DESIGN, SYNTHESIS, AND EVALUATION OF NOVEL ANTIMICROBIALS FOR THE ERADICATION OF BIOFILMS

by

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A dissertation submitted in partial fulfilment
of the requirements for the degree
of

Doctor of Philosophy

in

Organic Chemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2020

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DEDICATION

This dissertation is dedicated to my father, Dr. Thomas J. Walsh.

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Tom Livinghouse for his dedication to my success as a chemist and his unyielding commitment in lab. I would also like to thank my co-advisor, Dr. Phil Stewart for his constant support and advice. Committee members as well as MSU staff, past and present, who have kindly supported me and provided me with continuous inspiration and encouragement over the years; Professors Tom Livinghouse, Phil Stewart, Mary Cloninger, Sharon Neufeldt, Darla Goeres and other dedicated mentors, Betsey Pitts, Scott Busse, Steve Holmgren, and Doreen Brown.

Ultimately, my success in graduate school could not have been achieved without the support of my thoughtful family & friends; Dr. Tom Walsh, Kathrine Walsh-Spurgin, Alison Walsh, Adrienne Arnold, Harrison Bly, Aoife Casey, Jonas David, Sharon Dorsey, Pieter Gerrits, Pieter's mom, Alex Hintz, Casey Kennedy, Collin Miller, Sam Phalan, Billie Smith, Martha Welander, and Sam Ziegler.

I would also like to thank past and present group members; Yenny Chase-Bayless, Greg Durling, Khoi Hong, Ky Mickelsen, Brian Pettygrove, Jeff Simkins and Sean Zabawa. Montana State University, National Institute of Health, and Juan Valdez for the Endowment of the Sciences have all contributed to this work.

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ABSTRACT

The majority of microorganisms live in association with surfaces as biofilms. Biofilm communities are encased in a robust, extracellular matrix that reduces their susceptibility to antimicrobial agents. This poses a health concern due to the potential for pathogenic bacteria to cause serious infections. For example, hospital-acquired infections are among the top ten leading causes of death in the U.S. and are responsible for nearly 23,000 deaths per year. The goal of my research is to develop efficient antimicrobial agents capable of eradicating biofilms.

In this project, I have focused on three different derivatizations of small, phenolic compounds in effort to increase efficacy towards biofilms. An initial study compared the potency of small, naturally occurring phenols and their corresponding allyl, propyl, and methallyl derivatives against bacteria. This study showed that in parent and derivative pairs potency increased towards free floating cells but decreased towards biofilms. This illustrated the importance of evaluating antimicrobial efficacy toward biofilms when the bacteria they intend to treat has the propensity to form biofilms. This was in contrast to a second studyishowing that trichloromethylsulfenate ester derivatives generally increased potency towards both biofilms and planktonic cells.

In a third study, we found that iminodiacetoxy-methylester (AM) appendages increase potency towards planktonic cells and biofilms. AM appendages are ester groups that are employed as part of a prodrug design. Prodrugs are biologically inactive compounds until metabolized. Ester groups are commonly used in prodrug intracellular dyes, where, once inside the cell, ester groups are cleaved enzymatically, resulting in a negatively charged dye that is retained in the cell. Similarly, after the cleavage event, the AM antimicrobial compound will concentrate within the cell. This design serves two functions to increase potency: increasing permeability towards the biofilm matrix and achieving cellular retention. We have shown that the efficacy of antimicrobial agents towards biofilms can be increased through this strategic design. This class of prodrugs presents a wide array of potential applications, from controlling hospital-acquired infections to incorporation into household cleaning products and addresses the need for novel treatments of pathogenic bacteria.

CHAPTER ONE

1. GENERAL PROJECT BACKGROUND

1.1 Project Overview

In nature, most microorganisms live in association with surfaces as multicellular communities called biofilms. Biofilms begin to form when planktonic, or free-floating cells, adhere to a surface. Attached cells secrete proteins, extracellular DNA and polysaccharides which encase the biofilm colony in a protective, extracellular polymeric substance (EPS), reducing susceptibility to antimicrobial agents as well as the host's immune system[1-3]. Many antimicrobial agents exhibit diminished potency towards biofilms because they lack the ability to efficiently permeate through the EPS[4-5]. Another factor contributing to biofilm virulence is the tendency for indwelling cells to experience slow growth rates and become dormant, allowing them to persist when peripheral cells in the biofilm are killed. These 'persister' cells are able to regrow the biofilm, resulting in chronic infection and contributing to the refractory characteristics of biofilms[6]. This robustness exhibited by biofilms has created an urgency for the development of new antimicrobial agents for inhibition, control, and eradication.

Two bacteria were chosen for the experimental models throughout these studies: *P. aeruginosa*, which is a Gram-negative bacterium, and *S. epidermidis*, a Gram-positive bacterium; both with the propensity to form biofilms[7-8]. *P. aeruginosa* is a rod-shaped opportunistic pathogen that exhibits multidrug resistance[9-11]. Its ubiquity in hospital-acquired infection has provided impetus for advancements in treating infections and

diminishing the number of associated illnesses. *S. epidermidis* is a Gram-positive bacterium typically found on human skin and mucosa. This pathogen is known for causing infections in prosthetic joints and valves as well as in postoperative wounds and the urinary tract[12]. *S. epidermidis* is among the five most common organisms found to cause hospital-acquired infections[13]. Unlike *P. aeruginosa, S. epidermidis* is commonly found on skin and can be a harmless commensal bacterium, although its ability to form biofilms increases its persistence on medical devices[14]. Both bacteria pose a serious risk of infection for patients undergoing surgical procedures or those who are otherwise immuno compromised. This heightens the need for novel methods in treating biofilm-mediated infections of the organisms.

Phenols were chosen as model antimicrobials for this project because they constitute a well-studied class of antimicrobial agents that have been evaluated extensively against planktonic cells and biofilms of taxonomically diverse bacteria[15-22]. Phenols have been shown to disrupt bacterial cell membranes, causing cell lysis, and have been shown to attack cytoplasmic targets, denaturing proteins and inactivating enzymes by binding to them and forming inoperative complexes[15, 18]. Maddox *et al.,* demonstrated that low-molecular weight phenols inhibit the growth of *X. fastidiosa,* a Gram-negative bacterium and plant pathogen, in vitro[18]. Similarly, Alves *et al.*, studied phenolic compounds and their activity against *S. epidermidis, E. coli*, *Past. Multocida, N. gonorrhoeae*, methicillin resistant *S. aureus* (MRSA) and several other Gram-negative and Gram-positive bacteria[15], finding that many naturally occurring phenols have significant antimicrobial activity. Several phenolic essential oils have also been shown to present antimicrobial properties

against taxonomically diverse bacteria in assays toward both planktonic cells and biofilm[23-26]. These attributes made small, phenolic compounds good candidates for these studies.

In an initial study, three phenolic essential oils were selected for evaluation against biofilms (Chapter 2). In an effort to increase anti-biofilm efficacy, allyl, propyl and methallyl functional groups were added to either the 2 or 4 position on the aromatic ring. An allyl group was chosen in an effort to increase permeability, while propyl and methallyl derivatives were chosen for their structural similarity to the allyl chain. In a second study, a selection of 25 small phenolic compounds were functionalized with a trichloromethyl sulfonate group (Chapter 3). This sulfonate group was chosen due to its employment in the antifungal agents Captan and Folpet, fungicides with both preventative and curative properties that have been shown not to possess carcinogenic, mutagenic or teratogenic properties toward humans[27].

In a third study, 18 phenols were functionalized with iminodiacetoxymethyl ester (AM) groups, as well as several structural variations of the AM (Chapter 4). AM groups were employed to increase permeability and to implement a prodrug design in order to concentrate the antimicrobial within the cell. A prodrug is a compound that is activated once inside the cell; in this case the activation event is the cleavage of AM ester bonds, resulting in a negatively charged phenolic antimicrobial. For each study stated above, parent compounds and derivatives were evaluated against both planktonic cells and biofilms in order to better understand these novel antimicrobial agents.

1.2 General Background

1.2.1 Biofilm growth and development

There are four steps in the biofilm lifecycle: attachment, colony formation, maturation and dispersal. A biofilm begins to form when planktonic cells adhere to a surface. Bacteria use proteins such as pili or flagella to reversibly attach themselves but can quickly become irreversibly bound by employment of other extracellular adhesive appendages and the secretion of proteins and extracellular DNA[28]. The resulting matrix, or EPS, allows cells to mature under nutrient rich conditions while being protected from desiccation, host immune defenses and antimicrobial agents[29-32].

It has been shown that specific proteins are more abundant in the stages of biofilm formation and maturation, correlating the onset of protein production with the progression of the biofilm; this suggests biofilm formation is a genetically regulated process[33-34]. It has also been shown that cells which grow in a biofilm are affected morphologically, physiologically and metabolically via regulation of gene expression[35-39]. For example, indwelling cells experience a slower growth rate, causing them to become dormant, or metabolically inactive. These cells can persist when the bulk of cells are killed and are able to regenerate the biofilm, contributing greatly to the persistence of chronic infections and ongoing contamination[40-42]. The ability to modify gene expression also allows indwelling cells to become densely concentrated before dispersing into the environment[43-44].

Dispersal of bacteria is controlled through extracellular and intracellular signaling that regulate gene expression in response to internal and external pressures such as population density, shear stress, and fluctuations in nutrients or oxygen content[45-47]. This results in particulates of biofilm detaching into the surrounding environment. Three types of dispersal have been identified to date: seeding, erosion and sloughing. Seeding is an

active mechanism of dispersal which entails the rapid release of many small clusters of cells from hollow cavities within the biofilm[47-49]. Erosion and sloughing can be both active and passive processes of biofilm dispersal. Erosion involves the gradual release of either single cells or small clusters[50]. Sloughing refers to the sudden detachment of large clumps of cells[51]. All types of biofilm dispersal are strongly associated with the transmission of bacteria from environmental reservoirs to humans as well as the spread and exacerbation of infections of many Gram-negative and positive bacteria[52-55].

1.2.2 Biofilms in industry

Food processing plants and hospitals, which provide optimal environments for biofilm growth, are among the many industries affected by biofilms. Biofilms pose a threat to public health through the contamination of consumables and the spread of infectious disease but can also cause corrosion on surfaces such as water passage ways and food processing equipment[56]. Contamination of equipment can create blockages in membrane pores which not only reduces flow rate but also leads to metal corrosion causing financial loss through reduced hydraulic and heat exchange efficiencies[57-58].

Biofilms can form quickly in food industry environments where they are found on many abiotic and biotic surfaces. Dairy factories, for example, provide numerous structures that can act as excellent environments for biofilm formation such as raw milk tanks, butter centrifuges, cheese tanks, pasteurizers, and packing tools[59]. Many other factory surfaces are also at risk for contamination, including reverse osmosis membranes, tables, employee gloves, liquid pipelines, storage silos for raw materials, dispensing tubing and packing material. Contamination on such surfaces not only puts consumers at risk for the

transmission of illness but also cause spoilage and severe degradation of equipment that can become extremely costly for the company[60].

Another prime example of biofilms as a public health concern is biofilm contamination in hospitals. Hospital-associated infections (HAIs), which can lead to life-threatening illnesses, prolonged hospitalizations, surgical site infections, chronic wounds and implant rejection, are a worldwide problem[61-62]. According to the World Health Organization, on average 10.1% of patients in the US contract a HAI, with higher associations in less developed countries[61]. These issues are largely due to bacterial accumulation on surgical equipment and medical devices, such as catheters, prosthetic joints and tympanostomy tubes[63-65]. Biofilms residing in water systems also pose a threat to patients and have been likened to a large number of infectious disease outbreaks of *P. aeruginosa*[66-68]. Infections from biofilms can quickly become chronic due to the biofilm EPS which protects cells from the host's immune system and lowers susceptibility to antibiotic treatment[69-71]. There are currently many strategies for treating biofilms, although many have serious shortcomings or limited applications.

1.2.3 Current anti-biofilm methods

Several methods to prevent and inhibit biofilm formation have been proposed or implemented, including chemical and physical modification of surfaces and application of antimicrobial compounds. There are few antimicrobials with efficacy toward biofilms and, to our knowledge, there are no prodrug antimicrobials used commercially to control biofilms. This lack of biofilm-specific antimicrobials makes degradation and contamination hard to avoid and even harder to control once biofilm formation has begun.

Current solutions that involve the physical alteration of surfaces that bacteria bind to include applying paint coatings or a treatment of antibodies[72]. Some coatings work by interfering with the adherence of bacteria by diminishing surface roughness or by reducing hydrophilicity which ultimately determines the ability of bacteria to adhere to the surface. Others work by killing the bacteria once they have made contact. These are not long-term solutions though and the effectiveness is greatly reduced by the leaching of these reagents, making them impractical for application to surgical instruments or industrial equipment.

In hospitals, disinfectants and anti-microbial coatings are commonly used to prevent the spread of infectious disease, though these are not always effective towards biofilms or may exhibit toxicity towards humans. Routine disinfectants include hydrogen peroxide, sodium hypochlorite, chlorine and quaternary ammonium salts. Several studies have shown that *P. aeruginosa* and *E. coli* biofilms exhibit resistance toward hydrogen peroxide and that *S. aureus* and *P. aeruginosa* biofilms are tolerant toward many quaternary ammonium salts[73-78]. Chlorine and chlorine oxide are commonly used as disinfectants in water systems, although they are highly toxic towards humans[79-80].

In food manufacturing plants, there are many procedures in place to ensure sanitation, including choice of materials used, brushing of equipment and use of detergents and disinfectants, but these methods are, for the most part, only effective on planktonic cells[81]. Food manufacturers also utilize a variety of biofilm prevention techniques, such as surface modifications, quorum sensing inhibitors, chemical treatment, bacteriocins, and enzymes, with varying degrees of success[82]. Chemical methods of sanitation such as sodium hydroxide and ozone are less expensive than methods such as enzymatic

approaches or surface modification, although these chemical methods are less effective towards biofilms. The more effective implementations are also limited to few commercial applications, reducing their usefulness across industry. This leaves industries in need of methods that are just as efficient as these novel technologies, but applicable to diverse food processing plants and medical facilities.

Phenols are a well-studied class of antimicrobial compounds that have varying degrees of potency against a wide range of planktonic state bacteria[15-22]. The antimicrobial activity of phenols has generally been attributed to their ability to degrade the cytoplasmic membrane, damage membrane proteins, reduce ATP synthesis and disrupt the lipid bilayer[17, 83-86]. Although there has been little research on phenols against biofilms, recently phenols such as thymol, carvacrol, baicalein, *trans*-resveratrol and pterostilbene have shown to have inhibitory and eradication properties toward biofilms of multiple bacteria[87-89].

1.2.4 Prodrug applications

Prodrugs are pharmacologically inactive derivatives of drugs that undergo bioconversion into their active form once the site of action is reached, maximizing the efficiency of the drug. Prodrug design has largely been implemented in pharmaceuticals to overcome obstacles such as absorption, distribution, metabolism and excretion[90-91]. Prodrug medications are used to treat a wide range of medical conditions such as Parkinson's, diabetes, Alzheimer's disease, cancer and tuberculosis[92-94]. For example, the pharmaceutical Amprenavir, an HIV protease inhibitor, exhibits complications in delivery associated with absorption. Specifically, Amprenavir demonstrates inadequate dissolution

in the gastrointestinal tract. However, the corresponding prodrug, Fosamprenavir, contains a phosphate ester group and exhibits higher aqueous solubility and is thus better able to deliver the drug[95]. In another example, dopamine, which is unable to effectively cross the blood-brain barrier, is synthetically converted to the prodrug Levodopa which is functionalized with a carboxylic acid group. This group is catabolized by aromatic-L-amino-acid decarboxylase once the drug has crossed the blood brain barrier, producing dopamine[96].

A variety of structurally diverse functional groups are employed for different purposes, including phosphates, hemisuccinates, aryloxy phosphoramidates, phosphonooxymethyls, carbamates, aminoacyl conjugates, ethers and esters[97-99]. Ester functional groups are among the most common functional groups implemented in prodrug design and are often used to enhance lipophilicity and thus membrane permeability[90, 100-102]. Ester-containing prodrugs are not only implemented in pharmaceuticals but also in intracellular fluorescent dyes. Calcein AM is a fluorescein derivative which passively crosses the cell membrane of viable cells where it is then converted into Calcein. Calcein is retained within the cell due to its liberated, negatively charged carboxylic acid groups (Figure 1.1).[103-104].

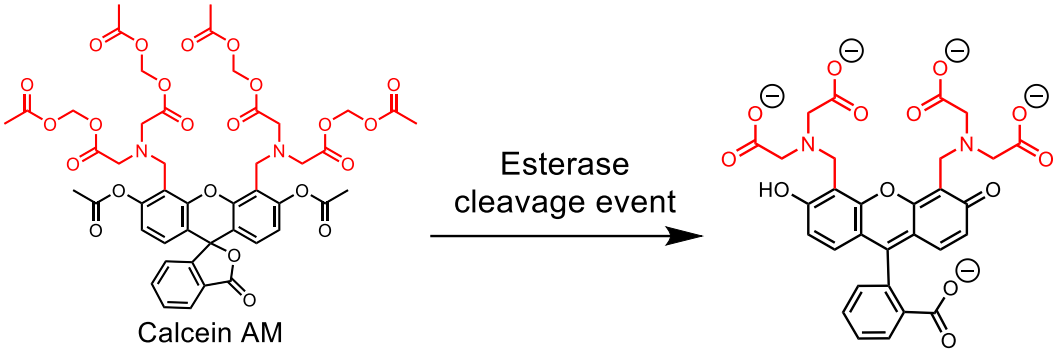


Figure 1.1. Calcein AM converted to its active form, Calcein.

Calcein has also been shown to stain biofilms in Gram-negative and Gram-positive bacteria such as *Streptococcus oralis, Streptococcus gordonii, S. mutans* and *P. aeruginosa*[105-109]. This class of fluorescent dyes has inspired a novel class of prodrug antimicrobials. Small, antimicrobial phenols that are employed with iminodiacetate (AM) groups are expected to permeate the biofilm membrane, accessing the indwelling cells. Once inside, the liberated form will become negatively charged and concentrated within cells. This class of prodrugs can be found in Chapter 4.

CHAPTER TWO

ANTIMICROBIAL ACTIVITY OF NATURALLY OCCURRING PHENOLS AND DERIVATIVES AGAINST BIOFILM AND PLANKTONIC BACTERIA

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Status of Manuscript:
____ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-reviewed journal
____ Accepted by a peer-reviewed journal
_X_ Published in a peer-reviewed journal

Frontiers in Chemistry, 2019. **7**(653). doi.org/10.3389/fchem.2019.00653

2.1 Abstract

Biofilm-forming bacteria present formidable challenges across diverse settings, and there is a need for new antimicrobial agents that are both environmentally acceptable and relatively potent against microorganisms in the biofilm state. The antimicrobial activity of three naturally occurring, low molecular weight, phenols and their derivatives were evaluated against planktonic and biofilm *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The structure activity relationships of eugenol, thymol, carvacrol and their corresponding 2- and 4-allyl, 2-methallyl, and 2- and 4-*n*-propyl derivatives were evaluated. Allyl derivatives showed a consistent increased potency with both killing and inhibiting planktonic cells but they exhibited a decrease in potency against biofilms. This result underscores the importance of using biofilm assays to develop structure-activity relationships when the end target is biofilm.

Keywords: Biofilm, antimicrobial, anti-biofilm, essential oil

2.2 Introduction

P. aeruginosa is a Gram-negative, rod shaped bacterium with a pronounced tendency to form biofilms. It is also an opportunistic pathogen that exhibits multidrug resistance [110]. Its ubiquity in hospital-acquired infection has provided impetus for advancements in treating infections and diminishing the number of associated illnesses. *S. epidermidis* is a Gram-positive bacterium typically found on human skin and mucosa. This pathogen is known for causing infections in prosthetic joints and valves as well as in postoperative wounds and the urinary tract, due to catheter use. *S. epidermidis* is also

among the five most common organisms found to cause hospital acquired infections [13]. Unlike *P. aeruginosa, S. epidermidis* is typically a harmless commensal bacterium, although its ability to form biofilms increases its persistence on medical devices. With recent advances in understanding biofilm development, including molecular mechanisms and cell surface proteins of *S. epidermidis*, this opportunistic pathogen is gaining increased interest within the medical field [14].

The majority of microorganisms in nature, including those responsible for hospital-acquired infections, live in association with surfaces as biofilms [111]. Due to the secretion of proteins, extracellular DNA and polysaccharides, biofilm communities are encased in a robust matrix which reduces their susceptibility to antimicrobial agents as well as the immune system [1-3, 112]. This poses a health concern due to the potential for these organisms to cause serious infections in patients with indwelling medical devices and those who are undergoing surgical procedures, stressing the need for novel methods in treating biofilm mediated infections [113-114]. According to the Agency for Health care Research and Quality, hospital-acquired infections are in the top ten leading causes of death in the United States, and are consequently responsible for nearly 100 thousand deaths per year [115]. Several methods to prevent and inhibit biofilm formation have been proposed or implemented, including chemical and physical modification of surfaces and application of antimicrobial compounds [116-118].

Phenols constitute an extensive class of compounds that have been shown to present antimicrobial properties against a wide range of bacteria [15-22]. Maddox et al. [18] demonstrated that low-molecular weight phenolic compounds inhibit the growth of *X.*

fastidiosa, a Gram-negative bacterium and plant pathogen, in vitro. Alves et al. [15] studied phenolic compounds and their activity against *S. epidermidis, E. coli*, *Past. Multocida, N. gonorrhoeae,* MRSA and several other Gram-negative and Gram-positive bacteria. Several essential oils have also been shown to present antimicrobial properties against taxonomically diverse bacteria both in planktonic and biofilm assays [23-26]. This includes a variety of phenolic essential oils that have been studied as therapeutic and antimicrobial agents, such as thymol (**1a**), carvacrol (**2a**) and eugenol (**3a**) (Figure 2.) which are plant metabolites [19, 119-124]. In a 2018 study, Pinheiro et al. [19] studied thymol (**1a**) carvacrol (**2a**), eugenol (**3a**), 'ortho-eugenol' (**3b**) and guaiacol (**3c**) as well as several chlorinated and allyl phenyl ether derivatives; these compounds were shown to be active towards several bacteria including *S. aureus* and *P. aeruginosa*. Eugenol has also been successfully evaluated for its antibacterial, antifungal, antiviral, anti-parasitic and anti-cancer activity [83, 125]. In another study by Friedman et al. [122] several bioactivities of carvacrol (**2a**), including cell membrane disruptive properties, are extensively evaluated. This article also concludes that carvacrol has great potential to be used as a therapeutic for human infection and disease.

A number of structurally diverse essential oils, including thymol (**1a**), carvacrol (**2a**) and eugenol (**3a**) have been evaluated for their antimicrobial and anti-biofilm properties. Essential oils have been shown to act as biofilm inhibitors against Staphylococci[126-128] as well as Pseudomonas[129-131]. Thymol (**1a**) and carvacrol (**2a**) have demonstrated anti-biofilm properties, both alone and as a mixture, against diverse bacteria including *Cryptococcus*[132], *Salmonella*[87], *Staphylococci*[133], *Enterococcus*[134] and

Escherichia[135]. Eugenol (**3a**) has also been shown to exhibit anti-biofilm properties against a variety of Gram-negative and Gram-positive bacteria including *Porphyromonas*[136], *Salmonella*[137], *Escherichia*[135] and *Listeria*[138].

The mechanism of action of several structurally varied naturally occurring phenols has been studied against a variety of microorganisms. The antimicrobial activity of essential oils has generally been attributed to a cascade of reactions involving the bacterial cell, as opposed to a single mode of action, which lead to degradation of the cytoplasmic membrane, damage of membrane proteins, reduced ATP synthesis, and increased membrane permeability [17, 83-84, 139]. It has also been well documented that the hydrophobicity of essential oils contributes to their antimicrobial activity by enabling them to disrupt the lipid bilayer in bacterial cells [85-86].

Carvacrol has been shown to destabilize the cytoplasmic membrane, increasing membrane fluidity causing leakage of ions, a decrease in the pH gradient across the cytoplasmic membrane and inhibition of ATP synthesis in *Bacillus cereus* [140]. The importance of the hydroxyl group on the aromatic ring in carvacrol has also been demonstrated by comparing carvacrol with similar compounds such as carvacrol methyl ester, methanol and cymene; which lack the hydroxyl group that carvacrol possesses [140-141]. Ultee et al. observed that carvacrol is able to diffuse through the cytoplasmic membrane, becoming deprotonated and then binding to a monovalent cation such as potassium it is able to diffuse out of the cytoplasm where it again takes up a proton from the external environment, there for acting as a transmembrane carrier of monovalent cations [140]. Another study by Knobloch et al. [83] discusses the antimicrobial activity of

essential oils as causing damage to the biological membrane. Knobloch et al. also speculated that the acidity of the hydroxyl group on thymol and carvacrol may attribute to their antimicrobial activity as well. The effect of eugenol on the cell membrane has also been examined using *C. albicans* [142], showing that eugenol, like carvacrol, also targets the cytoplasmic membrane. Another study by Xu et al. [143], demonstrated that eugenol disrupts the cell wall of *S. aureus*, increasing permeability, causing leakage of cellular substituents and permanent damage to the cell membrane. Eugenol has also been shown to bind to proteins in *E. aerogenes* and inhibit the production of enzymes in *B. cereus*, causing degradation of the cell membrane [144-145].

A variety of phenolic essential oils and other aromatic alcohols that are not evaluated in this study, have also been studied for their mode of action. Wu et al. [146] reported the antimicrobial activity and mechanism of action of the natural occurring phenol, 3-p-trans-coumaroyl-2-hydroxyquinic acid. In this study it was shown that this phenol caused the loss of cytoplasmic membrane integrity, increased membrane fluidity and caused conformational changes in membrane proteins of *S. aureus.* Aromatic alcohols such as phenoxyethanol have also shown to increase permeability of the cytoplasmic membrane in *E. coli* [147-148].

In this communication, thymol (**1a**), carvacrol (**2a**) and eugenol (**3a**) as well as guaiacol are evaluated along with several 2- and 4- allyl, 2-methallyl and 2-*n*-propyl derivatives (Figure. 3.1). Thymol (**1a**), carvacrol (**2a**) and eugenol (**3a**) have also been evaluated for their ability to inhibit adherence and biofilm formation as well as biofilm eradication [23, 137, 149-161]. Oh et al. [161] has shown that thymol (**1a**) and carvacrol (**2a**) have

anti-biofilm effects on the formation of *E. coli* and *Salmonella*. Unlike previous studies, the parent phenolic compounds are being compared to their allyl, methallyl and propyl derivatives, which have not been extensively evaluated against either planktonic cells or biofilms.

Thymol (**1a**) and carvacrol (**2a**) are both monoterpenes and are constitutional isomers found in thyme, oregano, bergamot and other culinary herbs (Figure 2.). Both are used as a flavoring agents as well as in tinctures for their antifungal, antibacterial, and antiprotozoal properties [162-163]. Eugenol (**3a**) is an essential oil found in plants such as vanilla, clove, nutmeg and cinnamon. It is a flavoring agent utilized as well as for its antibacterial and anti-inflammatory properties [120, 164]. Guaiacol (**3c**) was also evaluated and is a naturally occurring phenol found in guaiacum, a shrub in the Zygophyllaceae family, and in creosote wood. It is structurally similar to eugenol, although it lacks the 4-allyl appendage. Guaiacol's ability to inhibit planktonic cell growth as well as biofilm formation in a mixture has also been evaluated [19, 165]. In this study, these four compounds and several of their derivatives have been assessed for potency towards inhibiting planktonic cell growth as well as their ability to eradicate biofilms.

The 2- and 4-allyl (**1b**, **2b, 1c** and **2c),** *n*-propyl (**1e** and **2e**) and 2-methallyl (**1d** and **2d**) derivatives of thymol and carvacrol as well as the 4-*n*-propyl derivative of eugenol (**3d**) and 2-allyl and 2-methallyl derivative of guaiacol (**3b** and **3g**) were evaluated, all of which are previously synthesized derivatives of these essential oils [166-169] (Figure 2.**Figure 2.**). None of these derivatives have previously been evaluated for their antimicrobial activity against biofilms. The corresponding allyl ether derivatives of thymol (**1a**) and

carvacrol (**1b**) have been studied, [19] although to our knowledge the 2- and 4- allyl derivatives as well as 2-methallyl and 2-propyl derivatives have yet to be evaluated against both planktonic cells and biofilms in the same study. This study not only evaluates the potency of the derivatives stated above against planktonic cells but against biofilms as well, illustrating the difference in potency and trends in potency between these two modes of microbial growth. The structures that are being evaluated here are allyl, methallyl or propyl groups and whether these groups increase potency of the selected essential oils. The addition of an allyl group was selected in effort to increase lipophilicity, and thus to increase permeability towards the cell membrane. Lacey et al,. [170] also demonstrated that ethylene binds to an ethylene binding protein in Synechocystis affecting pili, which are binding proteins. The 2-methallyl group was also selected to increase lipophilicity.

The simple analogues 2-allylphenol (**3e**) and 2-*n*-propylphenol (**3f**) were also evaluated for comparative purposes to the aforementioned 2-allyl derivatives of the selected essential oils. The purpose of this investigation was to develop structure activity relationships for naturally occurring phenol derivatives and to compare these relationships between planktonic and biofilm modes of bacterial growth.

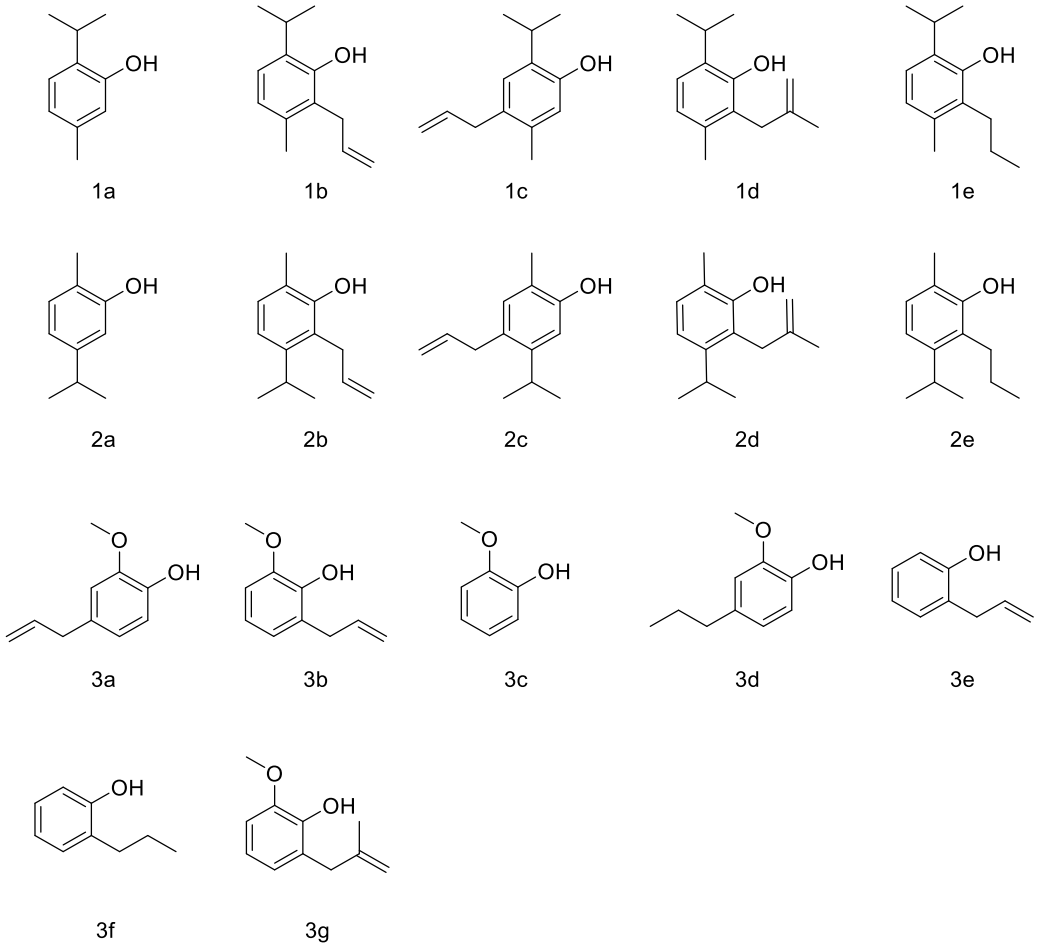


Figure 2.1 Structures of parent compounds, thymol (**1a**), carvacrol (**2a**) and eugenol (**3a**) as well as allyl (**1b/c**, **2b/c**, and **3b/e**), 2-methallyl (**1d, 2d** and **3g**) and propyl (**1e**, **2e**, and **3d/f**) derivatives.

2.3 Materials and Methods

2.3.1 Experimental general information

Thymol (**1a**) (99% pure), carvacrol (**2a**) (95% pure), guaiacol (99% pure), 2-allyl phenol (95% pure) and eugenol (**3a**) (99% pure) were purchased from Tokyo Chemical Industry Co. (TCI). All other reagents for chemical synthesis were purchased from commercial sources and used as received without further purification. Solvents for filtrations, transfers, and chromatography were certified ACS grade. Thin layer

chromatography was performed on Silicycle Glass Backed TLC plates, and visualization was accomplished with UV light (254 nm), and/or potassium permanganate. All ^{1}H NMR spectra were recorded on a Bruker DRX300. All ^{13}C NMR spectra were recorded on a Bruker DRX500, all NMR data was reported in ppm, employing the solvent resonance as the internal standard.

P. aeruginosa (PA01 and PA015442) and *S. epidermidis* (35984) were obtained from American Type Culture Collection (ATCC). All bacteria were sub-cultured onto tryptic soy agar (TSA) plates and incubated at 37 °C for 24 h. Single colonies were transferred from the plates and inoculated into 25 mL tryptic soy broth (TSB) in Erlenmeyer flasks. Culture were incubated 37 °C for 24 h and 10 µL of culture was transferred into 25 mL of TSB and the absorbance was read at 600nm using a spectrophotometer and standardized to 10^6-10^7 CFU/mL.

2.3.2 Efficacy of naturally occurring phenols and derivatives on inhibiting planktonic cells

The minimum inhibitory concentrations (MICs) of all compounds against *S. epidermidis* and *P. aeruginosa* were determined using a 96-well plate assay previously described by Xie [171]. The data from at least three replicates were evaluated for each compound tested. Samples were diluted in dimethyl sulfoxide (DMSO) and DMSO controls were conducted as the negative control. Experiments were done in biological triplicate and technical duplicates were done. Tests for statistical significance were calculated with a two-tailed t-test assuming unequal variances.

2.3.3 Efficacy of naturally occurring phenols and derivatives on killing planktonic cells

Parent compounds (**1a**, **2a** and **3a**) were used as reference standards for each synthesized derivative. Both strains were cultured as described above. Compounds were diluted in 9.9 mL Phosphate-buffered saline (PBS) and 0.1 mL DMSO. Each tube was inoculated and allowed to sit at room temperature for 5 hours, with sampling every hour. For sampling, three ten-fold dilutions were made in PBS. Each dilution was drop platted using 50 µL. Plates were incubated for 24 h and colony forming units (CFU) were counted. The concentration which showed no CFUs after 5 h was established as the lowest concentration which allowed for no bacterial growth. Negative controls with 9.9 mL PBS and 0.1 mL DMSO were done as well. Experiments were done in biological triplicate and technical duplicates were done. Standard deviations were determined by calculating the standard deviation for data from triplicate experiments. The mean log reduction was also determined for each compound evaluated using the following equation:

$$Log\ reduction = log10\left(\frac{A}{B}\right)$$

where A is the average number of CFU before treatment and B is the average number of CFU after treatment.

2.3.4 Efficacy of naturally occurring phenols and derivatives on biofilms

2.3.4.1 Biofilm eradication concentration assays: Parent compounds (**1a**, **2a** and **3a**) were used as reference standards for each synthesized derivative. Both strains were cultured as described above and biofilms were grown in Costar polystyrene 96-well plates at 37 °C. After 24 h of incubation, the planktonic-phase cells were gently removed and the wells washed three times with PBS. Wells were filled with 150 µL dilutions of the

compound being evaluated. The 96-well plates were incubated for an additional 24h at 37 °C. The media was gently removed and each well filled with 150 µL PBS and the biofilm broken up through stirring with sterile, wooden rods. Three tenfold dilutions of each sample were taken and drop plated on TSA plates and incubated for 24 h. The biofilm eradication concentration (BEC) was determined to be the lowest concentration at which no bacterial growth occurred. This procedure was modelled based on previously reported procedures according to Pitts [172]. Negative controls were also conducted with 150 µL PBS in the absence of antimicrobial agent. Experiments were done in biological triplicate and technical duplicates were done. Tests for statistical significance were calculated with a two-tailed t-test assuming unequal variances.

2.3.4.2 Center for disease control (CDC) biofilm reactor evaluation: The parent compound eugenol (**3a**) was used as a reference standards for the synthesized derivatives. A CDC biofilm reactor was also used to assess potency of compounds towards biofilms. American Society for Testing and Materials (ASTM) method E2562 – 17 which describes how to grow a biofilm in the CDC biofilm reactor under high shear and continuous flow, and ASTM method E2871 -13, a biofilm efficacy test generally known as the single tube method were used for this procedure. Formation of 48 h biofilms in a CDC reactor was formed on glass coupons (4.02 cm^2). A CDC reactor containing 340 mL of TSB (300 mg/L) was inoculated with 1 mL of a 3.21 x 10^8 CFU/mL overnight culture of *P. aeruginosa* (PA015442), which was grown in TSB (300 mg/L) overnight. The biofilm was grown in batch condition at room temperature at 125rpm for 24 h, and then for 24 h at room temperature under continuous flow with a feed rate of 11.25 mL/min at 125 rpm. The

continuous feed TSB was 100 mg/L. Coupons were then sampled from the reactor in triplicate. The mean log reduction in viable biofilms cells exposed to each compound for 1 h was quantitatively measured according to ASTM method E2871-13. After coupons were removed from the CDC reactor they were rinsed and transferred to separate, 50 mL conical tubes and 4 mL of a 100 mM solution of the antimicrobial compound being tested in sterile PBS buffer was added. The tubes were incubated at room temperature under static conditions or 1 h. After one h 36 mL DE broth was added and the biofilm was disaggregated by a series of vortexing and sonicating for 30 seconds each in the order of v/s/v/s/v. Each sample was diluted tenfold six times and the diluted samples were drop plated on (Reasoner's 2A agar) R2A agar plates, incubated overnight at 37°C and enumerated. Experiments were done in biological duplicate and technical duplicates were done. The mean log reduction was also determined for each compound evaluated using the following equation:

$$Log\ reduction = log10\left(\frac{A}{B}\right)$$

where A is the average number of CFU before treatment and B is the average number of CFU after treatment.

2.3.5 Chemical synthesis procedures

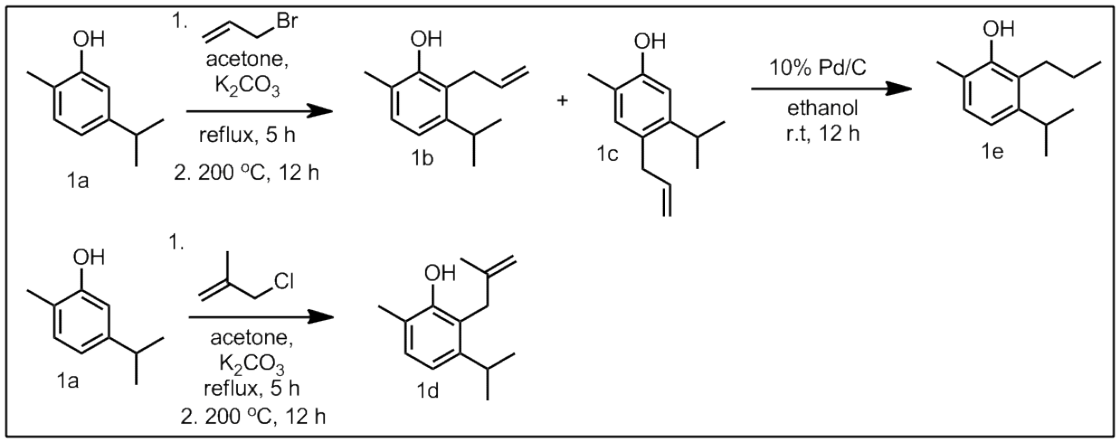
2.3.5.1 Preparation of 2-(2-propen-1-yl)-6-(1-methylethyl)-3-methylphenol (1b). Representative procedure. A 25 mL round-bottomed flask equipped with a magnetic stirring bar was charged with thymol **1a** (751 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux

and allyl bromide (0.5 mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo to remove solvent and the by-product of diallyl ether. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 742 mg (78%) of **1b** as a yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. ^{1}H NMR (300 MHz, $CDCl_3$) **1b**: δ 6.98 (d, *J* = 7.82 Hz, 1H), 6.77 (d, *J* = 7.82 Hz, 1H), 5.95 (m, 1H), 5.12 (m, 2H), 4.93 (s, 1H) 3.44 (d, *J* = 5.88 Hz, 2H), 3.16 (sept, *J* = 6.87 Hz, 1H) 2.26 (s, 3H), 1.24 (d, *J* = 6.87 Hz, 6H). C^{13} NMR (500 MHz, $CDCl_3$) **1b**: δ 19.2 (CH_2), 22.9 (CH_3), 26.8 (CH_2), 29.3 (CH), 115.6 (CH_2), 124.3 (C), 128.8 (CH), 132.1 (C), 134.7 (CH), 137.3 (CH), 139.4 (C), 150.7 (C). H^{1} NMR (300 MHz, $CDCl_3$) Spectral data and general procedures for compounds **1c**, **1d**, **2b**, **2c, 2d** and **3b** can be found in the supplementary data.

2.3.5.2 Preparation of *2-(2-n-propyl)-6-(1-methylethyl)-3-methylphenol* (1c). Representative procedure. A 10 mL round-bottomed flask was charged with 10% Pd/C (30 mg, 0.28 mmol, 0.1 equiv). The round-bottomed flask was put under an atmosphere of hydrogen and 100% ethanol (2 mL) was added. 2-allylthymol (**1b**) (300 mg, 1.5 mmol, 1 equiv) was added at room temperature and the reaction was stirred for 12 h. The resulting mixture was filtered through silica and concentrated in vacuo to afford 260 mg (87%) of **1e** as a light, yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the

following. ^{1}H NMR (300 MHz, $CDCl_3$) **1e**: δ 6.95 (d, *J* = 7.88 Hz, 1H), 6.81 (d, *J* = 7.88 Hz, 1H), 2.98 (sept, *J* = 6.81 Hz, 1H), 2.56 (t, *J* = 7.89 Hz, 2H), 2.25 (s, 3H) 1.59 (q, *J* = 7.89, 7.32 Hz, 2H), 1.36 (d, *J* = 6.81 Hz, 6H) 0.96 (t, *J* = 7.32 Hz, 3H). C^{13} NMR (500 MHz, $CDCl_3$) **1e**: δ 14.5 (CH_3), 19.4 (CH_3), 22.3 (CH_3), 22.7 (CH_2), 27.1 (CH_2), 28.8 (CH_2), 122.3 (CH), 123.1 (C), 126.5 (CH), 131.3 (CH), 134.7 (C), 150.8 (C). Spectral data and general procedures for compounds **2e**, **3d** and **3f** can be found in the supplementary data.

2.4 Results and discussion



Scheme 2.1. Representative synthesis, using carvacrol (**1a**) and derivatives (**1b**-**1d**).

In this study, four 2-allyl derivatives were synthesized, with the corresponding 4-allyl derivative as a secondary product (Scheme 2.1). This was accomplished through the synthesis of the corresponding allyl ether, followed by a thermal Claisen rearrangement. The allylated compounds **1b**-**3b**, **3a** and **3e** were then hydrogenated to give the propyl derivatives (**1e**, **2e**, **3d** and **3f**, **Figure 2.**). Compounds **1a**, **2a** and **3c** were also converted to the corresponding methallyl derivatives in an analogous manner.

In an initial study we assessed each naturally occurring phenol and its derivatives against planktonic cells. Studies have shown that thymol (**1a**) and carvacrol (**2a**) compromise the outer membrane of Gram-negative bacteria increasing the permeability of the cytoplasm [149]. The 2- and 4-allyl compounds for thymol (**1a**) and carvacrol (**2a**) all showed an increase in potency towards planktonic cells when compared to the parent compounds, as seen in Table 2.1. Interestingly, the 2-allyl derivatives (**1b** and **2b)** were more potent than the corresponding 4-allyl isomers (**1c** and **2c)** towards *P. aeruginosa*, whereas both isomers had identical MICs against *S. epidermidis* (Table. 2.1). The transposed isomer "ortho-eugenol" (**3b)** was more potent towards both *S. epidermidis* and *P. aeruginosa* than the parent eugenol (**3a**). 4-*n*-propyl-2-methoxyphenol (**3d**) was more potent than eugenol (**3a**). Guaiacol (**3c**), which does not possess an allyl appendage was less potent towards *S. epidermidis* when compared to eugenol (**3a**) (Table. 2.1). The methallyl derivate of carvacrol (**2d**) was more potent than the *n*-propyl derivative (**2e**) against *S. epidermidis,* though in the cases of methallyl thymol (**1d**) and methallyl eugenol (**3g**), the *n*-propyl derivatives (**1e** and **3f**) were more potent in comparison (Table 2.1).

Table 2.1: Minimum inhibitory concentrations in mM of parent compounds and derivatives against planktonic cells of *S. epidermidis* and *P. aeruginosa.*

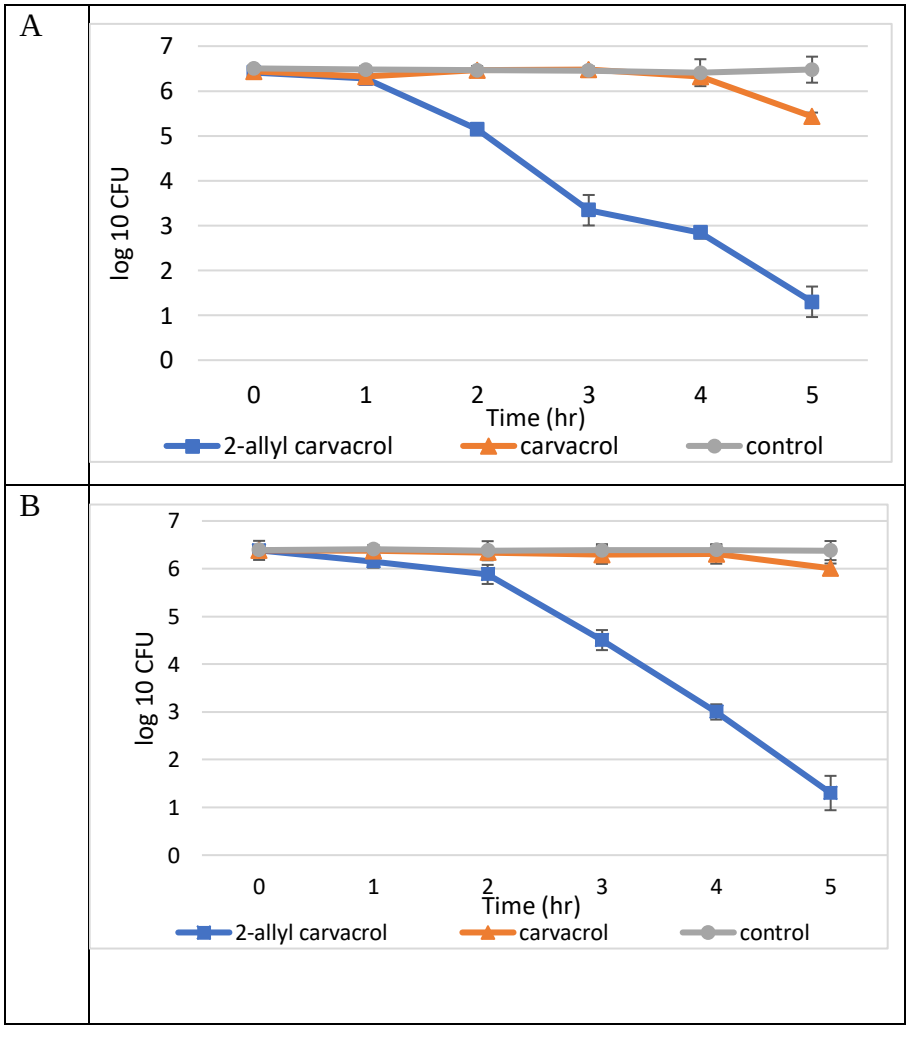
	MIC (mM)	
Compound	*S. epidermidis* (35984)	*P. aeruginosa* (PA01)
Thymol (**1a**)	2.5	3.9
2-allylthymol (**1b**)	0.12	0.25
4-allylthymol (**1c**)	0.12	0.97
2-methallylthymol (**1d**)	15	31.2
2-*n*-propylthymol (**1e**)	7.8	15.62
Carvacrol (**2a**)	2.5	3.9
2-allylcarvacrol (**2b**)	0.12	0.25
4-allylcarvacrol (**2c**)	0.12	0.97

2-methallylcarvacrol (**2d**)	3.9	31.2
2-*n*-propylcarvacrol (**2e**)	7.8	15.62
Eugenol (**3a**)	15	31.2
ortho-eugenol (**3b**)	7.8	7.8
Guaiacol (**3c**)	31.2	31.2
2-methoxy-4-*n*-propylphenol (**3d**)	7.8	15.62
2-allylphenol (**3e**)	7.8	7.8
2-*n*-propylphenol (**3f**)	15.62	15.62
2-methallyl-4-methoxyphenol (**3g**)	62.5	125

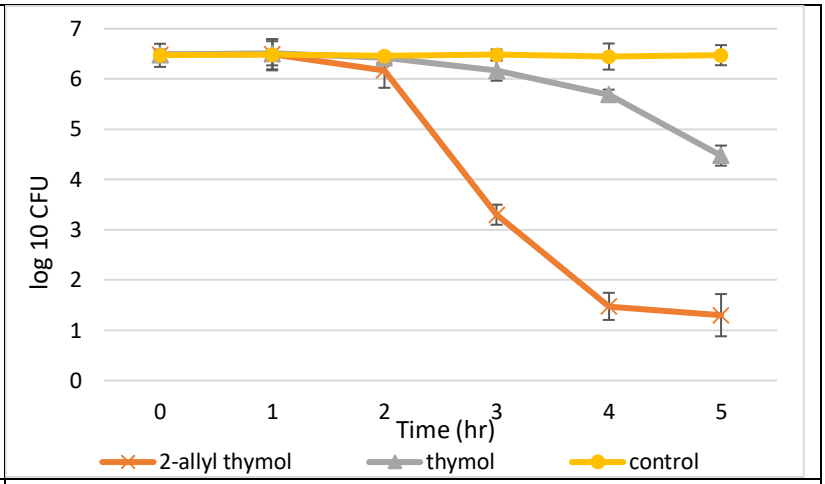
Planktonic MICs of allyl derivatives were generally statistically significantly lower than the MIC of the parent compound. For example, the *p*-values for parent compounds and their allyl derivatives were also calculated. The p-value of 1a/b against S. epidermidis is 0.041 and against P. aeruginosa is 0.025. The p-value of 2a/b against S. epidermidis is 0.0003 and against P. aeruginosa is 0.0005. Likewise, the p-value of 3a/c against S. epidermidis is 0.016 although against P. aeruginosa was calculated to be 0.42 due to the similarities in potency.

Time dependent killing data against planktonic bacteria were measured for all 2-allyl and parent compounds (Figure 2.2). It was shown that over the time period of five hours, 2-allyl carvacrol (**2b**) reduced bacterial growth by 79.80% against *S. epidermidis* and 79.63% against *P. aeruginosa*. The parent compound, carvacrol (**2a**), only reduced bacterial growth by 15.55% against *S. epidermidis* and 2.35% against *P. aeruginosa*. Similarly, 2-allyl thymol (**1b**) reduced the average bacterial growth by 79.00% for *S. epidermidis* and 77.93% for *P. aeruginosa,* while the average reduction of growth for thymol (**1a**) was 25.67% for *S. epidermidis* and 19.18% for *P. aeruginosa.* In the eugenol series, against *S. epidermidis*, eugenol (**3a**) and ortho eugenol (**3b**) had similar potency after 5 hours with a decrease in bacterial growth of 79.76% and 79.34% respectively.

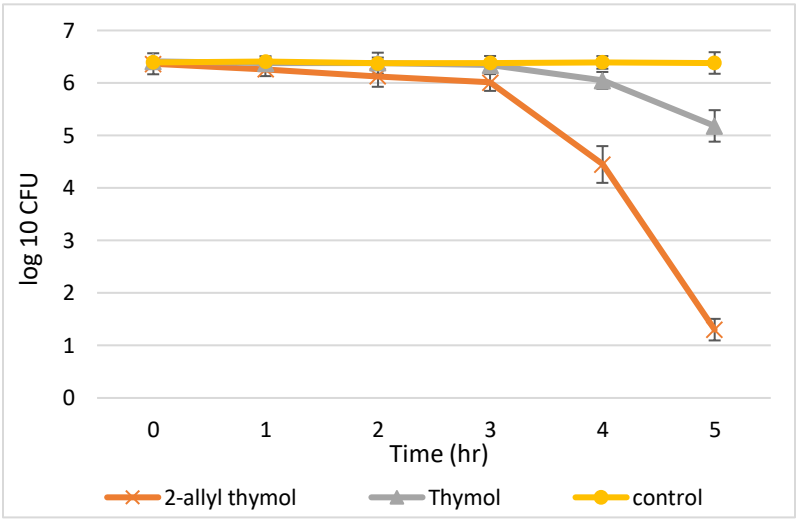
Although, at 4 hours, eugenol (**3a**) was able to decrease growth by 79.76% while ortho eugenol (**3b**) decrease growth by 53.89%. Against *P. aeruginosa*, eugenol (**1a**) reduced growth by 79.60%, while ortho eugenol (**3b**) reduced growth by 32.88%. Against both bacteria, guaiacol (**3c**) reduced growth by <2%. On average, the controls for each study showed a 0.079% decrease in growth for *S. epidermidis* and a 0.45% decrease in *P. aeruginosa*.



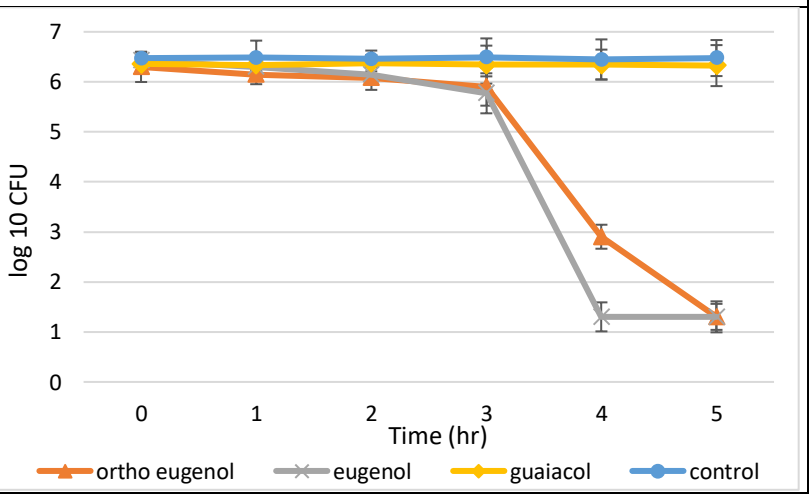
C


log 10 CFU
Time (hr)
2-allyl thymol
thymol
control

D


log 10 CFU
Time (hr)
2-allyl thymol
Thymol
control

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log 10 CFU
Time (hr)
ortho eugenol
eugenol
guaiacol
control

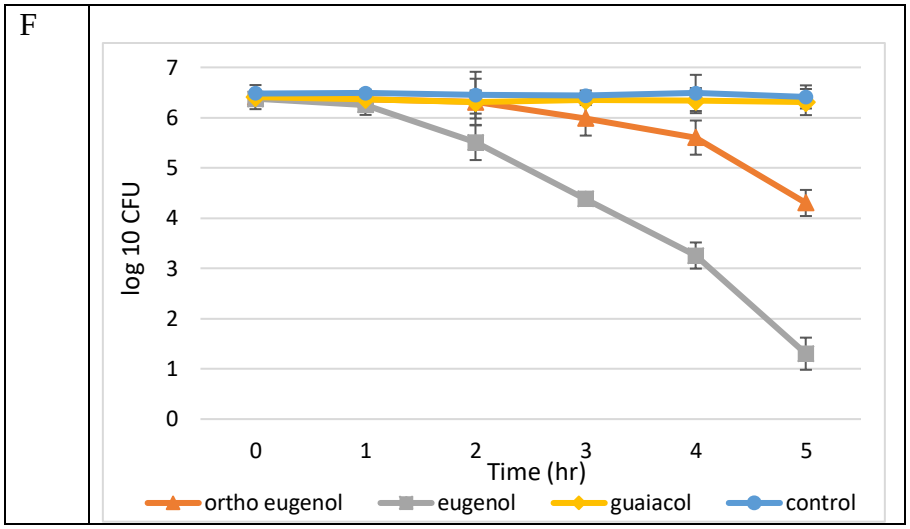


Figure 2.2 Time kill assays. Compounds were diluted in PBS and DMSO, (9.9:0.1), all controls were PBS and DMSO: (A) Carvacrol, 2-allyl carvacrol and a control with *S. epidermidis*, while the concentration of both carvacrol and 2-allyl carvacrol was 1.7 mM; (B) Carvacrol, 2-allyl carvacrol and a control with *P. aeruginosa*. While the concentration of both carvacrol and 2-allyl carvacrol was 15.6 mM; (C) Thymol, 2-allyl thymol and a control with *S. epidermidis*, at a concentration of 7.8 mM; (D) Thymol, 2-allyl thymol and a control with *P. aeruginosa*, at a concentration of 30 mM; (E) Eugenol, 'ortho eugenol', guaiacol and a control with *S. epidermidis*, at a concentration of 1.7 mM; (F) Eugenol, 'ortho eugenol', guaiacol and a control with *P. aeruginosa*, at a concentration of 15.6 mM

The mean log reduction after 5 h was recorded for all evaluated compounds as well (Table 2.). In this assay the 2-allyl derivatives of thymol and carvacrol (**1b** and **2b**) exhibited greater potency than the parent compound. Like 2-allylthymol and 2-allyl carvacrol, eugenol (**3a**) also exhibited a 5 log reduction after only 5 h, demonstrating that these allylate derivatives have bactericidal activity towards planktonic cells. Ortho-eugenol (**3b**) also exhibited a 5 log reduction against *S. epidermidis* after 5 h.

The lower mean log reduction further conveys the inferiority of thymol (**1a**) and carvacrol (**2a**) to their 2-allyl derivatives in killing bacteria (Table 2.2). This observation is consistent with the MIC data presented in Table 2.1. In the dynamic killing assay, eugenol (**3a**) was more potent than both guaiacol (**3c**) and "ortho-eugenol" (**3b**) with a

mean log reduction of 5.2 against *S. epidermidis* and 5.1 against *P. aeruginosa* (Table 2.2). This corresponds to a differing trend in activity when compared to the MICs in Table 2.1, where "ortho-eugenol" (**3b**) demonstrated a stronger growth inhibition than eugenol (**3a**).

Table 2.2: Mean log reduction after 5 h against of exposure. Concentrations of thymol and allyl thymol against *S. epidermis* was 7.8 mM and 30 mM against *P. aeruginosa*, while carvacrol, 2-allylcarvacrol, eugenol, ortho-eugenol and guaiacol was exposed at 1.7 mM against *S. epidermidis* and 15.6 mM against *P. aeruginosa*.

	S. epidermidis		*P. aeruginosa*	
Compound	Mean log reduction	Concentration (mM)	Mean log reduction.	Concentrations (mM)
thymol (**1a**)	1.9	7.8	1.2	30
2-allylthymol (**1b**)	5.1	7.8	5	30
carvacrol (**2a**)	0.09	1.7	0.37	15.6
2-allylcarvacrol (**2b**)	5.1	1.7	5	15.6
eugenol (**3a**)	5.2	1.7	5.1	15.6
ortho-eugenol (**3b**)	2.6	1.7	2.1	15.6
guaiacol (**3c**)	0.13	1.7	0.1	15.6

Efficacious concentrations varied greatly between MICs and BECs. BECs were consistently higher than MICs, conforming to the expected lower susceptibility of bacteria in the biofilm mode of growth. In addition, biofilm assays exhibited significant differences in the structure-activity relationship in comparison to planktonic results. Thymol (**1a**) and carvacrol (**2a**) continued to show a higher potency than their 2-*n*-propyl derivatives **1e** and **2e** (Table. 2.1), although they were more potent than their 2-allyl derivatives **1b** and **2b** against biofilms (Table. 2.3). The 4-allyl derivatives **1c** and **2c** however, did have an identical or a slightly lower BEC, against *P. aeruginosa* and thus were still more potent than their 2-allyl counterparts.

"Ortho-eugenol" (**3b**) continued to be more potent than eugenol (**3a**) against *P. aeruginosa* although the BECs for both compounds against *S. epidermidis* were identical.

Guaiacol (**3c**) was the most potent against both bacteria in a biofilm when compared to other eugenol derivatives, which was dissimilar to the trend in potency against both killing and inhibiting planktonic cells. The 4-*n*-propyl derivative of eugenol (**3d**) exhibited the same potency as "ortho-eugenol" (**3b**) against both bacteria (Table 2.3). It was interesting here that the MICs for the 4-*n*-propyl derivative **3d** were lower than eugenol (**3a**) against both bacteria tested, but the BEC against *S. epidermidis* was the same for both compounds (Table. 2.3). This information illustrates that it is not reliable to predict structure activity relationships against biofilms based on planktonic cell data.

Table 2.3: Biofilm eradication concentrations in mM of parent compounds and derivatives against *S. epidermidis* and *P. aeruginosa*.

	BEC (mM)	
Compound	*S. epidermidis*	*P. aeruginosa*
thymol (**1a**)	3.9	15.6
2-allylthymol (**1b**)	9.25	31.25
4-allylthymol (**1c**)	6.5	13
2-n-propylthymol (**1e**)	31.25	62.5
carvacrol (**2a**)	1.95	7.5
2-allylcarvacrol (**2b**)	9.25	31.25
4-allylcarvacrol (**2c**)	3.25	7.5
2-*n*-propylcarvacrol (**2e**)	31.25	62.5
eugenol (**3a**)	31.25	62.5
ortho-eugenol (**3b**)	31.25	31.25
guaiacol (**3c**)	7.8	15.6
2-methoxy-4-*n*-propylphenol (**3d**)	31.25	31.25

Unlike what was seen with MICs, the BECs of allyl derivatives were generally statistically significantly higher than the BECs of the corresponding parent compound. For example, the *p*-values for parent compounds and their allyl derivatives against biofilms were also calculated. The *p*-value of 1a/b were calculated to 0.022 be against *S. epidermidis*

and 0.019 against *P. aeruginosa*. The *p*-value of 2a/b were calculated to be 0.009 against *S. epidermidis* and 0.023 against *P. aeruginosa*. The p-value of 3a/c is 0.003 against S. epidermidis and 0.015 against P. aeruginosa.

A CDC Biofilm reactor assay was also used to substantiate the comparative efficacy of thymol and its allyl and n-propyl derivatives against *P. aeruginosa* (PA015442) (Table. 2.4). Here biofilms were grown in a high sheer environment as opposed to a static environment in 96-well plates as was done with BEC evaluations. This increases the biofilms adherence to the surface which it is grown. The CDC biofilm assay was chosen for this purpose. Methods were performed in accordance with ASTM; Designations: E 1054 – 08, E2562 - 17, and E2871 -13. The *P. aeruginosa* strain (PA015442) used in this experiment was used because it was the strain named in the ASTM procedures. Results with the CDC biofilm reactor was consistent with the relative efficacies determined in the BEC assay (Table 2. and Table 2.). Thymol (**1a**) had the highest mean log reduction, correlating with the highest potency (Table 2.4). The 2-allylthymol (**1b**) was less potent than the parent and the *n*-propyl derivative (**1e**).

Table 2.4: Mean log reductions of thymol, 2-allylthymol and 2-n-propylthymol against *P. aeruginosa* (PA015442)

Compound	Mean Log Reduction	Concentration (mM)
Thymol (**1a**)	4.48	100
2-allylthymol (**1b**)	0.13	100
2-*n*-propylthymol (**1e**)	0.21	100

MIC and BECs were also measured for strain PA015442 to compare with strain PA01 that was used with all other assays apart from the CDC biofilm reactor. As with strain

PA01, 2-allylthymol (**1b**) exhibited the highest degree of potency against planktonic cells of PA015442 with an MIC of 0.08 mM, whereas thymol (**1a**) had an MIC of 0.68 (Table 2.5). It was interesting that thymol (**1a**), 2-allylthymol (**1b**) and 2-*n*-propylthymol (**1e**) were more effective against PA015442 (Table 2.5) than against PA01 in biofilm assays; whereas thymol (**1a**) had a BEC of 15 mM, 2-allylthymol (**1b**) had a BEC of 31.25 mM and 2-*n*-propylthymol (**1e**) a BEC of 62.5 mM against the PA01 strain (Table 2.3). In planktonic cell assays, the three compounds evaluated against PA015442 were also more potent towards PA01 (Tables 2.3 and 2.5). Again, in accordance with previously observed BEC trends, thymol (**1a**) exhibited a lower BEC than both 2-allylthymol (**1b**) and 2-*n*-propylthymol (**1e**) as seen in Table 2.5.

Table 2.5: Biofilm eradication concentrations and minimum inhibitor concentrations of thymol, 2-allylthymol and 2-n-propylthymol against *P. aeruginosa* PA015442, as well as mean log reduction at 100 mM.

	Mean log reduction	MIC (mM)	BEC (mM)
Compound	PA015442		
Thymol (**1a**)	4.48	0.68	1.37
2-allylthymol (**1b**)	0.13	0.08	6.5
2-*n*-propylthymol (**1e**)	0.21	3.125	25

The addition of an allyl group to the naturally occuring phenols thymol (**1a**) and carvacrol (**2a**) increased the compounds potency towards both inhibiting and killing planktonic cells; although decreased their ability to eradicate biofilms. Similarly, the elimination of an allyl group from the essential oil eugenol (**3a**) decreased potency towards planktonic cells and increased potency towards biofilms.

Naturally occuring phenols such as thymol (**1a**), carvacrol (**2a**) and eugenol (**3a**) have been shown to present antimicrobial properties against both planktonic cells and biofilms [23, 137, 149-156]. Here we demonstrated that 2-allyl (**1b** and **2b**) and 4-allyl derivatives (**1c** and **2c)** of thymol (**1a**) and carvacrol (**2a**) showed an increase in potency in comparison to the parent compounds against planktonic cells in both growth inhibition and killing assays. In biofilm assays the opposite trend was always observed: the non-allylated, parent compounds exhibited a higher potency than the allyl derivatives. Similarly, the non-allylated guaiacol (**3c**) was less potent against planktonic bacteria but more potent than eugenol (**3a**) or ortho-eugenol (**3b**) against biofilm bacteria. These observations underscore that structure-activity relationships determined for planktonic bacteria can be completely different than those for biofilms formed by the same species.

The fact that structure-activity relationships diverge between planktonic and biofilm assays may indicate that these compounds experience different limitations to their efficacy against planktonic and biofilm forms of the bacteria. It can be for example, that the penetrations of the agents into the biofilm is rate-limiting. Alternatively it could be that the permeability of the compounds into the cytoplasm of the cell becomes rate-limiting in the biofilm mode of growth. A third possibility is that the expression of molecular targets differs between planktonic and biofilm cells. If these mechanisms were better understood, it might be possible to rationally design superior anit-biofilm anitmicrobial agents.

The 2-*n*-propyl derivatives (**1e** and **2e**) consistently were least potent compared to parent compounds and corresponding allyl derivatives. Here an allyl group will increase potency in comparison with a propyl group against both planktonic cells and biofilms. Both

thymol (**1a**) and carvacrol (**2a**) have two alkyl groups, which are weakly electron donating. Eugenol (**3a**) in comparison has a methoxy group which is strongly electron donating as well as an allyl group; studies showed that eugenol (**3a**) was less potent than thymol (**1a**) and carvacrol (**2a**) in assays evaluating potency towards killing biofilms and inhibiting planktonic cell growth. Although was more efficacious towards killing planktonic cells, this is likely due to the presence of an allyl group.

Thymol (**1a**) and carvacrol (**2a**) are constitution isomers and had identical MICs against both *S. epidermidis* and *P. aeruginosa* (Table 2.1). The same was observed with their 2-allyl derivatives (**1b** and **2b**), as well as both 4-allyl derivatives (**1c** and **2c**) (Table 2.1). The 2-allyl derivative of thymol (**1b**) though, was less efficient in killing, as shown in Table 2., where the mean log reduction of thymol is 5.1 at 7.8 mM but the mean log reduction for carvacrol is 5.1 at 1.7 mM, although carvacrol (**2a**) was less potent than thymol (**1a**) (Table 2.).

In biofilm eradication assays, carvacrol (**2a**) was more potent than thymol (**1a**) against both bacteria (Table 2.). 4-Allylcarvacrol (**2c**) was also more potent than 4-allylthymol (**1c**), although the 2-allyl derivatives exhibited the same BEC. Overall, there was very little difference in changing the allyl group from the 2 to the 4 position. The decreased potency of thymol against biofilms may result from the isopropyl group in the 2 position (Figure 2.), creating more steric hindrance around the phenol. This would suggest that steric hindrance around the phenol has more of an effect with assays involving biofilms. Steric hindrance may play two different roles here; inhibiting permeation through the biofilm extracellular matrix, and obstruction of the alcohol group. These also may

contribute to the observed decrease in potency with 2-allyl and 4-allyl derivatives against biofilms when compared to their parent compounds. The addition of an allyl group does slightly increase polarity, which may also inhibit the compounds ability to permeate through the biofilm matrix.

In the case of eugenol (**1b**), the 2-allyl derivative, "ortho-eugenol" (**3b**) was more potent in inhibiting planktonic cells of *S. epidermidis* although they shared the same BEC against *S. epidermidis.* **3b** also had lower BEC with *P. aeruginosa* with both planktonic cells and biofilms. This observation was in accordance with the trend found in thymol (**1a**) and carvacrol (**2a**) in which allyl derivatives were less affective against biofilms.

In this study, mammalian cells were not evaluated, although oral LD_{50} (median lethal dose) for carvacrol and thymol has been calculated in rats to be 810 mg/kg body weight and 980 mg/ kg body weight, respectively[173]. Cytotoxicity of carvacrol and thymol was also evaluated against intestinal cells (Caco-2), finding no cytotoxic effects of thymol although carvacrol was found to cause cell death[174]. A study by Machado et. al.[175] concluded that eugenol did not exhibit cytotoxicity in vitro towards mammalian cells at the IC_{50} determined for growth inhibition for *G. lamblia*.

Various structure activity relationships of antimicrobial compounds, including natural products and plant metabolites, and their potency towards planktonic cells and biofilms have been evaluated [176-184]. Richards et al. [178] synthesized and assayed a 50-compound library of oroidin-based natural products for their anti-biofilm activity against two strains of *P. aeruginosa,* classifying several compounds as inhibitors of biofilm formation.

Structural factors such as stereochemistry, alkyl chain length and subsitution patterns have also been examined in the context of biofilms [176, 181-182]. No uniform correlation of biofilm potency with planktonic potency is evident. Some studies show equipotent activity of compounds against planktonic cells and biofilms [180, 185], while other reports provide support that biofilms are more resistant to anitmicrobials than planktonic cells [1-2, 186-187] and still others have found that compounds exhibiting an increase in activity against planktonic cells also show increased potency against biofilms [181]. The present study demonstrates that essential oil derivatives exhibiting greater activity against planktonic cells were often less effective when tested against biofilms.

2.5 Conclusion

The presence of an allyl group in either the 2 or 4 position relative to the hydroxy phenol increases the potency of the small, phenolic essential oils thymol (**1a**) and carvacrol (**2a**) when evaluated against planktonic cells of both the Gram-positive *S. epidermidis* and the Gram-negative *P. aeruginosa*. In contrast, when the same compounds were evaluated against biofilms, the parent compounds were more potent. Similarly, eugenol (**3a**), which has an allyl group in the 4 position, was more potent than guaiacol (**3c**) against *S. epidermidis* in planktonic cell inhibition assays although less effective in killing planktonic cells and biofilms. The 2-methallyl derivatives (**1d, 2d** and **3g**) evaluated against planktonic cells were in all cases less potent than allyl; and when compared to propyl derivatives, were less potent the majority of the time. This study illustrates the importance of using biofilm assays to determine structure-activity relationships of antimicrobials when the end target is a biofilm.

2.6 Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

2.7 Acknowledgements

Generous funding for this research was provided by the National Institute for General Medical Science.

CHAPTER THREE

3. SULFENATE ESTERS OF SIMPLE PHENOLS EXHIBIT ENHANCED ACTIVITY AGAINST BIOFILMS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author and corresponding author: Danica J. Walsh

Contributions: Designed experiments, performed experiments, analyzed data. Organized, prepared and wrote manuscript.

Co-Author and corresponding author: Thomas Livinghouse

Contributions: Performed experiments, preparation of manuscript, corresponding author.

Co-Author: Greg M. Durling

Contributions: Performed research, analyzed data.

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Contributions: Performed research.

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Contributions: Performed research.

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Contributions: Designed experiments, preparation of manuscript.

Manuscript Information

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Status of Manuscript:
____ Prepared for submission to a peer-reviewed journal
_X_ Officially submitted to a peer-reviewed journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Accepted, ACS Omega, Feb. 2020

3.1 Abstract

The recalcitrance exhibited by microbial biofilms to conventional disinfectants has motivated the development of new chemical strategies to control and eradicate biofilms. The activity of several small phenolic compounds and their trichloromethylsulfenyl ester derivatives were evaluated against planktonic cells and mature biofilms of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Some of the phenolic parent compounds are well-studied constituents of plant essential oils, for example, eugenol, menthol, carvacrol and thymol. The potency of sulfenate ester derivatives was markedly and consistently increased both towards planktonic cells as well as biofilms. The mean fold difference between parent and derivative minimum inhibitory concentration against planktonic cells was 44 for *S. epidermidis* and 16 for *P. aeruginosa*. The mean fold difference between parent and derivative biofilm eradication concentration for twenty-two tested compounds against both *S. epidermidis* and *P. aeruginosa* was 3. This work demonstrates the possibilities of a new class of biofilm-targeting disinfectants deploying a sulfenate ester functional group to increase antimicrobial potency towards microorganisms in biofilms.

3. 1. Introduction

Biofilms are multicellular communities that form when planktonic cells adhere to a surface via cell adhesions structures such as pili or flagella [188-189]. Attached cells begin to secrete extracellular DNA, proteins and polysaccharides to form an extracellular polymeric substance (EPS), which traps nutrients while providing protection from antimicrobials, disinfectants and host immune defences [190-194]. In the biofilm interior, cells experience slow growth rates or become dormant and are able to persist when other cells in the biofilm are

killed. These persistent cells are able to regenerate the biofilm, resulting in chronic infection, and contribute greatly to the refractory characteristics of biofilms [6, 195-198]. Reactive antimicrobial agents may be retarded in their penetration if they are neutralized as they diffuse into the biofilm [199-203]. These factors all contribute to increased tolerance towards antibacterial agents and disinfectants [71, 197, 204-208]. It is traits such as these and biofilms' prominence in hospitals that lead to elevated efforts to control biofilms with small molecules [209].

Over the last two decades the number of hospital-acquired infections has increased by 36% in the US, further stressing the need for novel disinfectants [210]. Routine disinfectants that are currently used in hospitals include hydrogen peroxide, sodium hypochlorite, chlorine and quaternary ammonium salts; although many of these have serious shortcomings when treating biofilms. For example, several studies have shown that *P. aeruginosa* and *E. coli* biofilms exhibit resistance toward hydrogen peroxide [73-75]. Bacterial strains prevalent in hospitals such as *S. aureus* and *P. aeruginosa* have also been shown to exhibit tolerance toward many quaternary ammonium salts such as benzalkonium chloride, benzyldimethyltetradecylammonium chloride, and didecyldimethylammonium bromide[76-78]. Chlorine and chlorine dioxide have been shown to have limited potency toward biofilms due to their inability to fully penetrate through the robust EPS, thus being unable to reach the inner layers of the biofilm [4-5]. Essential oils such as thymol and eugenol are used as environmentally friendly disinfectants to control *S. aureus* biofilms, although are used at high concentrations in order to be effective [158].

Phenols are a well-studied class of organic compounds which have been shown to demonstrate varying degrees of antimicrobial activity [171, 211-212] and were chosen here due to a wide variety of structurally diverse phenols being previously evaluated for biological activity [15, 83, 139-140, 213-217]. Among these activities, phenols have been shown to disrupt the cell membrane causing cell lysis, resulting in cell death [17, 83-84, 139]. Phenols have also been shown to attack cytoplasmic targets by denaturing proteins and deactivating enzymes, thereby binding to them to form inoperative complexes [140, 217]. The majority of phenols selected for this study were done so for their previously known antimicrobial activity towards planktonic cells. The essential oils thymol, menthol, carvacrol and eugenol were chosen for their inhibitory and antimicrobial properties against a wide range of taxonomically diverse bacteria [19, 218-220]. These essential oils were also chosen due to being found in several edible herbs and products such as mouth wash, which reduces the likelihood derivatives will be significantly more cytotoxic to mammalian cells [132, 221] . Halogenated phenols were chosen because of their extensive evaluation and high activity [16, 214]. Select alkylphenols were chosen for their antimicrobial and antifouling activity [222-223]. Several alkoxyphenols were selected since a variety alkoxy phenols have been previously studied for antimicrobial activity [224-225]. Two non-phenolic compounds were also chosen for this study, menthol for its structural similarity to thymol and 5-fluoro-2-((trichloromethyl)thio)isoindoline-1,3-dione (**21b**) for its similarity to the fungicide Folpet.

The trichloromethylanesulfenimide group that has been employed here on select phenols is the active antimicrobial pharmacophore in the broad-spectrum commercial fungicides Captan and Folpet. Captan and Folpet are phthalimide derivatives and are

commonly used for protection of fruit and vegetable crops. Their activity is attributed to their reactivity with thiols and are active against a wide range of fungal diseases [226-227]. Significantly, these compounds have been shown *not* to possess carcinogenic, mutagenic, or teratogenic threats to humans [27].

In this study, the trichloromethylsulfenate esters of a variety of phenolic compounds were synthesized and evaluated against both planktonic cells and biofilms. The bacteria chosen for evaluation were *P. aeruginosa*, a Gram-negative bacterium and *S. epidermidis*, a Gram-positive bacterium. These bacteria were selected for their prevalence in hospitals [228-230] as well as their propensity to form biofilms [13-14, 231]. Although the concept of biofilms was presented as early as the 1960's, the study of behavioural variations in biofilms such as, nutrient uptake, gene expression and increased tolerance did not arise until more recently [70, 232-234]. Biofilm research is an emerging field which has been rapidly gaining interest in light of new technologies in 3D modelling, imaging, anti-biofilm strategies and analytical tools [235-237] as well as recent research emphasizing clinical relevance [2, 233]. These advancements shed light on the need for novel strategies to treat and eradicate of biofilms including antibacterial small molecules, peptides, and lipids [238-243].

3.2. Materials and Methods

3.2.1 Synthetic reagents and bacteria

All organic reagents for chemical synthesis were purchased from commercial sources and used as received without further purification. *P. aeruginosa* (PA01 and PA015542) and *S. epidermidis* (35984) were obtained from American Type Culture Collection (ATCC). All bacteria were sub-cultured onto tryptic soy agar (TSA) plates and

incubated at 37 °C for 24 h. Single colonies were transferred from the plates and inoculated into 25 mL tryptic soy broth (TSB) in Erlenmeyer flasks. Cultures were incubated at 37 °C for 24 h and 10 µL of culture was transferred into 25 mL of TSB. The absorbance was read at 600 nm (OD_{600}) using a spectrophotometer and standardized to 10^6-10^7 CFU/mL.

3.2.2 Efficacy of phenols and derivatives on inhibiting planktonic cells

Minimum inhibitory concentrations (MICs) of all compounds were evaluated using a 96-well plate assay previously described by Xie [171]. 96-well plates were inoculated with bacterial culture, prepared as stated in section 2.1, followed by exposure to the phenol or sulfenate. Plates were incubated at 37 °C for 12 h. A plate reader was used to analyze bacterial inhibition. Experiments were done in biological triplicate with technical duplicates. Tests for statistical significance were calculated with a two-tailed t-test assuming unequal variances.

3.2.3 Efficacy of phenols and derivatives on biofilms

As previously published by Walsh. et. al. [244]: "Both strains were cultured as described above and biofilms were grown in Costar polystyrene 96-well plates at 37 °C. After 24 h of incubation, the planktonic-phase cells were gently removed and the wells were washed three times with PBS. Wells were filled with 150 µL dilutions of the compound being evaluated. The 96-well plates were incubated for an additional 12 h at 37 °C. The media was gently removed and each well filled with 150 µL PBS and the biofilm broken up through stirring with sterile, wooden rods. Three tenfold dilutions of each sample were drop plated on TSA plates and incubated for 24 h. The biofilm eradication

concentration (BEC) was determined to be the lowest concentration at which no bacterial growth occurred. This procedure was modelled on previously reported procedures according to Pitts [172]. Negative controls were also conducted with 150 µL PBS in the absence of disinfecting agents. Experiments were done in biological triplicate with technical duplicates."

3.2.4 Measuring rate of hydrolysis of sulfenate derivatives

(4-Fluorophenoxy)trichloromethylsulfane (**15b**) (13 mg, 0.05 mmol) was dissolved in water (1 mL). An aliquot was taken every 12 h and dissolved in D_2O in an NMR tube. F^{19} NMR was performed to measure the appearance of the parent compound, *p*-fluorophenol (**15a**) in the sample. A 0 h ^{19}F NMR spectrum of the sulfane derivative (**15b**) was taken, as was a spectrum of the pure parent compound (**15a**) for reference (Figure 2). Technical triplicates were done.

3.2.5 Synthesis of (2,4-dimethylphenoxy)trichloromethyl sulfane (3b).

A 25 mL round-bottomed flask equipped with a magnetic stirring bar was charged with 2,4-dimethylphenol (610 mg, 5 mmol, 1 equiv) and anhydrous diethyl ether (10 mL). The mixture was cooled to 0 ^{o}C and anhydrous triethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added. To the stirred mixture was added trichloromethyl hypochlorothioite (0.57 mL, 5.25 mmol, 1.05 equiv) dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm to room temperature and stirred for an additional 12 h. To the resulting mixture was added pentane (5 mL) which was then filtered through celite and washed with *t*-butyl methyl ether (3 X 5 mL). The solvents were evaporated in vacuo to

provide the title compound (1.03 g, 76%) as a yellow oil. ^{1}H NMR (300 MHz, CDCl3): δ 7.23 (dd, *J* = 843, 2.62 Hz, 1H), 7.00 (dd, *J* = 2.62, 0.45 Hz, 1H), 6.98 (dd, *J* = 8.43, 0.45 Hz, 1H), 2.39 (s, 3H), 2.31 (s, 3H). C^{13} NMR (500 MHz, CDCl3): δ 16.16 (CH3), 20.55 (CH3), 116.19 (C), 121.49 (CH), 130 (C), 132.02 (C), 133.81 (CH), 136.73 (CH), 156.05 (C).

3.3 Results and discussion

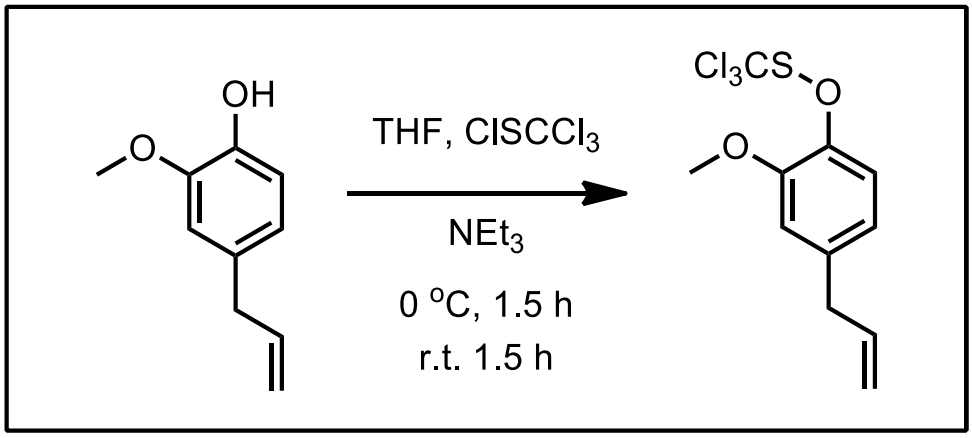


Figure 3.1 Representative synthesis, using eugenol (**8a**).

In this study, 25 sulfenate esters of small phenols were synthesized. This synthesis was accomplished by treating each phenol with trichloromethyl hypochlorothioite in either THF or diethyl ether with triethyl amine at 0 °C for 1.5 h and then allowing the reaction mixture to stir for 1.5 h at room temperature (Figure 3.1).

Sulfenate esters were more potent than their parent compounds 92 percent of the time. For example, on average, trichloromethylsulfenate esters were 9 times more potent than parent compounds against *S. epidermidis* and 17 times more potent towards *P. aeruginosa* in planktonic assays (Figure 3.2). Against biofilms, sulfenate esters were on

average 4 times more potent towards *S. epidermidis* and 3.8 times more potent towards *P. aeruginosa*. It was also observed that towards biofilms, phenols and sulfenates were less potent compared to planktonic cells, a phenomenon that has widely been observed in previous studies [190, 231, 234, 245-247]. The relative potencies of the precursor phenols and the corresponding sulfenate esters against both planktonic cells and biofilms will be discussed in turn.

3.3.1 Disinfectant activities in the planktonic state

3.3.1.1 Parent phenols The most potent parent phenols against planktonic cells were 4-heptyloxyphenol (**7a**), 4-chloro-2-methylphenol (**16a**), 3,4-dichlorophenol (**14a**), 2,4-dimethylphenol (**3a**), 6-(1-methylethyl)-3-methylphenol (thymol) (**1a**), and 3-(1-methylethyl)-6-methylphenol (carvacrol) (**4a**) against both *S. epidermidis* and *P. aeruginosa* (Figure 3.2). Compounds **16a** and **14a** both possess at least one chlorine group on the aromatic ring, and although *p*-fluorophenol (**15a**) also possesses a halogen on the aromatic ring, it was significantly less potent against both *S. epidermidis* and *P. aeruginosa* (Figure 3.). This is congruous with previous studies demonstrating that chlorine, which is more electron withdrawing than fluorine, increases the potency of the parent phenols to a greater extent[214]. Compounds **1a**, **3a** and **4a** all have either isopropyl or methyl groups in both the *ortho* and *para* positions while **7a** has a para heptyloxy group (Figure 3.1). In contrast to **1a**, **3a** and **4a**, compound **5a** (2,6-diisopropylphenol) has two isopropyl groups in the *ortho* positions, and possesses significantly lower potency. This is likely due to the higher degree of steric hindrance around the phenolic hydroxyl.

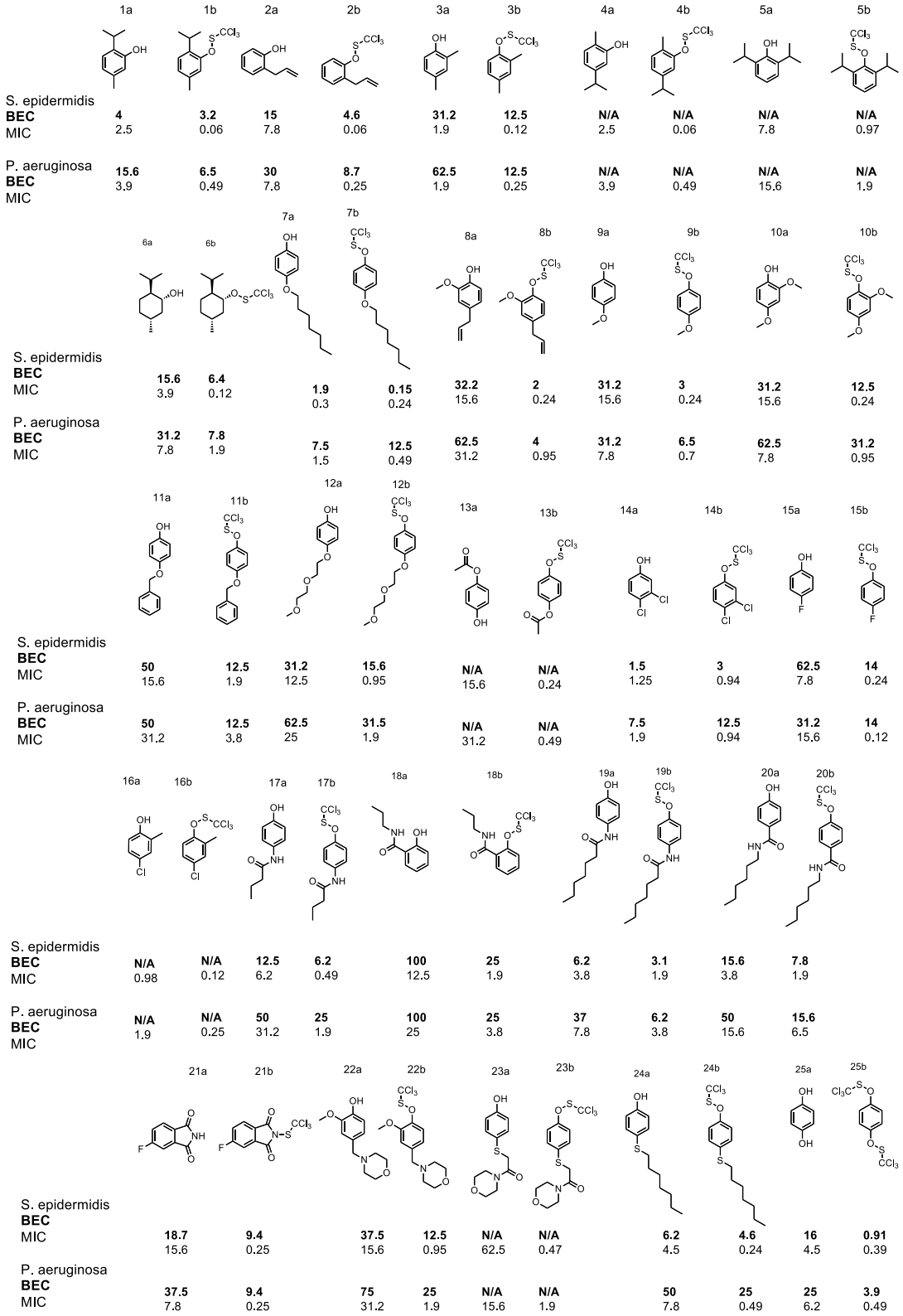


Figure 3.2 Parent phenols and corresponding sulfenate esters and their MICs and BECs

Compound **7a** had the lowest MIC towards both bacteria, making it of particular interest since it is considerably more active than the corresponding 4-methoxy and 2,4-dimethoxy derivatives, **9a** and **10a** (Table 3.1), which are less lipophilic. In light of this observation, two additional compounds in this series were synthesized and examined to evaluate the influence of lipophilicity on activity; these being 4-(benzyloxy)phenol (**11a**) and 4-(2-(2-methoxyethoxy)ethoxy)phenol (**12a**). Compound **12a** was chosen for its near identical side chain length, when compared to **7a**, and the increased hydrophilicity imparted by the oxygens within the side chain. Significantly, both of these structural alterations led to a marked decrease in activity compared to **7a** (Table 3.1). In addition, **24a,** which differs from **7a** solely by possessing a sulfur in place of oxygen, was evaluated and was found to be less potent than **7a** against both bacteria.

Table 3.1 MICs of phenols 7a, 9a, 10a, 11a, 12a and 24a.

Minimum Inhibitory Concentrations (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
7a	0.3	1.5
9a	15.6	7.8
10a	15.6	7.8
11a	15.6	31.2
12a	12.5	25
24a	4.5	7.8

Based on these results, a second SAR study was conducted with compounds **17a**, **19a** and **20a**, which all possess an amide chain in either the *para* or *ortho* position (Table 3.2). Compounds **17a** and **19a** were chosen to compare amide chain length, as **17a** has a 4-butanamide group while **19a** has a 4-heptanamide group, which is predicted to increase lipophilicity. Compound **20a** was chosen for the comparison of nitrogen placement in the

amide side chain, vis a vis "amide inversion". Accordingly, compound **19a** (N-(4-hydroxyphenyl) heptanamide) has the amide nitrogen on the aromatic ring, while **20a** (N-hexyl-4-hydroxybenzamide) has the carbonyl of the amide group attached to the aromatic ring. This structural alteration was performed to access effects of electron donating and electron withdrawing amide groups of the same length. An additional compound, **18a,** was also evaluated for similar reasons to compare to **17a**, although here the butanamide group was placed in the 2- position.

Table 3.2 MICs of phenols 17a, 18a, 19a, 20a.

Minimum Inhibitory Concentrations (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
17a	6.2	31.2
18a	12.5	25
19a	3.8	7.8
20a	3.8	15.6

Phenol **19a** was the most potent compound in this series overall, although **20a** shared the same potency against *S. epidermidis* (Table 3.2). It was observed that, **19a** was more potent than **17a** towards both bacteria, continuing the trend seen earlier that longer alkyl chain length does increase potency towards planktonic cells. Between **17a** and **18a**, **17a** was the more potent isomer against *S. epidermidis* but not against *P. aeruginosa*. This evaluation also suggests that the length of the alkyl chain has a greater effect than nitrogen placement with respect to the aromatic ring.

(-)-Menthol (**6a)** was selected due to its structural similarities to thymol (**1a)**, and since it is *non-phenolic*. Menthol, like thymol, has been studied for its antimicrobial activity [248-249]. It should be noted that **6a** was far less active than **1a** toward both bacterial strains used in this study.

3.3.1.2 Trichloromethylsulfenate esters It was observed that, in general, more potent parent phenols produced more potent sulfenate esters, although this trend could not reliably be used to predict activity in all cases. The most potent sulfenates against *S. epidermidis* were, in descending order, **1b**, **2b**, **4b**, **3b** and **16b.** For *P. aeruginosa* the most potent sulfenates were **15b**, **2b**, **3b** followed by **16b**. As seen with the parent phenols, the sulfenate esters **3b,** and **16b** are among the most potent disinfecting agents towards planktonic cells of both bacteria (Figure 3.2). Likewise, **1a**, **3a** and **4a** were also among the most potent towards planktonic *S. epidermidis*. In contrast, sulfenates **2b** and **15b** showed significant potency when their parent phenols did not. Conversely, phenols **7a** and **14a** showed exceptional potency towards both bacteria while the corresponding sulfenates **7b** and **14b** did not share this characteristic (Figure 3.2).

In consonance with the prior SAR study, the series of sulfenate esters **7b**, **9b**, **10b**, **11b**, **12b** and **24b** showed a similar trend to the corresponding phenols towards planktonic cells. Derivatives **7b, 9b, 10b** and **24b** shared the highest potency towards *S. epidermidis* while **7b** and **24b** were the most potent towards *P. aeruginosa* in this series (Table 3.3). Sulfenate **11b** was least potent towards both bacteria, as was seen with phenols. It was interesting to note that, in contrast to sulfenates, the 4-alkoxyphenol **7a** was significantly more potent than its 4-(thioalkyl) counterpart, **24a** (Table 3.1).

Table 3.3 MICs of sulfenates 7b, 9b, 10b, 11b, 12b, 24b.

Minimum Inhibitory Concentrations (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
7b	0.24	0.49
9b	0.24	0.7
10b	0.24	0.95

11b	1.9	3.8
12b	0.95	1.9
24b	0.24	0.49

In the SAR study involving amides, a trend consistent with parent compounds was not observed. Compound **17b** was the most potent sulfenate towards both bacteria, whereas the most potent parents were **19a** and **20a** against *S. epidermidis* and **19a** towards *P. aeruginosa* (Table 3.4). Similarly, **20b** was the least potent sulfenate in the amide series, while the least potent parent was **18a** towards *S. epidermidis* and **17a** towards *P. aeruginosa*. This further demonstrates that trends in parent compounds cannot necessarily be used to predict trends in their corresponding sulfenate ester derivatives.

Table 3.4 MICs of sulfenates 17b, 18b, 19b, 20b.

Minimum Inhibitory Concentrations (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
17b	0.49	1.9
18b	1.9	3.8
19b	1.9	3.8
20b	1.9	6.5

Compounds **6b** and **21b** were evaluated separately against planktonic cells. Compound **6b** was among the six most potent sulfenates against *S. epidermidis*, sharing an MIC with **3b** and **16b** (Figure 3.2). Compound **21b** was in the five most potent compounds towards *P. aeruginosa*, sharing an MIC with **2b**, **3b** and **16b**.

MICs of sulfenate esters were, in general, statistically significantly lower than the MIC of the parent phenols. A two-tailed t test was performed on four select phenol/sulfenate ester pairs; **3a**/**b**, **10a**/**b**, **13a**/**b** and **16a**/**b**. Compounds **10a**/**b** and **13a**/**b** were chosen due to the large discrepancy in potency observed between the parent phenol

and the sulfenate. Compounds **3a**/**b** and **16a**/**b** were chosen because the potency difference between parent phenols and sulfenate esters was the least dramatic of the 25 compound pairs evaluated. The *p*-value of **2a**/**b** against *S. epidermidis* was calculated to be 0.043 and against *P. aeruginosa* 0.0028. The *p*-value of **10a**/**b** against *S. epidermidis* is 0.039 and against *P. aeruginosa* is 0.031. The *p*-value of **13a**/**b** against *S. epidermidis* is 0.0039 and against *P. aeruginosa* was calculated to be 2.8×10^{-5}. The *p*-values for **16a**/**b** were 0.00051 and 0.0028 against *S. epidermidis* and *P. aeruginosa* respectively.

3.3.2 Disinfectant activity against biofilms

For comparison, the anti-parasitic drug nitazoxanide and the antibiotics metronidazole and tobramycin were evaluated for activity towards *S. epidermidis* and *P. aeruginosa* biofilms. Nitazoxanide is an antidiarrheal commonly used to treat strains of *Cryptosporidium*, *Blastocystis* and *Giardia* and is believed to interfere with pyruvate:ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction [250-252]. Nitazoxanide has also been shown to inhibit biofilm formation in S. epidermidis [253] and enteroaggregative *Escherichia coli* [254] as well as decrease the viability of *Clostridioides difficile* biofilms [255]. However, the efficacy of this drug to eradicate biofilms has yet to be evaluated towards *S. epidermidis* or *P. aeruginosa*. The BEC of nitazoxanide was found to be 50 mM towards *S. epidermidis* and 3.12 mM towards *P. aeruginosa.*

Metronidazole is a nitroimidazole used to treat a variety of bacterial and parasitic infections and is most commonly used to treat infections related to inflammatory disorders of the gastrointestinal tract. Metronidazole is often used to treat Gram-negative, Gram-positive and Gram-variable anaerobic bacteria, as well as protozoans such as *Giardia*

lamblia [256]. It has been shown to exhibit lower activity towards biofilms such as that of *Helicobacter pylori* [257] and *Clostridium difficile*, [258-259] although it has also been evaluated in tandem with several other antibiotics, resulting in improved activity against *Enterococcus faecalis* and *Candida albicans* biofilms [260]. Against *S. epidermidis* the BEC of metronidazole was 6.25 and against *P. aeruginosa* was 50mM.

Tobramycin is an antibiotic that inhibits protein synthesis, used to treat Gram-negative bacteria. Tobramycin is commonly used to treat bacterial *pneumonia*, bacterial eye infections and has been extensively studied against biofilms [261-262]. A study by Høiby et. al. showed that the inhibitory properties towards *P. aeruginosa* biofilms were lower than that towards planktonic cells, concluding that biofilms are tolerant to the clinically recommended dose of the antibiotic [263]. Sans-Serramitjana et. al. evaluated the antimicrobial activity of nanoencapsulated tobramycin; finding that nanoencapsulation did improve the ability of tobramycin to eradicate *P. aeruginosa* biofilms and suggested the strategy of lipid carries as delivery vehicles to overcome drug resistance to tobramycin [264]. Tobramycin was in part chosen due the known antimicrobial resistance of *P. aeruginosa*, making it a valuable comparison to this series of novel antimicrobials [265-267].

The BEC of nitazoxanide was 50 mM towards *S. epidermidis* and 3.12 mM towards *P. aeruginosa*, the BECs of metronidazole were 6.25 mM and 50 mM towards *S. epidermidis* and *P. aeruginosa* respectively. Tobramycin has a BEC of 18 mM towards *S. epidermidis* and 0.6 mM and *P. aeruginosa*. Tobramycin was found to have a BEC of 18 mM towards *S. epidermidis* and 0.6 mM and *P. aeruginosa*.

3.3.2.1 Parent phenols Based on the observations from planktonic cell assays, eighteen parent phenols and the corresponding sulfenate esters, were chosen for evaluation against biofilms. Compounds **1a**, **3a**, **7a**, and **14a** were chosen for their high potency towards planktonic cells (Figure 3.2). Compounds **2a**, **8a**, **15a, 10a, 17a** and **22a** were selected due to the large increase in potency between the moderately active parent phenols and corresponding sulfenate esters (Figure 3.2). Compounds **7a**, **9a**, **10a**, **11a**, **12a, 24a, 17a, 18a, 19a** and **20a** were selected for the purpose of continuing the two SAR studies conducted with planktonic cells. Compound **25a** was chosen since the corresponding sulfenate ester possess two trichloromethylsulfenate ester groups. Additionally, the non-phenolic compounds **6a** and **21a** were chosen, **6a** as an aliphatic alcohol bearing structural similarity to **1a** and **21a** for its similarity to the imide corresponding to the commercial fungicide Folpet. Neither compound showed significant activity towards either bacterium. As a continuation of the previous SAR study involving phenols **7a**, **9a**, **10a**, **11a**, **12a** and **24a** against planktonic cells**,** this series was subsequently evaluated against biofilms (Table 3.5). In accordance with planktonic trends, **7a** was the most potent phenol in this series towards both bacteria.

Table 3.5 BECs of phenols 7a, 9a, 10a, 11a, 12a and 24a.

Biofilm Eradication Concentration (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
7a	1.9	7.5
9a	31.2	31.2
10a	31.2	62.5
11a	50	50
12a	31.2	62.5
24a	6.2	50

In the SAR study involving amides, **19a** was the most potent phenol towards both bacteria. In contrast, **18a** was the least potent compound towards both bacteria, whereas in planktonic assays **17a** was least potent towards *P. aeruginosa*. In both SAR studies, the more potent phenols against planktonic cells did in general have lower BECs as well, though the trend in potency was not always predictable for all compounds (Table 3.6).

Table 3.6 BECs of phenols 17a, 18a, 19a and 20a.

Biofilm Eradication Concentration (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
17a	12.5	50
18a	100	100
19a	6.2	37
20a	15.6	50

Among the additional compounds selected for biofilm evaluations, the majority were alkyl phenols, along with two halophenols and hydroquinone (Table 3.7). The most potent phenols towards *S. epidermidis* biofilms were, in descending order, **14a, 7a**, **1a**, **19a** and **24a.** Against *P. aeruginosa*, the most potent phenols were **14a, 7a**, **1a**, **25a**, and **2a**; whereas here, **14a** and **7a** shared the same BEC. Out of these seven compounds, only three (**1a**, **7a** and **14a**) were among the active most against planktonic cells. These results further reveal that activity towards planktonic cells cannot be reliably used to predict potency towards biofilms.

Table 3.7 BECs for allyl- and halo-phenols as well as hydroquinone

Biofilm Eradication Concentration (mM)		
Compounds	S. epidermidis	P. aeruginosa
1a	4	15.6
2a	15	30
3a	31.2	62.5

8a	31.2	62.5
14a	1.5	7.5
15a	62.5	31.2
22a	37.5	75
25a	16	25

3.3.2.2 Sulfenate esters The most potent sulfenate esters towards *S. epidermidis* were **7b**, **25b**, **8b**, **9b** and **14b**. For *P. aeruginosa* the most active sulfenates were **25b**, **8b**, **19b**, **9b** and **1b.** Interestingly, out of these seven compounds, none were among the most potent towards planktonic cells, which was unexpected as it differs from the trend observed with parent phenols. However, there were similarities between most potent phenols and sulfenates towards biofilms. For example, the phenols corresponding to sulfenates **1b**, **7b**, **14b**, **19b** and **25b** were among the most potent parents.

In consonance with the previous SAR study involving 4-alkoxyphenols, sulfonates **7b, 9b, 10b, 11b, 12b** and the 4-(heptylthio)phenyl sulfenate **24b** were evaluated towards biofilms (Table 3.8). Sulfenate **7b** was the most potent compound against biofilms in this series. Against planktonic cells however, **7b** had the same MIC as **9b, 10b** and **24b** against *S. epidermidis* and **24b** against *P. aeruginosa* (Table 3.). This observation supports the finding that long, saturated alkoxy chains generally increase potency more so than a diethylene glycol derived chain or a benzyl group. It is also noteworthy that the replacement of the oxygen by sulfur (e.g. **7b** → **24b**) results in a substantial decrease in activity.

Table 3.8 BECs for sulfenates 7b, 9b, 10b, 11b, 12b and 24b.

Biofilm Eradication Concentration (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*

7b	0.15	2.5
9b	3	6.5
10b	12.5	31.2
11b	12.5	12.5
12b	15.8	31.2
24b	4.6	25

The SAR study involving amides showed that **19b** was the most potent derivative towards both bacteria, which is not congruent with what was observed with planktonic cells, where **17b** was the most active (Table 3.9). Compound **18b** was the least potent sulfenate in this series against biofilms while **20b** was least potent towards planktonic cells.

Table 3.9 BEC for sulfenates 17b, 18b, 19b and 20b.

Biofilm Eradication Concentration (mM)		
Compounds	*S. epidermidis*	*P. aeruginosa*
17b	6.2	25
18b	25	25
19b	3.1	6.2
20b	7.8	15.6

Sulfenates selected for biofilm evaluations which were not part of the two preceding SAR studies, are assembled in Table 3.10. Among these, monosulfenates **8b** and **1b** were the most active towards both strains of bacteria. It is not surprising that that bis(sulfenate) **25b** showed excellent activity towards biofilms as well. Sulfenate **22b**, which contains a basic morpholine group, exhibited low potency against both bacterial strains. In this case, it had been hoped that the presence of a basic amine might increase permeability by way of protonation, resulting in enhanced solubility. Non-phenolic sulfenates **6b** and **21b** were also evaluated towards biofilms (Figure 3.2). Neither compound showed significant activity, with **6b** showing only half the potency of **1b** towards *S. epidermidis*.

Table 3.10 BECs for allyl- and halo- sulfenates as well as the bis(sulfenate) 25b.

Biofilm eradication concentration (Mm)		
Compounds	*S. epidermidis*	*P. aeruginosa*
1b	3.2	6.5
2b	4.6	8.7
3b	12.5	12.5
8b	2	4
14b	3	12.5
15b	14	14
22b	12.5	25
25b	0.91	3.9

3.3.2.3 Comparison of phenols and sulfenates Among parents and sulfenates chosen for the alkoxy and alkylthio side chains SAR study, (**7a**/**b**, **9a**/**b**, **10a**/**b 11a**/**b, 12a**/**b** and **24b**) it was shown that the more potent phenols did typically produce more potent sulfenate esters when evaluated against biofilms. The exception to this being **12a** which has a lower BEC than **11a,** while **11b** has a lower BEC than **12b** against *S. epidermidis* biofilms. It is therefore evident that **7a**/**b** were the most potent compounds in this series overall. In the amide SAR study, between **17a**/**b** and **19a**/**b**, it was observed that **19a**/**b** was typically more potent than **17a**/**b** with the exception of **19b** being less potent towards planktonic *P. aeruginosa*. This relationship demonstrates that increasing alkyl chain length, as with **7a**/**b**, will in general increase potency of phenols and sulfenates. Between the isomers **19a**/**b** and **20a**/**b**, **19a**/**b** were generally the more potent isomers in both planktonic and biofilm assays, although possessing the same MICs towards *S. epidermidis*.

Overall, a correlation between increased potency towards planktonic cells leading to increased potency in biofilms was observed through evaluation of phenols and corresponding sulfenate esters, with the exception of **14a**/**b** and **7a/b** against *P. aeruginosa*

biofilms (Figure 3.). This type of relationship has been previously described by others as well [181], although it has also been observed that activity towards planktonic cells cannot reliably be used to predict that same compounds potency against biofilms. This has been demonstrated most recently by Walsh et. al. (2019) [244] and is further supported here by the foregoing results.

The BECs of sulfenate esters when compared to their corresponding parent phenols were generally statistically significantly lower. A two-tailed t test was performed on four select phenol/sulfenate ester pairs; **8a**/**b**, **9a**/**b**, **12a**/**b** and **17a**/**b**. Compounds **8a**/**b** and **9a**/**b** were chosen due to the large discrepancy in potency observed between the parent phenol and the sulfenate. Compounds 1**2a**/**b** and **17a**/**b** were chosen because the potency between parent phenols and sulfenate esters was the least dramatic of the 25 compound pairs evaluated. The *p*-value of **8a**/**b** against *S. epidermidis* was calculated to be 0.00012 and against *P. aeruginosa* 0.044. The *p*-value of **9a**/**b** against *S. epidermidis* is 0.044 and against *P. aeruginosa* is 0.0091. The *p*-value of **12a**/**b** against *S. epidermidis* is 0.0038 and against *P. aeruginosa* was calculated to be 0.019. The *p*-value for **17a**/**b** was 0.019 against both *S. epidermidis* and *P. aeruginosa*.

3.3.3 Analysis of sulfenate degradation

Sulfenate esters are expected to hydrolyze to the parent phenols in aqueous solutions via cleavage of the S-O bond. In a study to determine the hydrolytic stability of sulfenates, the decomposition of (4-fluorophenoxy)trichloromethylsulfane (**15b**) in water was monitored via ^{19}F NMR (Figure 3.3

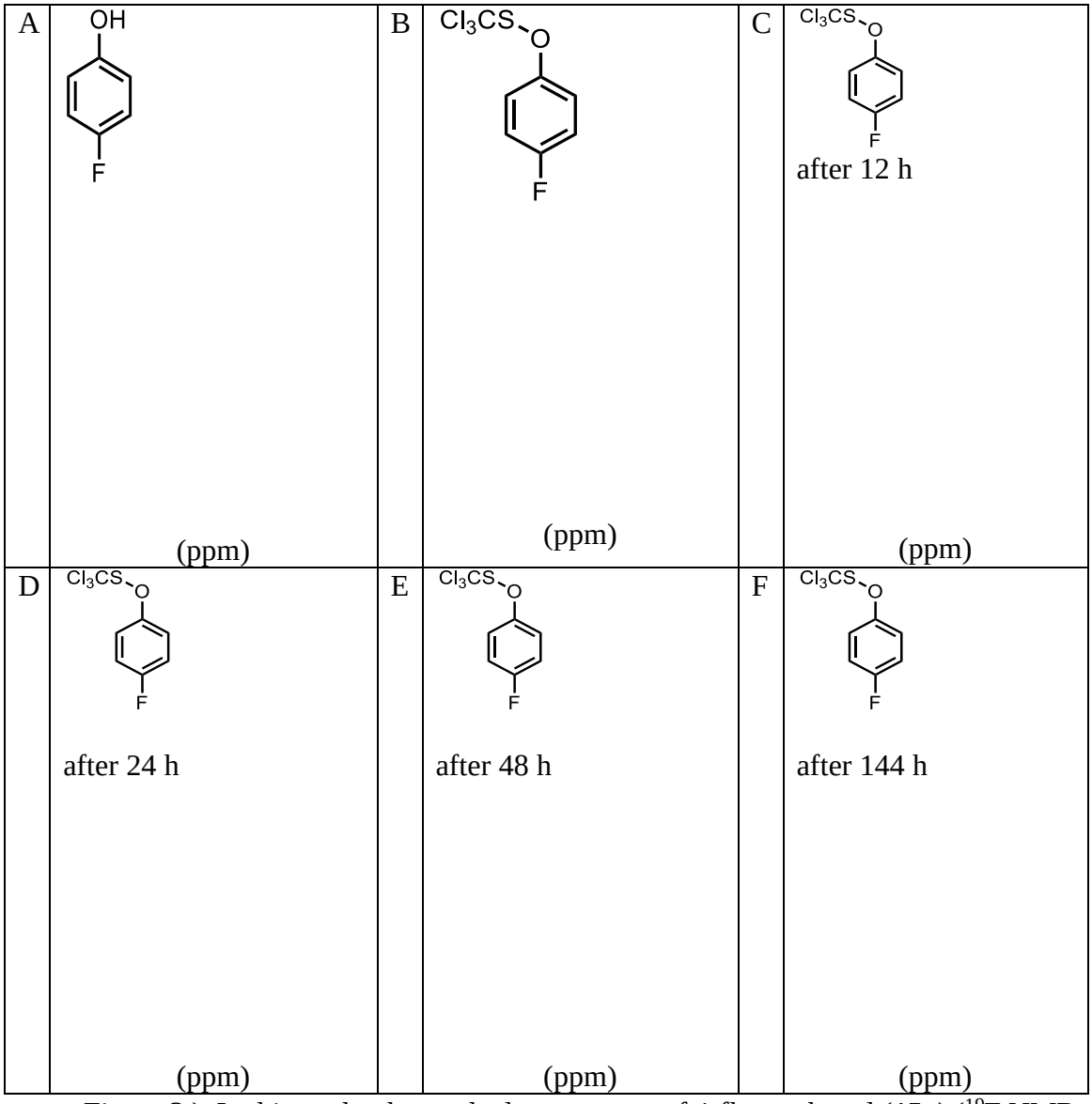
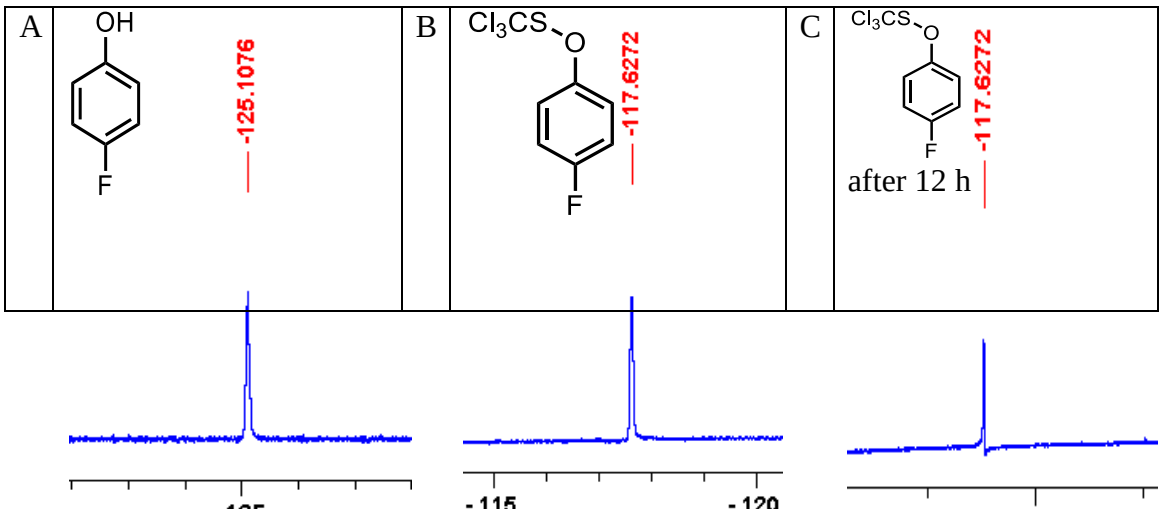


Figure 3.). In this study, the gradual appearance of 4-fluorophenol (**15a**) (^{19}F NMR δ: -125.1 ± 0.1) was clearly revealed.



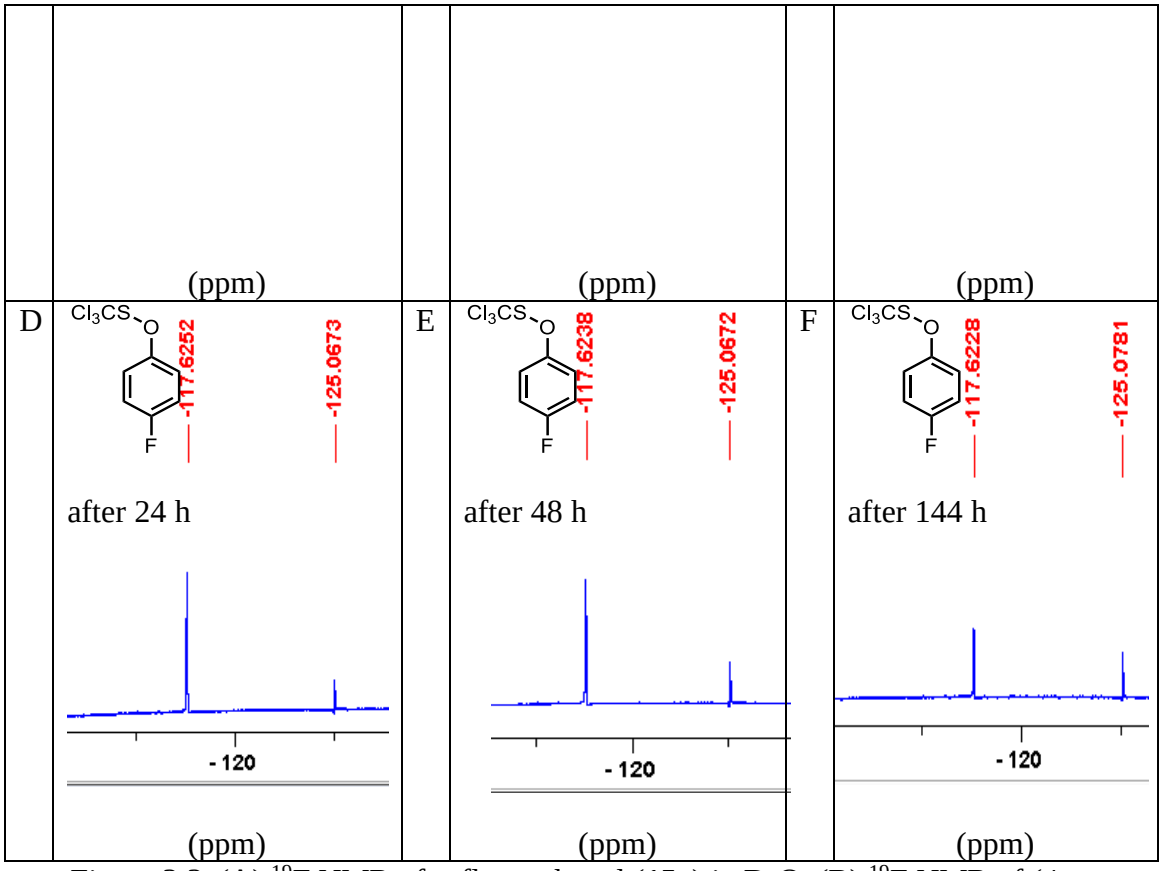


Figure 3.3. (A) ^{19}F NMR of p-fluorophenol (**15a**) in D_2O; (B) ^{19}F NMR of (4-fluorophenoxy)(trichloromethyl)sulfane (**15b**) in D_2O at 0 h; (C) ^{19}F NMR of (4-fluorophenoxy)(trichloromethyl)sulfane in D_2O after 12 h; (D) ^{19}F NMR of (4-fluorophenoxy)(trichloromethyl)sulfane in D_2O after 24 h; (E) ^{19}F NMR of (4-fluorophenoxy)(trichloromethyl)sulfane in D_2O after 48 h; (F) ^{19}F NMR of (4-fluorophenoxy)(trichloromethyl)sulfane in D_2O after 144 h.

After 12 h (**C**), there were no signs of decomposition of the sulfenate (**15b**). However, after 24 h (**D**), the sulfenate ester (**15b**) had begun to hydrolyze to the parent phenol. A continuation of this decomposition was observed (**E** and **F**), and after 144 h, the sulfenate ester approached a 1:1 ratio with the corresponding phenol (**F**). This shows that while sulfenate esters do hydrolyze in the presence of water, they are stable for up to 12 h. This is crucial since the biological assays employed here require a 12 h exposure time for

each phenol and sulfenate ester derivative in planktonic and biofilm assays. Accordingly, sulfenates should be robust for the entirety of exposure time.

3.4. Conclusion

This study has shown that sulfenate esters generally exhibit a significant increase in potency toward planktonic cells and biofilms of *S. epidermidis* and *P. aeruginosa* when compared to their phenolic counterparts. For example, it was found that on average sulfenates were 9 times more potent than the parent phenols against *S. epidermidis* and 17 times more potent towards *P. aeruginosa* in planktonic assays. Against biofilms, sulfenates were 4 times more potent towards both *S. epidermidis* and *P. aeruginosa*. The findings presented here also reveal that the most potent compounds towards planktonic cells are not always the most potent towards biofilms. Likewise, the most potent parent phenols do not consistently produce the most potent sulfenate esters. SAR studies have shown that placement, configuration, and alkyl chain length of functional groups does affect potency of the parent phenols as well as the derivatized sulfenates. An additional study, the monitored hydrolysis of **15b** by ^{19}F NMR, has shown that the stability of a representative sulfenate ester in aqueous solution is approximately 24 h. Further experimentation to determine clinical significance could be conducted with biofilms eradications measurements being determined with biofilms grown on different surfaces, such as metal and plastic, to mimic those found in clinical settings.

3.5 Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

3.6 Acknowledgements

Generous funding for this research was provided by the National Institute for General Medical Science. GM 116949

CHAPTER FOUR

2. ENHANCED ANTIMICROBIAL ACTIVITY OF PRODRUG PHENOLS AGAINST BIOFILMS AND PLANKTONIC BACTERIA

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Designed experiments, performed experiments, analyzed data. Organized, prepared and wrote manuscript.

Co-Author and corresponding: Thomas Livinghouse

Contributions: Performed experiments, preparation of manuscript, corresponding author.

Co-Author: Greg M. Durling

Contributions: Performed research, analyzed data.

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Contributions: Preparation of Manuscript.

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Manuscript Information

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Status of Manuscript:
____ Prepared for submission to a peer-reviewed journal
_X_ Officially submitted to a peer-reviewed journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Submitted 04/20

4.1 Abstract

Prodrugs are pharmacologically attenuated derivatives of drugs that undergo bioconversion into the active compound once reaching the targeted site, thereby maximizing their efficiency. This strategy has been implemented in pharmaceuticals to overcome obstacles related to absorption, distribution, and metabolism, as well as with intracellular dyes to ensure concentration within cells. In this study, we provide the first examples of a prodrug strategy that can be applied to simple phenolic antimicrobials to increase their potency against mature biofilms. The addition of iminodiacetoxymethyl ester groups has shown to increase the potency of small phenols. Biofilm-forming bacteria exhibit a heightened tolerance toward antimicrobial agents, highlighting the need for advancements in novel strategies to enhance activity towards biofilm forming bacteria.

4.2 Introduction

The majority of bacteria naturally reside in accumulations called biofilms, which exhibit a substantially increased resistant toward antimicrobials compared to free floating cells [268-269]. A biofilm is a community of bacteria that have adhered to a surface, either biotic or abiotic. Once attached, cells begin to secrete an extracellular polymeric substance (EPS) composed of extracellular DNA, polysaccharides and proteins. The EPS traps nutrients and water within the biofilm, allowing cells to mature under nutrient rich conditions while being protected from desiccation, host immune defenses and antimicrobial agents [29-32]. As a consequence of these defense mechanisms, biofilms are

associated with 3 out of 4 microbial infections in the body and are responsible for around 1.7 million hospital acquired infections per year, resulting in nearly 100 thousand deaths annually[158, 246].

One factor in treating biofilm-mediated infections is the impermeability of charged antimicrobial reagents engendered by the robust EPS matrix, thereby hindering complete access throughout the biofilm[199-203]. Another obstacle in treating biofilms is due to cells deep within the biofilms becoming dormant due to low nutrients and low levels of oxygen, therefore being less susceptible to antibiotics which work best on actively dividing cells[234, 270]. The inability of common antimicrobials to overcome biofilm tolerance has resulted in an urgent need for novel, efficacious anti-biofilm agents. Prodrug strategies has been effectively used to modify a wide array of structurally diverse pharmaceuticals to improve their physicochemical, pharmacokinetic, solubility and biopharmaceutical properties, or to circumvent issues such as premature drug metabolism [183, 271-274]. Synthetically, this is achieved through the incorporation of bio-reversible functional groups, which will be cleaved enzymatically upon delivery of the drug to the active site. A variety of structurally diverse functional groups have been employed for this purpose, including; phosphates, hemisuccinates, aryloxyphosphoramidates, phosphonooxymethyls, carbamates, aminoacyl conjugates, ethers and esters [97-99]. Ester functional groups in particular are employed to enhance lipophilicity and thus membrane permeability, and are used both in pharmaceuticals and intracellular fluorescent dyes, such as Calcein AM [90, 100-102].

Calcein AM is a fluorescein derivative which passively crosses the cell membrane of viable cells where it is then converted into Calcein, which is retained with in the cell

without compromising the cell membrane [103-104]. It has also been shown to stain biofilms of both Gram-negative and Gram-positive bacteria such as *Streptococcus oralis*, *Streptococcus gordonii*, *S. mutans* and *P. aeruginosa* as well as in endodontic biofilms [105-109]. We have chosen the AM group here to modulate polarity and cellular retention in order to increase potency of small phenols.

In this investigation, we demonstrate that the antibacterial activity of simple phenols is markedly increased by the incorporation of iminodiacetate (acetoxy)methyl (AM) groups. Differences in potency between the parent phenols and prodrugs derivatives were evaluated against both planktonic cells and biofilms of the model bacteria *S. epidermidis* (35984) and *P. aeruginosa* (PA01). Both bacteria were chosen for their propensity to form biofilms and prevalence in hospital-acquired infections [14, 275-278]. A collection of simple phenols have been chosen for this study based on conventional structure variations as well as documented antimicrobial activity [214, 222, 224, 244, 279-280]. Parent phenols such as eugenol (**1a**), 2-chloro-4-nitro phenol (**2a**), 2-methyl-4-chloro phenol (**4a**), 4-fluorophenol (**9a**), capsaicin (**14a**), 2,6-dichlorophenol (**17a**) and 4-chlorophenol (**18a**) were selected for their known antimicrobial properties [142, 280-283]. Several other phenols were chosen for structure activity comparison such as 2,4-dimethoxy phenol (**5a**), 4-methoxyphenol (**8a**), 2-allyl-4-methoxy phenol (**10a**) and 2-allyl-6-methoxy phenol (**12a**); where all compounds have at least one methoxy group. The phenols and their corresponding AM derivatives are assembled in Figure 4.1.

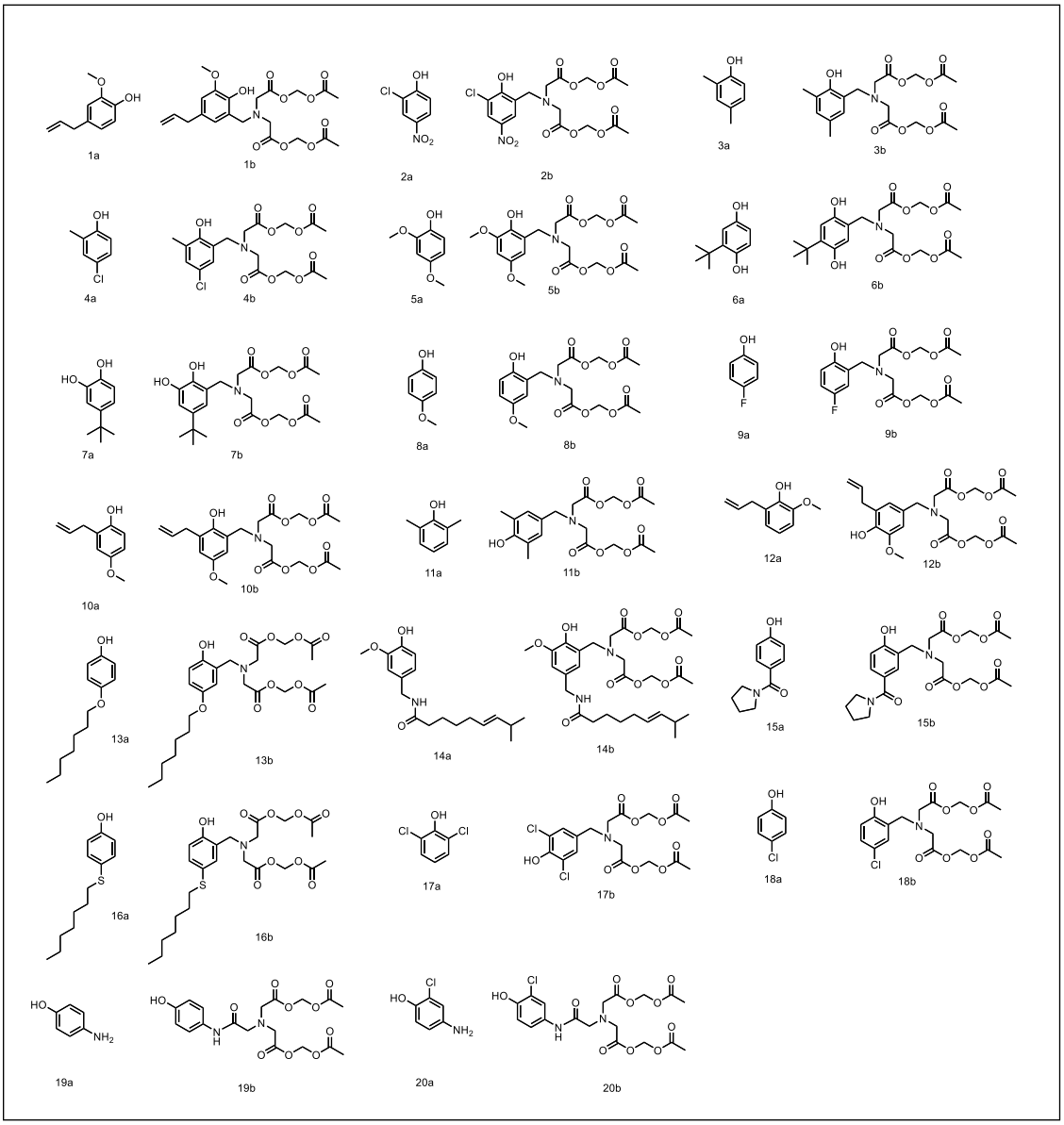


Figure 4.1 Parent compounds and AM derivatives

4.3 Materials and Methods

4.3.1 Synthetic reagents and bacteria

All chemical reagents purchased for chemical synthesis were purchased from commercial sources and used as received without further purification, unless stated in the supplementary information. Solvents for filtrations, transfers, and chromatography were

certified ACS grade. Thin layer chromatography was performed on Silicycle Glass Backed TLC plates, and visualization was accomplished with UV light (254 nm), and/or potassium permanganate. All ^{1}H NMR spectra were recorded on a Bruker DRX300. All ^{13}C NMR spectra were recorded on a Bruker DRX500, all NMR data was reported in ppm, employing the solvent resonance as the internal standard. HRMS was performed.

P. aeruginosa (PA01 and PA015542) and *S. epidermidis* (35984) were obtained from American Type Culture Collection (ATCC). All bacteria were sub-cultured onto tryptic soy agar (TSA) plates and incubated at 37 °C for 24 h. Single colonies were transferred from the plates and inoculated into 25 mL tryptic soy broth (TSB) in Erlenmeyer flasks. Culture were incubated 37 °C for 24 h and 10 µL of culture was transferred into 25 mL of TSB and the absorbance was read at 600nm using a spectrophotometer and standardized to 10^{6}-10^{7} CFU/mL.

4.3.2 Efficacy of naturally occurring phenols and derivatives on inhibiting planktonic cells

The minimum inhibitory concentrations (MICs) of all compounds against *S. epidermidis* and *P. aeruginosa* were determined using a 96-well plate assay previously described by Xie [171]. Data from at least three replicates were evaluated for each compound tested. Samples were diluted in dimethyl sulfoxide (DMSO), DMSO controls were also conducted. Experiments were done in biological triplicate and technical duplicates were done.

4.3.3 Efficacy of naturally occurring phenols and derivatives on biofilms

4.3.3.1 Biofilm eradication concentration assays: Both strains were cultured as described above and biofilms were grown in Costar polystyrene 96-well plates at 37 °C. After 24 h of incubation, the planktonic-phase cells were gently removed and the wells washed three times with PBS. Wells were filled with 150 µL dilutions of the compound being evaluated. The 96-well plates were incubated for an additional 24h at 37 °C. The medium was gently removed and each well filled with 150 µL PBS and the biofilm broken up through stirring with sterile, wooden rods. Three tenfold dilutions of each sample were taken and drop plated on TSA plates and incubated for 24 h. The biofilm eradication concentration (BEC) was determined to be the lowest concentration at which no bacterial growth occurred. This procedure was modelled based on previously reported procedures according to Pitts [172]. Experiments were done in biological triplicate and technical duplicates were done.

4.3.3.2 Center for disease control (CDC) biofilm reactor evaluation: A CDC biofilm reactor was also used to assess potency of compounds towards biofilms. American Society for Testing and Materials (ASTM) method E2562 – 17, which describes how to grow a biofilm in the CDC biofilm reactor under high shear and continuous flow, and ASTM method E2871 -13, a biofilm efficacy test generally known as the single tube method were used for this procedure. Formation of 48 h biofilms in a CDC reactor was formed on glass coupons (4.02 cm^2). A CDC reactor containing 340 mL of TSB (300 mg/L) was inoculated with 1 mL of a 3.21 x 10^8 CFU/mL overnight culture of *P. aeruginosa* (PA015542), which was grown in TSB (300 mg/L) overnight. The biofilm was grown in batch condition at room temperature at 125rpm for 24 h, and then for 24 h at room temperature under

continuous flow with a feed rate of 11.25 mL/min at 125 rpm. The continuous feed TSB was 100 mg/L. Coupons were then sampled from the reactor in triplicate. The mean log reduction in viable biofilms cells exposed to each compound for 1 h was quantitatively measured according to ASTM method E2871-13. After coupons were removed from the CDC reactor they were rinsed and transferred to separate, 50 mL conical tubes and 4 mL of a 100 mM solution of the antimicrobial compound being tested in sterile PBS buffer was added. The tubes were incubated at room temperature under static conditions or 1 h. After one h 36 mL DE broth was added and the biofilm was disaggregated by a series of vortexing and sonicating for 30 seconds each in the order of v/s/v/s/v. Each sample was diluted tenfold six times and the diluted samples were drop plated on (Reasoner's 2A agar) R2A agar plates, incubated overnight at 37°C and enumerated. Experiments were done in biological duplicate and technical duplicates were done. The mean log reduction was also determined for each compound evaluated using the following equation:

$$Log\ reduction = log10\left(\frac{A}{B}\right)$$

Where A is the average number of CFU before treatment and B is the average number of CFU after treatment.

4.3.4 Enzyme assay

A 30-microliter solution of bis(acetoxymethyl) 2,2'-((-2-hydroxy-5-methoxybenzyl)azanediyl)diacetate (**8b)** at a concentration of 50 μM in acetonitrile was dissolved in a 100 μL solution of (from porcine liver 72 units/mL in cold HEPE buffer, pH7.9). The mixture was incubated at 37 ^{o}C for the either 8, 16 or 24 minutes and then cooled to 0^{o}C to inhibit further enzyme activity. The sample was then extracted several

times with acetonitrile and analyzed via LCMS to determine if the cleaved product was present.

4.3.5 General chemical synthesis procedure

4.3.5.1 Preparation of diethyl 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (1c). A 10-mL round-bottomed flask equipped with a magnetic stirring bar and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL anhydrous acetonitrile. Eugenol (160 mg, 1.0 mmol, 1 equiv) was added with stirring, followed by paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv). The reaction was heated at reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography with 35% EtOAc in hexanes to give 401 mg (91%) of **1c** as a yellow oil.

4.4.5.2 Preparation of diethyl 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (1d) . A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with **1c** (0.5 mmol, 168 mg, 1 equiv), 2 mL of a 1:0.3 solution of methanol:water and lithium hydroxide monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was allowed to warm to room temperature and filtered to give a white powder which was then washed with water and dried to yield 121 mg (87%) of **1d** as a white powder.

4.3.5.3 Preparation of bis(acetoxymethyl) 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (1b). A 10-mL round-bottomed flask equipped with a

magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. Eugenol (164 mg, 1 mmol, 1 equiv) was added slowly with stirring and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((2-hydroxy-5-allyl-3-methoxybenzyl)azanediyl)diacetate, as a yellow solid. The same flask was equipped with a magnetic stirring bar and nitrogen balloon and charged with 3 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an addition 12 hr at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 3 mL). The organic layers were combined and washed with water (3 X 3 mL) and then with brine (3 X 3 mL). The organic layer was dried and concentrated in vacuo to afford 421 mg (93%) of **1b** as a viscous yellow oil.

4.4 Results and discussion:

In an initial study, the minimum inhibitory concentrations (MIC) and biofilm eradication concentrations (BEC) were evaluated for all parent phenols and their AM derivatives toward the Gram-negative bacteria *P. aeruginosa* and the Gram-positive bacteria *S. epidermidis.* AM derivatives (**1b**-**12b**, **14b**-**18b**) were typically more potent than their corresponding parent phenols (**1a**-**12a**, **14a**-**18a**) against planktonic cells with the exception of **4a** and **4b** against *S. epidermidis* (Table 4.1) The observation of **13b** demonstrating a lower potency compared to **13a** toward both bacteria is likely a result of

13b exhibiting a decrease in permeability towards the cell membrane. On average, AM derivatives were 25 times more potent than parent compounds toward *S. epidermidis* and 8 times more potent toward *P. aeruginosa* in the planktonic phase. This trend was expected due to the cleavage of the AM group via intracellular esterase, resulting in cellular retention and intracellular concentration of the antimicrobial.

Table 4.1. MICs for parent compounds and respective AM derivatives against *S. epidermidis* and *P. aeruginosa*.

Minimum Inhibitory Concentration (mM)				
	S. epidermidis		*P. aeruginosa*	
Compound	Parent (a)	Derivative (b)	Parent (a)	Derivative (b)
1a/b	15.62	0.7	31.2	1.3
2a/b	0.9	0.7	1.9	1.5
3a/b	1.9	0.1	1.9	0.5
4a/b	0.9	1.5	1.9	0.75
5a/b	15.6	0.23	7.8	1.9
6a/b	0.23	0.023	3.9	0.5
7a/b	0.23	0.12	7.8	0.9
8a/b	3.9	0.9	7.8	1.9
9a/b	15.6	0.1	7.8	0.9
10a/b	7.9	0.5	15.6	1.3
11a/b	1.9	0.5	1.9	0.1
12a/b	125	62.5	125	31.2
13a/b	0.3	1.9	1.5	3
14a/b	15.6	1.9	15.6	3.8
15a/b	15.6	7.8	31.2	25
16a/b	4.5	1.9	7.8	3
17a/b	3.1	0.6	3.9	0.9
18a/b	2.5	0.25	6.2	0.9

Against biofilms, AM derivatives (**1b**-**12b**, **14b**-**18b**) were again more potent than parent phenols (**1a**-**12a**, **14a**-**18a**) with the exception of **4a/b**, **10a**/**b** and **16a/b** where parent and AM shared the same BEC against *S. epidermidis* (Table 4.2). Compounds **13a**/**b** again

did not follow the commonly observed trend, instead the AM was less potent against both bacteria. On average, AM derivatives were 4.6 times more potent toward eradicating *S. epidermidis* biofilms, and 7 times more potent toward *P. aeruginosa* biofilms. This observation is likely due the cellular retention and intracellular accumulation of the cleaved prodrug. This shows that, in the majority of compounds tested, the addition of an AM group will increase the potency of small phenols towards biofilms and planktonic cells.

Table 4.2 BECs for parent compounds and respective AM derivatives against *S. epidermidis* and *P. aeruginosa*.

Biofilm Eradication Concentration (mM)				
	S. epidermidis		*P. aeruginosa*	
Compound	Parent (a)	Derivative (b)	Parent (a)	Derivative (b)
1a/b	31.2	2.7	62.5	2.7
2a/b	31	7.8	31.2	12.5
3a/b	31.2	6.2	62.5	12.5
4a/b	6.2	6.2	12.5	3.1
5a/b	31.2	3.1	62.5	6.2
6a/b	31.2	7.8	62.5	15
7a/b	15.6	7.8	31.2	15
8a/b	31.2	12.5	31.2	12.5
9a/b	62.5	6.2	31	12.5
10a/b	15.6	15.6	62.5	15.6
11a/b	62.5	25	31.2	1.5
12a/b	62.5	31.2	31.2	15.6
13a/b	1.9	12.6	7.5	25
14a/b	25	7.8	25	15.6
15a/b	50	12.6	50	15.6
16a/b	6.2	6.2	50	25
17a/b	6.2	3.0	12.5	6.2
18a/b	3.1	2.7	6.2	3.1

Compounds **1b**, **5b**, **17b**, and **18b** were the most potent compounds against *S. epidermidis* biofilms, while compounds **1b**, **4b**, **11b** and **18b** were most effective in eradicating *P. aeruginosa* biofilms. An AM derivative lacking all other functionalization

on the aromatic ring, including the phenolic OH, was also synthesized and was among the least potent AM derivatives with a BEC of 24 mM toward both bacteria.

It is also interesting to note here that the most potent parent compounds did not consistently result in the most potent AM derivatives against biofilms. Parent compounds **4a**, **13a**, **16a**, **17a** and **18a** were most potent toward *S. epidermidis*, while parent compounds **4a**, **13a**, **14a**, **17a**, and **18a** were most potent toward *P. aeruginosa* (Table 4.2 and Table 4.1). Out of these six compounds, the only corresponding AM derivatives with top potency were **4b** and **18b** toward *S. epidermidis* and **17b**, and **18b** toward *P. aeruginosa*. Parent compound **1a** was among the least potent toward both bacteria while **1b** was among the top five most potent towards both bacteria.

Compounds **1b** and **18b** were the most successful compounds against biofilms for both types of bacteria. Two isomers of **1b**, **10b** and **12b**, were also evaluated (Figure 4.1). However, these isomers demonstrated a substantial decrease in potency compared to **1b**. This trend was not seen in parent phenols where **10a** was the most potent isomer towards *S. epidermidis* and **12a** was the most potent isomer towards *P. aeruginosa.* In parent phenols, a dramatic difference in potency was not observed as it was with the prodrug derivatives (Table 4.3).

These results suggest that the positioning of functional groups around the aromatic ring can make a dramatic difference in potency for AM derivatives, which is also observed in the isomers **3b** and **11b** (Table 4.3). Compound **3b** has methyl groups is the 2 and 4 position while **11b** has methyl groups in the 2 and 6 positions. Here, **3b** is more potent

toward *S. epidermidis* and **11b** more potent toward *P. aeruginosa* (Table 4.3). This was also observed with the corresponding phenols (Table 4.2).

Table 4.3 BECs for sets of isomers 1b, 10b and 12b as well as 3b and 11b.

Biofilm Eradication Concentration (mM)			
S. epidermidis		*P. aeruginosa*	
1b	2.7	1b	2.7
10b	15.6	10b	15.6
12b	31.2	12b	15.6
3b	6.2	3b	12.5
11b	25	11b	1.5

It was observed that all compounds exhibited a higher potency toward planktonic cells when compared to biofilms (Table 4.1 and Table 4.2). Parent compounds were, on average, 26 times less potent toward *S. epidermidis* biofilms compared to planktonic cells and 10 times less potent toward *P. aeruginosa* biofilms. AM derivatives were on average 55 times less potent toward *S. epidermidis* and 11 times less potent toward *P. aeruginosa* biofilms compared to planktonic cells. This was expected due to the known higher susceptibility of planktonic cells. AM derivatives also experienced a larger disparity in potencies between planktonic cells and biofilms than was seen with parent phenols. This may be due to the ability for the activated prodrug to concentrate within cells, a characteristic that the parent phenol lacks, combined with the increase susceptibility of planktonic cells.

The majority of AM compounds possessed the AM group in the para position; the exception to this being **11b**, **12b** and **17b**. Two additional, ‘nontraditional’ AM derivatives (**20b** and **21b**) were also synthesized with the AM in the para position (Figure 4.1). Para substituted AM compounds (**11b**, **12b**, **17b**) did not consistently show either a heightened

or muted potency compared to ortho substituted AMs. The 'nontraditional' AMs **20b** and **21b** did not show significant potency towards either bacteria compared to the five most potent 'traditional AMs', although 19a/b demonstrated a dramatic increase in potency towards *P. aeruginosa* (Table 4.4).

Table 4.4 BECs of para substituted AMs and alternative AMs

Biofilm Eradication Concentration (mM)				
	S. epidermidis		*P. aeruginosa*	
Compound	*Parent (a)*	*Derivative (b)*	*Parent (a)*	*Derivative (b)*
11a/b	62.5	25	31.2	1.5
12a/b	62.5	31.2	31.2	15.6
17a/b	6.2	3	12.5	6.2
19a/b	50	15.6	100	15.6
20a/b	25	7.8	25	12.5

To compare the potency of these novel AM prodrugs to standard antimicrobial agents, three commercially available antimicrobials were evaluated for activity toward *S. epidermidis* and *P. aeruginosa* biofilms (Table 4.4). Metronidazole is a nitroimidazole derivative that was selected for its use in treating a variety of bacterial infections and has been shown to exhibit activity toward biofilms of *Helicobacter pylori* [257] and *C. difficile* [258-259]. Metronidazole had a BEC of 6.2 mM towards *S. epidermidis* and 50 mM towards *P. aeruginosa*. Tobramycin is an aminoglycoside that was chosen because it has been extensively studied for efficacy toward biofilms. A study by Høiby *et al*. showed that the inhibitory properties toward *P. aeruginosa* biofilms were lower than that toward planktonic cells, concluding that biofilms are tolerant to the clinically recommended dose of the antibiotic [263]. Sans-Serramitjana *et al*. evaluated the antimicrobial activity of nanoencapsulated tobramycin; finding that nanoencapsulation did improve the ability of

tobramycin to eradicate *P. aeruginosa* biofilms and suggesting the strategy of lipid carriers to deliver the drug, overcoming drug resistance to tobramycin [264]. Tobramycin had a BEC of 18 mM towards *S. epidermidis* and of 0.06 mM towards *P. aeruginosa*. Cephalothin sodium is a bactericidal beta-lactam that is efficacious towards Staphylococcal infections that have a high tolerance toward beta-lactamase [284]. Cephalothin sodium was selected for this and because it has been evaluated toward a range of bacterial biofilms including *S. epidermidis*, *P. aeruginosa*, and methicillin resistant *Staphylococcus aureus* (MRSA), with varying degrees of potency [285-287]. The BEC of Cephalothin towards *S. epidermidis* was 0.03 mM and 50 mM towards *P. aeruginosa*.

Against *S. epidermidis* biofilms, several AM compounds were more potent than metronidazole and tobramycin (Table 4.2 and Table 4.4). Compared to all other compounds cephalothin sodium had the lowest BEC towards *S. epidermidis*. This was expected due to its ability to treat resistant strains of Staphylococcus [288-289]. All 18 AM prodrugs exhibited a lower BEC toward *P. aeruginosa* when compared to metronidazole and cephalothin sodium, although here tobramycin exhibited the highest potency of all compounds evaluated. It was no surprise that several AMs were more potent towards both bacteria compared to metronidazole since metronidazole is used to treat anaerobic infections while both *S. epidermidis* and *P. aeruginosa* are both facultative anaerobes. When compared to these standard antimicrobials, only two parent phenols were more potent than metronidazole and tobramycin towards *S. epidermidis* biofilms. Seven out of the 18 parent phenols had a lower BEC toward *P. aeruginosa* compared to metronidazole and cephalothin sodium. As seen above, small phenols exhibit a much lower potency than the

known drugs evaluated here, although with the addition of an AM group, the potencies are much closer to those of tobramycin and cephalothin sodium.

In an additional study, a select number of iminodiacetic acid derivatives (**1c**-**4c**) were also synthesized and evaluated for potency (Figure 4.2). These derivatives are the active form of the drug; produced after esterase cleavage. The active form of the drug is not expected to be able to permeate through the biofilms or cross the cell membrane as efficiently as their AM counterparts, and thus produce higher BECs. By comparing the potency of the liberated drug to the corresponding prodrug, we gain further insight into the ester prodrug delivery vehicles' success.

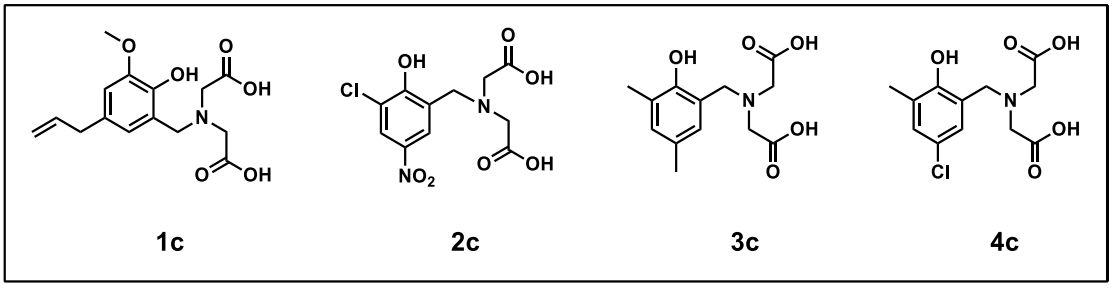


Figure 4.2. Diethyl prodrug derivatives and liberated, diacetic acid derivatives.

Compounds **1**-**4c** were significantly less potent than their corresponding AM prodrugs against biofilms (Table 4.5). AMs were, on average, 10 times more potent than the active form of the drug (**1**-**4c**) against *S. epidermidis* and 16 times more potent against *P. aeruginosa* biofilms. This observation suggests that the active drug is unable to penetrate the cell, likely due to the acetic acid appendages possessing a negative charge at a pH above 4. It also supports that the AM form of the drug is able to penetrate and eradicate cells within a biofilm. In comparison to corresponding parent phenols (**1**-**4a**), it was also observed that active drug derivatives (**1**-**4c**) were often less potent (Table 4.1, Table 4.5).

Table 4.5 BECs for AM prodrugs and liberated drugs against S. epidermidis and P. aeruginosa.

Biofilm Eradication Concentration (mM)				
	S. epidermidis		*P. aeruginosa*	
Compound	Derivative (b)	Derivative (c)	Derivative (b)	Derivative (c)
1b/c	2.7	62.5	2.7	125
2b/c	7.8	62.5	12.5	31.2
3b/c	6.2	125	12.5	>250
4b/c	6.2	62.5	3.1	125

Against planktonic cells, the liberated drug derivatives (**1**-**4c)** were again consistently less potent than their corresponding AMs (**1**-**4b**) (Table 4.5). In planktonic assays, AM derivatives were, on average, 42 times more potent against *S. epidermidis* and 10 times more potent against *P. aeruginosa*. Parent phenols (**1**-**4a**) again exhibited a higher potency compared to their active drug counterparts (**1**-**4c**). This suggests that the negatively charged iminodiacetic acid groups which are used to concentrate the active drug within the cell also inhibit it from entering. These negative charges could also weaken their antimicrobial affects.

Table 4.6 MICs for AM prodrugs and liberated drugs against S. epidermidis and P. aeruginosa.

Minimum Inhibitory Concentration (mM)				
	S. epidermidis		*P. aeruginosa*	
Compound	Derivative (b)	Derivative (c)	Derivative (b)	Derivative (c)
1b/c	0.7	53.7	1.3	26.9
2b/c	0.7	7.8	1.5	2
3b/c	0.1	7.8	0.5	3.9
4b/c	1.5	2	0.75	7.8

In effort to better explore additional synthetic options for derivations of iminodiacetate functionalized prodrugs, five variations of ester prodrugs (**1d-h**, Figure 4.3) using eugenol as a molecular backbone were synthesized and evaluated against planktonic cells and biofilms. This derivatization was done in effort to explore alternative ester prodrugs. Eugenol (**1a**) was chosen as the backbone because the AM derivative (**1b**) was one of the top five most potent AMs toward biofilms in the series above (Table 4.2). Derivatives **1d**, **1e** and **1h** contain only two ester functional groups, as opposed to the AMs four. The PEG tail (**1h**) was selected to increase hydrophilicity, since eugenol itself has low water solubility. Ethyl and allyl groups (**1d** and **1e**) were chosen for their structural simplicity. Derivatives **1f** and **1g** are structurally similar to the AM group, possessing a butyryl and pivaloyl ester in place of the acetate respectively. Both were selected for their variation in carbon chain at the terminus of the iminodiacetate group. The bis-pivaloyloxymethyl ester group was also selected due to its use in prodrugs to improve bioavailability[290].

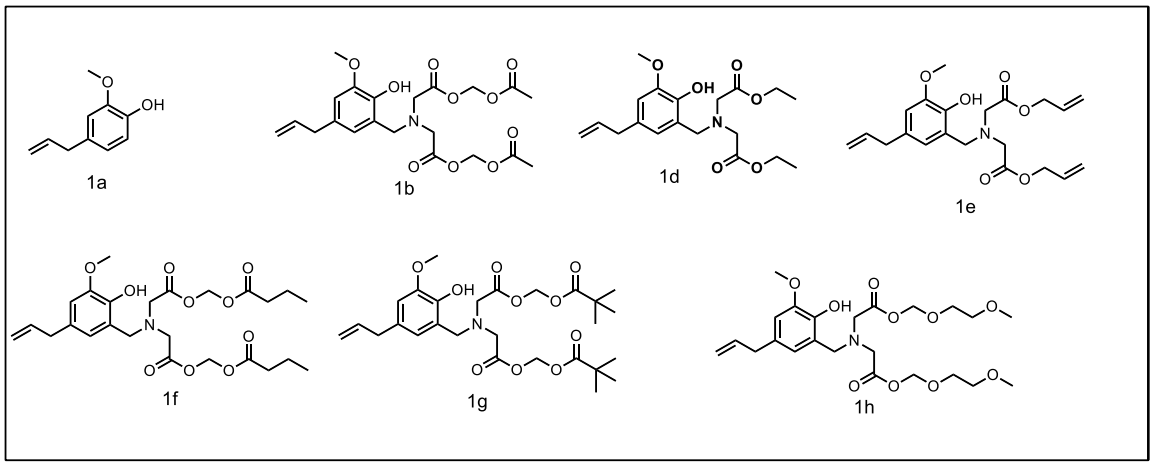


Figure 4.3 Eugenol (1a) with alternative derivatives (1e and 1f) and the AM prodrug (1b).

Against planktonic cells, prodrug derivative **1f** exhibited the highest potency against *S. epidermidis*, while **1b** showed the highest potency toward *P. aeruginosa* (Table 4.6). The ethyl and allyl ester derivatives (**1d**, **1e**) were the least potent prodrugs overall with MICs of 31.2 mM towards both bacteria. Against *S. epidermidis* **1d** and **1e** were also less potent than the parent phenol (**1a**).

Table 4.7 MIC (mM) for eugenol (1a) and the alternative prodrug derivatives (1e and 1f) compared to the AM derivative (1b).

Minimum Inhibitory Concentration (mM)			
S. epidermidis		*P. aeruginosa*	
1a	15.6	1a	31.2
1b	0.68	1b	1.3
1d	31.2	1d	31.2
1e	31.2	1e	31.2
1f	0.12	1f	3.9
1g	1.9	1g	15.6
1h	1.9	1h	31.2

Against biofilms, the AM derivative (**1b**) had the highest potency against both bacteria (Table 4.8). Compound **1e** was the least potent toward *S. epidermidis* while **1h** was the least potent toward *P. aeruginosa*. This suggests that the AM derivative (**1b**) is either more permeable to the biofilm or the ester bonds present in this group are more readily cleaved by esterase or both.

Table 4.8 BEC (mM) for eugenol (1a) and the alternative prodrug derivatives (1e and 1f) compared to the AM derivative (1b).

Biofilm Eradication Concentration (mM)			
S. epidermidis		*P. aeruginosa*	
1a	31.2	1a	62.5
1b	2.75	1b	2.75
1d	31.2	1d	62.5
1e	50	1e	50

1f	15.6	1f	62.5
1g	20.6	1g	41.2
1h	7.8	1h	125

A CDC Biofilm reactor assay was also used to substantiate the comparative efficacy of eugenol (**1a**) with its corresponding AM derivative (**1b**) against *P. aeruginosa* (PA015542). Here, unlike the static condition of the 96-well plate, biofilms were grown in a high shear environment. This method increases the biofilms adherence to the surface on which it is grown and causes the biofilm to produce a more robust EPS. This method was chosen because it has been standardized by the ASTM. Similar to the static 96-well plate assays, the potency of the AM derivative (**1b**) was greater than the parent compound (**1a**) using the CDC biofilm reactor. Eugenol (**1a**) demonstrated a mean log reduction of 1.68 ± 0.12 while its corresponding AM (**1b)** had a mean log reduction of 5.81 ± 0.53. This has shown that the AM (**1b**) is significantly more potent than the parent (**1a**) against biofilms grown in both static and high shear environments.

Once AM derivatives have penetrated the biofilm's extracellular matrix and the membrane of indwelling cells, they are predicted to be acted upon by intracellular esterase, liberating the active form of the prodrug (Figure 4.4). The resulting, highly charged antimicrobial is then retained within the cell. The observed increase in potency supports that AMs are being acted upon by esterase once inside the cell, but additional experimentation was also performed to further support this hypothesis. In order to determine if esterase will cleave these AM groups, **8b** was exposed to esterase in vitro and samples were viewed via mass spectrometry to determine if the liberated drug (**8c**) was present.

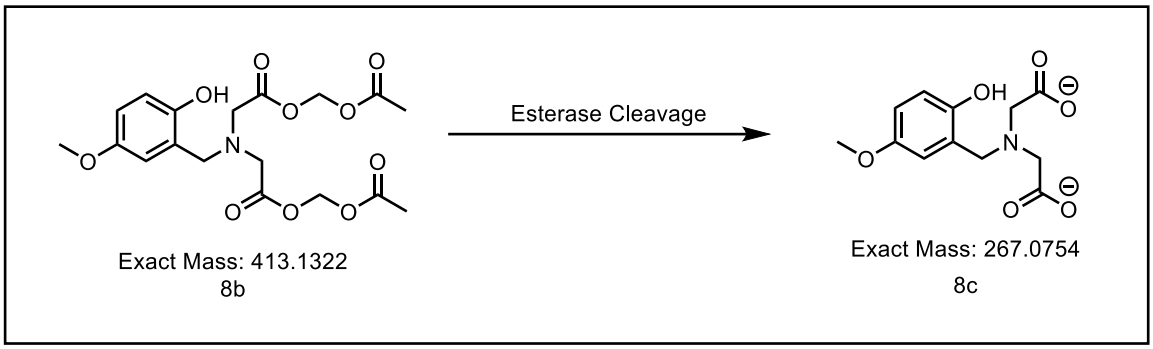


Figure 4.4. The AM derivative **8b** and the esterase cleavage product 8c.

The AM derivative of 4-methoxyphenol (**8b)** was exposed to esterase in cold HEPE buffer and LC-MS was performed to determine the amount of the liberated product, 2,2'-((2-hydroxy-5-methoxybenzyl)azanediyl)diacetate (**8c**), present. The exact mass of **8b** is 413.1322 amu, with a predicted $[M]^-$ of 413.1322 amu and the exact mass of **8c** is 267.0745 amu with predicted $[M - 2H]^{2-}$ of 132.5366 amu. The exact mass of the protonated derivative of **8c** is 269.0889 amu with a predicted $[M]^-$ of 268.0816. Both the mono and di-anionic product are expected to be present in the esterase exposed samples evaluated here.

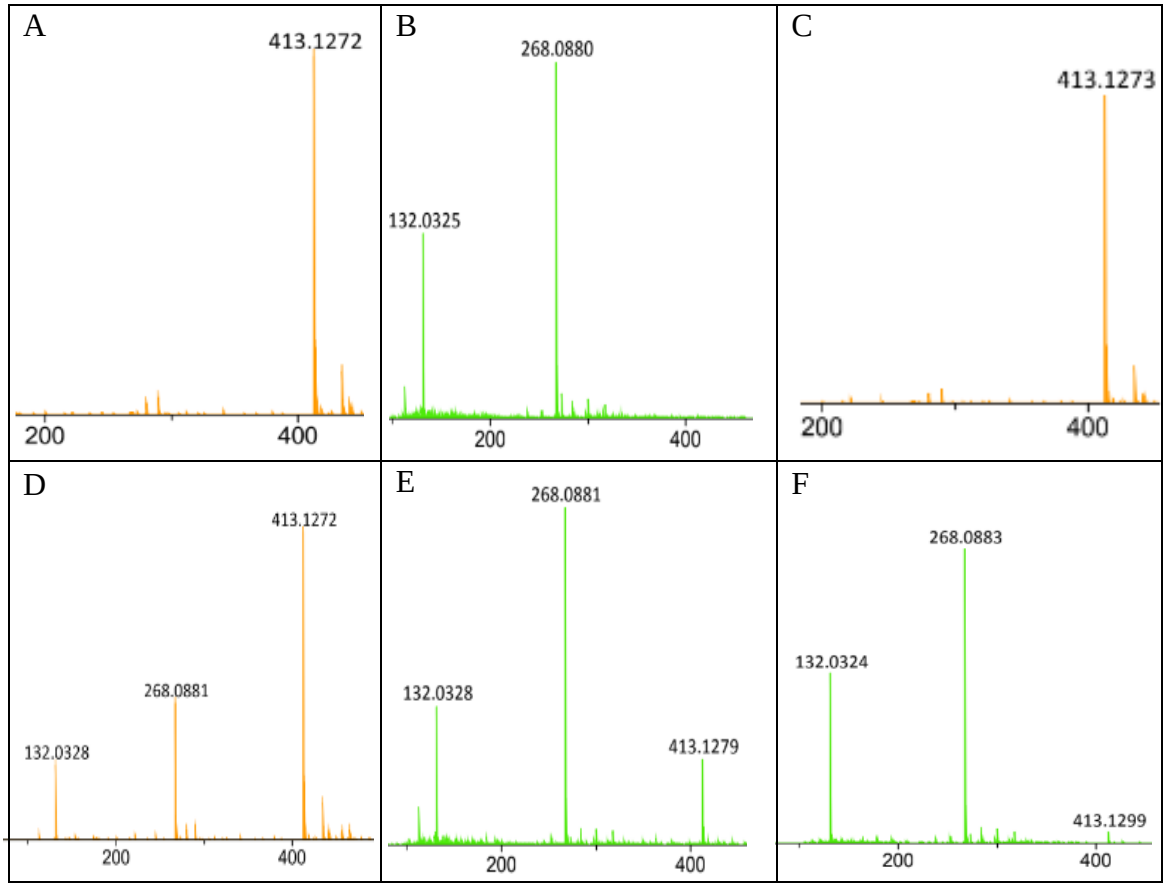


Figure 4.5 Liquid chromatography–mass spectrometry of parent AM **8b** before and after exposure to esterase, run in negative mode. **A**) The pure AM derivativev **8b B**) The pure protonated derivative of of **8c C**) **8b** after being exposed to HEPE buffer with no esterase, **D**) **8b** after being exposed to esterase for 8 min in HEPE buffer, **E**) **8b** after being exposed to esterase for 16 min in HEPE buffer, **F**) **8b** after being exposed to esteraes for 24 min in HEPE buffer.

Spectra were taken of the pure AM compound **8b** (**A**) and the liberated derivative **8c** (**B**) (Figure 4.6). In frame **B** both peaks for the mono and di anionic species can be observed. The AM **8b** was exposed to esterase in HEPE buffer for 8 (**D**), 16 (**E**) and 24 (**F**) minutes, extracted, and then analyzed via LCMS (Figure 4.6). The masses of **8b** and **8c** were found were within two decimals of the predicted masses for each compound. A control of **8b** in HEPE without esterase was also included to ensure that HEPE does not influence the prodrug (Figure 4., **C**). This assay has shown that, in vitro, the AM derivatives

are in fact acted upon by esterase. This suggests that the increase in potency is in part due to the prodrug being able to penetrate the biofilm and transform within the cell to release the liberated drug. This is further supported by the observation that the diacid (**1d**-**4d**) compounds are significantly less active than the prodrugs toward biofilms. An in vivo study has yet to be conducted although Calcein AM has been used extensively to successfully stain biofilms [105-106, 291-293].

4.4 Conclusion

It has been shown that AM's (14 out of 18 AMs against *S. epidermidis* and 18 out of 18 AMs against *P. aeruginosa*) are more potent than corresponding parent phenols toward biofilm eradication. This study proposes a prodrug method to increase to the potency of antimicrobials toward biofilms. Here it was shown that the addition of an AM appendage typically increased to potency of small phenols toward both planktonic cells and biofilms. For example, AM derivatives were on average 25.40 times more potent than parent compounds toward *S. epidermidis* and 8.44 times more potent toward *P. aeruginosa* in planktonic assays. Toward biofilms, AM derivatives were on average 4.64 times more potent toward *S. epidermidis* and 7.00 times more potent against *P. aeruginosa*.

4.5 Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

4.6 Acknowledgements

Generous funding for this research was provided by the National Institute for General Medical Science. GM 116949

CHAPTER FIVE

5. CURRENT AND FUTURE WORK

5.1. Expansion of current AM prodrug evaluation

One possibility for future work for this project is to expand the body of knowledge of current AM prodrugs from chapter 4. Valuable pursuits include elucidating their mode of action, evaluating toxicity towards mammalian cells and determining chelating properties of liberated AMs, and evaluating if the release of formaldehyde contributes to potency.

5.1.1. AM prodrug mode of action

One method for determining the mode of action is evaluating the effects of AM prodrugs on the lipid-membrane. If AM compounds accumulate in the cytoplasmic membrane, changes in the lipid membrane, such as expansion, will occur. This could be done through the labelling of a lipid-membrane with a self-quenching, fluorescent tag (such as octadecyl Rhodamine B chloride [294]) and measuring the increase in fluorescence, which is caused by the expansion of the cell membrane. This will be conducted with varying concentrations of AM compound. This technique has been previously used to assess the expansion of lipid-membranes in the presence of carvacrol [140].

Another beneficial study would be to investigate the effects of AMs on membrane potential ($\Delta\psi$). Here, the use of voltage-sensitive dyes, where the change in fluorescence can be visualized, can be used to observe changes in membrane potential with the addition of AM compound. This can be accomplished by exposing cells to a fluorescent membrane

dye such as 3,3-dipropylthiacarbocyanine. A stable fluorescent reading can be reached by energizing the cells with glucose, diminishing the pH gradient across the cytoplasmic membrane, while measuring $\Delta\psi$ with a spectrofluorometer, until a steady fluorescent reading is reach. The cells will then be exposed to different concentrations of the AM compound while $\Delta\psi$ is continuously measured. This technique has been used to determine that carvacrol reduces membrane potential of *Bacillus cereus* at concentrations as low as 0.01 mM and caused dissipation of membrane potential at 0.15 mM [295].

5.1.2. Toxicity toward mammalian cells

Evaluating AM compounds, particularly that of the essential oil eugenol, for toxicity towards mammalian cells would provide insight into their possible applications. The AM of eugenol would be of particular interest for this experiment due to eugenol gaining interest as an antimicrobial agent as well as being considered safe by the Food and Drug Administration (FDA) and possessing no mutagenic or carcinogenic potential [296-298]. For this, a Sulforhodamine B (SRB) assay[299-301] could be used to test compounds against a cancer cell line such as HeLa or HCT-116. This will be done by growing the cells in Trypsin-EDTA and seeding them in 96-well plates followed by treatment with the AM compound for a set amount of time. After being fixed and washed, the cells will then be exposed to a SRB solution for the set amount of time and washed again with glacial acetic acid. Plates will be dried overnight and dissolved in Tris-HCl. The color intensity will be measured at 540 nm.

5.1.3. Chelation effects of liberated AM prodrugs

In addition to the above biological experiments, a study could be conducted to assess the chelating abilities of liberated AM compounds. Several compounds which contain an iminodiacetic acid functional group (**1a**) have been studied for their chelating properties (Figure 5.1). Peng et al.[302] demonstrated that organic compounds with a Ethylenediaminetetraacetic acid (EDTA, **1b**) or Diethylenetriamine pentaacetic acid (DTPA, **1c**) backbone chelate Mg^{2+}, Zn^{2+} and Ca^{2+} (Figure 5.1). This was done measuring metal ions with 19F chemical exchange saturation transfer magnetic resonance imaging (19F CEST MRI). Chelators such as EDTA are often used as food preservatives and have been shown to potentiate the effects of weak acids and act as permeabilizing agents of the membrane in Gram-negative bacteria [303-304].

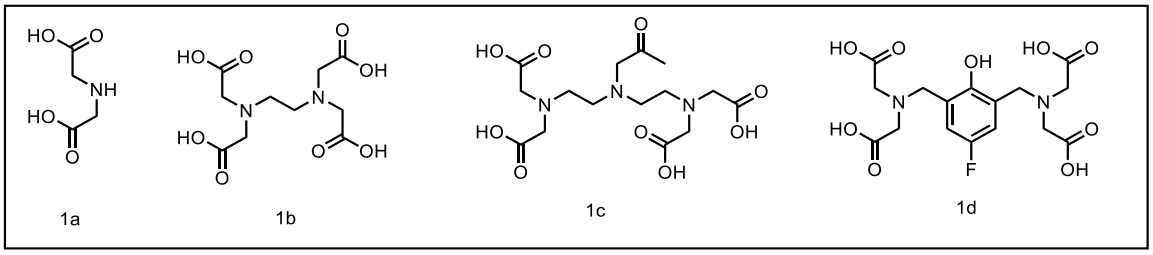


Figure 5.1 Previously studied chelating agents

The equilibrium binding of Fe(II)/Mn(II) of 2,2',2",2"'-(((5-fluoro-2-hydroxy-1,3-phenylene)bis(methylene))-bis(azanetriyl))tetraacetic acid (**1d**) has also been studied, showing that **1d** binds to Fe^{2+} and Mn^{2+} (Figure 5.1) [305]. Several lanthanide (Ln) binding peptides with iminodiacetic acid (**1a**) appendages have also been studied as well [306-309], showing that this groups efficiently binds Ln (III). This was done through monitoring chemical shifts via ^{19}F NMR.

To expand on characterization of current AM prodrugs through the evaluation of the chelating effects of corresponding di-acetic acids, chelation can be monitored via

fourier-transform infrared spectroscopy attenuated total reflection (FTIR-ATR). In a study by Miller et al., FTIR-ATR was used to measure the percent chelation of citric acid and malic acid with Ca^{2+}, Mg^{2+} and Zn^{2+} [310]. This technique used overlaid spectra of the di-acid and the analogous spectra as the metal was added, measuring the formation of the corresponding metal acetate. The same technique could be used here with the di-acetic acid derivatives of current AMs, such compounds **2a-d** in Figure 5.2.

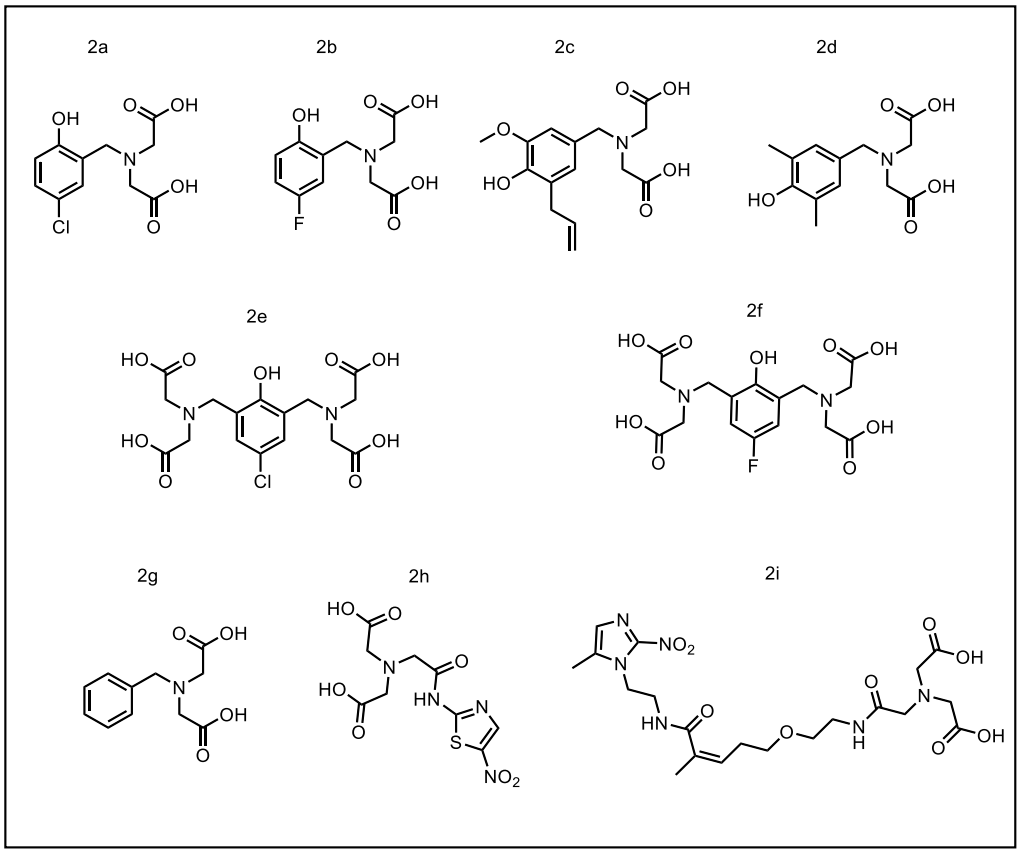


Figure 5.2. Examples of di-substituted derivatives

Compounds **2a**/**b** were chosen because they have the diacid in the *ortho* position while **2c**/**d** were chosen because the diacid is in the *para* position. For this study two di-substituted di-acetic acid derivatives have been synthesized as well (**2e** and **2f** in Figure 5.2). These compounds would be valuable to assess for comparison with their

corresponding mon-substituted counterparts. Additional compounds that have yet to be synthesized are **2h** and **2i** (Figure 5.2). Compounds **2g**, **2h** and **2i** also lack the phenolic hydroxyl group, and evaluation of these has potential to shed light on if the hydroxyl group participates in chelation.

The role chelation plays in the potency of AM compounds can also be elucidated through comparing the potency of compounds **3a** and **3b** as well as the chelating properties of **3c** and **3d** (Figure 5.3). Both compounds **3a** and **3b** possess ester groups, although once cleaved, only **3c** is expected to chelate divalent cation. The chelating properties of **3c** and **3d** will also be evaluated in order to insure **3d** is not a chelating agent. Comparing the potency of these prodrugs will give insight into whether chelation plays a role in potency.

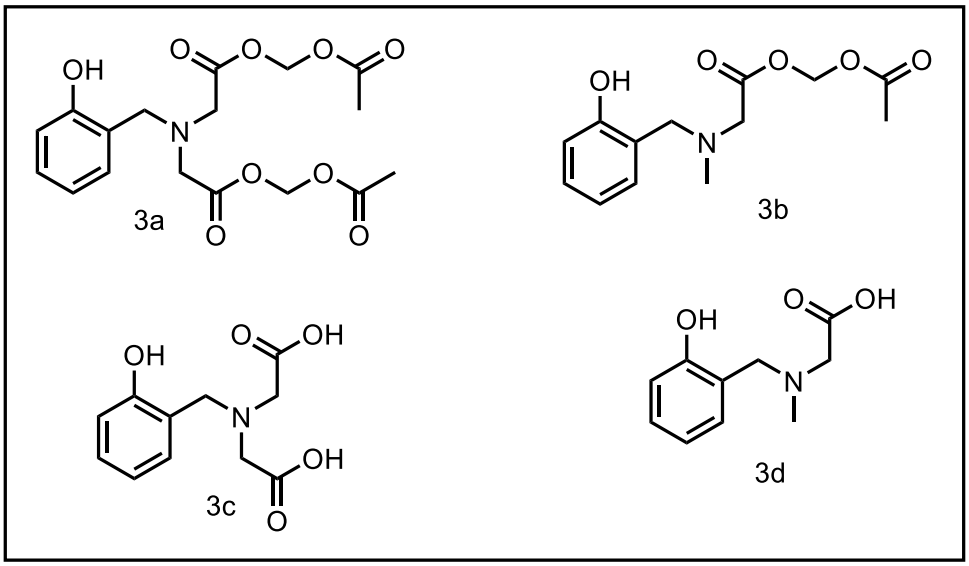


Figure 5.3 Compounds to comparison to determine the effect of chelation on potency

5.1.4 Determination of formaldehyde releasing AM groups

AM prodrugs are predicted to be formaldehyde releasing agents. It is our hypothesis that when AMs are cleaved by esterase within the cell the liberated drug (**4b**) is released as

well as acetic acid (**4c**) and formaldehyde (**4d**) (Figure 5.4). This could very well play a role in the potency of these compounds.

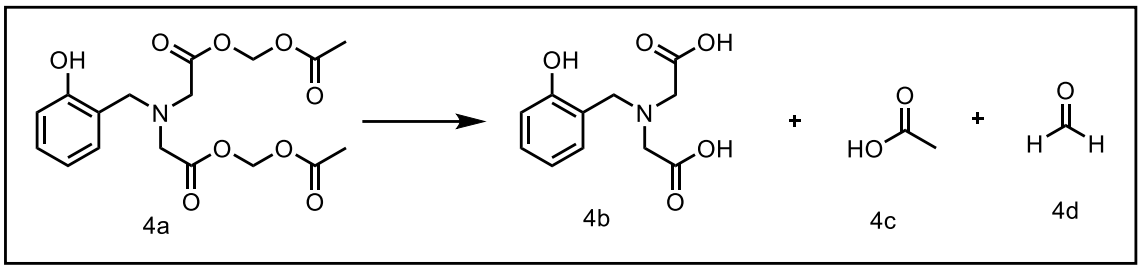


Figure 5.4 products produces upon AM cleavages

In order to determine the effects that the release of formaldehyde has on potency, the compounds shown in Figure 5.5 could be evaluated against biofilms. Compound **4e** will produce the corresponding liberated drug as well as **4c** and **4d**, while **4f** will only produce **4c** and **4b**. This will allow us to determine the degree of potency that the release of formaldehyde is contributing.

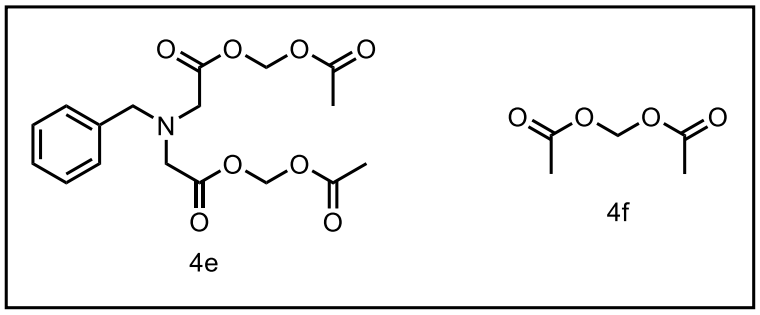


Figure 5.5 structures to be evaluated for release of formaldehyde

Compound **4e** will be evaluated as opposed to **4a** to eliminate the participation of the phenol the in killing of cells. To ensure that formaldehyde is released by these two compounds, **4e** and **4f** may also be exposed to esterase and then quantified via LCMS in the same procedure used in section 4.3.4. Spectra for pure parent compounds (**4e** and **4f**) will be obtained. The parent compounds will then be exposed to esterase and the resulting fragments evaluated.

5.2 AM prodrug synthesis on known antimicrobials

The synthesis and evaluation of AM prodrugs with a nitazoxanide (**5a**) backbone is currently underway by the Livinghouse group and will serve as a critical section of future work for this project (Figure 5.6). Nitazoxanide is a broad-spectrum anti-parasitic and anti-viral drug in the class of thiazolides, which interfere with the pyruvate:ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction [311]. This redox-active prodrug is active towards many bacterial, protozoan, and macro-parasitic species [253, 312-315].

In literature, several analogues have been synthesized to optimize nitazoxanide by replacing the nitro group with halogens as well as adding a number of other groups to the aromatic ring with fluoro, chloro, cyano, nitro and methoxy substituents [316-317]. These analogues aimed to increase antibacterial efficacy towards microorganisms that utilize PFOR, although they were evaluated exclusively towards planktonic cells of *Helicobacter pylori, Campylobacter jejuni, Clostridium difficile* as well as the protozoa *Giardia lamblia* and *Cryptosporidium parvum*. In the studies mentioned above, it was shown that *p*-Cl and *p*-methoxy substituents increased activity dramatically. Although there has been very limited research of nitazoxanide against biofilms, it has been shown to inhibit biofilm formation in *E. coli* and *S. epidermidis,* and decrease cell viability of *C. difficile* biofilms [248-249, 255].

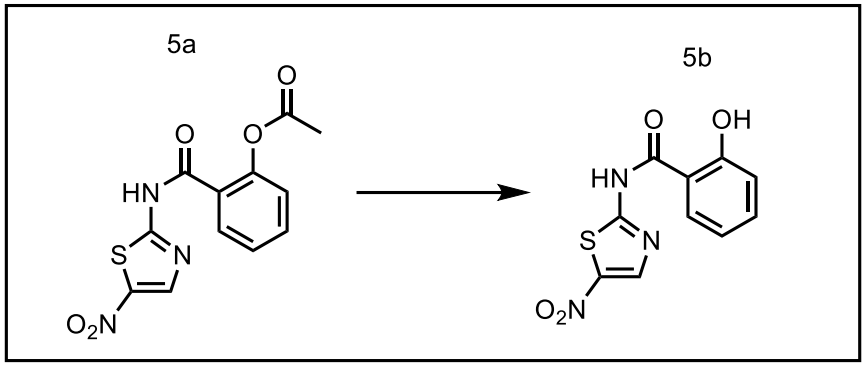


Figure 5.6 The anti-parasitic prodrug, nitazoxanide (**5a**) and its liberated form, tizoxanide (**5b**).

A series of nitazoxanide analogues are currently being synthesized and evaluated against biofilms of *S. epidermidis* and *P. aeruginosa* (**5c-5j**, Figure 5.7). These analogues will assess the effects of acetate placement on the aromatic ring as well as the effects of an additional chlorine or methoxy group. Variations of the nitrothiazole (**5k-5n**) group are also being evaluated (Figure 5.7). These derivatives will also go on to be further synthesized into AM prodrugs, as seen in Figure 5.8.

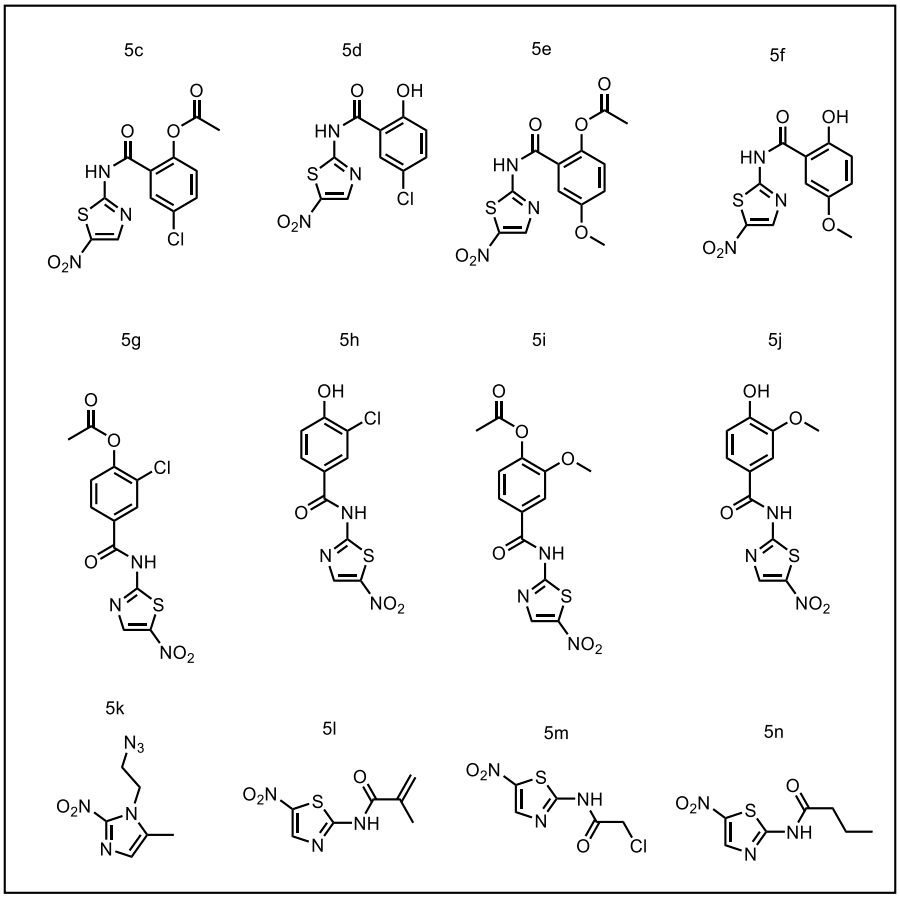


Figure 5.7 Currently synthesized nitazoxanide-analogues

To begin this study, nitazoxanide (**5a**) and the corresponding phenol tizoxanide (**5b**), were evaluated toward *S. epidermidis* and *P. aeruginosa* biofilms (Table 5.1). Tizoxanide was evaluated here to determine the effect of the acetate group on potency. BEC results for compounds that have been synthesized and evaluated thus far are included in Table 5.1. Analogues **5b, 5c** and **5g** exhibited a significant increase in potency compared to nitazoxanide (**5a**) against *S. epidermidis*, although were less potent towards *P. aeruginosa*. The prodrug **5c** exhibited a higher potency than its corresponding de-acetylated counterpart, **5d** towards *S. epidermidis* biofilms, although had the same BEC towards *P. aeruginosa*. This trend was not seen with nitazoxanide (**5a**) and tizoxanide (**5b)**,

where the BECs for the de-acetylated derivative were greater than 100 mM towards both bacteria. Compound **5g** is currently the only analogue with the acetate group in the para position as opposed to ortho. Compounds **5e, 5f, 5h, 5i** and **5j** are currently in the process of being synthesized.

Table 5.1 BECs of nitazoxanide (3a) as well as analogues 3b, 3c and 3e.

	Biofilm Eradication Concentration (mM)	
Compound	*S. epidermidis*	*P. aeruginosa*
5a	50	3.12
5b	>100	>100
5c	<0.19	12.5
5d	6.2	12.5
5g	0.78	>100

Due to this observation, it could be very valuable for these compounds to be evaluated towards additional Gram-positive bacteria such as *S. aureus*, *Streptococcus pneumonia* and *Enterococcus faecalis*. These three bacteria are likely options because of their prevalence in nosocomial infections [318]. Since nitazoxanide possessed the highest BEC toward *P. aeruginosa* it would be beneficial to test these compounds against additional strains for Gram-negative bacteria. *C. difficile* and *H. pylori* should be used as well due to extensive literature on nitazoxanide for its treatment [319-322]. This would assist in the characterizing of these compounds as viable antimicrobial agents towards Gram-positive bacterial biofilms.

Additionally, since nitazoxanide is not a conventional antibiotic, an interesting addition here would be the assessment of analogues against protozoa in the genera of

Cryptosporidium and *Giardia*. These genera are suggested due to nitazoxanide routinely being used for treatment of *C. parvum* and *G. lamblia* [323].

Analogues will undergo further synthesis into AM prodrugs (Figure 5.8). These new AM derivatives will be evaluated against planktonic cells as well as biofilms, to assess the alterations in activity compared to their corresponding, non-AM analogues as well as nitazoxanide (**5a**). Based on previous results, summarized in chapter 4, it is expected that AM groups will increase potency towards biofilms.

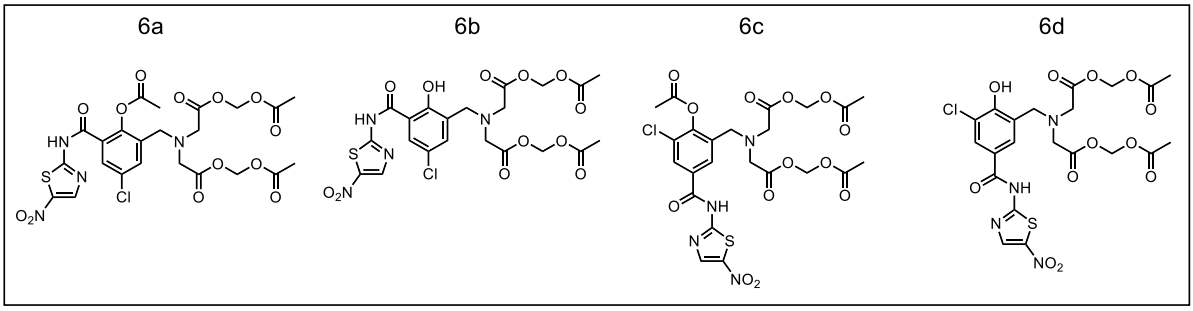


Figure 5.8 Examples of nitazoxanide AM-analogues.

5.3 Additional nitrile oxide AM prodrugs

An additional direction for this project is the design, synthesis and biological evaluation of nitrile oxide AM prodrugs such as compound **8a** (Figure 5.9). In this series, the nitrothiazole (**9a**) or nitrofuran (**9b**) side chain will serve as a 'warhead' on the AM prodrugs. Thiazolides, and more specifically nitrothiazoles, are a class of antimicrobial agents, such as nitazoxanide (**5a,** Figure 5.6) and are used to treat a wide range of pathogens [324-327]. The exact mode of action of thiazolides is currently under investigation, although compounds in this class such as nitazoxanide have been shown to interfere with the (PFOR) enzyme-dependent electron transfer reaction [311].

Nitrofurans encompass a diverse class of drugs commonly used to treat a wide range of bacteria, fungi and protozoa [328-330]. Nitrofurans are active towards both Gram-negative and Gram-positive bacteria and have been shown to interfere with gene expression, inhibiting mRNA [331]. Nitrofurans such as nitrofurantoin, nifuroxazide and furylfuramide have been used as antimicrobials since the 1960's but nitrofurans have only recently begun to be evaluated towards biofilms, with varying degrees of success [332-334].

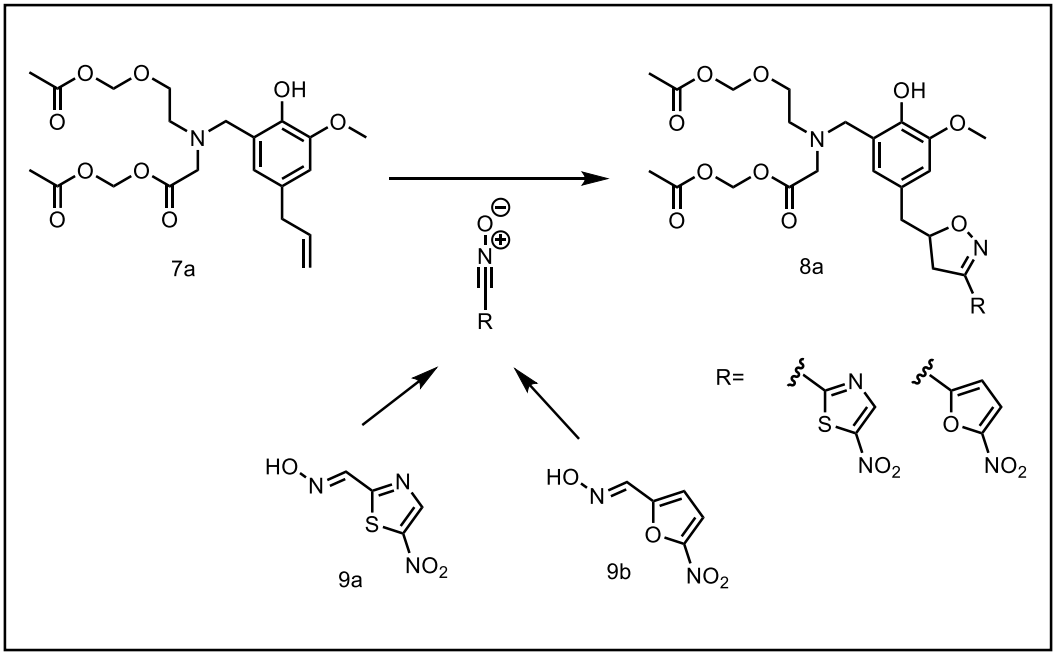


Figure 5.9 a representative of the synthesis of the nitrile oxide of "eugenol AM" (**8a)**.

This generation of AM prodrugs combines known antimicrobial functional groups with the currently synthesized AMs seen in chapter 4, aiming to dramatically increase potency towards biofilms. "Eugenol AM" (**7a**) will be the first in the series to be synthesized into this new generation of prodrug AMs due to its para-allyl group. The nitroheterocyclic 'warheads' **9a** and **9b** will be employed through the cycloaddition

mechanism shown in Figure 5.9. This new class of prodrug antimicrobials will be evaluated against both Gram-negative and positive bacteria in the planktonic and biofilm states.

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APPENDICES

APPENDIX A:

FULL EXPERIMENTAL

1 A Biological experiment

1.1A General Bacterial Growth Procedure

P. aeruginosa (PA01 and PA015542) and *S. epidermidis* (35984) were obtained from American Type Culture Collection (ATCC). All bacteria were sub-cultured onto tryptic soy agar (TSA) plates and incubated at 37 °C for 24 h. Single colonies were transferred from the plates and inoculated into 25 mL tryptic soy broth (TSB) in Erlenmeyer flasks. Culture were incubated 37 °C for 24 h and 10 µL of culture was transferred into 25 mL of TSB and the absorbance was read at 600nm using a spectrophotometer and standardized to 10^6-10^7 CFU/mL.

1.2A Efficacy of compounds on inhibiting planktonic cells

Minimum inhibitory concentrations (MICs) of all compounds evaluated here against were determined using a 96-well plate assay previously described by Xie [171]. 96-wellplates were inoculated with bacterial culture, prepared as stated in section 2.1, followed by exposure to the phenol or sulfenate. Plates were incubated at 37 °C for 12 h. A plate reader was used to analyze bacterial inhibition. Experiments were done in biological triplicate with technical duplicates. Tests for statistical significance were calculated with a two-tailed t-test assuming unequal variances.

1.3A Biofilm eradication assays

1.3.1A Biofilm eradication 96-well plate assay

Both strains were cultured as described above and biofilms were grown in Costar polystyrene 96-well plates at 37 °C. After 24 h of incubation, the planktonic-phase cells were gently removed, and the wells were washed three times with PBS. Wells were filled with 150 µL dilutions of the compound being evaluated. The 96-well plates were incubated for an additional 12 h at 37 °C. The media was gently removed and each well filled with 150 µL PBS and the biofilm broken up through stirring with sterile, wooden rods. Three tenfold dilutions of each sample were drop plated on TSA plates and incubated for 24 h. The biofilm eradication concentration (BEC) was determined to be the lowest concentration at which no bacterial growth occurred. This procedure was modelled on previously reported procedures according to Pitts [172]. Negative controls were also conducted with 150 µL PBS in the absence of antimicrobial agent. Experiments were done in biological triplicate with technical duplicates.

1.3.2A Center for disease control (CDC) biofilm reactor evaluation

A CDC biofilm reactor was also used to assess potency of compounds towards biofilms. American Society for Testing and Materials (ASTM) method E2562 – 17, which describes how to grow a biofilm in the CDC biofilm reactor under high shear and continuous flow, and ASTM method E2871 -13, a biofilm efficacy test generally known as the single tube method were used for this procedure. Formation of 48 h biofilms in a CDC reactor was formed on glass coupons (4.02 cm^2). A CDC reactor containing 340 mL of TSB (300 mg/L) was inoculated with 1 mL of a 3.21 x 10^8 CFU/mL overnight culture of *P. aeruginosa* (PA015542), which was grown in TSB (300 mg/L) overnight. The biofilm was grown in batch condition at room temperature at 125rpm for 24 h, and then for 24 h at

room temperature under continuous flow with a feed rate of 11.25 mL/min at 125 rpm. The continuous feed TSB was 100 mg/L. Coupons were then sampled from the reactor in triplicate. The mean log reduction in viable biofilms cells exposed to each compound for 1 h was quantitatively measured according to ASTM method E2871-13. After coupons were removed from the CDC reactor they were rinsed and transferred to separate, 50 mL conical tubes and 4 mL of a 100 mM solution of the antimicrobial compound being tested in sterile PBS buffer was added. The tubes were incubated at room temperature under static conditions or 1 h. After one h 36 mL DE broth was added and the biofilm was disaggregated by a series of vortexing and sonicating for 30 seconds each in the order of v/s/v/s/v. Each sample was diluted tenfold six times and the diluted samples were drop plated on (Reasoner's 2A agar) R2A agar plates, incubated overnight at 37°C and enumerated. Experiments were done in biological duplicate and technical duplicates were done.

1.4A Enzyme assays

A 30 microliter solution of 8b at a concentration of 50 µM in acetonitrile was dissolved in a 100 microliters solution of esterase (from porcine liver 72 units/mL in cold HEPE buffer, pH7.9). The mixture was incubated at 37 oC for the either 8, 16 or 24 minutes and then cooled to 0oC to inhibit further enzyme activity. The sample was then extracted several times with acetonitrile and analyzed via LCMS to determine if the cleaved product was present.

2A Chemical synthesis and experiments

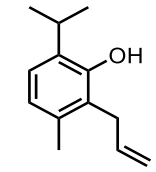
2.1A General procedure

Reactions employed oven-dried glassware under nitrogen unless otherwise noted. All reagents and starting materials were purchased from commercial suppliers and used as received, unless otherwise stated. External oil bath temperatures were used to record reaction mixture temperatures. Solvents for filtrations, transfers, and chromatography were certified ACS grade. Thin layer chromatography was performed on Silicycle Glass Backed TLC plates, and visualization was accomplished with UV light (254 nm), and/or potassium permanganate. All ^{1}H NMR spectra were recorded on a Bruker DRX300. Chemical shifts (δ) are ^{13}C NMR were recorded on a Bruker DRX500 reported in ppm, employing the solvent resonance as the internal standard. Splitting patterns are designated as: s (singlet), d (doublet), app d (apparent doublet), t (triplet), q (quadruplet), qu (quintet) dd (doublet of doublets), ddd (double double doublet), dddd (double, double, double, doublet), sept (septuplet), tq (triplet of quadruplets) and m (multiplet).

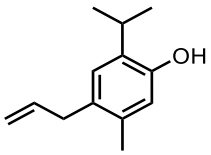
2.2A F ^{19} NMR monitoring rate of hydrolysis of
(4-fluorophenoxy) (trichloromethyl) sulfane

(4-fluorophenoxy)(trichloromethyl) sulfane (**15b**) (13 mg, 0.05 mmol) was dissolved in water (1 mL). An aliquot was taken every 12 hours and dissolved in D_2O in an NMR tube. F^{19} NMR was performed to measure the appearance of the parent compound, *p*-fluorophenol (**15a**) in the sample. A 0 h F^{19} NMR of the sulfane derivative (**15b**) was taken, as was of the pure parent compound (**15a**) for reference (Figure 2). Technical triplicates were done.

2.3A Synthesis of allyl, methallyl and propyl derivatives of naturally occurring phenols (seen in section 2)



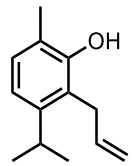
(Major product)



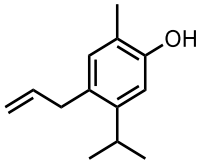
(Minor product)

Preparation of 2-(2-propen-1-yl)-6-(1-methylethyl)-3-methylphenol and 4-(2-propen-1-yl)-6-(1-methylethyl)-3-methylphenol (Section 2. 1b and 1c). A 25 mL round-bottom flask equipped with a magnetic stirring bar was charged with thymol (751 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux and allyl bromide (0.5mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 741 mg (78%) of **1b** and 95 mg (10%) of **1c**, both as light yellow oils. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **^{1}H NMR** (300 MHz, $CDCl_3$) **1b**: δ 6.98 (d, *J* = 7.82 Hz, 1H), 6.77 (d, *J* = 7.82 Hz, 1H), 5.95 (m, 1H), 5.12 (m, 2H), 4.93 (s, 1H) 3.44 (d, *J* = 5.88 Hz, 2H), 3.16 (sept, *J* = 6.87 Hz, 1H) 2.26 (s, 3H), 1.24 (d, *J* = 6.87 Hz, 6H). **C^{13} NMR** (500 MHz, $CDCl_3$) **1b**: δ 19.2 (CH_2), 22.9 (CH_3), 26.8 (CH_2), 29.3 (CH), 115.6 (CH_2), 124.3 (C), 128.8 (CH), 132.1 (C), 134.7 (CH), 137.3 (CH), 139.4 (C),

150.7 (C). H[1] NMR (300 MHz, $CDCl_3$). **^{1}H NMR** (300 MHz, $CDCl_3$) **1c**: δ 6.99 (s, 1H), 6.75 (s, 1H), 5.73 (m, 1H), 4.8 (m, 2H), 3.13 (app d, *J* = 6.1 Hz, 2H) 2.96 (sept, *J* = 6.97 Hz, 1H), 2.29 (s, 3H) 1.21 (d, *J* = 6.97 Hz, 6H). **C^{13} NMR** (500 MHz, $CDCl_3$) **1c**: δ 19.1 (CH_3), 23.12 (CH_3), 26.83 (CH), 33.81 (CH_2), 112.94 (CH_2), 123.53 (C), 128.91 (CH), 131.09 (CH), 134.76 (C), 137.5 (C), 143.48 (C), 152.31 (C).



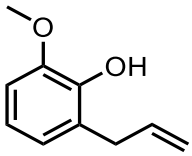
(Major product)



(Minor product)

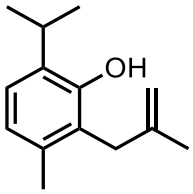
Preparation of 2-(2-propen-1-yl)-3-(1-methylethyl)-6-methylphenol and 4-(2-propen-1-yl)-3-(1-methylethyl)-6-methylphenol (Section 2. 2b and 2c). A 25 mL round-bottom flask equipped with a magnetic stirring bar was charged with carvacrol (751 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux and the allyl bromide (0.5 mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 703 mg (74%) of **2b** and 104 mg (11%) of **2c**, both as yellow oils. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **^{1}H NMR** (300 MHz, $CDCl_3$) **2b**: δ 7.01 (d, *J* = 7.88 Hz, 1H), 6.81 (d, *J* = 7.88 Hz, 1H), 5.94 (m, 1H), 5.10 (m, 2H), 4.82

(s, 1H) 3.46 (app d, *J* = 5.67 Hz, 2H), 3.07 (sept, *J* = 6.84 Hz, 1H) 2.20 (s, 3H), 1.19 (d, *J* = 6.84 Hz, 6H). **C^{13} NMR** (500 MHz, $CDCl_3$) **2b**: δ 15.6 (CH_3), 23.56 (CH_3), 29.59 (CH_2), 29.73 (CH), 115.47 (CH_2), 121.3 (C), 122.34 (CH), 125.28 (C), 136.76 (CH), 137.3 (CH), 139.25 (C), 151.63 (C). **H^1 NMR** (300 MHz, $CDCl_3$) **2c**: δ 6.85 (s, 1H), 6.67 (s, 1H), 5.93 (m, 1H), 4.97 (m, 2H), 4.75 (s, 1H), 3.31 (app d, *J* = 6.12 Hz, 2H), 3.05 (sept, *J* = 6.87 Hz, 1H) 2.17 (s, 3H), 1.16 (d, *J* = 6.87 Hz, 6H). **C^{13} NMR** (500 MHz, $CDCl_3$) **2c**: δ 15.8 (CH_3), 22.42 (CH_3), 29.83 (CH), 33.76 (CH_2), 112.94 (CH2), 121.53 (C), 122.4 (CH), 125.60 (CH), 136.06 (C), 139.54 (C), 143.46 (C), 152.31(C).



Preparation of 2-(2-propen-1-yl)-6-methoxyphenol (Section 2. 3b). A 25 mL round-bottom flask equipped with a magnetic stirring bar was charged with guaiacol (620 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux and allyl bromide (0.5mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 730 mg of **3b** as a light yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **H^1 NMR** (300 MHz, $CDCl_3$): δ 6.95 (t, *J* = 8.61 Hz, 8.07, 1H), 6.79 (d, *J* = 8.07 Hz, 2.76,

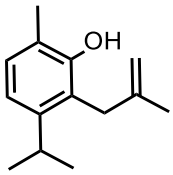
1H), 6.64 (d, *J* = 8.61 Hz, 2.76, 1H), 5.66 (m, 1H), 4.86 (m, 2H) 3.69 (s, 3H), 3.24 (d, *J* = 6.29 Hz, 2H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 33.9 (CH_2), 56.16 (CH_3), 109.02 (CH), 115.39 (CH_2), 122.08 (CH), 125.55 (CH), 131.13 (C), 136.73 (CH), 141.61 (C), 146.29 (C).



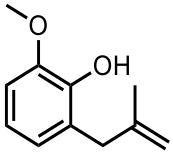
Preparation of 2-methallyl-6-isopropyl-3-methyl phenol (Section 2. 1d).

A 25 mL round-bottom flask equipped with a magnetic stirring bar was charged with thymol (751 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux and methallyl chloride (0.6 mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 887 mg (87%) of **1d** as a light yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **H^1 NMR** (300 MHz, $CDCl_3$): δ 6.99 (d, *J* = 7.82 Hz, 1H), 6.75 (d, *J* = 7.82 Hz, 1H), 4.86 (s, 1H), 4.70 (s, 1H), 3.37 (s, 2H), 2.93 (sept, *J* = 6.87 Hz, 1H) 3.26 (s, 3H), 1.59 (s, 3H) 1.23 (d, *J* = 6.87 Hz, 6H). **C^{13} NMR** (500 MHz,

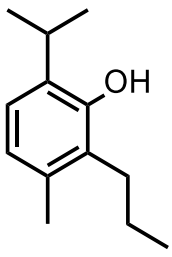
$CDCl_3$): δ 19.1 (CH_3), 22.42 (CH_3), 23.12 (CH_3), 26.83 (CH), 33.81 (CH_2), 112.94 (CH_2), 123.53 (C), 128.91 (CH), 131.09 (CH), 134.76 (C), 137.4 (C), 143.5 (C), 157.4 (C).



Preparation of 2-methallyl-3-isopropyl-6-methyl phenol (Section 2. 2d). A 25 mL round-bottom flask equipped with a magnetic stirring bar was charged with carvacrol (751 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux and methally chloride (0.6 mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 867 mg (85%) of **1d** as a light yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **H^1 NMR** (300 MHz, $CDCl_3$): δ 6.99 (d, *J* = 7.86 Hz, 1H), 6.81 (d, *J* = 7.86 Hz, 1H), 4.84 (s, 1H), 4.60 (s, 1H), 3.39 (s, 2H), 3.06 (sept, *J* = 6.81 Hz, 1H) 2.19 (s, 3H), 1.80 (s, 3H), 1.17 (d, *J* = 6.81 Hz, 6H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 15.1 (CH_3), 22.42 (CH_3), 23.59 (CH_3), 29.83 (CH), 33.81 (CH_2), 112.94 (CH2), 121.53 (C), 122.91 (CH), 126.60 (CH), 136.06 (C), 139.54 (C), 143.46 (C), 150.61 (C).

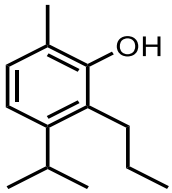


Preparation of 2-methallyl-6-methoxy phenol (Section 2. 3g). A 25 mL round-bottom flask equipped with a magnetic stirring bar was charged with guaiacol (620 mg, 5 mmol, 1 equiv) and anhydrous acetone (5 mL) was added. Finely pulverised potassium carbonate (1.4 g, 10 mmol, 2 equiv) was then added at room temperature with stirring. The reactant mixture was heated at reflux and methallyl chloride (0.6 mL, 6 mmol, 1.2 equiv) was added. The reactant mixture was heated at reflux for 5 h. The resulting mixture was cooled and filtered through celite, washed with brine and concentrated in vacuo. The crude phenyl ether was dissolved in N,N-diethylaniline (2 mL) and heated to 200 ºC with stirring for 12 h. N,N-diethylaniline was subsequently removed by washing the mixture with 10% sulfuric acid and extracting with ethyl acetate. The residue was purified via column chromatography (25% EtOAc/Hexane for eluation) to afford 801 mg (90%) of **3g** as a light yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **H[1] NMR** (300 MHz, $CDCl_3$): δ 6.95 (d, *J* = 7.88 Hz, 1H), 6.79 (m, 3H), 5.71 (s, 1H), 4.83 (s, 1H), 4.72 (s, 1H), 3.90 (s, 3H) 3.39 (s, 2H), 1.77 (d, 3H). **C[13] NMR** (500 MHz, $CDCl_3$): δ 22.40 (CH_3), 37.58 (CH_2), 55.99 (CH_3), 108.60 (CH_3), 111.28 (CH_2), 119.24 (CH), 122.81 (CH), 125.52 (C), 143.76 (C), 144.71 (C), 146.42 (C).



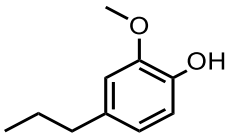
Preparation of 2-*n*-propyl-6-isopropyl-3-methyl phenol (Section 2. 1e). A 10 mL round-bottom flask was charged with 10% Pd/C (30 mg, 0.28 mmol, 0.1 equiv).

The round-bottom flask was put under an atmosphere of hydrogen and 100% ethanol (2 mL) was added. 2-(2-propen-1-yl)-6-(1-methylethyl)-3-methylphenol (**1b)** (285 mg, 1.5 mmol, 1 equiv) was added at room temperature and the reaction was stirred for 12h. The resulting mixture was filtered through silica and concentrated in vacuo to afford 251 mg (87%) of **1e** as a light, yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 6.95 (d, *J* = 7.88 Hz, 1H), 6.81 (d, *J* = 7.88 Hz, 1H), 2.98 (sept, *J* = 6.81 Hz, 1H), 2.56 (t, *J* = 7.89 Hz, 2H), 2.25 (s, 3H) 1.59 (q, *J* = 7.89, 7.32 Hz, 2H), 1.36 (d, *J* = 6.81 Hz, 6H) 0.96 (t, *J* = 7.32 Hz, 3H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 14.5 (CH_3), 19.4 (CH_3), 22.3 (CH_3), 22.7 (CH_2), 27.1 (CH_2), 28.8 (CH_2), 122.3 (CH), 123.1 (C), 126.5 (CH), 131.3 (CH), 134.7 (C), 150.8 (C).

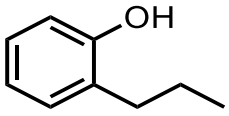


Preparation of 2-*n*-propyl-3-isopropyl-6-methyl phenol (Section 2. 2e). A 10 mL round-bottom flask was charged with 10% Pd/C (30 mg, 0.28 mmol, 0.1 equiv). The round-bottom flask was put under an atmosphere of hydrogen and 100% ethanol (2 mL) was added. 2-(2-propen-1-yl)-3-(1-methylethyl)-6-methylphenol (**2b)** (285 mg, 1.5 mmol, 1 equiv) was added at room temperature and the reaction was stirred for 12h. The resulting mixture was filtered through silica and concentrated in vacuo to afford 263 mg, (91%) of **2e** as a light, yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 6.95 (d, *J* = 7.86 Hz, 1H), 6.78 (d, *J* = 7.86 Hz, 1H), 3.09 (sept, *J* = 6.81 Hz, 1H), 2.61 (t, *J* = 7.95 Hz, 2H), 2.19 (s, 1H) 1.53 (q, J = 7.95, 7.29 Hz,

2H), 1.15 (d, *J* = 6.81 Hz, 6H), 1.0 (t, *J* = 7.29 Hz, 6H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 14.50 (CH_3), 15.81 (CH_3), 23.55 (CH_3), 24.22 (CH_2), 27.81 (CH_2), 28.83 (CH_2), 117.17 (CH), 119.81 (C), 125.49 (CH), 128.12 (CH), 146.13 (C), 151.64 (C).



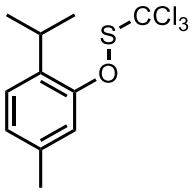
Preparation of 2-*n*-propyl-2-methoxy (Section 2. 3d). A 10 mL round-bottom flask was charged with 10% Pd/C (30 mg, 0.28 mmol, 0.1 equiv). The round-bottom flask was put under an atmosphere of hydrogen and 100% ethanol (2 mL) was added. Eugenol (**3a**) (246 mg, 1.5 mmol, 1 equiv) was added at room temperature and the reaction was stirred for 12h. The resulting mixture was filtered through silica and concentrated in vacuo to afford 249 mg, (93%) of **3d** as a light, yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **H^1 NMR** (300 MHz, $CDCl_3$): δ 6.79 (d, J = 8.58 Hz, 1H), 6.66 (m, 2H), 3.86 (s, 3H), 2.49 (t, J = 7.63, 2H), 1.60 (m, J = 7.63, 7.32, 2H), 1.22 (t, J = 7.32, 3H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 12.49 (CH_3), 24.67 (CH_2), 37.47 (CH_2), 56.16 (CH_3), 110.86 (CH), 114.89 (CH), 123.59 (CH), 135.39 (C), 147.13 (C), 147.81 (C).



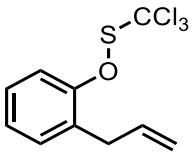
Preparation of 2-*n*-propyl phenol (Section 2. 3f). A 10 mL round-bottom flask was charged with 10% Pd/C (30 mg, 0.28 mmol, 0.1 equiv). The round-bottom flask was put under an atmosphere of hydrogen and 100% ethanol (2 mL) was added. 2-allylphenol (**3e)** (201 mg, 1.5 mmol, 1 equiv) was added at room temperature and the reaction was stirred for 12h. The resulting mixture was filtered through silica and

concentrated in vacuo to afford 184 mg, (90%) of **3f** as a light, yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following. **H^{1} NMR** (300 MHz, $CDCl_3$): δ 7.08 (m, 2H), 6.88 (t, *J* = 7.41 Hz, 1H), 6.74 (d, *J* = 7.89 Hz, 1H), 4.58 (s, 1H), 3.56 (t, *J* = 7.7 Hz, 2H) 1.64 (sep, *J* = 7.7, 7.34 Hz, 2H), 0.98 (t, *J* = 7.34 Hz, 3H). **C^{13} NMR** (500 MHz, $CDCl_3$): δ 12.39 (CH_3), 23.18 (CH_2), 31.97 (CH_2), 116.11 (CH), 120.7 (CH), 128.69 (CH), 130.32 (C), 131.89 (C), 153.39 (C).

2.4A Sulfonate chemistry (seen in section 4)

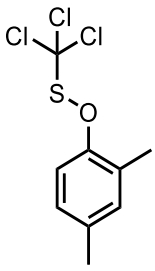


Preparation of (2-isopropyl-5-methylphenoxy)(trichloromethyl)sulfane (Section 3. 1b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 2-isopropyl-5-methylphenol (751 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. The resulting mixture was filtered through cilite and washed with *t*-butyl methyl ether. The solvents were evaporated in vacuum to provide 1.2 g (83%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 7.23 (s, 1H), 7.17 (d, *J* = 4.65 Hz, 1H), 6.94 (d, *J* = 4.65 Hz, 1H), 3.54 (m, 1H), 2.35 (s, 3H), 1.29 (d, *J* = 4.14 Hz 6H). **C13 NMR** (500 MHz, CDCl3): δ 21.10 (CH3), 23.10 (CH3), 26.42 (CH), 99.53 (C), 116.32 (CH), 125.20 (CH), 126.46 (C), 135.02 (CH), 136.64 (C), 156.20 (C). **HRMS** (**ESI**) calcd for $C_{11}H_{13}Cl_3OS^+$ ([M + H]$^+$), 297.9753, found, 297.9724.



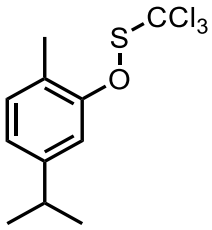
Preparation of (2-allylphenoxy)(trichloromethyl)sulfane (Section 3. 2b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was

charged with 2-allylphenol (671 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.2 g (80%) of the title compound in as a colorless oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 7.38 (d, *J* = 8.25 Hz, 1H), 7.38 (m, 2H), 7.38 (t, *J* = 7.02 Hz, 1H), 6.00 (m, 1H), 6.00 (m, 1H), 5.09 (m, 2H), 3.55 (d, *J* = 6.60 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 34.13 (CH2), 99.45 (C), 115.96 (CH), 116.25 (CH2), 124.41 (CH), 127.43 (C), 129.80 (CH), 130.38 (CH), 135.33 (CH), 136.14 (CH), 157.18 (C). **HRMS** (**ESI**) calcd for $C_{10}H_9Cl_3OS^+$ ([M + H]$^+$), 284.4910, found, 284.9483.



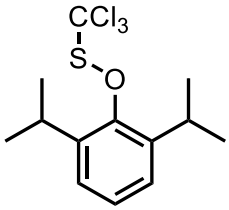
Preparation of (2,4-dimethylphenoxy)(trichloromethyl)sulfane (Section 3. 3b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 2,4-dimethylphenol (610 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 oC and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring,

mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.0 g (76%) of the title compound as a yellow oil. ^{1}H NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 7.23 (dd, *J* = 843, 2.62 Hz, 1H), 7.00 (dd, *J* = 2.62, 0.45 Hz, 1H), 6.98 (dd, *J* = 8.43, 0.45 Hz, 1H), 2.39 (s, 3H), 2.31 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 16.16 (CH3), 20.55 (CH3), 116.19 (C), 121.49 (CH), 130 (C), 132.02 (C), 133.81 (CH), 136.73 (CH), 156.05 (C). **HRMS** (**ESI**) calcd for $C_9H_9Cl_3OS^+$ ($[M + H]^+$), 271.9410, found, 271.9440.



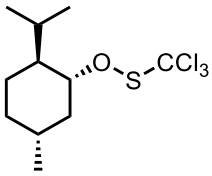
Preparation of (5-isopropyl-2-methylphenoxy)(trichloromethyl)sulfane (Section 3. 4b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 5-isopropyl-2-methylphenol (751 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous triethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then

filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.2 g (79%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 7.24 (s, 1H), 6.96 (d, *J* = 8.15 Hz, 1H), 6.74 (d, *J* = 8.15 Hz, 1H), 3.29 (m, 1H), 2.24 (s, 3H), 1.23 (d, *J* = 6.91 Hz 6H). **C13 NMR** (500 MHz, CDCl3): δ 19.91 (CH3), 23.91 (CH3), 29.75 (CH), 99.89 (C), 116.88 (CH), 126.82 (CH), 129.76 (C), 135.02 (CH), 142.08 (C), 148.19 (C). **HRMS** (**ESI**) calcd for $C_{11}H_{13}Cl_3OS^+$ ($[M + H]^+$), 297.9753, found, 297.9722.

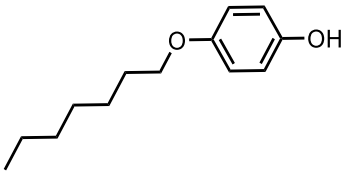


Preparation of (2,6-diisopropylphenoxy)(trichloromethyl)sulfane (Section 3. 5b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 2,6-diisopropyl phenol (891 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^oC and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^oC for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.2 g (75%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 7.15 (m, 3H), 3.55 (m, 2H), 1.29 (d, *J* = 4.38 Hz 6H). **C13 NMR** (500 MHz, CDCl3): δ 23.76 (CH3), 27.66 (CH), 99.32

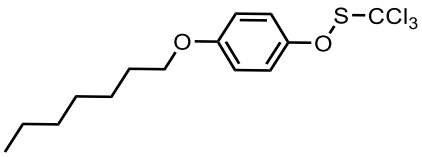
(C), 124.46 (CH), 125.9 (CH), 140.12 (C), 154.97 (C). **HRMS** (**ESI**) calcd for $C_{13}H_{17}Cl_3OS^+$ ([M + H]$^+$), 328.0036, found, 328.0066.



Preparation of (((1S,2R,5S)-2-isopropyl-5-methylcyclohexyl)oxy)(trichloromethyl)sulfane (Section 3. 6b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with (1R,2S,5R)-2-isopropyl-5-methylcyclohexanol (781 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^oC and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^oC for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.3 g (87%) of the title compound in as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 4.10 (q, *J* = 4.43 Hz, 1H), 2.34 (m, 2H), 1.64 (m, 2H), 1.39 (m, 2H), 1.02 (m, 2H), 0.937 (d, *J* = 6.78 Hz, 6H), 0.855 (d, *J* = 6.87 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 16.26 (CH6), 21.18 (CH3), 22.08 (CH2), 23.12 (CH), 25.31 (CH), 34.06 (CH2), 41.91 (CH2), 49.34 (H), 89.95 (CH), 100.64 (C). **HRMS** (**ESI**) calcd for $C_{11}H_{19}Cl_3OS^+$ ([M + H]$^+$), 307.0193, found, 307.0266.

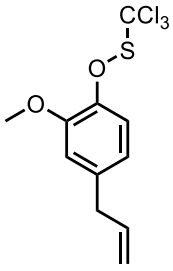


Preparation of 4-heptyloxyphenol (**Section 3. 7a**). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with hydroquinone (1.6 g, 15 mmol, 1.5 equiv), potassium carbonate (2 g, 15 mmol, 1.5 equiv) and 10 mL anhydrous ethanol. The reaction mixture was heated at reflux and heptylbrominde (1.7 mL, 10 mmol, 1 equiv) was added. The reaction was allowed to reflux for 12 h. The mixture was concentrated and purified via column chromatography in 35% EtOAc in hexanes to provide 2.5 g (79%) of **7a** as a white solid. **1H NMR** (300 MHz, CDCl3): δ 6.79 (ddd, *J* = 5.37 Hz, 4H), 2.07 (t, *J* = 3.96 Hz, 2H), 1.76 (quint, *J* = 4.80 Hz, 2H), 1.45 (m, 2H), 1.34 (m, 6H), 0.91 (t, *J* = 4.2 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 14.07 (CH3), 22.60 (CH2), 26.01 (CH2), 29.08 (CH2), 29.39 (CH2), 31.79 (CH2), 68.73 (CH2), 115.61 (CH), 115.98 (CH), 149.28 (C), 153.42 (C).



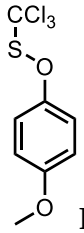
Preparation of (4-(octyloxy)phenoxy)(trichloromethyl)sulfane (Section 3. 7b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-octyloxyphenol (1.0 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional

12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.4 g (81%) of the title compound as a white solid. **1H NMR** (300 MHz, CDCl3): δ 7.20 (d, *J* = 9.09 Hz, 2H), 7.81 (d, *J* = 9.12 Hz, 2H), 3.89 (t, *J* = 6.54, Hz, 2H), 1.74 (quint, *J* =6.51 Hz, 2H), 1.44 (m, 4H), 1.32 (m, 8H), 0.91 (t, *J* = 4.2 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 14.08 (CH3), 22.61 (CH2), 26.02 (CH2), 29.09 (CH2), 29.39 (CH2), 31.79 (CH2), 29.35 (CH2), 31.80 (CH2), 45.78 (CH2), 68.52 (CH2), 96.14 (C), 114.96 (CH), 118.55 (CH), 149.32 (C), 153.37 (C). **HRMS** (**ESI**) calcd for $C_{14}H_{19}Cl_3O_2S^+$ ($[M + H]^+$), 358.0142, found, 358.0171.

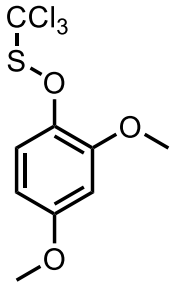


Preparation of (4-allyl-2-methoxyphenoxy)(trichloromethyl)sulfane (Section 3. 8b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-allyl-2-methoxyphenol (821 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^oC and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^oC for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.3 g (84%) of the title

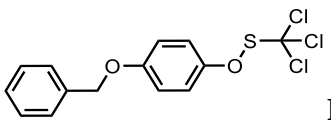
compound in as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 6.87 (dd, *J* = 5.1, 2.54 Hz, 1H), 6.7 (dd, *J* = 3.81, 1.29 Hz, 2H), 5.98 (m, 1H), 5.49 (s, 1H), 5.08 (m, 1H), 3.90 (s, 3H), 3.16 (d, *J* = 4.02 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 39.97 (CH2), 56.10 (CH3), 100.0 (C), 111.10 (CH), 114.23 (CH2), 115.52 (CH), 121.18 (CH), 131.93 (C), 137.82 (CH), 143.91 (C), 146.43 (C). **HRMS** (**ESI**) calcd for $C_{11}H_{11}Cl_3O_2S^+$ ([M + H]$^+$), 314.9561, found, 314.9589.



Preparation of (4-(methoxy)phenoxy)(trichloromethyl)sulfane (Section 3. 9b). A 25 mL round- bottomed flask equipped with a magnetic stir bar was charged with 4-methoxyphenol (620 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvents were evaporated in vacuum to provide 1.2 g (86%) of the title compound as an orange oil. **1H NMR** (300 MHz, CDCl3): δ 7.33 (d, *J* = 5.28 Hz, 1H), 6.53 (d, *J* = Hz, 1H), 1.55 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 55.66 (CH3), 99.49 (C), 114.37 (CH), 118.60 (CH), 153.55 (C), 156.43 (C). **HRMS** (**ESI**) calcd for $C_8H_7Cl_3O_2S^+$ ([M + H]$^+$), 271.9232, found, 271.9242.

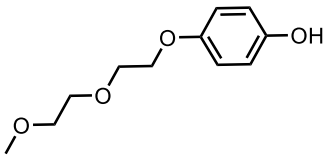


Preparation of (2,4-dimethoxy)(trichloromethyl)sulfane (Section 3. 10b). A 25 mL round- bottomed flask equipped with a magnetic stir bar was charged with 2,4-dimethoxyphenol (751 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.3 g (83%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 7.33 (d, *J* = 8.80 Hz, 1H), 6.53 (d, *J* = 2.80 Hz, 1H), 6.40 (dd, *J* = 8.80, 2.80 Hz 1H), 3.92 (s, 3H), 3.81 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 55.64 (CH3), 56.08 (CH3), 100.19 (CH), 103.22 (C), 121.34 (CH2), 143.59(C), 151.23 (C), 157.74 (C). **HRMS** (**ESI**) calcd for $C_9H_9Cl_3O_3S^+$ ([M + H]$^+$), 304.9309, found, 304.9382.



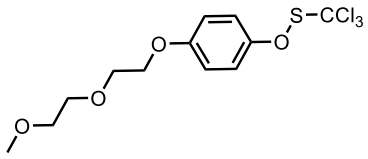
Preparation of (4-benzyloxy)(trichloromethyl)sulfane (Section 3. 11b) - A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-benzyloxy phenol (1.0 g, 5 mmol, 1 equiv) and 10 mL

anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.6 g (93%) of the title compound as a yellow oil. **1H NMR** (300 MHz, CDCl3): δ 7.39 (m, 4H), 6.88 (dd, *J* = 8.95 Hz, 2H), 3.84 (s, 2H). **C13 NMR** (500 MHz, CDCl3): δ 70.77 (CH2), 96.13 (C), 116.03 (CH2), 116.06 (CH2), 127.48 (CH2), 127.90 (CH2), 128.55 (CH), 137.26 (C), 149.62 (C), 153.06 (C). **HRMS** (**ESI**) calcd for $C_{14}H_{11}Cl_3O_2S^+$ ([M + H]$^+$), 349.9545, found, 349.9561.

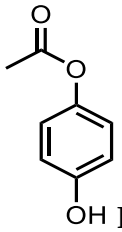


(4-(2-(2-methoxyethoxy)ethoxy)phenol (Section 3. 12a). A 50 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with hydroquinone (2.2 g, 20 mmol, 1 equiv), 2-methoxyethyl 4-methylbenzensulfonate (2.30 g, 10 mmol, 0.5 equiv), potassium hydroxide (1.1 g, 20 mmol, 1 equiv) and 20 mL anhydrous ethanol. The reaction mixture was heated to reflux. After 24 h, the reaction mixture was allowed to cool to room temperature and concentrated under vacuum. The resulting mixture was titrated with 10 mL water and acidified to pH 2 with 2 M HCl. The product was extracted using DCM and concentrated to give 3.0 g (71%) of **12a** as a light orange oil. **1H NMR** (300 MHz, CDCl3): δ 6.76 (s, 2H), 4.04 (t, *J* = 3.03 Hz, 2H), 3.84 (t,

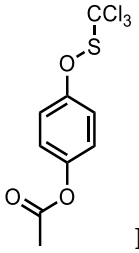
J = 3.03 Hz, 2H), 3.74 (t, J = 2.85 Hz, 2H), 3.63 (t, *J* = 2.85 Hz, 2H), 3.42 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 59.00 (CH3), 68.03 (CH2), 69.92 (CH2), 70.60 (CH2), 71.95 (CH2), 119.72 (CH4), 149.99 (C), 152.69 (C).



Preparation of (4-(2-(2-methoxyethoxy)ethoxy)phenoxy)(trichloromethyl)sulfane (Section 3. 12b) – A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-(2-(2-methoxyethoxy)ethoxy)phenol (1.1 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.5 g (81%) of the title compound as a light yellow oil. **1H NMR** (300 MHz, CDCl3): δ 7.23 (d, *J* = 9.20 Hz, 2H), 6.88 (d, *J* = 9.15 Hz, 2H), 3.84 (t, *J* = 5.00 Hz, 2H), 3.87 (t, J = 5.00 Hz, 2H), 3.743 (t, *J* = 4.55 Hz, 2H), 3.60 (t, *J* = 4.55 Hz, 2H), 3.41 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 59.09 (CH3), 67.93 (CH2), 69.78 (CH2), 70.78 (CH2), 71.97 (CH2), 99.50 (C), 115.19 (CH2), 118.55 (CH2), 153.66 (C), 155.66 (C). **HRMS** (**ESI**) calcd for $C_{12}H_{15}Cl_3O_4S^+$ ([M + H]$^+$), 361.6580, found, 361.9727.

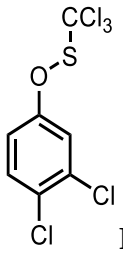


Preparation of 4-hydroxyphenyl acetate (**Section 3. 13a**). A 25 mL round-bottomed flask equipped with a magnetic stir bar was charged with hydroquinone (1.6 g, 15 mmol, 1.5 equiv), acetic anhydride (1.0 g, 10 mmol, 1 equiv) and 10 mL acetic acid. The reaction was allowed to reflux for 12 h. The mixture was concentrated and purified via column chromatography in 35% EtOAc in hexanes to provide 2.5 g (79%) of **13a** as a white solid. **1H NMR** (300 MHz, CDCl3): δ 7.10 (dd, *J* = 98.76 Hz, 2H), 6.98 (dd, *J* = 8.76 Hz, 2H), 2.05 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 169.10 (C), 151.91 (C), 150.03 (C), 116.43 (CH2), 116.10 (CH2), 20.80 (CH3).

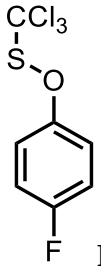


Preparation of 4-(((trichloromethyl)thio)oxy)phenyl acetate (Section 3. 13b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-hydroxyacetophenone (626 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl

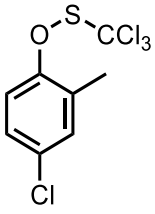
methyl ether. The solvent was evaporated in vacuum to provide 1.2 g (80%) of the title compound in as a white solid. **1H NMR** (300 MHz, CDCl3): δ 7.30 (dd, J = 9.09 Hz, 2H), 7.03 (dd, J = 9.09 Hz, 2H), 2.27 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 169.10 (C), 151.79 (C), 150.33 (C), 116.43 (CH2), 114.86 (CH2), 99.68 (C), 20.83 (CH3). **HRMS** (**ESI**) calcd for $C_9H_7Cl_3O_3S^+$ ([M + H]$^+$), 302.9152, found, 302.9225.



Preparation of (3,4-dichlorophenoxy)(trichloromethyl)sulfane (Section 3. 14b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 3,4-dichlorophenol (815 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.3 g (83%) of the title compound as a light-yellow oil. **1H NMR** (300 MHz, CDCl3): δ 7.43 (d, *J* = 2.94 Hz, 1H), 7.39 (d, *J* = 8.91 Hz, 1H), 7.17 (dd, *J* = 2.94, 8.91 Hz 1H). **C13 NMR** (500 MHz, CDCl3): δ 99.01 (C), 116.85 (CH), 119.48 (C), 128.19 (CH), 130.82 (CH), 133.19 (C), 158.05 (C). **HRMS** (**ESI**) calcd for $C_7H_3Cl_5OS^+$ ([M + H]$^+$), 312.8318, found, 312.8390.

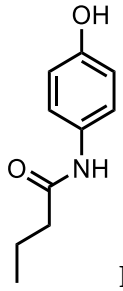


Preparation of (4-fluorophenoxy)(trichloromethyl)sulfane (Section 3. 15b). A 25 mL round- bottomed flask equipped with a magnetic stir bar was charged with 4-fluorophenol (561 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.0 g (78%) of the title compound as a yellow oil. **1H NMR** (300 MHz, CDCl3): δ 7.25 (ddd, *J* = 8.17, 2.38, 0.54, Hz, 2H), 7.02 (ddd, *J* = 8.17, 1.42, 0.54 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 98.10 (C), 115.92 (CH), 116.12 (CH), 151.48 (C), 151.72 (C), 160.1 (C). **HRMS** (**ESI**) calcd for $C_7H_4Cl_3FOS^+$ ($[M + H]^+$), 262.9003, found, 262.9076.



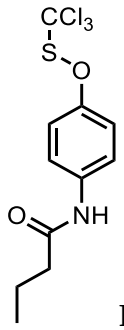
Preparation of (4-chloro-2-methylphenoxy)(trichloromethyl)sulfane (Section 3. 16b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-chloro-2-methylphenol (713 mg, 5 mmol, 1 equiv) and 10 mL

anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.2 g (81%) of the title compound as a light yellow oil. **1H NMR** (300 MHz, CDCl3): δ 7.30 (d, *J* = 5.1 Hz, 1H), 7.18 (s, 1H), 7.16 (d, *J* = 5.1 Hz, 1H), 2.41 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 16.17 (CH3), 99.34 (C), 117.43 (CH), 126.7 (C), 129.16 (C), 129.74 (CH), 130.85 (CH), 156.54 (C). **HRMS** (**ESI**) calcd for $C_8H_6Cl_4OS^+$ ([M + H]$^+$), 292.8893, found, 292.8937.

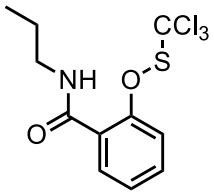


Preparation of N-(4-hydroxyphenyl)butyramide (Section 3. 17a). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with n-butylamine (1.8 g, 25 mmol, 2.5 equiv) and 4-hydroxymethyl benzoate (1.5 g, 10 mmol, 1 equiv). The reaction mixture was heated at reflux and allow to reflux for 24 h, concentrated and purified via column chromatography in 30% EtOAc in hexanes to give 3.8 g (85%) of **17a** as a yellow oil. **1H NMR** (300 MHz, CDCl3 and D_6 DMSO): δ 7.71 (d, *J* = 4.35 Hz, 2H), 6.63 (d, *J* = 4.35 Hz, 2H), 2.19 (t, *J* = 3.57 Hz, 2H), 1.78 (tq, *J* = 3.57,

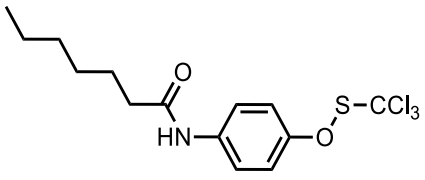
3.69 Hz, 2H), 0.89 (t, *J* = 3.69, Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 13.30 (CH3), 18.54 (CH2), 38.26 (CH2), 114.61 (C), 120.92 (CH), 130.47 (CH), 152.89 (C), 170.64 (C).



Preparation of N-(4-(((trichloromethyl)thio)oxy)phenyl)butyramide (Section 3. 17b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with *N*-(4-hydroxyphenyl)butyramide (896 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.3 g (79%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3 and D_6 DMSO): δ 7.51 (d, *J* = 5.43 Hz, 2H), 7.29 (d, *J* = 5.43 Hz, 2H), 2.35 (t, *J* = 4.44 Hz, 2H), 1.78 (tq, *J* = 4.44, 4.41 Hz, 2H), 1.03 (t, *J* = 4.41, Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 13.75 (CH3), 19.03 (CH2), 39.61 (CH2), 99.39 (C), 117.91 (CH), 120.90 (CH), 155.78 (C), 171.06 (C). **HRMS** (**ESI**) calcd for $C_{11}H_{12}Cl_3NO_2S^+$ ([M + H]$^+$), 327.9654, found, 327.9710.

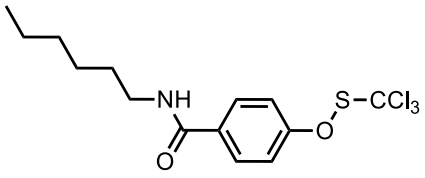


Preparation of N-propyl-2-(((trichloromethyl)thio)oxy)benzamide (Section 3. 18b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 2-hydroxy-*N*-propylbenzamide (1.4 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.9 g (87%) of the title compound as a beige oil. **1H NMR** (300 MHz, CDCl3 and D_6 DMSO): δ 7.15 (t, *J* = 6.85 Hz, 1H), 7.51 (d, *J* = 6.40 Hz, 1H), 7.51 (d, *J* = 6.85, Hz, 1H), 6.89 (t, *J* = 6.55 Hz, 1H), 2.45 (t, *J* = 4.41 Hz, 2H), 1.82 (tq, *J* = 4.44, 4.41 Hz, 2H), 1.06 (t, *J* = 4.44, Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 13.62 (CH3), 19.23 (CH2), 38.88 (CH2), 96.13 (C), 120.28 (CH), 122.06 (CH), 125.52 (CH), 127.28 (C), 148.94 (C), 173.42 (C). **HRMS** (**ESI**) calcd for $C_{11}H_{11}Cl_4NO_2S^+$ ($[M + H]^+$), 362.9265, found, 361.9235.



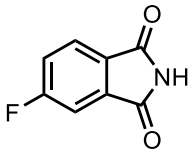
Preparation of N-(4-(((trichloromethyl)thio)oxy)phenyl)hepanamide (Section 3. 19b). A 25 mL round-

bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with *N*-(4-hydroxyphenyl)heptanamide (1.1 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.5 g (81%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3 and D_6 DMSO): δ 7.63 (d, *J* = 8.7 Hz, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 3.44 (t, *J* = 5.9 Hz, 2H), 1.78 (tt, *J* = 7.10, 6.8 Hz, 2H), 1.32 (m, 6H), 0.90 (t, *J* = 6.95, Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 14.00 (CH3), 22.55 (CH2), 26.65 (CH2), 29.59 (CH2), 31.48 (CH2), 40.27 (CH2), 96.13 (C), 115.61 (CH2), 125.75 (CH2), 128.76 (C), 159.95 (C), 168.17 (C). **HRMS** (**ESI**) calcd for $C_{14}H_{18}Cl_3NO_2S^+$ ([M + H]$^+$), 369.0124, found, 371.0094.

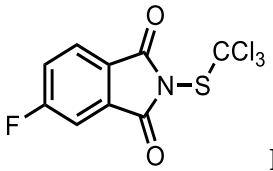


Preparation of N-hexyl-4-(((trichloromethyl)thio)oxy)benzamide (Section 3. 20b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with *N*-hexyl-4-hydroxybenzamide (1.1 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring,

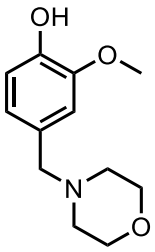
mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ºC for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.6 g (89%) of the title compound as a beige oil. **1H NMR** (300 MHz, CDCl3 and D_6 DMSO): δ 7.54 (d, *J* = 8.80 Hz, 2H), 7.04 (d, *J* = 8.85 Hz, 2H), 3.44 (t, *J* = 7.45 Hz, 2H), 1.78 (tt, *J* = 7.45, 6.32 Hz, 2H), 1.32 (m, 6H), 0.91 (t, *J* = 6.30, Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 14.40 (CH3), 22.47 (CH2), 25.67 (CH2), 28.83 (CH2), 31.51 (CH2), 36.72 (CH2), 95.87 (C), 115.41 (CH2), 121.28 (CH2), 131.52 (C), 153.52 (C), 170.95 (C). **HRMS** (**ESI**) calcd for $C_{14}H_{18}Cl_3NO_2S^+$ ([M + H]$^+$), 369.0124, found, 370.0131.



Preparation of 5-fluoroisoindoline-1,3-dione (Section 3. 21a). A 10 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-fluorophthalic anhydride (2.0 g, 12 mmol, 1 equiv) in toluene (1.5 mL) and urea (800 mg, 13.2 mmol, 1.1 equiv). The reaction mixture was stirred at refluxed for 16 h. The reaction mixture was allowed to cool to room temperature, concentrated under vacuum and triturated with water (5 mL) to afford 190 mg (96%) of 5-fluoro-isoindole-l,3-dione as a white solid. **1H NMR** (300 MHz, CDCl3): δ 11.22 (s, 1H), 7.93 (m, 1H), 7.78 (m, 1H), 7.45 (m, 1H). **C13 NMR** (500 MHz, CDCl3): δ 168.34 (C), 167.19 (C), 165.16 (C), 135.95 (C), 129.06 (C), 125.67 (CH), 121.25 (CH), 110.81 (CH). **F19 NMR** (500 MHz, CDCl3): δ -104.57.

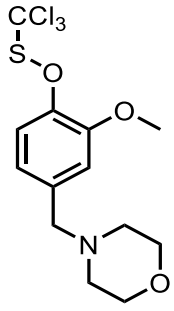


Preparation of 5-fluoro-2-((trichloromethyl)thio)isoindoline-1,3-dione (Section 3. 21b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 5-fluoroisoindoline-1,3-dione (826 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.3 g (84%) of the title compound as a beige powder. **1H NMR** (300 MHz, CDCl3): δ 11.44 (s, 1H), 7.89 (m, 1H), 7.69 (m, 1H), 7.64 (m, 1H). **C13 NMR** (500 MHz, CDCl3): δ 168.34 (C), 167.21 (C), 165.20 (C), 136.13 (C), 129.29 (C), 126.15 (CH), 121.66 (CH), 111.16 (CH), 95.87 (C). **HRMS** (**ESI**) calcd for $C_9H_3Cl_3FNO_2S^-$ ([M - H]$^-$), 312.8934 found, 312.8968.



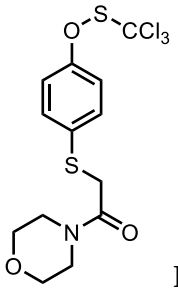
Preparation of 4-(morpholinomethyl)phenol (Section 3. 22a). – A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with morpholine (435 mg, 5 mmol, 1 equiv) with 5 mL anhydrous methanol. The mixture was stirred while concentrated HCl (0.7 mL) was added dropwise. Vanillin (760 mg, 5 mmol,

1 equiv) was added to the stirring mixture, the reaction mixture was stirred at room temperature until the vanillin was completely dissolved. The solution was heated at reflux for 2 h, allowed to cool to room temperature and 10 mL water was added. The reaction was stirred for 5 minutes, where upon it was extracted with DCM (3 X 10 mL). The organic layers were combined and concentrated under vacuum. The crude product was purified by column chromatography using 35% EtOAc in hexanes to provide 902 mg (82%) of the title compound as a white powder. **1H NMR** (300 MHz, CDCl3): δ 6.87 (dd, *J* = 8.49, 2.64 Hz, 1H), 6.82 (dd, *J* = 8.49, 1.11 Hz, 1H), 6.79 (dd, *J* = 2.64, 1.11 Hz, 1H), 3.91 (s, 3H), 3.72 (ddd, *J* = 2.79, 5.61 Hz, 4H), 3.44 (s, 1H), 2.45 (ddd, *J* = 2.58, 5.1 Hz, 4H). **C13 NMR** (500 MHz, CDCl3): δ 146.49 (C), 144.79(C), 129.67 (C), 122.14 (CH), 113.91 (CH), 111.55 (CH), 67.03 (CH2), 63.34 (CH2), 55.95 (CH3), 53.58 (CH2).



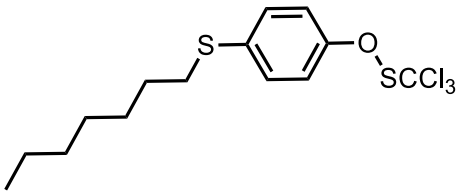
Preparation of 4-(3-methoxy-4-(((trichloromethyl)thio)oxy)benzyl)morpholine (**Section 3. 22b**). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 2-methoxy-4-(morpholinomethyl)phenol (1.1 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up

to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.5 g (81%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 6.87 (dd, *J* = 8.49, 2.64 Hz, 1H), 6.82 (dd, *J* = 8.49, 1.11 Hz, 1H), 6.79 (dd, *J* = 2.64, 1.11 Hz, 1H), 3.91 (s, 3H), 3.72 (ddd, *J* = 2.79, 5.61 Hz, 4H), 3.44 (s, 1H), 2.45 (ddd, *J* = 2.58, 5.1 Hz, 4H). **C13 NMR** (500 MHz, CDCl3): δ 150.28 (C), 148.34 (C), 136.07 (C), 120.74 (CH), 120.33 (CH), 113.08 (CH), 99.98 (C), 67.02 (CH2), 63.34 (CH2), 55.95 (CH3), 53.58 (CH2). **HRMS** (**ESI**) calcd for $C_{13}H_{16}Cl_3NO_3S^+$ ([M + H]$^+$), 371.9994, found, 371.9982.



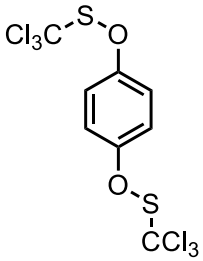
Preparation of 1-morpholino-2-(4-(((trichloromethyl)thio)oxy)phenoxy)ethan-1-one (Section 3. 23b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 2-((4-hydroxyphenyl)thio)-1-morpholinoethanone (1.3 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl

methyl ether. The solvent was evaporated in vacuum to provide 1.8 g (89%) of the title compound as a white solid. **1H NMR** (300 MHz, CDCl3): δ 6.71 (d, *J* = 8.97 Hz, 2H), 6.62 (d, *J* = 8.97 Hz, 2H), 4.62 (s, 3H), 3.52 (m, 4H), 3.41 (m, 4H). **C13 NMR** (500 MHz, CDCl3): δ 49.59 (CH2), 63.55 (CH2), 98.59 (C), 115.98 (CH), 133.32 (CH), 134.56 (C), 153.67 (C), 165.62 (C). **HRMS** (**ESI**) calcd for $C_{13}H_{14}Cl_3NO_3S_2^+$ ($[M + H]^+$), 402.9451, found, 402.9481.



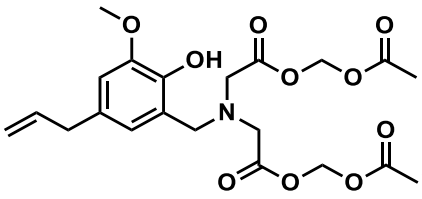
Preparation of heptyl(4-(((trichloromethyl)thio)oxy)phenyl)sulfane (Section 3. 24b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with 4-(heptylthio)phenol (1.1 g, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^oC and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (0.57 mL, 5.25 mmol, 1.05 equiv) was added dropwise. The reactant mixture was stirred at 0 ^oC for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.6 g (87%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 7.21 (d, *J* = 7.05 Hz, 2H), 6.70 (d, *J* = 7.05 Hz, 2H), 2.73 (t, *J* = 6.2 Hz, 2H), 1.49 (m, 4H), 1.31 (m, 2H), 1.19 (m, 4H), 0.80 (t, *J* = 5.95 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 150.28 (C), 148.34 (C), 136.07 (C),

120.74 (CH), 120.33 (CH), 113.08 (CH), 99.98 (C), 67.02 (CH2), 63.34 (CH2), 55.95 (CH3), 53.58 (CH2). **HRMS** (**ESI**) calcd for $C_{13}H_{16}Cl_3NO_3S^+$ ([M + H]$^+$), 371.9994, found, 371.9982.



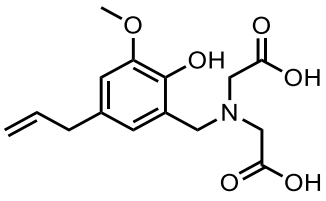
Preparation of 1,2-bis(((trichloromethyl)thio)oxy)benzene (Section 3. 25b). A 25 mL round-bottomed flask equipped with a magnetic stir bar, and an N2 inlet was charged with hydroquinone (550 mg, 5 mmol, 1 equiv) and 10 mL anhydrous diethyl ether was subsequently added. The reactant mixture was cooled to 0 ^{o}C and anhydrous trimethylamine (0.72 mL, 5.5 mmol, 1.1 equiv) was added dropwise. To the stirring, mixture trichloromethylsulfenyl chloride (1.14 mL, 10.5 mmol, 2.1 equiv) dropwise. The reactant mixture was stirred at 0 ^{o}C for 1.5 h and allowed to warm up to room temperature and let stir for an additional 12 h. To the resulting mixture was added 5 mL pentane. The mixture was then filtered through cilite and washed with *t*-butyl methyl ether. The solvent was evaporated in vacuum to provide 1.8 g (92%) of the title compound as a colorless oil. **1H NMR** (300 MHz, CDCl3): δ 7.21 (s, 4H). **C13 NMR** (500 MHz, CDCl3): δ 151.88 (C), 118.80 (CH), 96.33 (CH). **HRMS** (**ESI**) calcd for $C_{13}H_{16}Cl_3NO_3S^+$ ([M + H]$^+$), 371.9994, found, 371.9982.

2.5A Synthesis of Iminodiacetoxymethyl ester (AM) and other prodrug derivatives (seen in section 5)

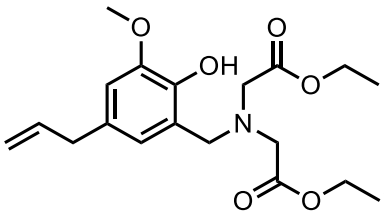


Preparation of bis(acetoxymethyl) 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (Section 4: 1b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (309 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the reaction mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide of the dicesium salt, cesium 2,2'-((2-hydroxy-5-allyl-3-methoxybenzyl)azanediyl)diacetate as a white solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 421 mg (93%) of **1b** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 6.65 (s, 1H), 6.42 (s, 1H), 5.88 (m, 1H), 5.76 (s, 4H), 5.04 (m,

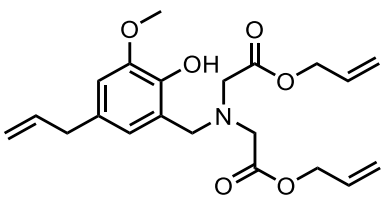
2H), 3.9 (s,1H), 3.84 (s, 2H), 3.59 (s, 3H), 2.10 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.50 (C), 159.37 (C), 147.99 (CH), 144.56 (CH), 137.70 (CH), 130.96 (CH2), 121.52 (CH), 115.54 (CH2), 112.34 (CH), 79.38 (CH2), 56.06 (CH2), 55.53 (CH3), 53.38 (CH2), 39.73 (CH2), 20.68 (CH3). **HRMS** (**ESI**) calcd for $C_{21}H_{27}NO_{10}^{+}$ ([M + H]$^{+}$), 454.1669 found, 454.1635.



Preparation of 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (Section 4: 1c). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (182 mg, 0.5 mmol, 1 equiv), 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was filtered to give a white powder which was subsequently washed with water and dried to yield 268 mg (87%) of **1d** as a white powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (500 MHz, D_6MSO): δ 6.70 (s, 1H), 6.49 (s, 1H), 5.92 (m, 1H), 5.03 (dd, *J*= 2.85 Hz, 2H), 3.80 (s, 2H), 3.73 (s, 3H), 3.41 (s, 4H), 3.24 (d, *J*= 4.02 Hz, 2H). **C13 NMR** (500 MHz, D_6MSO): δ 172.78 (C), 147.89 (C), 144.54 (CH), 138.54 (C), 130.14 (C), 121.93 (CH2), 115.86 (CH), 112.58 (CH4), 56.09 (CH3), 54.52 (CH2), 53.83 (CH2). **HRMS** (**ESI**) calcd for $C_{15}H_{19}NO_6^{-}$ ([M - H]$^{-}$), 308.1212, found, 308.1275.

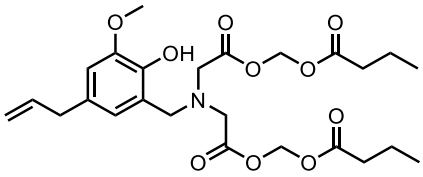


Preparation of diethyl 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (Section 4: 1d). A 10 mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture. Eugenol (164 mg, 1.0 mmol, 1 equiv), triethylamine (0.06 mL, 0.5 mmol, 0.5 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux and let reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes to give 400 mg (91%) of a **1c** as a yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 6.69 (s, 1H), 6.45 (s, 1H), 5.95 (m, 1H), 4.22 (q, *J*= 4.23 Hz, 4H), 3.98 (s, 2H), 3.89 (s, 3H), 3.56 (s, 4H), 1.29 (t, *J*= 4.29, 6H). **C13 NMR** (500 MHz, CDCl3): δ 170.59 (C), 148.05 (C), 144.92 (CH), 137.79 (C), 130.63 (C), 121.44 (CH2), 115.49 (CH), 112.34 (CH4), 60.97 (CH4), 56.97 (CH3), 53.90 (CH2), 39.73 (CH2), 14.19 (CH6). **HRMS** (**ESI**) calcd for $C_{19}H_{27}NO_6^+$ ($[M + H]^+$), 366.1838, found, 366.1891.



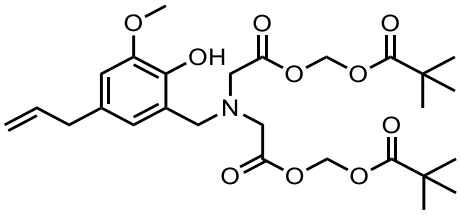
Preparation of diallyl 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (Section 4: 1e). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((5-allyl-2-

hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (309 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide of the dicesium salt, cesium 2,2'-((2-hydroxy-5-allyl-3-methoxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and allyl bromide (0.18 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 365 mg (94%) of **1e** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.69 (s, 1H), 6.45 (s, 1H), 5.95 (m, 1H), 5.29 (m, 4H), 5.09 (m, 2H), 4.66 (d, *J*= 5.85 Hz, 4H), 3.99 (s, 2H), 3.89 (s, 3H), 3.60 (s, 4H), 3.29 (d, *J*= 6.75 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 170.33 (C), 148.07 (C), 144.86 (CH), 137.77 (C), 131.68 (CH2), 130.69 (C), 121.46 (CH4), 118.89 (CH2), 115.51 (CH), 112.37 (CH4), 65.56 (CH4), 56.07 (CH3), 53.74 (CH2), 39.73 (CH2). **HRMS** (**ESI**) calcd for $C_{21}H_{27}NO_6^+$ ([M + H]$^+$), 390.1838 found, 390.1872.



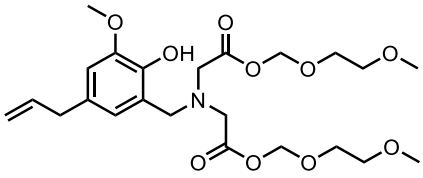
Preparation of ((2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)bis(acetyl))bis(oxy))bis(methylene) dibutyrate (Section 4: 1f). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (309 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide of the dicesium salt, cesium 2,2'-((2-hydroxy-5-allyl-3-methoxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl butyrate (380 mg, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The resulting yellow oil was purified via column chromatography in 35 % EtOAc in hexane, and concentrated in vacuo to afford 463 mg (91%) of **1f** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 6.69 (s, 1H), 6.46 (s, 1H), 5.94 (m, 1H), 5.80 (s, 4H), 5.06 (dd, *J*= 1.05 Hz, 2H), 3.98 (s, 2H), 3.89 (s, 3H), 3.63 (s, 4H), 3.30 (d, *J*= 6.65 Hz, 2H), 2.37 (t, *J*= 7.40 Hz, 4H), 1.67 (q, *J*= 7.40 Hz, 4H), 0.97 (t, *J*= 7.40 Hz, 6H). **C13 NMR** (500 MHz,

CDCl3): δ 172.16 (C), 169.45 (C), 147.98 (C), 144.57 (CH), 137.70 (C), 130.94 (CH), 121.54 (CH2), 115.58 (CH), 112.34 (CH4), 79.35 (CH4), 56.05 (CH3), 53.35 (CH2), 39.73 (CH2), 35.72 (CH4), 18.05 (CH4), 13.51 (CH6). **HRMS** (**ESI**) calcd for $C_{25}H_{35}NO_{10}^{+}$ ([M + H]$^{+}$), 510.2261, found, 501.2295.



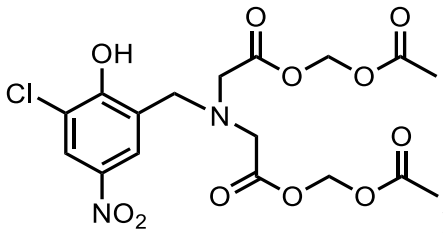
Preparation of ((2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)bis(acetyl))bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (Section 4: 1g). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (309 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the reaction mixture was stirred at room tempurature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide of the dicesium salt, cesium 2,2'-((2-hydroxy-5-allyl-3-methoxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and chloromethyl pivalate (0.3 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic

layer was dried and concentrated in vacuo to afford 484 mg (90%) of **1g** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.69 (s, 1H), 6.46 (s, 1H), 5.93 (m, 1H), 5.80 (s, 4H), 5.06 (m, 2H), 3.97 (s, 1H), 3.88 (s, 2H), 3.62 (s, 3H), 2.23 (s, 18H). **C13 NMR** (500 MHz, CDCl3): δ 177.00 (C), 169.46 (C), 148.01 (C), 144.61 (C), 137.70 (CH), 130.90 (C), 127.93 (C), 121.52 (CH), 115.58 (CH2), 112.36 (CH), 79.75 (CH4), 56.04 (CH4), 55.36 (CH3), 53.32 (CH2), 39.72 (CH2), 38.76 (C), 26.83 (CH18). **HRMS** (**ESI**) calcd for $C_{27}H_{39}NO_{10}^{+}$ ([M + H]$^{+}$), 538.2574 found, 538.2608.



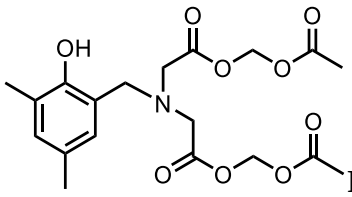
Preparation of bis((2-methoxyethoxy)methyl) 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetate (Section 4: 1h). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((5-allyl-2-hydroxy-3-methoxybenzyl)azanediyl)diacetic acid (309 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the reaction mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide of the dicesium salt, cesium 2,2'-((2-hydroxy-5-allyl-3-methoxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and 1-(iodomethoxy)-2-methoxyethane (454 mg, 2.1 mmol, 2.1 equiv) was added dropwise. The

reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The resulting yellow oil was purified via column chromatography in 35 % EtOAc in hexane, and concentrated in vacuo to afford 441 mg (91%) of **1e** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.64 (s, 1H), 6.42 (s, 1H), 5.89 (m, 1H), 5.35 (s, 4H), 5.06 (dd, *J*= 1.65 Hz, 2H), 3.96 (s, 2H), 3.84 (s, 3H), 3.76 (t, *J*= 4.67 Hz, 4H), 3.58 (s, 4H), 3.53 (t, *J*= 4.67 Hz, 4H), 3.35 (s, 6H), 5.06 (d, *J*= 6.63 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 171.39 (C), 146.55 (C), 141.64 (CH), 136.58 (C), 129.76 (C), 121.67 (CH2), 115.61 (CH), 95.9 (CH4), 72.76 (CH4), 68.07 (CH4), 58.61 (CH6), 56.91 (CH4), 54.52 (CH3), 52.69 (CH2), 39.90 (CH2). **HRMS** (**ESI**) calcd for $C_{23}H_{35}NO_{10}^{+}$([M + H]$^{+}$), 486.2261, found, 486.2295.



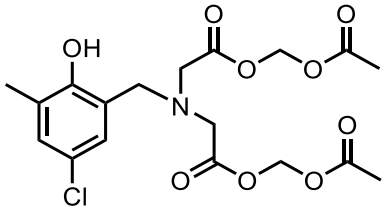
Preparation of bis(acetoxymethyl) 2,2'-((3-chloro-2-hydroxy-5-nitrobenzyl)azanediyl)diacetate (Section 4: 2b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((3-chloro-2-hydroxy-5-nitrobenzyl)azanediyl)diacetic acid (318 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried

under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3-chloro-2-hydroxy-5-nitrobenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 402 mg (87%) of **2b** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.97 (s, 1H), 7.72 (s, 1H), 5.50 (s, 4H), 3.81 (s, 2H), 3.55 (s, 4H), 2.05 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 170.61 (C), 159.40 (C), 139.75 (C), 126.10 (C), 123.79 (C), 122.57 (C), 122.21 (CH), 61.60 (CH), 55.44 (CH4), 53.92 (CH4), 56.06 (CH2), 14.15 (CH3). **HRMS** (**ESI**) calcd for $C_{17}H_{19}ClN_2O_{11}^{+}$ ([M + H]$^{+}$), 464.0677 found, 464.0648.



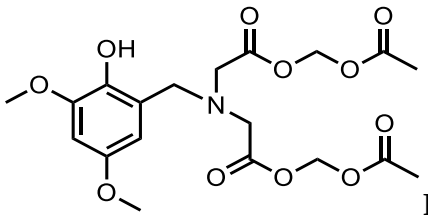
Preparation of bis(acetoxymethyl) 2,2'-((2-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate (Section 4: 3b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((2-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetic acid (267 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the reaction mixture stirred at room

temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((2-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate as a white solid. The same 10 mL round-bottomed flask was equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 386 mg (94%) of **3b** as a viscous, colorless oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 6.88 (s, 1H), 6.58 (s, 1H), 5.76 (s, 4H), 3.90 (s, 2H), 3.57 (s, 4H), 2.18 (s, 6H), 2.15 (s, 3H), 2.10 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 169.62 (C), 169.48 (C), 152.93 (C), 13.58 (CH), 127.98 (C), 127.67 (CH), 125.28 (C), 125.28 (C), 79.39 (CH4), 56.17 (CH4), 53.39 (CH2), 34.80 (CH6), 20.66 (CH3), 15.73 (CH3).



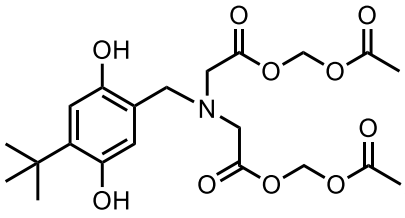
Preparation of bis(acetoxymethyl) 2,2'-((5-chloro-2-hydroxy-3-methylbenzyl)azanediyl)diacetate (Section 4: 4b). A 10-mL round-bottomed flask was equipped with a magnetic stirring, and an N_2 inlet bar was charged with 2,2'-((5-chloro-2-hydroxy-3-methylbenzyl)azanediyl)diacetic acid (287 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 5.68M

CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the mixture stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((5-chloro-2-hydroxy-3-methylbenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask was equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 366 mg (85%) of **4b** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.05 (s, 1H), 6.78 (s, 1H), 5.77 (s, 4H), 3.91 (s, 2H), 3.57 (s, 4H), 2.20 (s, 3H), 2.11 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.81 (C), 169.30 (C), 152.49 (C), 130.89 (CH), 127.13 (C), 126.86 (CH), 125.28 (C), 122.14 (C), 79.43 (CH4), 57.11 (CH4), 51.61 (CH2), 20.66 (CH6), 15.22 (CH3). **HRMS** (**ESI**) calcd for $C_{18}H_{22}ClNO_9^-$ ([M – H]$^-$), 431.0983, found, 431.0954.



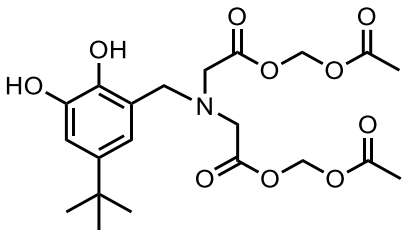
Preparation of bis(acetoxymethyl) 2,2'-((2-hydroxy-3,5-dimethoxybenzyl)azanediyl)diacetate (**Section 4**: **5b**). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-

azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 2,4-dimethoxyphenol (154 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux and allowed to heat for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((2-hydroxy-3,5-dimethoxybenzyl)azanediyl)diacetate as a light beige solid**.** The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 425 mg (96%) of **5b** as a viscous, colorless oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.50 (s, 1H), 6.21 (s, 1H), 5.79 (s, 4H), 3.99 (s, 2H), 3.88 (s, 4H), 3.76 (s, 3H), 3.64 (s, 3H), 2.14 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.50 (C), 169.45 (C), 152.78 (C), 148.70 (CH), 140.36 (C), 121.15 (CH), 105.16 (C), 100.09 (C), 79.37 (CH4), 65.85 (CH4), 56.08 (CH2), 53.37 (CH6), 20.66(CH3), 15.27 (CH3). **HRMS** (**ESI**) calcd for $C_{19}H_{25}NO_{11}^{+}$ ($[M + H]^{+}$), 444.1428, found, 444.1485.



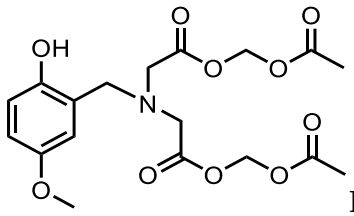
Preparation of bis(acetoxymethyl) 2,2'-((4-(tert-butyl)-2,5-dihydroxybenzyl)azanediyl)diacetate (Section 4: 6b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 4-hydroxy-3-tertbutylphenol (166 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((4-(tert-butyl)-2,5-dihydroxybenzyl)azanediyl)diacetate, as a light orange solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 1.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was filtered, and the filtrate diluted with *t*-butyl methyl ether (2 mL) and extracted with water (3 X 2 mL) and then with brine (3 X 2 mL), and the organic layer was then purified through column chromatography in 35% EtOAc in hexane. The organic layer was dried and concentrated in vacuo to afford 423 mg (93%) of **6b** as a light orange oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.75 (s, 1H), 6.27 (s, 1H), 5.71 (s, 4H), 3.80 (s, 2H), 3.53 (s, 4H), 3.06 (s, 6H), 1.32 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 171.17 (C), 144.68 (C), 141.19

(C), 119.84 (C), 117.34 (C), 79.46 (C), 60.41 (CH), 56.43 (CH), 53.36 (CH4), 49.45 (CH4), 34.12 (CH2), 31.46 (C), 26.99 (CH3), 20.75 (CH3), 20.67 (CH3), 14.20 (CH6). **HRMS** (**ESI**) calcd for $C_{21}H_{29}NO_{10}^{+}$ ([M + H]$^{+}$), 456.1791, found, 456.1852.



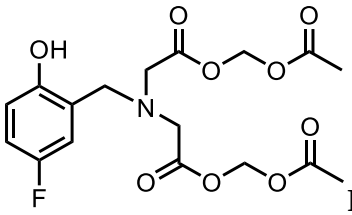
Preparation of bis(acetoxymethyl) 2,2'-((5-(tert-butyl)-2,3-dihydroxybenzyl)azanediyl)diacetate (Section 4: 7b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 2-hydroxy-4-tertbutylphenol (166 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((5-(tert-butyl)-2,3-dihydroxybenzyl)azanediyl)diacetate as a yellow solid. A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N2 inlet and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was filtered, and the filtrate diluted with *t*-butyl methyl ether (2 mL) and extracted with water (3 X 2 mL) and then with brine (3 X 2 mL), and the organic layer was then purified through column chromatography in 35% EtOAc in hexane. The organic layer was dried and concentrated in vacuo to afford 419 mg (92%) of **7b** as a yellow oil. ^{1}H and

^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.75 (s, 1H), 6.27 (s, 1H), 5.71 (s, 4H), 4.06 (q, *J* = 5.9, 2H), 3.80 (s, 2H), 3.53 (s, 3H), 2.06 (s, 2H), 1.30 (s, 6H), 1.17 (t, *J* = 5.9, 3H). **C13 NMR** (500 MHz, CDCl3): δ 171.20 (C), 169.61 (C), 150.37 (C), 146.88 (C), 137.78 (C), 118.29 (C), 115.52 (CH), 79.47 (CH), 60.42 (CH4), 55.19 (CH4), 53.40 (CH2), 34.47 (C), 29.55 (CH3), 21.07 (CH3), 20.71 (CH3), 14.20 (CH6). **HRMS** (**ESI**) calcd for $C_{21}H_{29}NO_{10}^{+}$ ([M + H]$^{+}$), 456.1791, found, 456.1823.



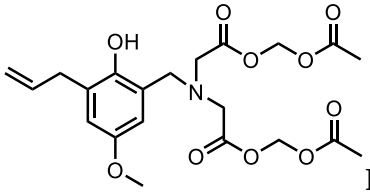
Preparation of bis(acetoxymethyl) 2,2'-((2-hydroxy-5-methoxybenzyl)azanediyl)diacetate (Section 4: 8b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 4-methoxyphenol (124 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((2-hydroxy-5-methoxybenzyl)azanediyl)diacetate as a white solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was

dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 379 mg (92%) of **8b** as a yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.77 (dd, *J* = 7.68, 2.73, 2H), 6.53 (s, 1H), 5.76 (s, 4H), 3.94 (s, 2H), 3.72 (s, 4H), 3.59 (s, 3H), 2.11 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.51 (C), 152.69 (C), 151.07 (C), 121.42 (C), 117.19 (CH), 115.43 (CH), 114.52 (CH), 79.40 (CH4), 55.73 (CH4), 53.38 (CH3), 26.99 (CH2), 20.66 (CH6). **HRMS** (**ESI**) calcd for $C_{18}H_{23}NO_{10}^{+}$ ([M + H]$^{+}$), 414.1322, found, 414.1380.



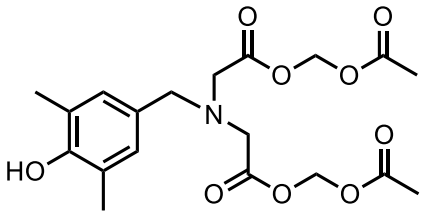
Preparation of bis(acetoxymethyl) 2,2'-((5-fluoro-2-hydroxybenzyl)azanediyl)diacetate (Section 4. 9b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 4-fluorophenol (111 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((5-fluoro-2-hydroxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2

mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 °C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 356 mg (89%) of **9b** as a viscous, yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.94 (ddd, *J* = 5.65, 3.05, 1H), 6.85 (dd, *J* = 4.80, 4.1, 1H), 6.72 (dd, *J* = 5.65, 3.05, 1H), 5.81 (s, 4H), 3.98 (s, 2H), 3.62 (s, 4H), 2.15 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.48 (C), 157.03 (C), 155.03 (C), 153.22 (C), 121.63 (CH), 117.56 (CH), 115.99 (CH), 79.43 (CH4), 55.66 (CH4), 53.44 (CH2), 20.64 (CH6). **F19 NMR** (500 MHz, CDCl3): δ -125.40. **HRMS** (**ESI**) calcd for $C_{17}H_{20}FNO_9^-$ ([M - H]$^-$), 400.1122, found, 399.1962.



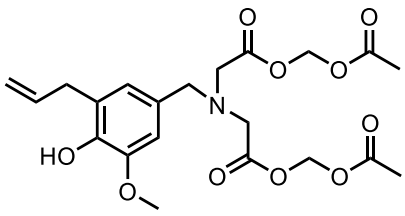
Preparation of bis(acetoxymethyl) 2,2'-((3-allyl-2-hydroxy-5-methoxybenzyl)azanediyl)diacetate (Section 4. 10b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 2-allyl-4-methoxyphenol (164 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3-allyl-2-hydroxy-5-

methoxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 21.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 421 mg (93%) of **10b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.70 (s, 1H), 6.44 (s, 1H), 6.03 (m, 1H), 5.80 (s, 4H), 4.09 (m, 2H), 3.98 (s, 2H), 3.75 (s, 4H), 3.63 (s, 3H), 2.15 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.52 (C), 152.37 (C), 148.67 (CH), 136.83 (C), 128.48 (C), 121.09 (CH), 155.40 (CH2), 113.26 (CH), 79.38 (CH4), 55.68 (CH4), 53.32 (CH3), 34.12 (CH2), 26.98 (CH2), 20.65 (CH6). **HRMS** (**ESI**) calcd for $C_{21}H_{27}NO_{10}^{+}$ ([M + H]$^{+}$), 454.1696, found, 454.1635.



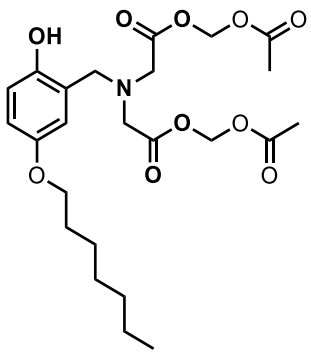
Preparation of bis(acetoxymethyl) 2,2'-((4-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate (Section 4. 11b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 2,6-dimethylphenol (122 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The

resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((4-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate, as a light purple solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 32 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 382 mg (93%) of **11b** as a viscous, yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.88 (s, 1H), 6.72 (s, 1H), 4.25 (s, 4H), 3.63 (s, 2H), 3.29 (s, 4H) 3.28 (s, 6H), 2.17 (s, 3H), 2.13 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 151.78 (C), 150.35 (C), 133.42 (C), 129.73 (C), 128.55 (CH2), 122.87 (C), 74.85 (CH4), 57.85 (CH2), 40.29 (CH4), 30.50, (CH6), 15.91 (CH6). **HRMS** (**ESI**) calcd for $C_{19}H_{25}NO_9^+$ ([M + H]$^+$), 411.1529, found, 411.1563.



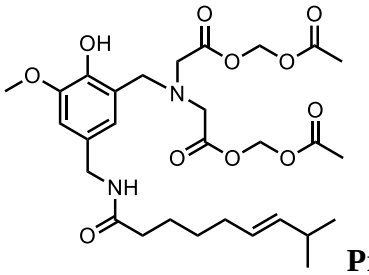
Preparation of bis(acetoxymethyl) 2,2'-((3-allyl-4-hydroxy-5-methoxybenzyl)azanediyl)diacetate (Section 4. 12b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2’-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH

was subsequently added. To the stirring reactant mixture 6-allyl-2-methoxyphenol (164 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3-allyl-4-hydroxy-5-methoxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 21.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 407 mg (90%) of **12b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.69 (s, 1H), 6.46 (s, 1H), 6.95 (m, 1H), 5.77 (s, 4H), 5.07 (m, 2H), 3.98 (s, 2H), 3.88 (s, 4H), 3.62 (s, 3H), 1.26 (s, 3H), 1.23 (s, 3H). **C13 NMR** (500 MHz, CDCl3): δ 172.16 (C), 169.45 (C), 147.99 (CH), 144.58 (C), 137.70 (C), 130.95 (CH), 121.54 (CH2), 115.58 (CH), 112.34 (CH4), 56.05 (CH4), 53.35 (CH3), 39.72 (CH2), 18.05 (CH2), 13.51 (CH6). **HRMS** (**ESI**) calcd for $C_{21}H_{27}NO_{10}^{+}$ ($[M + H]^{+}$), 454.1669, found, 454.1635.



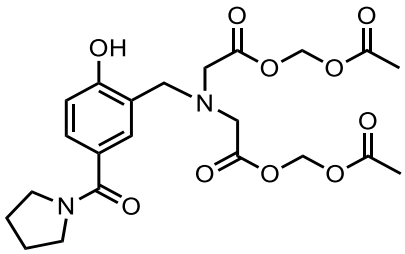
Preparation of bis(acetoxymethyl) 2,2'-((2-hydroxy-5-heptyloxybenzyl)azanediyl)diacetate (Section 4. 13b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 4-heptyloxyphenol (208 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((2-hydroxy-4-heptyloxybenzyl)azanediyl)diacetate as a white solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 21.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 462 mg (93%) of **13b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.84 (d, *J* = 8.7 Hz, 1H), 6.80 (d, *J* = 8.7 Hz, 1H), 6.57 (s, 1H), 5.80 (s,

4H), 3.97 (s, 2H), 3.88 (m, 2H), 3.62 (s, 4H), 2.15 (s, 6H), 1.75 (q, *J* = 6.9 Hz, 6H), 1.45 (m, 2H), 1.32 (m, 8H), 0.90 (t, *J* = 7.0 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 169.52 (C), 152.23 (C), 150.96 (C), 121.38 (C), 117.12 (CH), 116.18 (C), 115.15 (CH), 79.38 (CH), 68.67 (CH4), 56.23 (CH2), 53.37 (CH2), 31.79 (CH2), 29.26 (CH2), 26.04 (CH2), 22.61 (CH2), 20.66 (CH6), 14.08 (CH3). **HRMS** (**ESI**) calcd for $C_{21}H_{27}NO_{10}^{+}$ ([M + H]$^{+}$), 498.2261, found, 498.2295.



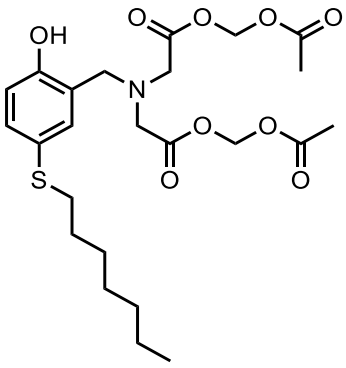
Preparation of (E)-bis(acetoxymethyl) 2,2'-((2-hydroxy-3-methoxy-5-((8-methylnon-6-enamido)methyl)benzyl)azanediyl)diacetate (Section 4. 14b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture (E)-N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-enamide (305 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium as a white solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 21.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in

water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 517 mg (87%) of **14b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.80 (s, 1H), 6.58 (s, 1H), 5.80 (s, 4H), 5.37 (m, 2H), 4.35 (s, 2H), 3.98 (s, 4H), 3.63 (s, 3H), 2.23 (m, 3H), 2.14 (s, 6H), 2.01 (m, 2H), 1.67 (m, 3H), 1.28 (m, 3H), 0.90 (d, *J* = 6.7 Hz, 3H), 0.90 (d, *J* = 6.7 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 169.48 (C), 148.23 (C), 145.71 (C), 138.09 (C), 129.49 (CH), 126.50 (C), 121.18 (CH), 111.68 (C), 79.47 (CH), 56.11 (CH), 55.27 (CH4), 53.48 (CH4), 43.37 (CH3), 38.96 (CH2), 36.87 (CH2), 32.24 (CH2), 30.96 (CH2), 29.33 (CH2), 27.93 (CH), 25.79 (CH2), 22.63 (CH6), 20.68 (CH6). **HRMS** (**ESI**) calcd for $C_{29}H_{42}N_2O_{11}^{+}$ ([M + H]$^{+}$), 595.2789, found, 594.2282.



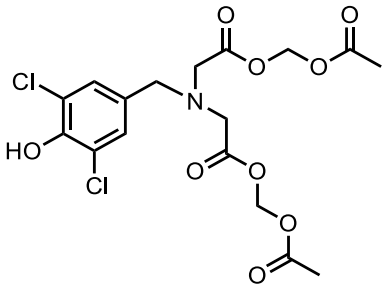
Preparation of bis(acetoxymethyl) 2,2'-((2-hydroxy-5-(pyrrolidine-1-carbonyl)benzyl)azanediyl)diacetate (Section 4. 15b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture (4-hydroxyphenyl)(pyrrolidin-1-yl)methanone (191 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly

dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((2-hydroxy-5-(pyrrolidine-1-carbonyl)benzyl)azanediyl)diacetate, as a light beige solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 21.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 437 mg (91%) of **15b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.41 (d, *J* = 8.7 Hz, 1H), 6.89 (d, *J* = 8.4 1H), 6.82 (d, *J* = 8.7 1H), 5.79 (s, 4H), 4.01 (s, 2H), 3.63 (m, 6H), 3.50 (m, 4H), 2.14 (s, 6H), 1.90 (m, 2H). **C13 NMR** (500 MHz, CDCl3): δ 170.19 (C), 169.50 (C), 158.90 (C), 129.21 (C), 127.66 (C), 120.55 (C), 116.22 (CH), 115.17 (CH), 79.46 (CH), 55.97 (CH4), 53.47 (CH4), 49.95 (CH2), 46.48 (CH2), 26.46 (CH4), 24.45 (CH4), 20.68 (CH6). **HRMS** (**ESI**) calcd for $C_{28}H_{22}N_2O_{10}^{+}$ ([M + H]$^{+}$), 481.1744, found, 418.1777.



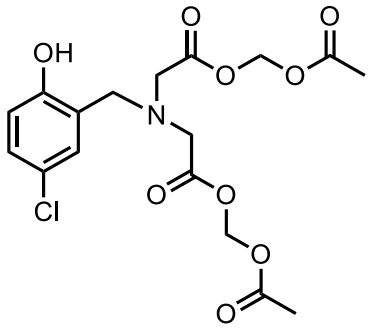
Preparation of bis(acetoxymethyl) 2,2'-((5-(heptylthio)-2-hydroxybenzyl)azanediyl)diacetate (Section 4. 16b). A 10-mL round-bottomed flask

equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 4-(heptylthio)phenol (224 mg, 1.0 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((5-(heptylthio)-2-hydroxybenzyl)azanediyl)diacetate, as a light beige solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^oC in an ice bath and bromomethyl acetate (0.2 mL, 21.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^oC and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 457 mg (89%) of **16b** as a viscous, light yellow oil. ^1H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.32 (d, *J* = 8.4 Hz, 1H), 6.87 (s, 1H), 6.79 (d, *J* = 6.7 Hz, 1H), 5.81 (s, 4H), 3.98 (s, 2H), 6.62 (s, 4H), 2.83 (m, 2H), 2.15 (s, 6H), 1.59 (m, 3H), 1.40 (s, 2H), 1.28 (m, 8H), 0.90 (t, *J* = 3.4 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 169.50 (C), 156.49 (C), 133.21 (C), 133.09 (C), 130.36 (CH), 121.99(C), 121.39 (CH), 117.43(CH), 115.94 (CH4), 79.43 (CH2), 55.91 (CH2), 53.42 (CH2), 35.90 (CH2), 31.72 (CH2), 22.59 (CH2), 20.68 (CH6), 14.06 (CH3). **HRMS** (**ESI**) calcd for $C_{24}H_{35}NO_9S^+$ ([M + H]$^+$), 513.2033, found, 513.2066.



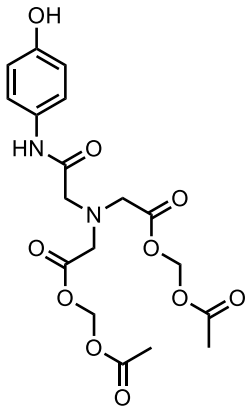
Preparation of bis(acetoxymethyl) 2,2'-((3,5-dichloro-4-hydroxybenzyl)azanediyl)diacetate (Section 4. 17b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 2,6-dichloro phenol (163 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3,5-dichloro-4-hydroxybenzyl)azanediyl)diacetate, as a light yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 1 h at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 275 mg (61%) of **17b** as a viscous, yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.31 (s, 1H), 7.13 (s, 1H), 5.74 (s, 4H), 4.39 (s, 2H), 3.41 (s, 4H)

2.14 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 170.05 (C), 137.70 (C), 129.27 (C), 128.44 (C), 127.83 (CH), 87.95 (CH), 58.47 (CH4), 53.80 (CH2), 39.66 (CH4), 20.94 (CH3), 14.20 (CH3). **HRMS** (**ESI**) calcd for $C_{17}H_{19}Cl_2NO_9^+$ ([M + H]$^+$), 153.0437, found, 153.0407.



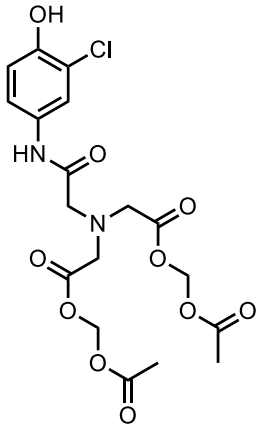
Preparation of bis(acetoxymethyl) 2,2'-((5-chloro-2-hydroxybenzyl)azanediyl)diacetate (Section 4. 18b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 4-chloro phenol (128 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((5-chloro-2-hydroxybenzyl)azanediyl)diacetate, as a light yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^oC in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^oC and let stir for an additional 32 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2

mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 304 mg (73%) of **18b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 6.89 (s, 1H), 6.82 (d, *J* = 8.0 Hz, 1H), 6.67 (d, *J* = 8.0 Hz, 1H), 5.77 (s, 4H), 3.93 (s, 3H), 3.58 (s, 4H), 2.11 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 169.46 (C), 157.53 (C), 155.38 (C), 152.75 (C), 117.54 (C), 115.92 (CH), 87.42 (CH), 79.43 (CH4), 55.74 (CH4), 53.45 (CH2), 20.64 (CH6). **HRMS** (**ESI**) calcd for $C_{17}H_{20}ClNO_9^+$ ([M + H]$^+$), 417.0827, found, 417.0797.



Preparation of bis(acetoxymethyl) 2,2'-((2-((4-hydroxyphenyl)amino)-2-oxoethyl)azanediyl)diacetate (Section 4. 19b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2-(2,6-dioxomorpholino)acetic acid (173 mg, 1 mmol, 1 equiv) and 2 mL anhydrous THF was subsequently added. To the stirring reactant mixture 4-aminophenol (109 mg, 1 mmol, 1 equiv) was added and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2 mL anhydrous MeOH. CsOH (0.35 mL, 2 mmol, 2 equiv) was added and the raction

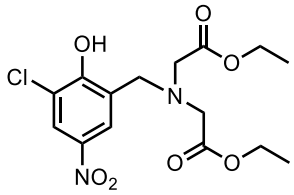
mixture was allowed to stir for 12 h at room temperature. The reaction was concentrated in vacuo to provide the dicesium salt cesium 2,2'-((2-((3-chloro-4-hydroxyphenyl)amino)-2-oxoethyl)azanediyl)diacetate. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to and column chromatography was performed using a solution of 25% EtOAc, 25% hexanes, 49% DCM and 1% MeOH to afford mg (__%) of **20b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.49 (d, *J* = 4.2 Hz, 2H), 7.27 (d, *J* = 4.2 Hz, 2H), 5.81 (s, 4H), 3.68 (s, 4H), 3.568 (s, 2H), 2.17 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 170.18 (C), 169.69 (C), 155.38 (C), 168.01 (C), 152.21 (C), 131.21 (CH2), 121.19 (CH2), 115.56 (CH4), 79.48 (CH2), 60.10 (CH4), 55.90 (CH6), 20.66 (CH6). **HRMS** (**ESI**) calcd for $C_{18}H_{22}N_2O_{10}^{+}$ ([M + H]$^{+}$), 426.3747, found, 426.1801.



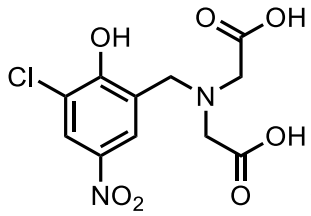
Preparation of bis(acetoxymethyl) 2,2'-((2-((3-chloro-4-hydroxyphenyl)amino)-2-oxoethyl)azanediyl)diacetate (Section 4. 20b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2-(2,6-dioxomorpholino)acetic acid (173 mg, 1 mmol, 1 equiv) and 2 mL anhydrous THF was subsequently added. To the stirring reactant mixture 4-amino-2-chlorophenol (143 mg, 1 mmol, 1 equiv) was added and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2 mL anhydrous MeOH. CsOH (0.35 mL, 2 mmol, 2 equiv) was added and the raction mixture was allowed to stir for 12 h at room temperature. The reaction was concentrated in vacuo to provide the dicesium salt cesium 2,2'-((2-((3-chloro-4-hydroxyphenyl)amino)-2-oxoethyl)azanediyl)diacetate. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers

were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to and column chromatography was performed using a solution of 25% EtOAc, 25% hexanes, 49% DCM and 1% MeOH to afford mg (__%) of **19b** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.55 (s, 1H), 7.28 (d, *J* = 7.4 Hz, 1H), 6.82 (d, *J* = 7.4 Hz, 1H), 5.82 (s, 4H), 3.74 (s, 4H), 3.53 (s, 2H), 2.14 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 170.2 (C), 169.58 (C), 168.43 (C), 153.03 (C), 146.28 (C), 130.45 (C), 129.31 (CH2), 121.44 (CH2), 115.67 (CH4), 79.51 (CH2), 59.90 (CH4), 55.91 (CH6), 20.66 (CH6). **HRMS** (**ESI**) calcd for $C_{18}H_{21}ClN_2O_{10}^{+}$ ($[M + H]^{+}$), 460.0885, found, 460.1330.

2.6A Compounds not found in the above papers

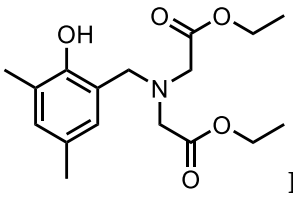


Preparation of diethyl 2,2'-((3-chloro-2-hydroxy-5-nitrobenzyl)azanediyl)diacetate (Appendix 2.6A: 1a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture. 2-chloro-4-nitrophenol (172.5 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes and concentrated to give 303 mg (81%) of **1a** as a yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 8.24 (s, 1H), 7.85 (s, 1H), 4.22 (q, *J*= 7.14 Hz, 4H), 4.05 (s, 2H), 3.52 (s, 4H), 1.27 (t, *J*= 7.14 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 170.61 (C), 159.40 (C), 139.76 (C), 126.10 (C), 123.79 (CH), 122.21 (C), 61.59 (CH4), 55.43 (CH4), 53.92 (CH2), 14.15 (CH6). **HRMS** (**ESI**) calcd for $C_{15}H_{19}ClN_2O_7^+$ ([M + H]$^+$), 376.0881 found, 376.0851.



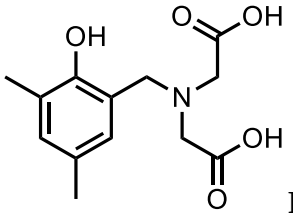
Preparation of 2,2'-((3-chloro-2-hydroxy-5-nitrobenzyl)azanediyl)diacetic acid (Appendix 2.6A: 1b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl 2,2'-

((3-chloro-2-hydroxy-5-nitrobenzyl)azanediyl)diacetate (374 mg, 1 mmol, 1 equiv) and 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was filtered to give a white powder which was washed with water and dried to yield 273 mg (86%) of **1b** as a white powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (500 MHz, D_6MSO): δ 8.23 (s, 1H), 8.02 (s, 1H), 4.04 (s, 2H), 3.48 (s, 4H). **C13 NMR** (500 MHz, D_6MSO): δ 172.78 (C), 159.74 (C), 139.43 (C), 125.32 (C), 124.94 (C), 124.68 (CH), 120.76 (CH), 55.08 (CH4), 54.05 (CH2). **HRMS** (**ESI**) calcd for $C_{17}H_{19}ClN_2O_{11}^-$ ([M - H]$^-$), 318.0225 found, 318.0338.

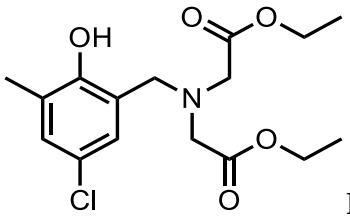


Preparation of diethyl 2,2'-((2-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate (Appendix 2.6A: 2a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture. 2,4-diemthlyphenol (121 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were added with stirring. The reaction was heated at reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes and concentrated to give 284 mg (88%) of **2a** as a yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the

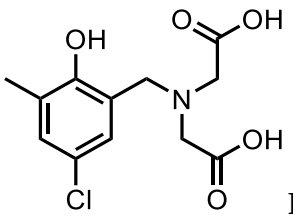
following; **1H NMR** (300 MHz, $CDCl_3$): δ 6.92 (s, 1H), 6.62 (s, 1H), 5.81 (s, 4H), 3.94 (s, 2H), 3.60 (q, *J*=7.15, 4H), 2.23 (t, *J*= 8.20 Hz, 2H), 2.14 (s, 1H). **C13 NMR** (500 MHz, CDCl3): δ 169.50 (C), 152.95 (C), 131.58 (CH), 127.66 (CH), 125.29 (C), 119.83 (CH4), 79.39 (CH4), 56.18 (CH2), 53.38 (CH3), 20.65 (CH3) 15.72 (CH3). **HRMS** (**ESI**) calcd for $C_{17}H_{25}NO_5^+$ ($[M + H]^+$), 324.1733, found, 324.1766.



Preparation of 2,2'-((2-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetic acid (Appendix 2.6A: 2b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl 2,2'-((2-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate (323 mg, 0.5 mmol, 1 equiv), 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^oC and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was filtered to give a white powder which was subsequently washed with water and dried to yield 229 mg (86%) of **2b** as a white powder. ^1H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (500 MHz, D_6MSO): δ 6.84 (s, 1H), 6.61 (s, 1H), 3.78 (s, 2H), 3.41 (s, 4H) 2.14 (s, 3H), 2.10 (s, 3H). **C13 NMR** (500 MHz, D_6MSO): δ 172.90 (C), 153.08 (C), 131.14 (CH), 128.09 (C), 127.09 (CH), 124.15 (C), 212.46 (C), 55.51 (CH4), 53.07 (CH2), 20.48 (CH3), 16.18 (CH3). **HRMS** (**ESI**) calcd for $C_{13}H_{17}NO_5^-$ ($[M - H]^-$), 266.1107, found, 266.1140.

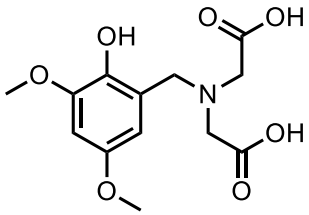


Preparation of diethyl 2,2'-((5-chloro-2-hydroxy-3-methylbenzyl)azanediyl)diacetate (Appendix 2.6A: 3a). A 10 mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture. 4-chloro-2-methlyphenol (142 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes and concentrated to give 292 mg (85%) of **3a** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 7.02 (s, 1H), 6.78 (s, 1H), 4.20 (q, *J*= 6.90 Hz, 4H), 3.89 (s, 2H), 3.50 (s, 2H), 2.20 (s, 1H), 1.53 (s, 3H), 1.26 (t, *J*= 7.90 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 170.08 (C), 154.20 (C), 130.22 (C), 127.43 (CH), 126.42 (C), 123.02 (CH), 121.98 (C), 61.16 (CH4), 55.68 (CH4), 53.86 (CH2) 15.72 (CH6), 14.17 (CH3). **HRMS** (**ESI**) calcd for $C_{16}H_{22}ClNO_5^-$ ([M – H]$^-$), 342.1187, found, 342.1425.



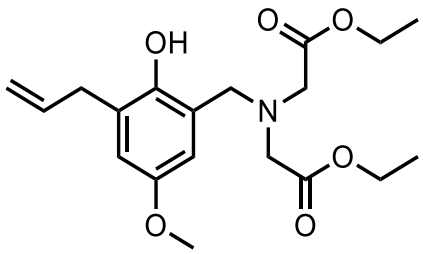
Preparation of 2,2'-((5-chloro-2-hydroxy-3-methylbenzyl)azanediyl)diacetic acid (Appendix 2.6A: 3b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with **3a** (343 mg, 0.5 mmol, 1 equiv), 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide

monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was filtered to give a white powder which was washed with water and dried to yield 241 mg (84%) of **3b** as a white powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, D_6MSO): δ 7.10 (s, 1H), 6.90 (s, 1H), 3.82 (s, 2H), 3.42 (s, 4H) 2.13 (s, 3H). **C13 NMR** (500 MHz, D_6MSO): δ 172.85 (C), 154.36 (C), 129.78 (CH), 126.99 (C), 126.94 (CH), 123.78 (C), 122.06 (C), 54.99 (CH4), 53.81 (CH2), 16.05 (CH3). **HRMS** (**ESI**) calcd for $C_{12}H_{14}ClNO_5^-$ ([M – H]$^-$), 286.0561, found, 286.0531.

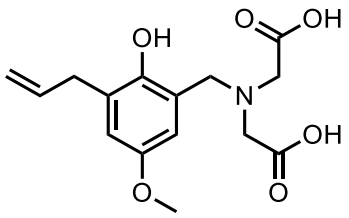


Preparation of 2,2'-((2-hydroxy-3,5-dimethoxybenzyl)azanediyl)diacetic acid (Appendix 2.6A: 4a). A 10 mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl 2,2'-((2-hydroxy-3,5-dimethoxybenzyl)azanediyl)diacetate (355 mg, 0.5 mmol, 1 equiv), 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was filtered to give a white powder which was subsequently washed with water and dried to yield 263 mg (88%) of a white powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, D_6MSO): δ 6.05 (s, 1H), 5.99 (s, 1H), 3.70 (s, 2H), 3.40 (s, 4H) 3.33 (s, 6H). **C13 NMR**

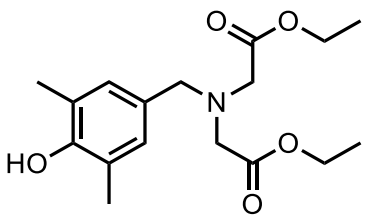
(500 MHz, D_6MSO): δ 172.78 (C), 169.96 (C), 160.81 (CH), 159.09 (C), 94.28 (CH), 90.28 (C), 55.96 (C), 55.41 (CH4), 53.91 (CH2), 46.76 (CH6). **HRMS** (**ESI**) calcd for $C_{13}H_{17}NO7^-$ ($[M - H]^-$), 300.1005, found, 300.1039.



Preparation of diethyl 2,2'-((3-allyl-2-hydroxy-5-methoxybenzyl)azanediyl)diacetate (Appendix 2.6A: 5a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture. 2-allyl-4-methoxyphenol (164 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes and concentrated to give 324 mg (89%) of a viscous yellow oil. ^1H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 7.19 (s, 1H), 6.90 (s, 1H), 4.10 (q, *J*= 5.95 Hz, 4H), 3.69 (s, 2H), 3.45 (s, 2H), 2.16 (s, 3H), 1.20 (t, *J*= 5.95 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 171.30 (C), 151.49 (C), 129.50 (C), 122.81 (CH), 60.40 (C), 57.22 (CH), 54.11 (CH4), 15.85 (CH3), 14.27 (CH6). **HRMS** (**ESI**) calcd for $C_{19}H_{27}NO_6^+$ ($[M + H]^+$), 366.1838, found, 366.1872.

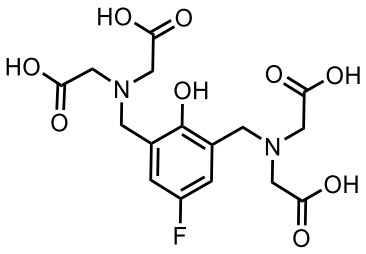


Preparation of 2,2'-((3-allyl-2-hydroxy-5-methoxybenzyl)azanediyl)diacetic acid (Appendix 2.6A: 5b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-azanediyldiacetic acid (133 mg, 1 mmol, 2 equiv) and 4 mL of acetonitrile. 2-allyl-4-methoxy phenol (164 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for 12 h. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes to give 219 mg (71%) of **5b** as a viscous, light beige powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (500 MHz, D_6MSO): δ 6.57 (s, 1H), 6.42 (s, 2H), 5.95 (m, 1H), 5.01 (m, 2H), 3.82 (s, 2H), 3.64 (s, 3H), 3.40 (s, 4H), 3.29 (s, 2H). **C13 NMR** (500 MHz, D_6MSO): δ 172.77 (C), 151.95 (C), 148.72 (C), 137.32 (CH), 127.43 (C), 122.59 (C), 115.74 (CH), 114.79 (CH2), 113.33 (CH), 55.60 (CH4), 53.72 (CH3), 34.24 (CH2), 31.08 (CH2). **HRMS** (**ESI**) calcd for $C_{15}H_{19}NO_6^-$ ($[M - H]^-$), 308.1005, found, 308.1039.



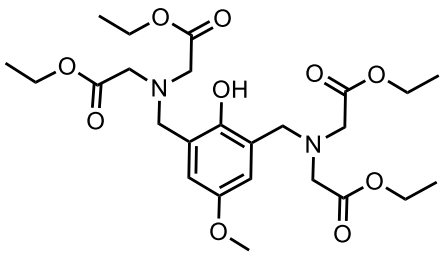
Preparation of diethyl 2,2'-((4-hydroxy-3,5-dimethylbenzyl)azanediyl)diacetate (Appendix 2.6A: 6a). A 10 mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture.

2,6-diemthlyphenol (122 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were added with stirring. The reaction was heated at reflux for three days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 35% EtOAc in hexanes to give 245 mg (76%) of **6a** as a yellow oil. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, CDCl3): δ 7.19 (s, 1H), 6.90 (s, 1H), 4.10 (q, *J* = 6.0, 4H), 3.69 (s, 2H), 3.45 (s, 4H), 2.16 (s, 6H), 2.23 (t, *J* = 6.0 Hz, 2H). **C13 NMR** (500 MHz, CDCl3): δ 170.70 (C), 156.92 (C), 155.04 (CH), 153.46 (CH), 122.20 (C), 117.35 (CH4), 115.75 (CH4), 61.14 (CH2), 55.81 (CH3), 53.87 (CH3) 14.16 (CH3). **HRMS** (**ESI**) calcd for $C_{17}H_{25}NO_5^+$ ($[M + H]^+$), 324.1733, found, 324.1806.



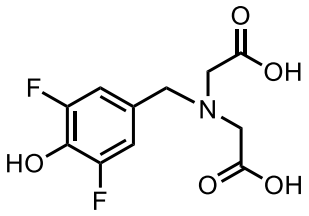
Preparation of 2,2',2'',2'''-(((5-fluoro-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetic acid (Appendix 2.6A: 7b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2',2",2'"-(((5-fluoro-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetate (128 mg, 0.25 mmol, 1 equiv) and 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 8 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 8 equiv) was added with stirring. The mixture was filtered to give a white powder which was washed with 1:1

THF in acetonitrile and dried to yield 68 mg (68%) of **7b** as a white powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, D_6MSO): δ 8.23 (s, 1H), 8.02 (s, 1H), 4.04 (s, 2H), 3.48 (s, 4H). **C13 NMR** (500 MHz, D_6MSO): δ 172.78 (C), 159.74 (C), 139.43 (C), 125.32 (C), 124.94 (C), 124.68 (CH), 120.76 (CH), 55.08 (CH4), 54.05 (CH2). **HRMS** (**ESI**) calcd for $C_{16}H_{19}FN_2O_9^-$ ($[M - H]^-$), 401.1075, found, 401.1108.

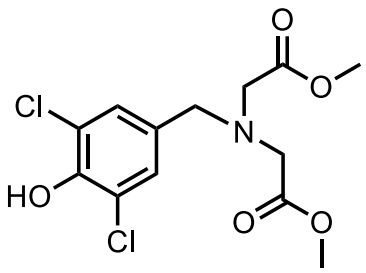


Preparation of tetraethyl 2,2',2'',2'''-(((2-hydroxy-5-methoxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetate (Appendix 2.6A: 8a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (430 mg, 2 mmol, 2 equiv) and 8 mL of a 1:2 water methanol mixture. 4-methoxyphenol (124 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for six days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 30% EtOAc in hexanes and concentrated to give 121 mg (23%) of **8a** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.45 (s, 2H), 7.28 (s, 1H), 3.89 (s, 2H), 3.72 (s, 4H). **C13 NMR** (500 MHz, CDCl3): δ 171.14 (C), 152.28 (C), 149.55 (C), 123.89 (C), 114.86 (CH8), 60.67 (CH8), 55.70 (CH3), 54.32

(CH4), 14.06 (CH12). **HRMS** (**ESI**) calcd for $C_{25}H_{38}N_2O_{10}^+$ ([M + H]$^+$), 527.2526, found, 527.2560.

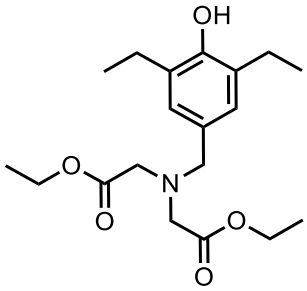


Preparation of 2,2'-((3,5-difluoro-4-hydroxybenzyl)azanediyl)diacetic acid (Appendix 2.6A: 9a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl 2,2'-((3,5-difluoro-4-hydroxybenzyl)azanediyl)diacetate (166 mg, 0.5 mmol, 1 equiv), 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 4 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^{o}C and 28 M HF (0.07 mL, 2 mmol, 4 equiv) was added with stirring. The mixture was filtered to give a white powder which was washed with 1:1 THF in acetonitrile and dried to yield 103 mg (72%) of **9a** as a white powder. ^{1}H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, D_6MSO): δ 7.01 (s, 1H), 6.92 (s, 1H), 3.73 (s, 2H), 3.31 (s, 4H). **C13 NMR** (500 MHz, CDCl3): δ 172.81 (C), 153.60 (C), 153.55 (C), 132.21 (C), 112.40 (CH), 56.55 (CH2), 54.20 (CH4). **HRMS** (**ESI**) calcd for $C_{11}H_{11}F_2NO_5^-$ ([M - H]$^-$), 274.0605, found, 274.0639.

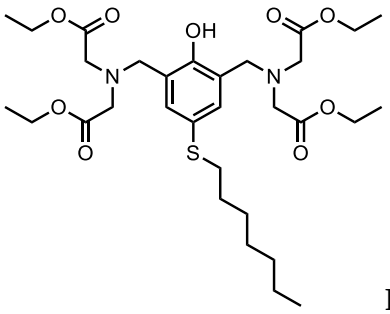


Preparation of dimethyl 2,2'-((3,5-dichloro-4-hydroxybenzyl)azanediyl)diacetate (Appendix 2.6A: 10a). A 10-mL round-bottomed

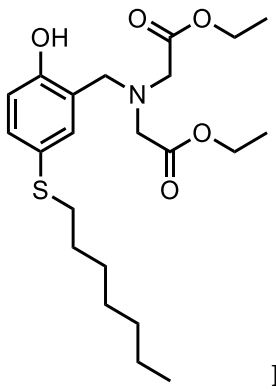
flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with cesium 2,2'-azanediyldiacetate (436 mg, 1.1 mmol, 1.1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture 2,6-dichlorophenol (163 mg, 1 mmol, 1 equiv) was added slowly and the reaction mixture was heated at reflux for 12 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3,5-dichloro-4-hydroxybenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with t-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 255 mg (76%) of **10a** as a yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.10 (s, 1H), 3.91 (s, 6H), 3.80 (s, 4H), 3.74 (s, 2H). **C13 NMR** (500 MHz, CDCl3): δ 171.31 (C), 150.51 (C), 129.78 (C), 129.04 (C), 127.66 (CH2), 60.37 (CH2), 57.47 (CH2), 54.13 (CH4), 23.07 (CH6), 14.28 (CH6). **HRMS** (**ESI**) calcd for $C_{13}H1_5Cl_2NO_5^+$ ($[M + H]^+$), 514.2327, found, 514.2360.



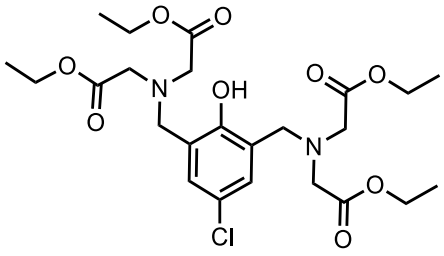
Preparation of diethyl 2,2'-((3,5-diethyl-4-hydroxybenzyl)azanediyl)diacetate (Appendix 2.6A: 11a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1 mmol, 2 equiv) and 8 mL of a 1:2 water methanol mixture. 2,6-diethylphenol (150 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for six days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 25% EtOAc in hexanes and concentrated to give 249 mg (71%) of **11a** as a viscous, light yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.01 (s, 2H), 4.20 (q, *J* = 7.1 Hz, 4H), 3.83 (s, 2H), 3.55 (s, 4H), 2.64 (q, *J* = 7.5 Hz, 4H), 1.29 (t, *J* = 7.1 Hz, 4H), 1.26 (t, *J* = 7.5 Hz, 4H). **C13 NMR** (500 MHz, CDCl3): δ 171.31 (C), 150.51 (C), 129.78 (C), 129.03 (CH), 127.66 (C), 60.37 (CH4), 57.47 (CH2), 54.13 (CH4), 14.28 (CH6), 14.03 (CH6). **HRMS** (**ESI**) calcd for $C_{19}H_{29}NO_5^+$ ($[M + H]^+$), 352.2046, found, 352.2079.



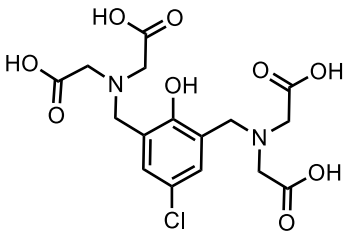
Preparation of tetraethyl 2,2',2'',2'''-(((5-(heptylthio)-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetate (Appendix 2.6A: 12a). A 1- mL round-bottomed flask equipped with a magnetic stirring bar was, and an N_2 inlet charged with diethyl iminodiacetate (440 mg, 2 mmol, 2 equiv) and 8 mL of a 1:2 water methanol mixture. 4-(heptylthio)phenol (224 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for nine days, monitored by TLC. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 25% EtOAc in hexanes to give 62 mg (10%) of **12a** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.21 (s, 1H), 7.28 (q, *J* = 7.1 Hz, 4H), 3.97 (s, 4H), 3.57 (s, 8H), 2.82 (t, *J* = 7.3 Hz, 2H), 1.58 (m, 4H), 1.37 (m, 4H), 1.30 (m, 12H), 0.89 (t, *J* = 6.8 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 171.08 (C), 154.98 (C), 132.71 (C), 125.06 (CH), 123.87 (CH8), 60.71 (CH8), 54.32 (CH4), 53.79 (CH2), 34.75 (CH2), 31.73 (CH2), 29.34 (CH2), 28.86 (CH2), 28.71 (CH2), 22.59 (CH2), 14.22 (CH12), 14.06 (CH3). **HRMS** (**ESI**) calcd for $C_{31}H_{50}N_2O_9S^+$ ([M + H]$^+$), 627.3237, found, 627.3271.



Preparation of diethyl 2,2'-((5-(heptylthio)-2-hydroxybenzyl)azanediyl)diacetate (Appendix 2.6A: 13a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (215 mg, 1.2 mmol, 1.2 equiv) and 8 mL of a 1:2 water methanol mixture. 4-(heptylthio)phenol (224 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (36 mg, 1.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for six days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 25% EtOAc in hexanes to give 366 mg (86%) of **13a** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.28 (d, *J* = 7.3 Hz, 1H), 7.06 (s, 1H), 6.85 (d, *J* = 8.3 Hz, 1H), 4.23 (m, 4H), 4.00 (s, 2H), 3.55 (s, 4H), 2.80 (m, 2H), 1.57 (m, 2H), 1.38 (m, 3H), 1.30 (m, 8H), 0.89 (t, *J* = 6.5 Hz, 3H). **C13 NMR** (500 MHz, CDCl3): δ 170.75 (C), 156.80 (C), 133.15 (C), 125.12 (CH), 121.96 (C), 117.33 (CH), 61.11 (CH), 53.99 (CH4), 54.33 (CH4), 53.86 (CH2), 36.03 (CH2), 31.71 (CH2), 29.35 (CH2), 28.66 (CH2), 22.58 (CH2), 14.17 (CH6), 14.06 (CH3). **HRMS** (**ESI**) calcd for $C_{22}H_{352}O_5S^+$ ($[M + H]^+$), 426.2236, found, 426.2263.

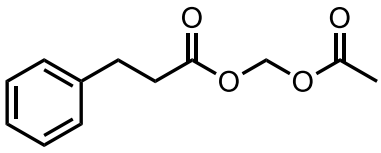


Preparation of tetraethyl 2,2',2'',2'''-(((5-chloro-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetate (Appendix 2.6A: 14a). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with diethyl iminodiacetate (440 mg, 2 mmol, 2 equiv) and 8 mL of a 1:2 water methanol mixture. 4-chlorophenol (128 mg, 1.0 mmol, 1 equiv) and paraformaldehyde (66 mg, 2.2 mmol, 1.2 equiv) were then added with stirring. The reaction was heated at reflux for six days. The resulting mixture was cooled, concentrated in vacuo and purified via column chromatography in 20% EtOAc in hexanes to give 153 mg (29%) of **14a** as a viscous, light orange oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.16 (s, 2H), 4.22 (q, *J* = 6.0 Hz, 8H), 3.96 (s, 4H), 3.57 (s, 8H), 1.30 (t, *J* = 6.0 Hz, 12H). **C13 NMR** (500 MHz, CDCl3): δ 171.04 (C), 154.31 (C), 129.04 (C), 123.72 (C), 86.89 (CH2), 60.79 (CH4), 54.39 (CH8), 53.57 (CH8), 14.20 (CH12). **HRMS** (**ESI**) calcd for $C_{24}H_{35}ClN_2O_9^+$ ([M + H]$^+$), 529.2031, found, 529.2002.



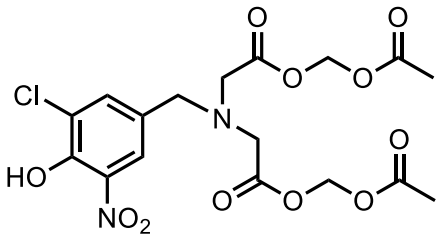
Preparation of 2,2',2'',2'''-(((5-chloro-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetic acid (Appendix 2.6A: 14b). A

10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with tetraethyl 2,2',2",2"'-(((5-chloro-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl))tetraacetate (133 mg, 0.25 mmol, 1 equiv) and 2 mL of a 1:0.3 solution of methanol water and lithium hydroxide monohydrate (84 mg, 2 mmol, 8 equiv). The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was cooled to 0 ^oC and 28 M HF (0.07 mL, 2 mmol, 8 equiv) was added with stirring. The mixture was filtered to give a white powder which was subsequently washed with 1:1 THF in acetonitrile and dried to yield 268 mg (64%) of **14b** as a white powder. ^1H and ^{13}C NMR data taken in D_6MSO and analytical data included the following; **1H NMR** (300 MHz, D_6MSO): δ 7.09 (s, 2H), 3.79 (s, 4H), 3.62 (s, 8H), 3.36 (s, 8H). **C13 NMR** (500 MHz, CDCl3): δ 173.36 (C), 128.32 (C), 126.27(C), 125.89 (C), 67.48 (CH2), 53.23 (CH6), 25.60 (CH8). **HRMS** (**ESI**) calcd for $C_{16}H_{19}ClN_2O_9^-$ ([M - H]$^-$), 418.7831, found, 418.0770.



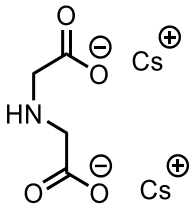
Preparation of acetoxymethyl 3-phenylpropanoate (Appendix 2.6A: 15b). A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 3-phenylpropanoic acid (150 mg, 1 mmol, 1 equiv) and 1.5 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (175 µL, 1 mmol, 1 equiv) was added slowly and the mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3-chloro-4-hydroxy-5-nitrobenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-

bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.1 mL, 1.1 mmol, 1.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 175 mg (79%) of **15b** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.22 (m, 5H), 5.71 (s, 1H), 2.95 (t, J = 6.0 Hz, 2H), 2.67 (t, 2H) 2.10 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 171.61 (C), 169.68 (C), 139.99 (C), 128.29 (CH), 126.40 (CH2), 86.69 (CH2), 85.48 (CH2), 35.77 (CH2), 30.55 (CH2), 20.72 (CH3). **HRMS** (**ESI**) calcd for $C_{12}H_{14}O_4^+$ ($[M + H]^+$), 222.0892 found, 222.0583.



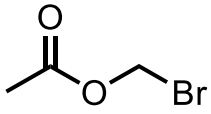
Preparation of bis(acetoxymethyl) 2,2'-((3-chloro-4-hydroxy-5-nitrobenzyl)azanediyl)diacetate (Section 4. 16a) A 10-mL round-bottomed flask equipped with a magnetic stirring bar, and an N_2 inlet was charged with 2,2'-((3-chloro-4-hydroxy-5-nitrobenzyl)azanediyl)diacetic acid (318 mg, 1 mmol, 1 equiv) and 2 mL anhydrous EtOH was subsequently added. To the stirring reactant mixture, 5.68 M CsOH (0.35 mL, 2 mmol, 2 equiv) was added slowly and the mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo and thoroughly dried

under high vacuum for 12 h to provide the dicesium salt, cesium 2,2'-((3-chloro-4-hydroxy-5-nitrobenzyl)azanediyl)diacetate as a yellow solid. The same 10 mL round-bottomed flask equipped with a magnetic stirring bar and charged with 2.5 mL anhydrous DMF. The stirring reactant mixture was cooled to 0 ^{o}C in an ice bath and bromomethyl acetate (0.2 mL, 2.1 mmol, 2.1 equiv) was added dropwise. The reactant mixture was stirred for 30 min at 0 ^{o}C and let stir for an additional 12 h at room temperature. The resulting mixture was dissolved in water and extracted with *t*-butyl methyl ether (3 X 2 mL). The organic layers were combined and washed with brine (2 X 2 mL). The organic layer was dried and concentrated in vacuo to afford 379 mg (82%) of **16a** as a viscous yellow oil. ^{1}H and ^{13}C NMR data taken in $CDCl_3$ and analytical data included the following; **1H NMR** (500 MHz, CDCl3): δ 7.97 (s, 1H), 7.72 (s, 1H), 5.50 (s, 4H), 3.81 (s, 2H), 3.55 (s, 4H), 2.05 (s, 6H). **C13 NMR** (500 MHz, CDCl3): δ 170.61 (C), 159.40 (C), 139.75 (C), 126.10 (C), 123.79 (C), 122.57 (C), 122.21 (CH), 61.60 (CH), 55.44 (CH4), 53.92 (CH4), 56.06 (CH2), 14.15 (CH3). **HRMS** (**ESI**) calcd for $C_{17}H_{19}ClN_2O_{11}^{+}$ ([M + H]$^{+}$), 464.0677 found, 464.0497.



Preparation of cesium 2,2'-azanediyldiacetate. A 50 mL round-bottomed flask equipped with a magnetic stirring bar and N^2 inlet was charged with the iminodiacetic acid (1.33 g, 10 mmol, 1 equiv) and 30 mL anhydrous EtOH. To the stirring reactant mixture aqueous cesium hydroxide (5.68 M) was added (610 mg, 20 mmol, 2 equiv) slowly and the reaction mixture was heated at reflux for 1 h. The resulting mixture was

concentrated in vacuo and thoroughly dried under high vacuum for 12 h to provide 3.8 mg (97%) of a white powder.



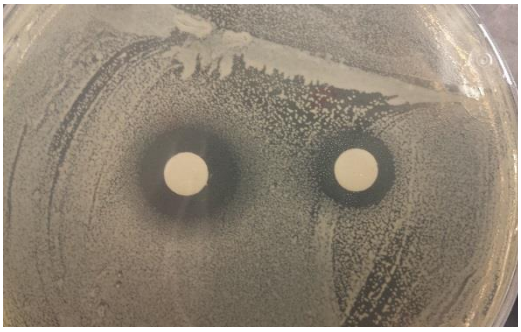
Improved p**reparation of bromomthyl acetate.** A 25 mL round-bottomed flask equipped with a magnetic stirring bar was charged with the acetyl bromide (3.7 mL, 50 mmol, 1 equiv) and stannic chloride (60 µL, 0.5 mmol, 0.01 equiv). To the stirring reactant mixture paraformaldehyde (1.3 g, 45 mmol, 0.9 equiv) was added portion-wise at 0 ^{o}C. The resulting mixture was distilled to yield 6.4 g (84%) of a yellow liquid.

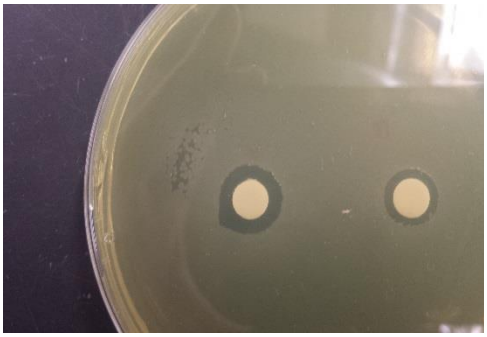
APPENDIX B:

UNINCLUDED AND UNSUCESSFUL EXPERIMENTS

1B Disc diffusion assay

Overnight cultures of *S. epidermidis* and *P. aeruginosa* were grown at 37 ºC as mentioned in "General Bacterial Growth Procedure" and 0.1 mL of the standardized culture was spread on an agar plate using sterile glass spreader. A sterile disc was placed on each plate with either 30 µL of eugenol or eugenol AM, both at a concentration of 10 mM diluted in 1% DMSO and PBS. Agar plates were incubated at 37 ºC for 24 h. The zone of inhibition was then measured. The zone of inhibition is defined as the area around the disc which exhibited no bacterial growth. Against *S. epidermidis*, eugenol had an annular radius of 0.3 cm while eugenol AM's was 0.5 cm (**1A**). Against *P. aeruginosa*, eugenol had an annular radius of only 0.1 cm and eugenol AM had an annular radius of 0.2 cm (**1B**). This assay has demonstrated to us that eugenol AM is a more potent antimicrobial than its parent phenol, Eugenol, which correlates with data from previous assays. This data was not presented in the prodrug paper because it was reserved for use in the event that reviewers requested additional experiments.

1A 

1B 

2B Live/Dead stains on eugenol and eugenol AM

A live/dead stain was performed in an attempt to show a ratio of live to dead cells after exposure to eugenol and eugenol AM using a stain mixture of Syto 9/PI. Syto 9 is a green fluorescent dye that binds to both live and dead cells, while PI is a red dye that binds exclusively to dead cells. In the mixture, PI will out compete Styo 9 for binding to dead cells, staining them red while live cells will remain green.

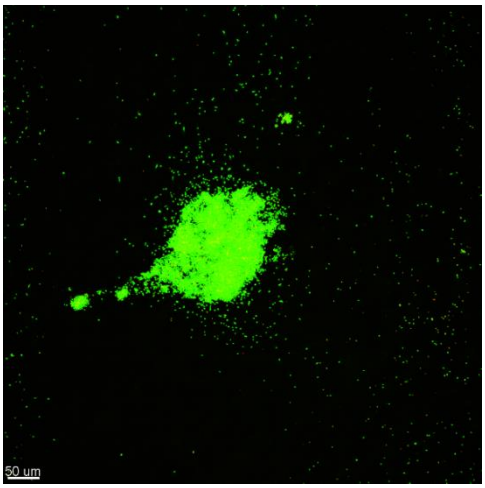
An overnight culture of *S. epidermidis* was grown at 37 ^{o}C, the absorbance was read at 600 nm and adjusted to 0.05 OD. 0.1 mL of the inoculum was pipetted into each fourth of a glass bottom dish and incubated at 37 ^{o}C for 30 min. The medium was extracted and replaced with 1 mL fresh TSB and incubated for 6 h. The media was pulled off and the biofilm washed 3x with phosphate buffer, pH 7.4. The biofilm was exposed to either 0.3 mL Eugenol (50 mM in 1% DMSO in PBS) or Eugenol AM (50 mM in 1% DMSO in PBS). As solution of 1% DMSO in PBS was used as a negative control and 70% ethanol in water was used as a positive control. The biofilms were incubated for an additional 1 h at 37 ^{o}C.

Biofilms were then gently washed with PBS 3x and 10 µL of a 1:1 mixture of Syto 9/PI was then added and incubated in the dark at 37 ^{o}C, for 10 min. Each biofilm was subsequently washed with filter-sterilized water and three different areas of each biofilm were imaged on an upright confocal. A snapshot is taken with both green and red fluorescence and the two images are imposed on each other so that a ratio of red to green can be determined.

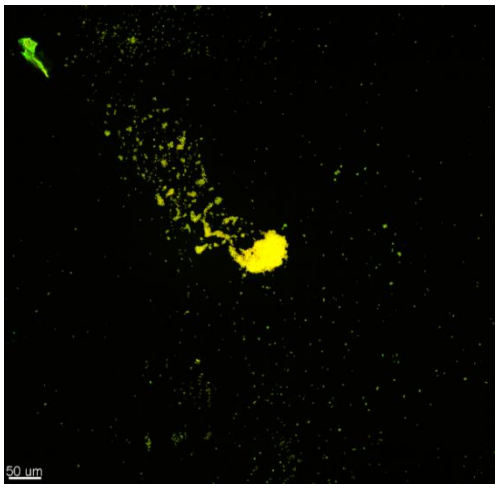
In the 1% DMSO control (**2A**), there is a higher ratio of green to red cells but in the ethanol control (**2B**) there is a higher ratio of red, causing the overlapped picture to look

yellow and showing significant killing. Eugenol did demonstrate some killing, although not to the extent of ethanol. Cell death cause by eugenol AM was unable to be accurately determined due to residual compound on the biofilms obstructing the abilities of the microscope (**2C**).

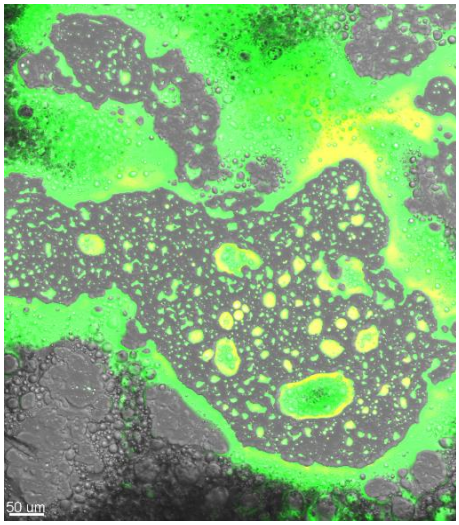
2A



2B



2C



In subsequent live/dead assays, the biofilm was washed more vigorously in order to remove the AM, although resulted in too much of the biofilm was removed. A thicker biofilm was also grown with incubate times of 12, 10 and 8 h as opposed to 6 h. Biofilms

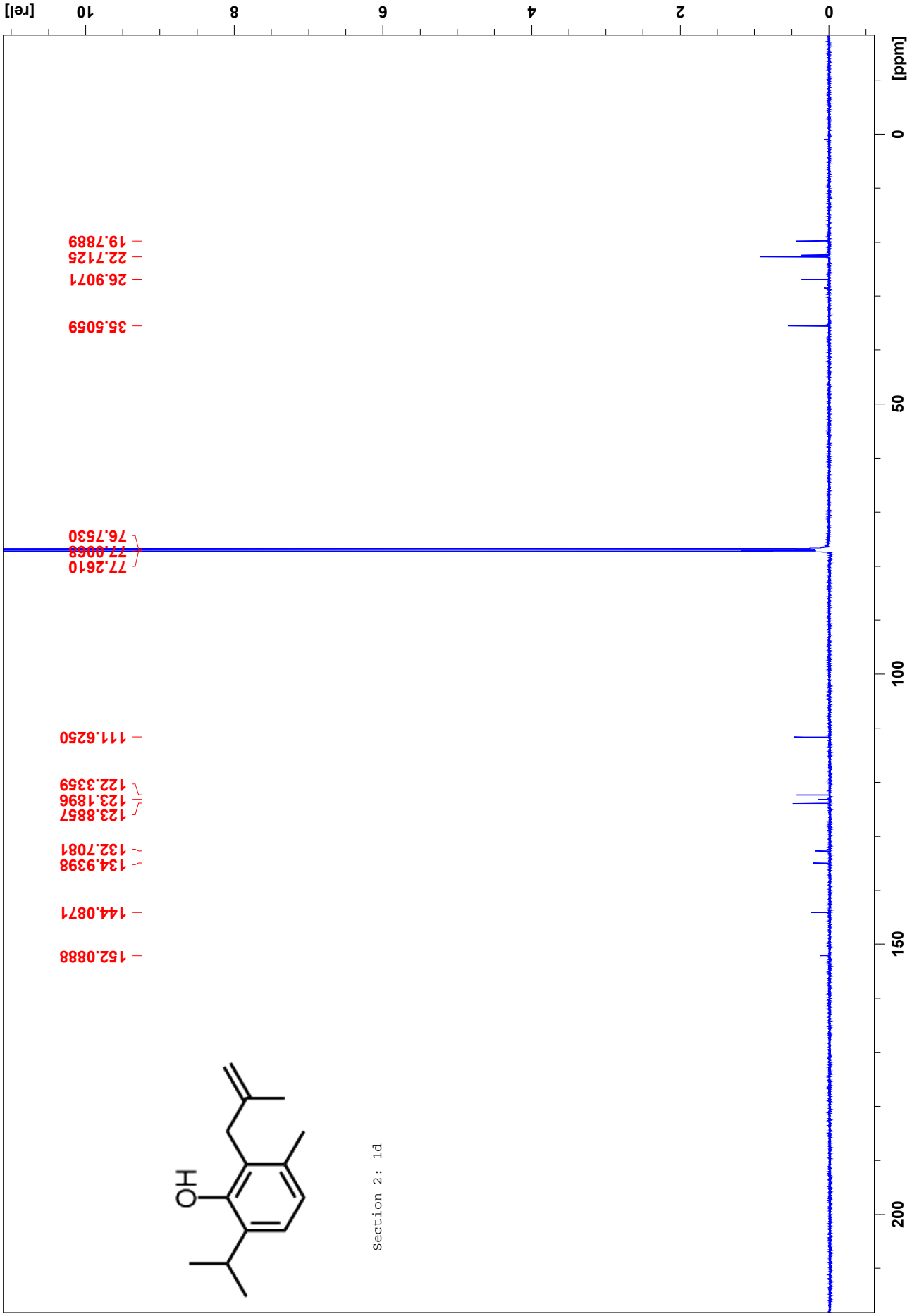
grown for longer than 6 h were able to be washed more vigorously without removing the biofilm although were too thick to view accurately under the microscope.

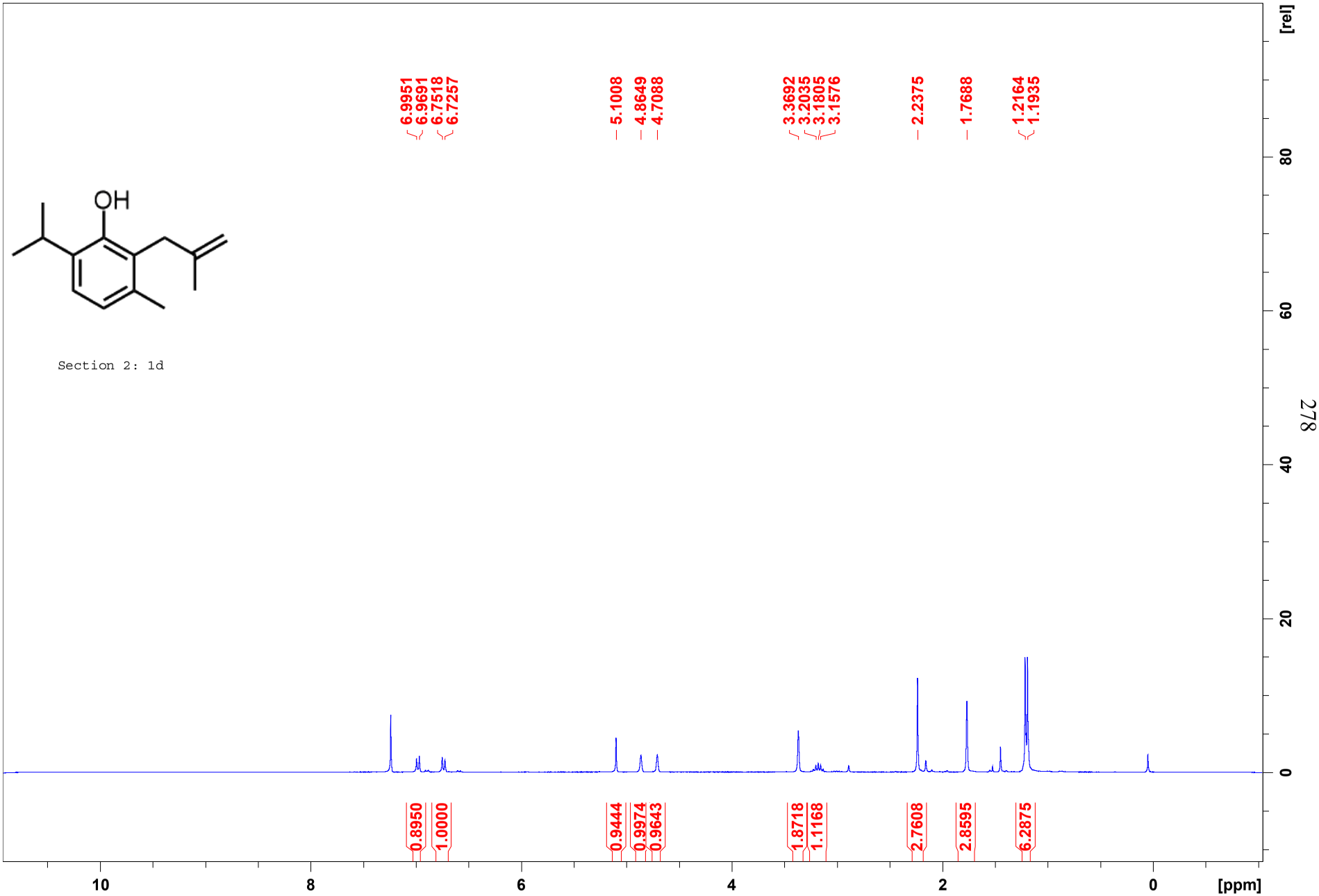
3B Variations of the procedure "Biofilm eradication 96-well plate assay" (1.3.1A)

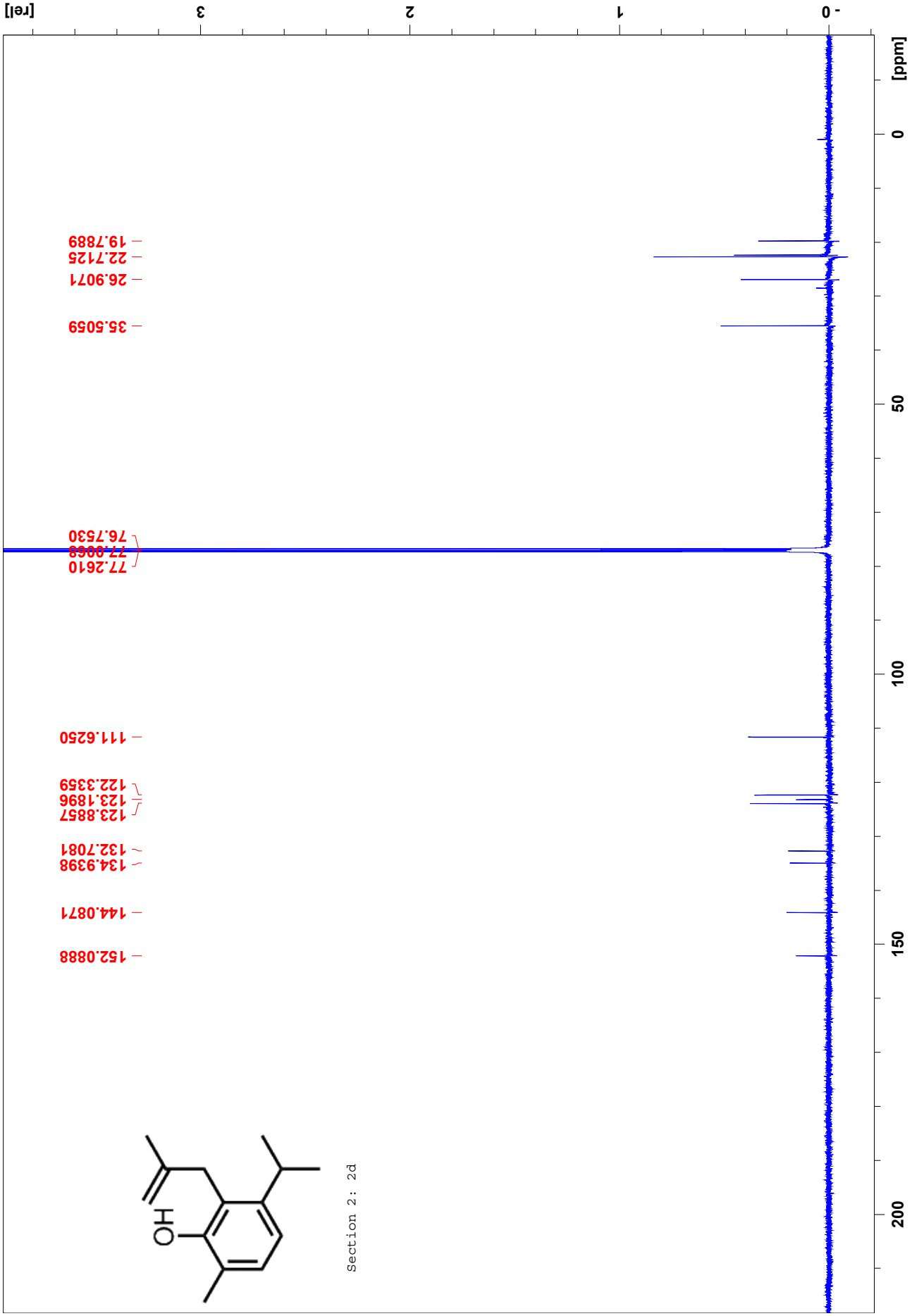
As stated in the above procedure on biofilm eradication, 1.3.1A, "The media was gently removed and each well filled with 150 µL PBS and the biofilm broken up…" At this step, a series of additional washing steps with PBS and/or sterile water were attempted. In this; the media was gently removed and either PBS or sterile water was used to wash the biofilms by being gently applied (150 µL) and again gently removed. If washed once, there was no difference in results as with using the aforementioned procedure, although if the biofilm was washed twice or three times the results became less reliable and varied more often in subsequent assays with the same compound, each experiment was done in triplicate. Therefore, this washing step was left out and is not worth revisiting.

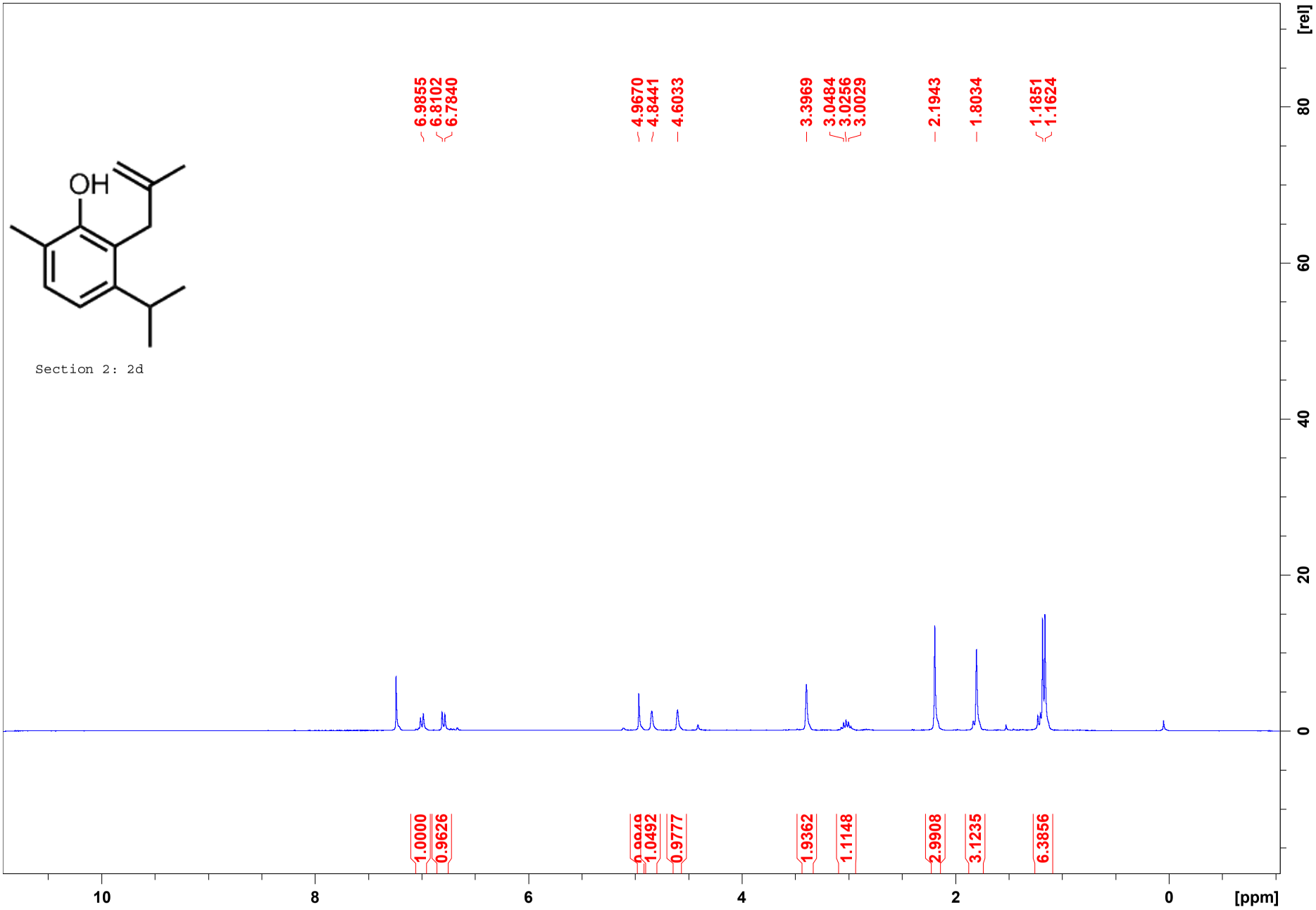
APPENDIX C:

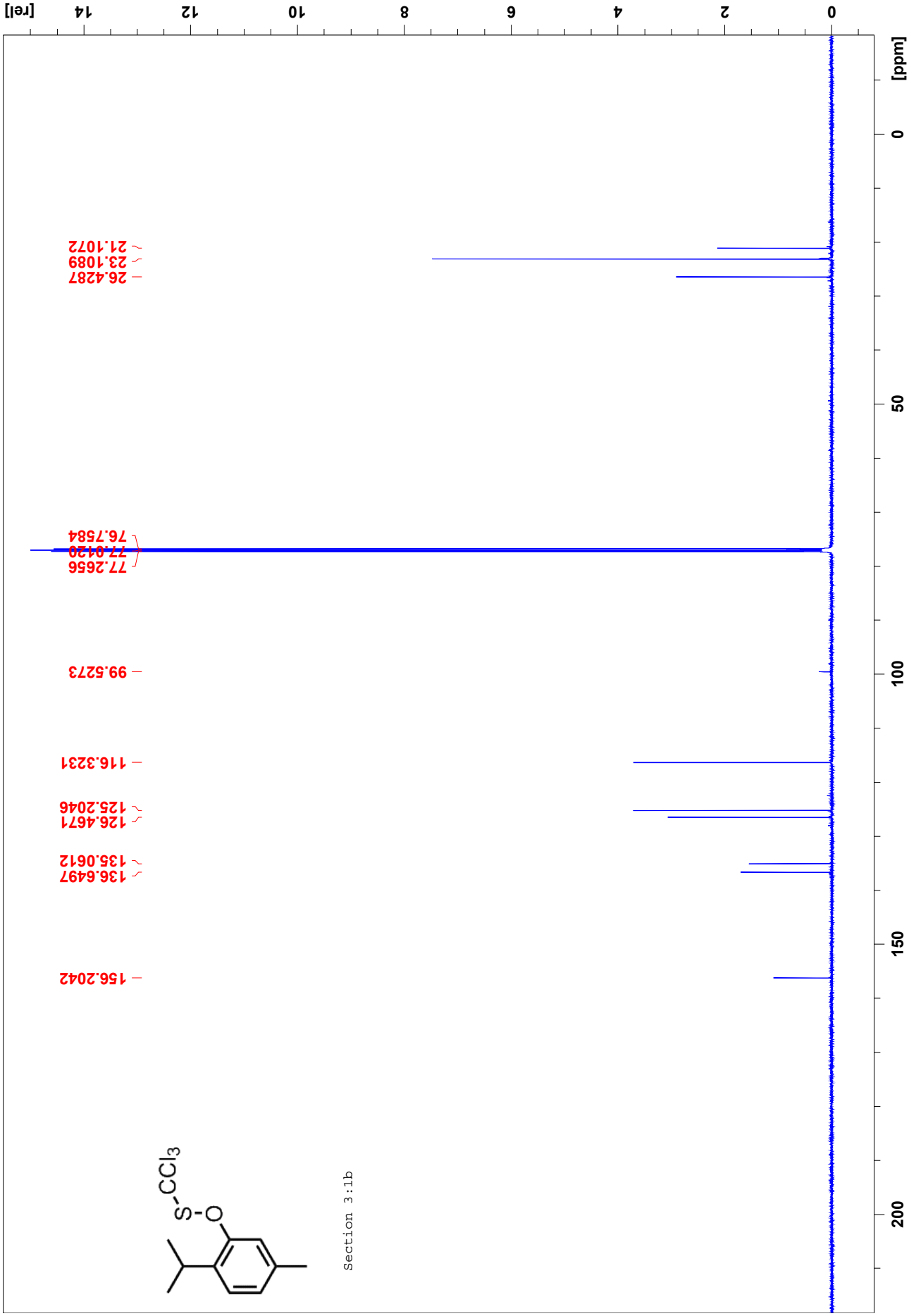
SPECTRAL DATA

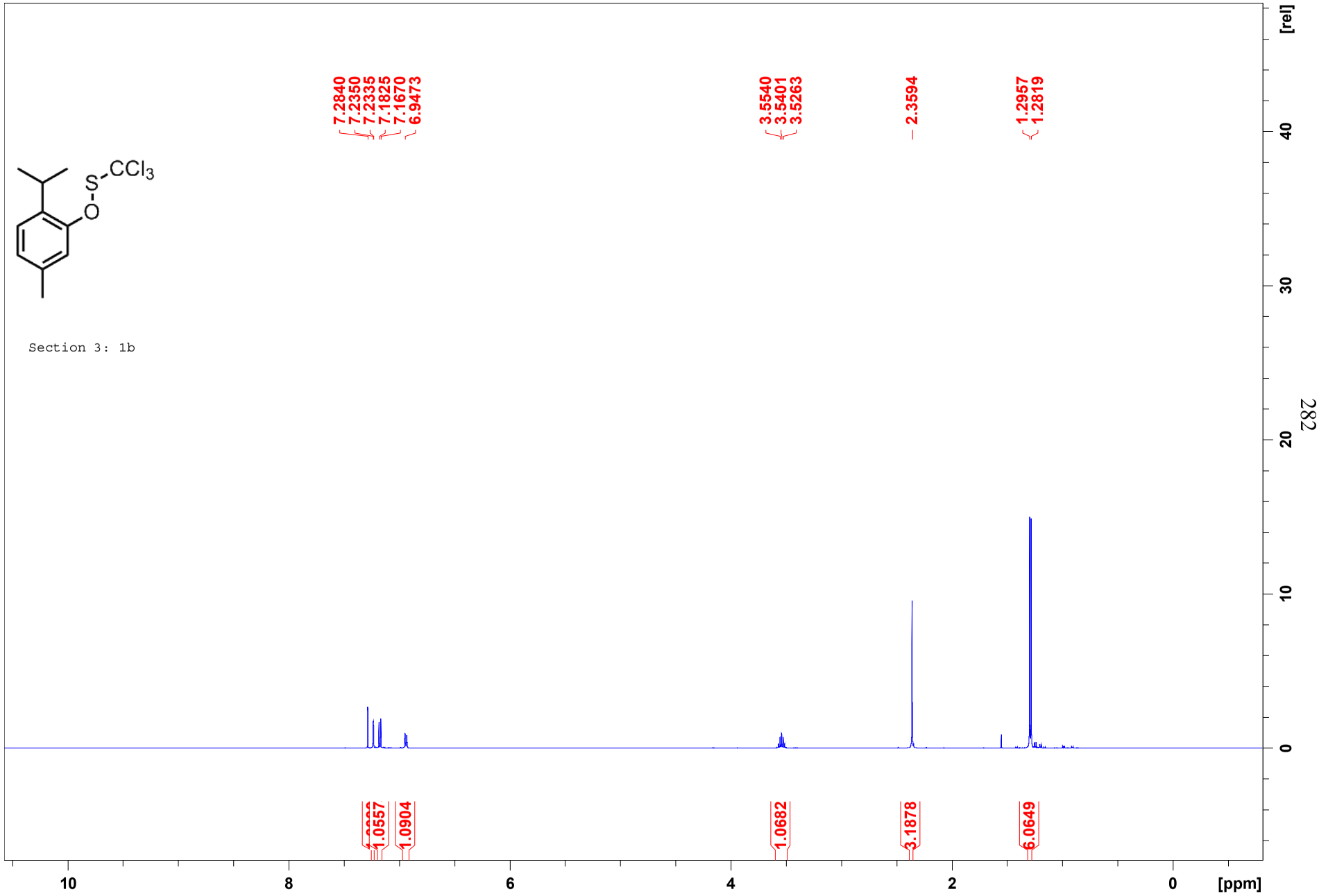

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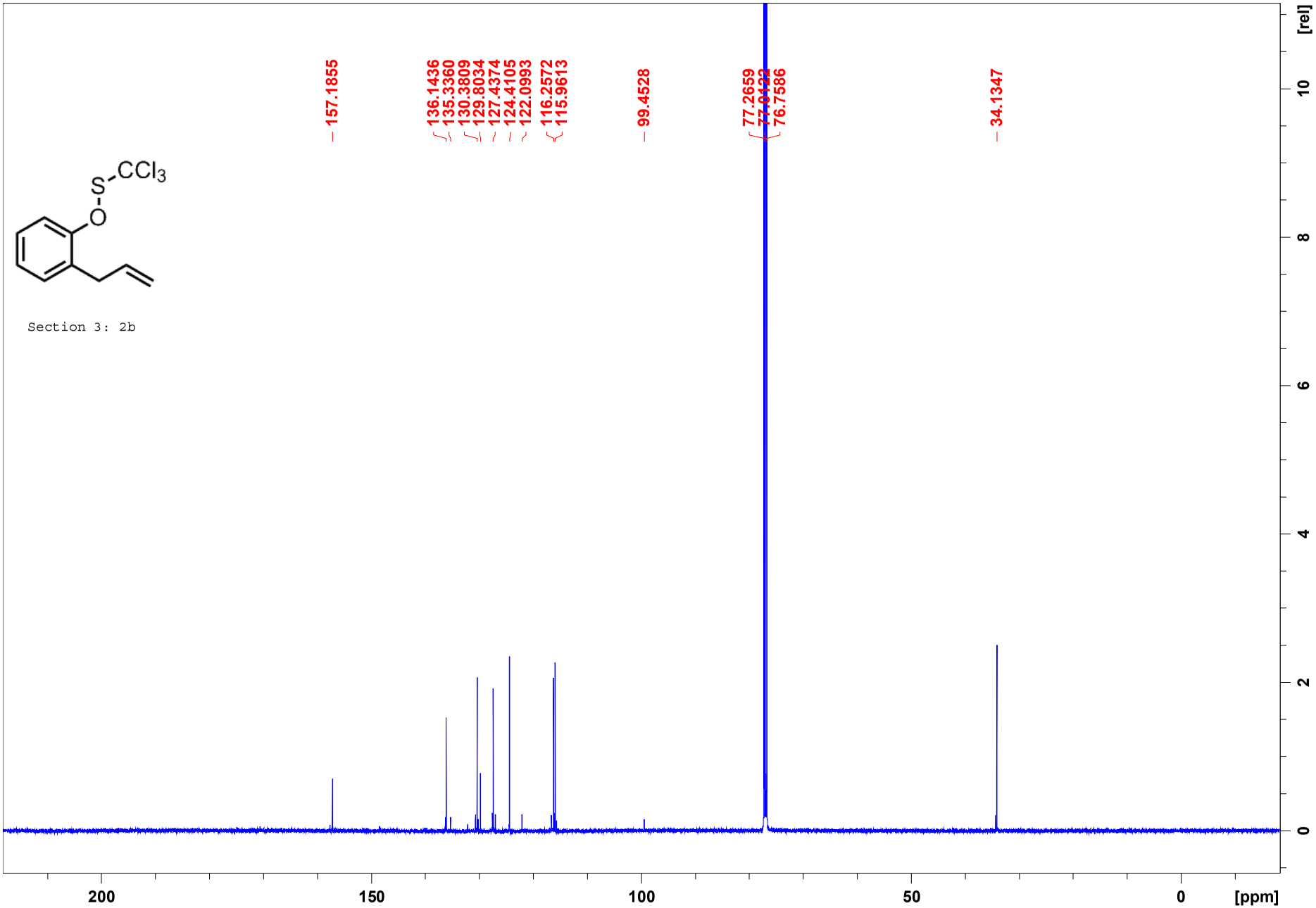

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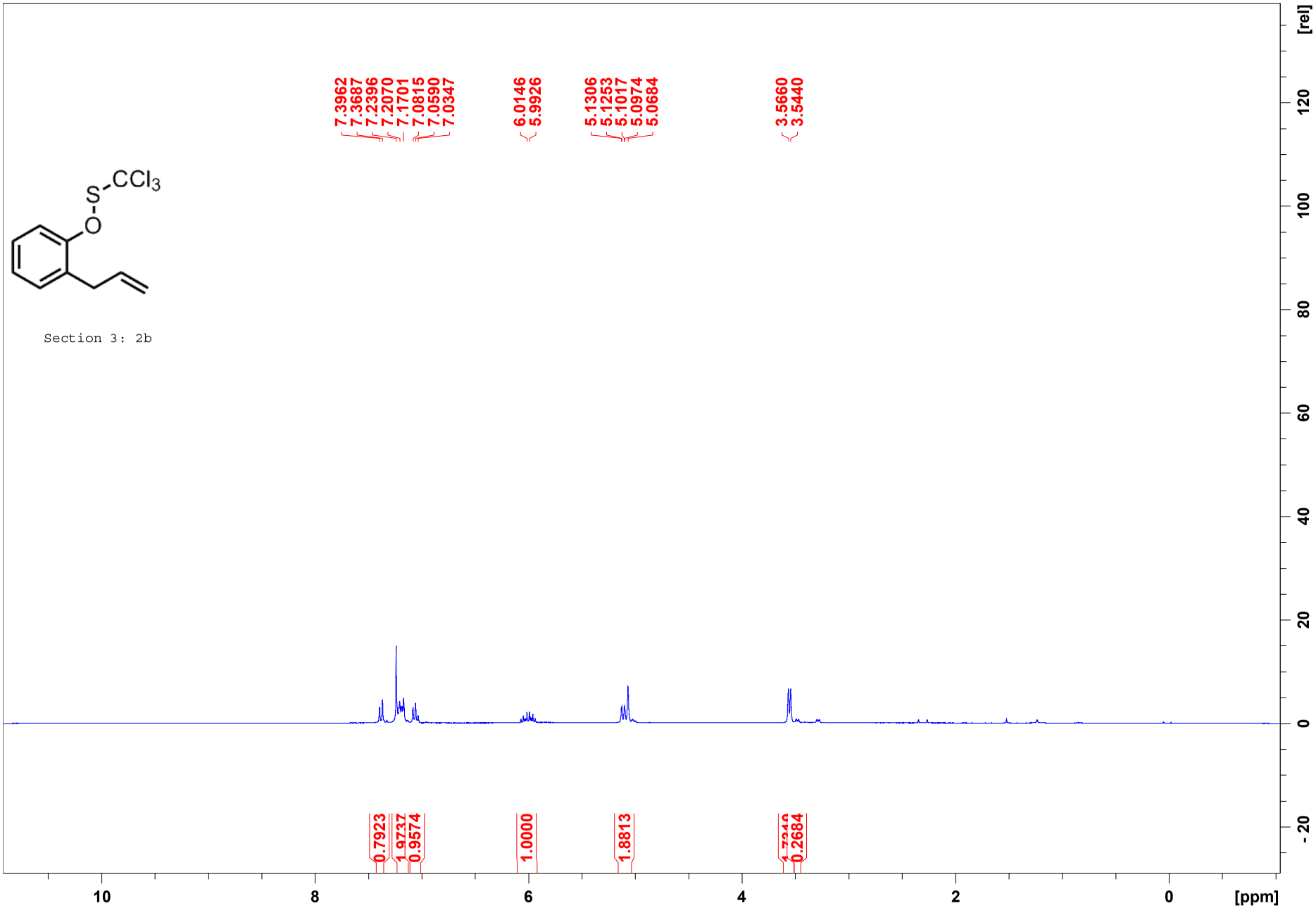

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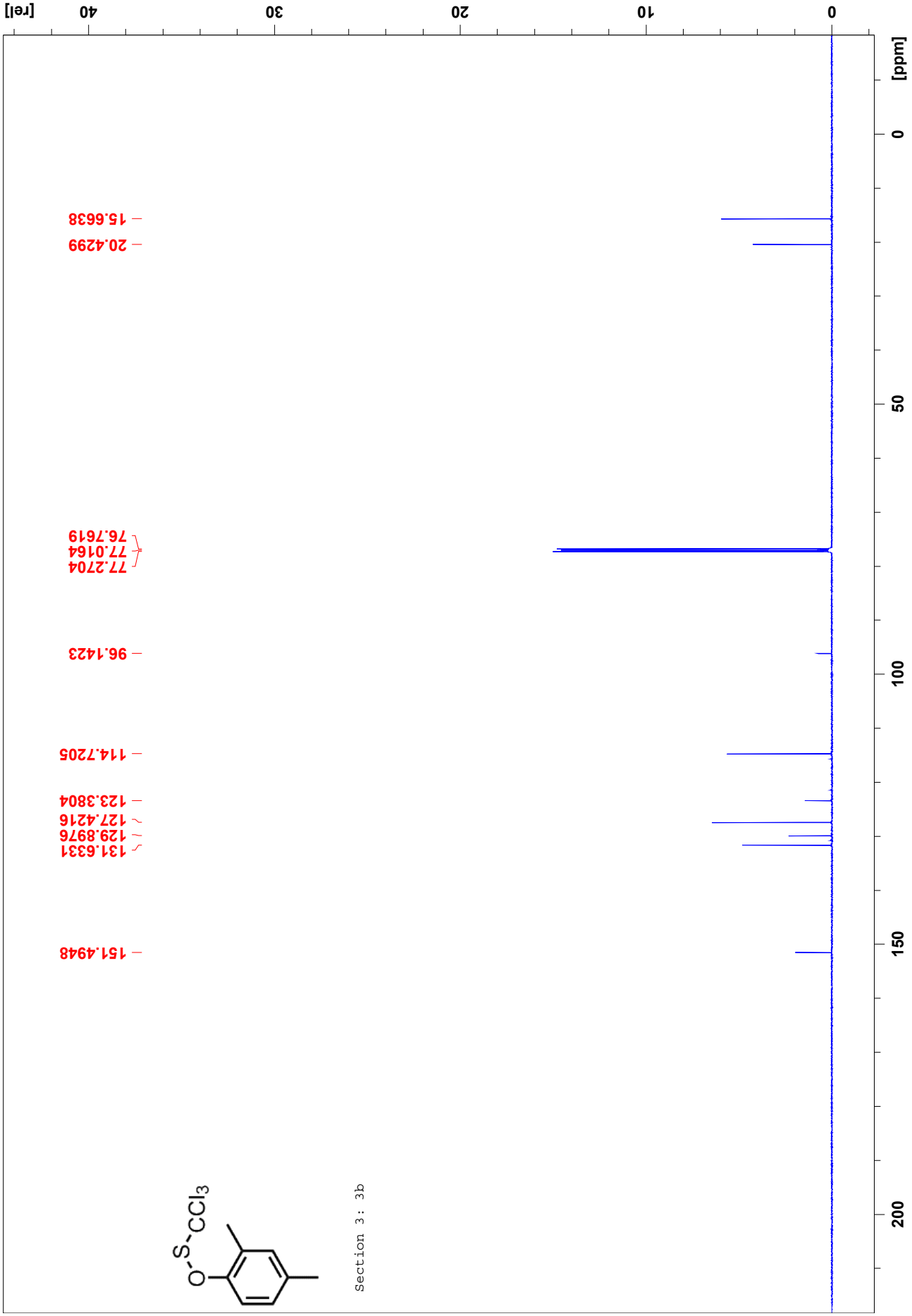

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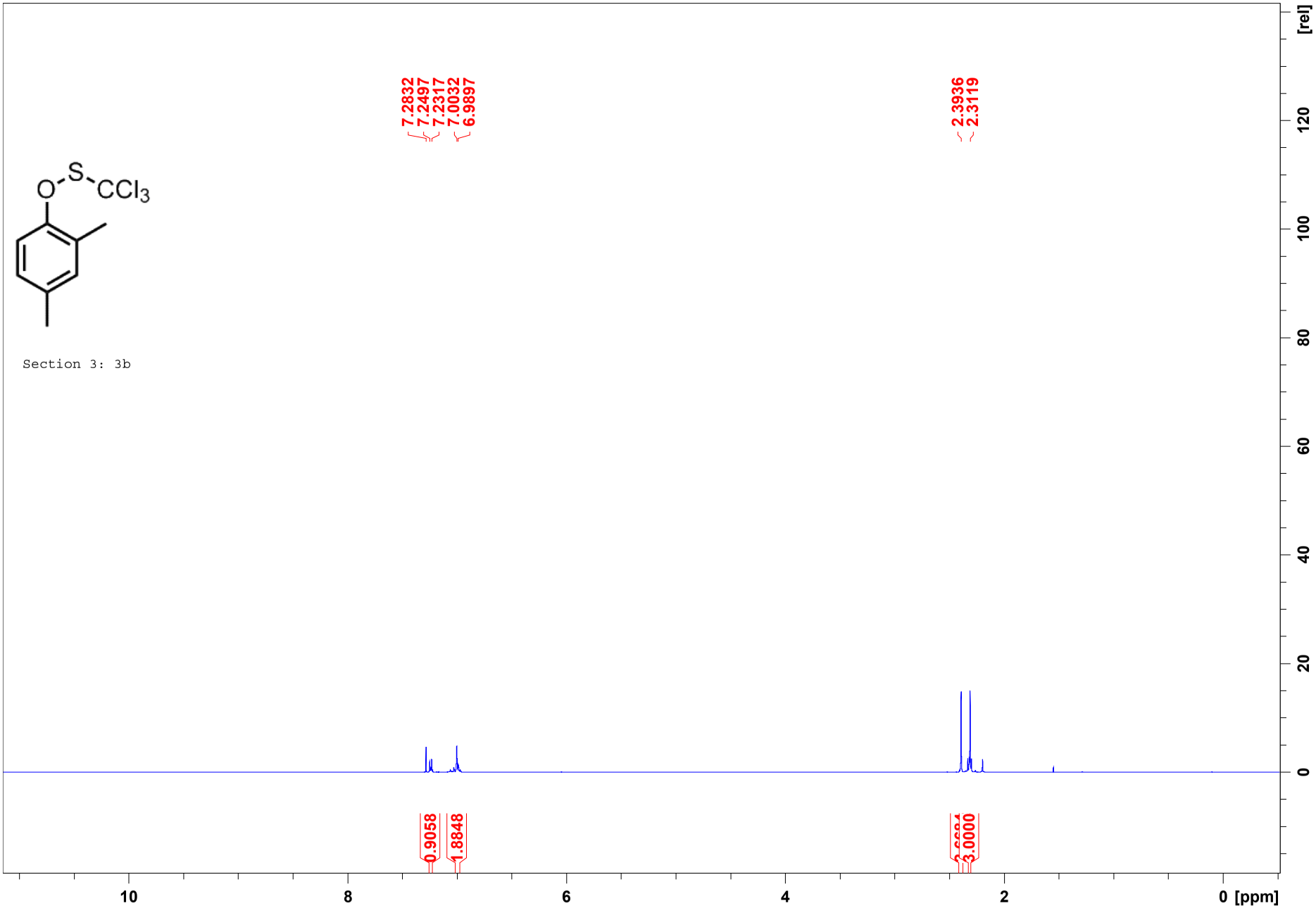

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156.2042
Section 3:1b

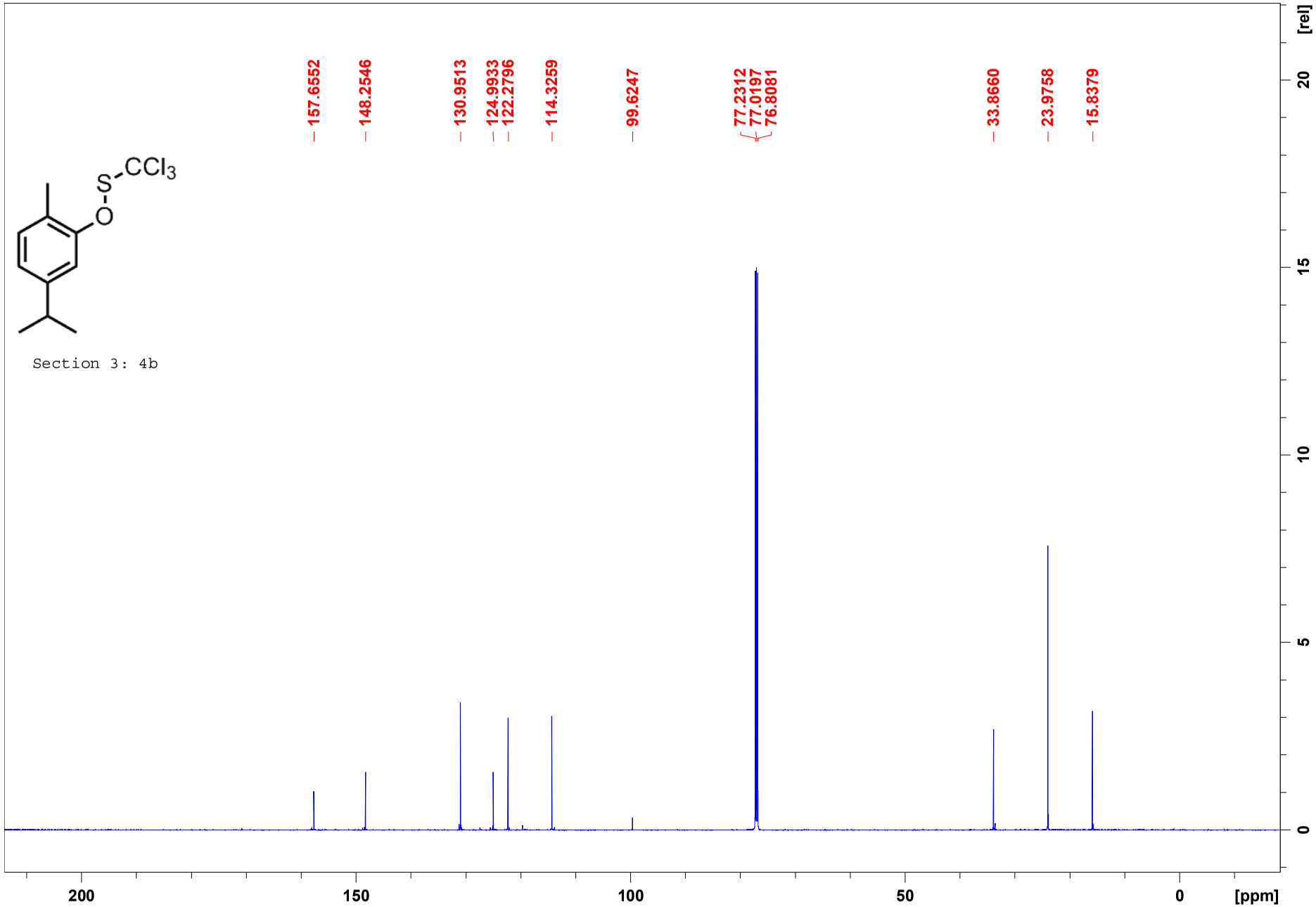

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1.2819
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CCl3
O
Section 3: 1b
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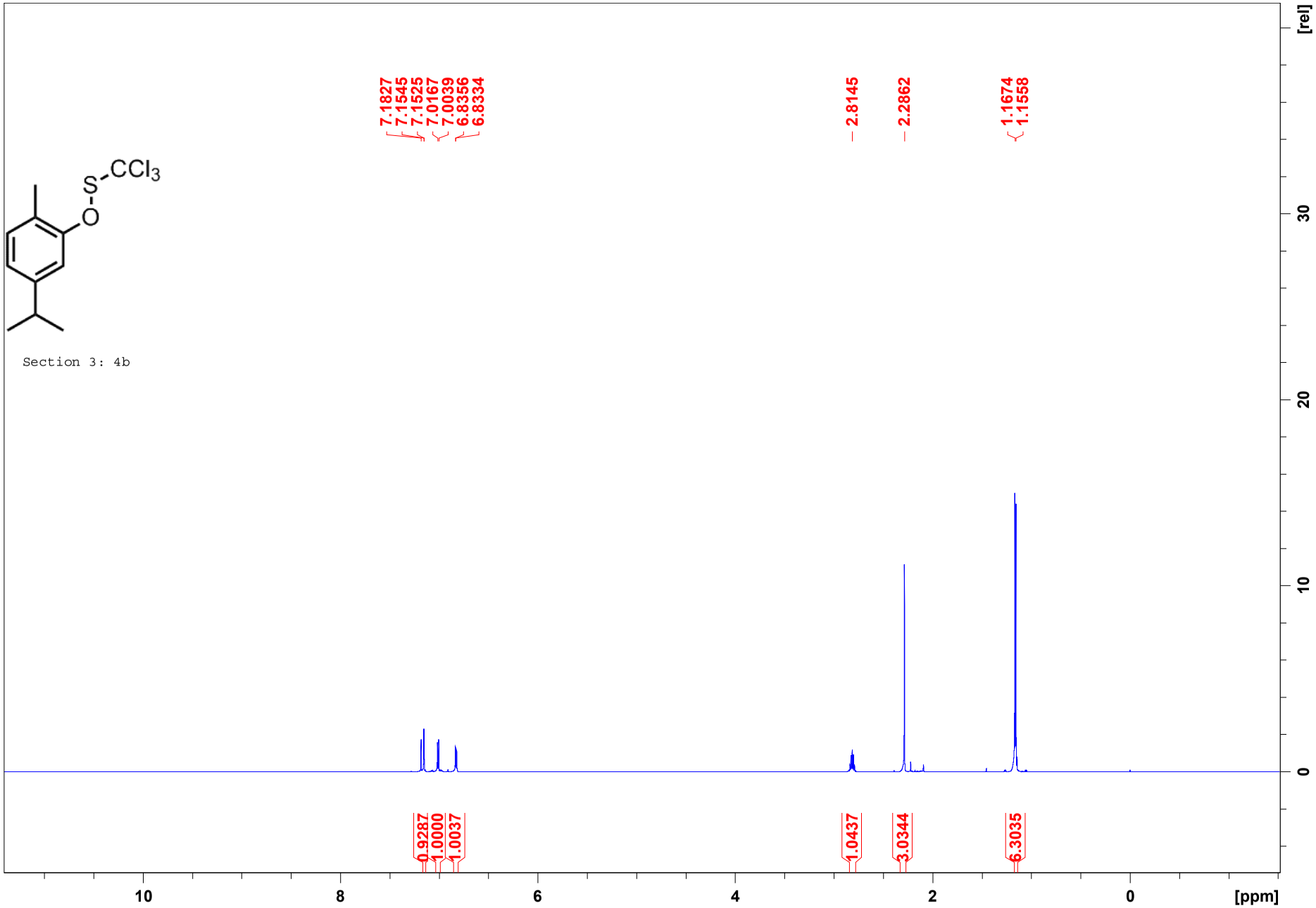

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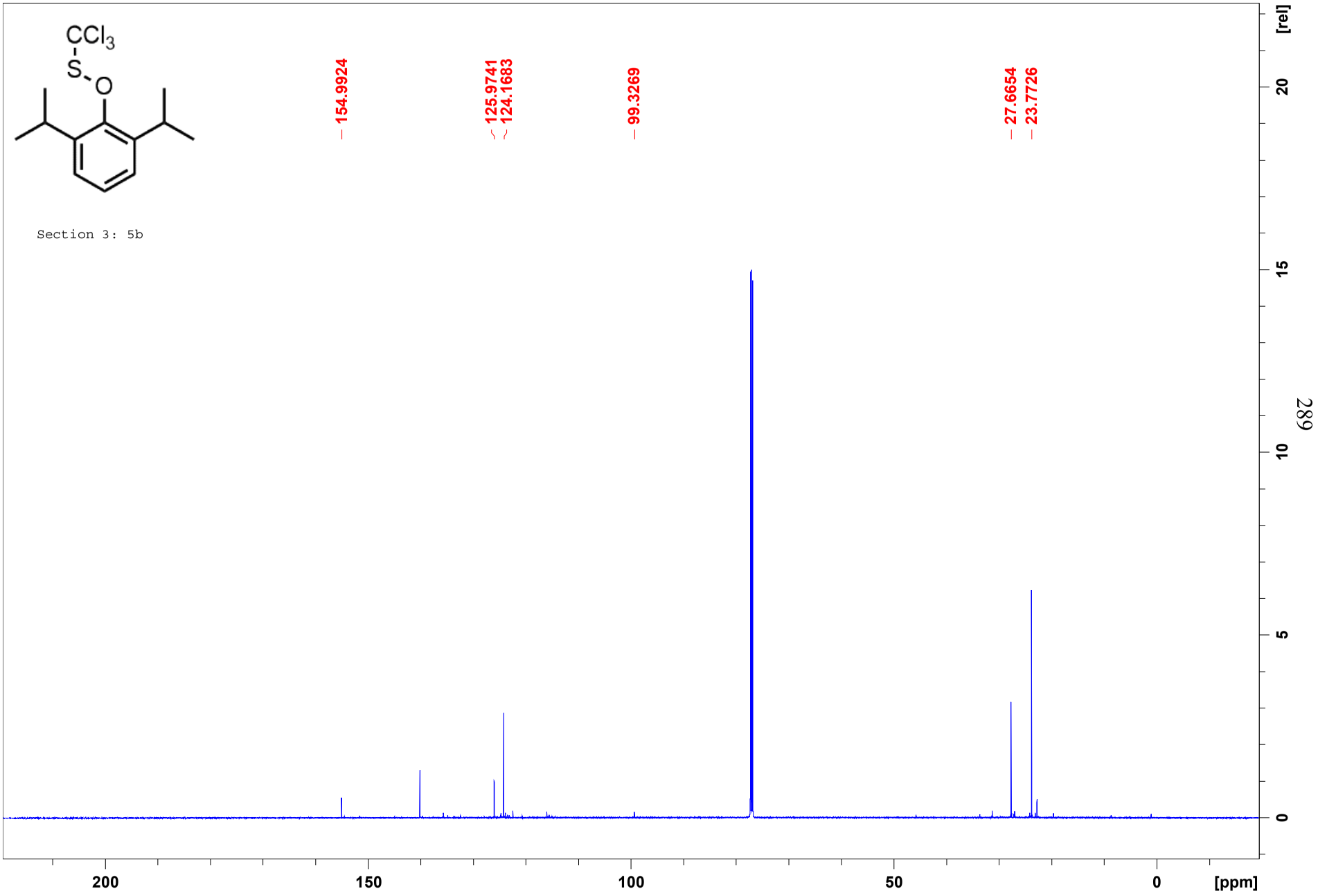

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S
CCl3
O
Section 3: 2b
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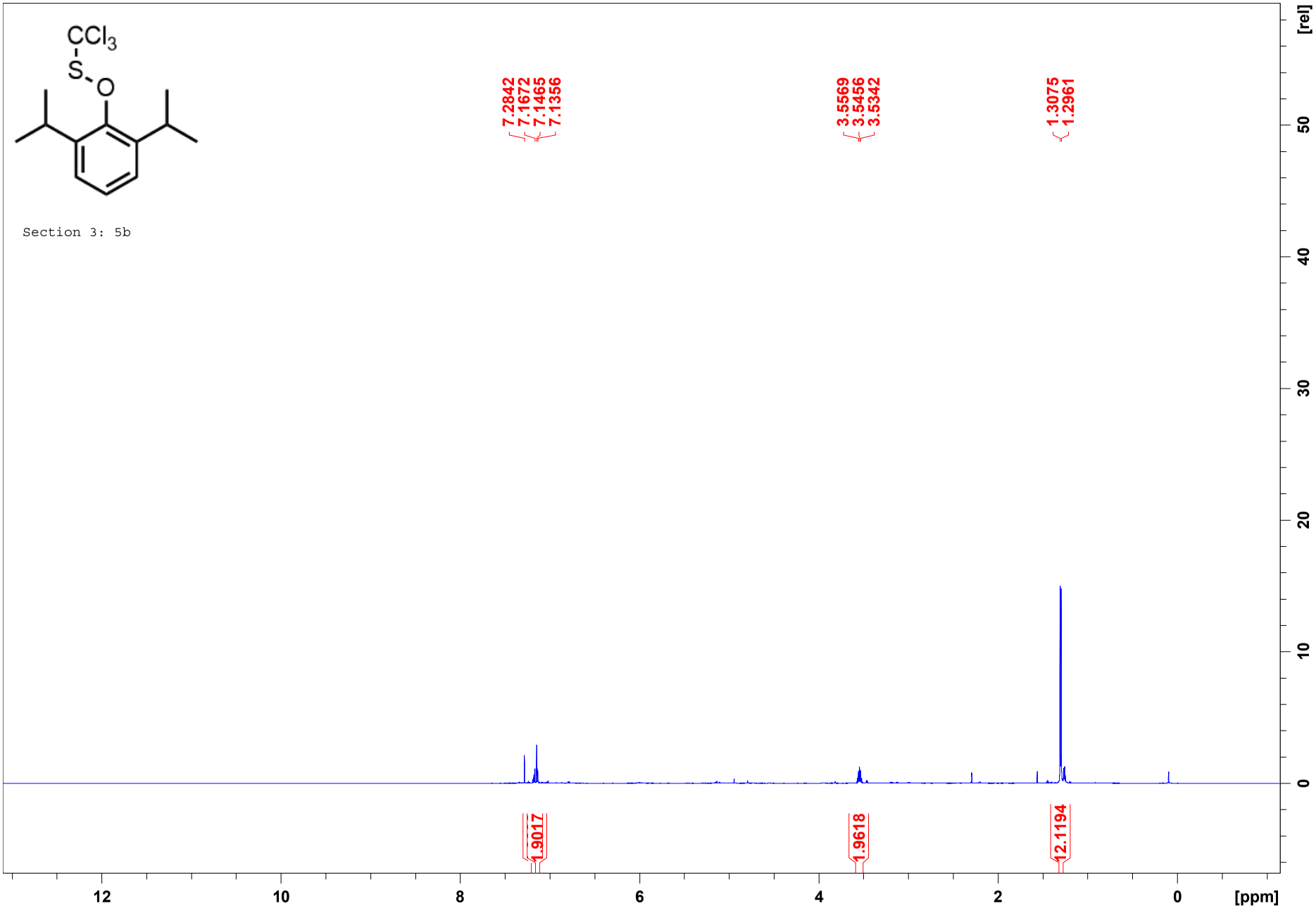

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Section 3 : 3b
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S
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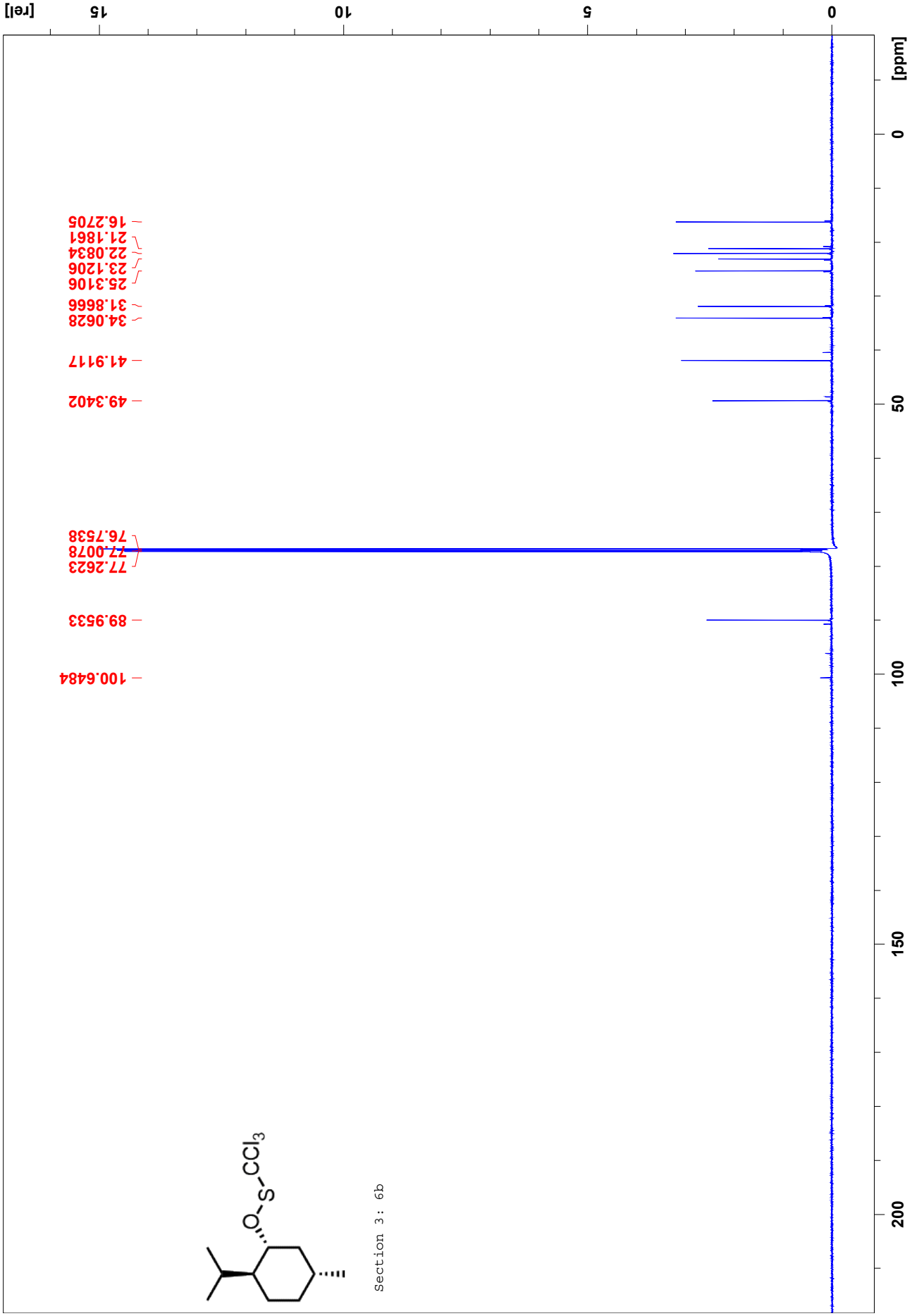

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S
CCl3
Section 3: 3b
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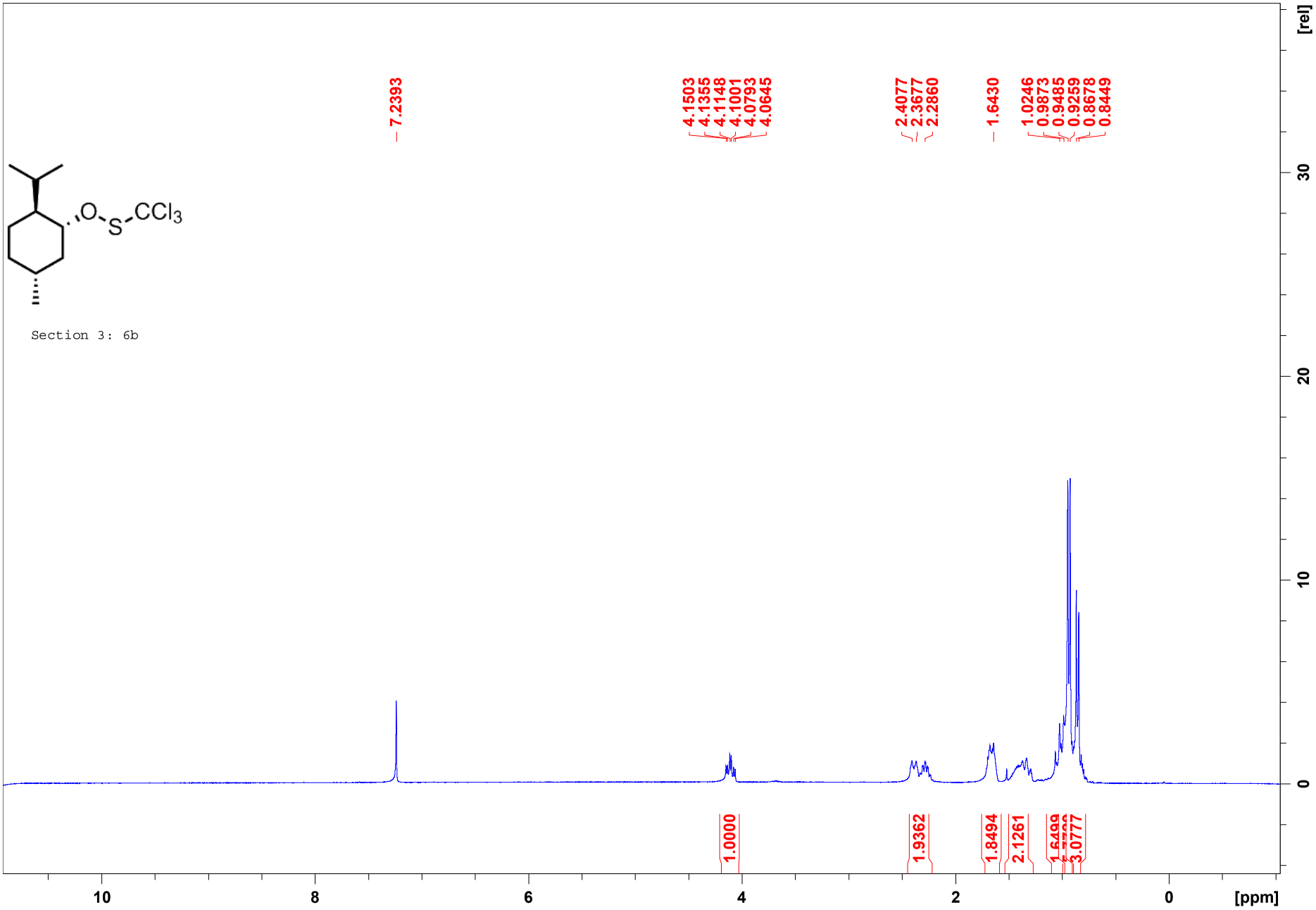

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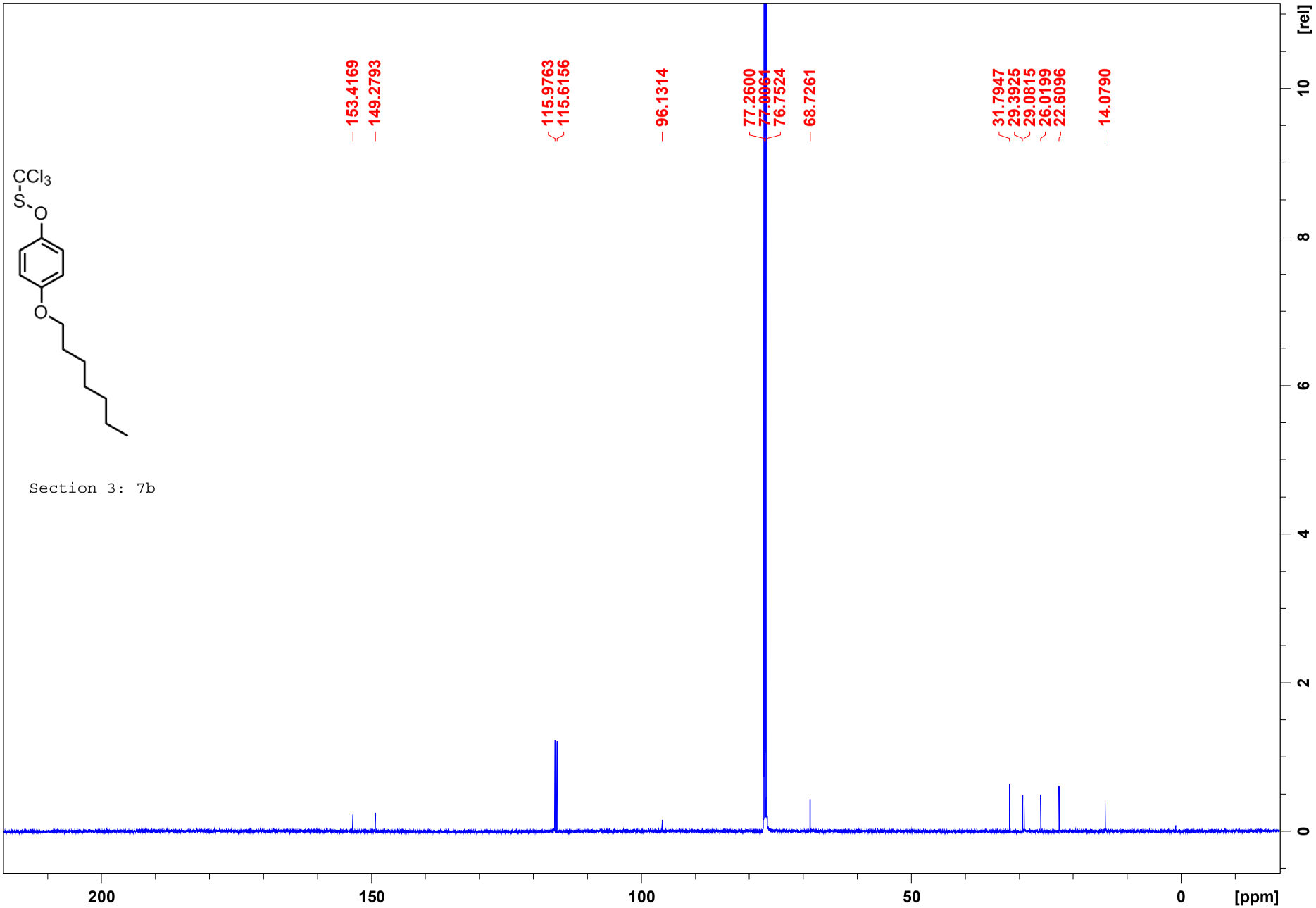

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S
CCl3
O
Section 3: 4b
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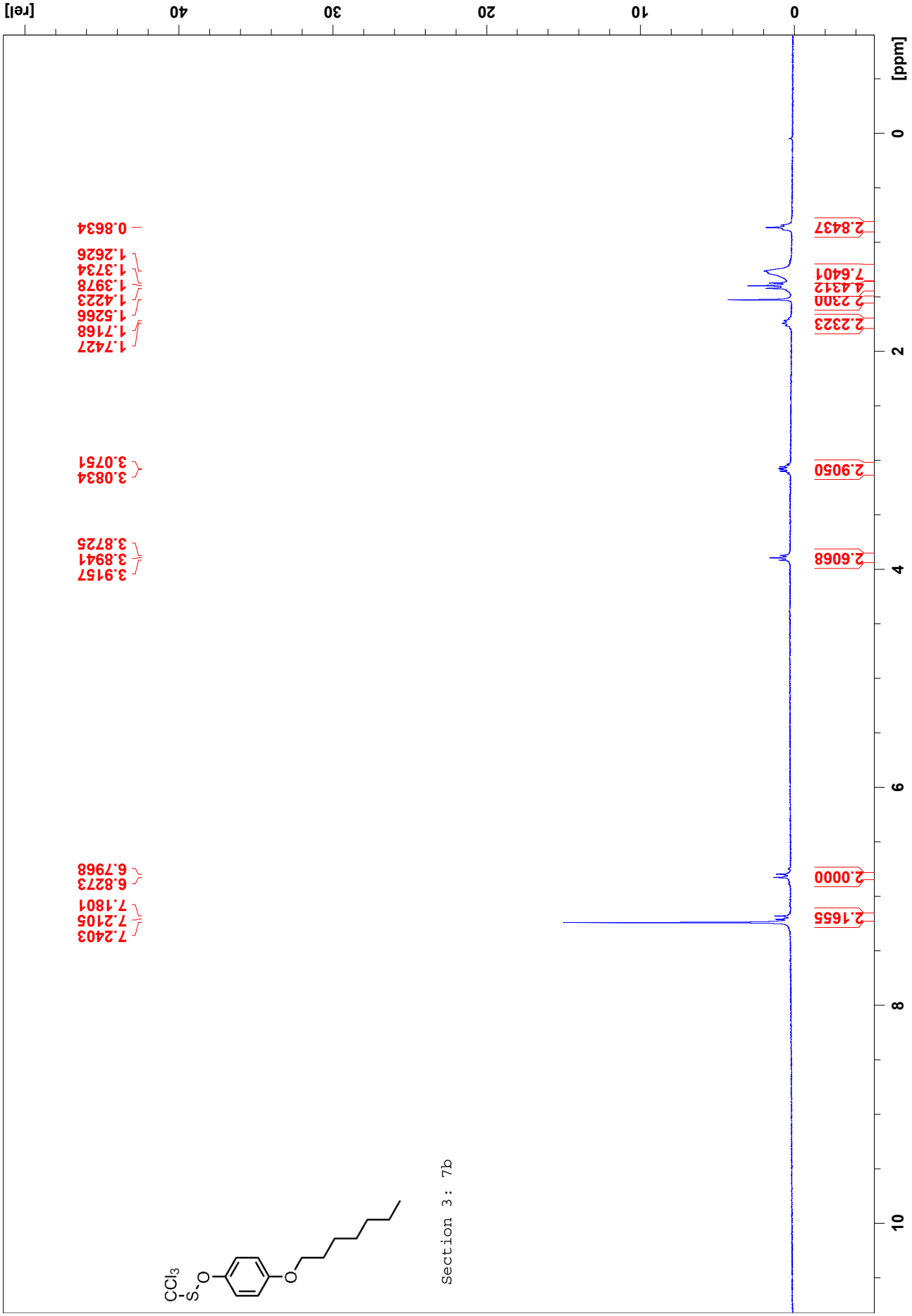

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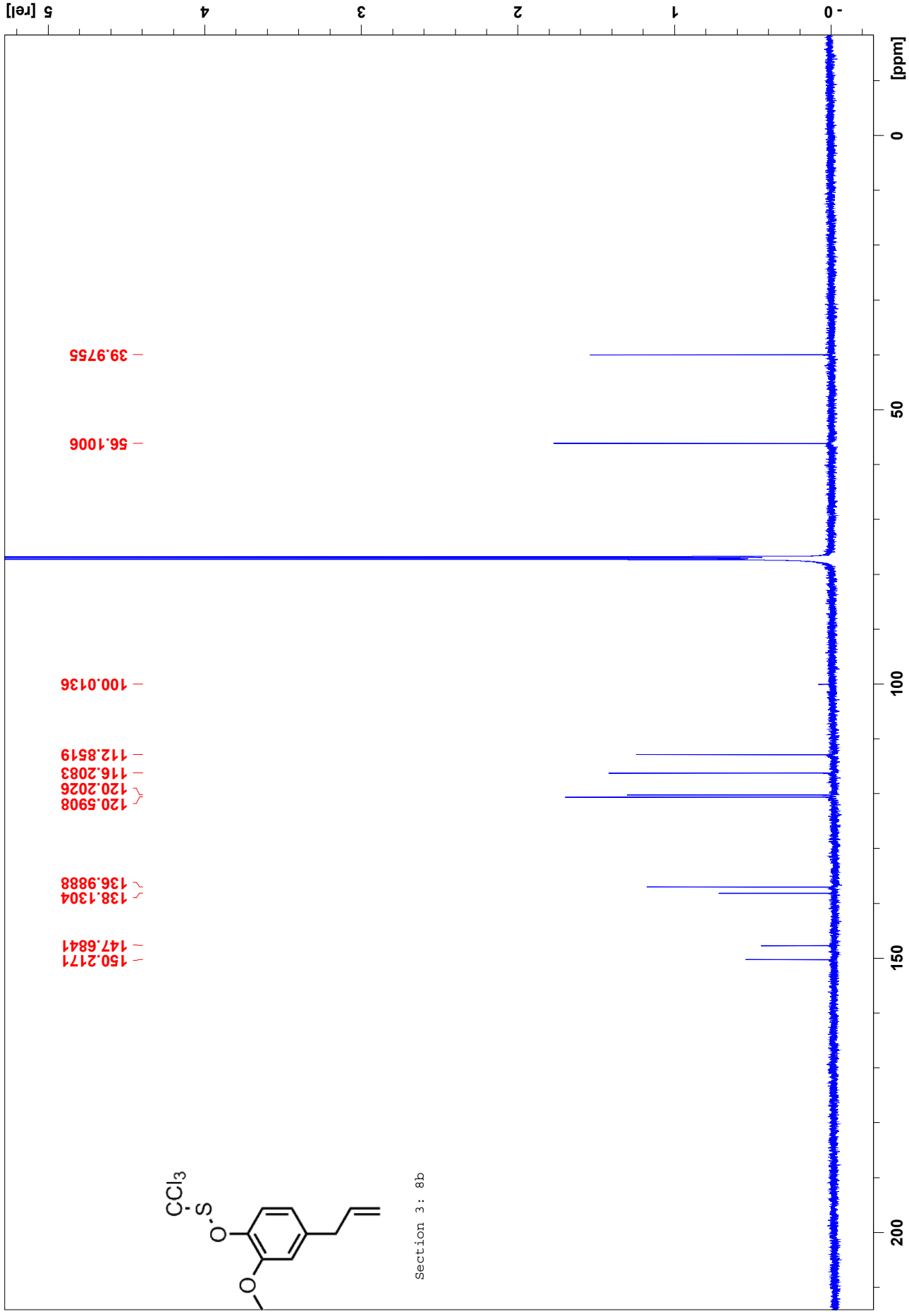

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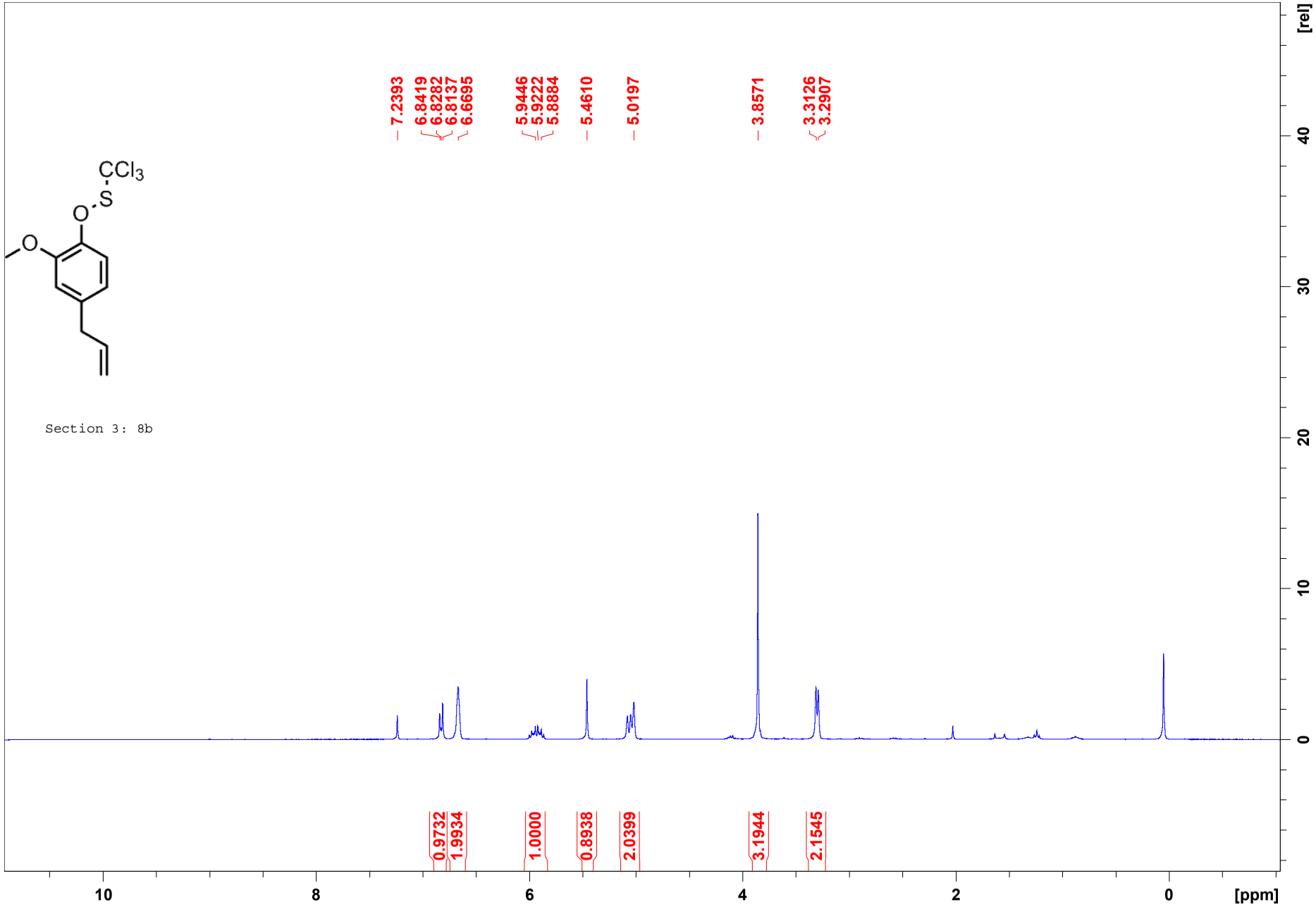

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Section 3 : 6b
O
S
CCl3

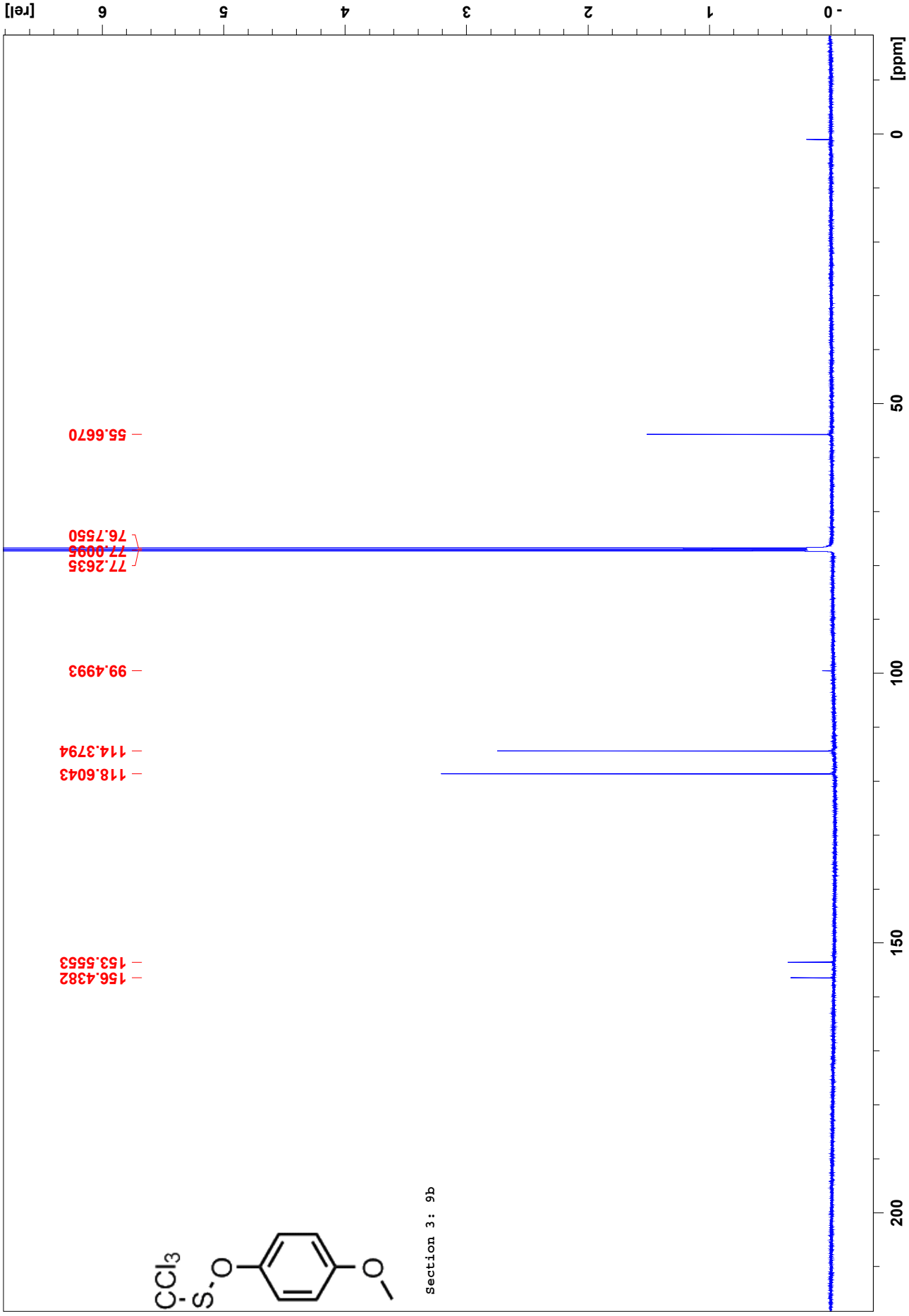

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0.9485
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0.8678
0.8449
O
S
CCl3
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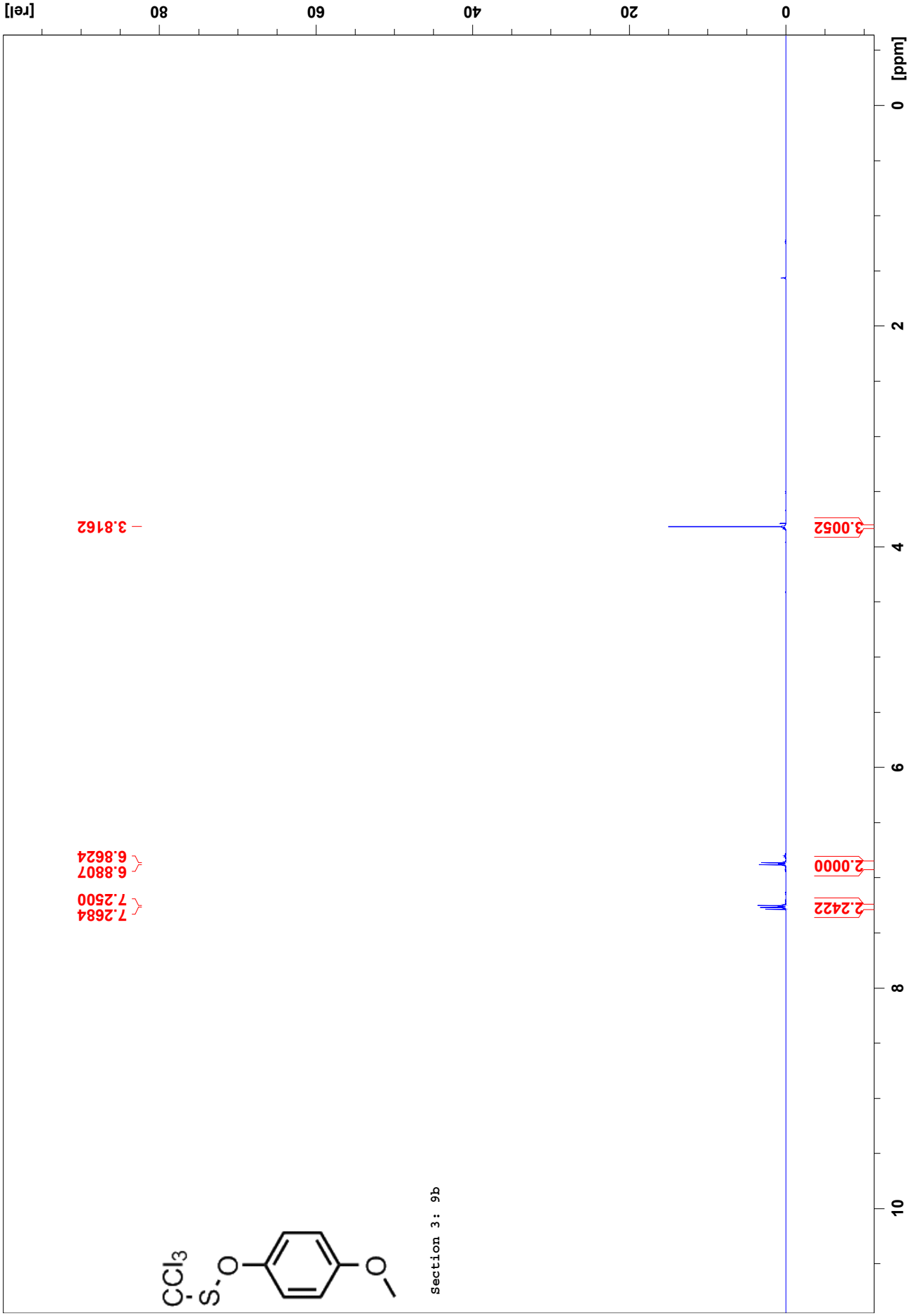

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29.0815
26.0199
22.6096
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CCl3
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O
Section 3: 7b
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6.8273
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7.2105
7.2403
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Section 3: 7b
CCl3
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O
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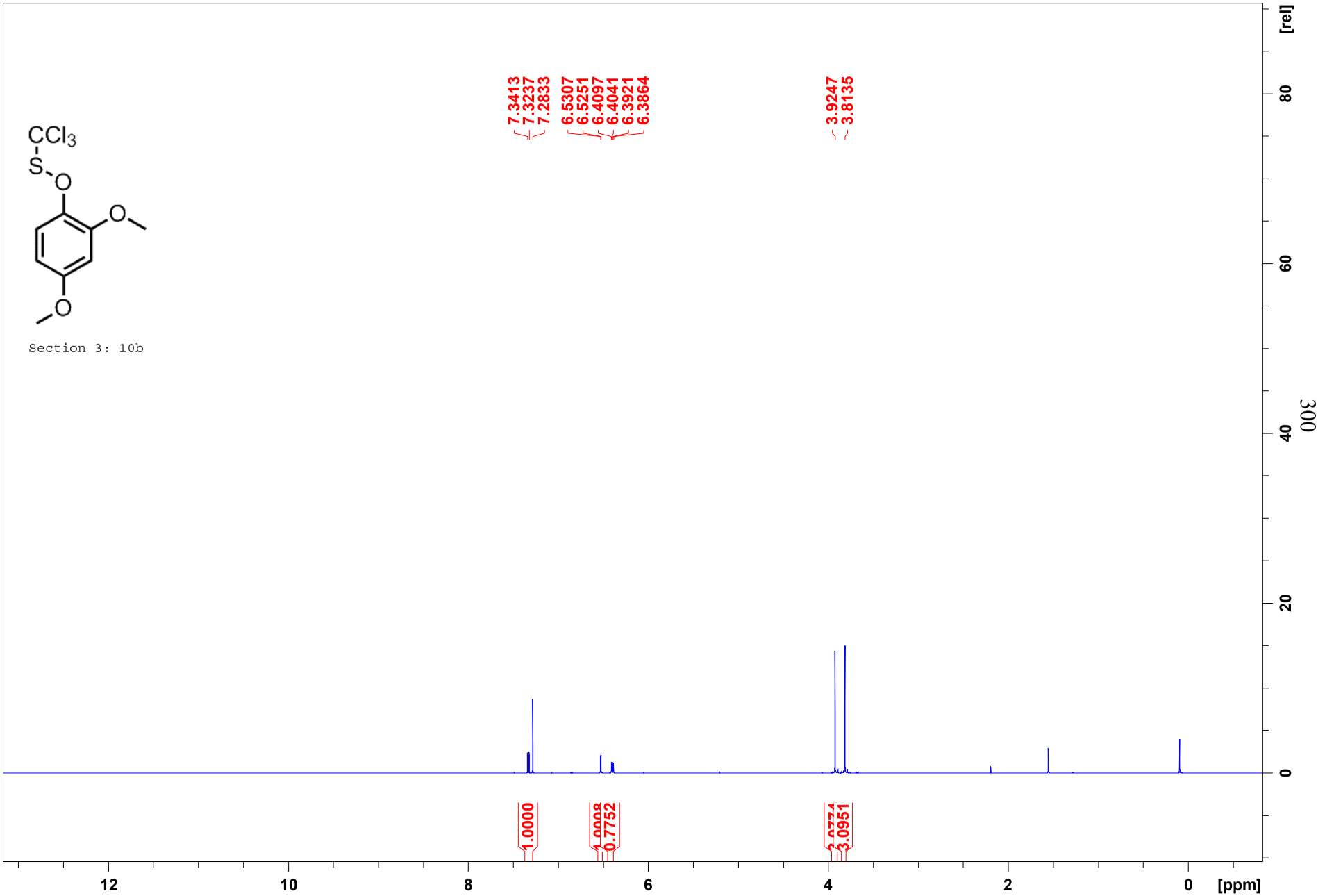

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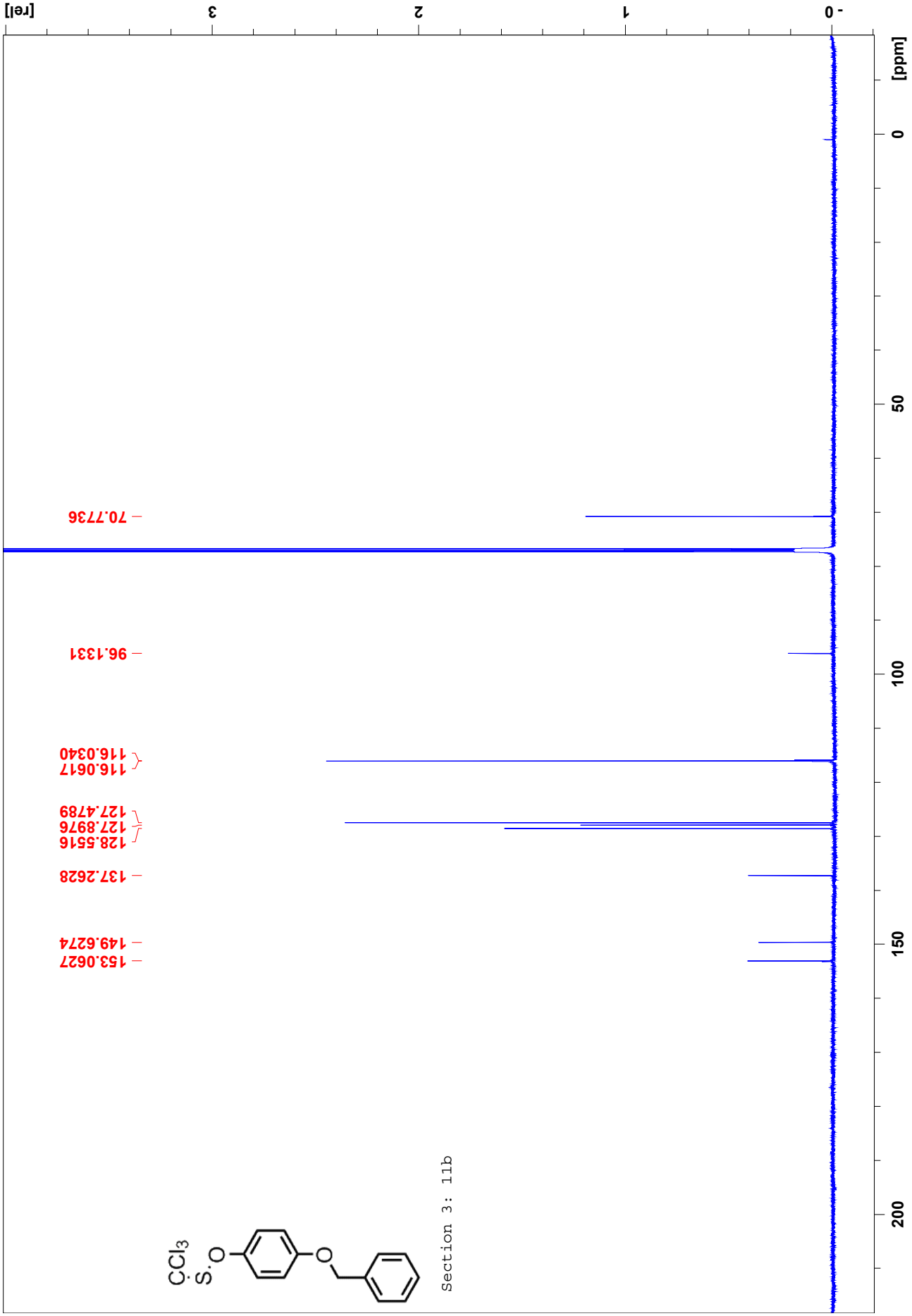

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CCl3
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Section 3: 8b
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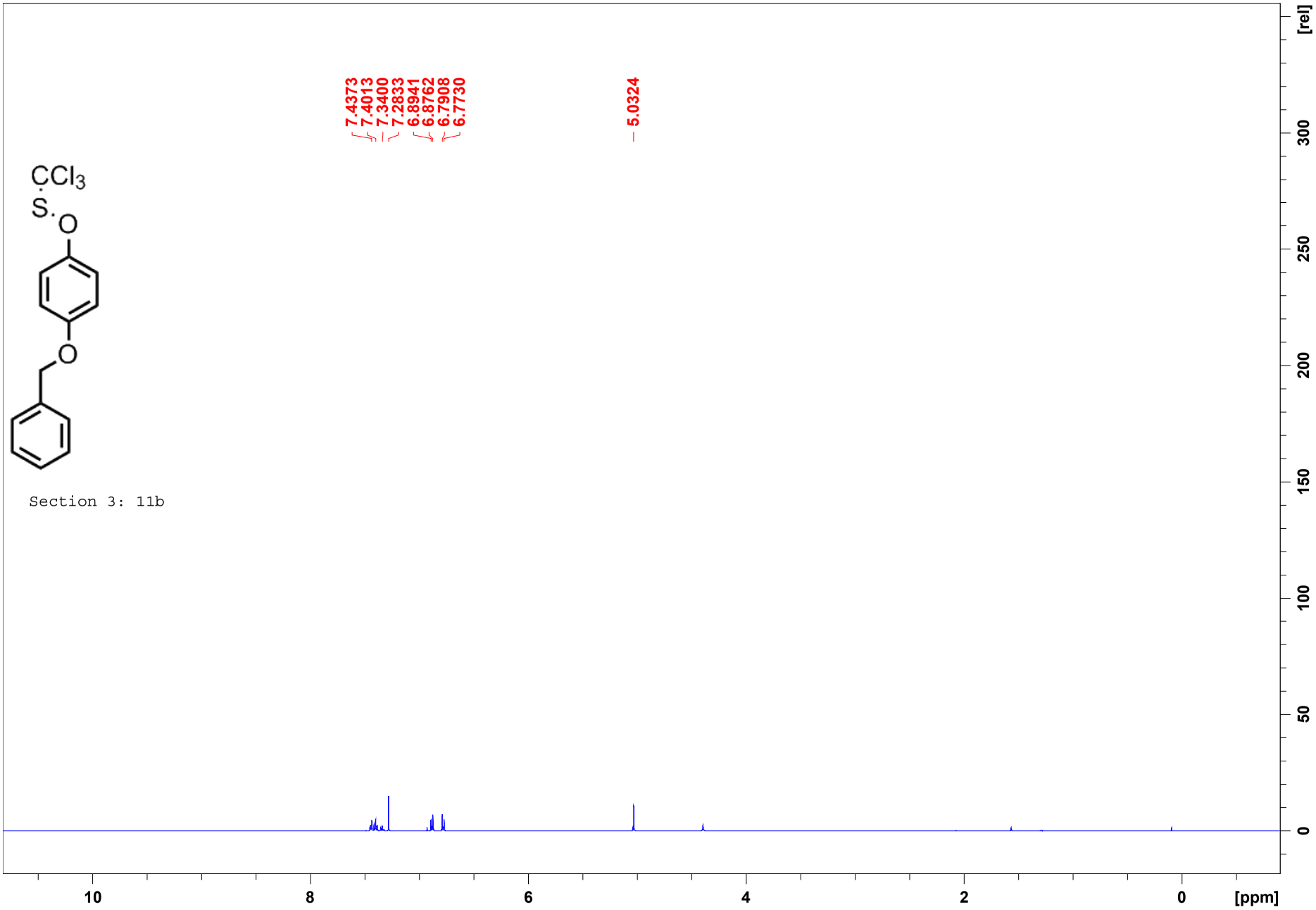

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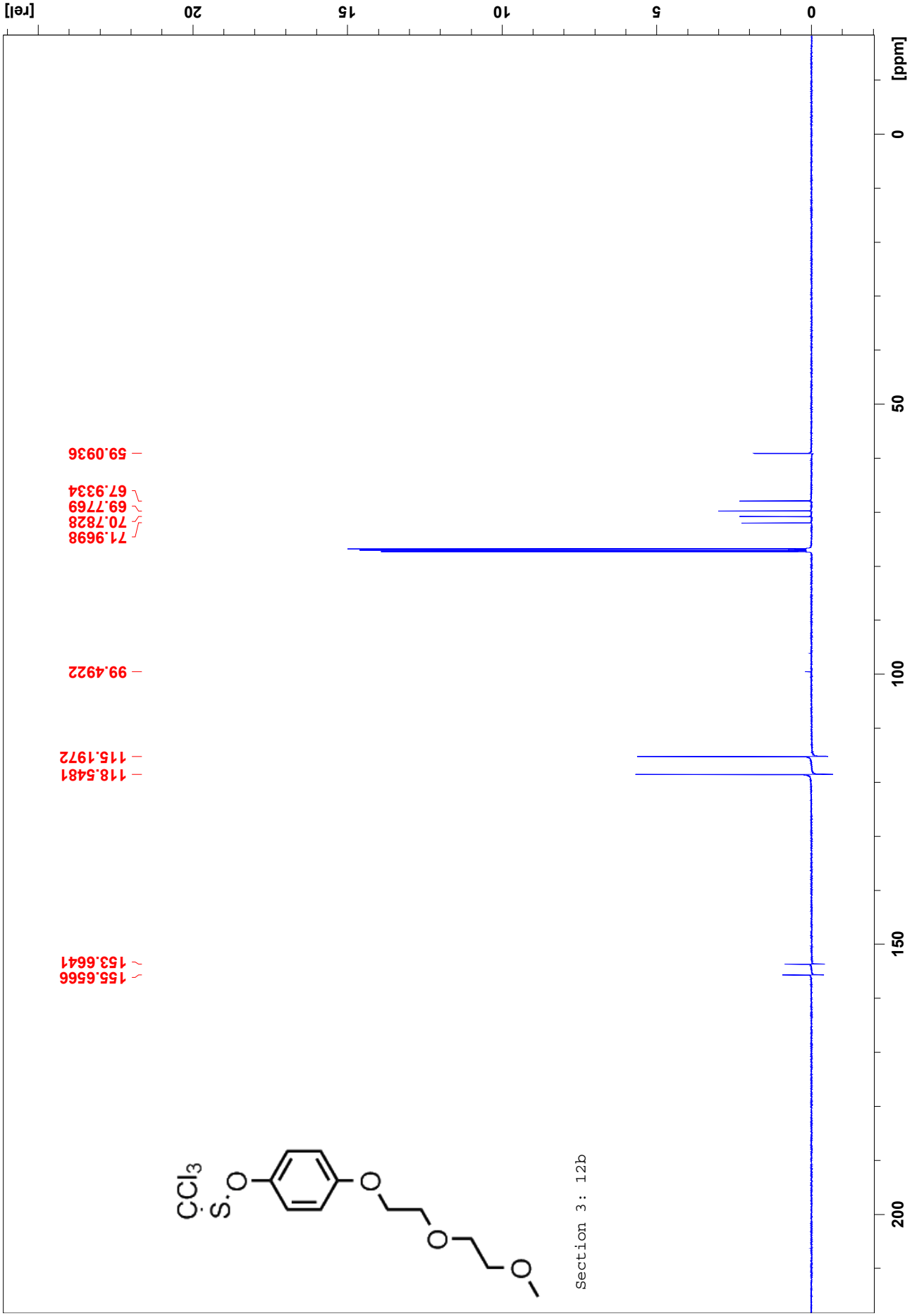

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Section 3: 9b

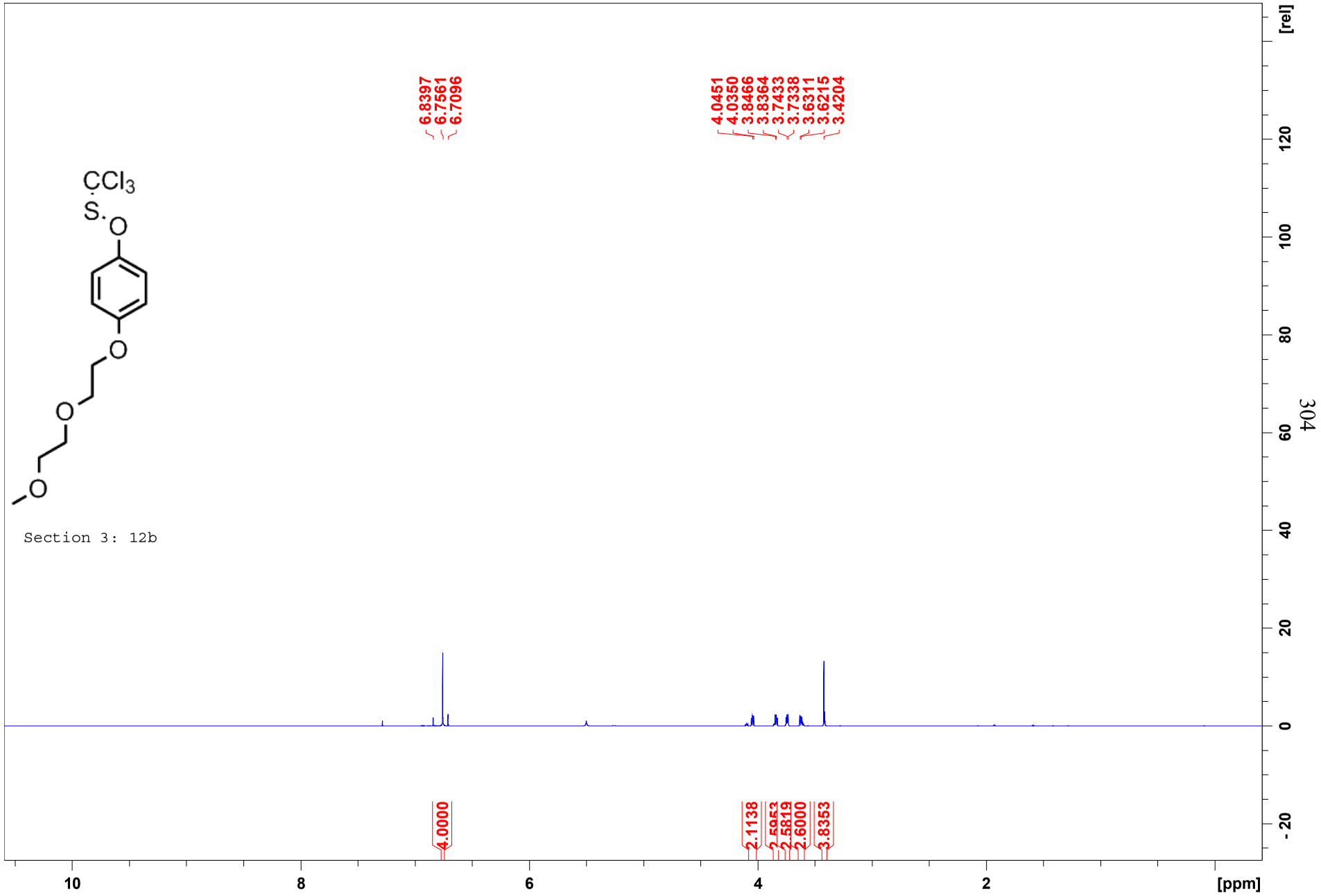
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O
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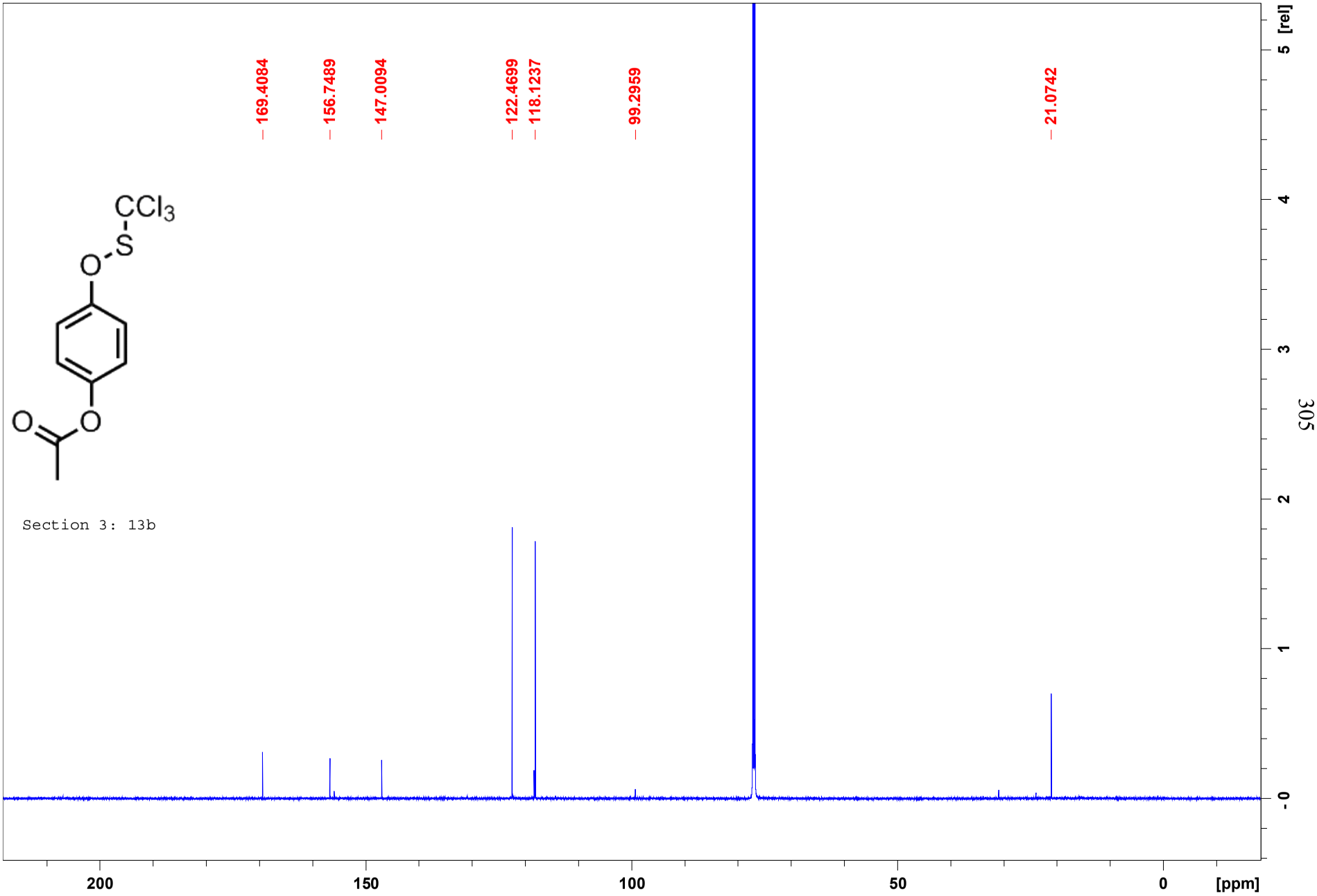

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6.4041
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O
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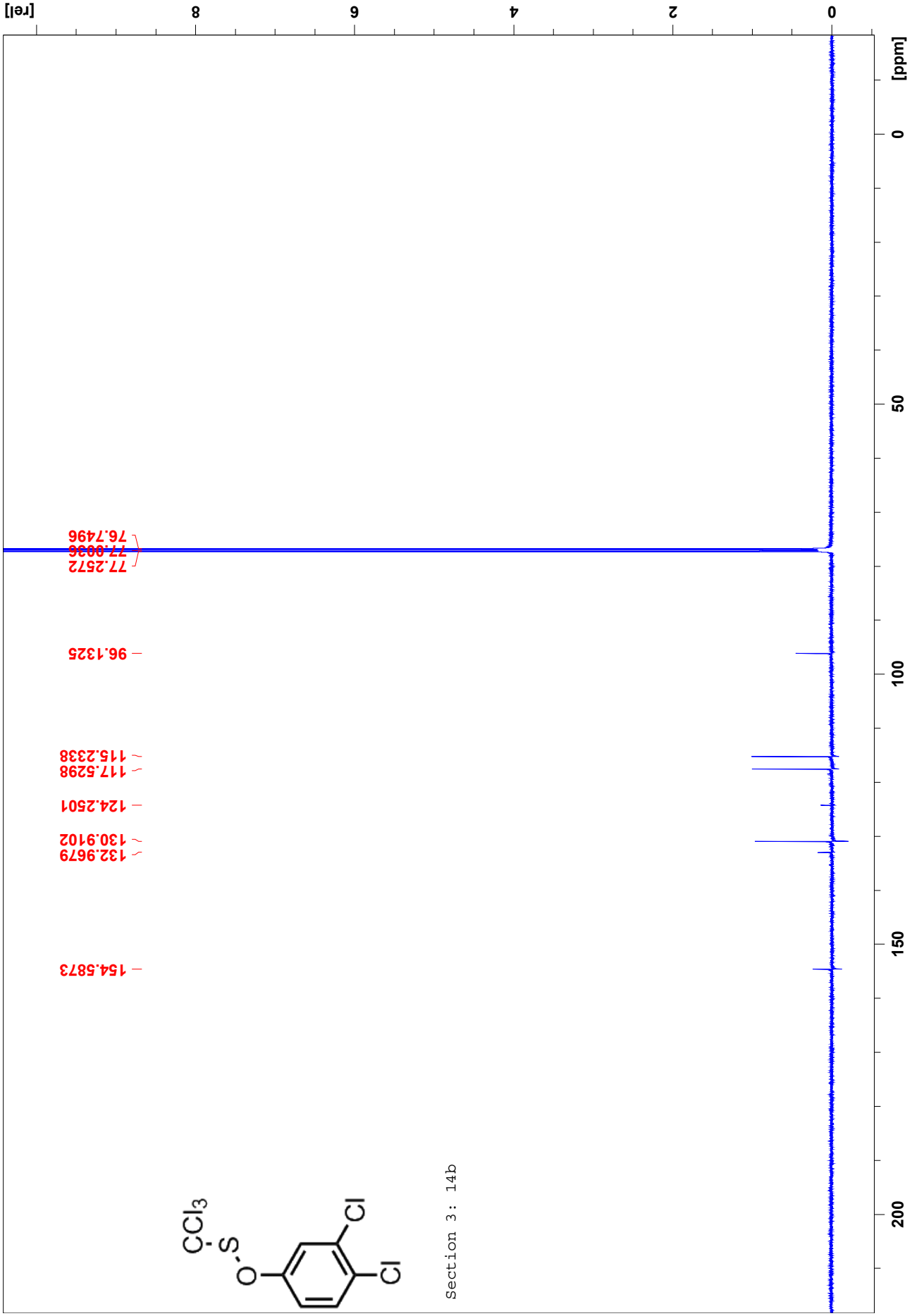

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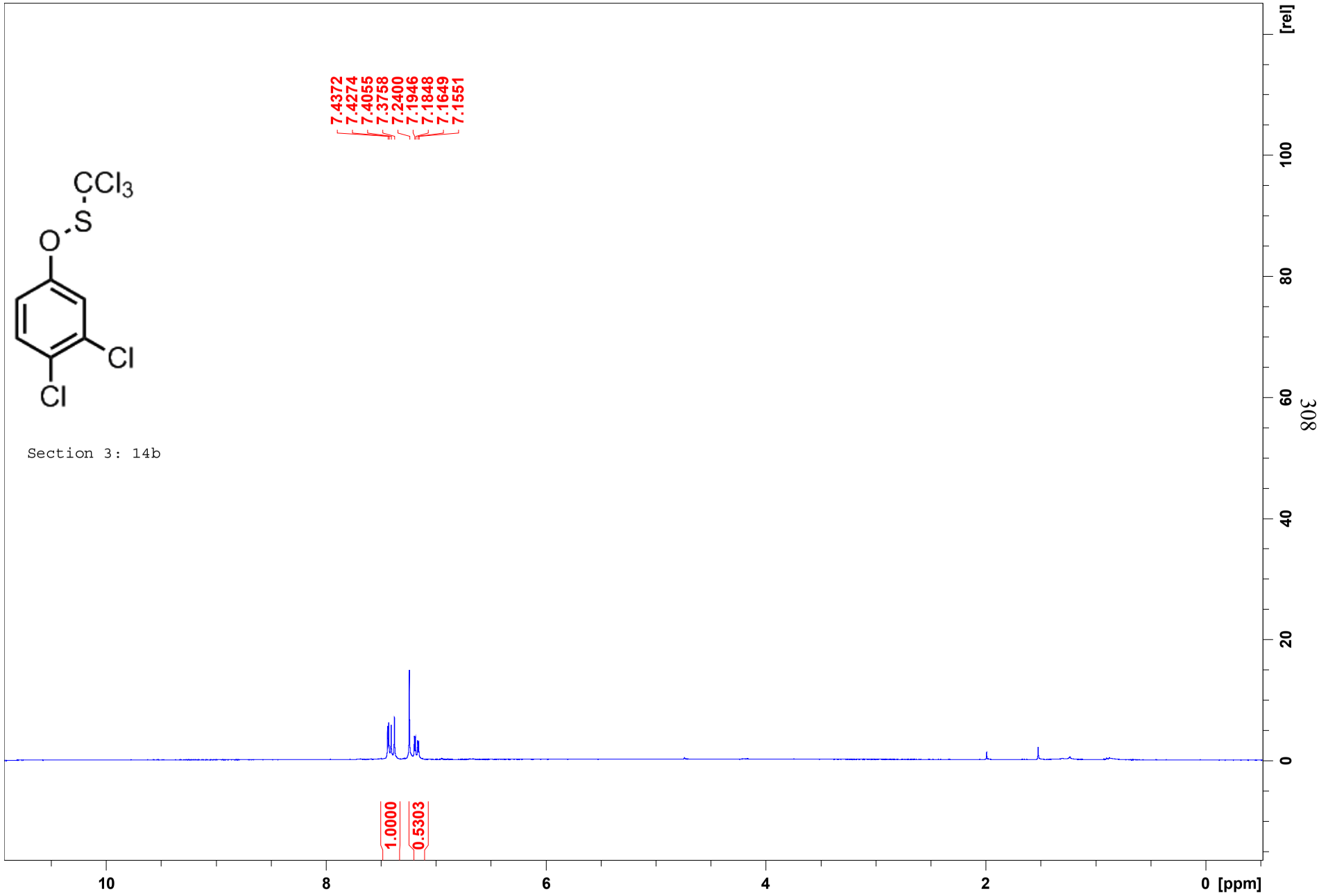

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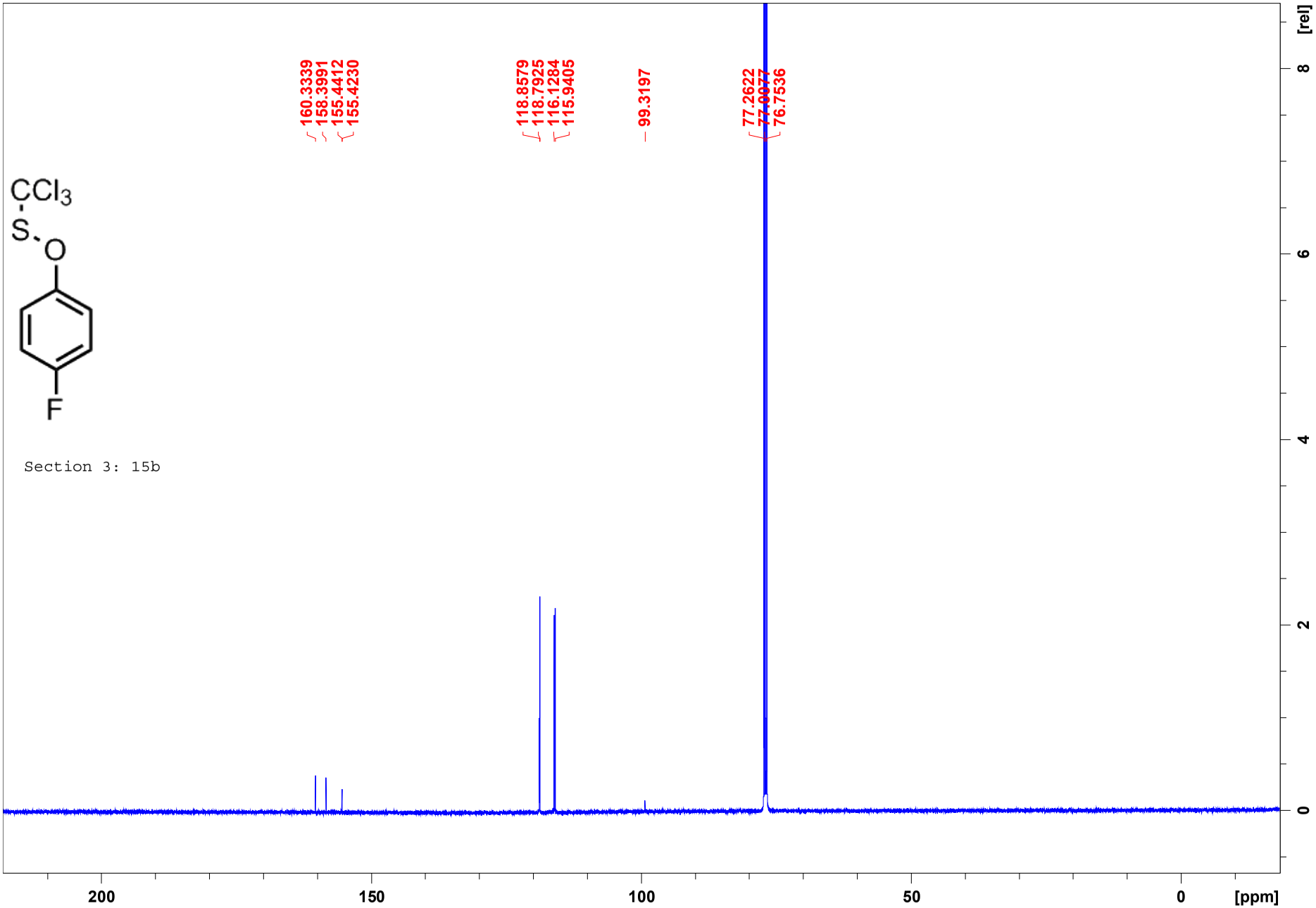

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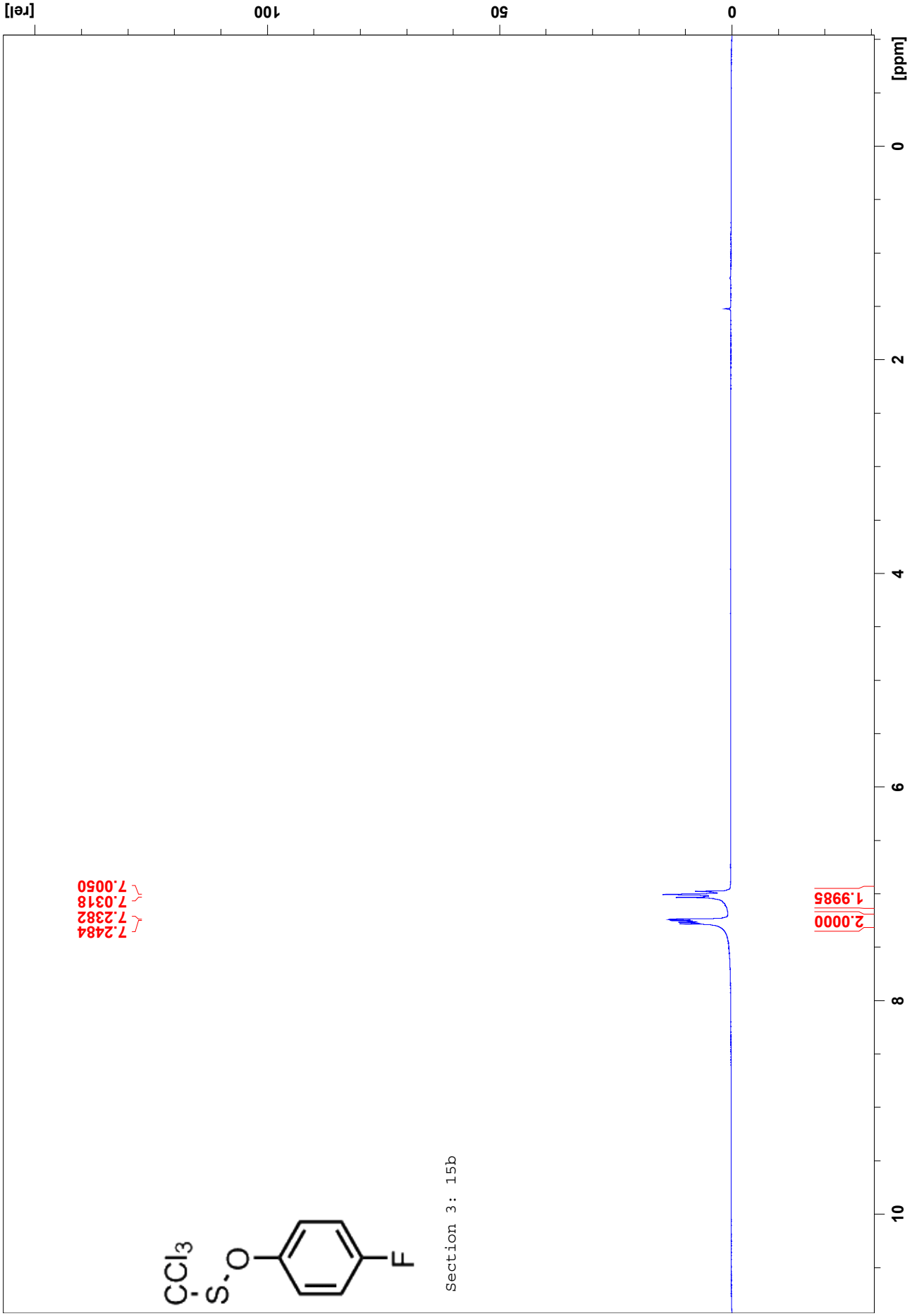

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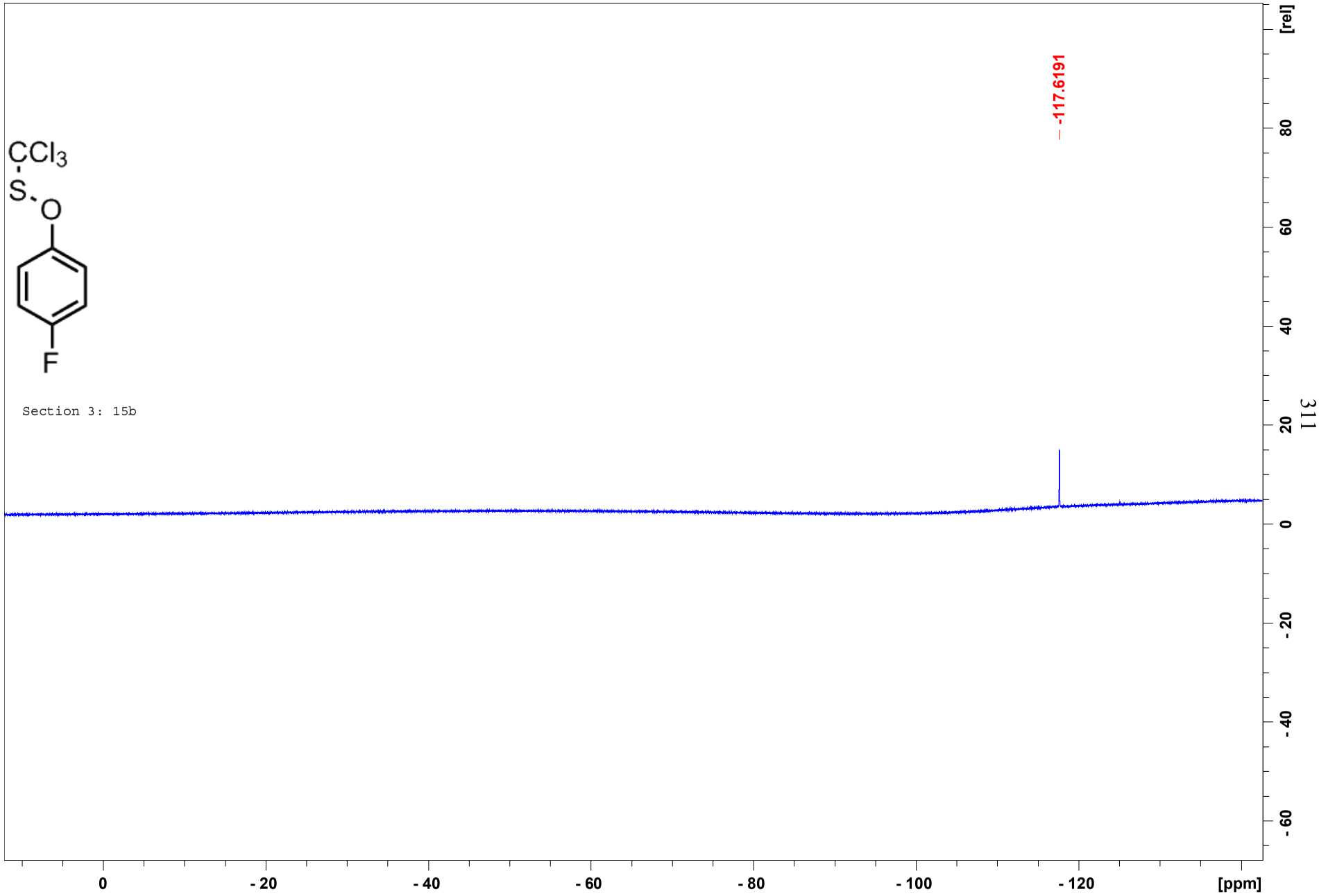
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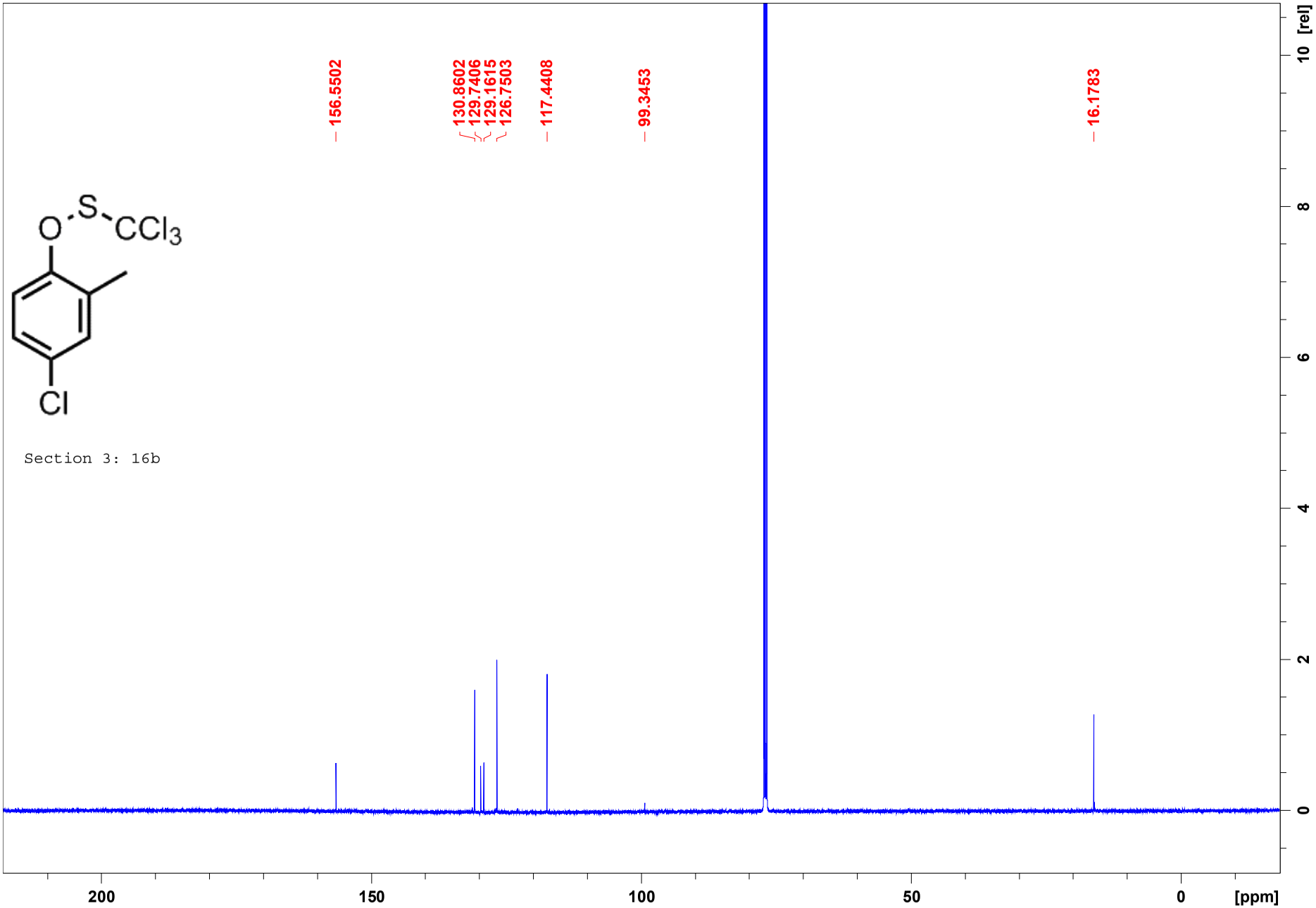

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Cl

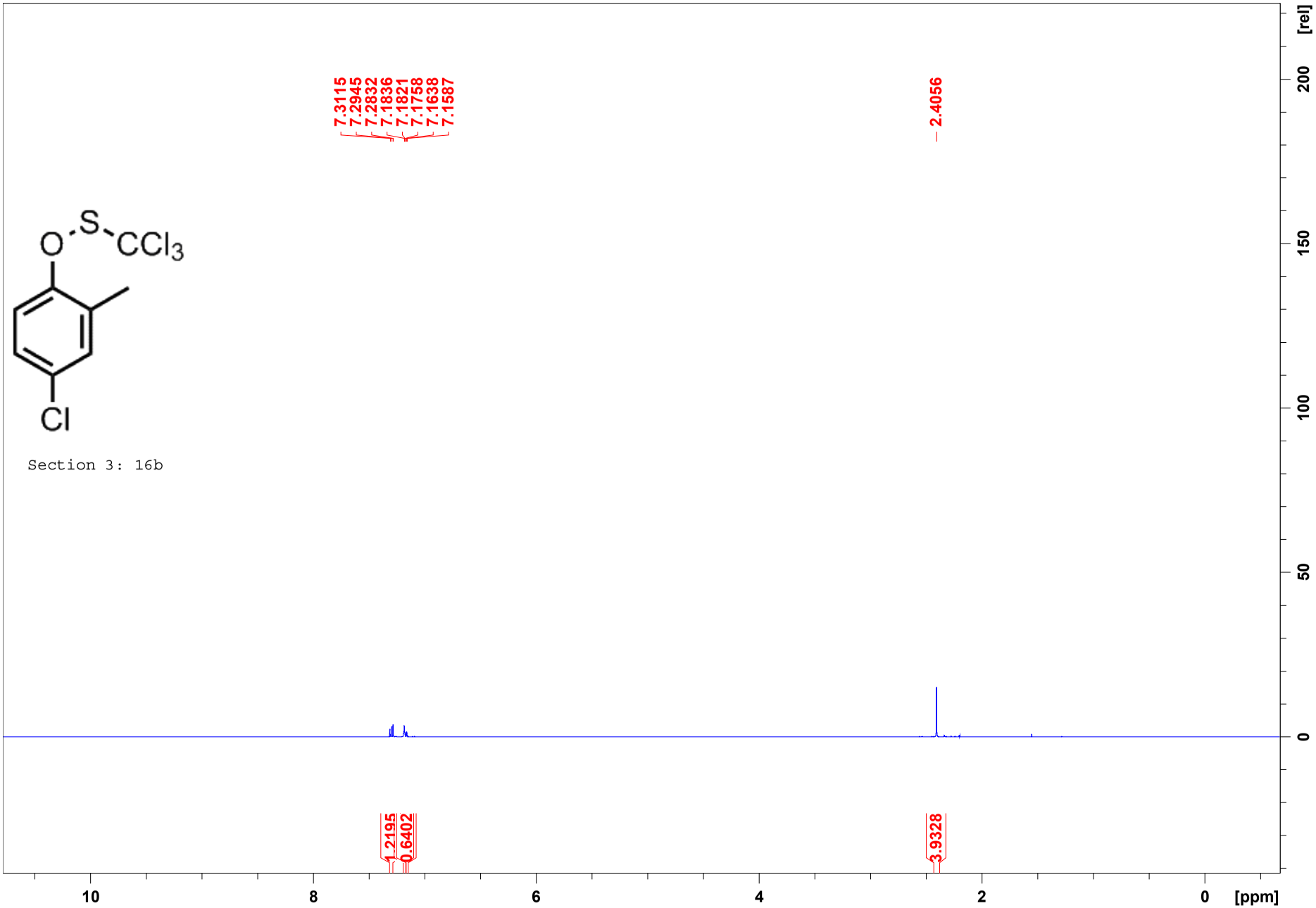

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O
Cl
Cl
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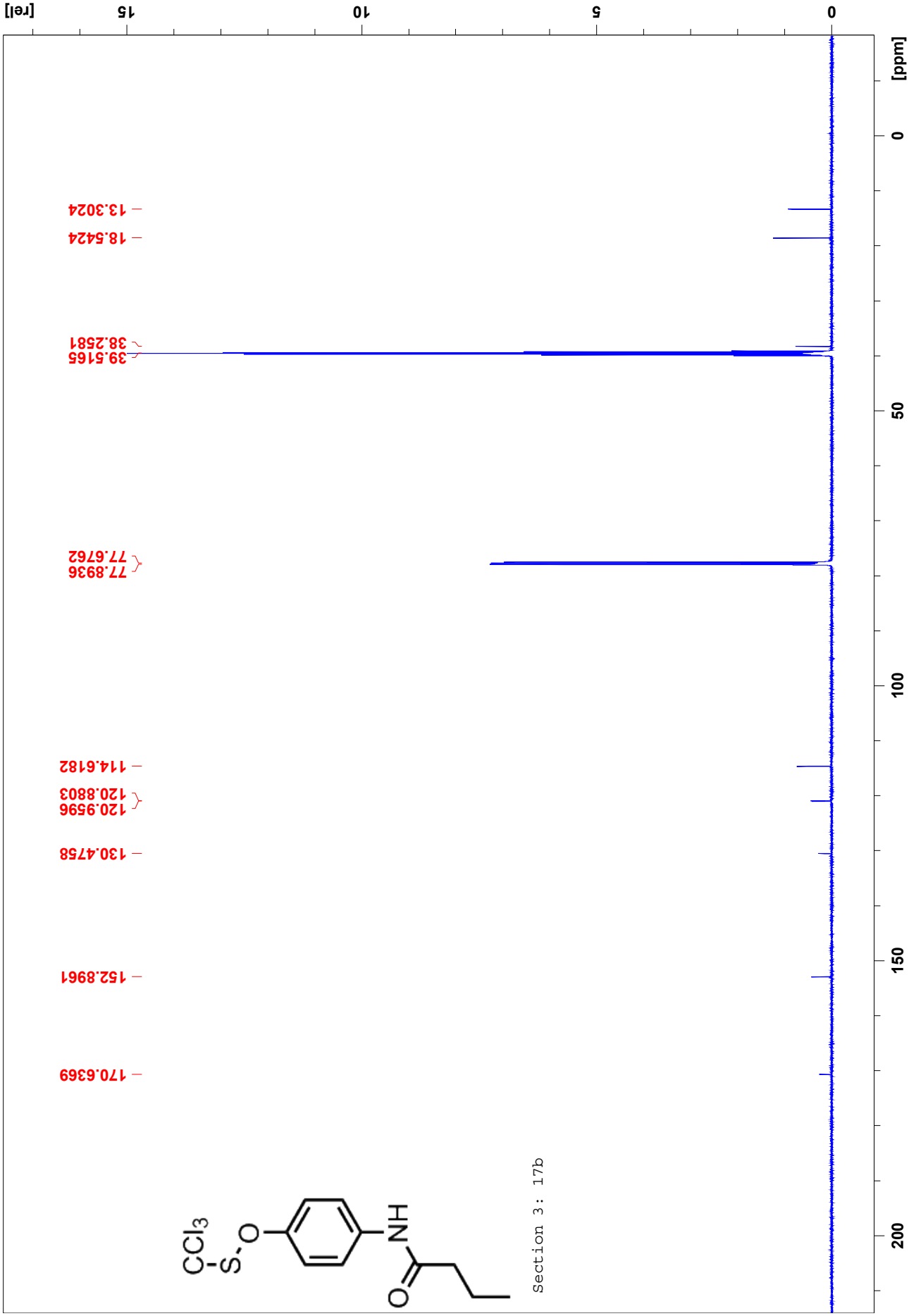

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F
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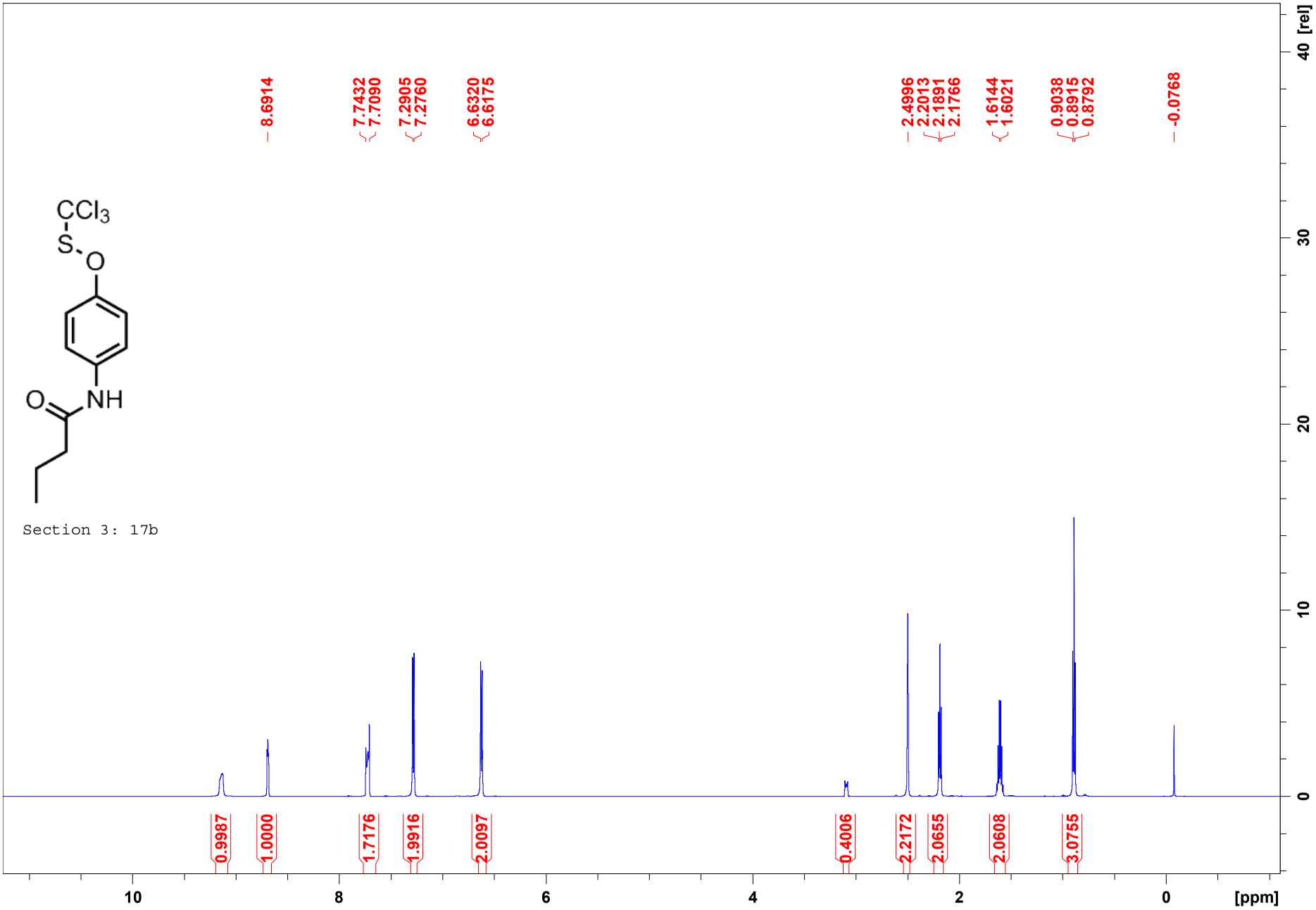

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O
F

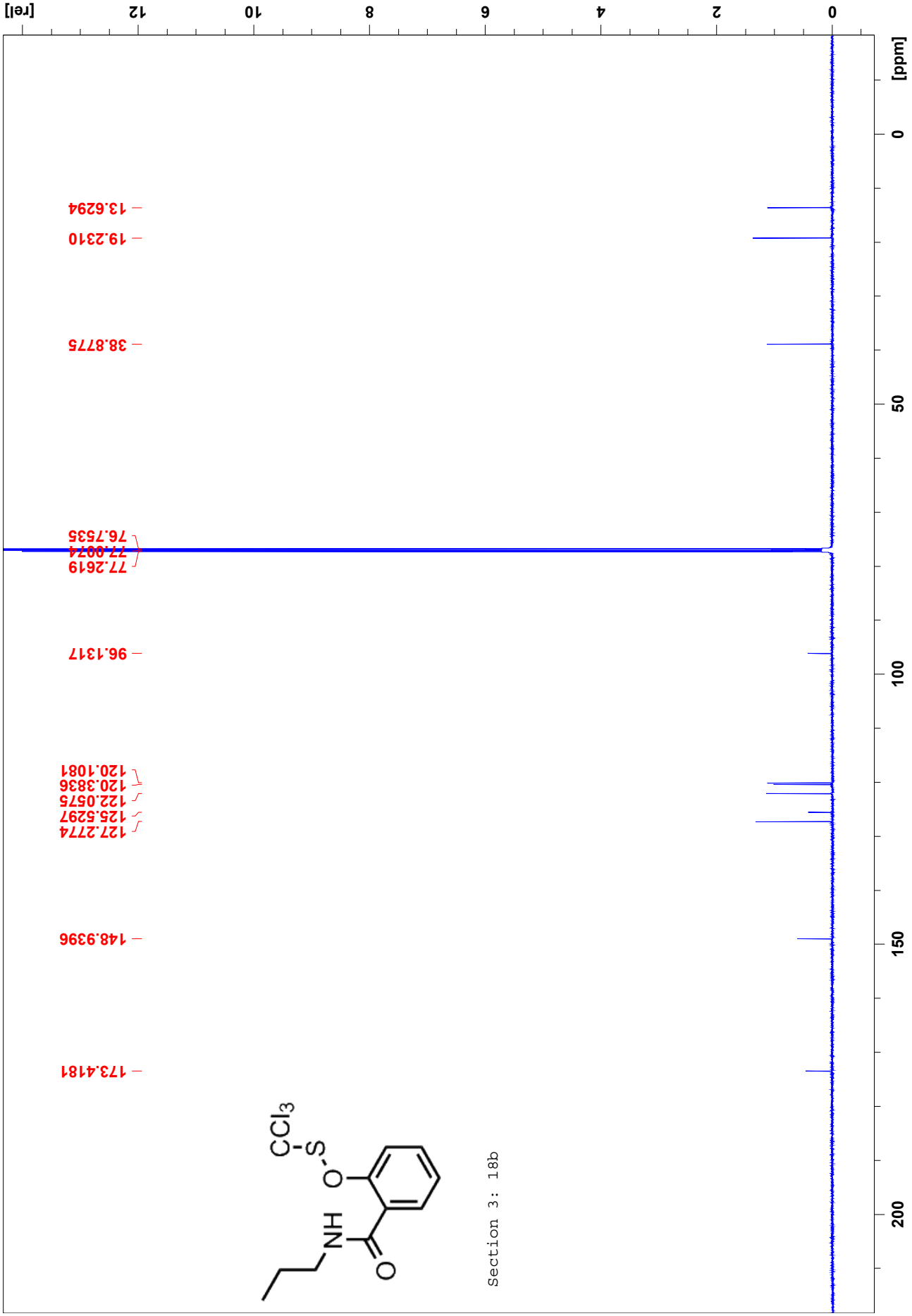

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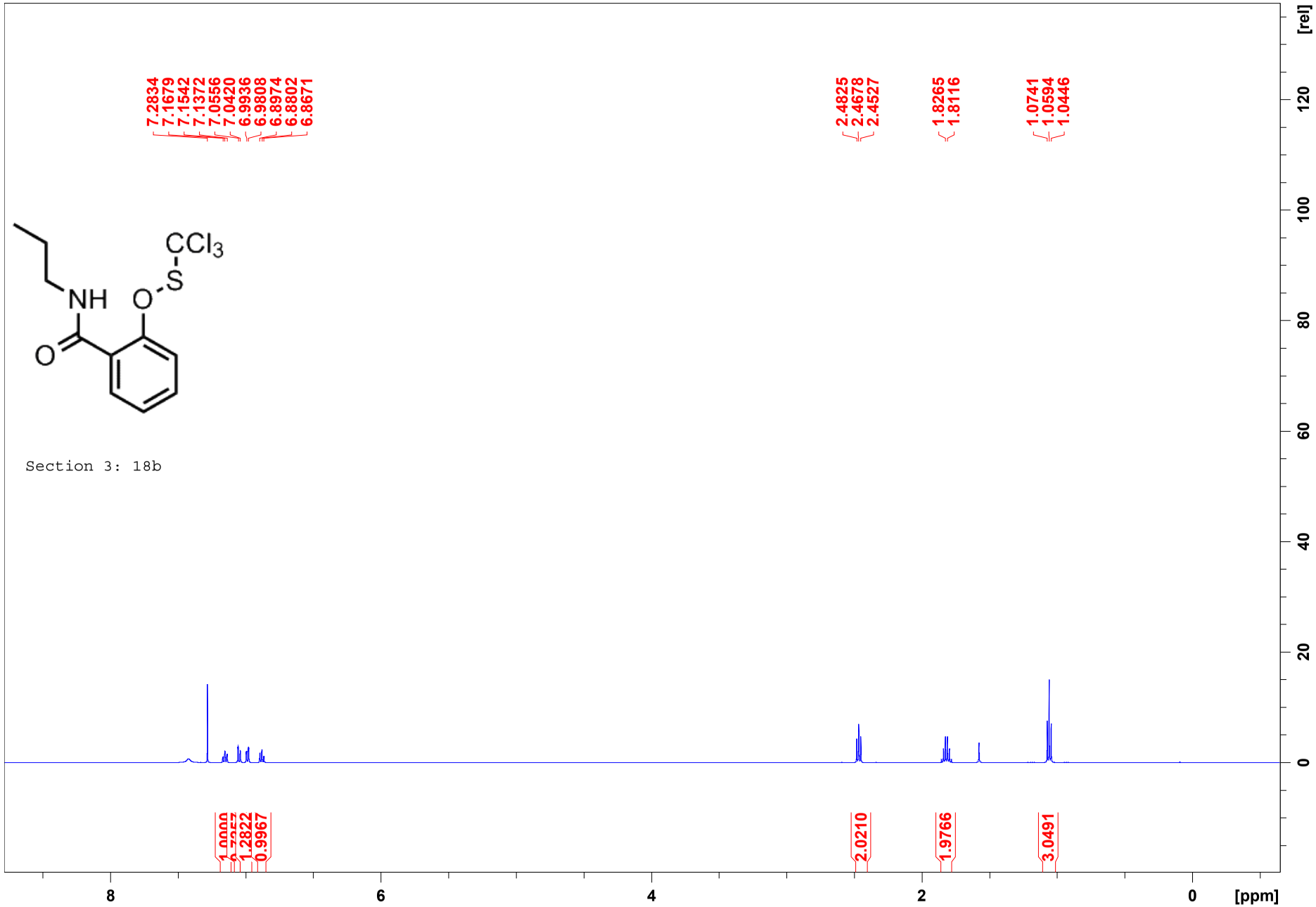

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16.1783
O
S
CCl3
Cl
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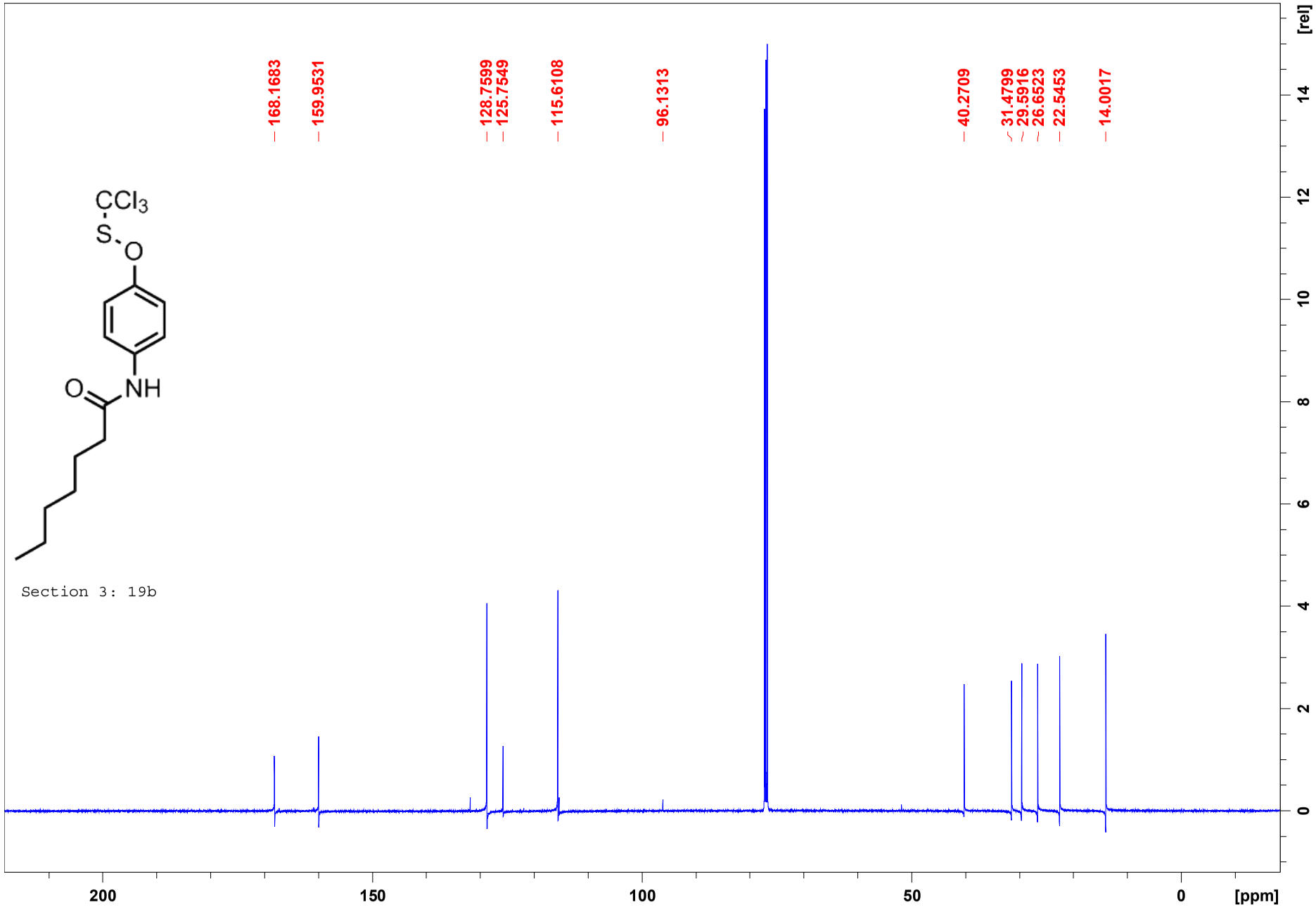

7.3115
7.2945
7.2832
7.1836
7.1821
7.1758
7.1638
7.1587
2.4056
O
S
CCl3
Cl
Section 3: 16b
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3.9328
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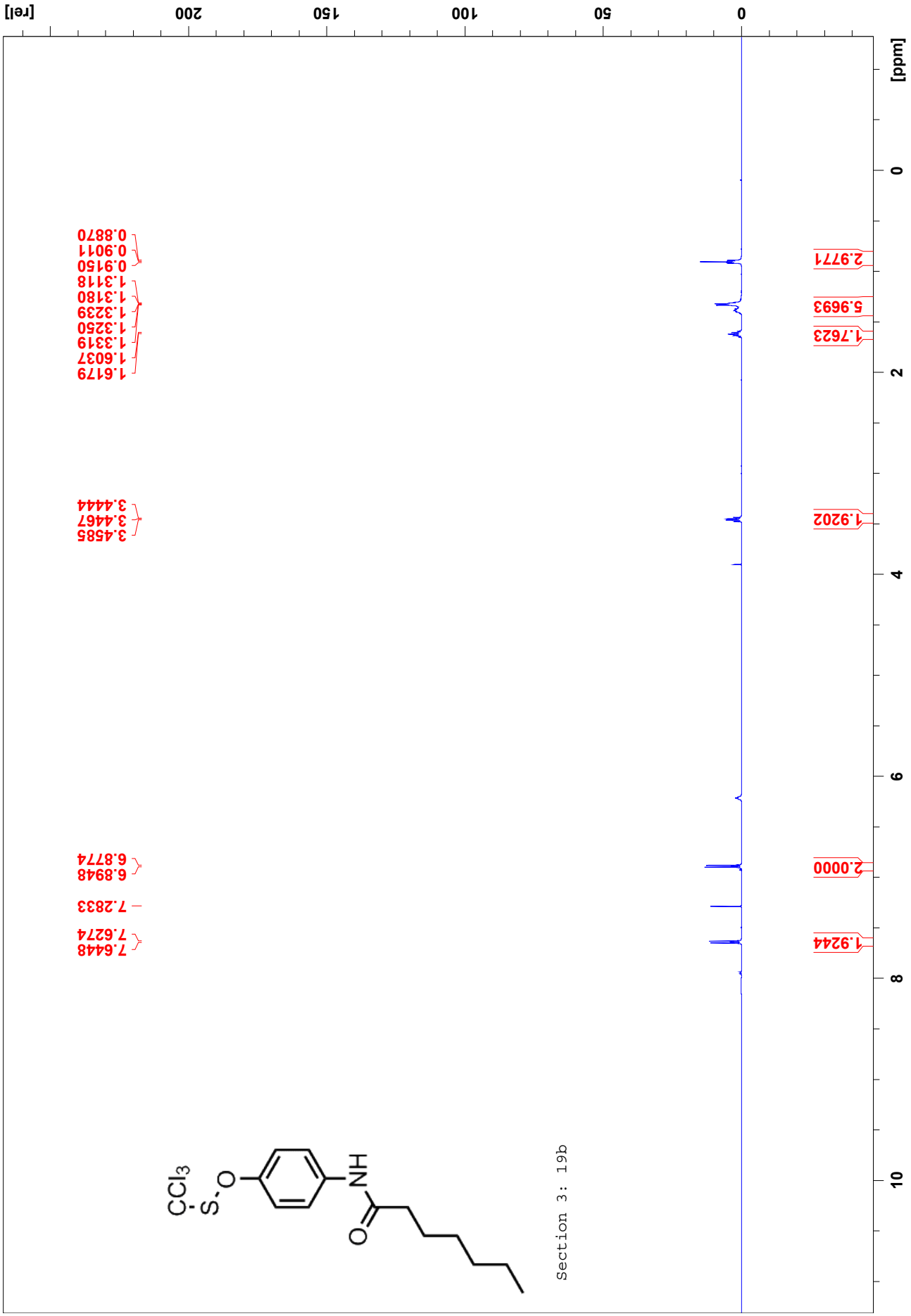

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114.6182
120.9596
120.8803
130.4758
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170.6369
Section 3 : 17b

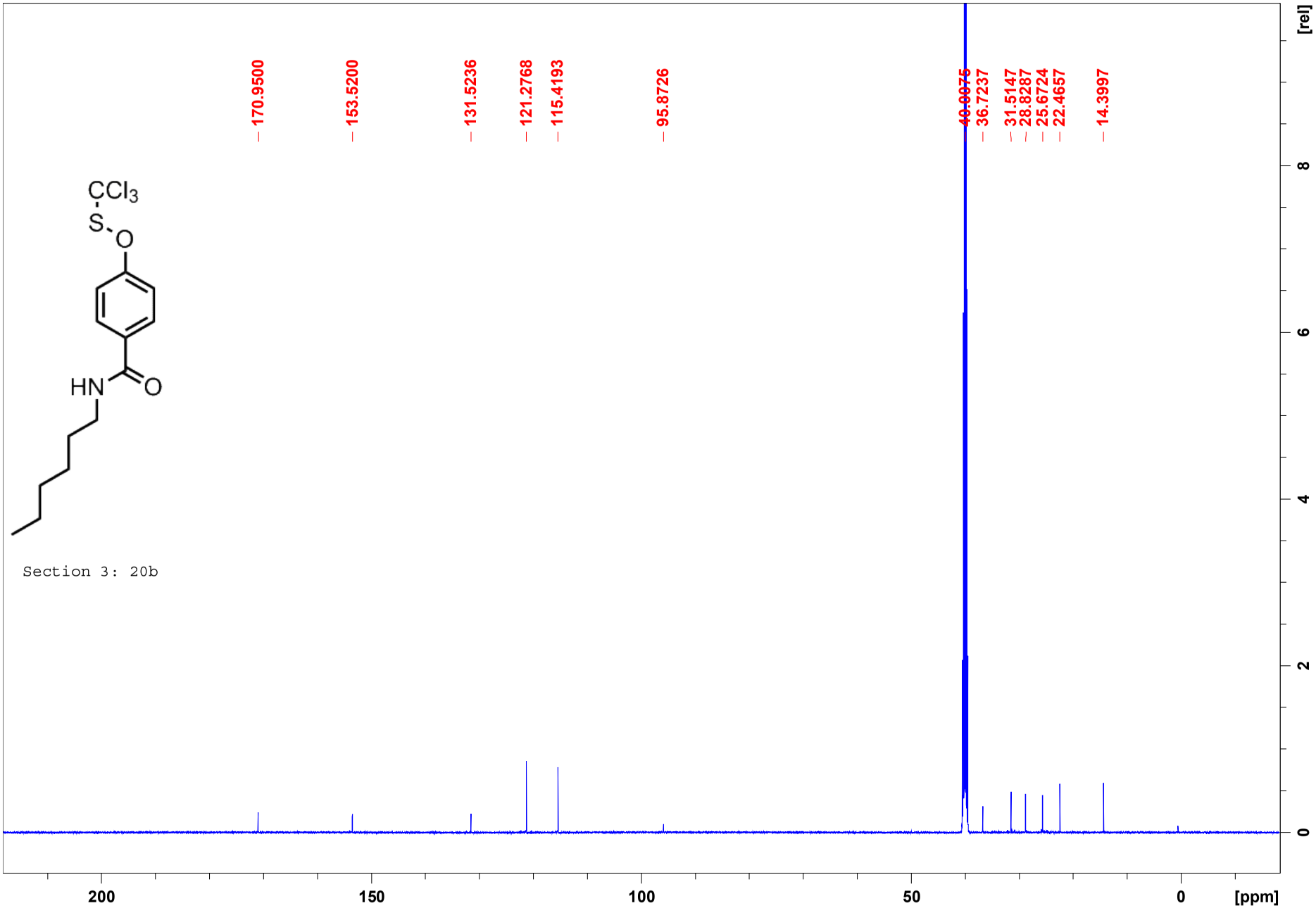

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7.2905
7.2760
6.6320
6.6175
2.4996
2.2013
2.1891
2.1766
1.6144
1.6021
0.9038
0.8915
0.8792
-0.0768
CCl3
S
O
O
NH
Section 3: 17b
0.9987
1.0000
1.7176
1.9916
2.0097
0.4006
2.2172
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3.0755
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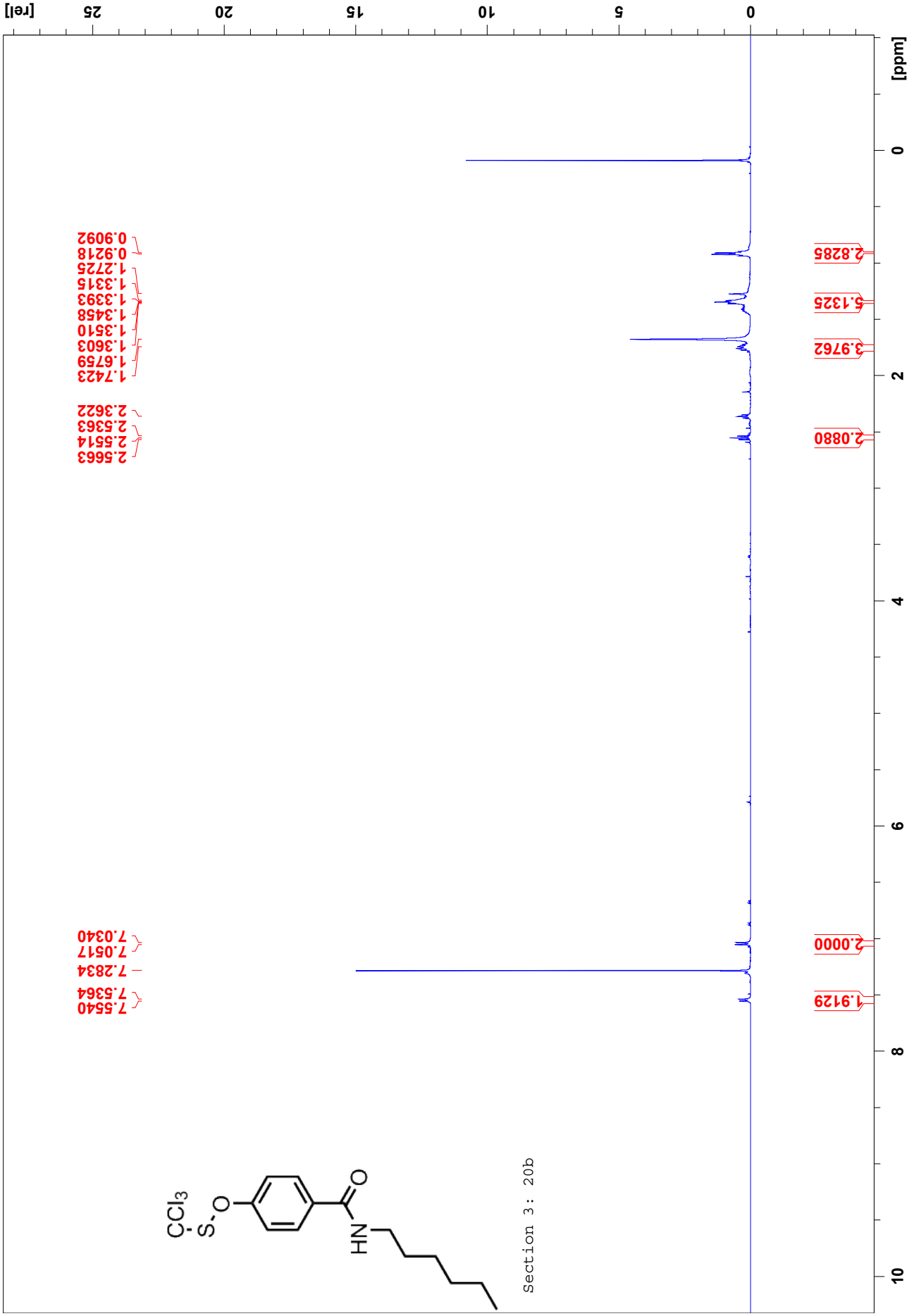

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38.8775
19.2310
13.6294
Section 3: 18b
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O
NH
O

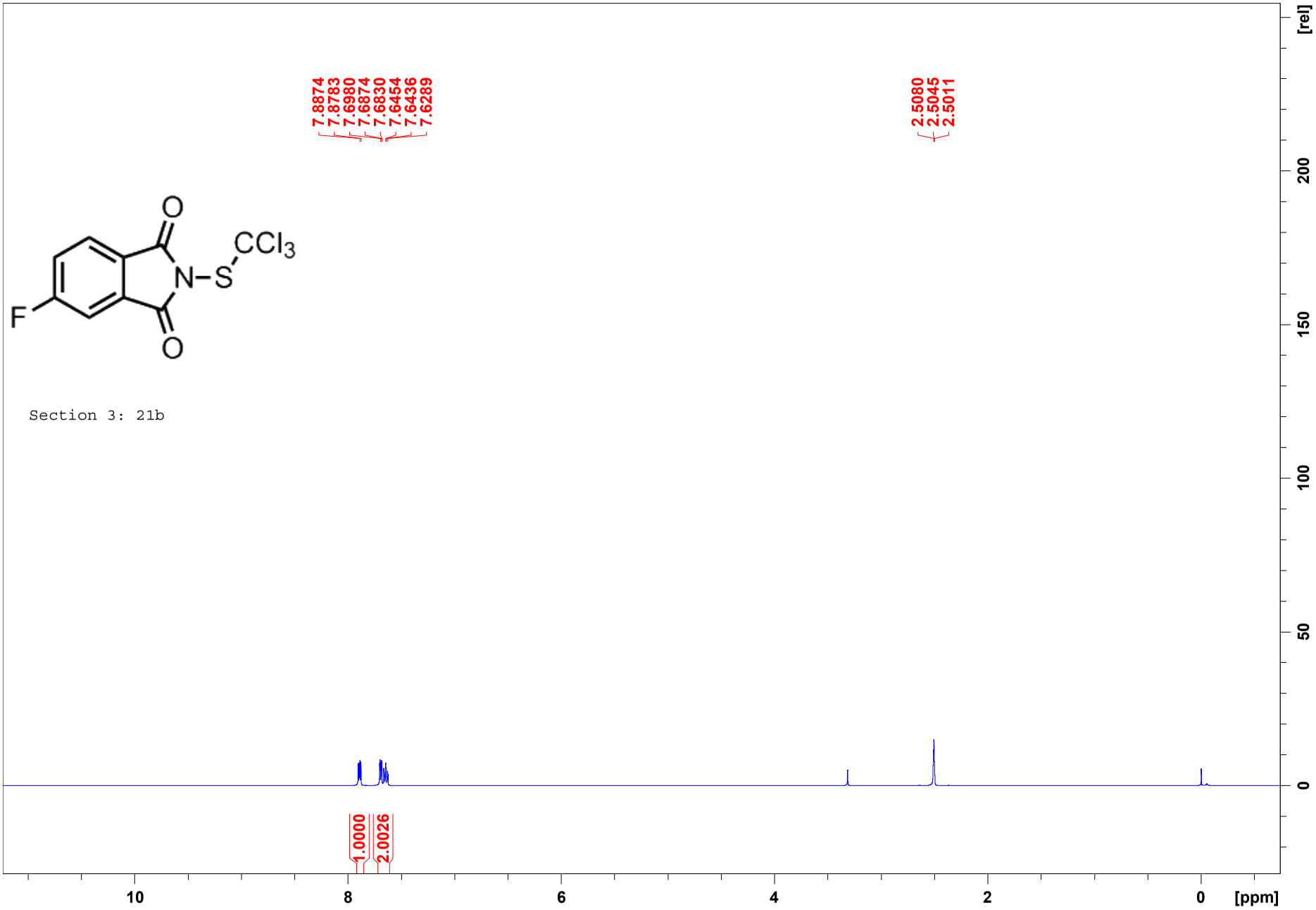

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7.1542
7.1372
7.0556
7.0420
6.9936
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6.8974
6.8802
6.8671
2.4825
2.4678
2.4527
1.8265
1.8116
1.0741
1.0594
1.0446
CCl3
S
O
NH
O
Section 3: 18b
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1.2822
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2.0210
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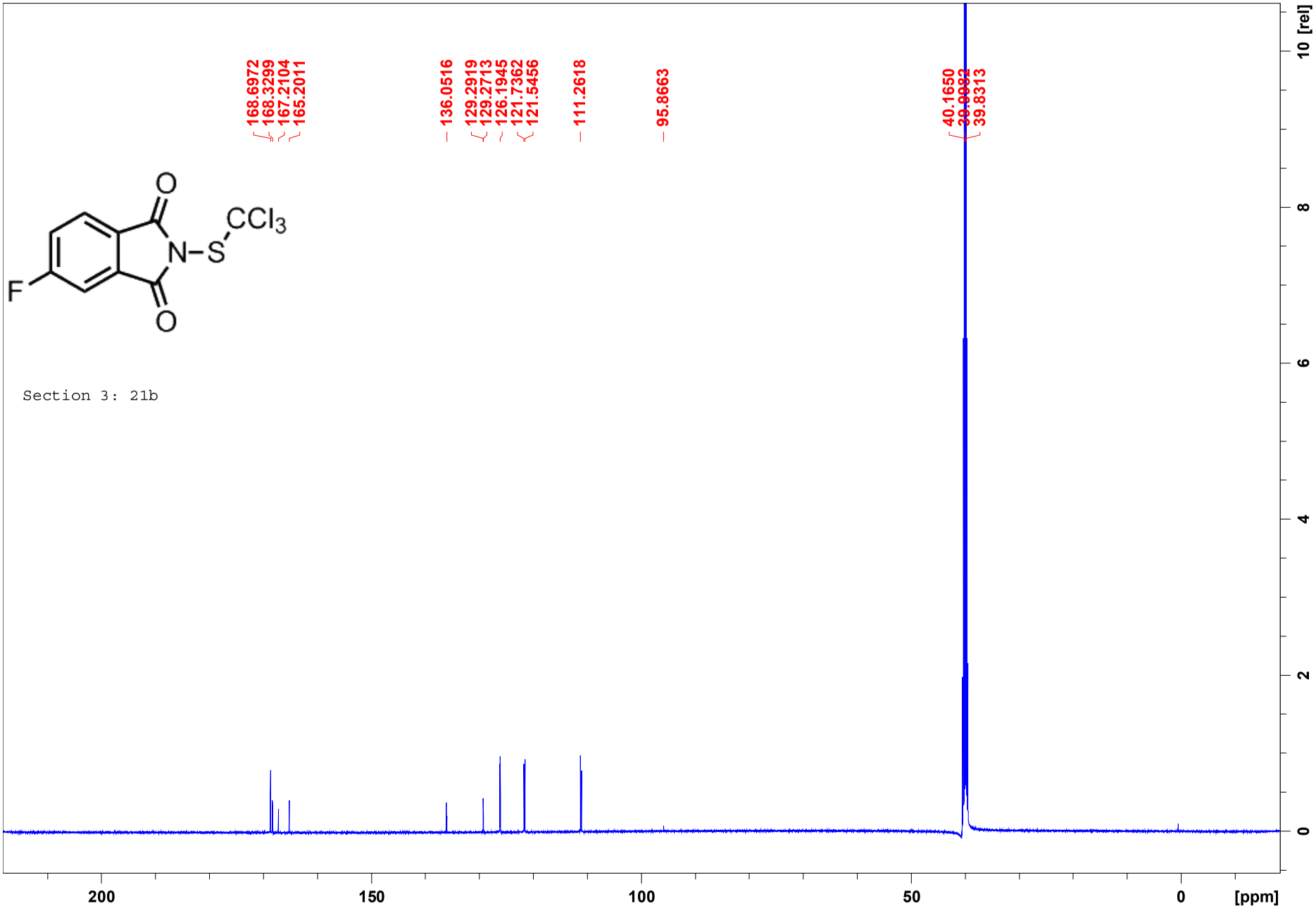

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128.7599
125.7549
115.6108
96.1313
40.2709
31.4799
29.5916
26.6523
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O
NH
Section 3: 19b
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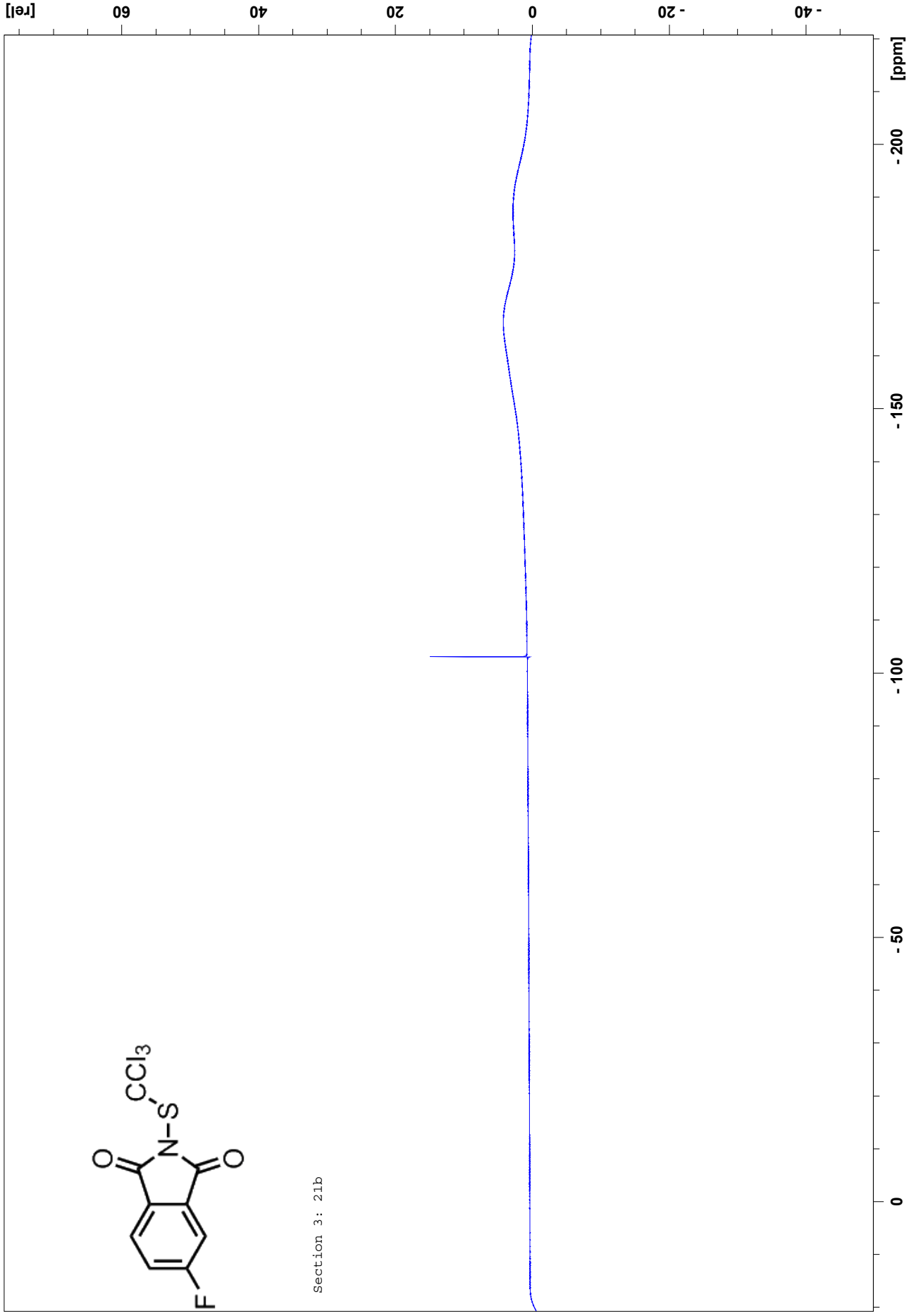

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2.0000
1.9244
0.8870
0.9011
0.9150
1.3118
1.3180
1.3239
1.3250
1.3319
1.6037
1.6179
3.4585
3.4467
3.4444
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6.8774
7.2833
7.6448
7.6274
Section 3: 19b
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S
O
NH
O

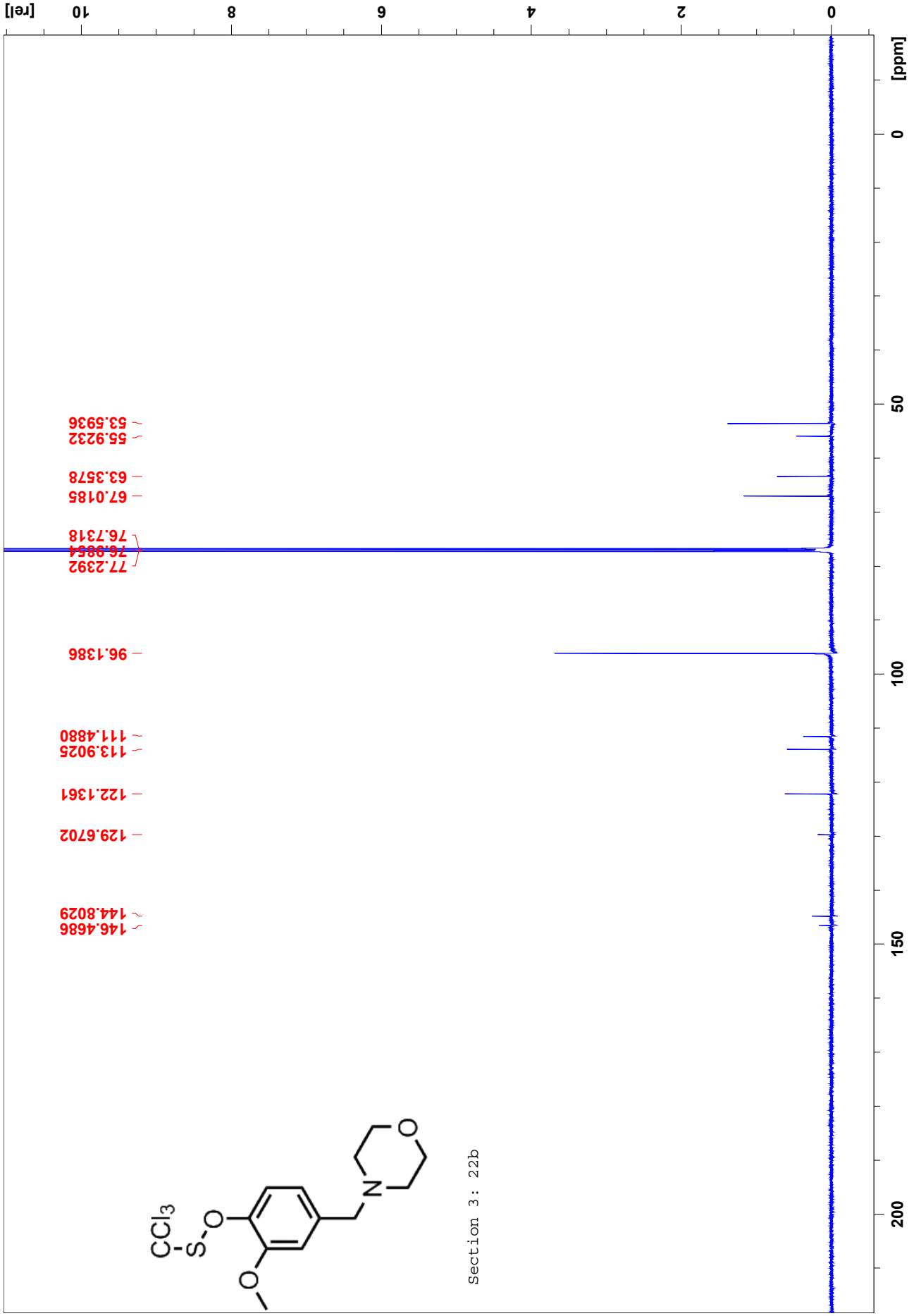

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131.5236
121.2768
115.4193
95.8726
40.0075
36.7237
31.5147
28.8287
25.6724
22.4657
14.3997
CCl3
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O
HN
O
Section 3: 20b
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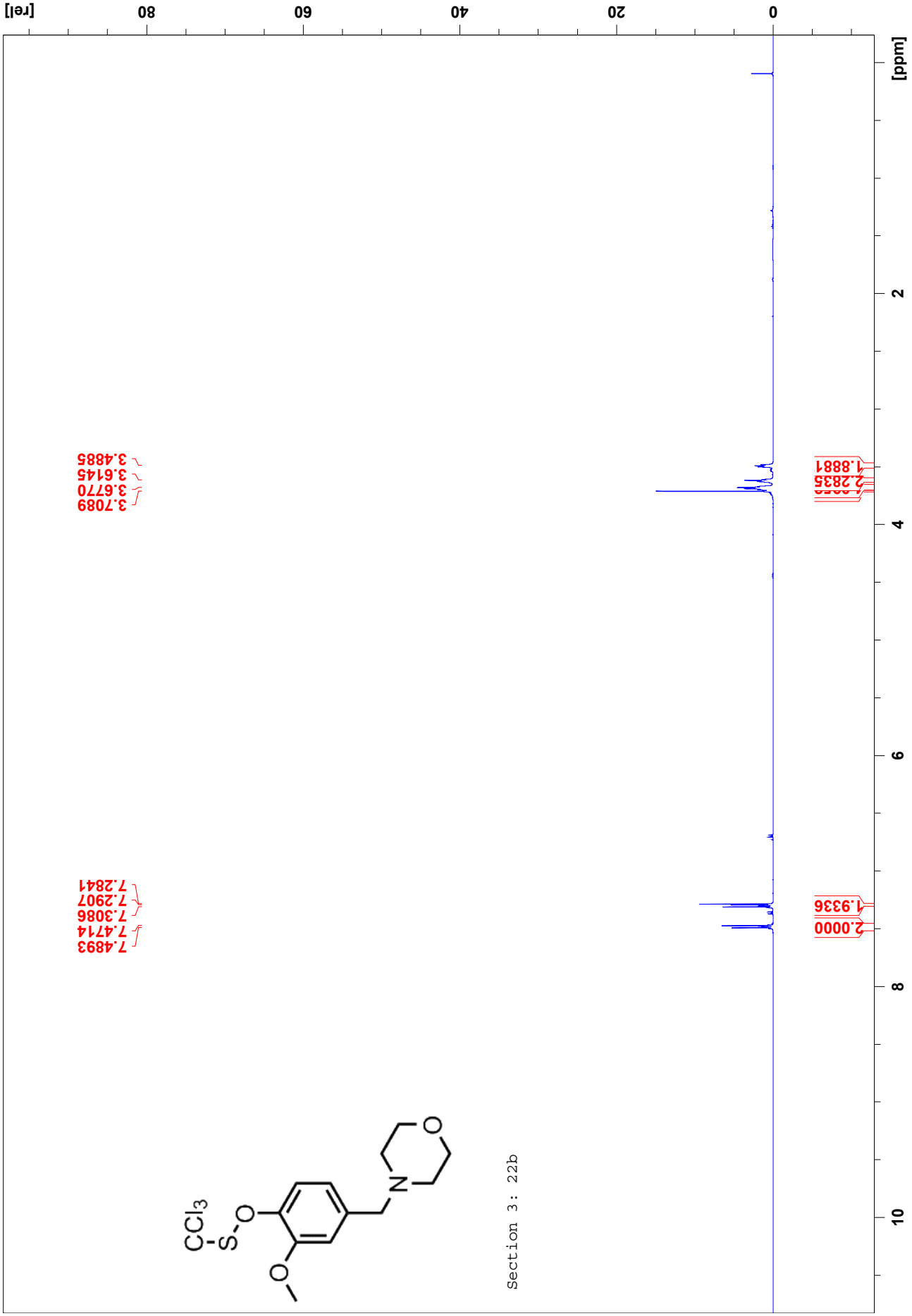

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7.0517
7.0340
2.5663
2.5514
2.5363
2.3622
1.7423
1.6759
1.3603
1.3510
1.3458
1.3393
1.3315
1.2725
0.9218
0.9092
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2.8285
Section 3: 20b
CCl3
S
O
O
HN

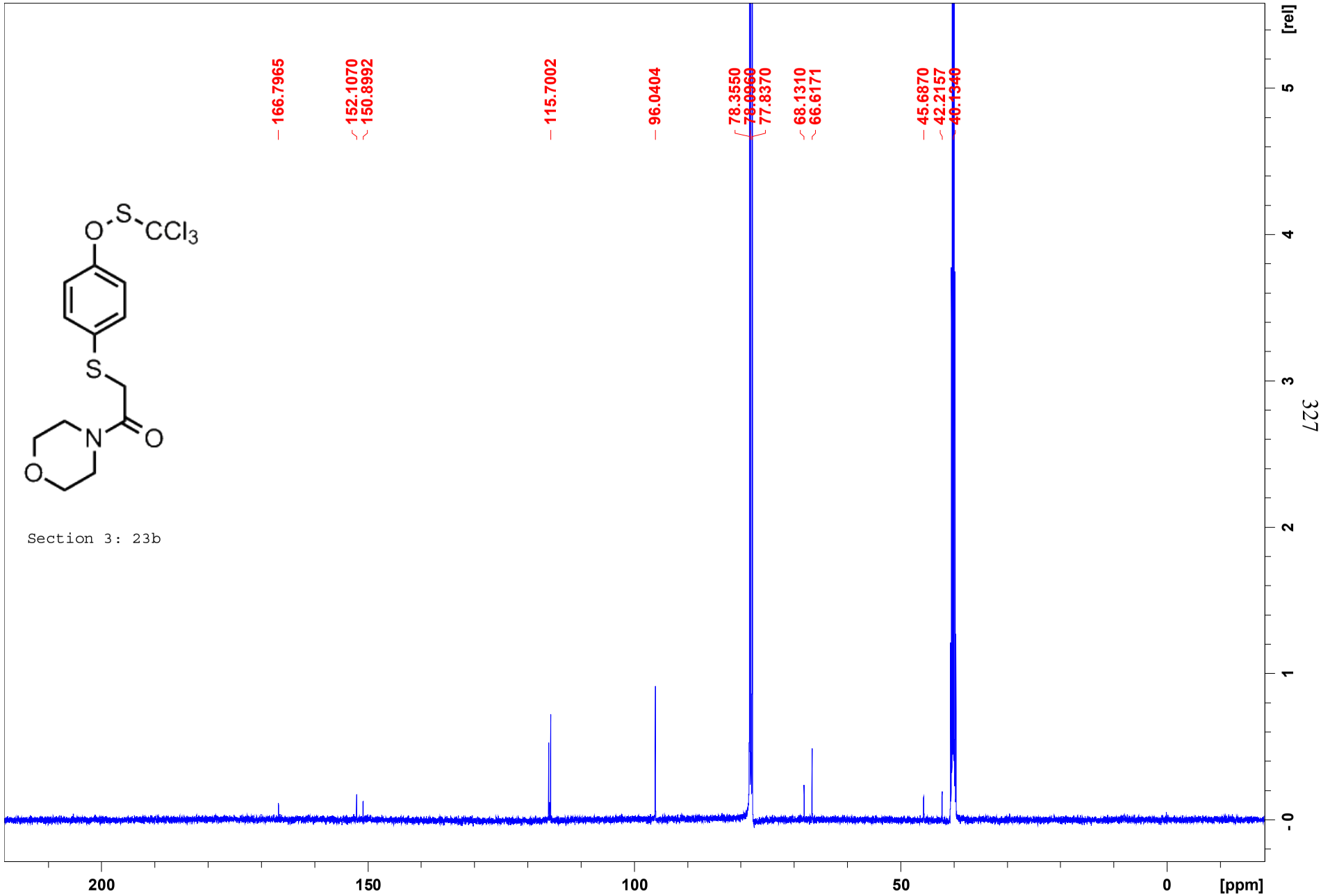

7.8874
7.8783
7.6980
7.6874
7.6830
7.6454
7.6436
7.6289
2.5080
2.5045
2.5011
O
N–S
CCl3
F
O
Section 3: 21b
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2
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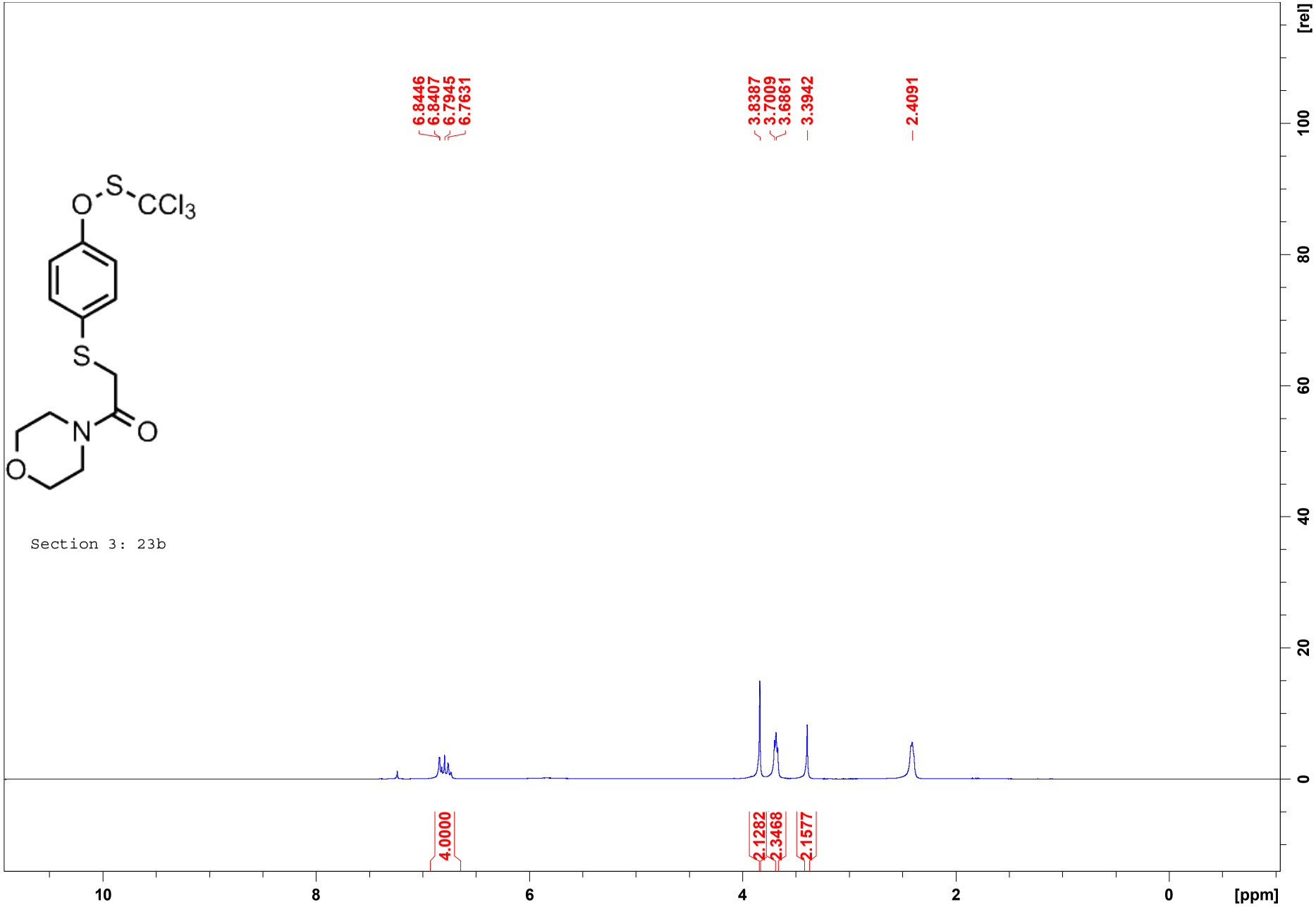

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167.2104
165.2011
136.0516
129.2919
129.2713
126.1945
121.7362
121.5456
111.2618
95.8663
40.1650
39.8313
O
O
O
N–S
CCl3
F
Section 3: 21b
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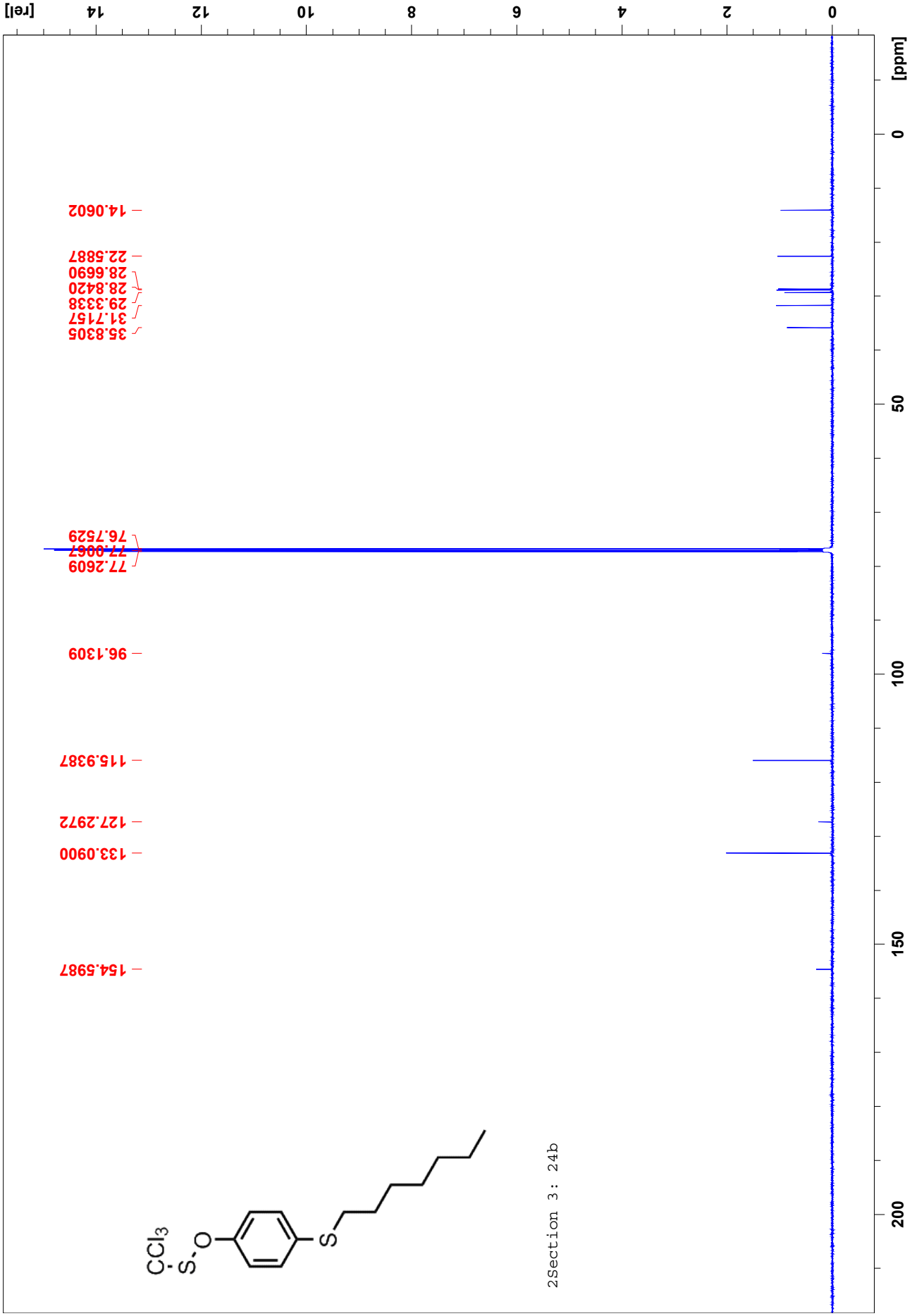

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- 40
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- 100
- 150
- 200
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N
O
O
F

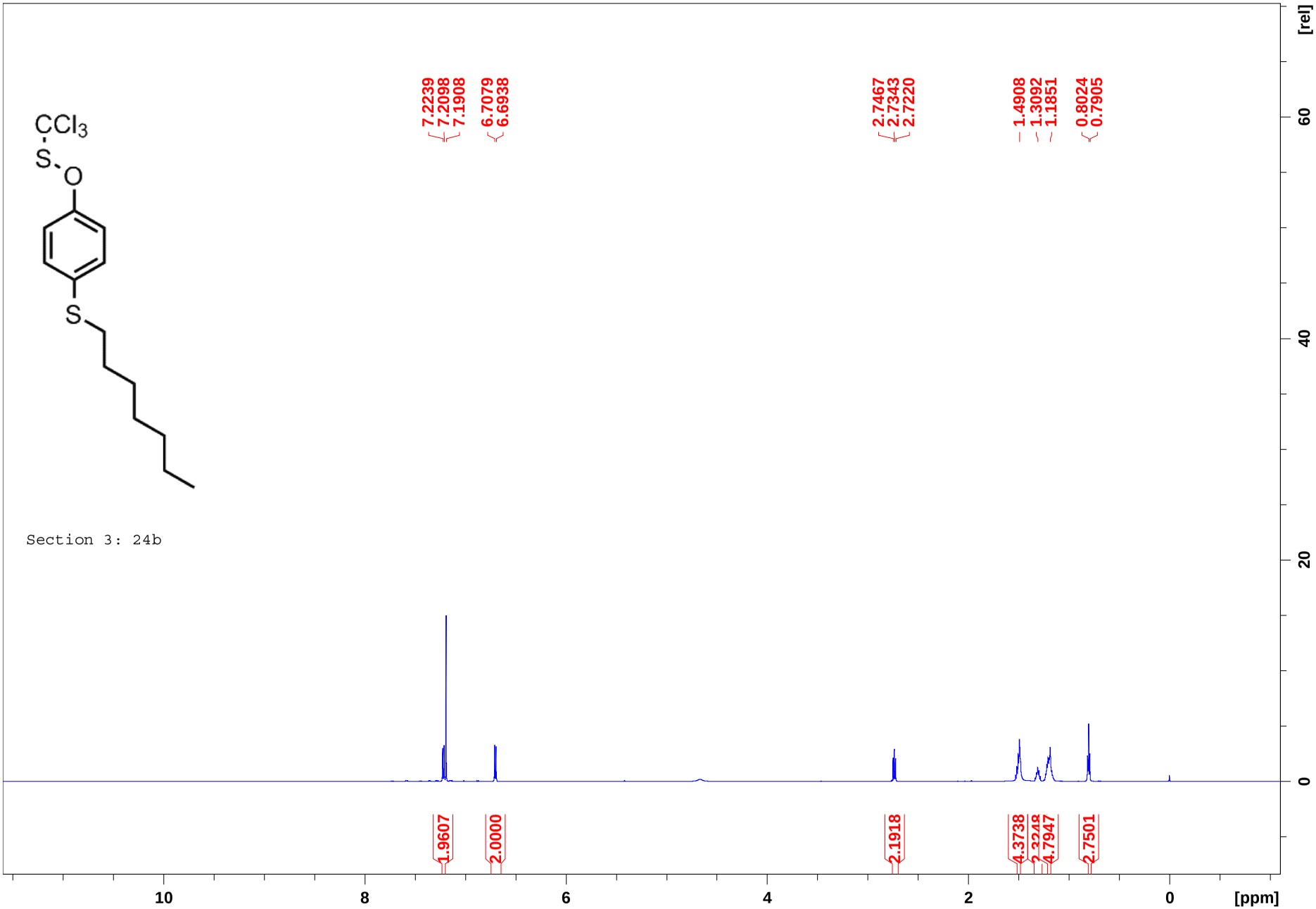

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67.0185
76.7318
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77.2392
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111.4880
113.9025
122.1361
129.6702
144.8029
146.4686
Section 3: 22b

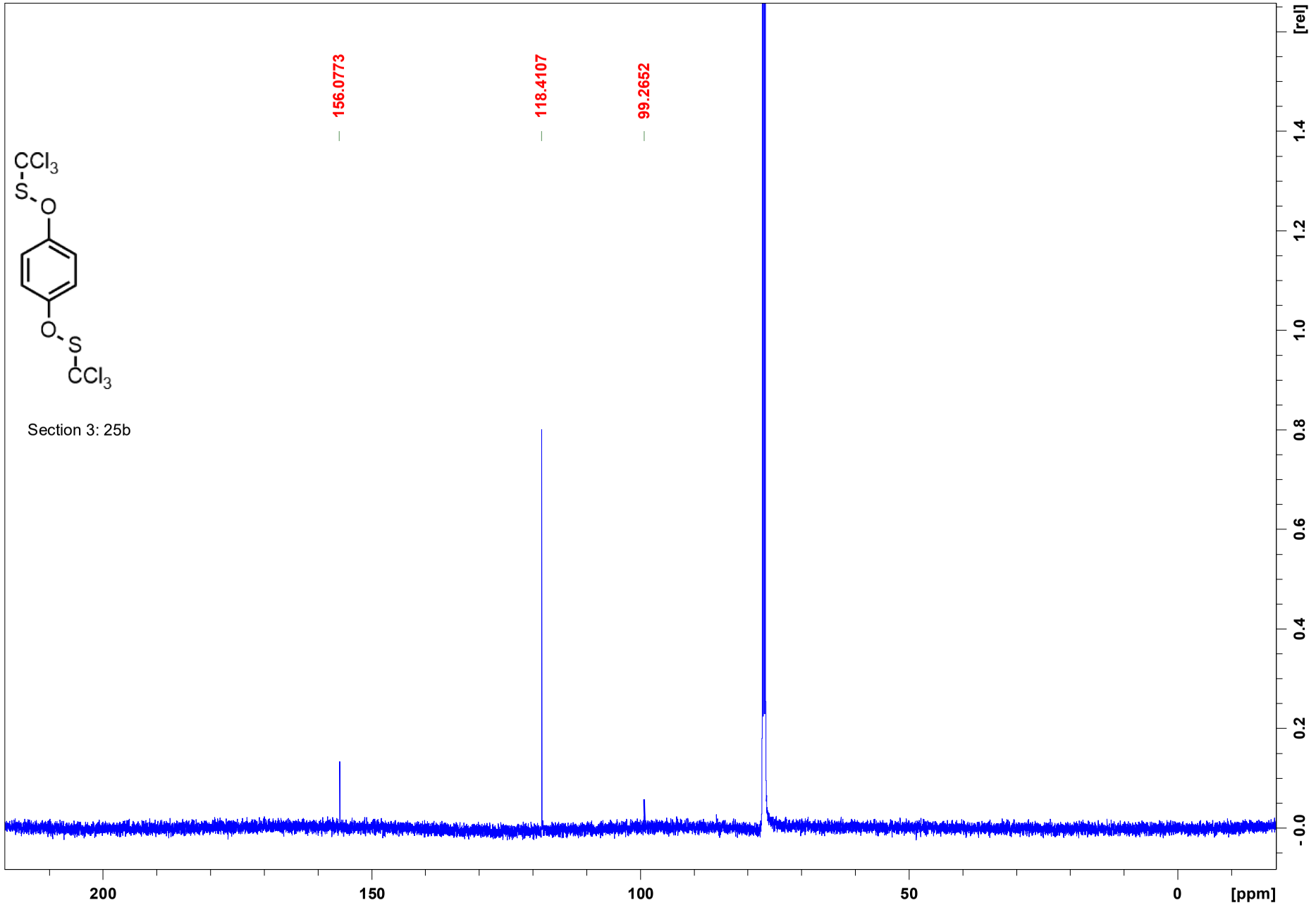

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3.6770
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1.9336
2.2835
1.8881
Section 3: 22b
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O
O
N
O

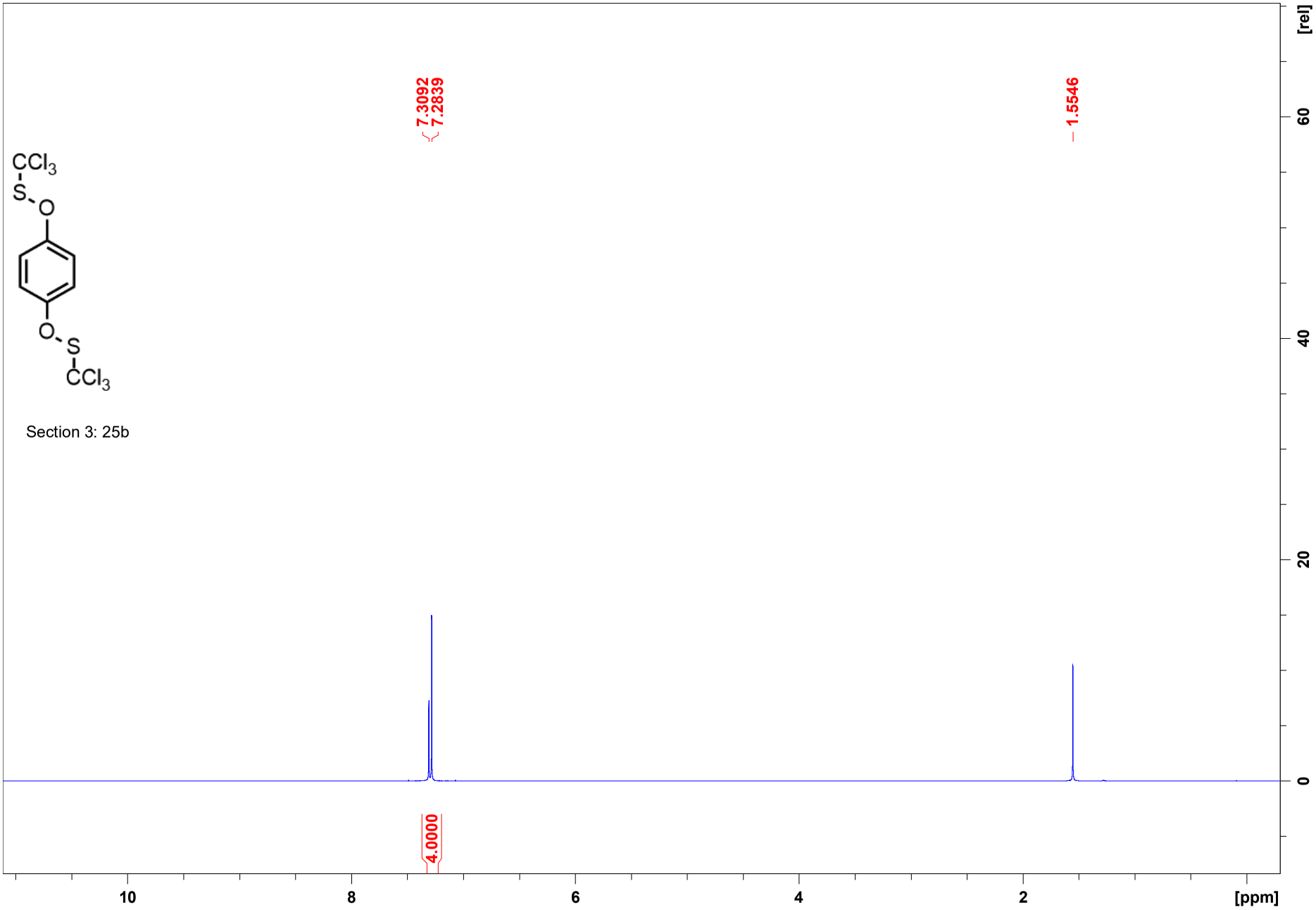

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115.7002
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78.3550
78.0060
77.8370
68.1310
66.6171
45.6870
42.2157
40.1340
O
S
CCl3
S
N
O
O
Section 3: 23b
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5
4
3
2
1
-0
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150
100
50
0
[ppm]


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6.7945
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3.8387
3.7009
3.6861
3.3942
2.4091
O
S
CCl3
S
N
O
O
Section 3: 23b
4.0000
2.1282
2.3468
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8
6
4
2
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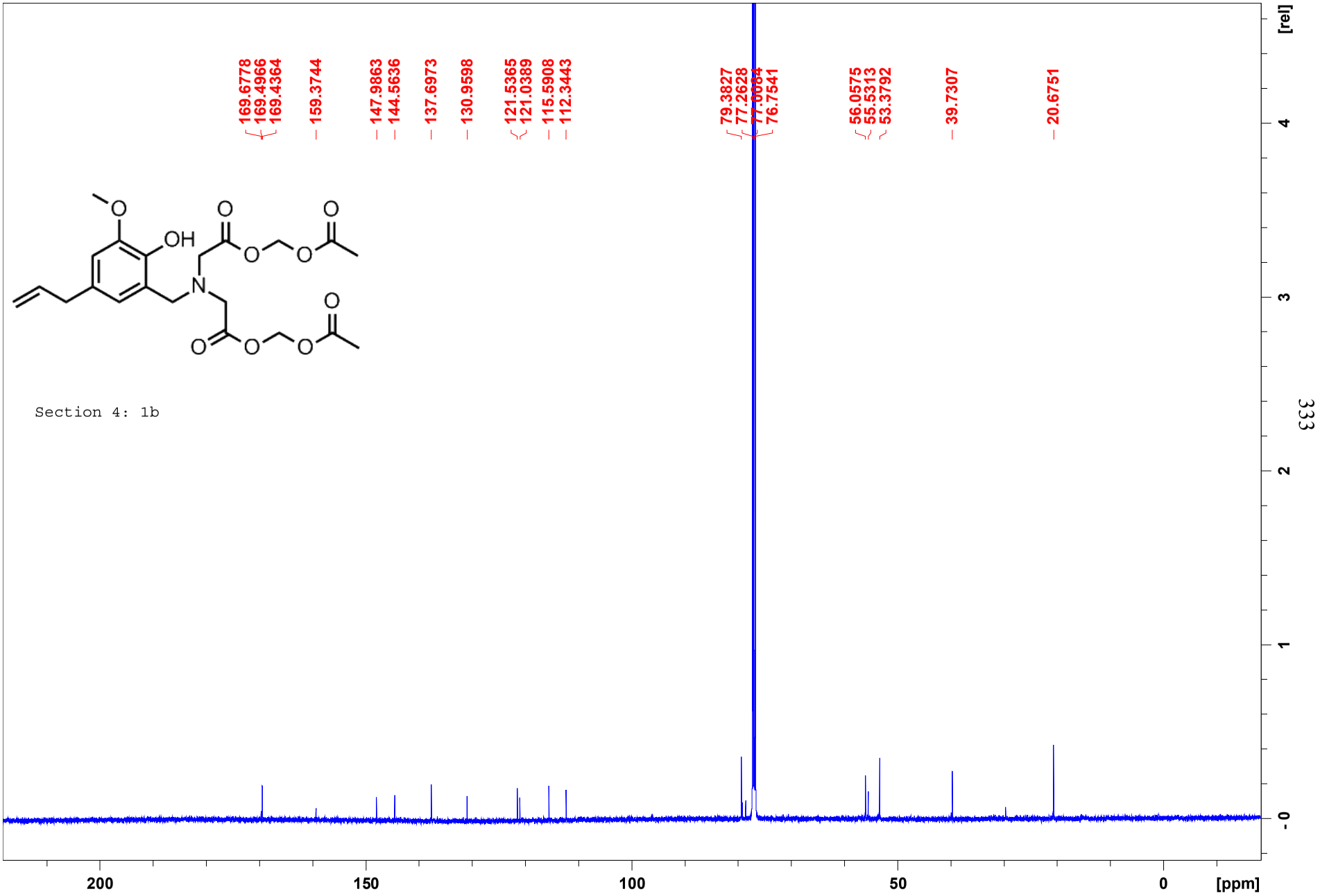

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29.3338
31.7157
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96.1309
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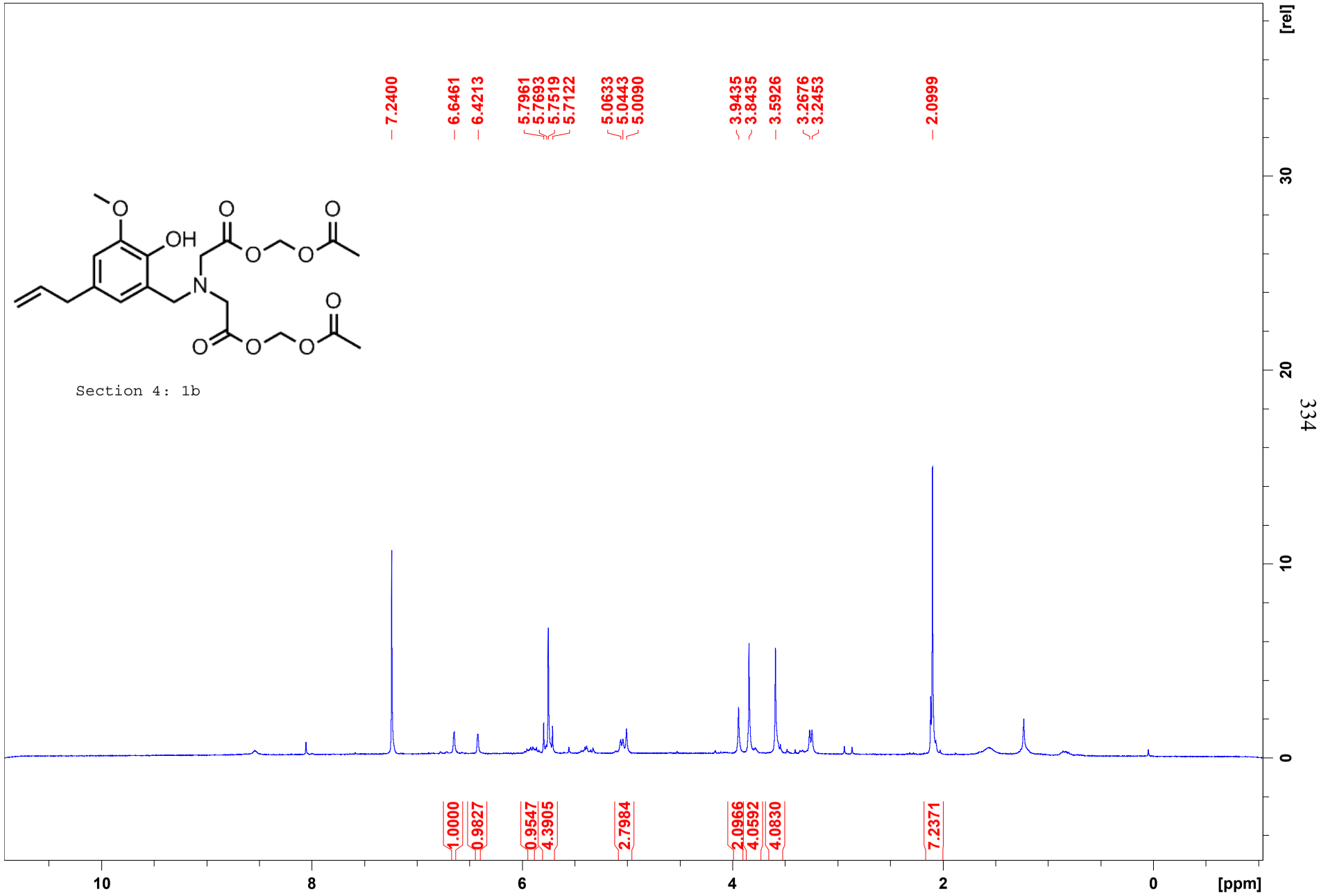

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S
O
S
Section 3: 24b
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7.2098
7.1908
6.7079
6.6938
2.7467
2.7343
2.7220
1.4908
1.3092
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0.8024
0.7905
1.9607
2.0000
2.1918
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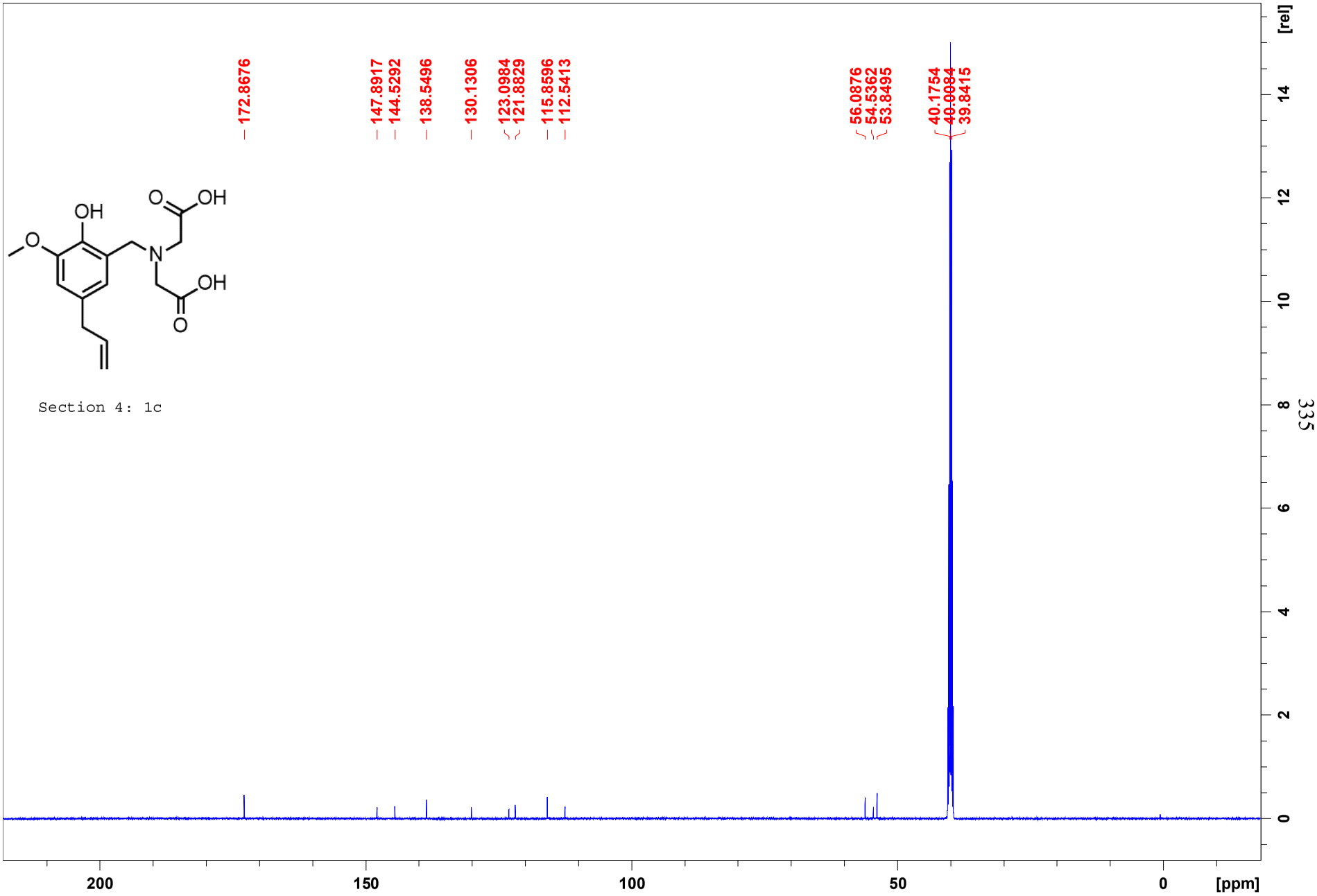

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S
O
O
S
CCl3
Section 3: 25b
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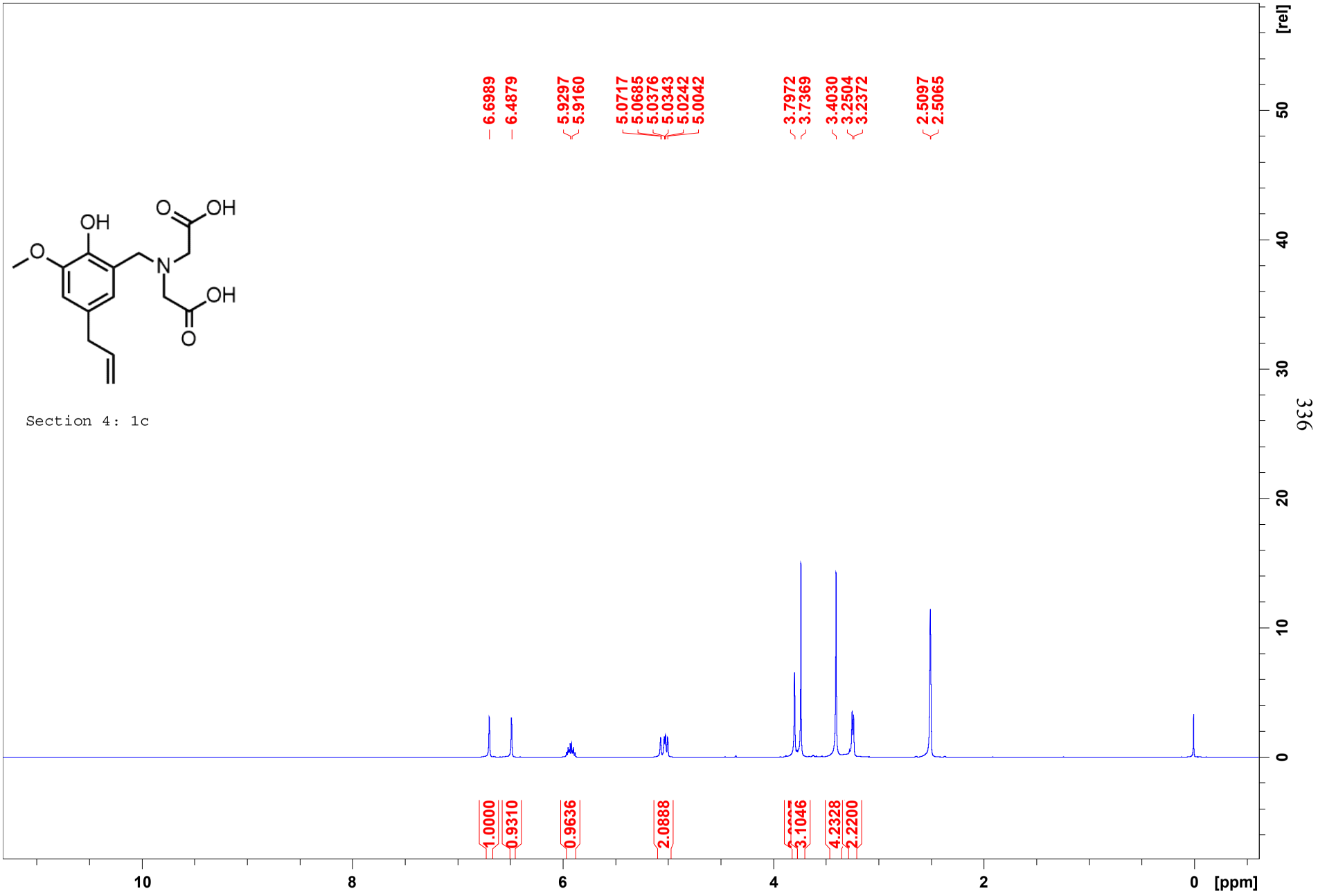


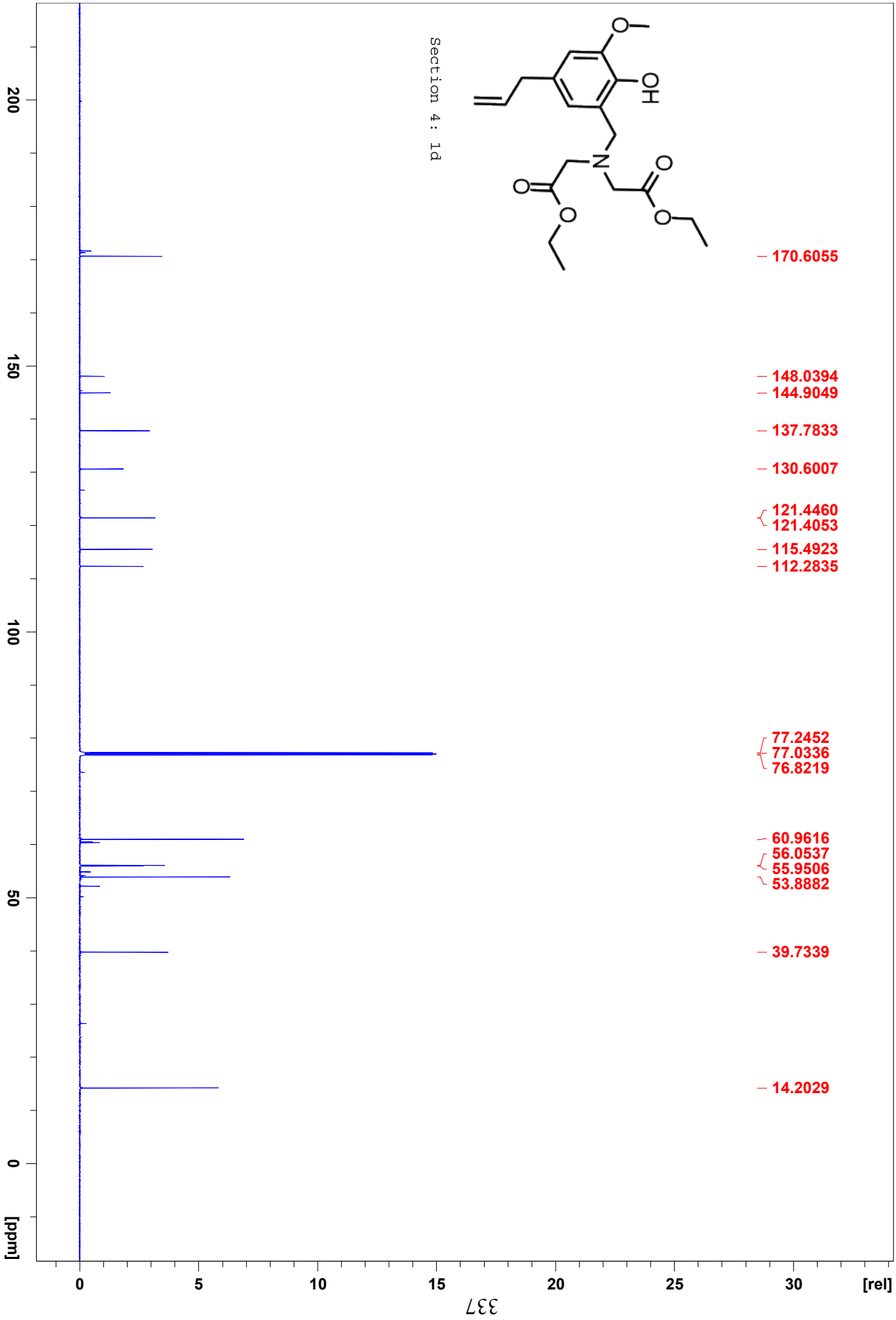
Section 3: 25b

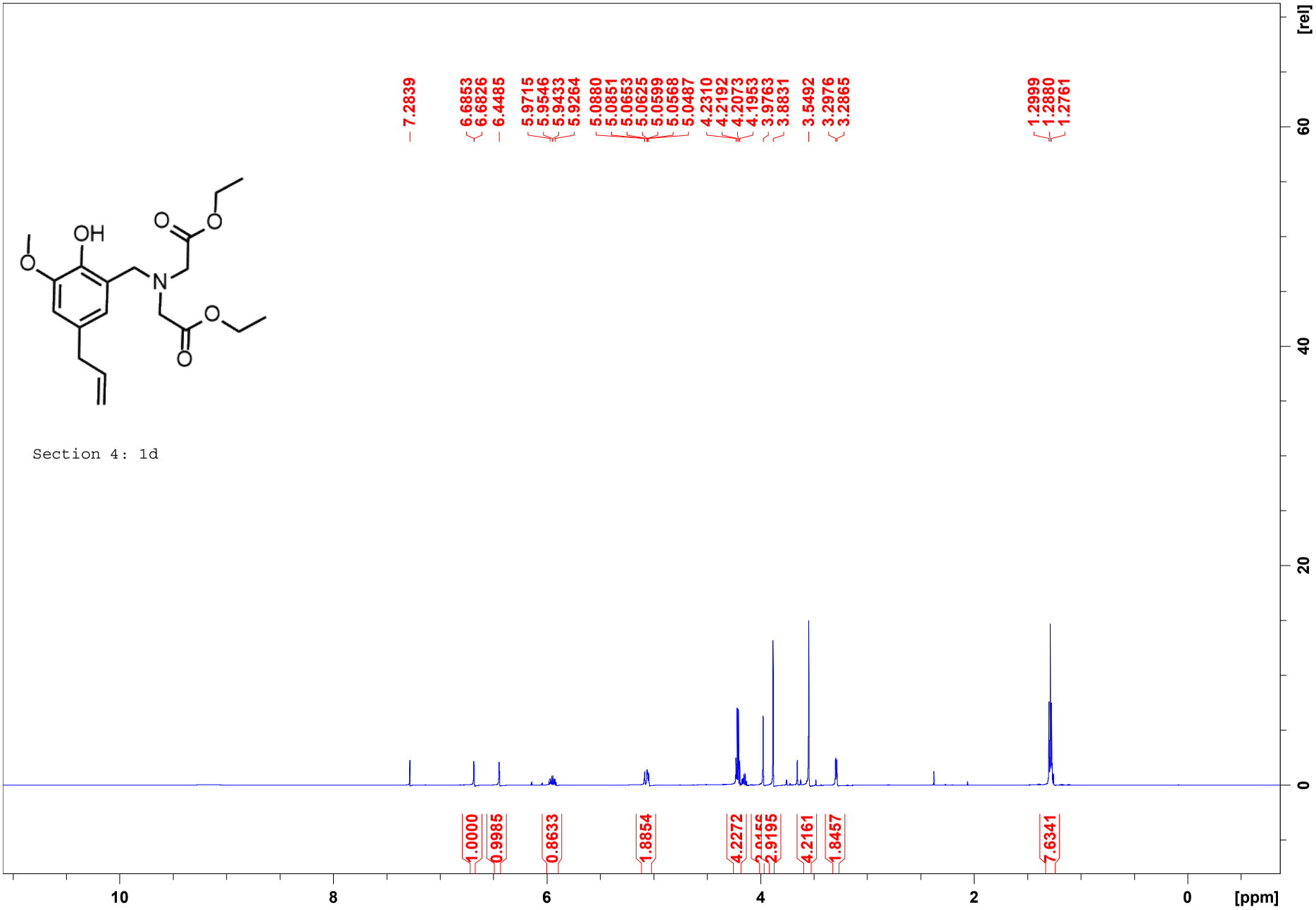

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112.3443
79.3827
77.2628
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76.7541
56.0575
55.5313
53.3792
39.7307
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50
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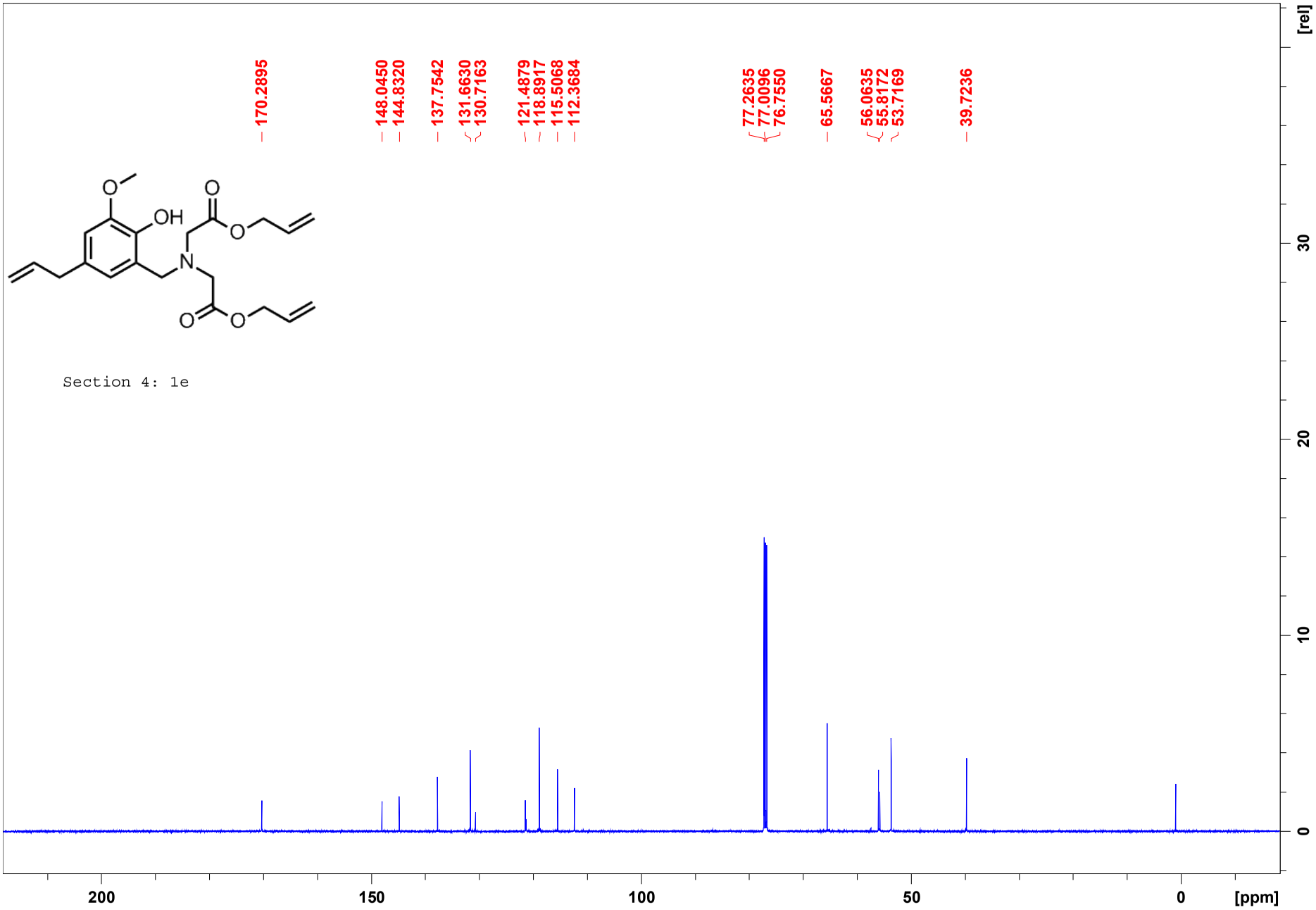

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5.7122
5.0633
5.0443
5.0090
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3.8435
3.5926
3.2676
3.2453
2.0999
OH
N
O
Section 4: 1b
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2.7984
2.0966
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6
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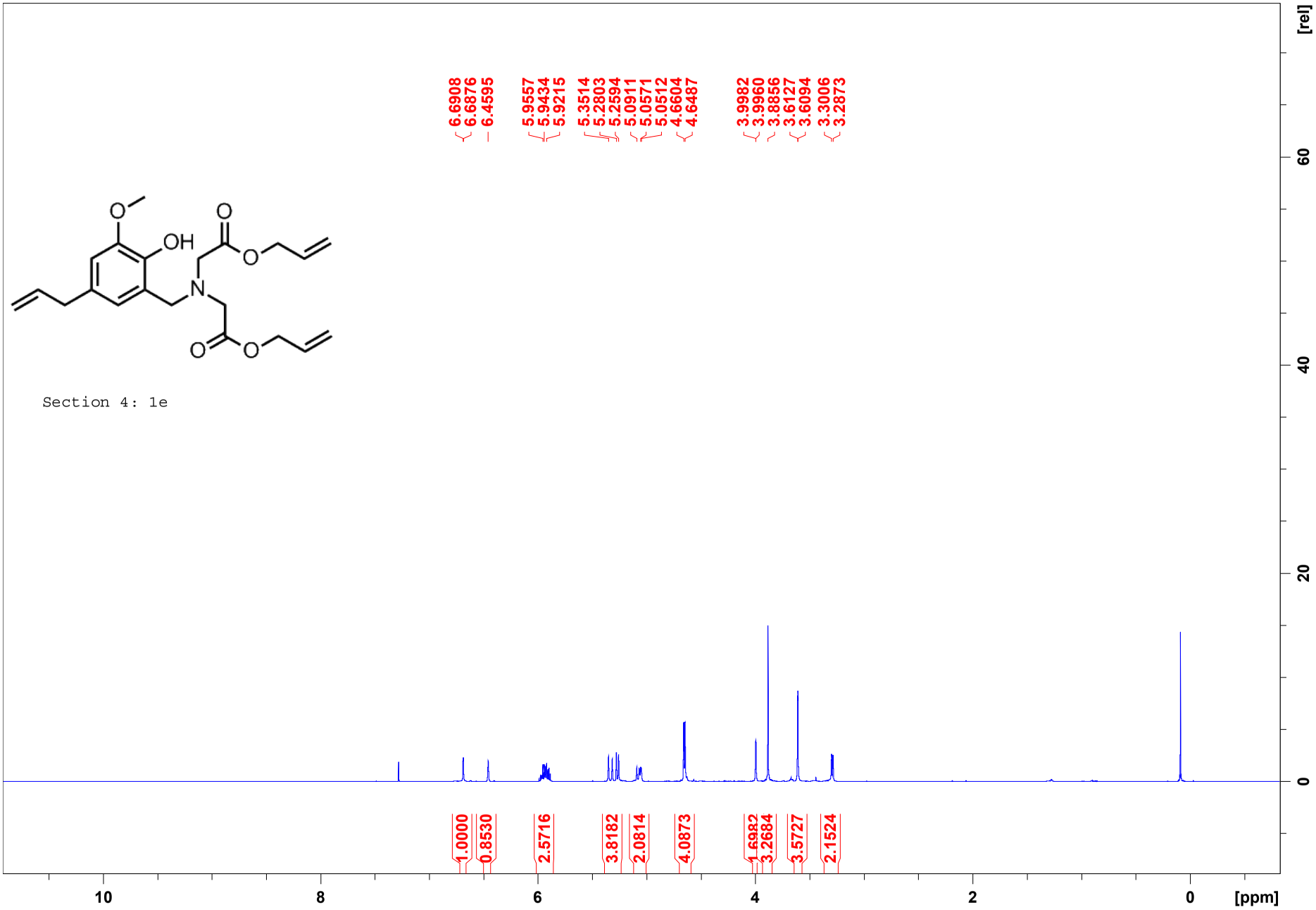

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OH
N
OH
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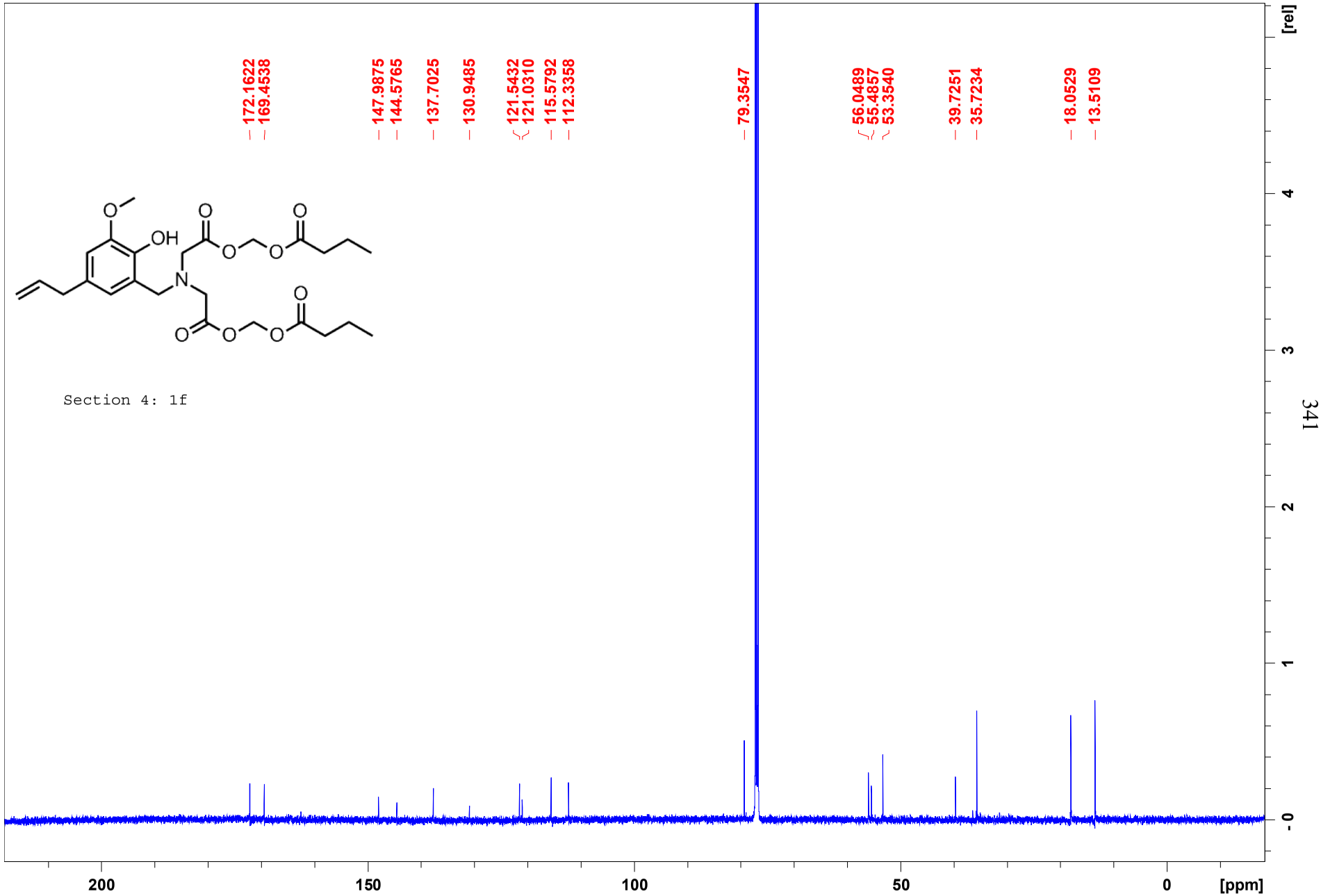

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5.0376
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5.0042
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3.7369
3.4030
3.2504
3.2372
2.5097
2.5065
OH
O
OH
N
OH
O
Section 4: 1c
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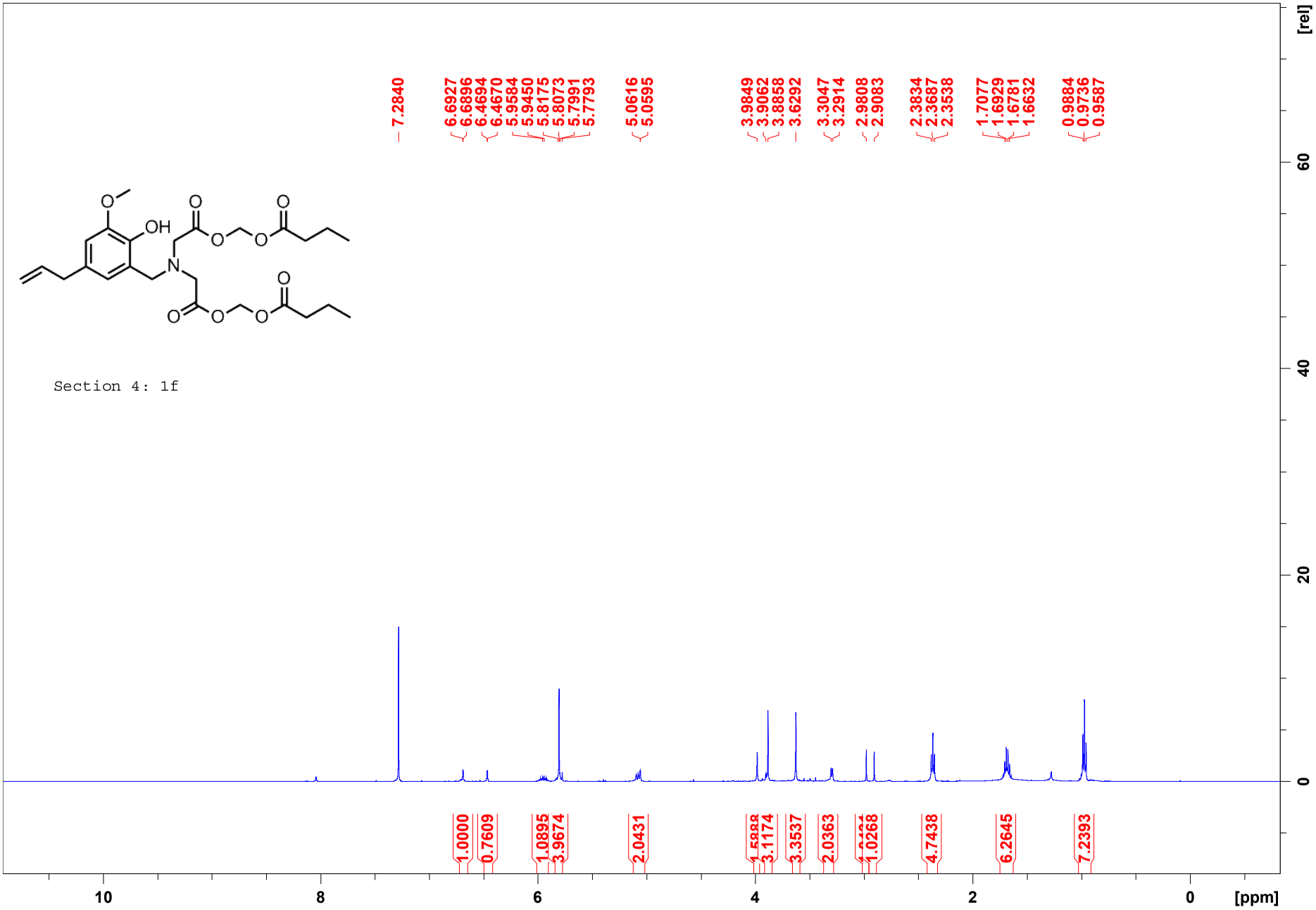

Section 4: 1d
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137.7833
130.6007
121.4460
121.4053
115.4923
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77.2452
77.0336
76.8219
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56.0537
55.9506
53.8882
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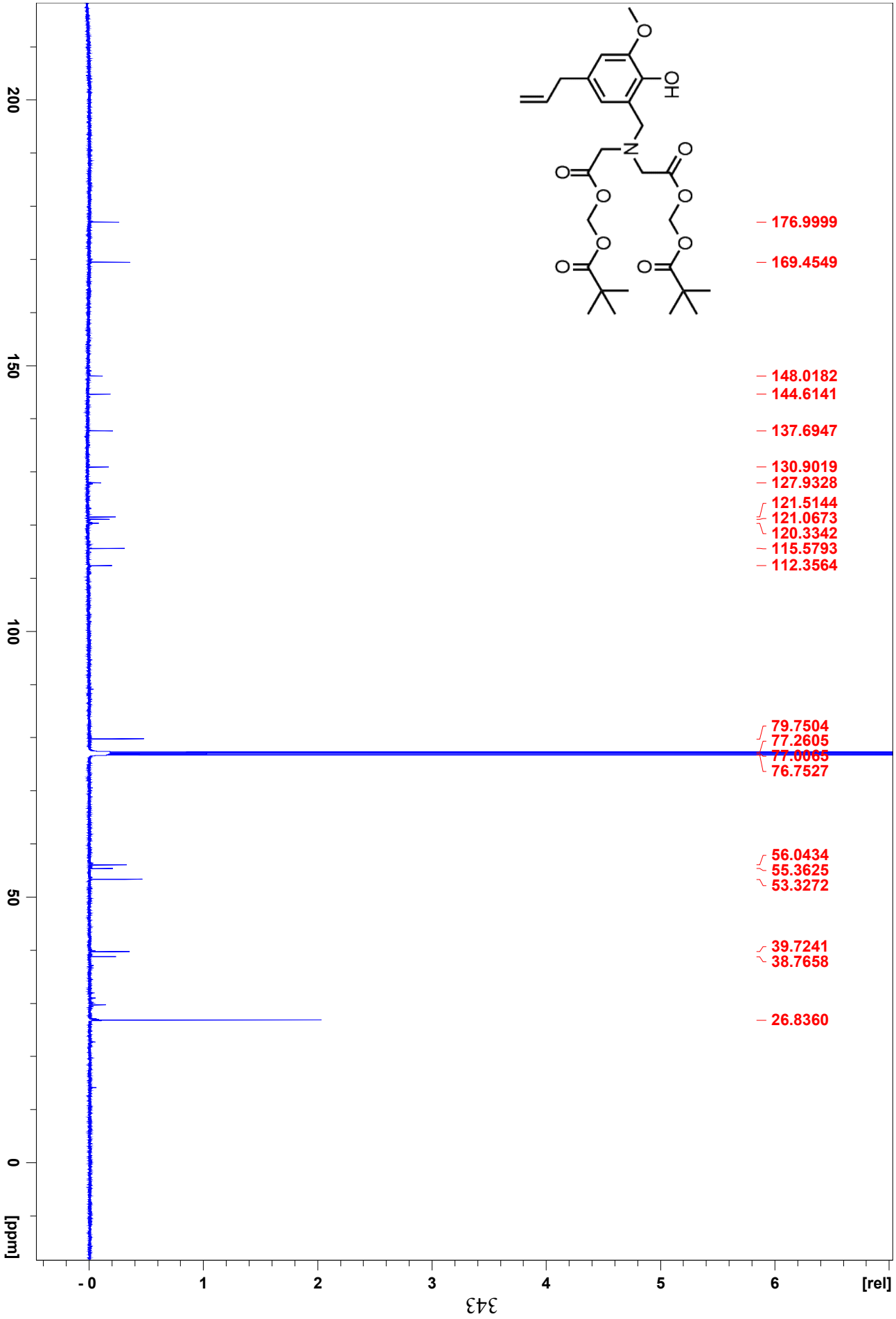

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5.0625
5.0599
5.0568
5.0487
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4.2073
4.1953
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3.5492
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3.2865
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OH
O
N
Section 4: 1d
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6
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0
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O
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O
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O
O
Section 4: 1e
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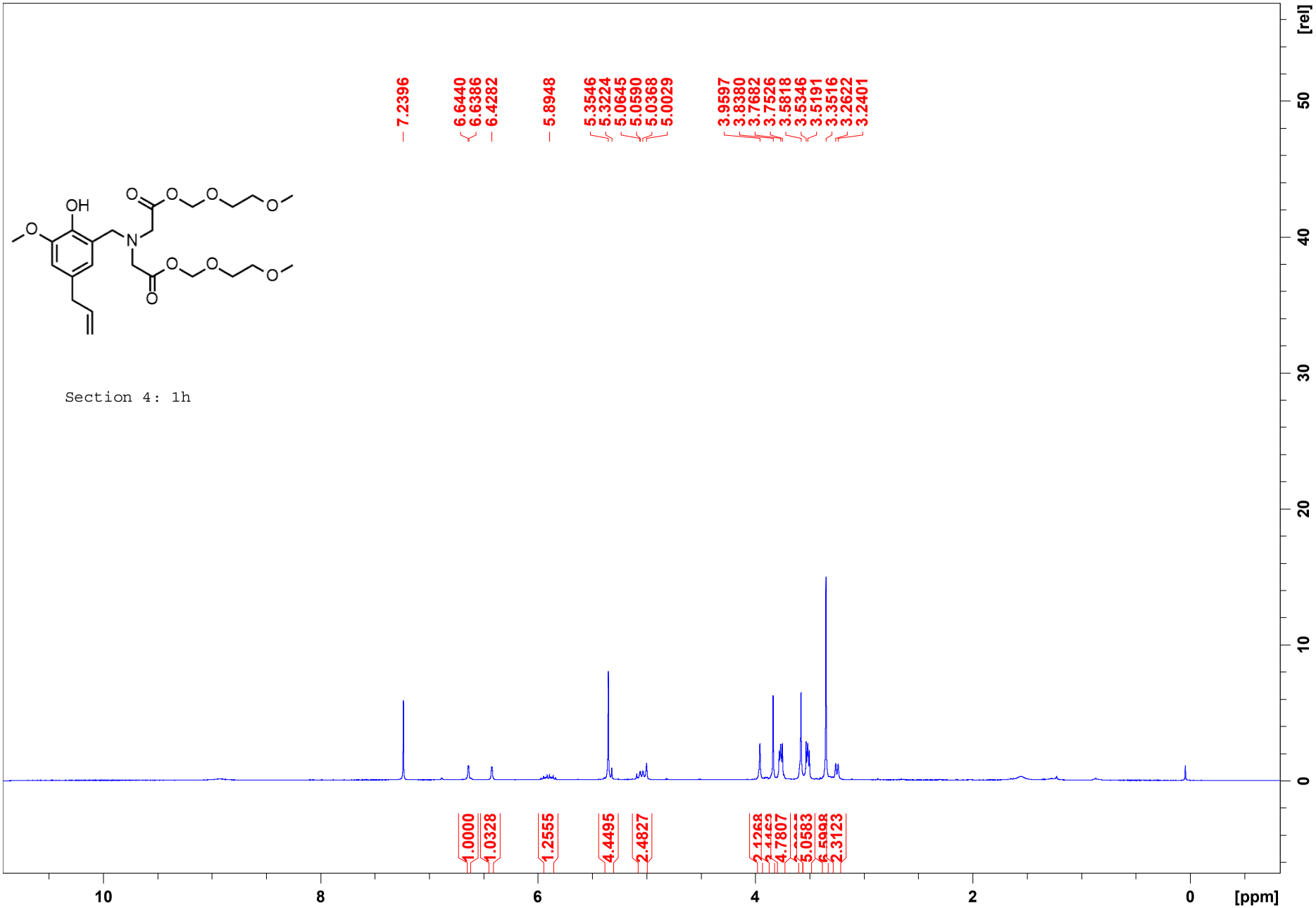

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3.6127
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3.3006
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O
OH
O
O
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O
O
Section 4: 1e
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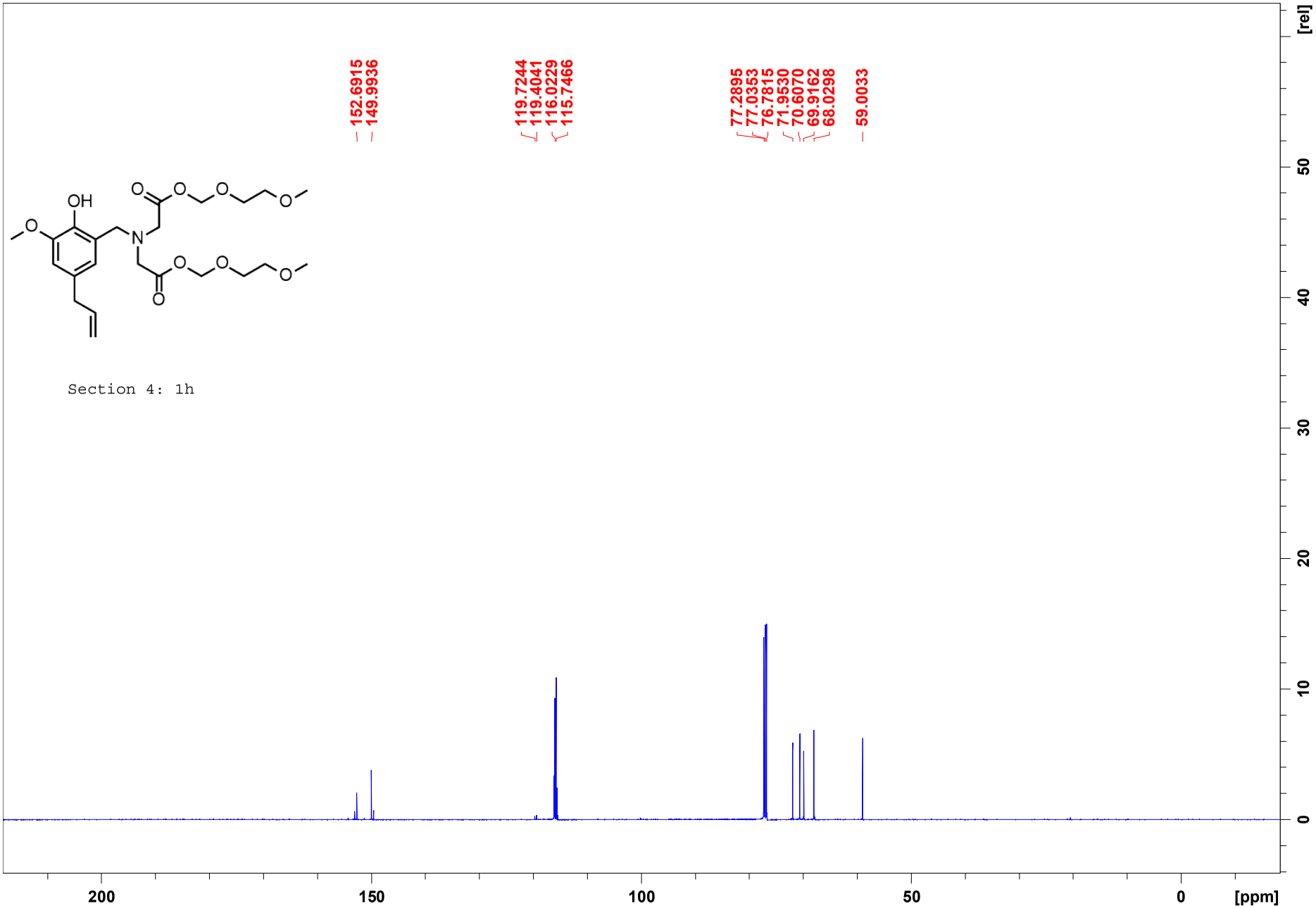

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53.3540
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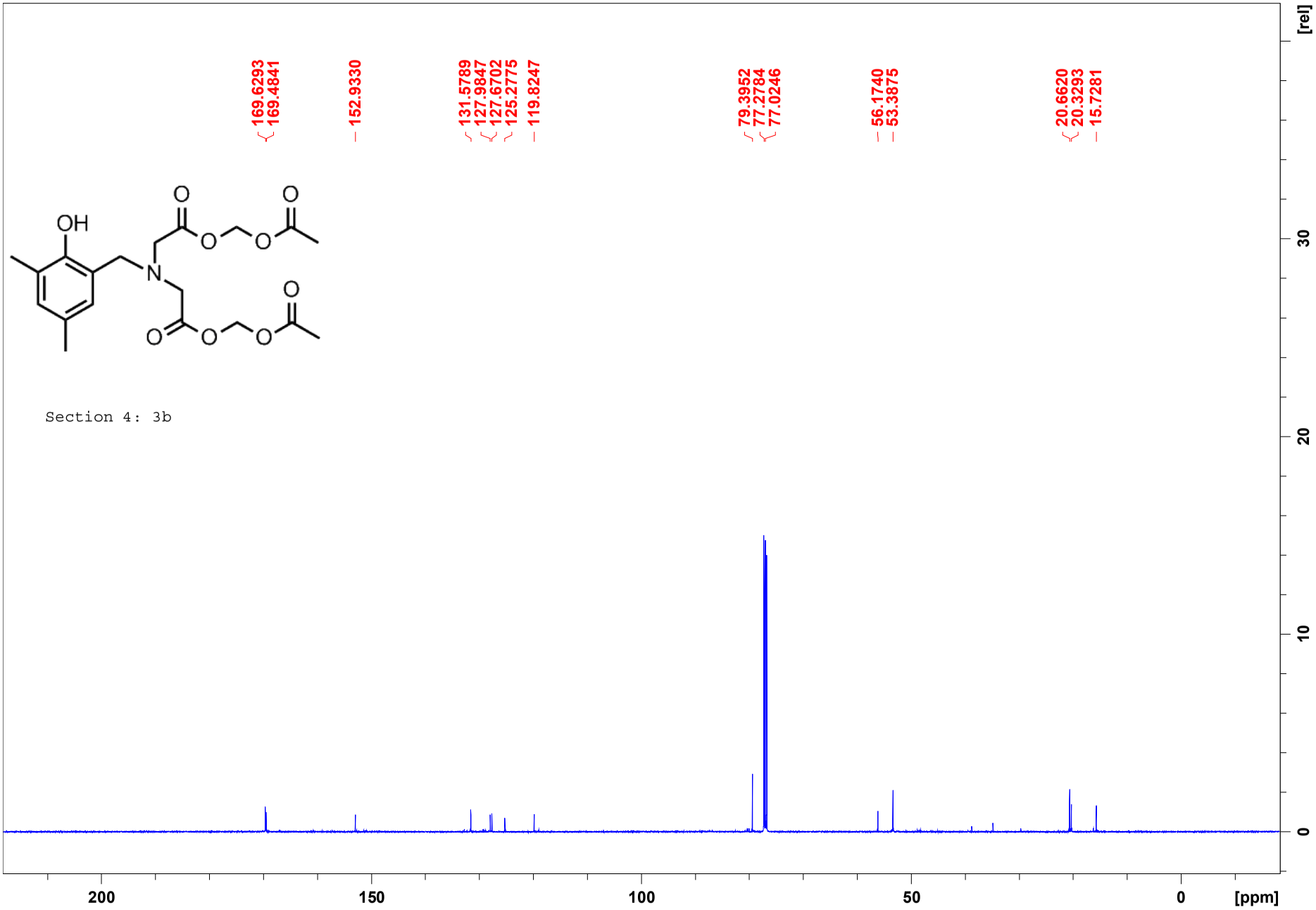

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5.0616
5.0595
3.9849
3.9062
3.8858
3.6292
3.3047
3.2914
2.9808
2.9083
2.3834
2.3687
2.3538
1.7077
1.6929
1.6781
1.6632
0.9884
0.9736
0.9587
O
OH
N
Section 4: 1f
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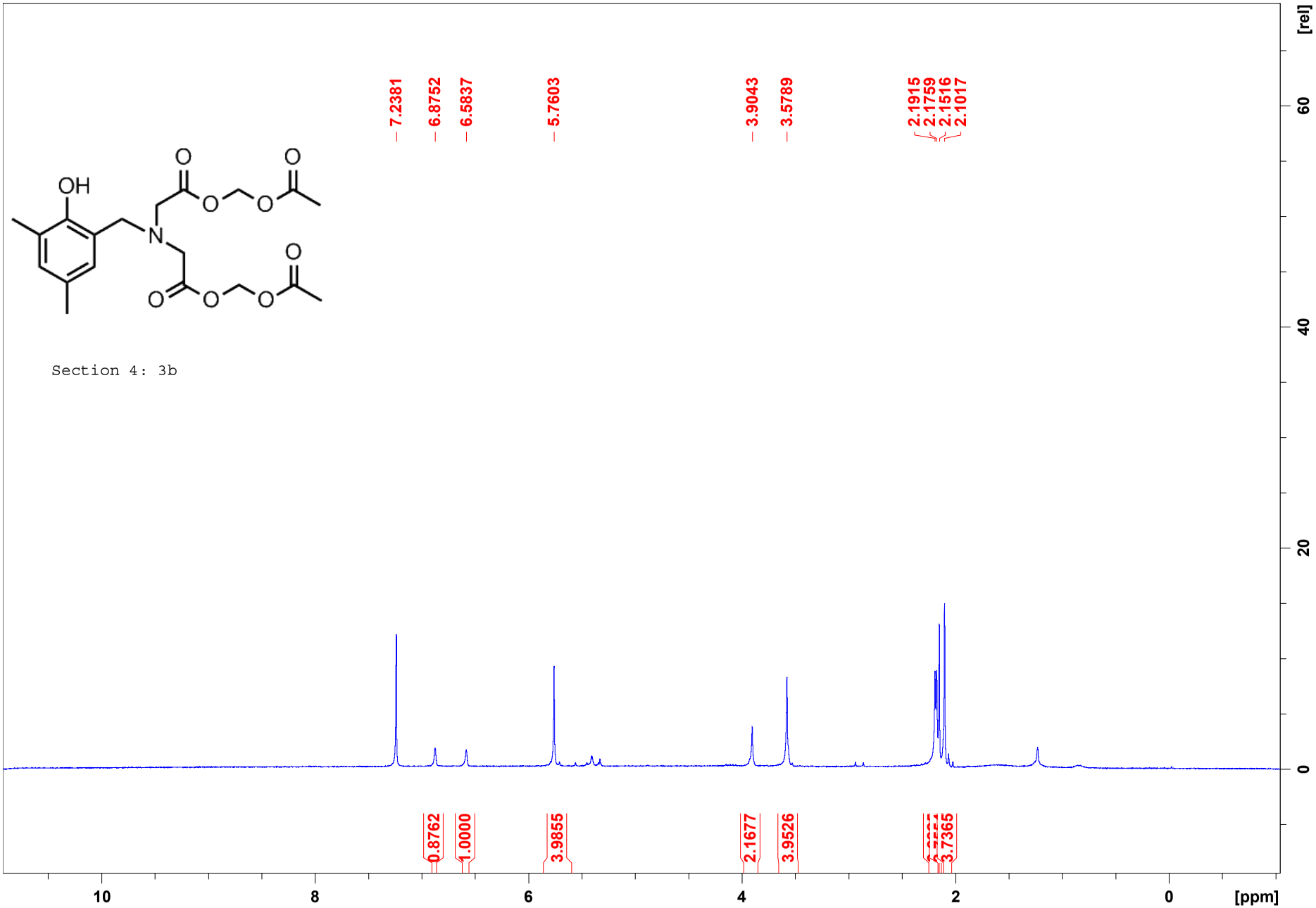

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112.3564
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53.3272
39.7241
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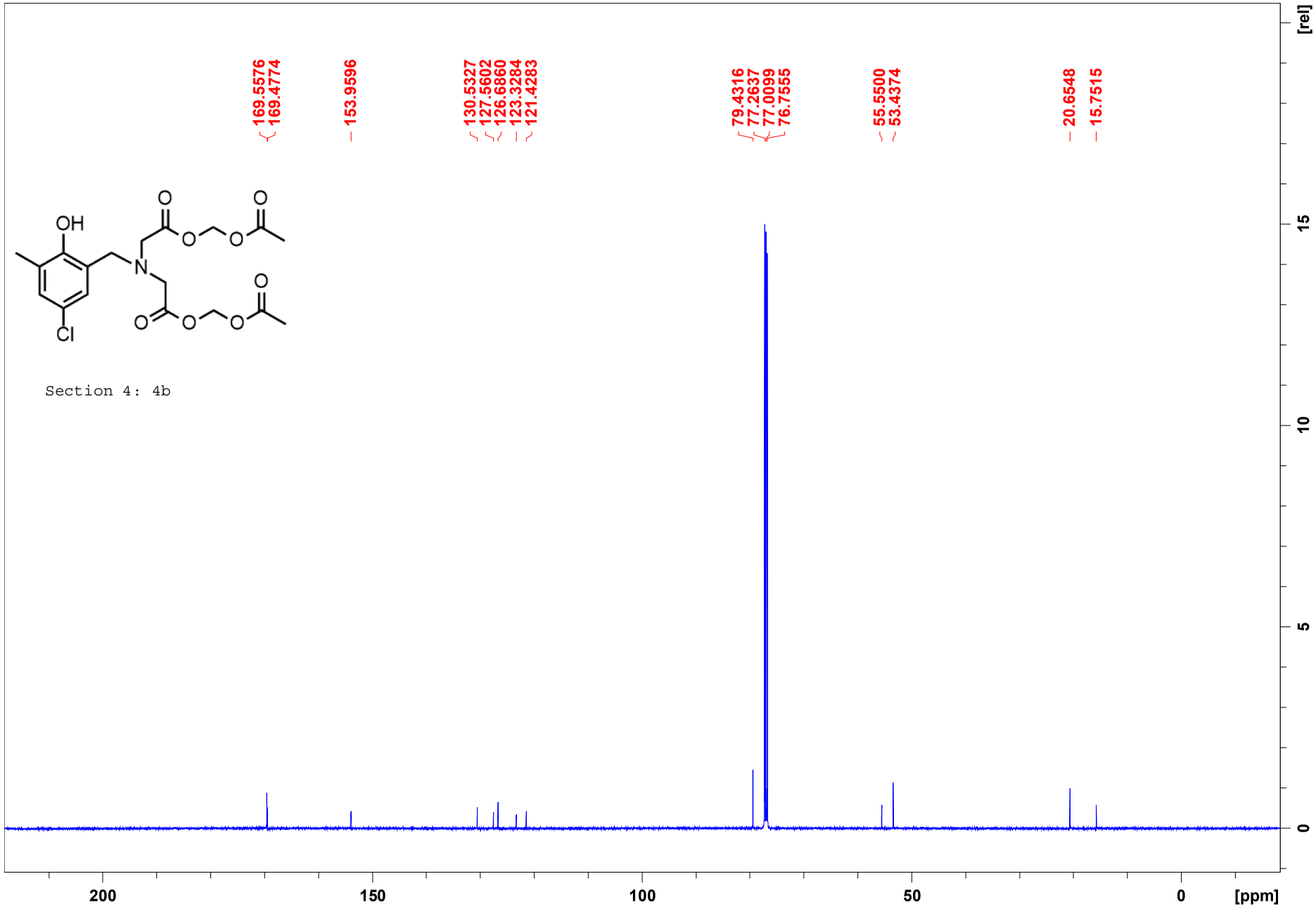
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N
HO
O

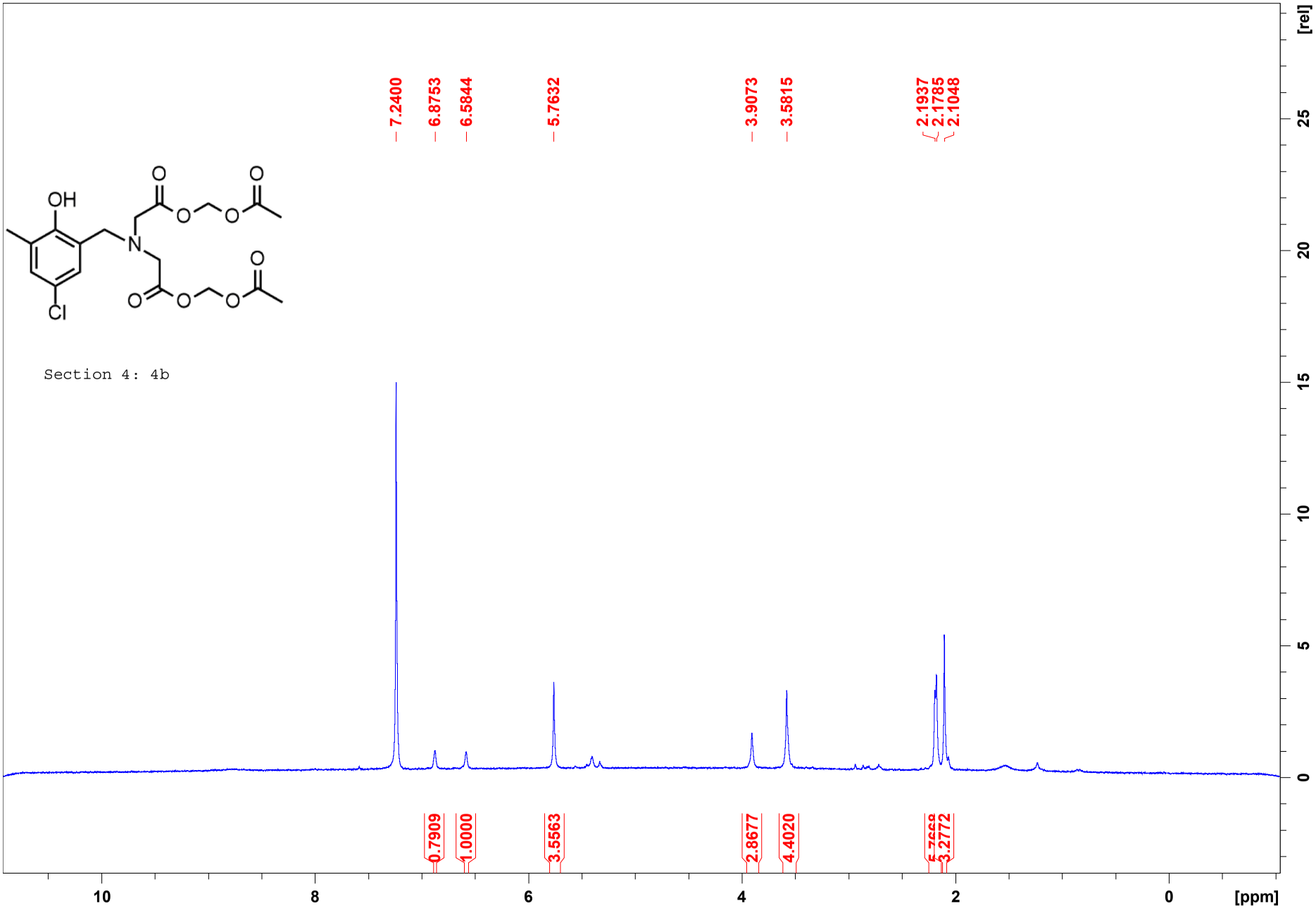

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3.7682
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3.5818
3.5346
3.5191
3.3516
3.2622
3.2401
OH
O
N
Section 4: 1h
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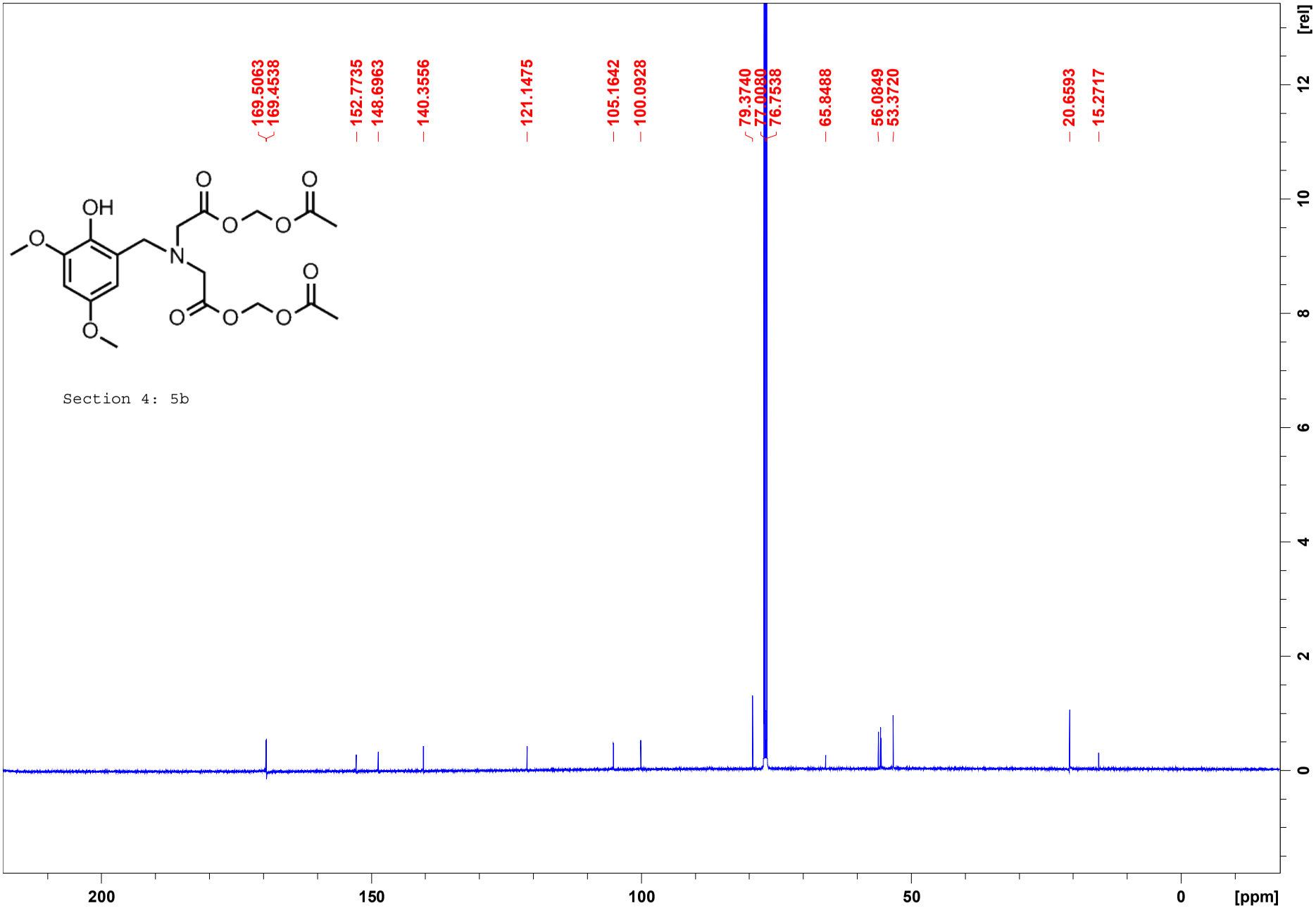

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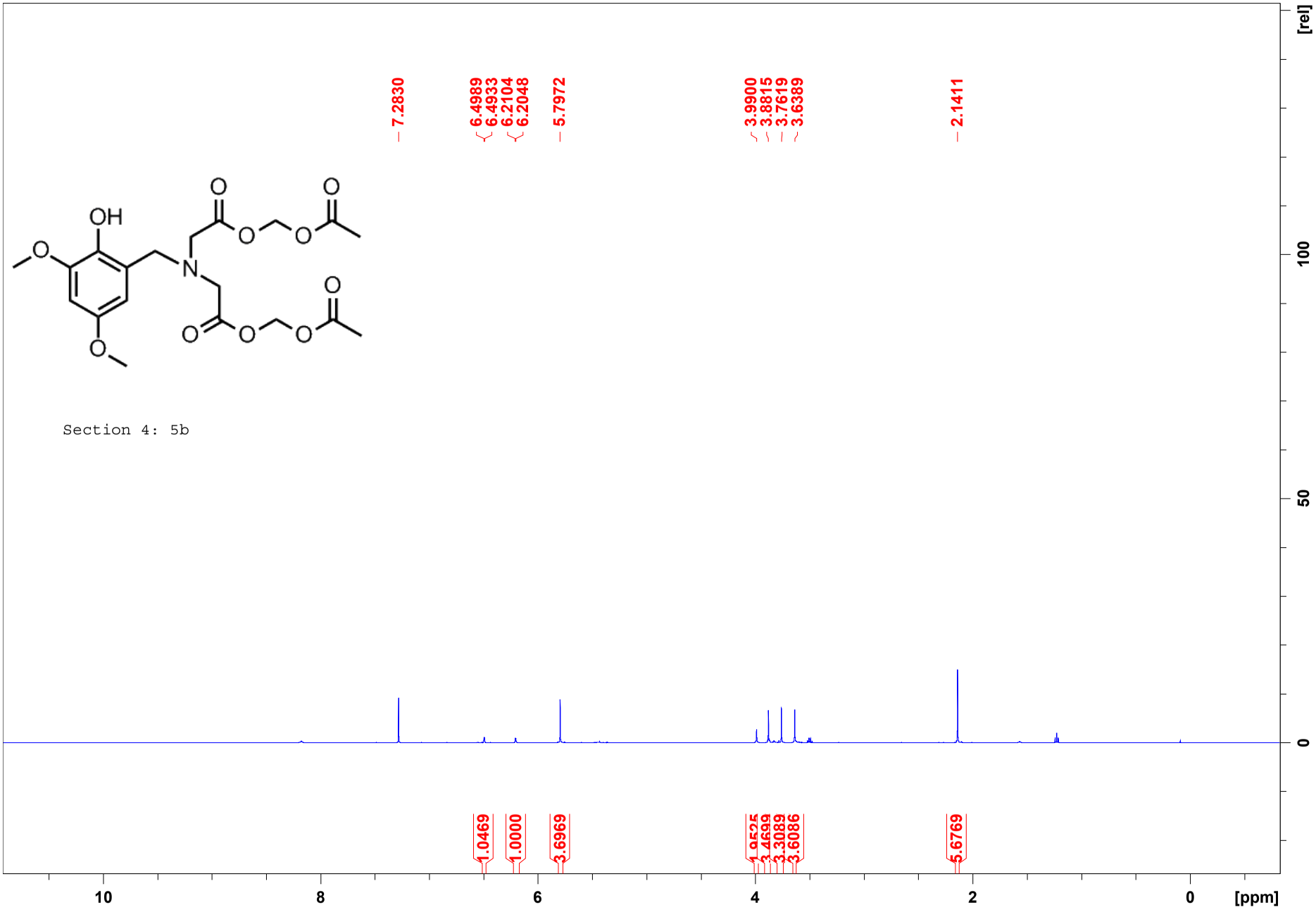

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53.3875
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20.3293
15.7281
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N
O
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3.9043
3.5789
2.1915
2.1759
2.1516
2.1017
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N
O
Section 4: 3b
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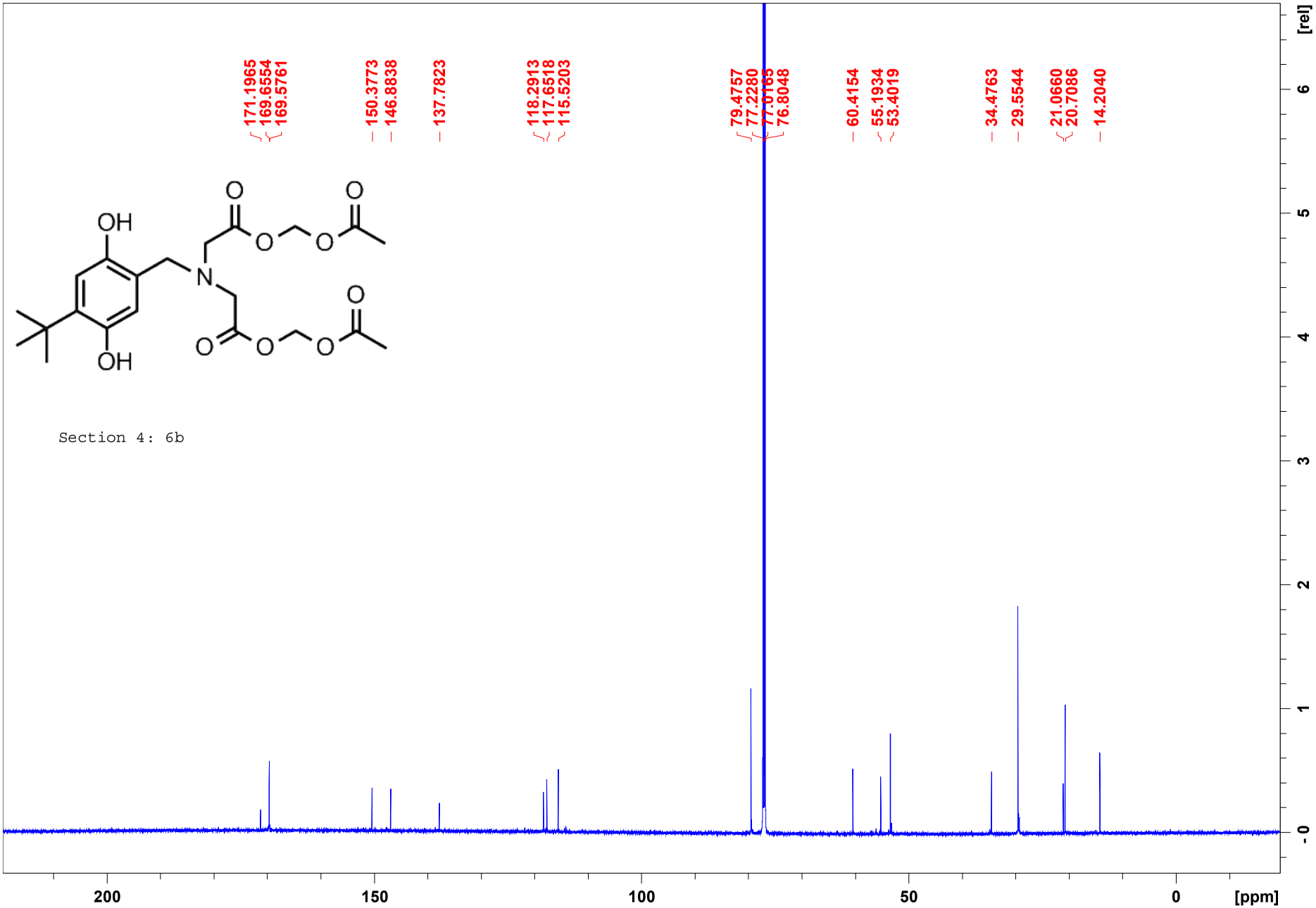

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76.7555
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53.4374
20.6548
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O
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Section 4: 4b
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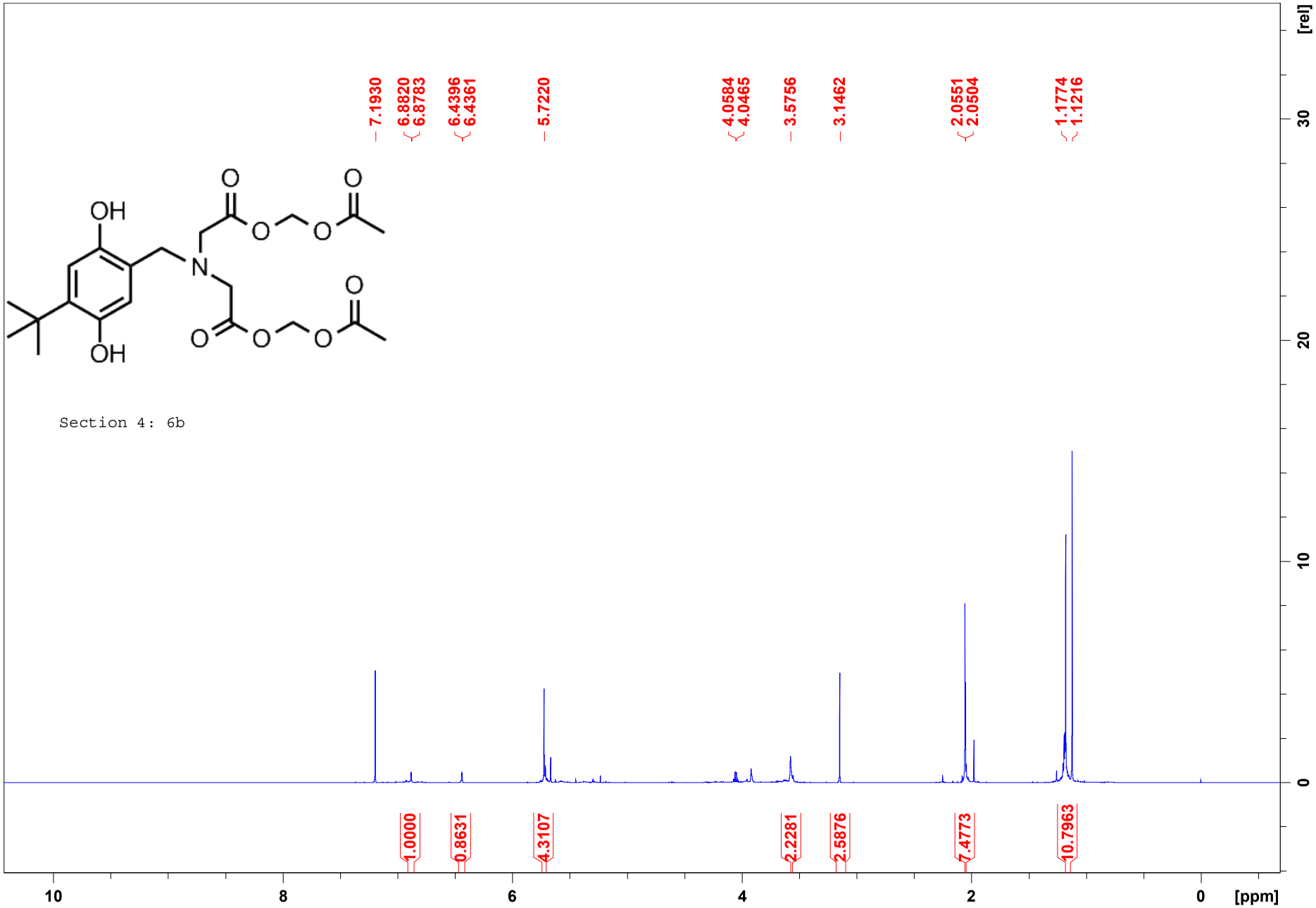

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6.5844
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3.9073
3.5815
2.1937
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O
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O
Cl
Section 4: 4b
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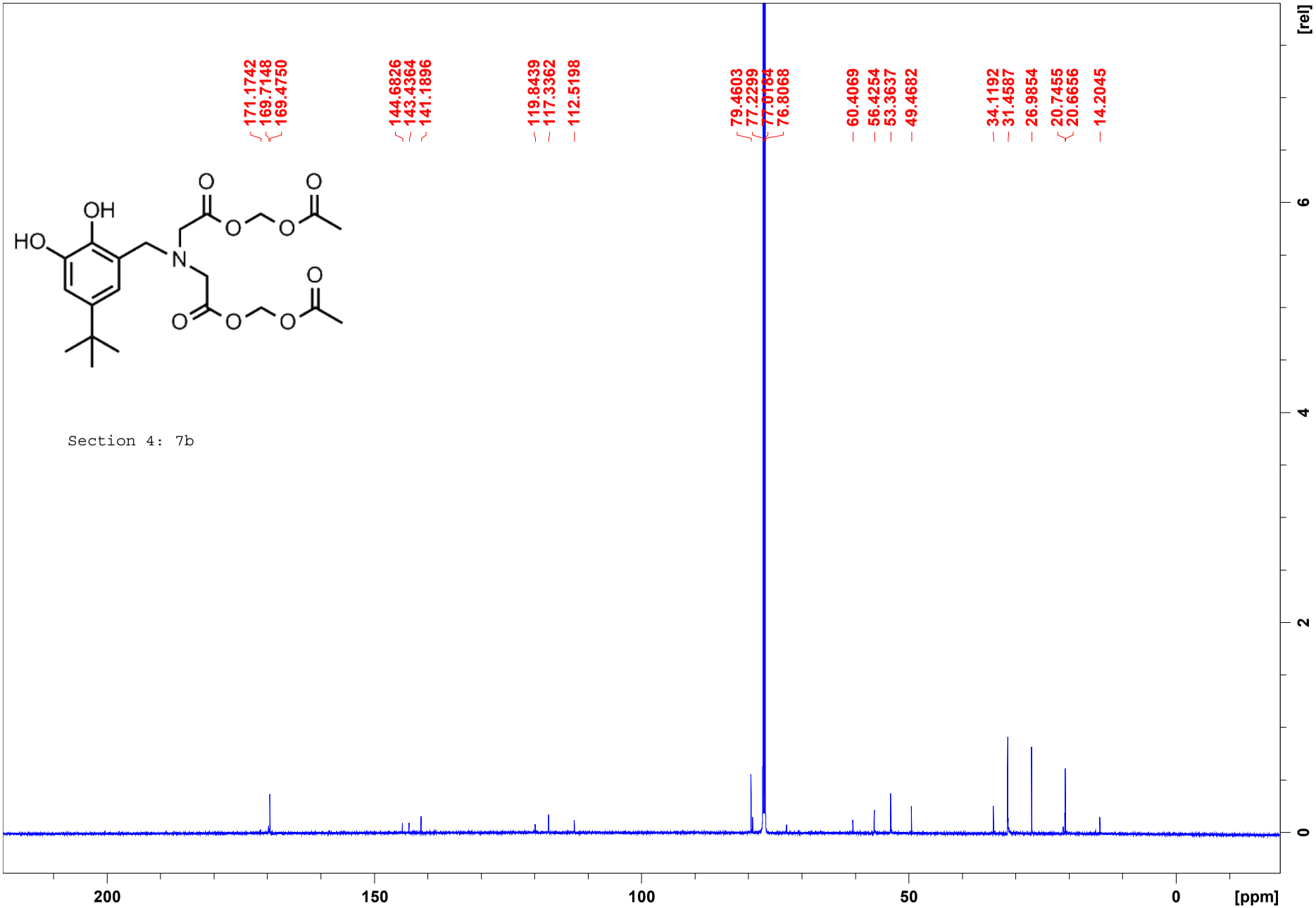

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56.0849
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15.2717
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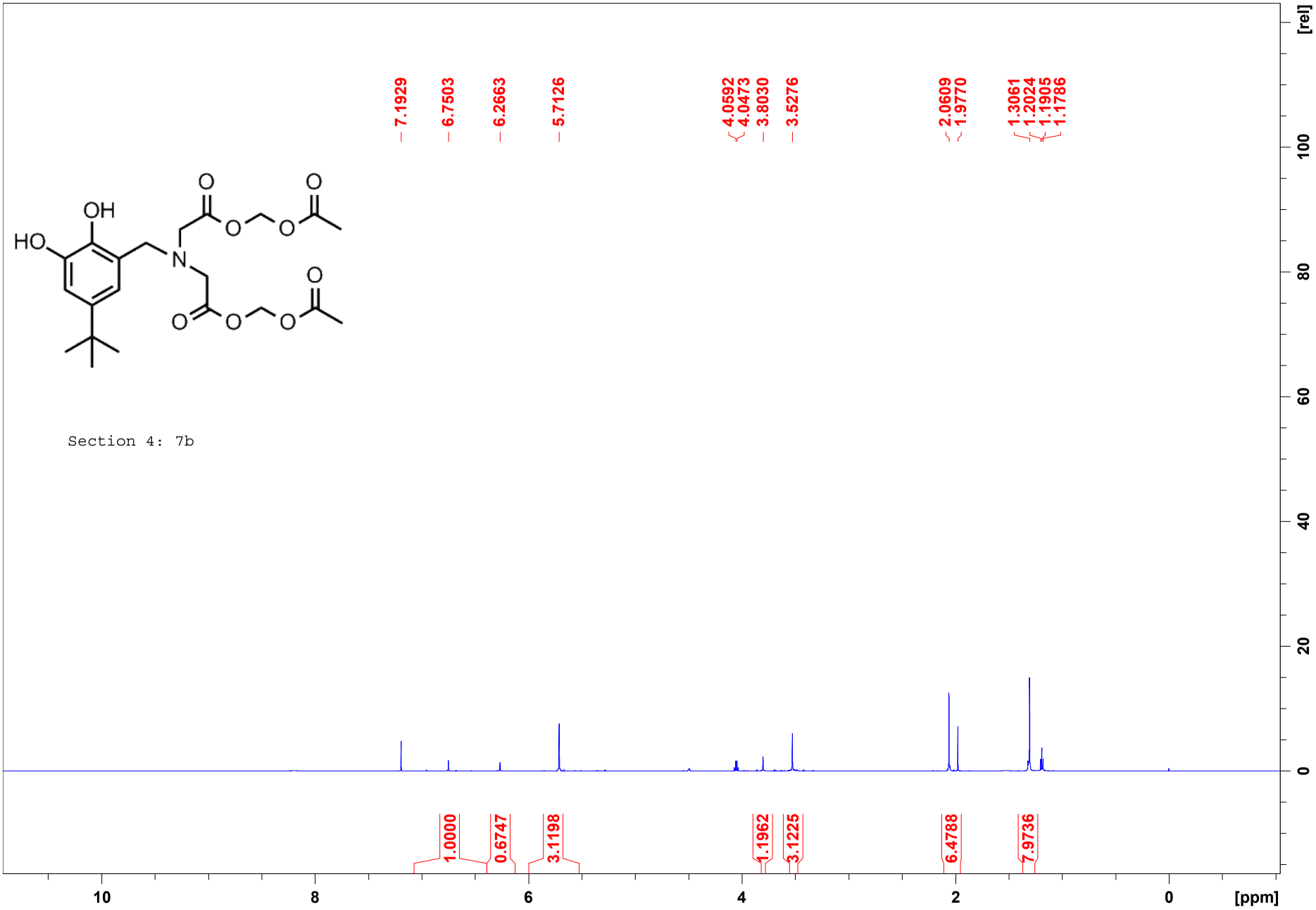

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3.7619
3.6389
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O
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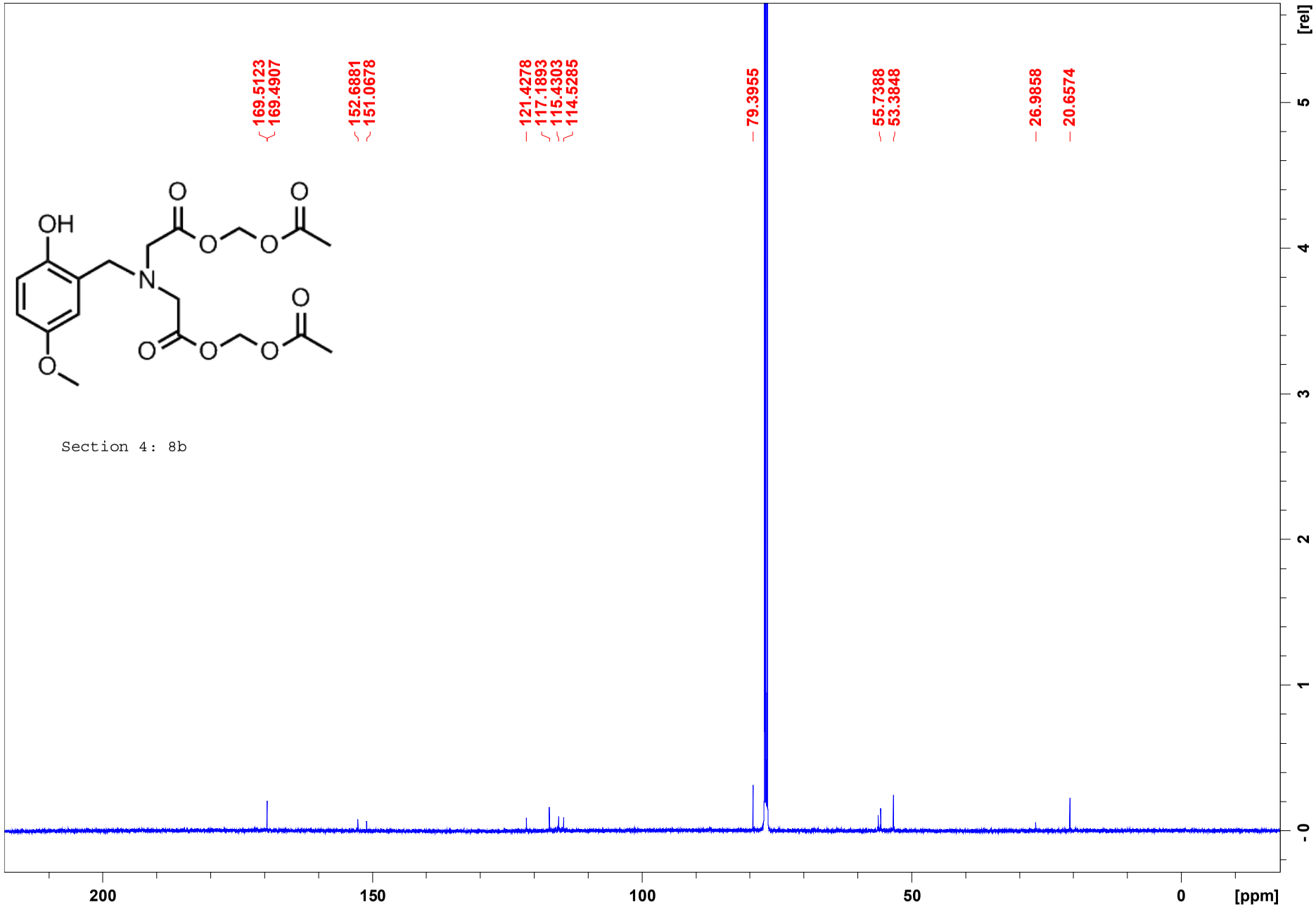
Section 4: 5b

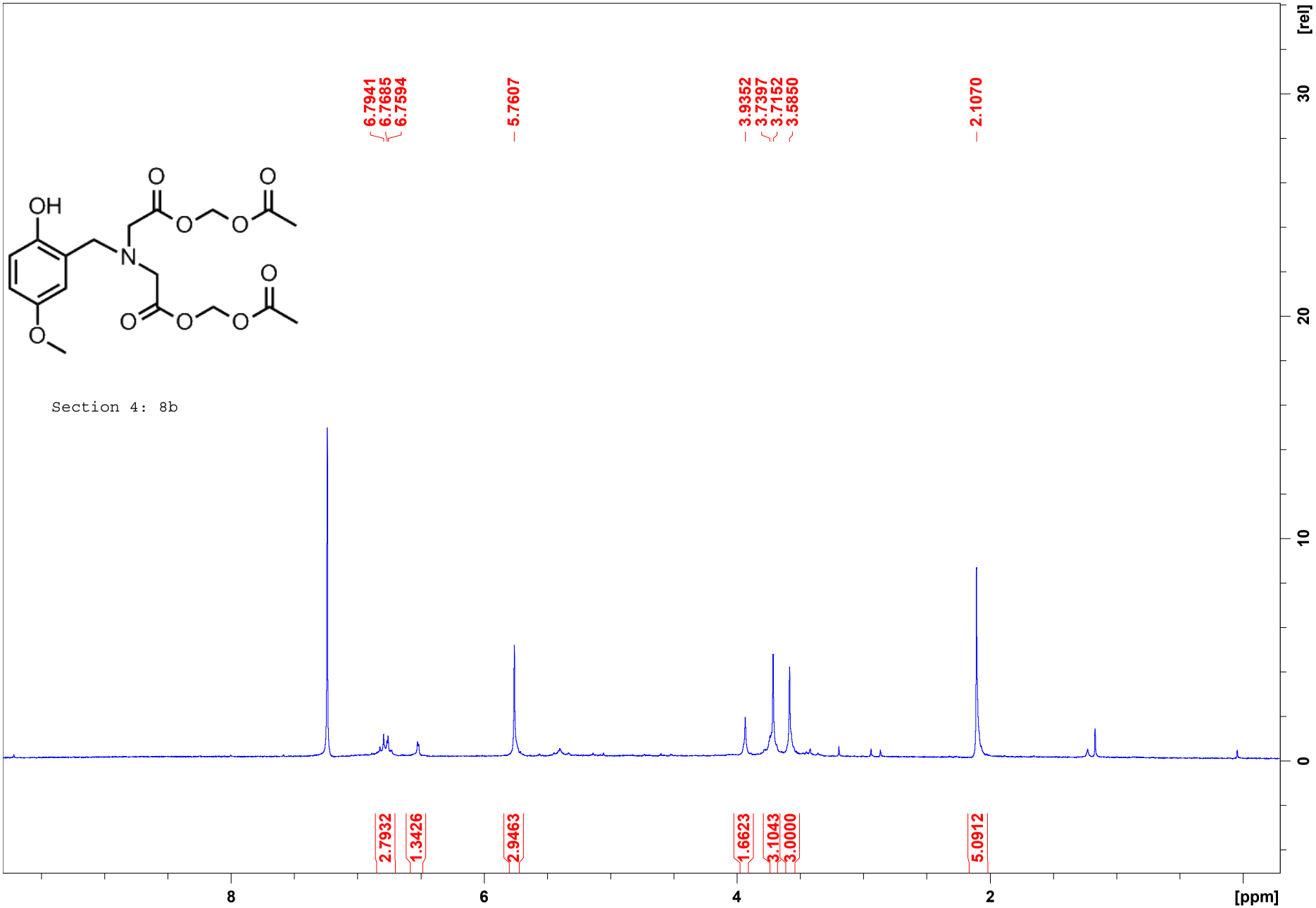

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77.0165
76.8048
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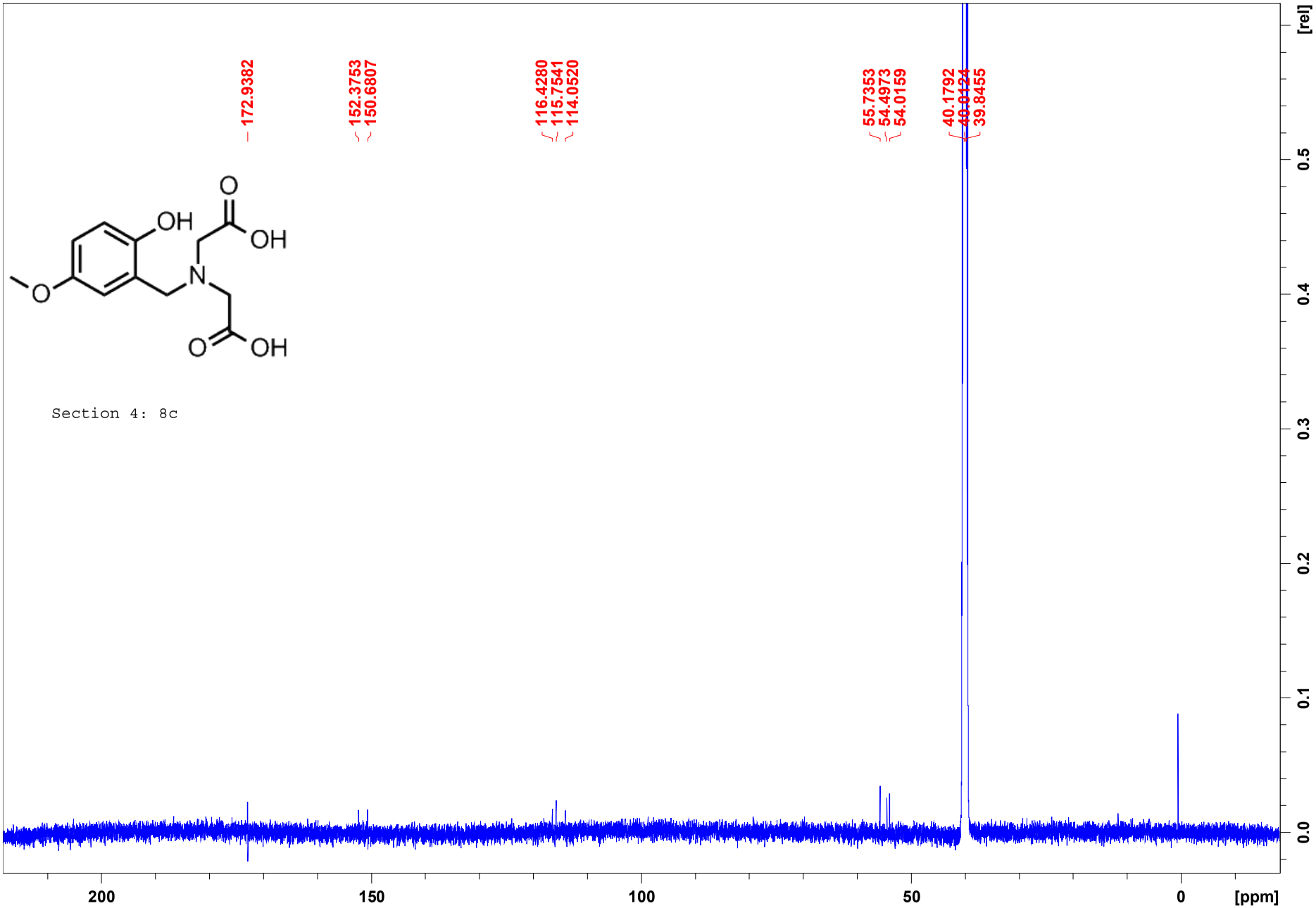

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2.0551
2.0504
1.1774
1.1216
OH
OH
O
O
O
O
O
O
N
Section 4: 6b
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2.2281
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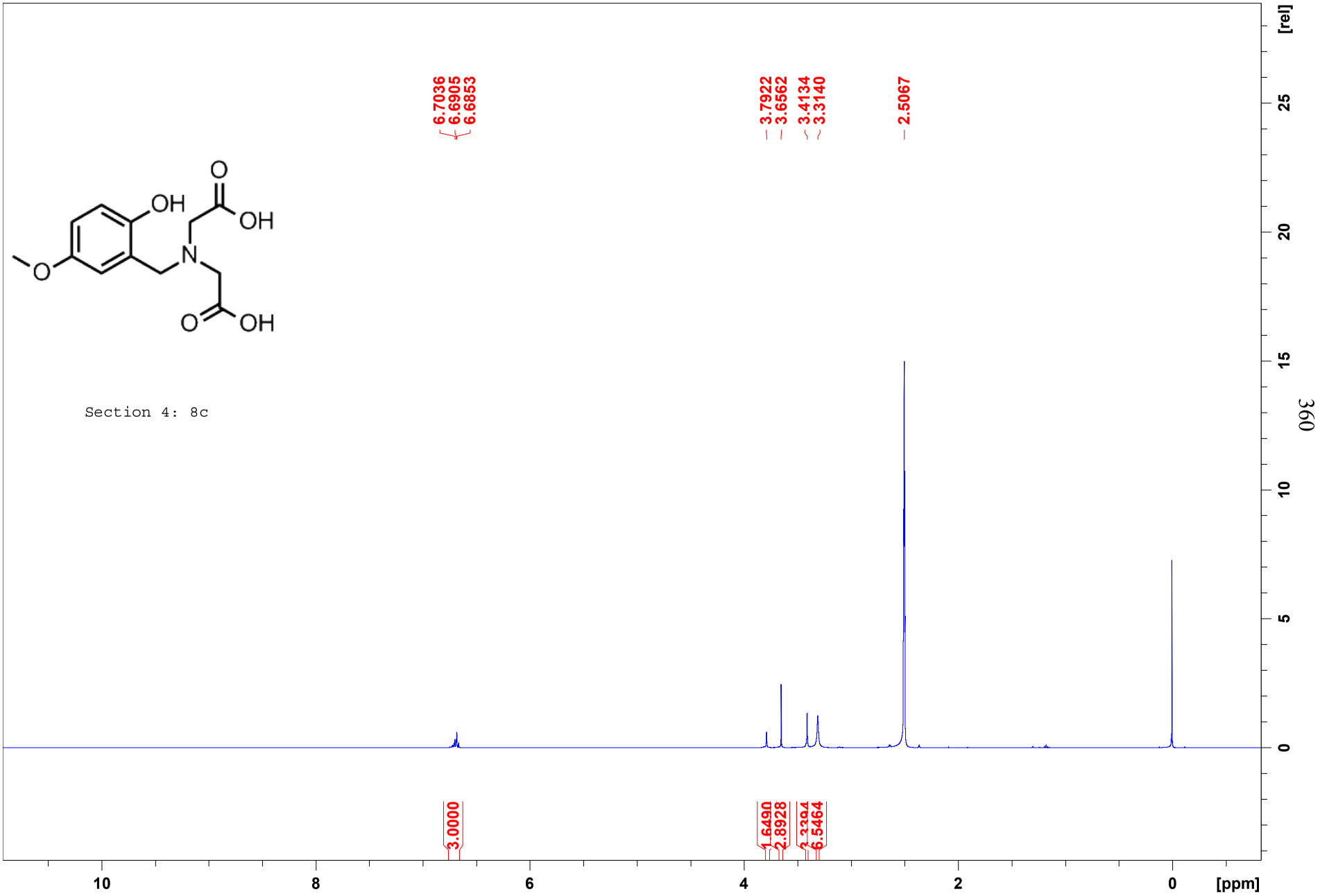

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76.8068
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49.4682
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20.6656
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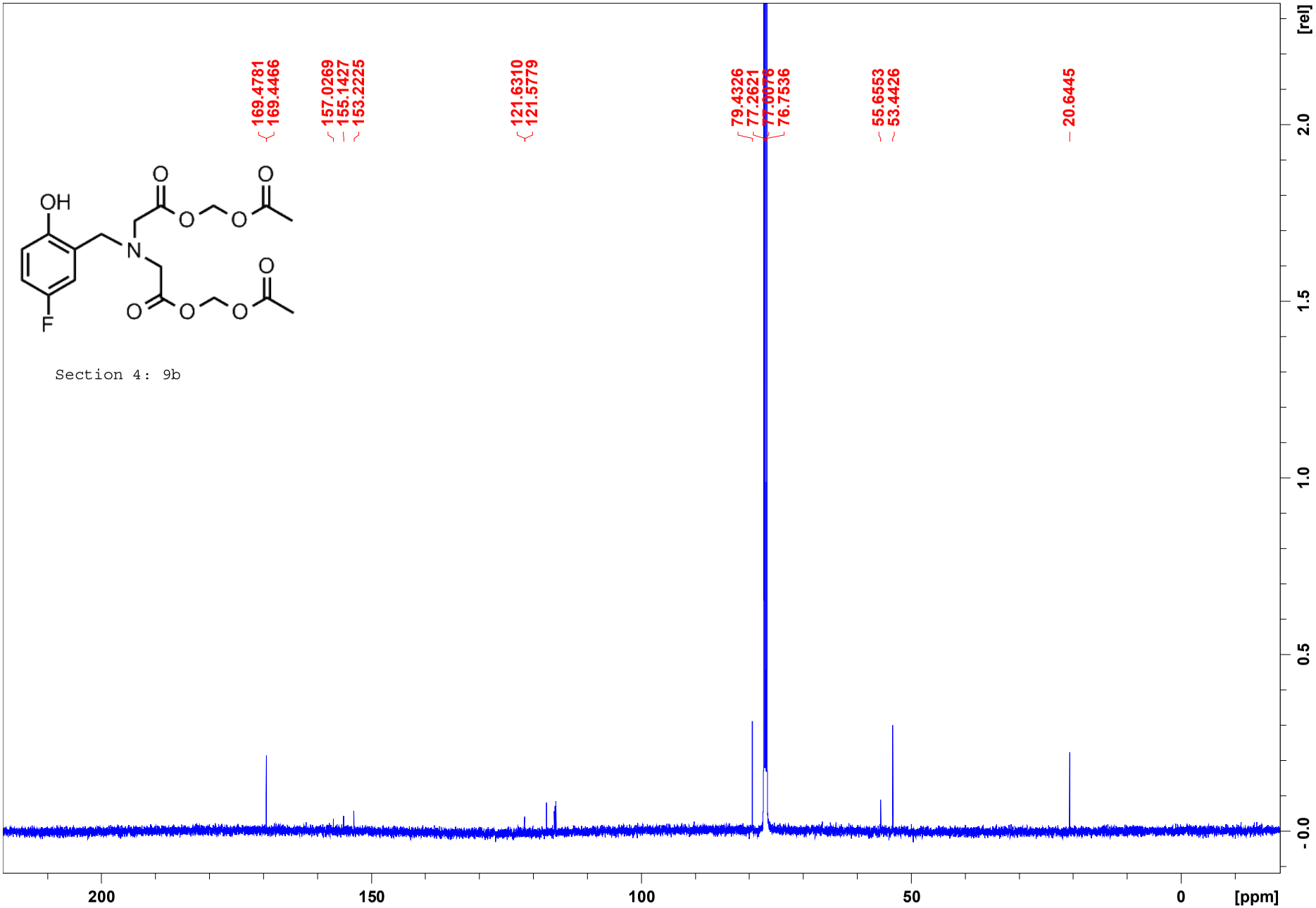

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1.1786
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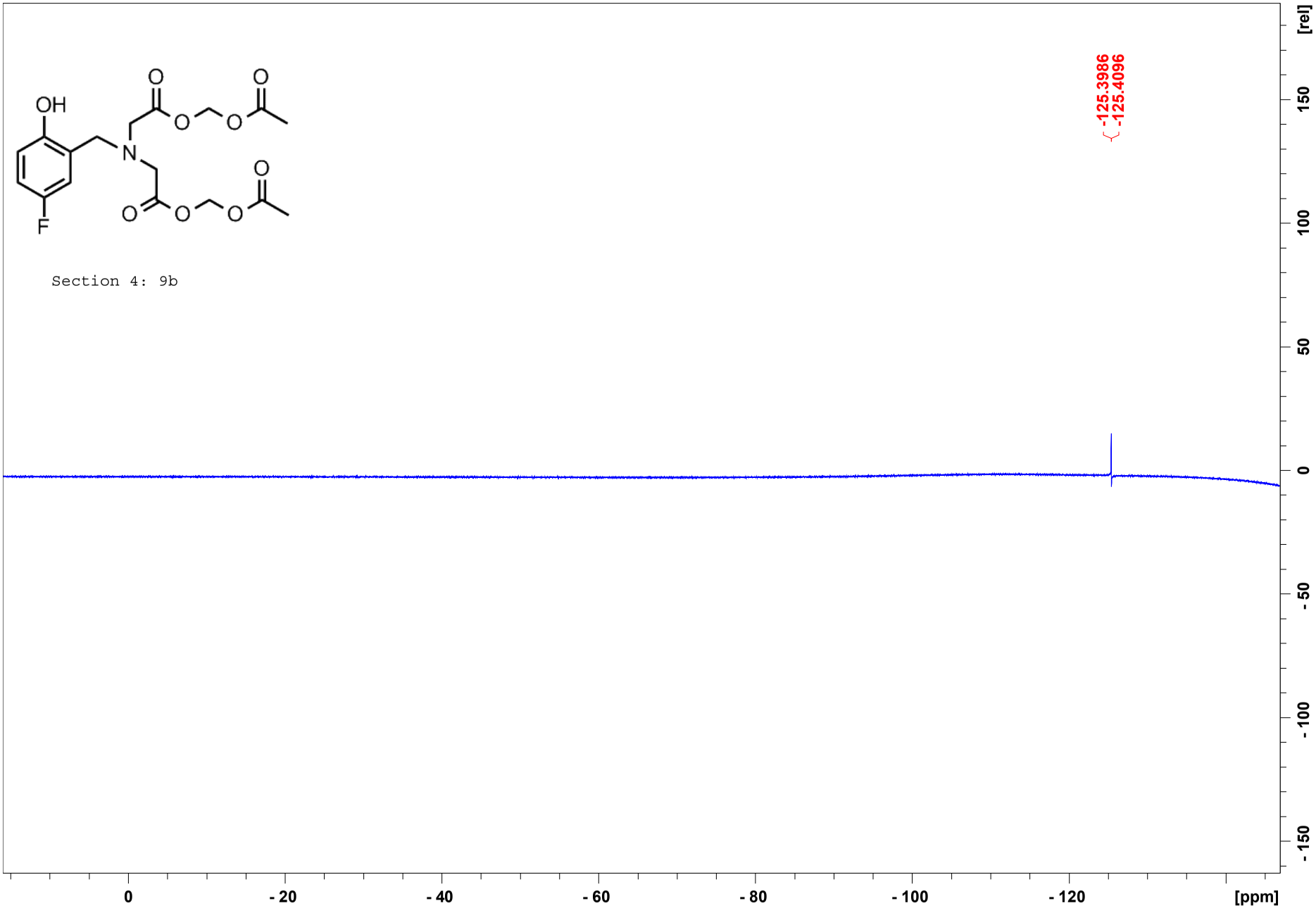

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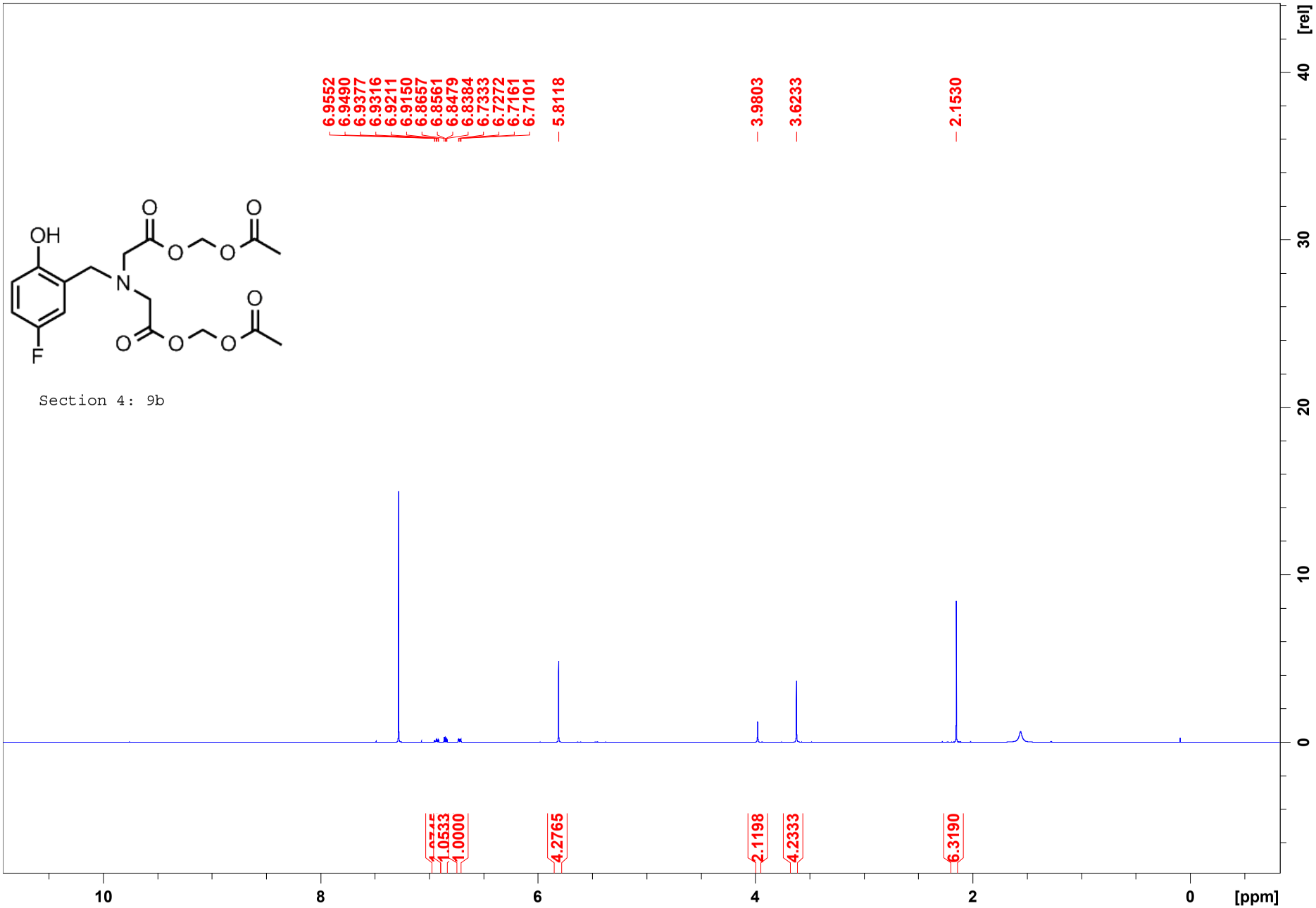

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O
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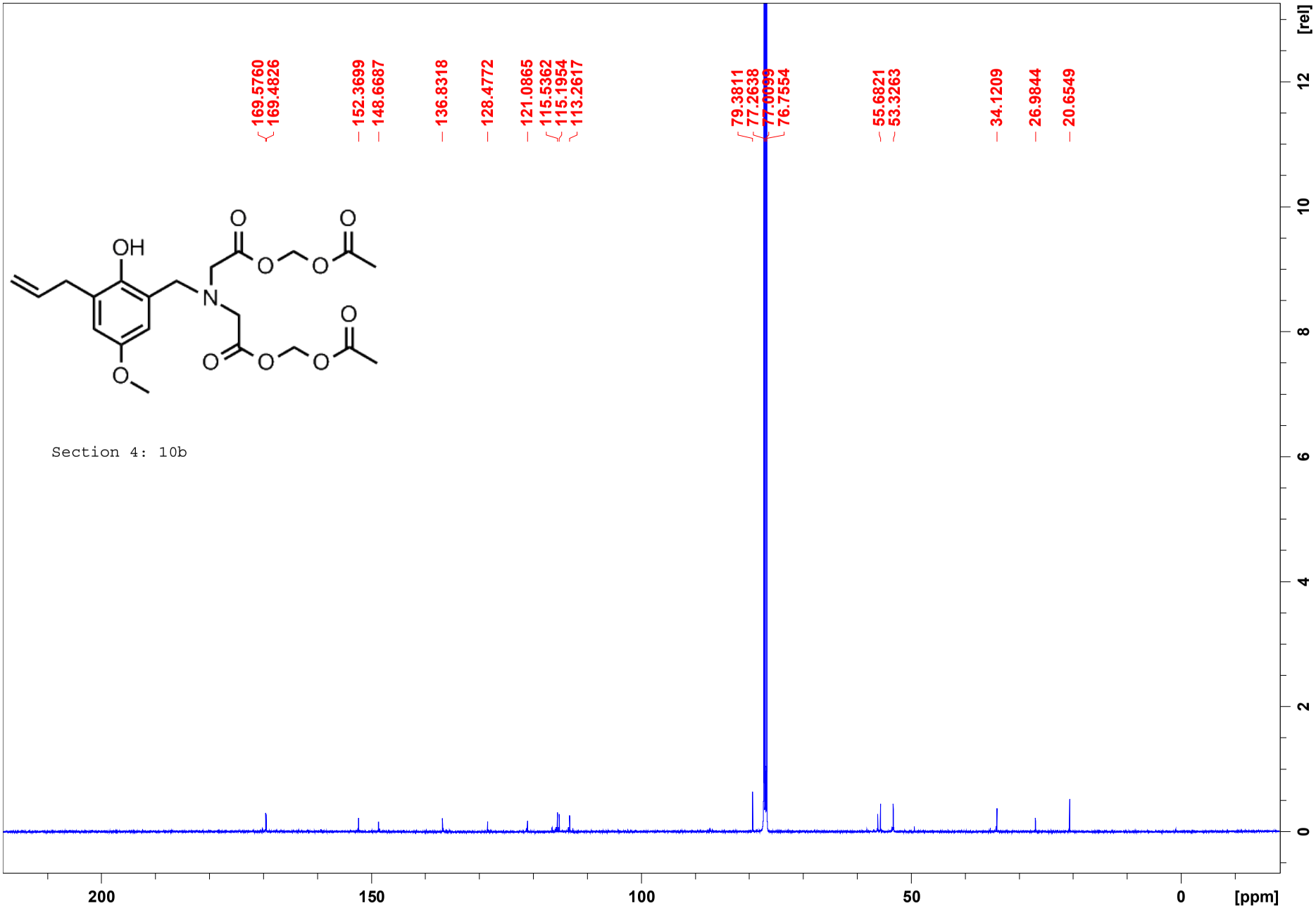

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O
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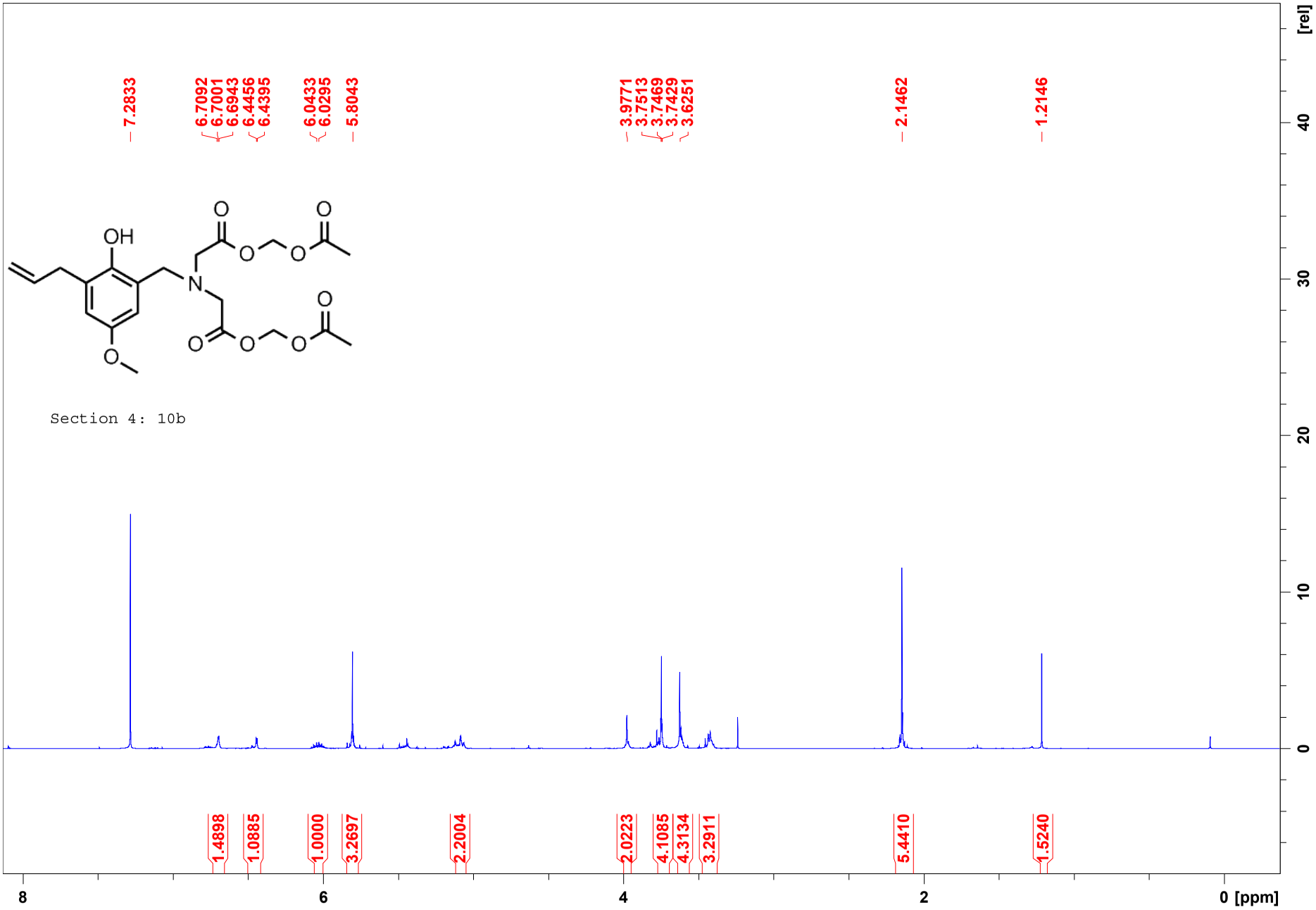

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OH
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O
OH
Section 4: 8c
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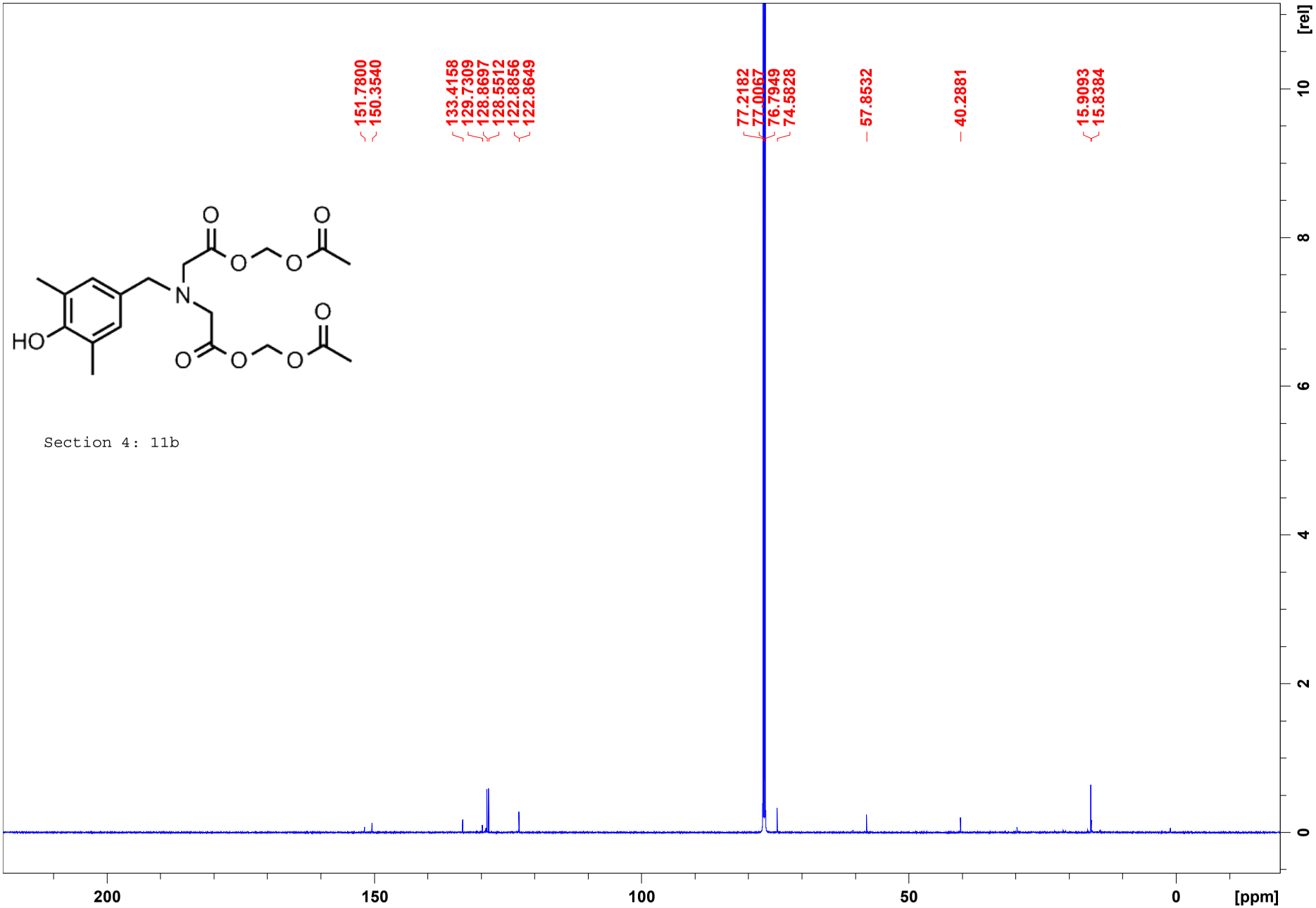

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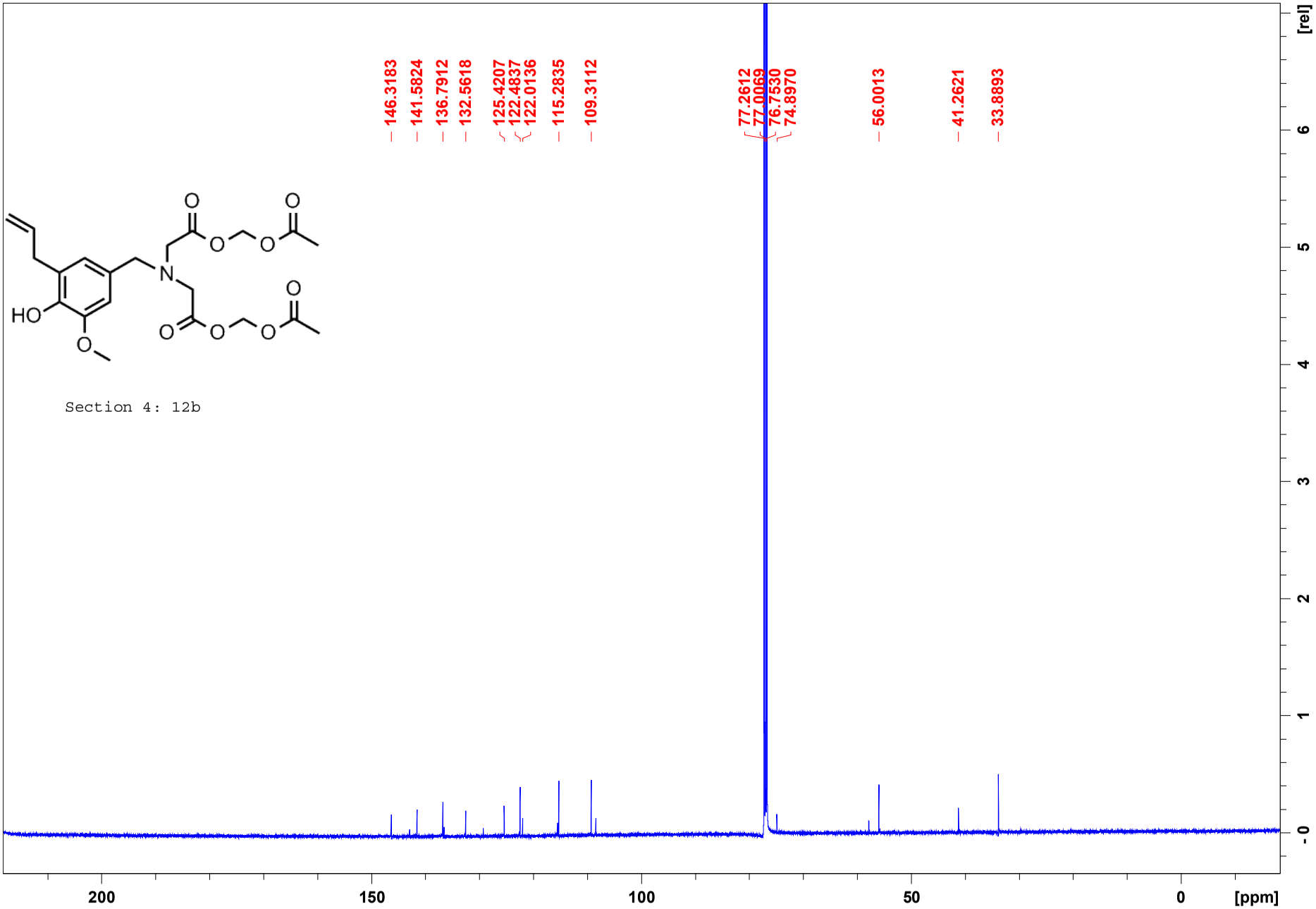

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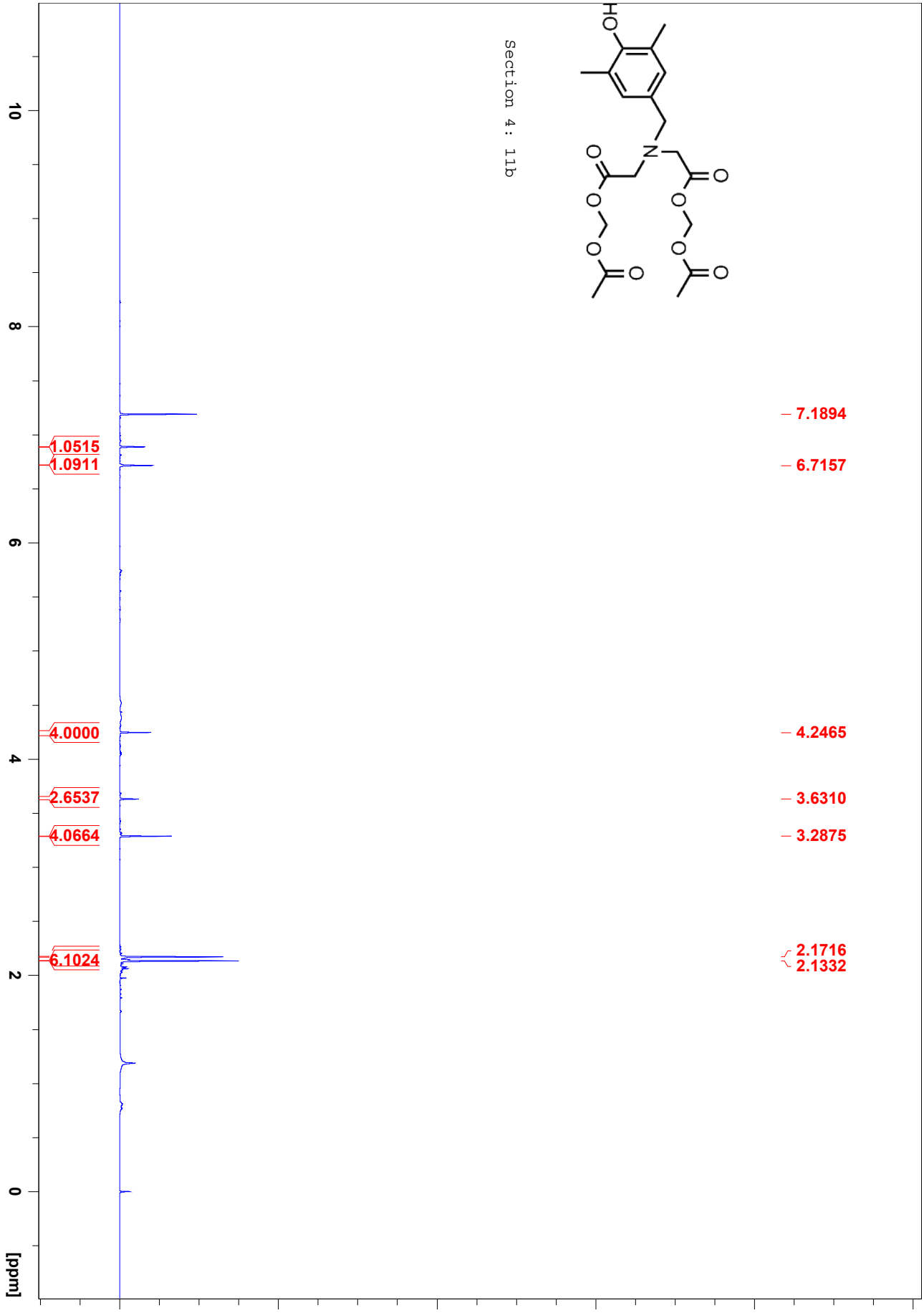

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6.7333
6.7272
6.7161
6.7101
5.8118
3.9803
3.6233
2.1530
OH
O
O
O
O
N
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O
O
F
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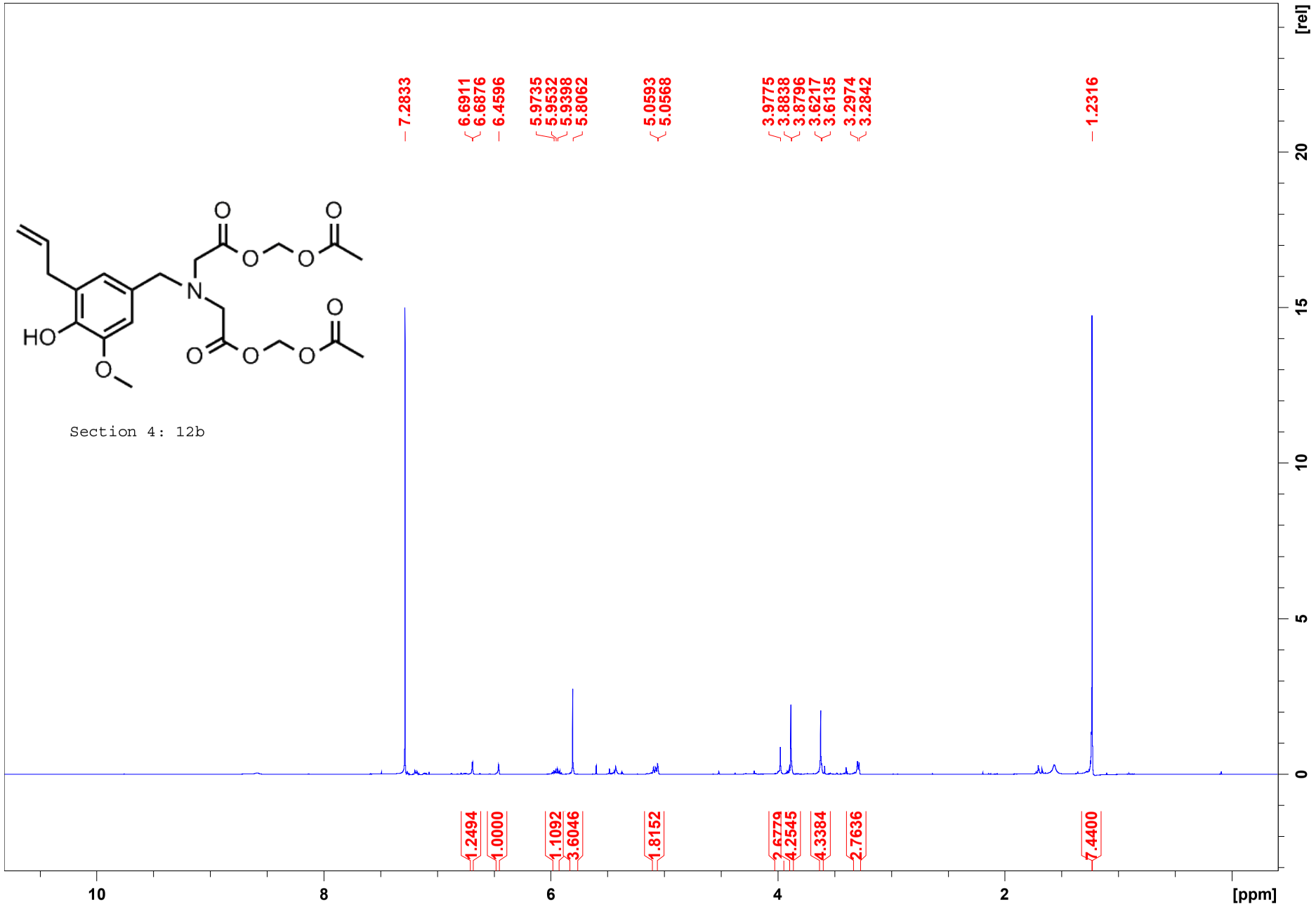

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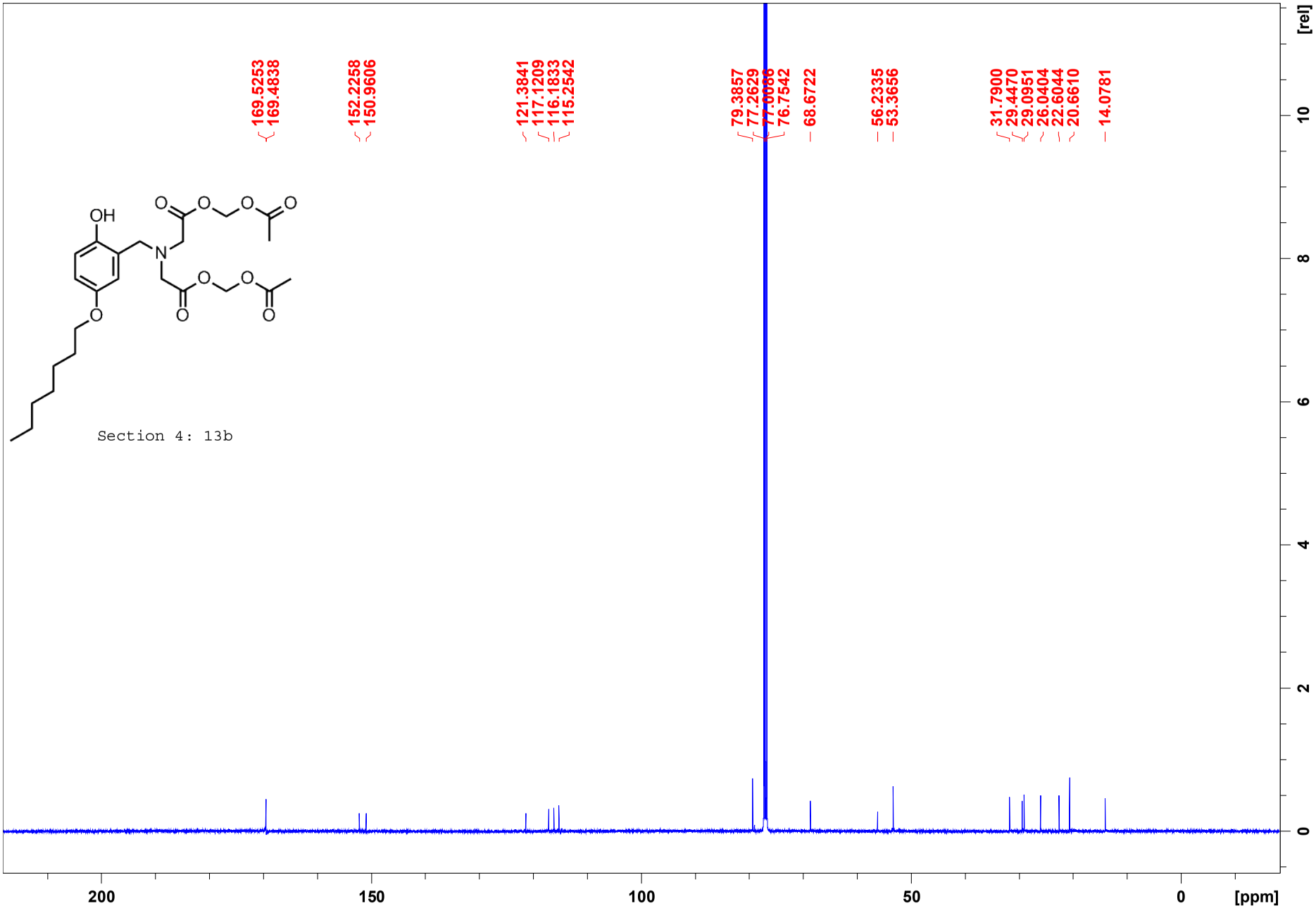

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N
Section 4: 10b
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3.2697
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2.0223
4.1085
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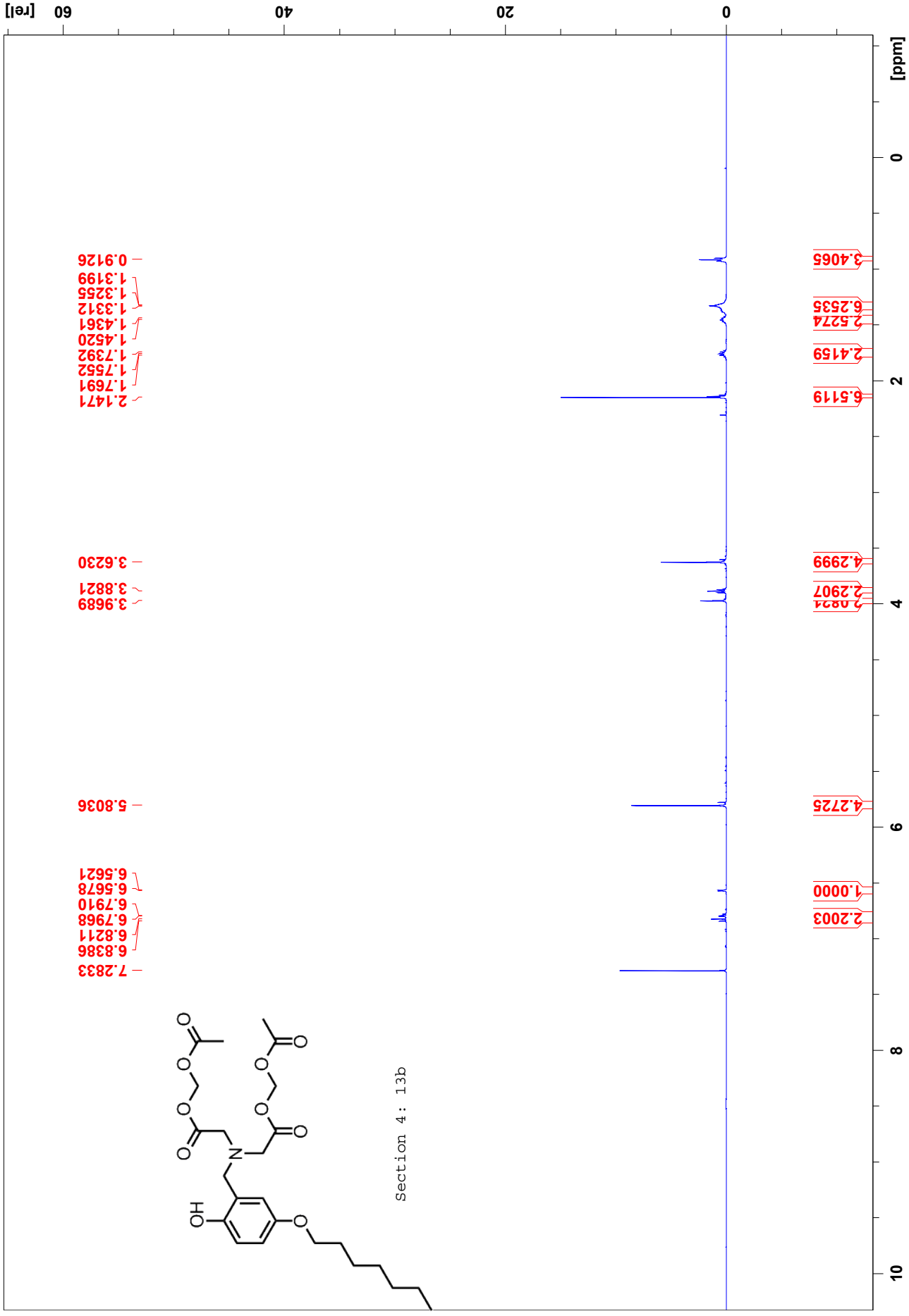

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O
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Section 4: 11b
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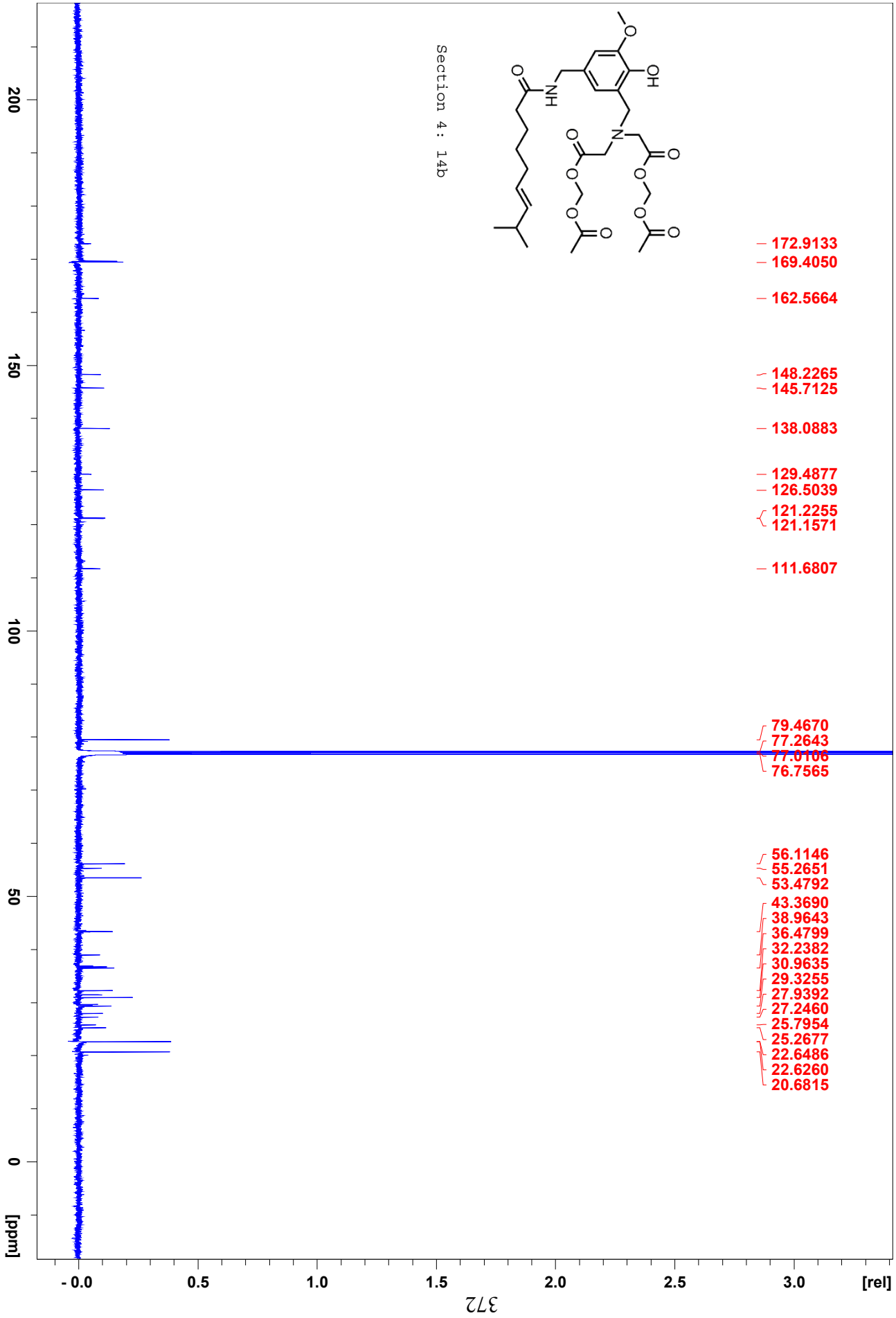

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74.8970
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33.8893
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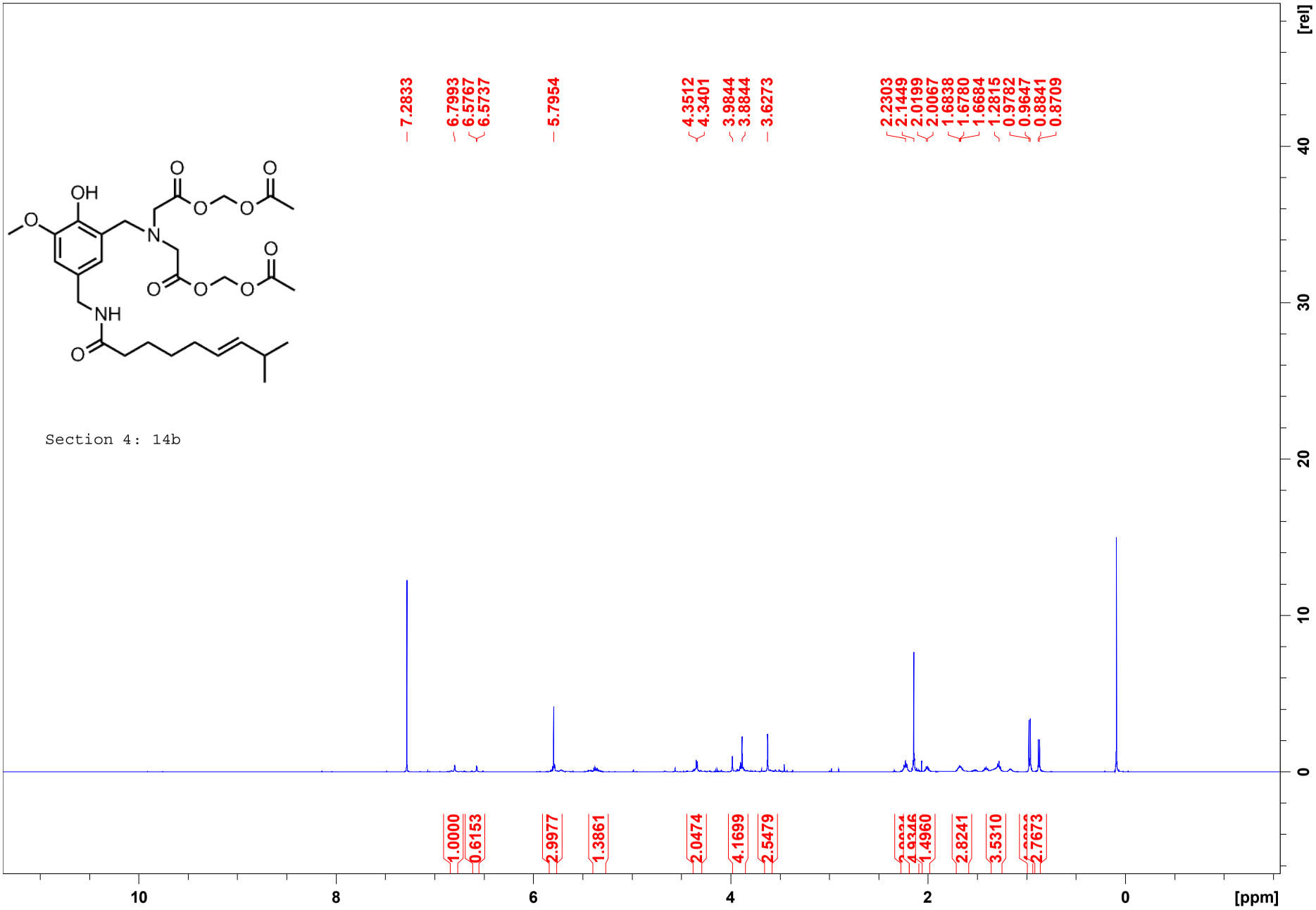

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O
O
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4.2465
3.6310
3.2875
2.1716
2.1332
1.0515
1.0911
4.0000
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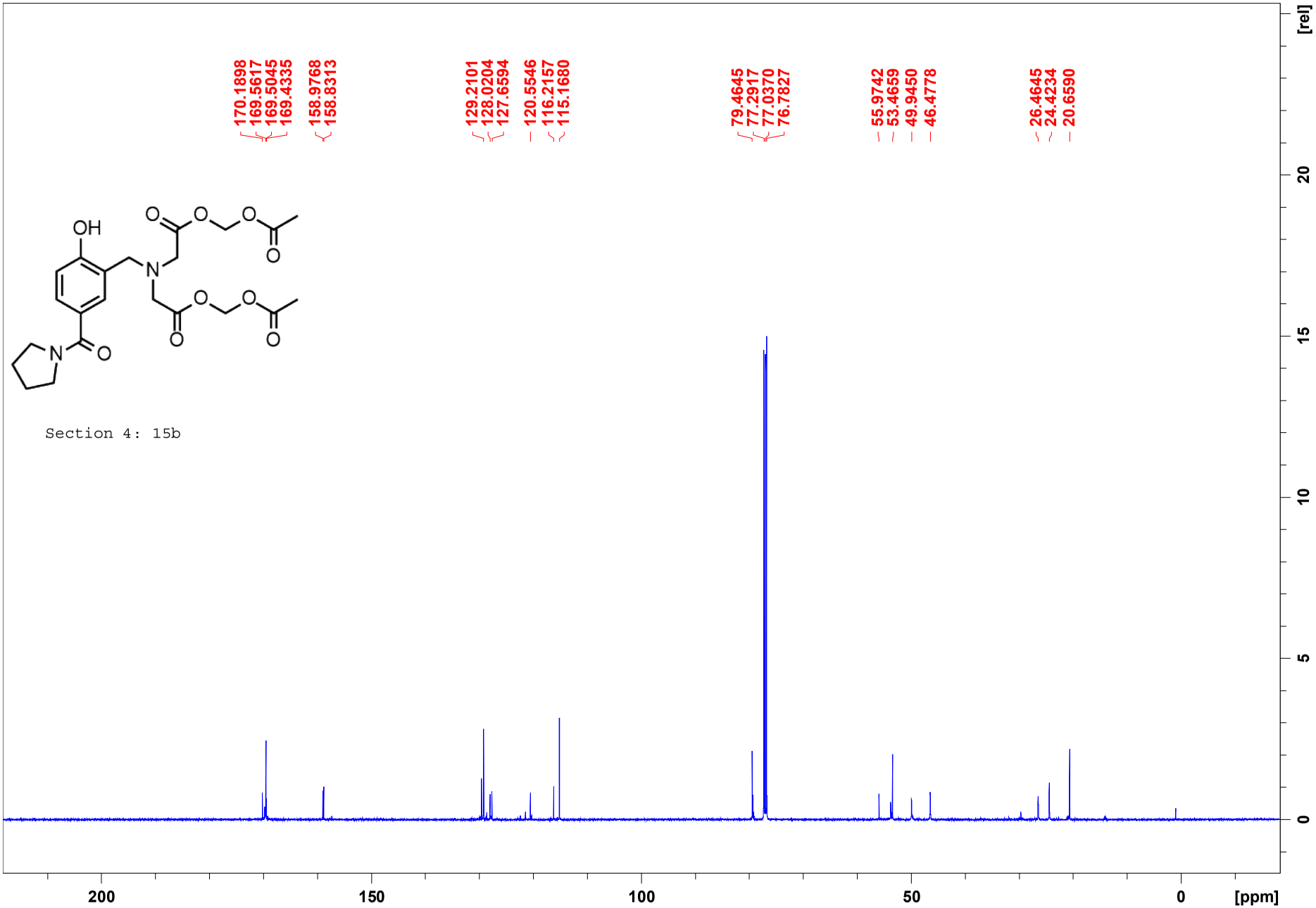

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5.0568
3.9775
3.8838
3.8796
3.6217
3.6135
3.2974
3.2842
1.2316
1.2494
1.0000
1.1092
3.6046
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2.6779
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4.3384
2.7636
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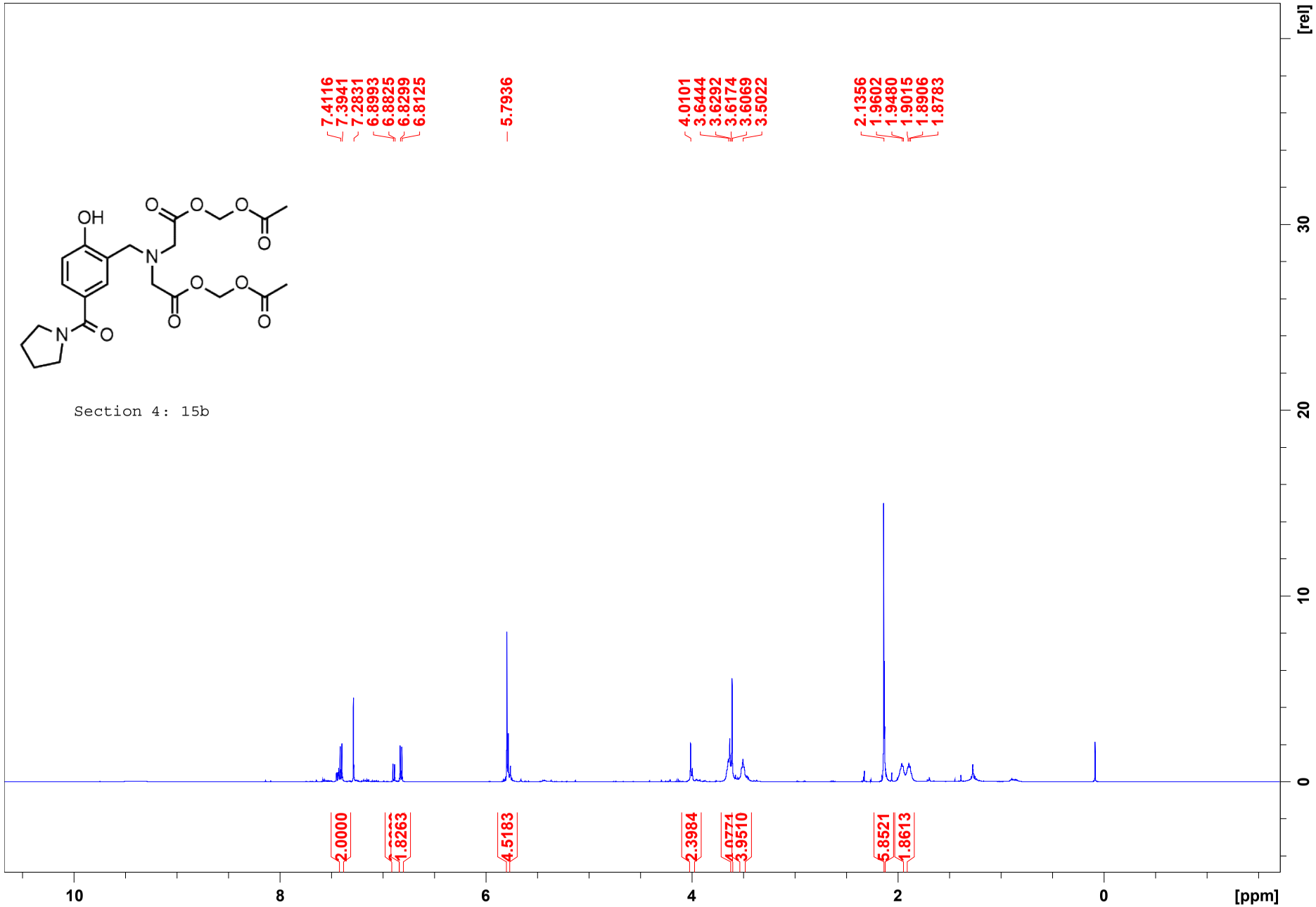

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117.1209
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76.7542
68.6722
56.2335
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31.7900
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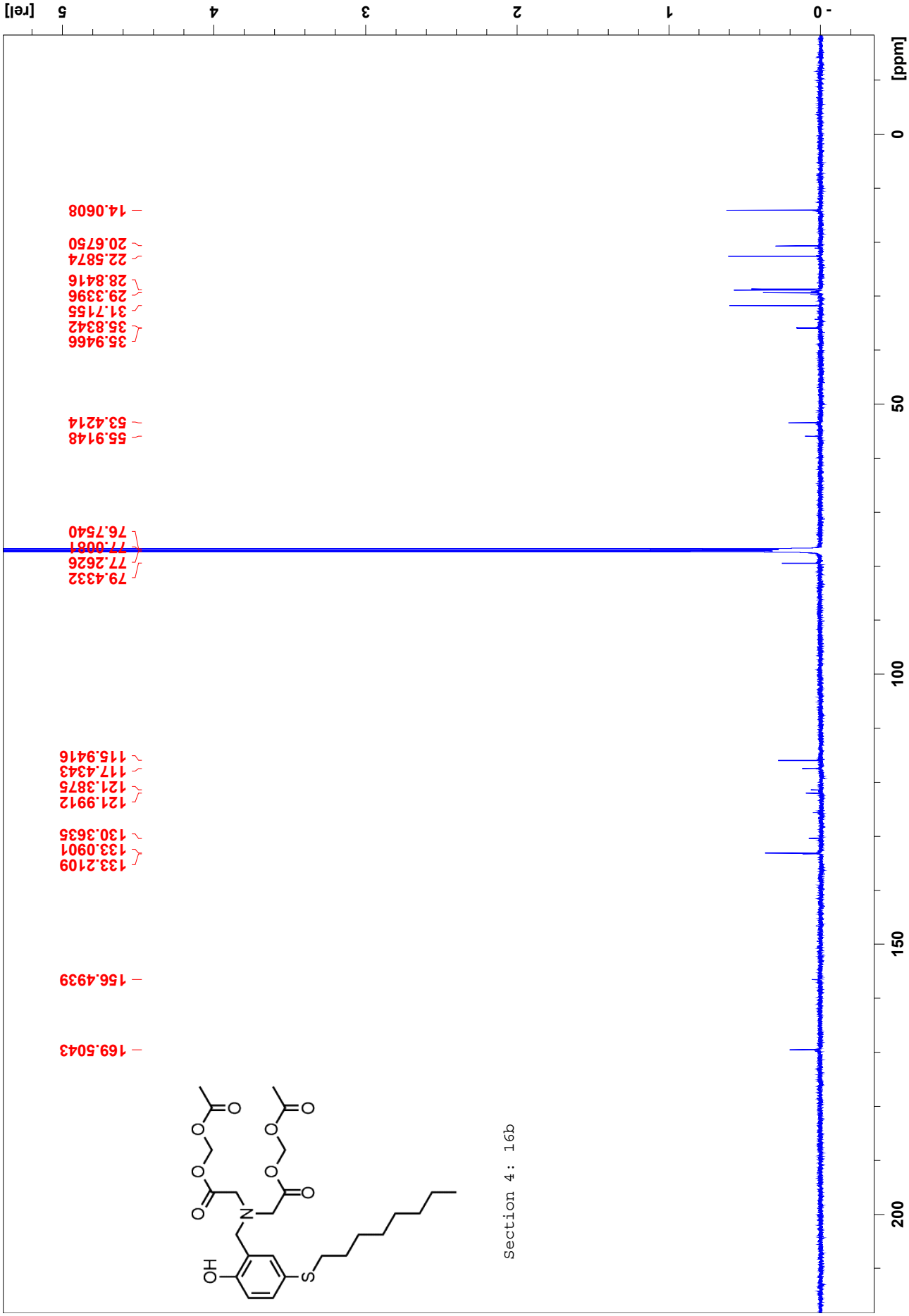

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2.2907
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4.2725
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2.2003
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1.3312
1.4361
1.4520
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1.7552
1.7691
2.1471
3.6230
3.8821
3.9689
5.8036
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6.8211
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Section 4: 13b
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N
O

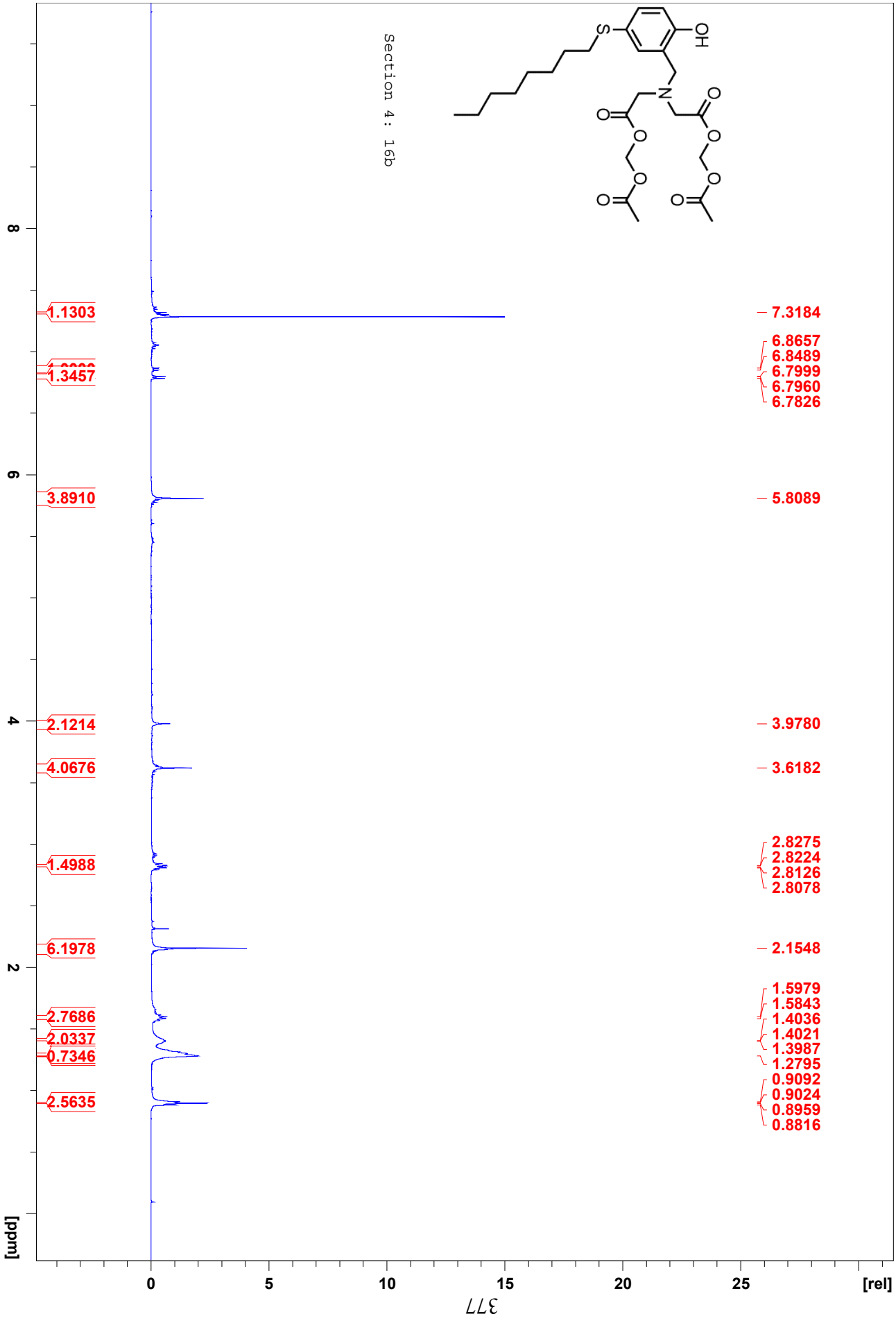

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126.5039
121.2255
121.1571
111.6807
79.4670
77.2643
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76.7565
56.1146
55.2651
53.4792
43.3690
38.9643
36.4799
32.2382
30.9635
29.3255
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27.2460
25.7954
25.2677
22.6486
22.6260
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3.0
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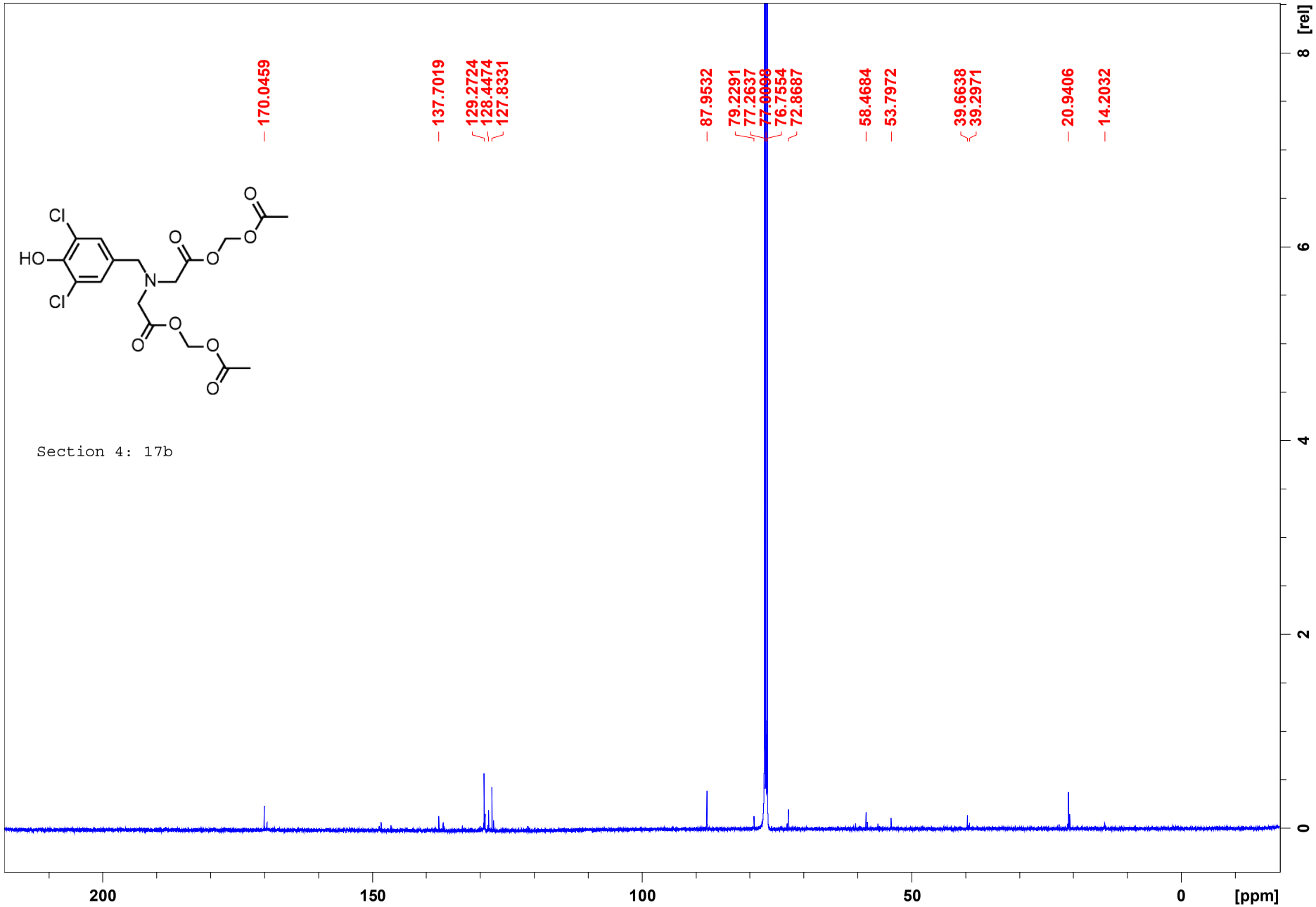

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6.5737
5.7954
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3.6273
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2.1449
2.0199
2.0067
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1.6780
1.6684
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0.8841
0.8709
OH
O
N
NH
Section 4: 14b
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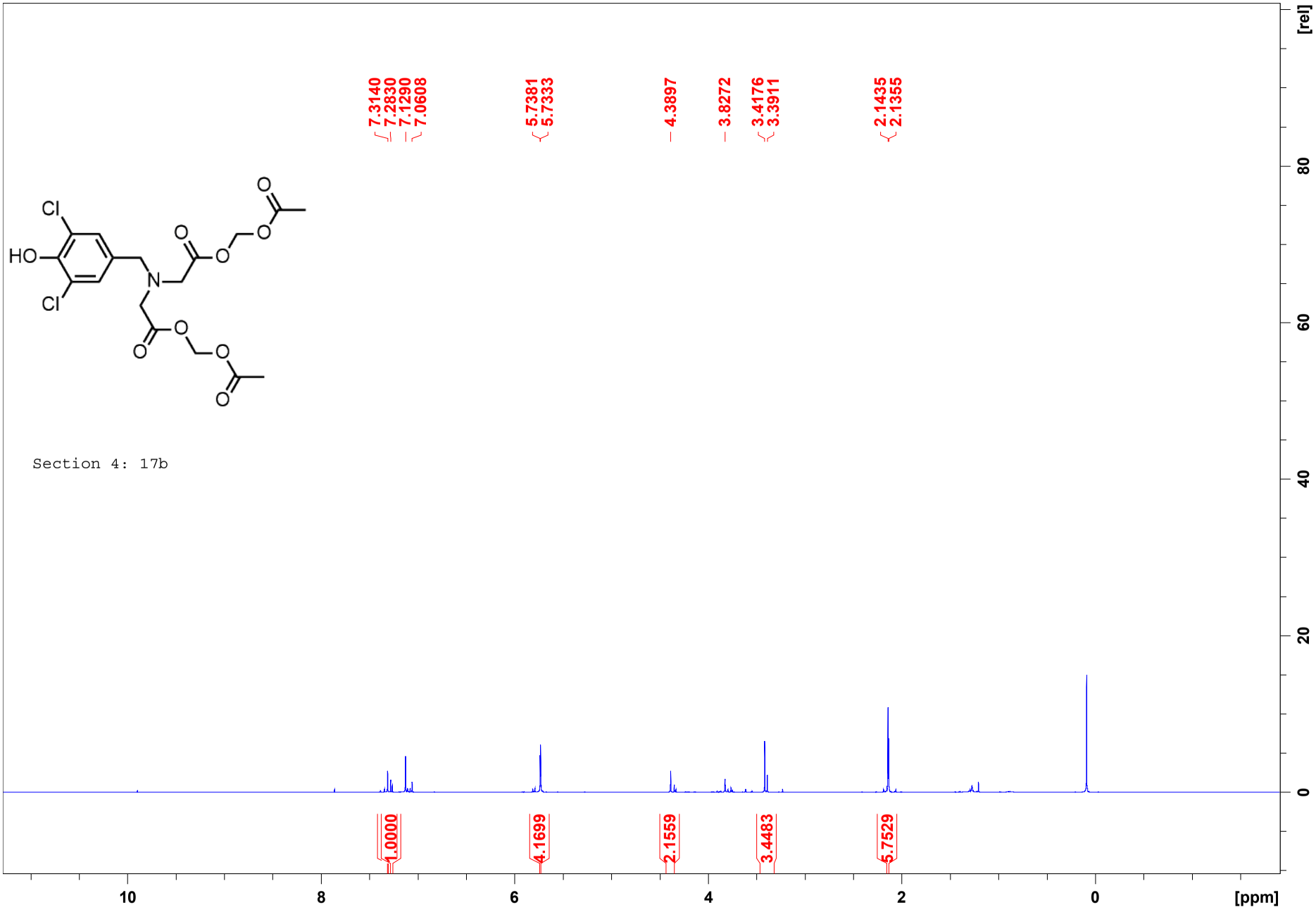

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120.5546
116.2157
115.1680
79.4645
77.2917
77.0370
76.7827
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46.4778
26.4645
24.4234
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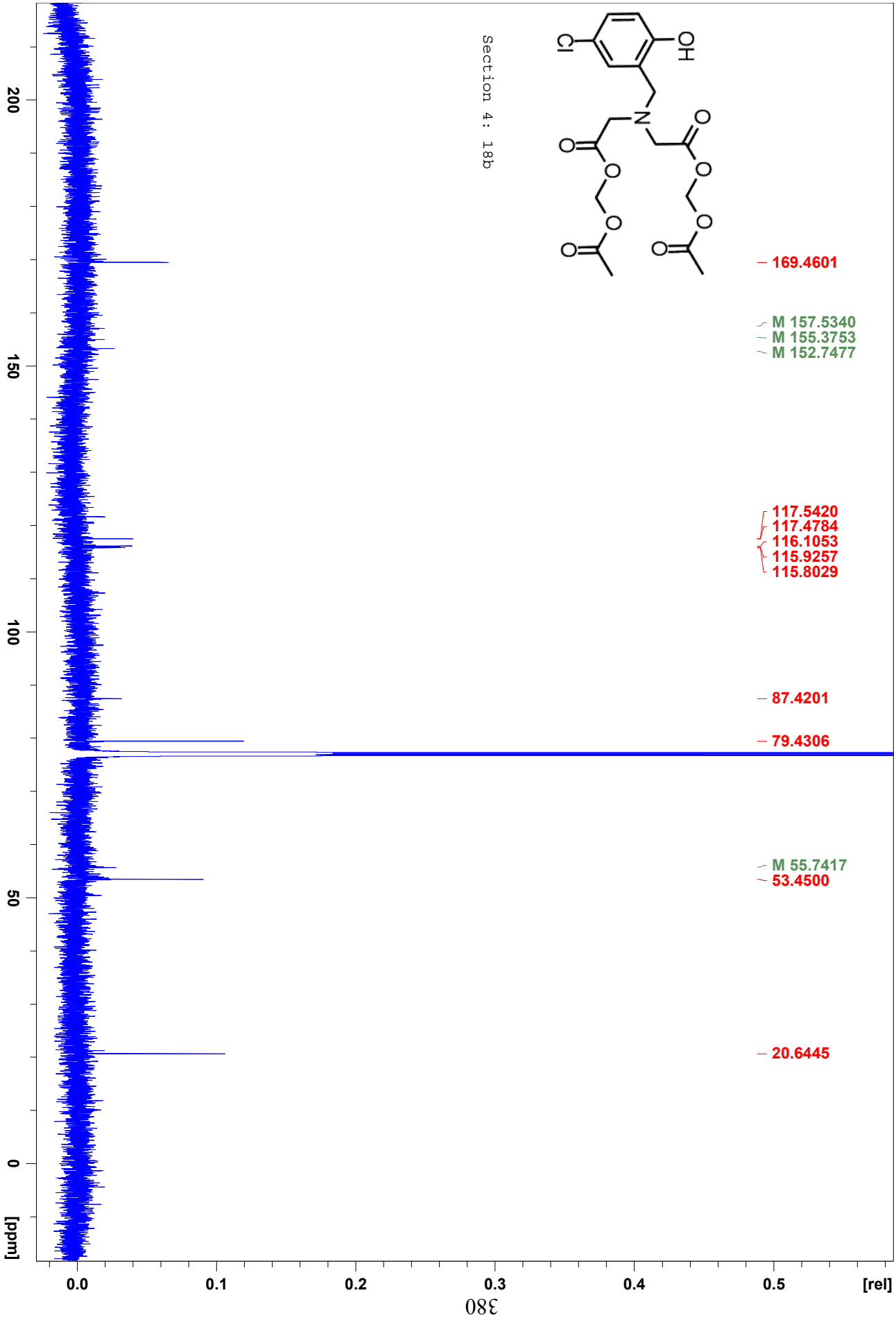

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6.8299
6.8125
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4.0101
3.6444
3.6292
3.6174
3.6069
3.5022
2.1356
1.9602
1.9480
1.9015
1.8906
1.8783
OH
O
N
Section 4: 15b
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2.3984
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1.8613
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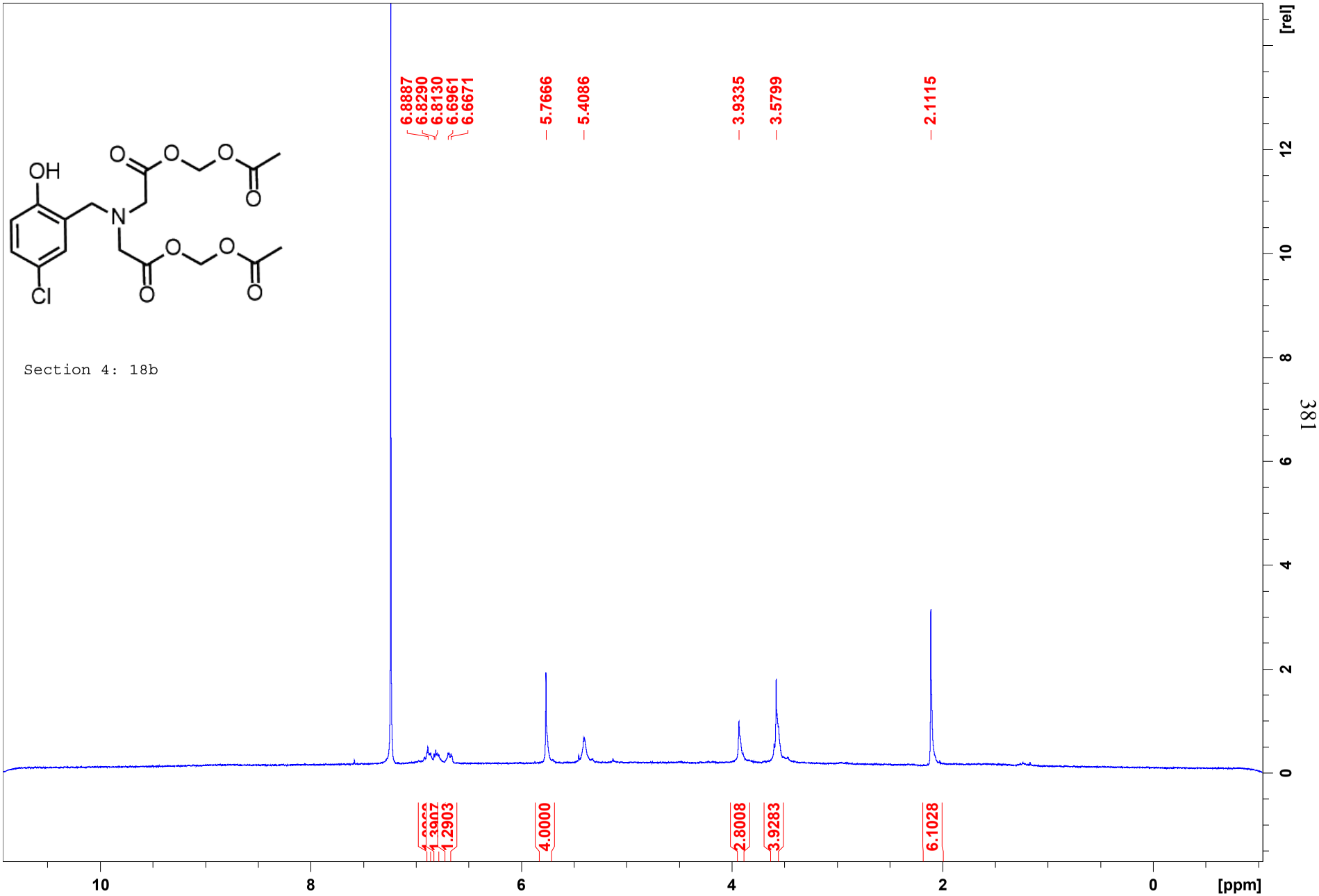

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77.2626
79.4332
115.9416
117.4343
121.3875
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133.0901
133.2109
156.4939
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S
N
O
Section 4: 16b

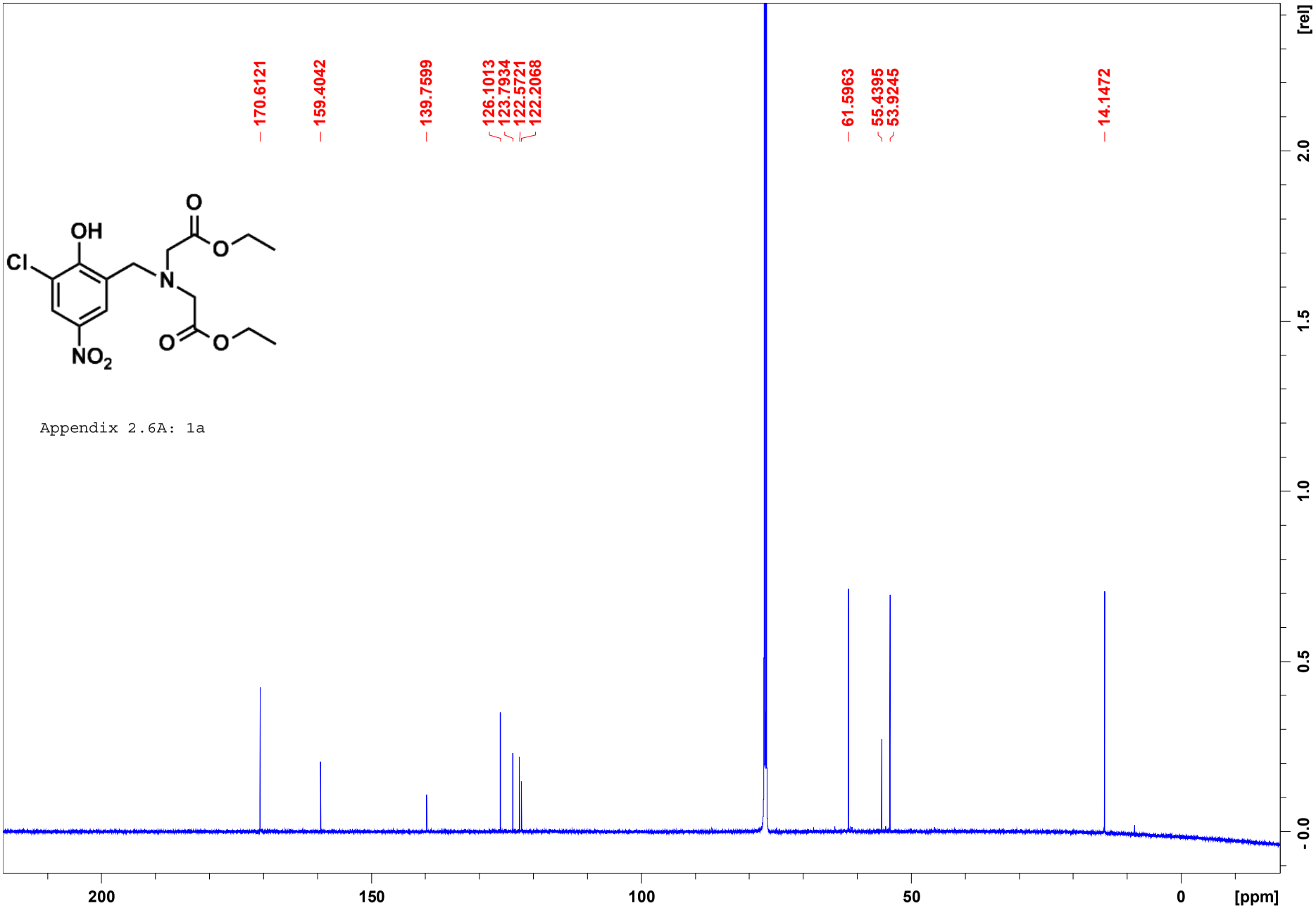

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OH
N
O
O
O
O
O
O
O
O
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2.1214
4.0676
1.4988
6.1978
2.7686
2.0337
0.7346
2.5635
7.3184
6.8657
6.8489
6.7999
6.7960
6.7826
5.8089
3.9780
3.6182
2.8275
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2.8126
2.8078
2.1548
1.5979
1.5843
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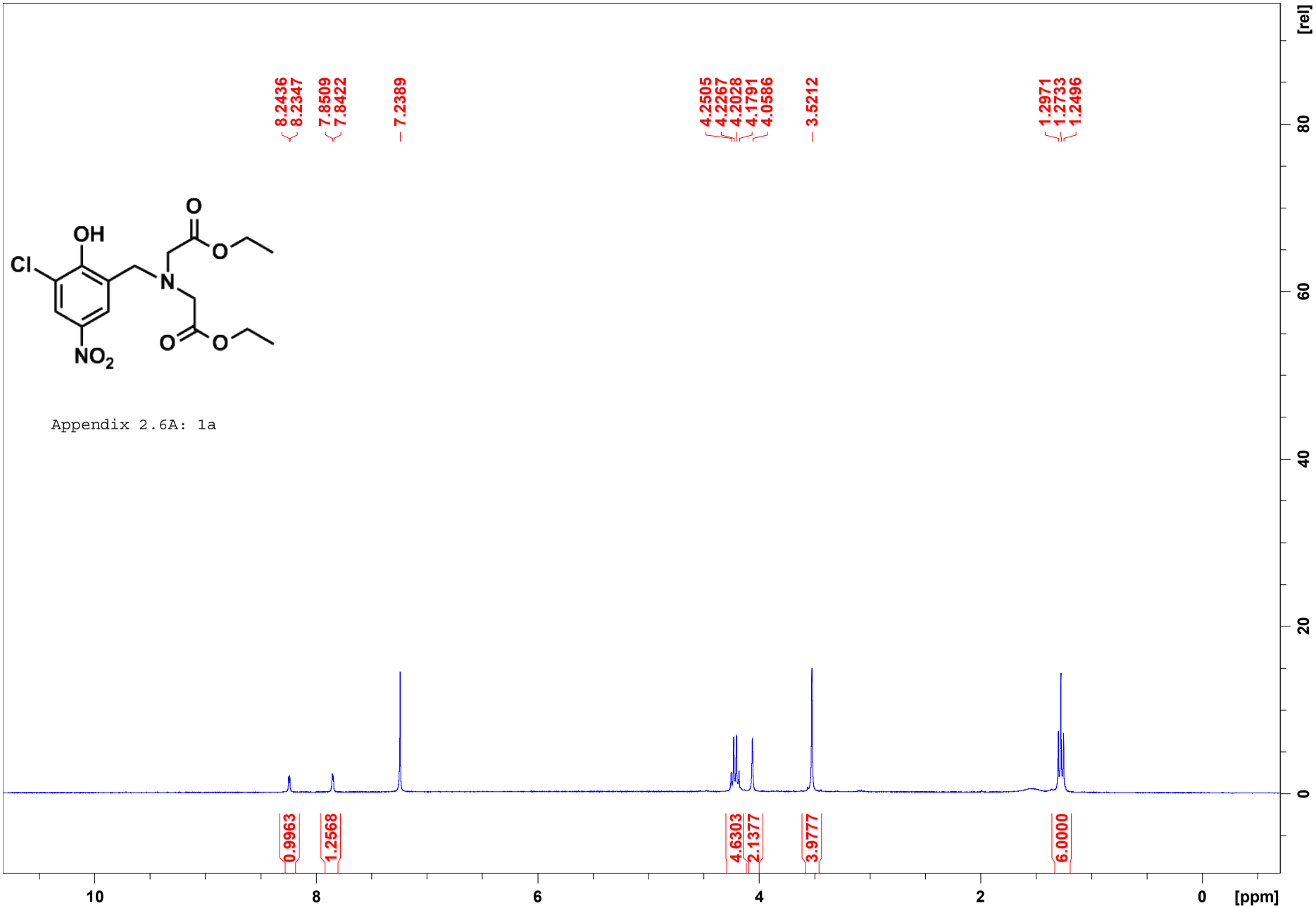

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76.7554
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58.4684
53.7972
39.6638
39.2971
20.9406
14.2032
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HO
Cl
O
O
O
O
O
O
O
O
N
Section 4: 17b
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4
6
8
[rel]


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5.7333
4.3897
3.8272
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3.3911
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2.1355
Cl
HO
Cl
N
O
O
O
O
O
O
O
O
Section 4: 17b
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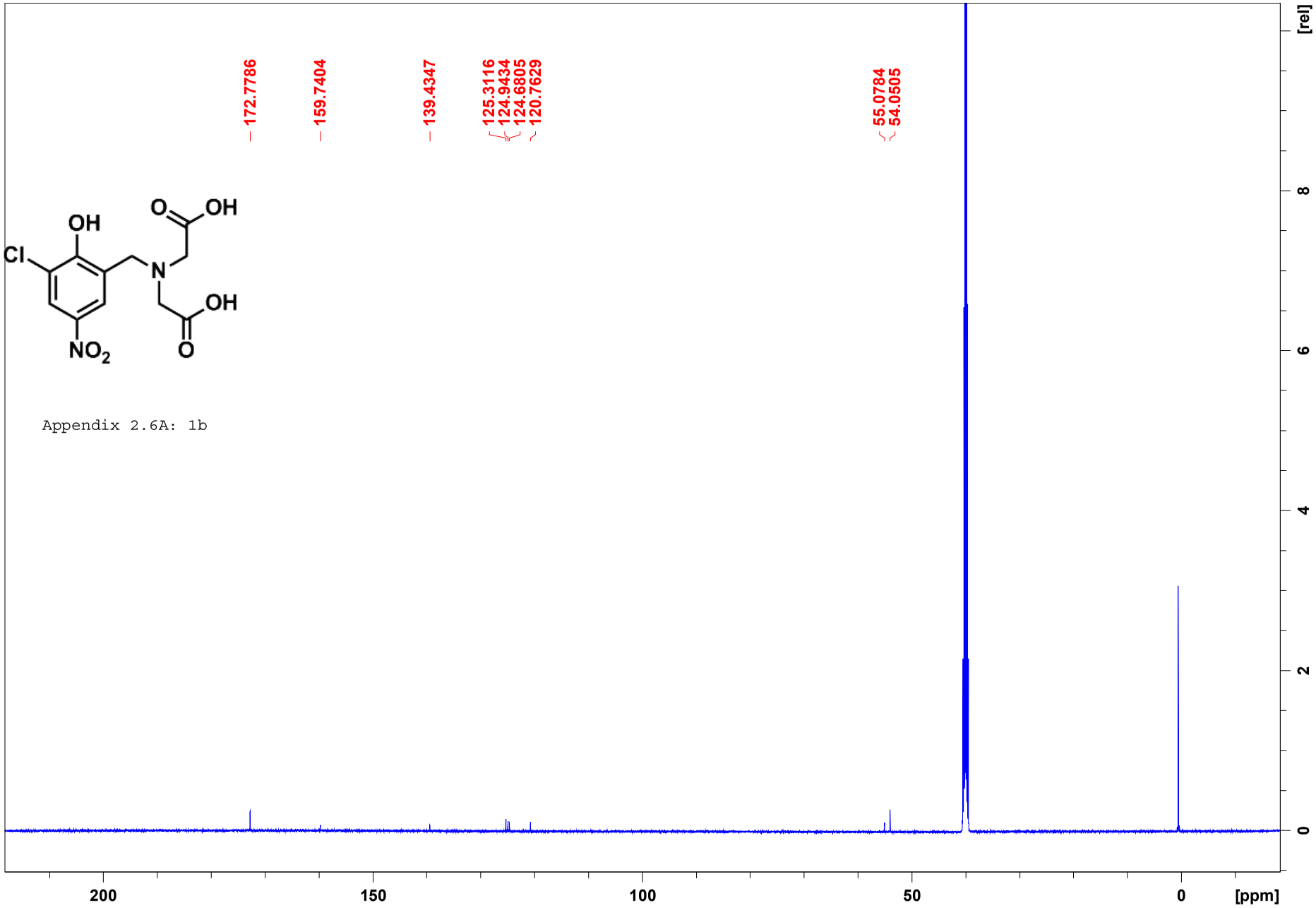

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M 155.3753
M 152.7477
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117.4784
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115.9257
115.8029
87.4201
79.4306
M 55.7417
53.4500
20.6445
200
150
100
50
0
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0.2
0.3
0.4
0.5
[rel]


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O
O
O
N
O
O
O
O
Cl
Section 4: 18b
6.8887
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6.8130
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3.9335
3.5799
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1.2903
4.0000
2.8008
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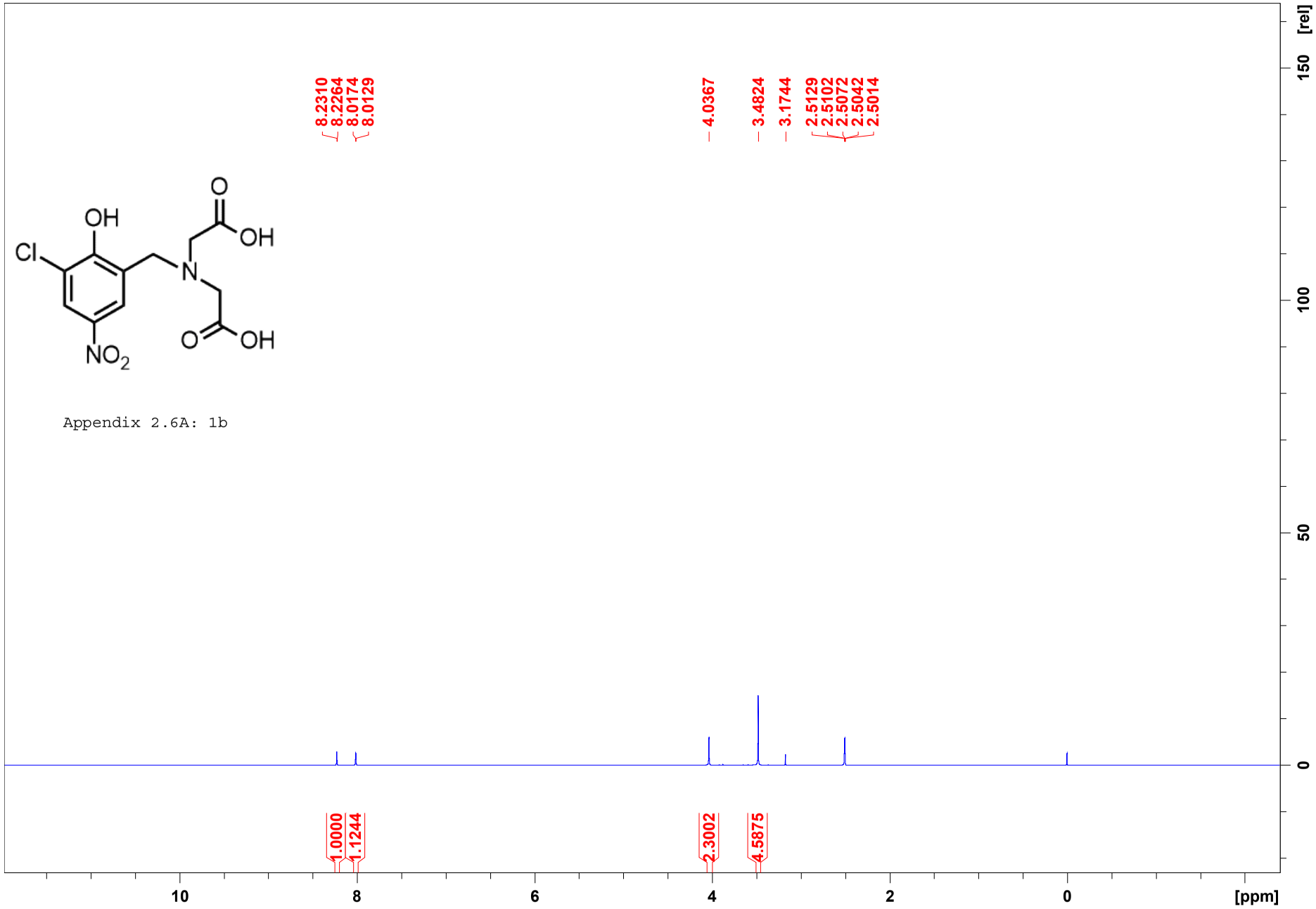

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122.5721
122.2068
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53.9245
14.1472
OH
Cl
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O
O
O
O
NO2
Appendix 2.6A: 1a
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50
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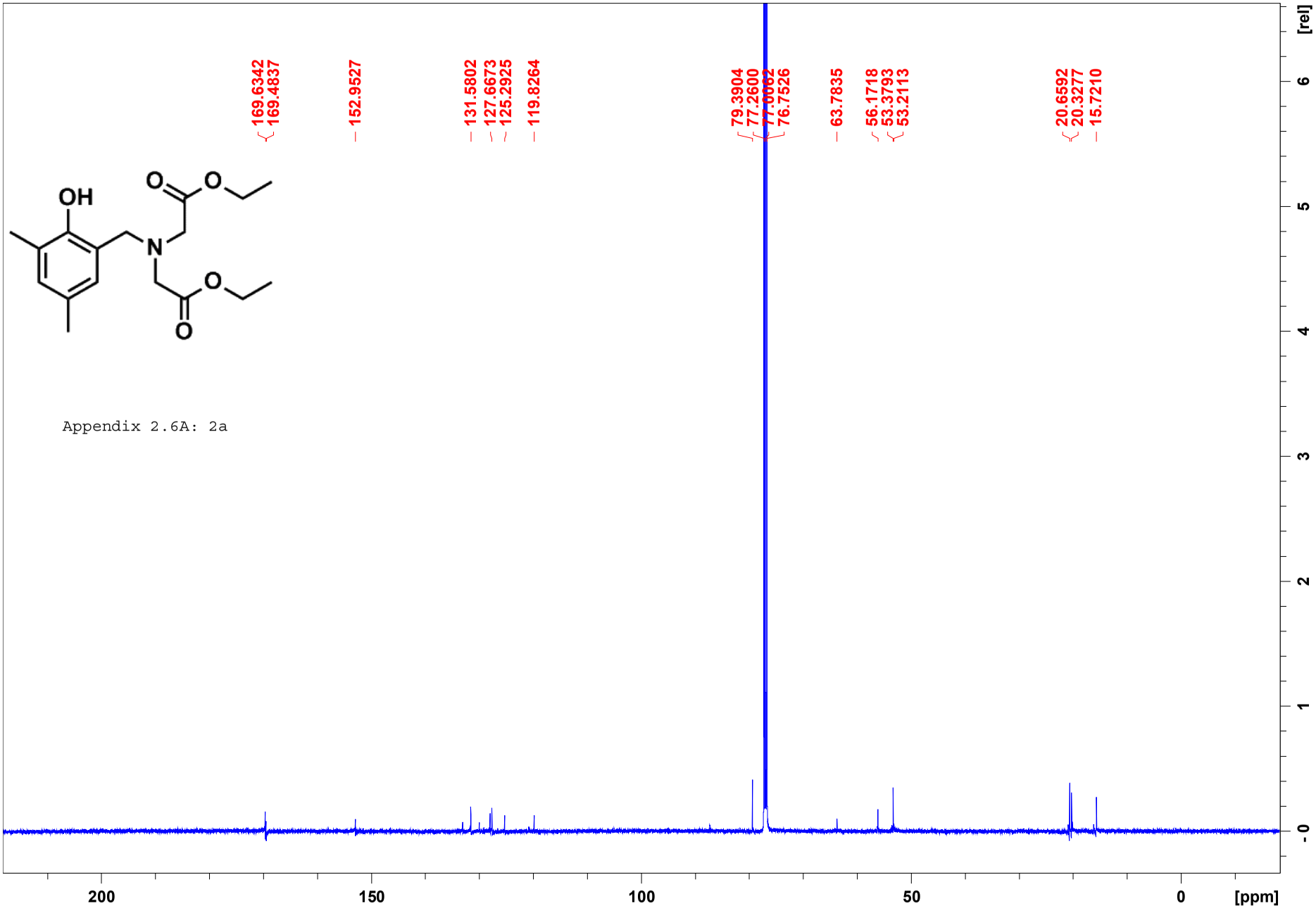
Appendix 2.6A: 1a

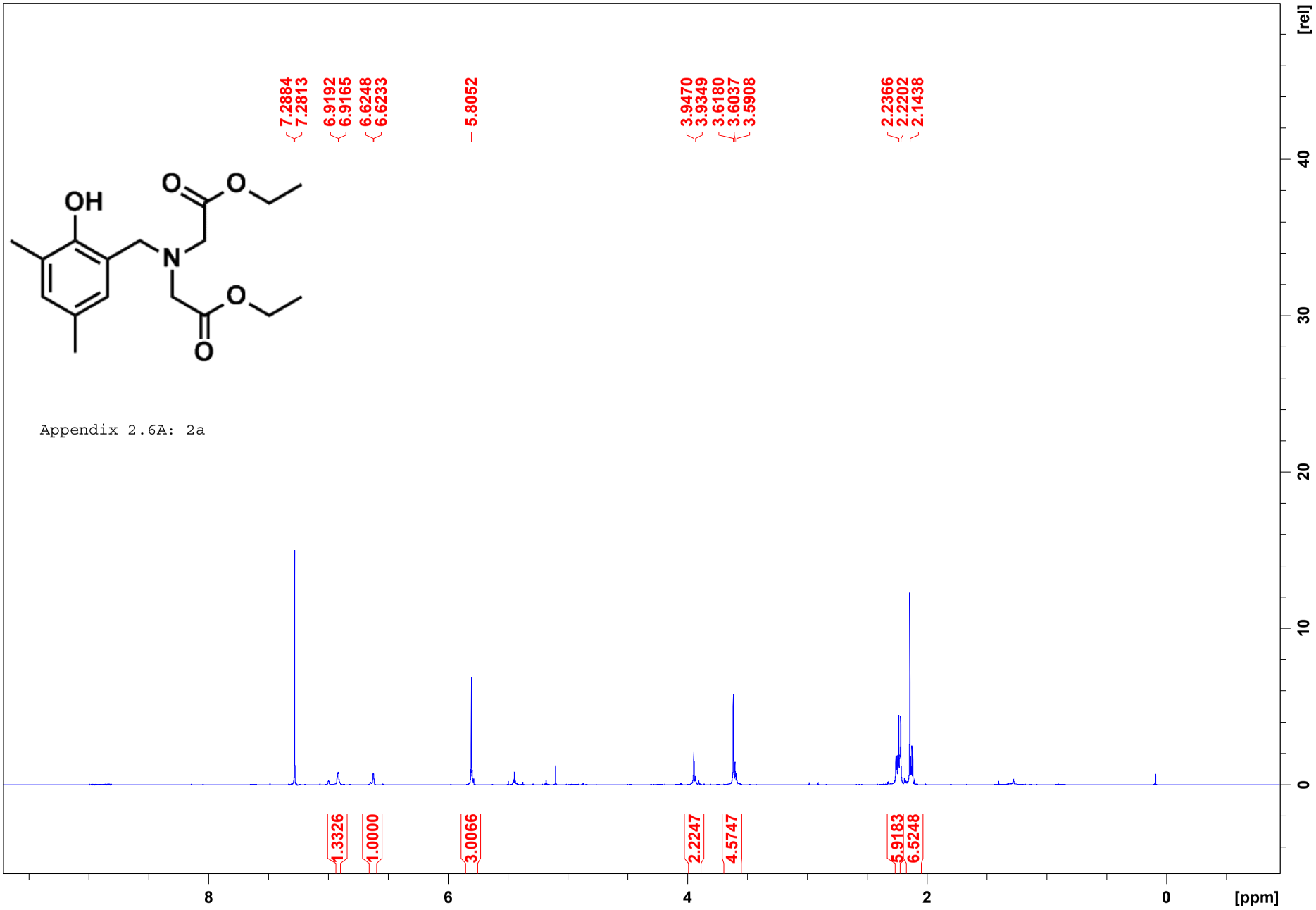


Appendix 2.6A: 1b

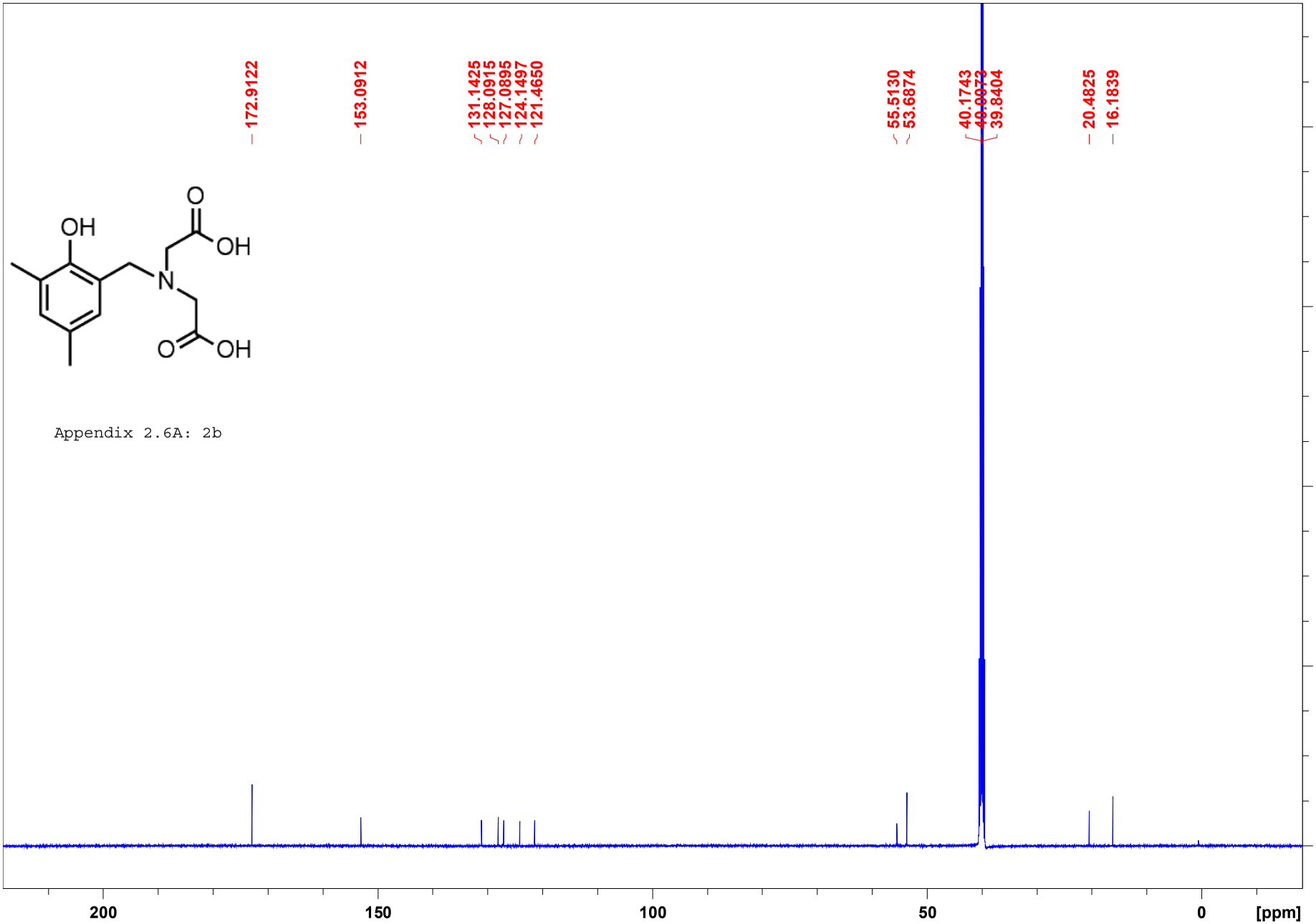


Appendix 2.6A: 1b

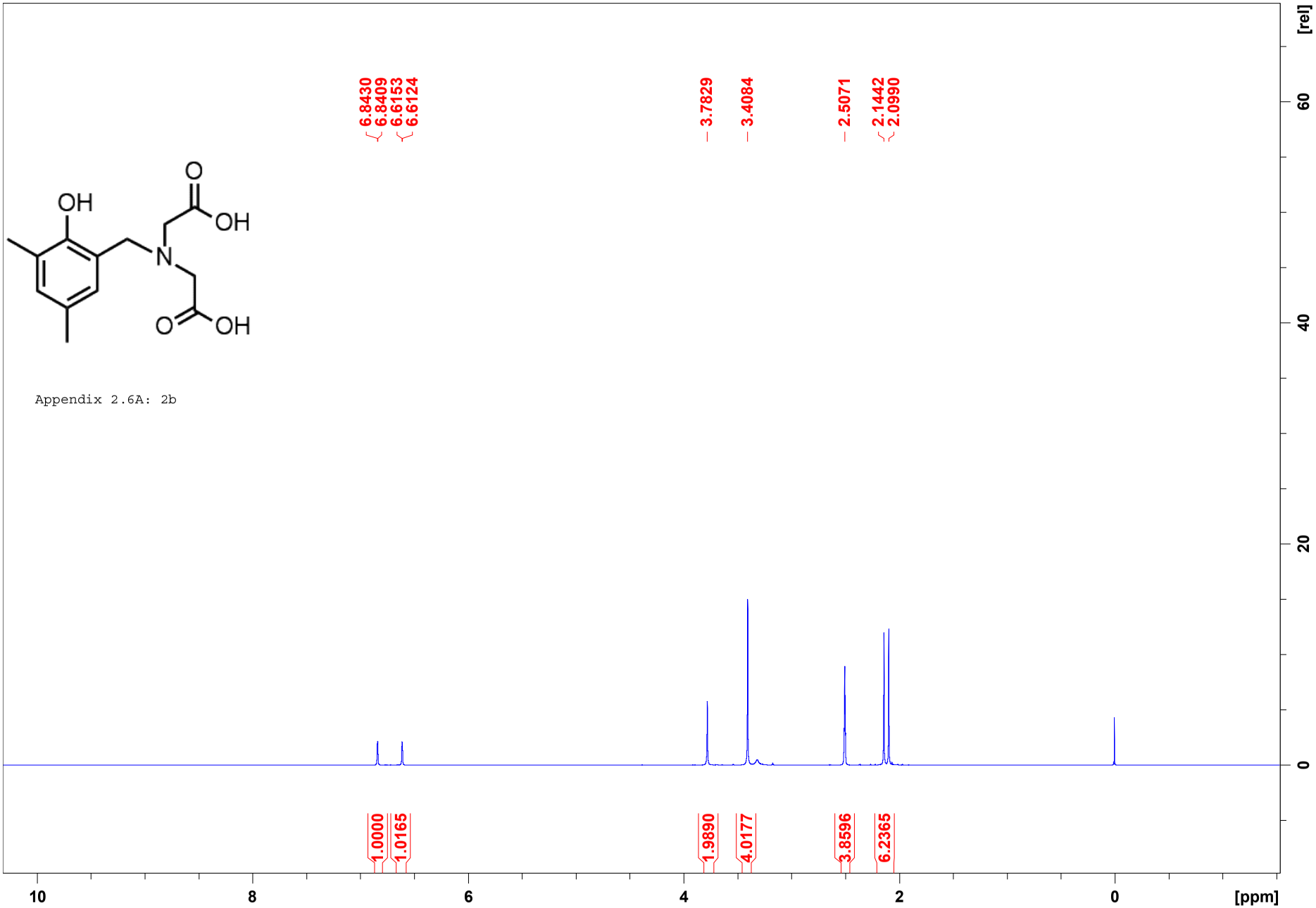

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76.7526
63.7835
56.1718
53.3793
53.2113
20.6592
20.3277
15.7210
OH
O
O
N
O
O
Appendix 2.6A: 2a
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150
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50
0
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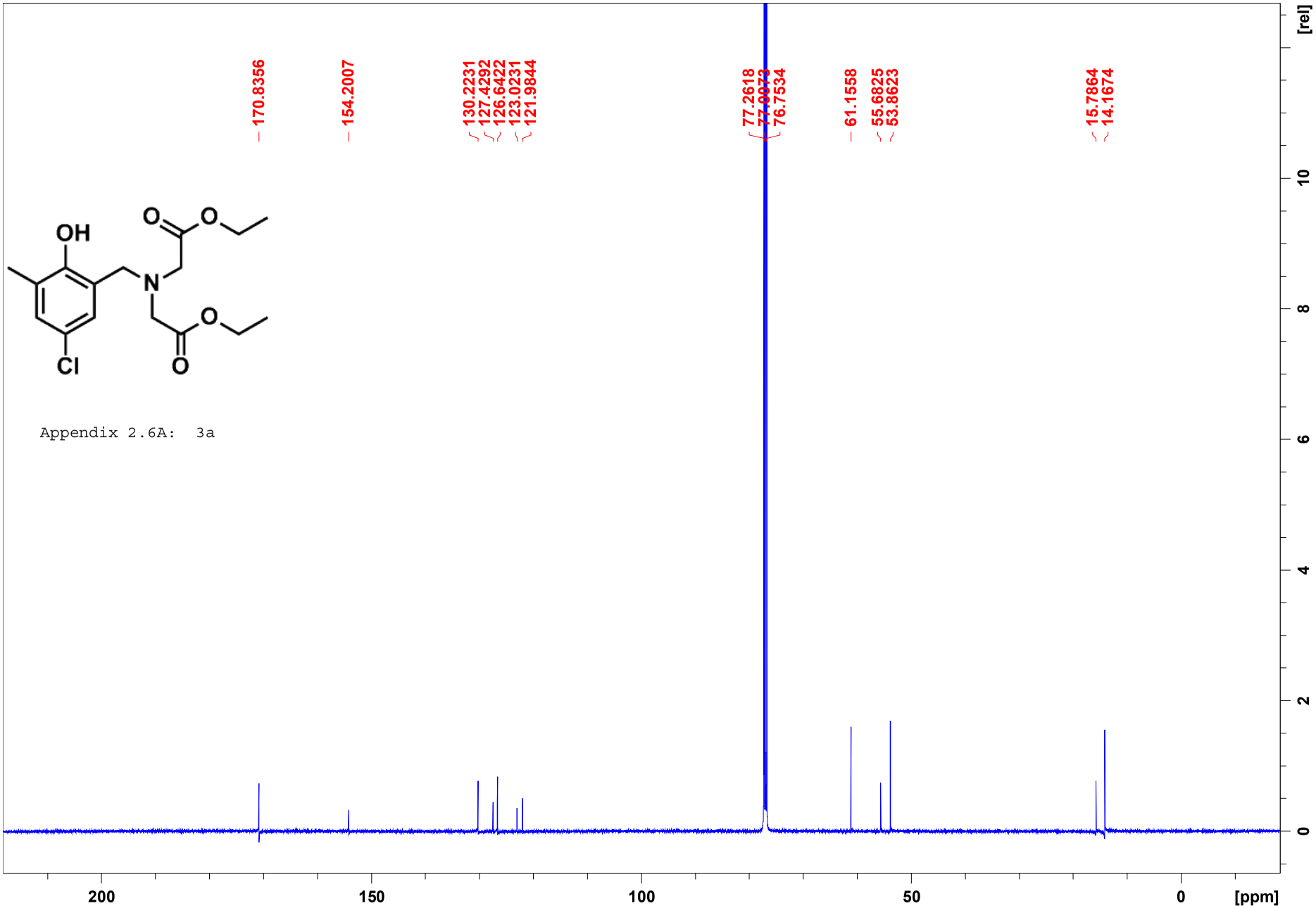
Appendix 2.6A: 2a



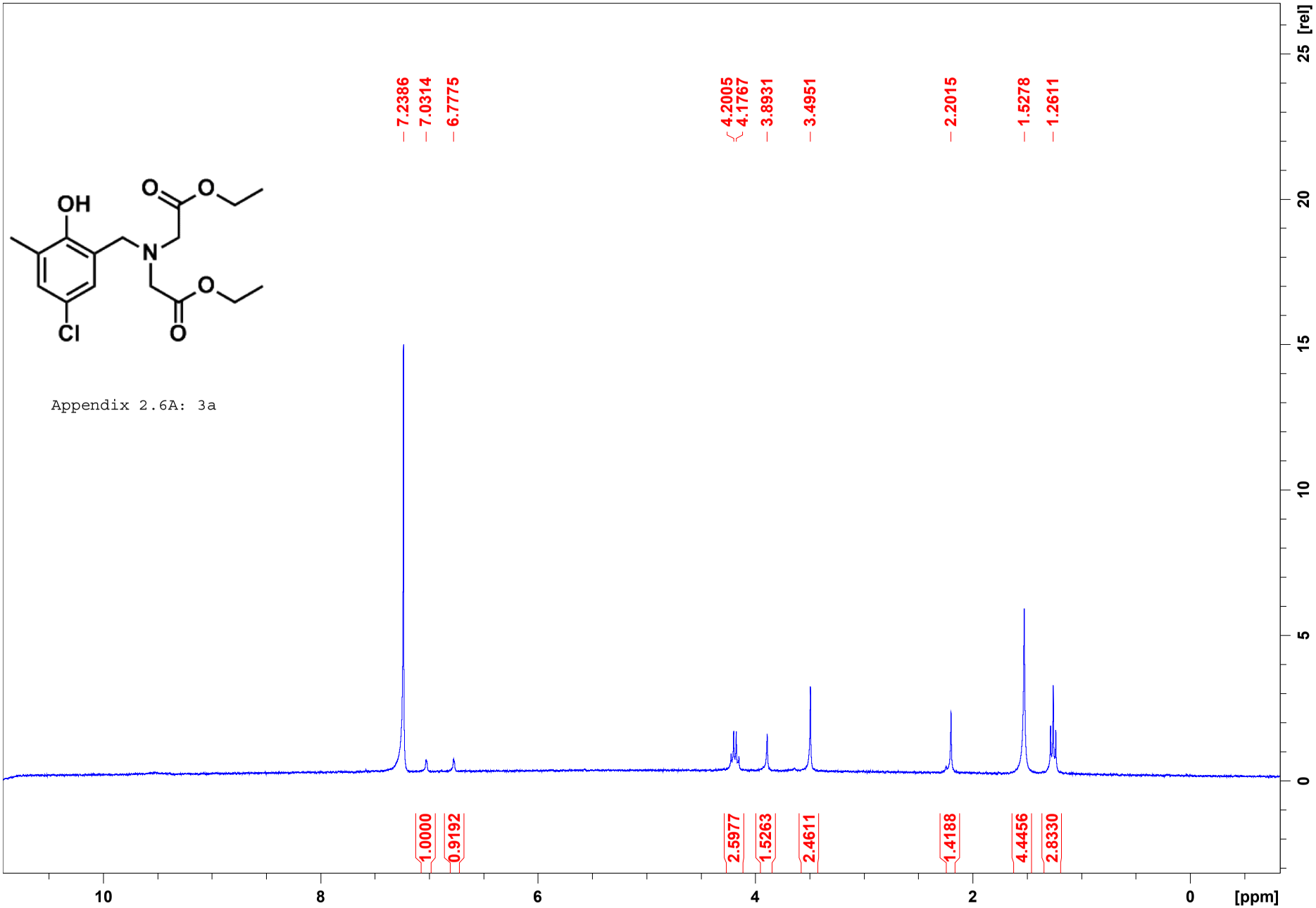
Appendix 2.6A: 2b



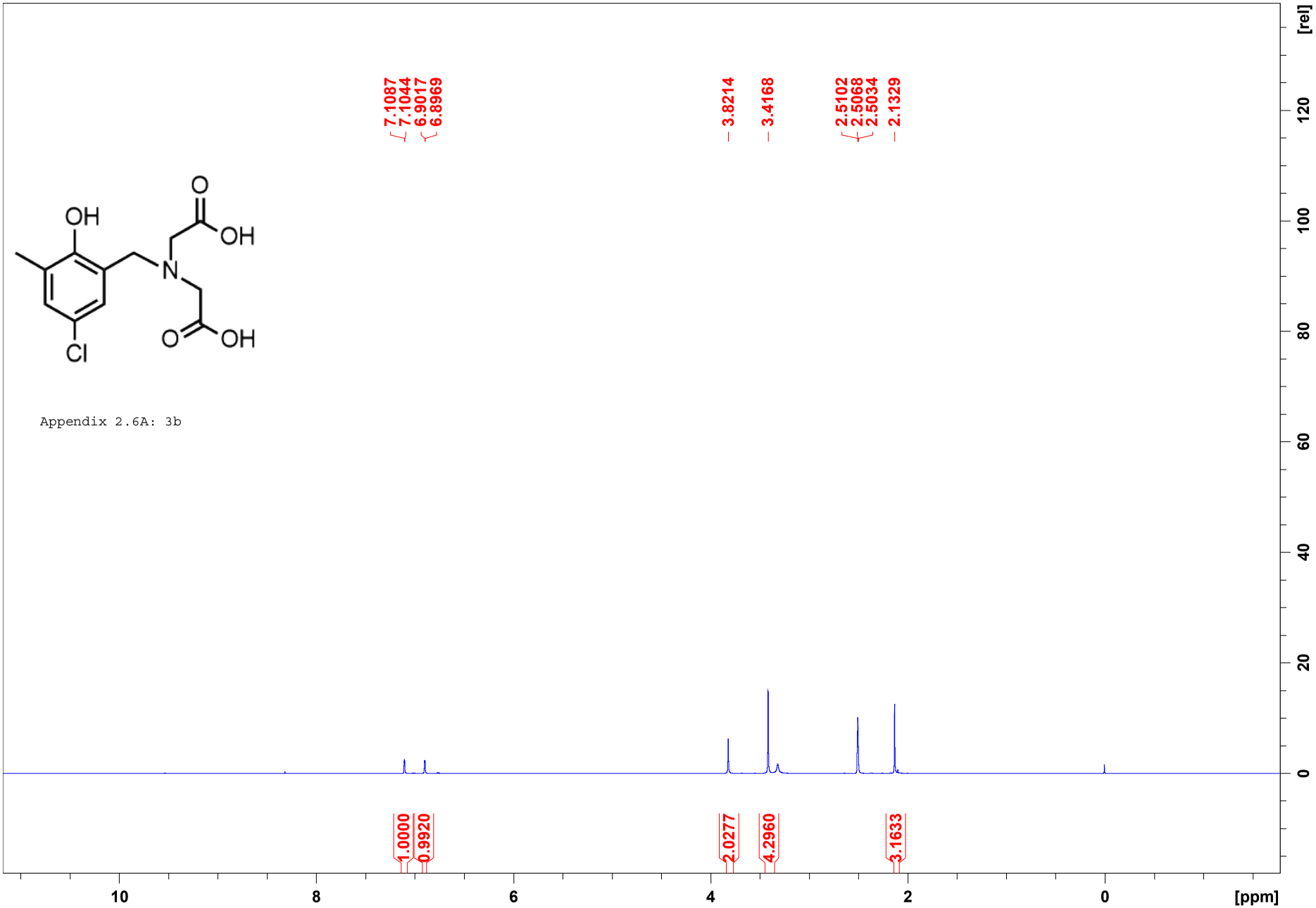
Appendix 2.6A: 2b



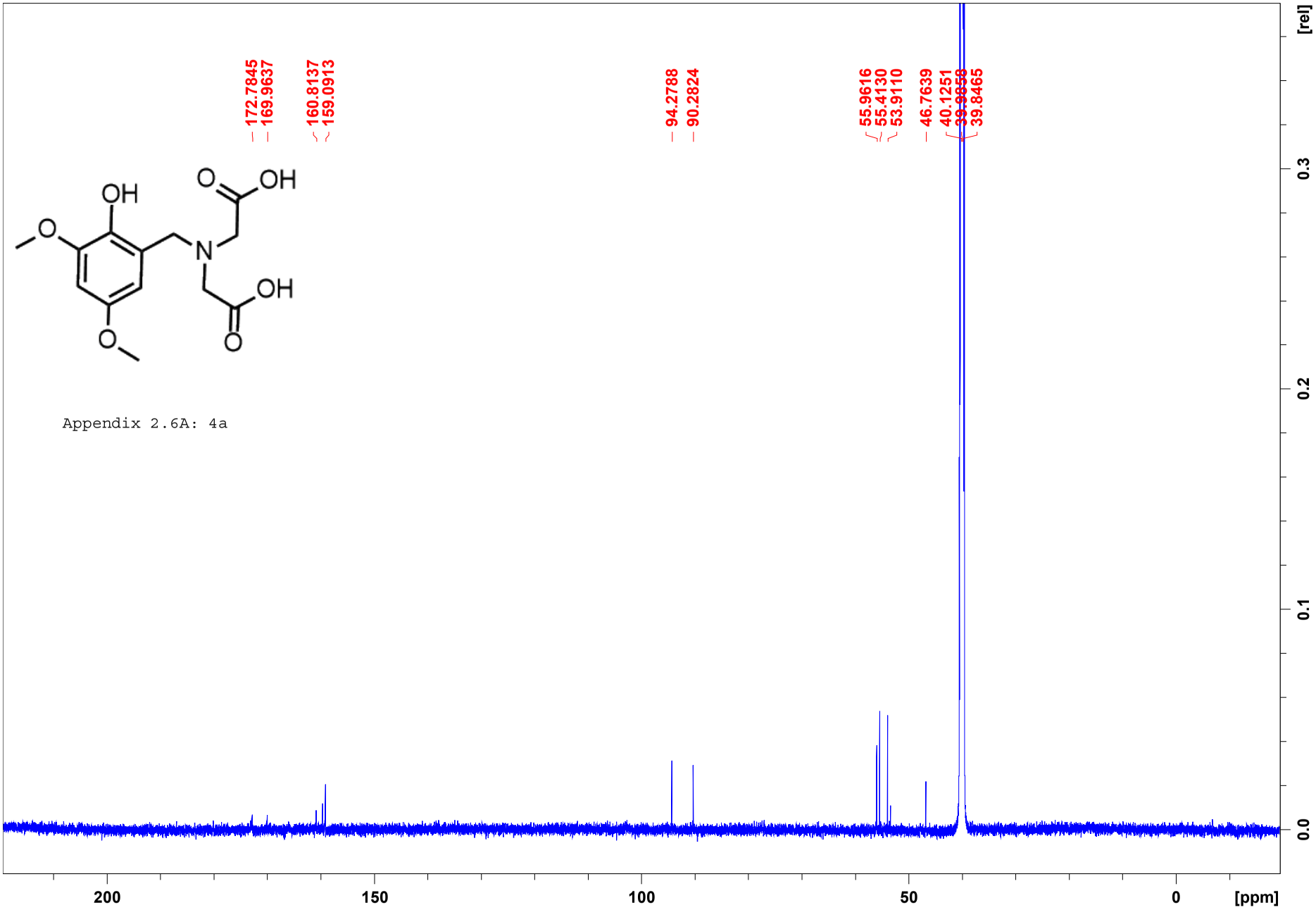
Appendix 2.6A: 3a



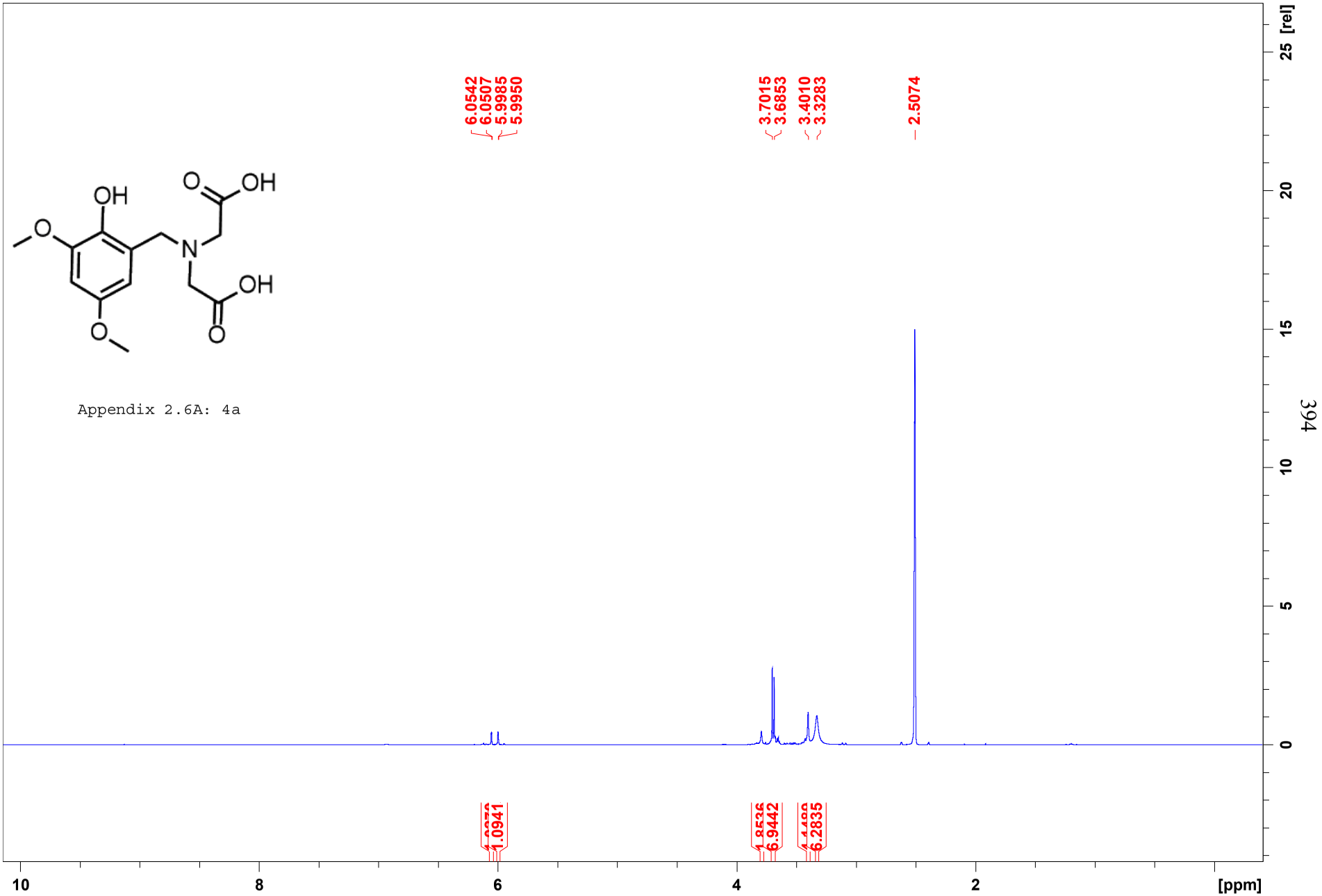
Appendix 2.6A: 3a



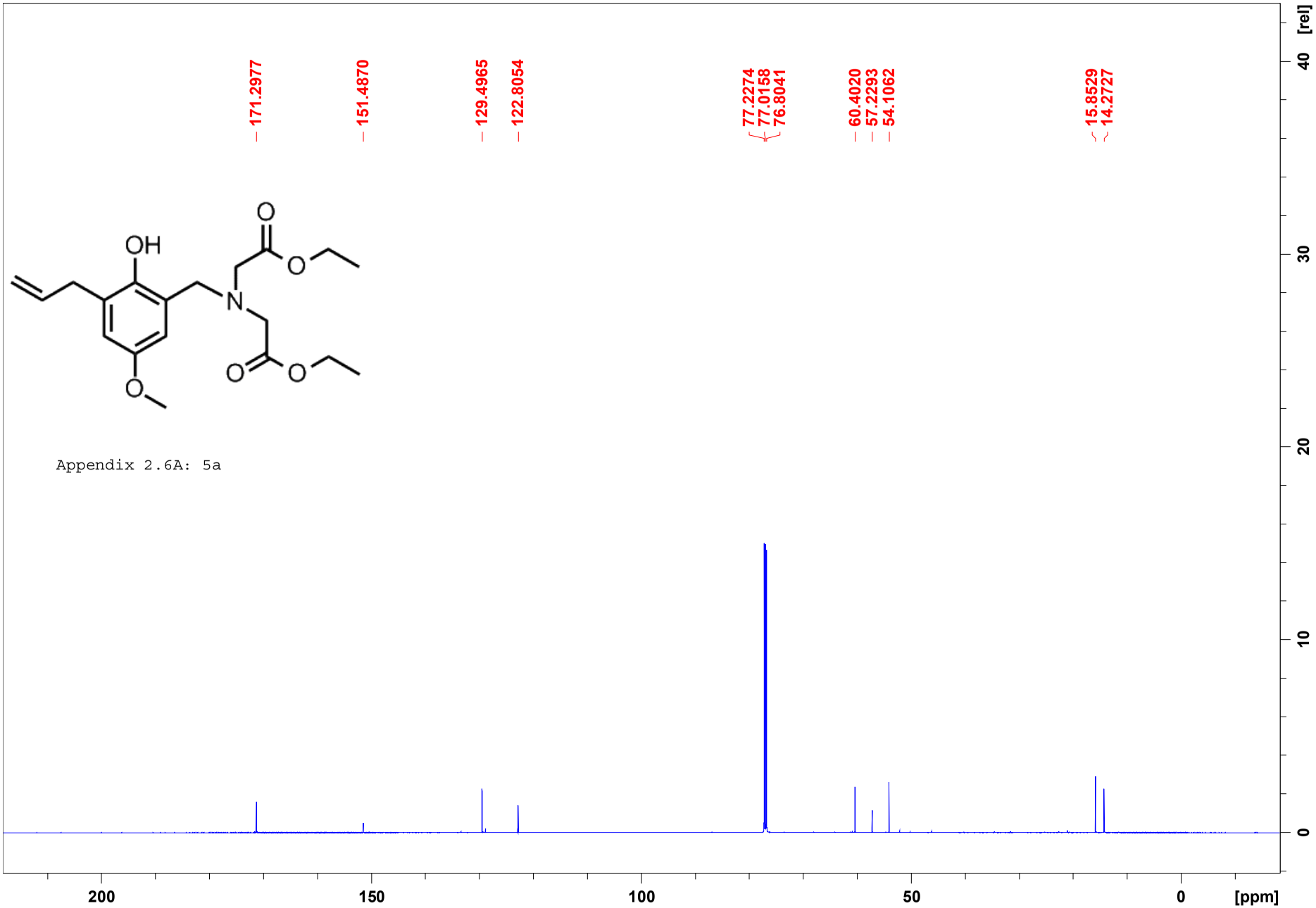
Appendix 2.6A: 3b



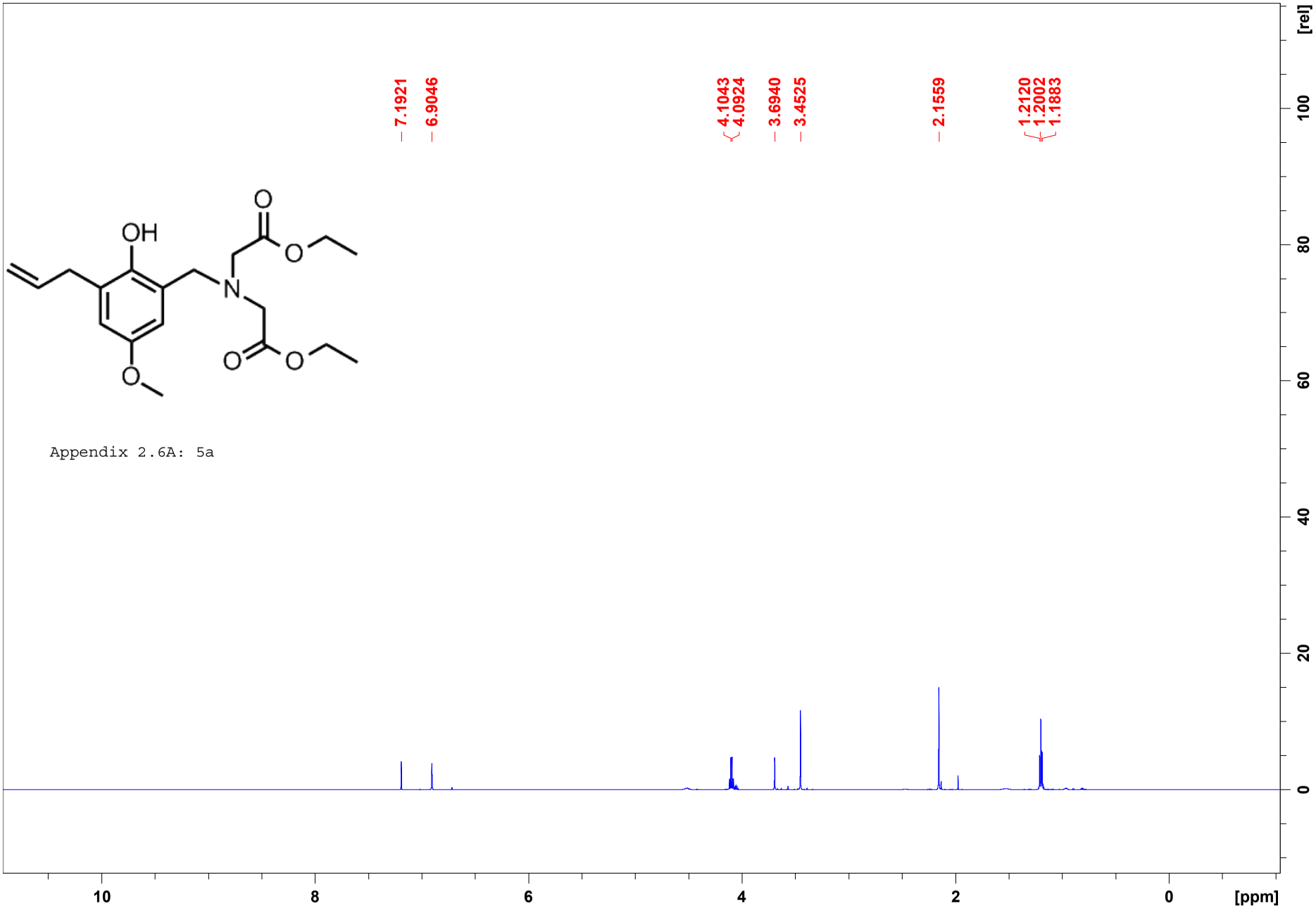
Appendix 2.6A: 4a



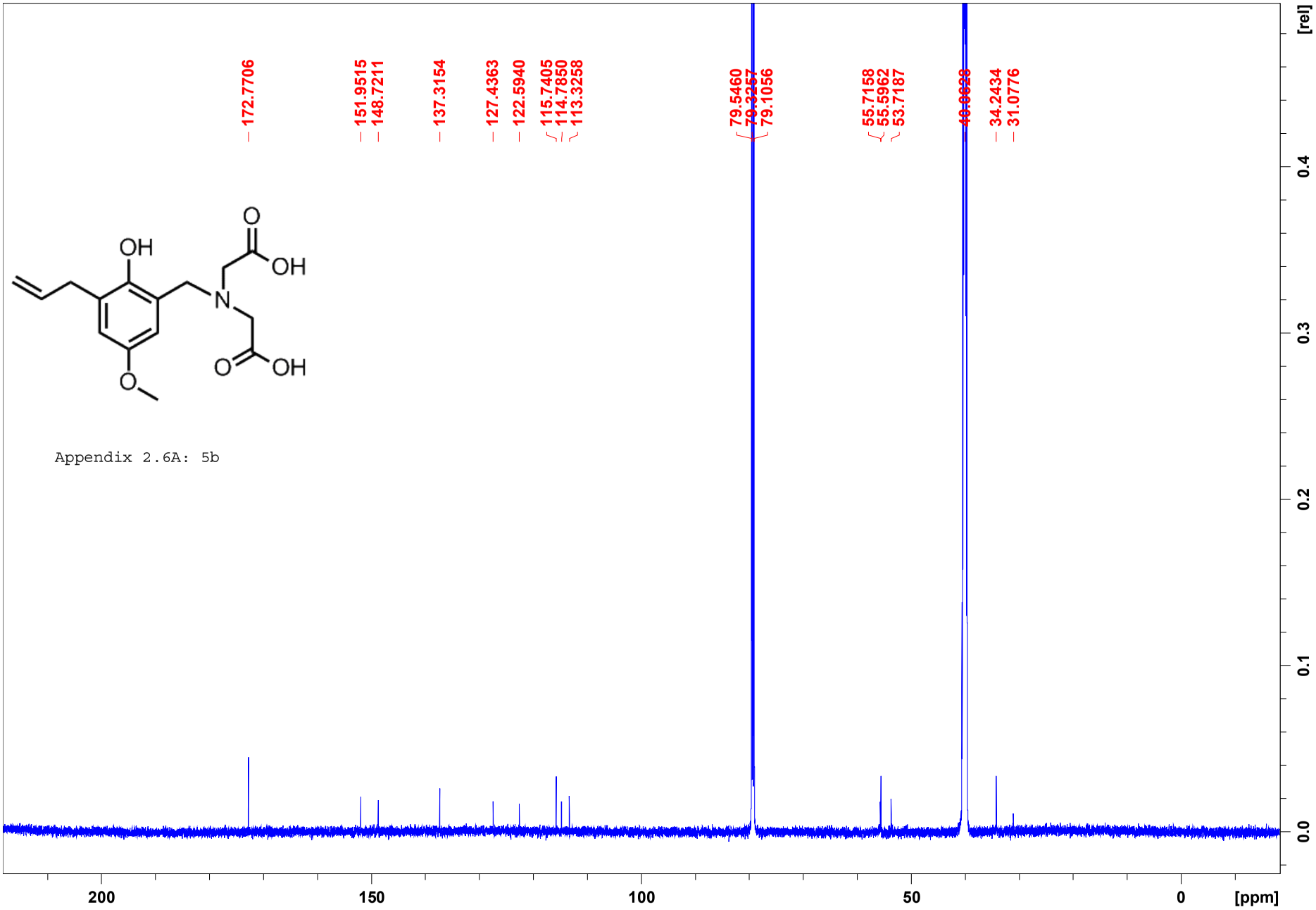
Appendix 2.6A: 4a



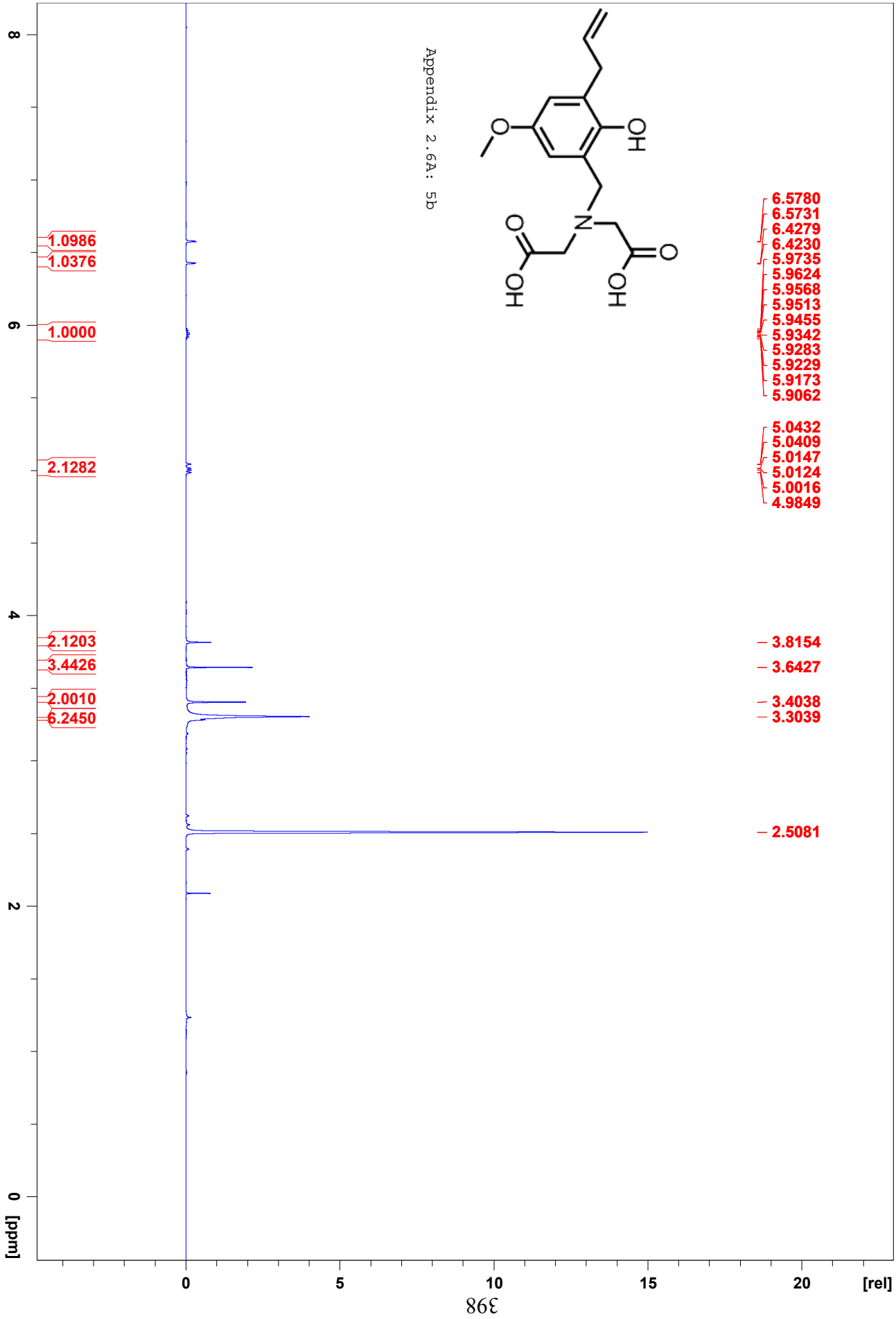
Appendix 2.6A: 5a

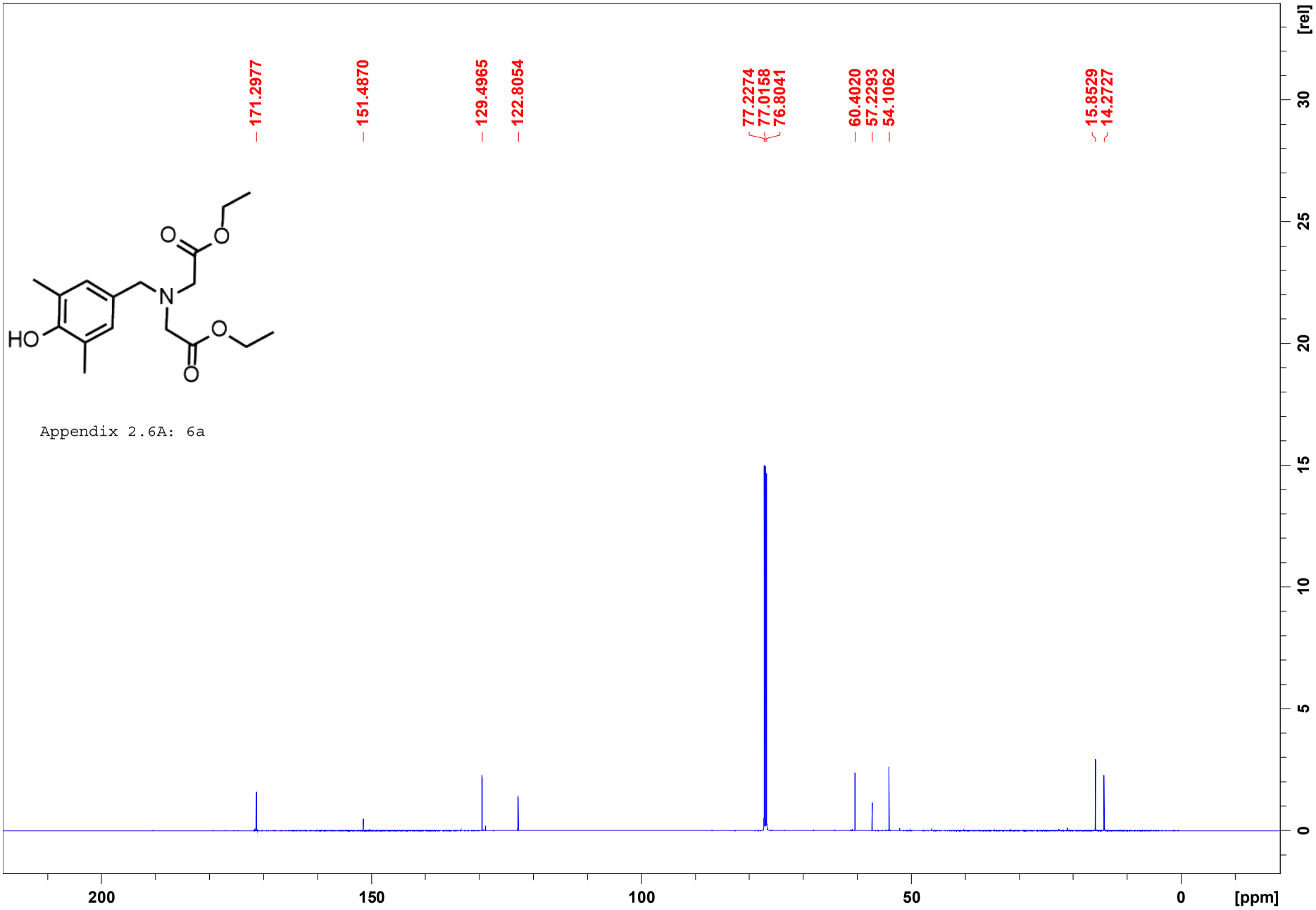


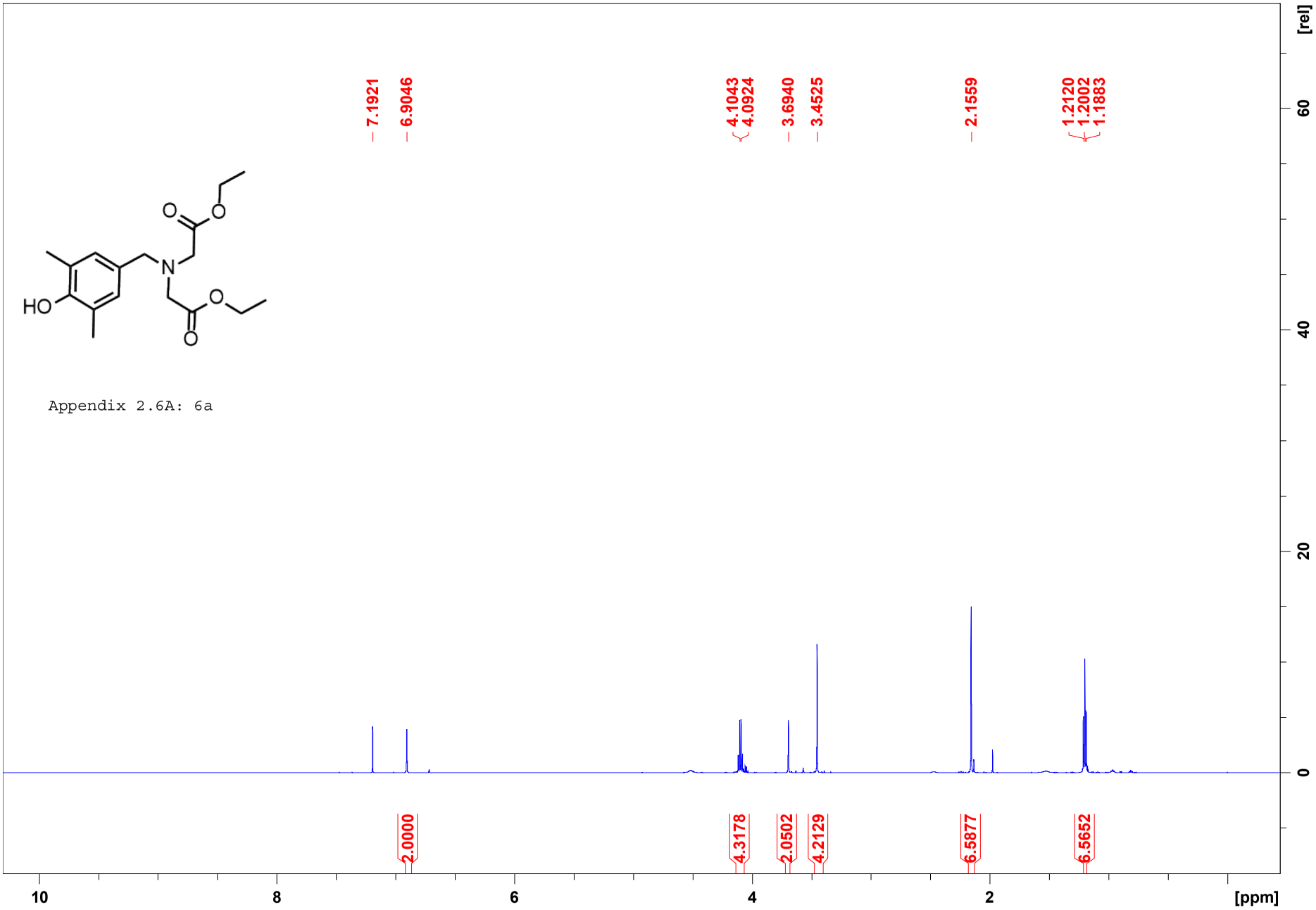
Appendix 2.6A: 5a



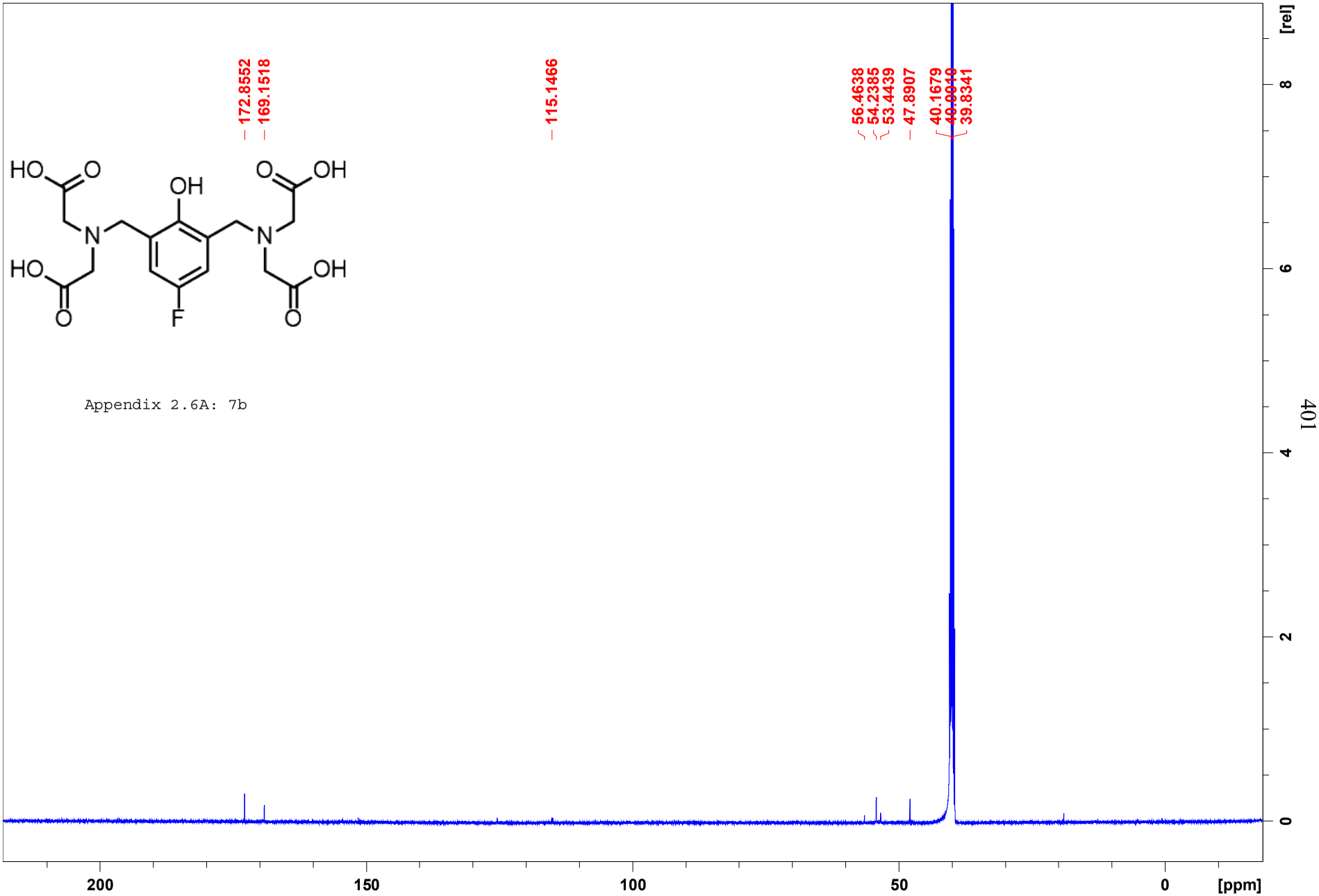
Appendix 2.6A: 5b


Appendix 2.6A: 5b
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5.9568
5.9513
5.9455
5.9342
5.9283
5.9229
5.9173
5.9062
5.0432
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5.0147
5.0124
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4.9849
3.8154
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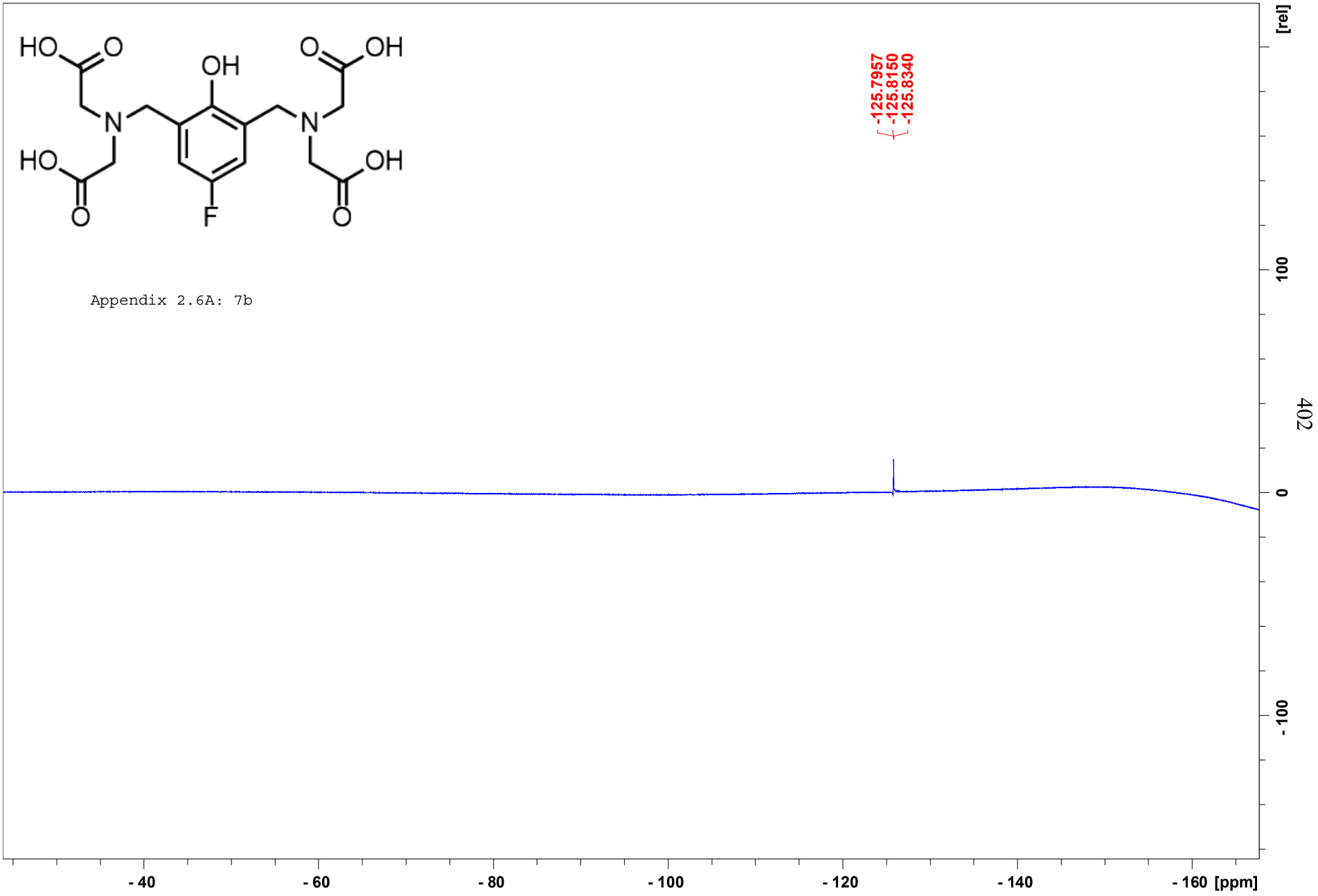

171.2977
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14.2727
HO
O
O
N
O
O
Appendix 2.6A: 6a
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50
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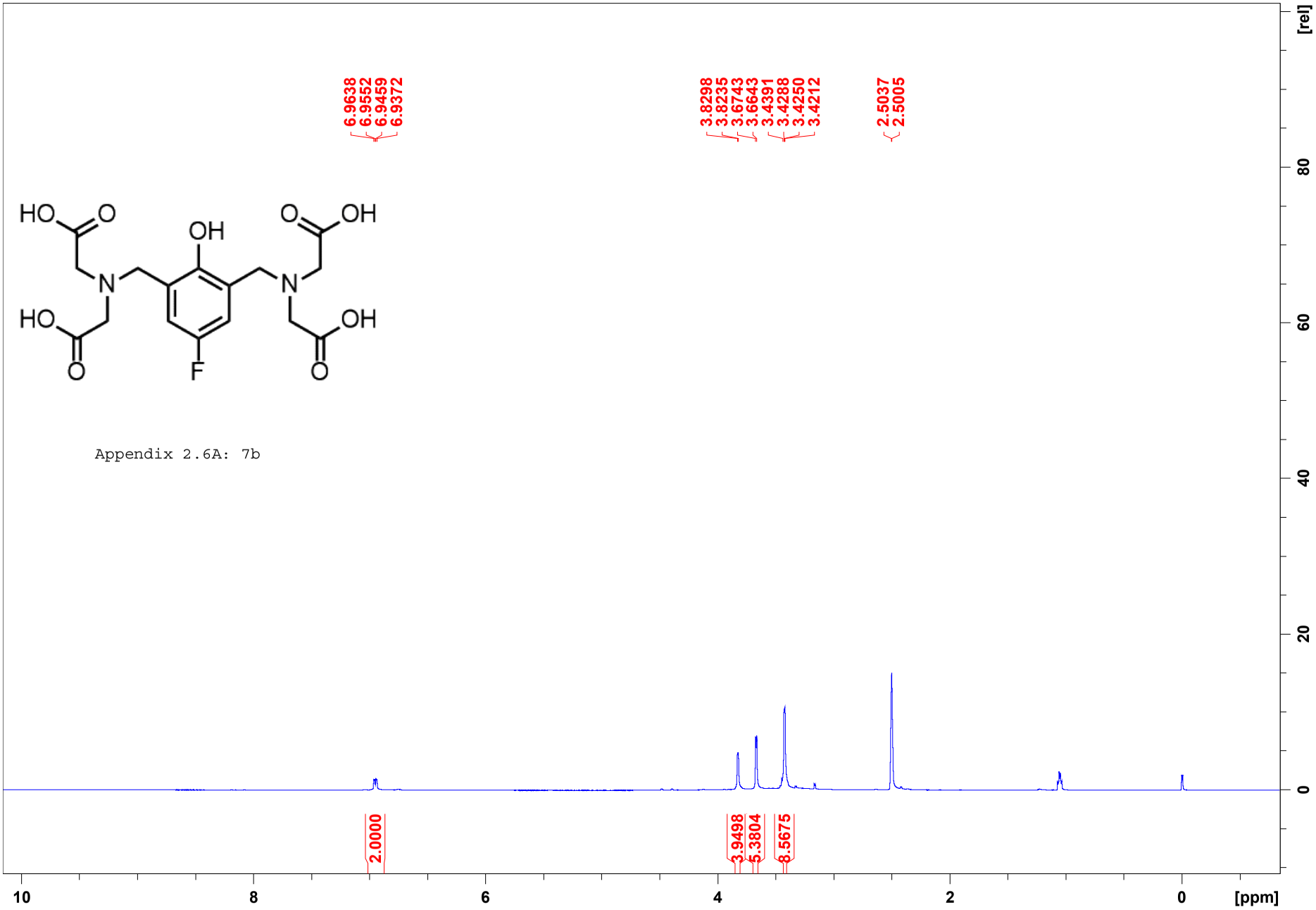
Appendix 2.6A: 6a



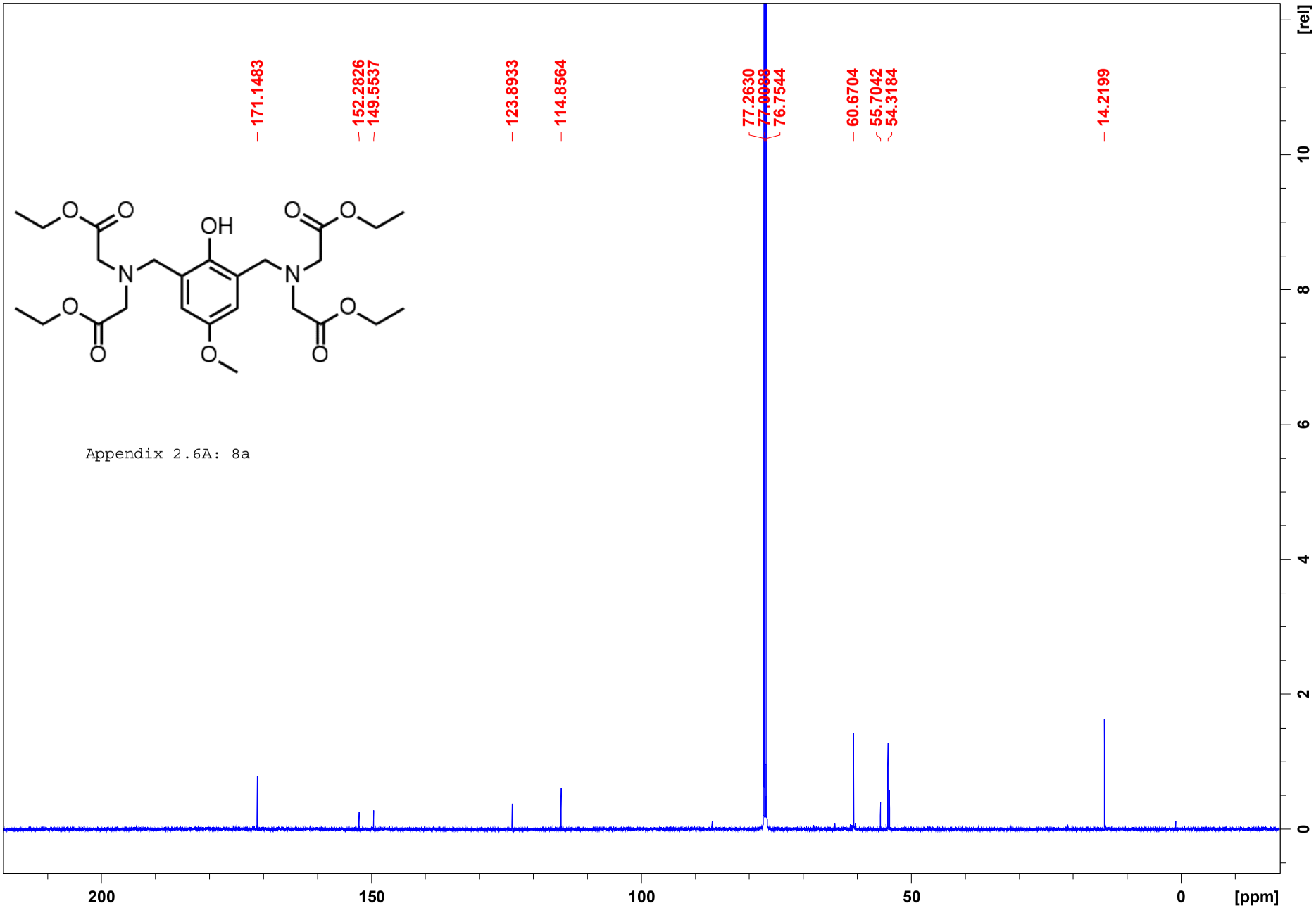
Appendix 2.6A: 7b



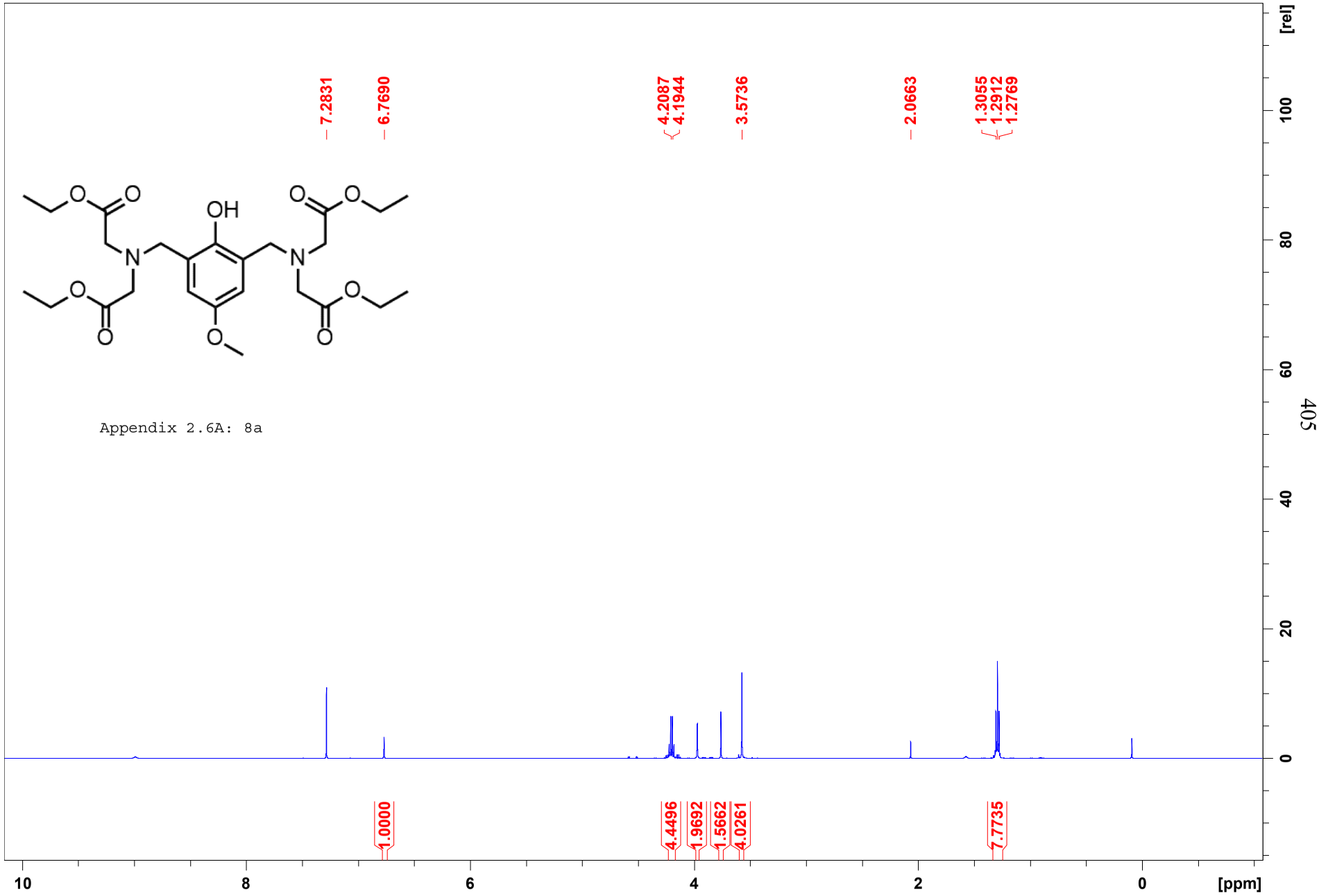
Appendix 2.6A: 7b



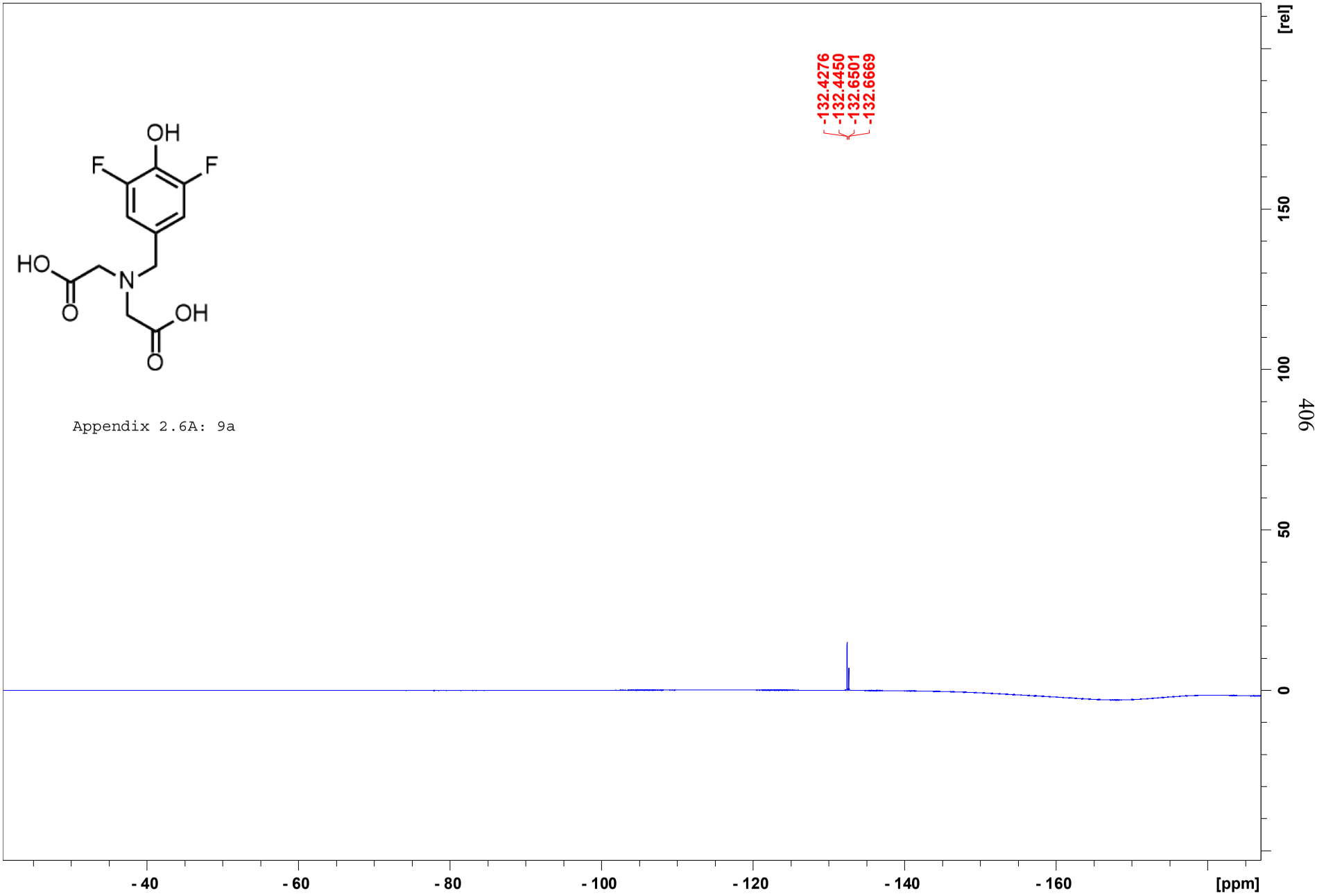
Appendix 2.6A: 7b



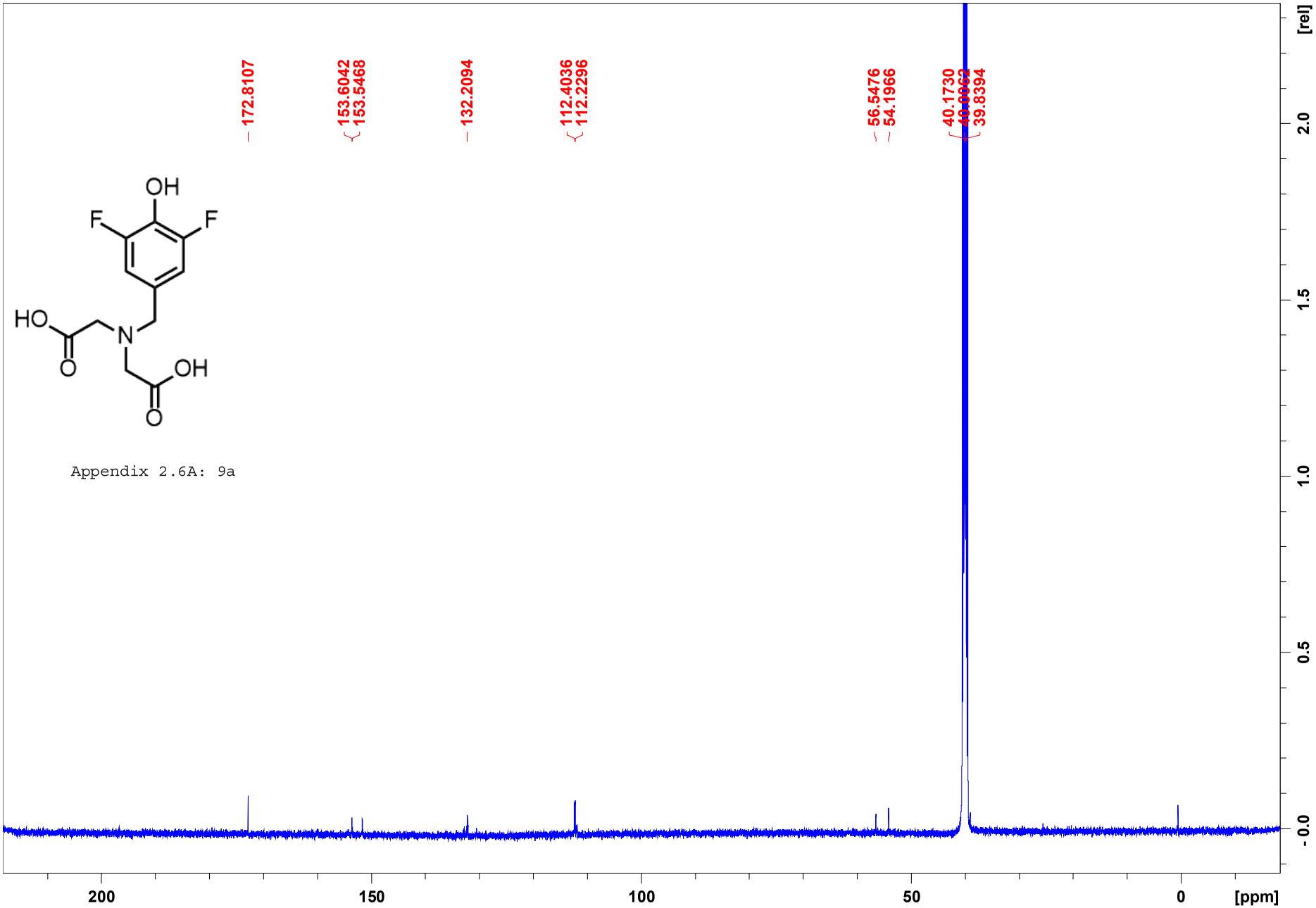
Appendix 2.6A: 8a



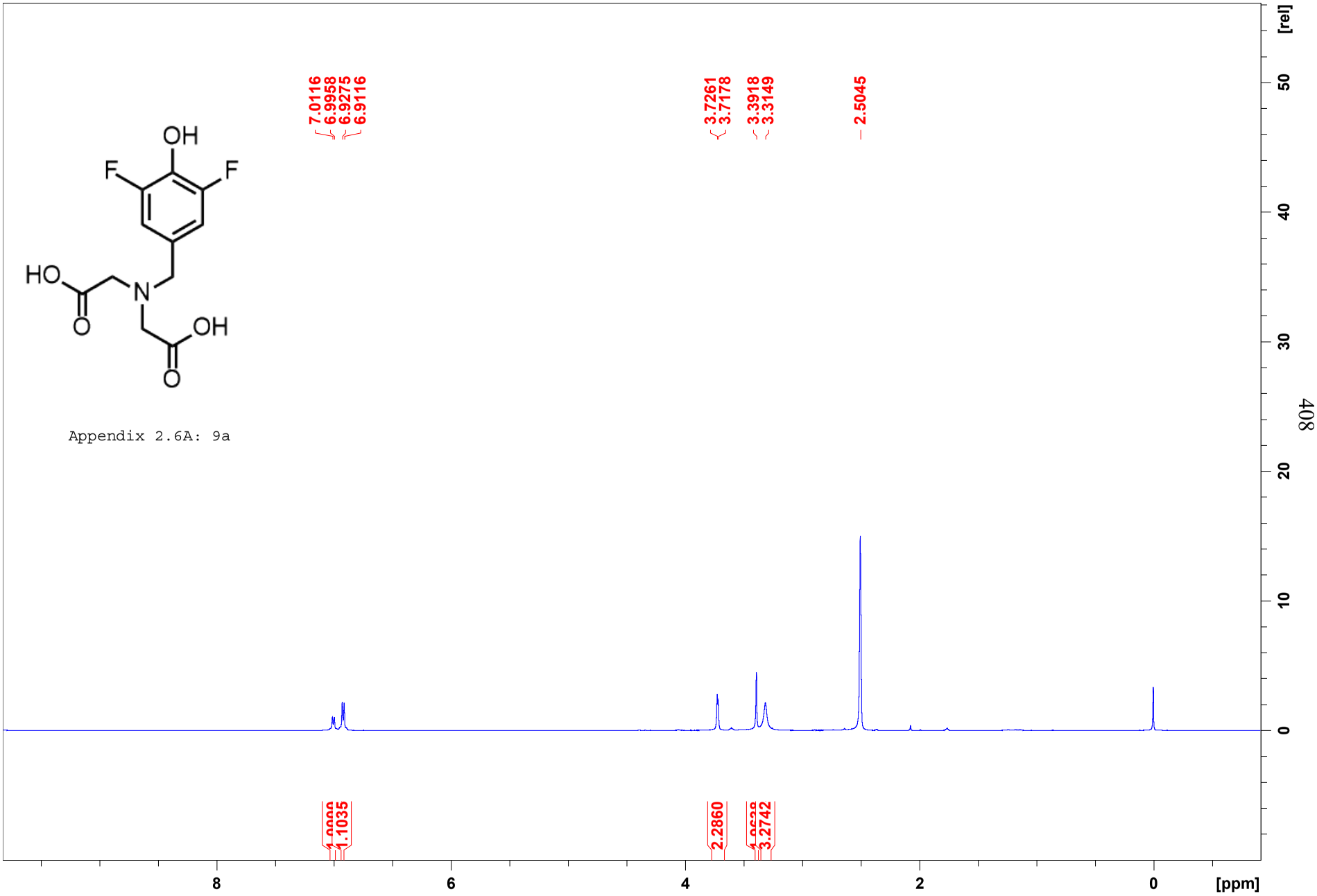
Appendix 2.6A: 8a



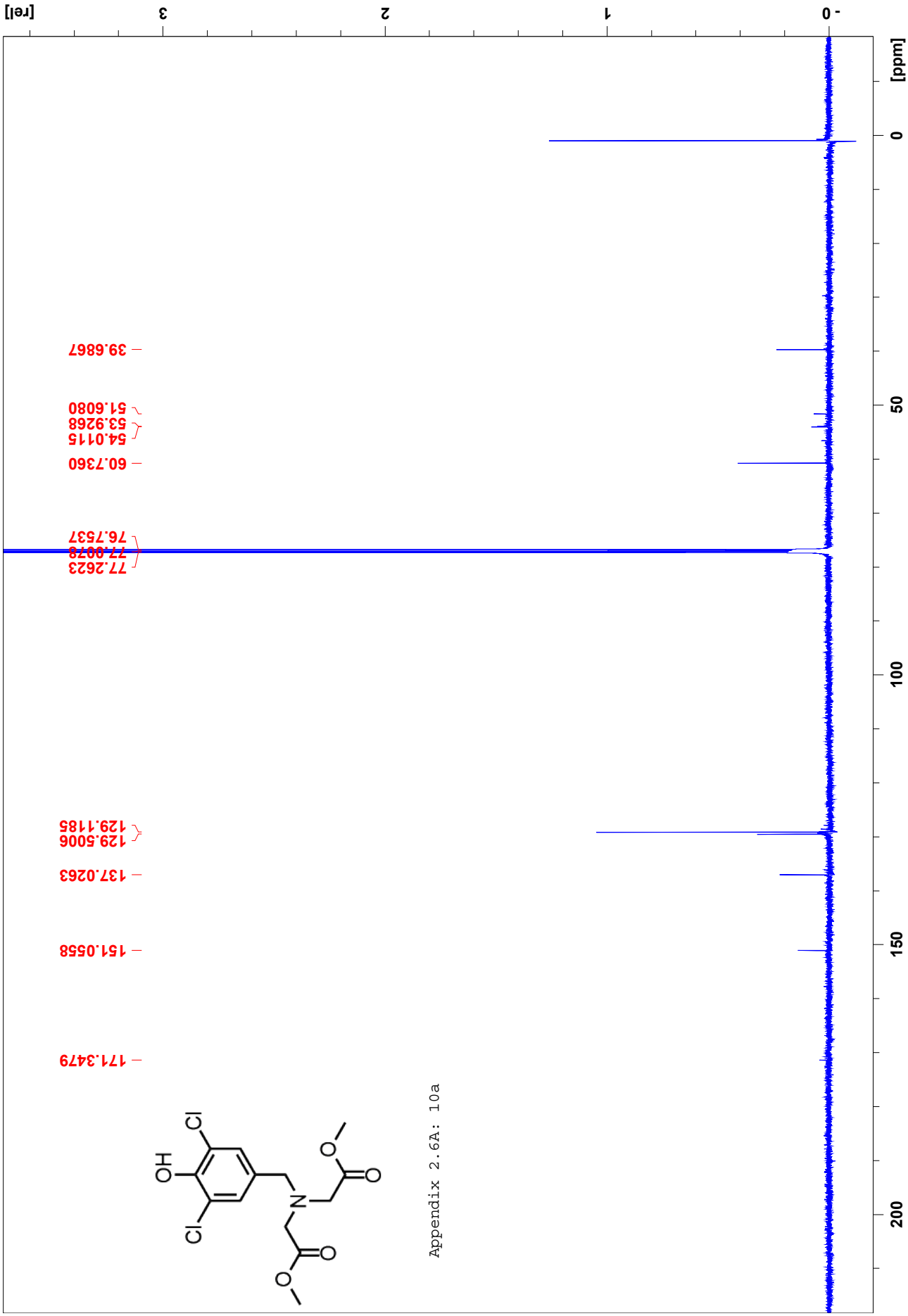
Appendix 2.6A: 9a

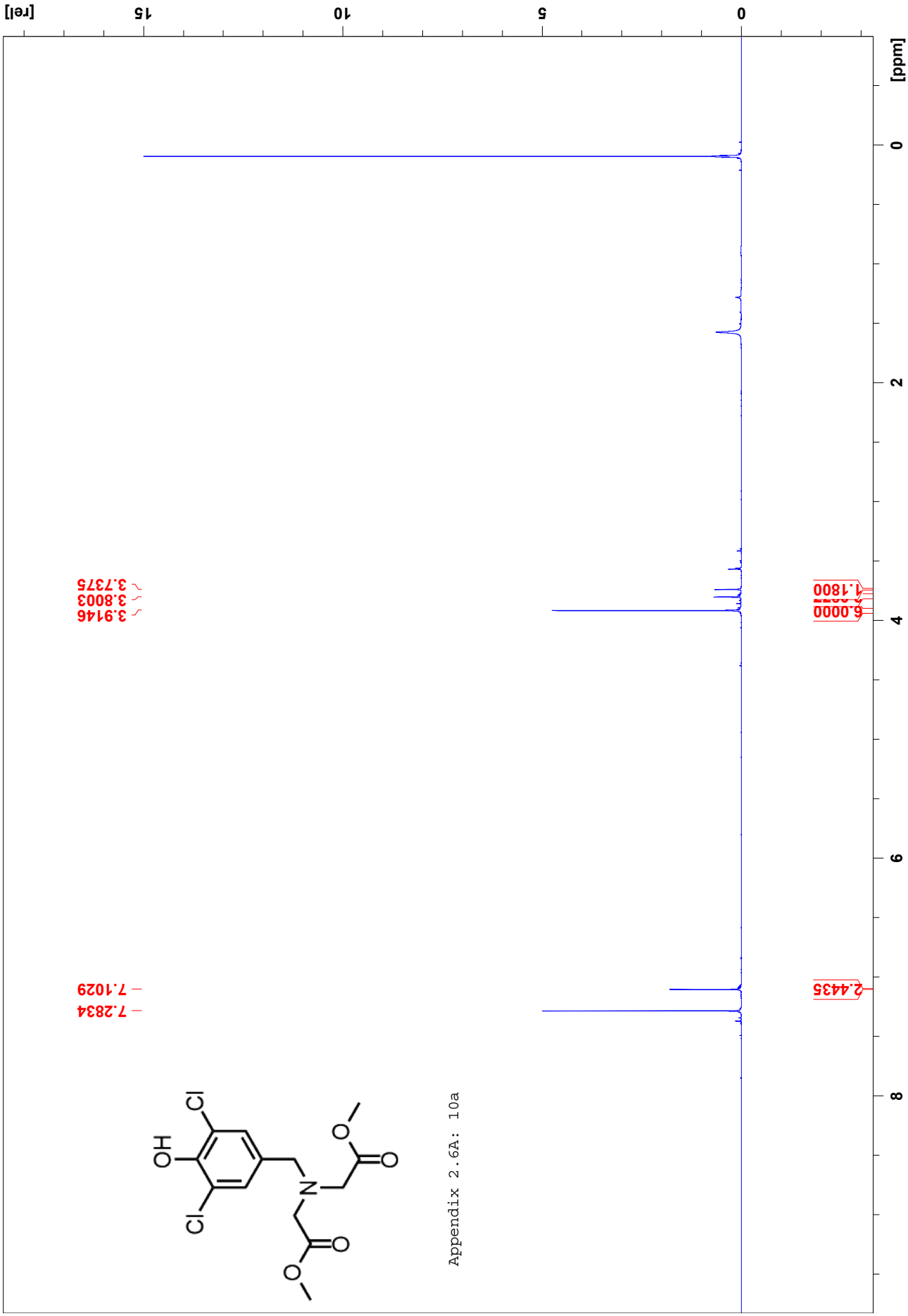


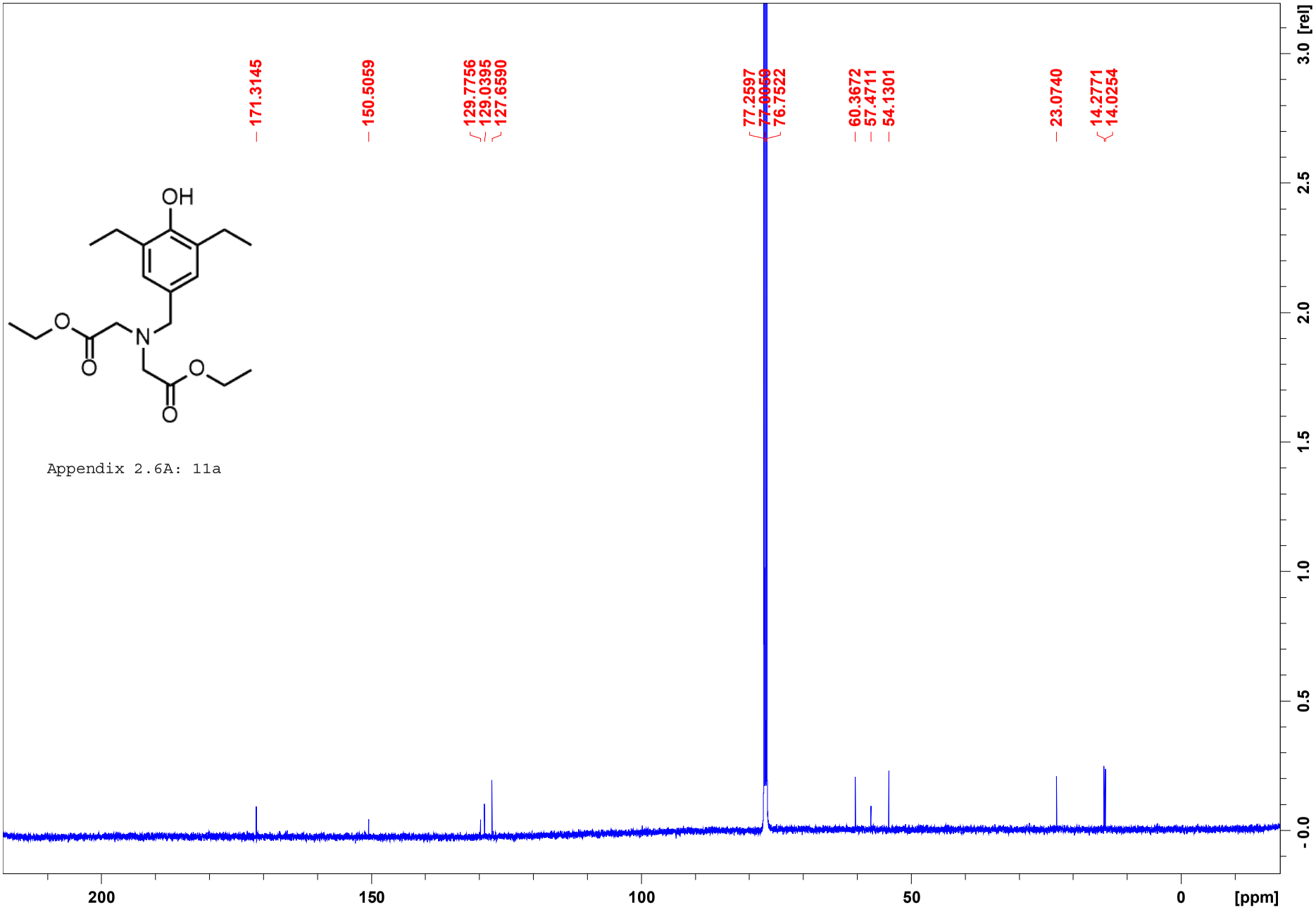
Appendix 2.6A: 9a

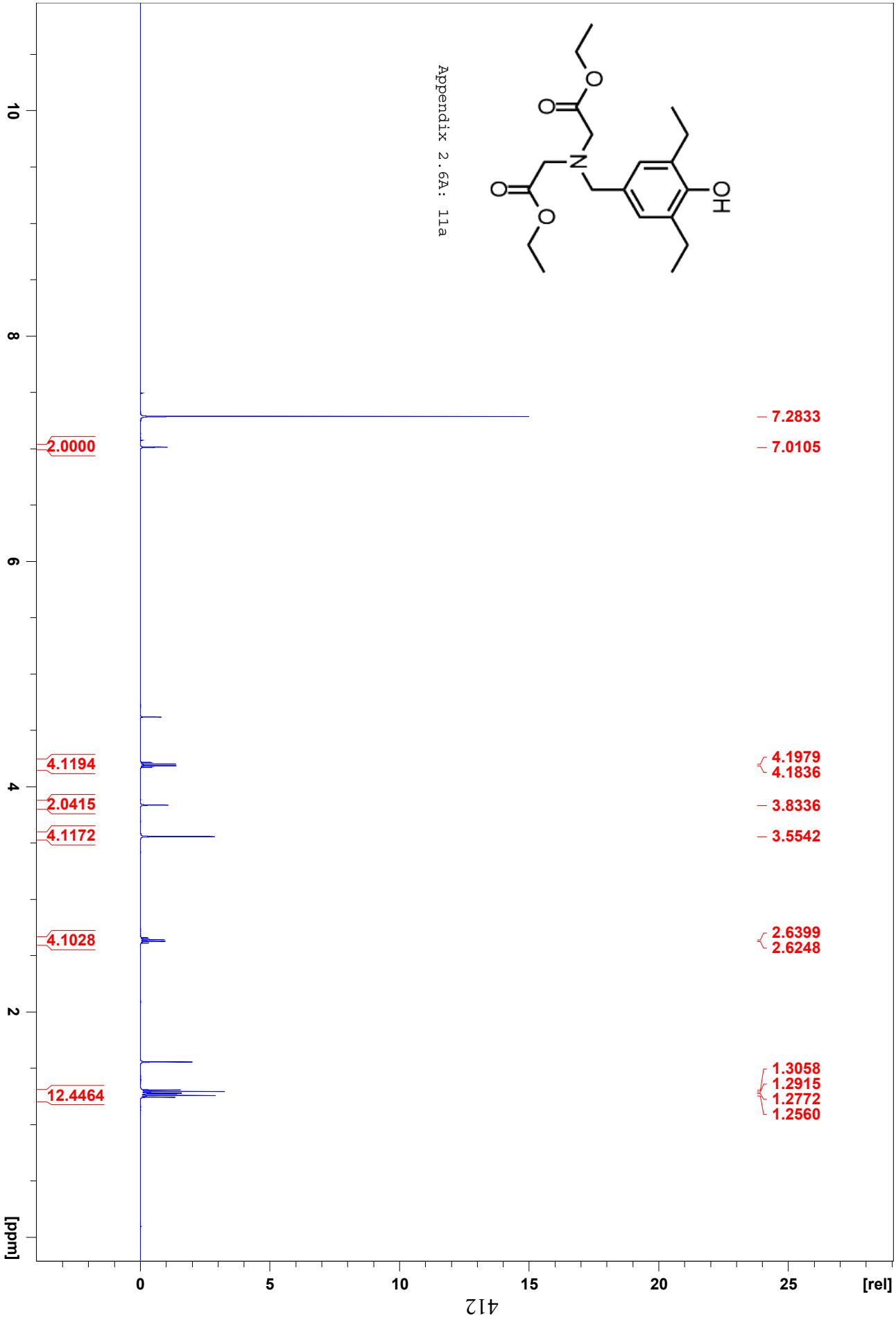


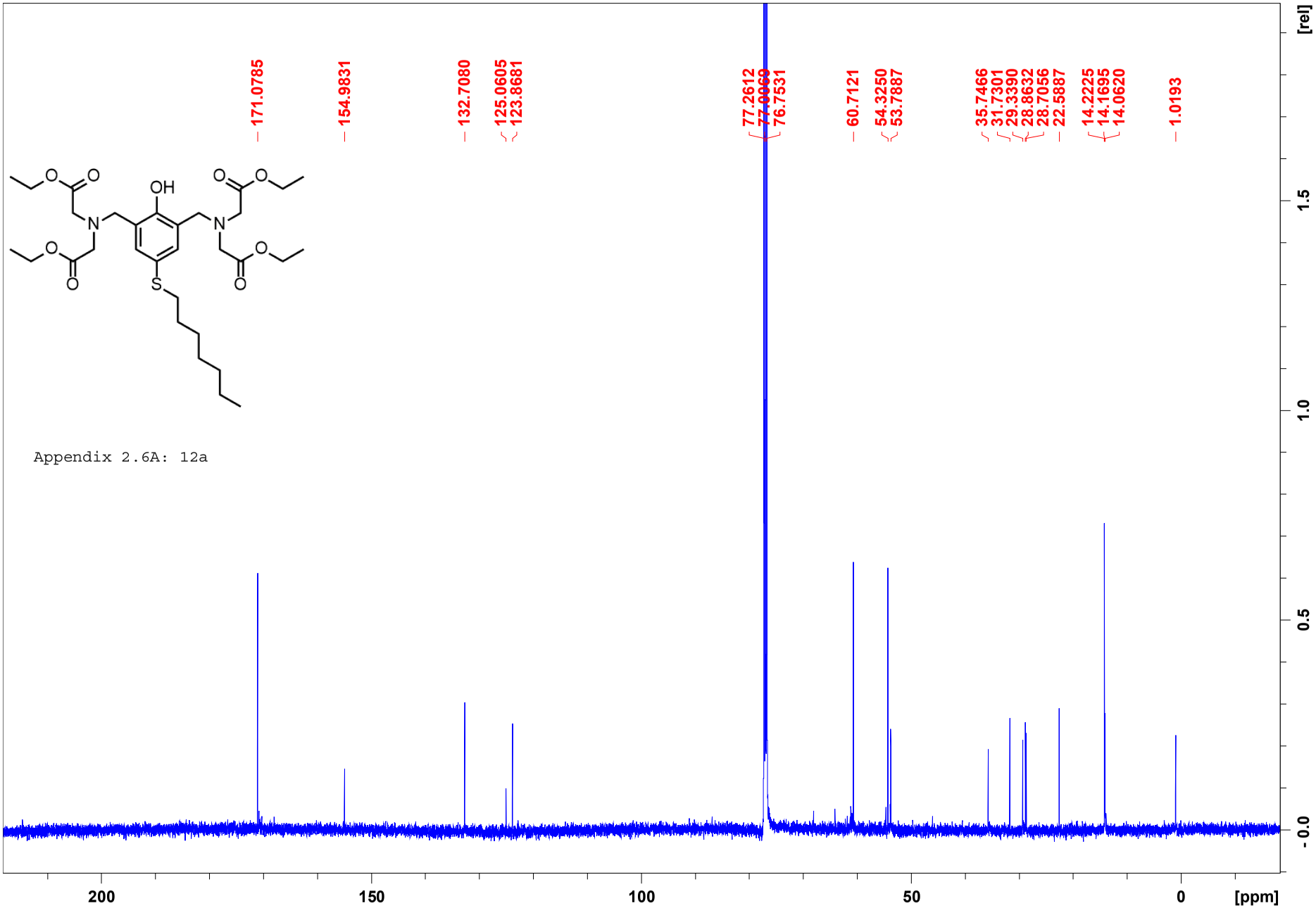
Appendix 2.6A: 9a


Appendix 2.6A: 10a
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137.0263
129.5006
129.1185
77.2623
77.0078
76.7537
60.7360
54.0115
53.9268
51.6080
39.6867
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[ppm]
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100
150
200
1
2
3
Cl
OH
Cl
N
O
O
O
O

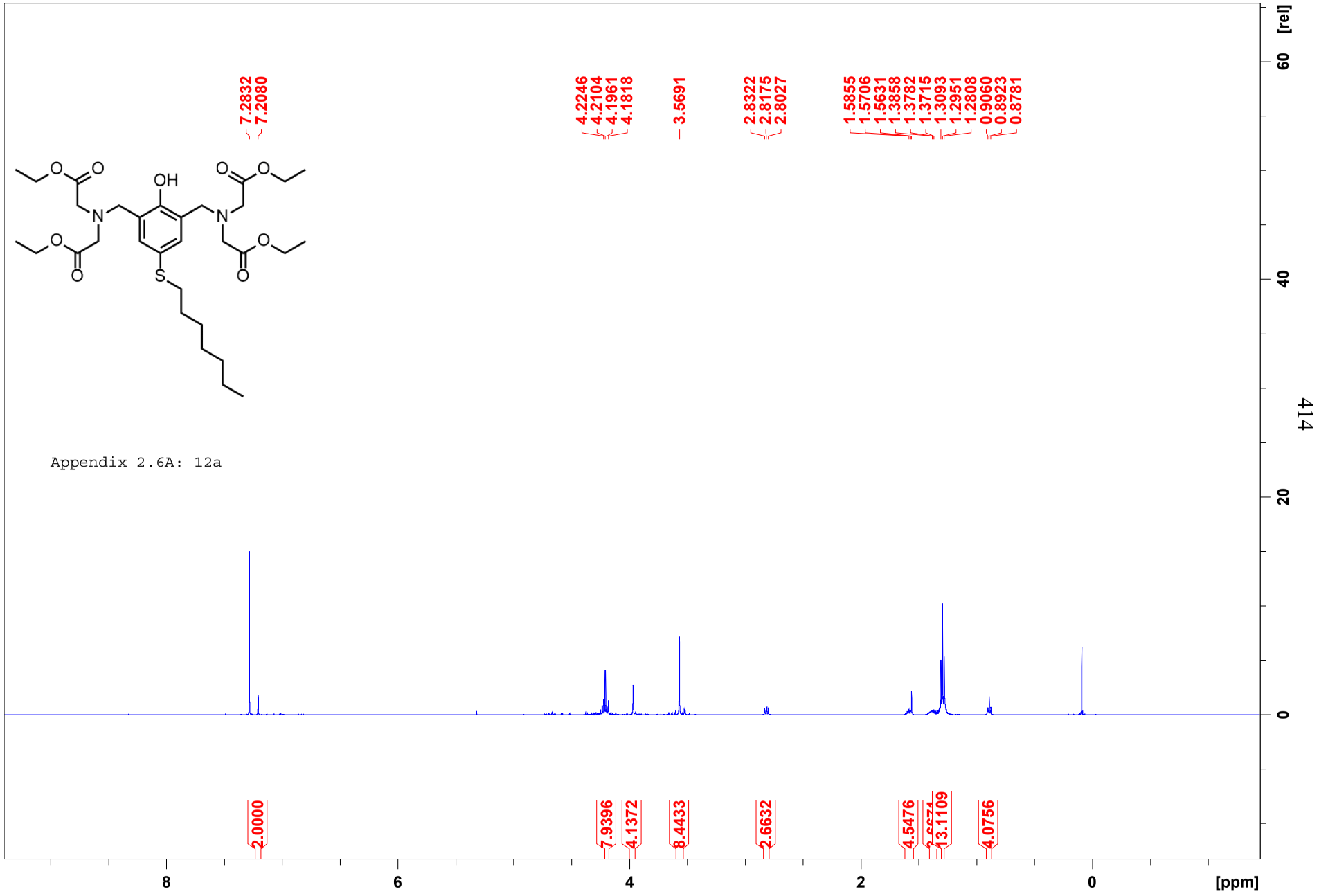

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5
0
[ppm]
0
2
4
6
8
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7.1029
2.4435
6.0000
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3.8003
3.7375
Cl
OH
Cl
N
O
O
O
O
Appendix 2.6A: 10a

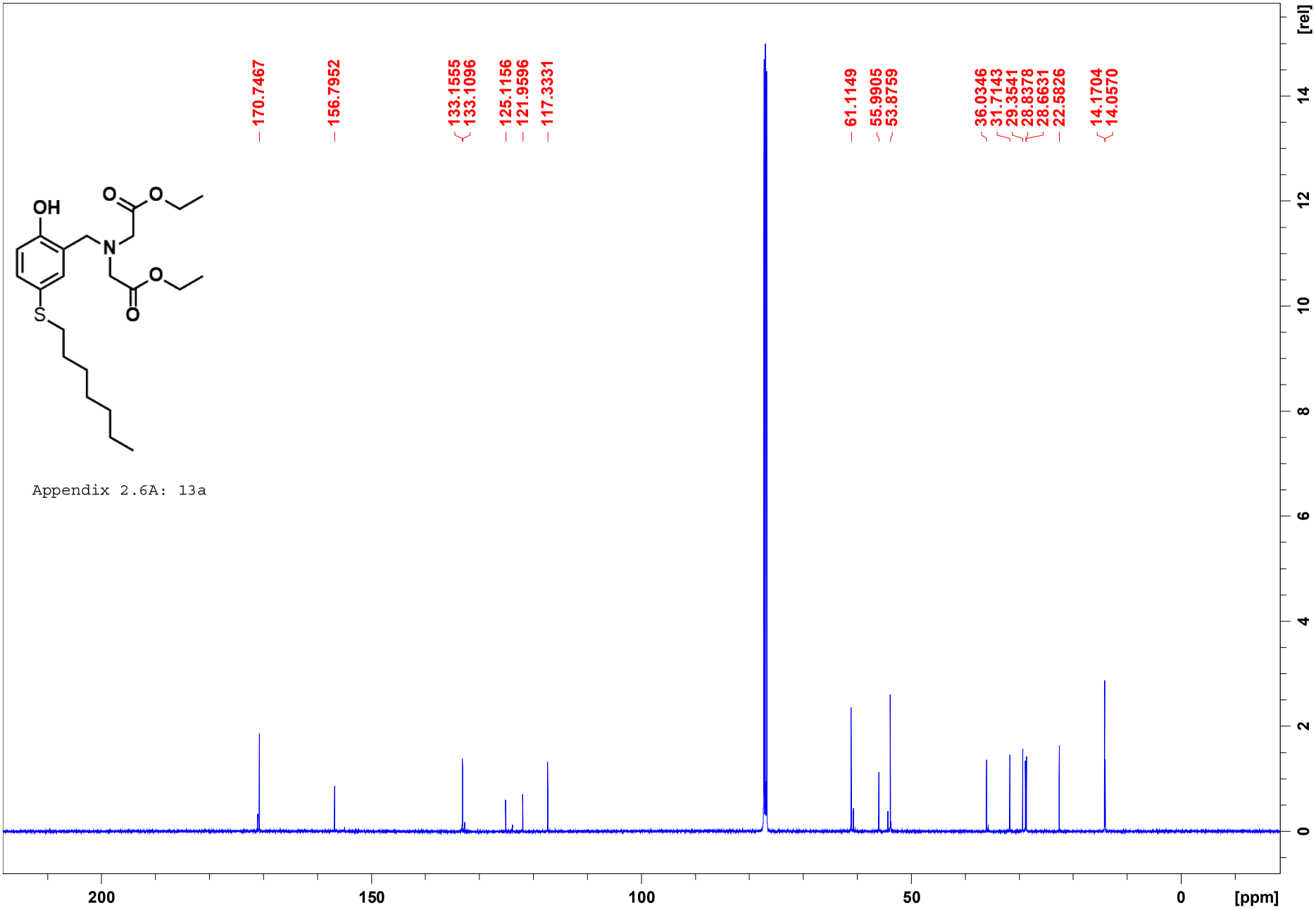

171.3145
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129.7756
129.0395
127.6590
77.2597
77.0050
76.7522
60.3672
57.4711
54.1301
23.0740
14.2771
14.0254
OH
O
N
O
O
O
Appendix 2.6A: 11a
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150
100
50
0
[ppm]
- 0.0
0.5
1.0
1.5
2.0
2.5
3.0 [rel]


Appendix 2.6A: 11a
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O
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O
O
OH
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7.0105
4.1979
4.1836
3.8336
3.5542
2.6399
2.6248
1.3058
1.2915
1.2772
1.2560
2.0000
4.1194
2.0415
4.1172
4.1028
12.4464
10
8
6
4
2
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5
10
15
20
25
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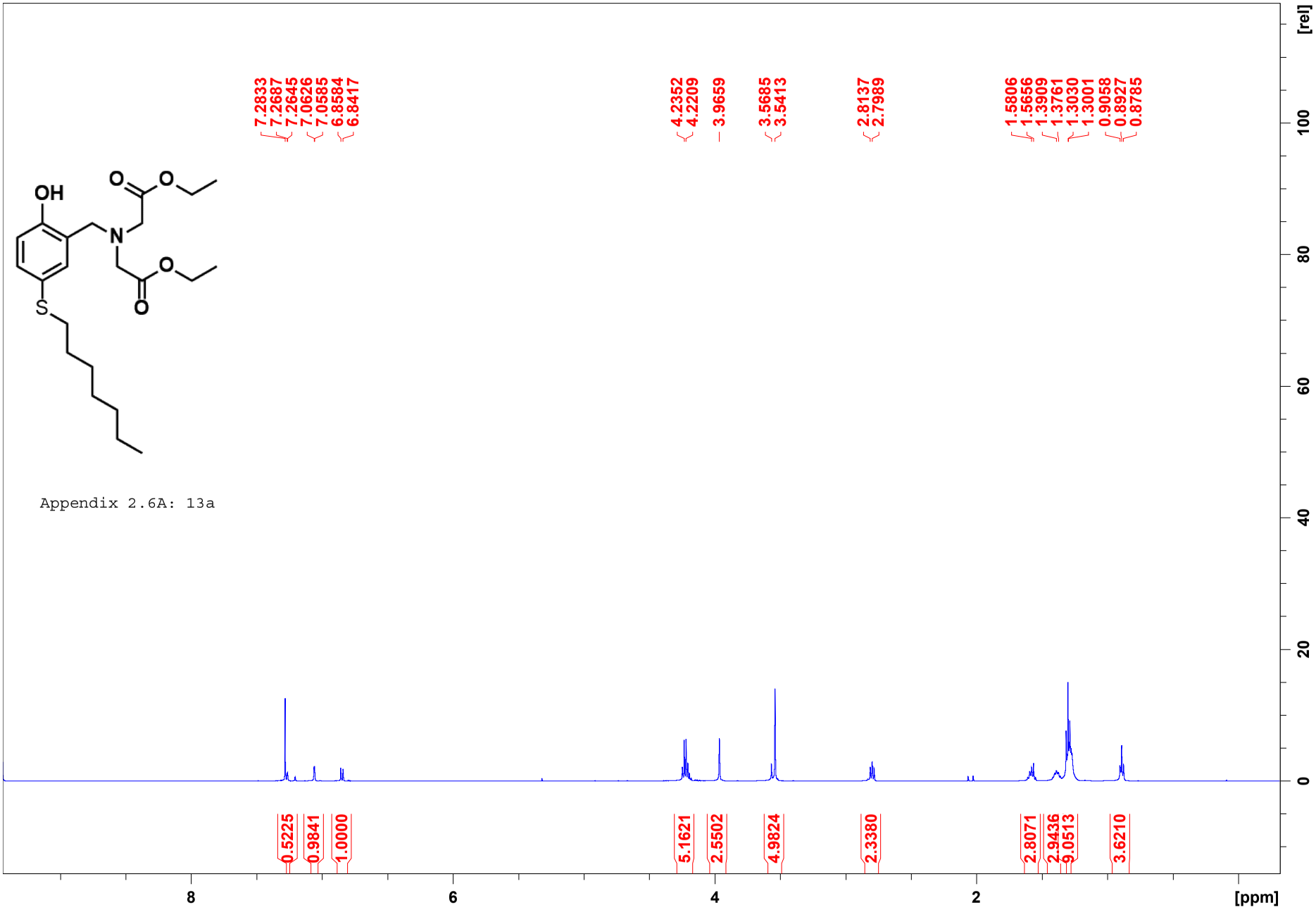


Appendix 2.6A: 12a

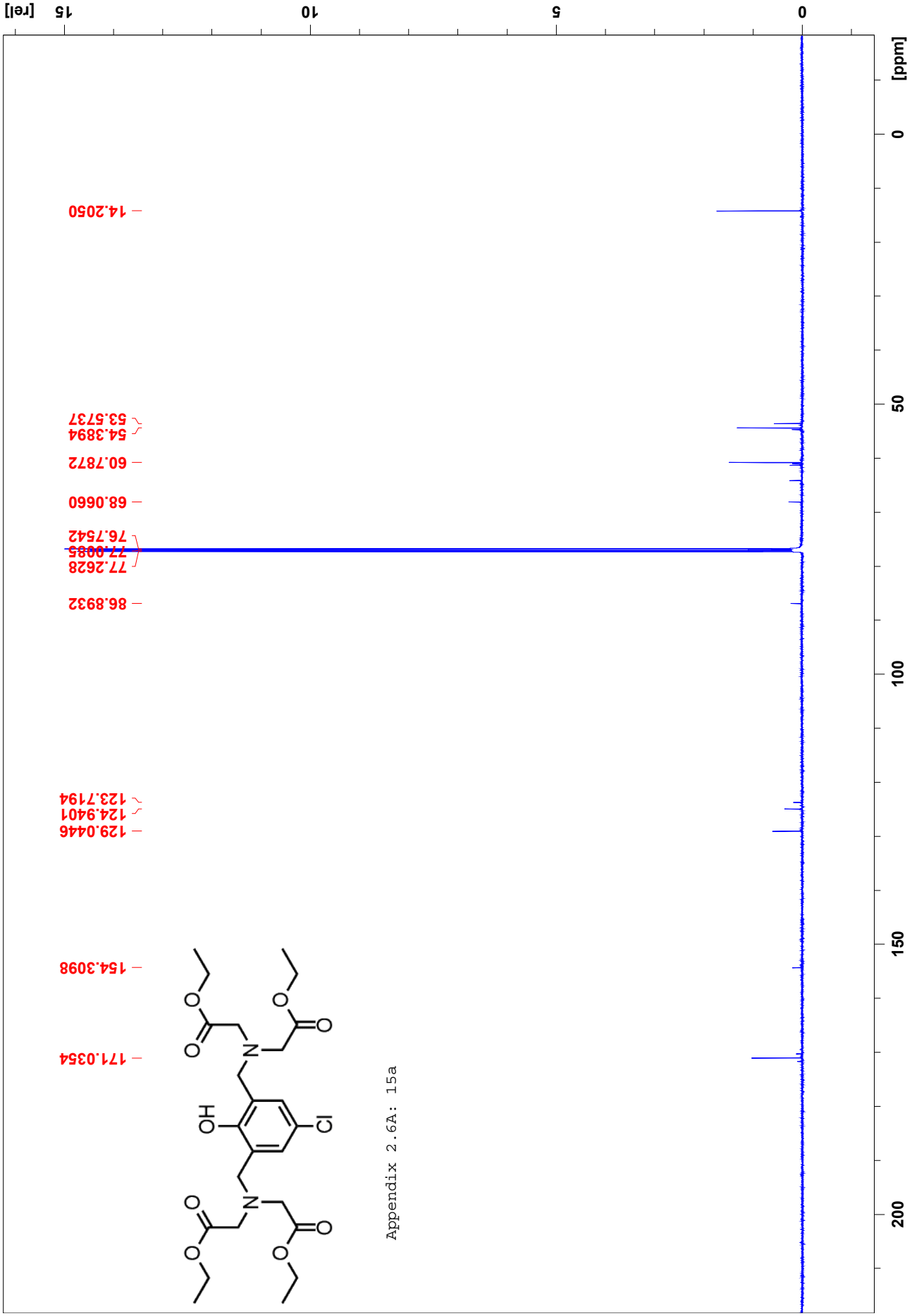

7.2832
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4.2246
4.2104
4.1961
4.1818
3.5691
2.8322
2.8175
2.8027
1.5855
1.5706
1.5631
1.3858
1.3782
1.3715
1.3093
1.2951
1.2808
0.9060
0.8923
0.8781
OH
O
N
S
Appendix 2.6A: 12a
2.0000
7.9396
4.1372
8.4433
2.6632
4.5476
13.1109
4.0756
8
6
4
2
0
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20
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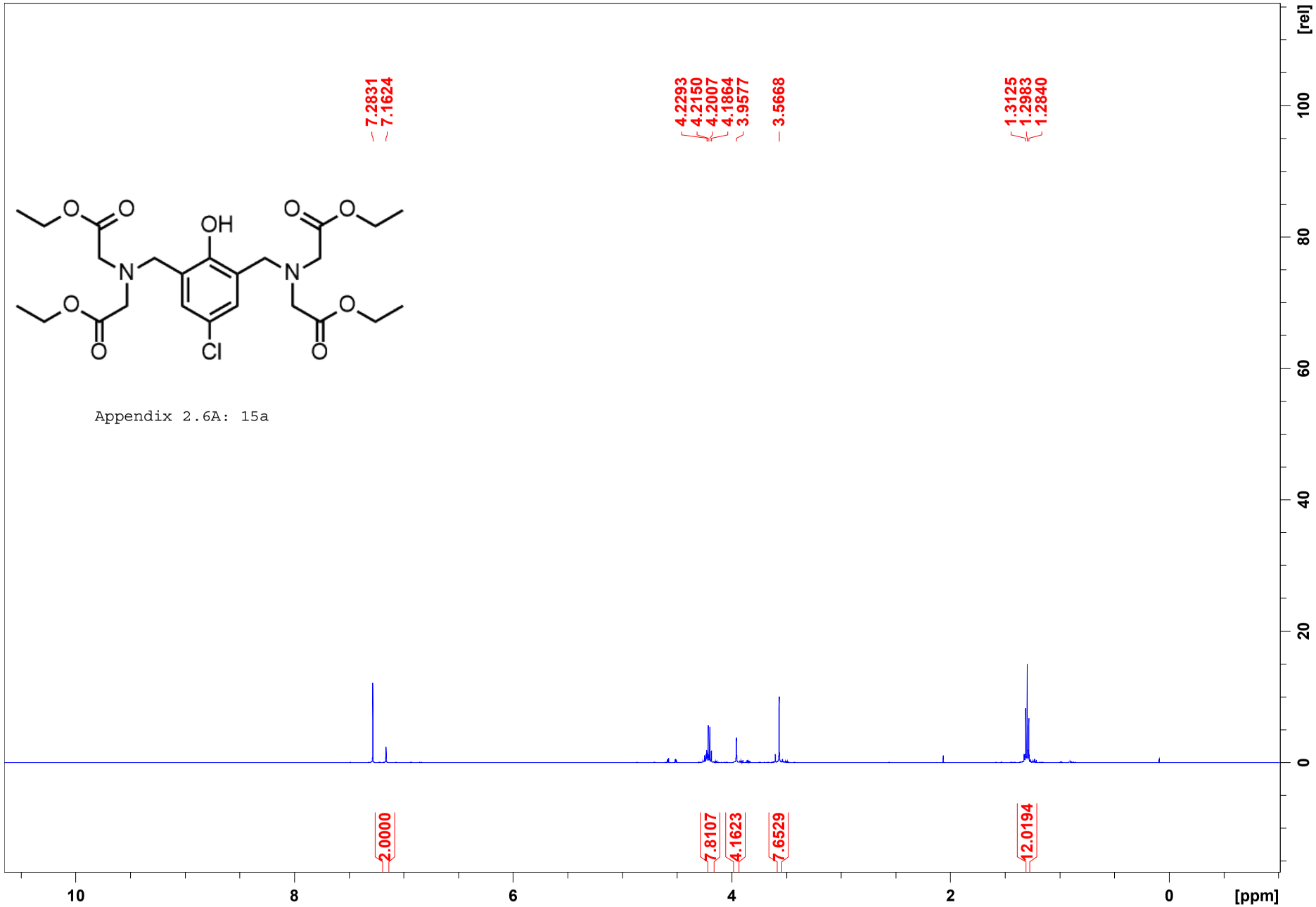
Appendix 2.6A: 13a



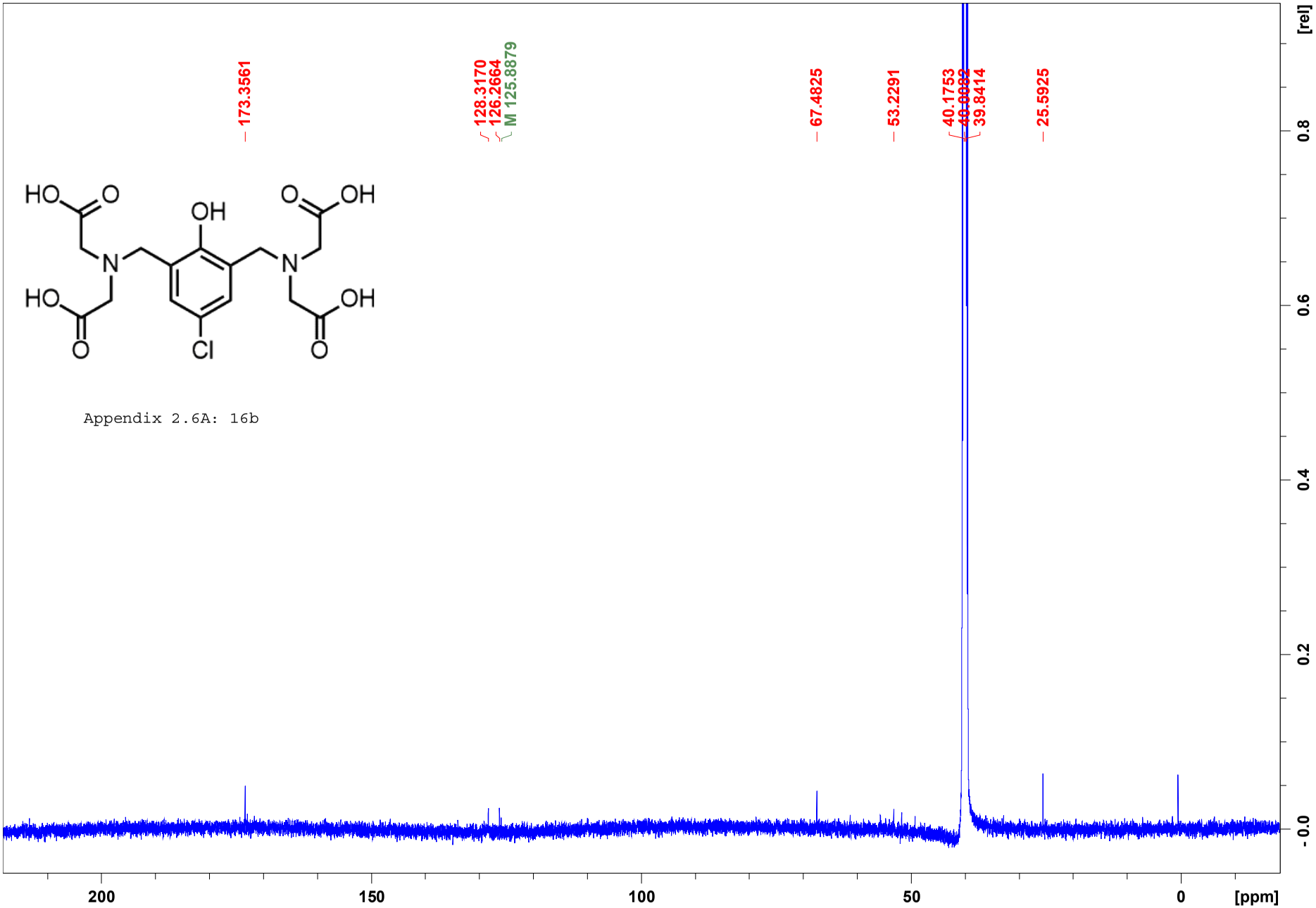
Appendix 2.6A: 13a



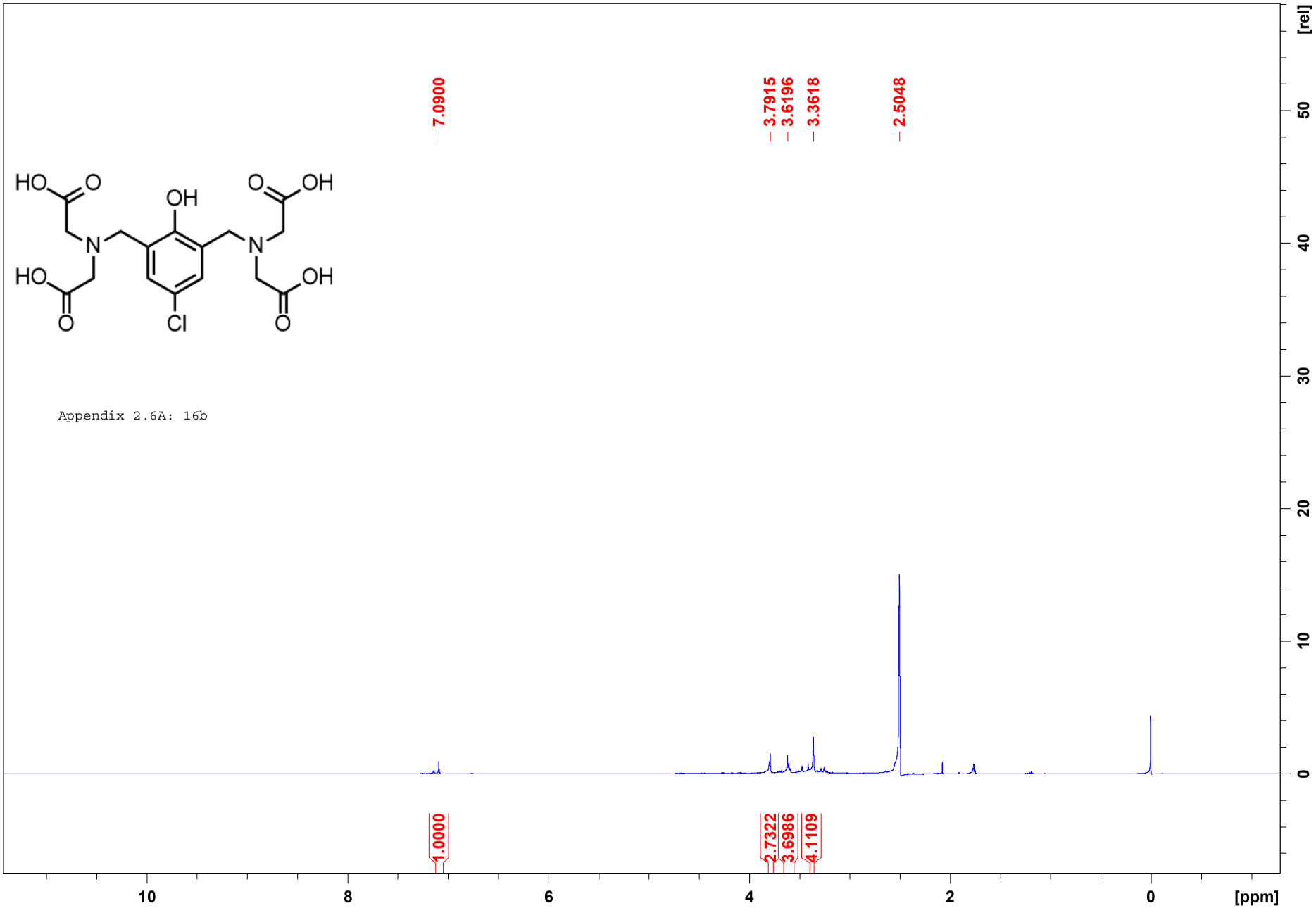
Appendix 2.6A: 15a



Appendix 2.6A: 15a



Appendix 2.6A: 16b


7.0900
3.7915
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3.3618
2.5048
HO
O
OH
O
OH
N
N
HO
O
OH
Cl
O
Appendix 2.6A: 16b
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3.6986
4.1109
10
8
6
4
2
0
[ppm]
[rel]
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40
30
20
10
0