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Light- and chemo-responsive organic molecules with biological application

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Light- and chemo-responsive organic molecules with biological application

by

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ABSTRACT

Part I. Self-immolative linkers are dynamic molecules which connect a cleavable mask to an output cargo molecule. Upon an input reaction that cleaves the mask, the self-immolative linker releases the output cargo. The scope of my research is synthesis of a new class of self-immolative linkers—aryl phthalate esters–sensitive to various inputs and able to release various cargo molecules, including within S2 cells.

In Chapter 1, fluoride sensitive aryl phthalate esters containing a phenolic output cargo molecule were synthesized. The fluoride sensitive 2-(trimethylsilyl)ethyl ether group was used as the mask molecule for each ester. The output cargo molecules were phenol, 7-hydroxycoumarin, and 3-(2-benzothiazolyl)-7-hydroxycoumarin. Full release of the cargo molecules were followed by NMR and fluorescence spectroscopy. The 7-hydroxycoumarin containing phthalate ester showed a 730-fold increase in fluorescence upon complete fluoride deprotection, making these compounds potential fluoride sensors.

In Chapter 2, self-immolative aryl phthalate esters conjugated with cleavable masking groups sensitive to light and hydrogen peroxide are reported. By altering the masking group, the phthalate linker releases the fluorescent dye 7-hydroxycoumarin upon exposure to stimuli such as light or hydrogen peroxide, respectively, leading to an increase in fluorescence. The light-sensitive aryl phthalate ester is demonstrated as a pro-fluorophore in cultured S2 cells.

Part II. BODIPY dyes can be *meso*-substituted to provide a new class of photoremovable protecting groups (PPGs). A PPG is the term used to describe a moiety (also known as a photocage) that has a deactivating influence on the biological substrate to which it is covalently

attached. Once the covalent bond is broken, the substrate is released and its reactivity or function is regained. Ideally, the cage detaches only through the action of light, giving investigators precise temporal and spatial control.

In Chapter 3, photoremovable protecting groups derived from meso-substituted BODIPY dyes release acetic acid with green wavelengths >500 nm, and photorelease is demonstrated in cultured S2 cells. The photocaging structures were identified by our lab's previously proposed strategy of computationally searching for carbocations with low-energy diradical states as a potential indicator of a nearby conical intersection. The superior optical properties of these photocages make them promising alternatives to the popular o-nitrobenzyl photocage systems.

In Chapter 4, a *meso*-substituted BODIPY photoremovable protecting group from Chapter 3 has been red-shifted by extending the conjugation of the BODIPY structure using a Knoevenagel condensation reaction. Release of acetic acid from the BODIPY photocage is successful using >600 nm light, making these photocages promising for use in photorelease studies in whole tissues or animals.

INTRODUCTION FOR PART I

SELF-IMMOLATIVE LINKERS

More than a century ago, Paul Ehrlich, a German immunologist and the founder of chemotherapy, established his "magic bullet" ideology.[1] Ehrlich reasoned that if a compound could be constructed to selectively target a diseased area of an organism, then a toxin for the disease could be delivered along with the targeting molecule and kill the disease.[1] A more modern and widely used term for this type of system is "pro-drug". A pro-drug is a medication that is administered in an inactive form, and is then converted to its active form through a bond cleavage event using a stimulus such as an enzyme.[2]

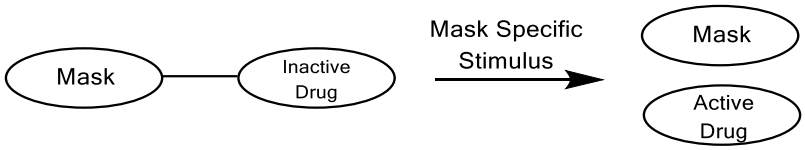

Figure 1. Un-masking and drug release of pro-drug

The concept of starting with an inactive molecule and activating it using a specific stimulus has received much attention from the scientific community. In the midst of developing a "magic bullet," many other avenues of research have spawned, including the development of pro-fluorophores, molecular probes, degradable polymers, and chemical sensors.

Self-immolative Linkers. One potential way to allow targeted drug delivery is through the use of self-immolative linkers. Conceptually, connecting a cargo molecule directly to the masking molecule (as shown in Fig. 1) can result in switching off bioactivity. A self-immolative linker can

then be used to help aid in stability, solubility, biodistribution, pharmacokinetics, bioavailability and disease-targeted activation. Such self-immolative linkers are also highly useful in the design of chemical sensors, wherein a reporter molecule is released upon a mask-cleaving reaction by an analyte.

A self-immolative linker is a molecule that attaches a cleavable masking molecule to an output cargo molecule. Upon introduction of the specific stimulus, the mask-linker bond is broken, and, in turn, the linker-cargo bond is broken. Typical input stimuli can include enzymatic activity, chemical cleavage, irradiation, or change in pH. Typical cargo molecules are drugs, reporter molecules, dyes, or other biomolecules.

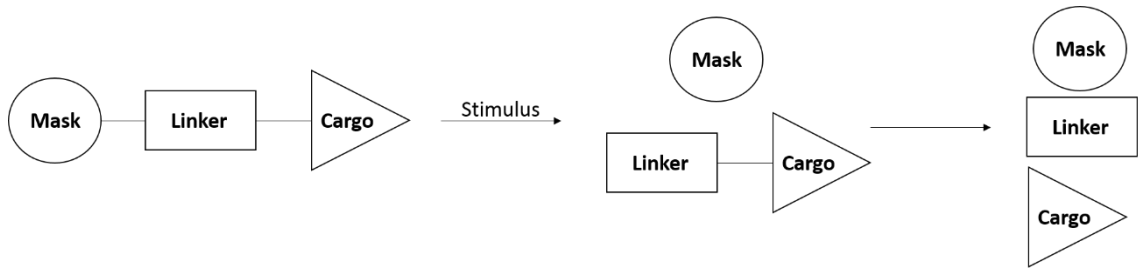

Figure 2. Un-masking and cargo release of self-immolative linker

Quinone Methide. The most common type of self-immolative linker to date is para-aminobenzyl alcohol (PABA), most recently championed by the lab of Shabat. This type of linker is able to undergo both 1,4 and 1,6 elimination via a quinone methide intermediate.[3] In the presence of water or a nucleophile, the quinone methide is quickly restored back to an aryl amine (see Figure 3). [3]

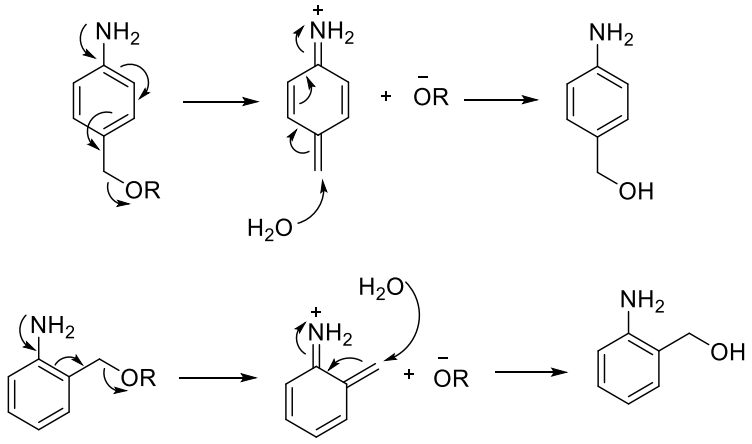

Figure 3. Quinone methide cargo release mechanism of PABA

Commonly an ester linkage between the self-immolative linker and the cargo molecule is used, releasing CO_2 and helping to drive the reaction forward.[3]

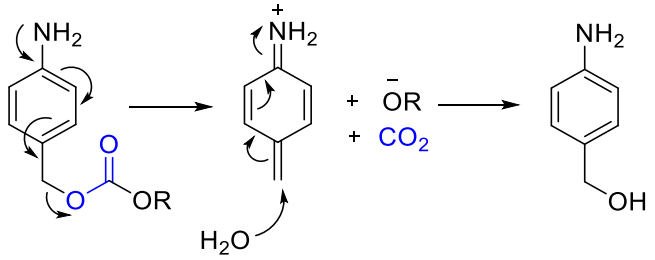

Figure 4. Use of ester linkage helps drive the release of cargo forward

The PABA linker was first introduced as a delivery system by Carl [4] in 1981 where an N-Boc-Lys group was used as the mask, trypsin was the input stimulus, and para nitroaniline was the cargo molecule. Carl was also the first to propose the term "self-immolative connector" to describe a connector linkage of this type.

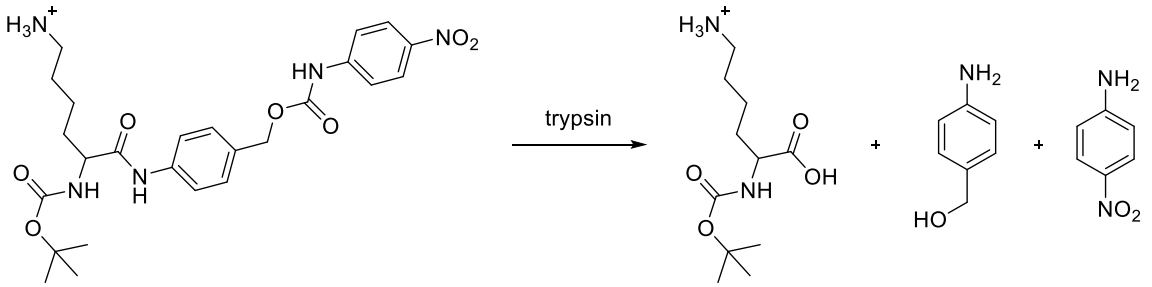

Figure 5. Initial PABA-based self-immolative linker

The system was found to be fairly stable in water at pH 6.9 in the absence of trypsin with a half-life of 40 hours at 25 °C. Under the same conditions, but in the presence of trypsin the half-life decreased to 11 minutes. Since the introduction of PABA, chemists utilized it to deliver drugs, fluorescent dyes, and biomolecules.

Senter, et. al.[5] reported the development of a prodrug strategy based on the reactivity of benzyl carbamate disulfide drug derivatives toward mild reducing agents. Upon disulfide bond reduction, appropriately substituted benzyl carbamates were shown to undergo fragmentation, and the amine-containing element of the carbamate was released. Mitomycin C, a chemotherapy drug, was used as one of the amine-containing elements.

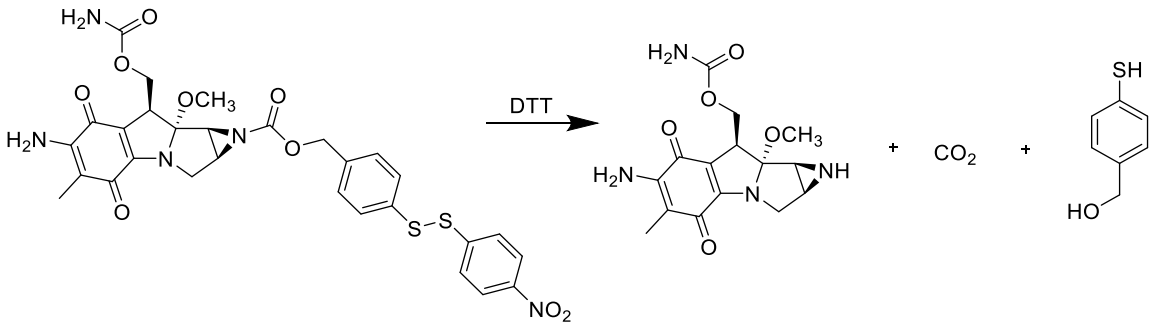

Figure 6. Disulfide bond reduction leads to the release of the chemotherapy drug Mitomycin C

De Groot et. al.[6] developed a system to release anthracyclines (doxorubicin and daunorubicin). Release occurred in the presence of the tumor-associated serine protease plasmin. A similar prodrug was synthesized which did not contain the self-immolative linker; however, inefficient drug release was observed. It was thought that attaching the drug directly to the tri-peptide caused too much steric bulk and the enzyme was not able to effectively access the tri-peptide.[7] This is a prime example of the benefit of having a self-immolative linker in a cargo release system.

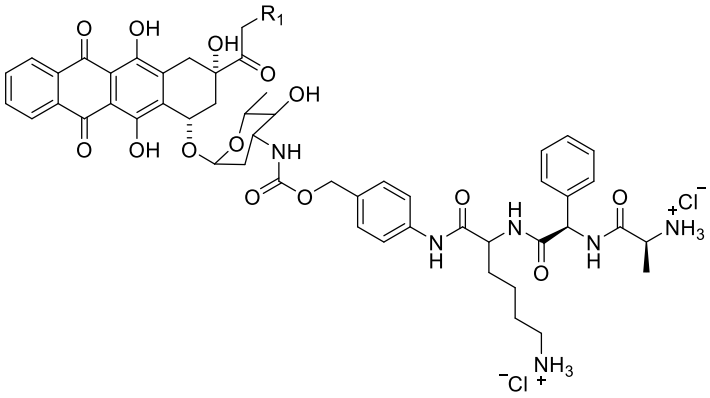

Figure 7. A serine protease plasmin sensitive prodrug

De Groot's lab[8] used a quinone-methide elimination in an extended conjugated 1,8 fashion to demonstrate a bioreductive paclitaxel prodrug. The 1,8-linker showed better stability towards enzymatic hydrolysis than its 1,4 analogue making it a more viable option for a prodrug. The researchers also attempted to use a naphthalene and biphenyl spacer system to undergo a 1,8 and 1,10 elimination, respectively. However, the desired eliminations were not observed.[9]

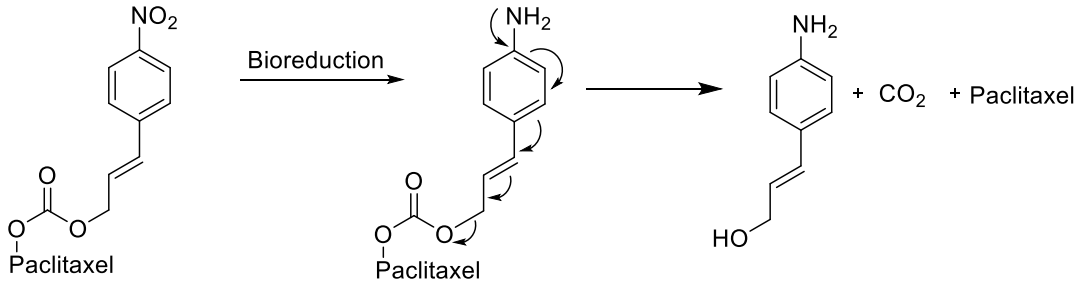

Figure 8. Bioreduction of an aryl nitro group yields active Paclitaxel

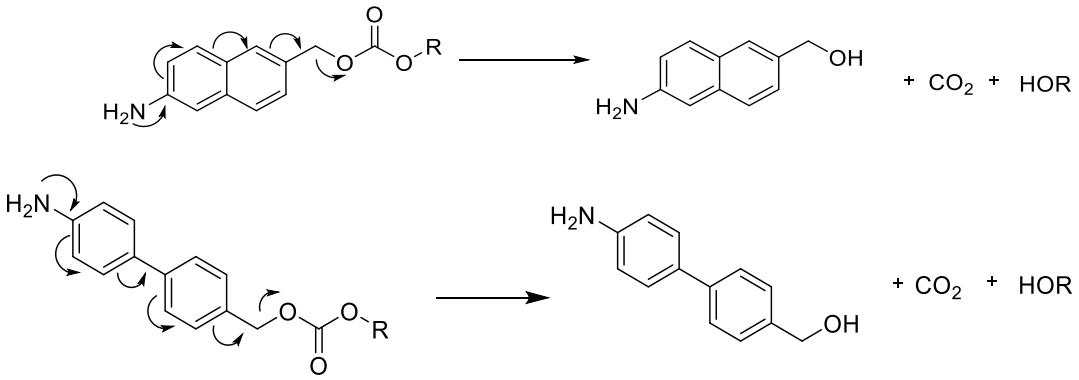

Figure 9. Use of naphthalene and biphenyl spacers did not result in elimination of cargo

Springer, et al.[10] designed four potential self-immolative prodrugs derived from phenol and aniline nitrogen mustards, all activated by the enzyme carboxypeptidase (CPG2). The analogue shown in Fig. 10 was found to be the most promising prodrug of the series. Nitrogen mustards form cyclic aziridinium ions by intramolecular displacement of the chlorine by the amine nitrogen. The aziridinium group alkylates DNA once it is attacked by a base pair of DNA.[11] This type of trauma leads to cell apoptosis.[11] The half-life for this particular pro-drug at a concentration of 10 mM in DMSO is 48 minutes in the absence of carboxypeptidase G2 (CPG2) and 7.6 minutes in

the presence of CPG2. The IC_{50} for this compound was found to be 0.46 µM. (The IC_{50} is the amount of a particular drug that is needed to inhibit a certain biological response by half.)

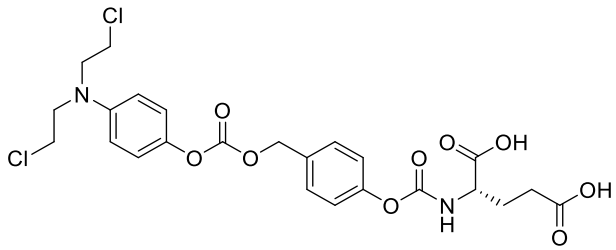

Figure 10. Nitrogen mustard prodrug

Papot, Renoux, et. al.[12] introduced a glucuronide prodrug of cyclopamine designed to selectively target the Hedgehog signaling pathway of cancer cells. The prodrug included a self-immolative linker containing a hydrophilic side chain that can be easily introduced via "click chemistry". In the prescence of β-glucuronidase, the prodrug exhibited quick release of cyclopamine and antiproliferative activity in U87 glioblastoma cells.

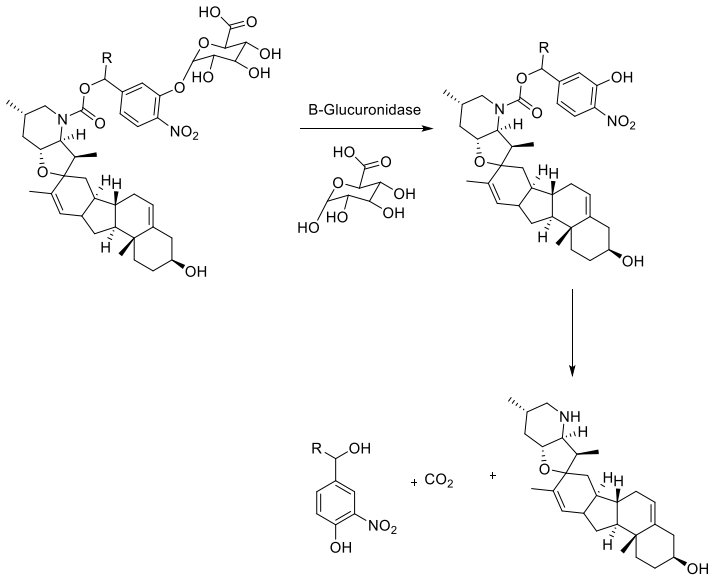

Figure 11. Cyclopamine prodrug; R= hydrophilic side chain

An amplifying effect of the above linker shown in Fig. 11 was achieved using both 1,6 and 1,4 elimination of a PABA linker.[13] The system is composed of five units including a targeting ligand for folate receptor positive tumor cells, an enzymatic trigger sensitive to β-galactosidase, a self-immolative linker and two doxorubicin compounds expressed around a chemical amplifier. The assembly is able to recognize a selected population of cells, penetrate into the intracellular medium through endocytosis and transform a single enzymatic activation into the release of two active drugs. Papot's self-immolative linker units are PABA derivatives which contain a nitro group in the ortho position of the aromatic ring. The strategic positioning of this strongly electron withdrawing group allows for faster kinetics of cargo release.[14]

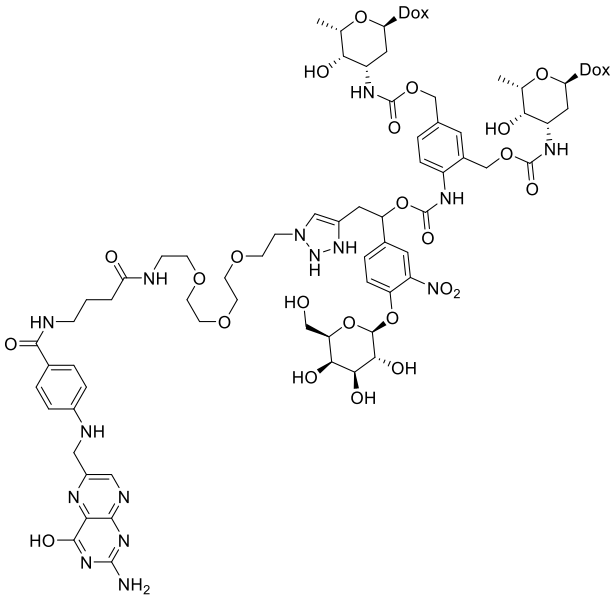

Figure 12. Self-immolative doxorubicin amplifier

Shabat, et al. has done extensive work using PABA to release multiple cargo molecules for a single triggering event. He has coined the term "self-immolative dendrimers" for these systems. The unique structural dendrimers can release all of their tail units, through a self-immolative chain fragmentation, which is initiated by a single cleavage at the dendrimer's core.[15] First generation dendritic prodrugs released doxorubicin and camptothecin as tail units and a retro-aldol retro-Michael focal trigger, which can be cleaved by catalytic antibody 38C2.[16] With these systems, a single cleavage event leads to the release of multiple cargo molecules.

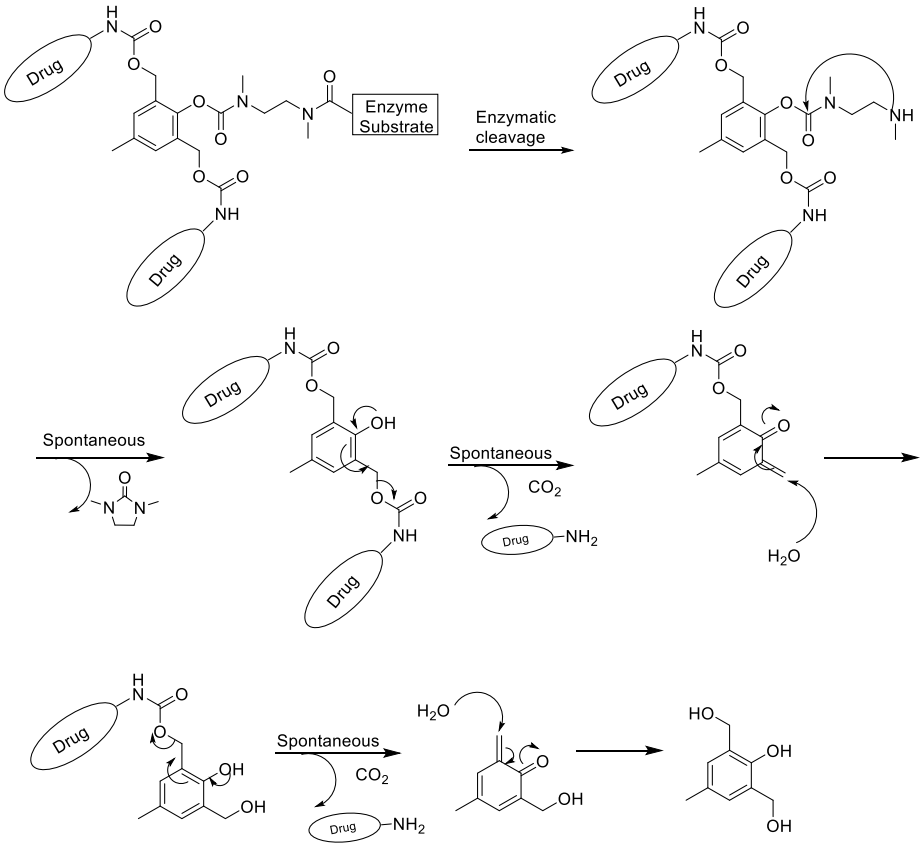

Figure 13. Shabat's first-generation dendridic prodrug

Shabat's group has since developed systems which can release three[17] and six[18] cargo molecules for every cleavage event at the dendrimer's core. The effect of swapping a benzene ring for a pyridine ring in the dendrimer's core has also been studied.[19] Shabat[20] has also introduced a molecular design for a theranostic prodrug based on a self-immolative linker attached to a pair of FRET dyes that produce a fluorescent signal upon disassembly.

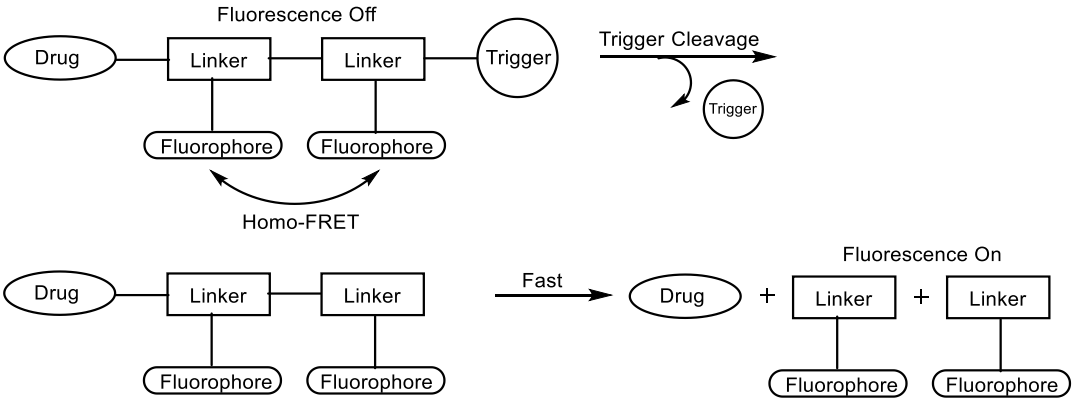

Figure 14. Self-immolative system containing FRET dyes

A turn-ON fluorescent diagnostic signal accompanies the disassembly of the prodrug and allows for monitoring of active drug release. The drug used in this system was camptothecin and activation was by the enzyme PGA. They found good correlation between the emitted fluorescence and the amount of free drug released.

McCarley et. al.[21] has more recently shown a cloaked fluorophore self-immolative linker system composed of a reporter molecule, naphthalimide, whose fluorescence is efficiently quenched by it being bound to a "trimethyl lock" trigger group through a PABA based, N-methyl-p-aminobenzyl alcohol, self immolative linker. Activation of the trigger group was achieved by

chemical and enzymatic means which ulitimately resulted in release of naphthalimide and an intense red-shifted emission.

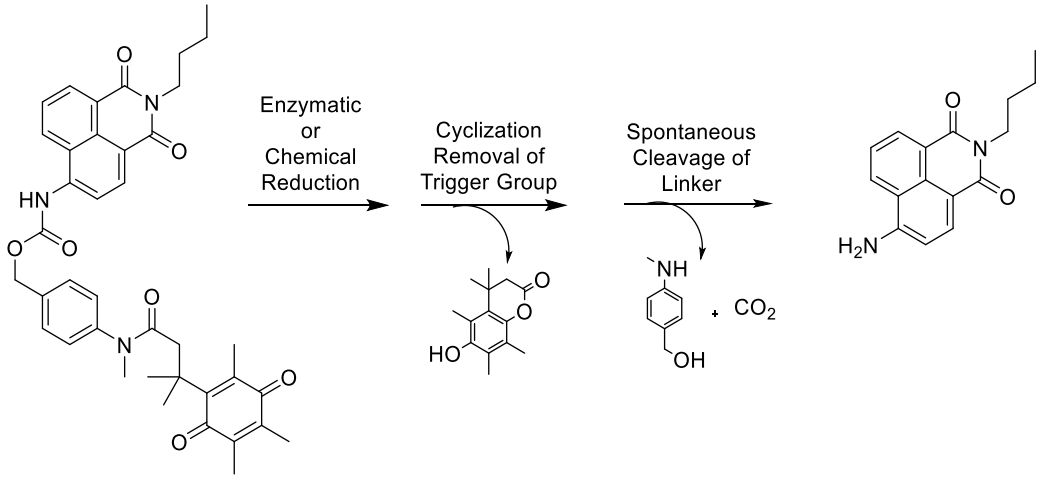

Figure 15. Release of the fluorescent dye naphthalimide (λ_{max}= 540 nm) through enzymatic or chemical reduction of a "trimethyl lock"[22] precursor

Romieu et. al.[23] used PABA as a key component in the design of a protease-sensitive fluorgenic probe whose parent coumarin fluorophore is released in the presence of penicillin amidase and caspase-3 protease.

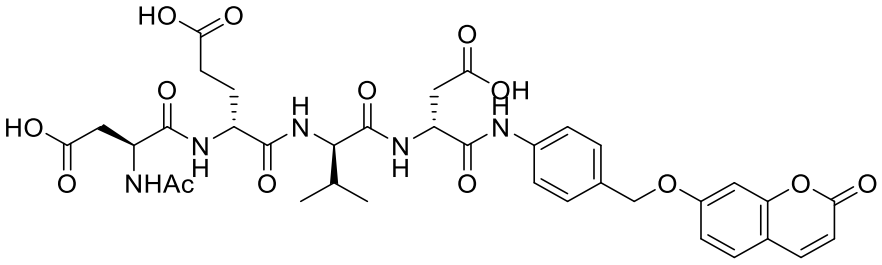

Figure 16. A protease-sensitive fluorgenic probe

A self-immolative dendritic probe which detects triacetone triperoxide through amplification of a single cleavage event initated by one molecule of hydrogen peroxide into multiple release of fluorogenic end-groups was introduced by Shabat's group.[24]

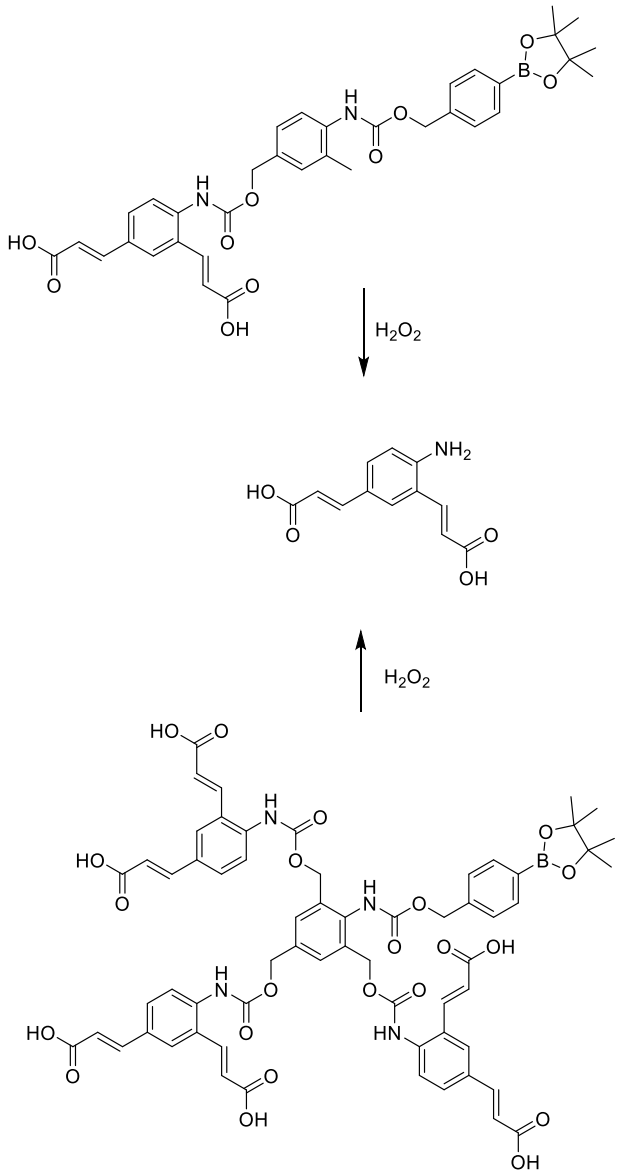

Figure 17. A self-immolative dendritic probe which detects triacetone triperoxide

Trimethyl Lock. Aside from the PABA-based self-immolative linkers, self-immolative linkers which undergo cyclization reactions in order to release a cargo molecule are popular. One example

is the "trimethyl lock quinone" which undergoes an intramolecular lactonization and leads to the release of a cargo molecule. The trimethyl lock is an o-hydroxy-cinnamic acid derivative in which unfavorable interactions between three methyl groups encourage rapid lactonization.[22]

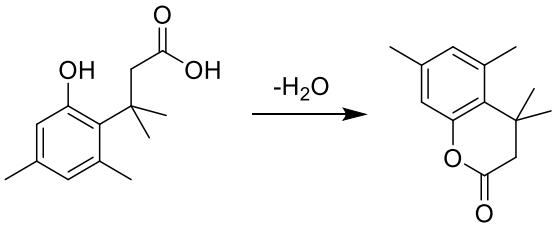

Figure 18. Tri-methyl lock

The inspiration for the tri-methyl lock came from Cohen and co-workers from the National Institute of Health in the 1960s.[25] They developed a model to test whether ubihydroquinone, which was suspected to be a key cofactor in the electron-transport chain, could be esterified by protein carboxylates to produce a high energy intermediate upon oxidation. The high energy intermediate would be able to activate inorganic phosphate to form a phosphoanhydride. Phosphoanhydrides transfer a phosphoryl group to adenosine 5'-diphosphate (ADP), generating ATP. [22]

Ronald Borchardt et. al.[22] introduced the "trimethyl lock" as a bioreversible option for release of an amine. In general, amides hydrolyze too slowly to be useful as prodrugs. However, Borchardt used the trimethyl lock to mask amines. The utility is apparent in the acetyl ester of the trimethyl lock, which releases p-methoxyaniline upon ester hydrolysis.

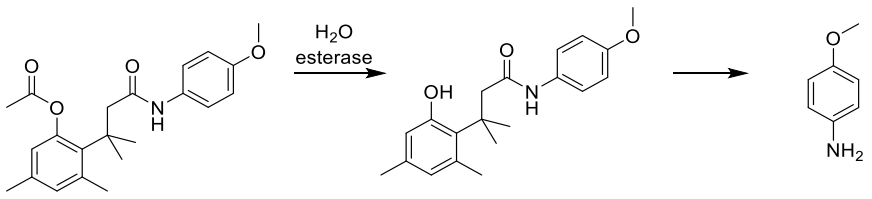

Figure 19. Circumventing sluggish amide hydrolysis with trimethyl lock

Borchardt, Nicoleu, et. al.[22] used the trimethyl lock to increase the water solubility of the otherwise insoluble Paclitaxel chemotherapy drug. It was found that the trimethyl lock analogue's solubility in water was >10mg mL^{-1} at 37 degrees Celsius, compared to Paclitaxel with is about 2mg mL^{-1} in water at 37 degrees Celsius.

Ronald Rains has done extensive work using "trimethyl lock" to release fluorescent molecules. The example in Fig. 18 used "trimethyl lock" as a fluorogenic probe for esterase.[22]

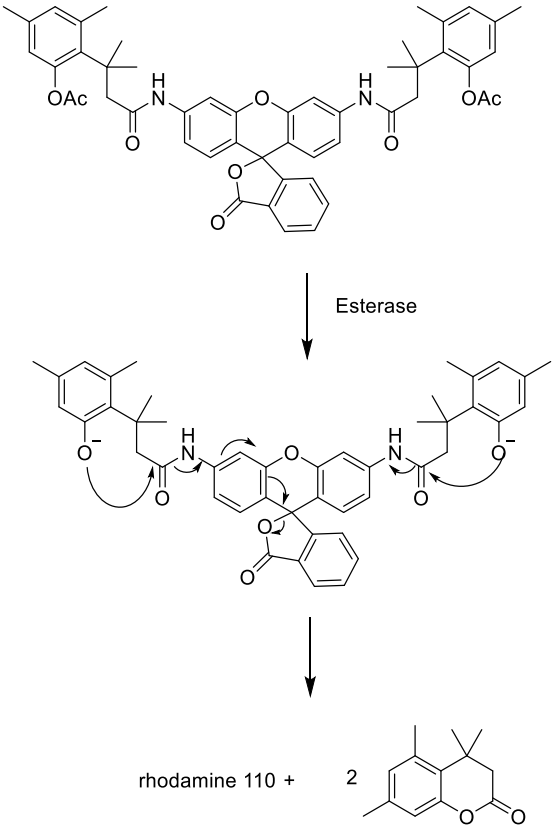

Figure 20. An esterase-sensitive fluorogenic probe

In this example, rhodamine 110 is conjugated to two trimethyl lock moieties through amide linkages rendering it virtually non-fluorescent. However, in the presence of esterase, hydrolysis

of the acetate groups leads to cyclization of the trimethyl locks and release of the rhodamine 110 fluorescent dye. Anilino fluorphores, in contrast to their hydroxy analogues, are not as prone to hydrolysis and their fluorescence intensity is not dependent on pH.[26] However, because they contain an amide, hydrolysis is slow. Adding the tri-methyl lock allows for faster ester hydrolysis followed by the release of a highly-fluorescent dye which is not dependent on pH.

Self-immolative linkers have also been incorporated into polymer systems. Greenwald et. al.[27] synthesized various poly(ethylene glycol) prodrugs of amino-containing compounds containing drugs such as Danorubicin. The addition of the PEG spacer aids in solubilization of insoluble drugs, extending plasma circulating half-lives and, in the case of anticancer agents, apparent tumor accumulation.[28]

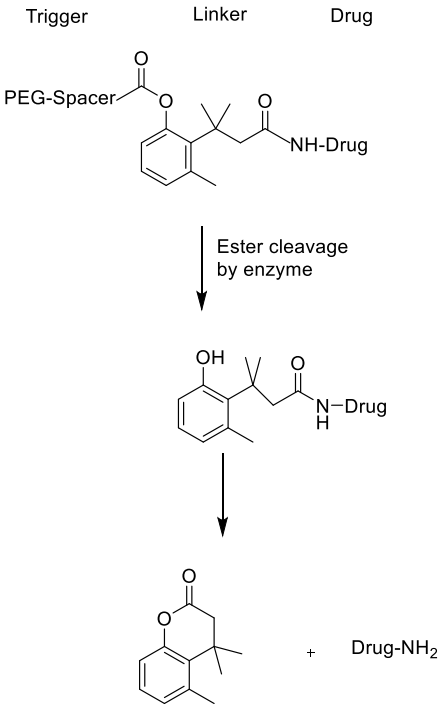

Figure 21. Polymeric self-immolative linker system

Zhou et. al.[29] recently synthesized a highly sensitive self-cleavable trimethyl lock quinone-luciferin substrates for diaphorase designed to measure NAD(P)H in biological samples. Quinones are known substrates for oxidoreductases and were reduced by accepting two electrons from NAD(P)H in a reactions catalyzed by diaphorase.[29] Upon reduction of the quinone the release of the biololuminescent luciferin molecule was observed using a luciferin detection reagent and a luminometer. Zhou reports that this bioluminescent assay provides advantages over current methods that quantify NAD(P)/NAD(P)H in biological samples because these other methods involve complicated preparation techniques.

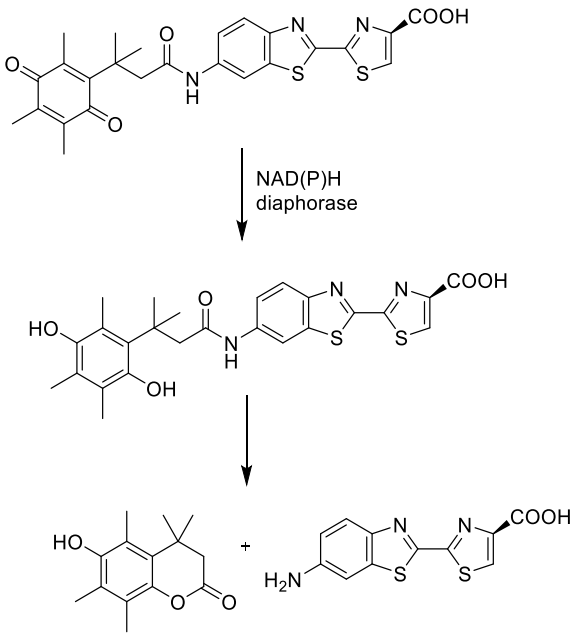

Figure 23. An NAD(P)H detector

Other Cyclization Mechanisms. Ojima et. al.[30] made a tumor-targeting drug delivery system using a tumor-targeting molecule (biotin: vitamin H or vitamin B-7), a mechanism based self-immolative linker and a taxoid (SB-T-1214) as the cytoxic agent.

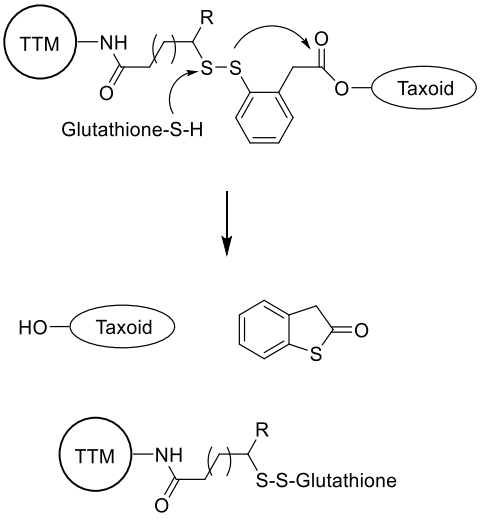

Figure 24. Use of a tumor targeting module (TTM) allows for selective release of taxoid

De Groot et. al.[8] synthesized analogous paclitaxel prodrugs one with a quinone methide linker and one with a cyclization linker. It was found that using the quinone methide linker led to a less cytotoxic pro-drug. Activation for the prodrugs was achieved using the tumor-associated enzyme plasmin.

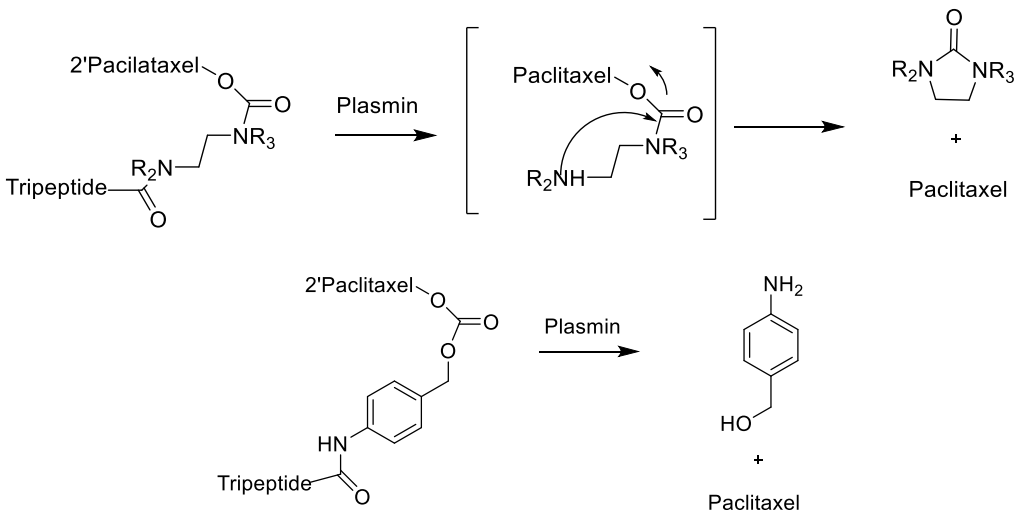

Figure 25. Analogous quinone methide based linker and cyclization linker prodrugs

Many of the currently utilized self-immolative linkers suffer from slow kinetics and solubility issues. It would be very beneficial if there were a self-immolative linker which possesses the following traits:

1. Kinetics which are on the time scale of the biological event being probed or desired time frame of drug delivery
2. Stable to conditions which do not include the input stimulus
3. Straight-forward synthesis with few steps
4. Once the linker has performed function, its byproduct should be benign
5. Inexpensive to synthesize
6. Aqueous compatibility for biological application

The following chapters describe the use of a new and promising class of self-immolative linker based on phenyl ester phthalate.

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CHAPTER 1

SELF-IMMOLATIVE ARYL PHTHALATE ESTERS

Taken in part from: Mahoney, K. M.; Goswami, P. P.; Winter, A. H., *J. Org. Chem.*, 2013, **78**, 702.

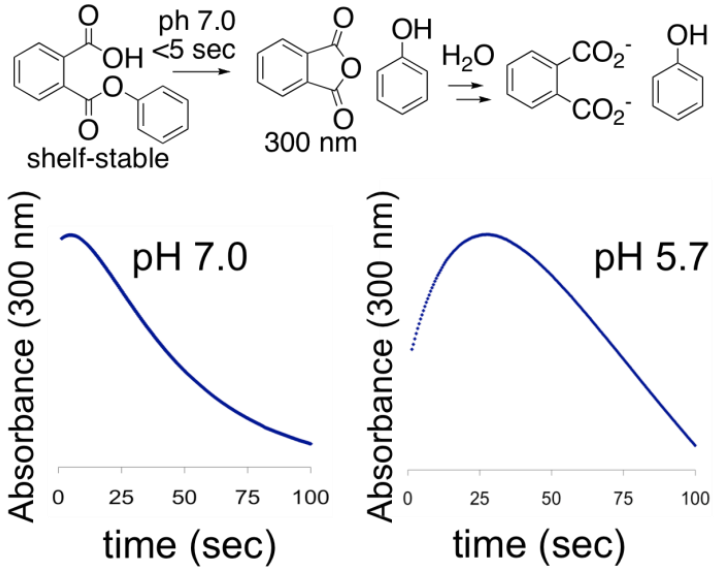
Introduction

Self-immolative linkers have become indispensible molecules for connecting a cleavable mask to an output cargo molecule.[1−3] Upon an input reaction that cleaves the mask, self-immolative linkers release their output cargo. Despite their unsavory moniker, self-immolative linkers have proven to be extremely useful in enzyme-activated prodrugs,[4−12] chemical sensors,[2,13−16] traceless linkers,[17−20] biological probes,[21−24] and degradable polymers.[1,25−33] Released chemical cargoes are often biomolecules, drugs, or reporters such as fluorescent dyes. Linker structure can aid prodrugs by improving stability, solubility, biodistribution, pharmacokinetics, bioavailability and activation.

The ideal self-immolative linker does not impose: It is simple, stable, compatible with water, and transforms into a benign byproduct upon releasing the output cargo. Furthermore, such linkers should be easy to conjugate, readily adaptable to a variety of inputs and outputs, and quickly release the output cargo upon the input reaction. In particular, some common self-immolative linkers suffer from slow release of their output cargo. New linkers that incorporate these desirable features would be highly useful.

The hydrolysis of phenyl hydrogen phthalate is a classic case of neighboring group participation, the mechanism of which has seen extensive investigation.[34−37] Phenyl hydrogen phthalate is a shelf-stable compound when stored away from moisture, but this compound

hydrolyzes rapidly in water (Scheme 1). It has been determined that the fast ester hydrolysis of this compound is a case of intramolecular catalysis wherein the neighboring carboxylate group displaces the phenol to generate a water-unstable anhydride that in turn spontaneously hydrolyzes to phthalic acid. In neutral water, release of phenol is too fast to obtain accurate rate constants using standard UV−vis studies ($\tau < 5$ s), but the rate of release is slowed in more acidic water ($\tau =$ 23 s, pH 5.7). The known favorable kinetics of this hydrolysis led us to test aryl phthalate esters for use as self-immolative linkers.



Scheme 1. Fast hydrolysis of the classic phenyl hydrogen phthalate hydrolysis in water followed by monitoring growth and decay of phthalic anhydride

Incorporating a classic reaction into a self-immolative linker. The hydrolysis of phenyl hydrogen phthalate is a classic case of neighboring group participation. [31-34] Phenyl hydrogen phthalate is a shelf-stable compound when stored away from moisture, but this compound hydrolyzes rapidly in water (Scheme 1). The astounishingly fast ester hydrolysis of this compound is an exemplary case of intramolecular catalysis wherein the neighboring carboxylate group displaces the phenol to generate a water-unstable anhydride that in turn spontaneously hydrolyzes

to phthalic acid. In neutral water, release of phenol is too fast to obtain reliable rate constants using standard UV-Vis studies (t < 5 sec), but the rate of release is slowed in more acidic water (t = 23 sec, pH 5.7). The favorable kinetics of this hydrolysis led us to test aryl phthalate esters for use as self-immolative linkers.

Using a fluoride-sensitive 2-(trimethylsilyl)ethyl ether to mask the catalytic carboxyl group, in combination with three phenolic cargos (phenol **1** plus the fluorescent dyes 7-hydroxycoumarin **2** and 3-(2-Benzothiazolyl)-7-hydroxycoumarin **3**), we find that aryl phthalate esters can indeed be exploited as self-immolative linkers. We show that these linkers can be conjugated easily starting from phthalic anhydride, a cheap industrial starting material in the manufacture of plastics, and "self-immolate" to ultimately yield phthalic acid as a biologically benign byproduct upon release of the phenolic output.

Results and Discussion

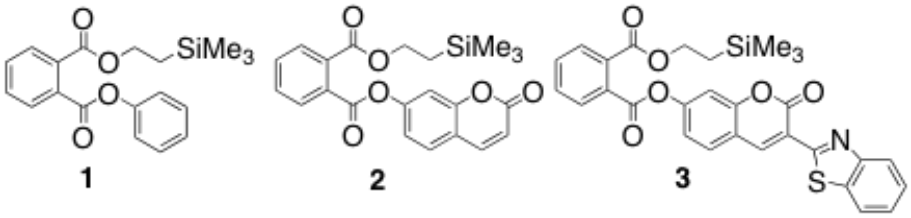


Figure 1. Aryl phthalate esters described in this study.

Fluoride titrations studies and product analysis. Compounds **1-3** were synthesized and titrated with fluoride ion (Scheme 2) in pH 7 buffer. The titration of **1** was followed by ^{1}H NMR spectroscopy and titrations of **2** and **3** were followed with fluorescence spectroscopy. The titration of compound **2** is remarkable because we observe a 730-fold increase in fluorescence upon complete fluoride deprotection as a consequence of the release of the highly fluorescent 7-

hydroxycoumarin dye. Thus, compound **2** is an exquisite fluoride sensor. Curiously, compound **3** shows a decrease in fluorescence during the titration even though the highly fluorescent free coumarin dye is released. This decrease in fluorescence is due to the starting ester **3** being highly fluorescent, whereas ester **2** is essentially non-fluorescent.

Chemical stability, product analysis, and release mechanism. Compounds **1**-**3** are stable in water in the absence of fluoride, with no decomposition observed after 1 day at room temperature (see Supporting Information). Additionally, NMR product analysis after fluoride deprotection indicates that the organic end products are the free phenolic compound and phthalic acid. These results lead us to postulate the mechanism of release shown in Scheme 2. Surprisingly, our titrations indicate that compounds **2,3** require three equivalents of fluoride to achieve complete deprotection, while **1** requires the expected 1 eq. of fluoride. This "excess" F^- required is puzzling since the presumed mechanism for TMSE deprotection involves a single fluoride ion adding to the silicon to eliminate ethane gas and trimethylsilylfluoride. Possibly, deprotection of **2** and **3** proceed through a hypervalent silicon mechanism, although further work would be needed to verify this mechanistic possibility.

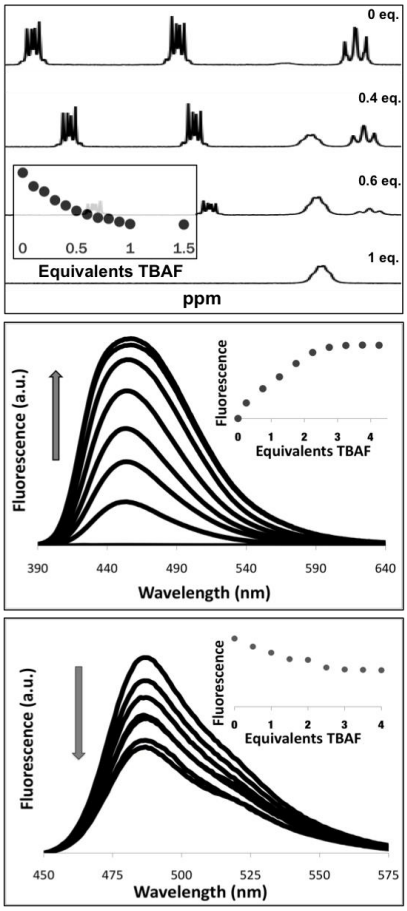
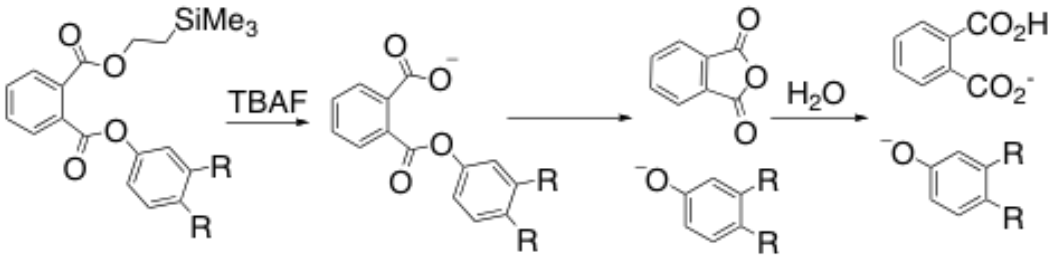
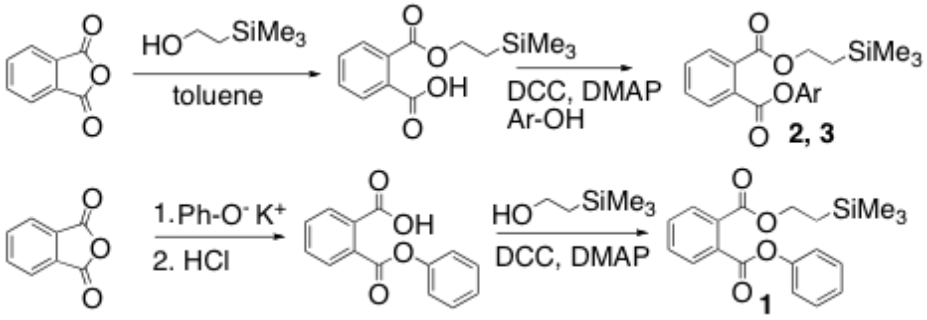


Figure 2. Fluoride titrations by NMR for **1** (top) and by fluorescence detection for **2** (middle) and **3** in pH 7.0 buffer. Plot inserts depict fluorescence (or NMR integration) at the emission maxima vs. equivalents of tetrabutyl ammonium fluoride.



Scheme 2. Putative mechanism of decomposition of **1-3** with F^- ion.

Compounds **1-3** were prepared from phthalic anhydride (See Scheme 3). Addition of TMSE to phthalic anhydride yields the TMSE-protected acid ester, which was further converted to aryl esters **2,3** using the Stieglich DCC/DMAP coupling procedure. For **1**, esterification of phenyl hydrogen phthalate was accomplished in a similar way using DCC/DMAP conditions.



Scheme 3. Synthesis of **1-3**.

Experimental

Phenyl hydrogen phthalate, [34] 2-(trimethylsilyl)ethyl hydrogen phthalate, [35] and 3-(2-benzothiazolyl)-7-hydroxycoumarin [36] were prepared by published procedures. All NMR matched the known spectra.

Synthesis of phenyl 2-(trimethylsilyl)ethyl phthalate **1.** Phenyl hydrogen phthalate (1.50 g, 6.21 mmol), 2-trimethylsilylethanol (1 mL, 6.98 mmol) and 4-N,N-dimethylaminopyridine (0.085 g, 0.69 mmol) were dissolved in dry DMF (4 mL), followed by continuous stirring of the solution. N,N-dicyclohexylcarbodiimide (1.54g, 7.45 mmol), dissolved in dry DMF (2 mL), was next added to the reaction mixture and the reaction was stirred under an argon atmosphere overnight. The dicyclohexylurea byproduct was filtered off as a white solid. The solvent was then removed under reduced pressure to yield the crude product as a yellow oil. Flash chromatography

(Hex/EtOAc, 90:10) gave the pure final product (0.595 g, 28%) as a colorless oil. (^{1}H NMR, CD_3OD, 400 MHz) δ 7.90 (m, 1H), 7.83 (m, 1H), 7.70 (m, 2H), 7.46 (m, 2H), 7.29 (m, 3H), 4.44-4.40 (m, 2H), 1.09 (m, 2H), 0.04 (s, 9H); (^{13}C NMR, CD_3OD, 100MHz) δ 169.1, 167.9, 152.5, 133.9, 133.1, 132.9, 132.7, 130.7, 130.4, 130.2, 127.3, 122.7, 65.4, 18.3, -1.4; High-res MS(ESI) calculated for formula $C_{19}H_{23}O_4Si$ (M+1) requires 343.1287; found 343.1360.

Synthesis of 7-hydroxycoumarinyl 2-(trimethylsilyl)ethyl phthalate **2**. 2-(trimethylsilyl)ethyl hydrogen phthalate (1.29 g, 4.83 mmol), 7-hydroxycoumarin (1.21 g, 4.83 mmol), and 4-dimethylaminopyridine (0.65 g, 5.3 mmol) were dissolved in a mixture of anhydrous methylene chloride (15 mL) and anhydrous DMF (9 mL). N,N-dicyclohexylcarbodiimide was quickly added to the reaction mixture and stirred under argon overnight. Dicyclohexyl urea was filtered off and the filtrate was diluted 10 mL of methylene chloride. The solution was washed with brine and then dried over anhydrous $MgSO_4$. The crude product was collected by evaporation under reduced pressure and then purified by flash chromatography on silica gel (Hex/EtOAc, 70:30) to yield **2** (0.65g, 33%) as a white solid: (^{1}H NMR $CDCl_3$, 400 MHz) δ 7.87, (m, 2H), 7.73 (d, 1H, J = 8 Hz), 7.64 (m, 2H), 7.57 (d, 1H, J = 8 Hz), 7.34 (s, 1H), 7.30 (s, 1H), 6.43 (d, 1H, J = 8 Hz), 4.43 (t, 2H, J = 8 Hz), 1.11 (t, 2H, J = 8 Hz), 0.06 (s, 9H); (^{13}C NMR $CDCl_3$, 100 MHz) δ 167.2, 166.2, 160.7, 155.1, 153.7, 143.2, 132.0, 131.8, 131.7, 129.6, 129.4, 128.9, 118.7, 117.2, 116.5, 110.8, 64.7, 17.7, -1.1. High-res MS (ESI) calcd. for formula $C_{22}H_{22}O_6Si$ (M+1) requires 411.1186; found, 411.1258.

Synthesis of 3-(benzo[d]thiazol-2-yl)-7-hydroxycoumarinyl-2-(trimethylsilyl)ethyl phthalate **3.** 2-(trimethylsilyl)ethyl hydrogen phthalate (50 mg, 0.34 mmol), 3-(2-benzothiazolyl)-7-hydroxycoumarin (99 mg, 0.34 mmol), and 4-dimethylaminopyridine (4 mg, 0.034 mmol) were

dissolved in DMF (5 mL). N,N-dicyclohexylcarbodiimide (69 mg, 0.34 mmol) was quickly added to the reaction mixture and was stirred under argon for 12 h. The white solid was filtered off and the DMF was removed by evaporation under reduced pressure. The crude mixture was purified by preparatory thin-layer chromatography (200 microns) using a (Hex/EtOAc, 70:30) eluent followed by an additional prep TLC purification using (Hexane/EtOAc, 50:50) to yield the product **3** (37 mg, 20%) as a yellow solid: (^{1}H NMR $CDCl_3$, 400 MHz) δ 9.11 (s, 1H), 8.12 (d, 1H, J=8 Hz), 8.01 (d, 1H, J = 8 Hz), 7.89 (m, 2H), 7.81 (d, 1H, J = 8 Hz), 7.66 (m, 2H), 7.56 (t, 1H, J = 8 Hz), 7.48 (s, 1H), 7.45 (s, 1H), 7.40 (dd, 2H, J = 4 Hz), 4.45 (t, 2H, J = 8 Hz), 1.12 (t, 2H, J = 8 Hz), 0.07 (s, 9H); (^{13}C NMR $CDCl_3$, 100 MHz) δ 167.1, 166.2, 160.0, 159.9, 154.9, 152.7, 141.24, 137.2, 132.3, 132.1, 131.9, 131.8, 130.6, 129.7, 129.5, 126.9, 125.8, 123.3, 122.1, 120.1, 119.7, 117.3, 110.68, 64.8, 17.7, -1.1. High-res MS (ESI) calcd. for formula $C_{29}H_{26}NO_6SSi$ (M+1) requires 544.1172; found, 544.1245.

^{1}H NMR titration of 1. A stock solution of **1** was prepared (9.05 x 10^{-2} M) in DMSO-D_6 and distributed equally (97 μL) into 12 vials. To these vials was added varying equivalents of a second stock solution made of 1M TBAF/THF (7.44 x 10^{-2}M) in DMSO-D_6. 0.5 ml D_2O was then added to each vial. ^{1}H NMR spectra of each was then recorded. The titration was repeated three times and the results were averaged. Conversion was calculated by measuring the ratio of DMSO-D6 signal integration with the integration of the $–CH_2$ peak (δ 4.42 ppm) in **1**.

Fluorescence titration of 2 and 3. A stock solution of **2** was prepared (7.68 x 10^{-5}M) in acetonitrile and distributed equally (52 μL) into vials. These samples were titrated using varying equivalents of a 1M TBAF in THF solution. The samples were then diluted with 1 mM phosphate buffered (pH = 7.0) water to 3.0 mL. Excitation was carried out at 370 nm with all

excitation and emission slit widths at 2 nm. The titration was repeated three times and the data were averaged. The same experimental procedure was used in the titration of compound **3** except the stock solution (2.3×10^{-6} M) was prepared in DMF, and the excitation of these scans was carried out at 440 nm.

See Appendix I for NMR and MS of synthesized compounds, stability tests, and product studies.

Conclusions

In conclusion, we have shown that aryl phthalate esters are robust self-immolative linkers in water using a fluoride sensitive mask as a test case and phenolic outputs. The phthalate scaffold also appears to be highly promising for latent fluorophores, given the ~10^3 fluorescence enhancement upon releasing 7-hydroxycoumarin. Ester **2** represents an exquisite water-compatible fluoride sensor. The advantages of this linker include a simple synthesis from inexpensive starting materials, aqueous stability and compatibility, but most importantly very fast release kinetics that lead to a biologically benign byproduct. The possibility of tuning the rate of release by chemical substitutions to the phthalate ring system, as well as the scope of this linker for different masking groups and output cargos, is currently under investigation in our laboratory. These phthalate esters appear to be highly promising for use in biological and materials applications.

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CHAPTER 2

SELF-IMMOLATIVE PHTHALATE ESTERS SENSITIVE TO HYDROGEN PEROXIDE AND LIGHT

Taken in part from: Mahoney, K. M.; Goswami, P. P; Syed, A.; Kolker, P.; Shannan, B; Smith, E. A.; Winter, A. H., *J. Org. Chem*. 2014, **79**, 11740.

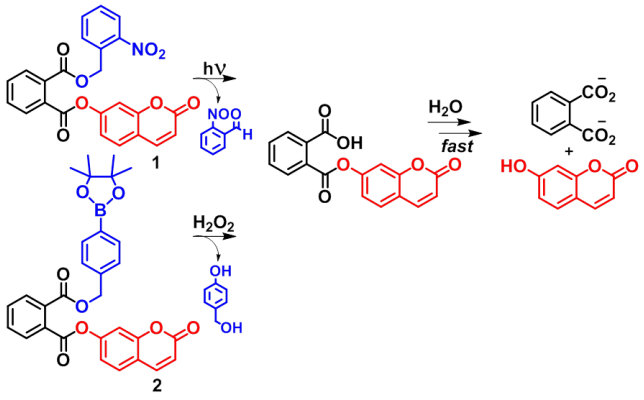
Introduction

Self-immolative linkers have proven to be useful for connecting a cleavable masking molecule to an output cargo molecule.[24,26,37-39] Upon exposure to an input stimulus that cleaves the mask, self-immolative linkers release their cargo. Self-immolative linkers have found applications in enzyme-activated prodrugs,[10,20,40-46] chemical sensors,[26,47] traceless linkers,[48-51] biological probes,[52-55] and degradable polymers.[3,56,57] Released chemical cargos are often biomolecules, drugs, or reporters such as fluorescent dyes. Ideally, self-immolative linkers should be simple in design, stable, compatible with water, and transformed into a benign byproduct upon releasing the output cargo. Additionally, they should be easy to conjugate, readily adaptable to a variety of inputs and outputs, and quickly release the output cargo. A drawback to known self-immolative linkers is that cargo release rates can be slow,[58] leading to loss of temporal resolution.

Our group has recently reported aryl phthalate esters as fast-releasing self-immolative linkers.[38] In this previous work, we demonstrated that a fluoride-sensitive masking group could release cargo phenols and phenolic-based fluorescent dyes. Phthalate self-immolative linkers exploit the rapid hydrolysis of esters with adjacent catalytic carboxylate moieties, a classic case of neighboring group participation[32,59,60] (phenyl hydrogen phthalate releases phenol in < 5 sec in neutral water[38]). Here, we demonstrate that phthalate esters masked with light- and peroxide-

sensitive groups can release a coumarin dye upon exposure to light or peroxide. Peroxide is an important biological signaling molecule, whereas light-releasable fluorescent dyes (pro-fluorophores) have found application in monitoring dynamic events in real time [61-66] as well as recording images with sub-diffraction resolution at the nanometer level.[67-70]

Results and Discussion



Scheme 1. General unmasking scheme

Both **1** and **2** were synthesized by the addition of the trigger molecule to phthalic anhydride followed by the addition of 7-hydroxycoumarin by either a DCC/DMAP or EDC/DMAP coupling.

Compounds **1** and **2** were synthesized and exposed to UV light and hydrogen peroxide, respectively. The reactions were monitored using fluorescence and ^{1}H NMR spectroscopy. The titration of Compound **1** resulted in an 18-fold increase in fluorescence intensity and Compound **2** showed an 8-fold increase as a result of releasing the free fluorescent dyes.

The titrations of **1** and **2** were followed by fluorescence spectroscopy (Figure 1). To aid with solubility, experiments with **1** were carried out by first dissolving the compound in DMF and

exposing the resulting solution to 350 nm light, Asmall aliquot (7 μL) of the solution was then injected into buffered water (3.0 mL, pH 7.0, 1 mM phosphate buffer) and fluorescence was followed as a function of time. Experiments for **2** were carried out by first dissolving the compound in DMF and titrating with increasing amounts of hydrogen peroxide. This procedure was followed by injection of a small aliquot of the DMF/H_2O_2 solutions into buffered water for the fluorescence analysis.

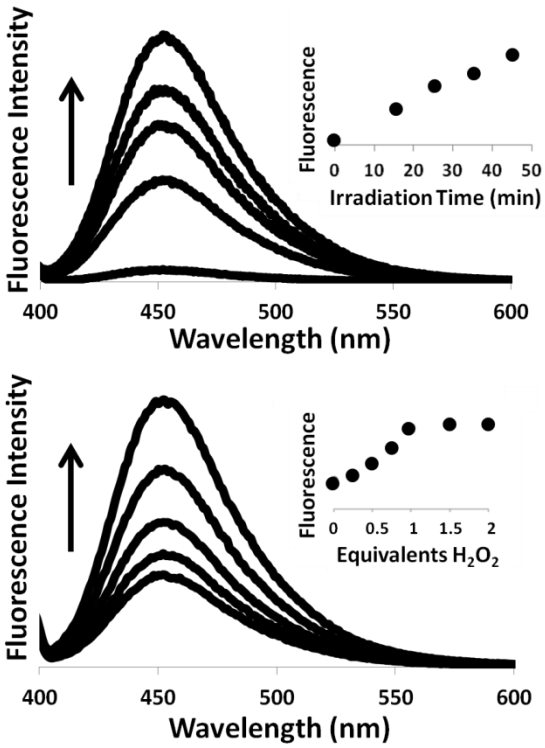


Figure 1. Fluorescence of compound **1** as a function of irradiation time (top); fluorescence of **2** as a function of peroxide (bottom) in pH 7.0 buffer. Plot inserts depict fluorescence at the emission maxima (453 nm) vs. time of irradiation or equivalents of hydrogen peroxide.

Compound **1** was stable in water/DMF mixtures in the absence of light for at least 1 day at room temperature (see SI for details). Compound **2** did show some instability, as seen by a small

increase in fluorescence after a 16-hour period in a water/DMF mixture in the absence of hydrogen peroxide (see SI for spectra). Additionally, it is noteworthy that this structure **2** is quite unstable under the seemingly mild conditions required to synthesize it (e.g. DCC/DMAP ester coupling), possibly the result of the boronate ester under the reaction conditions catalyzing a spontaneous ester hydrolysis (**2** is stable as a solid or dissolved in a solution void of hydrogen peroxide, however). Additionally, NMR product studies after exposure to light and hydrogen peroxide indicate that the organic products are the expected free 7-hydroxy coumarin as well as phthalic acid. The toxicity of phthalic acid has been studied due to its industrial use in the synthesis of phthalate plastics and esters; it has not been found to be very toxic in mice (LD_{50} (mouse) is 2.53 g/kg).[71,72]

Because **1** showed the largest increase in fluorescence intensity and the greatest stability during *in vitro* studies, we chose to use it for cellular experiments. Compound **1** was incubated with Drosophila *S2* cells and dye release was monitored using fluorescence microscopy. See Figure 2.

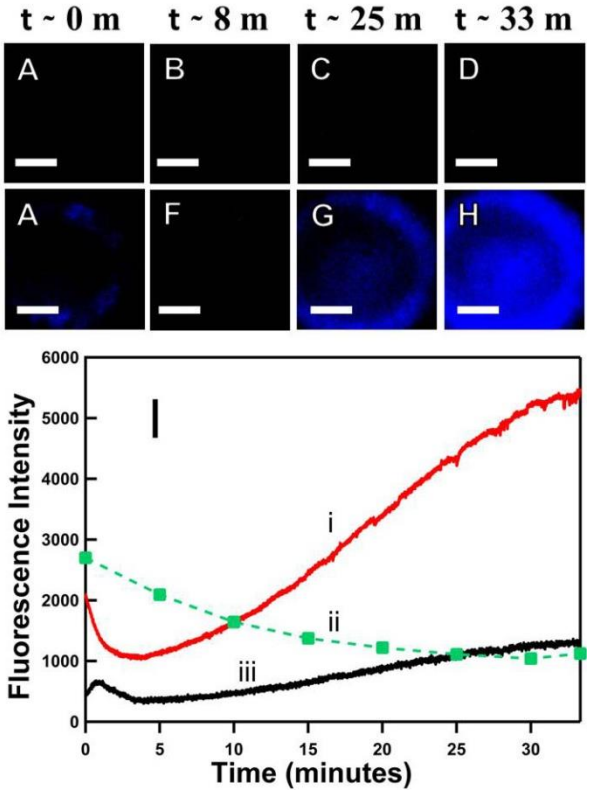


Figure 2. Fluorescence images of a cell with no compound 1 (A-D) and cell incubated with compound 1 (50 µM) (E-H) as function of irradiation time. I) average fluorescence intensity as a function of time for i) four cells incubated with compound 1 and exposed to continuous irradiation for 35 minutes ii) four cells incubated with compound 1 and only exposed to irradiation briefly every 5 minutes to obtain an image iii) a cell without compound 1 and exposed to continuous irradiation for 35 minutes. Scale bar represents 5 µm in all images.

The Drosophila *S2* cells were loaded with **1** and subjected to continuous irradiation with 335 nm light. Fluorescence images were collected every 500 ms for a total of 33 minutes (Figure 2). Fluorescence emission was observed at 450 nm. Fluorescence intensity for Figure 2, i-iii, was taken from the periphery of the cell where the concentration of **1** was highest. As shown in Figure 2 E-H, at the beginning of the experiment there was minimal fluorescence; however, after exposure

to light a gradual increase in fluorescence is seen for up to 33 minutes. The initial fluorescence seen at time zero can be attributed to cellular auto fluorescence, which undergoes initial bleaching prior to significant release of the free coumarin dye. Control studies were performed to make sure the fluorescence was due to the controlled release of 7-hydroxycoumarin by irradiation. Figure 2, A-D shows that there is minimal change in fluorescence of cells when irradiated without being loaded with **1**. Another control study (Figure 2, ii) was performed with cells incubated with **1** but not exposed to irradiation. There was an initial fluorescence signal due to cellular auto fluorescence; however, a decrease in fluorescence signal is seen, eventually leveling off to an intensity similar to that of the unloaded cells, indicating that the **1** does not release the dye in the absence of irradiation.

The cytotoxicity of **1** in the cells was determined by incubating the cells (1×106 cells/mL) with different dilutions (100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM and 3.125 μM) of **1** in phosphate buffer saline (PBS, pH=7.1) for an hour. At a compound concentration of 50 μM, 83% of the cells remained viable after an hour and this concentration was used in all fluorescence imaging cell studies.

Experimental

Synthesis of 2-(Nitrobenzyl) Hydrogen Phthalate. Phthalic acid (0.100 g, 0.675 mmol) and 2-nitrobenzyl alcohol (0.103 g, 0.675 mmol) were refluxed in toluene under argon overnight. The crude product was collected by evaporation under reduced pressure. The resulting mixture was dissolved in ethyl acetate and the product was extracted with aqueous sodium bicarbonate followed by acidification with 1 M aqueous hydrogen chloride. Final collection of a white solid was performed by vacuum filtration. The product was dried under vacuum and used without any

further purification (0.203 g, 36%); ^{1}H NMR (400 MHz, DMSO-d6) δ 13.35 (s, 1H), 8.15 (dd, *J* = 8.1, 1.2 Hz, 1H), 7.85 – 7.60 (m, 7H), 5.63 (s, 2H). δ; ^{13}C (DMSO-d6, 100 MHz) ^{13}C NMR (100 MHz) δ 168.4, 167.6, 147.8, 134.6, 132.1, 131.9, 131.9, 131.5, 129.9, 129.8, 129.4, 128.8, 125.3, 64.1; mp 142-145°C; HRMS (ESI) *m/z*: $[M + Na]^+$ for $C_{15}H_{11}NNaO_6$ requires 324.0479, found 324.0480

Synthesis of 7-Hydroxycoumarinyl 2-(Nitrobenzyl) Hydrogen Phthalate **1.** 2-(nitrobenzyl) hydrogen phthalate (0.200 g, 0.664 mmol), *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (0.126 g, 0.657 mmol), and 4-*N,N*-dimethylaminopyridine (0.089 g, 0.728 mmol) were dissolved in dry DCM (10 mL), followed by continuous stirring of the solution. 7-hydroxycoumarin potassium salt (0.132 g, 0.660 mmol) and 18-crown-6 (0.174 g, 0.660 mmol) were added next to the reaction mixture, and the reaction was stirred under an argon atmosphere for 12 h. The product was washed with an aqueous saturated sodium bicarbonate solution. The solvent was then removed under reduced pressure to yield the crude product as a white solid. Flash chromatography (Hex/EtOAc, 30:70 → Chloroform/MeOH 95:5) gave the pure final product (0.123 g, 42%). ^{1}H NMR (DMSO-d6, 400 MHz) δ 8.16 – 8.08 (m, 2H), 8.06 – 7.91 (m, 2H), 7.90 – 7.72 (m, 5H), 7.67 – 7.56 (m, 1H), 7.34 (d, *J* = 2.2 Hz, 1H), 7.23 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.53 (d, *J* = 9.6 Hz, 1H), 5.74 – 5.69 (m, 2H), 1.24 (s, 1H), 0.84 (t, *J* = 6.8 Hz, 1H); ^{13}C (DMSO-d6, 100 MHz) δ 166.3, 165.7, 160.0, 154.5, 153.0, 148.0, 144.2, 134.5, 132.8, 131.0, 130.3, 130.0, 129.9, 129.8, 125.3, 118.6, 117.4, 116.2, 110.2, 79.6, 79.4, 79.1, 64.4; mp >260°C; HRMS (ESI) calcd for formula $C_{24}H_{15}NO_8$ $[M + H]^+$ requires 446.0870, found 446.0872.

Synthesis of 4-(Hydroxymethyl)benzeneboronic Acid Pinacol Hydrogen Phthalate.

Phthalic anhydride (0.049 g, 0.294 mmol), 4-(hydroxymethyl)benzeneboronic acid pinacol (0.077 g, 0.329 mmol) were refluxed in toluene overnight. The crude product was collected by evaporation under reduced pressure. The resulting mixture was dissolved in ethyl acetate and the product was extracted with aqueous sodium bicarbonate followed by acidification with hydrogen chloride. Vacuum filtration was used to collect the white solid. The product was dried under vacuum and used without any further purification (0.045 g, 40%); ^{1}H NMR (DMSO-d6, 400 MHz) δ 7.85 (s, 1H), 7.72 (m, 6H), 7.52 (d, 1H, J = 0.8 Hz), 1.36 (s, 12H); ^{13}C (DMSO-d6, 100 MHz) δ 168.4, 167.9, 139.4, 137.0, 135.0, 134.7, 132.6, 131.9, 129.4, 128.8, 127.8, 127.4, 84.2, 67.1, 25.1; mp 121-123°C; HRMS(ESI) calcd for formula $C_{21}H_{23}BO_6$ $(M - H+)^-$ requires 380.1551, found 380.1550 (mass calculated using boron isotope ^{10}B).

Synthesis of 7-Hydroxycoumarinyl 2-(4-Hydroxymethyl)benzeneboronic Phthalate **2**. 4-(hydroxymethyl)benzeneboronic acid pinacol hydrogen phthalate (0.100 g, 0.262 mmol), *N,N*-dicyclohexylcarbodiimide (0.068 g, 0.314 mmol), and 4-*N,N*-dimethylaminopyridine (0.011 g, 0.087 mmol) and 18-crown-6 ether (0.069 g, 0.262 mmol) were dissolved in dry DMF (3 mL), followed by continuous stirring of the solution. 7-hydroxy coumarin potassium salt (0.052 g, 0.262 mmol) was next added to the reaction mixture, and the reaction was stirred under an argon atmosphere overnight. The dicyclohexylurea byproduct was filtered off as a white solid. The solvent was then removed under reduced pressure to yield the crude product as a white solid. Flash chromatography (Hex/EtOAc, 50:50) gave the pure final product (5.6 mg, 4.1%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.10 (d, J = 9.6 Hz, 1H), 8.02 – 7.90 (m, 2H), 7.87 – 7.75 (m, 3H), 7.62 (dd, J = 7.0, 1.3 Hz, 2H), 7.43 (d, J = 7.6 Hz, 2H), 7.32 (d, J = 2.1 Hz, 1H), 7.18 (ddd, J = 8.5, 2.2, 0.8 Hz, 1H), 6.52 (dd, J = 9.6, 0.8 Hz, 1H), 5.40 (s, 2H), 1.27 (d, J = 0.8 Hz, 12H); ^{13}C (DMSO-d6, 100 MHz) δ 166.6, 165.8, 160.3, 154.7, 153.3, 142.8, 138.2, 135.1, 131.8, 131.6, 131.5, 129.5,

129.2, 128.6, 127.5, 118.4, 116.8, 116.2, 110.4, 80.9, 67.6, 24.9; mp; HRMS(ESI) calcd for formula $C_{30}H_{27}BO_8$ $(M + H)^+$ requires 526.1904, found 526.1908. (mass calculated using boron isotope ^{10}B).

See Appendix II for NMR and MS of compounds, stability tests, and product studies.

Conclusions

In conclusion, we have shown that aryl phthalate self-immolative linkers are easily conjugated with the light sensitive 2-nitrobenzyl ethanol group and the hydrogen peroxide sensitive group 4-(hydroxymethyl)phenylboronic acid pinacol ester. Compound **2** could be potentially useful as a hydrogen peroxide sensor. Compound **1** is of interest because it is able to deliver cargo in a temporally and spatially controlled manner using irradiation. Release of caged 7-hydroxycoumarin occurs upon irradiation within *S2* cells. We note finally that the fast rates of hydrolysis of phthalate self-immolative linkers may make these structures good candidates for domino self-immolative linkers,[17,18,73-75] wherein a single input reaction results in the spontaneous release of numerous cargo molecules. Current domino self-immolative linkers tend to have slow kinetics of release.[58] A recent method to synthesize aryl mellitic acid esters[76] may enable these structures to be used within fast-releasing domino self-immolative systems.

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GENERAL CONCLUSIONS FOR PART 1

Aryl phthalate esters are fast and robust self-immolative linkers in water. This linker is easy to conjugate and releases output phenols within seconds upon cleaving a fluoride-sensitive mask to yield a benign phthalic acid byproduct, making these linkers useful as fluoride sensors and promising for use in biological and materials applications.

Self-immolative aryl phthalate esters were conjugated with cleavable masking groups sensitive to light and hydrogen peroxide. The phthalate linker releases the fluorescent dye 7-hydroxycoumarin upon exposure to light or H_2O_2 leading to an increase in fluorescence. The light sensitive aryl phthalate ester is demonstrated as a pro-fluorophore in cultured S2 cells.

INTRODUCTION FOR PART II

PHOTOCLEAVABLE PROTECTING GROUPS

Photoremovable protecting groups, sometimes called photocages or phototriggers, are popular light-sensitive chemical moieties that mask substrates through covalent linkages that render the substrates inert. Upon irradiation, the masked substrates are released, restoring their reactivity or function.

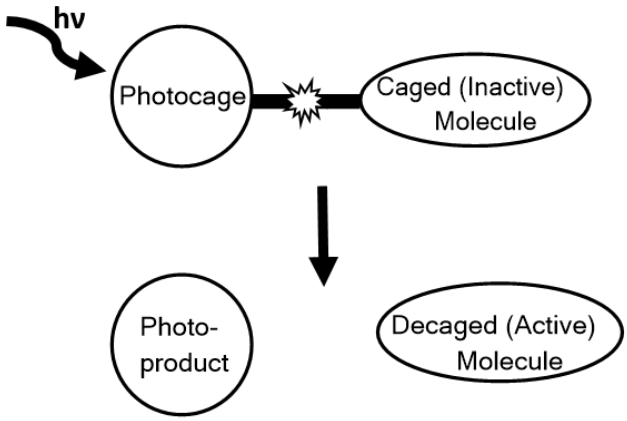

Figure 1. Un-caging scheme of photoremovable protection groups

Barltrop et al.[1] were among the first to introduce a photochemical deprotection reaction of a biologically significant substrate, glycine was released from N-benzyloxycarbonyl glycine.

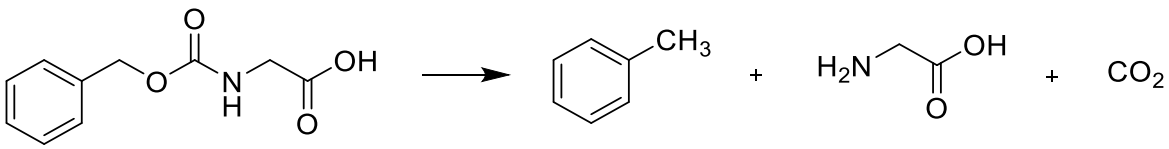

Figure 2. Barltrop's photochemical deprotection of glycine

This discovery lead to an outpouring of several additional photoremovable protecting groups (PPGs). The most commonly utilized include: o-nitrobenzyl,[2-4] phenacyl,[5] acridinyl,[6] benzoinyl,[7,8] coumarinyl,[9] xanthenyl,[10] and o-hydroxynaphthyl[11] structures.

Kaplan et al.[4] introduced the term "cage" in the 1970s to describe a photocage's deactivating influence on the biological substrate to which it is covalently attached. Ideally, the cage detaches only through the action of light allowing for good temporal and spatial control. It is also important that the photoremovable protecting group possess several other desirable properties. Several researches in the field, including Sheehan and Umezawa[12] and Lester and Nerbonne[13] developed a list of properties the ideal photocage would possess[14]:

1. The substrate, caged substrate, and photoproducts have good aqueous solubility for biological studies. For synthetic applications, this requirement is relaxed.

2. The photochemical release must be efficient (e.g., $\Phi > 0.10$).

3. The departure of the substrate from the protecting group should be a primary photochemical process (i.e., occurring directly from the excited state of the cage chromophore).

4. All photoproducts should be stable to the photolysis environment.

5. Excitation wavelengths should be longer than 300 nm and must not be absorbed by the media, photoproducts, or substrate.

6. The chromophore should have a reasonable absorptivity (a) to capture the incident light efficiently.

7. The caged compounds, as well as the photoproduct from the cage portion, should be inert or at least benign with respect to the media, other reagents, and products.

8. A general, high-yielding synthetic procedure for attachment of the cage to the substrate must be available.

9. In the synthesis of a caged substrate, the separation of caged and uncaged derivatives must be quantitative. This is also necessary for the deprotection process for synthetic applications

It may be the case that a photocage not fitting all of the criteria above could be very useful; however, if a photocage lacks many of the traits above then there is a good chance it is not a reasonable photocage.[14]

Many photoremovable protecting groups have been synthesized that cage a target agent for biochemical or biological studies. There have been many uses of caged molecules in biology including caged ATP, [4,3] nuerotransmitters, [15,16] pharmaceuticals,[17] etc.

Photochemistry. Photocleavage of a molecule occurs when the molecule absorbs light of the right intensity and wavelength resulting in a promotion of a single electron from a bonding or non-bonding orbital (HOMO) to an unoccupied molecular orbital (LUMO).

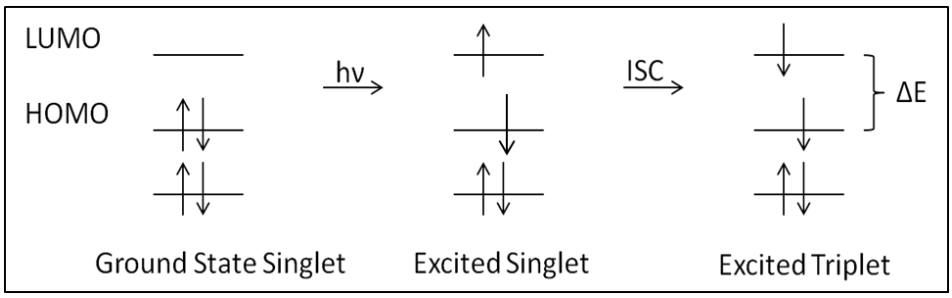

Figure 3. A schematic of promotion of an electron via light from ground state to excited state followed by intersystem crossing (ISC) to the triplet excited state.

The initial excited state is a singlet state which can undergo intersystem crossing (ISC) leading to an excited triplet state.[18] Photocleavage can occur from either the excited singlet or excited triplet state by mechanisms such as bond homolysis, heterolysis, solvolysis, electron transfer, photocyclization, and photooxygenation.[18]

The photocages o-nitrobenzyl, coumarin-4-ylmethyl, and xanthenyl structures are some of the most common and promising to date.

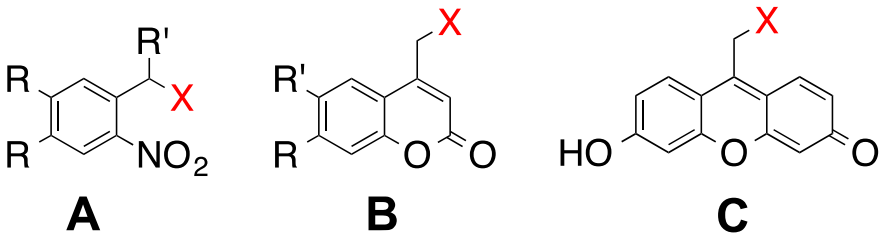

Figure 4. Common photocage backbones

O-nitrobenzyl. O-nitrobenzyl and its derivatives are by far the most commonly used PPG in spite of having many disadvantages. They absorb light in the UV region (λ_{max} = 250-350, typically) making them toxic to cellular structures. Also, photolysis leads to potentially toxic nitroso byproducts that can absorb light strongly.[19] One attractive feature is that cleavage quantum yields of up to 0.49–0.63 has been reported in the literature (releasing 1-(2-nitrophenyl)ethyl phosphate esters).[20]

Much work has been done to improve the o-nitrobenzyl system in terms of quantum yield, rate of release, and increasing light absorbance to longer wavelengths. Many studies have been

done to probe the mechanism of release [21, 22,4] in order to better understand the system and improve it. In general, substitution of the benzylic position affects quantum yield [23,24,25]. However, it also introduces a chiral center, which can be a drawback when protecting chiral molecules such as amino acids and carbohydrates.[26] Modification of the aromatic ring tends to affect the absorbance. Addition of two methoxy groups on the aromatic ring increases the absorbance to longer wavelengths (λ >350 nm). Other substituents have been studied to red-shift the absorption wavelength.[27,28,29,30] Extending the aromatic core as a naphthalene[31] or 7-methoxynapthalene[32] shifted the absorption wavelength to 380 nm however, it is still not in the biological window.

Coumarinyl. As an alternative to the o-nitrobenzyl cages, several coumarinyl cages (Fig. 4, B) have been established. Coumarinyl PPGs were introduced by Givens as a photoactivatable 7-methoxy coumarinyl-4-methyl releasing a diethyl phosphate.[9] Appealing aspects of coumrinyl cages include large molar absorption coefficients at longer wavelengths than the nitrobenzyl cages, fast release rates, and they are fluorescent allowing for monitoring of reaction progress.[26] They absorption maxima from 320 nm to 400 nm depending on the substitutents on the coumarin backbone structure.

Time resolved absorption studies have found the heterolytic bond cleavage to be very fast with rate constants near 2×10^{10} s^{-1} (releasing a phosphate ester).[33] This is one of the most rapid photorelease rates for any caged compound. One downfall is that recombination of the tight ion pair regenerates the ground state derivative leading to a non-productive pathway. Also, because coumarin is a fluorescent dye, fluorescence is another competing pathway to productive release of the caged compound.[34]

The mechanism of coumarinyl photorelease begins with an initial absorption of light followed by relaxation to the lowest π, π^* singlet excited state.[26] At this point, there can be

radiationless decay, fluorescence, or productive heterolytic C-X bond cleavage.[26] The coumarinylmethyl cation formed through heterolytic cleavage can then react with nucleophiles or solvent to form a new stable coumarinyl product (Figure 14).[26]

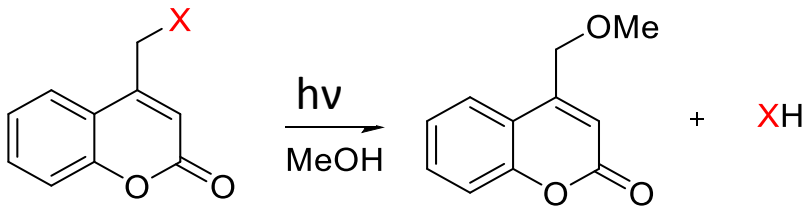

Figure 5. Coumarinyl photorelease scheme

Xanthenyl. A recently studied and potentially useful PPG is based on a xanthene backbone (Fig. 4, C). Wirz, Klan, and co-workers[10] have shown that (6-hydroxy-3-oxo-3H-xanthen-9-yl)methyl and derivatives release diethyl phosphate or carboxylic acid upon irradiation with visible light (over 500 nm) and quantum yields of 0.005 – 0.04.[10] However, these systems are synthetically challenging and have undesirable protonation and tautomerization equilibria.[10] The fate of the xanthene based PPGs is an unfortuante one because a photoremovable protecting group that absorbs at wavelengths >500 nm would tremendously increase PPG application in biological systems.

Structure- Reactivity Relationship. It is clear that photocages are important tools in chemistry and biology, however, most have been serendipitously discovered. It seems rather challenging to rationally design a cage that has a productive uncaging pathway versus fluorescence pathway or other non-productive pathway. This difficulty comes from a lack of understanding of the structure-reactivity relationship that leads to photoheterolysis in the excited state. Recently, Winter et al.[35] has addressed this issue and has developed a structure- reactivity relationship to predict excited

state photo-heterolysis based on the presence of a nearby conical intersection. A conical intersection is a geometry where the ground state and the excited state have the same energy, in other words, a point where two potential energy surfaces intersect.[34] Fig. 6 A shows a representation of a conical intersection (purple sunburst) where the ground state (blue) and excited state (red) potential energy surfaces intersect. This intersection allows an efficient path from the excited state to the ground state ion pair.[34] The closer in energy the gap between the excited state surface and the ground state surface is, the faster and more efficient this path will be.[34] It has been found computationally that carbocations favored from photoheterolysis tend to have nearby, low-energy conical intersections, while stable carbocations from thermal heterolysis tend to have high-energy, distant conical intersections.[35]

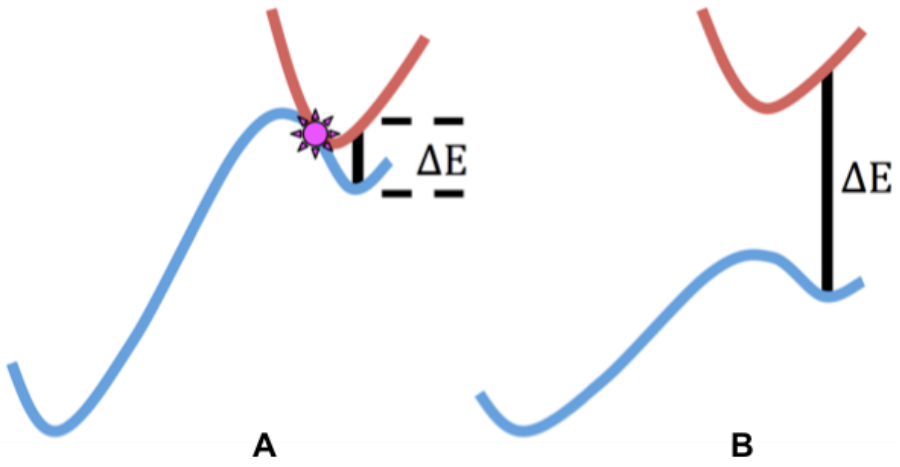

Figure 6. Schematic of Winter's hypothysis that a destabilized ground state and a stabilized excited state can lead to a favorable, nearby conical intersection (A), whereas it is unlikely that a stabilized ground state will have a nearby conical intersection (B).[35]

The idea that these photoheterolysis reactions may be governed by conical intersection control could facilitate the design of new photocages with improved light absorbing properties by searching for substrates leading to carbocations with a favorable built-in conical intersection. Furthermore, the structures that have nearby conical intersections tend to undergo photoheterolysis reactions releasing leaving groups to generate destabilized carbocations (anti-aromatic ions, donor unconjugated ions, dicoordinated aryl/vinyl cations, the opposite of *thermal* heterolysis preferences.

The following is a list developed by the Winter Lab that presents ideal photocage properties:

1. The photocage must be uncaged with visible light, preferably in the biological window.
2. The photocage must be thermally stable in the dark
3. The photocage must be biologically benign and convert to benign byproducts
4. The photocage must have a potent light-absorbing chromophore and high quantum yields of release.
5. The photocage must have fast photochemical release of the substrate from the excited state
6. The photocage must be water-soluble and water compatible
7. The photocage must be able to release a variety of functional groups
8. The photocage must be easily synthesizable

BODIPY. A computational investigation by Winter, et. al.[36] found that *meso*-substituted BODIPY structures would most likely undergo photoheterolysis in the excited state by way of a conical intersection.

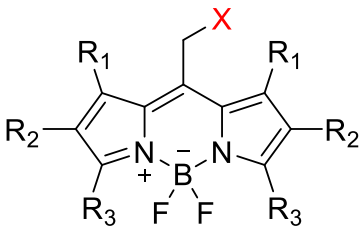

Figure 7. *meta*-substituted BODIPY dyes are computationally reasoned to undergo heterolysis in the excited state.

BODIPY dyes have excellent optical properties and would be an ideal photoremovable protecting group candidate. They absorb in the visible to near-IR, they have large molar absorbtivities ($\varepsilon > 60{,}000\ M^{-1}\ cm^{-1}$) and sharp absorption peaks. Also, the syntheses of BODIPYs are well established and the core structure is easy to modify. The following chapters describe the synthesis and use of *meso*-substituted BODIPY dyes as a photocage for acetic acid.

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CHAPTER 3

BODIPY-DERIVED PHOTOREMOVABLE PROTECTING GROUPS UNMASKED WITH GREEN LIGHT

Taken in part from: Goswami, P.P.; Syed, A.; Beck, C.L.; Albright, T.R.; Mahoney, K.M.; Unash, R.; Smith, E.A.; Winter, A.H. *J. Am. Chem. Soc.*, **2015**, 137, 3787.

Introduction

Photoremovable protecting groups, sometimes called photocages or phototriggers, are popular light-sensitive chemical moieties that mask substrates through covalent linkages that render the substrates inert. Upon irradiation, the masked substrates are released, restoring their reactivity or function. While photocages have important applications in areas such as organic synthesis,[1-3] photolithography,[4,5] and light-responsive organic materials,[6-8] these structures are particularly prized for their ability to trigger biological activity with high spatial and temporal resolution[9-13]. Examples of such chemical tools include photocaged proteins,[14-16] nucleotides,[17,18] ions,[19-23] neurotransmitters,[24,25] pharmaceuticals,[26,27] fluorescent dyes,[28-30] and small molecules[31,32] (e.g., caged ATP). These biologically relevant caged molecules and ions can be released from the caging structure within particular biological microenvironments using pulses of focused light. The most popular photocages used in biological studies are the *o*-nitrobenzyl systems[31-33] and their derivatives, but other photocages that see significant use

include those based on the phenacyl,[34] acridinyl,[35] benzoinyl,[36,37] coumarinyl,[38] and *o*-hydroxynaphthyl structures.[39] Unfortunately, with few exceptions described below, [40,41] a serious limitation of most popular photocages is that they absorb mostly in the ultraviolet where the limited penetration of UV light into tissues largely restricts these studies to fixed cells and thin tissue slices. Furthermore, prolonged exposure of cells or tissues to UV light can lead to cellular damage or death.

Consequently, new photocaging structures that absorb visible light are urgently needed. Advantages of visible light irradiation include diminished phototoxicity compared to UV light and deeper optical penetration into tissue. Additionally, visible light photolysis can be performed with cheap lamps and Pyrex glassware, while UV photolysis requires expensive UV sources. Unfortunately, the major problem that has hindered the development of new photocages that absorb visible light is the lack of a structure-reactivity relationship for excited state heterolysis. That is, it is difficult to predict *a priori* which structures, when irradiated with light, will undergo an efficient photoheterolysis reaction. Thus, attempts to prepare visible light absorbing photocages have mostly bypassed this problem by using metal-ligand photoreleasing systems[41-43] or by using creative indirect schemes. Examples of such creative schemes include upconverting nanoparticles with surface-attached UV-absorbing photocages[44-46] or release mediated by photoinduced electron transfer with a sacrificial electron donor.[47]

However, visible light absorbing organic structures that offer simple photorelease schemes and structures would potentially make a more compelling case for widespread use in biologically-oriented labs.[48] A recent computational study performed in our lab suggested the hypothesis that photoheterolysis reactions may be under conical intersection control.[49] That is, photoheterolysis of C-LG (carbon—leaving group) bonds to generate ion pairs[50] may be favored

if the ion pair has access to a nearby productive conical intersection that provides an efficient channel for the excited state of the photoprecursor to decay to the ground-state ion pair. Because conical intersections are challenging to compute, we further suggested using the vertical energy gap of the carbocation to its first excited state as a simple predictor of a nearby conical intersection (CI). A low S_0-S_1 energy gap of the cation would suggest the possibility of a nearby CI between the S_0 and S_1 surfaces, and the potential for a productive mechanistic channel for the photochemistry to proceed from the excited state of the photocaged precursor to the ion pair.

Thus, to find visible light absorbing photocages we searched for potential photocaging structures that would generate carbocations with low-lying diradical states. A time-dependent density functional theory (TD-DFT) computational investigation of carbocations attached to the BODIPY scaffold at the *meso* position indicated that these ions have low-lying excited states. For example, the TD-DFT computed S_0-S_1 vertical energy gap of the carbocation derived from C-O scission of **2** is 8 kcal/mol (TD-B3LYP/6-311+G (2d,p), suggesting a near-degenerate diradical configuration. Indeed, all of the cations derived from C-O scission of **1-6** have vertical gaps < 13 kcal/mol (see SI for computational details), and have singlet states with considerable diradical character. Large singlet stabilizations upon switching from restricted → spin-purified unrestricted singlet computations indicate that the singlet states can be described as diradicals or possessing considerable diradical character (see SI for details). Thus, the exact vertical energies from the TD-DFT computations are to be viewed with suspicion, but it is clear that there are low-energy diradical forms for these ions, suggesting a CI between the closed-shell singlet and singlet diradical forms of the carbocations in the vicinity of the ion pair geometry. Further, the singlet-triplet gaps of all the carbocations derived from **1-6** are ~5 kcal/mol in favor of the triplet state, suggesting that

the "carbocations" produced by heterolysis of **1-6** may in fact be better described as ion diradicals in their thermodynamic ground state than by traditional closed-shell carbocation structures.[51]

Results and Discussion

Encouraged by these computational studies, we synthesized structures 1-6 as photocages for acetic acid. Advantages of the BODIPY scaffold include simple syntheses, a compact structure, known biological compatibility,52 and high extinction coefficients in the visible.53 Photorelease studies, described below, indicate that these structures release carboxylic acids upon photolysis with wavelengths >500 nm.

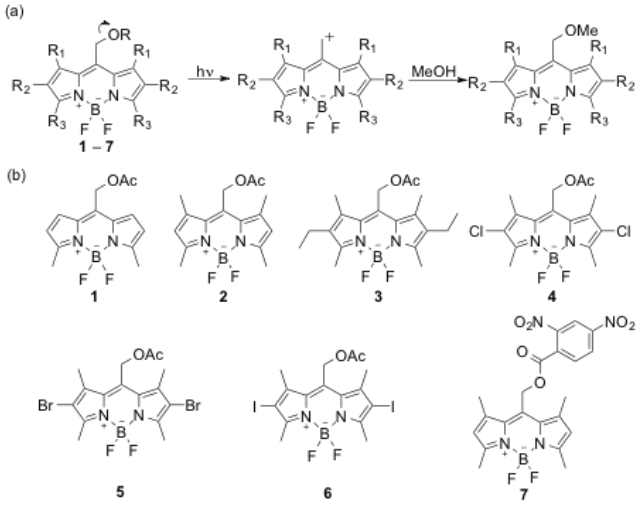

Figure 1. (a) Possible pathway for the photolysis of photocaged acetic acid; (b) Substrates described in this study.

The observed substrate release rate as a function of photolysis time is quantified by the quantum efficiency parameter (ε Φ), which is the product of the extinction coefficient at the irradiation wavelength (ε) and the quantum yield of release (Φ). Extinction coefficients for 1-6 were determined by UV-Vis spectroscopy (see Table 1). To compute the quantum yields of photorelease (Φ), the flux of a 532 nm laser exci-tation beam (ND:YAG, 1st harmonic) was

determined using potas-sium ferrioxalate actinometry. Release of acetic acid as a function of laser irradiation time in MeOH was followed by quantitative LC/UV (see SI for details). Each quantum yield reported is the average of 3 separate runs. Identical actinometry measurements performed after photolysis demonstrated high flux stability of the laser. Additionally, repeating the quantum yield measurement for 2 on a different day with a different laser power setting (in triplicate) gave essentially the same value for the quantum yield, indicating reproducibility. A preparative photolysis of 2 in MeOH gave a meso-substitued methyl ether adduct as a stable photoproduct of the photocaging moiety, suggestive of solvent trapping of an intermedi-ate carbocation. Additionally, lamp photolysis of 2 showed no major difference in release of acetic acid under argon or air atmos-phere. Curiously, unlike 1-4 and 6, the brominated compound 5 was found to be unstable. It decomposes after 1 day stored on the shelf in the dark, and photolysis of freshly prepared and purified 5 led to secondary products in addition to acetic acid release, and photolysis was accompanied by rapid solution bleaching. Conse-quently, we were not confident in our quantum yield measurements for 5 and excluded it from Table 1. Probably, 5 also has access to alternative photochemical pathways (e.g., C-Br homolysis) and thermal degradation channels. Photocaged compounds 1-4 and 6 are thermally stable in the dark. Boiling these compounds in MeOH for 1 h in a foil-wrapped vessel led to no change in the 1H NMR spectrum.

In general, the quantum efficiencies for 1-4 and 6 are lower or comparable with the popular caged o-nitrobenzyl or coumarinyl systems.9 Quantum yields for 1-4 are lower than those for typical o-nitrobenzyl photocaged structures or coumarinyl systems, but this lower quantum yield is compensated by the much higher extinction coefficients of the BODIPY chromophores compared to the o-nitrobenzyl chromophore, leading to reasonable quantum

efficien-cies. The iodinated derivative 6 has the largest quantum efficiency, comparable to that of some caged o-nitrobenzyl systems, but with a λmax at ~550 nm rather than in the UV (the parent o-nitrobenzyl system has a λmax of ~280 nm while a popular dimethoxy analog has a λmax of ~350 nm). A plausible explanation for the higher quantum yield of 6 is that the iodine atoms promote intersystem crossing (ISC) to a triplet state, which are usually longer lived than singlet excited states, giving more time for release. For example, the phenacyl photocage derivatives described by Givens undergo pho-torelease in the triplet state.34 The plausibility of a rapid ISC event is supported by the very weak fluorescence of solutions of 5 and 6, compared to solutions of 1-4.

Table 1. Optical properties and quantum efficiencies of 1-6. Quantum yields of acetic acid release (Φ) determined by ferrioxalate actinometry in MeOH with a 532 nm ND:YAG laser source and release followed using quantitative LC-UV (Φ values are the average of 3 runs).

	λ_{max} (nm)	λ_{em} (nm)	ε (x 10^4 M^{-1} cm^{-1})	Φ (x 10^{-4})	ε Φ ($M^{-1}cm^{-1}$)
1	519	527	5.7	6.4	37
2	515	526	7.1	9.9	70
3	544	560	6.2	9.5	59
4	544	570	4.8	4.0	19
5	545	575	--	--	--
6	553	576	4.9	23.8	117

The UV-Vis spectra and fluorescence spectra of 1-6 are shown in Figure 2. These structures absorb be-tween 515 nm and 553 nm (and emit between 520 nm and 580 nm), typical of simple BODIPY dyes, and feature large extinction coeffi-cients (~50,000-70,000 M-1cm-1).

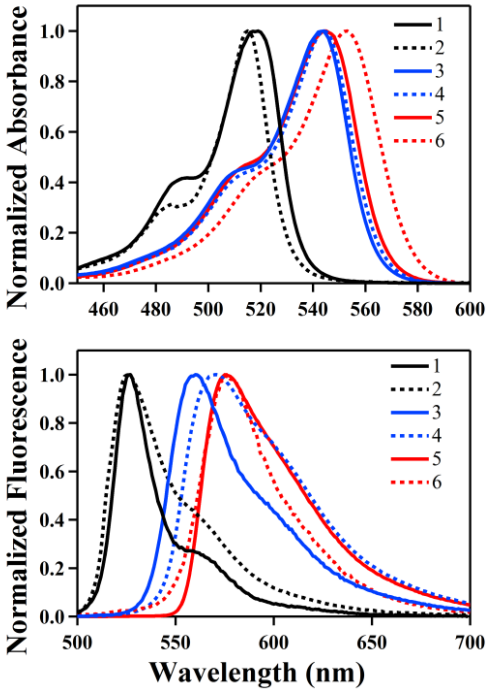

Figure 2. Normalized absorbance and fluorescence spectra of 1-6.

To test the viability and usefulness of the BODIPY derived photocages in biological systems, compound 7 was synthe-sized. 2,4-dinitrobenzoic acid is a known54 fluorescence quencher for BODIPY dyes. This quencher was coupled with our BODIPY moiety using a standard DCC/DMAP ester coupling reaction. We anticipated that 7 would be weakly fluorescent, but upon photore-lease of the quencher the fluorescence would increase. Indeed, when 7 was irradiated with a mercury lamp (excitation = 500 nm, see SI) in a cuvette and its fluorescence was plotted over time (Fig-ure 3 N), there was a growth in fluorescence attributed to release of the quencher. Photorelease of the quencher was confirmed by 1H NMR photolysis studies. As a control, similar steady state fluores-cence measurements were performed over time for compound 7 in the dark without light exposure, leading to essentially no change in fluorescence.

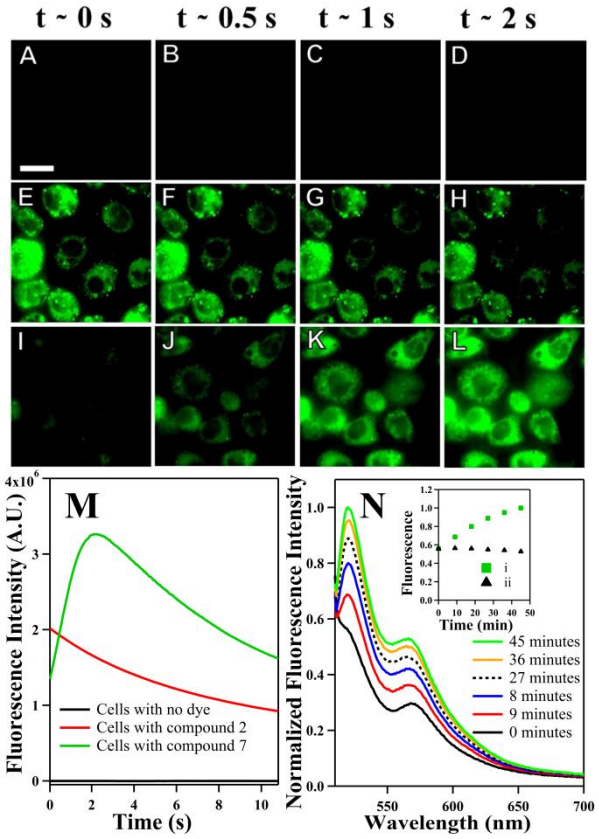

Figure 3. Fluorescence images of S2 cells with no BODIPY com-pound (A-D), cells incubated with compound 2 (E-H) and cells incubated with compound 7 (I-L) as a function of irradiation time (top). Scale bar is 20 µm (shown in panel A) and is the same for all the images. Images were adjusted to same contrast in each row. Average of at least 32 cells fluorescence intensity profile versus irradiation time using 100% lamp power for excitation in cells (M). Increase in free BODIPY fluorescence signal over time with quencher release from compound 7 in BES buffer (N). Plot insert (N) depicts the difference in growth of fluorescence vs time for compound 7 with (i) and without (ii) light irradiation in a cuvette.

Compound 2 and 7 were then incubated with Drosophila S2 cells and monitored using fluorescence microscopy (Figure 3 A-L). The Drosophilia S2 cells loaded with 2 and 7 were irradiated continu-ously with 500 nm light. Fluorescence images were collected every 36 ms for

a total of 10.8 seconds. The fluorescence intensity for compound 7 inside cell as shown in Figure 3I-L increases rapidly. This increase in fluorescence can be attributed to the release of the quencher. The same fluorescence study with 2 as a control in Fig-ure 3E-H shows no such increase in fluorescence. For 2, the leav-ing group is acetate, which is not a quencher. Thus, little change in the fluorescence would be anticipated upon photorelease of acetic acid from this moiety. The background decay in fluorescence for both 2 and 7 can be attributed to photobleaching under the intense focused light. Parts A-D of Figure 3 show that there is a minimal change in fluorescence of cells when they are irradiated without being loaded with compound 2 or 7. Figure 3M shows the fluores-cence intensity change over time for cells incubated with compound 2, 7, and the control experiments without any compound. Cytotoxicity of compounds were measured with trypan blue exclu-sion assay. All values are normalized with the control cells which were not incubated with any compound. At a compound concentra-tion of 25 µM, 97% for compound 2 and 92% for compound 7 remained viable after 1h.

Experimental

Compounds 1, 2, 3, and 4 were synthesized as previously described. (All spectra for these compounds matched those previously reported.)

Synthesis of 5. Compound **2** (0.1 g, 0.31 mmol, 1 equiv), was dissolved in 3 mL of dry THF under argon and cooled to -78 °C. N-Bromosuccinimide (0.23 g, 1.25 mmol, 4 equiv) dissolved in 2 mL of dry THF was added dropwise to the solution. The reaction mixture was stirred for 15 min at -78 °C, after which it was warmed to room temperature and stirred for an additional 5 h. The solvent was evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with hexane-ethyl acetate 90:10 vol/vol to give **5** as dark red crystals

(0.14 g, 95% yield). Mp 230°C (decomp); ^{1}H NMR (600MHz, $CDCl_3$): δ 5.32 (s, 2H), 2.63 (s, 6H), 2.40 (s, 6H), 2.15 (s, 3H); ^{13}C NMR (150MHz, $CDCl_3$) δ 170.45, 155.29, 138.93, 133.85, 131.87, 113.10, 58.04, 20.69, 14.94, 14.08; High-res MS (ESI) for formula $C_{16}H_{17}BBr_2F_2N_2O_2Na^+$, Calc. 497.9646, Found 497.9646.

Synthesis of 6. Compound **2** (0.1 g, 0.31 mmol, 1 equiv), was dissolved in 3 mL of dry THF under argon and cooled to -78 °C. N-Iodosuccinimide (0.18 g, 2.5 mmol, 4 equiv) dissolved in 2 mL of dry THF was added dropwise to the solution. The reaction mixture was stirred for 15 min at -78 °C, after which it was warmed to room temperature and stirred for an additional 5 h. The solvent was evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with dichloromethane to give 6 as dark purple crystals (0.07 g, 39% yield). Mp 210°C; ^{1}H NMR (600MHz, $CDCl_3$): δ 5.31(s,2H), 2.59(s, 6H), 2.38(s, 6H), 2.14(s, 3H); ^{13}C NMR (150MHz, $CDCl_3$) δ 170.43, 158.06, 143.60, 132.92, 132.70, 87.38, 58.35, 20.68, 18.29, 16.47; MS (ESI) for formula $C_{16}H_{17}BI_2F_2N_2O_2Na^+$, Calc. 593.9369, Found 593.9378.

Synthesis of 7. 2,4 dinitrobenzoic acid (0.054g, 0.194mmol, 1 equiv) was dissolved in 3ml of dry DCM under argon in room temperature. DCC (*N*,*N*'-Dicyclohexylcarbodiimide) (0.048mg, 0.233mmol, 1.2 equiv) dissolved in 3ml of dry DCM was added dropwise to the solution. 4-DMAP (4-Dimethylaminopyridine) (0.001g, 0.007mmol, 0.04equiv) was added to this solution. Next, *7a* (0.049, 0.233mmol, 1.2equiv) dissolved in 3ml of dry DCM was slowly added to the solution. The reaction mixture was stirred for 16 h until its completion. The reaction mixture was filtered to get rid of DCU (Dicyclohexyl Urea) by-product. The filtrate was evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with hexane-ethyl acetate 80:20 vol/vol to give 7 as a dark orange crystals (0.08g, 91% yield). ^{1}H

NMR (600MHz, $CDCl_3$): δ 8.86(d, J = 2.2Hz, 1H), 8.54(dd, J = 8.4Hz, 1H), 7.86(d, J = 8.4Hz, 1H), 6.11(s, 2H), 5.69(s, 2H), 2.53(s, 6H), 2.44(s, 6H); ^{13}C NMR (150MHz, $CDCl_3$) δ 163.79, 157.46, 149.27, 141.54, 132.59, 132.72, 131.20, 130.97, 128.07, 122.88, 120.10, 60.28, 15.89, 14.89; MS (ESI) for formula $C_{21}H_{19}BF_2N_4O_6Na^+$, Calc. 495.1258, Found 498.1271.

See Appendix III for product studies of the compounds.

Conclusions

BODIPY-derived photocages unmask carboxylic acids with green light excitation >500 nm and photocleavage can be carried out in living cells. These photocages are promising alternatives for the popular *o*-nitrobenzyl photocaging systems, being easy to synthesize, utilizing a biocompatible chromophore, and having superior optical properties to the most popular photocages in current use. More generally, our strategy of identifying new photocages by searching for carbocations with low-energy diradical states seems to be a promising one. BODIPY derivatives that release functional groups other than carboxylic acids and that have red-shifted absorptions into the biological window (~600-1000 nm) are currently under investigation.

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CHAPTER 4

SHIFTING BODIPY PHOTOREMOVABLE GROUPS INTO THE RED

Introduction

Photoremovable protecting groups (also known as photocages, phototriggers, photoreleasable and photocleavable protecting groups) are light-sensitive moieties that allow for spatial and temporal control over the release of a masked substrate by light-induced cleavage of a covalent PPG-substrate bond resulting in the restoration the substrate's function. Photocages are particularly useful for the release of biologically relevant substrates, such as proteins[1-3], nucleotides[4,5], ions[6-10], neurotransmitters[11-12], pharmaceuticals[13-14], and fluorescent dyes[15-17], and small molecules[18,19].

The most popular photocages used in biological studies are *o*-nitrobenzyl[31,33] and derivatives, but others include those based on the phenacyl[21], acridinyl[22], benzoinyl[23,24] coumarinyl[25], and *o*-hydroxynaphthyl[26] structures. A major limitation to many of these photocages and especially to the popular o-nitrobenzyl photocages is that they absorb in the ultraviolet region of the spectrum where tissue penetration is limited restricting studies for fixed cells and thin tissue slices. In addition, exposure of the cells or tissues to UV light can lead to cellular damage or death.

Recently, our lab developed a new class of protecting group derived from *meso*-substituted BODIPY dyes with heterolytic bond cleavage occurring at green wavelengths >500 nm.[27] This BODIPY structure was first computationally explored to suggest it would undergo heterolytic bond cleavage in the excited[29] and was then empirically investigated. This is the first example of a rationally designed photocage releasing a cargo molecule using visible light making meso-substituted BODIPY dyes a promising alternative to the popular *o*-nitrobenzyl photocage systems. A photocage which cleaves within the biological window of light would be

exceptionally valuable for cell and tissue studies. The biological window identifies the range of wavelengths from 650 nm to 1130 nm where light can more efficiently penetrate biological tissue because these tissues scatter and absorb less light at longer wavelengths.[28]

It has been well-established that extending the conjugation of BODIPY dyes allows for a red-shift in absorption maximum.[30] Here we use a Knoevenagel condensation reaction to extend the conjugation on the highly-acidic 3,5-methyl groups of our previously synthesized BODIPY photocage structure.

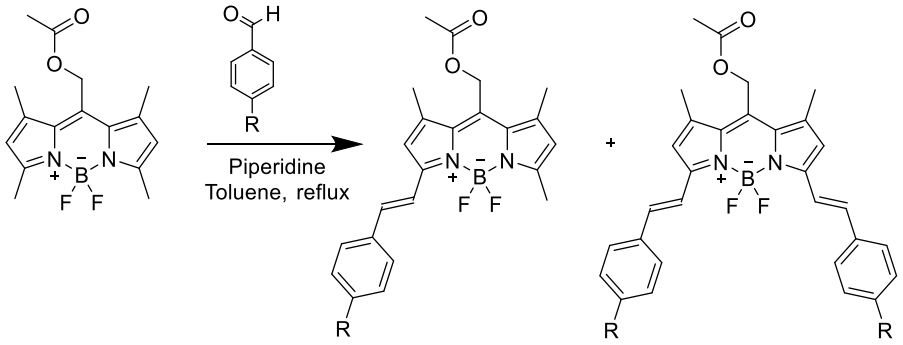

Figure 1. Knoevenagel condensation reaction was used to red shift the absorption maximum of BODIPY **2** from Chapter 3

The following photocages were prepared:

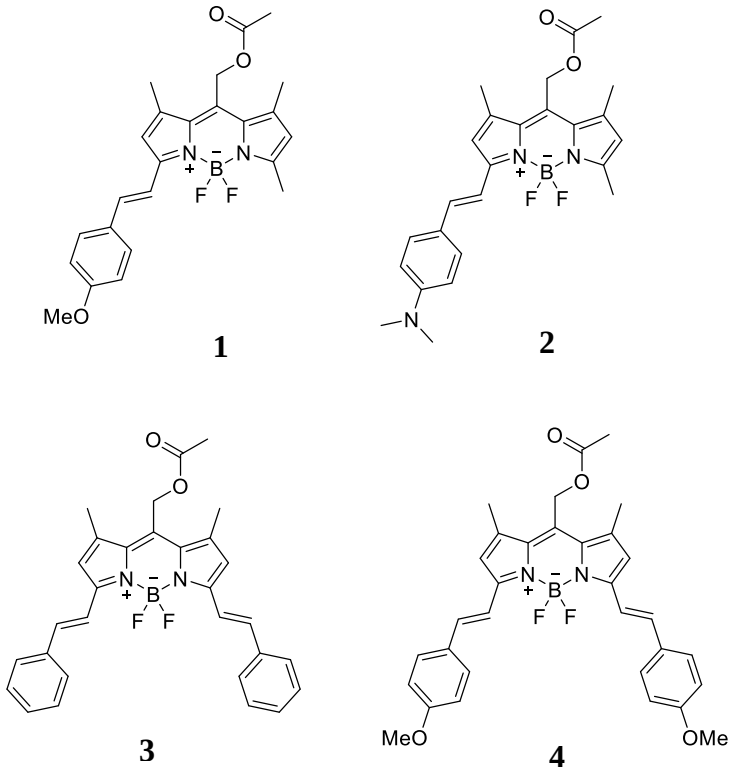

Figure 2. BODIPY photocages with extended conjugation

Results and Discussion

To demonstrate that **1-4** could indeed release acetic acid during light irradiation, NMR was used to follow the release progress over time.

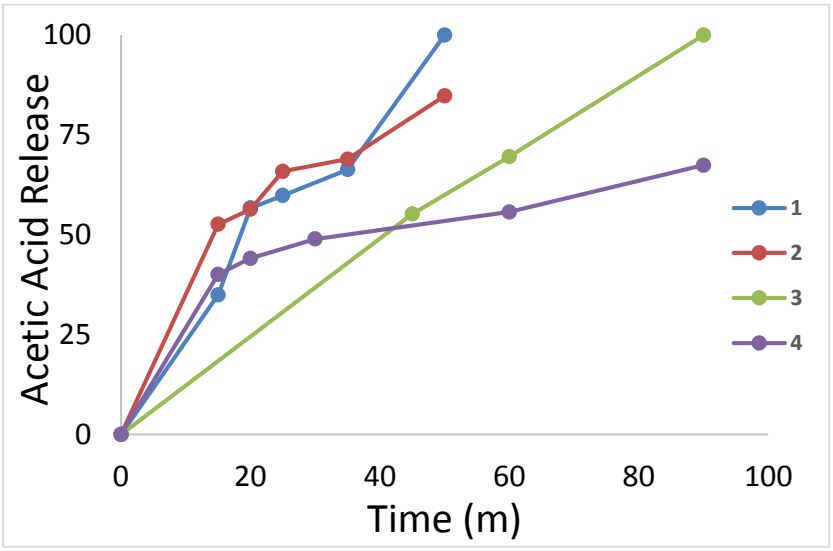

Figure 3. Release of acetic acid over time. 2 mM NMR samples were prepared and irradiated with a Xenon lamp.

The thermal stability of the dyes was tested by heating to 60 ^{o}C for 1 hour in the dark. No acetic acid release was observed.

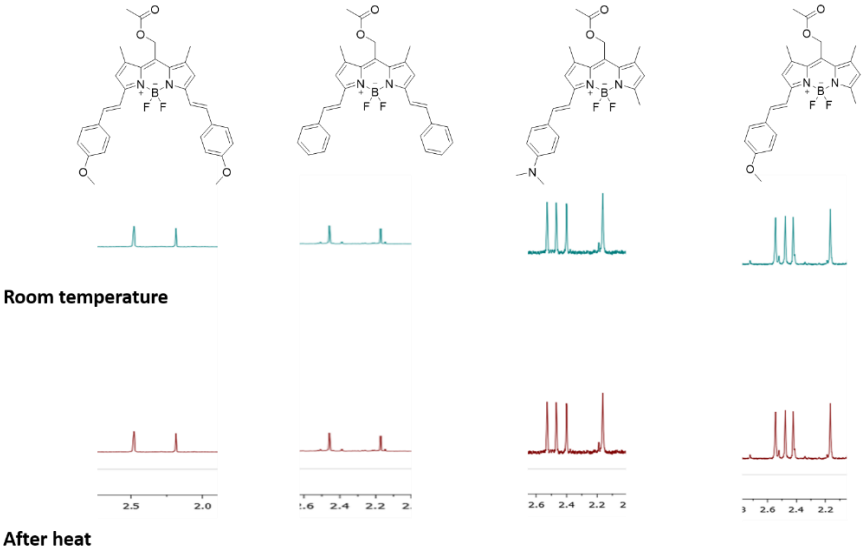

Figure 4. Thermal stability test of **1-4**; NMR scans were taken at room temperature (blue) and the samples were heated for one hour at 60^{o}C and NMR scans were taken again (red); No acetic acid (2.04ppm) release was observed. The NMR spectrum has been cropped for clarity, however, there was no change in any NMR peaks after heating.

The optical properties of **1-4** are shown in Fig. 5. The absorption maxima of the compounds range from 586 nm to 661 nm and the fluorescence ranges from 607-684 nm. Photocages **2**,**3**, and **4**, absorb within the biological window of visible light making them powerful alternatives the o-nitrobenzyl photocage which absorbs in the UV. The extinction coefficients of the photocages are ~60,000 M^{-1} cm^{-1}. The quantum yields are XXX, which are similar to the BODIPY dyes from Chapter 3. These values are relatively low, however, the

high extinction coefficients of **1-4** make the quantum efficiencies similar to that of the popular o-nitrobenzyl photocages.

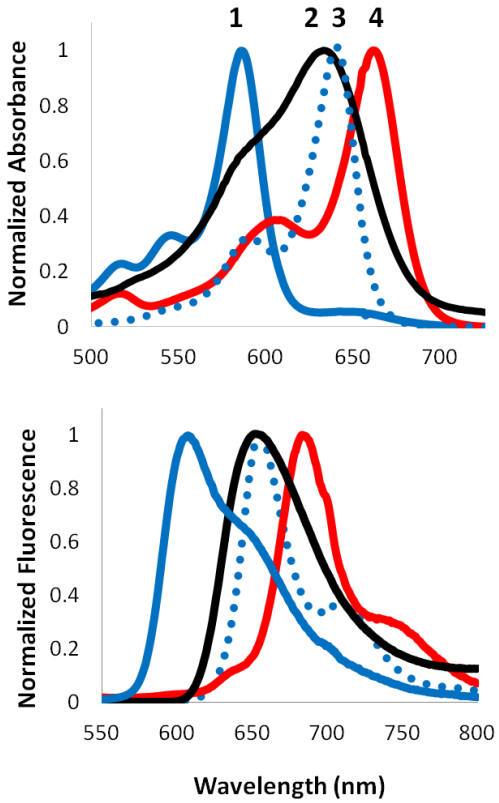

Figure 5. Normalized absorption and fluorescence spectra of compounds **1-4**

Table 1. Absorption (λ_{max}) and fluorescence (λ_{em}) maxima, extinction coefficients (ε), quantum yields (Φ) and quantum efficiencies (ε Φ**)** of compounds **1-4**.

	λ_{max} (nm)	λ_{em} (nm)	ε (x 10^4 M^{-1} cm^{-1})	Φ (x 10^{-4})	ε Φ ($M^{-1}cm^{-1}$)
1	**586**	**607**	**6.1**	**9.8**	**6.0**
2	**633**	**650**	**6.0**	**6.9**	**4.1**
3	**640**	**656**	**6.5**	**4.5**	**2.9**
4	**661**	**684**	**6.5**	**4.1**	**2.7**

Work is currently underway to take **4** and replace the acetate leaving group with the 3,5 -dinitrobenzoic acid fluorescence quencher as seen in Compound **7** from Chapter 3. Similar biological studies within S2 cells are to be performed.

Experimental

8-Acetoxymethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate was synthesized as previously reported. (Spectra for this compound matched those previously reported.)

Synthesis of Compound 1. 8-Acetoxymethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (50 mg, 0.016 mmol, 1 equivalent) and 4-methoxybenzaldehyde (4.4 mg, 0.032 mmol, 2 equiv.) were added to 8 mL of ethanol which had been previously dried over 3 Å molecular sieves for 24 h. This suspension was then placed in a dry, glass microwave reaction vessel. Both acetic acid (120 µL) and piperidine (120 µL) were then added and the vessel was sparged with argon. The microwave vessel was irradiated for 10 min at 113°C and 800 W. The solvent was evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with 50:50 hexanes:ethyl acetate. The dark purple product was recovered and further purified using a prep TLC plate and 80:20 hexanes:ethyl acetate. The product was obtained in 58% yield (32 mg, 0.009 mmol). 1HNMR (400 MHz, CDCl3): δ = 7.55 (d, J = 4 Hz, 2H), 7.52 (d, J = 8 Hz, 1H), 7.24 (d, J = 8 Hz, 1H), 6.91 (d, J = 4 Hz, 2H), 6.71 (s, 1H), 6.11 (s, 1H), 5.33 (s, 2H), 3.85 (s, 3H), 2.58 (s, 3H), 2.43 (s, 3H), 2.28 (s, 3H), 2.15 (s, 3H) ppm; 13CNMR (200 MHz, CDCl3): δ = 170.77, 160.84, 155.56, 154.57, 141.15, 140.41, 137.32, 135.35, 134.46, 132.94, 131.39, 129.37, 122.18, 118.87, 116.95, 114.45, 58.16, 55.24, 20.82, 16.05, 15.74, 14.94 ppm; Hi-res MS (ESI) for formula C24H25BF2N2O3, Calc. 438.2035, Found 438.2038.

Synthesis of Compound 2. 8-Acetoxymethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (50 mg, 0.016 mmol, 1 equiv) and 4-(dimethylamino)benzaldehyde (4.8 mg, 0.032 mmol, 2 equiv)

were added to 8-mL of ethanol which had been previously dried over 3 Å molecular sieves for 24 hours. This suspension was then placed in a microwave reaction vessel. Both acetic acid (120 µL) and piperidine (120 µL) were then added and the vessel was sparged with argon. The microwave vessel was irradiated for 20 minutes at 113°C and 800 W. The solvent was evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with 80:20 hexanes:ethyl acetate to give 9.1 mg of **2** as a dark blue solid (24% yield). ^{1}HNMR (400 MHz, $CDCl_3$): δ = 7.50 (d, *J* = 8Hz, 2H), 7.45 (d, *J* = 16 Hz, 1H), 7.24 (d, *J* = 16 Hz, 1H), 6.72 (s, 1H), 6.68 (d, *J* = 8Hz, 2H), 6.07 (s, 1H), 5.32 (s, 2H), 3.04 (s, 6H), 2.57 (s, 3H), 2.41 (s, 3H), 2.36 (s, 3H), 2.15 (s, 3H) ppm; ^{13}CNMR (200 MHz, $CDCl_3$): δ = 170.85, 156.07, 153.42, 151.41, 141.39, 139.20, 138.64, 134.93, 132.28, 129.70, 124.56, 121.37, 119.21, 114.25, 112.12, 58.31, 40.36, 24.02, 20.85, 16.12, 15.60 ppm; Hi-res MS (ESI) for formula $C_{25}H_{28}BF_2N_3O_2$, Calc. 451.2352, Found 451.2339.

Synthesis of Compound 3. 8-Acetoxymethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (50 mg, 0.016 mmol, 1 equiv) and benzaldehyde (3.4 mg, 0.032 mmol, 2 equiv) were added to 8 mL of ethanol which had been previously dried over 3 Å molecular sieves for 24 h. This suspension was then placed in a microwave reaction vessel. Both acetic acid (120 µL) and piperidine (120 µL) were then added and the vessel was sparged with argon. The microwave vessel was irradiated for 20 minutes at 113°C and 800W. The solvent was evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with 80:20 hexanes:ethyl acetate to give 23.7 mg of **3** as a dark blue solid (38% yield). ^{1}HNMR (400 MHz, $CDCl_3$): δ = 7.71 (d, J = 8 Hz, 2H), 7.64, (d, J = 4 Hz, 4 H), 7.42 (t, *J* = 8 Hz, 4H), 7.34 (t, *J* = 8 Hz, 2H), 7.30 (d, *J* = 8 Hz, 2H), 6.77 (s, 2H), 5.37 (s, 2H), 2.44 (s, 6H), 2.17 (s, 3H) ppm; ^{13}CNMR (200 MHz, $CDCl_3$): δ = 170.62, 153.35, 140.42, 137.06, 136.43, 134.79, 130.23,

129.18, 128.82, 127.68, 118.94, 58.02, 22.71, 15.88, 14.14 ppm; Hi-res MS (ESI) for formula $C_{30}H_{27}BF_2N_2O_2Na^+$, Calc. 519.2026, Found 519.2041.

Synthesis of Compound 4. 8-Acetoxymethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (50 mg, 0.016 mmol, 1 equivalent), 4-methoxybenzaldehyde (9.6 mg, 0.064 mmol, 4 equivalents) were added to a 10 mL Erlenmeyer flask. Dry toluene (3 mL) was added to the flask, followed by piperidine (1 mL). The flask was heated until the toluene evaporated. More toluene (1 mL) was then added and solvent allowed to evaporate again. The solvent was completely evaporated under reduced pressure. The solid residue was then loaded onto a silica gel flash column and eluted with 50:50 hexanes:ethyl acetate to give **4** as a dark blue solid. The product was further purified using a prep TLC plate and 80:20 hexanes: ethyl acetate providing 17.8 mg of **4** (26% yield). ^{1}HNMR (400 MHz, $CDCl_3$): δ = 7.59 (d, *J* = 4Hz, 4H), 7.58 (d, *J* = 8 Hz, 2H), 7.24 (d, *J* = 8 Hz, 2H), 6.94 (d, *J* = 4 Hz, 4H), 6.73 (s, 2H), 5.35 (s, 2H), 3.87 (s, 6H), 2.43 (s, 6H), 2.16 (s, 3H) ppm; ^{13}CNMR (200 MHz, $CDCl_3$): δ = 170.82, 160.73, 153.52, 140.06, 136.71, 134.73, 129.58, 129.35, 118.76, 117.24, 114.46, 58.28, 55.54, 20.87, 15.99 ppm; Hi-res MS (ESI) for formula $C_{32}H_{31}BF_2N_2O_4$, Calc. 556.2454, Found 556.2451.

See Appendix IV for NMR and MS of compounds, stability tests, and product studies.

Conclusions

Four new BODIPY based photocages were synthesized, **1-4**. They were found to successfully release acetic acid when irradiated with white light. They were also found to be thermally stable for 1 hour at 60ºC. The optical properties of these photocages are outstanding with absorbances of 586 nm to 661 nm and extinction coefficients ~60,000 M^{-1} cm^{-1}. The quantum efficiencies were found to be XX which is on-par with the common 2-nitrobenzyl photocages making **1-4** potentially powerful alternatives. Compounds **2**,**3**, and **4** absorb within

the coveted biological window of light making them promising candidates for applications in cells and tissues. Studies are currently underway to show the bio-compatibility of a BODIPY dye within S2 cells.

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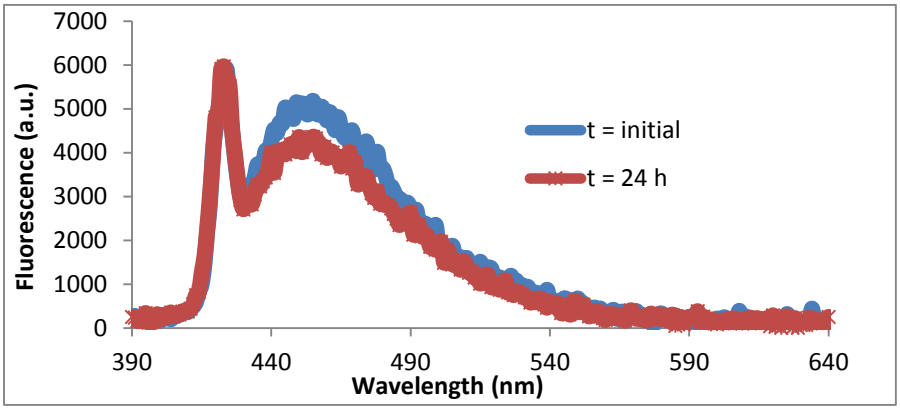
GENERAL CONCLUSIONS FOR PART 2

BODIPY-derived photocages unmask carboxylic acids with green light excitation >500 nm and photocleavage can be carried out in living cells. These photocages are promising alternatives for the popular *o*-nitrobenzyl photocaging systems, being easy to synthesize, utilizing a biocompatible chromophore, and having superior optical properties to the most popular photocages in current use. More generally, our strategy of identifying new photocages by searching for carbocations with low-energy diradical states seems to be a promising one.

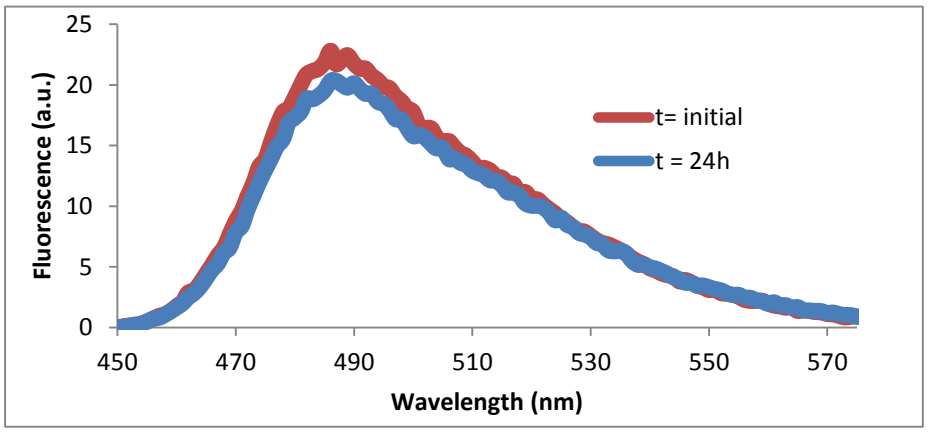
The BODIPY photocages' absorbances are red-shifted into the "biological window" using a Knoevenagal condensation reaction. These new photocages release acetic acid with red light excitiation >600 nm making them very promising for biological assays. The red-shifted photocages were found to be thermally stable and have high extinction coefficients. Studies are currently underway to show the bio-compatibility of a BODIPY dye within S2 cells and the release of different cargo bio-molecules.

APPENDIX I: SUPPLEMENTAL INFORMATION CHAPTER 1

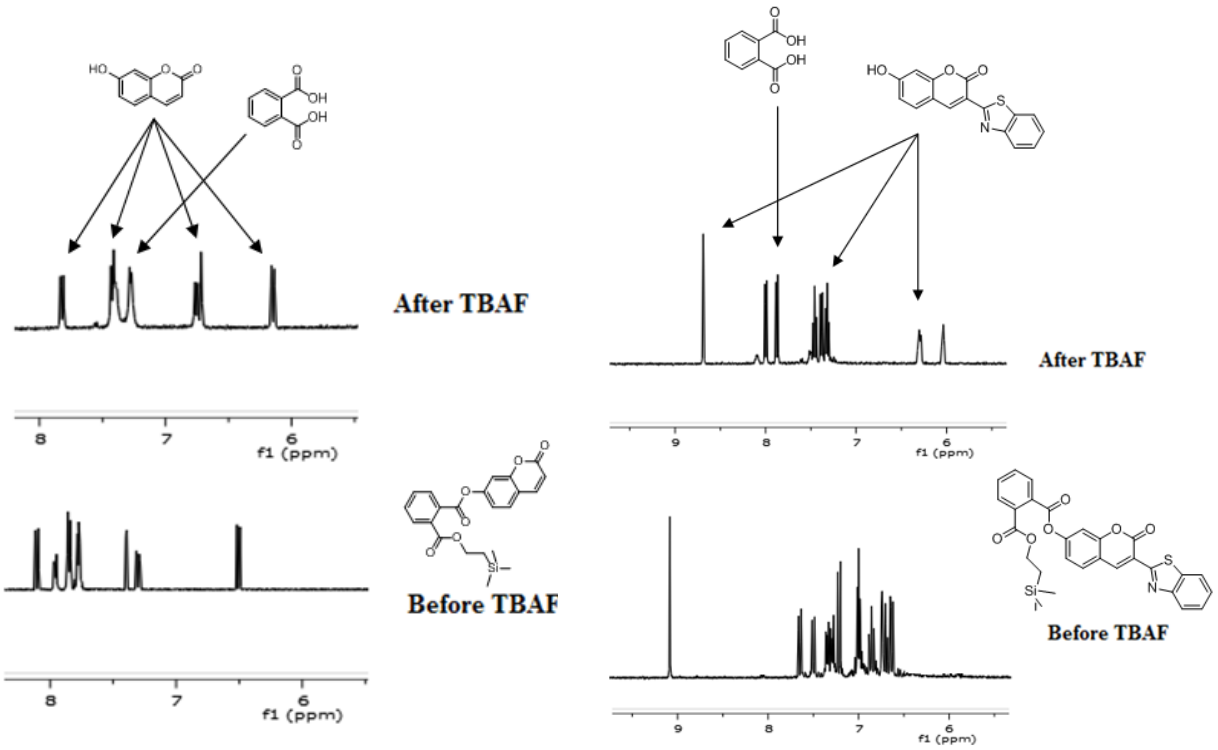
Stability Tests Compound **2** and **3**



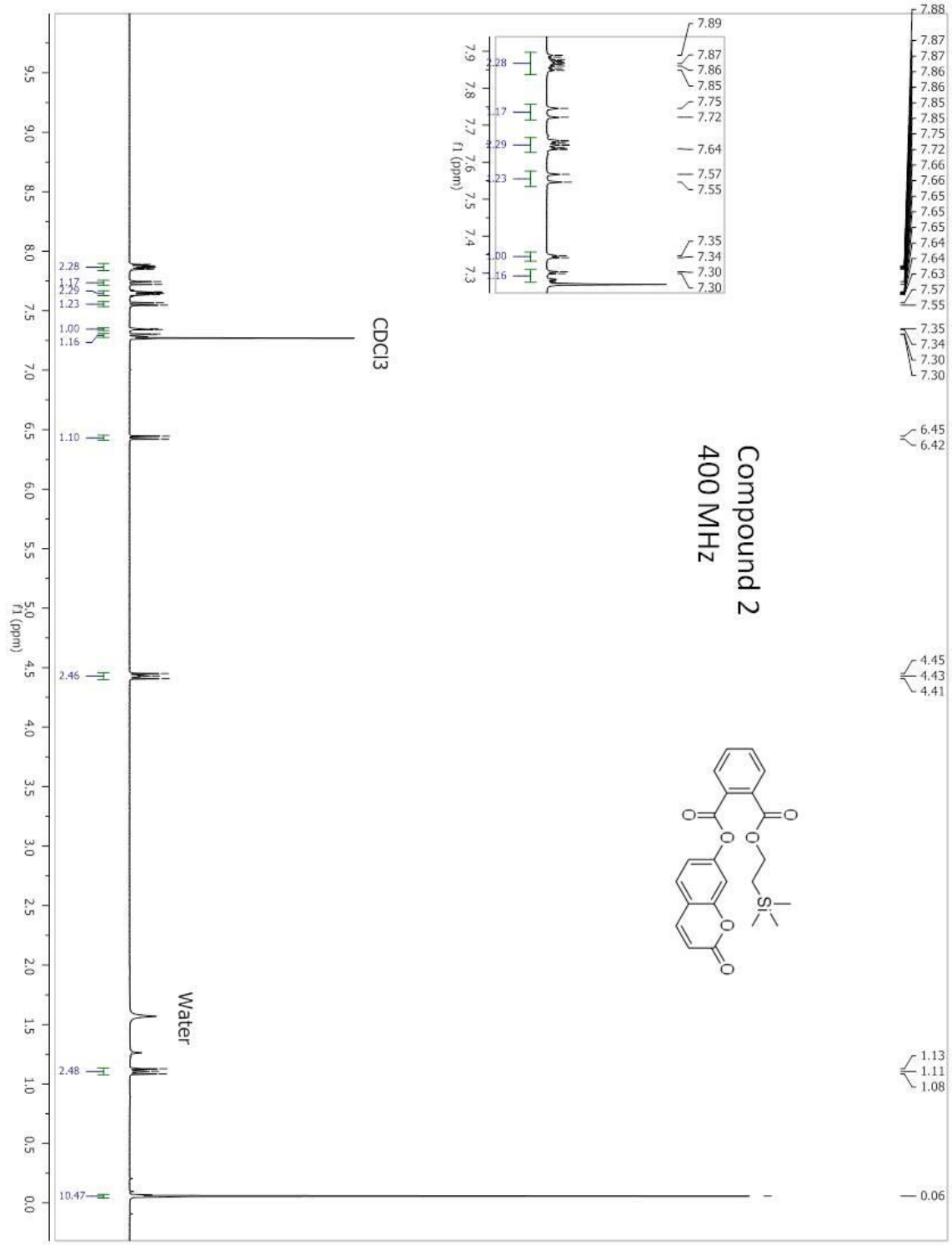
Stability study of compound **2** (top) in water in the absence of a fluoride source. The fluorescence brightness of **2** is so small that the water Raman band is seen. No significant change in fluorescence intensity was observed over a 24 hour period. Similar results for compound **3** (bottom).

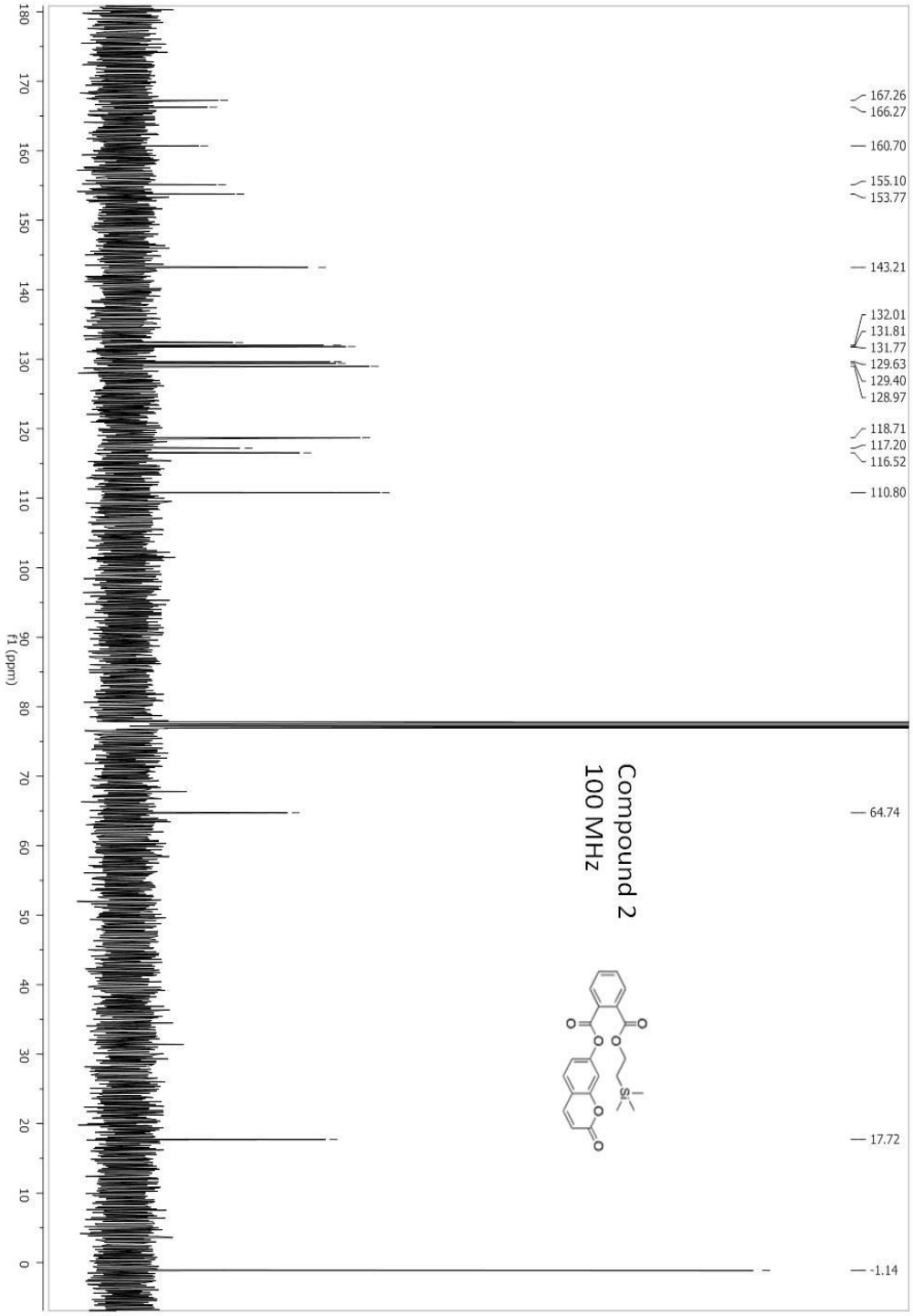


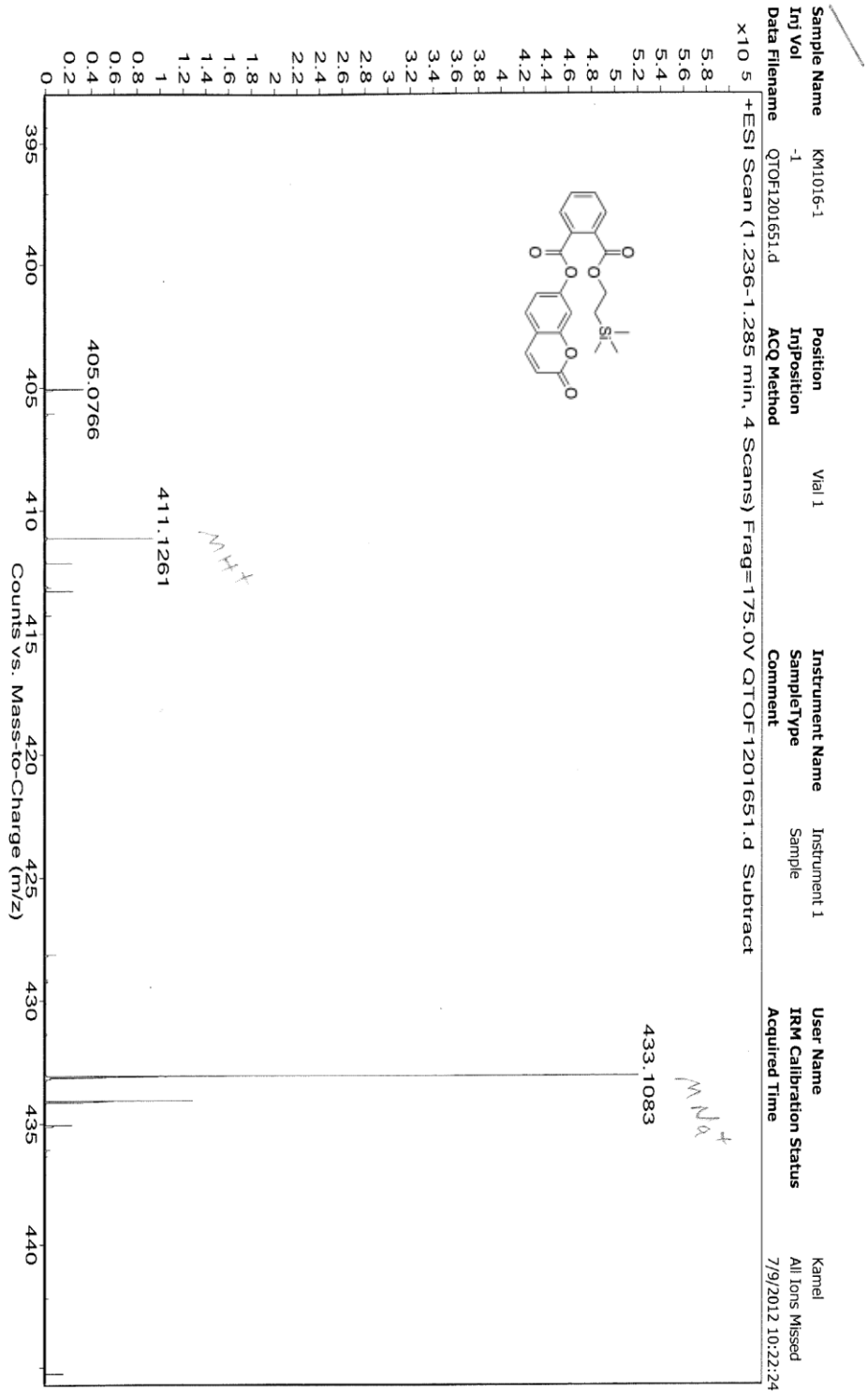
Product Studies Compound 2 and 3



Product analysis of **2** and **3** after addition of fluoride confirms formation of phthalic acid and the free dye. For **3**, the free dye was confirmed by spiking the NMR sample with 3-(2-benzothiazolyl)-7-hydroxycoumarin and observing the increase in the peaks labeled as free dye.





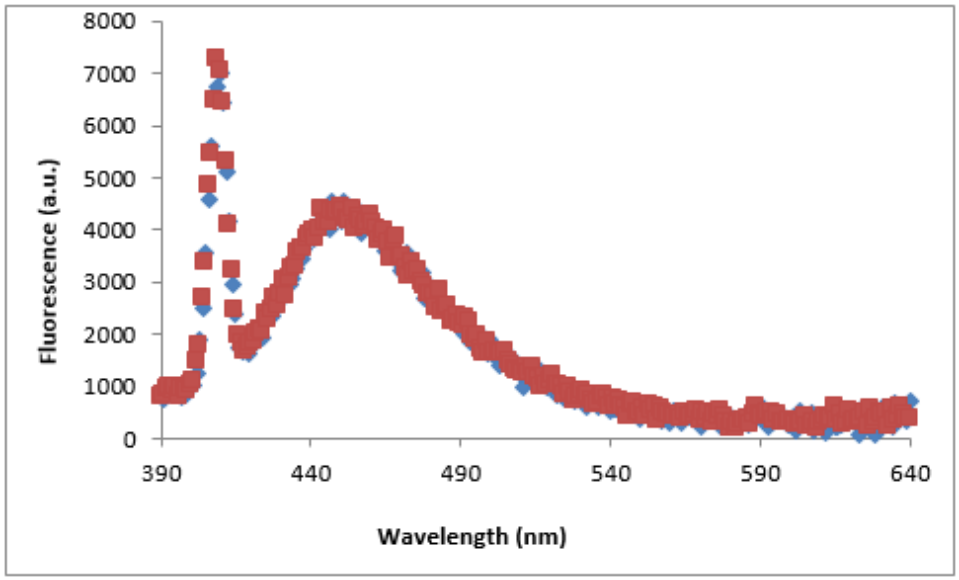


Appendix II: SUPPLEMENTAL INFORMATION CHAPTER 2

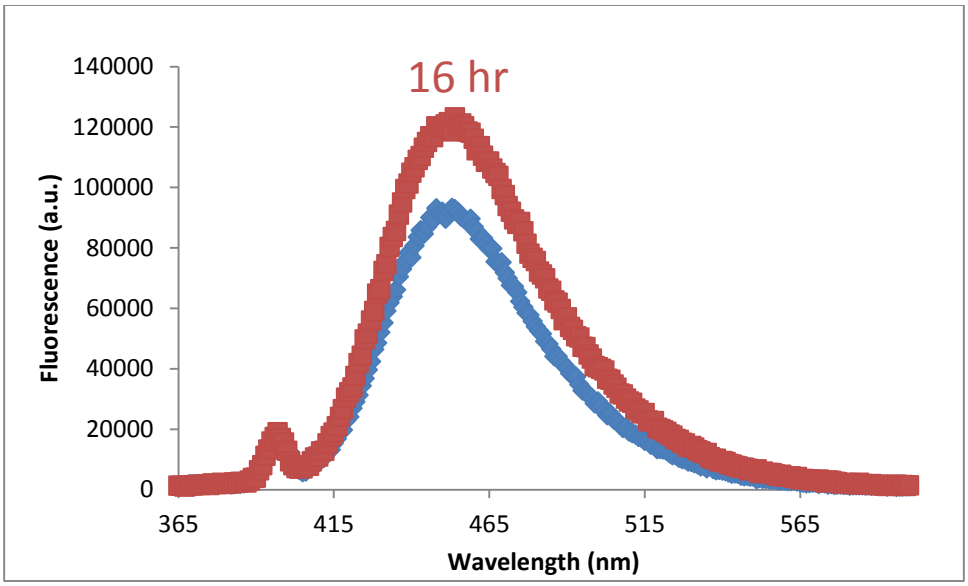
Fluorescence Titration Procedure

A stock solution (6.27 x 10^{-6} M) of 7-hydroxycoumarinyl 2-(nitrobenzyl) hydrogen phthalate **1** was prepared in DMF. These samples were irradiated with varying lengths of time. The samples were then diluted with 1 mM phosphate buffered (pH = 7.0) water to 3.0 mL. Excitation was carried out at 350 nm with all excitation and emission slit widths at 2 nm. The titration was repeated three times, and the data were averaged. The same experimental procedure was used in the titration of Compound **2** except it was titrated using 30% hydrogen peroxide aqueous solution.

Compound Stability Tests



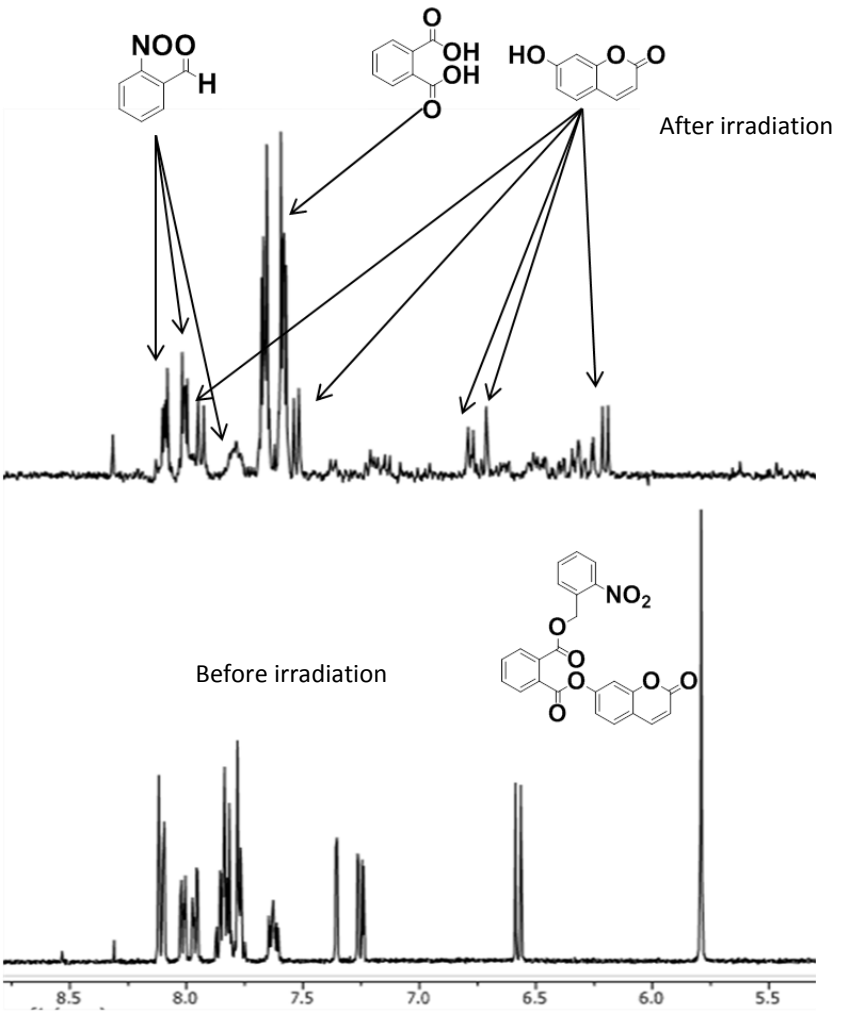
7-hydroxycoumarinyl 2-(nitrobenzyl) hydrogen (Compound **1**). Red line is initial scan and blue line is after 24 hours.



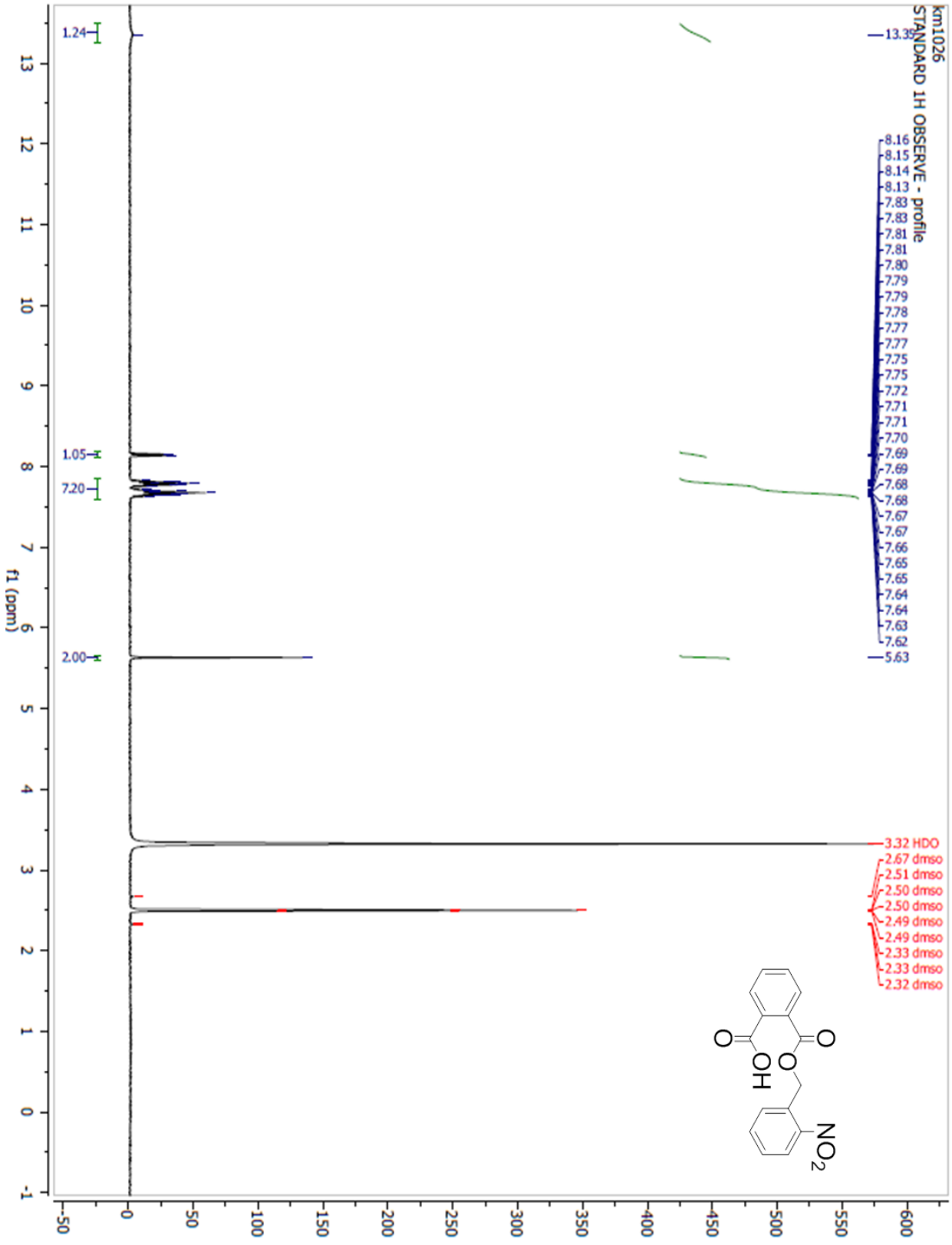
7-hydroxycoumarinyl 2-(4-hydroxymethyl) benzenebo-ronic phthalate (Compound **2**). Red line is the initial scan and blue line is after 16 hours.

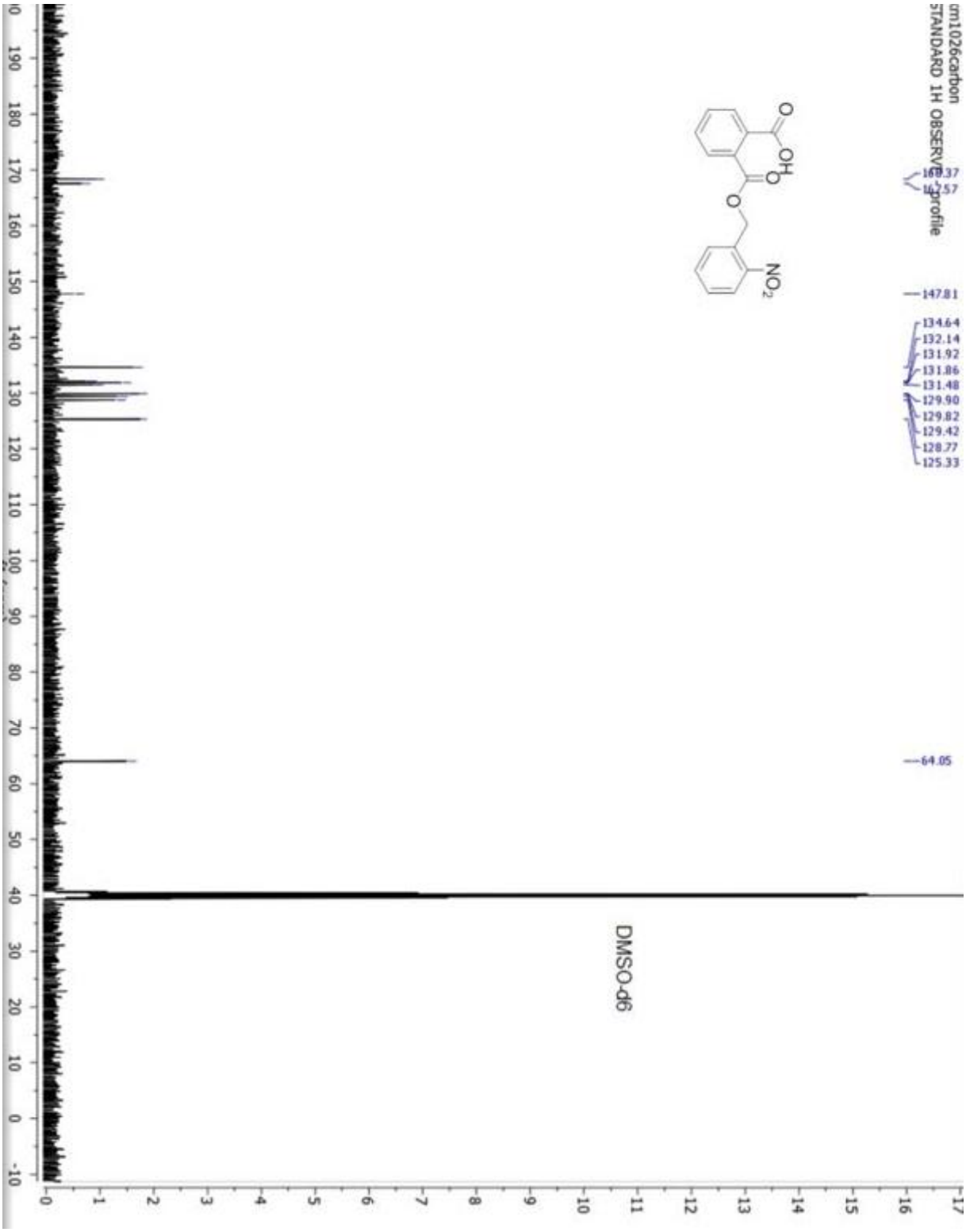
Stability study of 7-hydroxycoumarinyl 2-(nitrobenzyl) hydrogen phthalate (top) and 7-hydroxycoumarinyl 2-(4-hydroxymethyl)benzeneboronic phthalate (bottom) in water in the absence of light and hydrogen peroxide. The fluorescence intensity is so small that the water Raman band is seen at 397 nm. No significant change in fluorescence intensity was observed over a 24 hour period for Compound 1. A small increase in fluorescence was seen in Compound 2 after a 16 hour period (red is initial scan and blue is after 16 hours), but insignificant compared to the increase in fluorescence intensity due to free dye.

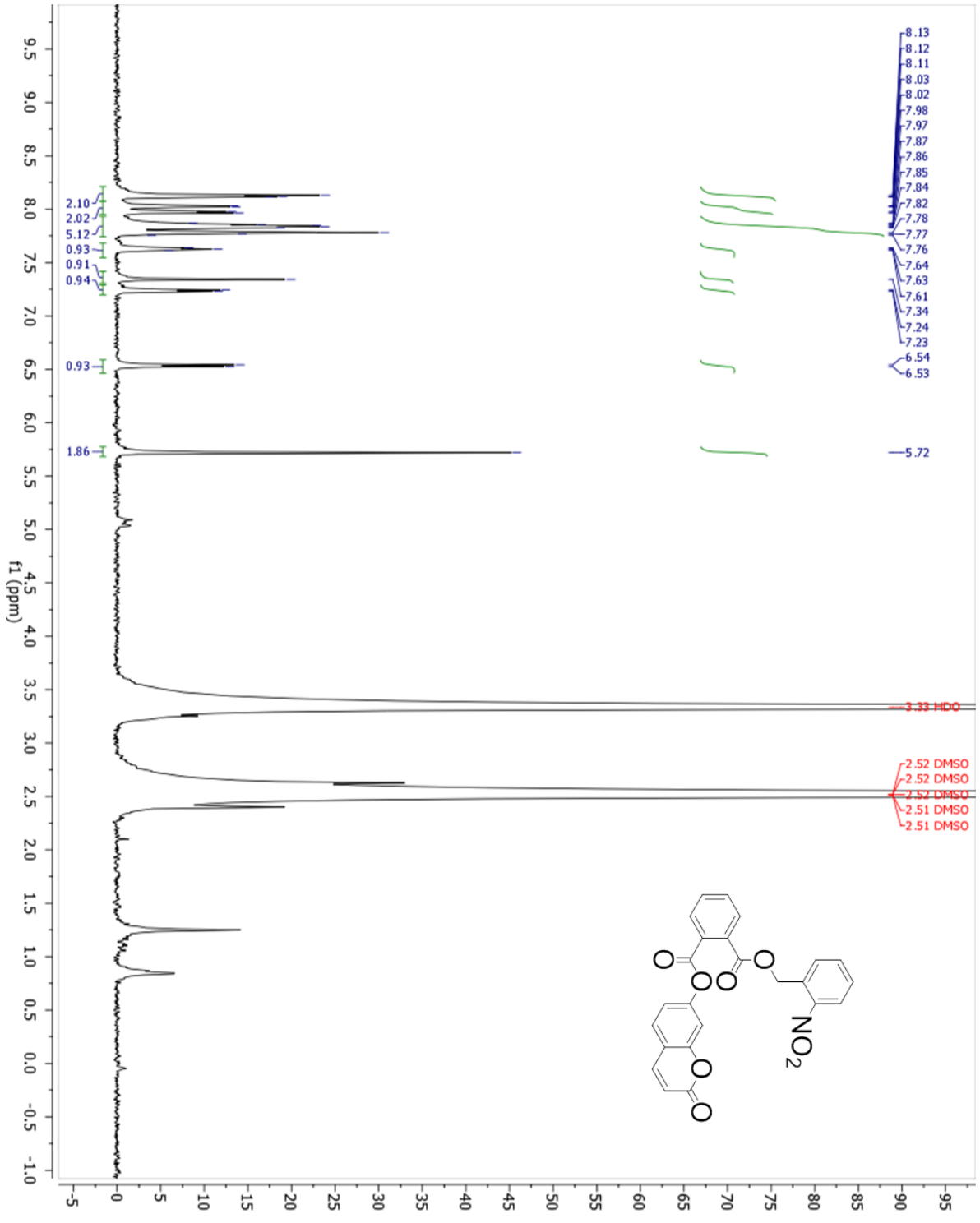
Product Study Compound 1

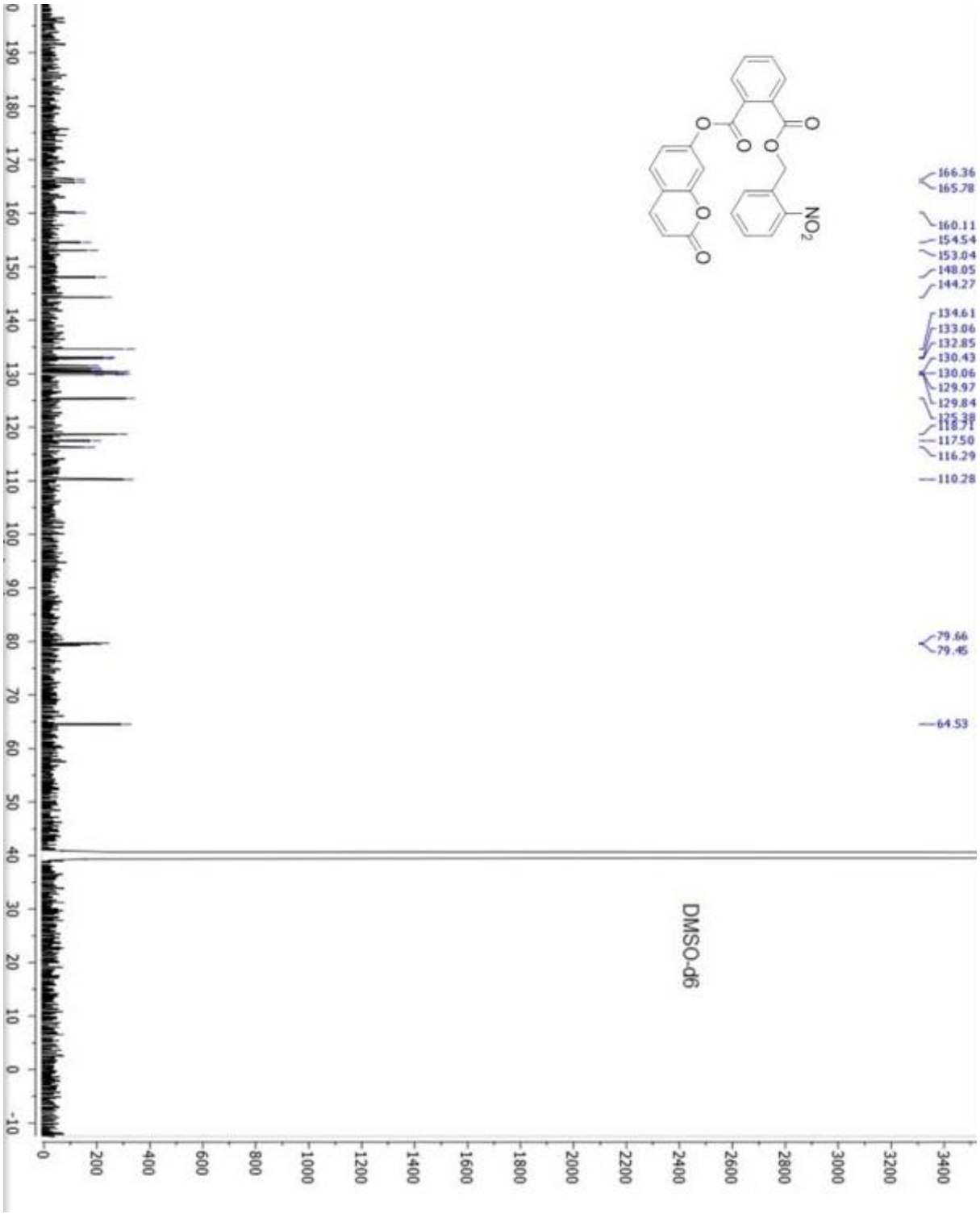


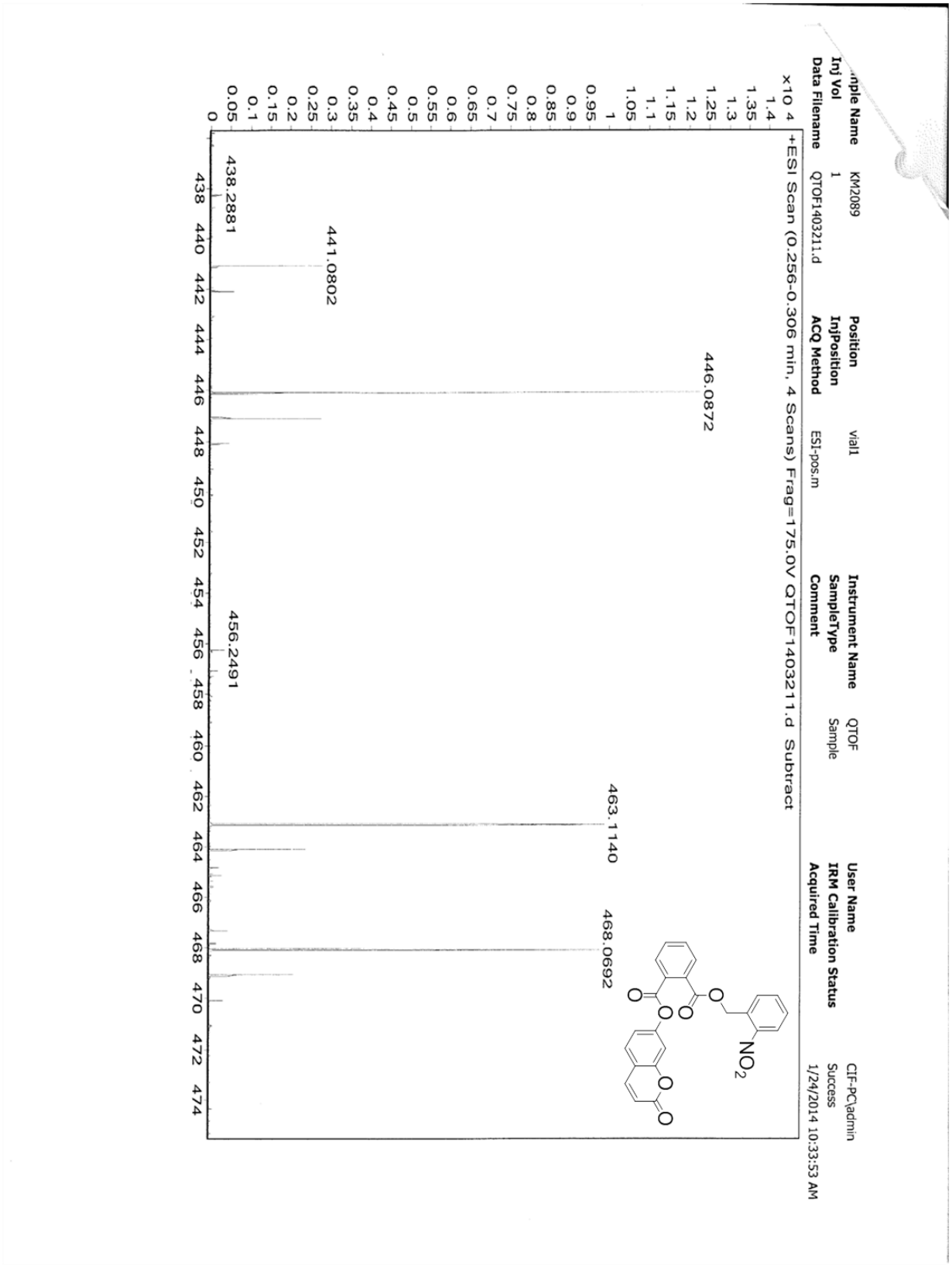
Product analysis of Compound 1 after exposure to light confirms formation of phthalic acid and the free dye.





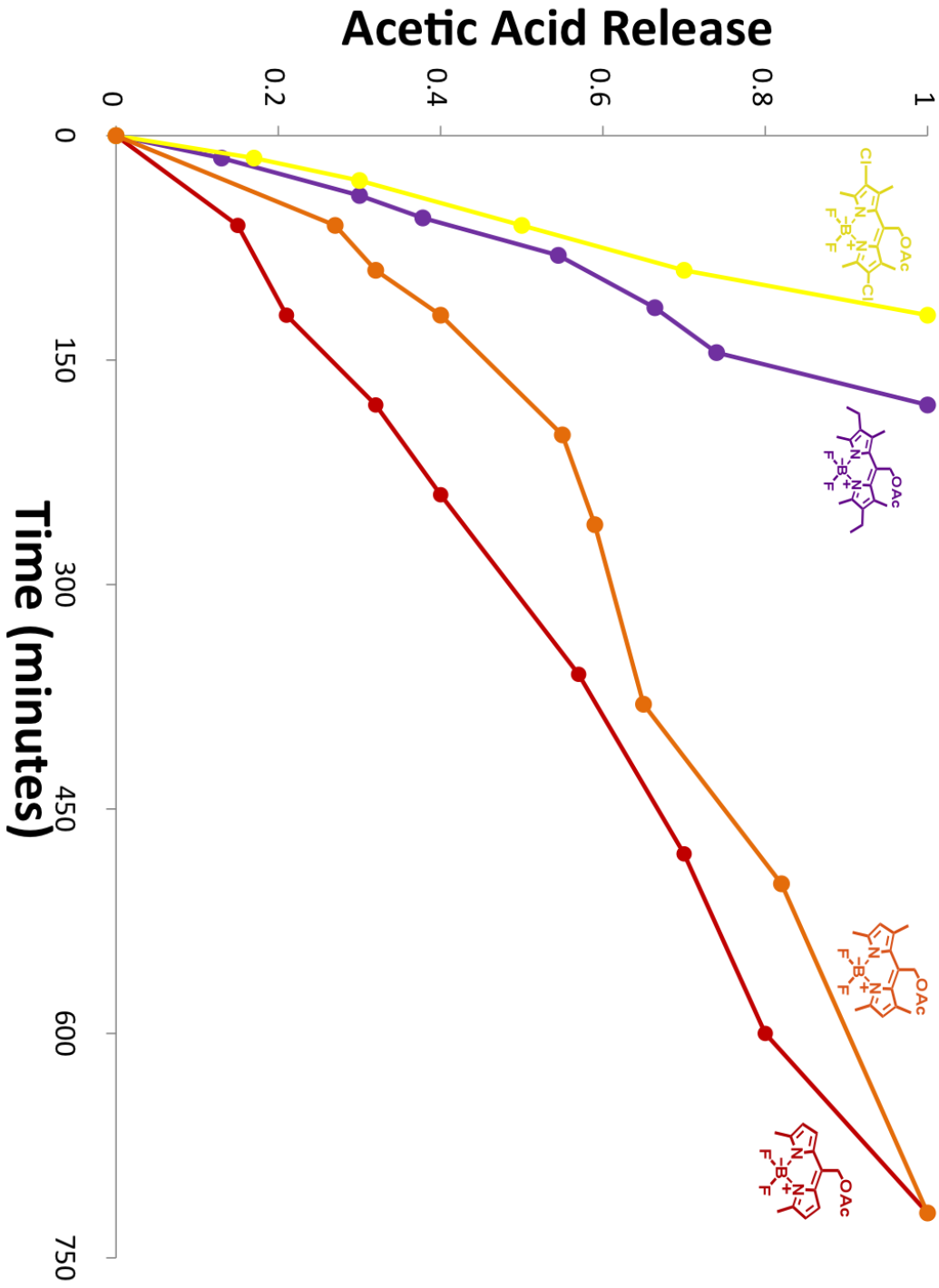




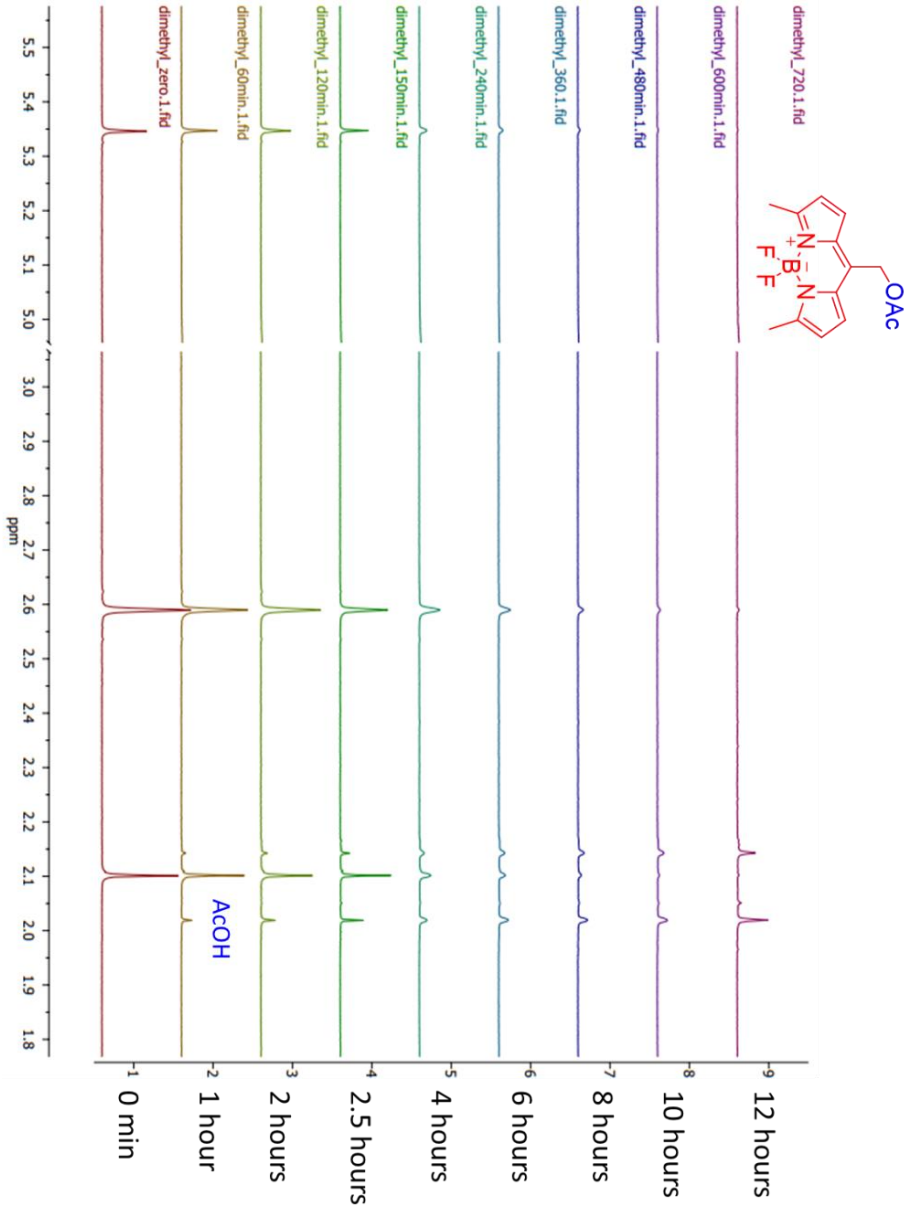


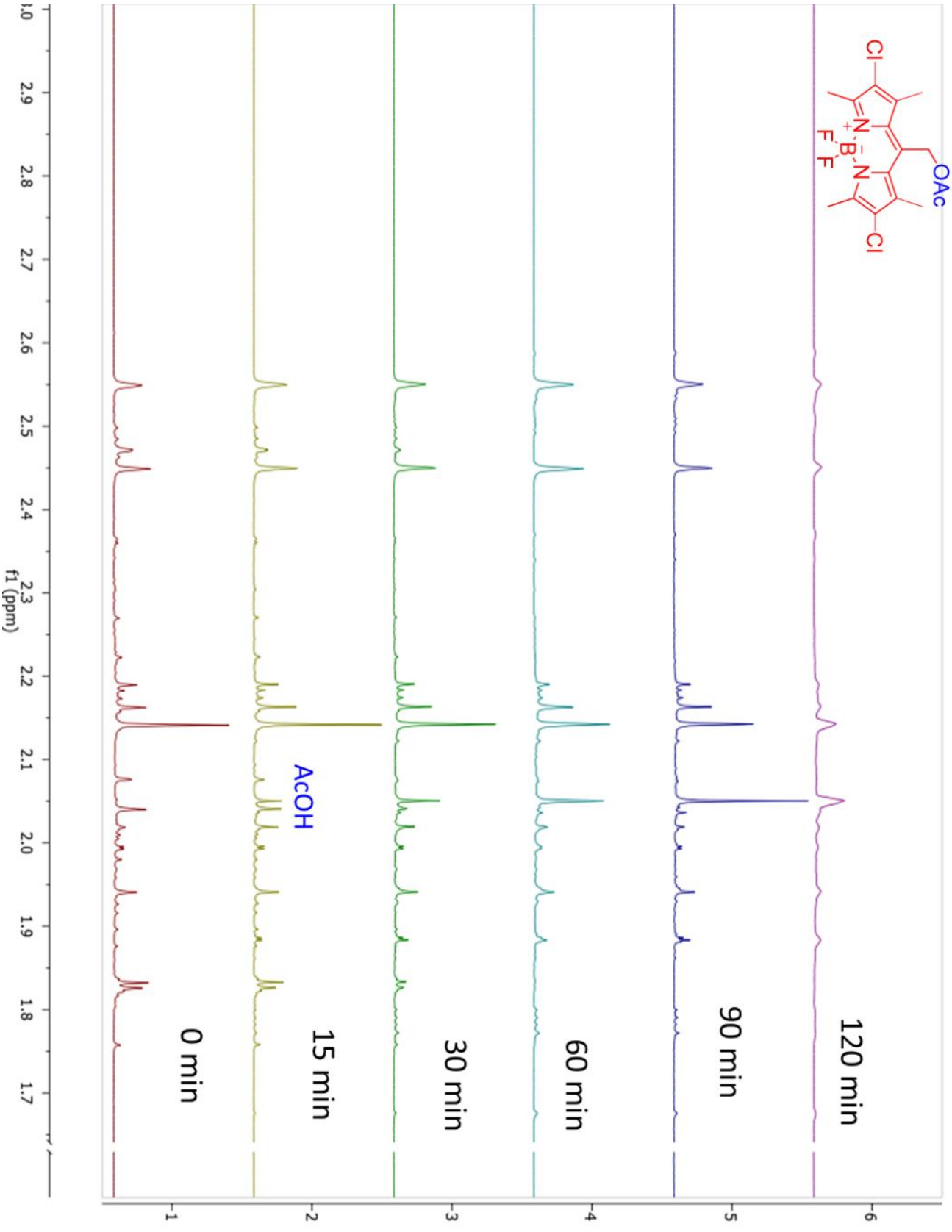
APPENDIX III: SUPPLEMENTAL INFORMATION CHAPTER 3

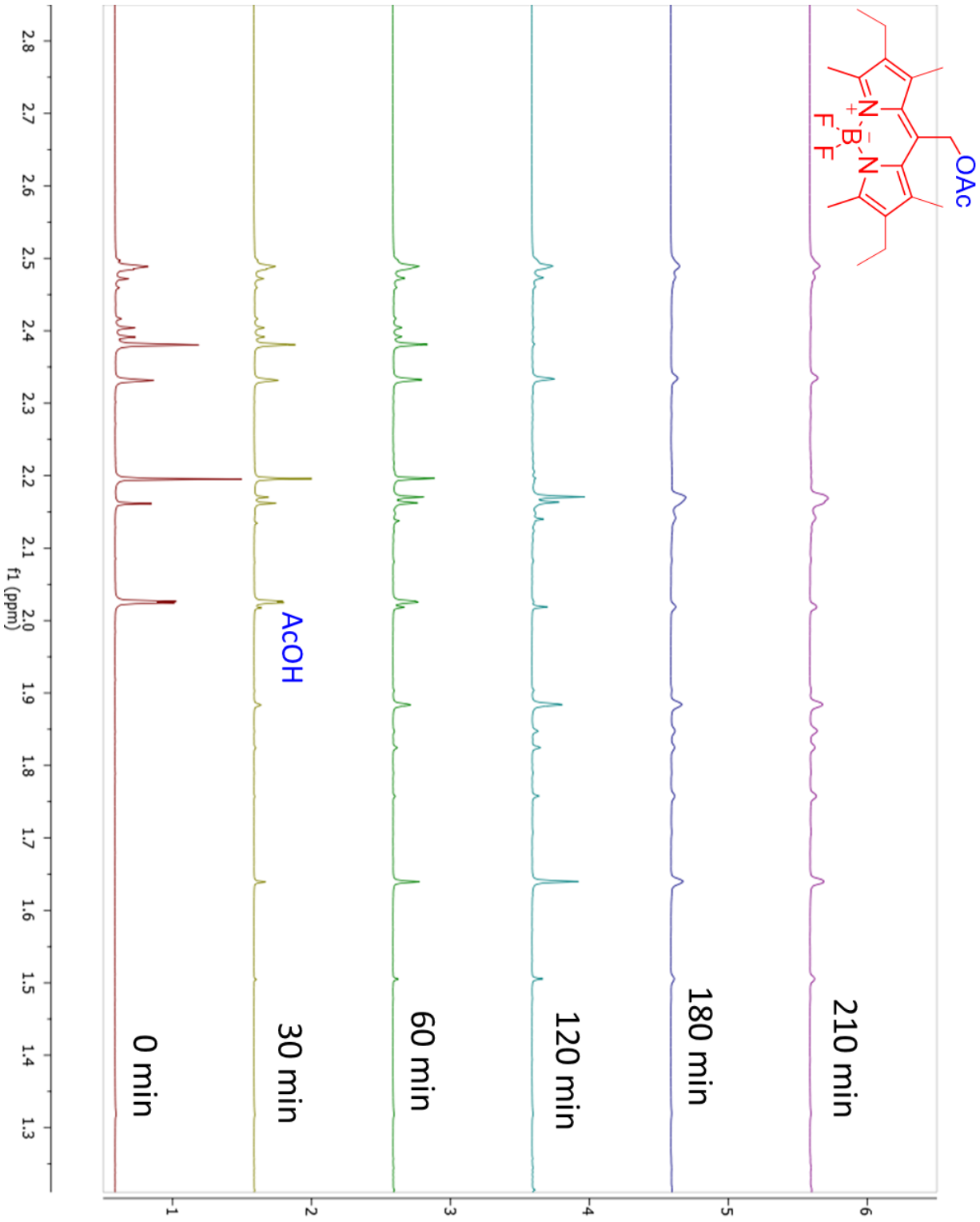
Acetic acid growth over time followed by NMR

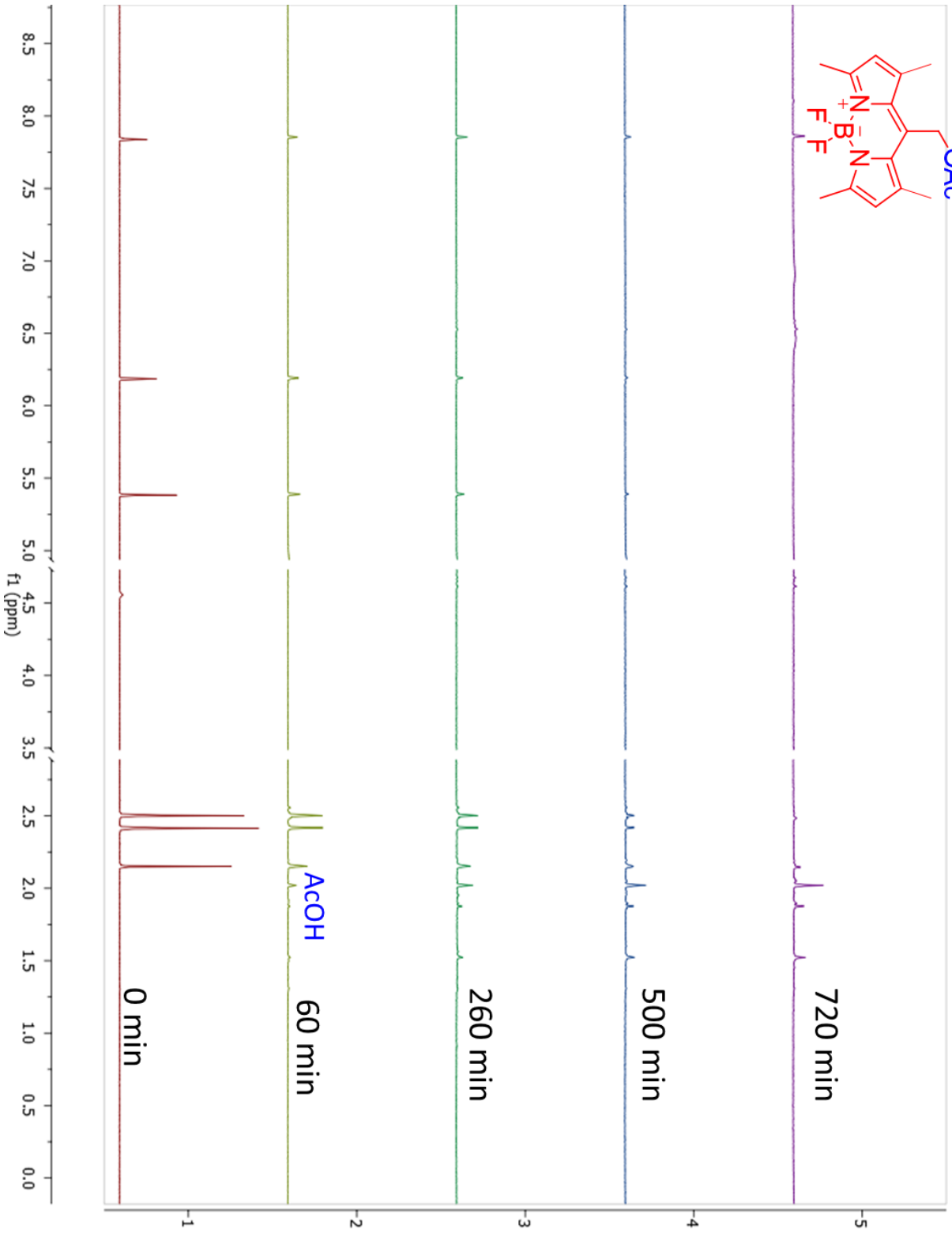


The BODIPY compound was dissolved in minimum amount of $CDCl_3$ to dissolve and then MeOD was added to it to make a 600 µl of 2 mM solution. A halogen lamp (500W) with a water IR cutoff filter was used to irradiate the sample and it was followed by NMR over time. Acetic acid release was plotted by relative integration ratio of caged to free acetic acid.



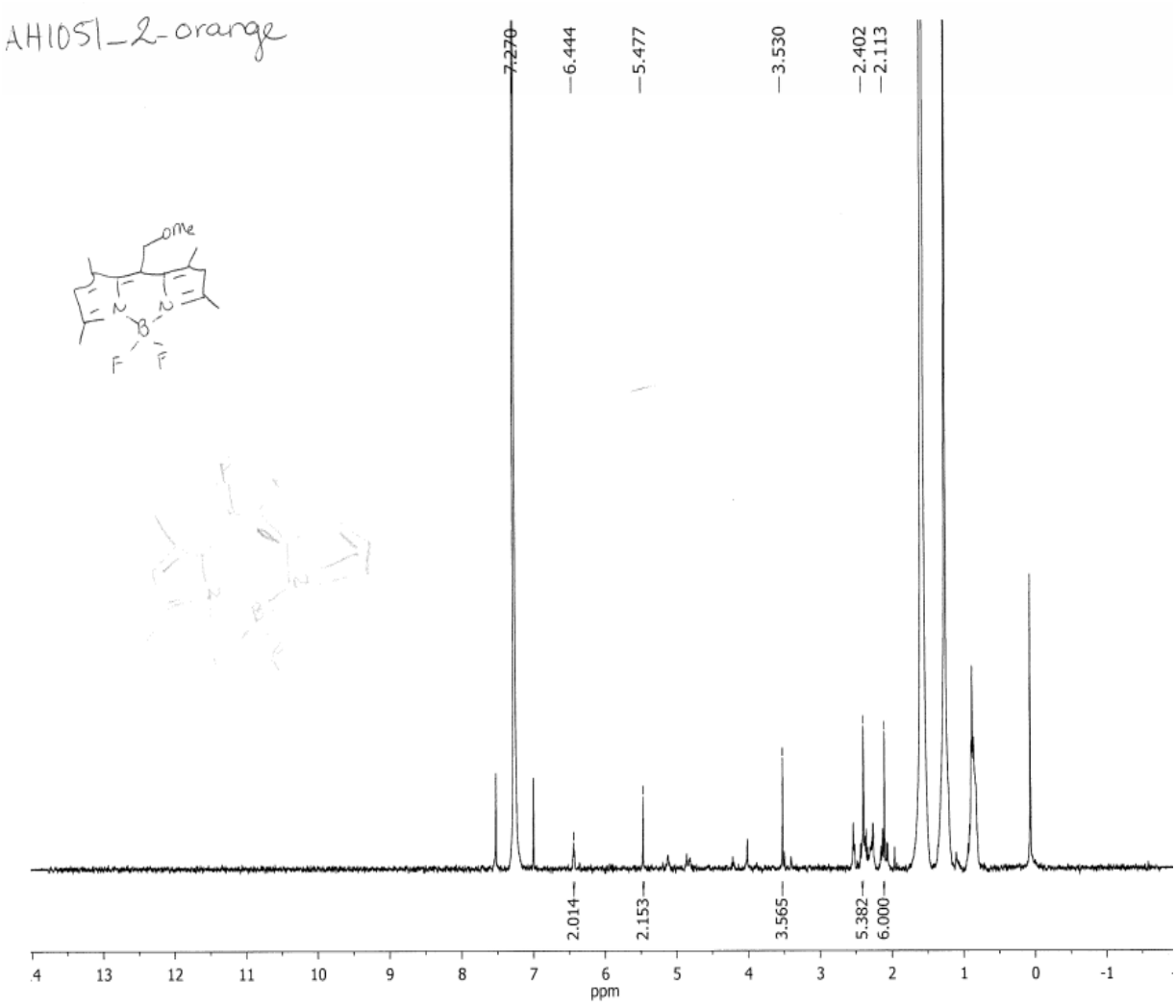


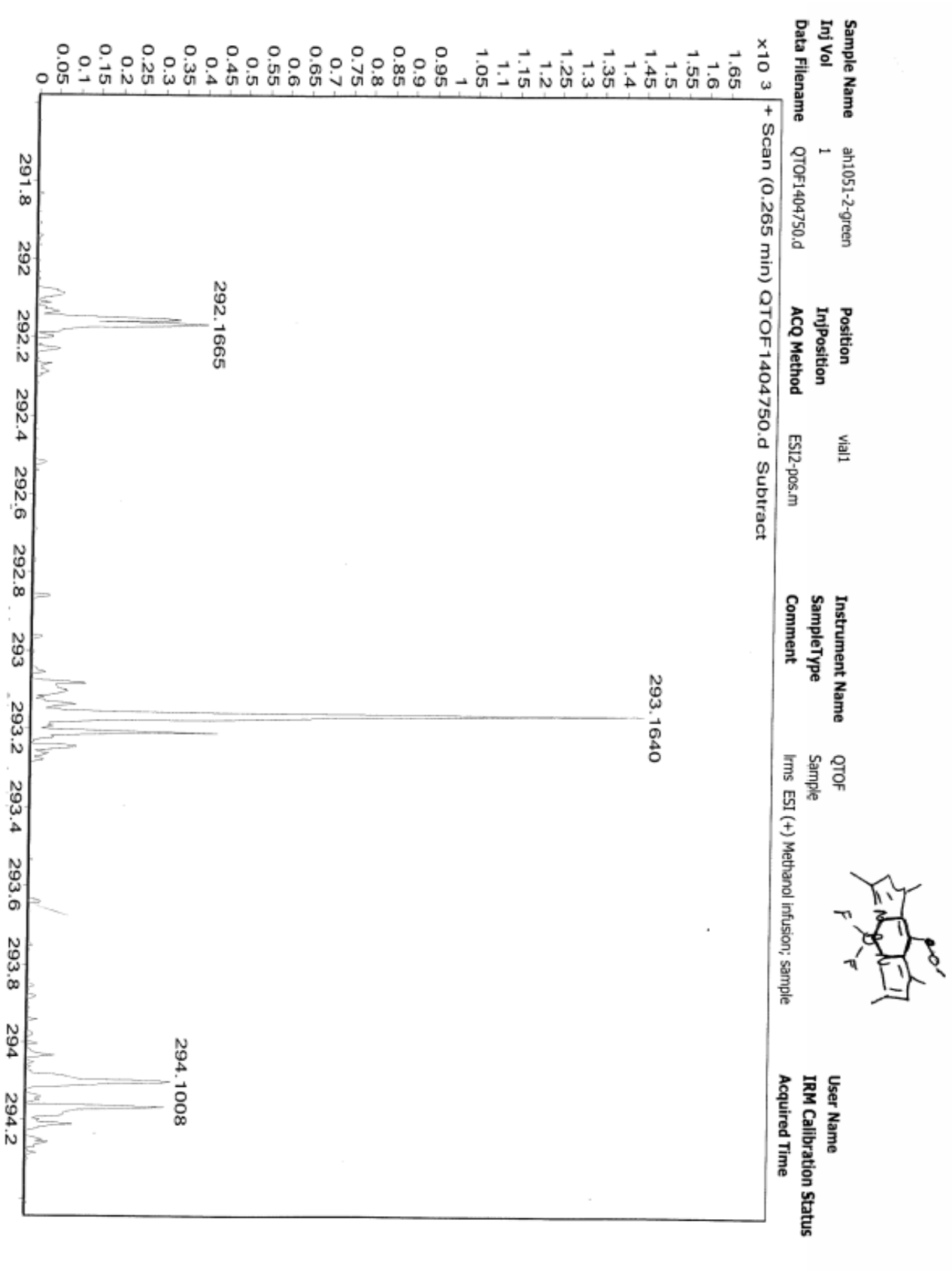




Preparative photolysis study of compound 2

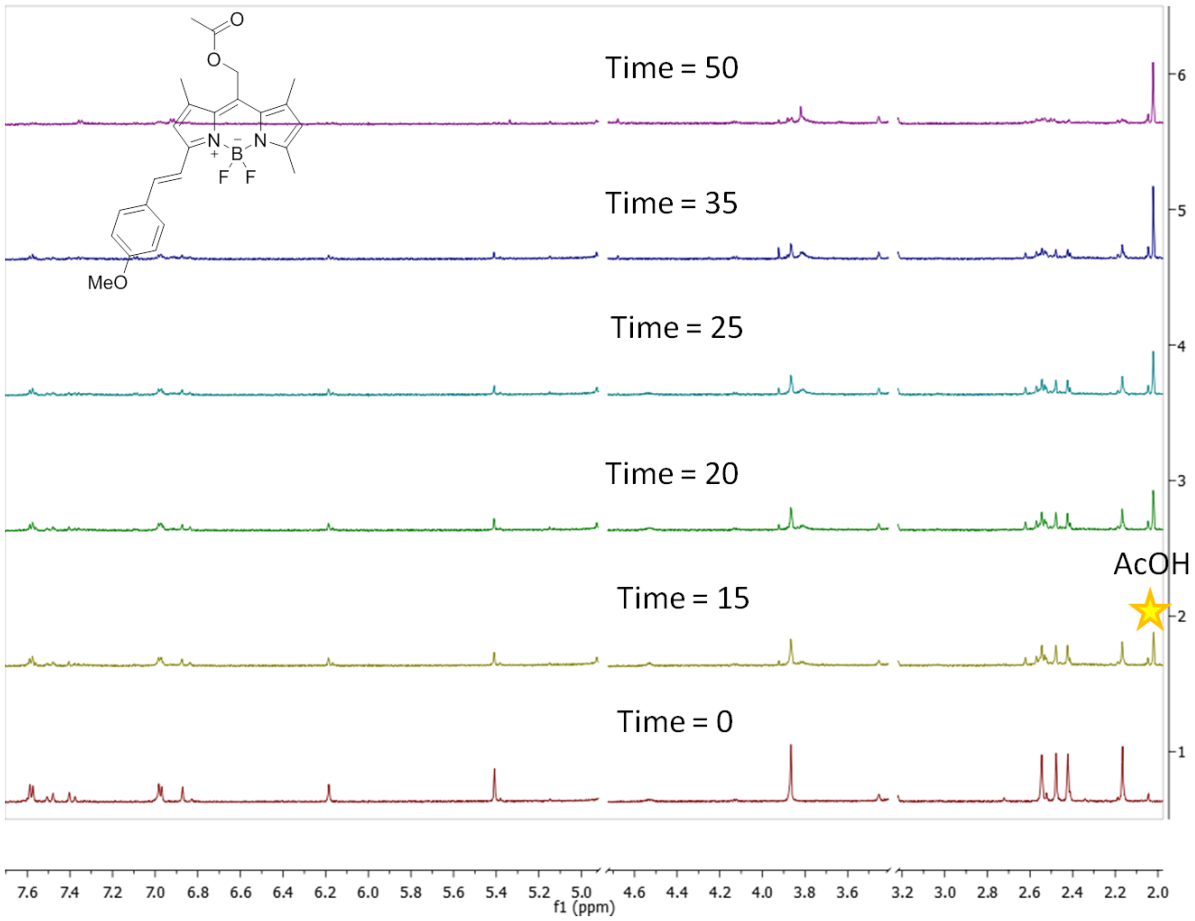
A 2 mM solution of compound 2 in a chloroform/methanol mixture was made and distributed evenly into multiple NMR test tubes and irradiated with a Xenon arc lamp. The solutions were combined and concentrated under vacuum and redissolved in $CDCl_3$. Photolysis progress was monitored by NMR until all starting compound 2 was gone. ^1H NMR and mass spectrometry was used to confirm the formation of methyl ether adduct as the photolysis product.

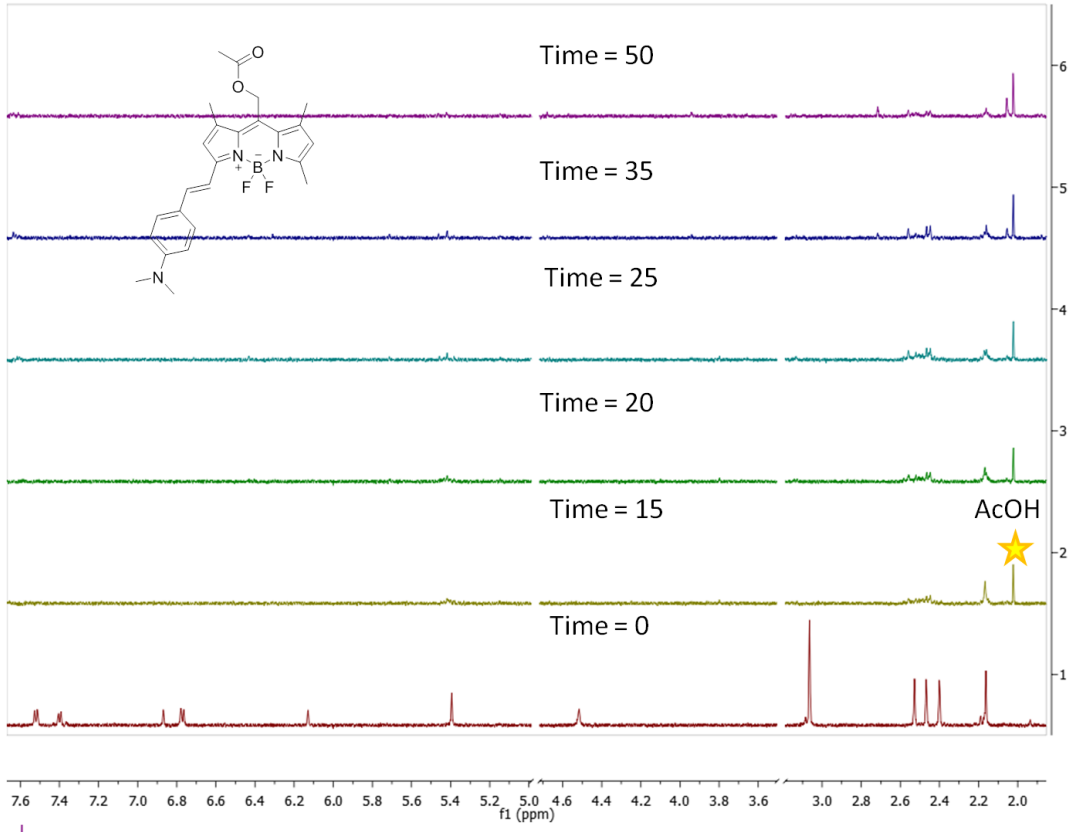


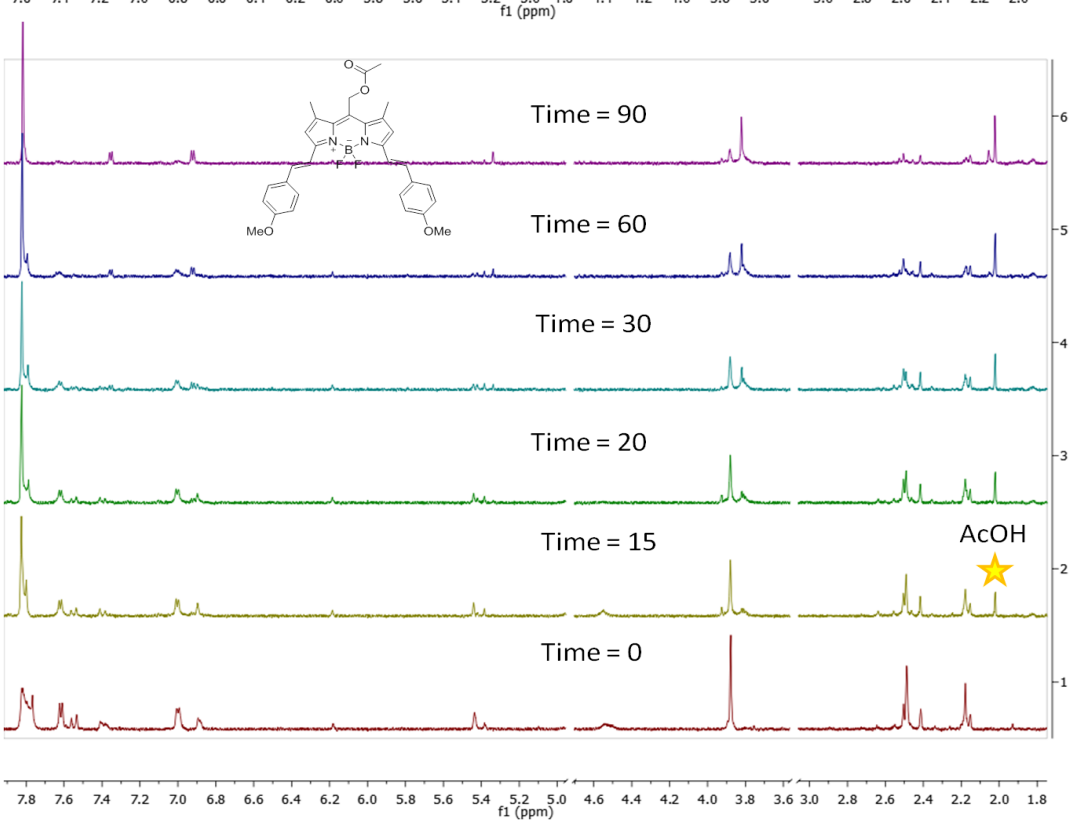


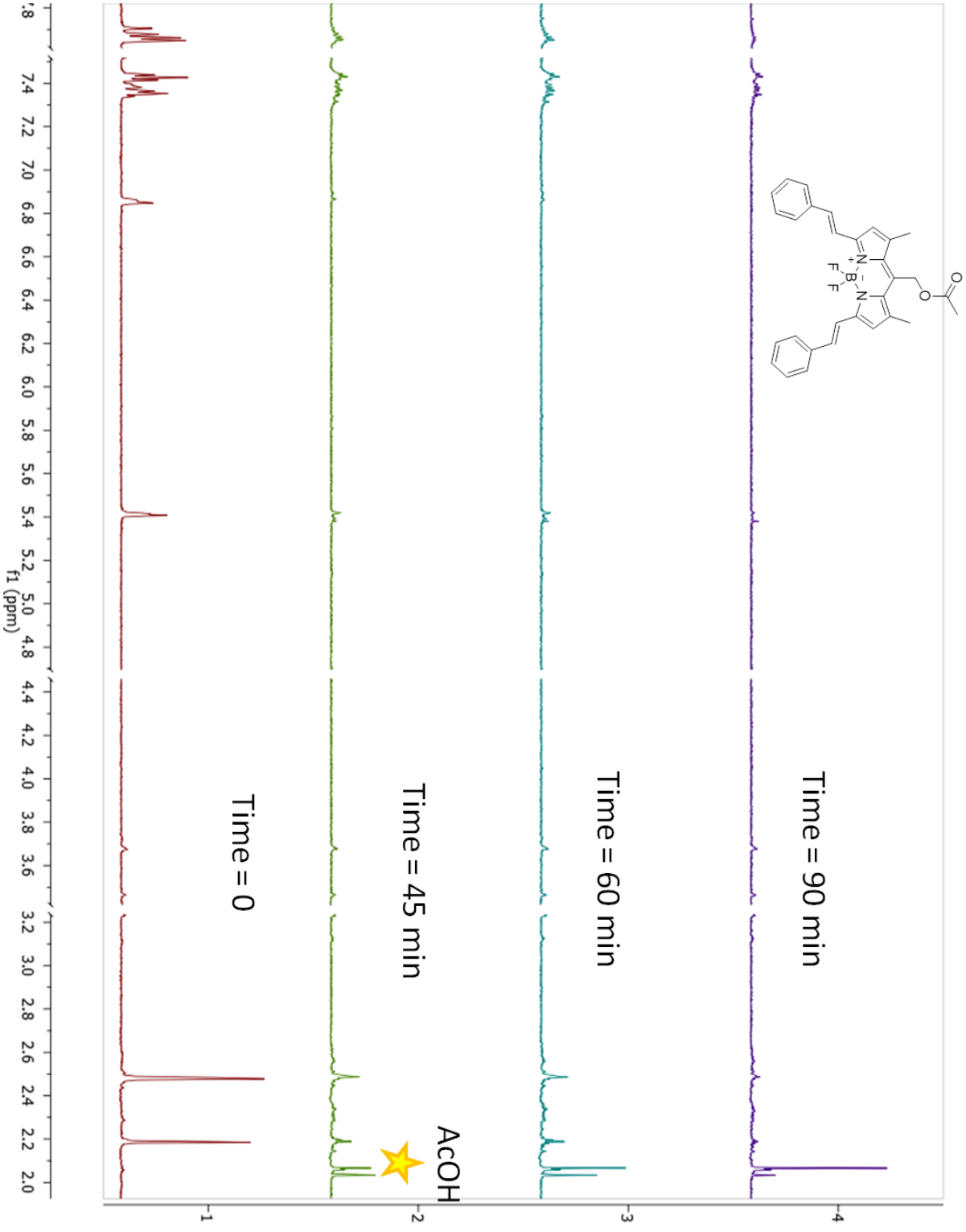
APPENDIX IV: SUPPLEMENTAL INFORMATION CHAPTER 4

The BODIPY compound was dissolved in minimum amount of CDCl3 to dissolve and then CD3OD was added to it to make a 600 µl of 2 mM solution. A Xenon arc lamp was used to irradiate the sample and it was followed by NMR over time.



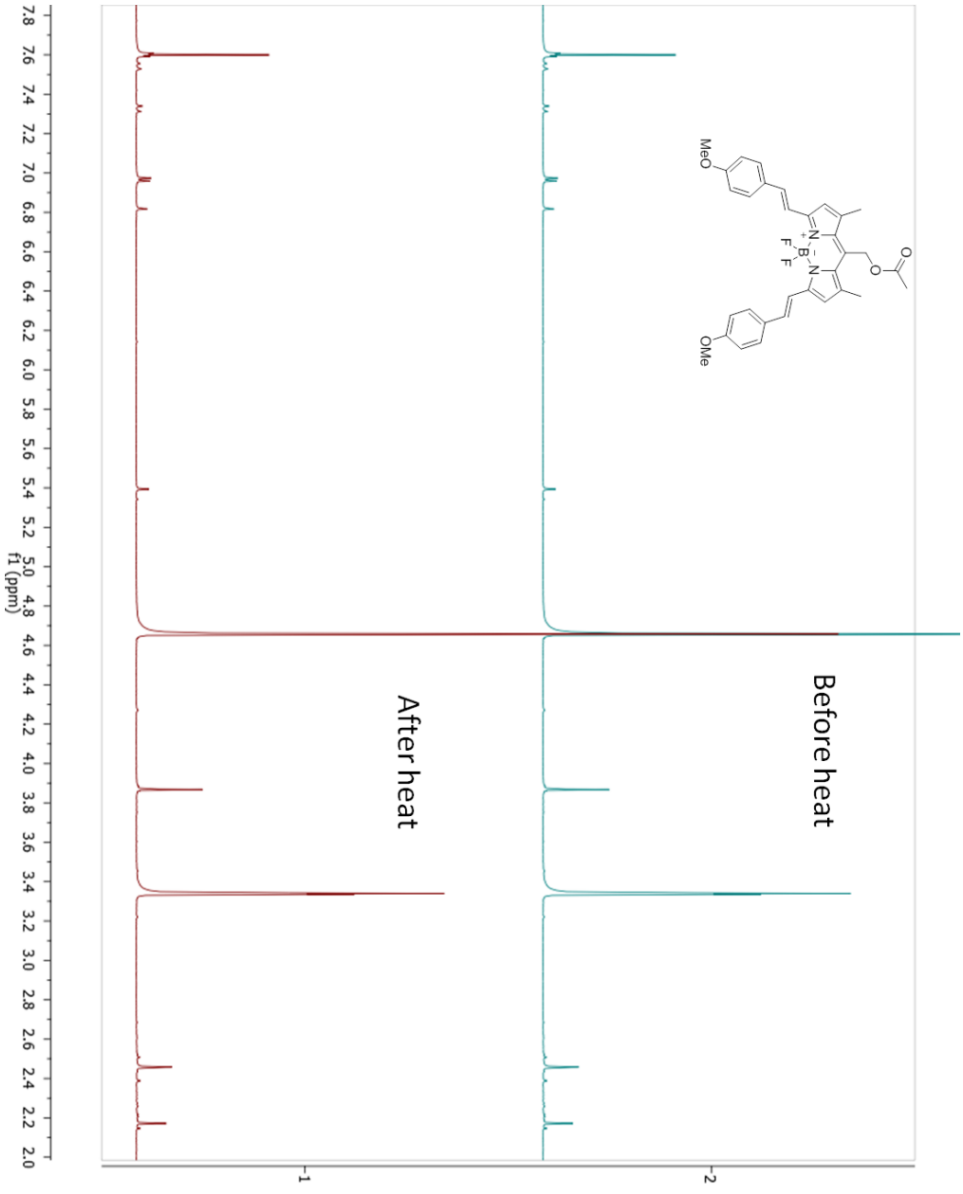


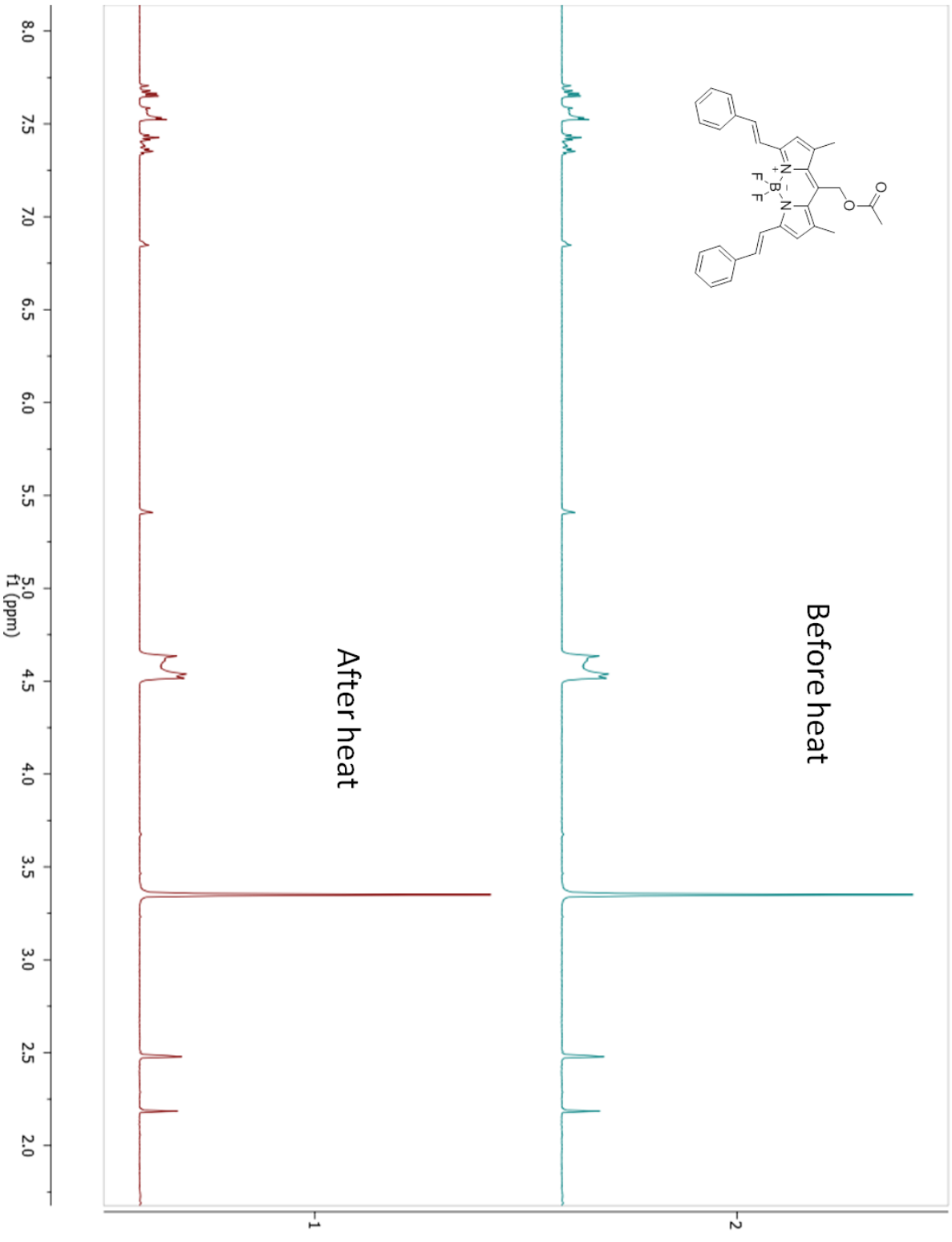


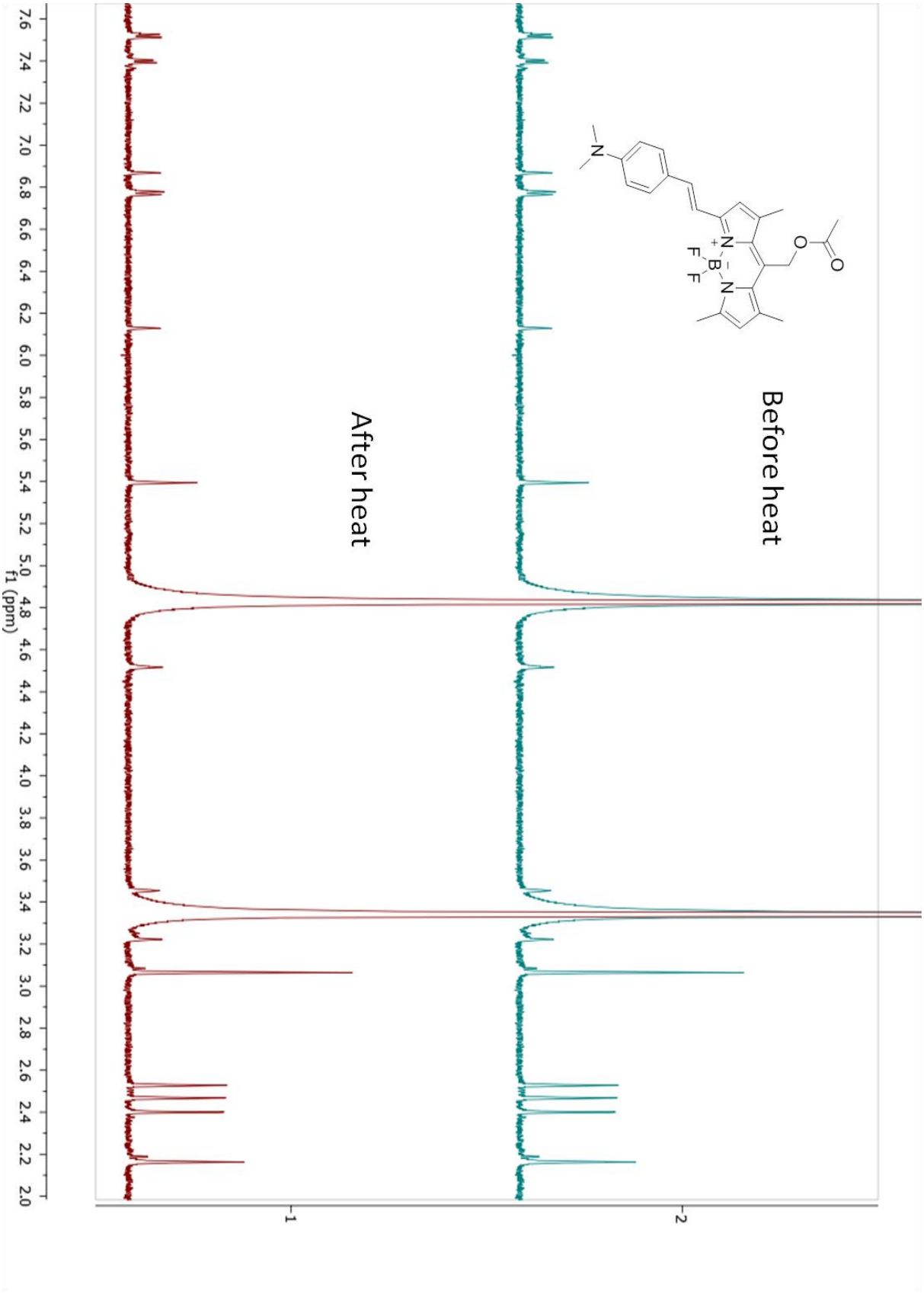


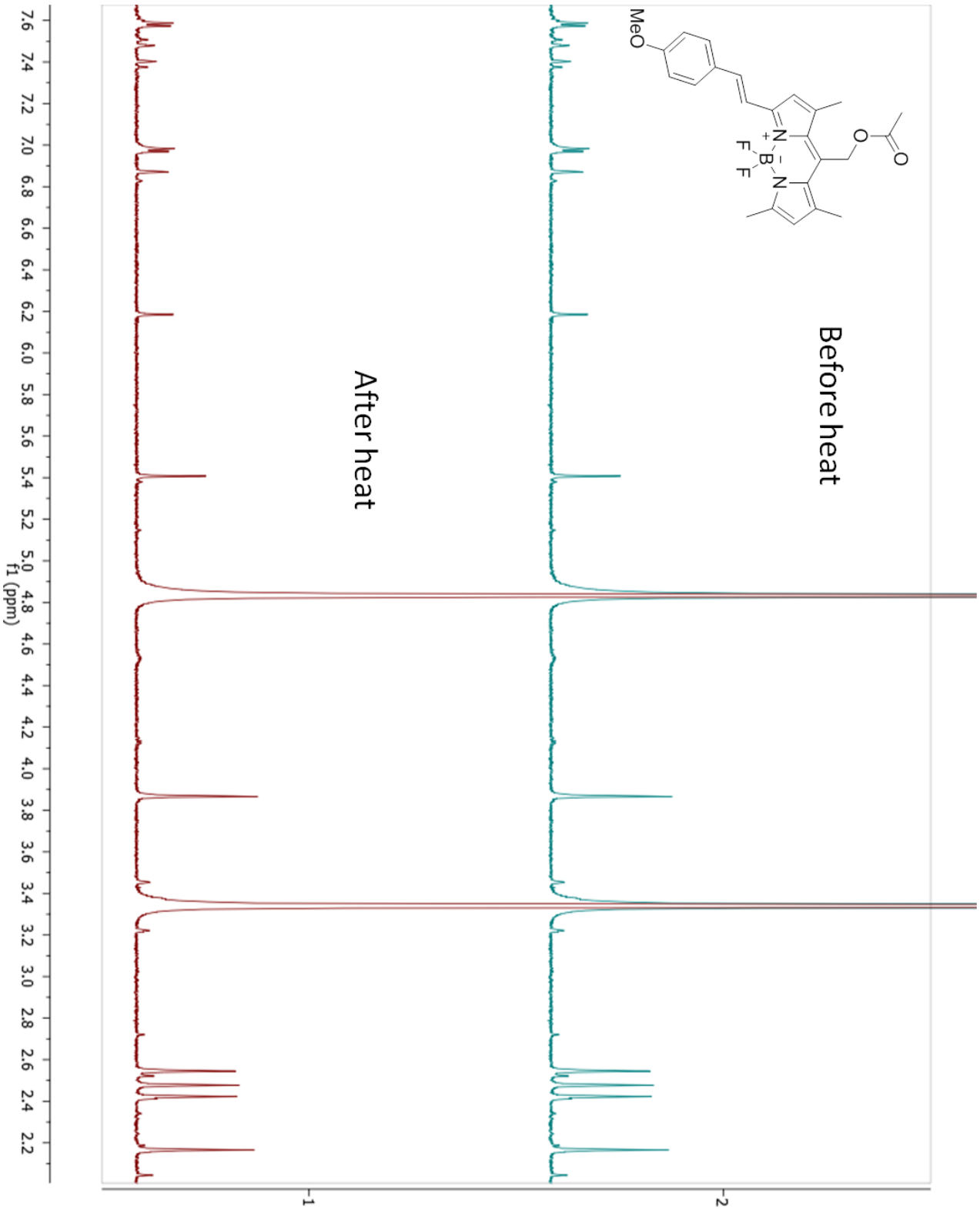
Stability Tests Compounds **1-4**

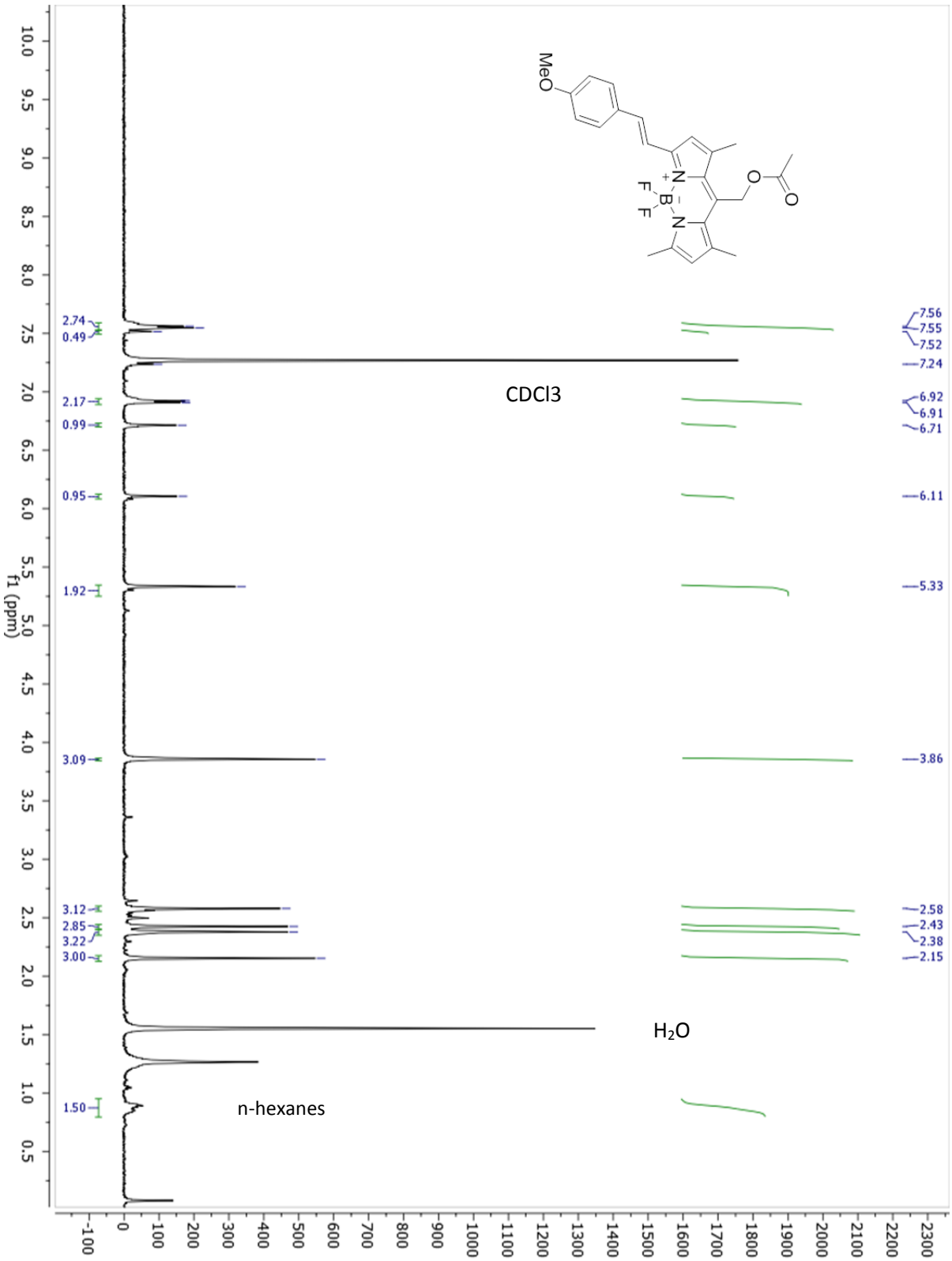
1 mg of all compounds were dissolved in 20 µL of CD_3OD and 600 µL of MeOD. 1 H NMR (600MHz) were recorded for these compounds at room temperature. They were then heated at 60 °C in the dark for 1 hour. 1 H NMR was then retaken to compare with the earlier NMRs.

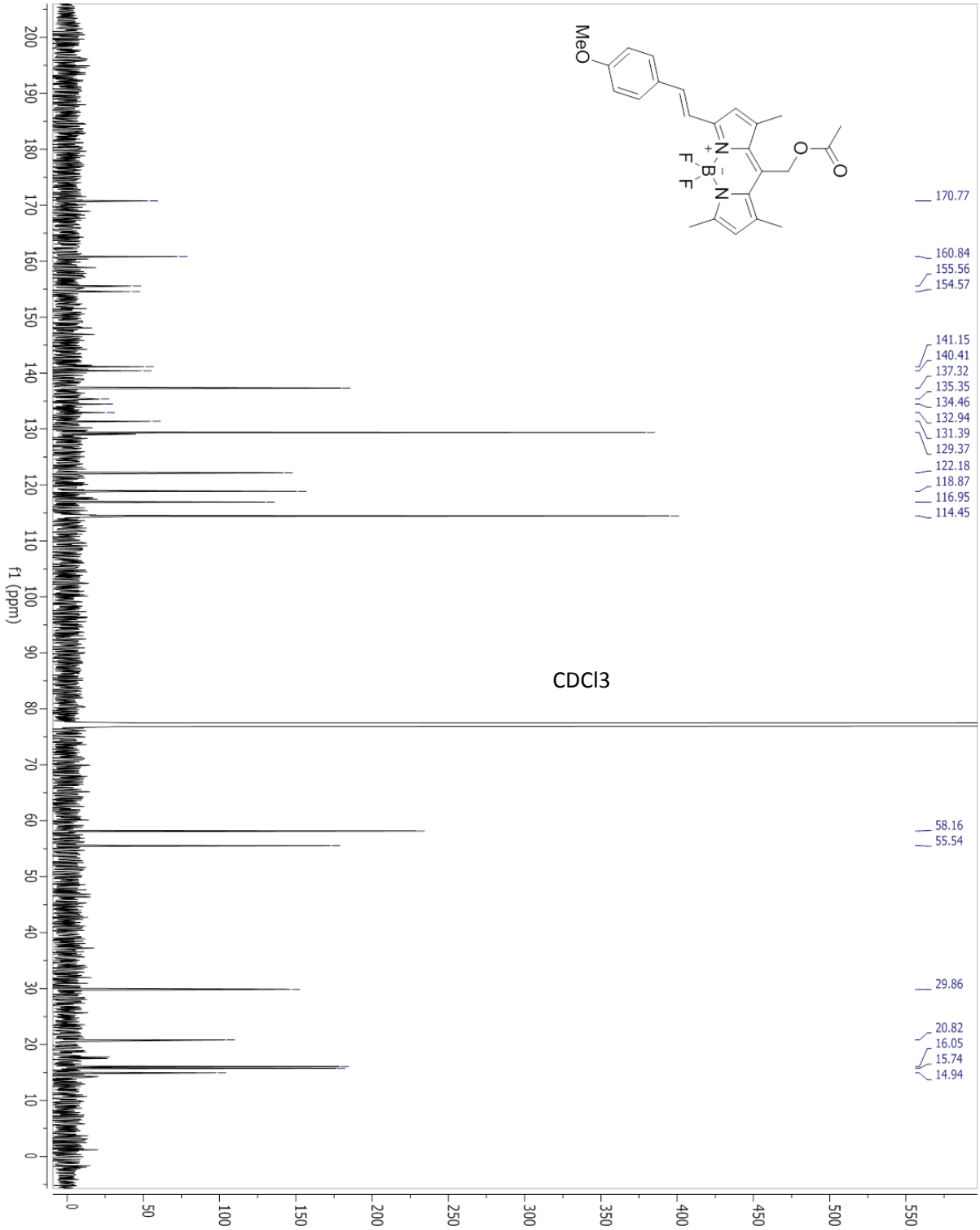












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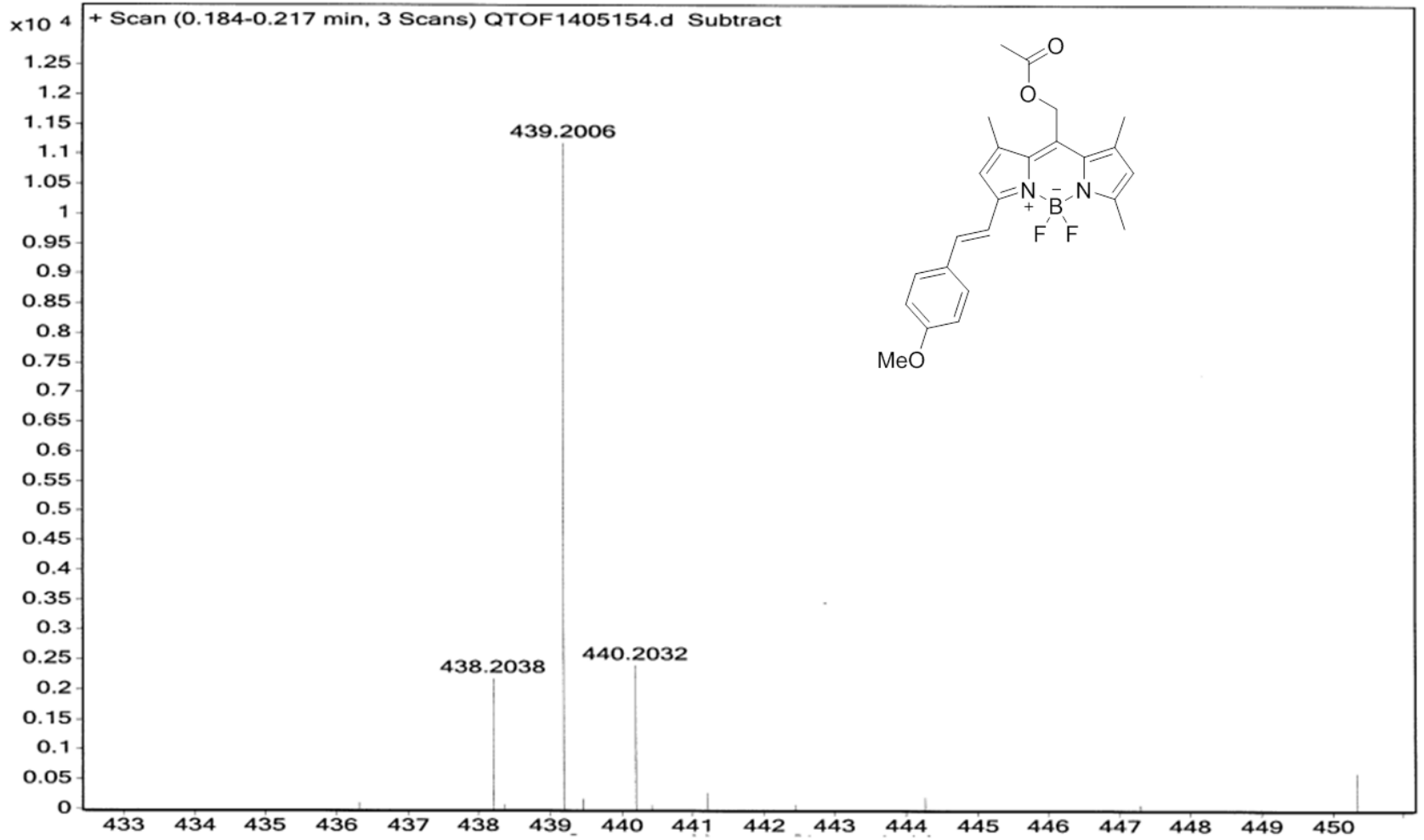

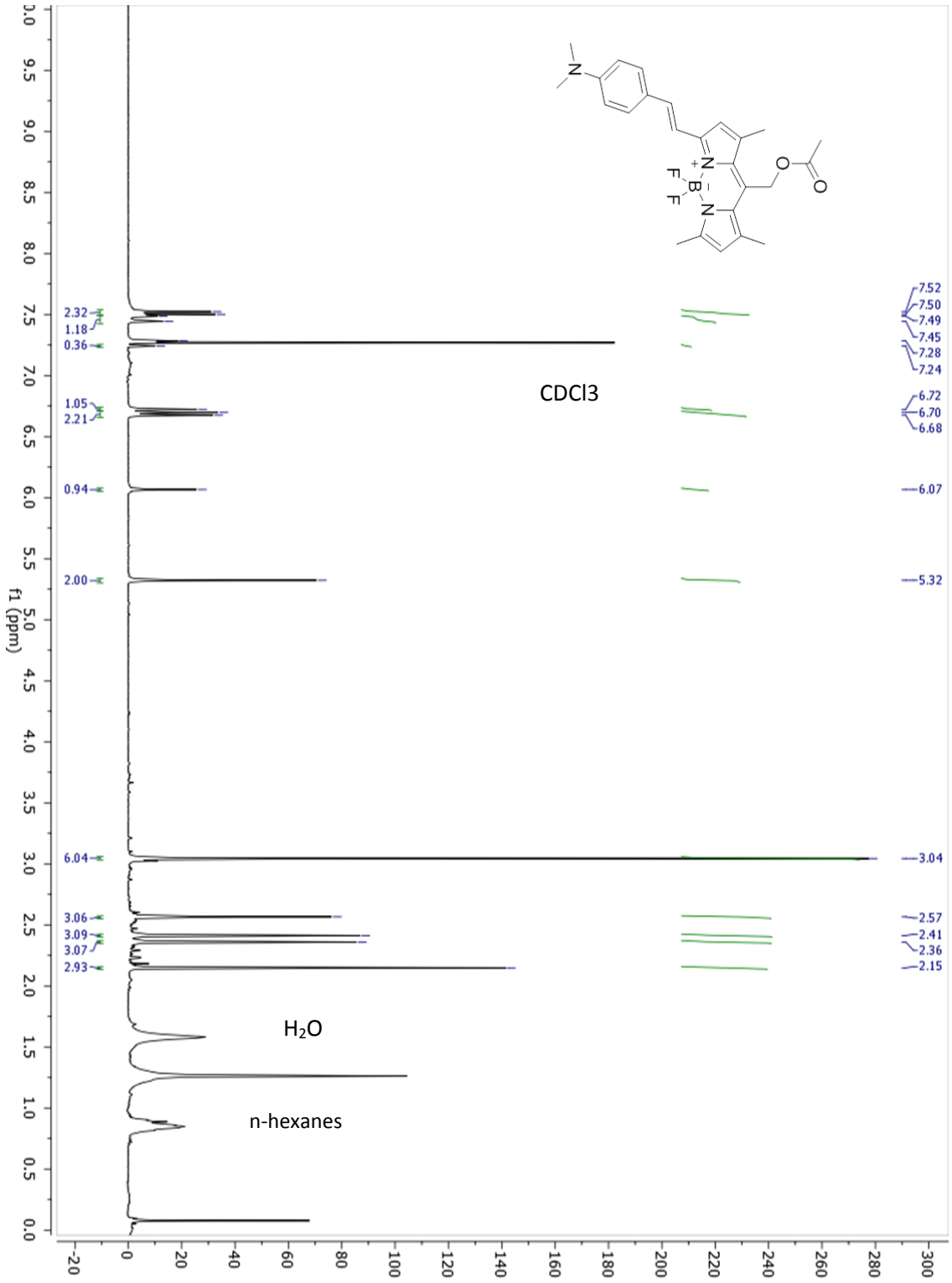

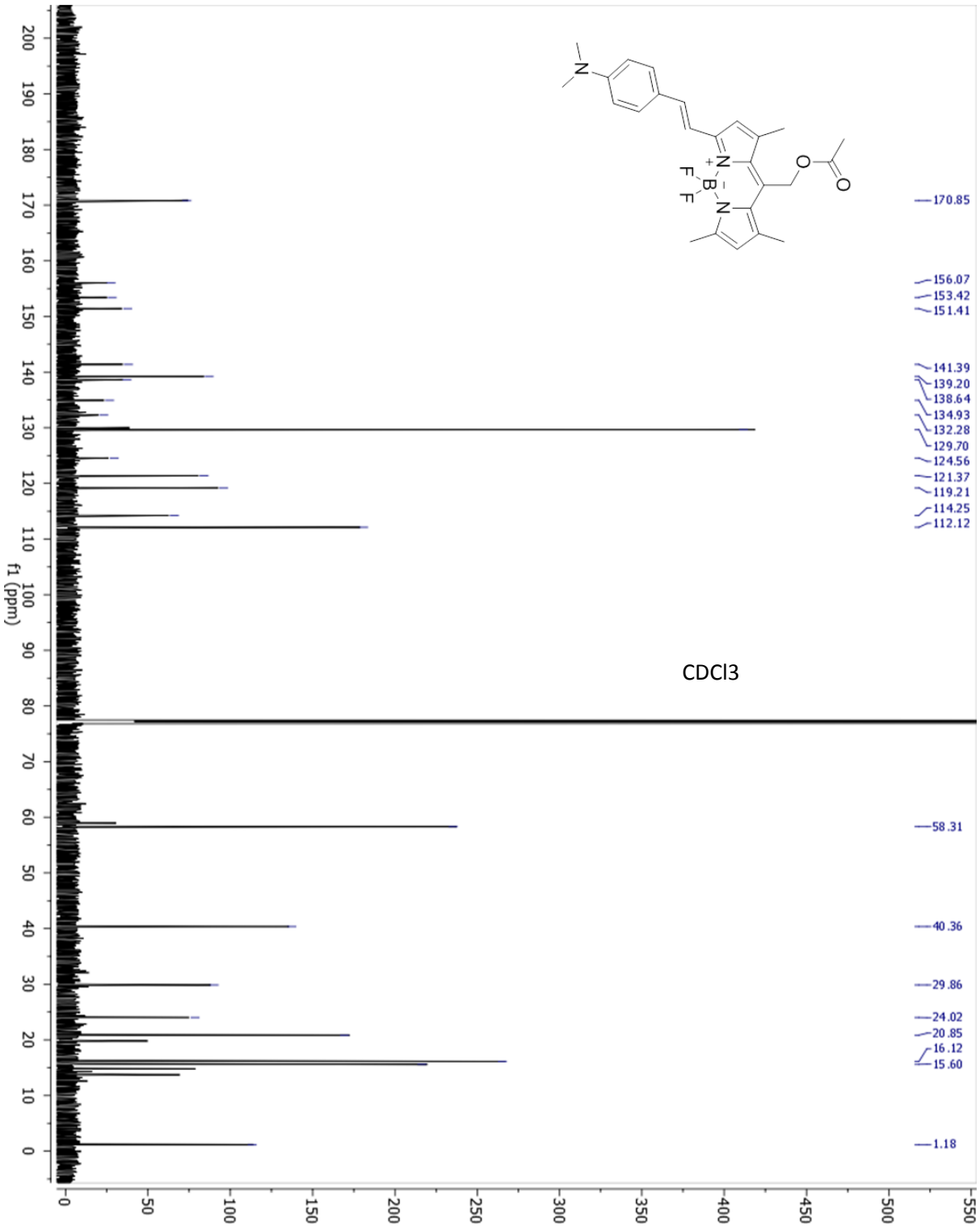

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