

**Addressing Solubility Limitations in Small-Molecule Ice Recrystallization Inhibitors and
Evaluating their Use in Hematopoietic Stem Cell and Red Blood Cell Cryopreservation**

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Dedicated to my family and friends who have continuously supported me, and to the little black girl who aspires to be great. Do not let anything or anyone stop you.

*“For I know the plans I have for you,”
declares the LORD, “plans to prosper you
and not to harm you, plans to give
you hope and a future.”
- Jeremiah 29:11, NIV*

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Abstract

Cryopreservation is a method used to preserve the quality of various cell types over long periods of time (up to several years). Using this preservation method can vastly improve cellular therapies and regenerative medicine by allowing the creation of biobanks containing high-quality cell products. For example, biobanks of red blood cells (RBCs) would be beneficial for cellular therapies such as RBC transfusions, which are used to treat patients suffering from hemorrhages, anemias, and to replace blood loss after traumatic/surgical events. RBCs are currently preserved via hypothermic storage which limits their shelf life to 42 days. Similarly, biobanks of hematopoietic stem cells (HSCs) from umbilical cord blood would be beneficial for regenerative medicine therapies such as HSC transplantations, which offer treatment for blood- and immune-related diseases by reconstituting hematopoiesis. The outcome of these transplantations depends greatly on the quality of the cell product; therefore, it is important for preserved HSCs to have a minimum loss of viability and functionality.

The cryopreservation of cells at low sub-zero temperatures (-80 to -196 °C) in a cryoprotectant solution allows for long-term storage. Common cryoprotectants used are 40% glycerol for the cryopreservation of RBCs and 10% dimethyl sulfoxide (DMSO) for the cryopreservation of HSCs. Before clinical use of cryopreserved products, DMSO and glycerol must be removed as they are severely toxic to patients upon infusion. The removal of 40% glycerol from RBCs is complicated, time consuming, and can result in a significant amount of cell damage. DMSO and glycerol also do not address the occurrence of ice recrystallization, which is the main cause of cellular damage during cryopreservation. Ice recrystallization describes the process of ice crystals growing larger and replacing smaller ice crystals, and significantly contributes to the

damage of cells post-thaw. Therefore, methods to decrease the concentration of cryoprotectants to improve their removal process while also mitigating ice recrystallization is of interest.

In nature, antifreeze proteins and glycoproteins (AF(G)Ps) are found in animals that can survive below-freezing temperatures. The Ben laboratory has used the structural components of AF(G)Ps to design several small-molecule carbohydrates that exhibit ice recrystallization (IRI) activity. *O*-aryl- β -D-glucosides and *N*-aryl-D-gluconamides are two classes of IRIs developed that have been used as supplemental additives to DMSO and glycerol to improve the post-thaw viabilities and functionalities of RBCs and HSCs. While many structure-activity relationship studies have been performed amongst these classes, one area of improvement is their solubilities to facilitate their use as cryoprotectants.

This thesis focuses on the design of a new class of effective IRIs that have high solubilities (>100 mM in phosphate-buffered saline). Previous studies on the structure of small-molecule IRIs have demonstrated the importance of balancing the hydrophobicity and hydrophilicity within a molecule, making it difficult to achieve high solubilities. This thesis further explores this point by the design and synthesis of IRIs with polar functional groups possessing an overall molecular charge. *N*-aryl-D-gluconamides bearing amino- and azido-substituents were designed, however their synthesis was unsuccessful. Instead, this work revealed a synthetically facile route towards *N*-xylo-L-furanosyl amide and ester compounds. Phosphonate-substituted carbohydrates were also designed and synthesized as a means to obtain highly soluble IRIs. All of these compounds displayed high solubilities, however the majority of the compounds exhibited moderate IRI activities.

While there are many assays used to measure IRI activity, this thesis also evaluates two of the most common IRI assays and their effect on IRI activity. In addition, the effect that

cryoprotective agents (CPAs) like DMSO and glycerol have on IRI activity was also evaluated. In both cases, the type of assay used and the addition of CPAs affected the quantitative values describing IRI activity. Notably, DMSO and glycerol, had an antagonistic effect on the IRI activity of *N*-aryl-D-gluconamides and antifreeze protein type I. This was a significant observation since these IRIs are sufficient cryoprotectants in the presence of DMSO or glycerol.

Lastly in this thesis, phosphonate-substituted IRIs and antifreeze (glyco)proteins (AF(G)Ps) were evaluated as cryoprotectants for the cryopreservation of RBCs and/or HSCs. These studies showed that phosphonate IRIs and AF(G)Ps were not toxic to RBCs and/or HSCs, however the concentrations evaluated were unable to improve the post-thaw viability and/or functionality of these cell types.

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Abbreviations

δ	Chemical shift
1D	One dimensional
^1H	Proton
^{13}C	Carbon-13
2D	Two dimensional
3D	Three dimensional
^{31}P	Phosphorus
Å	Angstrom
Ac ₂ O	Acetic anhydride
AcOH	Acetic acid
Ala	Alanine
AFGP	Antifreeze glycoprotein
AFGP8	Antifreeze glycoprotein fraction-8
AFP	Antifreeze protein
ANOVA	Analysis of variance
aq	Aqueous
ATP	Adenosine triphosphate
AuNP	Gold nanoparticle
BFU-E	Blast-Forming-Unit - Erythroid
BM	Bone marrow
bp	Boiling point
br	Broad
Bu ₂ BOTf	Dibutyl boron trifluoromethanesulfonate
CB	Cord blood
c _i	Concentration at inflection point
CDCl ₃	Deuterated chloroform
CFC	Colony Forming Cell
CFU	Colony Forming Unit

CFU-GEMM	Colony-Forming Unit-Granulocyte/Erythrocyte/Monocyte/Megakaryocyte
CFU-GM	Colony-Forming Unit –Granulocyte/Monocyte
CFM-Mk	Colony-Forming Unit –Megakaryocyte
CI	Confidence interval
cm ⁻¹	Wavenumber (units for IR spectroscopy)
COSY	Correlation spectroscopy
CPA	Cryoprotective agent
d	Doublet
D ₂ O	Deuterated water
dH ₂ O	Distilled water
ddH ₂ O	Distilled deionized water
dd	Doublet of doublets
DIS	Dynamic ice shaping
DMAP	4-Dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMP	Dess-Martin periodinane
DMSO	Dimethyl sulfoxide
DMSO-d ₆	Deuterated dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
eq.	Equivalents
ESI	Electrospray ionization
FADH	Flavin adenine dinucleotide
FADPH	Flavin adenine dinucleotide phosphate
G1CP	Glucose-1-phosphonate
G6CP	Glucose-6-phosphonate
h	Hour
HEK293	Human embryonic kidney cells
HepG2	Human hepatocellular carcinoma cell line
HLA	Human leukocyte antigen
HMBC	Heteronuclear multiple bond correlation
HRMS	High resolution mass spectrometry

HSC	Hematopoietic stem cell
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IC ₅₀	Half maximum inhibitory concentration
I _h	Hexagonal ice
INA	Ice nucleation activity
IR	Infrared
IRI	Ice recrystallization inhibitor
kDa	Kilo Daltons
LC/MS	Liquid chromatography mass spectrometry
LD ₅₀	Median lethal dose
LRMS	Low resolution mass spectrometry
LTC-IC	Long-term culture-initiating cell
Lys	Lysine
m	multiplet
M	Molarity
MeOD	Deuterated methanol
MEM	Minimum essential media
MGS	Mean grain size
MHz	Megahertz
min	Minute
mL	Millilitre
mM	Milimolar
mm	Millimetre
mol	Mole
ms	Millisecond
MSA	Molecular surface area
MsCl	Methanesulfonyl chloride
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
m/z	Mass/charge
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NIS	<i>N</i> -iodosuccinimide
nm	Nanometre
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser effect spectroscopy
PB	Peripheral blood
PBS	Phosphate-buffered saline
Pd/C	Palladium on carbon
PCC	Pyridinium chlorochromate
ppm	Parts per million
PSA	Polar surface area
pyr	Pyridine
q	Quartet
QLL	Quasi-liquid layer
RBC	Red blood cells
R _f	Retention factor
RFU	Relative fluorescent units
RI	Recrystallization inhibition
rpm	Revolutions per minute
s	Singlet
SAR	Structure-activity relationship
SCA	Splat cooling assay
SD	Standard deviation
SEM	Standard error of mean
S _N 2	Bimolecular nucleophilic substitution
SSA	Sucrose sandwich assay
t	Triplet
TBAF	Tetrabutylammonium fluoride
TBAI	Tetrabutylammonium iodide
TBDPSCI	<i>Tert</i> -butyldiphenylsilyl chloride

Tf ₂ O	Trifluoromethanesulfonyl chloride
TH	Thermal hysteresis
THF	Tetrahydrofuran
Thr	Threonine
TIPSCl	Triisopropylsilyl chloride
TLC	Thin layer chromatography
TNC	Total nucleated cell
TsCl	<i>p</i> -Toluenesulfonyl chloride
μL	microlitre
UV	Ultraviolet
v	volume
WRL68	Human embryonic liver cells
wt	weight

Chapter 1. Introduction

1.1 Cryopreservation in modern medicine

As the fields of cellular therapy and regenerative medicine continue to advance, meeting the demands to deliver quality cellular materials to patients becomes challenging. The challenge often arises in the number of donors available, as well as the ability to bank and preserve cellular material for long periods. Typically, biological samples are stored for a short-term at low temperatures (4 to 8 °C), however, there is a greater degree of deterioration after long periods (weeks to months).¹ Preserving biological samples at low sub-zero temperatures is a method that allows for prolonged storage. At sub-zero temperatures, cellular activity and metabolic processes slow down, and eventually come to a halt as the temperature decreases below -130 °C.¹ Decreasing the rate of deterioration that occurs in biological samples over time, would allow for the ability to create biobanks. Specifically, creating biobanks of red blood cells (RBCs) and hematopoietic stem cells (HSCs) would greatly increase the number of successful blood transfusions and stem cell transplants respectively. For RBCs, a common challenge is identifying quality methods for long-term storage. The current standard for RBC preservation entails hypothermic storage between 1 and 6 °C for a duration of 42 days, and this can still result in blood shortages.² Likewise, the main challenge for HSC preservation is maintaining the product quality, defined by the overall cell viability and functionality over long periods at sub-zero temperatures.³ Mitigating both issues would serve as a breakthrough in the field of long-term preservation.

1.1.1 Types of long-term preservation

Currently, there are three main methods for long-term preservation of biological samples (Fig. 1.1): vitrification,⁴⁻⁶ freeze drying,^{7,8} and cryopreservation.^{9,10} Vitrification is the process of freezing by applying a high cooling rate to samples submerged in a viscous solution with a high concentration of solutes. The high cooling rate and high viscosity of the sample allows for the solution to transition directly from a liquid state to a “glass-like” solid state by bypassing the process of ice nucleation. Freeze-drying requires cells to be completely dehydrated prior to cooling to low temperatures. By removing all water from the cell sample, this method also avoids the formation of ice. The third method, cryopreservation, is the process by which biological entities are cooled in a cryoprotective medium to very low temperatures (typically -196 °C) via controlled cooling rates. All three methods have been successfully used to preserve various cell types^{5,6,8,11-15}; however, their caveats are also apparent. The use of high solute concentrations and relatively slow warming rates during vitrification can be cytotoxic and damaging to cells^{16,17}; organelle protection and overall recovery are a continuous issue for freeze-drying¹⁸⁻²⁰; and the balance between mitigating intracellular ice formation and solute effects remains a challenge for cell viability with cryopreservation.^{21,22}

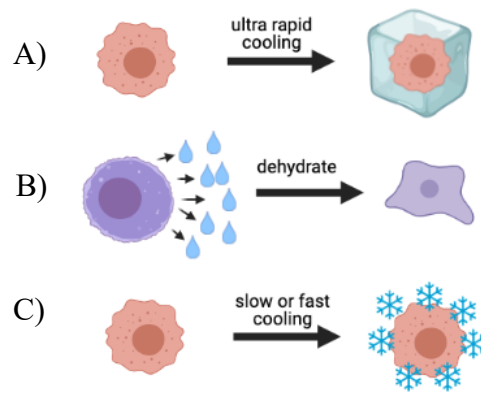


Figure 1.1 Graphical representation of the three different types of long-term preservation methods. A) vitrification; B) freeze-drying; C) cryopreservation. Figure created with BioRender.

1.2 Principles of cryopreservation

Cryopreservation involves the freezing of biological samples to sub-zero temperatures via the following steps:

- i. Addition of cryoprotective agents (CPAs) to the biological sample
- ii. Cooling of the biological sample to a low sub-zero temperature
- iii. Storage of the biological sample for a duration of time
- iv. Warming of the biological sample, when required for clinical use
- v. Removal of the CPAs before clinical use (if applicable)

The type of CPA used, and the rate at which the temperature of the sample is altered (i.e. cooling or warming) are important variables that are dependent on the cell type and can determine the success of cryopreservation. The efficiency of cryopreservation can be quantified by the percentage of viable and functional cells available before clinical use (post-thaw), and by the percentage of cells that experience cellular injury. Cell damage during cryopreservation can result in morphological changes to the cell, production of reactive oxygen species, cytoskeleton disruption, and activation of cell death pathways (apoptosis and necrosis).²³ These injuries, and

potential cell death, can arise at different stages of cryopreservation, but are most common during the cooling and warming stages.

1.2.1 Cryopreservation – The cooling process

The rate at which a biological sample is cooled is an important contributing factor to its viability post-thaw. As the temperature of a biological sample decreases, the intracellular and extracellular environments are affected differently. Due to the high osmolarity of the cytoplasm, the intracellular environment can remain unfrozen and in a supercooled state until temperatures of -10 to -15 °C.²⁴ In contrast, the extracellular environment undergoes ice nucleation and freezes at higher sub-zero temperatures (0 to -5 °C).²⁴ As ice crystals form in the extracellular environment, solute molecules are excluded from crystal formation resulting in an increase in solute concentration in the unfrozen fraction outside the cell. The uneven solute gradient between the intracellular and extracellular environment, increases the osmotic pressure across the membrane causing water to efflux, leading to dehydration of the cell.^{22,24} The efflux rate of water is dependent on both the cell membrane permeability and the cooling rate.^{21,22} Cells with a highly permeable cell membrane to water (e.g. red blood cells) equilibrate at low temperatures by allowing water to easily pass out of the intracellular environment, resulting in dehydration. Cells that have a low permeability to water (e.g. yeast cells) do not dehydrate as easily and therefore will equilibrate by the formation of intracellular ice.²⁴

Regarding the cooling rate, a slow cooling rate allows time for cells to dehydrate whilst cells cooled rapidly are unable to undergo dehydration and as a result its intracellular components freeze (Fig. 1.2).²⁴

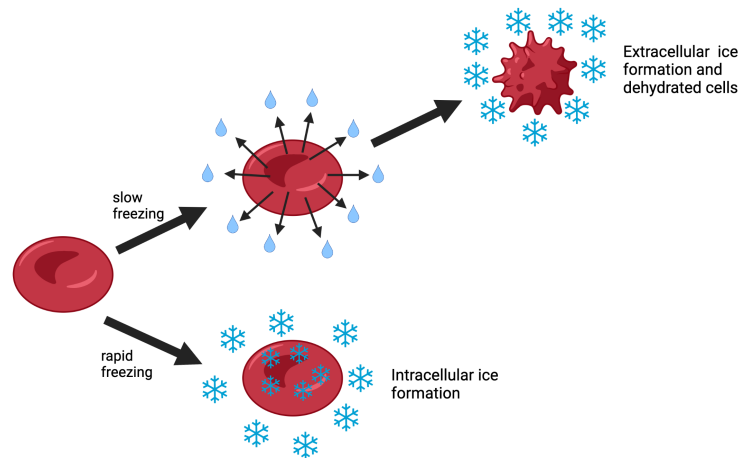


Figure 1.2. A depiction of the events that occur during slow and rapid cooling processes.^{21,22,24} Figure created with BioRender.

Both cell membrane permeability and cooling rate can cause cellular injury. Cells with a high permeability to water or that undergo slow cooling are susceptible to cellular damage caused by exposure to high solute concentrations from dehydration. Whereas cells that have low permeability to water or that undergo fast cooling rates can experience cell injury via intracellular ice damage.^{21,24} As a result of these observations, “a two-factor hypothesis” was established by Peter Mazur,²² which describes an optimal cooling rate for each cell that will result in minimal damage from exposure to high solute concentrations and intracellular ice (Fig. 1.3). For example, yeast cells which have a low membrane permeability can mitigate intracellular ice formation with slow cooling rates, however, cooling rates slower than its optimum will result in cell death from high solute concentrations. In essence, when the cooling rate is faster than the optimal, cell injury arises from intracellular ice formation, and slower cooling rates result in cell injury from high solute concentrations.²⁴

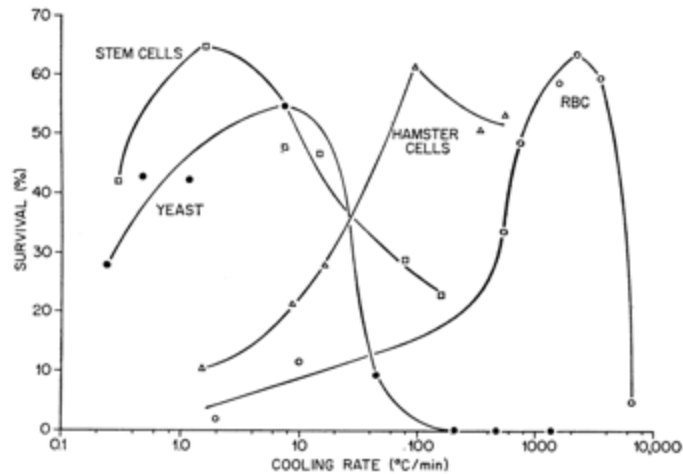


Figure 1.3. Mazur's two-factor hypothesis for different cell types. Image reprinted with permission from *Cryobiology* 1970, 168 (3934), 939–949. Copyright © 1970 The American Association for the Advancement of Science.

1.2.2 Cryopreservation – The warming process

Similar to the freezing process, the warming rate can also have an impact on the amount of cellular injury that occurs during cryopreservation. The extent of cellular damage that occurs during the warming process depends on the initial cooling rate.^{21,25,26} Dehydrated cells that result from slow cooling processes are simply rehydrated with minimal damage at both slow and fast warming rates. However, for cells that are cooled rapidly and form intracellular ice, the rate of warming can cause significant damage to the cells. During warming, the intracellular ice undergoes recrystallization (the process of small ice crystals growing into larger ice crystals), and this has been shown to be a major contributor to cellular injury.^{21,25,27} Cell damage via ice recrystallization worsens at slower warming rates and thus for rapidly cooled cells, faster warming rates are ideal.

1.2.3 Cryoprotective agents (CPAs)

Cryoprotective agents are often used during the cryopreservation of biological samples to help mitigate cryoinjury. There are two classes of CPAs that exist: penetrating and non-

penetrating. Penetrating CPAs are osmotically active and are able to pass through the cell membrane. Due to their colligative properties, penetrating CPAs provide protection against cell injury that occurs during dehydration. When water travels out of the cell during slow cooling, penetrating CPAs move freely into the cell and decrease the effective concentration of solutes both in the intra- and extracellular environment. This creates a protective effect against the increase in solute concentration in the unfrozen fraction during freezing. In addition, since penetrating CPAs have a lower freezing point than water, the overall amount of ice decreases and the unfrozen fraction increases, further adding to its protective effect.^{28,29}

Common penetrating CPAs that are used in cellular applications are glycerol and dimethyl sulfoxide (DMSO).^{11,30,31} Glycerol was the first cryoprotectant used for cellular cryopreservation¹¹ and is typically added in concentrations of 10 – 40% to cryopreserve cell types such as RBCs,^{32–36} embryos,^{15,37} oocytes,^{38–42} and sperm.^{40,43,44} DMSO is also among the early cryoprotectants reported and has been successfully used to cryopreserve oocytes,^{13,45,46} T-cells,^{47–53} and HSCs^{3,54,55} in concentrations of 5 – 15%.

Non-penetrating CPAs are also commonly used in cryopreservation to protect against cellular injury. These CPAs are usually larger in molecular weight, which makes it more difficult for them to pass through the cell membrane. Instead, these concentrate in the extracellular environment, increasing its osmolality, and promote dehydration. Due to the high extracellular osmolar concentration, the freezing rate of the extracellular environment decreases, allowing the cell to dehydrate and in turn significantly reduces intracellular ice formation.²⁹ Common non-penetrating CPAs include hydroxyethyl starch,^{14,56} polyvinylpyrrolidone,^{57,58} and sucrose.^{59,60}

Although penetrating and non-penetrating CPAs can protect against cell injury from freezing, they can also be inherently toxic. Factors such as the amount of time they are exposed to

the cell, the concentration used, and the temperature of exposure can all contribute to their lethality.⁶⁰ For example, the toxicity of DMSO has been noted in various studies when used at high concentrations and at temperatures above freezing.^{60–62} Cell membrane swelling,⁶³ protein unfolding,⁶⁴ and dissociation between the plasma membrane and cytoskeleton⁶⁵ are a few of the detrimental events that have been observed with increasing amounts of DMSO. In addition, the requirement to “wash out” CPAs before clinical use can be another limiting factor. The deglycerolization process of RBCs, for example, is necessary to prevent post-thaw osmotic hemolysis before transfusion and this is usually done by several washes with a saline solution.⁶⁶ This process can be quite lengthy and can result in up to 15% cell loss.⁶⁷

Overall, CPAs are able to replace water and protect against osmotic damage, however a common caveat is their inability to mitigate cellular damage which is caused majorly by ice growth.^{21,25,27} These limiting factors suggest the need to develop newer methods that will support the use of reduced concentrations of CPAs and to develop new CPAs that can prevent against ice-related damage.

1.3 Ice recrystallization

Ice recrystallization is the prominent cause of ice-related damage during cryopreservation.^{21,68,69} The exact mechanism by which this occurs remains unclear, however, the formation and growth of intracellular ice is deemed responsible.^{68,69} Although CPAs may assist in dehydrating cells to prevent the formation of intracellular ice, ice existing at low temperatures is inevitable and solutions to mitigate this issue are warranted.

1.3.1 The structure of ice

As water cools below 0 °C, it becomes supercooled (a liquid state of water existing below its freezing point) and eventually nucleates to form ice.^{70,71} Ice nucleation is an exothermic process that arises when water molecules arrange in an orderly fashion via hydrogen bonding to form ice crystals. Water molecules order to form various shapes of ice, with the hexagonal shape (I_h) being the most common form below 0 °C and atmospheric pressure.⁷¹ In the hexagonal ice structure, each water molecule forms a hydrogen bond to four other neighbouring water molecules. The oxygen atom hydrogen bonds to two hydrogen atoms and hydrogen atom hydrogen bonds to one oxygen atom (Fig 1.4).⁷¹

Between the formed ice crystals and liquid bulk water, an interface is present known as the quasi-liquid layer (QLL), which is a semi-ordered layer of water existing in varying thicknesses around the planes of ice.^{72,73} When ice grows, during nucleation or recrystallization events, growth occurs into the QLL versus the bulk water layer.⁷²

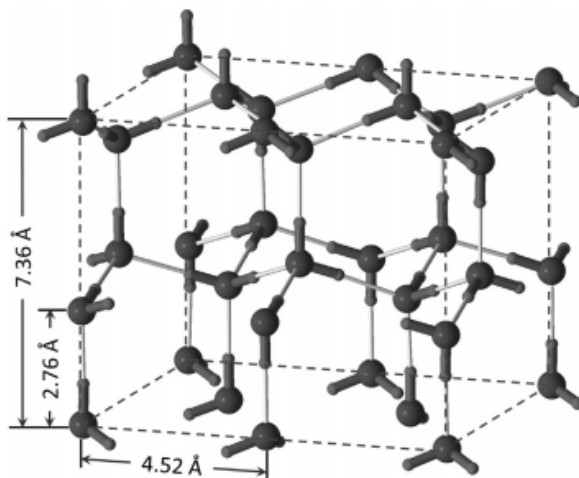


Figure 1.4. The hydrogen bonding network of water molecules in hexagonal ice. Large spheres are oxygen atoms and small spheres are hydrogen atoms. Image reprinted with permission from *Handbook of Crystal Growth*; Elsevier B.V, 2015; pp 1061–1112. Copyright © 2015 Elsevier.

1.3.2 *Mechanisms of ice recrystallization*

As temperatures begin to rise, ice recrystallization occurs as a mechanism to reduce the overall energy of the system. During ice recrystallization, ice crystals grow larger at the expense of smaller ice crystals (Fig. 1.5A), and this happens via one of two different mechanisms: grain boundary migration^{74,75} or Ostwald ripening.^{76,77} Grain boundary migration describes the transfer of water molecules from one ice grain to another. The interface where ice grains meet is called the grain boundary, which is curved, and its degree of curvature is dependent on the size of the ice grain. Large ice grains form a concave boundary resulting in a low surface energy, while smaller ice grains have a higher degree of curvature and form a convex boundary resulting in a higher surface energy. In order to decrease the overall energy of the system, grain boundaries will migrate to decrease its overall curvature.^{36, 38-39} Specifically, grain boundaries will migrate from the center of the convex crystal to the center of the concave crystal (Fig. 1.5B).

In systems with a large percentage of unfrozen fraction, the Ostwald ripening mechanism describes the recrystallization of ice and takes into account the QLL. Since larger ice crystals have a smaller surface area to volume ratio than smaller crystals, large ice crystals are more thermodynamically stable. Ostwald ripening describes the thermodynamically driven process in which water molecules travel from smaller ice crystals, through the QLL to larger ice crystals in order to reduce the ice crystal/bulk water interface and overall energy of the system.^{36, 40-41} During this process, the overall ice volume is constant, thus the number of crystals decrease as the overall size of crystals increases. Regardless of the mechanism of action, ice recrystallization is the most common cause for mechanical cell damage, therefore methods to inhibit this process are exceptionally valuable to improving cryopreservation outcomes.

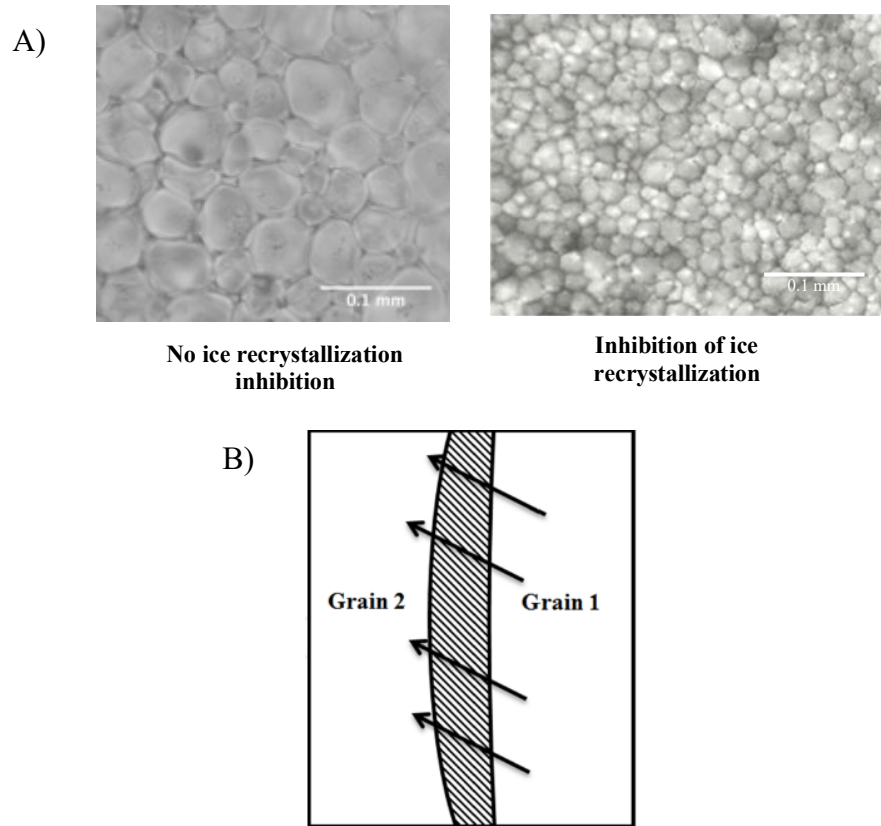


Figure 1.5. A pictorial representation of ice recrystallization inhibition (A) and grain boundary migration (B). Pictures were reprinted from *Wilson, P., Ed.; InTech, 2013; pp 177–224*. Copyright © 2013 InTechOpen.

1.4 Controlling ice recrystallization

1.4.1 Biological antifreezes

In nature, many organisms have the ability to survive in sub-zero temperature environments and to protect themselves against cryoinjury. Natural antifreezes are present in the form of antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs), and their ability to bind to ice allows them to control ice growth. The first report of a natural antifreeze was in deep sea Teleost fish found in the Antarctic ocean.⁷⁸ The antifreeze activity of the fish was attributed to the high levels of amino acids in their blood,⁷⁹ and these proteins and glycoproteins (AF(G)Ps) have since been reported in arthropods, plants, algae, fungi, yeast, and bacteria.^{80–86} There are four

classes of antifreeze proteins (type I–IV) based on their structural differences and eight classes of antifreeze glycoproteins based on their molecular mass. Antifreeze protein type I is an alanine-rich, amphiphilic, α -helix protein⁸⁷; type II AFPs contain high cysteine content and are sensitive to sulfhydryl reagents⁸⁸; type III AFPs are a series of four β -strands with one 3_{10} helical turn⁸⁹; and type IV AFPs contain an α -helix bundle.⁹⁰ Although type IV AFPs are able to bind to ice crystals and depress the freezing point of water, they are typically present alongside type I AFPs and are in too low a concentration to generate notable antifreeze effects on blood.⁹¹ Structurally, all AFGPs contain repeating (Ala–Ala–Thr)_n units with a disaccharide attached to the secondary hydroxyl group of the threonine residue, however their masses can vary from 2.6–34 kDa.⁹²

AF(G)Ps exhibit two types of “antifreeze” activities: thermal hysteresis (TH) and ice recrystallization inhibition (IRI). Thermal hysteresis is defined as a temperature gap between the nonequilibrium melting point and freezing point of a solution. At temperatures within the gap, ice crystals are unable to grow or melt as a result of an adsorption of the AF(G)P to the ice surface. AF(G)Ps adsorb along the a-axis (prism plane) of the hexagonal ice crystal lattice, preventing the addition of more water molecules onto the prism plane (Fig. 1.6A). Since ice growth typically occurs as water molecules bind to the a-axis, the presence of AF(G)Ps causes ice to grow along the c-axis to form needle-like, spiculated ice crystals (Fig. 1.6B).⁹³ As temperatures decrease, water molecules are unable to bind to the AF(G)P-bound areas on the ice crystal, therefore the ice front grows between adjacent AF(G)Ps, increasing the radius of curvature (Fig. 1.6C). Consequently, adding additional water molecules to the curved ice lattice becomes energetically unfavorable leading to a localized freezing point depression (Kelvin effect).⁹³ The mechanism by which AF(G)Ps adsorb to ice has been discussed with theories focusing on the formation of hydrogen bonds between AF(G)Ps and ice (“induced fit” model),⁹⁴ and the importance of

hydrophobic surfaces of AFPs for binding to the ice lattice.^{95,96} The second antifreeze property of AF(G)Ps is their IRI activity at high nanomolar to low micromolar concentrations. Their strong IRI activity can be attributed to their ability to adsorb onto the non-basal planes and prevent ice from growing at its grain boundaries.⁹⁷ Most naturally occurring AF(G)Ps exhibit both TH and IRI activity and these properties have been shown to be independent of each other.^{97–99}

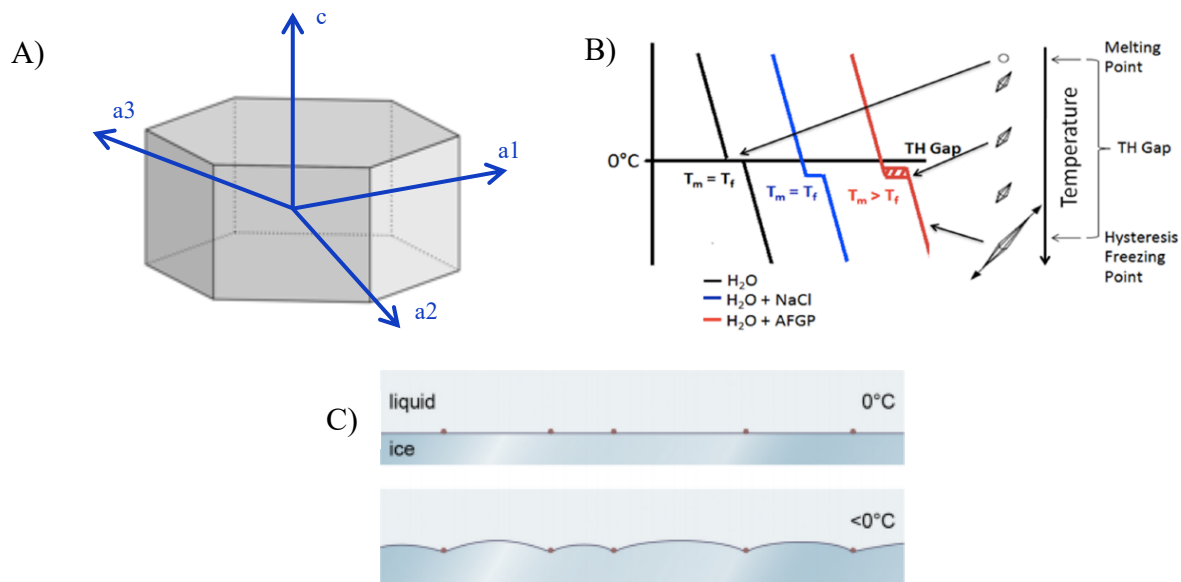


Figure 1.6. A) A display of the different axes and ice planes on the hexagonal ice lattice. B) The difference in ice crystal habit in solutions of water (H_2O), H_2O + salt ($NaCl$), and H_2O + AFP. C) Representation of the Kelvin effect. At $0^\circ C$ in the H_2O and H_2O and $NaCl$ solutions, ice crystal shape is normal. In the H_2O + AFP solution, the ice crystal shape is needle-like at temperatures below the TH gap. AFPs bound at the melting temperature ($0^\circ C$) inhibit the addition of water molecules and lead to a depressed freezing temperature. Image B reprinted with permission from *Wilson, P., Ed.; InTech, 2013; pp 177–224*. Copyright © 2013 InTechOpen. Image C reprinted with permission from *Biophys. J. 1991, 59, 409–418*. Copyright © 1991 Elsevier.

Due to their antifreeze abilities, AF(G)Ps have been used as cryo-additives to cryopreserve different cell types.^{100–104} In some cases, supplementing cryopreservative media with AF(G)Ps has resulted in minimal cell damage,^{105–110} and in other cases an extensive amount of cell lysis has been observed.^{103,111} The determining factor for cell survival during cryopreservation with

AF(G)Ps has been linked to their TH activity.¹⁰² As stated previously, at temperatures within the TH gap, spiculated ice crystals are formed which can consequently lyse cells (Fig. 1.6B). Therefore, suppressing TH activity by using low concentrations of AF(G)Ps can provide cryoprotective effect, however, high concentrations can be detrimental.¹⁰³

1.4.2 Synthetic AFGPs

Although AF(G)Ps are very IRI active, their inherent TH activity and challenges with their isolation and purification creates a need to develop synthetic mimetics that will exhibit a similar range in IRI activity without the limitations. Over the past two decades, the Ben laboratory has investigated AFGP analogues that are easier to synthesize than the native AFGPs. Initial studies observing the importance of each component of the native AFGP structure (AFGP8) included modifying the glycosidic linkage, the monomers of the polypeptide chain, and the distance between the saccharide and the polypeptide chain (Fig. 1.7).¹¹²⁻¹¹⁹

In the initial C-linked analogs (**1.1-1.4**), β -D-galactose, L-lysine, and glycine (Lys-Gly-Gly) were used in place of the native disaccharide, L-threonine, and L-alanine, respectively.¹¹⁸ Replacing the native carbon-oxygen linkage with a carbon-carbon linkage and changing the amino acid sequence to a monomer of the tripeptide unit (Lys-Gly-Gly) (**1.2**) abolished IRI activity; however, varying the length of the polypeptide chain (**1.2-1.4**) resulted in C-AFGP analogues that possessed both IRI and TH activity. Hexamer (**1.3**) and nonamer (**1.4**) analogs each contained six and nine repeating tripeptide units respectively and displayed a greater degree of IRI and TH activity than shorter constructs, although they were still not as active as the native AFGP. This trend compliments those observed with native AFGPs, where the higher molecular mass AFGPs are more active than lower molecular mass AFGPs.¹²⁰ In addition, the distance between

the saccharide moiety and the polypeptide backbone was evaluated using C-linked galactosyl serine analogues (**1.5-1.7**). It was observed that the proximity of the two moieties is important for IRI activity, since the distance of two or more carbons between the saccharide moiety and polypeptide backbone led to a decrease in IRI activity.¹¹² Synthesizing this class of C-linked AFGPs demonstrated that IRI activity and TH activity are independent of each other as **1.7** displayed the same level of IRI activity as the native AFGP, but lacked TH activity.¹¹² Comparatively, the L-ornithine and L-lysine analogues (**1.8-1.11**) displayed the opposite trend, where greater proximities between the two moieties had greater IRI activity, while also emphasizing the significance of the amide bond. Out of this class, the best AFGP mimetic exhibiting the highest degree of IRI activity, was the L-ornithine analogue possessing three carbons between the amide bond and the peptide backbone (**1.9**).¹¹² While the synthesis of C-linked AFGPs was notably easier than native AFGPs, the solid phase synthesis of these analogues was not amenable to large-scale applications.

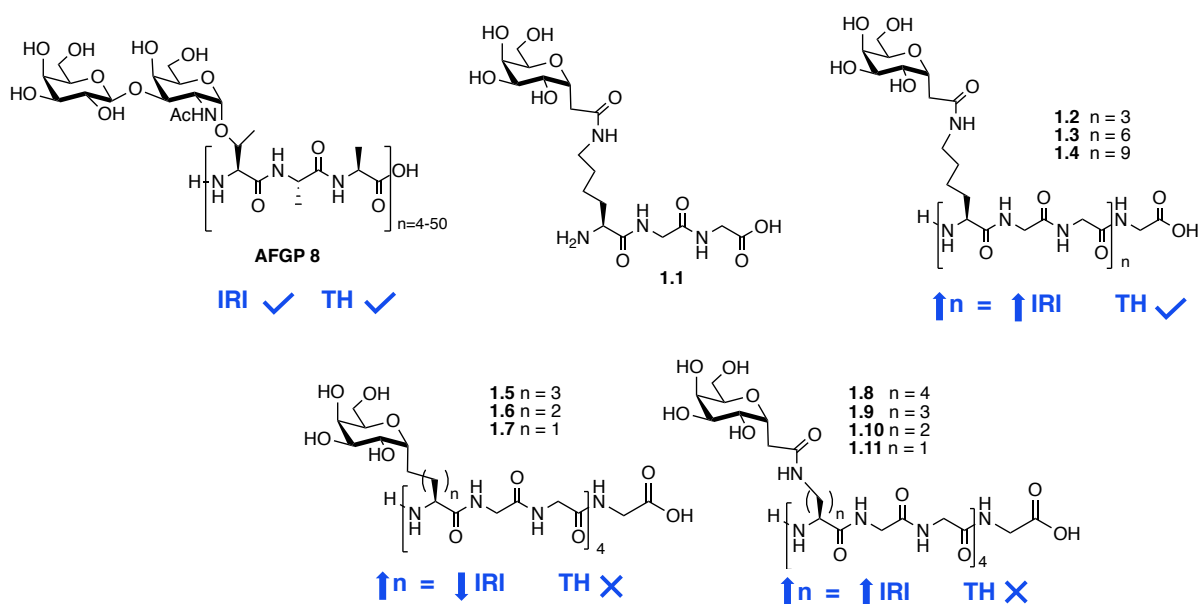


Figure 1.7. Chemical structures of the native AFGP8 and the synthetic C-linked AFGP analogs.

1.4.3 *Small molecule IRIs*

1.4.3.1 *The importance of the carbohydrate moiety*

The Ben laboratory has performed other structure–activity studies using C-linked analogues of AFGPs to better understand the role of carbohydrate hydration in IRI activity. A notable discovery by Galema et al. revealed a positive correlation between hydration of carbohydrates and their structure.^{121,122} The study demonstrated that orientation of the hydroxyl groups at C2 and C4, influenced the three-dimensional (3-D) hydrogen bond network of water as a result of their molar compressibility, the volume of space occupied by the molecule upon hydration.¹²² D-galactose fit poorly into the 3-D hydrogen-bonded network of water and allowed for the most re-organization of bulk water for hydration, whereas D-talose exhibited a better fit in the hydrogen bonding network of water.¹²¹ To investigate how this would translate into IRI activity, monosaccharides D-galactose, D-glucose, D-mannose, and D-talose were used to synthesize several C-linked AFGP analogues (Fig. 1.8). The D-galactose C-AFGP analogue was the most IRI active out of the monosaccharides whereas D-talose C-AFGP analogue, displayed the least IRI activity.¹¹⁴ These observations suggested that IRI activity is proportional to the ability of a compound to disrupt the 3-D hydrogen bonded network of bulk water.

Although this metric defined a trend amongst simple monosaccharides with galactose exhibiting the greatest IRI activity, it did not accurately translate to disaccharides (Fig. 1.8). In general, disaccharides exhibited much greater levels of hydration than monosaccharides, however, their IRI activity was synonymous to monosaccharides (Fig. 1.9A). As a result, the partial molar volumes of carbohydrates were also taken into consideration and a new parameter, the hydration index, was birthed, describing the number of tightly bound water molecules per molar volume of carbohydrate (Fig. 1.9B).¹²³ Mechanistically, carbohydrates with a larger

hydration index will form a greater hydration layer, leading to a greater distribution on the ordering of bulk water surrounding the hydration shell. This distribution will interrupt the quasi-liquid layer (QLL)/bulk water interface between two adjacent ice crystals and therefore inhibit ice recrystallization.¹²³

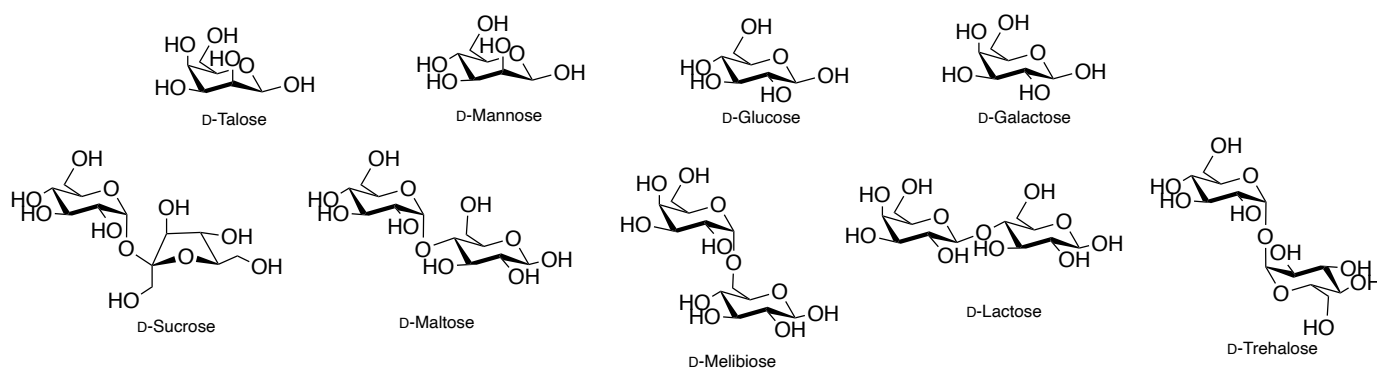


Figure 1.8. Structures of the monosaccharides and disaccharides evaluated to determine correlations between hydration number and hydration index with IRI activity.

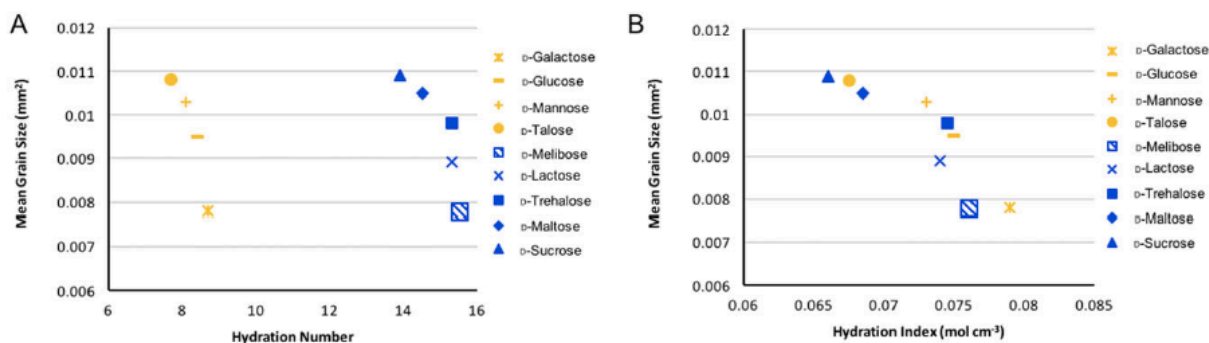
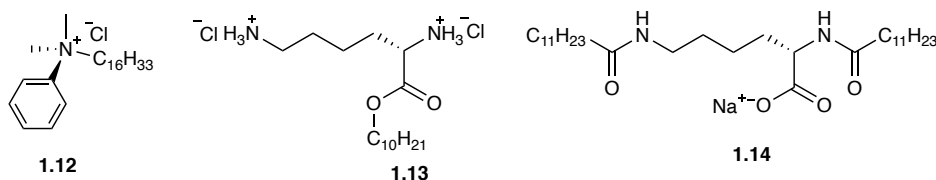


Figure 1.9. IRI activity of mono- and disaccharides plotted against their A) hydration number and B) hydration index (hydration number/partial molar volume). Figure reprinted with permission from *Peptide Science*, 2019, 111, 1–12. Copyright © 2019, Wiley.

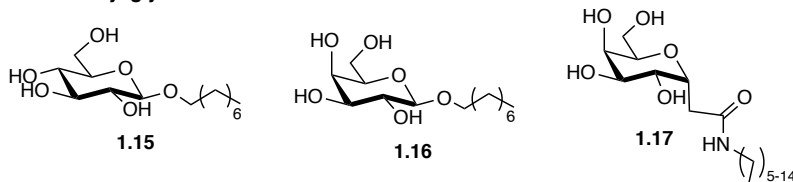
1.4.3.2 *The importance of balancing hydrophilicity and hydrophobicity*

Another notable discovery was that surfactants and hydrogelators (**1.12-1.14**) exhibited IRI activity,¹²⁴ therefore the Ben laboratory investigated the contribution of hydrophobic and hydrophilic components to IRI activity (Fig.1.10). The evaluation of alkyl glycosides (**1.15-1.17**) and *N*-alkyl aldonamides (**1.18** and **1.19**) suggested that increasing the length of the alkyl group also increased the IRI activity in both classes.¹⁰⁴ It was also observed that the D-glucose analogues (**1.18**) were more IRI active than the D-galactose (**1.19**) analogues in the aldonamide class.^{125,126} The relationship between hydrophilicity/hydrophobicity and IRI activity was further analyzed by observing the ratio of polar surface area to molecular surface area (PSA/MSA) of molecules that had a range of IRI activities.¹²⁷ In addition, another metric, the CH_n/OH ratio (the number of carbon atoms in the alkyl chain to the number of hydroxyl groups in the polyol portion) was employed. Results from this study showed that as the PSA/MSA ratio increased, IRI activity decreased, suggesting that IRI activity decreases with increasing net polarity of the molecule. Similarly, as the CH_n/OH ratio decreased, IRI activity also decreased, indicating that IRI activity decreases as the hydrophobic fraction decreases. Both results implied that having a balance between hydrophobicity and hydrophilicity in a molecule is important for IRI activity.

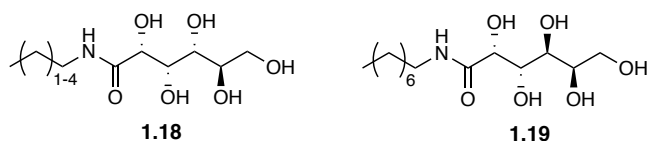
IRI active surfactants and hydrogelators:



IRI active alkyl glycosides:



IRI active *N*-alkyl aldonamides:



Trends:

- ♦ Galactosides are more active than glucosides
- ♦ Gluconamides are more active than galactamides
- ♦ Increasing alkyl chain length results in increasing IRI activity

Figure 1.10. Structures of surfactants and hydrogelators (**1.12-1.14**), alkyl glycosides (**1.15-1.17**) and *N*-alkyl aldonamides (**1.18 & 1.19**) tested for IRI activity.

1.4.3.3 Aryl pyranose and gluconamide IRIs

Building on the trends that were observed with the early classes of glycosides and aldonamides, a new class of *O*-aryl-β-D-glycosides (Fig. 1.11) and *N*-aryl-D-gluconamides (Fig. 1.12) were investigated. These substrates were desirable as ice recrystallization inhibitors for the cryopreservation of cells as they exhibited hydrophobic properties but lacked the long alkyl chains that are often detrimental to cell membranes.¹²⁸ Out of these classes, *p*-bromophenyl-β-D-glucoside (**1.22**) and *N*-2-fluorophenyl-D-gluconamide (**1.25**) display a high level of IRI activity and have showed several advantages when used in biological models.^{33,55,129} To develop a library of IRIs with similar activities, several SAR studies were conducted.

In the glycoside class, para-methoxyphenyl-β-D-glycosides (β-PMP Gly) were tested as inhibitors for ice recrystallization, and unlike previous observations, the glucose derivative β-PMP-Glc (**1.20**) was significantly more active than its galactose derivative β-PMP-Gal (**1.21**).³²

Further studies included converting the anomeric oxygen to a nitrogen or sulfur, which reduced the IRI activity of the β -PMP analogues immensely.¹³⁰ Due to the activity of β -PMP-Glc, other para-substituted derivatives of D-glucose and D-galactose have been evaluated for IRI activity.¹³⁰ The results suggested that there was no specific trend in the substituted derivatives and that electronic effects do not influence the IRI activity of *O*-aryl-glycosides in any predictable manner. Strongly electron withdrawing substituents ($-F$, $-Br$, $-Cl$, $-CF_3$) resulted in moderate to high activity, but para-substitution with $-NO_2$ resulted in minimal IRI activity. Out of the group of aryl glucosides tested, only derivatives possessing a halogen or a methoxy group in the para position possessed IRI activity, with $-Br$ (**1.22**) and $-OMe$ (**1.20**) being the most active; substitution with any other group was detrimental to activity. The aryl galactoside group had a different activity profile; only para-substituted derivatives with $-Br$ (**1.23**) and $-Cl$ (**1.24**) possessed IRI activity, while other groups, including $-F$ and $-OMe$ displayed no significant IRI activity. Another notable observation was that changing the anomeric position to an α -linkage resulted in a significant decrease in IRI activity.¹³⁰

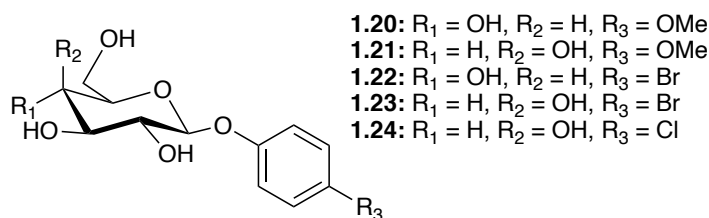


Figure 1.11. Structure of IRI active *O*-aryl-D-glycosides developed in the Ben laboratory.

In the *N*-aryl-D-gluconamide series, several substituted phenyl analogues were synthesized and evaluated for their IRI activity.⁵⁴ In accordance with the observations of the previous alkyl aldonamide studies, only the D-glucose class of aryl aldonamides were synthesized

since the D-galactose class was deemed relatively inactive.¹³¹ IRI activity in this class of compounds displayed that the regiochemistry of the substituent around the phenyl ring was crucial to IRI activity, as 2-fluorophenyl (**1.25**), 4-chlorophenyl (**1.26**), and 4-methoxyphenyl (**1.30**) substituted gluconamides were much more IRI active than 4-fluorophenyl (**1.28**), 2-chlorophenyl (**1.29**), and 2-methoxyphenyl (**1.27**) gluconamides respectively.

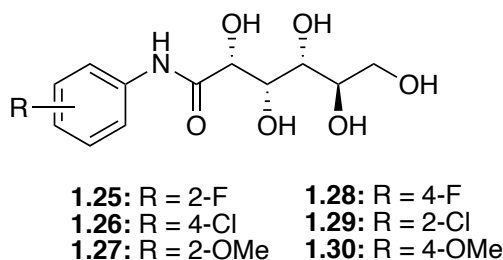


Figure 1.12. Structure of IRI active *N*-aryl-D-gluconamides developed in the Ben laboratory.

Several structure-activity relationship (SAR) studies have been performed in the *O*-aryl-D-glucoside and *N*-aryl-D-gluconamide classes and many of the IRI active compounds have been tested as additives to cryopreserve various cell types ranging from red blood cells to neuronal cells. There has been no specific trend observed relating IRI activity to the SAR work, and thus there is room to investigate different analogues of these classes as well as other novel classes of IRIs; these efforts will be discussed in more detail in the following chapters.

1.4.4 Assessing ice recrystallization inhibitors

As the discovery of IRI active compounds continues, methods to assess their activity are of importance. One of the more common assays used to evaluate the IRI activity of compounds, which is also the assay used in the Ben laboratory, is the splat cooling assay.¹³² In this assay, compounds dissolved in a phosphate-buffered saline (PBS) solution are dispensed onto an

aluminum block, cooled down to $-78\text{ }^{\circ}\text{C}$ and then annealed for 5 minutes at $-6.4\text{ }^{\circ}\text{C}$ (Fig. 1.13). It is during this annealing time that ice recrystallization occurs most rapidly. After this period, photographs are taken of the frozen sample under a microscope and the photos are analyzed, using ImageJ software, to determine the ice crystal grain size. The ice crystals are categorized into “bins” according to their sizes where the smallest ice grain (size corresponding to the size of ice grain at $t = 0\text{ min}$) is considered “bin 1” and the following bin sizes increase in 0.001 mm^2 increments. The proportion of ice crystals in each bin is used to determine the initial rate of ice recrystallization and is normalized against the PBS control (v_{norm}).¹³³ Different concentrations of each compound are tested to determine the compound-dependent rates of ice recrystallization and normalized rates are plotted and fitted onto a four-parameter non-linear regression to generate a dose response curve. Calculated IC_{50} values (the concentration that reduces the rate of ice recrystallization by 50%) are used as a metric to determine the quantitative inhibition activity of the IRI. In essence, the lower the IC_{50} value, the more IRI active the compound is at inhibiting ice recrystallization. IRIs that have been tested and deemed as IRI active by the Ben laboratory and have been used as cryo-additives in biological systems typically have an IC_{50} value of 20 mM or less.

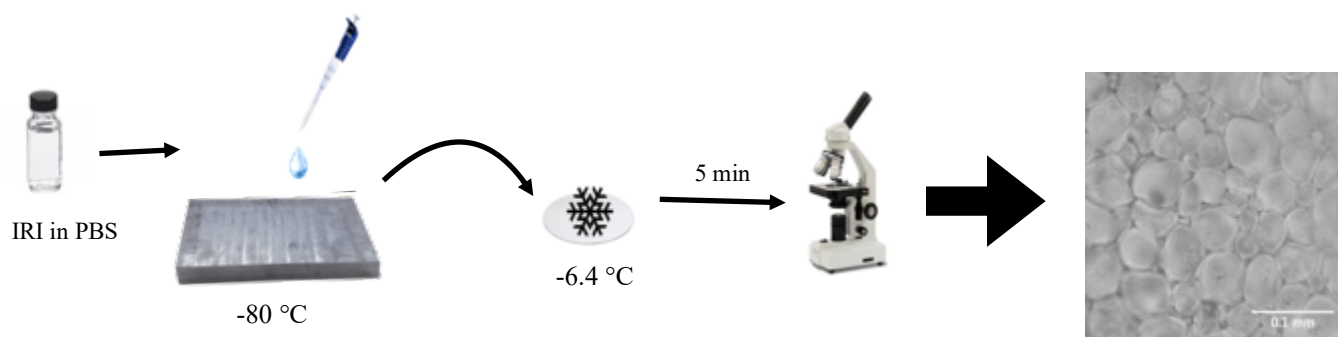


Figure 1.13. Graphical description of the splat cooling assay used to measure ice recrystallization inhibition.

Another metric used to quantify IRI activity is percent mean grain size (%MGS). To determine % MGS, the average ice grain size in the presence of a compound at a specific concentration is calculated and compared to the average ice grain size of PBS. This value describes the mean grain size of ice crystals after a specific annealing time, with respect to the PBS positive control for ice recrystallization. This metric is often used as a facile initial screening method for compounds.

Aside from the splat cooling assay, the sucrose-sandwich assay (SSA)¹³⁴ is another common assay used to evaluate IRI activity. The assay varies from the splat cooling assay such that 40% sucrose is used as the standard solution and positive control for ice recrystallization (instead of PBS), and 1 μ L of a compound dissolved in 40% sucrose is sandwiched between two coverslips prior to freezing. The quantification of ice recrystallization is usually performed by analyzing ice crystal sizes generated from the SSA and calculating % MGS values.

An in-depth comparison between these two assays and a description of other reported IRI assays (the capillary method,¹³⁵ the gold nanoparticle assay,¹³⁶ high-throughput sapphire slide assay,¹³⁷) will be discussed in **Chapter 5**.

1.4.5 Caveats with IRI active compounds

As mentioned previously, the appropriate balance of hydrophobicity and hydrophilicity is a major contributing factor to IRI activity. This results in compounds with high IRI activity often having low solubilities in aqueous media due to their hydrophobic component. The limited solubility of these compounds can become problematic during formulation procedures when the working concentration is near its maximum solubility. For example, **1.22** has an IC₅₀ value of 15 mM, however at this concentration the rate of ice recrystallization is reduced by 50%. To reduce the rate of ice recrystallization by 100% (complete inhibition of ice recrystallization), compound

1.22 would have to be used at higher concentrations (> 45 mM), which is close to its maximum solubility (55 mM) (Fig. 1.14). Since compounds have reduced solubility at low cryopreservation temperatures, compounds could precipitate out of solution, lowering the overall concentration in solution. Since the exact mechanism of IRIs is still unknown, it is difficult to design a tailored synthesis towards compounds that are highly soluble while also IRI active. Perfecting the balance of solubility and IRI activity would ease the usability of IRIs for several of its applications.

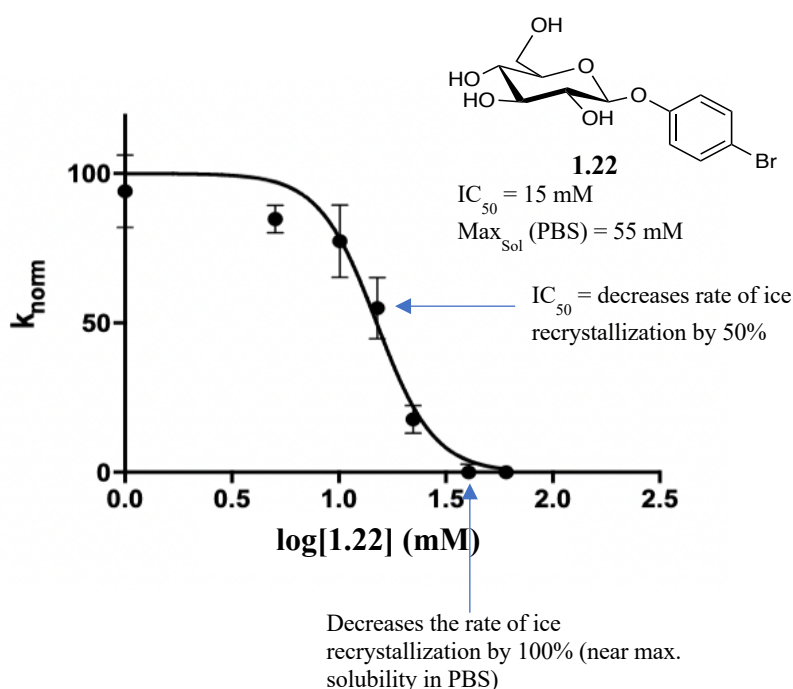


Figure 1.14. The IRI activity of **1.22** at its IC₅₀ concentration and its maximum solubility concentration in PBS. Figure reprinted with permission from University of Ottawa, 2015. Copyright © 2015, Stephanie Abraham¹³⁹

1.4.6 Other applications for IRIs

Aside from their benefits in cellular cryopreservation, IRIs can be useful in other applications where ice growth is problematic. In the frozen foods industry, IRIs can increase the quality of frozen foods by decreasing the amount of freeze damage.^{83,140,141} Foods such as ice

cream, in which the texture is important, decrease in quality when it goes through many freeze-thaw cycles. Variations in temperature enhance the recrystallization of ice crystals which results in an unpleasant grainy texture to the ice cream. IRIs, specifically AFPs with IRI activity, have been shown to decrease the size of ice crystals and therefore can improve the texture of ice cream products.^{142–144} Similarly, freezing and thawing can also decrease the nutrients in frozen meat via “drip loss.”¹⁴⁵ Drip loss occurs when large ice crystals form intracellularly that burst the cells in frozen meats. When the cells burst, their nutrients are lost in the liquid water that forms upon thawing; therefore molecules able to inhibit ice recrystallization can reduce the crystal size and the amount of nutrients lost.¹⁴⁰ IRIs have been used as additives for several industrial applications to lower the energy costs of dense ice slurries, used for long-distance transport, by lowering the freezing point and controlling the growth of ice crystals within the slurry.^{146,147} Considering all of these applications, the ability to inhibit ice recrystallization is highly valuable and beneficial to several industries.

1.5 Thesis goal

Overall, this thesis touches on many aspects of synthesizing new classes of IRIs with high solubilities, improving the cryopreservation procedures of various cell types by inhibiting ice recrystallization, and investigating the techniques used to measure IRI activity. Specifically, the following chapters will discuss: various methods to synthesize new classes of active IRIs possessing high solubilities (**Chapter 3** and **4**); efforts made to determine the effect of different assays on IRI activity (**Chapter 5**); and finally, this thesis will evaluate the ability of AF(G)Ps and newly synthesized IRIs to improve the cryopreservation methods of RBCs and HSCs (**Chapter 6**).

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Chapter 2. Goals and Objectives

2.1 Introduction

The requirement for better cryopreservation methods is increasing as the development of new cellular therapies arise. For cellular therapies to be successful, cellular material must be of high quality and be delivered to patients in a timely manner.¹ In order for this to occur, cryopreservation, as a method of long-term storage, is beneficial under optimum conditions and can allow for an on-demand supply of cellular material that has not deteriorated over time.

As outlined in **Chapter 1**, cryopreservation entails cooling cellular material to low sub-zero temperatures (between -80 °C and -196 °C) at which all cellular activity and metabolic processes are halted.¹ Although this method of long-term storage is currently used for the preservation of various cell types, such as red blood cells (RBCs)²⁻⁵ and hematopoietic stem cells (HSCs),⁶⁻⁸ its main limitation involves the onset of cryoinjury at low temperatures. Cryoinjury can occur during the cooling and/or warming stages of cryopreservation. Several studies focusing on efforts to mitigate cryoinjury involve close attention to cooling and warming rates and also the use of cryoprotective agents (CPAs), as described in **Chapter 1**.⁹⁻¹³ Conventional CPAs, like glycerol and DMSO, are able to provide protection by colligatively reducing the freezing point of cryosolutions and by preventing harm from increasing solute concentrations upon ice formation.^{10,11,14,15} Conversely, at high concentrations, CPAs are often harmful and can exhibit high levels of cytotoxicity, while also requiring complete removal before clinical use.¹⁶ The latter often involves several washes with saline solutions, which can also decrease the overall cell viability. Another limitation of CPAs is their inability to protect against ice recrystallization, which is a great contributor to cryoinjury.

One method of inhibiting ice recrystallization is through the use of ice recrystallization inhibitors (IRIs) as cryo-additives to conventional CPAs. To date, the Ben laboratory has identified many small molecule carbohydrates that are able to inhibit ice recrystallization and consequently improve the cryopreservation of various cell types.^{7,17-21} This work originated by the investigation of synthetic antifreeze glycoproteins (AFGPs), which exhibit ice recrystallization inhibition activity and have great potential as cryo-additives. Synthetic AFGPs were of interest due to their comparable IRI activity to the native AFGP and their absence of thermal hysteresis (TH) activity, which is detrimental to cells during cryopreservation.^{22,23} A deeper investigation into the key structural elements of AFGPs that are responsible for IRI activity, led to the synthesis of various classes of glucose-derived IRIs that were more synthetically accessible.²⁴⁻²⁷ In addition, several structure-activity relationship (SAR) studies of small molecule IRIs revealed the importance of balancing the hydrophobic and hydrophilic components. This gave rise to *O*-aryl- β -D-glucosides and *N*-aryl-D-gluconamides with high IRI activity.^{7,28} As a result of their IRI activity, these compounds have displayed great potential as cryo-additives in the cryopreservation of various cell types.^{7,17,19,28,29} However, due to the necessity of hydrophobic groups, IRIs from the *O*-aryl- β -D-glucoside and *N*-aryl-D-gluconamide classes often exhibit limited solubilities. Thus, improving solubility while maintaining their IRI activity is an area for improvement. Therefore, the overall goal of this thesis is to identify novel small molecule carbohydrates that possess high IRI activity and high solubility while also assessing their *in vitro* cytotoxicity and their ability to be used as cryo-additives. This goal will be achieved by performing a step-by-step process used by the Ben laboratory to identify novel IRIs (Fig. 2.1). The first step entails the synthesis and characterization of a small molecule carbohydrate with potential IRI activity. Potential IRI candidates are selected using previous SAR studies. Once the compound is purified and characterized, it will be evaluated

for IRI activity using the splat cooling assay.³⁰ From the splat cooling assay, a dose-response can be generated for compounds exhibiting IRI activity. The dose-response pertains to the compounds ability to inhibit (or reduce) the rate of ice recrystallization, and from a full dose-response curve, an IC₅₀ value can be obtained. Compounds that have an IC₅₀ value less than 20 mM are of great interest and are eligible to move on to *in vitro* testing. A low IC₅₀ value (< 20 mM) implies that only low concentrations are required to inhibit the rate of ice recrystallization and therefore the compound is an effective IRI. However, in some cases compounds possessing an IC₅₀ value greater than 20 mM are evaluated further. The *in vitro* cytotoxicity of IRI active compounds is assessed in HepG2 cells and compounds that display relatively low cytotoxicity at and around their IC₅₀ value for ice recrystallization will be further evaluated for their cryopreservative ability in different cell lines. Cryopreservation studies involve the supplementation of clinically established cryomedia with various concentrations of IRI active compounds. For example, to observe the cryoprotective ability of an IRI towards red blood cell (RBC) cryopreservation, 15% glycerol is supplemented with the IRI active compound. Similarly, for hematopoietic stem cell (HSC) cryopreservation, 10% DMSO supplemented with an IRI active compound is evaluated. Further cryopreservation studies in the Ben laboratory have also been conducted in other cell types such as mesenchymal stem cells,³¹ T-cells and neuronal cells (unpublished work).

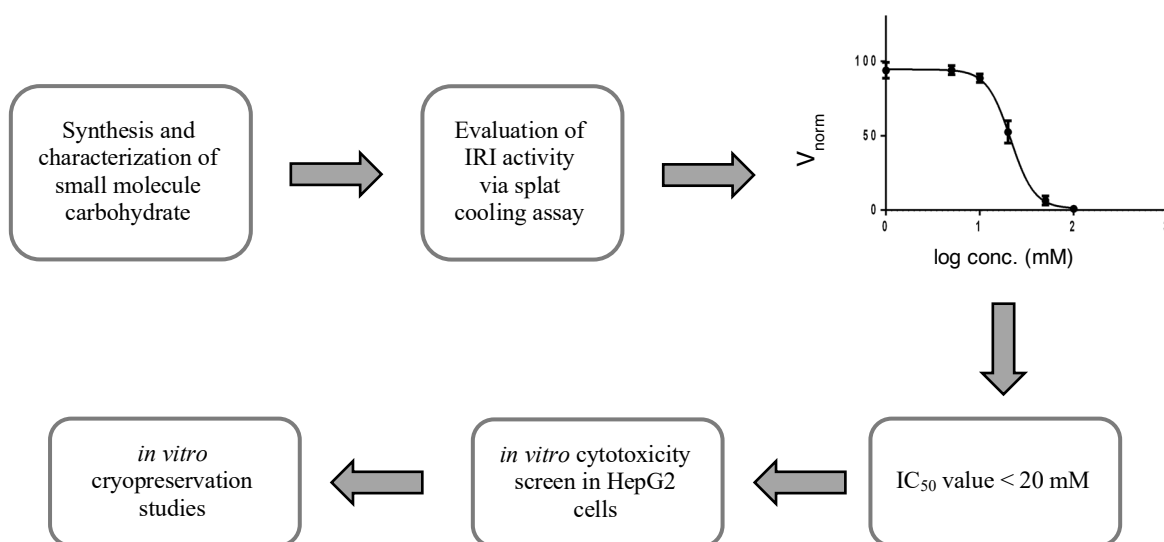


Figure 2.1. The step-by-step approach to identify compounds exhibiting IRI activity and *in vitro* cryopreservative ability.

As such, this thesis will begin by first designing novel IRIs with high solubilities. Specifically, the synthesis of amino-substituted *N*-aryl-D-gluconamides were hypothesized to provide increased solubility due to the polar group, while potentially maintaining high IRI activity. This hypothesis will be further explored by synthesizing various phosphonate-containing small molecule carbohydrates. Before evaluating some of these synthesized IRIs *in vitro*, the current methods used to evaluate IRI activity will be discussed and compared. There are several assays that can be used to evaluate the efficiency of an IRI, therefore it is essential to determine their translatability and the consistency in results when used amid conventional CPAs. Finally, the *in vitro* evaluation of novel IRIs and IRIs with high activity will be assessed. This will be important for further clinical use of these IRIs for the cryopreservation of cellular material. These four goals will be discussed thoroughly in the following chapters.

2.2 Objective 1 – Synthesis of 5-amino-*N*-aryl-D-gluconamides as next generation IRIs

N-aryl-D-gluconamides have been studied extensively in the Ben laboratory to identify trends owing to their IRI activity.^{7,32,33} Their core structure consists of a hydrophobic aryl ring bound to a hydrophilic open-chain carbohydrate via an amide bond. Investigations into this class revealed that the balance of the hydrophobic aryl ring with the hydrophilic carbohydrate component, are major contributors to their high IRI activity. Therefore, modifications to the hydrophobic/hydrophilic balance of the chemical structure could be detrimental to their IRI activity. In addition, several SAR studies show that the electronics of the aryl substituent (-F, -Cl, -Br, -OMe, etc.) and the regiochemistry of the aryl substituent also alter IRI activity, and from these studies *N*-(2-fluorophenyl)-D-gluconamide (**1.25**) was identified as the most active IRI in this class.^{32,33} Due to the requirement of a hydrophobic/hydrophilic balance, *N*-aryl-D-gluconamides often have poor solubilities; however, it is unknown if methods to increase their solubility would have a great impact on their IRI activity. To this point, objective 1 (**Chapter 3**) focuses on synthesizing an analogue of **1.25** by replacing the *C*5-hydroxyl group with a *C*5-amino group in an effort to increase its solubility (Fig. 2.2). At neutral pH, the amino group would possess a positive (+1) charge, therefore increasing the polarity of the overall molecule. The amino-substituted analogue, **3.28**, will be synthesized via an azido-substituted intermediate, **3.27**, which will also be evaluated for its solubility and IRI activity. It is hypothesized that **3.27** may also be IRI active due to the zwitterion azide substituent, which could contribute the hydrophilicity of the overall molecule.

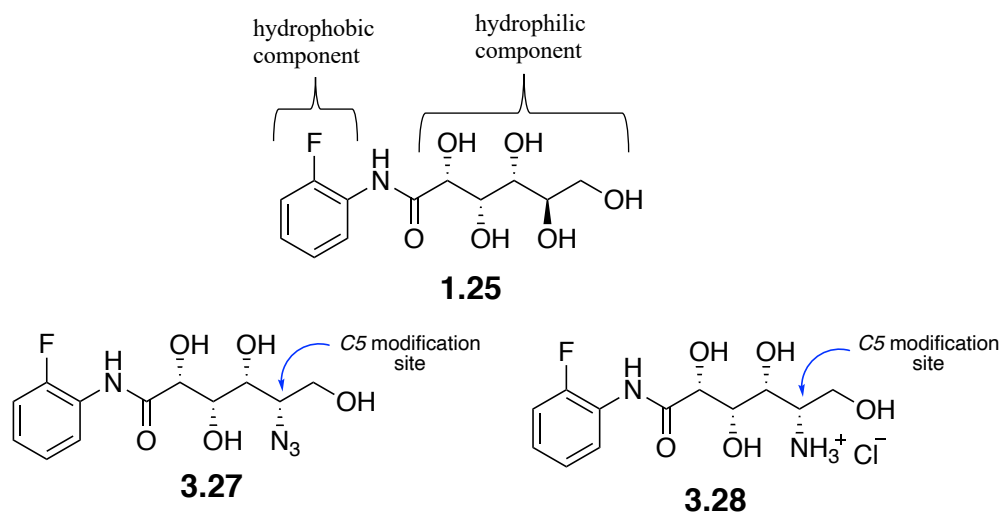


Figure 2.2. Chemical structure of *N*-(2-fluorophenyl)-D-gluconamide (**1.25**) and its azido- and amino-analogues (**3.27** and **3.28**).

The synthesis of the amino- and azido-substituted analogues will be explored using three different synthetic routes (Fig. 2.3): The first route is via a direct displacement of a leaving group at C5 of the aryl gluconamide, by a nucleophilic azide; the second route is similar to the first, however, the nucleophilic displacement by an azide ion will be performed on a glucono-ester before late-stage amidation with an aryl amine; finally, the third route will be via an aldol asymmetric synthesis leading to the desired product. In the asymmetric aldol synthesis, an azide-substituted butanal synthon and Evan's chiral auxiliary will generate a glucitol-derived oxazolidinone before cleavage of the chiral auxiliary to generate the desired gluconamide.

From route 1 and route 2, a new class of compounds was discovered, and a detailed characterization is performed to identify them as L-xylofuranosyl amides and esters. The scope and the proposed mechanism of the reaction is verified by evaluating various aryl/alkyl substituents and by altering the configuration of the nucleophilic hydroxyl group that drives the

mechanism. Finally, in the last section of **Chapter 3**, the IRI activity of this new class of compounds is also explored via the splat cooling assay.

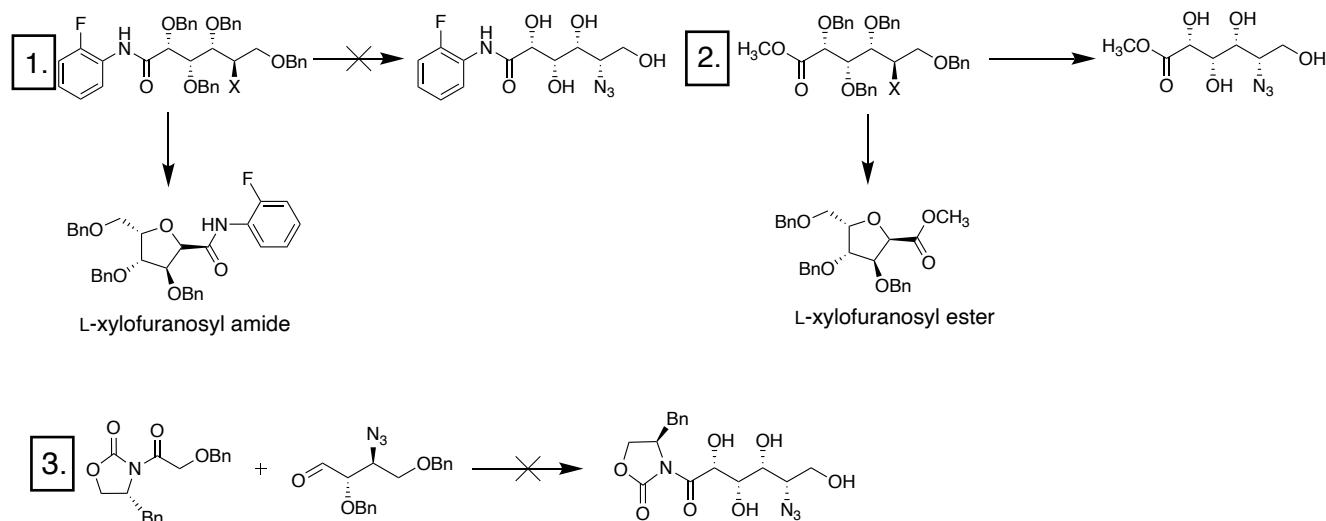


Figure 2.3. An overview of synthetic routes 1-3 and their formed and/or expected products.

2.3 Objective 2 – Assessing the solubility and IRI activity of IRIs containing a phosphonate functional group

The second objective of this thesis is to further the search in discovering IRI active compounds with high solubilities. Only a limited number of carbohydrate-derived compounds possessing a polar functional group with an overall molecular charge (+1/-1) have been evaluated for IRI activity.^{31,34} Out of these surveyed compounds, phosphate-derived monosaccharides displayed high solubilities (> 100 mM) and moderate IRI activities.³¹ Therefore, it was hypothesized that incorporating a phosphate functional group into the molecular structure of an IRI active compound could further increase its solubility. Since phosphates are hydrolyzed easily in aqueous environments, implementing a stable phosphonate analogue (possessing a carbon-phosphorus bond instead of an oxygen-phosphorous bond) will be explored. To this end, **Chapter 4** outlines

the synthesis of phosphonate-derived carbohydrates and evaluates their ability to inhibit ice recrystallization. This study will commence by the synthesis of glucose-1-phosphonates (**4.14-4.17**) and glucose-6-phosphonates (**4.18 & 4.19**) and their IRI activity will be evaluated via the splat cooling assay. Different positively charged counterions will also be surveyed in this section to balance the negatively charged phosphonates, and to investigate their contributions towards IRI activity. To verify the contributions of the counterions, chloride salts of the positive counterions will also be evaluated for IRI activity as a comparison. From there, the synthesis of phosphonate analogues of IRIs from the *O*-aryl- β -D-glucoside class (**4.34-4.37**), the *N*-aryl-D-gluconamide class (**4.38 & 4.39**), and the *N*-alkyl-D-gluconamide class (**4.40 & 4.41**) will be explored. As observed in previous studies, the regiochemistry of functional groups can have a great impact on IRI activity, and SAR studies are often useful to determine the regioisomer with the greatest activity.^{7,28} However, for this preliminary study, a *C6*-phosphonate derivative of *O*-aryl glucoside and *N*-aryl gluconamide will be synthesized due to their ease of synthesis. For the *N*-alkyl gluconamide, the phosphonate group will be incorporated at the end of the alkyl chain in an attempt to temper its surfactant properties (a property that is detrimental to cell membranes). Finally, the phosphonate analogues will be assessed to determine the effect of a phosphonate functional group on solubility and IRI activity with respect to the parent IRI.

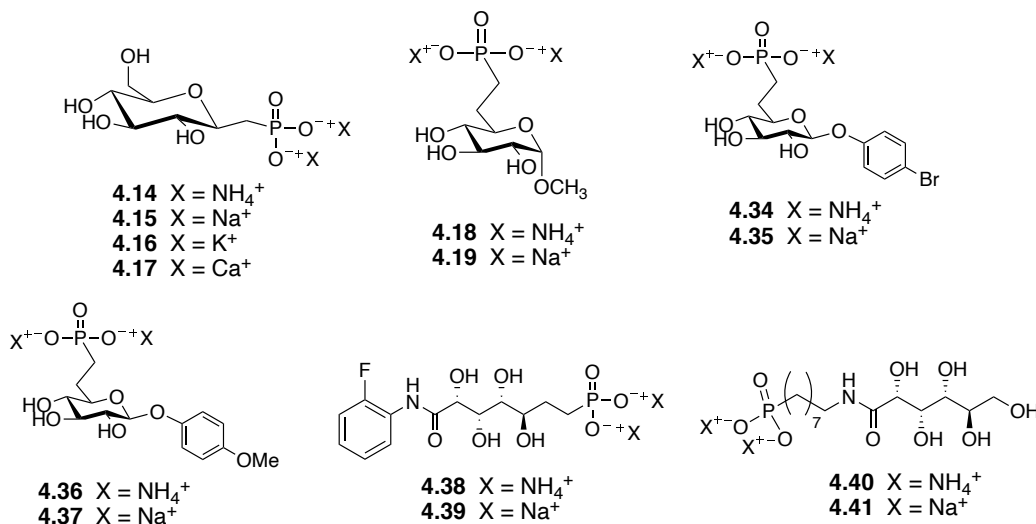


Figure 2.4. Structures of target carbohydrate phosphonate analogues to be synthesized and assessed for IRI activity and solubility.

2.4 Objective 3 – Evaluating the effects of different assays on IRI activity

Preventing the process of ice recrystallization is necessary to increase the outcomes of cryopreservation therefore the discovery of ice recrystallization inhibitors is important. There are a variety of assays that can be used to evaluate the ability of a compound to inhibit ice recrystallization. All of these assays use different techniques, temperatures, standard solutions, and different time periods to measure the growth of ice crystals either qualitatively or quantitatively. Ice recrystallization is highly dependent on factors such as the temperature, addition of solutes, and ice volume fraction, therefore the different IRI assays can result in varying qualitative and quantitative results. Thus, the first goal of **Chapter 5** is to determine the extent of the difference between two of the most commonly used IRI assays: the splat cooling assay (SCA)³⁰ and the sucrose sandwich assay (SSA).³⁵ The main differences between the SCA and SSA are the sample preparation and the standard solution used as the positive control for ice recrystallization. As such, the difference in ice crystal structure and crystal growth rate in the absence of an IRI will be

analyzed. In addition, to investigate the interchangeability of the assays, the quantitative values generated (IC_{50} value and the percent mean grain size) will be compared for an IRI active antifreeze protein.

Not only is it important to assess the interchangeability of commonly used IRI assays, but it is also important to verify if their results reflect the IRI's ability to inhibit ice recrystallization amidst conventional CPAs. As cryo-additives, IRIs are typically used alongside conventional CPAs such as DMSO and glycerol to prevent ice recrystallization during cryopreservation. Thus, the second goal of **Chapter 5** is to conduct a study to evaluate the IRI activity of several IRI active compounds in the presence of DMSO or glycerol. In this study, the splat cooling assay will be used to measure the IRI activity of an *O*-aryl glucoside and *N*-aryl gluconamide in increasing concentrations of DMSO or glycerol from 0.001% to 2.5%. IRI activity will be quantified as an IC_{50} value and if the IRI activity of the *O*-aryl glucoside and *N*-aryl gluconamide is unaffected by the presence of a CPA, its IC_{50} value should remain unchanged. The results from this chapter will afford a better understanding of the translatability of IRI assays as well as the activity of IRIs amidst conventional CPAs.

2.5 Objective 4 - Evaluating the cryopreservative ability of IRIs for red blood cell and hematopoietic stem cell preservation

The cryopreservation of red blood cells (RBCs) is valuable in providing a solution for long-term storage and for the creation of biobanks. This is beneficial since having an on-demand supply of RBCs can facilitate the faster delivery of blood for transfusions to treat blood-related diseases. In Canada and the US, the accepted cryopreservation conditions of RBCs involve the use of 40% glycerol and slow cooling procedures (1 °C/min). Due to the toxicity of glycerol to patients,

glycerol must be completely removed from the cryopreserved RBCs before clinical use. Two of the major drawbacks of this protocol is that the process of deglycerolization is time consuming (limiting the clinical applications of cryopreserved blood), and the added glycerol does not protect the cells from ice recrystallization. In previous studies, the Ben laboratory has demonstrated that supplementation of IRIs in samples cryopreserved with reduced glycerol concentrations can increase the post-thaw viability of RBCs.^{17,18,28} Until now, only compounds from the *O*-aryl glucoside class have demonstrated cryoprotective ability towards RBCs, thus the first objective of **Chapter 6** is to assess the cryoprotective ability of IRI active compounds from other classes towards the cryopreservation of RBCs.

Prior to evaluation in RBCs, IRI active compounds, from the AF(G)P and glucose phosphonate class (**4.14**, **4.15**, **4.18**, and **4.19**) will first be evaluated for their cytotoxicity in HepG2 cells. Finally, using two different cooling protocols, AF(G)Ps and glucose phosphonates (**4.14** and **4.15**) will be evaluated as cryo-additives in the cryopreservation of RBCs. The % hemolysis of RBC samples will be assessed post-thaw, to measure the cryopreservative ability of the evaluated IRIs.

Following the analysis of IRI active compounds in RBCs, the second objective of **Chapter 6** will be to evaluate glucose-1-phosphonate (**4.14**) as a cryo-additive to hematopoietic stem cell (HSC) cryopreservation. HSCs isolated from umbilical cord blood are often used in HSC transplantations due to its ability to reconstitute hematopoiesis and proliferate into various blood cells.³⁶ For HSC transplantations to be successful, cells must be stored properly to prevent high levels of apoptotic cells and to prevent decreases in their functional capacity. Currently, HSCs are cryopreserved in 10% DMSO using slow cooling rates, however, current procedures still result in less viable cryopreserved cells or cells with a reduced functional capacity. To improve these

procedures, the Ben laboratory has investigated supplementing IRIs in 10% DMSO and have observed increases in the functionality and engraftment potential of HSCs.^{7,21} To this point, the viability and functionality of cryopreserved HSCs with compound **4.14** will be evaluated.

2.6 Summary of goals and objectives

Overall, the goal of this thesis is to improve the solubility of ice recrystallization inhibitors by developing IRIs with polar functional groups while also verifying the type of assessment used to determine IRI activity. This goal will be achieved by exploring the following objectives:

1. To design IRIs with polar functional groups to increase their maximum solubility limit.
2. To determine the impact of the type of IRI assay used or the addition of CPA on the activity of IRIs.
3. To investigate new classes of IRIs in the cryopreservation of RBCs and HSCs.

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Chapter 3. Synthesis of 5-Amino-*N*-Aryl-D-Gluconamides as Next Generation IRIs

3.1 Previous structure-activity studies on *N*-aryl-D-gluconamides

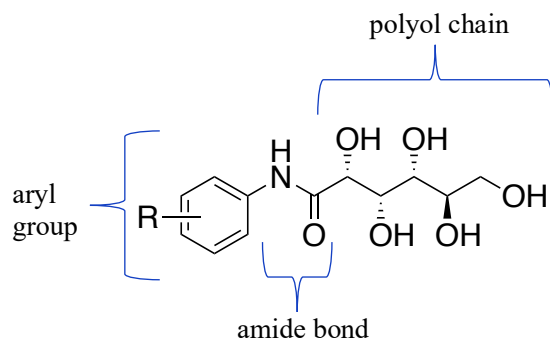


Figure 3.1. The structural components of *N*-aryl-D-gluconamides.

The *N*-aryl-D-gluconamide class of IRIs has been extensively studied in the Ben laboratory for their strong IRI activity and simple synthesis.¹ The general structure of *N*-aryl-D-gluconamides consists of an open-chain hydrophilic polyol attached to a hydrophobic aryl group via an amide linkage to the C1 of the carbohydrate polyol (Fig. 3.1). Prior to this, several gluconamides with *N*-alkyl substituents of different chain lengths were evaluated for IRI activity. This previous study was conducted to better understand the importance of amphiphilicity toward IRI activity. To assess the amphiphilicity of a molecule, two different metrics were used: the ratio of polar surface area to molecular surface area (PSA vs. MSA), and a ratio describing the number of carbons in the hydrophobic alkyl chain to the number of hydroxyl groups (CH_n/OH).² IRI activity was correlated to the amphiphilicity of the molecule (Fig. 3.2). A negative linear correlation between the PSA/MSA ratio and IRI activity was observed, and a positive linear correlation between the CH_n/OH ratio and IRI activity was observed. *N*-hexyl gluconamide was the only molecule to not follow the trend and was considered an outlier. The high IRI activity

observed with *N*-hexyl gluconamide was hypothesized to be a result of the formation of a different conformation or a transient aggregate structure in solution, however this could not be confirmed.² Both of these results suggested that IRI activity decreases with increasing polarity of a molecule. Stated another way, IRI activity increases as the hydrophobic fraction increases, further suggesting the hypothesis that a hydrophobic/hydrophilic balance is important for IRI activity.

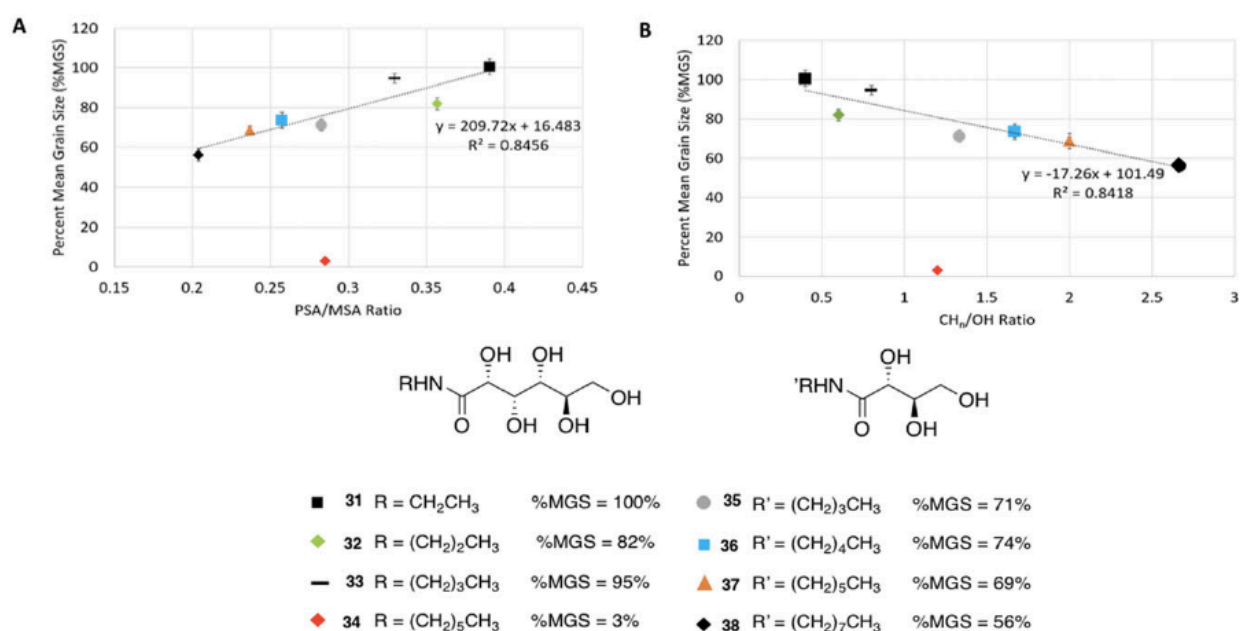


Figure 3.2. Correlation of IRI activity to PSA/MSA ratio (A) and IRI activity to CH_n/OH ratio (B) of *N*-alkyl gluconamides and *N*-alkyl erythonamides. Figure reprinted with permission from *Peptide Science* 2019, 111 (1), 1–12. Copyright 2019 Wiley.

Due to the surfactant-like properties of the *N*-alkyl gluconamides, which can be detrimental to the cell membrane,³ the *N*-aryl gluconamide class of IRIs was designed by a previous graduate student, Dr. Jennie Briard. Similar to the alkyl group, the aryl group contributes to the hydrophobicity of the compound and has the same number of carbons as the *N*-hexyl gluconamide, therefore maintaining the optimum hydrophobic/hydrophilic balance. The structural

components of the *N*-aryl gluconamide class of IRIs were studied to identify trends that will facilitate building a library of compounds that possess IRI activity. The SAR study of this class concluded that changing the substituent and the position of the substituent on the aryl ring had a great effect on IRI activity (Fig 3.3).⁴ Specifically, changes from *N*-2-fluorophenyl (**1.25**) to *N*-3-fluorophenyl (**3.02**), to *N*-4-fluorophenyl (**1.28**) resulted in a sequential decrease in IRI activity. Similarly, for the *N*-bromophenyl analogues, the *N*-3-bromophenyl analogue (**3.03**) was more active than the *N*-4-bromophenyl analogue (**3.04**); the *N*-2-bromophenyl analogue was not evaluated. However, in contrast, for the methoxy-substituted analogues, the *N*-4-methoxy analogue (**1.30**) was more active than the *N*-2-methoxy analogue (**1.27**); the *N*-3-methoxyphenyl analogue was not evaluated. Other aryl substituents were also evaluated (**1.26**, **1.29**, **3.05–3.07**), however there was no definitive trend that could be confirmed.⁵ Out of all the substituents analyzed, **1.25** was found to be the best inhibitor of ice recrystallization.

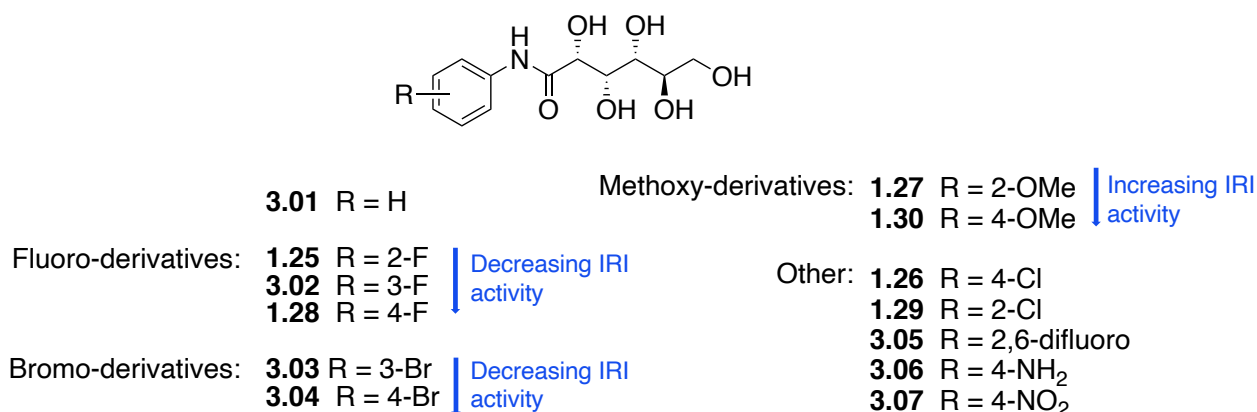


Figure 3.3. *N*-aryl-D-gluconamides previously synthesized and evaluated for IRI activity.

Another former graduate student in the Ben laboratory, Dr. Madeleine Adam, explored how making modifications to the C6-hydroxyl group affected IRI activity.⁵ Dr. Adam performed these modifications by either changing the C6 oxidation state or converting it into an azide (Fig.

3.4). Changing the oxidation state of C6 (**3.08** and **3.09**) led to a significant decrease in IRI activity compared to its parent compound (**1.30**). A similar decrease in IRI activity was observed when the C6-hydroxyl group was converted to an azide (**3.10-3.12**); suggesting the importance of the C6-hydroxyl group toward IRI activity.⁵ Although replacement of the C6-hydroxyl group with an azide decreased IRI activity, azides are still considered attractive functional groups as the presence of a zwitterion could contribute toward the hydrophilicity of the compound.^{2,6} Azides are also biocompatible and can be used as a detectable probe to label compounds⁷⁻¹⁰; a characteristic that may be useful to fully understand the *in vitro* mechanism of IRIs. From previous studies, it is evident that changing the position of a functional group on the carbohydrate portion can alter its IRI activity,^{1,11,12} thus, exploring other azide regioisomers of the *N*-aryl-D-gluconamide class is warranted. This chapter will discuss efforts in synthesizing these analogues.

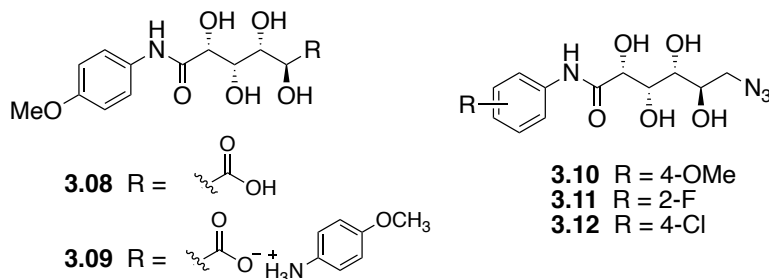


Figure 3.4. *N*-aryl-D-gluconamide compounds previously synthesized with modifications to the C6-hydroxyl group.⁵

3.2 Previous studies towards azido and amino analogues of IRIs

The contribution of azides to IRI activity has further been evaluated in the *O*-aryl-D-glucoside class. In this study, an azide functional group was moved to different positions in the pyranose ring (**3.13-3.21**).¹² C2, C3, C4, and C6 azide analogues of 4-methoxyphenyl and 4-bromophenyl β -D-glucosides were synthesized as well as a 4-azidophenyl- β -D-glucoside (Fig.

3.5A). In most cases, azido glucosides had limited solubility, and could not attain a full dose-response curve that is necessary to generate an IC_{50} value; the only exceptions were the C3-azido analogues (**3.17** and **3.18**) and the 4-azidophenyl glucoside (**3.21**) which exhibited IC_{50} values of 6 mM, 0.7 mM and 26 mM, respectively. When evaluated for its cryopreservation ability in RBCs, **3.21** significantly increased the post-thaw recovery of RBCs, and reduced cellular damage during the deglycerolization process¹²; a required post-thaw process that separates cryopreserved RBCs from glycerol.¹³ These observations suggested that azide analogues of pyranose IRIs have the potential to not only inhibit ice recrystallization but also work well *in vitro* as cryoprotective agents (CPAs).

Since amines are easily generated via the reduction of azides, amino *O*-aryl glucosides (**3.22-3.26**) were also explored as a method to increase solubility. C3- and C6-amino analogues of 4-methoxyphenyl (**3.22** and **3.25**) and 4-bromophenyl (**3.23** and **3.24**) β -D-glucosides were synthesized as chloride and formate salts, as well as a 4-aminophenyl β -D-glucoside (**3.26**) (Fig. 3.5B). As expected, these compounds were found to be more soluble than their respective azido precursors, however, the surveyed amino compounds displayed less IRI activity, with the most IRI active analogue (**3.24**) possessing an IC_{50} value of 16 mM.

Identifying IRIs with solubilities that are several times higher than their working concentration would be beneficial for their cryopreservation applications. For example, **1.25**, the most active IRI in the *N*-aryl gluconamide class has an IC_{50} value of 3 mM and a maximum solubility of 12 mM in PBS. IRI **1.25** is often used as an effective CPA at concentrations close to or at its maximum solubility.^{1,5,14} Using concentrations close to the maximum solubility can be problematic since low temperatures are employed during the process of cryopreservation, leading to a reduction in compound solubility. This can lead to a lower amount of IRI available in solution

and therefore, a less effective CPA. Thus, exploring the synthesis of amino-substituted aryl gluconamides for their high solubilities and moderate-to-high activities, could potentially be a method of mediating this issue, and could provide a class of novel IRIs.

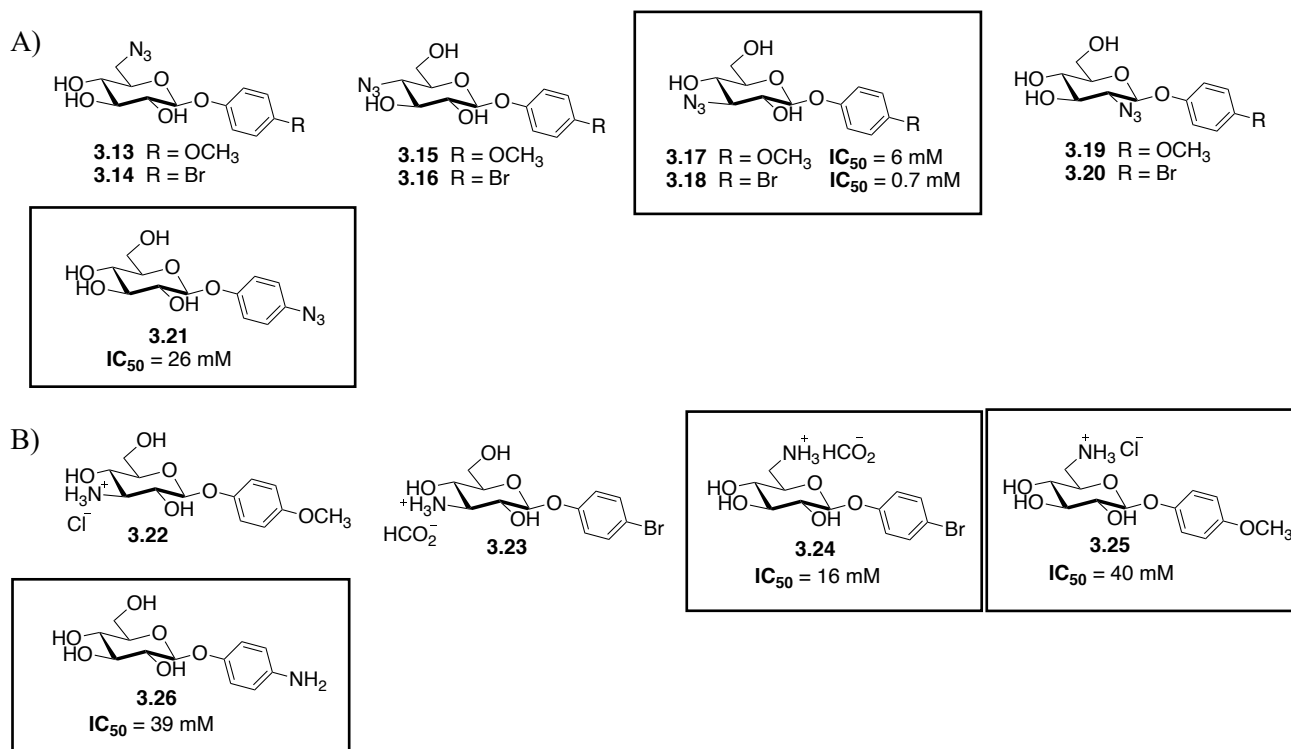


Figure 3.5. The structures and IC₅₀ values of previously synthesized azido (A) and amino (B) *O*-aryl- β -D-glucosides.¹² Boxed compounds generated IC₅₀ values from dose response curves.

3.3 Synthesis of 5-azido and 5-amino analogues of *N*-2-fluorophenyl-D-gluconamide

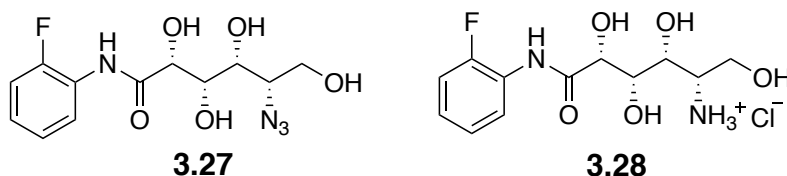


Figure 3.6. Chemical structures of 5-azido (3.27) and 5-amino (3.28) substituted D-gluconamides.

In this section, several attempts to synthesize 5-azido (**3.27**) and 5-amino (**3.28**) analogues of *N*-2-fluorophenyl-D-gluconamide (Fig. 3.6) will be discussed. The aryl substituent chosen for this synthesis was 2-fluorophenyl, since the parent compound, *N*-2-fluorophenyl-D-gluconamide (**1.25**), is the most IRI active gluconamide in its class.

The effect of modifications to C5 of aryl gluconamides on IRI activity has not yet been studied in the Ben laboratory. However, upon discovery that modifying C6 decreases IRI activity,⁵ it is of interest to determine how functionalization of C5 would compare. The C5-hydroxyl group is easily accessed from the opening of a protected gluconolactone, therefore synthesizing 5-azido and 5-amino derivatives (**3.27** & **3.28**) were hypothesized as an excellent starting point to investigate positional modifications in this class (Fig. 3.7). Previous azido glycosides were obtained via an S_N2 reaction with an azide nucleophile and electrophilic carbon at the desired position,^{5,12} followed by reduction to form the amino derivative.¹² Thus, the same approach was initially attempted to synthesize **3.27** and **3.28**. It was envisioned that the azide product could be generated from the respective gluconamide with a leaving group at C5, which can be generated from the C5-alcohol derivative (Fig 3.7, route 1). Unfortunately, the initial approach was unsuccessful, and an unexpected product was formed; the details of this synthetic route will be addressed in *Section 3.3.1*.

A second approach to synthesize **3.27** and **3.28** was developed. In this approach, the *N*-aryl amide bond was formed late in the synthesis (Fig. 3.7, route 2) as this bond was hypothesized to be the cause of the failed reaction in route 1. Formation of the amide could proceed via an amidation reaction with an *N*-aryl amine and a glucono-ester containing an azide at C5. The glucono-ester azide product could be synthesized via the same approach as route 1. This detailed

synthetic route will be described further in *Section 3.3.2*. However, attempts using this route were also unsuccessful.

In a final attempt to synthesize **3.27** and **3.28** an aldol asymmetric synthesis approach was employed (Fig. 3.7, route 3). In this scheme, it was envisioned that the gluconamide could be formed by cleavage of a glucitol-derived oxazolidinone. To construct the glucitol containing the C5-azide, an asymmetric aldol reaction with the respective azide-substituted butanal (originating from 1,4-butyne-1,3-diol) and Evan's chiral auxiliary would be performed. *Section 3.3.3* will discuss this synthetic route in more detail.

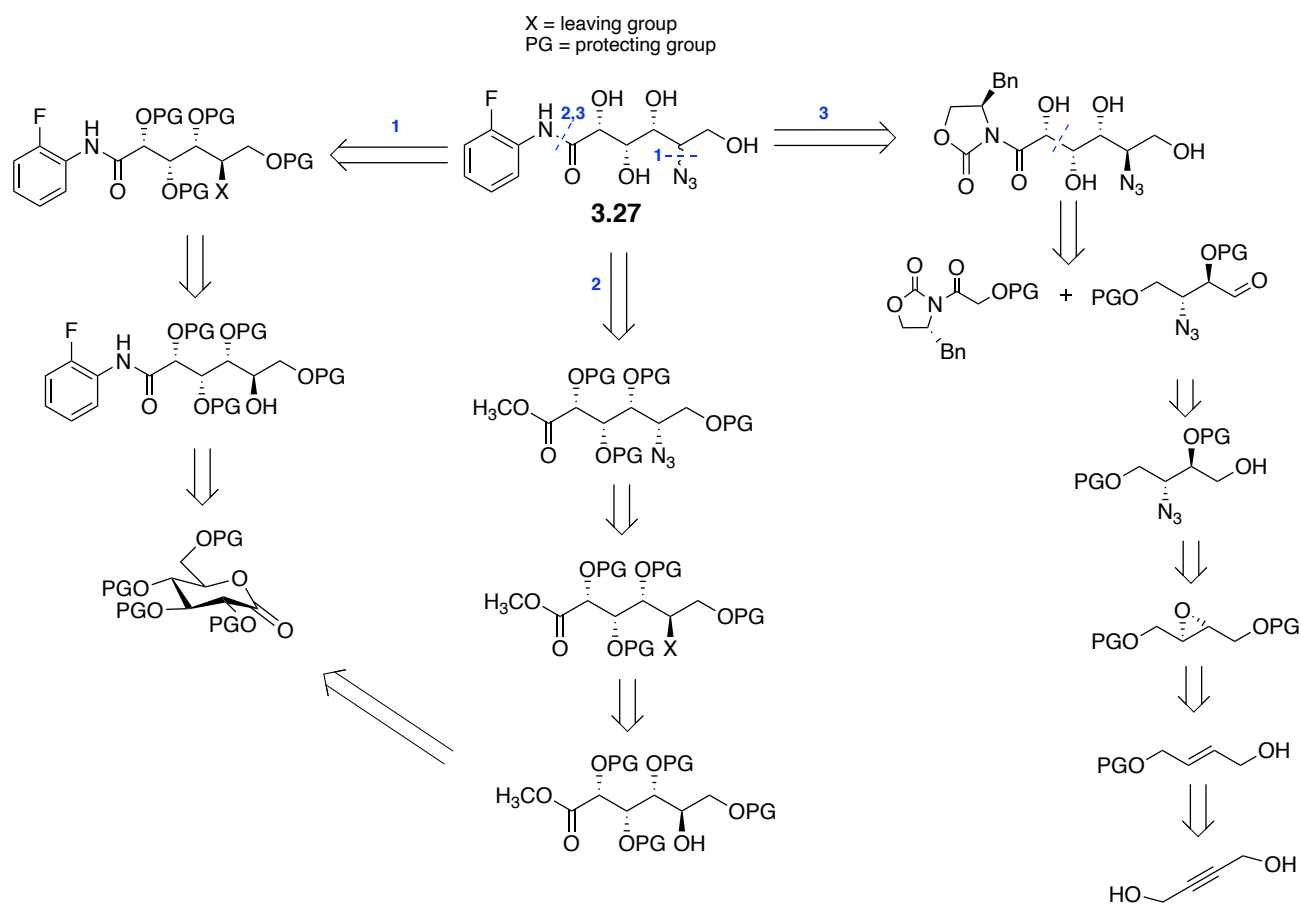
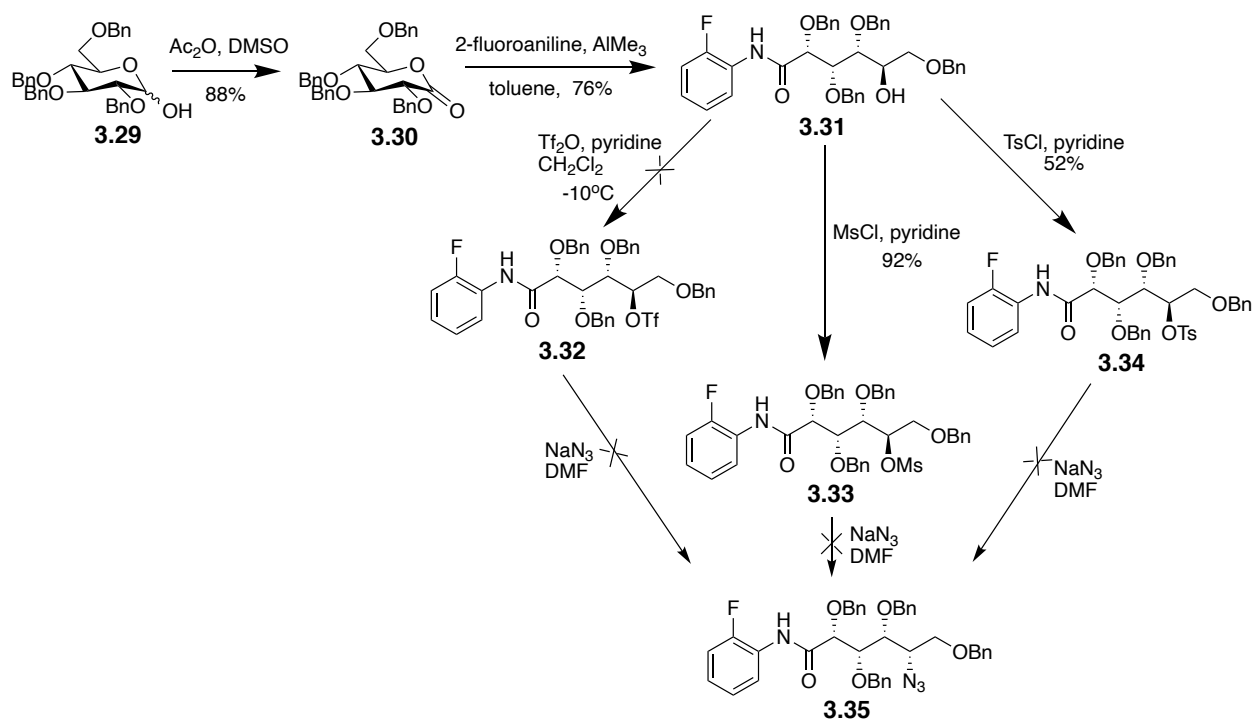


Figure 3.7. Retrosynthetic analysis for the synthesis of **3.27**.

3.3.1 Synthesis of **3.27** and **3.28** via a gluconamide electrophile (Route 1)

As presented in Scheme 3.1, the synthesis of **3.27** and **3.28** was initiated by the Albright-Goldman oxidation¹⁵ of commercially available 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (**3.29**) to form 2,3,4,6-tetra-*O*-benzyl-D-gluconolactone (**3.30**), followed by a AlMe₃-promoted amidation¹⁶ with 2-fluoroaniline to produce the benzyl-protected aryl gluconamide (**3.31**). The ring-opening of the benzyl protected saccharide resulted in a deprotected hydroxyl group at C5, which was further functionalized to enable the S_N2 reaction with an azide nucleophile. Typically, S_N2 substitutions, and specifically azide substitutions, at secondary carbons of carbohydrates are quite difficult and their success is dependent on factors such as the configuration of the hydroxyl group and their regiochemistry.¹⁷



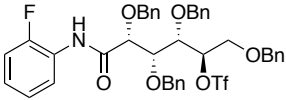
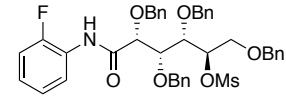
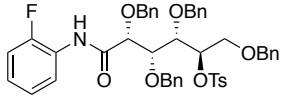
Scheme 3.1. Synthetic pathways attempted to obtain the 5-azido substituted aryl gluconamide via a gluconamide electrophile.

The first attempt at a nucleophilic displacement of a leaving group at C5 with an azide, was performed by converting the hydroxyl group into a triflate ester (**3.32**), followed by a

concomitant displacement with sodium azide. A triphenylphosphine–ninhydrin TLC stain¹⁸ was used to detect the presence of an organic azide, and the initial test of reaction product failed to indicate the presence of the azido group. The absence of an azide was also confirmed using IR spectroscopy, as there was no azide stretch observed from 2160 - 2120 cm⁻¹. Only one product was isolated and purified. Further characterization by ¹H and ¹³C NMR spectroscopy indicated that a product containing one less benzyl (-CH₂Ph) substituent had formed. Specifically, the ¹H NMR showed two less benzylic protons and five less phenyl protons and the ¹³C NMR showed one less benzylic ¹³C resonance. Mass spectrometry also confirmed this as the mass of the unknown product was 91 atomic mass units less, compared to compound **3.31**. In lieu of the failed attempt, other reaction conditions were explored for the nucleophilic displacement of **3.32** with an azide (Table 3.1). In the literature, nucleophilic azide displacements on primary triflate esters are usually performed at room temperature,¹⁹ however, due to the fact that azide substitution reactions on secondary carbons are quite challenging, changes in the reaction temperature and time were applied. First, the reaction was performed at room temperature for 48 h, then on a second attempt, the reaction temperature was raised to 50 °C after 24 h. Under both conditions, the formation of the azide functional group was not observed. In another attempt, the temperature of the reaction was increased to 100 °C and dimethyl sulfoxide (DMSO) was used as the solvent. DMSO has been reported to increase the nucleophilicity of azide anions for substitution reactions,²⁰ however the organic azide product was not formed. Since triflate esters are highly labile, it was speculated that **3.32** may have been hydrolyzed or undergone an intramolecular attack in the presence of heat, therefore, other leaving groups were investigated. A mesylate ester (**3.33**) and tosylate ester (**3.34**) derivative were successfully isolated and subjected to various azide nucleophilic displacement conditions (Table 3.1). A reaction with **3.33** and sodium azide at 65 °C resulted in a similar product

as observed previously. An increase in temperature and time to the reaction of **3.34** and sodium azide did not generate the desired azide product. In all cases, an organic azide product was not detected despite the type of electrophile used, but rather, the same unknown product (with a loss of CH₂Ph) was evident, which will be further explored in *Section 3.4.1*.

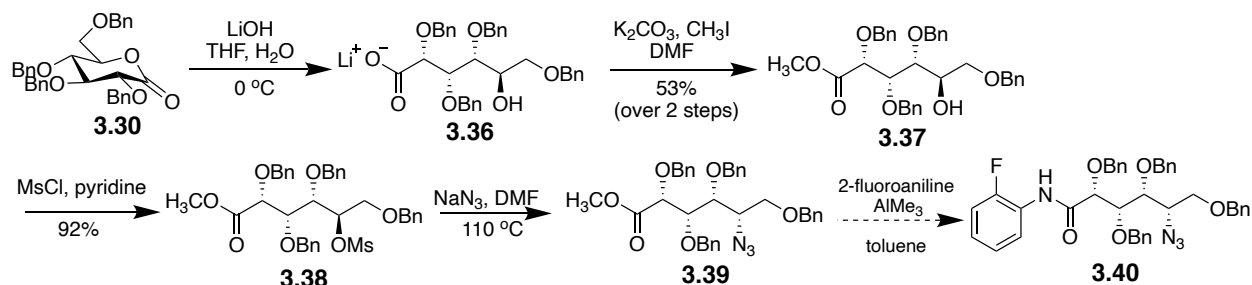
Table 3.1. Various azide substitution reaction conditions attempted with different gluconamide electrophiles. (RT = room temperature)

Starting material	Reagents	Temperature	Time	Product
 3.32	5 eq. NaN ₃ , DMF	RT	48 h	Unknown product
	5 eq. NaN ₃ , DMF	RT / 50 °C	24 h / 24 h	
	5 eq. NaN ₃ , DMF	50 °C	20 h	
	5 eq. NaN ₃ , DMSO	100 °C	20 h	
 3.33	5 eq. NaN ₃ , DMF	65 °C	35 h	
 3.34	5 eq. NaN ₃ , DMF	65 °C	35 h	
	5 eq. NaN ₃ , ACN	60 °C	72 h	
	5 eq. NaN ₃ , DMF	130 °C	20 h	

3.3.2 Synthesis of 3.27 and 3.28 via a glucono-ester (Route 2)

After the unsuccessful attempts at synthesizing **3.27** and **3.28**, another approach was implemented, in which the azide nucleophilic displacement was performed on a glucono-ester before amidation with the aryl amine (Scheme 3.2). Literature precedent suggested that the presence of the amide bond in electrophiles **3.32-3.34** could have prompted the formation of a

lactam product.²¹ Given this knowledge, we proposed that a nucleophilic displacement of the leaving group at C5 by the amide nitrogen could have occurred, therefore performing the amidation reaction in the final step was hypothesized to be an attractive alternative. As described in Scheme 3.2, synthesis of the azide-substituted glucono-ester was initiated with the base-catalyzed ring opening reaction²¹ of **3.30**, followed by subsequent methylation of the crude lithium carboxylate (**3.36**).²² Compound **3.37** was converted into a mesylate ester (**3.38**) which was successfully isolated and confirmed by ¹H NMR.



Scheme 3.2. Synthesis of azide-substituted gluconamide via a glucono-ester.

The ¹H NMR of **3.38** (Fig. 3.8) showed the presence of a mesylate ester on C5 from the deshielded multiplet at δ 4.97-4.99 ppm (assigned as H5 by analysis of the 2D COSY), and a singlet which integrated to three protons at δ 2.84 ppm, characteristic of the methanesulfonyl protons. Compound **3.38** was subjected to the same reaction conditions reported in the literature,²¹ which resulted in the formation of the azide derivative (**3.39**) as the minor product in only 5% yield. The formation of **3.39** was confirmed by 1D and 2D NMR, as well as mass spectroscopy. The ¹H NMR of **3.39** (Fig. 3.8) suggested the disappearance of the mesylate on C5 by observing the chemical shift change of H5 from δ 4.97 ppm to δ 3.19 ppm (characteristic of a proton alpha to an azide functional group), and the disappearance of the singlet at δ 2.84 ppm. Upon analysis,

it was discovered that the major product of the reaction did not possess an azide, and was missing one benzyl group, similar to the unknown product observed in *Section 3.3.1*. Due to the low yield of the desired azide product, this pathway was not pursued any further. A full analysis of the major product will be presented in *Section 3.4.3*.

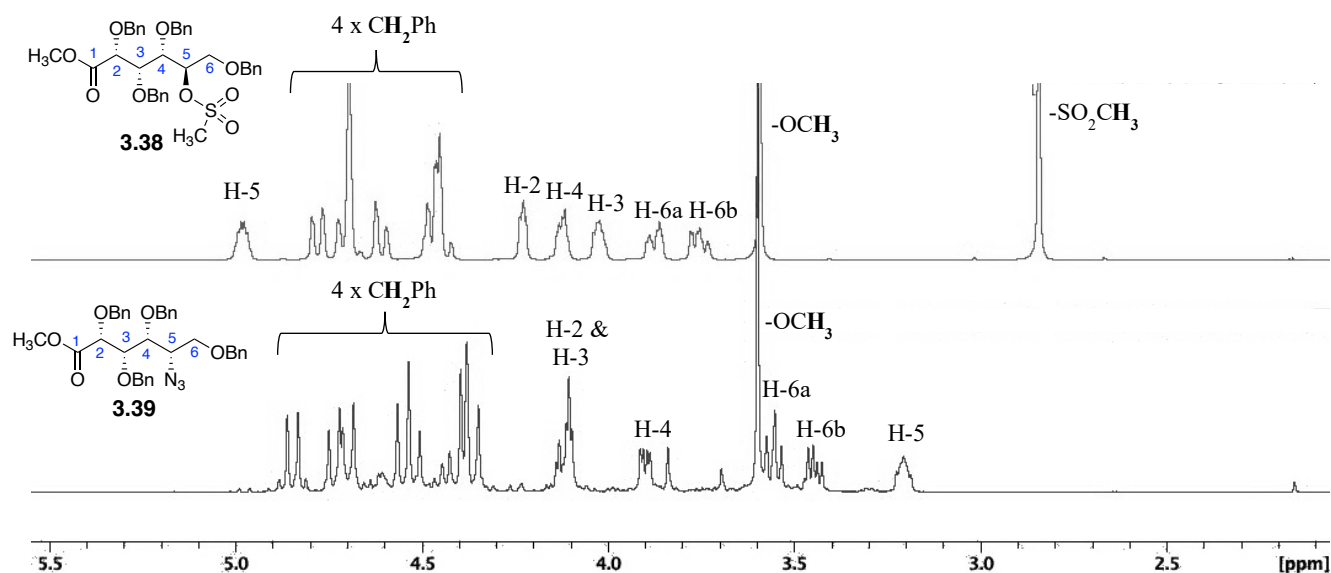
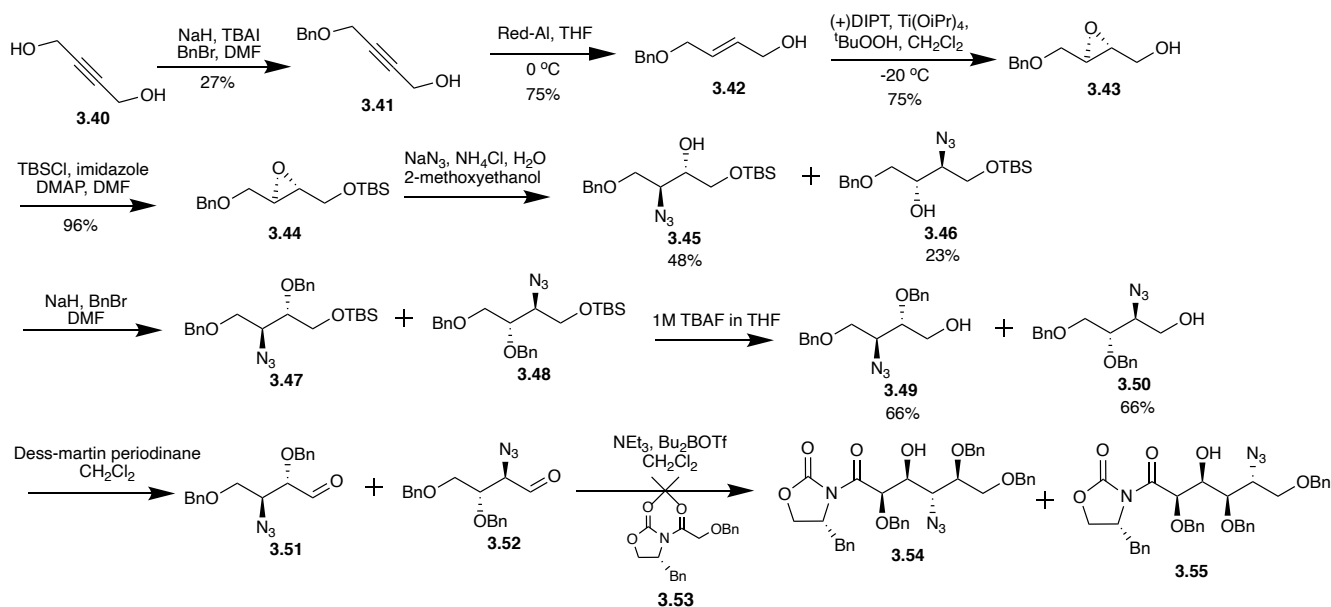


Figure 3.8. ^1H NMR characterization of compounds **3.38** and **3.39**.

3.3.3 Synthesis of 3.27 and 3.28 via a glucitol-derived oxazolidinone (Route 3)

Due to the complications observed from the direct nucleophilic displacement of a mesylate at C5 with an azide, a more novel approach was employed. Functionalization of hydroxyl groups on alditols are difficult due to protecting group chemistry and a required attention to chirality. In spite of this, several asymmetric approaches have been designed to generate functionalized alditol derivatives such as chemoenzymatic approaches,^{23,24} Diels Alder reactions,^{25,26} alkene dihydroxylations,^{27,28} chiral Lewis acid catalysis,²⁹ and aldol chemistry.^{30–32} An asymmetric aldol reaction³² was attempted for the synthesis of the 5-azido and 5-amino

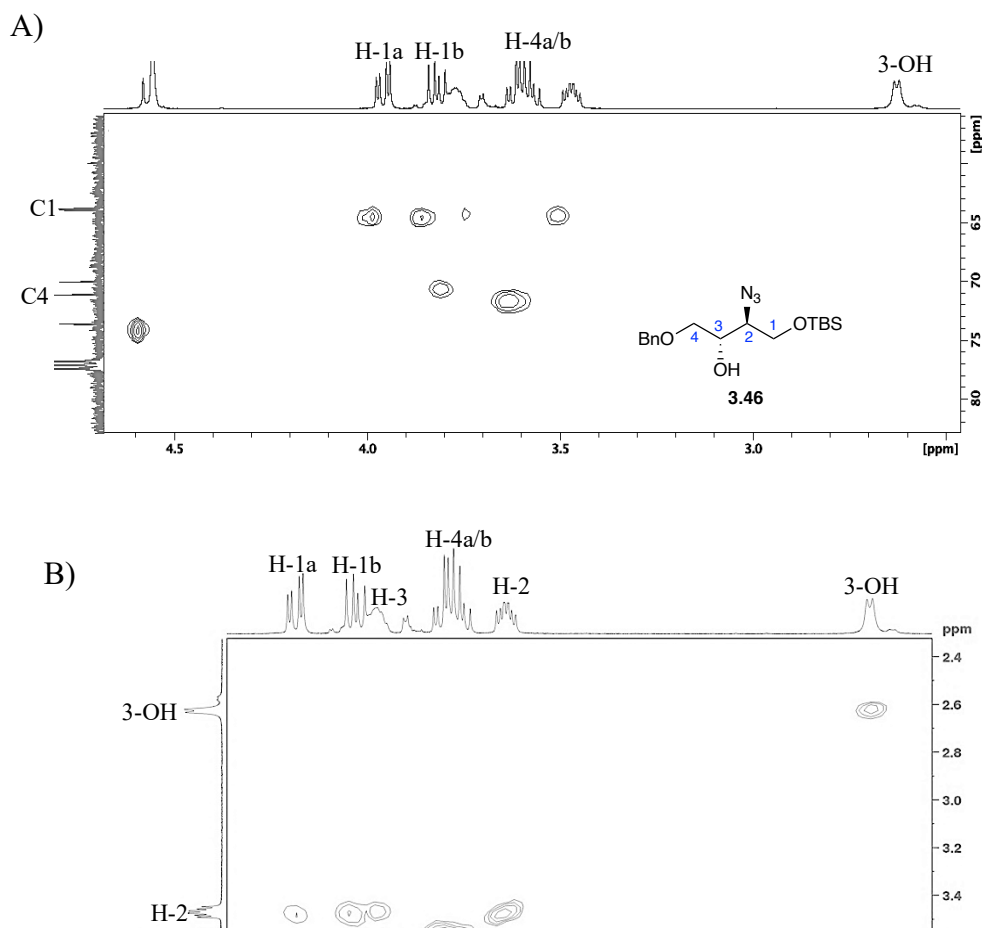
substituted gluconamides due to its reliability and selectivity. To begin, a nucleophilic displacement with an azide was performed on a silyl- and benzyl-protected, 2,3-epoxy butanol (**3.44**), which was derived via a 4-step synthesis from 1,4-butanediol (**3.40**).^{33,34} It was anticipated that the desired stereochemistry of the epoxy alcohol (**3.43**) required to generate the correct configuration of the azide-substituted butanol (**3.45** and **3.46**) would be accessed via an asymmetric Sharpless epoxidation.³⁵ A 2,3-*trans* disubstituted epoxide was desired from the *trans* allylic alcohol (**3.42**) and thus, a catalytic amount of titanium (IV) isopropoxide and (+)-diisopropyl-L-tartrate were used in the presence of *tert*-butyl hydroperoxide. The nucleophilic epoxide opening generated a mixture of azide-substituted butanol isomers (**3.45** and **3.46**) that could be easily isolated by silica gel column chromatography. Resultingly, the triphenylphosphine–ninhydrin TLC stain¹⁸ indicated the presence of an organic azide in the crude reaction mixture which was further confirmed after purification of the isomers.



Scheme 3.3. Synthesis of glucitol-derived oxazolidinone **3.54** and **3.55**.

¹H-¹H COSY and ¹H-¹³C HSQC NMR correlations were used to determine the structures of the two isomers, **3.45** and **3.46** (Fig. 3.9). For **3.46**, the methylene protons on C1 and C4 were

identified by observing the correlation of two ^1H resonances to one ^{13}C resonance in the ^1H - ^{13}C HSQC spectrum, and the hydroxyl proton was identified by the absence of a ^{13}C correlation (Fig. 3.9A). From their ^{13}C chemical shifts, C1 (δ 63.9 ppm) was assigned as the carbon with the silyl ether and C4 (δ 71.1 ppm) was assigned as the carbon with the benzyl ether. Benzyl ethers are more electron withdrawing than silyl ethers due to their inductive effect, therefore, the carbon directly bonded to the benzyl ether would be more deshielded and have a higher chemical shift. From these initial assignments, the ^1H - ^1H COSY spectrum (Fig. 3.9B) was used to identify the proton coupled to the hydroxyl proton, which was assigned as H3 since correlations were also observed to H4. The proton adjacent to the azide was assigned as H2 and its chemical shift (δ 3.45-3.49 ppm) agreed with the typical chemical shift of an organic azide alpha proton. The same type of analysis was performed for **3.45** and all spectral assignments were in agreement with the structure reported in the literature.³⁶



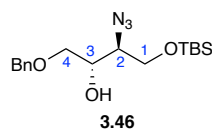
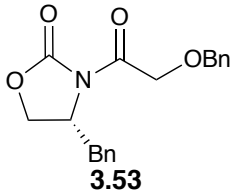
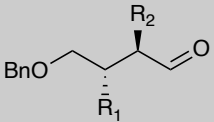
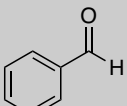
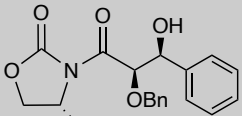
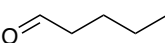
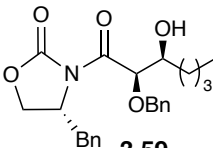


Figure 3.9. ^1H - ^{13}C HSQC (A) and ^1H - ^1H COSY spectrum (B) of **3.46**. Only notable ^1H and ^{13}C resonances are labelled.

In order to generate the aldehyde synthon for the aldol reaction, subsequent protection and deprotection of **3.45** and **3.46** afforded a terminal hydroxyl group that was oxidized using Dess-Martin periodinane (DMP).³⁷ The *R*- configuration of Evans-type oxazolidinone (**3.53**) was chosen as the chiral auxiliary to test because literature precedent suggests that it may provide the desired *syn*-aldol product (**3.54** and **3.55**) through a boron-mediated reaction. Unfortunately, the initial aldol reaction was unsuccessful. In the first attempt, triethylamine was used as the base and dibutylboron triflate (Bu_2BOTf) was used as the Lewis acid. Unreacted oxazolidinone was isolated and there was no indication of product formation by TLC or NMR spectroscopy. In other attempts (Table 3.2), the number of equivalents of aldehyde was increased to 2 eq., which did not change the result of the reaction. An additional increase to 4 eq. of aldehyde, 1.5 eq. of base, and 1.3 eq. of Lewis acid also failed to generate the desired aldol product. A final attempt using a stronger Lewis acid, titanium tetrachloride (TiCl_4), also resulted in no product formation. To determine the cause of the failed reactions, a test reaction was performed using oxazolidinone, dibutylboron triflate, triethylamine and distilled benzaldehyde (**3.56**). The *syn*-aldol product (**3.57**) was formed and isolated under normal reaction conditions. Pentaldehyde (**3.58**) was also used as a test

aldehyde and under the original aldol reaction conditions the *syn*-aldol product was generated (3.59).

Table 3.2. Various aldol reaction conditions used to synthesize aldol product.

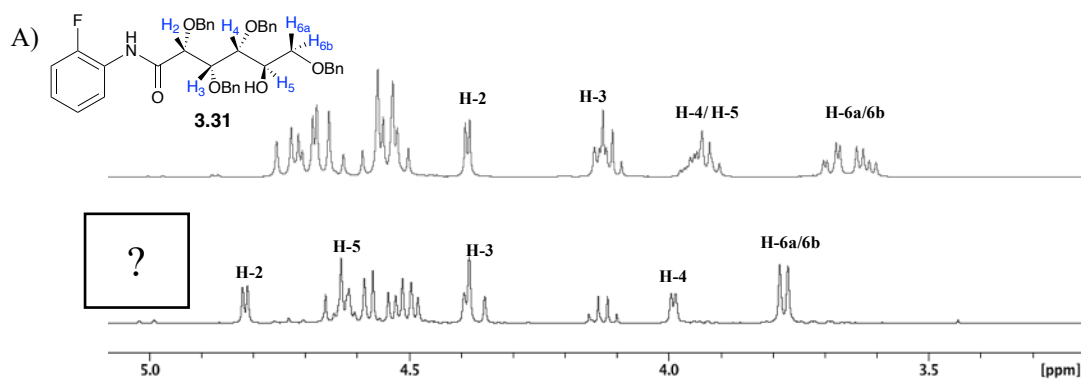
Oxazolidinone	Aldehyde	Eq. of aldehyde	Base	Lewis Acid	Product
 3.53	 3.51 R ₁ = N ₃ , R ₂ = OBn 3.52 R ₁ = OBn, R ₂ = N ₃	1 eq.	1.2 eq. NEt ₃	1.1 eq. Bu ₂ BOTf	No reaction
		2 eq.	1.2 eq. NEt ₃	1.1 eq. Bu ₂ BOTf	No reaction
		4 eq.	1.5 eq. NEt ₃	1.3 eq. Bu ₂ BOTf	No reaction
		2 eq.	1.5 eq. NEt ₃	1.3 eq. TiCl ₄	No reaction
	 3.56	4 eq.	1.5 eq. NEt ₃	1.3 eq. Bu ₂ BOTf	 3.57 76 % yield
	 3.58	1 eq.	1.2 eq. NEt ₃	1.1 eq. Bu ₂ BOTf	 3.59 58 % yield

3.4 A Serendipitous synthesis of L-xylofuranosyl amides and esters

3.4.1 Characterization of unknown product from Section 3.3.1

As forementioned, the nucleophilic displacement at C5 of the *N*-aryl gluconamides (**3.32**-**3.34**) with an azide resulted in a product absent of an azide group. It was hypothesized that due to the presence of an electrophile at C5, an intramolecular reaction may have occurred to generate a cyclic structure via an S_N2-like attack by an innate nucleophile.

The unknown product observed from Route 1 was characterized via 1D and 2D NMR spectroscopy and the ¹H NMR peaks were assigned using a 2D COSY and ¹H -¹³C HSQC spectra (Section 3.6.4). Identifying the H6a and H6b peaks by observing coupling to a single ¹³C resonance, and progressively connecting the coupling correlations afforded the full assignment of ¹H and ¹³C NMR resonances. To further investigate the chemical structure of the unknown product, the ¹H and ¹³C NMR of starting material **3.31** and the unknown product were compared (Fig. 3.11). A notable difference in the ¹H chemical shift of H2 and H5 were observed, and in both cases they were shifted downfield by ~0.5 ppm in the unknown product, indicating the presence of a nearby electron withdrawing group. H3 and H6 were also shifted slightly downfield as well. The ¹H NMR (Fig. 3.11A) was also missing two benzylic protons and five phenylic protons, suggesting the loss of a benzyl (-CH₂Ph) substituent. This was further confirmed by mass spectrometry and the ¹³C NMR spectrum by observing the loss of a benzylic carbon. C4 and C5 were also greatly deshielded in the ¹³C NMR (Fig. 3.11B) of the unknown product, placing C2, C3, C4, and C5 in a similar chemical environment.



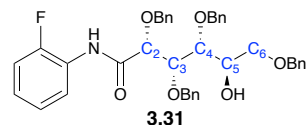


Figure 3.11. ^1H NMR (A) and ^{13}C NMR (B) spectra of **3.31** and unknown product.

To determine from which oxygen the benzyl substituent was lost, ^1H - ^{13}C HMBC correlations were examined (Fig. 3.12). A long-range coupling was observed between the benzylic carbons and H3, H4, and H6, however, there was no correlation observed with H2. This evidence strongly suggested that the benzylic group on O2 may have been lost during the course of the reaction, leaving O2 with a negative charge and available for an intramolecular nucleophilic attack. To verify this hypothesis, a 2D NOESY (Fig. 3.13) experiment was also performed. Weak NOE correlations were observed between H2 and H6. This suggested that H2 and H6 are in close proximity, and the formation of a ring may have occurred via an intramolecular nucleophilic attack by the participation of O2.

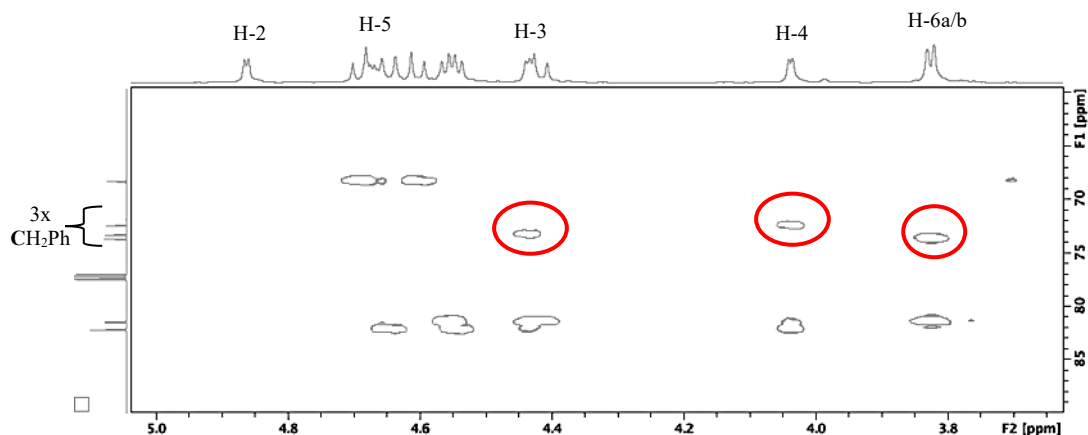


Figure 3.12. ^1H - ^{13}C HMBC spectra of the unknown product. Red circles indicate HMBC correlations between the sugar protons and benzylic carbons.

Benzyl ether oxygens acting as nucleophiles with the concomitant release of a resonance-stabilized benzylic group have been previously reported in the literature.^{40–42} In one of the cases, a five-membered ring was formed from an eight-membered ring via an intramolecular attack of a triflate by a benzyl ether oxygen.⁴² Based on the observed evidence and literature precedence, it was proposed that the chemical structure of the unknown product was an *N*-aryl xylo-L-furanosyl amide (**3.63**) that was formed as a result of an intramolecular attack of the C5 leaving group by the benzyl ether oxygen at C2 (Fig. 3.14). The NOESY spectra (Fig. 3.13) inferred the configuration of the product, as strong NOE signals were observed between H2 and H3, and between H4 and H5, suggesting that these proton pairs were situated on the same plane. There were also no apparent NOE signals between H2 and H4, and H3 and H5, suggesting that these proton pairs were on opposite planes.

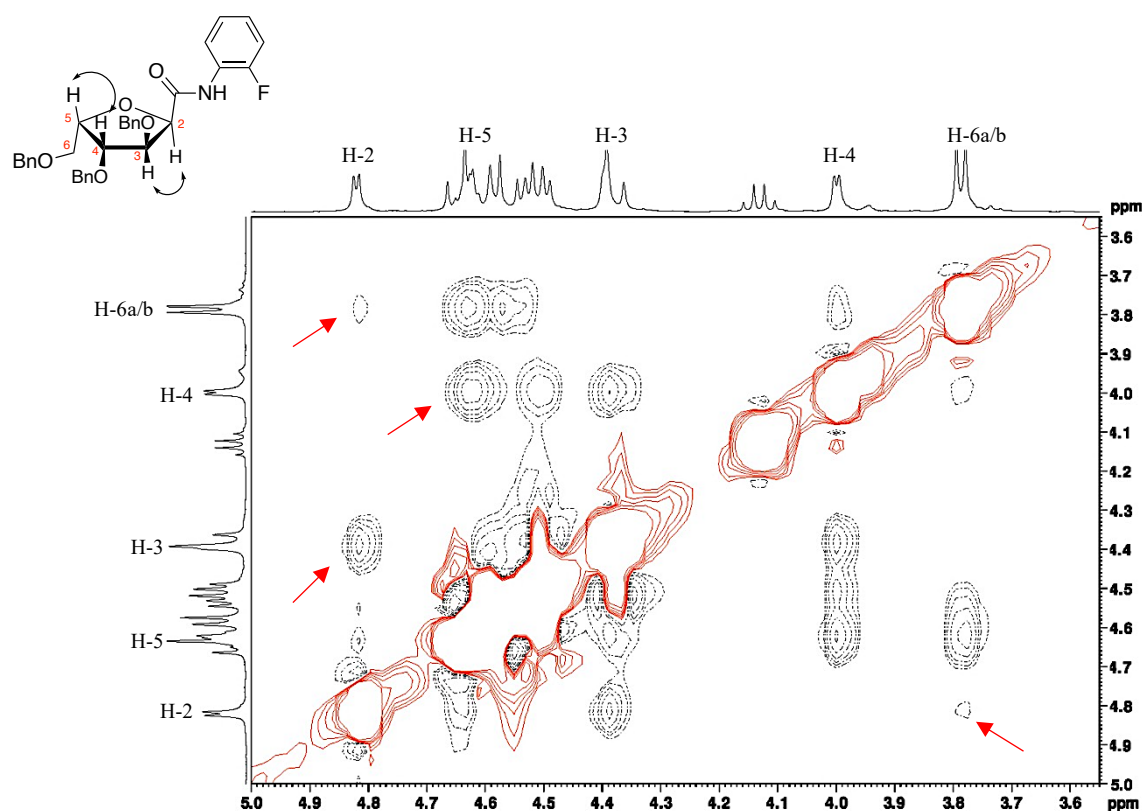


Figure 3.13. NOESY spectra of the unknown product. Red arrows represent notable NOE correlations between H-2 and H-6, H-4 and H-5, and H-2 and H-3.

The initial step of the reaction was hypothesized to occur from an intramolecular nucleophilic substitution between the benzyl ether oxygen at C2 and the electrophilic carbon (C5), forming a five-membered ring intermediate **3.62**. The position of the oxygen at C2 was in the proper orientation for an S_N2-like attack, therefore facilitating the reaction. During this process, the benzyl substituent at C2 in **3.62** is removed with the assistance of a nucleophile, such as pyridine under these reaction conditions. A closer look at the reaction revealed that **3.32** was simply an intermediate of the triflate reaction and could not be isolated. The nucleophilicity of the benzyl ether oxygen and the lability of the triflate leaving group enabled the immediate formation of **3.63** in the presence of pyridine. Contrarily, the mesylate and tosylate electrophiles **3.33** and **3.34** could be isolated and only formed the cyclic product, **3.63**, in the presence of heat.

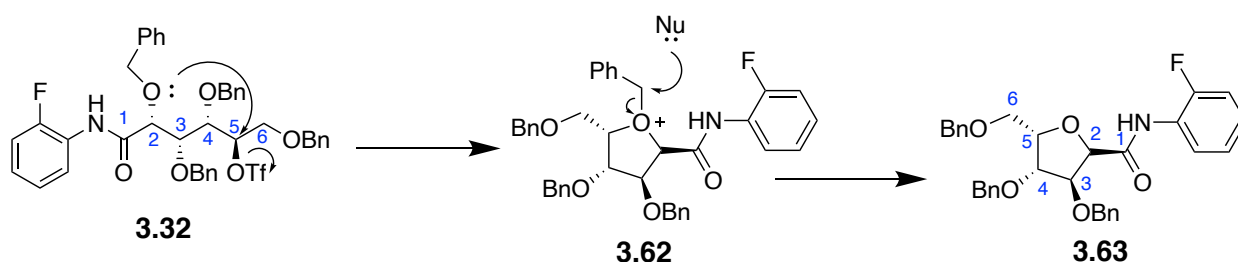


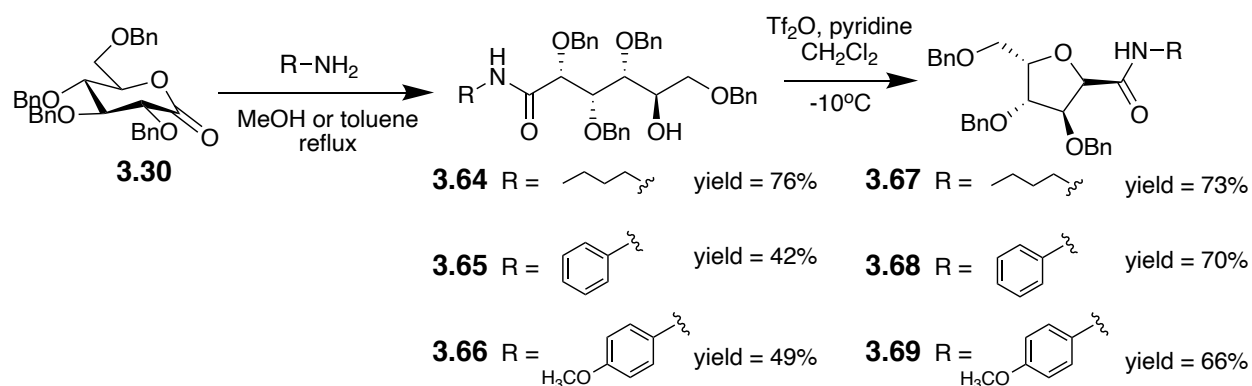
Figure 3.14. Proposed mechanism of the synthesis of *N*-aryl L-xylofuranosyl amides via gluconamide electrophiles; Nu = nucleophile.

According to the literature, L-xylofuranosyl derivatives are rarely observed and there have been no reports of this type of synthesis towards furanosyl amides. Rather, gluconamides with

electrophiles at C5 have been reported to form lactam^{21,43} and lactone⁴⁴ products, as a result of the presence of the amide. Therefore, we sought to investigate the scope of the reaction to form L-xylofuranoses.

3.4.2 *Elucidating the role of the aryl substituent in the proposed mechanism*

In order to confirm that the aryl substituent was not a significant contributor to the ring-closing mechanism proposed for the formation of **3.63**, different gluconamides were synthesized possessing an aryl or alkyl substituent with various electronic properties. All D-gluconamides were expected to form their respective L-furanosyl amide products despite their aryl or alkyl substituent. *n*-Butylamine, aniline, and *p*-anisidine were used, as a preliminary study, to form gluconamides **3.64-3.66**. The desired products were synthesized via reflux in methanol for the alkylamine and toluene for the arylamines. These reaction conditions were more mild than the organoaluminium reaction previously used, however it was slightly lower yielding, presumably due to the low nucleophilicity of the arylamines. The resulting alkyl and aryl gluconamides were then treated with triflic anhydride in the presence of pyridine, which generated one major product evident by TLC. Characterization of these products concluded that the furanose product had formed to generate *N*-alkyl-L-xylofuranosyl amide **3.67** and *N*-aryl-L-xylofuranosyl amides **3.68** and **3.69** in similar yields that were observed for the formation of **3.63**. The formation of these products suggested that the electronics of the aryl/alkyl substituent does not contribute to the mechanism of the formation of L-xylofuranosyl amides, and that this reaction is suitable for the synthesis of various *N*-alkyl and *N*-aryl-L-xylofuranosyl amides.



Scheme 3.4. Synthesis of L-xylofuranosyl amides **3.67-3.69** containing *N*-alkyl and *N*-aryl substituents.

3.4.3 Elucidating the contribution of the amide functional group in the proposed mechanism

Up to this point, the mechanism to synthesize furanoses from *N*-alkyl and *N*-aryl gluconamides has held true regardless of the aryl/alkyl substituent, however, the necessity of the amide functional group toward the reaction mechanism was unknown. Gratien et al. had reported evidence of a similar cyclized product when a C5 mesyl-substituted glucono-ester was subjected to various substitution conditions.⁴⁵ In order to investigate this further, the synthesis in *Scheme 3.2* was re-analyzed. As previously stated, the azide product (**3.39**) had formed in small quantity and another major product was evident. Analysis of the major product revealed that an intramolecular attack had occurred to generate a furanosyl ester product, **3.71**, with a similar configuration to that observed in **3.63**. It was hypothesized that the mechanism was similar to that shown in Fig 3.14, in which an intramolecular nucleophilic attack between the benzyl ether oxygen at C2 and the electrophilic carbon (C5) generated a five-membered ring intermediate (**3.70**) followed by the removal of the benzyl group to generate **3.71** (Fig. 3.15). The formation of the furanose product (**3.71**) and azide product (**3.39**) differs from what was observed in the gluconamide class in which

only one product was formed and there was no evidence of the azide product in any of the cases. These results may be attributed to the contribution of the carbonyl to the reactivity of the benzyl ether oxygen (O2). Specifically, the electron-withdrawing ability of the ester group in **3.38** decreases the nucleophilicity of O2 compared to the amide group, therefore allowing the azide substitution product to form. It also suggests that the nature of the carbonyl (amide or ester) has a greater contribution to the reaction mechanism than the alkyl/aryl amine substituent.

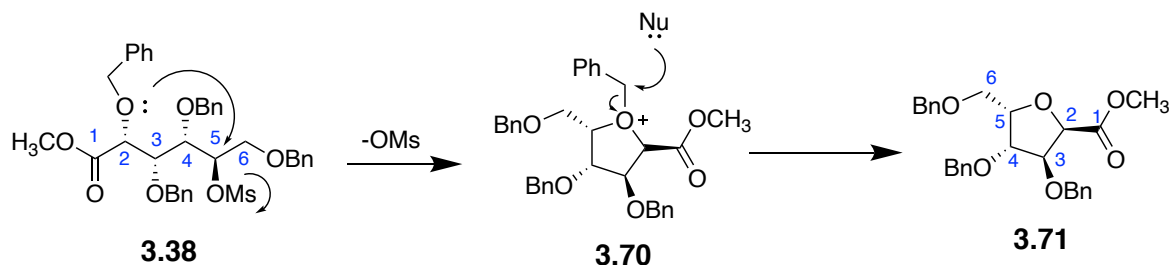
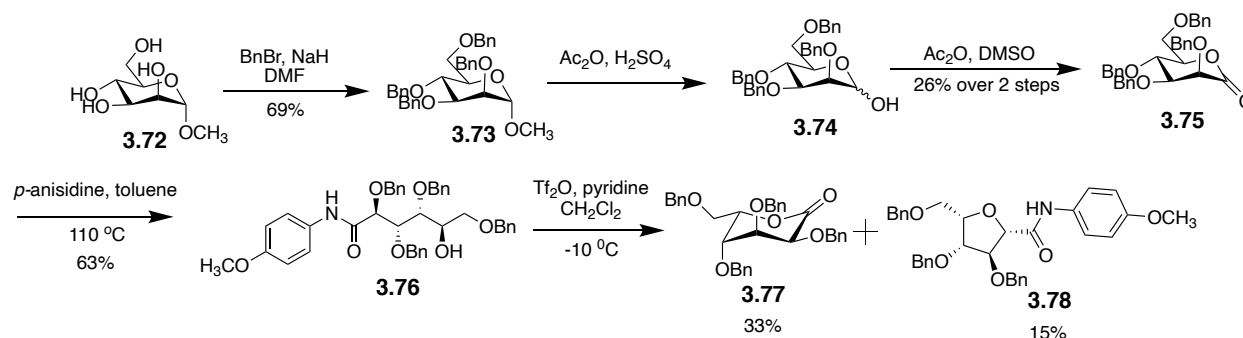


Figure 3.15. Proposed mechanism of the formation of the L-xylofuranosyl ester **3.71**.

3.4.4 Investigating the influence of the benzyloxy group configuration at C2 in L-xylofuranosyl amides

In the proposed mechanism for the formation of L-xylofuranose products, the C2-oxygen was the participating nucleophile in the reaction mechanism and was proposed to undergo a S_N2-like attack due to its favourable position with respect to the leaving group. In the previous experiments, glucose derivatives have been used to generate the L-xylofuranosyl products, therefore, to further understand the scope of the reaction, the dependency of the configuration at C2 was investigated. If the proposed reaction mechanism is stereospecific, the mannose

derivative, possessing the opposite configuration at C2, would not be able to undergo the same mechanism and would resultingly form a different major product. Therefore, benzylated mannonolactone (**3.75**), synthesized from α -methyl-D-mannopyranoside (**3.74**), was subjected to a ring-opening reaction with *p*-anisidine to form **3.76**. When the mannonamide was treated with triflic anhydride in the presence of pyridine, two products were evident, and isolated (**3.77** and **3.78**).



Scheme 3.5. Synthesis of mannose derivatives **3.77** and **3.78**.

As an initial analysis, an IR spectrum was taken of both products (Section 3.6.4). The IR spectrum of each product was similar, with the exception of the carbonyl stretch. In the spectrum of the major product, a carbonyl signal at 1735 cm^{-1} was evident, indicative of an ester functional group. The spectrum of the minor product showed a carbonyl signal at 1680 cm^{-1} and another notable signal at 3340 cm^{-1} , indicative of an amide functional group. Further analysis of the 1D and 2D NMR spectra revealed that the major product (33% yield) was benzylated L-gulono-1,5-lactone **3.77** and the minor product (15% yield) was a L-xylofuranosyl amide product **3.78**. It was proposed that **3.77** was formed as the major product due to the configuration of C2 placing the benzyl ether oxygen at an unfavourable position for an $\text{S}_{\text{N}}2$ -like reaction with C5. In order to generate the furanose product, **3.76** would have to reorganize its structure to position O2

antiperiplanar to the leaving group at C5 (Fig. 3.16A). As an alternative, the planar carbonyl oxygen at C1 can nucleophilically attack C5 to form a six-membered oxocarbenium ring (Fig. 3.16B). The half-chair conformations of oxocarbenium ions for pyranose derivatives have been studied extensively,^{46–49} and show that steric and electronic effects (along with the protecting groups) greatly contribute to the adopted configuration at each carbon in the transition state.^{46,49} Computational studies have revealed that for benzyloxy protecting groups, the most favourable conformation adopted places C3 and C4 benzyloxy groups in a pseudoaxial position and C2 and C5 benzyloxy groups in a pseudoequatorial position.^{48–50} Therefore for the mannose derivative, the ³H₄ half-chair conformation would be the lowest energy transition state and an available nucleophile (i.e. water) could attack from the axial position to generate an addition product and eventually the lactone **3.77** (Fig. 3.16B). These observations suggest that the stereochemistry at C2 is crucial in the proposed mechanism towards L-xylofuranosyl amides and esters. These findings also further increase the confidence in the assignment and configuration of the observed product and in the proposed mechanism.

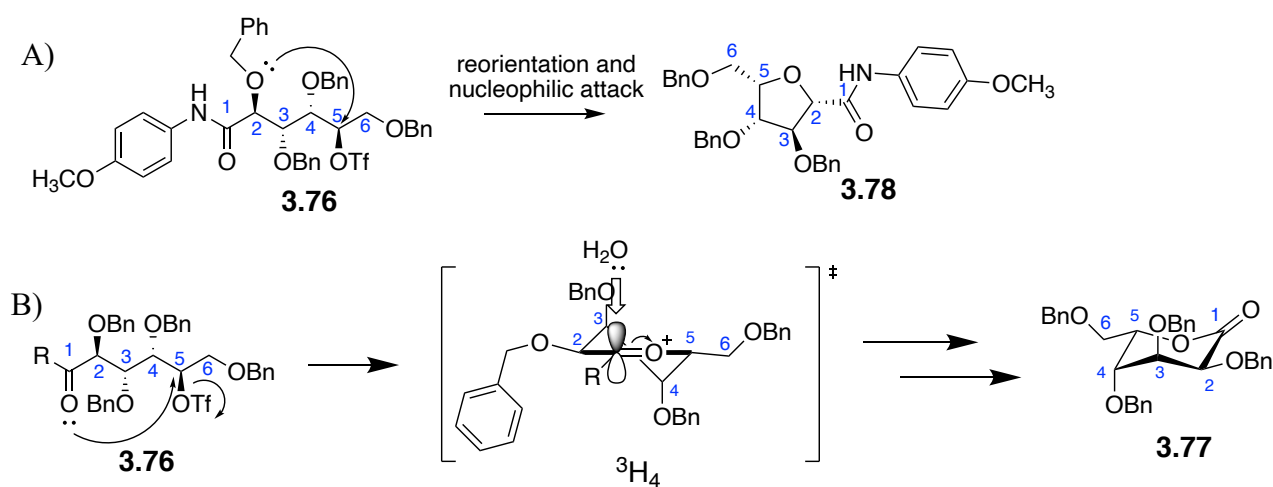
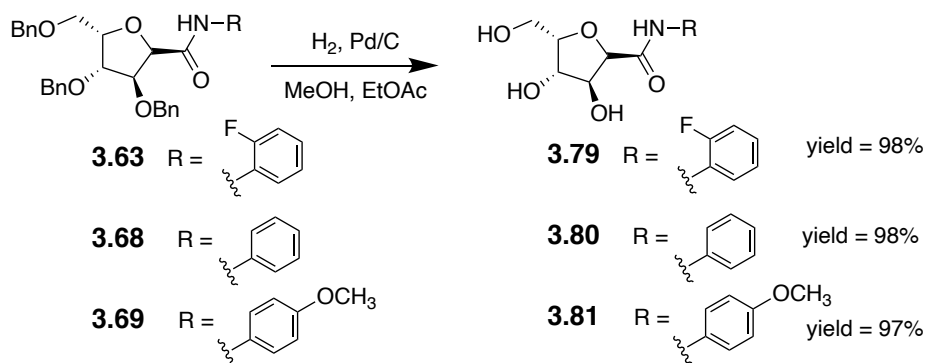


Figure 3.16. A) Intramolecular attack of C2-oxygen preceded by a reorientation of atoms to generate furanose product **3.78**. B) Pyranose oxocarbenium transition state conformation of the mannose

derivative; nucleophilic attack of water occurs at C1 of oxocarbenium intermediate to generate lactone product **3.77**. R= *N*-aryl amide.

3.4.5 IRI activity of *N*-aryl-*L*-xylofuranosyl amides

Several different monosaccharides and disaccharides have been extensively investigated for IRI activity in the Ben laboratory, but not furanoses. Therefore, since there are no data available of the IRI activity of molecules in this class, it was worthwhile to evaluate *N*-aryl-*L*-xylofuranosyl amides for IRI activity. *L*-Xylofuranosyl amides **3.63**, **3.68**, and **3.69** were subjected to hydrogenolysis (Pd/C) conditions to afford the fully deprotected compounds **3.79-3.81** prior to testing.



Scheme 3.6. Synthesis of deprotected *L*-xylofuranosyl amides **3.79-3.81**.

IRI activity is evaluated in the Ben laboratory through the splat cooling assay and can be quantified either with IC₅₀ values describing the rate of ice recrystallization or by % mean grain size (% MGS) describing the size of ice crystals at a specific concentration.^{51,52} Obtaining an IC₅₀ value from a full dose response curve provides information about concentrations that reduce the rate of ice recrystallization and is a suitable quantitative analysis of an IRI. However, in the case that full dose-response curves cannot be generated due to the inactivity of a compound or poor solubility, % MGS is an alternative way to quantify ice recrystallization inhibition. % MGS values

describe the average ice crystal size at a specific compound concentration in relation to the ice crystal size of PBS.

The 2-fluorophenyl derivative, **3.79**, and the phenyl derivative, **3.80**, were soluble in PBS up to 20 mM, and the 4-methoxyphenyl derivative, **3.81** was soluble in PBS up to 30 mM (Table 3.3). At their maximum solubilities, **3.79-3.81** were not effective inhibitors of ice recrystallization (30% MGS or less is considered a strong IRI). Based on the % MGS measured at their maximum solubility, the 2-fluorophenyl derivative **3.79** was the most active (76% MGS) and was significantly different from the PBS control ($p < 0.01$; one-way ANOVA) (Fig. 3.17). The 4-methoxyphenyl derivative **3.81** (85% MGS) and the phenyl derivative **3.80** (92% MGS) did not show any significant difference in their mean grain size to the PBS control (Fig. 3.17). These results are consistent with the trends observed in the *N*-aryl gluconamide class, as *N*-2-fluorophenyl-D-gluconamide (**1.25**) is more IRI active than the *N*-4-methoxyphenyl derivative (**1.30**) and the phenyl derivative (**3.01**). The limitations in solubility and IRI activity of the *N*-aryl L-xylofuranosyl amides were grounds to not further pursue the synthesis of this class for our applications. However, xylofuranose derivatives may be valuable in other applications such as carbohydrate hydration studies,^{53,54} antifungal compounds⁵⁵ and precursors for enzyme inhibitors.⁵⁶

Table 3.3. The maximum solubility and % MGS of compounds **3.84-3.86**.

Compound	Maximum Solubility in PBS (mM)	% Mean Grain Size
3.79	20	76 ± 4 %
3.80	20	92 ± 10 %
3.81	30	85 ± 6 %

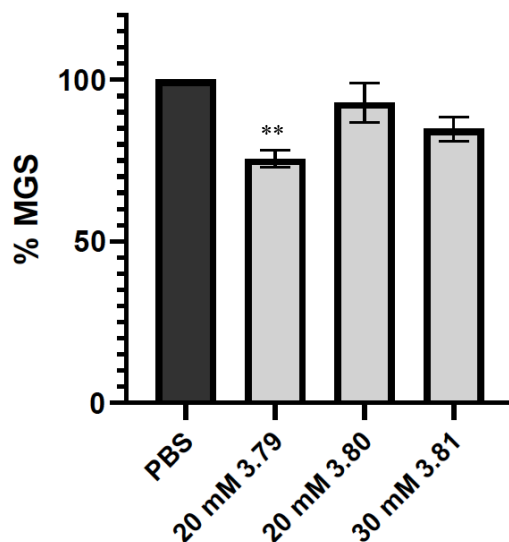


Figure 3.17. The IRI activity of **3.79-3.81** at their maximum solubility described by the % mean grain size (% MGS). % MGS are calculated in comparison to the PBS standard. Error bars indicate $\pm$ % SD (n=3). Asterisks indicates statistical significance from control condition (PBS) using the one-way ANOVA with Dunnett's multiple comparison test (** p < 0.01).

3.5 Summary

Sections 3.3 and *3.4* describe the attempts made to synthesize 5-azido and 5-amino derivatives of *N*-aryl-D-gluconamides but then embark on the discovered synthetic pathway towards L-xylofuranosyl amides. In *Section 3.3*, several attempts were made to synthesize the 5-azido and 5-amino compounds through direct nucleophilic substitution at the electrophilic C5 position. The first attempt involved the synthesis of various electrophiles via benzyl protected *N*-2-fluorophenyl gluconamide **3.31**. A triflate, mesylate, and tosylate group were all sampled as leaving groups, however, nucleophilic substitution with sodium azide did not result in the desired azide substitution product. Though evidence of the triflate intermediate was not apparent, the mesylate (**3.33**) and tosylate (**3.34**) intermediates were successfully isolated. In all three cases, an unknown product was formed which would be later characterized in *Section 3.4* as the L-

xylofuranosyl amide product, **3.63**. Other attempts were made to synthesize the 5-azido and 5-amino *N*-aryl gluconamides, which included a direct azide substitution on glucono-ester **3.38** and an asymmetric synthesis approach outlined in *Section 3.3.3*. Substitution of an azide nucleophile onto a glucono-ester electrophile resulted in two products: the azide-substituted glucono-ester, **3.39**, which was the minor product and the L-xylofuranosyl product, **3.71**, which was formed in a higher yield. The asymmetric synthesis approach outlined in Scheme 3.3 would have led to 4-azido and 5-azido products **3.54** and **3.55**. Unfortunately, after many trials and using a wide scope of conditions, the aldol reaction between azide aldehydes **3.51/3.52** and oxazolidinone **3.53** was unsuccessful as a result of the purity and reactivity of the synthesized aldehyde. Since the xylofuranosyl products were formed in high yield under nucleophilic substitution conditions with gluconamide (**3.32-3.34**) and glucono-ester (**3.38**) electrophiles, these products were further investigated. A full characterization using 1D and 2D NMR, and MS confirmed the structure of the *N*-2-fluorophenyl-L-xylofuranosyl amide product as **3.63** and the L-xylofuranosyl ester product as **3.71**. The proposed mechanism involved the intramolecular nucleophilic substitution at C5 with the C2 benzyl ether oxygen as the nucleophile. The scope of the reaction was probed by altering the amide substituent (alkyl vs. aryl), the nature of the carbonyl (amide vs. ester), and the configuration at C2 (glucose vs. mannose derivative). The L-xylofuranosyl amide (**3.63, 3.67-3.69**) and ester (**3.71**) products suggested that the amide substituent and the nature of the carbonyl did not greatly affect the reaction mechanism. However, the scope of the reaction was limited by discovery that the mannose derivative **3.76**, generated the xylofuranosyl amide, **3.78**, in very low yield compared to another lactone product, **3.77**. This discovery shed more light on the reaction mechanism and suggested that the position of O2 in mannose was unfavoured for the intramolecular substitution to occur; therefore, a bond rotation would have to occur in order to

generate **3.78**. Alternatively, the carbonyl oxygen can attack C5 to form an oxocarbenium intermediate leading to the lactone product, **3.77**. Lastly, the IRI activity of L-xylofuranosyl amides **3.79-3.81** were evaluated, and all exhibited poor IRI activity at their maximum solubilities in PBS.

Therefore, the several attempts made to synthesize 5-azido and 5-amino derivatives of *N*-aryl-D-gluconamides were unsuccessful, but its failure gave birth to a new class of L-xylofuranoside compounds. These compounds are rare and the mechanism by which they are synthesized is novel. Overall, **Chapter 3** presents a detailed case study of the synthesis and mechanism of these compounds while also looking at their reaction scope. Since IRIs with high solubilities could not be generated from this chapter, a different class of molecules will be investigated in the following chapters in hopes of solving this issue.

3.6 Experimental Procedures and Compound Characterization

3.6.1 Ice Recrystallization Assay

To measure the IRI activity of compounds **3.79-3.81**, a modified splat cooling assay was used.⁵¹ Samples were dissolved in phosphate buffered saline (PBS) and a 10 μ L drop of the solution was dispensed from a height of 2 metres, onto a polished aluminum block cooled to -78 °C with dry ice. The frozen wafer was carefully removed from the block, placed on a coverslip and transferred to a Cryostage at -6.4 °C connected to a cooling bath (Digital One RTE7 from Thermo Scientific NESLAB), a Peltier unit (Series 800 from Alpha Omega Instruments), a fish pump (SAX1 from Pen Plax), and a drying tube. The wafer was allowed to anneal at this temperature for 5 minutes. After the annealing time, the wafer was photographed between crossed polarizing filters using a digital camera (OEM Microscope Camera purchased from Nikon) fitted to the microscope (20X magnification). Images from the microscope were viewed and captured

using NIS Elements D software (version 5.30.01) with the exposure set to 8 ms. Images were then analyzed using ImageJ software, by circling each ice crystal with well-defined boundaries using the polygon function to generate the grain areas. Areas were inputted into Microsoft Excel where the average grain size of triplicate experiments was compared to the PBS positive control to generate a % mean grain size.

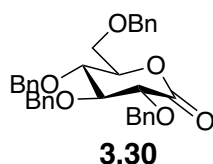
3.6.2 General Experimental

All reagents used in the outlined synthesis were purchased commercially. Anhydrous reactions were performed using a flame-dried flask flushed with argon gas and anhydrous reagents were transferred either using an oven-dried canula or an oven-dried glass needle and syringe. Anhydrous solvents such as tetrahydrofuran (THF), dichloromethane (DCM), toluene, and acetonitrile were distilled via a benchtop solvent purification system (SPBT-1, LC Technology Solutions Inc. Salisbury, MA). All other anhydrous reagents were purchased and stored under argon and over 4Å molecular sieves. Chemical reactions were monitored using thin layer chromatography (TLC) with a 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). TLC plates were visualized by a short-wavelength (254 nm) ultra-violet lamps or by staining with *p*-anisaldehyde stain or triphenylphosphine–ninhydrin stain. All column chromatography was performed with E. Merck silica gel 60 (230-400 mesh). All 1D and 2D NMR spectroscopy was performed on a Bruker AVANCE 400 or Bruker AVANCE 600 spectrometer. Deuterated NMR solvents used included deuterated DMSO (DMSO- d_6), deuterated chloroform ($CDCl_3$) or deuterated water (D_2O). The solvent peak was used as the internal standard of all NMR spectra and chemical shifts were reported in ppm. Splitting patterns are assigned as either s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet; or br, broad. Low-resolution mass

spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed on a Micromass Quatro-TOF I Electrospray Ionization mass spectrometer LC/MS with a pump rate of 20 μ L/min.

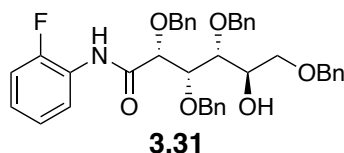
3.6.3 Compound Characterization

2,3,4,6-tetra-*O*-benzyl-D-gluconolactone (**3.30**)



Compound **3.29** (10 g, 18.5 mmol) was dissolved in anhydrous DMSO (47.4 mL), followed by the addition of acetic anhydride (36.5 mL). The mixture was stirred at room temperature overnight. After completion, the reaction mixture was diluted with water and the aqueous layer was extracted with diethyl ether. The organic layers were combined and dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude mixture was purified by silica gel flash column chromatography (4:1 hexane/ethyl acetate) which afforded pure **3.30** (8.76 g, 88% yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.43-7.18 (m, 20H, aromatic H), 5.00 (d, J =11.4 Hz, 1H, CHPh), 4.74 (d, J =11.3 Hz, 1H, CHPh), 4.71 (d, J =11.2 Hz, 1H, CHPh), 4.64 (d, J =11.4 Hz, 1H, CHPh), 4.60 (d, J =11.4 Hz, 1H, CHPh), 4.57 (d, J =11.9 Hz, 1H, CHPh), 4.52 (d, J =10.7 Hz, 2H, CH_2Ph), 4.46-4.50 (m, 1H, H-5), 4.14 (d, J =6.8 Hz, 1H, H-2), 3.91 (m, 2H, H-3/H-4), 3.73 (dd, J =3.1, 11.0 Hz, 1H, H-6a), 3.67 (dd, J =2.5, 11.0 Hz, 1H, H-6b) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 169.4 (C-1), 137.6, 137.5, 137.5, 136.9 (4 x quaternary aromatic C), 128.5-127.8 (20 x aromatic CH), 81.0 (C-3), 78.2 (C-5), 77.4 (C-2), 76.1 (C-4), 74.0, 73.8, 73.8, 73.6 (4 x CH_2Ph), 68.2 (C-6) ppm. LRMS (ESI $^+$): m/z calcd. for $\text{C}_{34}\text{H}_{36}\text{O}_6$ $[\text{M} + \text{Na}]^+$, 561.2 found 561.2.

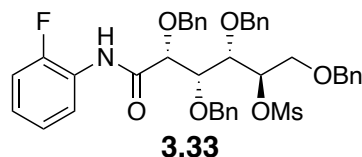
***N*-2-Fluorophenyl-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (3.31)**



To a flame dried argon-flushed round bottom flask was added 2-fluoroaniline (1.09 mL, 11.25 mmol) and dry toluene (30 mL). To the suspension was added a solution of 2.0 M trimethylaluminum in toluene (1.00 mL, 11.25 mmol). The reaction mixture was stirred at room temperature for 30 min. Compound **3.30** (2.02 g, 3.75 mmol) was added in one portion and the reaction was stirred at room temperature for 16 h. The reaction mixture was quenched with methanol (5 mL) drop wise. The mixture was extracted with water and the organic layer was dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude mixture was purified by silica gel flash column chromatography (3:1 hexane/ethyl acetate) which afforded pure **3.31** (1.85 g, 76% yield). ¹H NMR (400 MHz, CDCl₃): δ = 8.78 (d, *J*_{H-F}=2.1 Hz, 1H, NH), 8.28 (td, *J*=1.2, 7.8 Hz, 1H, aromatic H), 7.38-7.06 (m, 23H, aromatic H), 4.73 (d, *J*=11.2 Hz, 1H, CHPh), 4.68 (d, *J*=11.1 Hz, 1H, CHPh), 4.67 (d, *J*=11.0 Hz, 1H, CHPh), 4.63 (d, *J*=11.2 Hz, 1H, CHPh), 4.56 (d, *J*=11.1 Hz, 1H, CHPh), 4.53 (d, *J*=11.1 Hz, 1H, CHPh), 4.52 (d, *J*=11.0 Hz, 1H, CHPh), 4.50 (d, *J*=11.1 Hz, 1H, CHPh), 4.38 (d, *J*=3.1 Hz, 1H, H-2), 4.12 (t, *J*=2.7 Hz, 1H, H-3), 3.90 (m, 2H, H-4, H-5), 3.67 (dd, *J*=2.8, 9.8 Hz, 1H, H-6a), 3.60 (dd, *J*=5.3, 9.8 Hz, 1H, H-6b), 2.56 (broad s, 1H, OH) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 169.6 (amide C), 151.3 (d, ²*J*_{C-F}=244.5 Hz, aromatic C-F), 138.2, 138.1, 137.5, 136.4 (4 x quaternary aromatic C), 128.7-127.7 (20 x aromatic CH), 125.8 (d, ²*J*_{C-F}=10.0 Hz), 124.5 (d, ³*J*_{C-F}=2.2 Hz), 114.8 (d, ²*J*_{C-F}=18.9 Hz), 80.9 (C-3), 80.2 (C-2), 77.8 (C-5), 75.4, 74.3, 74.2, 73.5 (4 x CH₂Ph), 71.4 (C-4), 71.1 (C-6) ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = -130.4 ppm.

LRMS (ESI⁺): m/z calcd. for C₄₀H₄₀FNO₆ [M + Na]⁺, 672.3 found 672.0.

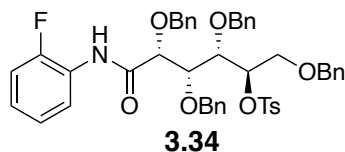
***N*-2-Fluorophenyl-2,3,4,6-tetra-*O*-benzyl-5-methanesulfonyl-D-gluconamide (3.33)**



Compound **3.31** (0.100 g, 0.154 mmol) was dissolved in pyridine (1.25 mL) and methanesulfonyl chloride (0.04 mL, 0.462 mmol) was added dropwise with stirring over 5 min. The reaction was allowed to proceed for 30 min. After the reaction had gone to completion, ethyl acetate (4 mL) was added. The solution was washed with 1 M HCl (2 mL), and the organic layer was dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude mixture was purified by silica gel flash column chromatography (3:1 hexane/ethyl acetate) which afforded pure **3.33** (0.104 g, 92% yield). ¹H NMR (400 MHz, CDCl₃): δ = 8.70 (d, J_{H-F}=2.5 Hz, 1H, NH), 8.30 (td, J = 1.2, 7.8 Hz, 1H, aromatic H), 7.40-7.26 (m, 16 H, aromatic H), 7.15-7.04 (m, 7H, aromatic H) 5.00-4.97 (m, 1H, H-5), 4.78 (d, J=11.1 Hz, 1H, CHPh), 4.73 (d, J=11.0 Hz, 1H, CHPh), 4.70 (d, J=11.1 Hz, 1H, CHPh), 4.70 (d, J=11.1 Hz, 1H, CHPh), 4.63 (d, J=11.2 Hz, 1H, CHPh), 4.55 (d, J=11.2 Hz, 1H, CHPh), 4.52 (d, J=11.1 Hz, 1H, CHPh), 4.50 (d, J=11.1 Hz, 1H, CHPh), 4.25 (dd, J=3.1, 7.2 Hz, 1H, H-3), 4.17 (d, J=3.1 Hz, 1H, H-2), 4.03 (dd, J=3.4, 7.2 Hz, 1H, H-4), 3.90 (dd, J=4.1, 6.4 Hz, 1H, H-6a), 3.87 (dd, J=4.1, 11.0 Hz, 1H, H-6b), 2.91 (s, 3H, SO₂CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 168.8 (amide C), 137.9, 137.4, 137.3, 136.1 (4 x quaternary aromatic C), 128.7-127.7 (20 x aromatic CH), 125.8, 124.5, 121.4, 114.8 (d, ²J_{C-F}=18.9 Hz), 81.9 (C-5), 80.2 (C-3), 79.8 (C-4), 79.6 (C-2), 75.8, 75.2, 74.6, 73.6 (4 x CH₂Ph), 68.6 (C-6), 38.6 (SO₂CH₃) ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = -130.4 ppm.

LRMS (ESI⁺): m/z calcd. for C₄₁H₄₂FNO₈S [M + Na]⁺, 750.2, found 750.3.

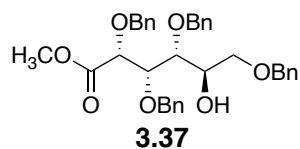
***N*-2-Fluorophenyl-2,3,4,6-tetra-*O*-benzyl-5-toluenesulfonyl-D-gluconamide (3.34)**



Compound **3.31** (0.163 g, 0.251 mmol) was dissolved in pyridine (0.89 mL) under argon atmosphere and was cooled down to 0 °C. *p*-toluenesulphonyl chloride (0.148 g, 0.777 mmol) was added and the reaction was slowly warmed to room temperature and stirring continued overnight. After 22 h, the reaction was diluted with CH₂Cl₂ and washed with ice cold water. The organic phase was washed sequentially with 10% CuSO₄ solution, saturated aqueous NaHCO₃, and water. The organic layer was collected and dried over sodium sulfate, filtered, and concentrated under reduced pressure. Silica gel flash column chromatography afforded pure **3.34** (0.105 g, 52% yield). ¹H NMR (400 MHz, CDCl₃): δ = 8.70 (d, *J*_{H-F}=2.5 Hz, 1H, NH), 8.31 (t, *J*=7.8 Hz, 1H, aromatic H), 7.72 (d, *J*=7.8 Hz, 2H, aromatic H), 7.40-7.00 (m, 25 H, aromatic H), 4.96-4.92 (m, 1H, H-5), 4.73 (d, *J*=11.3 Hz, 1H, CHPh), 4.72 (d, *J*=10.6 Hz, 1H, CHPh), 4.67 (d, *J*=10.5 Hz, 2H, CHPh), 4.60 (d, *J*=10.6 Hz, 1H, CHPh), 4.44 (d, *J*=10.6, 1H, CHPh), 4.35 (d, *J*=11.6 Hz, 1H, CHPh), 4.34 (d, *J*=11.6 Hz, 1H, CHPh), 4.33 (dd, *J*=3.2, 7.5 Hz, 1H, H-4), 4.17 (d, *J*=3.2 Hz, 1H, H-2), 4.04 (dd, *J*=3.2, 7.5 Hz, 1H, H-3), 3.85 (dd, *J*=4.3, 10.9 Hz, 1H, H-6a), 3.74 (dd, *J*=6.1, 10.9 Hz, 1H, H-6b), 2.34 (s, 1H, ArCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 168.8 (amide C=O), 153.6 (d, *J*_{C-F}=244.4 Hz, quaternary aromatic C) 144.6, 138.2, 137.6, 137.5, 136.3, 134.1 (6 x quaternary aromatic C), 129.6, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.8, 127.7, 127.6 (24 x aromatic CH), 125.9 (d, *J*_{C-F}=10.1 Hz, aromatic CH), 124.5 (d, *J*_{C-F}=5.6 Hz, aromatic CH), 121.4 (aromatic CH), 114.9 (d, *J*_{C-F}=19.0 Hz, aromatic CH), 81.3 (C-5), 80.5 (C-2), 80.1 (C-

3), 79.7 (C-4), 75.9, 75.2, 74.6, 73.3 (4 x CH₂Ph), 67.9 (C-6), 21.6 (ArCH₃) ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = -130.3 ppm.

Methyl 2,3,4,6-tetra-*O*-benzyl-D-gluconate (**3.37**)



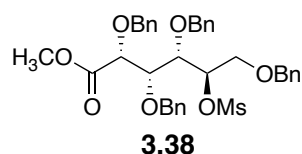
Lithium hydroxide (0.045 g, 1.88 mmol) was added to a solution of compound **3.30** (0.500 g, 0.928 mmol) in THF (4 mL) and water (2 mL) at 0 °C. The stirring was continued at the same temperature for 2-3 h. The reaction mixture was allowed to warm to room temperature and the crude product was acidified with 1 M HCl until pH=7-8. Solvents were evaporated under reduced pressure and the product was lyophilized to yield compound **3.36** as a dry crude powder that was used in the following step.

Compound **3.36** (0.102 g, 0.189 mmol) was dissolved in DMF (0.65 mL) at room temperature and methyl iodide (0.02 mL, 0.283 mmol) was added followed by potassium carbonate (0.026 g, 0.189 mmol). The resulting mixture was stirred for 20 min, then the solvent was evaporated, and the residue was dissolved in CH₂Cl₂ and filtered. The mixture was concentrated under reduced pressure and silica gel flash column chromatography afforded pure **3.37** (0.057 g, 53% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.33-7.24 (m, 20H, aromatic H), 4.87 (d, J=11.7 Hz, 1H, CHPh), 4.78 (d, J=10.3 Hz, 1H, CHPh), 4.75 (d, J=10.7 Hz, 1H, CHPh), 4.65 (d, J=11.0 Hz, 1H, CHPh), 4.61 (d, J=11.0 Hz, 1H, CHPh), 4.54-4.49 (m, 3H, 3 x CHPh), 4.22 (d, J=4.8 Hz, 1H, H-2), 4.17 (t, J=5.3 Hz, 1H, H-3), 4.12 (t, J=5.3 Hz, 1H, H-4), 3.84 (dd, J=3.8, 10.6 Hz, 1H, H-6a), 3.63 (dd, J=4.1, 10.6 Hz, 1H, H-6b), 3.51-3.48 (m, 1H, H-5), 3.32 (s, 3H, OCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 173.7 (C=O), 138.4, 138.1, 137.4, 137.0 (4 x quaternary aromatic C), 128.5, 128.5,

128.4, 128.4, 128.4, 128.3, 128.1, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5 (20 x aromatic CH), 80.3 (C-5), 79.8 (C-3), 77.6 (C-4), 77.2 (C-2), 75.4, 74.8, 73.5, 73.4 (4 x CH₂Ph), 67.7 (C-6), 57.3 (OCH₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₃₅H₃₈O₇ [M + Na]⁺, 593.2 found 593.0.

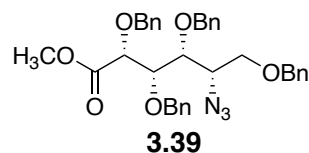
Methyl 2,3,4,6-tetra-*O*-benzyl-5-methanesulfonyl-D-gluconate (**3.38**)



Compound **3.37** (0.100 g, 0.154 mmol) was dissolved in pyridine (1.25 mL) and methanesulfonyl chloride (0.04 mL, 0.462 mmol) was added dropwise with stirring over 5 min. The reaction was allowed to proceed for 30 min. After the reaction had gone to completion, ethyl acetate (4 mL) was added. The solution was washed with 1 M HCl (2 mL), and the organic layer was dried with sodium sulfate, filtered, and concentrated under reduced pressure. The crude mixture was purified by silica gel flash column chromatography (3:1 hexane/ethyl acetate) which afforded pure **3.38** (0.092 g, 92% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.35-7.22 (m, 20H, aromatic H), 4.97-4.92 (m, 1H, H-5), 4.73 (d, J=11.1 Hz, 1H, CHPh), 4.70 (d, J=11.0 Hz, 1H, CHPh), 4.66 (d, J=11.1 Hz, 1H, CHPh), 4.63 (d, J=11.1 Hz, 1H, CHPh), 4.56 (d, J=11.2 Hz, 1H, CHPh), 4.44 (d, J=11.2 Hz, 1H, CHPh), 4.42 (d, J=11.1 Hz, 1H, CHPh), 4.39 (d, J=11.1 Hz, 1H, CHPh), 4.19 (d, J=4.4 Hz, 1H, H-2), 4.07-4.12 (m, 1H, H-4), 3.97 (dd, J=4.5, 6.4 Hz, 1H, H-3), 3.82 (dd, J=4.1, 11.0 Hz, 1H, H-6a), 3.69 (dd, J=7.0, 11.0 Hz, 1H, H-6b), 3.57 (s, 3H, OCH₃), 2.82 (s, 3H, SO₂CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 170.5 (C-1), 137.8, 137.7, 137.5, 136.9 (4 x quaternary aromatic C), 128.7-127.8 (20 x aromatic CH), 81.9 (C-5), 79.3 (C-3), 79.1 (C-4), 78.0 (C-2), 74.9, 74.7, 73.4, 73.2 (4 x CH₂Ph), 68.7 (C-6), 51.9 (OCH₃), 38.4 (SO₂CH₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₃₆H₄₀O₉S [M + Na]⁺, 648.8 found 593.0 (loss of mesyl ester group).

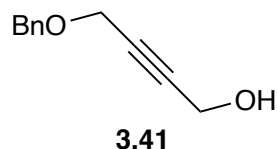
Methyl 2,3,4,6-tetra-*O*-benzyl-5-azido-D-gluconate (3.39)



Sodium azide (0.109 g, 1.672 mmol) was added to a solution of compound **3.38** (0.050 g, 0.077 mmol) in DMF (1 mL). The reaction mixture was heated to 65 °C for 35 h. The solvent was evaporated, and crude product was purified by silica gel flash column chromatography affording pure **3.39** (0.011 g, 5% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.35-7.30 (m, 20 H, aromatic H), 4.86 (d, J=11.9 Hz, 1H, CHPh), 4.75 (d, J=11.6 Hz, 1H, CHPh), 4.71 (d, J=11.6 Hz, 1H, CHPh), 4.56 (d, J=11.9 Hz, 1H, CHPh), 4.53 (d, J=11.6 Hz, 1H, CHPh), 4.42 (d, J=11.9 Hz, 1H, CHPh), 4.38 (d, J=12.5 Hz, 2H, 2 x CHPh), 4.14-4.10 (m, 2H, H-3, H-2), 3.91 (dd, J=3.2, 7.4 Hz, 1H, H-4), 3.60 (s, 3H, OCH₃), 3.58 (t, J=8.9 Hz, 1H, H-6a), 3.47-3.42 (m, 1H, H-6b), 3.22-3.18 (m, 1H, H-5) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 170.6 (ester C=O), 137.9, 137.9, 137.7, 136.6 (quaternary aromatic C), 128.9, 128.7, 128.6, 128.5, 128.5, 128.4, 128.3, 128.3, 128.3, 128.1, 128.0, 127.7, 127.6 (aromatic CH), 80.1 (C2), 78.1 (C4), 77.2 (C3), 75.1, 74.9, 73.2, 72.9 (4 x CH₂Ph), 69.3 (C6), 60.7 (C5), 51.9 (OCH₃) ppm.

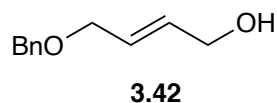
LRMS (ESI⁺): *m/z* calcd. for C₃₅H₃₇N₃O₆ [M + Na]⁺ 618.2, found 618.3

4-benzyloxy-2-butyne-1-ol (**3.41**)



To a suspension of 60% sodium hydride in mineral oil (3.95 g, 0.099 mol) in DMF (116 mL) was added compound **3.40** (10 g, 0.116 mol) and a catalytic amount of TBAI and the reaction was stirred at room temperature for 3 h. Benzyl bromide (11.7 mL, 0.099 mol) was then added, and the reaction continued to stir at room temperature for 18 h. The reaction was quenched with water (20 mL) and extracted with ether (300 mL). The organics were washed with brine and dried over anhydrous MgSO_4 . Purification by silica gel flash column chromatography (2:1 hexane/ethyl acetate) afforded the pure oil, **3.41** (5.62 g, 29% yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.28-7.34 (m, 5H, aromatic H), 4.57 (s, 2H, PhCH_2), 4.27 (t, J =1.8 Hz, 2H, H-2a&b), 4.19 (t, J =1.8 Hz, 2H, H-1a&b), 2.56 (1H, broad s, OH) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 137.2 (quaternary aromatic C), 128.5, 128.1, 128.0 (5 x aromatic CH), 85.0 (C-2), 81.5 (C-3), 71.8 (PhCH_2), 57.4 (C-1), 50.9 (C-4) ppm.

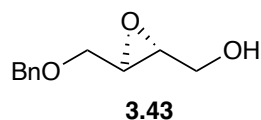
(*E*)-4-benzyloxy-2-buten-1-ol (**3.42**)



A solution of compound **3.41** (2.3 g, 0.013 mol) in THF (10.5 mL) was cooled to 0 °C before Red-Al (5.09 mL, 0.017 mol) was added slowly over a 1 h period. After complete addition the reaction mixture was immediately transferred into a solution of 20% H_2SO_4 (17 mL) at 0 °C. Toluene (14 mL) was added, and the mixture was vigorously stirred for 5 min. The two phases were separated,

and the aqueous layer was extracted with toluene. The combined organic layers were washed with saturated aqueous sodium bicarbonate and brine, dried with anhydrous Na₂SO₄ then concentrated under reduced pressure. An orange, brown liquid was obtained which was distilled under reduced pressure (bp = 110 °C) to obtain pure compound **3.42** (2.33 g, 98%). ¹H NMR (400 MHz, CDCl₃): δ = 7.24-7.34 (m, 5H, aromatic H), 5.78-5.92 (m, 2H, H-2, H-3), 4.50 (s, 2H, PhCH₂), 4.11 (dd, J=1.0, 4.8, 2H, H-4), 4.01-4.02 (m, 2H, H-1), 2.06 (1H, broad s, OH) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 138.2 (quaternary aromatic C), 132.4 (C-2), 128.4, 127.8, 127.7 (5 x aromatic CH), 127.7 (C-3), 72.3 (PhCH₂), 70.1 (C-1), 62.9 (C-4) ppm.

(2*R*,3*S*)-4-benzyloxy-2,3-epoxybutan-1-ol (3.43)

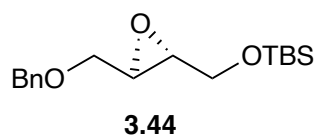


A -20 °C stirred suspension of powdered 4Å molecular sieves (2.02 g) in dry CH₂Cl₂ (130 mL) under argon was added titanium isopropoxide (0.79 mL, 2.60 mmol), L-(+)-diisopropyl tartrate (0.62 mL, 3.60 mmol) and tert-butyl hydroperoxide (4.83 mL, 48.0 mmol). The slurry was stirred at the same temperature for 20 min before the compound **3.42** (2.33 g, 13.1 mmol) was added and the reaction was stirred at -20 °C for 30 min, then kept at -20 °C water bath overnight. Water (15 mL) was added, and the solution was stirred at room temperature for 40 min before 30% NaOH in brine (5 mL) was added and stirred for another 30 min. The powdered sieves were filtered off and the aqueous layer was extracted with CH₂Cl₂ and dried over anhydrous MgSO₄ which was then concentrated over reduced pressure. Silica gel flash column chromatography (6:4 hexane/ethyl acetate) was performed to afford the pure compound **3.43** (1.91 g, 75% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.25-7.37 (m, 5H, aromatic H), 4.59 (d, J=11.9 Hz, 1H, PhCH), 4.53 (d, J=11.9 Hz,

1H, PhCH), 3.91 (dq, J=2.5, 12.6 Hz, 1H, H-1a), 3.75 (dd, J=3.0, 11.5 Hz, 1H, H-4a), 3.62 (ddd, J=4.1, 7.2, 12.6 Hz, 1H, H-1b), 3.50 (dd, J=5.4, 11.5 Hz, 1H, H-4b), 3.22 (quintet, J=2.7 Hz, 1H, H-3), 3.08 (dt, J=2.5, 4.1 Hz, 1H, H-2), 1.93 (t, J=6.5 Hz, 1H, -OH) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 137.8 (quaternary aromatic C), 128.5, 127.8, 127.8 (5 x aromatic CH), 73.4 (PhCH₂), 69.6 (C-1), 61.2 (C-4), 55.8 (C-2), 54.3 (C-3) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₁₀H₁₂O₃ [M + Na]⁺, 217.1 found 217.1.

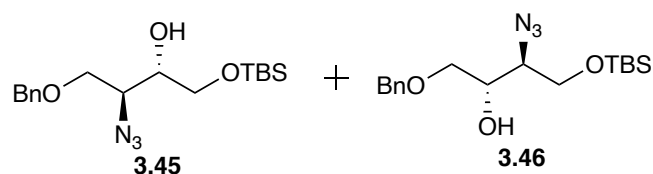
(2*R*,3*S*)-4-benzyloxy-2,3-epoxybutan-1-((*tert*-butyldimethylsilyl)oxy)butane (3.44)



Tert-butyldimethylsilyl chloride (2.35 g, 15.6 mmol) in DMF (17 mL) was added dropwise to a mixture of compound **3.43** (2.76 g, 14.2 mmol), imidazole (2.13 g, 31.2 mmol) and DMAP (0.17 g, 1.42 mmol) in DMF (8 mL) at 0 °C. The mixture was stirred for 2 h with warming to room temperature. The reaction was quenched with saturated aqueous sodium bicarbonate at 0 °C and the stirring was continued for 1 h with warming to room temperature. The mixture was extracted with ether (75 mL) and the extract was washed with 5% HCl solution (5 mL), 5% NaHCO₃ (5 mL) solution, and water (10 mL), then dried with anhydrous magnesium sulfate. The crude product was purified via silica gel flash column chromatography (9:1 hexane/ether) to afford pure **3.44** (4.16 g, 95% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.25-7.34 (m, 5H, aromatic H), 4.58 (d, J=12.0 Hz, 1H, CHPh), 4.52 (d, J=11.9 Hz, 1H, CHPh), 3.82 (dd, J=3.1, 12.0 Hz, 1H, H-1a), 3.73 (dd, J=3.1, 11.6 Hz, 1H, H-4a), 3.65 (dd, J=4.6, 11.9 Hz, 1H, H-1b), 3.45 (dd, J=5.6, 11.5 Hz, 1H, H-4b), 3.09 (q, J=2.7 Hz, 1H, H-3), 2.98 (q, J=2.7 Hz, 1H, H-2), 0.09 (s, 9H, SiC(CH₃)₃), 0.06 (s, 3H, SiCH₃), 0.05 (s, 3H, SiCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 137.9 (quaternary aromatic C), 128.4,

127.8, 127.7 (5 x aromatic CH), 73.3 (CH₂Ph), 70.0 (C-1), 62.9 (C-4), 56.0 (C-3), 54.5 (C-2), 25.9 (C(CH₃)₃), 18.4 (SiC(CH₃)₃), -5.3 (2 x SiCH₃) ppm. Characterization was in agreement with literature.³⁶

[(2*R*,3*S*)-3-azido-4-(benzyloxy)-2-hydroxybutoxy] (tert-butyl)dimethylsilane (3.45**) and [(2*S*,3*R*)-2-azido-4-(benzyloxy)-3-hydroxybutoxy](tert-butyl)dimethylsilane (**3.46**)**



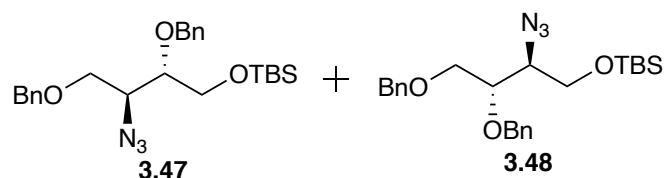
A solution of **3.44** (1.00 g, 3.2 mmol) in 8:1 methanol (26 mL)-water (3 mL) was treated with sodium azide (0.97 g, 14.9 mmol) and ammonium chloride (0.35 g, 6.5 mmol). The resulting reaction mixture was stirred at 85 °C for 18 h. The crude mixture was cooled to room temperature then diluted with ether (50 mL) and extracted. The washed organic solution was dried over anhydrous MgSO₄. The solvent was evaporated, and the crude mixture of products was purified by silica gel flash column chromatography (9:1 hexanes/ether) to afford **3.45** in a 23% yield and **3.46** in a 48% yield. Characterization for **3.45**: ¹H NMR (400 MHz, CDCl₃): δ = 7.25-7.37 (m, 5H, aromatic H), 4.55 (d, J=9.7 Hz, 2H, CH₂Ph), 3.84 (dd, J=2.8, 9.9 Hz, 1H, H-4a), 3.67-3.71 (m, 3H, H-1a,b, H-4b), 3.58-3.61 (m, 2H, H-2, H-3), 2.58 (d, J=5.1, 1H, OH), 0.09 (s, 9H, SiC(CH₃)₃), 0.07 (s, 3H, SiCH₃), 0.06 (s, 3H, SiCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 137.8 (quaternary aromatic C), 128.5, 127.8, 127.6 (5 x aromatic CH), 73.5 (CH₂Ph), 70.9 (C-2), 70.4 (C-4), 63.6 (C-1), 62.1 (C-3), 25.8 (C(CH₃)₃), 18.3 (SiC(CH₃)₃), -5.3 (2 x SiCH₃) ppm.

Characterization for **3.46**: ¹H NMR (400 MHz, CDCl₃): δ = 7.27-7.37 (m, 5H, aromatic H), 4.55 (d, J=9.7 Hz, 2H, CH₂Ph), 3.94 (dd, J=3.6, 10.6 Hz, 1H, H-1a), 3.79 (dd, J=6.5, 10.6 Hz, 1H, H-1b), 3.74-3.80 (m, 1H, H-3), 3.60 (dd, J=3.5, 9.7 Hz, 1H, H-4a), 3.55 (dd, J=5.7, 9.6 Hz, 1H, H-

4b), 3.45 (ddd, $J=3.6, 6.2, 10.1$ Hz, 1H, H-2), 2.64 (d, $J=6.2$, 1H, OH), 0.09 (s, 9H, $\text{SiC}(\text{CH}_3)_3$), 0.07 (s, 6H, 2 x SiCH_3) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 137.7 (quaternary aromatic C), 128.5, 128.0, 127.9 (5 x aromatic CH), 73.6 (CH_2Ph), 71.1 (C-4), 70.0 (C-3), 63.9 (C-1), 63.8 (C-2), 25.8 ($\text{C}(\text{CH}_3)_3$), 18.2 ($\text{SiC}(\text{CH}_3)_3$), -5.6 (2 x SiCH_3) ppm.

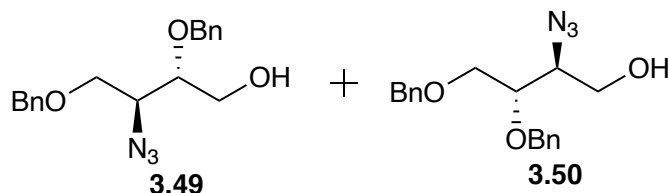
Characterization was in agreement with literature.³⁶

(2*R*,3*S*)-3-azido-2,4-(dibenzoyloxy)-1-((tert-butyldimethylsilyl)oxy)butane (3.47) and (2*S*,3*R*)-2-azido-3,4-(dibenzoyloxy)-1-((tert-butyldimethylsilyl)oxy)butane (3.48)



To a stirred solution of the azido-alcohol (**3.45** or **3.46**) (0.693 g, 2.24 mmol) in DMF (13 mL) was added sodium hydride (60% mineral oil dispersion) (0.081 g, 3.36 mmol) at 0 °C followed by the addition of benzyl bromide (0.29 mL, 2.46 mmol). After stirring for 2 h the reaction was quenched by the addition of ice. The aqueous layer was extracted 3x with ethyl acetate and the combined organic layer was washed with water, brine, and dried over Na_2SO_4 . The solvent was evaporated, and the crude mixture of products were pushed to the next step.

**(2*R*,3*S*)-3-azido-2,4-(dibenzoyloxy)-1-butanol (3.49) and
(2*S*,3*R*)-2-azido-3,4-(dibenzoyloxy)-1-butanol (3.50)**

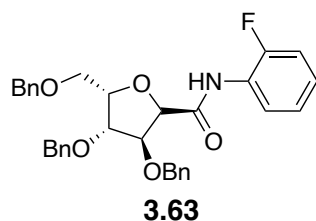


1 M tetrabutylammonium fluoride in THF (2.5 mL, 2.45 mmol) was added by syringe to a stirred solution of compound **3.47** or **3.48** (0.932 g, 2.33 mmol) in THF (4 mL). The mixture was stirred for 30 min at room temperature, then poured on ice water and extracted with ethyl acetate. The organic extract was then washed with 5% citric acid, brine, 5% aqueous sodium bicarbonate, and water. The organic extract was dried over anhydrous MgSO_4 and concentrated under reduced pressure. Purification by silica gel flash chromatography afforded the pure **3.49** in 51% yield. Characterization of **3.49**: ^1H NMR (400 MHz, CDCl_3): δ = 7.32-7.42 (m, 5H, aromatic H), 4.57-4.65 (m, 4H, 2 x CH_2Ph), 3.80-3.85 (m, 2H, H-4a,b), 3.77 (dd, J =2.0, 3.6 Hz, 1H, H-2), 3.67-3.75 (m, 2H, H-1a,b), 3.60-3.63 (m, 1H, H-3), 2.49 (broad s, 1H, OH) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 137.8 (quaternary aromatic C), 137.7 (quaternary aromatic C), 128.6 128.5, 128.1, 128.0, 127.8, 127.9 (10 x aromatic CH), 78.5 (C-3), 73.5 (CH_2Ph), 72.7 (CH_2Ph), 69.6 (C-1), 61.2 (C-4), 61.1 (C-2) ppm.

Characterization of **3.50**: ^1H NMR (400 MHz, CDCl_3): δ = 7.27-7.37 (m, 5H, aromatic H), 4.67 (d, J =11.9, 1H, CHPh), 4.54-4.58 (m, 3H, 3 x CHPh), 3.75-3.84 (m, 2H, H-4a,b), 3.67-3.75 (m, 3H, H-1b, H-2, H-3), 3.58-3.63 (m, 1H, H-1a), 2.12 (broad s, 1H, OH) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 137.6 (2 x quaternary aromatic C), 128.6, 128.5, 128.0, 127.9, 127.8 (10 x aromatic CH), 78.1 (C-2), 73.6 (CH_2Ph), 72.6 (CH_2Ph), 68.8 (C-1), 63.5 (C-3), 62.3 (C-4) ppm.

Characterization was in agreement with literature.³⁶

***N*-2-fluorophenyl-2,3,5-tri-*O*-benzyl- α -L-xylofuranosyl amide (**3.63**)**

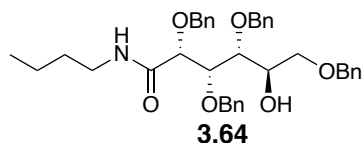


Pyridine (0.08 mL, 1.06 mmol) was dissolved in anhydrous dichloromethane (4.3 mL) and cooled to -10 °C. Trifluoromethanesulfonic anhydride (0.15 mL, 0.924 mmol) was added at the same temperature. The mixture was stirred for 15 min at -10 °C. In a separate flask, compound **3.31** (0.3 g, 0.462 mmol) was dissolved in anhydrous dichloromethane (2 mL) and added dropwise to the reaction. The reaction was stirred at the same temperature for 30 min, diluted with dichloromethane and quenched with water. The organic layer was separated, washed 2x with water, dried with sodium sulfate, filtered and purified by silica gel flash column chromatography to afford the pure **3.63** (0.190 g, 76% yield). ¹H NMR (600 MHz, CDCl₃): δ = 8.78 (br s, 1H, amide NH), 8.38 (t, J=7.7 Hz, 1H, aromatic H), 7.37-7.22 (m, 15H, aromatic H), 7.15-7.07 (m, 4H, aromatic H), 4.82 (d, J=3.4 Hz, 1H, H-2), 4.67 (d, J=11.8 Hz, 1H, CHPh), 4.63-4.61 (m, 1H, H-5), 4.63 (d, J=10.6 Hz, 1H, CHPh), 4.57 (d, J=11.8 Hz, 1H, CHPh), 4.52 (d, J=11.8 Hz, 1H, CHPh), 4.51 (d, J=11.8 Hz, 1H, CHPh), 4.40 (dd, J=3.0, 3.4 Hz, 1H, H-3), 4.38 (d, J=12.0 Hz, 1H, CHPh), 4.00 (d, J=3.0 Hz, 1H, H-4), 3.79 (d, J=6.8 Hz, 2H, H-6a/b) ppm. ¹³C NMR (152 MHz, CDCl₃): δ = 167.8 (amide C=O), 153.6 (d, J_{C-F}=245.0 Hz, quaternary aromatic C), 138.1, 137.5, 137.4 (3 x quaternary aromatic C), 128.6, 128.6, 128.5, 128.4, 128.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.8 (15 x aromatic CH), 125.8 (d, J_{C-F}=10.2 Hz, aromatic CH), 124.7 (d, J_{C-F}=7.8 Hz, aromatic CH), 124.5 (d, J_{C-F}=3.6 Hz, aromatic CH), 121.9 (aromatic CH), 115.0 (d, J_{C-F}=19.0 Hz, aromatic CH), 82.1

(C2, C3), 81.4 (C4), 81.3 (C5), 73.6, 73.2, 72.3 (3 x CH₂Ph), 68.2 (C6) ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = -130.0 ppm.

LRMS (ESI+) *m/z* calcd. for C₃₃H₃₂FNO₄ [M + Na]⁺ 564.6, found 564.3.

N-*n*-butyl-2,3,4,6-tetra-*O*-benzyl-*D*-gluconamide (**3.64**)



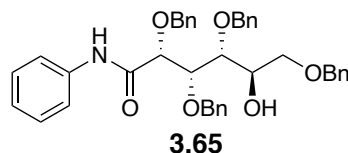
Compound **3.30** (0.328 g, 0.609 mmol) was added to a round bottom flask and dissolved in methanol (1.5 mL). *n*-Butylamine (0.12 mL, 1.217 mmol) was added, and the mixture was allowed to reflux for 12 h. After cooling, the mixture was diluted with ethyl acetate, washed with 1 M HCl, water, and brine. The organic phase was dried over anhydrous sodium sulfate, concentrated, and purified via silica gel flash chromatography to afford the pure **3.64** (0.283 g, 76% yield).

¹H NMR (400 MHz, CDCl₃): δ = 7.36-7.26 (m, 20 H, aromatic H), 6.68 (t, *J*=6.0 Hz, 1H, amide H), 4.75 (d, *J*=11.2 Hz, 1H, PhCH), 4.71 (d, *J*=11.2 Hz, 1H, PhCH), 4.63 (d, *J*=11.2 Hz, 1H, PhCH), 4.59 (d, *J*=11.2 Hz, 1H, PhCH), 4.59 (d, *J*=10.6 Hz, 1H, PhCH), 4.56 (d, *J*=10.6 Hz, 1H, PhCH), 4.56 (d, *J*=11.5 Hz, 1H, PhCH), 4.53 (d, *J*=11.5 Hz, 1H, PhCH), 4.29 (d, *J*=3.2 Hz, 1H, H-2), 4.11 (dd, *J*=3.2, 5.8 Hz, 1H, H-3), 3.97-3.94 (m, 1H, H-5), 3.91 (dd, *J*=5.8, 7.6 Hz, 1H, H-4), 3.69 (dd, *J*=3.0, 9.7 Hz, 1H, H-6a), 3.64 (dd, *J*=5.4, 9.7 Hz, 1H, H-6b), 3.34-3.35 (m, 1H, NHCH), 3.17-3.09 (m, 1H, NHCH), 2.98 (d, *J*=2.9 Hz, 1H), 1.43-1.36 (m, 2H, NHCH₂CH₂), 1.33-1.24 (m, 2H, NHCH₂CH₂CH₂), 0.90 (t, *J*=7.2 Hz, 3H, NHCH₂CH₂CH₂CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 170.8 (amide C), 138.3, 138.2, 138.0, 136.9 (4 x quaternary aromatic C), 128.7, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7, 127.7 (20 x aromatic CH), 80.8 (C-3), 80.2 (C-2), 77.7 (C-4), 75.2 (CH₂Ph), 74.1 (CH₂Ph), 74.0 (CH₂Ph), 73.4 (CH₂Ph), 71.5 (C-5),

71.2 (C-6), 38.9 (NHCH₂), 31.6 9 (NHCH₂CH₂), 20.1 (NHCH₂CH₂CH₂), 13.7 (NHCH₂CH₂CH₂CH₃) ppm.

LRMS (ESI⁺) *m/z* calcd. for C₃₈H₄₅NO₆ [M + Na]⁺ 634.3, found 634.2.

N-phenyl-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (**3.65**)

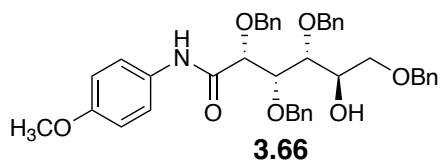


Compound **3.30** (0.500 g, 0.928 mmol) was added to a round bottom flask and dissolved in toluene (7.5 mL). Aniline (0.33 mL, 3.712 mmol) was added, and the mixture was allowed to reflux for 12 h. After cooling, the mixture was diluted with ethyl acetate, washed with 1 M HCl, water, and brine. The organic phase was dried over anhydrous sodium sulfate, concentrated, and purified via silica gel flash column chromatography to afford the pure **3.65** (0.243 g, 42% yield).

¹H NMR (400 MHz, CDCl₃): δ = 8.41 (br s, 1H, amide NH), 7.44-7.41 (m, 2H, aromatic H), 7.35-7.09 (m, 23H, aromatic H), 4.73 (d, J=11.2 Hz, 1H, CHPh), 4.68 (d, J=11.1 Hz, 1H, CHPh), 4.67 (d, J=11.0 Hz, 1H, CHPh), 4.63 (d, J=11.2 Hz, 1H, CHPh), 4.56 (d, J=11.1 Hz, 1H, CHPh), 4.53 (d, J=11.1 Hz, 1H, CHPh), 4.52 (d, J=11.0 Hz, 1H, CHPh), 4.50 (d, J=11.1 Hz, 1H, CHPh), 4.37 (d, J=2.9 Hz, 1H, H-2), 4.13 (t, J=2.7 Hz, 1H, H-3) 3.93-3.88 (m, 2H, H-4, H-5), 3.69 (dd, J=2.8, 9.8 Hz, 1H, H-6a), 3.63 (dd, J=5.3, 9.8 Hz, 1H, H-6b), 2.82 (br s, 1H, OH) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 169.3 (C-1), 138.1, 138.1, 137.4, 137.3, 136.5 (5 x quaternary aromatic C), 129.0-127.7 (23 x aromatic CH), 124.4, 119.5 (aromatic CH), 81.0 (C-3), 80.4 (C-2), 77.8 (C-5), 75.5, 74.3, 74.2, 73.5 (4 x CH₂Ph), 71.5 (C-4), 71.1 (C-6) ppm.

LRMS (ESI⁺) *m/z* calcd. for C₄₀H₄₁NO₆ [M + Na]⁺ 654.3, found 654.3.

***N*-4-methoxyphenyl-2,3,4,6-tetra-*O*-benzyl-D-gluconamide (3.66)**

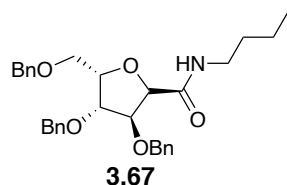


Compound **3.30** (0.500 g, 0.928 mmol) was added to a round bottom flask and dissolved in toluene (7.5 mL). Aniline (0.46 g, 3.712 mmol) was added, and the mixture was allowed to reflux for 12 h. After cooling, the mixture was diluted with ethyl acetate, washed with 1 M HCl, water, and brine. The organic phase was dried over anhydrous sodium sulfate, concentrated, and purified by silica gel flash column chromatography to afford the pure **3.66** (0.303 g, 49% yield).

^1H NMR (400 MHz, CDCl_3): δ = 8.41 (br s, 1H, amide NH), 7.44-7.41 (m, 2H, aromatic H), 7.35-7.09 (m, 23H, aromatic H), 4.73 (d, J =11.2 Hz, 1H, CHPh), 4.68 (d, J =11.1 Hz, 1H, CHPh), 4.67 (d, J =11.0 Hz, 1H, CHPh), 4.63 (d, J =11.2 Hz, 1H, CHPh), 4.56 (d, J =11.1 Hz, 1H, CHPh), 4.53 (d, J =11.1 Hz, 1H, CHPh), 4.52 (d, J =11.0 Hz, 1H, CHPh), 4.50 (d, J =11.1 Hz, 1H, CHPh), 4.37 (d, J =2.9 Hz, 1H, H-2), 4.13 (t, J =2.7 Hz, 1H, H-3), 3.93-3.88 (m, 2H, H-4, H-5), 3.69 (dd, J =2.8, 9.8 Hz, 1H, H-6a), 3.63 (dd, J =5.3, 9.8 Hz, 1H, H-6b), 2.82 (br s, 1H, OH) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 169.3 (C-1), 138.1, 138.1, 137.4, 137.3, 136.5 (5 x quaternary aromatic C), 129.0-127.7 (23 x aromatic CH), 124.4, 119.5 (aromatic CH), 81.0 (C-3), 80.4 (C-2), 77.8 (C-5), 75.5, 74.3, 74.2, 73.5 (4 x CH_2Ph), 71.5 (C-4), 71.1 (C-6) ppm.

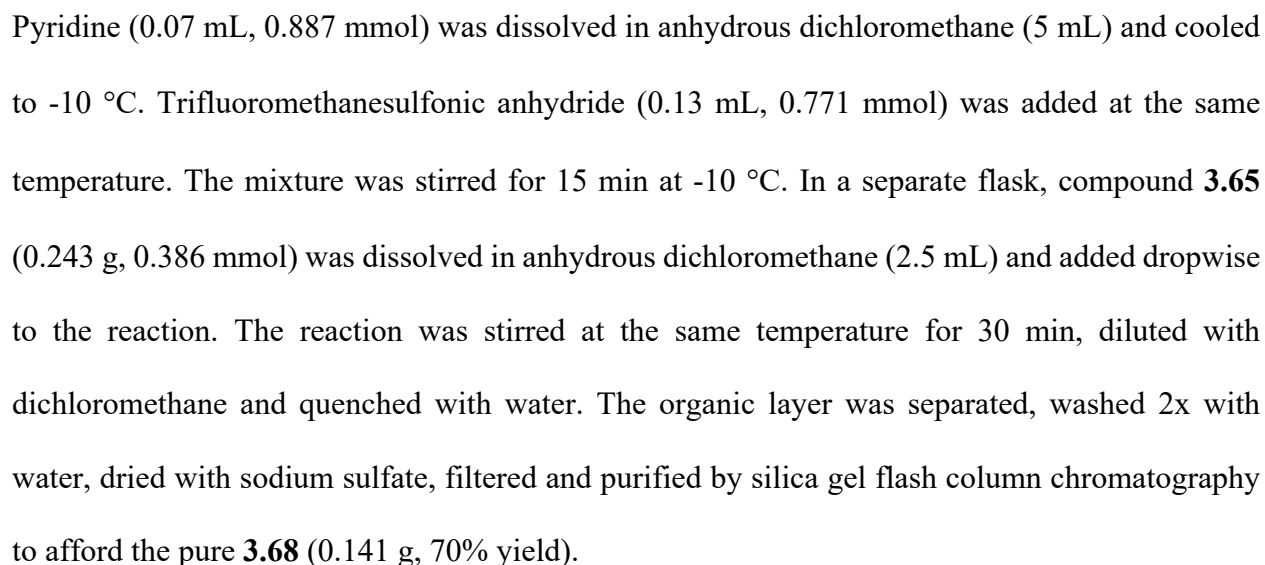
LRMS (ESI $^+$) m/z calcd. for $\text{C}_{41}\text{H}_{43}\text{NO}_7$ $[\text{M} + \text{Na}]^+$ 684.3, found 684.3.

***N*-*n*-butyl-2,3,5-tri-*O*-benzyl- α -L-xylofuranosyl amide (**3.67**)**



Pyridine (0.04 mL, 0.521 mmol) was dissolved in anhydrous dichloromethane (3 mL) and cooled to -10 °C. Trifluoromethanesulfonic anhydride (0.08 mL, 0.452 mmol) was added at the same temperature. The mixture was stirred for 15 min at -10 °C. In a separate flask, compound **3.64** (0.153 g, 0.226 mmol) was dissolved in anhydrous dichloromethane (1.5 mL) and added dropwise to the reaction. The reaction was stirred at the same temperature for 30 min, diluted with dichloromethane and quenched with water. The organic layer was separated, washed 2x with water, dried with sodium sulfate, filtered and purified by silica gel flash column chromatography to afford the pure **3.67** (0.083 g, 73% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.36-7.17 (m, 15H, aromatic CH), 6.75 (t, J=5.8 Hz, 1H, amide H), 4.67 (d, J=3.5 Hz, 1H, H-2), 4.62 (d, J=11.8 Hz, 1H, CHPh), 4.60 (d, J=11.6 Hz, 1H, CHPh), 4.52 (d, J=11.6 Hz, 1H, CHPh), 4.50-4.44 (m, 1H, H-5), 4.49 (d, J=12.2 Hz, 1H, CHPh), 4.47 (d, J=11.8 Hz, 1H, CHPh), 4.31 (d, J=3.5 Hz, 1H, H-3), 4.31 (d, J=12.2 Hz, 1H, CHPh), 3.91 (d, J=3.3 Hz, 1H, H-4), 3.70-3.67 (m, 2H, H-6a/b), 3.36-3.18 (m, 2H, H-7a/b), 1.49-1.42 (m, 2H, H-8a/b), 1.34-1.25 (m, 2H, H-9a/b), 0.87 (t, J=7.3 Hz, 3H, H-10) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 169.0 (amide C), 138.0, 137.7, 137.4 (3 x quaternary aromatic C), 128.45, 128.4, 128.4, 128.0, 127.9, 127.8, 127.8, 127.8, 127.7 (15 x aromatic CH), 81.8 (C-2), 81.6 (C-3), 81.5 (C-4), 81.0 (C-5), 73.6, 73.0, 72.1 (3 x CH₂Ph), 68.7 (C-6), 38.7 (C-7), 31.7 (C-8), 20.0 (C-9), 13.7 (C-10) ppm.

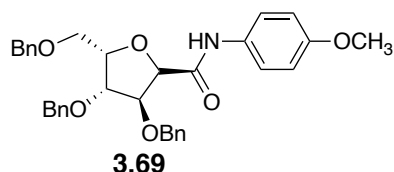
***N*-phenyl-2,3,5-tri-*O*-benzyl- α -L-xylofuranosyl amide (3.68)**



105

LRMS (ESI+) m/z calcd. for $C_{33}H_{33}NO_5$ $[M + K]^+$ 562.2, found 562.3 $[M + K]^+$

***N*-4-methoxyphenyl-2,3,5-tri-*O*-benzyl- α -L-xylofuranosyl amide (**3.69**)**



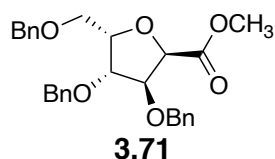
Pyridine (0.09 mL, 1.053 mmol) was dissolved in anhydrous dichloromethane (5 mL) and cooled to -10 °C. Trifluoromethanesulfonic anhydride (0.15 mL, 0.916 mmol) was added at the same temperature. The mixture was stirred for 15 min at -10 °C. In a separate flask, compound **3.65** (0.303 g, 0.458 mmol) was dissolved in anhydrous dichloromethane (2.5 mL) and added dropwise to the reaction. The reaction was stirred at the same temperature for 30 min, diluted with dichloromethane and quenched with water. The organic layer was separated, washed 2x with water, dried with sodium sulfate, filtered and purified by silica gel flash column chromatography to afford pure **3.69** (0.167 g, 66% yield).

^1H NMR (400 MHz, CDCl_3): δ = 8.33 (br s, 1H, amide NH), 7.46-7.43 (m, 2H, aromatic H), 7.37-7.17 (m, 15H, aromatic H), 6.86-6.83 (m, 2H, aromatic H), 4.76 (d, J =3.6 Hz, 1H, H-2), 4.63 (d, J =11.8 Hz, 1H, CHPh), 4.60 (d, J =11.8 Hz, 1H, CHPh), 4.59-4.55 (m, 1H, H-5), 4.54 (d, J =11.9 Hz, 1H, CHPh), 4.50 (d, J =10.6 Hz, 1H, CHPh), 4.47 (d, J =10.6 Hz, 1H, CHPh), 4.37 (d, J =3.7 Hz, 1H, H-3), 4.33 (d, J =11.8 Hz, 1H, CHPh), 3.95 (dd, J =3.6 Hz, 1H, H-4), 3.78 (s, OCH_3), 3.76 (dd, J =6.6, 10 Hz, 1H, H-6a), 3.71 (dd, J =5.7, 10.0 Hz, 1H, H-6b) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 167.2 (amide C=O), 156.5 (quaternary aromatic C), 138.0, 137.5, 137.4 (3x quaternary aromatic C), 130.4, 128.5, 128.5, 128.4, 128.0, 127.9, 127.8, 127.8, (18 x aromatic CH), 122.0

(aromatic CH), 114.1 (aromatic CH), 81.9 (C-2 & C-3), 81.5 (C-4), 81.3 (C-5), 73.6, 73.2, 72.2 (3 x CH₂Ph), 68.6 (C-6), 55.5 (OCH₃) ppm.

LRMS (ESI⁺) *m/z* calcd. for C₃₄H₃₅NO₆ [M + Na]⁺ 576.2, found 576.3.

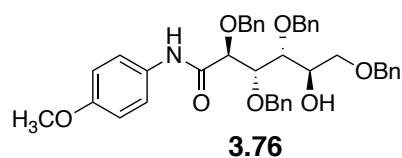
Methyl 2,3,5-tri-*O*-benzyl- α -L-xylofuranosyl carboxylate (3.71)



Compound **3.38** (0.217 g, 0.335 mmol) was dissolved in anhydrous DMF (12 mL) and sodium azide (0.109 g, 1.67 mmol) was added in one portion. The reaction was stirred at 110 °C overnight. The reaction mixture was diluted with dichloromethane, and the organic layer was extracted with water. The organic layer was dried with magnesium sulfate, concentrated, and purified by silica gel flash column chromatography to afford major product, **3.71** (0.028 g, 17% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.35-7.21 (m, 15H, aromatic H), 4.80 (d, J=5.1 Hz, 1H, H-2), 4.60 (d, J=11.9 Hz, 1H, CHPh), 4.55-4.51 (m, 1H, H-5), 4.53 (d, J=11.8 Hz, 1H, CHPh), 4.51 (d, J=11.7 Hz, 1H, CHPh), 4.50 (d, J=11.9 Hz, 1H, CHPh), 4.46 (d, J=12.0 Hz, 1H, CHPh), 4.44 (d, J=12.0 Hz, 1H, CHPh), 4.26 (dd, J=1.5, 5.0 Hz, 1H, H-3), 4.03 (dd, J=1.5, 3.9 Hz, 1H, H-4), 3.78 (dd, J=6.9, 9.4 Hz, 2H, H-6a), 3.74 (dd, J=5.9, 9.4 Hz, 1H, H-6b), 3.72 (s, 3H, OCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 170.2 (C-1), 138.1, 137.6, 137.3 (3 x quaternary aromatic C), 128.5-127.7 (15 x aromatic CH), 82.8 (C-3), 80.8 (C-4), 80.3 (C-5), 80.0 (C-2), 73.5, 72.6, 72.4 (3 x CH₂Ph), 67.5 (C-6), 51.9 (OCH₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₂₈H₃₀O₆ [M + Na]⁺ 485.2, found 485.2.

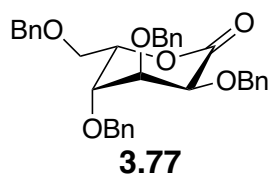
***N*-4-methoxyphenyl-2,3,4,6-tetra-*O*-benzyl-D-mannonamide (3.76)**



Compound **3.75** (0.349 g, 0.649 mmol) was added to a flame-dried round bottom flask and dissolved in dry toluene (8.5 mL). *p*-anisidine (0.359 g, 2.919 mmol) was added and the mixture was refluxed for 12 h. After cooling, the mixture was diluted with ethyl acetate, washed with 1 M hydrochloric acid, water, and brine. The organic phase was dried over anhydrous sodium sulfate, concentrated and purified by silica gel flash column chromatography (3:1 hexane/ethyl acetate) to afford pure **3.76** (0.271 g, 63% yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.36-7.15 (m, 22H, aromatic CH), 6.85-6.79 (m, 2H, aromatic CH), 4.77 (d, J =11.4 Hz, 2H, 2 x CHPh), 4.69 (d, J =11.6 Hz, 1H, CHPh), 4.65 (d, J =11.6 Hz, 1H, CHPh), 4.62 (d, J =11.4 Hz, 1H, CHPh), 4.57 (d, J =11.6 Hz, 1H, CHPh), 4.54 (d, J =11.1 Hz, 1H, CHPh), 4.52 (d, J =11.9 Hz, 1H, CHPh), 4.45 (d, J =3.6 Hz, 1H, H-2), 4.22 (dd, J =3.6, 5.7 Hz, 1H, H-3), 4.09-4.03 (m, 1H, H-5), 3.94 (dd, J =5.8, 7.0 Hz, 1H, H-4), 3.79 (s, 3H, OCH_3), 3.67 (d, J =4.6 Hz, 2H, H-6a/b) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 168.3 (amide C), 156.5, 138.2, 138.1, 138.1, 137.0 (5 x quaternary aromatic C), 130.5, 128.6, 128.4, 128.4, 128.3, 128.2, 128.1, 128.1, 128.1, 128.0, 127.9, 127.7, 127.6, 121.5, 114.1 (24 x aromatic CH), 81.7 (C-3), 80.6 (C-2), 79.1 (C-4), 74.7, 74.5, 73.4, 73.1 (4 x CH_2Ph), 71.2 (C-5), 71.0 (C-6), 55.5 (OCH_3) ppm.

LRMS (ESI+) m/z calcd. for $\text{C}_{41}\text{H}_{43}\text{NO}_7$ $[\text{M} + \text{Na}]^+$ 684.3, found 684.3.

2,3,4,6-tetra-*O*-benzyl-L-gulono-1,5-lactone (3.77)



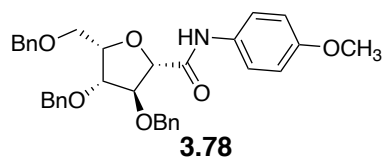
Pyridine (0.08 mL, 0.941 mmol) was dissolved in anhydrous dichloromethane (6 mL) and cooled to -10 °C. Trifluoromethanesulfonic anhydride (0.14 mL, 0.818 mmol) was added at the same temperature. The mixture was stirred for 15 min at -10 °C. In a separate flask, compound **3.76** (0.271 g, 0.409 mmol) was dissolved in anhydrous dichloromethane (3 mL) and added dropwise to the reaction. The reaction was stirred at the same temperature for 30 min, diluted with dichloromethane and quenched with water. The organic layer was separated, washed 2x with water, dried with sodium sulfate, filtered and purified by silica gel flash column chromatography to afford a major product (**3.77**, 0.074 g, 33% yield) and a minor product (**3.78**, 0.034 g, 15% yield).

Characterization of **3.77**: ¹H NMR (400 MHz, CDCl₃): δ = 7.33-7.23 (m, 18 H, aromatic H), 6.99-6.96 (m, 2H, aromatic H), 5.06 (d, J=12.2 Hz, 1H, CHPh), 4.85-4.80 (m, 1H, H-5), 4.82 (d, J=11.4 Hz, 1H, CHPh), 4.63 (d, J=12.2 Hz, 1H, CHPh), 4.57 (d, J=12.2 Hz, 1H, CHPh), 4.55 (d, J=11.4 Hz, 1H, CHPh), 4.47 (d, J=11.7 Hz, 1H, CHPh), 4.37 (d, J=11.7 Hz, 1H, CHPh), 4.33 (d, J=2.9 Hz, 1H, H-2), 4.30 (d, J=11.7 Hz, 1H, CHPh), 3.93 (dd, J=2.9, 4.3 Hz, 1H, H-3), 3.77-3.73 (m, 1H, H-4), 3.62-3.72 (m, 2H, H-6a/6b) ppm. . ¹³C NMR (101 MHz, CDCl₃): δ = 170.5 (ester C=O), 137.7, 137.7, 137.5, 137.0 (4 x aromatic quaternary C), 128.5, 128.5, 128.5, 128.5, 128.4, 128.2,

128.1, 128.0, 128.0, 128.0, 127.9, 127.9 (20 x aromatic CH), 77.6 (C-5), 75.6 (C-3), 74.2 (C-2), 73.8 (C-4), 73.7, 73.6, 73.4, 73.0 (4 x CH₂Ph), 67.5 (C-6) ppm.

LRMS (ESI⁺) *m/z* calcd. for C₃₄H₃₄O₆ [M + Na]⁺ 561.2, found 561.2.

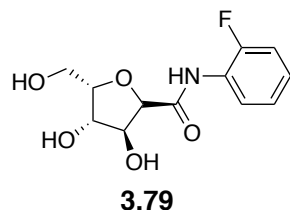
***N*-4-methoxyphenyl-2,3,5-tri-*O*-benzyl-β-*L*-xylofuranosyl amide (3.78)**



Characterization of **3.78**: ¹H NMR (400 MHz, CDCl₃): δ = 8.43 (amide H), 7.39-7.07 (m, 17H, aromatic H), 6.82-6.78 (m, 2H, aromatic H), 4.79 (d, *J* = 11.8 Hz, 1H, CHPh), 4.63-4.57 (m, 3H, 3 x CHPh, H-2), 4.53-4.44 (m, 3H, CHPh, H-5, H-3), 4.31 (d, *J* = 11.5 Hz, 1H, CHPh), 3.98 (dd, *J* = 0.7, 3.3 Hz, 1H, H-4), 3.83 (d, *J* = 5.7 Hz, 2H, H-6a/b), 3.78 (s, 3H, OCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 168.5 (amide C), 156.5, 137.9, 137.4, 137.1 (4 x quaternary aromatic C), 130.4, 128.5, 128.5, 128.3, 127.9, 127.9, 127.8, 127.8, 127.7, 121.6, 114.0 (19 x aromatic CH), 84.6 (C-3), 83.6 (C-2), 81.5 (C-5), 81.3 (C-4), 73.5, 71.6, 71.6 (3 x CH₂Ph), 68.2 (C-6), 55.5 (OCH₃) ppm.

LRMS (ESI⁺) *m/z* calcd. for C₃₄H₃₅NO₆ [M + Na]⁺ 576.2, found 576.2.

***N*-2-fluorophenyl-α-*L*-xylofuranosyl amide (3.79)**

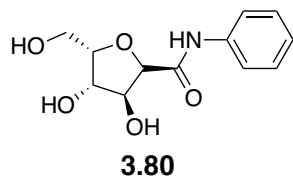


Compound **3.63** (0.1632 g, 0.602 mmol) and 5% palladium on carbon (0.102 g, 0.958 mmol) in ethyl acetate (7 mL) and methanol (7 mL) was degassed under vacuum and saturated with hydrogen gas via a hydrogen gas filled balloon. Suspension was stirred at room temperature for 20

h, filtered through a pad of celite and concentrated to give pure **3.79** (0.161 g, 98% yield). ^1H NMR (400 MHz, DMSO- d_6): δ = 8.90 (br s, 1H, amide NH), 8.04 (td, J =2.2, 7.8 Hz, 1H, aromatic H), 7.27-7.21 (m, 1H, aromatic H), 7.16-7.08 (m, 1H, aromatic H), 5.47 (d, J =4.7 Hz, 1H, 3-OH), 5.20 (d, J =4.0 Hz, 1H, 4-OH), 4.62 (t, J =5.2 Hz, 1H, 6-OH), 4.48 (d, J =3.7 Hz, 1H, H-2), 4.24 (td, J =2.7, 6.0 Hz, 1H, H-5), 4.17 (t, J =3.7 Hz, 1H, H-3), 3.95 (br s, 1H, H-4), 3.64-3.51 (m, 2H, H-6a/b) ppm. ^{13}C NMR (101 MHz, DMSO): δ = 169.0 (amide C=O), 154.5 (d, $J_{\text{C-F}}$ =244.3 Hz, aromatic CH), 126.1 (d, $J_{\text{C-F}}$ =11.2 Hz, aromatic CH), 125.5 (d, $J_{\text{C-F}}$ =8.1 Hz, aromatic CH), 125.0 (d, $J_{\text{C-F}}$ =3.1 Hz, aromatic CH), 123.1 (aromatic CH), 115.9 (d, J =18.8 Hz, aromatic CH), 83.5 (C-5), 82.2 (C-2), 78.0 (C-3), 76.2 (C-4), 59.9 (C-6) ppm. ^{19}F NMR (376 MHz, DMSO): δ = 128.9 ppm.

HRMS (ESI $^{+}$): m/z calcd. for $\text{C}_{12}\text{H}_{14}\text{NO}_5\text{F}$ $[\text{M} + \text{Na}]^{+}$ 294.0754, found 294.0750.

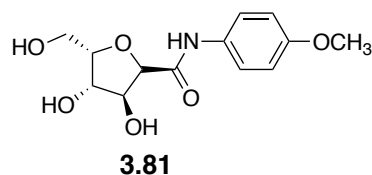
***N*-phenyl- α -L-xylofuranosyl amide (**3.80**)**



Compound **3.68** (0.1407 g, 0.556 mmol) and 5% palladium on carbon (0.09 g, 0.846 mmol) in ethyl acetate (6 mL) and methanol (6 mL) was degassed under vacuum and saturated with hydrogen gas via a hydrogen gas filled balloon. Suspension was stirred at room temperature for 20 h, filtered through a pad of celite and concentrated to give pure **3.80** (0.082 g, 98% yield). ^1H NMR (400 MHz, DMSO- d_6): δ = 9.19 (br s, amide H), 7.66 (d, J =8.0 Hz, 2H, aromatic H), 7.28 (t, J =7.4 Hz, 2H, aromatic H), 7.03 (t, J =7.4 Hz, 1H, aromatic H), 5.37 (d, J =4.3 Hz, 1H, 3-OH), 5.14 (d, J =3.0 Hz, 1H, 4-OH), 4.54 (t, J =5.4 Hz, 1H, 6-OH), 4.42 (d, J =3.6 Hz, 1H, H-2), 4.24-4.21 (m,

1H, H-5), 4.17 (t, J=3.6 Hz, 1H, H-3), 3.95 (br s, 1H, H-4), 3.64-3.52 (m, 2H, H-6a/b) ppm. ¹³C NMR (101 MHz, DMSO-d₆): δ = 169.0 (amide C), 138.8 (quaternary aromatic C), 129.0, 123.9, 120.2 (5 x aromatic CH), 83.0 (C-2), 82.0 (C-5), 78.2 (C-3), 76.2 (C-4), 59.7 (C-6) ppm.
LRMS (ESI⁺): *m/z* calcd. for C₁₂H₁₅NO₅ [M + Na]⁺ 276.1, found 276.1.

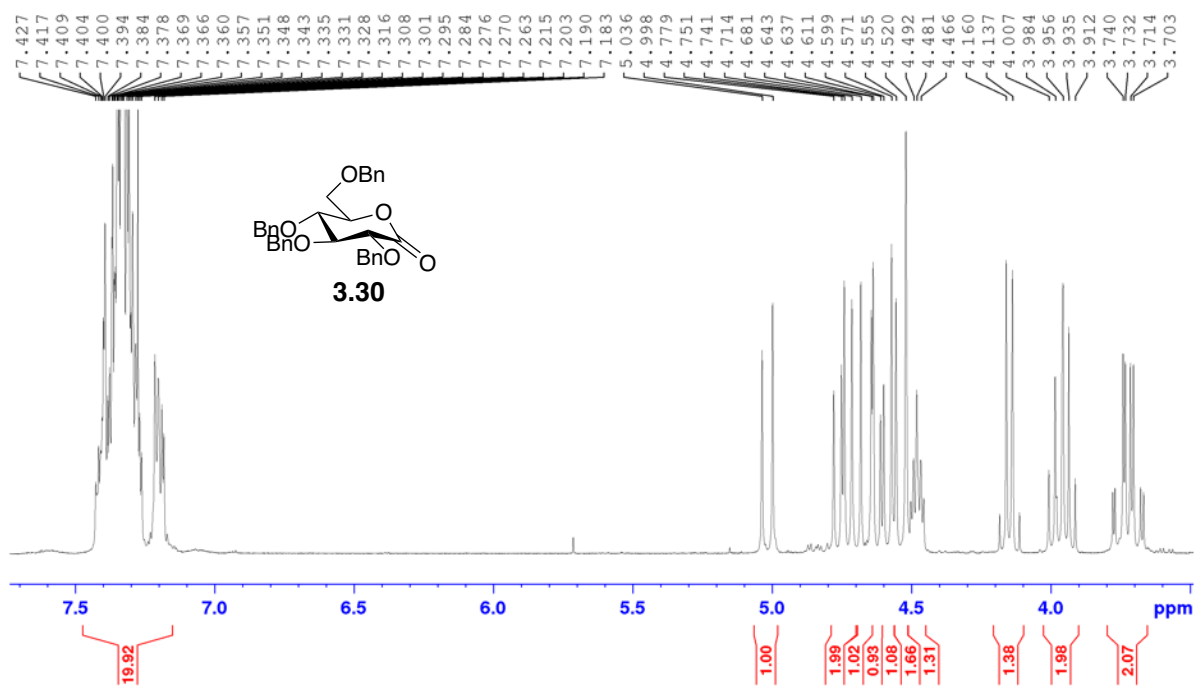
***N*-4-methoxyphenyl-α-L-xylofuranosyl amide (**3.81**)**



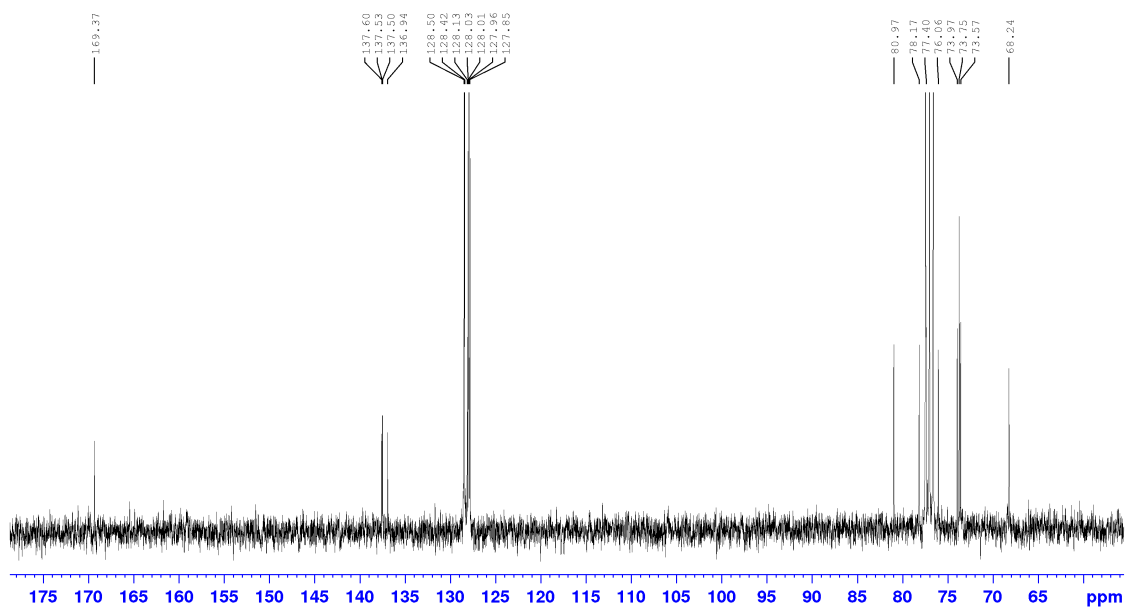
Compound **3.69** (0.083 g, 0.293 mmol) and 5% palladium on carbon (0.054 g, 0.507 mmol) in ethyl acetate (4 mL) and methanol (4 mL) was degassed under vacuum and saturated with hydrogen gas via a hydrogen gas filled balloon. Suspension was stirred at room temperature for 20 h, filtered through a pad of celite and concentrated to give pure **3.81** (0.111 g, 98% yield). ¹H NMR (400 MHz, DMSO-d₆): δ = 9.09 (br s, amide H), 7.57 (d, J=9.1 Hz, 2H, aromatic H), 6.84 (d, J=9.1 Hz, 2H, aromatic H) 5.34 (d, J=4.5 Hz, 1H, 3-OH), 5.13 (d, J=4.1 Hz, 1H, 4-OH), 4.52 (t, J=5.4 Hz, 1H, 6-OH), 4.39 (d, J=3.8 Hz, 1H, H-2), 4.23-4.19 (m, 1H, H-5), 4.15 (t, J=3.6 Hz, 1H, H-3), 3.94 (br s, 1H, H-4), 3.68 (s, 3H, OCH₃), 3.63-3.52 (m, 2H, H-6a/b) ppm. ¹³C NMR (101 MHz, DMSO-d₆): δ = 168.5 (amide C), 155.8, 132.0 (2 x quaternary aromatic C), 121.8, 114.1 (4 x aromatic CH), 83.0 (C-2), 82.0 (C-5), 78.1 (C-3), 76.2 (C-4), 59.7 (C-6), 55.6 (OCH₃) ppm.
LRMS (ESI⁺): *m/z* calcd. for C₁₃H₁₇NO₆ [M + Na]⁺ 306.1, found 306.1.

3.6.4 NMR Spectra

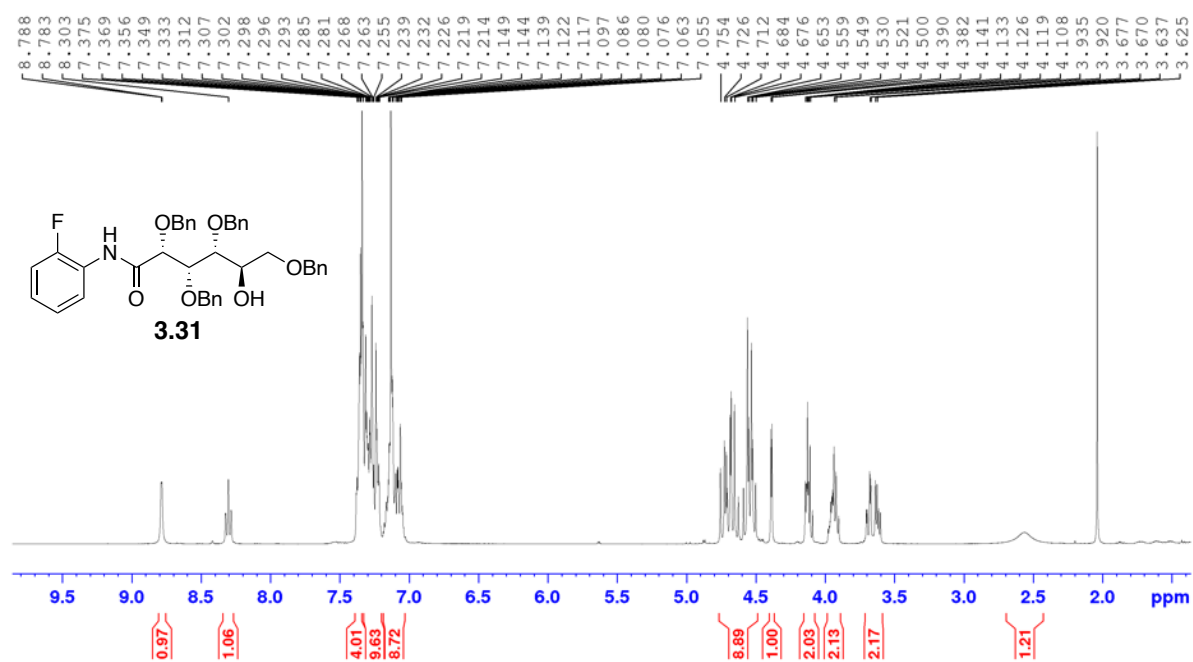
^1H NMR



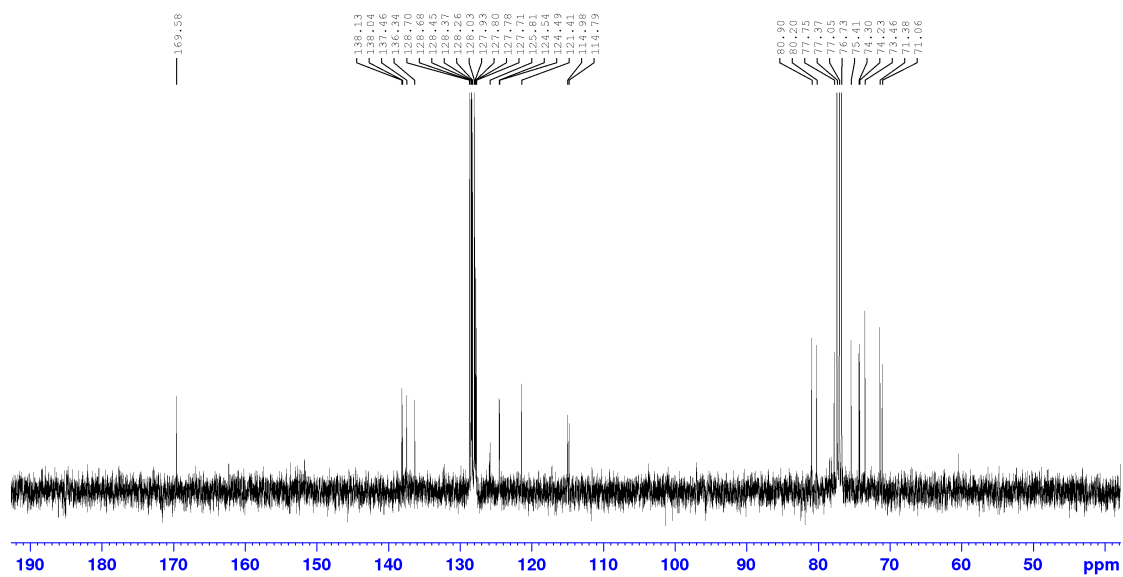
^{13}C NMR



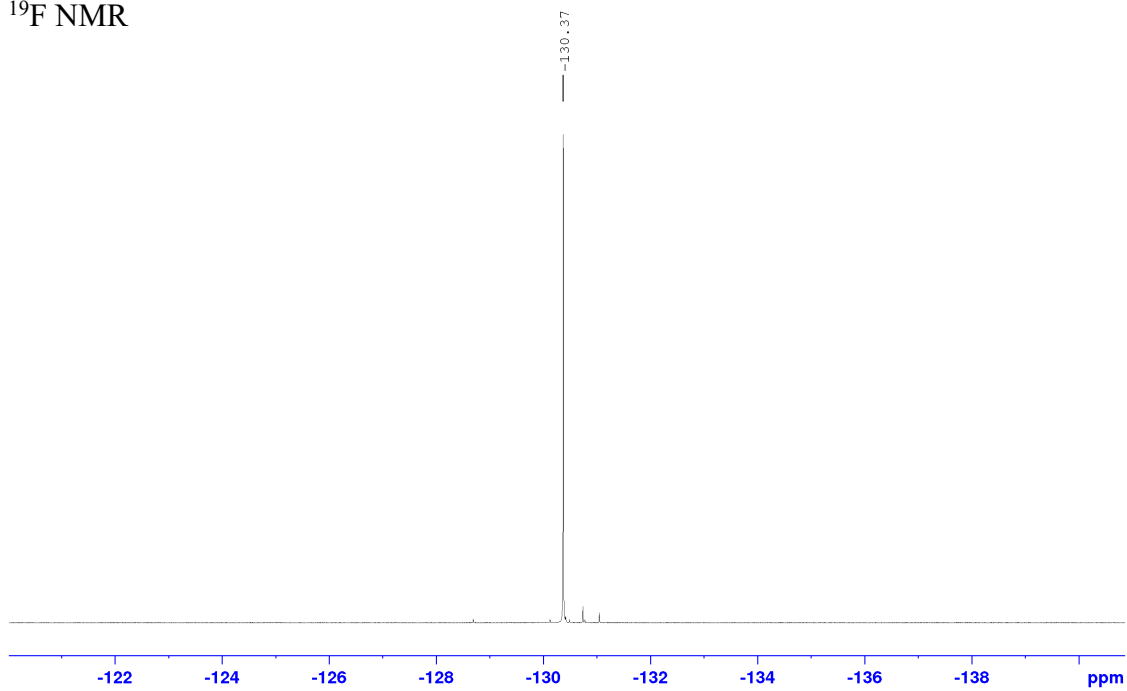
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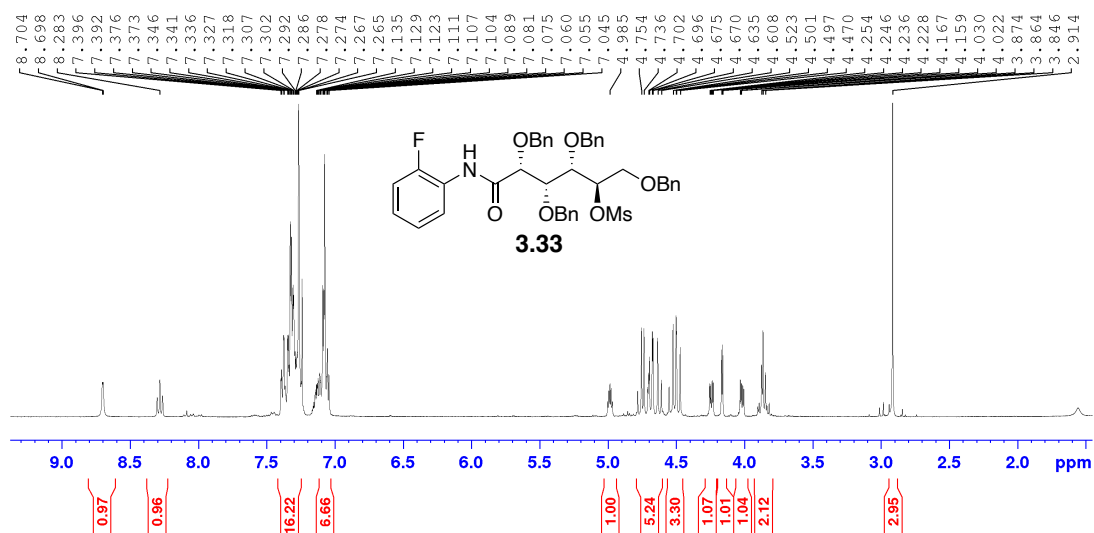
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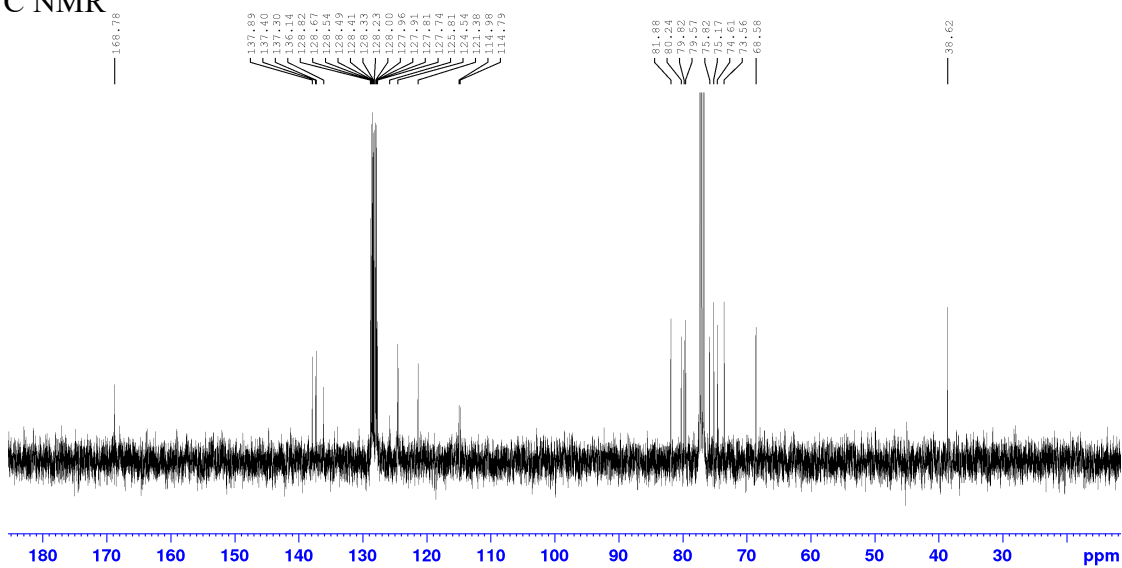
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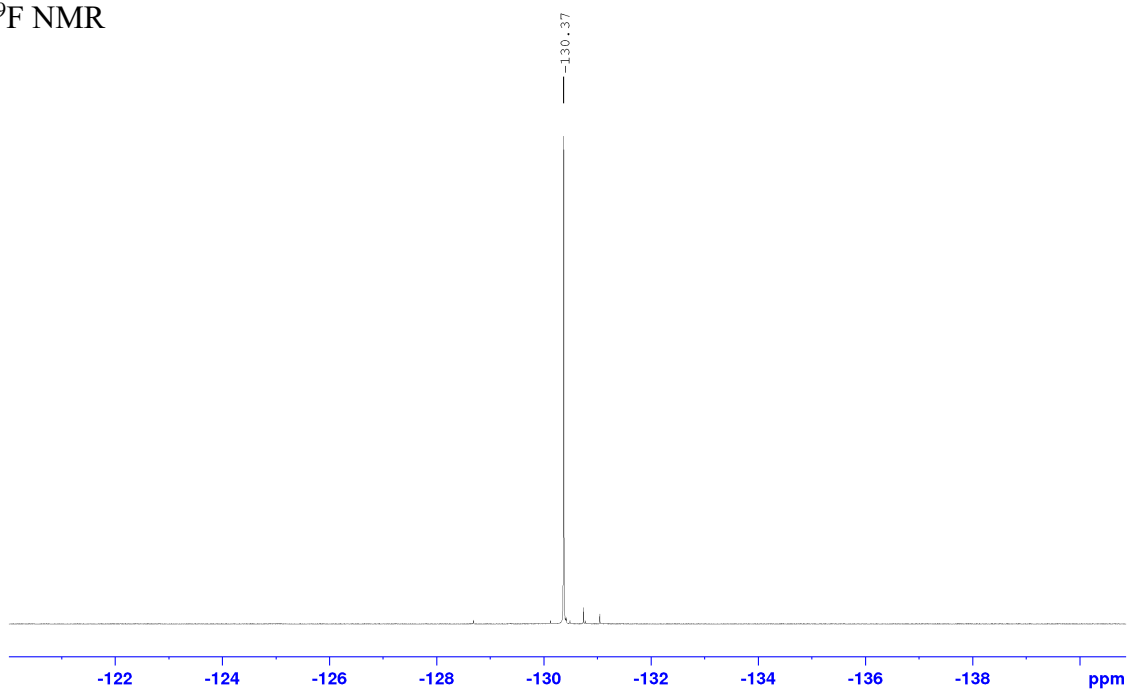
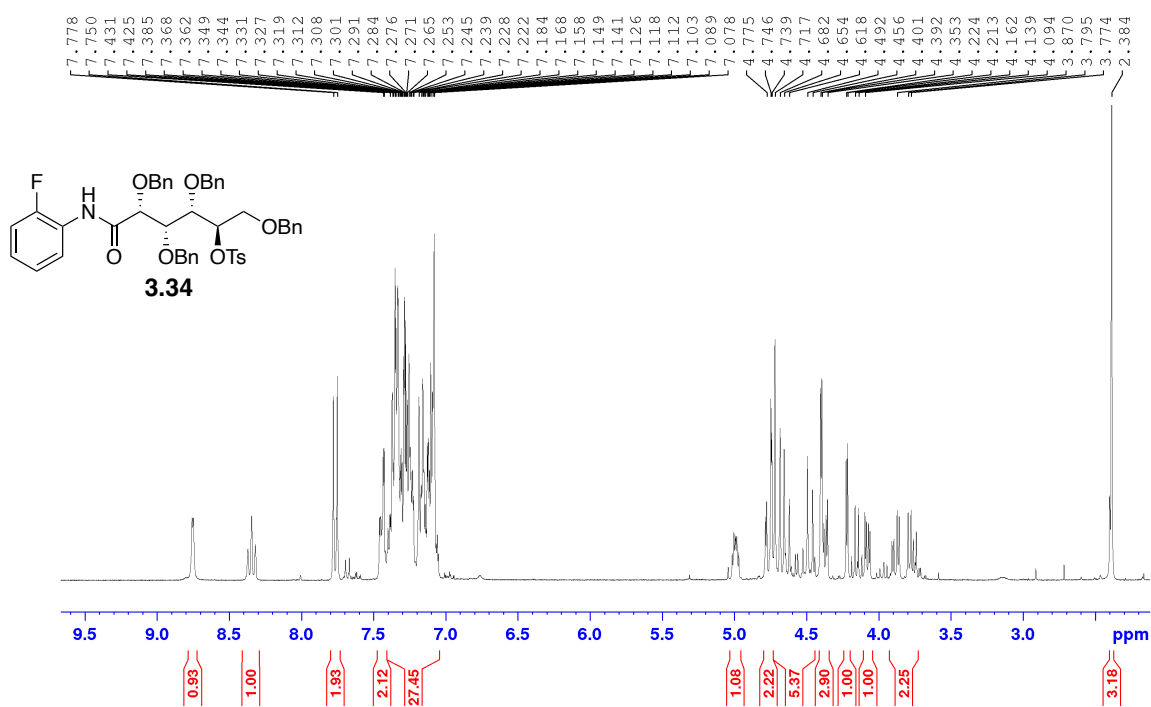


^1H NMR

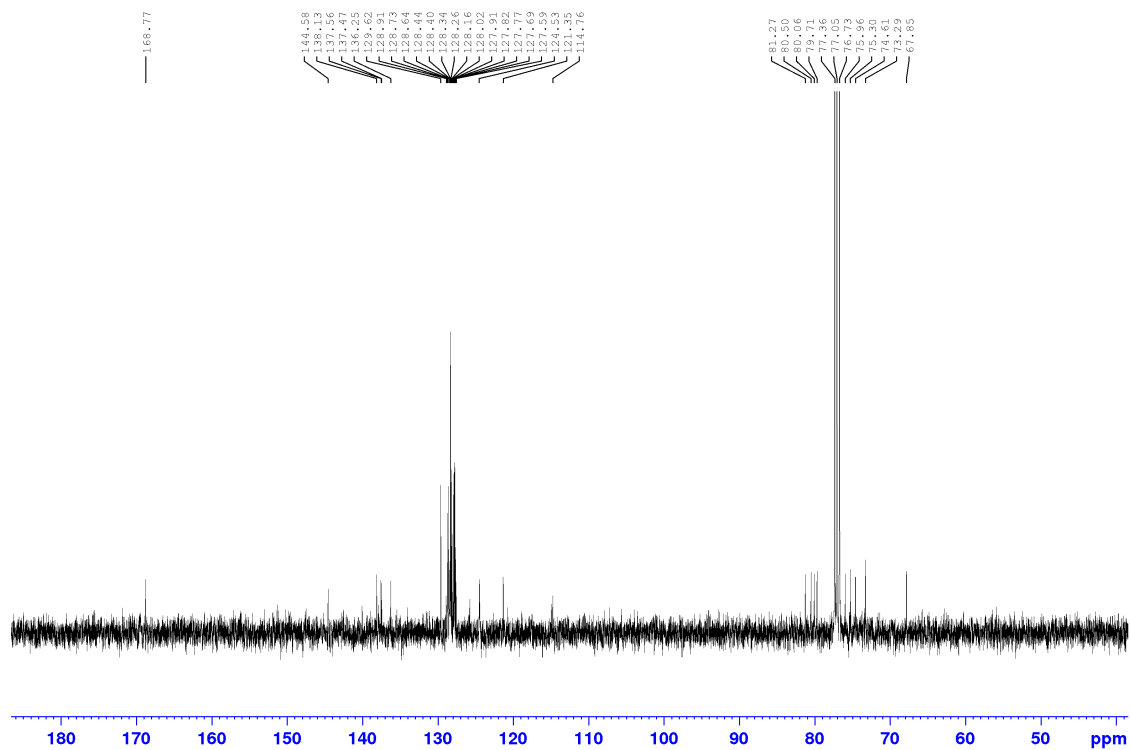


¹³C NMR

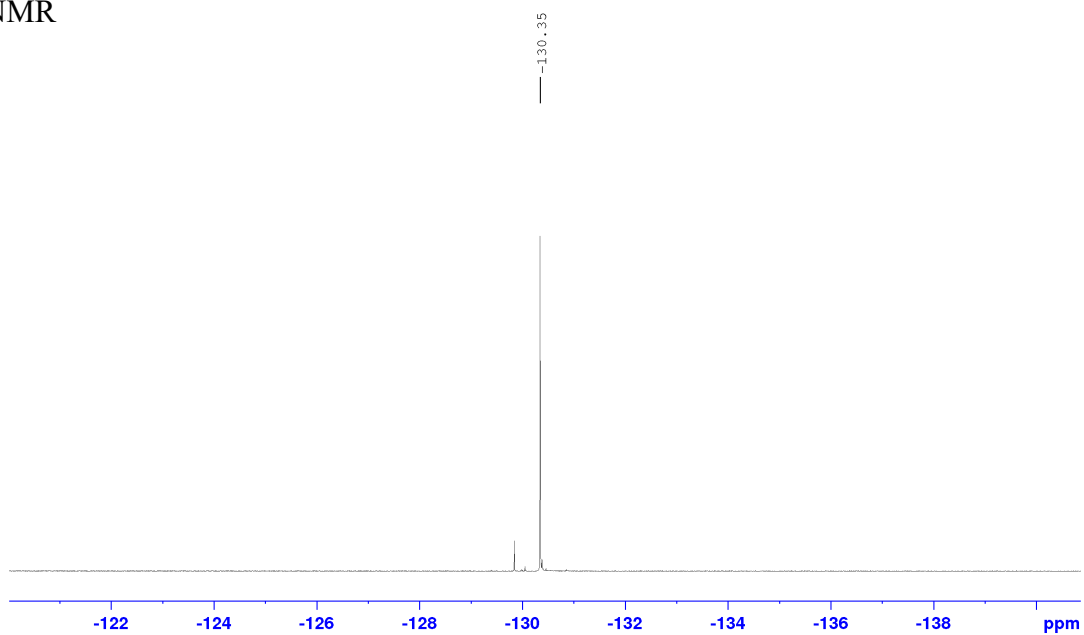


¹⁹F NMR¹H NMR

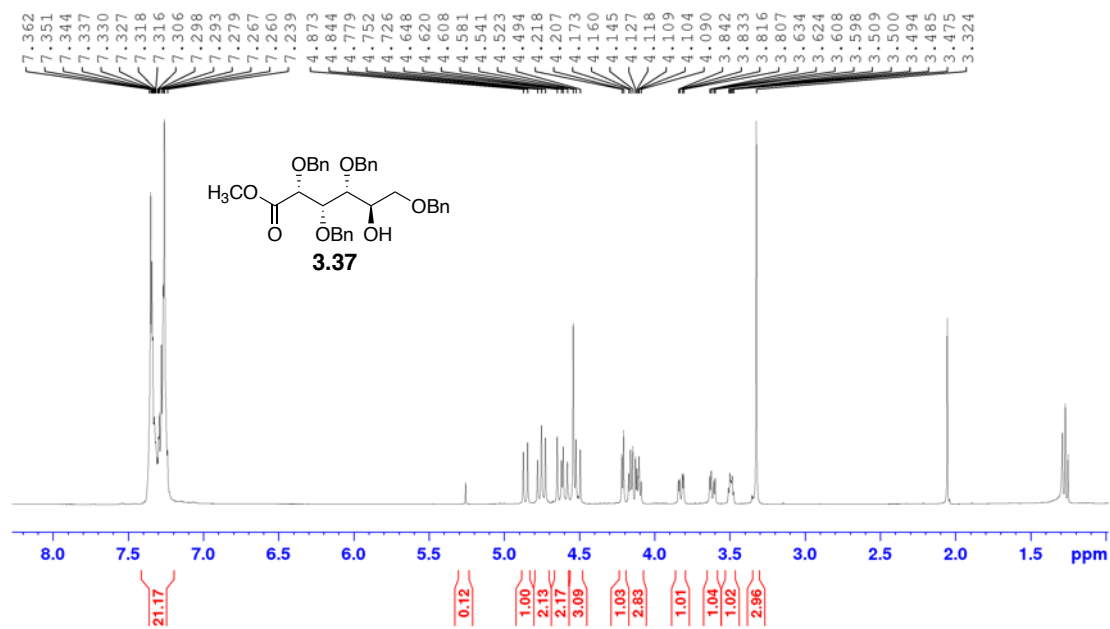
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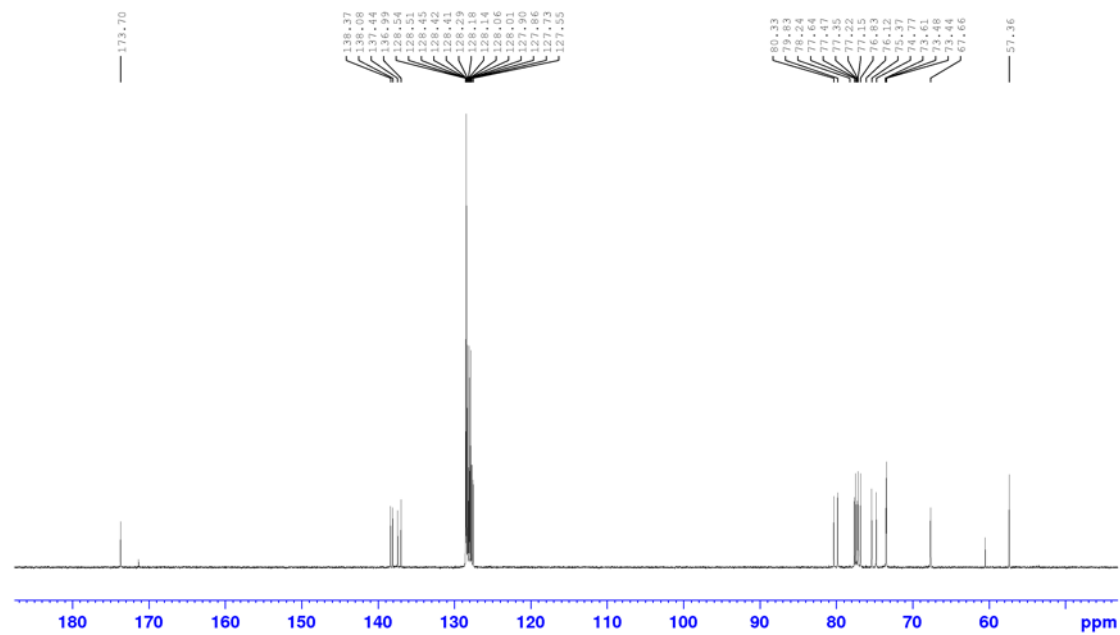
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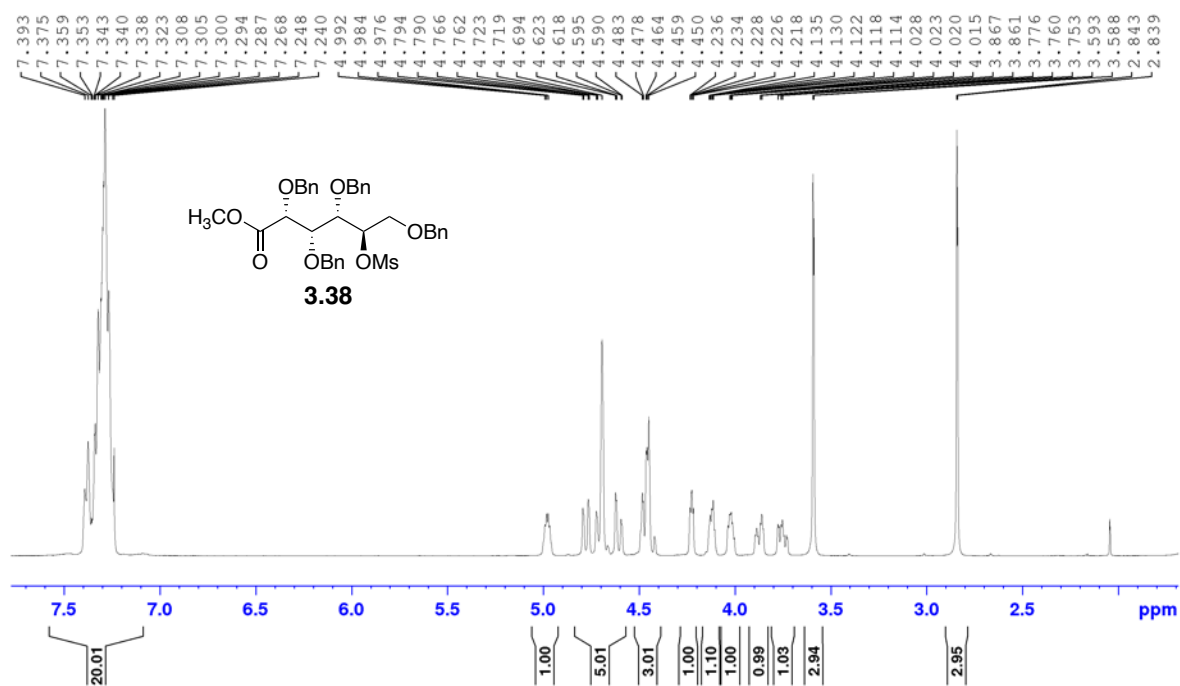
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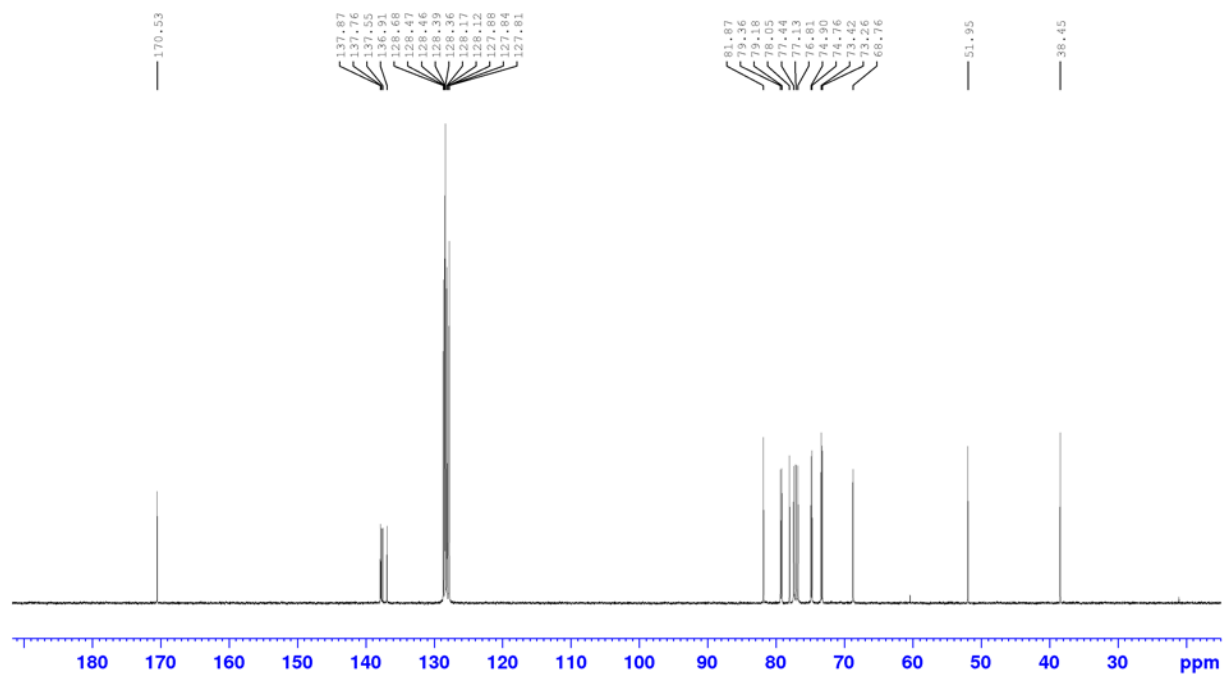
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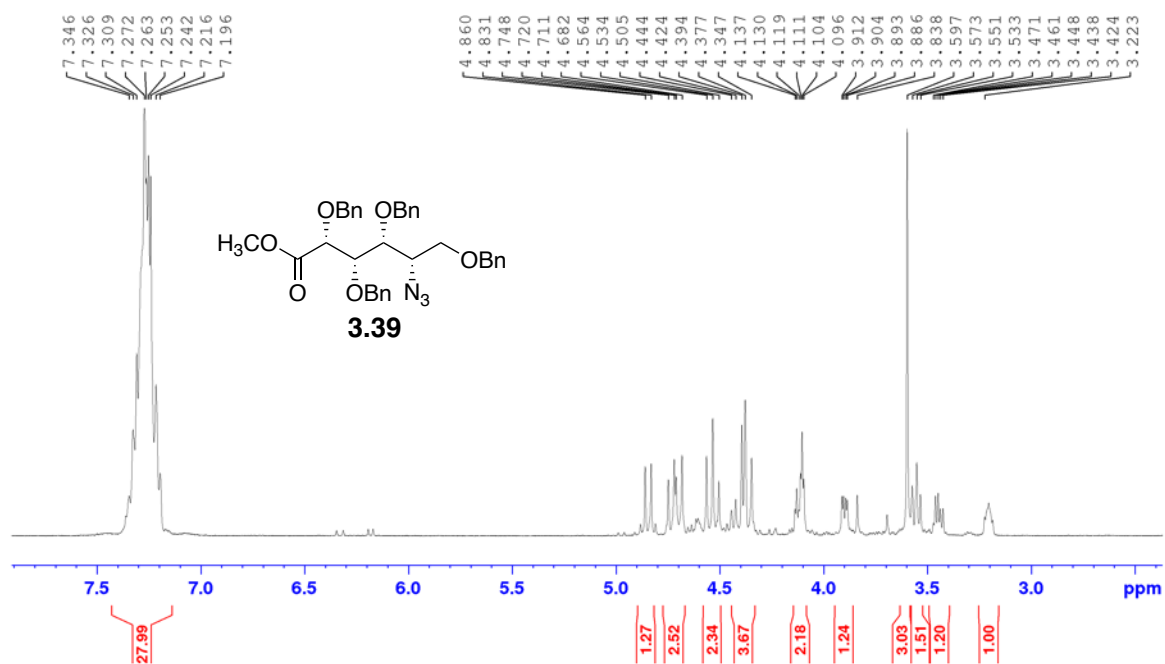
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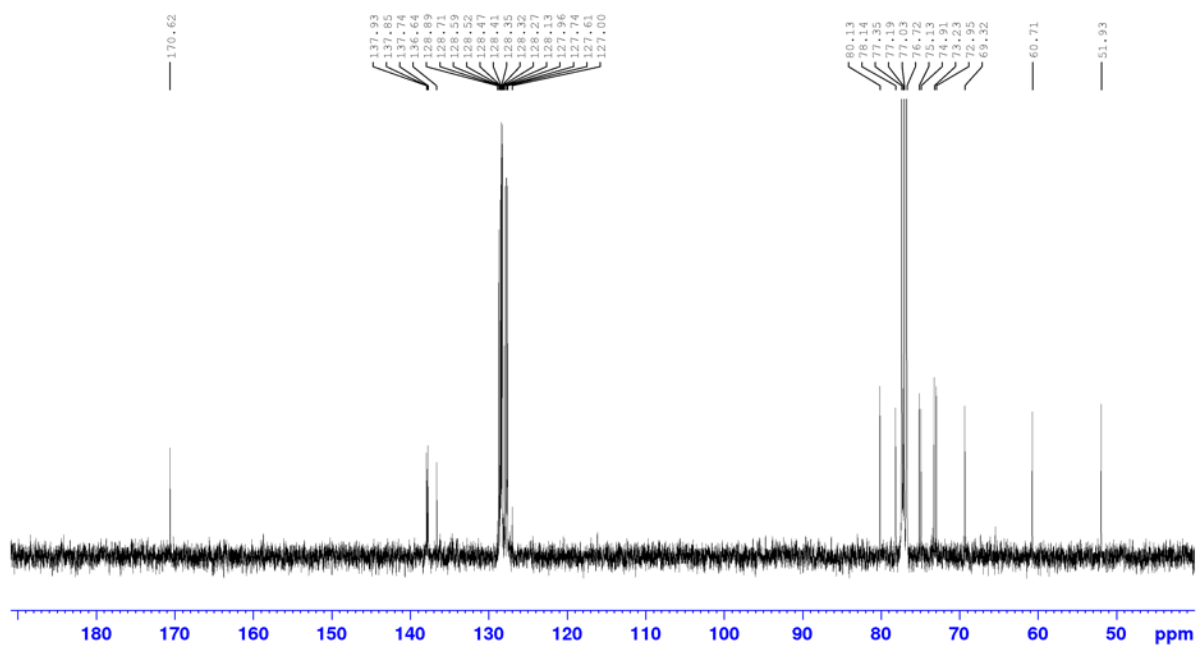
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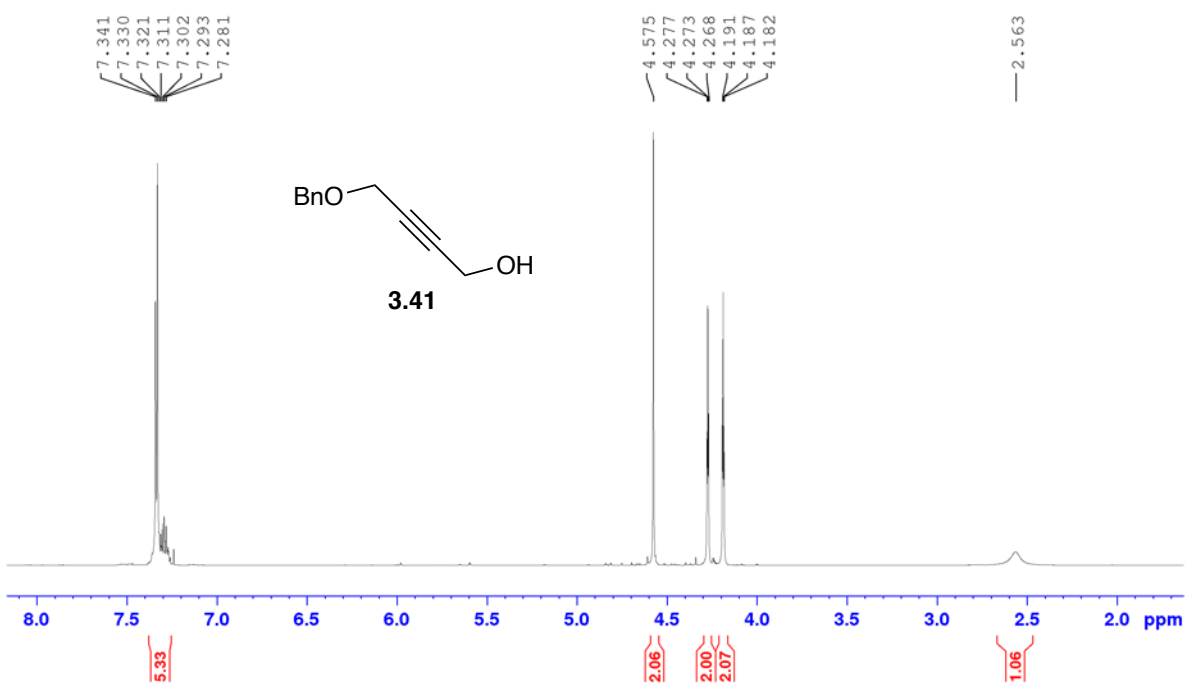
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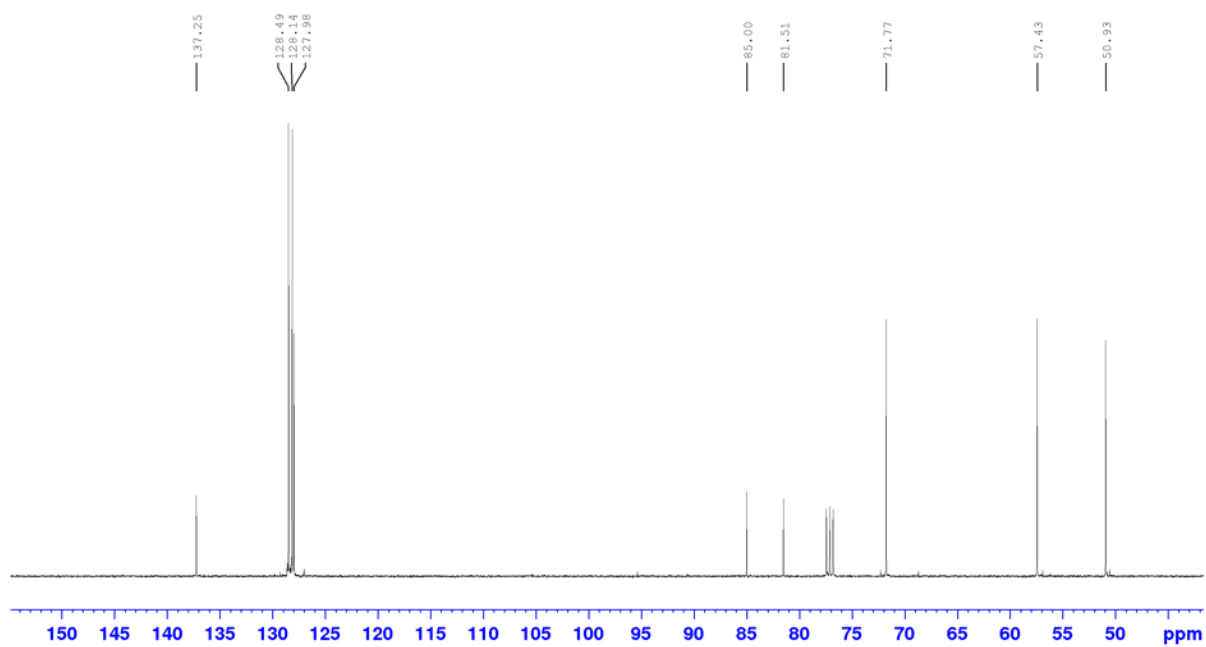
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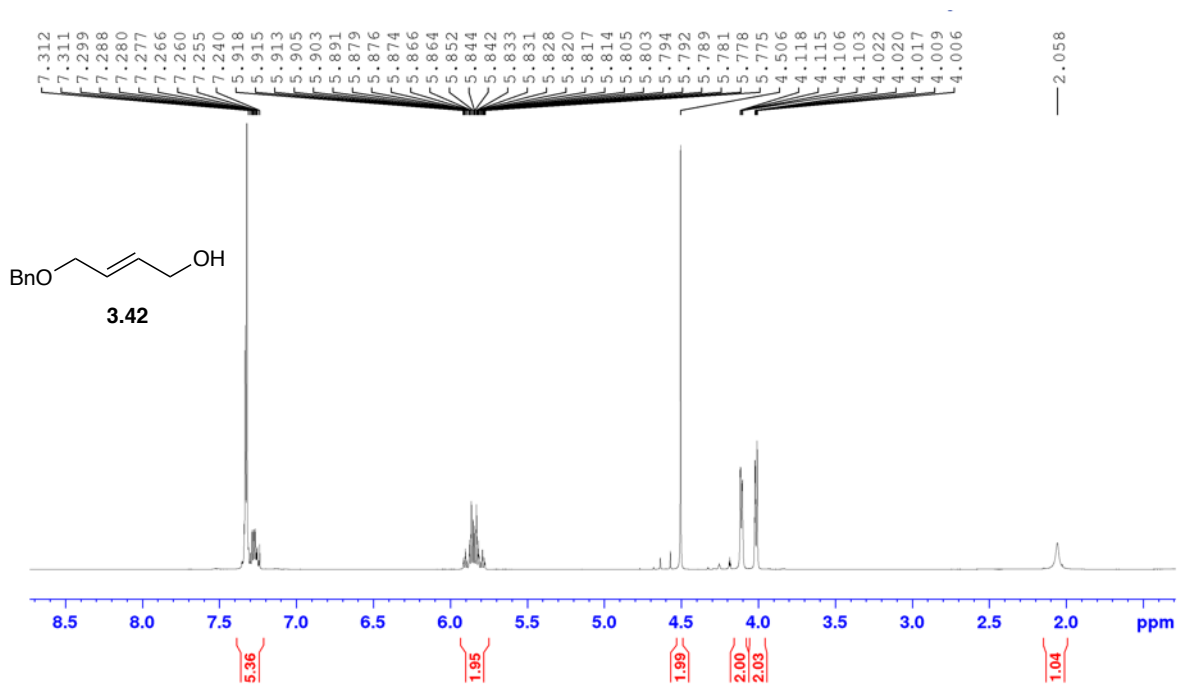
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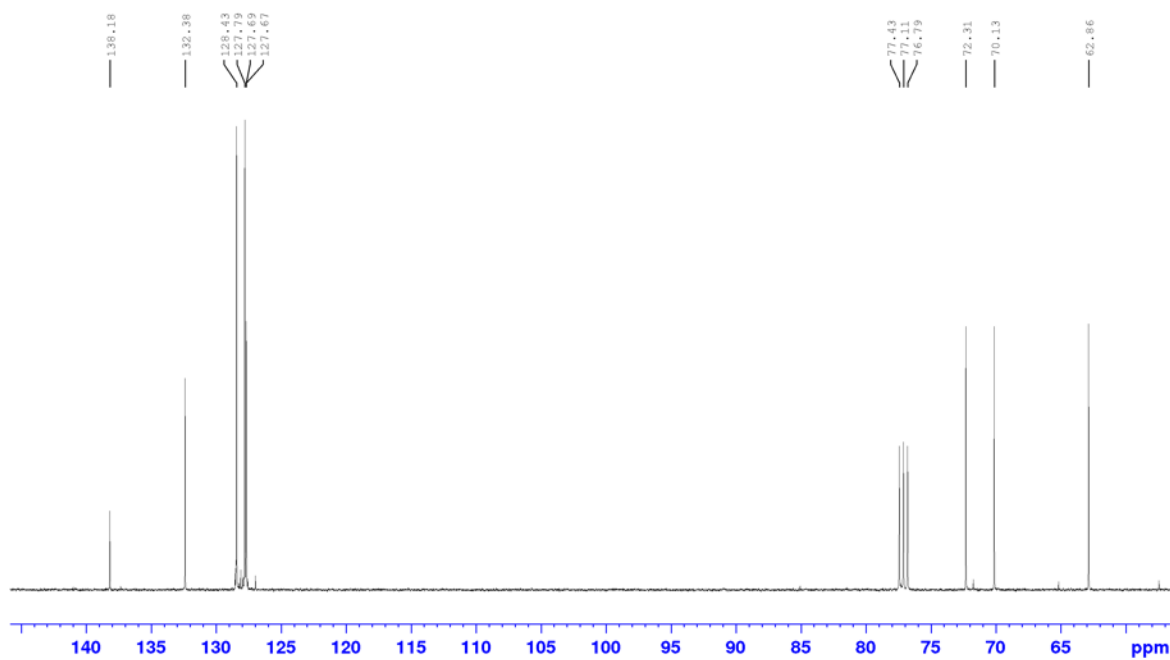
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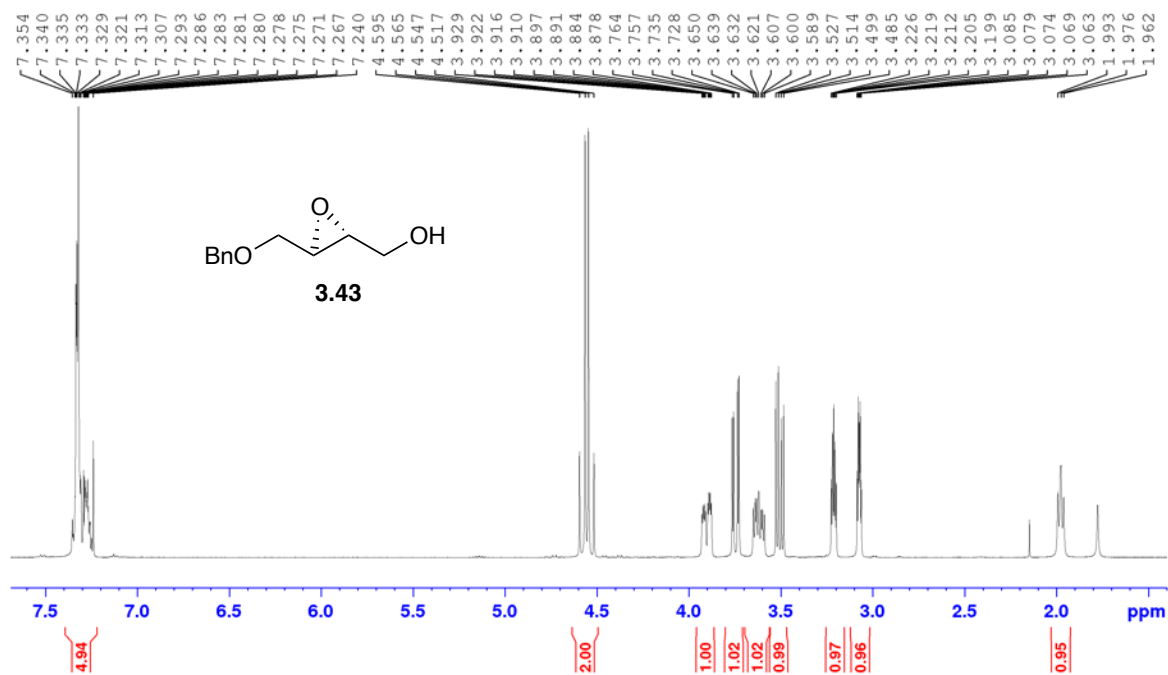
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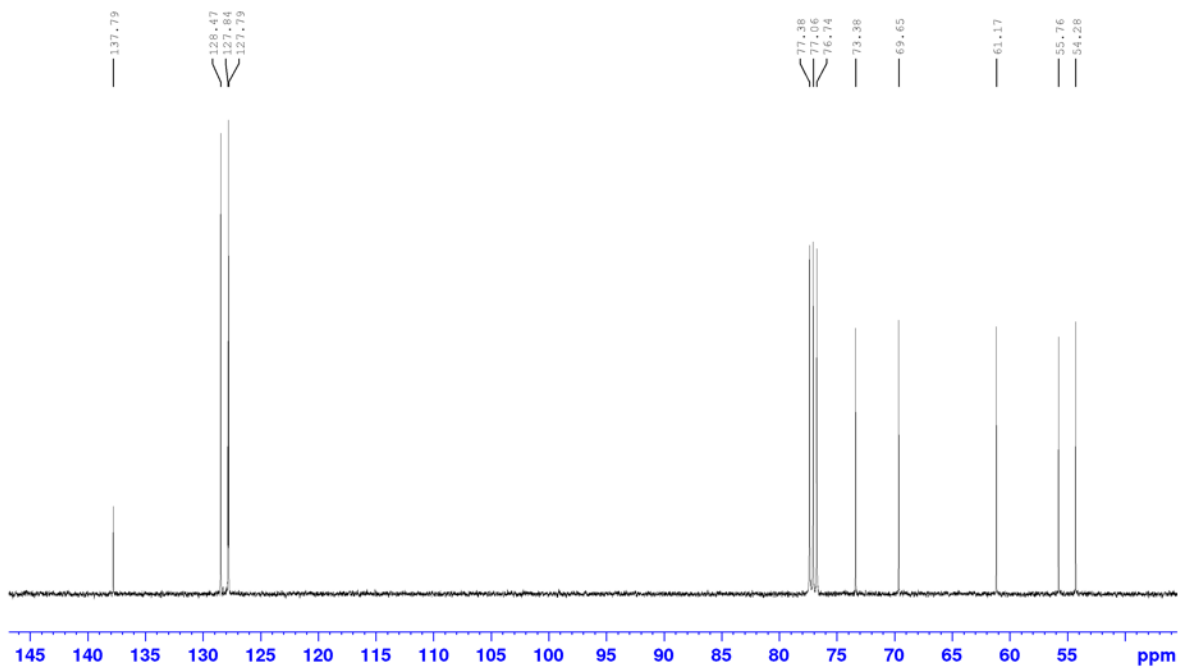
¹³C NMR



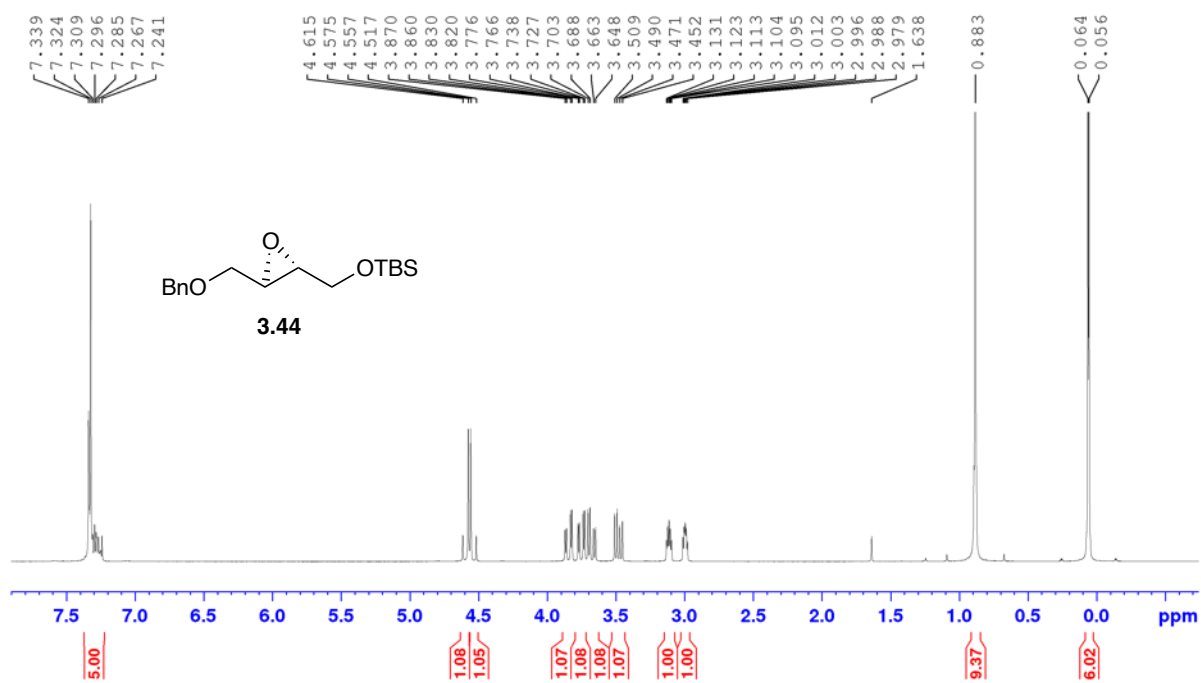
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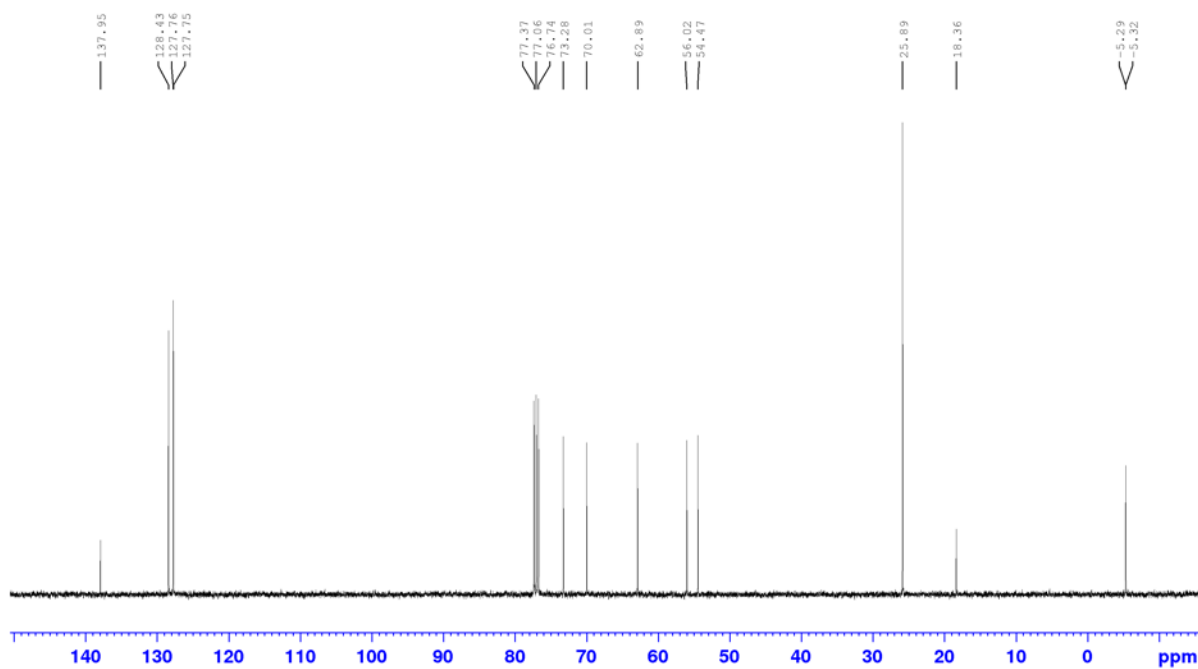
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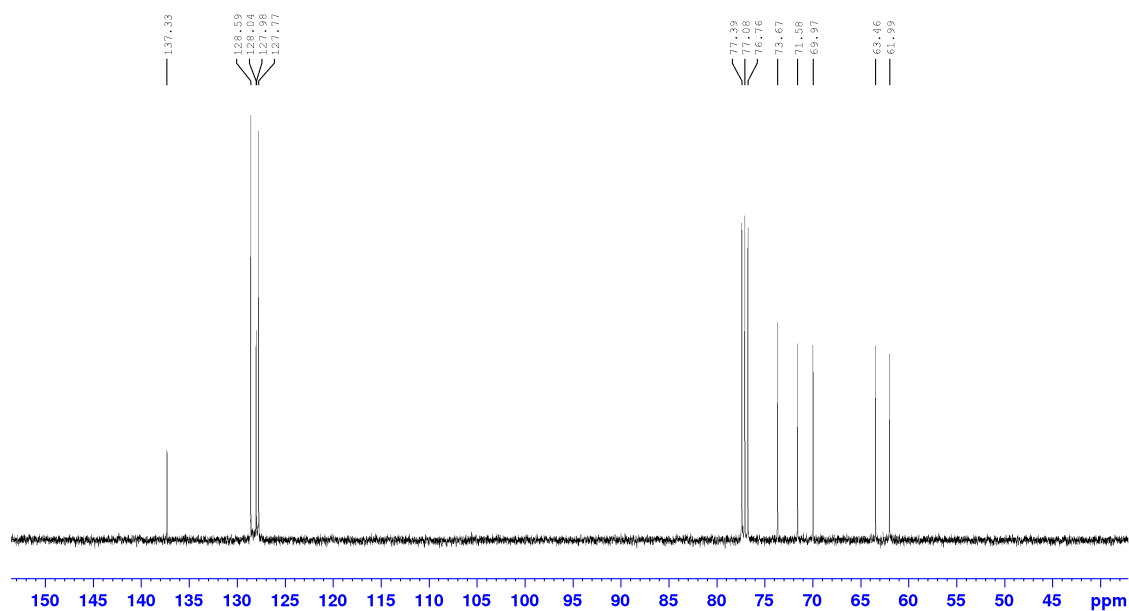


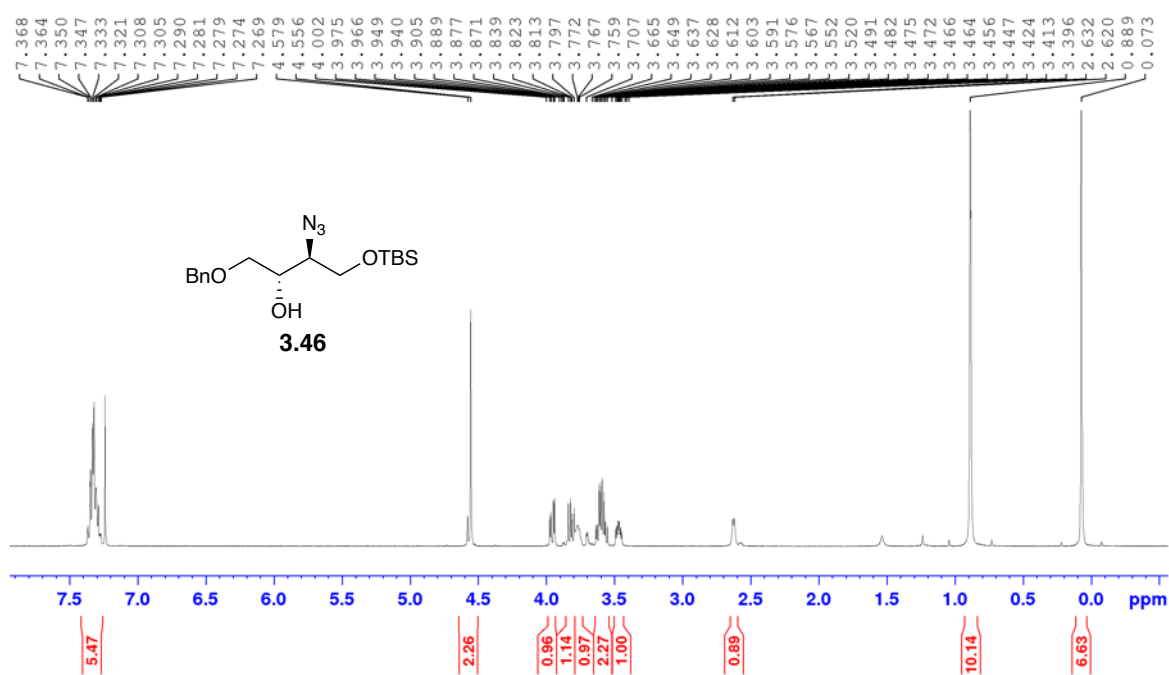
^1H NMR



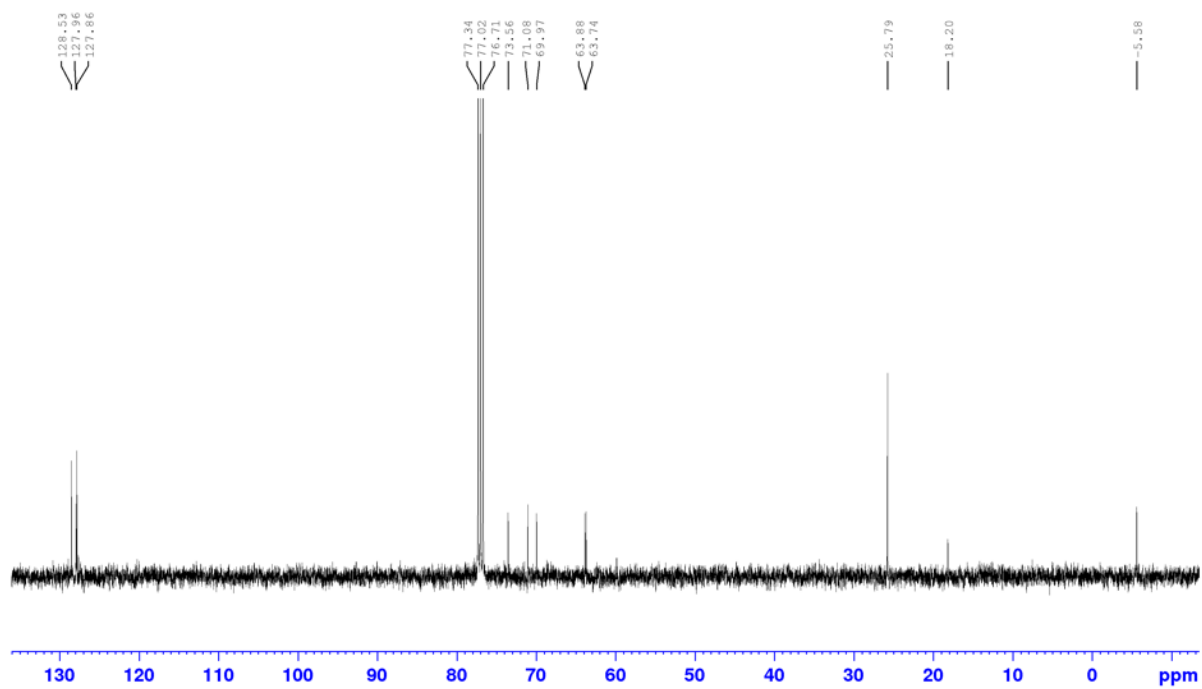
¹³C NMR



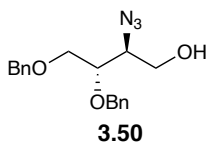
¹H NMR¹H NMR



¹³C NMR

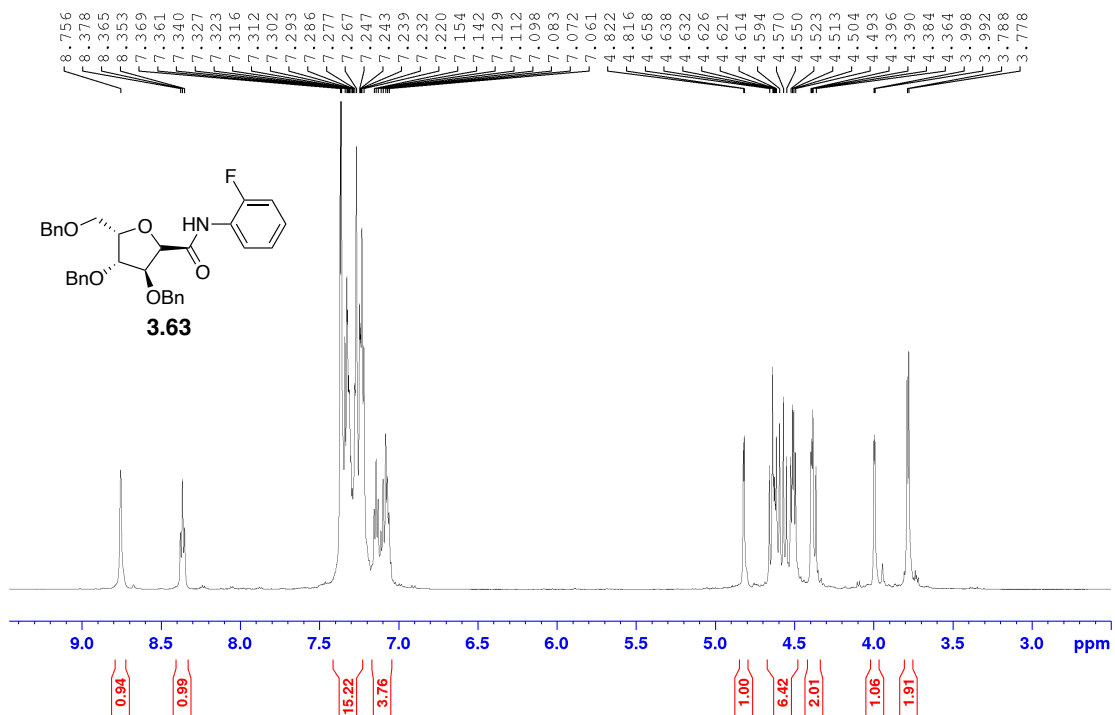


¹H NMR

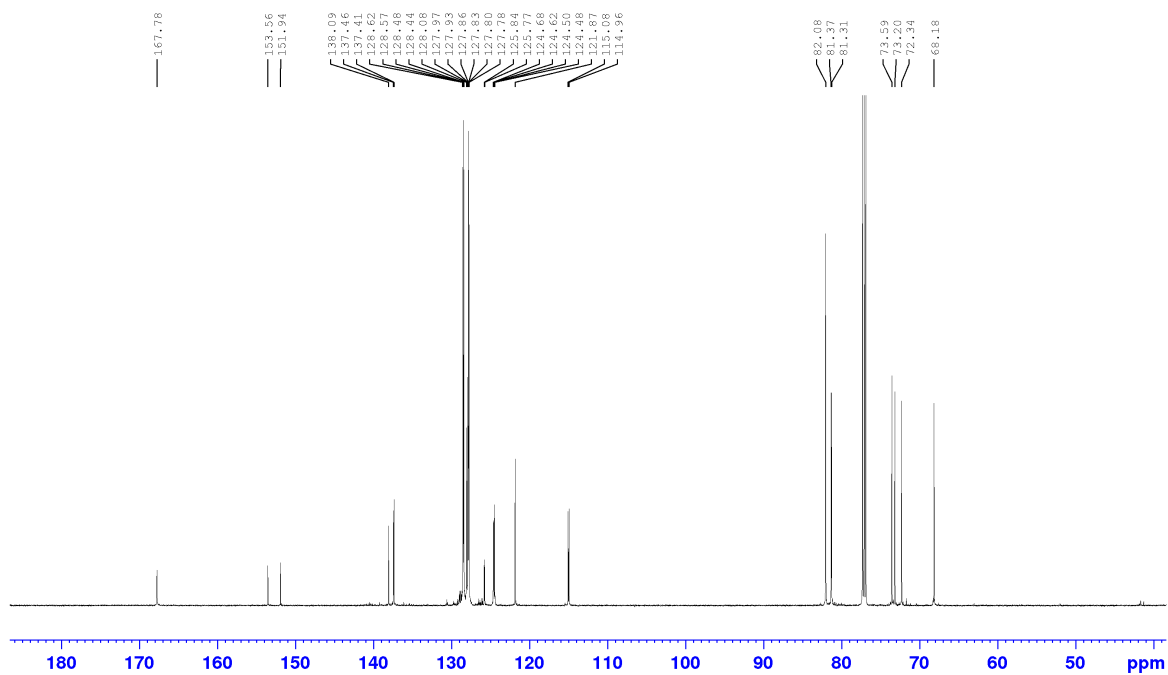


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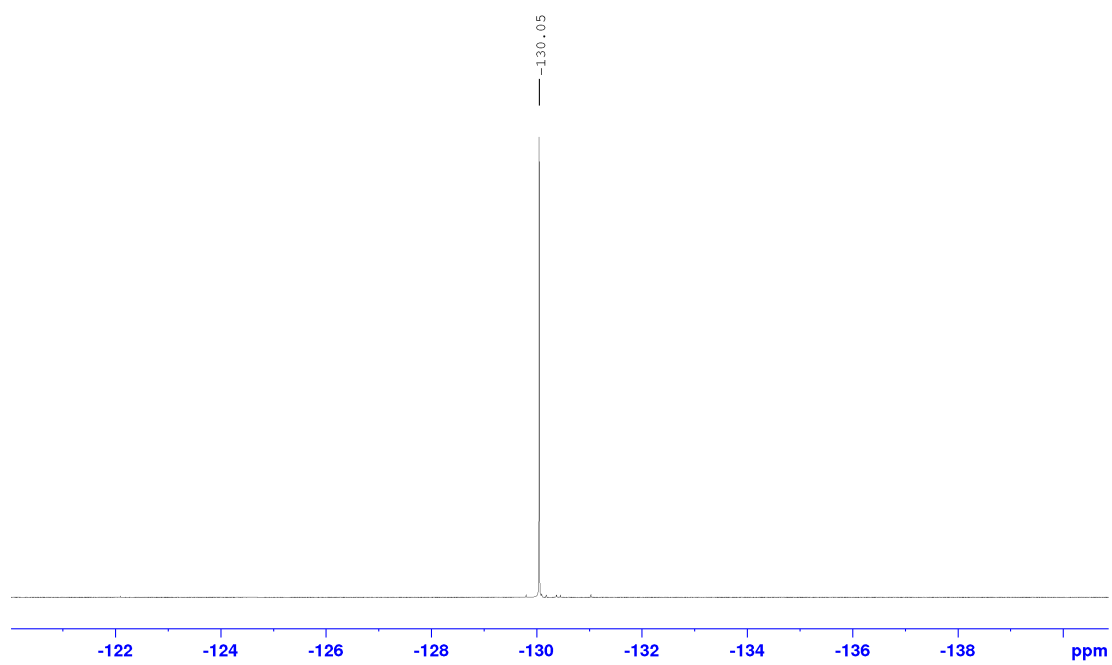
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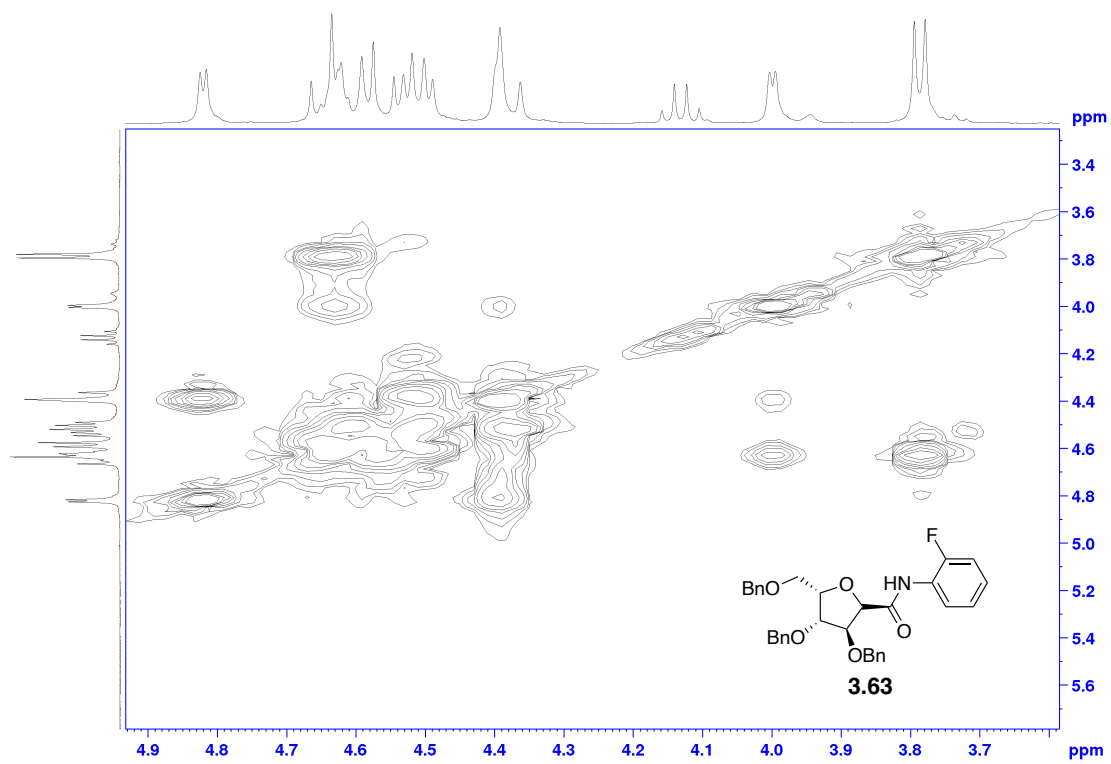
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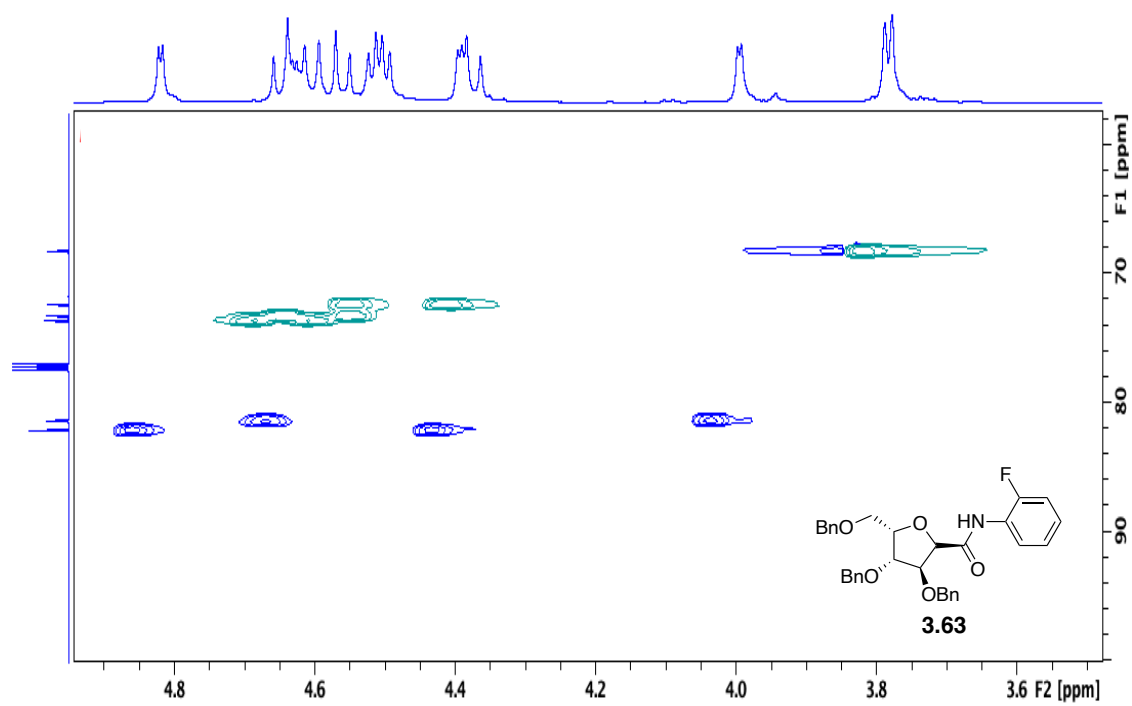
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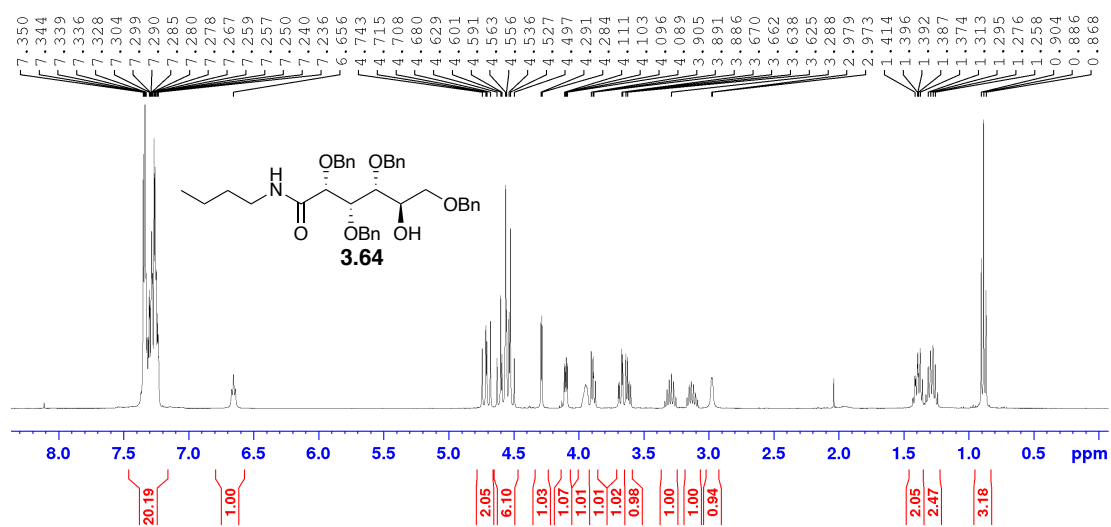
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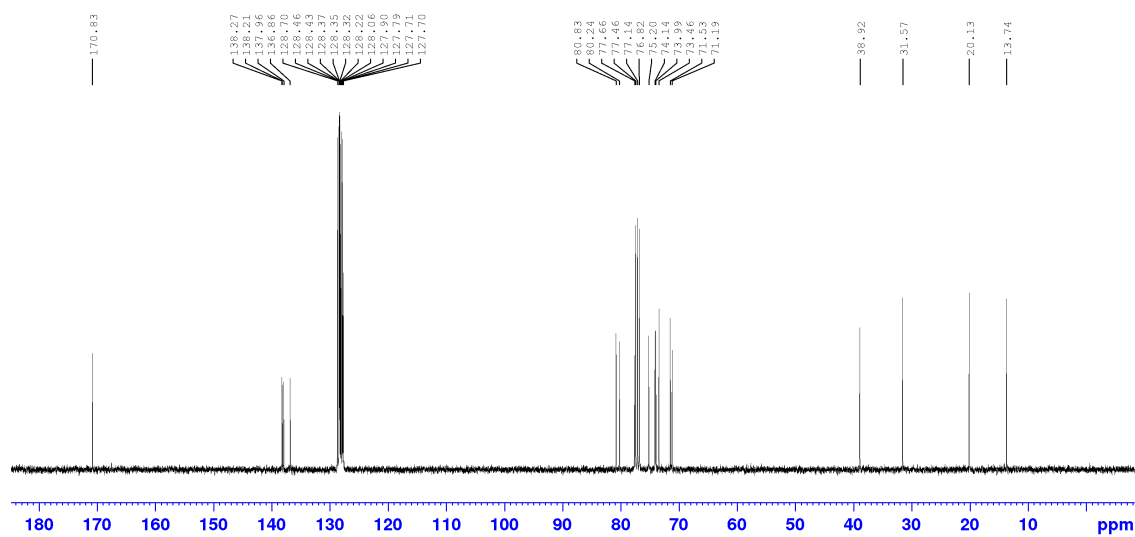
Edited ^1H - ^{13}C HSQC



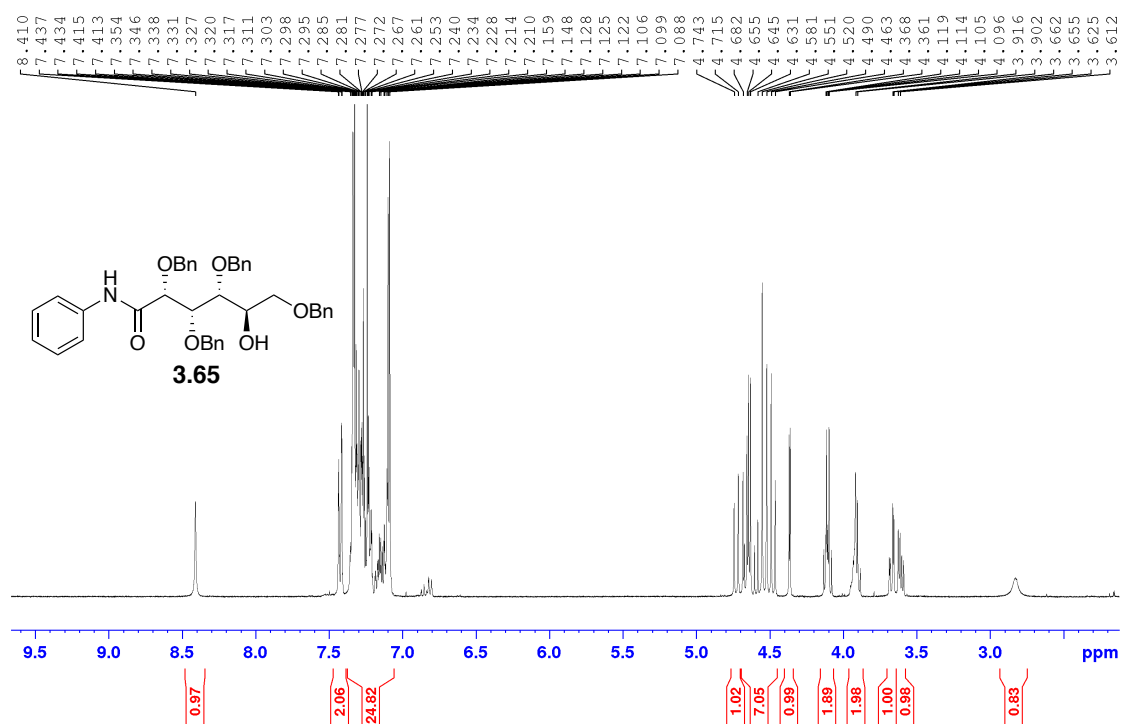
^1H NMR



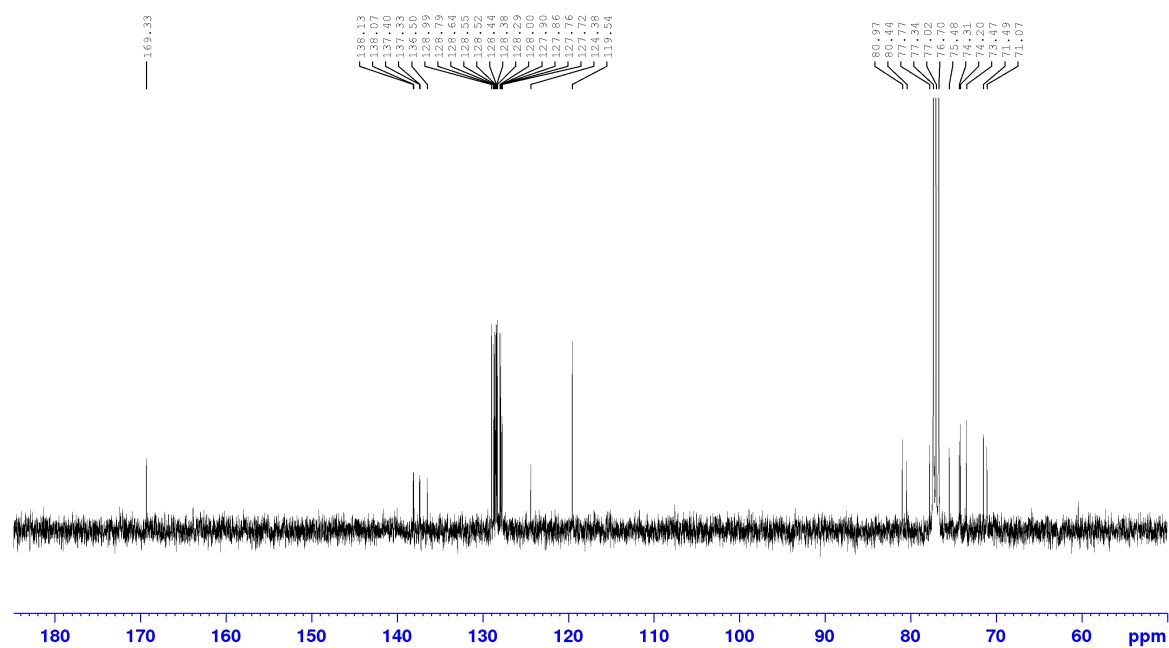
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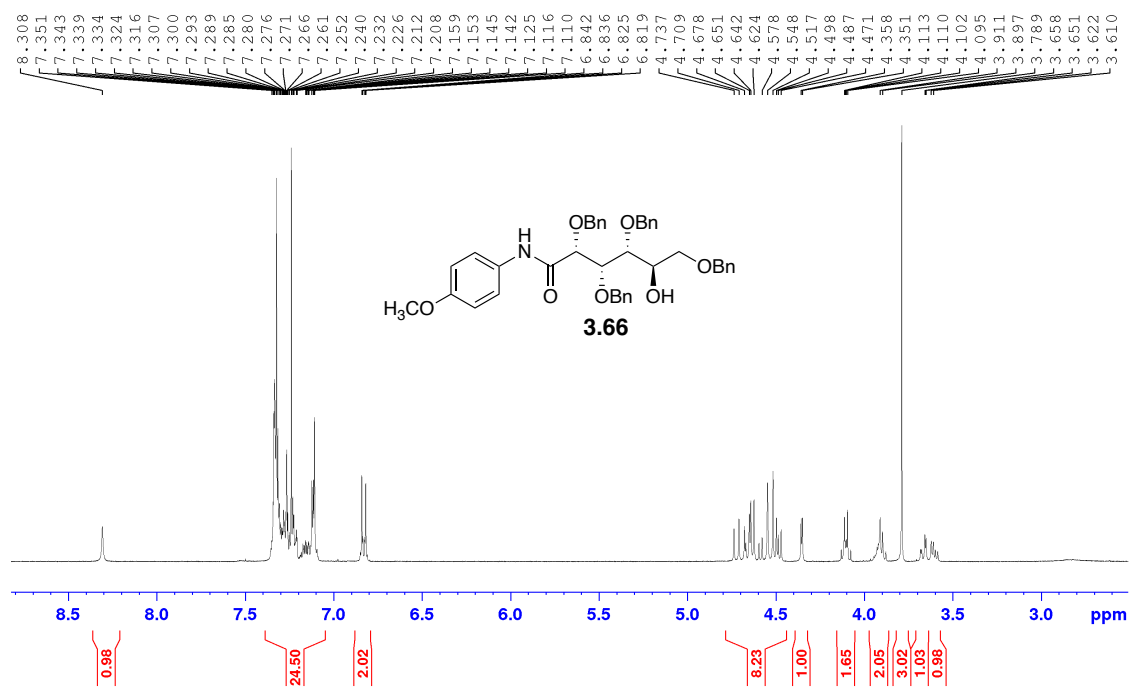
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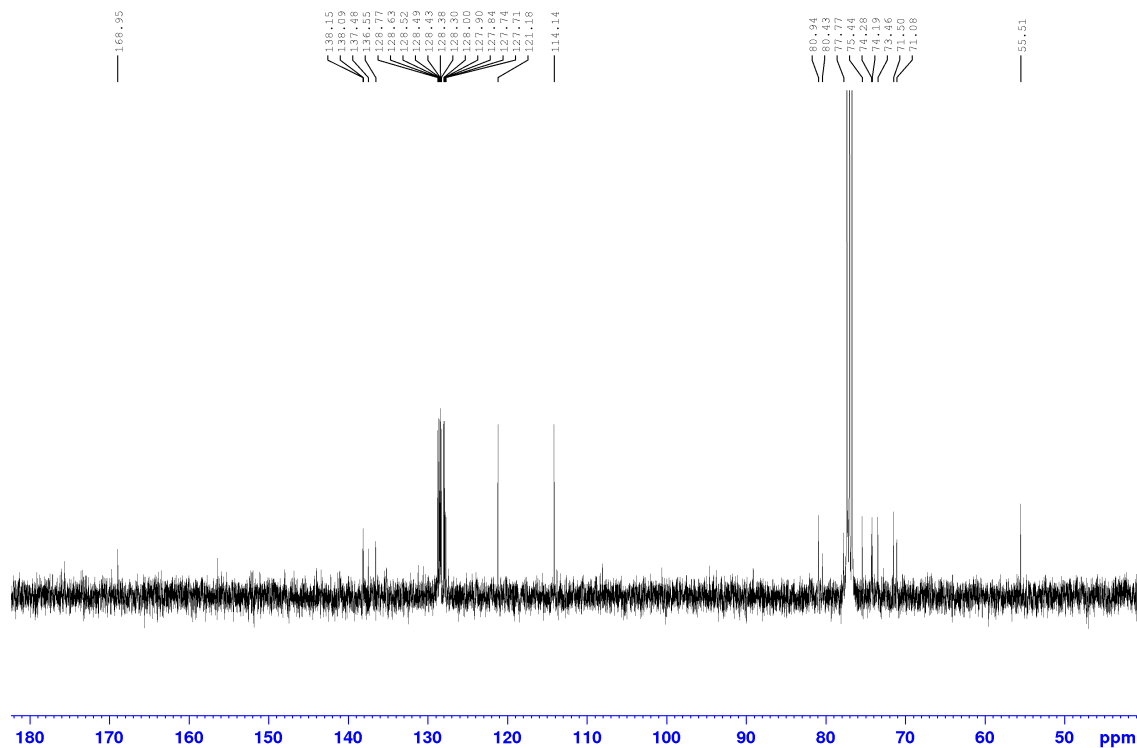
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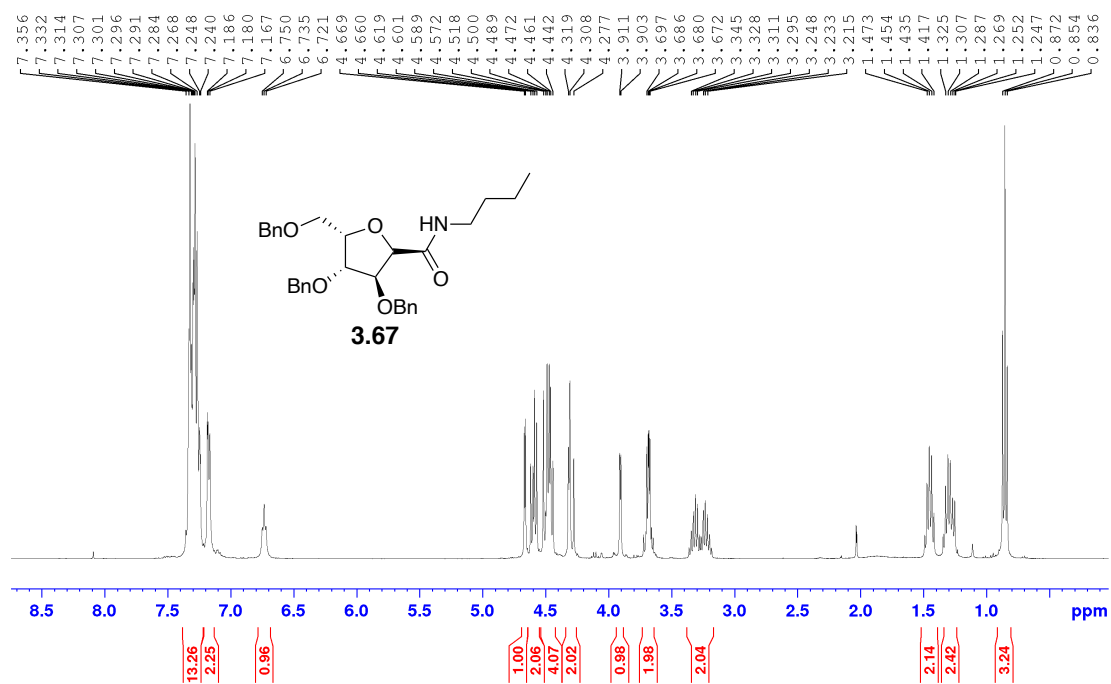
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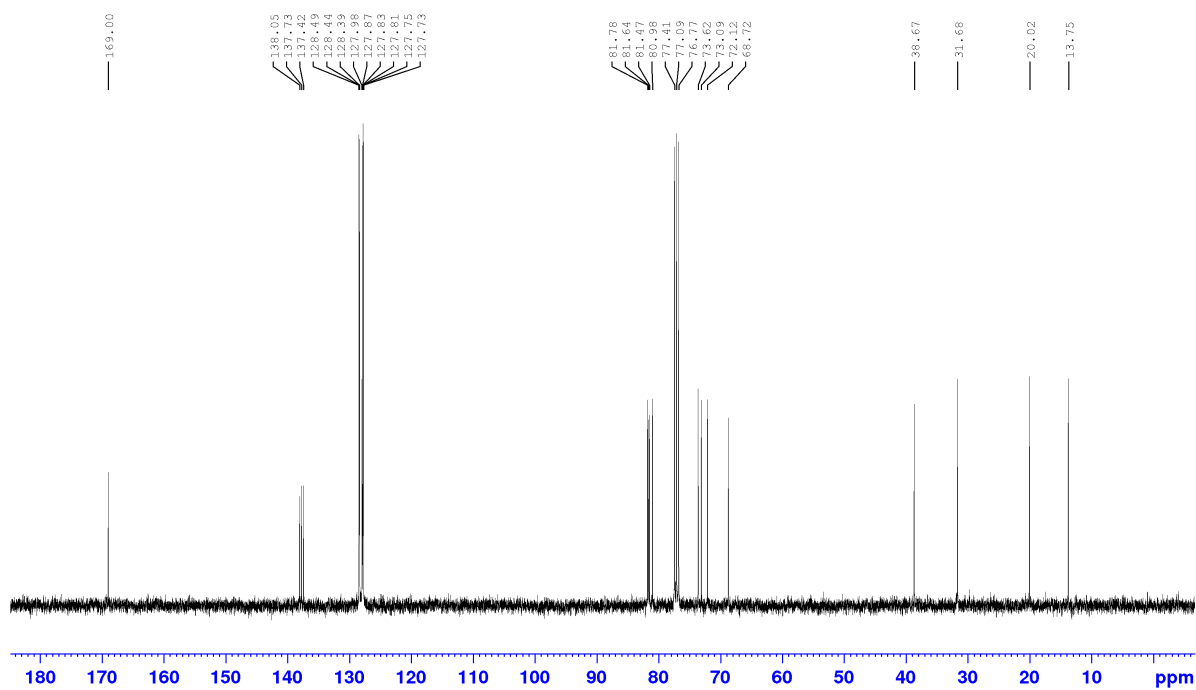
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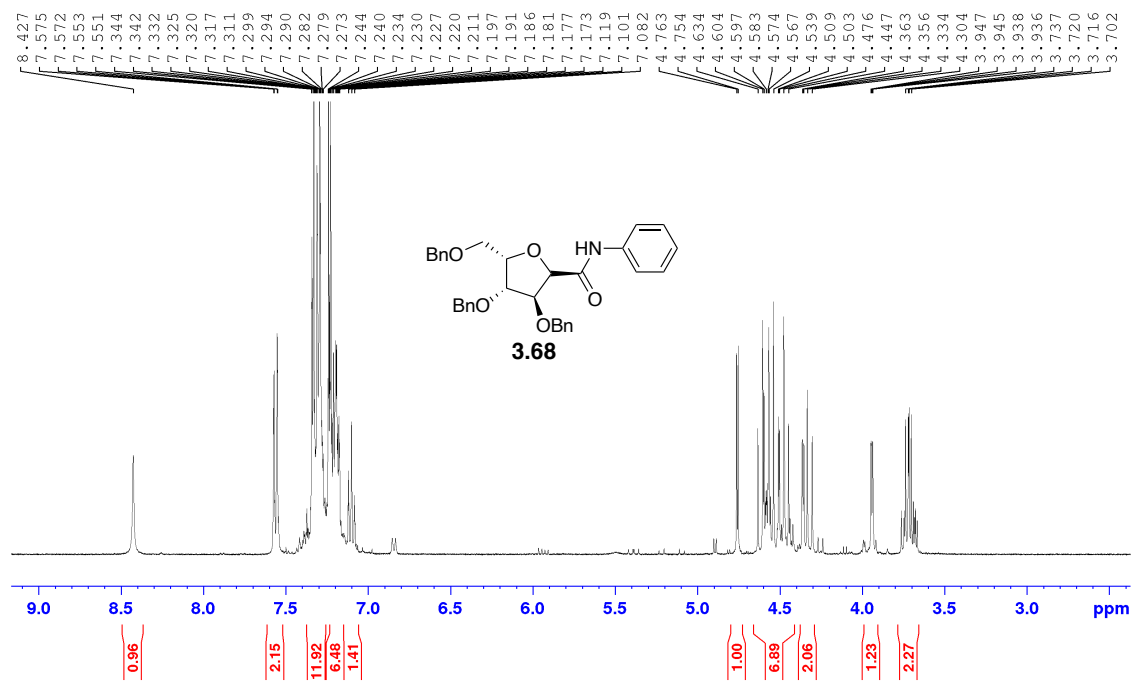
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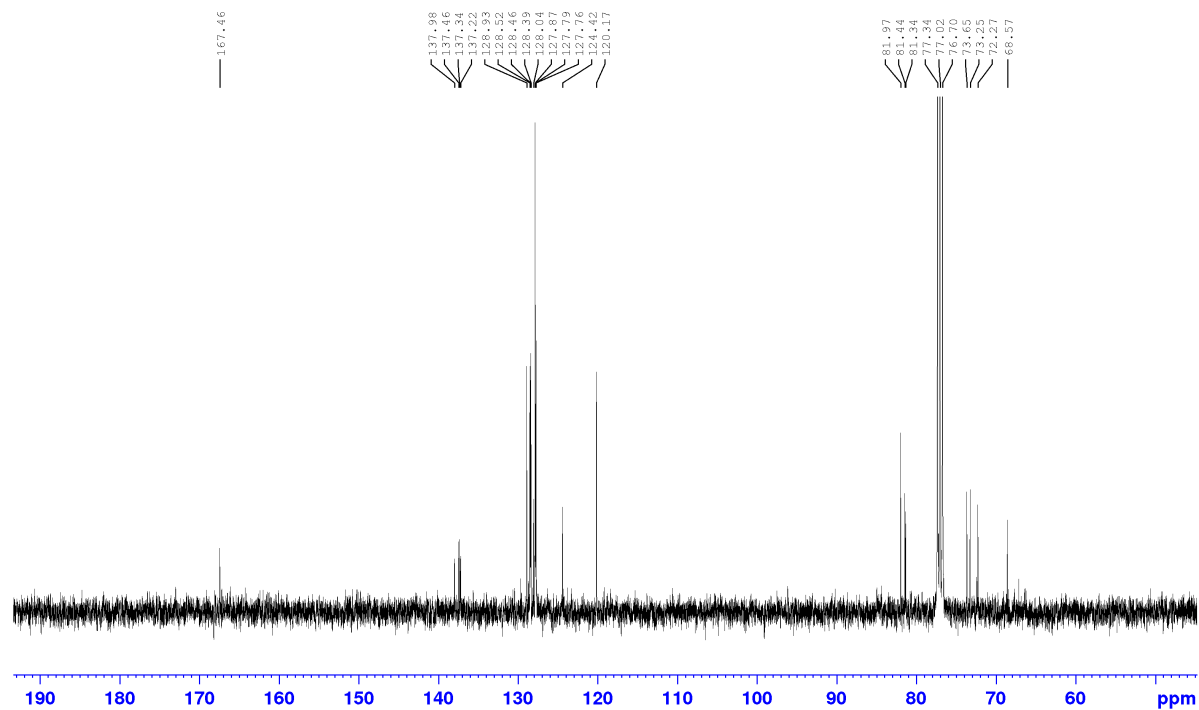
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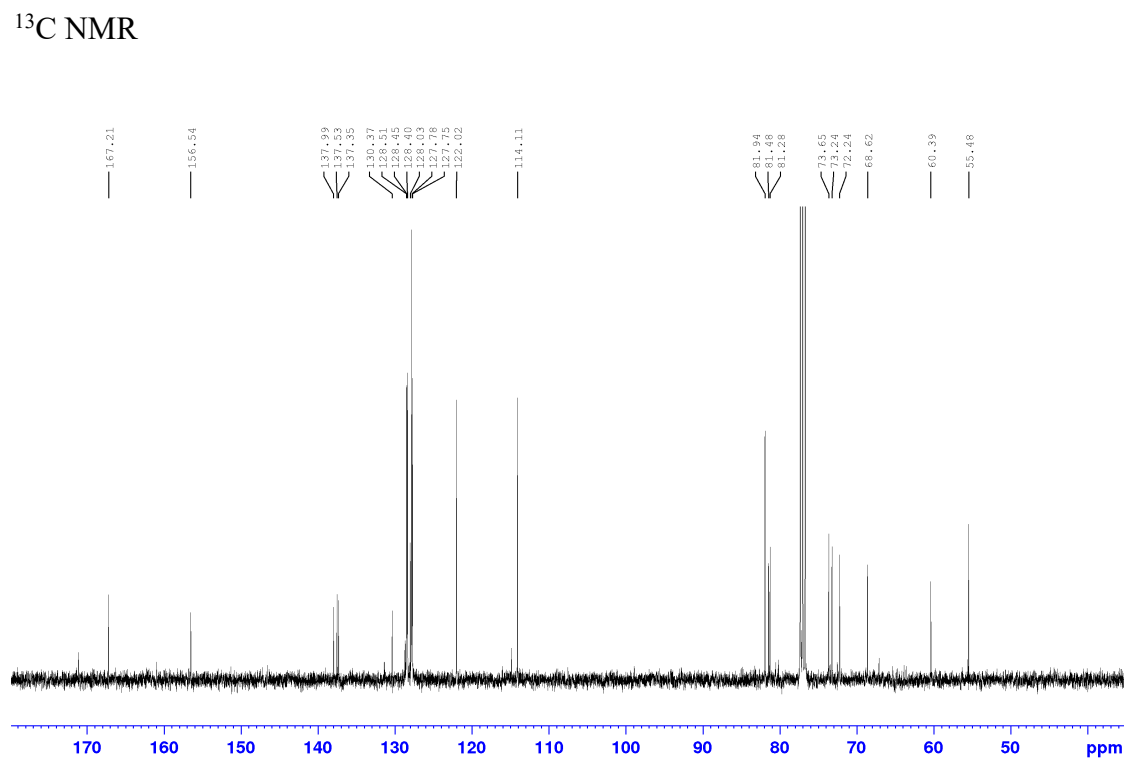
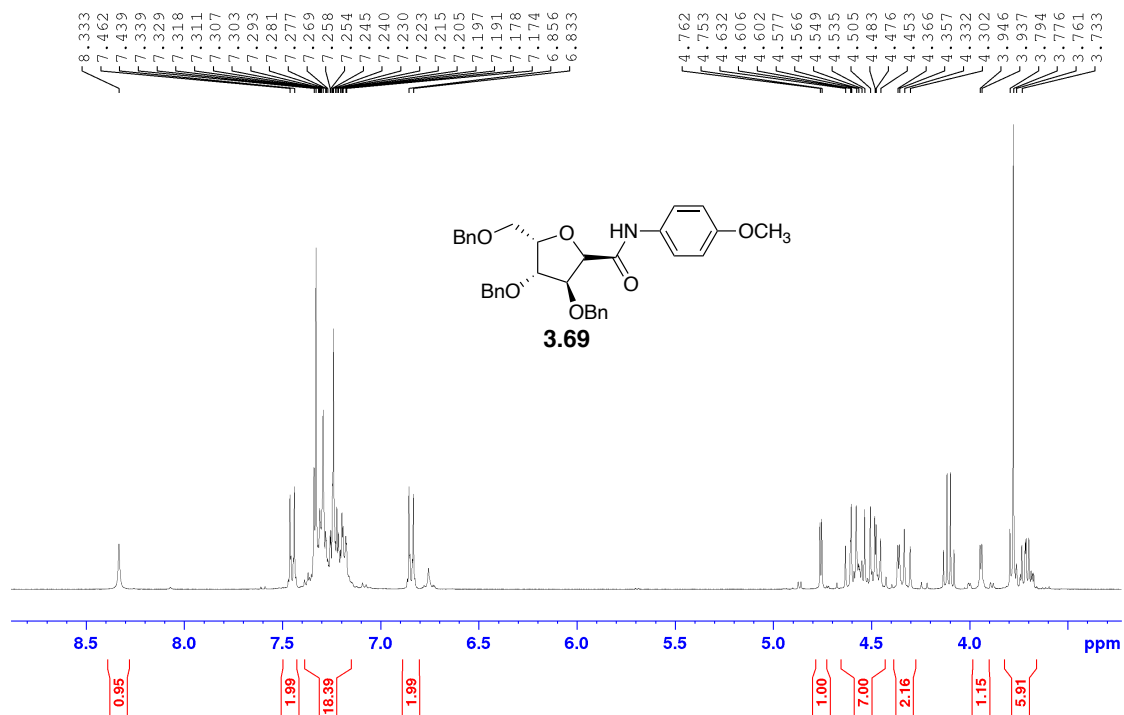
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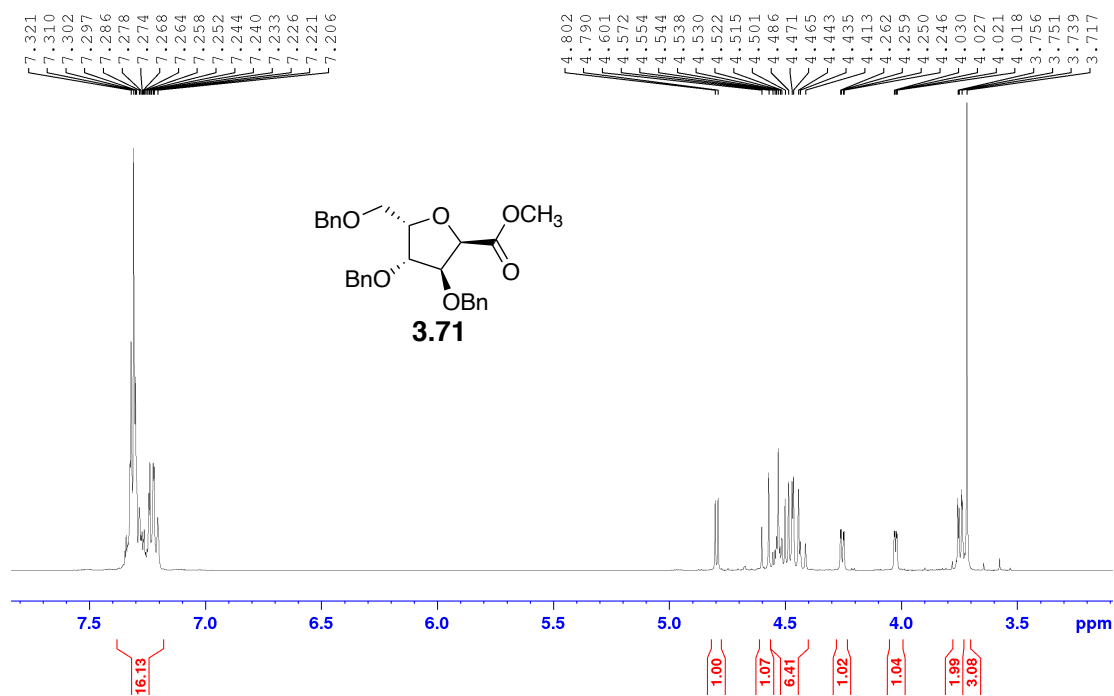
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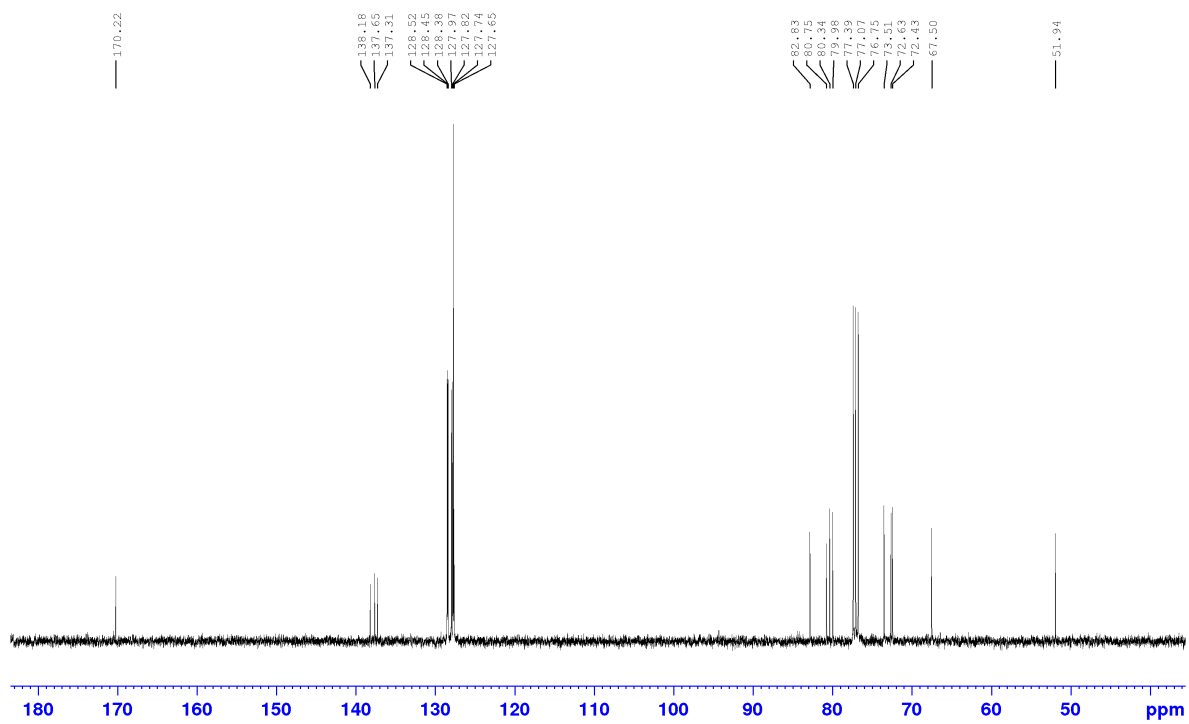
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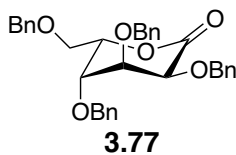
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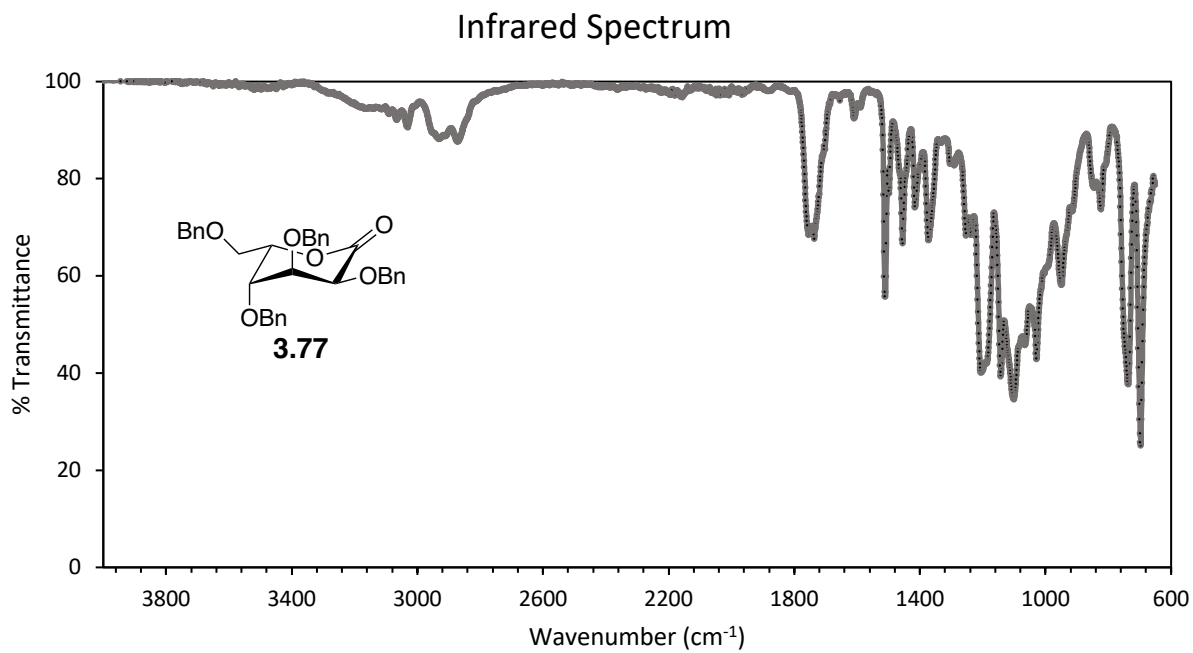


¹³C NMR

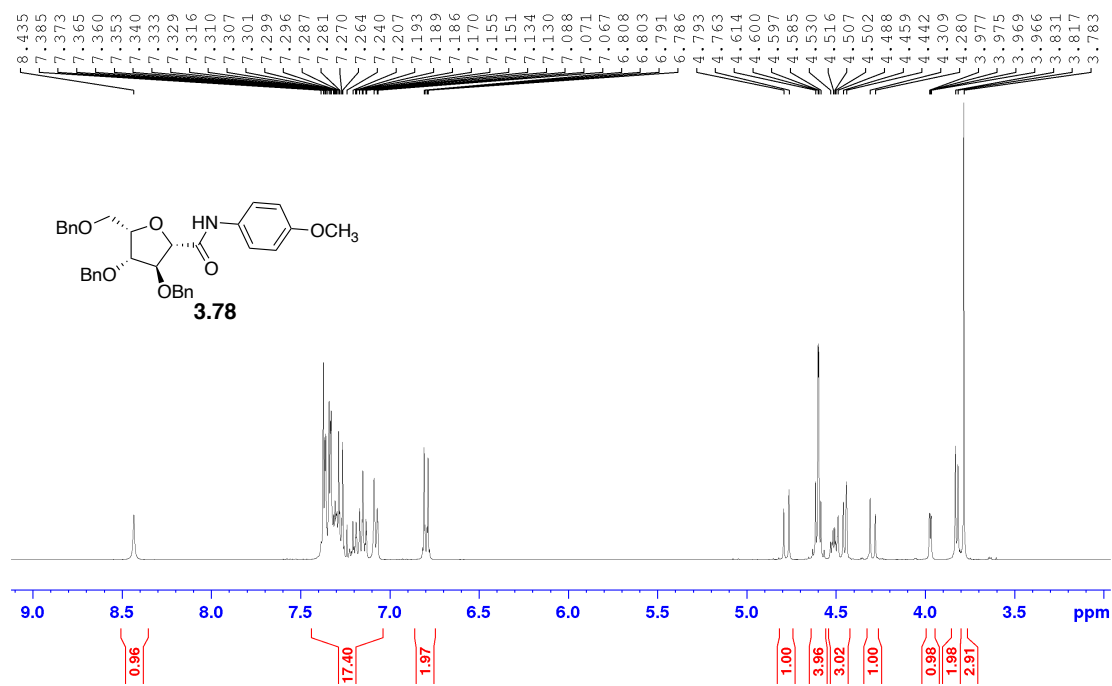


¹H NMR

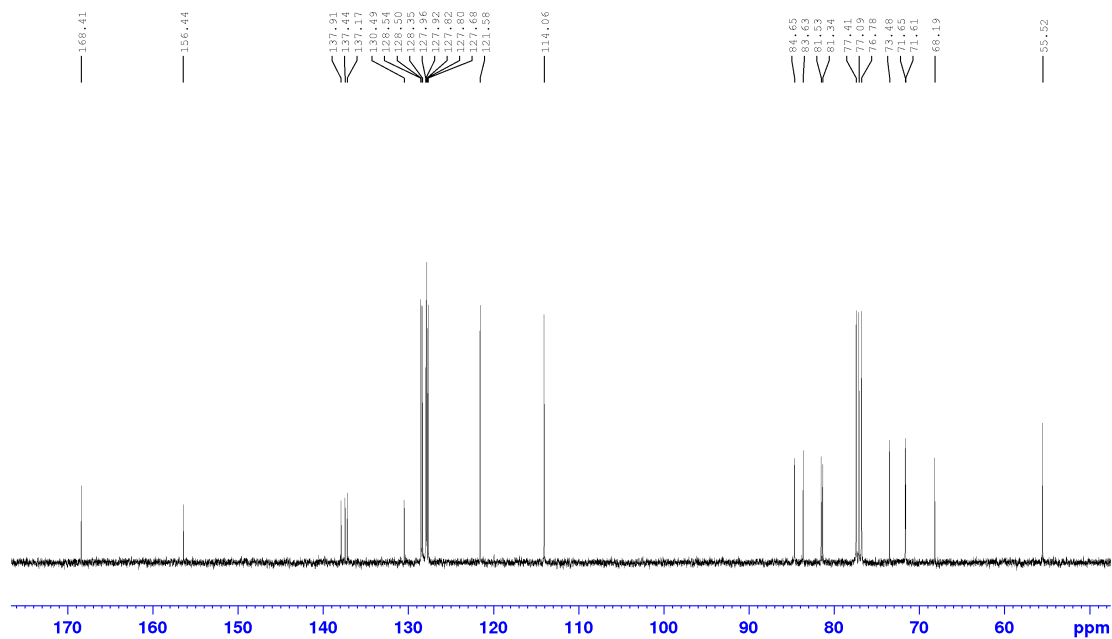
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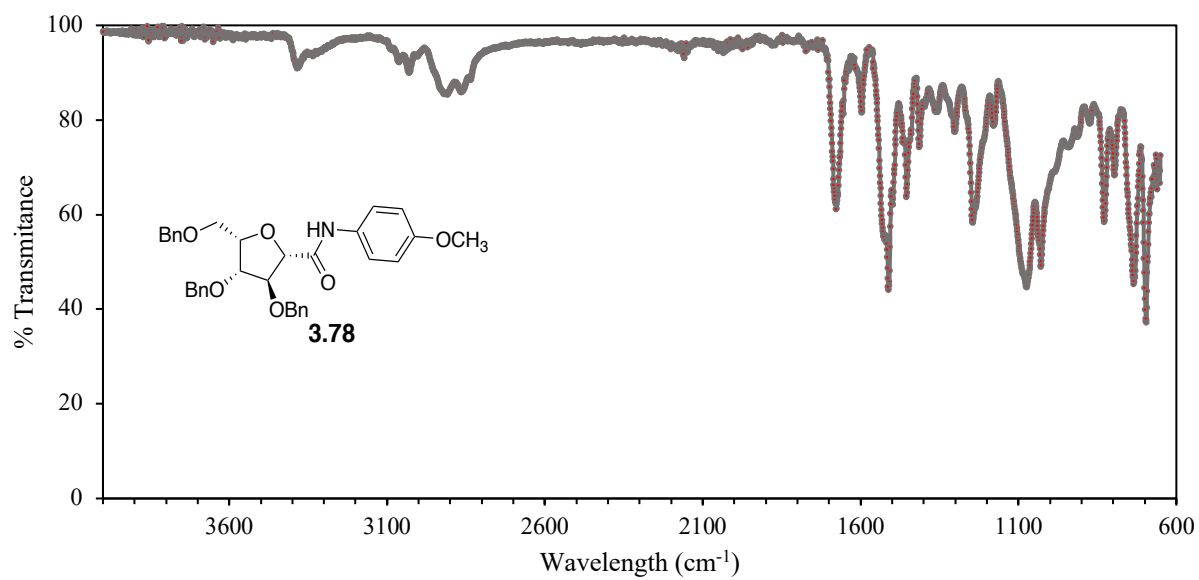
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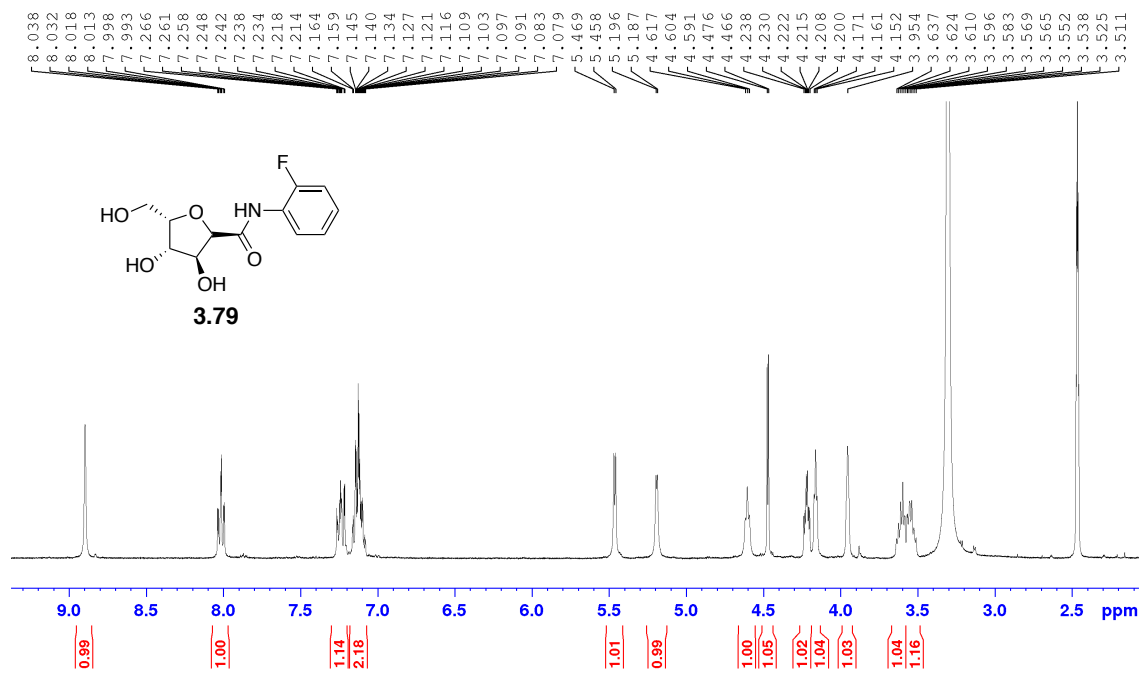
¹³C NMR



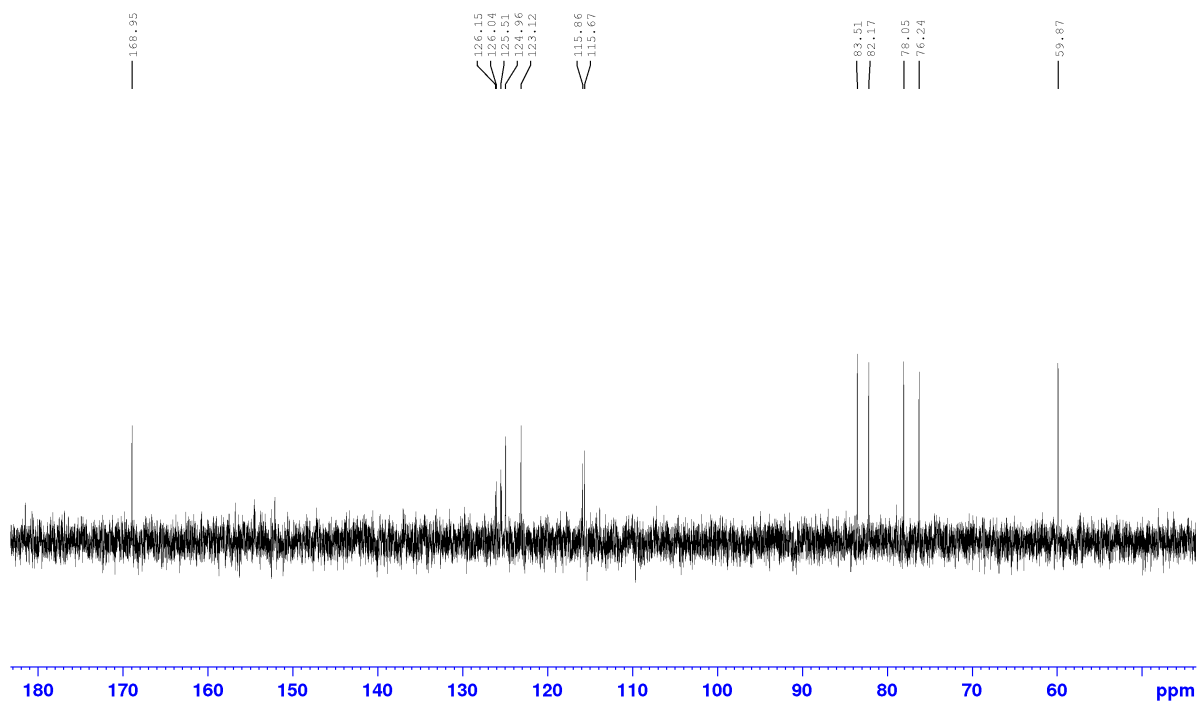
Infrared Spectrum



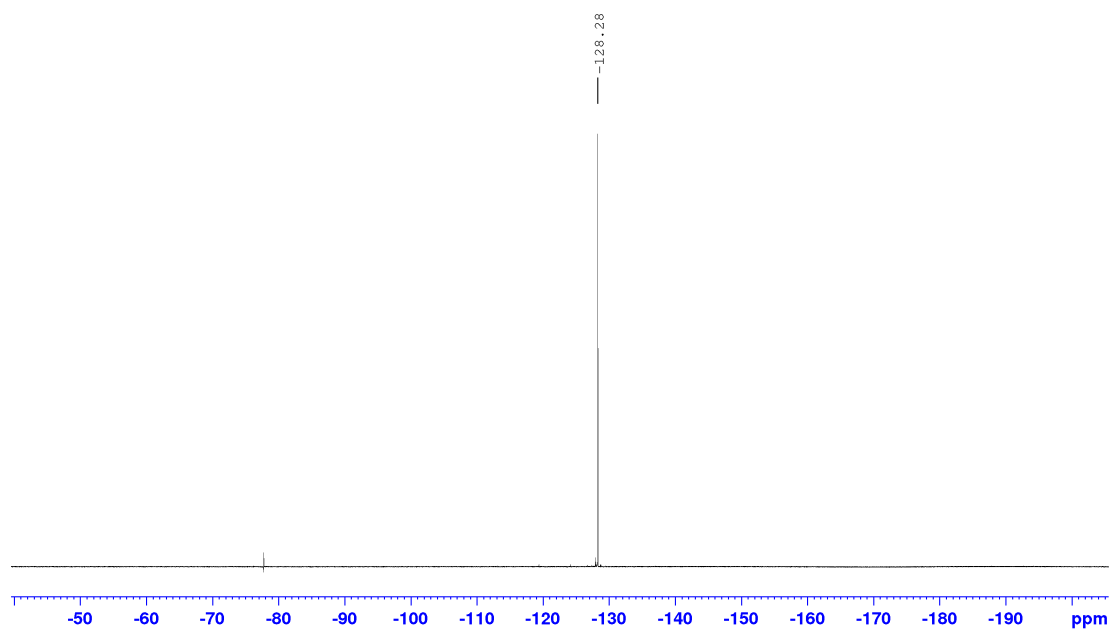
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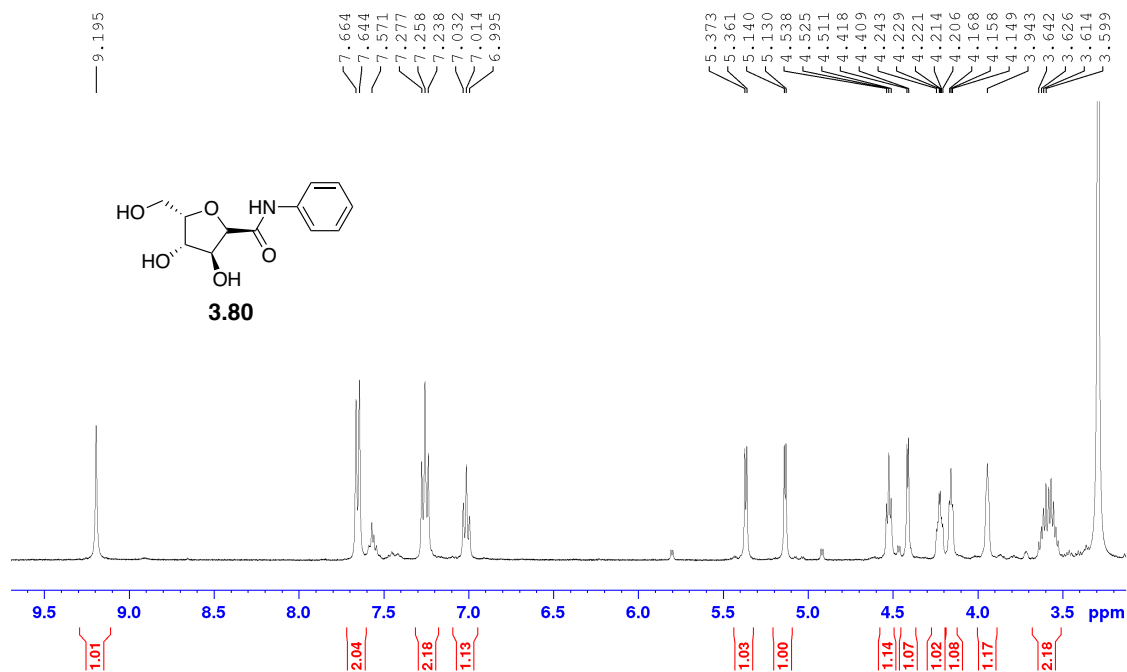
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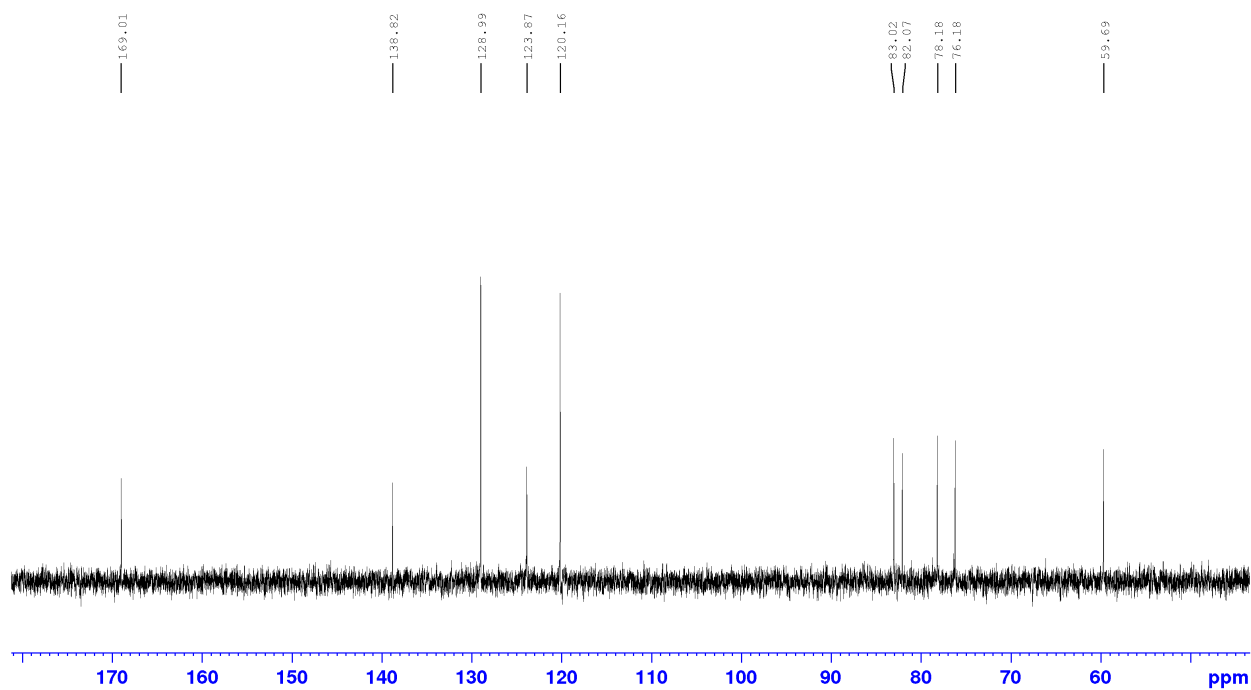
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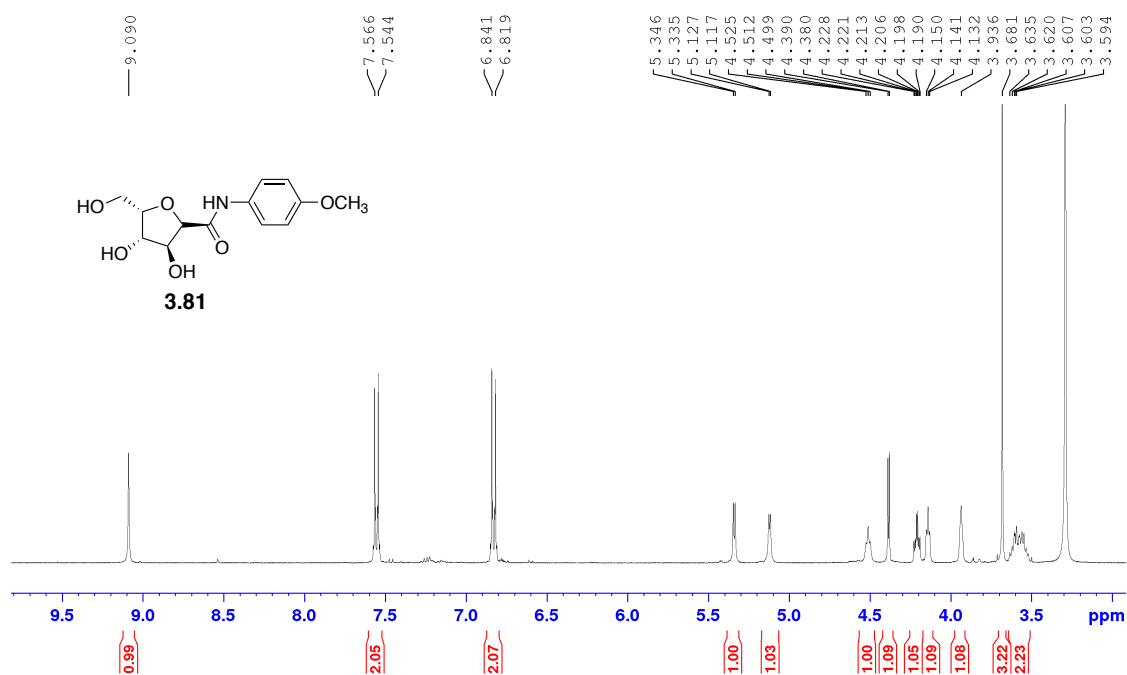
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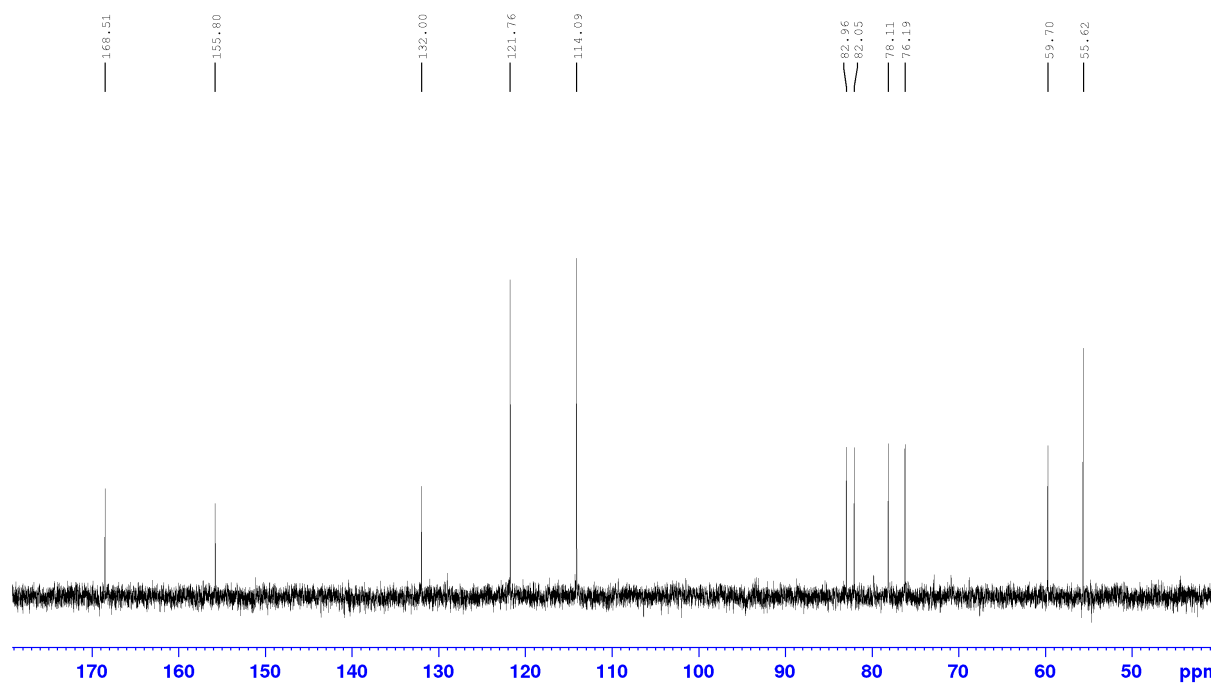
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¹H NMR



^{13}C NMR



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Chapter 4. Assessing the Solubility and IRI Activity of IRIs Containing a Phosphonate Functional Group

4.1 Introduction

The Ben laboratory has conducted several studies evaluating the important structural components of small molecules that contribute to IRI activity. As a result of these studies, several classes of small molecule carbohydrates have been identified as IRI active molecules and have been used in the cryopreservation of different cell types.¹⁻⁶

As described in **Chapter 1**, studies on synthetic AFGPs and their carbohydrate component revealed that the hydration of the carbohydrate can be correlated to its ability to inhibit ice recrystallization.^{7,8} The hydration of a carbohydrate is described by the degree of tightly formed hydrogen bonds to bulk water surrounding the carbohydrate, resulting in a non-exchangeable hydration layer. Carbohydrate hydration has been linked to the configuration of the hydroxyl groups, where the hydroxyl group on C2 and C4 substituents are great contributors to a compound's degree of hydration.^{9,10} Initially, the ability for a carbohydrate to disturb the 3-D hydrogen bonded network of water was correlated to IRI activity (i.e. a larger hydration number resulted in greater IRI activity).⁷ However, a more accurate parameter, the hydration index, describing the number of tightly bound water molecules per molar volume of carbohydrate, was found to have a strong positive correlation with IRI activity for monosaccharides and disaccharides.⁸

The correlation between the hydration index of carbohydrates and IRI activity was also positive for galactose and glucose derivatives containing a *C*-allyl group at C1,⁸ but not for *O*-aryl glycosides.¹¹ The hydration index for these compounds have not been determined however, according to the larger hydration index associated with galactose, it was expected that *O*-aryl

galactoside derivatives would be more IRI active than *O*-aryl glucoside derivatives. Since *O*-aryl glycosides did not follow this trend, this suggests that hydration is an important contributing factor for IRI activity, but other factors may also be involved in this complex phenomenon.

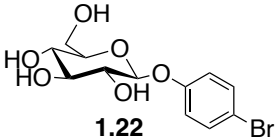
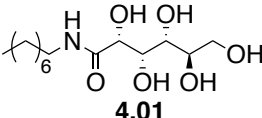
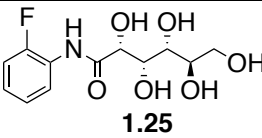
Alongside the hydration of a molecule, the contribution of hydrophobicity towards ice recrystallization inhibition has also been investigated in the Ben laboratory. A former graduate student, Dr. Anna Balcerzak, assessed many small molecules containing hydrophobic moieties for their IRI activity.¹² Anti-ice nucleating agents (anti-INAs) and surfactants/hydrogelators were investigated for their ability to sequester bulk water and prevent the movement of individual water molecules, a perceived requirement for IRIs. This class of molecules demonstrated that some cationic anti-INAs and some anionic surfactants/hydrogelators exhibited IRI activity and their hydrophilic and hydrophobic parts were both important for IRI activity.¹² Similarly, *N*-aryl and *N*-alkyl D-gluconamides were investigated due to their amphiphilicity: their hydrophobic aryl/alkyl group and hydrophilic polyol chain (described in the introduction of **Chapter 3**). However, not all *N*-aryl and *N*-alkyl D-gluconamides exhibited IRI activity but it appeared that the position and type of substituent on the aryl group, and the chain length of the alkyl group altered their activity.^{6,13–15}

In conclusion, these studies showed that several factors contribute to IRI activity, which depend on the structural attributes of the molecule. It is hypothesized that these structural attributes are responsible for the disordering of water molecules leading to ice recrystallization inhibition. In addition, for compounds composed of hydrophobic and hydrophilic parts, the requirement for hydrophobic parts like aryl groups or long-chain alkyl groups has made it difficult to yield compounds that have high IRI activity and also high solubility.

4.2 The challenges of IRI solubility

The requirement of amphiphilicity for IRI activity is observed amongst classes of IRIs synthesized in the Ben laboratory. As a result of the need for a hydrophobic component, the solubility of an IRI can be reduced in aqueous media. For example, out of the *O*-aryl glycoside class, **1.22**, has the best IC₅₀ value (15 mM) and a maximum solubility of 55 mM in PBS.¹⁶ Out of the *N*-alkyl gluconamide class, **4.01** has the best IC₅₀ value (0.09 mM) and a maximum solubility of 1 mM in PBS, and of the *N*-aryl gluconamide class, **1.25** has the best IC₅₀ value (3 mM) and a maximum solubility of 12 mM in PBS (Table 4.1).¹⁴ Although IC₅₀ values provide a dose-response, these IRIs can often be used in biological applications at concentrations higher than the IC₅₀ value. For example, kinetic studies on **1.22** showed that 15 mM **1.22** reduces the rate of ice recrystallization by 50%, while 40 mM **1.22** reduces the rate of ice recrystallization by 100% over a 60 min period (compared to the PBS control).¹⁶

Table 4.1. IC₅₀ values and solubilities of previously synthesized IRIs in the Ben laboratory. IC₅₀ values are reported with 95% confidence interval.

IRI	IC ₅₀ (mM)	Solubility (mM)
 1.22	15 ± 3	55
 4.01	0.09 ± 0.03	1
 1.25	3 ± 1	12

In addition, obtaining the IRI activity profile of some compounds can be challenging as a result of their poor solubility. In those cases, the percent mean grain size (% MGS) at the maximum solubility concentration is usually obtained to define the compound's ability to inhibit ice recrystallization. For example, in **Chapter 3**, L-xylofuranosyl amide, **3.79**, had a limited solubility, therefore the full scope of its concentration-dependent activity could not be obtained, and an IC_{50} value could not be generated (Fig. 4.1). Therefore, its IRI activity was measured as the % MGS at its maximum solubility (76% MGS at 20 mM), which indicated **3.79** had weak IRI activity at this concentration. Thus, the Ben laboratory has attempted to develop new classes of IRIs that have high IRI activity ($IC_{50} < 20$ mM) while also possessing high solubility (solubility > 100 mM). One approach to do this involves incorporation of charged polar functional groups.

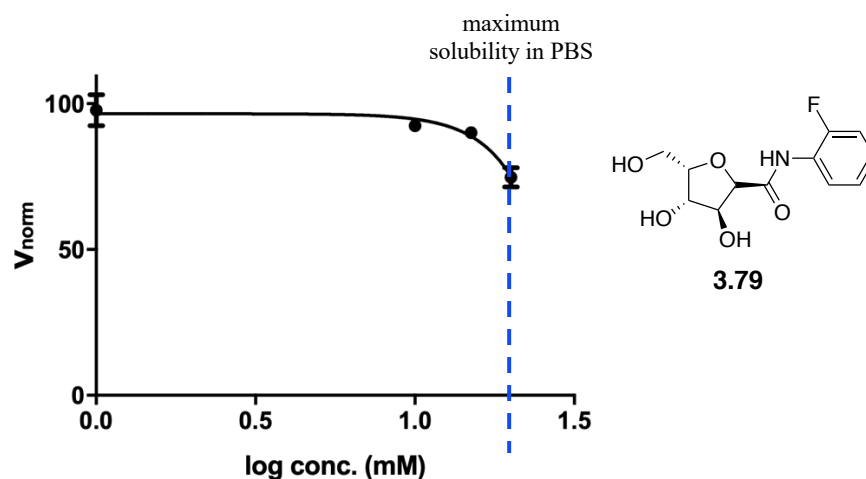
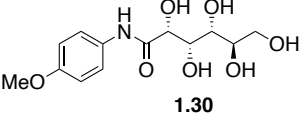
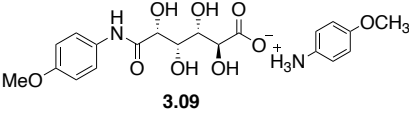
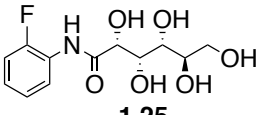
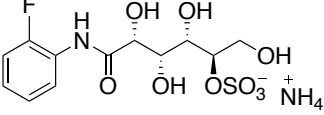
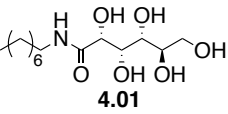
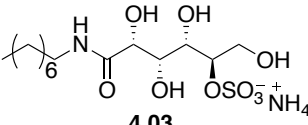
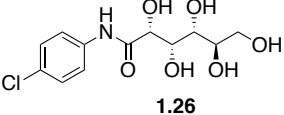
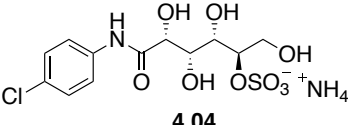
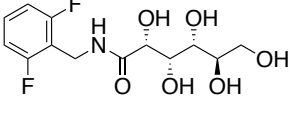
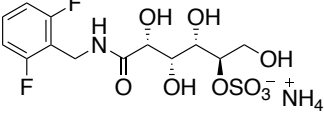


Figure 4.1. Normalized rate of ice recrystallization of **3.79** up to its maximum solubility. Blue dotted line represents the maximum solubility concentration of **3.79** in PBS. Error bars represent $\pm$ % SD for $n=3$ replicates.

4.2.1 *The solubility and IRI activity of previously synthesized “charged” analogues of IRIs from D-gluconamides*

Synthesizing IRIs possessing an overall molecular charge (+1/+2 or -1/-2) could provide a means to increase their solubility. Previous studies in the Ben laboratory have investigated the impact of substituting a charged polar functional group into IRI active compounds belonging to the D-gluconamide class (Table 4.2). As mentioned in **Chapter 3**, Dr. Madeleine Adam investigated modifying the oxidation state of C6 in the *N*-aryl-D-gluconamide class by synthesizing a carboxylate analogue (**3.09**) of *N*-4-methoxyphenyl-D-gluconamide (**1.30**).¹⁴ Although the analogue possesses an overall negative charge in solution, its solubility in PBS was only 20 mM and at this concentration its % MGS was 69%. In comparison to the parent compound (**1.30**) whose % MGS was 20% at 5 mM, analogue **3.09** was not an effective IRI (compounds with a % MGS less than 30% are deemed as strong IRIs). Also, amongst the D-gluconamide class, sulfate analogues were evaluated by Dr. Thomas Charlton, a previous graduate student in the Ben laboratory.¹⁷ Sulfated C5 analogues (**4.02-4.04**, **4.07**) of *N*-2-fluorophenyl (**1.25**), *N*-octyl (**4.01**), *N*-4-chlorophenyl (**1.26**), and *N*-2,6-difluorobenzyl (**4.06**) D-gluconamides were synthesized and all exhibited high solubilities (> 100 mM), in comparison to their parent compounds (Table 4.2). Due to the high solubility of the sulfated compounds, a full IRI activity profile was performed and IC₅₀ values were generated from dose-response curves. The impact of the sulfate group on IRI activity was variable, and in comparison, to their parent compounds, only the 4-chlorophenyl sulfate analogue **4.04** displayed an increase in IRI activity (Table 4.2).

Table 4.2. IRI activities and solubilities of charged gluconamide analogues compared to the IRI activity and solubility of parent compounds.^{14,17}

Parent compound	IRI activity	Solubility in PBS (mM)	Charged polar analogue	IRI activity	Solubility in PBS (mM)
 1.30	%MGS = 20% (5 mM)	20 mM	 3.09	%MGS = 69% (20 mM)	20 mM
 1.25	IC ₅₀ = 3 mM	25 mM	 4.02	IC ₅₀ = 38 mM	>100 mM
 4.01	IC ₅₀ = 0.09 mM	1 mM	 4.03	IC ₅₀ = 17 mM	>100 mM
 1.26	IC ₅₀ = 12 mM	20 mM	 4.04	IC ₅₀ = 5 mM	>100 mM
 4.06	IC ₅₀ = 11 mM	55 mM	 4.07	IC ₅₀ = 70 mM	>100 mM

4.2.2 The solubility and IRI activity of previously synthesized “charged” analogues of IRIs from D-glycosides

Analogues of D-glycosides possessing charged functional groups were also investigated by Dr. Jessica Poisson, a former graduate student in the Ben laboratory (Fig. 4.2).¹⁸ As mentioned in **Chapter 3**, Dr. Poisson synthesized amino analogues of 4-methoxyphenyl and 4-bromophenyl glucosides. These studies revealed that the position of the amino group depicted the IRI activity of the charged analogues such that the C6-amino analogues (**3.24** & **3.25**) displayed moderate IRI

activity, and the C3-amino analogues (**3.22** & **3.23**) could not generate a proper sigmoidal dose response curve. Regarding their solubility, **3.24** and **3.25** were more soluble than **3.22** and **3.23**.

Dr. Poisson also investigated the IRI activity of phosphate monosaccharides.¹⁸ Mannoheptose (**4.08** & **4.09**), mannose (**4.10** & **4.11**), and glucose (**4.12** & **4.13**) analogues were evaluated for their IRI activity and solubility. All of the phosphate monosaccharides exhibited the characteristic dose response curve and displayed moderate activity with the exception of the heptose derivative **4.08**, which was only soluble up to 2 mM. Compounds **4.09-4.13** were quite soluble in PBS with α -D-glucose-1-phosphate (**4.12**) displaying the highest solubility of 200 mM. These phosphate monosaccharides provide good precedence for charged compounds that possess high solubilities and moderate IRI activities. Therefore, incorporating a phosphate moiety into an active IRI ($IC_{50} < 20$ mM) with low solubility could potentially increase their solubility while maintaining a degree of their IRI activity. This current chapter will discuss the synthesis of phosphonate (a stable analogue of phosphate) derived glycosides and gluconamides in an attempt to generate IRI active compounds with high solubilities.

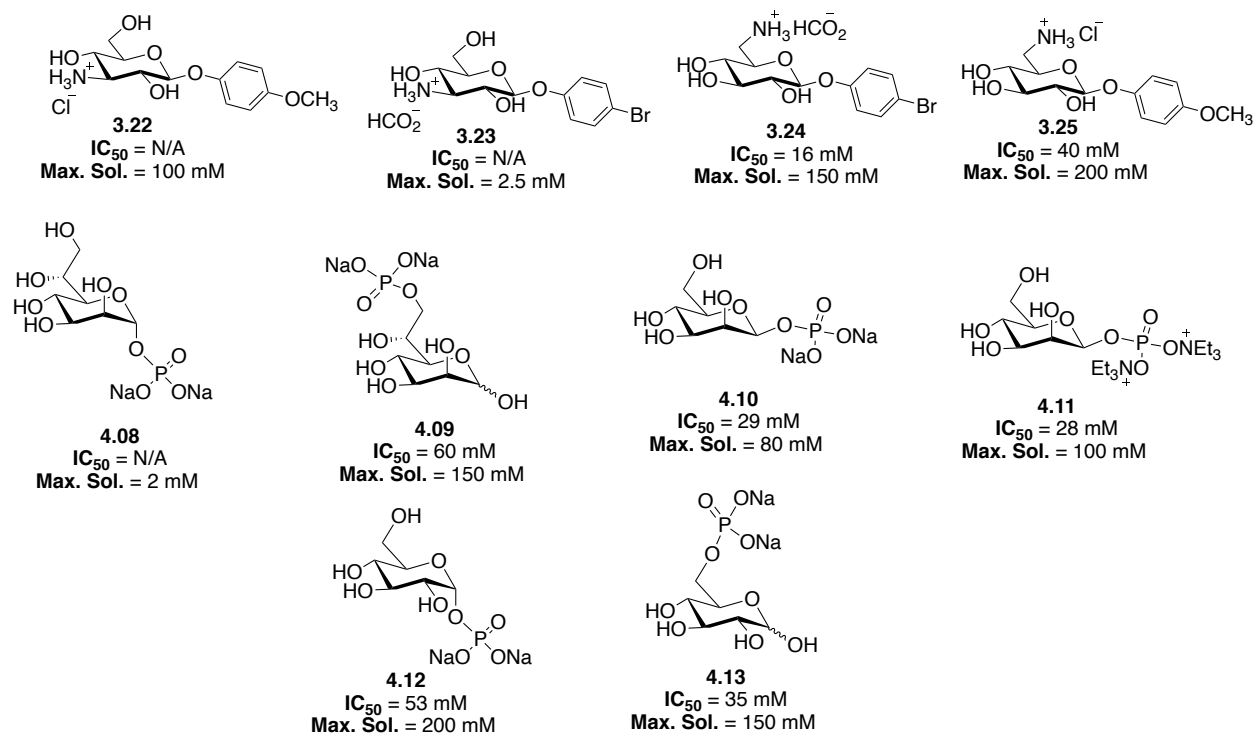


Figure 4.2. IC_{50} and maximum solubilities of charged amino (3.22-3.25) and phosphate (4.08-4.13) monosaccharides evaluated by Dr. Jessica Poisson.¹⁸ Maximum solubilities are measured in PBS.

4.3 Phosphonates as analogues of the phosphate group

Phosphonic acid is an analogue of phosphoric acid, and it differs by a phosphorous-carbon linkage compared to a phosphorus-oxygen linkage (Fig 4.3). Phosphonic acid functional groups are widely used in applications to mimic naturally occurring phosphoric acids, due to their resistance to hydrolysis in aqueous media and their stability toward enzymatic reactions.¹⁹ Similar to phosphoric acid, phosphonic acid has a high degree of polarity and is highly soluble in water, especially in its deprotonated phosphonate state. As such, it has been used as a means to improve the solubility of organocatalysts^{20,21} and drug-chelate supramolecular assemblies,^{22,23} while also being biologically accepted as a phosphate mimetic for several drug applications.²⁴⁻²⁷ Phosphonic acid contains two acidic protons with a $\text{p}K_{a1}$ range of 1.1 – 2.3 and a $\text{p}K_{a2}$ range of 5.3 – 7.2. Their definite $\text{p}K_a$ s are highly dependent on the electronic properties of their substituents, but at

physiological pH, phosphonic acid exists in its fully deprotonated state.¹⁹ For the purpose of the following studies, and to maximize solubility, the phosphonic acid compounds synthesized in this chapter were converted to phosphonate salts via the addition of a base.



Figure 4.3. The generic chemical structure of phosphonic acid (left) and phosphoric acid (right).

4.4 The synthesis and IRI activity of glucose phosphonate derivatives

To investigate phosphonate-containing IRIs, glucose-derived phosphonates were synthesized and evaluated for IRI activity. Recall that previous work with phosphates demonstrated that 1-phosphate (**4.12**) and 6-phosphate (**4.13**) glucose analogues were highly soluble and had moderate IRI activities. Keeping this in mind, synthesizing glucose derivatives containing a *C1*-phosphonate or *C6*-phosphonate were envisioned (Fig. 4.4). For the previously synthesized phosphate monosaccharides (**4.08-4.13**), sodium or triethylamine were used as counterions. In the case of the glucose-1-phosphonate (G1CP) derivatives, four different counterions were surveyed to evaluate for counterion effects on IRI activity, as was previously observed with the anionic surfactants.¹² In the surfactant class, changing the counterion to sodium, lithium, or potassium had different effects on IRI activity depending on the surfactant structure. For example, in some cases, a surfactant with a sodium counterion exhibited stronger IRI activity compared to the same surfactant with a lithium or potassium counterion.¹² In other cases, changing the counterion to sodium, lithium, or potassium had little effect on IRI activity.¹² Therefore, sodium and potassium counterions were chosen, to observe if there would be any notable

differences in IRI activity of the phosphonates. Ammonium was also of interest due to its ability to form multiple hydrogen bonds with water and thus increase hydration. In addition, the divalent nature of calcium ion made it attractive as it could potentially lead to the formation of phosphonate complexes in solution. The glucose-6-phosphonate (G6CP) derivative was only converted to the ammonium and sodium salts (**4.18-4.19**) after the initial analysis of **4.14-4.17**. Kayla Newell, an Honour's student who worked with me, assisted in the synthesis and IRI activity analysis of these compounds.

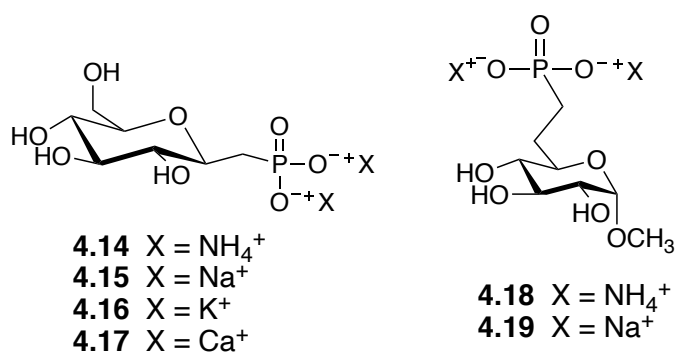
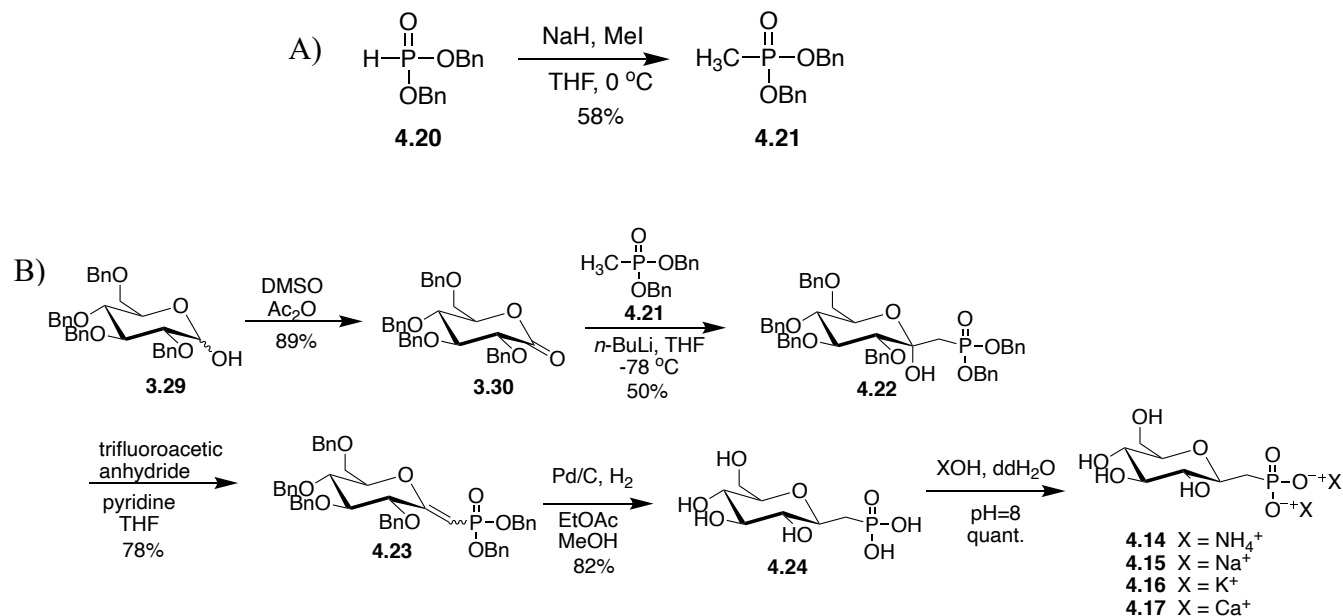


Figure 4.4. Chemical structures of glucose *C1*- and *C6*-phosphonate derivatives.

4.4.1 Synthesis of glucose-1C-phosphonate derivatives 4.14-4.17



Scheme 4.1. Synthesis of A) dibenzyl methyl phosphonate (**4.21**) and B) glucose-1C-phosphonate derivatives **4.14 - 4.17**.

The *beta*-anomer of D-glucose-1C-phosphonate (G1CP) (**4.14-4.17**), was synthesized since the *beta*-anomer of D-glucose derivatives is typically more IRI active than the *alpha*-anomer; a trend that has been observed in the *O*-aryl glucoside class.¹¹ The *beta*-anomer has also been previously reported in the literature and used as an inhibitor for glucose phosphotransferase enzymes.²⁸ The synthesis of the *beta*-anomer of G1CP (Scheme 4.1B) was initiated via an Albright-Goldman oxidation²⁹ of commercially available **3.29** to generate the gluconolactone analogue **3.30**. Treatment of the lactone with freshly synthesized dibenzyl methylphosphonate (**4.21**), afforded the α -hydroxy derivative **4.22**, which was confirmed by the appearance of two doublet of doublet peaks located upfield on the ¹H NMR (Fig. 4.5). The two doublet of doublet peaks are characteristic of methylene phosphonate compounds as a result of the methylene proton coupling to its geminal proton and coupling to the adjacent phosphorus atom. Elimination of the

α -hydroxyl group in **4.22** with trifluoroacetic anhydride afforded **4.23**. This was followed by benzyl deprotection and alkene reduction via hydrogenolysis (Pd/C) to provide the phosphonic acid derivative, **4.24**.

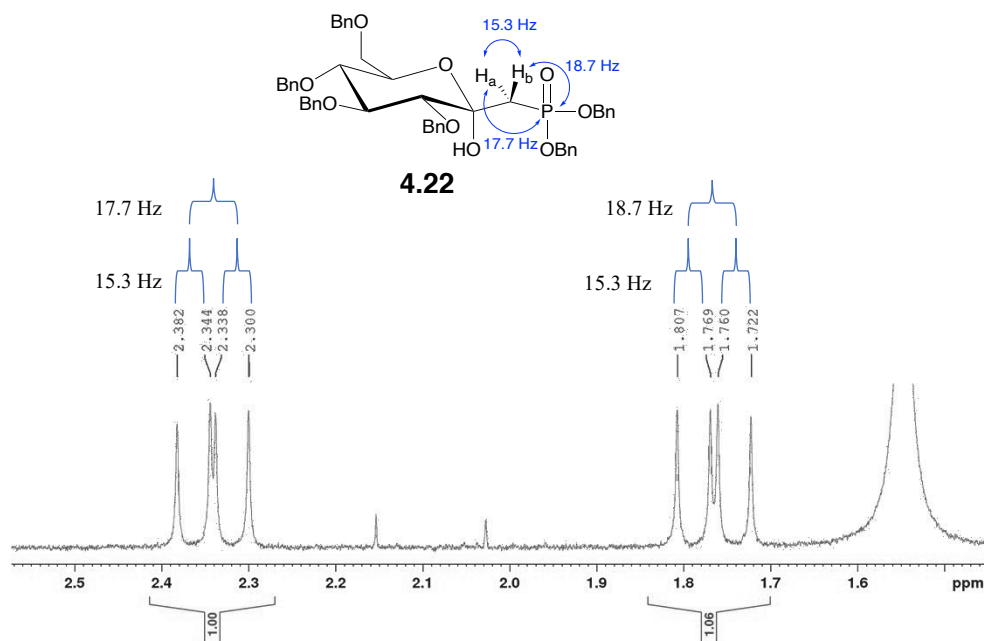


Figure 4.5. A section of the ^1H NMR spectrum of **4.22**. Coupling constants between the methylene protons and the phosphorous atom are highlighted.

Recall that phosphonic acid has a $\text{p}K_{a1}$ range of 1.1 – 2.3 and a $\text{p}K_{a2}$ range of 5.3 – 7.2, therefore **4.24** was treated with a base until a pH of 8 was reached to generate the fully deprotonated derivative. Ammonium hydroxide, sodium hydroxide, potassium hydroxide, and calcium hydroxide, were used to furnish their corresponding dibasic phosphonate salts (**4.14-4.17**). All resonances for **4.14** were assigned and corresponded to the literature spectra of the G1CP *beta*-anomer.²⁸

4.4.2 Solubility and IRI activity of GICP derivatives 4.14-4.17

As expected, compounds **4.14-4.17** exhibited high solubility in PBS (Table 4.3). There were only slight variances in solubility based on the counterion; the maximum solubility tested for all compounds was 500 mM and the ammonium (**4.14**), sodium (**4.15**), and potassium (**4.16**) phosphonates were all soluble up to that concentration. The calcium phosphonate derivative (**4.17**), however, was only soluble up to 200 mM. Using the splat cooling assay, the IRI activity of **4.14-4.17** was evaluated and the rate of ice recrystallization was determined for each compound. The rates were plotted and fitted onto a non-linear regression curve and a characteristic dose-response was observed for each set of data (Fig. 4.6), indicating that compounds **4.14-4.17** displayed IRI activity. The GICPs possessing the sodium, potassium, and calcium counterions (**4.15-4.17**) all exhibited similar curves, however, there was a noticeable difference in the curve generated by the ammonium phosphonate derivative (**4.14**). IC₅₀ values were obtained from each curve and the most IRI active analogue, **4.14**, had an IC₅₀ of 3.0 ± 0.5 mM (values reported with 95% confidence interval). Analogues **4.15-4.17** all exhibited moderate IRI activity, and taking into consideration their 95% confidence interval, their IC₅₀ values were essentially the same (Table 4.3). Since the only difference between the GICP derivatives evaluated were their counterions, the variances observed in IRI activity, specifically with the ammonium derivative, may be attributed to the ammonium counterions present in solution. The following section discusses in more detail the effect that the counterions have on IRI activity.

Table 4.3. IC₅₀ values and solubilities of **4.14-4.17**. IC₅₀ values are reported with 95% confidence intervals.

G1CP derivatives	IC ₅₀ (mM)	Maximum solubility in PBS (mM)
4.14	3.0 ± 0.5	>500
4.15	51 ± 8	>500
4.16	60 ± 12	>500
4.17	41 ± 8	200

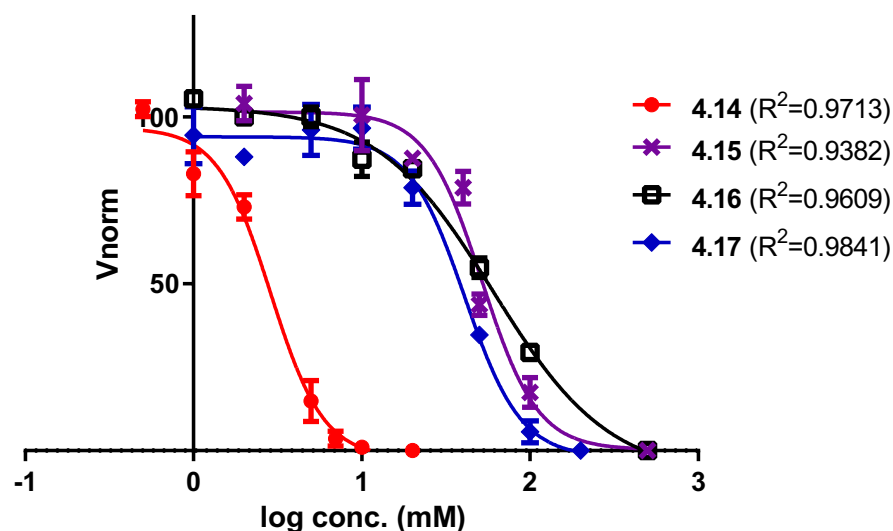


Figure 4.6. IRI activity of G1CP derivatives **4.14-4.17**. A non-linear regression line was fitted to points to generate IC₅₀ values. Error bars represent ± % SD for n=3 experiments.

4.4.3 The effect of counterions on IRI activity

Out of the G1CP derivatives **4.14-4.17**, the phosphonate with the ammonium counterions (**4.14**) appeared to be significantly more IRI active than the derivatives with sodium, potassium, or calcium counterions. To investigate this counterion effect further, several inorganic ammonium salts were evaluated for their IRI activity using the splat cooling assay.³⁰ Ammonium phosphate dibasic ((NH₄)₂HPO₄), ammonium carbonate ((NH₄)₂CO₃), ammonium chloride (NH₄Cl), and ammonium sulfate ((NH₄)₂SO₄), all generated dose-response curves, similar to that of **4.14** (Fig.

4.7A), and their IC₅₀ values were essentially the same (Table 4.4). These results suggested that ammonium on its own is very IRI active, and that the activity observed from **4.14** may be a result of the contributing activity of the ammonium counterions. Since all the ammonium salts displayed similar IRI activity, and the anions of the ammonium salts did not seem to significantly contribute to its activity, only the chloride salts of sodium, potassium, and calcium were evaluated. Both sodium chloride (NaCl) and potassium chloride (KCl) did not generate full dose-response curves, but rather, the data could be fitted to a linear regression with a slope that did not significantly deviate from zero (Fig. 4.7B&C). Calcium chloride (CaCl₂) was evaluated at 100 mM and exhibited only minor IRI activity such that its mean grain size was 75% with respect to PBS (Fig. 4.7D). The insignificant IRI activity observed for sodium chloride, potassium chloride, and calcium chloride, suggested that the activity observed for **4.15**, **4.16**, and **4.17** was therefore due to the pyranose phosphonate portion. This is significant since it indicates that G1CP is IRI active, and its “true” IC₅₀ value is that observed of **4.15-4.17**. The IRI activity of the ammonium ions overshadow the activity of the glucose phosphonate **4.14**.

Table 4.4. IRI activity of ammonium, sodium, potassium, and calcium salts. IC₅₀ values are reported with 95% confidence interval. N/A indicates an IC₅₀ value could not be obtained for the respective compound.

Salt	IRI activity
(NH ₄) ₂ HPO ₄	IC ₅₀ = 1.7 ± 0.3 mM
(NH ₄) ₂ CO ₃	IC ₅₀ = 1.9 ± 0.5 mM
NH ₄ Cl	IC ₅₀ = 3.0 ± 0.8 mM
(NH ₄) ₂ SO ₄	IC ₅₀ = 3.1 ± 0.5 mM
NaCl	N/A
KCl	N/A
CaCl ₂	75% MGS at 100 mM

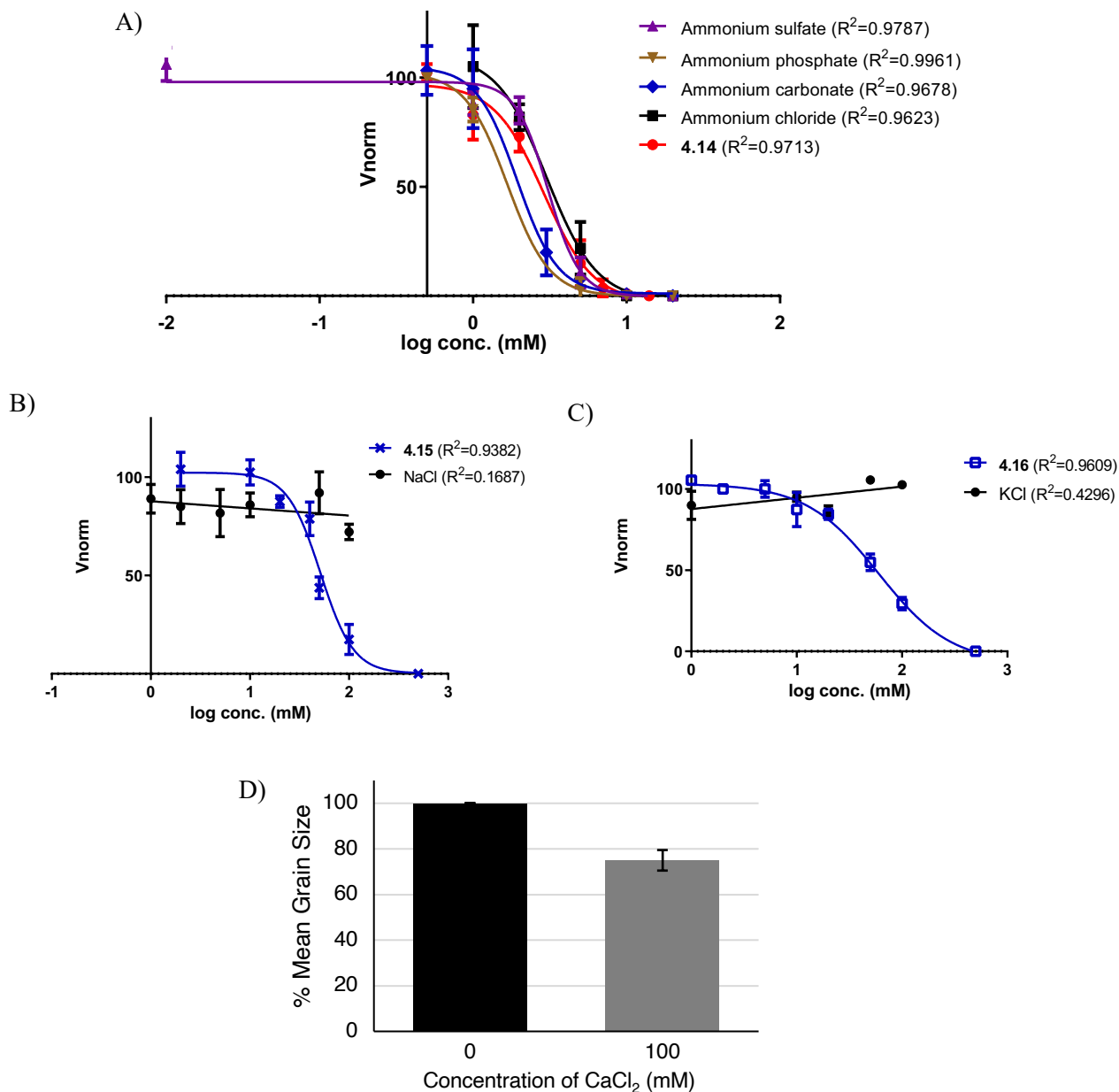


Figure 4.7. IRI activity of A) ammonium salts and 4.14, B) sodium chloride and 4.15, C) potassium chloride and 4.16, and D) calcium chloride. A non-linear regression line was fitted to points to generate IC_{50} values. Slope of linear regression for sodium chloride and potassium chloride does not significantly deviate from zero (p value (NaCl) = 0.1391; p value (KCl) = 0.2298). Error bars represent % SD for $n=3$ experiments.

In a study by Surís-Valss and Voets³¹ the growth rate of ice crystals in a sample containing an antifreeze protein or glycoprotein (AFPIII or AFGP₁₋₅) was significantly smaller

upon the addition of salts compared to in the absence of salts. It was concluded that the addition of anions increased the IRI activity of AFPIII and AFGP₁₋₅, according to the Hofmeister series for anions.

The Hofmeister series³² is the classification of ions for their effect on the physical behaviour of several aqueous properties, most notably, protein stabilization and precipitation. In this series, the effect of anions has been found to be more pronounced than the cation effect, and many theories have arisen describing the mechanism and cause of the effect. Initial theories stemmed from the ion's ability to alter the hydrogen bonding network of water. Ions that are highly hydrated and integrate well into the structure of water are called "kosmotropes" and have a stabilizing and salting-out effect on macromolecules and proteins; whereas ions that break the structure of water are called "chaotropes" and are known to destabilize proteins and have a salting-in effect.³³⁻³⁶ While this theory is still debated, other theories involving the direct interaction of ions and macromolecules and their effect on bulk water are more accepted.^{37,38} As IRIs with high hydration indices are more active, the kosmotrope/chaotrope theory would suggest that strongly hydrated ions (i.e. kosmotropic), such as ammonium, would exhibit increased IRI activity compared to ions that are chaotropic. If this theory is valid, it could potentially explain the high IRI activity observed amongst the ammonium phosphonate analogue (**4.14**) and ammonium salts, compared to the sodium, potassium, and calcium phosphonate analogues (**4.15-4.17**) and respective salts, which are neither strongly kosmotropic nor chaotropic (Fig. 4.8).

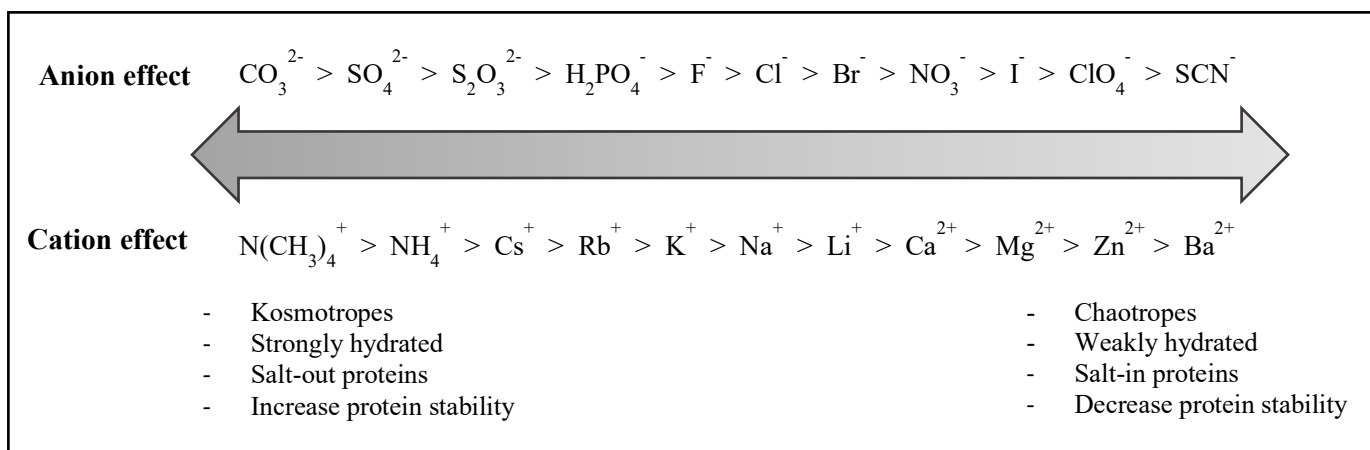


Figure 4.8. The Hofmeister series of anions and cations. Figure adapted with permission from *ACS Omega*, 2020, 5, 6229–6239. Copyright © 2020 American Chemical Society.

In the Surís-Valss and Voets study,³¹ the increased IRI activity with certain salts present was hypothesized to be a result of the salting-out of proteins, leading to an enhanced adsorption of proteins to ice, therefore preventing the addition of water molecules to ice and further heightening the inhibition of ice recrystallization.³¹ Unlike AF(G)Ps, phosphonate compounds are hypothesized to not bind to the ice lattice, thus, the justification for the correlation between IRI activity and the Hofmeister series observed amongst AF(G)Ps may not be an accurate way to explain the ion effect observed amongst the phosphonates. To verify if the Hofmeister series could be used to explain the counterion effect observed amongst **4.14-4.17**, a similar experiment to that reported by Surís-Valss and Voets was conducted.³¹ In their experiment, ice crystal growth over a 90-min period was measured using the sucrose sandwich assay.³⁹ The greatest difference in ice crystal size of the AF(G)P solutions containing the Hofmeister series anions was observed at 90-min.³⁹ Therefore for the sake of comparison, the sucrose sandwich assay was used to measure the ice crystal size of the phosphonate salts **4.14** and **4.15** at 90-min. The reader will recall from **Chapter 1**, that the sucrose sandwich assay has a slightly different protocol than the splat cooling

assay, and a 40% sucrose solution is used to make IRI solutions and is the positive control for ice recrystallization, instead of PBS. In the literature experiment, the presence of kosmotropic anions significantly decreased the ice crystal size in an AF(G)P solution compared to the sucrose solution and solutions containing chaotropic anions.³¹ Thus, if the IRI activity observed for the phosphonate derivatives correlates to the Hofmeister series, then the ice crystal size in a solution of 20 mM **4.14** would be significantly smaller than that observed for 20 mM **4.15** and the 40% sucrose control. The mean grain size of ice crystals in each solution was measured and the results were not statistically different from one another (one-way ANOVA) (Fig. 4.9). As expected, this is different from what was observed in the literature as the grain size of ice crystals containing the various anions with AFPIII and AFGP₁₋₅ were significantly smaller after 90-min in accordance with the Hofmeister series.³¹ These results suggest that the phosphonate counterion effect may not be a result of an enhanced ice binding mechanism caused by the Hofmeister effect, as observed with the AF(G)Ps.

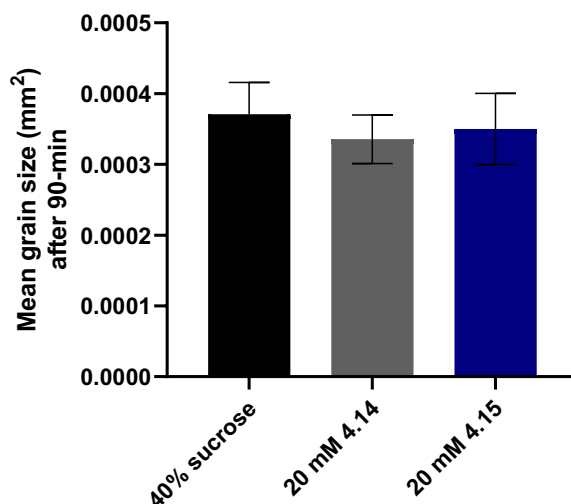


Figure 4.9. Mean grain sizes of ice crystals in 40% sucrose, 40% sucrose with 20 mM **4.14**, or 40% sucrose with 20 mM **4.15** measured after 90-min annealing times at -6.4 °C. Error bars represent SD for n=3 experiments. One-way ANOVA test was used to verify a lack of statistical significance between data.

To further investigate the kosmotrope/chaotrope theory of the Hofmeister series, the IRI activity of tetramethylammonium chloride ($\text{N}(\text{CH}_3)_4\text{Cl}$) was evaluated. If IRI activity did correlate with the Hofmeister series, it was hypothesized that tetramethylammonium chloride would also display strong IRI activity, similar to that observed for the ammonium salts since it is more kosmotropic than ammonium (Fig. 4.8). Using the splat cooling assay, the rates of ice recrystallization were obtained at different concentrations of tetramethylammonium chloride in PBS and were plotted. The characteristic dose response was not observed, but a nonlinear and linear regression were both fit to the data points (Fig. 4.10). Comparing IRI activity, tetramethylammonium chloride was less active than ammonium chloride such that at 20 mM, the rate of ice recrystallization was 100% for tetramethylammonium chloride and 0% for ammonium chloride. Although tetramethylammonium chloride did display some IRI activity, these results suggested that the activity of cations may not correlate with the Hofmeister series and the kosmotrope/chaotrope theory does not justify the observed IRI activity of the cations.

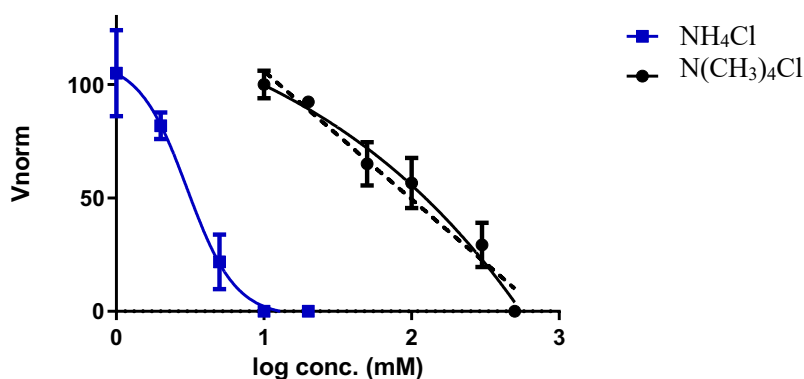


Figure 4.10. IRI activity of tetramethylammonium chloride and ammonium chloride. Tetramethylammonium chloride is fitted to both a nonlinear regression (solid black line; $R^2=0.9532$) and linear regression (dotted black line; $R^2=0.9357$). Ammonium chloride is fitted to a nonlinear regression ($R^2=0.9623$). Error bars represent % SD for $n=3$ experiments.

4.4.4 IRI activity of glucose-1-phosphonic acid 4.24

Considering that variable results were observed for IRI activity depending on the counterion of the G1CPs, the IRI activity of glucose-1-phosphonic acid (**4.24**) (in the absence of counterions) was also evaluated to observe if there would be a significant difference. The pK_{a1} and pK_{a2} values of **4.24** have been reported as 2-3 and 6.94 respectively,²⁸ thus in PBS (pH=7.4), it was hypothesized that **4.24** would be in its fully deprotonated state (**4.26**) and could complex with an available cation in solution (Fig. 4.11). Since PBS is composed of sodium and potassium salts, it was predicted that **4.24** would convert to a sodium or potassium salt in solution and would therefore exhibit similar IRI activity to the sodium and potassium G1CP derivatives **4.15** or **4.16**.

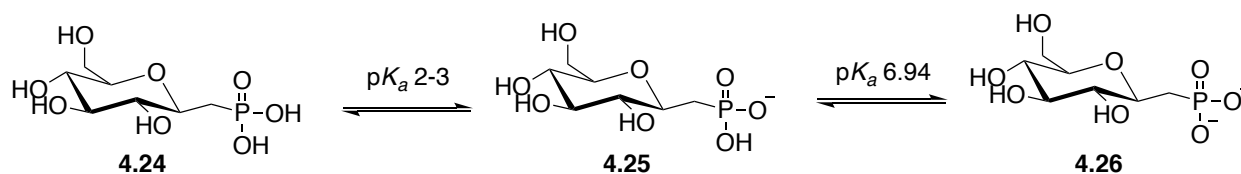


Figure 4.11. The dissociation equilibrium of glucose-1-phosphonic acid, **4.24**.

Using the splat cooling assay, **4.24** was evaluated at concentrations of 500 mM, 300 mM, 100 mM, 50 mM, 20 mM, and 10 mM (Fig. 4.12). The pH of each these solutions were determined as 2, 2, 2, 3, 5, and 5, respectively. Therefore, at 500 mM, 300 mM, and 100 mM concentrations, the fully protonated neutral compound, **4.24**, is present in solution, while at concentrations of 50 mM, 20 mM, and 10 mM, **4.25**, an overall charge of “-1” is present in solution (Fig. 4.11). The Ben laboratory has previously observed that pH does not affect IRI activity,¹² thus any difference in IRI activity between **4.24** and **4.15/4.16** can be assumed to result from the protonated/deprotonated state of the phosphonate. The IC_{50} value of **4.24** was 90 ± 27 mM (error is reported as 95% confidence interval). Considering the large 95% CI, the difference in IC_{50} values

obtained for **4.24** and the potassium phosphonate, **4.16** ($IC_{50} = 60 \pm 12$ mM), are not statistically significant. This suggests that there is little difference in IRI activity between glucose-1-phosphonate in its protonated and deprotonated state.

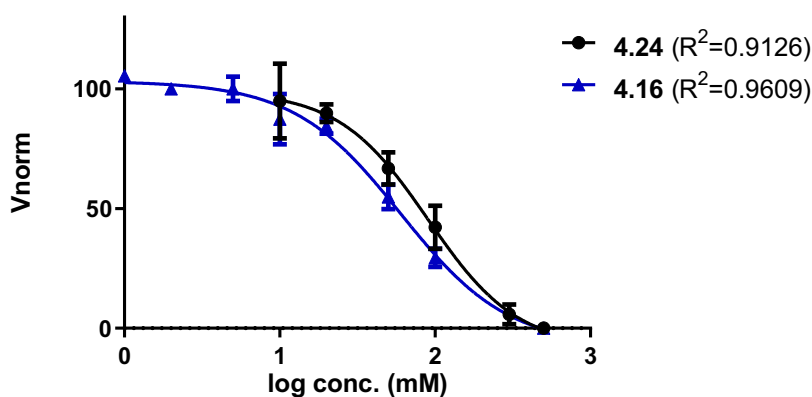


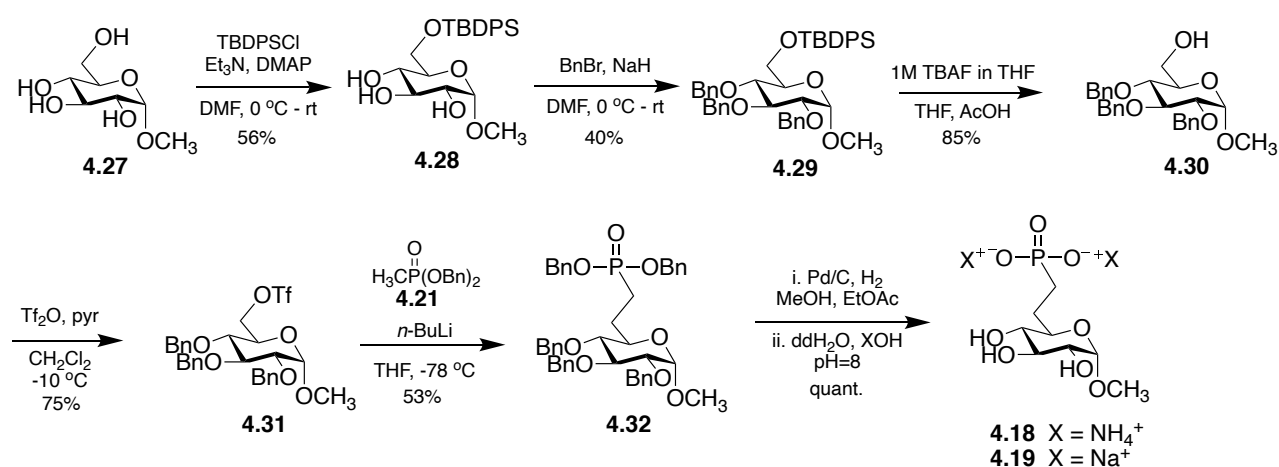
Figure 4.12. IRI activity of glucose-1-phosphonic acid derivative **4.24** and potassium derivative **4.16**. A non-linear regression was fitted to points to generate IC_{50} values. Error bars represent % SD for $n=3$ experiments.

From herein, phosphonate compounds were synthesized as both ammonium and sodium salts. This way, it could be determined if the strong IRI activity observed for the ammonium salts was consistent with all substrates and observing the activity of the sodium salts could provide the IRI activity of the phosphonate compound without the interference of counterions.

4.4.5 Synthesis and IRI activity of glucose-6-phosphonate derivatives **4.18** and **4.19**

The ammonium and sodium glucose-6-phosphonate (G6CP) derivatives (**4.18** & **4.19**) of α -methyl glucoside (**4.27**) were synthesized via a six-step synthesis (Scheme 4.2). The synthesis was initiated by the silyl protection of the C6-primary alcohol under basic conditions with *tert*-butylchlorodiphenylsilane (TBDPSCl) to form **4.28**, followed by benzylation of C2, C3, and C4 hydroxyl groups to form orthogonally protected **4.29**. Silyl deprotection with tetrabutylammonium

fluoride (TBAF) afforded alcohol **4.30**, which was converted to a triflate ester, **4.31**, then substituted with lithiated dibenzyl methylphosphonate (**4.21**) to form **4.32**. Deprotection of the benzyl groups via hydrogenolysis (Pd/C) followed by addition of ammonium or sodium hydroxide until pH=8, furnished the corresponding ammonium and sodium G6CP derivatives **4.18** and **4.19**.



Scheme 4.2. Synthesis of glucose-6-phosphonate derivatives **4.18** and **4.19**.

Similar to the G1CP derivatives (**4.14** and **4.15**), **4.18** and **4.19** were both soluble up to 500 mM. Their IRI activity was evaluated using the splat cooling assay, and dose-response curves were generated from the rates of ice recrystallization measured (Fig. 4.13). Consistent with the trend of the other ammonium salts, the ammonium phosphonate derivative, **4.18**, displayed an IC₅₀ value of 1.6 ± 0.2 mM. The sodium derivative, **4.19**, had an IC₅₀ value of 28 ± 7 mM, making it more active than the G1CP sodium derivative **4.15**. Although a direct comparison cannot be made between G1CP and G6CP, (the latter possess an α-methoxy group at C1) a similar trend was also observed in previous work amongst the phosphate class in which the glucose-6-phosphate analogue was more active than the glucose-1-phosphate analogue.¹⁸

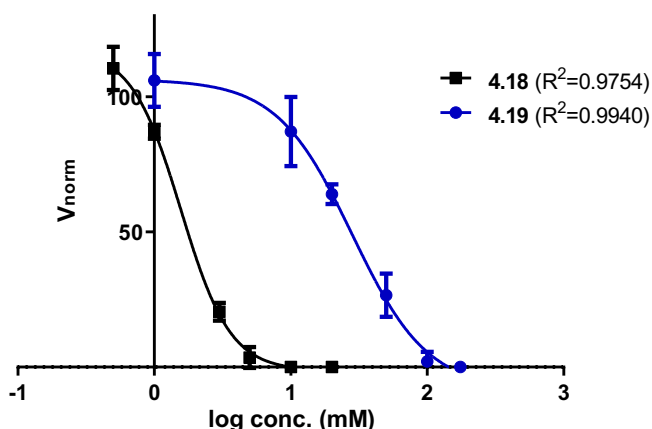


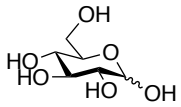
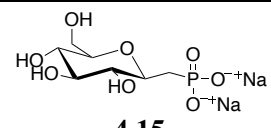
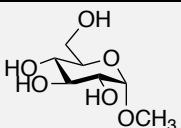
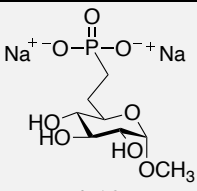
Figure 4.13. IRI activity of G6CP derivatives **4.18** and **4.19**. A non-linear regression line was fitted to points to generate IC₅₀ values. Error bars represent % SD for n=3 experiments.

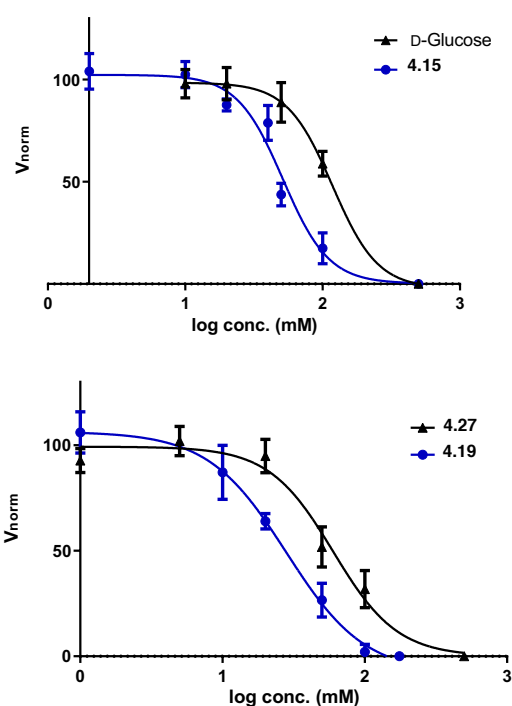
4.4.6 Evaluating the impact of a phosphonate group at C1 and C6 of glucose

Previous studies on simple monosaccharides have emphasized the importance of the C1- and C6-hydroxyl groups towards IRI activity.^{11,41} Evaluating monosaccharide reducing sugars, *O*-methyl monosaccharides, and β -C-allyl glycosides suggested that the anomeric substituent and their relative configuration has a profound impact on IRI activity.⁴¹ Most of the C1-substituted monosaccharides, including β -C-allyl glucoside, were found to display less IRI activity than their corresponding reducing sugar, however, α -methoxy glucoside was an exception as it was more active than glucose.⁴¹ Similarly, the C6-hydroxyl group was identified as a key contributor to IRI activity. Modifications such as alkylations, oxidations, or removal of the C6-hydroxyl group abolished activity for both glucose and galactose derivatives.⁴¹ In these studies, compounds were evaluated for IRI activity using a preliminary screening approach where compounds are compared solely by their % MGS at 22 mM. Therefore, to make a proper comparison to their phosphonate analogues for the purpose of this work, the IRI activity of D-glucose and α -methoxy glucoside (**4.27**) was re-evaluated by generating dose-response curves. A dose-response was obtained for D-

glucose and **4.27**, and IC₅₀ values were generated and compared to the IC₅₀ values of sodium phosphonate analogues **4.15** and **4.19** (Table 4.5). In both cases, converting the C1- or C6-hydroxyl group to a phosphonate moiety resulted in an approximately 2-fold increase in IRI activity.

Table 4.5. IRI activity of sodium G1CP (**4.15**) and G6CP (**4.19**) and their parent compounds D-glucose and **4.27**. Errors for IC₅₀ values are reported as 95% confidence interval. Dose-response curves of each compound are shown to the right of the table.

	Compound structure	IC ₅₀ (mM)
Parent compound	 D-glucose	118 ± 24
Sodium phosphonate analogue	 4.15	51 ± 8
Parent compound	 4.27	60 ± 14
Sodium phosphonate analogue	 4.19	28 ± 7



These results conflict with the previous observations that modifications to C1 or C6 are detrimental to IRI activity. An explanation for this could be the enhanced ability of the phosphonates (**4.15** & **4.19**) to form strong hydrogen bonds.^{19,42} In the previous modifications, a hydrogen bond donor (i.e. hydroxyl group) was replaced with groups that lacked hydrogen bonding ability.^{11,41} However, in the case of **4.15** and **4.19**, a hydrogen bond donor (i.e. hydroxyl group) is replaced with three hydrogen bond acceptors (i.e. phosphonate oxygens). Several studies have

examined the intramolecular and intermolecular hydrogen bonding ability of the individual hydroxyl groups of glucose and have emphasized the importance of O6 in the hydration of glucose.^{43–46} One study in particular by Suzuki,⁴³ used molecular dynamics simulations to determine the strength of the hydrogen bonding interactions between each hydroxyl group of glucose and water. The *O6*-hydroxyl group was found to form the greatest number of acceptor hydrogen bonds with water and was therefore the most hydrated hydroxyl group. Thus, substituting the *C6*-hydroxyl group to a more polar phosphonate group with three hydrogen bond acceptors, would increase the overall hydrogen bonding capacity of the saccharide. This increase in hydrogen bond capacity could improve IRI activity based on the previous hydration trends observed.⁸ In the same study, Suzuki also proposed that the anomeric oxygen (O1) is among the poorest hydrogen bond acceptors.⁴³ Calculations showed that the lone pairs on O1 were more tightly bound to the oxygen due to it being a hemiacetal alcohol, therefore its lone pairs are less available to form hydrogen bonds with water. In the case of **4.15**, O1 is replaced by a methyl phosphonate thus replacing a poor hydrogen bond acceptor with a stronger hydrogen bond acceptor; a potential cause for the increase in IRI activity.

From these initial results, the indication that IRI activity can be increased by integrating a polar functional group, such as sodium phosphonate, into the saccharide core structure of a poor ice recrystallization inhibitor is promising. Therefore, it is of interest to evaluate the effect of phosphonates into compounds with high IRI activity. The following section will discuss the synthesis and evaluation of phosphonate analogues of previously synthesized IRIs.

4.5 The synthesis and IRI activity of phosphonate analogues based upon previously identified IRI active compounds

O-Aryl- β -D-glucosides and *N*-alkyl/*N*-aryl D-gluconamides are two classes of compounds that the Ben laboratory has studied for their strong IRI activity. As previously mentioned, some of the most IRI active compounds in these classes have poor solubilities which can be a limitation for their use. Observing the increase in IRI activity amongst the glucose phosphonate compounds in *Section 4.4* sets a good precedence for synthesizing phosphonate derivatives of highly active IRIs as a means to increase their solubility while maintaining or even improving IRI activity. *O*-Aryl- β -D-glucosides **1.22** and **1.20**, *N*-aryl-D-gluconamide **1.25**, and *N*-alkyl-D-gluconamide **4.01**, were good candidates for this study as they possess the requirement of having an $IC_{50} < 20$ mM and a limited solubility.

The importance of the aryl group and aryl substituent towards IRI activity have been emphasized amongst the *O*-aryl glucoside class, therefore, installing a phosphonate moiety on the aryl group or changing the position of the aryl group could be detrimental to IRI activity.¹¹ Since it was observed that substitution of a phosphonate at C6 of a glucose derivative increases its IRI activity, it was hypothesized that C6-phosphonate derivatives of **1.22** and **1.20** may produce similar results. The C6-phosphonate derivative of *N*-aryl gluconamide **1.25** was also envisioned as a sufficient starting point as the C6 position of the gluconamides is a synthetically facile position to manipulate. The phosphonate derivative of *N*-alkyl gluconamide **4.01**, was designed to have the phosphonate moiety on the terminal carbon of the alkyl chain. This was envisioned as an ideal starting point since manipulating the alkyl chain is synthetically easier than making changes to the saccharide chain. In addition, one of the caveats of *N*-alkyl gluconamides as cryoprotective agents is their surfactant-like properties making them detrimental to cell membranes.⁴⁷ Therefore, it was hypothesized that adding a hydrophilic moiety at the end of the hydrophobic alkyl chain could help

mediate this issue. To this end, **4.34-4.41** were synthesized and evaluated for their solubility and IRI activity (Fig. 4.14). Kayla Newell, a Honour's student working on this project, assisted in the synthesis and IRI analysis of **4.36**, **4.37**, and **4.01**.

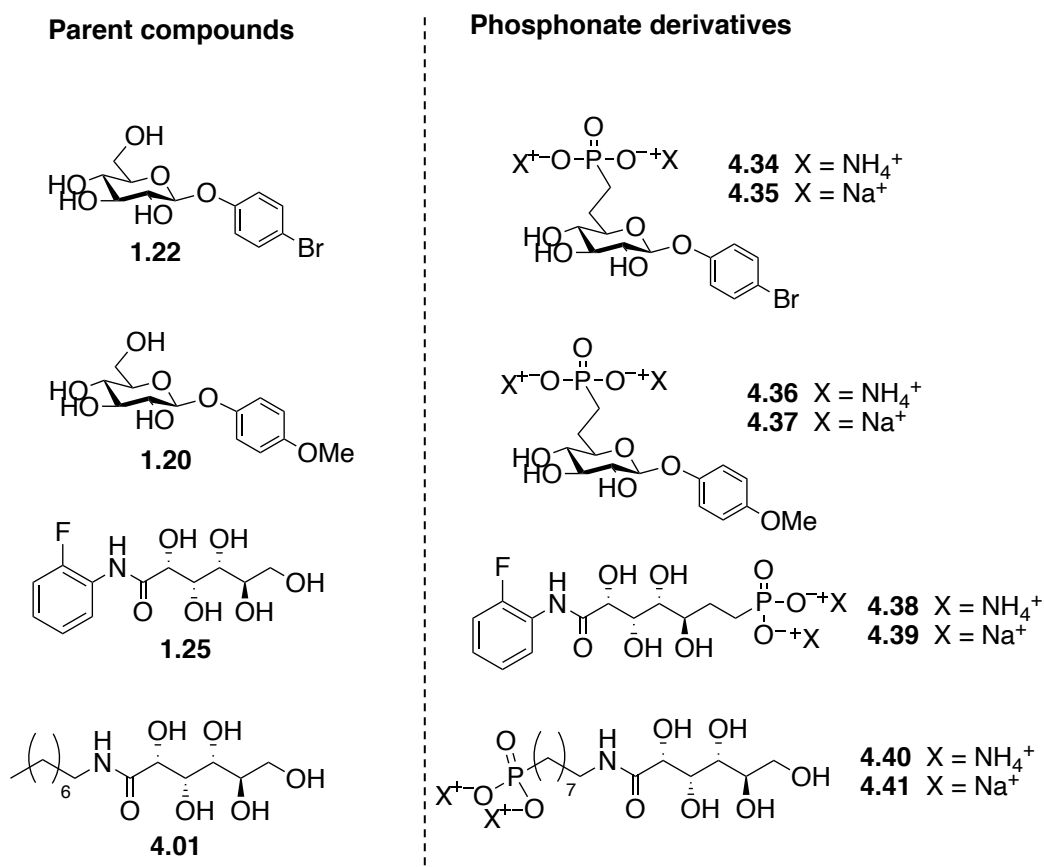
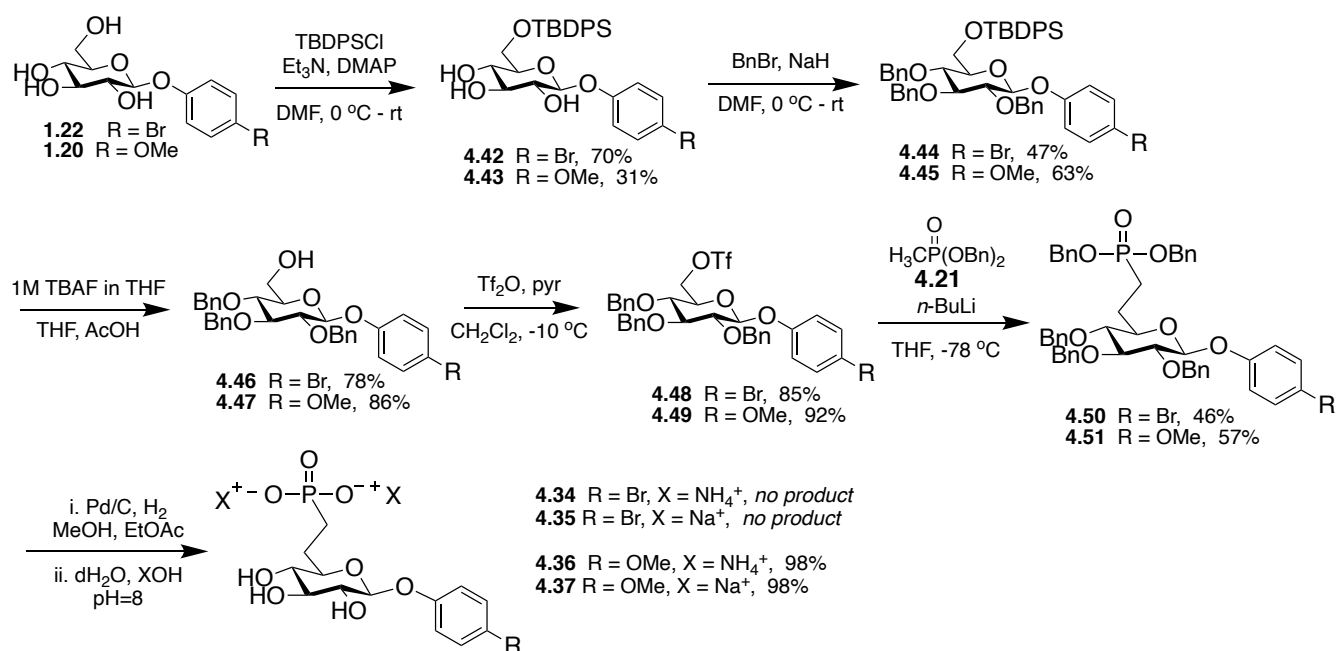


Figure 4.14. Envisioned phosphonate derivatives of parent compound IRIs.

4.5.1 Synthesis of *O*-aryl- β -D-glucoside phosphonate derivatives 4.34-4.37

To synthesize the C6-phosphonate derivative of 4-bromophenyl and 4-methoxyphenyl glucosides **1.22** and **1.20**, a similar synthetic approach to that of the G6CP was employed. The synthesis initiated from the parent compounds, **1.22** and **1.20**, which were synthesized with the

standard procedure published by the Ben laboratory.¹¹ The C6-position was then protected with a silyl ether followed by benzyl protection of C2, C3, and C4 resulting in derivatives **4.44** and **4.45**. Hydrolysis of the silyl ether with a 1M solution of tetrabutylammonium fluoride afforded the 6-hydroxyl derivatives **4.46** and **4.47**, which were then converted to triflate esters (**4.48** and **4.49**). Substitution with freshly made lithiated dibenzyl methylphosphonate (**4.21**) furnished the C6-phosphonate derivatives. Benzyl deprotection, under hydrogenolysis (Pd/C) conditions, of the 4-methoxyphenyl derivative (**4.51**), followed by treatment with ammonium hydroxide or sodium hydroxide yielded the C6-phosphonate products, **4.36** and **4.37**. The hydrogenolysis (Pd/C) of the 4-bromophenyl derivative (**4.50**), however, led to a mixture of products that could not be recovered or identified by NMR spectroscopy. It was speculated that this may be due to the strong affinity of palladium to bromine to form a palladium bromine product. In another attempt, boron trichloride-dimethyl sulfide complex (BCl₃-SMe₂) was used as a milder reagent, however, after using the literature reaction conditions⁴⁸ on compound **4.50**, the reaction did not go to completion. After the previous attempts, only a small quantity of **4.50** remained, therefore further attempts were not continued and only the 4-methoxyphenyl derivatives **4.36** and **4.37** were analyzed for IRI activity.

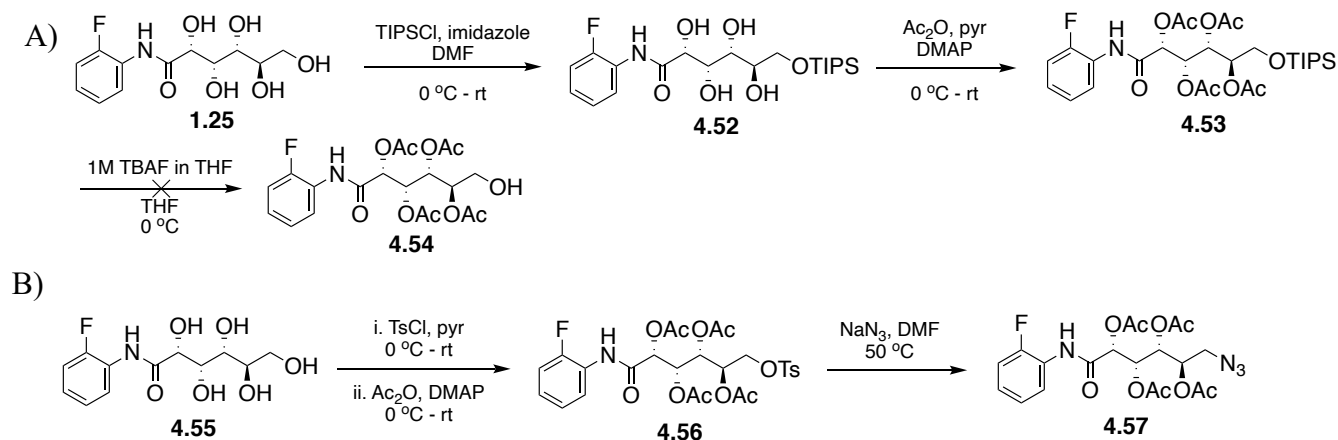


Scheme 4.3. Synthesis of C6-phosphonate derivatives of 4-bromophenyl and 4-methoxyphenyl glucoside (4.34-4.37).

4.5.2 Attempted synthesis of *N*-aryl-*D*-gluconamide phosphonate derivatives 4.38-4.39

To synthesize the C6-phosphonate derivative of **1.25**, several different reaction schemes were envisioned. Manipulating the C6 position of *N*-aryl gluconamides has been attempted in the past by a former graduate student in the Ben laboratory, Dr. Madeleine Adam.¹⁴ Dr. Adam attempted to oxidize the C6-hydroxyl group by a selective silyl protection with triisopropylchlorosilane (TIPSCl) followed by global acetylation (Scheme 4.4A). Unfortunately, removal of the TIPS group led to acetyl migration and an inseparable mixture of tetra-acetylated products. In another attempt, manipulation of the C6 position of *N*-aryl gluconamides by substitution of an azide nucleophile was performed via a late-stage nucleophilic displacement approach (Scheme 4.4B).¹⁴ A toluenesulfonyl group was installed as a leaving group at C6,

followed by global acetylation. Substitution with an azide nucleophile was successful to yield the *C6*-azide derivative, **4.57**.



Scheme 4.4. Previous synthetic routes attempted to manipulate the *C6*-hydroxyl group of *N*-aryl gluconamides.¹⁴

It was anticipated that this synthetic approach to generate the *C6*-phosphonate derivatives could be problematic due to the alkaline environment required for the phosphonate substitution and the presence of the alkali-labile acetyl protecting groups. As a solution, using benzyl ethers as protecting groups would be the best approach, however, global benzylation of gluconamides has been unsuccessful in the Ben laboratory. Instead, *N*-aryl gluconamides with benzyl protecting groups must be synthesized from benzyl-protected gluconolactones under reflux conditions as observed in **Chapter 3**, thus, the synthesis towards **4.38** and **4.39** was tailored such that the phosphonate group would be installed at *C6* on a benzylated saccharide prior to formation of the gluconamide product (Fig. 4.15).

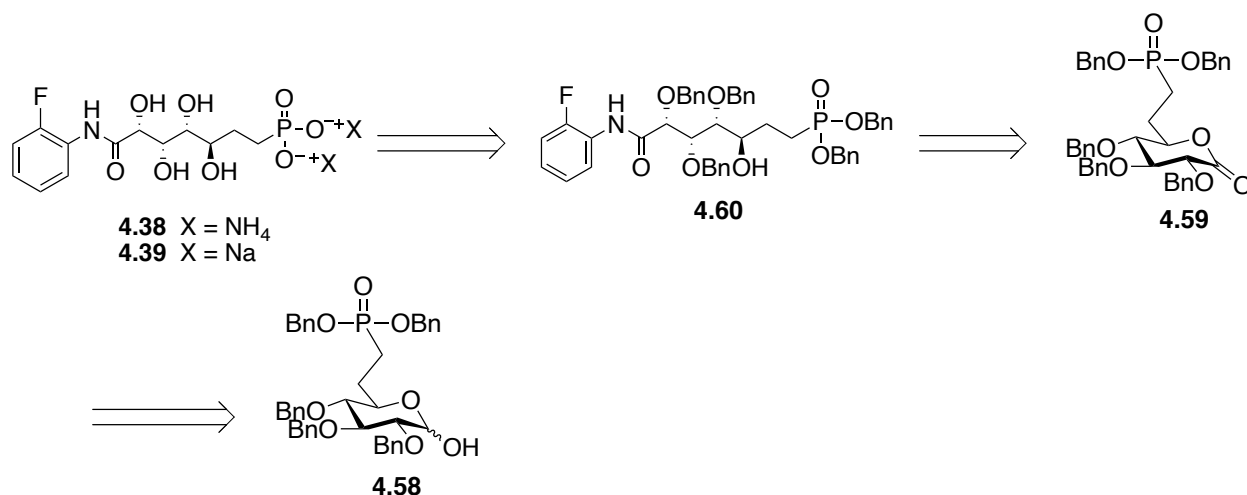
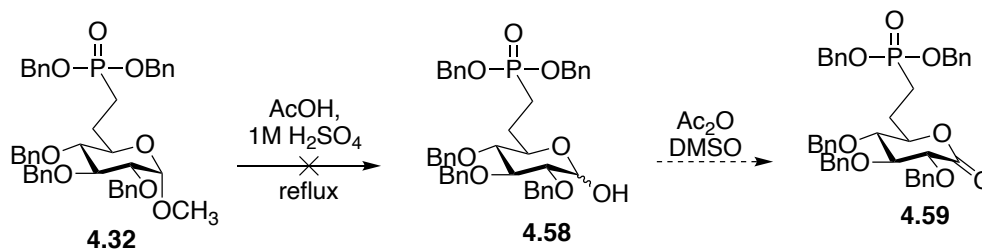


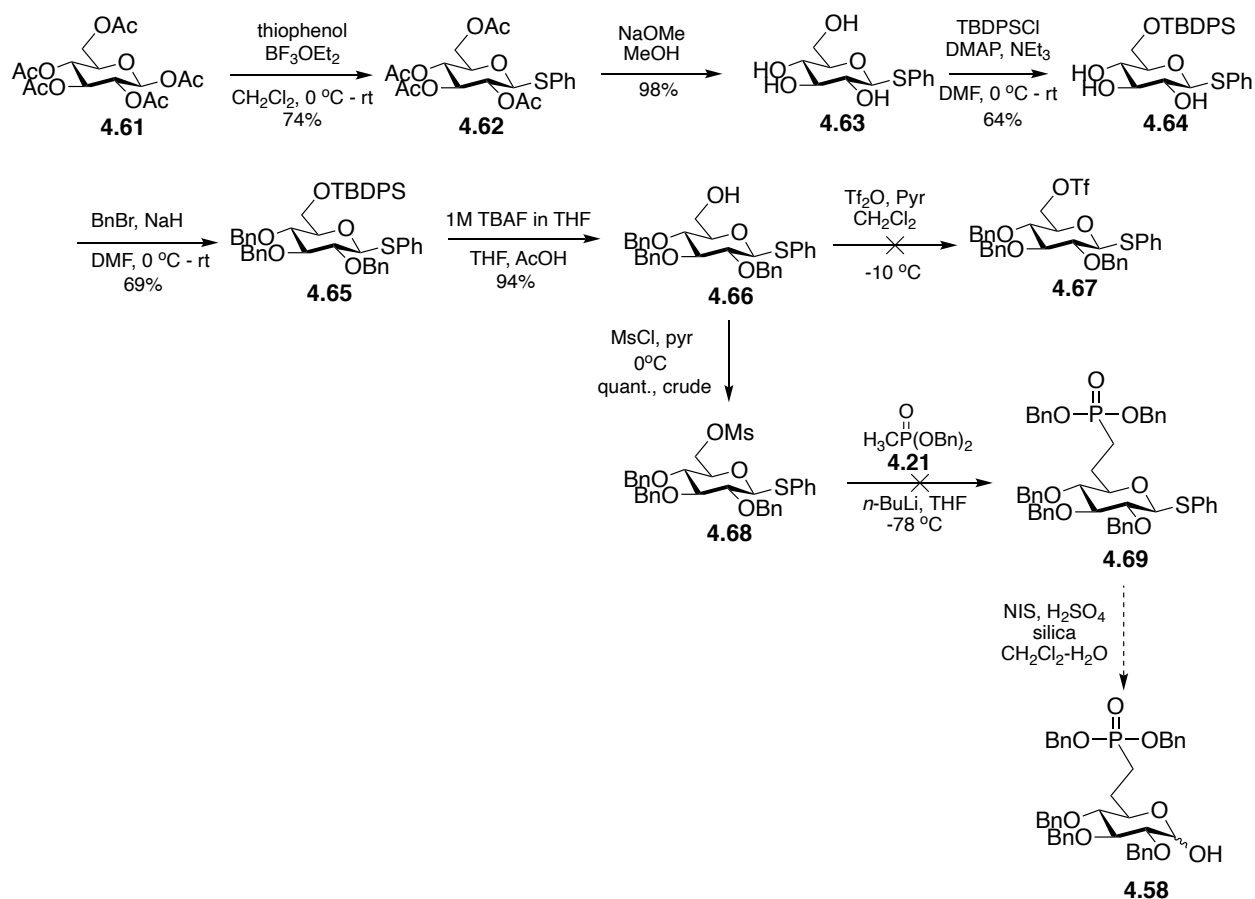
Figure 4.15. Proposed retrosynthesis of **4.38** and **4.39**.

To generate, gluconolactone **4.59**, the C6-phosphonate benzyl protected intermediate (**4.32**), used to synthesize the C6-phosphonate derivatives **4.18** and **4.19**, was a good starting point before proceeding with gluconamide formation as the last step. To convert **4.32** into a reducing sugar (**4.58**), **4.32** was subjected to acidic conditions to hydrolyze the methyl ether at C1 followed by the Albright-Goldman oxidation to generate the gluconolactone compound **4.59** (Scheme 4.5). Unfortunately, attempted acid hydrolysis of **4.32** resulted in multiple products, observed by TLC, and a major spot visible under UV light was observed on the baseline of the TLC (eluted in 3:1 hexane/ethyl acetate). Increasing the polarity of the eluent did not increase the R_f of the major spot and it was thought that the harsh acidic reaction conditions may have hydrolyzed the benzyl ethers on the phosphorus resulting in a polar phosphonic acid product.



Scheme 4.5. First attempted synthesis of C6-phosphonate gluconolactone derivative **4.59**.

To avoid the use of acidic conditions, a thioglycoside was used instead of the methoxyglucoside. Hydrolysis of thioglycosides to form reducing sugars can be performed under mild reaction conditions using *N*-iodosuccinimide (NIS), sulfuric acid (H_2SO_4) and silica.⁴⁹ NIS acts as an iodide source (activating the thioglycoside), and the sulfuric acid immobilized on silica provides a mild source of protons. The synthesis for the thioglycoside derivatives initiated with the Lewis acid mediated thiolation of glucose pentaacetate (**4.61**) to form the acetylated thioglycoside, **4.62**. Subsequent protecting group manipulations were performed to afford compound **4.66**, the 6-hydroxy tribenzyl thioglycoside derivative. Forming the triflate ester, **4.67**, was problematic and resulted in a variety of products that were inseparable. This may have been due to the high affinity of triflate anhydride for sulfur as it is often used as a promoter in the glycosylation of thioglycosides.^{50,51} As an alternative, **4.66** was successfully converted into a mesyl ester (**4.68**) followed by concomitant substitution with lithiated dibenzyl methylphosphonate (**4.21**). Intermediate **4.68**, however, remained unreacted possibly due to its poor leaving group ability under the set reaction conditions. After quite a few unsuccessful attempts, this reaction was abandoned, and the focus was shifted towards the synthesis of the *N*-alkyl gluconamide phosphonate derivative.

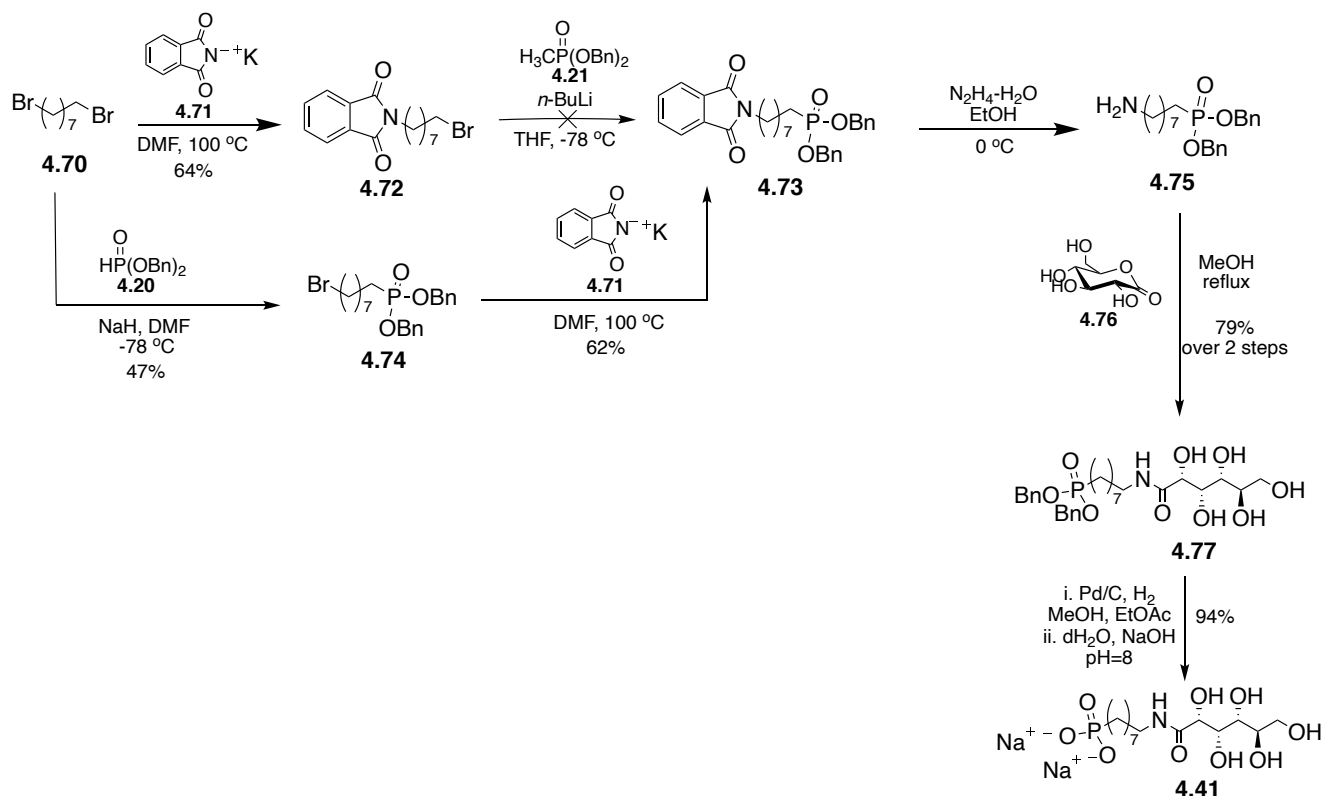


Scheme 4.6. Second attempted synthesis of C6-phosphonate gluconolactone derivative **4.59**.

4.5.3 Synthesis of *N*-alkyl-D-gluconamide phosphonate derivative **4.40-4.41**

In the Ben laboratory, *N*-alkyl gluconamides are typically synthesized via a one-step ring alkylation reaction between D-glucono- δ -lactone (**4.76**) and the appropriate alkyl amine.⁵² Therefore, the *N*-alkyl gluconamide phosphonate derivatives (**4.40** and **4.41**) were synthesized according to this method. Modifications to the alkyl amine by substitution of the phosphonate moiety were performed first on a “masked amine” via a modified Gabriel synthesis.⁵³ The synthesis commenced with a substitution reaction between 1,8-dibromooctane (**4.70**) and potassium phthalimide (**4.71**) to generate the phthalimide substituted alkyl bromide **4.72**. In an

attempt to generate the alkyl phosphonate derivative, **4.73**, dibenzyl methylphosphonate (**4.21**) was introduced under alkaline conditions, however reaction of the anionic phosphonate with **4.72** did not afford the desired substitution product.

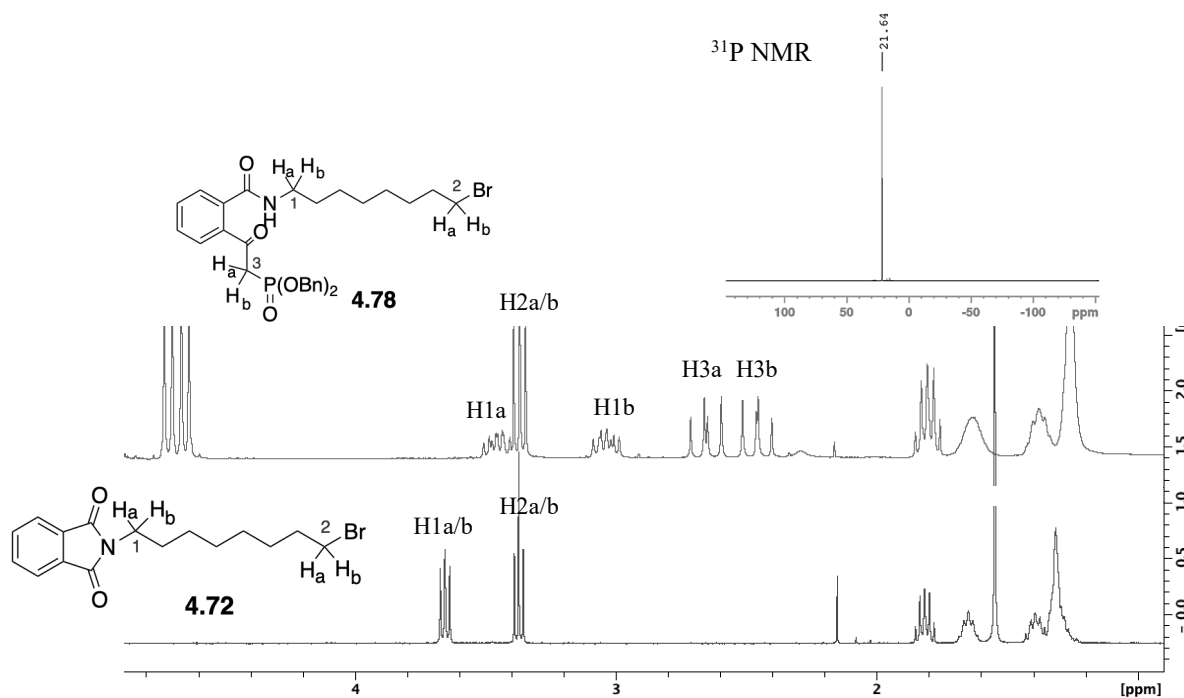


Scheme 4.7. Synthesis of *N*-alkyl gluconamide phosphonate derivative **4.41**.

A closer look at the ^1H NMR spectra of the formed product (Fig. 4.16A) indicated that the two bromomethylene protons at δ 3.37 ppm remained unchanged in comparison to the starting material (**4.72**). If **4.73** had been synthesized, a change in chemical shift and coupling was expected due to the presence of the newly formed phosphonate bond. In addition, the methylene protons adjacent to the amide nitrogen went from being equivalent in **4.72** to diastereotopic in NMR of the product, signifying that the achiral nitrogen in **4.72** is now a chiral atom. The two methyl phosphonate protons at δ 2.41 and 2.60 ppm and the presence of a phosphorus resonance via ^{31}P

NMR (Fig. 4.16A) indicated that **4.21** had reacted with **4.72**, however, the methyl phosphonate protons were fairly deshielded in comparison to the chemical shifts observed for the C1 and C6 glucose phosphonate products ($\delta \sim 1\text{--}2$ ppm). Also, analysis of the COSY spectra (Fig. 4.16B) indicated that the methyl phosphonate protons only coupled to themselves implying that the neighbouring carbon did not possess any hydrogen atoms. This spectral evidence suggested that the nucleophilic methyl phosphonate anion attacked the electrophilic amide carbon on the phthalimide, to form an addition product (**4.78**) (Fig. 4.17). To confirm this hypothesis, the mass of the addition product was analyzed and confirmed by mass spectrometry, also suggesting the formation of **4.78** ($m/z = 614.5$).

A)



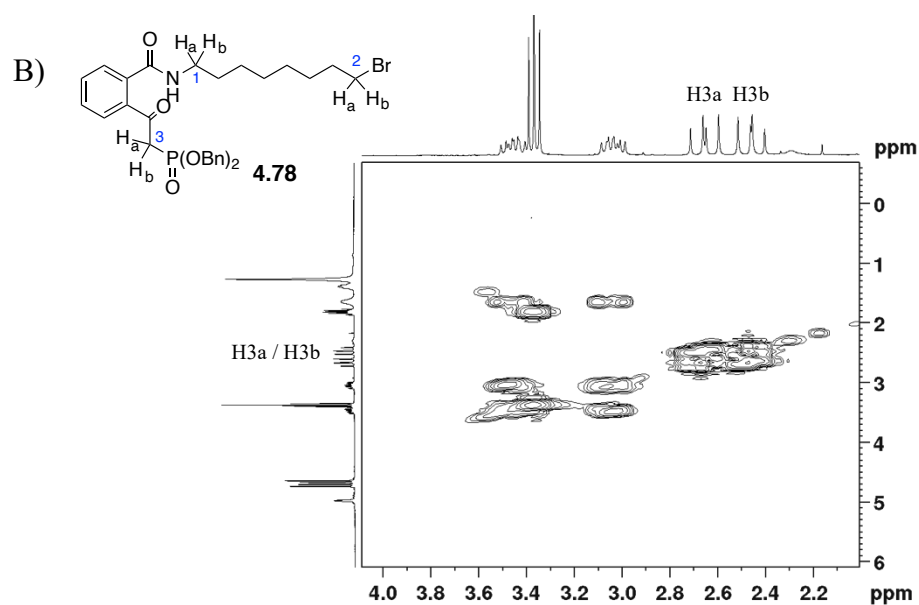


Figure 4.16. A section of the ¹H NMR spectra of **4.72** and ¹H and ³¹P NMR spectra of **4.78** (A) and a section of the COSY 2D NMR spectra of **4.78** (B).

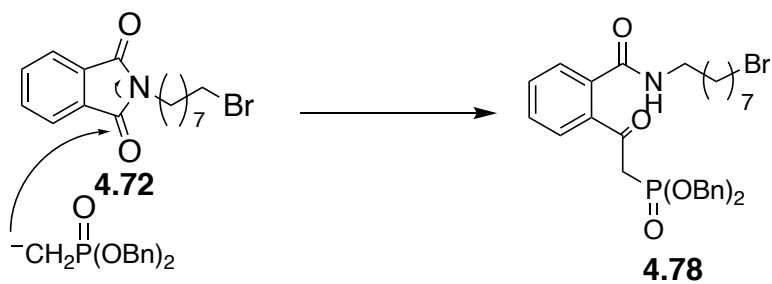


Figure 4.17. Proposed mechanism to afford addition product, **4.78**.

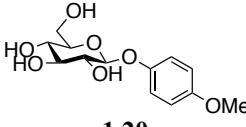
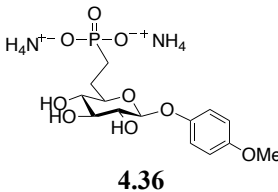
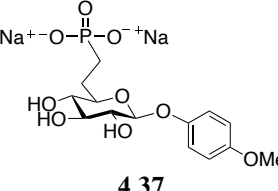
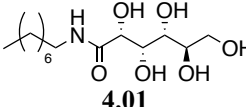
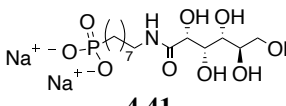
To prevent the formation of compound **4.78**, the phosphonate was substituted onto **4.70** first, to generate **4.74** followed by substitution onto the phthalimide ring to afford the desired phthalimide alkyl phosphonate product **4.73**. In this reaction, dibenzyl phosphite (**4.20**) was used

to prevent the elongation of the alkyl chain by one carbon. Treatment of **4.73** with hydrazine monohydrate yielded the desired amine **4.75**, which was refluxed with **4.76** to afford the *N*-alkyl gluconamide phosphonate derivative **4.77**. Compound **4.77** was subjected to hydrogenolysis (Pd/C) conditions, however, due to the small mass present, only the sodium phosphonate derivative **4.41**, was formed since it was expected that the IRI activity of the ammonium derivative **4.40** would be equivalent to the other ammonium phosphate compounds.

4.5.4 Evaluating the solubility and IRI activity of 4.36, 4.37, and 4.41

Due to the unsuccessful attempts at synthesizing some of the phosphonate derivatives, only compounds **4.36**, **4.37**, and **4.41** were evaluated for their solubility and IRI activity and compared against their parent compound (Table 4.6). All of the phosphonate derivatives were found to be remarkably more soluble than their parent compounds. Specifically, **4.36** and **4.37** were approximately 3x more soluble than **1.20**, and the highest increase in solubility was observed by **4.41** which was 250x more soluble than **4.01**.

Table 4.6. Solubility and IC₅₀ values of IRIs and their phosphonate derivatives. IC₅₀ values are reported with 95% confidence interval.

		Compound structure	Solubility in PBS (mM)	IC ₅₀ (mM)
4-methoxyphenyl- β -D-glucoside	Parent structure	 1.20	110	18 $\pm$ 1
	Ammonium C6-phosphonate derivative	 4.36	350	5 $\pm$ 1
	Sodium C6-phosphonate derivative	 4.37	300	51 $\pm$ 9
N-octyl-D-gluconamide	Parent structure	 4.01	1	0.09 $\pm$ 0.03
	Sodium phosphonate derivative	 4.41	250	21 $\pm$ 3

Using the splat cooling assay,³⁰ the IRI activity of the parent compounds and their phosphonate derivatives were analyzed. All compounds exhibited a full dose-response to ice recrystallization and IC₅₀ values were generated (Fig. 4.18). As expected, **4.36**, the ammonium C6-phosphonate analogue of **1.20** was the most IRI active and had an IC₅₀ value of 5 $\pm$ 1 mM. This agrees with the IC₅₀ values observed for the previous ammonium phosphonate derivatives and ammonium salts, implying that the potency of the ammonium ion towards IRI activity is still exhibited in the *O*-aryl glucoside class. Contrarily, **4.37**, the sodium phosphonate analogue of **1.20**, was much less active than the parent compound, exhibiting an IC₅₀ value of 51 $\pm$ 9 mM. Assuming

that there was no contribution from the sodium ions, these results show that substituting the *C6*-hydroxyl group of **1.20** with a sodium phosphonate moiety is detrimental to IRI activity. Former studies have investigated the significance of the *C6*-hydroxyl group to the IRI activity of **1.20**.¹ It was observed that replacing the *C6*-hydroxyl group in **1.20** with either a methoxy group, methanol, galactose, glucose, or forming the xylose derivative in which C6 is replaced with a hydrogen, completely abolishes IRI activity. Therefore, observing a diminished IRI activity for **4.37** follows this trend, however, these results conflict with those observed for **4.19**, in which substituting the *C6*-hydroxyl group for a phosphonate group increased the IRI activity of the parent compound. For the G6CP derivative, the cause of the increased activity was attributed to the presence of a stronger hydrogen bond acceptor, therefore improving IRI activity due to increased hydration potential. In the case of **1.20**, the *C6*-hydroxyl group may have an alternative contribution to IRI activity.

The *N*-alkyl gluconamide phosphonate derivative, **4.41** ($IC_{50} = 21 \pm 3$ mM), was also significantly less IRI active than its parent compound, **4.01** ($IC_{50} = 0.09 \pm 0.03$ mM). Trends in the *N*-alkyl gluconamide class have suggested that the alkyl chain length contributes to IRI activity and that a delicate balance between hydrophobic and hydrophilic moieties must be kept to maintain IRI activity.^{12,15,54} Thus, installing a phosphonate moiety at the end of the octyl chain in **4.01** may have deviated away from the ideal amphiphilicity required for strong IRI activity in this class. Although **4.41** is significantly less active than its parent compound, it is the most active out of all the sodium phosphonate derivatives and its activity is similar to IRIs that have been used in the Ben laboratory for cryopreservation purposes.^{14,18}

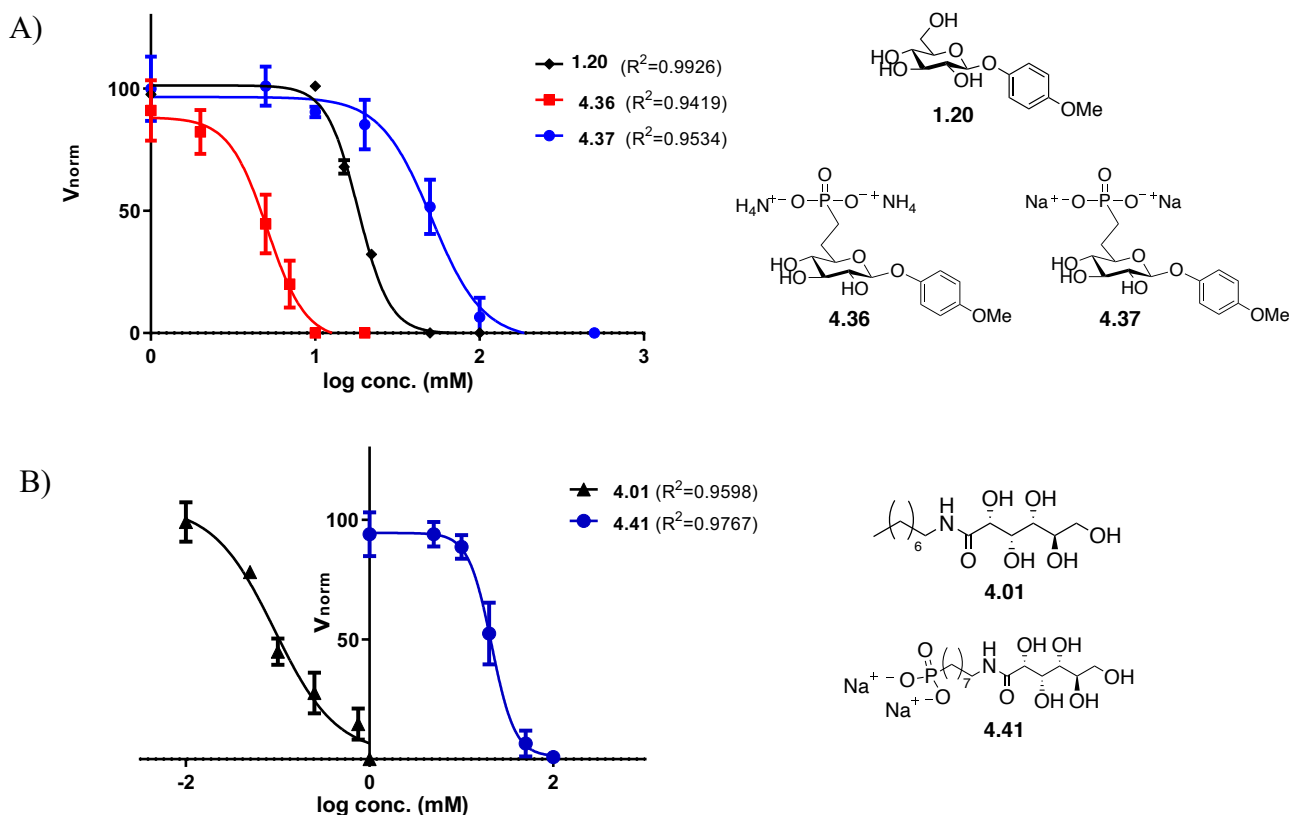


Figure 4.18. IRI activity of A) **1.20** and its phosphonate derivatives **4.36** and **4.37**; and B) **4.01** and its sodium phosphonate derivative **4.41**. A non-linear regression line was fitted to points to generate IC_{50} values. Error bars represent % SD for $n=3$ experiments.

Therefore, the synthesized phosphonate analogues, **4.36**, **4.37**, and **4.41**, provide further insight into the contributions of phosphonates towards the solubility and IRI activity of previously discovered IRI active compounds. In these specific cases (with exception of **4.36** due to the presence of the ammonium), an inverse relationship is observed between solubility and IRI activity, however, future studies should evaluate a larger scope of phosphonate compounds to arrive at more concrete conclusions.

4.6 Summary

In this chapter, compounds possessing a phosphonate functional group were synthesized and evaluated for IRI activity as a method of generating IRI active compounds with high solubilities. Phosphonate compounds of D-glucose and α -methoxy glucoside (**4.27**), were synthesized and evaluated for IRI activity and all compounds were soluble up to 500 mM. The IRI activity of the glucose-1-phosphonate derivatives varied depending on its counterion and out of ammonium, sodium, potassium, and calcium phosphonate derivatives (**4.14-4.17**), the ammonium phosphonate ($IC_{50} = 3.0 \pm 0.5$ mM) derivative was more IRI active than the sodium ($IC_{50} = 51 \pm 8$ mM), potassium ($IC_{50} = 60 \pm 12$ mM), and calcium ($IC_{50} = 41 \pm 8$ mM) phosphonate derivatives. After further analysis of various ammonium salts and the observation that all the ammonium salts have similar IRI activities, it was concluded that the strong IRI activity of the ammonium phosphonate derivative **4.14**, was a result of the presence of the ammonium ions. Thus, the similar IRI activities exhibited by the sodium, potassium, and calcium phosphonate derivatives (**4.15-4.17**), was considered the IRI activity of glucose-1-phosphonate without the interference of counterions. Due to the IRI activity of the ammonium ions, and the inactivity of the sodium, potassium, and calcium ions, the Hofmeister series was hypothesized to correlate to IRI activity. However, this theory was disproved by observation that tetramethylammonium chloride is more kosmotropic (i.e. more strongly hydrated) than ammonium and less IRI active. In comparison to their parent compounds, glucose *C1*- and *C6*- sodium phosphonate derivatives were approximately 2x more IRI active.

In light of these results, phosphonate derivatives of IRIs belonging to the *O*-aryl glucoside, *N*-aryl-D-gluconamide and *N*-alkyl-D-gluconamide class were synthesized. Unfortunately, the synthesis of *C6*-phosphonate derivative of *N*-2-fluorophenyl-D-gluconamide

(**4.38** & **4.39**) and *p*-bromophenyl- β -D-glucoside (**4.34** & **4.35**) were unsuccessful. Phosphonate derivatives of *p*-methoxyphenyl- β -D-glucoside (**4.36** & **4.37**) and *N*-octyl-D-gluconamide (**4.41**) were successfully synthesized and all were several times more soluble than their parent compounds. The ammonium phosphonate derivative **4.36** ($IC_{50} = 5 \pm 1$ mM) exhibited IRI activity, similar to that observed for the other ammonium phosphonate compounds and the sodium phosphonate derivatives, **4.37** ($IC_{50} = 51 \pm 9$ mM) and **4.41** ($IC_{50} = 21 \pm 3$ mM) were several times less active than their respective parent compounds. Overall, this study showed that incorporating a phosphonate functional group into the chemical structure of a compound can increase its solubility, however, it has variable impact on IRI activity. For compounds exhibiting low IRI activity (D-glucose, and α -methoxyglucoside), incorporating a sodium phosphonate group resulted in an IRI with higher activity (**4.15** and **4.19**); for compounds exhibiting high IRI activity (**1.20** and **4.01**) incorporating a phosphonate group decreased its IRI activity (**4.37**, and **4.41**). This confirms that for the *O*-aryl- β -D-glucoside and *N*-aryl-D-gluconamide classes, deviations away from the ideal hydrophobic/hydrophilic balance of IRIs is detrimental to their IRI activity.

4.7 Experimental Procedures and Compound Characterization

4.7.1 Ice Recrystallization Inhibition Assay

All compounds were measured for IRI activity using the modified splat cooling assay.³⁰ Samples were dissolved in phosphate buffered saline (PBS) and a 10 μ L drop of the solution was dispensed from a height of 2 metres, onto a polished aluminum block cooled to -78 °C with dry ice. The frozen wafer was carefully removed from the block, placed on a coverslip and transferred to a Cryostage at -6.4 °C connected to a cooling bath (Digital One RTE7 from Thermo Scientific NESLAB), a Peltier unit (Series 800 from Alpha Omega Instruments), a fish pump (SAX1 from

Pen Plax), and a drying tube. The wafer was allowed to anneal at this temperature for 5 minutes. After the annealing time, the wafer was photographed between cross polarizing filters using a digital camera (Nikon CoolPix 5000 or OEM Microscope Camera purchased from Nikon) fitted to the microscope (20X magnification). Images from the microscope camera were viewed and captured using NIS Elements D software (version 5.30.01) with the exposure set to 8 ms. Images were then analyzed using ImageJ software, by circling each ice crystal with well-defined boundaries using the polygon function to generate the grain areas. The ice crystals are categorized into “bins” according to their sizes where the smallest ice grain (size corresponding to the size of ice grain at $t = 0$ min) is considered “bin 1” and the following bin sizes increase in 0.001 mm^2 increments. The proportion of ice crystals in each bin was used to determine the initial rate of ice recrystallization and is normalized against the PBS control (v_{norm}). Different concentrations of each compound were tested to determine the compound-dependent rates of ice recrystallization and normalized rates were plotted and fitted onto a four-parameter non-linear regression to generate a dose response curve.

In *Section 4.43*, compounds **4.14** and **4.15** were evaluated for IRI activity using the sucrose sandwich assay.³⁹ Samples were dissolved in 40% sucrose and a 1 μL drop of the solution was sandwiched between two coverslips then transferred onto an aluminum block cooled to -78°C with dry ice. The sandwiched frozen wafer was transferred to a cryostage where it was allowed to anneal at -6.4°C for 40- or 90-min. After the annealing time, the wafer was photographed between crossed polarizing filters using a digital camera (OEM Microscope Camera purchased from Nikon) fitted to the microscope (20X magnification). Images from the microscope were viewed and captured using NIS Elements D software (version 5.30.01) with the exposure set to 8 ms. Images were then analyzed using ImageJ software, by circling each ice crystal with well-

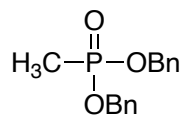
defined boundaries using the polygon function to generate the grain areas. Areas were inputted into Microsoft Excel where the average grain size of triplicate experiments was compared to the PBS positive control to generate a % mean grain size.

4.7.2 *General Experimental*

All reagents used in the outlined synthesis were purchased commercially. Anhydrous reactions were performed using a flame-dried flask flushed with argon gas and anhydrous reagents were transferred either using an oven-dried canula or an oven-dried glass needle and syringe. Anhydrous solvents such as tetrahydrofuran (THF), dichloromethane (DCM), toluene, and acetonitrile were distilled via a solvent purification system. Chemical reactions were monitored using thin layer chromatography (TLC) with a 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). TLC plates were visualized by a short-wavelength (254 nm) ultra-violet lamps or by staining with *p*-anisaldehyde stain or triphenylphosphine–ninhydrin stain. All column chromatography was performed with E. Merck silica gel 60 (230-400 mesh). The pH was tested using a wide-range pH test paper (Hydriion, pH 0.0 – 13.0). All 1D and 2D NMR spectroscopy was performed on a Bruker AVANCE 400 or Bruker AVANCE 600 spectrometer. Deuterated NMR solvents used included deuterated DMSO (DMSO- d_6), deuterated acetone (acetone- d_6), deuterated chloroform ($CDCl_3$) or deuterated water (D_2O). The solvent peak was used as the internal standard of all NMR spectra and chemical shifts were reported in ppm. Splitting patterns are assigned as either s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet; and br, broad. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed on a Micromass Quatro-TOF I Electrospray Ionization mass spectrometer LC/MS with a pump rate of 20 μ L/min.

4.7.3 Compound characterization

Dibenzyl methyl phosphonate (4.21)

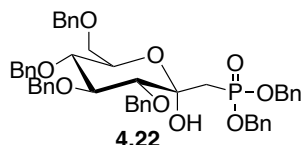


4.21

A suspension of 60% sodium hydride (0.99 g, 24.8 mmol) in anhydrous tetrahydrofuran (22 mL) was cooled to 0 °C. Dibenzyl phosphite (**4.20**) (5 g, 19.1 mmol) was added and the mixture was stirred for 1 h. Methyl iodide (1.5 mL, 24.8 mmol) was added, and the solution was stirred for an additional 2 h then quenched by the addition of water. The quenched reaction was diluted with diethyl ether and the aqueous layer was extracted with diethyl ether (4x). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. Purification by flash chromatography (30% ethyl acetate in hexanes to 100% ethyl acetate) afforded compound **4.21** (3.06 g, 58% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.35 (m, 10H, aromatic H), 4.93 (dd, J=8.9, 11.8 Hz, 4H), 1.44 (d, ²J_{H-P}=17.7 Hz, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 136.4, 136.3 (2 x quaternary C), 128.6, 128.4, 127.9 (10 x aromatic C) 67.1 (2C), 11.0 (d, ¹J_{C-P}=46 Hz) ppm. ³¹P NMR (162 MHz, CDCl₃): 28.8 ppm.

LRMS (ESI⁺): *m/z* calcd. for C₁₅H₁₇O₃P [M + Na]⁺ 299.1, found 298.9

2,3,4,6-Tetra-*O*-benzyl-*C*-(1'-dibenzylphosphonomethyl)-α-*D*-glucopyranoside (4.22)

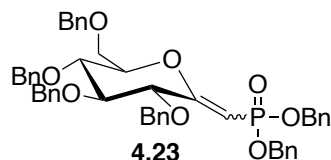


A solution of compound **4.21** (2.41 g, 8.73 mmol) in anhydrous tetrahydrofuran (40 mL) was cooled to -78 °C. 2.5 M *n*-butyllithium in hexanes (3.49 mL, 8.73 mmol) was slowly added and

the reaction was stirred for 30 min at the same temperature. A solution of compound **3.30** (2.35 g, 4.36 mmol) in THF (6 mL) was added to the mixture and the cold bath was removed. The stirring continued at to -78 °C for 2 h. The mixture was quenched by addition of saturated $\text{NH}_4\text{Cl}_{(\text{aq})}$ and the organic layer was separated. The organic layer was washed with water and the combined aqueous layers were extracted with dichloromethane (3x). The combined organic layers were dried with anhydrous sodium sulfate, filtered, and concentrated. Purification by silica flash column chromatography (4:1 hexane/ethyl acetate) afforded pure **4.22** (2.06 g, 58% yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.32-7.12 (m, 30H, aromatic H), 5.81 (br s, 1H, OH), 5.07 (d, $J=7.4$ Hz, 1H, CHPh), 5.07 (d, $J=7.8$ Hz, 1H, CHPh), 4.94-4.88 (m, 4H, CHPh), 4.84-4.79 (m, 2H, CHPh), 4.61 (d, $J=11.7$ Hz, 1H, CHPh), 4.54 (d, $J=11.0$ Hz, 1H, CHPh), 4.41 (d, $J=11.9$ Hz, 1H, CHPh), 4.29 (d, $J=12.1$ Hz, 1H, CHPh), 4.14 (t, $J=9.3$ Hz, 1H, H-3), 4.12 (m, 1H, H-5), 3.74 (dd, $J=3.8, 10.7$ Hz, 1H, H-6a), 3.66 (t, $J=9.8$ Hz, 1H, H-4), 3.55 (dd, $J=1.9, 10.9$ Hz, 1H, H-6b), 3.23 (d, $J=9.7$ Hz, 1H, H-2), 2.30 (dd, $J=15.2$ Hz, $^2J_{\text{H-P}}=17.6$ Hz, 1H, H-7b), 1.72 (dd, $J=15.0$ Hz, $^2J_{\text{H-P}}=18.8$ Hz, 1H, H-7a) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 138.6, 138.3, 137.9, 135.8 (6 x quaternary C), 128.7-127.7 (30 x aromatic C), 97.0 (d, $^2J_{\text{C-P}} = 8.2$ Hz, C-1), 83.3 (d, $^4J_{\text{C-P}}=4.0$ Hz, C-3), 82.7 (d, $^3J_{\text{C-P}}=13.8$ Hz, C-2), 78.4 (C-4), 75.7, 75.2, 74.9, 73.3 (4 x CHPh), 71.2 (C-5), 68.7 (C-6), 68.2 (d, $^2J_{\text{C-P}}=5.5$ Hz, P-O- CH_2PH), 66.9 (d, $^2J_{\text{C-P}}=5.9$ Hz, P-O- CH_2PH), 34.5 (d, $^1J_{\text{C-P}}=134.0$ Hz, C-7) ppm. ^{31}P NMR (162 MHz, CDCl_3): 29.5 ppm.

LRMS (ESI⁺): m/z calcd. for $\text{C}_{49}\text{H}_{51}\text{O}_9\text{P}$ $[\text{M} + \text{Na}]^+$ 837.3, found 837.0

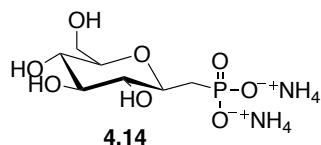
(E/Z)-C-(1-deoxy-2,3,4,6-tetra-O-benzyl-D-glucopyranosyl-2-ylidene) methanephosphonate (4.23)



Pyridine (3.4 mL, 41.9 mmol) and trifluoroacetic anhydride (3 mL, 20.9 mmol) were successfully added to a solution of compound **4.22** (2.28 g, 2.79 mmol) in anhydrous THF (29 mL) at 0 °C. The solution was stirred at room temperature for 2 h. The solution was concentrated, and dichloromethane was added. The organic mixture was extracted with saturated sodium bicarbonate, dried with anhydrous magnesium sulfate, filtered, and concentrated. Purification by silica flash chromatography (2:1 hexane/ethyl acetate) afforded pure compound **4.23** (1.73 g, 78% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.32-7.21 (m, 28H, aromatic H), 7.15-7.12 (m, 2H, aromatic H), 5.12 (dd, ¹J_{H-H} = 12.8 Hz, ²J_{H-P} = 0.9 Hz, 1H, CHPh), 5.05-4.96 (m, 4H, 4 x CHPh), 4.70 (d, J = 11.1 Hz, 2H, 2 x CHPh), 4.66 (d, J = 11.9 Hz, 1H, CHPh), 4.62 (d, J = 11.4 Hz, 1H, CHPh), 4.55 (d, J = 11.8 Hz, 1H, CHPh), 4.51 (d, J = 11.1 Hz, 1H, CHPh), 4.51 (d, J = 12.2 Hz, 1H, CHPh), 4.41 (d, J = 11.9 Hz, 1H, CHPh), 3.96 (dt, J = 2.9, 9.9 Hz, 1H, H-5), 3.91 (dt, J = 1.4, 6.6 Hz, 1H, H-2), 3.82 (dd, J = 7.0, 9.9 Hz, 1H, H-4), 3.73 (t, J = 6.6 Hz, 1H, H-3), 3.65-3.60 (m, 2H, H-6a/b) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 165.9 (C-1), 138.2, 138.1, 138.01, 137.34, 137.01 (d, ³J_{C-P} = 6.8 Hz), 137.90 (d, ³J_{C-P} = 6.3 Hz) (6 x quaternary aromatic C), 128.7-127.8 (30 x aromatic CH), 94.4 (d, ¹J_{C-P} = 191.4 Hz, C-7), 83.5 (C-3), 78.8 (C-2), 78.7 (C-5), 77.1 (C-4), 74.2, 74.1, 73.7, 72.9 (4 x CH₂Ph), 68.3 (C-6), 67.4 (d, ²J_{C-P} = 5.1 Hz, P-O-CH₂Ph), 67.1 (d, ²J_{C-P} = 5.1 Hz, P-O-CH₂Ph) ppm. ³¹P NMR (162 MHz, CDCl₃): 17.7 ppm.

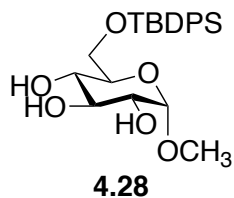
LRMS (ESI⁺): *m/z* calcd. for C₄₉H₄₉O₈PNa [M + Na]⁺ 819.3, found 819.3.

Ammonium C-(1-deoxy- β -D-glucopyranosyl) methanephosphonate (**4.14**)



Compound **4.23** (0.561 g, 0.705 mmol) and 5% palladium on carbon (0.031 g, 0.291 mmol) in ethyl acetate (24 mL) and methanol (24 mL) was degassed under vacuum and saturated with hydrogen gas via a hydrogen gas filled balloon. Suspension was stirred at room temperature for 12 h, filtered through a pad of celite and concentrated to give the phosphonic acid (**4.24**) as a colorless oil. The oil was then dissolved in water (1 mL), and adjusted to pH 8 with 2 M NH_4OH , then lyophilized to obtain compound **4.14** as an off-white foam (0.164 g, 78% yield). ^1H NMR (400 MHz, D_2O): δ = 3.77 (d, J =12.1 Hz, 1H, H-6a), 3.52-3.43 (m, 2H, H-6b, H-1), 3.35 (t, J =8.9 Hz, 1H, H-3), 3.31-3.26 (m, 1H, H-5), 3.21-3.11 (m, 2H, H-2, H-4), 1.76 (dd, J =5.6, 17.1 Hz, 2H, H-7a/b) ppm. ^{13}C NMR (101 MHz, D_2O): δ = 79.4 (C-5), 77.2 (C-3), 76.2 (C-2), 75.0 (d, J =8.3 Hz, C-1), 69.9 (C-4), 61.0 (C-6), 33.3 (d, J =128.9 Hz, C-7) ppm. ^{31}P NMR (162 MHz, D_2O): 17.3 ppm. LRMS (ESI $^+$): m/z calcd. for $\text{C}_7\text{H}_{15}\text{O}_8\text{P}$ [$\text{M} - \text{H}$] $^-$ 256.0, found 296.9 [$\text{M} + \text{K}$] $^+$.

Methyl-6-*O*-(*tert*-butyldiphenylsilyl)- α -D-glucopyranoside (**4.28**)

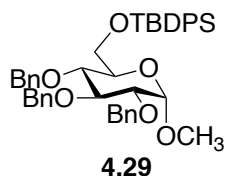


A flask containing **4.27** (5 g, 25.74 mmol) was charged with dry DMF (53 mL) and cooled to 0 $^{\circ}\text{C}$ under argon. To the stirring mixture was added DMAP (0.315g, 2.57 mmol), Et_3N (2.98 mL, 21.36 mmol) by syringe and *tert*butyl(chloro)diphenylsilane dropwise (4.42 mL, 16.99 mmol). The

reaction was warmed to room temperature and stirred for 1 h. The reaction was diluted with ethyl acetate, washed with water and brine and the organic layer was dried with anhydrous Na₂SO₄ and concentrated. The crude product was purified by recrystallization in ethyl acetate, yielding compound **4.28** as a white solid (6.23 g, 56% yield). ¹H NMR (400 MHz, CD₃OD) δ 7.77 – 7.69 (m, 4H, aromatic H), 7.43 – 7.33 (m, 6H, aromatic H), 4.71 (d, *J* = 3.7 Hz, 1H, H1), 3.97 (dd, *J* = 11.0, 2.1 Hz, 1H, H6), 3.86 (dd, *J* = 11.0, 5.6 Hz, 1H, H6), 3.69 – 3.62 (m, 2H, H3, H5), 3.47 – 3.35 (m, 5H, OCH₃, H2, H4), 1.04 (s, 9H, C(CH₃)₃) ppm. ¹³C NMR (101 MHz, CD₃OD) δ 136.74 (2 x quaternary aromatic C), 134.75, 134.68, 130.72, 130.70, 128.64 (10 x aromatic CH), 101.07 (C-1), 75.26 (C-3), 73.79 (C-4), 73.53 (C-5), 71.63 (C-2), 64.73 (C-6), 55.37 (OCH₃), 27.29 (C(CH₃)₃), 20.09 (C(CH₃)₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₂₃H₃₂O₆SiNa [M + Na]⁺ 455.2, found 455.4.

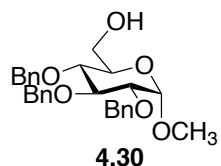
Methyl-2,3,4-tri-*O*-benzyl-6-*O*-(*tert*-butyldiphenylsilyl)-α-D-glucopyranoside (**4.29**)



Compound **4.28** (6.23 g, 14.39 mmol) was dissolved in DMF (83 mL) and cooled to 0 °C in an ice bath. 60% sodium hydride in mineral oil (3.57 g, 89.22 mmol) was added in parts over a 45 min period. Tetrabutylammonium iodide (0.464 g, 1.44 mmol) was added, then benzyl bromide (10.60 mL, 89.22 mmol) was syringed over a 30 min period. The cold bath was removed, and the reaction is warmed to room temperature and stirred overnight at 80 °C. The reaction was quenched with methanol and concentrated under pressure. The salts were re-dissolved in DCM and filtered off through celite. Product was purified by silica flash column chromatography to yield compound **4.29** as a pale-yellow oil (4.05 g, 40% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.72 – 7.68 (m, 4H,

aromatic H), 7.47 – 7.20 (m, 21H, aromatic H), 5.00 (d, J=10.9 Hz, 1H, CHPh), 4.91 (d, J=11.2 Hz, 1H, CHPh), 4.86 (d, J=10.9 Hz, 1H, CHPh), 4.85 (d, J=12.1 Hz, 1H, CHPh), 4.73 (d, J=12.1 Hz, 1H, CHPh), 4.68 (d, J=3.8 Hz, 1H, H-1), 4.63 (d, J=10.9 Hz, 1H, CHPh), 4.60 (d, J=10.0 Hz, 1H, CHPh), 4.60 (d, J=10.0 Hz, 1H, CHPh), 4.05(t, J=9.1 Hz, 1H, H-2), 3.91 – 3.85 (m, 2H, H-3, H-4), 3.74 – 3.71 (m, 1H, H-5), 3.65 – 3.56 (m, 2H, H-6a/b), 3.39 (s, 3H), 1.06 (s, 9H) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ 138.8, 138.3, 135.9, 135.7, 133.7, 133.4 (6 x quaternary aromatic C), 129.6, 129.6, 128.5, 128.5, 128.4, 128.4, 128.4, 128.2, 128.1, 127.9, 127.8, 127.7, 127.6 (25 x aromatic CH), 97.9 (C1), 82.3, 80.4, 77.9, 75.9, 75.1, 73.4, 72.2, 71.5, 63.0, 54.9, 26.9, 19.3 ppm. LRMS (ESI⁺): m/z calcd. for $\text{C}_{44}\text{H}_{50}\text{O}_6\text{SiNa}$ $[\text{M} + \text{Na}]^+$ 725.3, found 725.7.

Methyl-2,3,4-tri-*O*-benzyl- α -D-glucopyranoside (**4.30**)

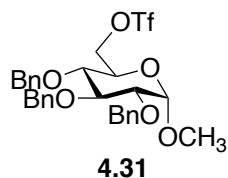


A stirring solution of compound **4.29** (3.97 g, 5.65 mmol) was treated with 1M TBAF in THF (16.95 mL, 16.95 mmol) and glacial acetic acid (1.07 mL, 18.65 mmol). The mixture was allowed to react overnight at room temperature. After completion, the mixture was concentrated under pressure and purified by silica flash column chromatography to yield compound **4.30** as a clear oil (2.23 g, 85% yield). ^1H NMR (400 MHz, CDCl_3) δ 7.44 – 7.28 (m, 15H, aromatic H), 5.02 (d, J= 10.9 Hz, 1H, CHPh), 4.91 (d, J= 10.9 Hz, 1H, CHPh), 4.87 (d, J= 10.9 Hz, 1H, CHPh), 4.82 (d, J = 12.1 Hz, 1H, CHPh), 4.68 (d, J = 12.1 Hz, 1H, CHPh), 4.67 (d, J = 10.9 Hz, 1H, CHPh), 4.60 (d, J = 3.6 Hz, 1H, H1), 4.04 (t, J = 9.3 Hz, 1H, H2), 3.82 – 3.65 (m, 3H, H6a, H6b, H3), 3.59 – 3.50 (m, 2H, H4, H5), 3.39 (s, 3H, OCH_3) ppm. ^{13}C NMR (101 MHz, CDCl_3) δ 138.79, 138.20, 138.17 (3 x quaternary aromatic C), 128.54, 128.47, 128.18, 128.09, 128.03, 128.01, 127.93,

127.69 (15 x aromatic CH), 98.22 (C-1), 82.01 (C-2), 80.03 (C-5), 77.45 (C-4), 75.81 (OCH₂Ph), 75.09 (OCH₂Ph), 73.48 (OCH₂Ph), 70.76 (C-3), 61.87 (C-6), 55.25 (OCH₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₂₈H₃₂O₆Na [M + Na]⁺ 487.2, found 487.4.

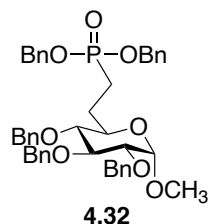
Methyl-2,3,4-tri-*O*-benzyl-6-*O*-trifluoromethanesulfonyl- α -D-glucopyranoside (**4.31**)



To a solution of pyridine (0.44 mL) in CH₂Cl₂ (21.0 mL) was added Tf₂O (0.79 mL, 4.7 mmol) at -10 °C under inert atmosphere. The mixture was stirred at -10 °C for 15 min and compound **4.30** (1.095 g, 2.36 mmol) was added in CH₂Cl₂ (10 mL). The mixture was stirred at -10 °C for 2 h, diluted with CH₂Cl₂ and washed with dH₂O. The organic layer was dried over Na₂SO₄, filtered, concentrated, and purified by silica flash column chromatography to afford compound **4.31** (1.06 g, 75% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.41 – 7.28 (m, 14H, aromatic H), 7.25 – 7.23 (m, 1H, aromatic H), 4.89 (m, 4H, (OCH₂Ph)₂), 4.63 (m, 2H, OCH₂Ph), 4.56 – 4.55 (m, 1H, H1), 4.53 (d, *J* = 2.1 Hz, 1H, H6a), 4.46 (dd, *J* = 10.7, 5.3 Hz, 1H, H6b), 4.02 (dd, *J* = 9.6, 8.8 Hz, 1H, H2), 3.86 (ddd, *J* = 10.1, 5.3, 2.0 Hz, 1H, H5), 3.53 (dd, *J* = 9.6, 3.5 Hz, 1H, H3), 3.42 (dd, *J* = 10.2, 8.8 Hz, 1H, H4), 3.38 (s, 3H, OCH₃) ppm. ¹³C NMR (101 MHz, CDCl₃) δ 138.51, 137.99, 137.57 (3 x quaternary aromatic C), 128.76 (C-Ar), 128.69, 128.62, 128.32, 128.25, 128.24, 128.22, 128.09, 127.91 (15 x aromatic C), 98.22 (C-1), 81.94 (C-2), 79.90 (C-3), 76.57 (C-4), 75.96 (OCH₂Ph), 75.22 (OCH₂Ph), 75.07 (C-6), 73.66 (OCH₂Ph), 68.37 (C-5), 55.66 (OCH₃) ppm.

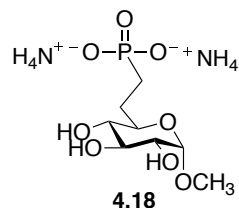
LRMS (ESI⁺): *m/z* calcd. for C₂₉H₃₁F₃O₈SNa [M + Na]⁺ 619.2, found 501.4. Due to dissociation of the triflate group.

Methyl-6-deoxy-6-(dibenzyl)phosphonomethyl-2,3,4-tri-*O*-benzyl- α -D-glucopyranoside (4.32)



To a solution of compound **4.21** (1.97 g, 7.12 mmol) and 2.5M *n*-BuLi (2.85 mL, 7.12 mmol) in THF (18.6 mL) at -78 °C, compound **4.31** (1.06 g, 1.78 mmol) was added slowly. After stirring for 1 h at -78 °C the reaction was quenched with saturated NH₄Cl. Diethyl ether was added to dilute the solution and the organic layer was washed with NH₄Cl, water and brine. The organic phases were combined, dried over anhydrous MgSO₄, filtered, concentrated, and purified by silica flash column chromatography, yielding compound **4.32** as a clear oil (2.73 g, 53% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.39 – 7.17 (m, 25H, aromatic H), 5.02 – 4.89 (m, 5H, 5 x CHPh), 4.83 (d, J=11.0 Hz, 1H, CHPh), 4.78 (d, J=11.0 Hz, 1H, CHPh), 4.77 (d, J=12.0 Hz, 1H, CHPh), 4.64 (d, J=12.0 Hz, 1H, CHPh), 4.53 (d, J=10.7 Hz, 1H, CHPh), 4.46 (d, J=3.5 Hz, 1H, H-1), 3.92 (t, J=9.4 Hz, 1H, H-3), 3.50-3.42 (m, 2H, H-2, H-5), 3.25 (s, 3H, OCH₃), 3.12 (t, J=9.1 Hz, 1H, H-4), 2.14-1.90 (m, 2H, H-6a, H-7a), 1.72-1.53 (m, 2H, H-6b, H-7b) ppm. ¹³C NMR (101 MHz, CDCl₃) δ 138.7, 138.2, 138.2, 136.4, 136.4 (5 x quaternary aromatic C), 128.3, 128.3, 128.2, 128.2, 128.1, 128.0, 128.0, 127.8, 127.8, 127.7, 127.7, 127.6, 127.5, 127.4 (25 x aromatic C), 97.7 (C-1), 81.9 (C-3), 81.5 (C-4), 80.0 (C-2), 75.7 (OCH₂Ph), 75.2 (OCH₂Ph), 73.4 (OCH₂Ph), 69.9 (d, J_{C-P}=18.1 Hz, C5), 67.2 (d, J_{C-P}=5.6 Hz, POCH₂Ph), 67.1 (d, J_{C-P}=5.6 Hz, POCH₂Ph), 55.1 (OCH₃), 24.7 (C6), 22.8 (C7) ppm. ³¹P NMR (162 MHz, CDCl₃): 33.2 ppm.

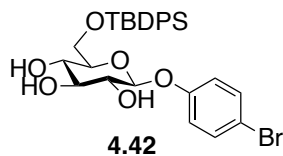
Methyl-6-deoxy-6-dihydroxyphosphonomethyl- α -D-glucopyranoside diammonium salt (4.18)



Compound **4.32** (686 mg, 0.95 mmol) was dissolved in the presence of 5% palladium on carbon (0.05 g, 0.470 mmol) in methanol (33 mL) and ethyl acetate (33 mL). The mixture was stirred overnight under H₂ gas. After completion, the solution was filtered through celite and concentrated to give the phosphonic acid which was treated with NH₄OH until pH = 8 to obtain the ammonium salt **4.18** (0.285 g, 98% yield). ¹H NMR (400 MHz, D₂O) δ 4.73 (m, 1H, H1), 3.60 – 3.48 (m, 3H, H2, H4, H5), 3.36 (s, 3H, OCH₃), 3.22 (t, J = 9.1 Hz, 1H, H3), 2.08 – 1.92 (m, 1H, H6a), 1.76 – 1.52 (m, 2H, H6b, H7a), 1.51 – 1.33 (m, 1H, H7b) ppm. ¹³C NMR (101 MHz, D₂O) δ 99.01 (C1), 72.94 (C2), 72.87 (C3), 71.74 (d, J_{C-P} = 16.7 Hz) (C5), 71.37 (C4), 54.89 (OCH₃), 25.39 (C-6), 24.19 (d, J_{C-P} = 114.6 Hz) (C-7) ppm. ³¹P NMR (162 MHz, D₂O) δ 23.32 ppm.

LRMS (ESI⁺): *m/z* calcd. for C₈H₁₇O₈PNa [M + Na]⁺ 295.1, found 295.1.

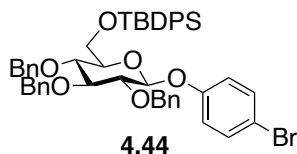
***p*-Bromophenyl-6-*O*-(*tert*-butyldiphenylsilyl)- β -D-glucopyranoside (4.42)**



Compound **1.22** (1.76 g, 5.26 mmol) was dissolved in DMF (11 mL) and cooled to 0 °C under inert atmosphere. To the stirred mixture 4-dimethylaminopyridine (0.064 g, 0.53 mmol), triethylamine (0.61 mL, 4.37 mmol), and *tert*-butyldiphenylsilyl chloride (0.90 mL, 3.47 mmol) was added. The reaction was warmed to room temperature and stirred for an additional hour. The

reaction was diluted with ethyl acetate and washed with water (2x) and brine (2x). The organic layer was dried with anhydrous sodium sulfate and concentrated to afford the crude product. Purification by flash column chromatography (8:2 ethyl acetate/hexane) afforded the pure **4.42** (0.595 g, 70 % yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.63-7.60 (m, 4H, aromatic H), 7.38-7.34 (m, 2H, aromatic H), 7.31-7.25 (m, 6H, aromatic H), 6.88-6.85 (m, 2H, aromatic H), 4.80 (dd, J =2, 5.3 Hz, 1H, H-1), 3.92 (dd, J =3.9, 10.9 Hz, 1H, H-6a), 3.85 (dd, J =5.5, 10.9 Hz, 1H, H-6b), 3.64-3.57 (m, 3H, H-2, H-3, H-4), 3.51-3.47 (m, 1H, H-5), 1.01 (s, 9H, 3 x CH_3) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 156.1, 135.6, 135.6, 132.9, 132.6, 132.4, 129.9, 127.8, 118.7, 115.3, 100.5 (C-1), 76.3 (C-3), 75.6 (C-5), 73.3 (C-2), 71.3 (C-4), 64.2 (C-6), 26.8 (3 x CH_3) ppm. LRMS (ESI $^+$): m/z calcd. for $\text{C}_{28}\text{H}_{33}\text{BrO}_6\text{Si}$ $[\text{M} + \text{Na}]^+$ 595.1, $[\text{M}+2 + \text{Na}]^+$ 597.1 found 595.0 and 597.0.

***p*-Bromophenyl-2,3,4-tri-*O*-benzyl-6-*O*-(*tert*-butyldiphenylsilyl)- β -D-glucopyranoside (**4.44**)**

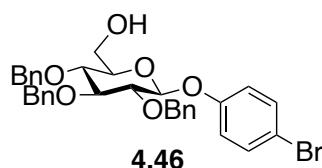


To compound **4.42** (1.22 g, 2.12 mmol) in DMF (12 mL) at 0 °C was added 60% sodium hydride in mineral oil (0.53 g, 13.2 mmol) and stirring continued for 5 min. Benzyl bromide (1.56 mL, 13.2 mmol) was added at the same temperature and the solution was stirred at room temperature overnight. Water was carefully added, and the product was extracted into diethyl ether and washed twice with water. Anhydrous magnesium sulfate was added, crude mixture was filtered and concentrated to afford crude mixture. Purification by silica flash column chromatography (10:1 hexane/ethyl acetate) afforded **4.44** (0.841 g, 47% yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.69-7.65 (m, 4H, aromatic H), 7.43-7.16 (m, 23H, aromatic H), 7.00 (d, J =8.9 Hz, 2H, aromatic H),

5.06 (d, $J=11.1$ Hz, 1H, CHPh), 4.99-4.97 (m, 1H, H-1), 4.97 (d, $J=10.8$ Hz, 1H, CHPh), 4.91 (d, $J=10.6$ Hz, 1H, CHPh), 4.87 (d, $J=10.8$ Hz, 1H, CHPh), 4.87 (d, $J=11.1$ Hz, 1H, CHPh), 4.69 (d, $J=10.7$ Hz, 1H, CHPh), 3.97 (dd, $J=1.9, 11.3$ Hz, 1H, H-6a), 3.91 (dd, $J=4.5, 11.3$ Hz, 1H, H-6b), 3.77-3.75 (m, 3H, H-2, H-3, H-4), 3.53-3.50 (m, 1H, H-5), 1.09 (s, 9H, 3 x CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 156.6, 138.4, 138.3, 138.0, 135.8, 135.6, 133.4, 132.8, 132.4, 129.7, 128.5, 128.2, 128.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.6, 118.8, 115.0, 101.7 (C-1), 84.6 (C-3), 82.3 (C-4), 77.4 (C-2), 76.2 (C-5), 76.0, 75.3, 75.2 (3 x CH₂Ph), 62.6 (C-6), 26.8 (3 x CH₃) ppm.

LRMS (ESI⁺): m/z calcd. for C₄₉H₅₁BrO₆Si [M + Na]⁺ 865.2, [M+2 + Na]⁺ 867.2 found 865.2 and 867.2.

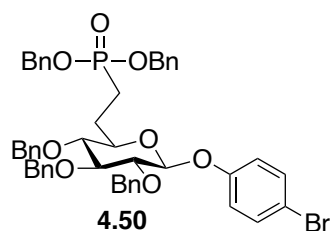
***p*-Bromophenyl-2,3,4-tri-*O*-benzyl- β -D-glucopyranoside (4.46)**



Compound **4.44** (0.408 g, 0.484 mmol) was treated with 1M tetrabutylammonium fluoride in THF (1.45 mL, 1.45 mmol) and glacial acetic acid (0.09 mL, 1.60 mmol). The mixture was allowed to stir overnight under inert atmosphere. The reaction mixture was then concentrated under reduced pressure and purified via flash column chromatography (4:1 hexane/ethyl acetate) to afford the pure **4.46** (0.229 g, 78% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.40 (d, $J=9.0$ Hz, 2H, aromatic H), 7.34-7.26 (m, 15H, aromatic H), 6.89 (d, $J=9.0$ Hz, 2H, aromatic H), 5.01 (d, $J=7.4$, 1H, H-1), 4.96 (d, $J=10.9$ Hz, 1H, CHPh), 4.95 (d, $J=10.8$ Hz, 1H, CHPh), 4.88 (d, $J=10.8$ Hz, 1H, CHPh), 4.84 (d, $J=11.1$ Hz, 1H, CHPh), 4.80 (d, $J=108$ Hz, 1H, CHPh), 4.66 (d, $J=11.1$ Hz, 1H, CHPh), 3.88 (d, 12.3 Hz, 1H, H-6a), 3.76-3.60 (m, 4H, H-2, H-3, H-4, H-6b), 3.50-3.45 (m, 1H, H-5), 1.79

(br s, 1H, OH) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 156.2, 138.0, 132.6, 128.6, 128.5, 128.2, 128.1, 128.0, 127.9, 127.8, 118.4, 101.4, 84.4 (C-2), 81.9 (C-3), 77.2 (C-4), 75.8 (C-5), 75.4, 75.2, 75.2 (3 x CH_2PH), 62.5 (C-6) ppm.

***p*-Bromophenyl-6-deoxy-6-(dibenzyl)phosphonomethyl-2,3,4-tri-*O*-benzyl- β -D-glucopyranoside (4.50)**



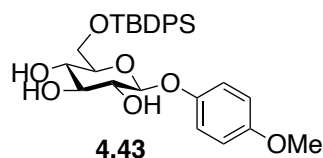
To a solution of pyridine (0.16 mL, 1.94 mmol) in dichloromethane (8 mL) was added triflic anhydride (0.3 mL, 1.69 mmol) at $-10\text{ }^{\circ}\text{C}$ under inert atmosphere. The mixture was stirred at $-10\text{ }^{\circ}\text{C}$ for 15 min, then a solution of **4.46** (0.56 g, 0.843 mmol) in dichloromethane (4 mL) was added. The mixture was stirred at $-10\text{ }^{\circ}\text{C}$ for 2 h, diluted with dichloromethane and washed with water (2x). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated. The crude product (85% yield) was immediately used for the next reaction.

Dibenzyl methyl phosphonate (0.72 g, 2.62 mmol) was dissolved in THF (7 mL) and cooled to $-78\text{ }^{\circ}\text{C}$. A 2.5 M solution of *n*-butyllithium in THF (1.05 mL, 2.62 mmol) was slowly added and the mixture was allowed to stir for 10 min at the same temperature. A solution of the crude triflate in THF was added into the reaction mixture dropwise and stirring continued for 30 min at $-78\text{ }^{\circ}\text{C}$. The reaction was monitored by TLC until disappearance of the triflate at which a saturated aqueous solution of NH_4Cl was added. Diethyl ether was added to dilute the solution and the organic layer was sequentially washed with saturated aqueous NH_4Cl , water, and brine. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated. Product purification by silica flash

column chromatography afforded the pure **4.50** (0.335 g, 46% yield). ^1H NMR (400 MHz, CDCl_3): δ = 7.38-7.24 (m, 28 H, aromatic H), 6.89 (d, J =8.8 Hz, 2H, aromatic H), 5.07-4.80 (m, 10H, 9 x CHPh, H-1), 4.63 (d, J =10.9 Hz, 1H, CHPh), 3.72-3.66 (m, 2H, H-2, H-3), 3.49 (td, J =2.6, 9.4 Hz, 1H, H-5), 3.32-3.25 (m, 1H, H-4), 2.29-2.20 (m, 1H, H-6a), 2.00-1.82 (m, 2H, H-7a/b), 1.80-1.64 (m, 1H, H-6b) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ = 156.1, 138.4, 138.1, 137.8, 136.4 (d, $^2J_{\text{C-P}}$ =3.8 Hz), 136.3 (d, $^2J_{\text{C-P}}$ =3.8 Hz), 132.5, 128.7, 128.5, 128.5, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 118.3, 115.1, 101.0 (C-1), 84.4 (C-2), 82.1 (C-3), 81.1 (C-4), 75.8, 75.3, 75.1 (3 x CH_2Ph), 74.3 (d, $^3J_{\text{C-P}}$ =14.7 Hz, C-5), 24.9 (d, $^2J_{\text{C-P}}$ =4.9 Hz, C-6), 21.1 (d, $^1J_{\text{C-P}}$ = 141 Hz, C-7) ppm. ^{31}P NMR (162 MHz, D_2O): 32.6 ppm.

LRMS (ESI⁺): m/z calcd. for $\text{C}_{48}\text{H}_{48}\text{BrO}_8\text{P}$ $[\text{M} + \text{Na}]^+$ 885.2, $[\text{M}+2 + \text{Na}]^+$ 887.2 found 885.0 and 887.0.

***p*-Methoxyphenyl-6-*O*-tert(butyldiphenylsilyl)- β -D-glucopyranoside (**4.43**)**

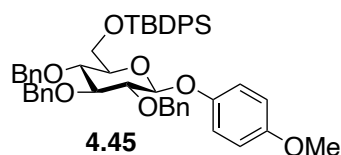


A flask containing **1.20** (2.044 g, 7.14 mmol) was charged with dry DMF (15 mL) and cooled to 0 °C under argon. To the stirring mixture was added DMAP (87.23 mg, 0.714 mmol), Et_3N (0.83 mL, 5.93 mmol) by syringe and tertbutyl(chloro)diphenylsilane dropwise (1.23 mL, 4.71 mmol). The reaction was warmed to room temperature and stirred for 1 h. The reaction was diluted with ethyl acetate, washed with water and brine and the organic layer was dried with Na_2SO_4 and concentrated. The crude product was purified by silica flash column chromatography (100% ethyl acetate) to yield **4.43** as a white solid (1.18 g, 31%). ^1H NMR (400 MHz, Acetone- d_6) δ 7.83 – 7.71 (m, 4H, aromatic H), 7.48 – 7.25 (m, 6H, aromatic H), 7.17 – 7.11 (m, 2H, aromatic H), 6.83

– 6.77 (m, 2H, aromatic H), 4.92 (d, $J = 7.4$ Hz, 1H, 1H), 4.82 (d, $J = 3.7$ Hz, 1H, OH), 4.68 (d, $J = 3.7$ Hz, 1H, OH), 4.49 (d, $J = 4.3$ Hz, 1H, OH), 4.13 (dd, $J = 10.9, 1.9$ Hz, 1H, H6a), 3.92 (dd, $J = 10.9, 6.1$ Hz, 1H, H6b), 3.71 (s, 3H, OCH₃), 3.69 – 3.64 (m, 1H, H5), 3.64 – 3.50 (m, 3H, H2, H3, H4), 1.07 (s, 9H, C(CH₃)₃) ppm. ¹³C NMR (101 MHz, Acetone-d₆) δ 155.60, 152.59, 136.18, 136.10 (4 x quaternary aromatic C), 134.19, 133.95, 130.25, 130.20, 128.30, 128.26, 118.60, 114.90 (14 x aromatic CH), 102.58 (C1), 77.75 (C5), 77.64 (C4), 74.45 (C3), 70.73 (C2), 64.31 (C6), 55.60 (OCH₃), 26.97 (C(CH₃)₃), 19.60 (C(CH₃)₃) ppm.

LRMS (ESI⁺): m/z calcd. for C₂₉H₃₆O₇SiNa [M + Na]⁺ 547.2, found 547.2.

***p*-Methoxyphenyl-2,3,4-tri-*O*-benzyl-6-*O*-tert(butyldiphenylsilyl)- β -D-glucopyranoside (4.45)**

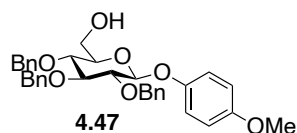


Compound **4.43** (1.18 g, 2.25 mmol) was dissolved in DMF (13 mL) and cooled to 0 °C in an ice bath. 60% NaH (405.2 mg, 10.13 mmol) was added portion wise. The mixture was stirred at 0 °C until gas subsided, then benzyl bromide (1.20 mL, 10.13 mmol) was syringed over a 5 min period. The reaction was stirred overnight at room temperature. The mixture was diluted with water while stirring, then extracted with DCM. The combined extracts were washed with brine, dried over Na₂SO₄, filtered, concentrated under pressure, and purified by silica flash column chromatography (9:1 Hex: EtOAc) to yield **4.45** as a white solid (1.12 g, 63%). ¹H NMR (400 MHz, CDCl₃): δ 7.80 – 7.73 (m, 4H, aromatic H), 7.49 – 7.20 (m, 21H, aromatic H), 7.18 – 7.11 (m, 2H, aromatic H), 6.90 – 6.84 (m, 2H, aromatic H), 5.17 (d, $J = 10.9$ Hz, 1H, OCH₂Ph), 5.05 – 4.89 (m, 5H, H1, OCH₂Ph), 4.72 (d, $J = 10.7$ Hz, 1H, OCH₂Ph), 4.05 – 3.93 (m, 2H, H6a, H6b), 3.82 (s, 3H, OCH₃),

3.82 – 3.79 (m, 3H, H2, H3, H4), 3.58 – 3.50 (m, 1H, H5), 1.13 (s, 9H, C(CH₃)₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 155.36, 151.79, 138.62, 138.55, 138.16, 135.97, 135.69 (7 x quaternary aromatic C), 133.63, 132.99, 129.73, 129.71, 128.56, 128.55, 128.28, 128.15, 128.10, 127.92, 127.87, 127.85, 127.83, 127.74, 118.67, 114.67 (29 x aromatic CH), 102.98 (C1), 84.85 (C2), 82.51 (C4), 77.67 (C3), 76.11 (C5), 76.06 (OCH₂Ph), 75.33 (OCH₂Ph), 75.22 (OCH₂Ph), 62.82 (C6), 55.78 (OCH₃), 26.91 (C(CH₃)₃), 19.39 (C(CH₃)₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₅₀H₅₄O₇SiNa [M + Na]⁺ 817.4, found 817.5.

***p*-Methoxyphenyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside (4.47)**

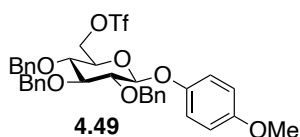


A stirring solution of **4.45** (578 mg, 0.73 mmol) was treated with 1M TBAF-THF (2.18 mL) and glacial acetic acid (0.137 mL, 2.41 mmol). The mixture was allowed to react overnight at rt. After completion, the mixture was concentrated under pressure and purified by silica flash column chromatography (3:1 Hex: EtOAc) yielding **4.47** as a clear oil (350 mg, 86%). ¹H NMR (400 MHz, CDCl₃): δ 7.71–7.26 (m, 15H, aromatic H), 6.98–6.94 (m, 2H, aromatic H), 6.84–6.79 (m, 2H, aromatic H), 5.03 (d, *J*= 11.1 Hz, 1H, OCHPh), 4.95 (d, *J*=10.9 Hz, 1H, OCHPh), 4.94 (d, *J*=7.7 Hz, 1H, H-1), 4.88 (d, *J*=11.1 Hz, 1H, OCHPh), 4.84 (d, *J*=10.8 Hz, 1H, OCHPh), 4.81 (d, *J*=10.8 Hz, 1H, OCHPh), 4.66 (d, *J*=10.8 Hz, 1H, OCHPh), 3.90–3.85 (m, 1H, H-6a), 3.76 (s, 3H, OCH₃), 3.75–3.60 (m, 4H, H-6b, H-2, H-3, H-4), 3.47–3.43 (m, 1H, H-5), 1.88 (t, *J*=6.7Hz, 1H, OH) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 137.9, 137.9, 137.9 (3 x quaternary aromatic C), 128.5, 128.5, 128.0, 127.9, 127.8, 118.1, 116.0, 114.8, 114.7 (19 x aromatic CH), 100.6 (C1), 76.9 (C5), 76.2

(C2), 76.1 (C4), 74.4 (C3), 72.1 (OCH₂Ph), 71.8 (OCH₂Ph), 71.2 (OCH₂Ph), 65.4 (C6), 56.6 (OCH₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₃₄H₃₆O₇Na [M + Na]⁺ 579.2, found 579.3.

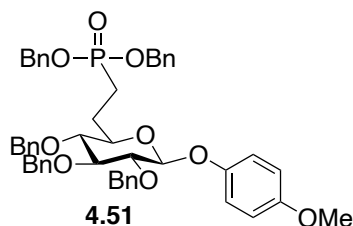
***p*-Methoxyphenyl-2,3,4-tri-*O*-benzyl-6-*O*-trifluoromethanesulfonyl-β-D-glucopyranoside (4.49)**



To a solution of pyridine (0.1 mL, 1.45 mmol) in CH₂Cl₂ (6.0 mL) was added Tf₂O (0.2 mL, 1.26 mmol) at -10 °C under inert atmosphere. The mixture was stirred at -10 °C for 15 min and **4.47** (0.35 g, 0.63 mmol) was added in CH₂Cl₂ (3 mL). The mixture was stirred at -10 °C for 2 h, diluted with CH₂Cl₂ and washed with dH₂O. The organic layer was dried over Na₂SO₄, filtered, concentrated, and purified by silica flash column chromatography (4:1 Hex: EtOAc) to afford pure product **4.49** (0.399 g, 92%). ¹H NMR (400 MHz, CDCl₃): δ 7.36-7.28 (m, 13H, aromatic H), 7.25-7.23 (m, 3H, aromatic H), 7.01 (d, *J*=9.1 Hz, 2H, aromatic H), 6.84 (d, *J*=9.1 Hz, 2H, aromatic H), 5.06 (d, *J*=11.0 Hz, 1H OCHPh), 4.99 (d, *J*=11.0 Hz, 1H OCHPh), 4.92 (d, *J*=11.0 Hz, 1H OCHPh), 4.85 (d, *J*=7.3 Hz, 1H, H-1), 4.82 (d, *J*=11.0 Hz, 1H OCHPh), 4.82 (d, *J*=11.0 Hz, 1H OCHPh), 4.58 (d, *J*=11.2 Hz, 1H OCHPh), 4.58 (dd, *J*=1.7, 10.4 Hz, 1H, H-6a), 4.41 (dd, *J*=6.1, 10.4 Hz, 1H, H-6b), 3.76 (s, 3H, OCH₃), 3.73 (t, *J*=7.5 Hz, 1H, H-2), 3.73 (t, *J*=8.4 Hz, 1H, H-3), 3.64-3.60 (m, 1H, H-5), 3.53 (dd, *J*=8.4, 9.8 Hz, 1H, H-4) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 155.8, 151.1, 138.1, 138.0, 137.1 (5 x quaternary aromatic C), 128.7, 128.5, 128.4, 128.3, 128.5, 127.9, 127.9, 127.9, 119.1, 114.6 (19 x aromatic CH), 103.1 (C1), 84.4 (C2), 81.8 (C3), 76.0 (C4), 75.8 (OCH₂Ph), 75.2 (OCH₂Ph), 75.1 (C6), 74.5 (OCH₂Ph), 72.4 (C5), 55.7 (OCH₃) ppm.

LRMS (ESI⁺): m/z calcd. for C₃₅H₃₅F₃O₉SNa [M + Na]⁺ 711.2, found 711.2.

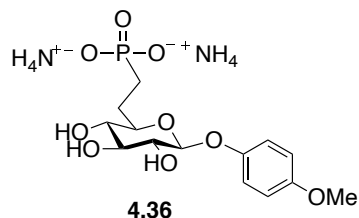
***p*-Methoxyphenyl-6-deoxy-6-(dibenzyl)phosphonomethyl-2,3,4-tri-*O*-benzyl- β -D-glucopyranoside (**4.51**)**



To a solution dibenzyl methylphosphonate (0.64 g, 2.31 mmol) and 2.5 M *n*-butyllithium (0.9 mL, 2.31 mmol) in THF (7 mL) at -78 °C, **4.49** (0.40 g, 0.579 mmol) was added slowly. After stirring for 1 h at -78 °C the reaction was quenched with saturated NH₄Cl. Et₂O was added to dilute the solution and the organic layer was washed with NH₄Cl, water and brine. The organic phases were combined, dried over MgSO₄, filtered, concentrated, and purified by silica flash column chromatography (1:1 Hex: EtOAc), yielding **4.51** as a clear oil (266 mg, 57%). ¹H NMR (400 MHz, CDCl₃): δ 7.33-7.19 (m, 25 aromatic H), 6.93-6.91 (m, 2 aromatic H), 6.80-6.77 (m, 2 aromatic H), 5.01-4.89 (m, 6H, 5x CHPh, H-1), 4.84-4.76 (m, 4H, 4xOCHPh), 4.58 (d, J =11.0, 1H, OCHPh), 3.74 (s, 3H, OCH₃), 3.64-3.62 (m, 2H, H-2, H-3), 3.37-3.32 (m, 1H, H-5), 3.27-3.22 (m, 1H, H-4), 2.23-2.15 (m, 1H, H-6b), 2.00-1.90 (m, 1H, H-7b), 1.86-1.65 (m, 2H, H-7a, H-6a) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 138.4, 138.3, 137.8 (3 x quaternary aromatic C), 128.6, 128.5, 128.4, 128.4, 128.2, 128.1, 127.9, 127.9, 127.8, 127.7, 118.1, 114.6 (29 x aromatic CH), 102.3 (C1), 84.5 (C2), 82.2 (C3), 81.1 (C4), 77.2 (OCH₂Ph), 75.8 (OCH₂Ph), 75.3 (OCH₂Ph), 74.3 (C5), 67.2 (J_{C-P} =2.7 Hz), 67.1(J_{C-P} =3.1), 55.6 (OCH₃) ppm. ³¹P NMR (162 MHz, CDCl₃): δ 32.8 ppm.

LRMS (ESI⁺): m/z calcd. for C₄₉H₅₁O₉PNa [M + Na]⁺ 837.3, found 837.2.

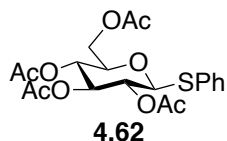
***p*-Methoxyphenyl-6-deoxy-6-dihydroxyphosphonomethyl- β -D-glucopyranoside
diammonium salt (**4.36**)**



Compound **4.51** (0.266 g, 0.326 mmol) was dissolved in the presence of 5% Pd/C (0.017 g, 0.160 mmol) in MeOH (11 mL) and EtOAc (11 mL). The mixture was stirred overnight under hydrogen gas. After completion, the solution was filtered through celite and concentrated to give the free acid. After the free acid was obtained, it was treated with NH_4OH until pH = 8 to obtain the ammonium phosphonate **4.36** (0.127 g, 98%). ^1H NMR (400 MHz, D_2O): δ 6.96-6.92 (m, 2H, 2xaromatic CH), 6.86-6.82 (m, 2H, 2xaromatic CH), 4.85-4.83 (m, 1H, H-1), 3.66 (s, 3H, OCH_3) 3.40-3.34 (m, 3H, H-2, H-4, H-5), 3.20-3.16 (m, 1H, H-3), 1.97-1.92 (m, 1H, H-7a), 1.61-1.46 (m, 2H, H-6a, H-7b), 1.41-1.33 (m, 1H, H-6b) ppm. ^{13}C NMR (101 MHz, D_2O): δ 154.6, 150.8 (2 x quaternary aromatic C), 118.0, 115.1 (2 x aromatic CH), 100.9 (C1), 76.1 (C2), 75.9 (C3), 75.4 (C4), 73.2 (C5, $J_{\text{C-P}}=6.4$ Hz), 55.8 (OCH_3), 25.3 (C6), 24.4 (C7) ppm. ^{31}P NMR (162 MHz, D_2O): δ 23.4 ppm.

LRMS (ESI⁺): m/z calcd. for $\text{C}_{14}\text{H}_{21}\text{O}_9\text{PNa}$ $[\text{M} + \text{Na}]^+$ 387.1, found 387.1.

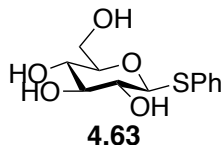
***S*-phenyl-2,3,4,6-tetra-*O*-acetyl- β -D-thioglucoside (4.62)**



Compound **4.61** (10 g, 0.026 mol) was dissolved in anhydrous dichloromethane (39 mL) and cooled to 0 °C. Boron trifluoride etherate (3.8 mL, 0.031 mol) was added dropwise followed by thiophenol (3.1 mL, 0.031 mol) dropwise. The reaction was warmed to room temperature and stirred overnight. Saturated aqueous NaHCO₃ (10 mL) was added, and the aqueous layer was extracted. The organic phase was washed with water (10 mL), followed by saturated aqueous NaHCO₃ (5 mL), then dried with Na₂SO₄, filtered, and concentrated under pressure. The crude reaction mixture was purified by silica flash column chromatography (7:3 hexane/ethyl acetate) to yield pure **4.62** (8.37 g, 74%). ¹H NMR (400 MHz, CDCl₃): δ 7.44-7.42 (m, 2H, aromatic H), 7.26-7.25 (m, 3H, aromatic H), 5.19 (t, J=9.4 Hz, 1H, H-3), 5.00 (t, J=9. Hz, 1H, H-4), 4.93 (t, J=9.4 Hz, 1H, H-2), 4.67 (d, J=10.1, 1H, H-1), 4.19-4.12 (m, 2H, H-6a/b), 3.69-3.67 (m, 1H, H-5), 2.02, 2.01, 1.95, 1.93 (s, 4 x C(O)CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 170.5, 170.1, 169.4, 169.2 (4 x C(O)CH₃), 133.1, 131.6, 128.9, 128.4 (6 x aromatic C), 85.6 (C1), 75.7 (C5), 73.9 (C3), 69.9 (C2), 68.2 (C4), 62.1 (C6), 21.0, 20.7, 20.7, 20.6 (4 x C(O)CH₃) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₂₀H₂₄O₉S [M + Na]⁺ 463.1, found 463.0.

***S*-phenyl- β -D-thioglucoside (4.63)**

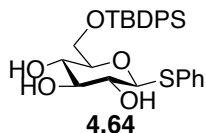


Compound **4.62** (8.37 g, 0.019 mol) was dissolved in methanol (400 mL) and 1 M sodium methoxide in methanol was added until the pH=12. The reaction was stirred for 2.5 h and monitored by TLC until completion. After completion, Amberlite-IR20 H⁺ was added until pH=6. The solution was filtered and concentrated to yield pure **4.63** as a white solid (6.47 g, 98%).

¹H NMR (400 MHz, DMSO-d₆): δ 7.43 (d, J= 7.5 Hz, 2H, aromatic CH), 7.28 (t, J=7.5 Hz, 2H, aromatic CH), 7.20 (t, J=7.2 Hz, 1H, aromatic CH), 5.29 (d, J=6.0 Hz, 1H, OH), 5.10 (d, J=5.1 Hz, 1H, OH), 4.99 (d, J=5.4 Hz, 1H, OH), 4.59-4.55 (m, 2H, OH, H-1), 3.68-3.64 (m, 1H, H-6a), 3.43-3.39 (m, 1H, H-6b), 3.22-3.16 (m, 2H, H-5, H-3), 3.10-3.00 (m, 2H, H-2, H-4) ppm. ¹³C NMR (101 MHz, DMSO-d₆): δ 135.4, 130.0, 129.3, 126.8 (6 x aromatic C), 87.5 (C1), 81.4 (C3/C5), 78.6 (C3/C5), 72.8 (C2/C4), 70.2 (C2/C4), 61.5 (C6) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₁₂H₁₆O₅S [M + Na]⁺ 295.1, found 295.0.

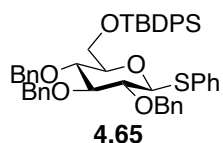
***S*-phenyl-6-*O*-tert(butyldiphenylsilyl)- β -D-thioglucoside (4.64)**



Compound **4.63** (6.47 g, 0.019 mol) was dissolved in DMF (40 mL) and cooled to 0 °C. DMAP (0.23 g, 0.002 mol), triethylamine (2.20 mL, 0.016 mol), and TBDPSCl (3.26 mL, 0.013 mol) were all added sequentially, and the mixture was allowed to stir and warm to room temperature. After 2 h, the reaction was diluted with ethyl acetate, washed with water and brine, and the organic layer

was dried with Na₂SO₄. The crude product was purified via silica flash column chromatography (10% ethyl acetate in hexane) to afford the pure **4.64** (6.21 g, 64%). ¹H NMR (400 MHz, CDCl₃): δ 7.70-7.67 (m, 4H, aromatic H), 7.52-7.50 (m, 2H, aromatic H), 7.43-7.34 (m, 6H, aromatic H), 7.25-7.19 (m, 3H, aromatic H), 4.51 (d, J=9.7 Hz, 1H, H-1), 3.96-3.89 (m, 2H, H-6a/b), 3.64-3.55 (m, 2H, H-4, H-3), 3.45-3.41 (m, 1H, H-5), 3.36 (t, J=9.1 Hz, 1H, H-2), 1.05 (s, 9H, 3 x CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 135.7, 135.6, 132.6, 129.9, 129.0, 128.1, 127.8, 127.7 (18 x aromatic C), 87.9 (C1), 78.9 (C5), 77.8 (C3), 71.6 (C2), 71.2 (C4), 64.3 (C6), 26.8 (3 x CH₃) ppm. LRMS (ESI⁺): *m/z* calcd. for C₂₈H₃₄O₅SSiNa [M + Na]⁺ 533.2, found 533.1.

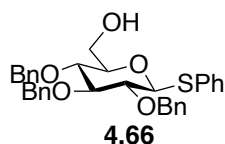
***S*-phenyl-2,3,4-tri-*O*-benzyl-6-*O*-tert(butyldiphenylsilyl)-β-D-thioglucoside (4.65)**



Compound **4.64** (0.84 g, 1.65 mmol) was dissolved in DMF (9.6 mL) and cooled to 0 °C in an ice bath. 60% sodium hydride in mineral oil (0.30 g, 7.44 mmol) was added portion wise and the mixture was stirred at the same temperature. Benzyl bromide (0.88 mL, 7.44 mmol) was added dropwise over a 5 min period and the reaction was stirred at room temperature overnight. After completion, the mixture was quenched with water while stirring and the mixture was extracted with CH₂Cl₂, brine, and dried with Na₂SO₄. The crude mixture was purified by flash column chromatography (9:1 hexane/ethyl acetate) to afford a clear oil as **4.65** (0.885 g, 69%). ¹H NMR (400 MHz, CDCl₃): δ 7.77-7.74 (m, 2H, aromatic H), 7.70-7.68 (m, 2H, aromatic H), 7.60-7.58 (m, 2H, aromatic H), 7.42-7.11 (m, 24H, aromatic H), 4.90 (d, J=10.7 Hz, 1H, PhCH), 4.90-4.83 (m, 4H, 4 x PhCH), 4.73 (d, J=10.3 Hz, 1H, PhCH), 4.69 (d, J=10.3 Hz, 1H, PhCH) 4.69 (d, J=9.8 Hz, H-1), 3.99 (dd, J=1.7, 11.3 Hz, 1H, H-6a), 3.94 (dd, J=3.7, 11.3 Hz, 1H, H-6b), 3.81 (t, J=9.4

Hz, 1H, H-4), 3.73 (t, J=8.7 Hz, 1H, H-3), 3.56 (dd, J=8.7, 9.8 Hz, 1H, H-2), 3.41-3.37 (m, 1H, H-5), 1.07 (s, 9H, 3 x CH₃) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 138.4, 138.2, 138.1, 135.9, 135.6, 131.7 (6 x quaternary aromatic C), 129.7, 129.6, 128.9, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 127.7 (30 x aromatic CH), 88.2 (C1), 87.7 (C3), 81.5 (C2), 80.4 (C5), 77.9 (C4), 63.3 (C6), 27.4 (3 x CH₃), 19.3 (C(CH₃)₃) ppm.

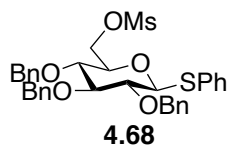
***S*-phenyl-2,3,4-tri-*O*-benzyl-β-D-thioglucoside (4.66)**



Compound **4.65** (0.88 g, 1.14 mmol) was dissolved in 1M TBAF-THF (3.4 mL, 3.40 mmol) and acetic acid (0.2 mL, 3.74 mmol). The reaction mixture was stirred at room temperature overnight under an inert atmosphere. After completion, the mixture was concentrated under reduced pressure and purified by silica flash column chromatography (4:1 hexane/ethyl acetate) to afford pure **4.66** (0.58 g, 94%). ¹H NMR (400 MHz, CDCl₃): δ 7.50-7.47 (m, 2H, aromatic H), 7.38-7.24 (m, 18H, aromatic H), 4.91-4.82 (m, 4H, 4 x PhCH), 4.76 (d, J=10.3 Hz, 1H, PhCH), 4.71 (d, J=9.8 Hz, 1H, H-1), 4.65 (d, J=11.0 Hz, 1H, PhCH), 3.87 (dd, J=2.0, 12.0 Hz, 1H, H-6a), 3.74 (t, J=8.8 Hz, 1H, H-3), 3.70 (dd, J=4.7, 12.0 Hz, 1H, H-6b), 3.58 (t, J=9.4 Hz, 1H, H-4), 3.49 (dd, J=8.8, 9.8 Hz, 1H, H-2), 3.39-3.35 (m, 1H, H-5) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 138.3, 137.9, 137.8, 133.5 (4 x quaternary aromatic C), 131.9, 129.1, 128.5, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7 (20 x aromatic CH), 87.6 (C1), 86.6 (C3), 81.1 (C4), 79.3 (C5), 77.6 (C2), 75.8 (OCH₂Ph), 75.6 (OCH₂Ph), 75.1 (OCH₂Ph), 62.2 (C6) ppm.

LRMS (ESI⁺): *m/z* calcd. for C₃₃H₃₄O₅SNa [M + Na]⁺ 565.2, found 542.1 [M + H]⁺ and 565.1 [M + Na]⁺

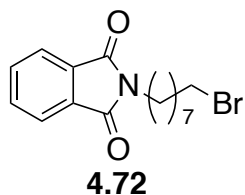
***S*-phenyl-2,3,4-tri-*O*-benzyl-6-*O*-methanesulfonyl- β -D-thioglucoside (4.68)**



Compound **4.66** (0.500 g, 0.921 mmol) was dissolved in pyridine (7.4 mL) before the dropwise addition of freshly distilled methanesulfonyl chloride (0.2 mL, 2.764 mmol) over 5 min. The reaction mixture was stirred for 30 min and then diluted with ethyl acetate. The organic layer was washed with 1 M HCl and separated. The organic layer was dried with anhydrous sodium sulfate, filtered, and evaporated. Silica gel flash column chromatography afforded pure **4.68** (0.469 g, 82%). ^1H NMR (400 MHz, CDCl_3): δ 7.55-7.53 (2H, m, aromatic H), 7.41-7.27 (17H, m, aromatic H), 4.96 (d, $J=11.0$ Hz, 1H, PhCH), 4.94 (d, $J=10.3$ Hz, 1H, PhCH), 4.91 (d, $J=10.6$ Hz, 1H, PhCH), 4.89 (d, $J=10.6$ Hz, 1H, PhCH), 4.78 (d, $J=10.3$ Hz, 1H, PhCH), 4.74 (d, $J=9.9$ Hz, 1H, H-1), 4.66 (d, $J=11.0$ Hz, 1H, PhCH), 4.47 (dd, $J=1.1, 11.3$ Hz, 1H, H-6a), 4.35 (dd, $J=4.6, 11.3$ Hz, 1H, H-6b), 3.77 (t, $J=8.6$ Hz, 1H, H-3), 3.56-3.49 (m, 3H, H-5, H-4, H-2), 2.98 (s, 3H, SO_2CH_3) ppm. ^{13}C NMR (101 MHz, CDCl_3): δ 137.5, 133.3, 132.0, 129.1 (4 x quaternary aromatic C), 128.7, 128.6, 128.5, 128.3, 128.2, 128.2, 128.0, 127.9, 127.9, 127.8 (19 x aromatic CH), 87.6 (C1), 86.5 (C3), 80.9 (C2), 77.0 (C5), 76.7 (C4), 75.9, 75.6, 75.3 (3 x CH_2Ph), 68.6 (C6), 37.8 (SO_2CH_3) ppm.

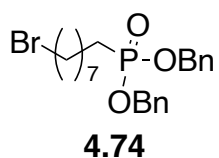
LRMS (ESI⁺): m/z calcd. for $\text{C}_{34}\text{H}_{36}\text{O}_7\text{S}_2\text{Na}$ $[\text{M} + \text{Na}]^+$ 643.2, found 634.2.

***N*-(8-bromooctyl)phthalimide (4.72)**



Compound **4.71** (0.200 g, 1.08 mmol) and **4.70** (1.47 g, 5.40 mmol) were dissolved in DMF (3 mL) and stirred at 100 °C for 16 h. Mixture was cooled to room temperature and solvent was evaporated under pressure to yield crude product. Product purification by silica flash column chromatography (9:1 hexane/ ether) afforded pure **4.72** (0.234 g, 64%). ¹H NMR (400 MHz, CDCl₃): δ 7.83-7.81 (m, 2H, aromatic H), 7.70-7.68 (m, 2H, aromatic H), 3.67 (t, J=7.0 Hz, 2H, NCH₂), 3.39 (t, J=7.0 Hz, CH₂Br), 1.85-1.78 (m, 2H, CH₂), 1.67-1.65 (m, 2H, CH₂), 1.43-1.28 (m, 8H, 4 x CH₂) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 168.5 (amide C), 133.9 (2 x quaternary aromatic C), 132.2, 123.2 (4 x aromatic CH), 38.0, 34.0, 32.7, 29.0, 28.6, 28.5, 28.0, 26.7 (8 x CH₂) ppm.

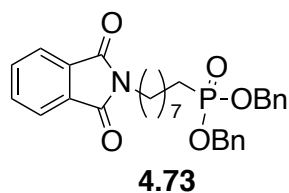
Dibenzyl-8-bromo-*n*-octylphosphonate (4.74)



60% sodium hydride in mineral oil (0.795 g, 19.91 mmol) was dissolved in DMF (41 mL) at 0 °C in an ice bath, followed by the dropwise addition of **4.20** (4.4 mL, 19.91 mmol). The reaction mixture was stirred for 2 h at room temperature. The solution was then cooled to 0 °C and compound **4.70** (3.4 mL, 18.11 mmol) was added dropwise and the mixture was stirred at room temperature overnight. The solvents were evaporated under pressure at 60 °C and the resulting oil was dissolved in ethyl acetate and extracted with water. The aqueous layer was extracted with

ethyl acetate (3x), dried with MgSO₄, and concentrated under reduced pressure. Pure **4.74** (3.93 g, 47%) as afforded by silica flash column chromatography (2:1 hexane/ethyl acetate). ¹H NMR (400 MHz, CDCl₃): δ 7.36-7.27 (m, 10 H, aromatic H), 5.06 (d, J=11.7 Hz, 1H, PhCH), 5.03 (d, J=11.7 Hz, 1H, PhCH), 4.96 (d, J=11.7 Hz, 1H, PhCH), 4.94 (d, J=11.7 Hz, 1H, PhCH), 3.38 (t, J=6.8 Hz, 2H, -CH₂Br), 1.83 (q, J=6.8 Hz, 2H, -CH₂CH₂Br), 1.76-1.67 (m, 2H, -CH₂P), 1.58-1.48 (m, 2H, CH₂CH₂CH₂P), 1.40-1.20 (m, 8H, BrCH₂CH₂CH₂CH₂CH₂CH₂CH₂CH₂P) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 136.4 (d, J_{C-P}=6.0 Hz, 2 x quaternary aromatic C), 128.5, 128.3, 127.8 (10 x aromatic CH), 66.9 (d, J_{C-P}=6.6 Hz, 2x CHPh), 33.9 (-CH₂Br), 32.6 (-CH₂CH₂Br), 30.3 (d, J_{C-P}=16.8 Hz, -CH₂CH₂P), 28.7, 28.3, 27.9, 26.6 (d, J_{C-P}=140.0 Hz, -CH₂P), 22.2 (d, J_{C-P}=5.5 Hz, -CH₂CH₂CH₂P) ppm. ³¹P NMR (162 MHz, CDCl₃): δ 33.7 ppm.

***N*-(8-dibenzylphosphonoctyl)phthalimide (4.73)**

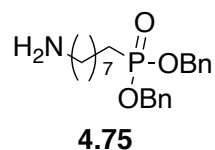


Compound **4.74** (3.929 g, 8.66 mmol) and **4.71** (0.321 g, 1.73 mmol) were dissolved in anhydrous DMF (4 mL) and stirred at 100 °C overnight. The reaction mixture was then concentrated under reduced pressure the crude product was purified by silica flash column chromatography (2:1 hexane/ethyl acetate) to yield the pure **4.73** (0.558 g, 62%). ¹H NMR (400 MHz, CDCl₃): δ 7.83-7.80 (m, 2H, aromatic H), 7.70-7.67 (m, 2H, aromatic H), 7.35-7.29 (m, 10 H, aromatic H), 5.07 (d, J=11.7 Hz, 1H, PhCH), 5.04 (d, J=11.7 Hz, 1H, PhCH), 4.98 (d, J=11.7 Hz, 1H, PhCH), 4.95 (d, J=11.7 Hz, 1H, PhCH), 3.67 (t, J=7.1 Hz, 2H, -CH₂N), 1.77-1.46 (m, 6H, 3 x CH₂), 1.32-1.17 (m, 8H, 4 x CH₂) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 168.5 (2 x C=O), 136.6 (d, J_{C-P}=6.0 Hz, 2

x quaternary aromatic C), 133.9 (2 x quaternary aromatic C), 132.2, 128.5, 128.3, 127.8, 123.2 (14 x aromatic CH), 67.0 (d, $J_{C-P}=6.6$ Hz, 2x $\underline{CH}Ph$), 38.0 ($-\underline{CH}_2N$), 30.3 (d, $J_{C-P}=17.3$ Hz, $-\underline{CH}_2CH_2P$), 28.9, 28.5, 26.7, 26.6 (d, $J_{C-P}=140.2$ Hz, $-\underline{CH}_2P$), 22.3 (d, $J_{C-P}=5.4$ Hz, $-\underline{CH}_2CH_2CH_2P$) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ 30.9 ppm.

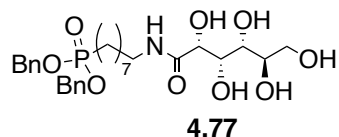
LRMS (ESI⁺): m/z calcd. for $C_{30}H_{34}NO_5PNa$ $[M + Na]^+$ 542.2, found 542.0.

Dibenzylphosphono octyl amine (4.75)



Compound **4.73** (0.258 g, 0.497 mmol) was dissolved in methanol (3 mL) and hydrazine hydrate (0.12 mL, 2.486 mmol) was added at 0 °C and stirred at the same temperature for 3 h. The ice bath was then removed and stirring continued for 5 h at room temperature. The reaction was monitored by thin layer chromatography and once completed; the methanol was evaporated. The crude material was dissolved in 1 M HCl (7.5 mL) and washed with diethyl ether (2x). The aqueous layer was then treated with ammonium hydroxide until a pH of 8, followed by extraction with ethyl acetate (2x). The organic extract was dried over sodium sulfate, and concentrated. The resulting product, **4.75**, was used crude.

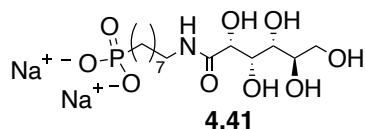
N-(8-dibenzylphosphonoctyl) gluconamide (**4.77**)



Compound **4.76** (0.028 g, 0.132 mmol) and **4.75** (0.063 g, 0.132 mmol) were dissolved in methanol (0.6 mL) and refluxed for 1 h. Reaction was cooled to room temperature and the solvent was evaporated. Crude mixture was purified by silica flash column chromatography (100% ethyl acetate) to afford pure **4.77** (0.091 g, 99%). ^1H NMR (400 MHz, DMSO- d_6): δ 7.57 (t, $J=6.0$ Hz, 1H, amide H), 7.34-7.29 (m, 10H, aromatic H), 5.33 (d, $J=5.1$ Hz, 1H, OH), 5.00-4.88 (m, 4H, 2 x OCH₂Ph), 4.52 (d, $J=4.8$ Hz, 1H, OH), 4.45 (d, $J=5.3$ Hz, 1H, OH), 4.37 (d, $J=7.3$ Hz, 1H, OH), 4.32 (t, $J=5.7$ Hz, 1H, OH), 3.94 (dd, $J=3.9, 4.8$ Hz, 1H, H₉), 3.87-3.84 (m, 1H, H₁₀), 3.55-3.51 (m, 1H, H_{13a}), 3.45-3.40 (m, 2H, H₁₁, H₁₂), 3.05-2.98 (m, 2H, H₈), 1.42-1.12 (m, 14H, H₁ - H₇) ppm. ^{13}C NMR (101 MHz, DMSO- d_6): δ 172.7 (amide C), 137.3, 137.2 (2 x quaternary aromatic C), 128.9, 128.6, 128.2 (5 x aromatic CH), 74.1 (C₉), 72.9 (C₁₁), 72.0 (C₁₂), 70.6 (C₁₀), 66.7 (OCH₂Ph), 66.7 (OCH₂Ph), 63.8 (C₁₃), 38.7, 32.6, 31.2, 30.2, 30.0, 29.6, 29.0, 28.9, 26.7 ppm. ^{31}P NMR (162 MHz, DMSO- d_6): δ 33.3 ppm.

LRMS (ESI⁺): m/z calcd. for C₂₈H₄₂NO₉P [M + Na]⁺ 590.2, found 590.3.

N-(8-phosphonoctyl) gluconamide disodium salt (**4.41**)



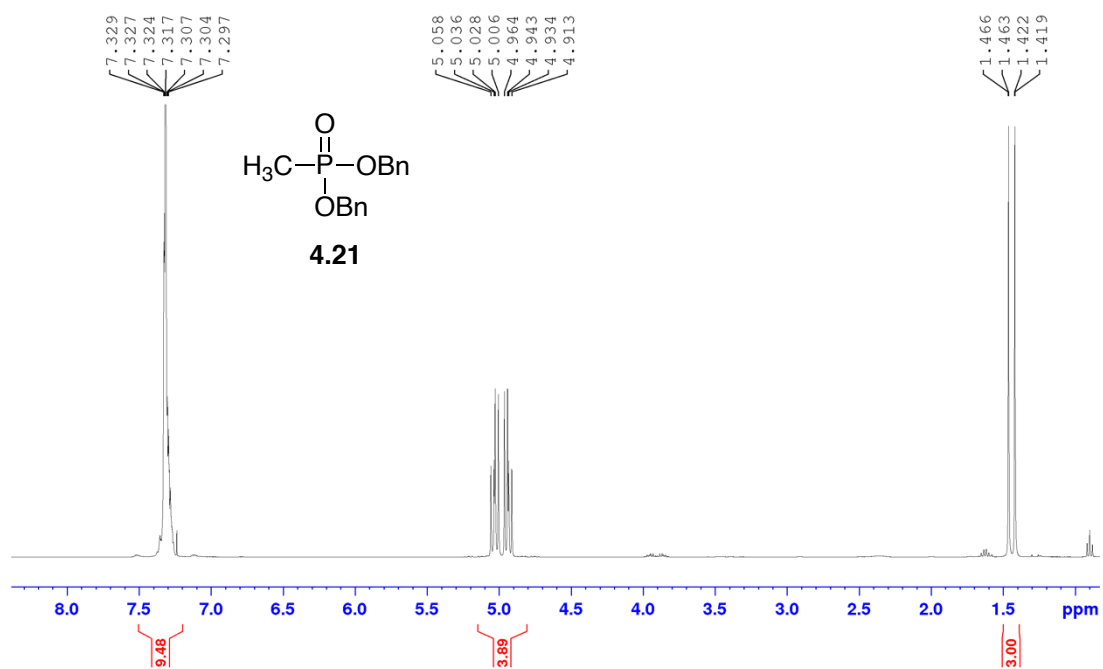
Compound **4.77** (0.090 g, 0.186 mmol) and 5% palladium on carbon (0.057 g, 0.538 mmol) were dissolved in a 1:1 mixture of ethyl acetate (3.5 mL) and methanol (3.5 mL). The mixture was

degassed and purged with hydrogen gas and allowed to stir for 16 h. The mixture was filtered through celite and concentrated to afford pure product without any further purification. The free acid was then titrated with concentrated NaOH(aq) until a pH of 8. The solvent was evaporated via lyophilization to afford **4.41** as a beige salt in 94% yield. ^1H NMR (400 MHz, D_2O): δ 4.0 (d, $J=3.7$ Hz, 1H, H-9), 3.90 (d, $J=3.3$ Hz, 1H, H-10), 3.70-3.67 (m, 1H, H-13a), 3.64-3.62 (m, 2H, H-11, H-12), 3.56-3.50 (m, 1H, H-13b), 2.48 (t, $J=7.0$ Hz, 1H, H-8), 1.33-1.18 (m, 14H, H-7 to H-1) ppm. ^{13}C NMR (101 MHz, D_2O): δ 178.7 (amide C), 74.2 (C9), 72.8 (C12), 71.3 (C11), 71.0 (C10), 62.7 (C13), 40.6 (C8), 31.8, 31.1, 30.9, 30.1, 28.8, 28.6, 28.5, 26.1, 24.2, 24.1 (C1-C7) ppm. ^{31}P NMR (162 MHz, D_2O): δ 23.3 ppm.

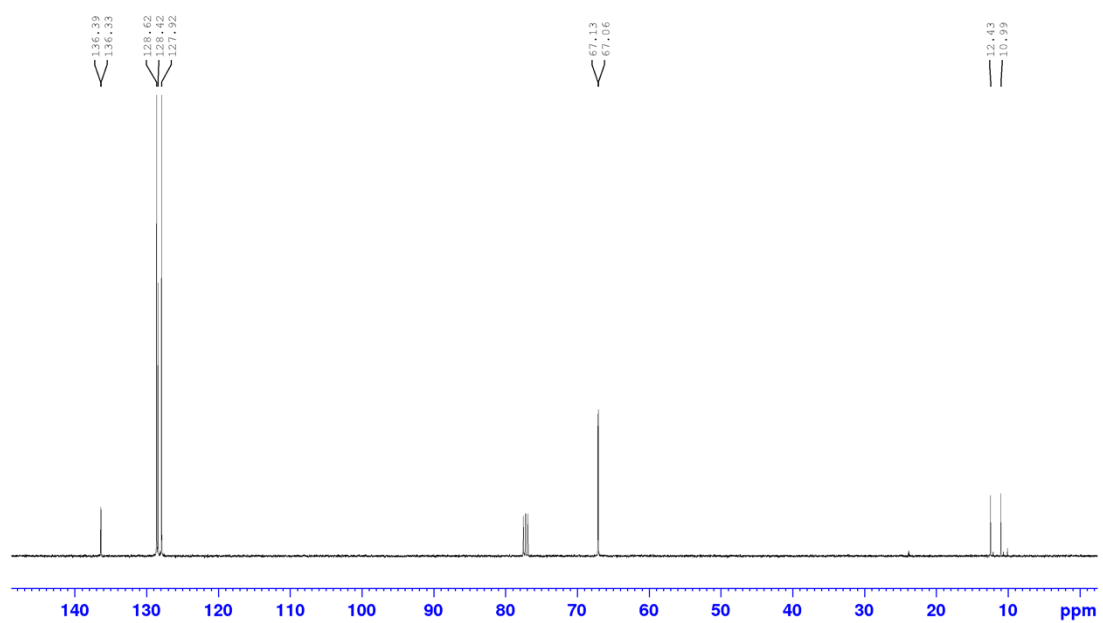
LRMS (ESI⁺): m/z calcd. for $\text{C}_{14}\text{H}_{30}\text{NO}_9\text{P}$ $[\text{M} + \text{Na}]^+$ 409.2, found 409.1.

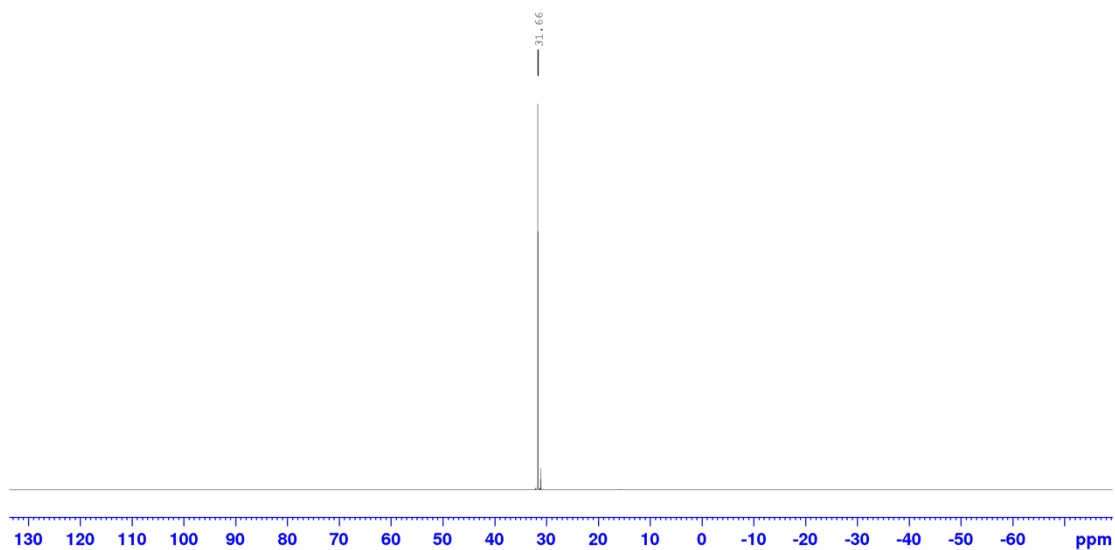
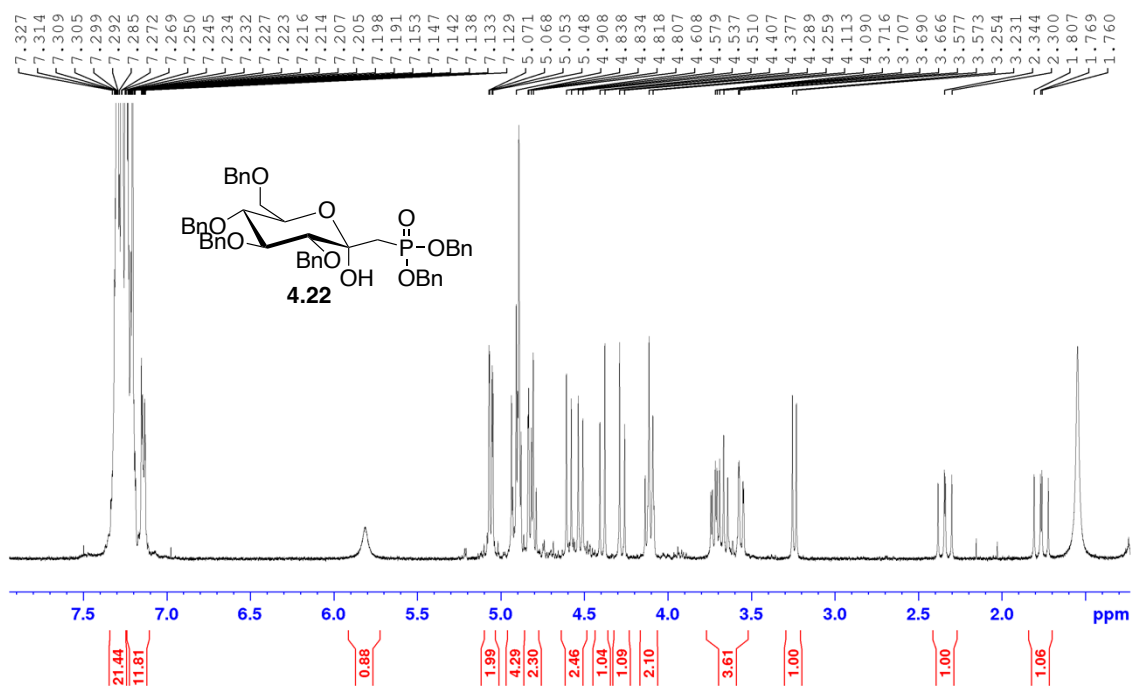
4.7.4 *NMR Spectra*

^1H NMR

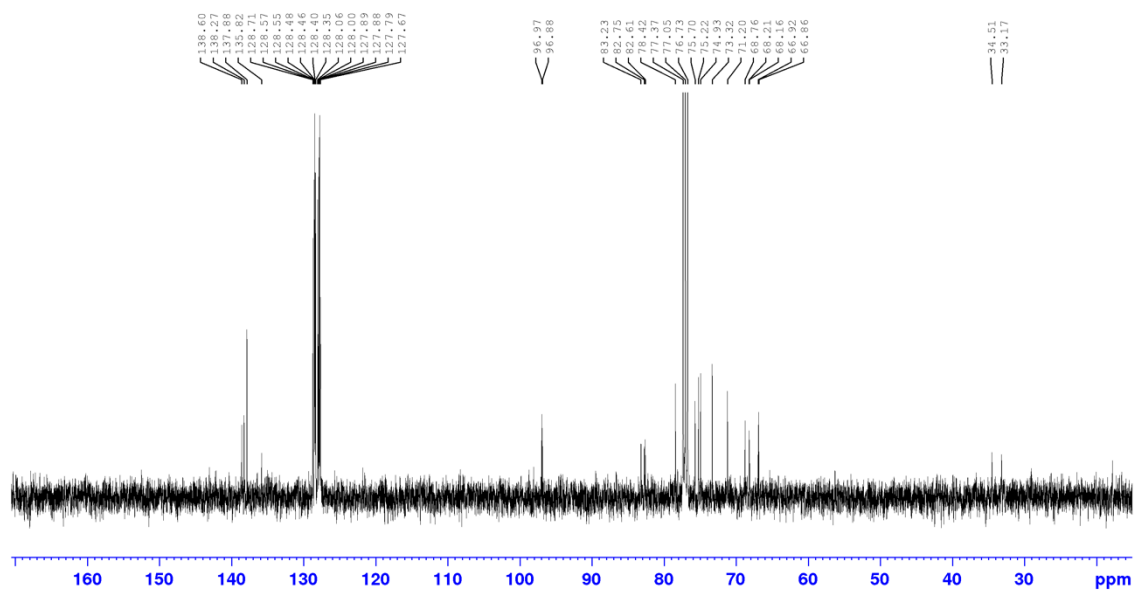


^{13}C NMR

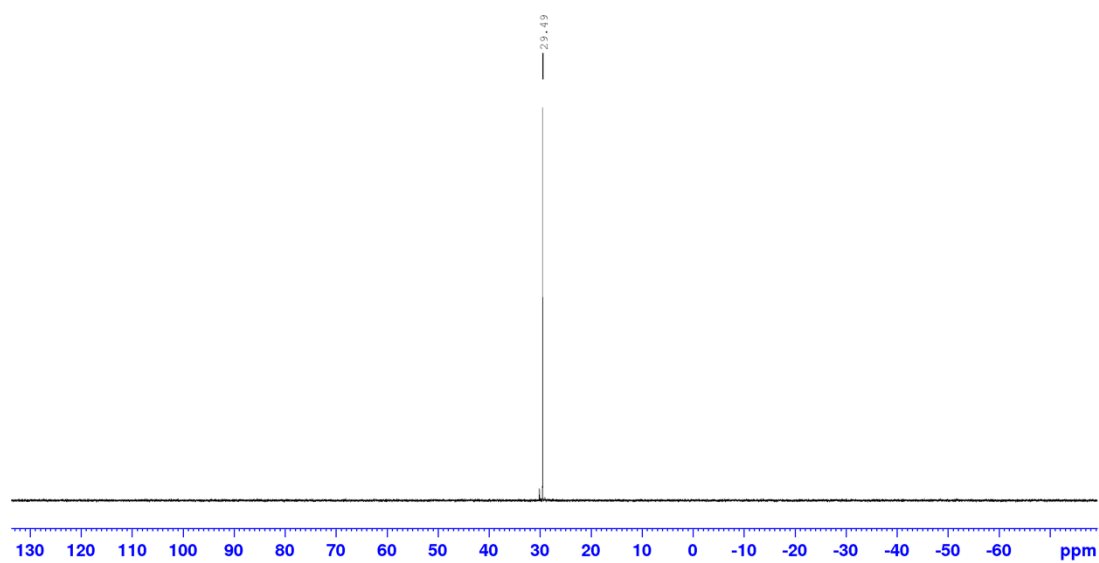


³¹P NMR¹H NMR

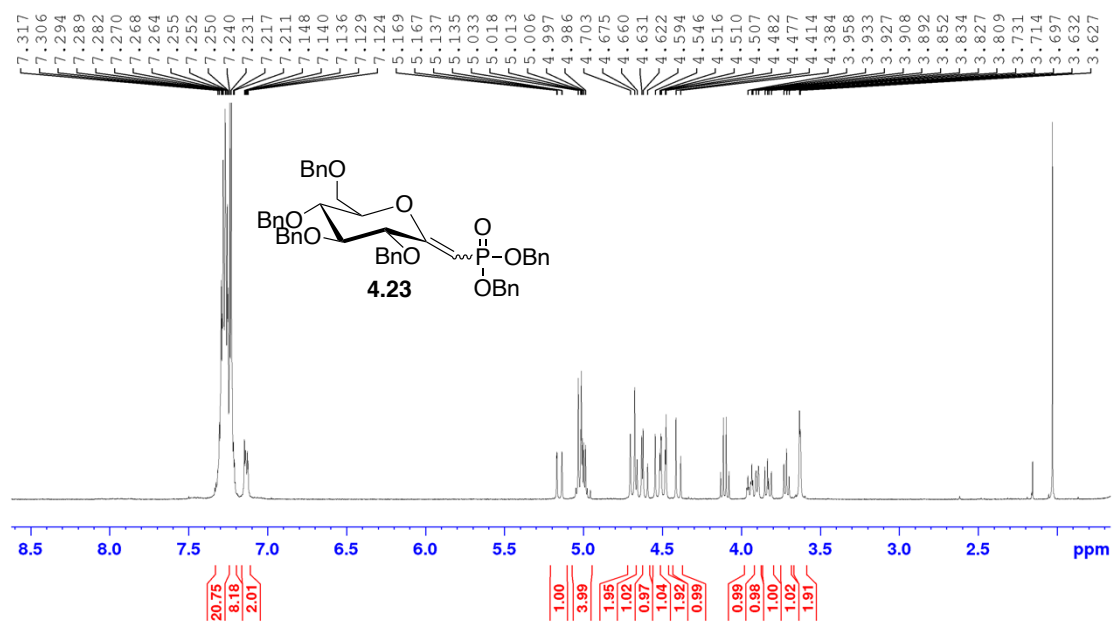
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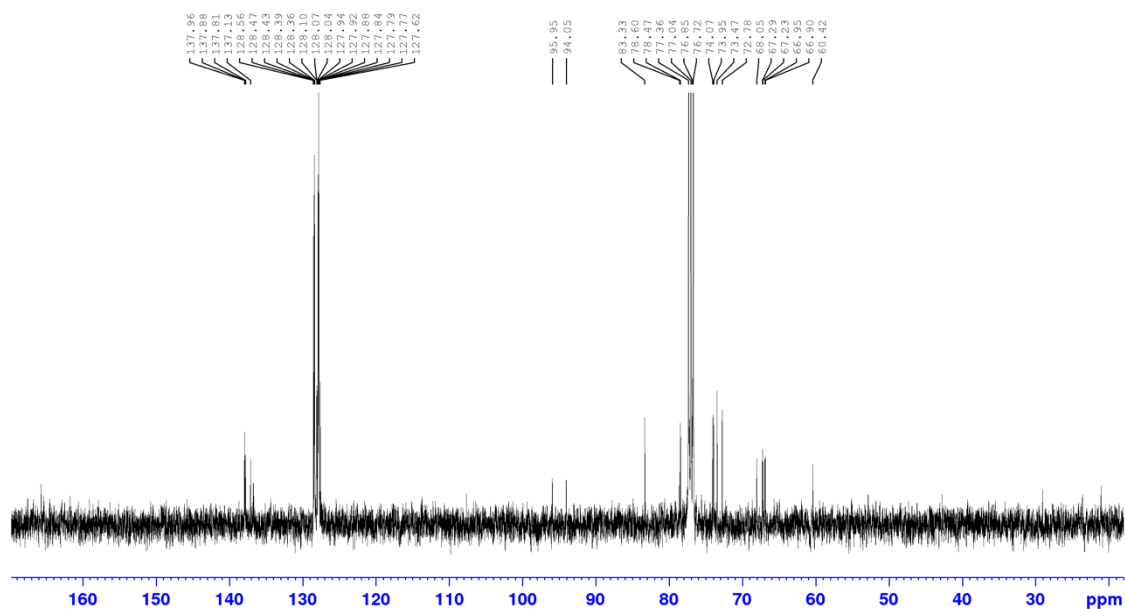
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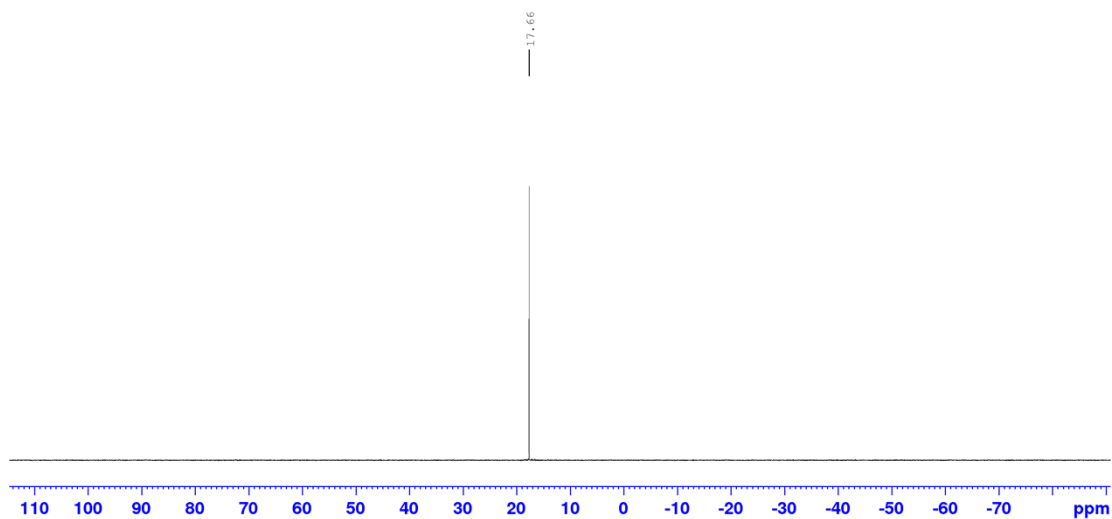
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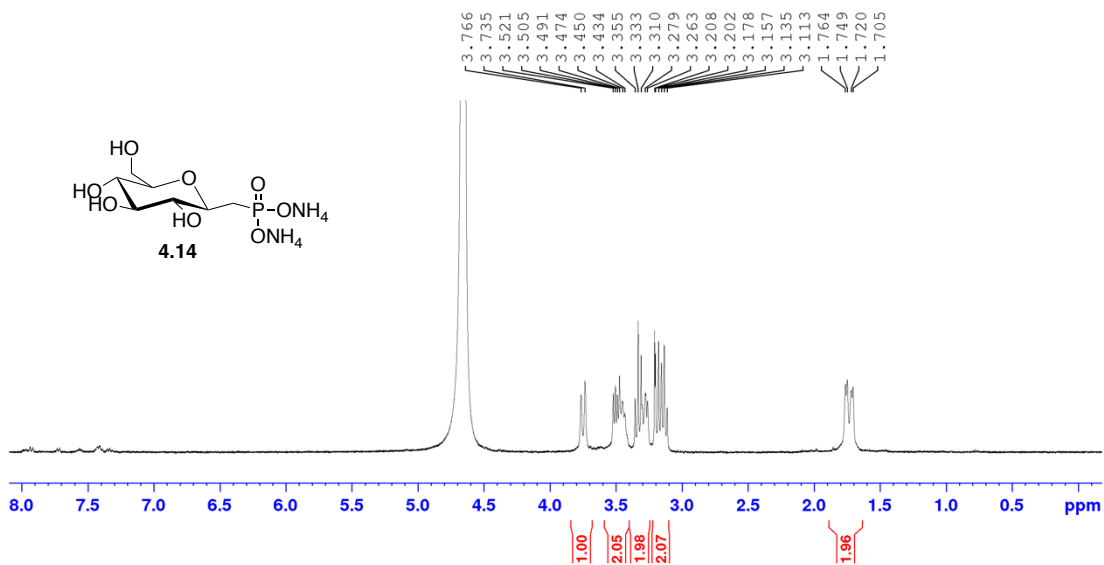
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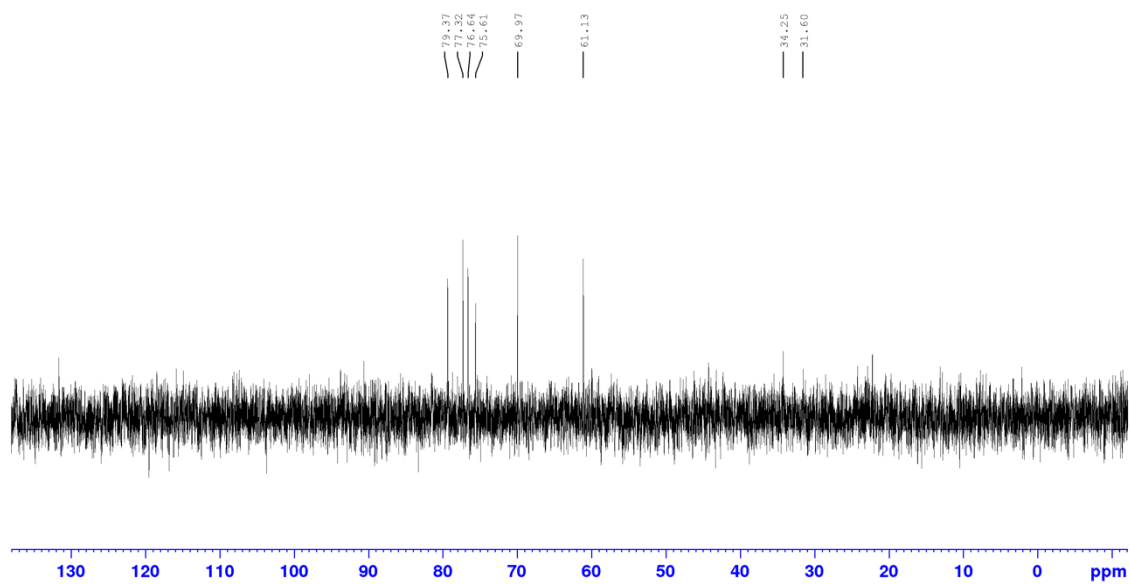
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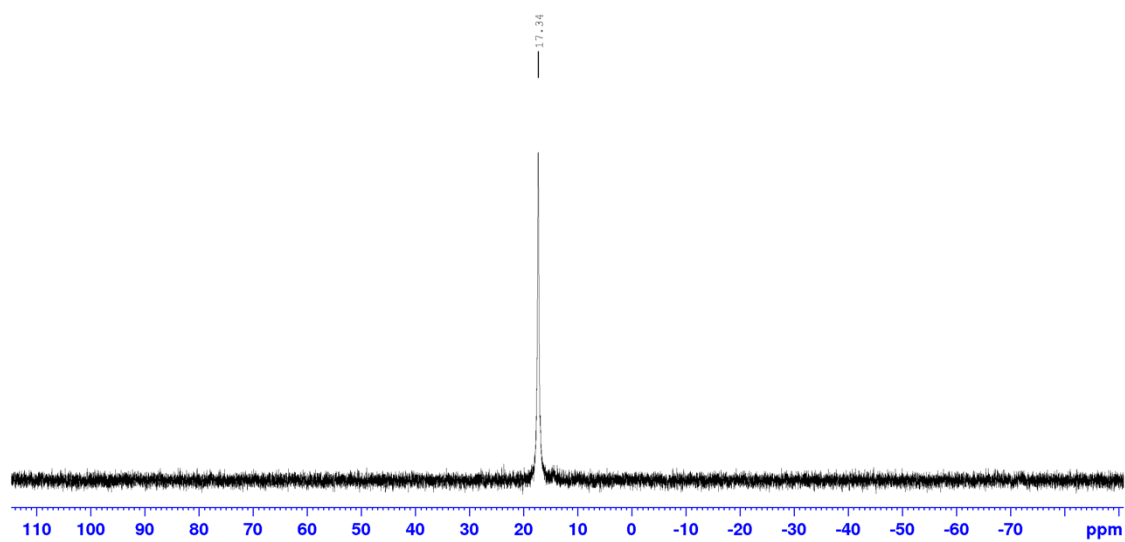
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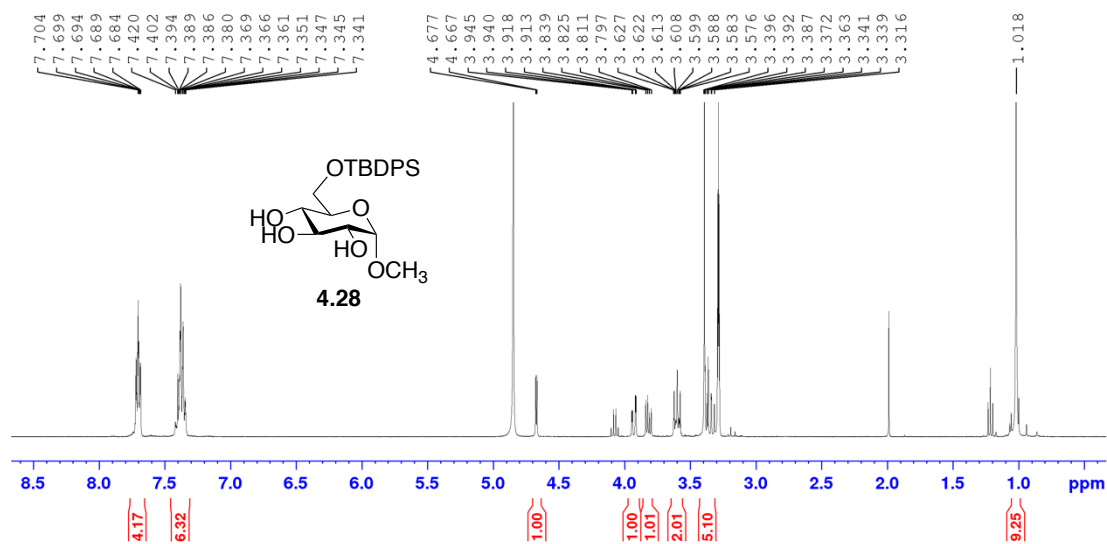
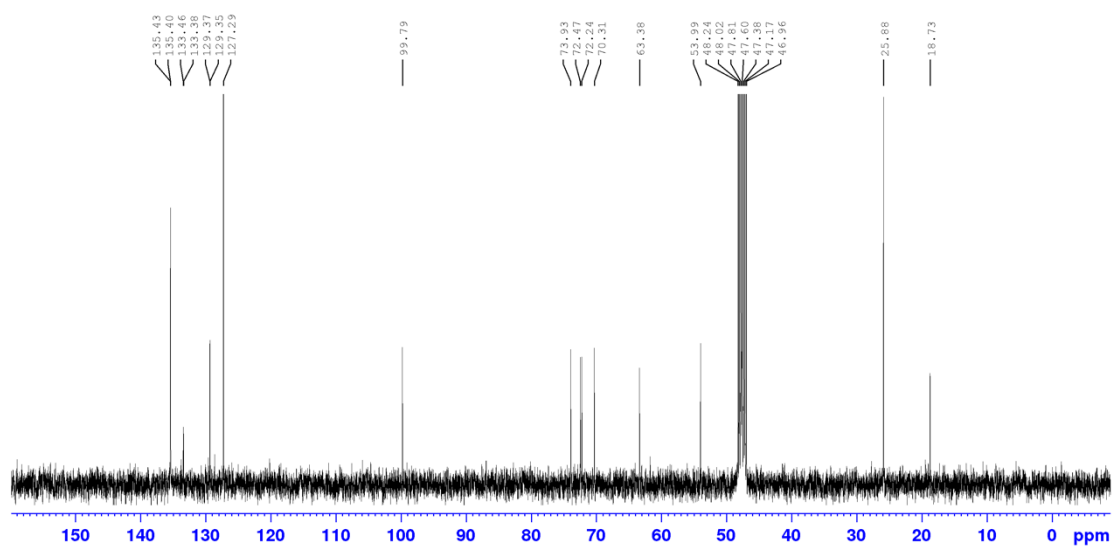


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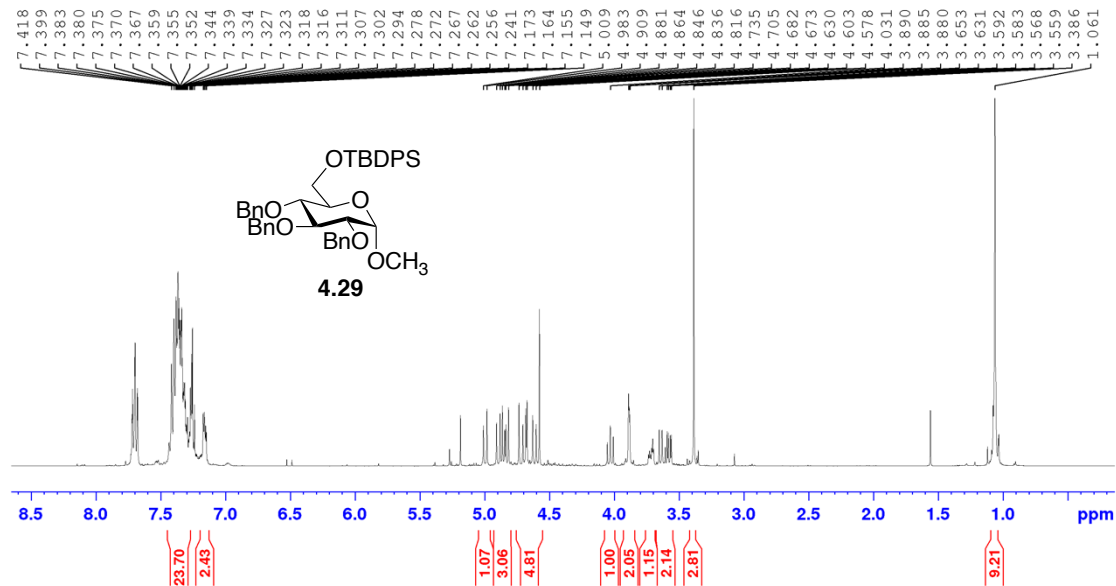


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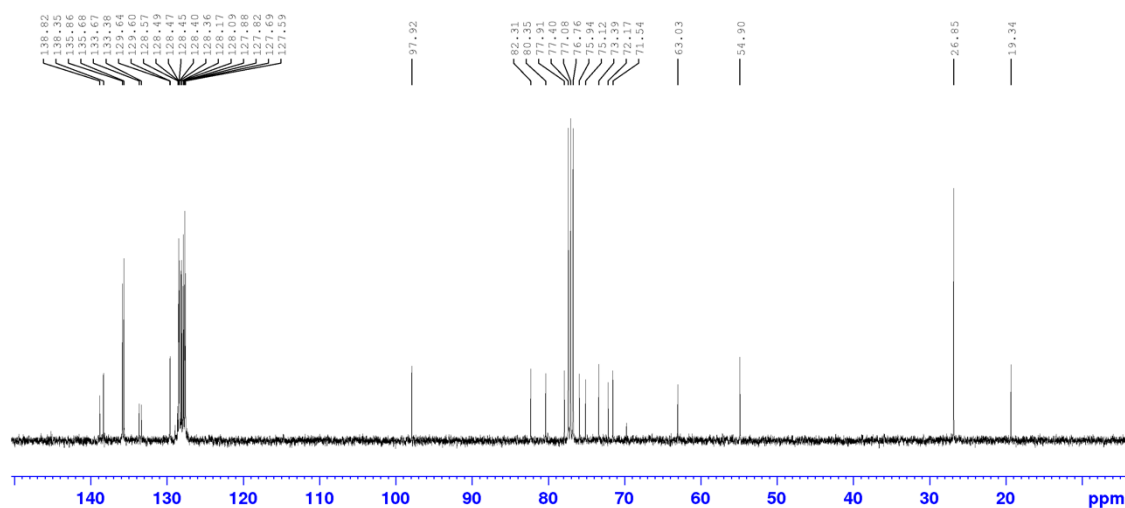


¹H NMR¹³C NMR

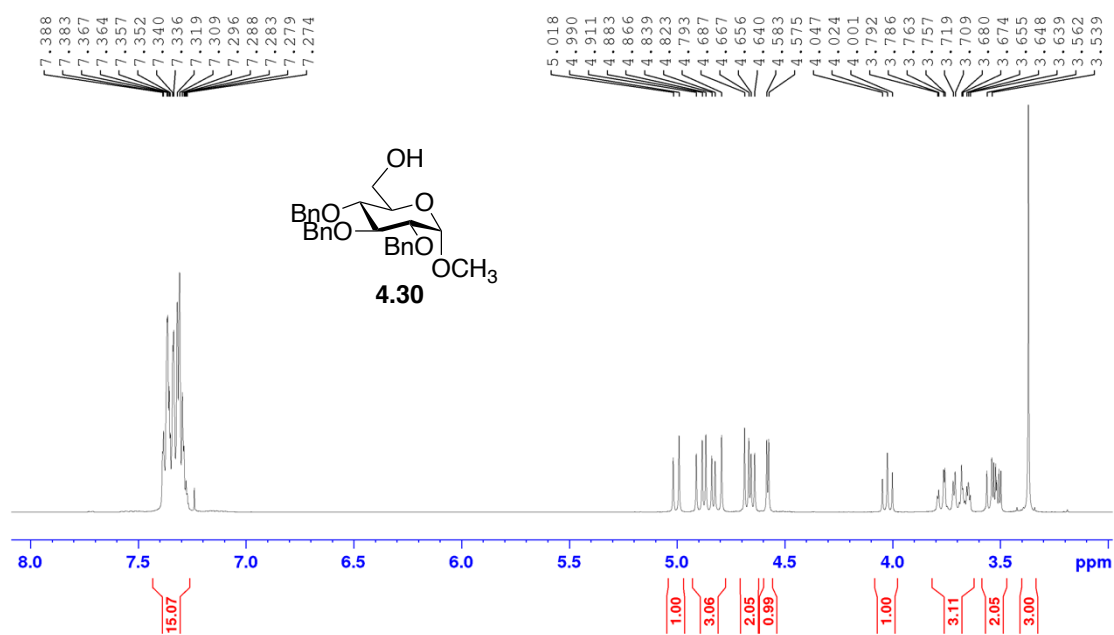
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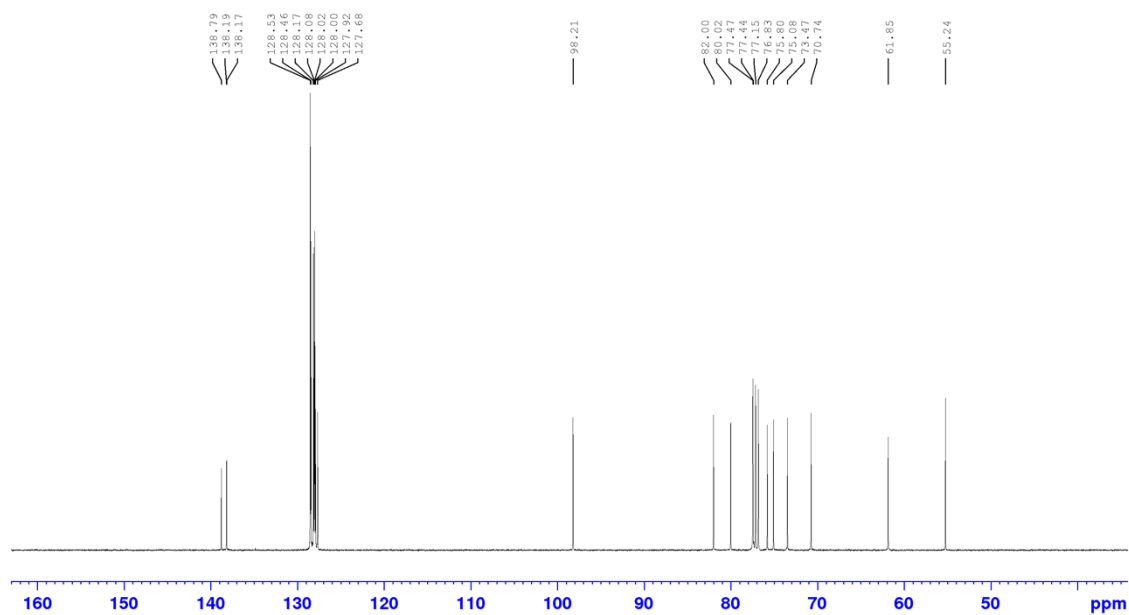
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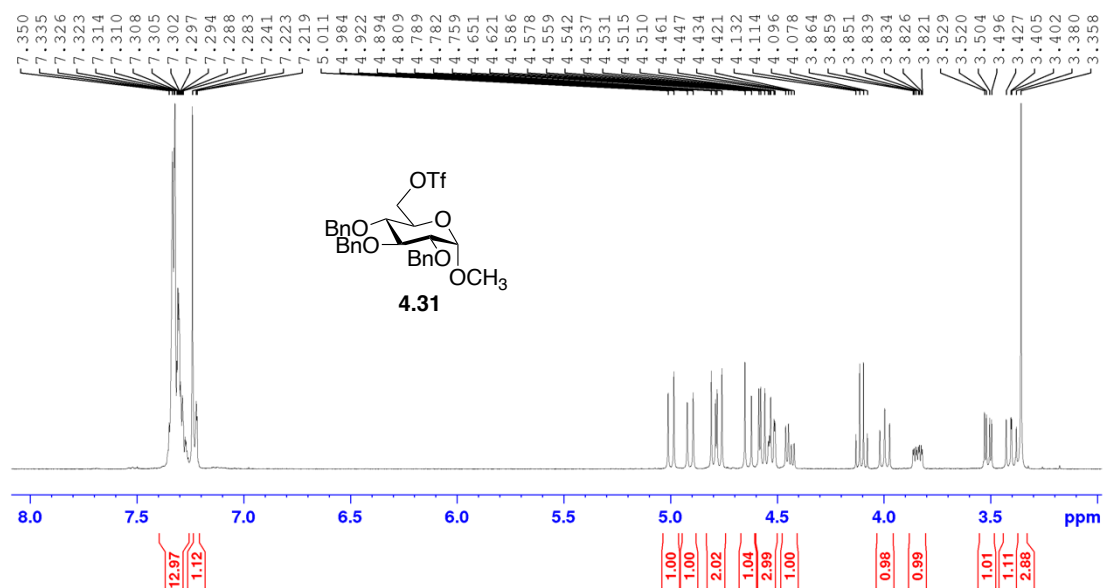
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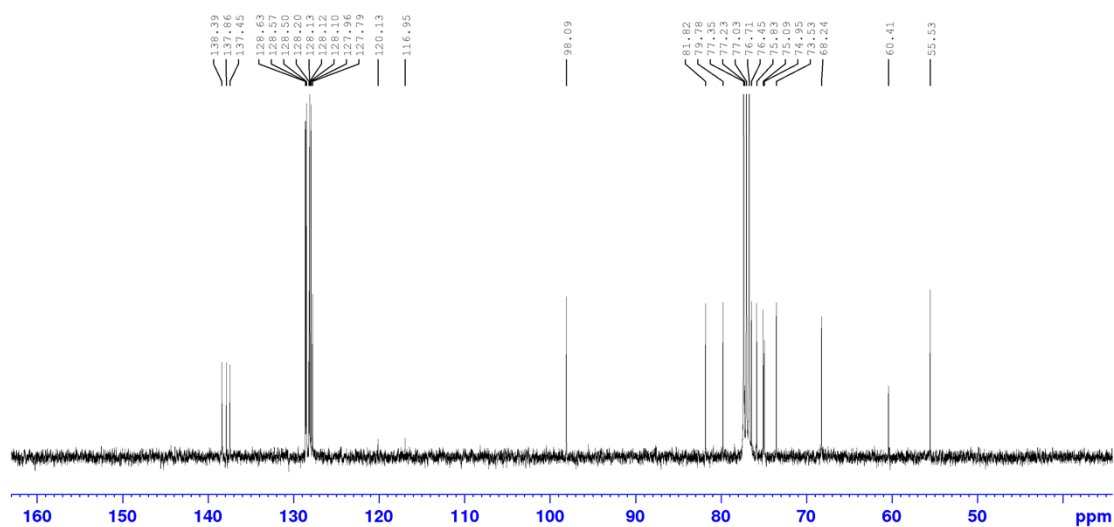
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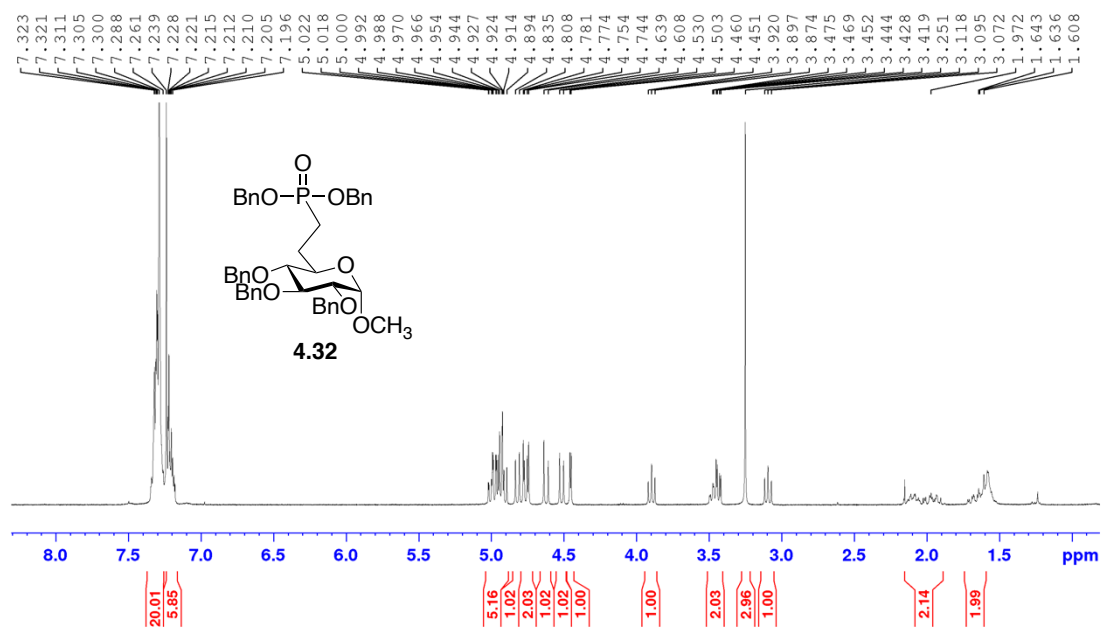
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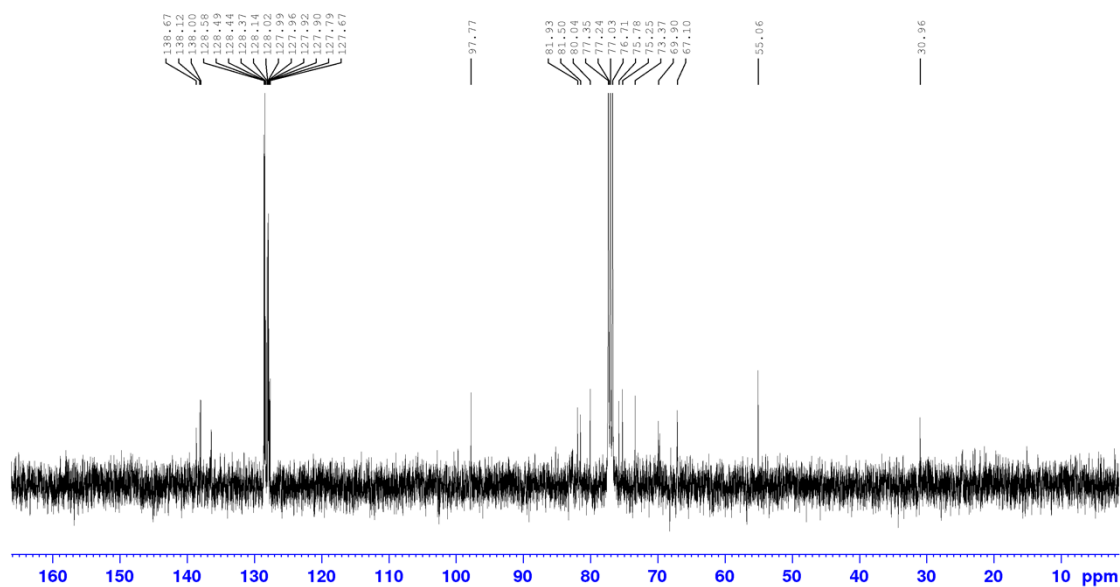
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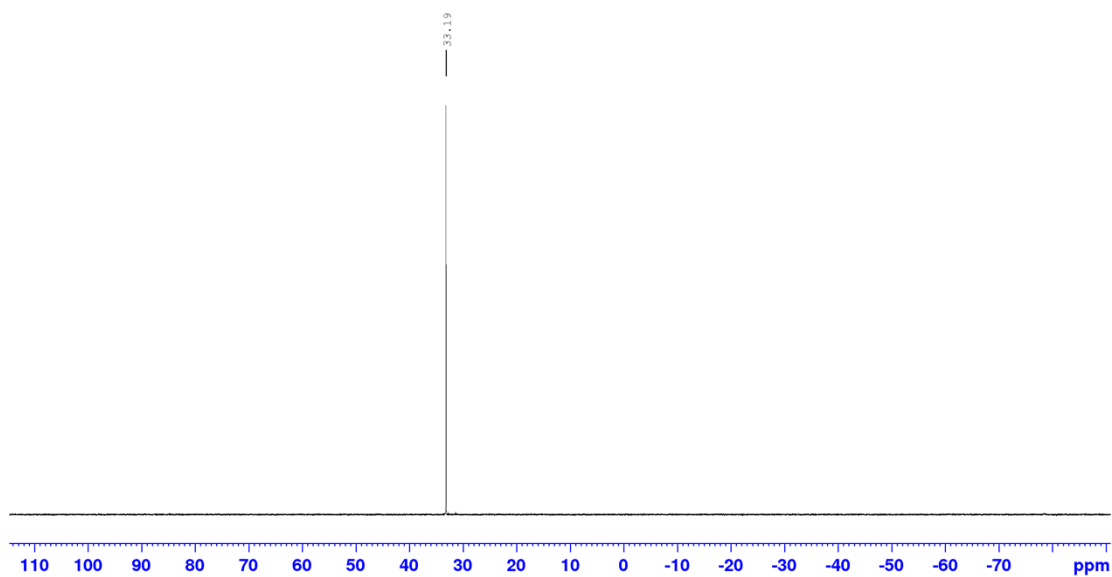
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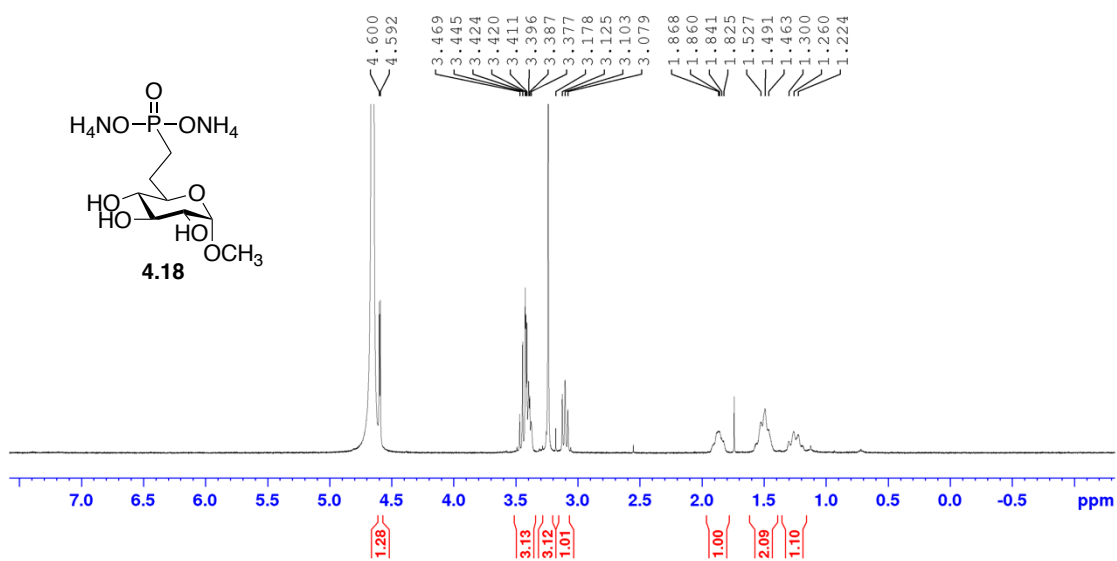
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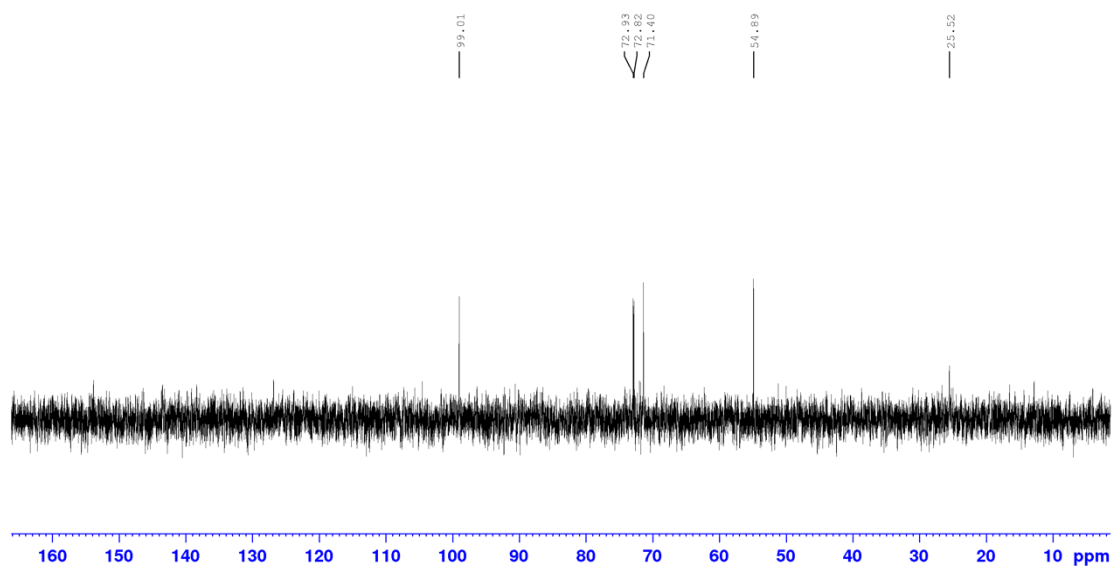
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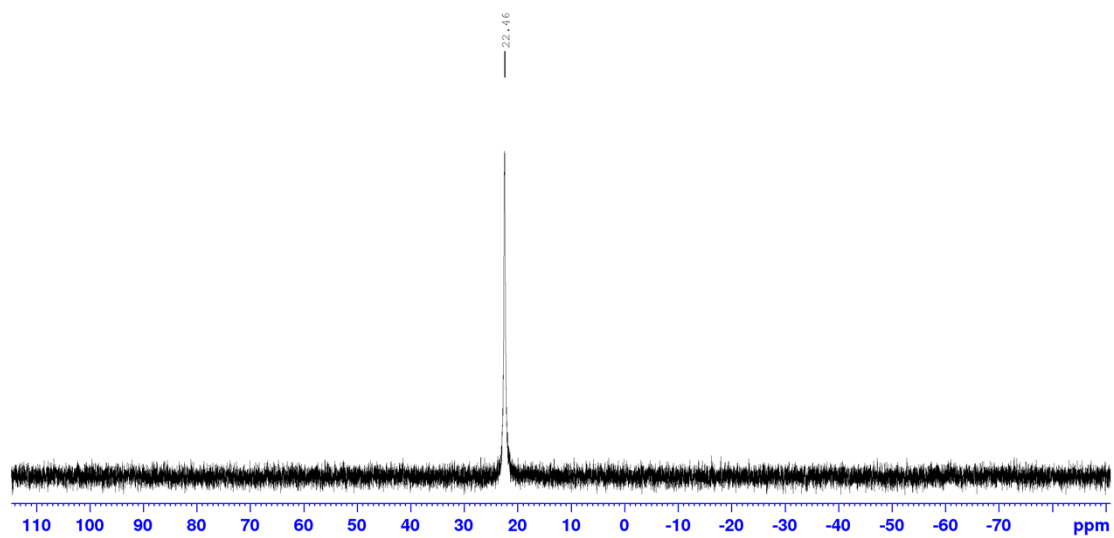
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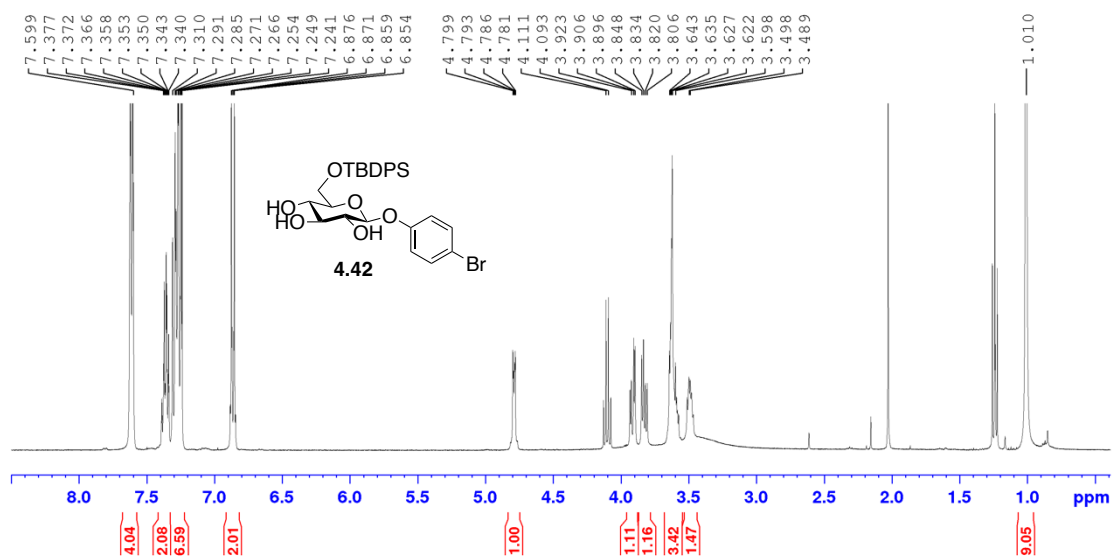
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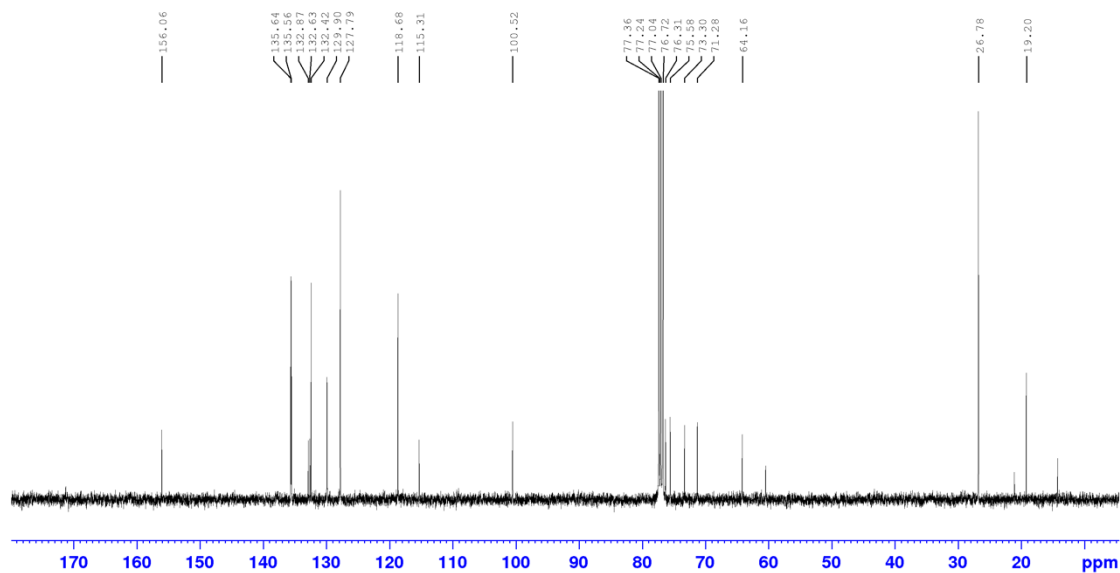
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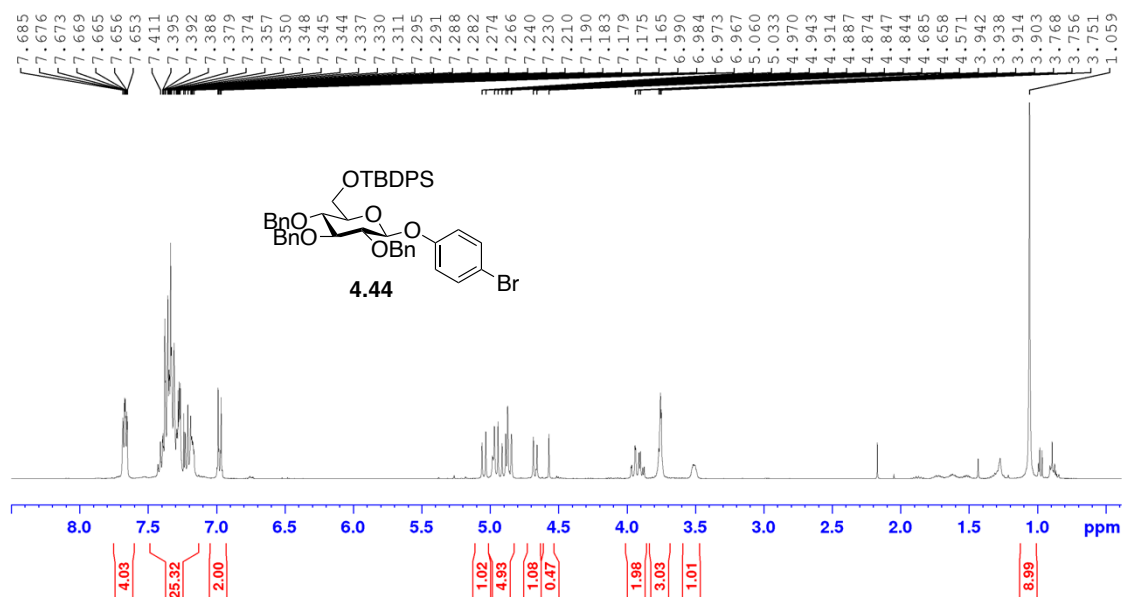
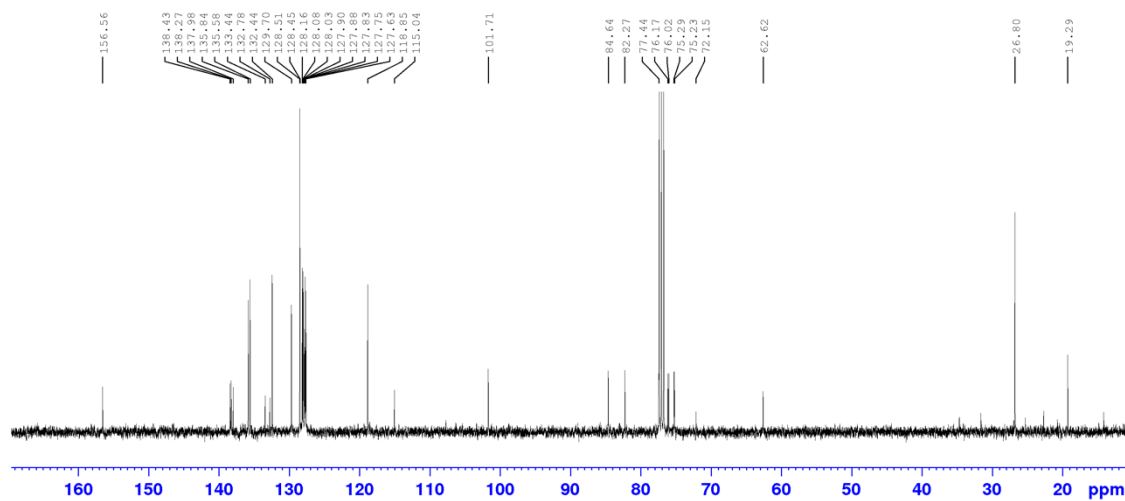


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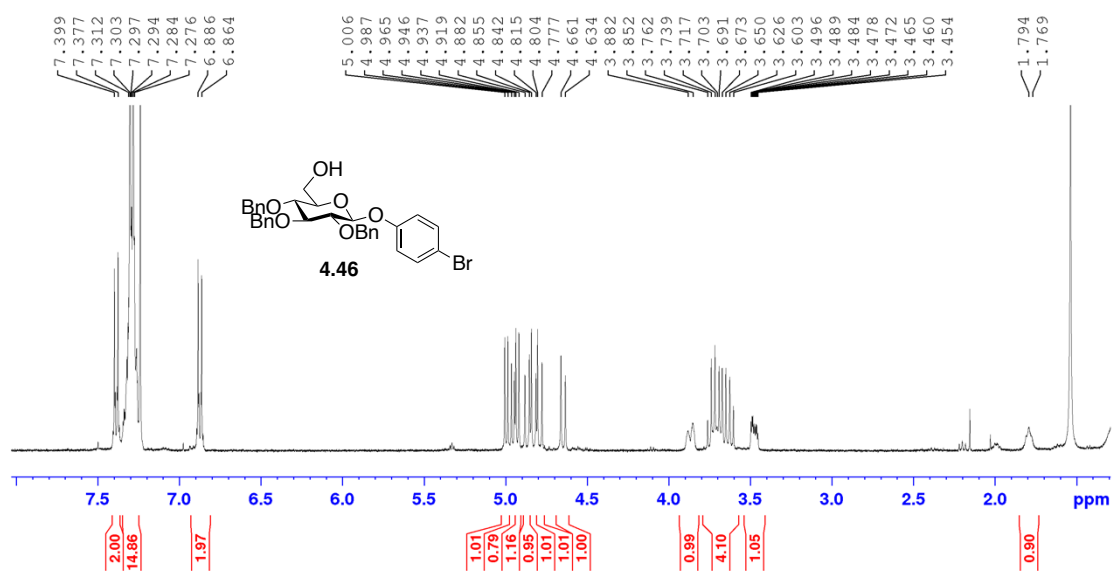


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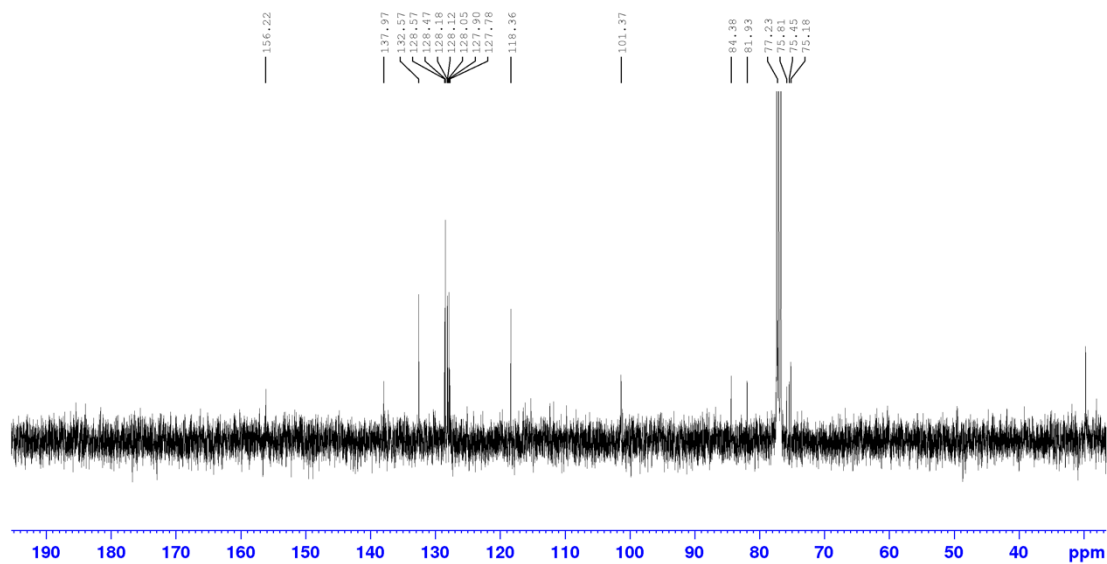


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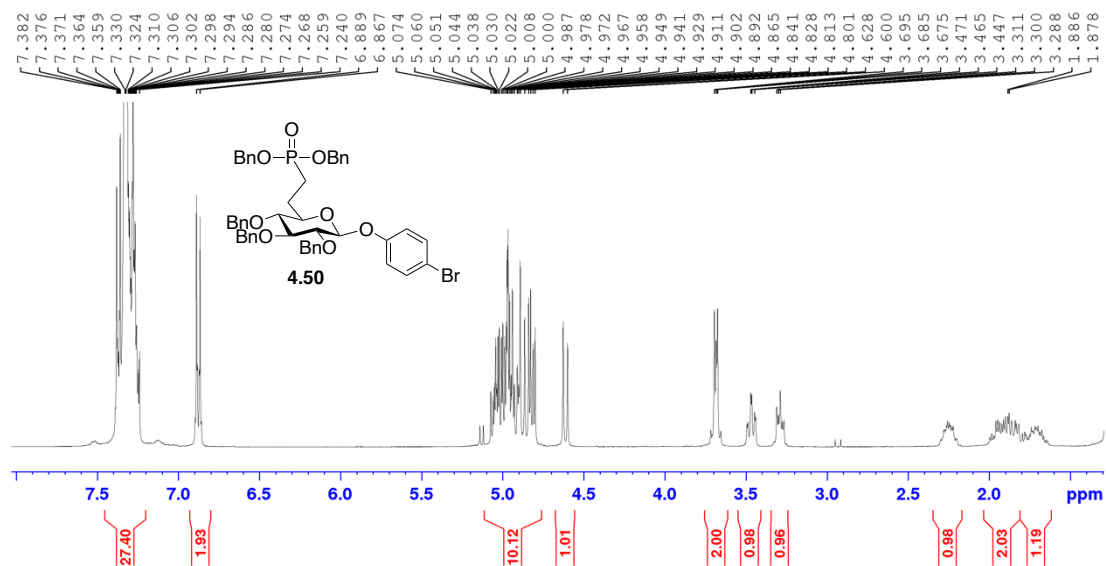
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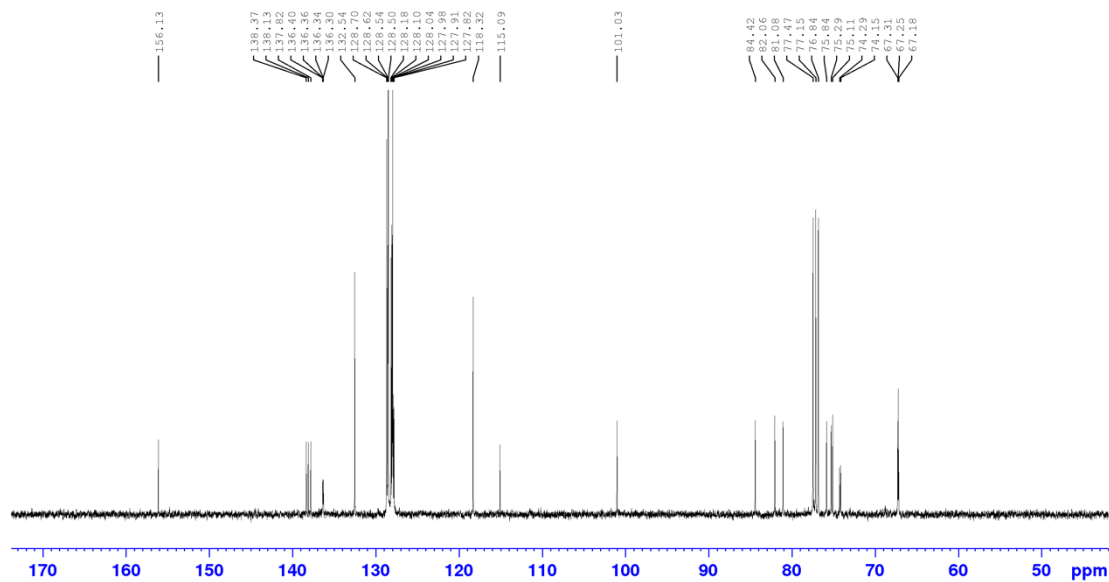
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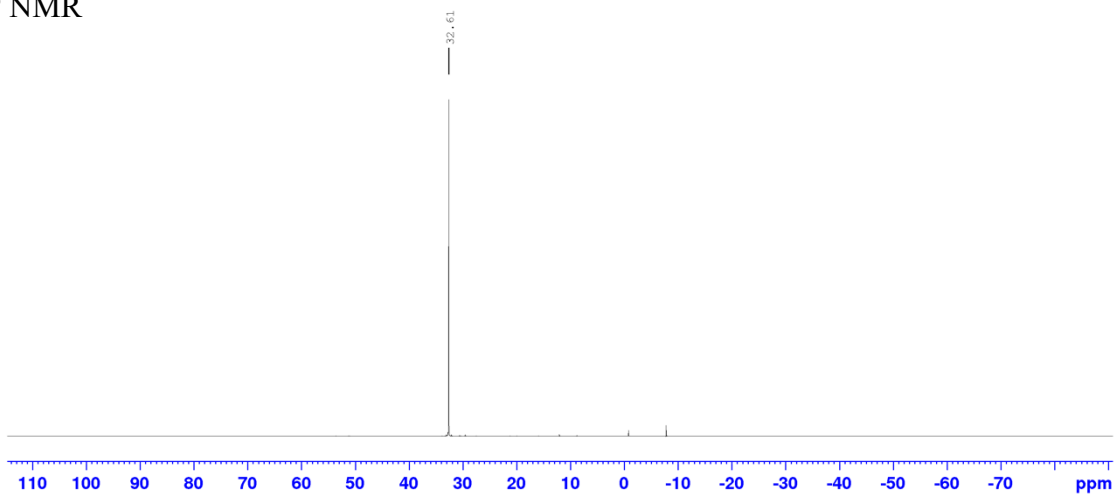
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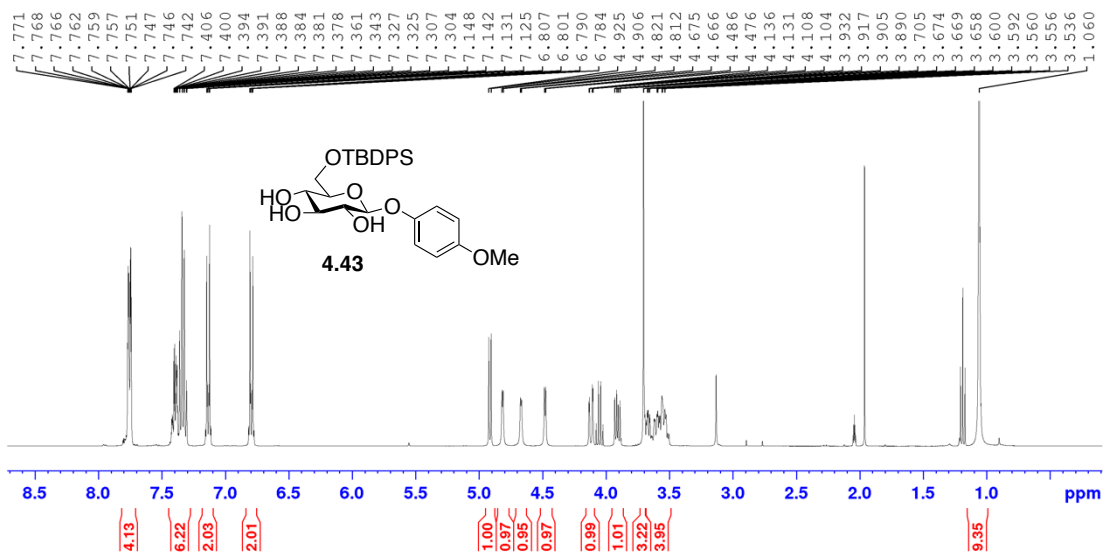
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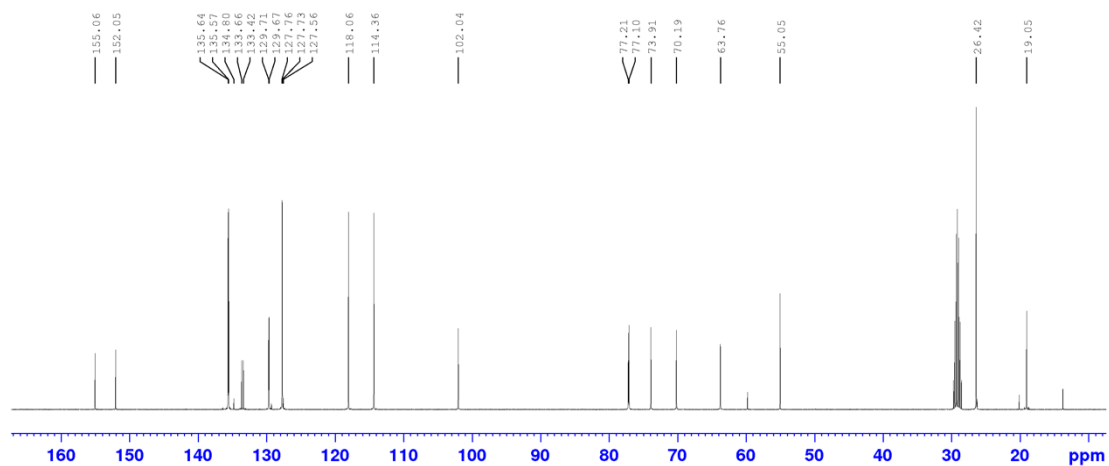
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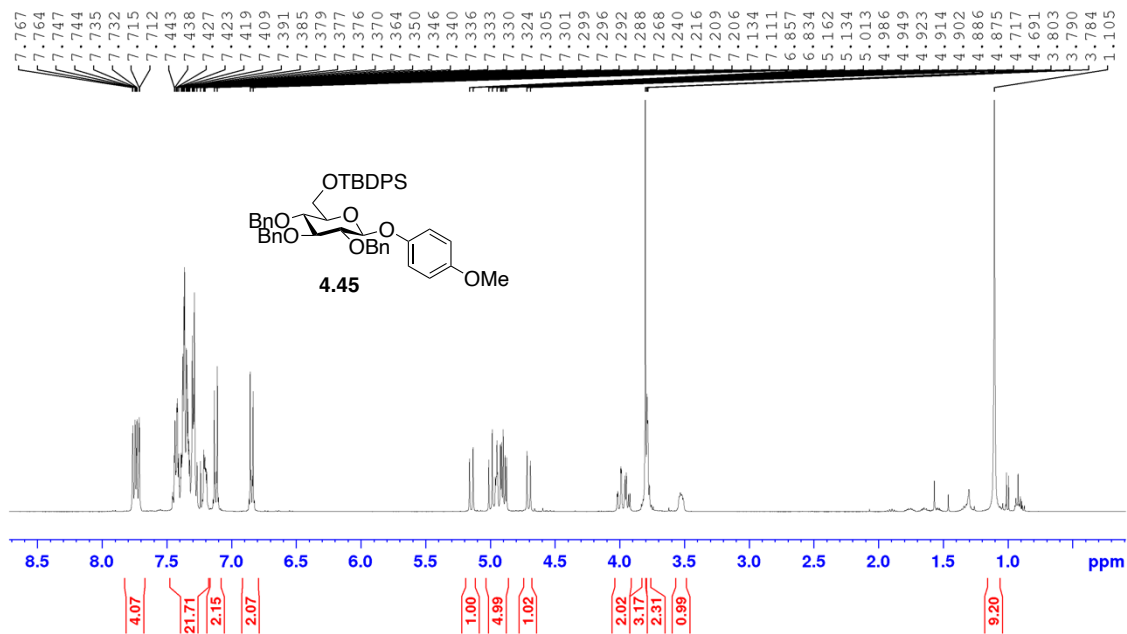
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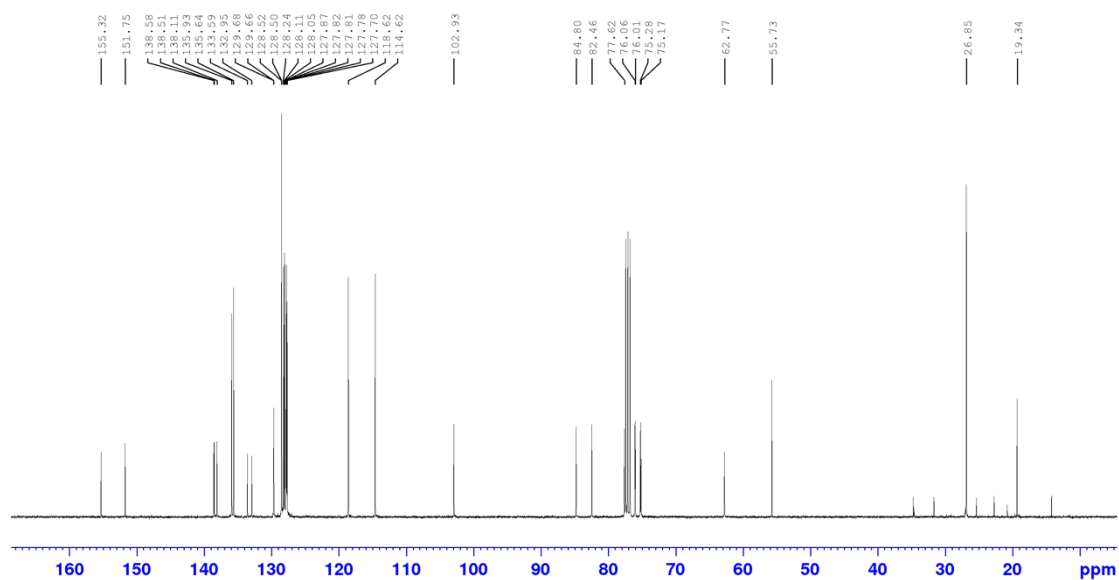
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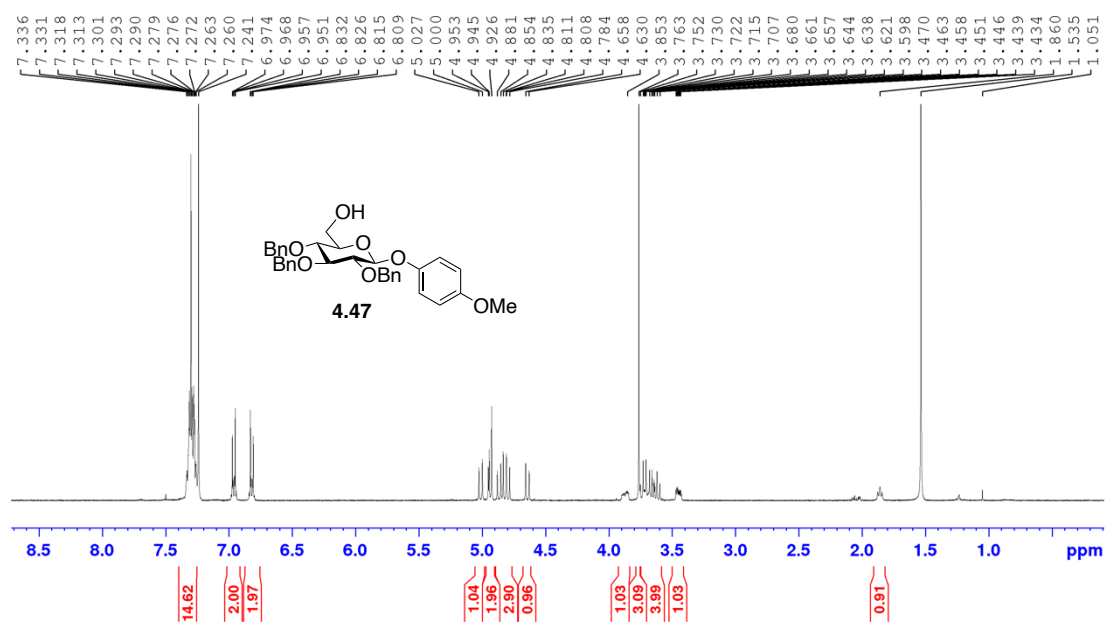
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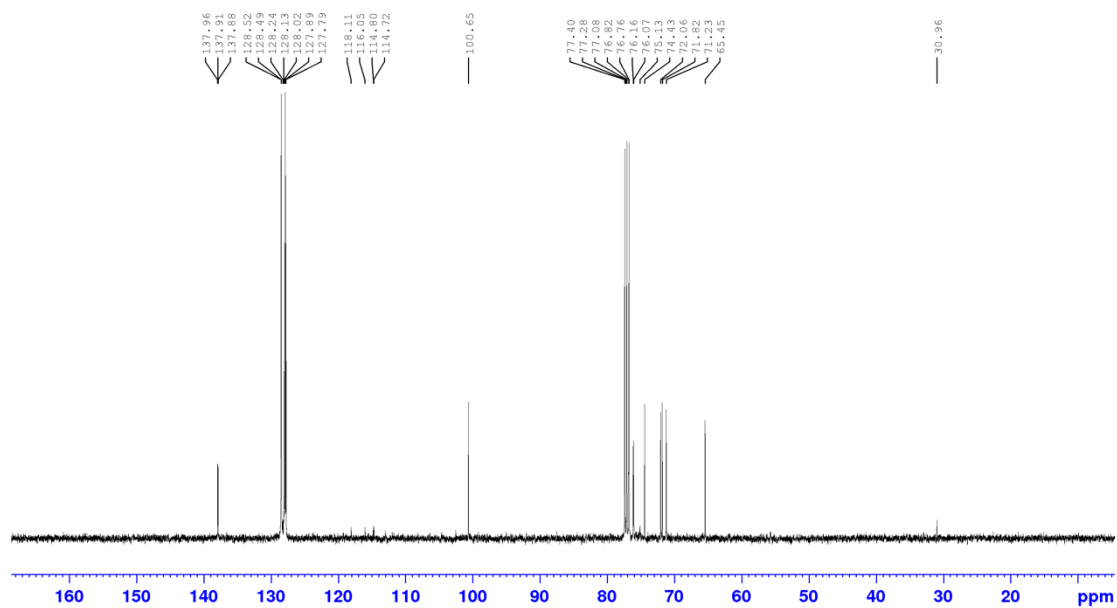
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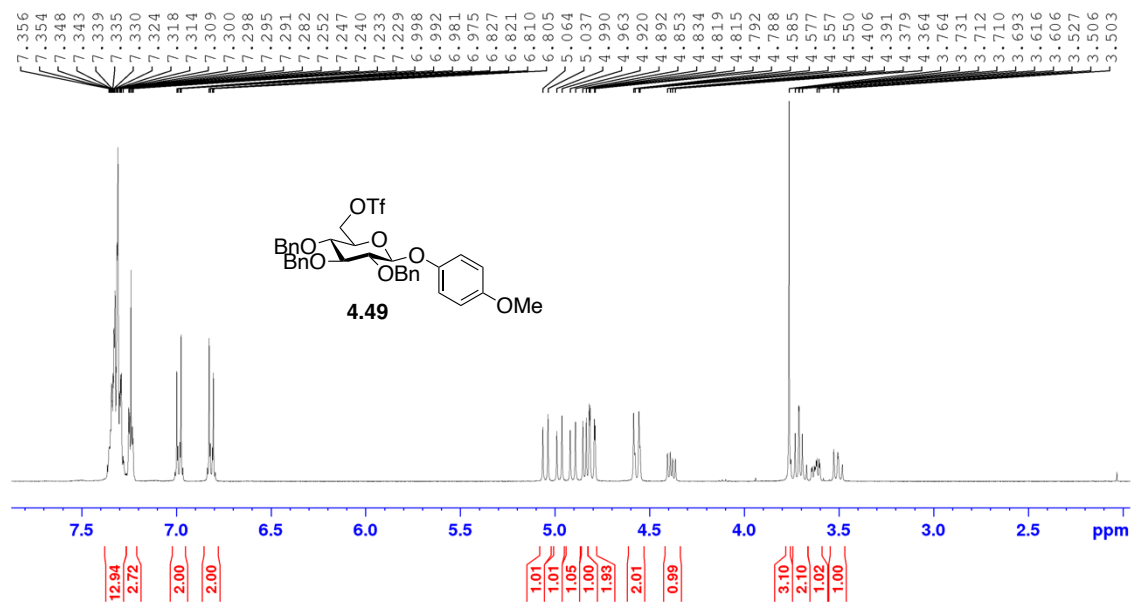
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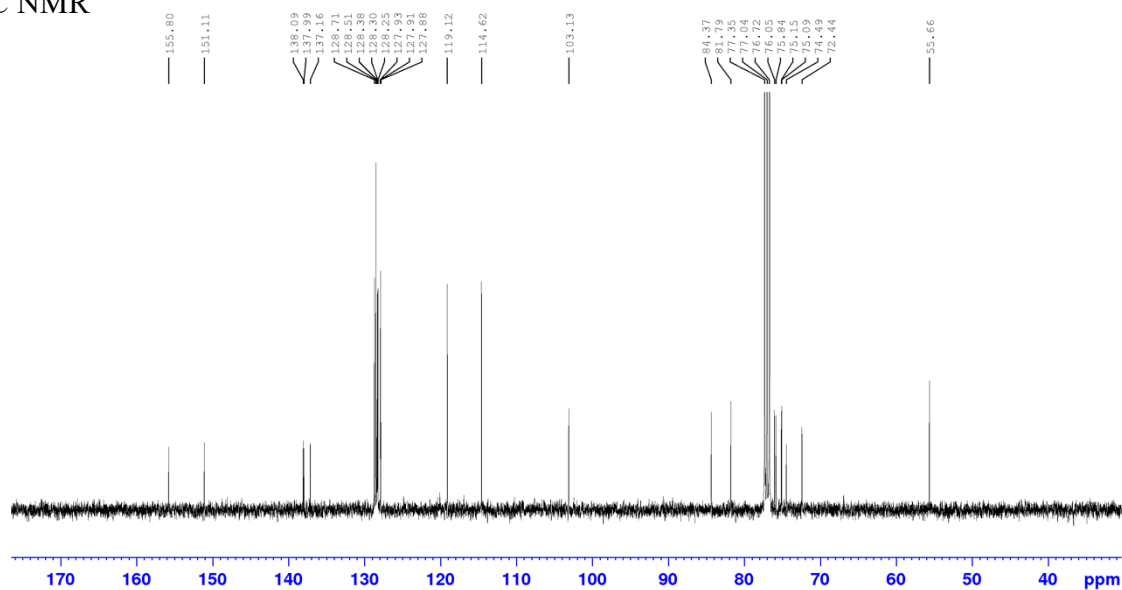
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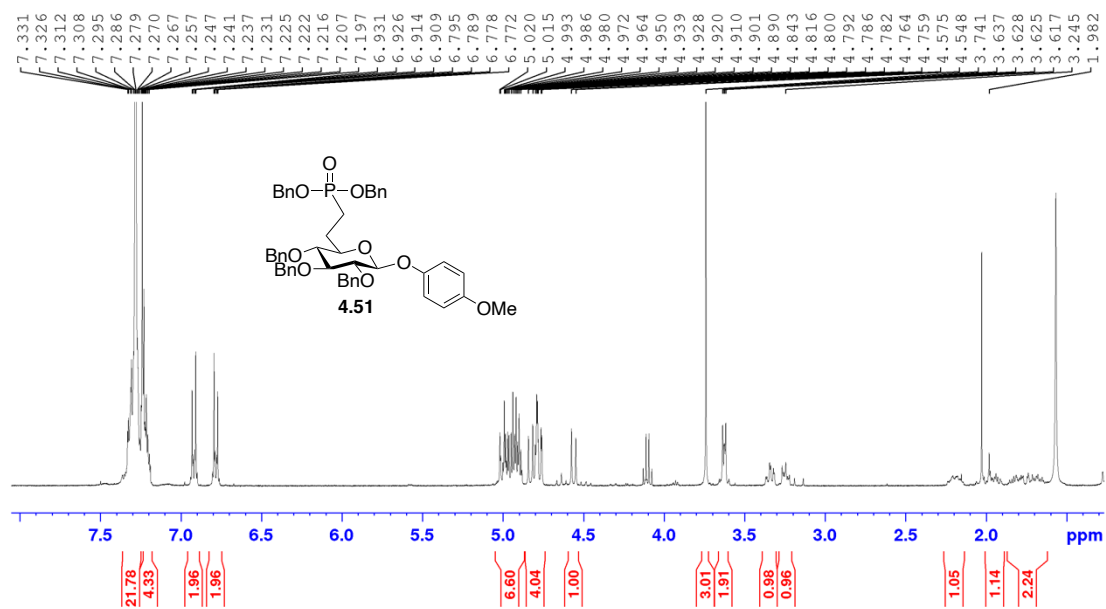
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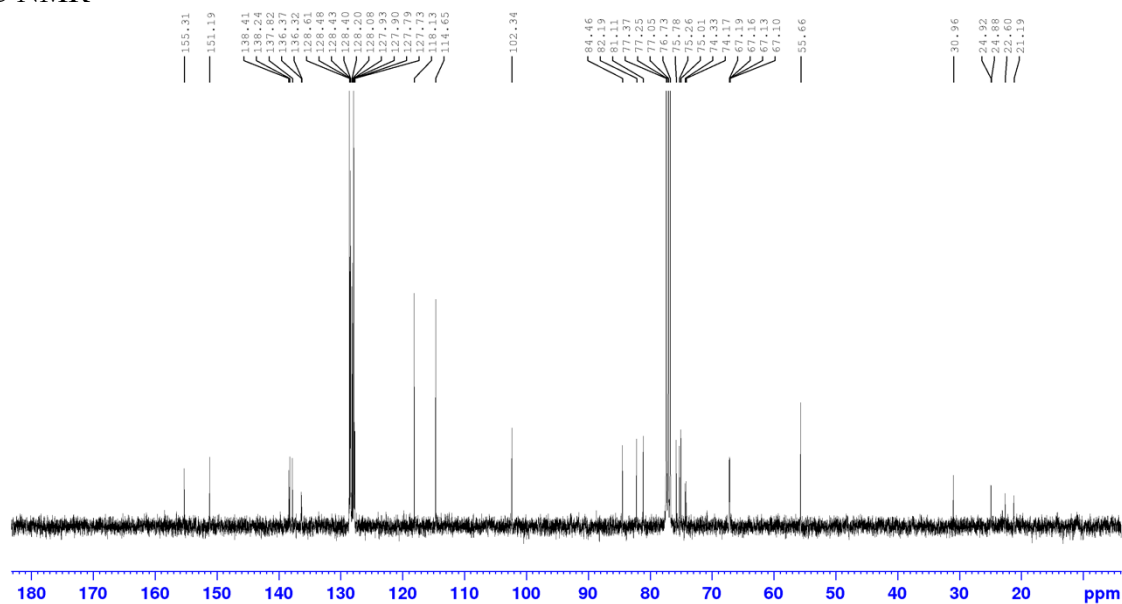
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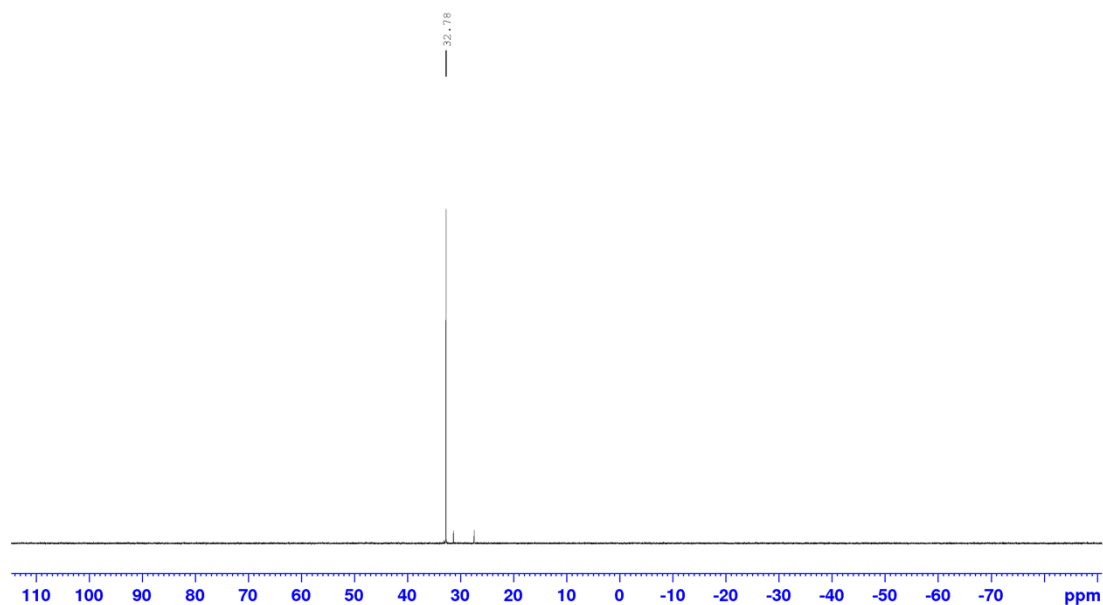
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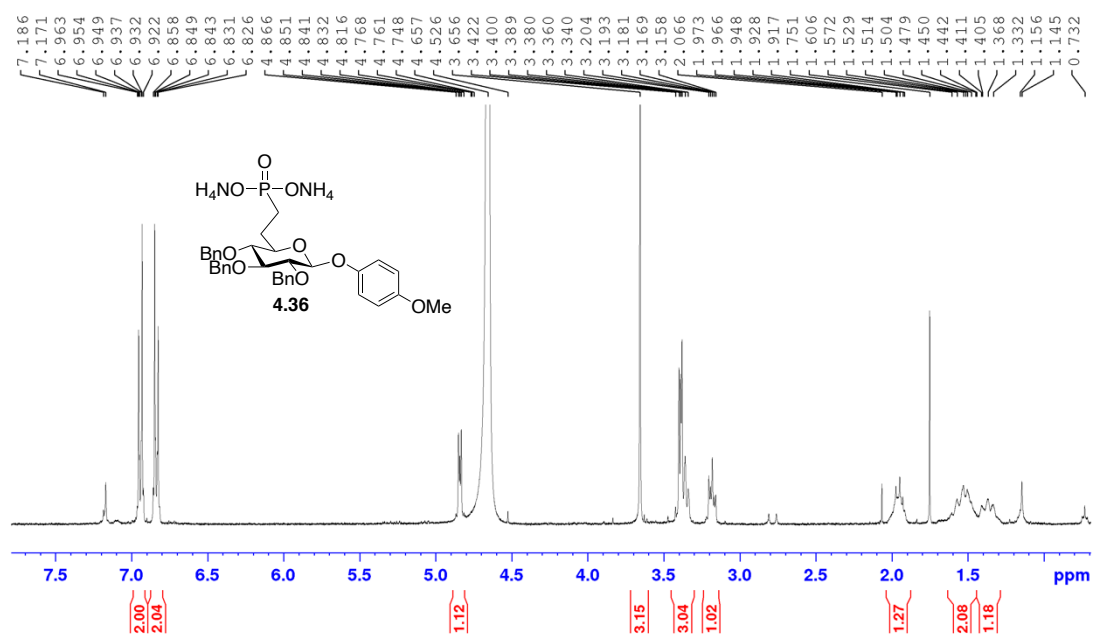
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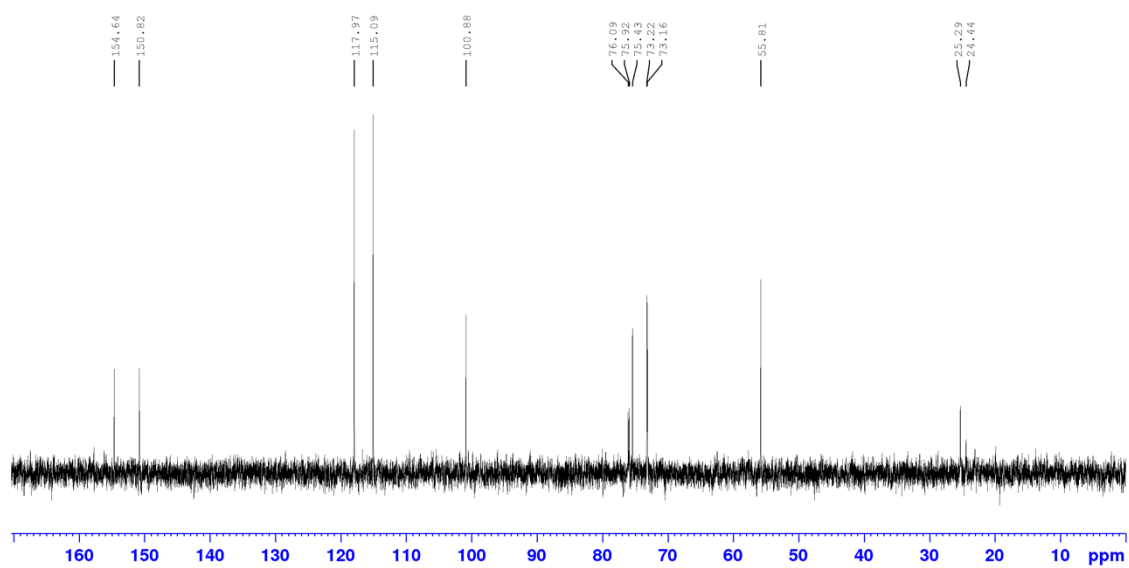
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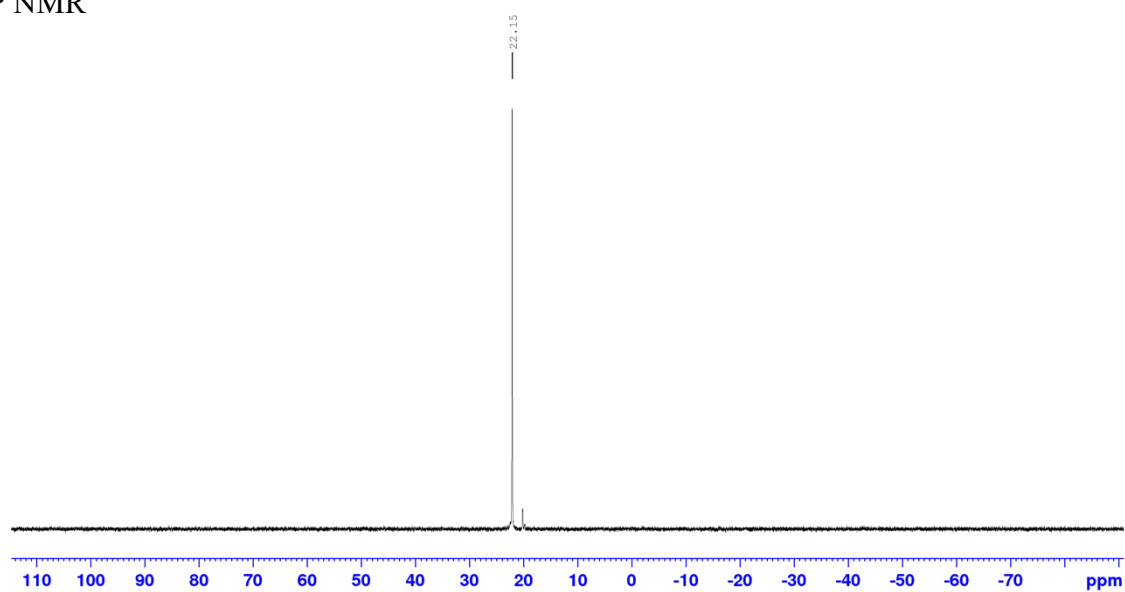
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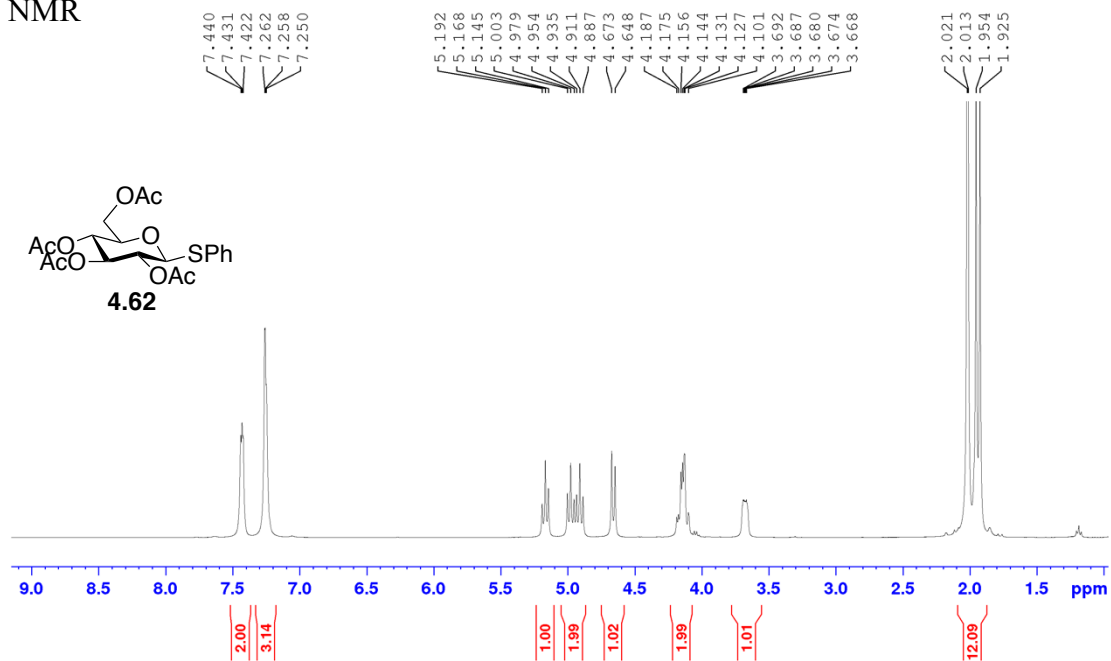
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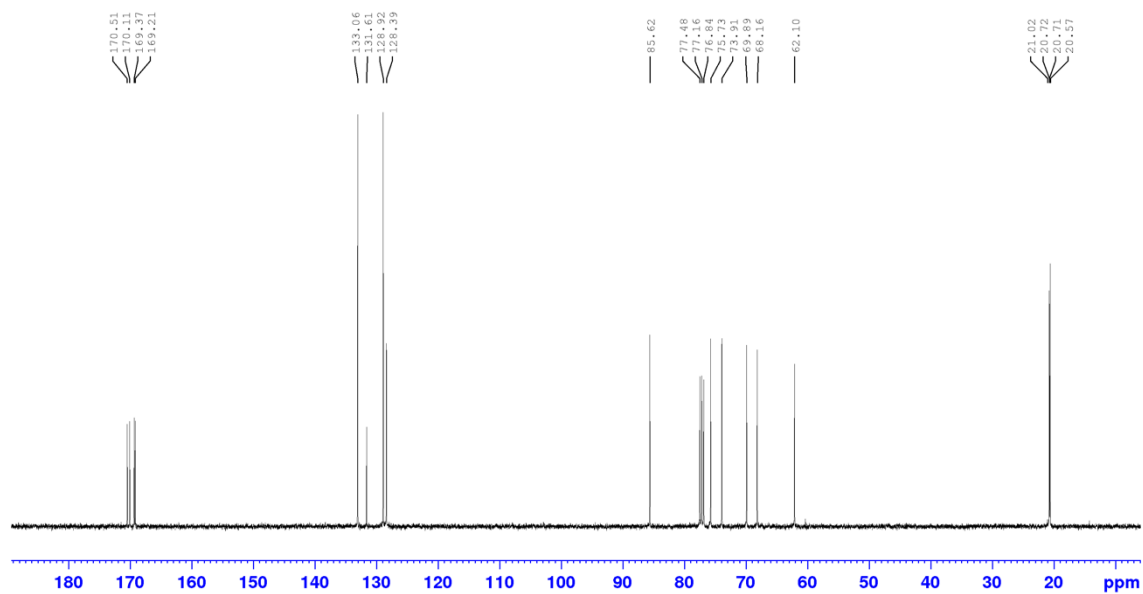
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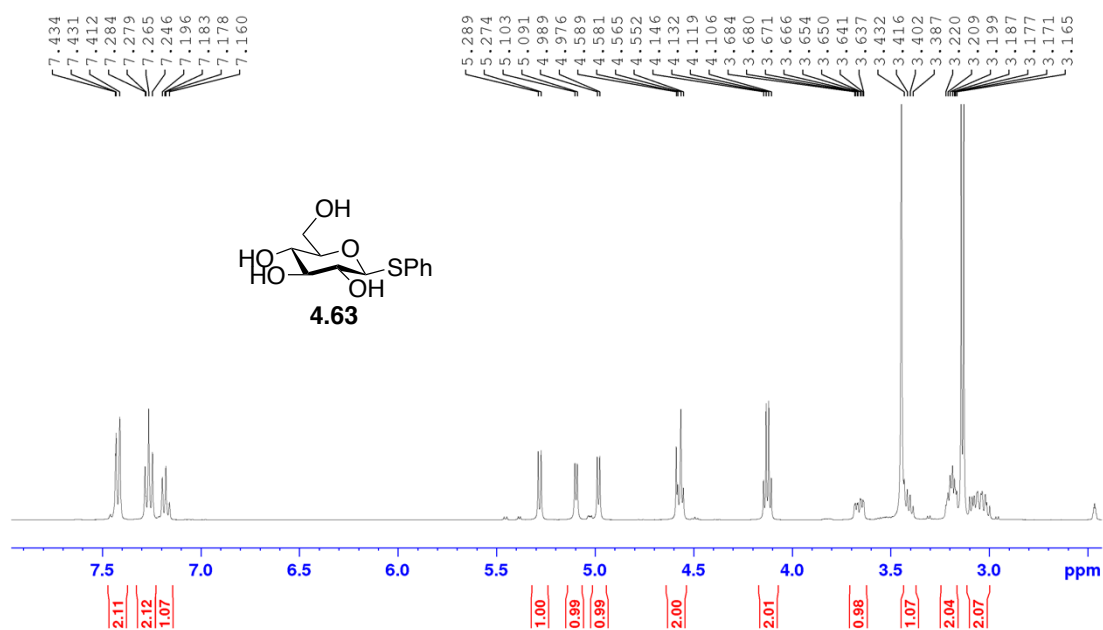
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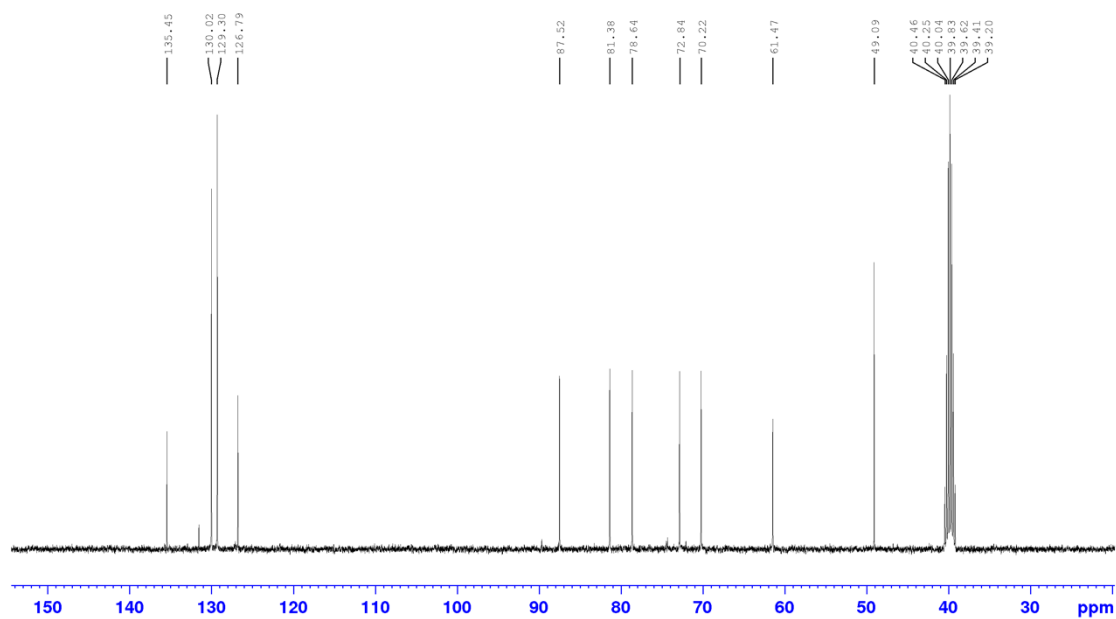
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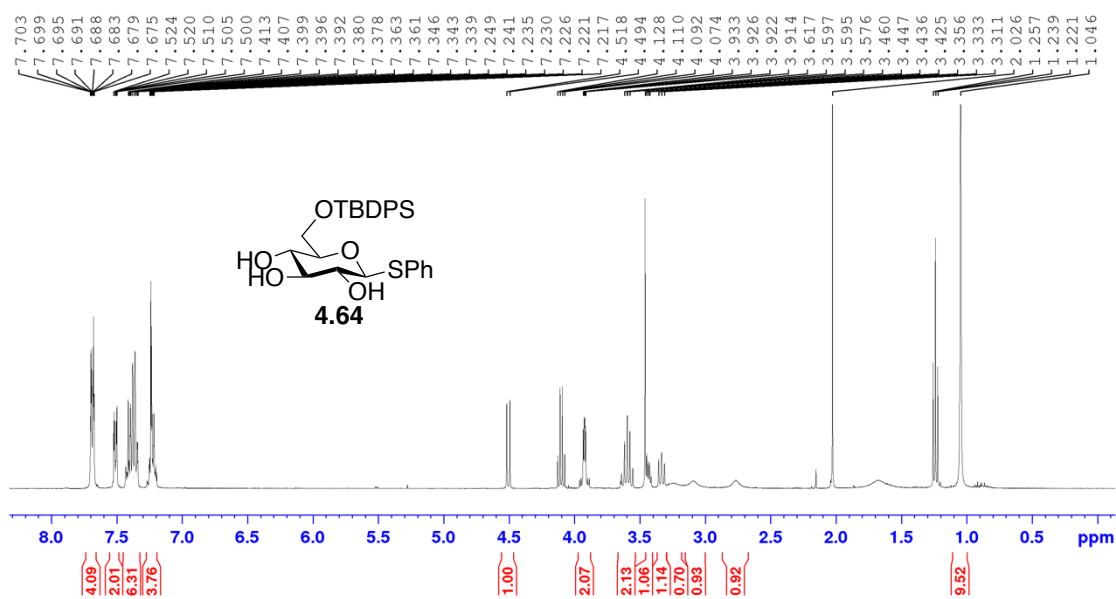
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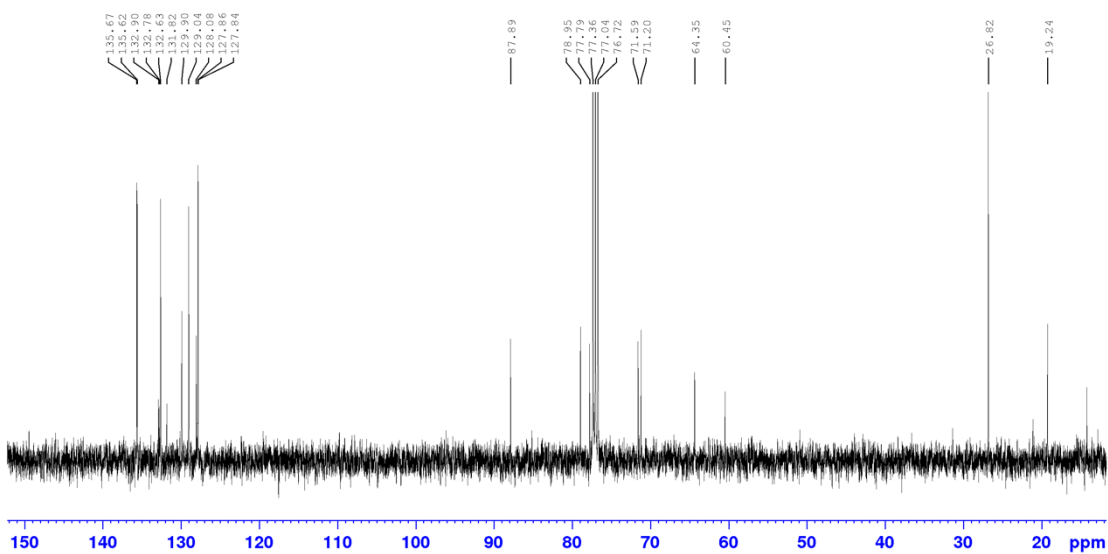
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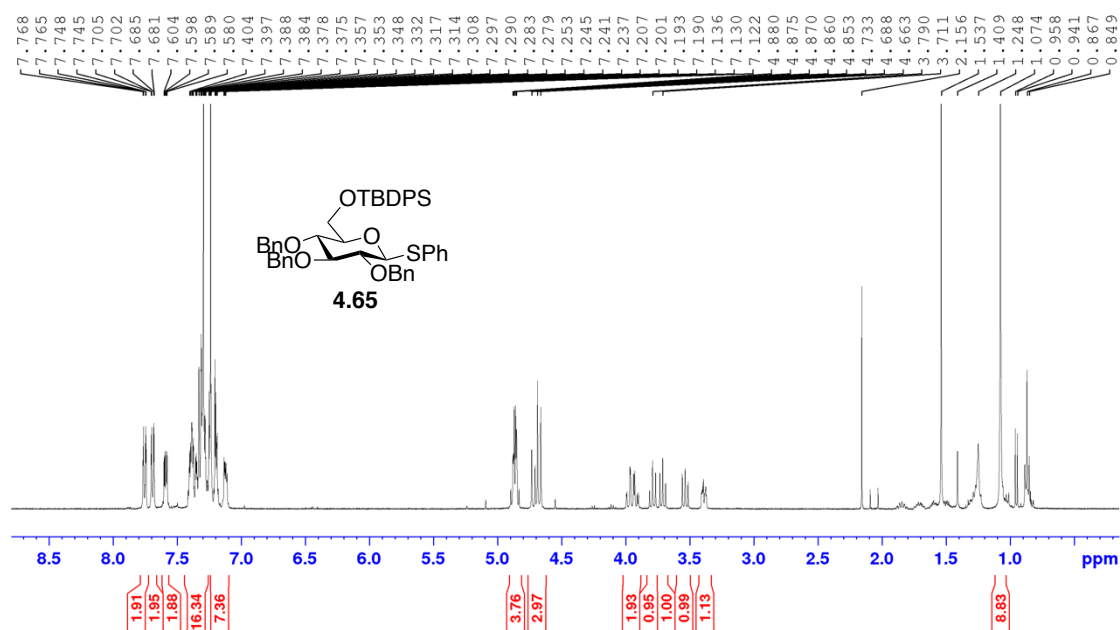
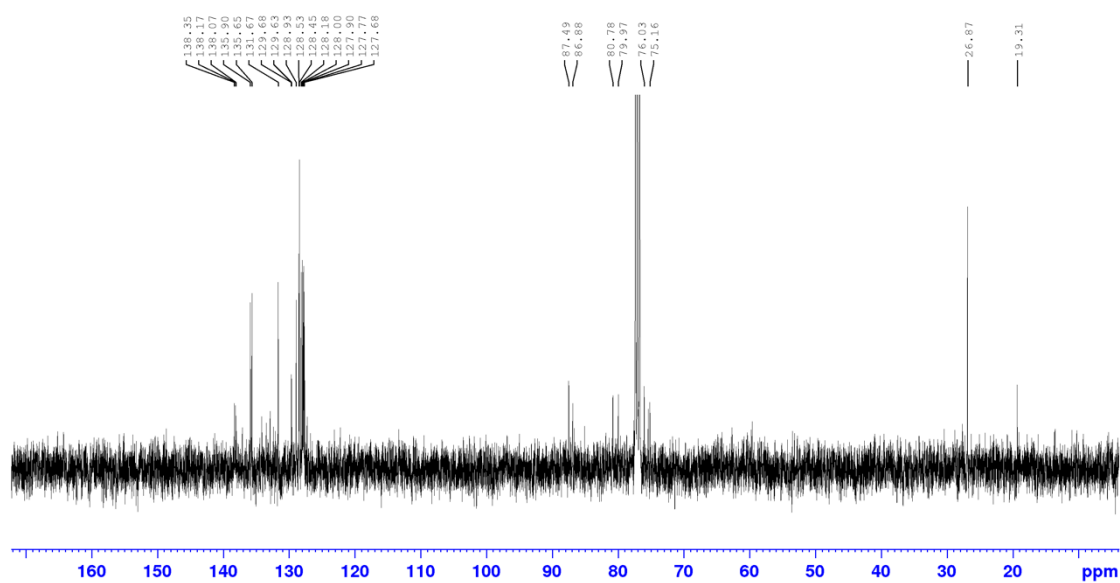


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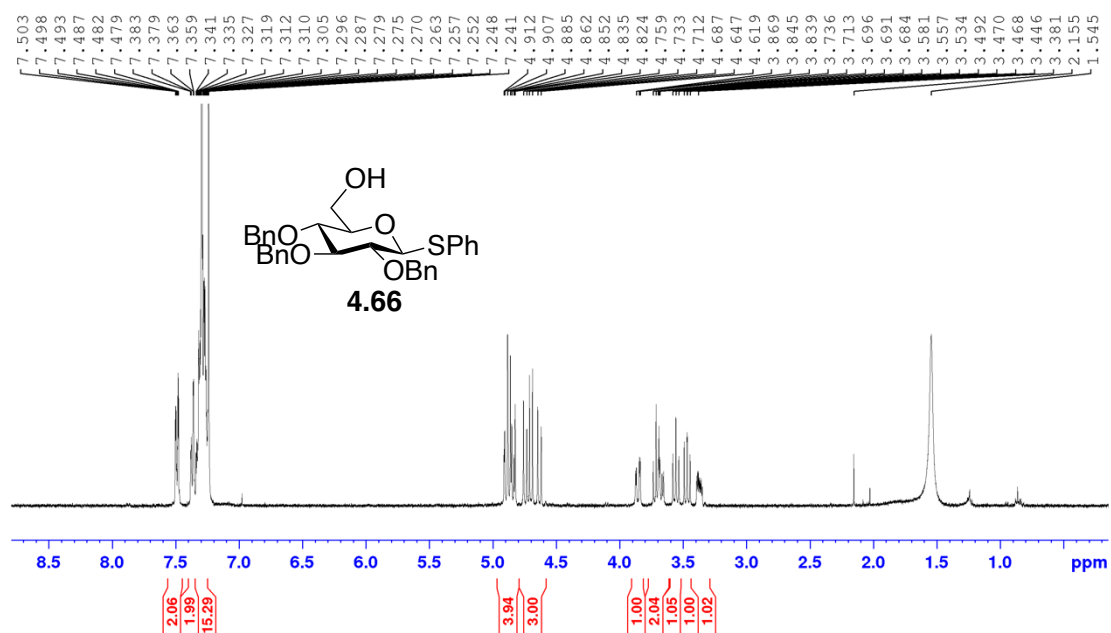


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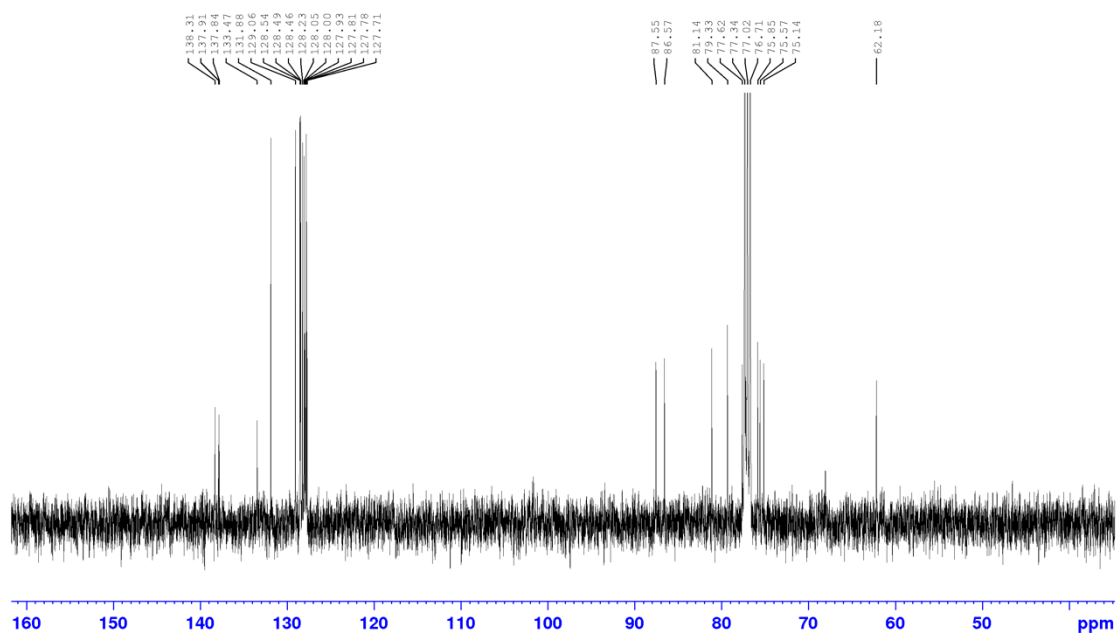


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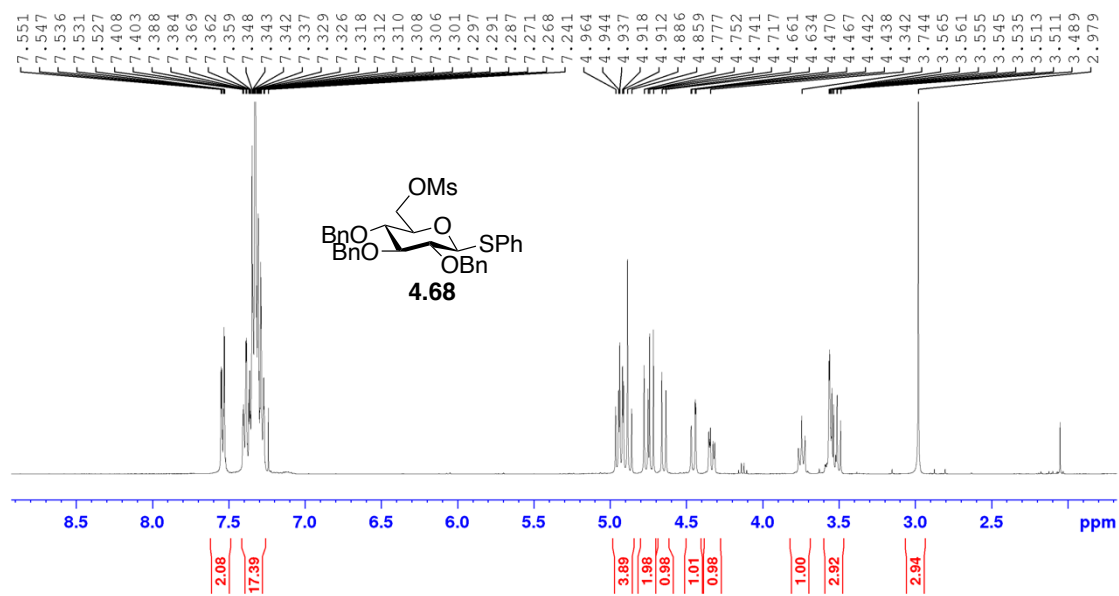
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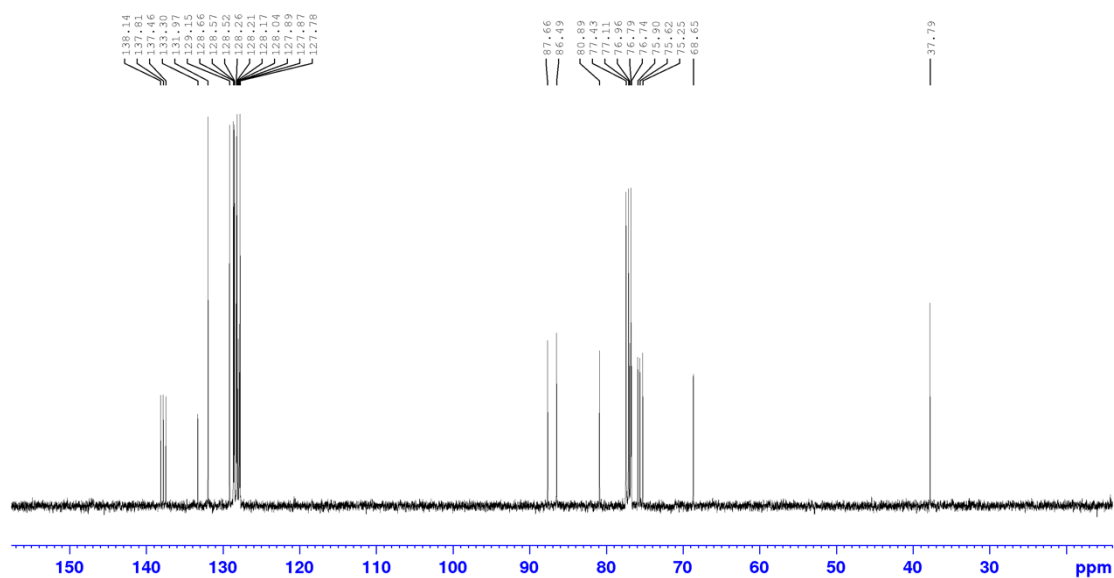
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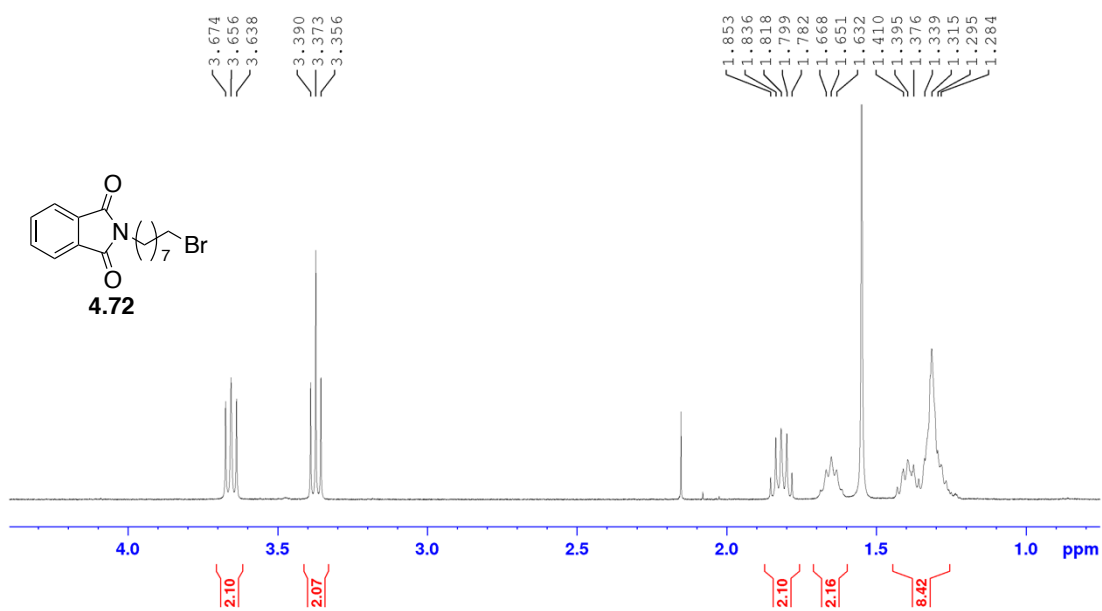
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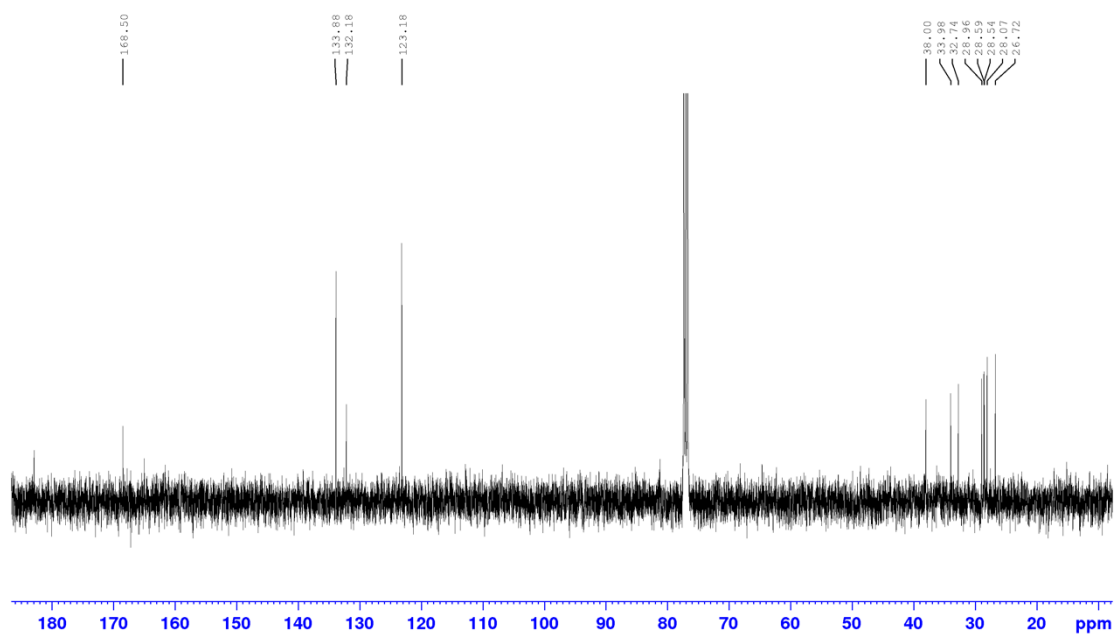
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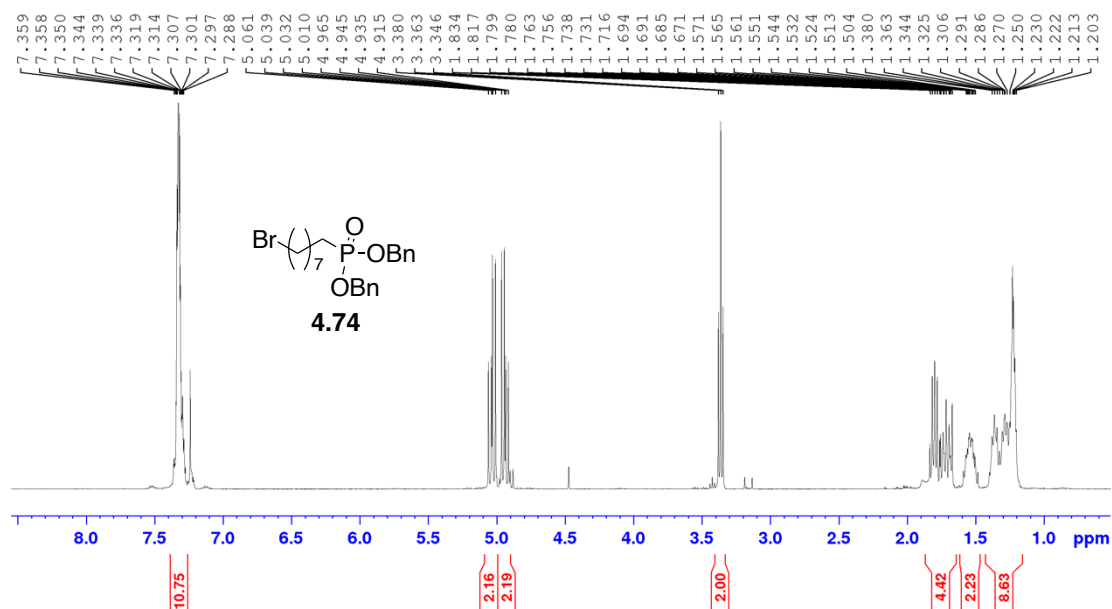
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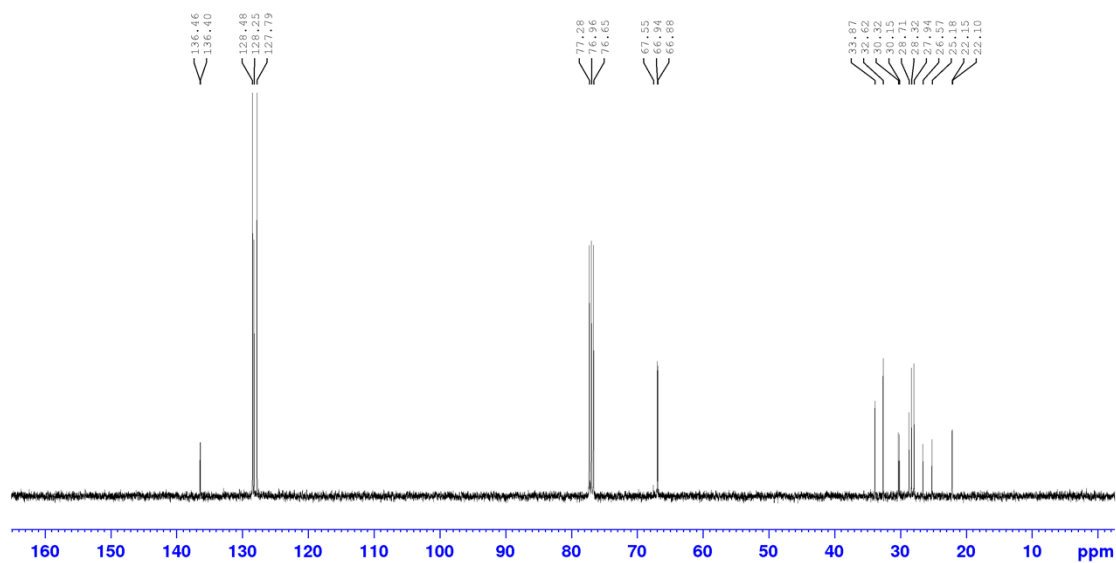
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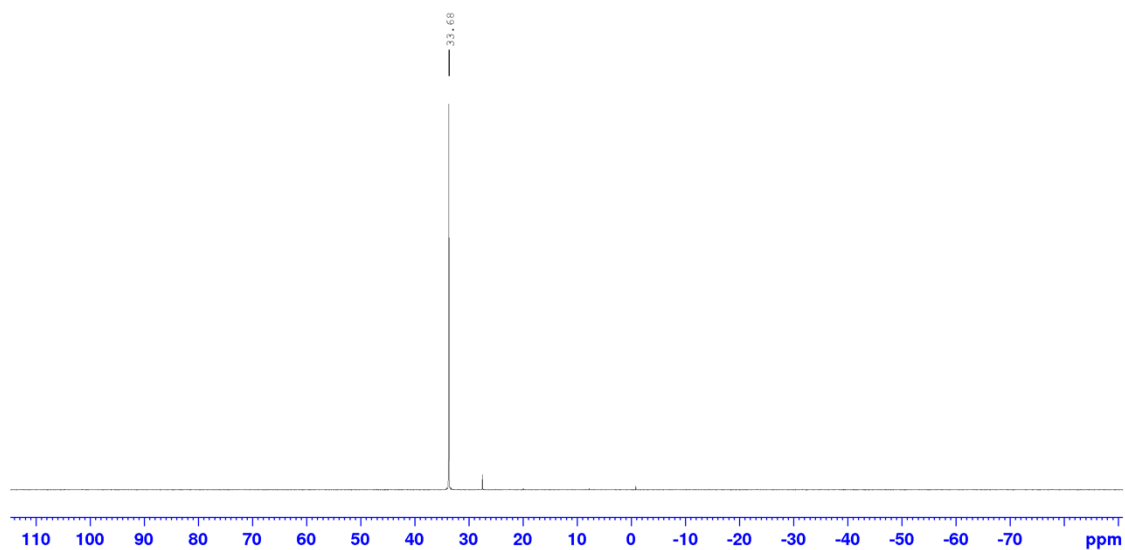
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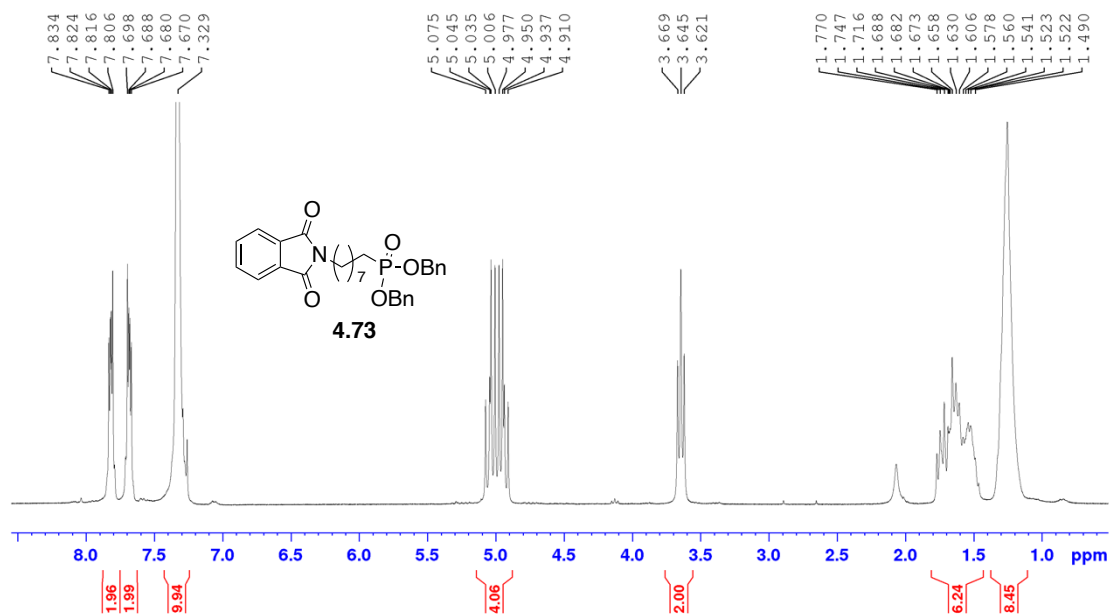
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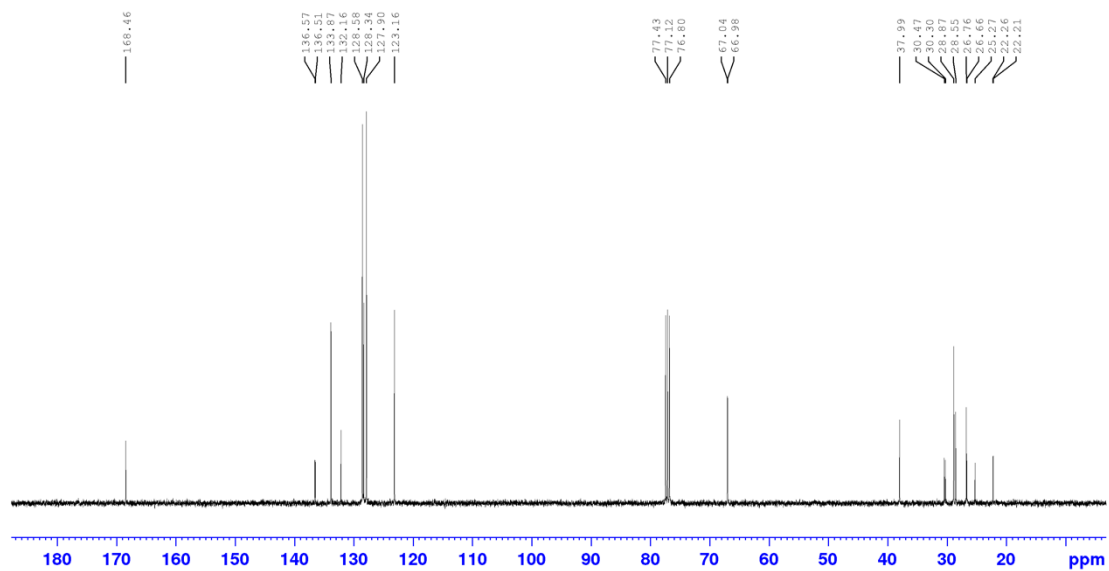
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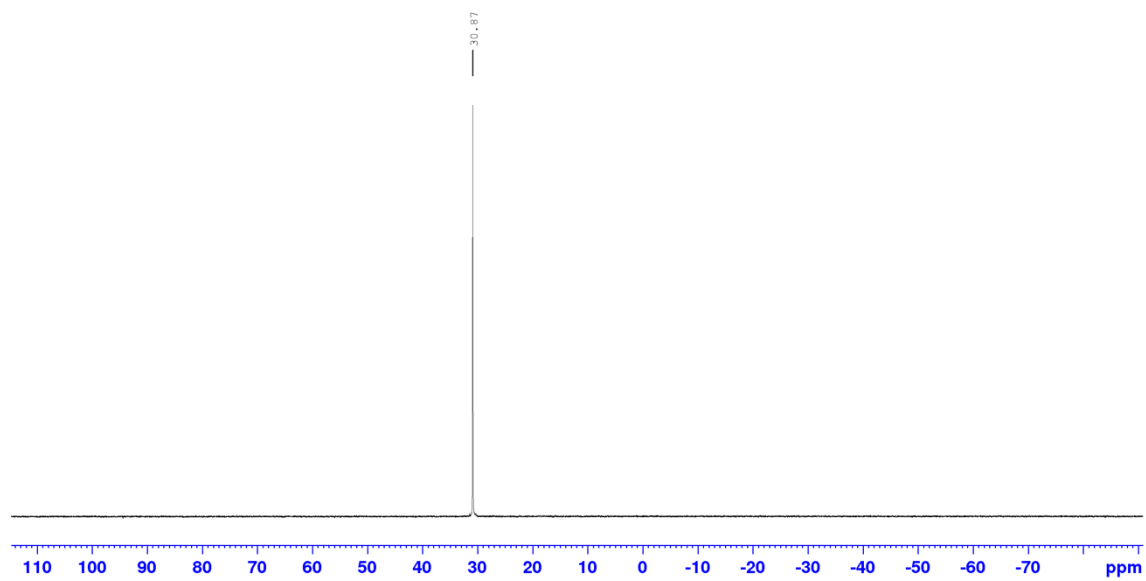
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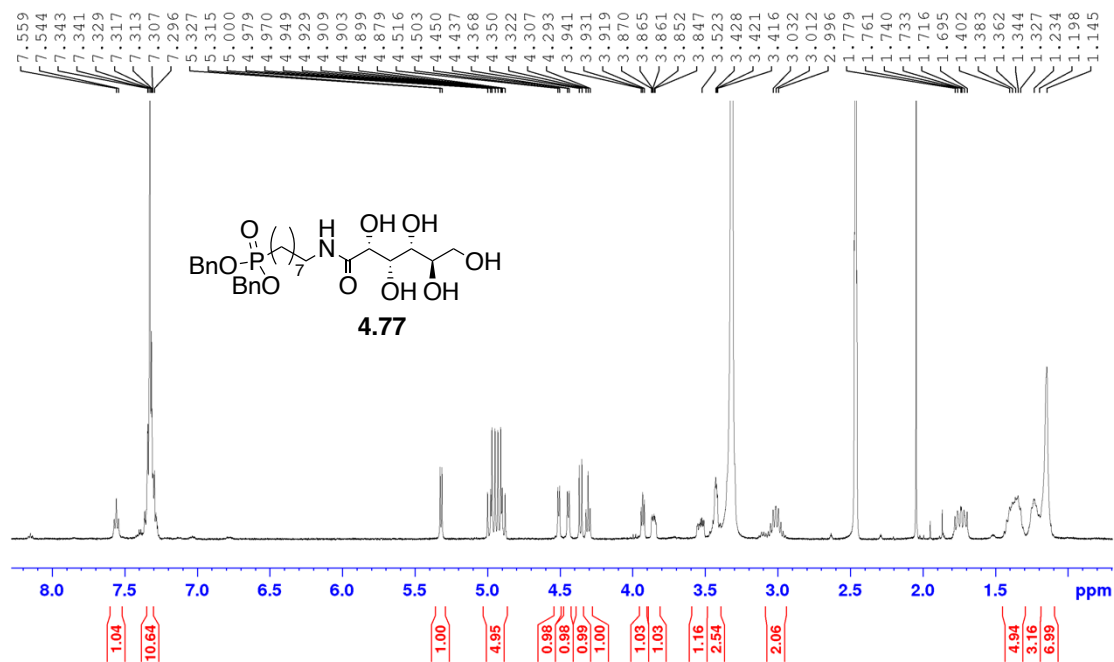
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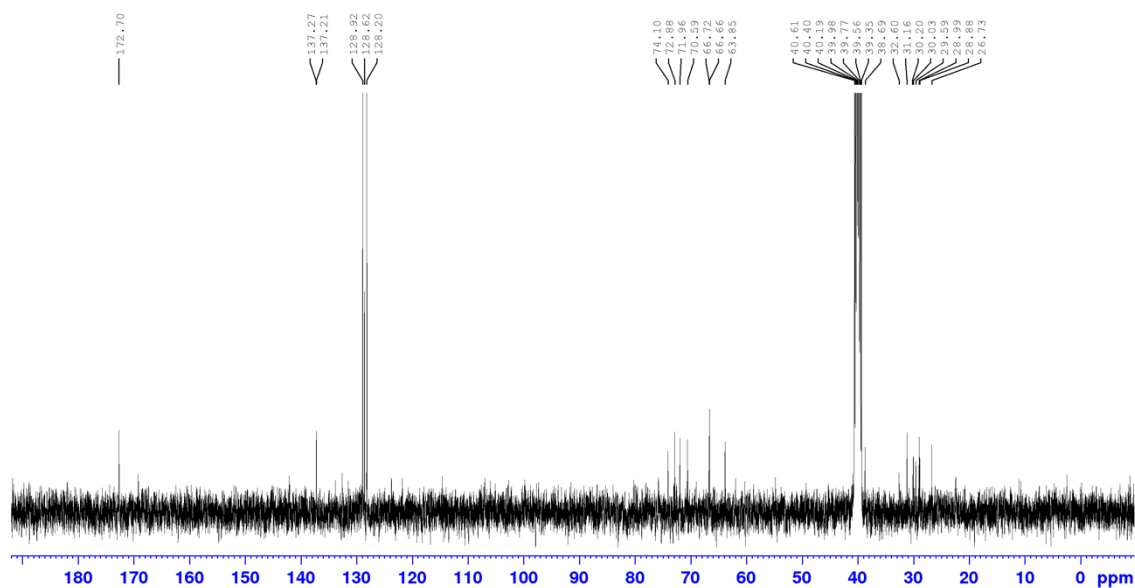
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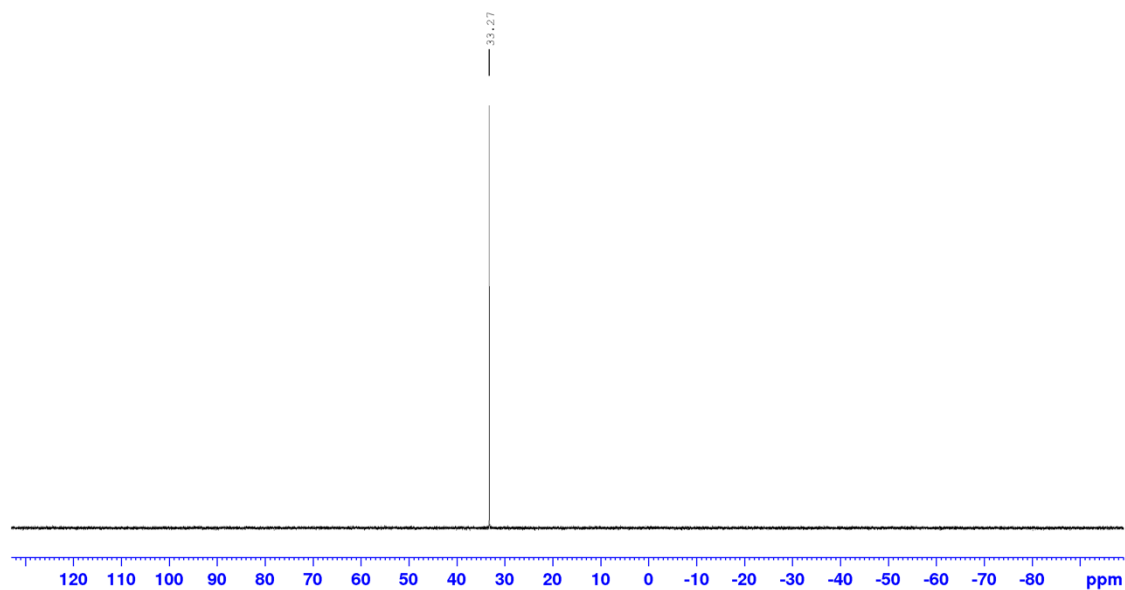
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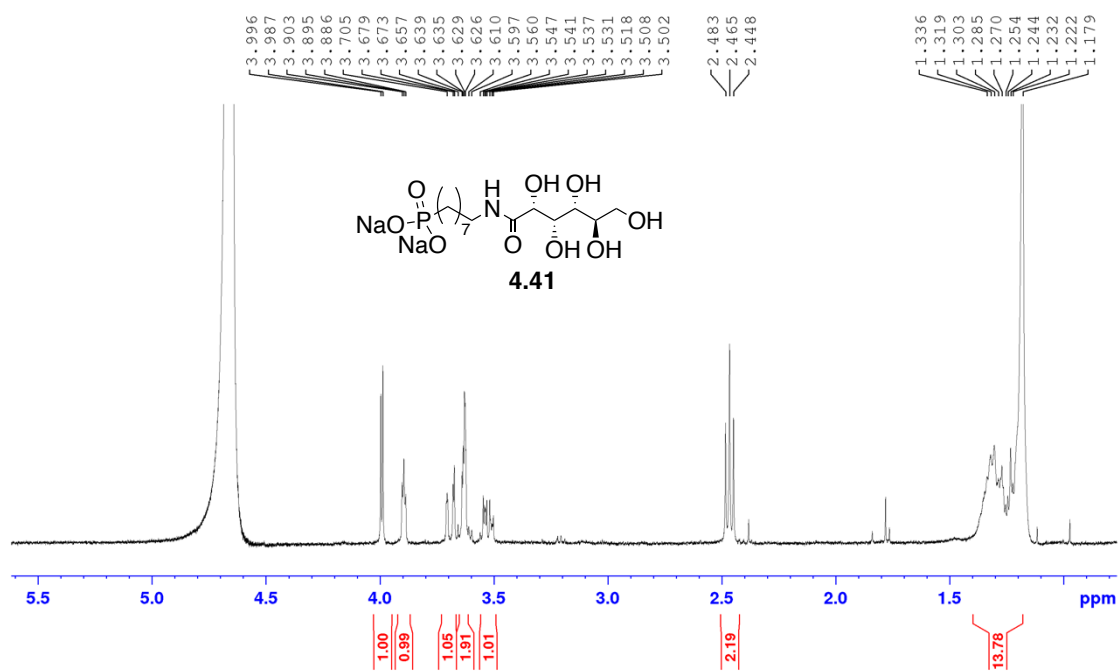
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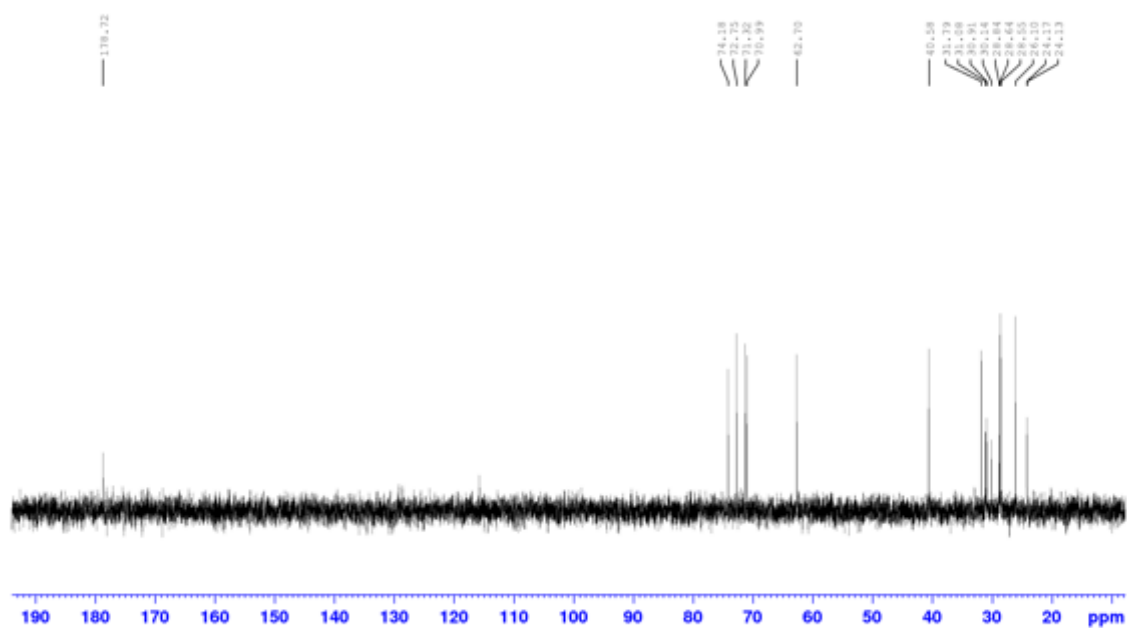
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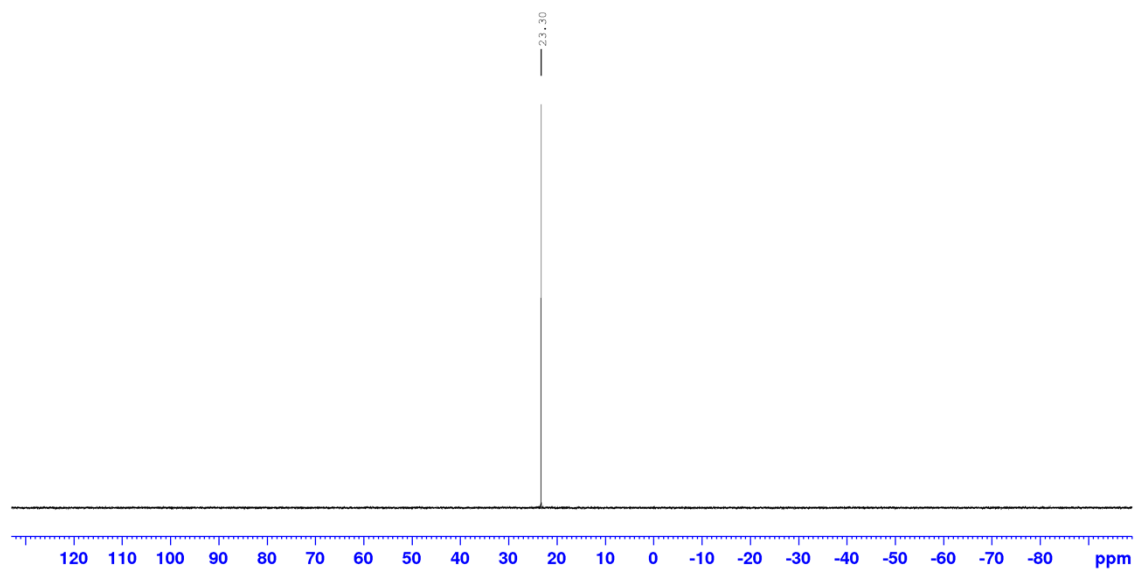
¹H NMR



^{13}C NMR



^{31}P NMR



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Chapter 5. Evaluating the effects of different assays on IRI activity

5.1 Introduction

5.1.1 *The importance of ice recrystallization inhibitors for cryopreservation*

There are a variety of mechanisms that cause cellular injury during cryopreservation, and ice recrystallization is the most prominent.¹ Recall that ice recrystallization is the process by which ice crystals grow larger at the expense of smaller ice crystals. This thermodynamically driven process is favoured as it results in an overall reduction in free energy as ice crystals grow larger.^{2–5} Typically in cryopreservation protocols, cryoprotective agents (CPAs), such as glycerol or dimethyl sulfoxide (DMSO), are used.^{6,7} These CPAs work by replacing intracellular water during cooling and by lowering the colligative freezing point of solutions however, they do not protect cells from mechanical ice damage caused by ice recrystallization.⁸ Thus, a plausible solution to this issue is the discovery of ice recrystallization inhibitors (IRIs).

Antifreeze proteins and glycoproteins (AF(G)Ps) were initially examined for their ability to bind to ice, leading to their two antifreeze properties, thermal hysteresis (TH) and ice recrystallization inhibition. Thermal hysteresis is the gap in temperature between the nonequilibrium melting point and freezing point, and this occurs as a result of AF(G)Ps binding along the $a1$, $a2$, and $a3$ axes representing the prism planes of ice (Fig. 5.1A).^{9,10} In the absence of AF(G)Ps water molecules bind to the prism planes at a much faster rate compared to the slow-growing basal planes (along the c axis). This is due to water molecules being favourably oriented along the prism planes for hydrogen bonding.¹¹ In the presence of high concentrations of AF(G)Ps below the freezing point, a burst in ice growth occurs only along the c axis leading to needle-like spiculated ice crystals (Fig. 5.1B), which have been shown to be detrimental to cells.^{10,12} Therefore,

the TH activity of AF(G)Ps limit its use in cryopreservation, as well as challenges with their isolation and purification. As a response, synthetic analogues of AF(G)Ps with various structural modifications have been discovered that lack TH activity but possess IRI activity. Synthetic antifreeze glycoproteins,^{13–20} small molecule carbohydrates,^{21–26} polymers^{27–33} and polyampholytes^{34–36} are all examples of AF(G)P analogues with IRI activity. Many of these IRIs have been applied to cells and tissues prior to freezing to regulate ice growth and have resulted in improved post-thaw viability and/or functionality.^{19,22,23,29,37–41}

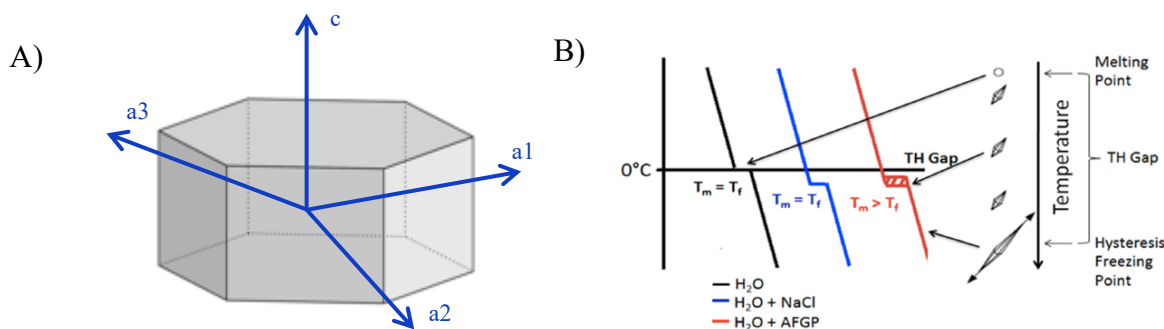


Figure 5.1. A graphical representation of ice crystal planes (A) and the needle-like ice crystal habit in an AFGP- H_2O solution compared to a H_2O , and H_2O -salt solution (B). Ice crystal axes $a1$, $a2$, and $a3$ represent the prism planes and ice crystal axis c represents the basal plane. Image B reprinted with permission from Wilson, P., Ed.; *InTech*, 2013; pp 177–224. Copyright © 2013 InTechOpen.

5.1.2 Measuring IRI activity via ice recrystallization assays

The ability to prevent ice recrystallization is valuable in cryopreservation, as well as applications in the food industry^{42–46} and transport industry.^{47,48} Thus, several assays have been designed to detect changes in ice recrystallization. In general, a compound is deemed “IRI active” if it can slow down the natural growth process of ice crystals over a period of time. Early studies identified AF(G)Ps as ice-binding proteins by observing the crystal habit and growth pattern of ice crystals using X-ray diffraction and single ice crystal growth experiments.^{49,50} In these

experiments, the ice crystal habit in the presence of AF(G)P were compared to ice crystals in pure water. As the field progressed, ice crystal growth experiments evolved to include solutes that mimic the natural environment of AF(G)Ps, and to confirm the non-colligative effects of AF(G)Ps on ice recrystallization.^{51,52} Other important variables that were also considered in these experiments were the cooling rate, warming rate, annealing temperature, and time. In most experiments, a supercooled environment is created after the initial freezing, by annealing ice crystals at high sub-zero temperatures to enhance the rate of ice recrystallization.⁵¹

Since then, different assays have been reported to assess ice recrystallization either qualitatively or quantitatively. Notable IRI assays include the microcapillary assay,⁵³ gold nanoparticle assay,⁵⁴ high-throughput sapphire slide assay,⁵⁵ splat cooling assay (SCA)⁵⁶ and the sucrose sandwich assay (SSA).⁵⁷ The previously mentioned assays will be described briefly in the following subsections.

5.1.2.1 Microcapillary assay

The microcapillary assay uses 10 μ L glass capillaries, each loaded with IRI sample dilutions in a saline buffer solution or 30% sucrose solution. The glass capillaries are snap-frozen to -50 °C, then held at -6 °C for 16 h and evaluated for ice recrystallization.⁵³ Through visual analysis, the concentrations of the sample that exhibit IRI activity can be determined. This method has been used to evaluate the IRI activity of AFPs, however, some major drawbacks of this method are its difficulty in sample preparation and in visually observing small differences in ice recrystallization in capillaries.

5.1.2.2 *Gold nanoparticle assay*

In the gold nanoparticle (AuNP) assay, the absorbance measurement change observed upon AuNP aggregation, as a result of ice recrystallization, is exploited. Serial dilutions of samples in the presence of AuNPs are prepared in 96-well plates and frozen to -20 °C, followed by slow thawing to room temperature. If ice recrystallization occurs and ice crystals grow larger, AuNPs are forced closer together in the unfrozen fraction leading to aggregation and a decrease in absorbance at 520 nm.⁵⁴ This is also accompanied by a distinct colour change from red to blue. One major caveat to this procedure is the frequency of false positives from the AuNP binding to the CPA (also resulting in aggregation), thus a splat assay must be used alongside as a control experiment.

5.1.2.3 *Sapphire slide assay*

In the search of an efficient high throughput IRI assay, the sapphire slide assay was developed.⁵⁵ This assay allows side by side comparison of many samples on a sapphire superhydrophobic slide. To perform this experiment, IRI samples are loaded into wells cut out of a superhydrophobic coating on sapphire slides and are sandwiched by another slide. The samples are flash-frozen to -196 °C, then incubated at -5.3 °C, and analyzed visually for ice recrystallization inhibition. One caveat to this assay is the expensive cost of the sapphire slides.

5.1.2.4 *Splat cooling assay and sucrose sandwich assay*

The splat cooling assay (SCA) and the sucrose sandwich assay (SSA) are two of the more commonly used assays for measuring IRI activity. The splat cooling assay (SCA) is

performed by dispensing a 10 μL drop of IRI sample, dissolved in PBS, onto an aluminum block pre-cooled to $-78\text{ }^{\circ}\text{C}$. A frozen wafer is instantaneously generated and after a specified annealing time at a high sub-zero temperature, ice recrystallization is evaluated by observing ice crystals under a microscope.⁵⁶ The sucrose sandwich assay (SSA) is performed by placing 1 or 2 μL of IRI sample dissolved in a concentrated sucrose solution (30-45% wt/v) between two coverslips, which is then frozen to a low sub-zero temperature ($-40\text{ }^{\circ}\text{C}$ to $-78\text{ }^{\circ}\text{C}$). The frozen sample is then allowed to warm to a high sub-zero temperature and is held at that temperature for a specified amount of time, after which ice recrystallization is evaluated by observing ice crystals under a microscope.⁵⁷

Despite their differences, all of these assays have been used extensively in the evaluation of ice recrystallization inhibition. Since ice recrystallization can be modulated by different factors such as the temperature, addition of solutes, and ice volume fraction,⁵⁸ it is imperative to discuss the differences in the qualitative and quantitative results generated from these ice recrystallization assays. August Sibthorpe, one of my former Honour's students, assisted in the experiments conducted in *Section 5.2*.

5.2 Exploring the differences in qualitative and quantitative results generated from the splat cooling assay and the sucrose sandwich assay

For all of the aforementioned assays, IRIs were analyzed in solutions containing either salt or sugar analytes. A high concentration of solutes can colligatively inhibit ice recrystallization, therefore, the addition of solutes to IRI assays prevents the occurrence of false positives from colligative effects.^{56,57} Compounds able to significantly inhibit ice recrystallization via their non-colligative effects in the presence of salt or sugar analytes are considered as IRIs. High

concentrations of salts or sugars are both sufficient solutes to use for IRI assays, however, the concentrations and procedures by which they are used may lead to varying results in the determination of IRI activity. For example, in experiments employing the capillary assay, Davies reported the recrystallization inhibition endpoint of AFPIII to be several-folds higher in a 30% sucrose solution than in a tris-buffered saline solution.⁵³ Since the SCA and SSA are more commonly used than the capillary assay, the extent of these differences was further analyzed in this work. In most cases, a saline buffer (PBS, TBS, etc.) is used for the SCA as a positive control for ice recrystallization and a 30-45% sucrose solution is used for the SSA.^{53,54} For the following experiments, phosphate-buffered saline (PBS) and 40% sucrose were used as positive controls for the SCA and SSA respectively.

5.2.1 Quantifying IRI activity using the SCA and SSA

The frozen wafers generated from freezing 10 μ L PBS using the SCA protocol and 1 μ L 40% sucrose using the SSA protocol, were photographed between cross-polarizing filters of a digital camera fitted to a microscope at 20x magnification. The images generated were analyzed by circling each defined ice grain boundary within the image frame to determine the average ice grain size. Each sample concentration was performed in triplicate and averaged to provide an overall mean grain size (% MGS) that was normalized against ice crystals generated in PBS for the SCA or 40% sucrose for the SSA.

5.2.2 Differences in ice crystal habit between the SCA and SSA

The ice solutions generated from the SCA and SSA (using the procedure described in *Section 5.1.2.4*) in the absence of an IRI were notably different. 10 μ L of PBS using the SCA method produced a frozen sample with a low liquid volume and a high ice volume. After a 40-min

annealing time at $-6.4\text{ }^{\circ}\text{C}$, the ice crystals recrystallized to a larger size (Fig. 5.2A). Contrarily, a $1\text{ }\mu\text{L}$ aliquot of 40% sucrose using the SSA method, resulted in a slurry containing smaller ice crystals surrounded in a high liquid volume after 40 -min of annealing at $-6.4\text{ }^{\circ}\text{C}$ (Fig. 5.2B). The formation of a slurry in the SSA method can be attributed to the high concentration of sucrose present in the liquid fraction creating an overall depressed freezing point. The freezing point of a 40% sucrose solution is $-4.9\text{ }^{\circ}\text{C}$,⁵⁹ and as the solution freezes, the sucrose particles are excluded, causing them to concentrate in the unfrozen fraction.⁶⁰ Thus, the concentration of sucrose in the unfrozen fraction is greater than 40%, leading to a further depressed freezing point and a larger volume of unfrozen fraction.

To determine if the difference in the appearance of ice crystals could be attributed to the assay method, different positive controls were used for each method. $1\text{ }\mu\text{L}$ of PBS was sandwiched between two cover slips, frozen to $-78\text{ }^{\circ}\text{C}$ and allowed to anneal for 40 -min at $-6.4\text{ }^{\circ}\text{C}$ (the SSA method). The liquid to ice volume ratio was still very low, but the ice crystal habit was inconsistent and less defined compared to the ice crystal habit of 40% sucrose (Fig. 5.2C). In most cases, the frozen $1\text{ }\mu\text{L}$ PBS solution resulted in a dendritic-like ice pattern upon warming to $-6.4\text{ }^{\circ}\text{C}$. When $10\text{ }\mu\text{L}$ of 40% sucrose was used for the SCA method, the high sucrose concentration led to the formation of an opaque ice wafer and ice crystals that were not visible under the microscope. This emphasizes the solution limitations of each assay, suggesting that the splat cooling method does not tolerate highly concentrated solutions while only concentrated solutions are feasible with the sandwich method.

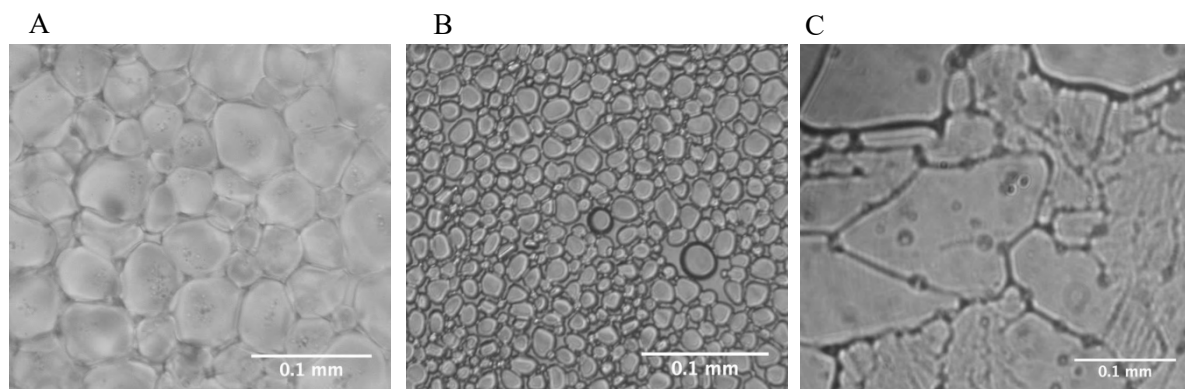


Figure 5.2. Photographs of ice crystals from a frozen wafer taken under a microscope (20x magnification). (A) A 10 μL drop of PBS using the SCA method; (B) a 1 μL drop of a 40 % sucrose solution using the SSA method frozen at a rate of 60 $^{\circ}\text{C}/\text{min}$ (C) a 10 μL drop of PBS using the SSA method frozen at a rate of 60 $^{\circ}\text{C}/\text{min}$. Wafers were allowed to warm (30 $^{\circ}\text{C}/\text{min}$) and anneal at -6.4 $^{\circ}\text{C}$ for 40 min. Figure reprinted with permission from *Methods in Molecular Biology*, Humana Press Inc., Cryopreser., 2021, vol. 2180, pp. 271–283. Copyright © 2021. Springer Nature.

5.2.3 Differences in the growth rate of ice crystals in the SCA and SSA

Since there was a noticeable difference in the appearance of ice crystals generated from the SCA and SSA, the innate inhibitory effect of the solutes was investigated. The sizes of the ice crystals in the PBS solution and 40% sucrose solution were measured as a function of time over a 40-min period. As expected, the SCA produced a high ice-to-liquid ratio sample, and the SSA produced a low ice-to-liquid ratio sample (i.e. a higher volume of unfrozen fraction). It has been previously noted that there are two mechanisms to ice recrystallization that can occur depending on the ice-to-liquid volume ratio.⁵⁸ Samples containing a low ice-to-liquid volume ratio undergo ice recrystallization via a bulk diffusion mechanism according to the Ostwald ripening theory.⁵⁸ During this process water molecules from smaller crystals diffuse through bulk water to larger ice crystals and the rate at which this occurs is dependent on the solution viscosity, temperature, and ice volume fraction.⁵⁸ For samples with high ice-to-liquid volume ratio, accretion, the mechanism of two ice crystals coalescing into one is preferential. These two mechanisms can occur sequentially during the ice recrystallization process, depending on the instantaneous ice-to-liquid

volume ratio, however in concentrated sucrose samples, the diffusion process dominates.⁵⁸ Diffusion has been observed to occur at a slower rate than accretion,^{58,61} thus, it was hypothesized that the growth rate of ice crystals would be slower in the SSA compared to the SCA with a low unfrozen fraction.

For both assays, photographs of the ice crystals were taken at 5-, 10-, 20- and 40-min time points (Fig. 5.3), and the images were analyzed to determine the mean grain size (mm^2) as a function of time (min) (Fig. 5.4). The splat cooling assay displayed a steady increase in grain size from 5 – 20 min, followed by a sudden increase in the growth rate from 20 – 40 min. The Ben laboratory has performed several experiments examining the mean grain size of ice crystals over time and have reported an exponential growth pattern.^{62,63} The annealing temperature used for those experiments was slightly greater and the ice crystal size was measured at more time points, which may account for the discrepancies observed. In comparison to the SCA, the crystals generated from the SSA were much smaller in grain size and the growth over time was minor. Crystals at an annealing time of 5-min were too small to observe under 20x magnification and could not be measured. The significantly slower rate of ice recrystallization in the sucrose assay could be a result of the mechanism of ice recrystallization. As previously described, samples with a low ice-to-liquid volume ratio undergo ice recrystallization via the slower bulk diffusion mechanism to which viscosity is a factor.⁵¹ Therefore, ice crystals in a viscous 40% sucrose solution would recrystallize at a much slower rate than ice crystals in a PBS solution (~1% solute wt/v).

Overall, these results suggest that the ice crystals in the PBS solution have a faster recrystallization rate than the ice crystals in the 40% sucrose solution. The crystals formed in the

sandwich method were small, however at 40-min, crystals were large enough to be measured under at 20x magnification.

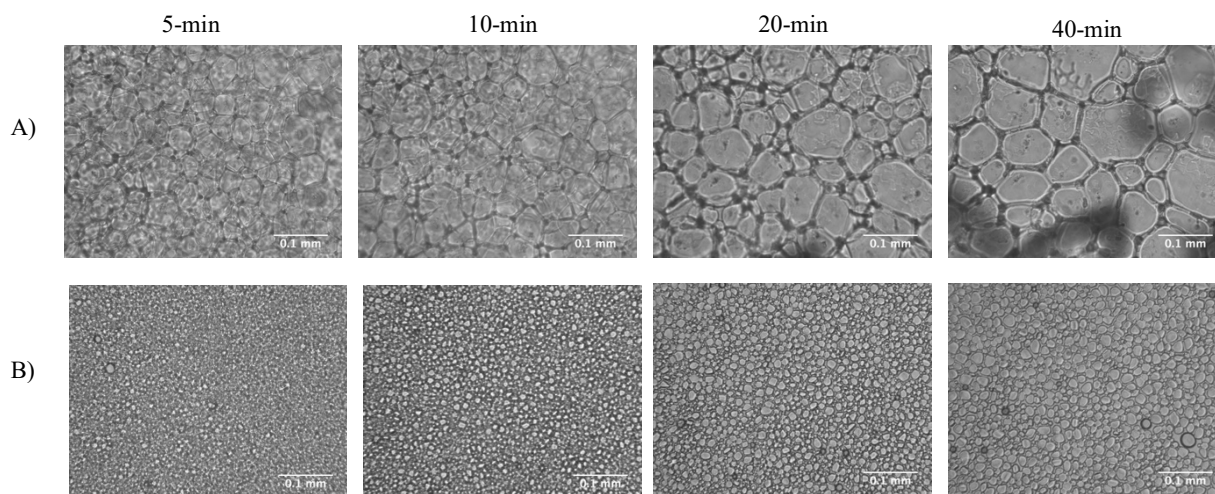


Figure 5.3. Photographs of ice crystals at 5-, 10-, 20-, and 40-min time intervals formed in PBS and 40% sucrose using the splat cooling assay (A) and sucrose sandwich assay (B) respectively.

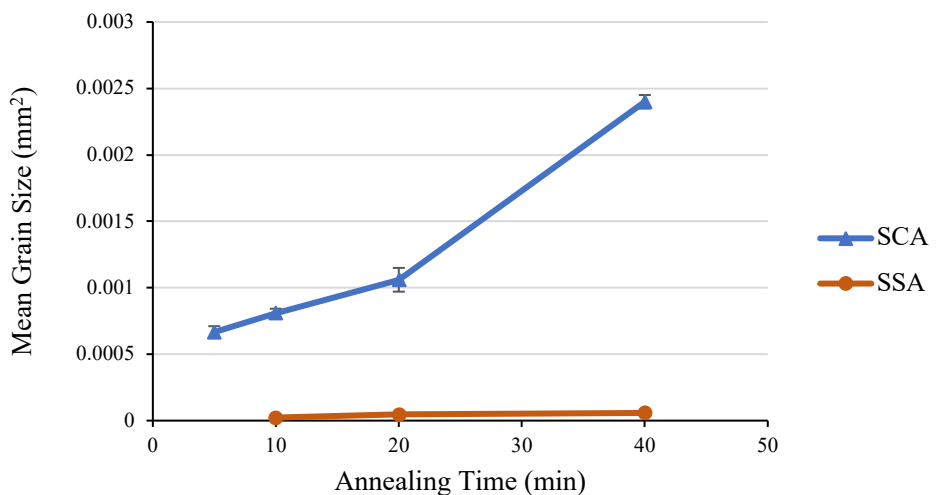


Figure 5.4 The ice recrystallization rate of a PBS and a 40% sucrose solution using the splat cooling assay (SCA) and sucrose-sandwich assay (SSA) respectively. Ice wafers were allowed to anneal at -6.4 °C for 5-, 10-, 20-, and 40-min time intervals before the determination of the MGS. Experiments were performed in triplicate. The error bars indicate SEM. Figure reprinted with permission from *Methods in Molecular Biology*, Humana Press Inc., *Cryopreser.*, 2021, vol. 2180, pp. 271–283. Copyright © 2021. Springer Nature.

5.2.4 *Differences in the ability of the SCA and SSA to quantify IRI activity*

In the literature, a variety of metrics have been used to describe IRI activity. Grain size,^{17,64–66} percent mean grain size,^{22,24,25,67} recrystallization inhibition (RI) endpoint (the concentration of sample that no longer displays IRI activity),^{53,55,68} and IC_{50} or c_i values (the concentration at the inflection point of a rate vs. concentration plot)^{9,69–71} have all been used to quantify the activity of various IRIs. Determining these metrics can provide knowledge of the active concentrations required to inhibit ice recrystallization for various applications, therefore it is important that these measurements are reliable and accurate. Considering that ice crystal habit and ice crystal growth rate are dependent on the type of assay and positive control used, evaluating the extent of the effect on quantitative values is warranted.

5.2.4.1 *Comparing IC_{50} and c_i values of antifreeze proteins and glycoproteins*

There are four classes of AFPs (type I–IV) based on their structural differences. Type I AFPs (AFPI) are short α -helices consisting of repeating Thr–(Ala)₂–Asn–(Ala)₇ sequences;⁷² type II AFPs (AFPII) are structurally different by their high cysteine content and their β -sheet secondary structure;⁷³ type III AFPs (AFPIII) have fewer repeating units and consist of short β -strands and one helical turn;⁷⁴ type IV AFPs (AFPIV) exist as a combination of helical bundles with high glutamine content.⁷⁵ There are eight classes of AFGPs based on their molecular mass and they all consist of repeating (Ala–Ala–Thr)_n units with a disaccharide attached to the secondary hydroxyl group of the threonine residue.⁷⁶

AF(G)Ps have been evaluated extensively for IRI activity using a variety of IRI assays and metrics,^{9,51,53,54,66,71,77–79} and in a few cases, c_i values were generated using the SSA.^{9,71} Olijve et al. and Rahman et al. evaluated AFPI, AFPIII, and AFGP for their IRI activity using the SSA

and generated c_i values describing the concentration at the inflection point of a rate vs. concentration plot (Table 5.1).^{9,71} The procedure used by Olijve et al. entailed a sample size of 1 μ L of AF(G)P in 30 % wt/wt sucrose solution in water, which was cooled to -40 °C at a rate of 20 °C/min and warmed to -7 °C at a rate of 10 °C/min, and further held at this temperature for 120-min. The procedure followed by Rahman et al. was similar, however, samples were in 40% wt/wt sucrose solutions in water and the annealing temperature was -6 °C. In both procedures, the rates were evaluated by measuring the size of ice crystals over the course of the annealing time and rate constants were plotted against AF(G)P concentrations.^{9,71} There were only minor differences observed in c_i values between Olijve et al. and Rahman et al. (Table 5.1), which may be due to the small differences in the SSA protocol (i.e. concentration of the sucrose solution and the annealing temperature). Both reported that AFPI and AFPIII were similar in activity, while AFGP was the most IRI active, exhibiting nanomolar activity.

Using the SCA method employed in the Ben laboratory (with an annealing temperature and time of -6.4 °C and 5-min), the initial rate of ice recrystallization, normalized to PBS, was plotted against the AFPI, AFPIII, and AFGP concentrations. The initial rates were obtained by the “binning analysis” method, as described in *Section 1.4.4*. IC_{50} values (the concentration that reduces the rate to 50%, also the concentration at the inflection point of the plot) in the nanomolar range were generated from dose response curves (Fig. 5.5). AFGP exhibited the strongest IRI activity ($IC_{50} = 2$ nM) and AFPIII exhibited the weakest IRI activity ($IC_{50} = 30$ nM) (Table 5.1). Comparing the values obtained from the SCA to those obtained from the two SSA experiments, shows a several-fold difference in which the SCA IC_{50} values are an order of magnitude smaller than the SSA c_i values. This discrepancy may be a result of the different methods used to calculate the rate of ice recrystallization in the SCA vs. SSA, such that rate constants were used to obtain c_i

values and initial rates normalized to PBS were used to obtain IC_{50} values. For a more direct comparison of the SCA vs. SSA, the same metric should be used to evaluate the IRI activity in both assays.

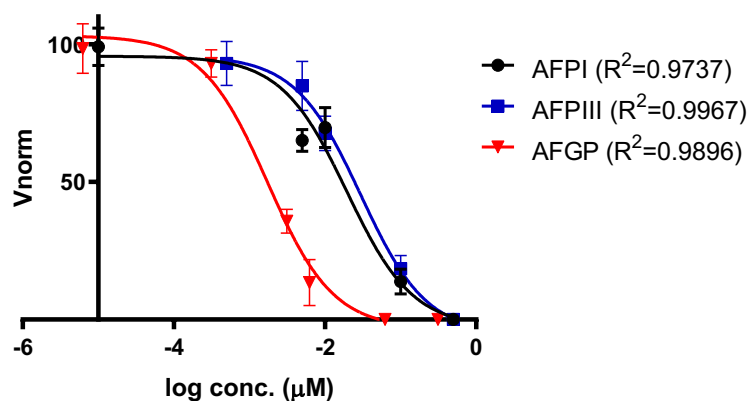


Figure 5.5. Plot of the normalized rate of ice recrystallization as a function of log concentration (μM). Points were fitted to a four-parameter non-linear regression curve to generate IC_{50} values for AFPI, AFPIII, and AFGP. Error bars represent % SD for $n=3$ experiments.

Table 5.1. Quantitative values obtained of the evaluation of AF(G)Ps as ice recrystallization inhibitors.

Antifreeze (glyco) protein type	IC_{50} values (μM) (SCA)	c_i values (μM) (Olijve et al.) ⁹	c_i values (μM) (Rahman et al.) ⁷¹
AFPI	0.015	5.8	4.69
AFPIII	0.030	5.9	3.00
AFGP	0.002	0.0091	0.06

5.2.4.2 Comparing percent mean grain size of AFPI in SCA and SSA

Measuring mean ice grain size is one of the more common metrics used to quantitatively describe IRI activity,^{17,21,22,25,64–67} however, it may not be translatable from assay to assay as different assays use different standard solutions which can impact the growth of ice grains. To resolve this issue, mean ice grain sizes are also reported as a percent with respect to their standard

solution. To determine if percent mean grain size (% MGS) of an IRI varies between the SCA and SSA, six different concentrations of AFPI were tested using both the SCA and SSA method (annealing time and temperature of 40-min and -6.4 °C respectively). % MGS was calculated with respect to PBS for the SCA and 40% sucrose for the SSA and plotted against the logarithmic transformed concentrations of AFPI (Fig. 5.6). A notable difference in % MGS was observed between the two assays, specifically at concentrations generating crystals <50% MGS. In the SCA, lower concentrations of AFPI were required to generate a smaller % MGS compared to the SSA; in other words, lower concentrations of AFPI can inhibit ice recrystallization in the SCA. For example, the % MGS recorded for the SSA and SCA at 0.1 μ M AFPI is 60% and 42%, and at 1 μ M AFPI is 57% and 14% respectively. Therefore, to observe the same inhibitory effect on ice crystal size in the SSA, a higher concentration of AFPI is required. This discrepancy may be a result of the difference in solute concentrations (PBS vs. 40% sucrose) or ice-to-volume ratio, as the rate of ice recrystallization is largely dependent on these variables.⁵⁸ As discussed in *Section 5.2.3*, the size of ice crystals over a 40-min time period is greatly depressed in the SSA compared to the SCA, which may be due to the high solute concentration. As such, a larger concentration of AF(G)P would be required to observe IRI activity above the already established inhibitory effect of sucrose. Therefore, these data show that quantitative values like % MGS are not transferrable between the splat cooling and sucrose assay, and in order to obtain correct active concentrations for the required use, the assay type should be accounted for.

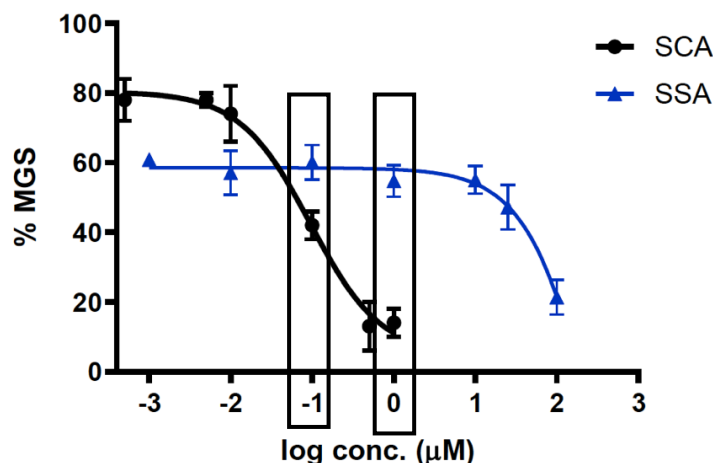


Figure 5.6. Plot of the % MGS as a function of log concentration of AFPI. A non-linear regression curve was fitted to the points (SCA $R^2=0.9942$; SSA $R^2=0.9806$); boxes represent notable data points. Error bars represent % SEM. Figure adapted with permission from *Methods in Molecular Biology, Humana Press Inc., Cryopreser.*, 2021, vol. 2180, pp. 271–283. Copyright © 2021. Springer Nature.

5.2.5 Summary

This study closely analyzed the difference between the splat cooling assay and the sucrose sandwich assay; assays which are used quite extensively to measure ice recrystallization inhibition. There were several differences observed in the ice crystal structure and grain size over time, such that the SCA generates crystals with a high ice-to-liquid volume and the SSA generates smaller crystals with a low ice-to-liquid volume. These disparities translated into the quantitative values generated by each assay in which the concentrations observed for % MGS values for AFPI were much greater using the SSA opposed to the SCA. Therefore, it is important to take these differences into account when using IRIs for various applications, such that the IRI assay method of choice should closely resemble the properties of its application. For example, in the case of cryopreservation applications, frozen samples are generated resulting in a high ice-to-liquid volume ratio, therefore, the splat cooling method would provide a more accurate analysis of the efficiency of the IRI in that system. Basing IRI concentrations off the values obtained by the sandwich method for cryopreservation purposes could lead to unnecessary high solute

concentrations and potential toxic effects. In contrast, for applications in which there is a low ice-to-liquid volume ratio, such as ice cream or slurry applications, the sucrose sandwich method would provide a more accurate quantitative analysis as those applications contain a larger unfrozen fraction. Using IRI concentrations based off the splat cooling method would result in underestimated concentrations and the IRI would not have its optimal effect in the system.

5.3 The effect of conventional cryoprotective agents on IRI activity

5.3.1 *The role of cryoprotective agents*

Cryoprotective agents (CPAs) are used during the cryopreservation of cells to provide protection against cryoinjury. Recall that two commonly used CPAs are DMSO and glycerol, and they are able to penetrate through the cell membrane. Their overall mechanism of protection is speculated, however, their ability to stabilize the cell membrane, form hydrogen bonds with water, and protect against osmotic stress via the “water replacement theory” has been noted as contributing factors.^{80–82} Specifically, studies have also shown that glycerol and DMSO interact with the polar head groups of the phospholipid bilayer and decrease the conformational disorder that occurs at sub-zero temperatures.^{83,84} In addition, preventing water molecules from forming ice crystals, by the CPA’s ability to form hydrogen bonds with water, has shown to be beneficial in protecting against cell damage at low temperatures.^{81,85–88} Although conventional CPAs provide a level of protection against low temperatures, a major caveat is their inability to protect against ice recrystallization. To solve this issue, compounds that inhibit ice recrystallization are often supplemented into cryoprotective media to reduce mechanical cell damage caused by ice.^{19,22,23,29,38,39,89–93} Attempts to cryopreserve red blood cells (RBCs) with glycerol supplemented with synthetic IRIs has been successful in the Ben laboratory. Using standard RBC freezing

conditions, *p*-bromophenyl-D-glucoside (**1.22**) supplemented in 15% glycerol at concentrations at or above its measured IC₅₀ value (15 mM), was able to yield ~60% less RBC hemolysis, compared to RBCs cryopreserved in 15% glycerol without **1.22**.²³ Similarly, supplementing *N*-2-fluorophenyl-D-gluconamide (**1.25**) or *N*-4-chlorophenyl-D-gluconamide (**1.26**) in 10% DMSO increased the post-thaw functionality of hematopoietic stem cells (HSCs) from umbilical cord blood as compared to 10% DMSO without **1.25** or **1.26**.²¹ These observations have been linked to the ability of **1.22**, **1.25**, and **1.26** to inhibit ice recrystallization, however, few studies analyzing the extent of IRI activity in the presence of glycerol and DMSO have been reported.

DMSO and glycerol depress the freezing point of water such that a 10% DMSO solution has a freezing point of -3 °C⁹⁴ and 15% glycerol solution has a freezing point of -4 °C.⁹⁵ Their ability to lower the freezing point of water at high concentrations contributes to the protective effect of CPAs, however, at temperatures lower than their freezing points, ice crystals are present and can recrystallize. A previous study by the Ben laboratory demonstrated, using cryo-microscopy, that supplementation of IRIs in 15% glycerol can protect red blood cells (RBCs) against extracellular ice damage that occurs during transient warming events.²² In this study, ice recrystallization was exacerbated by performing several cycles of temperature changes from -80 °C to -20 °C prior to thawing. A significant decrease in extracellular ice crystal size was observed in samples containing RBCs in 15% glycerol with IRI **1.22**, compared to samples without **1.22**.²² As a result, the RBC samples containing IRI **1.22** exhibited less post-thaw hemolysis than samples frozen in solely 15% glycerol. These results suggested that the IRI activity of **1.22** was still maintained in the presence of glycerol, however, this activity was not quantified or compared to the activity of **1.22** in the same system without glycerol.

In *Section 5.2* it was observed that the type of assay and standard solution implemented to measure IRI activity can impact the extent of the IRI activity observed. Therefore, since IRI assays typically analyze compounds in the absence of conventional CPAs (i.e. compounds are assessed in either concentrated salt or sugar solutions), it is of interest to investigate the impact of DMSO and glycerol on IRI activity using standard assays. The following studies in *Section 5.3.2* and *Section 5.3.3* were performed with Julia Meyer, a graduate student in the Ben laboratory, and aims to determine the effect of DMSO and glycerol on IRI activity to provide a better understanding of IRI activity *in vitro*.

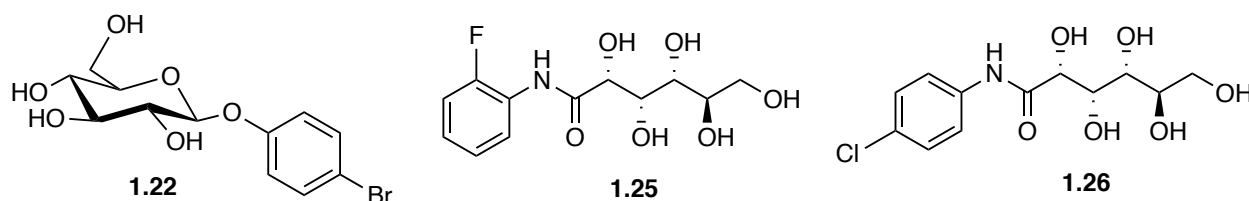


Figure 5.7. Previously synthesized IRIs *p*-bromophenyl-D-glucoside (**1.22**), *N*-2-fluorophenyl-D-gluconamide (**1.25**), and *N*-4-chlorophenyl-D-gluconamide (**1.26**) used as cryopreservative additives in the cryopreservation of RBCs and HSCs.

5.3.2 Evaluating the IRI activity of 1.22, 1.25, 1.26 and AFPI in the presence of DMSO

5.3.2.1 The effect of DMSO on the IRI activity of 1.25

To provide a deeper understanding of the effect of CPAs on IRI activity, a preliminary screen of IRIs was completed in the presence of DMSO. PBS supplemented with 5 mM of IRI **1.25** and five different concentrations of DMSO were evaluated for IRI activity via the splat cooling assay. Recall that cryopreservation applications using **1.25** have been in the presence of DMSO,^{21,70,90} therefore this CPA was selected as the starting point for this analysis. The IC₅₀ of **1.25** is 3 ± 1 mM,⁷⁰ thus a set concentration of 5 mM was initially used to ensure that a visible

difference in crystal size was apparent if DMSO further inhibited or promoted ice recrystallization. If DMSO amplified the IRI activity of **1.25**, the ice crystal size would be significantly smaller and difficult to observe under the microscope. If DMSO suppressed the IRI activity of **1.25**, the ice crystal size would be significantly larger and comparable to the PBS standard.

0.1%, 0.25%, 0.5%, 1%, and 2.5% DMSO titrations were made of 5 mM **1.25** in PBS and photographs of the ice crystals were taken after a 5-min annealing time at -6.4 °C (Fig. 5.8). DMSO concentrations greater than 2.5% resulted in samples with a large amount of unfrozen fraction at -6.4 °C and therefore were not analyzed for consistency purposes. It was apparent from the photographs that as the concentration of DMSO increased, the crystal size increased as well; so much that the ice crystal size of samples with 1%, and 2.5% DMSO (Fig. 5.8F&G) were visibly comparable to the PBS control (Fig 5.8A).

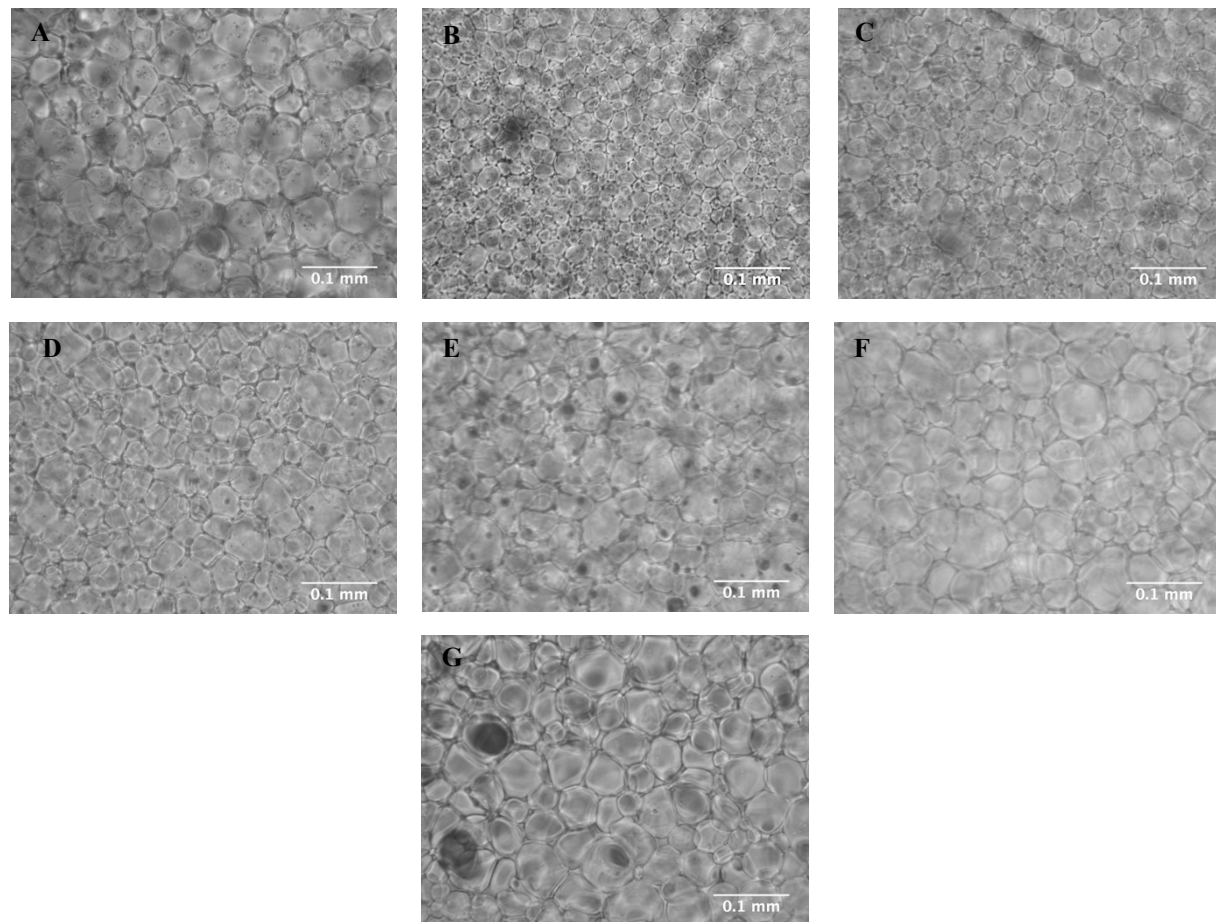


Figure 5.8. Photographs of ice crystals formed in the presence of 5 mM of **1.25** in PBS with varying concentrations of DMSO. Photos were taken of A) PBS, B) 5 mM **1.25**, C) 5 mM **1.25** in 0.1% DMSO, D) 5 mM **1.25** in 0.25% DMSO, E) 5 mM **1.25** in 0.5% DMSO, F) 5 mM **1.25** in 1% DMSO, and G) 5 mM **1.25** in 2.5% after an initial freezing to -78 °C and annealing at -6.4 °C for 5-min.

The ice crystal sizes generated in each sample were quantified and the % MGS was plotted against log concentration of DMSO (Fig. 5.9). A non-linear regression curve was fitted onto the data indicating that DMSO exhibits a full agonistic dose response for ice recrystallization. In the absence of DMSO, a 18% MGS was observed for 5 mM **1.25**, which is represented on the graph in Fig. 5.9A as the dotted horizontal line. At higher concentrations of DMSO, the % MGS gradually increased until the mean grain size was not statistically different from the PBS control

(one sample t-test), which occurred at a DMSO concentration of 2.5%. These results suggest that the IRI activity of 5 mM **1.25** is gradually suppressed with increasing concentrations of DMSO, until it is completely inactive. Since IRIs are antagonistic to ice recrystallization, it was hypothesized that changes in the IRI concentration could modulate the DMSO effect, such that higher concentrations of IRI may suppress the agonistic effects of DMSO. To investigate, the same experiment was performed with 1 mM, 10 mM, and 15 mM of **1.25** (Fig. 5.9B-D). In the absence of DMSO, 1 mM **1.25** generated ice crystals that were essentially 100% MGS, the same mean grain size as the PBS control (Fig. 5.9B). Serial titrations of DMSO up to 2.5% resulted in no significant increase or decrease in % MGS, indicating that DMSO does not temper the effect that the solutes in PBS have on ice recrystallization. Recall that solute particles can colligatively inhibit ice recrystallization; therefore, the results suggest that DMSO does not disturb or contribute to this process.

As expected, increasing the concentration of **1.25** decreased the agonistic effect of DMSO on ice recrystallization. Titrating DMSO into samples containing 10 mM **1.25** led to an increase in MGS from 12% in the absence of DMSO to 69% in 2.5% DMSO. Similarly, 15 mM **1.25** exhibited 10% MGS in the absence of DMSO and 71% MGS in 2.5% DMSO. The % MGS values of 10 mM and 15 mM **1.25** were statistically different ($p < 0.05$) from 100% MGS (Fig. 5.9C&D), implying that 2.5% DMSO did not completely abolish IRI activity. Nonetheless, the data points were fit to a four-parameter nonlinear regression curve with the minimum value equal to the % MGS at 0% DMSO and the maximum value equal to the % MGS at 2.5% DMSO. From these curves, the hill slopes and IC_{50} values were generated (Table 5.2). In these cases, the IC_{50} value describes the concentration of DMSO that generates a mean grain size at the midpoint between the highest % MGS and the % MGS at 0% DMSO (i.e. for 10 mM **1.25**, the IC_{50} value is the DMSO

concentration that results in 30% MGS). The IC_{50} values for 5 mM, 10 mM, and 15 mM **1.25** were 0.2%, 0.42% and 1.1% respectively; an IC_{50} value for 1 mM **1.25** could not be reported due to the absence of a sigmoidal dose-response curve. The direct proportionality of IC_{50} value to the concentration of **1.25** indicates that although DMSO suppresses the IRI activity of **1.25**, this effect can be decreased by increasing the concentration of **1.25**. Another notable observation is the hill slope values calculated for the plots of 5 mM, 10 mM, and 15 mM **1.25** in the presence of DMSO (Table 5.2). In all cases, the hill slopes were greater than one, suggesting that DMSO exhibits a cooperative-like mechanism for inhibiting IRIs.⁹⁶ The Ben laboratory has previously reported that there is a considerable amount of cooperativity associated with the inhibition mechanism of several IRIs, such that IRIs may form networks in solution that inhibit ice recrystallization.⁶² Therefore, DMSO may disrupt these networks in a cooperative-like manner so that the networks are more difficult to form.

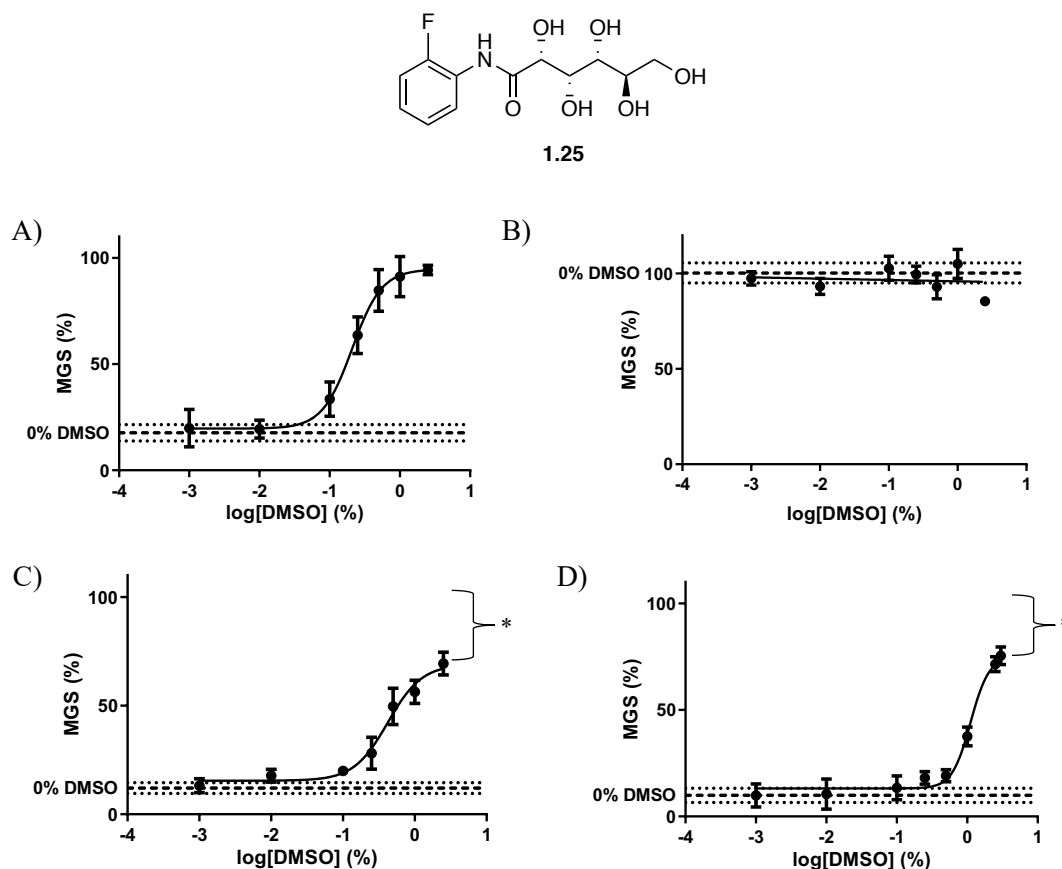


Figure 5.9. IRI activity of PBS solutions containing A) 5 mM, B) 1 mM, C) 10 mM, and D) 15 mM of **1.25** with increasing concentrations of DMSO. % MGS values of 10 mM and 15 mM **1.25** at 2.5% DMSO were statistically different from 100% MGS using the one sample t-test (* $p < 0.05$). Ice crystal wafer areas were normalized to PBS and fitted with a variable-slope four parameter nonlinear regression (A: $R^2=0.8865$; C: $R^2=0.8645$, D: $R^2=0.9262$) and a linear regression (B: slope=-0.6935). Dotted horizontal line represent the % MGS of **1.25** in PBS in the absence of DMSO. Error bars are represented by % SD ($n = 3$).

Table 5.2. The % MGS at the lowest and highest DMSO concentrations and the IC_{50} values and hill slope of the dose response curves for 1 mM, 5 mM, 10 mM, and 15 mM concentrations of **1.25**. IC_{50} values and hill slopes are reported with 95% confidence interval.

[1.25] (mM)	% MGS at 0% DMSO	% MGS at 2.5% DMSO	IC_{50} (% DMSO)	Hill slope
1	100	85	N/A	N/A
5	18	94	0.20 ± 0.10	2.0 ± 1.6
10	12	69	0.42 ± 0.15	1.8 ± 1.1
15	10	71	1.11 ± 0.21	3.3 ± 1.9

The inhibition of IRI activity by DMSO is an interesting observation, specifically for the fact that **1.25** has been previously used as an IRI in conjunction with 10% DMSO in several biological applications. At both 5 mM and 10 mM concentrations, **1.25** has yielded excellent post-thaw results in hematopoietic stem cells.^{21,90} Although the effect of 10% DMSO on IRI activity was not evaluated due to the experimental limitations, the observed trend suggests that at 10% DMSO, IRI **1.25** is not acting by inhibiting ice recrystallization, but it may still protect the cells by an alternative mechanism of action. In order to confirm this hypothesis, an experiment allowing the use of a 10% DMSO concentration would have to be designed.

5.3.2.2 The effect of DMSO on the IRI activity of 1.26

In light of the previous observations, IRI **1.26**, also from the gluconamide class, was examined in the presence of DMSO. The sole difference between **1.25** and **1.26** is the substituent and its position on the aryl ring; **1.25** contains a 2-fluorophenyl aryl ring and **1.26** contains a 4-chlorophenyl aryl group. Although these differences may be perceived as minor, the impact on IRI activity is unquestionable, such that **1.25** exhibits an IC_{50} of 3 ± 1 mM⁷⁰ and **1.26** exhibits an IC_{50} of 12 ± 4 mM⁹⁷ in PBS via the splat cooling assay.

The splat cooling assay was used to examine the effect of DMSO on the IRI activity of 10 mM **1.26**. Similar to the previous experiment, concentrations of DMSO were titrated into solutions of 10 mM **1.26** in PBS, up to 2.5% DMSO, and were plotted and fitted to a non-linear regression curve (Fig. 5.10). In the absence of DMSO, the ice crystal size generated was 36% MGS which increased and equilibrated to 70% MGS in the presence of 2.5% DMSO, which was statistically different from 100% MGS ($p < 0.01$) representing complete inactivation of the IRI. This was similar to the upper limit that was observed for 10 mM and 15 mM **1.25**. The data points

for **1.26** had a poor fit to the non-linear regression ($R^2=0.7166$), and its IC_{50} value for DMSO was $0.02 \pm 1.6\%$. These initial observations suggest that DMSO has a similar antagonistic effect on the IRI activity of **1.26** as observed with **1.25**, however, it is difficult to state if the effect is to the same degree. To understand the full scope of the DMSO effect on **1.26**, experiments using higher concentrations of **1.26** (above the IC_{50} value) would have to be evaluated.

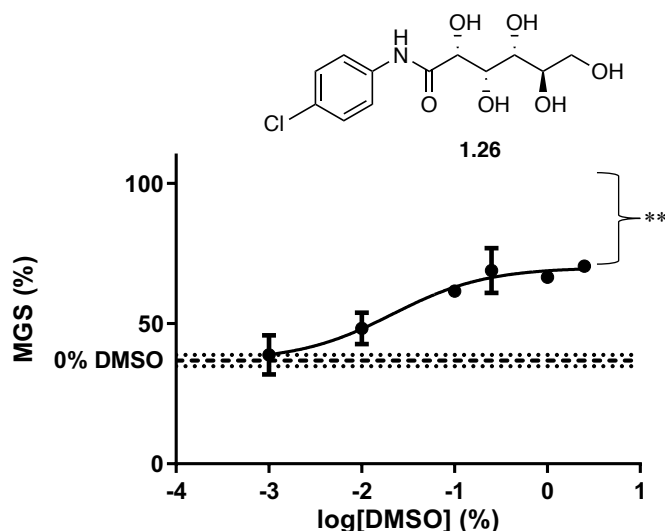


Figure 5.10. IRI activity of PBS solutions containing 10 mM **1.26** with increasing concentrations of DMSO. % MGS value at 2.5% DMSO was statistically different from 100% MGS using the one sample t-test (** $p < 0.01$). Ice crystal wafer areas were normalized to PBS and fitted with a variable-slope four parameter non-linear regression ($R^2=0.7166$). Dotted horizontal line represent the % MGS of **1.26** in PBS in the absence of DMSO. Error bars are represented by % SD ($n = 3$).

As the mechanism of small molecule IRIs synthesized in the Ben laboratory is still up for debate, the mechanism by which DMSO suppresses IRI activity is also uncertain. It has been speculated that IRIs inhibit ice recrystallization by disturbing the quasi-liquid layer (QLL) / bulk water interface between two ice crystals, making the ordering, and transferring of water molecules to ice crystals less favourable.⁹⁸ If this is the case, the presence of DMSO may prevent IRIs from interacting and disrupting the QLL. One hypothesis may be that DMSO is able to form hydrogen

bonds with the IRI, thereby preventing the IRI from forming networks or interacting with the QLL/bulk water interface. Previous studies on IRIs have indicated the significance of the amide bond for the potent IRI activity observed in the gluconamide class,⁹⁹ therefore, the DMSO effect could possibly stem from hydrogen bond interactions with the amide.

5.3.2.3 *The effect of DMSO on IRI activity of glycoside 1.22 and AFPI*

Up to this point, two members of the gluconamide class (**1.25** & **1.26**) have been evaluated in the presence of DMSO, and the results suggest that DMSO suppresses IRI activity in a dose-dependent manner. To determine if this effect is class dependent, IRI **1.22**, from the *O*-aryl glycoside class, was evaluated at 25 mM (its IC₅₀ value is 15 ± 3 mM) with increasing concentrations of DMSO. The splat cooling assay was used to analyze the ice crystals formed in the presence of 0.01%, 0.1%, and 1% DMSO, and the % MGS was plotted against the log concentration of DMSO (Fig. 5.11). Unlike previously observed, a dose-dependent response to increasing DMSO concentrations was not apparent, rather, the mean ice crystal size (23% MGS) remained constant up to 1% DMSO. In the DMSO experiments with **1.25** and **1.26**, the addition of 1% DMSO resulted in a significant increase in % MGS, therefore, not observing the same effect with **1.22** suggests that DMSO does not inhibit the IRI activity of **1.22** in the same manner that it does with gluconamides **1.25** and **1.26**. These observations may be a result of the differences of the structural components between the glycoside class and the gluconamide class, such that gluconamides possess an amide linkage and glycosides do not.

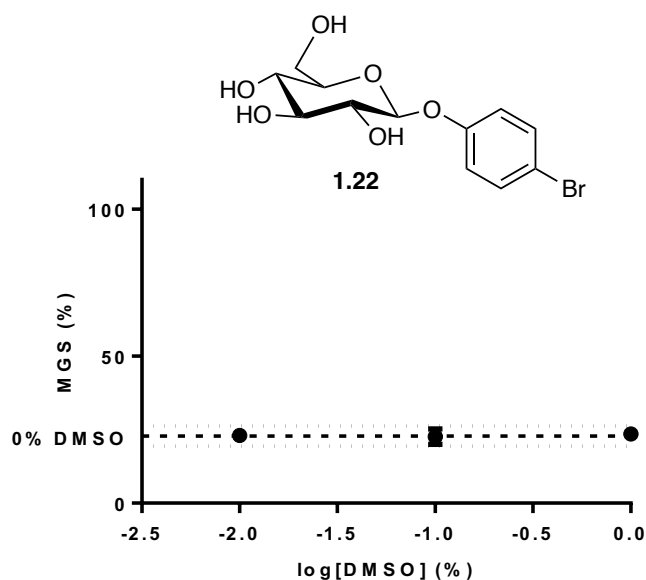


Figure 5.11. IRI activity of 25 mM **1.22** in PBS with 0.01%, 0.1%, and 1% DMSO. Ice crystal wafer areas were normalized to PBS. The dotted horizontal line represents the % MGS of 25 mM **1.22** in PBS in the absence of DMSO. Error bars are represented by % SD (n=3).

There have been several studies investigating the interaction between DMSO and amides in aqueous solutions that may explain these observations.^{100–104} Studies using molecular dynamic simulations and relaxation experiments, have revealed that in aqueous solutions of DMSO and small-molecule amides, DMSO forms a strong hydrogen bond between its oxygen, and the amide hydrogen of another molecule.^{100,104} Therefore in an aqueous solution, amides are more likely to hydrogen bond to DMSO rather than form hydrogen bonds with water. Similarly, it's been reported that the solvation shells formed between DMSO and amides are much more structured than solvation shells formed between water and amides.¹⁰¹ These studies, along with the observations above, infer that DMSO may be interacting with the gluconamides at the site of the amide bond while disturbing the interactions required between gluconamides and water for IRI activity. Since glycosides do not contain an amide bond, the strong DMSO-amide interactions affecting IRI activity cannot be observed. Likewise, the structural components that contribute to the IRI activity in the glycoside class, are clearly not affected by the presence of DMSO.

To further confirm this theory, 0.1 μM AFPI was evaluated in PBS via the splat cooling assay and was compared to its IRI activity in the presence of 1% DMSO (Fig. 5.12). AFPI contains several amide bonds which have been deemed as important contributors to IRI activity,^{13,105} therefore it was hypothesized that upon addition of DMSO, its IRI activity would be suppressed. Recall from Fig. 5.6 that 0.1 μM AFPI generated ice crystals around 50% MGS, therefore if the DMSO-amide interaction is valid, the % MGS of the ice crystals in 1% DMSO will be larger than observed. Similar to the gluconamides, a statistically significant decrease in IRI activity was observed such that 0.1 μM AFPI generated ice crystals with 42% MGS in the absence of DMSO and 57% MGS with 1% DMSO. These observations further strengthen the argument that the suppression in IRI activity could be due to the interaction between DMSO and amide functional groups. Although the mechanism by which gluconamides inhibit ice recrystallization is undefined, these results further emphasize the importance of the amide bond toward IRI activity in this class of compounds. Therefore, designing a new class of IRIs that do not possess an amide functional group would prevent the suppression of IRI activity in the presence of DMSO.

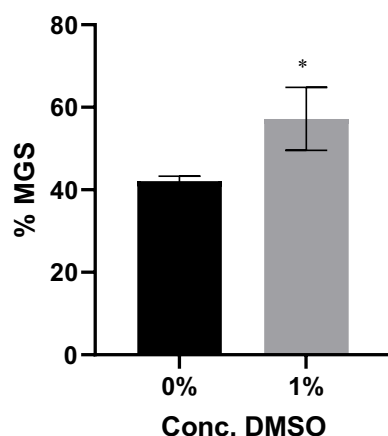


Figure 5.12. IRI activity of PBS solutions containing 0.1 μM AFPI with and without DMSO. Ice crystal wafer areas were normalized to PBS. Error bars are represented by % SD ($n = 3$). Significance testing was performed using an unpaired t-test with Welch's correction ($p = 0.0273$).

5.3.3 *Evaluating the IRI activity of 1.25 and 1.26 in the presence of glycerol*

The study performed in the previous sections demonstrates how the IRI activity of gluconamides **1.25** and **1.26** can be tempered by increasing amounts of DMSO, which was attributed to the potential for strong hydrogen bonding interactions between amides and DMSO. Since glycerol is another common CPA that is capable of forming hydrogen bonds, its effect on the IRI activity of **1.25** and **1.26** was investigated. The IRI activity of **1.25** and **1.26** were evaluated using the splat cooling assay in the presence of PBS and increasing amounts of glycerol. Unlike DMSO, glycerol exhibited a small amount of IRI activity, thus all data points were normalized to the appropriate PBS/glycerol control. A non-linear regression curve was fitted to the points, indicating that glycerol has an agonistic effect on ice recrystallization in the presence of 5 mM **1.25** and 10 mM **1.26** (Fig. 5.13). In both cases, titration with 2.5% glycerol did not completely inactivate the IRI activity of **1.25** and **1.26**, and were statistically different from 100% MGS, such that their % MGS was 82% ($p < 0.05$) and 64% ($p < 0.01$) respectively. This was also observed for 10 mM **1.26** with DMSO, however, the IRI activity of 5 mM **1.25** did completely diminish in the presence of 2.5% DMSO. IC_{50} values were used to compare the effect of glycerol to the effects observed with DMSO. For 5 mM **1.25**, the IC_{50} value of glycerol ($0.32 \pm 0.17\%$) (reported with 95% confidence interval) was slightly larger than the IC_{50} value for DMSO ($0.20 \pm 0.10\%$), however taking into consideration their 95% confidence interval, the IC_{50} values are in close range of one another. For 10 mM **1.26**, the IC_{50} value of glycerol was $0.48 \pm 0.13\%$ compared to the IC_{50} value of DMSO which was $0.02 \pm 1.6\%$ suggesting that DMSO suppresses the IRI activity of **1.26** to a greater degree than glycerol.

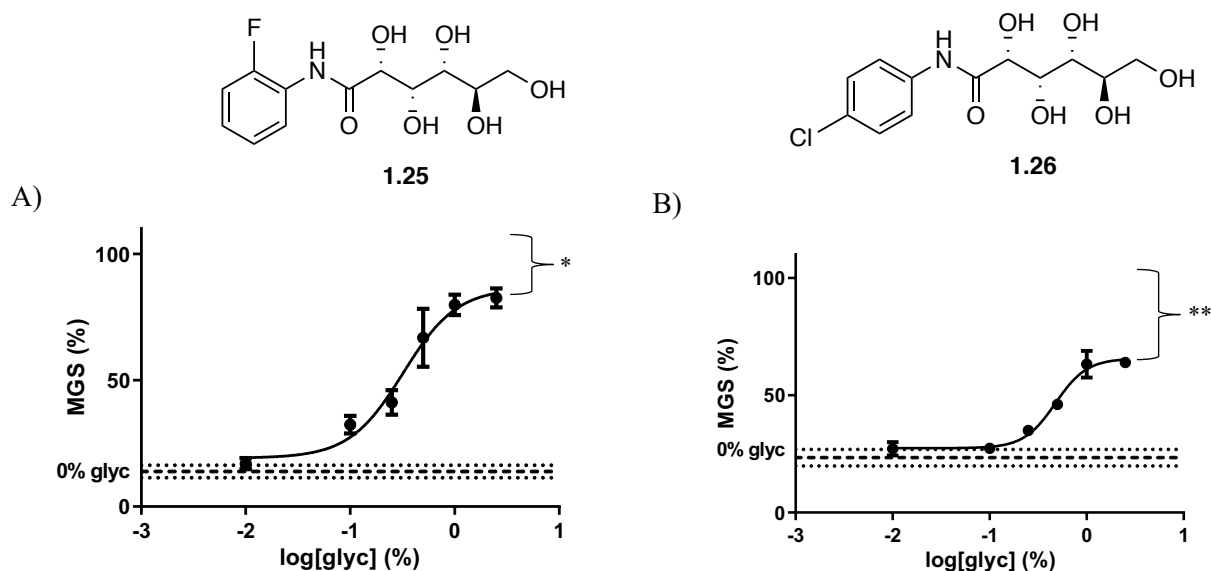


Figure 5.13. IRI activity of PBS solutions containing A) 5 mM **1.25** and B) 10 mM **1.26** with increasing concentrations of glycerol. % MGS values at 2.5% DMSO were statistically different from 100% MGS using the one sample t-test (* $p < 0.05$, ** $p < 0.01$). Ice crystal wafer areas were normalized to the appropriate glycerol/PBS solution and fitted with a variable-slope four parameter fit (A: $R^2=0.8834$; B: $R^2=0.9290$). Error bars are represented by % SD ($n=3$).

Table 5.3. IC_{50} values generated from dose response curves of gluconamides **1.25** and **1.26** in glycerol and DMSO. Values are reported with 95% confidence interval.

	IC_{50} (Glycerol)	IC_{50} (DMSO)
5 mM 1.25	0.32 ± 0.17 %	0.20 ± 0.10 %
10 mM 1.26	0.48 ± 0.13 %	0.02 ± 1.6 %

Observing that glycerol also has the ability to suppress the IRI activity of gluconamides **1.25** and **1.26** implies that similar to DMSO, glycerol inhibits the mechanism by which IRIs **1.25** and **1.26** act. As stated before, this may be a result of stronger hydrogen bonding interactions between CPA and IRI, therefore destroying interactions between water and IRI, or disruption of IRI networks that may form in solution. To definitively determine the cause of the suppression,

the mechanism of ice recrystallization inhibition by **1.22**, **1.25**, and **1.26** would have to be verified. The mechanism of IRIs may be different amongst each class of compounds as the IRI activity of *O*-aryl glucoside, **1.22**, was not affected by increasing concentrations of DMSO, unlike the *N*-aryl gluconamides, **1.25** and **1.26**. Future studies should include evaluating other IRI active compounds from the *O*-aryl glucoside class as well as observing their activity in the presence of glycerol. Overall, these results are quite interesting since IRIs **1.22**, **1.25**, and **1.26** have been used in the cryopreservation of various cell types with DMSO or glycerol and have yielded promising results indicating that they are sufficient cryo-additives,^{21–23,39,90} but **1.25** and **1.26** may not be acting by inhibiting ice recrystallization. Therefore, in light of these results, an in-depth analysis of the state of ice crystals in the presence of IRIs and CPAs would have to be conducted in order to verify the contributions of ice recrystallization inhibition *in vitro*.

5.4 Summary

There are several assays that have been established to measure the IRI activity of compounds. Amongst these assays, different techniques, cooling/warming rates, annealing times, temperatures, and positive controls are implemented, all of which have an effect on ice crystal size and the rate of ice recrystallization. *Section 5.2* investigated the qualitative and quantitative differences in two of the most common IRI assays, the splat cooling assay (SCA) and the sucrose sandwich assay (SSA). The differences in the size and shape of ice crystals generated from the technique and standard solutions were highlighted. Both assays exhibited solution limitations such that PBS could not generate uniform ice crystals using the SSA method and 40% sucrose was too concentrated to view ice crystals using the SCA method. The rate at which ice crystals recrystallized was significantly slower in the SSA, resulting in a discrepancy in the % MGS values

generated. Comparing these values for the IRI activity of AFPI suggested that a higher concentration of AFPI is required to produce the same inhibitory effect in the SSA compared to the SCA. Altogether, these results emphasize the importance of choosing IRI assays that have techniques and standard solutions similar to the expected application of the IRI, such that working concentrations will not be over- or under-estimated.

IRI assays were further analyzed in *Section 5.3* by investigating the contributions of CPAs, such as DMSO and glycerol that are often used alongside IRIs during cryopreservation. Compound **1.22**, from the *O*-aryl glucoside class, and **1.25** and **1.26**, from the *N*-aryl gluconamide class were evaluated for IRI activity via the splat cooling assay with increasing concentrations of DMSO or glycerol up to 2.5%. Both DMSO and glycerol were found to suppress the IRI activity of **1.25** and **1.26** in a concentration dependent manner, however, the IRI activity of **1.22** was unaffected by DMSO up to 1% (the maximum concentration tested). The agonistic dose-response to ice recrystallization observed by increasing amounts of DMSO and glycerol was speculated to be a result of hydrogen bonding interactions between the CPA and the IRI. Therefore, in the presence of DMSO and glycerol, IRIs with amide functional groups are less likely to interact with water and inhibit the process of ice recrystallization.

5.5 Experimental Procedures

5.5.1 *Ice Recrystallization Assay*

For compounds measured using the modified splat cooling assay,⁵⁶ samples were dissolved in phosphate buffered saline (PBS) and a 10 μ L drop of the solution was dispensed from a height of 2 metres, onto a polished aluminum block cooled to -78 °C with dry ice. The frozen wafer was carefully removed from the block, placed on a coverslip and transferred to a Cryo-stage

at -6.4 °C connected to a cooling bath (Digital One RTE7 from Thermo Scientific NESLAB), a Peltier unit (Series 800 from Alpha Omega Instruments), a fish pump (SAX1 from Pen Plax), and a drying tube. The wafer was allowed to anneal at this temperature for the specified time (5-min or 40-min). After the annealing time, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope (20x magnification). Images were then analyzed using ImageJ software, by circling each ice crystal with well-defined boundaries using the polygon function to generate the grain areas. Areas were inputted into Microsoft Excel where the average grain size of triplicate experiments was compared to the PBS positive control to generate a % mean grain size.

Compounds measured using the sucrose sandwich assay,⁵⁷ were dissolved in 40% sucrose and a 1 µL drop of the solution was sandwiched between two coverslips then transferred onto an aluminum block cooled to -78 °C with dry ice. The sandwiched wafer was carefully removed from the block, placed on a coverslip and transferred to a Cryo-stage at -6.4 °C connected to a cooling bath (Digital One RTE7 from Thermo Scientific NESLAB), a Peltier unit (Series 800 from Alpha Omega Instruments), a fish pump (SAX1 from Pen Plax), and a drying tube. The wafer was allowed to anneal at this temperature for 40 minutes. After the annealing time, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope (20x magnification). Images were then analyzed using ImageJ software, by circling each ice crystal with well-defined boundaries using the polygon function to generate the grain areas. Areas were inputted into Microsoft Excel where the average grain size of triplicate experiments was compared to the PBS positive control to generate a % mean grain size.

5.5.2 General experimental procedures

Small molecule IRIs (**1.22**, **1.25** and **1.26**) used in this chapter were synthesized as previously published.^{21,25} Antifreeze (glyco)proteins AFPI, AFPIII, and AFGP (Table 5.4) used in this chapter were a gift from Prof. Sakae Tsuda at the National Institute of Advanced Industrial Science and Technology (AIST) in Sapporo, Japan.

Table 5.4. Description and properties of AFPI, AFPIII, and AFGP.

	AFPI	AFPIII	AFGP
Source	Barfin Plaice, <i>Pleuronectes pinnifasciatus</i>	Notched-fin eelpout, <i>Zoarces Elongatus</i> Kner	Saffron Cod, <i>Eleginus gracilis</i>
Components	Mixture of 7 isoforms	Mixture of 13 isoforms	Mixture of 13-15 isoforms
Amino acid sequence	DTASDAAAAAAAAATAAA AAAAAAATAKAAAEAA AATAAAAR	NQESVVA AVLIPINT ALTVGMMTTRVVSP TGIPAEDIPRLISMQV NQVVPMGTTLMPD MVKGYAPA (11 th isoform)	ATA with disaccharide β -D galactosyl-(1 $\rightarrow$ 3)- α -N-acetyl-D-galactosamine
Molecular weight	3285.53 Da (40 residues)	7,000 (66 residues)	16,000 Da
Calculated pI	4.56	4.78	~4.5
Maximum thermal hysteresis	3.2 °C @ 500 mg/ml	2.1 °C @ 50 mg/ml	5.0 °C @ 1600 mg/ml
Heat stability	70% recovered after heating to 95 °C	70% recovered after heating to 95 °C	70% recovered after heating to 95 °C
Tolerable pH	3-11	3-11	3-11
Target ice plane	2ndary prism plane (whole planes if concentrated)	1 st prism plane and a pyramidal plane	1 st prism plane

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Chapter 6. Evaluating the Cryopreservative Ability of IRIs for Red Blood Cell and Hematopoietic Stem Cell Preservation

6.1 Introduction

The preservation of biological samples is important for several applications, most notably, cellular therapies and regenerative medicine.¹⁻⁴ To this end, storing biological samples at low temperatures is a commonly used method to reduce their rate of deterioration and therefore extend their shelf life. In **Chapter 1**, the three different types of long-term storage were presented with a focus on cryopreservation. Cryopreservation is the process where biological entities are frozen in a cryoprotective solution at low sub-zero temperatures (-80 °C to -196 °C) via controlled cooling rates. Although cryopreservation is a commonly used method, it has some disadvantages that include extensive cellular damage that can occur as a result of incorrect cooling/warming rates,⁵⁻⁹ and the type of cryoprotective agent (CPA) used.¹⁰⁻¹³

In the 1970s, the two-factor hypothesis was proposed by Peter Mazur, describing the optimal cooling and warming rates for different cell types that resulted in the least amount of cell damage.^{6,7} At slower than optimal cooling rates, cell damage is incurred as cells become dehydrated and are exposed to high solute concentrations, and at rapid cooling rates, intracellular ice can form; both result in cell lethality. Since then, cell viability has been maximized by close attention to freezing and warming protocols,^{6,9} and also by the inclusion of CPAs.^{12,14} The use of CPAs during the cryopreservation of biological materials presents many benefits, however, the inherent toxicity of some commonly used CPAs poses issues towards cell viability and patient safety.^{11,15,16} Thus, some post-thaw protocols require the removal of CPAs before use in clinical applications. For example, the removal of DMSO is required due to the possibility of gastrointestinal, cardiovascular, and neurological toxicity,¹⁷⁻²⁰ and the removal of glycerol is

required before blood transfusions due to the possibility of intravascular hemolysis.^{21,22} Removal techniques often involve centrifugation and several washes with concentrated solute solutions, which are not 100% effective, can be time consuming, and can be detrimental to cells.^{23–26}

Another caveat with commonly used CPAs is their inability to protect against mechanical cell damage caused by ice recrystallization, which is a major contributor to the reduction in post-thaw cell viability.^{6,27,28} Thus, conventional CPAs have many limitations. For the cryopreservation of cellular material, the Ben laboratory has investigated the use of small-molecule IRIs alongside conventional CPAs, as a method to mitigate cell damage caused by ice recrystallization.^{29–31} Likewise, due to their ability to mitigate the prominent cause of cell damage, IRIs can be used with reduced concentrations of CPA, and therefore lead to shorter CPA removal times.³²

The Ben laboratory uses a three-step process for identifying small molecule carbohydrates that are also potential cryo-additives. The process initially involves analyzing the IRI activity of a compound via the splat cooling assay³³ to determine its IC₅₀ value.³⁴ Compounds with an IC₅₀ value less than or equal to 20 mM, are considered effective inhibitors of ice recrystallization and are eligible for *in vitro* evaluation. Next, the cytotoxicity of the compound is assessed in mammalian HepG2 cells to provide a better understanding of its *in vitro* effects and to identify non-toxic working concentrations. Although cytotoxic effects may vary between cell lines, HepG2 cells are often widely used as a model for toxicity studies.^{35,36} Compounds exhibiting relatively high IRI activity and low cytotoxicity (LD₅₀ is above the IC₅₀ for IRI activity), can then be further evaluated for their cryoprotective ability in different cell lines.

The Ben laboratory has identified small molecules from the *O*-aryl glucoside class that have shown great cryoprotective ability in red blood cells (RBCs),^{29,32,37} and human umbilical vein endothelial cells³¹; also select *N*-aryl gluconamides have improved the post-thaw engraftment

ability of platelets³⁸ and the post-thaw recovery and functionality of hematopoietic stem cells (HSCs) isolated from umbilical cord blood.³⁰ In this chapter, IRI active compounds from **Chapter 4** and **Chapter 5** will be evaluated for their cryoprotective ability towards RBCs and HSCs as a preliminary measure of efficacy for cryopreservation.

6.2 The evaluation of IRIs in the cryopreservation of RBCs

6.2.1 *Current methods of RBC preservation*

Currently, the accepted method for RBC preservation is hypothermic storage in which RBCs are stored at a temperature lower than the normal physiological temperature, but higher than the freezing point (i.e. usually 2-4 °C).²⁶ Hypothermic storage of RBCs can extend their shelf life to 2-6 weeks, however, it does not fully preserve the biophysical, biochemical, and morphological properties of RBCs.²¹ An increase in hemolysis, depletion of ATP, and altered RBC aggregability are some of the complications that arise from hypothermic storage. These complications can lead to an increased infection rate, impaired tissue oxygenation, and proinflammatory effects upon clinical use.²¹ As blood transfusions are a highly accepted and a commonly used therapy method for several clinical diagnoses, these short storage periods and adverse effects can have a drastic impact on the blood supply chain and patient health.³⁹ Therefore, changing the accepted method of RBC storage to cryopreservation, so that the biological structure and function of RBCs can be preserved, is necessary.

Cooling and warming rates are important contributors to the survival of cryopreserved RBCs, where slower or faster than optimal cooling or warming rates can result in extensive cell damage.^{5,7} In addition, the type of CPA used can also contribute to cryopreserved RBC survival. The most accepted CPA used to cryopreserve RBCs is glycerol, due to its relatively low

cytotoxicity, its ability to easily permeate the cell membrane, and its ability to decrease the freezing point and the overall number of ice crystals that form.^{21,40,41} Two methods of glycerol RBC cryopreservation are currently accepted. RBCs can be cryopreserved in 40% glycerol using a slow cooling rate (1 °C/min) to -80 °C and rapid warming to 37 °C. This method is most common in Canada and the United States.⁴² RBCs can also be cryopreserved in 15-20% glycerol using a rapid cooling rate (>100 °C/min) to -196 °C, followed by rapid warming to 42 °C. This method is commonly used to cryopreserve RBCs in Europe.⁴² Employing either of these conditions, RBCs undergo dehydration during freezing due to their highly permeable membrane, however less dehydration occurs during rapid cooling rates.⁴³ The higher glycerol concentration used for slow cooling, provides protection against prolonged exposure to high solute concentrations, which is detrimental to unfrozen cells.

Under both methods, RBCs can be stored for up to 10 years,²¹ but must be deglycerolized before clinical use due to the toxicity of glycerol to patients. The deglycerolization process of cryopreserved RBCs must be performed carefully to ensure minimal damage to cells, and to ensure an intracellular glycerol concentration of <1%. Therefore, deglycerolizing cryopreserved units containing 40% glycerol can be quite time consuming (i.e. one RBC unit can take up to an hour to deglycerolize),³⁹ while units cryopreserved with 15-20% glycerol can take half the amount of time.⁴² In both cases, the formation of ice crystals and the events of ice recrystallization are problematic during the freezing and thawing of RBC units, resulting in RBC hemolysis and a reduced quality of blood product.

To reduce the amount of time needed for deglycerolization and to maintain a high quality of blood product, the Ben laboratory has evaluated the effect of IRIs towards the cryopreservation of RBCs using reduced amounts of glycerol.^{32,44} In these experiments, a cooling rate similar to that

used for standard RBC cryopreservation in Canada and the United States (1 °C/min) and a cooling rate similar to that used in Europe (>100 °C/min) were employed (Fig. 6.1).^{9,32} Ice nucleation was controlled in both protocols by manual induction after initial slow cooling to -5 °C. The temperature when ice nucleates is an important variable in cryopreservation that can lead to variances in cell viability and functionality.⁴⁵ Therefore, controlling the nucleation temperatures between samples will avoid post-thaw damage from inconsistencies in nucleation temperatures.

The protocol for slow cooling (Fig. 6.1A) involves cooling RBC samples at a rate of 1 °C/min to -40 °C, followed by rapid warming to 37 °C. The protocol for rapid cooling (Fig 6.1B) involves cooling RBC samples by immersion in dry ice (-78 °C) followed by rapid warming to 37 °C. The rapid cooling protocol typically generates a higher level of RBC recovery in 15% glycerol compared to slow cooling. This is because rapidly cooling RBCs allows less exposure to high solute concentrations and less damage from ice recrystallization.^{7,43}

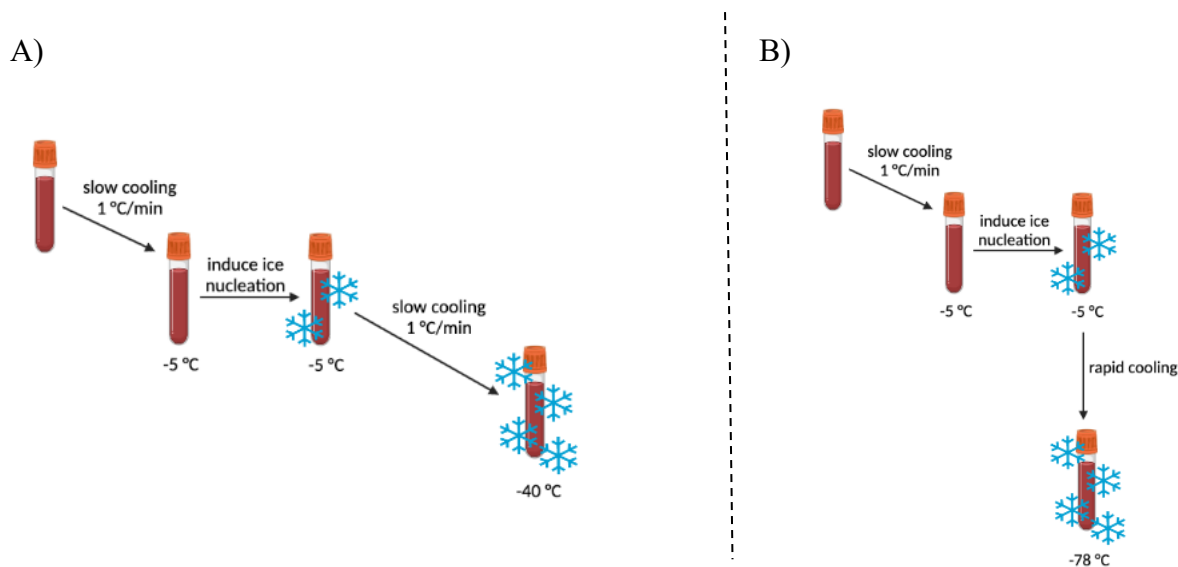


Figure 6.1. Graphical depiction of the slow cooling (A) and the rapid cooling (B) protocols. Figure created with BioRender.

Previous experiments using these two protocols, showed that IRIs from the *O*-aryl glucoside class (**1.20**, **1.22**, and **3.21**) in combination with 15% glycerol, significantly reduced the percentage of hemolysis and therefore increased the overall post-thaw cell viability (~70-80% RBC integrity) compared to RBCs in 15% glycerol (Fig. 6.2).^{44,46} Therefore, if other IRIs are sufficient CPAs, similar results would be expected.

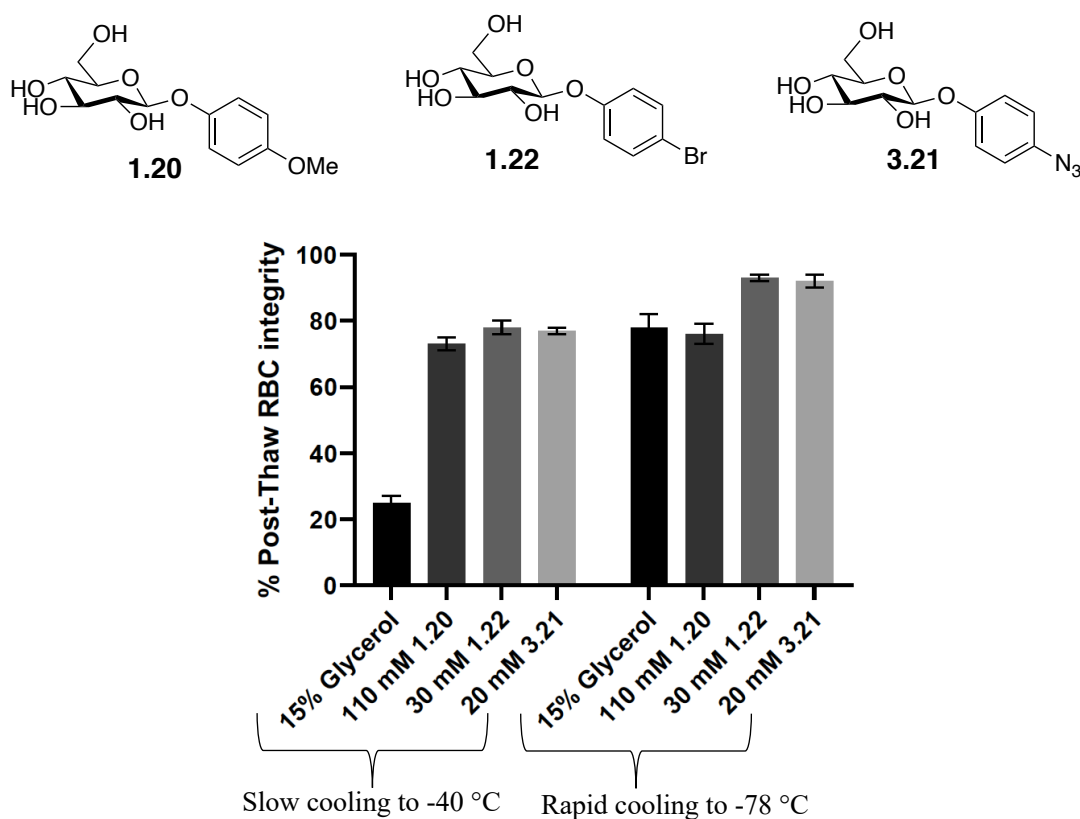


Figure 6.2. Post-thaw RBC integrity of cryopreserved RBCs in 15% glycerol supplemented with *O*-aryl glucosides **1.20**, **1.22**, and **3.21**. Samples were frozen by either slow cooling (1 °C/min) to -40 °C or rapid cooling (>100 °C) to -78 °C. Error bars represent SEM.

For both protocols, the impact of IRIs on the cryopreservation of RBCs is quantified by the percentage of RBCs intact (% post-thaw RBC integrity), which was calculated from the % hemolysis determined using the Drabkin's assay.^{47,48}

$$\% \text{ post-thaw RBC integrity} = 100 - \% \text{ hemolysis}$$

In the Drabkin's assay, Drabkin's reagent (containing potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), potassium cyanide (KCN), and sodium bicarbonate (NaHCO_3)), reacts with hemoglobin, resulting in the reduction product, potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$), and the oxidized product, methemoglobin. The methemoglobin reacts with cyanide to produce a stable pigment, cyanmethemoglobin which is quantified by its absorbance at 540 nm, and the absorbance is directly proportional to the hemoglobin concentration.⁴⁸ The % hemolysis is further determined by the ratio of supernatant hemoglobin to total hemoglobin.

Overall, these results were significant and displayed the importance of IRIs in improving the viabilities of cryopreserved RBCs, which could resultingly improve the efficacy of therapeutic blood transfusions. As such, the following section continues in the study of improving the cryopreservation of RBCs with both AF(G)Ps and phosphonate-containing IRIs.

6.2.2 *The in vitro evaluation of AF(G)P cytotoxicity and cryopreservative ability*

As observed in **Chapter 5**, antifreeze proteins and glycoproteins, AFPI, AFPIII, and AFGP are effective IRIs at low concentrations according to the splat cooling assay. Their IC_{50} values were recorded as 15 nM, 30 nM, and 2 nM for AFPI, AFPIII, and AFGP respectively. Having such strong IRI activity makes AF(G)Ps attractive as CPAs since only low concentrations are required to inhibit ice recrystallization, therefore limiting any *in vitro* high solute-induced toxicity. AF(G)Ps have previously been evaluated as CPAs in the cryopreservation of mammalian cell monolayers, sperm cells, embryos, and mammalian livers.^{49–52} Generally, AF(G)Ps were able

to protect against cryoinjury when used at low concentrations, however, higher concentrations led to extensive damage.⁵³

As discussed in **Chapter 1**, AF(G)Ps possess thermal hysteresis (TH) activity in addition to their ability to inhibit ice recrystallization. Thermal hysteresis is a phenomenon where ice neither grows or melts between the nonequilibrium melting point and freezing point, as a result of the adsorption of AF(G)Ps to the ice lattice. At temperatures below the freezing point, a sudden burst of ice growth occurs and ice crystals grow in a needle-like, spiculated manner (described as dynamic ice shaping).⁵⁴ It has been hypothesized that the TH gap and dynamic ice shaping (DIS) ability of AF(G)Ps increases with concentration and can exacerbate cellular damage,⁵⁵⁻⁵⁷ thus, using high concentrations of AF(G)Ps to cryopreserve biologics has been unsuccessful.^{53,58} For example, a few studies have been reported in which AF(G)Ps were used to cryopreserve RBCs. Rubinsky and DeVries observed that the cryopreservation of RBCs (slow cooling at 1 °C/min) in 20% glycerol supplemented with 40 mg/mL (~2.5 mM) of an AFGP mixture, led to spiculated ice crystals which damaged the cell membrane.⁵⁷ Contrarily, Chao et. al. observed that low concentrations of AFPI (5-150 µg/mL or ~1.5-4.5 µM) in the presence of hydroxyethyl starch were able to enhance the survival of cryopreserved RBCs (rapid cooling/warming, >100 °C/min) by inhibiting ice recrystallization.⁵⁹ Therefore, it was expected that using concentrations around the measured IC₅₀ values of AF(G)Ps to cryopreserve RBCs would provide sufficient protection against cryoinjury, while using higher millimolar concentrations would be lethal due to the DIS ability of AF(G)Ps.

Additionally, Rahman et al, reported the crystal habit of ice in the presence of AFPs at different concentrations.⁶⁰ The binding of AFPI, AFPII, and AFPIII, to the prism plane resulted in hexagonal ice crystals at concentrations lower than their IRI endpoint: the minimum concentration

at which the rate of ice recrystallization is zero (1.5 μM , 0.8 μM , 1.4 μM respectively, measured using the SSA).⁶⁰ The lethal bipyramidal shaped ice crystals were only apparent at much higher concentrations (50 μM and greater, Fig 6.3).⁶⁰ These results also confirmed that the detrimental bipyramidal ice shape is only a cause for concern at higher micromolar concentrations, and that high concentrations of AFPs are not required for maximum IRI activity (obtaining a zero rate of ice recrystallization). To this end, it was expected that evaluating AF(G)Ps as *in vitro* CPAs at high nanomolar to low micromolar concentrations should not be detrimental to cells as a result of their DIS ability.

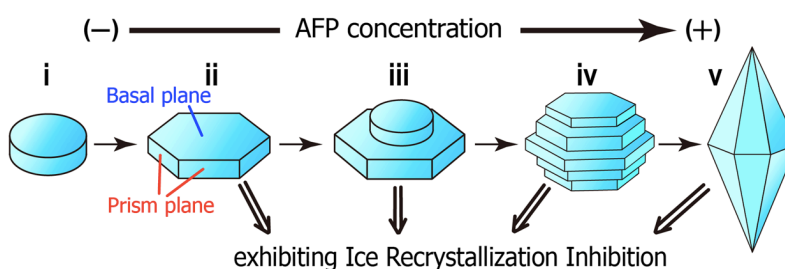


Figure 6.3. The variations in ice crystal growth habits at increasing concentrations of AFPs. In the absence of AFP, ice crystals are round disks (i) and upon binding of AFPs to the prism plane, ice crystals become hexagonal (ii). Increasing concentrations of AFPs results in layering (iii and iv) until bipyramidal ice crystals (v) form at higher AFP concentrations. Crystal habits were determined by observation of a single ice crystal through a photomicroscope. Figure reprinted with permission from A. T. Rahman, et.al., *Sci. Rep.*, 2019, 9, 1–9. Copyright © 2019, Springer Nature.

6.2.2.1 Assessing the cytotoxicity of AFPI, AFPIII, and AFGP in HepG2 cells

The Ben laboratory has previously evaluated the cytotoxicity of native AFGP 8 in human embryonic liver cells (WRL 68) and human embryonic kidney cells (HEK 293).⁶¹ Using an *in vitro* cytotoxicity assay (MTT assay⁶²), AFGP 8 was found to be cytotoxic at concentrations higher than 2 mg/mL (~0.8 mM) in WRL 68 cells and at concentrations higher than 0.63 mg/mL (~0.2 mM) in HEK 293 cells.⁶¹ These results suggest that the cytotoxicity of AFGP 8 is concentration-

dependent and varies between cell line. Since WRL 68 cells are morphologically and functionally similar to HepG2 cells,⁶³ similar results were expected such that cytotoxicity would not be observed at low milimolar concentrations. Thus, AFPI, AFPIII, and AFGP were assessed for their cytotoxicity using a HepG2 model cell line. HepG2 cells are an immortalized liver-derived cell line that are highly differentiated and display very similar genotypic features to normal hepatocytes, therefore they are a good model to examine cell metabolism.³⁵

The cytotoxicity of AFPI, AFPIII, and AFGP in HepG2 cells was evaluated using a fluorescent based Aquabluer™ assay that monitors the reducing environment of living cells and is directly proportional to cell viability. The Aquabluer™ assay contains the active reagent, resazurin, which has been used extensively in the determination of cell viability and cytotoxicity in various biological systems.^{64–66} Resazurin is a blue, non-fluorescent, water-soluble dye that easily permeates through cell membranes and acts as an intermediate electron acceptor for coenzymes NADPH, FADH, FMNH, NADH, and the cytochromes. As resazurin is metabolized by live cells, it accepts electrons and is reduced to resorufin, a pink, fluorescent dye that has an excitation wavelength of 540 nm and emission wavelength of 590 nm (Fig. 6.4). Measuring the fluorescence of resorufin provides a direct measurement of cell metabolism and therefore viability. Regarding its reliability, Aquabluer™ is comparable to other common bioassays such as the MTT assay and the WST-8 assay.⁶⁷

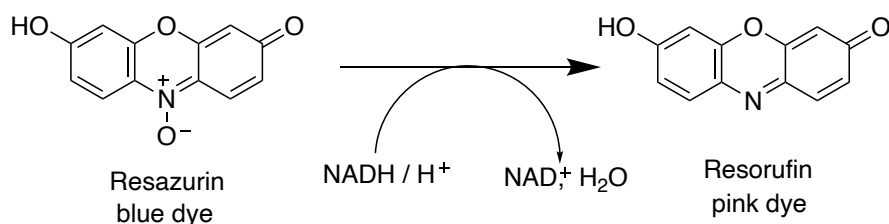


Figure 6.4. The reduction of resazurin to resorufin.

AFPI, AFPIII, and AFGP were evaluated in HepG2 cells at concentrations lower and higher than their IC₅₀ values, up to 100 nM (Fig. 6.5). Using the Aquabluer™ assay, relative fluorescent unit (RFU_{AF(G)P}) readings were obtained, and cell viabilities were calculated by the following equation:

$$\% \text{ cell viability} = \frac{RFU_{AF(G)P} - RFU_{neg}}{RFU_{pos} - RFU_{neg}} \times 100\%$$

The RFU readings (RFU_{pos}) of the positive control measures the metabolic activity of HepG2 cells in Eagle's Minimum Essential Media (MEM) and is representative of cell viability. The RFU readings (RFU_{neg}) of the negative control measures the metabolic activity of HepG2 cells in the presence of 1% Triton X, a surfactant that causes cell death, and is representative of cell viability.

Initial evaluation of cytotoxicity revealed that AFPI and AFPIII were cytotoxic at low nanomolar concentrations (one-way ANOVA with Dunnett's multiple comparison test). AFPI concentrations of 0.5 nM, 1 nM, 2 nM, and 100 nM displayed a significant amount of cytotoxicity, however, the cell viability at those concentrations was still around 80%. The greatest level of cytotoxicity was observed for AFPIII at 100 nM (39% cell viability). However, considering the IC₅₀ is 30 nM, concentrations at or above 100 nM should not be required for high IRI activity. Low nanomolar concentrations of AFGP were not cytotoxic (with the exception of 2 nM AFGP) compared to AFPI and AFPIII. However, 100 nM AFGP was significantly cytotoxic ($p < 0.01$) compared to the control condition (0 nM). Toxicity at 100 nM is not of concern, as AFGP displayed the strongest IRI activity (IC₅₀ = 2 nM), thus high nanomolar concentrations are not required for high IRI activity. Since HepG2 cells are nucleated, it is difficult to predict if the cytotoxic effects observed would be similar to that observed in anucleate RBCs. However, it can be confirmed that the cytotoxicity of AFPI, AFPIII, and AFGP in HepG2 cells are relatively low at low nanomolar

concentrations. This is beneficial as AF(G)Ps also portray strong IRI activity at low nanomolar concentrations.

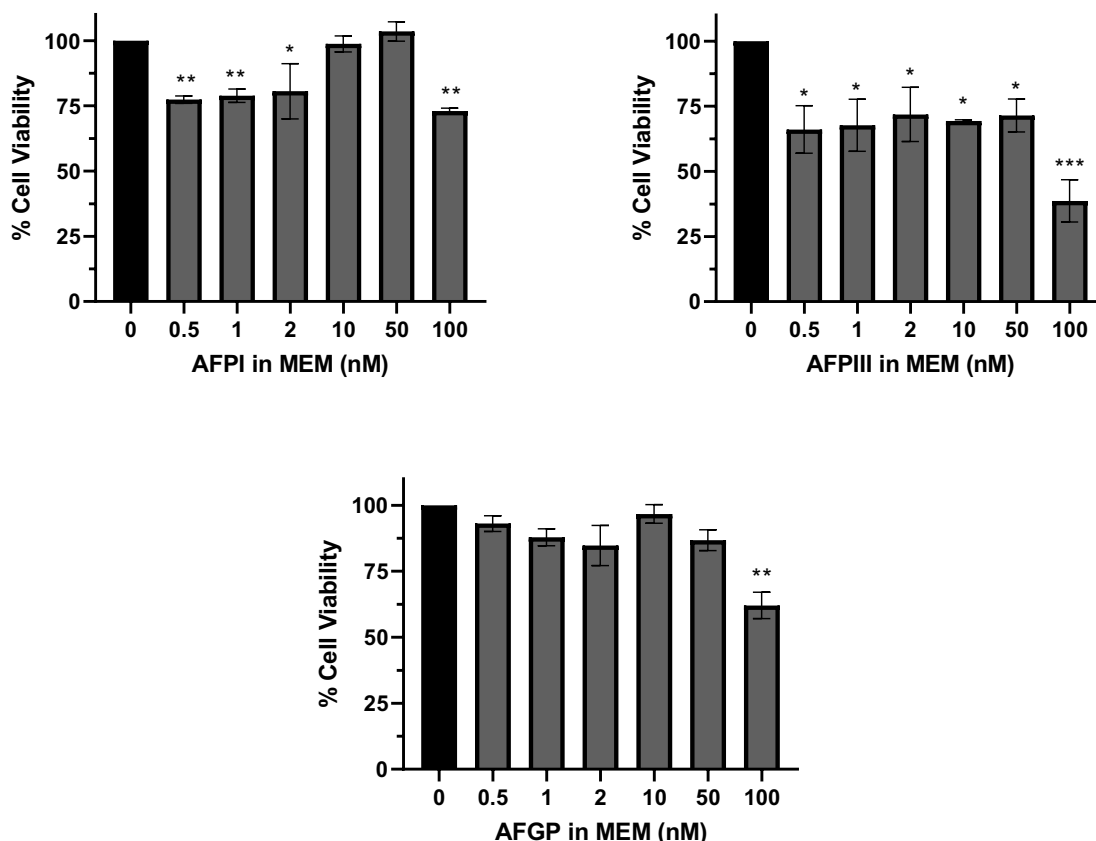


Figure 6.5. Cell viability of HepG2 cells in the presence of AFPI, AFPIII, or AFGP in Eagle's Minimum Essential Media (MEM). Cell viability was assessed using the Aquabluer™ assay. Data plotted is the mean of two plates each with n=6 wells. Error bars represent % SD for n=12 replicates. Asterisks indicates statistical significance from control condition (0 nM) using the one-way ANOVA with Dunnett's multiple comparison test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

6.2.2.2 The cryoprotective ability of AF(G)Ps in RBCs

As a preliminary analysis, RBCs were cryopreserved in 15% glycerol with AFPI, AFPIII, or AFGP at their IC₅₀ concentration (obtained from the splat cooling assay) and at a higher nanomolar concentration. Higher concentrations of AFPI, AFPIII, and AFGP that exhibited a minimal rate of ice recrystallization were chosen according to the respective dose-response curve.

To examine the cryopreservative ability of AF(G)Ps, the slow cooling method and the rapid cooling method described previously, were employed.

As expected, for RBCs cryopreserved in 15% glycerol using the slow cooling rates resulted in a high level of hemolysis (~28% intact RBCs post-thaw, Fig. 6.6) that is comparable to previously obtained values.³² Supplementation of AFPI at 15 nM or 300 nM did not result in a significant increase or decrease in post-thaw hemolysis (one-way ANOVA with Dunnett's multiple comparisons test). Similar levels of hemolysis were observed with 30 nM and 60 nM AFPIII, and 2 nM and 30 nM AFGP. It was suspected that the IRI activity of AFPI, AFPIII, and AFGP may be suppressed at these concentrations due to the presence of 15% glycerol. Recall that 1% DMSO had an inhibitory effect on the IRI activity of AFPI in **Chapter 5**, and it was proposed that glycerol also exhibits the same inhibitory effect. Thus, 1 μ M of AFPI and AFGP in 15% glycerol were tested; higher concentrations of AFPIII were not evaluated due to its previously observed cytotoxicity. Increasing the concentration of AFPI and AFGP to 1 μ M did not significantly increase or decrease post-thaw hemolysis compared to the 15% glycerol control.

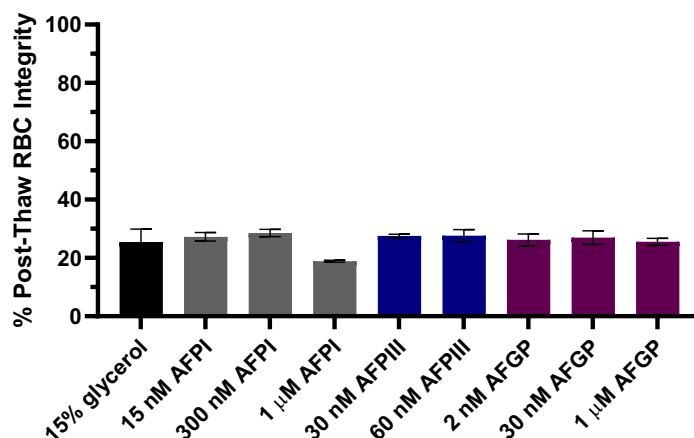


Figure 6.6. Cryopreservation of RBCs with AFPI, AFPIII, and AFGP via slow cooling to -40 °C. The percent intact of RBCs post-thaw was measured by determining the % hemolysis via the Drabkin's assay.⁴⁷ Cryosolutions of AFPI, AFPIII, and AFGP were made in 15% glycerol. Error bars represent the SD for n=2 replicates. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test (compared against 15% glycerol control).

The rapid cooling method was employed using the same concentrations of AFPI, AFPIII, and AFGP used for the slow cooling method (Fig. 6.7). Supplementation of 15 nM and 30 nM AFPI did not significantly increase or decrease the amount of RBC hemolysis compared to the 15% glycerol control (one-way ANOVA with Dunnett's multiple comparison test). Further increasing the concentration of AFPI to 1 μM also did not change these results. Similarly, the test concentrations of AFPIII (30 nM and 60 nM) and AFGP (2 nM, 30 nM, and 1 μM) did not exhibit any change to the amount of intact RBCs recovered compared to the 15% glycerol control. Observing that AFPI, AFPIII, and AFGP do not improve or worsen the amount of RBC hemolysis, regardless of the freezing protocol, suggests that AFPI, AFPIII, and AFGP are neither protecting RBCs from cryoinjury nor causing cell damage via their DIS ability. Future experiments should assess higher concentrations of AFPI, AFPIII, and AFGP to determine the concentration that provides RBCs with maximum protection from ice damage without causing lethality from DIS.

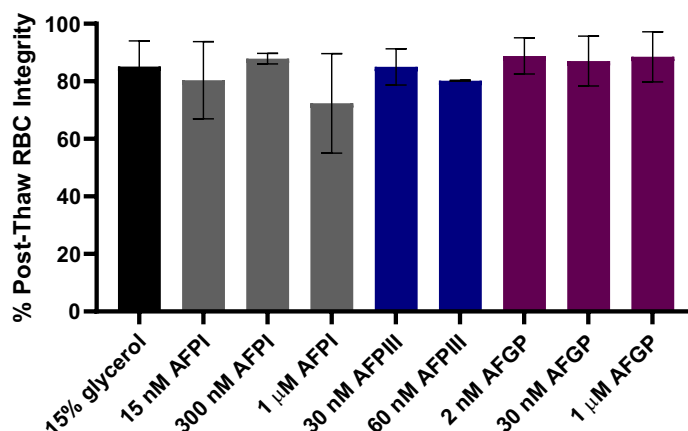


Figure 6.7. Cryopreservation of RBCs with AFPI, AFPIII, and AFGP via rapid cooling to -78 °C. The percent intact of RBCs post-thaw was measured by determining the % hemolysis via the Drabkin's assay.⁴⁷ Cryosolutions of AFPI, AFPIII, and AFGP were made in 15% glycerol. Error bars represent the SD for n=2 replicates. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test (compared against 15% glycerol control).

6.2.3 The *in vitro* evaluation of IRIs 4.14, 4.15, 4.18, and 4.19

As discussed in **Chapter 4**, glucose phosphonate compounds were synthesized as a method to generate IRIs of high solubilities. Phosphonate compounds **4.14**, **4.15**, **4.18** and **4.19** all exhibited high solubilities in PBS (>500 mM) however, their IRI activity varied (Table 6.1). Ammonium phosphonate compounds **4.14** and **4.18** had strong IRI activity due to the presence of the ammonium ion; this was verified by observing the same level of IRI activity for other ammonium salts. The sodium counterion did not exhibit IRI activity, thus the activities of **4.15** and **4.19** were considered the “true” activity without interference of the counterion (Table 6.1). The variability in IRI activity as a result of their counterion makes the glucose phosphonates interesting compounds to evaluate *in vitro*, as the presence of an active or inactive counterion could potentially alter the cytotoxicity and cryopreservative ability of the phosphonate compounds. Since the ammonium phosphonate compounds **4.14** and **4.18** were more IRI active than the sodium

phosphonate compounds **4.15** and **4.19**, their potential as better CPAs for RBC cryopreservation was assessed.

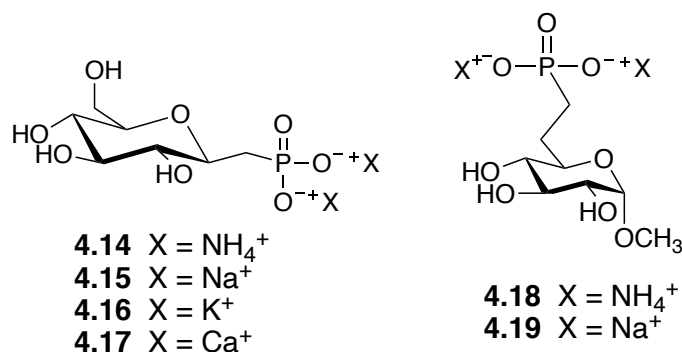


Figure 6.8. Glucose phosphonate compounds evaluated *in vitro*.

Table 6.1. IRI activities and maximum solubilities of glucose phosphonate compound **4.14**, **4.15**, **4.18** and **4.19**. IC_{50} values are reported with their 95% confidence interval.

Glucose phosphonate derivatives	IC_{50} (mM)	Maximum solubility in PBS (mM)
4.14	3.0 ± 0.5	>500
4.15	51 ± 8	>500
4.18	1.6 ± 0.2	>500
4.19	28 ± 7	>500

6.2.3.1 *The evaluation of the cytotoxicity of glucose phosphonates 4.14, 4.15, 4.18, and 4.19 in HepG2 cells*

Phosphonates are used quite extensively in medicinal chemistry as bioisosteric replacements for phosphates. Several nucleoside phosphonates have been synthesized as nucleotide mimetics and have displayed low *in vitro* cytotoxicity allowing their use as antiviral therapeutics.^{68–70} Therefore, it was expected that the phosphonate moieties in compounds **4.14**,

4.15, **4.18** and **4.19** would be biologically accepted and exhibit low cytotoxicity as well. To this point, the cytotoxicity of **4.14**, **4.15**, **4.18** and **4.19** were evaluated in HepG2 cells. The AlamarBlue™ assay, another resazurin based assay, was used and the cytotoxicity was calculated using the same method as described in Section 6.2.2.1. The Ben laboratory has used the AlamarBlue™ assay to evaluate the cytotoxicity of various *O*-aryl glycosides and *N*-aryl aldonamides, and it has proven to generate consistent and reproducible results.^{38,44,71}

Concentrations lower and higher than the IC₅₀ values were evaluated, and a nonlinear regression was fitted to the data points, generating a dose-response curve (Fig. 6.9). Since glucose phosphonates **4.14**, **4.15**, **4.18** and **4.19** displayed a full dose-response, LD₅₀ values were generated describing the dose of the compound required for 50% cell lethality. Compound **4.14** displayed a LD₅₀ of 16 ± 3 mM (reported with 95% CI), which is several times greater than the IC₅₀ value of **4.14**. The cytotoxicity of ammonium chloride (NH₄Cl) was also evaluated to determine if the ammonium ions were a major contributor to the cytotoxicity observed for **4.14**. It was hypothesized that if ammonium did contribute to the cytotoxicity of **4.14**, NH₄Cl would show a similar cytotoxicity to **4.14**, and little discrepancy between the dose-response curves would be observed when overlayed. Studies on the cytotoxicity of NH₄Cl towards primary hepatocytes, HepG2 cells, and other cell lines reported that exposure to NH₄Cl at concentrations of 20 mM and greater resulted in less than 50% cell viability (measured using MTT assay).⁷² Similar results were observed with HepG2 cells, using the AlamarBlue™ assay, such that concentrations greater than 20 mM resulted in a cell viability less than 50% (Fig. 6.9). A dose-response curve was generated for NH₄Cl, similar to that of **4.14**, however, at concentrations greater than 20 mM, compound **4.14** was more cytotoxic than NH₄Cl. Therefore, the cytotoxicity observed for **4.14** may be a combined contribution from the cytotoxicity of the glucose phosphonate and ammonium ions. Sodium

phosphonate derivative **4.15** was less cytotoxic than ammonium phosphonate **4.14**, as its LD₅₀ value was 32 ± 11 mM (reported with 95% CI), further suggesting that the ammonium ions may enhance the cytotoxicity of **4.14**. At the IRI IC₅₀ value of **4.15**, cells were only 15% viable, therefore treatment with higher concentrations would cause complete cell death. The G6CP derivatives **4.18** and **4.19** were non-cytotoxic (100% viability) at their IC₅₀ values, and their LD₅₀ values were 24 ± 2 mM and 38 ± 3 mM (reported with 95% CI) respectively. Similar to the G1CP derivatives, the ammonium phosphonate **4.18**, was more cytotoxic than the sodium phosphonate derivative **4.19**, possibly due to the toxicity of the ammonium ions. Another major difference was the hill slope of the dose-response curves of **4.18** and **4.19** which were much greater than the hill slopes of **4.14** and **4.15** (Table 6.2). Hill slope differences has been correlated to different mechanisms of action in ligand-receptor interactions,^{73–75} suggesting that G1CP and G6CP undergo different mechanisms of toxicity in HepG2 cells. Overall, low millimolar concentrations of **4.14**, **4.15**, **4.18** and **4.19** display low cytotoxicity in HepG2 cells, therefore the evaluation of their cryopreservative ability is warranted.

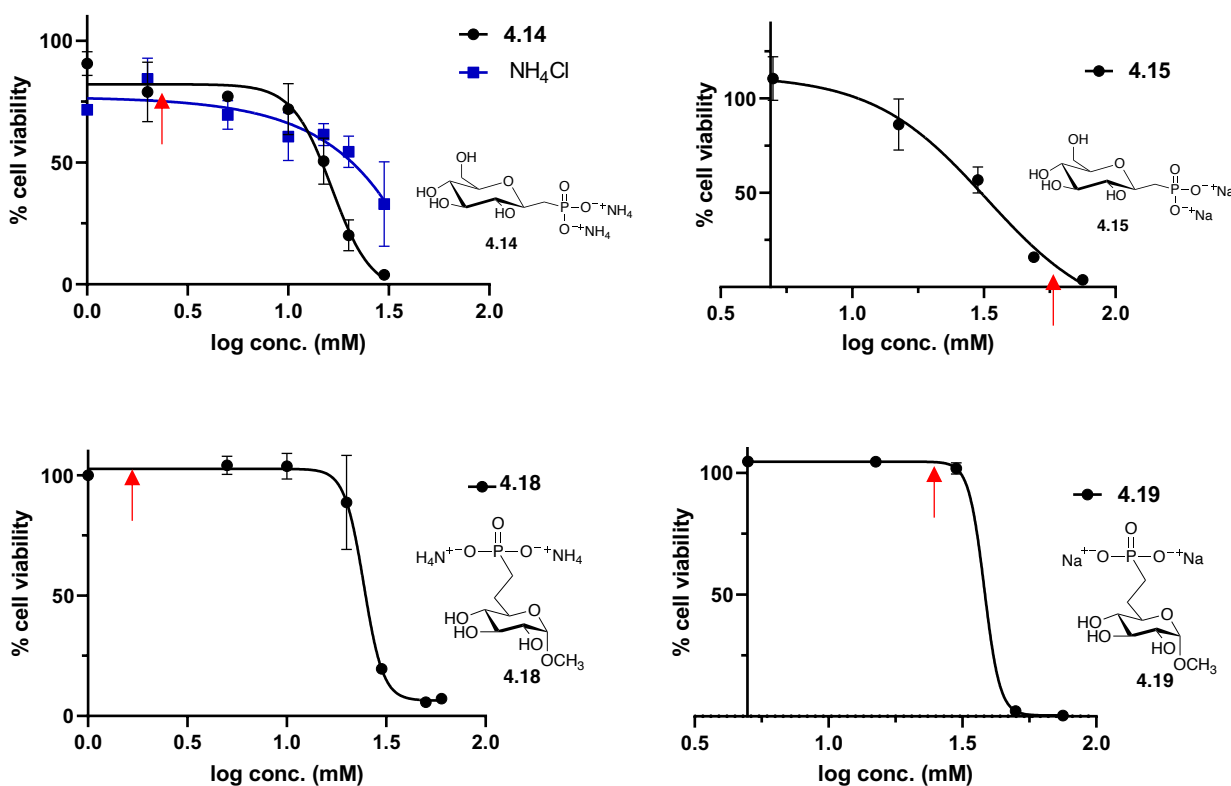


Figure 6.9. Cell viability of HepG2 cells in the presence of glucose phosphonate derivatives **4.14**, **4.15**, **4.18** and **4.19**. A non-linear regression curve was fitted to points to generate LD₅₀ values. Plates were run in duplicate with n=7 samples. Red arrows represent IRI IC₅₀ values (splat cooling assay). Error bars represent % SD.

Table 6.2. Cytotoxicity of phosphonate compounds **4.14**, **4.15**, **4.18** and **4.19** in HepG2 cells. LD₅₀ values and hills slopes are reported with 95% confidence interval.

Glucose phosphonate derivatives	LD ₅₀ (mM)	Hill slopes
4.14	16 ± 3	-4 ± 3
4.15	32 ± 11	-2 ± 5
4.18	24 ± 2	-9 ± 3
4.19	38 ± 3	-15 ± 2

6.2.3.2 *The cryopreservation of RBCs with IRIs 4.14, 4.15, and 4.24*

IRIs **4.14**, **4.15**, and **4.24** were evaluated as CPAs in RBCs using the same protocols and analysis as described in *Section 6.2*. The ammonium phosphonate (**4.14**), sodium phosphonate (**4.15**), as well as the phosphonic acid derivative (**4.24**) were evaluated to determine the impact of the ammonium and sodium counterions towards their cryopreservative ability and the impact of the ionic phosphonate vs. neutral phosphonic acid towards their cryopreservative ability. As an initial analysis, compounds **4.14**, **4.15**, and **4.24** were tested at 2 mM, 10 mM, and/or 50 mM concentrations. Lower concentrations (2 mM and 10 mM) of compound **4.14** were used since ammonium chloride is a common reagent used for RBC lysis, therefore higher concentrations of ammonium were expected to cause cell death.⁷⁶ Employing slow cooling rates, compounds were dissolved in 15% glycerol in the presence of RBCs and allowed to incubate for 10 minutes at room temperature before cooling down to -5 °C. At -5 °C, nucleation was induced, followed by continual slow cooling to -40 °C, then rapid warming to 37 °C. Following this protocol, compound **4.14** displayed no significant increase or decrease in the percentage of intact RBCs up to 10 mM (Fig. 6.10). The same results were observed for compound **4.15** at 10 mM and 50 mM. However, the phosphonic acid derivative **4.24**, exhibited a significant increase ($p < 0.05$) at 2 mM and 10 mM concentrations. This was surprising since compound **4.24** is acidic. Deviating from the physiological pH has a large effect on the structure and function of RBCs.⁷⁷ Specifically, a pH < 5.7 causes RBC hemolysis due to induced oxidative stress on the cell membrane.⁷⁸ As described in **Chapter 4**, 10 mM **4.24** has a pH of 5 in PBS, therefore it was expected that cryopreservation with 10 mM **4.24** would result in extensive cell damage. Additionally, **4.24** was the least IRI active ($IC_{50} = 90 \pm 27$ mM, reported with 95% CI) compared to **4.14** and **4.15**, and the concentrations of **4.24** evaluated in RBCs were several times below its IC_{50} value. Since **4.24** does not exhibit high

IRI activity at 2 mM and 10 mM (the measured rate of ice recrystallization was 100% in **Chapter 4**), its IRI activity may not have been the basis of the increase in RBC viability. To verify this hypothesis, further studies involving the evaluation of ice in a frozen sample of RBCs with the cryoprotective solution (15% glycerol supplemented with 2 mM or 10 mM **4.24**) is required.

Although **4.14** and **4.15** did not increase or decrease the post-thaw viability of RBCs, the increase in post-thaw viability caused by **4.24** is similar to that observed in the cryopreservation of RBCs with *O*-aryl glycoside, **1.20** (Fig. 6.2).⁴⁶ Thus, concentrations between 10 mM and 50 mM should be evaluated to determine the optimum concentration for maximum cell viability.

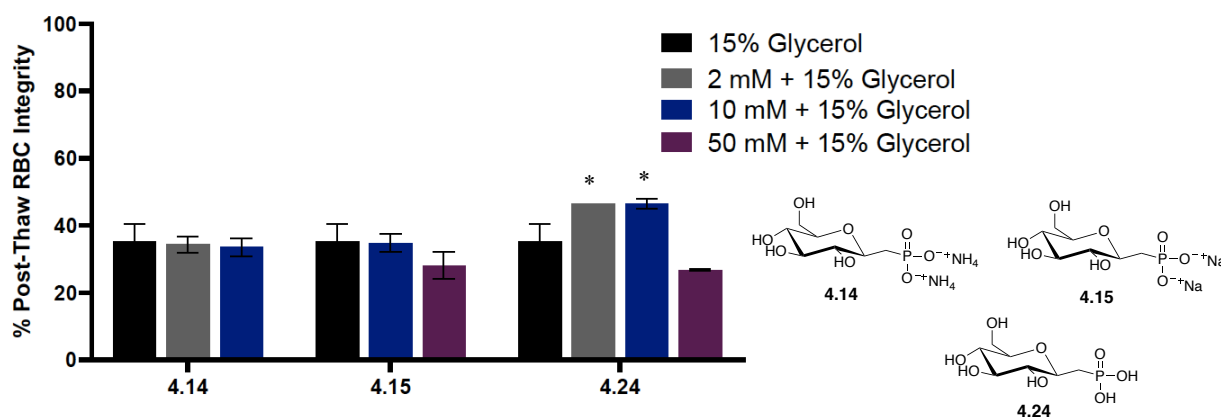


Figure 6.10. Cryopreservation of RBCs with **4.14**, **4.15**, and **4.24** via slow cooling to -40 °C. The percent intact of RBCs post-thaw was measured by determining the % hemolysis via the Drabkin's assay.⁴⁷

Cryosolutions of **4.14**, **4.15**, and **4.24** were made in 15% glycerol. Error bars represent the SD for n=2 replicates. Asterisks indicates statistical significance from control condition (15% glycerol) using the one-way ANOVA with Dunnett's multiple comparison test (* p < 0.05).

Using rapid cooling rates, compounds **4.14**, **4.15** and **4.24** in 15% glycerol were incubated with RBCs for 10 minutes, before cooling down to -5 °C. At this temperature, the samples were nucleated before rapid cooling to -78 °C. Using this protocol to cryopreserve RBCs in 15% glycerol led to 92% of RBCs intact post-thaw,⁷⁹ and the addition of 2 mM and 10 mM of **4.14** did

not deviate from those high viabilities (Fig. 6.11). Similarly, 2 mM of **4.24** and 10 mM of **4.15** and **4.24** also did not significantly increase or decrease the percentage of intact RBCs post-thaw. However, increasing the concentration of **4.15** and **4.24** to 50 mM resulted in a significant reduction ($p < 0.01$) of intact RBCs post-thaw to 40% and 71% respectively. This suggests that toxic effects of **4.15** and **4.24** are more pronounced by rapidly cooling RBCs to $-78\text{ }^{\circ}\text{C}$, compared to slow cooling to $-40\text{ }^{\circ}\text{C}$. For future studies, performing a concentration screen of **4.15** and **4.24** and evaluating intermediate concentrations between 10 mM and 50 mM would provide more information about their performance as cryo-additives. Also, since the G6CP derivatives are more IRI active than the G1CP derivatives, the cryopreservation ability of **4.18** and **4.19** should be evaluated in RBCs as well.

Therefore, from the results obtained, it can be concluded that the counterion and the ionic state of phosphonates **4.14**, **4.15**, and **4.24** did not alter their ability to cryopreserve RBCs according to their IRI activity. Overall, supplementing 15% glycerol with **4.14**, and **4.15** at the concentrations tested did not demonstrate any improvement in the cryopreservation of RBCs compared to 15% glycerol. Although 50 mM concentrations of **4.15** and **4.24** resulted in significant RBC hemolysis under rapid cooling conditions, 2 mM and 10 mM of **4.24** increased the post-thaw viability of RBCs under slow cooling conditions, as compared to 15% glycerol.

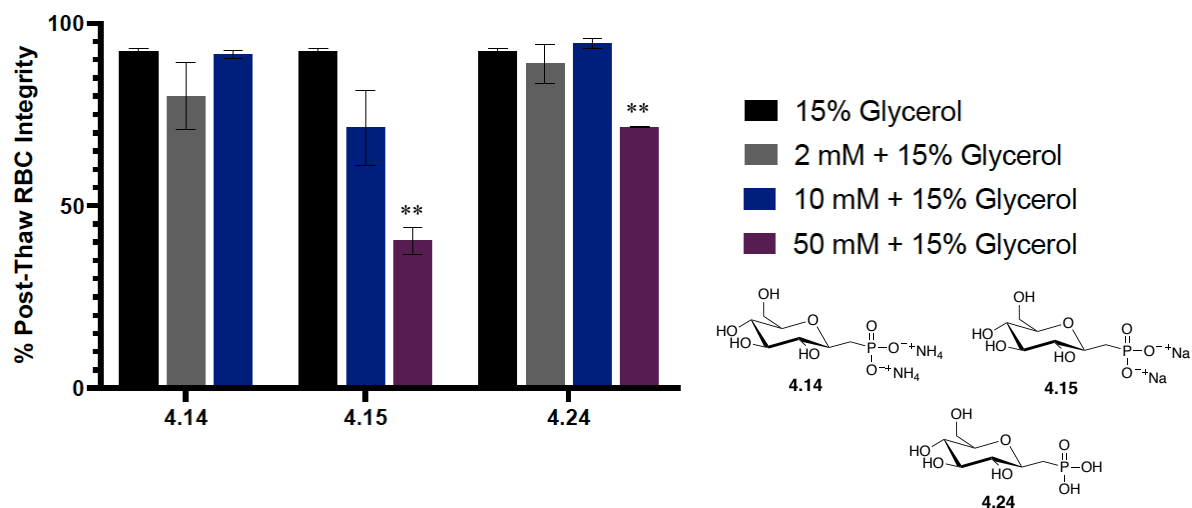


Figure 6.11. Cryopreservation of RBCs with **4.14**, **4.15**, and **4.24** via rapid cooling to -78°C . The percent intact of RBCs post-thaw was measured by determining the % hemolysis via the Drabkin's assay.⁴⁷ Cryosolutions of **4.14**, **4.15**, and **4.24** were made in 15% glycerol. Error bars represent the SD for $n=2$ replicates. Asterisks indicates statistical significance from control condition (15% glycerol) using the one-way ANOVA with Dunnett's multiple comparison test (** $p < 0.01$).

6.3 The evaluation of IRIs in the cryopreservation of hematopoietic stem cells

Hematopoietic stem cells (HSCs) are valuable due to their potential to reconstitute hematopoiesis and proliferate into various blood cells (Fig. 6.12). To this end, they are extensively used in regenerative medicine to treat blood- and immune-related diseases,⁸⁰ neurological diseases, cardiac disease, and diabetes.⁸¹ Sources of HSCs include bone marrow (BM), peripheral blood (PB), and cord blood (CB).⁸⁰ BM is a major site of hematopoiesis in humans, however, methods to isolate HSCs from bone marrow can be quite invasive for the patient; PB is also used for HSC transplantations; however, its HSC content is limited.⁸⁰ For patients requiring allogeneic transplantations, CB is often a significant source due to its lower number of activated lymphocytes compared to BM and PB stem cells.⁸² Therefore, an exact human leukocyte antigen (HLA) match is not required for CB transplantations. CB transplantation is also advantageous because of its lower incidence of chronic graft-vs-host-disease leading to an overall increase in survival rate.⁸³

However, unlike allogenic transplants of matched unrelated donors, transplantation of CB often results in a slower rate of platelet and neutrophil engraftment.⁸³

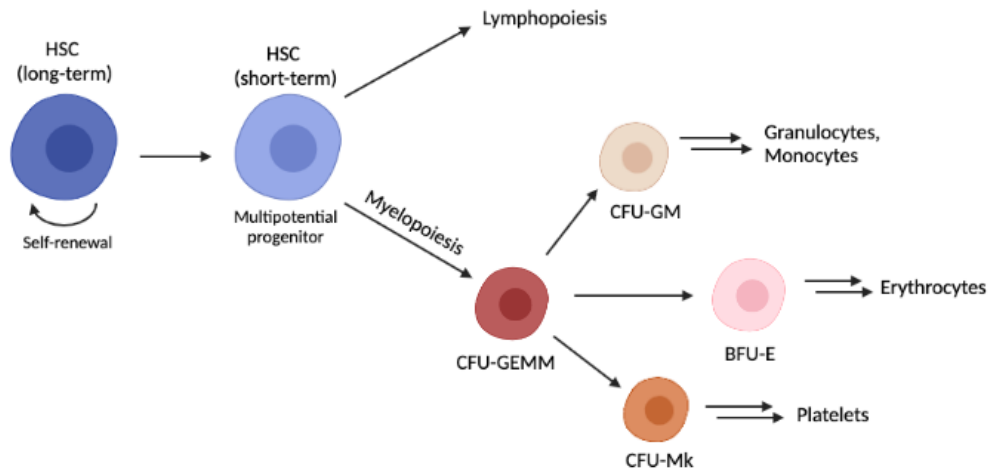


Figure 6.12. A representation of the proliferation and differentiation of hematopoietic stem cells (hematopoiesis). Selected cell populations shown include Colony-Forming Unit – Granulocyte/Erythrocyte/Monocyte/Megakaryocyte (CFU-GEMM) population, Colony-Forming Unit – Granulocyte/Monocyte (CFU-GM) population, Burst-Forming Unit – Erythroid (BFU-E) population, and Colony-Forming Unit – Megakaryocyte (CFU-Mk) population. Figure created with BioRender.

The success of engraftment has been linked to the level of apoptotic CD34⁺ cells, and the ability of HSCs to proliferate and differentiate into colony forming units (CFU).⁸⁰ The viability and functionality of HSCs is often impacted by the processing and the storage of stem cells from CB. Currently, HSCs are stored by slow cooling (1 °C/min) to -160 °C (in a mechanical freezer with vaporized liquid nitrogen) or to -196 °C (in a liquid nitrogen Dewar).³ To preserve the cell volume, and reduce damage from excessive dehydration, CPAs are used during the cryopreservation of HSCs. The most common cryoprotective medium used to cryopreserve HSCs is 5-10% DMSO with 1% dextran.⁸⁴ Supplementation of DMSO with trehalose,^{85,86} hydroxyethyl

starch,⁸⁷ and sucrose^{86,88} have also been effective in increasing the success of HSC cryopreservation, by increasing the recovery of HSC progenitors. Also, to reduce the effects of DMSO toxicity, DMSO is usually added to pre-cooled HSCs (at 0 °C) in a timely manner before freezing.

Moreover, the Ben laboratory has investigated the use of small-molecule IRIs in the cryopreservation of CB.^{30,71,88} In a previous study, leukocyte concentrates comprising of total nucleated cells (myeloid cells, lymphoid cells, and HSCs) were cryopreserved in 10% DMSO and supplemented with *N*-aryl gluconamides.³⁰ An improvement in the post-thaw recovery of colony forming units (CFUs) and of immature multipotent progenitors (measured by the long-term culture-initiating cell (LTC-IC) assay) was observed in samples supplemented with **1.25** and **1.30**, compared to samples with only 10% DMSO (Fig. 6.13).³⁰ Since an increase in the CFU strongly correlates to a greater success of engraftment and patient prognosis,⁸⁹ compound **1.25** was further evaluated for its ability to improve the engraftment activity of CB units. Cryopreservation of CB in 10% DMSO supplemented with **1.25** maintained the viability of total nucleated cell populations and improved platelet and BM engraftment in primary recipients that received CB grafts.³⁸

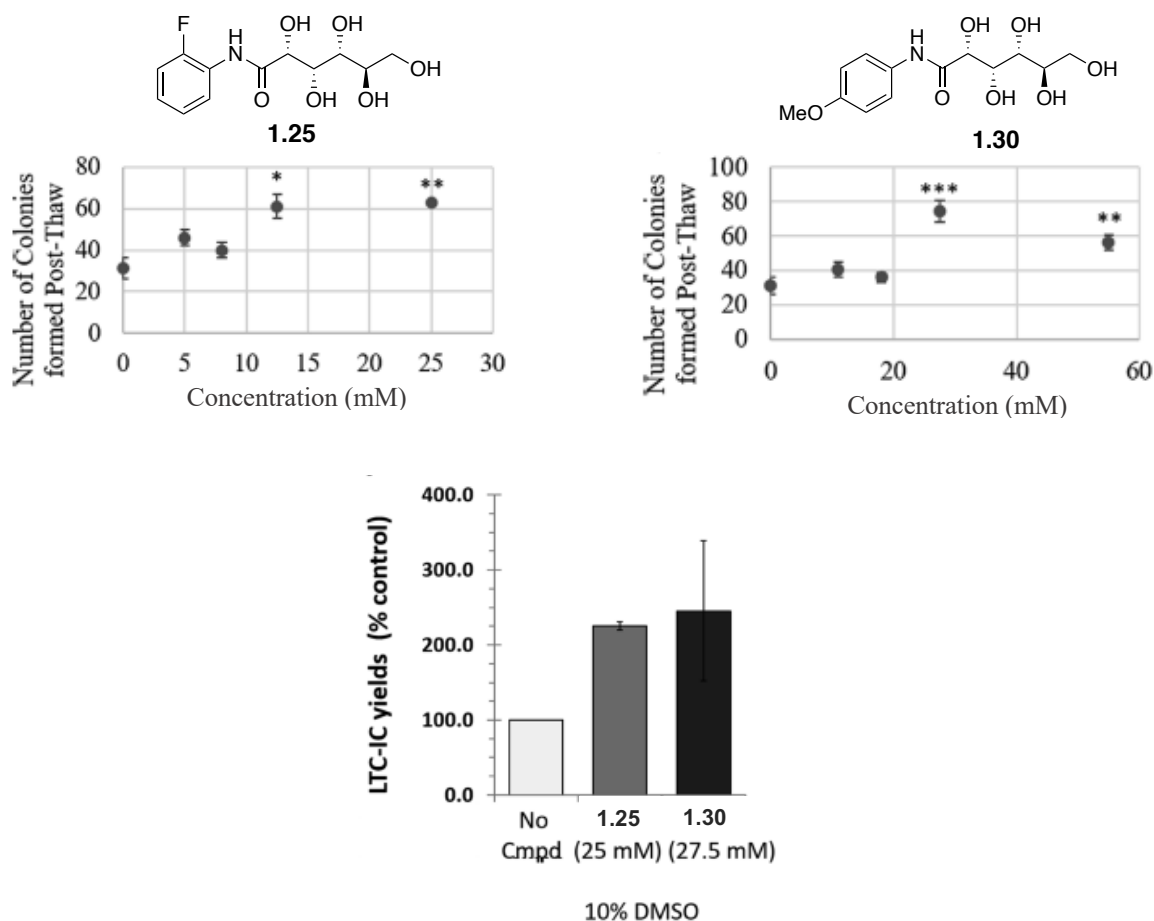


Figure 6.13. IRI active *N*-aryl aldonamides and their ability to improve the total number of colonies formed post-thaw and the total number of long-term culture-initiating cells (LTC-IC) of CB leukocyte concentrates. Compounds **1.25** and **1.30** were supplemented in 10% DMSO. Figure adapted with permission from *ACS Omega*, 2016, 1, 1010–1018. Copyright © American Chemical Society.

Until now, select *N*-aryl gluconamides have shown the ability to improve the post-thaw recovery and functionality of HSCs, however compounds exhibiting IRI activity from other classes have never been evaluated. In the following section, a study describing the evaluation of glucose phosphonate **4.14**, to cryopreserve HSCs from CB units will be discussed.

6.3.1 *The cryopreservation of HSCs with IRI 4.14*

The ammonium G1CP derivative (**4.14**) was assessed as a cryoprotective agent for the cryopreservation of HSCs isolated from CB. Two CB units were processed within 48 hours of collection from informed and consenting donors, according to the Canadian Blood Services standard. CB processing was performed in collaboration with Richa Kaushal, a graduate student at Canadian Blood Services. The CB units were assessed to ensure that the hospital volume was over 50 mL, and the hospital total nucleated cell (TNC) count was between 0.9×10^9 and 1.5×10^9 . To instigate RBC depletion from the CB units, Hespan[®] (6% hetastarch in 0.9% sodium chloride) was added to CB samples and the plasma and buffy coat layer (containing stem cells) were separated from the unwanted RBCs. Further processing was performed to isolate the plasma from the wanted leukocyte concentrate containing the TNCs. Cryopreservation of the TNCs at a concentration of 10×10^6 TNCs/mL was performed in either 10% DMSO (with 5% w/v dextran in 0.9% saline) or 10% DMSO (with 5% w/v dextran in 0.9% saline) supplemented with **4.14**. Cryovials containing 1 mL samples of the supplemented TNCs were cryopreserved (1 °C/min) to -80 °C, then transferred to liquid nitrogen vapours for prolonged storage (days to weeks) before thawing quickly in a 37 °C water bath. Post-thaw processing and analysis of the cryopreserved samples were performed by Richa Kaushal.

6.3.1.1 *The post-thaw viability of CD34⁺ and CD45⁺ cell populations cryopreserved in the presence of IRI 4.14*

Besides evaluating the TNC count, assessing the post-thaw viability of the CD34⁺ and CD45⁺ cell populations is valuable in determining the quality of HSCs and predicting their engraftment success.^{89,90} Flow cytometry was used to determine the viability of CD34⁺ and CD45⁺ cell populations. CD34 is expressed on the surface of human HSCs. CD34⁺ cell populations

include some LTC-IC, CFU-GEMM, BFU-E, and CFU-GM, and therefore it is an important marker for measuring the viability of HSCs.⁸⁰ CD45 is expressed on all leukocytes, therefore CD45⁺ populations are also a good indicator of the viability of HSCs.⁹¹ Alongside the viability of CD34⁺ and CD45⁺ cell populations, the number of necrotic and apoptotic CD34⁺ and CD45⁺ cells were assessed by flow cytometry. Necrotic cell populations were discriminated against apoptotic cell populations by the use of Sytox-AADvanced and Annexin V staining kits. The Sytox-AADvanced stain is an indicator for cells that have compromised membranes (have undergone necrosis) as the stain binds to nucleic acids with high affinity. The Annexin V stain, however, binds to the cell marker, phosphatidylserine, which appears only on the external leaflet of the cell membrane of apoptotic cells.

Supplementation of 0.3 mM, 1 mM, 5 mM, 10 mM, 30 mM, and 100 mM concentrations of **4.14** did not have any significant effect on the proportion of viable CD34⁺ cells (Fig. 6.14). However, CD34⁺ cells cryopreserved with 100 mM **4.14** displayed a greater ($p < 0.01$) proportion of apoptotic cells compared to cells cryopreserved in 10% DMSO. High levels of apoptotic CD34⁺ cells have been correlated to a decrease in the ability of cells to differentiate and proliferate.^{90,91} The proportion of necrotic CD34⁺ cells cryopreserved with varying concentrations of **4.14** were not statistically different from the cells cryopreserved in 10% DMSO.

Overall, supplementation of **4.14** in 10% DMSO did not deviate from the proportion of viable CD34⁺ cells observed with only 10% DMSO. An increase in the proportion of apoptotic CD34⁺ cells was observed only after cryopreservation in 100 mM **4.14**, therefore indicating that low concentrations of **4.14** (up to 30 mM) are not toxic to HSCs.

The effect of phosphonate **4.14** towards the cytotoxicity of the leukocyte (CD45⁺) population was similar to the effect on the CD34⁺ population, where supplementation of 100 mM

4.14 resulted in a significant decrease in the proportion of viable CD45⁺ cells ($p < 0.01$), and a significant increase in the proportion of apoptotic CD45⁺ cells and necrotic CD45⁺ cells ($p < 0.05$). Supplementation of 30 mM **4.14**, also resulted in a significant increase in the proportion of apoptotic cells ($p < 0.01$). This further suggests that low concentrations of **4.14** (up to 30 mM) are not toxic and do not affect CD34⁺ and CD45⁺ cell populations relative to 10% DMSO.

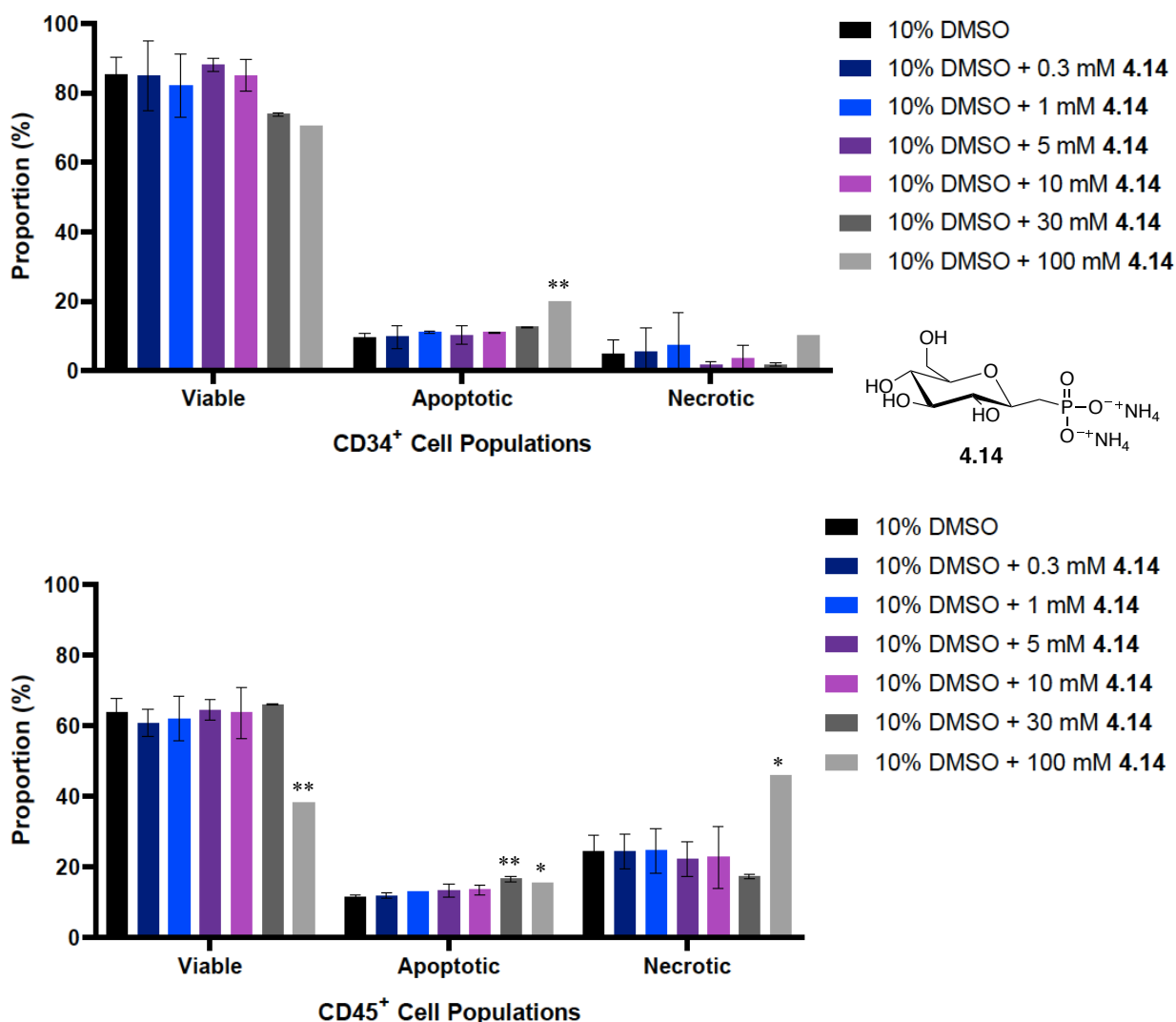


Figure 6.14. Post-thaw recovery of CD34⁺ and CD45⁺ cell populations cryopreserved in 10% DMSO, or 10% DMSO supplemented with **4.14**. Data plotted is the mean of $n=2$ CB units. Error bars represent % SD and asterisks indicates statistical significance from control condition (10% DMSO) using the one-way ANOVA with Dunnett's multiple comparison test (* $p < 0.05$; ** $p < 0.01$).

6.3.1.2 *The post-thaw functionality of HSCs cryopreserved in the presence of IRI 4.14*

The functional capacity and graft quality of HSCs can be predicted by observing the colony forming units (CFUs).⁸⁰ The colony-forming-cell (CFC) assay was performed to assess the clonogenicity of HSCs in the presence of ammonium G1CP, **4.14**. Cryopreserved HSCs were plated in ideal conditions to support the proliferation and differentiation of HSC progenitors,⁹² resulting in the formation of discrete colonies that can be identified by their morphological and phenotypic criteria. The different types of differentiated colonies consisted of Colony-Forming Unit – Granulocyte/Erythrocyte/Monocyte/Megakaryocyte (CFU-GEMM), Colony-Forming Unit – Granulocyte/Monocyte (CFU-GM) and Burst-Forming Unit – Erythroid (BFU-E). CFU-GEMM colonies are immature multipotential progenitors that give rise to erythroid cells and granulocyte, macrophage, and megakaryocyte cells. CFU-GM colonies are a more mature progenitor that further proliferate into granulocytes and monocytes. BFU-E colonies are a primitive erythroid progenitor that give rise to erythrocytes.

The clonogenicity of HSCs from CB was maintained after cryopreservation in 10% DMSO supplemented with 0.3 mM, 1 mM, 5 mM, 10 mM, or 30 mM **4.14** (Fig. 6.15). However, supplementation of 100 mM **4.14** prevented the growth of CFU-GEMM, CFU-GM, and BFU-E colonies, indicating that at high concentrations of **4.14**, HSC functionality is completely inhibited. This could possibly be due to high solute concentrations disturbing the ideal culture conditions required for the growth of colonies.

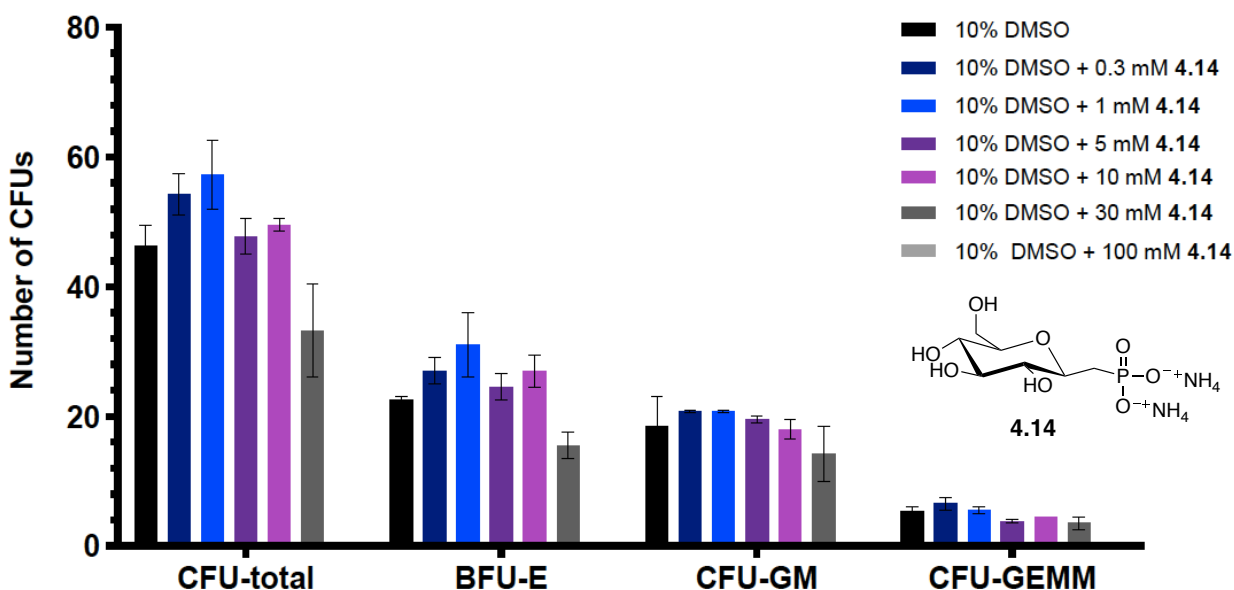


Figure 6.15. The clonogenicity of HSCs described by the number of CFUs observed post-thaw. CFUs were assessed using the CFC assay and the sub colonies of the total colonies (CFU-total) observed consisted of BFU-E, CFU-GM, and CFU-GEMM colonies. Compared to the control (10% DMSO), no significant difference was observed amongst the different treatments, using the one-way ANOVA with Dunnett's multiple comparison test (* $p < 0.05$). Treatment with 100 mM **4.14** resulted in an absence of colonies.

Overall, the lack of significant difference observed in HSCs that were cryopreserved in 10% DMSO with supplementation of relatively low concentrations of **4.14** implies that **4.14** is well tolerated by the cells and is able to maintain viability and functionality. These results are comparable with those observed for the cryopreservation of HSCs from CB with *N*-aryl gluconamide **6.9**, which resulted in an improvement of platelet engraftment.^{30,38} Further experiments should include assessing phosphonates **4.15** and **4.24** in the cryopreservation of HSCs from CB. Since the IRI activity of G1CPs is altered by the counterion and the ionic state of the phosphonate, evaluating **4.15** and **4.24** in HSC cryopreservation could reveal the contribution of IRI activity of G1CPs to the viability and functionality of HSCs.

6.4 Summary

The use of IRIs to cryopreserve various cell types offers many advantages as observed previously with select *O*-aryl glucosides and *N*-aryl aldonamides.^{29–32,37} As a result, compounds that exhibit IRI activity are also assessed for cryopreservative ability. Therefore, this chapter describes the evaluation of IRI active compounds, AF(G)Ps and glucose phosphonates as CPAs for the cryopreservation of RBCs and HSCs.

Before assessing the cryopreservative ability of IRIs *in vitro*, their cytotoxicity was assessed in HepG2 cells. AFPI, AFPIII, and AFGP exhibited relatively low cytotoxicity at low nanomolar concentrations, however, higher concentrations of AFPIII were more toxic than AFPI and AFGP. The cytotoxicity of glucose phosphonate compounds **4.14**, **4.15**, **4.18** and **4.19** generated a dose-response at millimolar concentrations. The ammonium phosphonate derivatives were more cytotoxic than the sodium phosphonate derivatives, and this was hypothesized to be a result of the cytotoxicity of the ammonium ion. Also, G6CP derivatives generated much larger hill slopes suggesting that they undergo different mechanisms of toxicity. Overall, at their IC₅₀ values, **4.14**, **4.15**, **4.18** and **4.19**, displayed low cytotoxicity to HepG2 cells.

Improvement to the cryopreservation of RBCs is necessary due to the high concentration of glycerol that is currently used, resulting in a long deglycerolization time required before the clinical use of cryopreserved RBCs. Previous work in the Ben laboratory displayed that IRIs can be supplemented to reduce glycerol concentrations and improve cell viability, therefore AFPI, AFPIII, and AFGP were evaluated due to their strong IRI activity. Nanomolar concentrations of AFPI, AFPIII, and AFGP did not significantly increase or decrease the cell viability of RBCs when supplemented into 15% glycerol. Despite exhibiting high IRI activity at these concentrations, it was hypothesized that due to the presence of glycerol, the IRI activity of the AF(G)Ps may have

been suppressed (as observed in **Chapter 5**). Next, the ammonium and sodium G1CP derivatives (**4.14** and **4.15**) as well as the phosphonic acid derivative (**4.24**) were assessed as CPAs for RBC cryopreservation. Supplementation of **4.14** and **4.15** into 15% glycerol also did not result in any substantial increase or decrease in cell viability, however, **4.24** provided an increase in post-thaw viability under slow cooling conditions. Cryopreservation with high concentrations of **4.15** and **4.24** exhibited a significant increase in RBC hemolysis.

The cryopreservative ability of ammonium G1CP derivative **4.14** was also assessed in HSCs. Cryopreserving HSCs in 10% DMSO supplemented with low concentrations (up to 30 mM) of **4.14** maintained the viability and functionality of HSCs. A significant decrease in HSC viability and functionality was only observed in HSCs cryopreserved with 100 mM **4.14**, indicating that relatively low concentrations of **4.14** are not cytotoxic to HSCs.

Therefore, considering the discussions of this chapter, it is of interest to continue the evaluation of AF(G)Ps and glucose phosphonates as cryo-additives in the cryopreservation of RBCs and HSCs. Future studies should include more extensive concentration scans to identify the ideal concentrations that provide maximum cryoprotection but limit cytotoxicity in RBCs and HSCs. Overall, these studies were a sufficient starting point to identify new IRIs that are also effective cryo-additives.

6.5 Experimental procedures

6.5.1 General experimental procedures

AFPI, AFPIII, and AFGP used for cytotoxicity studies and for the cryopreservation of RBCs were a gift from Prof. Sakae Tsuda at the National Institute of Advanced Industrial Science and Technology (AIST) in Sapporo, Japan. The description and properties of AF(G)Ps are the

same as outlined in the *Experimental Section* of **Chapter 5**. Phosphonate compounds **4.14**, **4.15**, **4.18**, **4.19** and **4.24**, were synthesized according to the synthetic procedure outlined in the *Experimental Section* of **Chapter 4**.

6.5.2 *HepG2 cell culture*

HepG2 cells (human hepatocellular carcinoma cells, ATCC, HB-8065) were plated at a frequency of 1.5×10^6 cells, in a Corning® U-shaped T75 flask (Fisher, 07-202-000), and cultured in Eagle's Minimum Essential Media (MEM, Sigma M4655) supplemented with 10% Fetal Bovine Serum (FBS, heat inactivated US), 1% penicillin-streptomycin (Fisher, HyClone 100X solution, SV30010), 1% non-essential amino acids (Sigma, M7145), and 0.1% 1M sodium pyruvate. The HepG2 cell culture proceeded at 37 °C and 5% CO₂. The cells were washed every two days with Gibco™ DPBS (without Ca and Mg, Fisher 14190136) and the culture media was changed until 80% cell confluency was reached. At 80% confluency, washed cells were detached with Accutase (Sigma, A6964) (incubated at 37 °C for 15 min) and a cell count was obtained by making a 1:4 dilution with Trypan Blue dye (Sigma, T8154) and using a hemocytometer.

6.5.3 *Aquabluor™ assay with HepG2 cells*

The cultured HepG2 cells were made into a 600,000 cells/mL stock solution and 100 µL was transferred to the wells of a 96-well plate to give a final concentration of 6,000 cells/well. The plate was incubated for 20-24 h at 37 °C and 5% CO₂, then centrifuged at 1000 rpm for 5 min. The supernatant in each well was removed via aspiration and either media (positive control), media supplemented with the compound to be tested, or media supplemented with 1% Triton X (negative control) was added to the appropriate wells as displayed in Fig. 6.16. The plate was incubated for

20-24 h at 37 °C and 5% CO₂, then centrifuged at 1000 rpm for 5 min. The supernatant in each well was removed via aspiration and 100X dilution of Aquabluer™ in Gibco™ Minimum Essential Media (without glutamine and phenol red, Fisher 51200038) was added to each well. The plate was incubated for 4 h at 37 °C and 5% CO₂. The fluorescence (540 excitation/590 emission) was measured by a SpectraMax Absorbance microplate reader to obtain RFU readings.

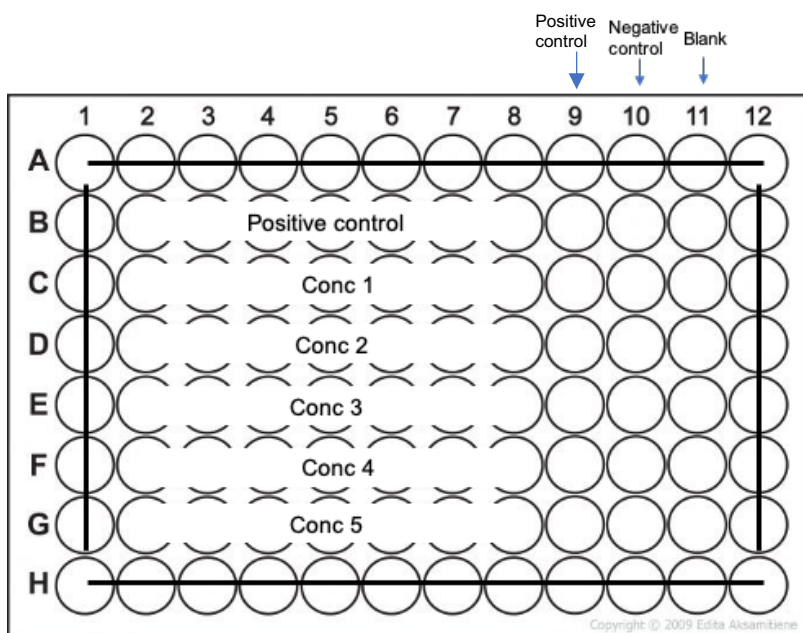


Figure 6.16. Representation of the 96-well plate setup used to evaluate the cytotoxicity of IRIs with HepG2 cells. Black lines represent wells filled with sterile ddH₂O.

6.5.4 AlamarBlue™ assay with HepG2 cells

The cultured HepG2 cells were distributed in a 96-well plate at a concentration of 10,000 cells/well. The plate was incubated for 20-24 h at 37 °C and 5% CO₂, then centrifuged at 1000 rpm for 5 min. The supernatant in each well was removed via aspiration and either media (positive control), media supplemented with IRI, or media supplemented with 1% Triton X (negative control) was added to the appropriate wells as displayed in Fig. 6.16. The plate was incubated for

20-24 h at 37 °C and 5% CO₂. 10 µL of 12.5% w/v resazurin (Sigma, R7017) in Gibco™ DPBS (without Ca and Mg, Fisher 14190136) was added to each well and the plates were incubated for 4 h at 37 °C and 5% CO₂. The fluorescence (530 excitation/590 emission) was measured by a SpectraMax Absorbance microplate reader to obtain RFU readings.

6.5.5 RBC collection

RBC samples were obtained from healthy volunteers via NetCAD Vancouver, BC (Canadian Blood Services' Network Centre for Applied Development). Before the collection of whole blood, informed consent was obtained from the volunteers and ethics approval was obtained from the Research Ethics Board (REB) at CBS and the University of Alberta. The blood units (ABO, Rh matched) were processed by NetCAD to generate SAGM RBC units and used within 14 days of collection. Upon receiving the processed units, the blood units were pooled together and split to reduce donor to donor variability.

6.5.6 RBC freezing and thawing experiments

Cryosolutions were made by dissolving the test compound (2X) in a 30% glycerol solution made from 57 Glycerolyte (Baxter), and 0.9% saline/0.2% dextrose. An equal volume of the cryosolution was added to an equal volume of the SAGM RBC sample to make a 1X compound solution in 15% glycerol. The RBC-cryosolution mixture was aliquoted into cryovials (n=2 for each freezing condition) and incubated for 10 min at room temperature. Cryovials were then transferred to a methanol bath at -5 °C and maintained at this temperature until the thermocouple (inserted in a RBC/15% glycerol sample) indicated that the internal temperature of the samples were -5 °C. All samples were nucleated with frozen forceps (cooled with liquid nitrogen) and held

at -5 °C for 5 min. Samples were then either slow cooled (1 °C/min) to -40 °C or rapidly cooled to -78 °C by transferring them to dry ice. After the internal temperature of all samples was at their designated temperature, samples were transferred to a 37 °C water bath until completely thawed.

6.5.7 RBC post-thaw analysis

Cryopreserved RBC samples were analyzed for their % hemolysis by determination of the hematocrit and hemoglobin content. The hematocrit content (Hct) was determined by transferring a small amount of the cryopreserved sample into a microhematocrit tube, which was centrifuged to observe the hematocrit levels. The remainder of the cryopreserved RBC samples were centrifuged (2200 g, 10 min) and Drabkin's reagent⁴⁸ was added to both the supernatant and the cell pellet. The hemoglobin content in the supernatant (Hb_S) and the total RBCs (Hb_T) was quantified by measuring the absorbance of the samples at 540 nm. Once the hemoglobin and hematocrit values were determined, % hemolysis was calculated from the following equation:

$$\% \text{ hemolysis} = \frac{(100 - Hct) \times Hb_S}{Hb_T}$$

6.5.8 CB Processing

CB units were collected from informed donors and their consent was obtained for research use at Canadian Blood Services. The collected units were stored at room temperature, and processed within 48 h. The hospital volume and TNC count of the CB unit was obtained and confirmed to be at least 50 mL and 0.9 x 10⁹ and 1.5 x 10⁹ TNCs. The unit was split evenly into three and processed by first adding Hespan[®] (6% hetastarch in 0.9% sodium chloride) to separate the RBCs from the plasma/buffy coat layer. The plasma/buffy coat layer was further centrifuged

(1390 rpm, 10 min) and only the buffy coat layer was kept. The TNC count from the buffy coat layer was obtained using a pocH-100i hematology analyzer (Sysmex Corporation, Ontario, Canada), and the TNC were split into tubes (total count of 10×10^6 TNC/tube) for each cryopreservation condition.

6.5.9 CB freezing experiment

Cryosolutions were prepared by dissolving the test compound in a 10% DMSO cryomedium (in 0.9% saline with 5% w/v dextran). Cryosolutions were prepared on the same day as CB processing. Tubes containing 10×10^6 TNC count were centrifuged (1390 rpm for 5 min), and the cell pellet was resuspended in freshly made cryosolutions. 1 mL of the supplemented TNCs was transferred to cryovials and slow cooled ($1^\circ\text{C}/\text{min}$) to -80°C before transfer to liquid nitrogen storage for long-term preservation.

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Chapter 7. Thesis Conclusions and Future Work

7.1 Conclusions

The overall focus of this thesis was to synthesize ice recrystallization inhibitors with high solubilities, and to assess their ability as cryo-additives. Identifying small-molecule carbohydrates that are able to inhibit ice recrystallization and are not limited by their solubility would improve the overall use of IRIs for cryopreservation. Towards this goal, small-molecule carbohydrates possessing a charged functional group in their molecular structure were synthesized and evaluated for IRI activity. As part of the evaluation process, various established assays used to measure IRI activity were investigated, and the qualitative and quantitative results generated by these assays were compared. Finally, a selection of IRI active compounds (including ones with polar functional groups) were evaluated *in vitro* for their cytotoxicity and cryopreservative ability.

The first objective of this thesis was achieved by exploring the synthesis of amino- and azido-substituted *N*-aryl-D-gluconamides (**Chapter 3**) and phosphonate-substituted small-molecule carbohydrates (**Chapter 4**). Modification to the *N*-aryl-D-gluconamides was performed at the *C5*-position, however this position was difficult to manipulate. Several attempts to nucleophilically displace a leaving group at the *C5*-position with a nucleophilic azide were unsuccessful and did not result in an azide product. Another approach involving an asymmetric aldol reaction between an azide-substituted butanal, and Evan's chiral auxiliary was attempted, however the azide-substituted butanal remained unreacted. Although these reactions were unsuccessful, the former attempts at synthesizing 5-azido and 5-amino derivatives of *N*-aryl-D-gluconamides demonstrated a novel route to synthesizing L-xylofuranosyl amides and esters. These unexpected products were a result of an intramolecular nucleophilic substitution at the *C5*-

position. Upon evaluating their IRI activity, the L-xylofuranosyl amide products displayed low activity at their maximum solubilities. Nonetheless, this study demonstrated a novel synthetic route towards L-xylofuranose products that has not yet been detailed in the literature.

Following the unsuccessful attempt to synthesize amino- and azido-substituted *N*-aryl-D-gluconamides, the goal of generating small-molecule IRIs with charged functional groups was further explored by the synthesis of phosphonate-substituted compounds (**Chapter 4**). The series of phosphonate-substituted small-molecule carbohydrates included glucose-1-phosphonate, a C6-phosphonate derivative of α -methoxyglucoside and *O*-(4-methoxyphenyl)- β -D-glucoside, and a phosphonate derivative of *N*-octyl-D-gluconamide. Surveying different counterions to generate phosphonate salts revealed that ammonium ions display strong IRI activity and therefore increase the overall IRI activity of the carbohydrate phosphonate. Sodium, potassium, and calcium ions, however, did not influence the IRI activity of the carbohydrate phosphonate. The sodium salt of glucose-1-phosphonate and the C6-phosphonate derivative of α -methoxyglucoside were found to maintain high solubilities and were more IRI active than their respective parent compounds. The sodium salt of the C6-phosphonate derivative of *O*-(4-methoxyphenyl)- β -D-glucoside and the phosphonate derivative of *N*-octyl-D-gluconamide were found to be more soluble but exhibit less IRI activity than their respective parent compounds. These results suggested that the impact of a phosphonate functional group on IRI activity varies. Specifically, for IRI active compounds such as *O*-(4-methoxyphenyl)- β -D-glucoside and *N*-octyl-D-gluconamide, incorporating a phosphonate into their molecular structure may deviate away from the ideal hydrophobic/hydrophilic balance required for IRI activity, resulting in reduced activities.

Before the evaluation of select phosphonate compounds as cryo-additives, the current methods used to assess IRI activity were evaluated (**Chapter 5**). Since there are various assays

established to qualitatively and quantitatively measure IRI activity, a direct comparison between the two most commonly used assays (splat cooling assay and sucrose sandwich assay) was performed. Major differences were observed in the ice crystal structure, the ice crystal growth rate, and the quantitative values generated from the splat cooling assay and sucrose sandwich assay. These results showed the importance of selecting an IRI assay that is closely related to its application, to ensure that the IRI activity observed in the assay will closely resemble the activity observed in the application. To that end, the impact of various concentrations of conventional CPAs (DMSO or glycerol) on IRI activity was evaluated. Increasing concentrations of DMSO or glycerol decreased the IRI activity of *N*-(2-fluorophenyl) and *N*-(4-chlorophenyl) gluconamides, and AFPI, however there was no impact on the IRI activity of *O*-(4-bromophenyl) glucoside. This work was significant and suggested that the concentrations of DMSO and glycerol used in cryopreservation (10% and 15-40% respectively) may significantly suppress the IRI activity of *N*-aryl-D-gluconamides and AFPI. Therefore, their ability to improve the post-thaw viability and functionality of certain cell types may not be a result of their IRI activity.

Chapter 6 achieved the objective of evaluating select IRIs *in vitro* to improve the cryopreservation of RBCs and HSCs. Despite their IRI activities, the supplementation of 15% glycerol with AFPI, AFPIII, AFGP or glucose-1-phosphonate did not result in an increase or decrease in the post-thaw viability of RBCs. Similarly, the supplementation of 10% DMSO with glucose-1-phosphonate did not increase or decrease the post-thaw viability or functionality of HSCs. These results suggested that the ability of these compounds to inhibit ice recrystallization is not contributing to the viability or functionality of cryopreserved RBCs and HSCs, or as described in **Chapter 5**, the *in vitro* IRI activity may be suppressed by the presence of the CPA.

Overall, the objectives of this thesis were achieved and have demonstrated the potential of small-molecule IRIs with charged functional groups as a method to increase solubility. Although these compounds did not improve the cryopreservation method of RBCs and HSCs, this study shows that these compounds are biologically accepted and do not exhibit significant toxicities towards cryopreserved RBCs and HSCs.

7.2 Future Work

Collectively, the contents of this thesis have provided further insight into the synthesis and IRI activity of novel small-molecule IRIs possessing an overall molecular charge. From the work covered in this thesis it is evident that compounds exhibiting strong IRI activity have a unique hydrophobic/hydrophilic balance and deviating away from that balance by incorporating a charged functional group, is detrimental to their IRI activity. To further identify how this hydrophobic/hydrophilic balance contributes to IRI activity, determining the definite mechanism of IRIs is warranted.

As shown in **Chapter 4**, the sodium glucose phosphonate compounds (**4.15** and **4.19**) were more IRI active than their weakly active parent compounds. On the other hand, the sodium phosphonate analogues (**4.37**, and **4.41**) of IRIs with strong activity ($IC_{50} < 20$ mM) were less active than their parent compounds. Future studies should include the synthesis of various compounds with phosphonates incorporated at different positions on the saccharide, to understand the full scope of the effect of a phosphonate functional group on IRI activity. Alongside, other polar functional groups possessing an overall charge should be investigated in the scope of IRI modifications. Unfortunately, pursuing this by attempting to synthesize 5-amino substituted *N*-aryl-D-gluconamides in **Chapter 3** was unsuccessful. Since manipulating the C5-

position of an *N*-aryl-D-gluconamide was challenging via a direct nucleophilic displacement and an asymmetric synthesis approach, other synthetic routes should be attempted. One possible approach may be to oxidize the *C*5-hydroxyl group and to arrive at the *C*5-amine via the formation of an imine.

The work outlined in **Chapter 5** provided insight about the assays used to evaluate IRI activity and displayed how the measurement of IRI activity can be influenced by several variables (i.e. type of solute present, annealing temperature, and annealing time). Future work on the influence of CPAs, such as DMSO and glycerol, on IRI activity would be valuable to gain a full understanding on how IRIs are performing *in vitro*. To start, the IRI activity of several other *N*-aryl-D-gluconamides and *O*-aryl- β -D-glucosides that are also sufficient cryoprotectants should be evaluated in the presence of DMSO and glycerol. Further, an *in vitro* analysis of ice recrystallization in the presence of IRI supplemented DMSO or glycerol, should be performed using cryomicroscopy. Specifically, assessment of *N*-aryl-D-gluconamides, **1.25** and **1.26** in 10% DMSO *in vitro* would verify the hypothesis proposed in this chapter that they may not be exhibiting IRI activity *in vitro*. If this is the case, these studies should be accompanied with a full analysis of the cell physiology before and after cryopreservation with IRIs to determine other possible beneficial contributions of **1.25** and **1.26**. Further mechanistic insight of **1.25** and **1.26** as ice recrystallization inhibitors, would be helpful in understanding how DMSO and glycerol suppress their IRI activity.

In **Chapter 6**, RBCs cryopreserved in 15% glycerol supplemented with glucose-1-phosphonate derivatives or AF(G)Ps showed no significant increase or decrease in its post-thaw viability despite the strong activity of the IRIs. These preliminary studies suggested that the IRI concentrations evaluated may not be high enough to provide cryoprotection *in vitro*. Therefore, a

complete concentration scan should be performed, evaluating higher concentrations, to determine the concentrations (if any) that increase post-thaw viability and the concentrations that are lethal. Specifically, AF(G)Ps exhibited strong IRI activity at nanomolar concentrations, thus were hypothesized to improve the cryopreservation of RBCs at those concentrations. However, observing the suppressive effect of DMSO on the IRI activity of AFPI in **Chapter 5**, suggests that glycerol may also exhibit a similar effect and higher concentrations of AF(G)Ps may be required to override this.

In the HSC cryopreservation experiments, a full concentration scan of ammonium phosphonate **4.14** was performed up to 100 mM, and a significant decrease in HSC viability and functionality was observed at 100 mM. However, lower concentrations (0.3 mM – 30 mM) of **4.14** were able to maintain the viability and functionality of post-thaw HSCs. Further experiments should include the evaluation of the sodium phosphonate derivative **4.15** and the phosphonic acid derivative **4.24** to determine if the observed counterion effect on IRI activity influences the cryopreservative ability of these compounds. Furthermore, since the glucose-6-phosphonate derivatives, **4.18** and **4.19**, were more IRI active than the glucose-1-phosphonate derivatives, their *in vitro* cryoprotective ability should also be evaluated in HSCs.

Contributions to Original Research

1. Synthesis and analysis of IRI activity of *N*-xylo-L-furanoses as described in **Chapter 3**.
2. Synthesis and analysis of IRI activity of phosphonate-substituted IRIs **4.14-4.19**, **4.36**, **4.37**, and **4.41** to generate effective IRIs with high solubilities, and to investigate the importance of amphiphilicity toward IRI activity.

3. Investigating the effect of various IRI assays and cryoprotective agents on measured values defining IRI activity (% MGS and IC₅₀ values) as described in **Chapter 5**.
4. Analysis of the cytotoxicity of AFPI, AFPIII, AFGP, **4.14**, **4.15**, **4.18**, and **4.19** with HepG2 cells.
5. Analysis of the cryopreservative ability of AFPI, AFPIII, AFGP, **4.14**, **4.15**, and **4.24** with RBCs and the cryopreservative ability of **4.14** with HSCs obtained from umbilical cord blood.

Publications Resulting from this Thesis

1. Ampaw, A. A.; Ben, R. N. A Serendipitous Route to *N*-xylo-L-furanoses. *Carb Res.* **2022**. Manuscript in preparation.
2. Ampaw, A. A.; Newell, K.; Ben, R. N. Investigating the Solubility and Activity of a Novel Class of Ice Recrystallization Inhibitors. *Processes.* **2021**, *9* (10), 1781.
3. Ampaw, A. A.; Sibthorpe, A.; Ben, R. N. Use of Ice Recrystallization Inhibition Assays to Screen for Compounds That Inhibit Ice Recrystallization. In *Methods in Molecular Biology*; Humana Press Inc., 2021; Vol. 2180, pp 271–283.
4. Ampaw, A.; Charlton, T. A.; Briard, J. G.; Ben, R. N. Designing the next Generation of Cryoprotectants – From Proteins to Small Molecules. *Peptide Science* **2019**, *111* (1), e24086.