

Synthesis of Novel Charged Ice Recrystallization Inhibitors

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Abstract

With emerging trends of new cellular therapies, the need for quick access to cellular components is necessary. For most applications genetically compatible biological components are essential to prevent adverse immune responses and graft-versus host disease (GVHD). Since these biological components have a limited window to be used, techniques for long-term storage are needed. Cryopreservation is essential for this in the field of biobanking and regenerative medicine to allow for long-term storage of cell products. During this process, ice recrystallization is the major contributor to cell death and decreased cell viability post-thaw. Due to this, controlling ice growth and recrystallization is imperative to increasing cell survival and function.

The Ben lab is focused on the synthesis of small molecule, carbohydrate-based cryoprotectants that function as ice recrystallization inhibitors (IRIs). Previously, many IRIs have been synthesized showing varying degrees of ice recrystallization inhibition (IRI). Through the structure-function work, a delicate balance between hydrophobic and hydrophilic portions on the same molecule must be met. These compounds are believed to disrupt hydrogen bonding networks present in the formation of ice, and control ice growth. While numerous types of functional groups on carbohydrate derivatives have been explored, many highly solvated functional groups (amines, sulfates, phosphates, etc.) have not been thoroughly investigated. Highly solvated functional groups should disrupt hydrogen bond networks due to their solvation and in theory, should illicit an IRI response.

Sulfate groups have not previously been studied, but are present in several different biological processes, such as immune response and blood coagulation. This suggests that sulfated carbohydrates should be well tolerated biologically. Sulfate groups can also be easily installed on

existing IRI active molecules through orthogonal protecting group chemistry. The first part of this thesis is focused on the synthesis and IRI activity of sulfated carbohydrates based upon previously synthesized, IRI active pyranose derivatives. When compared to their parent compounds, most of the sulfated derivatives were less active, but all compounds were incredibly soluble in aqueous media. These derivatives did not show much promise as new IRIs due to the length of their synthesis and reduced IRI activity compared to their parent compounds.

The Ben lab has also developed a new class of IRI active carbohydrates: aldonamide derivatives. These compounds are open-chain carbohydrates with an amide bond, arising from the ring opening of a carbohydrate lactone with a substituted amine. While many of these compounds displayed high degrees of IRI activity, many were incredibly insoluble in aqueous systems (many with solubility limits under 50 mM). Since sulfate groups were able to greatly increase solubility with some derivatives retaining IRI activity, installing sulfate groups on existing aldonamide-based IRIs should increase their solubility. Additionally, since many of these derivatives display high degrees of IRI activity, a reduction in IRI activity can be tolerated. Similarly, to the sulfated pyranose derivatives, the presence of a sulfate group reduced the IRI activity compared to the parent compounds in most derivatives. Though some sulfated derivatives possessed a higher degree of IRI activity, all the derivatives experienced a drastic increase in solubility (over 200 mM in PBS). Some of the sulfated aldonamide derivatives were assessed for their ability to protect red blood cells (RBCs) during freezing with reduced glycerol concentrations (15% glycerol), although none of the tested derivatives showed an improvement over existing IRIs explored by the Ben lab.

Since the introduction of sulfate groups to existing IRIs drastically increased solubility in aqueous systems, but resulted in reduced IRI activity in most compounds, focus was switched to

the addition of different hydrophilic functional groups. Amino functional groups were briefly explored with galactose-based pyranose IRIs, aldonamide derivatives had not been explored. Amino groups are present on many biological carbohydrates and should be well tolerated biologically. The addition of amino groups to aldonamide derivatives should increase solubility, with the amino derivatives ideally retaining some IRI activity. The amino aldonamide derivatives synthesized had high solubilities (>500 mM in PBS), but did possess lower degrees of IRI activity. Due to the high solubility these derivatives were initially assessed in the cryopreservation of RBCs with reduced glycerol concentrations. Initial experiments showed improvements over current IRIs, and the compounds were assessed in a number of other biological cryopreservation scenarios including articular cartilage, platelets, and hematopoietic stem/progenitor cells (HSPCs). While the compounds showed toxicity in these cell types, more studies need to be conducted for the cryopreservation of RBCs.

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List of Abbreviations

2FA	<i>N</i> -(2-fluorophenyl)-D-gluconamide
α	Alpha
β	Beta
δ	Delta
ϵ	Epsilon
AFGP	Antifreeze glycoprotein
AFGP-8	Antifreeze glycoprotein fraction 8
AFM	Atomic force microscopy
AFP	Antifreeze protein
ANOVA	Analysis of variance
BA	Biological antifreeze
BH ₃ THF	Borane tetrahydrofuran complex
BF ₃ ·OEt ₂	Boron trifluoride diethyl etherate
BFU-E	Burst-forming unit-erythroid
br	Broad
C-AFGP	C-linked antifreeze glycoprotein
CB	Cord blood
CD (CD34)	Cluster of differentiation
CDCl ₃	Deuterated chloroform
CFC	Colony-forming cell
CFU	Colony-forming unit
CFU-GEMM	Colony-forming-granulocyte, erythrocyte, monocyte, and megakaryocyte
CFU-GM	Colony-forming unit-granulocyte, monocyte
CMC	Critical micelle concentration
CPA	Cryoprotective agent
CoCl ₂	Cobalt chloride
CuSO ₄	Copper sulfate (anhydrous)
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
dddd	Doublet of doublet of doublet of doublets
dH ₂ O	Distilled water
DIAD	Diisopropyl azodicarboxylate
DIS	Dynamic ice shaping
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d ₆	Deuterated dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
ESI	Electrospray ionization mode
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gal	Galactose

Glc	Glucose
GPVI	Glycoprotein VI (in platelets)
GVHD	Graft-versus-host disease
HCO ₃ ⁻	Bicarbonate ion concentration
H ₂ SO ₄	Sulfuric acid
HepG2	Human hepatocellular carcinoma cell line
HES	Hydroxyethyl starch
HI	Hydration index
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
hPLT	Human platelet
HSPC	Hematopoietic stem and progenitor cell
I _c	Cubic ice
IC ₅₀	Half-maximal ice recrystallization inhibition concentration
I _h	Hexagonal ice
I _{sd}	Stacking-disordered ice
IIF	Intracellular ice formation
IMDM	Iscove's Modified Dulbecco's Media
IR resin	Amberlite ion exchange resin
IRI	Ice recrystallization inhibitor
LRMS	Low resolution mass spectrometry
m	Multiplet
M+	Molecular ion (e.x. for LRMS calculations)
Man	Mannose
MEM	Minimum essential media
%MGS	Percent mean grain size
MSC	Mesenchymal stem cell
NAD (H)	Nicotinamide adenine dinucleotide (+ hydrogen)
<i>N</i> -MeIm	<i>N</i> -methylimidazole
NMR	Nuclear magnetic resonance
pCO ₂	Partial CO ₂ pressure
PBS	Phosphate-buffered saline
pet. ether	Petroleum ether
PEG	Polyethylene glycol
Phth	Phthalimide
PI	Propidium iodide
PMA	<i>N</i> -(4-methoxyphenyl)-D-gluconamide
PMP	<i>para</i> -Methoxyphenyl
pO ₂	Partial O ₂ pressure
PPh ₃	Triphenylphosphine
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
q	Quartet
QLL	Quasi-liquid layer
RBC	Red blood cells
Rf	Retention factor

rt (RT)	Room temperature
s	Singlet
SAR	Structure-activity relationship
sat.	Saturated
SD	Standard deviation
SDS	Sodium dodecyl sulfate
% SEM	Percent standard error of the mean
sO ₂	Oxygen saturation
t	Triplet
TBAF	Tetrabutylammonium fluoride
TCE	Trichloroethyl
TCO ₂	Total CO ₂
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
T _g	Glass transition
TH	Thermal hysteresis
THF	Tetrahydrofuran
TIPS	Triisopropylsilyl
TLC	Thin-layer chromatography
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TNC	Total nucleated cell
TsCl	4-Toluenesulfonyl chloride
tt	Triplet of triplets
UCB	Umbilical cord blood
UV	Ultraviolet
UV-vis	Ultraviolet-visible
vWF	von Willebrand factor

Chapter 1. Introduction

1.1 Cellular Therapies

Each year, over 50,000 patients receive potentially life-saving cellular transplants for several diseases and disorders.¹ These transplants are examples of cellular therapies, which refer to the administration of cells or tissues to an individual in order to elicit a therapeutic response or to prevent future illness.^{1,2} Several cell and tissue types are being used as cellular therapies, originating from a patient's own cells (autologous) or a donor (allogenic) depending on the illness.^{1,3,4}

Cellular therapies are responsible for saving thousands of lives per year, with blood transfusions being one of the most common. Between 2017-2018 Canadian blood services shipped out over 700,000 red blood units to hospitals.⁵ Transfusion of red blood cells (RBCs) are used to treat accident victims, patients with anemias, and individuals with certain blood diseases including sickle cell anemia and malaria.^{1,6-12} While many of these patients receive transfusions, not all hospitals have adequate access to enough blood in times of crisis (natural disasters, etc.).¹³⁻¹⁵ The ability to store RBC units for the general population ensures RBC units are available when needed.

More recently, transplantation of various types of stem cells has become a common practice for regenerative medicine therapies.^{1,4,16} Transplantation of hematopoietic stem and progenitor cells (HSPCs) has been used to treat various conditions such as hematological diseases, immunodeficiencies, lymphomas, and leukemias.^{4,17-20} HSPCs can be obtained from three separate sources: bone marrow, peripheral blood, and umbilical cord blood. While all these sources contain HSPCs, the number of stem/progenitor cells and ease of isolation varies greatly.

HSPCs are used due to their ability to go through hematopoiesis; the generation of the cellular components of blood.²¹

While many cellular therapies rely on the administration of specific cells into a patient, tissues or other more complex structures can be used as well. Due to the common occurrence of cartilage damage, cartilage transplant has been an increasing area of study. Articular cartilage injury occurs in thousands of Canadians per year²² and leads to severe joint pain and inflammation.²³ This can be treated by several surgical procedures such as: arthroscopic lavage, microfracture surgery, and hydrogel implants.²⁴ While these methods are often successful, these procedures are generally limited to small injury sites (10-20 mm). In order to treat larger injury sites (>20 mm), regrowth or replacement of the cartilage is needed. Cell-based repairs are done by removing a small portion of the patients own cartilage in a non-load bearing area and culturing the chondrocytes. The chondrocytes are proliferated before being transplanted back into the injured area. Whole sections can also be transplanted into the affected areas either by osteochondral autografts (patient's own cartilage) or osteochondral allografts (outside patients' cartilage).^{24,25}

With emerging cellular therapies and the potential, they have to improving both survival and quality of life, there is a fundamental challenge associated with the storage of cellular therapy products. With the development of biobanks, cells and tissues for research and therapy are more widely obtainable, although samples of sufficient quality are not always available at the time they are needed. Due to this, cryopreservation of these cells and tissues is essential in order to meet the increasing need for these products.²⁶⁻²⁹

1.2 Cryopreservation

1.2.1 Importance of Cryopreservation

With the increased demand for cellular therapy products in the past few decades, innovative techniques are needed to meet this demand.^{27,28,30} One way to ensure these products are available when they are needed is to isolate and bank the necessary cells/tissues in a process known as biobanking. Biobanking of cellular therapy products allows adequate time for human leukocyte antigen (HLA) typing for the matching of donors and recipients, screening samples for transmissible diseases, and transportation of samples between hospitals and transplant centers.²⁸ Cryopreservation allows samples to be stored for long periods of time and subsequently thawed when necessary. Although many advancements in cryopreservation techniques have been developed, the rapid development of new cellular therapies requires novel methods to preserve these products. Currently, cells and tissues are preserved using one of four main methods of preservation: lyophilization, hypothermic storage, cryopreservation, and vitrification.

1.2.2 Lyophilization

Lyophilization (or freeze-drying) is a technique where a sample is frozen and held under very low pressure.^{31,32} This results in sublimation of the water, leaving an intact, dry sample afterwards. Due to the removal of water, samples often can be stored at room temperature. This technique is commonly used to preserve bacteria along with other cells and tissues such as platelets, RBCs, and sperm cells.³²⁻³⁴ This can be performed with or without additives to protect the sample throughout the process.³⁵⁻³⁷ While this technique is useful in limited applications, the process can cause extensive structural damage which greatly reduces its use in clinical applications.

1.2.3 Hypothermic Storage

Hypothermic storage is used for storing cells and tissues for short periods of time ranging from hours to sometimes weeks.^{38,38,39} These samples are stored at between 1-10 °C often without the addition of any cryoprotectant.³¹ Using hypothermic storage without the addition of a cryoprotectant allows immediate usage of the sample without lengthy processing steps. This type of storage is predominantly used for short term storage and transportation of patient biopsy samples, but also has been used for storage of RBCs, placental MSCs, and stem cell-derived cardiomyocytes.^{29,39-43} While storage at these temperatures partially suppresses biological activity, it is not completely halted resulting in cellular damage during extended storage times. As a result, reductions in cell viability and functionality are often observed and are directly correlated to the length of storage.^{39,43,44} For these reasons, hypothermically-stored samples must be used as soon as possible, and long-term storage is very limited.

1.2.4 Cryopreservation

Cryopreservation refers to the long-term storage of biological materials at subzero temperatures typically ranging from -20 to -196 °C. Storage at these temperatures essentially halts biological activity, allowing samples to be stored for months to years.⁴⁵⁻⁴⁷ Protocols for cryopreservation require extensive optimization due to significant variabilities between cell types. This includes, but is not limited to cooling and warming rates, cell concentrations, storage temperature, cryoprotectant mechanism and concentration, and pre-freeze incubation time.^{26,39,44,48-52} Many cryoprotectants that are currently used in clinical applications exhibit toxicity, and generally must be removed prior to use of the sample to prevent adverse reactions. These cryoprotectants also do not protect the samples from ice growth or recrystallization, which is a major source of cryoinjury during the warming of frozen samples. This and other mechanisms of cryoinjury are further discussed in **Section 1.2.7**.

Cryopreservation can also be carried out at higher temperatures generally ranging from -5 to -30 °C, known as high sub-zero cryopreservation.^{53–55} This type of cryopreservation has been used for the cryopreservation of platelets, oocytes, and RBCs.^{55–58} The need for this type of cryopreservation is necessary to preserve these organisms during the winter where they experience sub-zero temperatures. These organisms survive by accumulating high concentrations of extracellular trehalose, glucose, and sucrose.^{34,50,59,60}

Although cryoprotectants are used in order to help protect samples from the freezing and thawing processes, damage known as cryoinjury still occurs and affects post thaw viability and functionality of cryopreserved samples.

1.2.5 Vitrification

One of the major sources of damage during typical cryopreservation techniques is the formation and recrystallization of ice crystals.^{27,48,58,61–63} Vitrification avoids the formation of ice in the sample upon freezing by using ultra-fast cooling rates (>2000 °C/min in some cases). This glass formation happens as the viscosity of the solution drastically increases due to the fast-cooling rate and high concentrations of cryoprotectant. Rapid cooling allows the sample to surpass the glass transition temperature (T_g) of the solution, resulting in a solid glass which contains no ice crystals.^{64,65}

Vitrification is an attractive cryopreservation technique for more complex samples, including oocytes, embryos, cartilage, and organs.^{47,66–70} By using vitrification rather than more traditional cryopreservation techniques, some of the problems encountered with more complex cells can be avoided. More complex cells as well as tissues often are much less permeable to cryoprotectants, meaning that the interiors of the sample are susceptible to cryoinjury.^{48,65} Vitrification has

proven superior to conventional cryopreservation for oocytes^{57,64,71} due to slow permeation times of water and cryoprotectants into the tissue.^{68,72,73}

Although vitrification has many advantages, there are some inherent disadvantages and limitations. Along with the ultra-fast cooling rates needed for vitrification, high concentrations of cryoprotectants are also used to increase the viscosity of the solution and protect the sample during vitrification.^{26,66,74} Generally, DMSO in concentrations ranging from 1 to over 6 M is used to displace water within the sample (further discussed in **Section 1.4**).^{50,66,72,75} Due to the cytotoxicity of DMSO, pre-vitrification incubation times must be short to limit decreased viability post-thaw.

Currently, vitrification is limited to small volume samples due to the inability of achieving uniformly fast cooling rates larger samples.⁶⁶ One of the major drawbacks to vitrification is the warming (or devitrification) of the sample. Once the sample is removed from storage and begins to devitrify, ice crystals form as the temperature of the sample increases above the T_g of the solution.^{76,77} After ice crystals begin to form and the temperature increases further, these crystals go through high degrees of ice recrystallization (further discussed in **Section 1.5**). As previously discussed, current cryoprotectants used in vitrification do not adequately prevent ice recrystallization, leaving a large potential for new cryoprotectants with more desirable properties.

1.2.6 Mechanism and Action of Cryoprotectants

While post-thaw cell viability can be improved by employing optimal cooling and warming rates (**Figure 1.2**), cell survival remains <70% for most cell types.^{46,78} In some cases, survival can be increased to >90%, though the required cooling rates are impractical in clinical

situations.^{79–81} In order to increase cell viability during cryopreservation, cryoprotectants can be added to samples prior to freezing.

There are two major categories of cryoprotectants currently used: permeating and non-permeating. Permeating cryoprotectants, including dimethyl sulfoxide (DMSO) and glycerol, permeate cell membranes and internalize within cells. This protects cells from cryoinjury associated with slower than optimal cooling rates by displacing water within cells which helps maintain cell volumes during freezing.^{37,77,82} Permeating cryoprotectants can also reduce solution effects resulting from slow cooling by diluting solutes within cells. Non-permeating cryoprotectants are generally polymers such as polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP), hydroxyethyl starch (HES), and dextran, although disaccharides including trehalose and sucrose have also been used. These cryoprotectants cannot cross cell membranes and remain in the extracellular solution. This increases the osmolality of the extracellular solution, and aids in dehydration of the cell^{83–85} and this is beneficial in scenarios where fast cooling rates are used in order to dehydrate the cell prior to freezing and reduce the formation of intracellular ice.⁸⁴

Vitrification also requires the addition of cryoprotectants during the vitrification/devitrification process. Generally, high concentrations (>3 M DMSO) are employed along with various other glycol-based CPAs that make up sometimes complex cryosolutions.^{31,65}

While all the CPAs mentioned in this section do protect from some forms of cryoinjury, none are able to significantly prevent the recrystallization of ice during freezing and thawing.^{61,86–91} Ice recrystallization is a major source of damage to cells during cryopreservation and compounds able to prevent this phenomenon have previously been shown to work effectively as CPAs.^{61,89,92} Therefore, CPAs that prevent ice recrystallization and are also non-toxic need to be investigated

for improvement of current cryopreservation protocols.

1.2.7 Mechanisms of Cryoinjury

The process of cryopreservation is comprised of three major steps: 1) addition of a cryoprotective agent (CPA); 2) cooling the sample to the appropriate temperature (-80 °C or -196 °C); and 3) warming (thawing) of the sample. In some applications, the cryoprotectant is removed in a fourth step. Damage to the sample can occur at any of these four steps, resulting in reduced viability and/or functionality. Due to the cytotoxicity of many CPAs, incubation of the sample with the CPA must be minimized. Damage also occurs during the freezing process, mainly due to the rate at which samples are frozen (**Section 1.3.2**). During the thawing of frozen samples, a process known as ice recrystallization occurs, causing damage to biological samples. This is further elaborated in **Section 1.3.3**. Finally, due to cytotoxicity of CPAs, they are often removed from the samples prior to use. The process to remove CPAs from samples generally includes multiple washing and centrifugation steps, resulting in reduced cell recovery.

1.2.8 Cryoinjury During Freezing

In order to cryopreserve biological samples, they must be frozen in a cryosolution, which consists of a cryoprotectant and an aqueous medium. During the cooling process, the sample does not immediately freeze but remains in a super-cooled liquid state until between -5 °C to -15 °C, when extracellular ice spontaneously nucleates.^{93,94} As extracellular ice forms, solutes are excluded from the crystal lattice of ice,⁹⁵ increasing the concentration of solutes in the non-frozen phase. Cells will compensate for the increased extracellular solute concentration by releasing intracellular water via osmosis³⁷ and thus will shrink in size. The rate at which the samples are cooled influences the type of damage resulting from solvent effects. If the cooling rate is too slow, high concentrations of extracellular solutes build up and cause severe

dehydration of cells.^{48,78} This results in significant decreases in cell volume, membrane disruption, and changes in pH and electrolyte concentration. These effects are collectively referred to as “solution effects” and result in cell lysis upon thawing due to the subsequent uptake of water.^{48,78} In order to minimize these solution effects, cooling rates must be fast enough to freeze the solution before extensive dehydration of the sample. However, if the cooling rate is too fast, damage to samples can still occur as a result of insufficient levels of intracellular water loss resulting in intracellular ice formation (IIF). IIF occurs in small amounts regardless of cooling rates, large amounts result in damage during freezing and thawing, although the lethality of IIF has recently been debated.^{48,96–98}

In order to minimize damage to samples during the freezing process, a cooling rate that is fast enough to minimize solution effects while also being slow enough to minimize IIF must be used (**Figure 1-1**).⁵² This “optimal” cooling rate can be determined through experimentation and differs greatly between different cell types and tissues.^{48,52,78} This cooling rate is dependent on the membrane of the cell and its permeability to water and solutes.

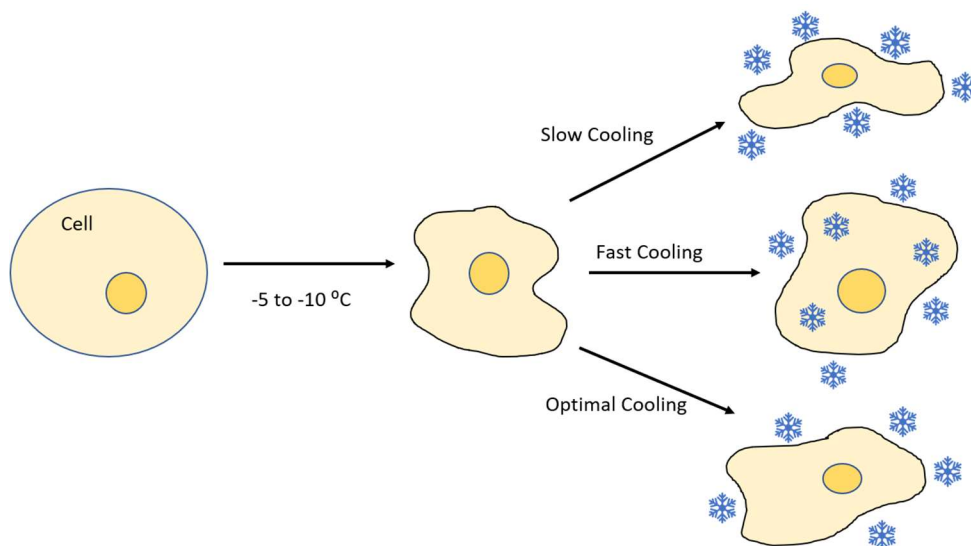


Figure 1.1: Representation of ice formation during freezing of cells.⁵²

The theory of an optimal cooling rate for different cell types was experimentally determined by Peter Mazur in 1972, which was described as the “two-factor hypothesis” (Figure 1.2).⁷⁸ This shows an inverted U-shaped curve for cell survival post-thaw as a function of cooling rate.

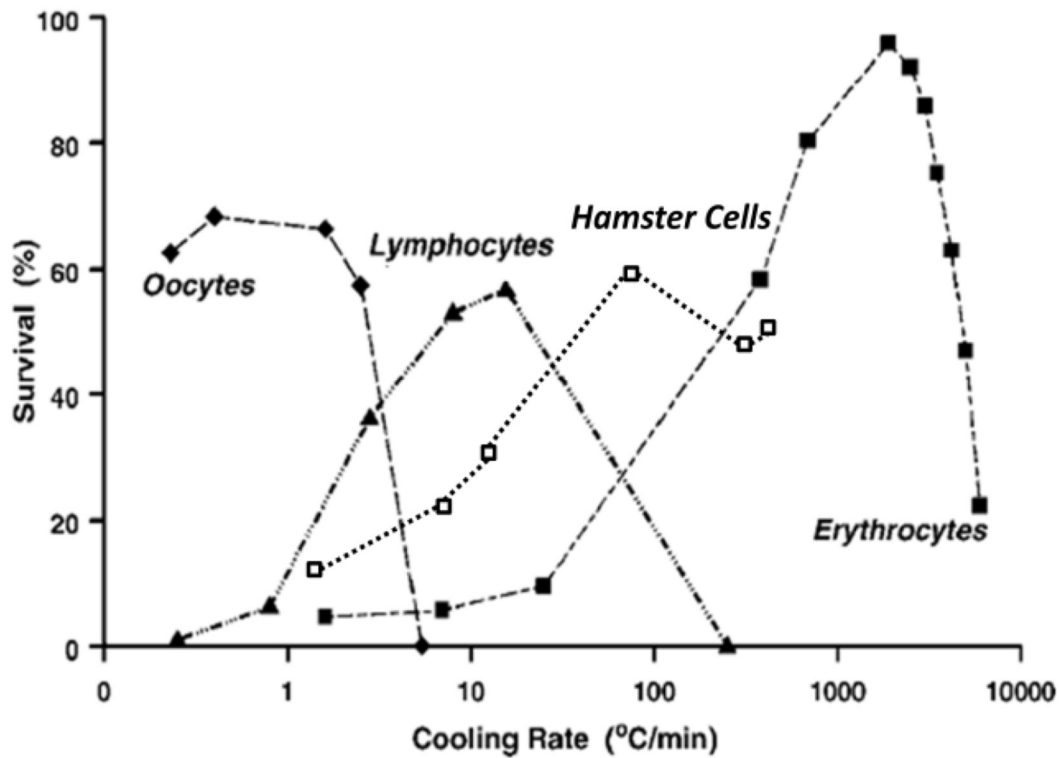


Figure 1-2. Survival of different cell lines post-thaw using various freezing rates.⁷⁸

1.2.9 Cryoinjury During Thawing

Cryoinjury that occurs during the thawing of biological samples can also be controlled depending on the rate at which the sample is thawed. As the sample warms, the ice crystals grow larger due to ice recrystallization (further discussed in **Section 1.5**) which results in mechanical damage to cells. If rapid thawing rates are utilized, the damage due to ice recrystallization can be reduced. However, the warming rates needed to avoid ice recrystallization are difficult to achieve, particularly in samples of large volume. Although ice recrystallization and resulting

cryoinjury cannot be eliminated completely, a reduction in ice recrystallization has been directly correlated to increased post-thaw viabilities in a number of cell types.^{58,95}

1.3 Structure of Ice and Ice Recrystallization

1.3.1 Structure of Ice

As ice forms during cryopreservation, several factors must be understood in order to explain the process of ice recrystallization. Ice can exist in many different forms, depending on the temperature and pressure at which it forms. At atmospheric pressure, the predominant form is hexagonal ice (I_h), which consists of 6 individual water molecules arranged in alternating chair- and boat-like orientations (**Figure 1-3**).⁹⁵

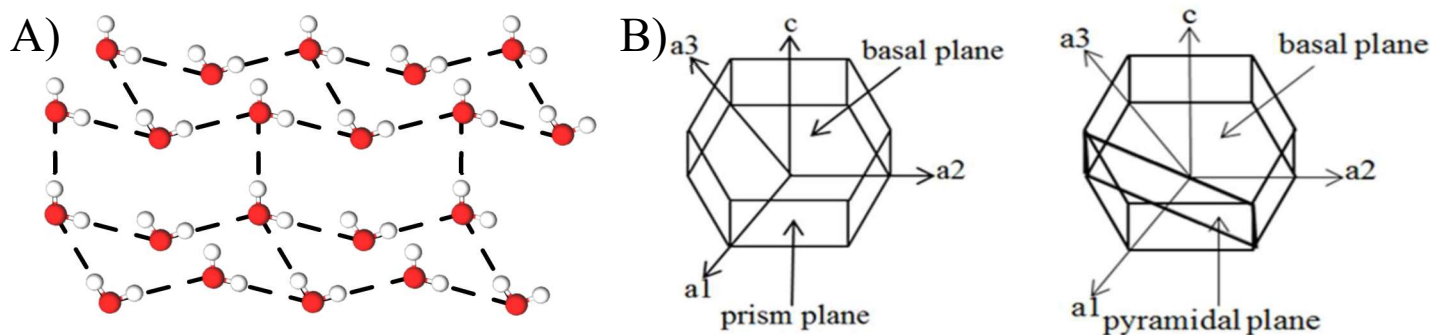


Figure 1-3. A) Structure of I_h (hexagonal ice) displaying the 3D hydrogen bond network that exists between water molecules and B) Different planes and axis present within I_h .⁹⁵

This results in the formation of an extensive hydrogen bonding network, where each water molecule participates in hydrogen bonding with four other water molecules in the ice lattice.

Typically, within the lattice the distance between oxygen nuclei is 2.76 Å with a bond length of 1.00 Å to the bound hydrogen. The hydrogen bond to the adjacent water molecule makes up the remaining 1.76 Å (**Figure 1-3 A**).⁹⁵ The basal plane (top and bottom) of ice crystals is comprised of the chair-like configuration of water molecules, while the prism plane (6 equivalent side

faces) consists of the boat-like configuration. The resulting crystal lattice in I_h has four axes: a_1 , a_2 , a_3 , and c depicted in (Figure 1-3 B).⁹⁵

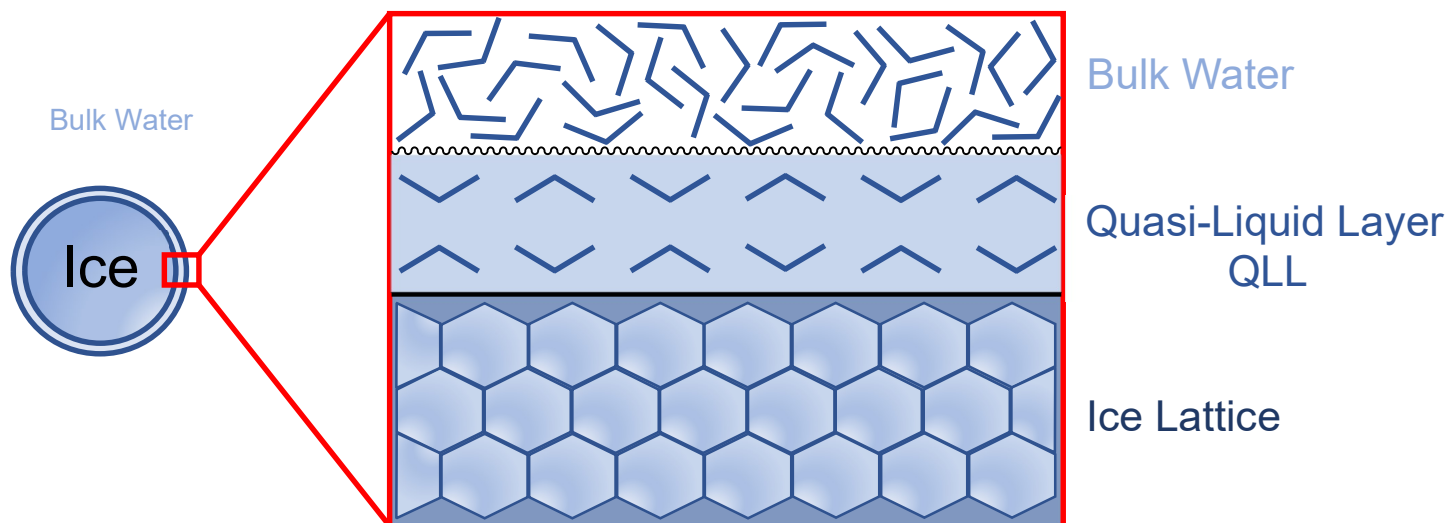


Figure 1-4. Representation of the Quasi-Liquid Layer (QLL) between bulk water and ice.

During normal ice growth at 0 °C and atmospheric pressure, ice growth occurs along the a -axis, producing hexagonal sheets of ice.⁹⁵ In the frozen state, water molecules are highly ordered within the crystal lattice although the transition from ice crystal to unfrozen (bulk) water is not as dynamic as once believed. Separating the ice crystal and the bulk water phase exists a layer of semi-ordered water molecules, referred to as the quasi-liquid layer (QLL). The size of the QLL is variable depending on temperature ranging from 40 monolayers of water (15 nm) at -0.03 °C to less than one monolayer at -10 °C. Although the QLL has been extensively studied, it is currently not well understood. Some studies have shown the QLL to be more water-like while others have suggested it to be more ice-like. Overall, the exact nature of the QLL and the equilibrium between water and ice is complicated and presently not well defined.^{99,100}

1.3.2 Ice Recrystallization

Ice recrystallization can occur in several different ways, although migratory ice recrystallization is defined as the growth of large ice crystals at the expense of smaller ones.^{88,95,101,102} This type of ice recrystallization is the major type associated with decreased cell survival and function post-thaw and will be the only type of ice recrystallization discussed. Migratory ice recrystallization is thought to occur via two mechanisms: Ostwald ripening and grain boundary migration.^{89,95,101,103,104}

Ostwald ripening is a thermodynamically mediated process in which small crystals or particles in a colloid solution dissolve and are redeposited on the surface of larger crystals or particles. This results in the growth of larger crystals or particles from smaller ones. The molecules on the surface of crystals result in higher internal energy than molecules within a crystal, and therefore larger crystals will be more energetically favored than small ones due to their lower surface area to volume ratio.¹⁰³ Specifically, during the freezing process, water molecules in the QLL cannot form the ideal amount of hydrogen bonds and are higher in energy than water molecules within the ice lattice.⁹⁵ The free energy of the system is decreased by the

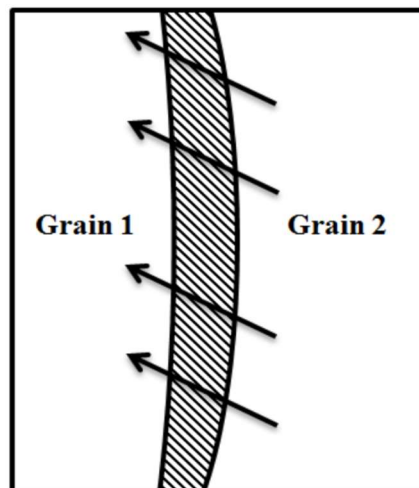


Figure 1-5. Grain boundary migration between 2 grains of ice with liquid (shaded area) between grains. Large grains with concave boundaries (grain 2) grow larger while smaller grains with convex boundaries (grain 1) decrease in size. Arrows show direction of boundary migration.⁸⁹

migration of water molecules from small ice crystals to larger ones. In order to do so, the water molecules travel through the QLL of the small ice crystal to bulk water and then to the QLL of the larger ice crystal. The result is an overall reduction in free energy within the system while the total volume of ice remains constant.^{89,95}

Migratory ice recrystallization also occurs via grain boundary migration. At equilibrium in I_h ice, each grain of ice will have six neighboring I_h grains with grain boundaries of 120° resulting in all edges being straight. Larger grains generally have more than 6 neighboring grains due to their large surface area while smaller grains have fewer. The number of neighboring grains also dictates grain boundary shape. Larger grains with more than 6 neighbors tend to have a concave curvature (**Figure 1-5**, Grain 2) while grains with fewer than 6 neighbors tend to have convex grain boundaries (**Figure 1-5**, Grain 1). During the process of grain boundary migration, water molecules will transfer from grains with a higher surface energy to grains with a lower surface energy in order to reduce the degree of curvature and overall energy in the system before reaching equilibrium.^{95,104}

Since ice recrystallization directly contributes to decreased cell viability/functionality post-thaw, there has been an increasing effort in investigating the inhibition of ice recrystallization.

1.4 Cryopreservation of Biological Samples

1.4.1 Red Blood Cells

While hypothermic storage of red blood cells (RBCs) allows for their direct transfusion (**Section 1.2.3**), it limits their shelf life to 42 days post-collection.^{7,42} Due to the limited storage time and reliance on blood donors, shortages of blood supplies can occur when high numbers of blood transfusions are needed and in rural areas.^{9,14} For longer storage times, RBCs must be

cryopreserved.^{6,34,91} This is currently limited to rare blood phenotypes, military applications, and autologous RBC storage.⁹⁵ In North America and most of the world, clinical RBC cryopreservation conditions follow a high glycerol/slow freeze protocol, which uses a high concentration of glycerol (40% v/v) and slow cooling rates of 1 °C/min to -80 °C.⁹¹ These cryopreserved units are stored at -80 °C for up to 10 years while still retaining a high recovery of RBCs post-thaw.¹⁰⁵

While 40% glycerol is an excellent cryoprotectant for RBCs over long periods of time and multiple freeze-thaw cycles, it must be removed to <1% prior to transfusion to prevent intravascular hemolysis. The deglycerolization process requires specialized equipment and is time consuming, resulting in a delay between thawing and transfusing. This process can damage RBCs, resulting in up to a 20% loss of volume after deglycerolization. Units must also be transfused or discarded within 24 hours of deglycerolization, meaning units cannot be thawed in anticipation of a traumatic event and creates logistical problems with transportation within the time restraints.^{7,91,106}

Many European countries use low glycerol (20 % v/v) concentrations which necessitates fast cooling (>50 °C/min) rates and with storage at -196 °C.^{106,107} While this protocol uses reduced glycerol concentrations, the liquid nitrogen needed for freezing and storage is expensive for large-scale operations and breakage of blood bags resulting in the loss of the sample is a problem at the temperatures used this also produces problems for the transportation of frozen units.

1.4.2 Hematopoietic Stem/Progenitor Cells

Hematopoietic stem and progenitor cells (HSPCs) are often used in regenerative medicine for the treatment of hematological cancers such as leukemias and lymphomas.^{20,53,108,109} HSPC transplantation can also be used to treat patients suffering from other hematological diseases, immunodeficiencies, and anemias.^{110,111} HSPCs can be identified by the stage-specific transmembrane phosphoglycoprotein CD34. CD34 is expressed at the early stages of hematopoietic differentiation, although the exact function the protein is not fully known.^{112,113} There are several sources that contain CD34 positive (CD34+) cells, including umbilical cord blood (UCB), bone marrow, and peripheral blood.¹¹⁴ Each cell type contains different percentages of CD34+ populations, with peripheral blood containing the smallest percentage at an average of 0.15% CD34+ cells. UCB contains approximately 1.2-1.5% overall, while bone marrow contains the highest population at 1.7%. Although bone marrow transplantation delivers the highest percentage of CD34+ cells, there are many risks associated with this. Graft-versus-host disease (GVHD) is a major concern with bone marrow transplants as well as risks associated with the surgery for both the donor and recipient.¹¹⁵ Isolated HSPC transplantation reduces both risks as well as the risk of blood-borne infections. HSPCs also have less stringent human leukocyte antigen (HLA) matching requirements, allowing for easier matching for transplants.¹¹⁶

Autologous stem cell transplantation can occasionally be done on fresh cells, within 72 hours post-harvest if a match is available. However, nearly all allogenic and autologous transplants use cryopreserved cells. This is especially true for HSPCs isolated from UCB because they are harvested at birth and must be stored until they are needed.¹¹⁷ The major factor in the success of HSPC transplantation is the number and quality of CD34+ cells transplanted.

Consequently, cryopreservation protocols must be highly optimized in order to preserve the quality of cells and is a significant issue for HSPC cryopreservation in UCB banks.^{17,19,114}

Currently the standard procedure for cryopreservation of HSPCs involves the use of 10% DMSO as a cryoprotectant in saline and dextran.¹¹⁸ Although this results in high levels of cell viability and functionality post-thaw, there are several disadvantages to using it as a cryoprotectant. The major disadvantage is the cytotoxicity of DMSO, affecting the cardiovascular, central nervous, renal, hepatic, respiratory, and gastrointestinal systems.¹¹⁹ Due to the cytotoxicity, several side effects are often associated with HSPC transplantations containing DMSO. These include nausea, vomiting, headache, hypertension, and more severe ones such as anaphylactic shock, among others.¹¹⁹ In some situations, DMSO can be removed prior to transplantation, but is expensive, time consuming, and often results in reduced cell survival and function. Therefore, DMSO is usually left in samples for transplantation. Not only is DMSO toxic to the transplant recipient, but also to the HSPC sample itself: prior to freezing, the sample must be cooled to 4 °C before the DMSO is added and must be frozen within 10 minutes after addition to avoid toxicity.¹²⁰ Regardless of the cytotoxicity and negative patient side effects, DMSO remains the “gold standard” cryoprotectant for HSPCs and other cell types.

1.4.3 Articular Cartilage

Articular cartilage plays a major role in joint health and regular function.^{24,25} Degradation due to age or injury has severe implications in loss of mobility and reduced quality of life. For these reasons, repair of articular cartilage is an important area of study. There are currently several methods of repairing or replacing cartilage, all with their own benefits and disadvantages. One of the most common techniques is osteochondral autograft transplantation, where the patient’s own cartilage from a non-weightbearing area is used for transplant.^{22,24} Isolated

chondrocytes from a patient can also be isolated, cultured, then transplanted back into the patient.¹²¹ For smaller lesions, the growth of new cartilage can be promoted by drilling, microfracture, or abrasion of the affected area.¹²² These procedures work by creating a new blood supply to the affected area, allowing chondrocytes (cartilage-producing cells) to be transported to the lesion and begin creating new cartilage. Due to the slow regeneration times of cartilage, these methods are limited to small lesions.

For larger lesions that cannot be repaired via stimulating blood supply or autograft, allografts can be used. Allografts refer to whole sections of cartilage taken usually from a cadaver, which is then transplanted to replace the damaged cartilage in the patient.¹²³ The major drawback to this procedure is the need for fresh donor cartilage. This means that a suitable donor cartilage must be available at the time of transplantation. Due to the complexity of cartilage tissue and its short lifetime after removal, banking of cartilage samples for later use is not possible with current techniques. While whole cartilage cryopreservation is an emerging field, many limitations currently exist.⁷⁰

Current cryopreservation protocols for whole cartilage often use 1-1.4 M DMSO and a two-stage cooling process. Samples are first cooled slowly at 1 °C/min to -20 °C, followed by more rapid cooling at 4 °C/min to -70 °C.^{47,124} A major disadvantage of this method is that the samples must be incubated with DMSO prior to freezing as it must permeate through the sample. The incubation time, temperature, and DMSO concentrations must be carefully controlled as to limit cytotoxicity of DMSO. Vitrification of whole cartilage has also been conducted, using high concentrations (3-6 M) of DMSO and ultra-fast cooling rates.^{70,74,125}

While both procedures can preserve whole cartilage sections, post-thaw viabilities are suboptimal and range from 40-60%. Post-thaw functionality also varies from 50-80% depending

on the procedure used. Once thawed, the DMSO must also be removed before transplantation, requiring long incubation steps to fully remove the DMSO.^{47,70,74,126}

1.4.4 Platelets

Platelets (thrombocytes) are a component of blood responsible for blood clotting at wound sites.¹²⁷ Platelets number between 150,000 to 450,000 per μL of blood in healthy individuals and make up less than 1% of whole blood.^{127,128} Platelets can be transfused in patients who have low platelet levels, often occurring due to various cancer treatments as well as during organ transplants and surgeries. While platelets can be isolated from whole blood, acquiring enough platelets for a patient would require multiple donors which could lead to complications from improper HLA matching.¹²⁹ To isolate larger quantities of platelets, plateletpheresis is used. This process uses apheresis to separate the platelets from RBCs and plasma, which is then returned to the donor. Once isolated, platelets are stored at 22 °C with constant agitation which prevents clumping of the unit. Platelet units can be stored this way for up to 5 days, and in rarer cases up to 7 days, although the risk of bacterial contamination can increase during extended storage.^{128,130} For longer storage of platelets, cryopreservation can be used, although limitations exist for this process.

The most employed cryopreservation protocols use between 5-6% DMSO, with freezing at -80 °C and storage between -65 to -80 °C or lower. As with other cryopreservation protocols, DMSO must be removed prior to transfusion, which requires longer periods of time and results in lower number of platelets.^{128,131} Newer protocols remove the DMSO prior to freezing, although decreases in platelet number and functionality are experienced post-thaw.

1.5 Natural Cryoprotectants

1.5.1 Antifreeze (glyco)proteins

While studying Teleost fish in the Arctic during the 1950's Scholander, observed that these fish are able to survive in water where the temperature drops below the freezing temperature of their blood (-1.9 °C).^{132,133} The isolation of various proteins present in the blood of these fish revealed certain glycoproteins that were credited to their survival. These glycoproteins were labelled biological antifreezes (BAs) due to their protection to organisms in sub-zero temperatures. Since then, several other BAs have been discovered and have been separated into two classes: antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) depending on their structure.⁹⁵

AF(G)Ps protect organisms from sub-zero temperatures through two antifreeze activities: ice recrystallization inhibition (IRI) and thermal hysteresis (TH). As mentioned in **Section 1**, ice recrystallization is the growth of large ice crystals at the expense of smaller ones. While these AF(G)Ps do exhibit IRI activity, which is desirable for cryopreservation, they also possess TH activity. TH is only beneficial to the organisms while they are at temperatures above their freezing point.

1.5.2 Thermal Hysteresis

TH can be defined as a selective depression of freezing point that is different than the melting point of a solution.¹³⁴ For example, pure water has a freezing and melting point of 0 °C. While this point can be lowered with the addition of salts, the freezing point is still equal to the melting point. With the addition of a compound exhibiting TH, the freezing point can be selectively lowered below the melting point. This means there is a small temperature where ice

crystals will not grow but will not melt until the temperature is raised over the melting point. This is achieved by the AF(G)P binding to a newly formed ice crystal, preventing the ice crystal from growing until colder than the depressed freezing point. Once the temperature decreases below the depressed freezing point there is a rapid growth in the ice crystal, usually forming spicules that cause a high degree of damage to cells (**Figure 1-6**).⁹⁵

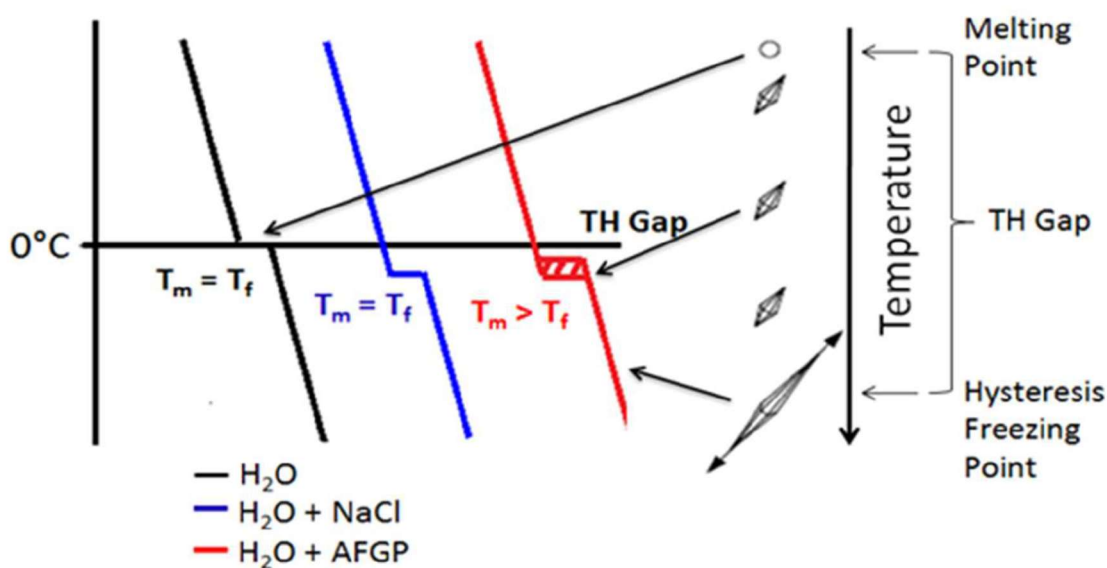


Figure 1-6. Temperature graph showing the decrease in freezing point relative to the melting point (red line), a process referred to as thermal hysteresis (TH). In this graph, TH is due to AFGP addition.⁹⁵

Since TH is detrimental at temperatures needed for cryopreservation, CPAs for clinical applications should not show any TH activity, while possessing a high degree of IRI activity. In order to eliminate TH activity while maintaining IRI activity, the Ben laboratory generated AFGP analogues followed by truncated derivatives, eventually leading to the investigating simple monosaccharides for IRI activity.

1.6 Small Molecule Ice Recrystallization Inhibitors (IRIs)

1.6.1 Naturally occurring monosaccharides and disaccharides

IRIs used for cryopreservation should ideally be commercially available or easily synthesized due to the large quantities needed for clinical cryopreservation. By synthesizing AFGP analogues followed by derivatization of these analogues, the Ben lab explored simple monosaccharides as new IRIs. D-galactose displayed the highest IRI activity, while D-mannose and D-talose showed the lowest^{135,136} This was hypothesized to be due to the hydration of the monosaccharide itself, D-galactose being the most hydrated and IRI active. Although these monosaccharides had known hydration numbers, the IRI activity had not been assessed. Interestingly, the IRI activity follows the same trend as the hydration number for these monosaccharides (**Figure 1-7**).¹³⁶

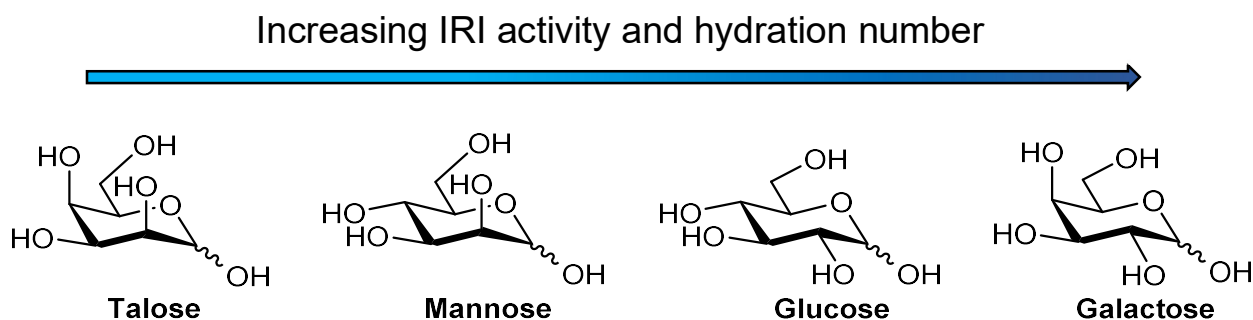
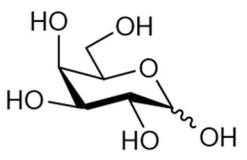
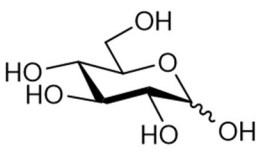
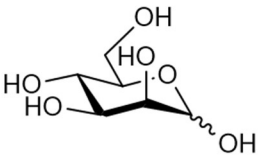
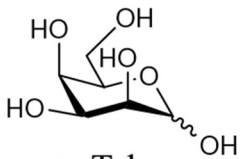


Figure 1-7. The relationship between Increasing IRI activity in simple monosaccharides with greater hydration.¹³⁶

The hydration number of a molecule represents the number of water molecules that can interact closely with the solute.^{135,137} The hydration number of many mono- and disaccharides were determined via density ultrasound measurement along with kinetic experiments in the 1990s by Galema *et al.* These values are summarized in **Table 1-1**.¹³⁷ Along with the hydration numbers, isentropic molar compressibilities for these carbohydrates were also determined.

These were determined using ultrasound measurements and kinetic experiments and were used to assess the “compatibility” of a carbohydrate in the hydrogen bond network of bulk water.

Table 1-1. Hydration number with associated uncertainty of various monosaccharides.¹³⁷

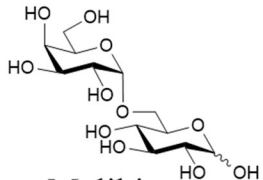
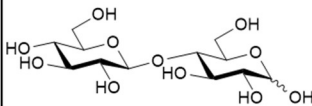
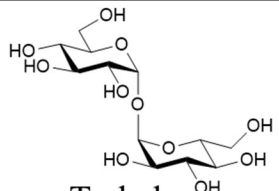
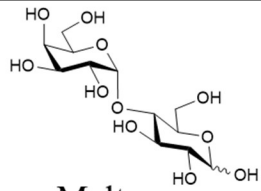
Carbohydrate	Hydration Number	Carbohydrate	Hydration Number
 D-Galactose	8.7 ± 0.2	 D-Glucose	8.4 ± 0.2
 D-Mannose	8.1 ± 0.2	 D-Talose	7.7 ± 0.2

The compatibilities of the carbohydrates are directly related to the C2 and C4 stereochemistry of the carbohydrate. D-Talose, which has the lowest hydration number and highest isentropic compressibility, displays the best fit in the hydrogen bonding network of bulk water and the lowest IRI activity. Like D-talose, D-galactose possesses an axial C4 hydroxyl group, but it exhibits the highest IRI activity. Comparing these two monosaccharides, stereochemistry of the C4 position alone cannot be used to predict IRI activity and therefore other hydroxyl groups must be considered. The second least IRI active monosaccharide is D-mannose, with an axial C2 hydroxyl and an equatorial C4 hydroxyl. This suggests that the relationship between the C2 and C4 hydroxyl groups can be used to gauge the IRI activity of simple monosaccharide reducing sugars.^{135–137}

Unfortunately, this correlation is not maintained for disaccharides, which display greater hydration numbers and isentropic compressibilities but lower IRI activities (**Table 1-2**).¹³⁸ To account for this observation, the Ben lab devised new measure, the “hydration index”. This uses the hydration number divided by the molar volume and eliminated the discrepancies between simple mono- and disaccharides.¹³⁶

Although these studies have provided insight into relationships between hydration number/index, isentropic compressibility, and IRI activity, hydration and compressibility are not easily obtained and are not able to predict IRI activity for all carbohydrate derivatives. Other metrics to correlate IRI activity to structure need to be developed in order to elucidate key features to increase IRI activity.

Table 1-2. Hydration number with associated uncertainty of various disaccharides.¹³⁹

Carbohydrate	Hydration Number	Carbohydrate	Hydration Number
 Melibiose	15.5 ± 0.3	 Lactose	15.3 ± 0.3
 Trehalose	15.3 ± 0.3	 Maltose	14.5 ± 0.3

1.6.2 Current Status of Small Molecule Ice Recrystallization Inhibitors

Numerous functional groups were explored in order to find a fine balance between hydrophobicity and hydrophilicity that appears to be necessary for IRI activity. In these derivatives, alkyl chains were replaced by 6-membered aromatic rings in order to maintain hydrophobicity without the resulting surfactant-like properties.^{62,140} The use of substituted phenols also allows for simple coupling to the pyranose ring with a high degree of structural modifications. Many of these substituted phenol derivatives are commercially available and can be directly coupled in a one-step reaction with β -D-glucose pentaacetate via Lewis acid

catalysis.⁶² The final derivatives were then generated by acetate removal under Zemplén conditions.

To assess IRI activity between compounds, the Ben lab developed a “splat-cooling” assay which, in brief, freezes the compound dissolved in PBS followed by incubation at -5.4 °C to induce ice recrystallization. The resulting ice crystals are photographed and compared to PBS frozen without IRI which represents a negative control for ice recrystallization. The activity of the compound is presented as a percent mean grain size (%MGS) relative to PBS (100 %).

The first derivatives synthesized used phenol to generate the β -phenyl derivatives **126** and **127** (Figure 1-8). While both derivatives **125** and **126** failed to retain the IRI activity of compound **123**, the glucose derivative **125** showed similar IRI activity to D-galactose, **124** was

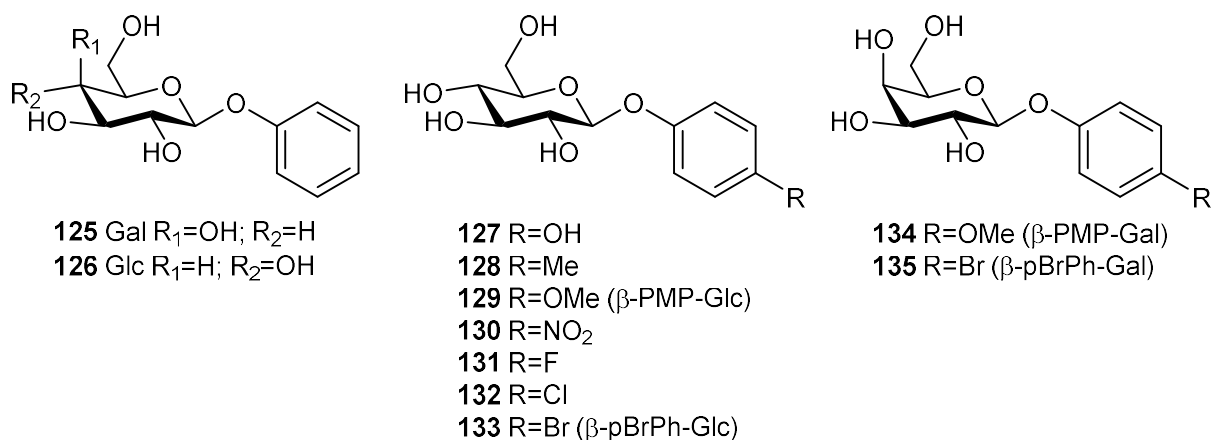


Figure 1-8. Structures of C1 phenyl-substituted β -D-glucose/galactose derivatives synthesized by the Ben lab.

much less active, showing the opposite trend to the previous derivatives. Next, functionalized phenol rings were explored, first examining *para*-substituted β -D-glucose (**127-133**) derivatives. When comparing these derivatives, the hydroxyl- (**127**) and methyl- (**128**) substituted compounds showed little IRI activity and were comparable to D-galactose (~60% MGS at 22 mM). Interestingly, when a *para*-methoxy substituent was used (**129**), the IRI activity was greatly increased to approximately 20% MGS. Numerous other functional groups (nitro [**130**],

amines, esters, carboxylic acids, etc.) were tested, none of which exhibited appreciable IRI activity at 22 mM.^{140,141} Halogen-substituted phenol derivatives **131-133** however were more IRI active than **129** (Br>Cl>F), with activities similar to the surfactant **123**. This shows that remarkably small changes to functional groups can have drastic effects on IRI activity for these molecules. To explore the effect of stereochemical changes of the C4 position on IRI, compounds **134/135** (β -D-galactosyl derivatives) were synthesized, although no discernable trend was observed between D-glucose derivatives **129/133** and D-galactose derivatives **134/135**.

Further testing of compounds **129** and **133** was conducted in the cryopreservation of RBCs in order to reduce the amount of glycerol in the sample. Rather than using the standard 40% glycerol solution for freezing RBCs, instead using 15% glycerol supplemented with **129/133**. By supplementing 15% glycerol with these compounds along with slow cooling rates showed drastic increases in post-thaw RBC integrity over the control. By reducing the glycerol concentration, deglycerolization times can be drastically reduced, allowing RBC units to be transfused in less time.

1.6.3 Substituted Aldonamides as Ice Recrystallization Inhibitors

The majority of all IRI active compounds contained a pyranose ring with various C1 modifications until this point. While both α - and β -linked pyranose derivatives have been previously synthesized and tested, open chain derivatives have not. Open chain derivatives can easily be synthesized starting from commercially available δ -gluconolactone and an amine to

generate gluconamides (**Figure 1-9**).^{62,89,142} The first synthesized were the alkyl- and cycloalkyl-gluconamides **140-149**.

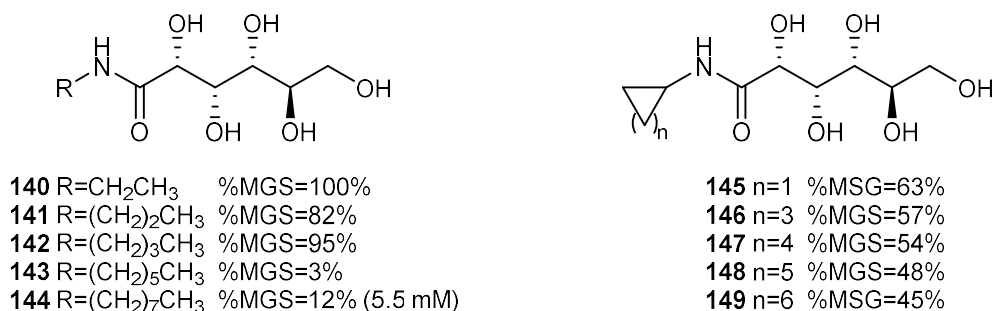


Figure 1-9. Structures of alkyl-substituted gluconamides (**140-144**) and cycloalkyl-substituted gluconamides (**145-149**).^{62,89,142}

Following a similar trend to compounds **119-122**, the IRI activity of compounds **140-144** increased as the alkyl chain was lengthened. The surfactant-like properties also increased with the increase in chain length, although a drastic decrease in solubility was observed with **144**. All cycloalkyl-gluconamide derivatives expressed low IRI activity even with small ring sizes in compounds **145-148**. Compound **149** with a ring size of 8-carbon atoms did show a greatly reduced activity than the linear hexyl chain in compound **143**. As with other compounds, the aldonamides also demonstrate the fine balance between hydrophobicity and hydrophilicity for IRI active molecules exists, but a way of predicting IRI activity is still unavailable.

Although some of the alkyl-gluconamides displayed a high degree of IRI activity, their surfactant-like properties mean they cannot be used for cryopreservation of biological samples. In order to eliminate the surfactant properties of the active compounds, the alkyl chain was replaced with a phenyl ring, in a similar strategy used with the phenyl pyranosides.⁸⁶ To install the phenyl ring, a commercially available aniline was used to generate the resulting amides. Several substituted phenyl-aldonamides have been previously synthesized^{86,120} with large variability in IRI activity and no definite trend between them (**Figure 1-10**). Although there was

no trend between all molecules, it appears substitution on the *ortho* and *para* positions on the phenyl ring appear to be more active than on the *meta* position. In compounds **150** and **151**, slight modifications to the substituent in the *ortho* position plays a major role in the molecules IRI activity. The compound is highly active with a fluorine substituent, but inactive with a methoxy group in the same position. This observation does not hold true for the *para* position, where the fluorine (**152**) substituted molecule is inactive, while the methoxy (**153**) substituted molecule is highly active.

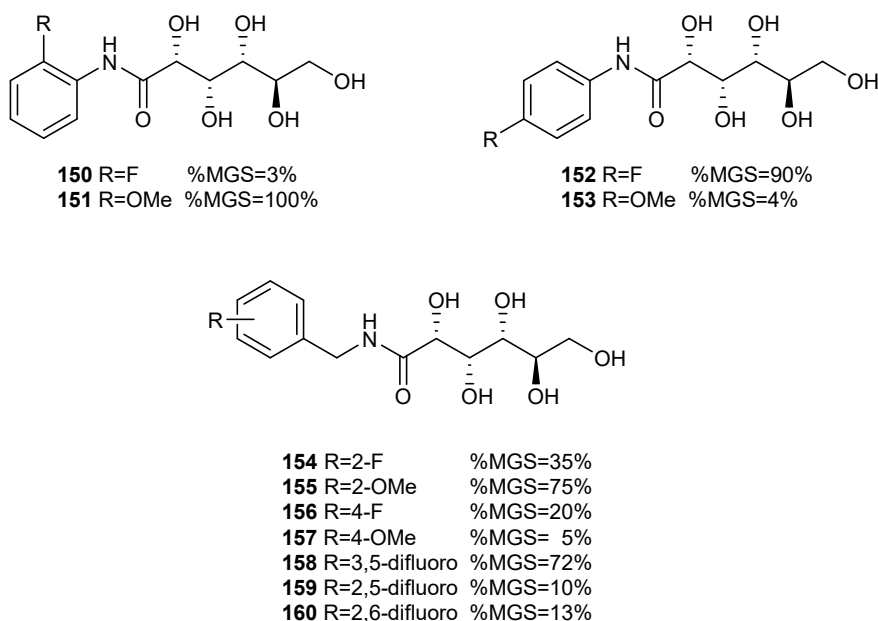


Figure 1-10. Structure and corresponding %MGS values for various phenyl- and benzylamine substituted gluconamides.¹⁴³

Similarly, substituted benzyl amide derivatives were synthesized to assess the effect of a single carbon spacer in these molecules. Of the monosubstituted derivatives **154-157**, compound **157** (4-methoxy) had the highest degree of IRI activity. Interestingly, compound **157** and **155** follow the same trend as their corresponding phenyl derivatives where the 2-methoxy substituted derivative is less active than the 4-methoxy isomer. Unlike the phenyl derivatives, neither of the singly fluorinated derivatives showed a high degree of IRI activity. Difluorinated derivatives

158-160 reinforced that small changes in regiochemistry can have a large impact on IRI activity. Compound **158** (3,5-difluoro) displayed no IRI activity, while both the 2,5- and 2,6-difluoro derivatives were active (**Figure 1-10**).¹⁴³

Due to their high degree of IRI activity, compounds **150**, **153**, and **160** were selected for further biological testing for the cryopreservation of HSPCs.¹²⁰ With the addition of these compounds in the typical cryosolution, post thaw recovery of HSPCs was increased compared to the control without an IRI. HSPCs that were frozen in the presence of an IRI also displayed increased functionality post-thaw over the control. These results suggest that supplementing HSPC cryosolutions with IRIs could increase total cell numbers post-thaw, and in turn increase engraftment of these samples in a clinical setting.

1.7 Chapter Summary

In summary, cryopreservation is an essential technique required for long-term storage of biological products such as: RBCs, HSPCs, cartilage, and platelets. While protocols currently exist for these cell types, there are limitations stemming from the use of the necessary cryoprotectant among other issues. This has resulted in the need for new techniques and cryoprotectants which can improve upon already existing protocols. Cryoprotectants possessing IRI activity are particularly attractive for these applications due to the detrimental nature of ice crystal formation. Up to this point a number of small molecule, carbohydrate-based molecules have been shown to possess a high degree of IRI activity, although there is still a need to expand the types of IRIs to better suit specific situations over others. Current IRIs can be modified to modulate their properties, or “custom tailor” IRIs for specific applications. By modifying specific properties of IRIs, many different cryopreservation applications can be explored and

improved upon. The development of more specific IRIs can have a significant impact on a number of both clinical and commercial cryopreservation applications for biological samples.

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Chapter 2 – Goals and Objectives

2.1 - Introduction

With the ongoing development of new cellular therapies, the need for readily available biological components is urgently required. These components must be correctly matched to the patient using the human leukocyte antigen (HLA) system to avoid graft-versus-host disease (GVHD) in transfused/transplanted biological components. Efficient transportation of components from medical facilities is also required to minimize damage post-removal to the components themselves. In the case of frozen samples, thawing and processing time must be minimal to maintain cell viability, and in turn increase the likelihood of a positive prognosis.¹⁻³ Overall, biobanking of these components with reliable cryopreservation techniques is necessary but is logistically difficult. While different types of cell and tissues can currently be cryopreserved for later use, it is usually reserved for rare phenotypes, military applications, and more recently space travel.⁴⁻⁶ Widespread biobanking for everyday use is not normally done due to the high cost associated with storage of samples and logistical problems with the cryopreservation protocols, transportation, and post-thaw processing.^{3,7} Many current cryopreservation techniques require storage at sub-zero temperatures, commonly -80 °C or -196 °C. This proves difficult for transportation of these materials as they must be kept at this low temperature until thawing, slight variations in temperature also called “transient warming” often reduce recovery and functionality of the sample.^{8,9} Thawing samples prior to transportation is rarely possible due to the need for the thawed product to be used shortly after thawing.^{10,11} Another current problem with cryopreservation of cellular products is the post-thaw processing of samples.

Currently, many commonly used cryoprotectants used (such as DMSO and glycerol) are cytotoxic and cause adverse reactions to patients receiving treatment with the cellular product. For this reason, cryopreservation protocols must minimize the time prior to freezing with these cryoprotectants and remove most or all prior to transplant to reduce adverse side effects. The “gold standard” cryoprotectant DMSO is used in a number of different cryopreservation protocols for freezing cellular products such as: cartilage, platelets, hematopoietic stem/progenitor cells (HSPCs), and many others.^{12–15} DMSO is a cytotoxic, permeating cryoprotectant, meaning it is able to cross cell membranes and tissues. Due to its toxicity, incubation times at room temperature must be minimized, although for more complex tissues, permeation may take several hours.^{16–18} Extended incubation time results in reduced cell recovery and function. Additionally, DMSO can cause headaches, abdominal cramps, dyspnea, hypertension and other cardiovascular reactions if not removed prior to transplant/transfusion.^{15,16,19} While DMSO can be removed post-thaw, it generally results in reduced cell recovery and function.

With blood being one of the most commonly used biological components via transfusion, the need for long-term storage of blood is essential. Cryopreservation can be preformed with the use of glycerol as a cryoprotectant. Using glycerol in high concentrations (40% w/v) coupled with slow cooling temperatures, RBCs are able to be successfully frozen and thawed with high post-thaw recoveries (>95%). This method is used in North America and some parts of Europe. Other parts use less glycerol (20% w/v) and fast cooling and can achieve similar post-thaw recoveries.^{20–23} Glycerol must be removed before transfusion of RBC units to prevent intravascular hemolysis.^{24,25} The deglycerolization process is time-consuming, which makes it impractical for urgent clinical uses.⁸ The disadvantages of these commonly used cryoprotectants

stresses the need for new, non-cytotoxic cryoprotective agents as alternatives, or to be used in conjunction with lowered concentrations of currently used cryoprotectants.

One major source of cellular damage during cryopreservation in addition to cryoprotectant toxicity is ice recrystallization.^{26–29} This is the tendency of small ice crystals to combine with larger crystals, forming even larger crystals during the freezing and thawing processes. While this process is detrimental to cells and tissues, some molecules have the ability to inhibit this process; these molecules are referred to as ice recrystallization inhibitors (IRIs).^{26,30,31} The ability to inhibit ice recrystallization and control ice growth is a desirable property for a cryoprotectant as conventional cryoprotectants do not inhibit ice recrystallization and cannot control ice growth. The Ben lab has been focused on the synthesis and characterization of many classes of small molecule, carbohydrate-based ice recrystallization inhibitors. These classes include simple monosaccharides³¹, antifreeze (glyco)proteins (AF(G)Ps)^{30,32–34}, pyranose derivatives^{35–37}, and aldonamide derivatives^{13,38,39} (**Figure 2-1**).

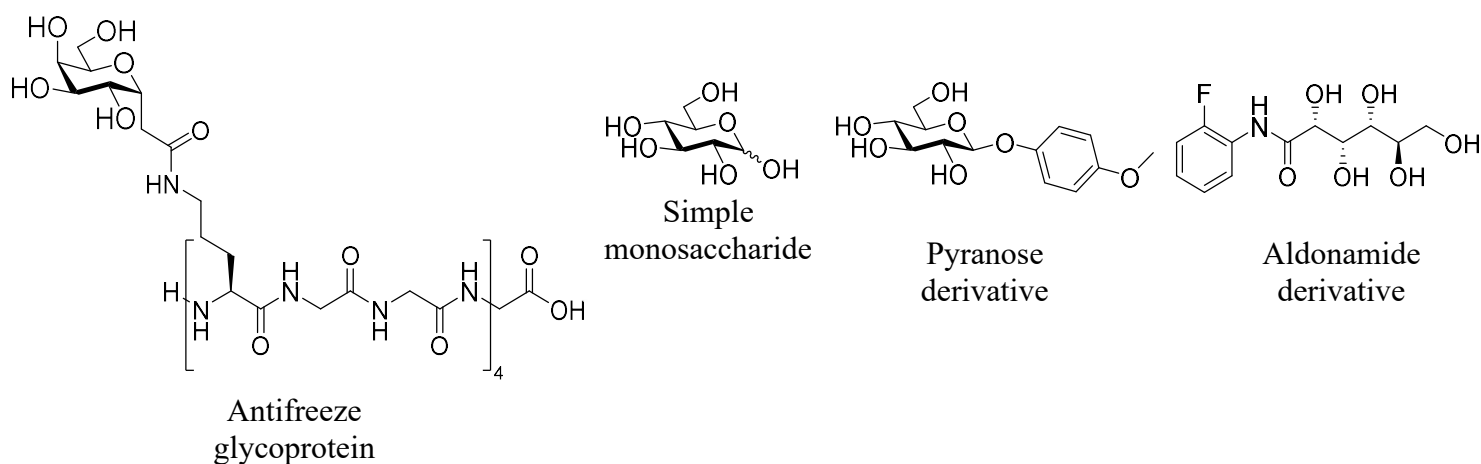


Figure 2-1. Structures of previously synthesized Ben Lab IRIs.

Initially, naturally derived AF(G)Ps were shown to display high degrees of ice recrystallization at low concentrations, but also showed thermal hysteresis (TH). This is where

ice growth halts over a small range of temperatures, but dramatically grows when the temperature is lowered under that range and is undesirable for IRIs (**Figure 2-1**).⁴⁰ Synthesis of modified AFGP analogues were able to custom-tailor the properties of these molecules to select for IRI activity with minimal TH activity.³²

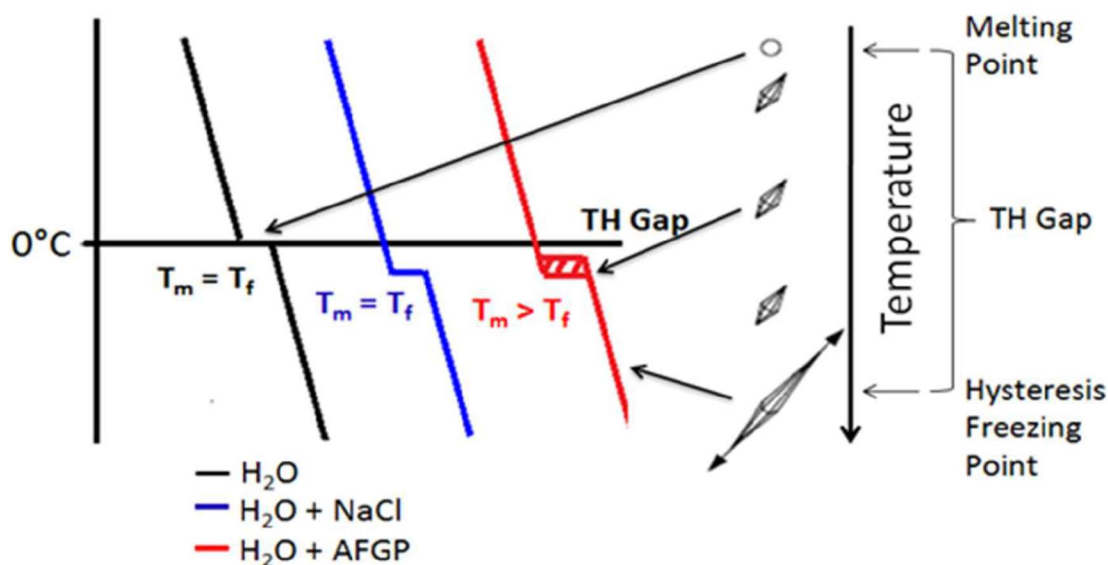


Figure 2-2. Temperature graph showing the decrease in freezing point relative to the melting point (red line), a process referred to as thermal hysteresis (TH). In this graph, TH is due to AFGP addition.⁴¹

Next, these AFGP analogues were further derivatized, leading to the discovery of C1 alkyl-substituted pyranose derivatives.³⁵ While these derivatives also showed IRI activity, the long alkyl chains resulted in surfactant-like properties that solubilized cell membranes.^{35,42}

From the synthesis and testing of many IRI active molecules, it was apparent a delicate balance between hydrophobic and hydrophilic moieties on the same molecule must be met for IRI activity. To balance these properties and generate more biologically compatible IRIs, C1 aryl-substituted derivatives were synthesized. These molecules retained some of the IRI activity present with the alkyl derivatives, but did not solubilize cell membranes, allowing their use in biological systems. These derivatives have been investigated for the cryopreservation of RBCs

^{8,37,43} and have proved successful for this application, allowing cryopreservation with reduced (15% w\ v) glycerol to be used.

Based on these compounds, open-chain aldonamide sugars were explored using similar *N*-linked aryl moieties. While these molecules show a high degree of IRI activity, the solubility of these molecules in aqueous media is very low (many under 15 mM). This severely limits the concentrations that they can be assessed at and requires extensive (>80 °C) heating to fully dissolve. In some scenarios this is acceptable, many cryosolutions contain heat-sensitive compounds that can degrade upon heating to the required temperatures to dissolve these IRIs, further limiting the concentrations that can be used. Another concern with the sparse solubility is that once fully dissolved, the IRIs may be precipitating during the freezing process further reducing the concentration of solubilized IRI.

This highlights the need for new, more soluble IRI active molecules for cryopreservation of biological samples. Consequently, the goal of this thesis is to explore the synthesis of more soluble, IRI active molecules for their use in cryopreservation of biological samples. Design and synthesis of these molecules will further progress our understanding of the balance of hydrophilic and hydrophobic moieties and their relation to IRI activity and subsequent cryopreservation in biological systems. An emphasis will be placed on IRIs bearing a charged functional group at physiological conditions as a way of increasing their solubility and in turn working concentrations in aqueous media. These charged IRIs will be investigated in the cryopreservation of a number of biological systems, including RBCs, cartilage, platelets, and HSPCs. Below are the specific objectives that will be investigated in this thesis.

2.2 Objective 1 – Synthesis and Evaluation of Sulfated Pyranose Derivatives

The Ben lab have shown the IRI activity of many *O*-aryl glycosides and their use as novel IRIs in the cryopreservation of RBCs. Previous to this, the role of hydration of a molecule (number of water molecules that can closely interact with the molecule) was compared to the IRI activity. It showed a strong correlation between increased hydration and IRI activity.³¹ While direct calculation of a molecule's hydration is difficult, incorporating a highly hydrated functional group on the molecule should theoretically increase the molecules hydration overall. Sulfate groups are an example of highly hydrated functional groups, capable of hydrogen bonding with numerous water molecules in complicated hydrogen bond networks.^{44–46} Sulfated carbohydrates are also prevalent in nature, comprising chondroitin sulfate, found in cartilage and is a major component of mucins, which give a secretions gel-like properties. Since these are naturally-occurring, the addition of sulfate groups to carbohydrate-based IRIs should not increase cytotoxicity.^{47–50}

Sulfate groups can also be easily installed on carbohydrates using a number of techniques in good yield.^{51–55} One notable example of sulfation of various carbohydrates with a novel bench-stable sulfating agent was developed by the Taylor lab, using sulfuryl imidazolium triflates (**Figure 2-3**).

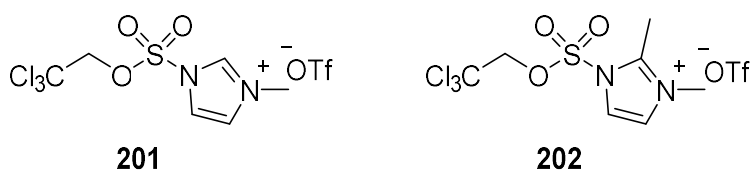


Figure 2-3. Structure of sulfuryl imidazolium triflates designed by the Taylor lab.^{51,52}

This method is compatible with many functional groups, and installs a 2,2,2-trichloroethanol protected sulfate on a free hydroxyl group, which can subsequently deprotected. Sulfate groups

can be added in different positions on pre-existing IRIs (**Figure 2-4**), and should theoretically increase their solubility, while ideally maintaining their IRI activity.

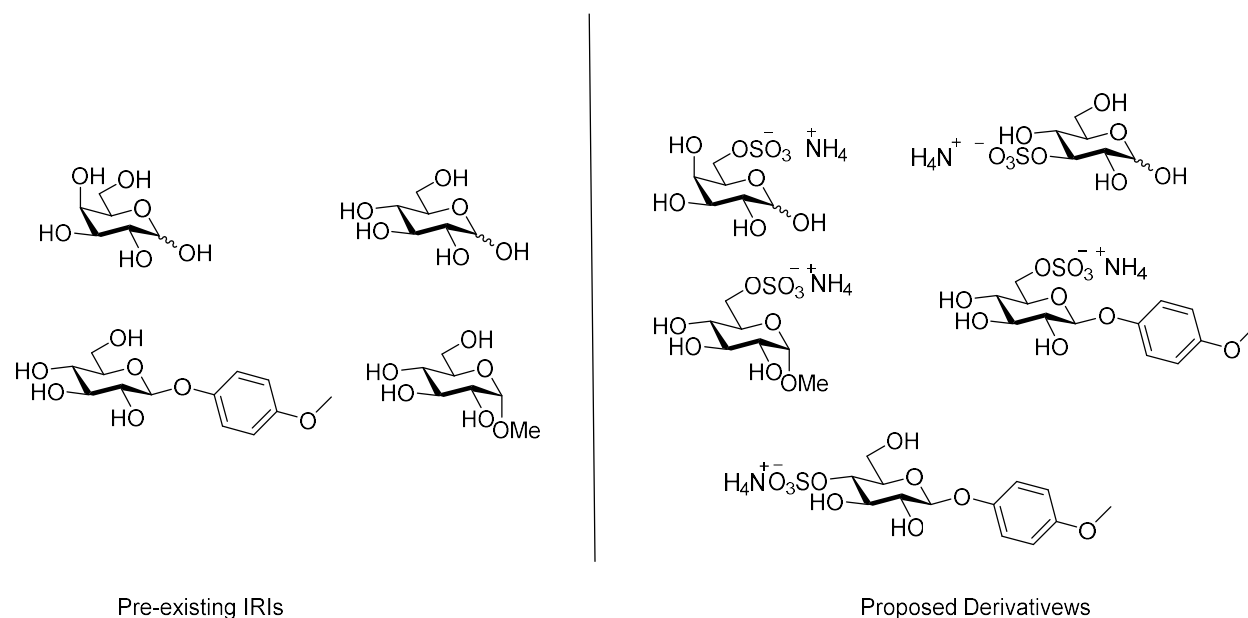


Figure 2-4. Structures of pre-existing IRIs and proposed sulfated derivatives.

2.3 Objective 2 – Synthesis and Evaluation of Sulfated Aldonamide Derivatives

Another class of IRI-active cryoprotectants developed by the Ben lab were substituted aldonamide derivatives. These open-chain carbohydrates have a hydrophobic moiety linked to the hydrophilic chain via an amide bond. These compounds display IRI activity in the low mM range, while having very low solubilities (0.5-55 mM in PBS). As previously mentioned, this greatly limits the working concentrations of these IRIs in biological systems, especially in cryopreservation protocols using heat-sensitive components. While all the current IRI active aldonamide derivatives are synthesized via a one-step ring opening reaction, the initial substrate can be modified prior to reaction, leaving the C5 hydroxyl unprotected after the ring opening. This serves as an ideal sulfation point of these derivatives and will be the focus in this chapter (**Figure 2-5**). Along with *N*-phenyl derivatives, *N*-benzyl, and *N*-alkyl derivatives will also be

explored. Although previously synthesized alkyl-substituted pyranose and aldonamide derivatives possess IRI activity, due to the extended alkyl chain, these derivatives often act as detergents, they will be included as a proof-of-concept experiment. The *N*-alkyl aldonamide derivatives are among the least soluble IRIs synthesized to date, and therefore would benefit most from an increase in solubility. This will also demonstrate the extent that solubility can be increased with sulfate groups.

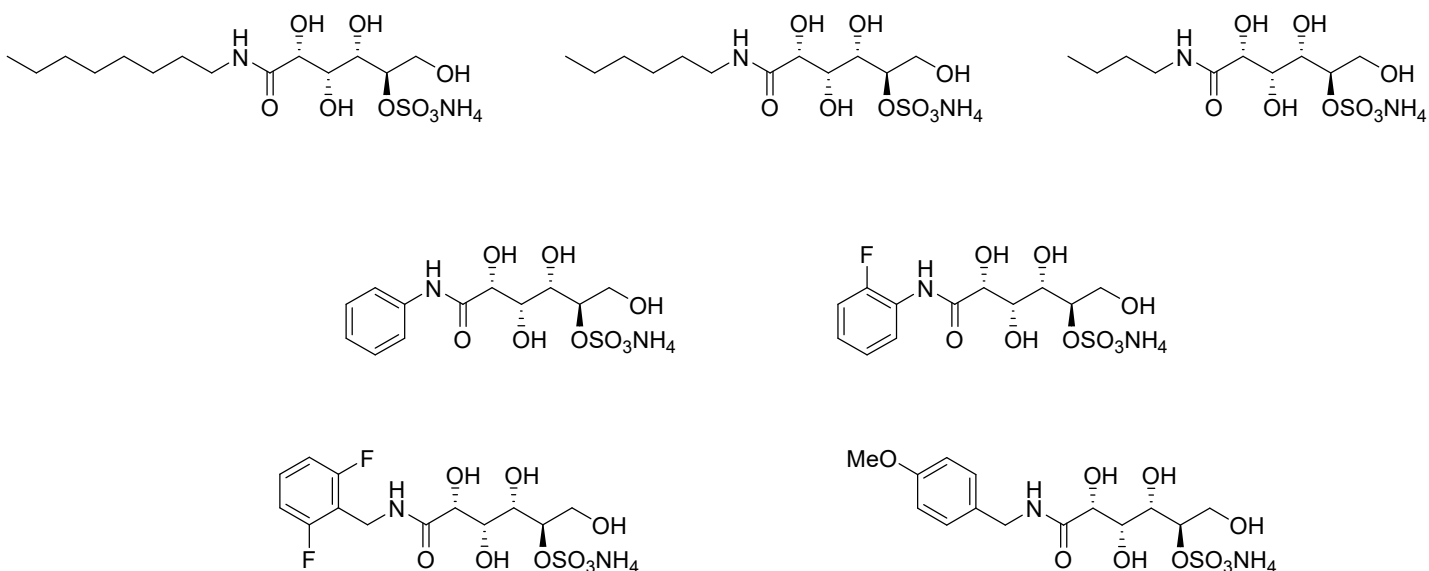


Figure 2-5. Structures of C5 sulfated aldonamide derivatives.

2.4 Objective 3 – Synthesis and Evaluation of Aminogluconamides

The addition of polar, charged functional groups has been shown to drastically increase solubility of previous insoluble IRI active compounds. To this point very few charged carbohydrate based IRIs have been explored.^{56,57} With more emphasis placed on anionic (at physiological pH) functional groups (carboxylates, sulfates) while few cationic IRIs have been explored. Since the introduction of a highly polar, anionic functional group was able to drastically increase the solubility of previous insoluble IRI active molecules, the increased solubility should be conserved with the introduction of cationic functional groups as well. To

further explore this, the introduction of an amino group was explored with previous IRI active gluconamides.

For these the derivatives, it was originally proposed to displace the C5 hydroxyl via a Mitsunobu reaction which would result in inversion of the C5 stereochemistry while installing an amino group. This process could then be repeated in order to invert the C5 again, to achieve the original stereochemistry of the C5 position on the gluconamide. Since this synthetic route was unable to be achieved, focus then switched to the C6 position. Using a triflate displacement on the C6 position of the corresponding gluconamide, an azide functional group can be installed. With subsequent deprotection and azide reduction, the C6 amino gluconamide derivatives could be synthesized (**Figure 2-6**).

These derivatives were synthesized on the principle that the inclusion of a polar (amino) functional group should drastically increase the solubilities of previously insoluble gluconamide derivatives. Since these derivatives are protonated at physiological pH, a similar effect of increasing solubility should be observed as with the sulfated gluconamides. Also, the addition of a cationic functional group may custom tailor the ability of these compounds to diffuse more easily through specific biological systems. Since tissue cryopreservation and subsequent biobanking is becoming more and more important, the need for new cryopreservation techniques is imperative.

One tissue that is commonly injured is cartilaginous tissue, primarily in the knees.⁵⁸ Cartilage bears an overall negative charge due to the presence of high concentrations of chondroitin sulfate that is distributed throughout the extracellular matrix of chondrocytes (cartilage producing cells).⁵⁰ Typically, current cryopreservation uses high concentrations of DMSO (>10%) that are required for successful cryopreservation.^{50,59-62} Due to the complex

nature of the tissue and overall negative charge, permeability of cryoprotectants is difficult, and often requires extended preincubation with cryoprotectant prior to cryopreservation..^{17,18} By using an IRI bearing a positive charge in conjunction with DMSO, should allow increased permeation by the IRI. This is beneficial due to the tendency of more complex tissues to freeze and/or thaw at different rates, depending on the thickness. These discrepancies in cooling/thawing rate can induce large amounts of ice recrystallization, causing damage to the interior of the sample.⁶³ If IRI active molecules could more easily permeate throughout tissues, the ice recrystallization induced by non-uniform cooling and warming could potentially be reduced, resulting in better quality tissues post-thaw.

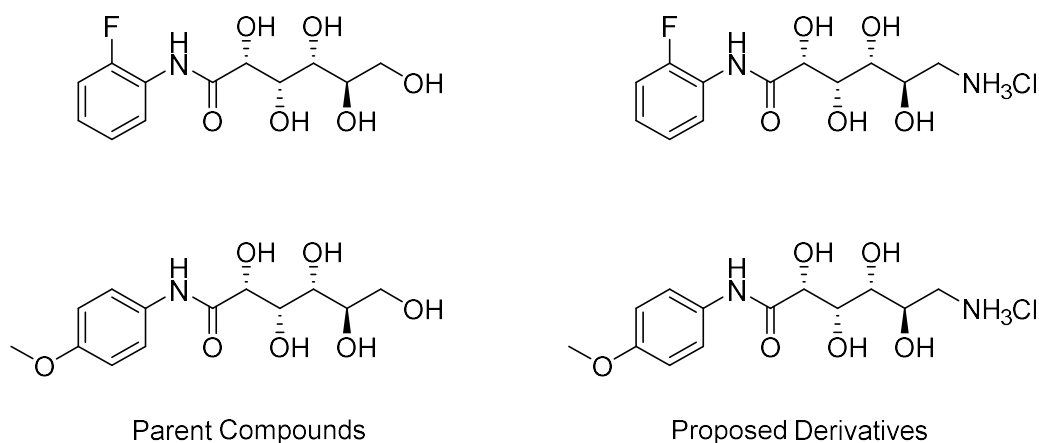


Figure 2-6. Proposed aminogluconamides and parent compounds.

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Chapter 3. Sulfated Pyranose Derivatives

3.1 Introduction

3.1.1 Hydration Analysis of Monosaccharides

Early research from the Ben laboratory has suggested that the ice recrystallization inhibition ability of simple monosaccharides is related to the hydration of the molecule itself.^{1,2} Hydration has been thoroughly studied for many monosaccharides, disaccharides, as well as modified monosaccharides by looking at a number of factors including: partial molar volume, hydration number, and isentropic partial molar compressibility.^{1,3-7} These studies have shown that molecules with low isentropic molar compressibility values disrupt the three-dimensional hydrogen bond network found in bulk water, which contributes to their effectiveness as ice recrystallization inhibitors.^{2,8} A key factor that determines the molecule's "fit" in bulk water is the stereochemistry of the carbohydrate, therefore by assessing the isentropic molar compressibilities as well as hydration number, a molecules fit into bulk water can be determined. Galema *et al.* have shown that the most important positions on carbohydrates is the stereochemistry at C4 and the relative position at C2.⁹ D-Galactose, with an axial C4 hydroxyl along with an equatorial C2 hydroxyl demonstrates the worst fit in bulk water, as opposed to D-talose with an axial C4 and C2 hydroxyl showing the best fit.

The hydration number, which represents the number of water molecules that are more strongly bound to the molecule than to surrounding water molecules, is another method of quantifying the "fit" of a molecule in bulk water.² D-Galactose has a hydration number of 8.7 ± 0.2 , while that of talose is 7.7 ± 0.2 . This suggests that molecules with greater hydration numbers disrupt bulk water to a greater extent. Considering this, the carbohydrate that disrupts bulk water

more should show a greater degree of IRI activity. This holds true for simple monosaccharides (**Figure 1.7**), though disaccharides do not follow the same trend. All previously tested disaccharides displayed larger hydration numbers and partial molar compressibilities (**Table 1.1**) although they are less IRI active than monosaccharides. While similar trends based on stereochemistry are present within the disaccharide series, the hydration numbers and partial molar compressibilities were not sufficient to describe their IRI activity. As a result, a measure of “hydration index” was described - the hydration index of a carbohydrate is defined as the hydration number divided by the molar volume. This eliminates the discrepancies observed between simple mono- and disaccharides.³

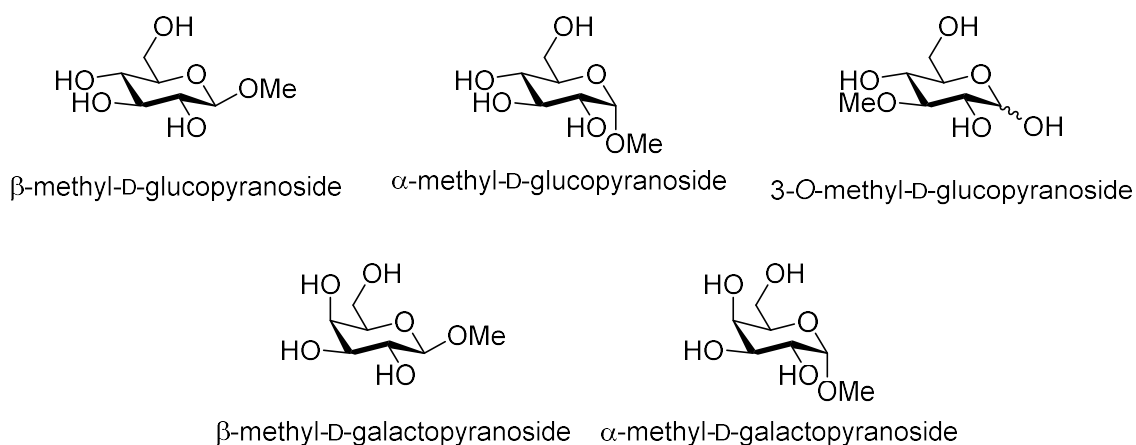


Figure 3-1. Structures of methylated pyranosides.

A number of α - and β -O-methyl-D-pyranosides along with 3-O-methyl glucopyranose were also studied to correlate any trends of hydroxyl group position and hydration number (**Figure 3-1**). Comparing the O-methyl-D-pyranosides to the non-methylated parent compounds, a similar stereochemical dependence on hydration is observed: methyl-D-galactopyranosides ($\alpha/\beta = 9.5$) were more hydrated and disrupt bulk water the more than O-methyl-D-glucopyranoside analogues (all hydration values taken from ^{8,10} and were determined using density ultrasound

measurements). In the case of 3-*O*-methylglucopyranoside, the hydration number of 8 was lower than both the α - and β -methyl-glucopyranosides (9.2 and 8.8 respectively) as well as D-glucose (8.4). This suggests that the C3 hydroxyl group has little impact on the hydration of D-glucose. Interestingly, when comparing the α - and β -methyl glucose analogues, the α -anomer shows a greater hydration number of 9.2 compared to the β -anomer (8.8), and therefore the α -anomer has a greater capacity to disrupt bulk water than the β -anomer^{8,10}. This suggests that in some carbohydrate derivatives, the anomeric position can play a role in the fit in bulk water, and consequently affect IRI activity as well.

3.1.2 Hydrogen Bonding and IRI Activity

Another metric to evaluate a compound's ability to disrupt bulk water is to modulate the hydrogen bond donating/accepting ability of individual hydroxyl groups in a compound. Hydrogen bond accepting atoms are electronegative atoms with a free lone pair of electrons that can form a hydrogen bond with a free hydrogen atom on another molecule. Hydrogen bond donors are electronegative atoms bonded to a hydrogen atom, which can freely interact with an acceptor atom. To further explore the differences between α/β -pyranose derivatives, the Ben laboratory synthesized a variety of α - and β -*C*-allylated pyranose derivatives. This study focused on the IRI activity, rather than the hydration index of the molecules to evaluate their fit into bulk water (**Chapter 1, Section 1.9**), as there were no published hydration numbers for these molecules. All α -*C*-allyl analogues tested followed the same trend and showed almost identical MGS values to their parent monosaccharides (**Figure 3-2**), while β -*C*-allyl-galactopyranose derivative showed an uncharacteristic decrease in IRI activity when compared to the α -anomer.

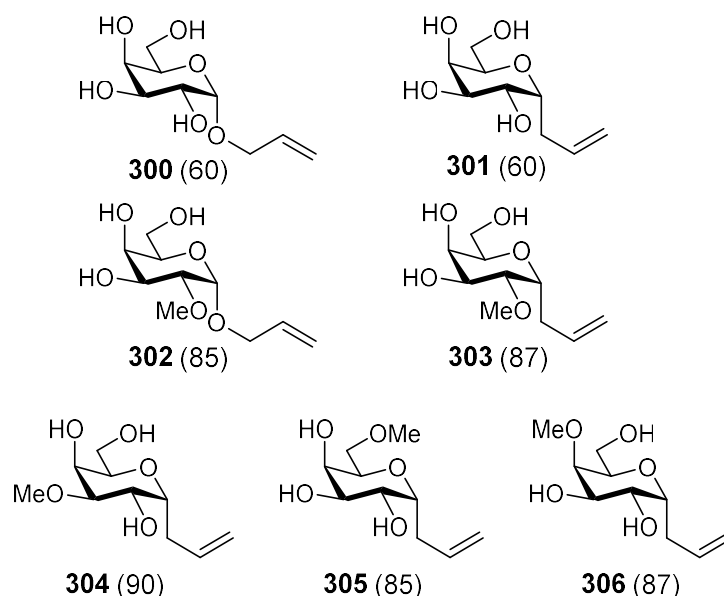


Figure 3-2. Structures of D-Gal based C-allyl-glycopyranosides previously synthesized and assayed for IRI activity. %MGS values shown in parentheses.¹¹

In attempts to further elucidate a structure-function relationship, the effect of different functional groups at other positions on the pyranose ring were investigated. By replacing the proton on a hydroxyl group with another atom (such as an alkyl substituent), the ability to hydrogen bond with other molecules is altered, allowing it to only function as a hydrogen bond acceptor but not donor depending on the atom it is replaced with.¹² As a result, the molecules fit in bulk water and IRI activity should be affected. Previous studies performed by Dashnau *et al.*¹³ and Simons *et al.*^{6,14,15} showed that the relative conformation of the carbon atoms in pyranose rings affect their intramolecular hydrogen-bonding ability, and their IRI activity (**Figure 3-3**). With removal of the hydrogen bond donor, the molecule's entire hydrogen bond network should be modified, not allowing it to "fit" within bulk water as well. This should result in more bulk water disruption and potentially an increase in IRI activity. In attempts to modulate IRI activity *via* elimination of hydrogen bond interactions, Chaytor synthesized several *O*-methylated α -C-

allyl-galactopyranose derivatives (**Figure 3-2**), selectively eliminating the hydrogen bond acceptor ability of the C2 (**303**), C3 (**304**), C4 (**306**), and C6 (**305**) positions.¹¹

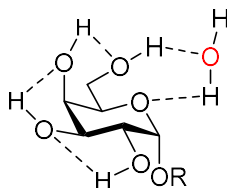


Figure 3-3. D-Galactose with corresponding water molecule insertion into the weakest intramolecular hydrogen bond. Note: multiple other water-carbohydrate and intermolecular carbohydrate-carbohydrate interactions not shown.

Although the hydration numbers of these derivatives could not be calculated, all methylated derivatives showed decreased IRI activity compared to α -C-allyl-galactose (**301**). While this suggested that intramolecular hydrogen bonding in pyranose rings is essential for IRI activity, and disrupting the network reduces IRI activity, this could be further explored with the synthesis of other compounds with similar properties.

3.1.3 Aminopyranosides as IRIs

Many different functional groups can be used as isosteres for hydroxyl groups, such as amines, which can be used to replace a hydroxyl group, but maintain similar physiochemical properties, focusing on hydrogen bond donating/accepting ability.¹⁶ These atoms have similar electronegativity values, meaning they can act as hydrogen bond acceptors similar to oxygen (pH dependent). All groups also contain an H atom, capable of acting as a hydrogen bond donor as well. By substituting a hydroxyl group with an amine, the size and hydrogen bond donating/accepting ability is left relatively unchanged, depending on the substituent and the pH of the solution.

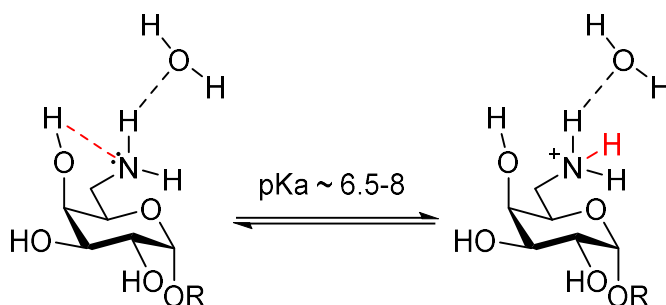


Figure 3-4. Hydrogen bond networks with water and aminogalactose at varying pH. Key pH - dependent interactions shown in red. Note: multiple other water-carbohydrate and intermolecular carbohydrate-carbohydrate interactions not shown.

Since amines are slightly basic, under physiological conditions, amines will become protonated, and thus the lone pair is occupied and cannot accept a hydrogen bond. Thus, we expect that substituting amines at various positions on the pyranose ring, hydrogen bond donating ability will be changed and this will result in a difference in IRI activity. Amino-substituted galactopyranoses were synthesized by Tonelli, installing amines at the C2, C3, C4, or C6 positions on the pyranose ring (**Figure 3-5**).¹⁷ The galactose derivatives with amines at the C2, C4, and C6 positions all showed slightly reduced IRI activity compared to galactose, although the C3 amine displayed a considerable increase in IRI activity. Interestingly, this is the first derivative with modification to the C3 position to show increased IRI activity. All compounds (**D-galactose, 306-309**) were analyzed as an α/β mixture (ratio not determined) which may have affected the determined MGS. This study was not repeated with C1 substituted carbohydrates to address this. This illustrates the importance of a hydrogen bond acceptor at all positions other than C3, which seems to tolerate having only a hydrogen bond donor at that position opposed to exclusively an acceptor such as a C3 methoxy group.^{10,12} As previously stated, the relative configuration of the C2 and C4 positions affect monosaccharide

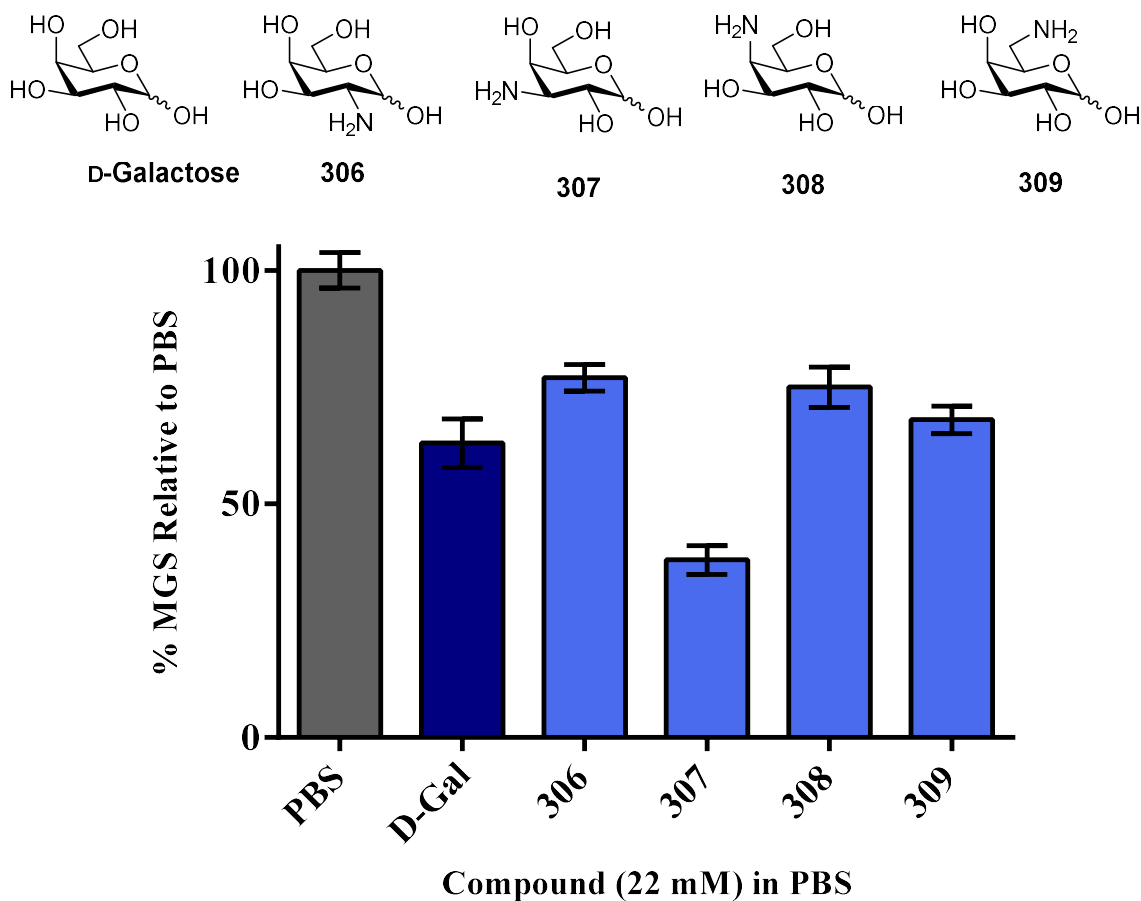


Figure 3-5. Structures of amino-deoxy-d-galactose derivatives and corresponding IRI activity at 22 mM. All compounds assayed as an undetermined α/β anomer ratio. %MGS shown relative to PBS negative control (100%) using the 30-minute drop assay. Error bars represent SEM, $n = 3$.

hydration, with galactose (C2 eq, C4 ax) being the most hydrated (hydration number = 8.7), compared to talose (C2 ax, C4 ax) being the least (hydration number = 7.7) (**Chapter 1 Figure 1.7**). The same cannot be said for the hydration of monosaccharides modified at the C3 position. Considering the previously tested monosaccharides, all maintained an equatorial hydroxyl at C3 except ribose. While ribose does have an axial C3 hydroxyl group, it lacks the axial hydroxyl at C4 and is also missing C5 and C6 in its preferred conformation in solution.¹⁸ Due to this, the effect of configuration of the C3 position cannot be concluded. Further studies such as comparing the hydration numbers could help explain some of these observations but have not been addressed at this time. Although the hydration numbers were not determined for the amino

galactose derivatives, it would be expected that the amines would be slightly less hydrated than their hydroxyl parent compounds.¹⁹ This is due to the ability of alcohols to form slightly more hydrogen bonds as a hydroxyl group being a better hydrogen bond acceptor than an amine acting as a hydrogen bond donor.²⁰

3.1.4 Azasugars as IRIs

Rather than assessing newly synthesized derivatives for their hydration values, structure-function relationships can be inferred through IRI activity. There are many examples of highly hydrated functional groups such as amides, sulfates, phosphates, and carboxylic acids that could be ideal candidates to potentially increase IRI activity. Some of these functional groups have already been explored, specifically C6 carboxylic acids and C1 lactams as seen in **Figure 3-6**. Firstly, both C6 carboxylic acid derivatives of glucose and galactose show negligible IRI activity. This may be due to the importance of the C6 hydroxyl both intra- and hydrogen bonding.^{7,14} Also, at physiological pH, **310/311** will exist as a carboxylate salt, meaning they cannot act as hydrogen bond donors. This likely affects the overall hydrogen bond network, resulting in the diminished IRI activity. Although, C6 amides have not been synthesized. These molecules would act as both hydrogen bond donors and acceptors because of their neutral charge in solution, and therefore should show IRI activity. Amide-containing compounds **312/313** replace the endocyclic ring oxygen with an amine, modifying the hydrogen bonding properties of the pyranose ring. The glucose azasugar derivative **313** did show IRI activity comparable to the C3 amino galactose derivative **307**, while the galactose derivative **312** was slightly less active. The differences in IRI activity between the two is likely due to differences in the overall hydrogen bond network due to the configuration of the compounds.

To further study what role the ring heteroatom has on the IRI activity of monosaccharides, additional azasugars (**314/315**) were synthesized. In azasugars, the ring oxygen is replaced with a nitrogen atom, which can both donate and accept hydrogen bonds. Compounds **312/313** possess a secondary amine (able to donate/accept hydrogen bonds) while tertiary amine compounds **314/315** replace the hydrogen atom with a phenyl derivative to remove hydrogen bond donation. These compounds were assessed for their IRI activity (**Figure 3-6**). Both secondary amine compounds displayed IRI activity that was higher than that of the non-substituted parent compounds (D-galactose and D-glucose). This suggests that both hydrogen bond donating and accepting ability of the endocyclic atom could contribute to increased IRI activity. The two tertiary amine derivatives both show lower activity than the parent compounds, suggesting that both hydrogen bond donating and accepting ability of the ring heteroatom contributes to IRI activity, while removing the donating ability results in reduced IRI activity.

Overall, hydrogen bonding ability plays an important role in IRI, and small modifications in hydrogen bond donating and accepting ability at different position on a molecule can modulate the IRI activity of a molecule. As there are no definitive trends, more research in this must be explored.

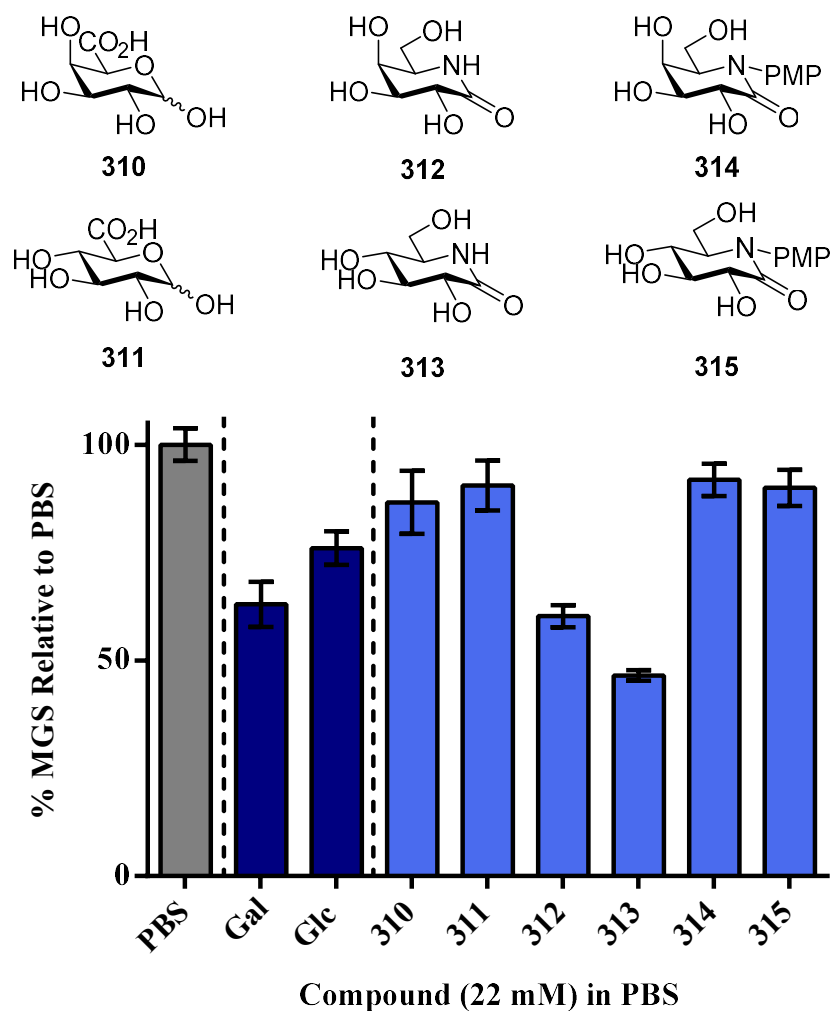


Figure 3-6. Structures of carbonyl-based derivatives of D-Gal and D-Glc and corresponding IRI activity at 22 mM. %MGS shown relative to PBS negative control (100%) using the 30-minute drop assay. Error bars represent SEM, n = 3.

3.1.5 Sulfated Carbohydrates as IRIs

Until this point, the only negatively charged, carbohydrate-based compounds that were tested for IRI activity by the Ben lab were the C6 carboxylic acid derivatives (carboxylate salt at physiological pH). To further investigate the effect of charged functional groups on IRI activity, sulfate groups was chosen to incorporate on to simple monosaccharides and pre-existing IRI

active carbohydrates. Sulfate functional groups were chosen to examine this due to their ability to bind multiple water molecules^{20–23}, as well as their general tolerance in biological systems.^{24–}
²⁶ Since sulfate groups ($-\text{OSO}_3^-$) have 3 terminal oxygen atoms, all which can act as hydrogen bond acceptors, multiple water molecules can hydrogen bond to them, forming large hydrogen bond networks.^{20–23} A number of naturally-derived, sulfated carbohydrates exist, that play a variety of biological roles. One of the most common sulfated carbohydrate-containing molecules are heparin sulfate. It is present on the surface of most animal cells and in the extracellular matrix. Heparin and heparin sulfate are able to inhibit serine proteases during blood coagulation, and are often medically used for these anticoagulant properties.^{25,26} These molecules contain repeating units of carbohydrates, with sulfate groups on the C6 or C3 residues, as well as sulfamates on glucosamine residues.

Mucins are another class of biological sulfated carbohydrates which are produced in various epithelial cells and contributes to gel-like properties of many secretions. These glycoproteins generally feature a C6 or C3 sulfated, terminal galactose residue, or C6 sulfated *N*-acetylglucosamine residues. With the addition of sulfate groups happens frequently in biological systems, sulfated carbohydrates should be relatively non-toxic in biological systems. There are many literature examples of biological and chemical synthesis of sulfated carbohydrates using many different techniques.²⁷

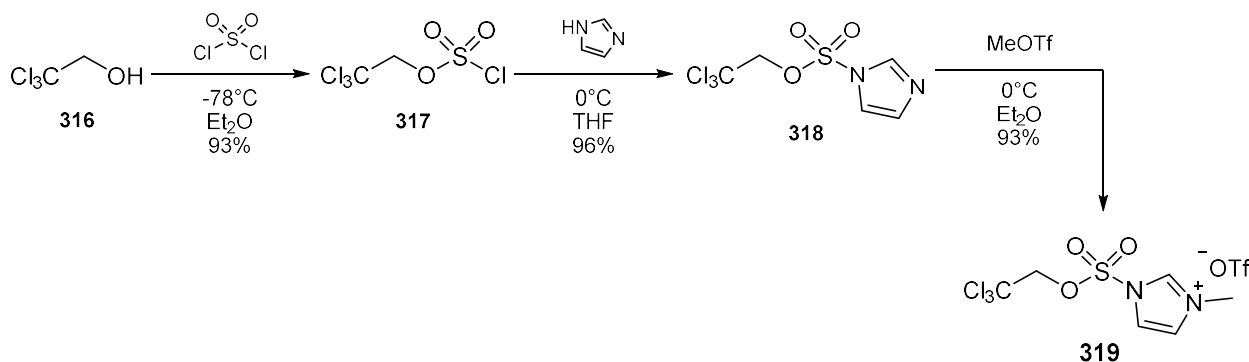
3.1.6 Synthesis of Sulfated Carbohydrates

Biologically, carbohydrate sulfation proceeds *via* a sulfotransferase, which in eukaryotic cells uses 3'-phosphoadenosine-5'-phosphosulfate as the sulfate source. Numerous sulfotransferases exist, each specific to the carbohydrate and the area of sulfation. Sulfation/desulfation is used to regulate the activity of various glycoproteins, either adding

sulfate or removing groups to terminal residues or removing the entire carbohydrate residue altogether.^{28–31}

In synthetic chemistry methodology, there exist many reactions that can install sulfate groups with varying applicability and efficiency. Some of the first methods employed sulfuric acid with or without various dehydration methods. Although moderate yields can be achieved with this method, it is not compatible with the acid-sensitive functional groups, which are commonly used as protecting groups in carbohydrate chemistry. To overcome this, sulfur trioxide (SO₃) based reagents can be used due to the increased functional tolerances and milder reaction conditions needed for addition. Free SO₃ (gas or liquid) can be used but is quite difficult to handle due to the oxidizing ability and difficulty to work with. Because of this, it is commonly used as a relatively safe, solid SO₃-amine complex.²⁷ These reagents can be used to install a sulfate on a free hydroxyl group (or sulfamate in the case of an amine) producing a polar, negatively charged group that can be difficult to isolate and purify, especially when multiple sulfate groups are installed. For these applications, installation of a protected sulfate group would seem ideal, followed by deprotection at the latest possible point in the synthesis. Many ways of achieving this have been developed, most notably the method by Taylor *et al.*, which uses a 2,2,2-trichlorosulfonyl imidazolium triflate salt to install a trichloroethyl-protected (TCE) sulfate on carbohydrates.³² Originally, Taylor's method employed 2,2,2-trichloroethyl chlorosulfate to install the protected sulfate on phenol derivatives, though when applied to carbohydrates, resulted in low yields and unwanted byproducts. To avoid these unwanted outcomes, the imidazolium triflate salt was added, resulting in a bench-stable reagent that can be readily synthesized (**Scheme 3-1**) and used with carbohydrates in good yield. Due to the reagent's

robustness, it was selected for the synthesis of sulfated galactose derivative **324** (Scheme 3-2) which was also assessed for its IRI activity.



Scheme 3-1. Synthesis of 2,2,2-trichloroethyl imidazolium triflate sulfating (**319**) reagent developed by Taylor.³²

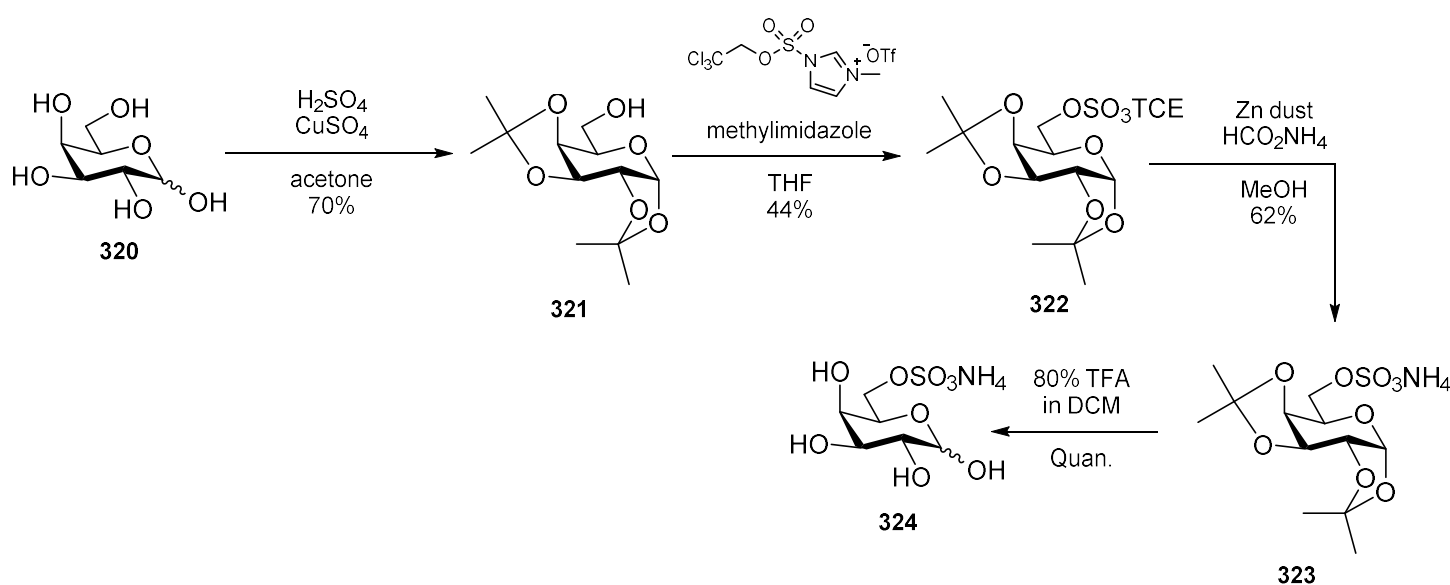
3.2 Synthesis of Sulfated Pyranose Derivatives as IRIs

3.2.1 Synthesis of 6-*O*-sulfo-D-galactose (**324**)

In order to assess the effect of replacing the C6 hydroxyl of D-galactose with a more hydrated sulfate functional group, efforts went toward synthesizing C6 sulfated D-galactose derivative **324**. This derivative is based on the C6 galactose residues present on mucin glycoproteins. This derivative can also be used to compare the effect of modulating the hydrogen bonding ability of the C6 position on galactose. By replacing the hydroxyl group with as sulfate, the hydrogen bond donating ability is removed, leaving only a hydrogen bond acceptor at C6 (like C6 carboxylates **310/311**). As the free sulfate is an anion, a counterion must be selected. The Ben lab has previously conducted studies looking at the effect counterions have on IRI activity, examining cationic ions Li^+ , Na^+ , and K^+ ³³ as well as NH_4^+ .^{34,35} From this, ammonium (NH_4^+) ions with simple counterions (chloride/formate) were able of inhibiting ice recrystallization. To ensure that a compounds IRI activity is due to the compound itself, and not

the corresponding salt, all compounds with an %MGS value of less than 10 % should be tested with an additional counterion and compared to the ammonium salt.

Additionally, using the sulfation addition/deprotection developed by Taylor, in order to deprotect the sulfate, a hydrogen source is necessary.³⁶ Ammonium formate is convenient in acting as a catalytic hydrogen transfer source. In doing so, the free sulfate is left as an ammonium salt. Due to this, all subsequent sulfated derivatives were synthesized as

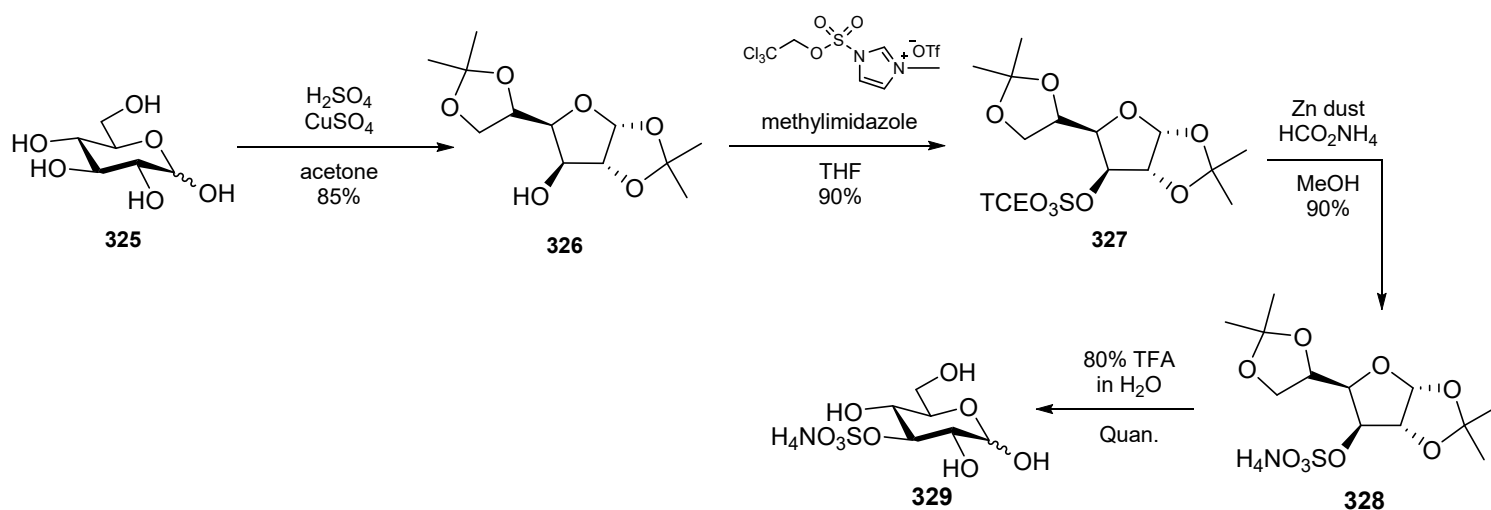


Scheme 3-2. Synthesis of 6-*O*-sulfo-D-galactopyranose (**324**).

ammonium salts. Commercially available D-galactose was converted into diisopropylidene **321** by treatment with H_2SO_4 , CuSO_4 , and acetone in a 70% yield (**Scheme 3-2**). With the C6 hydroxyl left unprotected the sulfation reaction was performed with sulfating reagent **319** along with *N*-methylimidazole (*N*-MeIm) in THF generating the protected C6 sulfate **322** in a 44% yield. Subsequent sulfate deprotection with zinc dust and ammonium formate in methanol resulted in the free sulfate as an ammonium salt in a 62% yield overall. The final step was the removal of the isopropylidene acetals, which was done using 80% TFA in DCM in a quantitative yield, producing C6 sulfated galactose derivative (**324**) in a 19% overall yield from D-galactose.

3.2.2 Synthesis of 3-*O*-sulfo-D-glucose (329)

Using a similar method starting with D-glucose, the C3 sulfated glucose product can be synthesized. This derivative is similar to the C3 sulfates found in some heparan sulfate glycoproteins. It can also be used to assess the effect of sulfation on the C3 position. In other derivatives, removing the hydrogen bond donating ability (-OMe) reduced IRI activity, while removing the hydrogen bond accepting ($-\text{NH}_3^+$) increased IRI activity. Although both trends were observed in galactose derivatives, glucose derivatives may show similar trends. Unlike the reaction with D-galactose, the C3 hydroxyl of glucose is left unprotected after an isomerization to the furanose form during the isopropylidene acetal installation (**Scheme 3-3**). The rest of the reaction steps remain the same as the galactose derivative, generating the final C3 sulfated glucose derivative in an overall yield of 69% from D-glucose.



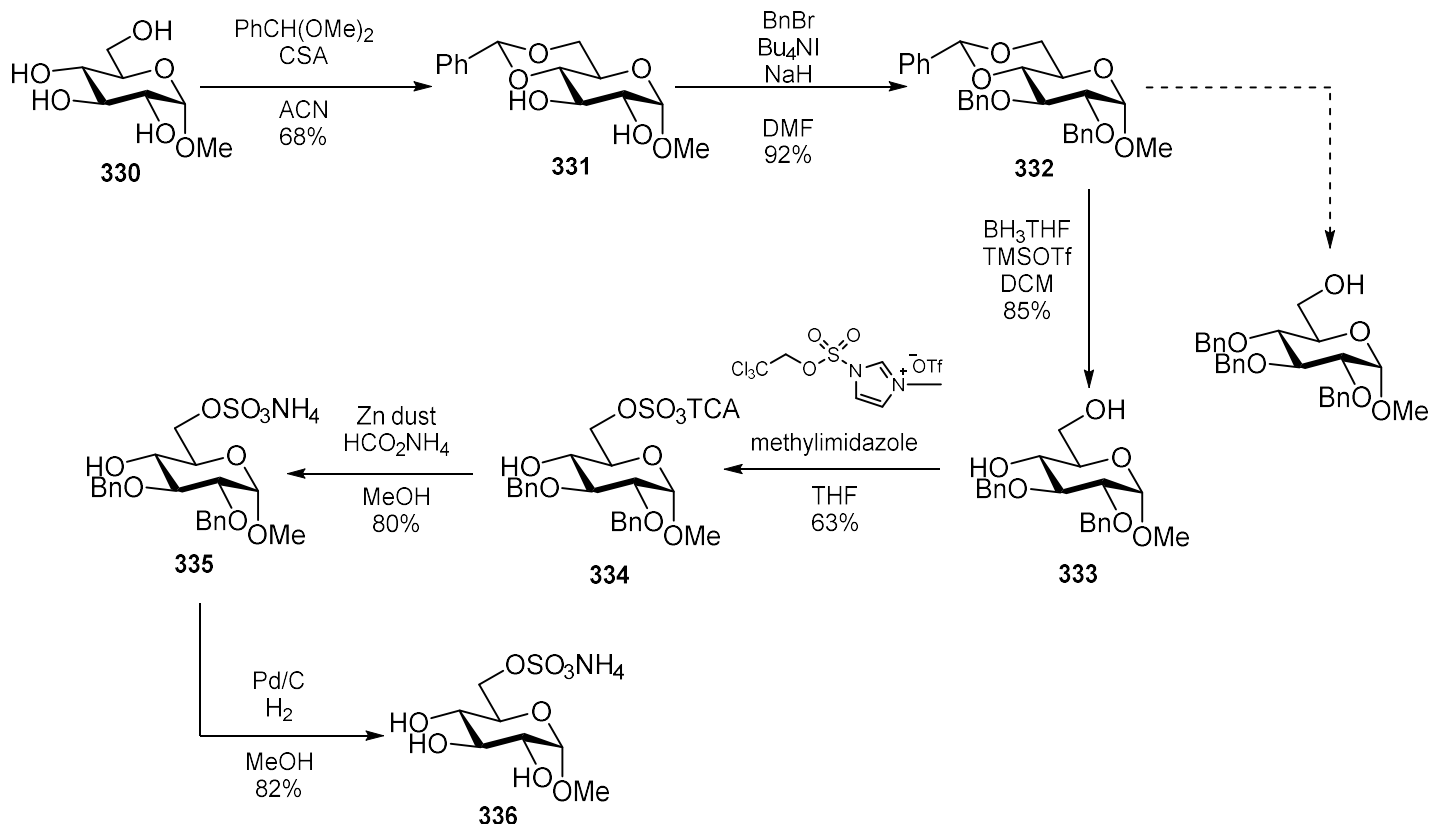
Scheme 3-3. Synthesis of 3-*O*-sulfo-d-glucopyranose (329).

3.2.3 Synthesis of Methyl-6-*O*-sulfo- α -D-glucopyranoside (336)

The next derivative to be synthesized was an α -methylglucose derivative (**Scheme 3-4**), bearing the sulfate at the C6 position. This derivative was synthesized to assess the effect that the anomeric hydroxyl group plays in IRI activity with sulfated derivatives. Firstly, commercially

available methyl α -D-glucopyranoside was reacted with benzaldehyde dimethyl acetal to form a benzylidene acetal, protecting the C4 and C6 hydroxyls on the glucose derivative in a 68% yield. Next, the free C2 and C3 hydroxyls were protected with benzyl groups installed in a 92% yield using benzyl bromide, tetrabutylammonium iodide, and sodium hydride in DMF (**332**). With the fully protected sugar in hand, the benzylidene acetal could then be selectively opened leaving the C6 hydroxyl free, using $\text{BH}_3 \cdot \text{THF}$ and TMSOTf in DCM. Surprisingly, the reaction did not proceed as intended and the protecting group was removed. Fortunately, the C4/C6 deprotected derivative was still of use due to the more reactive nature of the C6 position (**333**). This derivative was still able to be sulfated using the same sulfation conditions in a 63% yield to generate the C4 hydroxyl, C6 sulfated derivative **334**.

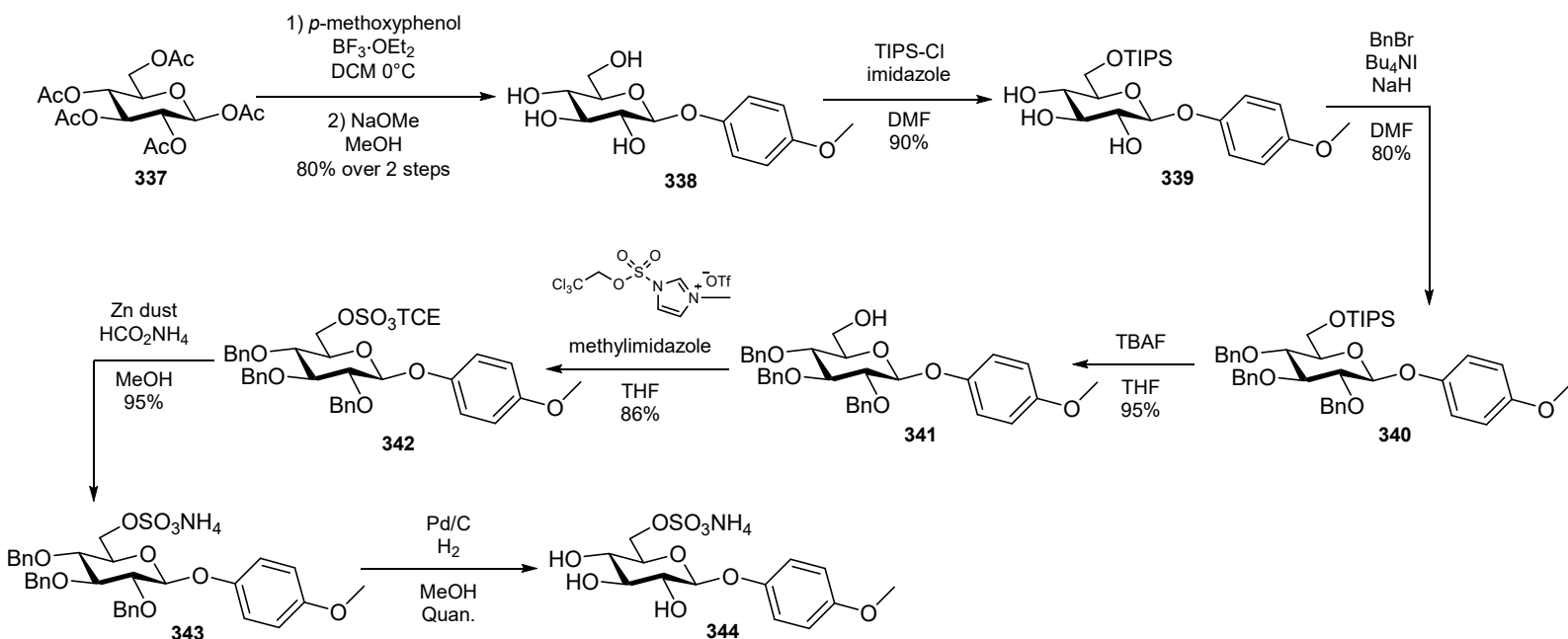
This derivative was then subjected to the same sulfate deprotection conditions as the previous compounds, followed by hydrogenolysis of the benzyl groups using Pd/C and hydrogen in ethanol to yield the C6 sulfated derivative **336** in 66% over two steps.



Scheme 3-4. Synthesis of methyl-6-*O*-sulfo- α -D-glucopyranoside (**336**).

3.2.4 Synthesis of Sulfated Phenolic Glycosides

The next three sulfated glucose derivatives are derived from the phenolic glycosides **134** and **135** due to some desirable properties of these compounds. Both compounds show IRI activity (23 and 12 % relative to PBS, respectively) and have been successfully used in the cryopreservation of RBCs.^{37–39} The C6 sulfated β -PMP-D-glucose (**134**) derivative was synthesized beginning with penta-*O*-acetyl- β -D-glucopyranose **337**. To install the anomeric β -linked *p*-methoxy phenyl group, Lewis acid ($\text{BF}_3\cdot\text{OEt}_2$) catalyzed glycosidation was used followed by Zemplén deacetylation conditions to generate compound **338** in 80% over two steps. Due to problems with the selective opening of the benzylidene acetal in the previous synthesis, a new



Scheme 3-5. Synthesis of *p*-methoxyphenyl-6-*O*-sulfo- β -D-glucopyranoside (**344**).

route where C6 could be selectively protected was envisioned using a silyl protecting group.

triisopropyl chloride was used along with imidazole as a base in DMF to selectively protect the

C6 alcohol in a 90% yield. Benzyl groups were then installed on the C2-4 positions using the

same conditions as were used to synthesize compound **333** in **Scheme 3-4**. In order to install the

sulfate group at C6, the TIPS group must first be removed. This was achieved via TBAF

deprotection which proceeded in a 95% yield. As with the other derivatives, the sulfate was then

installed using the same conditions as previously used followed by deprotection with Zn dust.

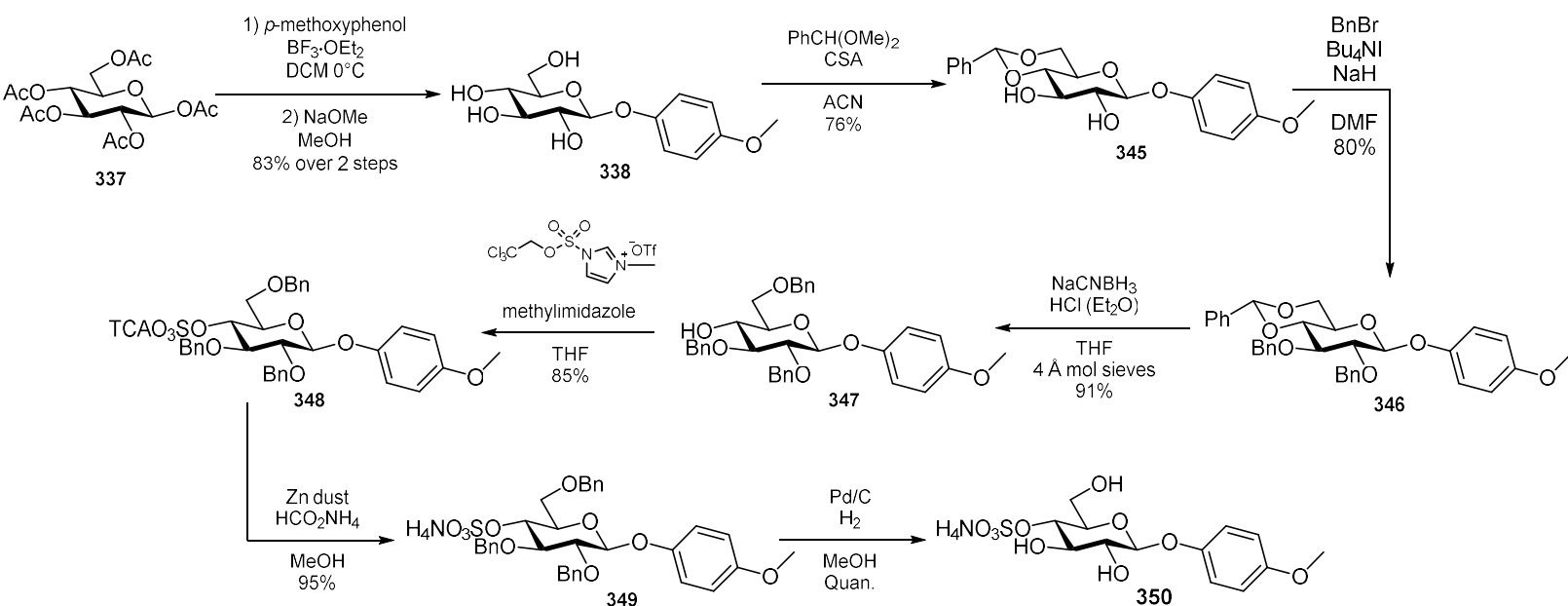
These two steps were carried out in an 82% overall yield, furnishing the C6 sulfate as an

ammonium salt. The final step in this synthesis was to remove the remaining benzyl groups with

palladium catalyzed hydrogenolysis, providing the final compound **344** in a yield of 45% overall

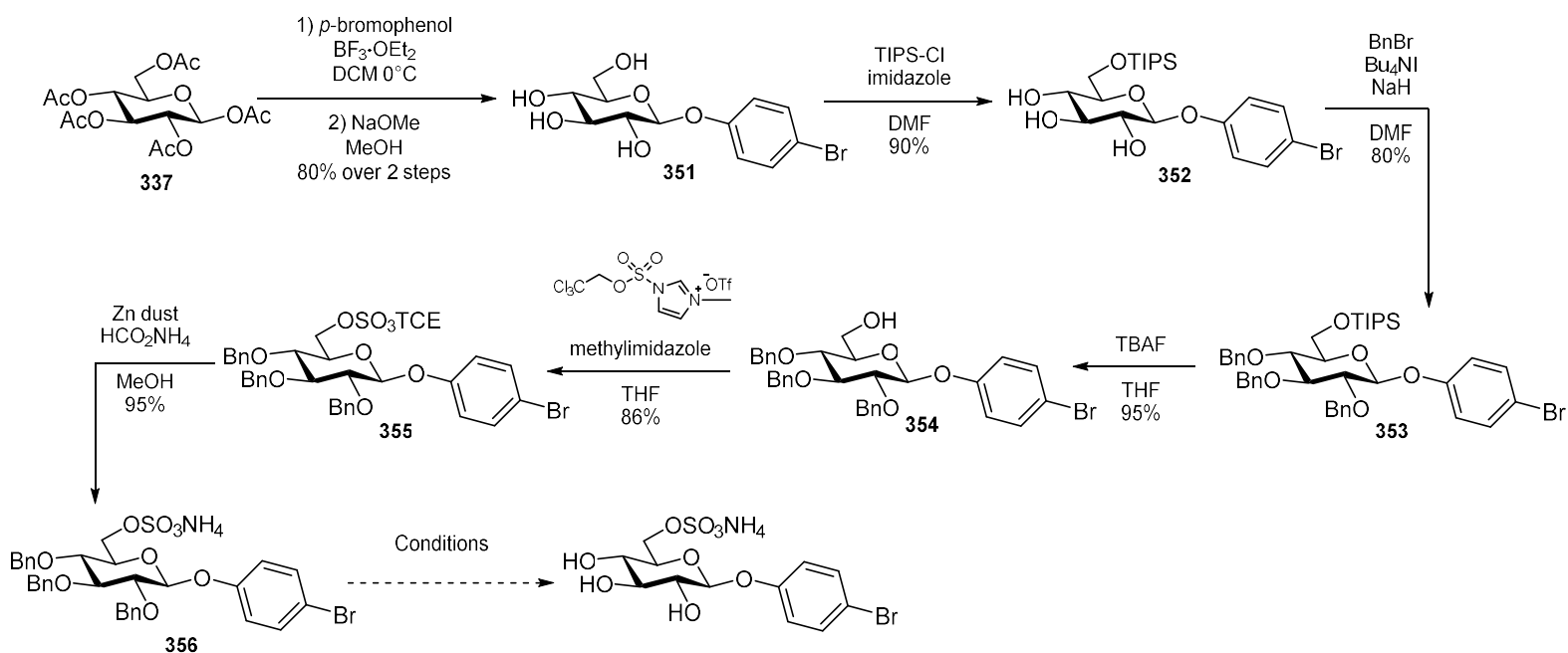
from **337**.

The next compound to be investigated was the C4 sulfated derivative of β -PMP-glc (135). This compound will assess the effect of sulfate regiochemistry on the pyranose ring. This should modify the hydrogen bond network surrounding the molecule, which should modulate IRI activity. As with the C6 sulfated PMP derivative, penta-*O*-acetyl- β -D-glucopyranose underwent a glycosidation reaction with *p*-methoxyphenol followed by Zemplén deacetylation. Instead of selective C6 protection, a benzylidene acetal was installed. This was to allow for selective opening, leaving the C4 hydroxyl free and C6 protected with a benzyl group. This was done using NaCNBH₃ and ethereal HCl in THF with 4 Å molecular sieves in a 91% yield, leaving the C4 free (347) to be sulfated under the typical conditions with a yield of 83%. The last two steps consisted of the sulfate deprotection followed by hydrogenolysis yielding the final C4 sulfated β -PMP-glc derivative 350 (Scheme 3-6) in 36% overall from 337.

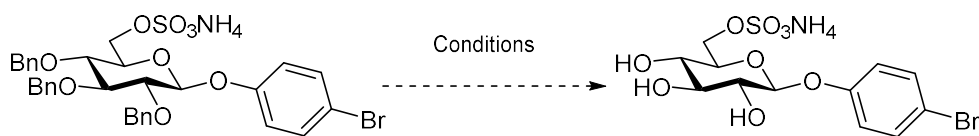


Scheme 3-6. Synthesis of *p*-methoxyphenyl-4-*O*-sulfo- β -D-glucopyranoside (350).

The final compounds to be synthesized was based upon β -*p*-Br-Glc derivative (**135**) bearing the sulfate group again at the C6 and C4 positions (**Scheme 3-7**). Compound **135** shows high degrees of IRI activity (12% relative to PBS) and has previously been successful in the cryopreservation of RBCs. Ideally, these beneficial properties would be retained with the addition of a sulfate group. By installing a sulfate on the molecule, the hydrogen bonding ability will be modified, and the relationship to IRI activity can be assessed. This synthesis was identical to that of the C6 sulfated β -PMP-Glc derivative **344**, only using *p*-bromophenol in the glycosidation rather than *p*-methoxyphenol. This pathway proceeded smoothly until the final hydrogenolysis, in which the typical conditions resulted in cleavage of the phenyl-bromine bond due to palladium insertion and elimination. Regardless of the conditions (**Scheme 3-8**) used to deprotect the remaining hydroxyls, no product was formed and due to the undesirable results obtained with all attempts, this route was abandoned. In the future, these derivatives could potentially be synthesized by means of alternative protecting group strategies.



Scheme 3-7. Synthetic efforts toward *p*-bromophenyl-6-*O*-sulfo- β -D-glucopyranoside.



Entry	Conditions	Result
1	Pd/C, H ₂ , EtOH	Bromide cleavage
2	BCl ₃ , DCM, -78°C	Complex mixture of products
3	FeCl ₃ , DCM, -20°C	No reaction
4	CCl ₄ , <i>hν</i> , MeOH, H ₂ O	No reaction

Scheme 3-8. Attempted benzyl deprotection conditions.

3.3 IRI Activity of Sulfated Pyranose Derivatives

Once the sulfated pyranose derivatives were synthesized, the IRI activity needed to be assessed. To do this, the MGS values compared to PBS were calculated using the previously described splat cooling assay (**Chapter 1.6.2**). Briefly, the analyte was dissolved in a 1x phosphate buffered saline (PBS) solution and a 10 μ L droplet of this solution was dropped from a micropipette from a height of two metres onto a block of polished aluminum precooled to approximately -80 °C (dry ice). The droplet immediately froze and was incubated at -6.4 °C for 30 min. Afterwards, pictures were taken and analyzed using a novel domain recognition software (doman3) to analyze ice crystal area. Areas were normalized to PBS (negative control of IRI) to generate a percent mean grain size (%MGS) compared to PBS. Lower %MGS are considered to show more IRI activity, relative to higher %MSG values, with PBS having a %MGS value of 100 %. The values for compounds **324** and **329** are presented in **Figure 3-7**.

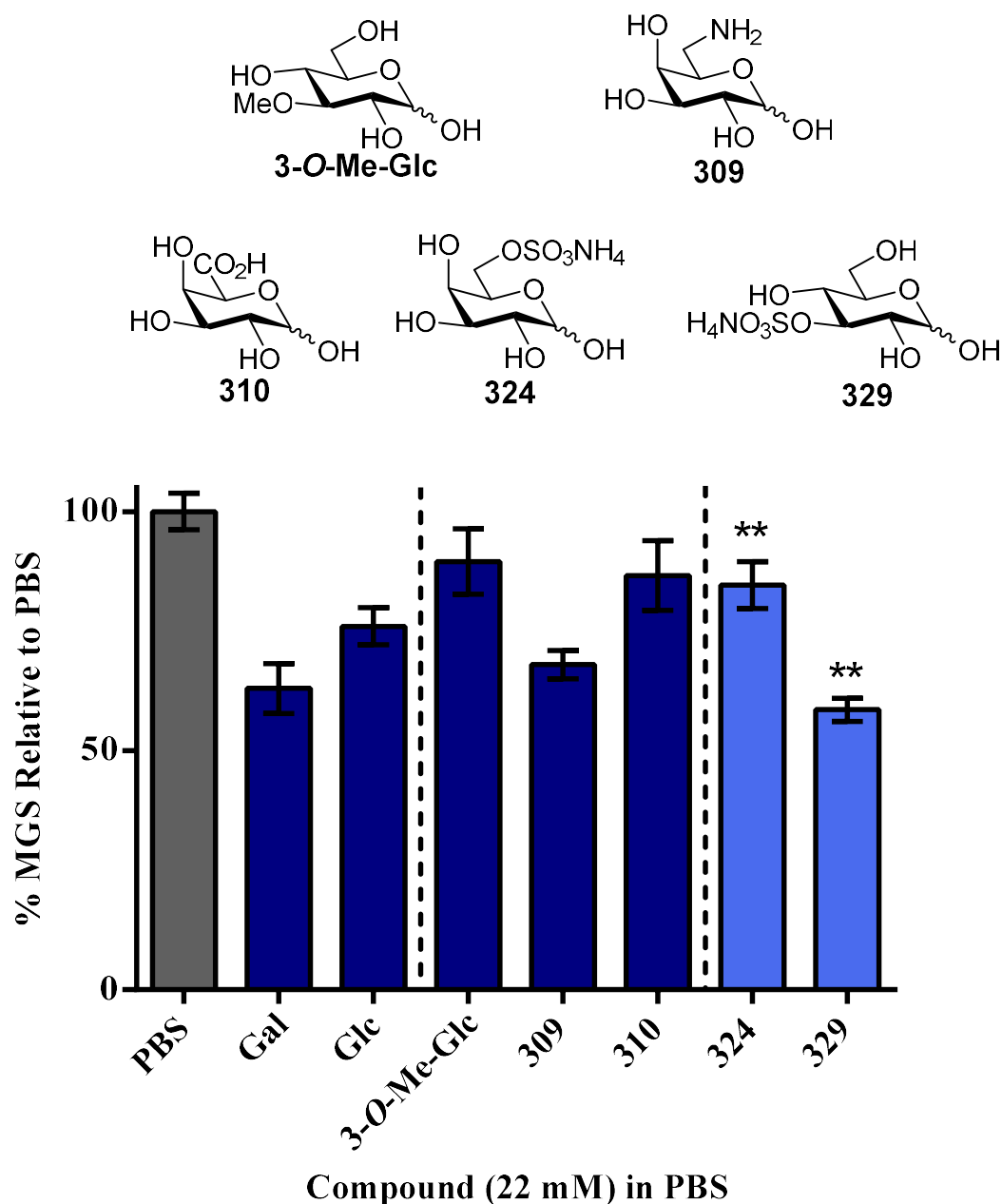


Figure 3-7. Structures of sulfated derivatives **324** and **329** and corresponding IRI activity at 22 mM. %MGS shown relative to PBS negative control (100%) using the 30-minute drop assay. Error bars represent SEM, n=3. Asterisk represents a statistical significance between compound and control (PBS) generated by student's t-test (*, $p < 0.05$ and **, $p < 0.01$).

D-Galactose based C6 sulfated derivative **324** showed negligible IRI activity at 22 mM compared to D-galactose. This was interesting because it was expected that the sulfated derivative **324**

should have a higher hydration than D-galactose due to the C6 sulfate and increase in hydrogen bonding capability. This being based on the ability of sulfate groups to bind multiple water molecules in large hydrogen bond networks.^{20,22,23} It is possible that the sulfate interrupts the intramolecular bond between the C6 hydroxyl and the preferred water binding site usually observed with D-galactose^{15,40} where the C6 hydroxyl group acts as both a hydrogen bond donor and acceptor. The sulfate only acts as an acceptor, and this may potentially modify the compounds ability to disturb bulk water. This would also explain the decreased IRI of other C6 modified compounds **309** and **310** (both charged at physiological pH), when compared to D-galactose. While **309** does not have a negatively charged functional at the C6 position, the amine will be protonated at physiological pH, removing the hydrogen bond acceptor. Compound **310** was used to examine the opposite effect of hydrogen bonding ability, as it is a carboxylate salt at physiological. This removes the hydrogen bond donor, which may more closely compare to the sulfated pyranose derivatives, which also express only hydrogen bond acceptors. Compound **310** shows a slight reduction in IRI activity over **311** (86% vs 68% relative to PBS), possibly showing hydrogen bond donors elicit a higher IRI response than hydrogen bond acceptors. This modification is still able to interrupt the hydrogen bond network in galactose by eliminating the hydrogen bond between the C4 and C6 hydroxyls observed with D-galactose. While compound **310** should retain this hydrogen bond but eliminate the bond between the donor and a water molecule acceptor. When looking at the sulfated D-glucose derivative **329**, the C3 position is replaced with the sulfate group rather than the C6 position. Interestingly, this compound displays a higher degree of IRI activity than both D-glucose as well another C3 modified glucose derivative: 3-*O*-methylglucoside (3-*O*-Me-Glc). As previously described, methylation of the C3 position of glucose does not increase hydration and in turn decreases IRI activity. Although the

hydration of derivative **329** was not assessed, it should be greater than that of both D-glucose and 3-*O*-Me-Glc due to the addition of the sulfate.^{20,22,23} The increased hydration could explain the slight increase in IRI activity over the other glucose derivatives. Due to this, other sulfated glucose derivatives were explored. All three derivatives synthesized possessed modifications at the anomeric position in both α - and β -configurations.

Compound **336**, bearing a methoxy group in the α -configuration on the anomeric carbon, was sulfated at the C6 position. This modification resulted in an increased activity compared to the non-sulfated parent compound as well as D-glucose (methyl Glc, **Figure 3-8**). The parent compounds both show a large %MGS compared to other IRI-active compounds, with D-Glc being slightly more active. While the trend between D-Glc and methyl D-Glc can be explained by hydration of the molecules (Glc = 8.4, methyl Glc = 8), the hydration numbers could not be calculated for the sulfated analogues. The increased IRI activity of compound **336** could be due to an increase in hydration and retention of the hydrogen bond acceptor needed for water insertion at the preferred site on glucose, as the key interaction bind to a hydrogen atom on water, which acts a hydrogen bond donor.^{15,40}

The next sulfated derivative was based on the β -linked, *p*-methoxyphenyl derivative (**134**) with the sulfate group replacing the C6 hydroxyl. The parent compound (**134**) compound has been previously shown to reduce RBC hemolysis with reduced concentrations of glycerol.³⁹ The introduction of a sulfate group to the molecule should be well-tolerated biologically and may increase the IRI activity. Since this is a glucose derivative, it was theorized that the addition of a sulfate group at the C6 position should be tolerated, as it does not interrupt key hydrogen bond interactions, which was demonstrated by compound **336**. The parent compound (**134**) itself displays a high degree of IRI activity with percent MGS of 23 % (22

mM), and while the C6 sulfated derivative (**344**) shows some IRI activity, the percent MGS is lower at 38 %. The difference between the IRI activities of the parent compound and sulfated derivative is much less with this derivative and while compound **344** is less active than the parent compound, IRI activity has not been eliminated. A similar trend is observed with compound **350**, which has the C4 hydroxyl replaced with a sulfate group and the same β -linked, *p*-methoxyphenyl anomeric group. While the C4 hydroxyl of glucose participates in the key hydrogen bond in glucose as both a hydrogen bond donor and acceptor, eliminating the donating ability could be used to assess the importance of having a donor at that position. The IRI activity is further reduced in this compound compared to **134** and **344**, resulting in a percent MGS of 48 %. The further reduction could be in part due to removal of the hydrogen bond donating ability of the C4 position, which is partially responsible for preferential water binding.^{15,40} Since PMP Glc shows a high degree of IRI activity inherently, structural changes to the molecule are not well tolerated, which has been observed for other C6 modified PMP Glc derivatives.³⁴

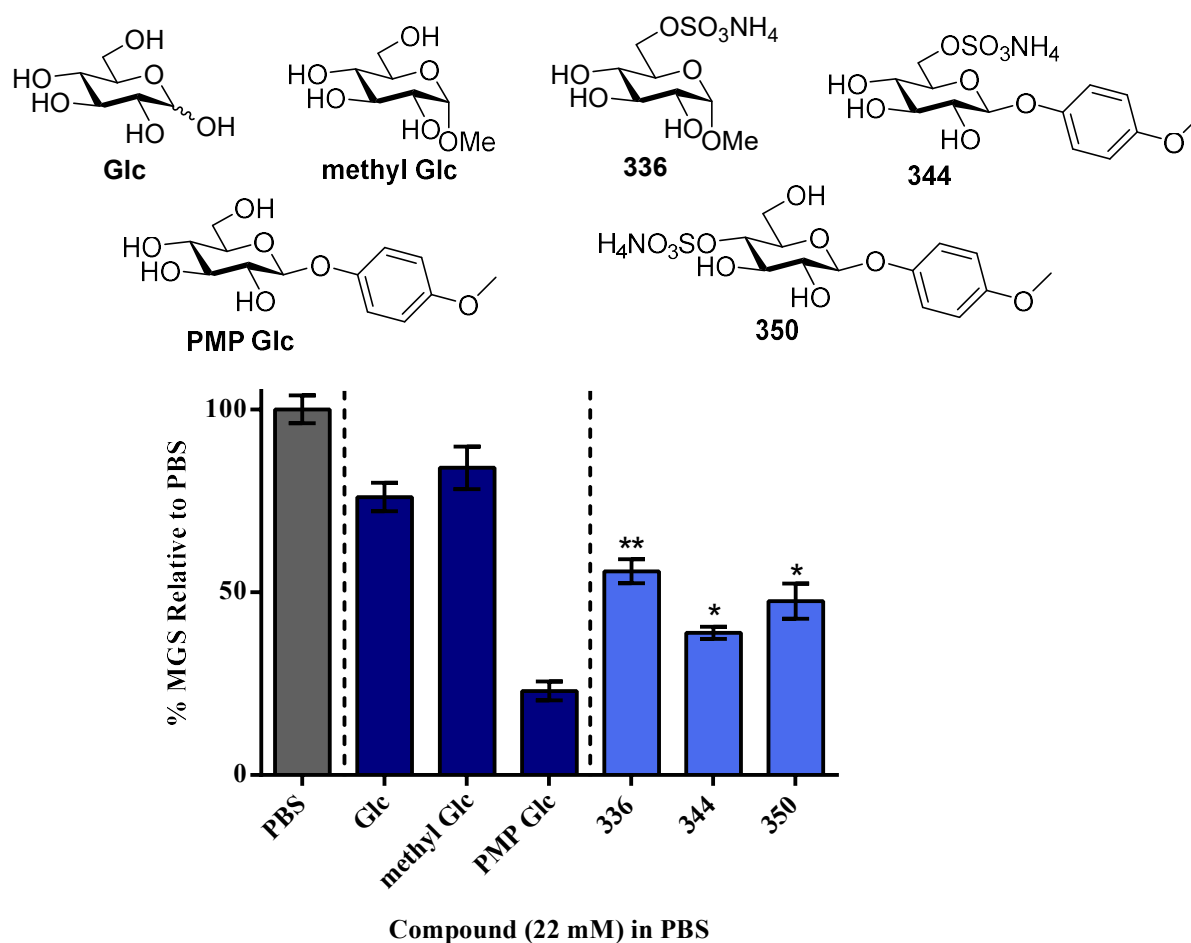


Figure 3-8. Structures of sulfated derivatives **336**, **344**, and **350** and corresponding IRI activity at 22 mM. %MGS shown relative to PBS negative control (100%) using the 30-minute drop assay. Error bars represent SEM, n=3. Asterisk represents a statistical significance between compound and control (PBS) generated by student's t-test (*, $p < 0.05$ and **, $p < 0.01$).

3.4 Dose-response Analysis of Compound 334

All previous IRI data shown was using the 30-minute splat cooling assay, where compounds were only assessed at 22 mM. While this assay is useful in comparing newly synthesized compounds to previous ones (all tested at 22 mM), testing at a single concentration greatly limits the scope of this assay. Testing over a range of concentrations is beneficial to see the whole IRI profile of a compound, while allowing dose-response measurements to be done.

To do this, a new kinetics based IRI assay was developed. This assay was essentially the same as the previously described assay (%MGS) but an incubation of only 5 minutes was used. Ideally, multiple concentrations spanning from no IRI activity ($v_{\text{norm}} = 100 = \text{PBS}$) to a concentration where all ice crystals are uniform and small ($v_{\text{norm}} = 0$) were used. The concentration value at half-maximal inhibition (IC_{50}) was obtained from a two-parameter sigmoidal curve that was fit to the data, with error bars representing SEM.⁴¹ While being able to compare IRI activity directly to previously tested compounds (at 22 mM) is convenient, it examines only a small part of a compounds IRI ability. Testing at multiple concentrations allows for much more data to be gathered about a specific compound, much of which could be missed otherwise. Previous testing also only calculates the average crystal size of the sample, which is problematic due to the nonhomogeneous nature of ice crystal size, especially at extended annealing times. During the annealing portion of the assay, ice recrystallization is promoted where large ice crystals become larger at the expense of smaller crystals. While not all of the small ice crystals are taken up by larger ones during this, they are not represented by the average ice crystal size. To resolve this, the newly developed assay took “bin” sizes into account, where all ice crystals present are separated into various bins according to their size to accurately depict the nonhomogeneous distribution of ice crystal size. The smallest bin size represents the ice crystal size at $t = 0$, which shows the maximum inhibition of ice recrystallization while the largest represents the average size of PBS (no IRI activity). Annealing time (at $-6.4\text{ }^{\circ}\text{C}$) of the ice wafer was assessed and reduced to 5 minutes (previously 30 minutes) which was sufficient to accurately obtain kinetic data.

Only the most active sulfated IRI (**344** – based on previous 30-minute drop assay) was analyzed using this new procedure. Below, **Figure 3-9** shows the IC_{50} curve for compound **344**.

Compound **344** displays a relatively low IC_{50} (4.7 mM) value compared to the 30-minute splat cooling assay at 22 mM (38% relative to PBS). This showcases the major disadvantage of the previous splat cooling assay, where some compounds may not seem as active at a single concentration. Comparing the IC_{50} of compound **344** to that of its parent compound PMP-Glc it is noteworthy that in the previous 30-minute assay, compound **344** performs slightly worse than PMP-Glc, but in the kinetic assay the IC_{50} value of 4.7 mM is much lower than that of PMP-Glc with an IC_{50} of 16 mM.^{42,43}

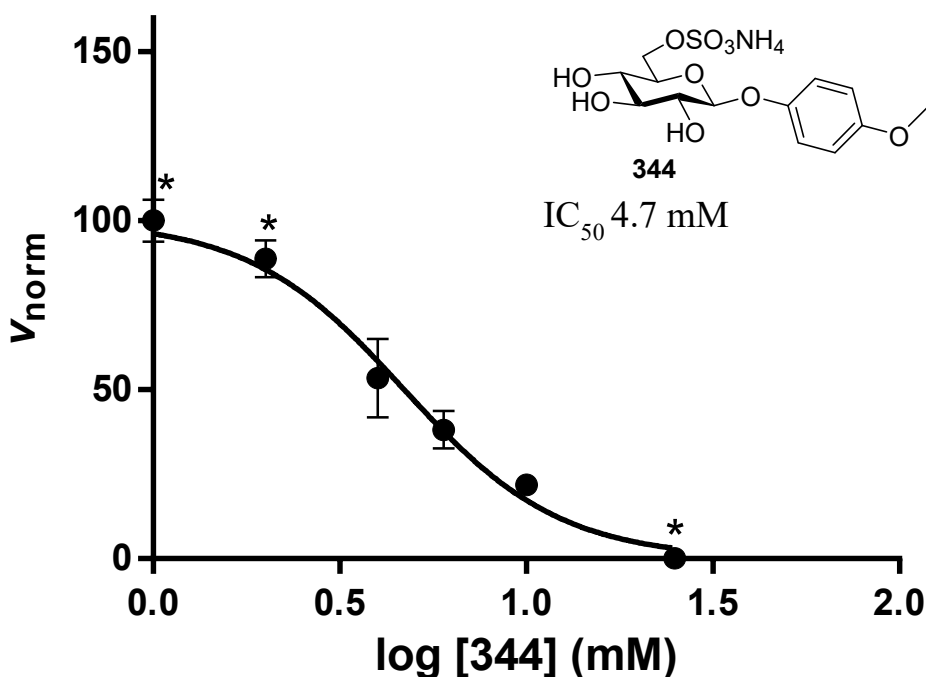


Figure 3-9. IC_{50} curve for compound **344** using 5-minute kinetic splat-cooling assay. $IC_{50} = 4.7$ mM for this compound, error bars represent SEM, $n = 3$. Asterisk represents a statistical significance between each compound and control (PBS) generated by a one-way ANOVA and Dunnett's post-test (*, $p > 0.05$).

3.5 Chapter Summary

In summary, the work described in this chapter details the addition of hydrophilic functional groups of sulfates to simple monosaccharides and the effect of this on IRI activity. Specifically, substituting the C6 hydroxyl with a sulfate group on a number of D-galactose and

C1 modified D-glucose derivatives, with compound **339** displaying the greatest IRI activity, although it is still less active at 22 mM compared to the parent compound. This is most likely due to modifications of PMP Glc being ineffective in increasing IRI activity. Sulfate groups were chosen due to their high hydration, ease of installation, and common occurrence in many naturally occurring carbohydrates. Previous structure-function work has suggested that hydration, as well as hydrogen bond accepting/donating ability may be important for IRI activity of carbohydrates. The addition of a sulfate group was used to further explore this theory. The synthesis of these sulfated carbohydrate derivatives was efficiently accomplished through the use of a hydroxyl group protection/deprotection strategy as well as selective benzylidene acetal openings. Many of these derivatives, while displaying IRI activity, were less active than their non-sulfated parent compounds. Compounds **329** and **336** were the only two derivatives to display greater IRI activity compared to their parent compounds, although were much less active than current IRIs. Due to this, the sulfation of carbohydrates, specifically pyranose rings, does not seem to be a beneficial modification to increase IRI activity.

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- (43) Briard, J. G. The Rational Design and Use of Novel Small-Molecule Ice Recrystallization Inhibitors for the Cryopreservation of Hematopoietic Stem Cells and Red Blood Cells, Ph.D. Dissertation, University of Ottawa 2016.

Chapter 4. Sulfated Gluconamide Derivatives as Ice Recrystallization Inhibitors

4.1 Introduction

Open-chain (alditol) carbohydrates exist in small amounts in the equilibrium between 6-membered ring (pyranose) and 5-membered ring (furanose) reducing sugars. These alditol conformations account for less than one percent of the equilibrium naturally (**Figure 4-1**).¹

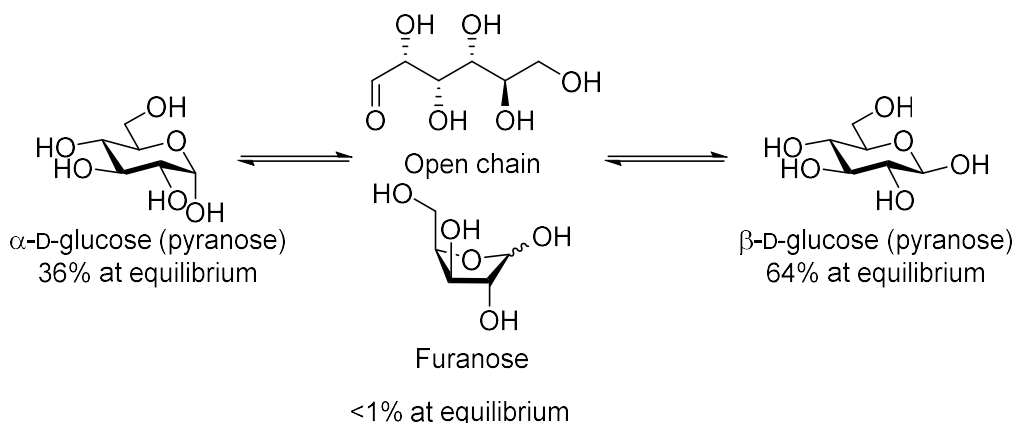


Figure 4-1. Equilibrium of D-glucose in water.

Due to the small percentage in equilibrium, ice recrystallization inhibition (IRI) activity of these molecules cannot be directly measured without modification, locking the molecules in their linear form. This is necessary to differentiate the IRI activity of different forms of carbohydrates. Since pyranose-form carbohydrate derivatives have been extensively studied, different forms may show different properties. One of the most important factors considered for new compounds developed by the Ben lab is the IRI activity of the compound. Little is known about the IRI activity of linear sugars, open-chain derivatives may possess similar IRI activities to non-linear pyranose derivatives. To lock these molecules in their linear (alditol) configuration, a substituted amine was used to prevent cyclization resulting in the aldonamide in solution (**Figure 4-2**). The linear structure of aldonamides allows for more freedom of movement which may further disrupt bulk water compared to their pyranose and furanose isomers and increase the IRI activity of

these molecules. The Ben lab has synthesized an extensive library of substituted alditols with various *N*-linked substituents (aldonamides) locking the molecules in a linear conformation.² To date we have prepared three classes of aldonamides (**Figure 4-2**) with varying degrees of IRI activity via a one step reaction.^{2,3} These reactions combine a substituted amine (alkyl-, aryl-, or benzylamine) with a carbohydrate-based lactone (gluconolactone, **Figure 4-2**) to generate the corresponding gluconamide. Due to the high degree of IRI activity observed in some of the previously synthesized extended alkyl chain pyranose derivatives (**123/124** – Chapter 1) alkylaldononamides were extensively explored.² Both *N*-octyl-D-gluconamide **144** and *N*-octyl-D-galactonamide **402** as well as their *N*-methyl analogues (**402** and **404** respectively) were assessed (**Figure 4-3**). Due to the insolubility of compounds **144/402** compared to their *N*-methylated analogues, all four derivatives could not be assessed at the same concentrations, with compounds **144/402** being

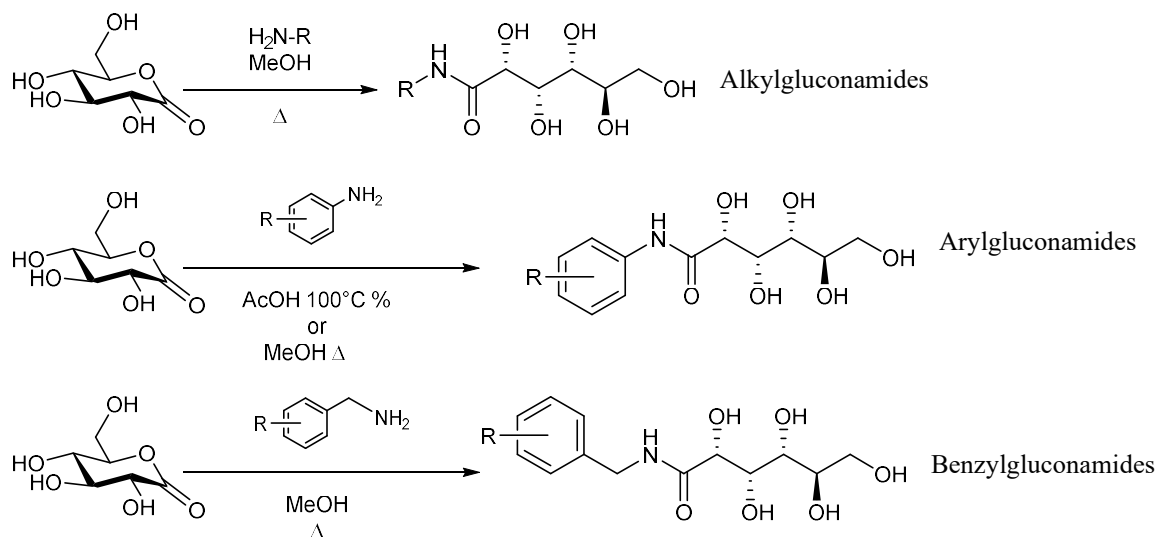


Figure 4-2. Three classes of aldonamides: alkylgluconamides, arylgluconamides, and benzylgluconamides.²

tested at 0.5 mM in PBS and the *N*-methylated analogues at 22 mM in PBS. Interestingly, compound **144** exhibited substantial IRI activity at the low concentration of 0.5 mM.³

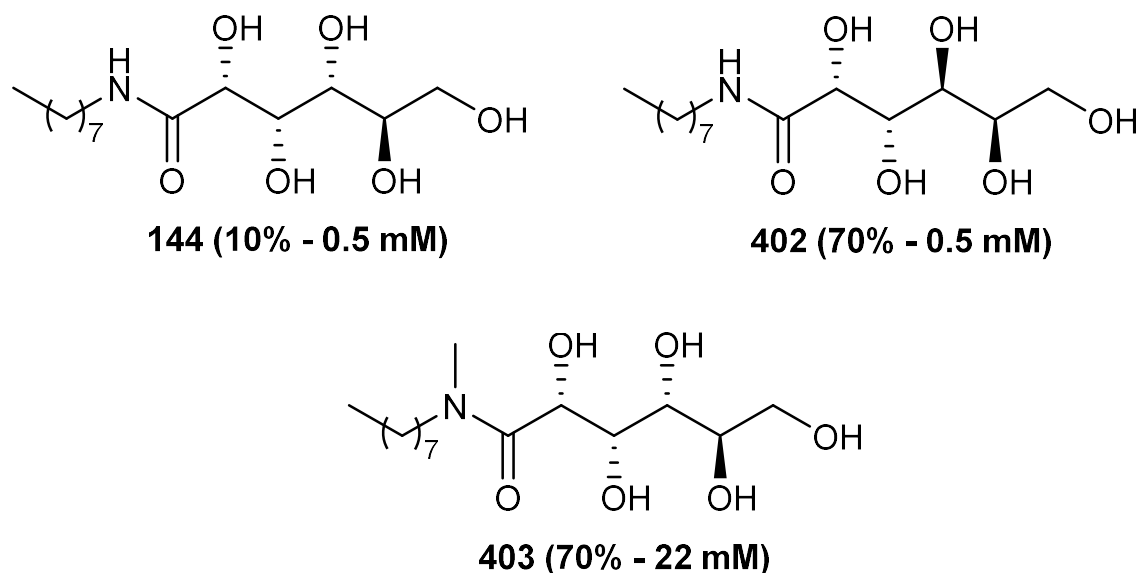


Figure 4-3. Structures of previously synthesized alkylaldonamides and corresponding IRI activities (% MGS compared to PBS, 30 min. drop) at specified concentrations.³

Neither compounds **402** nor **403** exhibited considerable (70% MGS for both compounds relative to PBS, 30 min. drop) IRI activity suggesting that the N-H bond of the amide is essential for IRI activity as well as slight differences in configuration can result in elimination of IRI activity. Ether-linked derivatives of compounds **144/402** were also synthesized also resulting in no considerable IRI activity, again suggesting that the N-H bond is essential for IRI activity in these derivatives. Shorter length alkyl chains were explored, although no discernible trend was observed.²⁻⁴

Although some of these derivatives display high IRI activity, their practical uses are limited because they possess surfactant-like properties, which eliminates their potential in biological systems. Surfactant-like compounds such as the above octylaldonamides, are able to

dissolve cell membranes, resulting in cell death. Due to this undesirable property, the alkyl chain was then replaced by a substituted phenyl rings, drawing inspiration from IRI-active *O*-linked phenyl glycoside derivatives that were previously synthesized by former graduate students in the Ben lab (Chantelle Capicciotti and Jennie Briard).^{2,5,6} These derivatives maintain the amphiphilic nature between the hydrophobic (phenyl ring) and hydrophilic (carbohydrate hydroxides) portions as previous molecules that has been shown is necessary for IRI activity.^{4,5,7} The phenyl ring contains a comparable number of carbon atoms while not possessing detergent-like properties due to the unsaturated phenyl ring. Allowing these derivatives to be more biologically compatible than their linear, unsaturated counterparts.

These derivatives were synthesized in similar fashion to the alkylaldonamides.^{2,6} Two major classes of gluconamides were explored: phenyl and benzylgluconamides (**Figure 4-4**).²

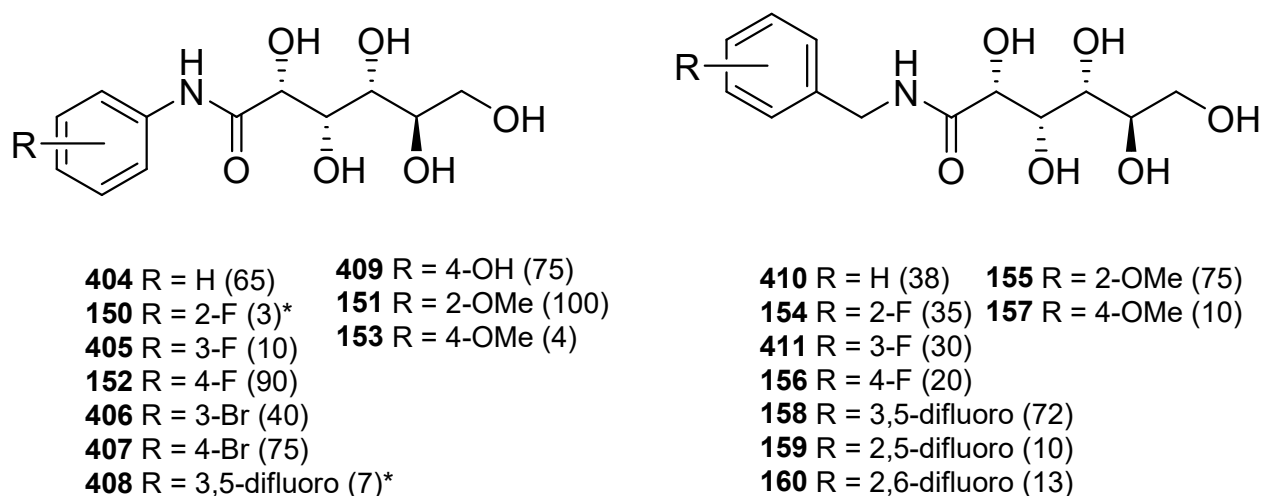


Figure 4-4. Structures of phenyl- and benzylgluconamides previously synthesized. % MGS at 22 mM relative to PBS control (100%) in parentheses. * Tested at 11 mM due to low solubility.²

Unsubstituted aniline **404** did not display a high degree of IRI activity (65% relative to PBS – 30 min drop), almost identical unsubstituted pyranose derivative **126** (introduction) (66

and 64% respectively – 30 min drop).^{3,8} Interestingly, by replacing one hydrogen atom with a halogen at various positions along the phenyl ring, IRI activity can be significantly modulated. While *para*-fluoro **152** derivative is less active than the phenyl gluconamide (90%), by moving the fluorine atom to the *ortho*- or *meta*-position (**150** and **405** respectively) the IRI activity drastically increases to 3 and 10%, respectively. The difluorinated analogue **408** retained the IRI activity the other compounds (7%), while replacing fluorine in the *meta*- or *para*-position with bromine decreases the IRI activity (40 and 70% respectively). Next, the halogen atom was substituted with either a hydroxyl or methoxy substituent. Both the *ortho*-methoxy (100%) and *para*-hydroxyl derivatives were inactive (75%); surprisingly, the *para*-methoxy was highly active (4%). This shows the opposite trend as with fluorine substitution where the *ortho*-fluoro derivative was much more active than the *para*-fluoro compound.² This suggested that electron withdrawing groups appear to enhance IRI activity in the *ortho*- and *meta*-positions, while electron donating groups are better tolerated in the *para*-position.

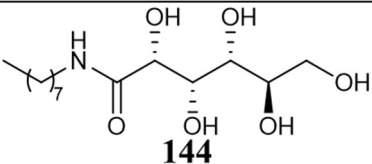
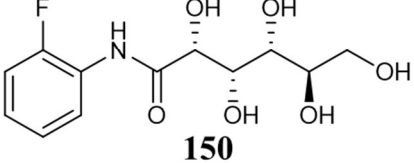
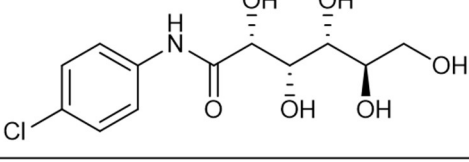
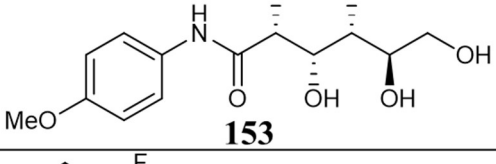
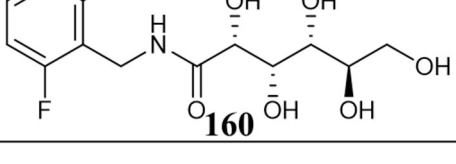
Since no clear trends with phenyl ring substitution was observed with the phenylgluconamides, benzylgluconamides were explored next. As with the phenylgluconamides, hydrogen atoms were replaced with either fluorine or a methoxy substituent. Overall, the benzylgluconamides displayed a moderate degree of IRI activity, only three showed comparable activity to the most active phenylgluconamides; both the 2,6- (**160** – 13%) and 2,5-difluoro (**159** – 10%) as well as the *para*-methoxy (**157** – 10%) analogues. With the erratic nature of substitution type/position and IRI activity, no apparent trends can be obtained, although single fluorine substitutions do not seem to be tolerated well and having a fluorine in the *para*-position for difluorinated analogues is necessary for IRI activity.

Although a number of phenyl and benzylgluconamides possess a high degree of IRI activity (%MGS and $IC_{50} < 20$ mM) and show some promise as biological cryoprotectants,^{2,9,10} one major disadvantage to these molecules are their solubility in aqueous systems. **Table 4-1** displays the solubility of the most IRI active gluconamides, many of which are below 20 mM.^{2,6} Higher solubilities could allow for higher concentrations of IRI to be used without the need for extensive heating.

While the gluconamide concentrations currently used (10-20 mM) are effective to inhibit ice recrystallization, testing at higher concentrations is impossible in some solutions due to low solubility of the IRIs. Furthermore, in order to dissolve many of these compounds at the maximum concentration, extensive heating of the solution is required (>80 °C). This is particularly a problem with the cryopreservation of hematopoietic stem/progenitor cells (HSPCs) with compounds **150**, **153**, and **160**. Compounds **150/160** have solubilities in water of 15 and 11 mM, respectively, while **153** is soluble up to 55 mM. Even in the case of using **153**, the cryosolutions must be extensively heated to fully dissolve the compound prior to cryopreservation.^{10,11} Precipitation of the IRI during the slow cooling (1 °C/min to -80 °C) is another potential disadvantage of using these IRIs near their solubility limit. As with the cryopreservation of HSPCs, if these samples are to be used in the cryopreservation of other biological materials, they must be fully dissolved in the cryosolution used to freeze the sample. This can be done with heating in some cases, although many cryosolutions contain components that are unstable or can degrade at the temperatures (>50 °C) needed to dissolve the cryoprotectant. In order to allow for additional testing at higher concentrations or in heat-sensitive cryosolutions, the solubility in aqueous systems needs to be increased. To accomplish

this, highly soluble functional groups can be installed on the gluconamides to increase solubility while ideally retaining the IRI activity of the parent compounds.

Table 4-1. Solubilities of IRI active gluconamides. ²

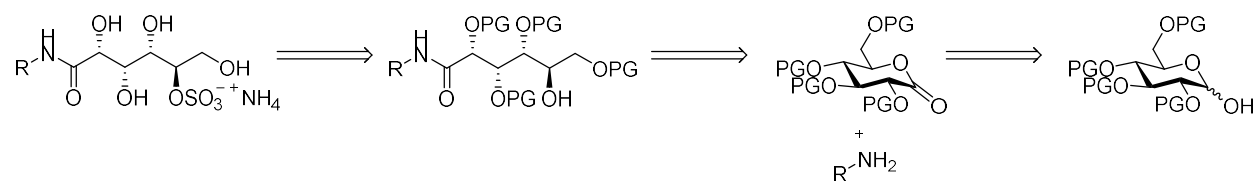
Compound	Solubility in H ₂ O (mM)	IC ₅₀ (mM)
 144	0.5	0.1
 150	15	4
 153	20	12
 153	55	5
 160	11	11

4.2 Synthesis of Sulfated Gluconamides as Ice Recrystallization Inhibitors

As previously discussed, one way to increase the solubility of gluconamides in aqueous media, is to incorporate highly water-soluble groups. Sulfate groups are able to bind multiple water molecules via a hydrogen bond network. This should drastically increase the solubility of these molecules, while ideally retaining the high degree of IRI displayed by the parent

compounds. Consequently, sulfate groups were chosen due to their high hydration and their ease of installation on carbohydrate derivatives.^{12,13} Sulfate groups are also present on a number of highly soluble bioactive compounds of both natural and synthetic origins.

While sulfate groups can be installed on various hydroxyl groups on the gluconamide chain or on the phenyl ring itself, the C5 hydroxyl located on the hydrophilic gluconamide chain was chosen due to the ease of synthesis (**Scheme 4-1**). In a similar lactone opening reaction to the unsubstituted aldonamide derivatives, a perbenzylated gluconolactone (**424**) can be used. The ring-opening leaves the C5 hydroxyl unprotected (**425**) and easily modified.



Scheme 4-1. Retrosynthesis of C5 sulfated aldonamide derivatives.

Consistent with the retrosynthetic analysis (**Scheme 4-1**), 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose was chosen as the starting material possessing an unprotected anomeric hydroxyl group, which was easily oxidized via an Albright-Goldman oxidation¹⁴ to generate the perbenzylated lactone derivative **424** in near quantitative yield. The crude product was then subjected to ring-opening conditions with either an alkyl- or benzylamine

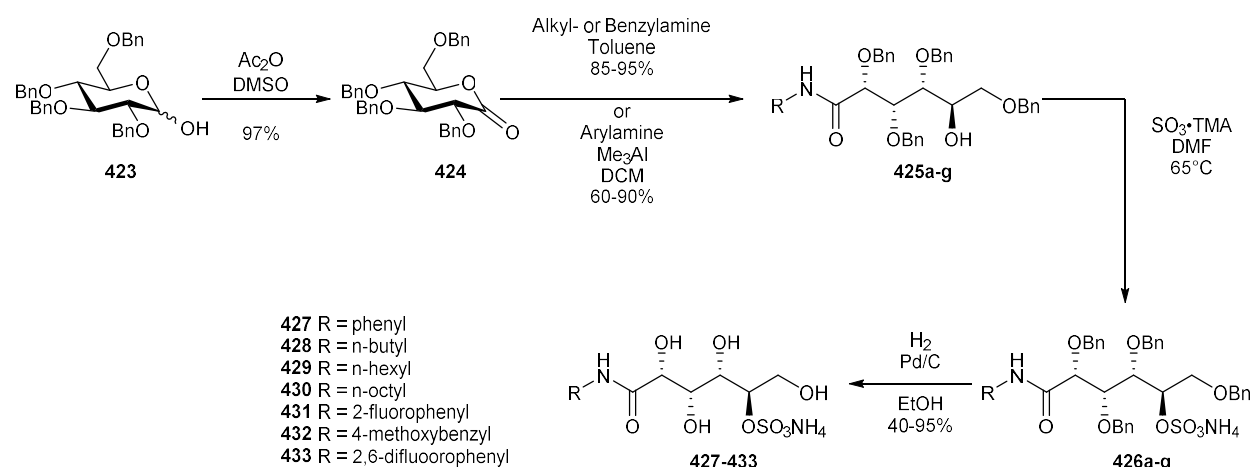


Figure 4-5. General synthetic route for C5 sulfated gluconamides.

in toluene or an aniline with trimethylaluminum in DCM^{3,15,16} due to their less reactive nature. This reaction conveniently leaves the C5 hydroxyl group unprotected **425** and able to be modified in the following reaction. The sulfate group was added using a sulfur trioxide trimethylamine complex in DMF at 65 °C, upon workup with ammonium hydroxide, the free sulfate **426** was produced as an ammonium salt. Finally, the 4 benzyl protecting groups were removed via hydrogenolysis with palladium on carbon in ethanol in good yield, affording the final product **427-433**. One problem encountered was during the synthesis of the 4-chloroaniline substituted aldonamide. During the final hydrogenolysis on **426f** with Pd/C the chlorine atom was also cleaved, resulting in a low yield due to indeterminate by-products. The resulting C5 sulfated aldonamide **427** was isolated and used for further testing.

The first three derivatives synthesized contained alkyl chains of varying length as a proof-of-concept investigation. Compound **430**, with an alkyl chain length of 8 carbons was

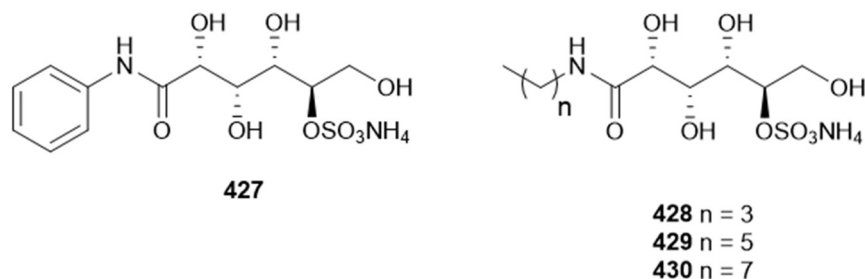


Figure 4-6. Synthesized **C5** sulfated gluconamides.

synthesized to compare to *N*-octylgluconamide **401** due to its IRI activity and very low solubility in aqueous systems. As anticipated, the addition of the sulfate group drastically increased solubility compared to the parent compound (0.5 mM and >100 mM respectively – **Table 4-2**). This same trend with increased solubility remained with the other two derivatives, confirming the theory that IRI solubility can be drastically increased with the addition of a highly polar functional group.

The next four compounds synthesized were based off the four most active phenyl- and benzylgluconamides previously synthesized in the Ben lab: phenylgluconamide (**427**), 2-fluorophenylgluconamide (**431**), 4-methoxybenzylgluconamide (**432**), and 2,6-difluorobenzylgluconamide (**433**). While some of these parent compounds were more soluble than the alkylgluconamides, many were still sparsely soluble in aqueous systems (15-65 mM in water with required heating >80 °C – **Table 4-3**). In all 4 compounds synthesized, the addition of a sulfate group at C5 increased the solubility of the compound compared to the parent compounds.

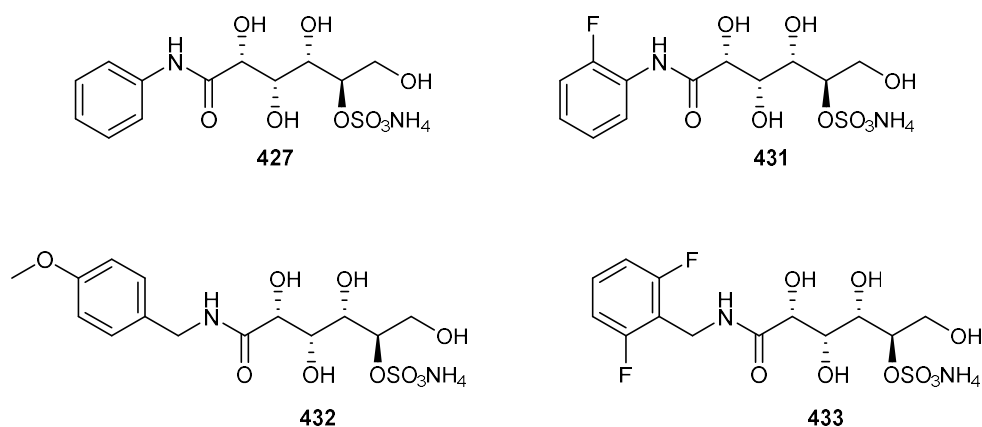


Figure 4-7. C5 sulfated phenyl- and benzylgluconamides synthesized, all showing solubilities over 100 mM in water.

Despite the fact that the addition of a sulfate group drastically increases the gluconamides solubility, not all sulfated compounds show promise as next-generation, more soluble IRIs. This is due to inherent, unwanted attributes displayed by some. The addition of a sulfate group to the alkylgluconamides produces detergent-like properties, capable of solubilizing cell membranes.

4.3 IRI Activity of Sulfated Gluconamides

Once the gluconamide derivatives were synthesized, testing for IRI activity was assessed using the method previously described (Chapter 3 – 5 min drop) in order to obtain the IC_{50} value for each parent compound and sulfated derivative. **Table 4-2** shows the IC_{50} values and solubilities for the sulfated derivatives and their parent compounds. With the addition of a sulfate group, the compound's solubility was drastically increased, generally at the cost of a reduction in IRI activity. The *N*-Octyl derivative **428** shows the greatest reduction in IRI activity, with an IC_{50} of 17 mM compared to the parent compound with an IC_{50} of 0.1 mM. While this is a large reduction over the parent compound **144**, an IC_{50} of 17 mM is comparable to those of previously synthesized gluconamides. This compound also shows the greatest increase in solubility (>100 mM compared to 0.5 mM for the parent compound), showcasing the ability of sulfate groups to

increase solubility. As previously mentioned, due to the long alkyl chain coupled with a polar sulfate, the compound shows detergent-like properties. Next, the *N*-hexyl derivative results in a slight increase in IRI activity ($IC_{50} = 4 \text{ mM}$ vs 7 mM for the parent compound) and remained much more soluble than its parent compound (**Table 4-2**). The unmodified *N*-butyl compound was the most soluble of the parent gluconamides due to its shorter alkyl chain though the addition of the sulfate group still increased the solubility. Although an IC_{50} value was not calculated for the parent compound, it does not display a high degree of IRI activity (MGS = 95% at 22 mM relative to PBS – 30 min. drop assay), ^{2,4} therefore the IRI activity is increased in the sulfated derivative.

Table 4-2. Solubilities and IC_{50} values of C5 sulfated alkylgluconamides in PBS. ²

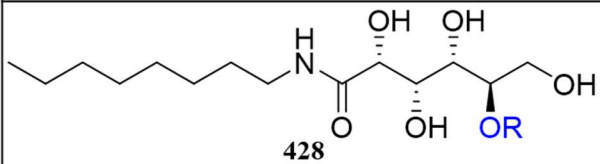
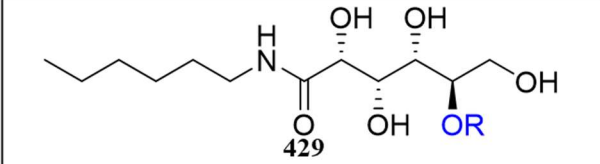
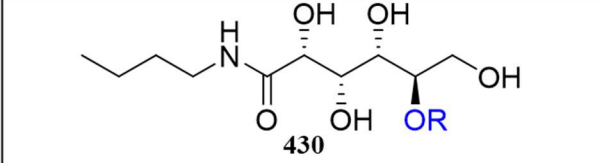
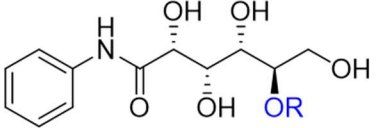
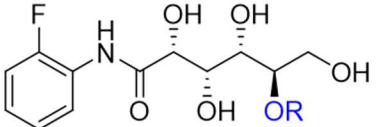
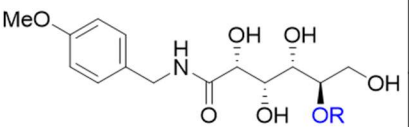
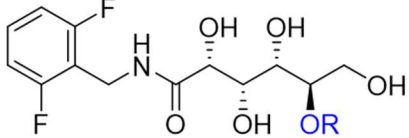
Compound	R	IC_{50} (mM)	Solubility (mM)
	H	0.1	0.5
	$-SO_3NH_4$	17	>100
	H	7	45
	$-SO_3NH_4$	4	>100
	H	N/A	75
	$-SO_3NH_4$	5	>100

Table 4-3. Solubilities and IC₅₀ values of C5 sulfated phenyl- and benzylgluconamides in PBS. ²

Compound	R	IC ₅₀ (mM)	Solubility (mM)
 427	H	N/A	65
	-SO ₃ NH ₄	5	>100
 431	H	4	15
	-SO ₃ NH ₄	38	>100
 432	H	5	55
	-SO ₃ NH ₄	42	>100
 433	H	11	55
	-SO ₃ NH ₄	70	>100

Next, the IRI activity and solubility for the phenyl and benzyl gluconamide compounds are shown in **Table 4-3**. With the addition of the sulfate, the solubility in aqueous media was increased in some cases drastically. With the exception of compounds **430** and **427**, the IRI activity was decreased in the sulfated gluconamides when compared to the parent compound. While a decrease in IRI activity is generally regarded as a disadvantage, the drastic increase in solubility is a particularly attractive property for biological IRIs. For this reason, select compounds were chosen for the cryopreservation of RBCs. Initially sulfated gluconamides **428** and **431** were chosen for this study. At the time of testing, the most IRI active sulfated alditol was compound **428**, and was selected for testing. The next most active compound was **431**, and since the parent compound was previously assayed for the cryopreservation of RBCs ^{2,9} and

HSPCs¹⁰, compound **431** was selected in hopes of showing similar properties. To compare the ability of these compounds to other ionic IRIs, compounds **339** and **345** were also tested alongside **428/431** (**Figure 4-7**). These two molecules are sulfated pyranose derivatives based off of PMP-Glc (**333**), which has shown previous success as an IRI with reduced glycerol concentrations.^{6,17}

4.4 Red blood Cell (RBC) Cryopreservation

Due to the high demand for blood products, a steady supply must be accessible at any given time. While RBC units can be clinically preserved hypothermically at 1-10 °C without cryoprotectant, this method of storage is limited to 42 days.¹⁸⁻²¹ This can lead to blood shortages in times of high demand in the event of natural disasters or for patients who experience an extensive loss of blood as the result of surgeries or traumas.^{22,23} In order to store RBC units for longer periods of time, they must be cryopreserved.^{18,20,24-26} Cryopreservation of RBCs typically uses glycerol in 40% v/v (most countries other than Europe) with a slow cooling rate of -1 °C coupled with storage at -80 °C.^{18,25,27} In some parts of Europe, 20% v/v glycerol is used coupled with a fast cooling rate (>50 °C/min) using liquid nitrogen for the cooling process as well as storage.¹⁷ The major drawback of this process is the need for liquid nitrogen, which could be costly for large-scale cryopreservation as well as problems with transportation. One major disadvantage to both procedures is the use of glycerol as a cryoprotectant.^{9,28} In order to transfuse a cryopreserved unit, the glycerol must be removed to <1% post-thaw to prevent intravascular hemolysis.^{17,26} This is a complicated procedure that can take up to one hour (40% glycerol) and can result in damage to the cells and a reduced volume post-deglycerolization.^{9,26} Deglycerolized units must also be used within 24 hours unless preformed in a closed system,²⁹

complicating the distance/transportation process. Currently, the cryopreservation of RBCs is limited to autologous RBC storage, rare blood types, and military applications.²³

To make cryopreservation of RBCs available for more practical applications such as the routine use of cryopreserved RBC units, the deglycerolization process must be reduced. This will allow RBC units to be readily thawed and transfused. The easiest way to achieve this is to reduce the amount of glycerol used for cryopreservation.^{9,26} Using regular slow cooling rates (1 °C/min to -80 °C) used in clinical settings with 40 % glycerol, decreasing the glycerol concentration results in damage to the cells and low post-thaw recovery.^{17,24,30} To combat this, an additional cryoprotectant or cryoprotectants are needed to retain the post-thaw recovery observed in higher glycerol samples. The Ben lab has previously synthesized numerous non-toxic IRIs that have been shown to be beneficial supplements to RBCs frozen with 15% v/v glycerol. Specifically, compounds (**333** and **346**, **Figure 4-8**) have been shown to increase the post-thaw recovery of RBCs to levels nearing the amount recovered with 40% glycerol^{9,31,32}. Using these compounds with 15% glycerol decreases deglycerolization times to near 10 minutes, which could allow for more widespread use in clinical applications.^{2,6,17}

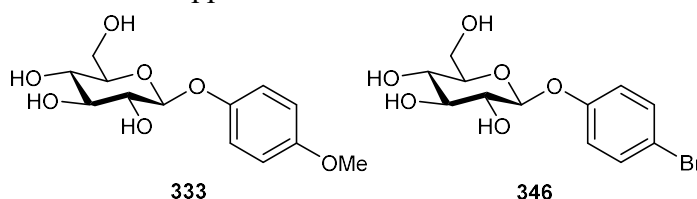


Figure 4-8. Phenolic glycoside IRIs used for the cryopreservation of RBCs.^{2,6,17}

Section 4.4.1 Sulfated Gluconamides as Cryoprotectants for RBCs

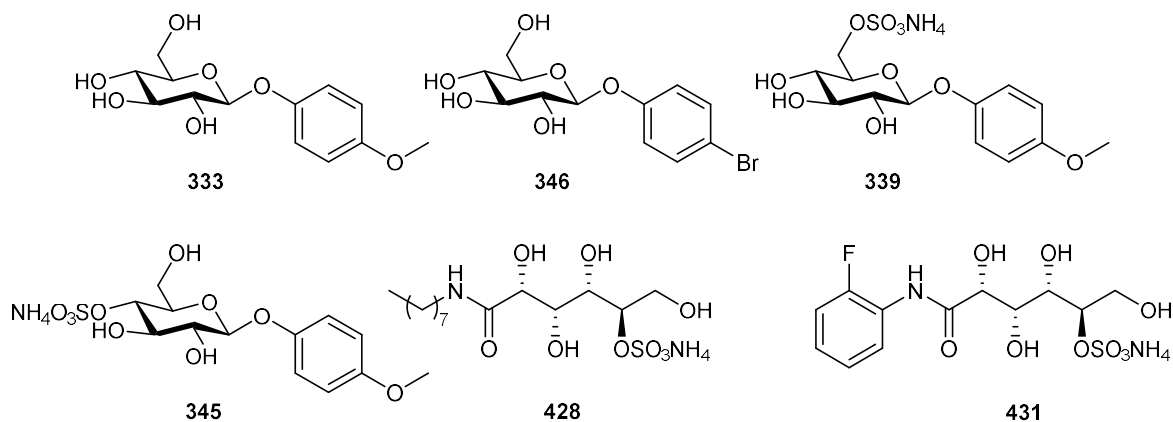
The similar IRI activity to previously synthesized compounds and high solubility of the sulfated aldonamide derivatives (**Table 4-2** and **4-3**) showed their potential for use in the cryopreservation of biological samples. Preliminary screening of compounds **428** and **431** was

conducted to assess their ability to improve the cryopreservation of RBCs in the presence of a lowered concentration of glycerol. This compound screen also included two sulfated pyranose derivatives (**339** and **345**) to compare cryopreservation ability between sulfated derivatives.

The preliminary screen uses 15% glycerol instead of the more common 40% glycerol in attempts to reduce the deglycerolization time post-thaw in order to be able to transfuse RBC units faster than is currently possible. This method was developed by the Acker/Ben labs to quickly screen new IRIs as cryoprotectants for RBCs. The compounds of interest were dissolved in 30% glycerol/saline/dextrose as a 2x solution before being mixed with the RBC samples in equal volumes as the cryosolution, ultimately resulting in 15% total glycerol and the concentration of interest of the IRI. Samples were incubated at room temperature for 10 minutes before they were cooled to -5°C (MeOH bath) and held for an additional 5 minutes at which point ice was nucleated via chilled forceps. Ice nucleation is induced to ensure all samples nucleate at the same temperature. Two different cooling methods were explored: cooling at 1 °C/minute to -40 °C or a dry ice dump (approx. -80 °C). The samples frozen by either method were thawed immediately after freezing by incubating in a water bath (37 °C) for 1-2 minutes after which the assessment of hemolysis was conducted.

Compounds that reduce RBC hemolysis post-thaw compared to the 15% glycerol control were considered to improve the cryopreservation of RBCs. The data of the concentration screen using the dry ice dump freezing method are summarized below (**Figure 4-9**). This freezing method is explored due to its similarity to the 20% glycerol fast-freeze procedure used in some parts of Europe.²⁴ Also, because this procedure is optimized for lower glycerol concentrations, increased hemolysis (lower % RBC intact) with compounds can be attributed to compound toxicity. **Figure 4-9** illustrates the amount of RBC hemolysis as % RBCs intact (100 –

hemolyzed RBCs) post-thaw. All compounds were dissolved in 15% glycerol and used as a 1:1 ratio of RBC sample:cryosolution. While 15% glycerol preforms similarly to the C1 substituted phenyl pyranose derivatives **333** and **346**, sulfated pyranose derivatives **345** and **339** perform poorly, resulting in moderate degrees of RBC hemolysis over the concentrations explored. This may be due to the lower IRI activity (38 and 48% respectively) compared to the parent compound (23%), resulting in slightly higher amount of ice recrystallization during freezing/thawing. Toxicity may also be an issue with these compounds, although LD₅₀ values were not calculated. This is apparent with compound **345**, which at 55 mM displays similar hemolysis to compound **339** (at 110 mM), although at 110 mM compound **345** displays more hemolysis than the lower concentration.



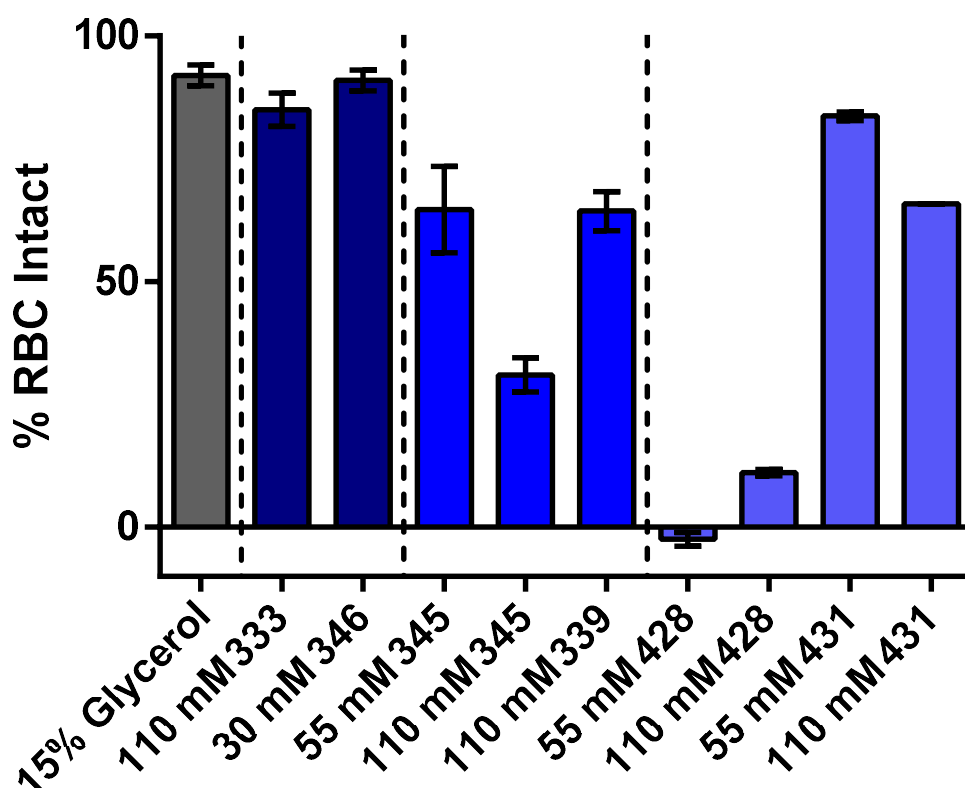


Figure 4-9. RBC recovery post-thaw following rapid freezing (1 °C/min) to -80 °C via dry ice dump. All compounds tested dissolved in 15% v/v glycerol. Error bars represent SEM, n = 2.

Sulfated octyl-substituted derivative **428** resulted in complete hemolysis of the sample as a result of the compound itself. Detergent-like properties are experienced with the long alkyl chain coupled with a polar sulfate group on the other end of the molecule, causing RBC membranes to lyse. While derivative **431** (55 mM) displayed %RBC intact values comparable to **333** and **346**, the length of the synthesis of the compound compared to the other compounds makes it less desirable as a cryoprotectant. Moreover, at higher concentrations (110 mM) **431** results in increased hemolysis compared to the lower concentration. Increased hemolysis at the higher concentration suggests compound toxicity. Toxicity studies were not performed for this compound, and therefore the hemolysis mechanism cannot be determined. This compound may

be capable of solubilizing RBC membranes at higher concentrations, although there is no alkyl chain present. Further studies would need to be performed to assess the toxicity of this compound.

While this fast-cooling procedure coupled with 15% glycerol can prevent most hemolysis without any IRI, this type of procedure is difficult on large-scale applications, and requires the use of special equipment.²⁶ The addition of an IRI does not improve percent intact RBCs and is not useful in this type of freezing procedure, although the hemolysis experienced with most compounds is a result of compound toxicity.

In order to determine whether or not the IRIs are able to prevent hemolysis when freezing RBCs with reduced glycerol concentrations, 15% glycerol coupled with slow cooling rates can be used (-1 °C/min to -40 °C). Typically, these slow cooling rates require 40% glycerol to prevent hemolysis due to toxic effects as a result of a buildup of extracellular solutes and subsequent dehydration during freezing.^{18,33,34} Lowering the glycerol concentration under these conditions will result in increased hemolysis, which will ideally be mitigated with the addition of an IRI. As previously mentioned, the focus of these experiments was to reduce the glycerol concentration needed for cryopreservation and subsequent deglycerolization times. For this reason, 15% glycerol and slow freezing rates were used.

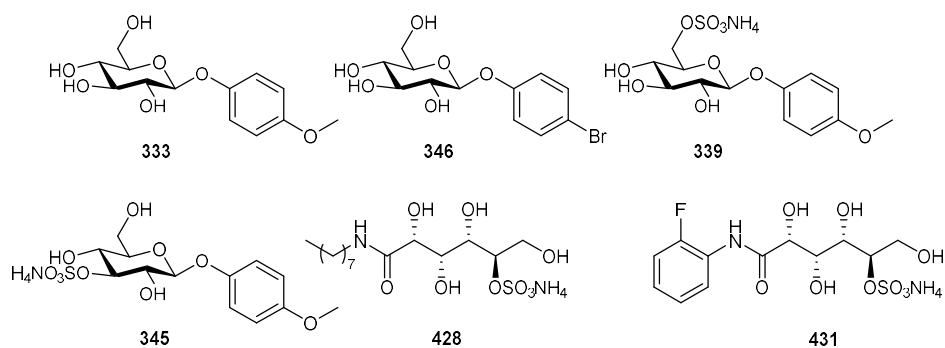
Using the slow-freeze method with reduced concentrations of glycerol, increased levels of hemolysis are expected due to the suboptimal concentration of glycerol used. Since glycerol can internalize within RBCs, it replaces water during the freezing process. With a suboptimal glycerol concentration, an insufficient amount of water may be displaced, causing intracellular ice formation. Adding a charged IRI could help draw water out of the cell and prevent intracellular ice formation and subsequent recrystallization.

While all the compounds tested show a greater degree of hemolysis than 40% glycerol, which is the clinical standard in North America and much of Europe, the deglycerolization time needed for this is often over one-hour post-thaw. In order to reduce the deglycerolization times, the concentration of glycerol can be reduced, these trials show the compound's ability to prevent hemolysis compared to the reduced 15% glycerol control. Many of the IRIs tested show some improvement over this glycerol control suggesting that the IRIs are preventing hemolysis. Compound **428** shows virtually no intact RBCs, although that is due to membrane solubilization from the compound as a result of it acting as a detergent. This compound was understandably eliminated as a cryoprotectant due to the hemolytic activity.

With a percent RBC intact values of greater than 60%, compounds **346**, **339**, and **431** all show an improvement over the 15% glycerol control (~40% intact). Interestingly, compound **431** shows a decrease in hemolysis at higher (110 mM – 69% intact) concentrations compared to the lower (55 mM – 36% intact) concentration. The decrease in hemolysis with 110 mM **431** over 55 mM indicates some level of protection from the compound at higher concentrations. The compound may be offering membrane stabilization during the freezing/thawing of the RBC units, although further studies would need to be conducted to confirm this hypothesis. The compound may also be assisting in water diffusion out of the cell during the freezing process, which is needed with low concentrations of glycerol. Using the rapid freezing procedure, 55 mM **431** showed less hemolysis compared to 110 mM, which suggested potential toxicity with the higher concentration. This is not the case for the slow-cooling procedure, unlike the rapid freeze procedure, 110 mM **431** shows an increase in intact RBCs compared to 55 mM (69% vs. 36% intact). The decreased intact RBC value for the rapid freeze trial was attributed to toxicity, as both 55 and 110 mM are lower than the glycerol control. This shows they offer no additional

protecting during this procedure over 15% glycerol. During the slow-cooling procedure, 55 mM shows almost identical percent intact values to 15% glycerol (37%), while 110 mM was higher at 69%. This shows an improvement over compound **333** and is almost identical to **346**. The increase in percent intact RBCs is most likely due to protection during freezing/thawing by compound **413**. If there is toxicity to RBCs associated with this compound (as seen in the rapid freeze trials), the protection during cryopreservation outweighs the toxicity with a slow-cooling rate.

Although these values are lower than optimized cooling procedure (40% glycerol, slow freeze – **Figure 4-10**) there is precedence for using sulfated IRIs along with reduced glycerol concentrations to cryopreserve RBCs. Compound **431** was able to reduce hemolysis in slow freezing conditions with 15% glycerol, with intact RBC values on par with current IRIs used for RBC cryopreservation (**346**). By modulating the substituents present, as well as the substitution pattern on the phenyl ring could better protect RBCs during cryopreservation. Having the sulfate group on the C5 position of the gluconamide shows potential, although the sulfate could be moved to other positions on the molecule. This being said, further optimization and testing of compounds and concentrations are required to maximize protection of RBCs during cryopreservation.



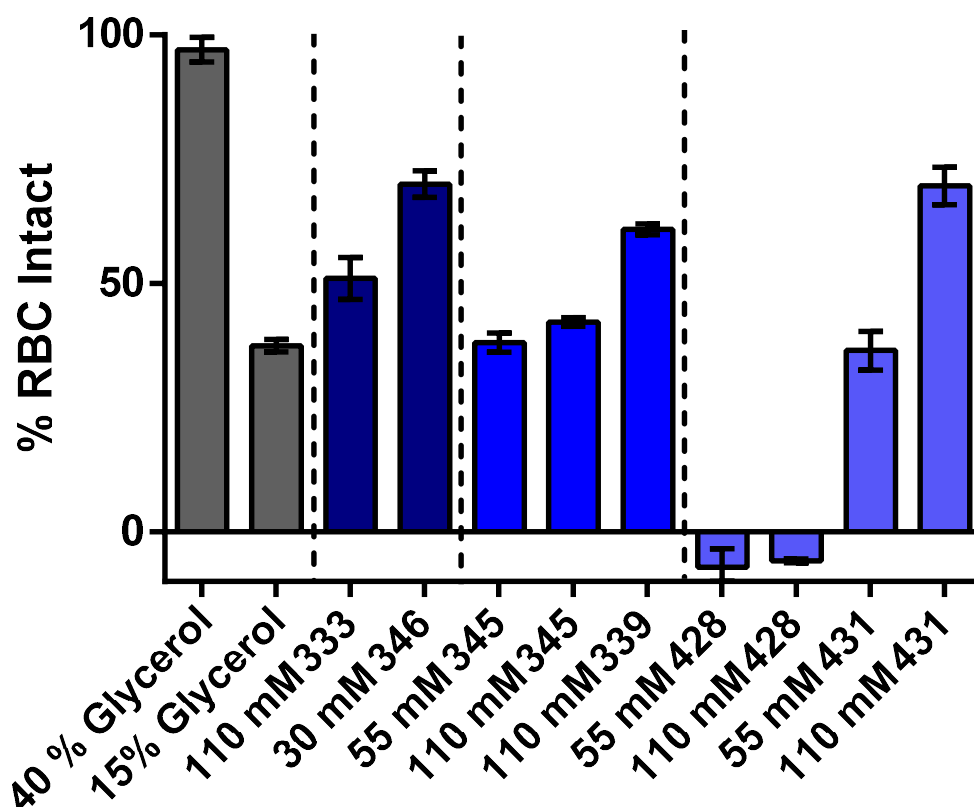


Figure 4-10. RBC recovery post-thaw following slow freezing (1 °C/min) to -40 °C via rate-controlled freeze. All compounds tested dissolved in 15% v/v glycerol. Error bars represent SEM n = 2.

4.5 Chapter Summary

One major problem encountered with some of the Ben lab IRIs is low solubility in aqueous media. If these IRIs are to be used in biological systems, solubility in aqueous solutions is imperative. Many of the previously synthesized alditol compounds are sparsely soluble in water, with solubilities often below 20 mM.² In order to be able to assess the compounds at higher concentrations, the solubilities must be increased by some means.

A simple way to increase the solubility of these compounds was to introduce highly polar functional groups onto the molecule itself. Sulfate groups were chosen for their ability to easily

be installed on carbohydrate derivatives. With the addition of a sulfate group, the solubility of a number of alditol derivatives was able to be drastically increased, although a loss of IRI activity was also observed in many of the sulfated derivatives. While the sulfated derivatives do not retain the IRI activity of the parent compounds, due to their solubility they have potential as IRIs in biological systems. Using a fast cool, low glycerol procedure, the sulfated derivatives do not protect the sample compared to the 15% glycerol control, although fast cooling is optimized to use low concentrations of glycerol. On the other hand, slow cooling ($-1^{\circ}\text{C}/\text{min}$) uses higher (40%) glycerol concentrations in order to minimize hemolysis. Using lower concentrations of glycerol results in large amounts of hemolysis. Some of this hemolysis can be prevented using IRIs, specifically compound **431** (110 mM). Using this compound along with 15% glycerol, the percent RBCs post-thaw was increased to nearly 70%. While this was lower than trials using optimized concentrations of glycerol, further optimization could yield better post-thaw recoveries of RBCs. Using lower concentrations of glycerol supplemented with an IRI greatly reduces the extended post-thaw deglycerolization time, resulting in shorter times between thawing and transfusion of an RBC unit.

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Chapter 5. Aminogluconamide Derivatives as IRIs

5.1 Introduction

While the Ben lab has focused recently on anionic small-molecule IRIs, few cationic derivatives have been previously synthesized. These compounds feature positively charged nitrogen atoms as either primary or quaternary amines.¹⁻⁵ With the partial success of the negatively charged sulfated pyranose and alditol derivatives (**Figure 5-1**), the next target chosen were cationic alditol derivatives. Similarly, to the previously synthesized compounds, nitrogen can easily be installed and modified to generate cationic alditol IRIs.

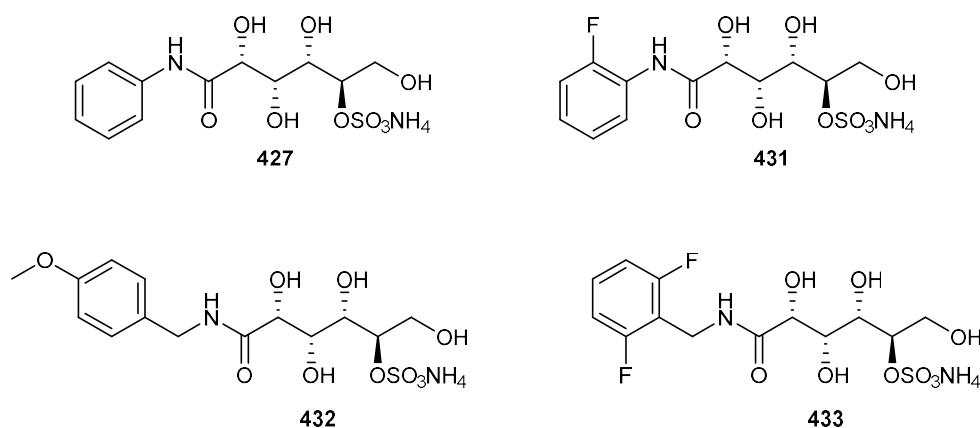


Figure 5-1. C5 sulfated phenyl- and benzylgluconamides synthesized, all showing solubilities over 100 mM in water.

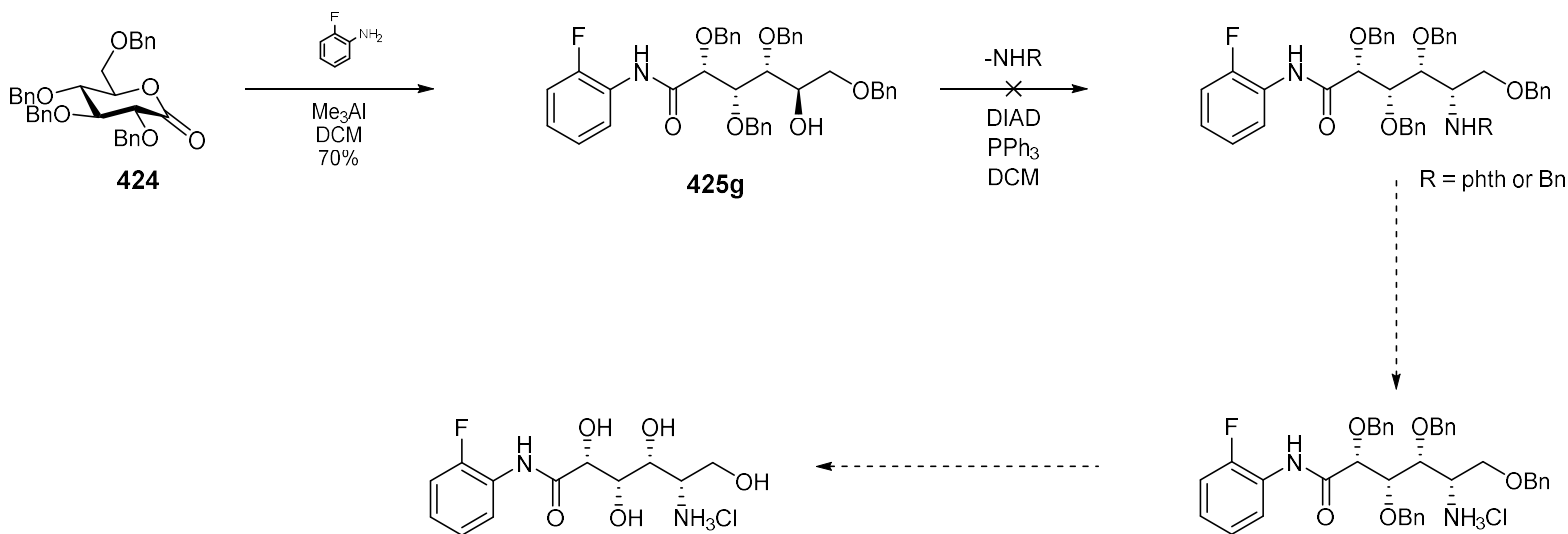
Having an IRI bearing a positive charge may be useful in many biological applications such as cryopreservation of red blood cells (RBCs), articular cartilage, platelets, and hematopoietic stem/progenitor cells (HSPCs).⁶⁻¹¹ Due to a positive charge on the IRI itself, it should more easily permeate certain tissues that have higher concentrations of negatively charged residues. This is especially important in more complex tissues (such as cartilage) where permeation of a cryoprotectant is essential in order to protect the whole sample during cryopreservation.¹²⁻¹⁴ Due to the cytotoxicity of some commonly used cryoprotectants such as

DMSO, incubation with the cryoprotectant must be limited.¹⁵ Finding a balance between incubation times to optimize cryoprotectant permeation versus cryoprotectant toxicity must be investigated. By having a cryoprotectant that can better permeate biological samples can simplify this process to maximize protection during freezing/thawing and minimize cytotoxicity. Additionally, more soluble cryoprotectants can be used at higher concentrations without the aid of heat to dissolve them. Currently, many novel cryoprotectants developed by the Ben lab have very low (0.5-15 mM for many gluconamide derivatives) solubilities in aqueous media. Meaning that the cryosolutions these IRIs are dissolved in must be heated (often >80 °C) to fully dissolve the IRI. This is problematic due to many cryosolutions containing growth media, which commonly have a series of heat-sensitive components. A highly soluble IRI would allow testing at much higher concentrations than are currently possible, without the need for extensive heating to dissolve the IRI in the cryosolution.

5.2 Synthesis of Aminogluconamides

Initially, the target amino alditols would feature a free amine at the same position as the sulfate in the C5 sulfated gluconamide derivatives, although with the opposite stereochemistry at the C5 position (**Scheme 5-1**), generating a substituted L-iodoamide derivative. This was due to the nature of the employed Mitsunobu reaction on the C5 position of compound **425g**. As seen with the C5 sulfated gluconamide derivatives (Chapter 4), including a polar functional group is capable of drastically increasing solubility. C5 was initially chosen due to the ease of synthesis, where it is left unprotected after ring opening of the protected pyranose (**Scheme 4-1**, Chapter 4). A second Mitsunobu reaction would result in double inversion at the C5 position resulting in the original configuration observed in the D-gluconamide derivatives. Attempts at this reaction with various amines resulted in degradation of the starting material and no discernable product

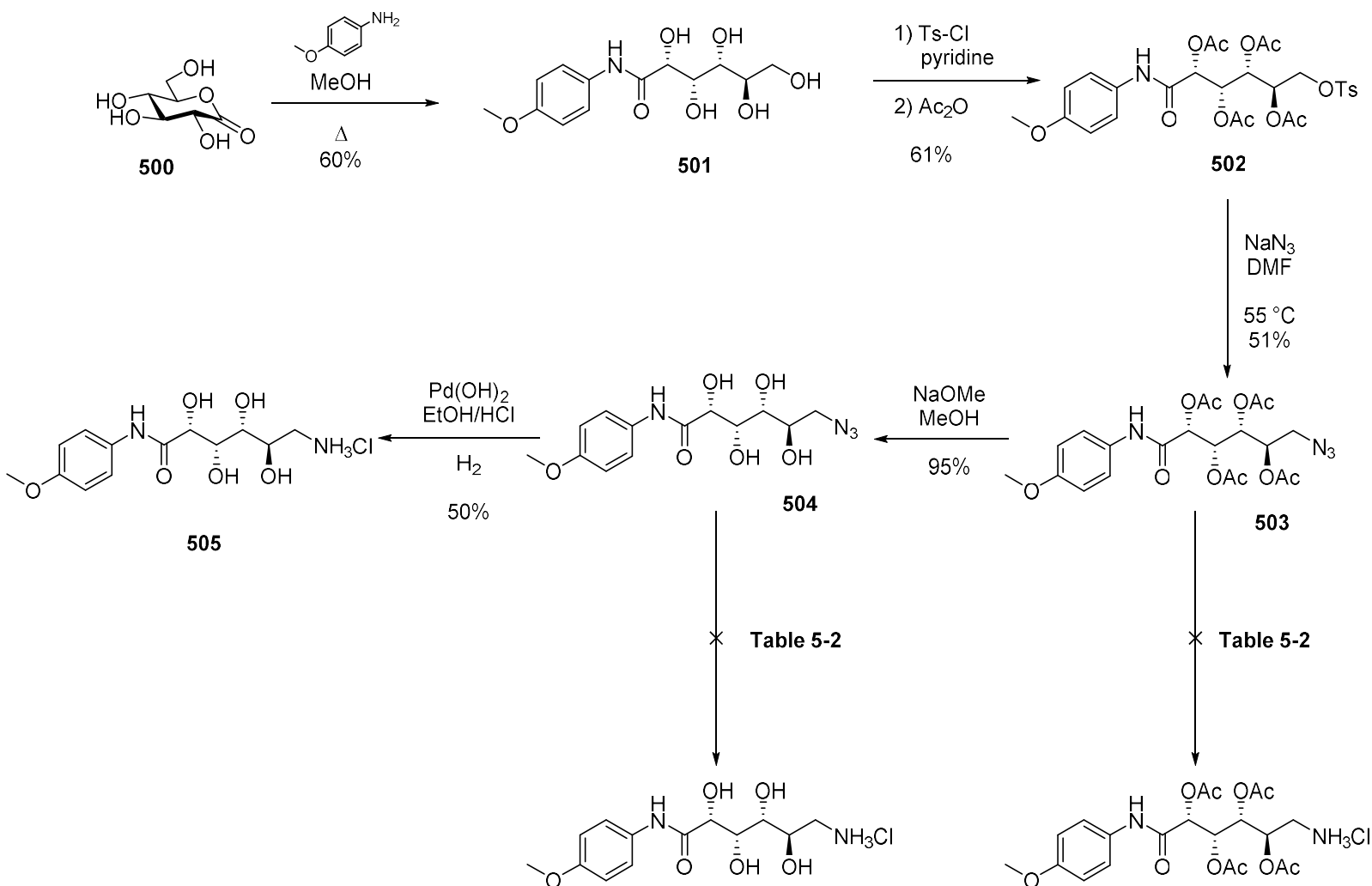
formation via NMR. Various reaction conditions were utilized (**Table 5-1**) with only degradation products and residual starting material being isolated.



Scheme 5-1. Initial proposed synthesis of *N*-(2-fluorophenyl)-6-amino-L-iodoamide.

Table 5-1. Mitsunobu reaction conditions.

Entry	-NHR (equivalents)	DIAD equivalents	Result
1	benzylamine (3)	3	No product formation, starting material isolated
2	phthalimide (1.3)	1.3	Degradation of product (indiscernible by NMR), some starting material reisolated
3	phthalimide (1.3)	2.3	Degradation of product (indiscernible by NMR)



Scheme 5-2. Synthesis of *N*-(4-methoxyphenyl)-D-gluconamide.

Due to the inability to successfully displace the C5 hydroxyl group with an amine, a new synthetic route that focused on the synthesis of a C6 amine was undertaken (**Scheme 5-2**). In order to be able to displace the C6 hydroxyl, the 4 remaining hydroxyl groups must be protected. This is achieved in the one-pot reaction generating compound **502** in 61 % yield, leaving the C6 hydroxyl protected with an easily displaceable tosyl (as a tosylate) group, and the remaining hydroxyl groups acetate protected. The tosylate group is displaced using NaN_3 producing the

acetate protected azide compound **503** in 51 % yield. The final two steps remaining are the reduction of the azide and removal of the acetyl groups. Initially, the azide would be reduced prior to acetate removal in order to make purification of the free amine more manageable. Attempts at reducing the azide with the acetate groups on derivative **503** proved unsuccessful using a variety of different conditions (**Table 5-2**).

Table 5-2. Azide reduction conditions on substrate **503** and **504**.

Entry	Substrate	Conditions	Result
1	503	PPh ₃ , THF, H ₂ O, 60°C	Product degradation
2	503	PPh ₃ , MeOH, 80°C	Product degradation
3	503	COCl ₂ ·6H ₂ O, NaBH ₄ , H ₂ O	No reaction
4	504	Zn dust, NH ₄ Cl, H ₂ O, EtOH, Δ	No reaction

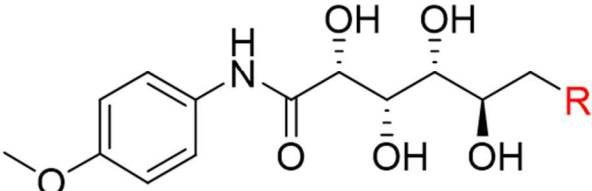
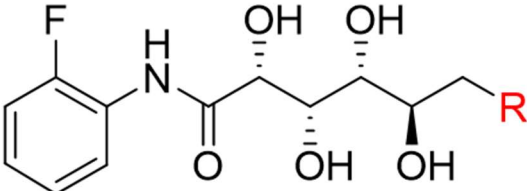
Due to this, the acetyl groups were removed employing Zemplén conditions, followed by reduction of the azide. After optimization, simple hydrogenation of the azide using Pd(OH)₂ and H₂ in MeOH was used, affording the final free amine **505** as an HCl salt in 50% yield. Overall, the *p*-methoxyphenyl derivative **505** was synthesized in 8.8 % yield, the 2-fluorophenyl (**505a**) was also synthesized in the same manner in a 5.7 % yield overall.

5.3 IRI Activity and Toxicity of Aminogluconamides

With the two C6 aminogluconamides synthesized, IRI testing had to be conducted on the derivatives using the same method as previously applied (5 min drop Chapter 3). IRI activities compared to the parent compounds are shown in **table 5-3**. While both aminogluconamides show

a reduction in IRI activity compared to the parent compounds, they still possess moderate degrees of activity compared to other compounds (33 and 39 mM respectively). Although there is a reduction in IRI activity with these compounds, they are drastically more soluble in aqueous media than their parent compounds, both being soluble over 500 mM compared to between 15 and 55 mM in PBS.

Table 5-3. IRI activity (IC_{50}) and solubilities of C6 amino compounds and parent compounds.

Compound	Entry	R	IC_{50} (mM)	Solubility in PBS (mM)
	153	-OH	5	55
	505	-NH ₃ Cl	39	>500
	150	-OH	4	15
	505a	-NH ₃ Cl	33	>500

The high solubility at room temperature of these compounds permits testing at very high concentrations (>100 mM in some cases) in various aqueous systems. This is of great importance in heat-sensitive media that would normally only allow low concentrations (<10 mM) of IRIs to be used.

One potential problem with the high solubility of the compounds is toxicity at these concentrations. In order to determine the toxicity of these compounds, an alamarBlue assay was undertaken. This assay is based off the ability of living cells to reduce resazurin (non-

fluorescent) to resorufin (fluorescent), eliciting a colour change that can be related to cell viability. In live cells, resazurin is reduced via the electron transport chain, without interrupting normal function. It may also be reduced in the mitochondrial cytoplasm via various reductases. ¹⁶

Once reduced to resorufin, fluorescence (or absorbance) can be measured using a 530-566 nm excitation and 590 nm emission wavelength. While this assay is not a direct measure of cell viability, only live cells will metabolize resazurin to resorufin and in doing so, is an indirect measure of viability.¹⁶

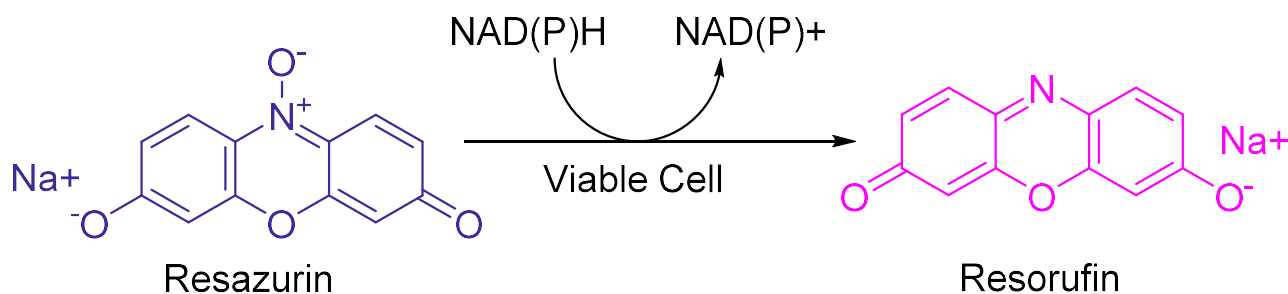
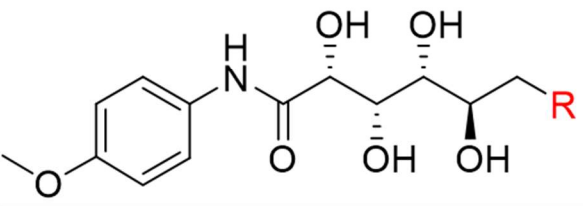
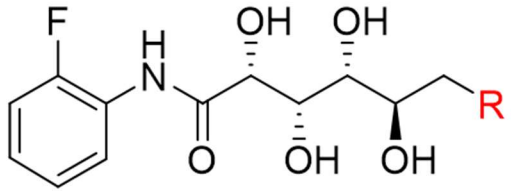


Figure 5-2. Conversion of Resazurin to Resorufin by metabolically active cells. ¹⁶

HepG2 (human liver carcinoma) cells have previously been used as model cells for toxicity of monosaccharides to be used as cryoprotectants.^{1,17} These cells are ideal due to their ability to model liver metabolism of chemical compounds. As shown in **Table 5-4**, both derivatives are quite toxic, with LD₅₀ values of 18 and 20 mM for the p-methoxy- and 2-fluorophenyl compounds, respectively. Since the IC₅₀ values are 39 and 33 mM respectively, the compounds would have to be used below their IC₅₀ value to minimize toxicity, which would result in lower than ideal IRI activity. Although these LD₅₀ values only apply to HepG2 cells, and other cell lines/tissues may have different toxicity values. While both LD₅₀ values are low, the toxicity was only assessed in HepG2 cells. The biological systems of interest are composed of different cells, although if these compounds were to be used in cryopreservation, the compound should be removed prior to introduction to avoid liver toxicity. With this considered the compounds were

tested in a number of biological systems due to their high solubility at room temperature as well as the cationic amine on the molecule.

Table 5-4. LD₅₀ values for C6 amino compounds and parent compounds obtained via alamarBlue assay in HepG2 cells.

Compound	Entry	R	LD ₅₀ (mM)	Solubility in PBS (mM)
	153	-OH	17	55
	505	-NH ₃ Cl	20	>500
	150	-OH	>15*	15
	505a	-NH ₃ Cl	18	>500

*LD₅₀ not reached at maximum solubility.

5.4 Applications

Since molecules of this nature had not been previously explored in many biological cryopreservation applications, the aminogluconamides could be beneficial during cryopreservation. The high solubility of these compounds allows testing at high concentrations, without the need to heat the sample. Heating (>80 °C) is required for many of the current small-molecule IRIs developed by the Ben lab which is not always possible for all freezing mediums. Many cryopreservation media include heat-sensitive molecules such as proteins, which can degrade upon heating to the temperatures needed to fully dissolve some of the current IRI active molecules. Another problem with current IRIs is the potential for the molecules to precipitate when the sample is being frozen, lowering the concentration of cryoprotectant in solution.

By supplementing cryosolutions with IRIs may also allow lower concentrations of commonly used cytotoxic cryoprotectants such as DMSO and glycerol. This would facilitate post-thaw processing generally required to remove the toxic components prior to use. In addition to lowering the concentrations of cytotoxic cryoprotectants, the incorporation of IRIs during cryopreservation should mitigate damage due to ice recrystallization which may result from reduced cryoprotectant concentrations. Finally, the charge on the IRI may aid in diffusion in more complex tissues, inhibiting ice recrystallization throughout. Incorporating charged, IRI active compounds in cryopreservation protocols should not only reduce cytotoxicity from high concentrations of cryoprotectants, but also protect from ice recrystallization. These factors should result in increased post-thaw cell viabilities as well as cell function.

5.4.1 Cryopreservation Red Blood Cells (RBCs)

The transfusion of red blood cells (RBCs) is essential in many medical scenarios, such as traumas, surgical operations, and patients with bleeding disorders. While blood can be stored at temperatures of $\sim 4^{\circ}\text{C}$ without preservatives, this is limited to 42 days post-collection. To lengthen the storage time and allow for biobanking, RBC units must be frozen and stored at sub-zero temperatures and high concentrations of glycerol as a cryoprotectant. In North America and some European countries 40% glycerol along with slow ($1^{\circ}\text{C}/\text{min}$) cooling temperatures to -80°C are used, while other European countries use 20% glycerol and fast cooling ($>50^{\circ}\text{C}/\text{min}$) to -196°C . This is generally only used with rare blood phenotypes as well as in military applications due to the lengthy post-thaw deglycerolization step. Deglycerolization is required pre-transplant (to $<1\%$) in order to prevent intravascular hemolysis. The deglycerolization process results in up to a 20% loss of volume, and the deglycerolized unit must be used within 24 hours of deglycerolization. In order to more readily cryopreserve and subsequently use RBC

units, glycerol concentrations need to be reduced in order to make the deglycerolization time practical in urgent situations.^{18–20} Using an even lower concentration of glycerol would further reduce deglycerolization times to acceptable levels for transfusion. The Ben lab uses 15% glycerol v/v coupled with IRIs to achieve deglycerolization times of <10 minutes.^{11,18}

Experiments were based off established protocols from the Acker lab (Center for Innovation – Canadian Blood Services, Edmonton) and conducted by Tracey Turner, and coworkers Julia Meyer and Jessica Poisson.

The first system in which that aminogluconamides were investigated was the cryopreservation of red blood cells (RBCs). Until this point, few charged IRIs have been studied for RBC cryopreservation, with most of them being neutral. An IRI bearing a positive charge may be beneficial during RBC cryopreservation as previously tested charged molecules (Chapter 4) have been beneficial. This is due to the high solubility of the IRIs meaning they can be tested in higher concentrations compared to existing IRIs. This is important because using IRIs at higher concentrations may offer more protection to the samples during freezing/thawing than low concentrations.

Initially, both compounds **505** and **505a** were studied at 37.5 and 75 mM due to their solubility. Both compounds were dissolved in 15% glycerol prior to cooling to -40 °C at a rate of 1 °C/min (slow cooling). **Figure 5-3** shows the percentage of intact RBCs post-thaw compared to the 15% glycerol control. While compound **505a** at 37.5 mM shows a decrease of 10% (17 vs 27%) over 15% glycerol, the *p*-methoxy derivative **505** performs much better in a dose-dependent manner. Since the compounds are dissolved in 15% glycerol, any increase or decrease in percent intact RBCs can be attributed to the IRI itself. The decrease observed with compound **505a** can most likely be attributed to toxicity from the compound, although both compounds

have similar LD₅₀ values in HepG2 cells (18 and 20 mM). Since the structures and LD₅₀ values for both compounds are so similar, the difference in percent intact RBCs could be due to the slight structural differences between the two compounds. Interestingly, **505** at 75 mM displays levels of intact RBC post-thaw near 80%, which is only slightly lower than optimized RBC cryopreservation using 40% glycerol (~95% intact). At half the concentration (37.5 mM) it shows a percent intact RBC value of 51%, three times higher than compound **505a** at the same concentration.

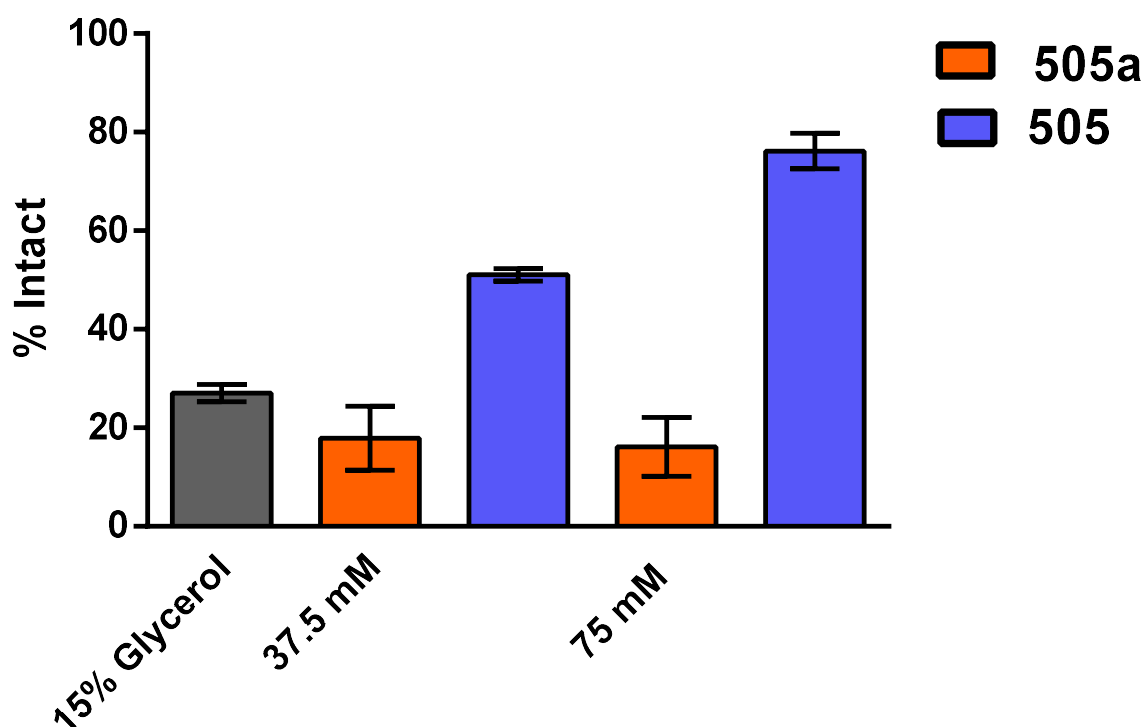


Figure 5-3. Initial concentration screen of compounds **505a** and **505** dissolved in 15% DMSO in RBCs and 15% glycerol control. % intact value represents the percentage of intact RBCs post-thaw. Error bars represent SEM, n = 2.

Due to the initial success with **505** in conjunction with 15% glycerol for the cryopreservation of RBCs, a more detailed concentration screen was conducted using both a slow cooling (1 °C/min to -40 °C) as well as a -80 °C dump freeze (fast cooling) freezing method. **Figure 5-4** shows the

slow cooling concentration screen below. Slow cooling of RBC units is optimized for 40% glycerol and using a reduced glycerol concentration (15%) results in large amounts of hemolysis. This is observed in the 15% glycerol control in figure 5-4. Due to the slow cooling most RBCs have lysed, but interestingly supplementing 15% glycerol with **505** drastically reduces hemolysis even at low concentrations. The percent intact RBCs increase in a concentration-dependent manner until 150 mM **505**, with subsequent slight reduction above this concentration (91% vs 74% for the highest concentration). This is likely due to the slight surfactant-like properties of **505** at higher concentrations. This was suspected due to highest

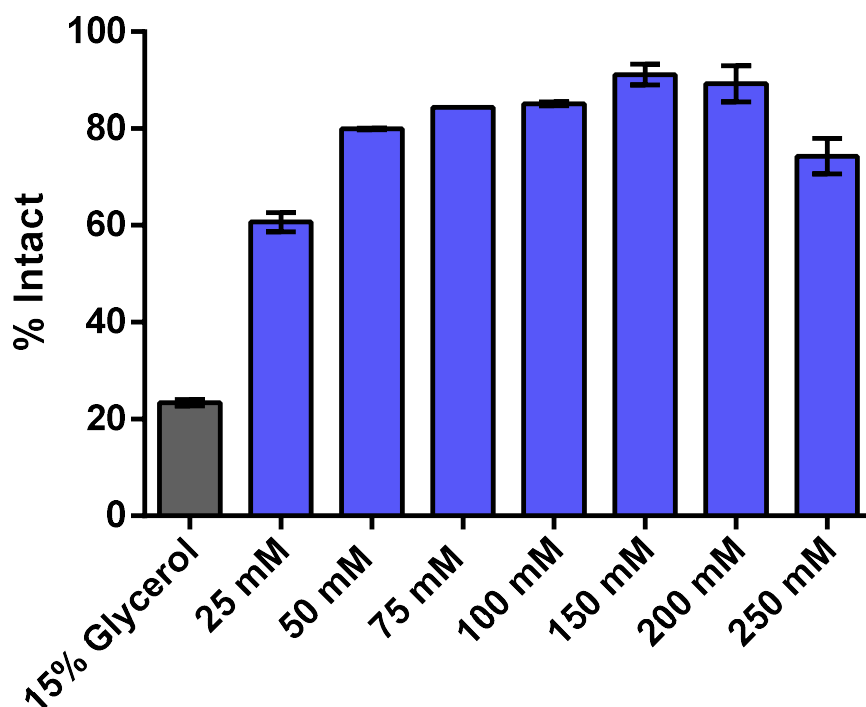


Figure 5-4. Full concentration screen of compound **505** in 15% glycerol alongside 15% glycerol control in RBCs. % intact value represents the percentage of intact RBCs post-thaw. Error bars represent SEM, n = 2.

two concentrations creating a foam upon mixing in the 15% glycerol solution, which was not observed for the lower concentrations, although further tests are required to confirm this hypothesis. These trials succeeded in showing that **505** shows potential as a novel cryoprotectant

for RBCs in conjunction with reduced glycerol levels. To further demonstrate **505**'s use as a cryoprotectant, additional experiments such as osmotic fragility, transient warming, and deglycerolization experiments need to be conducted as well.

5.4.2 Cryopreservation of Articular Cartilage

Proper cartilage function is necessary for normal joint health and movement.^{21,22} Articular cartilage degrades over time, which results in pain when moving and an overall reduced quality of life. Degradation due to sports injuries are also common and can occur to individuals at any age. Since cartilage degradation results in pain when moving and potentially a dependence on pharmaceuticals to alleviate it, cartilage repair has been extensively studied.^{21,23–27} There are a number of different solutions when repairing damaged cartilage, depending on the size and location of the cartilage. Repair can be done by creating a new blood supply to the affected area or by osteochondral autograft or allograft.^{13,25,27,28} In the case of an allograft, a section of cartilage is taken from a cadaver and implanted in the patient (Chapter 1 **Section 1.4.3**).²⁹ The major problem with this treatment is the necessity for a fresh cartilage sample. A sample that is a correct Human Leukocyte Antigen (HLA) match for the patient is rarely used, although the necessity of a correct HLA match is still debated.^{30,31} The lifetime of cartilage post-removal is very short, making allografts often difficult. Due to the complexity of cartilage, biobanking of cartilage is not common.

Current cartilage cryopreservation protocols use a two-stage cooling process along with DMSO as a cryoprotectant. Other cryoprotectants such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) can be used as well, although are generally toxic.³² The first cooling stage is a slow cool (1 °C/min) to -20 °C after a preincubation with 1-1.4 M DMSO followed by

a faster (4 °C/min) cooling rate to -70 °C. The preincubation of the cartilage sample with DMSO is necessary to allow the DMSO to fully permeate the cartilage sample. Decreased cell viability on the interior of cartilage sections is often observed due to slow cryoprotectant permeation throughout the sample, while cytotoxicity on the exterior of the sample is observed with increased incubation times. Overall incubation times must be carefully controlled along with incubation temperature to limit cytotoxicity.^{13,24,33–36}

Cartilage can also be cryopreserved using ultrafast cooling rates coupled with high concentrations (>2 M) of DMSO in order to vitrify the cartilage sample before storing. Due to the incubation time needed before freezing, vitrification has the same disadvantage of cryoprotectant cytotoxicity upon extended incubation times that other cryopreservation procedures experience.^{13,14,17,37,38}

While these two methods allow for the successful freezing of cartilage with minimal damage to the sample, the damage encountered during the thawing process results in low to moderate levels of post-thaw viability and functionality. During the devitrification process, ice crystals present can grow larger due to ice recrystallization, causing large amounts of mechanical damage to the sample. The rate at which samples are thawed determines how much ice recrystallization can occur, ideally a rapid warming rate would be used to minimize the ice recrystallization. Unfortunately, rapid enough warming rates are often unachievable due to sample structure, size, as well as the equipment available.^{34,39,40} When dealing with large samples, uniform, rapid warming rates are often unobtainable due to heat dissipation throughout the sample. New technologies such as nanoscale warming with magnetic nanoparticles are being developed to circumvent ice recrystallization during devitrification.⁴¹ The magnetic particles are used in conjunction with (often lower concentrations of) cryoprotectant, and uses an applied

magnetic field uniformly heat the particles throughout the sample, resulting in uniform temperatures throughout the sample. Disadvantages of this technique include biocompatibility and removal of nanoparticles, specific equipment for warming, and achieving a uniform dispersion of particles throughout the sample.⁴¹

Often with large samples, the outer layer of the sample will warm at a quicker rate than the center, resulting in cracking and ice recrystallization in the middle areas of the sample. The structure of cartilage also results in difficulties when freezing/thawing. Since cartilage is a complex tissue, if any part of the sample is damaged during freezing/thawing, the whole sample may be unusable or decrease drastically in functionality post-thaw. In order to protect the whole sample during warming, a cryoprotectant that can inhibit ice recrystallization can be used along with DMSO in hopes of protecting the interior of the sample. For this to be possible, the IRI must be able to fully permeate the sample, which can be achieved by modulating the properties of the IRI in question.

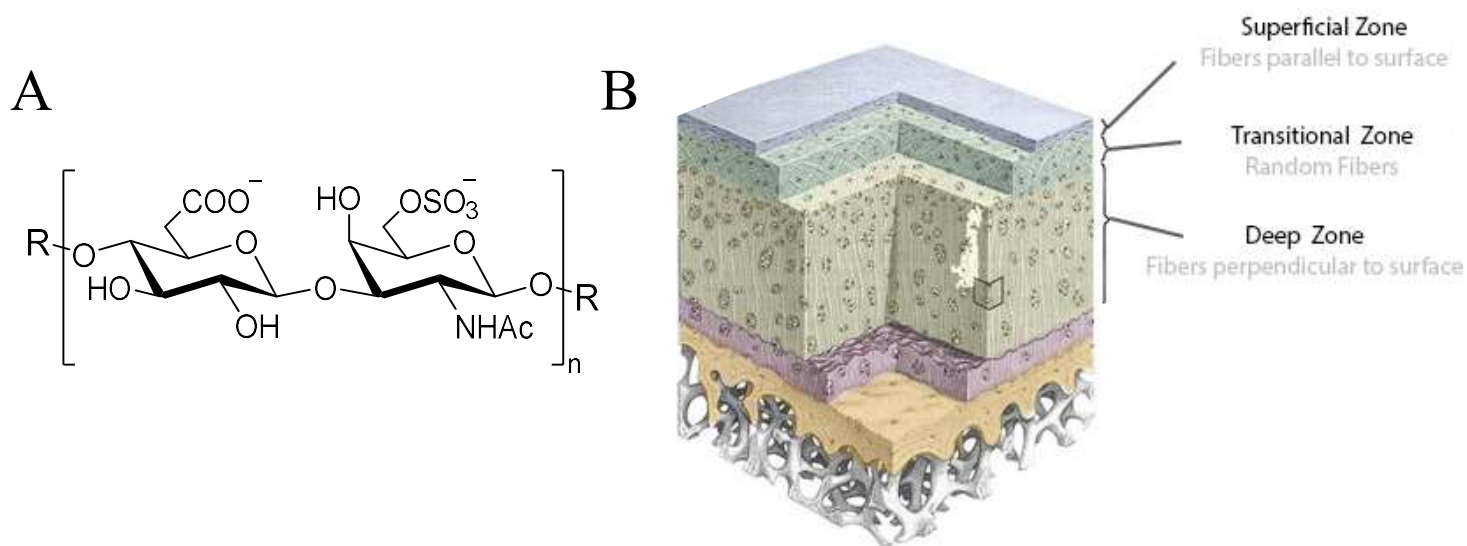


Figure 5-5. **A** – chondroitin sulfate subunit present in cartilage. **B** – Cross-section of articular cartilage showing chondrocyte distribution throughout layers.⁴²

Articular cartilage is comprised of proteoglycan and collagen fibers, which contain high concentrations of chondroitin sulfate, a negatively charged polysaccharide.⁴² There are three distinct layers of articular cartilage (**Figure 5-5 B**). The outermost layer is referred to as the superficial zone and is the thinnest of the three sections. In this layer proteoglycan and collagen fibers are tightly packed and run parallel to the surface. Its main purpose is to protect the deeper layers from shear stress and give cartilage its tensile properties. Chondrocytes (cartilage creating cells) are most abundant in this layer due to the stress placed on this layer.⁴² Underneath is the transitional zone, which has proteoglycan and thicker collagen fibers that are randomly dispersed through the layer. This layer acts as a bridge between the superficial and deep layers and contains less chondrocytes with slightly different morphology (spherical vs flat) than the superficial layer. The innermost layer is the deep layer, which has the thickest collagen fibers, arranged perpendicular to the surface. This results in this layer providing the most resistance to compressive forces. This layer is also responsible for anchoring cartilage to the bone. Chondrocytes in this layer are hypertrophic and low in number.⁴² All three layers contain high concentrations of both proteoglycan and collagen fibers, resulting in an overall negative charge throughout the tissue. While permeation through all layers is difficult with conventional cryoprotectants, a positively charged IRI used during the cryopreservation of cartilage should be able to diffuse throughout the sample. This should be beneficial to vitrification with lower concentrations of DMSO and should protect the interior of the sample from ice recrystallization during devitrification.

Initially, two IRI-active phenyl gluconamide derivatives **150** (*N*-(2-fluorophenyl)-D-gluconamide) referred to as 2FA (**2-FluoroAniline**) and **153** (*N*-(4-methoxyphenyl)-D-gluconamide) referred to as PMA (***p*-MethoxyAniline**) were chosen to assess their potential as

novel cryoprotectants for the cryopreservation of articular cartilage. The first experiment conducted was an incubation study using freeze substitution after vitrification of whole cartilage samples. This study used small Porcine cartilage discs (6 mm) to investigate the permeation and subsequent cryopreservation of cartilage with 2FA (5 mM and 11 mM) and PMA (25 mM). The study outline is shown in **Figure 5-6**. Porcine knee cartilage was used as a model due to its similarities to human cartilage. Knee cartilage was chosen because knee cartilage injury is one of the most common types of cartilage injury. This procedure was based on established protocols from the Jomha lab (University of Alberta) and were conducted by Kezhou Wu, Rachael Dong, Kevin Duong, and Leila Laouar (University of Alberta).

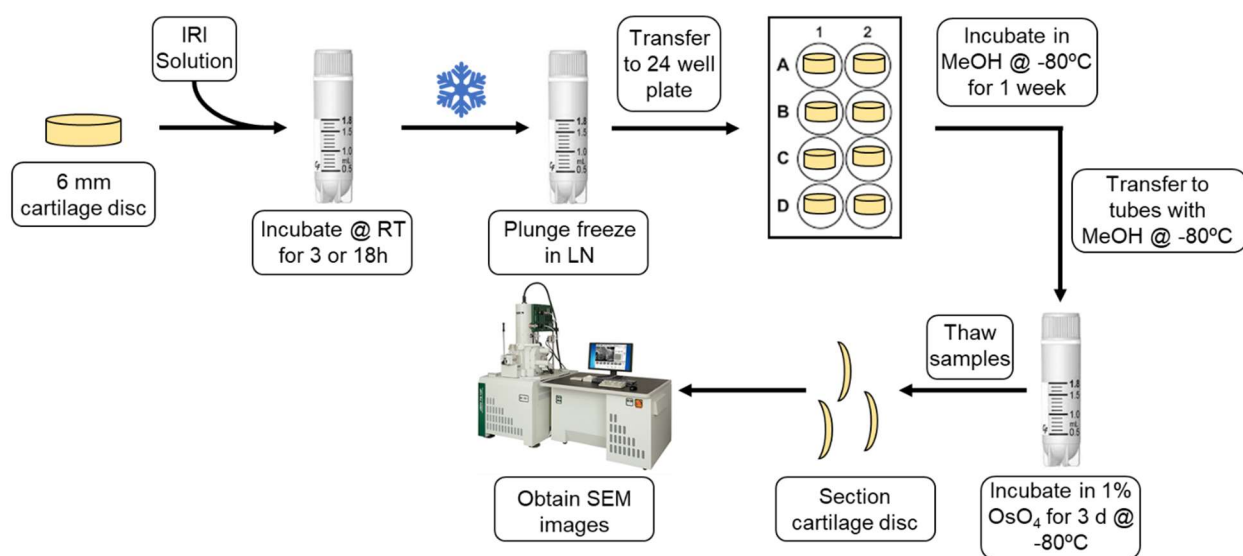


Figure 5-6. IRI incubation study with Porcine cartilage sections.

To allow the IRI solution (compound in PBS) to fully permeate throughout the sample, the cartilage discs were incubated in the IRI solution for either 3 or 18 hours at room temperature. After the incubation, the samples were plunge froze in liquid nitrogen to vitrify the sample. Afterwards, the cartilage samples were transferred to 24 well plates filled with MeOH at -80 °C for one week (MeOH changed daily). MeOH was used to displace any water left in the

sample in order to keep the sample morphology upon thawing. After MeOH incubation, the samples are moved to a 1% OsO₄ in MeOH solution at -80 °C for 3 days. OsO₄ is used as a fixative to maintain the frozen sample morphology after the sample has been thawed. This process is referred to as freeze substitution. Once the tissue was fixed, the cartilage samples were sectioned to view the 3 major sections of the cartilage disc (superficial, transitional, and deep zones) via SEM imaging.

The first images are of an unfrozen cartilage section as a control. Normal chondrocyte morphology can be observed in **Figure 5-7**. The chondrocytes (circled in red) take up the entire extracellular matrix around them in all 3 layers without shrinking or degradation.

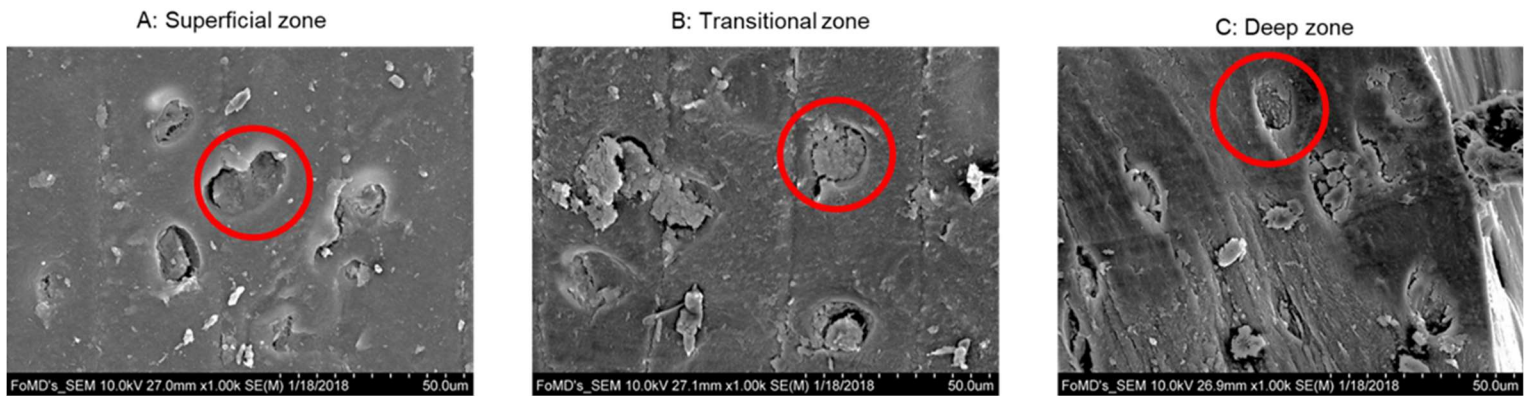


Figure 5-7. SEM images of unfrozen control cartilage sections. Red circles show intact chondrocytes within extracellular matrix of cartilage.

The next series of images (**Figure 5-8**) show the cartilage after being incubated with PBS, then frozen with no cryoprotectant as a negative control. PBS should offer no protection from the freezing process and the chondrocytes should be drastically smaller or completely degraded. Opposed to the unfrozen control, there are large voids in the cartilage extracellular matrix where the chondrocytes have either degraded (red circle) or drastically decreased in size (yellow circle) due to the freezing process. In subsequent trials where IRIs were incorporated

into the cryosolution, it is expected that there should be minimal shrinking of chondrocytes, and most, if not all should remain intact.

The first IRI to be investigated was 2FA at 5 mM with both a 3- and 18-hour preincubation before freezing and processing (**Figure 5-9** top two rows). The initial concentration of 5 mM was used, based on previous experiments conducted in human umbilical cord blood (hUBC) experiments.⁴³ The chondrocytes in this sample appear much more intact and have similar morphology to the unfrozen control, especially in the superficial zone of the 3- and

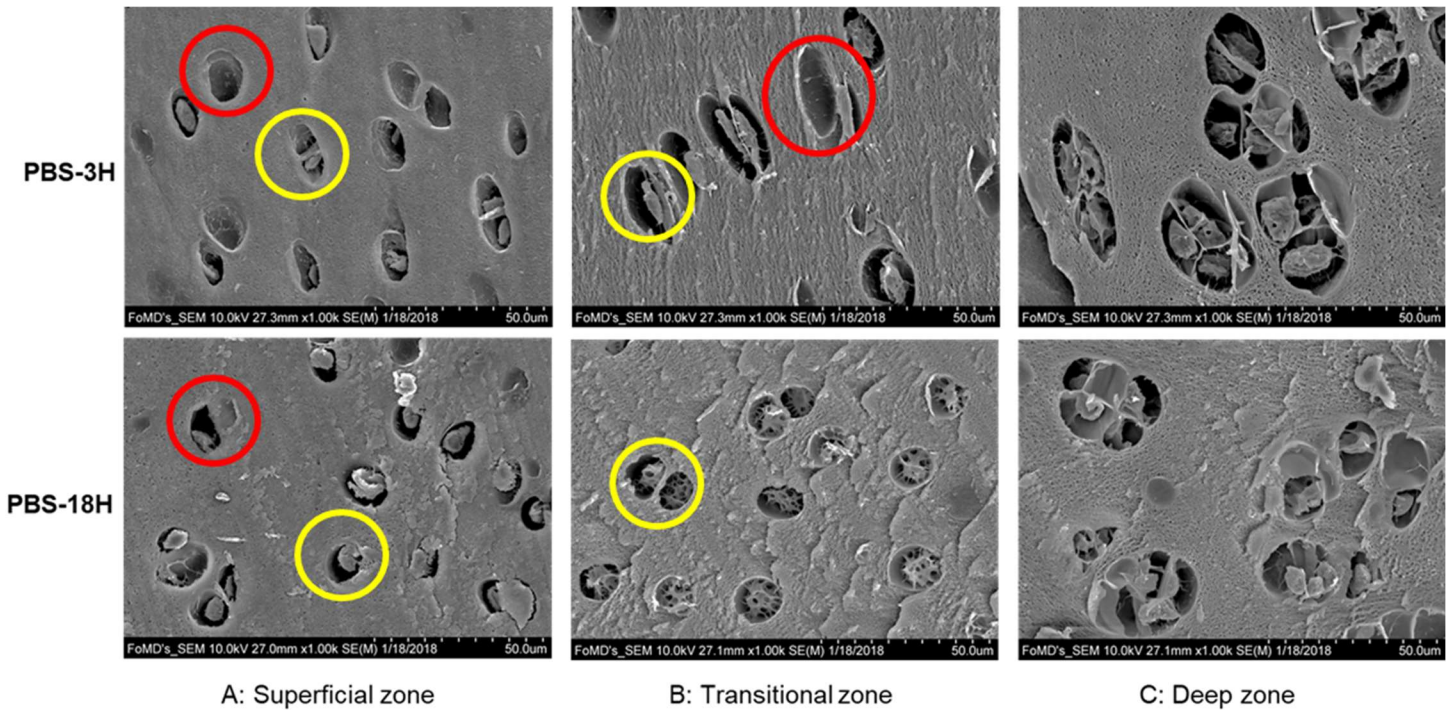


Figure 5-8. SEM images of cartilage sections frozen with PBS (negative) control post-thaw. Red circles show gaps in the extracellular matrix where chondrocytes have fully degraded, while yellow circles show size reduction of chondrocytes.

18-hour incubation. While most chondrocytes are intact in the transitional and deep zones for the 3-hour incubation, most are drastically reduced in size, indicating damage due to freezing. The 18-hour incubation appears to prevent much of this damage, as all the chondrocytes in all

three layers are intact and show minimal size reduction. This suggests that the longer preincubation is needed in order for the IRI to permeate the cartilage disc.

Since 5 mM 2FA showed improvement over PBS, it was tested near its solubility limit (15 mM) in order to assess what role concentration of the cryoprotectant plays in cartilage cryopreservation. 2FA was assessed at 11 mM using the same conditions with both a 3- and 18-hour incubation (**Figure 5-9** bottom two rows). With the use of a higher concentration of cryoprotectant, the chondrocytes appear more intact with both incubation times. The most intact and largest chondrocytes are located in both the superficial and transitional zone of the 3-hour incubation, although in the deep zone the chondrocytes appear intact but have lost some volume. This was consistent with the lower (5 mM) concentration trial for the 3-hour incubation. While the longer incubation time results in more cryoprotectant permeation, the chondrocytes in the superficial and transitional zones have lost volume compared to the shorter incubation trial. This may be in part due to toxicity of the cryoprotectant over the extended incubation time. For compound **150**, an 18-hour incubation with 5 mM is most beneficial, demonstrating its protective nature with fully intact chondrocytes with minimal size reduction.

A second IRI explored was PMA at 25 mM exclusively as this is the compounds maximum solubility. **Figure 5-10** shows the 3- and 18-hour incubation. The superficial zones of both the 3-and 18-hour incubation trials appear similar, with the longer incubation time showing slightly more intact chondrocytes (although additional images would be needed to confirm) than the 3-hour incubation. The opposite is observed in the transitional zones, where the chondrocytes show less size reduction in the 3-hour incubation compared to the 18-hour incubation. Moving to the deep zones, both incubation times show a mix of intact, reduced size, and fully degraded chondrocytes. This indicated that either there is some level of toxicity associated with PMA, or

the compound is unable to fully permeate the sample, even with the extended 18-hour incubation that was used.

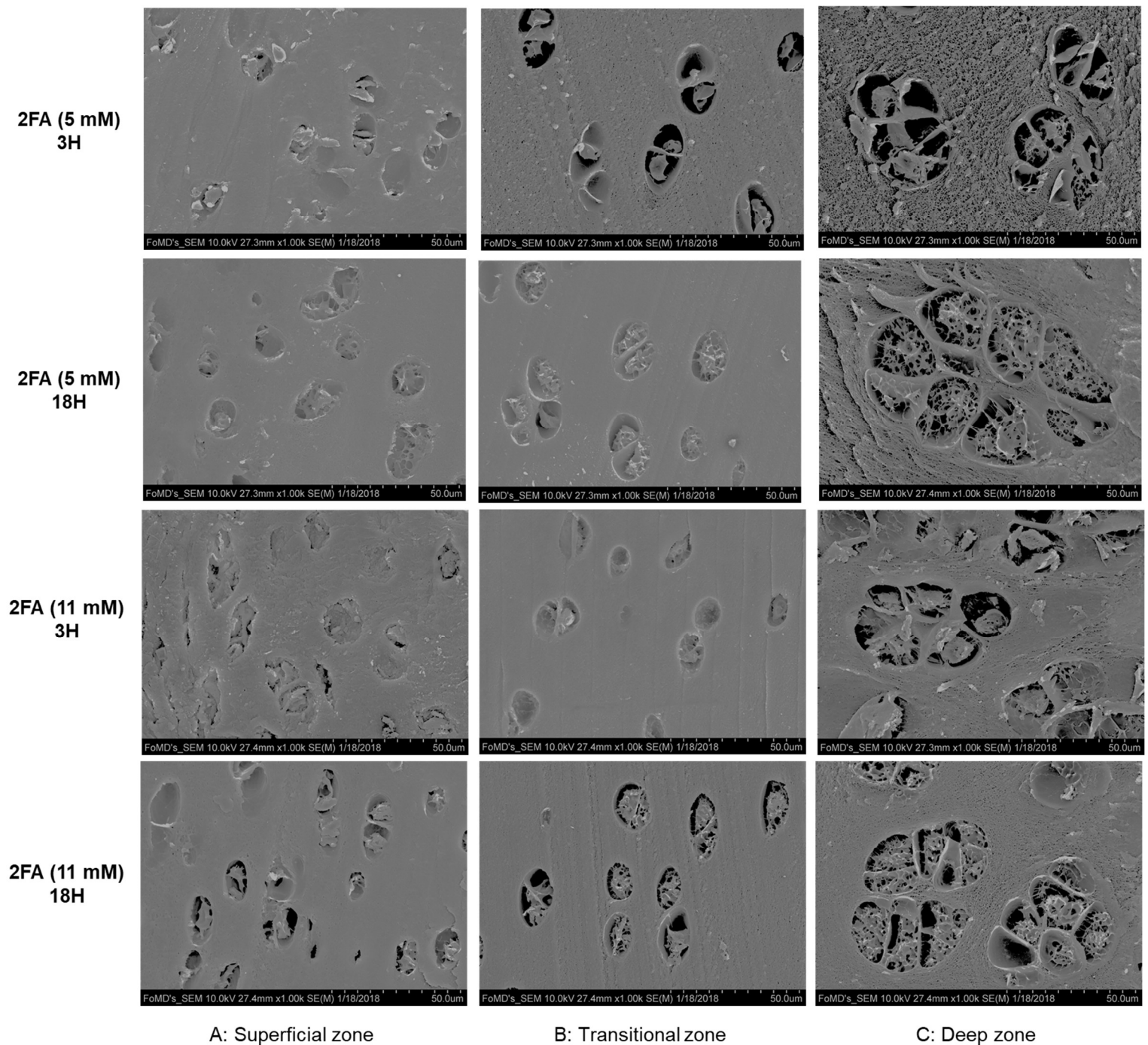


Figure 5-9. SEM images of cartilage sections incubated with 5 and 11 mM 2FA for 3 and 18 h.

Due to the initial success of using **505** as a cryoprotectant in RBCs as well as 2FA in cartilage cryopreservation, both compounds were selected for further experimentation in cartilage. **Figure 5-12** outline the experiment including points where the concentration of

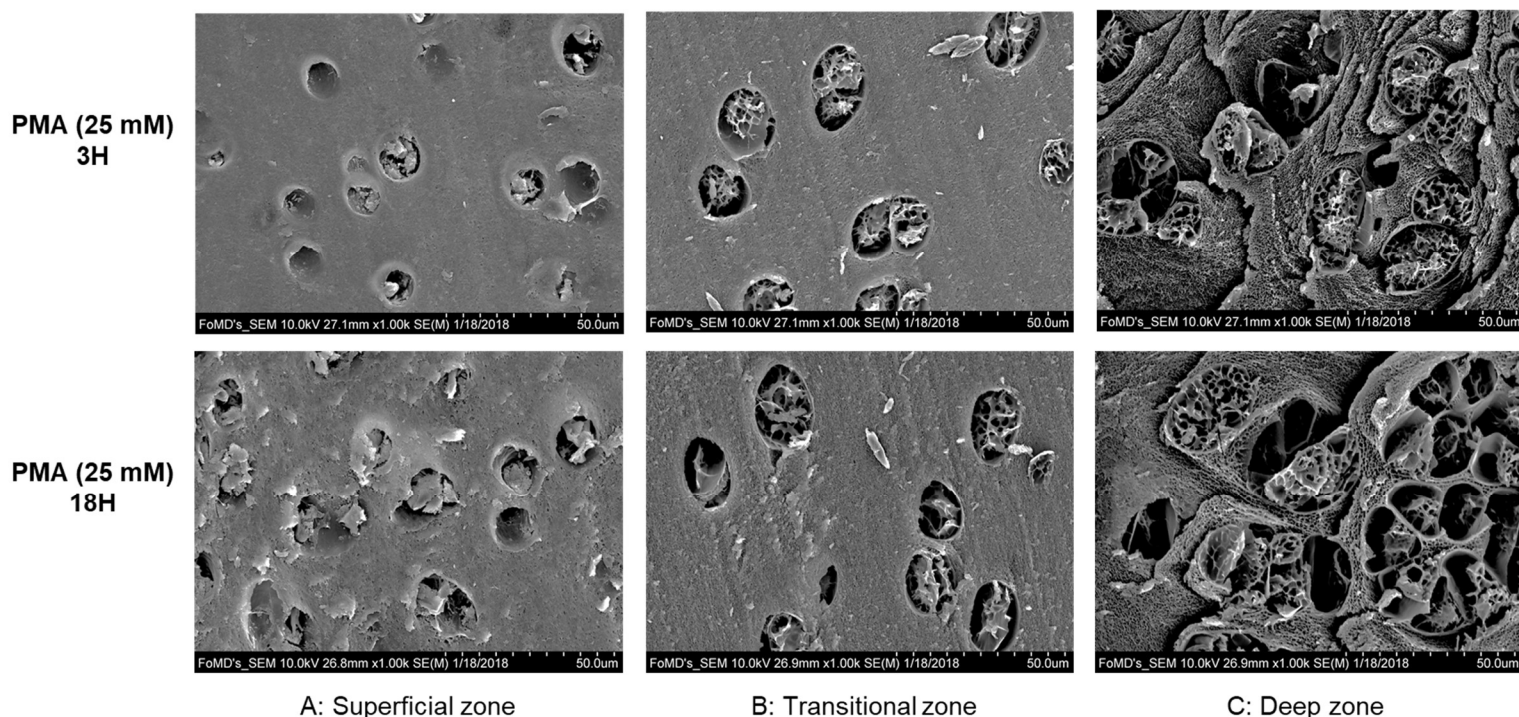


Figure 5-10. SEM images of cartilage sections incubated with 25 mM PMA for 3 and 18 h.

cryoprotectant is assessed. To allow sufficient time for the cryoprotectants to fully permeate the cartilage and to assess the toxicity of the compound, the cartilage disc was incubated at room temperature for 24 hours. After this, the disc was moved to PBS at room temperature for an additional 24 hours to allow removal of the IRI before staining and imaging.

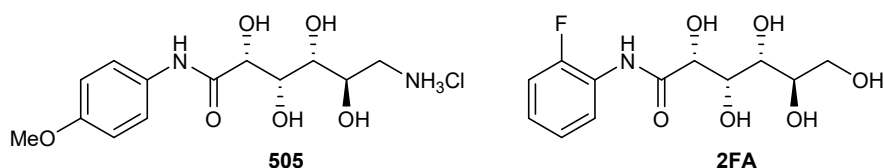


Figure 5-11. Structures of IRIs *N*-(4-methoxyphenyl)-6-amino-D-gluconamide (**505**) and *N*-2-florophenyl-D-gluconamide (**150 - 2FA**).

Samples of both the initial incubation sample solution (“sample”, **Figure 5-13**) and the washout solution (“washout”, **Figure 5-13**) were subjected to quantitative NMR measurements to see if firstly the compounds are, permeating into the cartilage disc, and secondly, that if the compounds are able to permeate through the cartilage disc, that they can be subsequently removed. By combining the sample and washout values the concentration that is retained in the

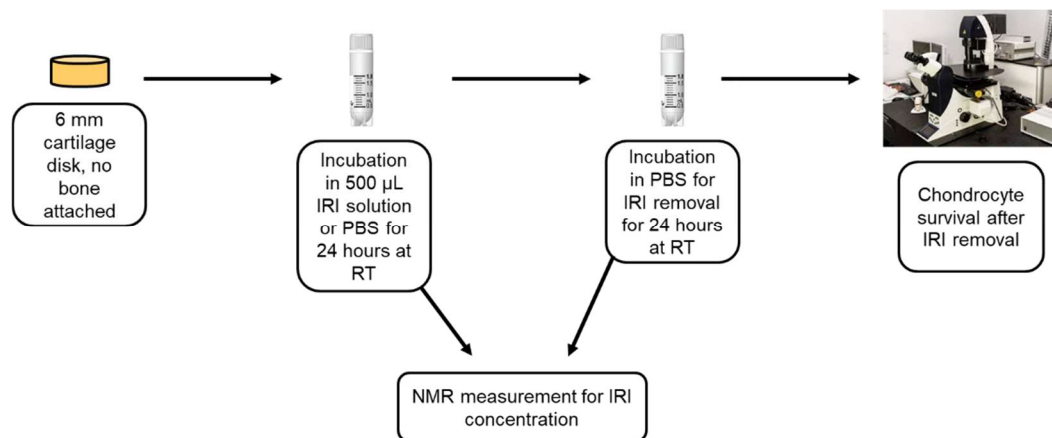


Figure 5-12. Modified experimental procedure to measure toxicity (and indirectly permeating ability) of 2FA and **505**.

cartilage disc can be observed by comparing it to the original concentration of cryoprotectant used. With both concentrations of 2FA and **505**, the cryoprotectants appear to permeate the cartilage disc to some extent and can be easily removed afterward. Taking the error associated with the measurements, little to no cryoprotectant is retained in the cartilage after washout. Although the study does not replicate standard cartilage processing but was designed to investigate the permeating ability of the two IRIs, it demonstrates that the cryoprotectants can permeate the cartilage sample and be fully removed afterward. Finally, chondrocyte survival was assessed using fluorescence microscopy. In order to assess the toxicity

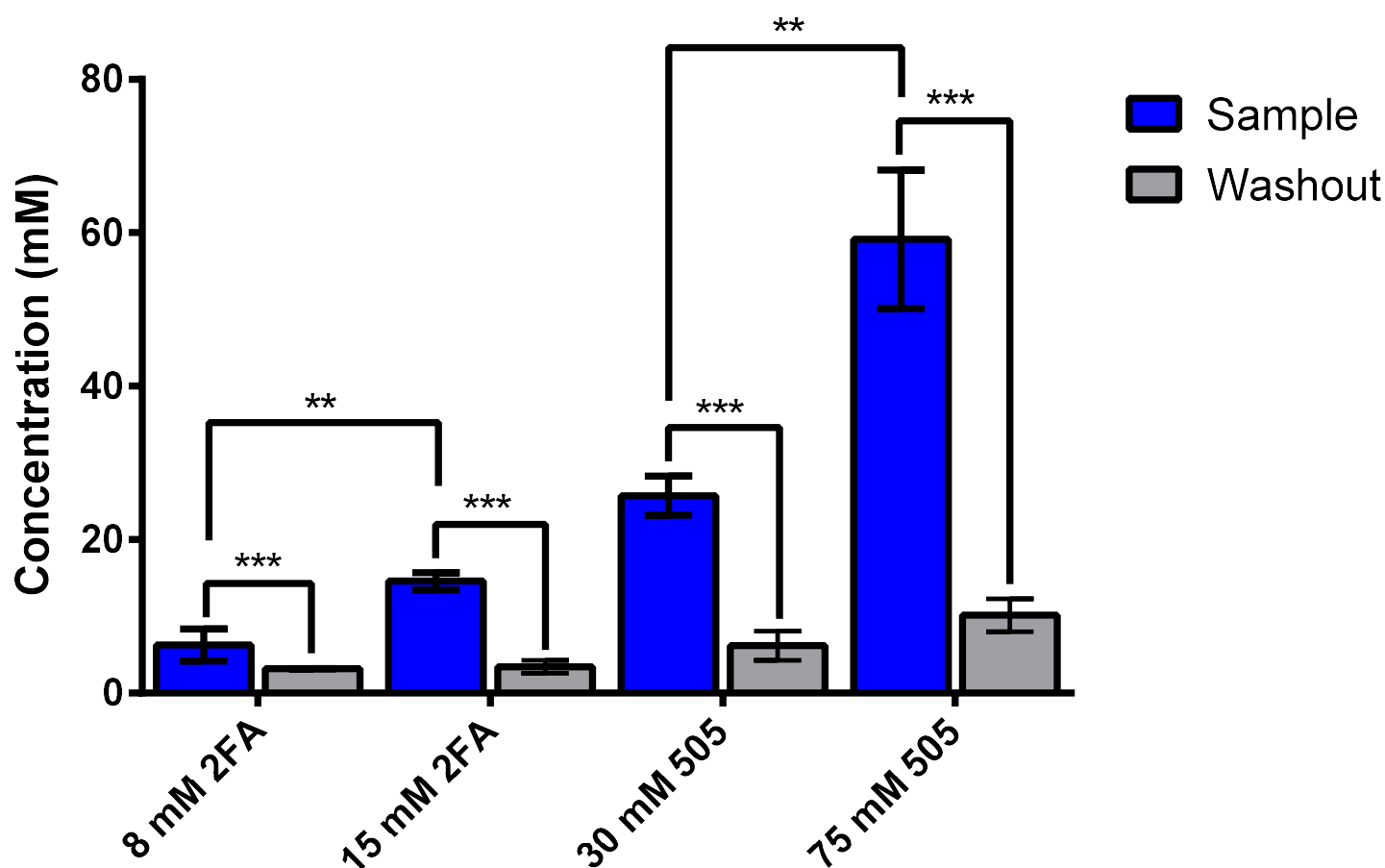


Figure 5-13. Residual concentration of 2FA (initial concentrations of 8 and 15 mM) and **505** (initial concentrations of 30 and 75 mM) in sample (initial incubation with compound) and washout (compound removed by PBS incubation). Error bars represent SEM n = 3. Asterisk represents a statistical significance between each compound and control (PBS) generated by a two-way ANOVA (**, $p > 0.01$, ***, $p > 0.001$).

and permeating ability of both compounds, a dual stain (live/dead cell) imaging study was performed. This study was conducted on whole cartilage sections to assess toxicity in the cells of interest (chondrocytes). This will also indirectly assess the difference in permeating ability of the two compounds. By looking at the localized toxicity throughout will show how far the IRIs have permeated through the cartilage sample. Using Syto-13, a green fluorescent nucleic acid stain which stains living cells coupled with propidium iodide (PI), a red fluorescent intercalating dye, a differential colour stain is achievable. This is possible due to the inability of PI to cross the cell

membrane, allowing it to only stain membrane-compromised cells.⁴⁴ No observable dead cells were detected (**Figure 6-1** Supporting Information) in any of the concentrations assessed, although this may be attributed to the whole cartilage, rather than sections being imaged. This is likely due to longer incubation times being required for whole cartilage sections. In order to confirm this, a similar experiment was performed where the cartilage disc is first sectioned into 85 μ M thick slivers before being incubated with cryoprotectant. **Figure 5-14** (top panel) shows the PBS control. Localized dead cells are observed on the superficial layer, with others spread throughout the transitional and deep zones, as well as localized dead cells on the surface of the deep zone resulting from removal of the cartilage disc from bone. Similar results are observed for both concentrations (8 and 15 mM) of 2FA, although there appears to be more toxicity in the middle of the sample compared to the control, suggesting that the cryoprotectant is able to permeate the cartilage sample. **505** was then tested at 30, 75, and 150 mM using the same conditions. While 30 mM displays little toxicity, at 75 mM more toxicity is apparent beginning from the superficial layer that travels through the transitional later to the deep layer. Similar to 2FA, **505** appears to permeate fully through the cartilage section based off the resulting toxicity. In order to confirm this, a concentration of 150 mM of **505** was also evaluated, resulting in large amounts of chondrocyte death throughout. The higher concentration of dead cells was near the superficial and deep zones, with less occurring in the center of the sample, indicating that the cryoprotectant will permeate the cartilage section, although it requires longer than 24 h to equilibrate throughout the sample. While **505** appears to be able to freely permeate cartilage sections, inherent toxicity of the compound at high concentrations results in large amounts of chondrocyte death. Altering the cationic portion of the molecule could eliminate toxicity while retaining the permeating ability of **505**.

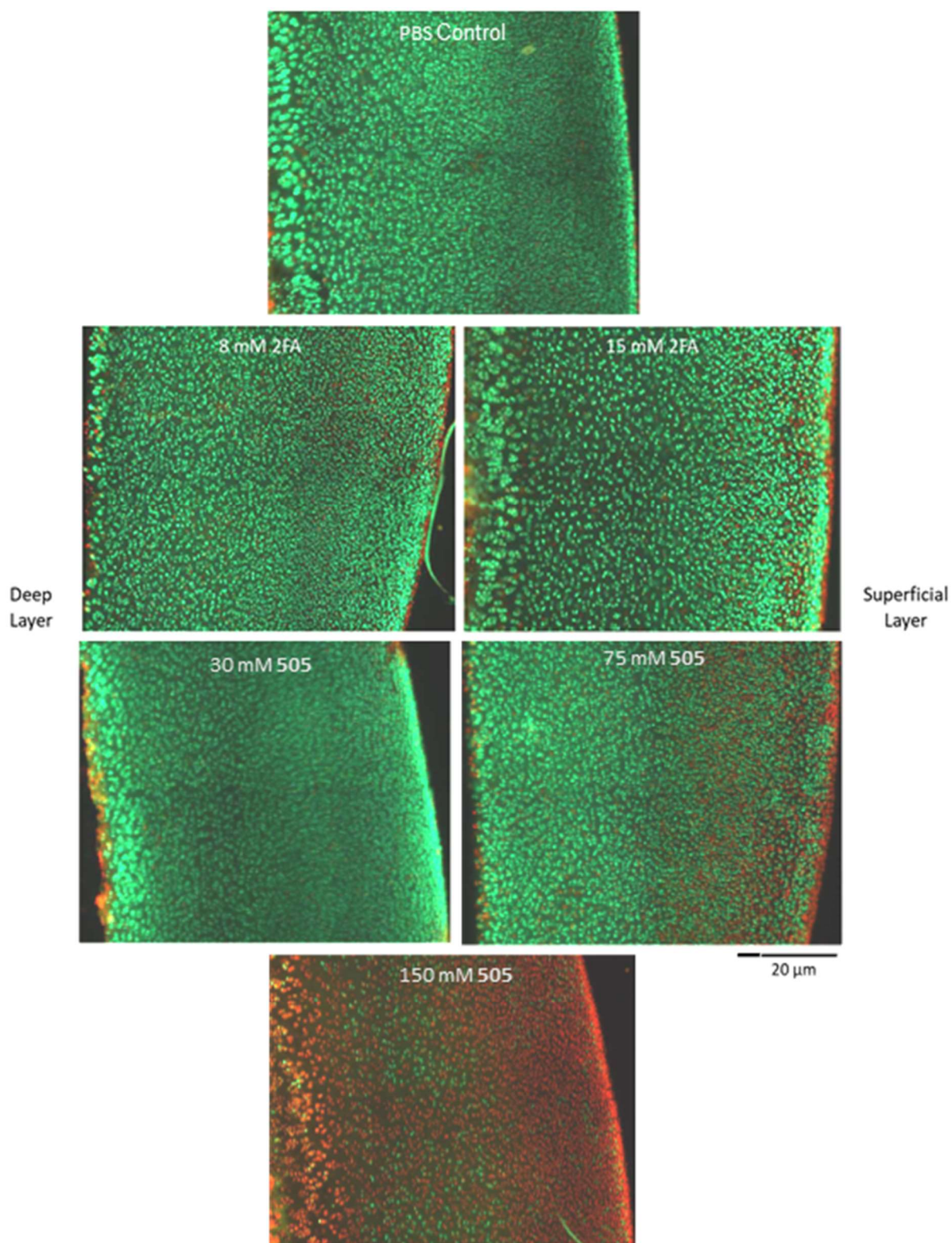


Figure 5-14. Fluorescence microscopy images of sectioned cartilage incubated with PBS (control) 2FA (8 and 15 mM) and **505** (30, 75, and 150 mM). Green fluorescence (Syto-13) shows live cells and red fluorescence shows dead (leaky – PI) cells.

5.4.3 Cryopreservation of Platelets

Platelets (thrombocytes) are a blood component necessary for clotting at wound sites.⁴⁵ As they make up only about 1% of whole blood in healthy adults, isolation of platelets is required in order to attain enough for a single unit used for transfusion.⁴⁵⁻⁴⁷ Platelet transfusion is most often used to prevent excess bleeding in patients who have low platelet numbers, usually due to cancer chemotherapy or suboptimal platelet function due to genetic conditions.^{20,48} Generally, platelets isolated from four separate are used along with plasma from one of the four donations for one unit of pooled platelets. To accomplish this, plateletpheresis is performed which isolates platelets from whole blood via centrifugation.^{46,49} Once the platelets have been isolated, they are stored at 22 °C with constant agitation for 5 days, due to risk of infection. In rare cases platelet units can be stored for up to 7 days, although the risk increases in these extended storage times. During this time, the platelet units are constantly agitated to prevent clumping of the unit, which promotes premature activation.

For longer storage or biobanking of platelets, cryopreservation is required, although this is not commonly done. The standard freezing process uses between 5-6% DMSO and cooling to -80 °C followed by storage between -65 °C to -80 °C for up to two years.^{46,50} Unlike the cryopreservation of other biological materials with DMSO, platelets are concentrated prior to freezing. This allows removal of some of the DMSO, although the frozen platelet sample retains some DMSO, which is subsequently transfused into the patient.^{46,50} The residual DMSO often results in skin irritation, mild fever, and headaches. In order to eliminate these symptoms, attempts⁴⁶ have been made in the past to reduce the amount of DMSO with the addition of another cryoprotectant, with limited success. While many additives were tried alongside DMSO, only one was tested that was an IRI. Polyvinyl alcohol (PVA) can inhibit ice recrystallization but

offered no additional benefits for platelet cryopreservation with 6% DMSO.^{46,51} Ice recrystallization during the slow cooling and warming times can be a major contributor to reduced recovery and functionality post-thaw. While PVA offered no protection during freezing/thawing, other non-polymer IRIs may increase post-thaw viability and functionality. With the addition of other IRIs, the DMSO concentration may be lowered, which in turn would lessen side effects in patients receiving platelet transfusions.

Due to the initial success of compound **505** in the increase of intact RBCs post-thaw using reduced (15%) glycerol concentrations, focus was switched to other components of blood. Blood can be split into 3 distinct components: plasma (55%), RBCs (45%), and white blood cells/platelets (<1%). Plasma is the most abundant component and can be easily cryopreserved and stored for long periods of time (~1 year) while white blood cells and platelets are present in very small quantities and have a short lifetime after isolation. This creates the need to improve the cryopreservation of platelets for long-term storage. The following experiments were based off established protocols and conducted by the Johnson lab (Australian Red Cross Lifeblood) by Lacey Johnson and Lauren Waters.

To assess platelet functionality post-thaw, several factors are typically assessed, these include unit weight\volume, pH, presence of specific glycoproteins or surface markers, as well as the presence of metabolic markers such as lactate and glucose.^{45,47,49} Compounds 2FA and **505** were selected as the two novel IRIs to be assessed in platelet cryopreservation. By



Figure 5-15. Whole platelet units with 0 (control), 30, 75, and 200 mM **505** at 0 hours post thaw (A), and 24 hours post thaw (B).

using a previously studied (neutral) IRI 2FA in conjunction with a new charged IRI (**505**) the differences in protection during cryopreservation can be compared between a known cryoprotectant and a novel one.

Prior to freezing, using standard platelet procedures various platelet units were ABO-matched and pooled, after which DMSO was added to achieve a final concentration of 5-6% DMSO. The pooling of plates from multiple donors is necessary to achieve enough platelets for transfusion. The platelets must be ABO-matched to avoid severe immune responses, this is done

by combining samples with the same surface antigen. Next, units were centrifuged to obtain a hyperconcentrated platelet component and the majority of the DMSO-containing supernatant was removed prior to freezing at -80 °C. After thawing, platelet units were reconstituted in a unit of plasma and stored at room-temperature with constant agitation prior to testing immediately after thawing and at 24-hours post-thaw.

The standard measurements for platelet cryopreservation post-thaw examines 3 categories of analysis: recovery of the platelets themselves, metabolism, and activation. The first category, recovery, can be assessed with the weight/volume of the sample as well as flow cytometry counts. pH is the major factor to be examined that relates to metabolism, along with pCO₂ (partial CO₂ pressure), pO₂ (partial O₂ pressure), HCO₃⁻ (bicarbonate ion concentration), TCO₂ (total CO₂), sO₂ (oxygen saturation), and lactate measurements. Finally, activation can be assessed by looking at the presence and concentration of a number of surface markers. ^{20,45,50,52}

Initial parameters investigated were the weight, volume, and colour of the different concentrations immediately and 24-hours post-thaw. Both weight and volume of all the units remained constant between units and incubation time post-thaw. Immediately after thawing of the units, all trials appeared to have the same colour, although upon the 24-hour incubation, the IRI-containing units darkened (green to brown) in a dose-dependent manner (**Figure 5-15**). The source of the colour change is currently unknown but could arise from degradation of the IRI over the incubation time by plasma enzymes or IRI-protein interactions.

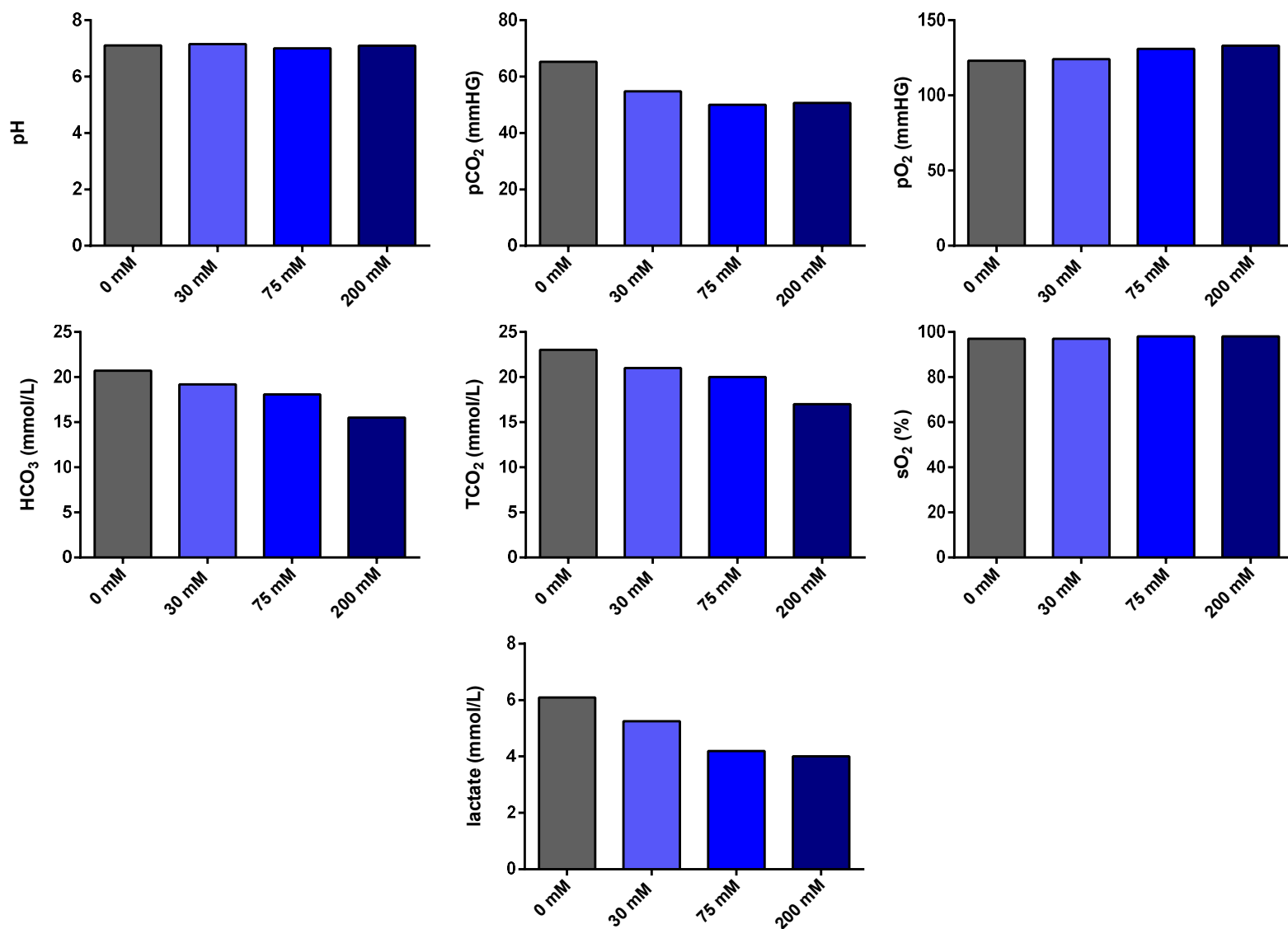


Figure 5-16. iSTAT measurements of pH (0 hours post-thaw), partial CO₂ (pCO₂), partial O₂ (pO₂), bicarbonate ion concentration (HCO₃⁻), total CO₂ (tCO₂), O₂ saturation (sO₂), and lactate concentration (lactate) all preformed 24 hours post-thaw. Using **505** as a cryoprotectant. iSTAT analysis or n = 1.

Next, the platelet recovery (cell count) and volume were assessed using two separate flow cytometry analyzers, cell-dyn ruby and emerald hematology analyzers.^{45,53} Emerald recovery showed similar recoveries between all trials, with a slight increase in recovery in a dose-dependent manner immediately after thawing, with the mean volume remaining constant between through all trials and incubation times (**Figure 6-2/6-3** Supporting Information). Upon

24-hour incubation, the cell count decreases as the concentration of **505** increases, suggesting that compound toxicity which could also be responsible for the colour change observed in the 24-hour period post thaw. In order to confirm regular platelet function after freezing (post-thaw), metabolism and function of the platelets must be measured. pH is a major factor considered with PSL and aggregation ability.^{45,49,50} Measurements were taken 24 hours after thawing and included pH, pCO₂ (partial CO₂ pressure), pO₂ (partial O₂ pressure), HCO₃⁻ (bicarbonate ion concentration), TCO₂ (total CO₂), sO₂ (oxygen saturation), and lactate measurements (**Figure 5-16**). While **505** did not affect many of these parameters, there were slight deviation in HCO₃⁻ and lactate levels which relate to TCO₂ levels. As the concentration of **505** increases, there is a slight decrease in partial CO₂, decrease in total CO₂ is also observed. The slight decrease is usually a result of hypoxia or pH imbalance. Since the pH remains constant between the control and all concentrations of **505**, the pCO₂ reduction is likely due to hypoxic conditions. Overall, the slight reductions in levels of CO₂ and lactate do not suggest changes in metabolism post-thaw.

One of the most important properties to consider during platelet cryopreservation is the activation post-thaw. Ideally, platelets should show similar or lower activity compared to the untreated controls. Premature activation of platelets is associated with decreased post-transfusion efficacy, in part due to decreased signaling function and circulation times. To assess the platelet activation post-thaw, a number of surface proteins/glycoproteins can be quantified. An increase of Annexin V, CD62P, CD42a suggest more activated platelets opposed to CD42b, CD61 and GPIIb/IIIa, which should remain constant pre- and post-activation. **Figure 5-17** shows the percent of platelets expressing each marker compared to an untreated platelet unit immediately after

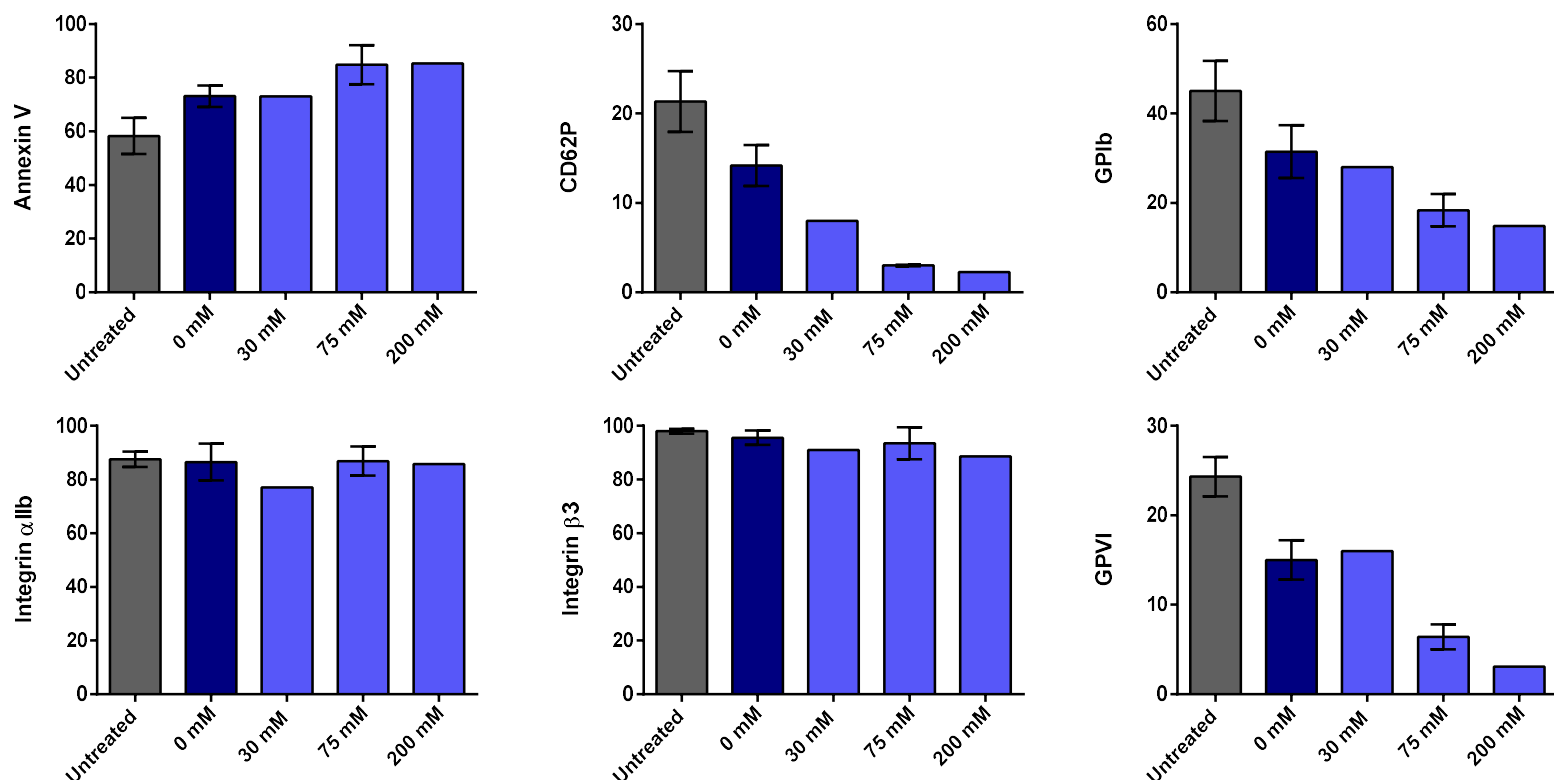


Figure 5-17. Percentage of platelets expressing Annexin V, CD26P, GPIb, Integrin αIIb, Integrin β3, and GPVI surface markers. Using **505** as a cryoprotectant. Error bars represent SD. Note, analysis of 30 and 200 mM only conducted on one platelet unit (n = 1). Otherwise n = 2.

thawing. The first panel shows Annexin V, a phosphatidylserine binding protein located on the surface of platelets necessary for blood coagulation.

An increase of expression of Annexin generally relates to increased platelet activation or apoptosis, due to its ability to bind procoagulant factors.^{54,55} The next platelet activation marker to be investigated was CD26P, also known as P-selectin. CD62P is another membrane glycoprotein associated with platelet clotting ability. Upon platelet activation, CD26P is expressed via exocytosis, and mediates interactions between platelets and leukocytes during clotting.⁵⁶⁻⁵⁸ As with Annexin V, an increase in expression suggests increased activation. Unlike Annexin V levels, CD62P expression is drastically reduced in a dose-dependent manner with **505**, which may show decreased platelet activity. Glycoprotein Ib (GPIb or CD42b) is

responsible for von Willebrand factor (vWF) recognition and binding, adhering platelets to the sub endothelium of wound sites during thrombosis.^{56,59} GPIb followed the same trend as CD26P, decreasing in a dose-dependent manner with **505**. While the decrease in CD26P suggests lower levels of activation, there should be no change in GPIb expression in response to platelet activation.^{60,61} The drop in expression could be indicative of microparticle (MP) formation, due to stresses of the freezing/thawing of the unit. MP formation is an undesirable effect of platelet cryopreservation and suggests that there is little protection from the cryopreservative.^{62,63}

Next, integrins α IIb and β_3 (CD41 and CD61 respectively) were analyzed. These two membrane glycoproteins form a calcium-dependent complex and are responsible for platelet aggregation via binding of vWB and fibrinogen, resulting in crosslinks between platelets at the wound site. As with GPIb, both integrin α IIb and β_3 expression remains constant during activation and therefore should have levels consistent to the untreated controls.^{58,64–66} While the levels of both integrins remain constant over the concentrations assessed, all values were larger than expected and were closer to values found on platelets in the presence of an agonist. While the presence of a platelet agonist would explain the elevated integrin levels, it is unlikely that there is one present in all trials. Finally, glycoprotein VI (GPVI), an important collagen receptor responsible in binding and once bound, secreting various compounds that assist in recruitment of additional platelets and platelet aggregation. The drastic decrease in GPVI is most likely a result of MP formation.^{58,61}

To fully assess platelet quality prior to cryopreservation, post-thaw unit composition should also be considered alongside changes in platelet activity. Microparticle release from platelets can easily be observed and is indicative of platelet activation, stress, and apoptosis. Microparticles (MPs) are small (0.1-1 μ m) phospholipid-based particles shed from platelets

undergoing activation, in response to external stresses, and during apoptosis. MPs retain some functional receptors present on platelets and are linked to increased platelet function during hemostasis and thrombosis. While MP release is considered beneficial during thrombotic events, generally MP release is regarded as disadvantageous during platelet storage and cryopreservation and is associated with increased thrombotic and inflammatory side-effects in patients receiving platelet units.^{62,63,67} Platelet composition can be measured using a number of techniques, with flow cytometry being one of the most common methods. **Figure 5-18** shows the number of MPs/unit and the unit composition post-thaw. Microparticle release drastically increases with the concentration of **505** likely as a result of either increased activation or stress due to cryopreservation. Both increased platelet activation and stress result in higher number of MPs, as both are experienced during cryopreservation with **505**, and therefore should be the reason for increase in MPs.⁶²

As for the platelet unit composition, the percentage of platelets versus MPs vary over the concentrations observed, with no observable trend. The percentage of microparticles was higher than expected for all trials, although large variation was observed between replicates.

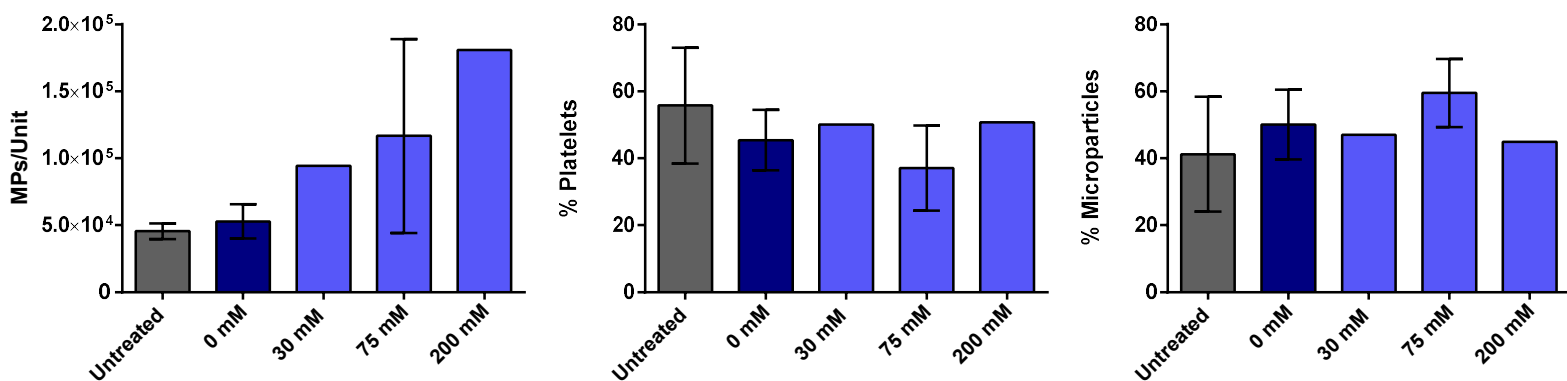


Figure 5-18. Microparticle number/unit and composition (% platelets + % microparticles = 100%) of platelet units 0 hours post-thaw. Using **505** as a cryoprotectant. Error bars represent SD. Note, analysis of 30 and 200 mM only conducted on one platelet unit. Otherwise n = 2.

While the increased platelet recovery at 0 h post-thaw as well as the reduction in CD26P suggest less activation and protecting from **505** during cryopreservation, all other factors assessed show either no affect or reduced quality of the platelets due to cryopreservation with **505**. This was also confirmed with the reduced recovery 24-hours post-thaw. Reduced externalization of CD26P is indicative of reduced platelet activation, but surface CD26P can also be reduced via cleavage of the protein or secretion via MP release. In order to assess whether or not CD26P reduction was due to less externalization or via cleavage/secretion, an ELISA for CD26P was performed.

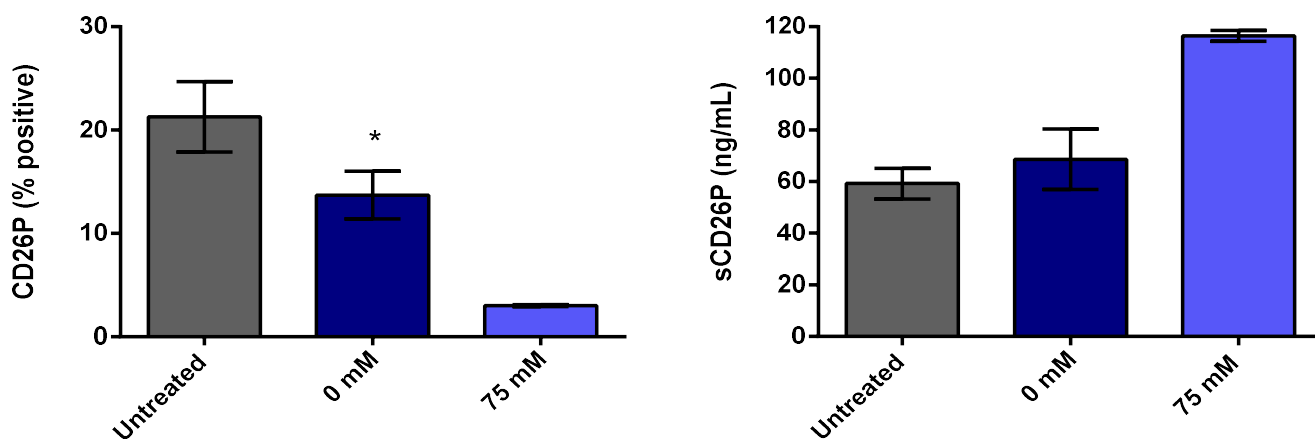


Figure 5-19. CD26P expression on platelets (A) and soluble CD26P levels 0 hours post-thaw. Error bars represent SD n = 4 (untreated and 0 mM) otherwise n = 2. Asterisk represents a statistical significance between compound and control (Untreated) generated by student's t-test (*, $p < 0.05$ and **, $p < 0.01$).

Figure 5-19 shows a drastic decrease in platelet bound CD26P in the presence of 75 mM **505** along with an increase in soluble (sCD26P) which represents the receptor being cleaved due to activation or MP formation.

Overall, **505** does not provide protection to platelets during the cryopreservation process. This is evidenced by the increase in shearing of CD26P, increased MP formation, and decreased

expression of CD41b/ GPVI, all of which are indicative of either increased platelet activation and/or increased damage due to cryopreservation or toxic effects of **505**.

5.4.4 Cryopreservation of Hematopoietic Stem/Progenitor Cells (HSPCs)

Hematopoietic stem and progenitor cells (HSPCs) are often used in regenerative medicine for the treatment of hematological cancers such as leukemias and lymphomas.^{68–70} HSPC transplantation can also be used to treat patients suffering from other hematological diseases, immunodeficiencies, and anemias. HSPCs can be isolated from a number of sources although umbilical cord blood (UCB) remains one of the simpler ways of isolation.^{71–73} One problem associated with HSPC transplantation is the availability of fresh samples with a positive HLA match. Isolated HSPC samples must either be used within 72 hours post-collection or cryopreserved for use at a later date.

Typical freezing procedures use 10% DMSO in saline/dextran coupled with slow (1 °C/min) cooling rate to -80 °C followed by storage at -196 °C. While this freezing procedure results in post-thaw viabilities around 70 %, the cytotoxicity of DMSO is a major disadvantage to this process. DMSO can be removed pre-transplant, although this process is time consuming, expensive, and results in decreased cell survival and function. A strong correlation between number of cells transplanted and engraftment rate in UCB samples has been shown in clinical trials, supporting the need for improved methods of cryopreservation.^{43,74}

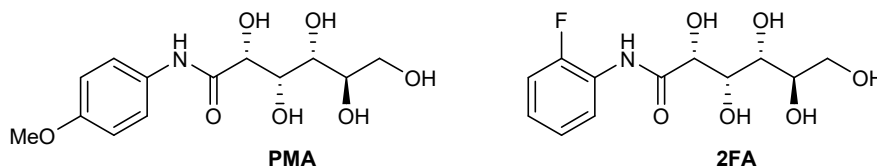


Figure 5-20. Structures of IRIs *N*-4-methoxyphenyl-D-gluconamide (**PMA**) and *N*-2-florophenyl-D-gluconamide (**2FA**).

Previously, the Ben lab has explored the use of reduced concentrations of DMSO coupled with novel IRIs^{43,75,76} for the cryopreservation of umbilical HSPCs. Using compounds PMA and 2FA with 10% DMSO, the post-thaw viability was increased even using low (5-11 mM) concentrations due to limited solubility. While these trials still used 10% DMSO, post-thaw recovery was increased above the levels of 10% DMSO alone. Using similar compounds that are more soluble would allow cryopreservation using elevated levels of IRI and potentially decreased concentrations of DMSO, minimizing cytotoxicity of DMSO.

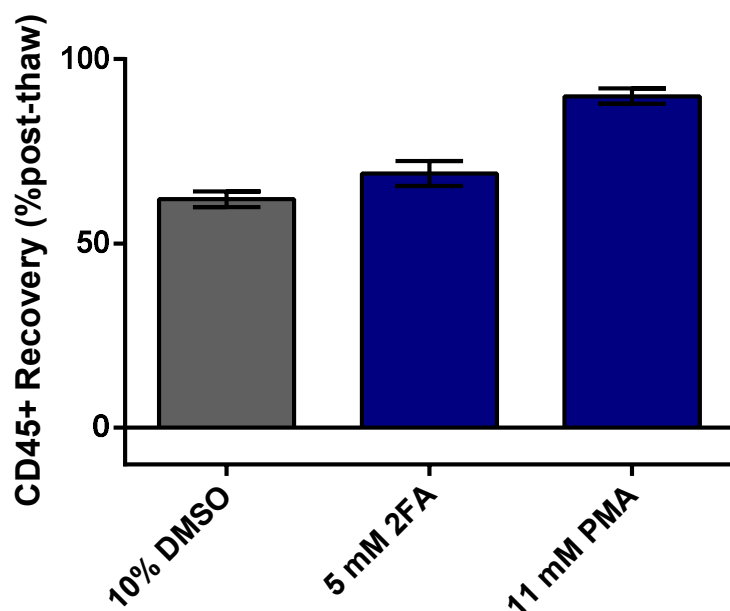


Figure 5-21. CD45+ cell recovery of HSPCs frozen with IRIs 2FA and PMA. Post-thaw percentage shown compared to the initial pre-frozen CD45+ cell values. Error bars represent SEM $n = 3$.^{43,75}

With the successes of previous IRIs (PMA and 2FA) for the cryopreservation of HSPCs,⁴³ new compounds showing IRI activity were explored. Subsequently, **505** was one compound selected due to its IRI activity ($IC_{50} = 39$ mM) and high solubility (>100 mM without heating). Similarly, to other cryopreservation procedures mentioned, the cryosolution must be heated to fully dissolve the IRI which is undesirable in most applications. As the previous IRIs were used

at their solubility limit, extensive heating is required, and due to the slow cooling method employed, the IRIs could precipitate out of solution as the sample is frozen. Due to the high solubility of **505**, no heating is required for concentrations >100 mM, making it ideal for HSPC cryopreservation.

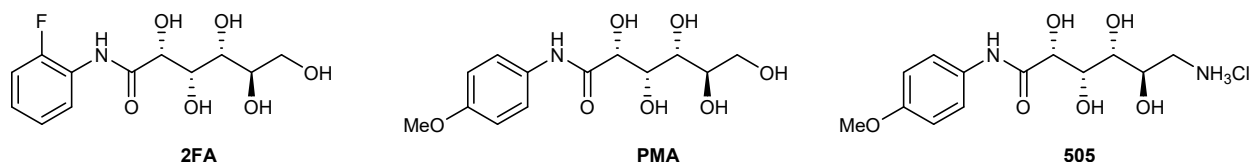


Figure 5-22. Structures of 2FA, PMA, and **505**.

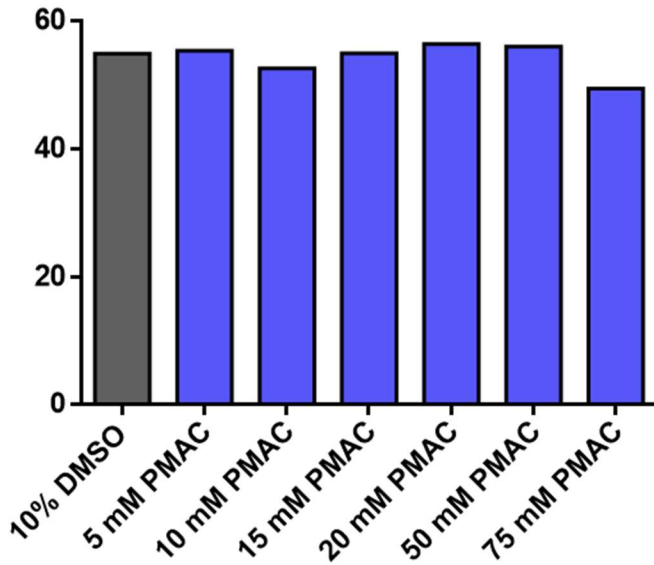
Compound **505** was assessed over a range of concentrations in two separate experiments, post-thaw viability and colony forming unit (CFU) assay. After isolating HSPCs from the umbilical cord blood unit (supporting information) it was split into aliquots before being centrifuged and resuspended in corresponding cryosolutions. Compound **505** was supplemented into the cryosolution at 5, 10, 15, 20, 50, and 75 mM, all containing 10% DMSO in saline/dextran. Once the cryosolution was added, samples were frozen to -80 °C at an approximate rate of 1 °C/min. After holding this temperature overnight, samples were transferred to N₂ (*l*) for storage. Upon thawing (37 °C water bath for 90 seconds) 900 µL Iscove's Modified Dulbecco's Media (IMDM - supplemented 10% FBS, 1% pen/strep) was added and samples were stored on ice until use.

First, the post-thaw recovery was assessed using flow cytometry, looking at both counts of total nucleated cells (TNC) as well as CD45⁺ cells as well as CD45⁺CD34⁺ stem cells. TNC is a useful measure due to its reproducibility between labs and is easily obtained but must be accompanied by other cell marker analysis. CD45 is a transmembrane receptor unique to hematopoietic cells that is responsible for signal transduction during hematopoiesis (formation of cellular blood elements).⁷⁷ To determine the recovery of stem cells, CD45⁺CD34⁺ cells are

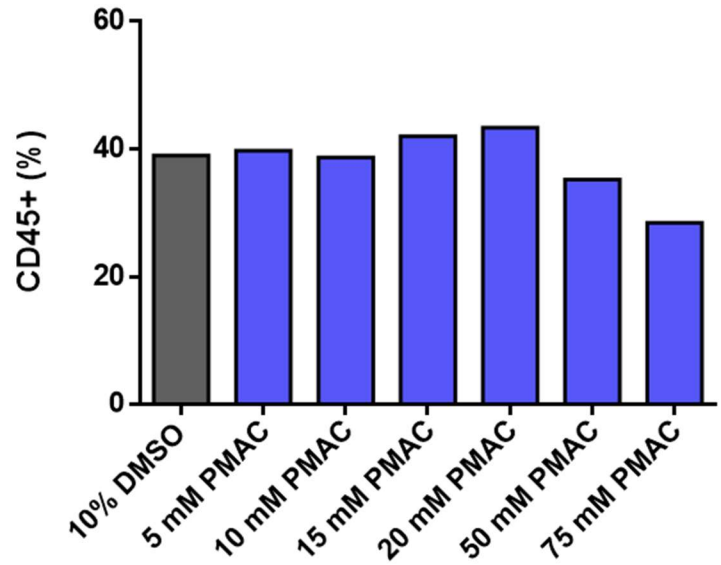
examined, and usually represent around 1% of the total cells found in UCB.^{43,71} CD34 is a transmembrane glycoprotein unique to specific stem/progenitor cells, including hematopoietic stem/progenitor cells found in UCB.⁷¹ CD34 along with CD45 (CD45+CD34+ cells) is used to quantify the population of stem/progenitor cells in UCB samples. Post-thaw viability represents the ability of **505** to protect during the cryopreservation process and is shown in **Figure 5-23**. Both recoveries were evaluated on a single UCB unit due to availability of UCB units and time/compound availability.

The amount of total nucleated cells (TNC) remains consistent between the control and all concentrations of **505** (**Figure 5-23 A**), showing no increase in recovery over 10% DMSO. Although there is no benefit of **505** on TNC, there does not appear to be any toxic effects to the cells, which have been observed in other cell types. This may be attributed to differences in cellular membrane, metabolism, or the minimal incubation time with **505** overall. Next, specifically CD45+ cells were assessed using CD45-allophycocyanine (APC) antibodies (**Figure 5-23 B**). CD45+ cells are a subset of the TNC and represent leukocyte cells bearing the CD45 transmembrane protein. CD45 acts as a tyrosine phosphatase in order to regulate signals during hematopoiesis.⁷⁷ As this is a subset of the TNC, the overall percentage is lower as expected, and similarly to the TNC is relatively consistent between all concentrations tested. With the highest two concentrations (50 and 75 mM) there may be a slight decrease in CD45+ cells, although additional testing would be required to determine if this is statistically significant. Finally, the last subset of cells; CD45+CD34+ cells were assessed. These represent the amount of stem cells as CD34+ is unique to hematopoietic stem/progenitor cells in UCB and bone marrow.^{7,68,78}

A



B



C

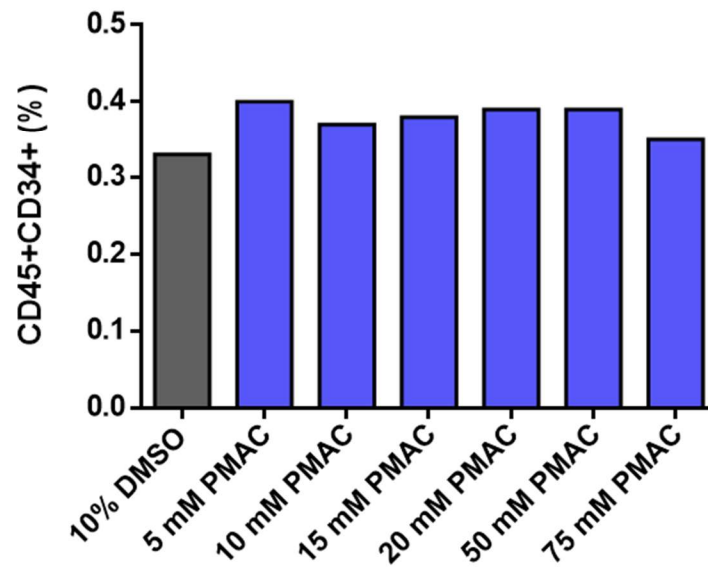


Figure 5-23. Percentage of total nucleated cells (TNC), CD45+ cells, and CD45+/CD34+ stem cells analyzed by flow cytometry. After cryopreservation with **505**. Analysis done on a single UCB unit, n = 1.

CD34 can also be expressed on various other stem/progenitor cells from other sources, although these types of cells should not contribute to the overall population in UCB samples. The

amount of CD45⁺CD34⁺ cells should be the smallest population of cells and should represent approximately 1% of the total number of cells in the original UCB unit.^{7,71,77} **Figure 5-23 C** shows the overall percentage of these cells, and similarly to the other trials the percentage is relatively constant between the concentrations of **505**, although they appear to be slightly higher than the DMSO control. While additional UCB units would be needed to validate this, compound **505** does not appear to be toxic to this population of cells. To assess functionality of the cells post-thaw, a colony-forming unit (CFU) assay was performed on the same unit with the same concentrations of **505** (0-75 mM) and is summarized in **figure 5-24**. This assay uses a methylcellulose-based semi-solid growth medium specifically formulated for hematopoietic stem/progenitor cells. In this experiment, thawed and washed samples were mixed with the growth medium and transferred to culture dishes and were allowed to proliferate over 14 days in an incubator before being scored.

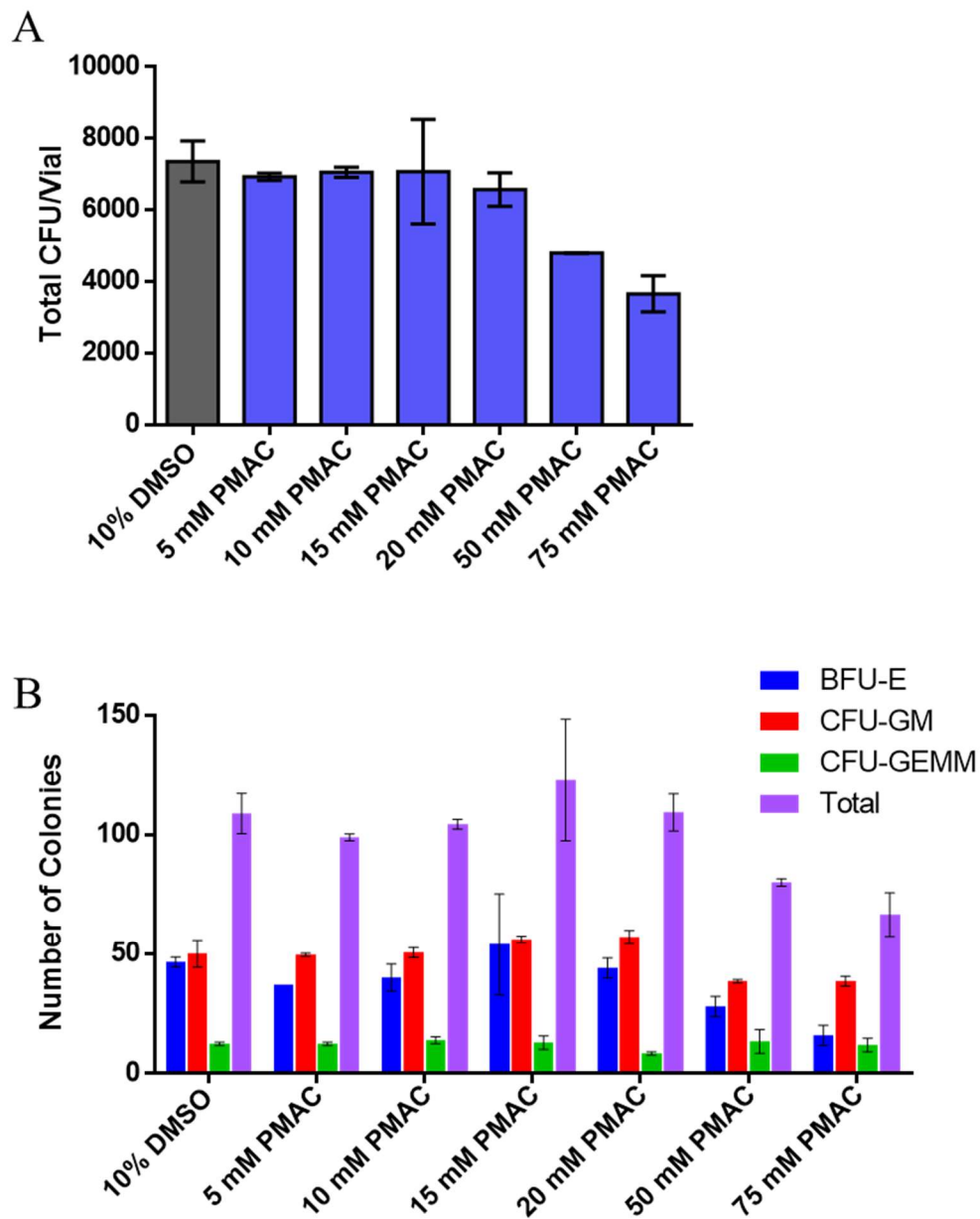


Figure 5-24. A - Total colony forming units (CFUs)/vial of 10% DMSO control, and 5, 10, 15, 20, 50, 75 mM of **505** in 10% DMSO. B – breakdown of specific colonies/plate for 10% DMSO (control) and 5, 10, 15, 20, 50, 75 mM of **505** in 10% DMSO. Error bars represent SEM, n = 2.

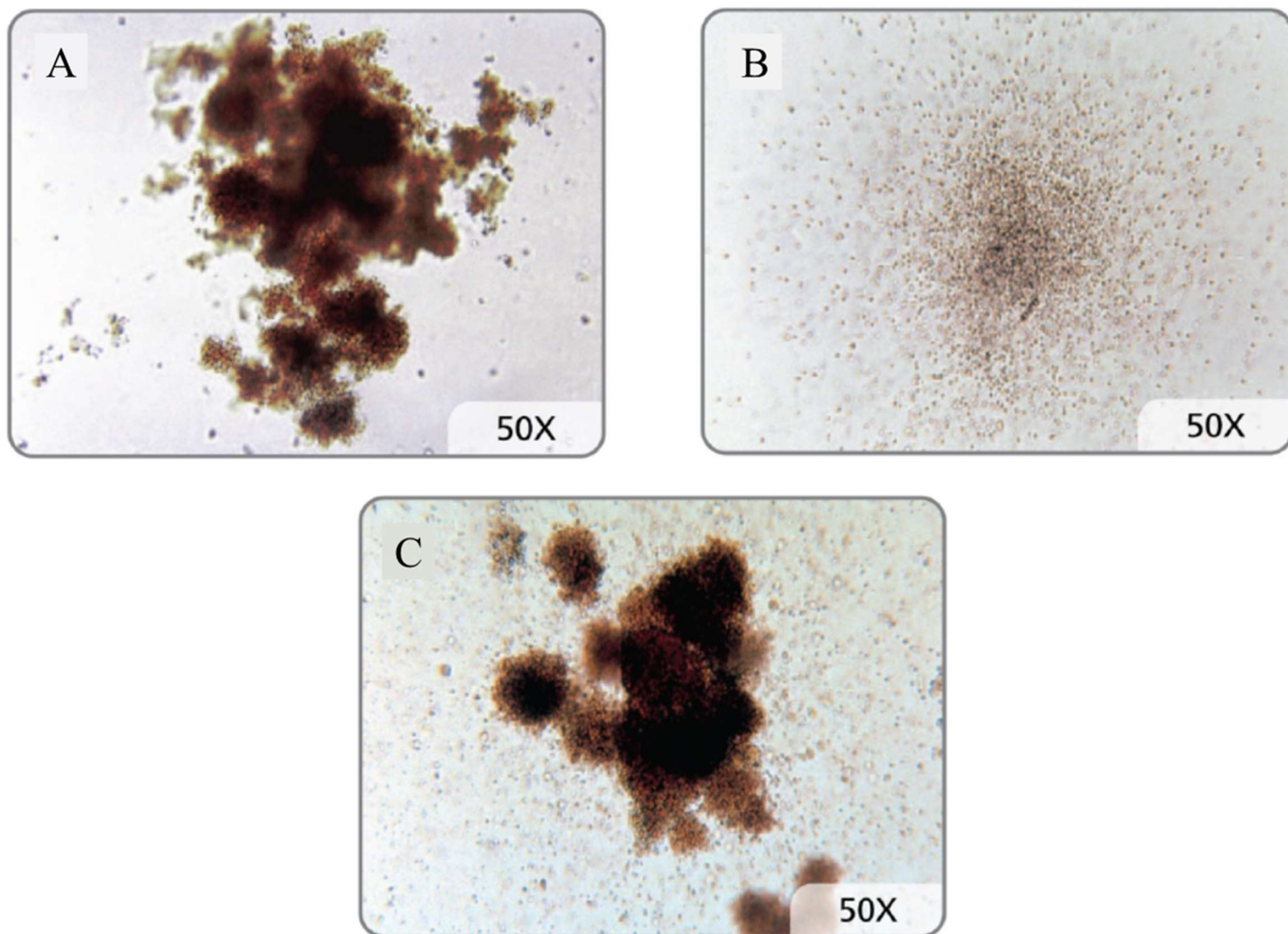


Figure 5-25. Morphologies of BFU-E (burst-forming unit-erythroid (A), CFU-GM (colony-forming unit-granulocyte, macrophage (B), and CFU-GEMM (colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (C) colonies.⁷⁹

Plates were scored for three specific colonies as well as total number of colonies/vial.

The colonies counted were BFU-E (burst-forming unit-erythroid, **Fig. 5-25 A**), CFU-GM (colony-forming unit-granulocyte, macrophage, **Fig. 5-25 B**), and CFU-GEMM (colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte, **Fig. 5-25 C**).⁷⁹

From the number of colonies counted/plate, the total colonies/vial of UCB could be calculated, shown in **Figure 5-25 A**. Compared to the DMSO control, 5-20 mM **505** appear to have consistent total CFUs/vial, while 50- and 75-mM decrease in a dose-dependent trend. The breakdown of specific types of colonies is pictures in **Figure 5-25 B**, along with the total number of colonies/plate. In most conditions, CFU-GM are the most numerous, followed by BFU-E, with CFU-GEMM being the least numerous. This distribution is typical in UCB CFU assays,⁴³ although the distributions in 50 and 75 mM are overall smaller, with both trials retaining the number of CFU-GEMM as the control and other trials.

While the overall decrease in number of colonies can be attributed to compound toxicity, distribution of specific types of colonies may offer more insight into their properties. Interestingly, while CFU-GEMM colonies are least numerous in all trials, they remain consistent throughout. The highest concentrations of IRI resulted in toxicity, and an overall reduction in colonies, although even in these trials CFU-GEMM remained consistent with the lower concentrations. Since CFU-GEMM represent the least differentiated colony type (first cells in hematopoiesis after HSPCs) they exist in small quantities compared to other colony types. As these cells are less differentiated, they are inherently more robust than more differentiated cell types. This may be the reason for the consistent levels of CFU-GEMM observed throughout all concentrations of IRI. The more differentiated colony types (BFU-E and CFU-GM) lose some of the inherent robustness and is likely the reason for the observed toxicity at the highest two (50 and 75 mM) concentrations.

In conclusion, while **505** does not offer any additional protection during cryopreservation compared to 10% DMSO, there seems to be minimal toxicity to HSPCs, in stark contrast to other cell types observed. This may be attributed to the overall more resilient nature of less

differentiated cell lines. Overall, **505** does not seem to have as severe toxic effects on HSPCs as other cell lines, but also does not provide additional protection over the standard currently used; 10% DMSO. Since **505** is much more soluble than current IRIs investigated for HSPC cryopreservation, modification of the amine portion of the compound could provide better protection during cryopreservation, while retaining the greatly increased solubility compared to current IRIs.

5.5 Chapter Summary

Solubility of current IRI active molecules developed by the Ben lab is an ongoing problem due to the need for a hydrophobic moiety needing to be present alongside a hydrophilic portion required for IRI activity. Many current gluconamide IRIs show promise as novel cryoprotectants in a number of biological systems, the low solubility requires extensive heating to fully dissolve in cryosolutions. As some cryosolutions contain heat sensitive components, heating may not be possible. The solubility also limits the concentrations of the compound that can be assessed. Increasing the solubility of these IRIs would eliminate the need for heating and allow much higher concentrations to be assessed. With the previous assessment of anionic IRIs (sulfates), the length of synthesis and resulting properties do not offer any benefits over current IRIs. Few cationic molecules have currently been investigated for cryopreservation, and the addition of a cationic functional should work to increase the solubility of current IRIs. Amine functional groups can easily be installed on sparsely soluble gluconamide derivatives and should increase the solubility of these compounds.

Overall, **505** appears to be very toxic in some of cell lines (HepG2, chondrocytes, platelets) and somewhat toxic in other cells (RBCs, HSPCs) but only in high concentrations. As all these cell types possess very different properties, metabolism, and function, a specific cause

of toxicity cannot be determined, although, it appears to be due to the C6 amine, as the parent compounds are not toxic. While the toxicity is a problem with most of the cell types, cryopreservation with reduced glycerol concentrations and **505** seems promising. Much more optimization of cryopreservation conditions, concentration, and possibly the addition of an additional cryoprotectant in conjunction with **505** and 15% glycerol need to be explored.

Since the C6 amine appears to be the source of the toxicity issues associated with **505**, modifications to that part of the molecule can be conducted in order to retain the increased solubility. Exploring additional functional groups that could be added to the amine portion should retain most of the solubility, while potentially reducing toxicity.

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Chapter 6. Thesis Conclusions

6.1 Conclusions

The overall goal of this thesis was to synthesize novel, charged, carbohydrate-based molecules to be used as ice recrystallization inhibitors (IRIs). This first involved installing highly polar sulfate groups on select pyranose derivatives in hopes of disrupting bulk water and in turn, displaying IRI activity. Also, by installing a sulfate onto a preexisting alcohol, the hydrogen bonding abilities were altered. All sulfated derivatives synthesized were assayed for their IRI activity, and the most promising compound(s) were tested for the cryopreservation of red blood cells (RBCs) with reduced concentrations of glycerol. Dose-response curves were also generated for promising compounds. Next, focus was switched to sulfating a new class of IRIs, aldonamide derivatives. Many of the aldonamides displayed IRI activity but were sparsely soluble in aqueous media. Sulfation of these derivatives was done to increase their solubility, while ideally retaining their IRI activity. Compounds with the highest IRI activity compounds were assayed for the cryopreservation of RBCs. Finally, rather than the addition of sulfate groups to previous IRI active molecules, amines were selected due to their high polarity. These amino aldonamides were examined in HepG2 cells to obtain LD₅₀ values and assess their toxicity. Compound **505** was then assayed for the cryopreservation of several biological products including RBCs, articular cartilage, platelets, and hematopoietic stem/progenitor cells (HSPCs).

The first objective of studying the effect of installing sulfate groups on preexisting IRI-active carbohydrates involved the synthesis of several glucose-derived IRIs and one galactose derivative. Sulfate groups were installed at various positions around the pyranose rings, with a focus on the C6 position. Many of the compounds displayed less IRI activity when compared to their parent compound, with only two compounds (**329** and **336**) showing higher IRI activity

than their parent compounds. This suggested that the hydrogen bond donating ability of alcohols may play an important role in modulating IRI activity. This was most apparent on compounds **344** and **350**. These sulfated compounds are derivatives of compound **129** (β -PMP-Glc), which displays a high degree of IRI activity. In both cases, the IRI activity is reduced, highlighting that small changes to IRI-active molecules can drastically alter their activity. While these derivatives did not show benefits over previous IRIs, they still showed promise as cryoprotectants. For this reason, dose-response curves were generated, and select compounds were tested for the cryopreservation of RBCs. Although some of the assayed compounds displayed similar benefits to previous IRIs, the increased length of synthesis of these molecules eliminated them as useful cryoprotectants. While the compounds investigated did not offer greater cryoprotection compared to previous IRIs, further studies could look at using these compounds in situations that may benefit from the use of a charged IRI.

Following the synthesis and determination of IRI activity of the sulfated pyranose derivatives, focus was shifted to an emerging class of IRIs, referred to as aldonamides. These aldonamides result from the ring opening of pyranoses with an amine, forming an open-chain derivative locked in the configuration by the resulting amide that is formed. Many of these aldonamide derivatives showed significant IRI activity, many were sparsely soluble in aqueous systems. This prevented their use at higher concentrations and required substantial heating (>80 °C) which greatly limits the application of these derivatives. To increase the solubility of the aldonamides, polar functional groups can be installed. Based on their use for pyranose derivatives, sulfate groups were ideal candidates for this. Due to ease of synthesis, the C5 position of the aldonamides were sulfated and a number of alkyl-, phenyl-, and benzylamines were used in the ring-opening. The addition of a sulfate was able to greatly increase the

solubility of the aldonamides, all displaying a solubility over 100 mM in water and PBS. While all of the derivatives were much more soluble than their parent compounds, many displayed a lowered degree of IRI activity. Since the parent compounds displayed such a high degree of IRI activity, a reduction in activity was tolerated. The most active of these compounds were then investigated for the cryopreservation of RBCs with reduced (15%) glycerol concentrations. Some of the sulfated aldonamides displayed comparable protection to existing IRIs used for RBC cryopreservation, others resulted in total RBC hemolysis because of the detergent-like properties. This arose from the combination of a long alkyl chain and polar sulfate. This eliminated them for cryoprotectants for biological systems. While the derivatives showing comparable benefits to existing IRIs, the length of synthesis eliminated their use as RBC cryoprotectants. Due to the solubility of these derivatives, they can show promise as cryoprotectants in applications using heat-sensitive components. Further study by using differently substituted amines may prove beneficial for new cryoprotectants.

Finally, due to the lack of promising targets arising from the sulfated IRI derivatives, focus was switched to other, highly polar functional groups. Since amino groups have not been well studied as IRIs, they were selected for their use in synthesizing amino aldonamides. Due to synthetic problems, the C6 position was chosen for the installation of the amino functional group, opposed to the C5 which was initially investigated. As with the sulfated IRI derivatives, the solubility was greatly increased when compared to the parent compound, with both amino aldonamides showing solubilities over 500 mM in water and PBS. This allowed for a wide range of concentrations to be investigated, and while these compounds were not as IRI active than their parent compounds, using a higher concentration was feasible. Although, using high concentrations of IRI can lead to cytotoxicity. HepG2 cells in an AlamarBlue assay were used to

generate LD₅₀ values of the compounds. Both compounds displayed a relatively low LD₅₀ between 30 and 40 mM, indicating that higher concentrations may generate problems in some cell types. Regardless, these compounds were first assessed for RBC cryopreservation, and compound **505** showed a large improvement over the 15% glycerol control. This compound was further studied in RBCs, with concentrations up to 200 mM being well-tolerated. Since the promise displayed with RBC cryopreservation, other biological systems were explored, including cartilage, platelet, and HSPC cryopreservation. Unfortunately, compound **505** proved to be toxic in cartilage and platelets and was eliminated as a cryoprotectant for these cell type. Is also did not provide any additional cryoprotection to HSPCs compared to existing cryopreservation methods and was eliminated as a cryoprotectant for HSPCs. Its use as an RBC cryoprotectant shows potential, and further studies using it alone, or in conjunction with other IRIs need to be conducted.

Overall, these studies promote further investigation of factors that contribute to IRI activity as well as the benefit they may demonstrate as biological cryoprotectants. While sulfation of current IRIs does not appear beneficial to IRI activity, the drastically increased solubility allows for much greater concentrations to be used and can potentially be useful where heat-sensitivity is an issue. The use of amino alditols in RBC cryopreservation has also been demonstrated, despite their cytotoxicity in other cell types. The success of some of the compounds investigated in this thesis have contributed to the overall field of cryopreservation and show promise in clinical and other applications.

6.2 Claims to Original Research

1. Synthesis and IRI analysis of sulfated pyranose derivatives presented in **Chapter 3**, including their assessment for RBC cryopreservation (conducted by Julia Meyer, Jessica Poisson, and Tracey Turner)
2. Synthesis and IRI analysis of sulfated aldonamide derivatives presented in **Chapter 4**, including their assessment for RBC cryopreservation (conducted by Julia Meyer, Jessica Poisson, and Tracey Turner)
3. Synthesis and IRI analysis of amino aldonamide derivatives presented in **Chapter 5**, including cytotoxicity assessment. Compound **505** was also investigated for the cryopreservation of RBCs, articular cartilage, platelets, and HSPCs.

6.3 Publications

Wiens, J.; Ho, R.; Brassinga, A. K.; Deck, C.A.; Walsh, P.J.; Ben, R.N.; McClymont, K.; Charlton, T.A.; Evans, A.N.; Anderson, W. G. Biosynthesis of 1 α -hydroxycorticosterone in the winter skate *Leucoraja ocellata*: evidence to suggest a novel steroidogenic route. *J. Fish Bio.* 2017, 91(1), 260.

Ampaw, A.; Charlton, T.A.; Briard, J.G.; Ben, R.N. Designing the next generation of cryoprotectants – From proteins to small molecules. *Pep. Sci.* 2018, 111(1), e24086.

Mousavifar, L.; Shiao, T.Z.; Charlton, T.A.; Roy, R. One-Pot Chemoselective S- vs O-Deacetylation and Subsequent Thioetherification. In preparation.

Suchy, M.; Charlton, T.A.; Ben, R.N.; Shuhendler, A.J. Synthesis of Natural/¹³C-Enriched D-Tagatose from Natural/¹³C-Enriched D-Fructose. *Carb. Res.* Accepted, June 15th, 2021.

Chapter 7. Experimental Procedures and Characterization Data

7.1 General Experimental Conditions

All anhydrous reactions were performed in flame-dried glassware under a positive pressure of dry argon. Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes or cannulae. All flash chromatography was performed with E. Merck silica gel 60 (230-400 mesh) or SiliaFlash F60 40-63 μm (230-400 mesh) silica gel (SiliCycle Inc.). All solution phase reactions were monitored using analytical thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra-violet light and/or staining (ceric ammonium molybdate, ninhydrin stain, potassium permanganate, or orcinol/sulfuric acid stain solution). All solvents used for anhydrous reactions were obtained from a SPBT-1 solvent purification (LC Technology solutions Inc.) or distilled. Tetrahydrofuran (THF), diethyl ether (Et_2O), dichloromethane (DCM), dimethylformamide (DMF), and acetonitrile were obtained from SPBT-1 solvent purification (LC Technology solutions Inc.).

^1H (300, 400, 500, or 600 MHz) and ^{13}C NMR (75 or 100, 125, or 150 MHz) spectra were recorded at ambient temperature on a Bruker Avance 300, Bruker Avance 400, Bruker Avance 500, Varian Inova 500, or Bruker Avance HD 600 spectrometers. Deuterated chloroform (CDCl_3), methanol (MeOD), DMSO ($\text{DMSO}-d_6$), acetone ($\text{acetone}-d_6$), or water (D_2O) were used as NMR solvents. Chemical shifts are reported in ppm downfield from trimethylsilane (TMS) or the solvent residual peak as an internal standard. Splitting patterns are designated as

follows: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; sext, sextet; m, multiplet, and br, broad. Low resolution mass spectrometry (LRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 $\mu\text{L}/\text{min}$ using electrospray ionization (ESI) in either positive or negative mode.

7.2 Ice Recrystallization Inhibition (IRI) Assay

IRI activity analysis was performed using the “splat-cooling” assay. In this assay, the analyte was dissolved in a 1x phosphate buffered saline (PBS) solution and a 10 μL droplet of this solution was dropped from a micropipette from a height of two meters onto a block of polished aluminum precooled to approximately $-80\text{ }^{\circ}\text{C}$ (dry ice). The droplet froze immediately upon contact with the block, and the resulting ice wafer was approximately 1 cm in diameter and 20 μm thick. This wafer was then transferred to a cryostage held at $-6.4\text{ }^{\circ}\text{C}$ (via a Peltier unit) for annealing (recrystallization). After an incubation of 30 minutes (at $-6.4\text{ }^{\circ}\text{C}$), the wafer was photographed between crossed polarizing filters using a Nikon CoolPix 5000 digital camera fitted to the microscope. A total of three images were taken from each wafer. During freezing, ice crystals spontaneously nucleated from the PBS/compound solution. The initial crystals were relatively small and homogeneous in size. During the annealing incubation, ice recrystallization occurred, which resulted in a dramatic increase in ice crystal size. Quantitative measurements of the difference in recrystallization inhibition of concentrations of a compound, or multiple compounds was the difference in ice crystal size distribution. Analysis of ice wafer pictures was performed using a domain recognition software (DRS) program (doman3). This program allows the storage of the vertices of ice domains in a digital micrograph, which was then used to calculate the domain areas. The resulting data were plotted via Microsoft Excel and used to

determine the IRI activity. This was done by comparing the mean grain size (MGS) (ice crystal size) of the compound of interest to the MGS of PBS (performed in the same day), and activity was presented as a percentage of MGS (%MGS) compared to PBS. Standard error of the mean was also generated and plotted with the %MGS. Overall, higher %MGS values denote poorer IRI activity, as PBS does not show any IRI activity (negative control).

7.3 Dose-response Measurement of IRI Activity

The “splat-cooling” assay was run according to **Section 6.2**. Although, annealing time was reduced from 30 to 5 minutes. The rates were determined for at least 5 concentrations/IRI in PBS, encompassing concentrations showing no IRI activity to concentrations where all ice crystals fall within the first bin (smallest crystal size). Three drops were done for each sample concentration, with a single image being analyzed/drop. ImageJ was used to analyze each image, circling all visible ice crystals in a single plane. Crystals cut off on the edges of each image were omitted. Crystal area was calculated using ImageJ and corrected for the corresponding objective lens’ magnification factor. Crystal areas were then sorted into bins, starting at $1.0 \times 10^{-3} \text{ mm}^2$, and increasing by $1.0 \times 10^{-3} \text{ mm}^2$ in each bin. At time = 0, all crystals should be within the first bin, and as recrystallization occurs, ice crystals will become bigger, and fall within larger bins. Area of each bin was calculated by dividing the sum of all areas within a bin by the sum of all ice crystals in each picture. Next, rates were determined for each concentration/compound being tested and then normalized with respect to the PBS control. From this, dose-response curves were generated for each compound using GraphPad and the normalized rate constants v_{norm} along with log values of the concentrations tested. The concentration value at half-maximal inhibition

(IC₅₀) was obtained from a two-parameter sigmoidal curve that was fit to the data, with error bars representing SEM.

7.4 Toxicity with HepG2 Cells

Initially, HepG2 cells (human hepatocellular carcinoma cells, ATCC, HB-8065) were cultured in Corning cell culture flasks (T75 U-shaped 275 mL flasks, Fisher 07-202-000) using minimum essential medium (Fisher, Gibco MEM, 11095080 or Sigma, Eagle MEM, M4655) supplemented with 10% fetal bovine serum (heat-inactivated), 1% penicillin-streptomycin, 1% non-essential amino acids (Sigma, M7145), and 0.1% 1M sodium pyruvate. Cells were incubated at 37 °C with 5% CO₂, with media changes every two days. At 75-85% confluency, cells were washed with DPBS (Gibco without Ca and Mg, Fisher 14190136) and detached with accutase (Sigma, A6964), and a cell count was obtained via hemocytometer and trypan blue (Sigma, T8154) as a stain.

96-well plates (Costar, Fisher 0720090) were plated at 10,000 cells/well with MEM and incubated overnight (16h) at 37 °C with 5% CO₂, then centrifuged at 1000 rpm for 5 minutes before the media was aspirated off. Next, 100 µL of MEM supplemented with the following: MEM only (blank wells), cells with MEM (positive controls), MEM and compound (in varying concentrations), and 1% Triton X-100 (Sigma, X100) in MEM (negative controls) was added. After 16 hours incubation, 30 µL of 0.01% resazurin (Sigma, R7017) in ddH₂O (in the dark) was added to each well, and the plates were incubated for 8 h before fluorescence reading (BioTek Synergy H1Multi-Mode Plate Reader). An excitation wavelength of 570 nm was used along with an emission wavelength of 585 nm, blanks (only MEM – 585 nm) were subtracted from all values. Viability values were presented as the value relative to controls.

7.5 Red Blood Cell (RBC) Compound Screening

RBC experiments were conducted by fellow lab members Julia Meyer and Jessica Poisson along with Tracey Turner at CBS Edmonton in the Acker lab. The compound of interest was prepared at 2x the working concentration in a 30 % (w/v) glycerol solution (final concentration 15% glycerol made from 57 Glycerolyte and 0.9% saline/0.2% dextrose). This was repeated for each concentration to be assessed, along with a 15% glycerol control. RBC samples from a red cell concentrate (RCC) was mixed with equal volumes of cryosolutions, incubated at room temperature for 10 minutes and then cooled to -5°C in a methanol bath. Samples were held at -5°C for five minutes before controlled ice nucleation. Samples were held for an additional five minutes at this temperature before either freezing to -40°C in the methanol bath (slow freezing) or frozen on dry ice (fast freeze). Once samples reached the desired temperature, they were thawed in a 37°C water bath before RBC hemolysis was examined using Drabkin's percent hemolysis method with the following calculation.¹⁻³

$$\text{RBC Hemolysis (\%)} = \frac{[(100 - \text{Hematocrit(\%)}) \times \text{supernatant hemoglobin (g/L)}]}{\text{total hemoglobin (g/L)}}$$

7.6 Cartilage Cryopreservation and Post-thaw Analyses

Cartilage experiments (other than NMR measurements) were designed and conducted by the Jomha lab (University of Alberta) and were conducted by Kezhou Wu, Rachael Dong, Kevin Duong, and Leila Laouar (University of Alberta).

7.6.1 Freeze Substitution and SEM imaging

Fresh porcine knee joints from the Meat Shop at Pine Haven Colony and were dissected with the chondrocytes being removed (isolated) and stored in PBS. A core sample of

6.9 mm osteochondral dowels was obtained from dissected condyles, leaving bone base intact. Dowels were washed with PBS with antibiotics/antimycotic (penicillin, streptomycin, amphotericin B) for 15 min before being stored in PBS with antibiotics. Dowels were incubated in PBS or a solution of PBS and IRI of interest for 3 or 18 hours at room temperature before being plunged into N_{2(l)} to freeze. While still frozen, a 24-well plate was filled with 2 mL of MeOH/well and cooled to -80 °C. Once cooled fully, the plate was removed and frozen dowels were added from N_{2(l)} (removed one at a time in order to prevent thawing) before the plate was placed in a -80 °C freezer. Every two days, dowels were transferred to another 24-well plate filled with MeOH at -80 °C for one week. After one week, dowels were transferred to 15 mL conical vials with 3 mL of MeOH (precooled) before 3 mL of 2% OsO₄ in MeOH was added. Samples were incubated on dry ice for 2-3 days (until the dry ice has completely evaporated) and the excess incubation solution was discarded. Dowels were then washed with MeOH (room temperature) four times before being sectioned. The vibratome 1000 Plus Sectioning System was used to obtain 85 µm sections. The sections were stored in MeOH and sent out for SEM imaging.

7.6.2 Chondrocyte Staining

Fresh porcine knee joints from the Meat Shop at Pine Haven Colony were dissected with the chondrocytes being removed (isolated) and stored in PBS. A core sample of 6.9 mm osteochondral dowels was obtained from dissected condyles, leaving bone base intact. Dowels were washed with PBS with antibiotics/antimycotic (penicillin, streptomycin, amphotericin B) for 15 min before being stored in PBS with antibiotics. Next, 85 µm sections were obtained via vibratome and stored in the fridge in PBS until staining. Samples were stained with 6.25 µM Syto 13 and 9 µM propidium iodide and were stained on ice for 10-15 minutes in the dark. Fluorescence microscopy images were then obtained (**Figure 6-1**).

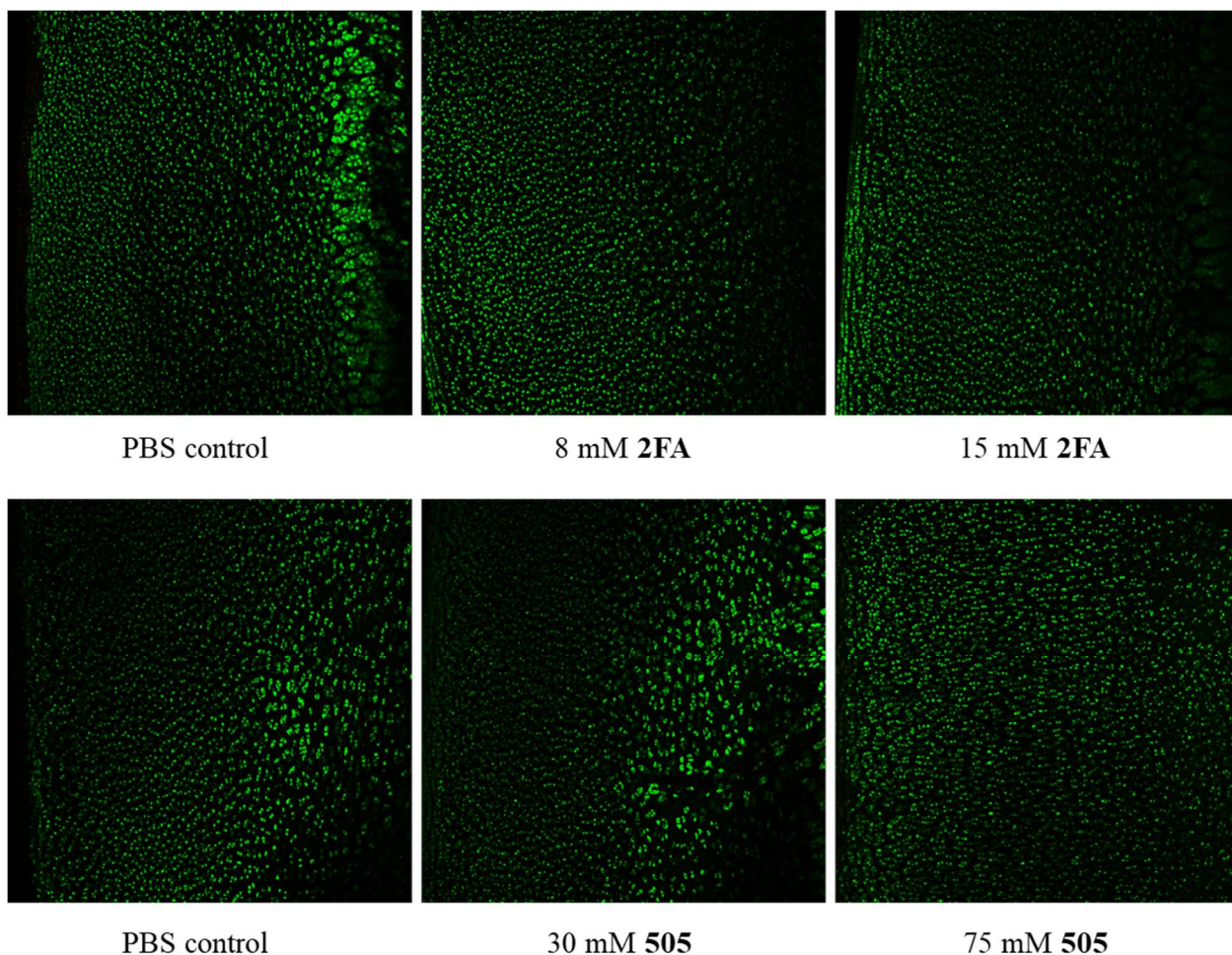


Figure 7-1. Fluorescence microscopy images of sectioned cartilage incubated with PBS (control) 2FA (8 and 15 mM) and 505 (30 and 75). Green fluorescence (Syto-13) shows live cells and red fluorescence shows dead (lysed – PI) cells.

7.6.3 Quantitative NMR Measurements

Quantitative NMR was performed based off of “Quantitative NMR” Technical Details and TraceCERT Certified Reference Materials – Sigma-Aldrich ⁴ using the following equation:

$$C_x = \frac{I_x}{I_{cal}} \cdot \frac{N_{cal}}{N_x} \cdot C_{cal}$$

Where C, I, and N are: C = concentration, I = integral area, and N = number of nuclei (protons) and x = compound of interest and cal = calibrating compound (internal standard). The internal standard used was 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS).

7.7 Platelet Cryopreservation

Experiments were designed and conducted in the Johnson lab (Australian Red Cross Lifeblood) by Lacey Johnson and Lauren Waters.

7.7.1 Platelet Processing

Four ABO-matched platelet units were combined and split to obtain one ABO-matched unit. This matched unit was then split into four equal parts and DMSO was added to achieve a final concentration of 5-6%. The platelet units were then centrifuged (1350 x g for 10 minutes) and the majority of DMSO-containing supernatant was removed to yield a hyperconcentrated platelet component, of approximately 25 mL. The hyperconcentrated platelet component was separated into 5 equal portions and the IRI **505** was added to three portions (30, 75, and 200 mM) while one has no compound, and one was not frozen (unfrozen control). Portions to be frozen were frozen and stored at -80 °C until thawing. Thawing was done using a standard protocol ⁵ and platelets were reconstituted in a unit of plasma, and stored at room temperature with constant agitation.

7.7.2 Emerald Platelet Recovery/Unit Volume

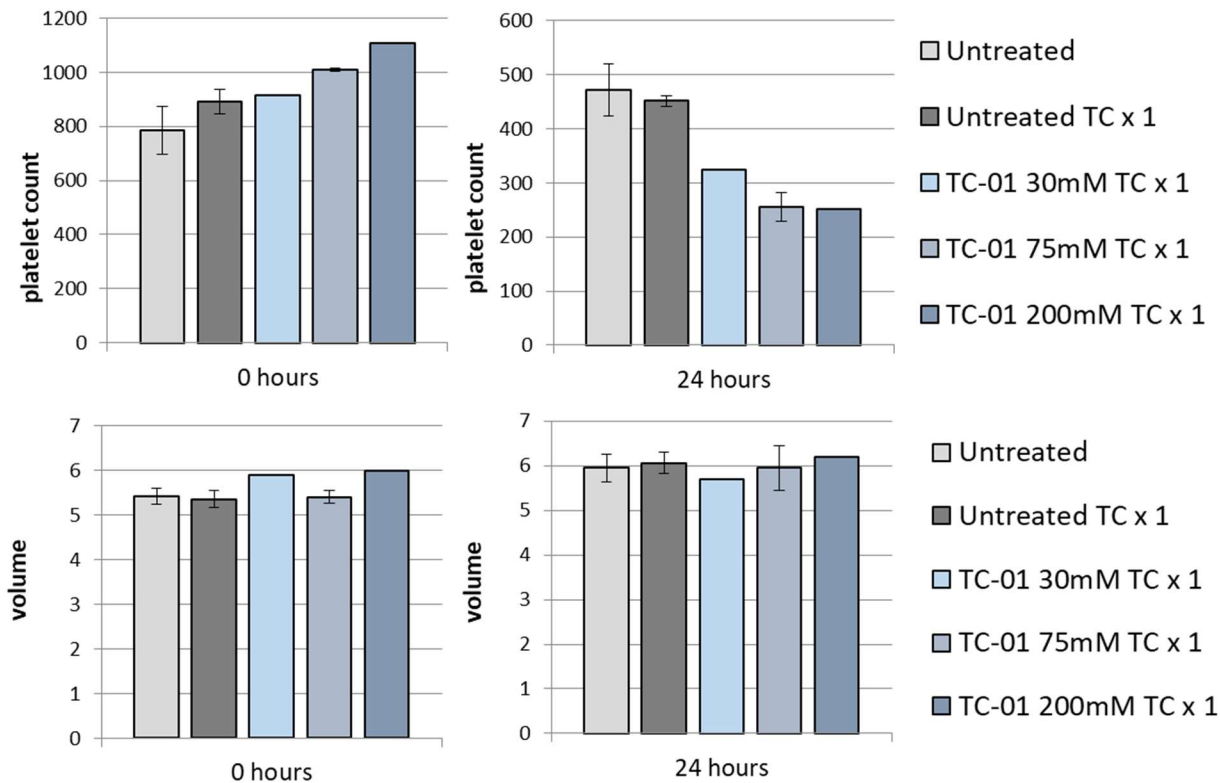


Figure 7-2. Emerald counts and volume measurements of platelets, 0 hours and 24-hours post-thaw.

7.7.3 Ruby Platelet Recovery

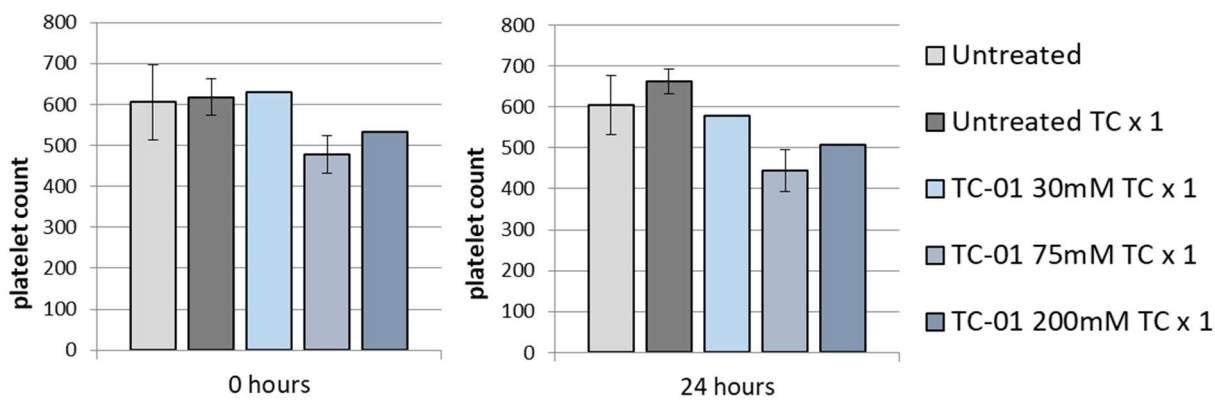


Figure 7-3. Ruby counts of platelets, 0 hours and 24-hours post-thaw.

7.8 Human Umbilical Cord Blood (hUCB) Processing, Cryopreservation, and Post-Thaw Analysis

Flow cytometry was performed by Javed Manesia at the Canadian Blood Services (Pineault lab).

7.8.1 UCB Processing and Cryopreservation

Cord blood units processed according to Canadian Blood Services and using their facilities. All units were donated with informed consent for research purposes. Cord blood units were received and processed within 48 hours post-collection and were stored at room temperature until processing. UCB units with a total nucleated cell (TNC) count between 0.9×10^9 and 1.5×10^9 and a volume of at least 50 mL Units were split into 2-3 equal portions into 50 mL Falcon tubes and diluted with Hespan (6% Hetastarch in 0.9% sodium chloride, B. Braun Medical Inc., Irvine, CA, USA) at a ratio of 167 $\mu\text{L/mLUCB}$. The mixture was inverted 10 times to mix and then incubated at room temperature for 90 minutes undisturbed. The buffy coat was carefully pipetted off and centrifuged at 1390 rpm ($450 \times g$) for 10 minutes at 10°C . Once complete, excess plasma was removed, and resulting pellet was resuspended in 10 mL plasma. This mixture was stored at 4°C while a TNC count was obtained via a pocH-100i hematology analyzer (Sysmex Corporation, Ontario, Canada). Using this count, cells were equally divided in 15 mL Falcon tubes in such that $\text{TNC} \sim 10 \times 10^6$ cells/tube before being centrifuged (1390 rpm [$450 \times g$]) at 10°C for 5 minutes. Excess plasma was removed, and pellets were resuspended in 1 mL corresponding cryosolution (compound, 10% DMSO, and saline/dextran) and transferred to 2.0 mL cryovials (VWR 2.0 mL cryotubes, 89092-262) before being transferred to a pre-cooled Mr. Frosty (isopropanol-based device, Thermo Fisher Scientific) and placed in a -80°C freezer. This generated an approximate cooling rate of -1°C/min , and cells were held at this temperature overnight, before being stored at -196°C (vapour-phase N_2) until used.

7.8.2 UCB Thawing

Thawing of UCB samples followed the thaw-and-dilute protocol optimized by Canadian Blood Services.³ Cryovials were placed in a 37 °C for approximately 90 seconds before being diluted with 1 mL of a solution consisting of PlasmaLyte A (Baxter, Deerfield, IL, USA) in 4% human albumin (Grifols Ltd, Barcelona, Spain). The sample was then transferred to a 15 mL falcon tube and an additional 0.5 mL of the previous solution was added and the mixture was incubated for 15 minutes at room temperature. Afterwards, 2.5 mL of the previous solution was added and was incubated again for 15 minutes at room temperature. This provides a 1/5 dilution of the original sample. Next, samples were centrifuged for 10 minutes (300xg) at room temperature and the supernatant removed. Pellets were resuspended in 2 mL of Iscove's Modified Dulbecco's Medium (Life Technologies) supplemented with 2% Fetal Bovine Serum (HyClone, Thermo Fisher Scientific) and a TNC count was obtained.

7.8.3 Post-thaw viability using flow cytometry

Flow cytometry was performed on a FACS-Attune flow cytometer (Thermo Fisher Scientific) by Javed Manesia at the Canadian Blood Services and myself, with data being analyzed by Javed Manesia. After thawing and TNC was obtained, samples were transferred into 15 mL Falcon tubes and red blood cells were lysed using 2 mL ammonium chloride solution (BD Biosciences). Before samples were centrifuged at 250 x g for 8 minutes and supernatant was then removed, and the pellets were resuspended in 1X Annexin Binding Buffer (1X, Thermo Fisher Scientific V13246). Staining was performed with the corresponding antibodies: CD34-PE (BD Biosciences PE mouse anti-human CD34, 555822), CD38-PECy-5, and CD45-266-allophycocyanine (APC) antibodies (BD Biosciences). Samples were mixed and incubated in the

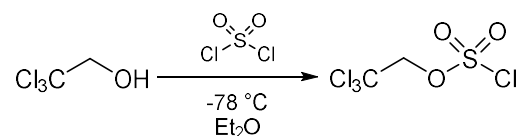
dark for 20 minutes prior to the addition of 1X annexin binding buffer. Samples were incubated on ice until data acquisition (performed within one hour).

7.8.4 Post-thaw Colony Forming Unit (CFU) Assay

Procedure for this assay was adapted from Stem Cell Technologies, Inc.⁶ Post-thaw cells were processed (**6.5.2 UCB Thawing**), using the TCN count, a 10x cell stock was prepared for each sample. Next, 300 μ L of this 10x solution was pipetted into a (pre-prepared) 3 mL methylcellulose medium (MethoCult, H4434 Classic, Stem Cell Technologies) and was repeated for each condition. To evenly distribute cells in the MethoCult, each tube was vigorously mixed prior to plating to eliminate bubbles in the media. A sterile 16-gauge blunt-end needle (28110, Stem Cell Technologies) along with a 3 mL syringe were used to draw up $\sim$ 2.6 mL of the cell/media mixture, avoiding bubbles. Using two 35 mm culture dishes (27150, Stem Cell Technologies), 1.1 mL of the media mixture was dispensed per plate (total of 2 plates/condition). To evenly distribute the media, plates were tilted, making sure the entire plate bottom was covered and any bubbles (if present) were removed. Both 34 mm plates were covered and placed in a larger plate along with another 35 mm plate containing sterile water (uncovered) to ensure proper hydration of the media during incubation. The larger plate was then covered, and samples were incubated for 14 days (37 °C, 5% CO₂ with 97% humidity) before plate scoring. Colony scoring was performed on an Olympus CKX41-inverted phase-contrast microscope (Olympus America) the following types of colonies were scored: burst-forming unit-erythroid (BFU-E), granulocyte-macrophage (CFU-G, CFU-M, CFU-GM), and the granulocyte, erythroid, macrophage, and megakaryocyte (CFU-GEMM) colonies.

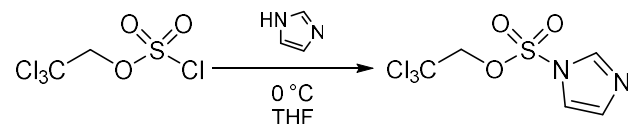
7.9 Synthesis and Characterization for Chemical Compounds

2,2,2-Trichloroethoxysulfonyl chloride (**312**)⁷



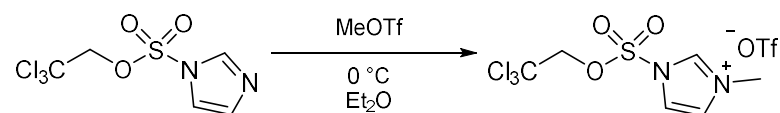
2,2,2-trichloroethanol (1 mL, 0.01 mol) was combined with pyridine (0.84 mL, 0.01 mol) in dry Et_2O (2 mL) at -78°C . To this solution was added freshly distilled SO_2Cl_2 (0.76 mL, 0.01 mol) drop wise. After addition, the solution was stirred for 1 h at -78°C then for an additional 3 h at room temperature. The crude product was vacuum filtered and washed with Et_2O , the filtrate was concentrated to a dark yellow oil. Product was purified by vacuum distillation (B.P: $60^\circ\text{C}/0.8$ mmHg) in a 70 % yield of **312**. ^1H NMR (300 MHz, CDCl_3) δ 4.70 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 91.4, 81.2. LRMS (ESI): m/z $\text{C}_2\text{H}_2\text{O}_3\text{SCl}_4\text{Na}$ $[\text{M}+\text{Na}]^+ = 268.2$. All spectral data were consistent with those reported in the literature.⁷

2,2,2-Trichloroethoxysulfonyl imidazole (**313**)⁷



Imidazole (15.7088 g, 0.231 mol) was added to dry THF (40 mL), this solution was stirred at 0°C . Next, 2,2,2-trichloroethylsulfonylchloride (28.6027 g, 0.115 mol) was added to dry THF (20 mL) and this solution was added drop wise via syringe pump to the imidazole solution. This mixture was stirred for 1 h at 0°C before being stirred at room temperature for an additional hour. Salts were then removed by suction filtration washed with THF and the filtrate was condensed to a pale-yellow oil with white crystals lining the flask. Flash chromatography (33:67 EtOAc:hex) yielded **313** as white crystals in a 96.5 % yield. ^1H NMR (300 MHz, CDCl_3) δ 8.02 (s, 1H), 7.38, 7.20 (2m, 2H), 4.65 (s, 2H); ^{13}C NMR (75 MHz, CDCl_3) δ 137.0, 131.7, 118.1, 91.6, 80.2. LRMS (ESI): m/z $\text{C}_5\text{H}_5\text{O}_3\text{N}_2\text{SCl}_3\text{Na}$ $[\text{M}+\text{Na}]^+ = 300.1$. All spectral data were consistent with those reported in the literature.⁷

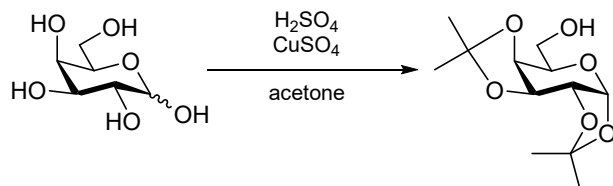
2,2,2-trichloroethoxysulfonyl-*N*-methylimidazolium triflate (**314**)⁷



To a solution of 2,2,2-trichloroethylsulfonylimidazole (14.9437 g, 0.5346 mmol) in dry Et_2O (200 mL) at 0°C was added MeOTf (6.9 mL, 60.94 mmol) dropwise under argon. The reaction was

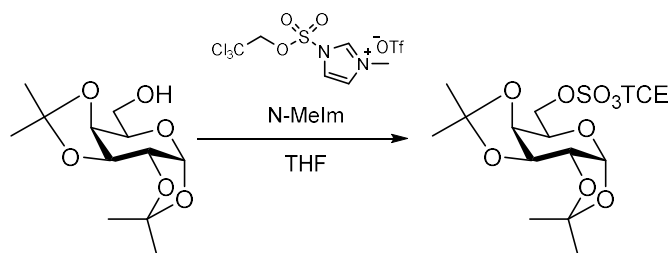
then stirred at 0 °C for 3 h while a white precipitate was formed. The product was isolated by suction filtration (filtrate was re-precipitated once) and washed with cold Et₂O to yield **314** as a white solid (93.4 %). ¹H NMR (300 MHz, CD₃OD) δ 9.97 (1, 1H), 8.30 (s, 1H), 7.89 (s, 1H), 5.39 (s, 2H), 4.04 (s, 3H). ¹³C NMR (75 MHz, CD₃OD) δ 141.4, 127.2, 122.3, 93.1, 83.7, 37.7. LRMS (ESI): m/z C₆H₈O₃N₂SCl₃Na [M+Na]⁺ = 316.2. All spectral data were consistent with those reported in the literature.⁷

1,2:3,4-Di-*O*-isopropylidene- α -D-galactopyranose (**316**)⁷



Anhydrous CuSO₄ (2.0g, 25mmol) was combined with D-galactose (1.0g, 5.5mmol) in acetone (25mL). To this solution, conc. H₂SO₄ (125μL) was added and let stir for 40h. The crude product was then filtered and washed with acetone before being neutralized with a saturated NaHCO₃ solution. Solvent was removed under vacuum and solid product was redissolved in DCM. Organic layer was washed with water (3 times) before drying over MgSO₄. Organic layer was filtered, and solvent removed under vacuum. Flash chromatography (6:4 hexanes: ethyl acetate) yielded **316** as a light-yellow oil in a 70% yield. ¹H NMR (400 MHz, CDCl₃): δ 5.52 (d, 1H, *J* = 5.0 Hz), 4.56 (dd, 1H, *J* = 7.9, 2.4 Hz), 4.29 (dd, 1H, *J* = 5.0, 2.4 Hz), 4.23 (dd, 1H, *J* = 7.9, 1.6 Hz), 3.86-3.80 (m, 2H), 3.75-3.70 (m, 2H), 1.49 (s, 3H), 1.40 (s, 3H), 1.29 (s, 6H). ¹³C NMR (100 MHz, CDCl₃): δ 109.5, 108.6, 96.3, 71.5, 70.7, 70.5, 68.1, 62.2, 26.0, 25.9, 24.9, 24.3. LRMS (ESI): m/z C₁₂H₂₀O₆Na [M+Na]⁺ = 283.1. All spectral data were consistent with those reported in the literature.⁷

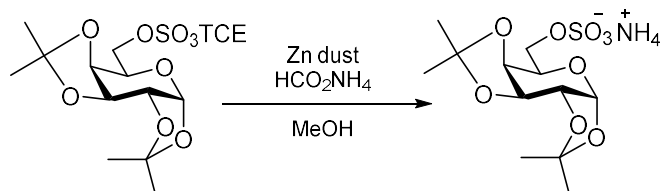
1:2,3:4-di-*O*-isopropylidene-6-*O*-trichloroethylsulfogalactose (**317**)⁷



1:2,3:4-diisopropylidene-galactose (0.25 g, 0.97 mmol), N-methylimidazole (0.19 mL, 2.39 mmol), and 2,2,2-trichloroethoxysulfonyl-N-imidazoliumtriflate (0.85 g, 1.92 mmol) were added to THF (4.2 mL) at 0 °C. The reaction mixture was stirred at 0 °C and allowed to warm to room temperature overnight. The mixture was stirred for 24 h before being quenched with H₂O. The crude mixture was extracted with ethyl acetate (x3) and organics were combined and washed with NaHCO₃, brine, before being dried over MgSO₄. The crude product was filtered and solvent

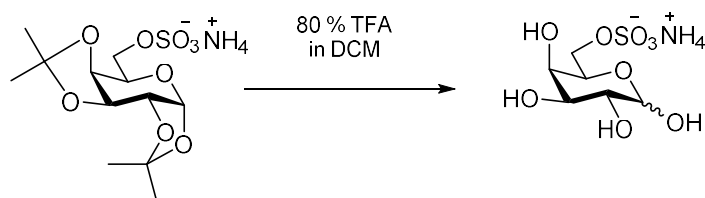
was removed under reduced pressure before being purified by flash chromatography (25:75 EtOAc:hex) yielding **317** in 44 %. ^1H NMR (300 MHz, CDCl_3) δ 5.55 (d, 1H, $J = 4.9$ Hz), 4.82, 4.79 (d, 2H, $J = 10.8$ Hz), 4.76 (dd, 1H, $J = 7.8, 2.5$ Hz), 4.51 (m, 2H, H6), 4.38 (dd, 1H, $J = 4.9, 2.5$ Hz, H2), 4.26 (dd, 1H, $J = 7.8, 1.7$ Hz, H4), 4.15 (m, 1H, H5), 1.56 (s, 3H, CH_3), 1.45 (s, 3H), 1.35 (s, 6H); ^{13}C NMR (75 MHz, CDCl_3) δ 109.9, 109.0, 96.0, 92.5, 79.4, 72.0, 70.5, 70.3, 70.1, 65.5, 25.9, 25.8, 24.7, 24.3. LRMS (ESI): m/z $\text{C}_{14}\text{H}_{21}\text{Cl}_3\text{O}_9\text{SNa}$ $[\text{M}+\text{Na}]^+ = 493.9$. All spectral data were consistent with those reported in the literature.⁷

1:2,3:4-di-*O*-isopropylidene-6-*O*-sulfo-galactose (**318**)⁷



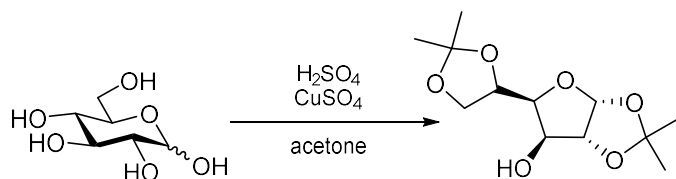
To a suspension of ammonium formate (0.16 g, 2.6 mmol) in MeOH (2.5 mL) was added **317** (0.20 g, 0.43 mmol) followed by zinc dust (0.22 g, 3.4 mmol). The reaction was stirred at room temp. for 6 h. The product was filtered through Celite and condensed to the crude product. Final product was then purified by flash chromatography (20:4:1 DCM:MeOH: NH_3OH) yielding **318** in a 62 % yield. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 1.27 (s, 3H), 1.28 (s, 3H), 1.34 (s, 3H), 1.43 (s, 3H), 3.71-3.81 (m, 2H), 3.94 (dd, 1H, $J = 5.7$ Hz), 4.22 (d, 1H, $J = 8.0$ Hz), 4.32 (dd, 1H, $J = 4.8, 2.1$ Hz), 4.57 (dd, 1H, $J = 8.0, 1.9$ Hz), 5.43 (d, 1H, $J = 5.0$ Hz), 7.09 (br, 4H); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 24.6, 25.3, 26.2, 26.3, 65.1, 66.3, 70.1, 70.3, 70.7, 95.9, 108.1, 108.6. LRMS (ESI): m/z $\text{C}_{12}\text{H}_{19}\text{O}_9\text{S}$ $[\text{M}]^- = 340.5$. All spectral data were consistent with those reported in the literature.⁷

6-*O*-sulfo-D-galactose (**319**)



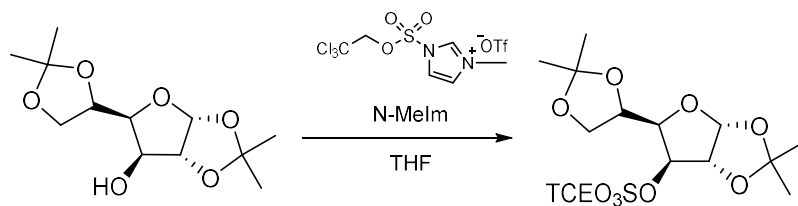
The protected carbohydrate **318** (10.4 mg, 0.03 mmol) was dissolved in 2 mL of 80 % TFA in H_2O . This was allowed to stir for 10 minutes while being tracked by TLC. After 10 minutes, no starting material was observed. The reaction was quenched with NH_4OH (aq.) and concentrated under reduced pressure. The product was recrystallized from MeOH/ Et_2O yielding **319** quantitatively as an α/β mixture. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 1.27 (s, 3H), 1.28 (s, 3H), 1.34 (s, 3H), 1.43 (s, 3H), 3.71-3.81 (m, 2H), 3.94 (dd, $J = 5.7$ Hz), 4.22 (d, 1H, $J = 8.0$ Hz), 4.32 (dd, 1H, $J = 4.8, 2.1$ Hz), 4.57 (dd, 1H, $J = 8.0, 1.9$ Hz), 5.43 (d, 1H, $J = 5.0$ Hz), 7.09 (br, 4H). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 24.6, 25.3, 26.2, 26.3, 65.1, 66.3, 70.1, 70.3, 70.7, 95.9, 108.1, 108.6. LRMS (ESI): m/z $\text{C}_6\text{H}_{11}\text{O}_9\text{S}$ $[\text{M}]^- = 257.9$.

1,2:5,6-Di-*O*-isopropylidene- α -D-glucufuranose (**321**)⁷



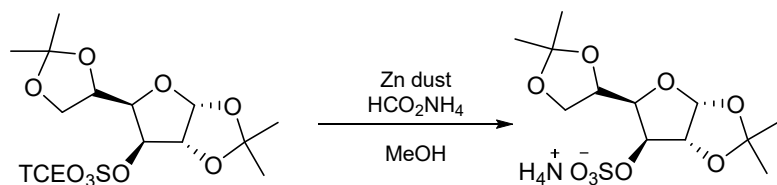
D-glucose (2.0 g, 11.0 mmol) was suspended in acetone (50 mL) before CuSO₄ (4.0 g, 50.0 mmol) and H₂SO₄ (0.25 mL) was added. Reaction was stirred overnight before being filtered through Celite and was washed with acetone. Saturated NaHCO₃ was added until the reaction was neutralized, and solvent was removed *in vacuo*. The crude product was then dissolved in DCM and washed with H₂O before being dried over MgSO₄ and the solvent removed under reduced pressure. The product was recrystallized from cyclohexane in an 80 % yield of **321**. ¹H NMR (400 MHz, CDCl₃) δ 5.94 (d, 1H, J = 3.6 Hz), 4.53 (d, 1H, J = 3.6 Hz), 4.36-4.31 (m, 2H), 4.16 (dd, 1H J = 6.1, 8.6 Hz), 4.06 (dd, 1H, J = 2.8, 7.6 Hz), 3.98 (dd, 1H J = 5.3, 8.5 Hz), 2.57 (d, 1H, J = 3.8 Hz), 1.49, 1.44, 1.35, 1.31 (4s, 12H). ¹³C NMR (100 MHz, CDCl₃) δ 111.8, 109.6, 105.3, 85.1, 81.1, 75.2, 73.5, 67.6, 26.8, 26.7, 26.2, 25.1. LRMS (ESI): m/z C₁₂H₂₀O₆Na [M+Na]⁺ = 283.2. All spectral data were consistent with those reported in the literature.⁷

1:2,5:6-di-*O*-acetone-3-*O*-trichloroethylsulfoglucufuranose (**322**)⁷



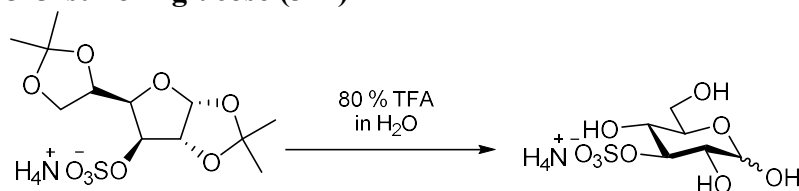
Compound **321** (500 mg, 1.92 mmol) was dissolved in THF (50 mL) and cooled to 0 °C. The sulfating group (1.7 g, 3.8 mmol) and *n*-MeIm (0.4 mL, 4.8 mmol) were added, and the reaction was allowed to stir overnight while warming to room temperature. The reaction was quenched with water and extracted with EtOAc (x3). Organics were combined and washed with NaHCO₃ and brine. The product was dried with Na₂SO₄, filtered, and the solvent was removed under reduced pressure. The product was purified via flash chromatography using 2:8 EtOAc:pet. ether in a 90 % yield of **322**. ¹H NMR (400 MHz, CDCl₃) δ 5.92 (d, 1H, J = 3.70), 5.07 (d, 1H, J = 10.57), 5.02 (d, 1H, J = 2.89), 4.97 (d, 1H, J = 3.70), 4.77 (d, 1H, J = 10.57), 4.27-4.24 (m, 1H), 4.16-4.07 (m, 3H), 4.04 (dd, 1H, J = 3.80, 8.96). ¹³C NMR (100 MHz, CDCl₃) δ 112.73, 110.0, 104.9, 92.51, 85.3, 82.5, 79.6, 79.6, 71.6, 67.4, 26.9, 26.5, 26.1, 25.0. LRMS (ESI): m/z C₁₄H₂₁Cl₃O₉SNa [M+Na]⁺ = 492.7. All spectral data were consistent with those reported in the literature.⁷

1:2,5:6-di-*O*-acetone-3-*O*-sulfoglucufuranose (**323**)⁷



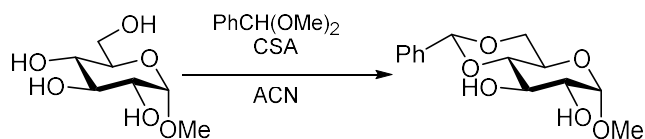
Compound **322** (1.0 g, 2.12 mmol) was dissolved in MeOH (50 mL) before ammonium formate (800 mg, 12.7 mmol) and Zn dust (970 mg, 14.84 mmol) were added. The reaction stirred for 5 h at room temperature before being filtered through celite and concentrated under reduced pressure. Product was purified via flash chromatography using 20:4:1 DCM:MeOH:NH₄OH, in 90 % yield of **323**. ¹H NMR (400 MHz, CDCl₃) δ 7.08 (t, 4H), 5.83 (d, 1H, *J* = 3.8 Hz), 4.75 (d, 1H, *J* = 3.9 Hz), 4.44 (d, 1H, *J* = 3.1 Hz), 4.25-4.18 (m, 2H), 3.95-3.90 (m, 1H), 3.83-3.77 (m, 1H), 1.38, 1.29, 1.24, 1.22 (4s, 12H); ¹³C NMR (100 MHz, CDCl₃) δ 110.7, 107.5, 104.4, 82.7, 79.3, 78.2, 73.0, 64.8, 26.6, 26.4, 26.1, 25.3. LRMS (ESI): *m/z* C₁₂H₁₉O₉S [M]⁻ = 339.9. All spectral data were consistent with those reported in the literature.⁷

3-*O*-sulfo-D-glucose (**324**)



Compound **323** (684 mg) was dissolved in 80 % TFA (10 mL) and was allowed to stir for 4h. The solvent was removed under reduced pressure and product was co-vaped with toluene (x3). ¹H NMR (400 MHz, D₂O) δ 5.29 (d, 1H, *J* = 3.7 Hz), 4.74 (d, 1H, *J* = 7.9 Hz), 4.50 (t, 1H, *J* = 9.3 Hz), 4.33 (t, 1H, *J* = 9.0 Hz), 3.94-3.87 (m, 3H), 3.85-3.75 (m, 3H), 3.75-3.71 (m, 1.8H), 3.65-3.61 (m, 2.6H), 3.57-3.52 (m, 1.5H), 3.46-3.42 (m, 1.5H); ¹³C NMR (100 MHz, D₂O) δ 95.5, 91.9, 84.2, 82.2, 75.3, 72.8, 71.1, 70.1, 68.2, 68.1, 60.6, 60.4. LRMS (ESI): *m/z* C₆H₁₁O₉S [M]⁻ = 258.4.

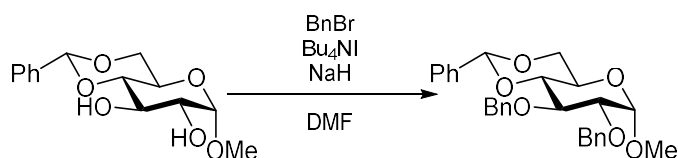
Methyl-4,6-di-*O*-benzylidene-α-D-glucopyranoside (**326**)



α-methoxy-D-glucose (1 g, 5.41 mmol) was dissolved in dry ACN (16 mL). Then PhCH(OMe)₂ (1.41 g, 9.26 mmol) was added, followed by CSA (60 mg, 0.26 mmol). The solution was brought to a reflux and left to stir for 20 min. The reaction was tracked by TLC and was left overnight to complete. Before the reaction was neutralized, TLC showed some residual starting material. The reaction was neutralized with Et₃N and diluted with DCM. This mixture was extracted with water and brine before drying (Na₂SO₄), filtering, and concentrating under reduced pressure. Product recrystallized from DCM and hexanes in 68 % yield of **326**. ¹H NMR (400 MHz,

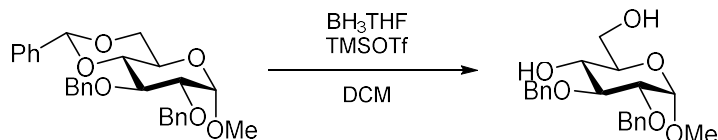
CDCl₃) δ 7.52–7.47 (m, 2H), 7.40–7.35 (m, 3H), 5.56 (s, 1H), 4.82 (d, 1H, J = 6.0 Hz), 4.31 (m, 1H), 3.94 (t, 1H, J = 6.0 Hz), 3.88–3.72 (m, 2H), 3.65 (dd, 1H, J = 6.0, 9.6 Hz), 3.49 (t, 1H, J = 6.0 Hz), 3.47 (s, 3H), 2.67 and 2.24 (2brs, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 137.3, 128.7, 128.1, 126.4, 101.8, 100.9, 81.2, 73.1, 71.5, 68.9, 62.9, 53.8. LRMS (ESI): m/z C₁₄H₁₈O₆ [M]⁺ = 282.7. All spectral data were consistent with those reported in the literature.⁸

Methyl-2,3-di-*O*-benzyl-4,6-di-*O*-benzylidene- α -D-glucopyranoside (**327**)⁸



To DMF (25 mL) at 0 °C, 60 % NaH (1.17 g, 29.44 mmol) was added. Next, compound **326** (1.0393 g, 3.6 mmol) was added over about an hour (~100 mg every 5-10 min.). Once added, BnBr (2.62 mL 22.10 mmol) was added dropwise before the addition of Bu₄AI (0.14 g, 0.368 mmol). The reaction was allowed to stir at 0 °C for 20 minutes, then room temperature overnight. The reaction was quenched with water and brine and extracted (x3) with DCM. Product was purified via flash chromatography using 5 % EtOAc in hexanes in a 92 % yield of **327**. ¹H NMR (400 MHz, CDCl₃) δ 7.49-7.26 (15H, m), 5.54 (1H, s), 4.90 (1H, d, J = 11.2 Hz), 4.85 (1H, d, J = 9.0 Hz), 4.82 (1H, d, J = 9.0 Hz), 4.69 (1H, d, J = 12.2 Hz), 4.58 (1H, d, J = 3.6 Hz), 4.25 (1H, dd, J = 10.1, 4.8 Hz), 4.03 (1H, t, J = 9.6 Hz), 3.81 (1H, m), 3.69 (1H, t, J = 10.0 Hz), 3.61-3.53 (2H, m), 3.39 (3H, s). ¹³C NMR (100 MHz, CDCl₃) δ 138.8, 138.2, 137.4, 128.9, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.6, 126.0, 101.3, 99.3, 82.2, 79.2, 78.6, 75.3, 73.8, 69.1, 62.3, 55.3. LRMS (ESI): m/z C₂₈H₃₀O₆Na [M+Na]⁺ = 485.1. All spectral data were consistent with those reported in the literature.⁸

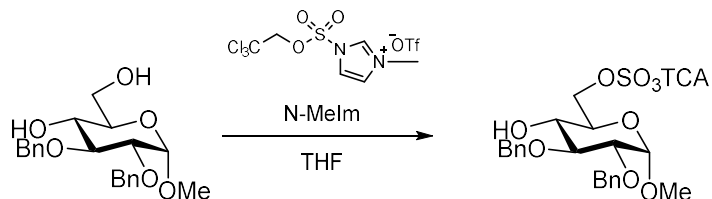
Methyl-2,3-di-*O*-benzyl- α -D-glucopyranoside (**328**)⁸



Compound **327** (1.1297 g, 1.13 mmol) was dissolved in DMC. Next, BH₃.THF (12.2 mL, 12.2 mmol) was added, followed by TMSOTf (74.68 mg, 0.336 mmol). The reaction was followed by TLC (10 min) and quenched with Et₃N (1.0 mL) then MeOH was added slowly until gas formation stopped. Product was purified via flash chromatography using 7:3 Hexanes:EtOAc in a yield of **328** in 85 %. ¹H NMR (400 MHz, CDCl₃) δ 7.27-7.15 (10H, m),

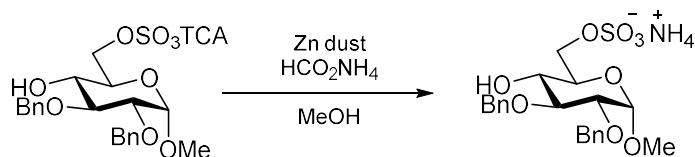
5.03 (1H, d, $J = 11.6$ Hz), 4.79-4.64 (m, 2H), 4.60 (1H, d, $J = 3.3$ Hz), 3.82-3.68 (3H, m), 3.63-3.56 (1H, m), 3.54-3.41 (2H, m), 3.26 (3H, s), 2.27 (1H, br s), 1.87 (1H, br s). ^{13}C NMR (100 MHz, CDCl_3) δ 137.9, 134.5, 129.9, 129.7, 128.9, 128.6, 128.5, 128.4, 128.0, 127.9, 127.8, 127.7, 98.15, 81.3, 79.7, 75.4, 73.1, 70.6, 70.3, 62.4, 55.2, 29.7. LRMS (ESI): m/z $\text{C}_{21}\text{H}_{26}\text{O}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 397.9$. All spectral data were consistent with those reported in the literature.⁸

Methyl-2,3-di-*O*-benzyl-6-*O*-trichloroethylsulfo- α -D-glucopyranoside (**329**)



Compound **328** (625.3 mg, 1.67 mmol) was dissolved in THF (12 mL), before *n*-methylimidazole (0.33 mL, 4.12 mmol) and sulfating agent **314** (1.482 g, 3.34 mmol) were added at 0 °C. The reaction was then allowed to warm to room temperature while stirring for 24 h. The reaction was quenched with water then extracted with EtOAc (x3), washed with NaHCO_3 , and brine before drying, filtering, and concentrating under reduced pressure. Product was purified via flash chromatography using 2:1 hex.:EtOAc yielding **329** in a 63 % yield. ^1H NMR (400 MHz, CDCl_3) δ 7.3-7.2 (m, 10H), 5.01 (d, 1H), 4.77-4.59 (m, 6H), 4.56 (dd, 1H, $J = 2.1, 10.7$ Hz), 4.51 (dd, 1H, $J = 4.9, 10.7$ Hz), 3.84-3.75 (m, 2H), 3.52-3.44 (m, 2H), 3.37 (s, 3H), 2.77 (br, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 138.4, 137.7, 128.5, 128.5, 128.0, 128.0, 127.9, 98.1, 92.5, 80.9, 79.4, 79.4, 75.3, 73.1, 72.63, 69.1, 86.6, 55.5. LRMS (ESI): m/z $\text{C}_{23}\text{H}_{27}\text{Cl}_3\text{O}_9\text{SNa}$ $[\text{M}+\text{Na}]^+ = 606.9$.

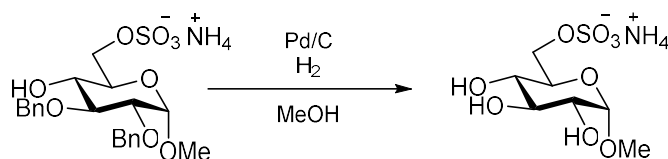
Methyl-2,3-di-*O*-benzyl-6-*O*-sulfo- α -D-glucopyranoside (**330**)



Compound **329** (585.5 mg, 1.05 mmol) was dissolved in MeOH (20 mL) and ammonium formate (395.3 mg, 6.27 mmol) and Zn dust (480.1 mg, 7.35 mmol) was added slowly. The reaction was stirred overnight before being filtered through Celite, dried, concentrated under reduced pressure, and **330** was recrystallized from DCM/MeOH in an 80 % yield. ^1H NMR (400 MHz, DMSO-d_6) δ 7.36-7.24 (m, 10H), 5.30 (d, 1H, $J = 6.7$ Hz), 4.81-4.72 (m, 3H), 4.16 (s, 2H), 4.01 (dd, 1H, $J = 1.6, 10.9$ Hz), 3.76 (dd, 1H, $J = 6.4, 10.9$ Hz), 3.59-3.50 (m, 2H), 3.37 (dd, 1H, $J = 3.5, 9.7$ Hz), 3.28-3.24 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 139.3, 138.6,

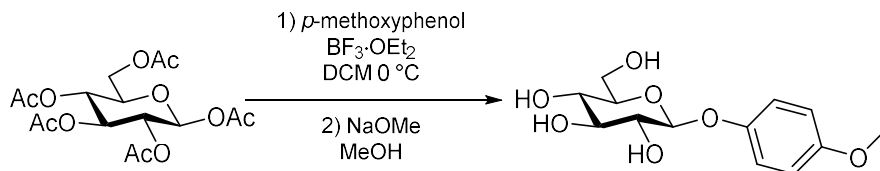
128.1, 127.9, 127.5, 127.4, 127.4, 127.1, 96.8, 81.1, 79.1, 74.0, 71.5, 70.4, 70.2, 65.5, 54.3.
LRMS (ESI): m/z $C_{21}H_{25}O_9SNa$ $[M+Na]^+ = 453.4$.

Methyl-6-*O*-sulfo- α -D-glucopyranoside (**331**)



Compound **330** (93 mg, 0.20 mmol) was dissolved in EtOH (0.5 mL). Next, $Pd(OH)_2/C$ was added and flask was evacuated of air. $H_2(g)$ was added, and flask was evacuated again (x3) before being left to stir overnight. Reaction mixture was then filtered through Celite, dried (Na_2SO_4), concentrated under reduced pressure and recrystallized from DCM/MeOH in an 82 % yield of **331**. 1H NMR (400 MHz, D_2O) δ 4.8 (d, 1H, $J = 3.6$ Hz), 4.3 (dd, 1H, $J = 2.2, 11.2$ Hz), 4.23 (dd, 1H, $J = 4.9, 11.2$ Hz), 3.85 (ddd, 1H, $J = 2.1, 7.2, 14.7$ Hz), 3.66 (dd, 1H, $J = 9.6$ Hz), 3.57 (dd, 1H, $J = 3.8, 9.7$ Hz), 3.45 (dd, 1H, $J = 9.1, 9.9$ Hz), 3.42 (s, 3H). ^{13}C NMR (100 MHz, D_2O) δ 99.2, 72.9, 71.0, 69.5, 69.1, 66.9, 55.1. LRMS (ESI): m/z $C_7H_{13}O_9SNa$ $[M+Na]^+ = 273.2$.

4-methoxyphenyl- β -D-glucopyranoside (**333**)⁹⁻¹¹

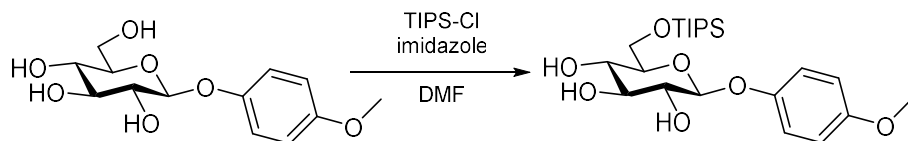


Pre-activated 4Å molecular sieves (powdered) were added to a flame-dried flask and allowed to cool before DCM (40 mL) was added and let stir for ~20 minutes. β -D-glucose pentaacetate (3 g, 7.68 mmol) was added, followed by 4-methoxyphenol (1.3345 g, 10.75 mmol) and $BF_3 \cdot OEt_2$ (1.25 mL, 9.98 mmol). The reaction was stirred overnight before being diluted with DCM and quenched with $NaHCO_3$. The organic layer was washed with $NaHCO_3$, brine, and water before being dried over Na_2SO_4 . The product was filtered and concentrated under reduced pressure. Product was recrystallized with EtOAc/heptane at $-20^\circ C$. 1H NMR (500 MHz, $CDCl_3$) δ 6.96-6.78 (m, 1H), 5.30-5.20 (m, 1H), 4.96-4.93 (m, 1H), 5.18-5.13 (m, 1H), 4.31-4.13 (m, 1H), 3.82-3.77 (m, 1H), 3.76 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H). ^{13}C (400 MHz, $CDCl_3$) δ 170.6, 170.2, 169.3, 169.3, 155.7, 150.8, 118.6, 114.5, 100.2, 72.7, 71.9, 71.2, 68.2, 61.9, 55.6, 20.6, 20.6, 20.6, 20.5. LRMS (ESI): m/z $C_{21}H_{26}O_{11}Na$ $[M+Na]^+ = 477.4$.

The purified product was suspended in a minimal amount of MeOH and NaOMe was added until a pH of 10 was achieved. The reaction stirred for 2 h, before being treated with Amberlite IR-120 (H^+ form) resin until neutral. The resin was filtered off and MeOH removed *in vacuo*. The product **333** was recrystallized from MeOH/ Et_2O in an 80% yield over 2 steps. 1H NMR (400

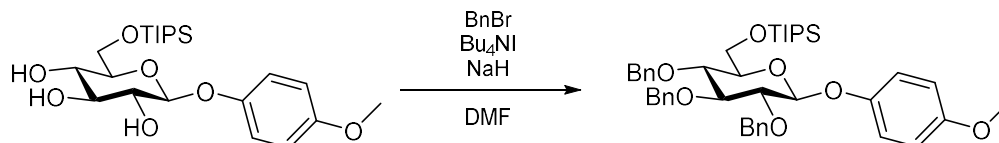
MHz, D₂O) δ 7.58-7.30 (m, 5H), 4.76-4.71 (d, 1H), 3.88-3.82 (dd, 1H), 3.73-3.64 (m, 1H), 3.57-3.46 (m, 2H), 3.46-3.30 (m, 3H). ¹³C NMR (400MHz, D₂O) δ 131.6, 129.3, 128.0, 87.3, 79.8, 77.2, 71.8, 69.3, 66.0, 60.8, 14.1. LRMS (ESI): m/z C₁₃H₁₈O₇Na [M+Na]⁺ = 309.2. All spectral data were consistent with those reported in the literature.⁹⁻¹¹

4-methoxyphenyl-6-*O*-(triisopropylsilyl)- β -D-glucopyranoside (**334**)



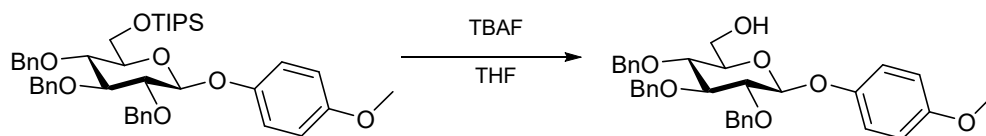
Compound **333** (0.9748 g, 3.43 mmol) was dissolved in DMF (7 mL) and imidazole (498.9 mg, 7.55 mmol) was added. TIPS-Cl (0.81 mL, 3.77 mmol) was added dropwise, and the reaction stirred O/N. Reaction was purified vis flash chromatography using 3:1 EtOAc: Pet ether, in 90 % yield of **334**. ¹H NMR (400 MHz, CDCl₃) δ 7.02-6.67 (m, 4H), 4.76-4.70 (m, 1H), 4.02-3.82 (m, 2H), 3.72 (s, 3H), 3.69-3.61 (m, 2H), 3.59-3.49 (m, 1H), 3.49-3.38 (m, 1H), 1.13-0.90 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 155.2, 151.2, 118.5, 114.4, 101.8, 75.3, 73.2, 72.2, 64.6, 55.5, 17.9, 11.7. LRMS (ESI): m/z C₂₂H₃₈O₇SiNa [M+Na]⁺ = 465.1.

4-methoxyphenyl-2,3,4-tri-*O*-benzyl-6-*O*-(triisopropylsilyl)- β -D-glucopyranoside (**335**)



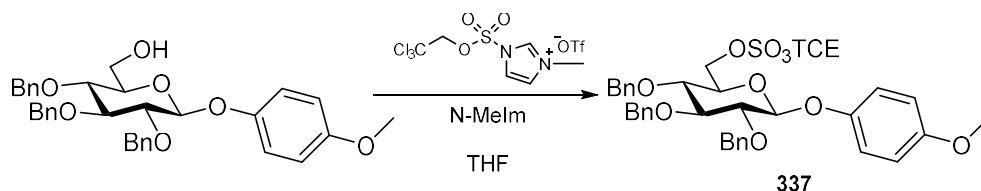
Into DMF (15 mL) 60% NaH (1.1443 g, 28.59 mmol), BnBr (2.6 mL, 21.43 mmol) and Bu₄AI (131.8 mg, 0.375 mmol) were added. Compound **334** (1.5816 g, 3.57 mmol) was dissolved in DMF (15 mL) and was added dropwise to the first flask. Reaction was allowed to stir overnight. Once complete, the reaction was then quenched with water and the product was extracted with EtOAc (x3). The organic layer was washed with 1 % HCl, Sat. NaHCO₃, water (x2) and brine. Organic layer was dried (Na₂SO₄), filtered, and concentrated under reduced pressure before being purified via flash chromatography (5 % EtOAc in pet. ether) in 80 % yield of **335**. ¹H NMR (300 MHz, CDCl₃) δ 7.47-7.30 (m, 15H), 7.14-6.83 (m, 4H), 5.17-4.98 (m, 2H), 4.99-4.86 (m, 4H), 4.79-4.73 (m, 1H), 4.07-4.01 (m, 1H), 3.96-3.89 (m, 1H), 3.81 (s, 3H), 3.80-3.69 (m, 3H), 3.52-3.45 (m, 1H), 1.17-1.07 (m, 21H). ¹³C NMR (300 MHz, CDCl₃) δ 155.3, 151.7, 138.0, 138.5, 138.3, 128.4, 128.4, 128.1, 128.02, 127.9, 127.8, 127.7, 127.6, 118.81 114.4, 103.2, 84.8, 82.3, 77.5, 76.3, 75.8, 75.0, 75.0, 62.6, 60.3, 55.4, 18.1, 18.0, 11.9. LRMS (ESI): m/z C₄₃H₅₆O₇SiNa [M+Na]⁺ = 735.9.

4-methoxyphenyl-2,3,4-tri-*O*-benzyl- β -D-glucopyranoside (**336**)



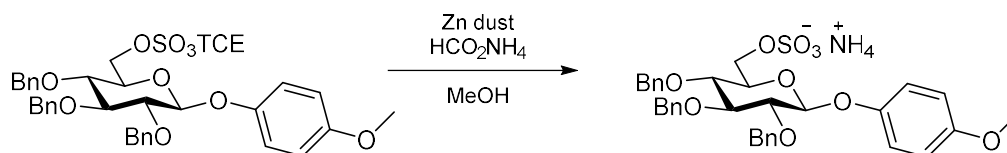
Compound **335** (0.3692 g, 0.51 mmol) was dissolved in THF (3 mL). TBAF (1.05 mL, 1.05 mmol) was added, and reaction was allowed to stir overnight. Reaction was then diluted with EtOAc and washed with 1 % HCl, NaHCO₃, and Brine before being dried (Na₂SO₄), filtered, and concentrated under reduced pressure. Product **336** was purified via flash chromatography using 5 % EtOAc in Pet Ether in a 95 % yield. ¹H NMR (500 MHz, CDCl₃) δ 7.38-7.27 (m, 14H), 7.01-6.80 (m, 4H), 5.05-5.01 (m, 1H), 4.98-4.94 (m, 2H), 4.91-4.80 (m, 3H), 4.69-4.65 (m, 1H), 3.93-3.86 (m, 1H), 3.78 (s, 3H), 3.77-3.61 (m, 4H), 3.50-3.44 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 154.7, 150.8, 118.1, 116.2, 115.0, 101.1, 76.0, 75.5, 72.9, 69.4, 60.5, 55.8. LRMS (ESI): m/z C₃₄H₃₆O₇Na [M+Na]⁺ = 578.9.

4-methoxyphenyl-2,3,4-tri-*O*-benzyl-6-*O*-trichloroethylsulfo-β-D-glucopyranoside (**337**)



Compound **336** (2.0 g, 3.6 mmol) was dissolved in THF (120 mL) at 0 °C before the sulfating agent **314** (6.39 g, 14.4 mmol) and n-methylimidazole (1.47 mL, 14.4 mmol) were added, and the reaction was allowed to stir 2 days. The reaction was quenched with H₂O and the product was extracted with EtOAc (x3). Organics were combined and washed with NaHCO₃ and brine, before being dried over MgSO₄. Crude product was filtered, condensed, and purified via flash chromatography (5 % EtOAc in Pet. Ether) in an 86 % yield of **337**. ¹H NMR (500 MHz, CDCl₃) δ 7.44-7.27 (m, 15H), 7.00-6.81 (m, 4H), 5.07-4.88 (m, 4H), 4.87-4.79 (m, 2H), 4.65-4.58 (d, 1H), 4.61 (s, 2H), 4.56-4.52 (m, 1H), 4.42-4.35 (m, 1H), 3.78 (s, 3H), 3.77-3.64 (m, 2H), 3.61-3.55 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 155.5, 150.9, 138.1, 137.9, 137.1, 128.6, 128.4, 128.4, 128.3, 128.2, 128.2, 127.9, 127.8, 118.1, 114.7, 102.3, 92.4, 84.2, 81.7, 79.6, 76.2, 75.7, 75.1, 75.1, 72.2, 71.9, 55.6, 29.6. LRMS (ESI): m/z C₃₆H₃₇Cl₃O₁₀SNa [M+Na]⁺ = 789.6.

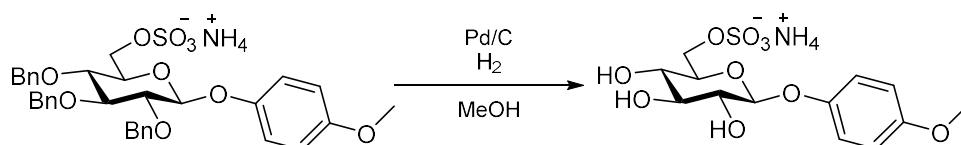
4-methoxyphenyl-2,3,4-tri-*O*-benzyl-6-*O*-sulfo-β-D-glucopyranoside (**338**)



Compound **337** (2.383 g, 3.1 mmol) dissolved in MeOH (70 mL) before Zn dust (1.42 g, 21.7 mmol) and ammonium formate (1.17 g, 18.5 mmol) were added. The reaction was allowed to stir for 24 h before being filtered through Celite and dried over Na₂SO₄. The crude product was then filtered and concentrated under reduced pressure before being purified via flash chromatography using 20 % MeOH in DCM, yield 95% of **338**. ¹H NMR (500 MHz, DMSO-d₆) δ 7.32-7.26 (m,

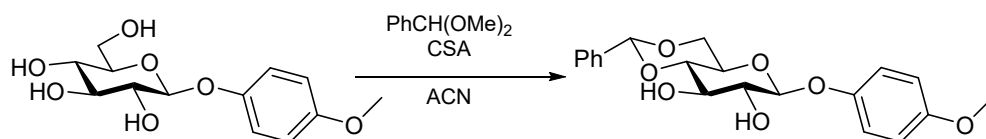
15H), 7.09 (brs, 1.4H, NH₄), 7.09-7.01 (m, 2H), 6.90-6.86 (m, 2H), 5.05 (d, 1H, *J* = 7.7 Hz), 4.93 (d, 1H, *J* = 11.3 Hz), 4.86-4.73 (m, 4H), 4.69 (d, 1H, *J* = 10.4 Hz), 4.04-3.98 (m, 2H), 3.77-3.68 (m, 2H), 3.71 (s, 3H), 3.54-3.50 (m, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 156.7, 154.3, 140.1, 139.9, 139.8, 129.8, 129.2, 129.1, 129.0, 128.8, 128.6, 128.4, 119.1, 115.5, 103.4, 84.2, 82.8, 78.3, 76.7, 76.0, 75.8, 74.3, 71.1, 56.0. LRMS (ESI): *m/z* C₃₄H₃₅O₁₀SNa [M+Na]⁺ = 635.0.

4-methoxyphenyl-6-*O*-sulfo-β-D-glucopyranoside (**339**)



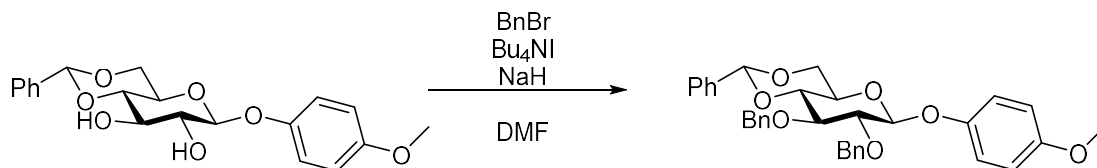
Compound **338** (2.77 g, 4.32 mmol) was suspended in EtOH (100 mL), and Pd/C (300 mg) was added before the atmosphere was removed and replaced with hydrogen. The reaction was stirred overnight when the atmosphere was evacuated and replaced with hydrogen again and stirred for an additional 3h. The reaction was then filtered through celite and concentrated under reduced pressure to generate **339** as a white solid in a 60 % yield. ¹H NMR (500 MHz, D₂O) δ 7.10-7.08 (m, 2H), 6.95-6.93 (m, 2H), 4.97 (d, 1H, *J* = 7.4 Hz), 4.33-4.31 (m, 1H), 4.20 (dd, 1H, *J* = 5.3, 11.3 Hz), 3.80-3.73 (m, 1H), 3.77 (s, 3H), 3.58-3.48 (m, 3H). ¹³C NMR (125 MHz, D₂O) δ 154.6, 150.8, 118.1, 114.9, 101.1, 75.2, 73.7, 72.7, 68.9, 66.7, 55.6. LRMS (ESI): *m/z* C₁₃H₁₇O₁₀SNa [M+Na]⁺ = 365.6.

4-methoxyphenyl-4,6-di-*O*-benzylidene-β-D-glucopyranoside (**340**)



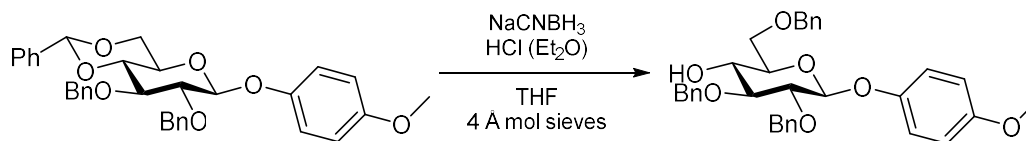
Compound **339** (10 g, 35.2 mmol) was dissolved in CAN (500 mL) before CSA (410 mg, 1.76 mmol) and PhCH(OMe)₂ (9.5 mL, 63.4 mmol) were added and the solution was heated to reflux. The solution was refluxed for 2 h, then cooled and left overnight. The reaction was quenched with Et₃N and the resulting solid product was filtered. The filtrate was washed with brine before being dried over Mg₂SO₄. The solvent was removed under reduced pressure and the crude product was recrystallized from DCM/hexanes in a 76 % yield of **340**. ¹H NMR (400 MHz, MeOD) δ 7.52-7.49 (m 2H), 7.36-7.32 (m, 3H), 7.03 (d, 2H, *J* = 9.1 Hz), 6.86-6.81 (d, 2H, *J* = 9.1 Hz), 5.60 (s, 1H), 4.91 (d, 1H, *J* = 7.6 Hz), 4.31 (dd, 1H, *J* = 4.2, 10.0 Hz), 3.82 (d, 1H, *J* = 10.0 Hz), 3.75 (s, 3H), 3.71 (d, 1H, *J* = 8.9 Hz), 3.61-3.49 (m, 3H). ¹³C NMR (100 MHz, MeOD) δ 129.0, 127.5, 119.4, 115.4, 82.1, 74.5, 69.6, 67.6, 56.0. LRMS (ESI): *m/z* C₂₀H₂₂O₇Na [M+Na]⁺ = 397.1.

4-methoxyphenyl-2,3-di-*O*-benzyl-4,6-di-*O*-benzylidene- β -D-glucopyranoside (**341**)



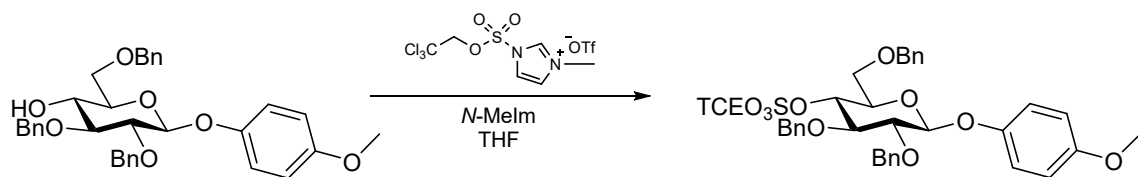
Compound **340** (9.5 g, 25.4 mmol) was dissolved in DMF (200 mL) before BnBr (7.6 mL, 63.4 mmol) was added. NaH (3.5 g, 88.8 mmol) was slowly added over ~30 min, and the reaction was allowed to stir overnight. The reaction was quenched with brine and the solvent was removed under reduced pressure. The crude product was recrystallized from DCM/hexanes in 80 % yield of **341**. ^1H NMR (400 MHz, CDCl_3) δ 7.51-7.49 (m, 2H), 7.41-7.29 (m, 13H), 7.02 (d, 2H, $J = 9.0$ Hz), 6.85 (d, 2H, $J = 9.1$ Hz), 5.60 (s, 1H), 5.02-4.94 (m, 3H), 4.86 (d, 1H, $J = 14.5$ Hz), 4.84 (d, 1H, $J = 15$ Hz), 4.38 (q, 1H, $J = 4.9, 5.4$ Hz), 3.88-3.71 (m, 7H), 3.54-3.48 (m, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 155.7, 151.3, 138.5, 138.3, 137.3, 129.1, 128.5, 128.4, 128.4, 128.3, 128.1, 127.9, 127.8, 126.1, 118.7, 114.7, 103.4, 101.3, 82.0, 81.4, 81.0, 75.6, 75.3, 68.9, 66.3, 55.8. LRMS (ESI): m/z $\text{C}_{34}\text{H}_{34}\text{O}_7\text{Na}$ $[\text{M}+\text{Na}]^+ = 557.8$.

4-methoxyphenyl-2,3,6-tri-*O*-benzyl- β -D-glucopyranoside (**342**)



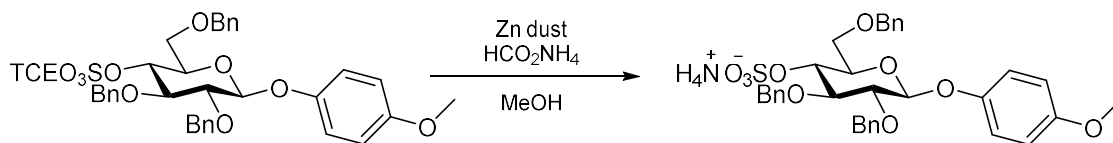
Compound **341** (2.13 g, 3.84 mmol) was dissolved in THF (80 mL) with 4 Å molecular sieves, before NaCNBH_3 (2.89 g, 46.1 mmol) was added. HCl in Et_2O was added until the formation of bubbles ceased (~30 mL). The reaction was allowed to stir for an additional 15 min before being diluted with DCM and H_2O . The reaction was then filtered, washed with sat. NaHCO_3 , dried over MgSO_4 , and concentrated under reduced pressure. Product **342** was passed through a silica plug and then recrystallized from Et_2O /Pet ether in a 91 % yield. ^1H NMR (400 MHz, CDCl_3) δ 7.37-7.28 (m, 15H), 7.01 (d, 2H, $J = 9.1$ Hz), 6.81 (d, 2H, $J = 9.1$ Hz), 5.06-4.90 (m, 3H), 4.81 (d, 1H, $J = 10.8$ Hz), 4.73 (d, 1H, $J = 11.3$ Hz), 4.56 (d, 2H, $J = 2.2$ Hz), 3.83-3.63 (m, 7H), 3.56-3.49 (m, 2H), 2.54 (brs, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 155.3, 151.6, 138.6, 138.4, 138.3, 128.7, 128.6, 128.5, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 118.4, 114.6, 103.0, 82.3, 79.4, 75.5, 75.0, 74.3, 73.5, 72.8, 62.0, 55.7. LRMS (ESI): m/z $\text{C}_{34}\text{H}_{36}\text{O}_7\text{Na}$ $[\text{M}+\text{Na}]^+ = 558.7$.

4-methoxyphenyl-2,3,6-tri-*O*-benzyl-4-*O*-trichloroethylsulfo- β -D-glucopyranoside (**343**)



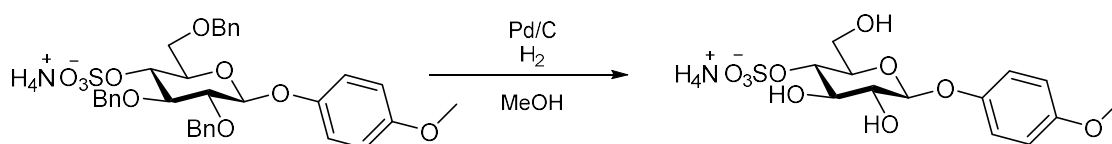
Compound **342** (1.96 g, 3.52 mmol) was dissolved in THF (125 mL) at 0°C before the sulfating agent **314** (3.12 g, 7.04 mmol) and *n*-methylimidazole (0.7 mL, 8.8 mmol) were added, and the reaction was allowed to stir for 2 days. The reaction was then quenched with H₂O and the product was extracted with EtOAc (x3). Organics were combined and washed with NaHCO₃ and brine, before being dried over MgSO₄. Crude product was filtered, condensed, and purified via flash chromatography (5 % EtOAc in Pet. Ether) in an 86 % yield of **343**. ¹H NMR (400 MHz, CDCl₃) δ 7.38-7.27 m (15H), 7.04-7.02 (m, 2H), 6.82-6.80 (m, 2H), 5.05 (d, 1H, *J* = 10.7 Hz), 4.97 (d, 1H, *J* = 10.7 Hz), 4.92 (dd, 1H, *J* = 2.0, 5.4 Hz), 4.90-4.85 (m, 1H), 4.80 (dd, 2H, *J* = 10.9, 12.0 Hz), 4.66-4.56 (m, 4H), 3.96-3.89 (m, 1H), 3.85-3.75 (m, 7H). ¹³C NMR (100 MHz, CDCl₃) δ 155.7, 151.2, 137.9, 137.7, 137.5, 128.6, 128.6, 128.4, 128.3, 128.1, 128.0, 127.8, 127.8, 118.6, 114.7, 102.7, 92.8, 81.8, 81.6, 81.0, 80.1, 75.4, 75.2, 73.8, 73.4, 68.6, 55.7. LRMS (ESI): *m/z* C₃₆H₃₇Cl₃O₁₀SNa [M+Na]⁺ = 789.5.

4-methoxyphenyl-2,3,6-tri-*O*-benzyl-4-*O*-sulfo-β-D-glucopyranoside (**344**)



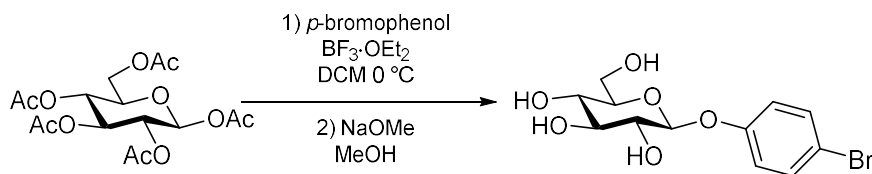
Compound **343** (159 mg, 0.21 mmol) was dissolved in MeOH (20 mL) before Zn dust (94 mg, 1.45 mmol) and ammonium formate (78 mg, 1.24 mmol) were added. The reaction was stirred for 6 h at room temperature before being filtered through celite. The crude mixture was concentrated under reduced pressure and purified via flash chromatography using 5 % MeOH in DCM. ¹H NMR (400 MHz, MeOD) δ 8.04 (brs, 1H), 7.47-7.39 (m, 2H), 7.37-7.18 (m, 15H), 7.03 (d, 2H, *J* = 9.0 Hz), 6.75 (d, 2H, *J* = 9.0 Hz), 5.16 (d, 1H, *J* = 10.3 Hz), 4.96-4.93 (m, 2H), 4.75 (d, 1H, *J* = 11.3 Hz), 4.68 (d, 1H, *J* = 10.4 Hz), 4.61-4.53 (m, 2H), 4.36 (t, 1H, *J* = 9.2 Hz), 4.21 (dd, 1H, *J* = 1.7, 11.2 Hz), 3.83-3.67 (m, 6H), 3.59 (dd, 1H, *J* = 7.8, 9.0 Hz). ¹³C NMR (100 MHz, MeOD) δ 156.7, 152.9, 140.0, 139.9, 139.8, 129.8, 129.2, 129.1, 129.0, 128.7, 128.5, 128.4, 119.8, 115.5, 103.4, 84.2, 82.8, 78.3, 76.7, 76.0, 75.8, 74.3, 71.1, 56.0. LRMS (ESI): *m/z* C₃₄H₃₅O₁₀S [M]⁻ = 635.5.

4-methoxyphenyl-4-*O*-sulfo-β-D-glucopyranoside (**345**)



Compound **344** (2.77 g, 4.32 mmol) was suspended in EtOH (100 mL) and Pd/C (300 mg) was added before the atmosphere was removed and replaced with hydrogen. The reaction was stirred overnight when the atmosphere was evacuated and replaced with hydrogen again and stirred for an additional 3 h. The reaction was then filtered through Celite and concentrated under reduced pressure to yield **345** as a white solid in 60 %. ^1H NMR (500 MHz, D_2O) δ 7.10-7.08 (m, 2H), 6.95-6.93 (m, 2H), 4.97 (d, 1H, J = 7.4 Hz), 4.33-4.31 (m, 1H), 4.20 (dd, 1H, J = 5.3, 11.3 Hz), 3.80-3.73 (m, 1H), 3.77 (s, 3H), 3.58-3.48 (m, 3H). ^{13}C NMR (125 MHz, D_2O) δ 154.6, 150.8, 118.1, 114.9, 101.1, 75.2, 73.7, 72.7, 68.9, 66.2, 55.6. LRMS (ESI): m/z $\text{C}_{13}\text{H}_{17}\text{O}_{10}\text{SNa}$ $[\text{M}+\text{Na}]^+ = 388.1$.

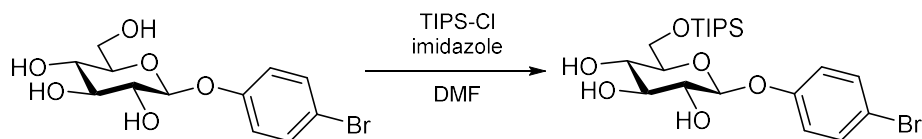
4-bromophenyl- β -D-glucopyranoside (**346**)⁹⁻¹¹



Glucose pentaacetate (15 g, 38.43 mmol) was dissolved in DCM (60 mL) before *p*-bromophenol (9.31 g, 53.80 mmol) was added and the reaction was cooled to 0 °C. $\text{BF}_3\cdot\text{OEt}_2$ (6.3 mL, 50 mmol) was then slowly added. The reaction stirred overnight before being quenched with sat. NaHCO_3 . The reaction was diluted with DCM and washed with H_2O and brine. The organic layer was dried over Na_2SO_4 , and the solvent was removed under reduced pressure. The product was recrystallized from EtOAc/hexanes to afford a white solid. ^1H NMR (400 MHz, CDCl_3) δ 7.40 (d, 2H, J = 8.8 Hz), 6.88 (d, 2H, J = 9.0 Hz), 5.31-5.22 (m, 2H), 5.18-5.13 (m, 1H), 5.03 (d, 1H, J = 7.7 Hz), 4.28 (dd, 1H, J = 5.1, 12.8 Hz), 4.16 (dd, 1H, J = 2.6, 12.4 Hz), 3.87-3.82 (m, 1H), 2.07, 2.06, 2.05, 2.04 (4s, 12H). ^{13}C NMR (100 MHz, CDCl_3) δ 171.8, 170.3, 169.2, 169.3, 156.3, 150.9, 118.5, 114.5, 100.4, 72.3, 71.9, 71.1, 68.5, 61.7, 55.3, 21.5, 20.3, 20.0, 19.9. LRMS (ESI): m/z $\text{C}_{20}\text{H}_{23}\text{O}_{10}\text{BrNa}$ $[\text{M}+\text{Na}]^+ = 527.9$.

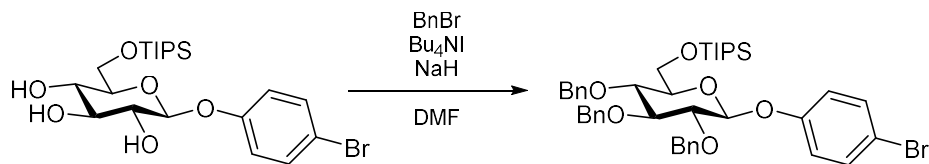
The recrystallized product (10.5 g) was dissolved in MeOH and NaOMe was added until a pH of ~ 12 . The reaction was stirred overnight before Amberlite resin (H^+ form) was added until a pH of ~ 6 . The solvent was removed under reduced pressure yielding 7.03 g of **346** (80 % yield over two steps). ^1H NMR (400 MHz, D_2O) δ 7.39 (d, 2H, J = 8.9 Hz), 6.92 (d, 2H, J = 8.9 Hz), 4.97 (d, 1H, J = 7.3 Hz), 3.79 (dd, 1H, J = 2.1, 12.3 Hz), 3.62 (dd, 1H, J = 5.8, 12.4 Hz), 3.52-3.34 (m, 4H). ^{13}C NMR (100 MHz, D_2O) δ 155.7, 132.5, 118.4, 114.9, 100.1, 76.1, 75.4, 72.8, 69.3, 60.4. LRMS (ESI): m/z $\text{C}_{13}\text{H}_{18}\text{O}_6\text{BrNa}$ $[\text{M}+\text{Na}]^+ = 357.6$. All spectral data were consistent with those reported in the literature.⁹⁻¹¹

4-bromophenyl-6-*O*-(triisopropylsilyl)- β -D-glucopyranoside (**347**)



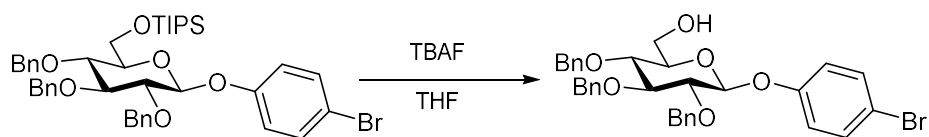
Compound **346** (7.02 g, 20.95 mmol) was dissolved in DMF (40 mL), and imidazole (2.79 g, 41.90 mmol) was added. The reaction was cooled to 0 °C before TIPS-Cl (4.7 mL, 21.99 mmol) was added dropwise. The reaction was allowed to warm to room temperature overnight before being quenched with H₂O. The product was extracted with EtOAc (x3) and dried over Na₂SO₄ before the product was filtered and precipitated using pet. ether in a 90 % yield of **347**. ¹H NMR (400 MHz, CDCl₃) δ 7.02-6.67 (m, 4H), 4.76-4.70 (m, 1H), 4.02-3.82 (m, 2H), 3.69-3.61 (m, 2H), 3.59-3.49 (m, 1H), 3.49-3.38 (m, 1H), 1.13-0.90 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ 156.3, 153.5, 120.3, 112.5, 101.8, 78.3, 75.6, 74.3, 65., 16.14, 12.4. LRMS (ESI): m/z C₂₁H₃₅O₇BrSiNa [M+Na]⁺ = 513.5.

4-bromophenyl-2,3,4-tri-*O*-benzyl-6-*O*-(triisopropylsilyl)- β -D-glucopyranoside (**348**)



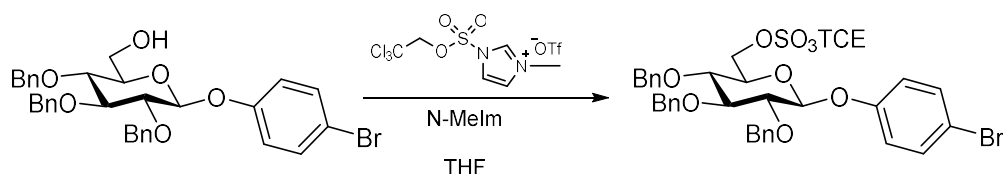
Compound **347** (12.4 g, 25.37 mmol) was dissolved in DMF (150 mL) before BnBr (12.0 mL, 101.4 mmol) was added. The flask was cooled to 0 °C before 60 % NaH (7.10 g, 177.6 mmol) was added in 4 equal portions. The reaction was allowed to warm to room temperature while stirring overnight. The reaction was quenched with brine and extracted with EtOAc (x3). The combined organics were dried over sodium sulfate, filtered, and concentrated under reduced pressure. Product was purified via flash chromatography using 2:8 EtOAc:pet. ether. ¹H NMR (300 MHz, CDCl₃) δ 7.51-7.35 (m, 15H), 7.24-6.84 (m, 4H), 6.18-5.11 (m, 2H), 4.99-4.86 (m, 4H), 4.79-4.73 (m, 1H), 4.07-4.01 (m, 1H), 3.96-3.89 (m, 1H), 3.76-3.59 (m, 3H), 3.49-3.38 (m, 1H), 1.22-1.09 (m, 21H). ¹³C NMR (300 MHz, CDCl₃) δ 155.7, 135.1, 135.2, 135.2, 127.3, 127.1, 127.3, 127.1, 125.1, 125.5, 125.3, 125.1, 116.21, 112.8, 101.1, 83.4, 81.6, 75.4, 74.3, 73.1, 73.7, 73.1, 60.1, 58.3, 19.3, 17.1, 10.6. LRMS (ESI): m/z C₄₂H₅₃O₇BrSiNa [M+Na]⁺ = 783.7.

4-bromophenyl-2,3,4-tri-*O*-benzyl- β -D-glucopyranoside (**349**)



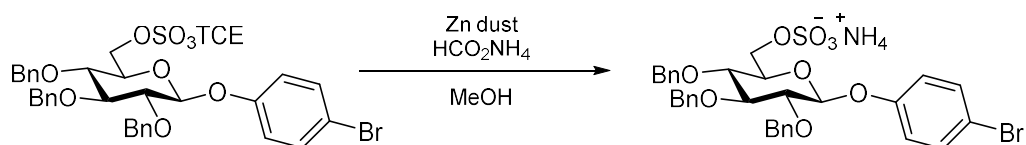
Compound **348** (25 mmol) was dissolved in THF (120 mL) before a solution of TBAF (1 M in THF) (25 mL) was added. The reaction was stirred for 5 h before being diluted with EtOAc. The mixture was washed with water, 10 % HCl, sat. sodium bicarbonate, and brine before being dried over sodium sulfate. The crude product was filtered, and the solvent was removed under reduced pressure. The product was recrystallized from MeOH. ^1H NMR (400 MHz, CDCl_3) δ 7.14 (d, 2H, J = 8.8 Hz), 7.35-7.29 (m, 15H), 6.90 (d, 2H, J = 8.8 Hz), 5.02 (d, 1H, J = 7.5 Hz), 4.96 (dd, 2H, J = 7.0, 11.0 Hz), 4.90-4.80 (m, 3H), 6.67 (d, 1H, J = 11.0 Hz), 3.89 (qd, 1H, J = 2.6, 6.0 Hz), 3.79-3.63 (m, 4H), 3.52-3.48 (m, 1H), 1.87 (dd, 1H, J = 6.0, 7.6 Hz). ^{13}C NMR (100 MHz, CDCl_3) δ 156.2, 138.6, 138.4, 138.3, 132.2, 128.2, 128.1, 127.8, 127.7, 127.6, 127.5, 127.4, 118.4, 113.7, 99.9, 83.5, 81.4, 77.2, 75.4, 74.5, 73.9, 59.9, 54.9, 30.6. LRMS (ESI): m/z $\text{C}_{33}\text{H}_{33}\text{BrO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 627.3$.

4-bromophenyl-2,3,4-tri-*O*-benzyl-6-*O*-trichloroethylsulfo- β -D-glucopyranoside (**350**)



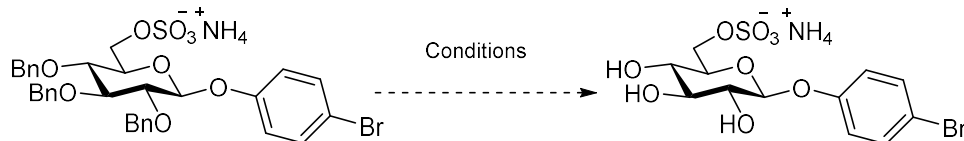
Compound **349** (1.09 g, 1.8 mmol) was dissolved in THF (100 mL) and cooled to 0 °C. The sulfating agent **314** (1.6 g, 3.6 mmol) and *n*-MeIm (0.36 mL, 2.5 mmol) were added, and the reaction was allowed to stir overnight while warming to room temperature. Once complete, the reaction was quenched with water and extracted with EtOAc (x3). Organics were combined and washed with NaHCO_3 and brine. Solvent was removed under reduced pressure and purified via flash chromatography using 2:8 EtOAc:pet. ether. ^1H NMR (400 MHz, CDCl_3) δ 7.45 (d, 2H, J = 8.8 Hz), 7.40-7.31 (m, 15H), 6.93 (d, 2H, J = 8.8 Hz), 5.07-5.02 (m, 3H), 4.97 (d, 1H, J = 11.0 Hz), 4.88 (t, 2H, J = 11.1 Hz), 4.67 (d, 1H, J = 10.3 Hz), 4.61 (s, 2H), 4.58 (dd, 1H, J = 1.9, 10.7 Hz), 4.41 (dd, 1H, J = 5.4, 10.6 Hz), 3.85-3.73 (m, 3H), 3.64-3.60 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 156.3, 155.9, 138.2, 138.1, 138.0, 137.8, 137.5, 137.1, 132.9, 132.6, 132.5, 132.3, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.9, 127.6, 118.8, 118.4, 115.6, 101.7, 101.2, 92.4, 84.4, 84.2, 81.9, 81.6, 79.8, 79.6, 78.2, 77.4, 77.3, 77.1, 76.8, 76.1, 75.8, 75.2, 75.0, 74.9, 72.5, 71.9, 60.4, 44.0, 21.1, 14.2. LRMS (ESI): m/z $\text{C}_{35}\text{H}_{34}\text{BrCl}_3\text{O}_9\text{SNa}$ $[\text{M}+\text{Na}]^+ = 837.8$.

4-bromophenyl-2,3,4-tri-*O*-benzyl-6-*O*-sulfo- β -D-glucopyranoside (**351**)



Compound **350** (1.68 g, 2.06 mmol) dissolved in MeOH (90 mL) before Zn dust (940 mg, 14.4 mmol) and ammonium formate (782 mg, 12.4 mmol) were added. The reaction was allowed to stir for 24 h before being filtered through Celite, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified using 20 % MeOH in DCM, yielding 95 %. ¹H NMR (400 MHz, MeOD) δ 7.38 (d, 2H, *J* = 8.8 Hz), 7.33-7.22 (m, 15H), 6.96 (d, 2H, *J* = 8.8 Hz), 5.02 (d, 1H, *J* = 7.5 Hz), 4.94-4.74 (m, 10H), 4.33 (dd, 1H, *J* = 1.8, 10.8 Hz), 4.26 (dd, 1H, *J* = 4.6, 10.8 Hz), 3.76-3.72 (m, 2H), 3.65-3.61 (m, 2H). ¹³C NMR (100 MHz, MeOD) δ 157.8, 139.8, 139.6, 139.5, 133.4, 129.3, 129.2, 129.1, 128.7, 128.7, 128.6, 128.5, 119.6, 115.7, 102.4, 85.5, 83.1, 78.5, 76.5, 75.9, 75.9, 74.7, 67.4. LRMS (ESI): *m/z* C₃₃H₃₂BrO₉S [M]⁻ = 683.9.

4-bromophenyl-6-*O*-sulfo-β-D-glucopyranoside (**352**)



Condition 1

Compound **351** (341 mg, 0.79 mmol) was suspended in EtOH (15 mL), and Pd/C (25 mg) was added before the atmosphere was removed and replaced with hydrogen. The reaction was stirred overnight when the atmosphere was evacuated and replaced with hydrogen again. Stirred for an additional 3h. The reaction was then filtered through Celite and concentrated under reduced pressure to a yellow solid. NMR showed removal of the bromine atom.

Condition 2

Compound **351** (100 mg, 0.14 mmol) was dissolved in DCM (3 mL) and was cooled to -78 °C. BCl₃ (1 mL, 1 mmol) was slowly added, and the reaction was stirred at -78 °C for one hour. The reaction was slowly quenched using DCM:MeOH (1:1) and the solvent was removed under reduced pressure. A complex mixture of products was generated, and no SM was reisolated.

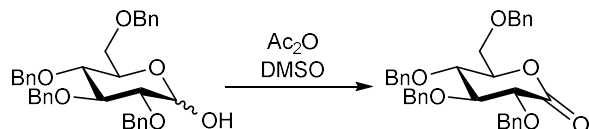
Condition 3

Compound **351** (126 mg, 0.17 mmol) was dissolved in DCM (4 mL) and was cooled to -78 °C. BCl₃ (261.3 mg, 1.61 mmol) was slowly added, and the reaction was stirred at -20 °C overnight. The reaction was slowly quenched using DCM:MeOH (1:1) and the solvent was removed under reduced pressure. No product formation was observed.

Condition 4

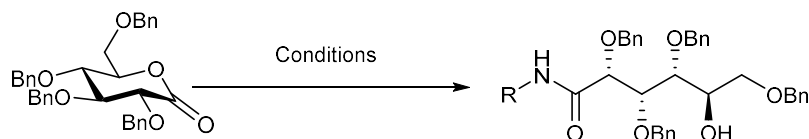
Compound **351** (100 mg, 0.14 mmol) was dissolved in MeOH (2 mL) before CCl₄ (0.5 mL, 0.79 mmol) and water (1 mL) was added. The reaction was exposed to 395 nm light (LED) overnight. Solvent was removed under reduced pressure. No product formation was observed.

2,3,4,6-tetra-*O*-benzyl-D-gluconic acid- δ -lactone (**424**)



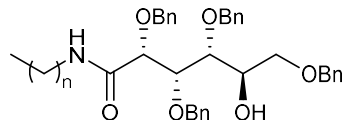
2,3,4,6-tetra-*O*-benzyl-D-glucose (2.0 g, 3.7 mmol) was dissolved in anhydrous DMSO (8.0 mL, 112.63 mmol) and stirred at room temperature for 30 mins. Ac₂O (6.0 mL, 63.59 mmol) was then added, and the reaction mixture stirred for 24 hours at room temperature. The solution was quenched with 3.3 M HCl and diluted with toluene. The organic layer was extracted with 2 M PBS (pH 7 x 2). The combined organic layers were dried with Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified via flash chromatography (15:75 EtOAc:pet. Ether) to produce yellow oil (95 % yield). ¹H NMR (400 MHz, C₆D₆) δ 7.35 (d, 2H, *J* = 7.1 Hz), 7.24-7.08 (m, 20H), 5.00 (d, 1H, *J* = 11.6 Hz), 4.61 (dd, 2H, *J* = 3.7, 11.5 Hz), 4.55 (d, 1H, *J* = 11.5 Hz), 4.42 (dd, 1H, *J* = 5.5, 11.2 Hz), 4.35-4.30 (m, 2H), 4.23 (d, 1H, *J* = 12.0 Hz), 4.11 (d, 1H, *J* = 6.2 Hz), 3.96 (dd, 1H, *J* = 6.8, 8.4 Hz), 3.85 (t, 1H, *J* = 6.5 Hz), 3.52-3.45 (m, 2H). ¹³C (100 MHz, C₆D₆) δ 168.8, 138.5, 138.4, 138.3, 137.8, 128.7, 128.6, 128.6, 128.5, 128.1, 128.1, 127.9, 81.4, 78.1, 78.0, 76.5, 73.8, 73.7, 73.6, 73.5, 68.6. LRMS (ESI): *m/z* C₃₄H₃₄O₆Na [M+Na]⁺ = 561.6.

2,3,4,6-tetra-*O*-benzyl-D-gluconamides (**425 a-g**)



Condition 1 (alkyl- and benzylgluconamides **425a-e**)

Lactone **424** (1 mmol) was added to the alkyl- or benzylamine (2.5 mmol) in toluene and the mixture was stirred overnight. The solution was quenched with H₂O (5 mL/g) and stirred for 20 minutes (x2). The combined organic layers were dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified via flash chromatography (30:70 EtOAc:pet. Ether) to produce a colourless oil (60-98 %)



426a *n* = 3
426b *n* = 5
426c *n* = 7

N-octyl-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (**425a**)

^1H NMR (400 MHz, DMSO- d_6) δ 0.81-0.85 (m, 3H), 1.16-1.33 (m, 11H), 1.36-1.46 (m, 2H), 3.00-3.10 (m, 1H), 3.15-3.26 (m, 1H), 3.50 (dd, 1H, J = 9.9, 5.5 Hz), 3.60-3.68 (m, 2H), 3.79-3.87 (m, 1H), 4.02-4.07 (m, 1H), 4.11 (d, 1H, J = 5.9 Hz), 4.44-4.50 (m, 4H), 4.58-4.64 (m, 3H), 4.67 (d, 1H, J = 11.0 Hz), 5.05 (d, 1H, J = 5.4 Hz), 7.15-7.42 (m, 21H), 7.98 (t, 1H, J = 5.8 Hz). ^{13}C (100 MHz, DMSO- d_6) δ 169.6, 138.8, 138.4, 137.7, 128.1, 128.12, 128.0, 127.9, 127.6, 127.5, 127.5, 127.2, 127.2, 127.1, 81.8, 80.3, 79.6, 74.8, 73.4, 72.4, 72.3, 71.7, 69.9, 38.4, 31.2, 29.0, 28.7, 28.6, 26.4, 22.0, 13.9. 86 % yield. LRMS (ESI): m/z $\text{C}_{42}\text{H}_{53}\text{NO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 690.8$.

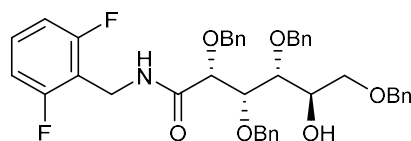
***N*-hexyl-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (425b)**

^1H NMR (400 MHz, CDCl_3) δ 7.37-7.23 (m, 20H), 6.66 (t, 1H, J = 5.8 Hz), 4.73-4.49 (m, 8H), 4.28 (d, 1H, J = 2.9 Hz), 4.09 (dd, 1H, J = 3.1, 5.7 Hz), 3.96-3.92 (m, 1H), 3.88 (dd, 1H, J = 5.7, 7.5 Hz), 3.67 (dd, 1H, J = 3.0, 9.7 Hz), 3.61 (dd, 1H, J = 5.3, 9.8 Hz), 3.33-3.22 (m, 1H), 3.15-3.07 (m, 1H), 2.93 (d, 1H, J = 3.9 Hz), 1.42-1.39 (m, 2H), 1.30-1.22 (m, 6H), 0.90-0.86 (m, 3H). ^{13}C (100 MHz, CDCl_3) δ 170.8, 138.3, 138.2, 138.0, 136.9, 128.7, 128.5, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7, 127.7, 80.8, 80.26, 77.7, 75.2, 74.2, 74.0, 73.5, 71.5, 71.2, 39.3, 31.5, 29.5, 26.7, 22.6, 14.1. 98 % yield LRMS (ESI): m/z $\text{C}_{40}\text{H}_{49}\text{NO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 662.1$.

***N*-butyl-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (425c)**

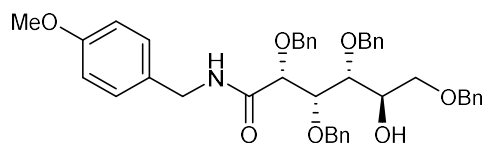
^1H NMR (400 MHz, CDCl_3) δ 7.20-7.37 (m, 20H), 6.64 (t, 1H, J = 5.8 Hz), 4.47-4.73 (m, 9H), 4.26 (d, 1H, J = 3.1 Hz), 4.05-5.09 (m, 1H), 3.84-9.92 (m, 1H), 3.57-3.69 (m, 2H), 3.23-3.34 (m, 1H), 3.06-3.16 (m, 1H), 2.90 (d, 1H, J = 3.6 Hz), 1.33-1.42 (m, 2H), 1.24-1.32 (m, 2H), 0.87 (t, 3H, J = 7.2). ^{13}C (100 MHz, CDCl_3) δ 170.8, 138.2, 138.1, 137.8, 136.8, 128.6, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 80.7, 80.1, 77.5, 75.1, 74.1, 73.9, 73.4, 71.4, 71.1, 38.8, 31.5, 20.1, 13.7. 60 % yield. LRMS (ESI): m/z $\text{C}_{38}\text{H}_{45}\text{NO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 634.3$.

***N*-(2,6-difluorobenzyl)-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (425d)**



^1H NMR (400 MHz, acetone- d_6) δ 7.69 (t, 1H, J = 5.6 Hz), 7.40-7.21 (m, 20H), 7.10-6.64 (m, 2H), 4.79 (d, 1H, J = 11.3 Hz), 4.71-4.46 (m, 9H), 4.34 (d, 1H, J = 4.7 Hz), 4.18 (t, 1H, J = 4.7 Hz), 4.30-3.99 (m, 2H), 3.92 (t, 1H, J = 5.8 Hz), 3.75 (dd, 1H, J = 2.9, 9.5 Hz), 3.65 (dd, 1H, J = 5.1, 9.8 Hz). ^{13}C NMR (100 MHz, acetone- d_6) δ 171.0, 163.7 (d, $J_{\text{C-F}}$ = 8.2 Hz), 161.2 (d, $J_{\text{C-F}}$ = 8.1 Hz), 140.1, 139.9, 139.7, 138.6, 130.6 (t, $J_{\text{C-F}}$ = 10.4 Hz), 129.1 (d, $J_{\text{C-F}}$ = 6.1 Hz), 128.9 (d, $J_{\text{C-F}}$ = 3.5 Hz), 128.6 (d, $J_{\text{C-F}}$ = 3.5 Hz), 128.5 (d, $J_{\text{C-F}}$ = 2.9 Hz), 128.2, 128.0 (d, $J_{\text{C-F}}$ = 3.9 Hz), 115.2 (t, $J_{\text{C-F}}$ = 19.2 Hz), 112.3 (d, $J_{\text{C-F}}$ = 6.7 Hz), 112.1 (d, $J_{\text{C-F}}$ = 6.4 Hz), 82.1 (d, $J_{\text{C-F}}$ = 29.2), 80.1 (d, $J_{\text{C-F}}$ = 2.9 Hz), 75.9, 74.7, 74.1, 73.7, 72.6 (d, $J_{\text{C-F}}$ = 2.0 Hz), 71.9 (d, $J_{\text{C-F}}$ = 10.1 Hz), 31.5 (t, $J_{\text{C-F}}$ = 3.9 Hz). 91 % yield. LRMS (ESI): m/z $\text{C}_{41}\text{H}_{41}\text{F}_2\text{NO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 704.4$.

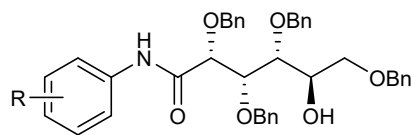
***N*-(4-methoxybenzyl)-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (425e)**



^1H NMR (400 MHz, CDCl_3) δ 7.32-7.15 (m, 20H), 7.08 (m, 2H), 6.86 (t, 1H, $J = 5.7$), 6.75 (m, 2H), 4.69-4.46 (m, 8H), 4.36 (dd, 1H $J = 6.3, 14.6$), 4.29 (d, 1H, $J = 3.07$), 4.20 (dd, 1H, $J = 5.4, 14.4$), 4.08-4.06 (m 1H), 3.93-3.82 (m 2H), 3.57 (s 3H), 3.64 (dd, 1H, $J = 3.0, 12.7$), 3.57 (dd, 1H, $J = 5.4, 9.6$). ^{13}C NMR (100 MHz, CDCl_3) δ 170.8, 159.1, 138.3, 138.2, 138.0, 136.7, 130.1, 129.3, 128.7, 128.6, 128.5, 128.4, 128.4, 128.2, 128.1, 128.0, 127.8, 127.8, 114.2, 80.8, 80.2, 77.6, 75.2, 74.2, 74.1, 73.5, 71.6, 71.2, 55.4, 42.9. 86 % yield. LRMS (ESI): m/z $\text{C}_{42}\text{H}_{45}\text{NO}_7\text{Na}$ $[\text{M}+\text{Na}]^+ = 699.1$

Condition 2 (arylgluconamides 425f, g)

Corresponding aniline (0.5 mmol) was dissolved in anhydrous toluene. Me_3Al (1.0 mmol) was added to the solution and stirred for an hour at room temperature. Then lactone (**424**) (0.5 mmol) was added, and the reaction mixture stirred overnight. The solution was slowly quenched with 10 % HCl (aq.). The solution mixture was filtered over Celite, and the organic layer was extracted with EtOAc (x3). The combined organic layers were dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified via flash chromatography (20:80 EtOAc :Pet Ether) to produce a faintly yellow oil (59-75 % yield).



425f R = 4-Cl
425g R = 2-F

***N*-(4-chlorophenyl)-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (425f)**

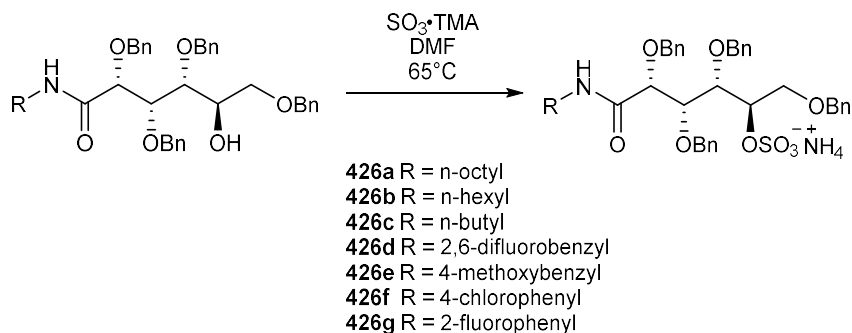
^1H NMR (400 MHz, DMSO) δ 3.52 (dd, 1H, $J = 9.9, 5.4$ Hz), 3.65 (dd, 1H, $J = 10.0, 3.3$ Hz), 3.72 (dd, 1H, $J = 6.4, 5.1$ Hz), 3.82-3.89 (m, 1H), 4.16 (t, 1H, $J = 5.3$ Hz), 4.31 (d, 1H, $J = 5.6$ Hz), 4.44-4.47 (m, 8H), 5.12 (d, 1H, $J = 5.5$ Hz), 7.14-7.45 (m, 22H), 7.73-7.79 (m, 2H), 10.06 (s, 1H). ^{13}C (100 MHz, DMSO) δ 169.4, 138.7, 138.5, 138.4, 137.4, 137.4, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5, 127.5, 127.3, 127.2, 127.2, 121.6, 82.2, 80.5, 79.6, 75.0, 73.5, 72.6, 72.3, 71.6, 70.0. 75 % yield. LRMS (ESI): m/z $\text{C}_{40}\text{H}_{40}\text{ClNO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 688.4$.

***N*-(2-fluorophenyl)-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (425g)**

^1H NMR (400 MHz, DMSO) δ 3.56 (dd, 1H, $J = 9.9, 5.4$ Hz), 3.68 (dd, 1H, $J = 9.9, 3.32$ Hz), 3.79 (dd, 1H, $J = 6.4, 5.5$ Hz), 3.86-3.94 (m, 1H), 4.19 (t, 1H, $J = 5.2$ Hz), 4.42 (d, 1H, $J = 5.1$), 4.46-4.78 (m, 8H), 5.18 (d, 1H, $J = 5.47$ Hz), 7.14-7.40 (m, 22H), 7.43 (dd, 2H, $J = 8.1, 1.6$ Hz),

7.85-7.92 (m, 1H), 9.48 (s, 1H). ^{13}C (100 MHz, DMSO) δ 170.2, 169.1, 154.9, 152.4, 138.9 (d, $J_{\text{C-F}} = 2.2$ Hz), 138.4, 137.4, 128.6 (d, $J_{\text{C-F}} = 1.2$ Hz), 128.0 (d, $J_{\text{C-F}} = 3.1$ Hz), 127.8 (d, $J_{\text{C-F}} = 2.1$ Hz), 127.7, 126.1, 126.0, 126.0, 125.9, 124.8 (d, $J_{\text{C-F}} = 3.2$ Hz), 124.3, 115.4, 115.2, 81.3, 80.6, 79.6, 75.0, 73.6, 72.9, 72.3, 71.6, 70.0, 59.7, 20.7, 14.0. 80 % yield. LRMS (ESI): m/z $\text{C}_{40}\text{H}_{40}\text{FNO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 672.29$.

2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-D-gluconamides (426 a-g)



Corresponding gluconamide (1 mmol) was dissolved in DMF. Next, $\text{SO}_3 \cdot \text{Me}_3\text{N}$ (10-20 mmol) was added to the solution and stirred overnight at 60 °C. The solution was quenched with MeOH, and the solvent was then removed under reduced pressure. The solution was then co-vaped with toluene, 99% EtOH, and DCM. The solution was then dissolved in minimal amount of MeOH and ammonium hydroxide was added, and the solids filtered off. The remaining crude product was dry packed on a column and purified with (10:90 MeOH:DCM + 1% NH_4OH) to produce a white solid (40-86 % yield).

N-octyl-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-D-gluconamide (426a)

^1H NMR (400 MHz, DMSO- d_6) δ 0.80-0.86 (m, 3H), 1.15-1.29 (m, 11H), 1.34-1.45 (m, 2H), 2.96-3.05 (m, 1H), 3.16-3.26 (m, 1H), 3.70 (dd, 1H, $J = 10.3, 3.5$ Hz), 3.97-4.08 (m, 2H), 4.09-4.16 (m, 2H), 4.10-4.15 (m, 2H), 4.45-4.75 (m, 9H), 7.11 (s, 3H), 7.16-7.40 (m, 19H), 7.44-7.57 (m, 2H), 7.76 (t, 1H, $J = 5.9$ Hz). ^{13}C (100 MHz, DMSO- d_6) δ 169.7, 139.0, 138.9, 138.5, 137.7, 128.6, 128.1, 128.0, 128.0, 127.8, 127.8, 127.6, 127.4, 127.2, 127.2, 127.1, 127.0, 81.2, 80.4, 78.7, 75.3, 74.7, 73.8, 72.7, 72.4, 68.3, 38.4, 31.2, 29.0, 28.7, 28.6, 26.4, 22.0, 13.9. 86 % yield. LRMS (ESI) $\text{C}_{42}\text{H}_{52}\text{NO}_9\text{S}$ $[\text{M}]^- = 769.8$.

N-hexyl-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-D-gluconamide (426b)

^1H NMR (400 MHz, DMSO- d_6) δ 7.77 (t, 1H, $J = 5.6$ Hz), 7.49-7.18 (m, 20H), 7.09 (brs, 4H), 4.73-4.46 (m, 9H), 4.14-3.99 (m, 4H), 3.71 (dd, 1H, $J = 3.5, 10.1$ Hz), 3.35 (s, 3H), 3.21 (sext, 1H, $J = 6.5$ Hz), 3.01 (sext, 1H, $J = 7.0$ Hz), 1.41-1.36 (m, 2H), 1.21 (brs, 6H). ^{13}C (100 MHz, DMSO- d_6) δ 170.2, 139.5, 139.4, 139.0, 138.2, 129.1, 128.6, 128.5, 128.5, 128.3, 128.3, 128.1,

127.9, 127.7, 127.7, 127.6, 127.5, 81.7, 80.9, 79.1, 75.7, 75.2, 74.3, 73.2, 72.9, 68.8, 38.9, 31.4, 29.5, 26.6, 22.4, 14.3. 88 % yield. LRMS (ESI): m/z $C_{40}H_{48}NO_9S$ $[M]^- = 741.1$.

***N*-butyl-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-*D*-gluconamide (426c)**

1H NMR (400 MHz, DMSO- d_6) δ 7.75 (t, 1H, $J = 6.1$ Hz), 7.16-7.51 (m, 20H), 7.07 (s, 4H), 4.42-4.75 (m, 9H), 4.08-4.16 (m, 2H), 3.97-4.07 (m, 2H), 3.69 (dd, 1H, $J = 3.6, 10.6$ Hz), 3.14-3.27 (m, 1H), 2.95-3.09 (m, 1H), 1.33-1.43 (m, 2H), 1.17-1.29 (m, 2H), 0.82 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR (100 MHz, DMSO- d_6) δ 169.7, 139.1, 138.9, 138.5, 137.7, 128.6, 128.1, 128.0, 128.0, 127.8, 127.6, 127.4, 127.2, 127.2, 127.1, 127.0, 81.3, 80.4, 75.2, 74.7, 73.8, 72.7, 72.4, 68.3, 38.0, 31.2, 19.5, 13.6. 87 % yield. LRMS (ESI): m/z $C_{38}H_{44}NO_9S$ $[M]^- = 713.4$.

***N*-(2,6-difluorobenzyl)-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-*D*-gluconamide (426d)**

1H NMR (400 MHz, acetone- d_6) δ 7.85 (t, 1H, $J = 5.7$ Hz), 7.38-6.92 (m, 27H), 4.81-4.62 (m, 7H), 4.56-4.43 (m, 5H), 4.37 (t, 1H, $J = 7.9$ Hz), 4.30 (dd, 1H, $J = 2.9, 7.5$ Hz), 4.12 (dd, 1H, $J = 3.3, 10.6$ Hz), 3.92 (dd, 1H, $J = 3.05, 10.4$ Hz). ^{13}C NMR (100 MHz, acetone- d_6) δ 171.0 (d, $J_{C-F} = 7.6$ Hz), 169.5, 163.6 (d, $J_{C-F} = 8.4$ Hz), 161.1 (d, $J_{C-F} = 8.2$ Hz), 140.0, 139.8, 139.6, 139.0 (t, $J_{C-F} = 9.6$ Hz), 138.5, 130.6 (t, $J_{C-F} = 10.5$ Hz), 129.6-128.3 (m, complex C-F coupling), 128.17, 128.0 (d, $J_{C-F} = 4.30$ Hz), 127.8, 115.1 (t, $J_{C-F} = 19.37$ Hz), 112.7-112.0 (m, complex C-F coupling), 82.2 (d, $J_{C-F} = 10.67$ Hz), 81.9, 81.38, 80.06, 78.6 (d, $J_{C-F} = 27.4$ Hz), 77.04, 75.8 (t, $J_{C-F} = 37.4$ Hz), 74.6, 74.15, 73.9 (d, $J_{C-F} = 6.7$ Hz), 73.74 (t, $J_{C-F} = 3.3$ Hz), 72.60, 71.93, 69.6 (d, $J_{C-F} = 19.4$ Hz), 31.5-31.3 (m, complex C-F coupling). 66 % yield. LRMS (ESI): m/z $C_{41}H_{40}F_2NO_9S$ $[M]^- = 760.8$.

***N*-(4-methoxybenzyl)-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-*D*-gluconamide (426e)**

1H NMR (400 MHz, acetone- d_6) δ 7.97 (t, 1H, $J = 6.1$ Hz), 7.53-7.37 (m, 6H), 7.29-7.16 (m, 18H), 6.75-6.71 (m, 2H), 4.84-4.29 (m, 14H), 4.10 (dd, 1H, $J = 3.2, 10.5$ Hz), 3.90 (dd, 1H, $J = 2.7, 10.4$ Hz), 3.71 (s, 3H). ^{13}C NMR (100 MHz, acetone- d_6) δ 172.6, 159.8, 140.2, 140.1, 139.9, 138.7, 131.7, 129.8, 129.6, 129.1, 129.0, 128.9, 128.8, 128.5, 128.5, 128.0, 127.9, 127.9, 114.6, 82.8, 82.4, 78.7, 77.8, 76.2, 75.4, 74.7, 73.9, 69.9, 55.5, 43.0, 42.9. LRMS (ESI): m/z $C_{42}H_{44}NO_{10}S$ $[M]^- = 755.3$.

***N*-(4-chlorophenyl)-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-*D*-gluconamide (426f)**

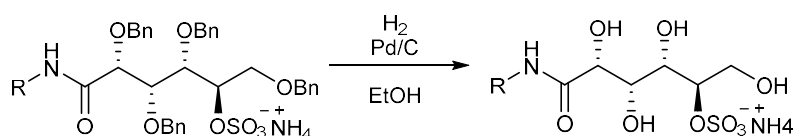
1H NMR (400 MHz, DMSO) δ 3.71 (dd, 1H, $J = 10.3, 3.3$ Hz), 4.00 (dd, 1H, $J = 10.2, 4.4$ Hz), 4.13-4.20 (m, 2H), 4.33 (d, 1H, $J = 3.9$ Hz), 4.43-4.46 (m, 4H), 4.60-4.70 (m, 4H), 4.72 (d, 1H, $J = 10.5$ Hz), 7.06-7.38 (m, 23H), 7.48-7.53 (m, 2H), 7.69-7.73 (m, 2H), 9.85 (s, 1H). ^{13}C (100 MHz, DMSO) δ 129.4, 128.8, 128.6, 128.5, 128.4, 128.3, 128.1, 127.8, 127.7, 127.7, 127.6, 122.0, 81.9, 81.2, 78.9, 75.5, 74.3, 73.4, 72.9, 68.9. 98 % yield. LRMS (ESI): m/z $C_{40}H_{39}ClNO_9S$ $[M]^- = 744.2$.

***N*-(2-fluorophenyl)-2,3,4,6-tetra-*O*-benzyl-5-*O*-sulfo-*D*-gluconamide (426g)**

1H NMR (400 MHz, DMSO) δ 3.75 (dd, 1H, $J = 10.1, 3.5$ Hz), 4.03 (dd, 1H, $J = 10.2, 5.0$ Hz), 4.18 (dd, 1H, $J = 7.2, 3.7$ Hz), 4.26 (dd, 1H, $J = 7.2, 5.1$ Hz), 4.42 (d, 1H, $J = 6.7$ Hz), 4.44 (s,

1H), 4.49-4.74 (m, 7H), 4.81 (d, 1H, $J = 10.4$ Hz), 7.03-7.36 (m, 21H), 7.37-7.41 (m, 2H), 7.49-7.54 (m, 2H), 7.98-8.05 (m, 1H), 9.24 (s, 1H). ^{13}C (100 MHz, DMSO) δ 169.2, 162.3, 154.3, 151.9, 138.9, 138.4 (d, $J_{\text{C-F}} = 7.8$ Hz), 137.3, 128.2 (d, $J_{\text{C-F}} = 5.4$ Hz), 128.0, 127.9, 127.8 (d, $J_{\text{C-F}} = 2.0$ Hz), 127.4, 127.2 (d, $J_{\text{C-F}} = 3.0$ Hz), 127.16, 125.6 (d, $J_{\text{C-F}} = 10.9$ Hz), 125.2 (d, $J_{\text{C-F}} = 8.0$ Hz), 124.4 (d, $J_{\text{C-F}} = 3.4$ Hz), 122.8, 115.2 (d, $J_{\text{C-F}} = 18.5$ Hz), 80.9 (d, $J_{\text{C-F}} = 11.6$ Hz), 78.9, 75.2 (d, $J_{\text{C-F}} = 24.0$ Hz), 74.1, 73.2, 72.4, 68.4, 44.2, 35.7, 30.7. 88 % yield. LRMS (ESI): m/z $\text{C}_{40}\text{H}_{39}\text{FNO}_9\text{S} [\text{M}]^- = 728.1$.

5-*O*-sulfo-D-gluconamides (427-433)



427 R = phenyl
 428 R = butyl
 429 R = hexyl
 430 R = octyl
 431 R = 2-fluorophenyl
 432 R = 4-methoxybenzyl
 433 R = 2,6-difluorophenyl

The benzyl-protected gluconamide (1 mmol) was dissolved in EtOH (99 %) and Pd/C (10 mol %) was added. The flask was then evacuated of air and filled with H_2 ; this process was repeated 5 times. Once completed the solution was run over celite and washed with ethanol. Solvent was removed under reduced pressure to yield off-white crystals (427,429-433) or a clear oil (429) in 40-95 % yield.

N-phenyl-5-*O*-sulfo-D-gluconamide (427)

^1H NMR (400 MHz, DMSO- d_6) δ 3.51-3.60 (m, 1H), 3.60-3.77 (m, 2H), 3.97-4.03 (m, 1H), 4.05-4.12 (m, 1H), 4.20 (t, 1H, $J = 3.3$ Hz), 4.44 (t, 1H, $J = 5.2$ Hz), 4.66 (d, 1H, $J = 5.2$ Hz), 4.84 (d, 1H, $J = 6.3$ Hz), 5.47 (d, 1H, $J = 4.0$ Hz), 7.00-7.16 (m, 5H), 7.29 (t, 2H, $J = 7.8$ Hz), 7.69 (d, 2H, $J = 7.8$ Hz), 9.55 (s, 1H). ^{13}C (100 MHz, DMSO- d_6) δ 171.5, 139.0, 129.0, 123.8, 119.9, 77.6, 74.5, 70.7, 70.2, 61.5. 43 % yield. LRMS (ESI): m/z $\text{C}_{12}\text{H}_{16}\text{NO}_9\text{S} [\text{M}]^- = 350.7$.

N-butyl-5-*O*-sulfo-D-gluconamide (428)

^1H NMR (400 MHz, DMSO- d_6) δ 7.63 (t, 1H $J = 5.7$ Hz), 7.10 (brs, 1H), 5.24 (d, 1H, $J = 3.7$ Hz), 4.81 (d, 1H, $J = 6.1$ Hz), 4.56 (d, 1H, $J = 4.9$ Hz), 4.41 (t, 1H, $J = 5.1$ Hz), 4.07-3.98 (m, 2H), 3.86 (brs, 1H), 3.73-3.64 (m, 2H), 3.56-3.51 (m, 1H), 3.17-3.0 (m, 2H), 1.42-1.35 (m, 2H), 1.29-1.24 (m, 2H), 0.85 (t, 3H, $J = 6.5$ Hz). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.2, 73.6, 72.4, 71.4, 70.1, 63.3, 22.3, 11.3. 40 % yield. LRMS (ESI): m/z $\text{C}_{10}\text{H}_{20}\text{NO}_9\text{S} [\text{M}]^- = 330.3$.

N-hexyl-5-*O*-sulfo-D-gluconamide (429)

¹H NMR (400 MHz, DMSO-d₆) δ 7.63 (t, 1H, *J* = 5.8 Hz), 7.08 (brs, 3H), 5.23 (d, 1H, *J* = 3.3 Hz), 4.81 (d, 1H, *J* = 5.6 Hz), 4.55 (d, 1H, *J* = 4.7 Hz), 4.41 (brs, 1H), 4.08-4.03 (m, 1H), 3.99 (brs, 1H), 3.74-3.62 (m, 2H), 3.55-3.51 (m, 1H), 3.14-3.01 (m, 2H), 1.43-1.34 (m, 2H), 1.30-1.21 (m, 2H), 0.86 (t, 3H, *J* = 7.3 Hz). ¹³C NMR (100 MHz, DMSO-d₆) δ 172.2, 73.6, 72.3, 71.4, 70.1, 63.3, 38.2, 31.0, 29.1, 26.0, 22.0, 13.9. 95 % yield. LRMS (ESI): *m/z* C₁₂H₂₄NO₉S [M]⁻ = 359.5.

***N*-(octyl-5-*O*-sulfo-D-gluconamide (430)**

¹H NMR (400 MHz, DMSO-d₆) δ 0.82-0.88 (m, 3H), 1.18-1.32 (m, 10H), 1.34-1.45 (m, 2H), 2.98-3.14 (m, 2H), 3.53 (dt, 1H, *J* = 11.5, 40.7 Hz), 3.62-3.75 (m, 2H), 3.83-3.89 (m, 1H), 3.99 (t, 1H, *J* = 4.3 Hz), 4.02-4.09 (m, 1H), 4.41 (dd, 1H, *J* = 6.6, 4.6 Hz), 4.56 (d, 1H, *J* = 5.2 Hz), 4.80 (d, 1H, *J* = 6.2 Hz), 5.23 (d, 1H, *J* = 4.3 Hz), 7.08 (s, 1H), 7.63 (t, 1H, *J* = 5.8 Hz). ¹³C (100 MHz, DMSO-d₆) δ 171.9, 77.0, 73.6, 70.5, 69.7, 61.2, 38.2, 31.2, 29.1, 28.7, 28.6, 26.3, 22.0, 13.9. 82 % yield. LRMS (ESI): *m/z* C₁₄H₂₈NO₉S [M]⁻ = 386.2.

***N*-(2-fluorophenyl)-5-*O*-sulfo-D-gluconamide (431)**

¹H NMR (400 MHz, DMSO-d₆) δ 3.43 (ddd, 1H, *J* = 6.9, 6.9, 5.0 Hz), 3.56 (dt, 1H, *J* = 11.6, 4.6 Hz), 3.69-3.82 (m, 3H), 4.00-4.05 (m, 1H), 4.06-4.12 (m, 1H), 4.27 (t, 1H, *J* = 3.2 Hz), 4.45 (dd, 1H, *J* = 6.4, 4.5 Hz), 4.80 (d, 1H, *J* = 5.2 Hz), 4.93 (d, 1H, *J* = 6.2 Hz), 5.73 (d, 1H, *J* = 3.1 Hz), 7.09-7.20 (m, 3H), 7.27 (ddd, 1H, *J* = 10.8, 7.7, 1.6 Hz), 7.95 (s, 1H), 8.11 (td, 1H, *J* = 8.0, 1.9 Hz), 9.22 (s, 1H). ¹³C (100 MHz, DMSO-d₆) δ 171.1, 162.3, 124.5, 122.1, 115.2 (d, *J*_{C-F} = 19.4 Hz), 76.9, 73.8, 70.3, 69.5, 61.0, 56.0, 35.7, 30.7, 18.5. 44 % yield. LRMS (ESI): *m/z* C₁₂H₁₅FNO₉S [M]⁻ = 368.0.

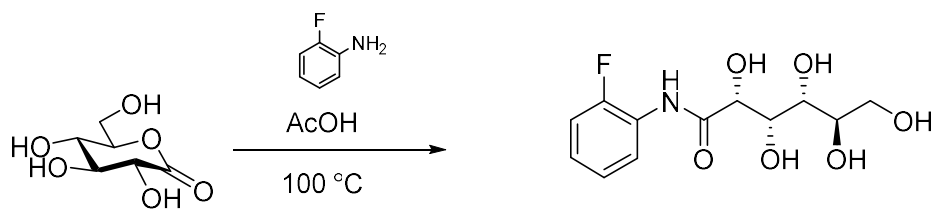
***N*-(4-methoxybenzyl)-5-*O*-sulfo-D-gluconamide (432)**

¹H NMR (400 MHz, DMSO-d₆) δ 8.08 (t, 1H, *J* = 6.2 Hz), 7.21-6.38 (m, 7H), 5.36 (dd, 1H, *J* = 4.9, 34.6 Hz), 4.85-3.90 (m, 9H), 3.71-3.47 (m, 7H). LRMS (ESI): *m/z* C₁₄H₂₀NO₁₀S [M]⁻ = 394.4.

***N*-(2,6-difluorobenzyl)-5-*O*-sulfo-D-gluconamide (433)**

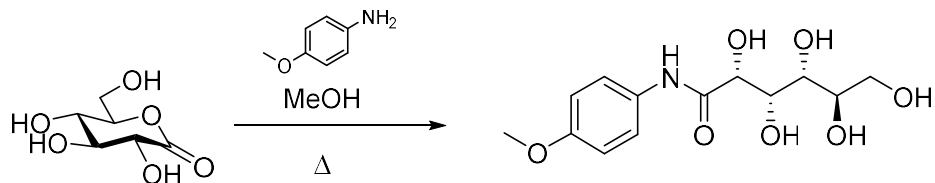
¹H NMR (400 MHz, D₂O) δ 7.44-7.34 (m, 1H), 7.07-6.99 (m, 2H), 4.55 (q, 2H, *J* = 14.6 Hz), 4.46-4.41 (m, 1H), 4.32 (d, 1H, *J* = 4.2 Hz), 4.09-3.99 (m, 3H), 3.87 (dd, 1H, *J* = 4.1, 12.7 Hz). LRMS (ESI): *m/z* C₁₃H₁₆F₂NO₉S [M]⁻ = 401.2.

***N*-(2-fluorophenyl)-D-gluconamide (500a) ¹²**



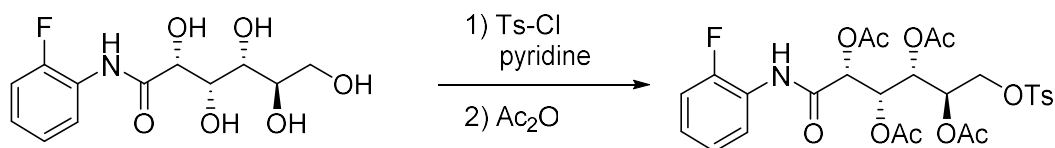
D-gluconic acid- δ -lactone (20 g, 112.3 mmol) was suspended in acetic acid (200 mL) and 2-fluoroaniline (32 mL, 336.9 mmol) was added. The mixture was stirred at 100 °C for 1 hour. The crude product was precipitated with hexanes and filtered to obtain a dark brown solid. The solid was recrystallized from EtOH to afford the product as a white solid (45 %). ^1H NMR (400 MHz, DMSO- d_6) δ 9.20 (br. s, 1H), 8.12 (td, 1H, J = 7.9, 2.1 Hz), 7.29-7.24 (m, 1H), 7.21-7.09 (m, 2H), 5.95 (d, 1H, J = 6.0 Hz), 4.67 (d, 1H, J = 6.9 Hz), 4.62 (dd, 2H, J = 8.4, 5.4 Hz), 4.38 (t, 1H, J = 5.7 Hz), 4.24 (dd, 1H, J = 4.9, 3.4 Hz), 4.04-4.01 (m, 1H), 3.62-3.49 (m, 3H), 3.42-3.33 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.5, 152, 125.9 (d, $J_{\text{C-F}}$ = 10.5 Hz), 124.6 (d, $J_{\text{C-F}}$ = 7.6 Hz), 124.5 (d, $J_{\text{C-F}}$ = 3.5 Hz), 122.0 (d, $J_{\text{C-F}}$ = 1.7 Hz), 115.2, 73.9, 72.2, 71.6, 70.2, 63.3. LRMS (ESI): m/z $\text{C}_{12}\text{H}_{15}\text{FNO}_6\text{Na}$ $[\text{M}+\text{Na}]^+ = 288.2$. All spectral data were consistent with those reported in the literature.¹²

***N*-(4-methoxyphenyl)-D-gluconamide (500)**¹²



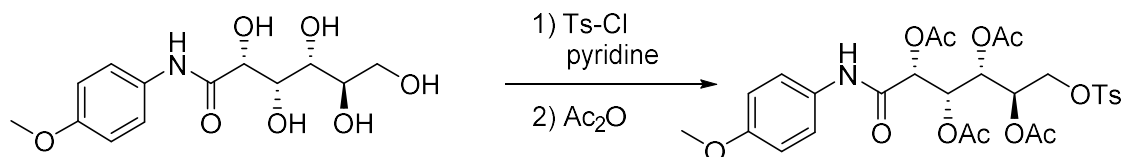
D-gluconic acid- δ -lactone (10 g, 56.13 mmol) was suspended in MeOH (280 mL) before *p*-anisidine (6.9 g, 56.13 mmol) was added. The mixture was stirred at reflux overnight. The solvent was removed under reduced pressure, and the residue was recrystallized from EtOH to afford 500 as a white solid (60 %). ^1H NMR (400 MHz, DMSO- d_6) δ 9.39 (s, 1H), 7.62 (d, 1H, J = 9.3 Hz), 6.88 (d, 2H, J = 9.0 Hz), 5.65 (d, 1H, J = 5.3 Hz), 4.58 (d, 1H, J = 5.1 Hz), 4.53-4.51 (m, 2H), 4.36 (t, 1H, J = 5.9 Hz), 4.14 (dd, 1H, J = 4.1, 5.2 Hz), 4.01-3.99 (m, 1H), 3.72 (s, 3H), 3.61-3.56 (m, 1H), 3.51-3.49 (m, 2H), 3.41-3.36 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.0, 155.3, 131.7, 121.0, 113.7, 74.1, 72.3, 71.5, 70.3, 63.3, 55.1. LRMS (ESI): m/z $\text{C}_{13}\text{H}_{18}\text{NO}_7\text{Na}$ $[\text{M}+\text{Na}]^+ = 300.4$. All spectral data were consistent with those reported in the literature.¹²

***N*-(2-fluorophenyl)-1,2,3,4-tetra-*O*-acetyl-6-*O*-(4-methylbenzenesulfonyl)-D-gluconamide (502a)**



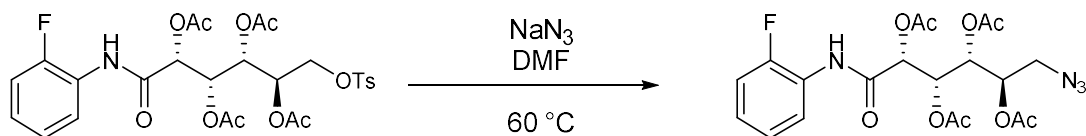
N-(2-Fluorophenyl)-D-gluconamide (19 g, 65.7 mmol) was dissolved in pyridine (450 mL) and cooled to 0 °C. Next TsCl (13.78 g, 72.3 mmol) in pyridine (70 mL) was added over 1 h at 0 °C, before warming up to room temperature over 4 h. Ac₂O (24 mL, 251.3 mmol) was added, and the reaction was stirred at room temperature overnight. The reaction was then poured over a mixture of ice/sat. sodium sulfate and precipitated over 2 h. The resulting solid was filtered and washed with water before being dissolved in DCM. Organics were washed with water (x2) and brine before being dried over sodium sulfate. The product was filtered, and the solvent was removed under reduced pressure to afford an off-white solid. Solid was recrystallized to afford a white solid (49 %). ¹H NMR (400 MHz, CDCl₃) δ 8.23 (m, 1H), 8.00 (d, 1H, *J* = 2.21 Hz), 7.08-7.75 (m, 2H), 7.38-7.30 (m, 2H), 7.13-7.07 (m, 3H), 5.60 (dd, 1H, *J* = 3.98, 5.47 Hz), 5.58 (dd, 1H, *J* = 3.94, 7.39 Hz), 5.35 (d, 1H, *J* = 5.47 Hz), 5.09-5.05 (m, 1H), 4.20 (dd, 1H, *J* = 3.40, 11.41 Hz), 4.41 (dd, 1H, *J* = 5.27, 11.41 Hz), 2.44 (s, 3H), 2.23 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 169.8 (d, *J*_{C-F} = 1.2 Hz), 169.4, 164.3, 145.3, 132.5, 130., 128.21, 125.3 (d, *J*_{C-F} = 7.6 Hz), 124.8 (d, *J*_{C-F} = 3.1 Hz), 122.1, 115.0 (d, *J*_{C-F} = 19.5 Hz), 71.6, 68.7, 68.3, 66.7, 21.8, 20.7, 20.7, 20.6, 20.5. Product not observable by mass spec.

***N*-(4-methoxyphenyl)-1,2,3,4-tetra-*O*-acetyl-6-*O*-(4-methylbenzenesulfonyl)-D-gluconamide (502)**



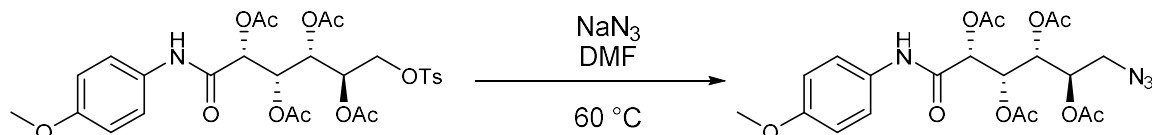
N-(4-methoxyphenyl)-D-gluconamide (32.44 g, 107.7 mmol) was dissolved in pyridine (500 mL) and cooled to 0 °C. TsCl (24.6 g, 129.2 mmol) in pyridine (100 mL) was added over 1 h at 0 °C, before warming up to room temperature over 4 h. Ac₂O (71 mL, 753.9 mmol) was added, and the reaction was stirred at room temperature overnight. The reaction was then poured over a mixture of ice/sat. sodium sulfate and precipitated over 2 h. The resulting solid was filtered, washed with water, and dissolved in DCM. Organics were washed with water (x2) and brine before drying over sodium sulfate. The solvent was removed under reduced pressure to afford an off-white solid. Solid was recrystallized to afford a white solid (61 %). ¹H NMR (400 MHz, CDCl₃) δ 7.83 (brs, 1H), 7.81-7.73 (m, 1H), 7.43-7.38 (m, 1H), 7.37-7.31 (m, 1H), 6.88-6.80 (m, 1H), 5.62 (dd, 1H, *J* = 4.02, 6.10 Hz), 5.46 (dd, 1H, *J* = 4.09, 7.03 Hz), 5.28 (d, 1H, *J* = 6.17 Hz), 5.09-5.04 (m, 1H), 4.21 (dd, 1H, *J* = 3.88, 11.34 Hz), 4.14 (dd, 1H, *J* = 4.88, 11.34 Hz), 3.82-3.77 (m, 4H), 2.43 (s, 3H), 2.21 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.98 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 169.5, 163.7, 156.8, 145.2, 132.2, 130.0, 128.0, 121.9, 114.1, 71.5, 68.8, 68.0, 66.4, 55.4, 21.6, 20.6, 20.4. Product not observable by mass spec.

***N*-(2-fluorophenyl)-1,2,3,4-tetra-*O*-acetyl-6-azido-D-gluconamide (503a)**



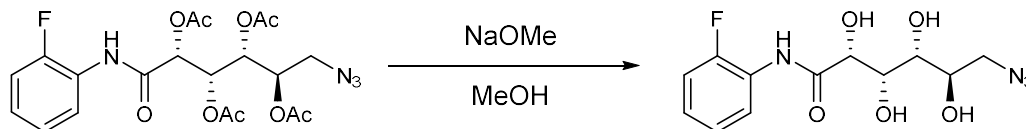
Compound **502a** (23.81 g, 38.93 mmol) was dissolved in DMF (390 mL) and NaN_3 (8.86 g, 136.26 mmol) was added. The reaction was warmed to 60 °C and was stirred overnight. Solvent was removed under reduced pressure and the resulting solid was dissolved in EtOAc. Organics were washed with water (x2) and brine before being dried over sodium sulfate, filtered, and the solvent was then removed under reduced pressure. Subsequent solid was then purified via recrystallization from EtOH to afford **503a** as a white solid (50 %). ^1H NMR (400 MHz, CDCl_3) δ 8.24 (m, 1H), 7.99 (d, 1H, $J = 2.06$ Hz), 7.14-7.07 (m, 3H), 5.64 (dd, 1H, $J = 4.33, 5.51$ Hz), 5.51 (dd, 1H, $J = 4.22, 6.92$ Hz), 5.42 (d, 1H, $J = 5.41$ Hz), 5.07-5.03 (m, 1H), 3.52 (dd, 1H, $J = 3.79, 13.41$ Hz), 3.41 (dd, 1H, $J = 6.37, 13.41$ Hz), 2.26 (s, 3H), 2.09 (s, 6H), 2.07 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 169.9 (d, $J_{\text{C-F}} = 7.4$ Hz), 169.4 (d, $J_{\text{C-F}} = 17.9$ Hz), 164.3, 125.3 (d, $J_{\text{C-F}} = 7.2$ Hz), 125.2, 124.9 (d, $J_{\text{C-F}} = 3.8$ Hz), 122.0, 115.0 (d, 19.1 Hz), 71.5, 69.5, 69.4, 68.8, 50.4, 20.8, 20.7, 20.5. LRMS (ESI): m/z $\text{C}_{20}\text{H}_{23}\text{FN}_4\text{O}_9\text{Na}$ $[\text{M}+\text{Na}]^+ = 505.1$.

***N*-(4-methoxyphenyl)-1,2,3,4-tetra-*O*-acetyl-6-azido-D-gluconamide (503)**



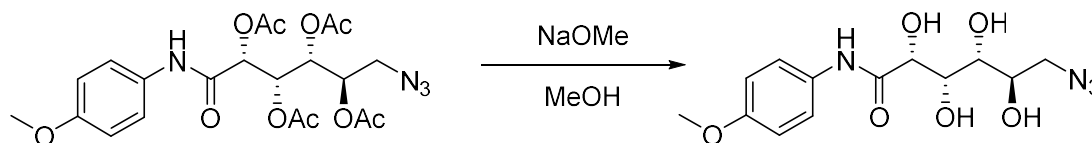
Compound **502** (22.19 g, 35.58 mmol) was dissolved in DMF (390 mL) and NaN_3 (8.86 g, 136.26 mmol) was added. The reaction was warmed to 60 °C and was stirred overnight. The solvent was removed under reduced pressure and resulting solid was dissolved in EtOAc. Organics were washed with water (x2) and brine before being dried over sodium sulfate. The product was filtered, and the solvent was then removed under reduced pressure. Subsequent solid was then purified via recrystallization from EtOH to afford a white solid (50 %). ^1H NMR (400 MHz, CDCl_3) δ 7.70 (brs, 1H), 7.41-7.37 (m, 2H), 6.87-6.83 (m, 2H), 5.64 (dd, 1H, $J = 3.94, 6.33$ Hz), 5.48 (dd, 1H, $J = 3.94, 7.05$ Hz), 5.35 (d, 1H, $J = 6.33$ Hz), 5.07-5.03 (m, 1H), 3.78 (s, 3H), 5.51 (dd, 1H, $J = 3.73, 13.61$ Hz), 3.40 (dd, 1H, $J = 3.41, 13.44$ Hz), 2.23 (s, 3H), 2.11 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 170.0, 169.8, 169.5, 169.3, 163.8, 157.0, 129.5, 122.0, 114.2, 71.6, 69.4, 69.4, 68.7, 55.5, 50.4, 20.7, 20.7, 20.4. LRMS (ESI): m/z $\text{C}_{21}\text{H}_{26}\text{N}_4\text{O}_{10}\text{Na}$ $[\text{M}+\text{Na}]^+ = 517.4$.

N-(2-fluorophenyl)-6-azido-D-gluconamide (**504a**)



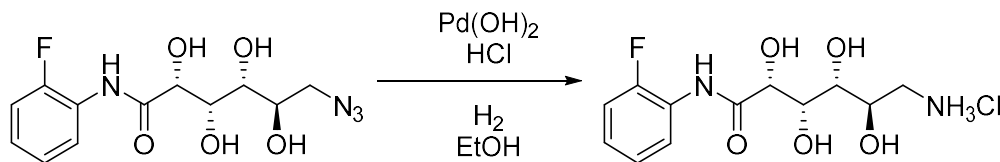
Compound **503a** (1.5 g, 3.1 mmol) was dissolved in MeOH and NaOMe was added until a pH of ~12. The reaction was stirred for 2 h before Amberlite resin (H⁺ form) was added until a pH of ~6. The solvent was removed under reduced pressure yielding **504a** in 92 % yield. ¹H NMR (400 MHz, DMSO-d₆) δ 9.91 (brs, 1H), 8.40-8.35 (m, 1H), 7.23-7.08 (m, 3H), 5.35 (brs, 1H), 4.57 (brs, 1H), 4.44 (brs, 1H), 4.34-4.26 (m, 3H), 3.94-3.83 (m, 2H), 3.56 (dd, 1H, *J* = 2.13, 12.46 Hz), 3.41 (dd, 1H, *J* = 6.38, 12.46 Hz). ¹³C NMR (100 MHz, DMSO-d₆) δ 173.5, 155.5 (d, *J*_{C-F} = 245.8 Hz), 128.2 (d, *J*_{C-F} = 8.0 Hz), 126.3 (d, *J*_{C-F} = 0.7 Hz), 124.6 (d, *J*_{C-F} = 3.7 Hz), 123.2 (d, *J*_{C-F} = 12.4 Hz), 116.0 (d, *J*_{C-F} = 19.5 Hz), 76.0, 73.2 (d, *J*_{C-F} = 37.6 Hz), 70.5 (d, *J*_{C-F} = 6.1 Hz), 68.9 (d, *J*_{C-F} = 83.5 Hz), 42.6. LRMS (ESI): *m/z* C₁₂H₁₅N₄O₅Na [M+Na]⁺ = 347.4.

N-(4-methoxyphenyl)-6-azido-D-gluconamide (**504**)



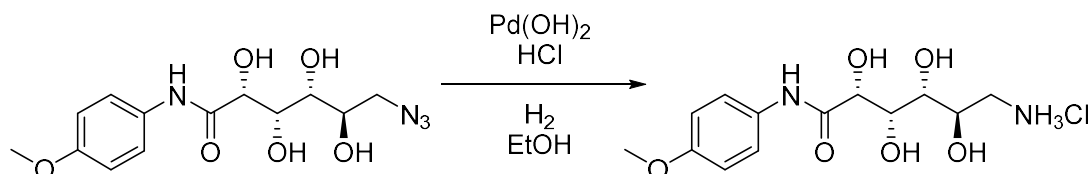
Compound **503** (19.13 g, 38.69 mmol) was dissolved in MeOH and NaOMe was added until a pH of ~12. The reaction was stirred for 2 h before Amberlite resin (H⁺ form) was added until a pH of ~6. The solvent was removed under reduced pressure yielding **504** in 95 % yield. ¹H NMR (400 MHz, DMSO-d₆) δ 9.45 (s, 1H), 7.62-7.58 (m, 2H), 6.89-6.85 (m, 2H), 5.70 (d, 1H, *J* = 5.3 Hz), 5.23 (d, 1H, *J* = 5.9 Hz), 4.65 (d, 1H, *J* = 6.2 Hz), 4.59 (d, 1H, *J* = 7.3 Hz), 4.14 (t, 1H, *J* = 4.5 Hz), 4.00-3.97 (m, 1H), 3.74-3.86 (m, 4H), 3.53-3.48 (m, 1H), 3.39 (dd, 1H, *J* = 2.8, 12.7 Hz), 3.27 (dd, 1H, *J* = 8.2, 15.0 Hz). ¹³C NMR (100 MHz, DMSO-d₆) δ 170.9, 155.3, 131.7, 121.1, 113.6, 73.8, 72.3, 70.2, 69.9, 55.1, 53.9. LRMS (ESI): *m/z* C₁₃H₁₈N₄O₆Na [M+Na]⁺ = 349.8.

N-(2-fluorophenyl)-6-amino-D-gluconamide (**505a**)



Compound **504a** (220 mg, 0.7 mmol) was dissolved in EtOH (99 %) and Pd(OH)₂ (10 mol %) was added. The flask was then evacuated of air and filled with H₂; this process was repeated 5 times. Once completed the solution was acidified with HCl (conc.) and **505a** precipitated upon cooling. The product was filtered and washed with cold EtOH to afford **505a** as a white powder in a 45 % yield. ¹H NMR (400 MHz, MeOD) δ 8.19-8.14 (m, 1H), 7.19-7.12 (m, 3H), 4.38 (d, 1H, *J* = 3.4 Hz), 4.18 (t, 1H, *J* = 2.8 Hz), 3.70 (dd, 1H, *J* = 2.6, 8.0 Hz), 3.67-3.63 (m, 1H), 2.93 (dd, 1H, *J* = 3.7, 13.2 Hz), 2.71 (dd, 1H, *J* = 6.7, 13.2 Hz). ¹³C NMR (100 MHz, MeOD) δ 173.5, 126.4, 126.3, 125.4 (d, *J*_{C-F} = 3.6 Hz), 123.7, 116.2, 116.0, 75.6, 75.2, 73.1, 72.1, 45.4. LRMS (ESI): *m/z* C₁₂H₁₈FN₂O₅ [M+H]⁺ = 289.9.

N-(4-methoxyphenyl)-6-amino-D-gluconamide (**505**)



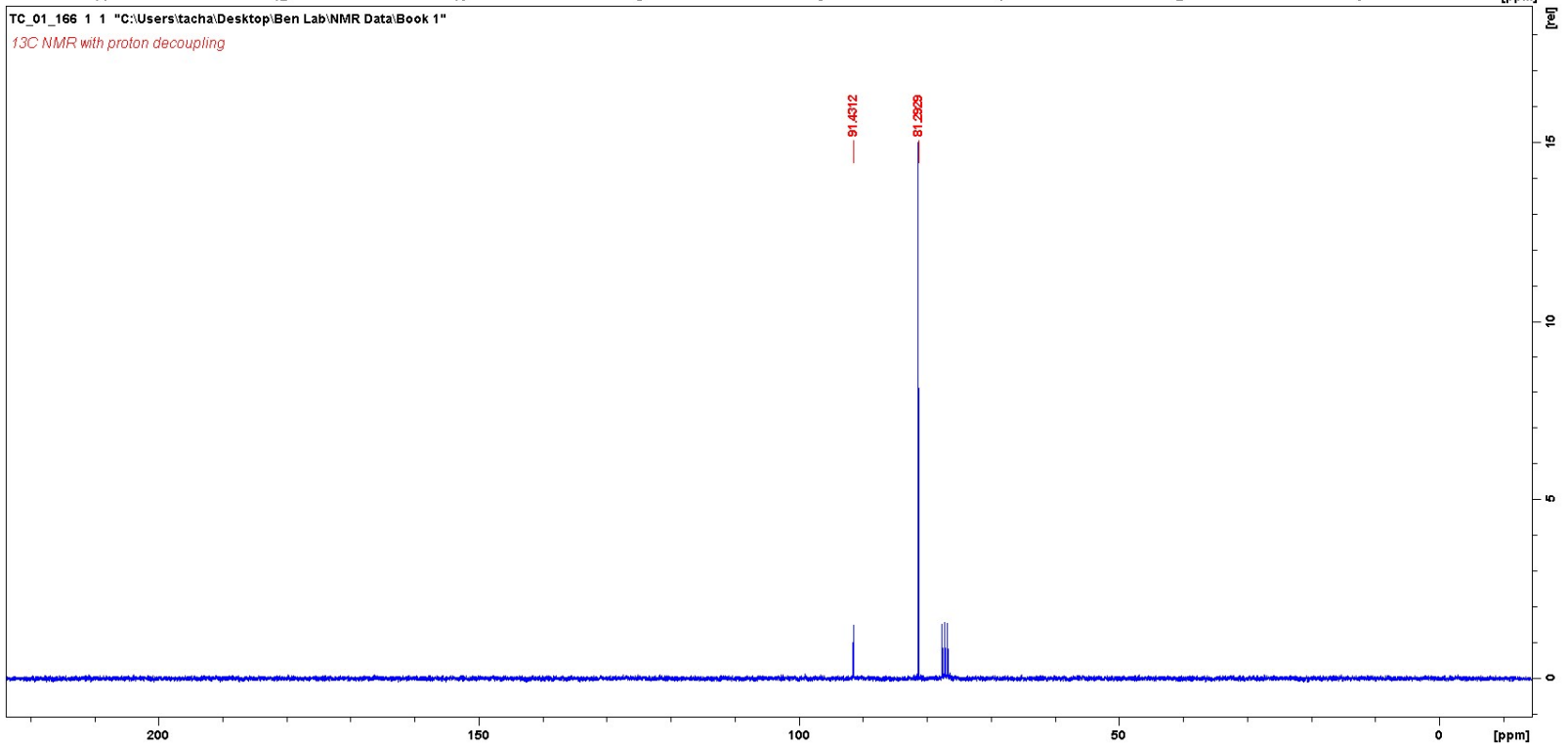
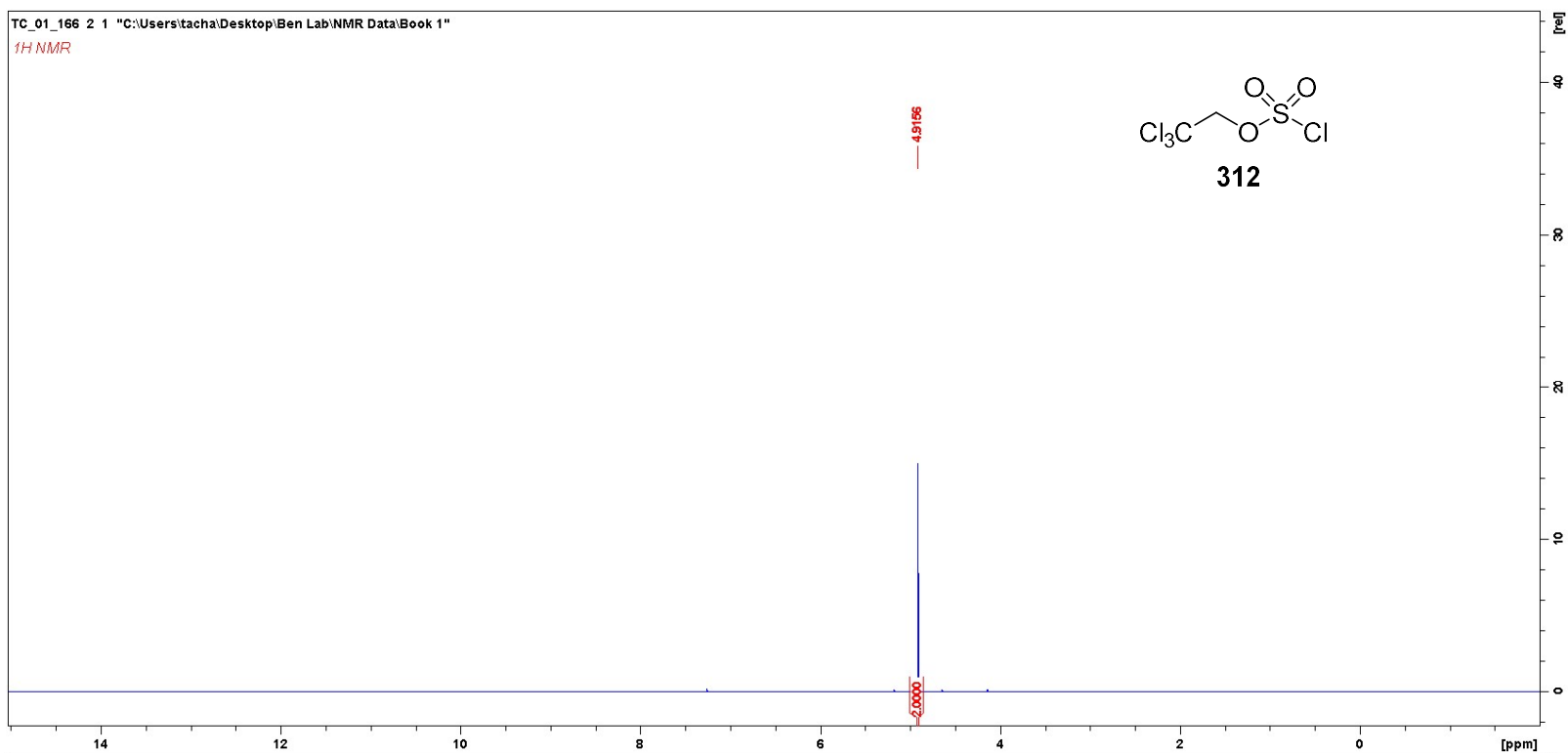
Compound **504** (11.47 g, 35.15 mmol) was dissolved in EtOH (99 %) and Pd/C (10 mol %) was added. The flask was then evacuated of air and filled with H₂; this process was repeated 5 times. Once completed the solution was acidified with HCl (conc.) and **505** precipitated upon cooling. The product was filtered and washed with cold EtOH to afford **505** as a white powder in a 50 % yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.53 (s, 1H), 7.77 (brs, 3H), 7.63-7.58 (m, 2H), 6.86-6.84, (m, 2H), 5.72 (d, 1H, *J* = 5.4 Hz), 5.39 (d, 1H, *J* = 6.2 Hz), 4.89 (d, 1H, *J* = 5.9 Hz), 4.70 (d, 1H, *J* = 7.1 Hz), 4.16 (t, 1H, *J* = 4.4 Hz), 3.98-3.93 (m, 1H), 3.80-3.74 (m, 1H), 3.71 (s, 3H), 3.54-3.49 (m, 1H), 3.06-3.01 (m, 1H), 2.74 (brs, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 170.8, 155.3, 131.7, 121.1, 113.6, 73.5, 73.2, 70.1, 67.4, 55.1, 42.1. LRMS (ESI): *m/z* C₁₃H₂₁N₂O₆ [M+H]⁺ = 301.4.

7.10 References

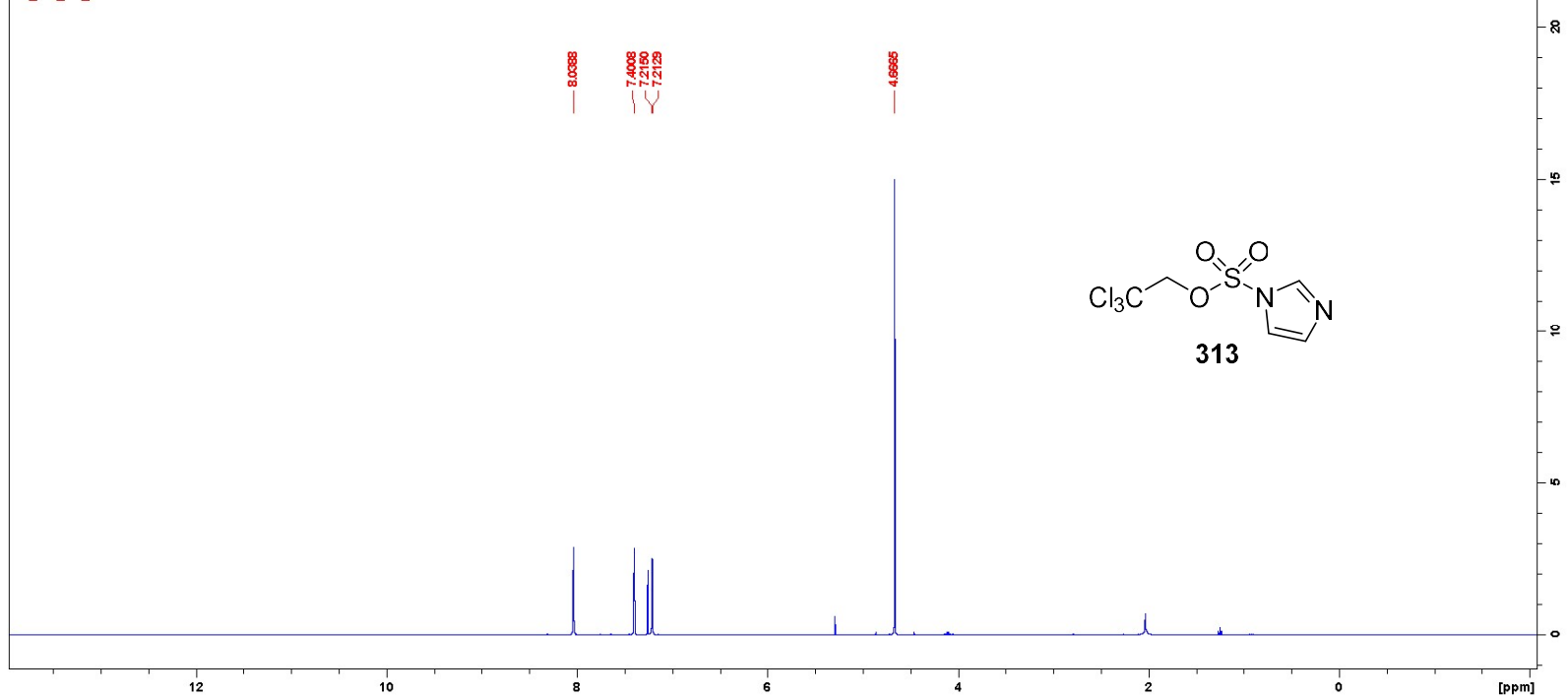
- (1) Sowemimo-Coker, S. O. *Transfus. Med. Rev.* **2002**, *16* (1), 46.
- (2) Acker, J. P.; Hansen, A. L.; Kurach, J. D. R.; Turner, T. R.; Croteau, I.; Jenkins, C. *Transfusion* **2014**, *54* (10), 2534.
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- (11) Briard, J. G. The Rational Design and Use of Novel Small-Molecule Ice Recrystallization Inhibitors for the Cryopreservation of Hematopoietic Stem Cells and Red Blood Cells, Ph.D Dissertation, University of Ottawa, 2016.
- (12) Briard, J. G.; Jahan, S.; Chandran, P.; Allan, D.; Pineault, N.; Ben, R. N. *ACS Omega* **2016**, 1 (5), 1010.

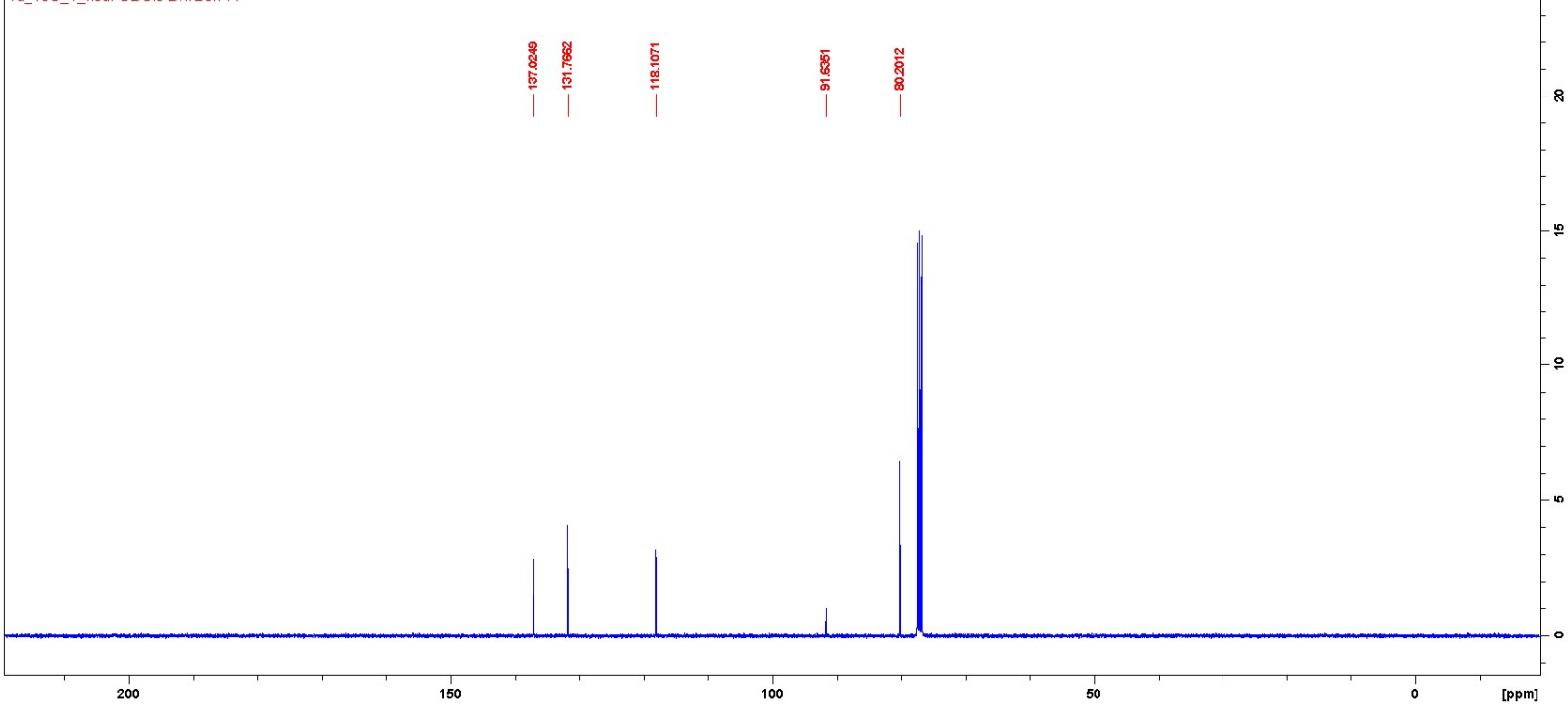
6.11 NMR Spectral Data

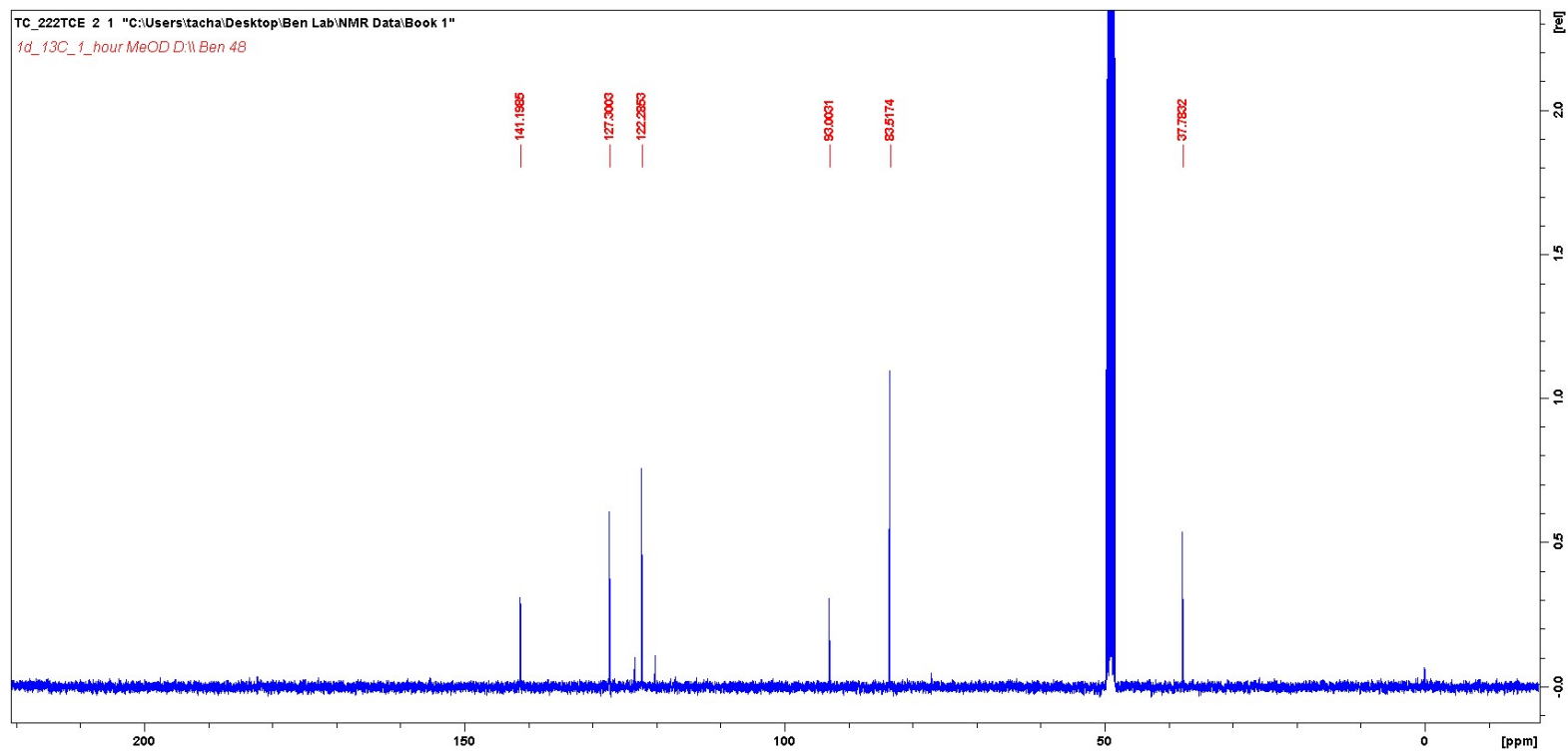
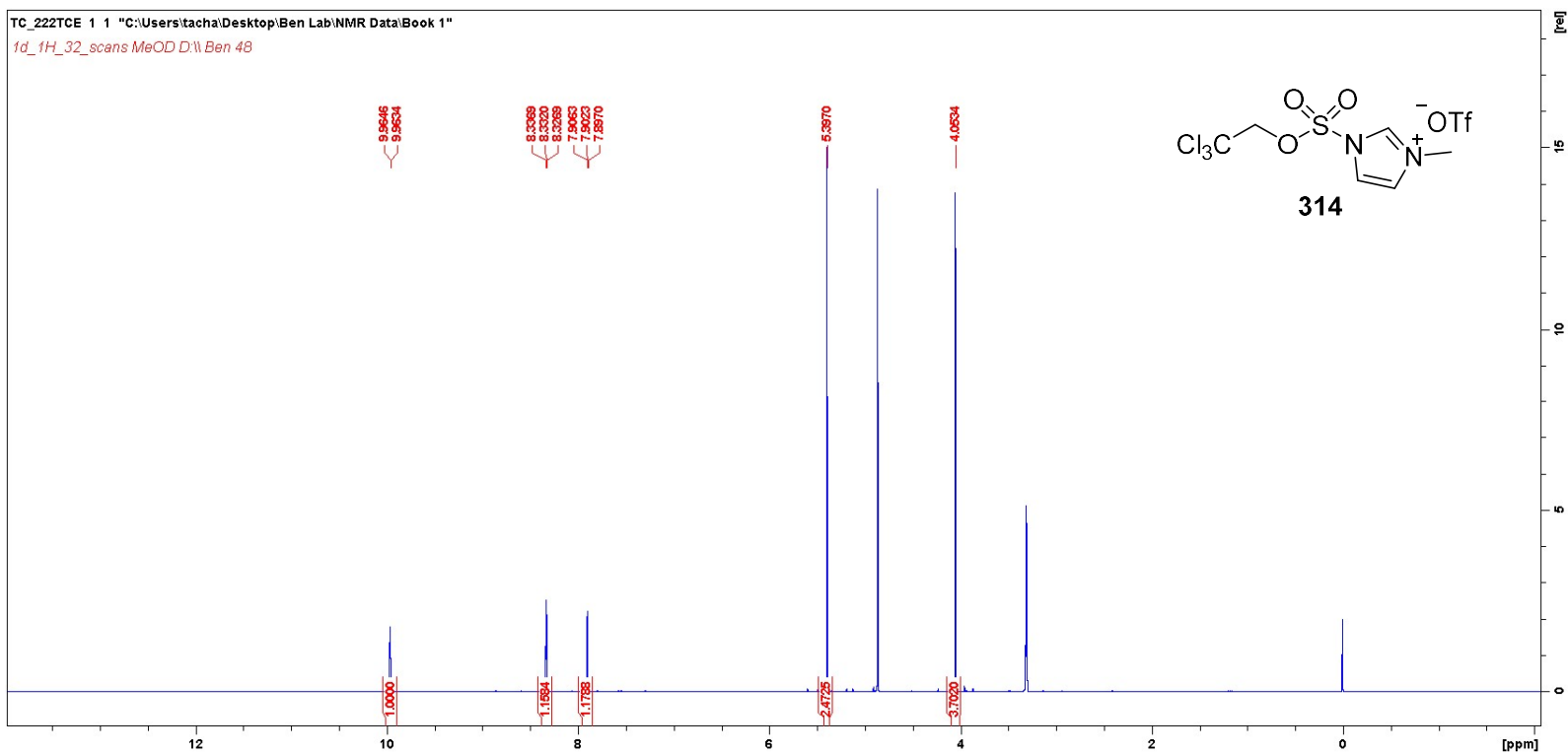


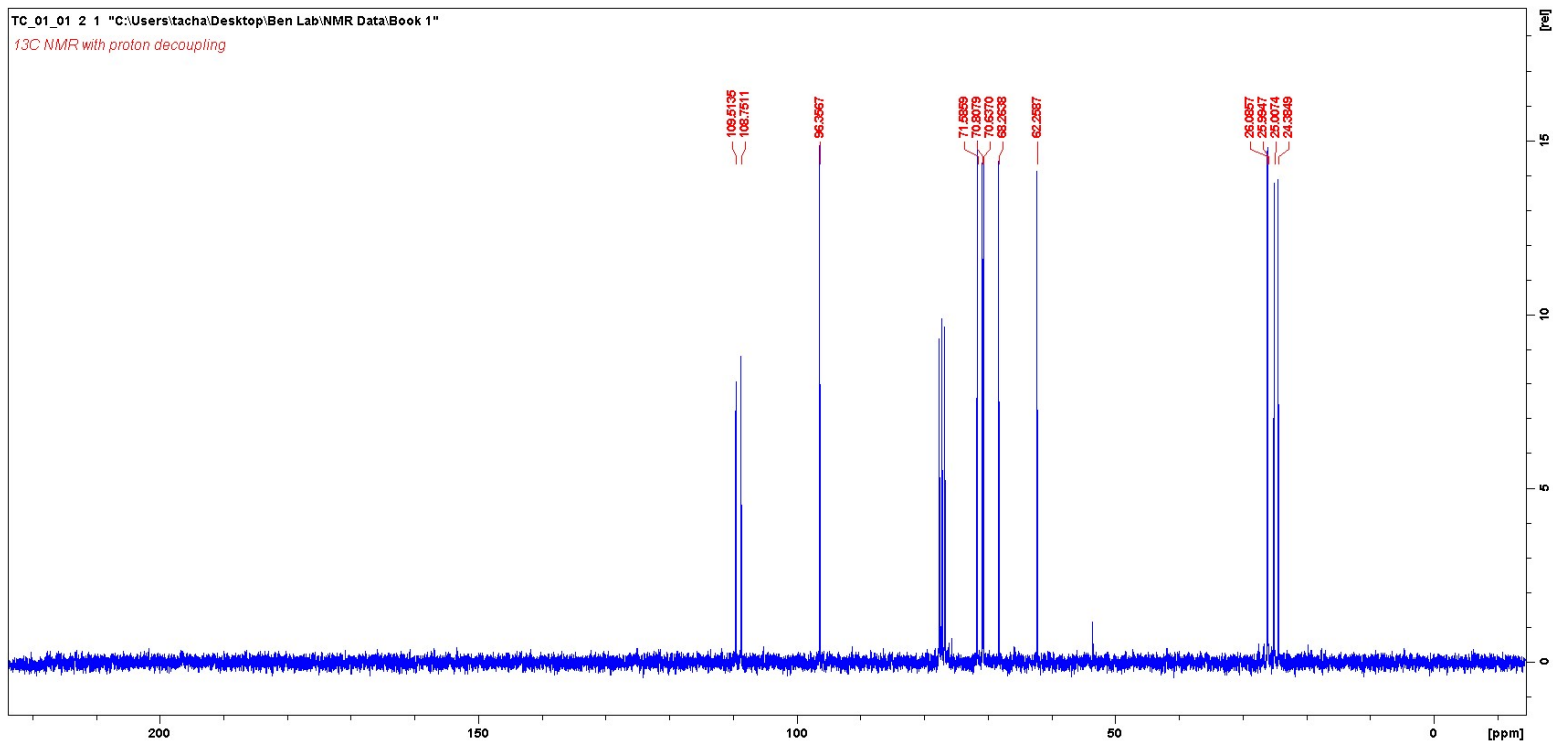
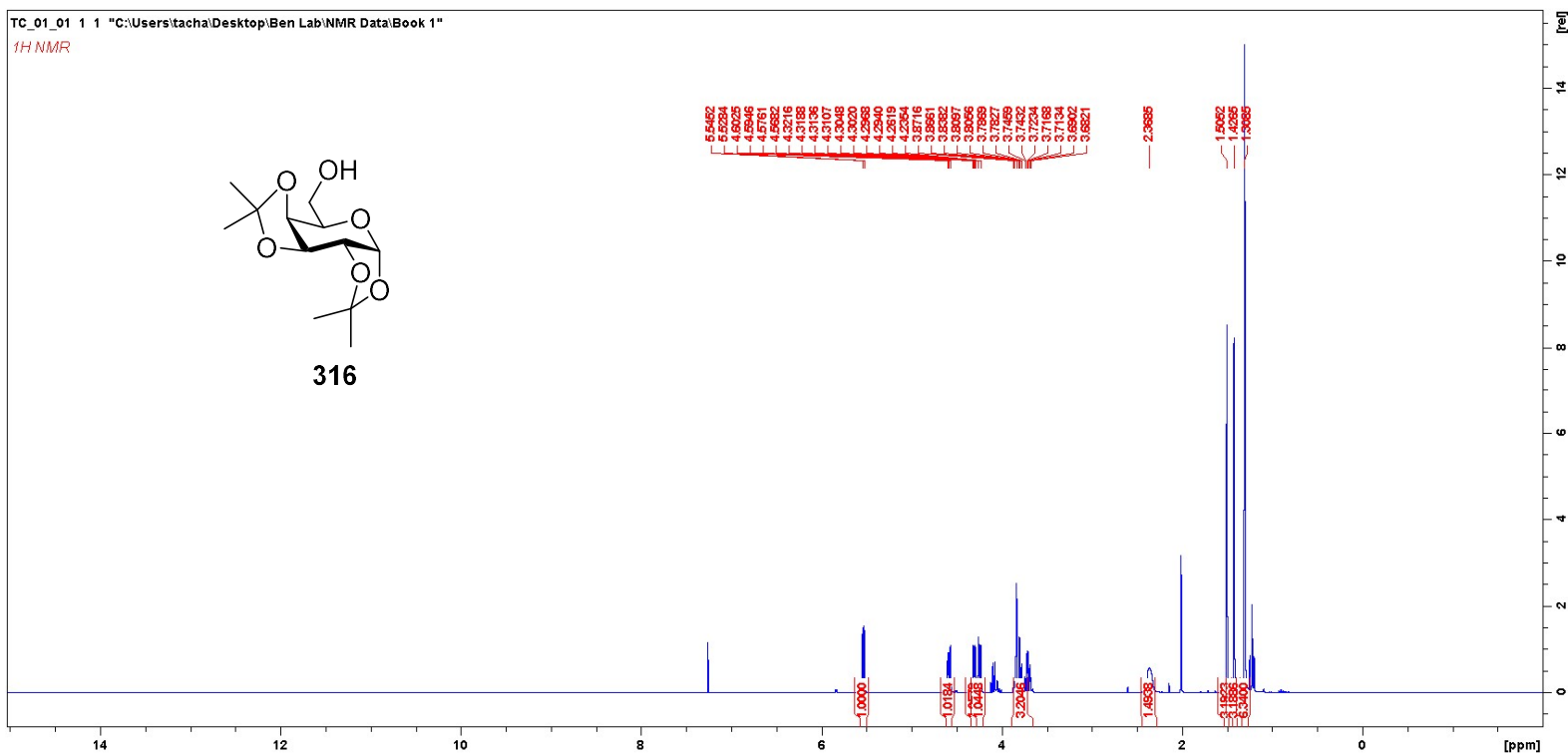
1d_1H_32_scans CDCl3 D:\Ben 11

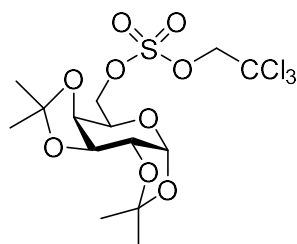


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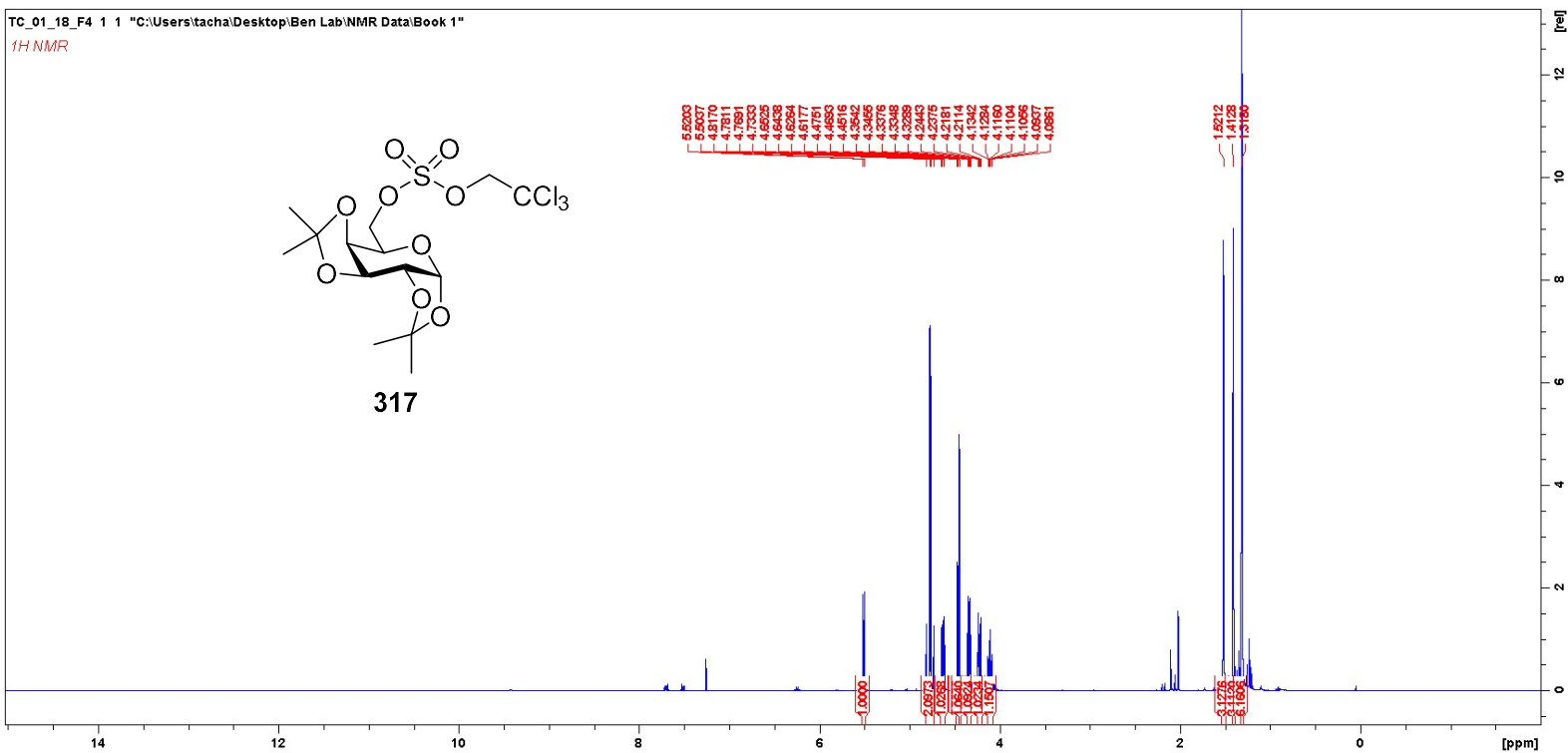


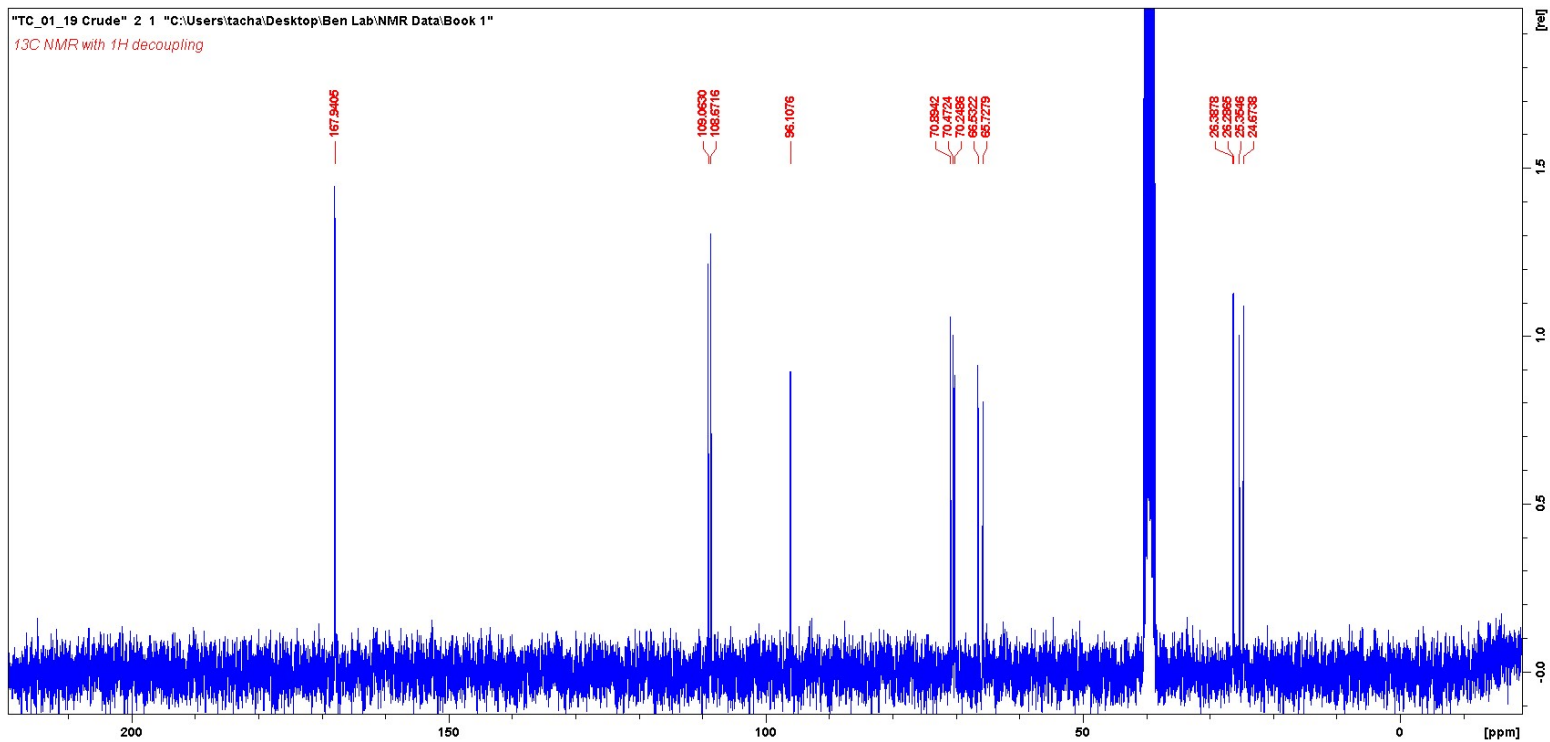
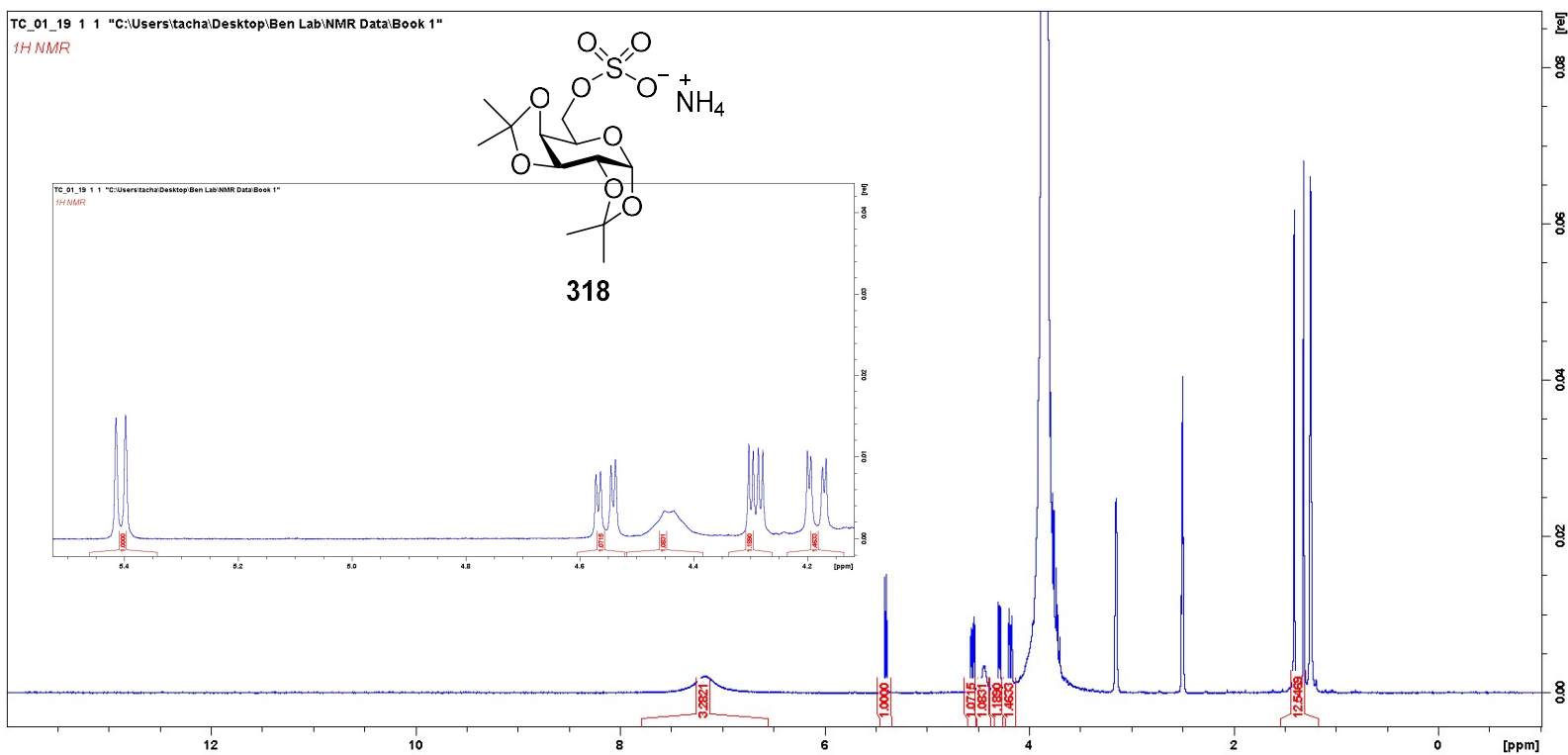


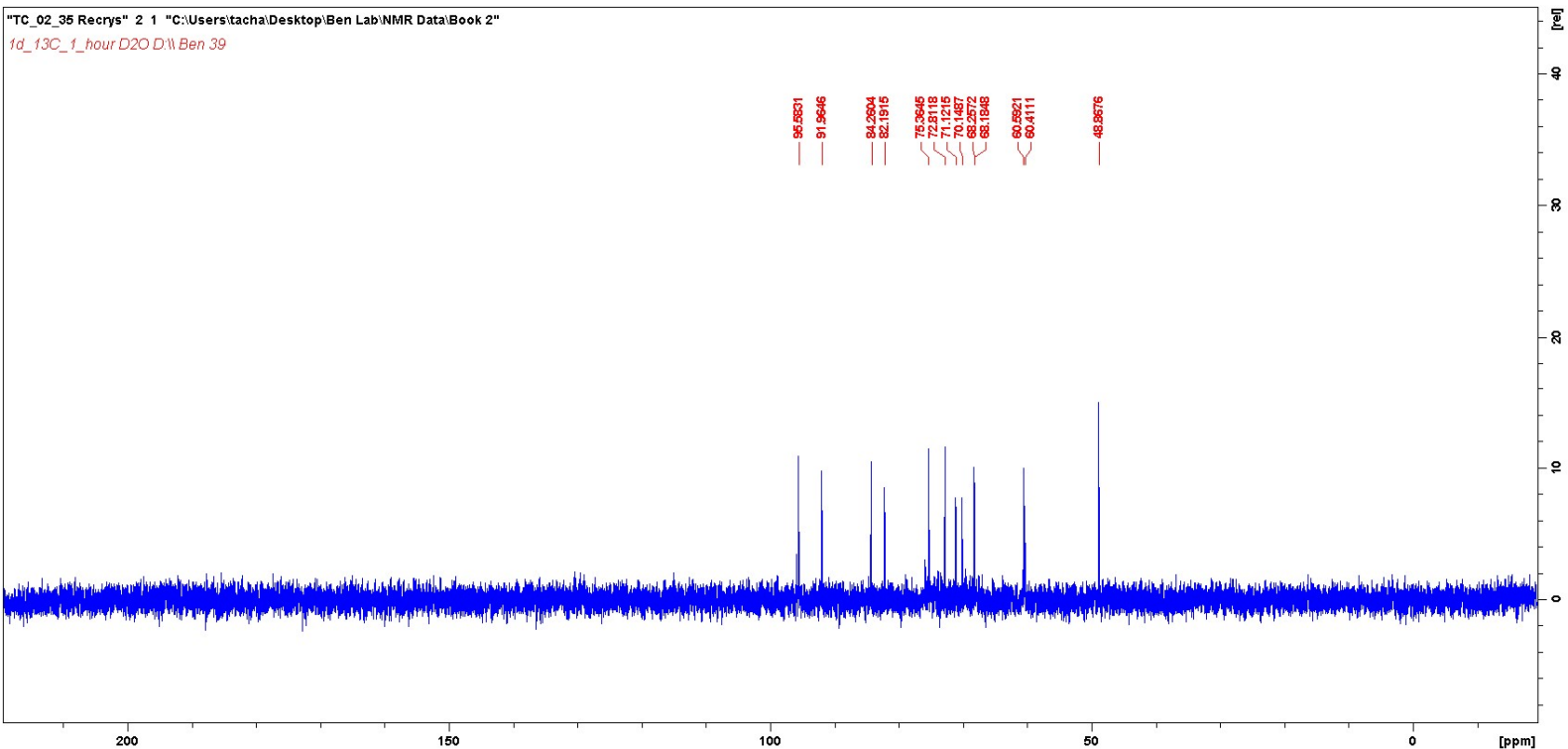
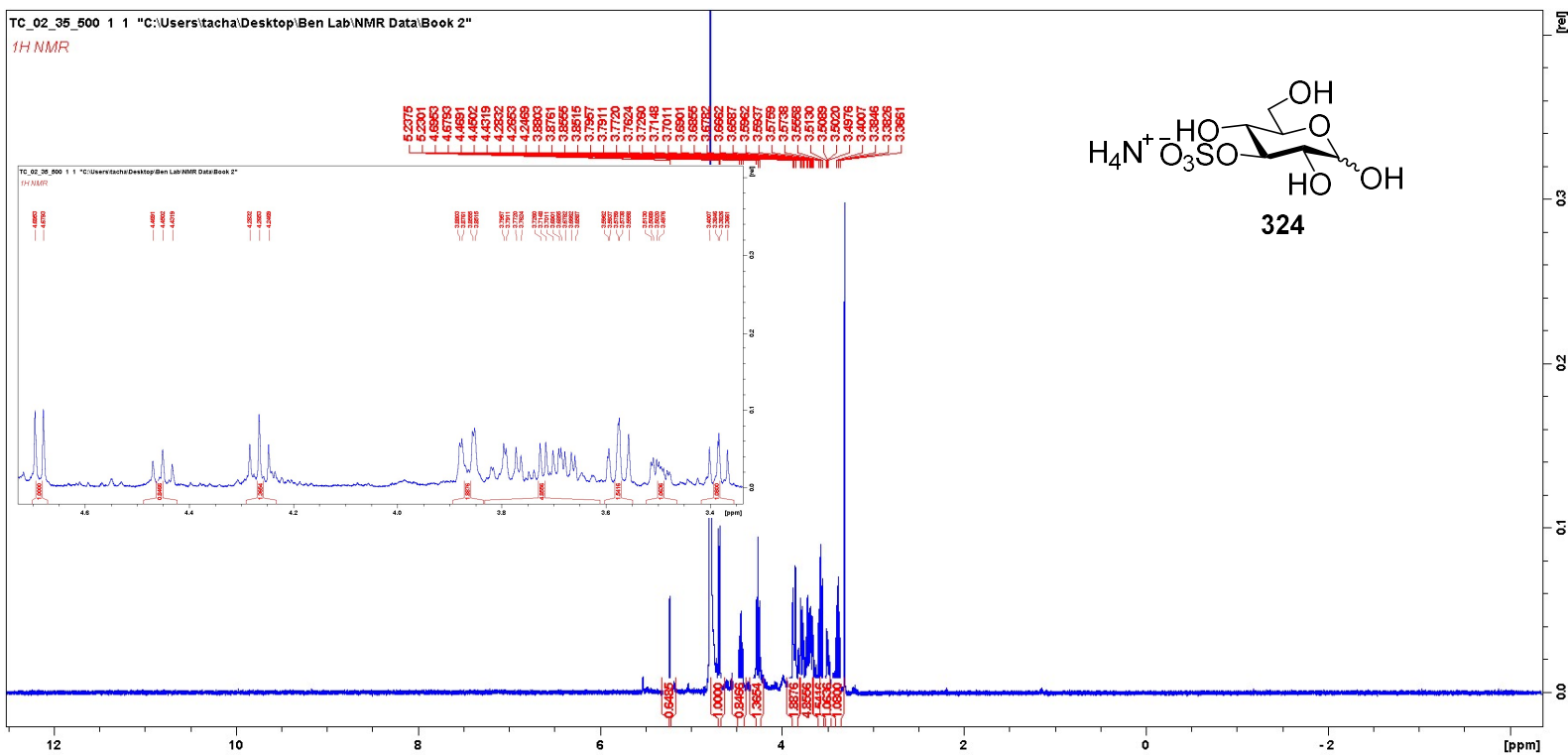


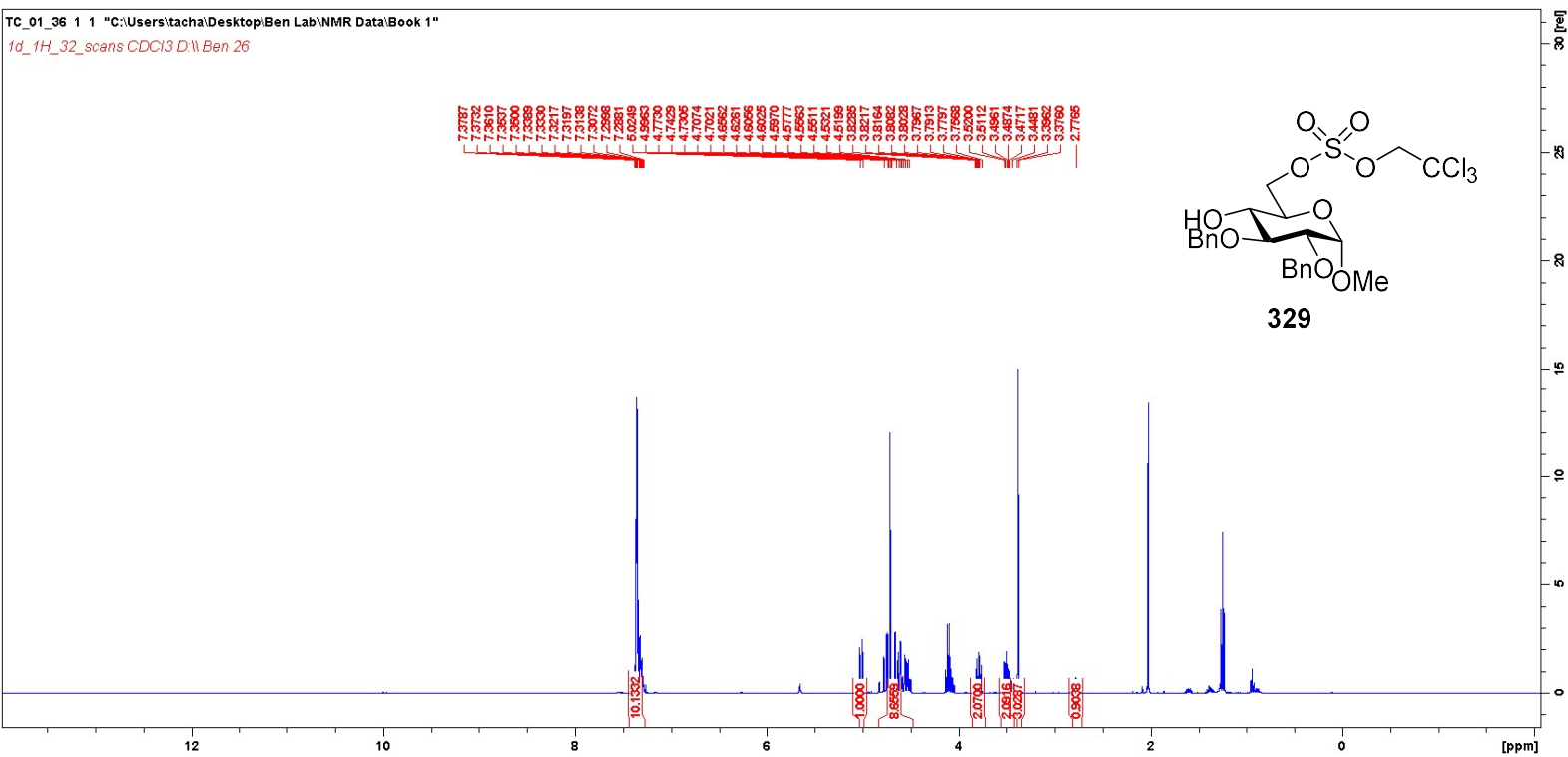
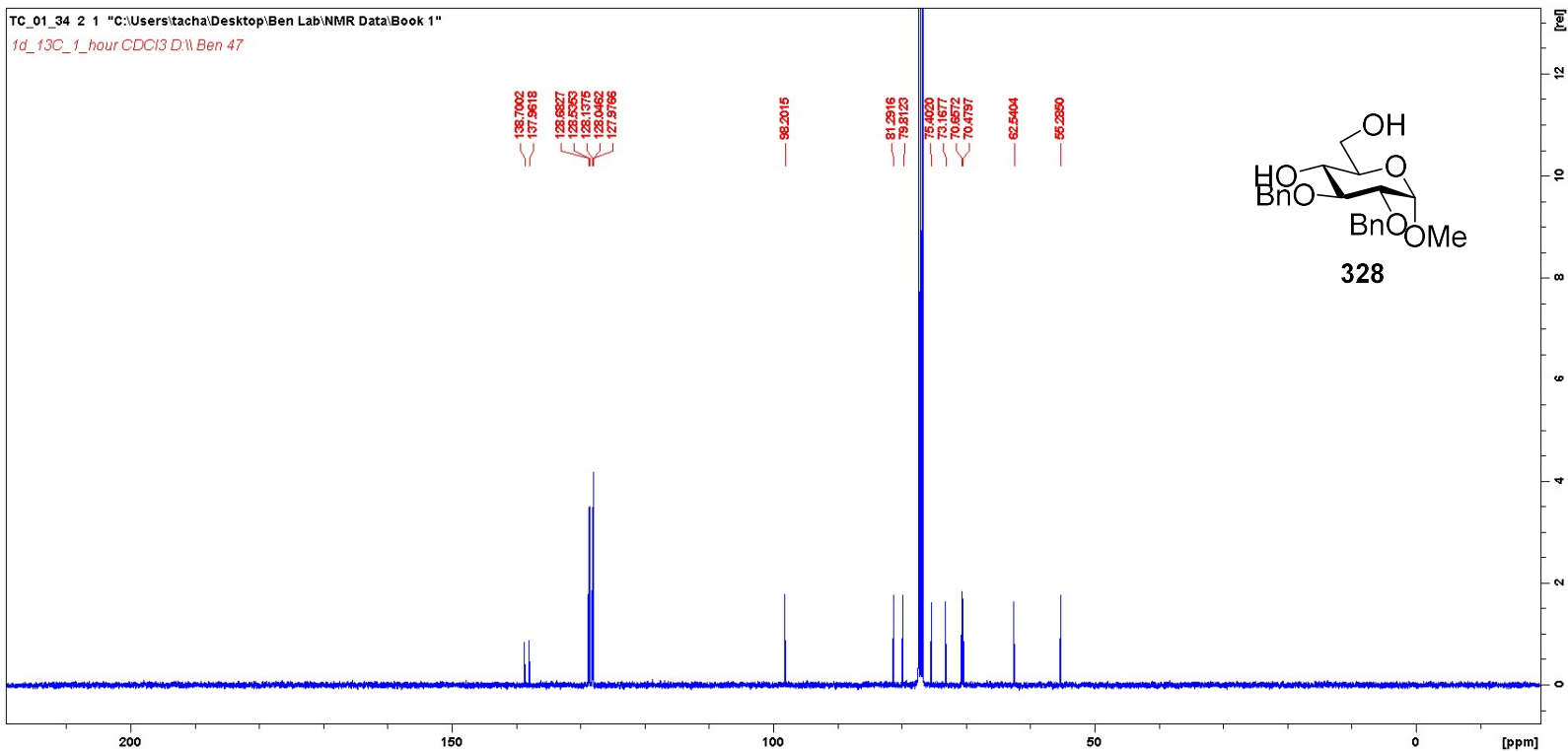
¹H NMR

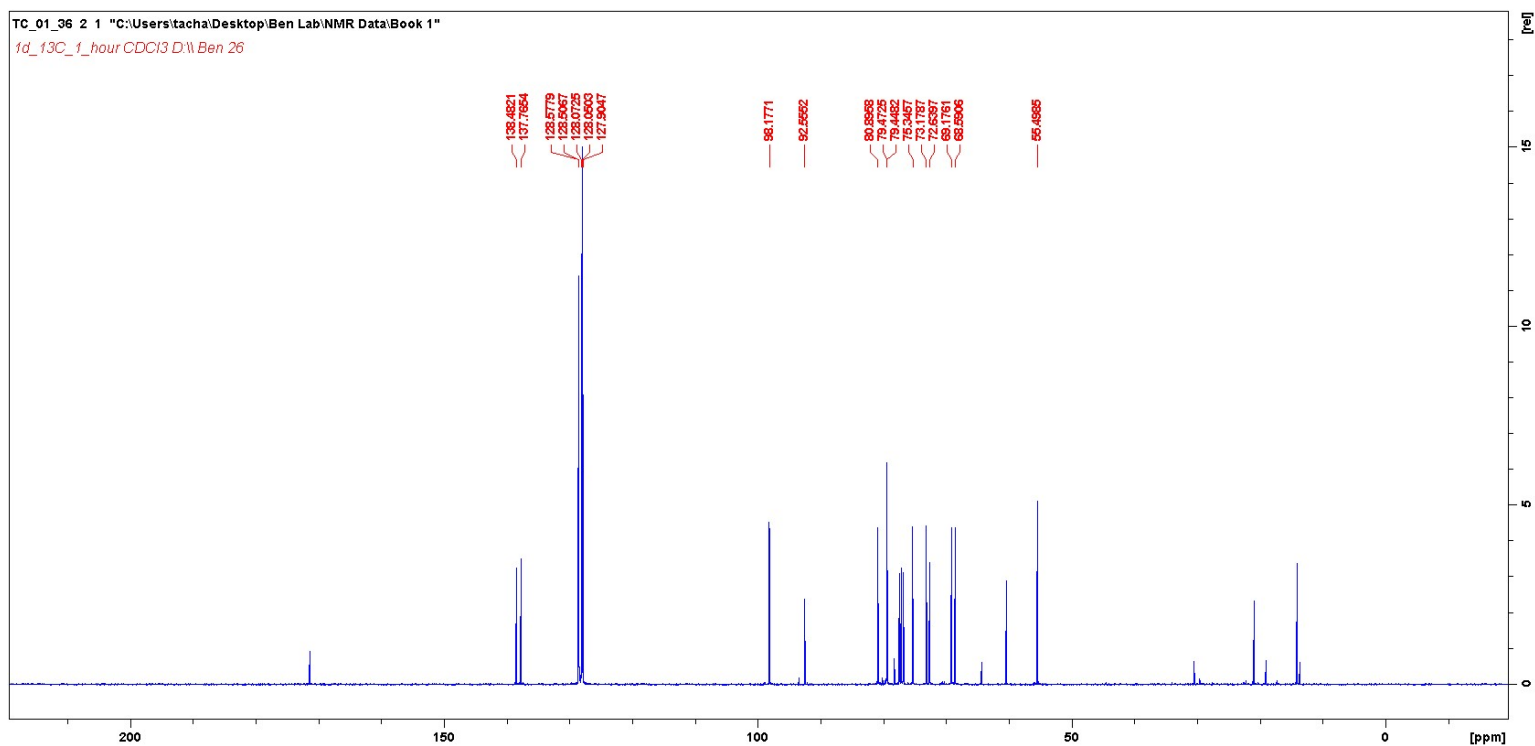
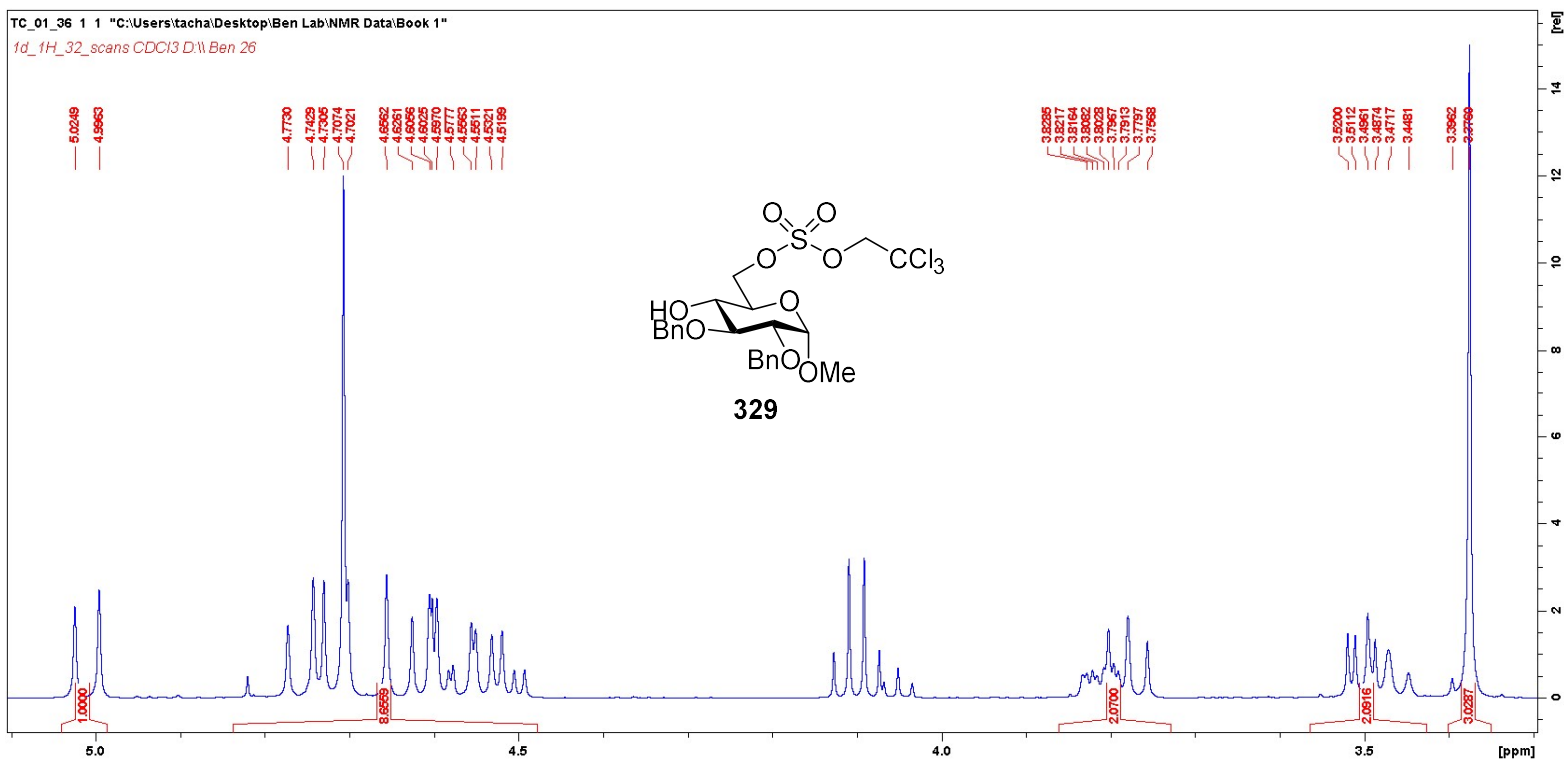
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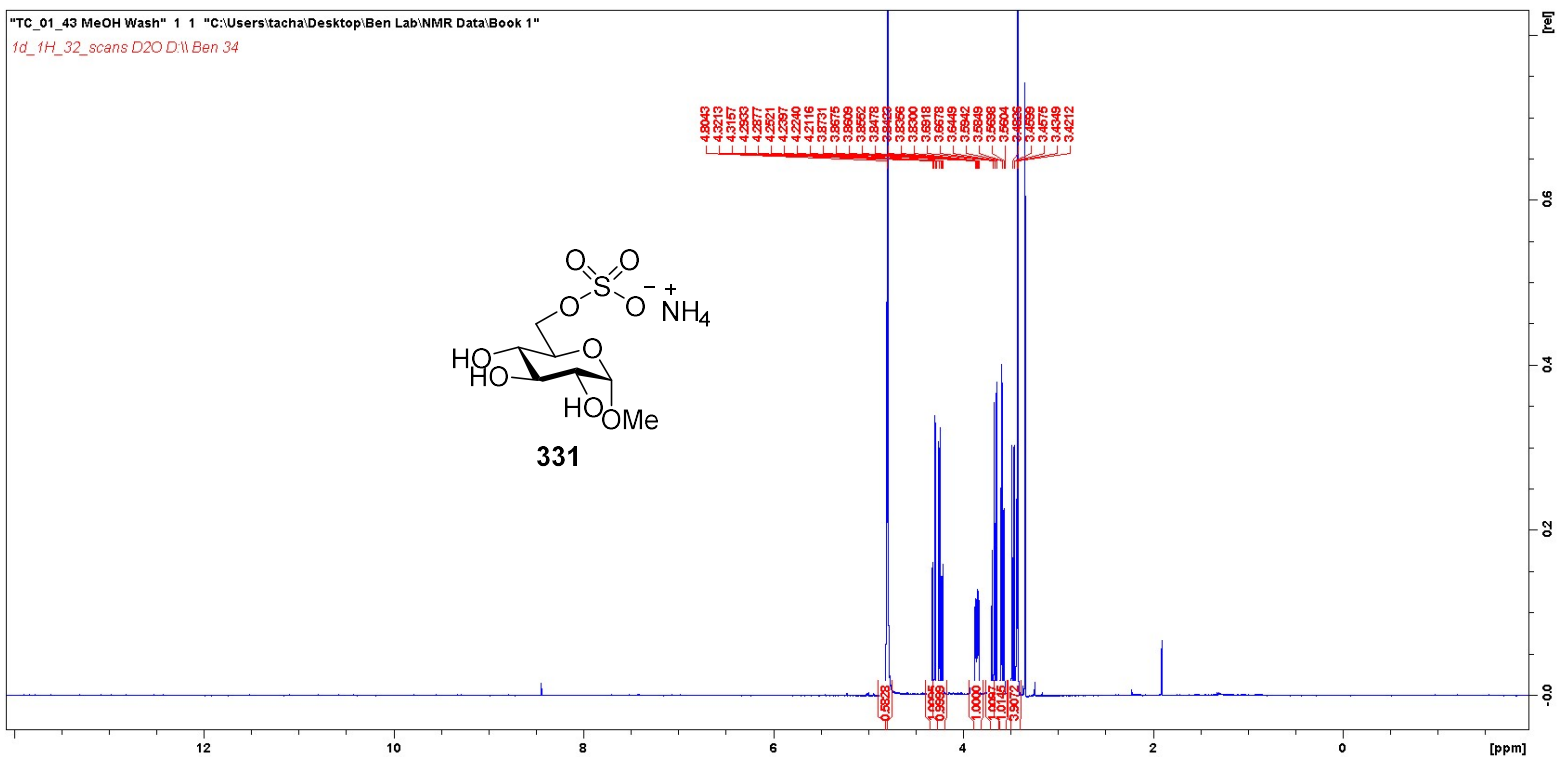
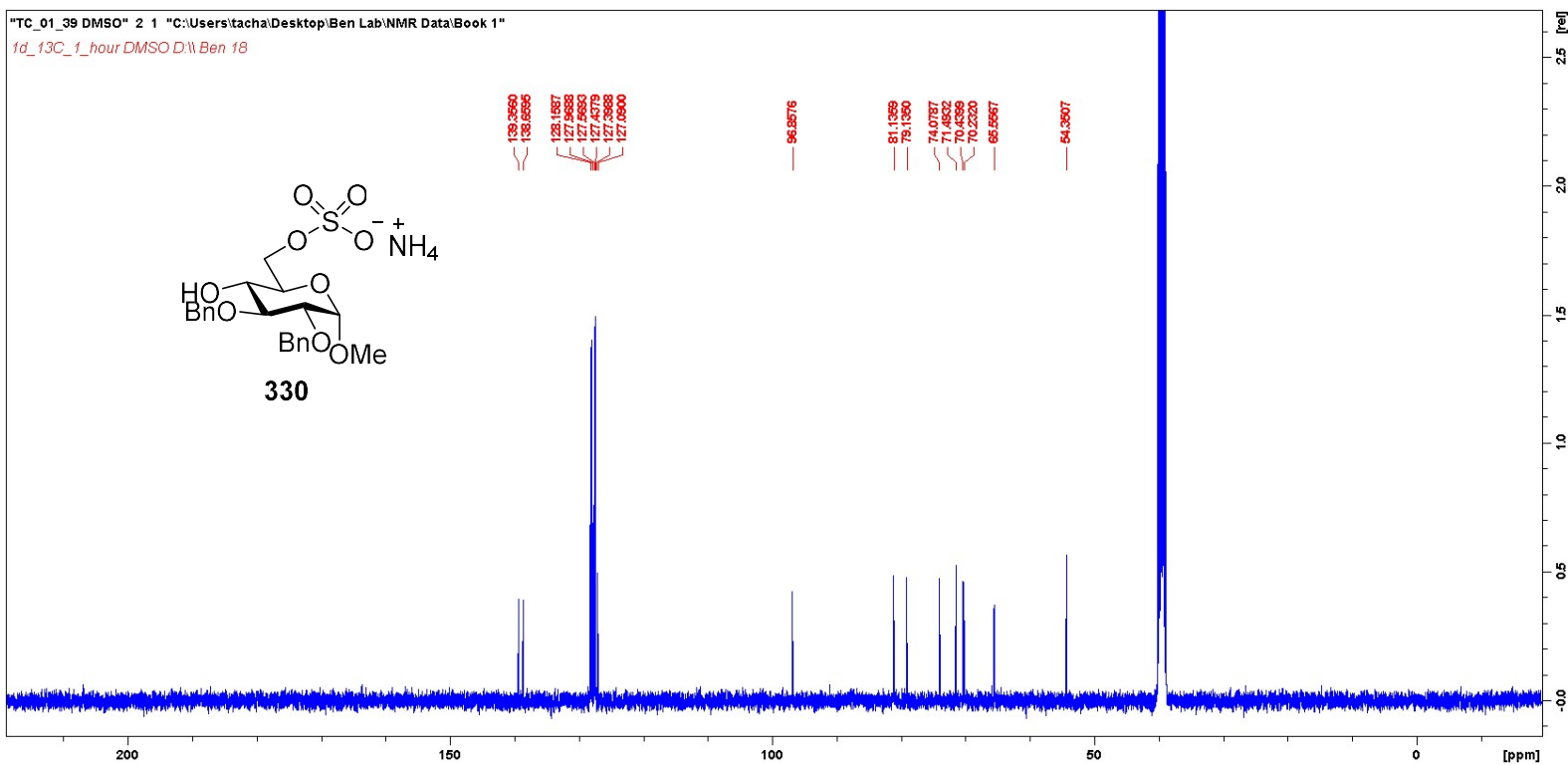


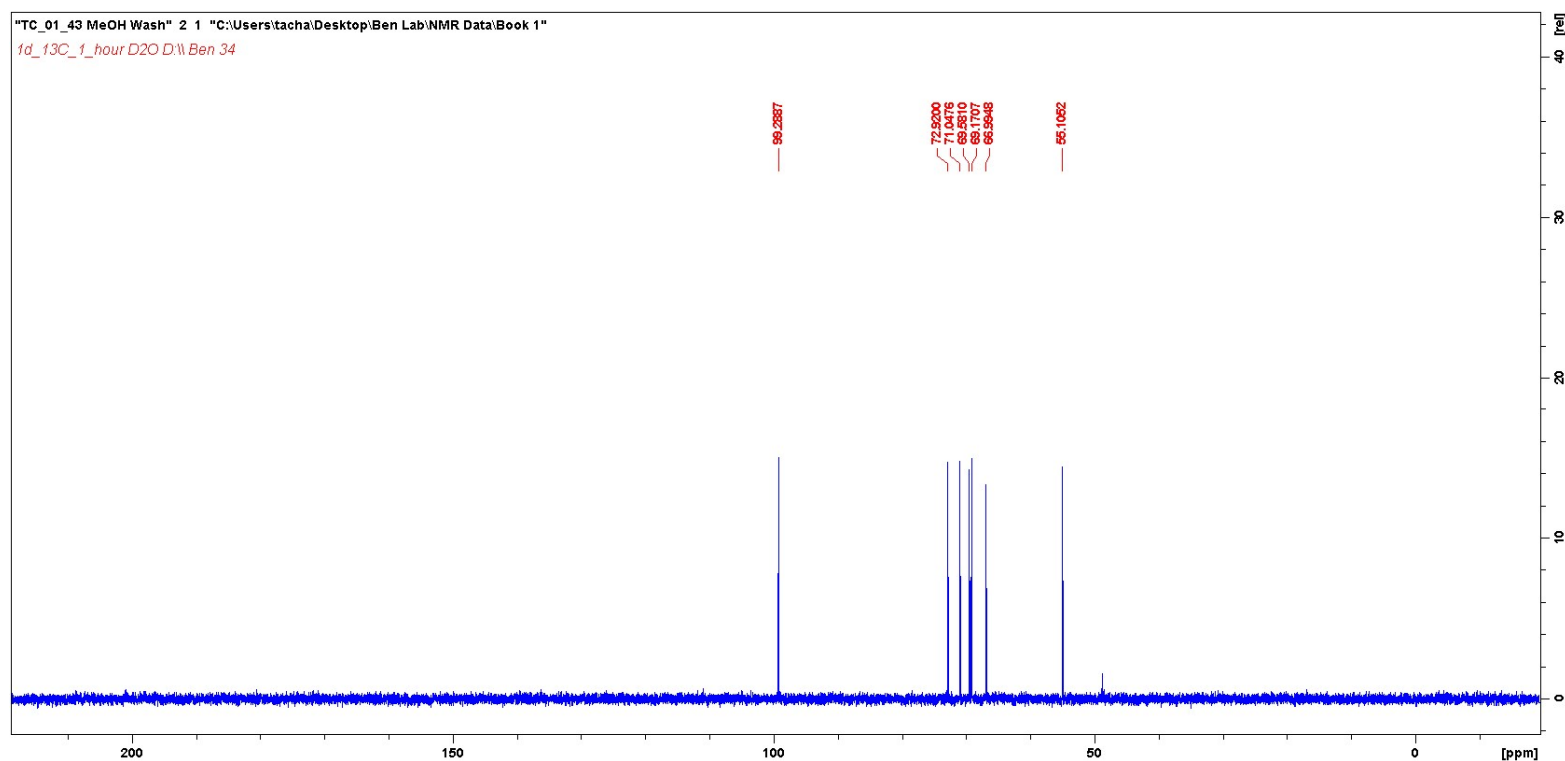
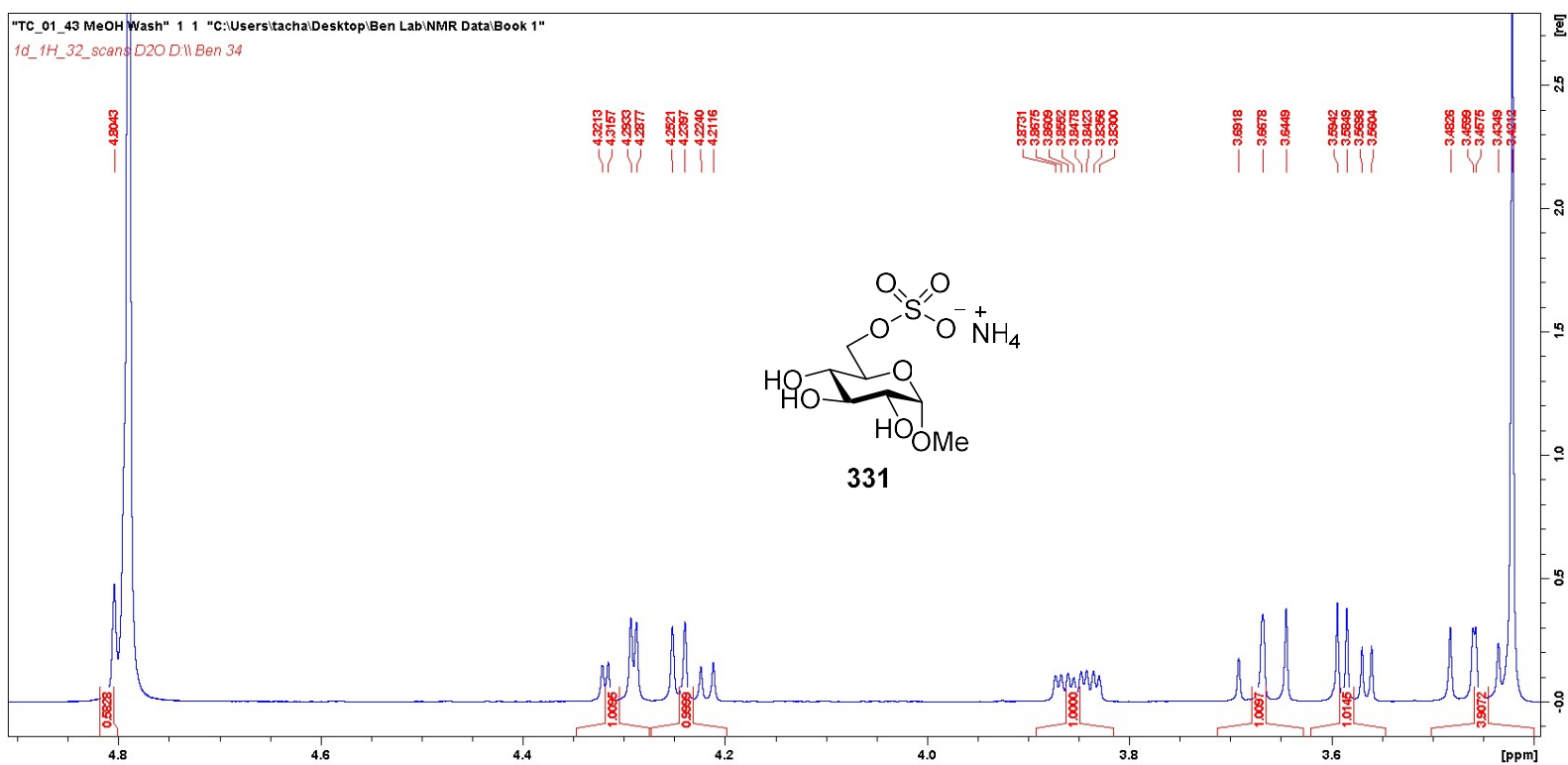


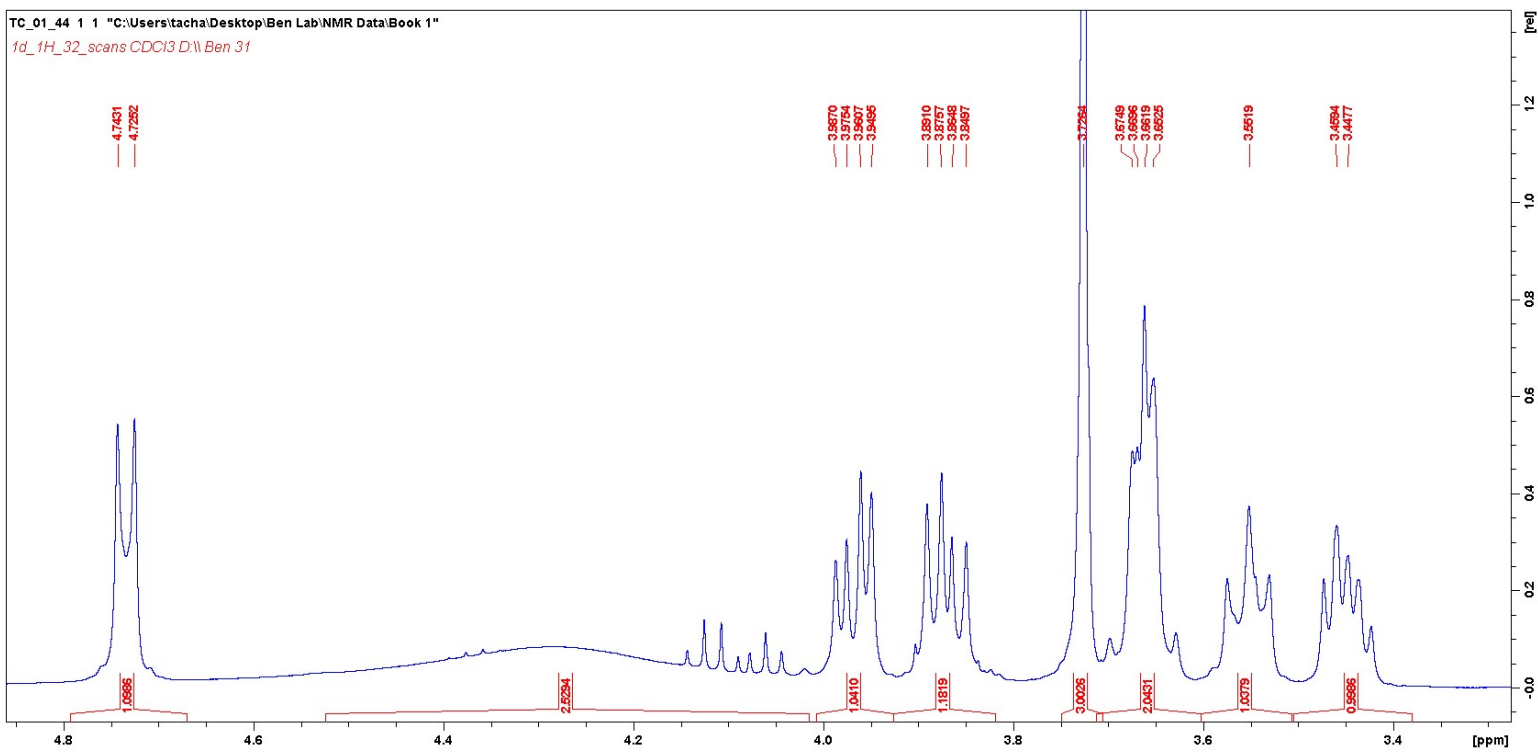
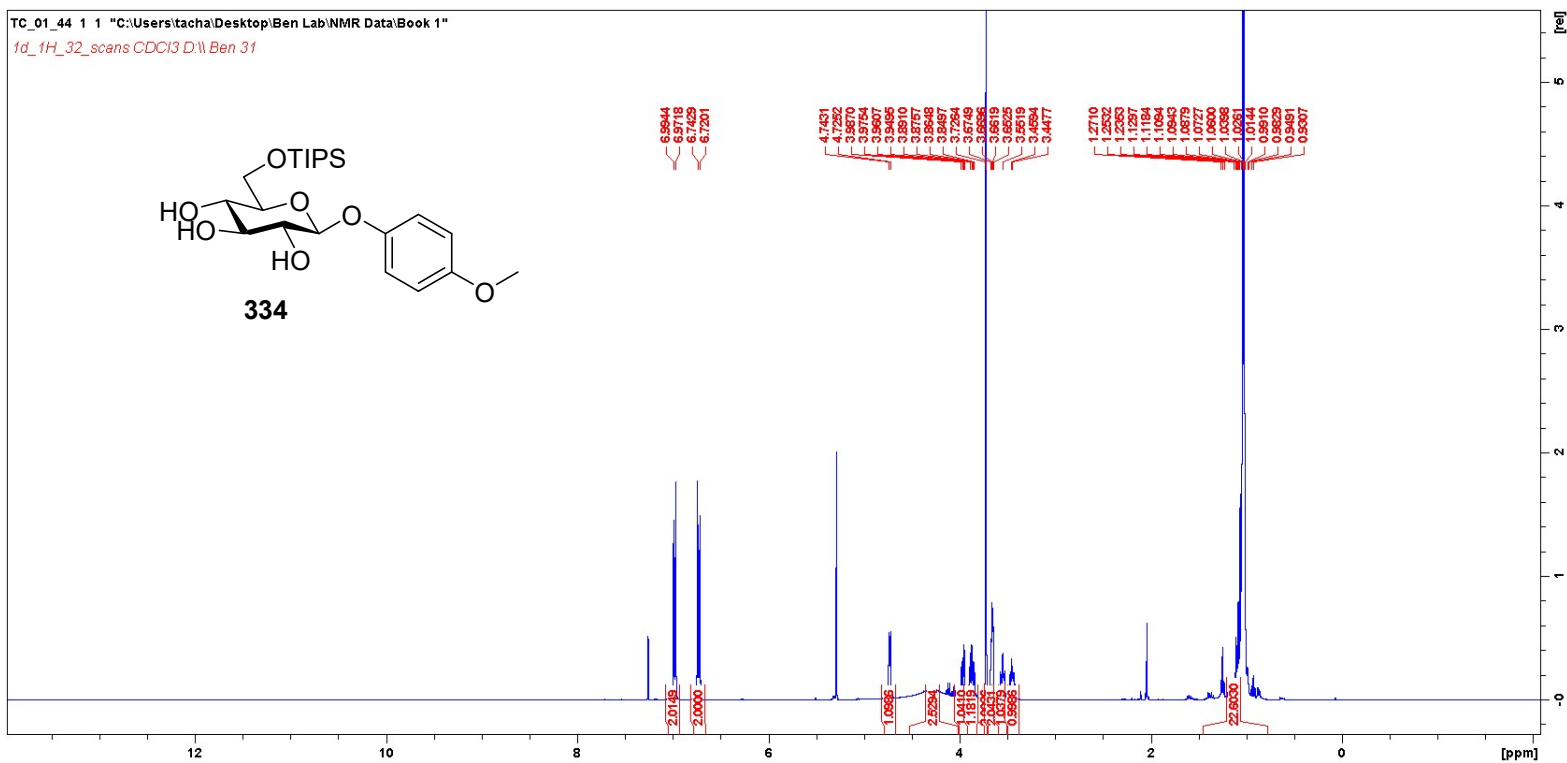






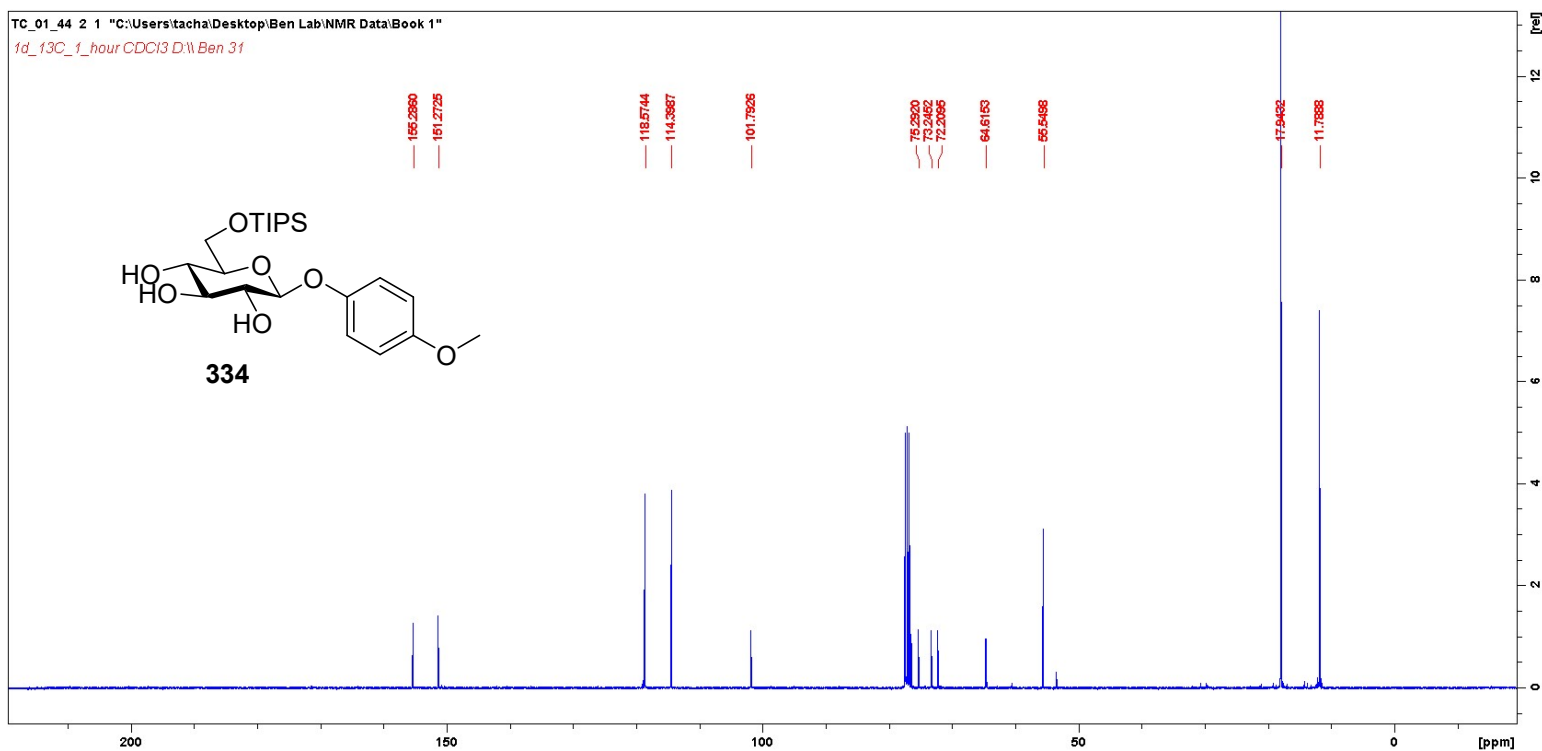






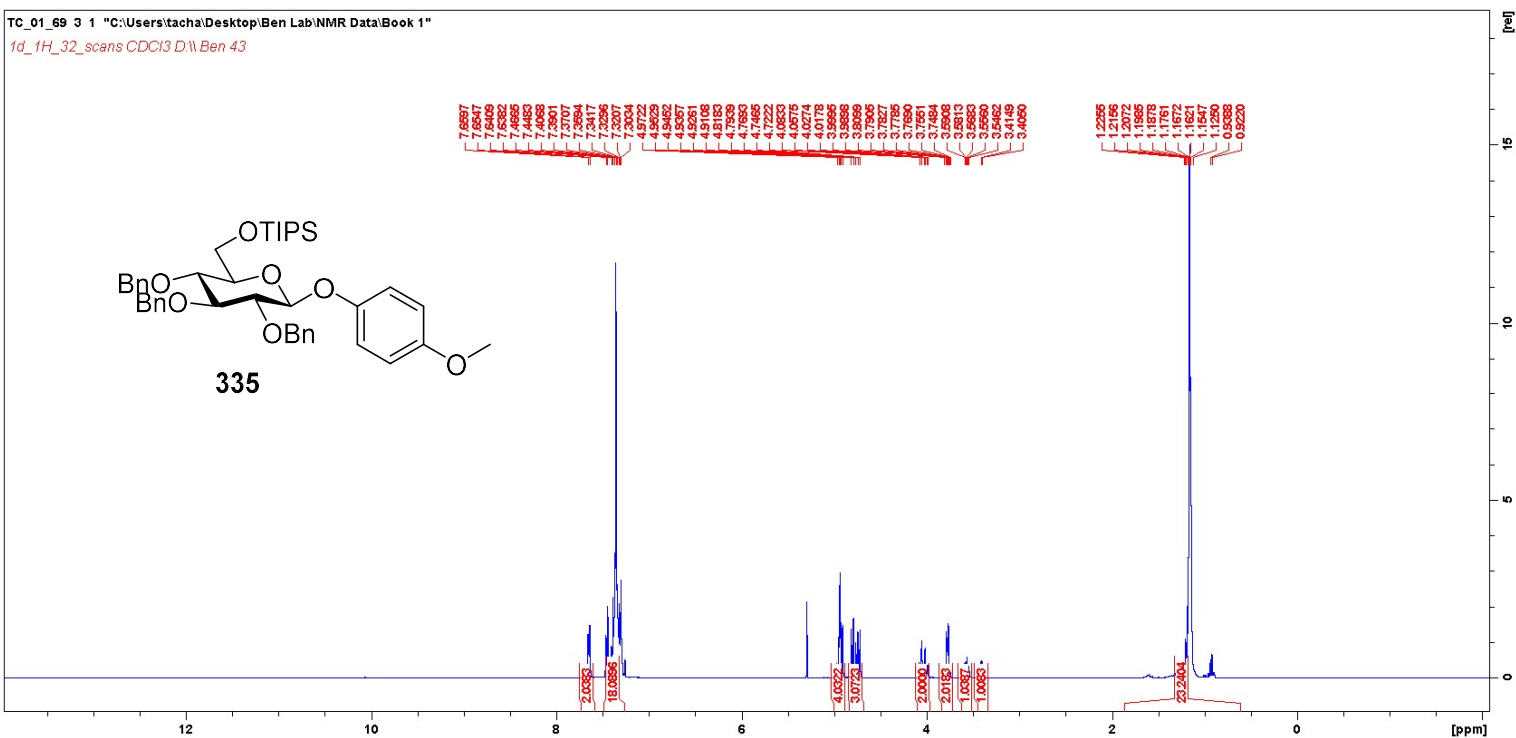
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1d_13C_1_hour CDCl3 D:\Ben 31



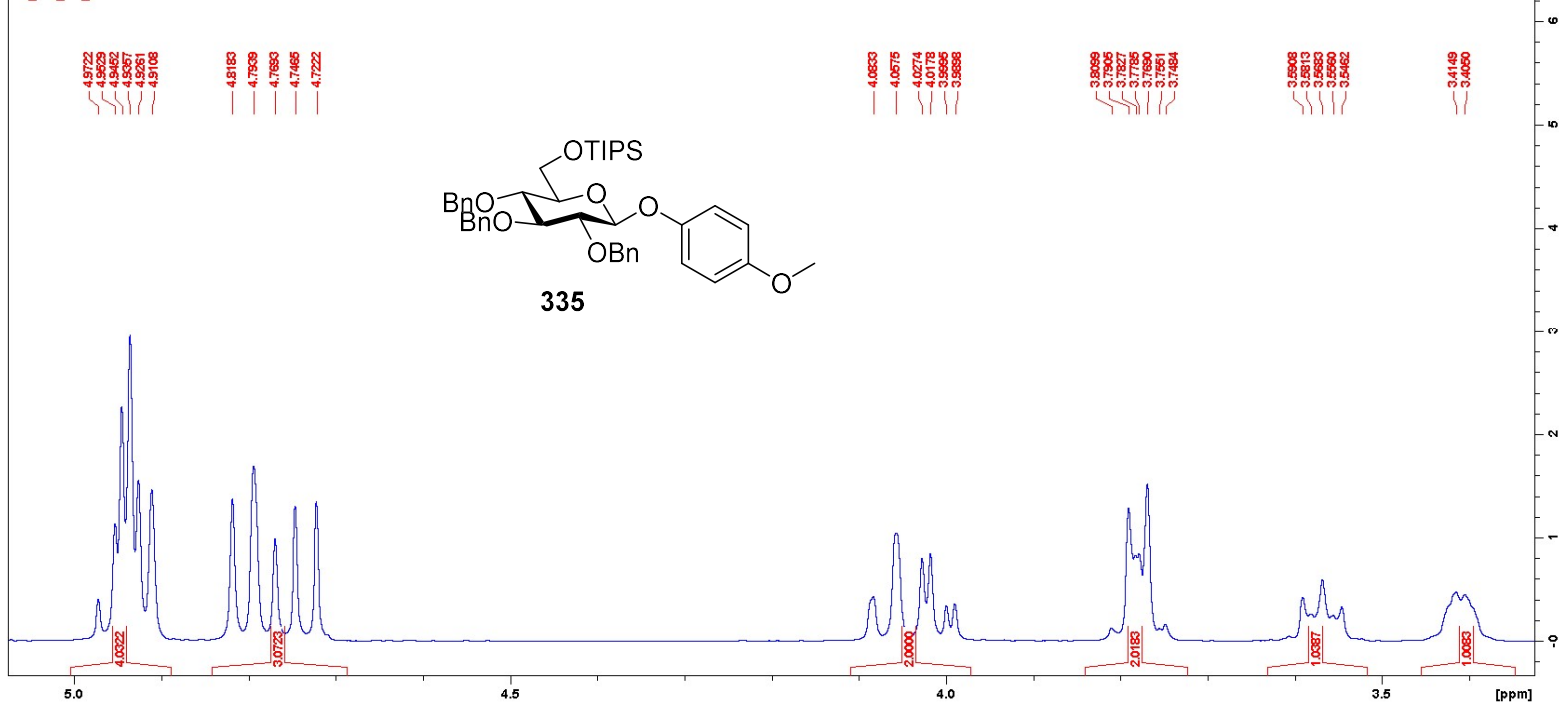
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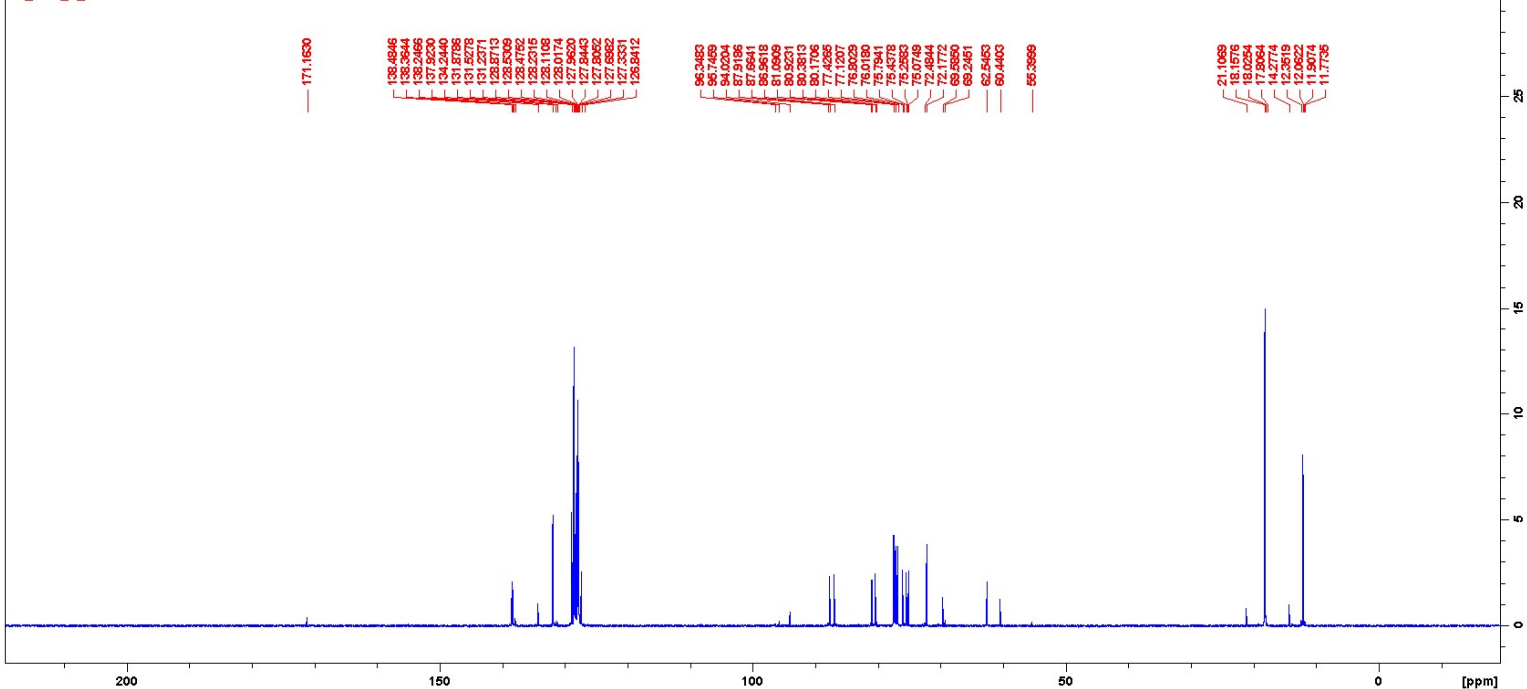
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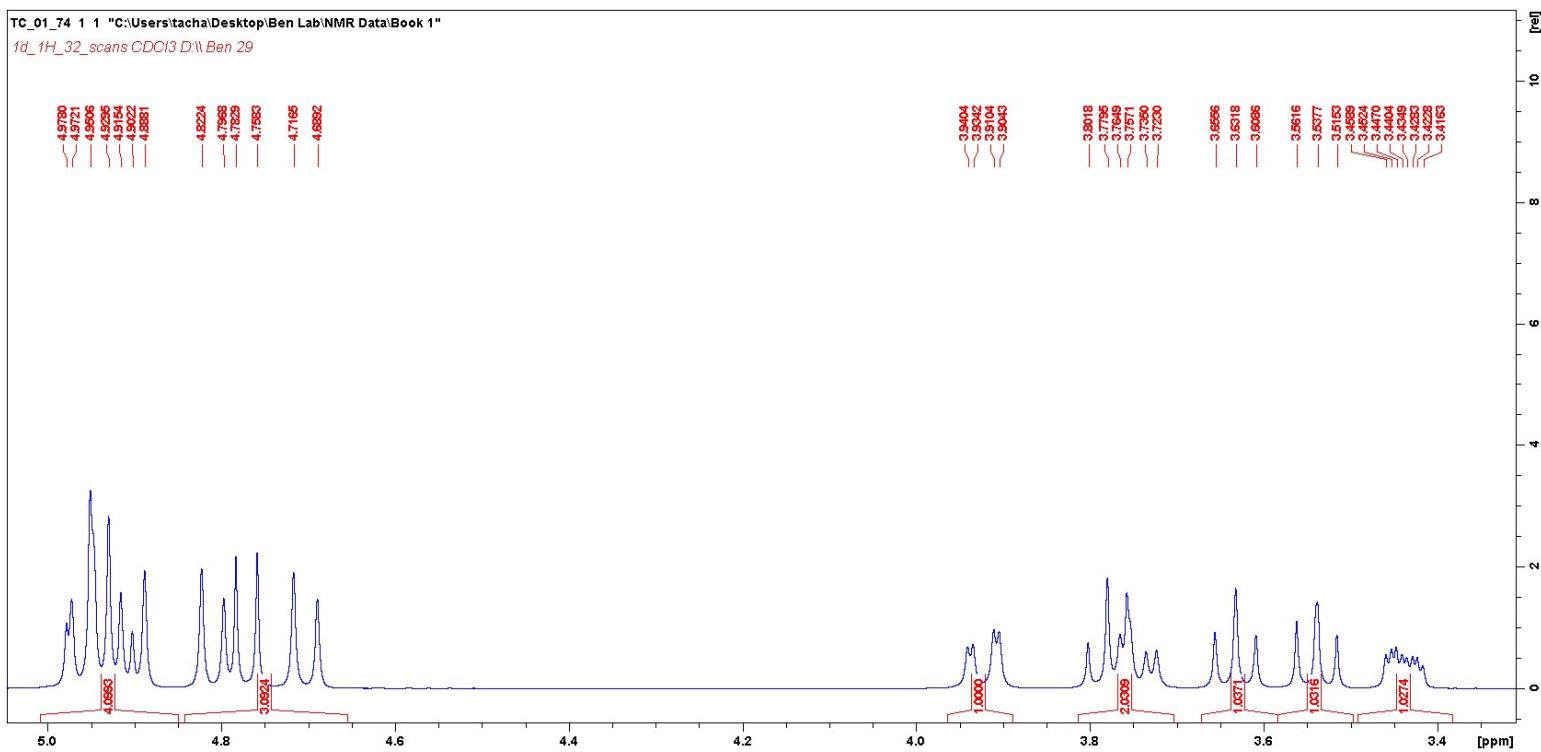
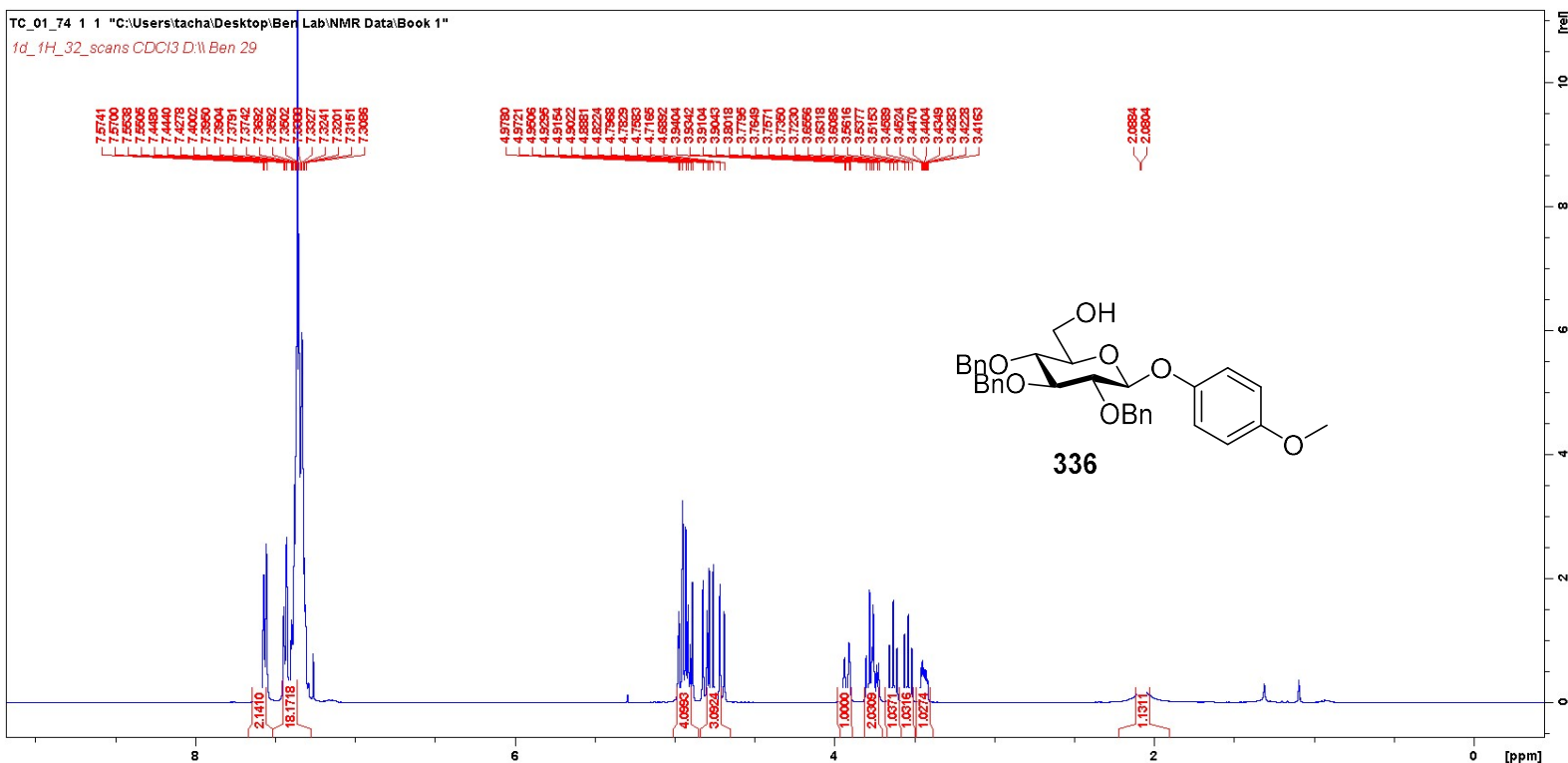
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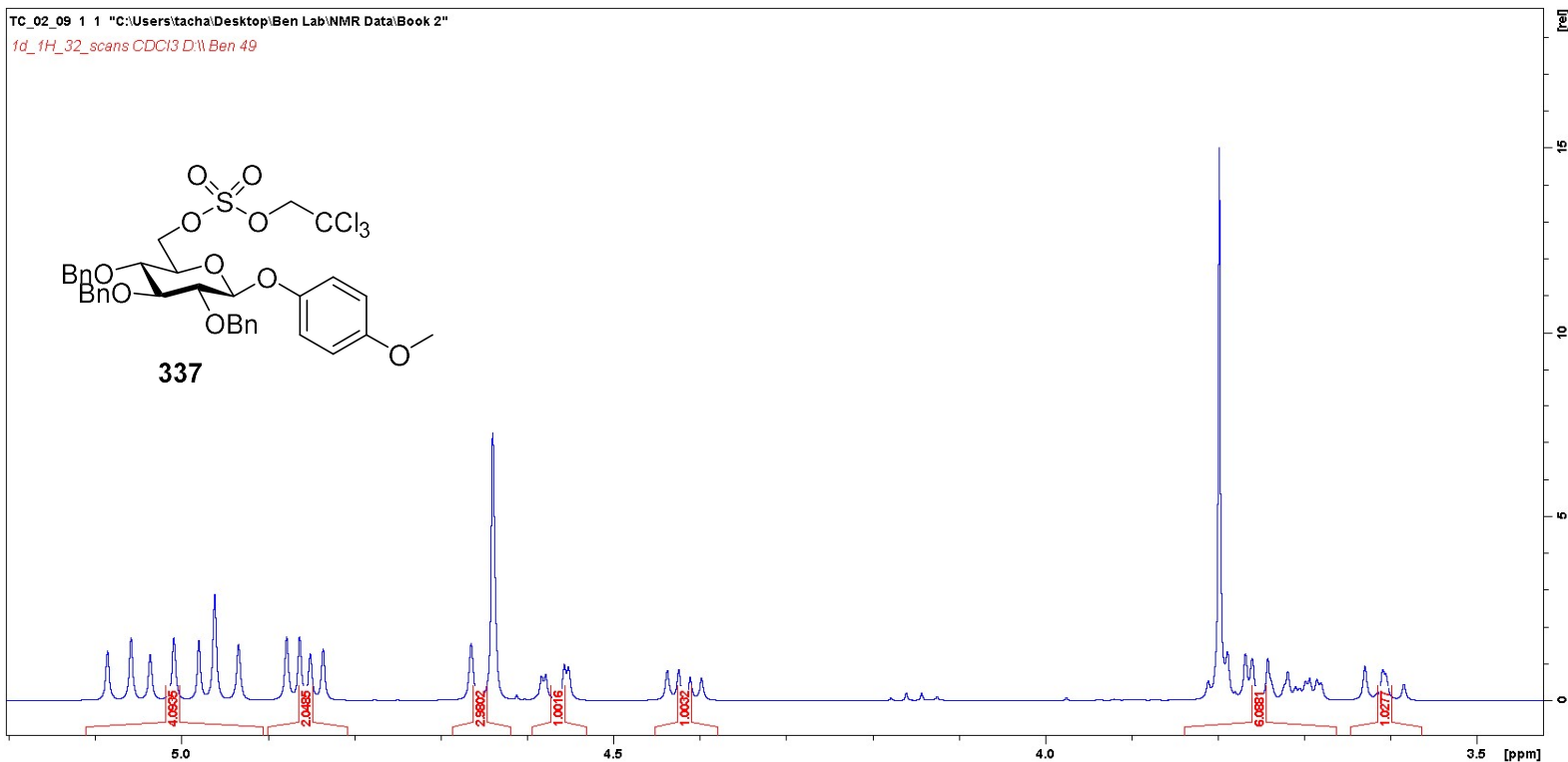
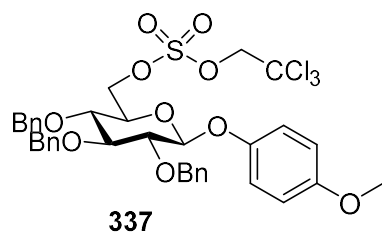
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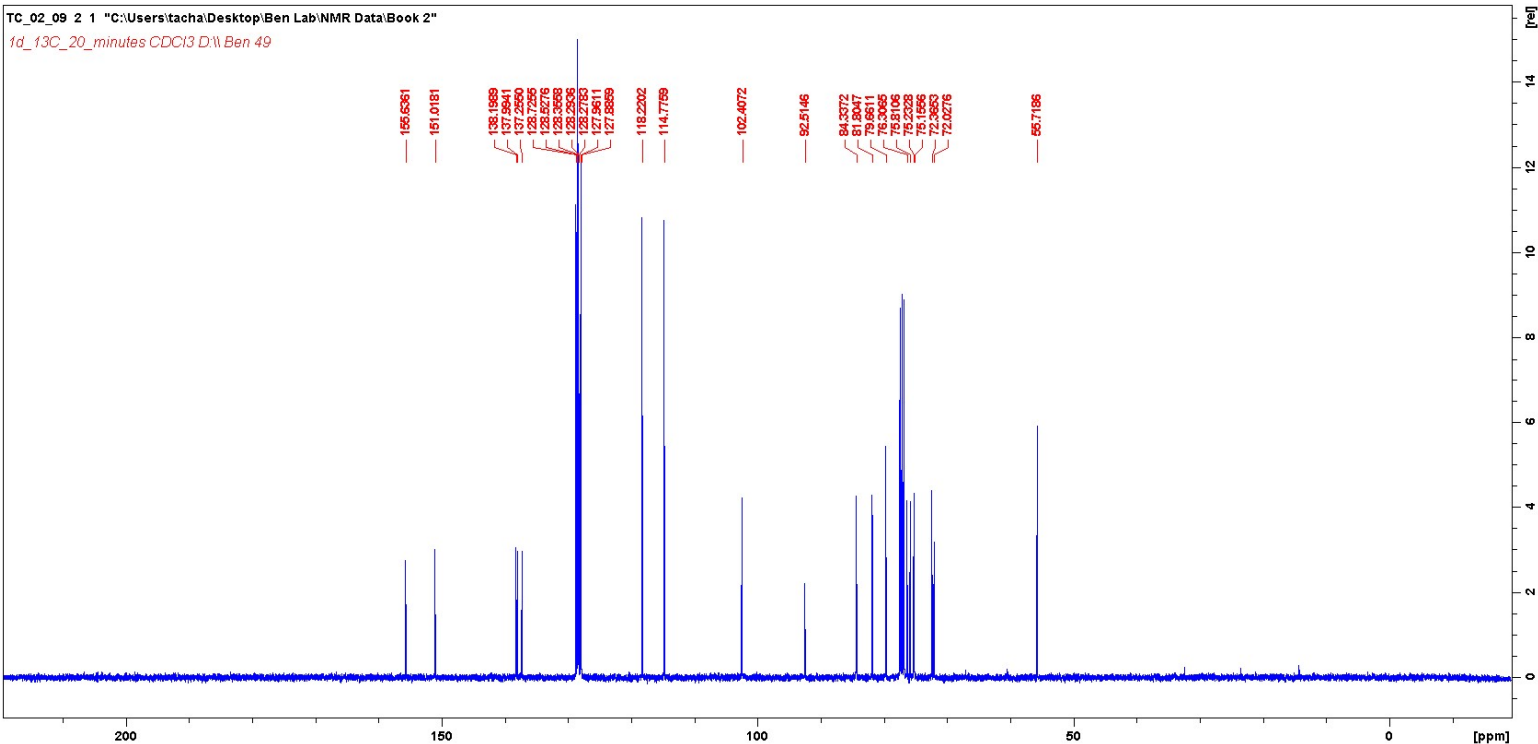
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1d_1H_32_scans CDCl3 D:\Ben 49



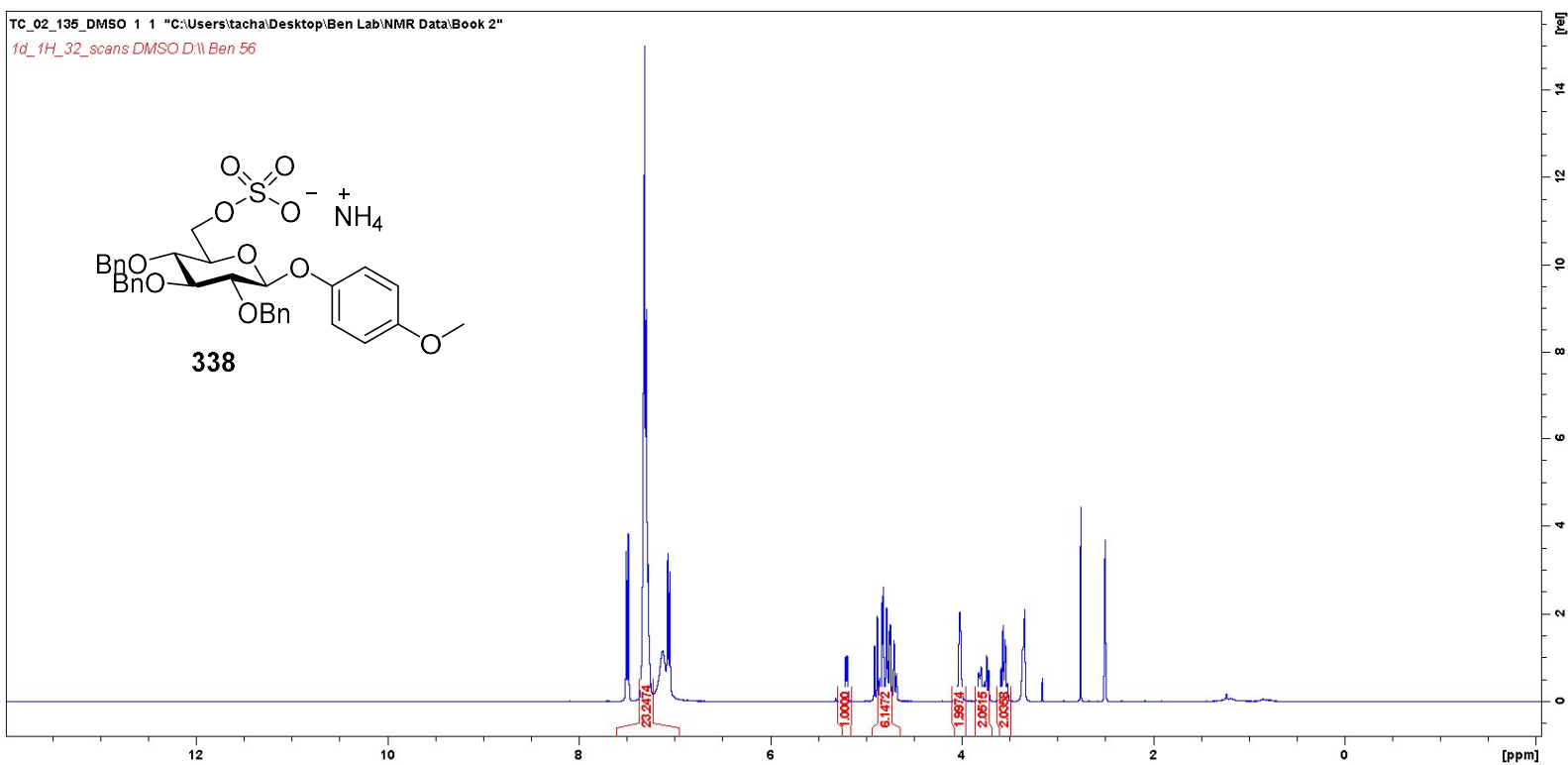
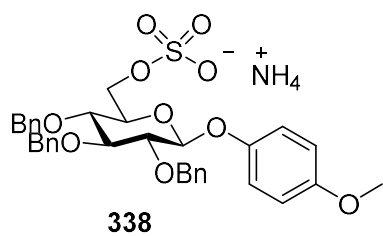
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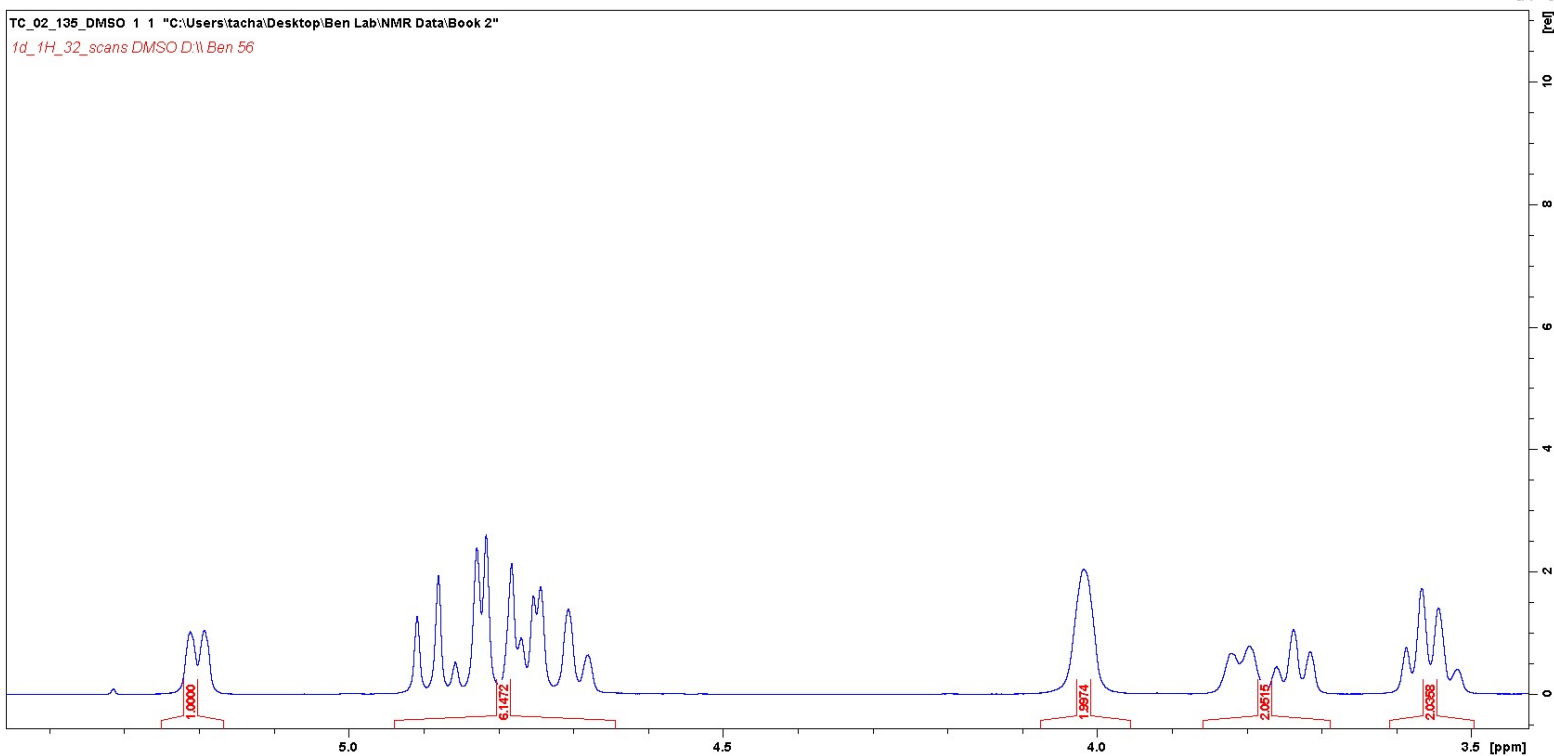
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1d_1H_32_scans DMSO D\ Ben 56



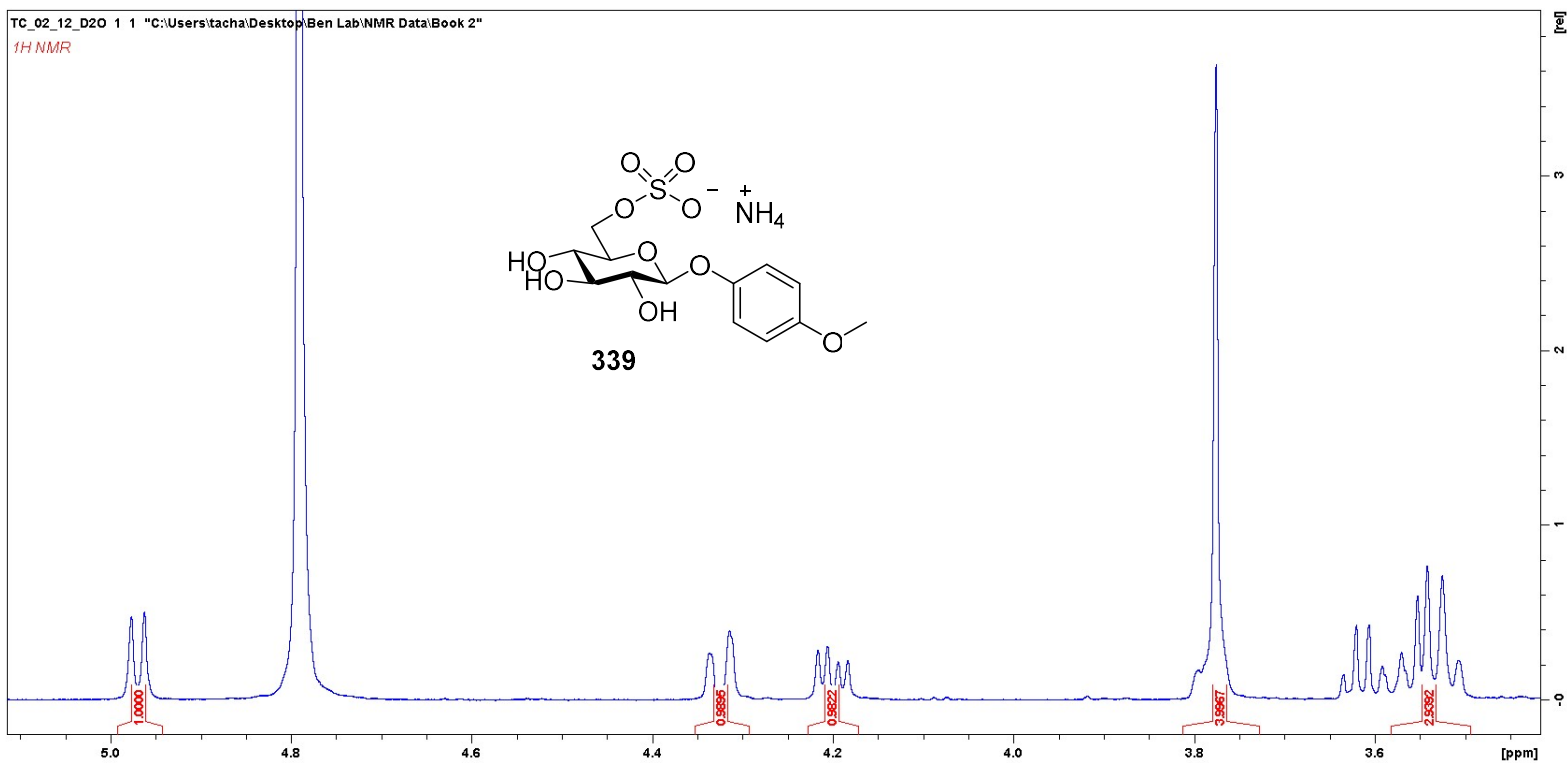
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1d_1H_32_scans DMSO D\ Ben 56



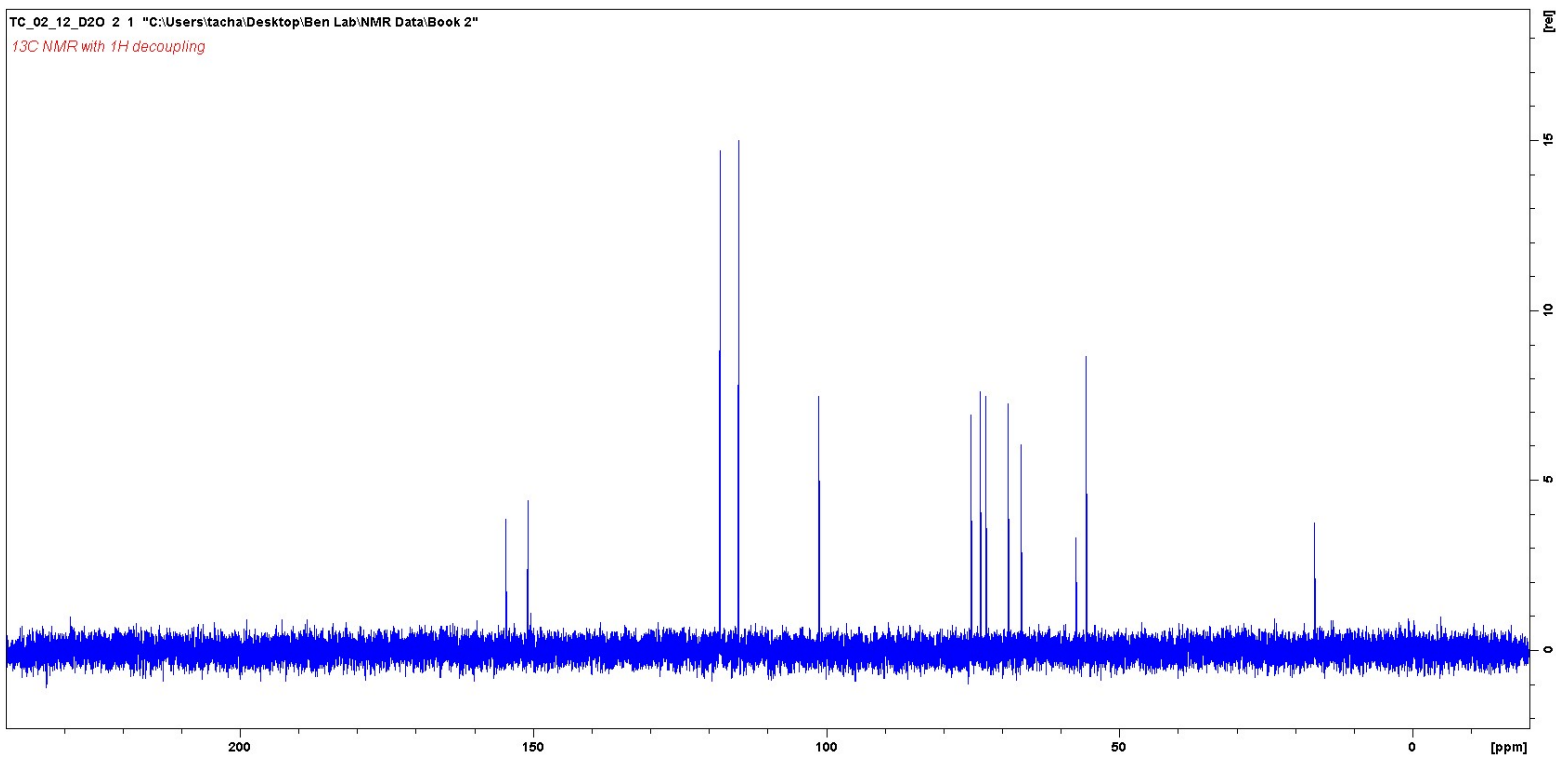
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¹H NMR



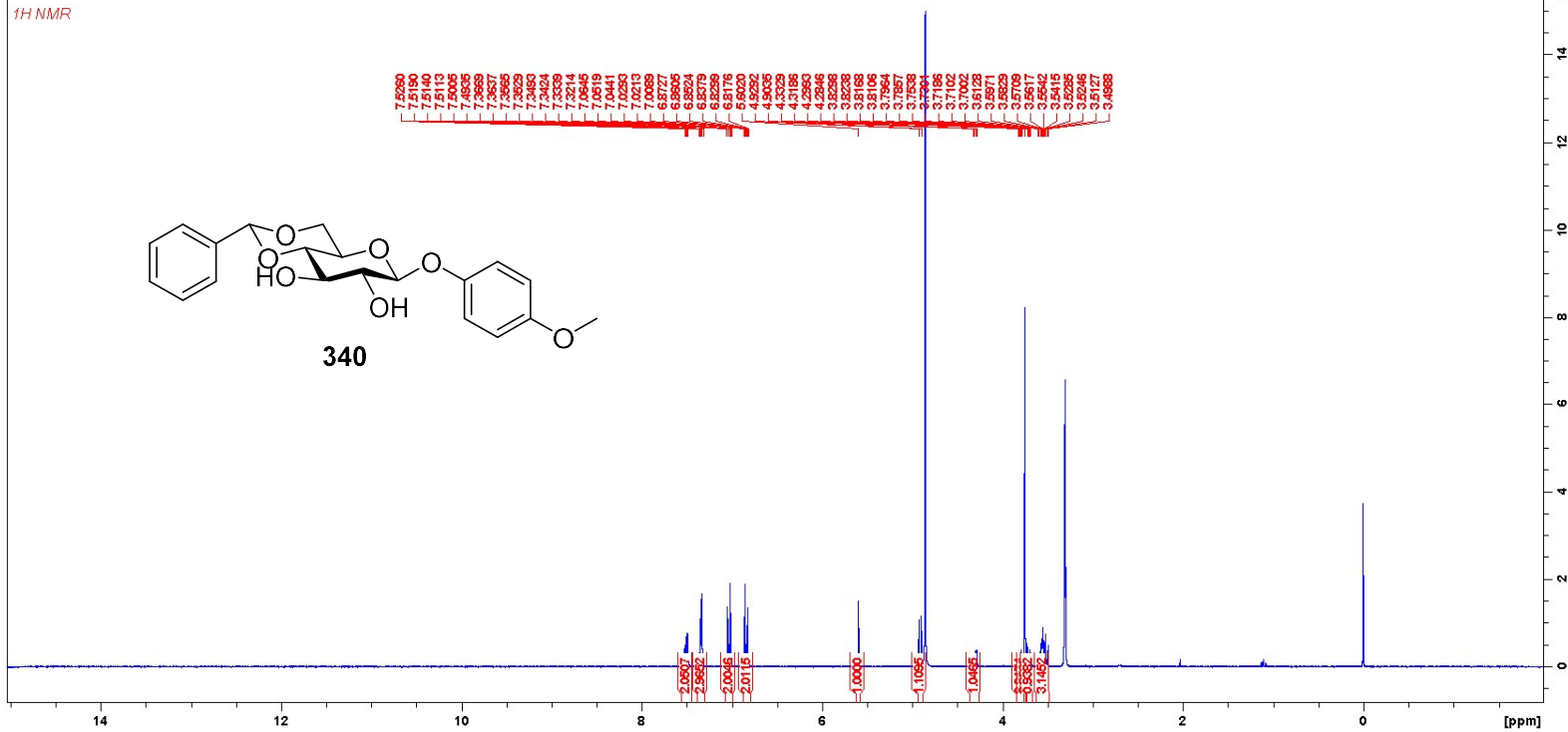
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¹³C NMR with ¹H decoupling



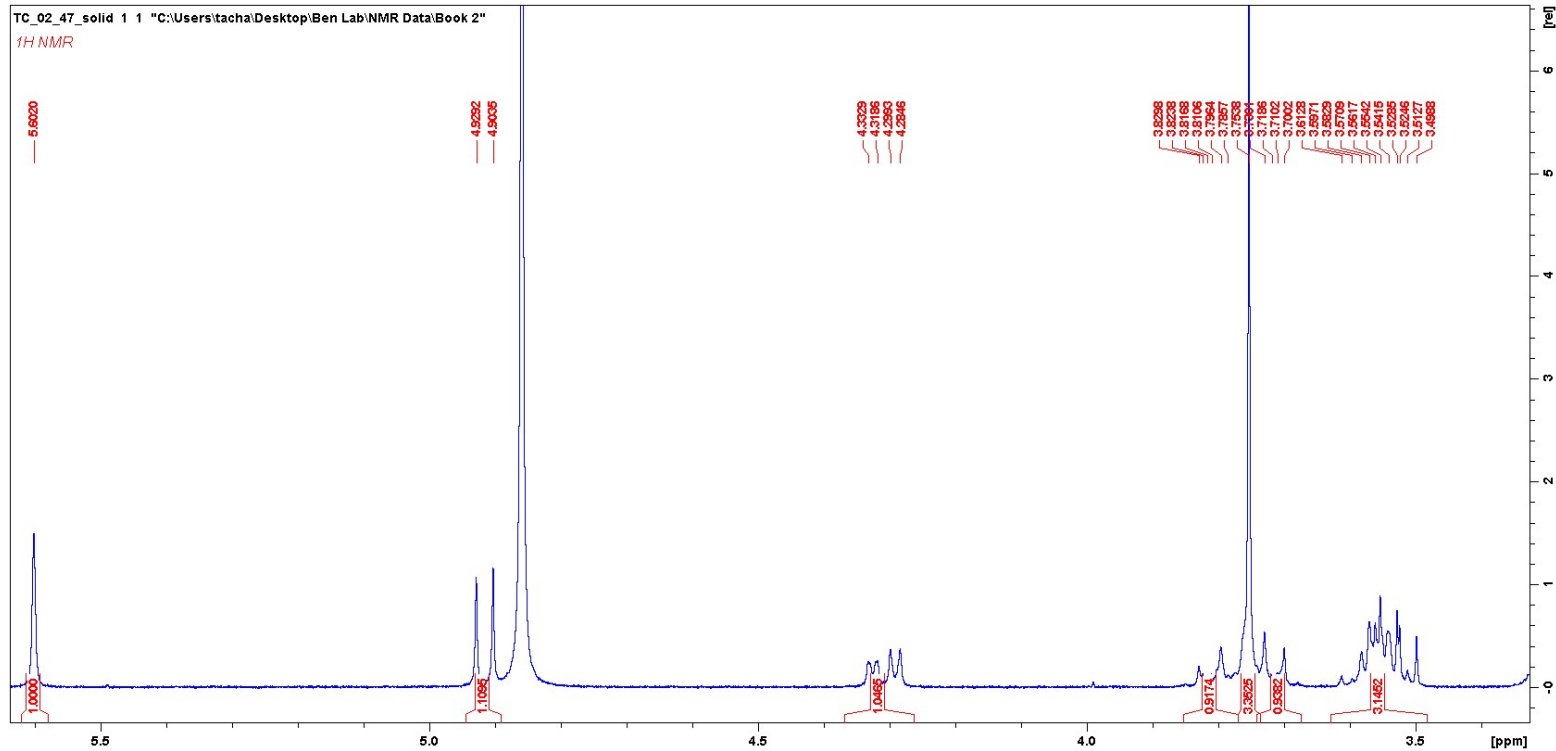
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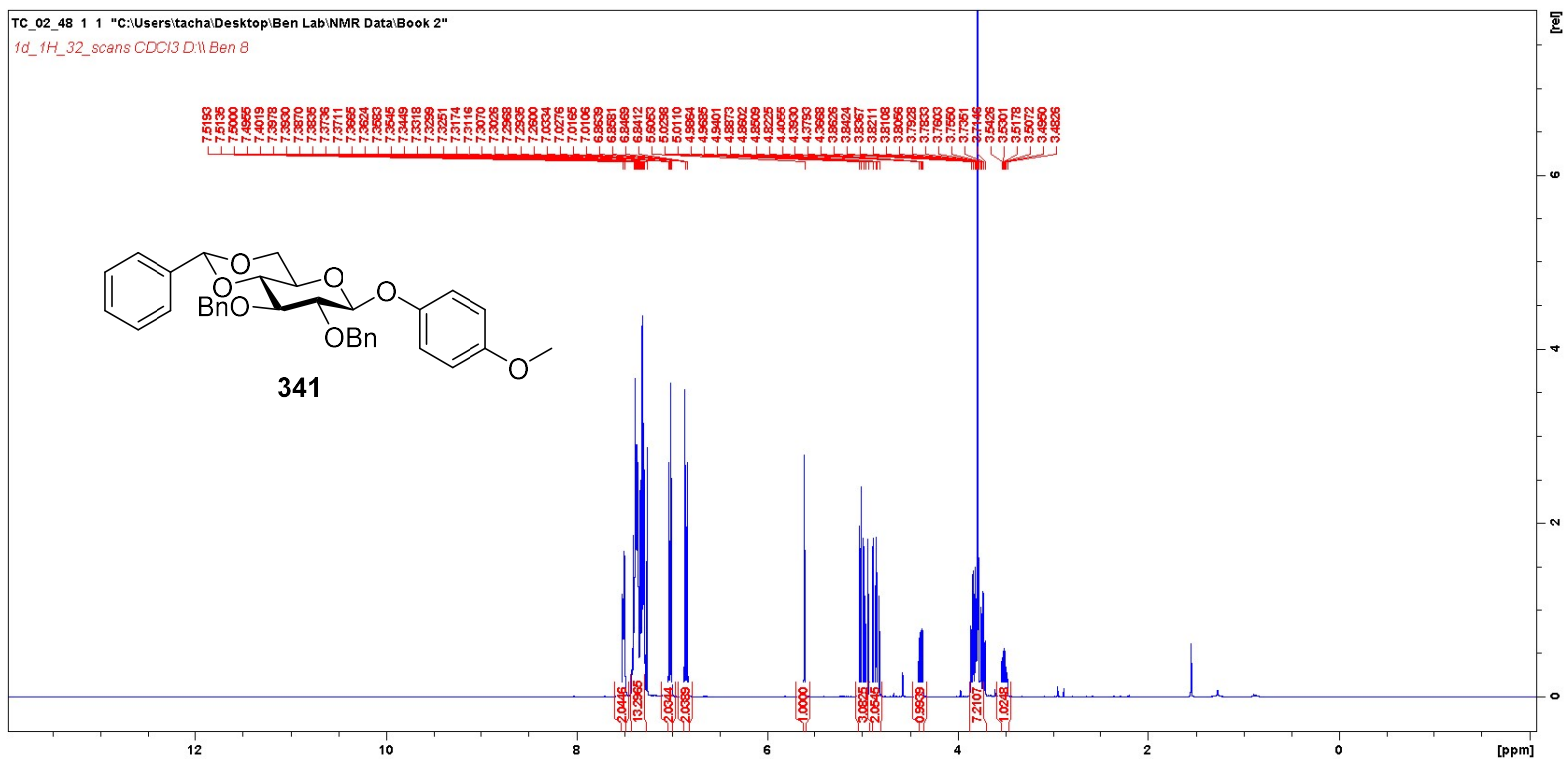
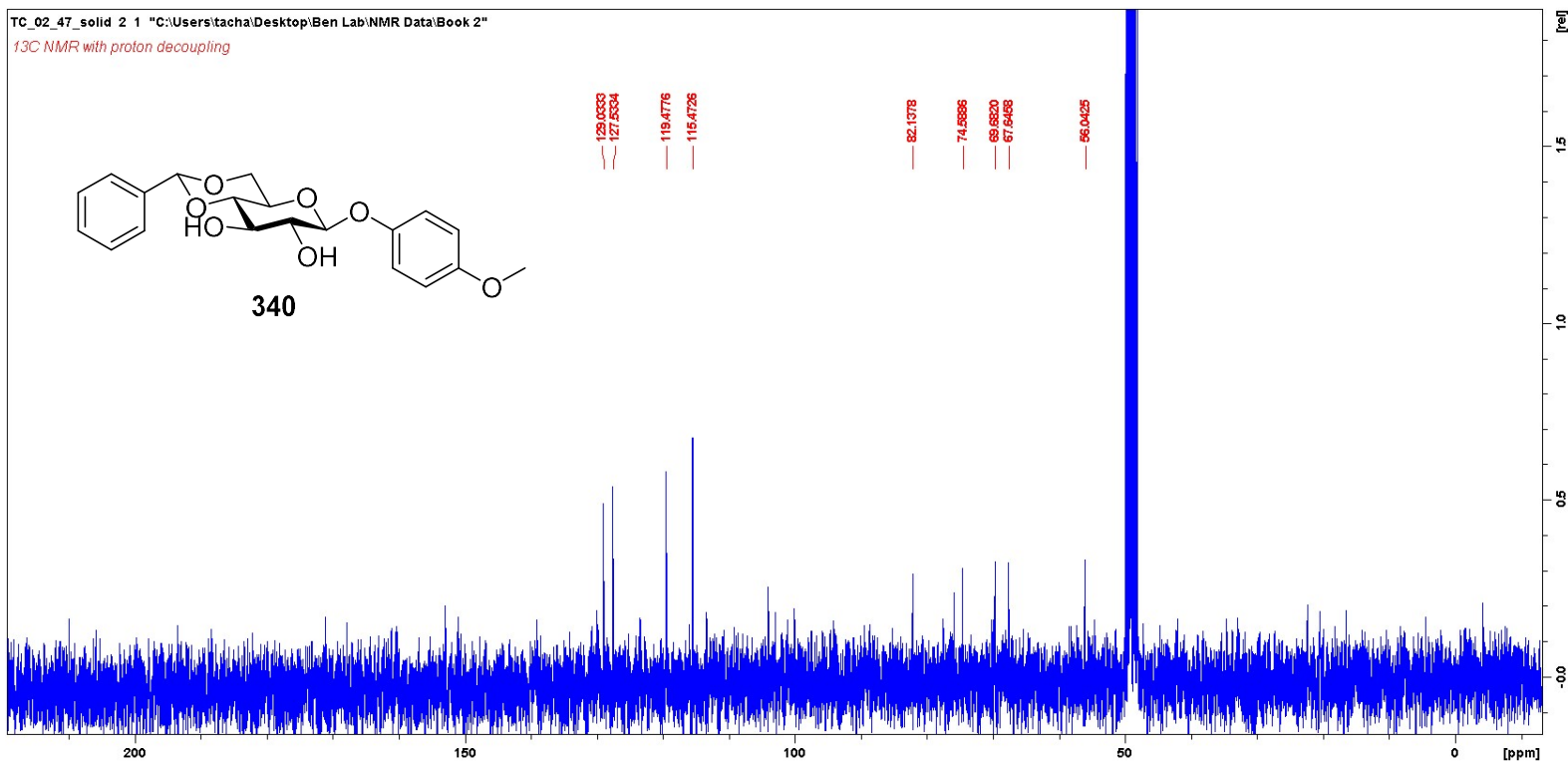
¹H NMR

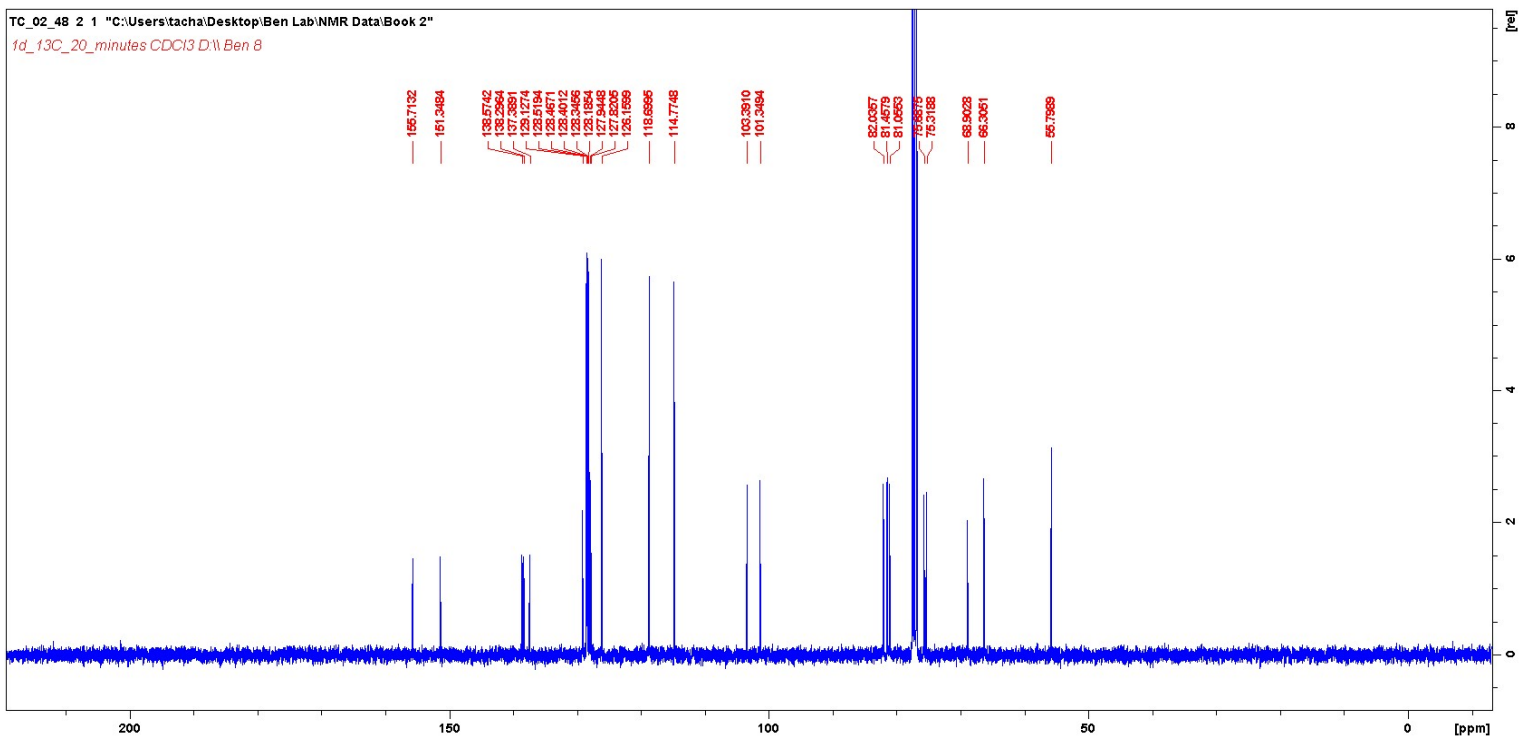
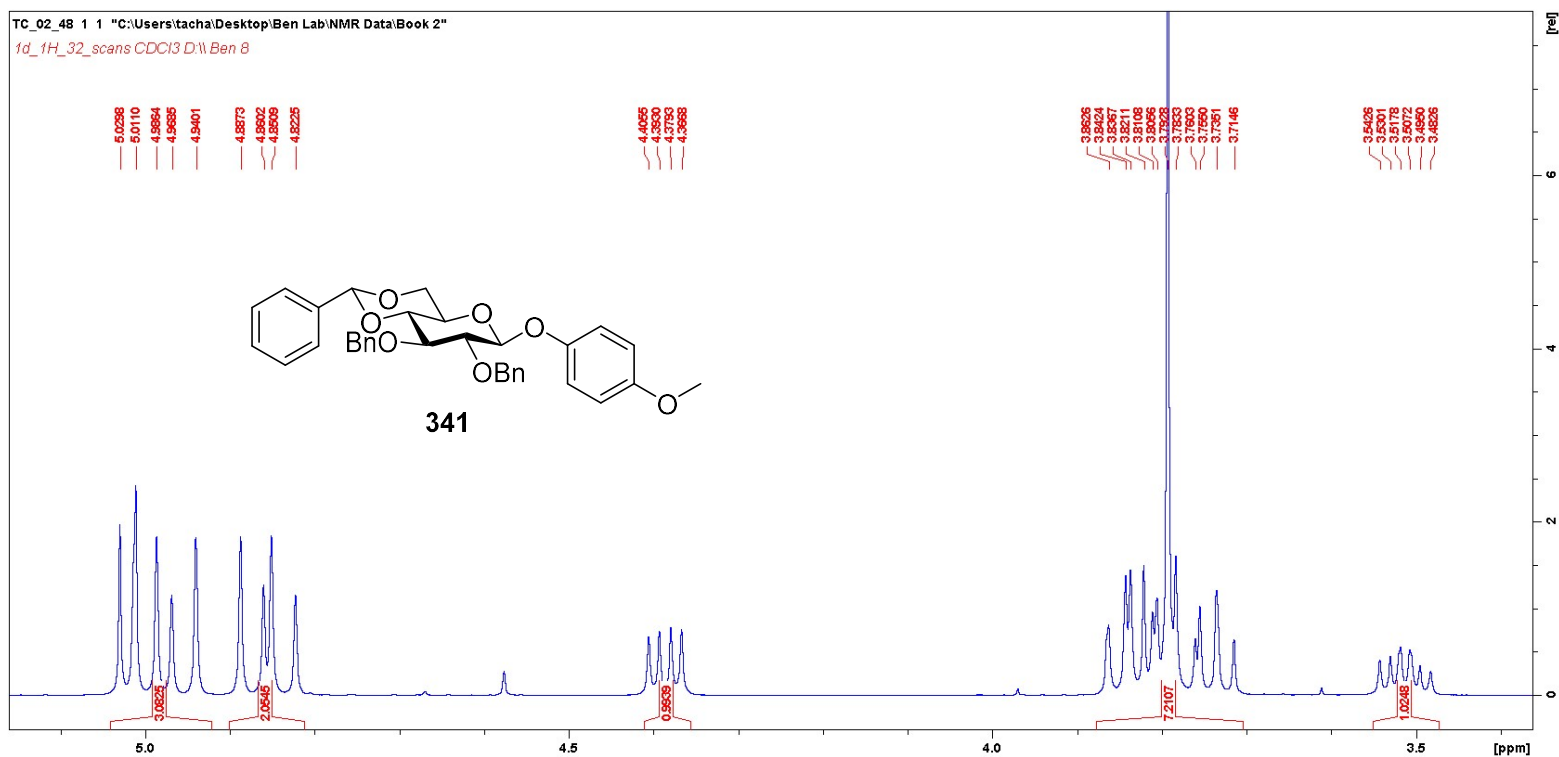


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¹H NMR

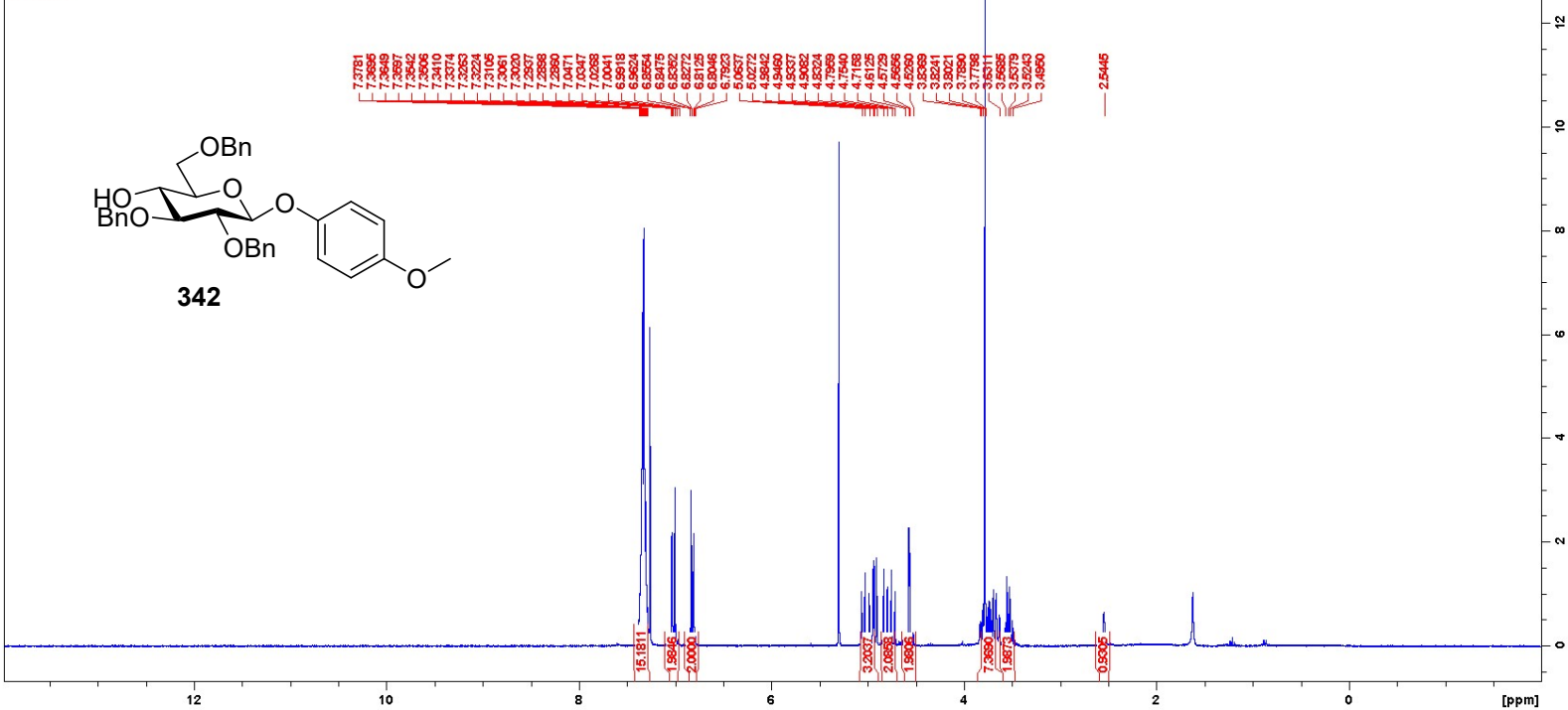






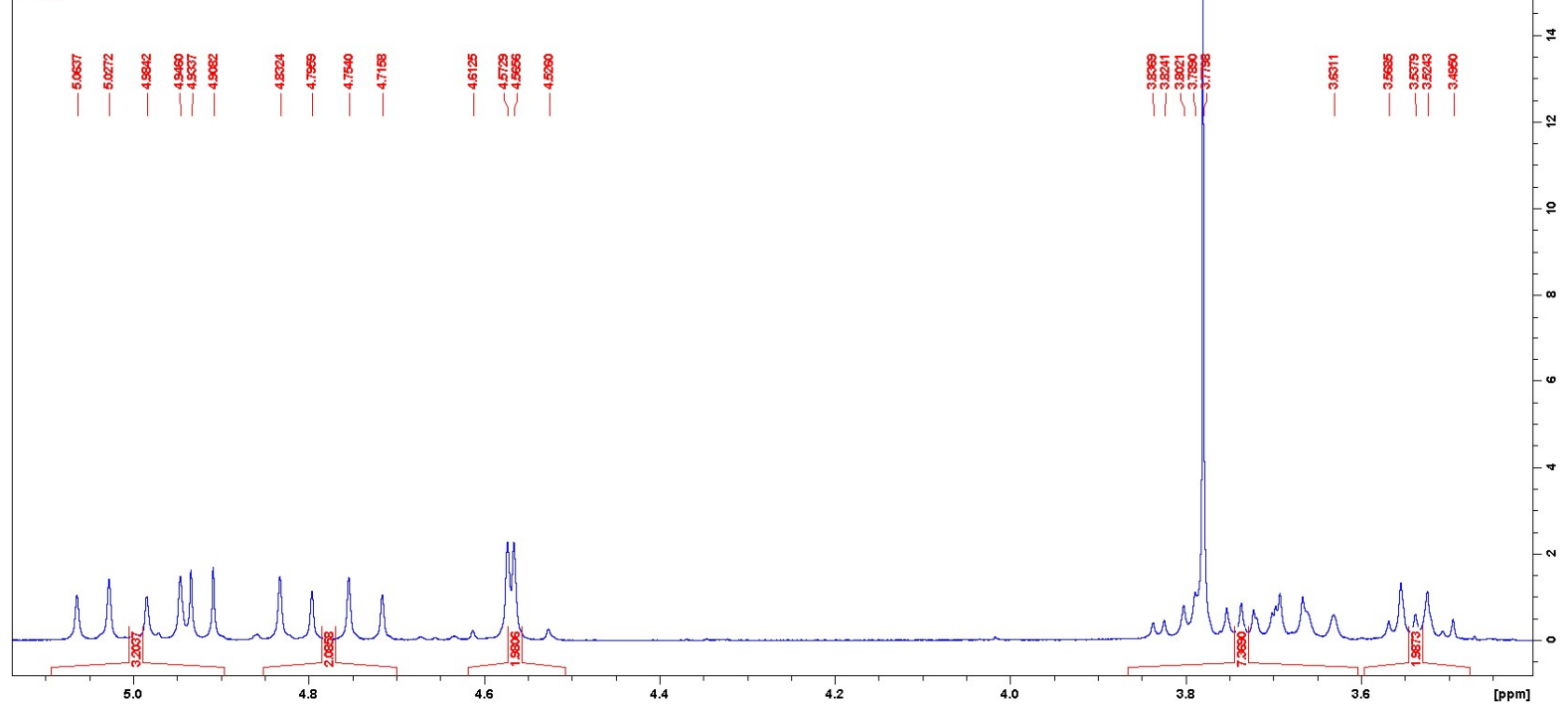
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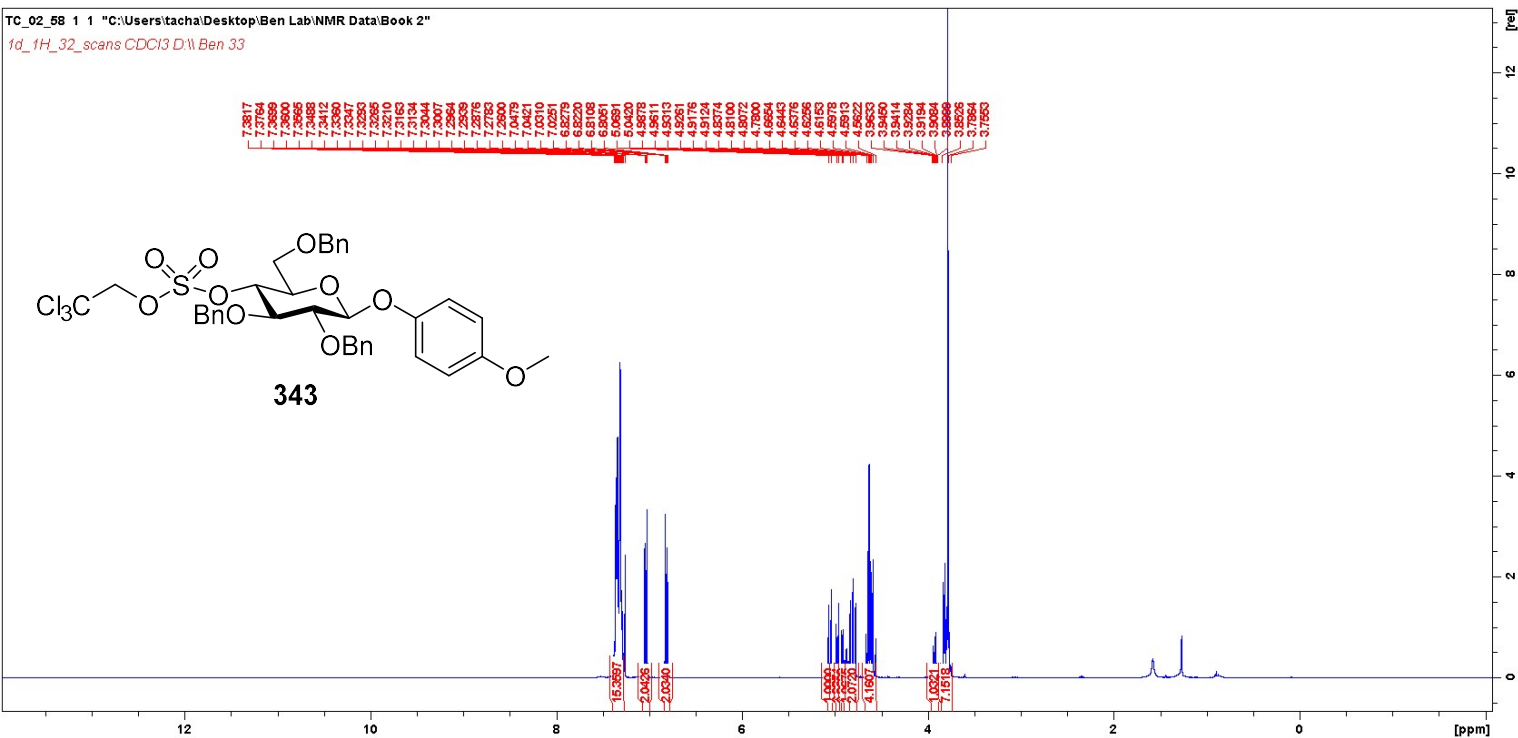
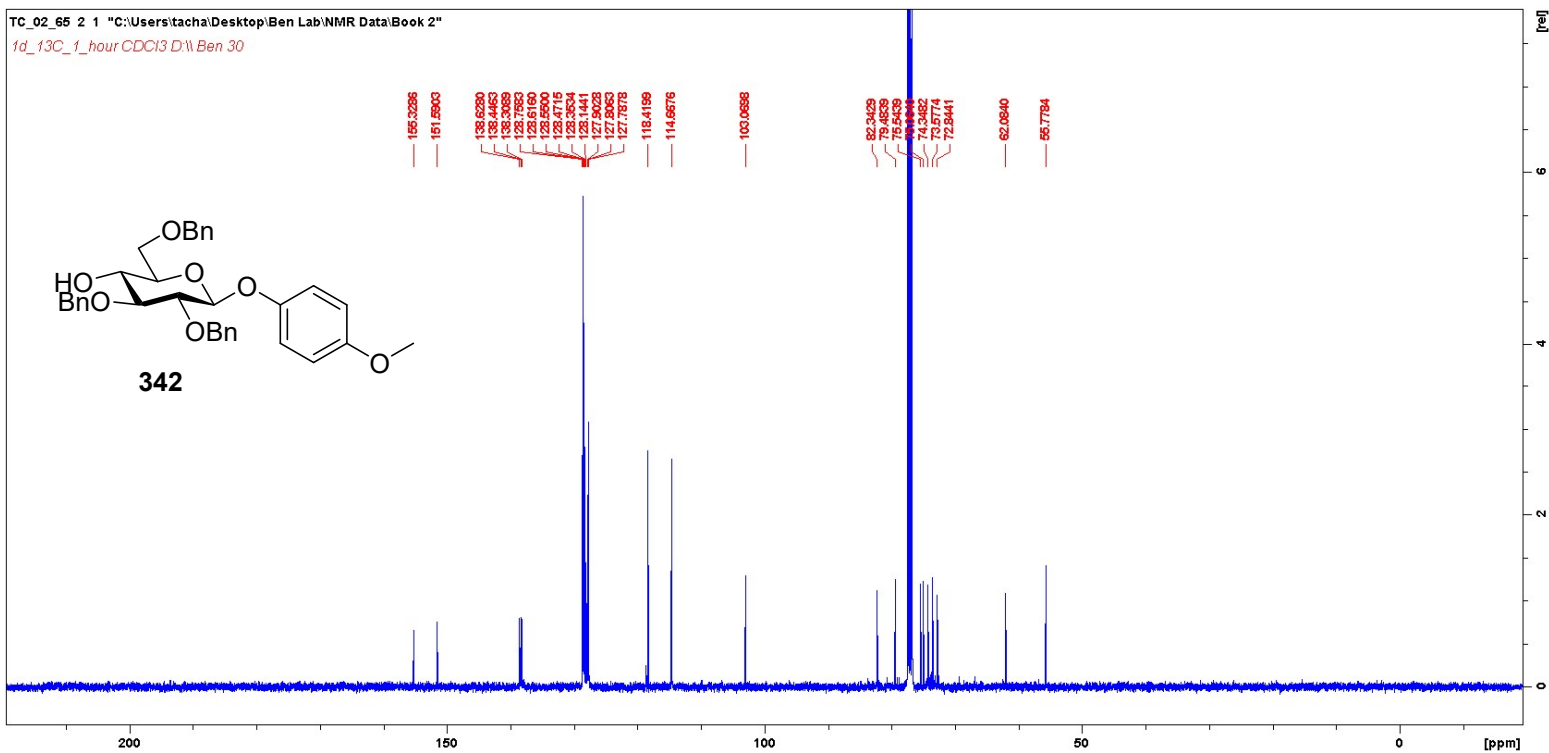
¹H NMR

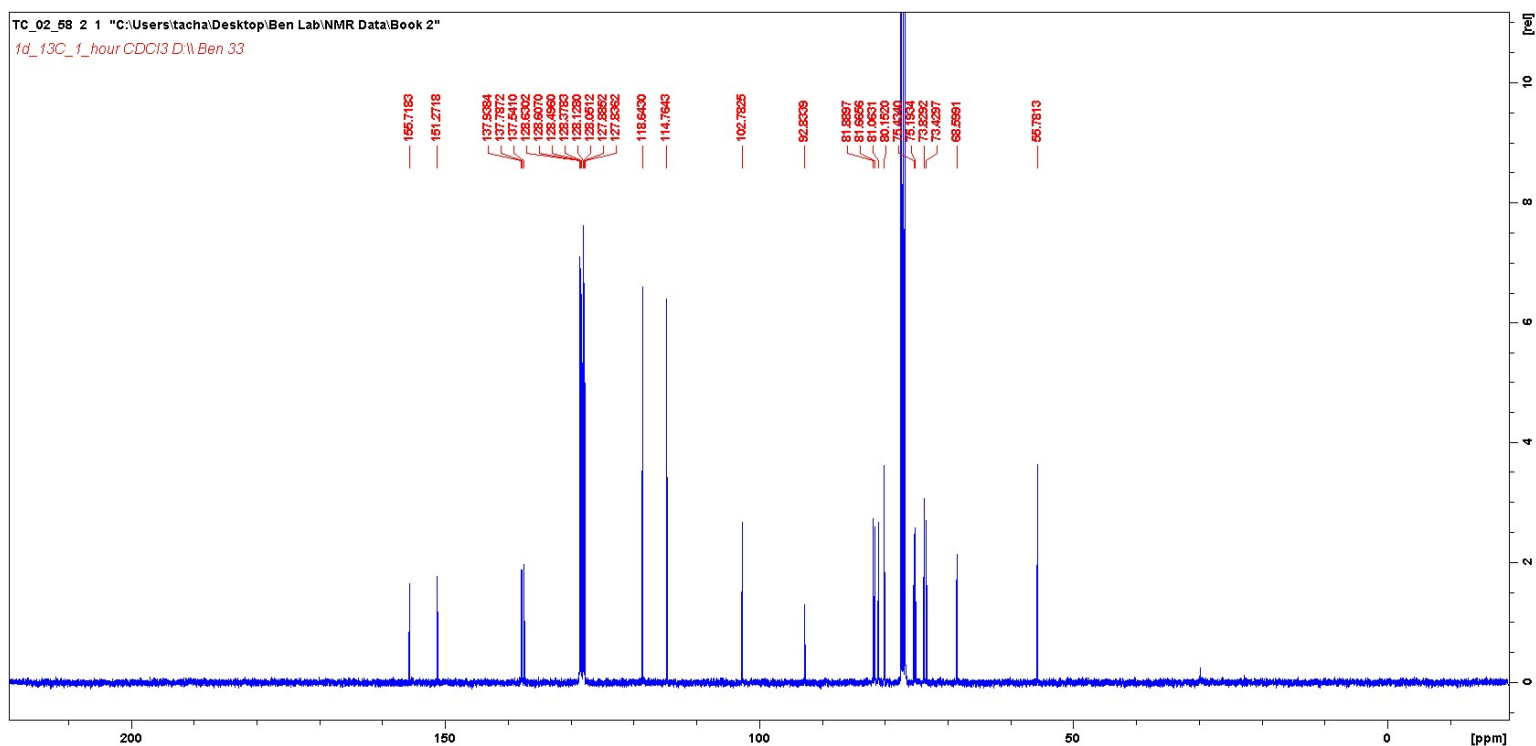
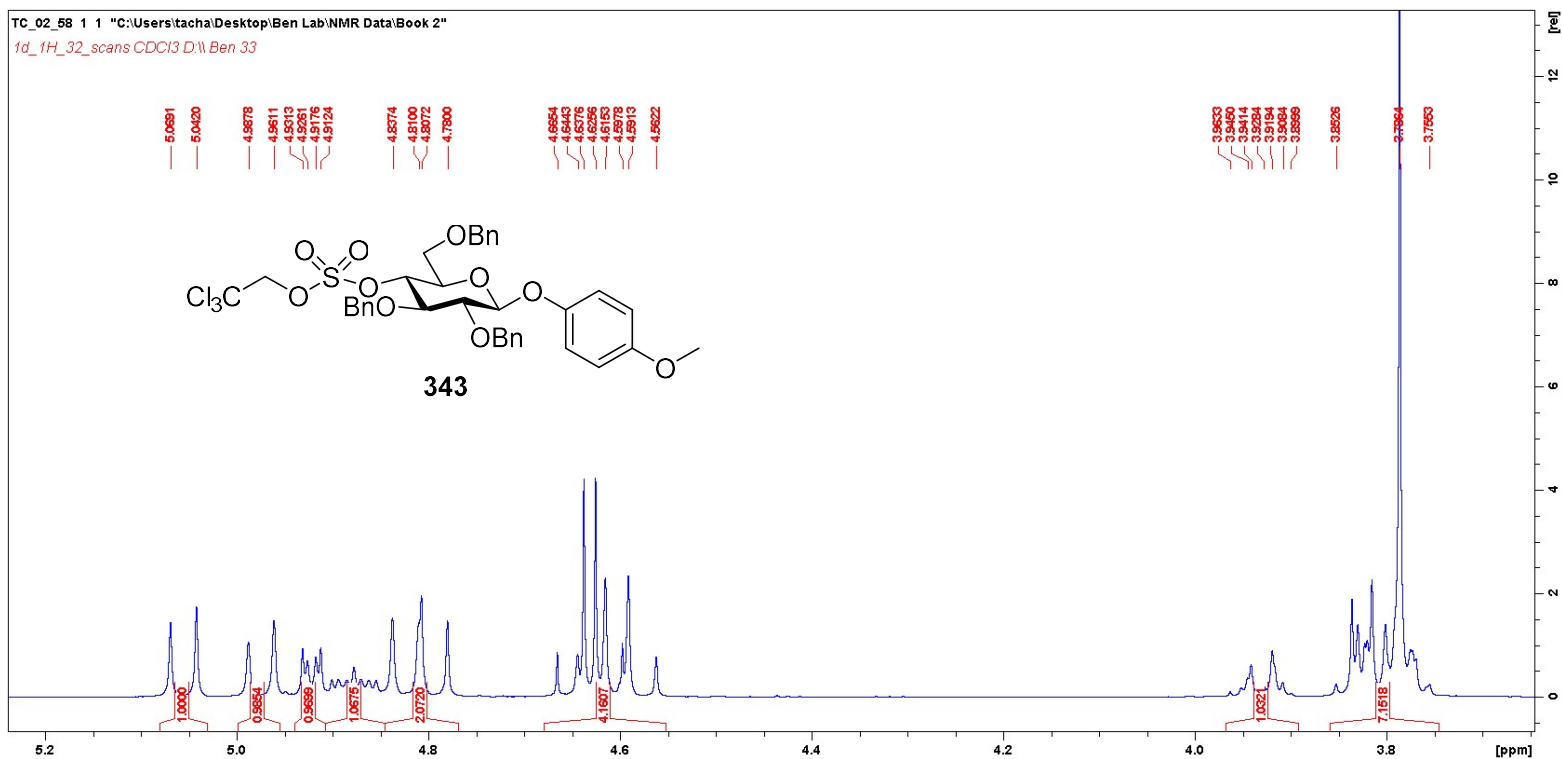


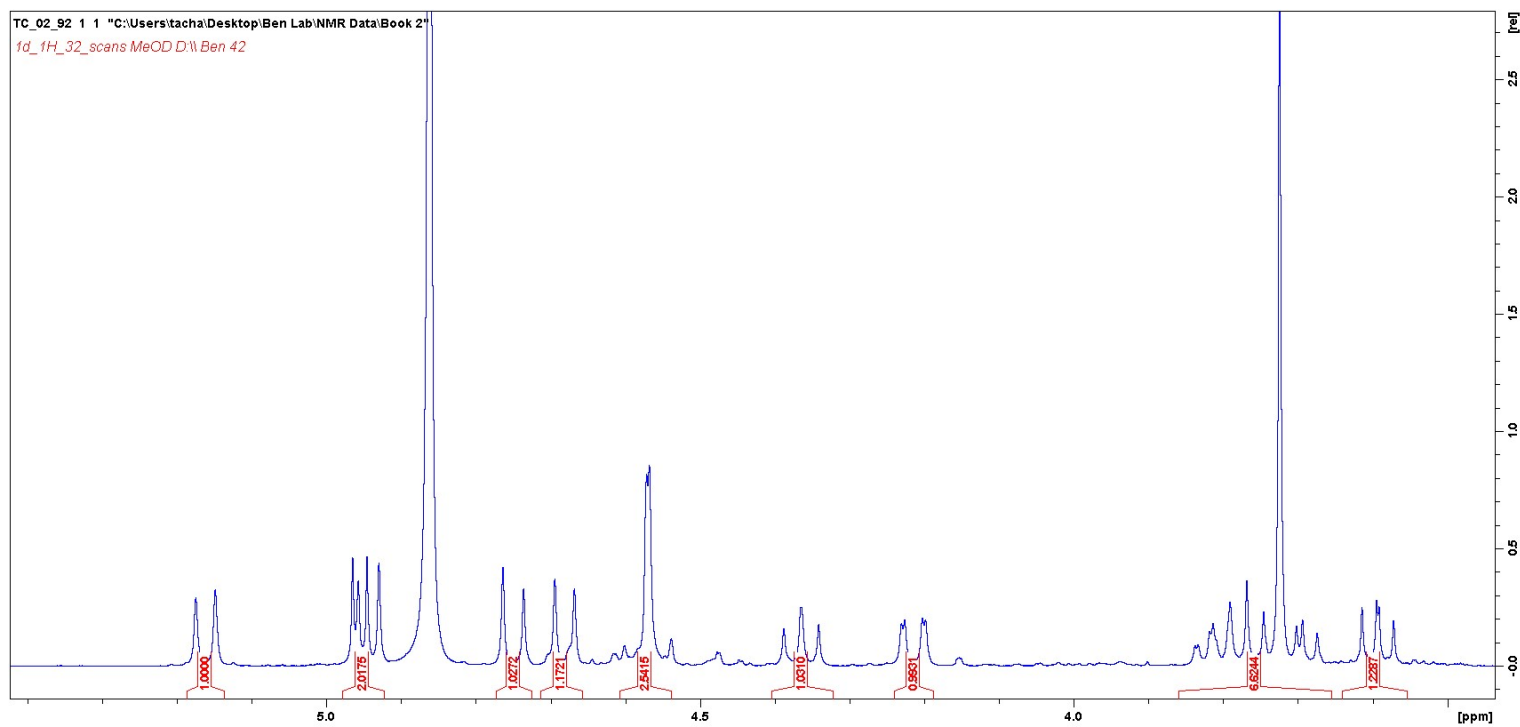
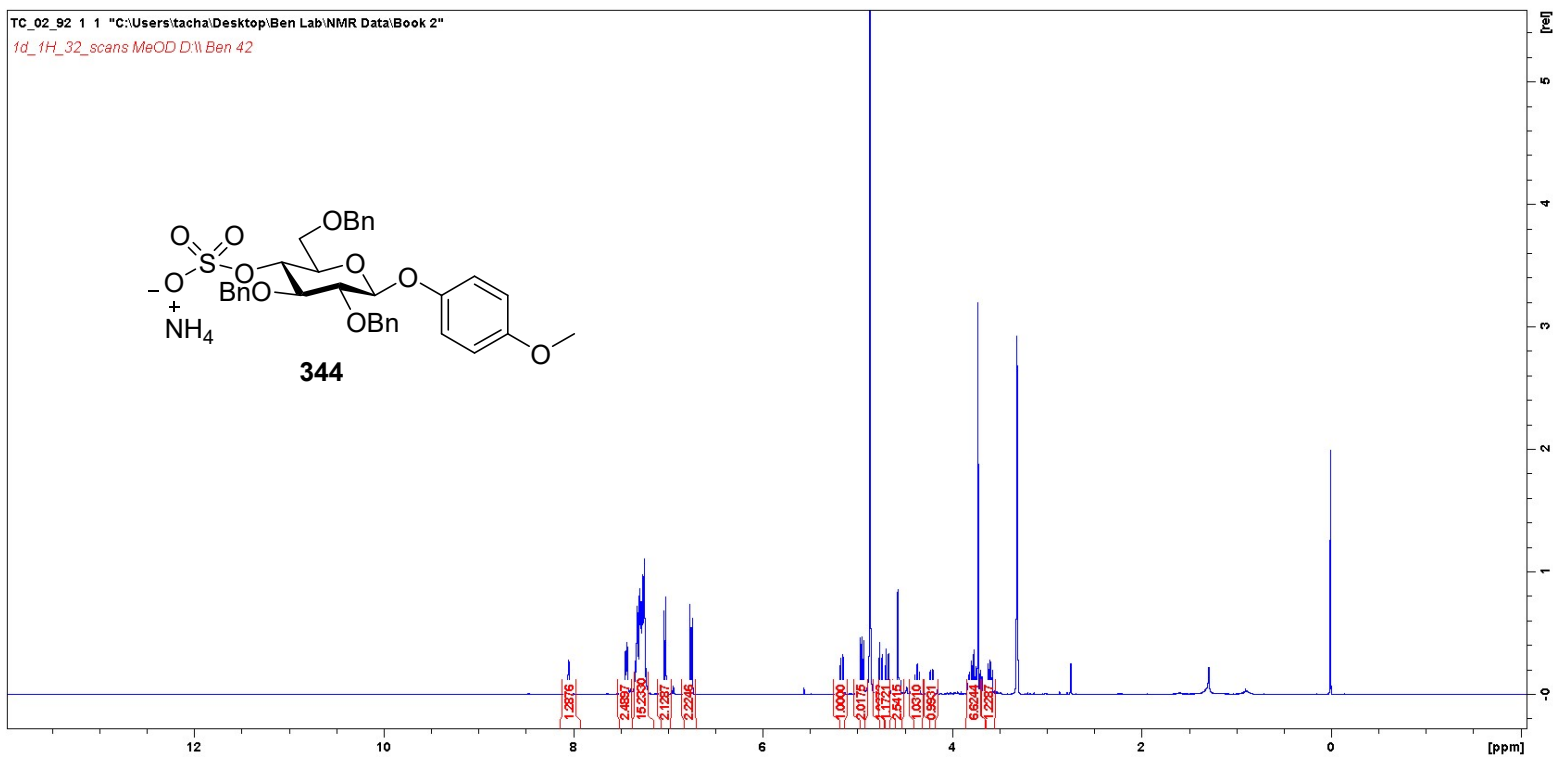
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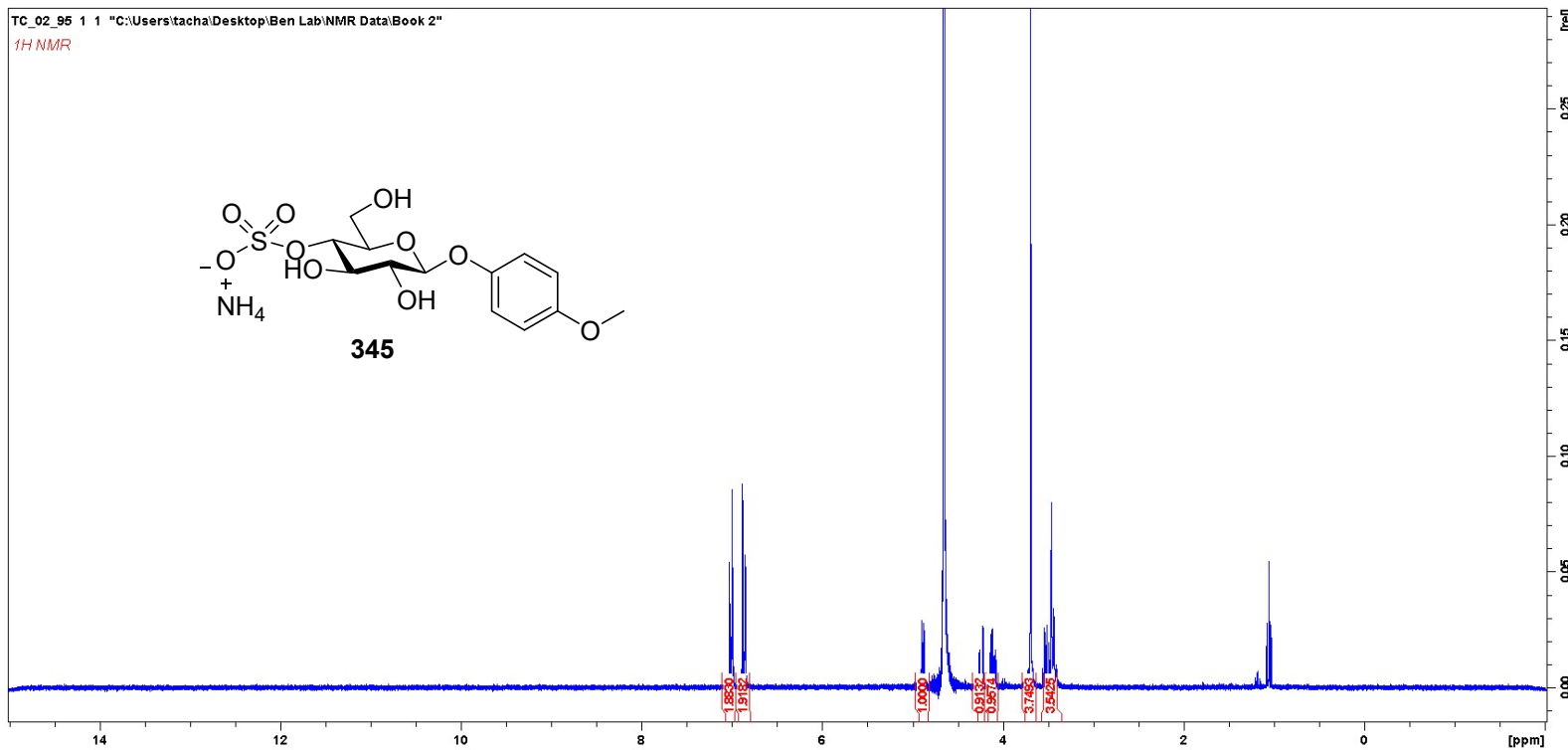
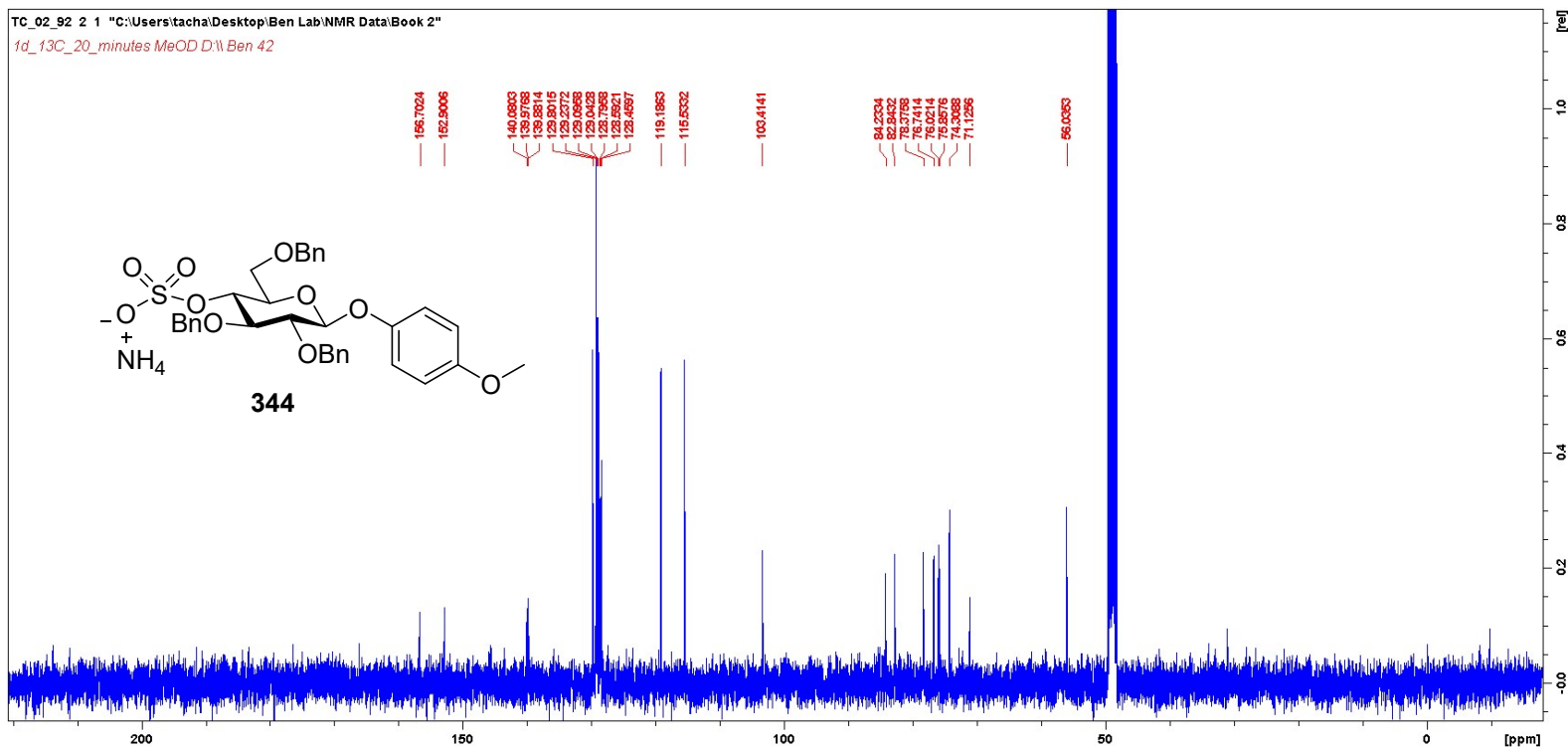
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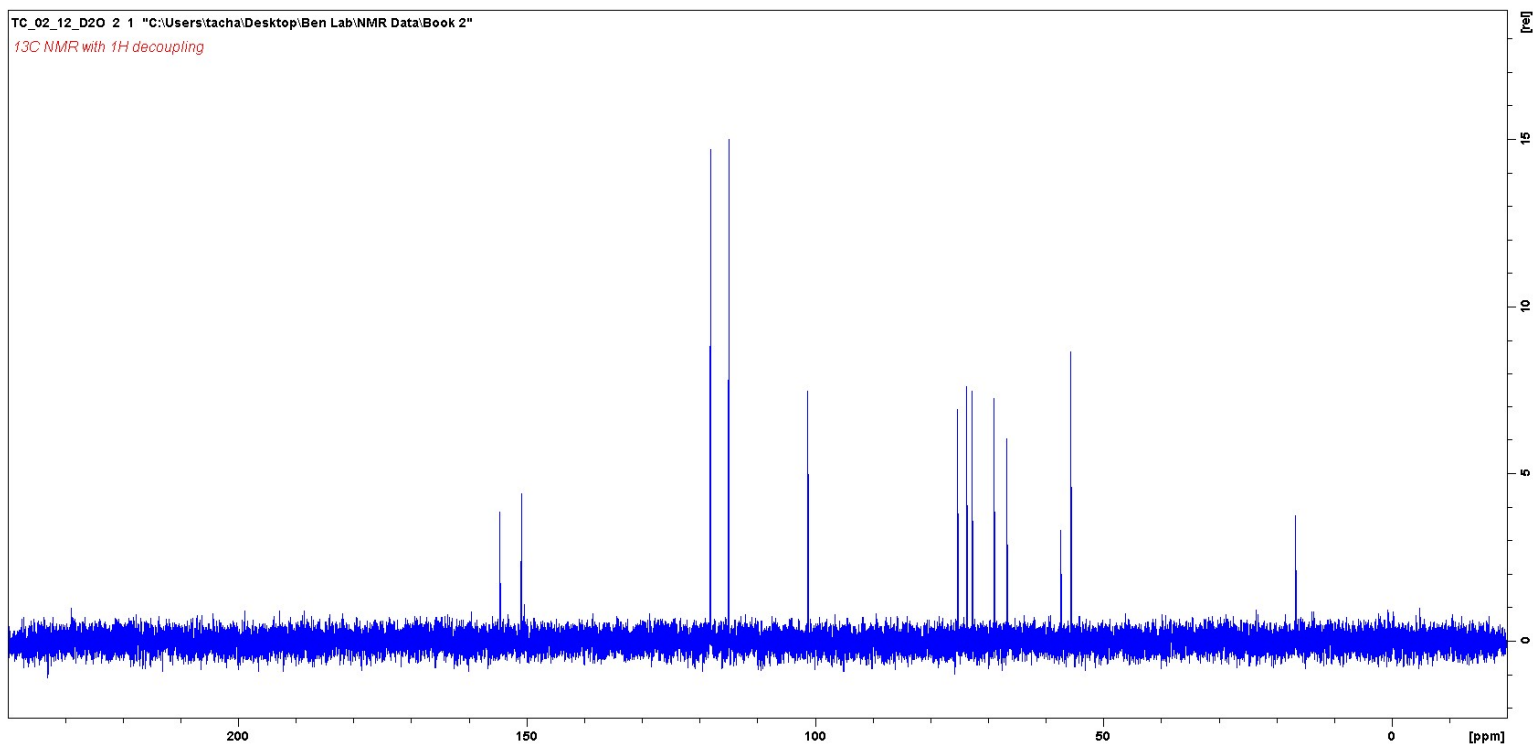
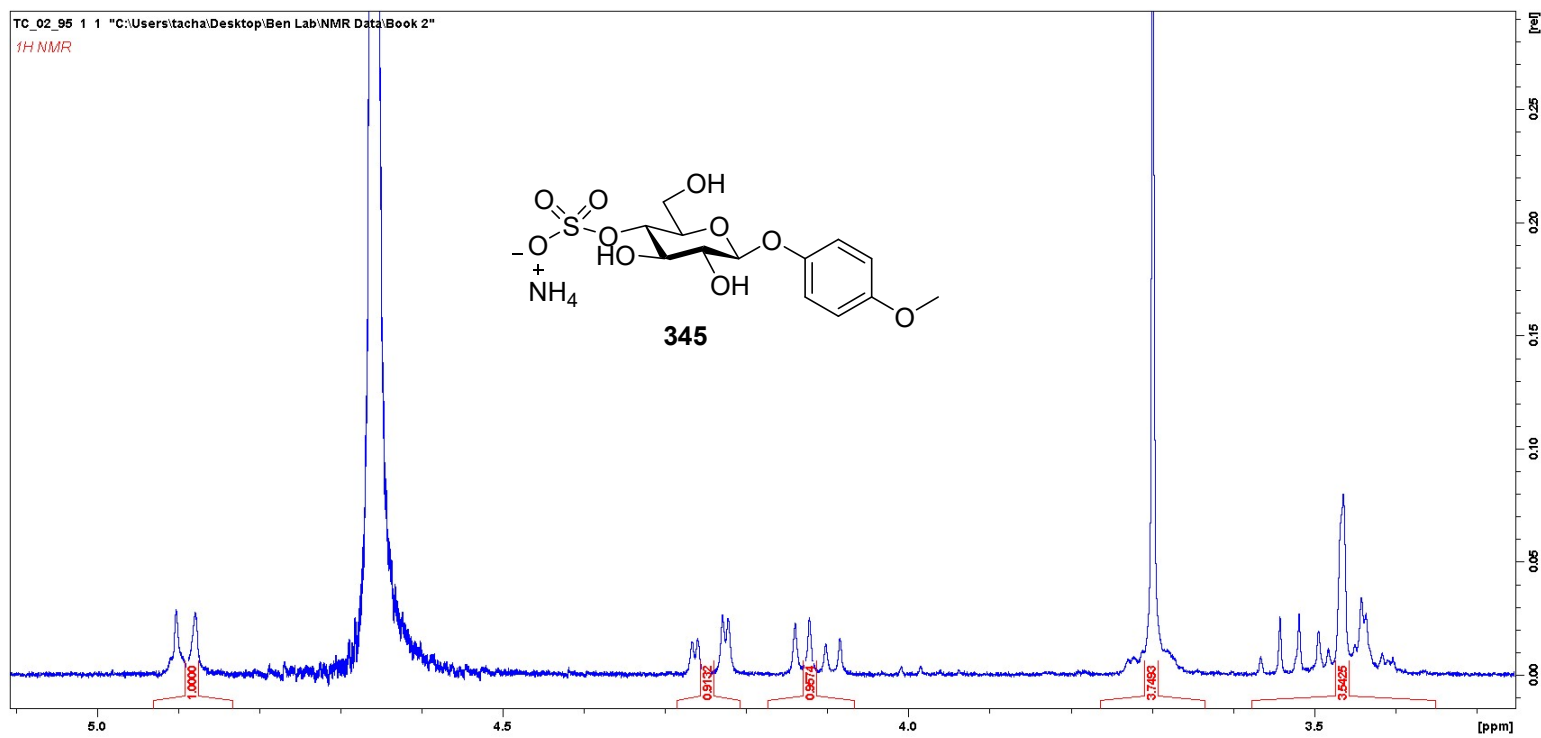


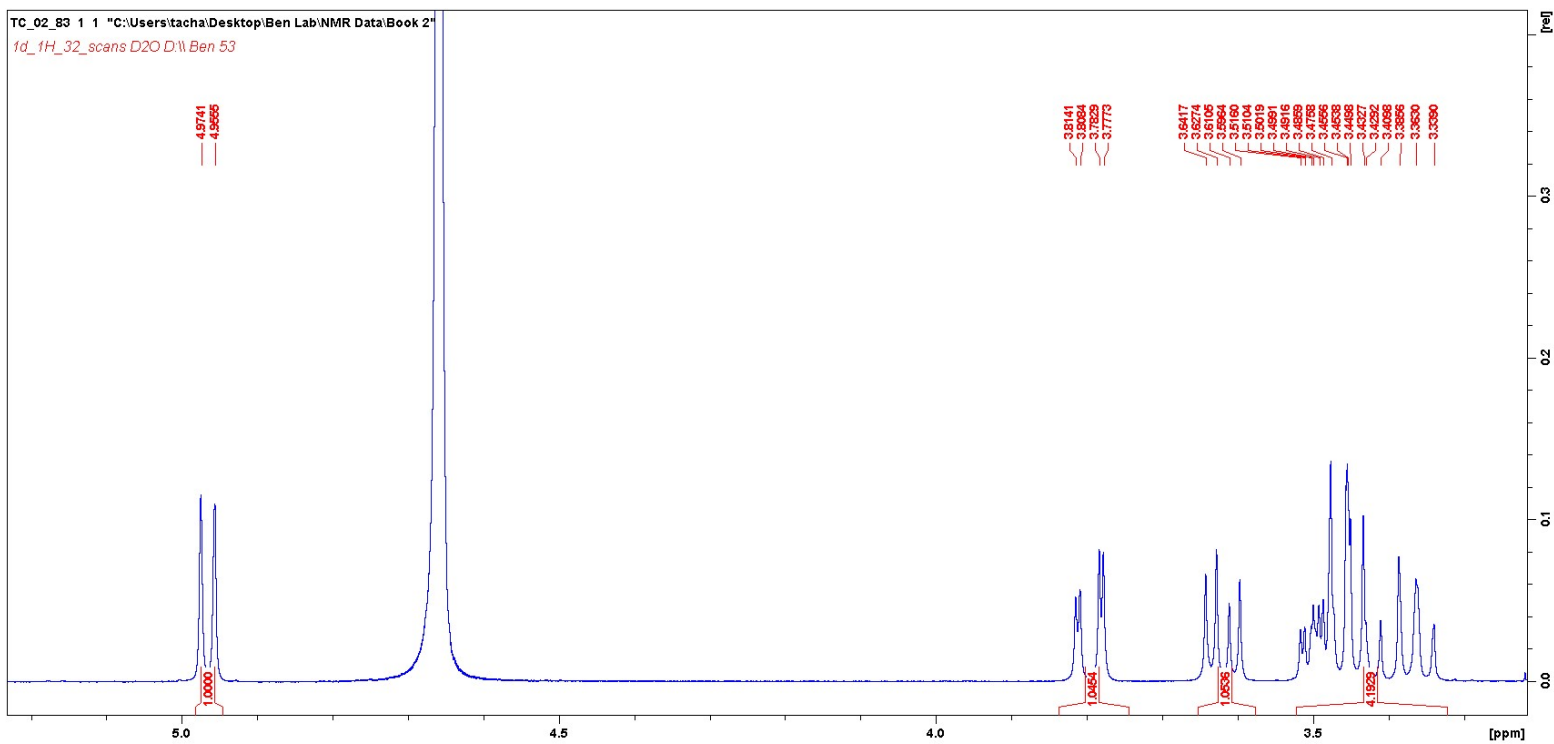
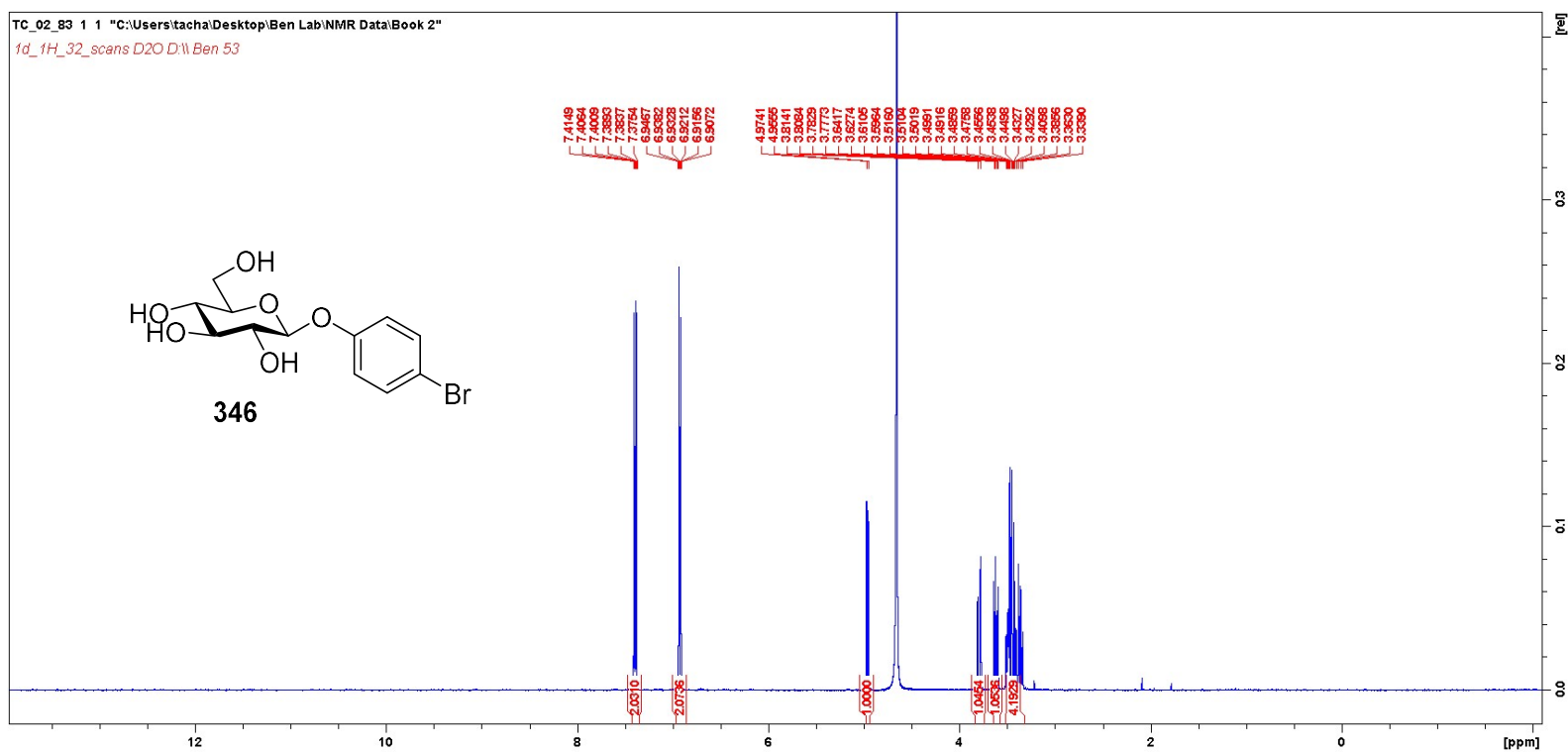


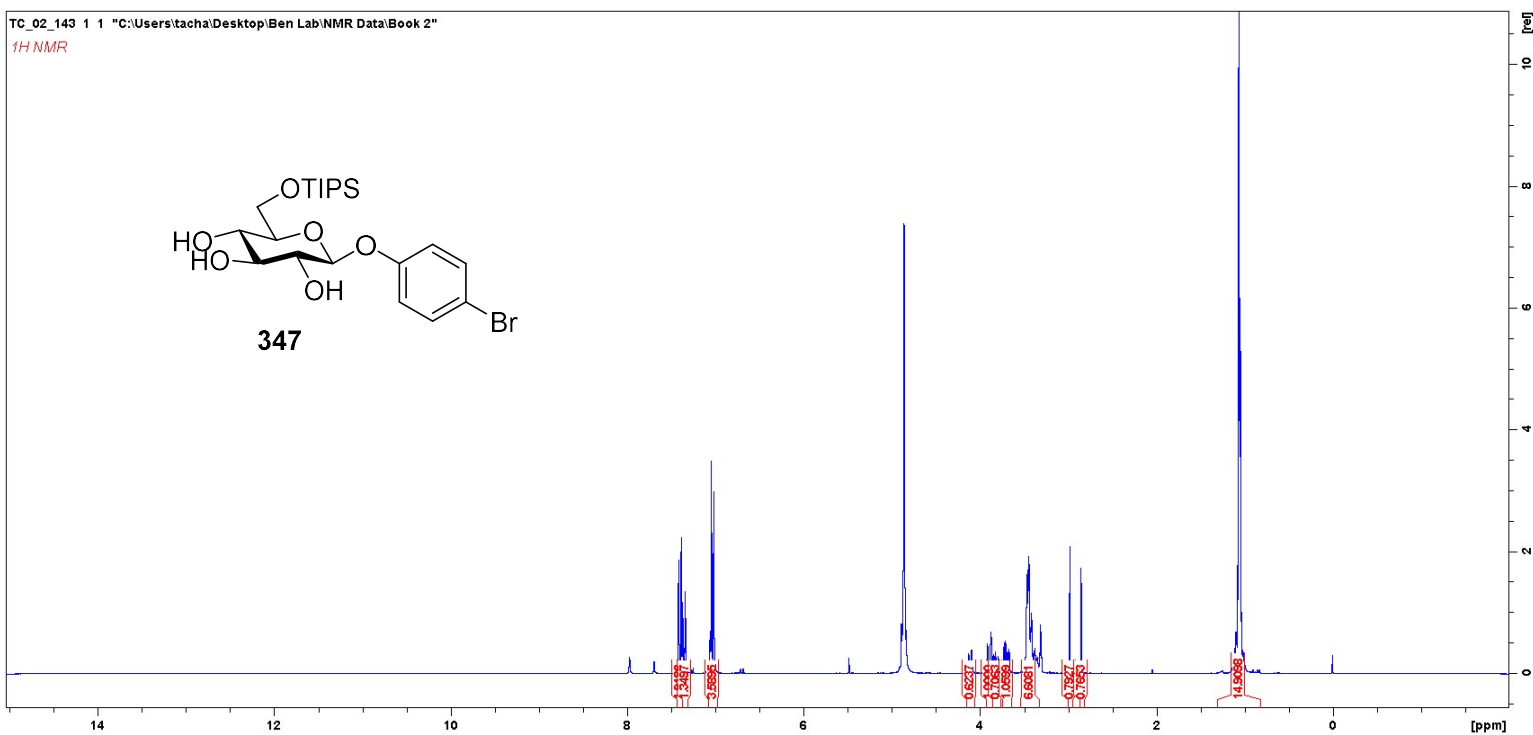
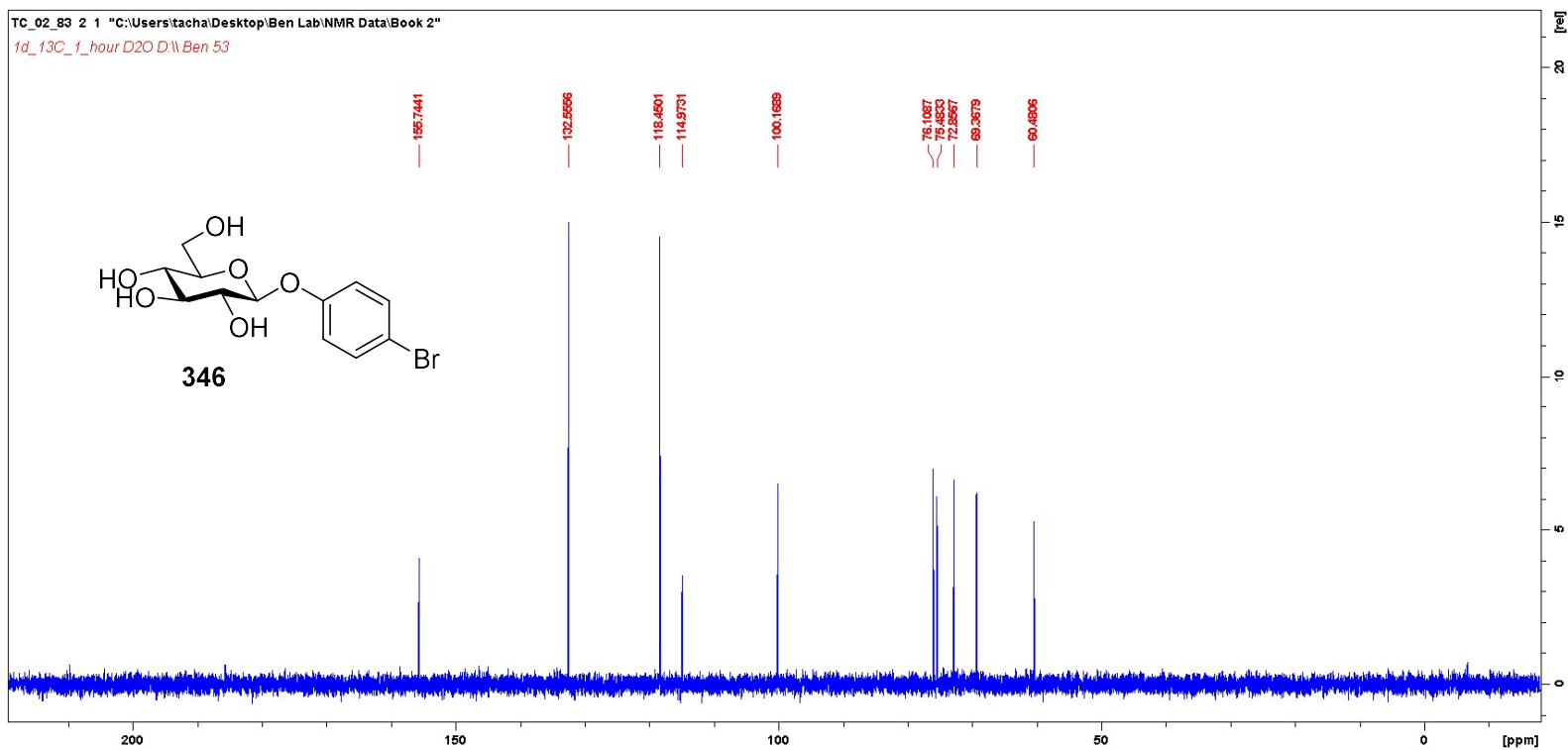






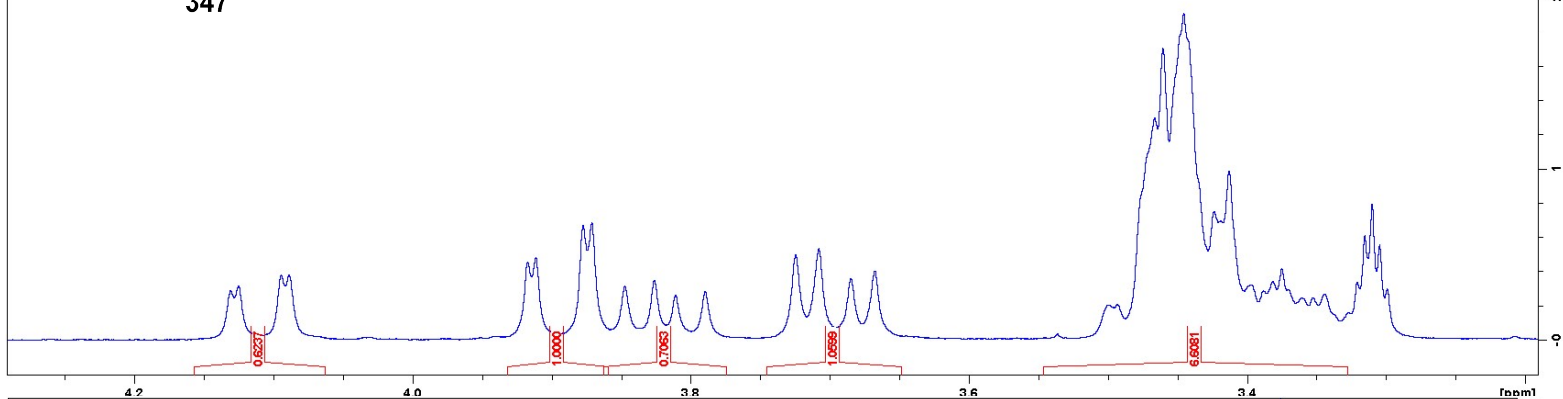
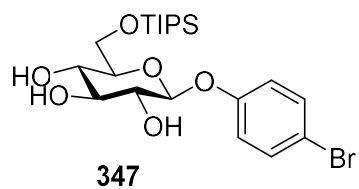






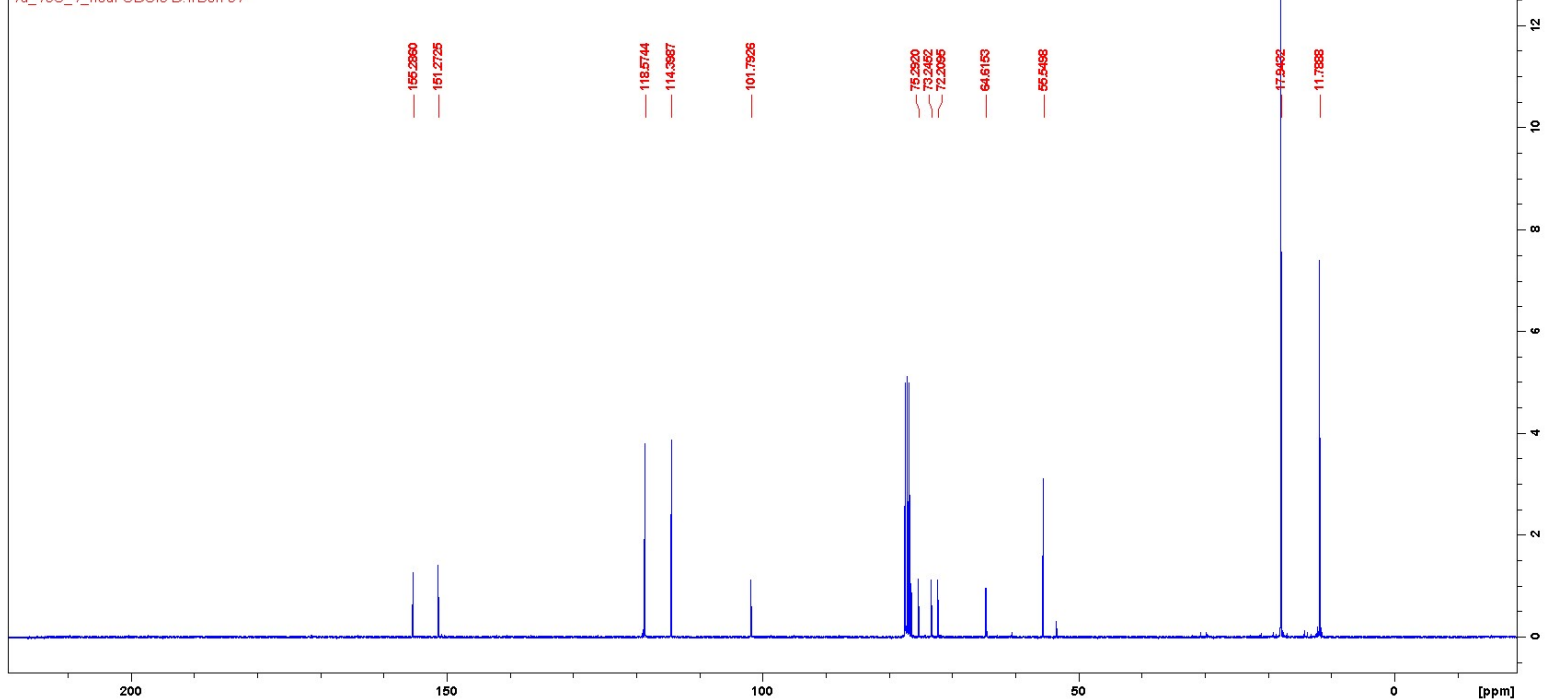
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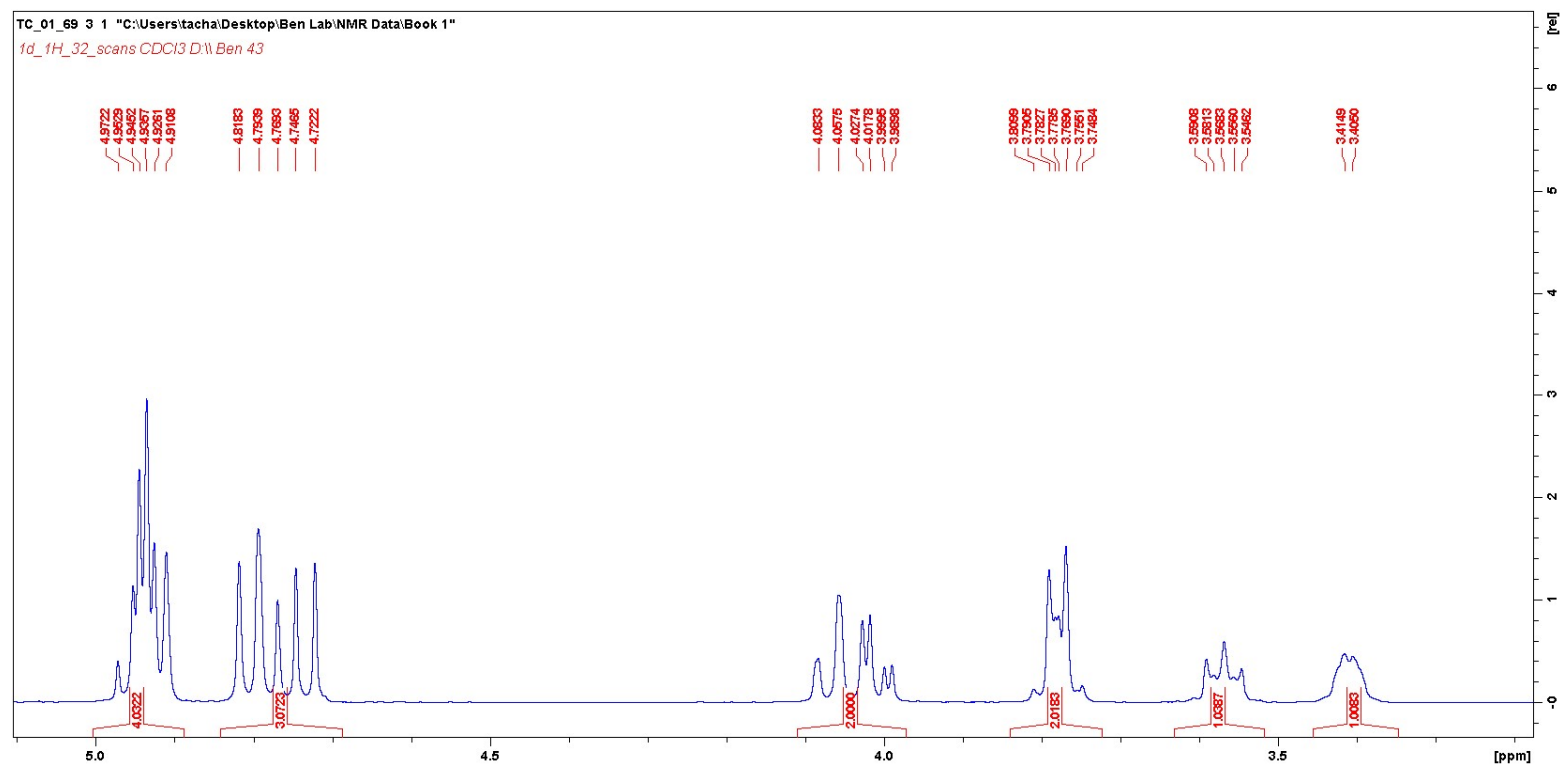
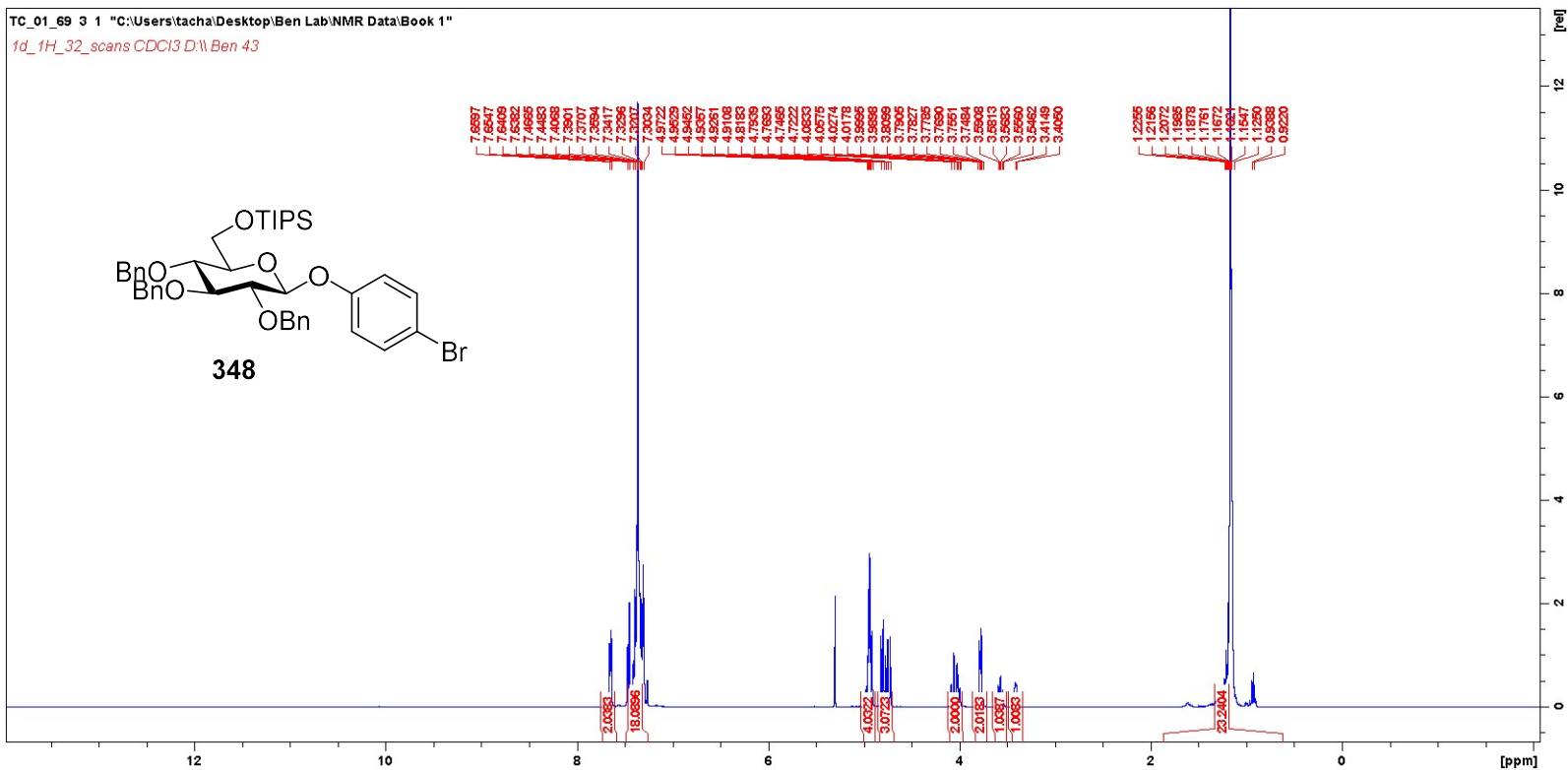
¹H NMR

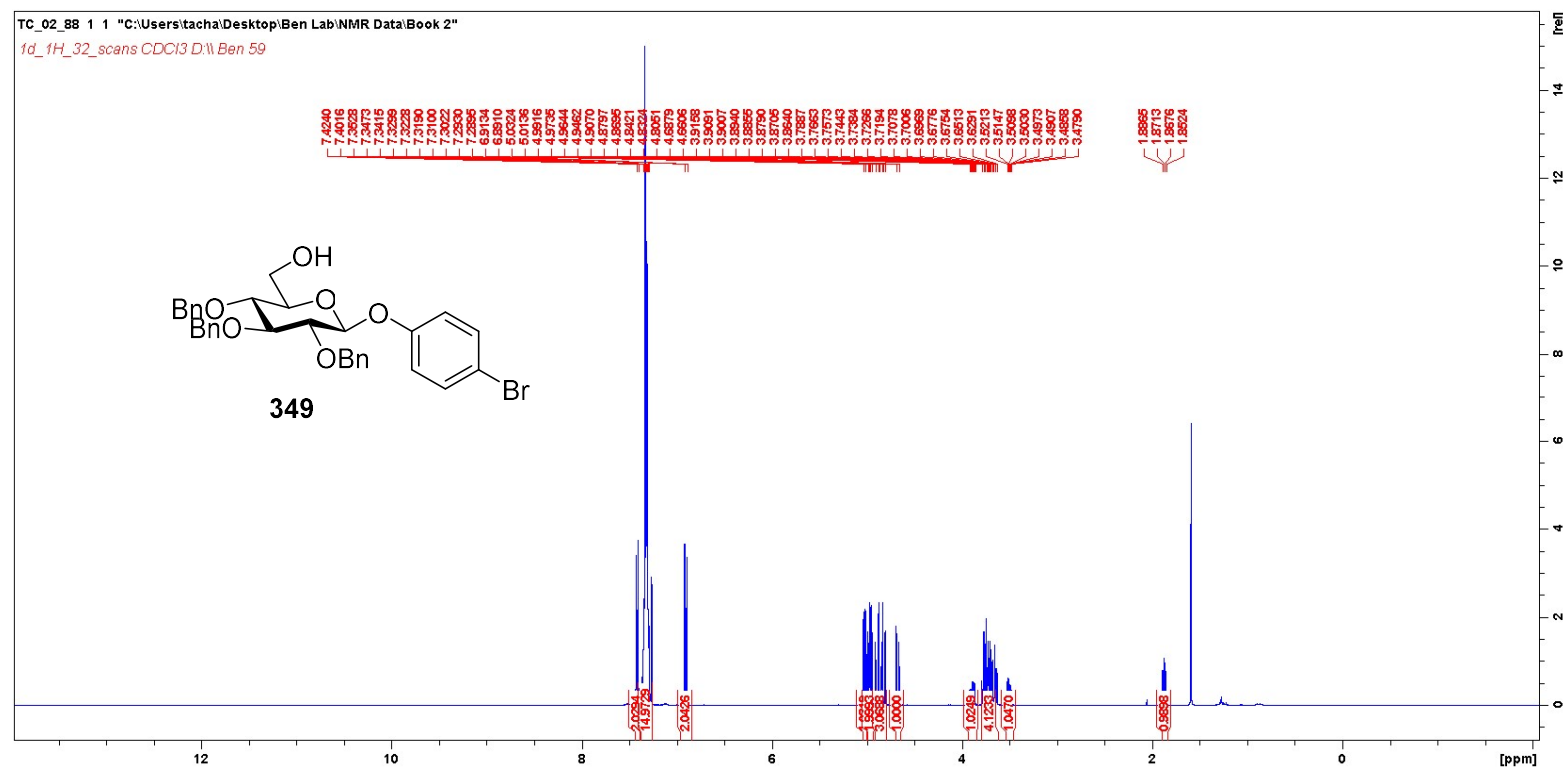
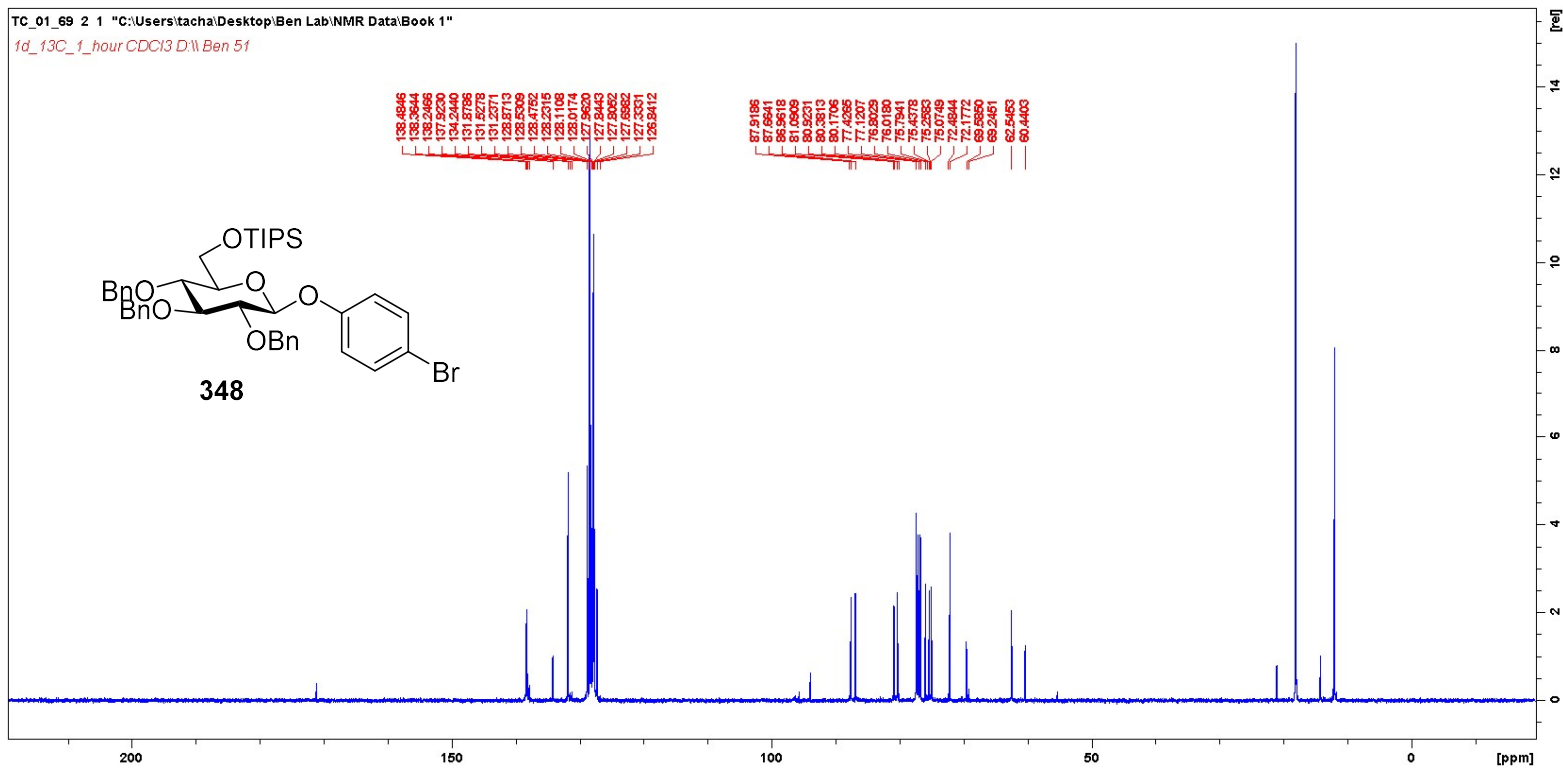


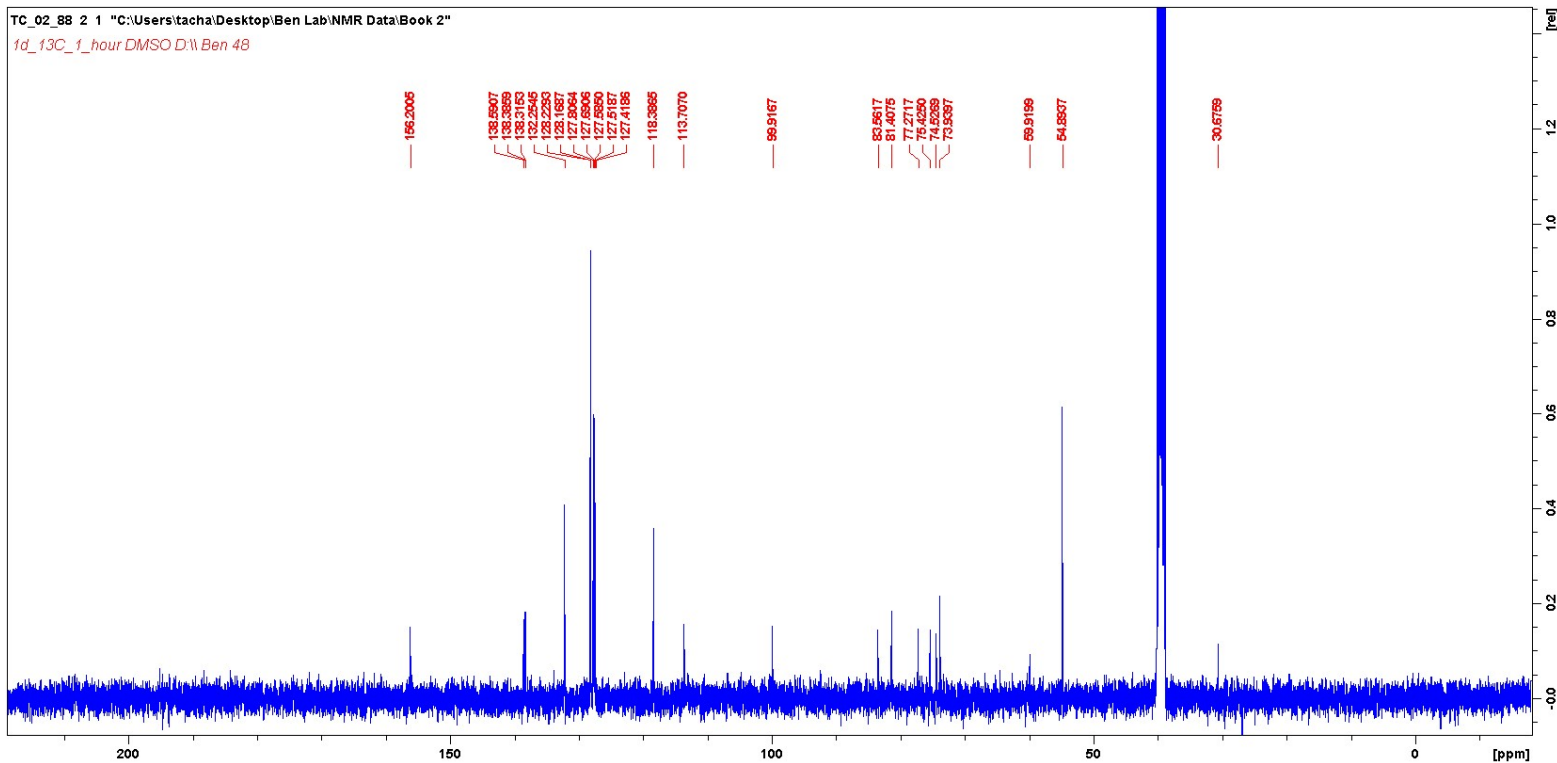
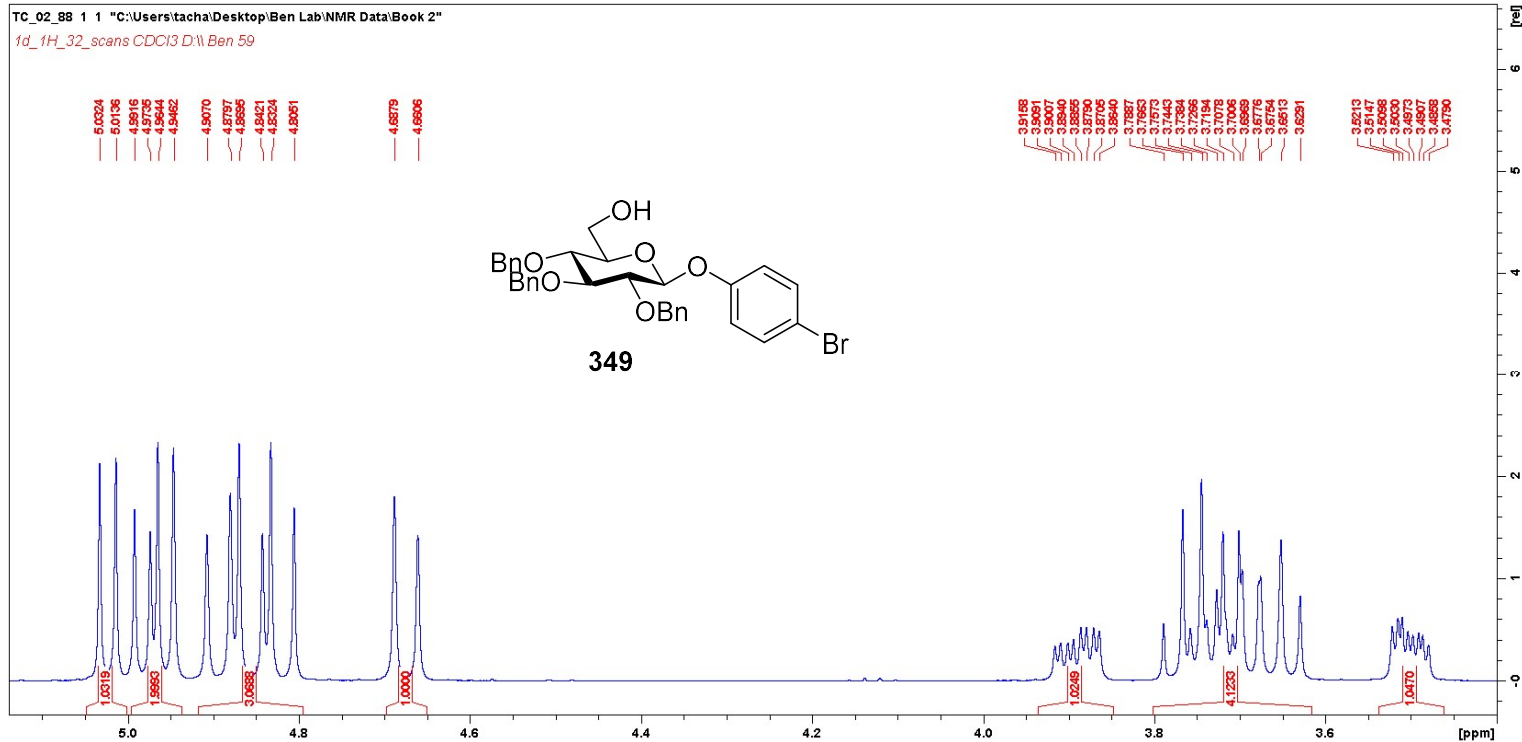
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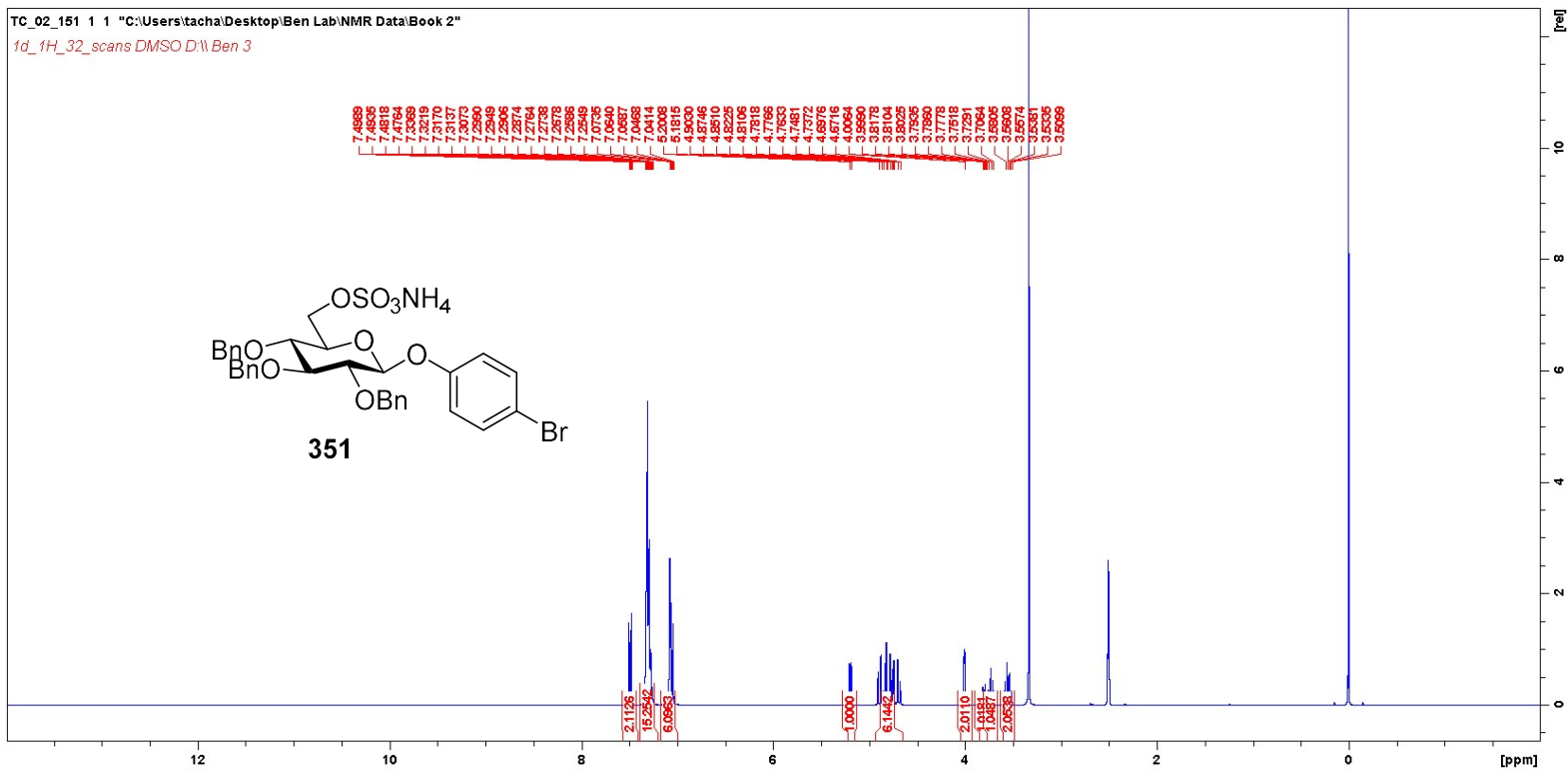
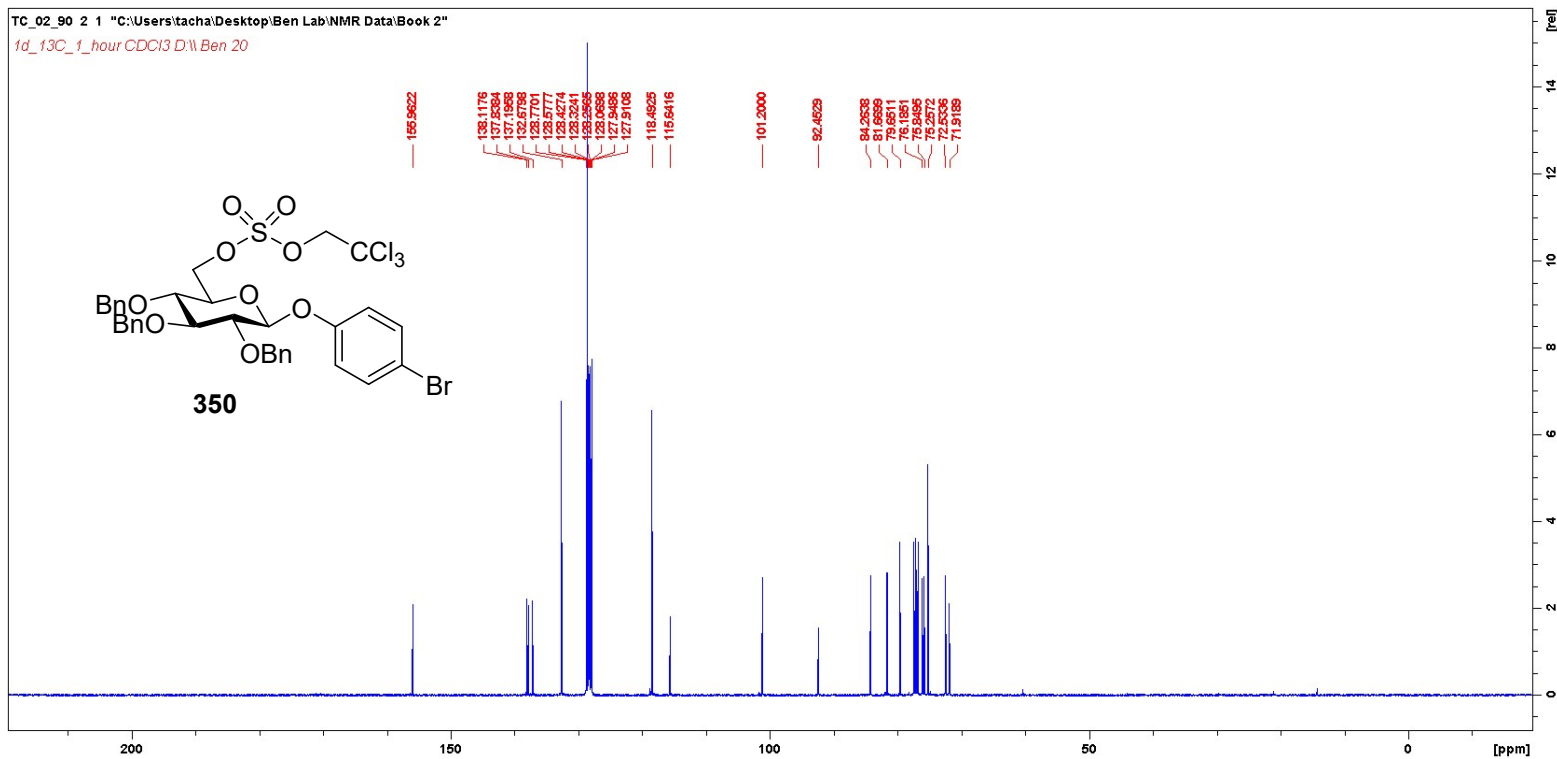
1d_13C_1_hour CDCl3 D\ Ben 31





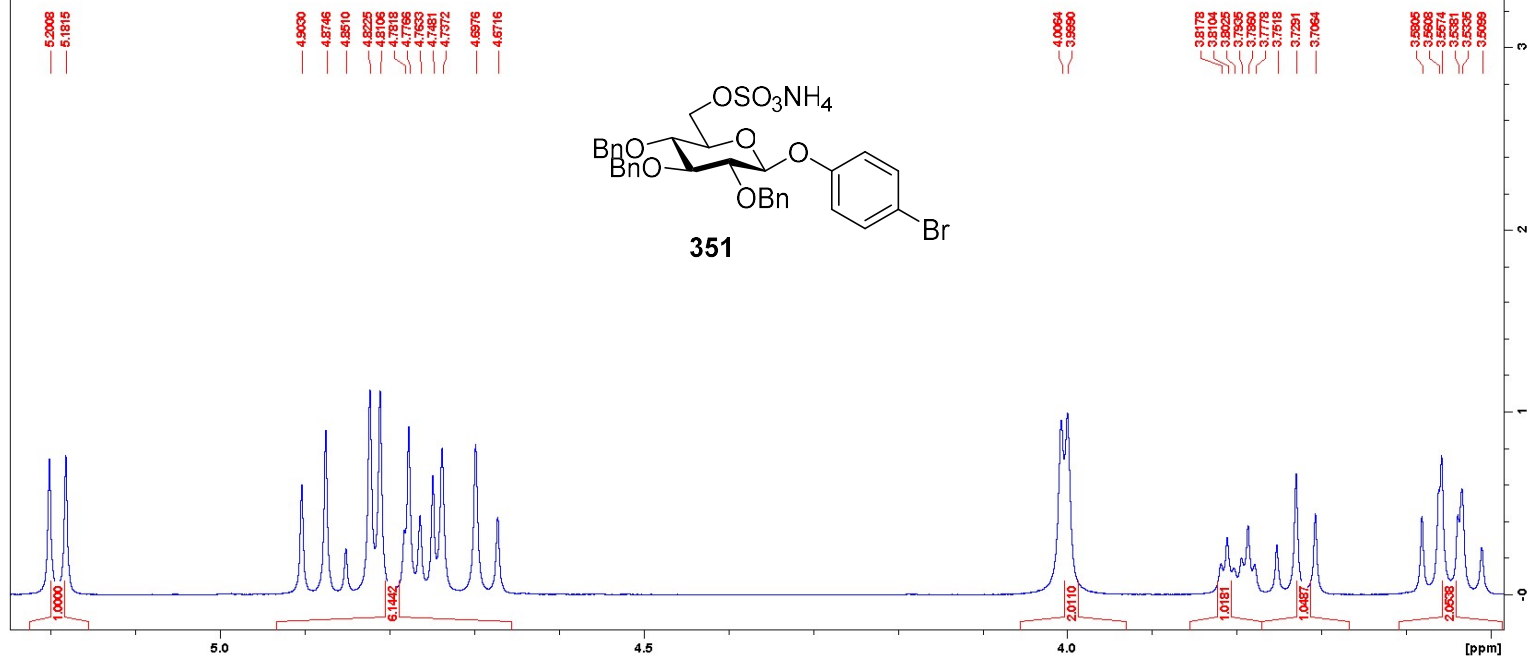






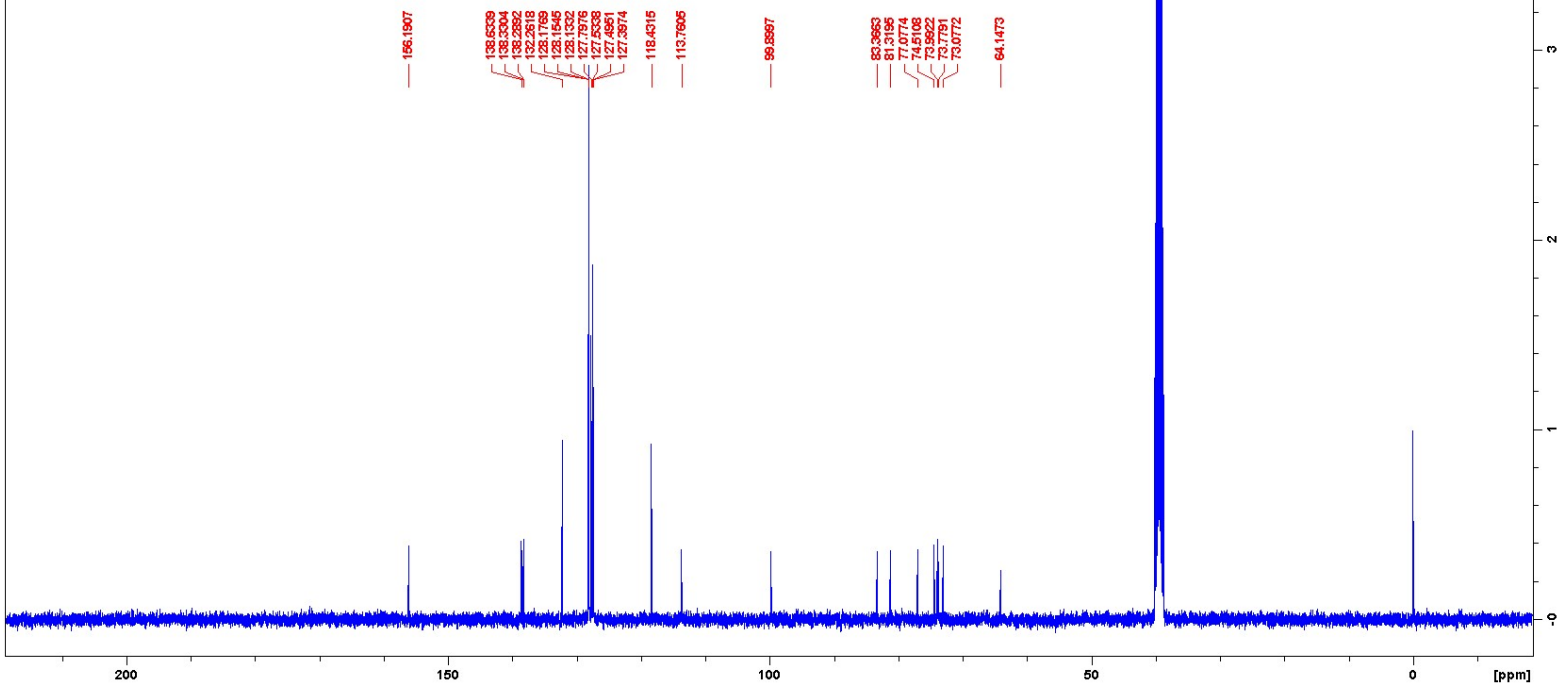
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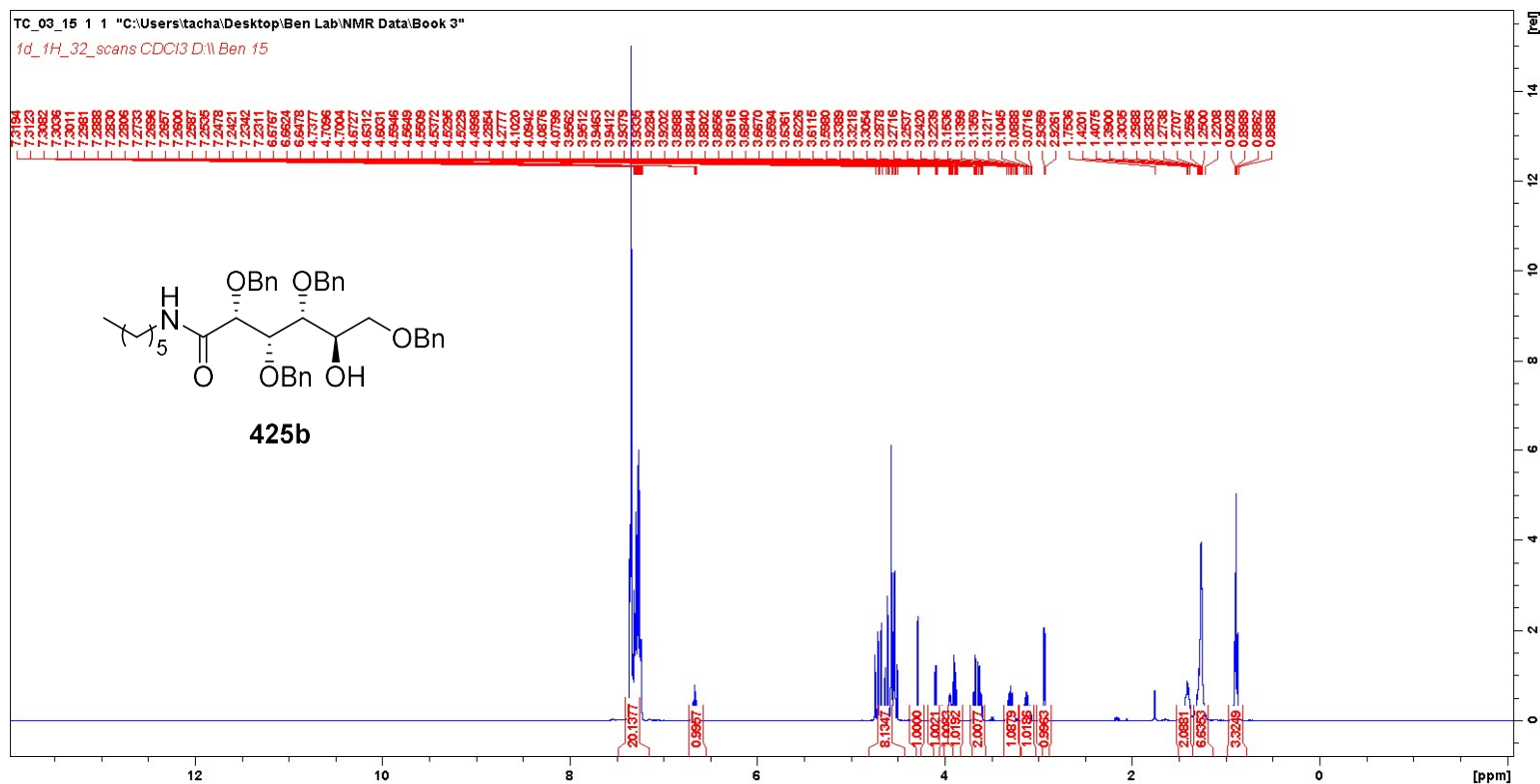
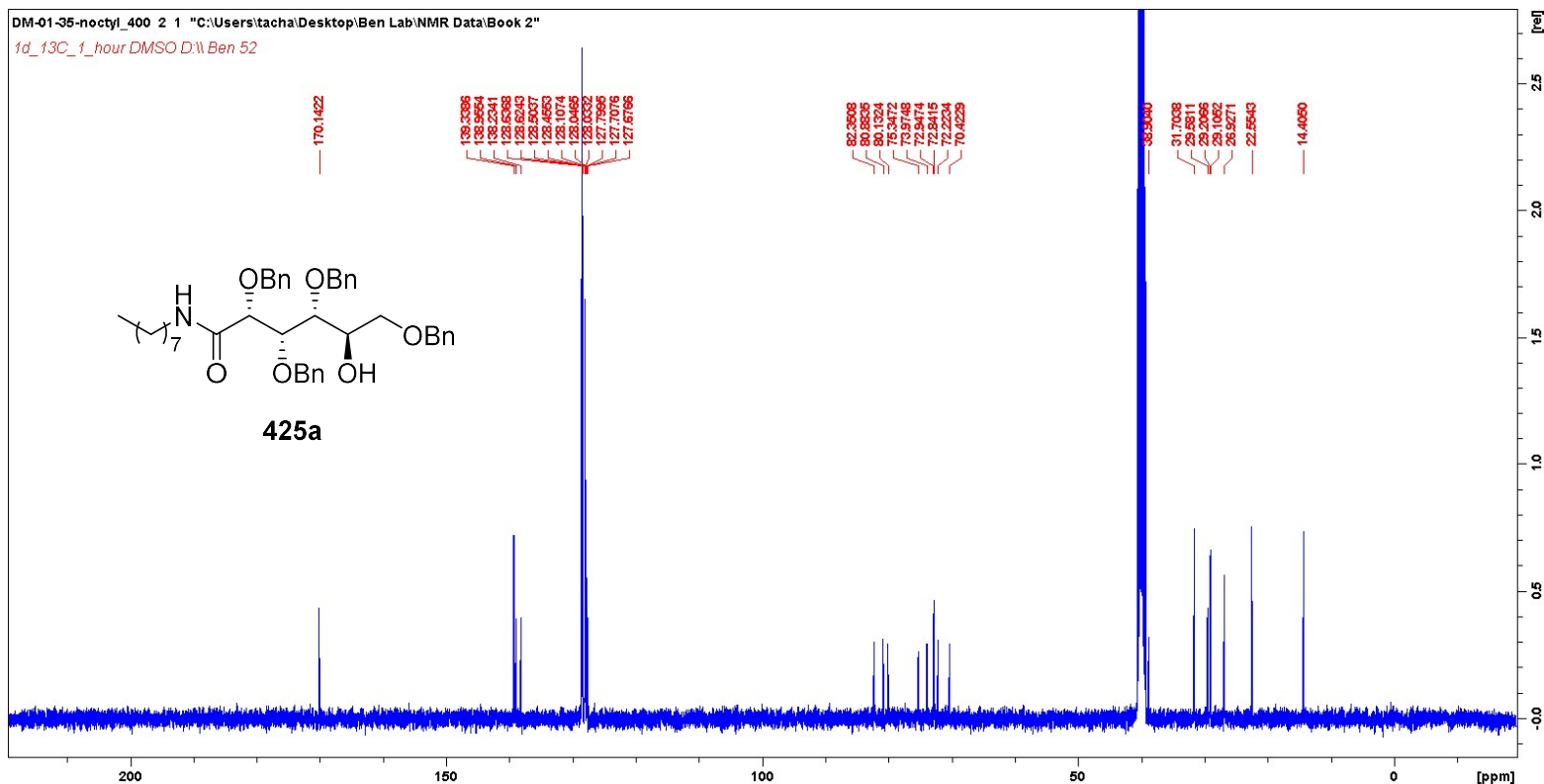
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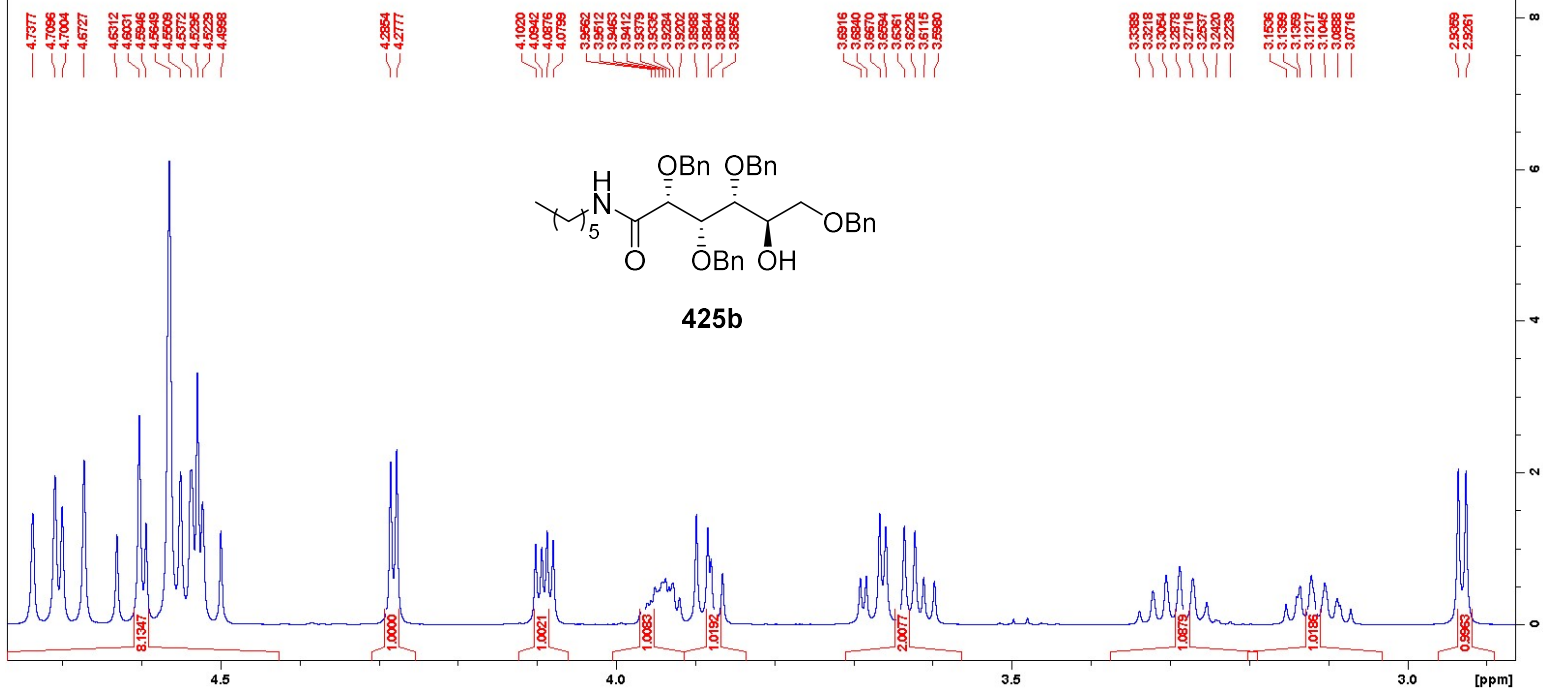
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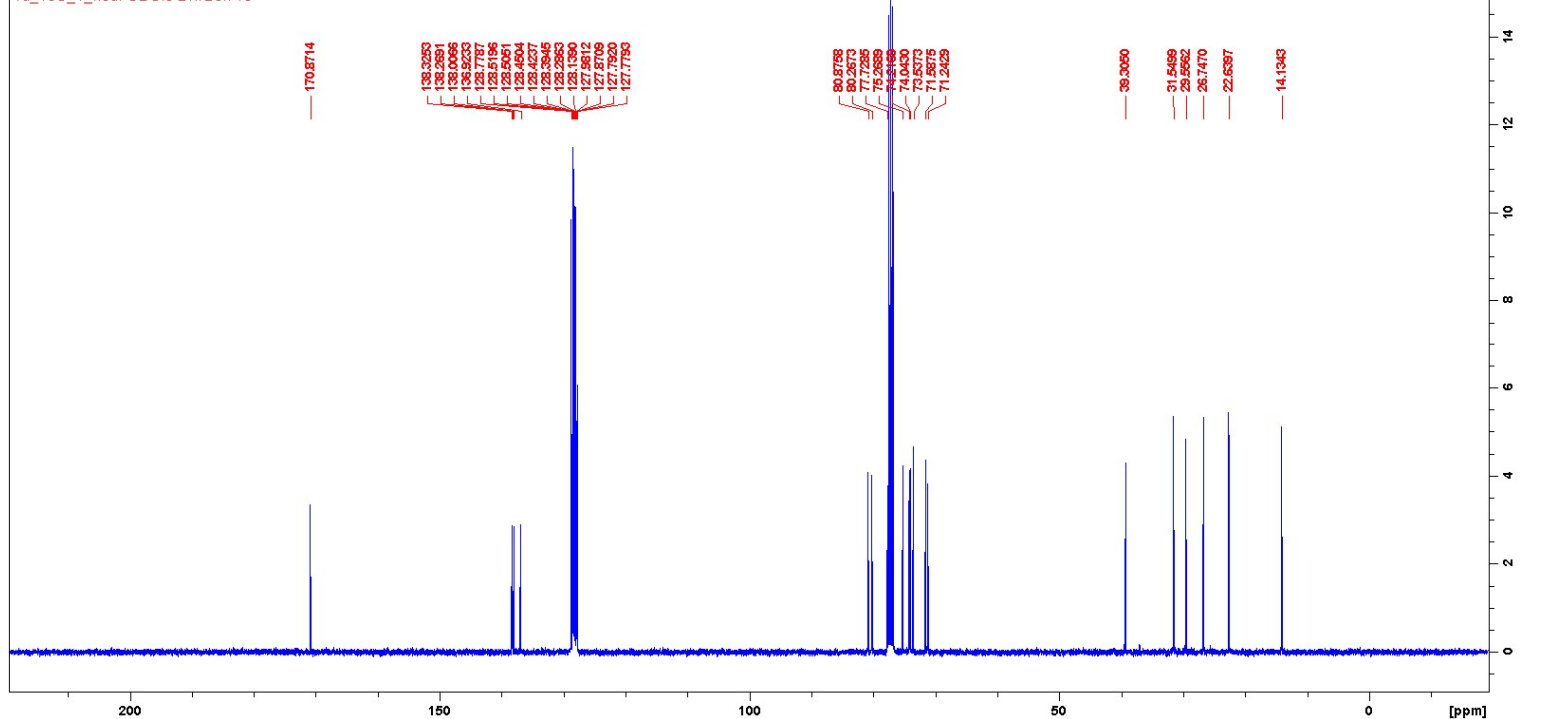
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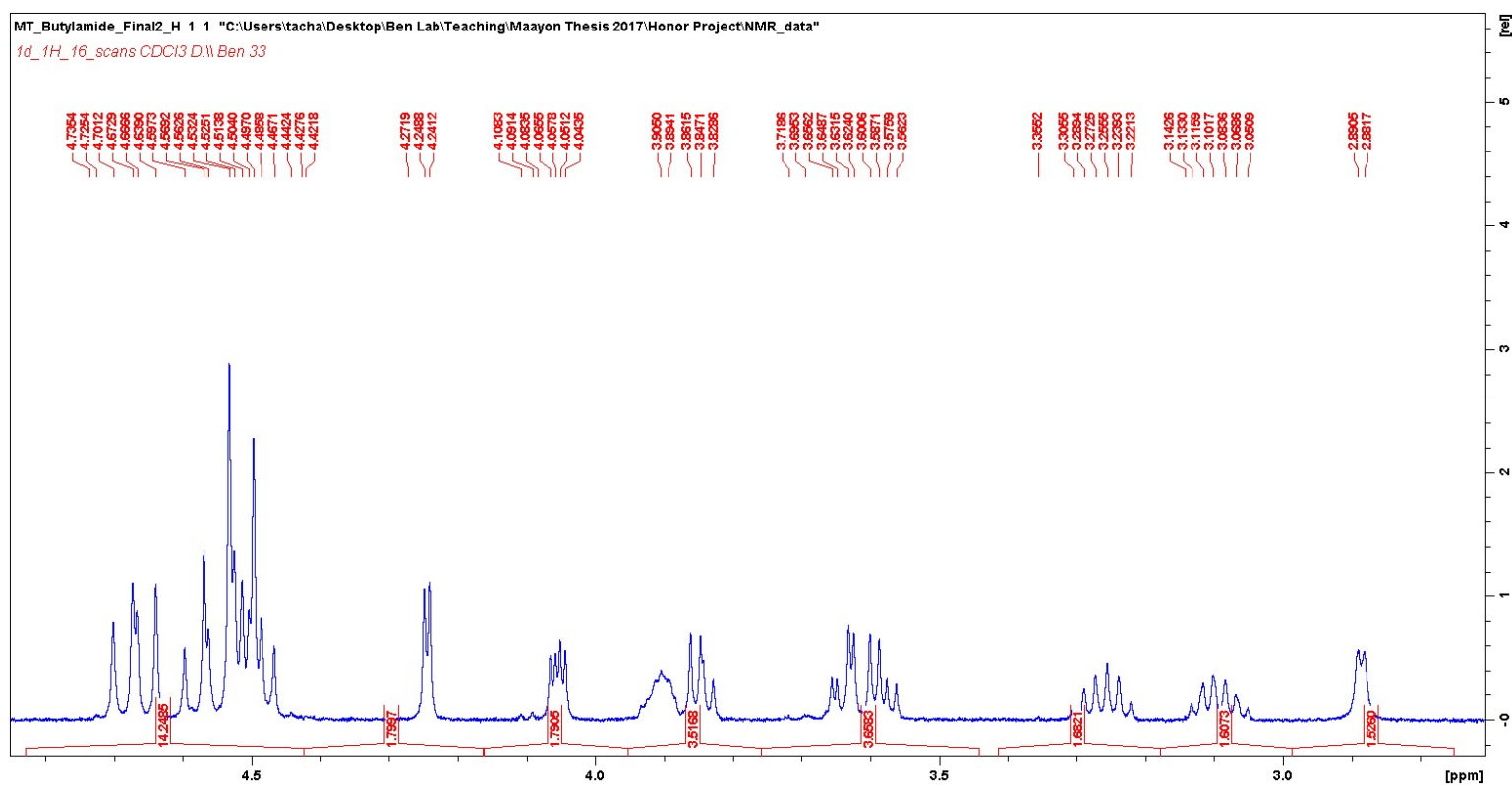
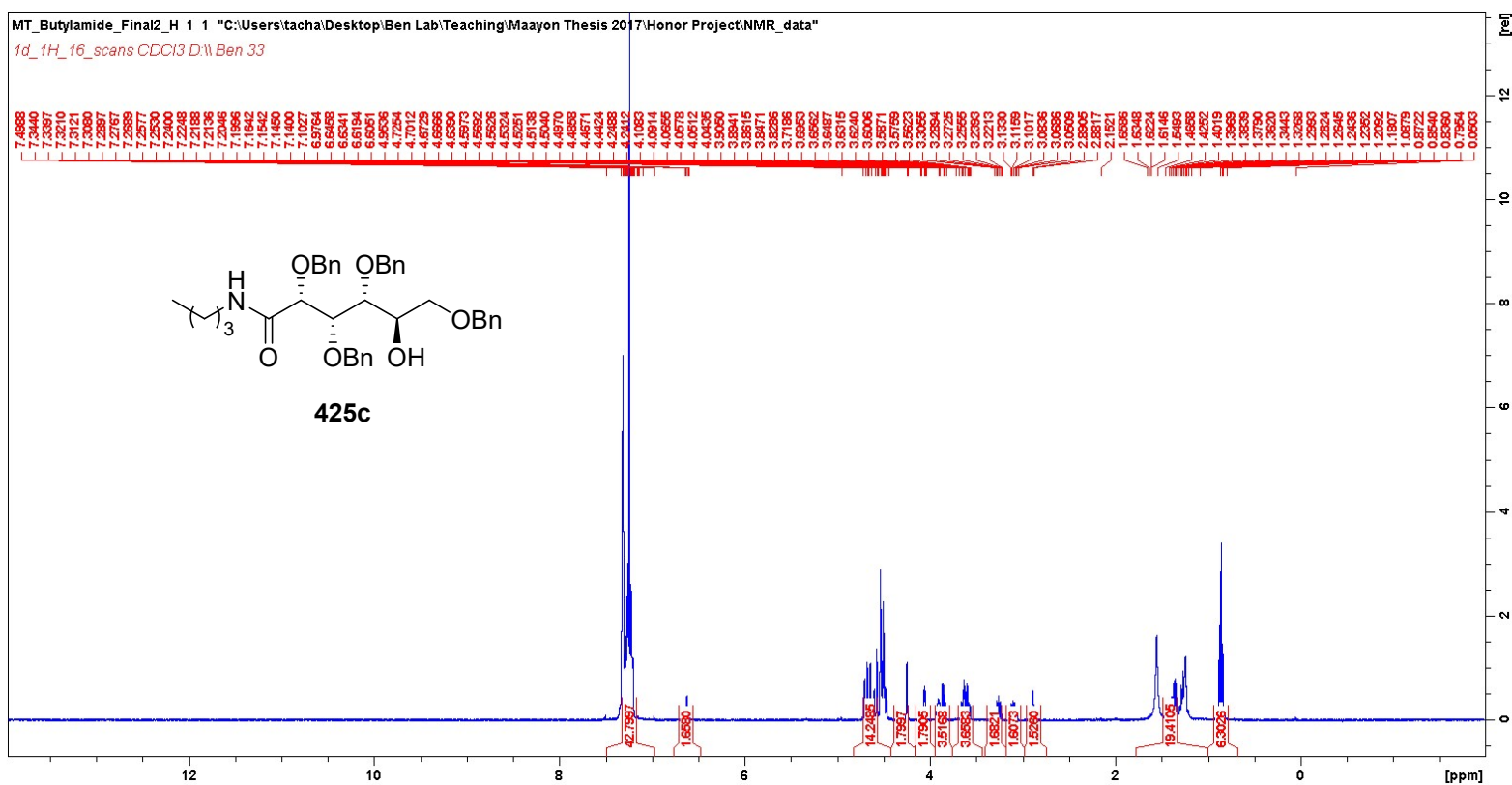
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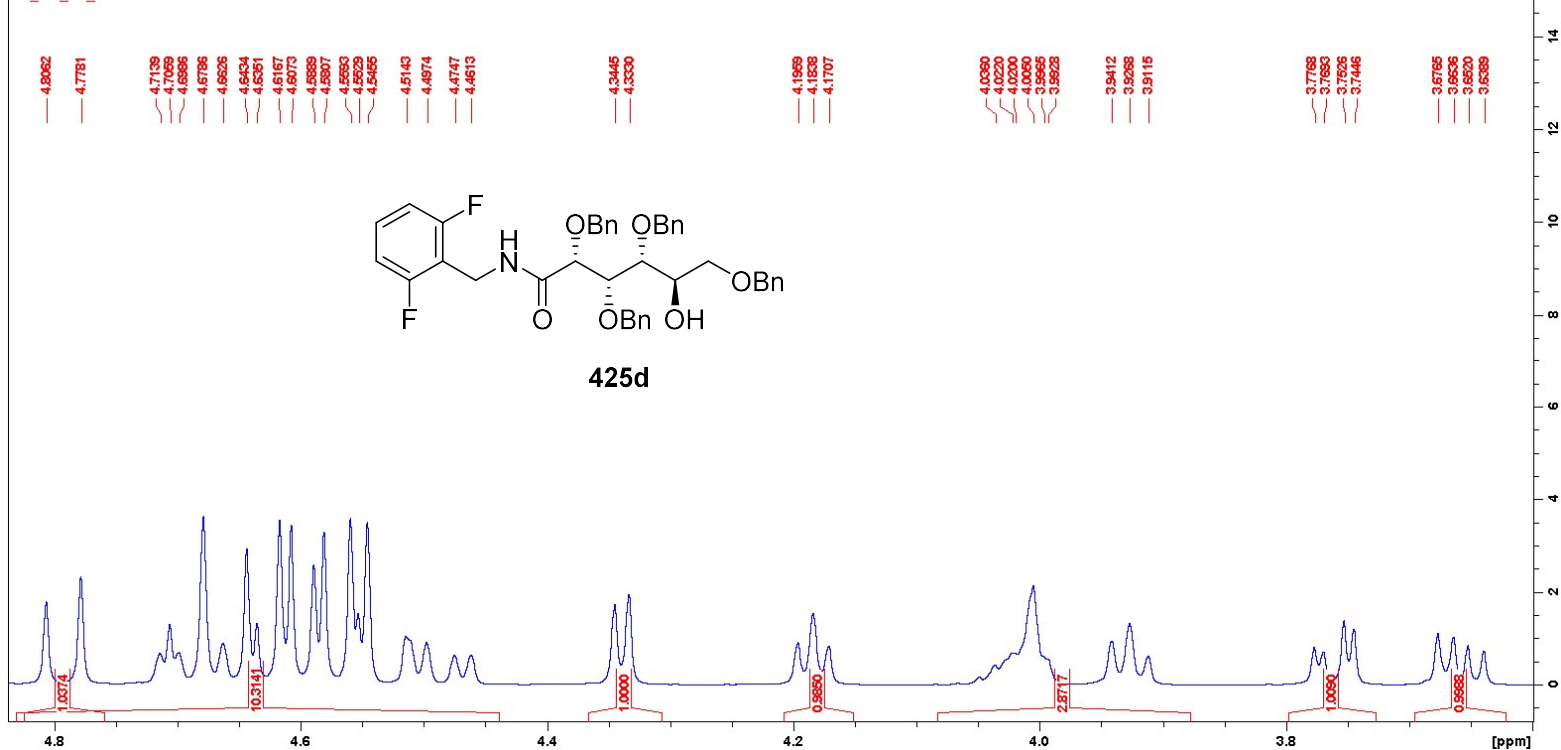
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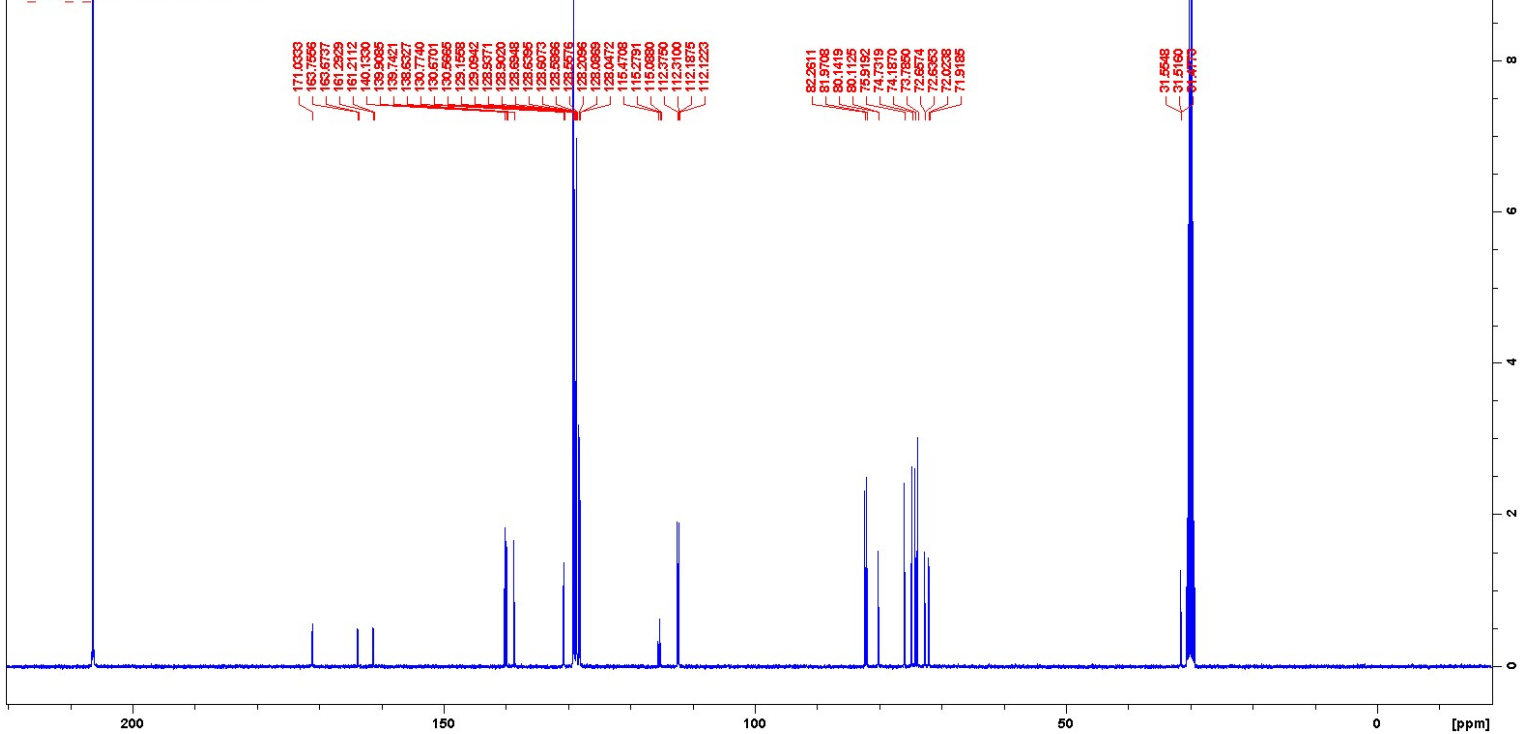
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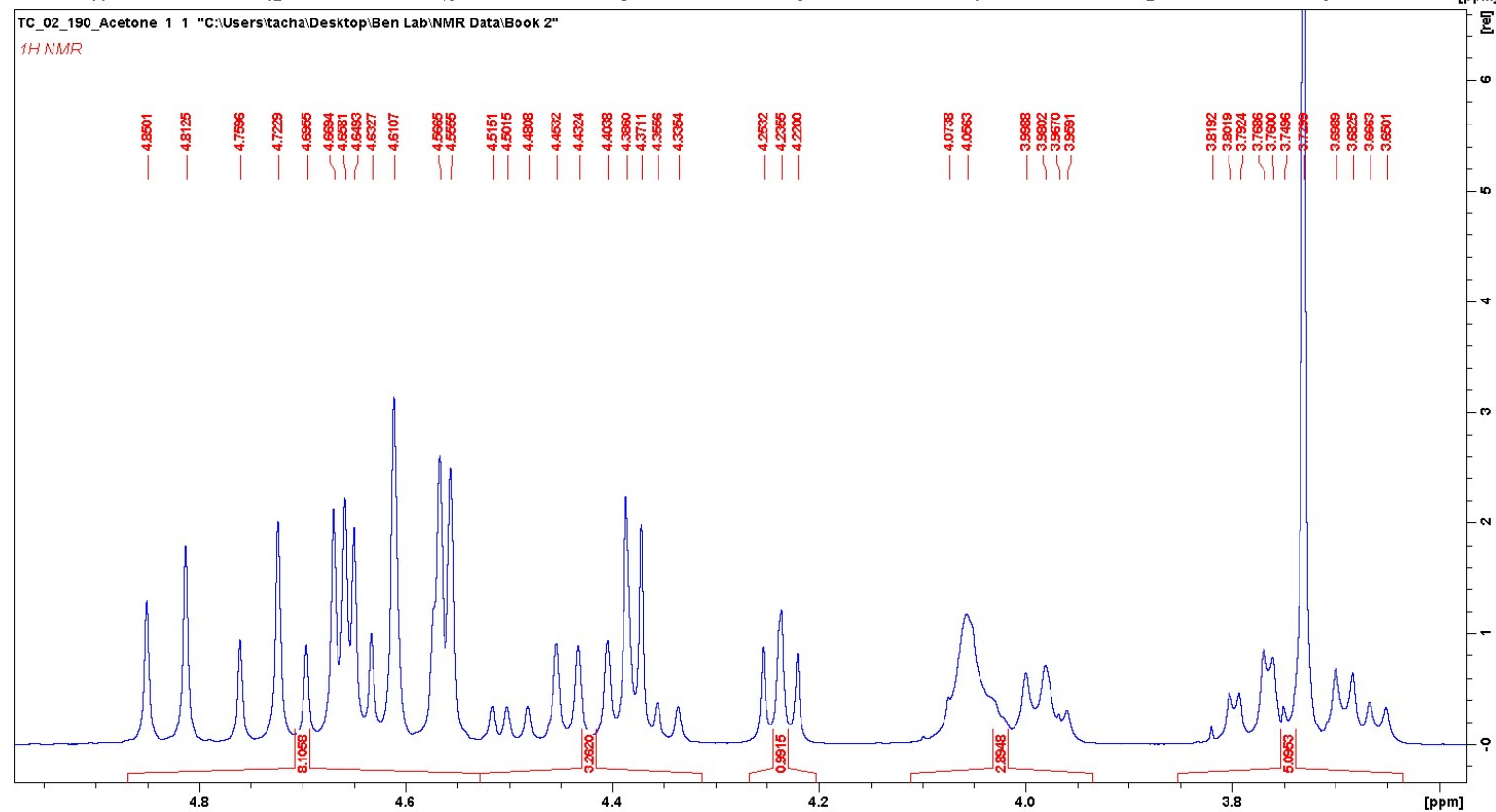
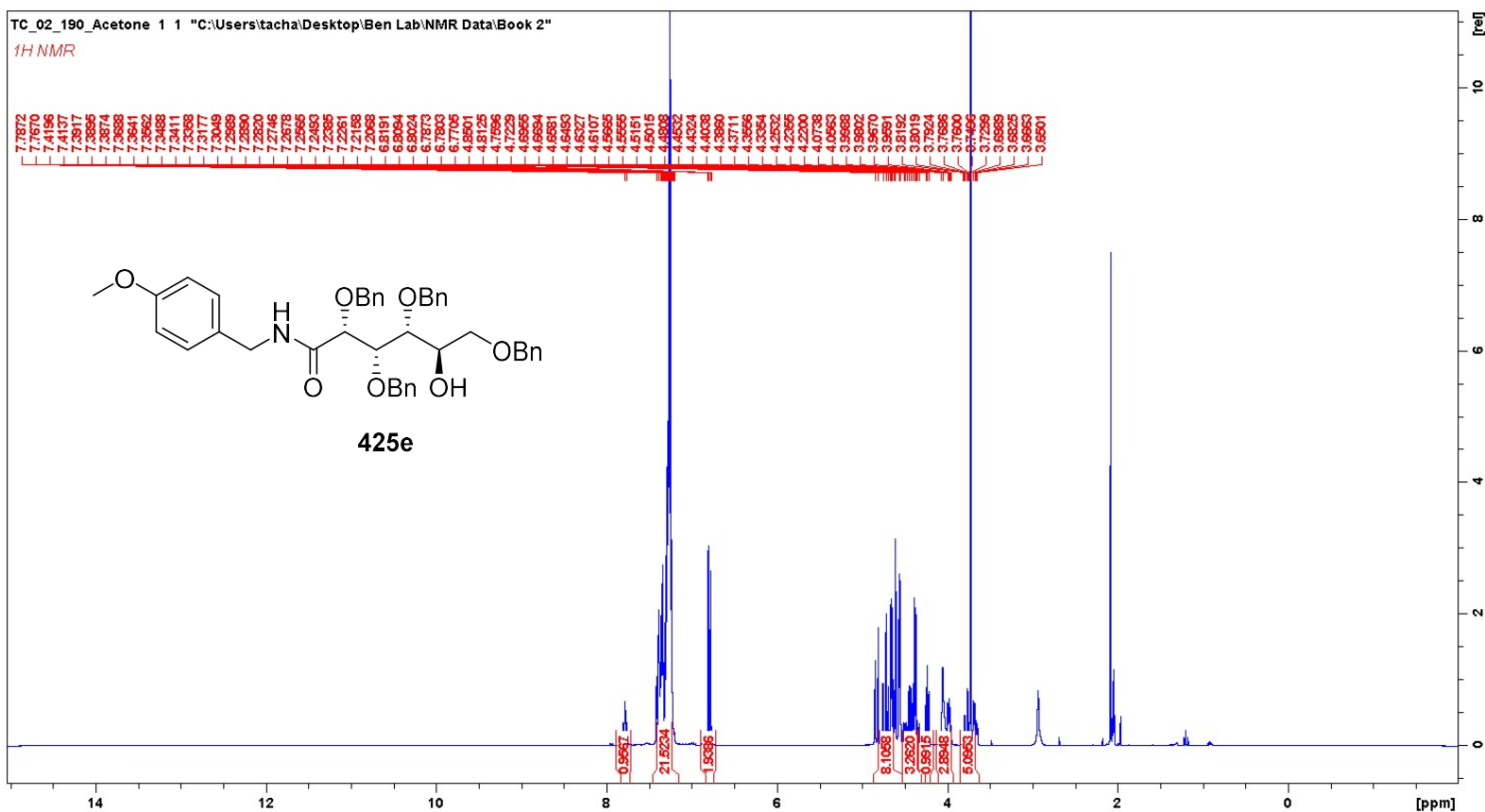
1d_1H_32_scans Acetone D\Ben 55

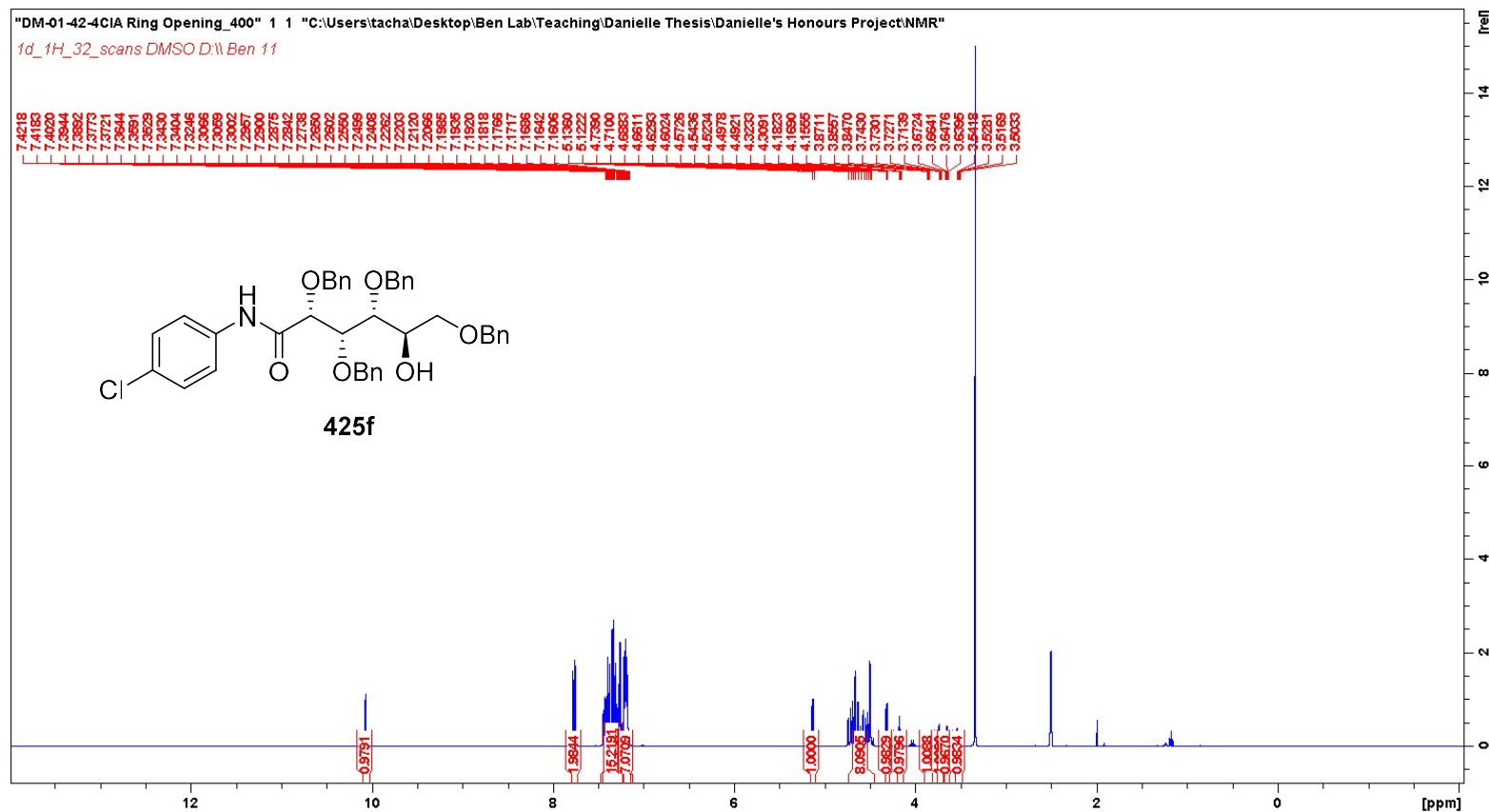
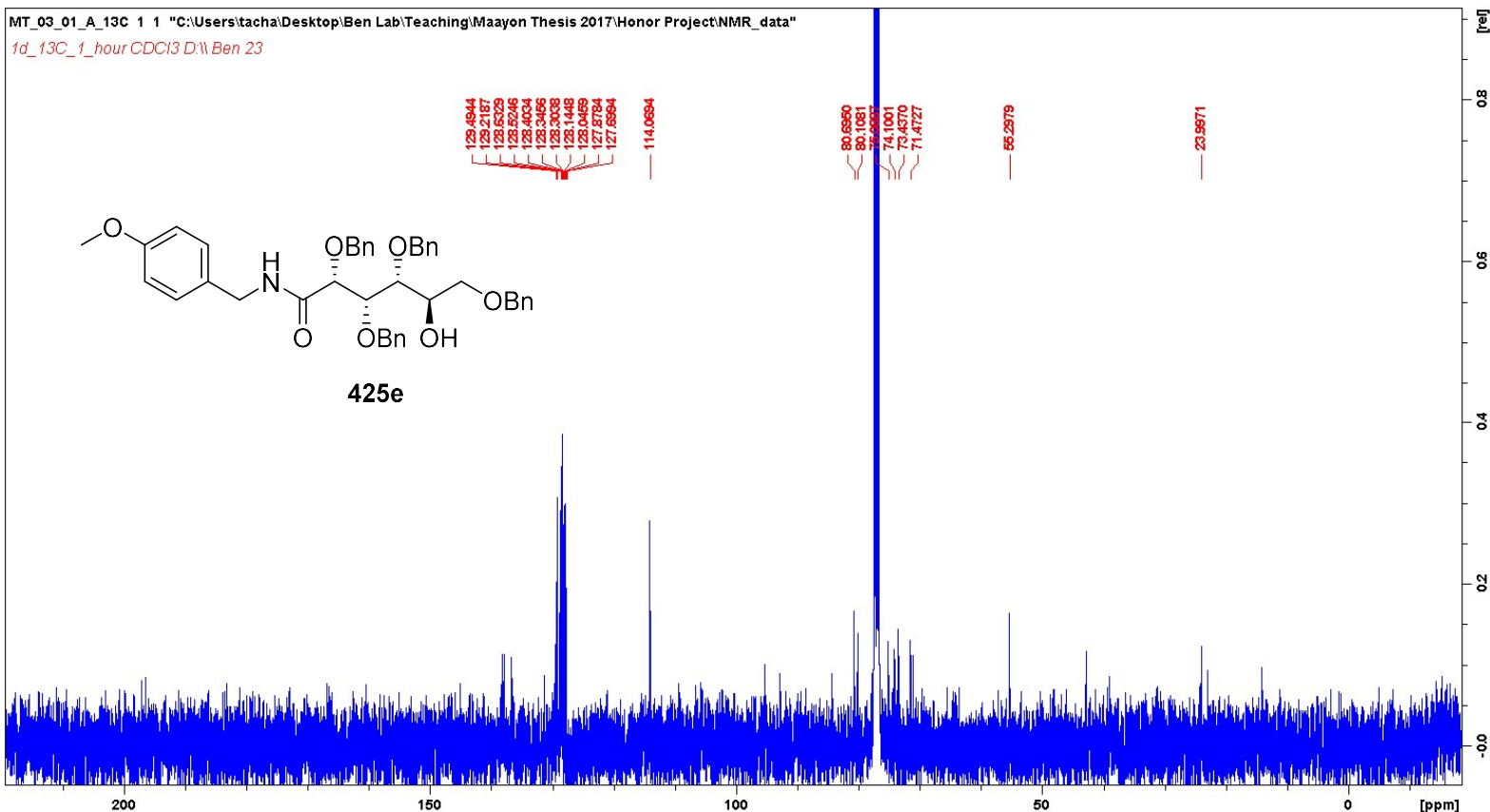


TC_02_185_Acetone 2 1 "C:\Users\tacha\Desktop\Ben Lab\NMR Data\Book 2"

1d_13C_1_hour Acetone D\Ben 55

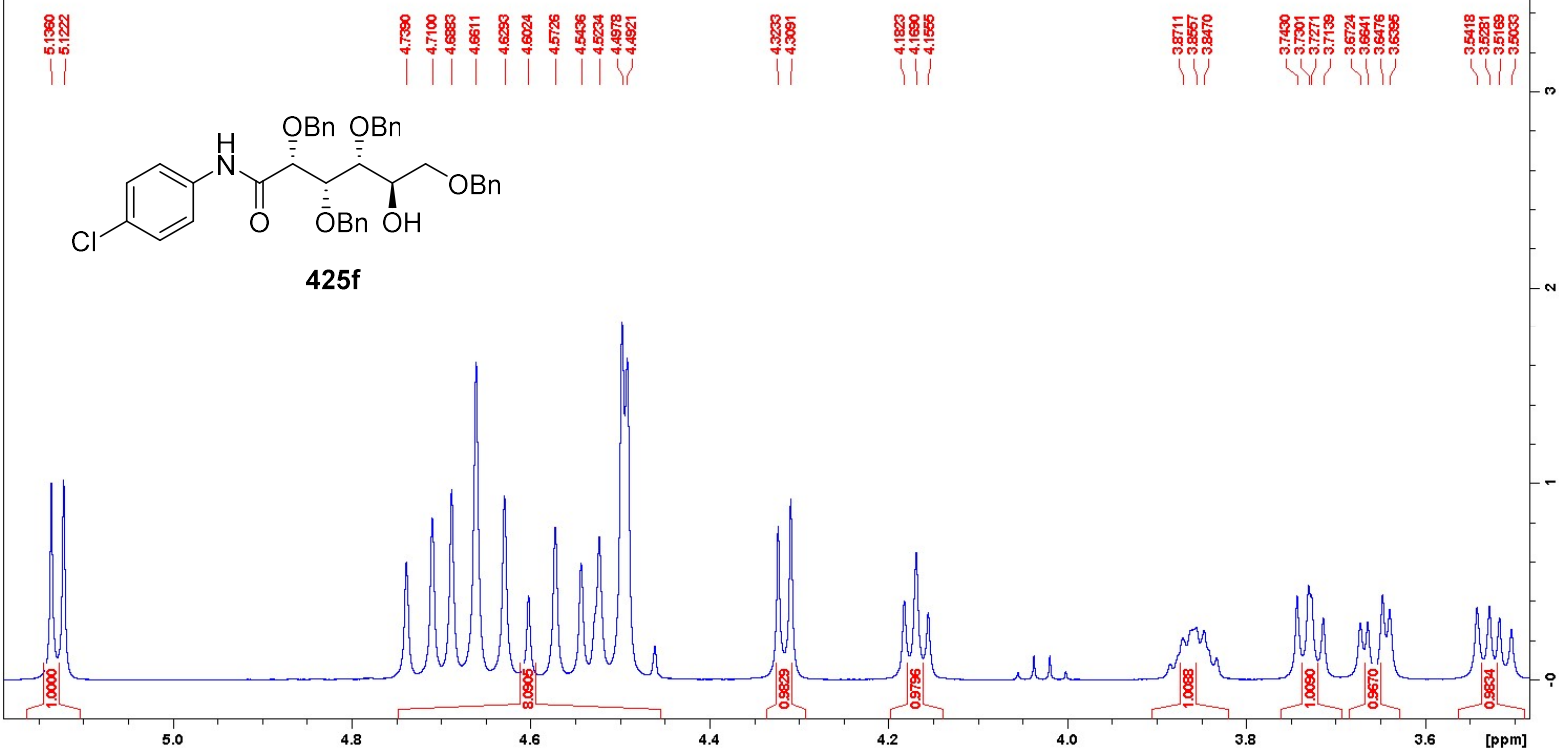






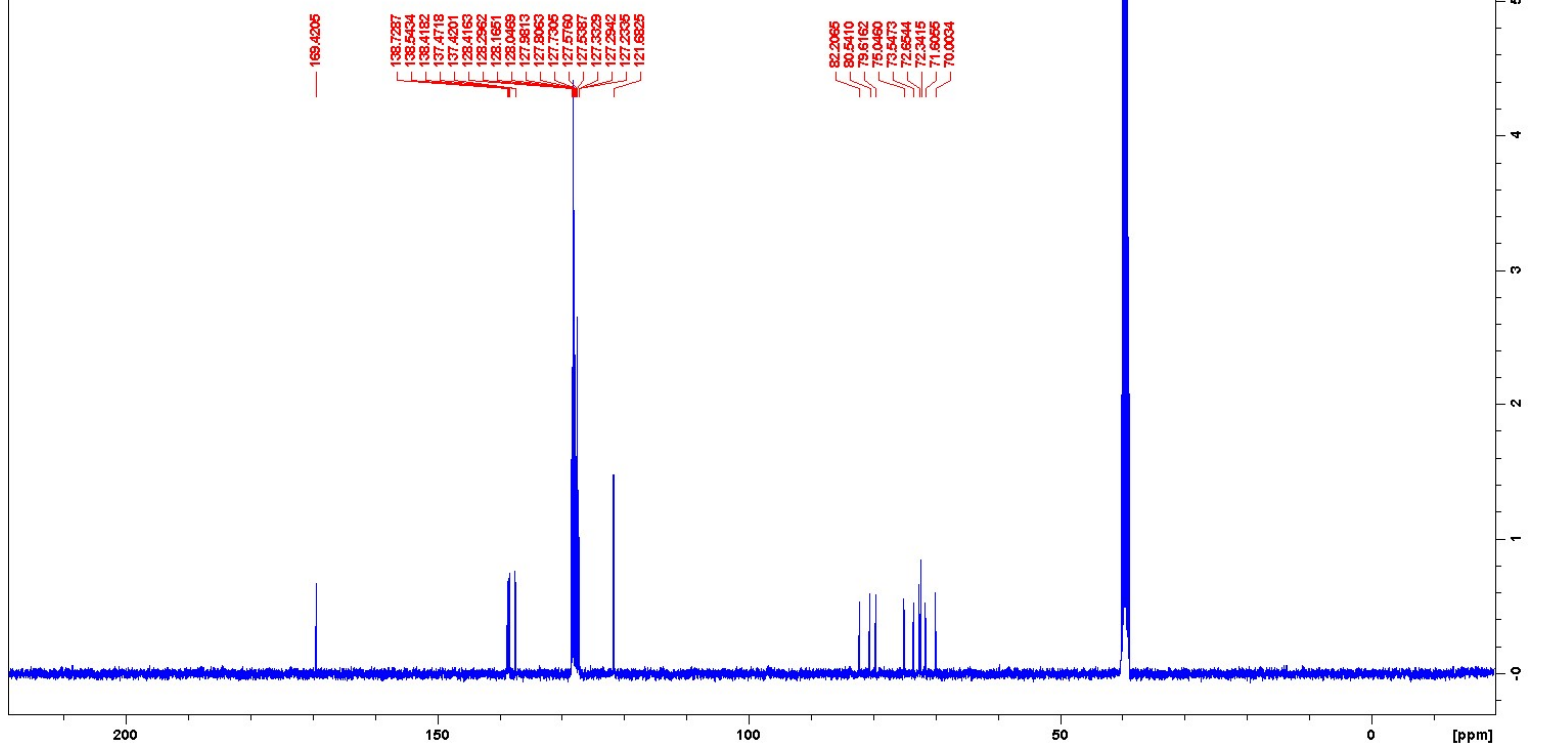
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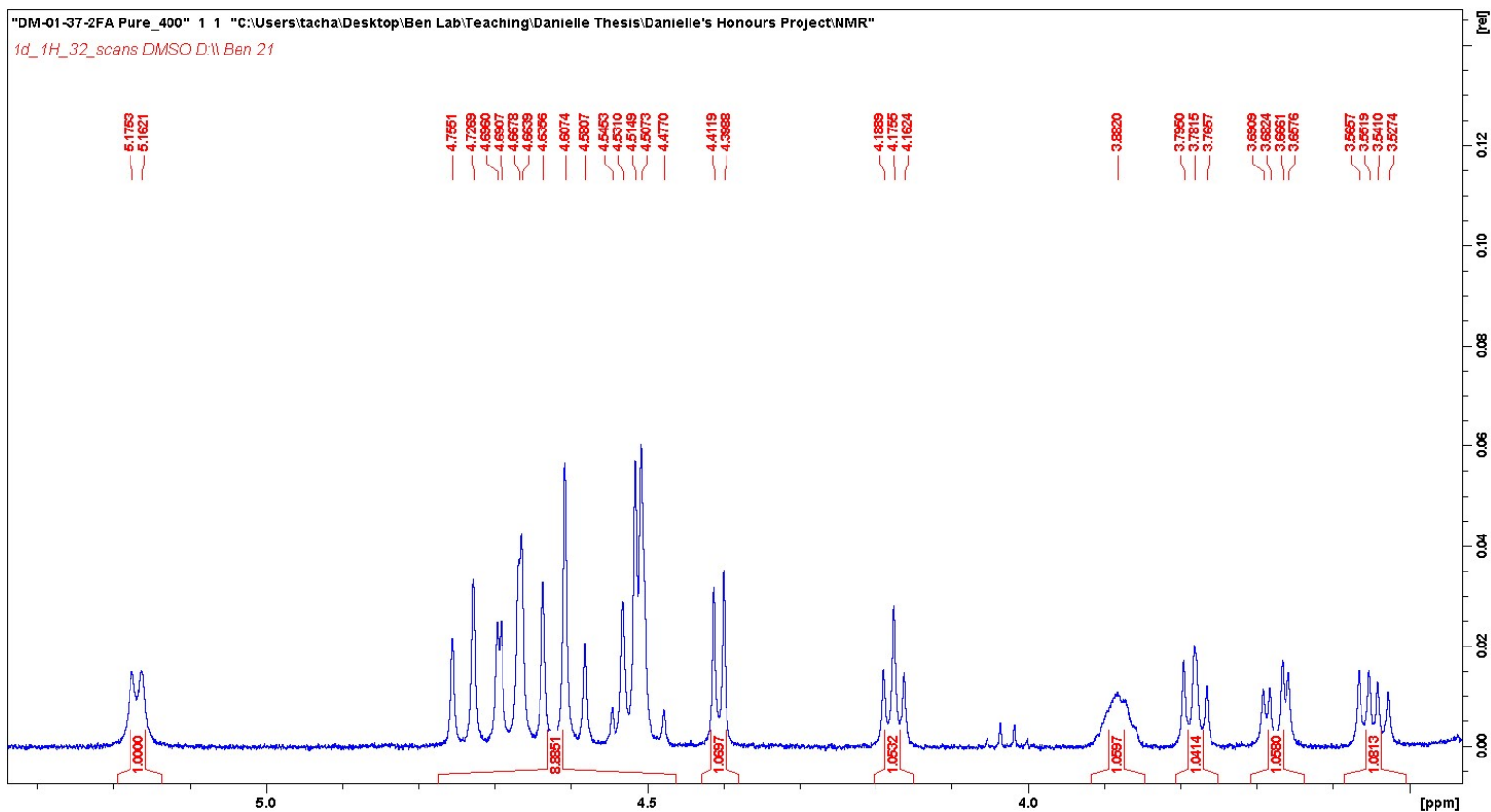
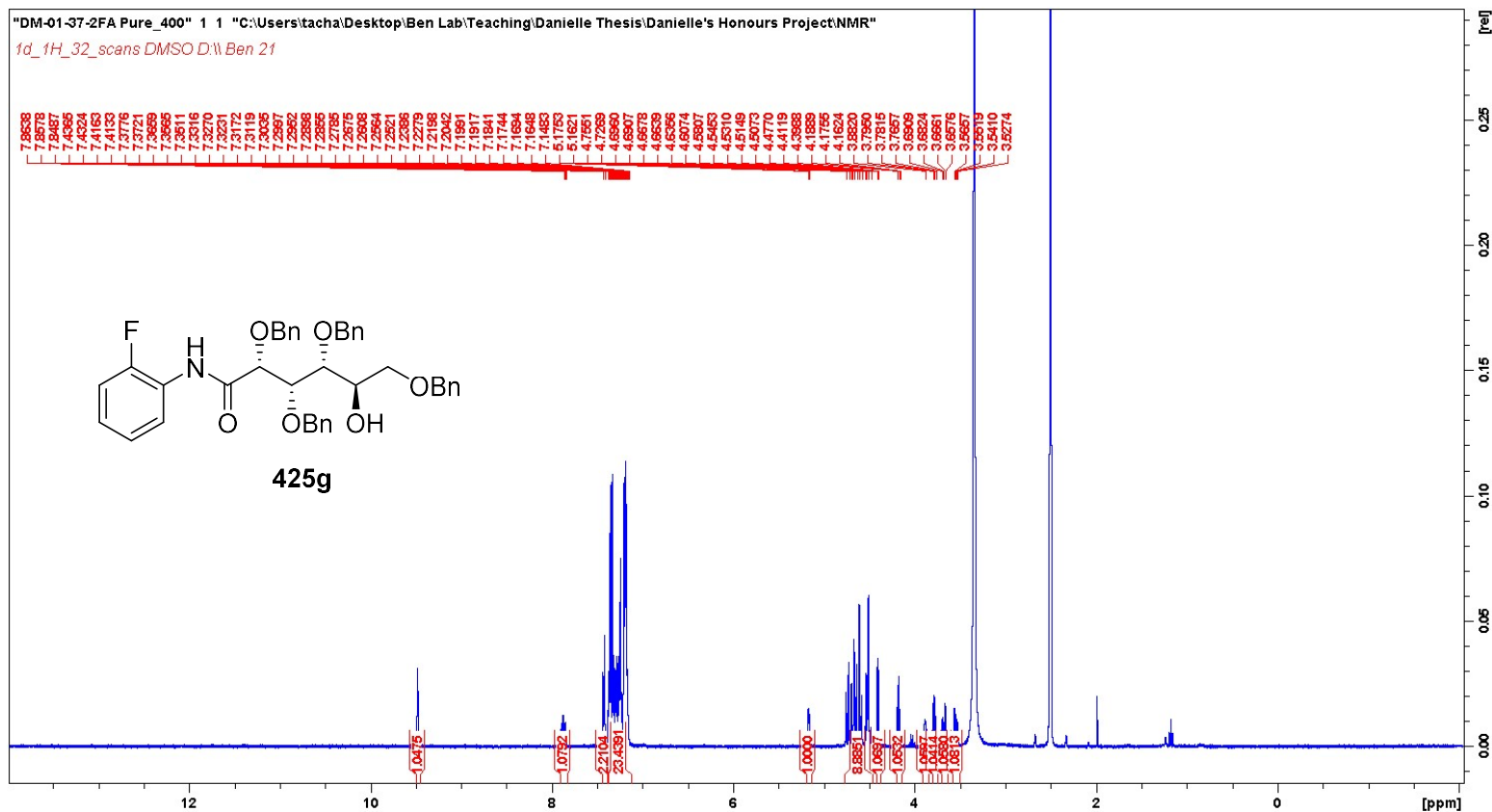
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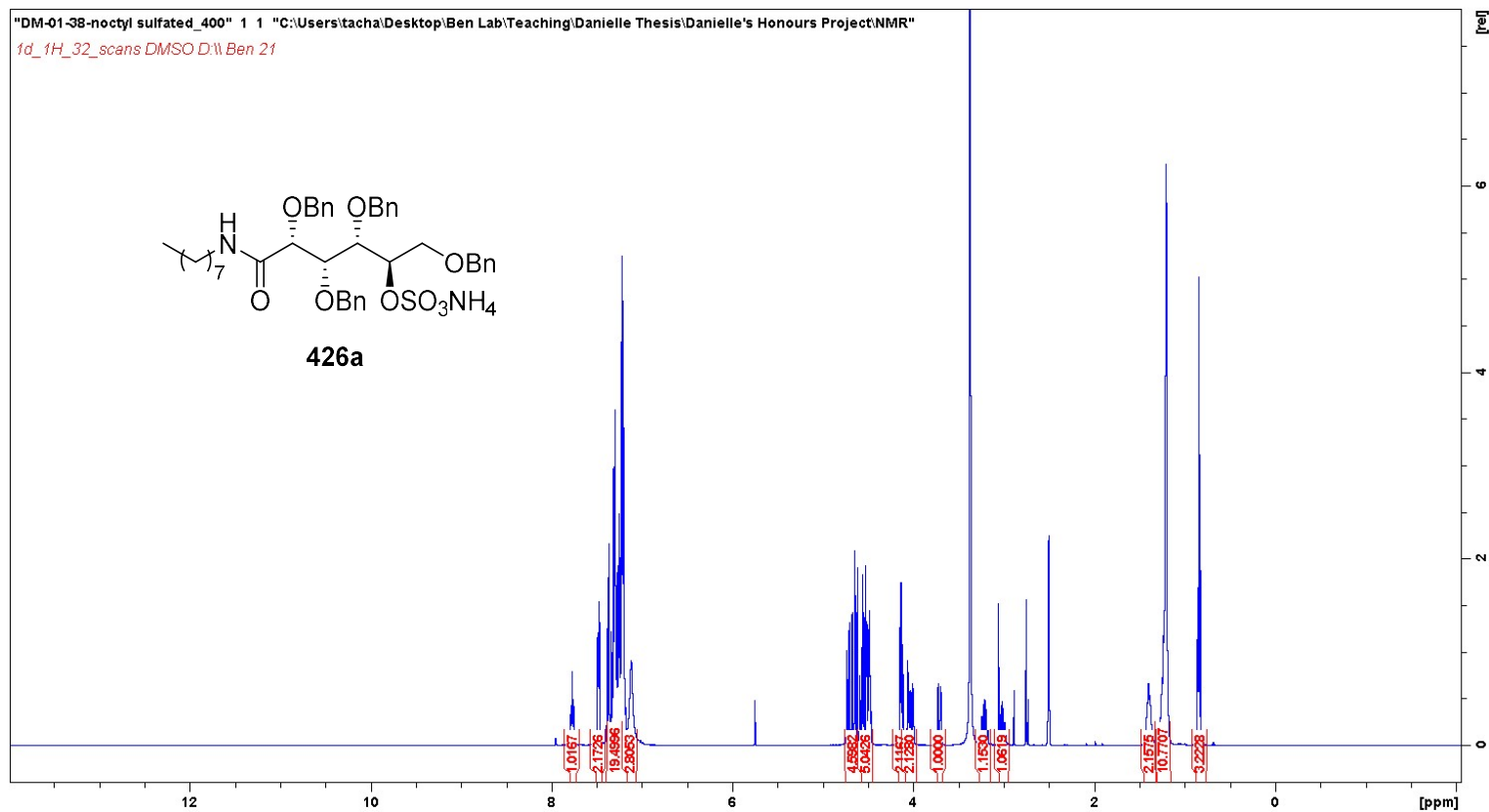
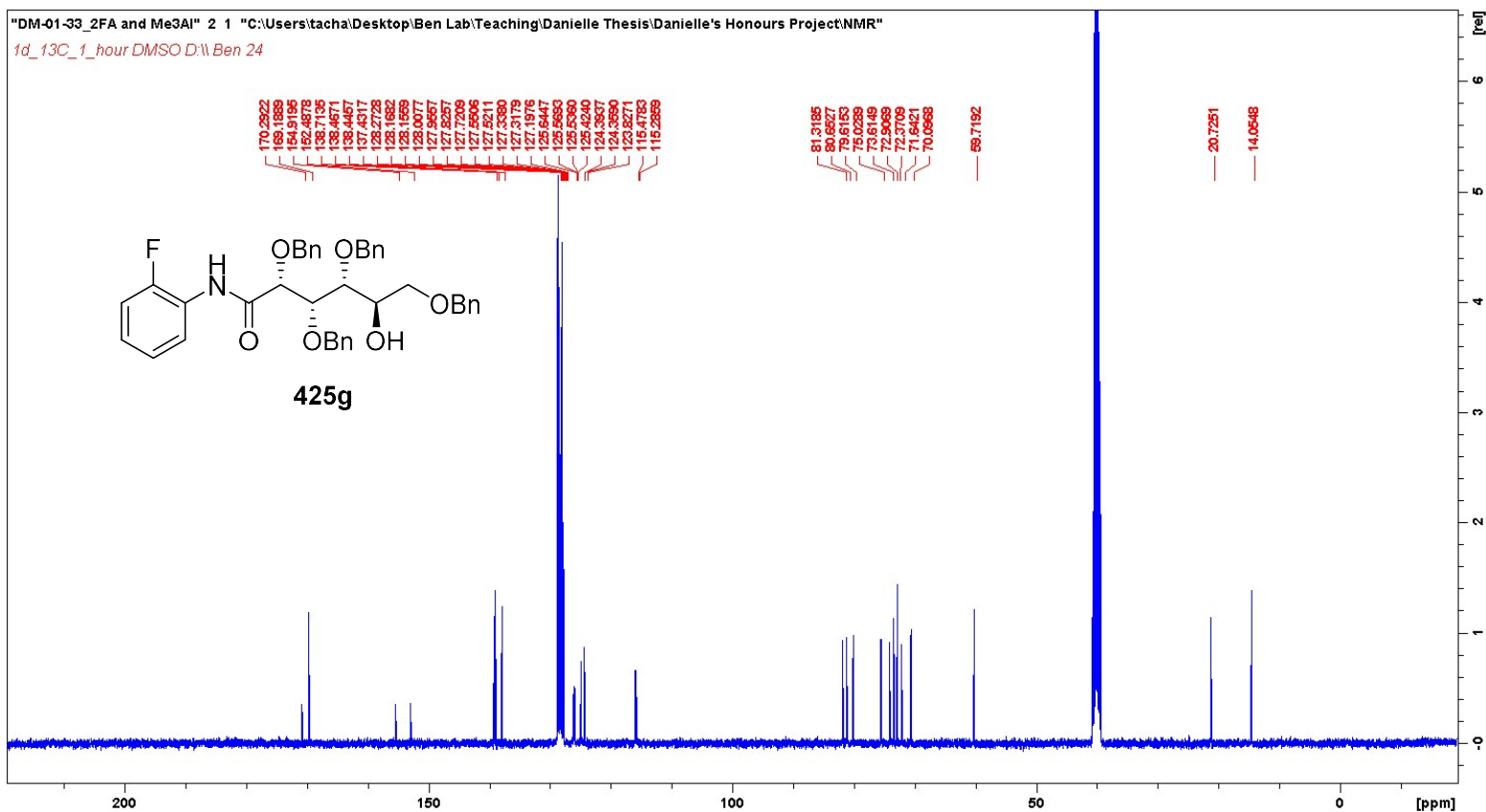


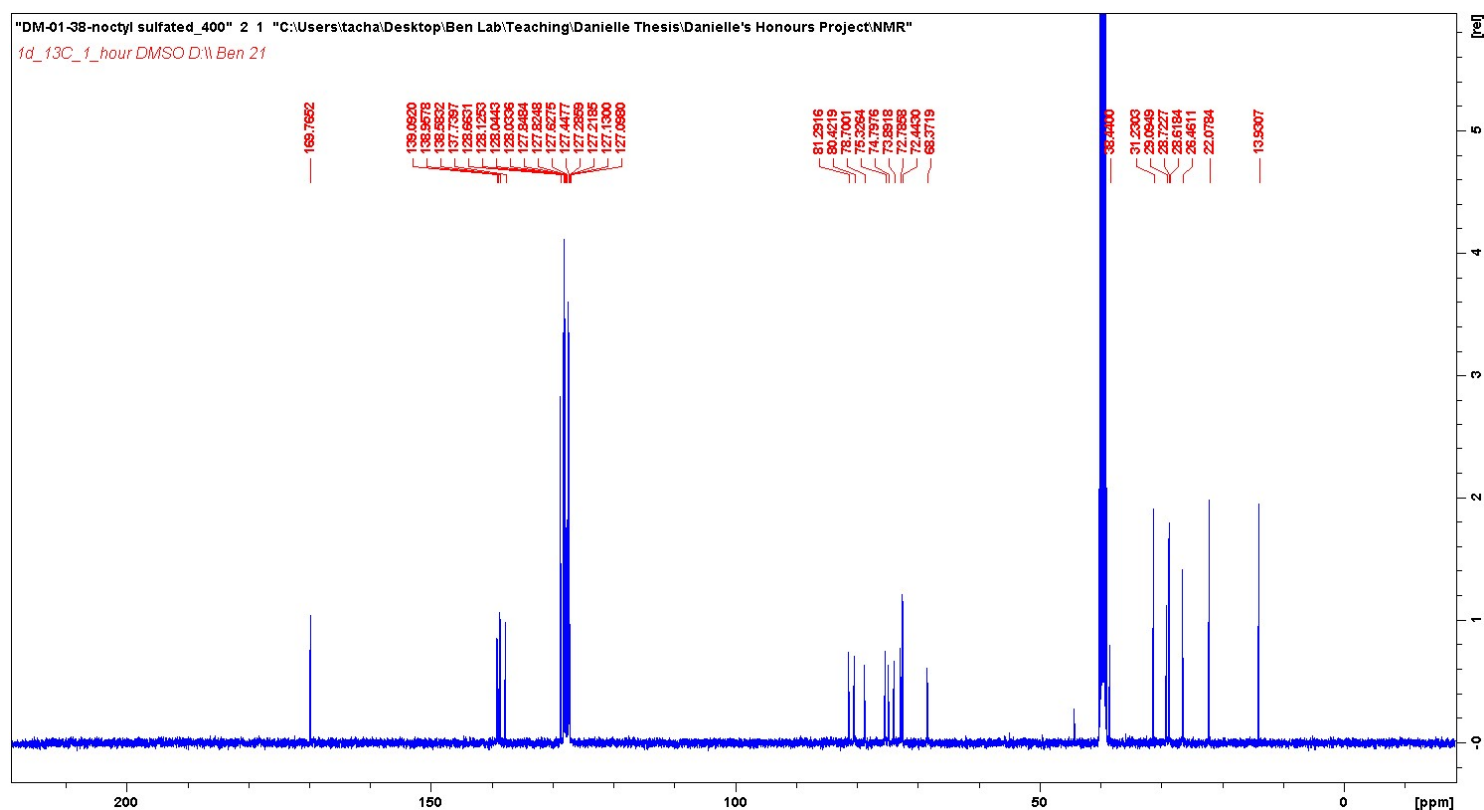
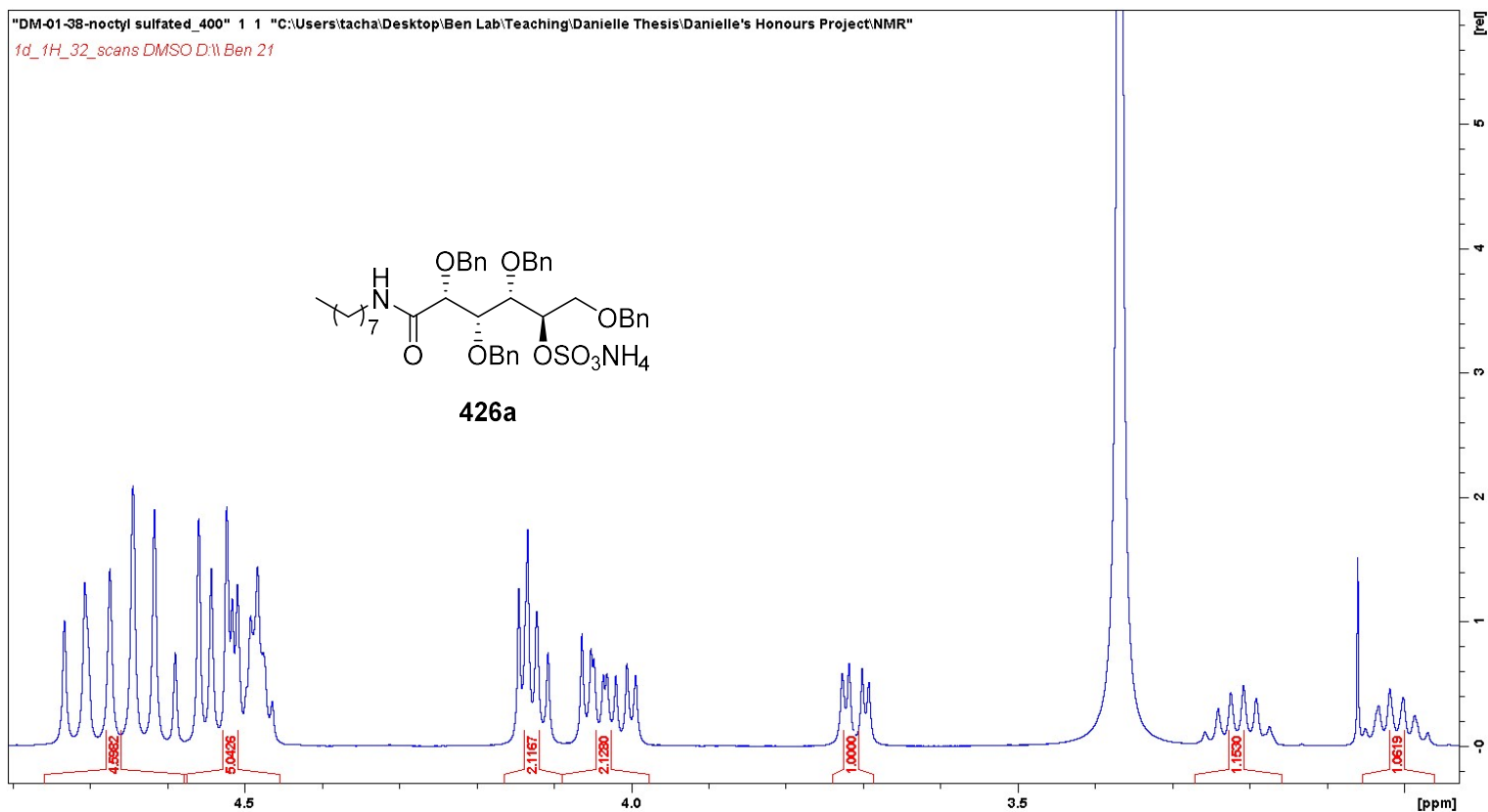
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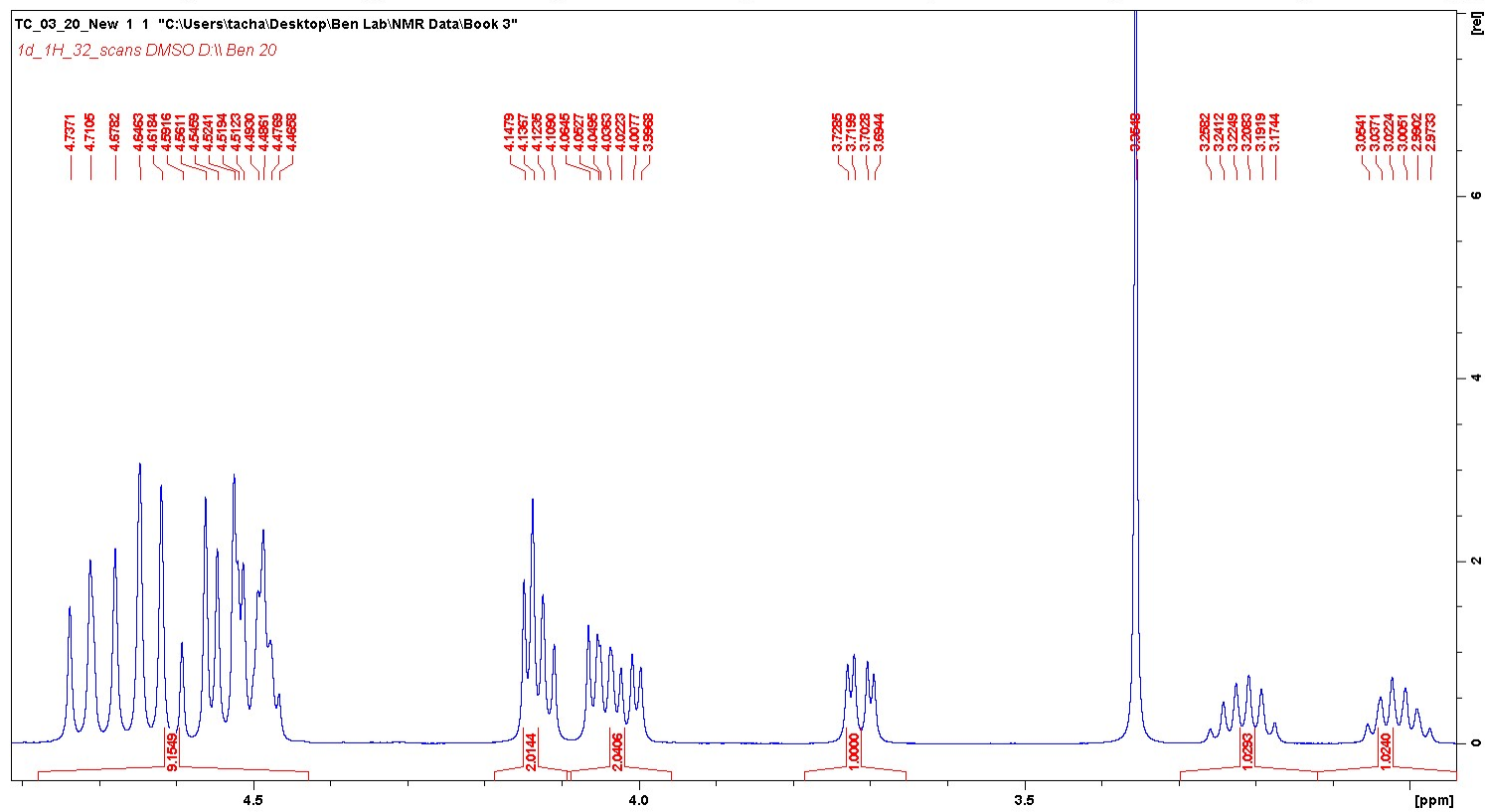
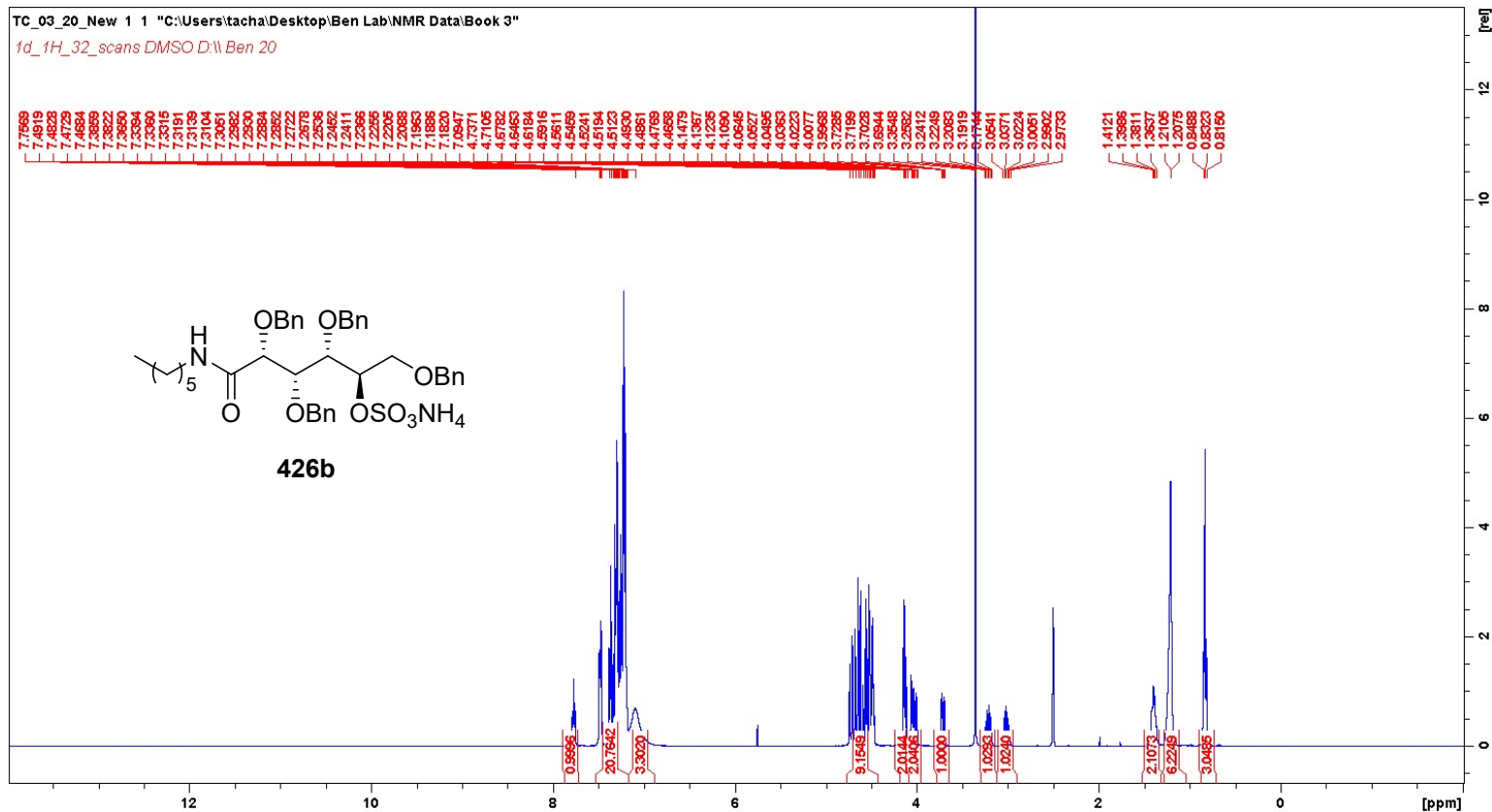
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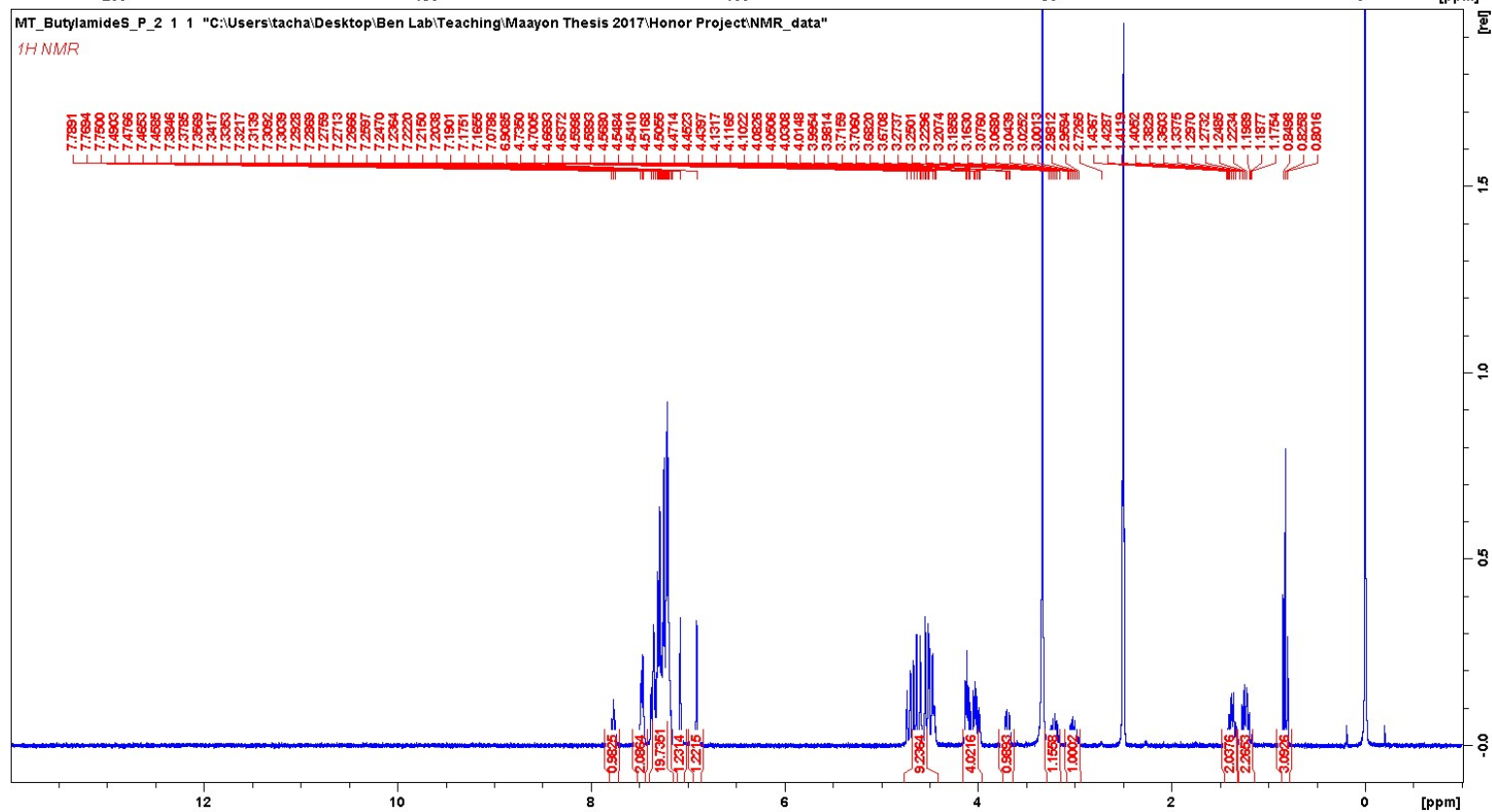
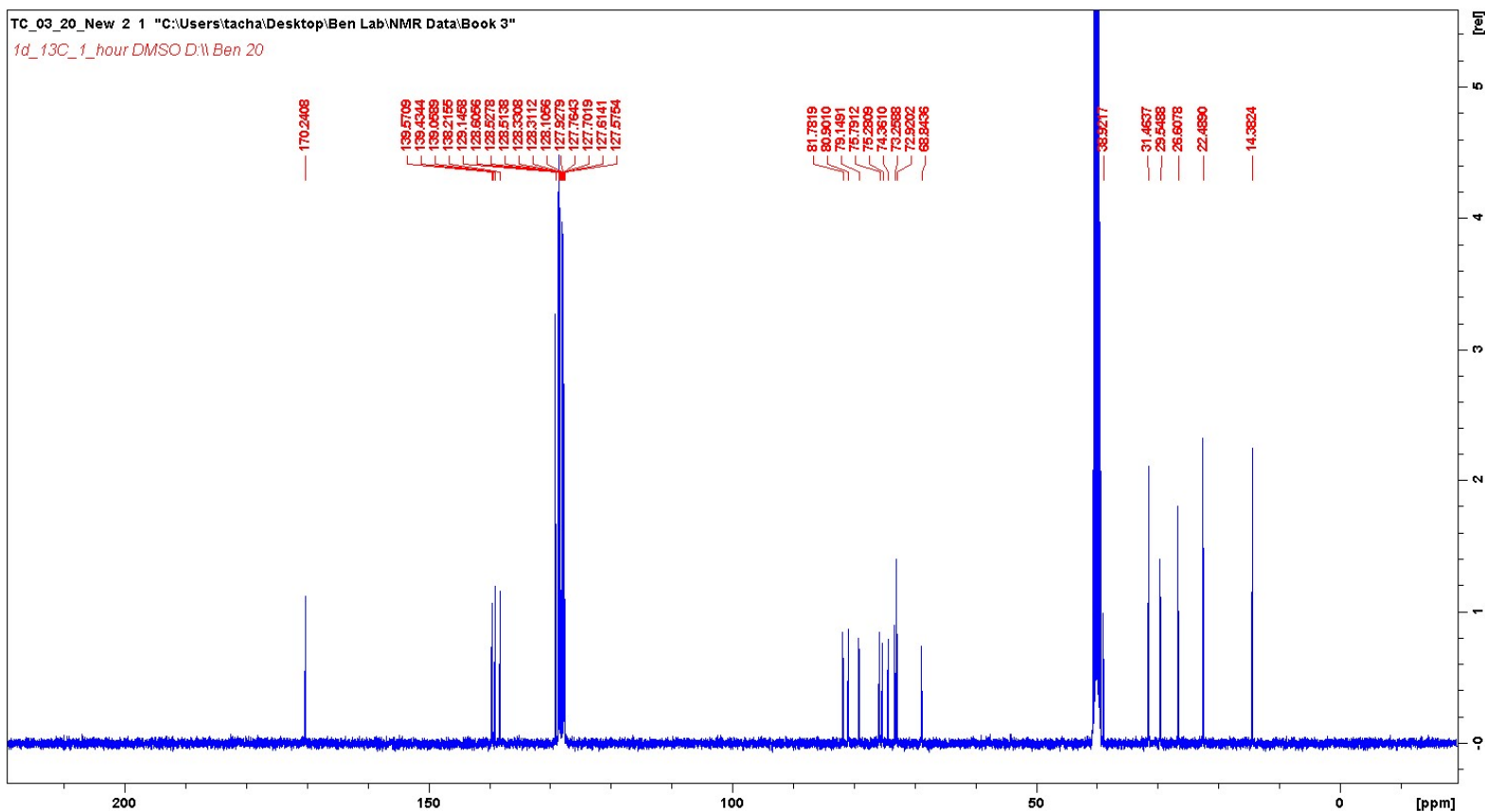


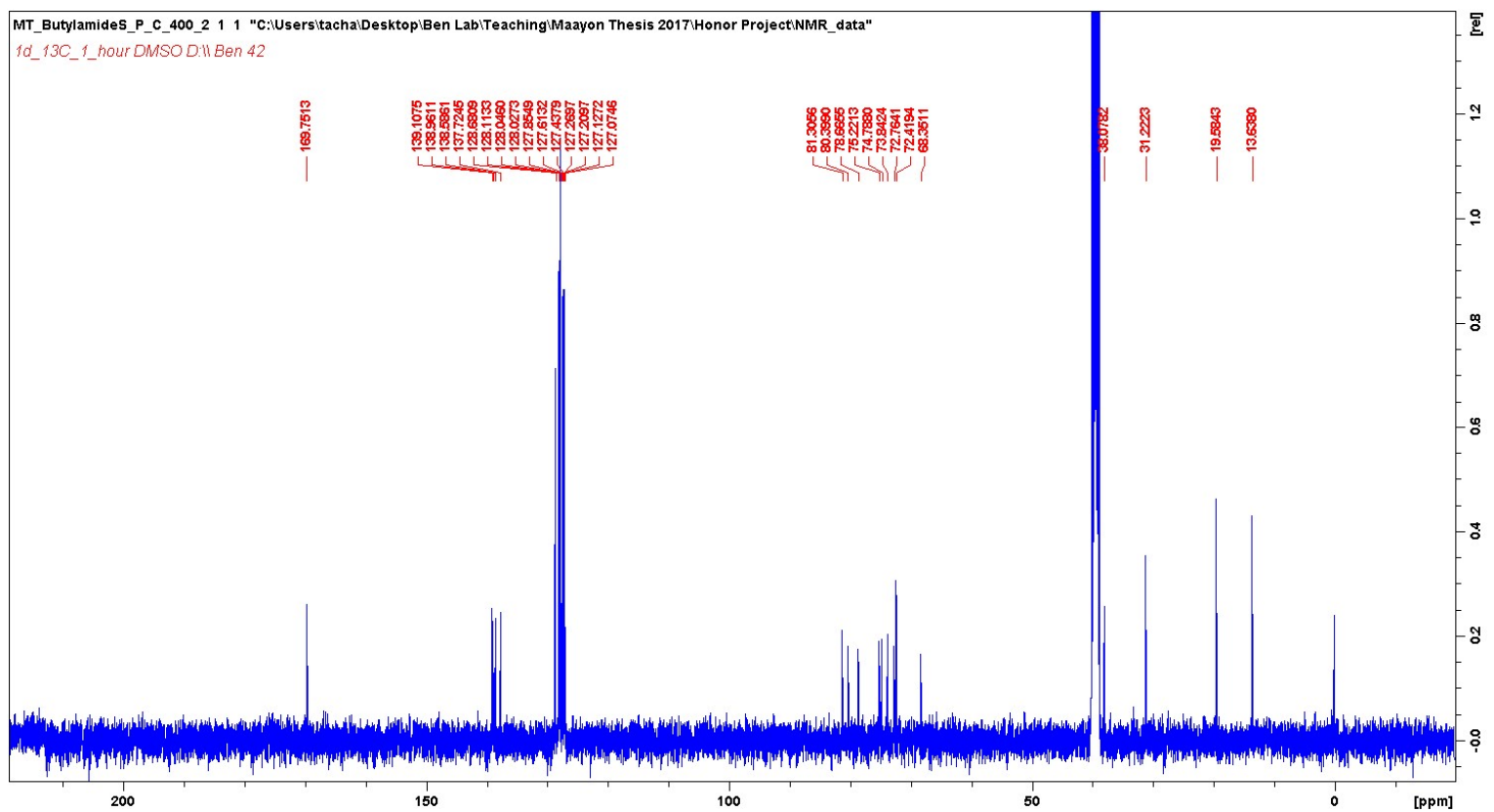
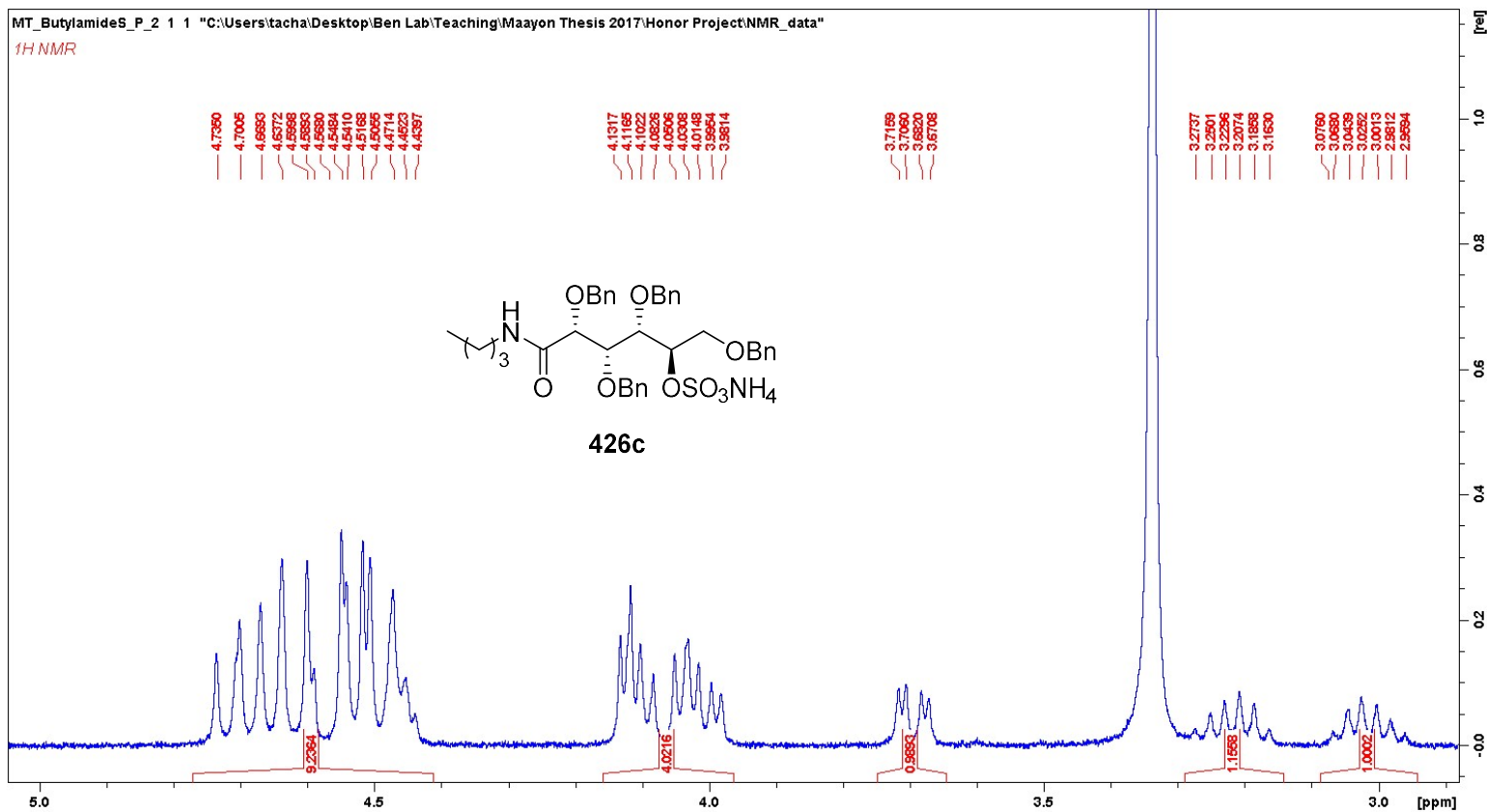






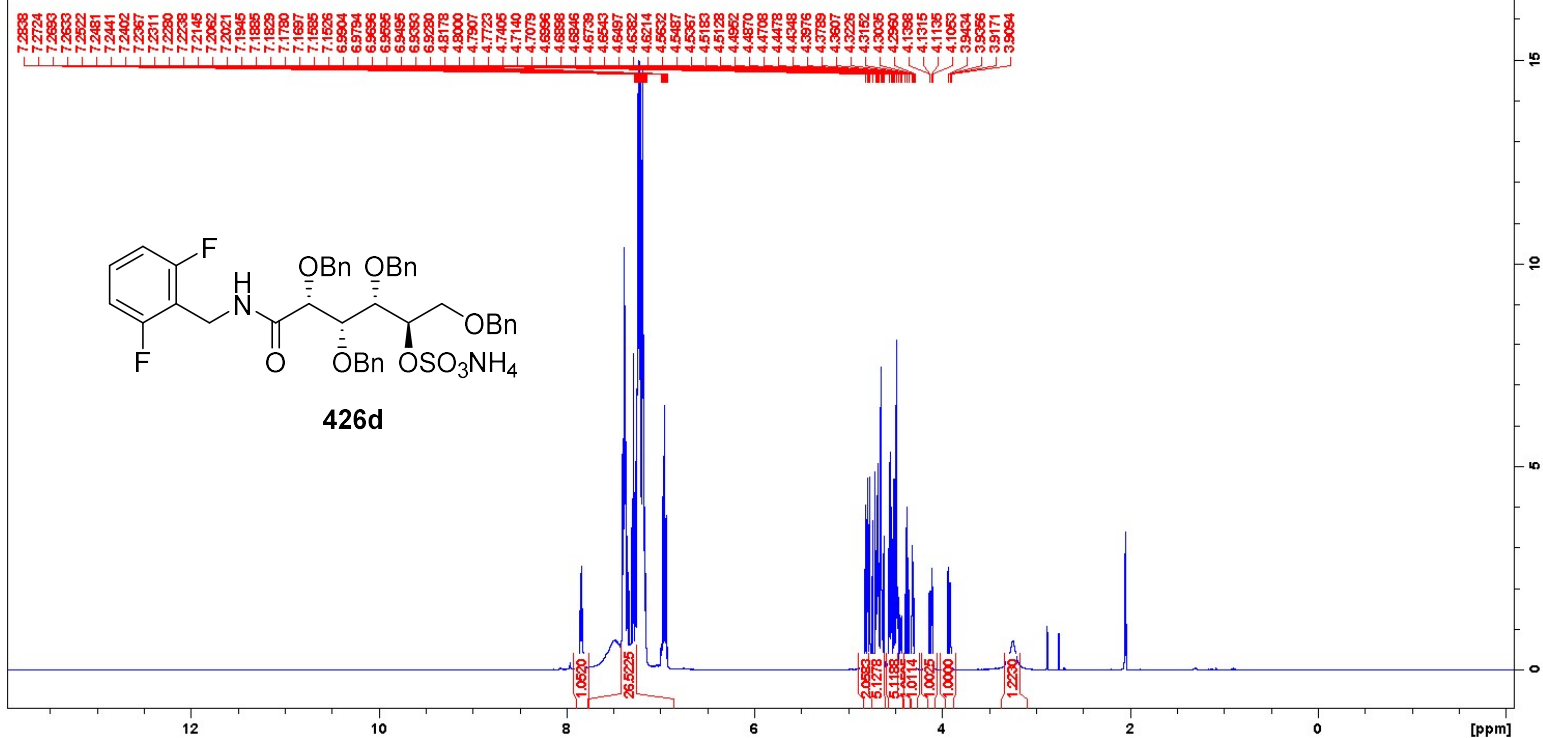






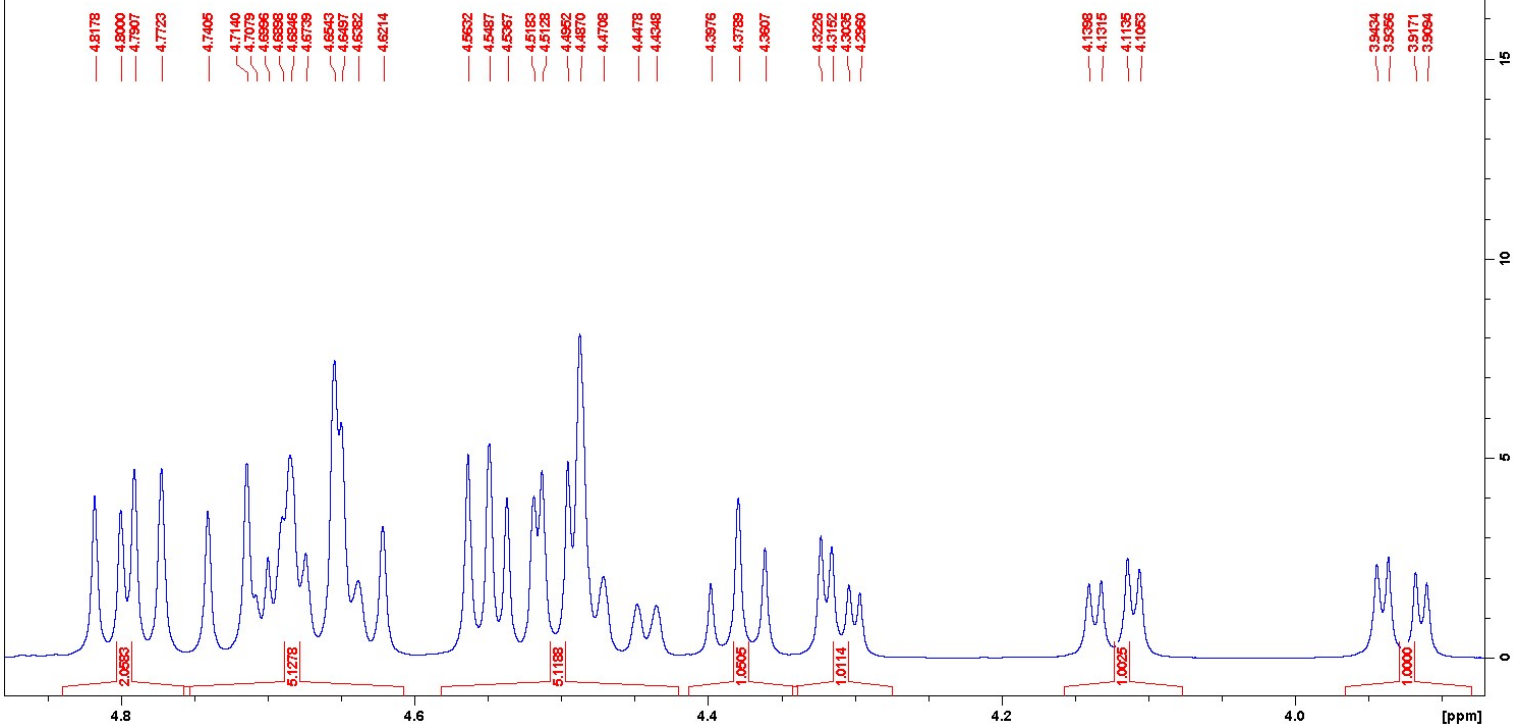
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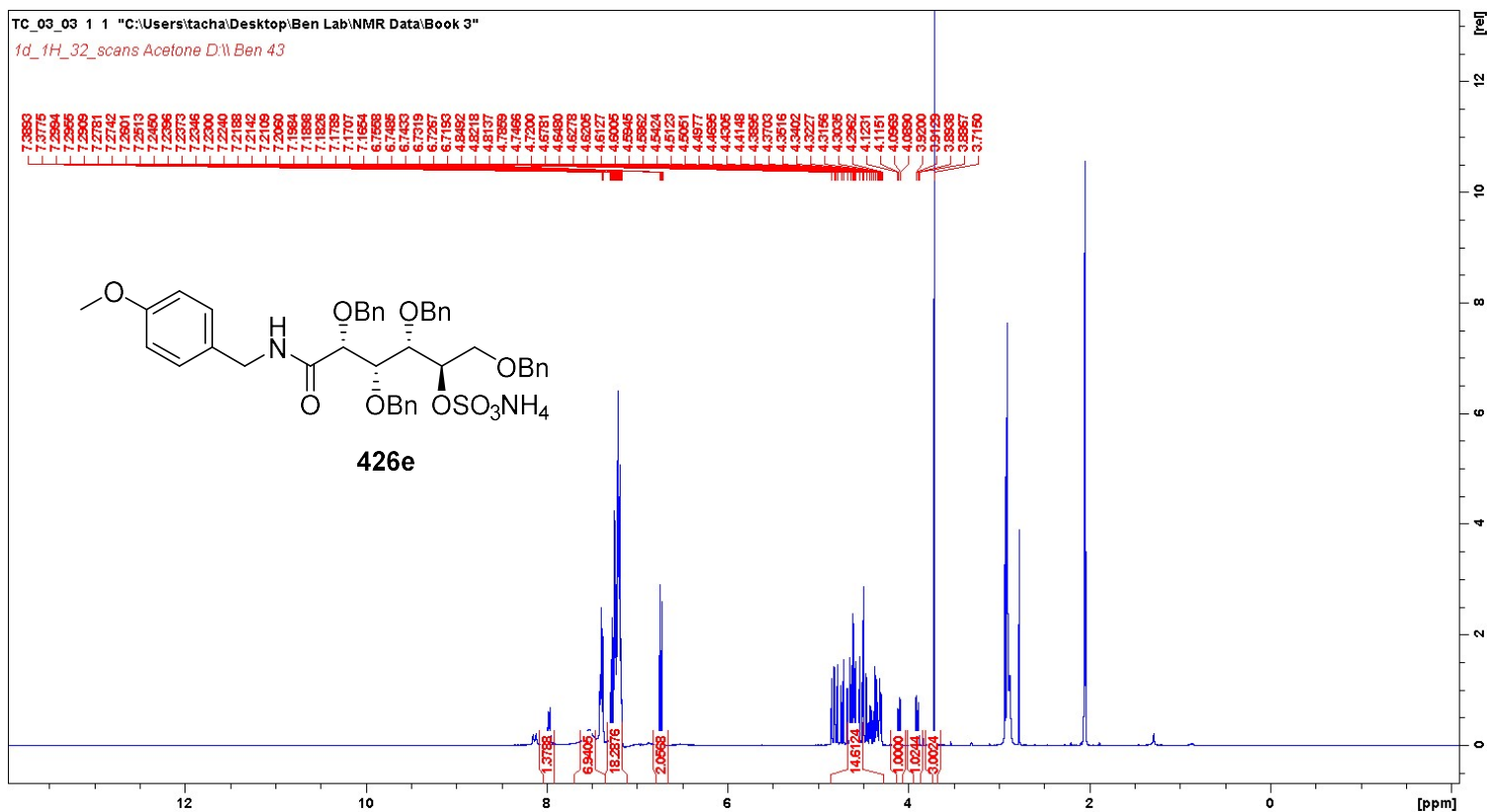
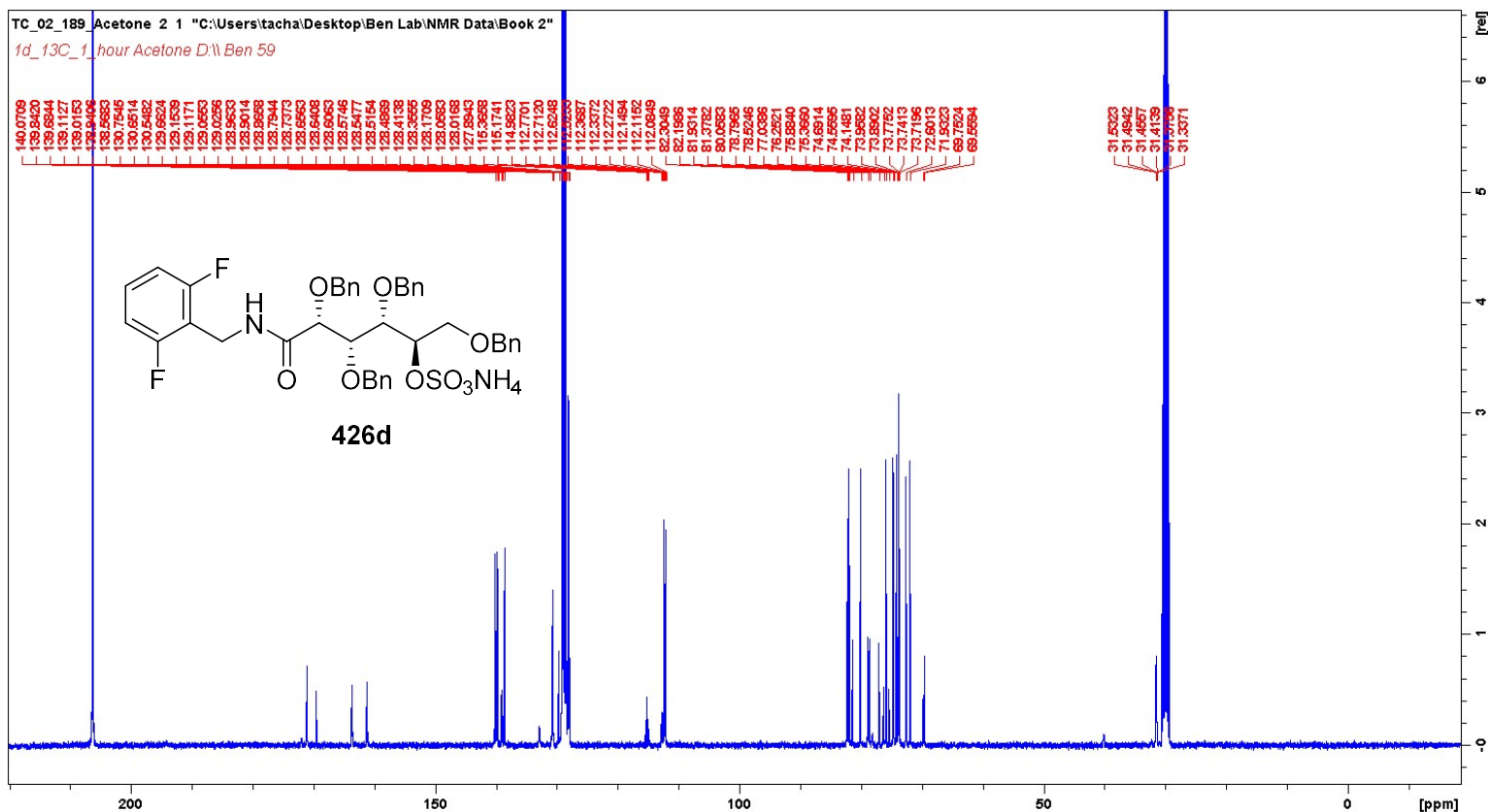
1d_1H_32_scans Acetone D:\Ben 59

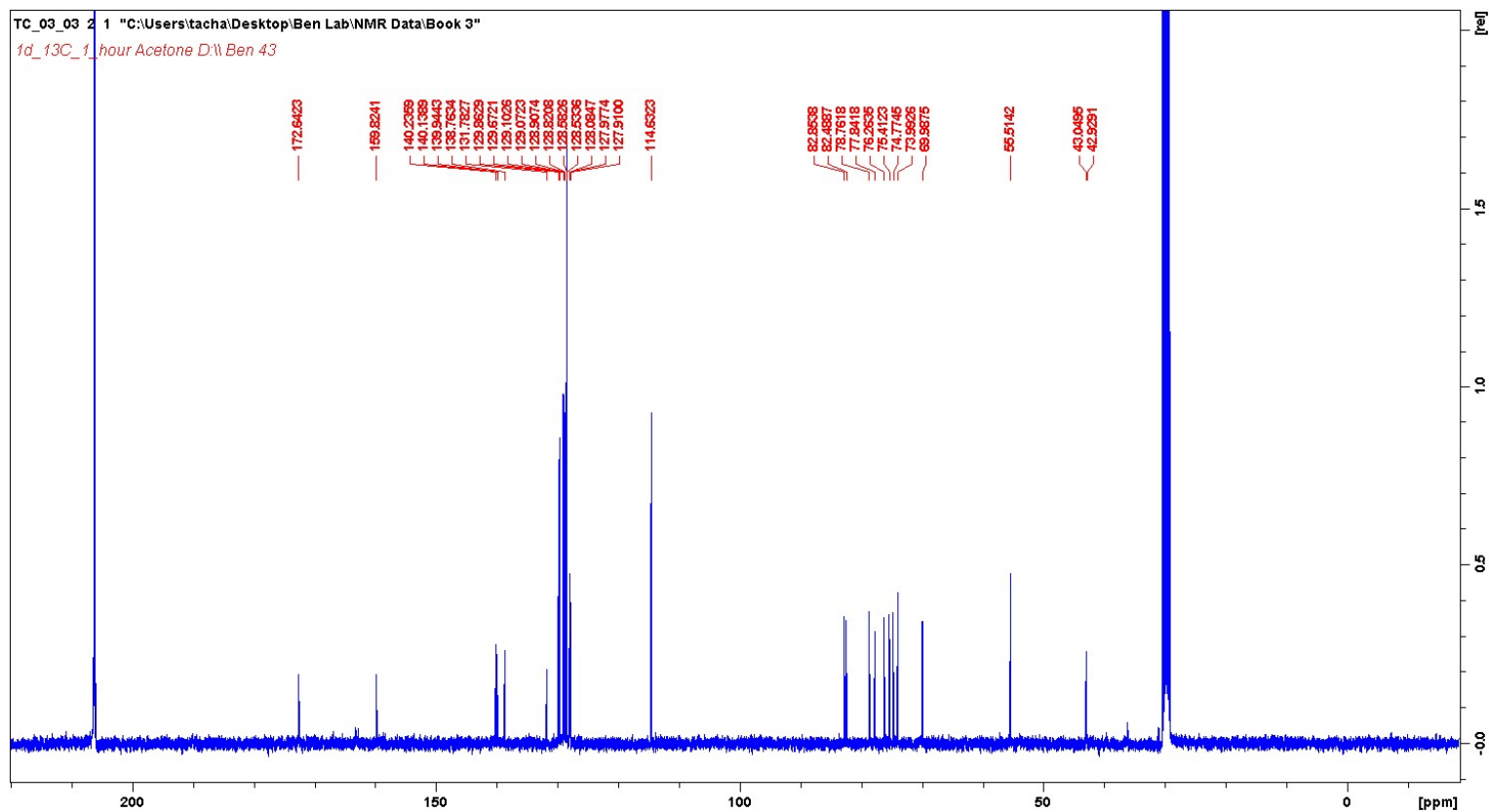
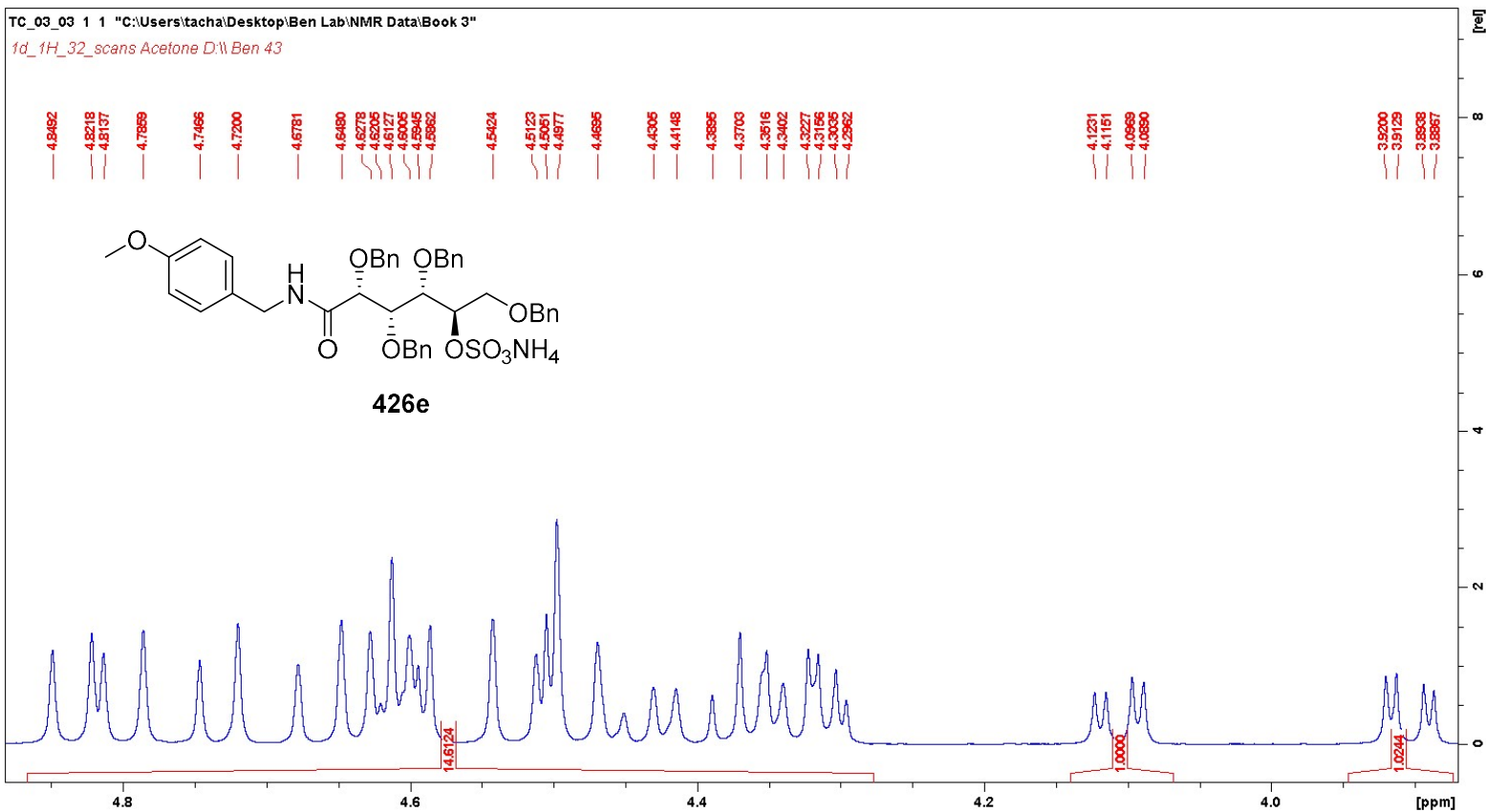


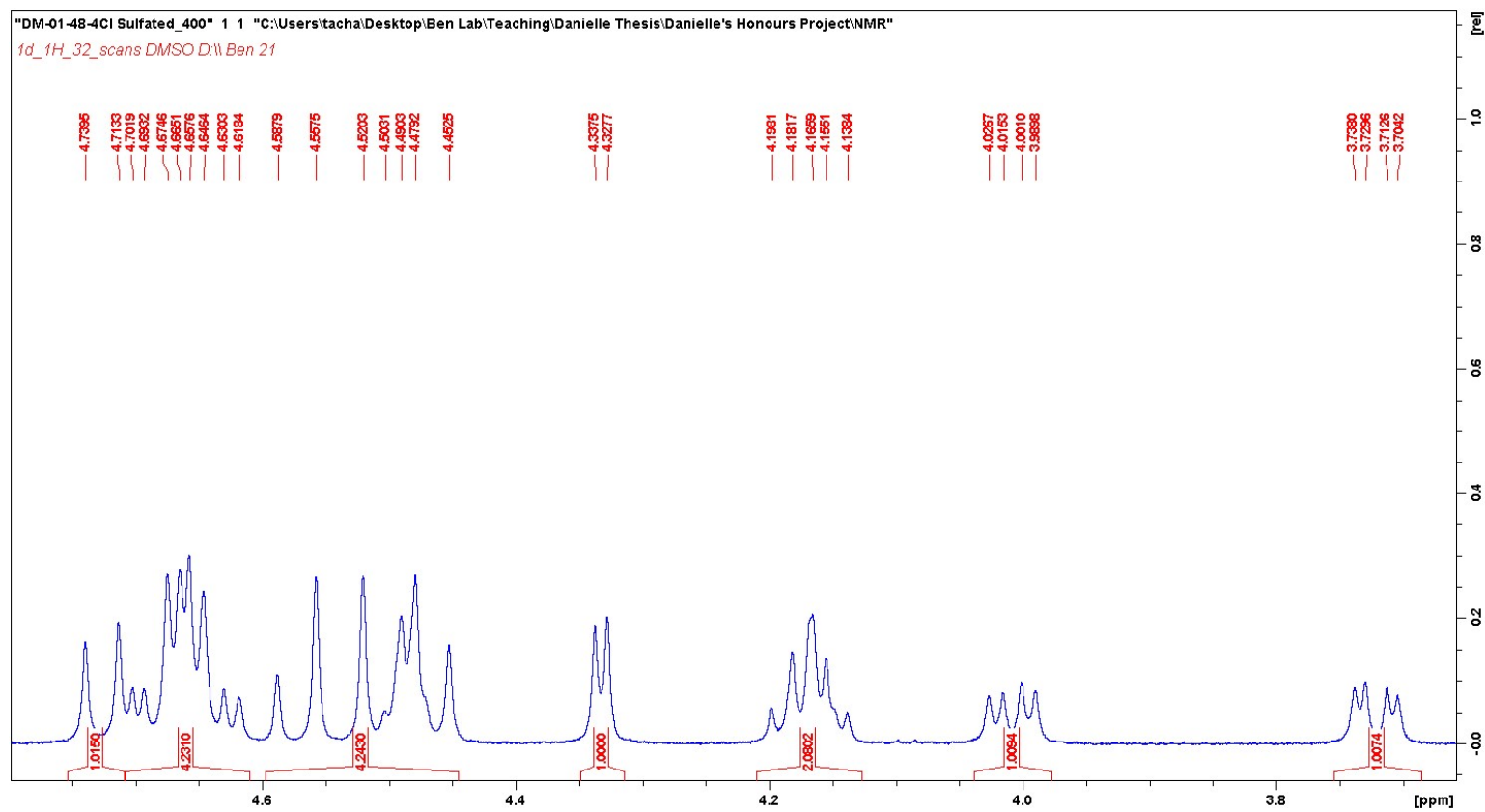
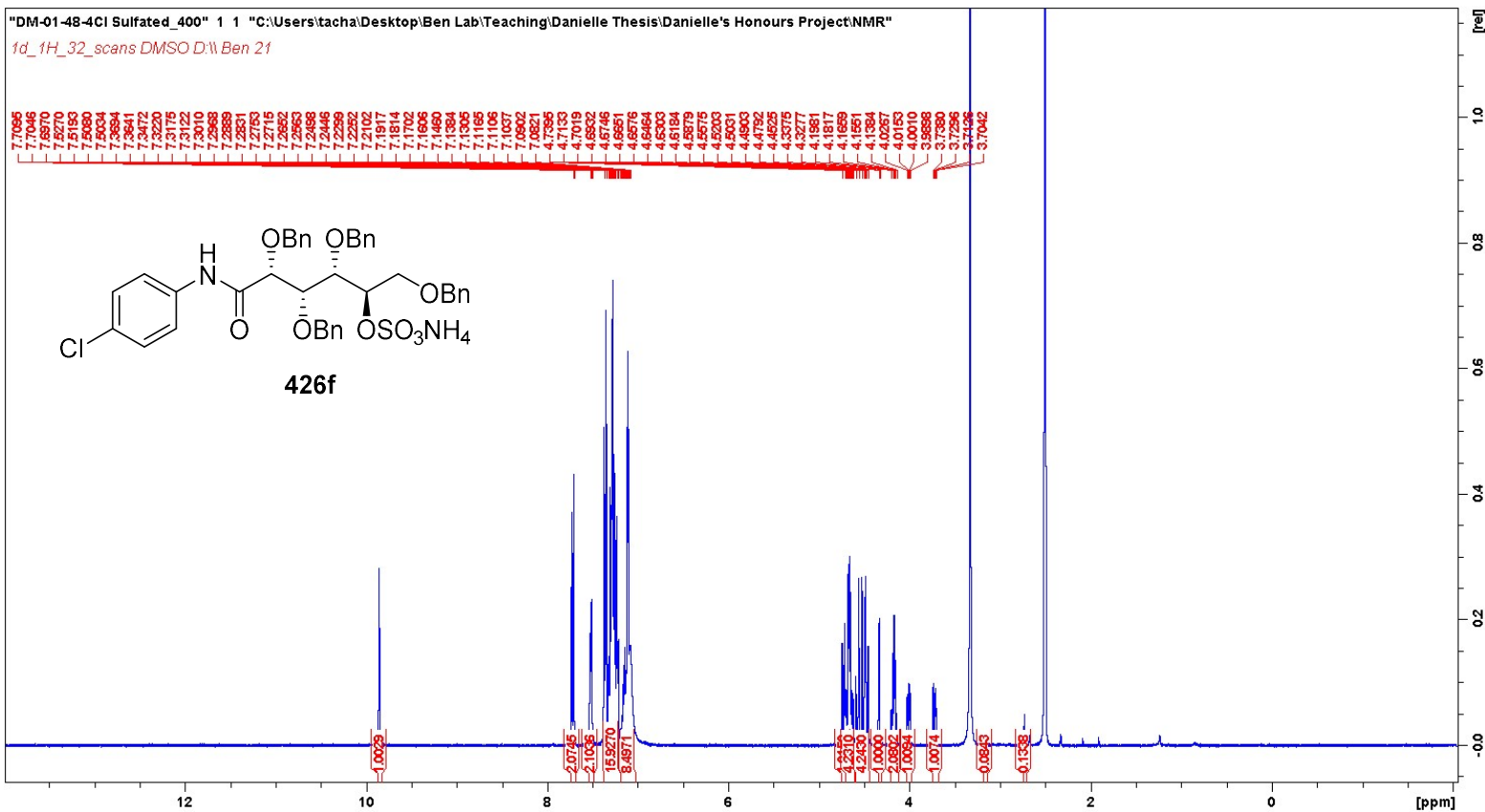
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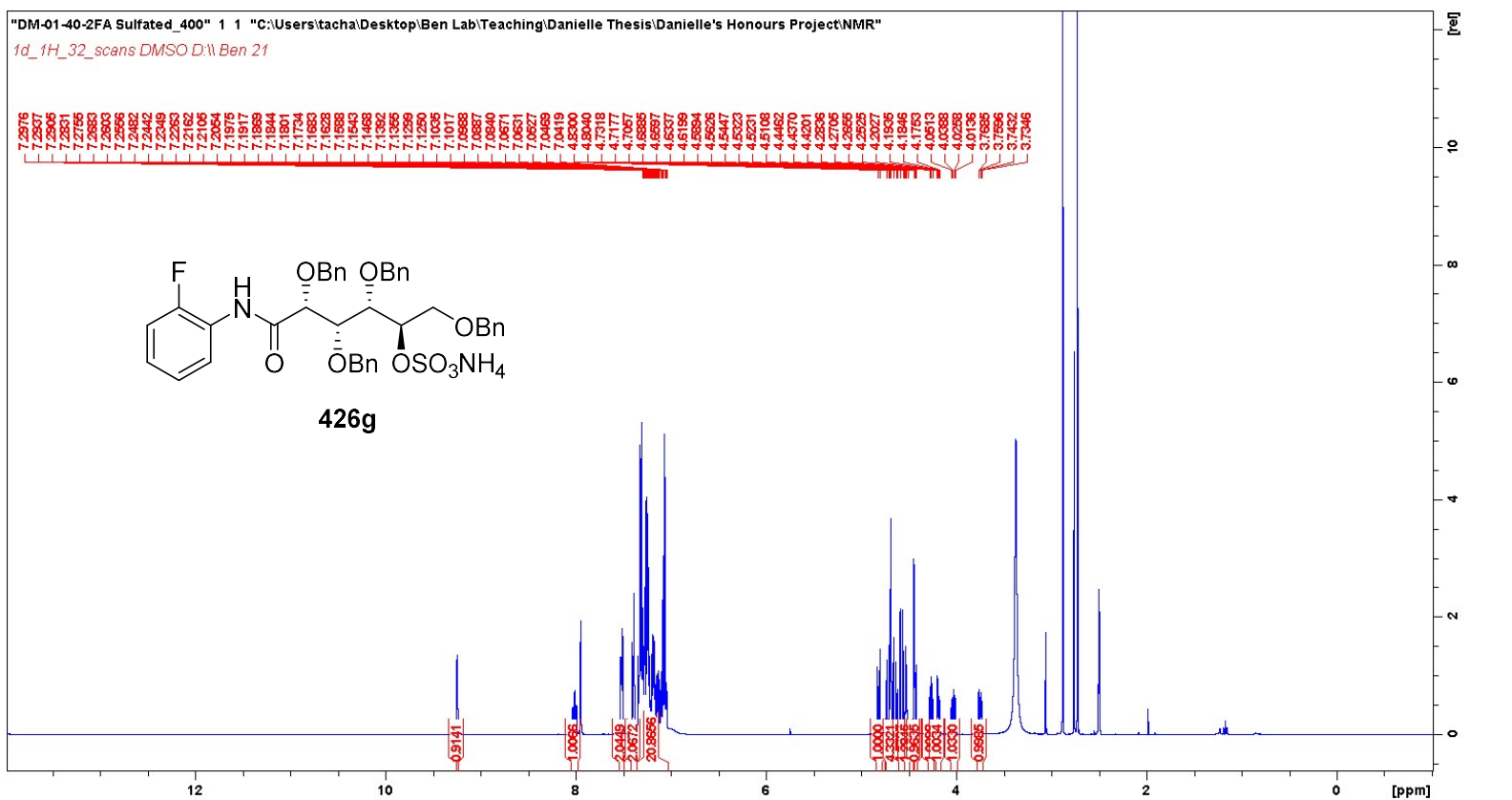
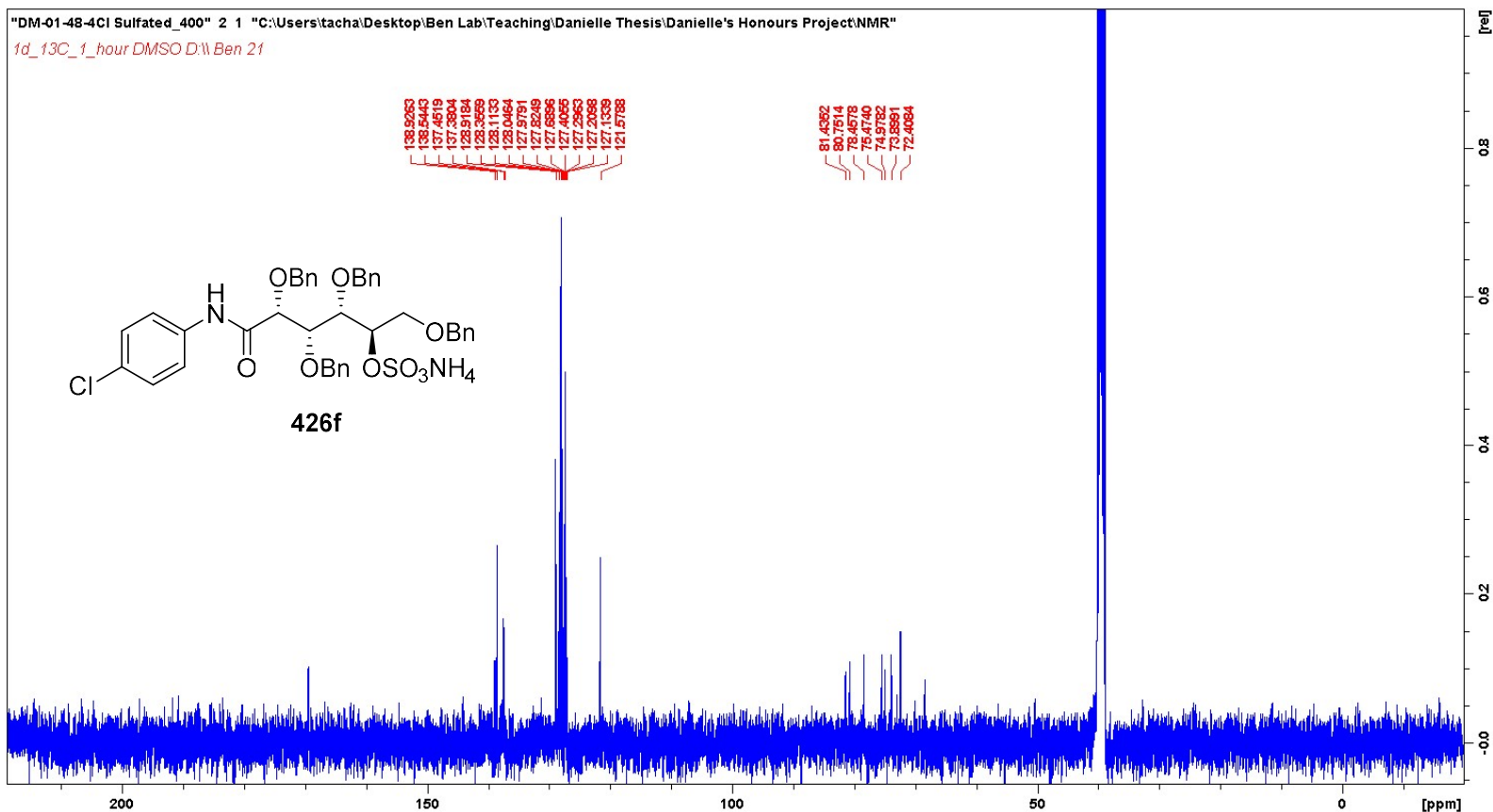
1d_1H_32_scans Acetone D:\Ben 59

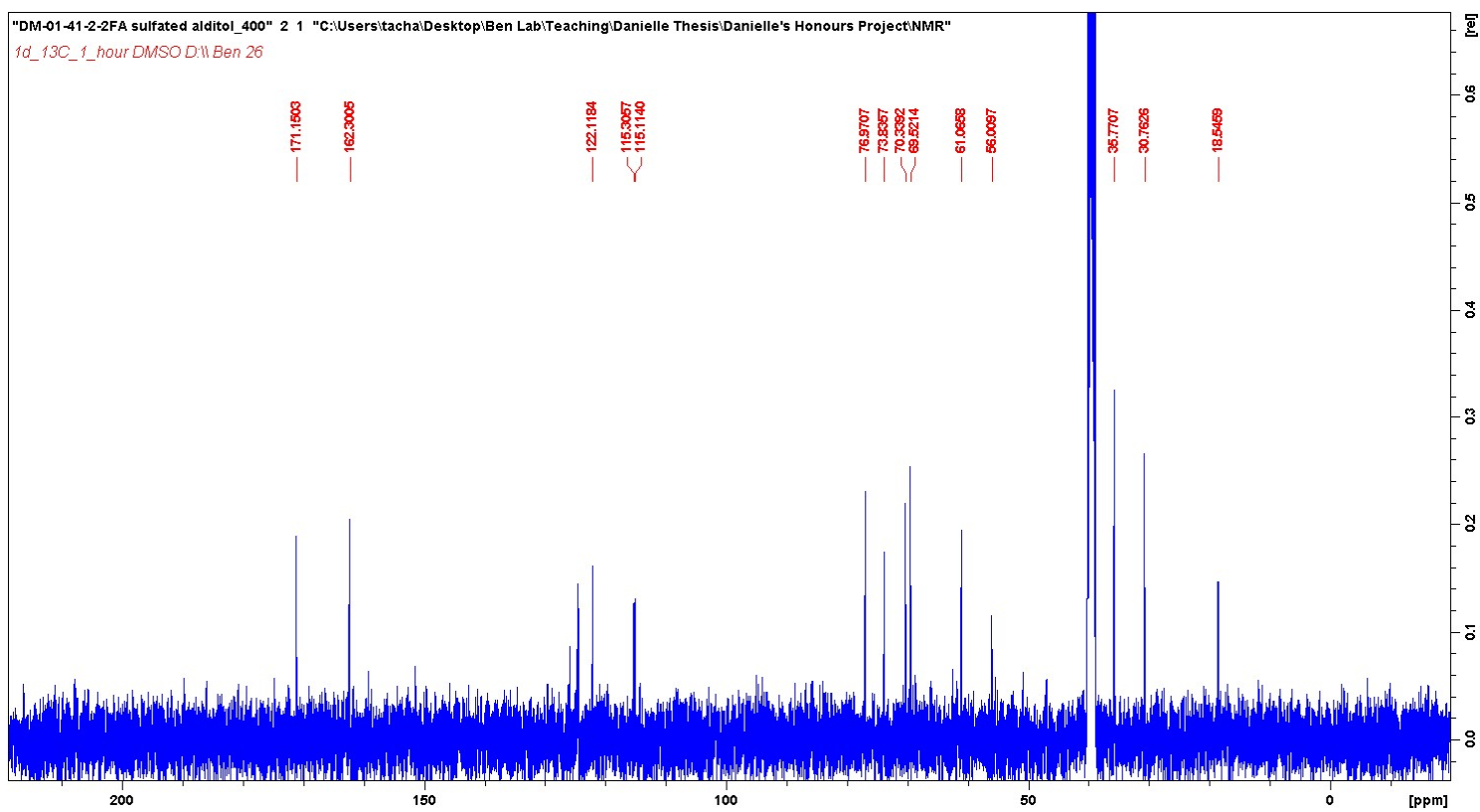
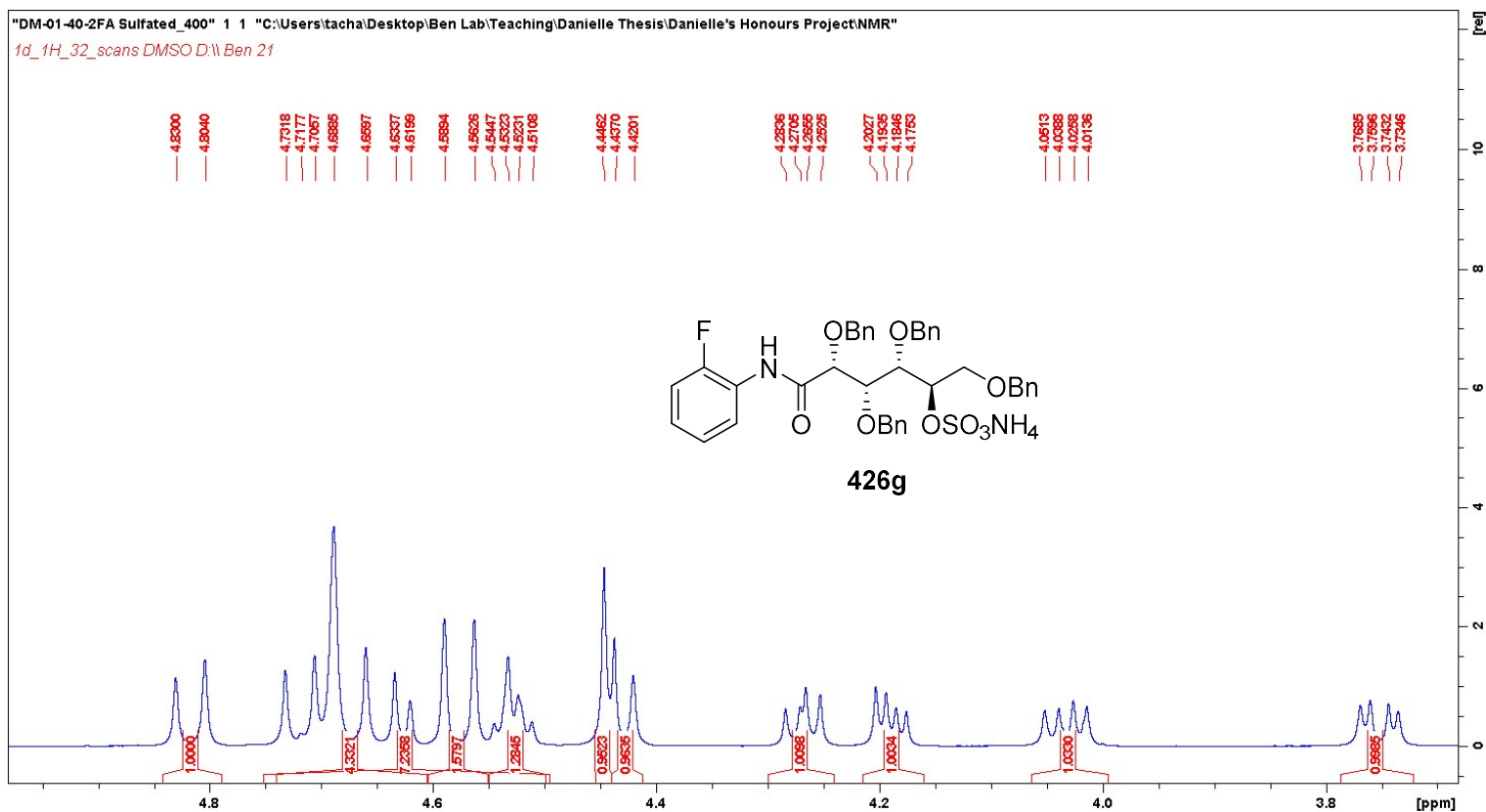






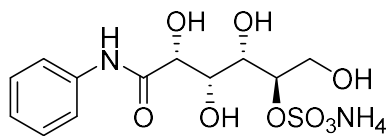




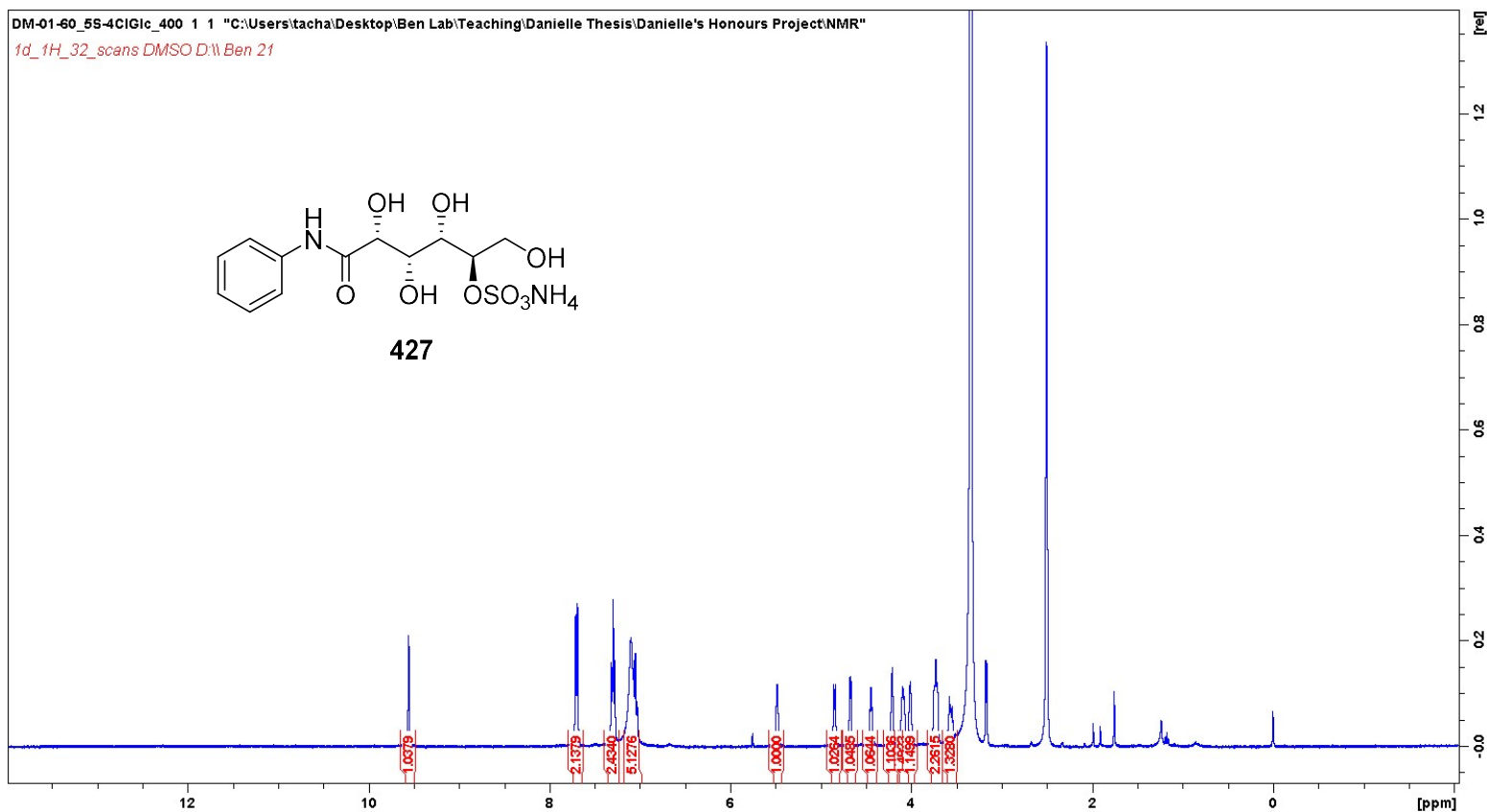


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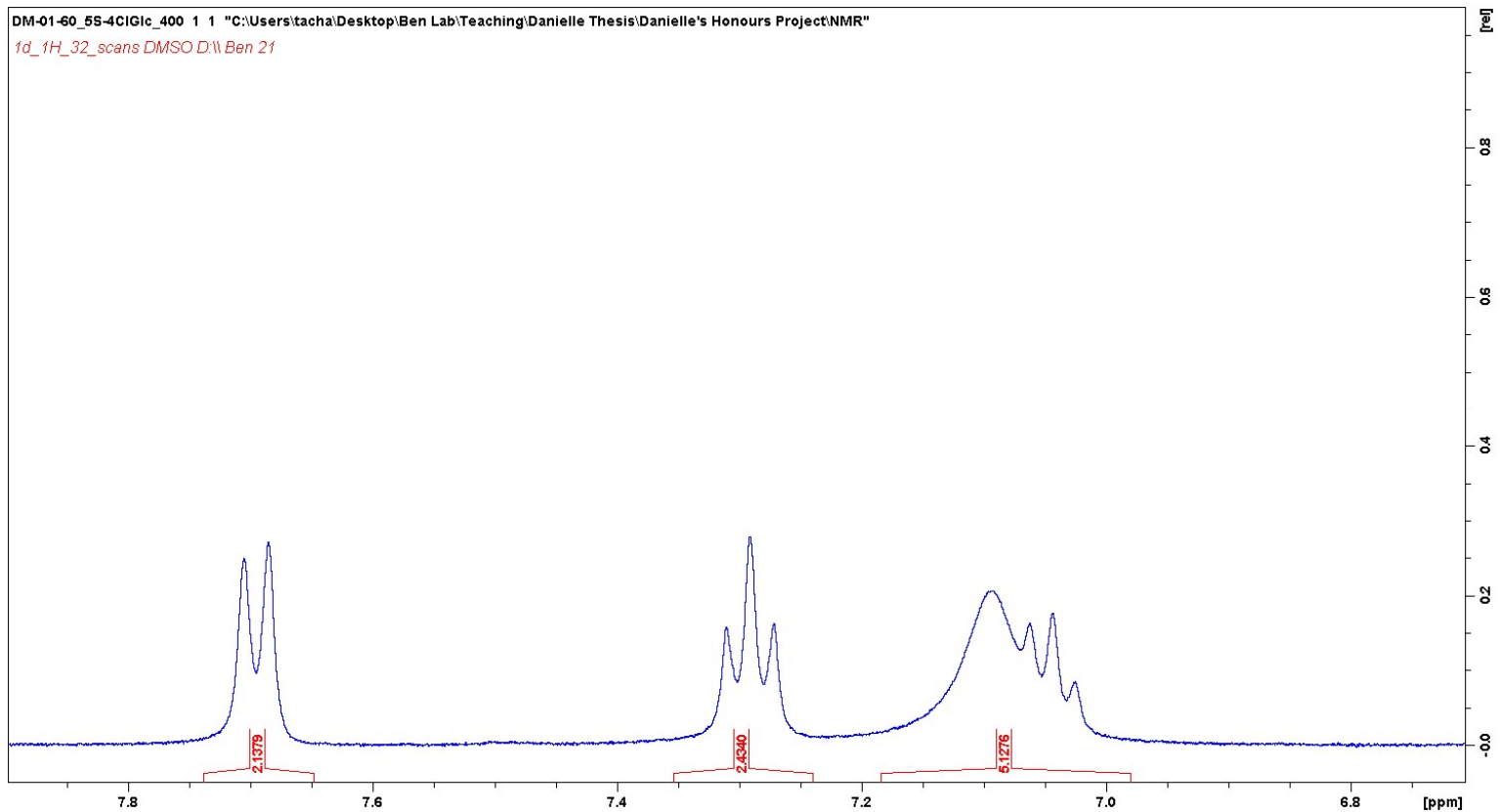


427



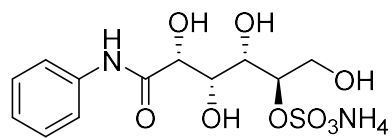
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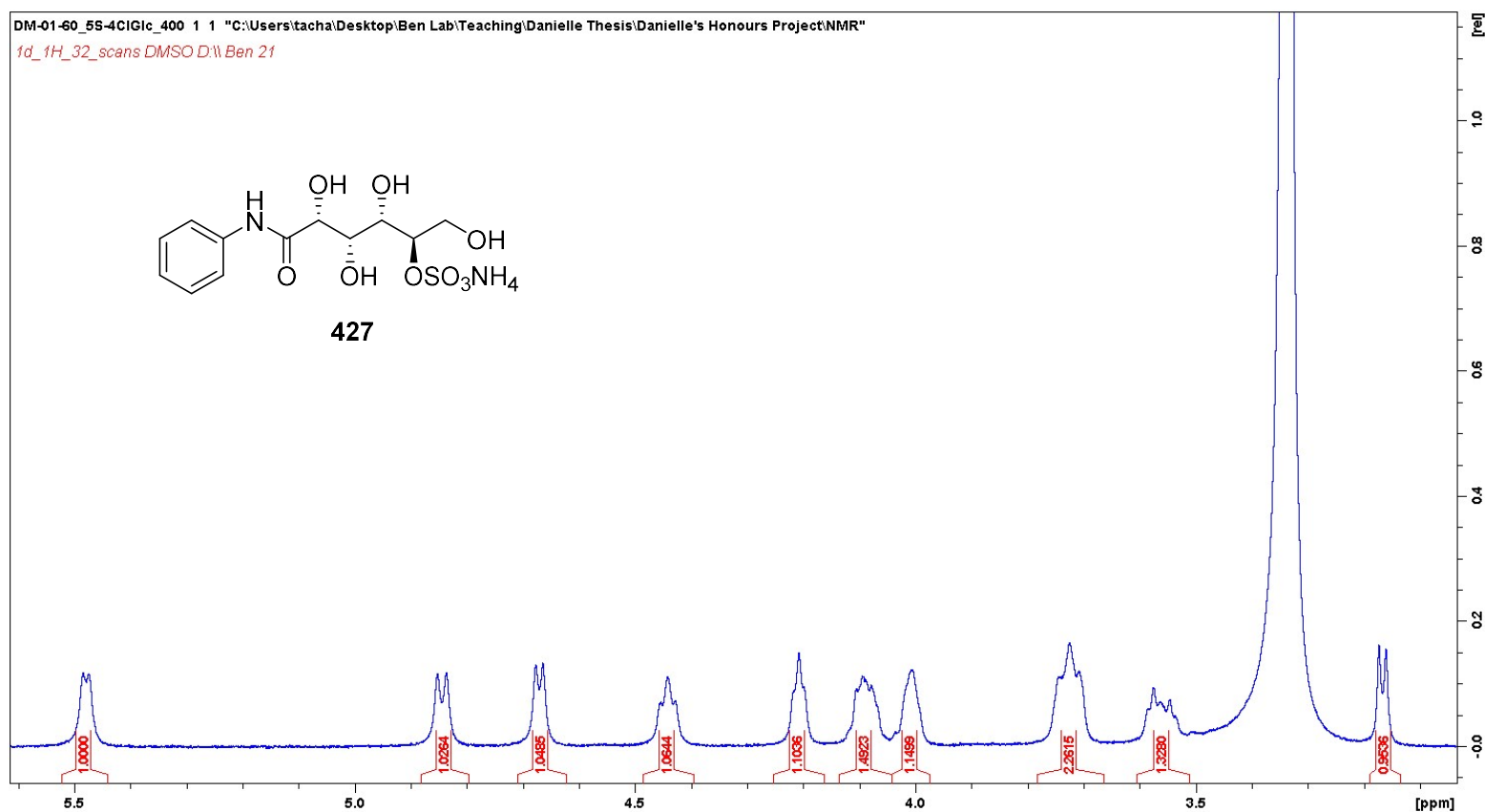


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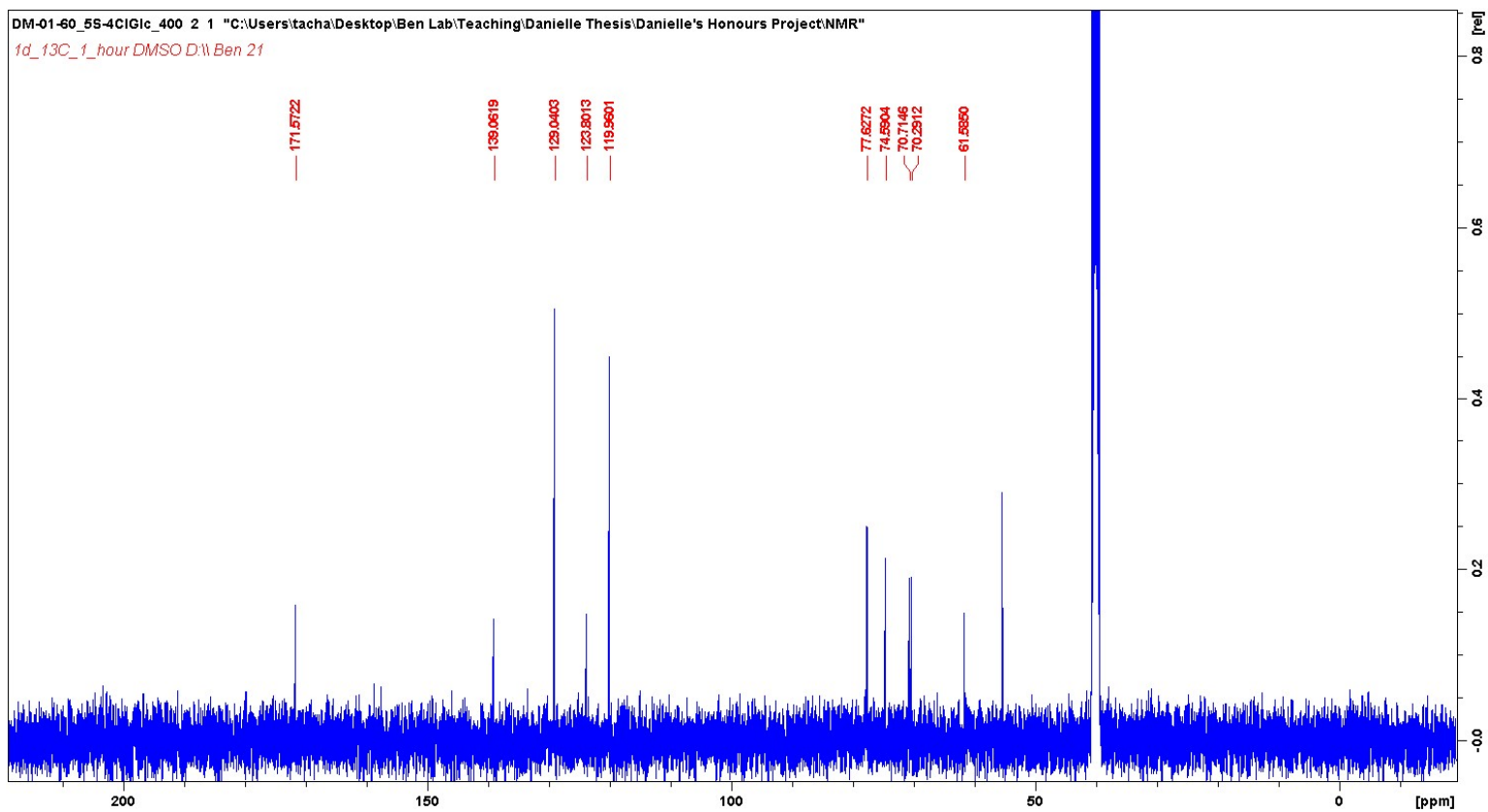


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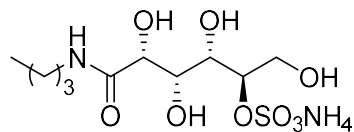
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1d_13C_1_hour DMSO D₆ Ben 2f

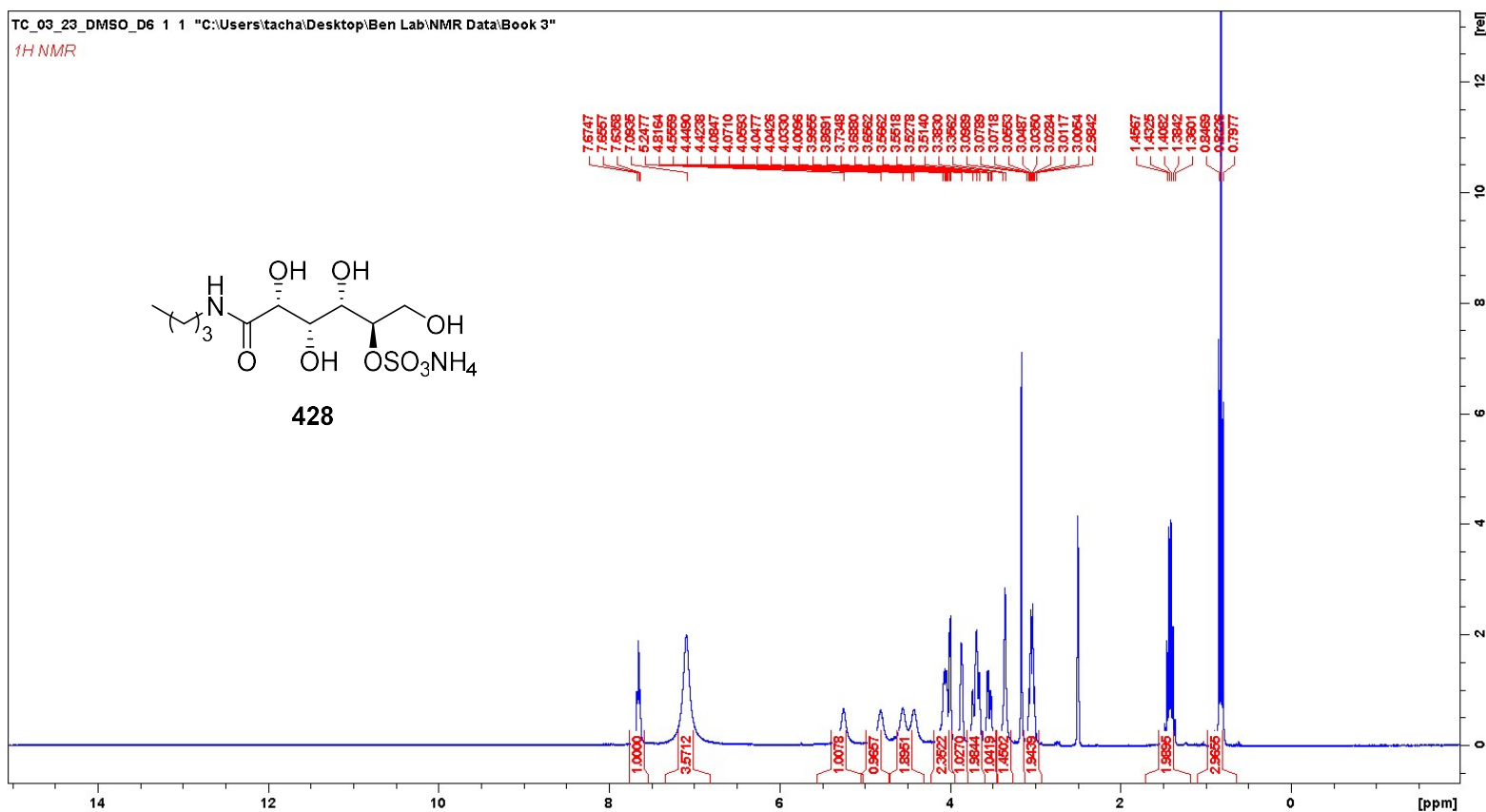


TC_03_23_DMSO_D6 1 1 "C:\Users\tacha\Desktop\Ben Lab\NMR Data\Book 3"

¹H NMR

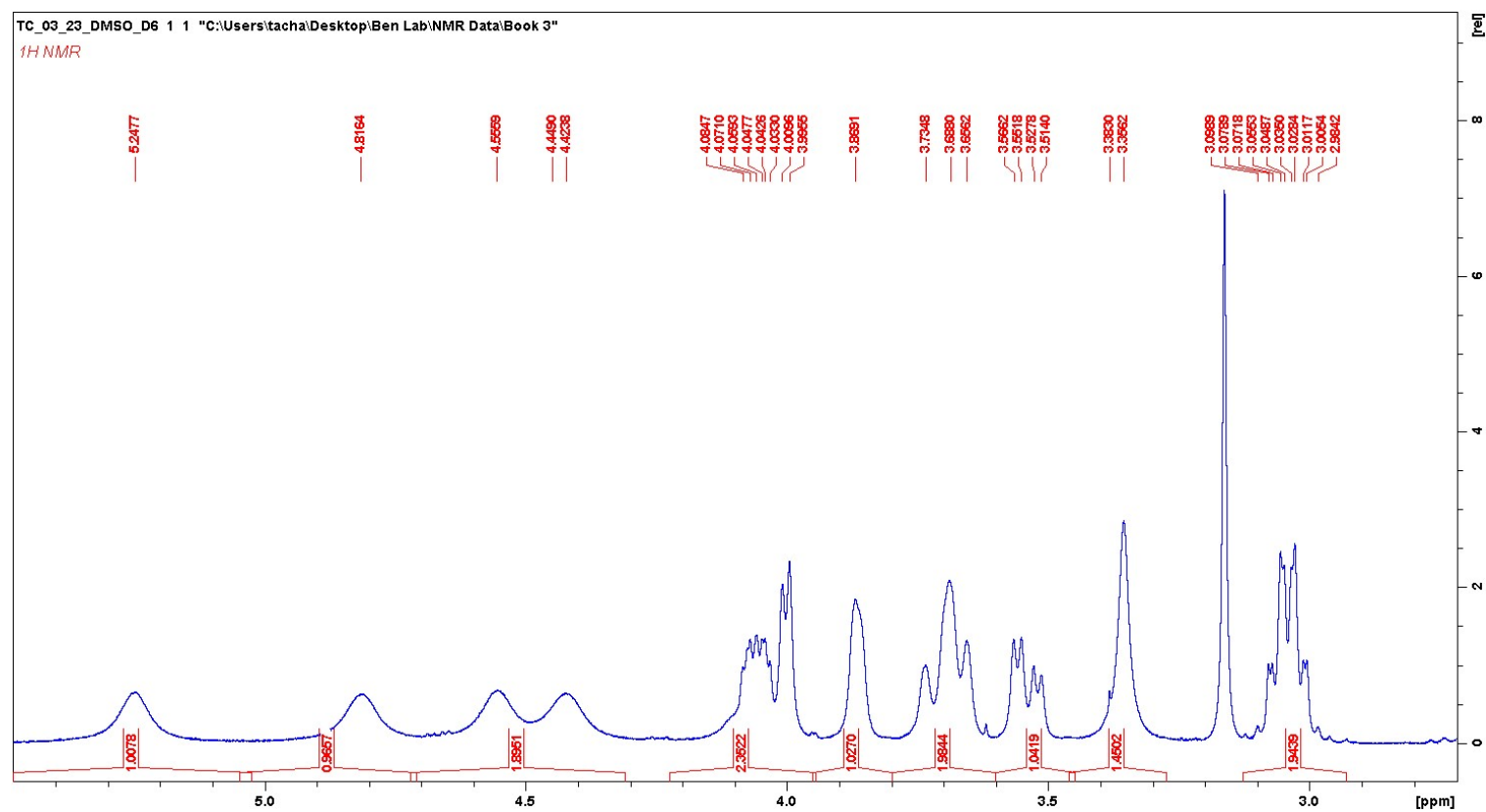


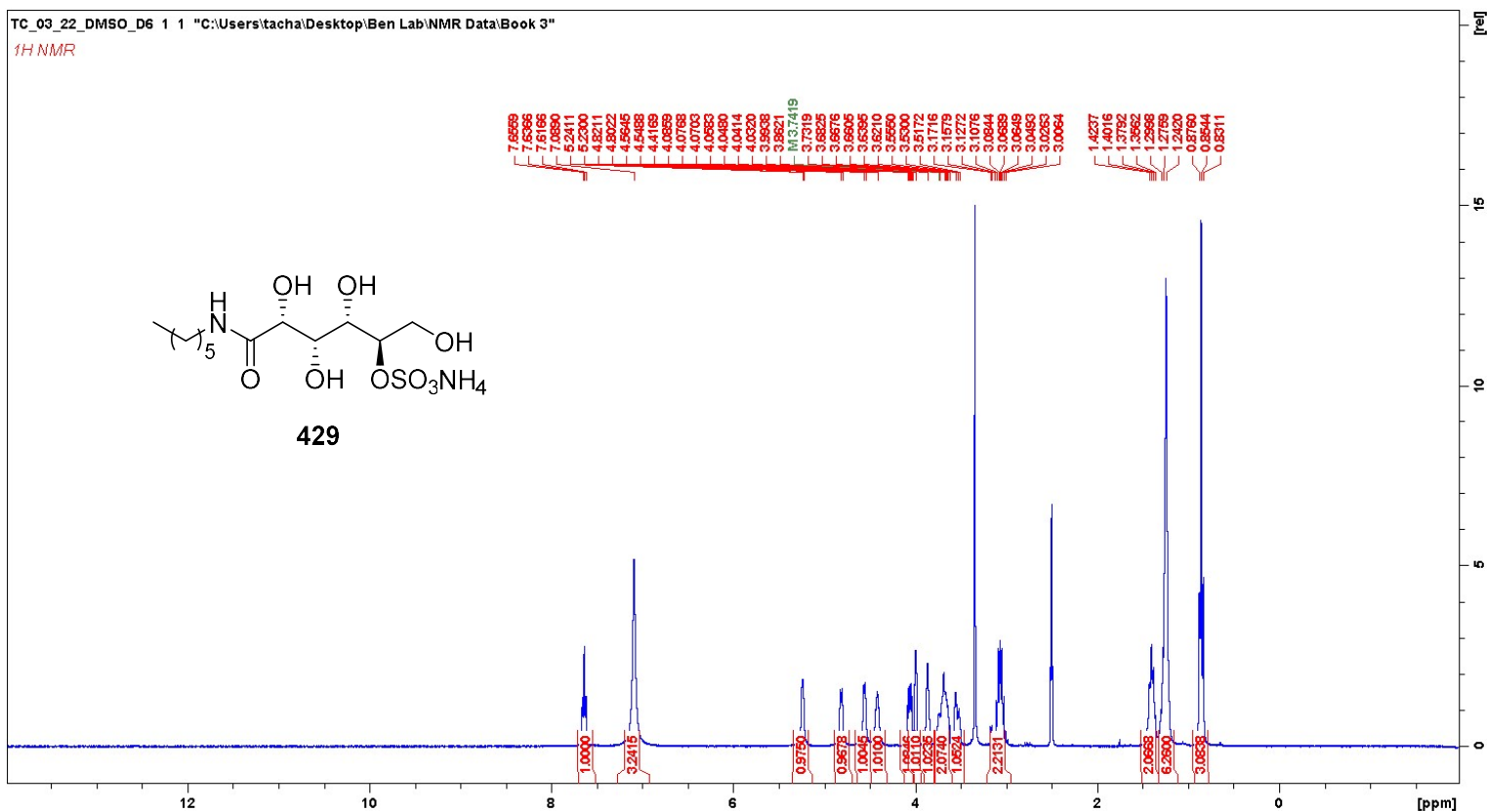
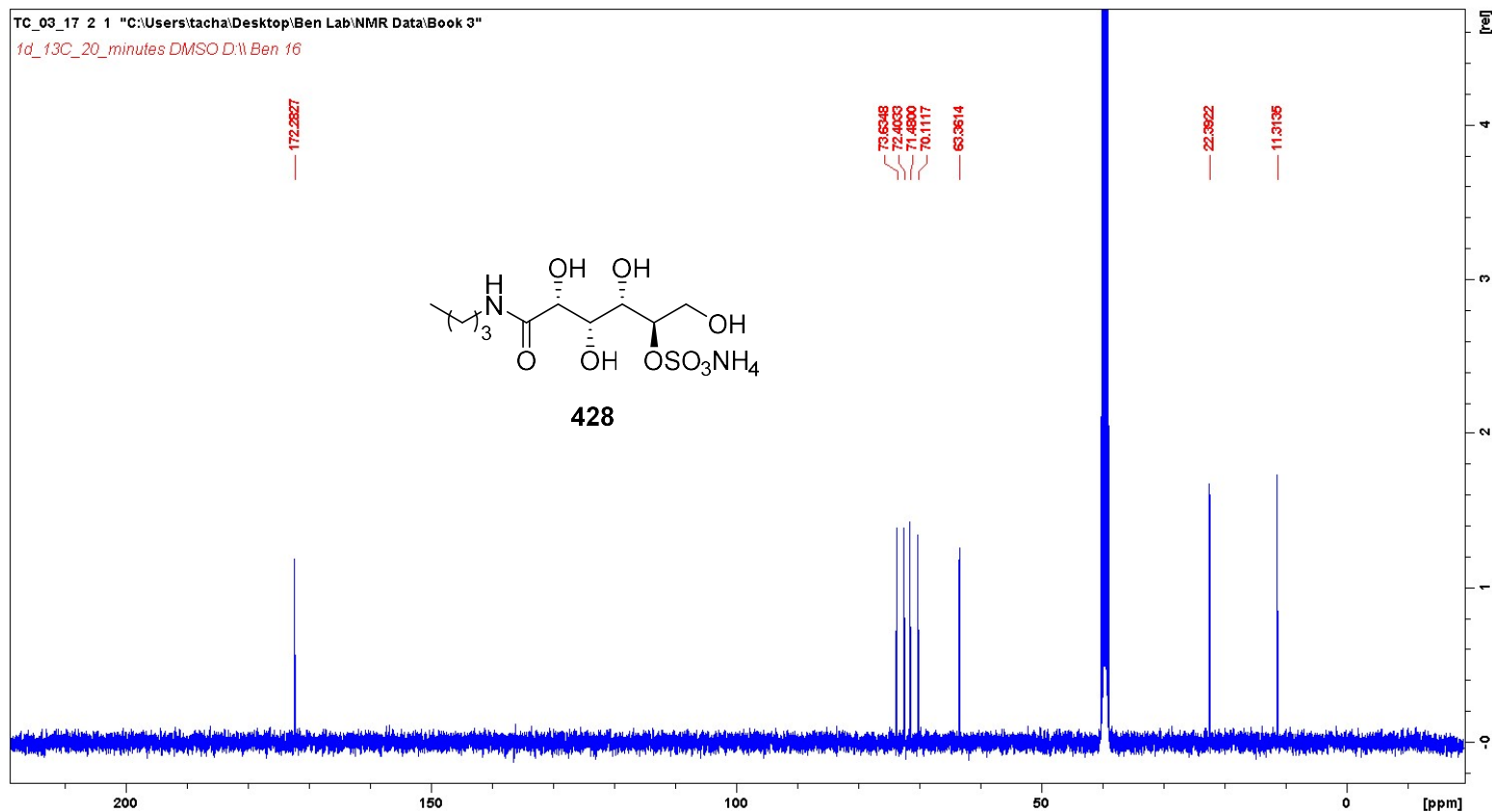
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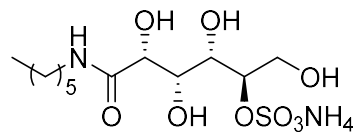
¹H NMR



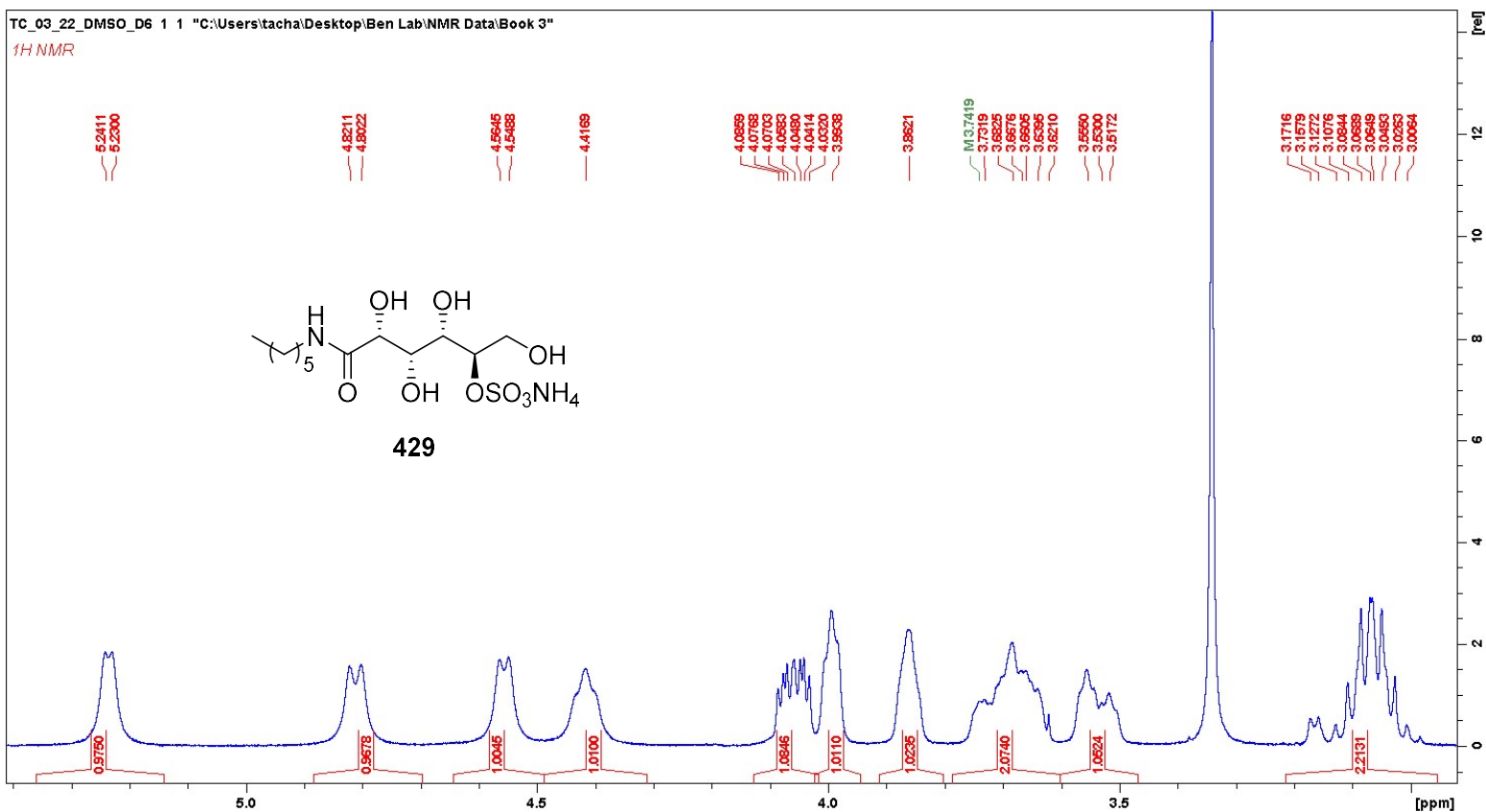


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¹H NMR

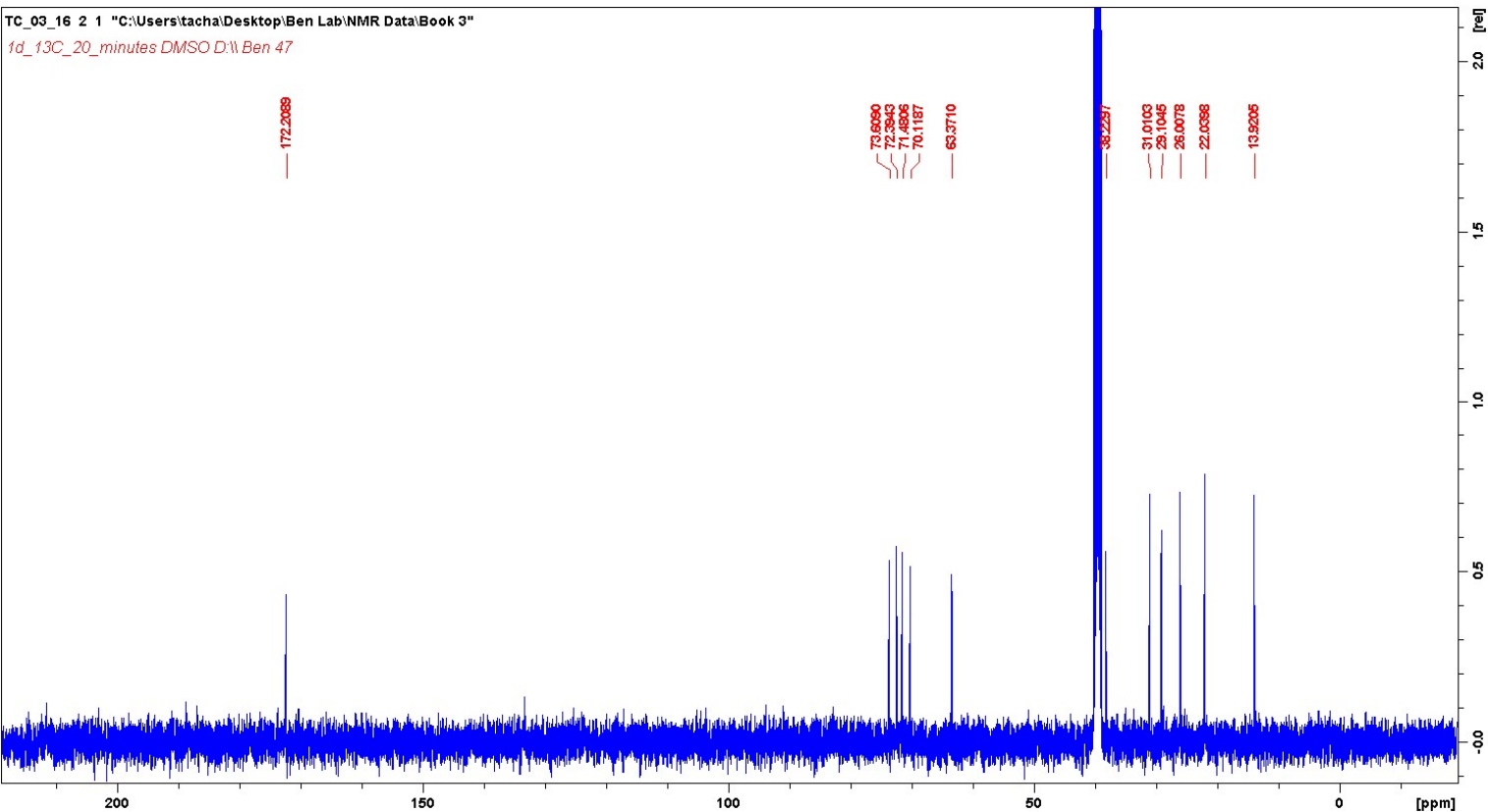


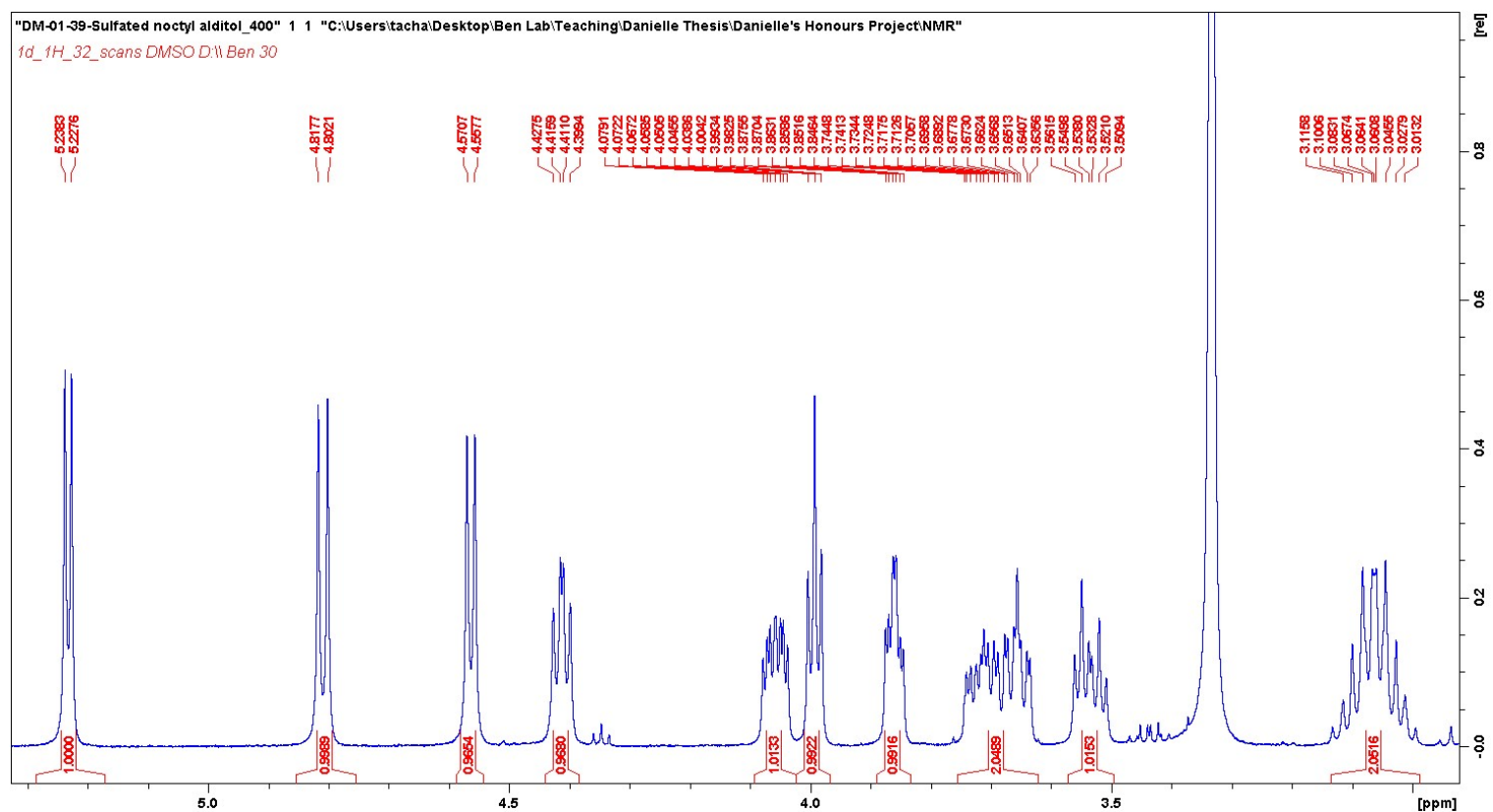
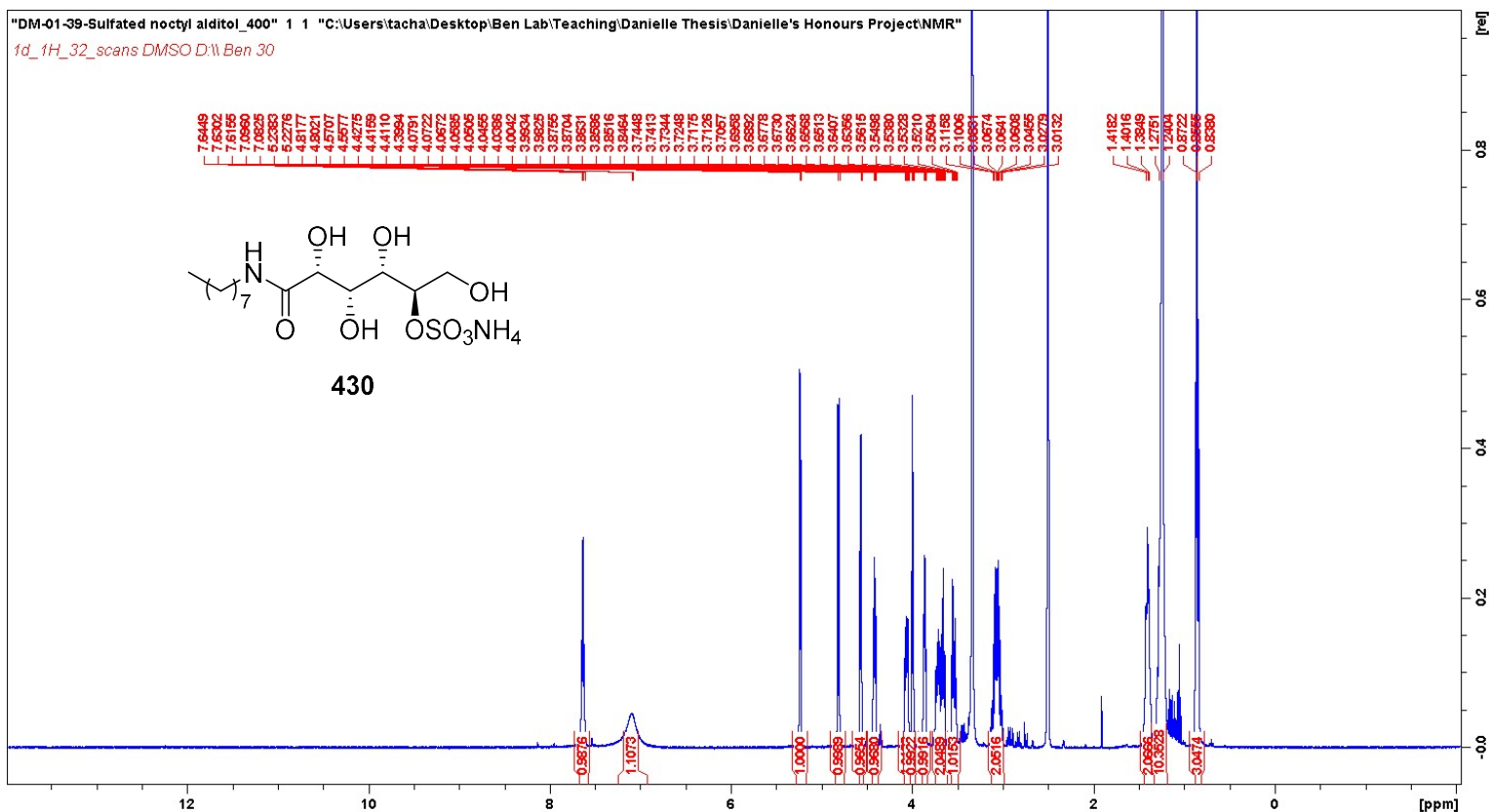
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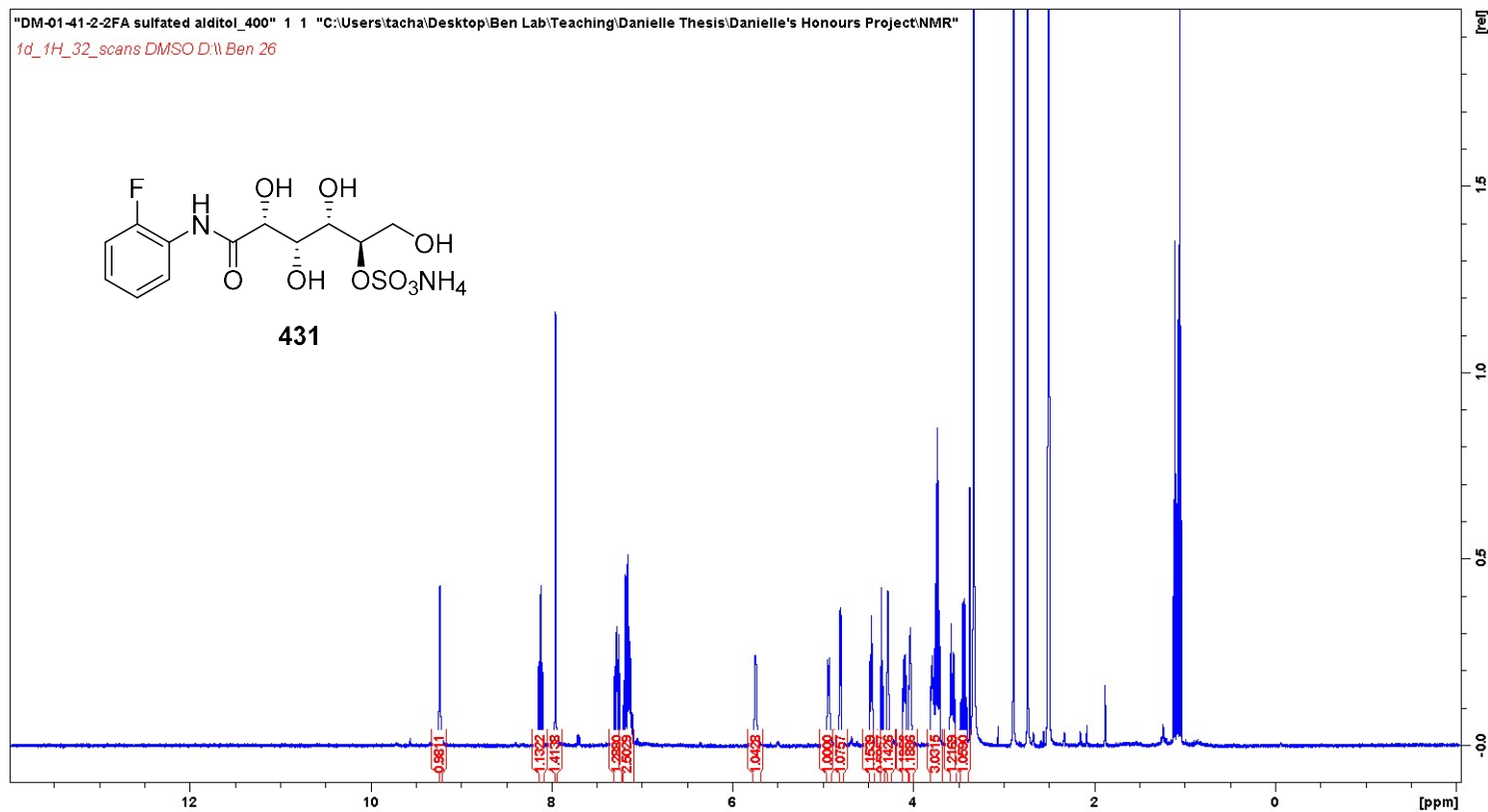
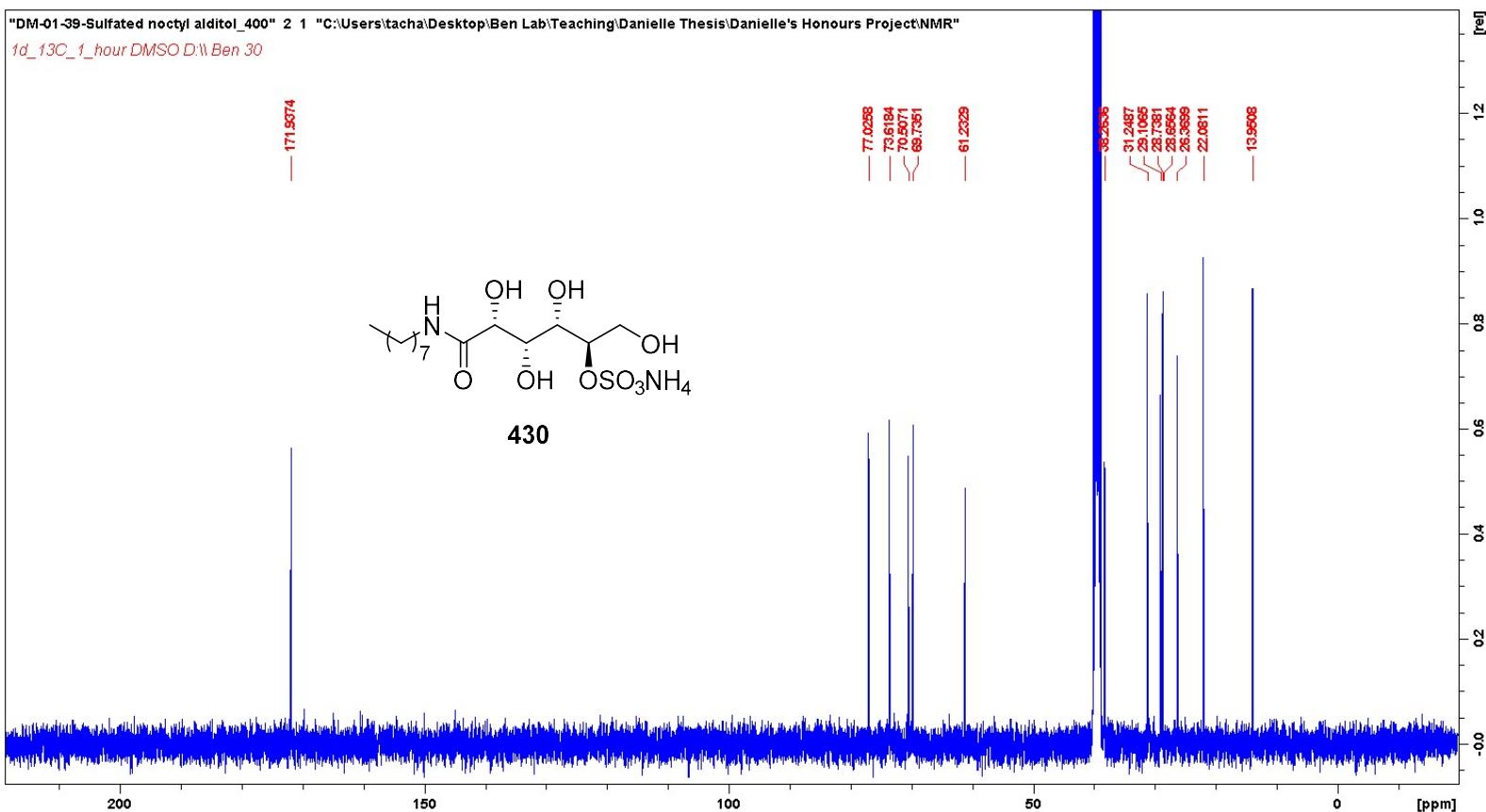


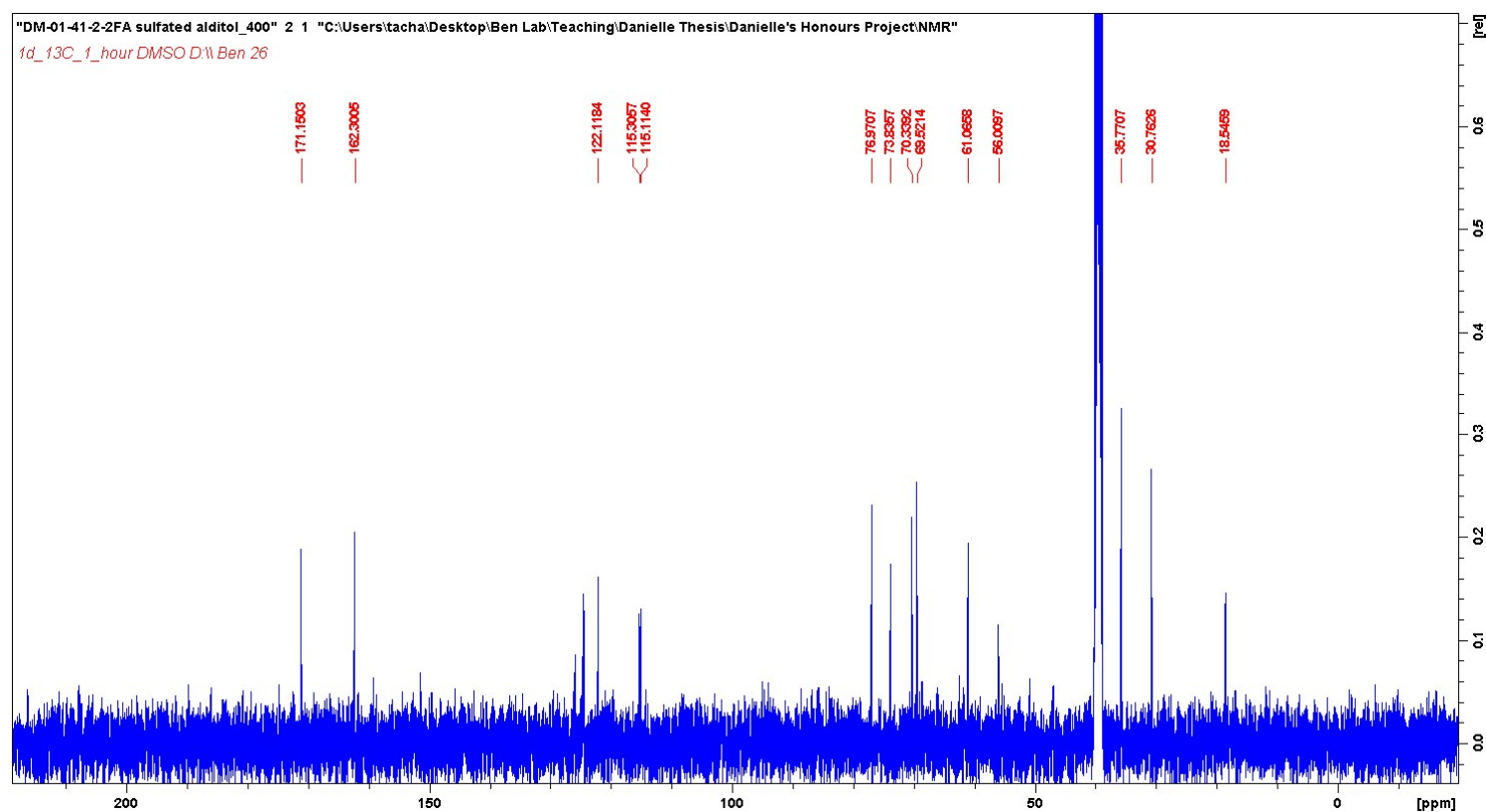
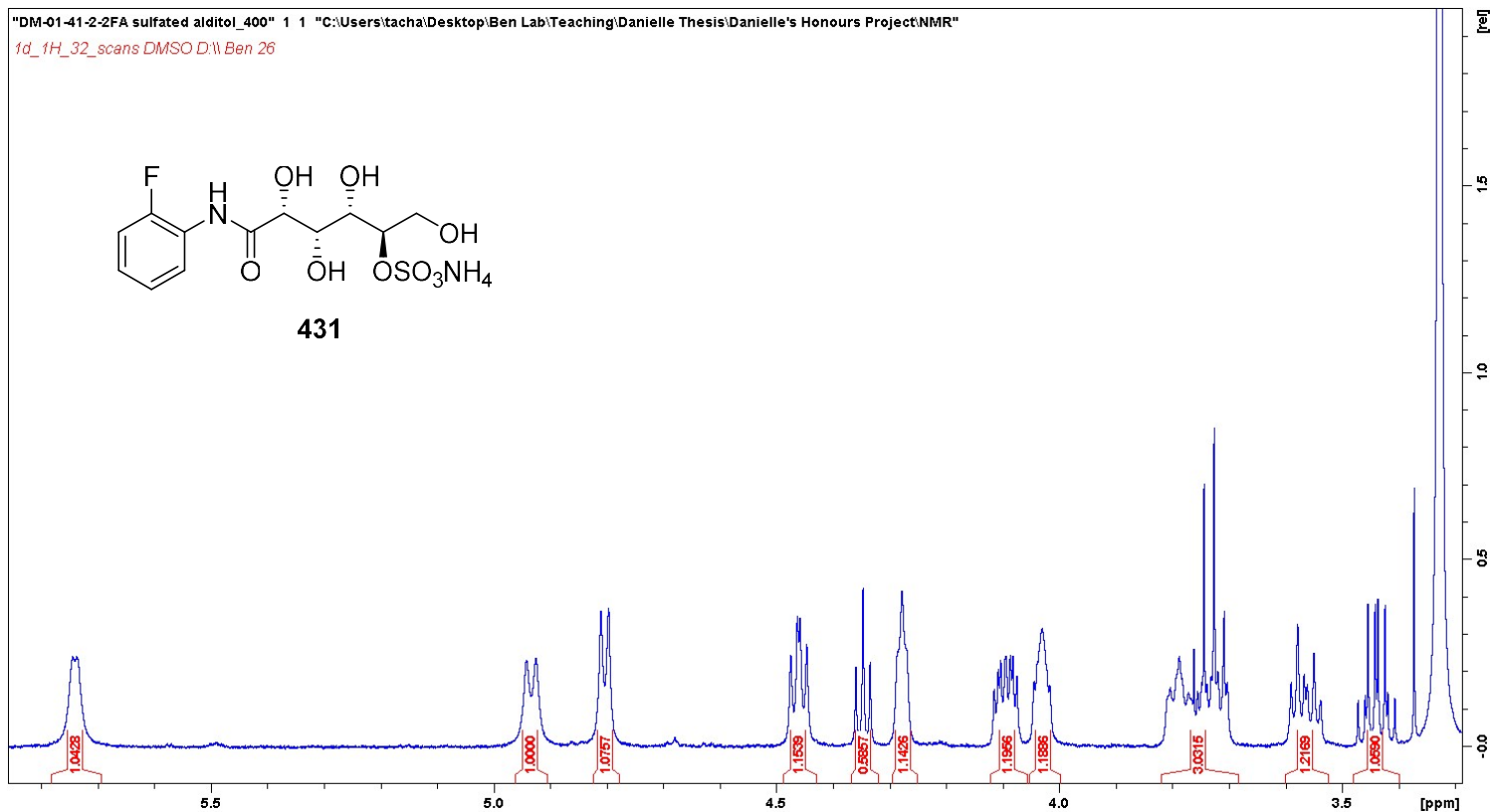
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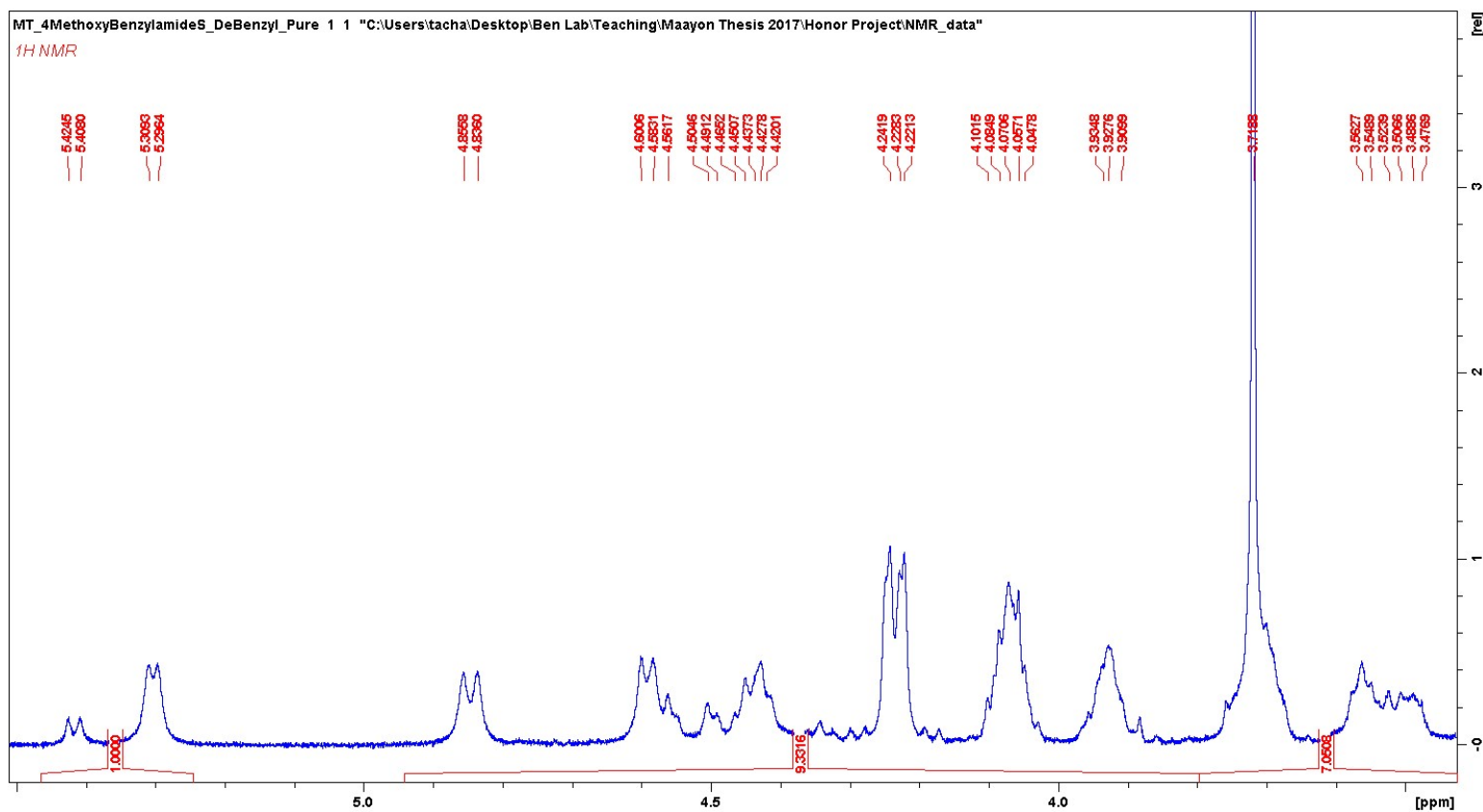
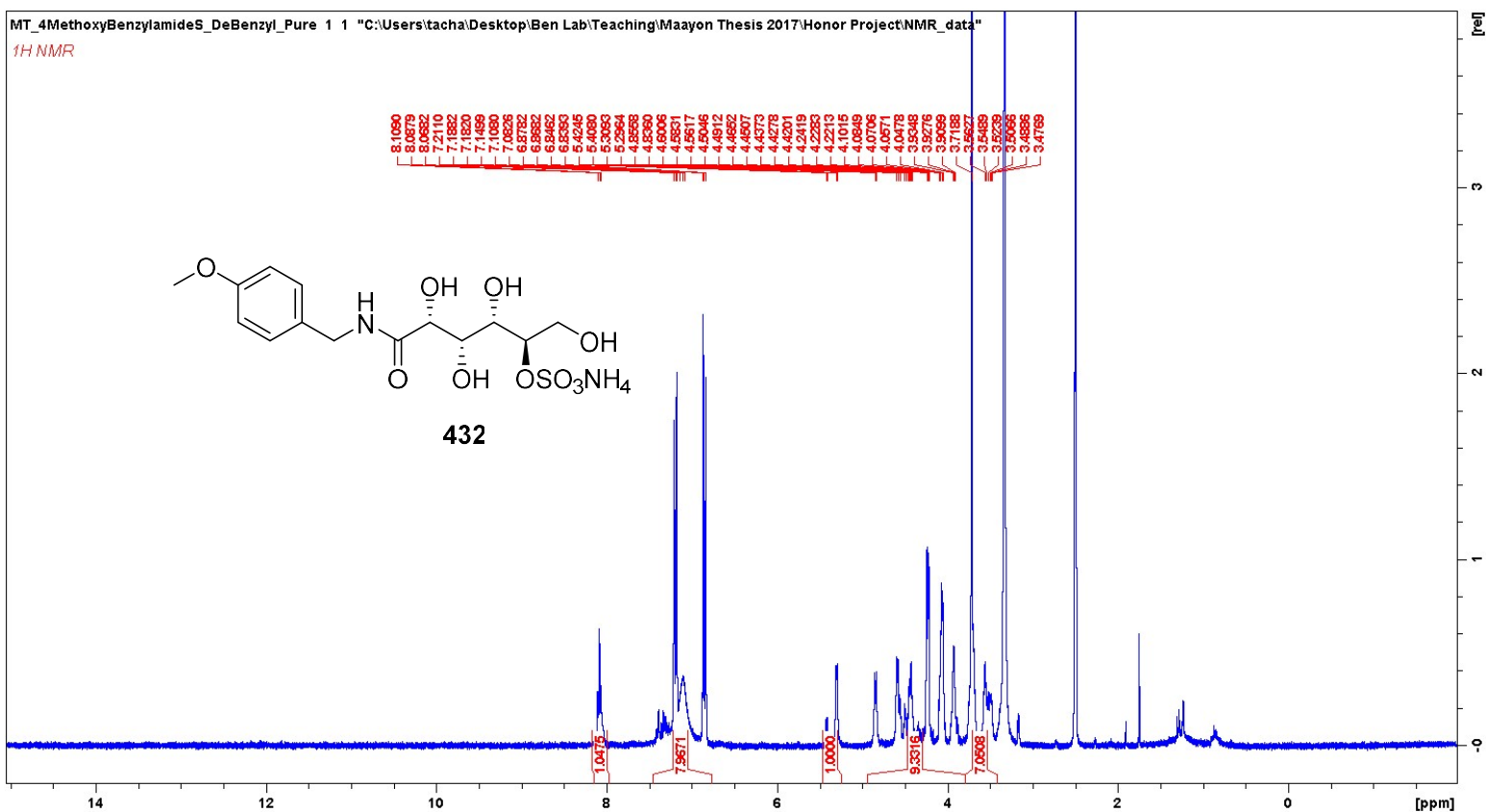
1d_13C_20_minutes DMSO D:\Ben 47

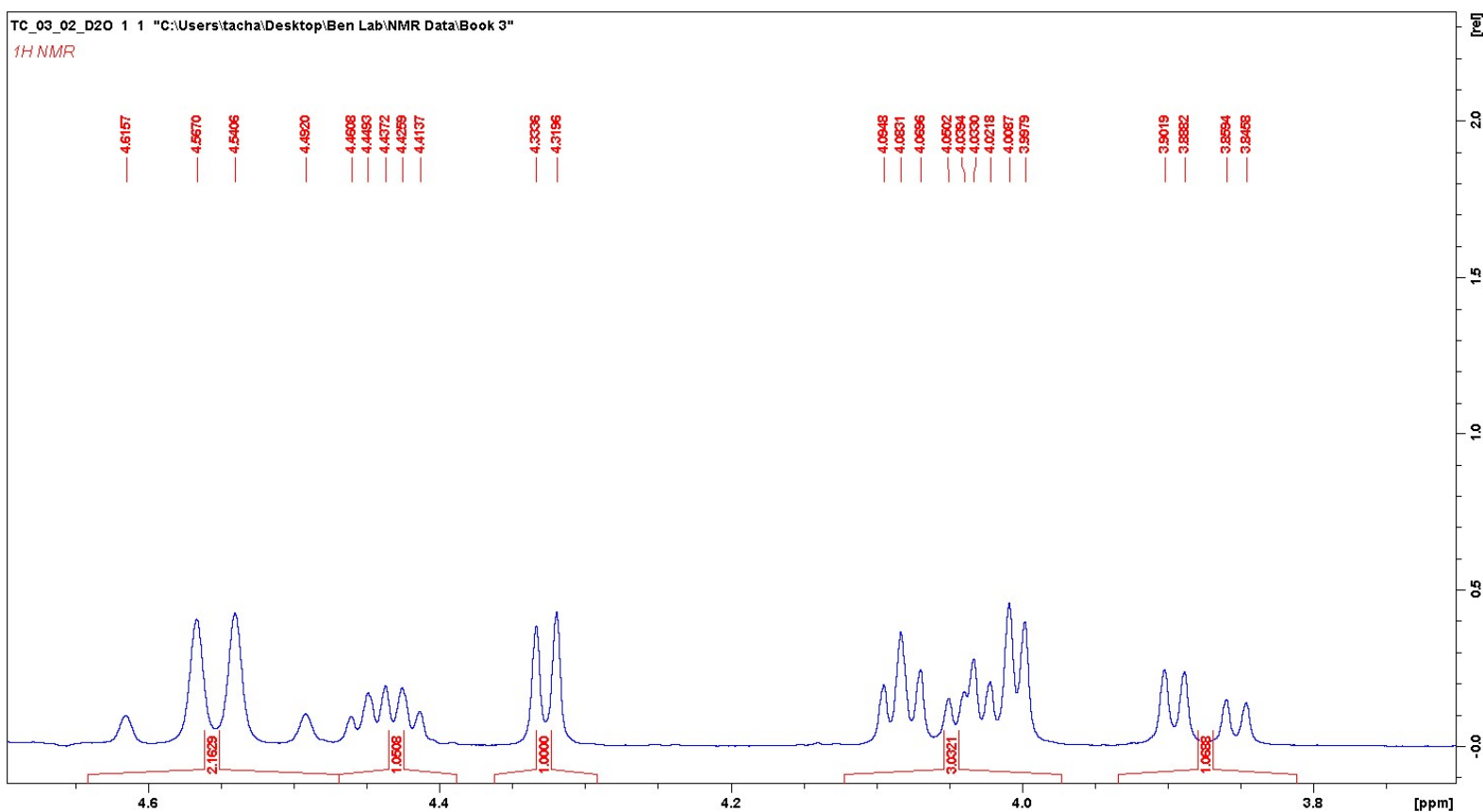
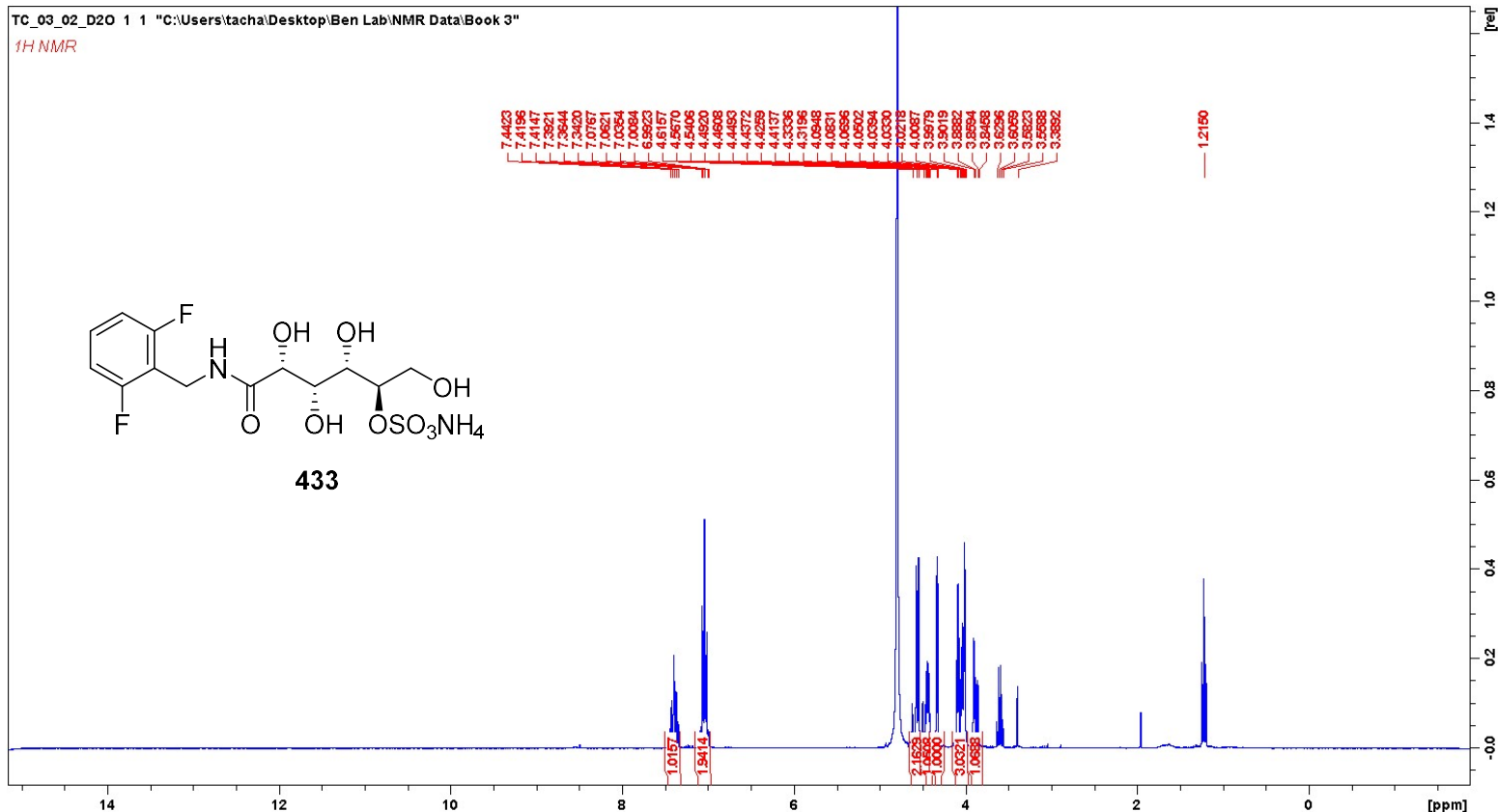


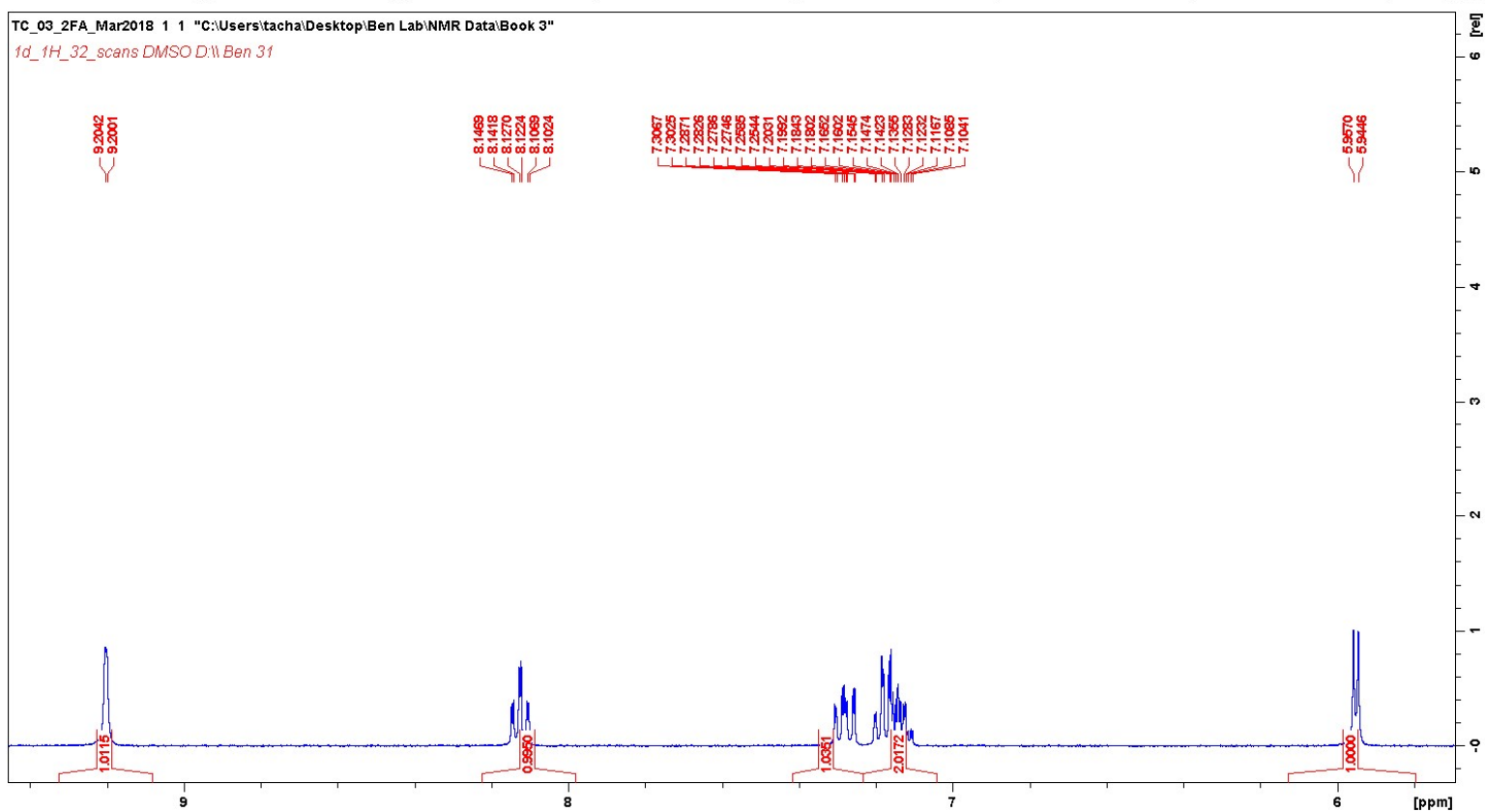
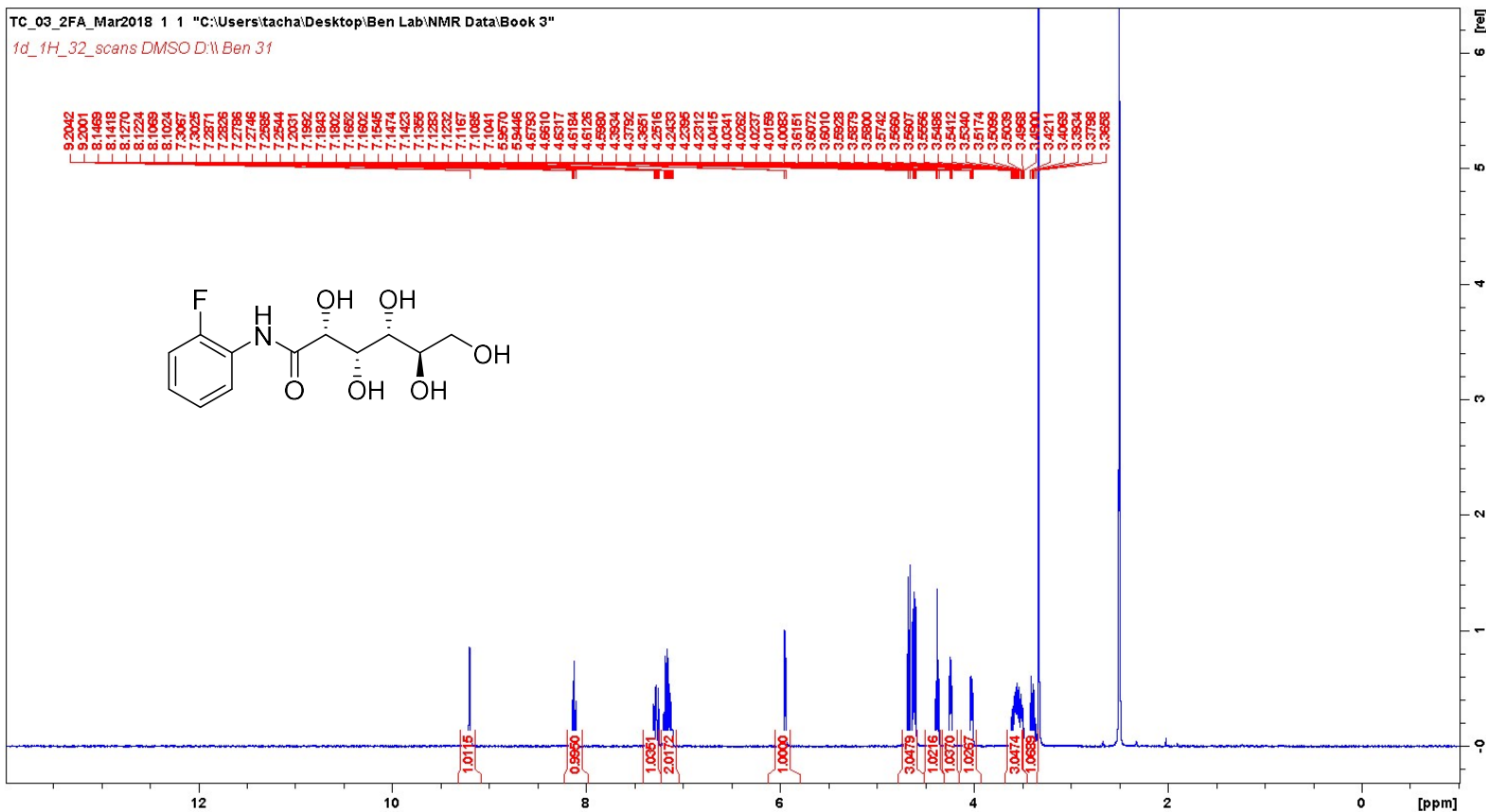






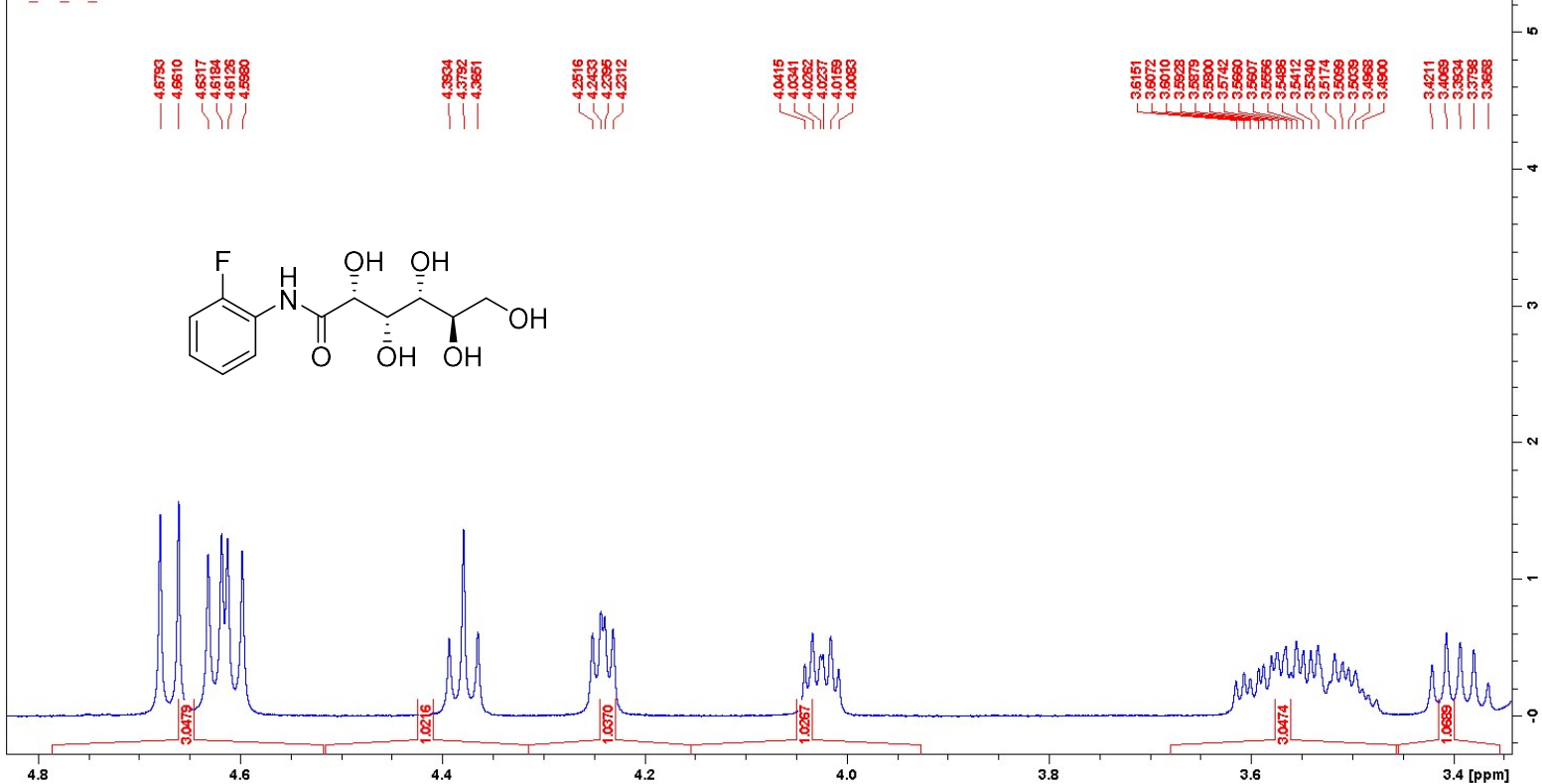






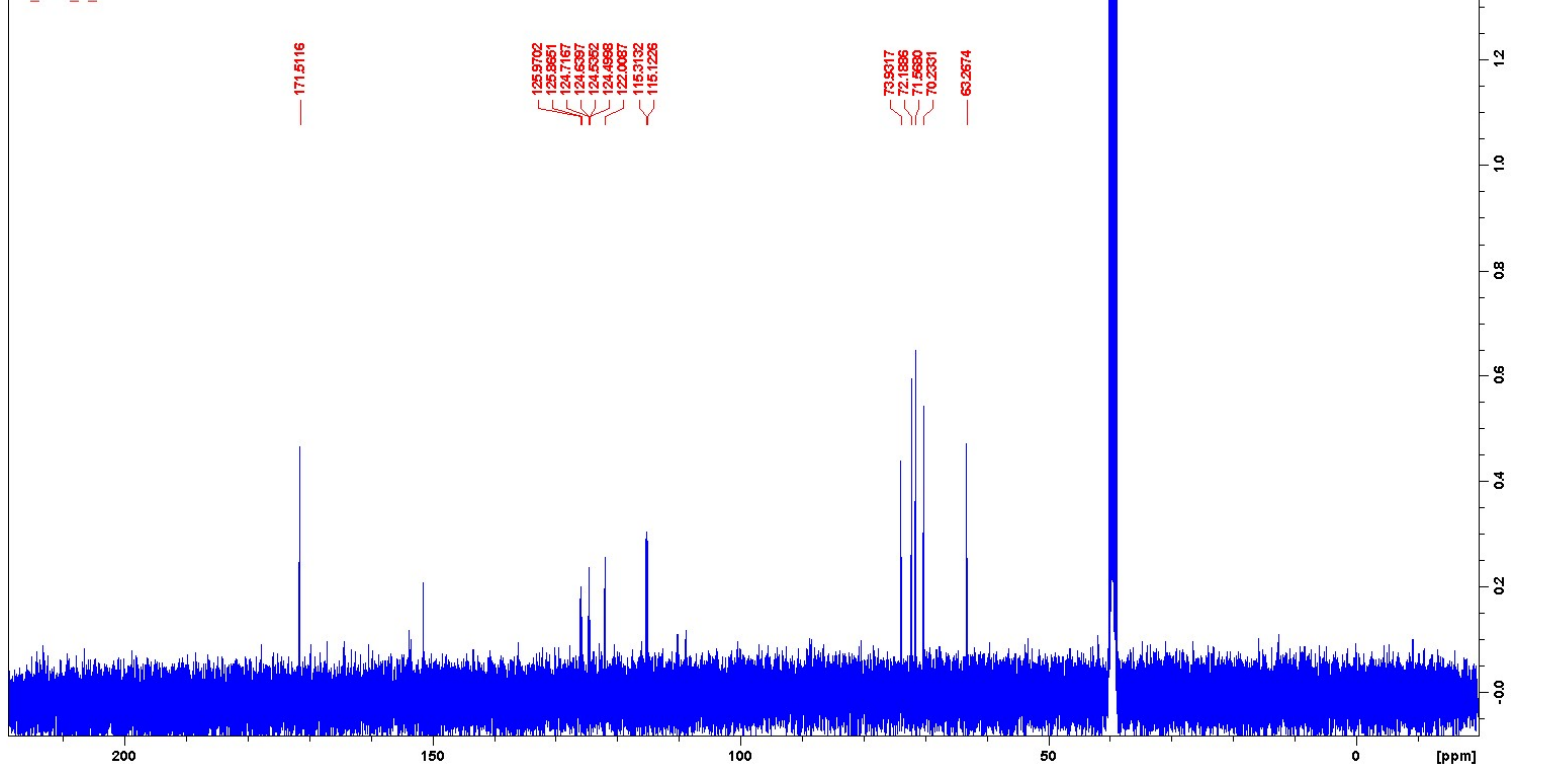
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1d_1H_32_scans DMSO D₆ Ben 31

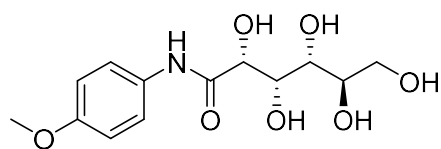


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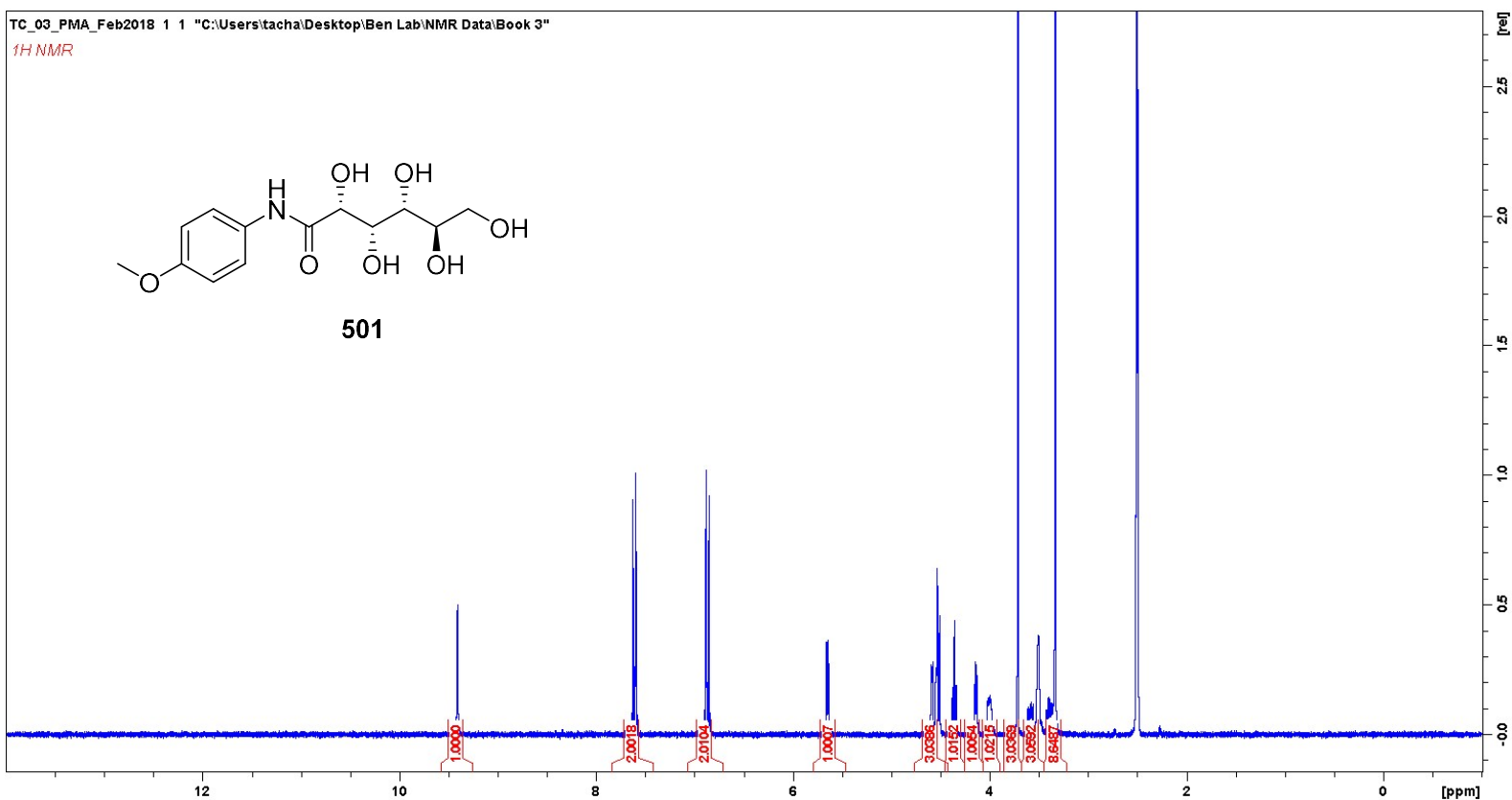
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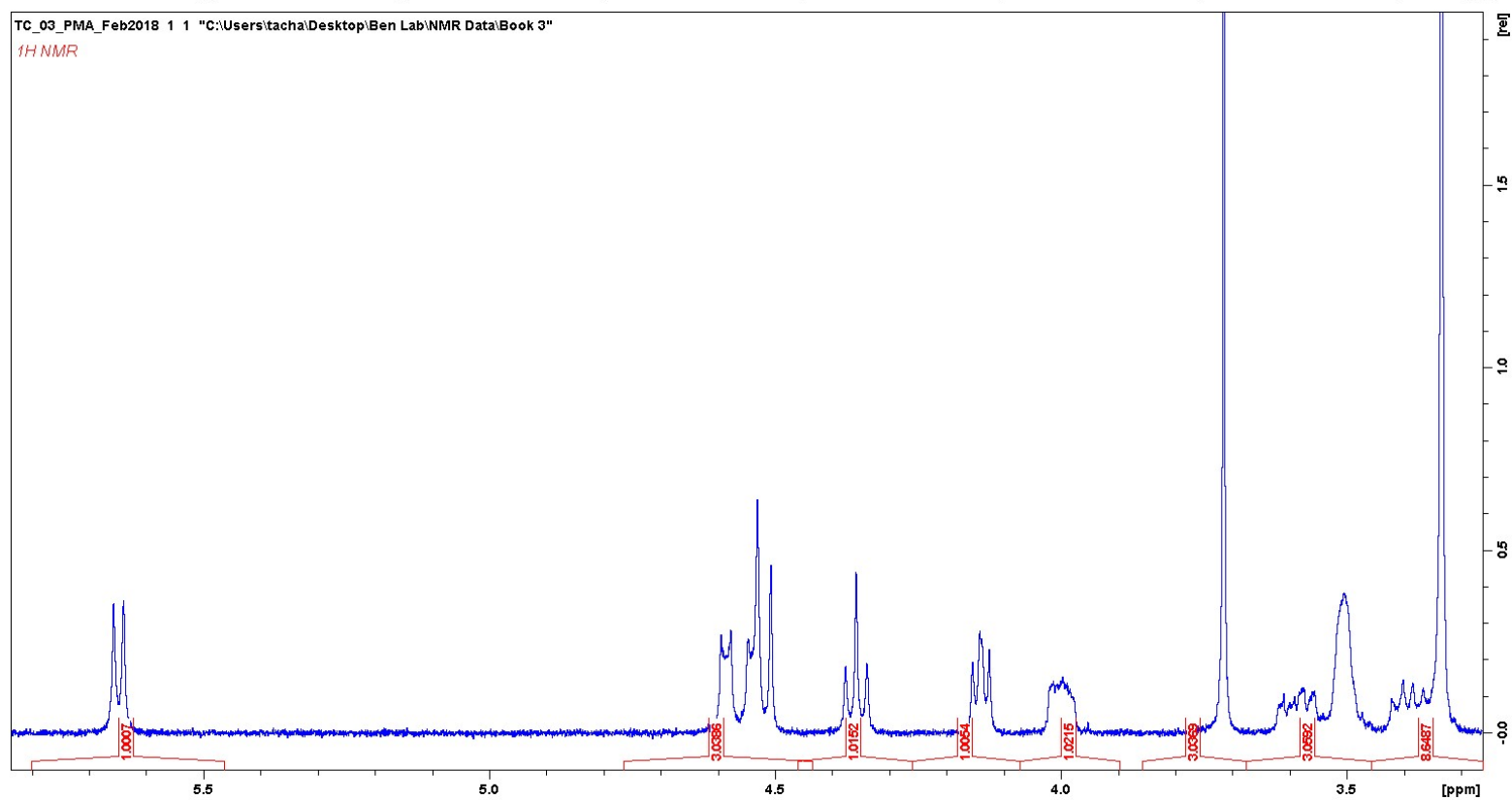
¹H NMR

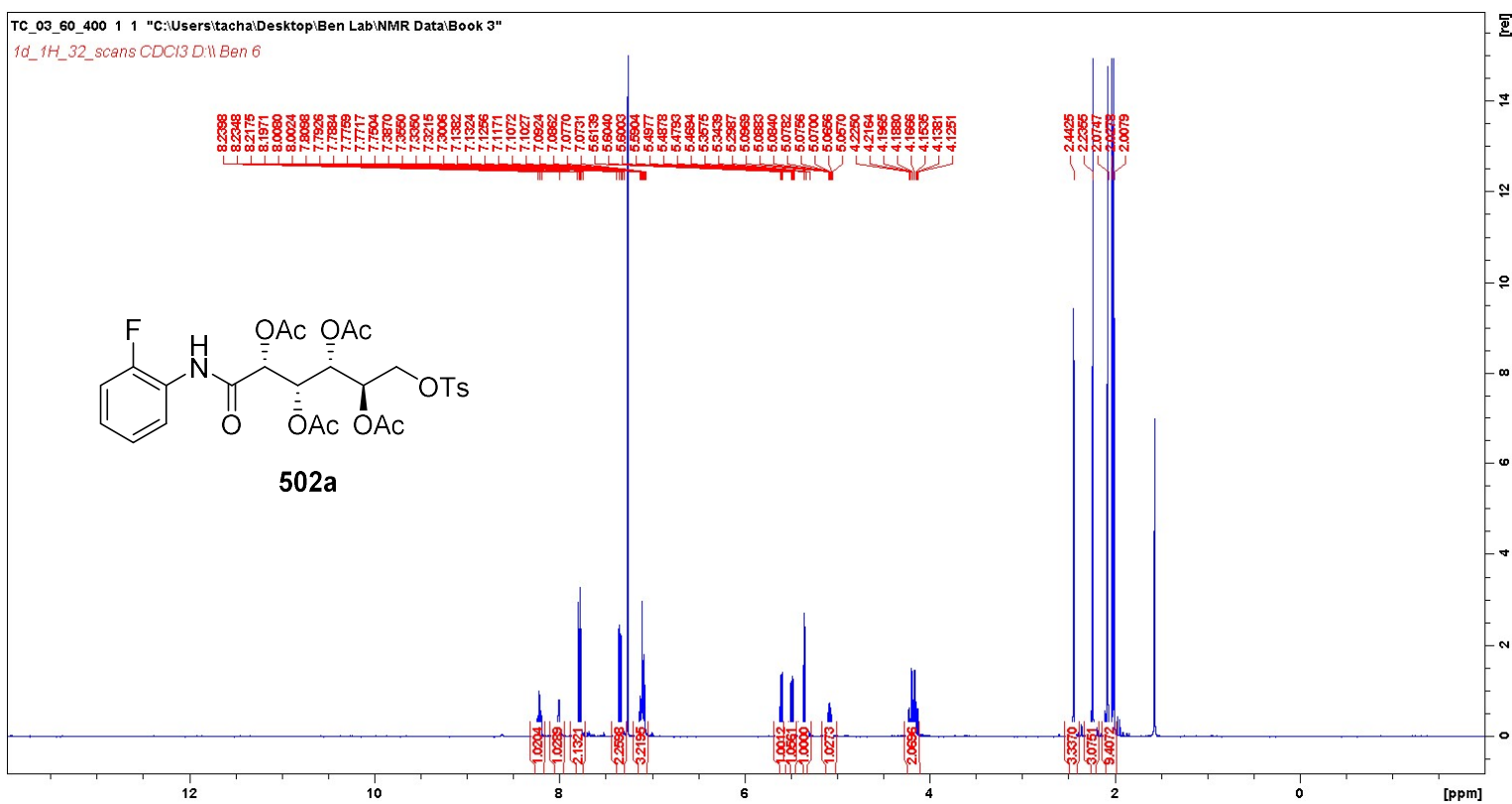
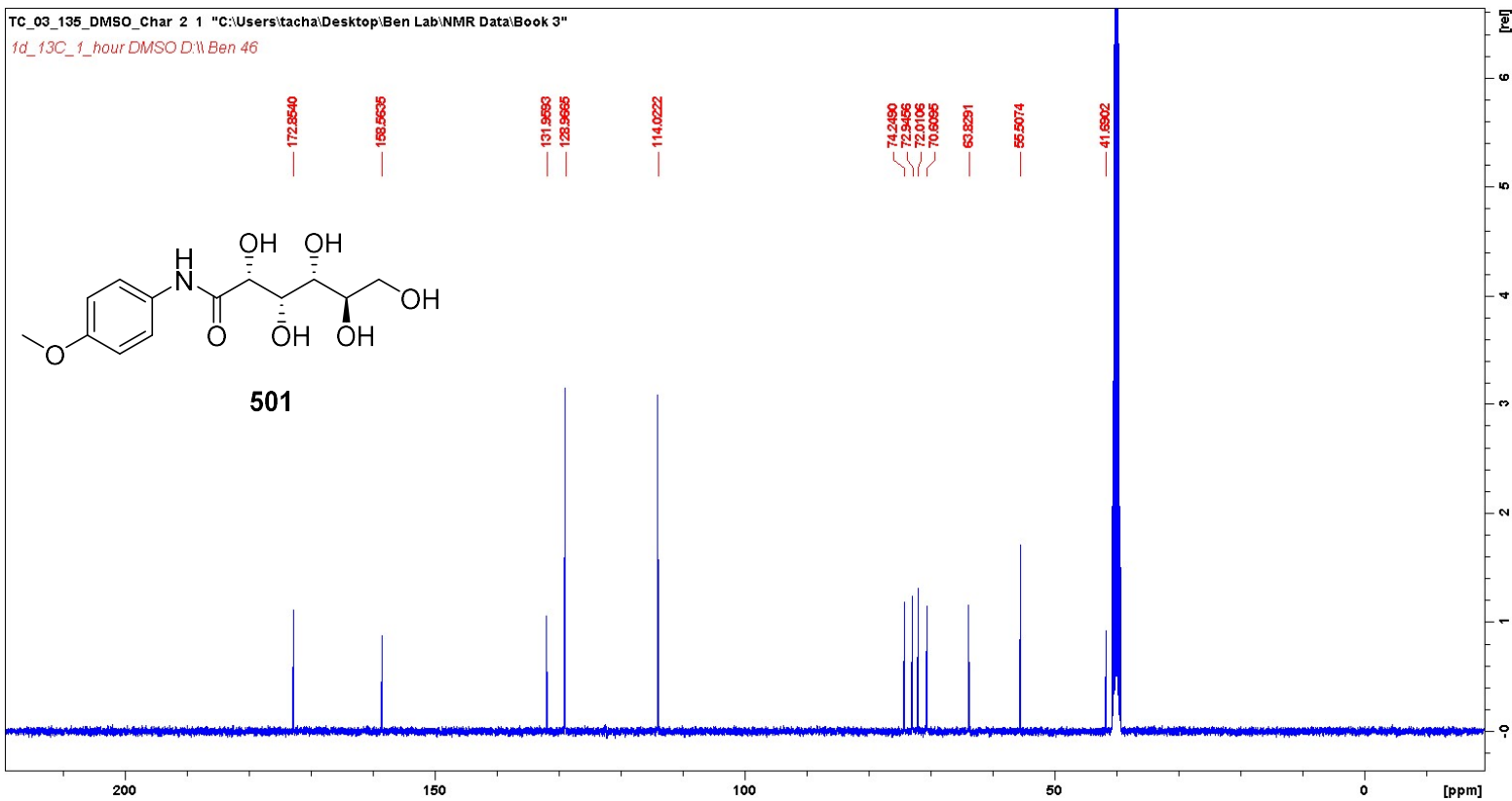


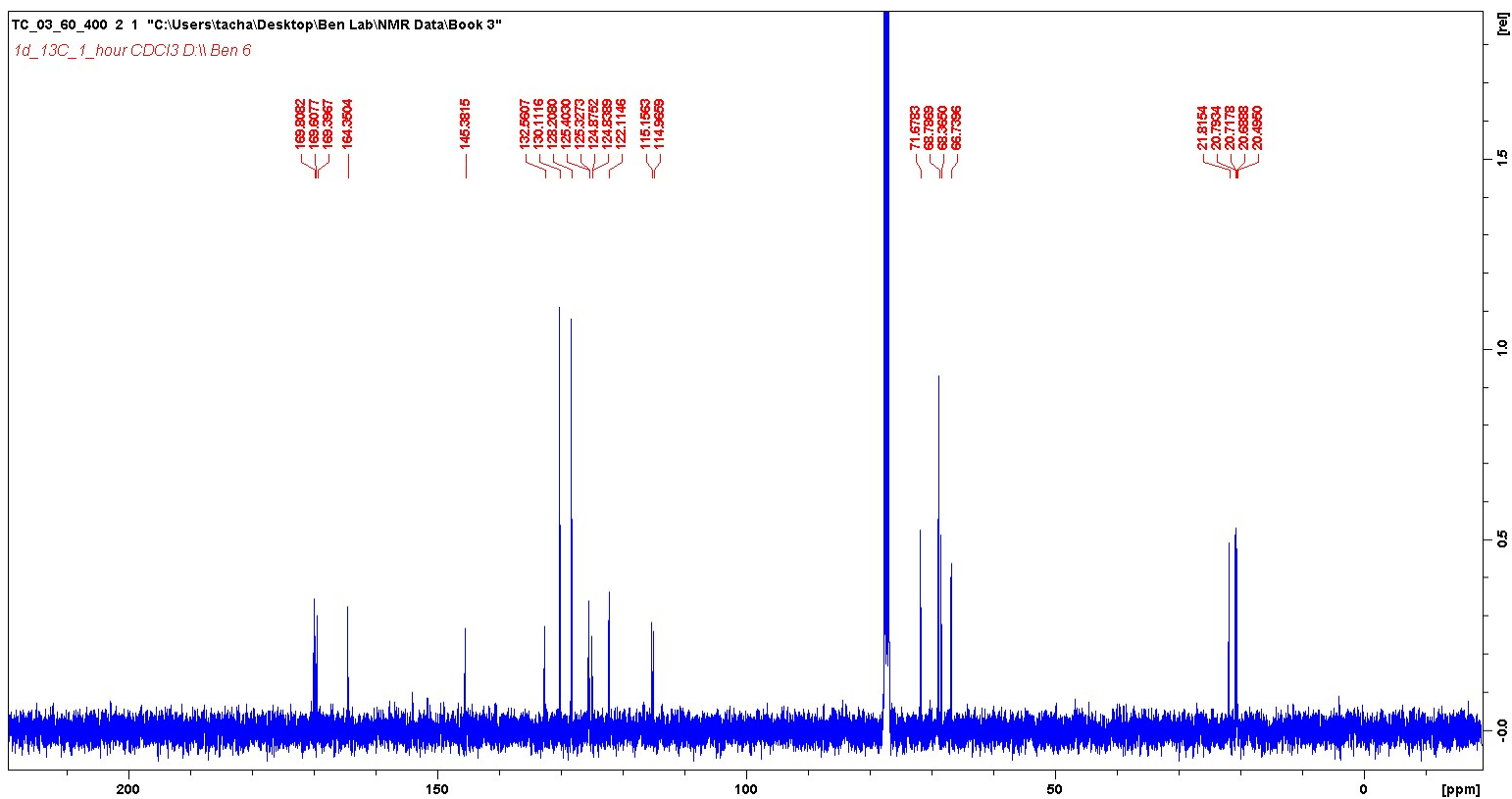
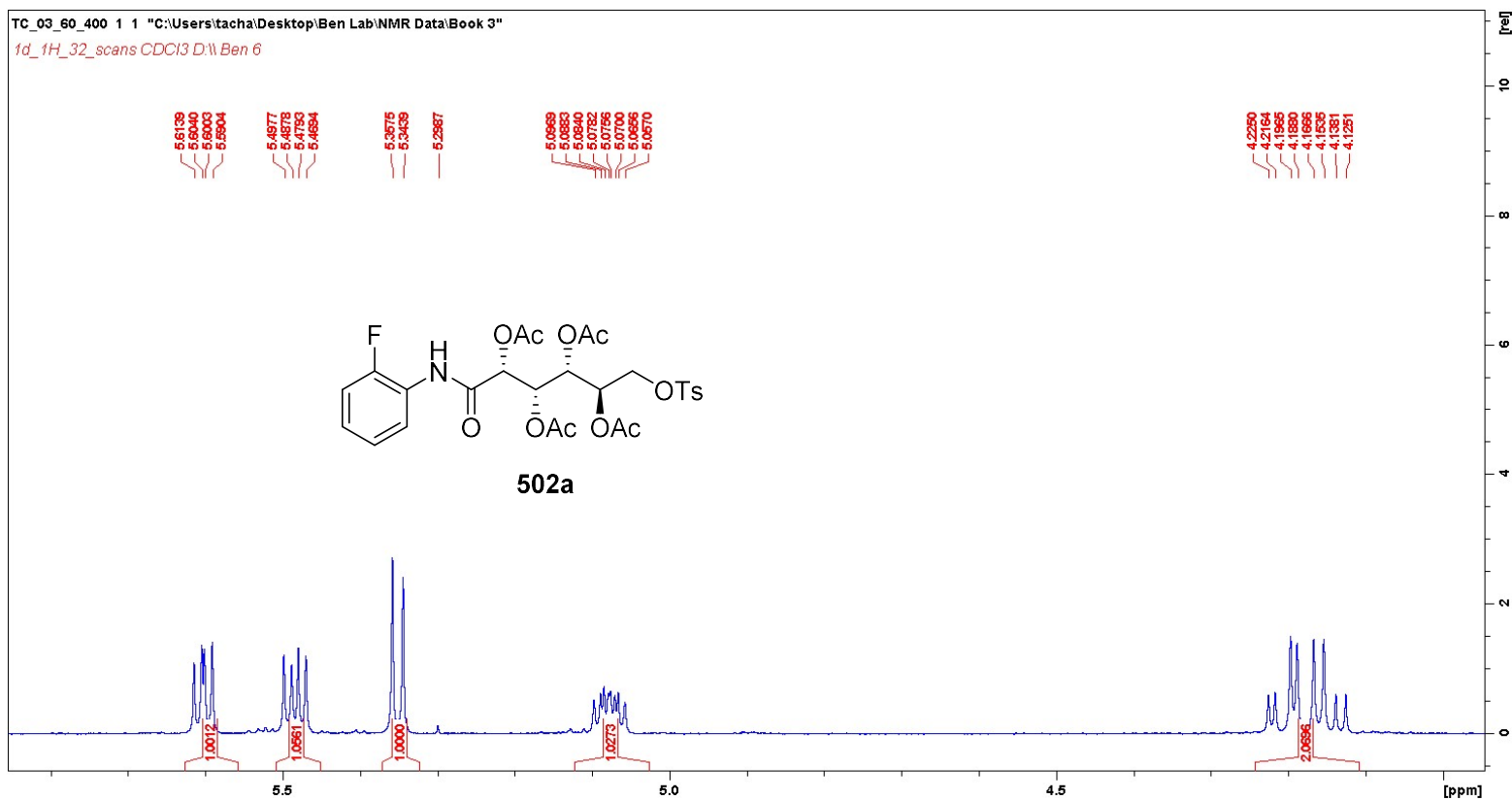
501



¹H NMR

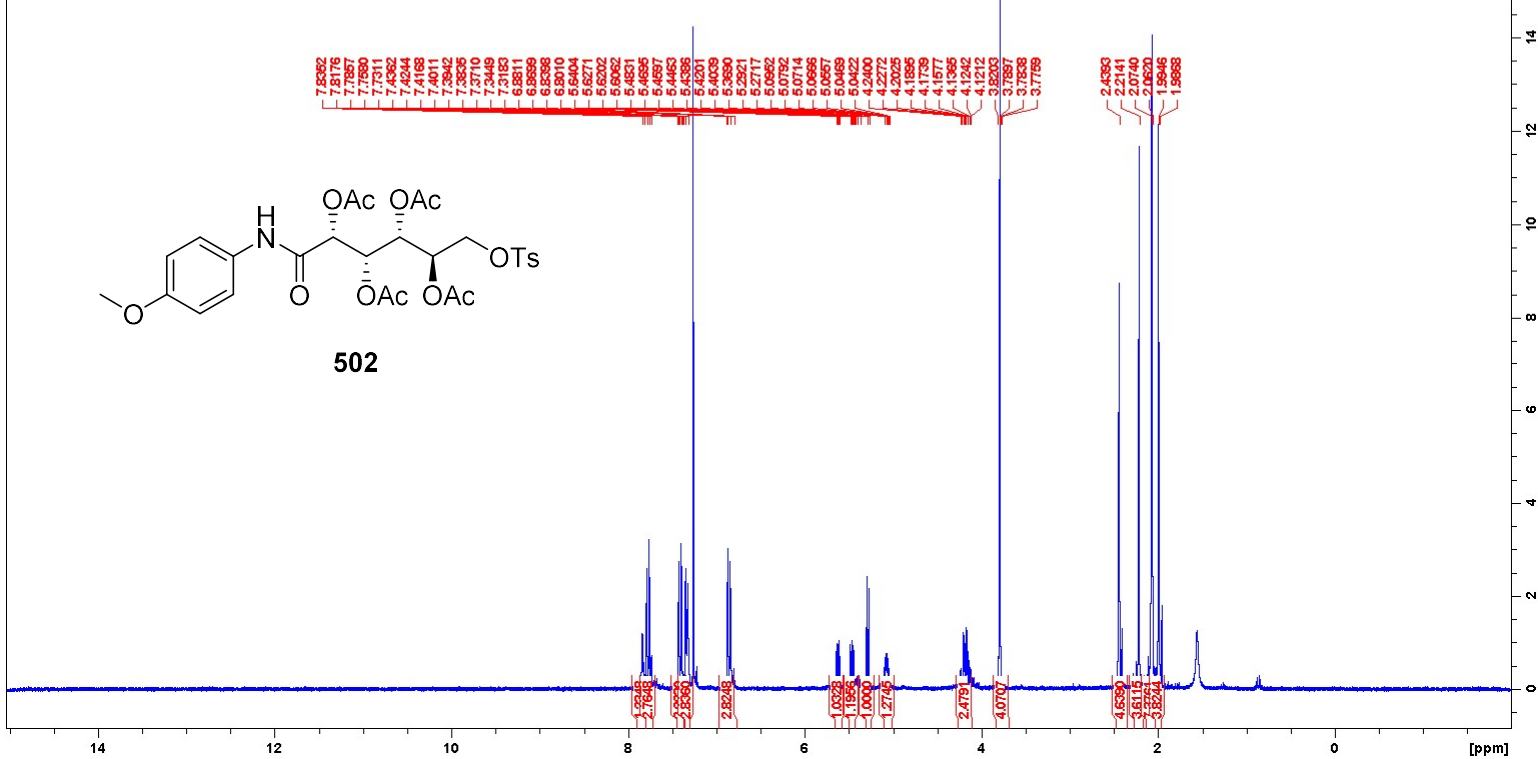






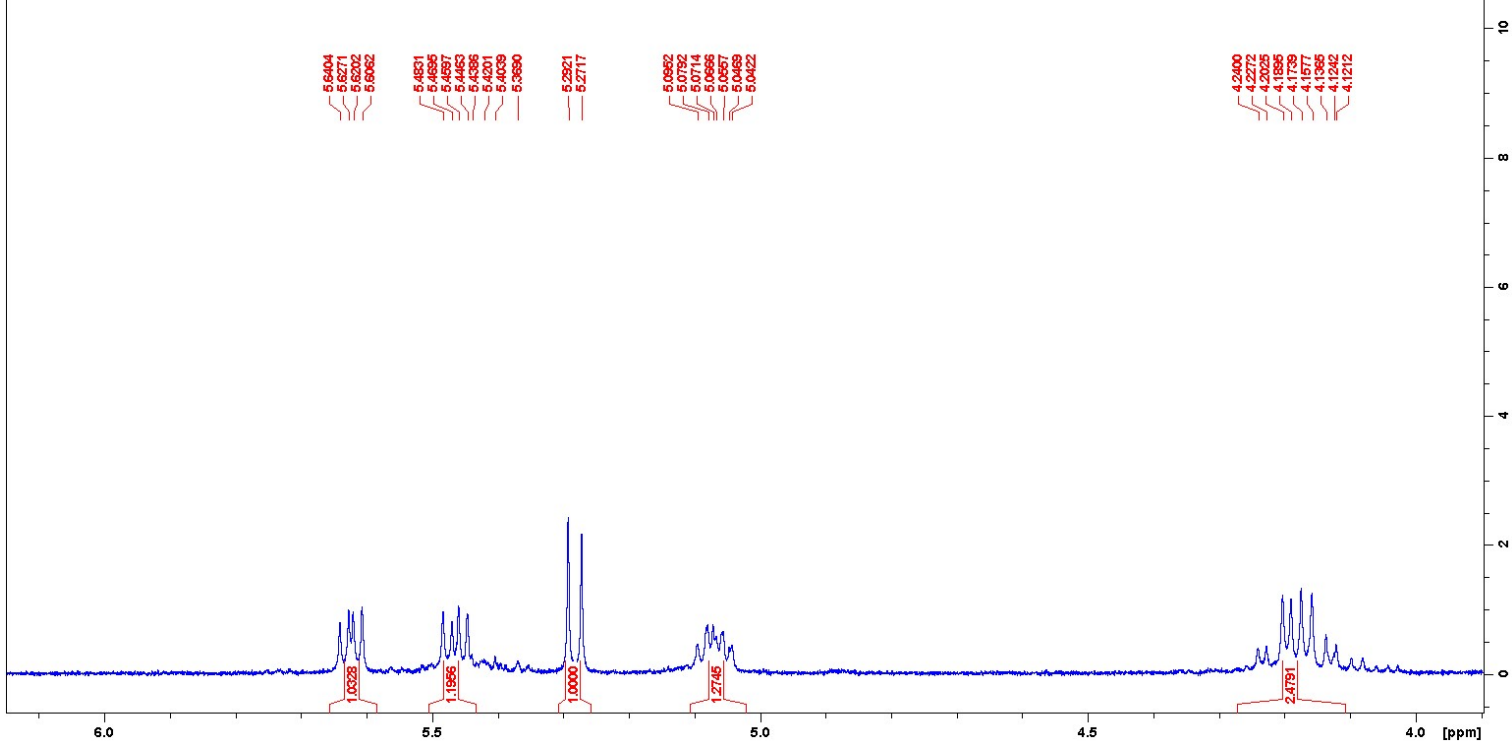
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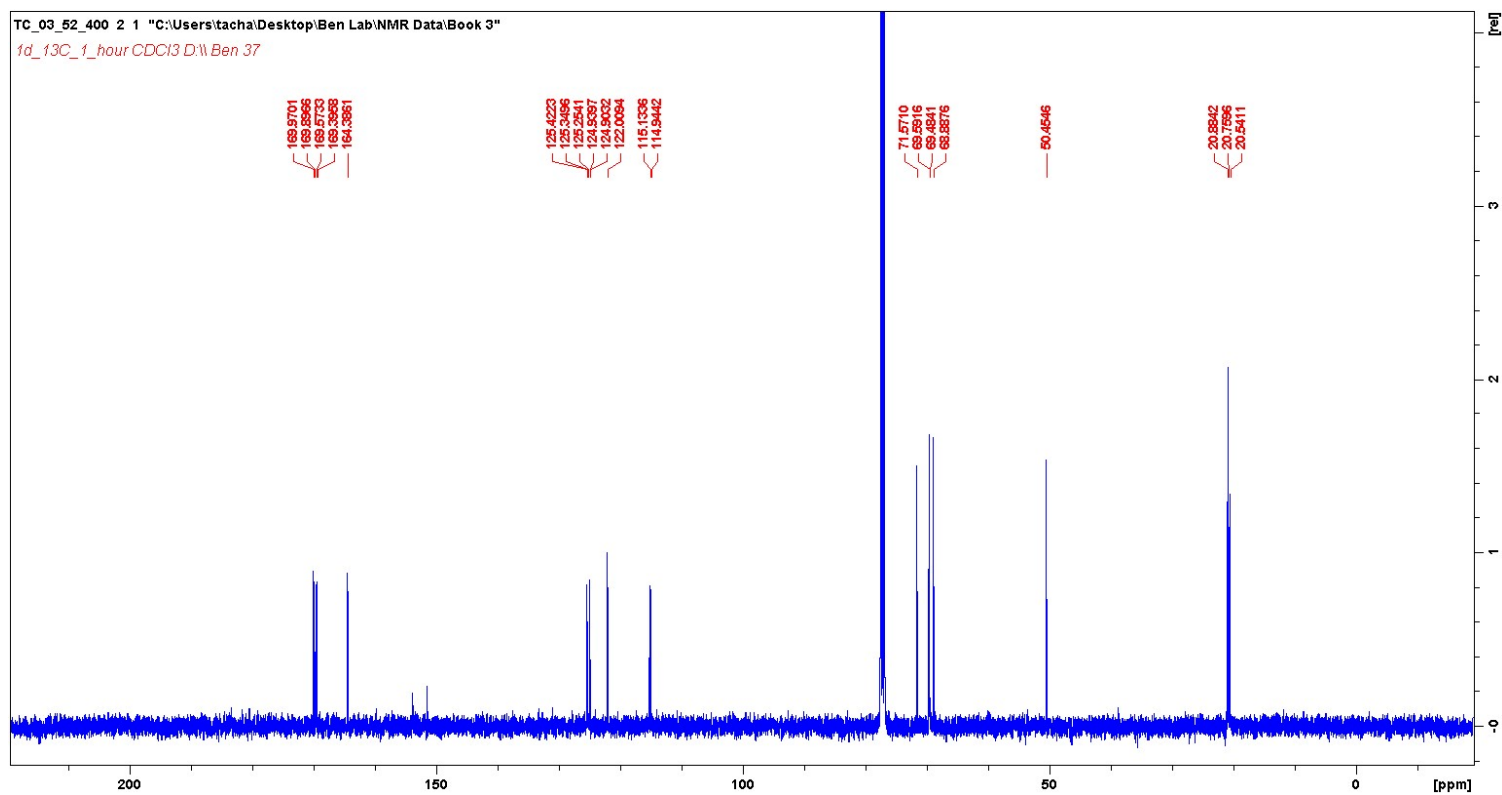
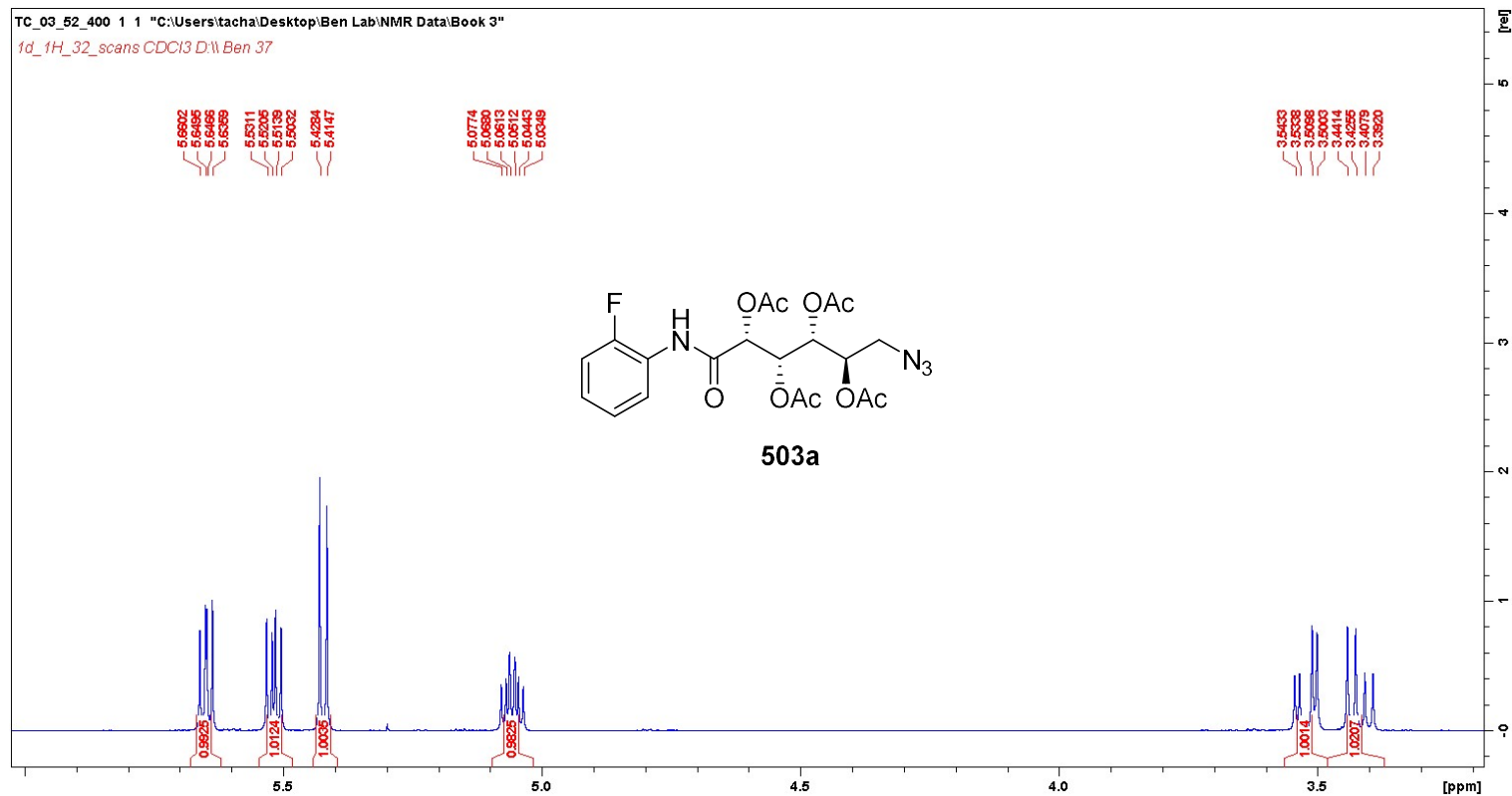
¹H NMR

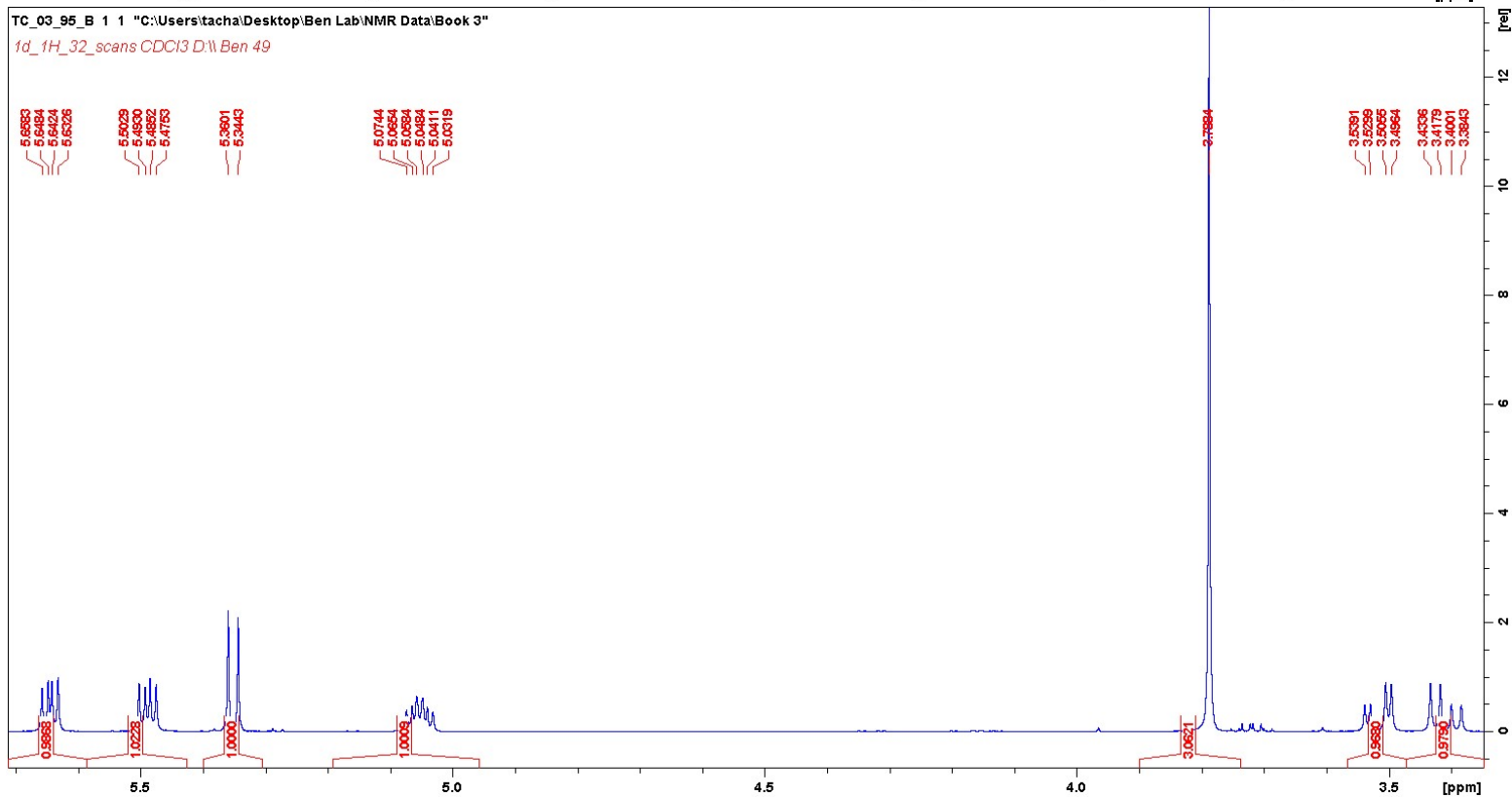
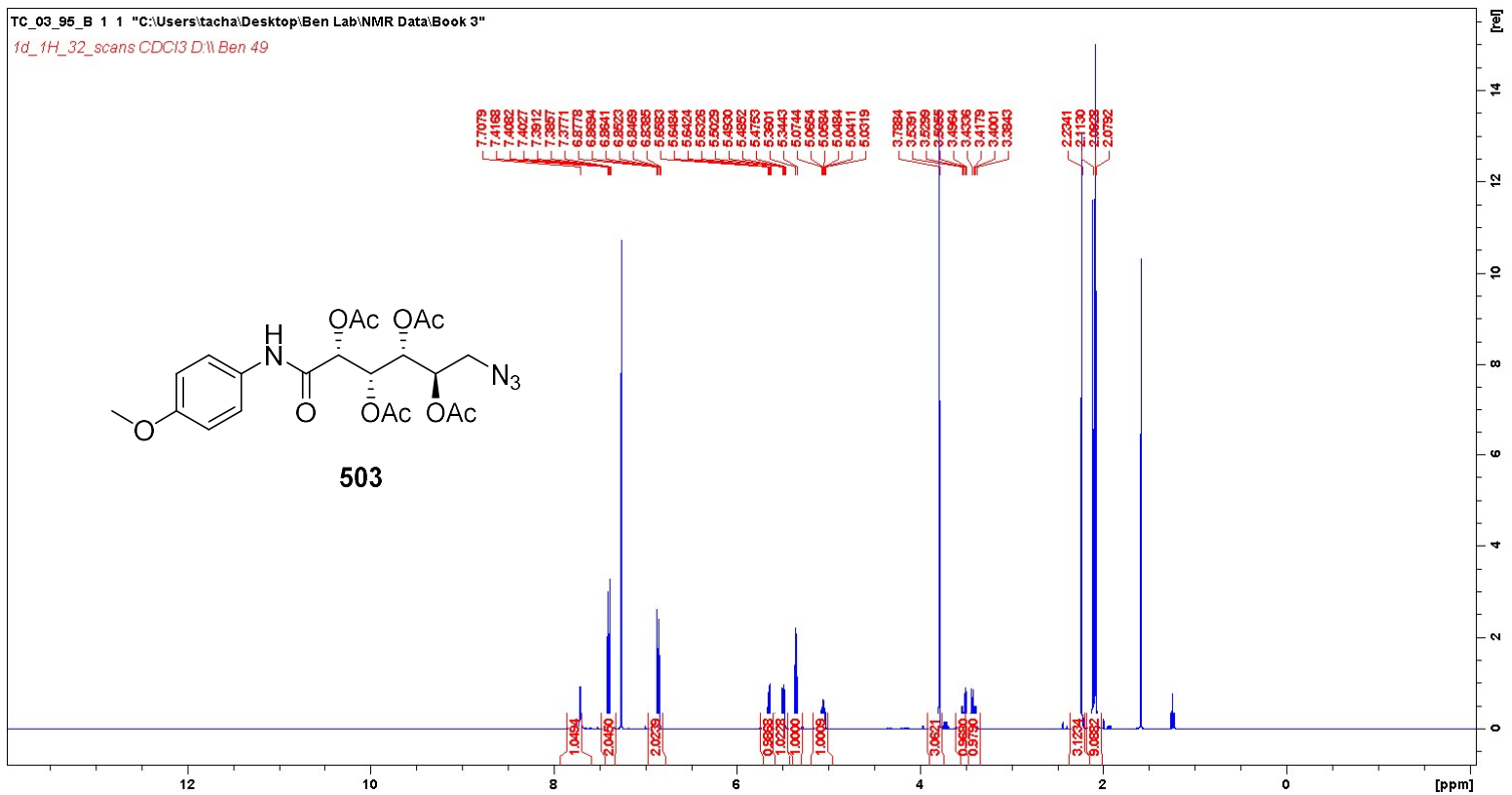


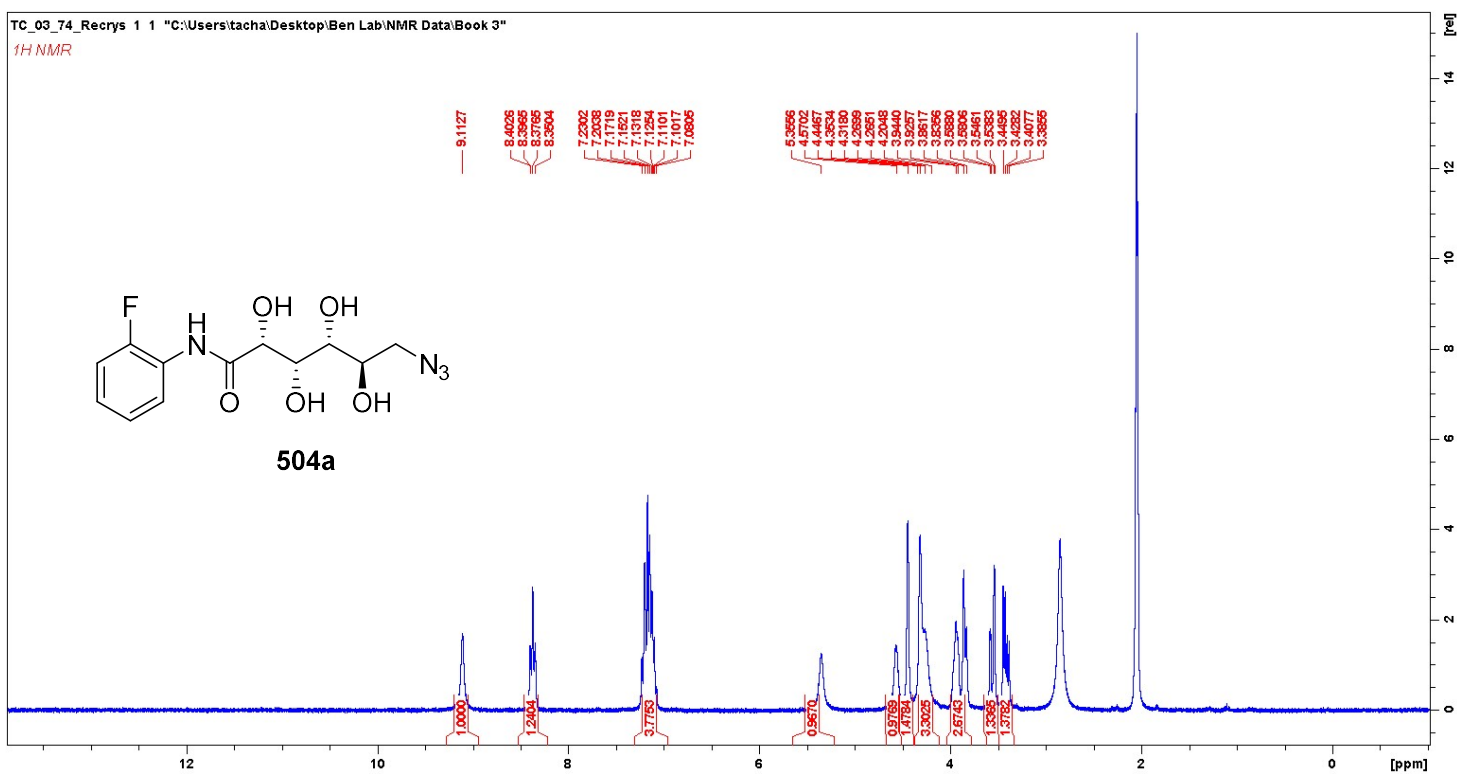
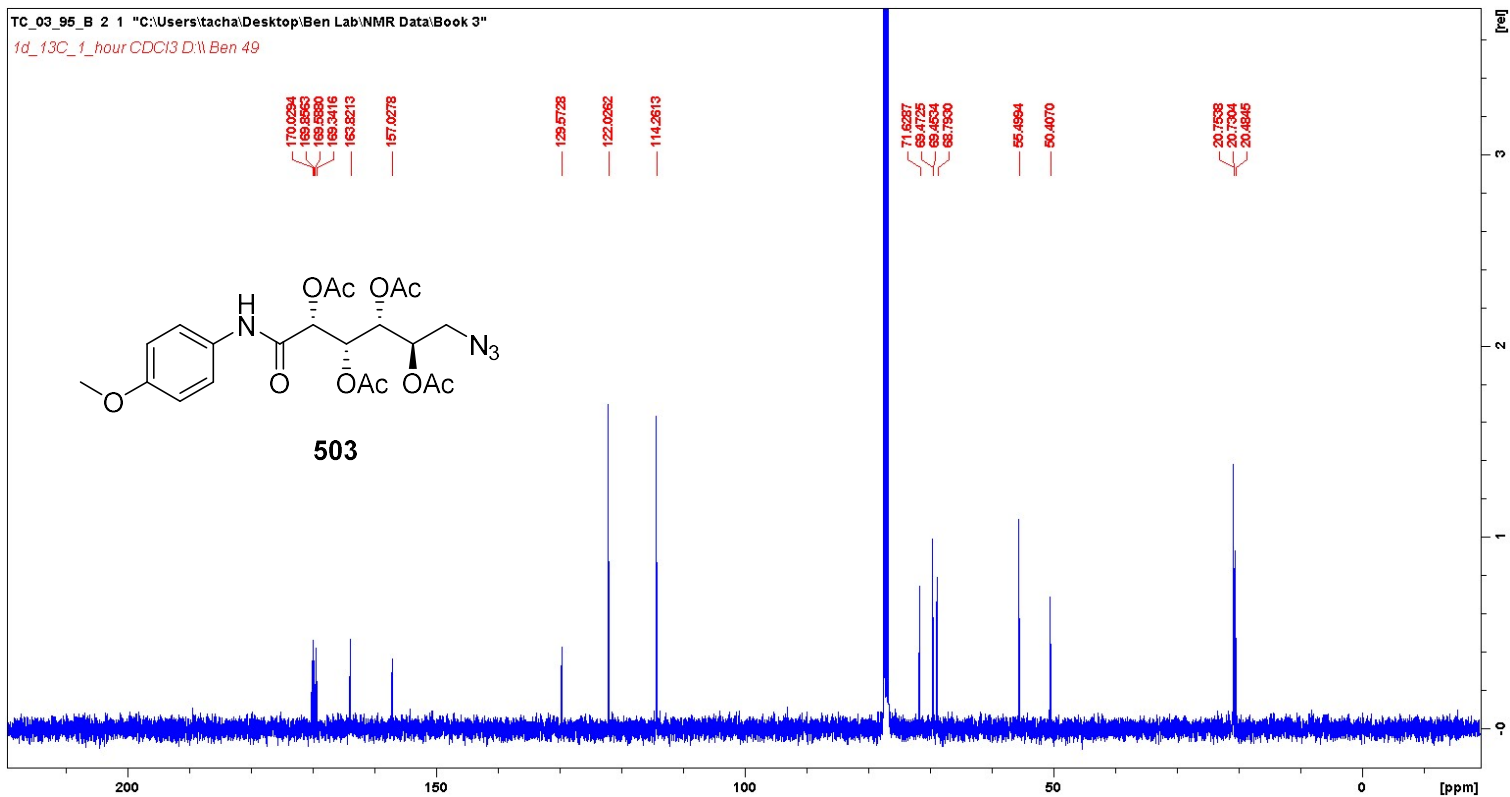
TC_03_132_CDCl3 1 1 "C:\Users\tacha\Desktop\Ben Lab\NMR Data\Book 3"

¹H NMR



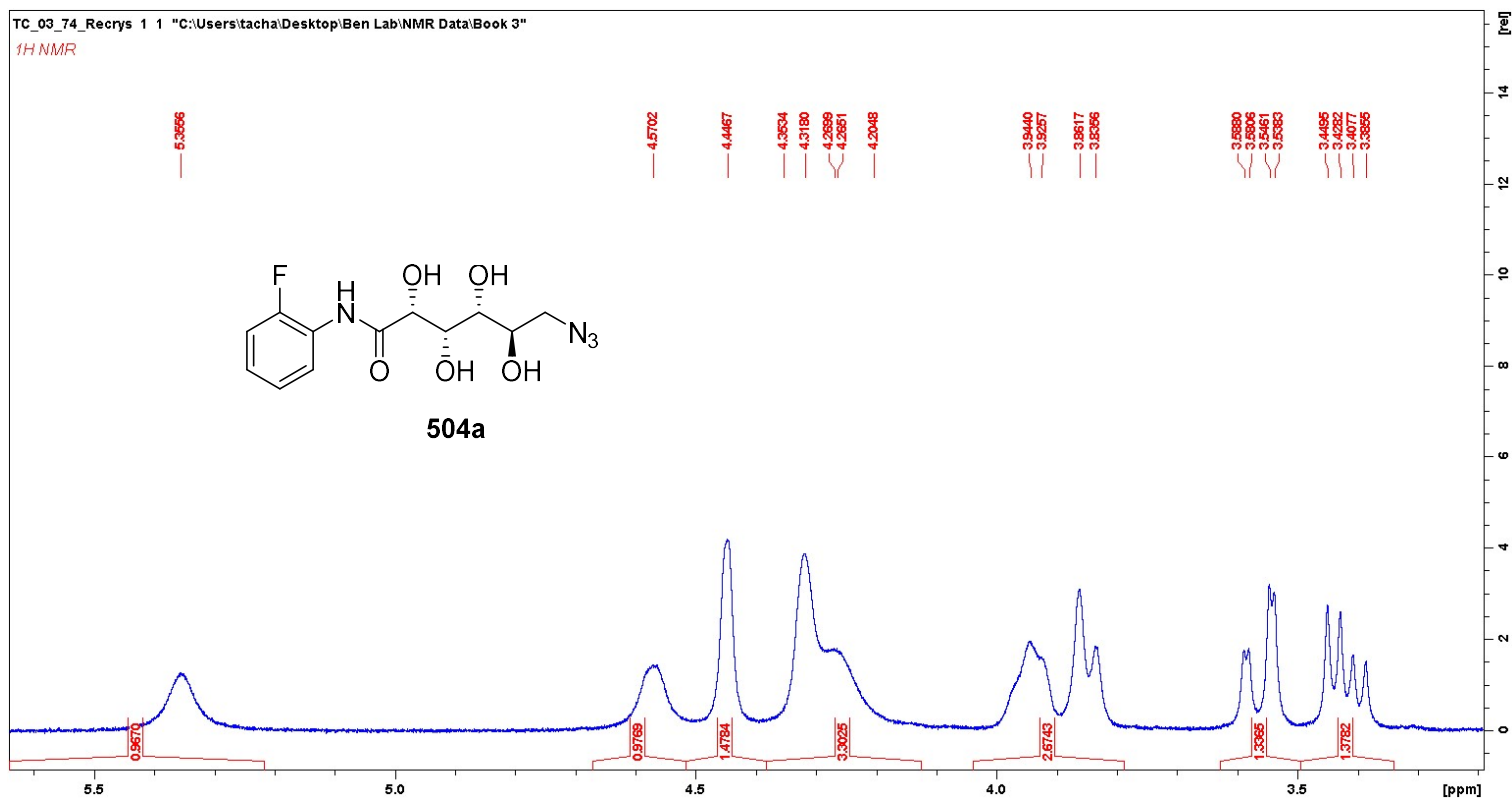






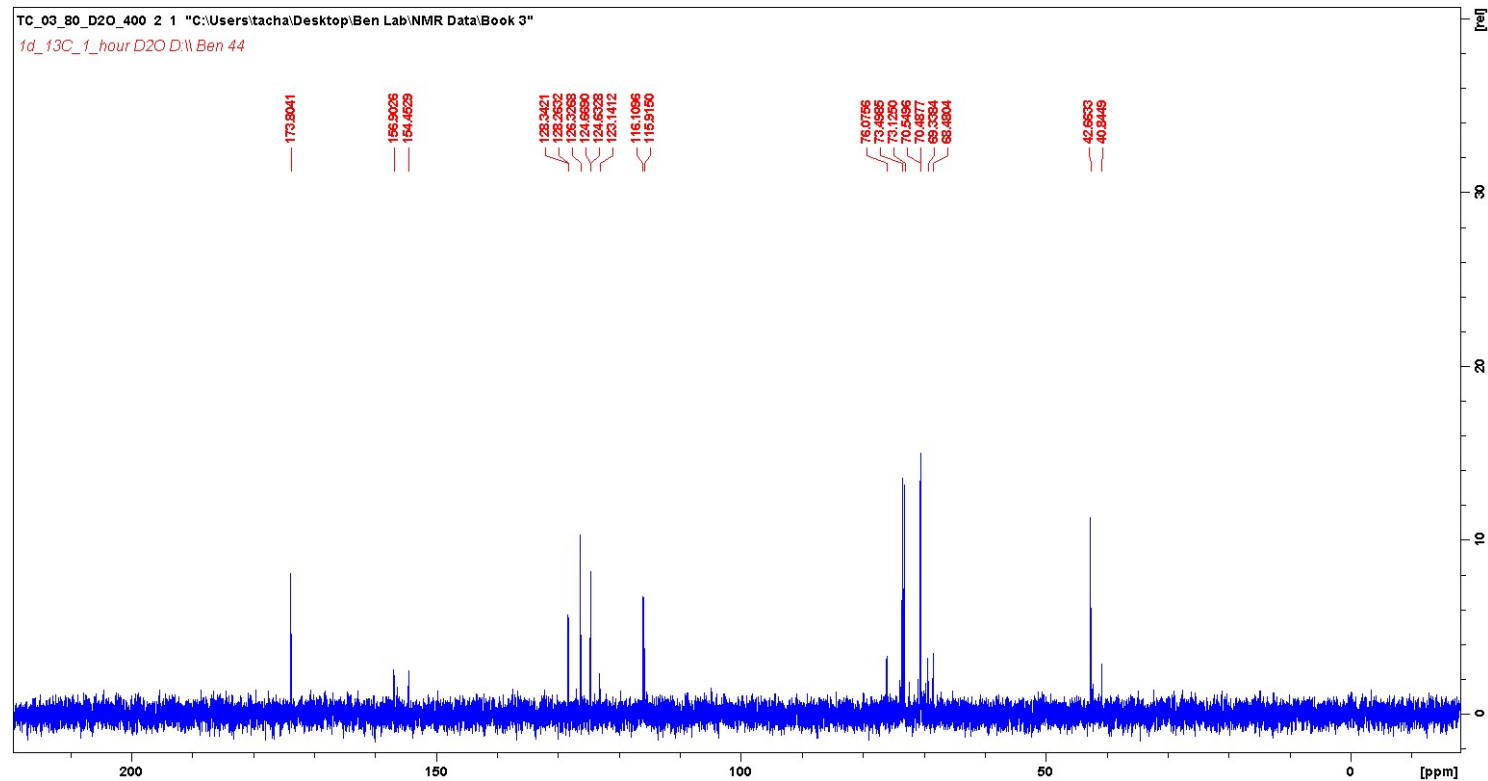
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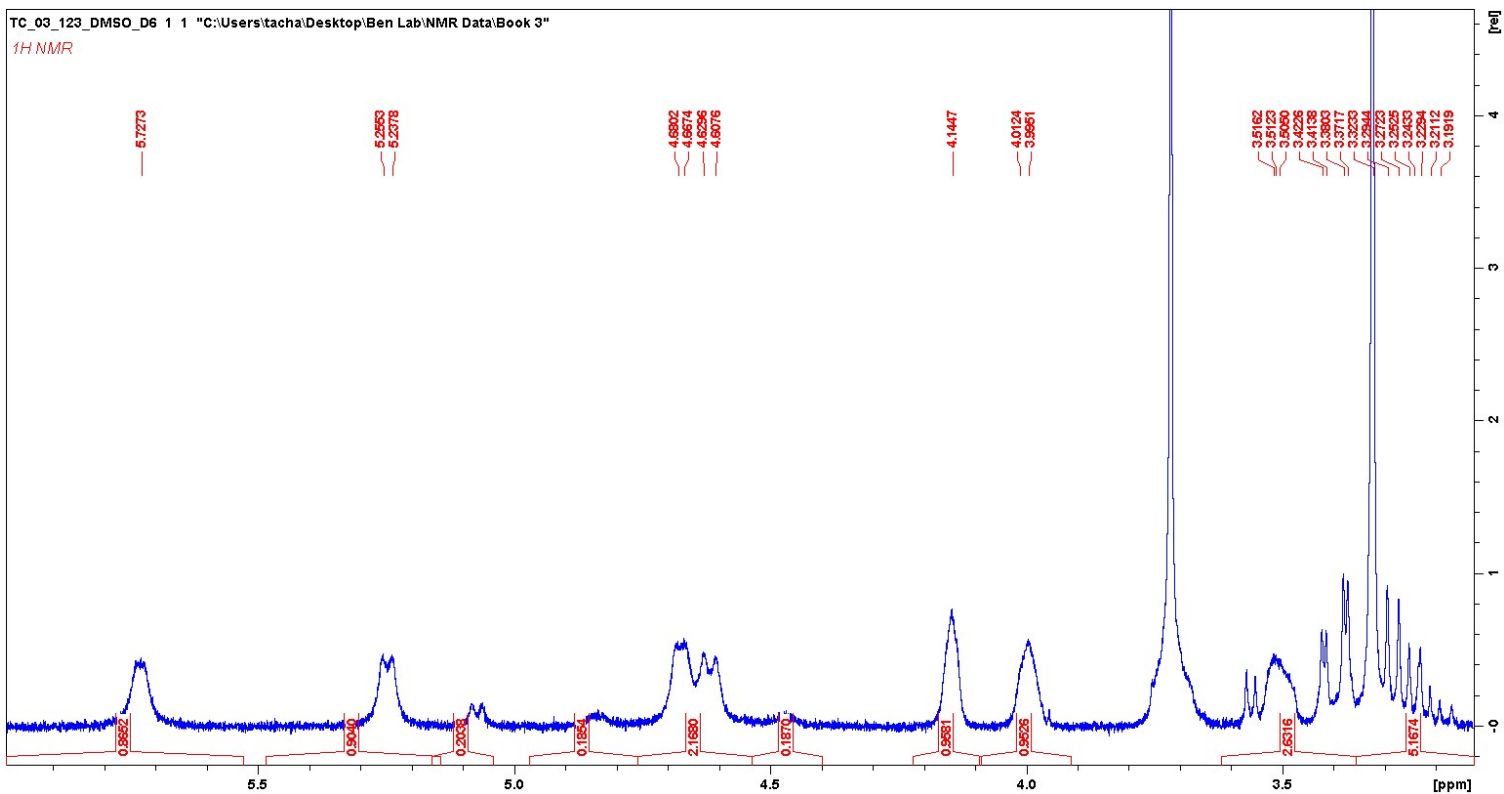
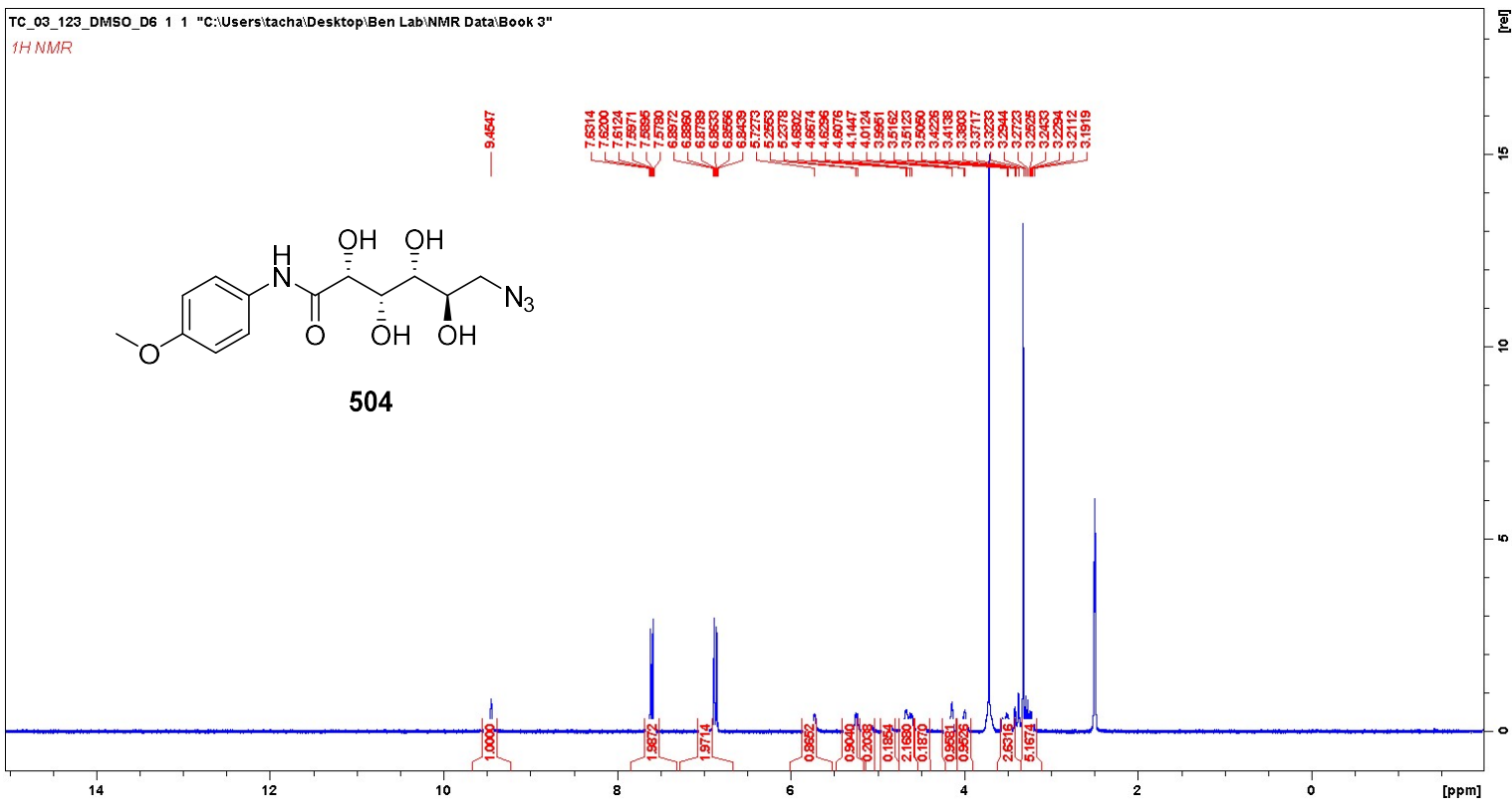
¹H NMR

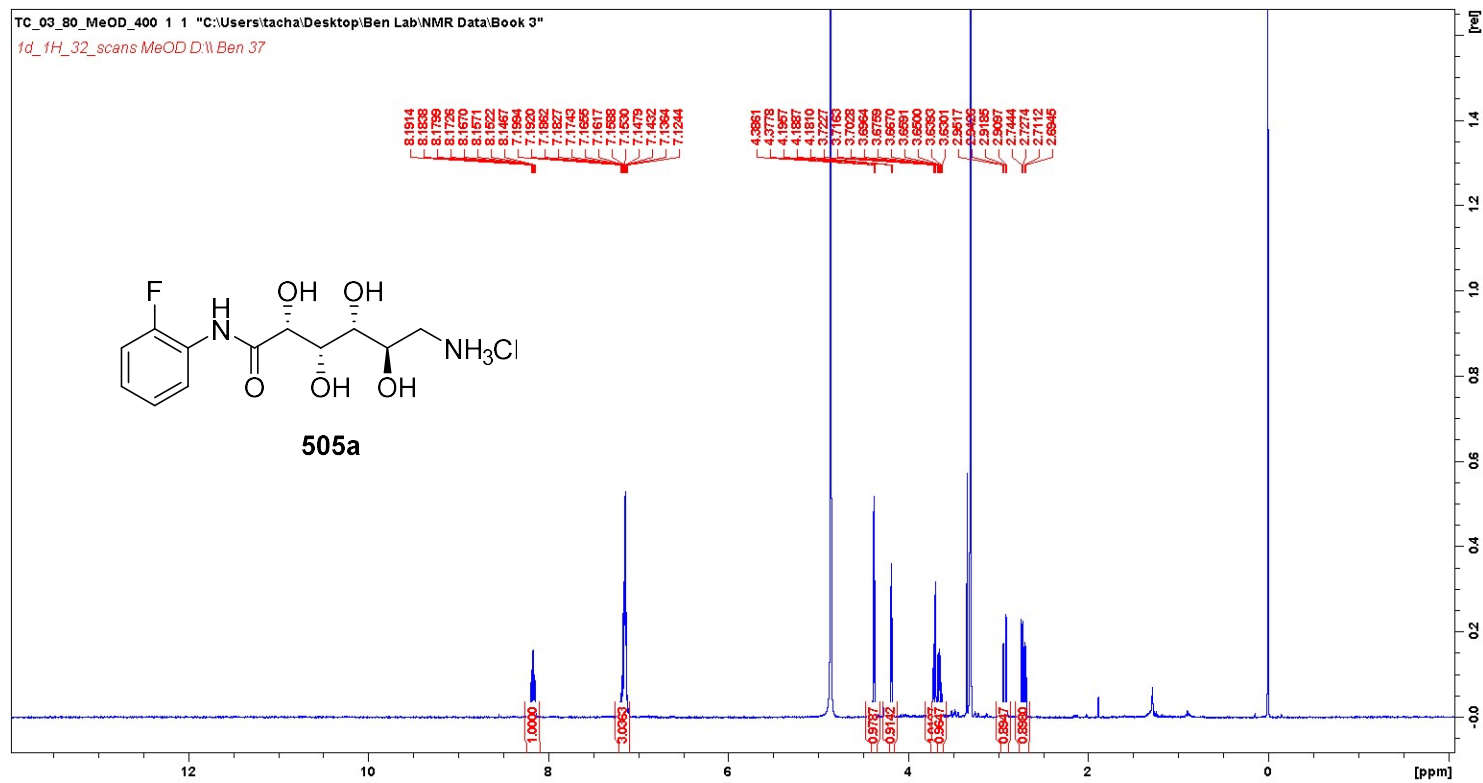
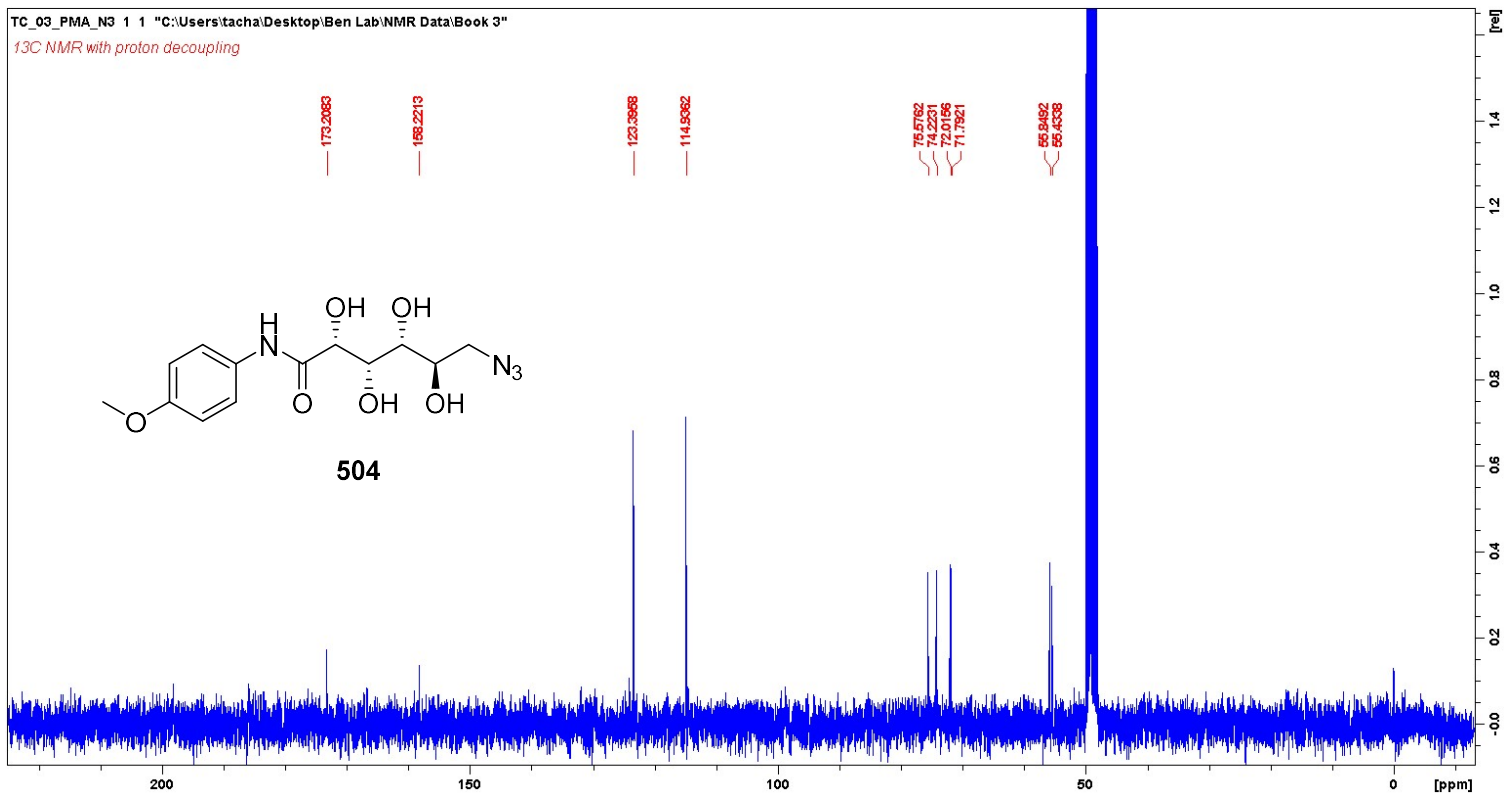


TC_03_80_D2O_400 2 1 "C:\Users\tacha\Desktop\Ben Lab\NMR Data\Book 3"

¹³C NMR

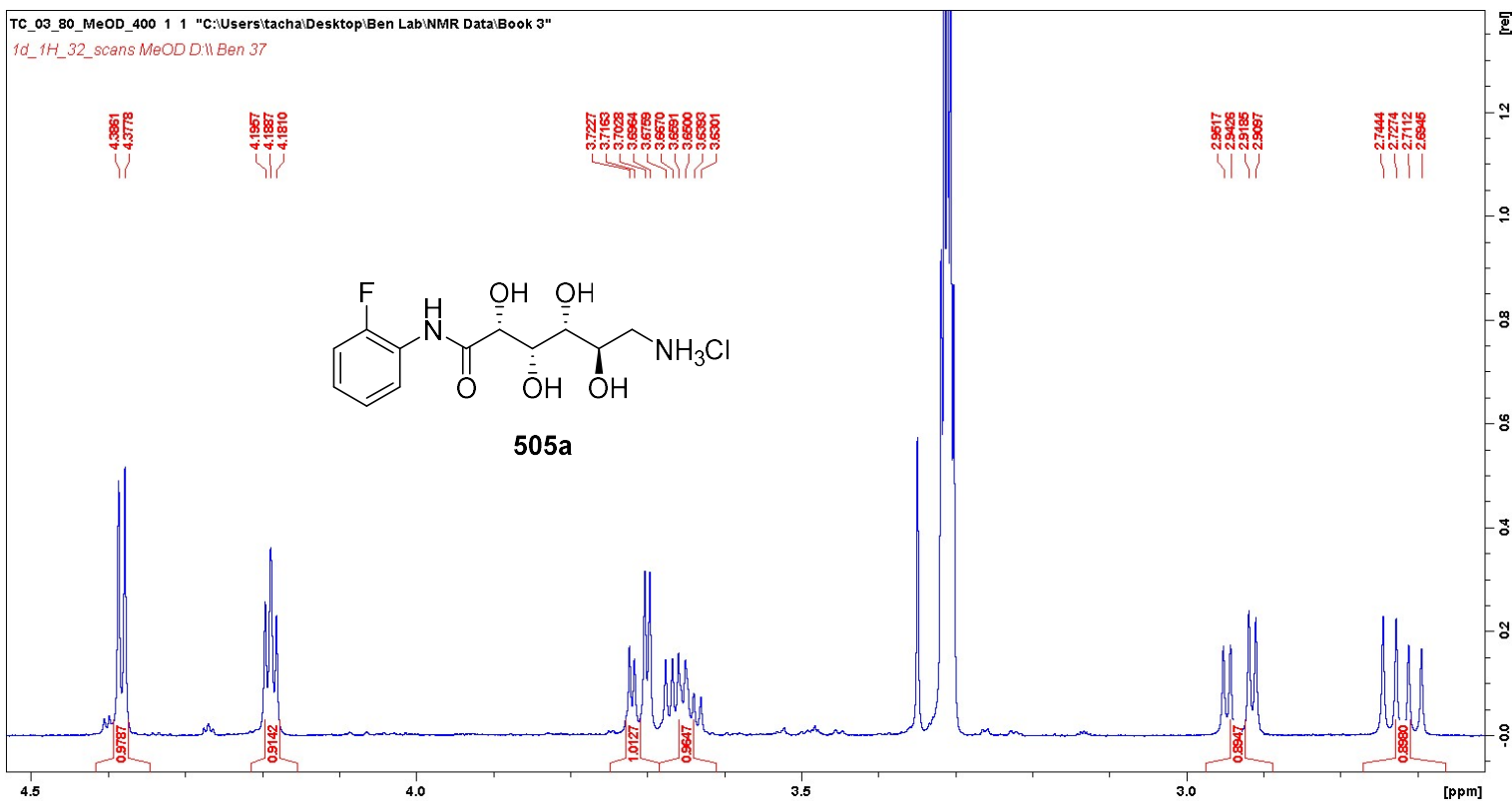






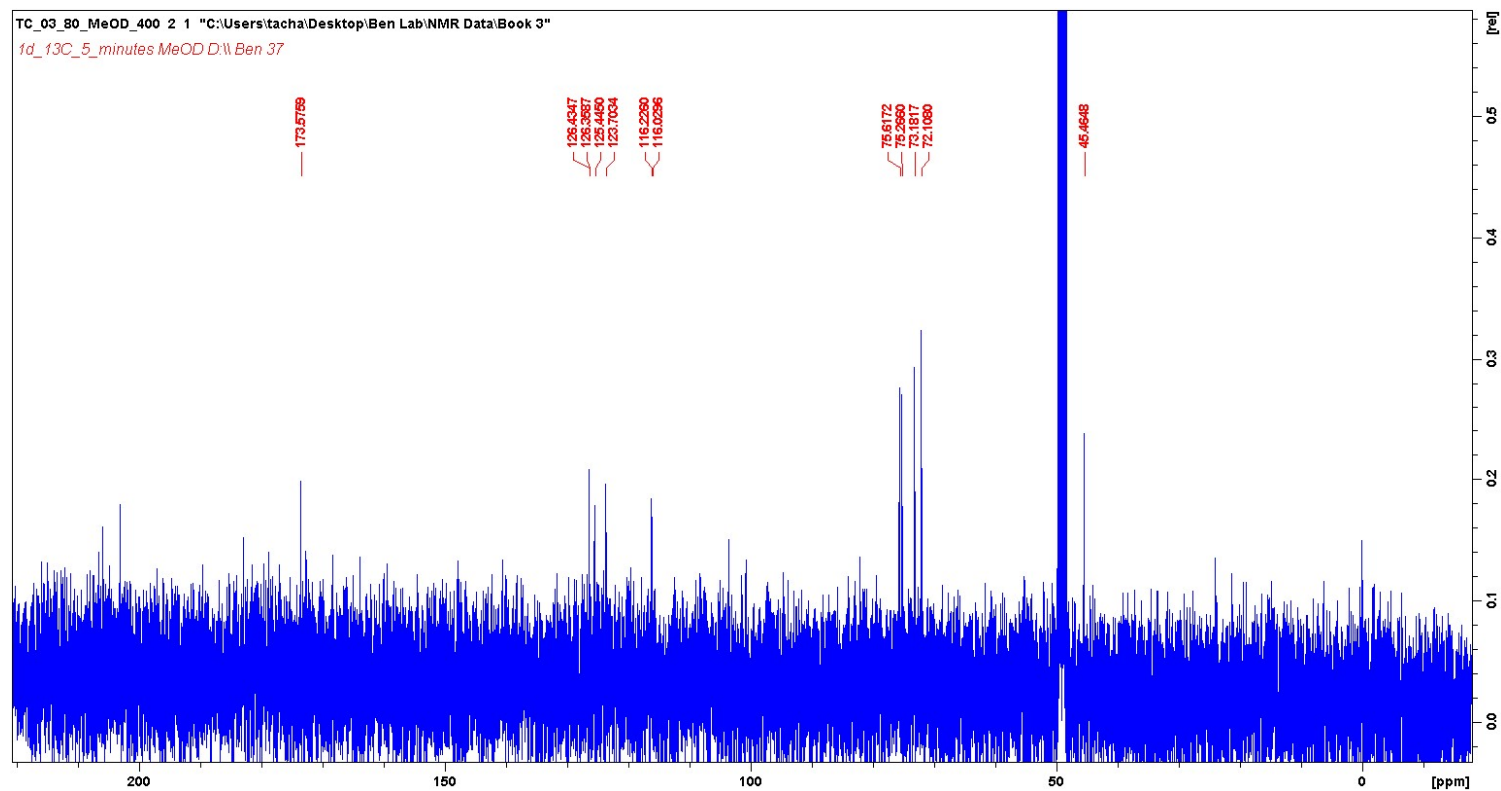
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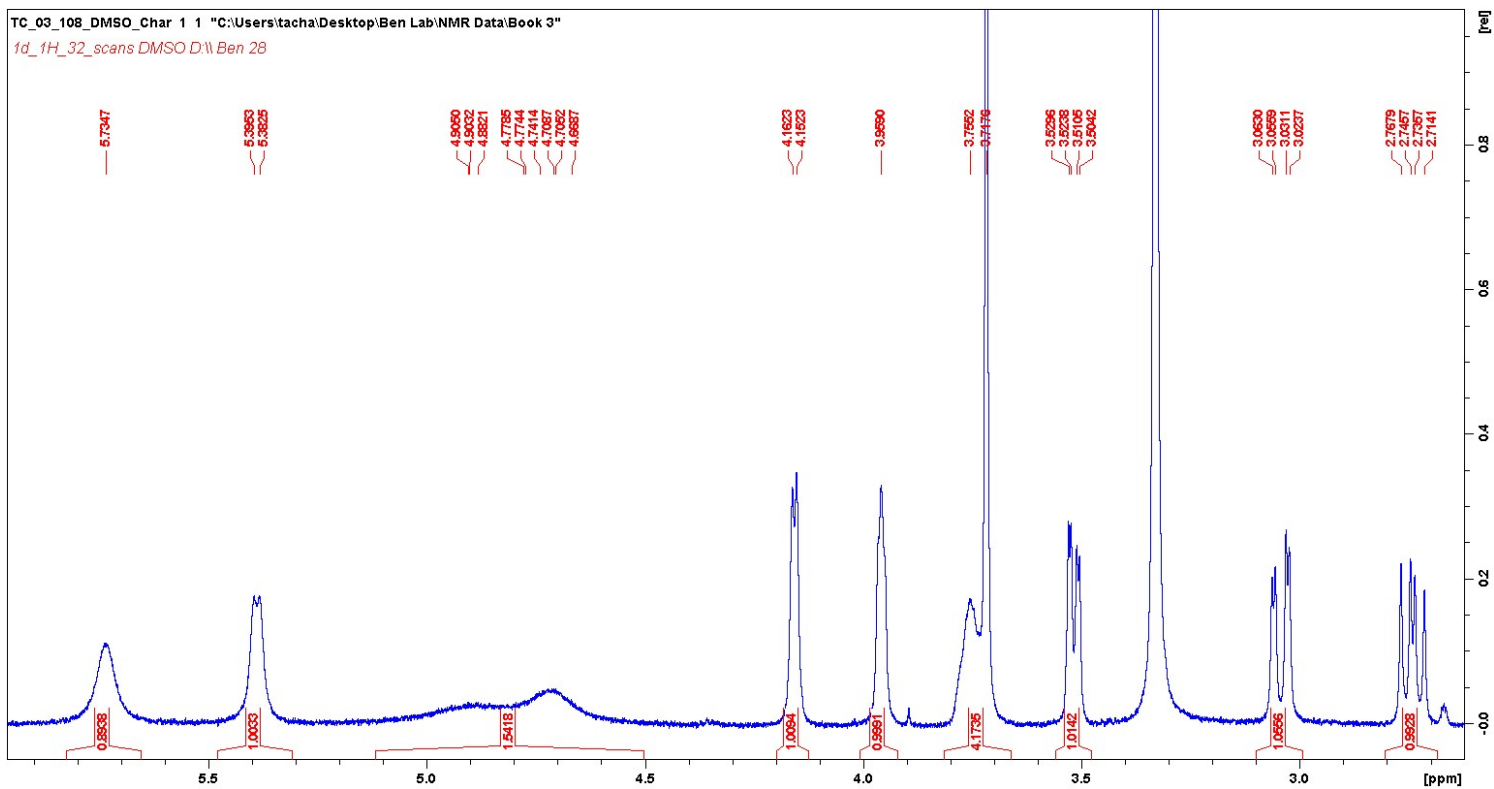
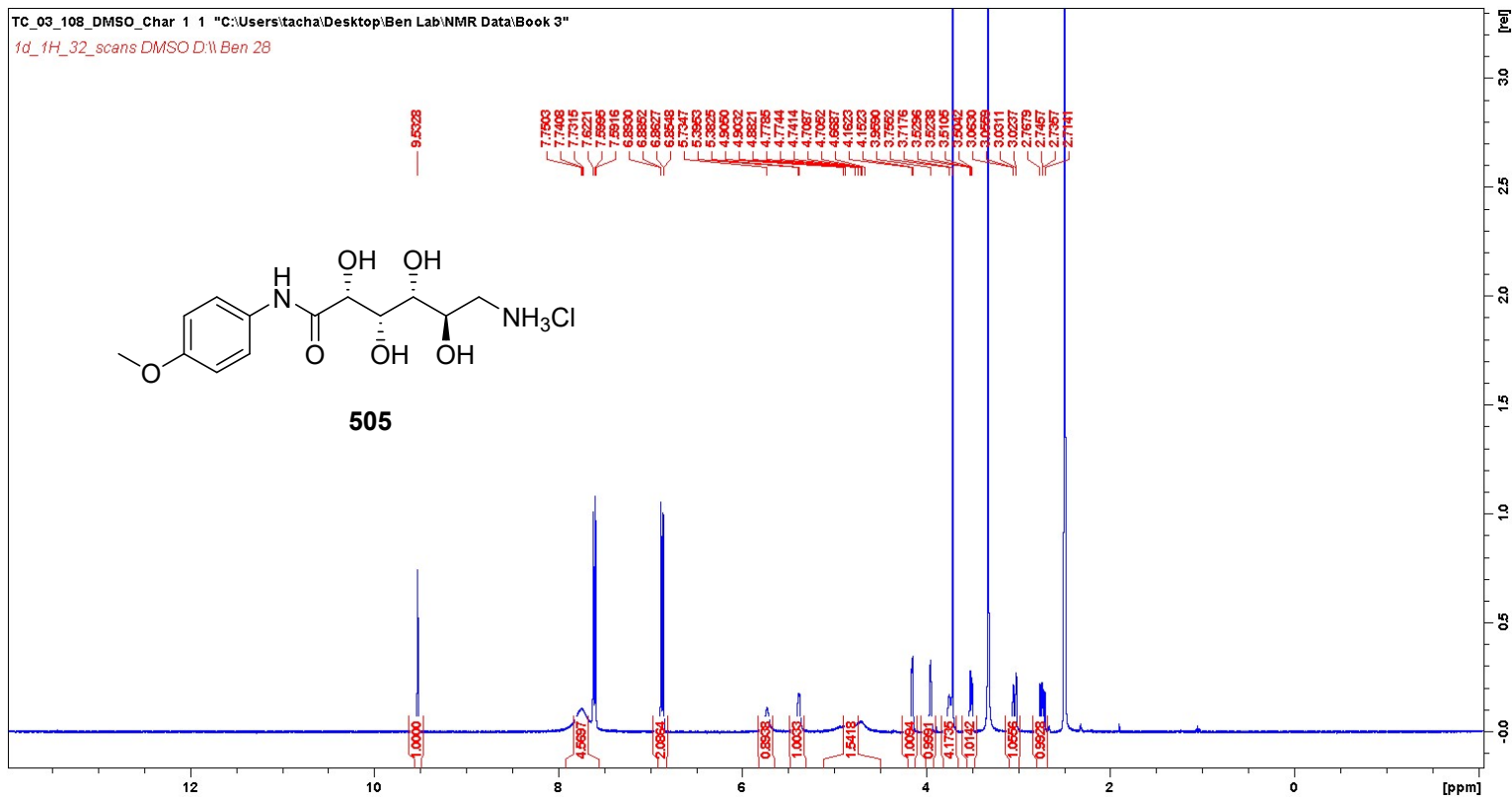
1d_1H_32_scans MeOD D\ Ben 37



TC_03_80_MeOD_400 2 1 "C:\Users\tacha\Desktop\Ben Lab\NMR Data\Book 3"

1d_13C_5_minutes MeOD D\ Ben 37





TC_03_108_DMSO_Char 2 1 "C:\Users\tacha\Desktop\Ben Lab\NMR Data\Book 3"

1d_13C_1_hour DMSO D\ Ben 28

