Design and Synthesis of Nitroreductase-activated HNO Donor

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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Supervisor: Prof. Harinath Chakrapani

Certificate

This is to certify that this dissertation entitled, '**Design and Synthesis of Nitroreductase-activated HNO Donor**' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Shayandeep Bhaumik at Indian Institute of Science Education and Research under the supervision of Prof. Harinath Chakrapani, Professor, Department of Chemistry, during the academic year 2023-2024.

C. Harinath

Prof. Harinath Chakrapani

Committee:

Supervisor – Prof. Harinath Chakrapani

TAC member – Dr. Amrita Hazra

This thesis is dedicated to Friends and Family

Declaration

I hereby declare that the matter embodied in the report entitled, '**Design and Synthesis of Nitroreductase-activated HNO Donor**' are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. Harinath Chakrapani and the same has not been submitted elsewhere for any other degree. Whereever others contribute, every effort is made to indicate this clearly, with due reference to the literate and acknowledgement of collaborative research and discussions.

Shayandeep Bhaumik

Date: 27-03-2024

List of Contents

1. Abbreviations

NMR..... Nuclear Magnetic Resonance

J..... Coupling Constant

Hz..... Hertz

M*Hz*..... Megahertz

EtOAc ..... Ethyl Acetate

DCM ..... Dichloromethane

DMF ..... Dimethylformamide

DMSO ..... Dimethyl Sulfoxide

mg ..... Milligram

g ..... Gram

mL ..... Millilitres

mmol ..... Millimoles

µM ..... Micromolar

µL ..... Microlitre

RT ..... Room temperature

RBF ..... Round Bottomed Flask

TLC ..... Thin Layer Chromatography

RotaVap ..... Rotary Evaporator

NMR ..... Nuclear Magnetic Resonance

HRMS ..... High Resolution Mass Spectrometry

EA ..... Ethyl Acetate

PE ..... Pet Ether/Hexane

NTR ..... Nitroreductase Enzyme

NADH ..... Nicotinamide Adenine Dinucleotide Hydride

2. Acknowledgement

First and foremost, I would like to thank my research advisor Prof. Harinath Chakrapani and my mentor, Dr. Laxman R. Sawase for their continuous guidance, supervision throughout my time in the lab. Their tremendous support continuously encouraged me to deal with various challenges in the completion of all the projects I worked on. None of my work would have been possible without their kind help and feedback.

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I would like to acknowledge the Chemistry Dept. of IISER Pune for providing me with all the facilities and atmosphere to carry forward promising scientific research and KVPY for the funding they provided to me. I also thank the instrument operators of NMR, HRMS for helping me get my data in a timely manner.

Above all, I am immensely grateful for the support I got from my mother and father and also the sacrifices they made in order for myself to just focus on studies and not worry about anything else. I am also grateful to my girlfriend, Moumita Pal who have been a constant support for me from the last 5 years during the lows and the highs, starting from the pandemic to the low times during my project last year. I am also thankful to all of my friends for always been there for me during my journey at IISER Pune, specially Pritam, Ranojoy and Hritwik.

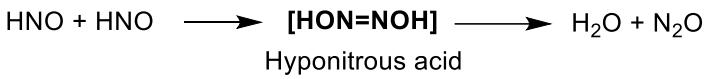
3. Abstract

HNO, which is the 1-e^- reduced form of NO, has recently been reported to enhance the antimycobacterial activity of anti-TB drugs like Rifampicin, enhance H_2O_2 mediated bacterial cell killing, increase the RSS levels in various bacterial cells such as *S. Aureus.* However, the HNO donor, Angeli's salt used in these studies concomitantly release Nitrite (NO_2^-) which may complicate the conclusions. Also, the esterase activated HNO donors reported from our lab, has shown high antibacterial activity (micromolar MIC values), however, also showed equal cytotoxic nature. We hypothesized that this cytotoxic nature of these donors can be bypassed by changing the trigger for HNO release from an ubiquitous esterase trigger to a more bacteria specific trigger, such as nitroreductase enzyme. In this project, we designed and synthesized nitroreductase activated HNO donors and evaluated the HNO release from them.

4. Introduction

4.1 Nitroxyl

HNO (nitroxyl) is a 1-e^- reduced and protonated form of NO (nitric oxide).[1] It was first reported by Angeli *et al* in 1896.[2] In solution form, it is a short-lived species which dimerizes rapidly to generate $H_2N_2O_2$ (hyponitrous acid), which then spontaneously decomposes to give N_2O (nitrous oxide) and H_2O (water).[3]



The ground state of HNO is a singlet while that of its conjugate base, NO^- is a triplet. This is thought to be the reason for a higher than expected *pKa* value of 11.4.[3] Hence, it is suggested that HNO is a weak acid and exists majorly as HNO at physiological pH.

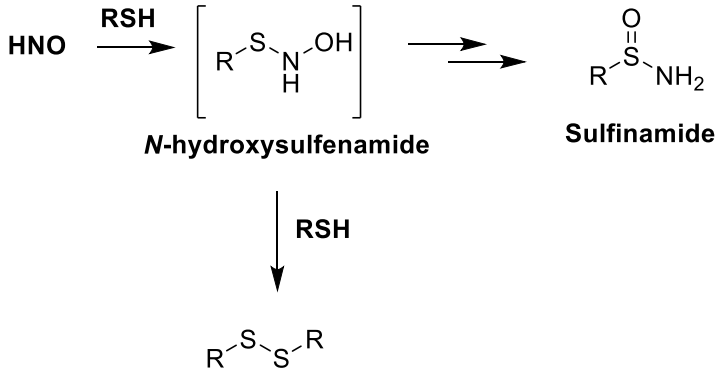
It is believed to be endogenously synthesized in cells via various biosynthetic pathways such as oxidation of hydroxylamine by heme proteins,[4] reaction between NO and H_2S,[5] reduction of NO by cytochrome C[6] etc.

4.2 Reactivity of HNO

4.2.1 Reaction of HNO with H_2S, persulfides and thiols

One of the distinct feature of HNO's reactivity is its reactivity with thiols. It can react with H_2S to generate polysufides via the *N*-mercaptohydroxylamine intermediate. Similarly, it can also react with persulfides to generate polysulfides.

Due to its thiophilic nature, it majorly targets the thiols and thiol proteins, resulting in a protein function.[7] The thiol groups in GSH, proteins etc attacks on the nitrogen (the electrophilic centre in HNO) of HNO generating the unstable *N*-hydroxysulfenamide intermediate which in presence of excess thiol can generate disulfide or can also rearrange to produce the sulfinamide depending on the concentration of thiols. (Scheme 1.1).[7]



Scheme 4.1. The reaction of HNO with thiols

An alternate reactive pathway between HNO and C-terminal thiols has also been reported which leads to the formation of thiosulfonate (RSO_2SR') and sulfohydroxamic acid (RSO_2NHOH) derivatives via a carboxyl assisted formation of sulfenic acid intermediate (RSOH).[8]

The reaction rates for the reaction of HNO with free cellular thiols such as glutathione[9] (GSH), *L*-cysteine[9], thiol residues of proteins such as oxymyoglobin[10], Cu/Zn superoxide dismutase[9] at physiological conditions are reported as 8 x 10^6 $M^{-1}s^{-1}$, 4.5 x 10^6 $M^{-1}s^{-1}$, 1 x 10^7 $M^{-1}s^{-1}$, 1 x 10^6 $M^{-1}s^{-1}$ respectively.

4.2.2 Reaction of HNO with metalloproteins

HNO reacts with ferrous or ferric heme proteins via substitution or redox reactions.

For example, HNO reacts with methemoglobin and metmyoglobin to give the ferrous nitrosyl complex where nitrosyl is oxidised to NO and Fe^{3+} centre is reduced to Fe^{2+}.[11]

While with ferrous heme proteins, HNO forms a stable adduct via coordinating with the metal centre via the Nitrogen atom.

$$MbFe^{3+} + HNO \longrightarrow MbFe^{2+}\text{- NO} + H^+$$

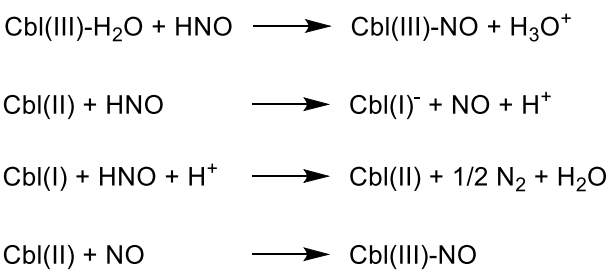
$$HbFe^{3+} + HNO \longrightarrow HbFe^{2+}\text{- NO} + H^+$$

$$HbFe^{2+} + HNO \longrightarrow HbFe^{2+}\text{- N(H)O}$$

Scheme 4.2. Reaction of HNO with Fe Heme proteins

Except transition metal-porphyrin complexes, Brasch and coworkers reported the reaction between HNO donor Angeli Salt (AS) and Co^{3+} and Co^{2+} in H_2O-Cbl^+(cobalamin) and H_2O-Cbl.[12] HNO reacts with H_2O-$Cbl(III)^+$ to form the

corresponding Cbl(III)-NO adduct. The reaction between HNO and H_2O-Cbl occurs in a stepwise manner where HNO reduces the Co^{2+} to Co^{+} while itself getting oxidised to NO. HNO then rapidly reacts with the Co^{+} to give Co^{2+} which reacts with the NO to generate Cbl(III)-NO adduct.[13]

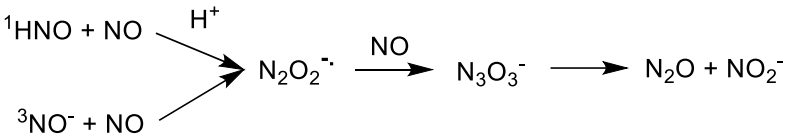


Scheme 4.3. Reaction of HNO with Cobalamin

HNO also reacts with Cu(II)-Zn superoxide dismutase to generate Cu(I) complex and NO. [14]

4.2.3 Reaction of HNO with NO and O_2

Both HNO and its conjugate base NO^- reacts with NO to ultimately generate N_2O and NO_2^-.[15] A stepwise mechanism is given in Scheme 4.4.



Scheme 4.4 Reaction of HNO with NO

According to a recent report by Sikora and coworkers, HNO is reported to react with O_2 at physiological pH to generate peroxynitrite which was detected via a boronate based probe.[16] However, the reaction rate for $^3NO^-$ is much faster than that of HNO due to spin forbidden process.

4.3 Chemical biology of HNO signalling

4.3.1 Anti-alcoholism

Nagasawa and coworkers reported that *N*-hydroxy derivative of cyanamide was able to inhibit aldehyde dehydrogenase.[17] Later it was shown that this inhibitory effect was modulated through the intermediacy of HNO. HNO was able to irreversibly modify the thiol residues in aldehyde dehydrogenase to sulfinamide groups.

4.3.2 Cardiovascular System

It was shown that HNO has both a positive inotropic (myocardial contractility) and lusitropic (myocardial contractility) effect.[18]

HNO has also been shown to promote vasodilation via its interaction with the regulatory Fe^{2+} Heme enzyme, sGC (soluble Guanylyl cyclase).[19]

Although the mechanism is unclear, treatment of Angeli's Salt (an HNO donor) before reperfusion provided considerable protection from the subsequent reperfusion injury.[20] Contrarily, treatment with Angeli's salt during the reperfusion process worsened the injury due to reperfusion.[21]

4.3.3 Cancer

HNO has been shown to demonstrate cytotoxic effects and hence anticancer effects. One of the mechanisms involve modification of the thiol residues of enzymes important to the functioning of cancer cells such as GAPDH (which is an important glycolytic enzyme),[22] PARP (involved in DNA damage repair).[23]

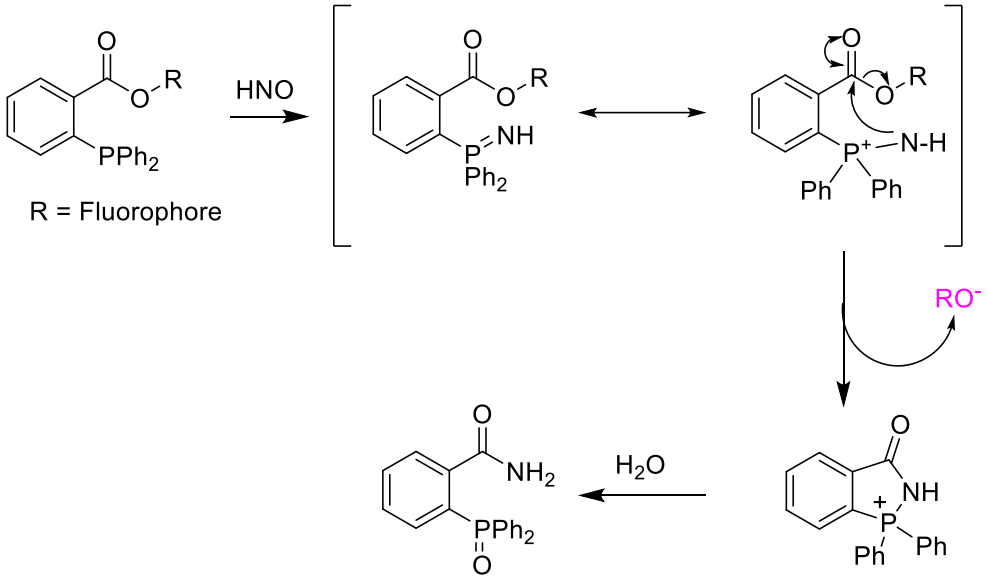
4.3.4 Antibacterial properties

HNO has also been reported to have antibacterial effects. HNO has been shown to synergistically enhance H_2O_2 mediated bacterial cell killing.[24] Angeli's salt (HNO donor) is reported to enhance the antimycobacterial activity of commonly used drugs such as Rifampicin at concentrations lower than its MIC value.[25] Angeli's salt is also reported to increase the cellular Reactive Sulfur Species (RSS) levels in *Staphylococcus aureus*.[26] Another report showed that PA-824 (a bicyclic nitroimidazole), which kills non-replicating Mtb via intracellular NO release, also generates HNO.[27]

4.4 Detection of HNO

4.4.1 Phosphine based probes

Phosphine base probes use the Staudinger ligation chemistry. The basic design of the probe includes a fluorophore which is linked to a scaffold containing a PPh_2 group in close proximity typically via an ester linkage. The reaction of this probe with HNO is accompanied by the release of the fluorophore and hence a fluorescent signal.[28]



Scheme 4.5 Proposed reaction mechanism of reaction of HNO with Phosphine based probes

The reaction starts by the attack of the nucleophillic phosphine group on the electrophilic N atom of HNO which is then followed by the attack of O^- on the P^+ of the phosphine group to give a 3 membered ring. This is then followed by an attack of the phosphorus atom of another molecule of the probe on the N atom to generate the shown phosphine aza-ylide (as shown in Scheme 4.5) and a molecule of phosphine oxide. This then leads to an intramolecular cyclisation reaction which results in the fluorophore being released and giving the fluorescent signal.

4.4.2 N_2O based detection by GC/MS

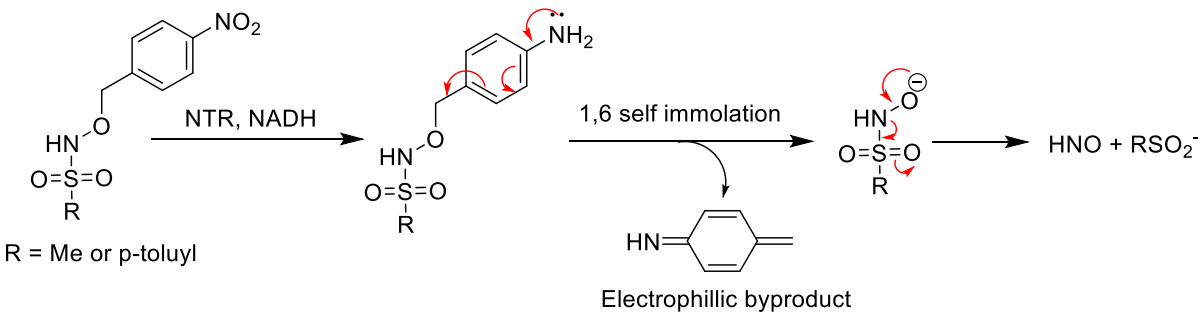
As already mentioned, in aqueous solution, HNO rapidly dimerizes to generate to form N_2O which is stable and can be detected using GC/MS (m/z = 44) and hence can be used as a proxy to detect the HNO levels in solution.

4.5 Design of nitroreductase activated HNO donor

HNO donors has been shown to demonstrate antibacterial properties but at the same time show cytotoxic effects to human cells. Also, from preliminary experiments done in our lab, we found that esterase activated HNO donors developed in our lab show antibacterial activity but are also equally cytotoxic which could be because of the nonselective release of HNO due to the ubiquitous presence of esterase enzyme in cells.

In this project, we hypothesized that by making a triggerable HNO donor-specific to bacterial cells, one could study the role of HNO to act as antibacterial agents. One such trigger which is specific to bacterial cells could be the nitroreductase enzyme.

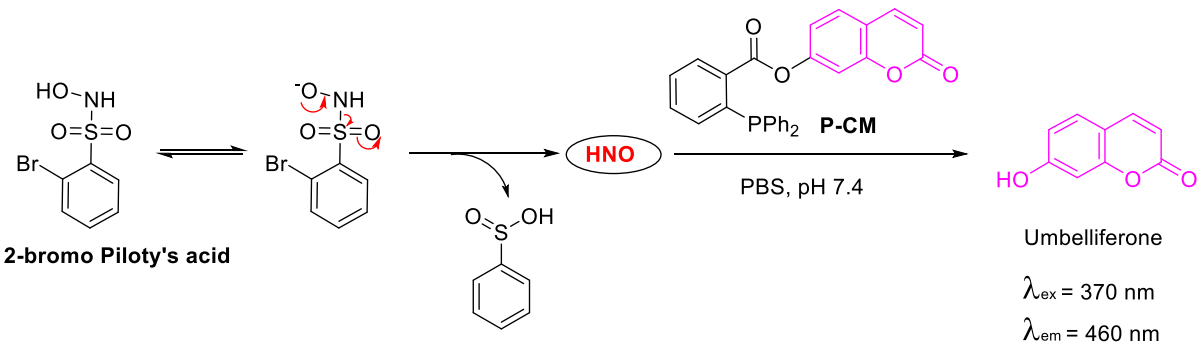
The most common scaffold used as a nitroreductase specific trigger is the 4-nitrobenzyl scaffold. (Scheme 4.6). Recently, Bruce King and coworkers reported a O-(p-nitro benzyl) substituted Sulfohydroxamic Acid derivate proposed to work as an NTR activated HNO donor.[33] (Scheme 4.6). However, they were unable to detect any N_2O generated (section 4.4.2) in presence of NTR, NADH. They proposed that this was due to the reaction of HNO with NADH where HNO gets reduced to hydroxylamine.[34]



Scheme 4.6 Nitroreductase activated HNO donor based on 4-nitrobenzyl scaffold

Besides that, there are other two limitations of this scaffold. First, the rate of self-immolation is low and as a result leads to low yields of the sulfohydroxamic acid scaffold. Second, it leads to the release of an electrophilic byproduct which can lead to cytotoxicity.

Now, in a preliminary experiment that I did, I observed that the side reaction of HNO with NADH doesn't lead to HNO not being detected from 2-bromo Piloty's acid (2-BrPA), an HNO donor which can release HNO when dissolved in pH 7.4 buffer (Scheme 4.7).



Scheme 4.7 Mechanism of HNO release from 2-BrPA in pH 7.4 buffer and its detection via P-CM probe

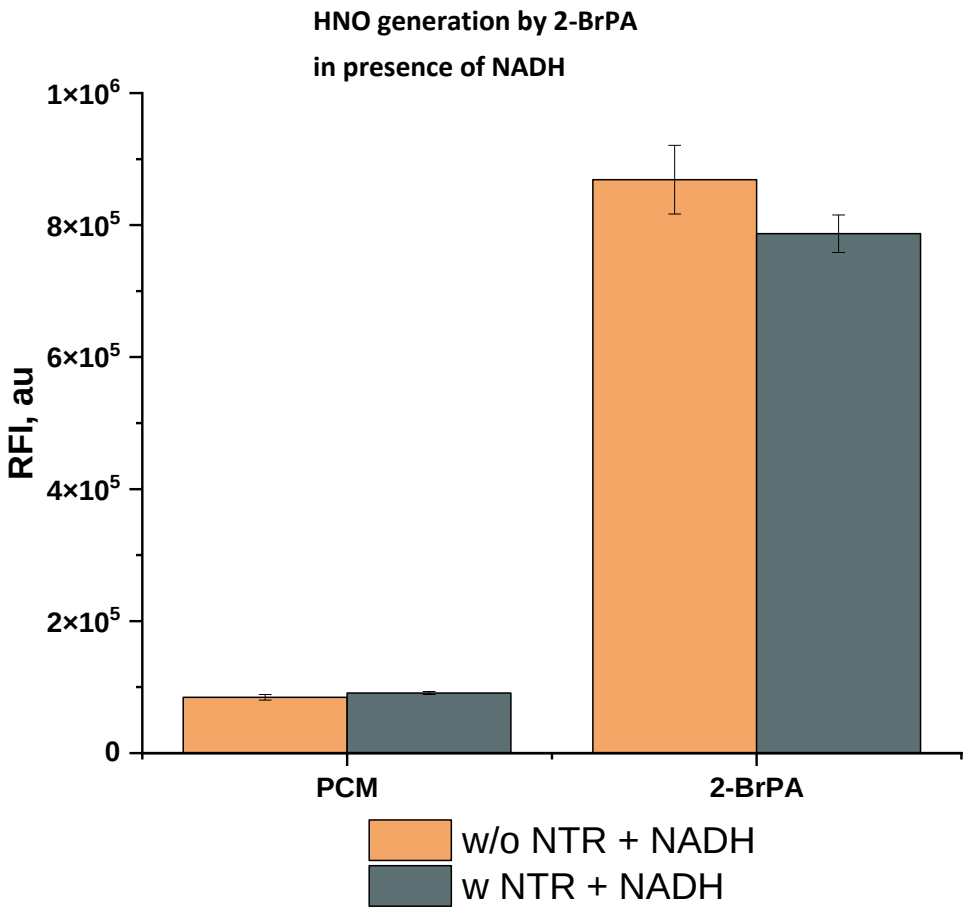
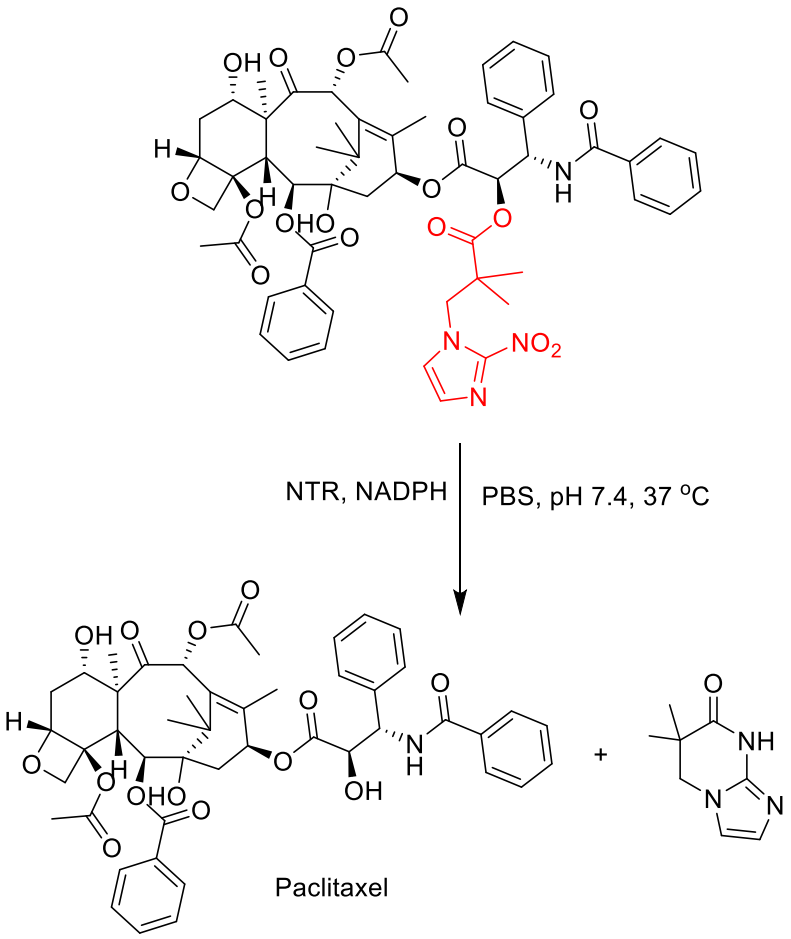


Figure 4.1 HNO detection from 2Br-PA in presence of NTR and NADH via fluorescence based P-CM probe

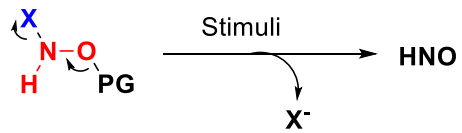
Hence, we hypothesized that a different NTR triggered scaffold which could release the sulfohydroxamic acid scaffold at a faster rate could be effective in releasing and detecting HNO in presence of Nitroreductase using the P-CM probe.

Recently, 2-nitroimidazole scaffold has been used by Zhang and coworkers as a hypoxia sensitive scaffold to deliver paclitaxel.[29] (Scheme 4.6). They showed that this scaffold was also able to release paclitaxel in presence of nitroreductase as a trigger. In presence of nitroreductase enzyme, the nitro group gets reduced to an amine group which releases the paclitaxel moiety via a thermodynamically favoured 1,6 intramolecular cyclization reaction. This scaffold doesn't lead to any obvious cytotoxic byproduct and we also think will have higher self-immolation rates due to the already thermodynamically favoured 1,6 intramolecular cyclization reaction accelerated by the Thorpe-Ingold effect.



Scheme 4.8 2-nitroimmidazole as a nitroreductase activated scaffold to release paclitaxel

Now, the general design of stimuli responsive HNO donors include a Protecting group (PG) attached to the O-atom of the hydroxyl amine type scaffold and a leaving group, X connected to the N-atom. (Scheme 4.8) The protecting group contains the stimuli responsive trigger which in response to the stimuli will get cleaved which releases the O^- ion which then leads to the elimination of X^- and generation of HNO.



Scheme 4.9 Stimuli Responsive HNO donors

For X = $ArSO_2$, the scaffold is called piloty's acid scaffold (or sulfohydroxamic acid scaffold). Based on these scaffolds, high yielding, Esterase activated HNO donors with tunable HNO release has been reported from our lab. [32]

Hence, with all these factors in mind, we designed the following molecule to act as a nitroreductase-activated HNO donor and evaluate the HNO release from this molecule.

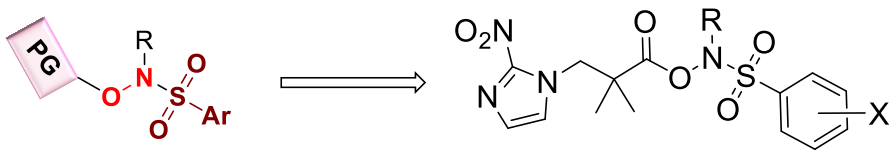
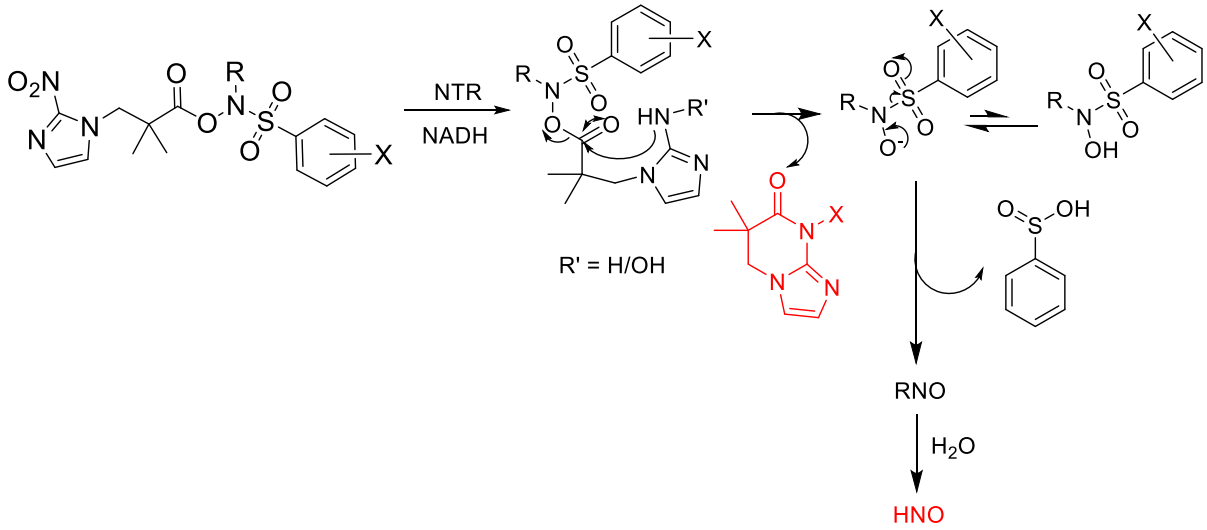


Figure 4.2 Molecular design of nitroreductase activated HNO donor

Here is the proposed mechanism by which it will release HNO in presence of nitroreductase enzyme as a trigger.



Scheme 4.10 Proposed mechanism of HNO release by the designed donor in presence of nitroreductase trigger

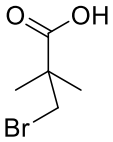
5. Materials and Methods

5.1 General methods

All reactions are conducted under a nitrogen atmosphere unless stated otherwise. All the chemicals and solvents were purchased from commercial sources and used as received unless stated otherwise. Column chromatography was performed using silica gel-Rankem (60-120 mesh). 1H and 13C NMR spectra were recorded on a Bruker 400 MHz (or 100 MHz for 13C) NMR spectrometer using residual solvent signals (CDCl3 δH = 7.26 ppm, δC = 77.2 ppm) and an internal tetramethylsilane (δH = 0.00, δC = 0.0) as standard. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The following abbreviations are used: bs (broad signal), m (multiplet), s(singlet), d (doublet), t (triplet), dd (doublet of doublets), dt (doublet of triplets), dq (doublet of quartets), td (triplet of doublets), tt (triplet of triplets), ddd (doublet of doublet of doublets), and ddt (doublet of doublet of triplets).

5.2 Synthesis

3-bromo-2,2-dimethyl propanoic acid (2) –

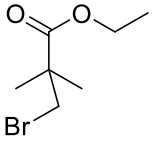


Compound **2** was synthesized according to a reported protocol.[30]

3-hydroxypivaloic acid (3.3 g, 27.93 mmoles) was taken in an RBF and dissolved in 33 ml of 48 % HBr (in water) solution. The reaction mixture was refluxed (130 ^{0}C) overnight for 18 hours. The reaction mixture was cooled to RT and diluted with water. Extraction was done using EtOAc (50 ml x 3) and, the organic layer was concentrated, and the crude reaction mixture was purified via column chromatography.

Yield (4.12 g, 81 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.52 (s, 2H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.12, 44.1, 40.75, 24.18

Ethyl 3-bromo-2,2-dimethyl propanoate (3) -



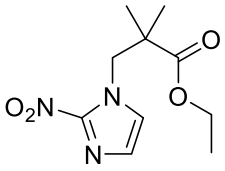
Compound **3** was synthesized according to a reported protocol.[30]

Compound **2** (4 g, 22.1 mmoles) was taken in an RBF and dissolved in 40 ml of EtOH and 4-5 drops of conc. H_2SO_4 was added. The reaction mixture was refluxed (80 ^{0}C) for 6-8 hours. The reaction mixture was cooled to RT, and the solvent was evaporated. The reaction mixture was dissolved in EtOAc and

washed with sat. $NaHCO_3$ solution (50 ml x 3). The organic layer was then evaporated to obtain the product.

Yield (3.2 g, 68 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.12 Hz, 2H), 3.50 (s, 2H), 1.31 (s, 6H), 1.27 (t, *J* = 7.12 Hz, 3H).

Ethyl 2,2-dimethyl-3-(2-nitro-1H-imidazole-1-yl)-propanoate (4) –

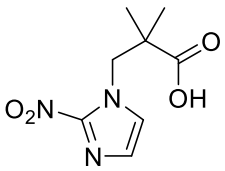


Compound **4** was synthesized according to a reported protocol.[29]

2-nitroimidazole (605 mg, 5.35 mmoles) was taken in an RBF and dissolved in 15 mL DMF, and Cs_2CO_3 (3.49, 10.7 mmoles) g was added, and the reaction mixture was stirred at RT for 20 minutes. Compound **3** (2.24 g, 10.7 mmoles) was then added to the solution, and the reaction mixture was heated at 100 ^{0}C for 24 h. The reaction mixture was filtered, and the filtrate was diluted with ice-cold water and extracted with EtOAc. Then the organic layer was concentrated, and the crude reaction mixture was purified via column chromatography.

Yield (1.01 g, 81%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (d, *J* = 0.92 Hz, 1H), 7.11 (d, *J* = 0.84 Hz, 1H), 4.74 (s, 2H), 4.14 (q, *J* = 7.12 Hz, 2H), 1.25 (t, *J* = 7.12 Hz, 3H), 1.21 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.47, 128.15, 126.31, 61.59, 55.31, 44.64, 22.97, 14.02.

2,2-dimethyl-3-(2-nitro-1H-imidazole-1-yl)-propanoic acid (5) –

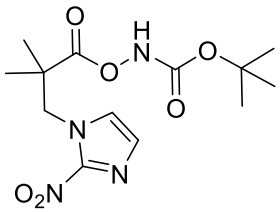


Compound **5** was synthesized according to a reported protocol.[29]

Compound **4** (850 mg, 3.52 mmoles) was dissolved in 10 ml of 1 M NaOH solution (EtOH:Water, 1:1(v/v)). The reaction mixture was stirred at RT for 4-5 hrs. The reaction mixture was evaporated to remove EtOH and then pH of the reaction mixture was adjusted to pH = 2 by using 1 N dil. HCl solution. Extraction was done using EtOAc. The organic layer was evaporated to obtain the product which was used without further purification.

Crude yield (665 mg, 87 %)

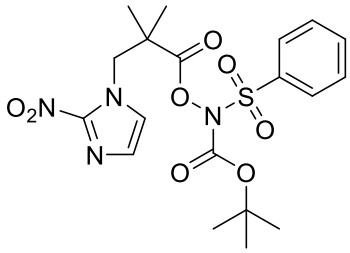
tert-butyl ((2,2-dimethyl-3-(2-nitro-1H-imidazol-1-yl)propanoyl)oxy)carbamate (6) –



Compound **5** (48 mg, 0.225 mmoles), EDC.HCl (65 mg, 0.337 mmoles), DMAP (83 mg, 0.675 mmoles), N-Boc hydroxylamine (30 mg, 0.270 mmoles) was charged into an RBF and dissolved in 5 mL of DCM and the reaction mixture was stirred overnight at RT. TLC was checked for the complete consumption of N-Boc hydroxylamine. Upon completion, the reaction mixture was evaporated and the crude reaction mixture was purified via column chromatography.

Yield (44 mg, 60 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.36 (d, *J* = 1.04 Hz, 1H), 7.13 (d, *J* = 1.08 Hz, 1H), 4.82 (s, 2H), 1.49 (s, 9H), 1.31 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.87, 155.41, 128.5, 126.79, 83.86, 60.42, 54.84, 44.03, 28.03, 22.65

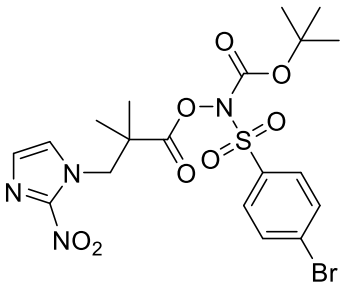
tert-butyl((2,2-dimethyl-3-(2-nitro-1H-imidazol-1-yl)propanoyl)oxy)(phenylsulfonyl) carbamate (7a) –



Compound **6** (18 mg, 0.054 mmoles) was dissolved in 3 mL THF. The reaction mixture was cooled to 0 ^{0}C. NaH (4.39 mg, 0.109 mmoles) (60% w/w suspension) was added, and the reaction mixture was stirred for 15-20 minutes. The ice bath was removed, and Benzene sulphonyl chloride (7.72 uL, 0.06 mmoles) was added, and the reaction mixture was stirred at RT for 4 h. Upon completion, the reaction mixture was concentrated, and extraction was done using DCM and H_2O. The organic layer was concentrated, and the crude reaction mixture was purified using prep TLC.

Yield (8 mg, 31 %) ^{1}H NMR (400 M*Hz*, $CDCl_3$) δ 8.01 (dd, J = 8.4, 1.1 *Hz*, 1H), 7.75 – 7.69 (m, 1H), 7.59 (dd, *J* = 10.8, 4.9 *Hz*, 1H), 7.47 (d, J = 1.1 Hz, 1H), 7.15 (d, *J* = 1.0 Hz, 1H), 4.93 (s, 2H), 1.39 (s, 9H), 1.38 (s, 6H). ^{13}C NMR (100 M*Hz*, $CDCl_3$) δ 172.34, 148.7, 137.49, 129.13, 128.81, 126.76, 87.29, 54.52, 44.39, 29.71, 27.85

tert-butyl((2,2-dimethyl-3-(2-nitro-1H-imidazol-1-yl)propanoyl)oxy)(4-bromophenylsulfonyl) carbamate (7b) –



Compound **6** (19 mg, 0.057) was dissolved in 3 mL THF. The reaction mixture was cooled to 0 ^{0}C. NaH (4.63 mg,

0.115 mmoles) (60% w/w suspension) was added, and the reaction mixture was stirred for 15-20 minutes. The ice bath was removed, and 4-bromobenzene sulphonyl chloride (16.26 mg, 0.063 mmoles) was added, and the reaction mixture was stirred at RT for 4 h. Upon completion, the reaction mixture was concentrated, and extraction was done using DCM and H_2O. The organic layer was concentrated, and the crude reaction mixture was purified using flash column chromatography.

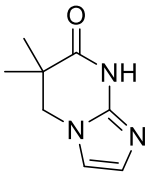
Yield (15 mg, 47 %) ^{1}H NMR (400 M*Hz*, $CDCl_3$) δ 7.89 – 7.84 (m, 2H), 7.75 – 7.71 (m, 2H), 7.43 (d, *J* = 1.1 Hz, 1H), 7.16 (d, *J* = 1.0 Hz, 1H), 4.92 (s, 2H), 1.42 (s, 9H), 1.38 (s, 6H). ^{13}C NMR (400 M*Hz*, $CDCl_3$) δ 172.55, 148.4, 136.56, 132.59, 130.26, 126.84, 87.72, 54.57, 44.49, 29.84, 28.02.

tert-butyl((2,2-dimethyl-3-(2-nitro-1H-imidazol-1-yl)propanoyl)oxy)(2-bromophenylsulfonyl) carbamate (7c) –

Compound **6** (21 mg, 0.064) was dissolved in 3 mL THF. The reaction mixture was cooled to 0 ^{0}C. NaH (5.12 mg, 0.128 mmoles) (60% w/w suspension) was added, and the reaction mixture was stirred for 15-20 minutes. The ice bath was removed, and 2-bromobenzene sulphonyl chloride (17.98 mg, 0.07 mmoles) was added, and the reaction mixture was stirred at RT for 4 h. Upon completion, the reaction mixture was concentrated, and extraction was done using DCM and H_2O. The organic layer was concentrated, and the crude reaction mixture was purified using flash column chromatography.

Yield (12 mg, 34 %) ^{1}H NMR (400 M*Hz*, $CDCl_3$) δ 8.28 – 8.25 (m, 1H), 7.82 – 7.78 (m, 1H), 7.55 – 7.50 (m, 3H), 7.15 (d, *J* = 0.9 Hz, 1H), 4.94 (s, 2H), 1.39 (s, 6H), 1.36 (s, 9H). ^{13}C NMR (400 M*Hz*, $CDCl_3$) δ 172.84, 147.97, 137.75, 135.95, 135.36, 133.75, 128.89, 127.69, 127.08, 120.85, 87.52, 54.54, 44.47, 29.83, 27.94.

6,6-dimethyl-5,6-dihydroimidazo[1,2-a]pyrimidin-7(8H)-one (9) -

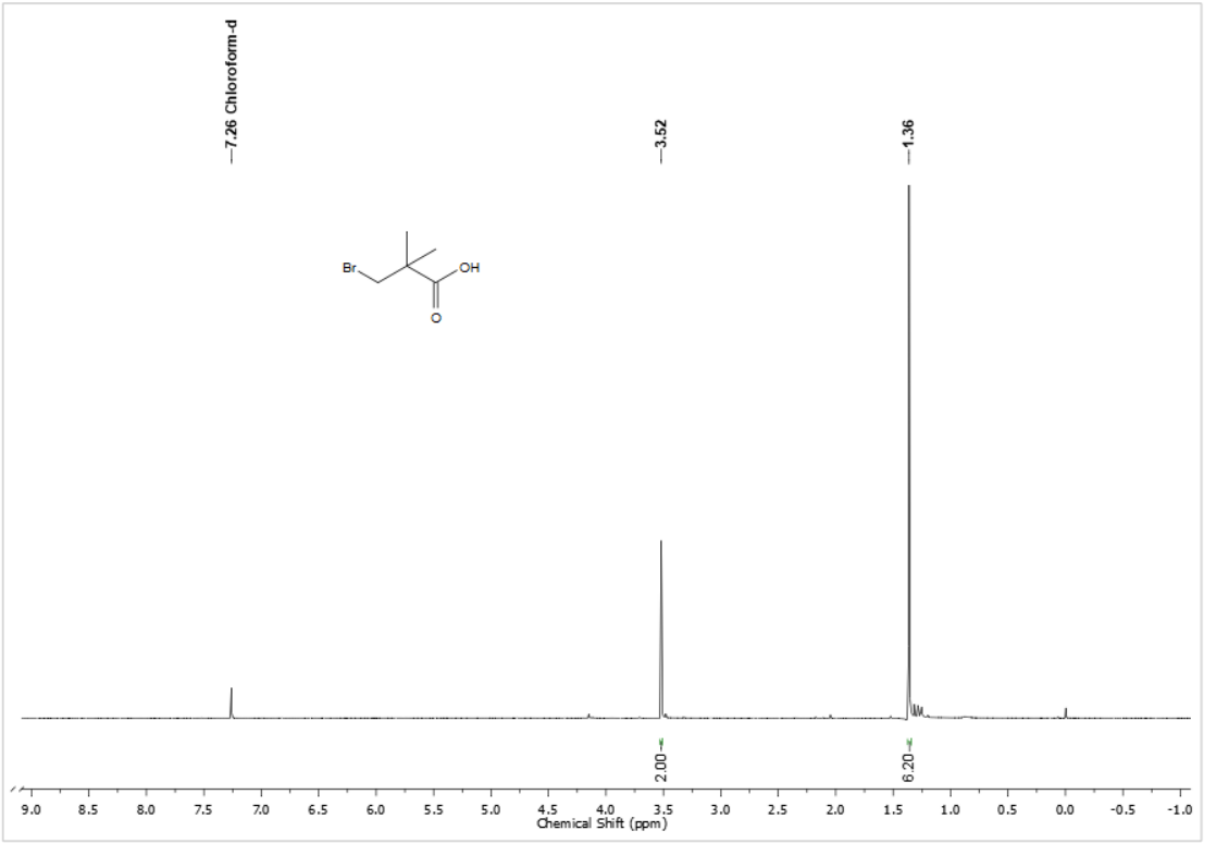


Compound 9 was synthesized according to a reported protocol.[29]

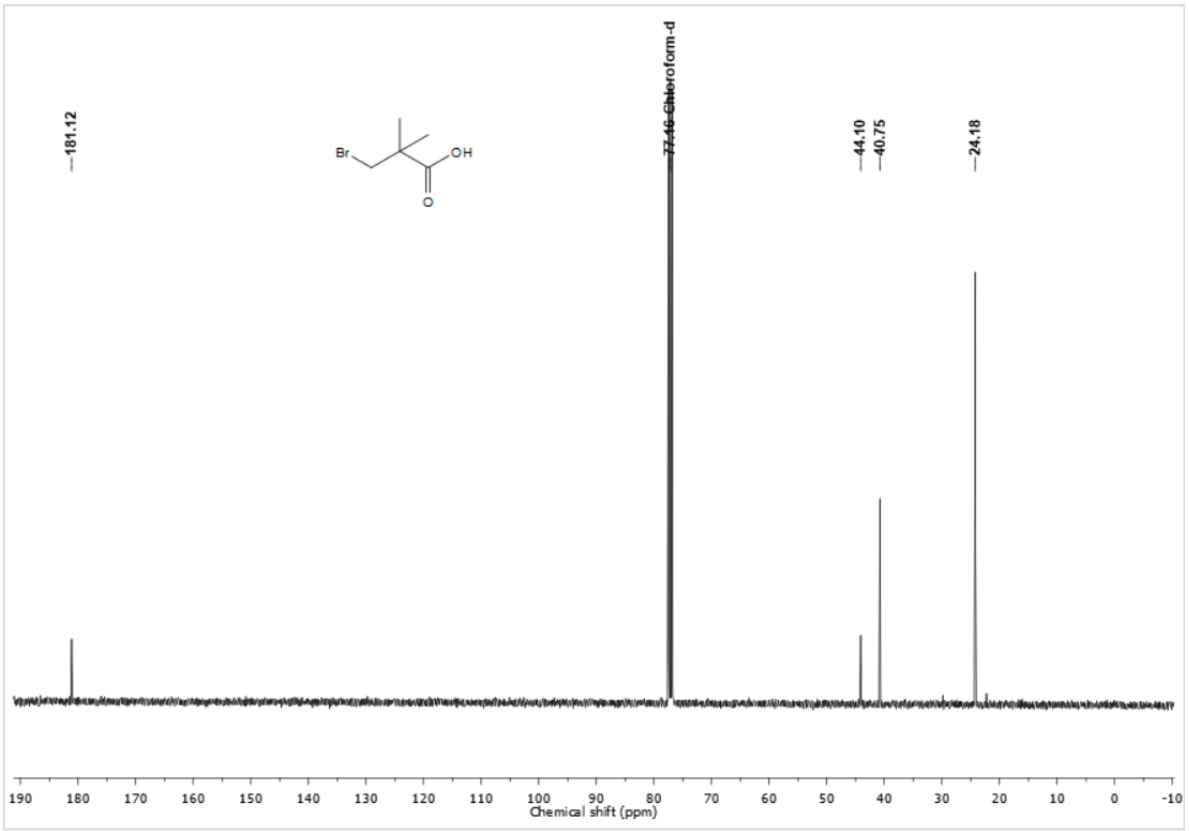
Compound 4 (10 mg) was taken in an RBF and dissolved in MeOH (1 mL). The mixture was then purged with N_2 for 30 min and then Pd/C (2 mg) was added and the reaction mixture was hydrogenated using a hydrogen ballon for 2 h. Upon completion, the reaction mixture was filtered and the solvent was evaporated to give compound 9. No further purification was done.

5.3 NMR spectra of compounds

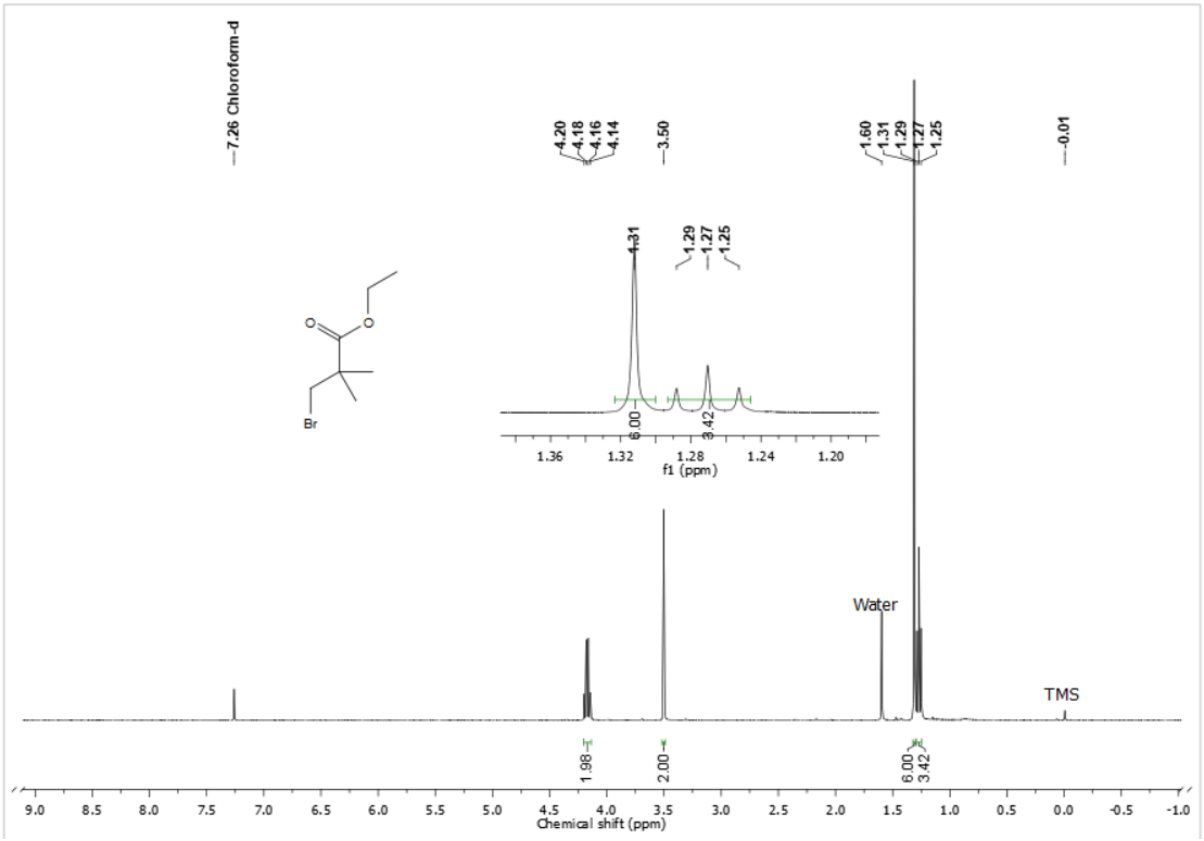
^{1}H NMR (400 MHz, $CDCl_3$) of compound 2



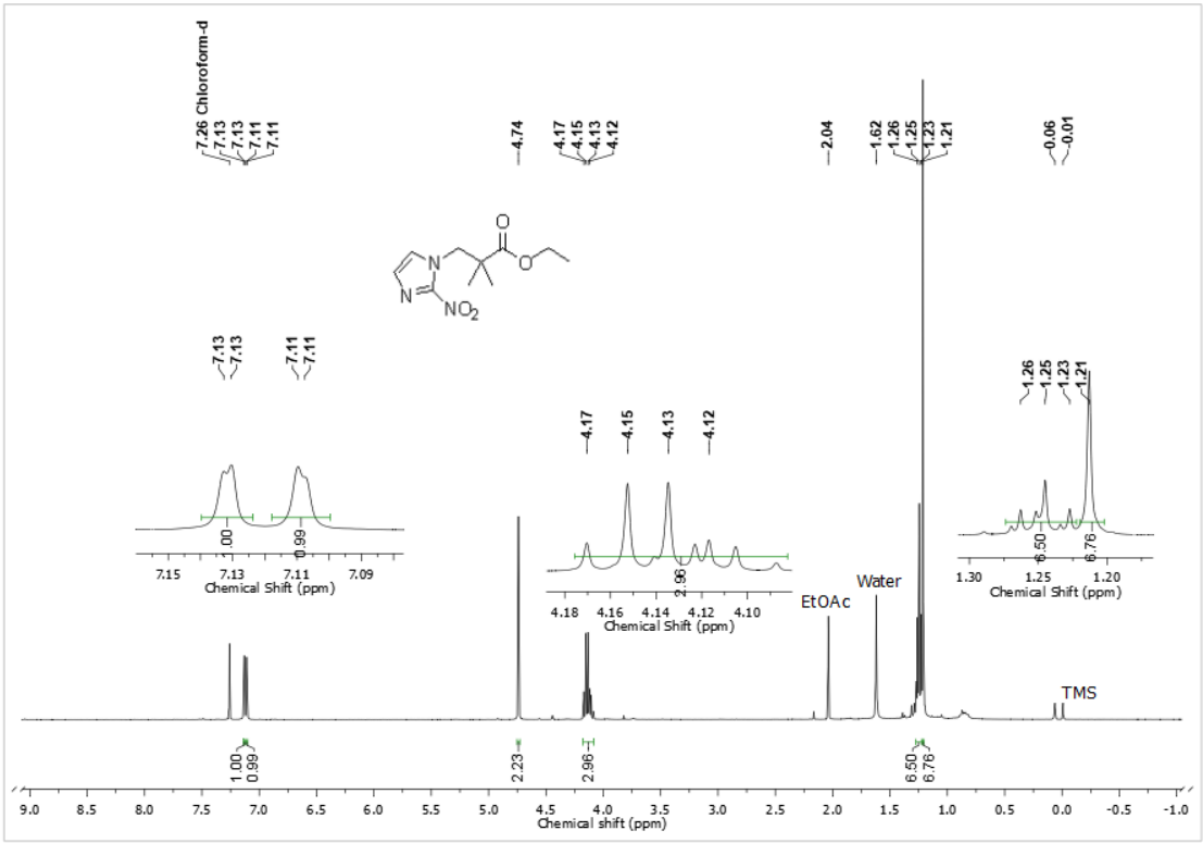
^{13}C NMR (100 MHz, $CDCl_3$) of compound 2



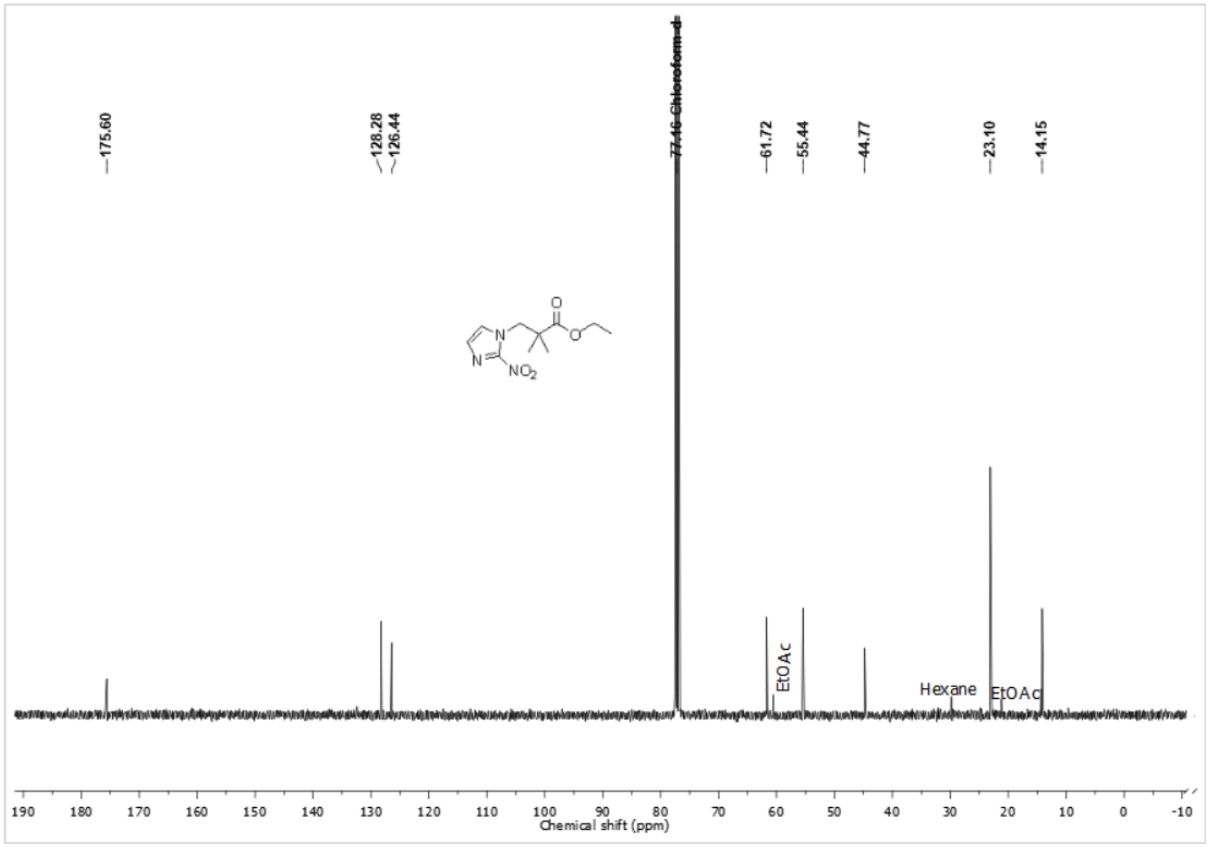
^{1}H NMR (400 MHz, $CDCl_3$) of compound 3



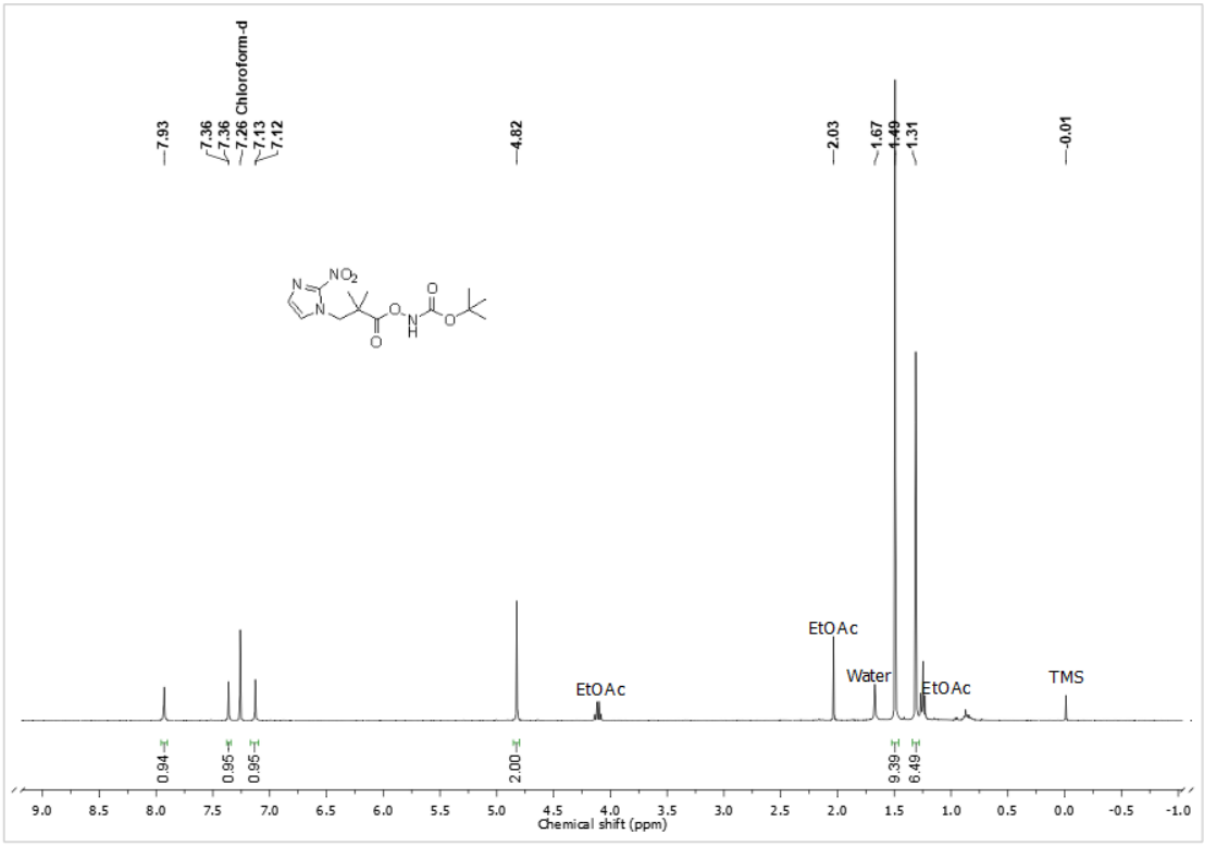
^{1}H NMR (400 MHz, $CDCl_3$) of compound 4



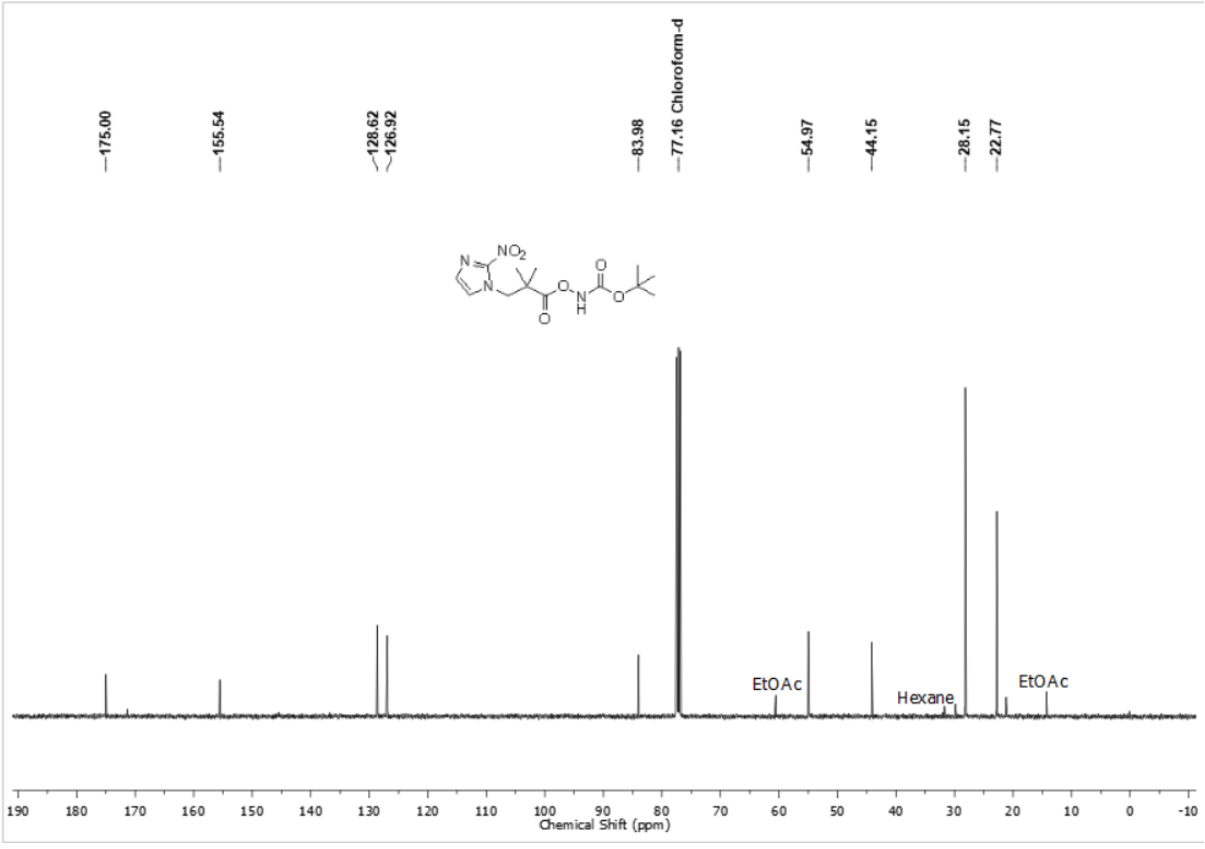
^{13}C NMR (100 MHz, $CDCl_3$) of compound 4



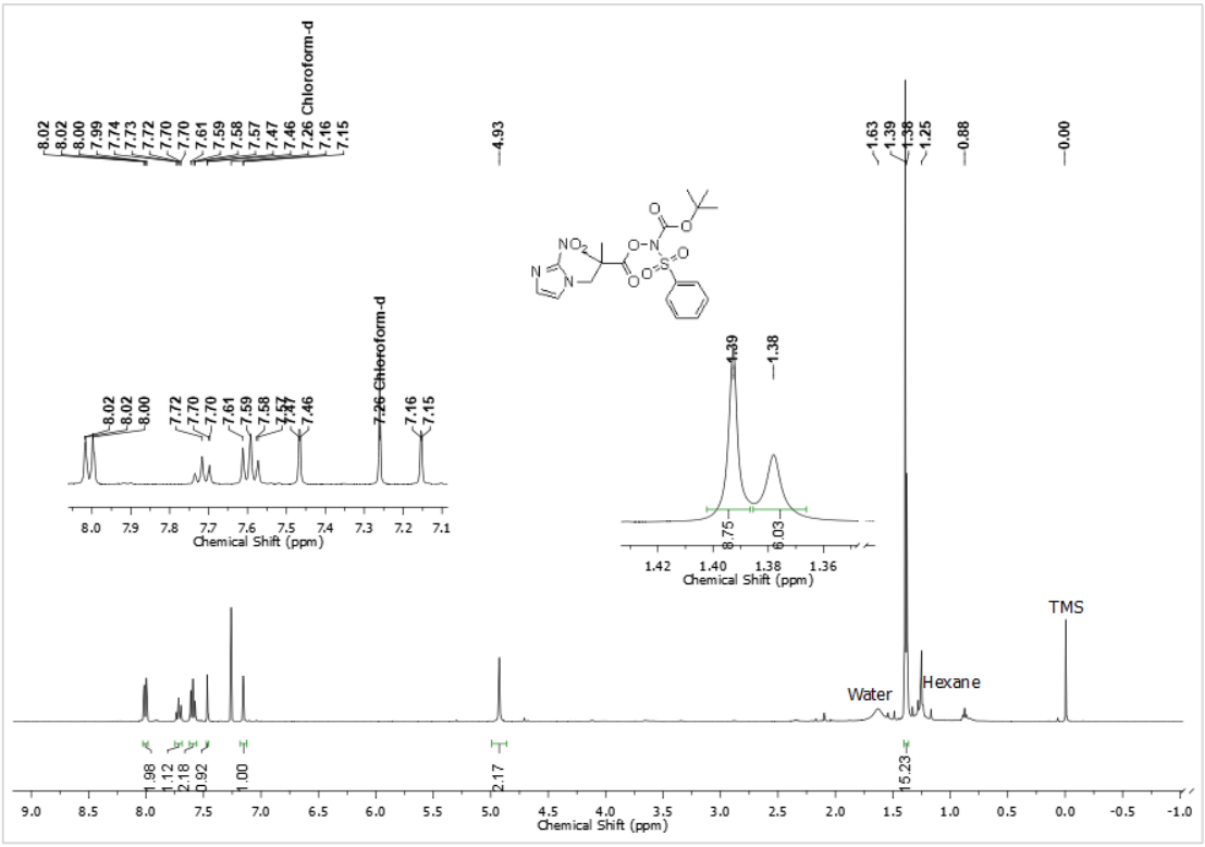
^{1}H NMR (400 MHz, $CDCl_3$) of compound 6



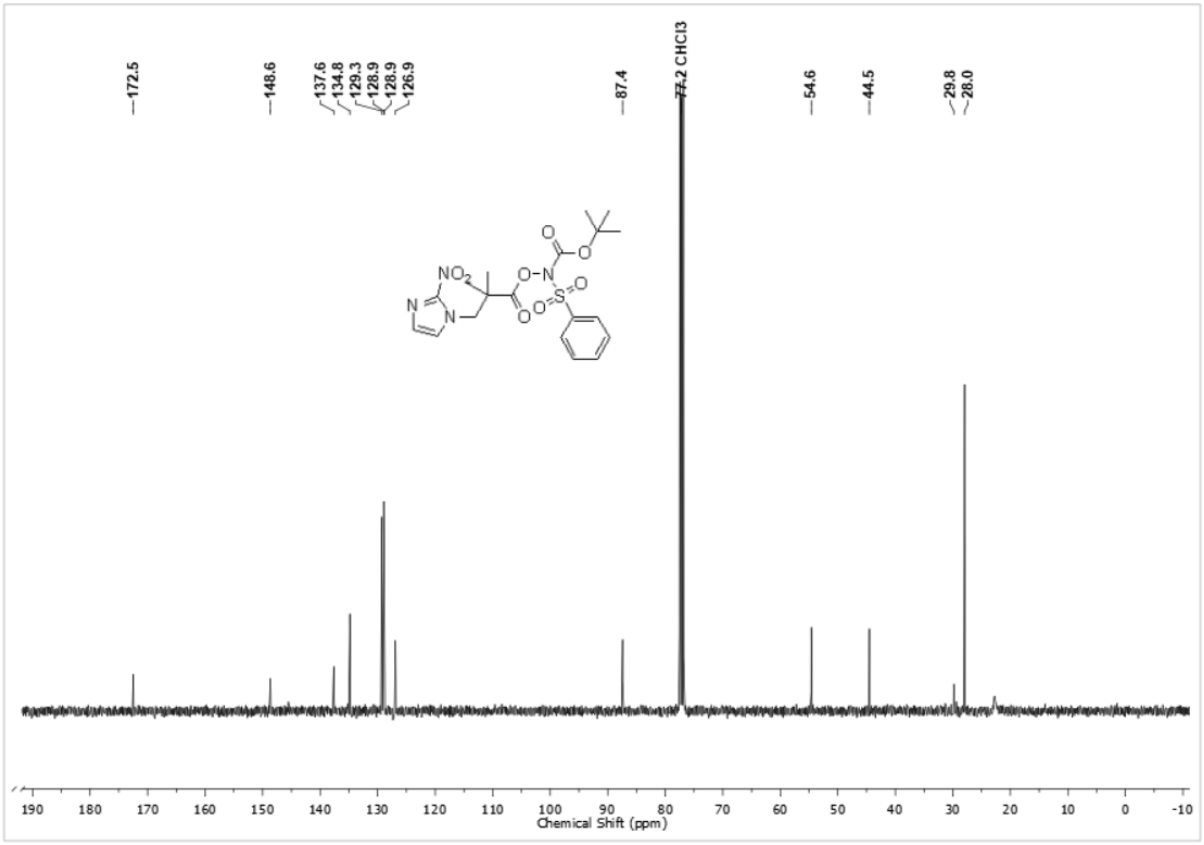
^{13}C NMR (100 MHz, $CDCl_3$) of compound 6



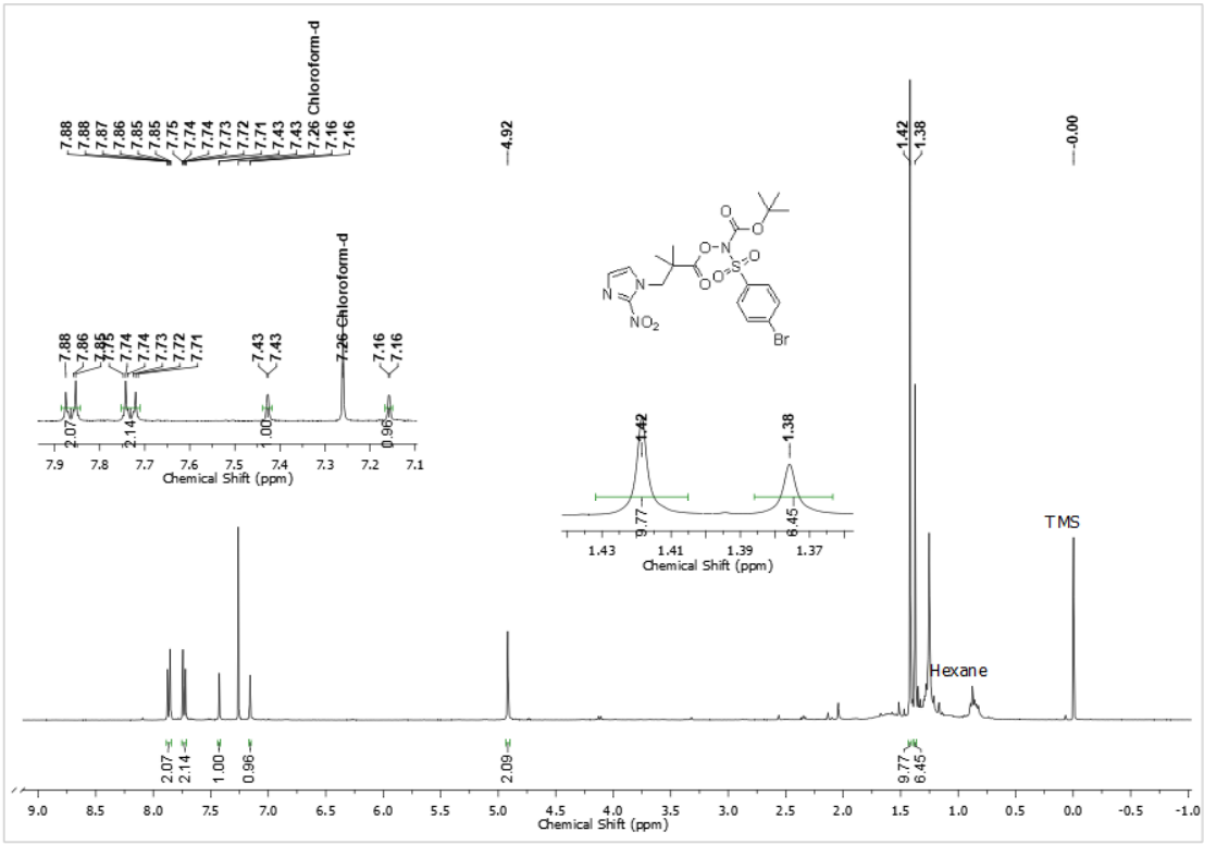
^{1}H NMR (400 MHz, $CDCl_3$) of compound 7a



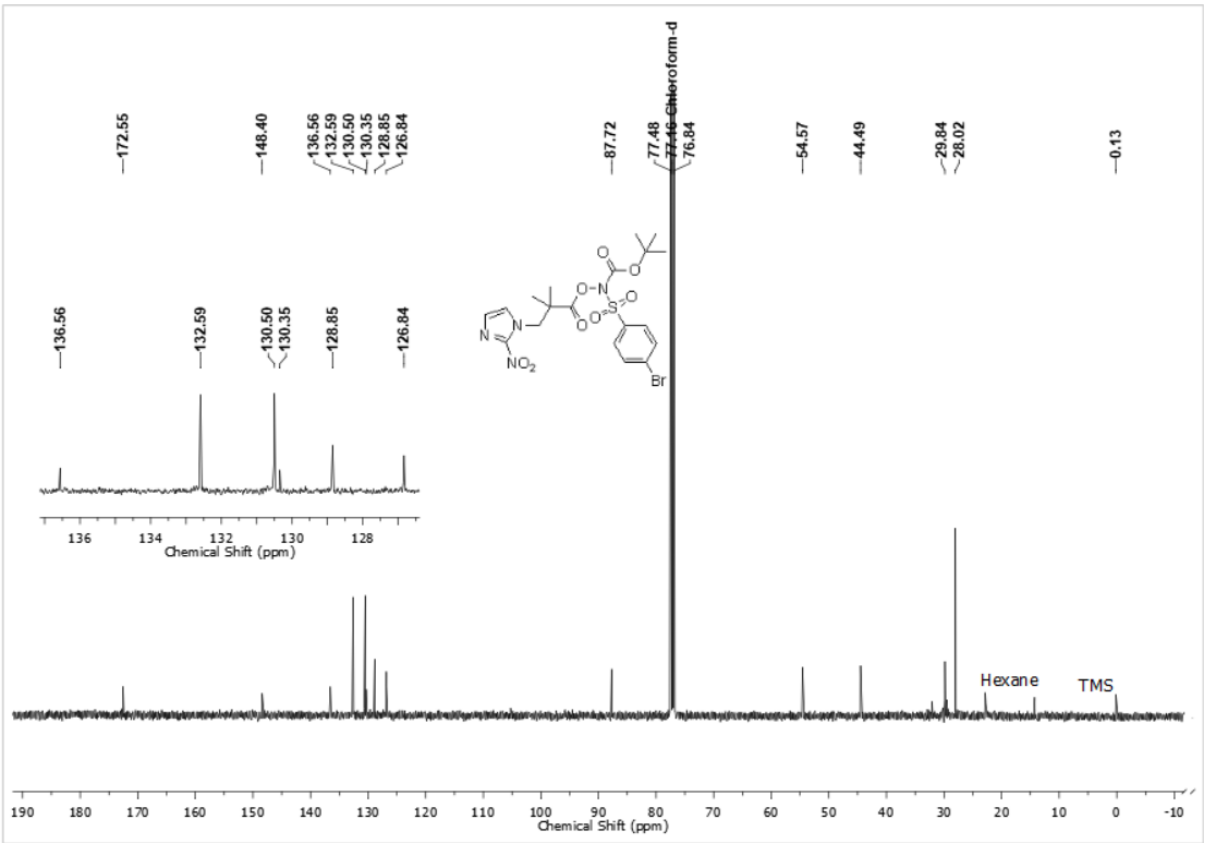
^{13}C NMR (100 MHz, $CDCl_3$) of compound 7a



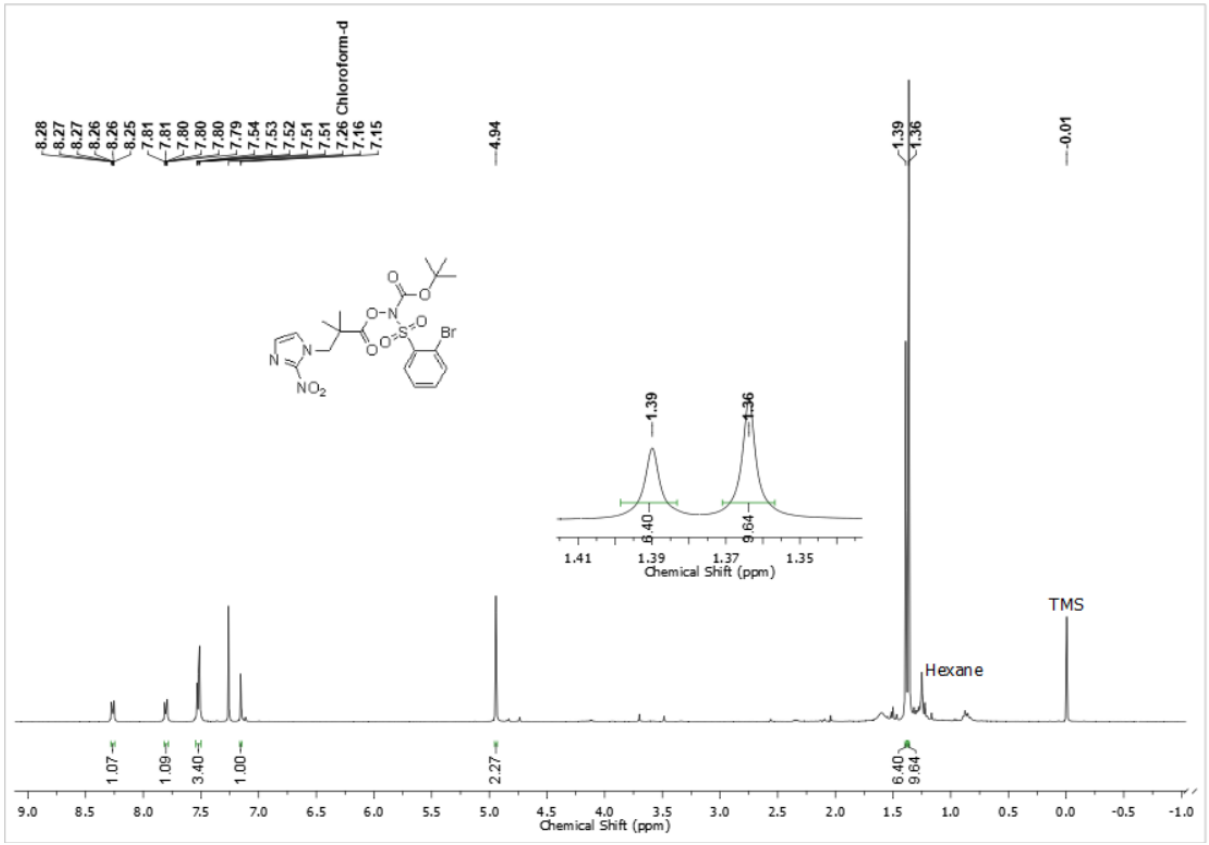
1H NMR (400 MHz, $CDCl_3$) of Compound 7b



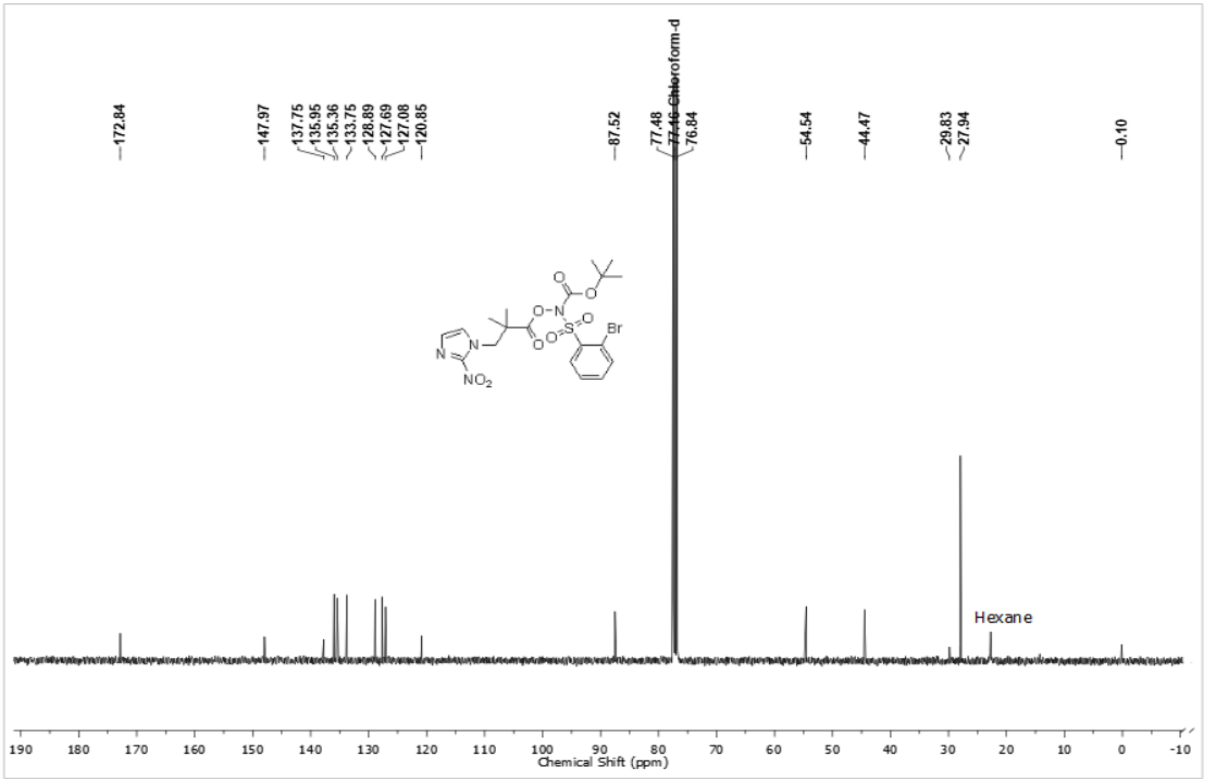
13C NMR (100 MHz, $CDCl_3$) of Compound 7b



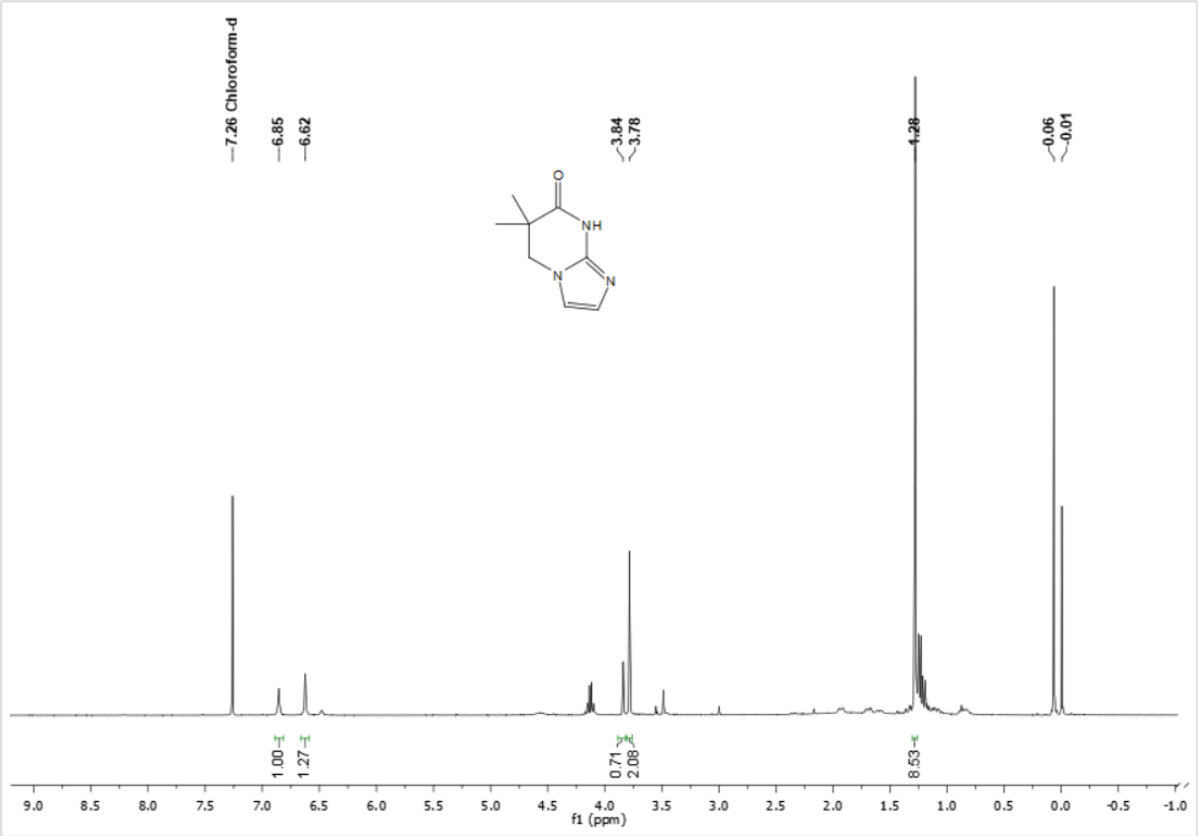
1H NMR (400 MHz, $CDCl_3$) of Compound 7c



13C NMR (100 MHz, $CDCl_3$) of Compound 7c



^{1}H NMR (400 MHz, $CDCl_3$) of compound 9



5.4 Chemoreduction assay to study decomposition of 7a-c

To study the activation of the synthesized HNO donors in reductive conditions, a chemoreduction assay was done using Zn dust and Ammonium Formate (AF) in 1:1 (by volume) H_2O:MeOH solvent system.[31] HNO donors (100 µM) were dissolved in 1 mL of H_2O:MeOH solvent system in glass vials and 150 mg of Zn and AF (300 µM) was added. The reaction mixture was incubated at 37 ^oC for 60 minutes. The reaction mixture was then centrifuged (9,391 x g for 2 min). The supernatant was taken in an RBF and the solvent was evaporated using RotaVap and the crude reaction mixture was dissolved in 100 uL of EtOAc and monitored via TLC.

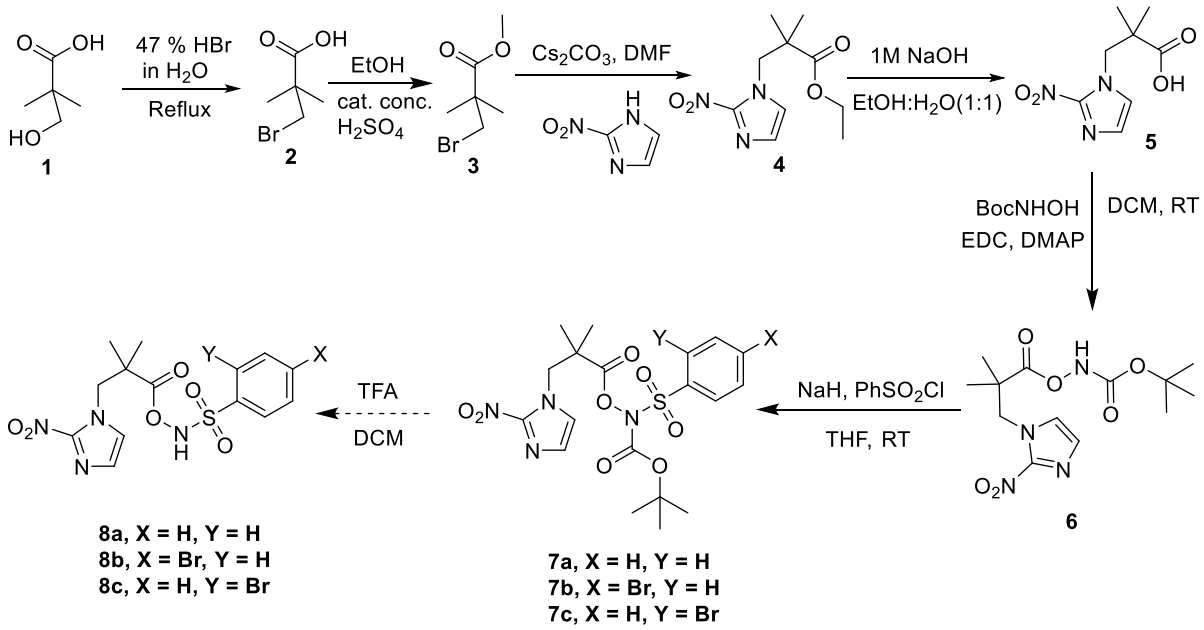
5.5 Quantification of HNO release from 7a-c using a fluoroscent dye

Next, a fluoroscence based assay was done to detect the HNO release from the synthesized HNO donors in presence of Nitroreductase enzyme (from *E. Coli*) and NADH.[31] HNO donors (50 µM) was dissolved in 200 µL of 1x PBS buffer (pH = 7.4) reaction mixture containing 50 µg/mL NTR, 150 µM NADH, and 10 µM PCM dye (Phosphine based dye for HNO detection using fluorescence). This reaction mixture was incubated at 37 ^oC in a 96 well plate and fluorescence (excitation 370 nm; emission 460 nm) was measured in every 5 min interval for 240 minutes.

6. Results and Discussion

6.1 Synthesis of compounds 7a-c as HNO donors

Compound **7a-c** were synthesized using the following synthetic scheme (Corresponding de-Boc HNO donors, **8** were not synthesized)



Scheme 6.1 Synthetic scheme used for the synthesis of NTR activated HNO donors

Compound **1** was brominated using HBr solution to give compound **2**. This was then made into an ethyl ester, **3**, which was then coupled to 2-nitroimidazole to afford compound **4**. The ethyl ester was then hydrolysed to give the corresponding acid **5**. This was then coupled to N-Boc hydroxyl amine to give compound **6**. Compound **6** was then N-sulphonylated to give compound **7a,7b** and **7c.**

6.2 TLC decomposition study of HNO donors in Chemoreductive assay

Compounds **7a-c** (100 µM) was incubated with excess Zn (150 mg) and AF (300 µM) for 60 min at 37 ^{o}C. The reaction mixture was centrifuged and the supernatant was spotted in a TLC.

Figure 6.1 TLC of chemoreduction of **7a**. First spot – 7a (100 µM) control, Second spot – 7a (100 µM) + Zn dust (150 mg) + AF (300 µM), Third spot – Compound 9. TLC run in 50 % EA:PE

Figure 6.2 TLC of chemoreduction of **7b**. First spot – Authentic 7b, Second spot – 7b (50 µM) control, Third spot – 7b (50 µM) + Zn dust (150 mg) + AF (150 µM), Third spot – Compound 9. Left picture – TLC run in 50 % EA:PE twice, Right Picture – TLC run in 50 % EA:PE twice then 70 % EA:PE

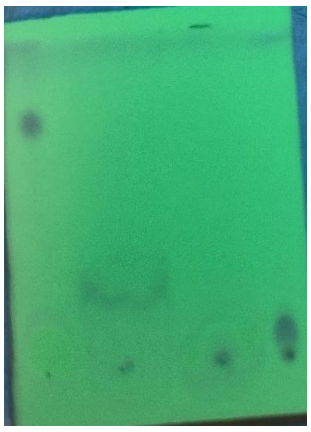


Figure 6.3 TLC of chemoreduction of **7c**. First spot – Authentic 7c, Second spot – 7c (50 µM) control, Third spot – 7c (50 µM) + Zn dust (150 mg) + AF (150 µM), Third spot – Compound 9. Left picture – TLC run in 50 % EA:PE twice, Right Picture – TLC run in 50 % EA:PE twice then 70 % EA:PE

From this experiment, we can see that all the compounds **7a-c** get decomposed completely within 60 minutes in the above mentioned chemoreductive conditions. Also, the compounds **7a-c** get boc deprotected in the control reaction mixture in an hour to generate corresponding **8a-c.**

6.3 Fluorescence studies - EnSight™ multimode plate reader (Perkin Elmer, India)

Compounds **7a-c** (20 µM) were incubated with *E. Coli* NTR enzyme and NADH at 37 ^{o}C and 7.4 pH, and fluorescence was measured every 5 minutes at 460 nm (excitation at 370 nm).

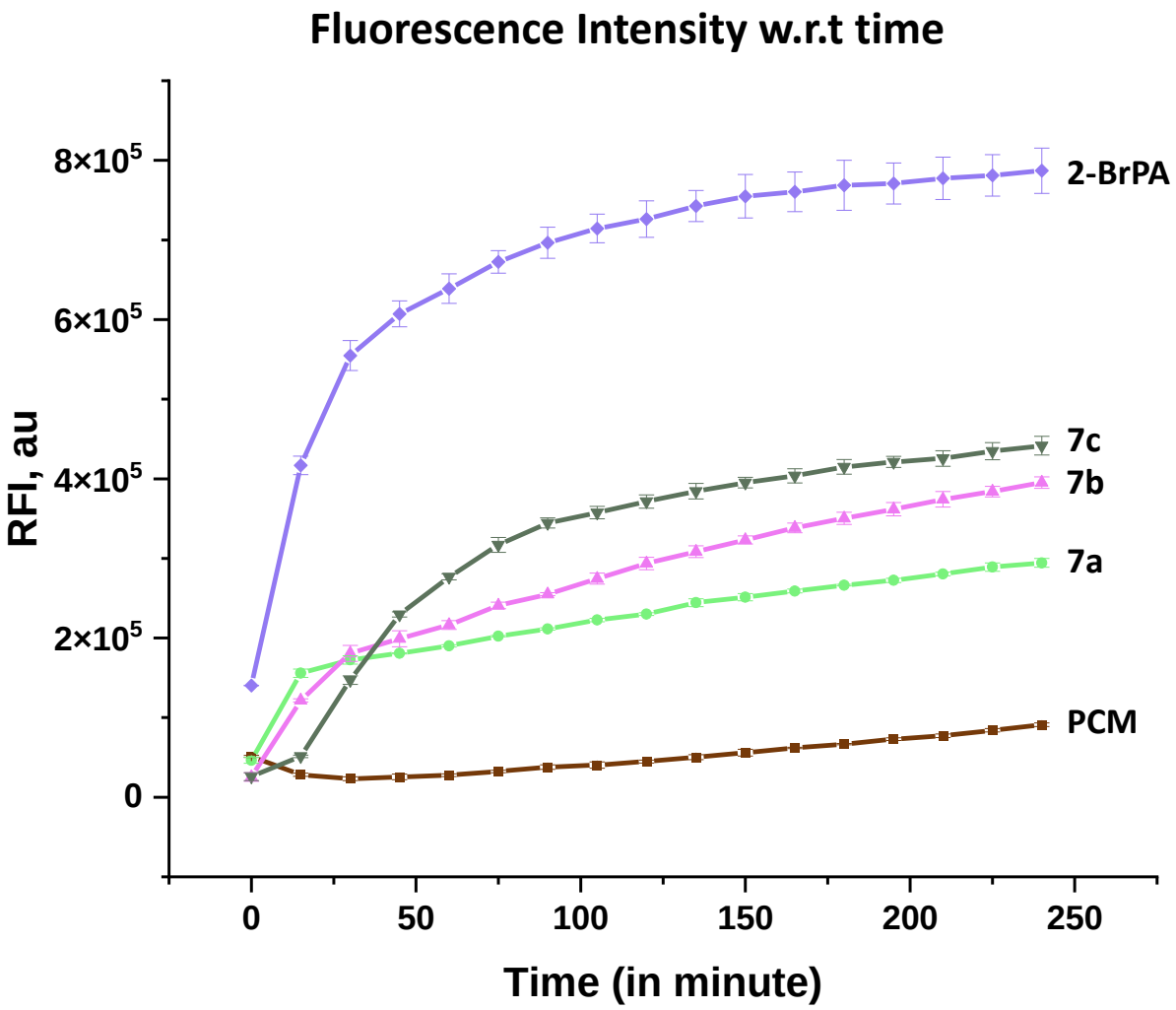


Figure 6.4 Fluorescence intensity of Compounds **7a-c** in presence of Nitroreductase and NADH with time (2BrPA – 2-Bromo Piloty's acid is used as a positive control for HNO release)

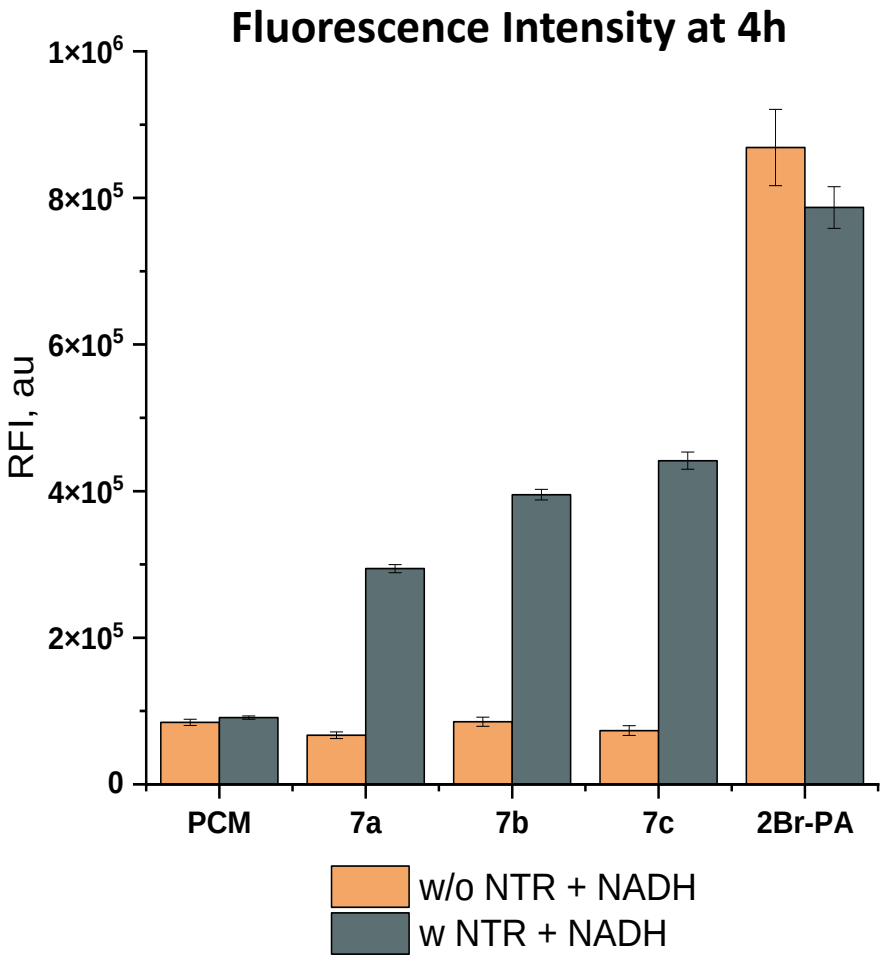


Figure 6.5 Fluorescence intensity of Compounds **7a-c** at the end of 60 minutes in presence and absence of Nitroreductase and NADH

We can see from the fluorescence data that all the three compounds **7a-c** show a significant increase in fluorescence in presence of NTR and NADH which suggests that these compounds are generating HNO in presence of nitroreductase and NADH which can be detected via the fluorescence based P-CM probe in presence of NADH which is the first time that HNO has been detected from a nitroreductase activated HNO donor in presence of NADH.

7. Conclusion and future outlook

Compounds **7a-c** have been synthesized as hypothesized NTR activated HNO donors.

The TLC decomposition experiments and the fluorescence based HNO quantification assays has been performed on these donors to validate them as NTR activated HNO donors. Then, in an in-vitro assay using NTR and NADH we showed that the designed and synthesized HNO donors **7a-c** produced HNO which was detected using a fluorescence based HNO probe, P-CM. However, the actual level of HNO release from these donors might be even more since the side reaction of HNO with NADH can decrease the level of HNO detected via the fluorescence probe, P-CM.

Further experiments to detect and quantify other byproducts of the proposed HNO generation mechanism of these donors have to be carried out to find out the actual HNO yield from these donors. The antibacterial activity and the cytotoxicity of these donors have also to be studied.

8. References

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