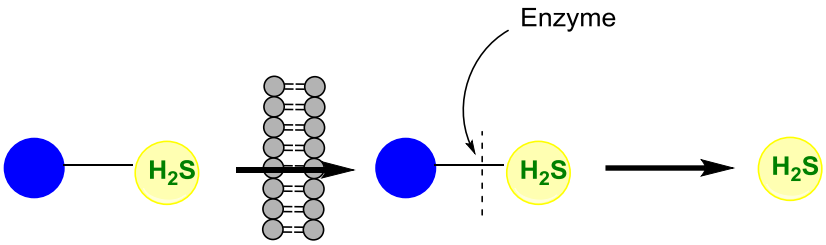
Earlier efforts made to study the role of H_2S in cells have used inorganic salts like Na_2S and NaHS which produce H_2S upon dissolution in water/buffer.[4] The advantage of using these salts is their low toxicity at high concentrations. However, their uncontrolled and burst release of H_2S upon dissolution is a major problem as a slow and sustained release of H_2S is essential for cellular studies. Moreover, they have very poor bio-availability. Dithiolthiones and GYY4137 were also used as H_2S donors.[6] However, extremely high concentrations of GYY4137 were used in order to observe significant effects limiting its therapeutic potential, while in the case of dithiolthiones, the mechanism of H_2S release is not very well understood and the release of H_2S is instantaneous leading to difficulties in its usage for studies. To address this, a number of organic small molecule H_2S donors have been developed. For the release of H_2S under physiological conditions, triggers like biological thiols, light, etc. have been employed. These organic H_2S donors can broadly be divided into four classes namely: thiol activated H_2S donors, light activated H_2S donors and polymer based H_2S donors and phosphodithiolate based H_2S donors. Thiol activated

H_2S donors are small molecules capable of releasing H_2S upon reaction with thiols like cysteine, glutathione, etc. analogous to that of the biosynthesis of H_2S.[1,7] Drawbacks of these donors mainly include:

- Using biological thiols as a trigger may not be a viable strategy, as the amount of thiols present inside the cells is huge and site specific activation may not be achieved with this trigger.
- Moreover, studies suggests that thiols themselves are targets of H_2S which prevent them in being used as a trigger.[8,9]
- Although, site specific activation has been achieved using light as a trigger, the intensity of light used may not be compatible with a few cellular studies.[10]
- Polymeric donors mainly implement the use of dithiolthione moiety which has a spontaneous H_2S release.[11]
- To study the role of H_2S in bacteria, mutant strains lacking H_2S synthesizing enzymes have been used.[4] Concentration-dependent effects of H_2S may not be possible to observe with such a strategy.

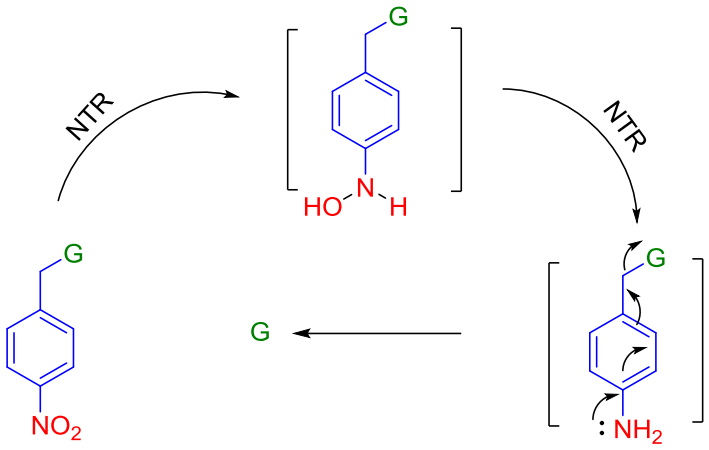
To achieve controlled release and site specificity, enzymes can be used as a trigger.[12] For example, nitroreductase enzyme (NTR) has been used for site specific drug delivery.[13]

Scheme 1: Scheme for the enzyme activated donor.



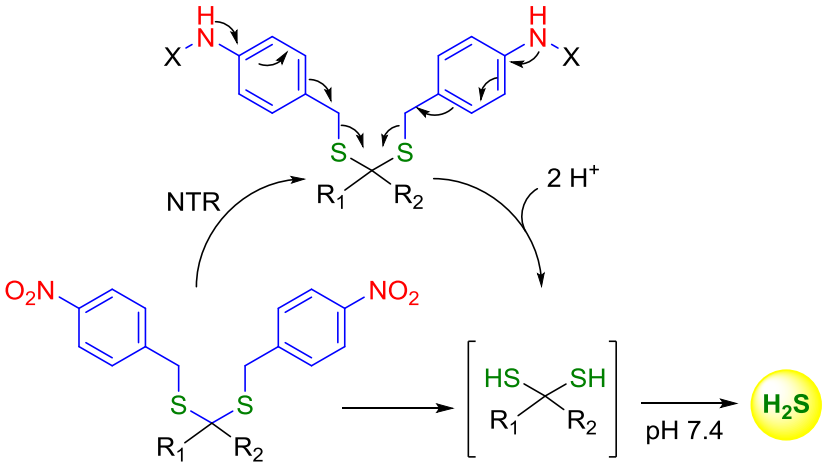
Nitroreductases are a class of enzymes present mostly in bacteria which are capable of reducing a variety of substrates like nitro compounds and quinones using NADH/NADPH as a cofactor. Early reports suggests that NTR mediated reduction of the nitro group leads to the formation of hydroxylamine intermediate which is further reduced to give an amine. After the formation of the amine, the lone pair of electrons in conjugation with the aromatic π-electron cloud reorganizes to release the drug molecule (Scheme 2).

Scheme 2: Scheme demonstrating the mode of action of NTR. G is the leaving group.



Our group has developed H_2S donors using the gem dithiol template which is known to release H_2S under physiological conditions (Design 1). These donors have low solubility in buffer which limit the concentration of compound that can be used for various studies.

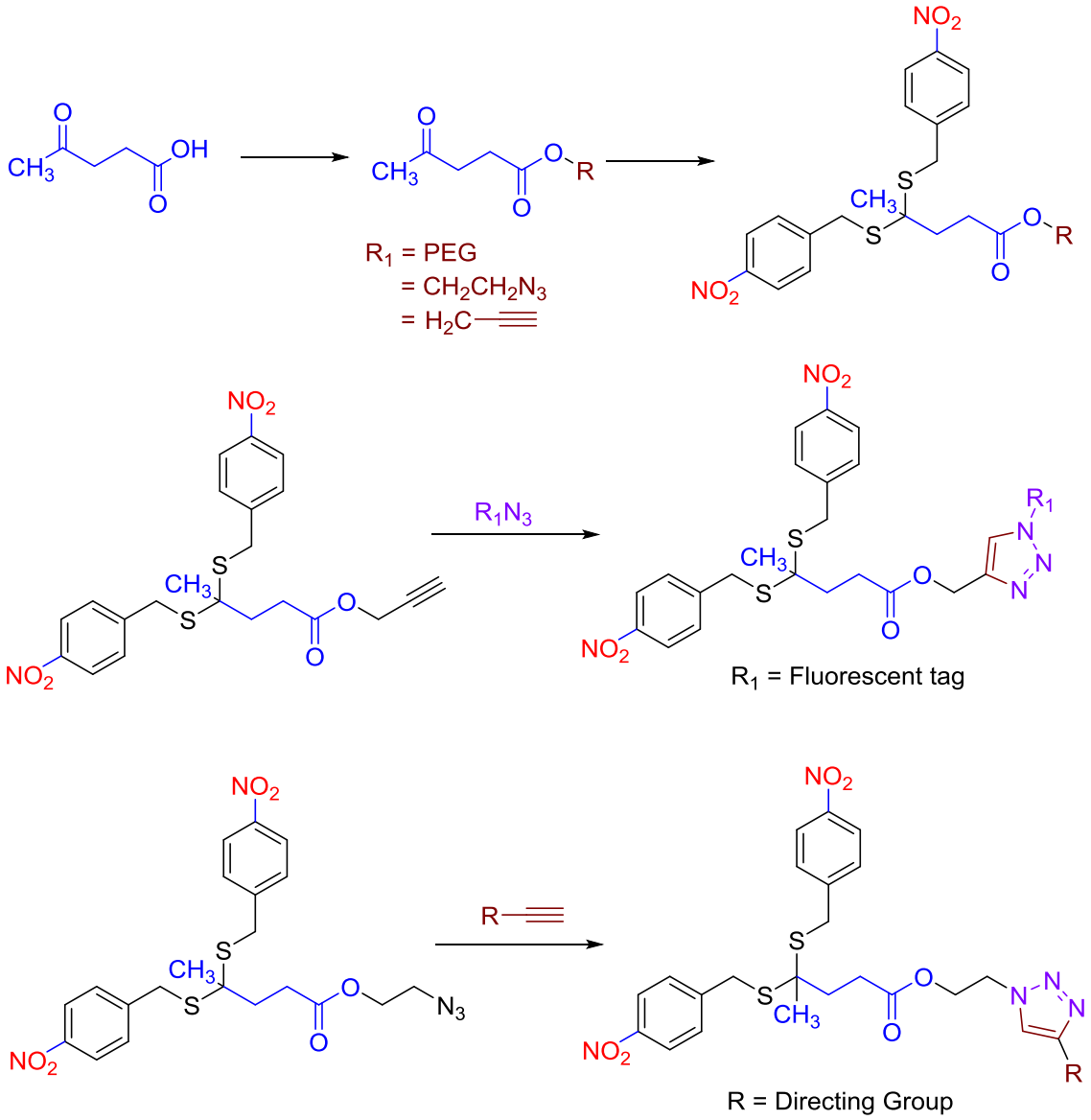
Design 1: Design of the NTR activated H_2S donor where X= H/ OH.



Functionalization of this donor can be achieved by using a linker (Scheme 3). The solubility of gem dithiol donors can be improved by incorporating polyethylene glycol chains. The site specificity of these donors can further be enhanced by functionalizing with a sugar molecule like mannose which can then be used to direct the donors towards the mannose receptors of *Mycobacterium tuberculosis* (MTB).[14,15] *Mycobacterium tuberculosis* is a well-known pathogenic bacteria which has a robust cell wall making it difficult to treat. The cell wall of MTB has mannosylated lipoarabinomannan which can bind with mannose and hence can be

targeted selectively. Functionalization with a fluorescent molecule can be used to study the localization of these donors. Moreover, by having a “turn on” fluorescent tag, direct detection of H_2S released by these donors can be monitored.

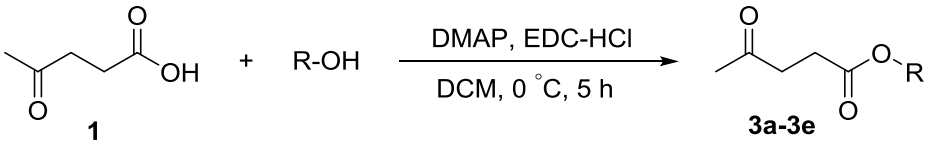
Design 2: Functionalization of gem dithiol donor.



The synthesis of gem dithiols with good water solubility and developing new synthetic strategies for the synthesis of H_2S donors is the main objective of this thesis.

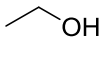
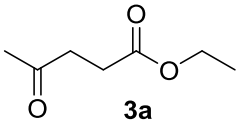
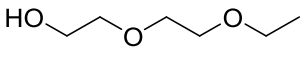
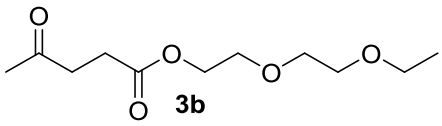
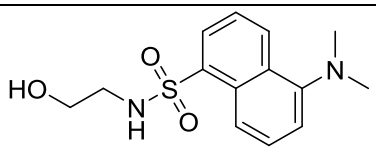
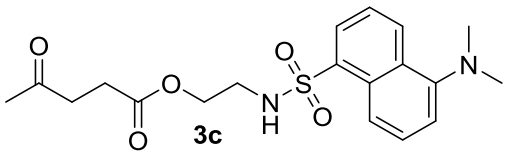
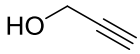
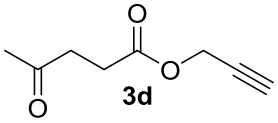
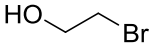
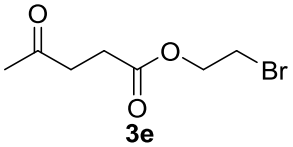
Results

Scheme 3



Gem dithiol moiety is susceptible towards decomposition under harsh conditions. In order to successfully functionalize the gem dithiol moiety a linker molecule must be incorporated in the structure which can be functionalized further under mild conditions, so as to not affect the gem dithiol moiety. In order to achieve this we chose to use levulinic acid (**1**) as the linker. The acid moiety can be easily functionalized using a variety of reagents under mild reaction conditions. We prepared a series of esters (**3a-3e**) with good yields using the reported protocol.[16]

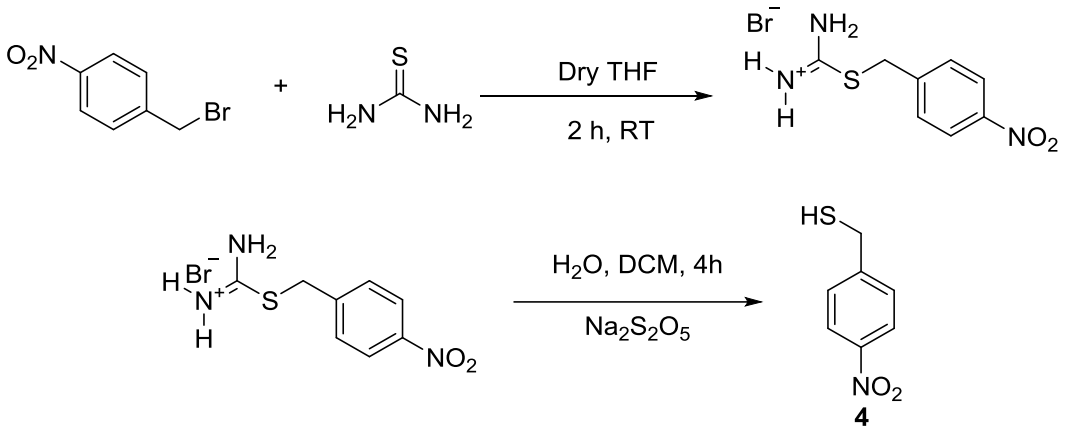
Table 1: Table with the yield of different esters prepared

SNo.	Reactant	Product	% Yield
1	 **2a**	 **3a**	61
2	 **2b**	 **3b**	60
3	 **2c**	 **3c**	61
4	 **2d**	 **3d**	62
5	 **2e**	 **3e**	44

The compounds were characterized by proton NMR and the characteristic ^{1}H NMR peak for the esters (**3a-3e**) is the singlet corresponding to 3 protons of the methyl group attached to the carbonyl in the chemical shift range of 2.10-2.20 ppm. **3b** was synthesized as a proof of concept for the synthesis polyethylene glycol derivatives, which can improve the water solubility of these donors. **3c**, a fluorescent molecule, was synthesized in order to have a fluorescent tag on the gem dithiol molecule. **3d** was prepared in order to achieve further functionalization using the azide alkyne coupling strategy. **3e** was synthesized to prepare an azide derivative on the gem dithiol moiety. This can again be used in azide alkyne coupling, where the synthesis of azide on the other substrate is difficult.

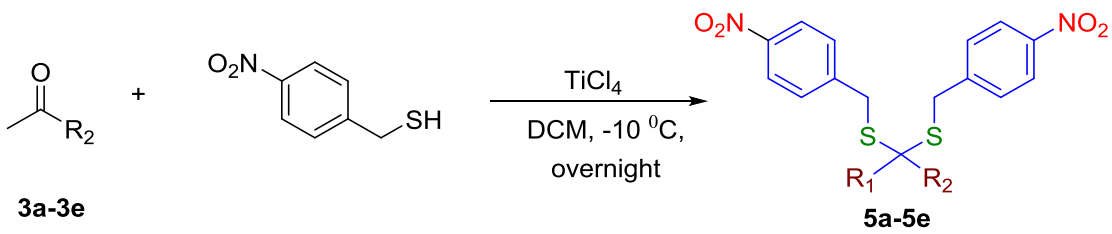
With esters in hand we moved on to the synthesis of (4-nitrophenyl)methanethiol (**4**). Starting from 1-(bromomethyl)-4-nitrobenzene, **4** was obtained in good yield in 2 steps.

Scheme 4: Scheme showing the synthesis of **4**.



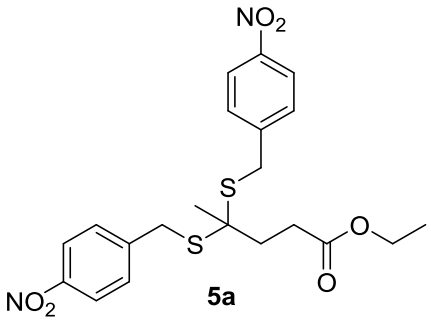
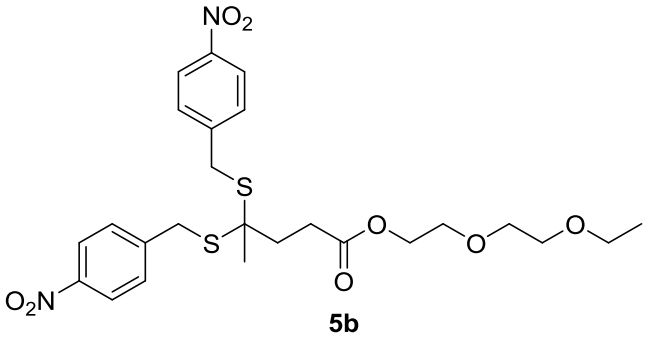
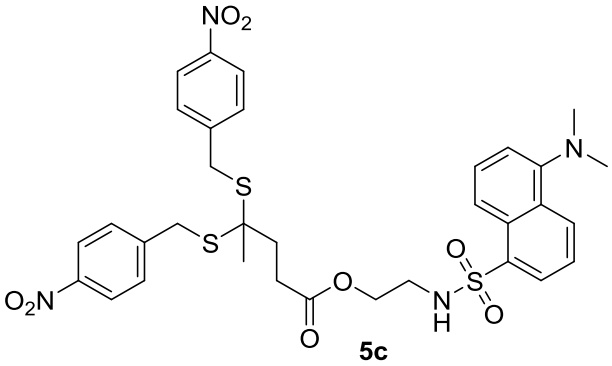
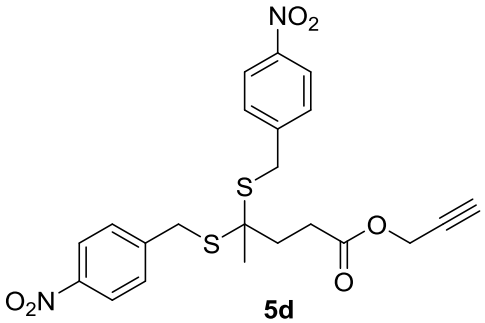
With both the required starting materials in hand we moved on to synthesize the gem dithiol molecules **(5a-5e)**.

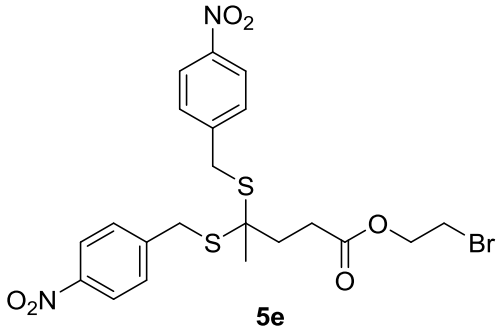
Scheme 5:



3a-3e **5a-5e**

Table 2: Table with all the gem dithiol molecules synthesized and their yields.

SNo.	Reactant	Product	% Yield
1	**3a**	 **5a**	59
2	**3b**	 **5b**	61
3	**3c**	 **5c**	42
4	**3d**	 **5d**	59

5	**3e**	 **5e**	61

The propargyl group in **5d** was further functionalized exploiting the azide-propargyl coupling strategy to obtain **7a** and **7b**.[17]

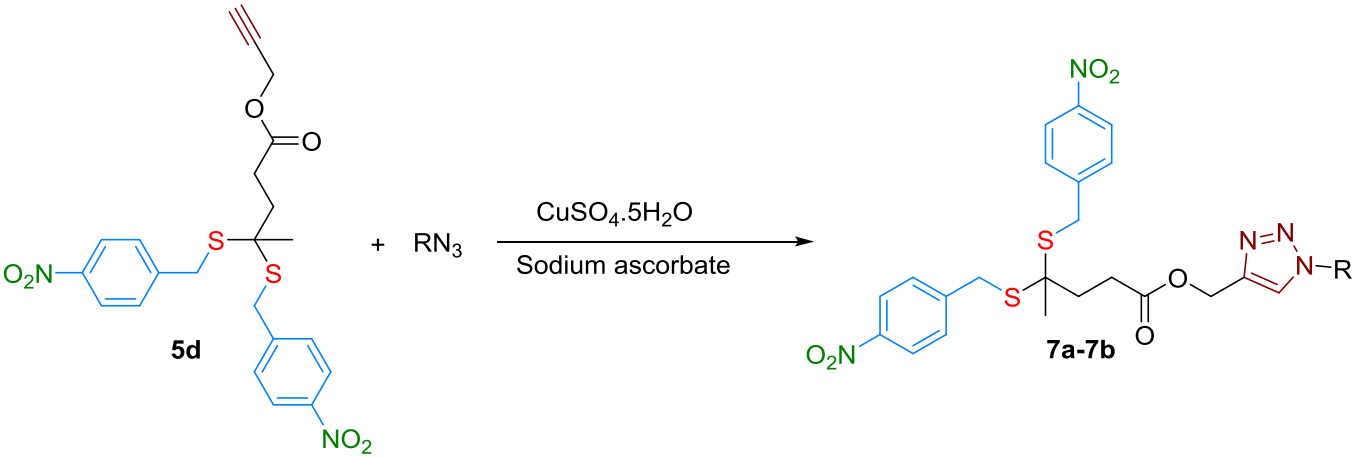
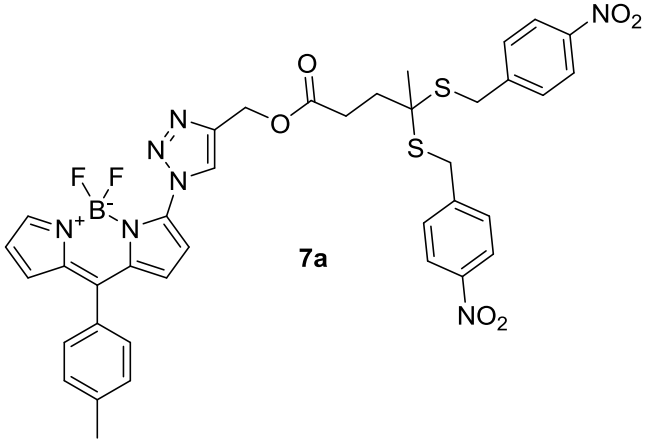
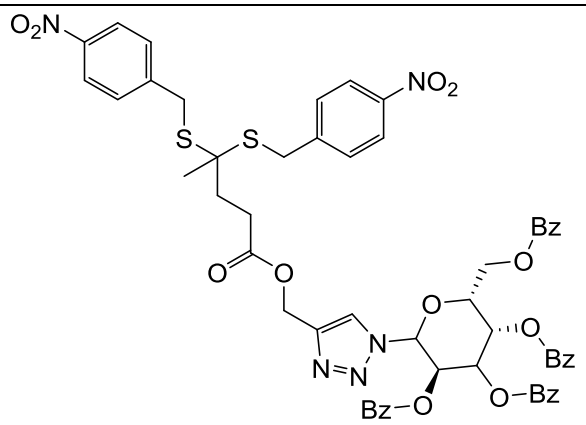


Table 3: Table with all the gem dithiol molecules synthesized and their yields.

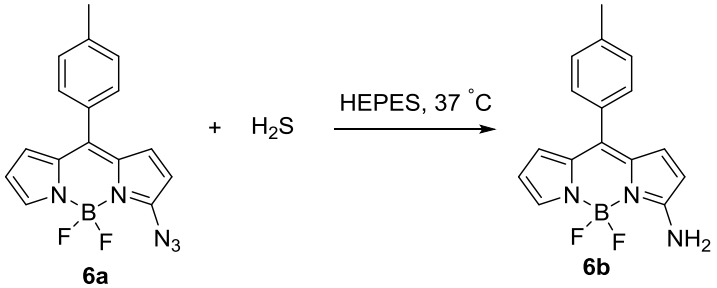
SNo.	Reactant	Product	% Yield
1	**5d**	 **7a**	73

2	**5d**	 **7b**	65

The precursor for compound **7a** was synthesized using a reported protocol and the precursor for the synthesis of compound **7b** was supplied by Dr Srinivas Hotha's group. Compound **7a** was found to be a fluorescent compound. The excitation and emission maxima in DMSO were found to be 373 nm and 536 nm respectively. Compound **7b** was synthesized in order to obtain a water soluble H_2S donor. It was observed that benzoyl groups in **7b** cannot be de-protected without hydrolyzing the ester. We have developed a new synthetic strategy for the functionalization of gem dithiol moiety.

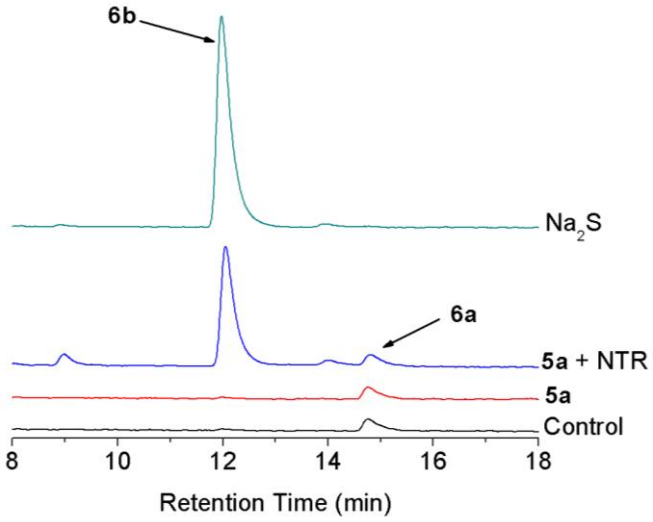
Detection of H_2S from 5a using HPLC:

Compound **5a** was chosen for the assay to verify the H_2S generating ability of the compounds. The assay was performed using *Escherichia coli* NTR and NADH as the cofactor. To detect the H_2S released, BODIPY azide dye (**6a**) was used as reporter molecule.[18] Sodium sulfide was used as a positive control. BODIPY azide upon reaction with H_2S gives the fluorescent BODIPY amine (**6b**) which can be detected using HPLC attached with fluorescent detector (Excitation maxima 444 nm; Emission maxima 520 nm).



6a **6b**

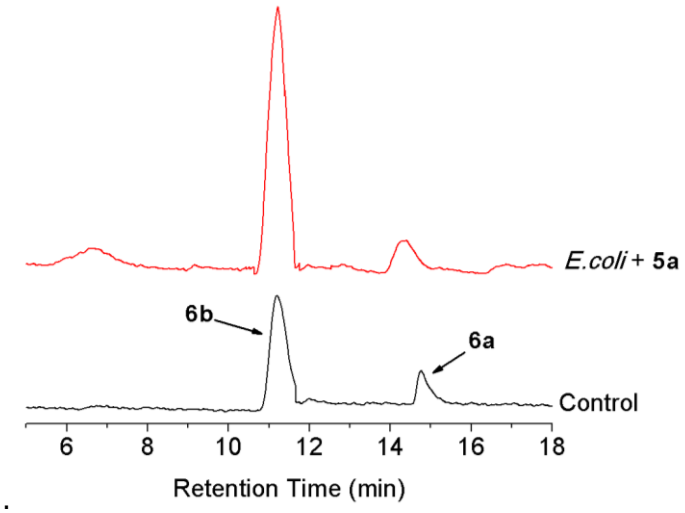
Figure 3: HPLC traces for H_2S detection using **6a** (20 μM). **5a** (50 μM) was incubated with NTR in HEPES buffer pH 7.4 for 15 min. Control is 6a (100 μM), Sodium sulfide was reacted with 6a and used as positive control.



From this assay we can say that the gem dithiol template is capable of generating H_2S only in the presence of NTR.

Intracellular detection of H_2S from 5a using HPLC: *Escherichia Coli* (ATCC 25922) was cultured in 5 mL of tryptone soya broth (TSB) medium at 37 ºC for 16 h. The cultured bacteria were centrifuged to aspirate out the medium and suspended to an O.D of 1.0 with fresh TSB medium. This bacterial solution was incubated with 50 μM of **5a** and 10 μM of **6a** for 60 min in dark by covering the falcon tube in an aluminum foil. The suspension was centrifuged to aspirate out any excess of the compound and/or **6a** in the medium. The bacterial pellet was washed with HEPES buffer pH 7.4 (1 mL × 3) and centrifuged. The collected bacterial pellet was re-suspended with acetonitrile and the cells were lysed by vortexing for 1 min. The cell lysate was then removed by centrifugation and the supernatant acetonitrile (50 μL) was injected in high performance liquid chromatography (HPLC) attached with a fluorescence detector (excitation at 444 nm; emission at 520 nm). The HPLC method used was as described previously.

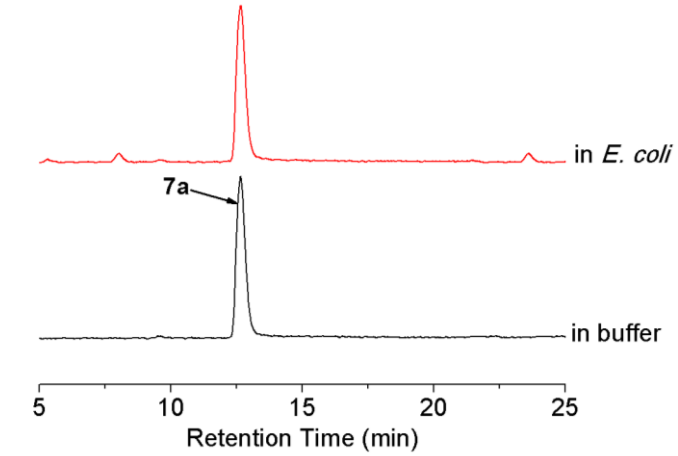
Figure 4: HPLC traces for detection of H_2S from **5a** (50 μM) in *E.coli* using **6a** (10 μM). **5a** (50 μM) was incubated with *E.coli* for 60 min. Control is 6a (10 μM) incubated with *E.coli* for 60 min.



From the above HPLC trace, it can be observed that the height of **6b** increased when **5a** was incubated with *E.coli* suggesting that H_2S was generated by **5a** inside the bacteria.

Intracellular assay to verify the permeability of 7a: *Escherichia Coli* (ATCC 25922) was cultured in 10 mL of tryptone soya broth (TSB) medium at 37 ºC for 16 h. The cultured bacteria were centrifuged to aspirate out the medium and re-suspended with fresh TSB medium to an O.D. of 1.0. This bacterial solution was incubated with 50 μM of **7a** and incubated for 60 min in dark by covering the falcon tube in an aluminum foil. The suspension was centrifuged to aspirate out any excess of the compound. The bacterial pellet was washed with HEPES buffer pH 7.4 (1 mL × 3) and centrifuged. The collected bacterial pellet was re-suspended with acetonitrile and the cells were lysed by vortexing for 1 min. The cell lysate was then removed by centrifugation and the supernatant acetonitrile (50 μL) was injected in high performance liquid chromatography (HPLC) attached with a fluorescence detector (excitation at 373 nm; emission at 536 nm). 50 μM of authentic **7a** was injected in the HPLC independently to verify the retention time of the compound.

Figure 5: HPLC traces demonstrating the bacterial cell permeability of **7a**. **7a** (50 μM) was incubated with *E.coli* for 60 min under dark conditions. A solution of **7a** in HEPES buffer was also injected as a control.



From the above HPLC traces, we can say that the compound **7a** permeated into the cell. This assay demonstrates that the gem dithiol moiety is capable of permeating the bacterial cell wall.

Discussion

Compound **5a** was used to demonstrate the H_2S generating ability of these donors in buffer as well as in *E.coli*. The solubility of these donors have been improved by functionalizing the gem dithiol moiety. Compounds synthesized earlier in our group (unpublished work) were poorly soluble in 2% DMSO-buffer solution, while **5b** was found to be soluble in 1% DMSO buffer solution. The cLogp values (Chembiodraw Ultra 14.0 was used to calculate the cLogP values) also came down from 6 to around 5 for the compound **5b**. These gem dithiols have been successfully tagged with a fluorescent molecule to obtain the fluorescent compound **7a** which may be useful in understanding the localization of the compound inside the cell. The gem dithiol molecules can be successfully functionalized to incorporate various directing molecules using the Huisgen coupling. As a proof of concept, **7b** was synthesized in good yield.

Summary

We have developed a new synthetic methodology for the preparation of gem dithiols using functionalized levulinic acid. We successfully synthesized gem dithiols which

are capable of releasing H_2S only in the presence of NTR, as demonstrated through **5a**. A H_2S donor with a fluorescent tag (**7a**) in the gem dithiol moiety was synthesized and this compound might facilitate tracking of the molecule within the cell.

Materials and Methods

All chemicals were purchased from commercial sources and used as received unless stated otherwise. Petroleum ether (PE) and ethyl acetate (EtOAc), for chromatography were distilled before use. THF and DCM were dried using a sodium wire and distilled before use. Column chromatography was performed using Merck silica gel (60-120/100–200 mesh) as the solid support. ^{1}H and ^{13}C spectra were recorded on JEOL 400 MHz (or 100 MHz for ^{13}C) spectrometer using either the residual solvent signal as an internal reference ($CDCl_3$ δH, 7.24 ppm, δC 77.1 ppm) (CD_3CN δH, 1.94 ppm, δC 1.4, 118.7 ppm) or tetramethylsilane (δH = 0.00, δC = 0.0) standard. Chemical shifts (δ) are reported in ppm. The following abbreviations are used: s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Mass spectra were obtained using HRMS-ESI-Q-Time of Flight LC-MS (Synapt G2, Waters) or MALDI TOF/TOF Analyser (Applied Biosystems 4800 Plus). Infrared spectra (IR) were obtained using NICOLET 6700 FT-IR spectrophotometer using a KBr disc. Melting points were measured using a VEEGO melting point apparatus in open glass capillary and values reported are uncorrected. High performance liquid chromatography (HPLC) was performed on Agilent Technologies 1260Infinity, attached with a C-18 column (Phenomenex, 5 µm, 4.6 × 250 mm). Fluorimetric and spectrophotometric measurements were performed using Thermo Scientific Varioscan microwell plate reader. The purity of all compounds synthesized in this study was ≥ 95% as determined by HPLC.

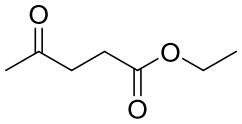
General Procedure A

To a solution of levulinic acid (**1**) in DCM (25 mL), 1 eq. of alcohol (**2a-2d**) and 1.5 eq. of DMAP were added. The mixture was cooled to 0 ˚C and 1.5 eq. of EDC·HCl was added. The resulting mixture was allowed to warm up to RT and was stirred for 5 h or until the consumption of the corresponding alcohol (monitored by TLC and Vanillin staining). The reaction mixture was diluted with water (25 mL) and extracted with DCM (3 x 20 mL). The combined organic layers were collected and dried over

anhydrous Na_2SO_4, filtered and concentrated. The concentrate was then purified using silica gel column chromatography with EtOAc/hexane as eluent to obtain the desired ester (**3a-3d**).

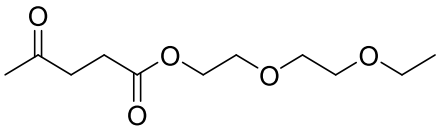
The general procedure A was used to synthesize **3a-3d**.

Ethyl 4-oxopentanoate (3a)



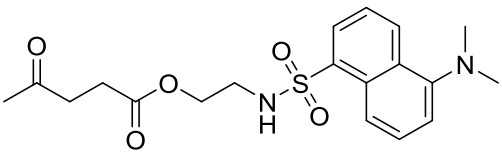
Compound **3a** was reported previously and our data is in agreement with the reported spectral data.[19]

2-(2-Ethoxyethoxy)ethyl 4-oxopentanoate (3b)



Starting from **2b** (0.57 g, 4.3 mmol), **3b** (0.60 g, 60%) was obtained as a colourless liquid. FTIR (ν, cm^{-1}): 2972, 2869, 1720, 1409, 1357, 1246, 946, 763; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.17 (t, *J* = 4.8 Hz, 2H), 3.63 (t, *J* = 4.8 Hz, 2H), 3.58 (t, *J* = 4.7 Hz, 2H), 3.54 (t, *J* = 4.7 Hz, 2H), 3.45 (q, *J* = 5.7 Hz, 2H), 2.69 (t, *J* = 6.4 Hz, 2H), 2.55 (t, *J* = 6.4 Hz, 2H), 2.13 (s, 3H), 1.15 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.6, 172.7, 70.6, 69.7, 69.0, 66.6, 63.7, 60.3, 37.8, 29.8, 27.9; HRMS (ESI): Calcd. $[C_{11}H_{20}O_5 + Na]^+$ $[M + Na]^+$: 255.1208. Found $[M + Na]^+$: 255.1211.

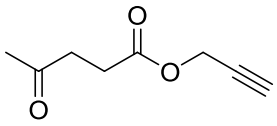
2-((5-(Dimethylamino)naphthalene)-1-sulfonamido)ethyl 4-oxopentanoate (3c)



Starting from **2c** (0.2 g, 0.68 mmol), **3c** (0.18 g, 61%) was obtained as a greenish yellow liquid. FTIR (ν, cm^{-1}): 2970, 2864, 1720, 1410, 1322, 1360, 1152, 946, 763, 704; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.52 (d, *J* = 4.7 Hz, 1H), 8.26 (d, *J* = 4.3 Hz, 1H), 8.22 (d, *J* = 4.3 Hz, 1H), 7.54 (t, *J* = 8.1 Hz, 1H), 7.49 (t, *J* = 8 Hz, 1H), 7.15 (d, *J* = 7.1 Hz, 1H), 5.37 (t, *J* = 6.2 Hz, 1H), 4.00 (t, *J* = 5.3 Hz, 2H), 3.16 (q, *J* = 8.3 Hz, 2H),

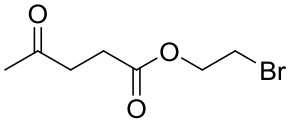
2.89 (s, 6H), 2.63 (t, *J* = 6.2 Hz, 2H), 2.32 (t, *J* = 6.6 Hz, 2H), 2.16 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.8, 172.4, 129.9, 129.4, 128.3, 123.1, 118.8, 115.2, 62.9, 51.7, 45.3, 42.1, 37.9, 29.7, 27.6; HRMS (ESI): Calcd. $[C_{19}H_{24}N_2O_5S + H]^+$ $[M + H]^+$: 393.1484. Found $[M + H]^+$: 393.1487.

Prop-2-yn-1-yl 4-oxopentanoate (3d)



Starting from **2d** (0.24 g, 4.31 mmol), **3c** (0.19 g, 62%) was obtained as a colourless liquid. FTIR (ν, cm^{-1}): 3273, 2925, 2128, 1740, 1410, 1356, 1204, 1029, 760; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.68 (d, *J* = 2.2 Hz, 2H), 2.78 (t, *J* = 6.5 Hz, 2H), 2.63 (t, *J* = 5.6 Hz, 2H), 2.49 (d, *J* = 2.5 Hz, 1H), 2.20 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.2, 171.8, 77.5, 74.8, 52.0, 37.7, 29.7, 27.7; HRMS (ESI): Calcd. $[C_8H_{10}O_3 + Na]^+$ $[M + Na]^+$: 177.0527. Found $[M + Na]^+$: 177.0531.

2-bromoethyl 4-oxopentanoate (3e)



Starting from **2e** (0.22 g, 1.72 mmol), **3e** (0.17 g, 44%) was obtained as a colourless liquid. FTIR (ν, cm^{-1}): 2965, 2132, 1743, 1401, 1351, 1209, 1019, 760;^{1}H NMR (400 MHz, $CDCl_3$): δ 4.36 (t, *J* = 6.1 Hz, 2H), 3.48 (t, *J* = 6.1 Hz, 2H), 2.27 (t, *J* = 6.2 Hz, 2H), 2.59 (t, *J* = 6.2 Hz, 2H), 2.17 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.4, 172.2,63.9, 37.9, 29.8, 28.6, 27.8; HRMS (ESI): Calcd. $[C_7H_{11}BrO_3 + Na]^+$ $[M + Na]^+$: 244.9789. Found $[M + Na]^+$: 244.9781.

Synthesis of 4

Compound **4** was synthesized with a yield of 72%. The spectral data collected is in agreement with the reported values.[20]

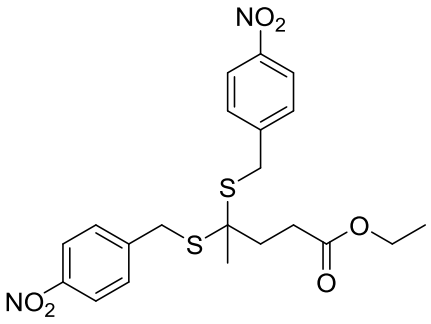
General Procedure B

To a solution of ketone (**3a**-**3d**) in $CHCl_3$ (25 mL), 2.5 eq. of **4** was added. The reaction mixture was cooled to -10 ˚C and 0.5 eq. of $TiCl_4$ was added. The reaction

mixture was allowed to warm up to rt and was stirred overnight or till the consumption of the ketone. The reaction mixture was then quenched by adding water (25 mL) and extracted with $CHCl_3$ (3 x 5 mL). The combined organic layers were collected and dried over anhydrous Na_2SO_4, filtered and concentrated. The concentrate was then purified by silica gel column chromatography to obtain **5a**-**5e**.

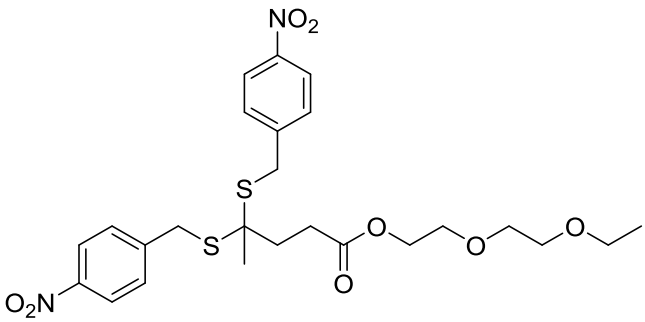
The general procedure B was used to synthesize **5a-5e**.

Ethyl 4,4-bis((4-nitrobenzyl)thio)pentanoate (5a)



Starting from **3a** (0.10 g, 0.70 mmol), **5a** (0.20 g, 59%) was obtained as a green liquid. FTIR (ν, cm^{-1}): 3744, 3076, 2979, 2928, 2856,1729, 1601, 1518, 1451, 1019, 858; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, *J* = 8.6 Hz, 4H), 7.49 (d, *J* = 8.6 Hz, 4H), 4.12-4.07 (m, 2H), 4.04 (s, 4H), 2.49 (t, *J* = 7.6 Hz, 2H), 2.10 (t, *J* = 7.6 Hz, 2H), 1.52 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.7, 147.1, 145.4, 130.0, 123.9, 61.5, 60.8, 35.7, 34.1, 29.7, 27.3, 14.2; HRMS (ESI): Calcd. $[C_{21}H_{24}N_2O_6S_2 + Na]^+$ $[M + Na]^+$: 487.0973.Found $[M + Na]^+$: 487.0978.

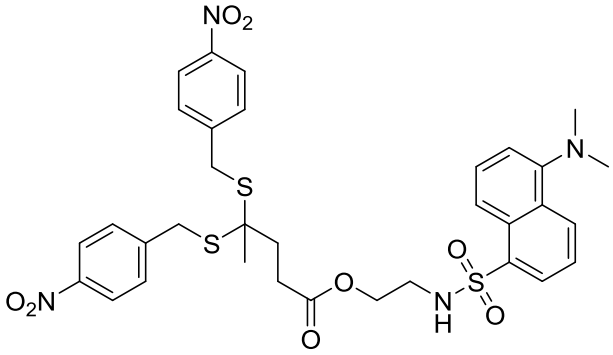
2-(2-Ethoxyethoxy)ethyl 4,4-bis((4-nitrobenzyl)thio)pentanoate(5b)



Starting from **3b** (0.10 g, 0.70 mmol), **5b** (0.20 g, 61%) was obtained as a green semi-solid. FTIR (ν, cm^{-1}): 3842, 3744, 3076, 2970, 2864, 1929, 1731, 1647, 1600, 1517, 1449, 1021, 855; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, *J* = 8.6 Hz, 4H), 7.49

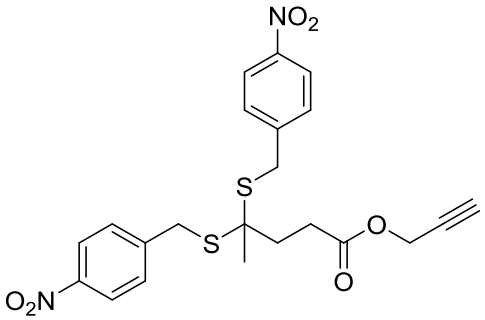
(d, *J* = 8.6 Hz, 4H), 4.21 (t, *J* = 4.7 Hz, 2H), 3.92 (s, 4H), 3.69 (t, *J* = 6.9 Hz, 2H), 3.64 (t, *J* = 5.0 Hz, 2H), 3.59 (t, *J* = 7.1 Hz, 2H), 3.52 (q, *J* = 7.0 Hz, 2H), 2.55 (t, *J* = 8.1 Hz, 2H),2.11 (t, *J* = 8.1 Hz, 2H), 1.52 (s, 3H), 1.21 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.6, 147.1, 145.3, 129.8, 123.8, 70.6, 69.7, 69.0, 66.6, 63.8, 61.4, 35.6, 34.0, 29.5, 27.2, 15.1; HRMS (ESI): Calcd. $[C_{25}H_{32}N_2O_8S_2 + Na]^+$ [M + Na]$^+$: 575.1497. Found [M + Na]$^+$: 575.1494.

2-((5-(Dimethylamino)naphthalene)-1-sulfonamido)ethyl4,4-bis((4-nitrobenzyl)thio)pentanoate (5c)



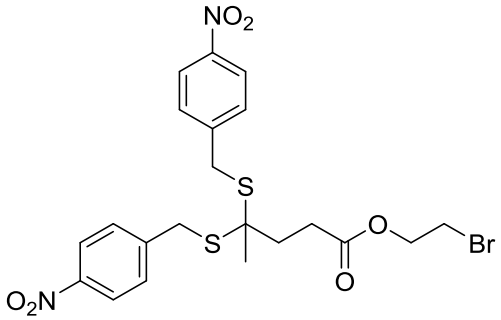
Starting from **3c** (0.20 g, 0.51 mmol), **5c** (0.14 g, 42%) was obtained as a greenish semi-solid. FTIR (ν, cm^{-1}): 3840, 3745, 3073, 2970, 2861, 1921, 1731, 1643, 1601, 1513, 1449, 1020, 855; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.54 (d, *J* = 8.5 Hz, 1H), 8.25 (t, *J* = 8.2 Hz, 1H), 8.23 (t, *J* = 8.2 Hz, 1H), 8.14 (d, *J* = 8.6 Hz, 4H), 7.54 (m, 2H),7.47 (d, *J* = 8.6 Hz, 4H), 7.17 (d, *J* = 7.5 Hz, 1H), 5.05 (br s, 1H), 4.01 (t, *J* = 5.4 Hz, 2H), 3.89 (s, 4H), 3.16 (q, *J* = 8.3 Hz, 2H), 2.87 (s, 6H), 2.38 (t, *J* = 6.2 Hz, 2H), 2.02 (t, *J* = 6.6 Hz, 2H), 1.49 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.4, 147.1, 145.2, 134.5, 130.7, 129.8, 129.6, 128.4, 123.8, 123.2, 118.4, 115.2, 114.0, 63.0, 61.2, 45.3, 42.2, 35.5, 34.0, 29.6, 29.3, 27.2, 22.6, 14.0; HRMS (ESI): Calcd. $[C_{33}H_{36}N_4O_8S_3 + Na]^+$ [M + H]$^+$: 713.1773. Found [M + H]$^+$: 713.1767.

Prop-2-yn-1-yl 4,4-bis((4-nitrobenzyl)thio)pentanoate (5d)



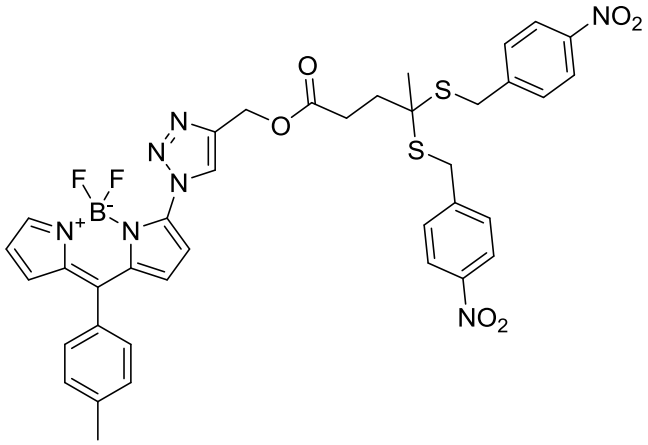
Starting from **3d** (0.12 g, 0.79 mmol), **5c** (0.16 g, 43%) was obtained as a white solid. MP 87-88 ˚C; FTIR (ν, cm^{-1}): 3891, 3857, 2922, 2851, 1908, 1735, 1639, 1421, 1130, 1020, 808; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, *J* = 8.7 Hz, 4H), 7.49 (d, *J* = 8.7 Hz, 4H),4.64 (d, *J* = 2.4 Hz, 2H), 3.91 (s, 4H), 2.54 (t, *J* = 8.0 Hz, 2H), 2.47 (t, *J* = 2.5 Hz, 1H), 2.10 (t, *J* = 7.7 Hz, 2H), 1.52 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.8, 147.1, 145.3, 129.9, 123.9, 77.2, 75.1, 61.3, 52.2, 35.6, 34.1, 29.4, 27.2; HRMS (ESI): Calcd. $[C_{22}H_{22}N_2O_6S_2 + Na]^+$ $[M + Na]^+$: 497.0816. Found $[M + Na]^+$: 497.0809.

2-bromoethyl 4,4-bis((4-nitrobenzyl)thio)pentanoate (5e)



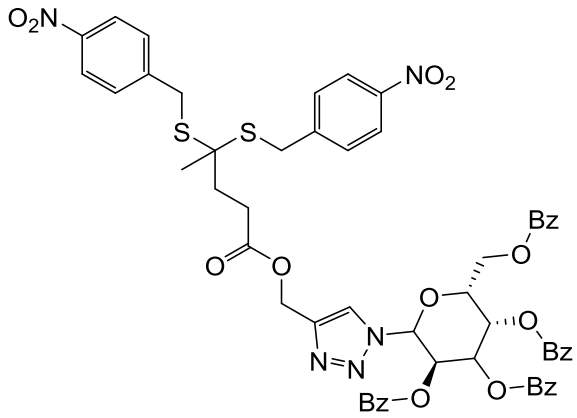
Starting from **3e** (0.12 g, 0.79 mmol), **5e** (0.16 g, Yield 43%) was obtained as a green liquid. FTIR (ν, cm^{-1}): 3857, 2922, 2965, 2132, 1743, 1421, 1351, 1209, 1019, 1130, 1020, 808, 760; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, *J* = 8.7 Hz, 4H),7.49 (d, *J* = 8.7 Hz, 4H),4.36 (t, *J* = 5.9 Hz, 2H), 3.92 (s, 4H),3.49(t, *J* = 5.9 Hz, 2H),2.55 (t, *J* = 8.0 Hz, 2H),2.11 (t, *J* = 7.7 Hz, 2H),1.53 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.1, 147.1, 145.2, 129.9, 123.8, 64.0, 61.3, 35.7, 34.1, 29.5, 28.7, 27.3; TLC-MS (ESI): Calcd. $[C_{21}H_{23}BrN_2O_6S_2 + Na]^+$ $[M + Na]^+$: 565. Found $[M + Na]^+$: 565.

(1-(5,5-Difluoro-10-(*p*-tolyl)-5*H*-5λ^4,6λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)-1*H*-1,2,3-triazol-4-yl)methyl 4,4-bis((4-nitrobenzyl)thio)pentanoate (7a)



To a mixture of **5d** (0.10 g, 0.22 mmol) and **6a** (0.08 g, 0.27 mmol) in DCM (5 mL), a solution of sodium ascorbate (0.07 g, 0.32 mmol) and copper sulphate pentahydrate (0.08 g, 0.32 mmol) in water (5 mL) was added at RT. The resulting mixture was stirred for 3 h under dark conditions. The reaction mixture was washed with DCM (3 x 5 mL). The combined organic layers were collected, dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The concentrate was purified by silica gel column chromatography using EtOAc/hexane as eluent to obtain **7a** (0.12 g, 73%) as a dark red solid. MP 115-117 °C; FTIR (ν, cm^{-1}): 3069, 2925, 2855, 1725, 1600, 1517, 1343, 1248, 1170, 976, 855;^{1}H NMR (400 MHz, CD_3CN): δ 8.90 (s, 1H), 8.15 (d, *J* = 8.7 Hz, 4H), 7.90 (s, 1H), 7.50–7.47 (m, 6H), 7.37 (d, *J* = 7.8 Hz, 2H),7.08 (d, *J* = 4.5 Hz, 1H), 7.05 (d, *J* = 4.5 Hz, 1H), 7.01 (d, *J* = 4.5 Hz, 1H), 6.60 (q, *J* = 6.1 Hz, 1H), 5.33 (s, 2H), 3.91 (s, 4H), 2.61 (t, *J* = 8.0 Hz, 2H), 2.5 (s, 3H), 2.19 (q, *J* = 7.7 Hz, 2H), 1.52 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.2, 149.7, 148.0, 147.0, 146.6, 146.3, 144.1, 143.2, 135.9, 134.1, 132.9, 132.0, 131.1, 130.3, 130.0, 126.6, 124.6, 121.2, 114.1, 61.9, 58.2, 36.3, 34.5, 30.4, 27.6, 21.5; HRMS (ESI): Calcd. $[C_{38}H_{34}BF_2N_7O_6S_2 + Na]^+$ $[M + Na]^+$: 820.1971. Found $[M + Na]^+$:820.1974.

(2*R*,3*S*,5*R*)-2-((Benzoyloxy)methyl)-6-(4-(((4,4-bis((4-nitrobenzyl)thio)pentanoyl)oxy)methyl)-1*H*-1,2,3-triazol-1-yl)tetrahydro-2*H*-pyran-3,4,5-triyl tribenzoate (7b)



To a mixture of **5d** (0.05 g, 0.11 mmol) **6b** (0.09 g, 0.15 mmol) in DCM (5 mL) a solution of sodium ascorbate (0.04 g, 0.16 mmol) and copper sulphate pentahydrate (0.04 g, 0.16 mmol) in water (5 mL) was added at RT. The mixture was stirred for 3 h. The reaction mixture is washed with DCM (3 x 5 mL) and the organic layers were collected, concentrated and dried over anhydrous sodium sulphate. The crude product was purified by silica gel flash column chromatography using EtOAc/hexane as eluent to obtain **7b** (0.08 g, Yield 65%) as a white solid. MP 132-134°C; FTIR (ν, cm^{-1}): 3846, 3745, 3113, 2926, 2855, 2252, 1922, 1735, 1602, 1514, 1371, 1262, 1133, 984, 853; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15–8.11 (m, 4H), 8.01–7.92 (m, 3H), 7.92 (d, *J* = 8.4 Hz, 2H), 7.82-7.75 (m, 4H), 7.55-7.50 (m, 2H), 7.46-7.41 (m, 8H), 7.39-7.35 (m, 3H), 7.31-7.26 (m, 3H), 6.26 (d, *J* = 9.4 Hz, 1H), 6.10 (d, *J* = 9.6 Hz, 1H), 5.93 (t, *J* = 9.5 Hz, 1H), 5.87 (t, *J* = 9.6 Hz, 1H), 5.18 (q, *J* = 12.8 Hz, 2H), 4.67 (d, *J* = 12.1 Hz, 1H), 4.51 (d, *J* = 12.2 Hz, 2H), 3.88 (s, 4H), 2.51 (t, *J* = 7.6 Hz, 2H), 2.09 (t, *J* = 8.2 Hz, 2H), 1.47 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.3,166.0, 165.5, 165.1, 164.7, 147.1, 145.3, 145.2, 143.3, 133.7, 133.5, 133.3, 130.o, 129.9, 129.8, 128.5, 128.4, 123.8, 122.3, 86.2, 75.7, 72.9, 68.8, 62.7, 61.3, 57.6, 35.4, 34.1, 29.7, 29.5, 27.2; HRMS (ESI): Calcd. $[C_{56}H_{49}N_5O_{15}S_2 + Na]^+$ $[M + Na]^+$: 1118.2564. Found $[M + Na]^+$: 1118.2529.

Detection of H_2S from 5a using HPLC: A stock solution of **5a** (2.5 mM) was prepared in DMSO and 10 mM NADH was prepared in HEPES buffer pH 7.4. A stock solution of commercially available *Escherichia Coli* nitroreductase (NTR) was prepared using 1 mg of a lyophilized powder dissolved in HEPES buffer (2 mL). A solution of **6a** (1 mM) was prepared in DMSO and stored under dark conditions. The reaction mixture was prepared by adding **5a** (4 µL, 2.5 mM), NADH (10 µL, 1 mM) and NTR (4 µL) in HEPES buffer (162 µL) pH 7.4. This mixture was incubated at 37

°C for 5 min. **6a** (20 µL, 1 mM) was added to this mixture. The resulting mixture was incubated at 37 °C for 10 min under dark conditions. The reaction mixture was filtered (0.22 mm) and injected (50 mL) in a high performance liquid chromatography (HPLC) attached with a fluorescence detector (excitation at 444 nm; emission at 520 nm). C-18 reversed phase column (250 mm × 4.6 mm, 5 mm) was used, the mobile phase was water: acetonitrile and a gradient starting with 60: 40 % →0 min, 60: 40 to 30: 70 → 0 - 10 min, 30: 70 to 0: 100 → 10 - 17 min, 0: 100 → 17 – 25 min, 0: 100 to 60: 40 → 25 – 28 min was used with flow of 1 mL/min. A solution of 50 mM of **6c** (retention time 11.7 min) was injected to confirm the formation of **6c** from **6a** by the H_2S generated from **5a**. Sodium sulfide (100 mM) was reacted with **6a** and served as a positive control.

NMR Spectra

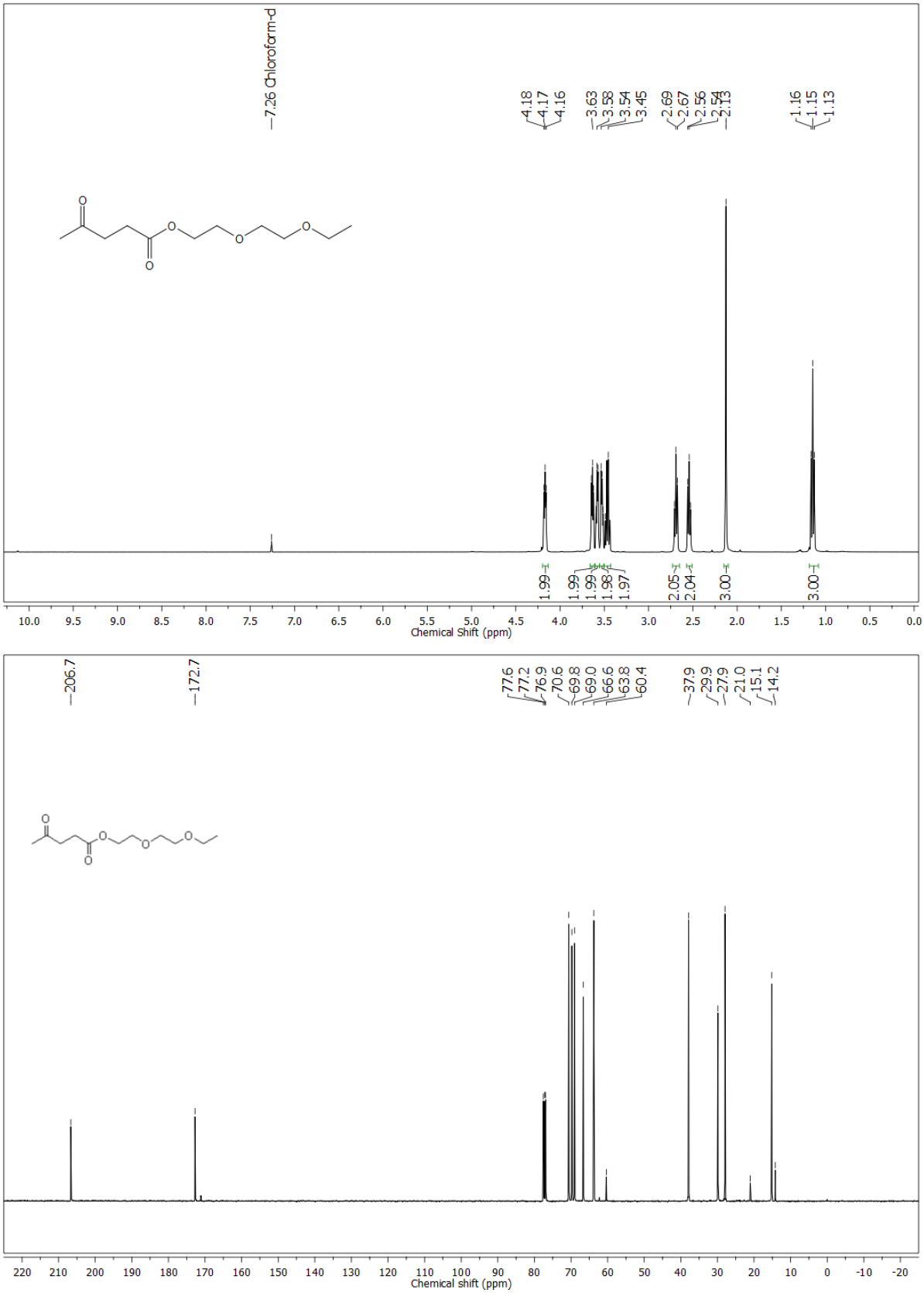


Figure 6. ^{1}H and ^{13}C NMR of **3b**

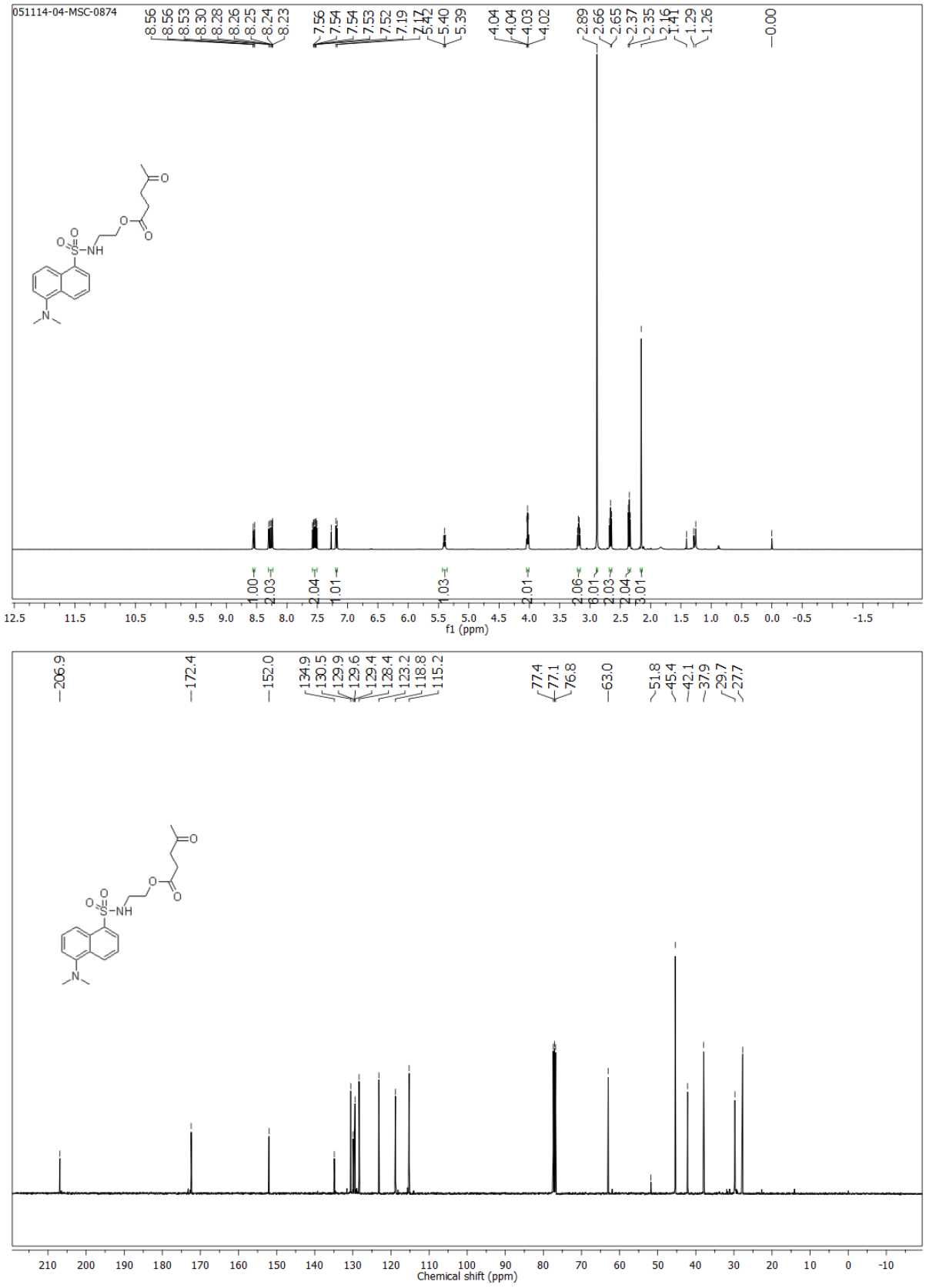


Figure 7 ^{1}H and ^{13}C NMR of **3c**

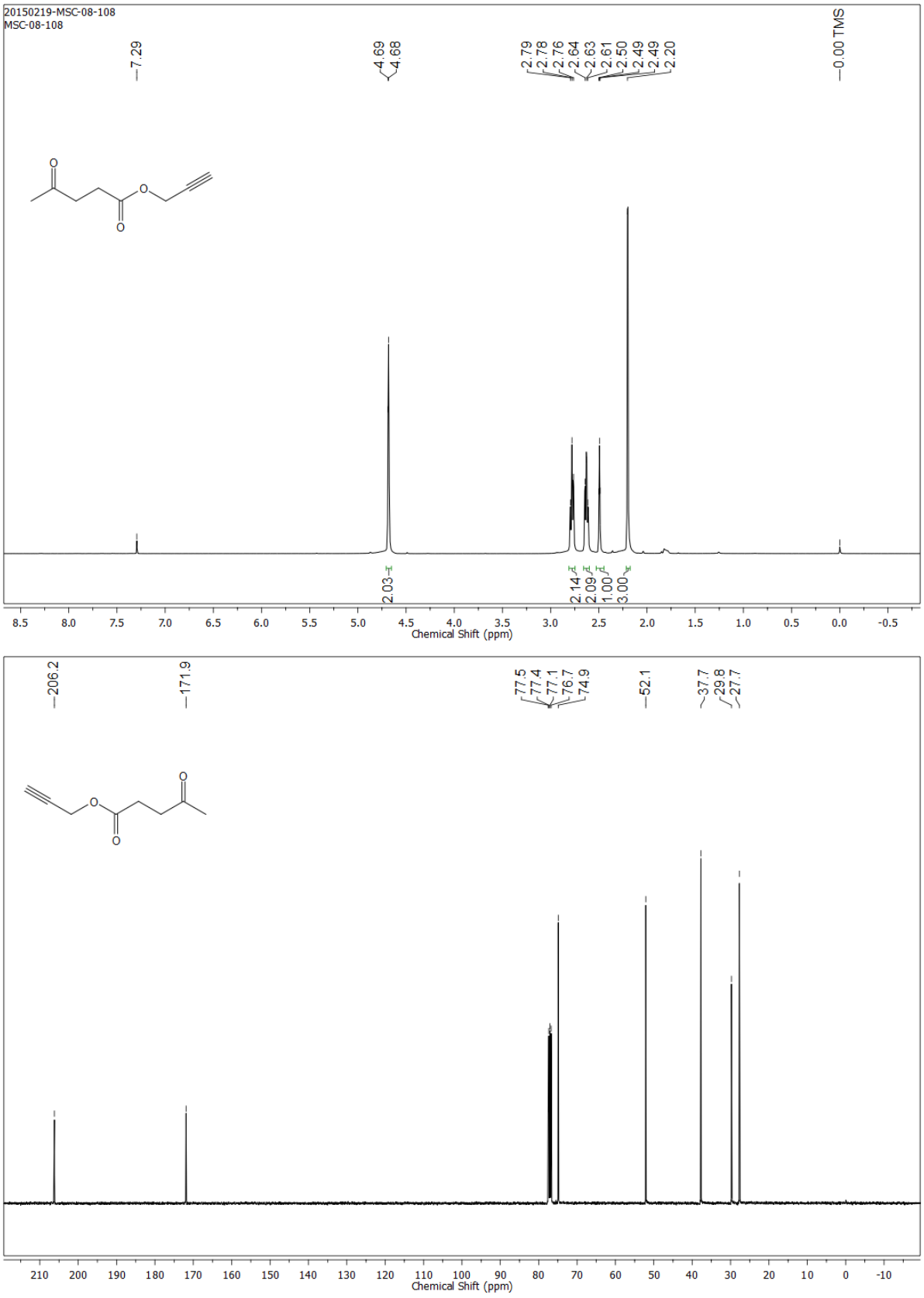


Figure 8 ^{1}H and ^{13}C NMR of **3d**

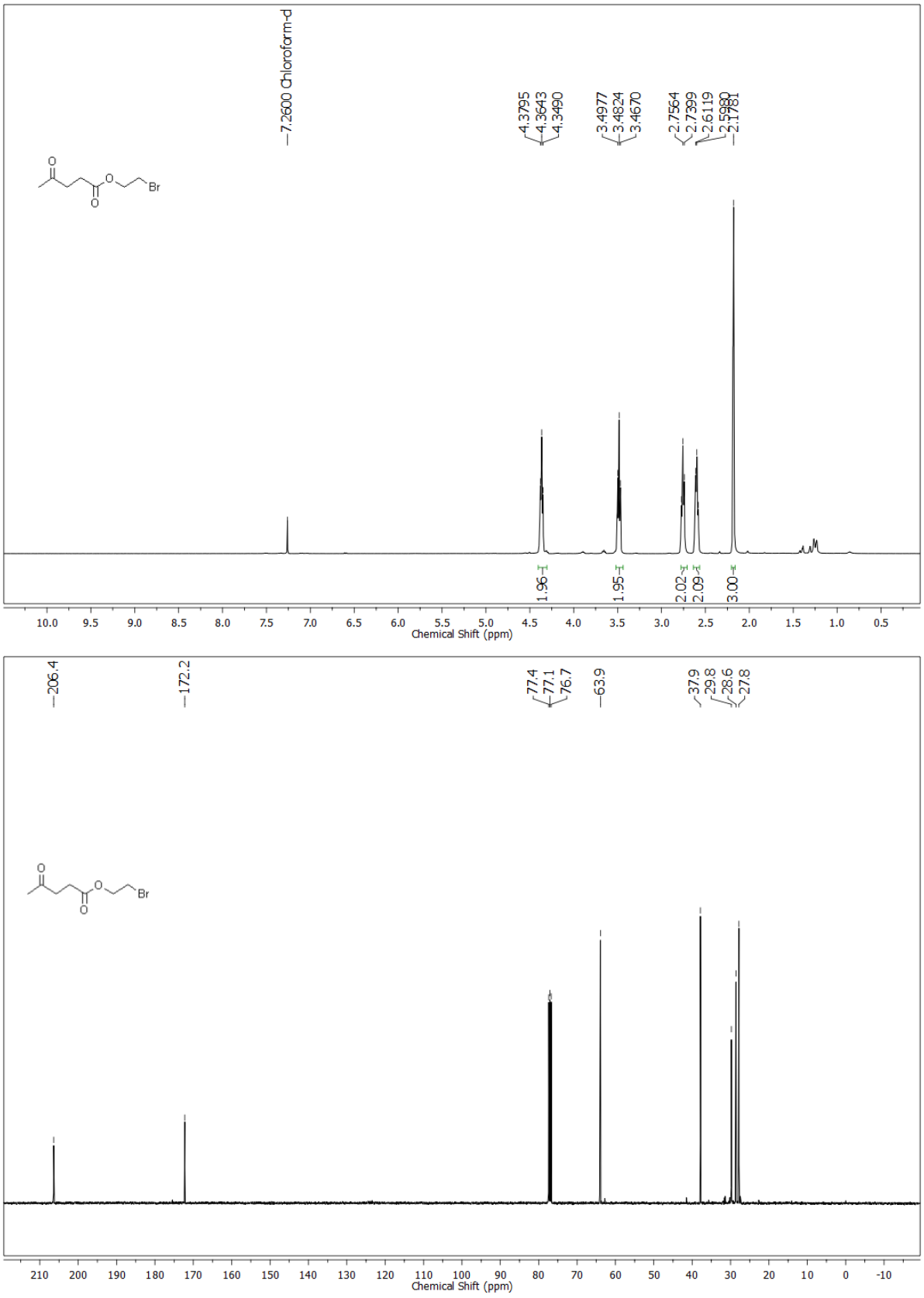


Figure 9 ^{1}H and ^{13}C NMR of **3e**

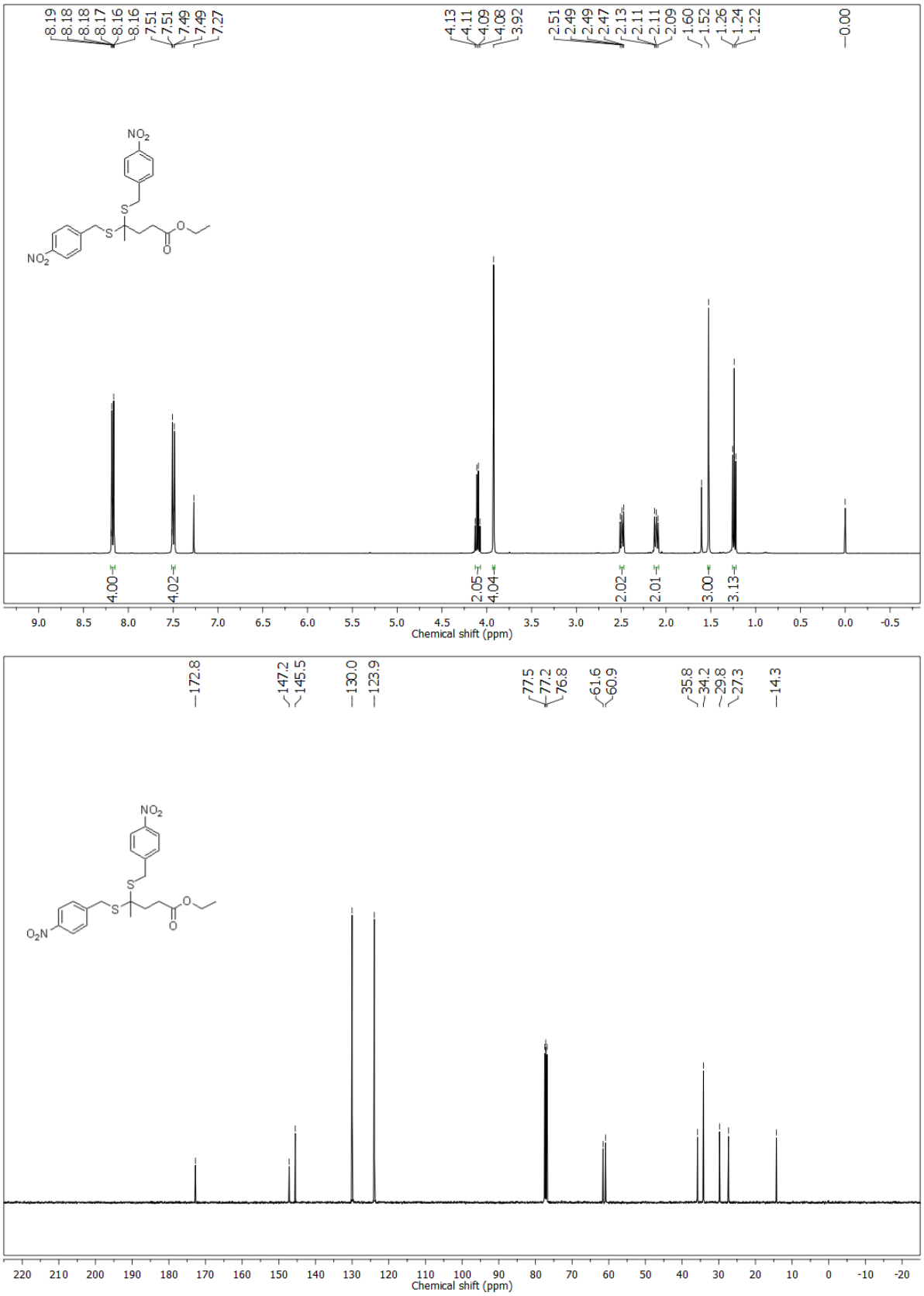


Figure 10 ^{1}H and ^{13}C NMR of **5a**

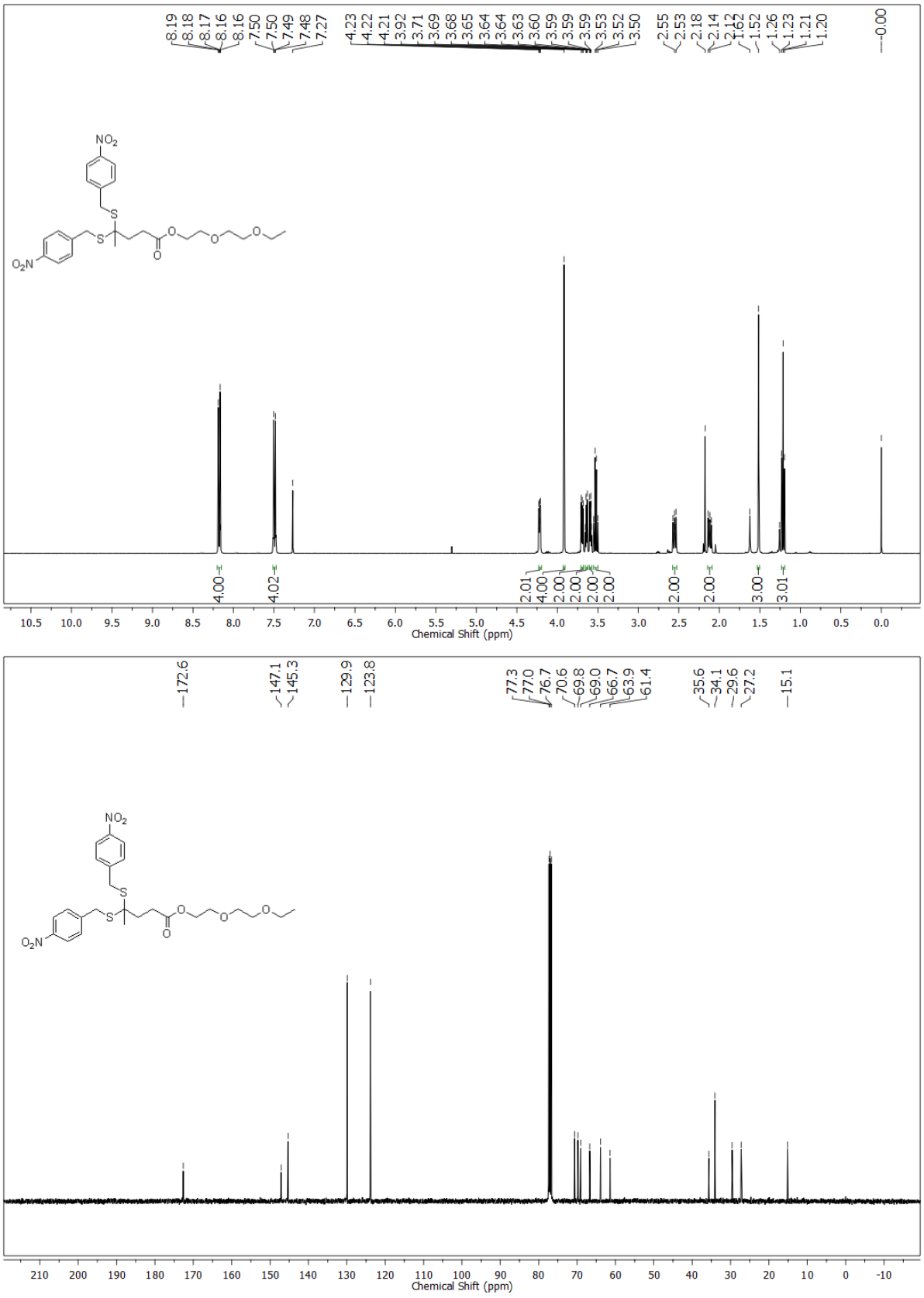


Figure 11 ^{1}H and ^{13}C NMR of **5b**

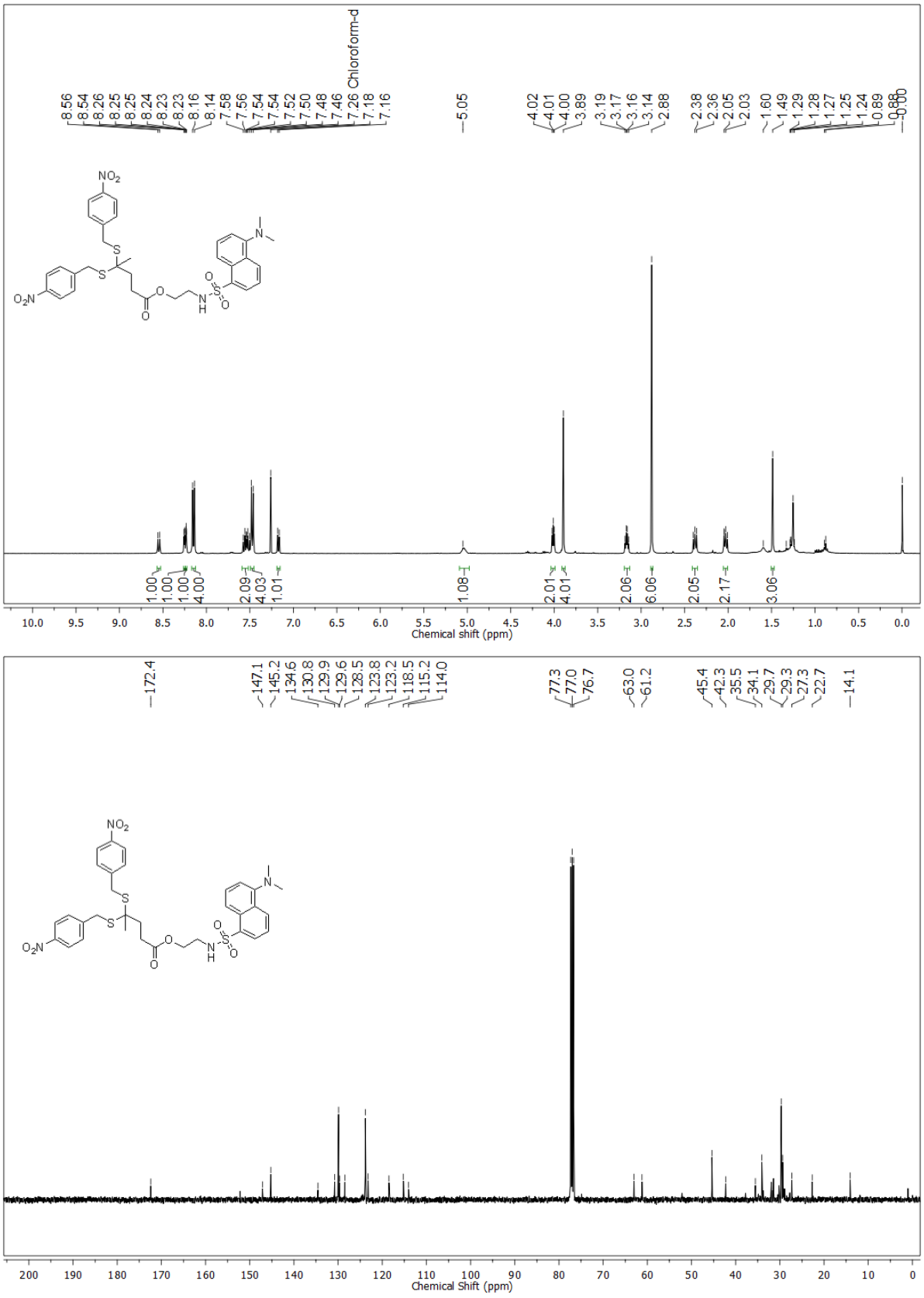


Figure 12 ^{1}H and ^{13}C NMR of **5c**

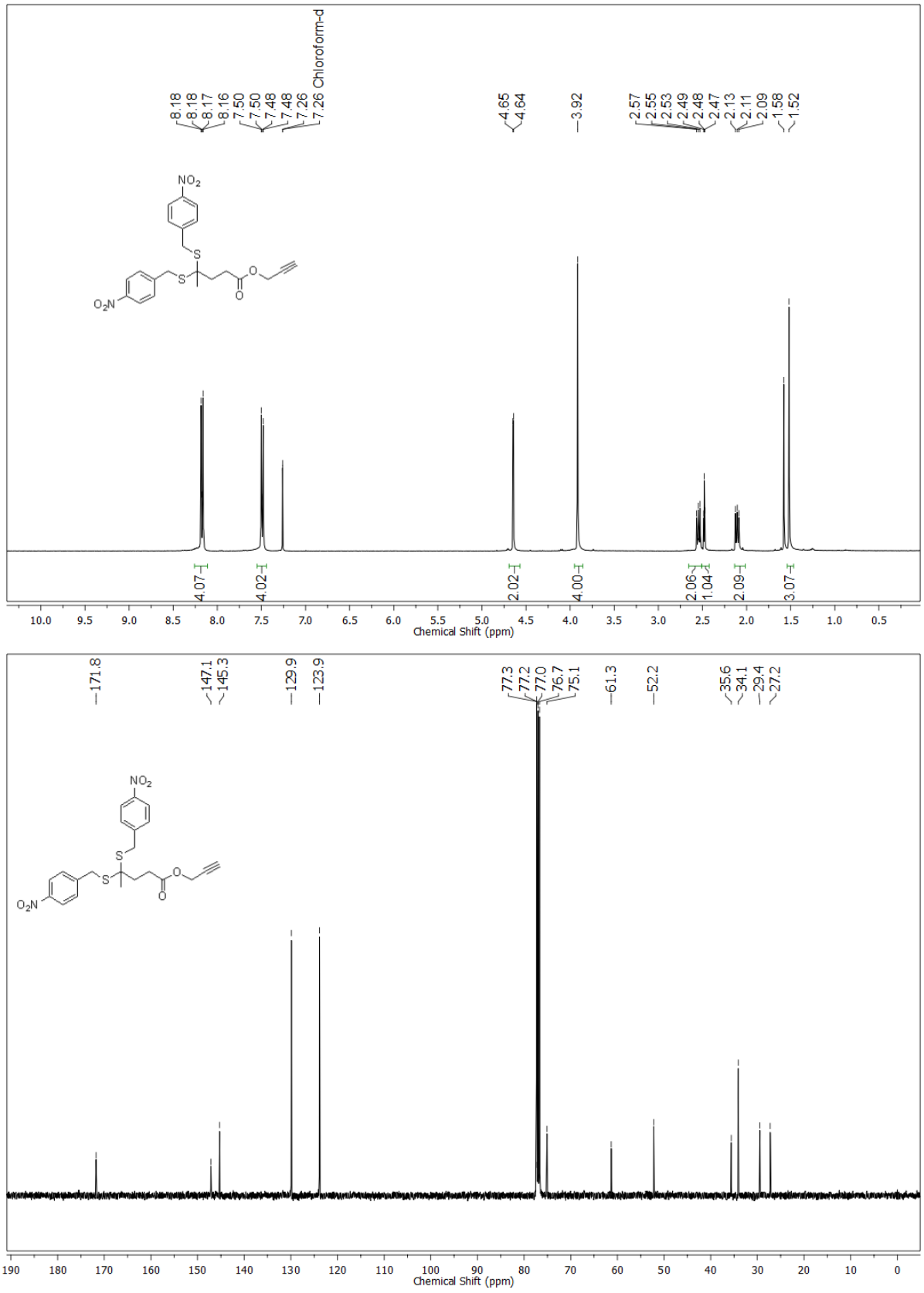


Figure 13 ^{1}H and ^{13}C NMR of **5d**

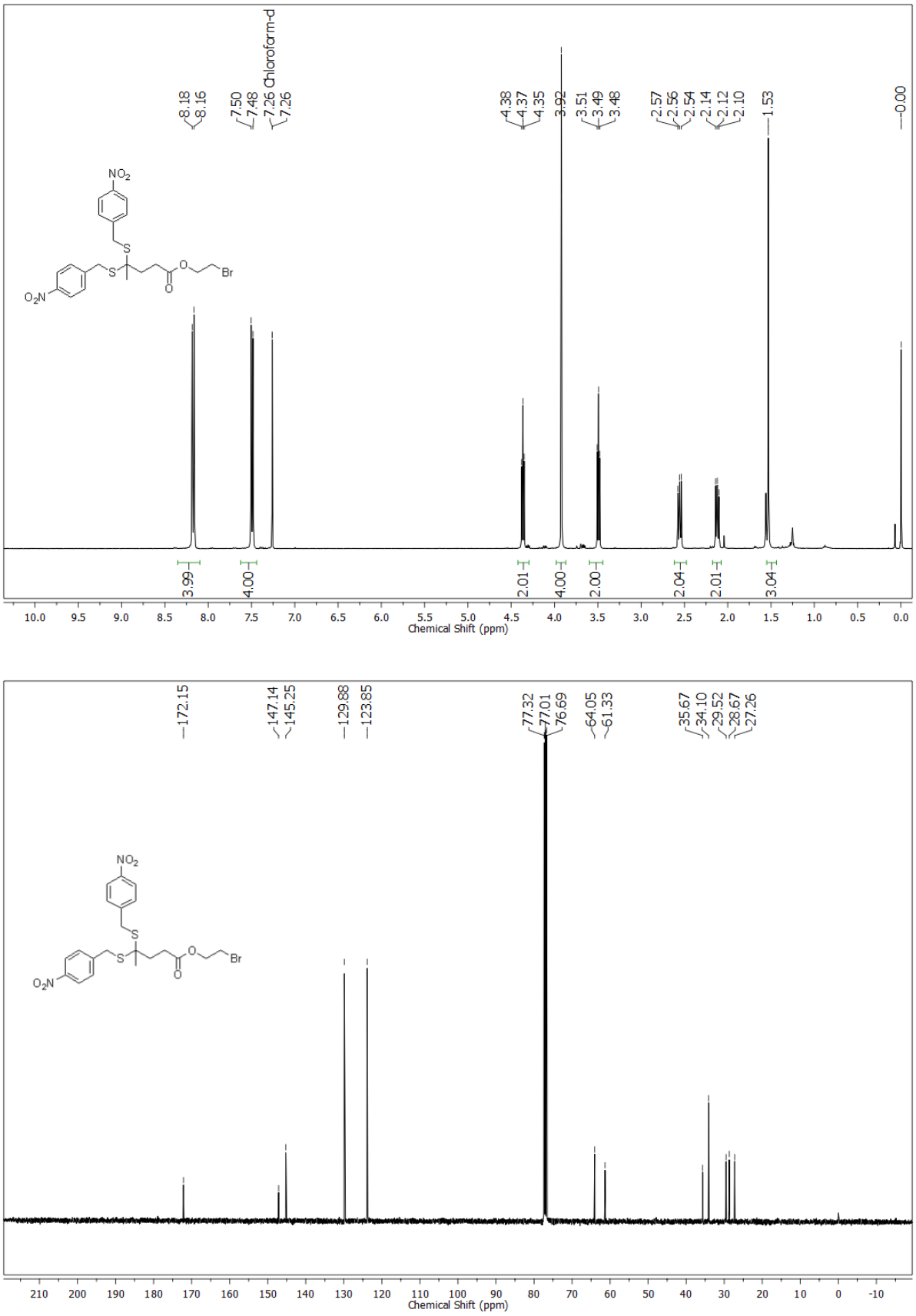


Figure 14 ^{1}H and ^{13}C NMR of **5e**

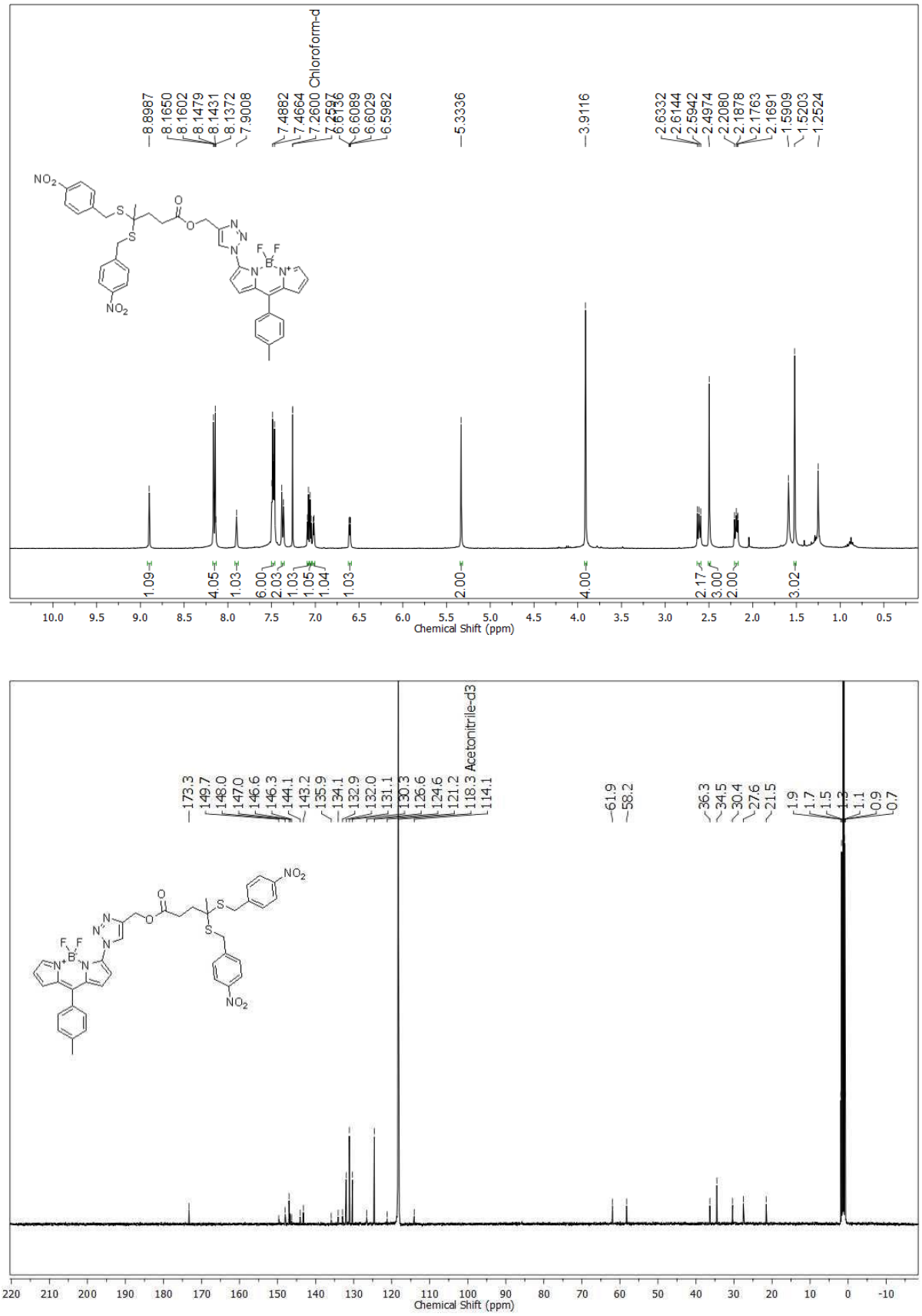


Figure 15 ^{1}H and ^{13}C NMR of **7a**

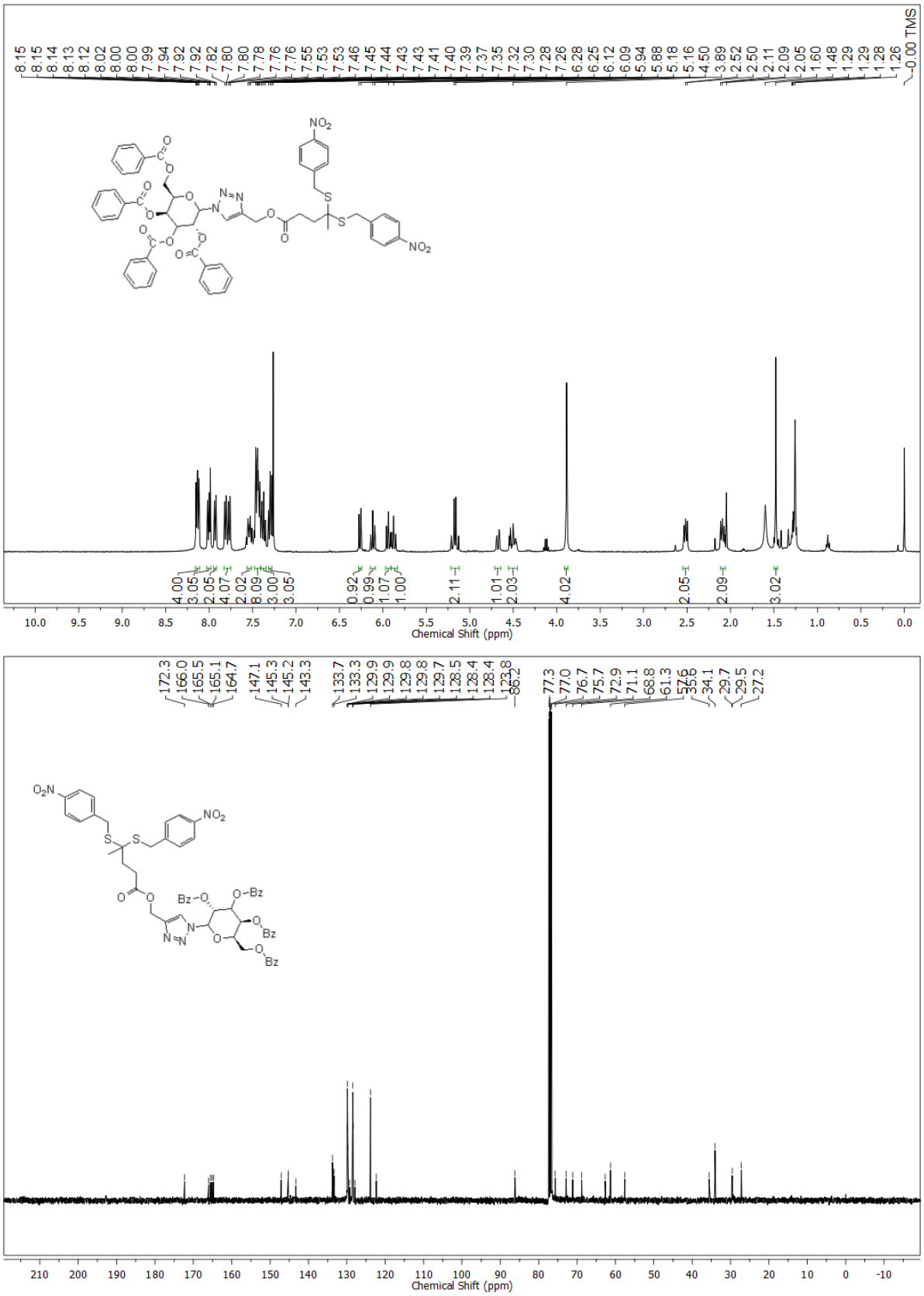


Figure 16 ^{1}H and ^{13}C NMR of **7b**

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