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Cell-Trappable Chemiluminescent Probes For Monitoring Hydrogen Sulfide in Living Cells

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CELL-TRAPPABLE CHEMILUMINESCENT PROBES

FOR MONITORING HYDROGEN SULFIDE

IN LIVING CELLS

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CELL-TRAPPABLE CHEMILUMINESCENT PROBES FOR MONITORING HYDROGEN SULFIDE IN LIVING CELLS

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Cell-Trappable Chemiluminescent Probes
For Monitoring Hydrogen Sulfide
In Living Cells

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Hydrogen sulfide (H_2S) is an important biological signaling molecule that has been recognized alongside nitric oxide and carbon monoxide as being a small gasotransmitter that is enzymatically produced and impacts multiple physiological functions. To detect hydrogen sulfide, there has been a focus on developing fluorescent probes to target particular analytes; however, fluorescent probes lack sensitivity and depth penetration due to background autofluorescence and light scattering. Chemiluminescence does not require light excitation, which greatly reduces the amount of autofluorescence and photoactivation.

In order to detect hydrogen sulfide in living systems with high sensitivity, a series of sterically stabilized 1,2-dioxetane chemiluminescent reduction-reaction based hydrogen sulfide probes have been synthesized. These probes are prepared in 10-11 synthetic steps, with key transformations including a Horner-Wadsworth-Emmons reaction to form a tetra-substituted enol ether, a Mitsunobu reaction to install the sensing trigger, and a versatile Heck reaction to append electron-withdrawing groups. The final preparation of the sterically crowded 1,2-dioxetane proceeds in high yields and is accomplished by a [2+2] cycloaddition with singlet oxygen generated by an energy pooling mechanism from triplet

oxygen and the triplet state of methylene blue accessed via visible light irradiation. The appendage of an acetoxymethyl ester enables cell-trappability and enhances utility for monitoring hydrogen sulfide in living cells. These newly synthesized hydrogen sulfide chemiluminescent probes have been tested at a physiological relevant pH both *in vitro* and in living systems.

This dissertation contains the synthesis and characterization of two new hydrogen sulfide chemiluminescent probes, CHS-4 and CHS-5-AM.

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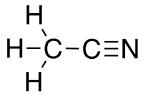
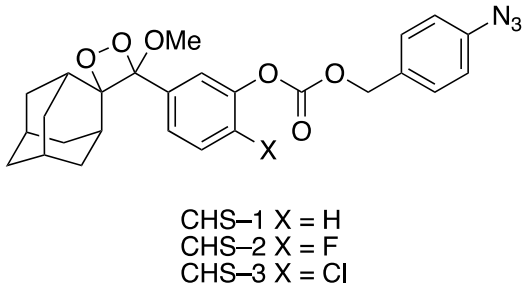
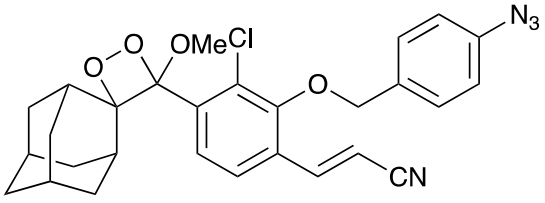
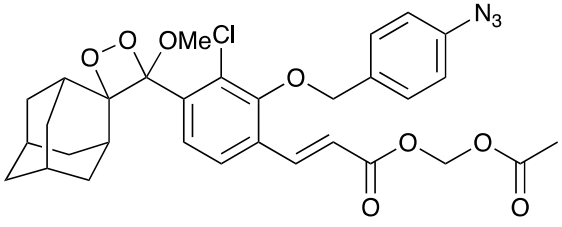
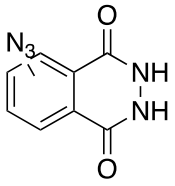
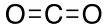
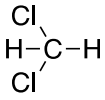
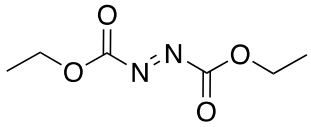
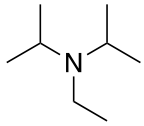
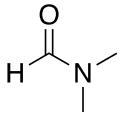
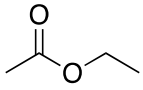
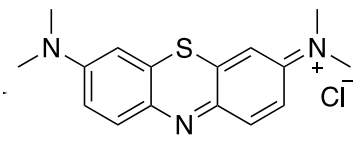
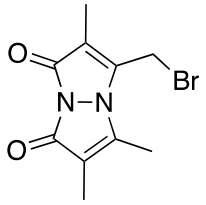
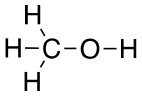
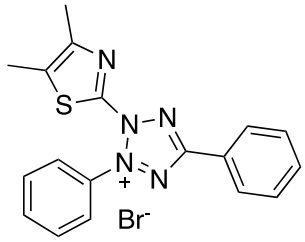
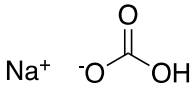
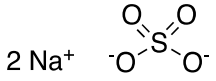
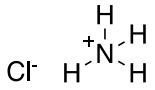
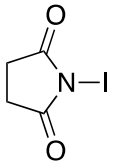
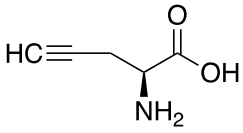
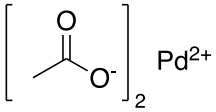
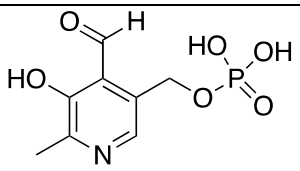
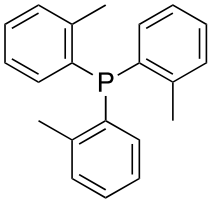
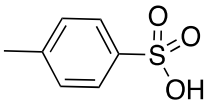
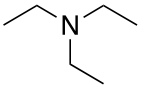
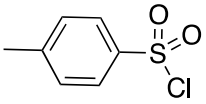
LIST OF ABBREVIATIONS

Abbreviation	**Name**	**Structure**
ACN	Acetonitrile	
A549	Human Lung Adenocarcinoma Epithelial Cells	
BET	Back Electron Transfer	
CAT	Cysteine Aminotransferase	
CBS	Cystathionine β-synthase	
CHS-1, CHS-2, CHS-3	Chemiluminescent Hydrogen Sulfide Probes 1-3	 CHS–1 X = H CHS–2 X = F CHS–3 X = Cl
CHS-4	Chemiluminescent Hydrogen Sulfide Probe 4	
CHS-5-AM	Chemiluminescent Hydrogen Sulfide Probe 5 with an Acetoxymethyl Ester	

CIEEL	Chemically Induced Electron Exchange Luminescence	
CLSS-1, CLSS-2	ChemiLuminescent Sulfide Sensors 1 and 2	 CLSS–1 N_3 = ortho CLSS–2 N_3 = meta
CO	Carbon Monoxide	
CO_2	Carbon Dioxide	
CSE	Cystathionine γ-lyase	
DCM	Dichloromethane	
DEAD	Diethyl Azodiocarboxylate	
DIPEA	*N*,*N*-Diisopropylethylamine	
DMF	Dimethylformamide	
EtOAc	Ethyl Acetate	
HPLC	High Performance Liquid Chromatography	
H_2S	Hydrogen Sulfide	
MB	Methylene Blue	

mBB	Monobromobimane	
MeOH	Methanol	
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	
$NaHCO_3$	Sodium Bicarbonate	
Na_2SO_4	Sodium Sulfate	
NH_4Cl	Ammonium Chloride	
NIS	*N*-Iodosuccinimide	
NO	Nitric Oxide	
N_2	Nitrogen	
PAG	Propargylglycine	
PBS	Phosphate-Buffered Saline	
Pd $(OAc)_2$	Palladium (II) Acetate	

PLP	Pyridoxal-Phosphate	
P (*o*-tolyl)$_3$	Tri (*o*-tolyl) phosphine	
pTSA	*p*-toluenesulfonic acid	
S_0	Ground Singlet State	
S_1	Excited Singlet State	
TEA	Triethylamine	
THF	Tetrahydrofuran	
TsCl	4-Toluenesulfonyl Chloride	
T_1	Excited Triplet State	
3-MST	3-Mercaptopyruvate Sulfurtransferase	

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I would like to thank my advisor, Dr. Lippert, for giving me the opportunity to work with his team here at Southern Methodist University. Working for Dr. Lippert has been such a blessing. As an advisor, he is driven, motivating, understanding, and encouraging to his students. Additionally, I would like to thank my committee members Dr. Son, Dr. Buynak, and Dr. Zoltowski.

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Lastly, I would like to thank my wonderful husband and family for all their support along the way.

Chapter 1

Chemiluminescent Hydrogen Sulfide Probes

1.1 Introduction

1.1.1 Introduction to Chemiluminescence

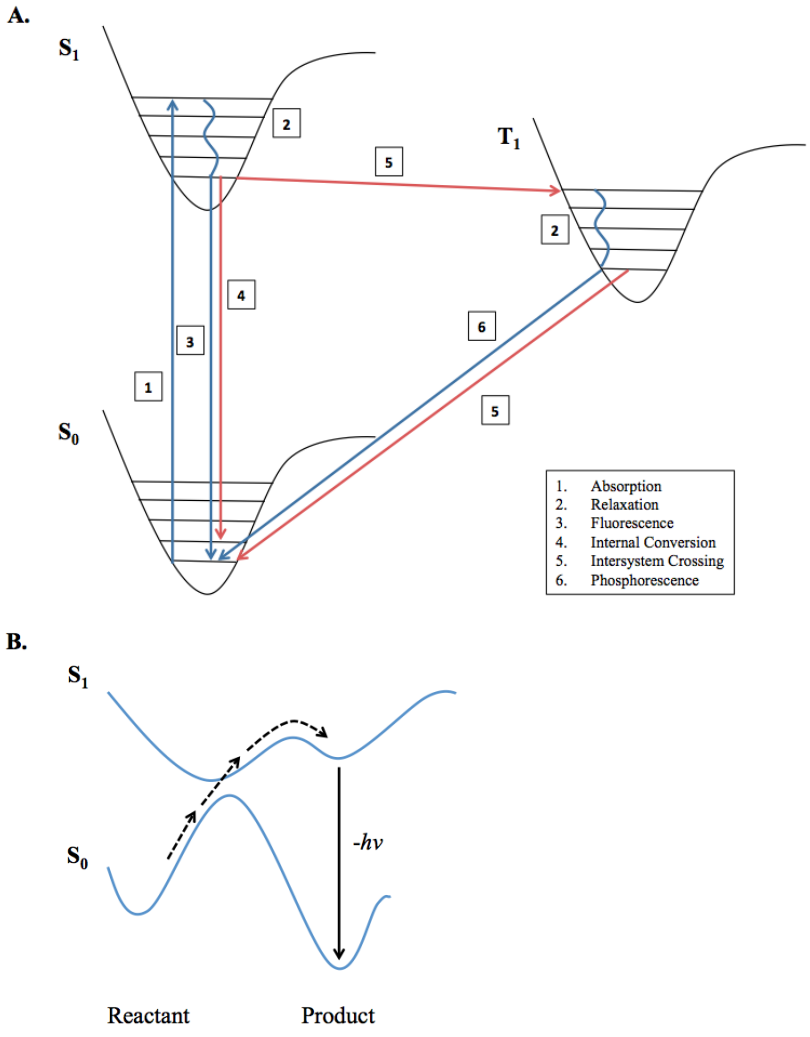


Figure 1. A. The Jablonski diagram depicting the photophysical process of fluorescence. **B.** Potential energy surface for a chemiluminescent reaction. (1)

The Jablonski diagram depicts three energy surfaces and highlights significant photophysical processes. Although the diagram simplifies the energy surfaces, each energy surface is a combination of all a molecules vibrational modes and possible interconversions between chemical structures. The y-axis denotes potential energy, while Morse potentials are at different locations along the x-axis.

Photochemistry starts at the ground state (S_0), and when a single molecule is exposed to light with an appropriate amount of energy, the molecule can be absorbed to the excited electronic state (S_1). Then relaxation occurs, which is radiationless conversion of the vibrational excited states to the ground vibrational state of S_1. From the ground vibrational state of S_1, conversion back to S_0 can occur with the emission of a photon known as fluorescence. If the excited state is accessed through a chemical reaction, then the emission of the photon is called chemiluminescence (Figure 1, B). If the conversion from S_1 to S_0 occurs without emitting a photon and heat is released instead, this is known as internal conversion. The third way to exit from S_1 is through intersystem crossing. This consists of the interconversion of different spin states to form the triplet excited state (T_1) without the release of a photon. Then, radiationless vibrational relaxation can occur to convert the excited states to the ground vibrational state of T_1. The transition from the triplet-excited state to the ground singlet state with the emission of a photon is known as phosphorescence (1) (Figure 1, A).

Unlike fluorescence, the excited state for a chemiluminescent reaction is not accessed through light absorption. A chemiluminescent thermal reaction begins by progressing upwards towards the transition state. Then near the transition state, the ground

state and the excited state are close enough in energy that essentially allows the system to hop over into the excited state. The reaction proceeds forward to the minimum of the excited state surface, and the product is produced there in the excited state. Lastly, the product relaxes down to the ground state with the emission of a photon (1)

(Figure 1, B).

1.1.2 Biological Roles of Hydrogen Sulfide

Hydrogen sulfide is commonly known as a toxic gas with a rotten egg odor and environmental pollutant, but in recent years hydrogen sulfide has been discovered to be an important biological signaling molecule and plays various roles in mammalian physiology. Hydrogen sulfide has joined nitric oxide (NO) and carbon monoxide (CO) as the third biological gasotransmitter. A gasotransmitter is a small gas molecule that is enzymatically produced and influences numerous physiological functions (2,3,4). For example, hydrogen sulfide plays a role in vasorelaxation (5), angiogenesis (6), neuromodulation (7), diabetes (8), and cancer (9). Understanding the biological role of H_2S is much more difficult compared to NO and CO due to its redox activity and nucleophilic properties. (10) In order to understand hydrogen sulfide signaling and deepen our knowledge to the impact it has, hydrogen sulfide detection tools are being developed.

The pK_a of hydrogen sulfide is 7.0 making it a weak acid. The pK_a of the deprotonated form (HS^-) is greater than 14, and under physiological conditions HS^- makes up about 80% of the speciation (2). The high nucleophilicity of HS^- allows for this gaseous

molecule to react with electrophilic functional groups on reactive nitrogen, oxygen, and sulfur species.

1.1.3 Enzymatic Production of Hydrogen Sulfide

The enzymatic production pathways of hydrogen sulfide involve three main enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST)/ cysteine aminotransferase (CAT) (2). Both CBS and CSE use vitamin B_6 as a cofactor, and each pathway uses either $_L$-cysteine or homocysteine to obtain the sulfur atom for hydrogen sulfide.

CBS can catalyze the production of hydrogen sulfide in the presence of $_L$-cysteine. Additionally, CBS catalyzes the formation of cystathionine with homocysteine and the cofactor vitamin B_6 (Figure 2). Each CBS subunit has 551 amino acids (11). The NH_2 terminal of CBS has binding cites for the cofactor pyridoxal-phosphate (PLP) and heme (12). The heme group plays a redox sensor role and PLP is the active form of vitamin B_6 and functions as the catalytic domain (13). The COOH terminal of CBS has a regulatory domain of about 140 residues (2). When this regulatory domain is deleted, CBS is activated (14). CBS expression is primarily found in the brain, and when the regulatory domain has mutations this can lead to an array of diseases (15,16).

Once CBS has catalyzed the production of cystathionine, CSE can generate the production of hydrogen sulfide and release ammonia and α-ketobutyrate (Figure 2). CSE is also capable of yielding hydrogen sulfide from $_L$-cysteine, while also producing ammonia and pyruvate. Furthermore, CSE can generate hydrogen sulfide by first

catalyzing L-cystine to produce thiocysteine, which can lead to the production of hydrogen sulfide with the use of CSE. Human deficiency of CSE can lead to metabolic disorders and increased risk of developing bladder cancer (17).

The last enzymatic hydrogen sulfide production pathway uses both CAT and MST. First, CAT converts L-cysteine to 3-mercaptopyruvate (Figure 2). Then MST uses 3-mercaptopyruvate to transfer the sulfur to sulfite or an available sulfur acceptor. The direct product of this production pathway is sulfane sulfur not hydrogen sulfide. To obtain hydrogen sulfide, atomic sulfur would need to be reduced or hydrogen sulfide could be released from thiosulfate or persulfides (18, 19,20). MST is found in the kidney, liver, heart, and brain, and defects with MST have not shown any disorders (21, 22).

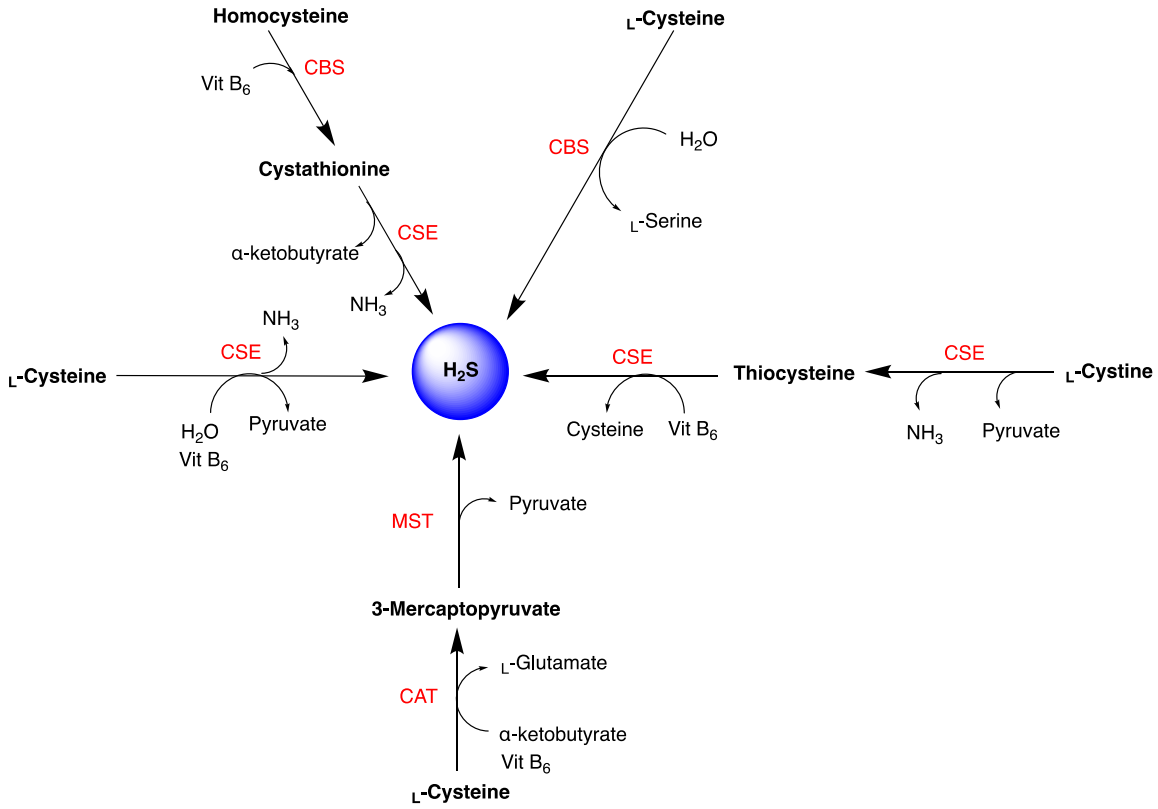
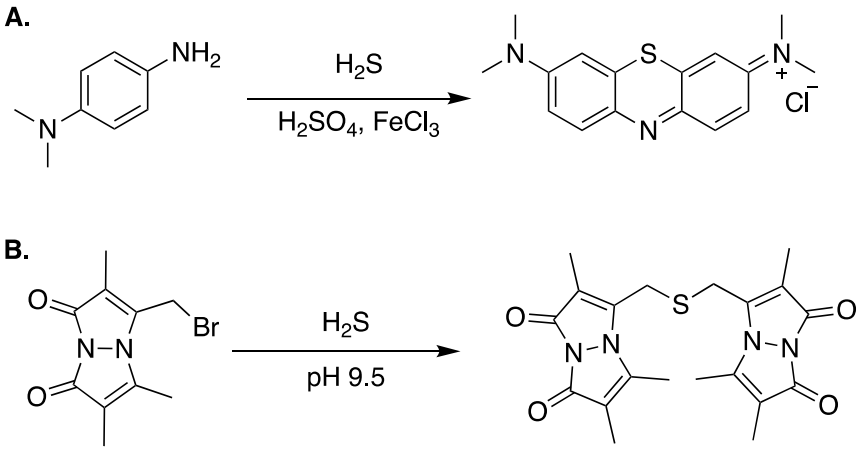


Figure 2. Enzymatic hydrogen sulfide production pathways in mammalian cells. CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CAT, cysteine aminotransferase; MST, 3-mercaptopyruvate sulfurtransferase.

When studying the production of hydrogen sulfide *in vivo*, experiments can be done using small-molecule inhibitors to modify the amount of hydrogen sulfide produced. For example, propargylglycine (PAG) is a common CSE inhibitor that is used to decrease the synthesis of hydrogen sulfide (23, 24). Opposite of inhibitors, enzymatic stimulators can be utilized to increase the release of hydrogen sulfide. A commonly used allosteric activator of CBS is *S*-adenosylmethionine (adoMet), which stimulates the production of more hydrogen sulfide (25, 26).

1.1.4 Current Methods to Detect Hydrogen Sulfide

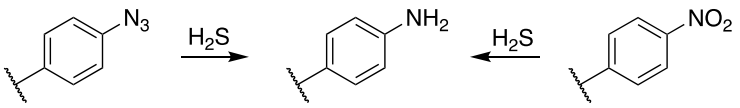


Scheme 1. **A.** Methylene blue (MB) and **B.** monobromobimane (mBB) hydrogen sulfide quantification methods.

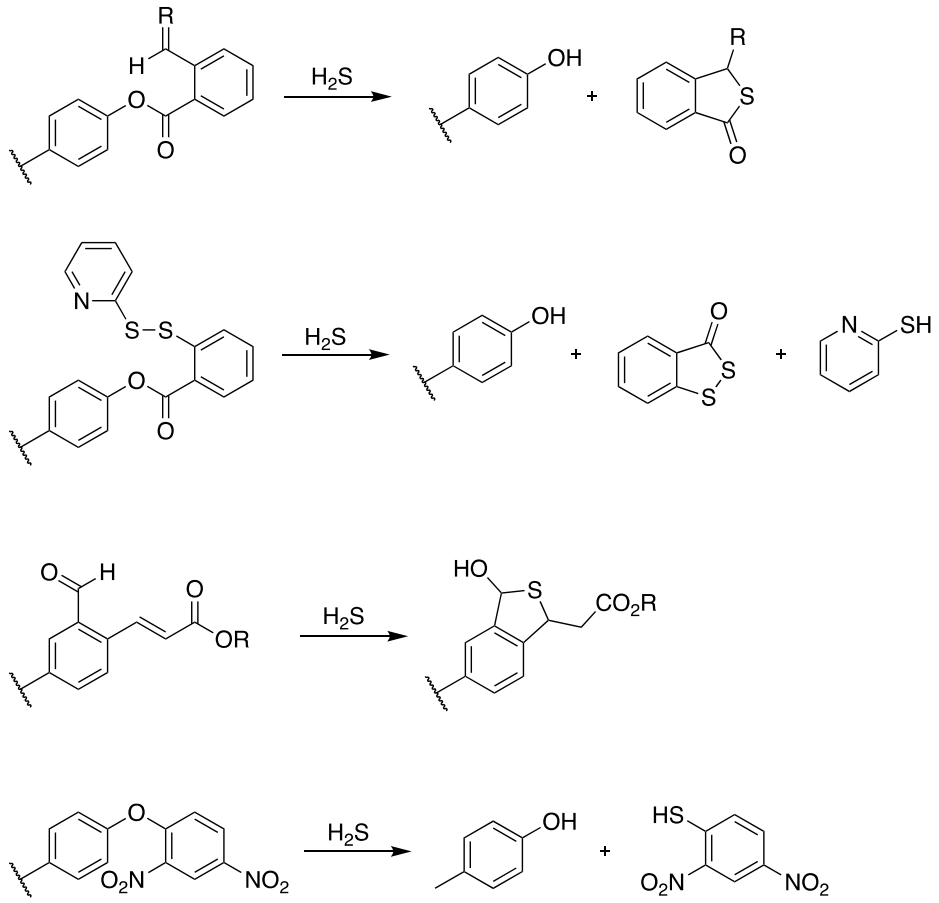
The most common hydrogen sulfide quantification methods currently used are the methylene blue (MB) assay and monobromobimane (mBB), which both require the sample to be homogeneous. These methods work for samples such as blood or serum but not for heterogeneous samples such as cells or living tissue. The methylene blue assay is a

commonly used method that uses iron (III) chloride catalyzed electrophilic aromatic substitution for hydrogen sulfide detection (Scheme 1, A). The product has a characteristic absorbance at 670 nm that allows for quantification (27). Drawbacks from this method include a limit of detection of only 2 μM, which makes it difficult to differentiate between sulfur species (28). Additionally, highly acidic conditions are needed, which can lead to sulfur extraction from other biological resources and decrease the accuracy (27). Another commonly used hydrogen sulfide quantification method is monobromobimane (Scheme 1, B). This process involves the reaction between two equivalents of mBB and one equivalent of hydrogen sulfide. Due to mBB reacting with both hydrogen sulfide and thiols, fluorescence HPLC is needed for separation and quantification (28). Although this method has a limit of detection as low as 2 nM, it cannot be used for cellular or tissue culture experiments (28). Overall, methylene blue and monobromobimane are useful tools to detect hydrogen sulfide, but different detection methods need to be investigated to expand the realm of biological hydrogen sulfide detection.

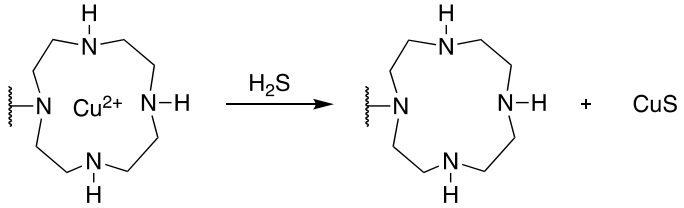
A. Reduction



B. Nucleophilic Attack



C. Metal Precipitation



Scheme 2. Common reaction-based detection strategies for hydrogen sulfide fluorescent and chemiluminescent scaffolds including **A.** reduction, **B.** nucleophilic attack, and **C.** metal precipitation.

Another hydrogen sulfide detection method is the application of reaction-based luminescent probes. Similar to the methylene blue assay and the monobromobimane method, hydrogen sulfide detection relies on the product after hydrogen sulfide reacts with the probe. Although this time the product is light. This offers much greater spatiotemporal data and allows for live cell and tissue imaging experiments. The reaction-based luminescent probe detection strategy requires a hydrogen sulfide reactive site that triggers light emission (Scheme 2). These hydrogen sulfide triggers can be categorized into three main groups: reduction, nucleophilic, and metal precipitation. The most commonly used detection strategy is azide reduction due to the selectivity over other reactive sulfur, oxygen, and nitrogen species (29). Additionally, numerous hydrogen sulfide triggers have been made utilizing the nucleophilicity of HS^-. This strategy requires hydrogen sulfide to attack an electrophilic functional group on the probe leading to the removal of the caging protecting group (29). Furthermore, metal precipitation can be used to detect hydrogen sulfide. For example, when a metal ion such as Cu^{2+} is ligated to a fluorescent molecule, the fluorescence is quenched. Then in the presence of hydrogen sulfide, CuS precipitates out and triggers the turn-on response from the fluorescent probe (30). Overall, reaction-based luminescent detection offers greater spatiotemporal data and allows for cellular and live tissue experiments.

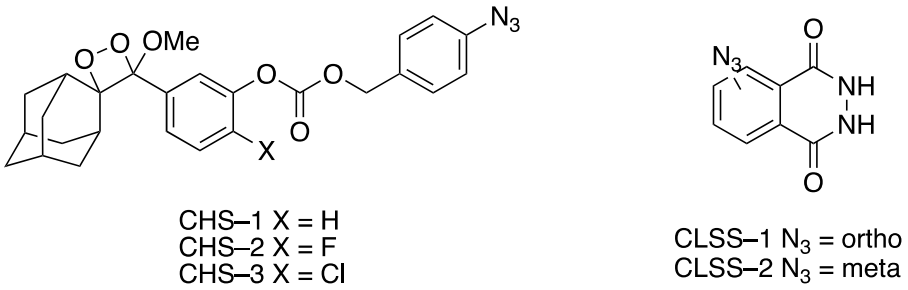
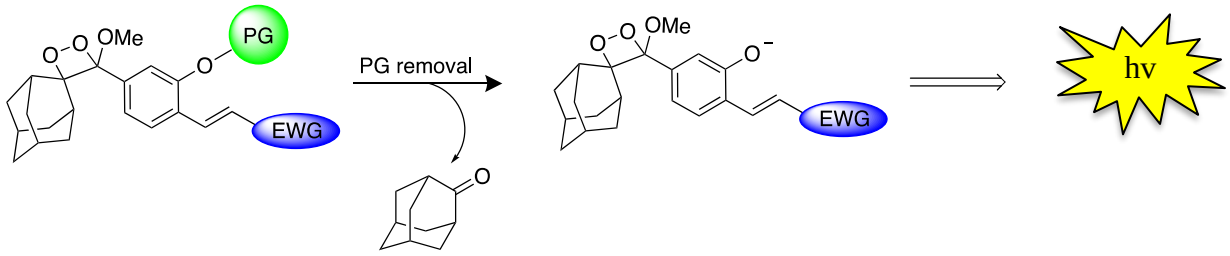


Figure 3. All current chemiluminescent hydrogen sulfide probes.

In just the past two years, over one hundred hydrogen sulfide detecting fluorescent probes have been synthesized. This number emphasizes the importance and need of having a sensitive and selective spatiotemporal hydrogen sulfide detecting device. On the other hand, only five chemiluminescent hydrogen sulfide probes have ever been reported (31, 32) (Figure 3). Chemiluminescence compared to fluorescence does not require light excitation. This notably reduces the background autofluorescence and photoactivation of azide functional groups. In 2013, the Pluth group published the first chemiluminescent hydrogen sulfide probe, which has a luminol azide structure (31). In the presence of hydrogen sulfide, the azide gets reduced to the amine and then subsequently reacts with H_2O_2 / HRP (horseradish peroxidase) to generate chemiluminescence. Then in 2015, the Lippert group published the first generation of chemiluminescent hydrogen sulfide probes based off of the chemically induced electron exchange luminescence (CIEEL) mechanism (32). Overall, chemiluminescent hydrogen sulfide probes are still in the beginning stages of development with much room for improvement in areas such as sensitivity, selectivity, and cell permeability.

1.1.5 Design of CHS-4 and CHS-5



Scheme 3. Design strategy for hydrogen sulfide chemiluminescent probe CHS-4 and CHS-5-AM.

This design strategy for developing highly sensitive, cell permeable chemiluminescent hydrogen sulfide probes consists of a scaffold with three main parts: a hydrogen sulfide specific protecting group, an electron withdrawing group, and a stable 1,2- dioxetane (33) (Scheme 3). The purpose of the protecting group is to provide high selectivity for hydrogen sulfide against other reactive nitrogen, oxygen, or sulfur species. The hydrogen sulfide trigger chosen for probes CHS-4 and CHS-5-AM was the azide-protecting group due to its selectivity and the strong reduction property of hydrogen sulfide. An electron-withdrawing group was added to the scaffold at the conjugated position to the phenolate to create a donor-acceptor pair to increase the final light emission. Lastly, the 1,2-dioxetane that is stabilized by the adamantane group plays an important role the chemically induced electron exchange luminescence (CIEEL) mechanism.

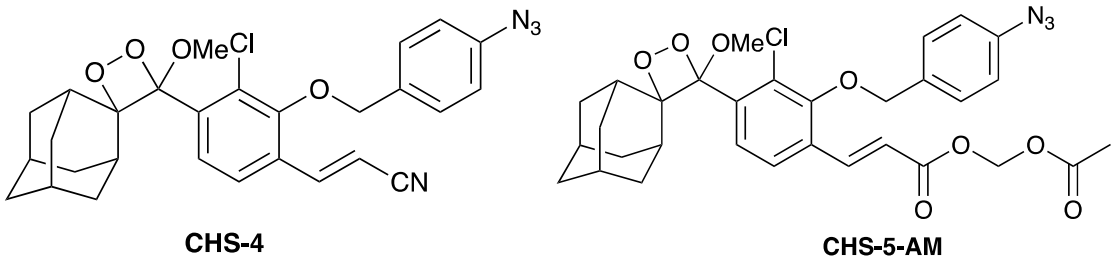


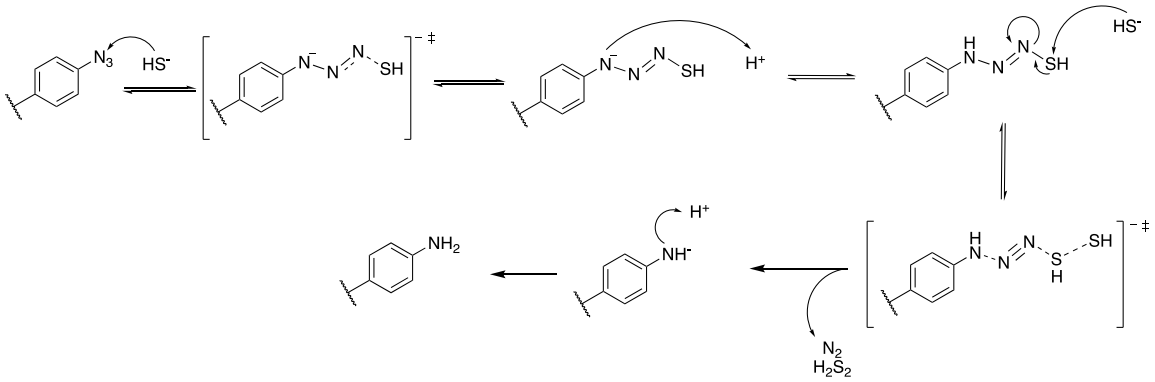
Figure 4. Chemiluminescent hydrogen sulfide probes CHS-4 and CHS-5-AM.

The final structure for CHS-4 and CHS-5-AM include an acrylonitrile and acetoxymethyl ester electron-withdrawing group, respectively (Figure 4). The acetoxymethyl ester makes the CHS-5-AM scaffold cell permeable, and intracellular esterase cleaves the acetoxymethyl ester once the probe is inside the cell in the cytoplasm. The addition of this particular functional group allows for cellular testing to be conducted without the need of a fluorescent enhancer. Furthermore, the chlorine substituent on both CHS-4 and CHS-5-AM reduces the pK_a of the phenolate and improves the phenolate's relative concentration (32). This results in the rate of chemiluminescent decomposition to be increased at a physiological pH.

Scheme 4. Mechanism of hydrogen sulfide detection for CHS-5-AM.

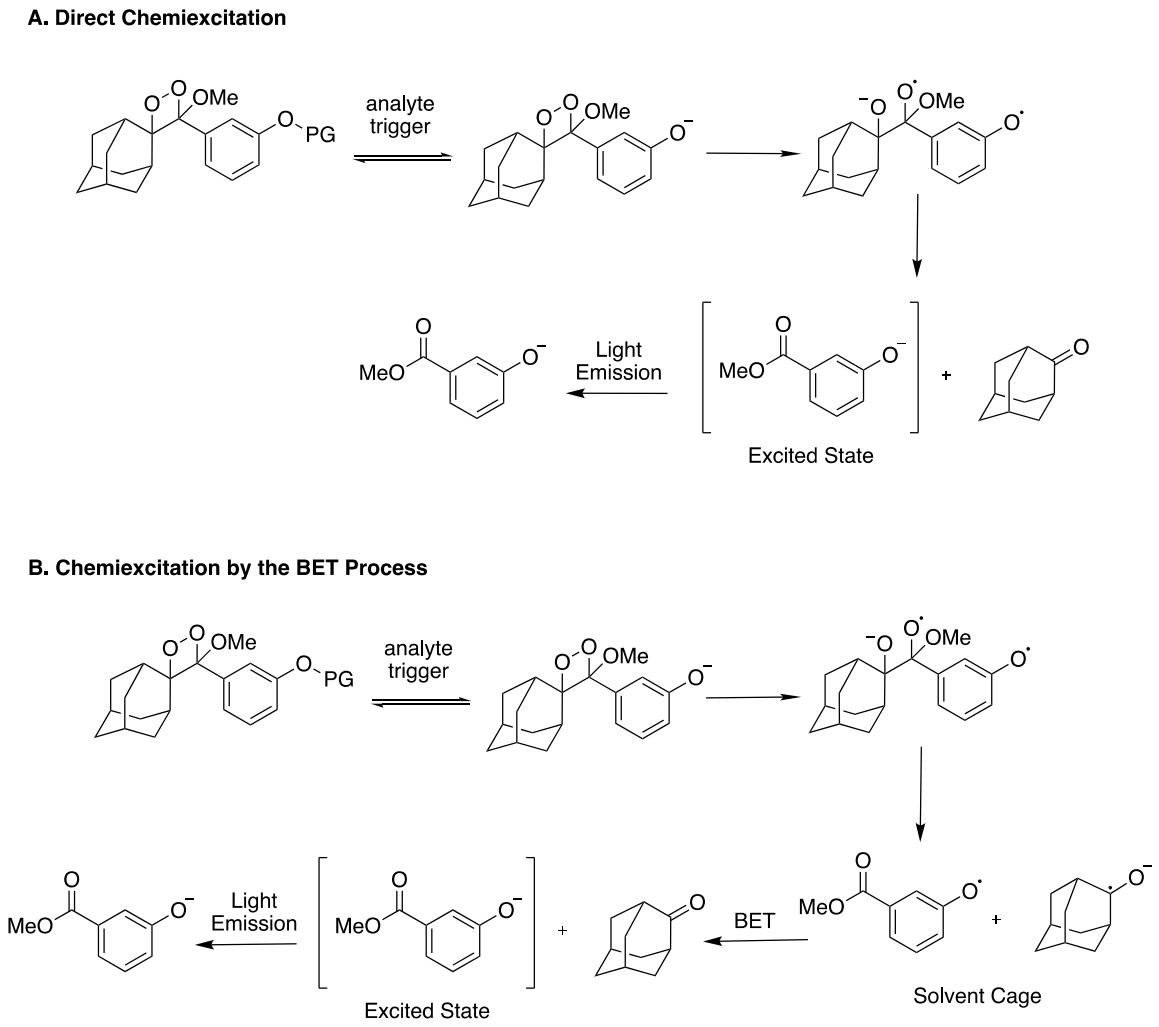
The mechanism of hydrogen sulfide detection begins with reduction of the azide-protecting group by hydrogen sulfide and is followed by self-immolative cleavage to yield the phenolate bearing the 1,2-dioxetane. The free phenolate will emit light spontaneously

after decomposition through the chemically induced electron exchange luminescence (CIEEL) mechanism. (Scheme 4)



Scheme 5. Mechanism for the reduction of the azide-protecting group by hydrogen sulfide.

The mechanism for the reduction of an azide-protecting group by hydrogen sulfide was recently investigated in 2015 by the Pluth group (34) (Scheme 5). The first step in the reduction of the azide-protecting group is the reversible attack on the terminal nitrogen of the azide by HS^-. The terminal nitrogen is the most electrophilic, and this nucleophilic attack is modeled in the Staudinger reaction mechanism as well (35). This yields an anionic azidothiol intermediate, which is then protonated to give a neutral compound. Next, the attack of a second equivalent of HS^- results in the formation of N_2, H_2S_2, and a deprotonated amine. The last step in the mechanism is a proton transfer reaction yielding the final amine.



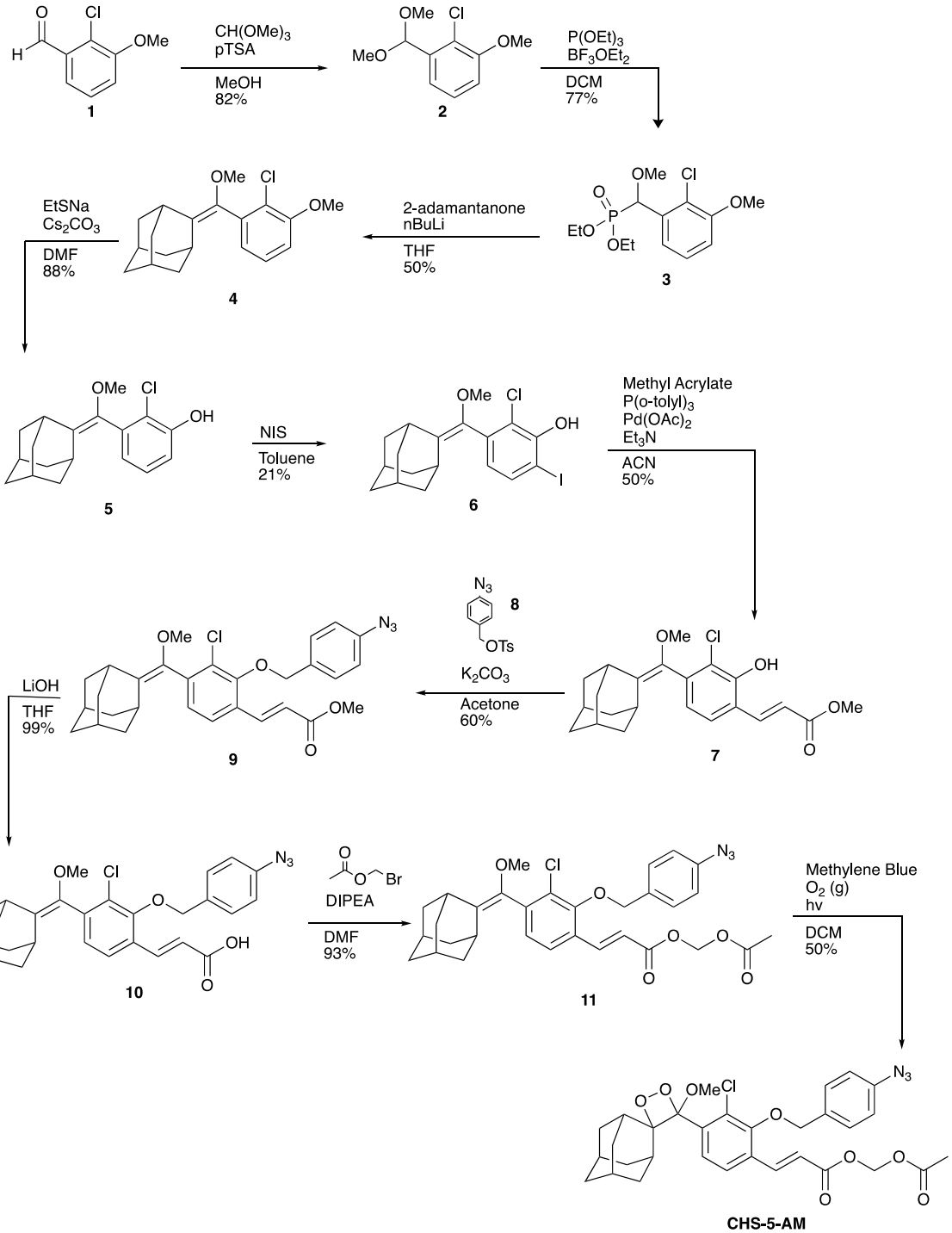
Scheme 6. Chemically Induced Electron Exchange Luminescence (CIEEL) mechanism through **A**. direct chemiexcitation and **B**. chemiexcitation by the Back Electron Transfer (BET) process.

The Chemically Induced Electron Exchange Luminescence (CIEEL) mechanism results in light emission through electron transfer and describes the general chemiluminescent process. A practical CIEEL system consists of a dioxetane that is thermally stabilized by spiroadamantane with a protected phenolate ion. The mechanism begins with the removal of the protecting group by a certain analyte trigger to release the phenolate ion. Next, intramolecular electron transfer occurs between the phenolate ion and the O–O bond. This step is the rate-limiting step due to the energy needed to remove one

electron from the phenolate ion and transfer it to the peroxide bond (36). From here, the mechanism can precede either directly or through Back Electron Transfer (BET), although research favors the mechanism that utilizes BET (37). This mechanism occurs in a solvent cage between two radical molecules, which is dependent on the solvent viscosity. An increase in chemiexcitation is observed with an increase in solvent viscosity (38). The final step in the mechanism leads to light emission from the excited methyl *m*-oxybenzoate relaxing down to the ground state. (Scheme 6)

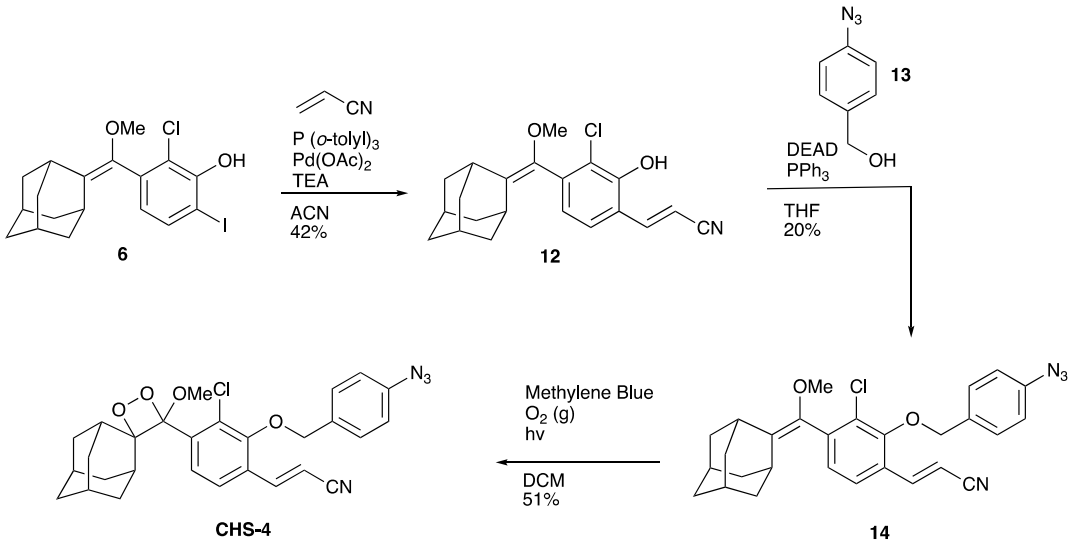
1.2 Results and Discussion

1.2.1 Synthesis of Chemiluminescent Hydrogen Sulfide Probes CHS-4 and CHS-5



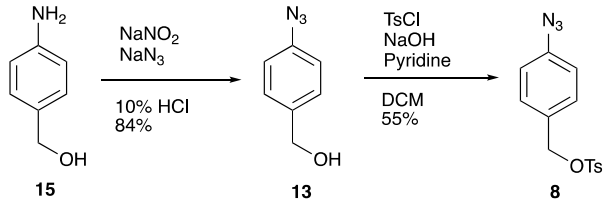
Scheme 7. Synthesis of CHS-5-AM.

The synthesis of CHS-5-AM began with 2-chloro-3-methoxybenzaldehyde being treated with trimethyl orthoformate in the presence of p-toluenesulfonic acid in methanol to yield the acetal compound **2**. This acetal was then treated with triethyl phosphite and boron trifluoride diethyl etherate in DCM to give the diethyl methoxy phosphonate compound **3**. Next, a Horner–Wadsworth–Emmons reaction was conducted by the addition of *n*-butyllithium and 2-adamantanone in THF to compound **3** to yield the enol ether product compound **4**. Then an S_N2 reaction was performed on compound **4** by treating it with sodium ethanethiolate and cesium carbonate in DMF. The resulting phenol product **5** is the leaving group of the S_N2 reaction. Next, the phenol was treated with *N*-iodosuccidimide in toluene to yield compound **6**. Then a Heck reaction was conducted with compound **6** by the addition of methyl acrylate, tri(*o*-tolyl)-phosphine, palladium (II) acetate, and triethyl amine in acetonitrile to form a new carbon-carbon bond yielding compound **7**. Then compound **7** underwent a S_N2 reaction attach to the hydrogen sulfide trigger by the addition of compound **8** and potassium carbonate in acetone to yield compound **9**. Next, the ester group on compound **9** was subjected to hydrolysis upon the addition of lithium hydroxide in THF to yield compound **10**. Then bromomethyl acetate and *N,N*-diisopropylethylamine were added to compound **10** in DCM to form the acetoxymethyl ester functional group, compound **11**. Finally, **CHS-5-AM** was obtained through the formation of the 1,2-dioxetane by singlet oxygen by dissolving the scaffold in DCM, adding the triplet sensitizer methylene blue hydrate, bubbling oxygen through, and shining light. (Scheme 7)



Scheme 8. Synthesis of CHS-4.

The synthesis of CHS-4 is only three steps from compound **6**. First, a Heck reaction is conducted with compound **6**, tri(*o*-tolyl)-phosphine, palladium (II) acetate, and triethyl amine in acetonitrile to yield compound **12**. Then the hydrogen sulfide azide trigger, compound **13**, is attached through a Mitsunobu reaction with the addition diethyl azodicarboxylate and triphenylphosphine in THF to yield compound **14**. Lastly, **CHS-4** is obtained through the use of singlet oxygen by dissolving compound **14** in DCM, adding the triplet sensitizer methylene blue hydrate, bubbling oxygen through, and shining light. (Scheme 8)



Scheme 9. Synthesis of the azide reagent.

To synthesize the hydrogen sulfide azide trigger reagents, 4-aminobenzyl alcohol was first reacted with sodium nitrite and sodium azide in HCl to yield compound **13**. Next, compound **8** was obtained through the addition of *p*-toluenesulfonyl chloride, pyridine, and sodium hydroxide in DCM to compound **13**. (Scheme 9)

1.2.2 Chemiluminescent Response of CHS-4 and CHS-5-AM

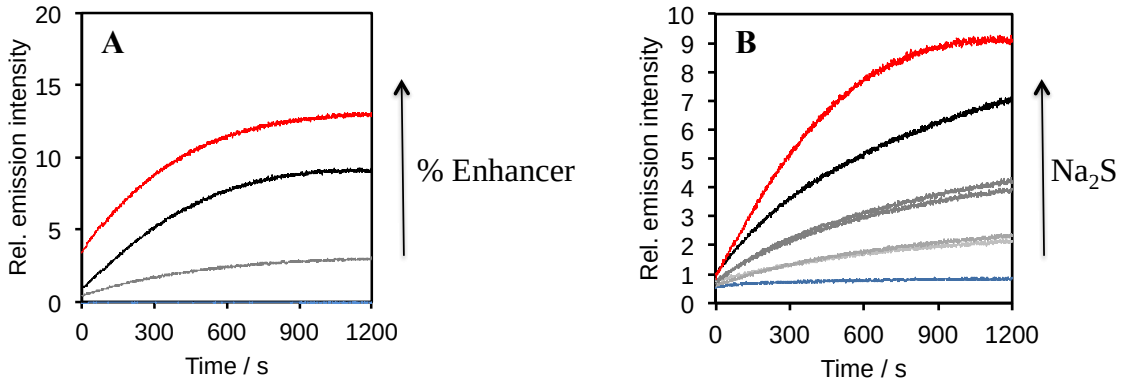


Figure 5. Hydrogen sulfide dose dependence in vitro with Emerald II Enhancer. **A.** Timescan for 20 μM CHS-5-AM, 1 mM Na_2S, and various percentages of Emerald II Enhancer (0, 0.2, 2, 10%) in PBS buffer (pH = 7.41). **B.** Timescan for 20 μM CHS-5-AM with 0, 50, 100, 200, 400, 800, and 1000 μM Na_2S with 2 % Emerald II Enhancer in PBS buffer (pH = 7.41). Three replicates of each were performed on a F-7000 Hitachi spectrophotometer.

The chemiluminescent response for CHS-5-AM was first tested using 20 μM CHS-5-AM, various amounts of Emerald II Enhancer (0, 0.2, 2, 10%), and 1 mM Na_2S. Twenty minute timescans were ran at a physiologically relevant pH using PBS buffer (pH = 7.41) with a F-7000 Hitachi spectrophotometer. Luminescence response appeared to increase with increasing amounts of the Emerald II Enhancer. Furthermore, at least 0.2% Emerald II Enhancer is necessary to see a response from 20 μM CHS-5-AM with 1 mM Na_2S on

this spectrophotometer (Figure 5, A). The chemiluminescent emission for CHS-5-AM was then tested with increasing concentrations of Na_2S holding the amount of enhancer constant at 2%. Twenty minute time scans were conducted using 20 μM CHS-5-AM with 0, 50, 100, 200, 400, 800, and 1000 μM Na_2S with 2% Emerald II Enhancer in PBS buffer (pH = 7.41). Instantaneous light emission was observed in a dose dependent manner under physiological relevant conditions (Figure 5, B).

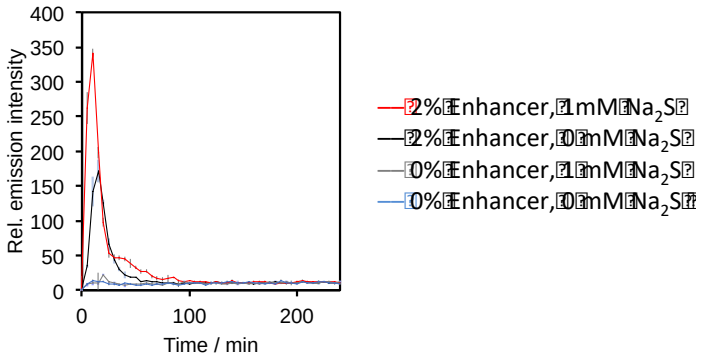


Figure 6. Detection of hydrogen sulfide in A549 cells with Emerald II Enhancer. Nine technical replicates across three biological replicates were performed using a Cytation 5 Bio Tek plate reader at 37 °C.

CHS-5-AM was tested in human lung adenocarcinoma epithelial (A549) to detect endogenous hydrogen sulfide. CHS-5-AM was used due to its cell permeability from the acetoxymethyl (AM) ester functional group. The cells were first incubated with 20 μM CHS-5-AM for 30 minutes. Then the cells were washed with PBS to remove any extracellular probe. Lastly, the cells were treated with Emerald II Enhancer (2, 2, 0, 0%) and Na_2S (1, 0, 1, 0 mM). The relative emission intensity was then recorded using a Cytation 5 Bio Tek plate reader at 37 °C. The cells treated with enhancer produced immediate light emission. The luminescence from the cells without addition of Na_2S and with enhancer could indicate that CHS-5-AM is detecting endogenous H_2S. Furthermore,

there was still observable light emission from the cells without the enhancer and treated with 1 mM Na_2S (Figure 6).

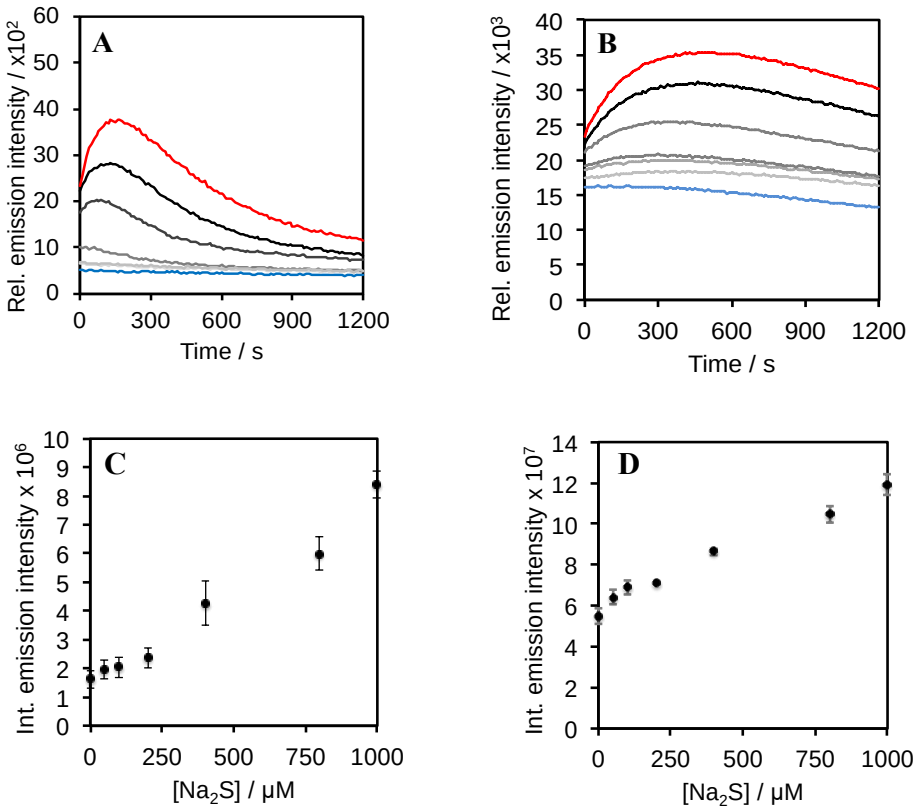


Figure 7. Time scans of the chemiluminescent emission of **A.** 20 µM CHS-5-AM and **B.** 20 µM CHS-4 and 0, 50, 100, 200, 400, 800, 1000 µM Na_2S in PBS buffer (pH = 7.41). The integrated emission intensity of the chemiluminescent response for CHS-5-AM and CHS-4 is depicted in **C.** and **D.** respectively. Three replicates of each were performed using a Cytation 5 Bio Tek plate reader.

The response of CHS-5-AM and CHS-4 was then tested without the enhancer *in vitro* with the Cytation 5 Bio Tek plate reader due to the noticeable sensitivity difference compared to the F-7000 Hitachi spectrophotometer. 20 µM CHS-5-AM and CHS-4 were treated with 0, 50, 100, 200, 400, 800, 1000 µM Na_2S in PBS buffer (pH = 7.41). Both CHS-5-AM and CHS-4 displayed instantaneous light emission in a dose dependent manner

in biologically relevant conditions (Figure 7, A, B). There was a 4-fold and 2-fold increase in integrated luminescence response from CHS-5-AM and CHS-4 respectively (Figure 7, C, D).

1.2.3 Detection of Cellular Hydrogen Sulfide with CHS-5-AM

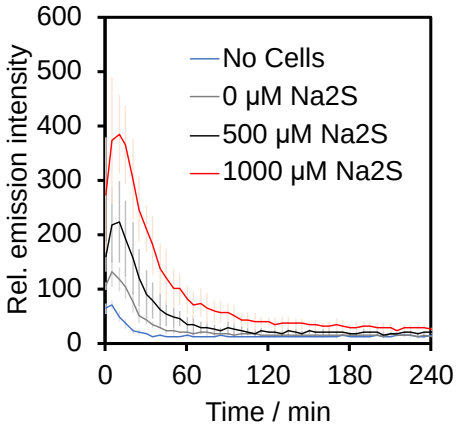


Figure 8. A549 cellular response for 20 µM CHS-5-AM with 0, 500, and 1000 µM Na_2S. Nine technical replicates across three biological replicates were performed using a Cytation 5 Bio Tek plate reader at 37 °C.

Detection of endogenous hydrogen sulfide was then tested without enhancer with CHS-5-AM. The cells were first incubated with 0, 500, and 1000 µM Na_2S for 1 hour. Then the cells were washed with PBS to remove any extracellular Na_2S. Next, the cells were treated with 20 µM CHS-5-AM and tested immediately with the plate reader at 37 °C. Two controls were used in this testing, one without any cells and one with cells. Both controls only have 20 µM CHS-5-AM and no additional Na_2S. An instantaneous luminescence response was observed and maintained for 1 hour in a dose dependent manner. The peak emission occurred around 15 minutes (Figure 8).

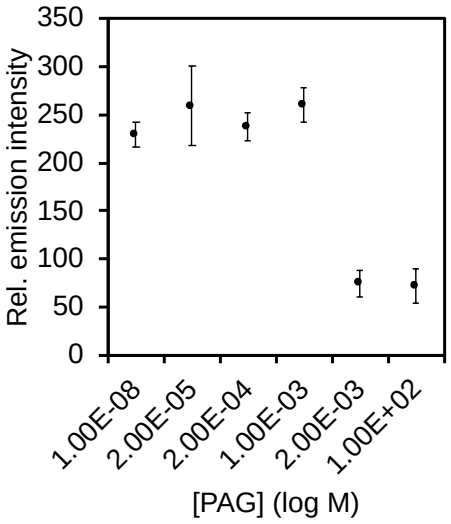


Figure 9. Integrated emission intensity of the chemiluminescent response for A549 cells treated with 20 μM CHS-5-AM and 0, 20, 200, 1000, and 2000 μM propargylglycine (PAG). Additionally, there was a control without A549 cells, only containing 20 μM CHS-5-AM and no addition of PAG. Seven technical replicates across four biological replicates were performed using a Cytation 5 Bio Tek plate reader at 37 °C.

Propargylglycine (PAG) is a small molecule competitive inhibitor for the hydrogen sulfide producing enzyme cystathionine *γ*-lyase (CSE). To examine the PAG inhibition of endogenous hydrogen sulfide, A549 cells were incubated with 0, 20, 200, 1000, and 2000 μM PAG for 40 minutes in complete F-12K media at 37 °C with 5% CO_2. Next, the cells were washed with PBS, and then 20 μM CHS-5-AM was added. The Cytation 5 Bio Tek plate reader recorded the relative emission intensity, and the integrated emission intensity vs. the log of the concentration of PAG was observed (Figure 9).

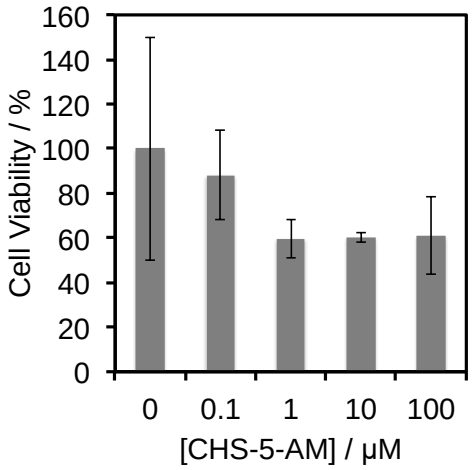


Figure 10. The MTT assay evaluating the cell viability of CHS-5-AM with 0, 0.1, 1, 10, and 100 μM of CHS-5-AM after an 18-hour incubation period. Three technical replicates across one biological replicate was performed using a Cytation 5 Bio Tek plate reader at 37 °C by Daphne Nguyen.

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted using human lung adenocarcinoma epithelial cells (A549) that were purchased from ATCC. The cells were first seeded into a 96-well plate giving a total volume of 120 μL / well and maintained in an incubator at 37 °C with 5% CO_2 for 12 hours. Once about 80% cell confluence was achieved, the complete F-12K media was removed, and the cells were washed with PBS. The cells were then treated with 0, 0.1, 1, 10, and 100 μM of CHS-5-AM and incubated for another 18 hours in 125 μL complete F-12K media per well. Next, each well was treated with 10 μL of the MTT reagent (Cayman Chemical, Ann Arbor, MI), mixed gently, and incubated for an additional 4 hours. Lastly, 100 μL of crystal dissolving solution was added to each well to dissolve the formazan crystals. Then the Cytation 5 Bio Tek plate reader was used to measure the absorbance at 570 nm and the cell viability was reported as a percent of the control (Figure 10).

1.3 Conclusions

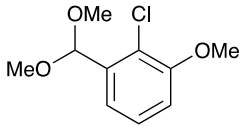
To summarize, the next generation of hydrogen sulfide probes (CHS-4 and CHS-5-AM) have been synthesized and characterized, expanding the general knowledge of using chemiluminescence to detect hydrogen sulfide both *in vitro* and *in vivo*. CHS-4 and CHS-5-AM produced instantaneous light emission in the presence of hydrogen sulfide in a dose dependent manner in PBS buffer at a physiologically relevant pH. CHS-5-AM proves the feasibility of developing chemiluminescent, cell-permeable probes that detect endogenous hydrogen sulfide without the need of a fluorescent enhancer. Propargylglycine (PAG) inhibition of cellular hydrogen sulfide production was also examined in living A549 cells with CHS-5-AM. Although improvement could be done on the scaffold design, CHS-5-AM serves as a good building block to now increase sensitivity and kinetics in the next generations of chemiluminescent hydrogen sulfide probes.

1.4 Experimental Methods

1.4.1 General Materials and Methods

All reactions were performed in dried glassware under an atmosphere of dry N_2. Silica gel P60 (SiliCycle) was used for column chromatography, and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light or by staining with iodine. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), and

Cayman Chemical (Ann Arbor, MI) and used without further purification. ^{1}H NMR for compounds **7,13, 14,** and CHS-4 were collected on a Bruker 400 MHz spectrometer in the Department of Chemistry at Southern Methodist University. ^{1}H NMR for compound **2, 3, 4, 5, 6, 8, 9, 10, 11,** CHS-5-AM and ^{13}C NMR for compounds **9, 10, 11**, and CHS-5-AM were measured on a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University. All ^{1}H and ^{13}C NMR spectra for characterization of new compounds and monitoring reactions were collected in $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA). All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of the deuterated solvent as an internal reference. Coupling constant units are in Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets.

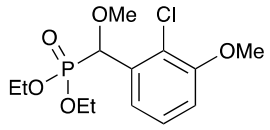


2

2-chloro-1-(dimethoxymethyl)-3-methoxybenzene (2).

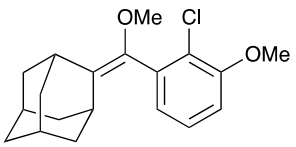
2-chloro-3-methoxybenzaldehyde (5 g, 29 mmol, 1.0 equiv.) was added to dry round-bottom flask, flushed with N_2, and dissolved in 15 mL of anhydrous methanol. P-toluenesulfonic acid (0.505 g, 2.93 mmol, 0.1 equiv.) and trimethyl orthoformate (3.2 mL, 29 mmol, 1 equiv.) were then added to the reaction mixture. The reaction was carried out at room temperature and allowed to run for 24 hours under N_2. After completion, the reaction was transferred to a separatory funnel and washed with 30 mL $NaHCO_3$. The

organic layer was then eluted with 3 x 30 mL EtOAc. Subsequently, the organic layers were combined and dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (10:1 hexanes: EtOAc) yielded compound (**2)** (5.181 g, 23.94 mmol, 82%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (s, 1H), 7.23 (s, 1H), 6.90 (d, 1H, *J* = 7 Hz), 5.64 (s, 1H), 3.88 (s, 3H), 3.37 (s, 6H).



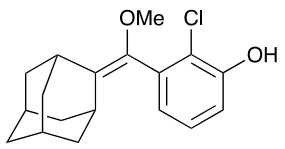
3

Diethyl ((2-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate (3). The acetal compound **(2)** (6.409 g, 29.61 mmol, 1.00 equiv.) was added to an oven-dried round-bottom flask, flushed with N_2, and dissolved in 10 mL anhydrous DCM. The reaction was then cooled to 0 °C in an ice bath. Boron trifluoride diethyl etherate (3.83 mL, 30.5 mmol, 1.03 equiv.) was then added dropwise to the flask. Then triethyl phosphite (5.23 mL, 30.5 mmol, 1.03 equiv.) was added dropwise to the mixture, and the reaction was stirred for 10 minutes at 0 °C. The reaction mixture temperature was then raised to 35 °C in an oil bath and stirred for 2 hours while being monitored by TLC. Upon completion, the reaction was cooled to room temperature and quenched with 30 mL of NH_4Cl. In a separatory funnel, the organic layer was then eluted with 3 x 30 mL of EtOAc. Then the organic layers were combined and dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (10:1 hexanes: EtOAc) yielded compound **(3)** (7.39 g, 22.9 mmol, 77 %) as a light brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (s, 1H), 6.89 (s, 1H), 5.16 (d, 1H, *J* = 16 Hz), 4.14 – 3.88 (m, 5H), 3.88 (s, 3H), 3.32 (s, 3H), 2.01 (s, 1H).



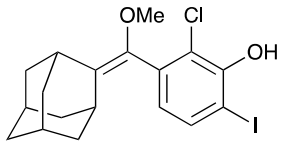
4

(1r,3r,5R,7S)-2-((2-chloro-3-methoxyphenyl)(methoxy)methylene)adamantine (4). Compound **(3)** (1.00 g, 3.09 mmol, 1.00 equiv.) was added to an oven-dried round-bottom flask, flushed with N_2, and dissolved in 10 mL anhydrous THF. The temperature was then brought down to –78 °C in a dry-ice acetone bath. 2.5 M n-BuLi (1.50 mL, 3.72 mmol, 1.20 equiv.) was then added slowly and dropwise over 10 minutes. Next, 2-adamantanone (0.577 g, 3.72 mmol, 1.20 equiv.) was dissolved in 5 mL anhydrous THF in a separate round bottom flask and then added to the reaction mixture dropwise over 10 minutes. The temperature is then raised to 35 °C in an oil bath and stirred for 2 hours. Subsequently, the temperature was raised to 90 °C and put under reflux for 1 hour. Upon completion, the reaction was cooled to room temperature and quenched with 30 mL of NH_4Cl. The mixture was transferred to a separatory funnel and the organic layer was eluted with 3 x 30 mL EtOAc. The combined organic layers were then dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (20: 1 hexanes: EtOAc) yielded compound **(4)** (0.683 g, 2.145 mmol, 69 %) as an amber oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19 (t, 1H, J = 16 Hz), 6.89 – 6.85 (m, 2H), 3.90 (s, 3H), 3.29 (s, 3H), 3.28 (s, 1H), 1.69 – 1.29 (m, 15H).



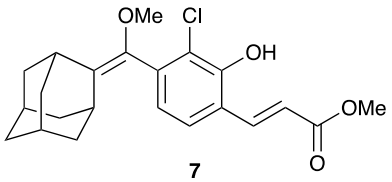
5

3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenol (5). The ether compound **(4)** (13.022 g, 40.897 mmol, 1.000 equiv.) was added to an oven-dried, N_2 filled flask and dissolved in 70 mL of anhydrous DMF. Sodium ethanethiolate (4.128 g, 49.076 mmol, 1.200 equiv.) and cesium carbonate (33.31 g, 102.2 mmol, 2.50 equiv.) were then added to the flask. The temperature was raised to 120 °C and stirred for 24 hours under reflux. TLC was used to determine the completion of the reaction, and then the reaction was quenched with 200 mL of NH_4Cl. The reaction contents were transferred to a separatory funnel, and the organic layer was eluted with 3 x 200 mL of EtOAc. Then the organic layer was washed with 3 x 250 mL of brine. Lastly, the organic layers were combined and dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (5:1 hexanes:EtOAc) was used for purification to give compound **(5)** (10.557 g, 34.635 mmol, 85%) as an off-white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.04 (t, 1H, *J* = 15 Hz), 6.91 (d, 1H, *J* = 6 Hz), 6.72 (d, 1H, *J* = 6 Hz), 3.22 (s, 3H), 3.19 (s, 1H), 2.01 – 1.84 (m, 16H).



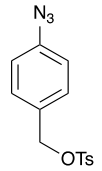
6

3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chloro-6-iodophenol (6). Compound **(5)** (1.00g, 3.28 mmol, 1 equiv.) and *N*-iodosuccinimde (0.74 g, 3.3 mmol, 1.0 equiv.) were added to a round-bottom flask, flushed with N_2, and then dissolved in 20 mL of toluene. The reaction was carried out at 0 °C in an ice bath for 30 minutes. There is a color change from light orange to red. Upon completion, the reaction mixture was transferred to a separatory funnel and washed with brine. A couple of sodium thiosulfate pentahydrate crystals were added to the separatory funnel to quench any remaining iodine. Here there was a color change from red to clear after shaking the separatory funnel containing the sodium thiosulfate pentahydrate crystals. If the color did not become clear, more crystals were added. The organic layer was eluted with 3 x 30 mL of EtOAc. Then the organic layers were combined, dried with Na_2SO_4, and concentrated under reduced pressure. For purification, the crude product was rinsed multiple times with hexanes. A solid white powder crashed out giving compound **(6)** (0.496 g, 1.151 mmol, 35%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (d, 1H, *J* = 8 Hz), 6.60 (d, 1H, *J* = 8 Hz), 3.28 (s, 3H), 3.24 (s, 1H), 2.07 – 1.71 (m, 15H).



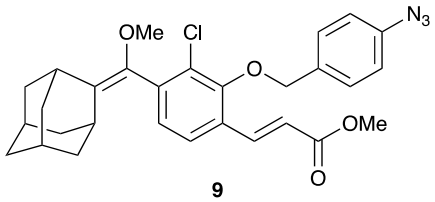
7

Methyl (E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylate (7). Compound **(6)** (0.123 g, 0.404 mmol, 1 equiv.), palladium (II) acetate (0.004 g, 0.02 mmol, 0.05 equiv.), and tri(*o*-tolyl)-phosphine (0.001 g, 0.004 mmol, 0.01 equiv.) were added to an oven-dried pressure flask and flushed with N_2. These reagents were then dissolved in 5 mL of anhydrous ACN. Then methyl acrylate (0.11 mL, 1.2 mmol, 3 equiv.) and triethylamine (0.10 mL, 0.61 mmol, 1.5 equiv.) were added to the pressure flask. The reaction was then tightly capped and heated to 120 °C for 2 hours. Upon completion, the reaction was cooled to room temperature and quenched with 30 mL NH_4Cl and 30 mL of brine. The reaction contents were transferred to a separatory funnel, and the organic layer was washed with 3 x 30 mL of EtOAc. Finally, the organic layers were combined, filtered, and concentrated under reduced pressure. Column chromatography (12:1 hexanes:EtOAc) yielded compound **(7)** (0.038 g, 0.098 mmol, 80%) as a brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 (d, 1H, J = 16 Hz), 7.40 (d, 1H, J = 8 Hz), 6.88 (d, 1H, J = 8 Hz), 6.63 (d, 1H, J = 16 Hz), 6.35 (s, 1H), 3.84 (s, 4H), 3.33 (s, 3H), 3.29 (s, 1H), 2.14 – 1.19 (m, 22H).



8

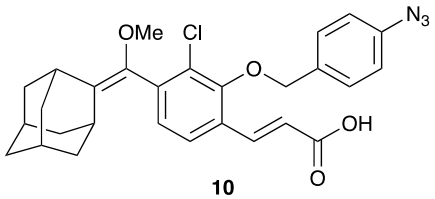
4-azidobenzyl 4-methylbenzenesulfonate (8) Compound **(13)** (1.01 g, 6.79 mmol, 1.0 equiv.) was dissolved in 15 mL DCM in an oven-dried round bottom flask under N_2 atmosphere and cooled to 0°C. p-Toluenesulfonic anhydride (3.323 g, 10.18 mmol, 1.5 equiv.) and pyridine (1.1 mL, 14 mmol, 2.0 equiv.) were added to the flask, stirred for 1 hour, and monitored by TLC. Upon completion, the crude reaction was washed with sat. NH_4Cl and brine, and eluted with 3 x 30 mL EtOAc. To purify, the crude product was dissolved in 10 mL diethyl ether and filtered through a cotton plug. It was then dissolved in 10 mL hexanes and cooled at 0°C for 1 hour. Then the supernatant was pipetted off and washed with 2 x 10 mL hexanes. Finally, the pure product was concentrated under reduced pressure yielding compound **(8)** (1.39 g, 4.58 mmol, 55%) and stored in the freezer. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72 (d, 2H, *J* = 9 Hz), 7.26 (d, 2H, *J* = 9 Hz), 7.17 (d, 2H, *J* = 9 Hz), 6.90 (d, 2H, *J* = 9 Hz), 4.95 (s, 2H), 2.38 (s, 3H).



9

Methyl(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-((4-azidobenzyl)oxy)- 3-chlorophenyl)acrylate (9) The precursor phenol compound **(7)** (0.0889g, 0.229 mmol, 1 equiv.) was added to an oven dried round bottom flask and dissolved in 5 mL anhydrous DMF under N_2 atmosphere. Compound **(8)** (0.0886 g, 0.292

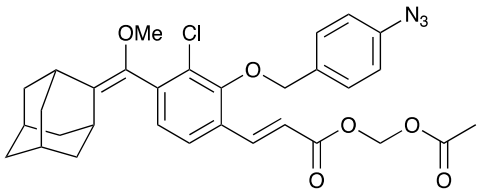
mmol, 1.5 equiv.) and K_2CO_3 (0.134 g, 0.970 mmol, 5 equiv.) were added and the reaction was stirred at room temperature overnight and monitored with TLC. After completion, the reaction was transferred to a separatory funnel and quenched with sat. NH_4Cl. The organic layer was eluted with 3 x 30 mL EtOAc, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (25:1 hexanes: EtOAc) yielded compound **(9)** (0.0204 g, 0.039 mmol, 20%) as an amber oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.88 (d, 1H, *J* = 18 Hz), 7.44 (d, 2H, *J* = 8 Hz), 7.41 (d, 1H, *J* = 10 Hz), 7.06 (d, 1H, *J* = 8 Hz), 7.01 (d, 2H, *J* = 9 Hz), 6.42 (d, 1H, *J* = 16 Hz), 4.97 – 4.95 (m, 2H), 3.79 (s, 3H), 3.30 (s, 3H), 3.25 (s, 1H), 1.94 – 1.57 (m, 15H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.18, 153.61, 140.44, 139.46, 138.89, 138.29, 132.75, 132.62, 130.59, 130.19, 129.9, 129.75, 127.96, 125.17, 119.99, 119.31, 119.20, 77.37, 77.12, 76.86, 57.37, 51.92, 39.29, 39.13, 38.74, 38.74, 38.68, 37.13, 33.02, 29.79, 28.43, 28.27.



10

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-((4-azidobenzyl)oxy)- 3-chlorophenyl)acrylic acid (10) Compound **(9)** (0.0204 g, 0.0392 mmol, 1 equiv.) was added to an oven dried round bottom flask and dissolved in 5 mL THF under a N_2 atmosphere. Then 5 mL of 1 M LiOH was added and the reaction was heated to 80 °C under reflux for 5 hours and monitored with TLC. Upon completion, the reaction was transferred to a separatory funnel and washed with 1 M HCl and eluted with 3 x 30

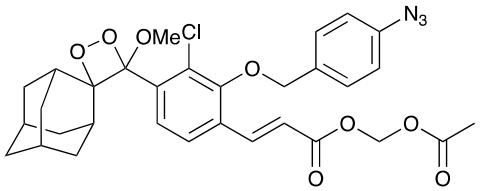
mL EtOAc. The organic layer was collected, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (8:1 hexanes:EtOAc) yielded compound **(10)** (0.0195 g, 0.385 mmol, 99%) as an amber oil. ^{1}H NMR (500 MHz, $CDCl_3$) **δ** 7.99 (d, 1H, *J* = 17 Hz), 7.45 – 7.47 (m, 3H), 7.10 (d, 1H, *J* = 9 Hz), 7.04 (d, 2H, *J* = 8 Hz), 6.44 (d, 1H, 16 Hz), 5.05 – 4.95 (m, 2H), 3.33 (s, 3H), 3.28 (s,1H), 2.01 – 1.60 (m, 15H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.96, 153.82, 141.10, 139.39, 138.78, 132.81, 132.61, 130.68, 129.98, 129.44, 128.02, 125.36, 119.34, 119.27, 77.38, 77.12, 76.87, 57.41, 39.29, 38.74, 37.12, 33.06, 29.81, 28.43, 28.28.



11

Acetoxymethyl(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)- 2-((4-azidobenzyl)oxy)- 3-chlorophenyl)acrylate (11). Compound **(10)** (0.02g, 0.04 mmol, 1 equiv.) was added to an oven dried round bottom flask and dissolved in 1 mL DMF under a N_2 atmosphere. Bromomethyl acetate (0.02 mL, 0.2 mmol, 4 equiv.) and DIPEA (0.024 mL, 0.14 mmol, 4 equiv.) were added, and the reaction stirred at room temperature for 4 hours while being monitored by TLC. Upon completion, the reaction was transferred to a separatory funnel and quenched with sat. NH_4Cl. The organic layer was eluted with 3 x 30 mL EtOAc, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (5:1 hexanes: EtOAc) yielded compound **(11)** (0.02g, 0.035 mmol, 92%)

as an amber oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.91 (d, 1H, *J* = 16 Hz), 7.39 – 7.35 (m, 3H), 7.02 (d, 1H, *J* = 10 Hz), 6.97 (d, 2H, *J* = 6 Hz), 6.36 (d, 1H, *J* = 14 Hz), 5.81 (s, 1H), 5.49 (s, 1H), 4.96 – 4.85 (m, 2H), 3.26 (s, 3H), 3.21 (s, 1H), 1.95 – 1.32 (m, 15H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.84, 165.25, 153.78, 140.75, 139.39, 138.83, 132.80, 132.55, 130.70, 129.97, 129.38, 128.01, 125.18, 119.27, 118.70, 77.38, 77.12, 76.87, 57.42, 39.28, 39.12, 38.73, 38.67, 37.11, 33.04, 29.80, 28.42, 28.26, 20.89.

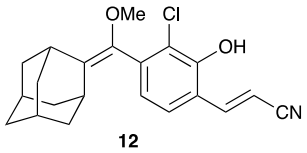


CHS-5-AM

acetoxymethyl(E)-3-(2-((4-azidobenzyl)oxy)-3-chloro-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane- 2,3'-[1,2]dioxetan]-4'-yl)phenyl)acrylate (CHS-5-AM)

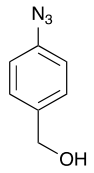
Compound **(11)** (10 mg, 0.017 mmol, 1 equiv.) and methylene blue hydrate (4 mg, 0.01 mmol, 0.7 equiv.) were added to a dry flask and dissolved in 2 mL DCM. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0°C. After 1.5 hours, TLC indicated no starting material remained, the reaction mixture was concentrated under reduced pressure. The product was purified by column chromatography (4:1 hexanes:EtOAc) yielding compound **(CHS-5-AM)** (3.8 mg, 0.0064 mmol, 37%) as an amber oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, 1H, *J* = 18 Hz), 7.55 (d, 1H, *J* = 12 Hz), 7.42 (d, 3H, *J* = 8 Hz), 7.03 (d, 3H, *J* = 8 Hz), 6.45 (d, 1H, *J* = 17 Hz), 5.86 (s, 3H), 4.89 (s, 3H), 3.21 (s, 3H), 2.99 (s, 1H), 0.83 – 2.02 (m, 15H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.79, 166.57, 164.98, 154.18, 140.72, 140.13, 135.81, 132.23, 131.30, 130.76, 129.20, 127.93, 125.37, 119.97, 119.36, 111.79, 96.77, 96.44,

77.37, 77.11, 76.86, 49.83, 36.65, 33.97, 33.68, 32.72, 32.29, 31.68, 31.64, 31.59, 29.79, 26.22, 25.90, 22.75, 20.87, 14.22.



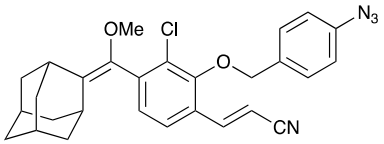
12

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylonitrile (12). Compound **(6)** (0.20 g, 0.46 mmol, 1.00 equiv.) and palladium (II) acetate (0.011 g, 0.046 mmol, 0.10 equiv.) were added to an oven-dried microwavable flask, flushed with N_2, and dissolved in 1 mL of acetonitrile. Then acrylonitrile (0.10 mL, 1.4 mmol, 3.0 equiv.) and triethylamine (0.10 mL, 0.70 mmol, 1.50 equiv.) were added to the reaction mixture. The flask was then capped and stirred in the microwave for 70 minutes at 120 °C. Upon completion, the reaction was cooled to room temperature and quenched with 15 mL of NH_4Cl and transferred to a separatory funnel. The organic layer was then eluted with 3 x 20 mL of EtOAc. The organic layers were then combined, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (9:1 hexanes: EtOAc) yielded compound **(12)** (0.069 g, 0.19 mmol, 42 %) as a brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, 1H, *J* = 17 Hz), 7.25 (d, 1H, *J* = 8 Hz), 6.86 (d, 1H, *J* = 8 Hz), 6.32 (s, 1H), 6.15 (d, 1H, *J* = 17 Hz), 3.29 (s, 3H), 3.24 (s, 1H), 2.02 – 1.15 (m, 15H).



13

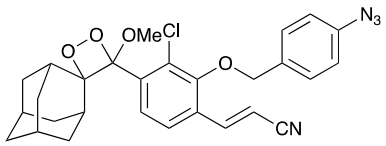
(4-azidophenyl)methanol (13) 4-aminobenzyl alcohol (1.0 g, 8.1 mmol, 1.0 equiv) was dissolved in 20 mL 10% HCl solution in a round bottom flask under N_2 atmosphere and cooled to 0°C. Sodium nitrite (0.672 g, 9.74 mmol, 1.2 equiv.) was dissolved in 10 mL water, added slowly and dropwise, and allowed to stir for 30 min. Then sodium azide (0.8278 g, 9.74 mmol, 1.2 equiv.) was dissolved in 10 mL water, added slowly and dropwise, and stirred for an additional 2 hrs. The reaction was monitored by TLC. Upon completion, the reaction was transferred into a separatory funnel, washed with brine, eluted with 3 x 50 mL EtOAc. The combined organic layer was dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by column chromatography (5:1 hexanes:EtOAc) yielded compound **(13)** (1.01 g, 6.79 mmol, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (d, 2H, *J* = 18 Hz), 7.02 (d, 2H, *J* = 18 Hz), 4.68 (s, 2H), 1.90 (s, 1H).



14

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-((4-azidobenzyl)oxy)- 3-chlorophenyl)acrylonitrile. (14) The precursor phenol, compound **(6)** (0.035 g, 0.098 mmol, 1 equiv.) was dissolved in 3 mL anhydrous THF under a N_2 atmosphere and cooled to 0°C. Compound **(13)** (0.0147 g, 0.098 mmol, 1 equiv.) and triphenylphosphine (0.031 g, 0.12 mmol, 1.2 equiv.) were added to the reaction mixture

and allowed to cool for 5 min. Then DEAD (0.02 mL, 0.1 mmol, 1.2 equiv.) was added and stirred for 1 hr while being monitored by TLC. Upon completion, the reaction was transferred to a separatory funnel and quenched with sat. NH_4Cl. The organic layer was eluted with 3 x 25 mL EtOAc, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Column chromatography (8:1 hexanes:EtOAc) yielded compound **(14)** (5.2 mg, 0.01 mmol, 11%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (d, 1H, *J* = 16 Hz), 7.43 (d, 4H, *J* = 23 Hz), 7.33 (d, 2H, *J* = 23 Hz), 7.15 – 7.02 (m, 5H), 5.93 (d, 2H, *J* = 16 Hz), 5.43 (s, 1H), 5.01 (s, 3H), 4.54 – 4.53 (m, 2H), 3.35 (s, 3H), 3.31 (s, 1H), 2.19 – 0.91 (m, 21H).



CHS-4

(E)-3-(2-((4-azidobenzyl)oxy)-3-chloro-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'- [1,2]dioxetan]-4'-yl)phenyl)acrylonitrile (CHS-4)

Compound **(14)** (5.2 mg, 0.01 mmol, 1 equiv.) and methylene blue hydrate (4 mg, 0.01 mmol, 0.7 equiv.) were added to a dry flask and dissolved in 2 mL DCM. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0°C. After 1.5 hours, TLC indicated no starting material remained, the reaction mixture was concentrated under reduced pressure. The product was purified by column chromatography (8:1 hexanes:EtOAc) yielding compound **(CHS-4)** (2.8 mg, 0.0005 mmol, 51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, 1H, *J* = 16 Hz), 7.54 (s, 1H), 7.50 (s, 1H), 7.42 (d, 2H, *J* = 8 Hz), 7.09 (d, 2H, *J* = 8 Hz), 5.98 (d, 1H, *J* = 20 Hz), 5.01 (s, 1H), 4.95 (s, 2H), 3.25 (s, 3H), 3.06 (s, 1H), 2.19 – 0.88 (m, 20H).

1.4.2 Chemiluminescent Response

The chemiluminescent response of CHS-5-AM with Emerald II Enhancer was done with a Hitachi-7000 Fluorescence Spectrometer (Hitachi, Tokyo, Japan, Figure 5) using the luminescence detection mode. 20 mM PBS buffer (pH 7.4), a 5 mM stock solution of CHS-5-AM (final concentration of 20 μM), and a 50 mM stock solution of Na_2S made immediately before the experiment were used. For Figure 5, A, various percentages of Emerald II Enhancer were used (0, 0.2, 2, 10%) with 1 mM Na_2S. For Figure 5, B, 2% enhancer was used with different concentrations of Na_2S (0, 50, 100, 200, 400, 800, 1000 μM). After the addition of PBS buffer, the probe, Na_2S, and the enhancer, the cuvette was shaken gently and the time scans was performed seconds later. All experiments were conducted at room temperature (25–28 °C) with three replicates of each.

The chemiluminescent response and time scans of CHS-4 and CHS-5-AM without enhancer were obtained with a Cytation 5 Bio Tek plate reader (Winooski, VT, Figure 7) using the luminescence detection method, endpoint read type with a gain set to 135, and conducted at room temperature (25–28 °C). A 5mM stock solution of each probe was used with a 20 μM final concentration. A 50 mM stock solution of Na_2S was made on the day of each experiment and the final concentration used varied (0, 50, 100, 200, 400, 800, 1000 μM). Experiments were performed in 20 mM PBS buffer (pH 7.4) in a multi-well plate. Scans were ran immediately following the addition of PBS buffer, probe, and Na_2S. Three replicates of each were obtained.

1.4.3 Cellular Experiments

Human lung adenocarcinoma epithelial cells (A549) were purchased from ATCC. The cells were cultured in Ham's F-12K (Kaighn's) medium with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cells were kept in a humidified incubator at 37 °C with 5% CO_2. One or two days before a cellular experiment was conducted, cells were plated onto a sterile multi-well plate and allowed to reach a confluence of 80–95% by the time of testing. All chemiluminescent responses for cellular testing were obtained using the Cytation 5 Bio Tek plate reader (Winooski, VT) at 37 °C.

APPENDIX

Cell Culture Protocol

Human lung adenocarcinoma epithelial cells (A549) were purchased from ATCC and is the only current cell line in the lab. Cells are kept in a humidified incubator at 37 °C with 5% CO_2.

The cells are cultured in Ham's F-12K (Kaighn's) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). To make complete F-12K medium for culturing A549 cells follow the following steps:

1. Turn on the UV for the hood 10 minutes prior to working in it.
2. Spray the hood down with the 70% alcohol mixture and wipe dry with paper towel.
3. Put the following items in the hood (after warming them up in the 37 °C water bath and then spraying with the alcohol mixture): a new bottle of incomplete F-12K (500 mL), FBS, and the penicillin.
4. Take out 55 mL of the incomplete F-12K media using a 55 mL pipette and transfer into a falcon tube. Label this falcon tube as "incomplete F-12K and the date".
5. Using another 55 mL pipette, add 50 mL of FBS to the remainder of the incomplete F-12K bottle.

6. Then using a 5 mL pipette, add 5 mL of penicillin to the incomplete F-12K bottle.
7. Swirl gently to ensure mixing without causing bubbles.
8. Label the bottle as “Complete and date and ARL”. The mixture will be a red color at first and then turn to a pink color over time.
9. Store the complete media in the fridge.
10. The FBS is stored in Dr. Zoltowski’s lab in the –80 °C freezer. Make 4 aliquots of 50 mL FBS in falcon tubes to keep in the fridge before storing it the freezer.

To passage A549 cells, follow the following steps:

1. Check the confluence of the cells. The confluence should be around 80–95% before passaging. Otherwise, wait another day or two to allow the cells to proliferate more.
2. Turn on the UV for the hood 10 minutes prior to working in it.
3. Spray the hood down with the 70% alcohol mixture and wipe dry with paper towel.
4. Put the following items in the hood (after warming them up in the 37 °C water bath and then spraying with the alcohol mixture): the complete F-12K media, trypsin, and PBS.
5. To start, remove the cell flask from the incubator and aspirate off the old media.
6. Wash away the dead cells by rinsing the cells with 10 mL of PBS (use a 10 mL pipette). Then aspirate off the PBS.

7. Add 2 mL of trypsin to the flask. Move the plate around to ensure all the cells are coated with trypsin. Then incubate for 10 minutes to allow the trypsin to break the bonds between the cell surface and the flask.
8. Then bring the flask back to the hood and add 10 mL of complete F-12K media. Swirl the flask slightly to make sure all the cells are in the media.
9. Using the same 10 mL pipette, draw up the entire contents of the flask back into the pipette and transfer into a falcon tube.
10. Tightly cap the flacon tube and put it into the centrifuge for 10 minutes at 1000 rpm.
11. Aspirate off the supernatant. Then add about 1 mL of complete media to the falcon tube. Disperse the cells evenly throughout by using a 1000 μL pipette and pipetting up and down multiple times.
12. Add 20 mL of complete F-12K media to a new T75 flask.
13. Add 20 μL of the cells to the flask and gently rock the flask back and forth to disperse the cells. Check the confluence of the cells. If more cells are needed, continue to add cells and check the confluence.
14. Lastly, label the flask with the your initials, the date, the cell line (A549), and the passage and generation number. Store in the incubator.

The frozen cells are kept in a liquid nitrogen tank and a binder is kept nearby with the inventory of the frozen cells. To freeze cells, follow the following steps:

1. Freezing more cells should be done after 2 or 3 passages from newly thawed cells. This allows the cells to grow enough before freezing again.

2. The confluence should be around 80–90 % before freezing.
3. Prepare 10 mL the freezing media (10% cell-culture grade DMSO in complete media). This will make 10 vials for freezing.
4. Follow all cell passaging steps like normal up until the centrifuge step.
5. After the centrifuge step, aspirate off the media.
6. Add 1 mL of the cell freezing media. Disperse the cells throughout the media by pipetting up and down with a 1000 μL pipette.
7. Then add the rest of the freezing media to the falcon tube and mix.
8. Use the cryopreservation vials to freeze the cells. Add 1 mL of the cell mixture to each vial. Cap the vials tightly and label with the cell type, generation and passage number, date, and initials. Parafilm the lids of the vials.
9. Put the vials in the Mr. Frosty vial container and put into the –80 °C freezer in Dr. Zoltowski's lab for 3 days.
10. After 3 days, transfer the vials into the liquid nitrogen container.
11. Fill out the cell inventory in the liquid nitrogen storage chart.

To thaw cells, follow the following steps:

1. Turn on the UV for at least 10 minutes prior to starting.
2. Put the complete F-12K media in the water bath and allow to reach 37 °C.
3. Check the inventory of the cells to locate the vials in the liquid nitrogen storage chart. Pull up the cells from the liquid nitrogen tank- labeled as B. Let the liquid nitrogen drip back into the tank before pulling it all the way out. Use the thick orange gloves when handling the storage containers in the liquid nitrogen tank.

4. Take out 2 vials and allow them to thaw on the counter for about 5–10 minutes.
5. Spray down the hood with the 70% alcohol spray.
6. In the hood, draw up the cells from the vials and transfer into a falcon tube.
7. Add complete F-12K media to the falcon tube so the total volume is about 10 mL.
8. Centrifuge for 10 minutes at 1000 rpm.
9. Then remove the media from the falcon tube, leaving the pellet of cells.
10. Add 1 mL of complete F-12K media to the falcon tube and pipette up and down to mix.
11. Add about 20 mL of complete F-12K media to a new T75 flask.
12. Add ALL the cells from the falcon tube into the T flask.
13. Label the flask with your initials, the date, the cell line (A549), and the passage and generation number. (If the cell vial thawed was labeled "P_2G_2", then this new flask would be labeled "P_1G_3". Store the cells in the incubator.
14. The next day, remove the media and wash the cells with 10 mL PBS. Then aspirate off the PBS and add 20 mL of complete F-12K media. The purpose of this step is to remove the dead cells that did not survive the freezing and thawing cycle.

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1.0
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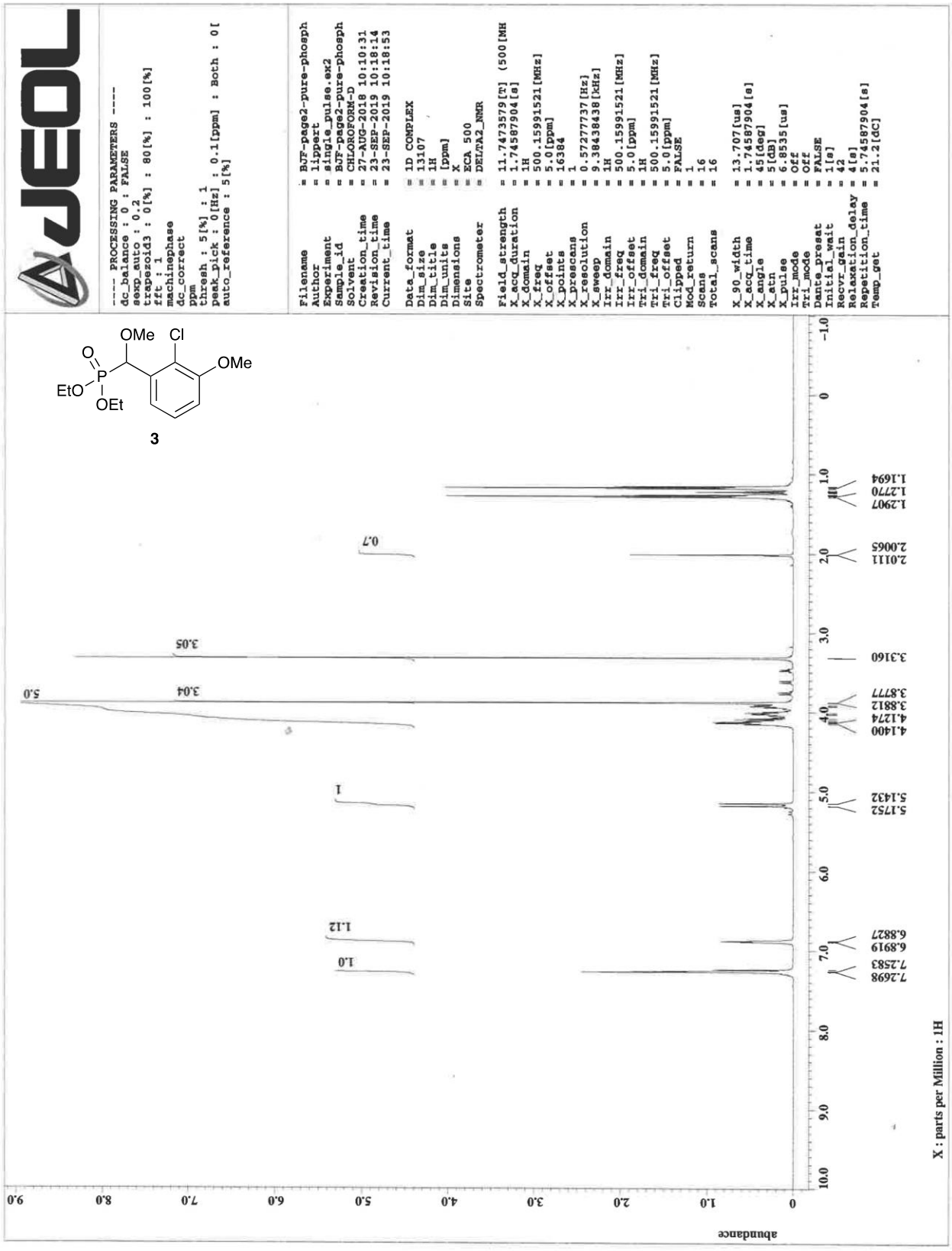
abundance
X : parts per Million : 1H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OMe
Cl
O
P
EtO
OEt
OMe
3
abundance
X : parts per Million : 1H
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7.2583
6.8919
6.8827
5.1752
5.1432
4.1400
4.1274
3.8812
3.8777
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2.0111
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Cl
OMe
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3.05
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7.2045
7.1885
7.1724
6.8747
3.9075
3.9041
3.2914
3.2868
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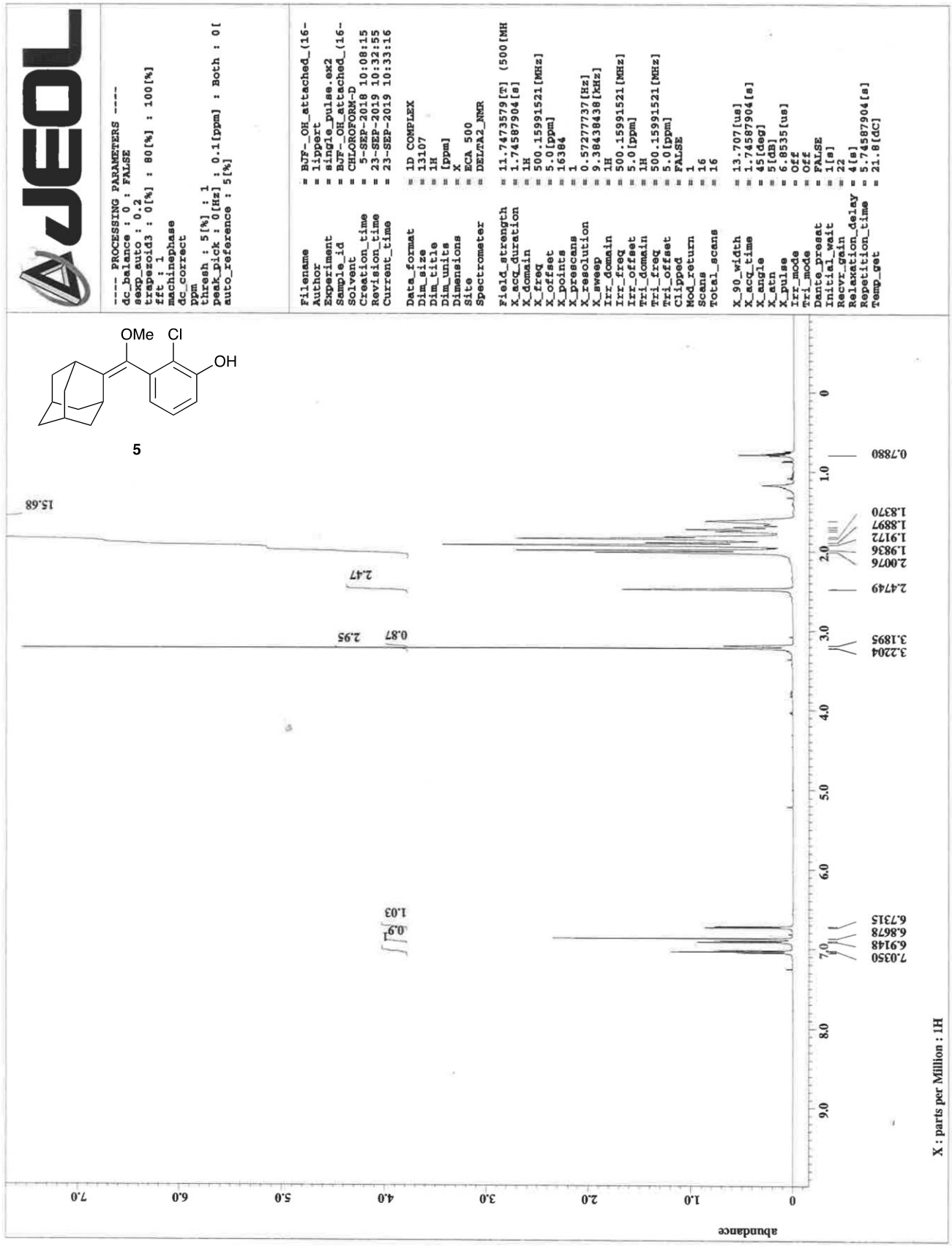
abundance
X : parts per Million : 1H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0.87
2.95
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0.9
1
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1.9172
1.9836
2.0076
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7.0350
abundance
X : parts per Million : 1H

JEOL
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1.7454
1.7145
abundance
X : parts per Million : 1H

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PROCNO 1
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TD0 1
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NUC1 1H
P1 11.25 usec
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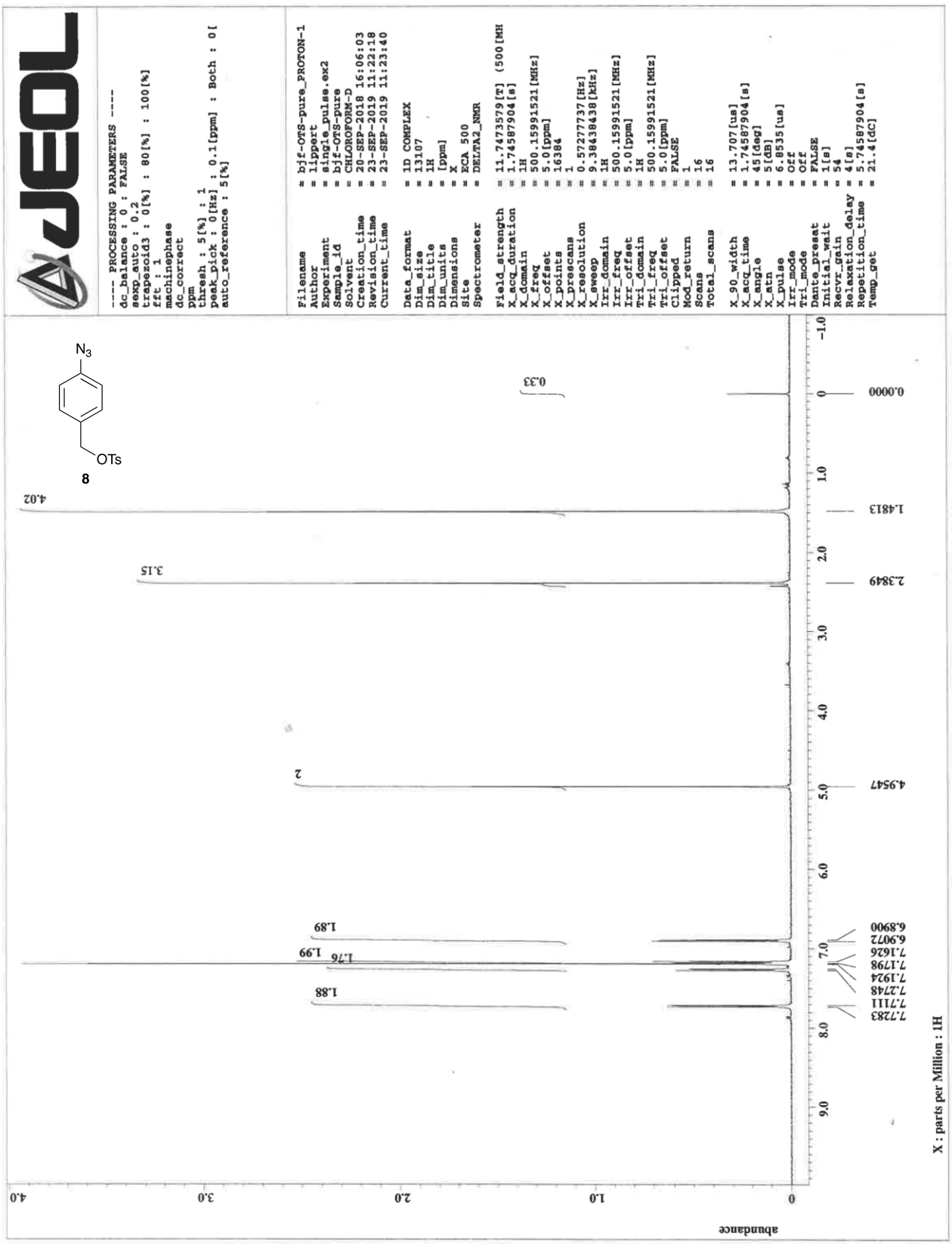
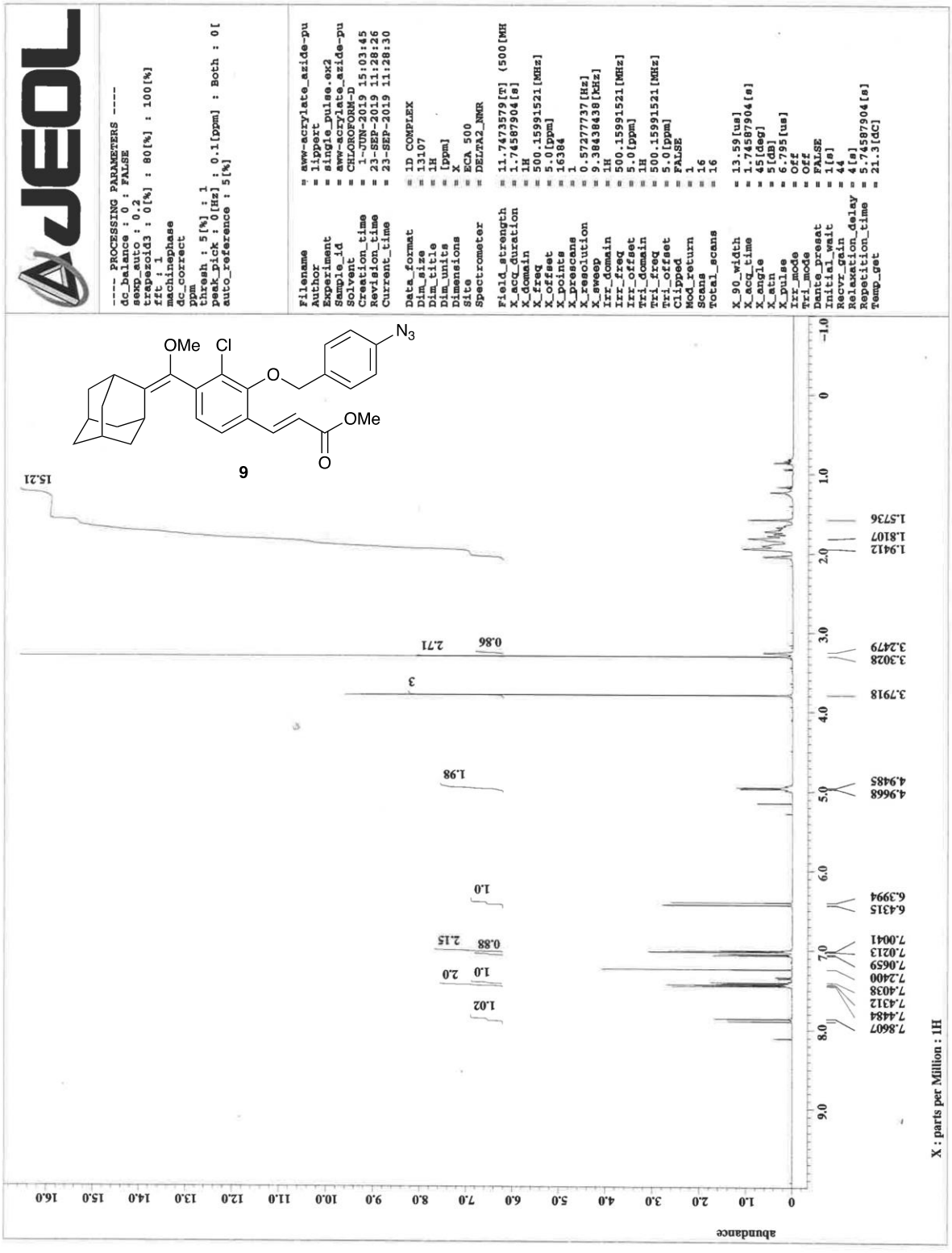
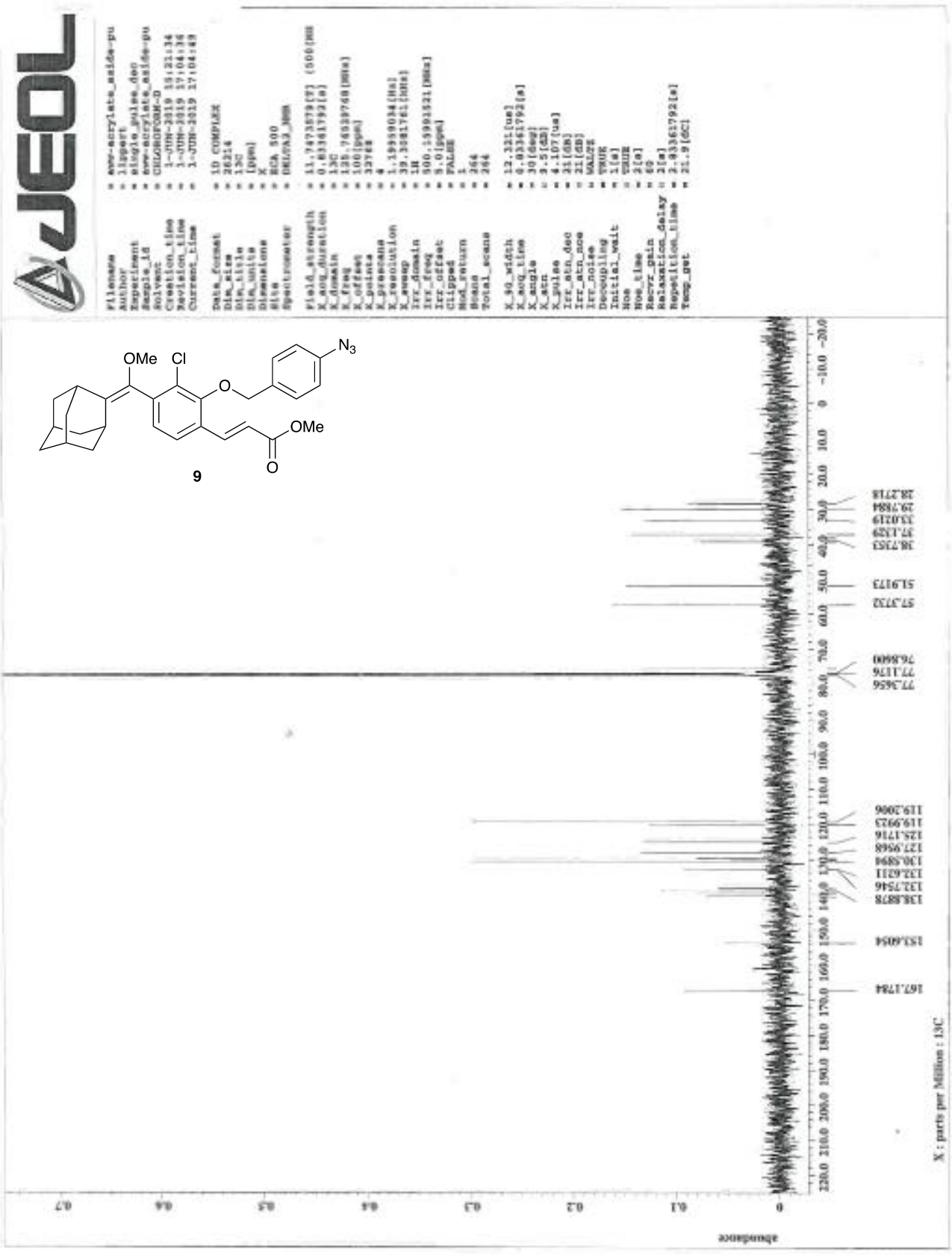
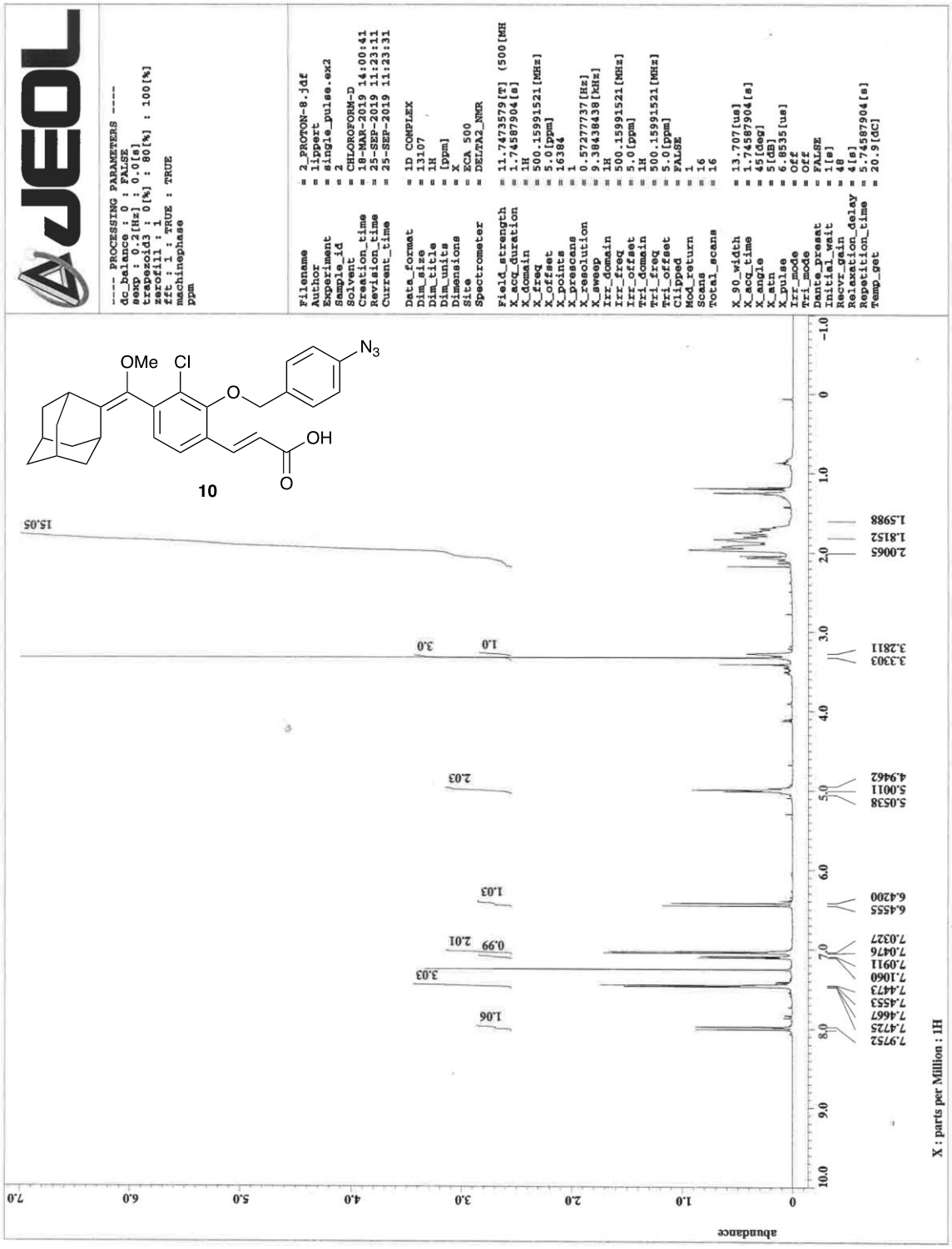
1.24
1.05
1.00
1.42
ppm

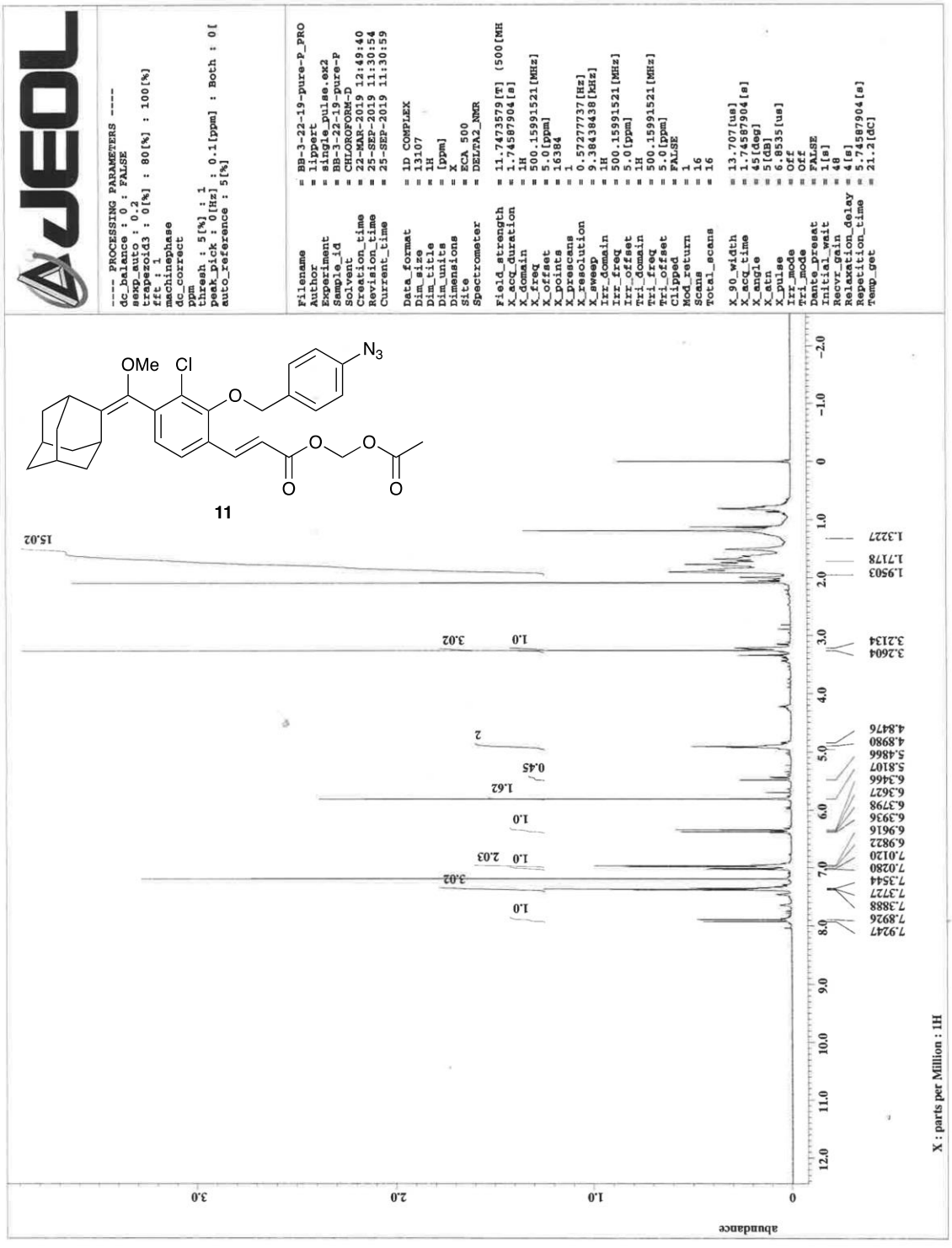

JEOL
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
Filename = bjf-OTS-pure_PROTON-1
Author = 11ppert
Experiment = single_pulse.ex2
Sample_id = bjf-OTS-pure
Solvent = CHLOROFORM-D
Creation_time = 20-SEP-2018 16:06:03
Revision_time = 23-SEP-2019 11:22:18
Current_time = 23-SEP-2019 11:23:40
Data_format = 1D COMPLEX
Dim_size = 13107
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 13.707[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 5[dB]
X_pulse = 6.8535[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Recvr_gain = 54
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_get = 21.4[dC]
N3
OTs
8
4.02
3.15
2
1.89
1.99
1.76
1.88
0.33
7.7283
7.7111
7.2748
7.1924
7.1798
7.1626
6.9072
6.8900
0.0000
1.4813
2.3849
4.9547
abundance
X : parts per Million : 1H


JEOL
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
exp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
Filename = aww-acrylate_azide-pu
Author = lippert
Experiment = single_pulse.ex2
Sample_id = aww-acrylate_azide-pu
Solvent = CHLOROFORM-D
Creation_time = 1-JUN-2019 15:03:45
Revision_time = 23-SEP-2019 11:28:26
Current_time = 23-SEP-2019 11:28:30
Data_format = 1D COMPLEX
Dim_size = 13107
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 13.59[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 5[dB]
X_pulse = 6.795[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Recvr_gain = 44
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_get = 21.3[dC]
OMe
Cl
O
N3
OMe
O
9
15.21
2.71
0.86
3
1.98
1.0
0.88
2.15
1.0
2.0
1.02
1.5736
1.9412
1.8107
3.3028
3.3479
3.7918
4.9668
4.9485
6.4315
6.3994
7.8607
7.4484
7.4312
7.4038
7.2400
7.0659
7.0213
7.0041
abundance
X : parts per Million : 1H

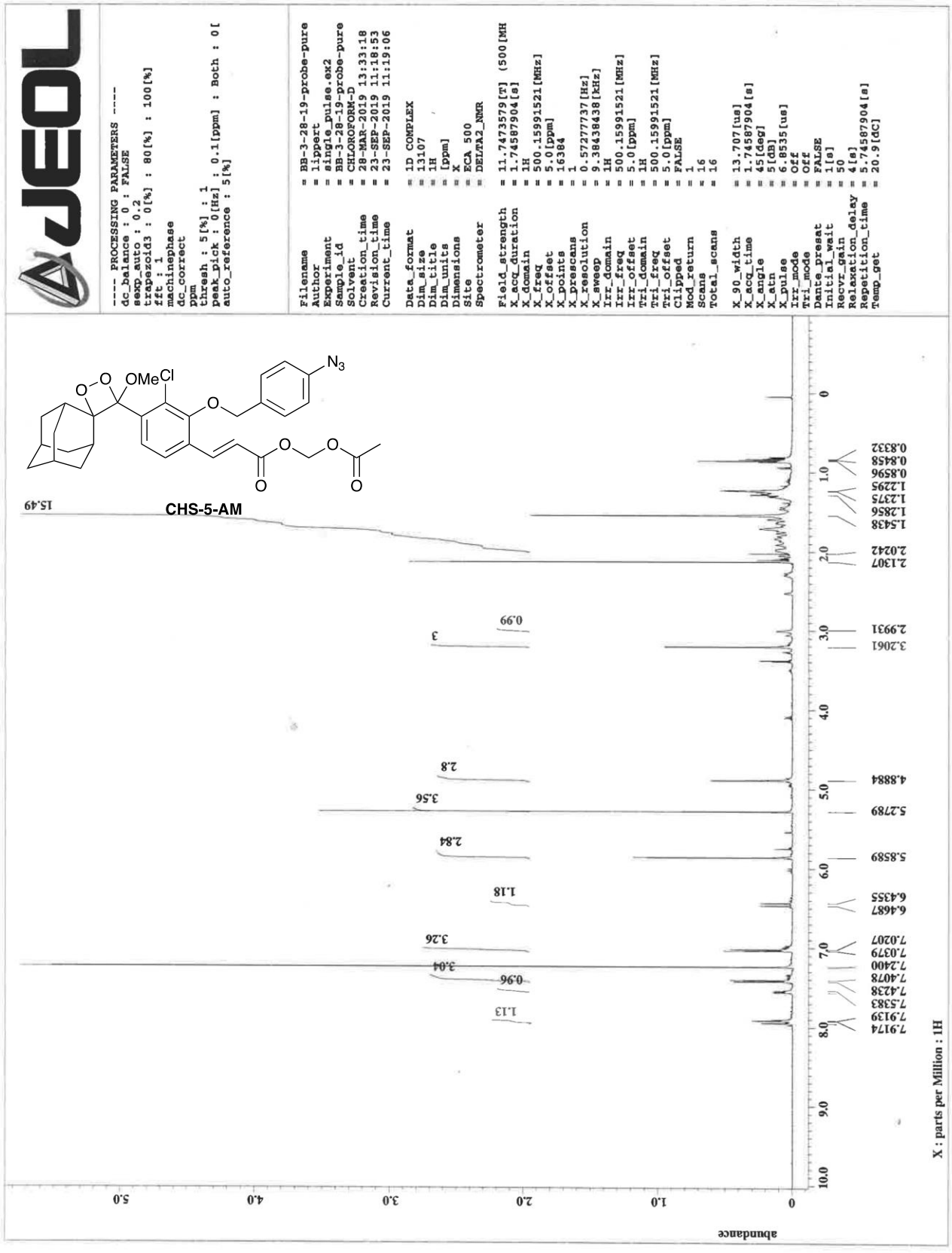

OMe
Cl
O
N3
OMe
O
9
JEOL
Filename = exv-acrylate_azide-pu
Author = ligpet
Experiment = single_pulse_dec
Sample_id = exv-acrylate_azide-pu
Solvent = CHLOROFORM-D
Creation_time = 1-JUN-2019 15:21:34
Revision_time = 1-JUN-2019 17:04:36
Current_time = 1-JUN-2019 17:04:49
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA3_NMR
Field_strength = 11.7473578[T] (500[MH
X_acq_duration = 0.83361792[s]
X_domain = 13C
X_freq = 125.7652976[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 8
X_resolution = 1.19999034[Hz]
X_sweep = 39.3681741[kHz]
Irr_domain = 1H
Irr_freq = 500.1599152[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 264
Total_scans = 264
X_90_width = 12.325[us]
X_acq_time = 0.83361792[s]
X_angle = 30[deg]
X_atn = 3.5[dB]
X_pulse = 4.107[us]
Irr_atn_dec = 21[dB]
Irr_atn_one = 21[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Now_time = 2[s]
Recyc_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 2.83361792[s]
Temp_get = 21.9[dC]
167.1784
153.4094
138.8878
132.7546
132.6211
130.5894
127.9568
125.1716
119.9923
119.2006
77.3656
77.1176
76.8600
57.3732
51.9173
38.7353
37.1329
33.0219
29.7884
28.2718
absorbance
X : parts per Million : 13C


JEOL
OMe
Cl
O
N3
OH
O
10
X : parts per Million : 1H
abundance

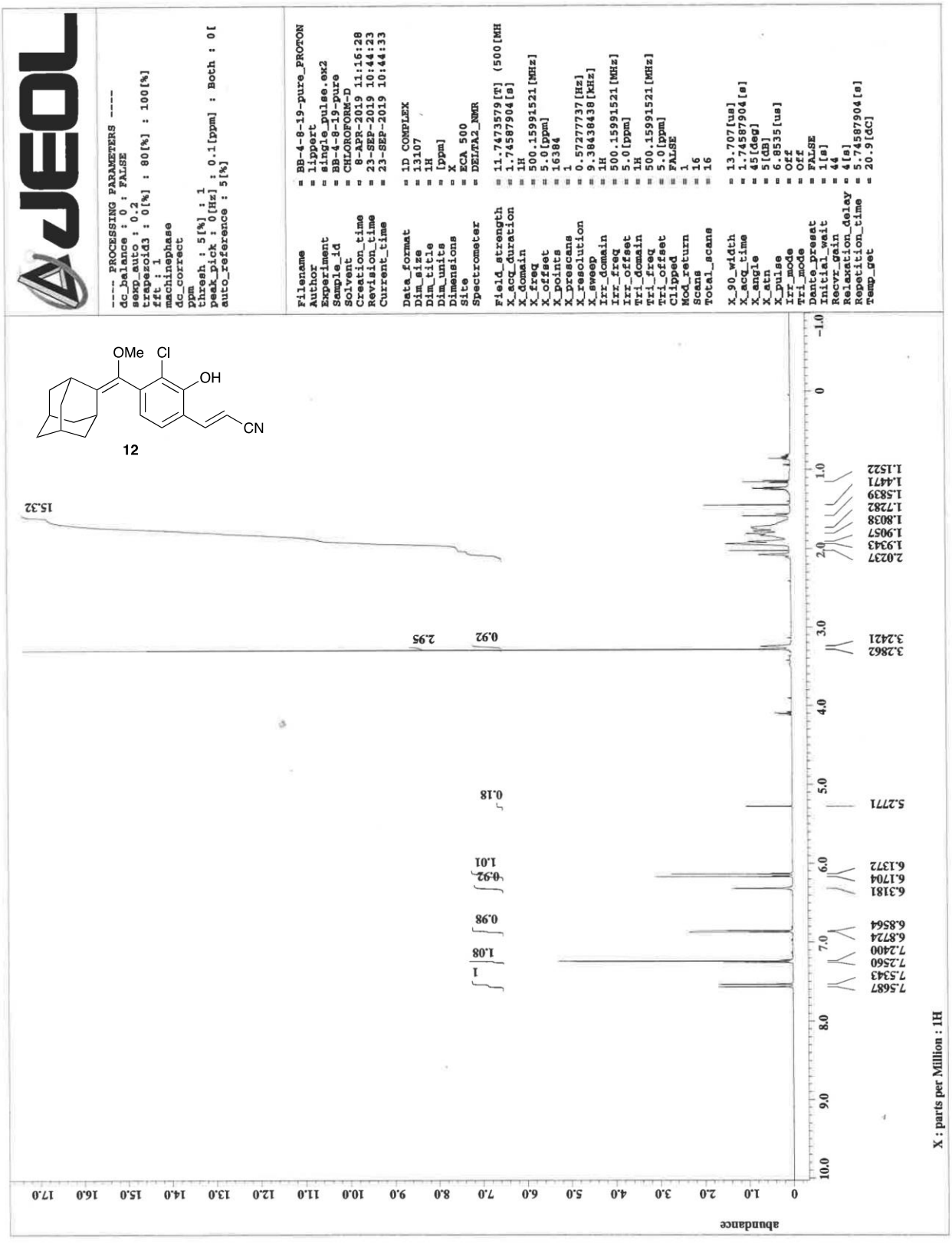
JEOL
Filename = wa-acrylate_carboxyli
Author = lippert
Experiment = single_pulse_dec
Sample_id = wa-acrylate_carboxyli
Solvent = CHLOROFORM-D
Creation_time = 4-JUN-2019 15:37:10
Revision_time = 4-JUN-2019 17:20:04
Current_time = 4-JUN-2019 17:20:41
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 0.83361792[s]
X_domain = 13C
X_freq = 125.76529768[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 156
Total_scans = 156
X_90_width = 12.321[us]
X_acq_time = 0.83361792[s]
X_angle = 30[deg]
X_atn = 9.5[dB]
X_pulse = 4.107[us]
Irr_atn_dec = 21[dB]
Irr_atn_noe = 21[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recyr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 2.83361792[s]
Temp_get = 22[dC]
OMe
Cl
O
N3
OH
O
10
171.9571
153.8152
141.1006
139.3933
132.6115
130.6752
128.0236
125.3624
119.3437
119.2674
77.3751
77.1176
76.8696
57.4114
38.7353
37.1234
29.8075
28.4339
28.2813
abundance
X : parts per Million : 13C


JEOL
---- PROCESSING PARAMETERS ----
dc_balence : 0 : FALSE
expg_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
Filename = BB-3-22-19-pure-P_PRO
Author = lippert
Experiment = single_pulse.ex2
Sample_id = BB-3-22-19-pure-P
Solvent = CHLOROFORM-D
Creation_time = 22-MAR-2019 12:49:40
Revision_time = 25-SEP-2019 11:30:54
Current_time = 25-SEP-2019 11:30:59
Data_format = 1D COMPLEX
Dim_size = 13107
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 13.707[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 5[dB]
X_pulse = 6.8535[us]
Irr_mode = Off
Tri_mode = Off
Dante_preset = FALSE
Initial_wait = 1[s]
Recvr_gain = 48
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_get = 21.2[dC]
OMe
Cl
O
N3
O
O
O
11
15.02
3.02
1.0
2
0.45
1.62
1.0
1.0
2.03
3.02
1.0
1.3227
1.7178
1.9503
3.2134
3.2604
4.8476
4.8980
5.4866
5.8107
6.3427
6.3466
6.3798
6.3936
6.9616
6.9822
7.0120
7.0280
7.3544
7.3727
7.3888
7.8926
7.9247
abundance
X : parts per Million : 1H

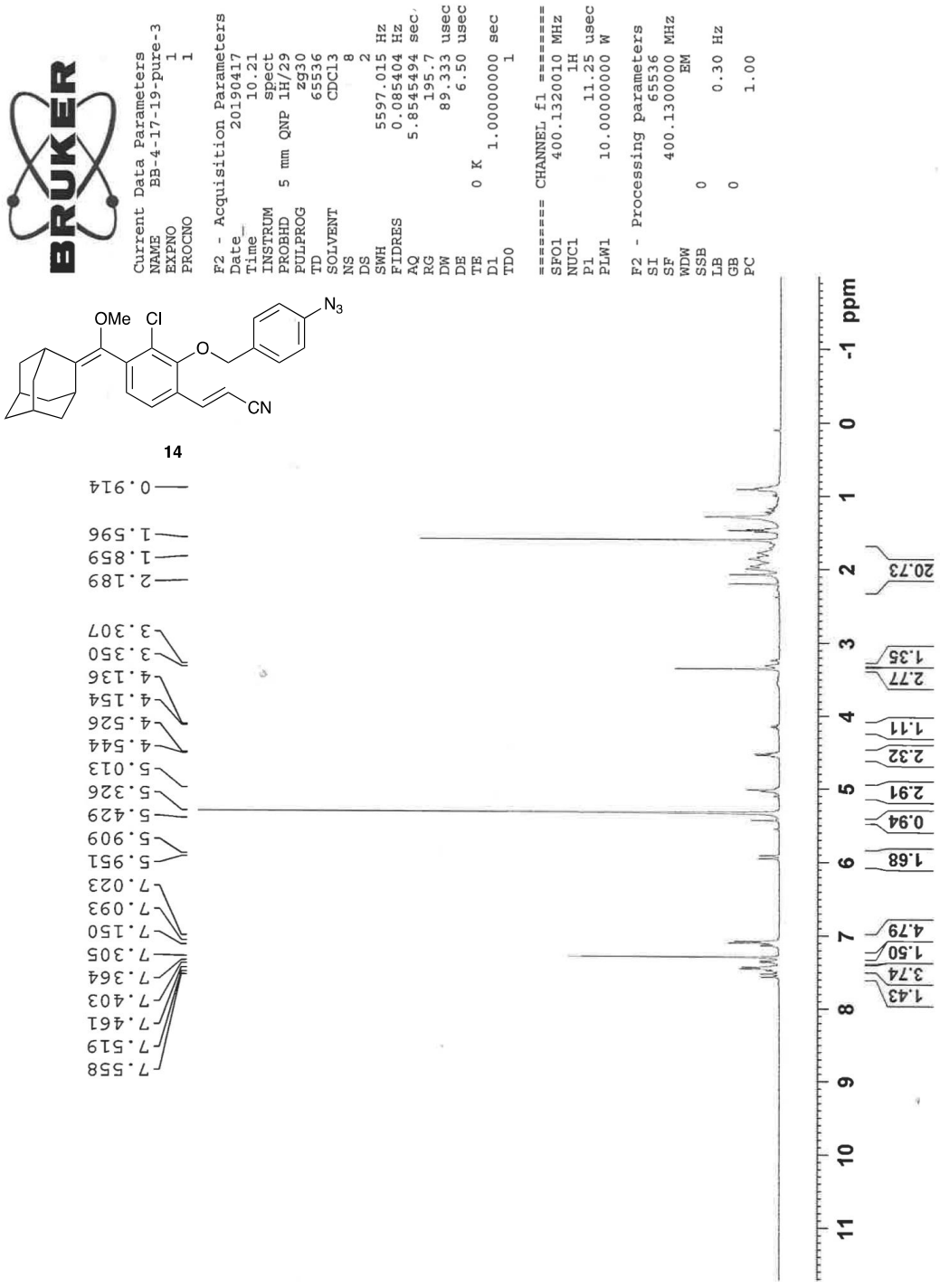
JEOL
Filename = aww-azide_am_ester-pu
Author = lippert
Experiment = single_pulse_dec
Sample_id = aww-azide_am_ester-pu
Solvent = CHLOROFORM-D
Creation_time = 5-JUN-2019 14:50:30
Revision_time = 5-JUN-2019 16:33:24
Current_time = 5-JUN-2019 16:33:29
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 0.83361792[s]
X_domain = 13C
X_freq = 125.76529768[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 155
Total_scans = 155
X_90_width = 12.321[us]
X_acq_time = 0.83361792[s]
X_angle = 30[deg]
X_atn = 9.5[dB]
X_pulse = 4.107[us]
Irr_atn_dec = 21[dB]
Irr_atn_noe = 21[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recvr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 2.83361792[s]
Temp_get = 21.9[dC]
OMe
Cl
N3
O
O
O
O
O
11
ABUNDANCE
X : parts per Million : 13C
169.8396
165.2517
153.7771
140.7477
139.3933
132.5543
130.7039
128.0141
125.1812
119.2674
118.6951
77.3751
77.1176
76.8696
57.4209
38.7258
37.1138
33.0410
29.7979
28.4244
28.2623
20.8891

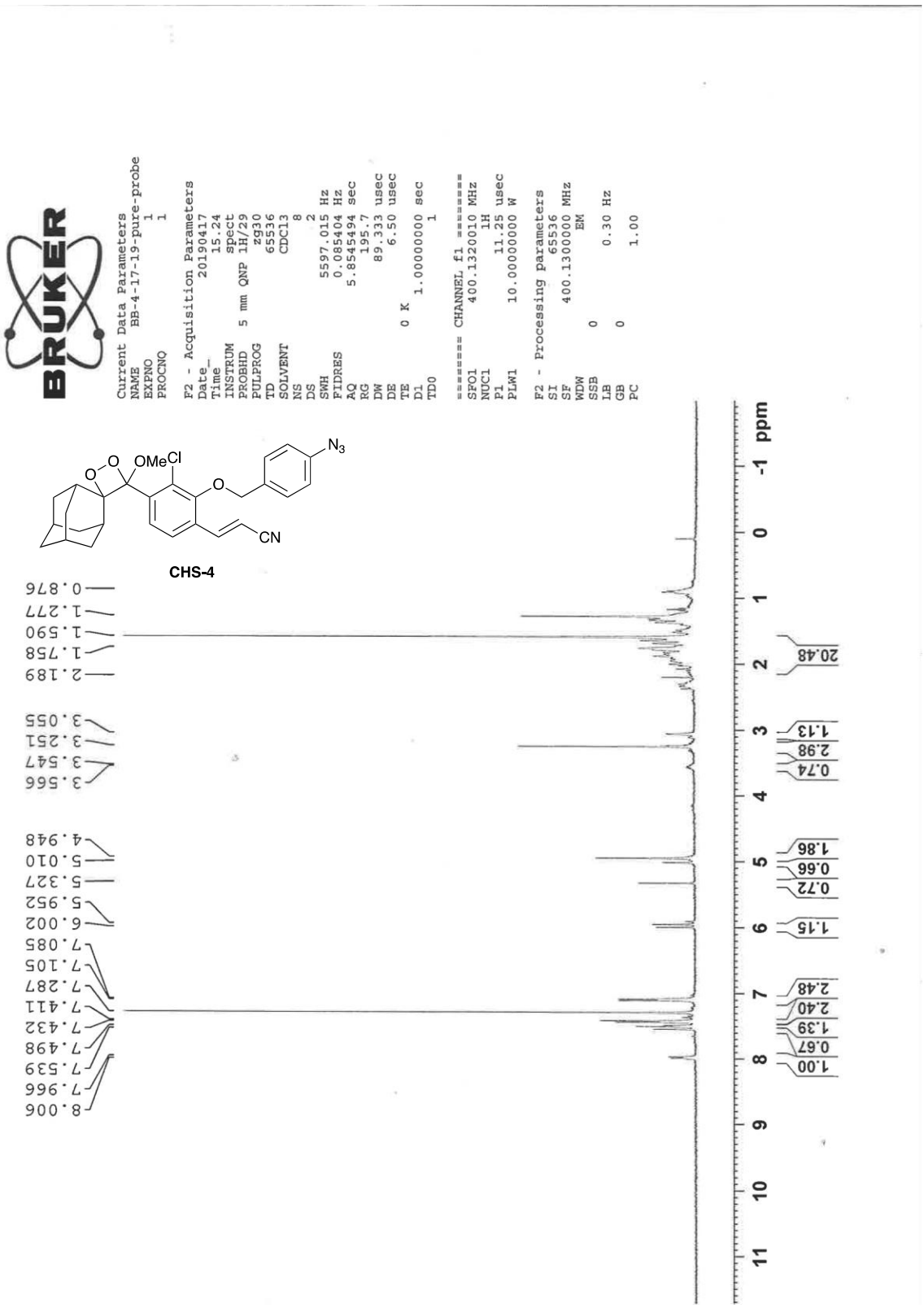

JEOL
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
exp_auto : 0.2
trapazoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
Filename = BB-3-28-19-probe-pure
Author = lippert
Experiment = single_pulse.ex2
Sample_id = BB-3-28-19-probe-pure
Solvent = CHLOROFORM-D
Creation_time = 28-MAR-2019 13:33:18
Revision_time = 23-SEP-2019 11:18:53
Current_time = 23-SEP-2019 11:19:06
Data_format = 1D COMPLEX
Dim_size = 13107
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 13.707[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 5[dB]
X_pulse = 6.8535[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Recvr_gain = 50
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_get = 20.9[dC]
CHS-5-AM
15.49
3
0.99
2.8
3.56
2.84
1.18
3.26
3.04
0.96
1.13
7.9174
7.9139
7.5383
7.4238
7.4078
7.2400
7.0379
7.0207
6.4687
6.4355
5.8589
5.2789
4.8884
3.2061
2.9931
2.1307
2.0242
1.5438
1.2856
1.2375
1.2295
0.8596
0.8458
0.8332
abundance
X : parts per Million : 1H

JEOL
Filename = aww-CHS-5-AM-probe_CA
Author = lippert
Experiment = single_pulse_dec
Sample_id = aww-CHS-5-AM-probe
Solvent = CHLOROFORM-D
Creation_time = 6-JUN-2019 15:06:35
Revision_time = 6-JUN-2019 16:50:53
Current_time = 6-JUN-2019 16:51:03
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.74735579[T] (500[MH
X_acq_duration = 0.83361792[s]
X_domain = 13C
X_freq = 125.76529768[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 222
Total_scans = 222
X_90_width = 12.321[us]
X_acq_time = 0.83361792[s]
X_angle = 30[deg]
X_atn = 9.5[dB]
X_pulse = 4.107[us]
Irr_atn_dec = 21[dB]
Irr_atn_noe = 21[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recyc_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 2.83361792[s]
Temp_get = 22[dC]
CHS-5-AM
abundance
X : parts per Million : 13C
14.2218
20.8701
25.8967
26.2211
31.6388
31.6770
32.2874
49.8284
76.8600
77.1080
77.3656
96.4422
96.7665
111.7894
119.3628
119.9733
125.3719
127.9282
129.1968
130.7611
131.2952
140.1277
154.1777
164.9751
166.5680
169.7919


JEOL
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
exp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
Filename = BB-4-8-19-pure_PROTON
Author = lippert
Experiment = single_pulse.ex2
Sample_id = BB-4-8-19-pure
Solvent = CHLOROFORM-D
Creation_time = 8-APR-2019 11:16:28
Revision_time = 23-SEP-2019 10:44:23
Current_time = 23-SEP-2019 10:44:33
Data_format = 1D COMPLEX
Dim_size = 13107
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = ECA 500
Spectrometer = DELTA2_NMR
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 13.707[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 5[dB]
X_pulse = 6.8535[us]
Irr_mode = Off
Tri_mode = Off
Date_preset = FALSE
Initial_wait = 1[s]
Recvr_gain = 44
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_get = 20.9[dC]
OMe
Cl
OH
CN
12
15.32
2.95
0.92
0.18
1.08
1
0.98
0.92
1.01
7.5687
7.5343
7.2560
7.2400
6.8724
6.8564
6.3181
6.1704
6.1372
5.2771
3.2862
3.2421
2.0237
1.9343
1.9057
1.8038
1.7282
1.5839
1.4471
1.1522
abundance
X : parts per Million : 1H

Current Data Parameters
NAME BB-4-12-19-pure
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20190412
Time 16.32
INSTRUM spect
PROBHD 5 mm QNP 1H/29
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 8
DS 2
SWH 5597.015 Hz
FIDRES 0.085404 Hz
AQ 5.8545494 sec
RG 173.8
DW 89.333 usec
DE 6.50 usec
TE 0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 400.1320010 MHz
NUC1 1H
P1 11.25 usec
PLW1 10.00000000 W
F2 - Processing parameters
SI 65536
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
N3
OH
13
1.900
4.676
7.403
7.357
7.286
7.048
7.004
1.20
2.04
2.00
2.00
ppm


Current Data Parameters
NAME BB-4-17-19-pure-3
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20190417
Time_ 10.21
INSTRUM spect
PROBHD 5 mm QNP 1H/29
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 8
DS 2
SWH 5597.015 Hz
FIDRES 0.085404 Hz
AQ 5.8545494 sec
RG 195.7
DW 89.333 usec
DE 6.50 usec
TE 0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 400.1320010 MHz
NUC1 1H
P1 11.25 usec
PLW1 10.00000000 W
F2 - Processing parameters
SI 65536
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
OMe
Cl
O
N3
CN
14
0.914
1.596
1.859
2.189
3.307
3.350
4.136
4.154
4.526
4.544
5.013
5.326
5.429
5.909
5.951
7.023
7.093
7.150
7.305
7.364
7.403
7.461
7.519
7.558
20.73
1.35
2.77
1.11
2.32
2.91
0.94
1.68
4.79
1.50
3.74
1.43
ppm


Current Data Parameters
NAME BB-4-17-19-pure-probe
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20190417
Time_ 15.24
INSTRUM spect
PROBHD 5 mm QNP 1H/29
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 8
DS 2
SWH 5597.015 Hz
FIDRES 0.085404 Hz
AQ 5.8545494 sec
RG 195.7
DW 89.333 usec
DE 6.50 usec
TE 0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 400.1320010 MHz
NUC1 1H
P1 11.25 usec
PLW1 10.00000000 W
F2 - Processing parameters
SI 65536
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
N3
OMe
Cl
O
O
O
CN
CHS-4
0.876
1.277
1.590
1.758
2.189
3.055
3.251
3.547
3.566
4.948
5.010
5.327
5.952
6.002
7.085
7.105
7.287
7.411
7.432
7.498
7.539
7.966
8.006
20.48
1.13
2.98
0.74
1.86
0.66
0.72
1.15
2.48
2.40
1.39
0.67
1.00
11 10 9 8 7 6 5 4 3 2 1 0 -1 ppm